NOVEL RESVERATROL ANALOGUES: SYNTHESIS, METABOLISM AND CELL PROLIFERATION

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

Charles Simon (MSc, Ingénieur Chimiste ESCOM – France)

Department of Chemistry

University of Leicester

November 2010

Table of Content

ACKNOWLEDGMENTS

First I would like to thank the University of Leicester, Prof Eric Hope and Prof Andrew Abbott, successive heads of department, for welcoming me in the Chemistry Department and for offering me this PhD. I also thank the different staff members and academics in the department.

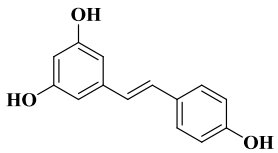
A very special thank you goes to the Organic Lab: Will, Steve, Helen, Kat, Youcef, Vanessa, Angelo, Ruggero, Michele, Chris, Sharifa and Andy for the fun, the parties, the friendships, scientific and general chats and their help, advice and support. I also want to show gratitude to the Cancer Studies and Molecular Medicine group members: Emma, Ankur, Isobel, Shaban and Glenn, but more especially to Mr Ketan Patel for his help and demonstration of different biology techniques especially in cell culture. Specific thanks go to Dr Hong Cai for her assistance and work in the metabolism studies and her corrections and to Dr Lynne Howells for reading the cell culture chapter and her help in the flow cytometry studies alongside Dr Christoph Boes.

I thank a lot Papa, Maman and my brother JB for their support and encouragements as well as my friends in France in no specific order Maupet, Pierro, Mouf, Greg, Dr Antoine, Juliette and Philippe and all others who encouraged me at some point during my PhD.

A massive thank you goes to Dr Rob Britton for his friendliness, precious help and recommendations in the lab and outside and for all the reading I've given him and the corrections he has done on the thesis... I would like to acknowledge Dr Karen Brown, my supervisor in the RKCSB building, for her advice, guidance and explanations in biology. And finally my biggest gratitude goes to Dr Paul R. Jenkins, my supervisor, for offering me this extremely good opportunity, for his help, guidance, support and friendship.

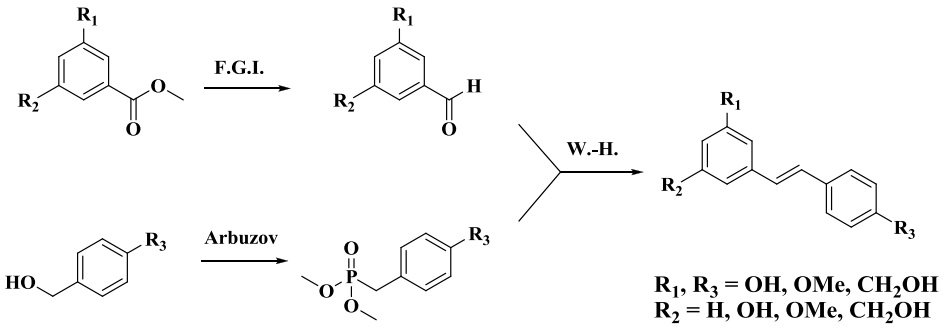
ABSTRACT

Resveratrol or *trans*-3,4',5-trihydroxystilbene is a naturally occurring phytochemical contained in red grapes skin, nuts and berries. It has been shown over the years to have different biological properties particularly in the chemoprevention of cancer. However, it is metabolised *in vivo* to sulfates and glucuronides within 1h and is active against different targets in a dose dependant manner.



A library of new analogues of resveratrol has been synthesised with the aim of stopping or at least slowing down the metabolism whilst keeping its activity on the inhibition of cancer cell proliferation.

Seven new analogues of resveratrol were synthesised in which the phenol substituents were systematically replaced by benzylic alcohols and/or methoxy groups. The library was then assessed in *in vitro* enzymatic metabolism with mouse and human liver fractions in the presence of a cofactor. The compounds were also evaluated as inhibitors of HCA-7 colorectal cancer cell proliferation.



ABBREVIATIONS

AcO	Acetate
AhR	Aryl hydrocarbon and dioxin receptor
ATP	Adenosine triphosphate
BaP	Benzo[a]pyrene
BRCA	Breast cancer gene
Cat.	Catalytic
CDK	Cyclin Dependant Kinase
CDKI	CDK Inhibitor
CoA	4-Coumarate:coenzyme A
COMT	Catechol O-Methyl Transferase
Conc.	Concentrated
COX	Cyclooxygenase
CYP450	Cytochrome P450
δ	Chemical shift
DCE	Dichloroethane
DCC	*N,N′*-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DHP	3,4-2*H*-Dihydropyran
DMAP	4-Dimethylaminopyridine
DMBA	7,12-bimethylbenz[a]anthracene
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid

ECM	Extracellular matrix
ED_{50}	Effective Dose at 50%
EGF	Epidermal Growth Factor
EI	Electronic impact
ESI	Electrospray ionisation
EWG	Electron withdrawing group
FACS	Fluorescence-Activated Cell Sorting
FCS	Foetal Calf Serum
F.G.I.	Functional Group Interconversion
FITC	Fluorescein isothiocyanate
4CL	4-CoA Ligase
GF	Growth Factor
HCA-7	Human Colon Carcinoma 7
HCEC	Human Colonic Epithelial Cell
HOBt	Hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
IAP	Inhibitor of Apoptosis Protein
IC_{50}	Inhibitory Concentration at 50%
IκB	Inhibitor of NF-κB
IR	Infrared
INK 4	Inhibitor of the cyclin dependant Kinase 4
i.v.	Intra-venous
KITC	*N*-Hydroxy-*N*'-(3,4,5-trimethoxyphenyl)-3,4,5-trimethoxybenzamidine
LAH	Lithium Aluminium Hydride
LC	Liquid Chromatography

LDL	Low density lipoprotein
Lit.	Literature
LRP	LDL Related protein
Mad	Mitotic arrest deficient
MAO	Monoamine oxidase
MeO	Methoxy
MeCN	Acetonitrile
MHz	Mega Hertz
min	Minute
mM	Millimolar
MMP	Matrix Metalloproteinase
mp	Melting point
MRM	Multiple Reaction Monitoring
MS	Mass Spectroscopy
MW	Microwave
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NF-κB	Nuclear Factor κ-B
NMR	Nuclear Magnetic Resonance
NSAID	Non steroidal anti-inflammatory drug
PAPS	3'-Phosphoadenosine-5'-phosphosulfate
PBS	Phosphate Buffered Saline
PCC	Pyridinium chlorochromate
PCD	Programmed Cell Death
PDGF	Platelet Derived Growth Factor
PG	Prostaglandin / Protecting group

PGE	Prostaglandin E
PI	Propidium Iodide
PKC	Protein kinase C
ppm	Parts per million
PPTS	Pyridinium *p*-toluenesulphonate
pRb	Protein Retinoblastoma
PS	Phosphatidyl serine
Py	Pyridine
ROS	Reactive Oxygen Species
rpm	Rotation per minute
r.t.	Room temperature
SD	Standard deviation
SN	Nucleophilic Substitution
STS	Stilbene Synthase
SULT	Sulfotransferase
t	Time
TBAI	*tert*-Butylammonium iodide
TBDMSCl	*tert*-Butyldimethylsilyl chloride
TEA	Triethylamine
TfO	Triflate
THF	Tetrahydrofuran
THP	Tetrahydropyranyl
TIMP	Tissue Inhibitor of MMP
TLC	Thin Layer Chromatography
TMS	Tetramethylsilyle

TNF	Tumour Necrosis Factor
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
Ts	Tosyl
TSG	Tumour Suppressor Gene
TsO	Tosylate
μM	Micromolar
UDPGA	Uridine Diphosphate Glucuronic Acid
uPA	Urokinase Plasminogen Activator
uPAR	uPA Receptor
UV	Ultra-violet
VEGF	Vascular Growth Factor

CHAPTER 1 - INTRODUCTION

CHAPTER 1: INTRODUCTION

1. Cancer

Recent statistics made by Cancer Research UK[1] show that an estimated 12.7 million people worldwide were diagnosed with cancer in 2008, 2% of them being in the UK, and that around 7.6 million people worldwide died of cancer that same year (mortality in the UK represents 2% of worldwide deaths). The most common diagnosed cancer in men in the UK is prostate cancer (24%) followed by lung and colorectal cancers (15 and 14%, respectively). The most common cause of mortality by cancer in men in the UK is lung cancer which represents around a quarter of the deaths. The most common diagnosed cancer in women in the UK is breast cancer (31%). Cervical, colorectal and liver cancers are less common. The most common cause of mortality by cancer in women in the UK is lung cancer (21%) followed by breast cancer.

1.1. Definitions

Cancer is a group of diseases characterised by an unregulated increase in cell number. Cancer cells can invade the tissues throughout the body.[2] The type of cancer is determined by the tissue of origin and can generally be classified as:

- ***Carcinoma***: cancer coming from epithelial cells (skin, gut, etc.),
- ***Sarcoma***: cancer coming from mesoderm cells (bone, muscles),
- ***Adenocarcinoma***: cancer developing from glandular tissues (breast cells),
- ***Leukaemia***: cancer emerging from haemapoeitic cells (blood).

Cancer cells are formed after the accumulation of DNA damage and mutations in “normal” cells. These mutations can be due to different environmental factors

coming from life style and diet and less commonly from genetic factors. Cancerous cells will grow in an anarchic manner leading to the formation of a solid tumour which will have the ability to create its own vascular system (angiogenesis). These cells will then be able to spread into the neighbouring tissues and invade the body (metastasis) whilst avoiding apoptosis (Programmed Cell Death).[3,4]

1.2. The hallmarks of cancer

Weinberg *et al.*[5] defined six hallmarks of cancer which are the phenotype of the disease (Figure 1). Cancer can be described by these six main characteristics.

- ***Self sufficiency in growth signals****:* the cells evade the cell cycle check-points and grow in an anarchic way. They can grow without any external growth signals.
- ***Insensitivity to anti-growth signals***: the cells evade the quiescent state (G_0) and continue to grow without differentiation, even if receiving signals telling them not to grow.
- ***Tissue invasion and metastasis***: creation of proteases which break the basal lamina (such as matrix metalloproteinases MMPs) and allow the tumour cells to enter the blood stream and invade other tissues. This succession of events is known as the metastatic cascade.[6,7]
- ***Limitless replication potential***: tumour cells do not stop replicating compared to normal cells, which stop and eventually die after a certain number of replications.
- ***Sustained angiogenesis***: creation of new blood vessels from existing one in the

direction of the tumour to supply oxygen and nutrient for the survival. It is due to the "angiogenic switch" between positive (angiogenin, angiopoeitin) and negative (angiostatin, MMP inhibitors) regulators of angiogenesis. [7-10]

- ***Evading apoptosis*:** normal cells die if there is too much DNA damage or after a certain time (Programmed Cell Death), but tumour cells evade the death pathways.

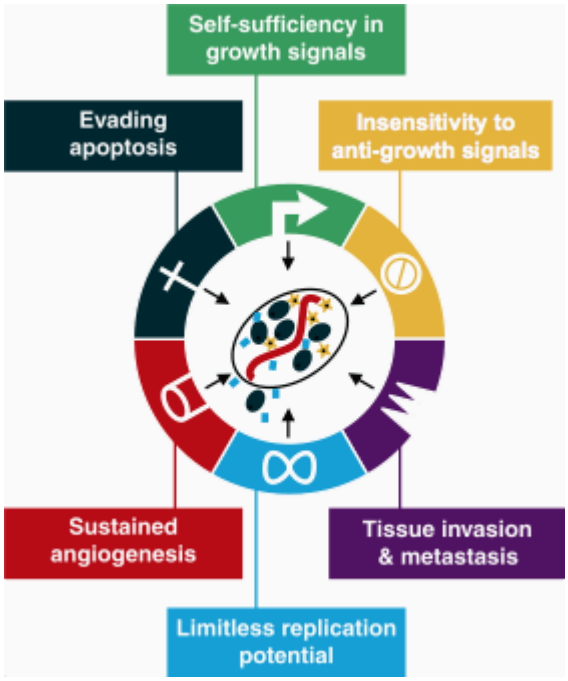


Figure 1: The Hallmarks of cancer by Weinberg and Hanahan[5]

Recently, Luo *et al.*[11] increased the number of hallmarks to 12, adding five new hallmarks describing the stress phenotype of cancer cells to the hallmark "evasion of immune surveillance" proposed earlier by Kroemer.[12] (Figure 2)

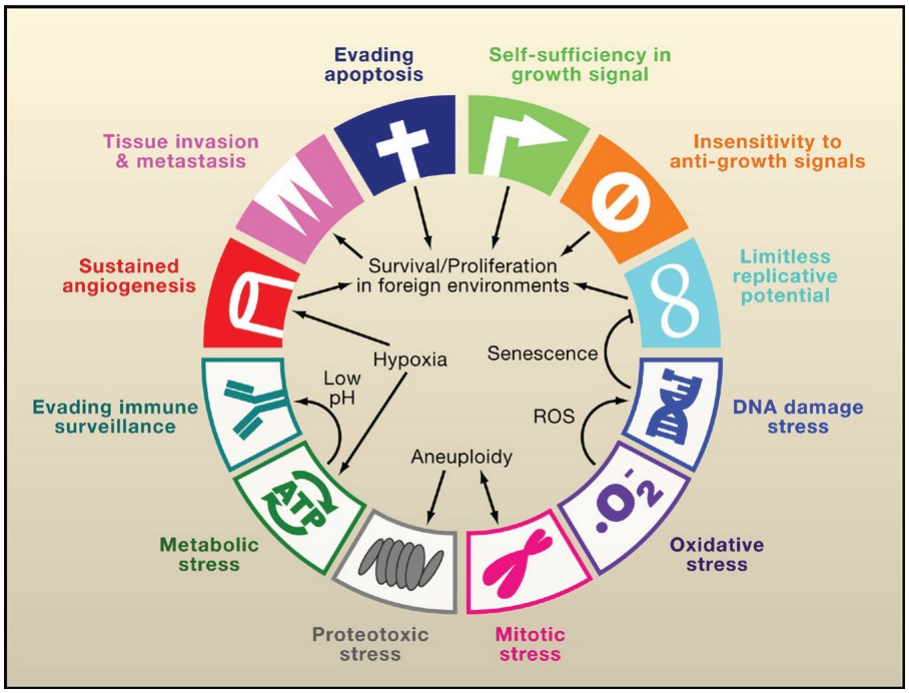


Figure 2: Hallmarks of cancer by Luo *et al.*[11]

- ***Evading immune surveillance***: cancer cells have the ability to inhibit the effects of anti-tumour effectors such as cytotoxic T-lymphocytes or of natural killer cells from its own metabolic micro-environment.[12]
- ***DNA damage and DNA replication stress***: cancer cells can continue to proliferate even in the presence of multiple types of damage such as point mutations, cross-links or mismatches.
- ***Proteotoxic stress***: cancer cells have the ability to survive in the presence of toxic-unfolded or non-destroyed proteins.
- ***Mitotic stress***: cancer cells can proliferate and divide even in the case of acute aneuploidy[a] and chromosome instability.
- ***Metabolic stress***: cancer cells have a higher intake of glucose and higher rate of glycolysis allowing them to survive in low-oxygen and more acidic

[a] Aneuploidy: an abnormal number of chromosomes in the cell.

environments.

- ***Oxidative stress***: cancer cells generate more Reactive Oxygen Species (ROS) than normal ones which will promote DNA damage leading to further transformation.

1.3. Oncogenes and tumour suppressor genes

DNA mutations can lead to the activation or deactivation of two types of genes, oncogenes and tumour suppressor genes (TSG).[13] Oncogenes are genes which encode for a protein whose increased activity or expression can promote oncogenesis such as Src, Myc and Ras.[14,15] Tumour suppressor genes are genes which when deleted or inactivated promote oncogenesis, such as p53, pRb and Ink4a. Only one mutation on the alleles of the chromosomes is necessary to activate an oncogene and to promote cancer, whereas two deletions or mutations (one on each allele) are needed to inactivate a TSG (Figure 3).[16] The activation of an oncogene can be due to:[17]

- ***A deletion or point mutation***: hyperactive protein made in normal amounts;
- ***Gene amplification***: normal protein greatly overproduced;
- ***Chromosome rearrangement***:
    - Nearby regulatory DNA sequence causes normal protein to be overproduced;
    - Fusion of the genes causes new gene to overproduce the fusion protein or the fusion protein produced is hyperactive.

The deactivation of a TSG can occur by the same events of the formation of an oncogene but with the opposite effects.

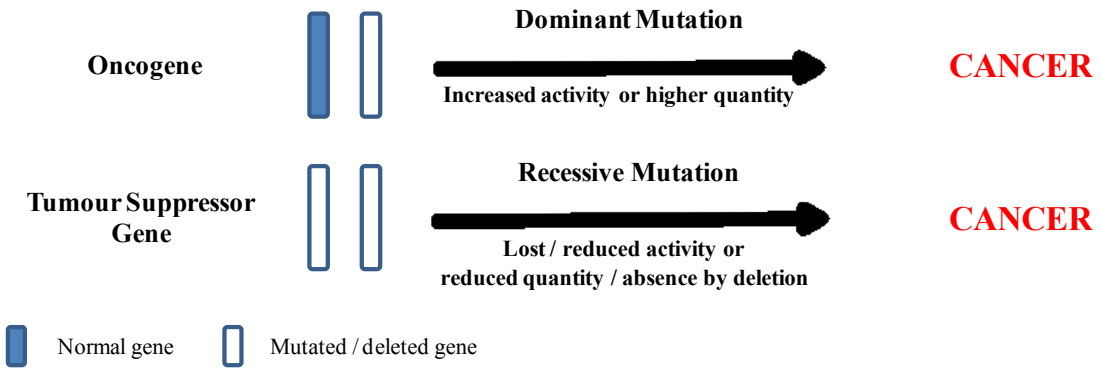


Figure 3: Roles of oncogene and tumour suppressor genes in cancer

1.4. Cell Cycle and cell cycle checkpoints

The cell cycle (Figure 4) is composed of five main phases: G_0 (quiescent state), G_1 (Growth phase), S (DNA Synthesis), G_2 (Growth phase) and M (Mitosis). The transition between each phase is controlled by regulators of the cell cycle, Cyclin Dependant Kinase (CDK)-Cyclin complexes. The transition between each phase is called a "checkpoint" and the evasion or loss of these controls is a characteristic of cancer cells.[3,18]

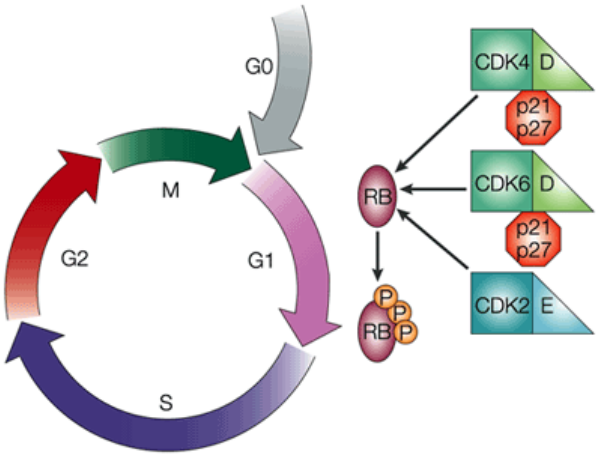


Figure 4: Cell cycle with the restriction point from Coleman *et al.*[19]

- ***G1/S checkpoint or restriction point:*** [18,20] this checkpoint controls the entry into the S phase. It is regulated by CDK-cyclin complexes such as CDK4/6-cyclin D complex, and by CDK inhibitors (CDKI) like p27. Evasion of this checkpoint leads to a continuous proliferation of the cells.[21]
- ***S/G2 checkpoint or DNA damage checkpoint***: loss of this checkpoint leads to an accumulation of genetic damage such as mutations and chromosomal translocation.[22]
- ***G2/M or DNA replication checkpoint:*** [18,22,23] this checkpoint controls the entry of cells into mitosis by verifying the level of duplication of DNA and checks that there is no damage in the form of either DNA strand breaks (single or double) or mutations which may create genetic instability. It is regulated by DNA-break sensor proteins like ATM and by DNA repair proteins such as BRCA1/2. Loss of this checkpoint can lead to loss of genetic material and breakage of DNA.[22]
- ***Exit of mitosis or mitotic spindle checkpoint:*** [18,24] this checkpoint controls the entry of the mitotic cells into anaphase by checking that all the chromosomes are properly attached to the mitotic spindle. It is regulated by Mitotic Arrest Deficient proteins Mad1/2 and proteases such as Separase and Aurora kinases. Problems at this checkpoint can lead to chromosome segregation errors, aneuploidy and chromosomal instability.[24]

When the cells are not growing nor dividing they are in G_0 phase, or quiescent state. Stimulation by growth factors (GF) such as Epidermal Growth Factor (EGF), Platelet Derived Growth Factor (PDGF) or Vascular Endothelial Growth Factor (VEGF), will make the cells move from the G_1 to S phase through the restriction point.

Tumour cells do not rest and can continue even if they do not receive any growth signals.[25]

1.5. Apoptosis

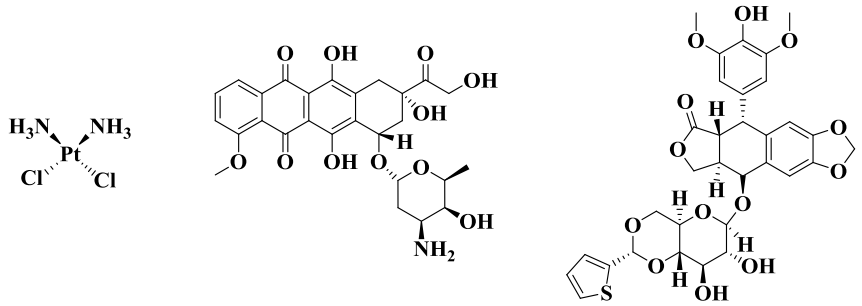
Apoptosis or programmed cell death (PCD) is a cell suicide mechanism that enables organisms to control cell number in tissues and to eliminate cells that threaten their survival. Tumour cells have the ability to evade apoptosis and to survive even if their genetic information is corrupted and cellular DNA is damaged. Apoptosis is different from necrosis which is an unregulated death of the cell after an injury. The cell degrades after mechanical damage or exposure to a toxic reagent. Apoptosis is essential for the development and survival of multi-cellular animals e.g. the formation of toes and fingers in the embryo requires the removal of connective tissues, which happens by apoptosis. Apoptosis is stimulated by positive signals or survival factors (cell detachment, removal of neighbouring cells, removal of growth factors) or by addition of negative signals or death signals (increased level of oxidants in cells, DNA damage, accumulation of unfolded proteins, death activators).[26] Apoptosis is promoted in mammalian cells by:

- the Bcl-2 family[27] which contains the anti-apoptotic proteins Bax, Fas and pro-apoptotic proteins such as Bad, p53 or Puma;
- caspases, initiators and effectors of apoptosis including caspases 2, 3, 8 or 9;
- death receptors and their ligands,[28] the Tumour Necrosis Factors (TNF) Fas or TNF-α;
- inhibitors of apoptosis proteins (IAPs) including cytochrome c or Smac/Diablo released by the mitochondrion.

All the steps of the carcinogenesis described here have been extensively studied and some are not completely elucidated yet. Many studies are still in progress to understand exactly how cancer progresses via these different molecular events in cells. An accurate knowledge of the pathways and possible target enzymes involved in cancer will be essential in the search for new cancer drugs.

1.6. Current treatments of cancer

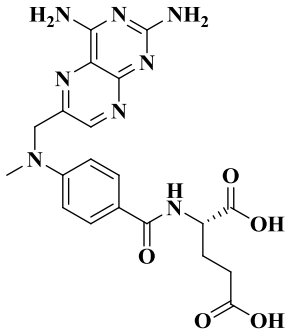
The current approaches to cancer management are surgery, chemotherapy and radiotherapy.[1] Traditional drugs target different enzymes which control the cell cycle or other hallmarks of cancer such as metastasis, angiogenesis or apoptosis. Targeting cell cycle arrest is one of the used approaches to cancer treatment and it is usually carried out by the administration of DNA-damaging drugs such as alkylating agents, platinum compounds such as *cis*-platin[29] or analogues (oxaliplatin)[30] or DNA replication enzyme inhibitors such as doxorubicin[31] or teniposide.[32] Increasing the DNA damage in the cancer cells may make them die by an over-accumulation of damage, especially if the cells have lost the DNA-damage checkpoint and DNA-repair pathways.



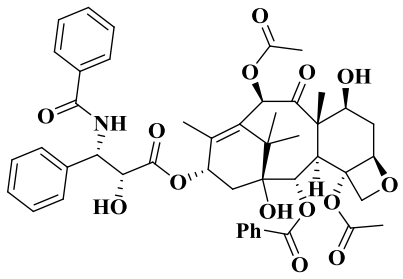
cis-platin Doxorubicin Teniposide

Cell cycle arrest is another way to treat cancer and can be targeted by inhibiting the synthesis of DNA building blocks (e.g. Methotrexate) or by inhibiting the formation

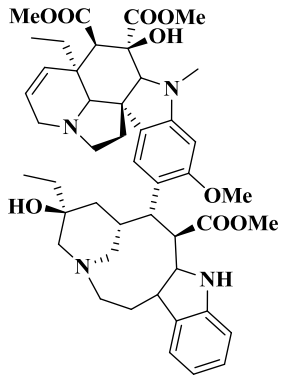
of the daughter cells by stopping the mitosis with anti-microtubules chemotherapeutics such as vinblastine or paclitaxel.[33,34]



Methotrexate



Paclitaxel



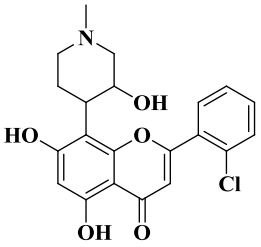
Vinblastine

Generally anti-cancer drugs act on the basis that cancer cells divide and grow more frequently than normal cells. A disadvantage of this feature is that these non-selective drugs are extremely toxic and are linked to numerous serious side effects such as nausea and vomiting, immune suppression and hair loss. These side effects are due to the fact that these chemotherapeutic drugs are not specific and attack “normal” cells as well as cancer cells.

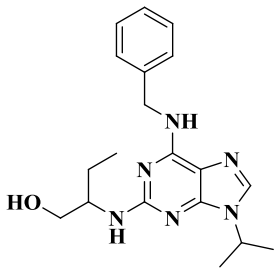
Nowadays, there is a considerable focus on the creation of target-specific-drugs such as the kinase inhibitors, notably on kinases which are linked to the cell cycle, the CDKs. Each cell cycle check point is ruled by specific types of CDKs/Cyclin complexes, inhibition of these complexes may be of great interest for cancer treatment. However, the kinase family contain around 600 members divided in sub-families, which makes the finding of new specific drugs very difficult.

Generally, kinase inhibitors such as flavopiridol, roscovitine or fascaplysin, are small molecules extracted from natural source. More experimental ones such as

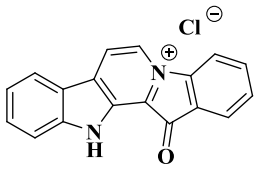
Gleevec (Imatinib), BMS-387032 or PD-0332991 are issued from rational drug designs. They are also generally flat hydrophobic molecules and would inhibit the kinase activity by competing with ATP for the ATP binding site. Many of these drugs are used in clinics and many others are still at the experimental stage or in clinical trials. Numerous analogues of these natural molecules roscovitine, fascaplysin and others have also been synthesised to improve their bioavailability or lower their toxicity.



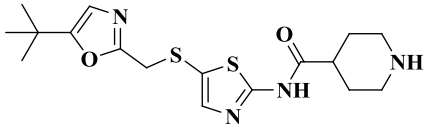
Flavopiridol



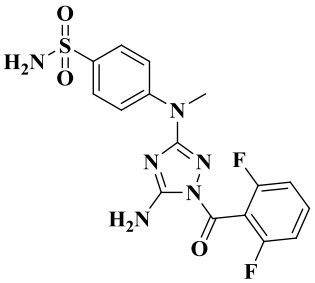
Roscovitine



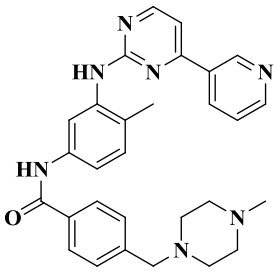
Fascaplysin



BMS-387032



PD-0332991

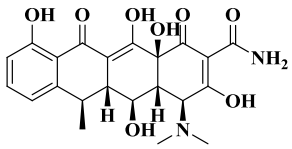


Imatinib (Gleevec©)

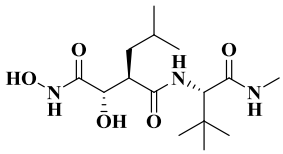
Extensive research in both academic and industrial laboratories is focused on the development of new small molecule drugs which inhibit key controlling enzymes in the cell cycle, metastasis and angiogenesis. Biological antigen agents (like Herceptin[35]) have also been developed and research is underway on finding new specific treatments for a wider range of cancers.[1]

Inhibition of MMPs may be a potential way of stopping metastasis. Natural inhibitors of metastasis are tissue inhibitors of MMP (TIMPs), but synthetic inhibitors

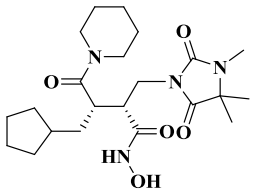
generally contain a chelating agent to chelate the Zn^{2+} ion of the MMPs. So far the only MMP inhibitor used clinically as experimental treatment for cancer is doxycyclin, an antibiotic which is a good MMP inhibitor at sub-antimicrobial doses.[36] Two other broad spectrum MMP inhibitors, marimastat[37] and cipemastat,[38] gave good results during the pre-clinical studies but are not used as drugs because of their toxicity.



Doxycyclin

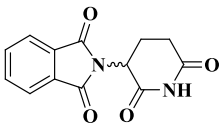


Marimastat

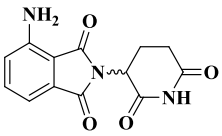


Cipemastat

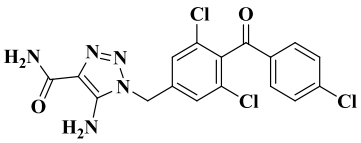
Angiogenesis is naturally inhibited by angiopoietins, and angiostatins (cf. 1.2.). It can also be inhibited by biological analogues of human anti-bodies (benvacizumab)[39], interferons (INF-α Human Type I interferon), platelet factors (Platelet factor-4) and by synthetic small molecules like Thalidomide[40] or a less toxic analogue lenalidomide[41,42] or by carboxyamidotriazole.[43]



Thalidomide



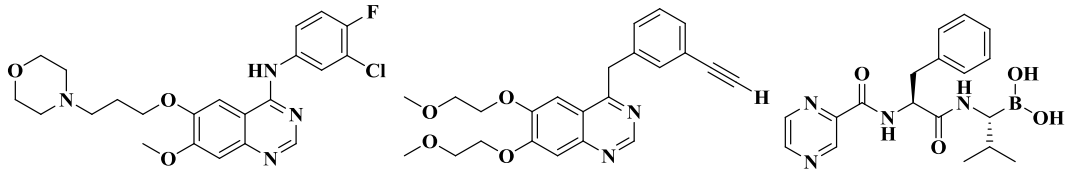
Lenalidomide



Carboxyamidotriazole

Apoptosis of cancer cells can be triggered by different death pathways, by an accumulation of DNA-damage or by stopping the cell cycle like by the inhibition of EGFR (Gefitinib and Erlotinib).[44,45] It can also be induced by inhibition of pro-apoptotic enzymes or by modifying the balance of pro-/anti- apoptotic enzymes. For

example, Bortezomib[46] is used to block proteosome 26S avoiding the degradation of pro-apoptotic enzymes.



Gefitinib **Erlotinib** **Bortezomib**

In summary we can say that cancer is a group of diseases which has extensively been studied and is still under investigation to understand all the possible mechanisms of action and how to fight them. The current treatments by chemotherapy are using natural or synthetic drugs more or less selective to specific targets or types of cancer and are generally creating different side effects. The other current treatments are radiotherapy and surgery.

A possible approach to the treatment of cancer could be chemoprevention from natural products or known synthetic products, and is going to be described further.

2. Chemoprevention of cancer

2.1. Definition

Chemoprevention of cancer can be defined as the use of natural products such as vitamins or phytochemicals[b], or synthetic drugs to prevent, retard or stop the process of carcinogenesis at an early stage.[47] This process can prevent the initiation of cancer, delay its onset and decrease the speed of its progression. It can also reduce the size of the tumour and slow down its growth or even kill tumour cells at the early stages of tumorigenesis.

Chemoprevention can be divided into three levels:[48]

- ***Primary:*** Prevention of cancer in healthy population with high risks of developing cancer. These patients can be people who have a cancer in their family or heavy smokers.
- ***Secondary:*** Prevention of the progression of cancer in patient with pre-neoplastic conditions. These patients generally have the first symptoms of cancer like an adenoma and the disease is at the very early stages.
- ***Tertiary:*** Prevention of the recurrence of cancer in successfully treated cancer patients.

Overall, the risk of cancer can be decreased by identifying carcinogenic agents linked with cancer, and limiting or completely eliminating exposure to them.[47] Population studies around the world have shown variations in the incidences of cancer in different areas. These disparities can be attributed to either genetic or environmental

[b] Phytochemical: naturally occurring compound that is considered beneficial to human health.

differences such as air and water quality, diet and lifestyle (Table 1).[49] Geographical incidences of cancer varies a lot between regions, and some cancers are more prevalent in developing countries (cervical and stomach) whilst some are more common in developed ones (lung, breast, prostate). The maps on Figure 5 show the geographical incidences of cancer worldwide (c.f. appendix).[50] It has been suggested that diet could be responsible of around 35% of cancer mortality,[51] which is the highest cause of the disease after smoking.[1,52] Epidemiological data and clinical trials also suggest that diet can be a source of compounds that protect against cancer.

Site of origin of cancer	High-Incidence Population		Low-Incidence Population	
	Location	***Incidence***	***Location***	***Incidence***
Lung	USA (New-Orleans)	**110**	India (Madras)	**5.8**
Breast	Hawaii	**94**	Israel (Non Jews)	**14.0**
Prostate	USA (Atlanta)	**91**	China (Tianjin)	**1.3**
Stomach	Japan (Nagasaki)	**82**	Kuwait	**3.7**
Liver	China (Shanghai)	**34**	Canada (Nova Scotia)	**0.7**
Colon	USA (Connecticut)	**34**	India (Madras)	**1.8**
Melanoma	Australia (Queensland)	**31**	Japan (Osaka)	**0.2**
Oesophagus	France (Calvados)	**30**	Romania (urban Cluj)	**1.1**
Bladder	Switzerland (Basel)	**28**	India (Nagpur)	**1.7**
Uterus	USA (San Francisco)	**26**	India (Nagpur)	**1.2**
Ovary	New-Zealand (Polynesia)	**26**	Kuwait	**3.3**
Rectum	Israel (US and European)	**23**	Kuwait	**3.0**
Pancreas	USA (Los Angeles)	**16**	India (Poona)	**1.5**
Kidney	Canada (NWT and Yukon)	**15**	India (Poona)	**0.7**

Incidence per 100,000 men aged 35-64 years old per year (uterus, ovary and breast: women)

Table 1: Geography and incidence of cancer adapted from Doll *et al.*[49]

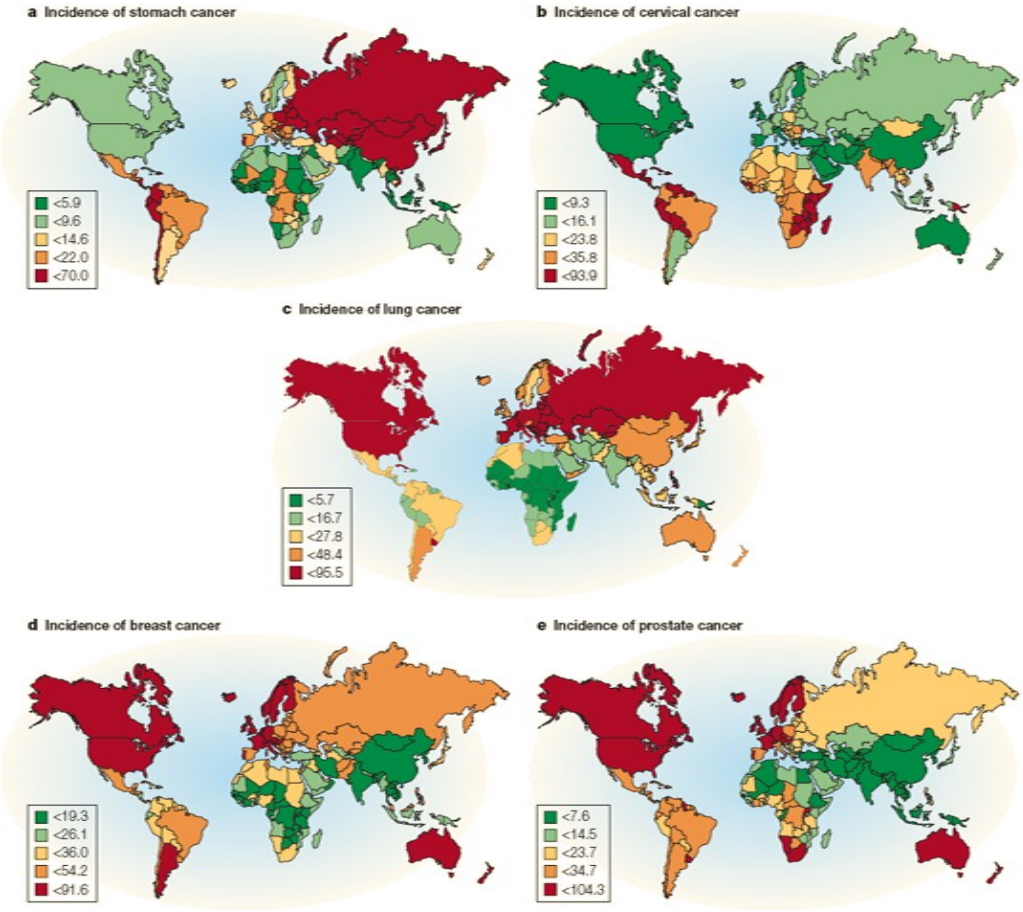


Figure 5: Global variations in cancer incidence for specific cancers from Rastogi *et al.*[50]

2.2. Diet and cancer incidence

Epidemiological studies suggested that different factors like diet could be responsible in the cancer incidence. Diet is generally specific to a geographical region and the religion and way of life in this region. A study carried out between 1970 and 1992 on Japanese populations shows that diet and environment may have an effect on the incidence of cancer. In Figure 6 we see a comparison of different cancer rates among Japanese living in Japan, Japanese living in Hawaii and caucasians living in Hawaii during different period of time.[53] The Osaka population of 1970-71 and 1988-92 have quite low rates of prostate (0.4 to 0.8%) and breast cancer (1 to 2.5%) whereas

caucasians in Hawaii, during the same years, have a high rate of these same cancers (6 to 14% and 9.5 to 11% respectively). On the other hand, the Japanese of Osaka have a very high rate of stomach cancer (7.5 to 12%), whereas Caucasian Hawaiians' is low (0.8 to 2.5%). The important information of this figure is the rate of these cancers in the Japanese population in Hawaii. It could have been supposed that the rates of cancer in these populations should be similar to Japanese of Osaka, but they are in fact very similar to Caucasian Hawaiians'. These results confirm the idea that the incidence is mainly affected by environmental and not genetic factors.

Another study reported recently by Takata *et al.*[54] on the same kind of panel showed that the rate of chronic diseases and cancers of the population studied is really linked to the diet. Indeed, Japanese Hawaiian women have a high intake of grains, meat and carbohydrates and low intakes of eggs and fish. This diet is in between that of Caucasian Hawaiian's (rich in dairy products, fruits, ethanol, vitamins but low in fish, carbohydrates and soy products) and Japanese's in Japan (rich in fish, eggs, vegetables, and soy products but low in fruits, dairy products, grains and meat). These results demonstrate that Japanese who have been living in Hawaii for a period of time tend to have the same cancer incidence as caucasian Hawaiians, which indicates that diet is linked to cancer.[51]

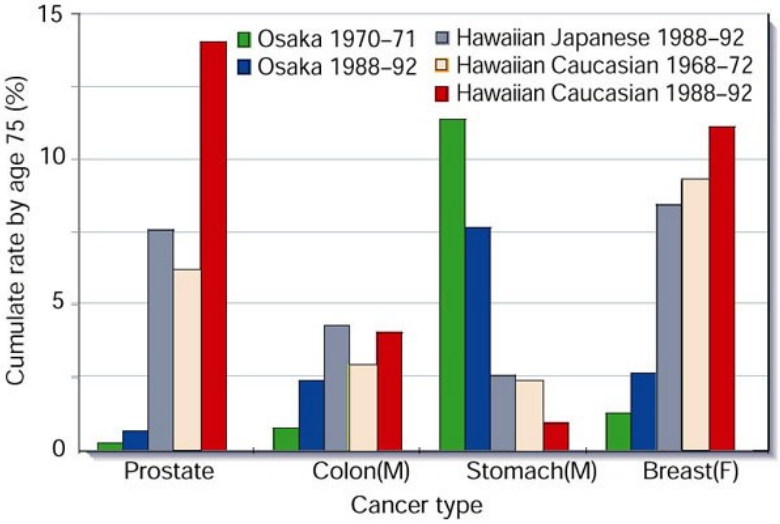


Figure 6 : Comparison of cancer rates between Japanese populations in Japan and in Hawaii taken from Peto *et al.*[53]

2.3. Chemoprevention of cancer from the diet

As explained previously, diet and environmental factors have an impact on the incidence of cancer. Dietary required and non required compounds such as vitamins and minerals or natural products are of interest as potential anti-cancer agents as an alternative to chemotherapeutic drugs.

Epidemiological results encourage research on new chemopreventive agents from the diet and many natural products contained in dietary compounds (Figure 7), mainly phytochemicals, have been found to have anti-cancer activity on all the different steps of cancer. [47] Some of these compounds have been extensively tested in different models of cancer using *in vitro* and *in vivo* systems, and some of these have shown very good activity against numerous pathways involved in carcinogenesis.

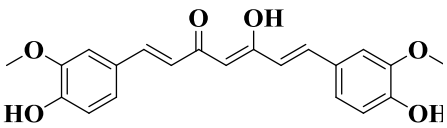
A natural phytochemical which had been extensively tested is resveratrol, a

natural phytoalexin[c] found in red grape skin (*Vinis vitifera*), nut oils and berries, and has been shown to be a very powerful antioxidant. This compound had been believed to be responsible for the "French Paradox", the fact that French populations tend to die less of cardiovascular diseases compared to other western countries despite a diet rich in saturated fats.[55] Resveratrol had been shown to have inhibiting properties on a large number of different pathways of tumorigenesis; its properties will be described and detailed further in this report.

Further studies on these phytochemicals and development of potential drugs from these compounds can be an alternative or a complement to the current treatments of cancer (chemotherapy, radiotherapy and surgery). Education of the population regarding their diet and way of life may also be a way to decrease the impact of cancer in today's society.

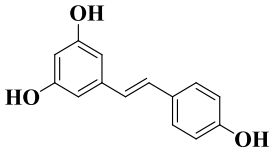
[c] Phytoalexin: antibiotic produced by plants that are under attack.

Turmeric



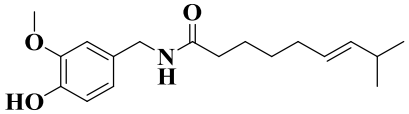
Curcumin[56]

Grapes



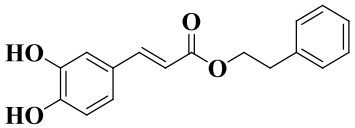
Resveratrol[57]

Chilli Peppers



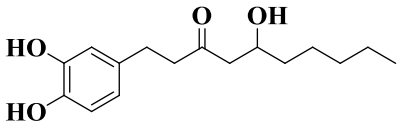
Capsaicin[58]

Honey



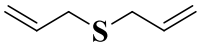
Caffeic acid phenethyl ester[59]

Ginger



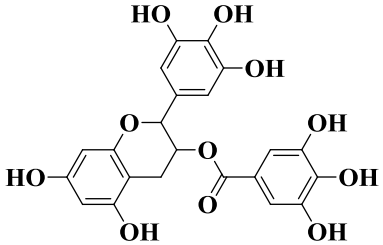
[6]-Gingerol[60]

Garlic



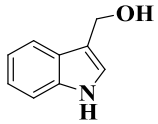
Diallyl sulphide[61]

Green Tea



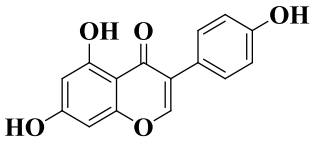
Epigallocatechin-3-gallate[62]

Cabbage



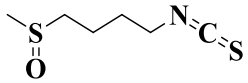
Indole-3-carbinol[63]

Soybeans



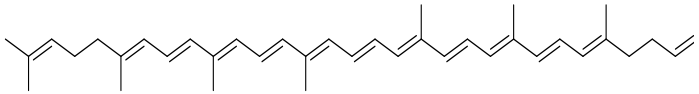
Genistein[64]

Broccolis



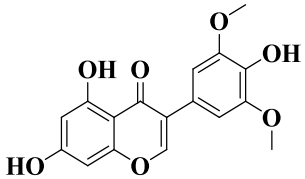
Sulphoraphane[65]

Tomatoes



Lycopene[66]

Rice bran



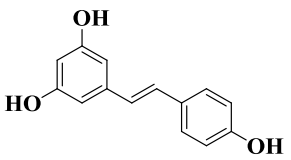
Tricin[67]

Figure 7: Examples of widely studied cancer chemopreventive phytochemicals and their dietary origin

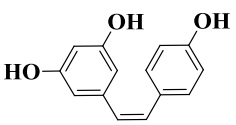
3. Resveratrol and chemoprevention of cancer

3.1. Resveratrol and the French paradox

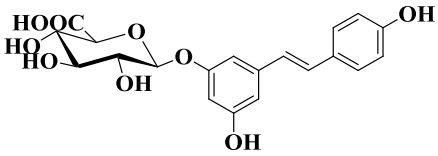
Resveratrol or 3,4',5-*trans*-hydroxystylbene **1** is a natural occurring phytochemical of the family of the phytoalexins produced by spermatophytes[d] such as grapes (v*inis vitifera*) and other common food stuffs such as nuts and berries. It exists in two isomeric forms, the *trans*- **1a** and the *cis*- **1a'** and it is also found in plants in the glucoside form (piceid), resveratrol 3-*O*-β glucoside **2**.



1a



1a'



2

The first published identification of resveratrol occurred in the early 1980s by Arichi *et al.*[68] who found it in Japanese knotweed (*Polygonum cuspidatum*), a dry root commonly used in Japanese and Chinese medicines. This root was used to treat different afflictions such as fungal diseases, skin inflammation and heart disease. In grapes, resveratrol was found to be produced by the plant in large amounts after fungal infection (*Botrytis cinerea*) or exposure to UV light. It acts as an antibiotic in the plants after infection by the pathogen.[69] Resveratrol is produced by the grapevine and contained in the skin of the grapes, it is found in red wine in substantial concentrations, varying from 4 to 20 mg/L depending on the origin of the grapes[69] (Table 2), but in lower concentrations in white or rosé wine. It is thought to be synthesised by the plant

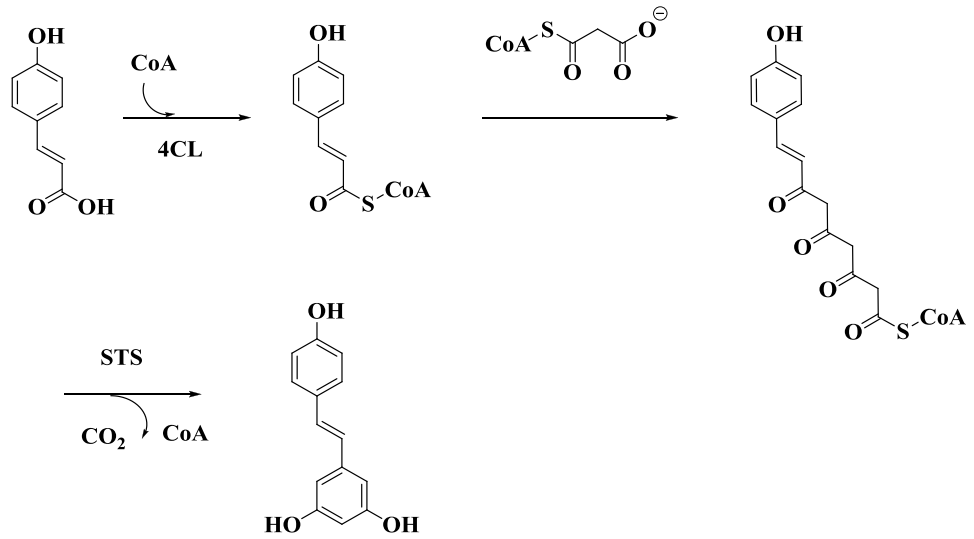
[d]Spermatophyte: Plant which produces seeds

from *p*-coumaric acid through the action of 4-coumarate:coenzyme A (CoA) ligase (4CL), in the presence of malonyl-CoA and the action of stilbene synthase (STS, Scheme 1).[70,71]

Region of origin of the grapes					
Europe			**Others**		
	Resveratrol	Piceid		Resveratrol	Piceid
France	0.3 – 7.1	0 – 14.5	***USA***	0.09 - 3	Up to 42
Italy	0.05 – 0.8		***Japan***	~4.4[b]	~2.5[b]
Spain	2.5 – 13.8[a]		***South America***	~1.8[a]	
Portugal	2.8				
Central Europe	3.1				
Switzerland	6.9				

[a] Resveratrol quantities not specified, [b] mean values

Table 2: Concentration of resveratrol and piceid in red wine in mg/L adapted from Frémont et al.[69]



Scheme 1: Biosynthesis pathway of resveratrol

Besides its antibiotic properties in plants, resveratrol has shown several interesting biological properties, which have been reviewed at different occasions and recently by Brown *et al.*[72], such as prevention and protection against heart diseases[73] thanks to its antioxidant properties, and it was first thought to be responsible of the so

called "French Paradox". The "French Paradox" is based on the observation that French people suffer less from coronary heart disease than other countries, especially the USA and UK, despite eating a diet very rich in saturated fat (which is known to cause heart disease).[74] The French population has a moderate intake of red wine, which contains between 0.4 and 20 mg of resveratrol per litre, which could be the explanation of this paradox. Indeed, heart disease can be originated by oxidised lipids, especially phospholipids created from low density lipoprotein (LDL). This oxidation pathway has been shown to be inhibited *in vitro* by flavonoids (type of antioxidants) contained in red wine including resveratrol and other polyphenols.

Resveratrol not only protects from heart disease but is also a potential cancer chemopreventive compound which acts on the three main steps of carcinogenesis: tumour initiation, promotion and progression.[57] It has also been shown to enhance the action of antiretroviral drugs in HIV treatment in its glucuronide form in *in vitro* human blood cell culture[75] and has been shown to increase the life-span of some species including fish and flies, varying from species to species.[76]

Inhibition by resveratrol of the three main stages of the carcinogenesis: initiation, promotion and progression have been extensively reviewed recently by Kundu and Surh.[77]

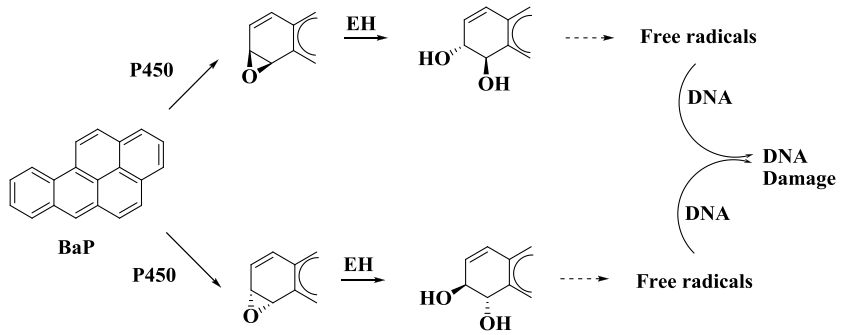
3.2. Anti-initiation activity[55]

Initiation of carcinogenesis can be defined as the step where an accumulation of DNA damage and cellular changes occur leading to the formation of the primary

tumour. Initiation is generally induced by carcinogens like free radicals or chemical carcinogens coming from external factors such as cigarette smoke or pollution.

Resveratrol is an anti-initiator of carcinogenesis. It has the ability to inhibit the activation of carcinogens like for example polyaromatic hydrocarbons (PAH) to free radicals and reactive oxygen species. PAH such as benzo[a]pyrene (BaP) or 7,12-dimethylbenz[a]anthracenes (DMBA)[57] are metabolically activated by cytochromes P450 super-family to highly mutagenic diol-epoxy derivatives and liberate free radicals which are responsible for oxidative damage to DNA and leading to cellular damage (Scheme 2).

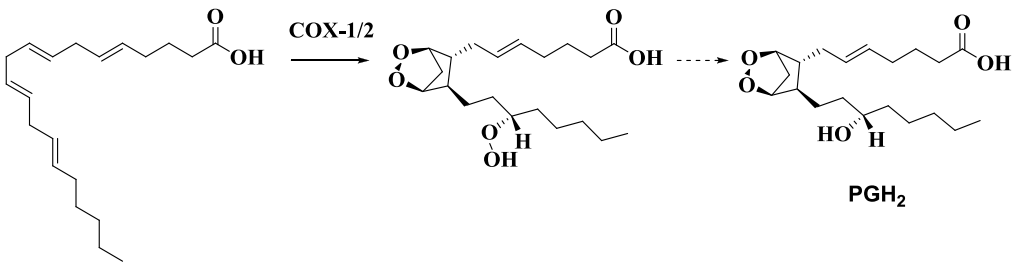
In addition to its properties of inhibition of carcinogen activation, resveratrol blocks the transcription activation and activity of phase I enzymes such as CYP450. It also induces phase II enzymes, such as quinone reductase, which are able to metabolically detoxify carcinogens (review by Savouret et *al.*[55]).



Scheme 2: Simplified schematic activation of BaP by cytochrome P450 and DNA damage formation. Taken from Fang *et al.*[78]

3.3. Anti-inflammatory and anti-promotion activity: inhibition of COX-1 and COX-2 activity

The enzyme cyclooxygenase (COX) also called prostaglandin H synthase is responsible for the formation of numerous biological mediators such as prostaglandins (PGs) which are thought to be involved in carcinogenesis.[79] COX is responsible for inflammation and pain and can stimulate tumour cell growth by catalyzing the formation of prostaglandins from arachidonic acid (Scheme 3) *via* cyclooxygenase and hydroperoxidase reactions. Different COXs exist but only COX-1 and COX-2 have been shown to have an effect on cancer promotion. COX-1 is the constitutive form and COX-2 is the inducible one associated with responses such as inflammation.[80] COX-1 and COX-2 are commonly found in certain regions of the brain, kidneys and in cancerous tissues (especially COX-2). The prostaglandins are one of the responsible for the tumour growth by increasing the cell proliferation and promoting angiogenesis.



Scheme 3: PGH_2 synthesis from arachidonic acid

COXs are generally inhibited by non-steroidal-anti-inflammatory-drugs (NSAIDs) like aspirin, indomethacin, piroxicam or sulindac. Resveratrol has been shown by Pezzuto *et al.* to inhibit cyclooxygenase activity of COX-1 with a relatively high efficient dose at 50% (ED_{50}) of 15 μM and the hydroperoxidase activity with an

ED_{50} of 3.7 μM.[57] Resveratrol also seems to inhibit the hydroperoxidase activity and over-expression of COX-2. Indeed, resveratrol suppresses the 12-myristate-13-acetate (PMA)-mediated activation of COX-2 transcription by interfering at different levels in the Protein Kinase C (PKC) pathway. All these results strengthen the hypothesis that one of the mechanisms through which resveratrol acts as a chemopreventive agent is *via* inhibition of COX-1 and COX-2 enzymes. Other oxidative enzymes or pro-inflammatory enzymes such as nitric oxide synthase, which have a link with the promotion and progression of cancer have been shown to be inhibited by resveratrol.[77]

3.4. Effect on the cell cycle and apoptosis

The three steps of the carcinogenesis are all linked to the accumulation of DNA damage, over-expression and/or deregulation of pro-carcinogenic enzymes. Arrest of cell cycle at the different phases is one of the strategies to stop the progression of cells. Increasing specific apoptosis and inhibiting the angiogenesis are also possibilities to stop the progression.

3.4.1. Cell cycle arrest

Down-regulation of the CDK/cyclin complexes and/or up-regulation of CDKI is one of the approaches to stop the cell cycle. Arrest at the different stages of the cell cycle can allow the cells to repair the DNA or go into apoptosis if there is too much damage.

Resveratrol has been shown to have important effects on cell cycle arrest at the S phase (DNA replication) through the G2/M transition in human histiocytic lymphoma

U937 cells.[81] Resveratrol can block the S/G2 transition in a concentration dependant manner (30-60 μM) but not with higher concentrations (90-120 μM) in those cells. This fact is highlighted by the accumulation of cyclins E and A, cyclins involved in the G_1/S checkpoint. The authors also showed that this process of cell cycle arrest in S phase was reversible after 18h. Cells were incubated with resveratrol for 12h after which time there was an observed decrease in cyclin A and E concentrations (cyclin E at the minimum after 6h).

In bovine pulmonary endothelial cells, the S/G_2 transition arrest is due to an increase of p53 and p21 levels (subsequently to p53 up-regulation) as shown by Hsieh *et al.*,[82] which is not the case of U937 cells. Hsieh *et al.* also showed that those same cells treated with resveratrol with high levels of p53 and p21 induced apoptosis.

3.4.2. Pro-apoptotic effects

Apoptosis is regulated by many different factors such as p53 (transcription factor and guardian of the genome) which can induce apoptosis, or by others like the Bcl-2 family which contains pro- and anti-apoptotic factors. Enhancing apoptosis in cancer cells can be done by either up-regulating pro-apoptotic proteins (Bax, Bid, Bad and Bak) or down-regulating/inhibiting anti-apoptotic ones (Bcl-2).

Ahmad *et al.*[83] showed that resveratrol inhibited the proliferation and the viability of human prostate cancer LNCaP by causing apoptosis but they also showed that resveratrol did not cause apoptosis in normal prostate HPEC cells, even with a high concentration of 50 μM. They showed that resveratrol was up-regulating the levels of pro-apoptotic proteins and down-regulating Bcl-2. Their results also illustrated that resveratrol inhibited the PI3K and Akt levels, upstream proteins of Bcl-2 and Bcl-2

family. Very recently, Juan *et al.*[84] proved that colon cancer cells (HT-29) had a reduced proliferation rate after exposure to 10-300 μM of resveratrol with a half maximum effect at 80 μM. They also indicated that resveratrol increased caspase-3 activity with a minimum concentration of 100 μM, which led to complete apoptosis of the cancer cells. Resveratrol has many chemopreventive properties as illustrated above and other studies on resveratrol targets and activity in the different steps of cancer are in progress.

3.5. Other effects linked to the inhibition of carcinogenesis

3.5.1. Inhibition of transcription factors

Transcription factors are important in the regulation of genes which control cell proliferation and cell survival when they are active such as the nuclear factor κ-B NF-κB or activator protein AP-1, two enzymes generally found in cancerous cells. These transcription factors are involved in tumorigenesis by trans-activating pro-inflammatory, anti-apoptotic and cell cycle genes.[77] Resveratrol has activity against NF-κB by diminishing the nuclear level of NF-κB and decreasing IκB (inhibitor of NF-κB) proteosomal degradation.[80] It induces apoptosis in human breast cancer MCF-7 cells[85] and in both rat and human pancreatic carcinoma cell lines.[86]

3.5.2. Anti-angiogenic effect

Angiogenesis is the formation of new capillaries from existing blood vessels and is very important in carcinogenesis as it allows the tumour access to nutrients and oxygen. The formation of these capillaries involves destruction of the extra-cellular matrix and their adhesion to the matrix is helped by different anchoring molecules such

as β-catenin or vascular endothelial (VE)-cadherin.

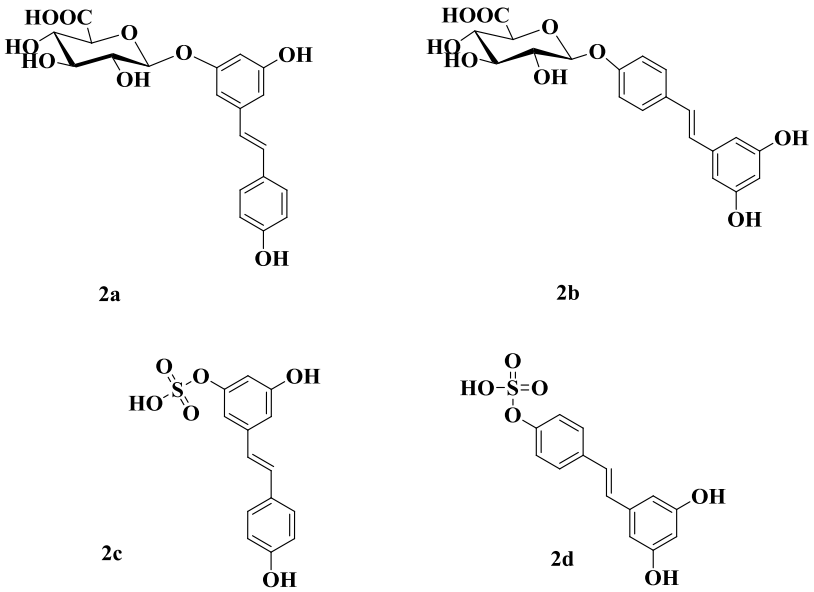
VE-cadherin forms a complex with catenins and plays a role in cell-cell contact. To perform its role completely it needs a phosphorylated tyrosine which can be achieved by Vascular Endothelial Growth Factor (VEGF). Lin *et al.*[87] showed that 1 and 2.5 μM of resveratrol could effectively block VEGF-mediated migration of the capillary tubes but not the cell proliferation. Resveratrol at the same concentration also abrogated VEGF-mediated tyrosine phosphorylation of VE-cadherin and β-catenin. Cao *et al.*[88] showed that at 40 μM of resveratrol the number of capillary tubes formed in *ex vivo* and *in vivo* rat aorta models decreased significantly compared to the control group. These results show that resveratrol has an effect in the inhibition of angiogenesis.

In summary, resveratrol interacts with different enzymatic pathways linked to the carcinogenesis in a dose dependant manner. It stops the initiation of cancer by scavenging free radicals; it stops the promotion by inhibiting PGH_2 synthesis by inhibition of COXs, effect coming from its anti-oxidant properties. It allows cell cycle arrest at the S/G_2 phase by interacting with CDK/cyclin levels. It also induces apoptosis by up-regulating pro-apototic enzymes, such as Bcl-2. However, these interesting biological properties of resveratrol are down-regulated *in vivo* by the low bioavailability and fast metabolism.

3.6. Metabolism and bioavailability of resveratrol

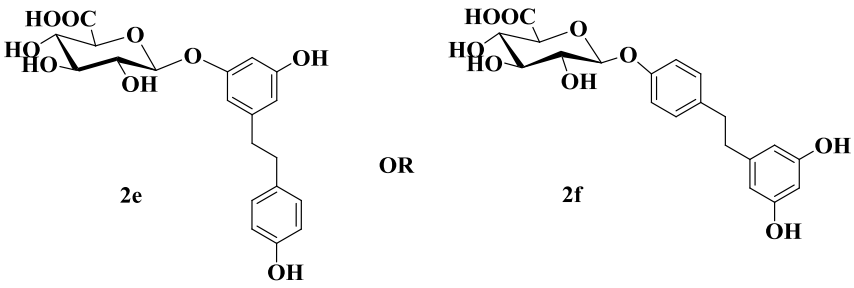
3.6.1. Metabolism of resveratrol

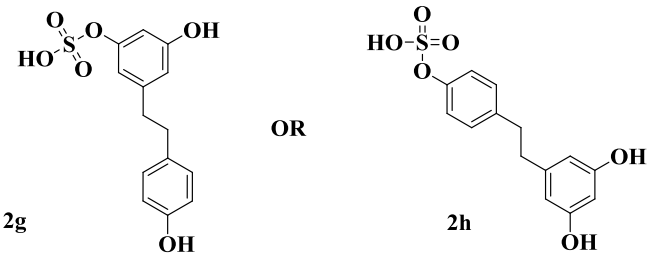
Resveratrol had been shown to be highly absorbed and rapidly and extensively metabolized through glucuronidation or sulfation by the intestine or liver in human, mouse and rat systems.[89,90] The main metabolites found come from phase II metabolism. Two glucuronide forms exists, the resveratrol-3-*O*-β-glucuronide **2a** and the resveratrol-4'-*O*-β-glucuronide **2b**. There are also two sulfate metabolites of resveratrol, resveratrol-3-*O*-sulfate **2c** and resveratrol-4'-*O*-sulfate **2d**.[91] Very recently our laboratory identified a glucuronide-sulfate metabolite and a disulfate in human colorectal tissues.[92]



No phase I metabolites resulting from oxidation, reduction or hydrolysis are generally found in the different systems studied. Walle *et al.*[93] surprisingly found a mono-glucuronide dihydroresveratrol **2e** or **2f** and a monosulfate dihydroresveratrol **2g** or **2h** as metabolites of resveratrol after giving 100 mg of resveratrol to one human

subject (Figure 8). These compounds were detected by LC/MS/UV in urine samples taken 12h after administration but their relative quantities could not be measured accurately.





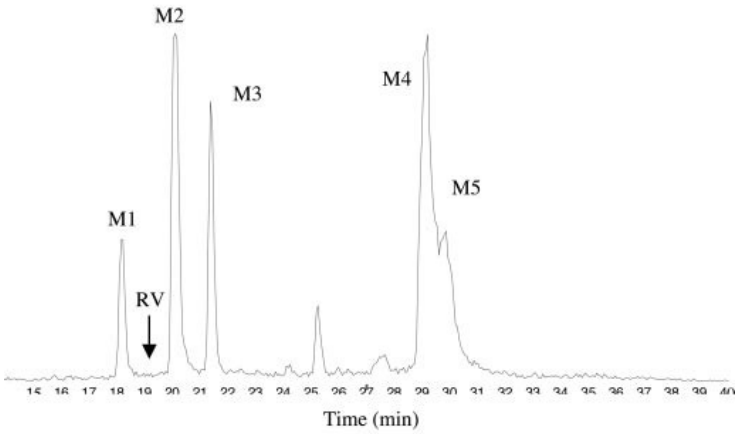
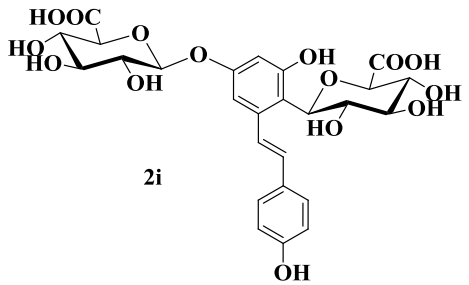
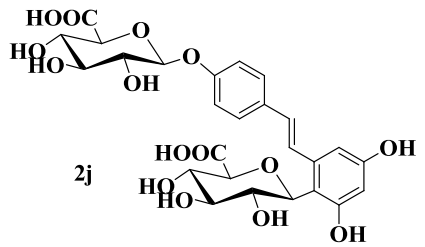


Figure 8: LC/MS tracing of urinary excretion of resveratrol (RV) metabolites (M1-M5) after 100mg unlabeled oral dose (0- to 12-h urine). Taken from Walle et al.[93] MS detection of the $[M-1]^{-1}$ ions for RV (*m/z* 227), M1 and M2 (*m/z* 403) are the glucuronides **2a** and **2b**, M3 (*m/z* 405) is a dihydroresveratrol glucuronide **2e** or **2f**, M4 (*m/z* 307) a monosulfate **2c** or **2d** and M5 (*m/z* 309), a dihydroresveratrol monosulfate **2g** or **2h**.

A very recent study of Burkon[94] indicated two other possible metabolites of resveratrol found in human plasma after giving 85.5 mg per 70 kg body weight of resveratrol to the volunteers. They found the classical metabolites described above but also two new ones, trans-resveratrol-*C/O*-diglucuronides **2i,j**. To confirm the structures and nature of the new metabolites, they used LC-MS/MS, HPLC-DAD and NMR after synthesising them from resveratrol and four equivalents of acetobromo-α-D-glucuronic acid methyl ester following Wenkel's *et al.*[95] method.



***trans*-resveratrol-2-*C*-β/5-*O*-β-diglucuronide**



***trans*-resveratrol-2-*C*-β/4'-*O*-β-diglucuronide**

3.6.2. Bioavailability of resveratrol

Bioavailability can be defined as the degree to which a compound/nutrient becomes available to the systemic circulation or the target tissue after administration. It can be determined by knowing the absorption, distribution and metabolism of the studied product.[90] The amounts of sulfate and glucuronide metabolites found in human, rats or mice are not the same and depend on the administrated dose and the administration way (intravenous (i.v.), intraperitoneal (i.p.) or gavage).

Breemen *et al.*[91] showed that resveratrol-3-*O*-sulfate **2c** was found in human hepatocytes after 4h incubation but seemed to be a minor metabolite compared to the formation of 3-*O*- and 4'-*O*-glucuronides **2a** and **2b**. In the same study, the most

abundant metabolite in rat hepatocytes was resveratrol-3-*O*-sulfate **2c**, but in rat urine after 2h, 3-*O*-glucuronide **2a** was the only metabolite found. When 20 mg/kg of resveratrol **1a** was given to mice through i.p. injection or gavage, blood samples were taken at regular intervals up to 4h. 3-*O*-Glucuronide **2a** (5 μM), the 3-*O*-sulfate metabolites **2c** (13 μM) were detected in the serum after 15 min along with traces of unconjugated resveratrol **1a**. Furthermore, after 1h neither resveratrol nor metabolites were detected in the blood samples (Figure 9A). Surprisingly, when the tests were carried out at a dose of 60 mg/kg by gavage, the same metabolites were detected 30 min after administration instead of 15 min (at 20 mg/kg). After 3h no more free resveratrol **1a** was detected but the metabolites were still present at very low levels (Figure 9B).

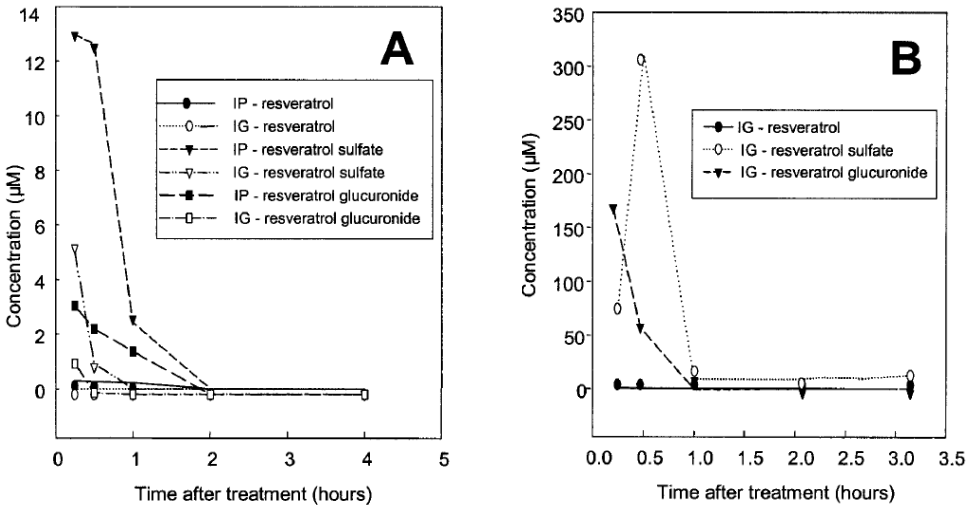


Figure 9: Concentration of resveratrol metabolites in mouse serum following doses of (A) 20 mg/kg via i.p. injection or gavage (IG) and (B) 60 mg/kg via IG administration. Taken from Yu *et al.*[91]

A recent phase I dose escalation pharmacokinetic study was performed on healthy volunteers to consider the safety of high-dosage resveratrol intake and assess the plasma concentration attained.[96] Volunteers took orally one dose of resveratrol at

0.5, 1.0, 2.5 or 5 g, then blood, urine and faecal samples were taken over a 24h-time period. The study showed no serious adverse effects associated with taking resveratrol at high dosage, indicating a single dose was safe. It also revealed the presence of six metabolites (detected by LC/MS/MS) quickly formed after administration. The major metabolites were resveratrol-3-*O*-sulfate **2c** and two mono-glucuronides (3-*O*- and 4'-*O*-) **2a** and **2b** and the minor ones were 4'-*O*-monosulfate **2d**, a disulfate, and a glucuronide-sulfate (Figure 10).[97]

Free resveratrol disappeared very quickly from the plasma with the highest concentration observed between 0.83 and 1.5h post-dose, depending on the dose administered (Figure 11). The major metabolite formed was resveratrol-3-*O*-sulfate **2c** with the highest mean average (172-1089 ng/mL or 0.56-3.5 μM) and peak plasma concentration (1135-4294 ng/mL or 3.7-14 μM) nearly 3-fold the two glucuronide metabolites concentrations.[97] The analysis of the levels of resveratrol and its metabolites in urine and feces indicated that, depending on the dose administered, between 0.5% and 11.4% of the dose administered was excreted from the body within 24h with 77% of this amount being excreted within the first 4 hours. The levels of free resveratrol recovered in the urines and feces were below 0.04% of the dose.[96]

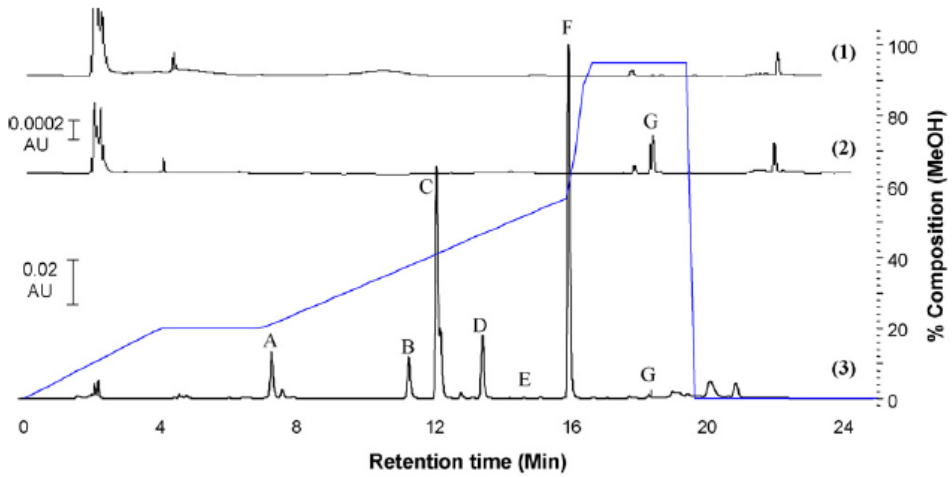


Figure 10: UV-HPLC chromatograms of human plasma samples with or without resveratrol. Taken from Boocock *et al.*[97] (1) Blank plasma, (2) plasma spiked with resveratrol (10 ng/mL) and (3) human plasma sample 1 h post-oral dosing with 1 g resveratrol showing: resveratrol (G) and its major conjugated metabolites. Putative identification by LC-MS/MS: (A) sulfate-glucuronide, (B and D) mono-glucuronides, (C) disulfate and (E and F) mono-sulfates.

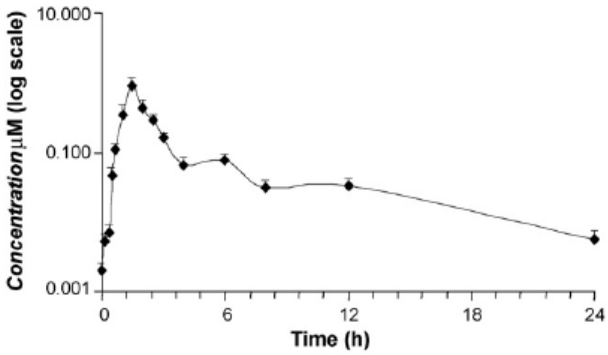


Figure 11: Plasma concentration versus time profile showing levels of resveratrol in human plasma after a single oral dose of 1 g. Taken from Boocock *et al.*[97]

Vitrac *et al.*[98] investigated the bioavailability of resveratrol in male Balb/c mice by administrating ^{14}C-labelled resveratrol. Their study indicated that radio-labelled resveratrol was found in the stomach, liver, kidneys, intestine, bile and urine. They also showed that free labelled-resveratrol and the corresponding phase II metabolites were

also detected in those same tissues.

A very recent report by Hoshino *et al.*[99] describes the selective synthesis of all the possible mono-, di- and tri-sulfate metabolites of resveratrol and shows the assessment of these five metabolites on known targets of resveratrol. For example, 3-*O*-sulfate exhibited better quinone reductase 1 induction (with an IC_{50} of 2.6 ± 0.38 μM) compared to 21.0 ± 0.46 μM for resveratrol as well as a slightly better inhibition of COX-1 (IC_{50} of 3.60 ± 0.8 μM vs. 6.65 ± 2.5 μM) and similar free radical scavenging (68.0 ± 1.9% inhibition vs. 65.2 ± 2.0%). The 3'-*O*-, 4-*O*- and 4'-*O*-sulfates were evaluated on the same targets and show some activity as well. However, the team also demonstrated that these sulfates do not display significant anti-proliferative properties compared to resveratrol on KB cells (nasopharyngal cancer) and MCF7 cells (breast cancer).

In summary, resveratrol disappears very rapidly after administration (oral, i.p. or i.v.) and is metabolised within an hour in a dose dependant manner in sulfate and glucuronide metabolites. The main metabolites formed in both human and rat are the 3-*O*-sulfate and 3-*O*-glucuronide resveratrol and their regio-isomers. Other metabolites like *O*-disulfates, *C/O*-diglucuronides and dihydroresveratrol-*O*-sulfates and dihydroresveratrol-*O*-glucuronides. Numerous studies have been carried out to evaluate these metabolisms, and some others are in progress to evaluate whether these metabolites have any role in resveratrol chemopreventive properties. Numerous analogues have been synthesised and their properties on enzyme inhibition and cancer cell proliferation have been evaluated and will be detailed further in this report.

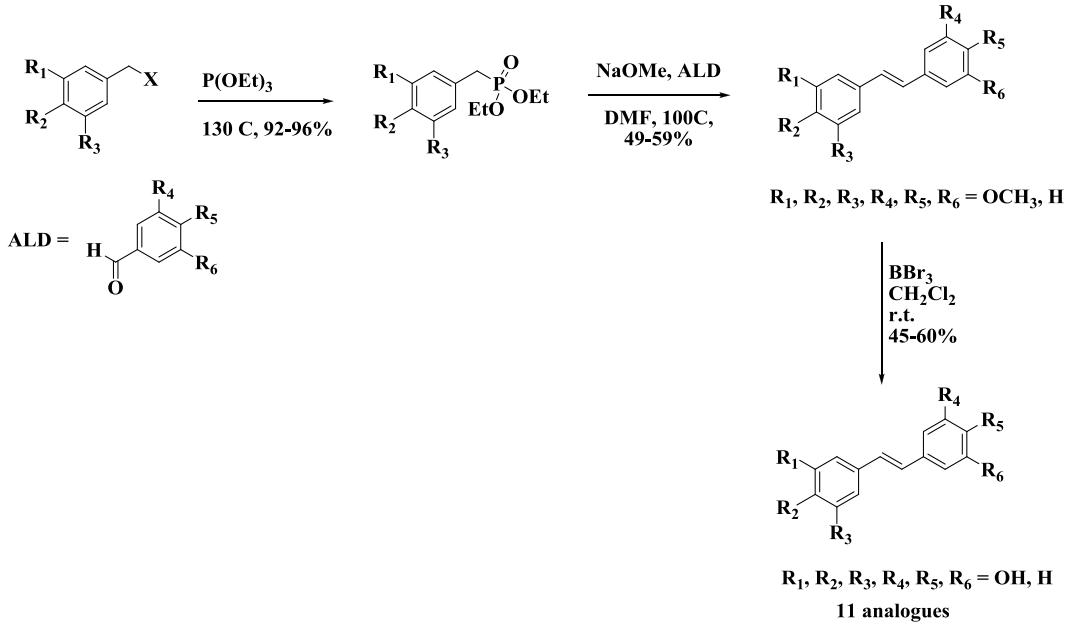
4. Analogues of resveratrol

Numerous studies have been carried out in the last decade involving the synthesis of new resveratrol analogues to improve the activity of resveratrol against known protein targets such as the CYP 450 family,[100,101] COX-1 and COX-2[102-106], NF-κB,[107,108] and tubulins[109-113], as well as enhancing the cytotoxicity and anti-proliferative effects on various cancer cell lines.[113-118] Different approaches have been taken, either by starting with resveratrol itself and its natural analogues,[101,102,104] like combrestatin A and piceatannol, or by developing new strategies to synthesise new series of analogues.[100,103,107-109,111,112,114-118] The structural variations on the different analogues are generally in the number of hydroxyl or methoxy substituents on the rings or the introduction of a different structure, linker or substituent.

4.1. Synthesis the analogues

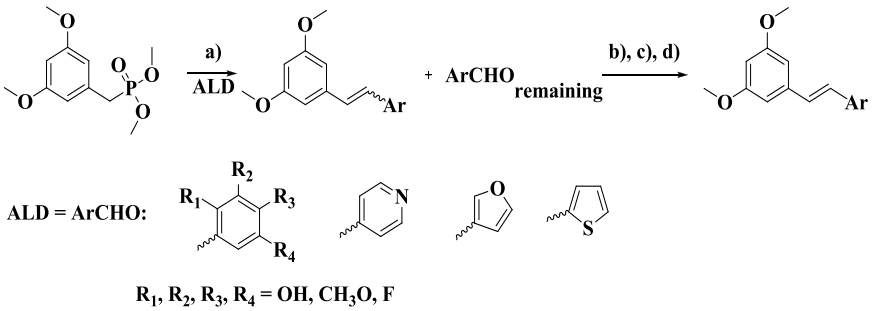
Most of the resveratrol analogues assessed on different biological targets were synthesised using the Wittig or Wittig-Horner-Emmons reaction to produce the *trans*-double bond. The Wittig reaction is the reaction between a phosphonium ylide and an aldehyde. Depending on the substituents on the ylide, a mixture of *cis*- and *trans*-double bonds products is obtained. The Wittig-Horner-Emmons reaction is the reaction between a phosphonate and an ester and generally gives the *trans*-double bond, especially if the phosphonate is linked to an electron-withdrawing group.

The difference between all the analogues is mainly in the number of hydroxy[109,119,120] and methoxy groups on the different rings (Scheme 4).[103,117]



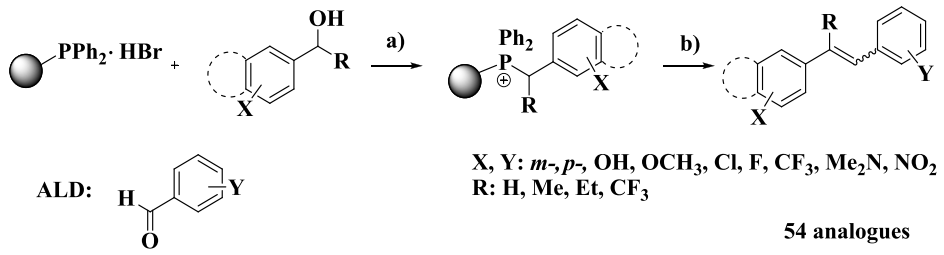
Scheme 4: Wittig-Horner-Emmons reaction by Murias *et al.*[103]

Other groups used either a Wittig/Wittig-Horner-Emmons reaction or a Heck coupling[112,121] to add different rings such as pyridines, pyrroles, thiophenes (Scheme 5)[100] or benzene rings with different substituents including fluorine,[112] nitro groups or anilines[114] by "normal" or by solid-phase synthesis (Scheme 6).[107,108]



a) **ALD**, KOH, CH_2Cl_2, 18-crown-6; b) Girard's reagent T, AcOH, CH_2Cl_2; c) cat. I_2, Heptane; d) $NaHSO_3$

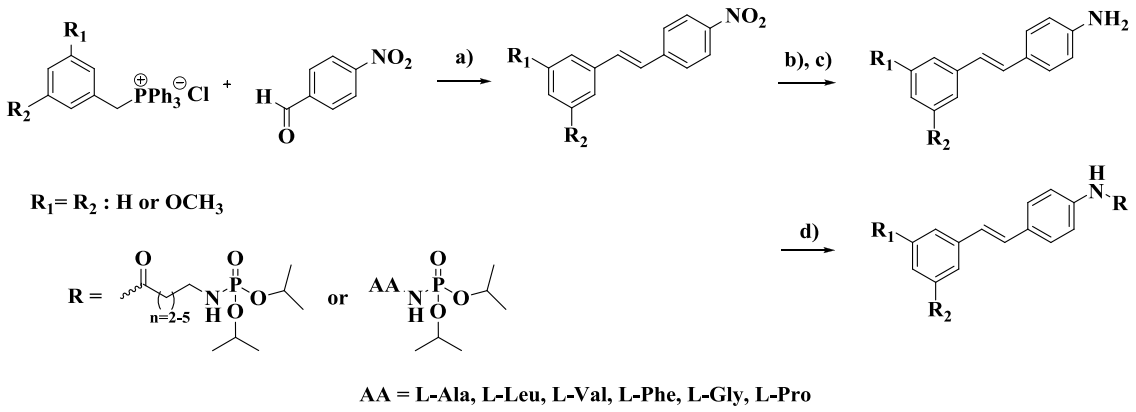
Scheme 5: Wittig-Horner-Emmons reaction by Kim *et al.*[100]



a) $CHCl_3$, 65 C, 8h; b) NaH, **ALD**

Scheme 6: Solid-phase synthesis leading to 54 analogues by Kal *et al.*[107]

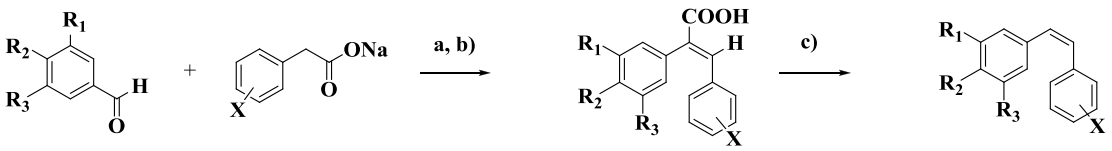
A substitution of the hydroxyl group in the 4'-position of resveratrol with an amine permitted Liu *et al.*[114] to add amino acids and to create N-phosphorylaminoacid derivatives of resveratrol (Scheme 7).



a) NaH, CH_2Cl_2, 0°C, 24h; b) I_2, $CHCl_3$,reflux, 24h; c) Zn, AcOH, r.t, 2h; d) HOBt, DCC, THF, r.t., 24h.

Scheme 7: Synthesis of phosphorylamino acid derivatives of resveratrol by Liu *et al.*[114]

Another group of analogues are based on natural analogues *cis*-resveratrol and combrestatin A. Solladié *et al.* described a modified Perkin reaction between benzaldehyde and diverse 2-phenyl acetic acid sodium salts, giving them aryl cinnamic acids and *cis*-resveratrol analogues after decarboxylation (Scheme 8).[122]

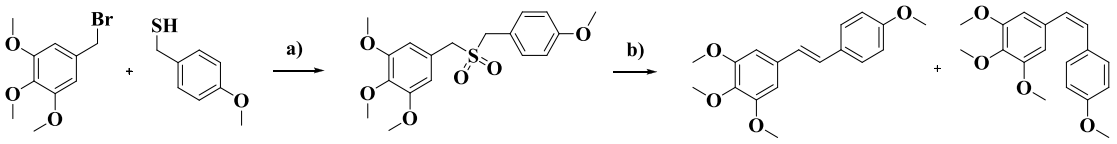


R_1, R_2, R_3 = OMe, OiPr, OH, H
X = o-, m-, p- H, OMe, OiPr, OH

a) TEA, Ac_2O, reflux, 15h; b) Deprotection: BCl_3, DCM, -78°C; c) $CuCr_2O$ – $BaCr_2O$/Quinoline, 230°C

Scheme 8: Formation of aryl-cinnamic acids and *cis*- resveratrol analogues by Solladié *et al.*[122]

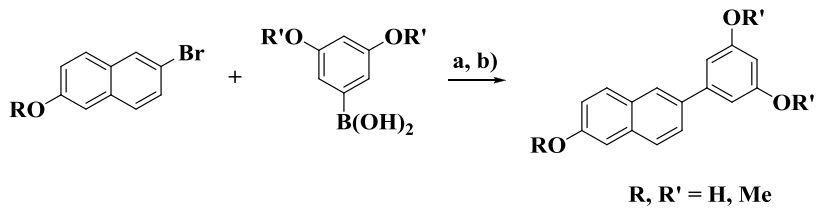
Robinson and Taylor synthesised both *cis*- and *trans*-stilbenes as analogues of Combrestatin A-4, an inhibitor of tubulin polymerisation, by a Ramberg-Bäcklung reaction (Scheme 9). They obtained different isomeric ratios of E:Z stilbenes depending on the conditions used, but obtained the *trans*- isomer as the main product.[123]



a) i)KOH, EtOH; ii) mCPBA, $NaHCO_3$, CH_2Cl_2; b) CCl_4, *t*-BuOH, KOH, H_2O → E: Z 42: 5

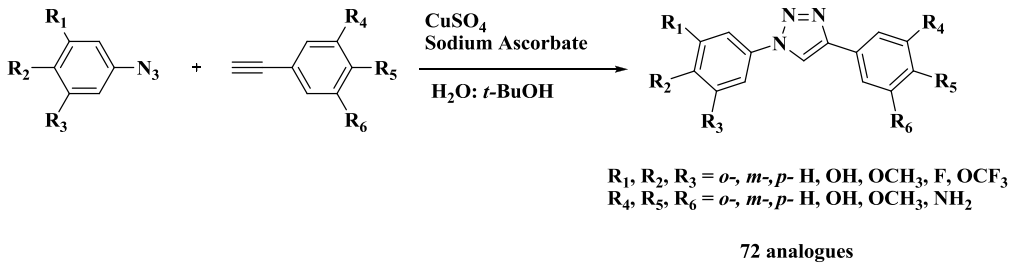
Scheme 9: Ramberg-Bäcklung reaction by Robinson *et al.*[123]

A third class of analogues was synthesised in which the double bond is replaced by another functionality. Minutolo *et al.*[116] synthesised analogues where they replaced the double bond by a naphthalene ring in order to overcome the metabolic and UV-instability of the *trans*-stilbene double bond. They synthesised four hydroxyl and methoxy-analogues by a Suzuki Pd-catalysed cross-coupling reaction (Scheme 10). Paglai *et al.*[115] synthesised a combinatorial-72-analogue library by using copper (I) click chemistry with the aim of establishing whether the double bond of resveratrol is amenable to bioisosteric substitution with the triazole moiety (Scheme 11).



a) $Pd(PPh_3)_4$, K_3PO_4 (aq), dioxane, 80°C, 16h; b) BBr_3, CH_2Cl_2, -78°C, 3h

Scheme 10: Suzuki reaction to form naphthalene analogues of resveratrol by Minutolo *et al.*[116]



Scheme 11: Click Chemistry synthesis by Paglai.[115]

4.2. Biological properties of the analogues

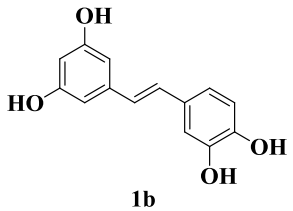
These synthetic analogues were assessed on different biological systems but most of them were designed to improve the inhibition of cancer, either on the enzymatic level or on cell proliferation inhibition and cytotoxicity. Some of the enzymatic and cell proliferation inhibition assays done on these analogues are described further.

4.2.1. Enzymatic assays

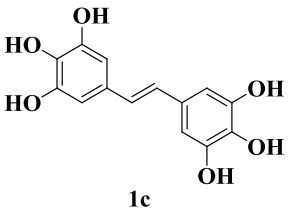
4.2.1.1. COX-1 and COX-2 Inhibition

Cyclooxygenases 1 and 2 (COX-1 and COX-2) are induced during the inflammatory response and transform arachidonic acid into prostaglandins PG.

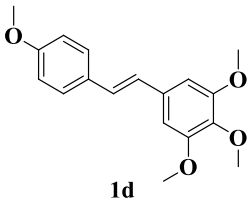
Increased levels of COX-2 and prostaglandin E-2 have been implicated in the development of cancer.[124] Murias *et al.*[103] studied hydroxylated and methoxylated analogues of resveratrol and piceatannol to improve the activity against COX-1 and COX-2 and the selectivity between them, and compared their results with Celecoxib, a COX-2 inhibitor currently used in clinics. They found that piceatannol **1b** and the hexa-hydroxy analogue **1c** have better activity against COX-2 (IC_{50} of 0.0113 μM and 0.00104 μM respectively) than resveratrol (IC_{50} 0.996 μM) and are much more selective towards COX-2 than COX-1. They also demonstrated that the methoxy analogues did not inhibit COX-2 and are not selective between COX-1 and COX-2.



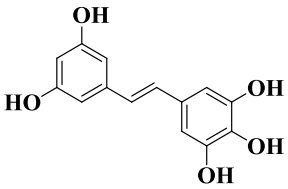
1b



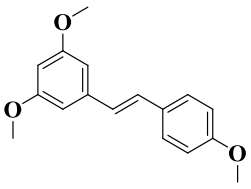
1c



1d



1e

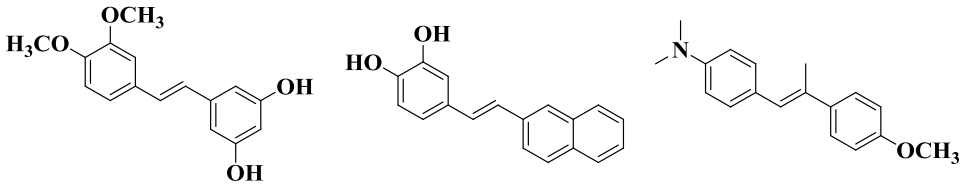


1f

The absence of activity of the methoxy analogues such as *trans*-tetramethoxy-3,4,4',5-stilbene DMU-212 **1d** against COX-2 has also been shown by Sale *et al.*[104] They compared the ability of **1d** and resveratrol to inhibit the expression of COX-2 and on the levels of PGE-2 in HCA-7 colon cancer cells and PMA-treated HCEC cells ; DMU-212 **1d** had no effect in both cell lines whereas resveratrol has an IC_{50} of 5-10 μM.

Zykova *et al.*[102] compared resveratrol with pentahydroxyresveratrol **1e** and trimethoxyresveratrol **1f** in different binding assaya as well as their effects on COX-$2^{+/+}$ and COX-$2^{-/-}$ cells. They concluded that resveratrol binds strongly to COX-2 and analogue **1e** was even better and that the trimethoxy analogue **1f** did not have any effect on the binding nor on the reduction of PGE-2 levels. From these results they suggested that the cancer chemopreventive effect of resveratrol could be due to this inhibition of COX-2.

The presence of phenol groups seems to be essential for the inhibition of COX-2 and has been confirmed recently by Kang *et al.*[107] who synthesised a combinatorial library of analogues of **1a**. Their most active analogues (with similar IC_{50} as resveratrol) all contain a catechol structure like **1g** or **1h**, except one **1i**. This could be explained by potentially required hydrogen bonding between the enzymes and the ligands.



1g **1h** **1i**

4.2.1.2. CYP450 family inhibition

Cytochrome P450's are a family of recombinant oxidising enzymes involved in the metabolism of all foreign and many endogenous compounds. This metabolism includes the activation of pro-carcinogens like polycyclic hydrocarbons into carcinogens and are linked to tumour initiation.[55] Some of the enzymes studied with resveratrol and its analogues are CYP 1A1, 1A2, 1B1 and 2E1.

It was generally found that the more lipophilic the analogue was, the more active it was in decreasing the expression of the CYPs. Mikstacka *et al.*[101] have studied the effect of the methoxy substitution of resveratrol towards the inhibition of CYP 1A2 and 2E1. They found that polymethoxy analogues like **1d** have a better activity on 1A2 than resveratrol. On the other hand they found that the more methoxylated the stilbene, the lesser the reduction of 2E1 levels and they noticed that a hydroxyl group on the 4'-position is essential to inhibit 2E1.

Kim *et al.* improved the activity and selectivity for CYP 1B1, which is thought to be important in estrogen-induced carcinogenesis and initiation of breast cancer.[125] They synthesised a library of analogues of resveratrol and rhapontigenin, a natural analogue of resveratrol, and obtained higher activity and specificity towards CYP 1B1. Their results demonstrated that a methoxy substituent on the 2'-position should be present for the analogue to be more specific to CYP 1B1 and more active.

4.2.1.3. Other enzymes

Numerous other studies on specific enzymes have been carried out, especially on the inhibition of NF-κB and of tubulin formation. NF-κB is a transcription factor generally involved in cancer and triggers the production of anti-apoptotic enzymes, inflammation, and immunity response.[47] Heynekamp *et al.*[108] synthesised different analogues with the *trans*-stilbene structure and different substituents (hydroxy, methoxy, fluorine or amine). No general pattern emerged on what is necessary to attain specificity towards NF-κB and to be a potent inhibitor. Their most active analogues do not contain any hydroxyl substituents. On the other hand, Kang *et al.*[107] showed that all the best analogues inhibiting NF-κB all contained a diphenol ring like **1h**.

Improving the inhibition of tubulin polymerisation was studied by synthesising

analogues of Combrestatin A4, a very good inhibitor of this biological process. Simoni *et al.*[118] designed new benzo-heterocycles like benzo[a]furan or benzo[a]thiophenes and have shown that these heteroaromatic rings are bioisosteres of importance for the inhibition of tubulin polymerisation and enables the products to be very good cytotoxic agents. On the other hand, Pettit *et al.*[111] synthesised β-(*E*)-nitrostyrenes analogues of combrestatins and found that they are relatively good tubulin polymerisation inhibitors, interact with numerous cancer cell lines and are good anti-fungal and microbial agents.

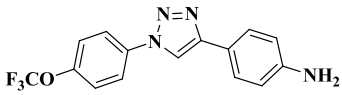
4.2.2. Cell proliferation inhibition and cytotoxicity

The resveratrol analogues synthesised have been tested on numerous cancer cell lines derived from breast, lymphoma, melanoma, colorectal and leukaemia. The anti-invasive or anti-metastatic as well as the pro-apoptotic effects were assessed to explain the possible mechanisms of the analogue on the growth and proliferation of the different cells. The conclusions drawn from the results allowed some of the research groups to formulate potential structure-activity relationships.

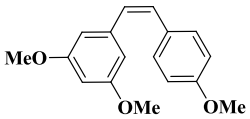
4.2.2.1. Growth inhibition and anti-proliferation effects

Most of the resveratrol analogues synthesised were tested against the proliferation of cancer cells. Paglai[115] tested a library of triazole analogues on neuroblastoma SH-SY5Y, breast cancer MDA-MB-231, basophilic leukaemia RBL-2H3 and human pancreatic carcinoma FG2 cells. They found that generally these analogues have a similar activity to resveratrol against cell proliferation and that the poly-methoxylated analogues tend to have a slightly better activity but not on all the cell lines. However, the best of the analogues **1j** is not methoxylated ($IC_{50} \geq 10$ nM) and

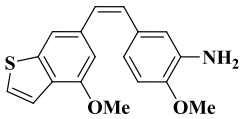
the mode of action is not clear from the results.



1j



1k



1l

Surprisingly, a study by Sale *et al.*[104] showed that DMU-212 **1d** (p.54) was less active than resveratrol at inhibiting intestinal adenomas formation in Apc^{Min+} mice. They found that DMU-212 did not affect the expression of COX-2 in HCA-7 and PMA-treated HCEC colorectal cells whereas resveratrol had an activity of 5-10 μM. Zykova[102] illustrated some similar results. The team evaluated the effect on the growth of colon cancer cell lines of a pentahydroxyresveratrol **1e** and of trimethoxyresveratrol **1f** and compared the results with resveratrol. They found that the pentahydroxyresveratrol was better at inhibiting the growth and the colony formation of HT-29 colon cancer cells. On the other hand, trimethoxyresveratrol **1f** did not have any activity. In the same study they showed that resveratrol activity and of its analogue **1e** may be linked to the inhibition of COX-2.

In contrast, the methoxylated analogues of *cis*-resveratrol and of combrestatins seem better anti-proliferative agents than *trans*-resveratrol. Simoni found that *cis*-trimethoxyresveratrol was a 33-fold better inhibitor of the proliferation of leukaemia cells HL-60.[117] Analysis of treated cells by Fluorescence Activated Cell Sorting (FACS) showed that this analogue increased the population of cells in the sub G_0/G_1 phase, and decreases these in the G_0/G_1 and G_2/M phases in a dose and time dependant manner. However, the inhibition is not phase specific as cells in all stages of the cycle are influenced by this analogue. Later another study[118] demonstrated that combrestatin A-4

analogues with a heteroaromatic ring and methoxy groups were better cytotoxics and tubulin polymerisation inhibitors. The synthesised analogues have similar activity to combrestatin A-4 against cell proliferation of colon cancer cells HT-29 and of non-small-cell (NCS) lung cancer H-460, except analogue **1l** which is 5-fold better on HT-29 (IC_{50} = 21± 1.3 μM).

4.2.2.2. Anti-invasive effects

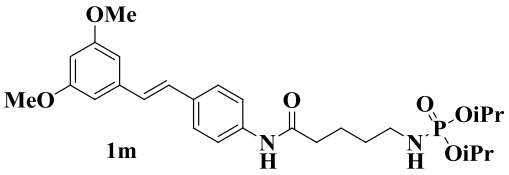
A couple of studies evaluated the effect of methoxylated resveratrol analogues on the inhibition of the metastasis. Invasion of cancer cells is generally linked to the over-expression of matrix metalloproteinases MMP-2 and 9. These enzymes break the extracellular matrix and allow the cells to invade the surrounding areas.

Young *et al.*[126] showed that *trans*-trimethoxyresveratrol **1f** inhibits the invasion of human lung adenocarcinoma cells A459 at a concentration of 0.5 μM. It also inhibits directly the activity of MMP-2 after treatment of 5 μM for 12h by observing a 40% reduction in MMP-2 activity.

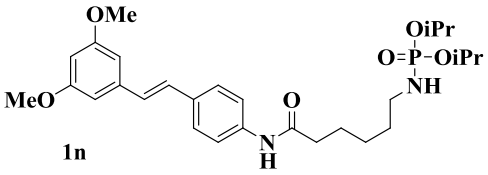
Similar results were obtained by Weng recently using human hepatocarcinoma cells HepG2 and Hep3B.[113] Both cell lines express MMP-2, Hep3B also expresses MMP-9 but HepG2 cells require PMA to induce MMP-9 expression. Resveratrol **1a** and **1f** both inhibited MMP-9 and MMP-2 in the two cell lines in a dose and time dependant manner. The invasion of Hep3B cells is reduced by both 50 μM resveratrol and 1 μM **1f** (27% and 42% respectively). Inhibition of MMP-2 was induced by the increased levels of TIMP-1/2, a natural endogenic inhibitor of MMP-2.

4.2.2.3. Pro-apoptotic effects

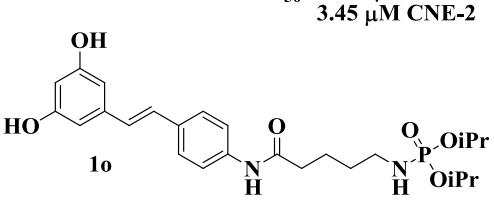
Liu *et al.*[114] designed *N*-phosphoryl-amino acid modified resveratrol analogues based on the idea that the more lipophilic the analogue relative to pterostilbene, a natural methoxylated analogue of resveratrol, the better the pro-apoptotic properties. Their design is also based on *trans*-4'-amino-3,5-dimethoxystilbene, a more potent anti-proliferative analogue of resveratrol. The library of resveratrol analogues was tested on human nasopharyngal carcinoma CNE-1 and CNE-2 cell lines and four analogues **1m-p** stood out with better anti-proliferative properties (IC_{50} ~ 6-8 μM on CNE-1 and 3-6 μM on CNE-2) than resveratrol (IC_{50} = 68 μM on CNE-1 and 53 μM on CNE-2). By analysing the results obtained on these two cell lines they concluded that the 3,5-dimethoxy substitution pattern on the left ring and the presence of the *N*-amino-phosphoryl group are important factors for the inhibitory activity. They also deduced that longer chains (more lipophilic) played a role in anti-proliferation. The best four analogues **1m-p** were tested on other immortal cervical cancer Hela cells and on myelogenous leukaemia and they obtained the same effects. A potential mechanism of anti-proliferation from the myelogenous leukaemia cell line was determined. The analogues were pro-apoptotic by arresting the cell cycle in the G_0/G_1 phase and by inducing the caspase 3 and caspase-9 apoptosis pathways.



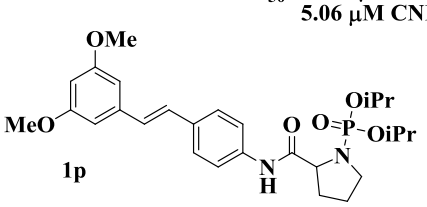
IC_{50} = 5.67 μM CNE-1
3.45 μM CNE-2



IC_{50} = 6.37 μM CNE-1
5.06 μM CNE-2



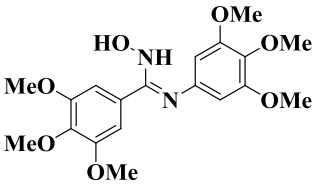
IC_{50} = 6.36 μM CNE-1
5.19 μM CNE-2



IC_{50} = 8.11 μM CNE-1
9.86 μM CNE-2

The methoxy analogues of resveratrol, especially trimethoxy-resveratrol **1f**, tend to have a good pro-apoptotic effect. Minutolo *et al.*[116] found out that **1f** is a 20-fold better proliferation inhibitor (IC_{50} of 1.2 μM) than resveratrol (20.5 μM) by inducing ceramide-mediated apoptosis in breast cancer cell lines MDA-MB-231 and MDA-MB-468. A similar conclusion for trimethoxyresveratrol was made by Weng[110] who found that it could induce apoptosis in A459 and CH27 lung cancer cell lines by increasing the Bax/Bcl-2 ratio, Bax being a pro-apoptotic enzyme, natural inhibitor of the anti-apoptotic/survival enzyme Bcl-2.

The analogue of resveratrol, KITC (*N*-hydroxy-*N*'-(3,4,5-trimethoxyphenyl)-3,4,5-trimethoxy-benzamidine) **1q**, a hexamethoxylated compound increased the number of sub G_0/G_1 phase (apoptotic) pancreatic cancer cells (AsPC-1 and BxPC-3).



1q

In conclusion, a number of analogues, both natural and synthetic, have been shown to inhibit cancer cells *in vitro*, with some exhibiting more potent effect than resveratrol. The research groups have tried to determine structure-activity relationships specific to a particular enzyme or cell line and generally with no protein structure information.

Some studies showed that trimethoxyresveratrol **1f** is a less good anti-proliferative agent, but on the other hand it is a good promoter of apoptosis and inhibitor of metastasis. It has also been explained previously that the presence of methoxy groups would make the analogues more specific to CYP450's family whereas hydroxylated analogues would be more specific to COX-2 family. Each enzyme and cell line reacts differently to the different analogues depending on the substitution. Some groups tried to create potentially more active and promising chemopreventive/chemotherapeutic agents and are still evaluating their results and continue their research in this direction.

However, methoxy analogues would be expected to either not be metabolised or to be transformed in a slower manner than parent resveratrol. None of the above studies, and none to our knowledge, have really tried to alter the metabolism of resveratrol as a primary aim, and they have not proven directly that their analogues have any effect on metabolism or bioavailability.

5. Aims of the PhD

Chemoprevention of cancer can be an alternative to the current treatments chemotherapy and radiotherapy which have very serious side effects. Population studies and studies on different phytochemicals brought about the discovery of resveratrol as a potential cancer chemopreventive agent. *In vivo*, *in vitro* and *ex vivo* studies showed that resveratrol has extensive chemopreventive properties as it inhibits all the steps of carcinogenesis, initiation, promotion and progression. Unfortunately, all the different studies on the metabolism of resveratrol and its behaviour in rat, mice or humans showed that it does not have good bioavailability due to the fast metabolism rate to sulfate and glucuronide metabolites.

It was decided to synthesise a library of new analogues of resveratrol in order to stop or at least slow down the metabolism of resveratrol into sulfates and glucuronides in *in vitro* systems. The analogues synthesised will also be assessed against the proliferation of colorectal cancer cells to see whether the modifications on the structure have an effect.

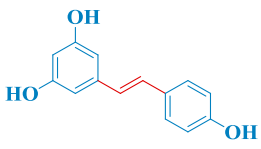
CHAPTER 2 – SYNTHESIS OF RESVERATROL ANALOGUES

CHAPTER 2 – SYNTHESIS OF RESVERATROL ANALOGUES

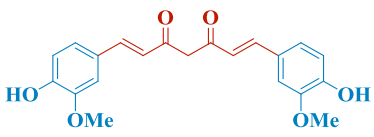
6. Design of the library

As described previously (cf. Chapter 1) resveratrol **1a**, curcumin **3**[127-130] and tricin **4**[67,131-133] are three naturally occurring polyphenols which show very good anticancer activity *in vitro* but whose activity drops dramatically in *in vivo* systems. Numerous natural and synthetic analogues of resveratrol have been extensively assessed on different cancer targets, but to the best of our knowledge, there is little work on metabolism studies to increase their bioavailability (cf.4. Analogues of resveratrol).

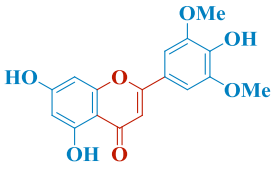
A closer look at the structures of **1a**, **3** and **4** shows that they all have two polyphenolic rings (blue) connected by a conformationally restricted linker (red) that varies in length and functionality, and that the phenols are either free or methyl-protected.



Resveratrol **1a**



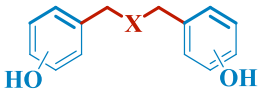
Curcumin **3**



Tricin **4**

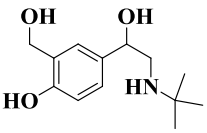
We decided to prepare a series of novel analogues which has been designed on the structural similarities of resveratrol, curcumin and tricin in order to produce compounds with a greater bioavailability without decreasing the activity. The general

structure of the new analogues will be two aromatic rings with hydroxyl substituents connected by a linker.

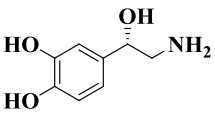


General Structure

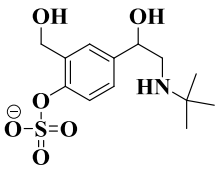
We also based our design on a previous study which led to the development of salbutamol **5a**, also known as Ventolin™. Salbutamol **5a**, a short-acting β_2-adrenergic receptor agonist used for the relief of bronchospasm like asthma, is an analogue of noradrenalin **6**. Noradrenaline **6** is quickly metabolised by catechol-*O*-methyl transferase (COMT) and monoamine transferase (MAO) and loses its activity (Scheme 12). Replacement of one of the phenols with a benzylic alcohol,[134] and protection of the amine allowed the formation of **5a** which is more bioavailable. The only metabolite found for **5a** is salbutamol sulfate **5b**,[135] where sulfation occurs on the phenol and not on the benzylic alcohol.



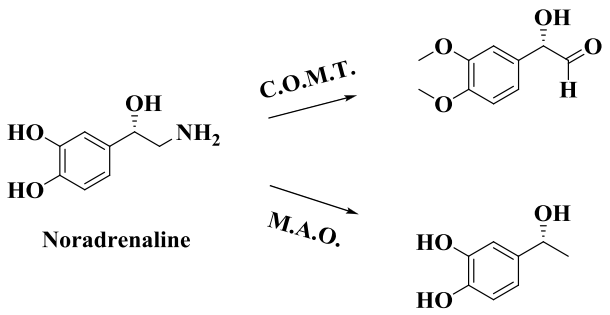
Salbutamol **5a**



Noradrenaline **6**

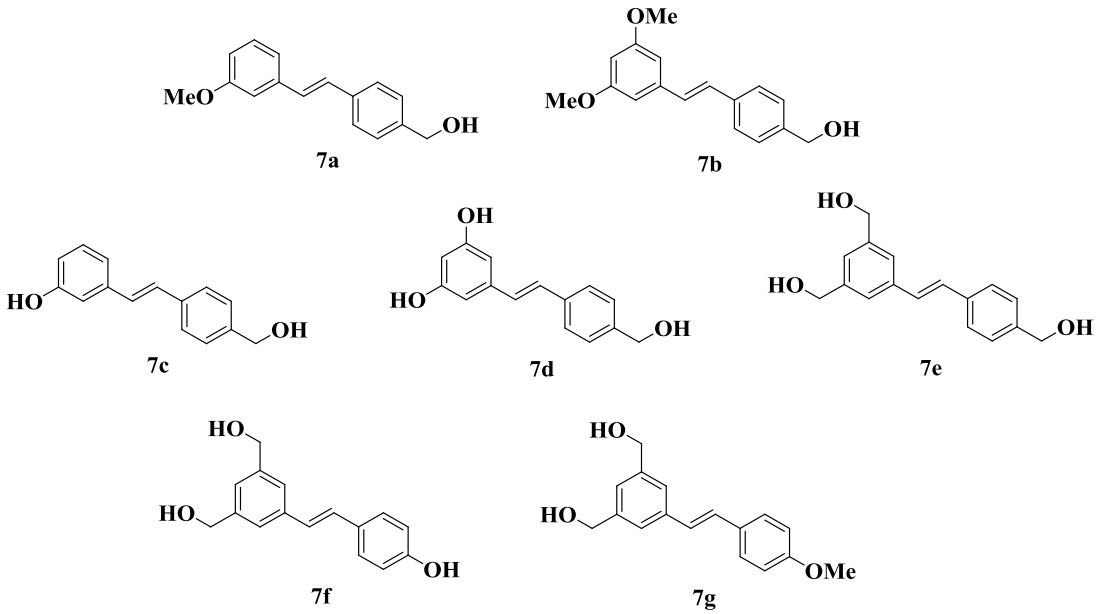


Salbutamol sulfate **5b**



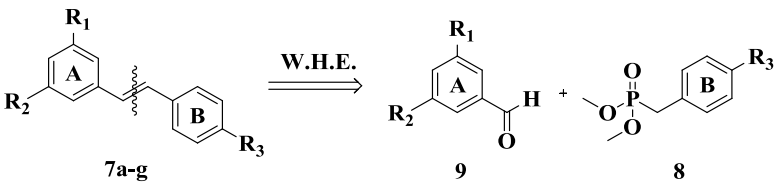
Scheme 12: Noradrenaline metabolism

Based on these observations, the synthesis of a series of novel resveratrol analogues **7a-e** where the *trans*-stilbene scaffold would be kept and the phenol would be systematically replaced by a benzylic alcohol and/or methoxy groups was undertaken.

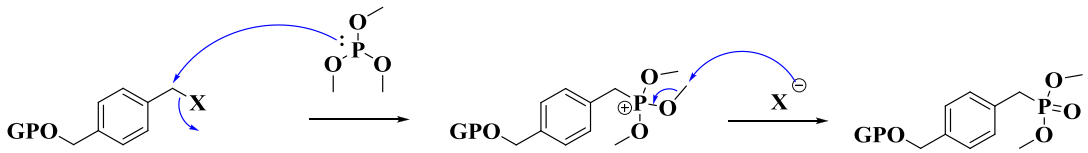


7. Retrosynthetic analysis

Most of the research groups who have synthesized analogues of resveratrol used a Wittig reaction giving the *E:Z* mixtures at the end of the reaction or a Wittig-Horner-Emmons (W.H.E.) reaction leading to the *trans*-product.[103,136] We decided to follow Murias *et al.*[103] method to construct the *trans*-double bond on the analogues **7a-g** using a W.H.E. reaction between a phosphonate **8** (Ring B) and an aldehyde **9** (Ring A).[137,138]



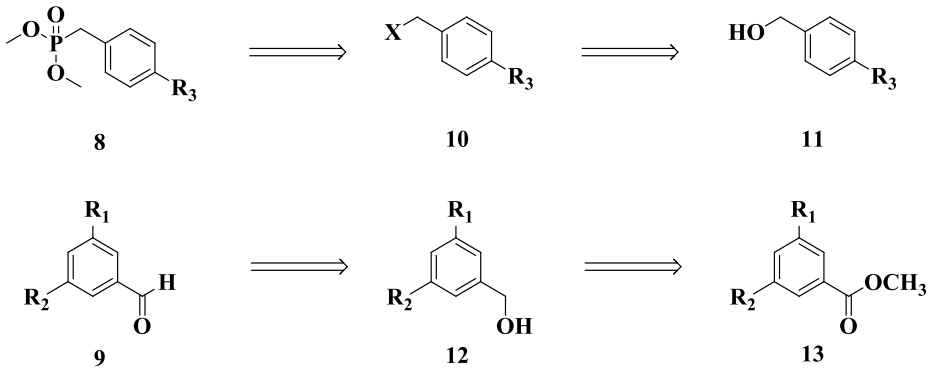
The Wittig-Horner[137] type reaction occurs between a phosphonate anion and an aldehyde. The phosphonates **8** can be obtained by a Michaelis-Arbuzov[139] reaction (Scheme 13) from a halogenomethylbenzene methanol or halogenomethyl-phenol, which can be made from the commercially available alcohols benzene-1,4-dimethanol and 4-hydroxymethylphenol respectively or from an intermediate containing a good leaving group X (Scheme 14).



X= Leaving group (Cl, I, etc.)

Scheme 13: Arbuzov reaction[139]

The aldehydes **9**, if not commercially available, can be produced *via* oxidation of a benzylic alcohol **12** which is made from the corresponding ester after reduction. (Scheme 14)

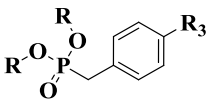


R_1, R_2: H, OH, OTHP, OCH_3, CH_2OH, CH_2OTHP; R_3: OH, OTHP, OCH_3

Scheme 14: Retrosynthetic analysis of phosphonates 8 and aldehydes 9

8. Synthesis of the phosphonates 8a-f

The Wittig-Horner-Emmons reaction occurs between a phosphonate and an aldehyde.[103,138,140] It is possible to make a phosphonate by a Michaelis-Arbuzov reaction between a halogenated compound and a trialkylphosphite (Scheme 13).[139] Table 3 summarises the phosphonates **8a-f** which have been synthesised.



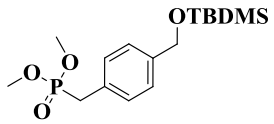
8a-f

Phosphonate	R_3	R
8a	$CH_2OTBDMS$	CH_3
8b	CH_2OTHP	CH_3
8c	OTBDMS	CH_3
8d	OTHP	CH_3
8e	OMe	CH_3
8f	OH	CH_3

Table 3: Phosphonates 8a-f

The phosphonates **8a,b** may be obtained from the commercially available benzene-1,4-dimethanol **11a**. **8c,d,f** and **8e** may be obtained from commercially available 4-hydroxybenzylalcohol **11b** and 4-methoxybenzylalcohol **11c** respectively. Functional group interconversion of one of the alcohols into a leaving group and a potential protection of the remaining one could give us the intermediate **10** which will then be submitted to an Arbuzov or Michaelis phosphorylation reaction.

8.1. Synthesis of the phosphonate 8a



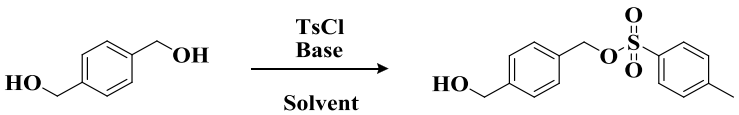
8a

Phosphonate **8a** may be obtained by different successive functional group interconversions from commercially available benzene-1,4-dimethanol **11a**. Different sequences of protection, direct phosphorylation or insertion of a leaving group were tried.

8.1.1. Benzylic alcohol activation

8.1.1.1. Direct tosylation

Hydroxyl groups are not very good leaving groups by themselves, so different attempts of activation of one of the benzylic alcohol were tried with or without protection of one of the benzylic alcohols.



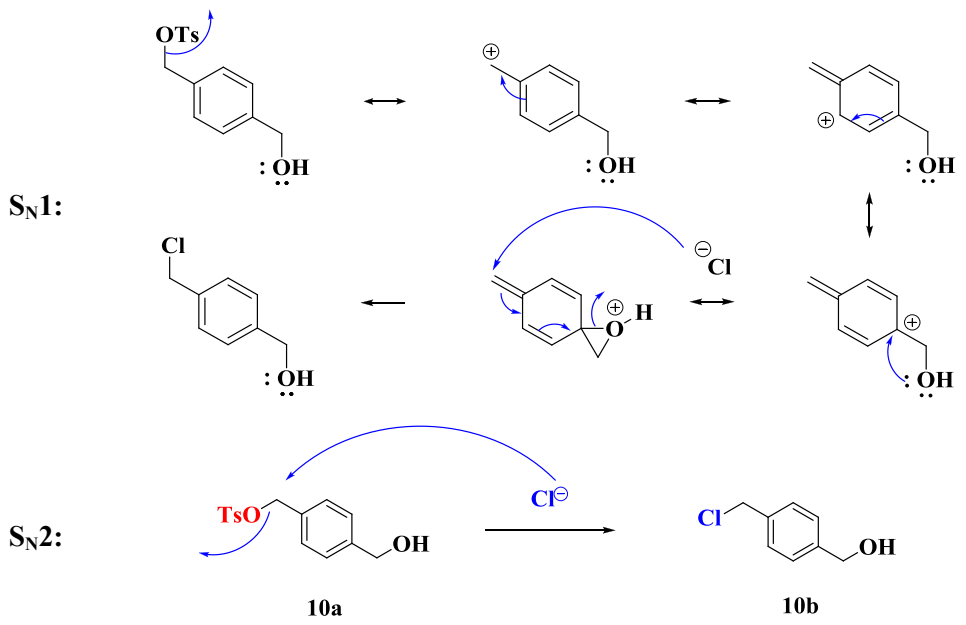
11a **10a**

Tosylation of **11a** to **10a** using toluene-4-sulfonyl chloride was attempted under a variety of conditions (Table 4). In each case where the starting material **11a** disappeared, the mono-tosylate **10a** was never recovered. The only product isolated was the 4-chloromethylbenzenemethanol **10b** (entry 3) but this was in low yield (10%). The

recovery of this compound could be explained by either an S_N1 or S_N2 reaction (Scheme 15). In the S_N1 possible mechanism, after the loss of the tosylate substituent, the positive charge is delocalised on the aromatic ring by resonance to form the oxonium cation. The mono substitution can be explained by the fact that the chloronium cation cannot be formed, so only one chlorination takes place. In the S_N2 reaction, the chloride ion substitutes the tosylate substituent which is a good leaving group compared to the hydroxyl group.

Entry	Base	Catalyst	Solvent	Time / T°C	Results
1	Pyridine[141] (2mL)	-	-	1h30 / 0°C	SM consumed no product recovered
2	KOH pellets	-	-	18h / r.t.	No reaction
3	Et_3N[142] (2 eq.)	DMAP (0.02 eq.)	THF	2h / 0-5°C then 18h at r.t.	Chlorinated product
4	NaH[143] (1.5 eq.)	-	THF	18h / r.t.	No reaction

Table 4: Conditions of formation of the tosylate 12a

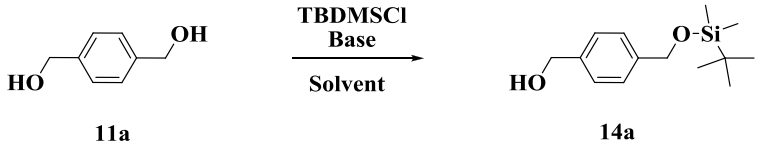


Scheme 15: Proposed S_N1 and S_N2 mechanisms of chlorination of benzene-1,4-dimethanol 11a

In order to avoid the chlorination, mono-protection of benzene-1,4-dimethanol with *tert*-butyldimethylsilyl chloride was attempted. TBDMS protecting group was chosen due to its stability in basic conditions and facile deprotection with fluoride ions.

8.1.1.2. Mono-protection of benzene-1,4-dimethanol with TBDMS

A mono-silylation of **11a** to intermediate **14a** with TBDMSCl in THF at room temperature with a base was carried out according to different literature procedures (Table 5).



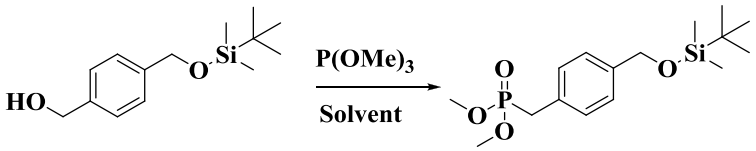
Entry	TBDMSCl	Base	Solvent	Time / T°C	Yield after purification
1	1 eq.	NaH[144] (1.0 eq.)	THF	18h / r.t.	16%
2	1 eq.	Et_3N (1.4 eq.) / DMAP (0.02 eq.)	THF	18h / r.t.	54%
3	1.1 eq.	Imidazole (0.75 eq.)	THF	18h / r.t.	19%

Table 5: Conditions of TBDMS mono-protection of benzene-1,4-methanol

The reaction in the presence of NaH as the base according to McDougal[144] gave a 16% yield (Entry 1). Other conditions were evaluated and the reaction with triethylamine/DMAP gave a better yield (Entry 2).

8.1.1.3. Formation of the phosphonate by direct phosphorylation

Direct phosphorylation from a benzylic alcohol with trimethylphosphite in toluene has been reported in the literature.[145,146] 4-((*tert*-Butyldimethylsilyloxy)methyl)-benzenemethanol **14a** was reacted with one equivalent of trimethylphosphite under different conditions as summarised in Table 6.



After heating overnight at 70°C the reaction was incomplete, and after a further 12h heating, the mixture was completely degraded (entry 1). The reaction was subjected to microwave activation without solvent and the desired phosphonate **8a** was recovered with 16% yield after purification (entry 2).

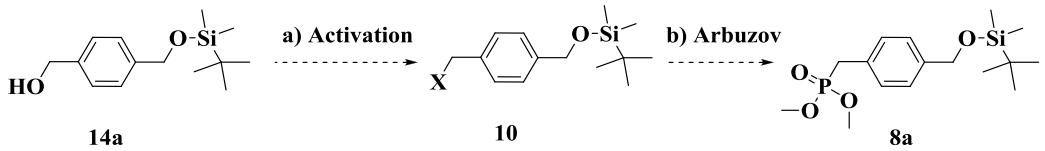
Entry	Solvent	Time / T°C	Result
1	Toluene	48h / 70°C	Decomposition
2	-	MW, 300W, 110°C, 45 min	16% yield

Table 6: Conditions of direct phosphorylation

8.1.2. Activation of the alcohol and subsequent phosphorylation

Direct phosphorylation of the intermediate **14a** was not satisfactory, so we

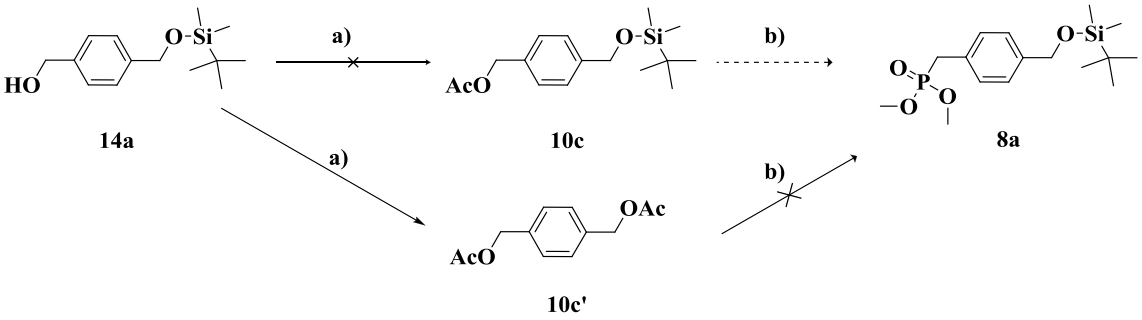
decided to activate the remaining benzylic alcohol. Different leaving groups were tried in place of the hydroxyl group (Scheme 16).



a) Leaving Group X: AcO, TsO, Halogen; b) $P(OMe)_3$, microwave, no solvent

Scheme 16: Proposed synthesis of phosphonate 8a

8.1.2.1. Use of acetate as activation group



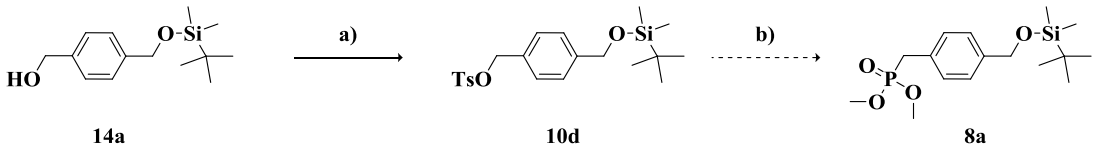
a) ZnO (10%), CH_3COCl, neat, r.t., 10 min, 0% ; b) $P(OMe)_3$, MW, neat

Scheme 17: Acetylation and phosphorylation sequence

According to F. Tamaddon *et al.*[147] it is possible to make the acetate of an alcohol using acetyl chloride, a catalytic quantity of Zinc oxide (ZnO) without solvent at room temperature for 10 to 120 minutes with very high yields (80-98%). This reaction was tested on 4-((*tert*-butyl-dimethylsilyloxy)methyl)benzenemethanol **14a** for 30 minutes and we recovered only the bis-acetate **10c'** in 70% yield. In this case acetyl chloride conditions seem incompatible with TBDMS protecting group, probably because of the experimental conditions where HCl is released which will allow the deprotection of the silyl ether and further acetylation. The phosphorylation on the bis-

acetate was tried with one equivalent of phosphite under microwave activation (300W, 45 min, 120°C), but no reaction occurred (Scheme 17).

8.1.2.2. Use of tosylate as activation group

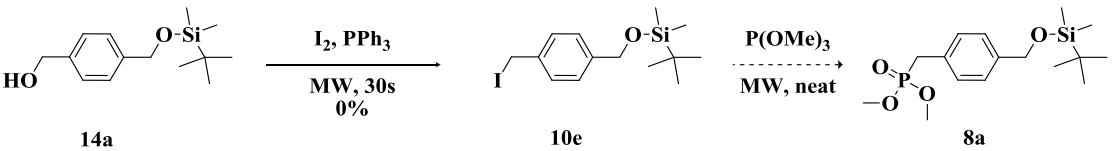


a) TsCl, Et_3N, DMAP cat., THF, r.t., 3 d, 0% ; b) $P(OMe)_3$, MW, neat

Scheme 18: Tosylation and phosphorylation sequence

Lai *et al.*[142] performed a mono-tosylation of primary alcohols with 1.1 equivalent of tosyl chloride in THF and triethylamine as base. We previously attempted this synthesis on the benzene dimethanol **11a** and obtained the 4-chloromethylbenzenemethanol **10b** by substitution of the tosylate group by chloride ion Cl^- (Scheme 15), which could have been due to the presence of a free para benzylic alcohol. In this case, the alcohol in para of the benzylic alcohol is protected by a TBDMS group, which might help the reaction to proceed. Alcohol **14a** was reacted with tosyl chloride in THF with triethylamine and a catalytic quantity of DMAP for 3 days at room temperature and reflux for one day, but no reaction occurred (Scheme 18).

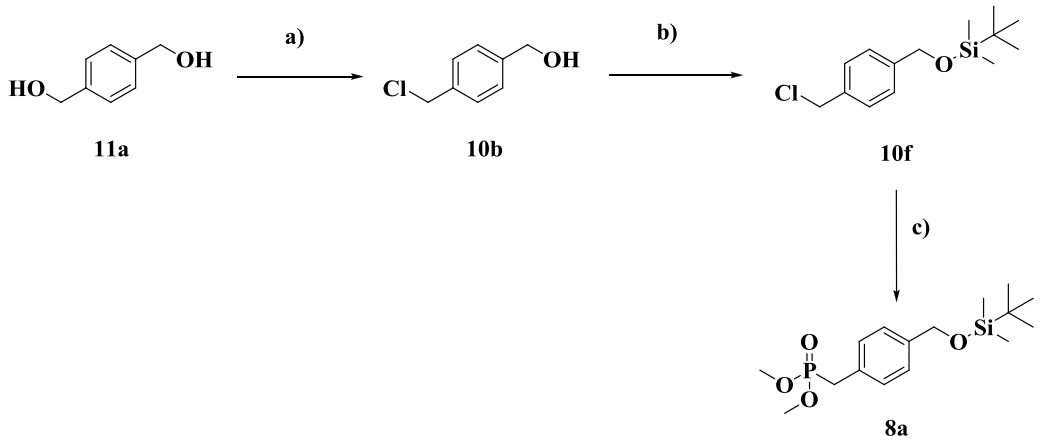
8.1.2.3. Use of halogen as activation group



Scheme 19: Iodination and phosphorylation sequence

Conversion of the alcohol into a halogen such as chlorine or iodine could be an alternative method to activate the benzylic position. Indeed, halogens are generally good leaving groups, especially iodine. Hajipour *et al.*[148] converted a benzylic alcohol into an iodide with I_2 and triphenylphosphine without solvent by microwave activation for 30 seconds with very high yields (75-94%). This reaction was attempted on the silylated benzenemethanol **14a**, but only degraded mixtures and none of the iodinated product **10e** was recovered (Scheme 19).

The use of chlorine as a leaving group and of TBDMS as a protecting group was still interesting. TBDMS seems to be sensitive to acidic conditions, so we decided to add the chlorine before protecting the remaining benzylic alcohol. Following the method of Breschi *et al.*[149], benzene-1,4-dimethanol **11a** was reacted with an excess of concentrated hydrochloric acid in toluene overnight at room temperature to give 4-chloromethylbenzenemethanol **10b** in 94 % yield. The latter was then reacted with 1.5 equivalent of TBDMSCl and 3 equivalents of imidazole in dry DMF to give **10f** in 60% yield. The chlorinated intermediate was then treated with one equivalent of sodium hydride and dimethylphosphite in dry THF and **8a** was obtained with 57% yield (Scheme 20).



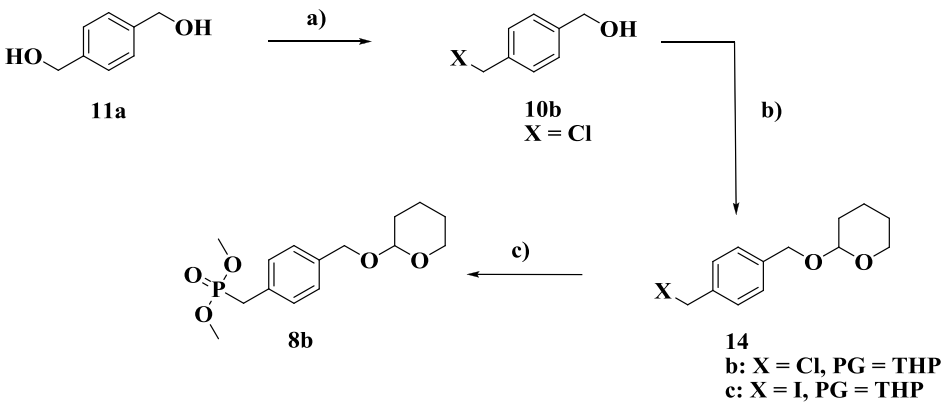
a) conc. HCl, Toluene, 18h, r.t. 94% ; b) TBDMSCl, Imidazole, dry DMF, 18h, r.t., 60% ; c) NaH, $P(O)(H)(OMe)_2$, dry THF, 0°C → r.t., 60%

Scheme 20: Alternative synthesis of 8a

8.2. Another synthetic route: synthesis of phosphonate 8b

According to the results described above, it seems to be important to have the benzylic halogen as the leaving group for the subsequent Michaelis-Arbuzov reaction. The benzylic alcohol *para* to the halogen can be protected before the Arbuzov reaction so that it will not interfere with the subsequent reactions. Different protecting groups can be envisaged, such as tetrahydropyranyl ether.

Phosphonate **8b** may be obtained from the mono-halogenation of the commercially available benzene-1,4-dimethanol **11a**. The remaining alcohol of the latter will then be protected before reacting it with a trialkylphosphite (Scheme 21).

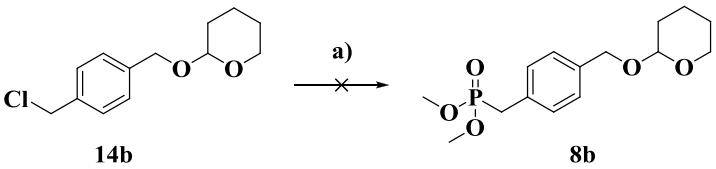


a) conc. HCl, toluene ; b) DHP, HCl aq. ; c) $P(OMe)_3$, Δ

Scheme 21: Potential synthesis of 8b

4-Chloromethylbenzene methanol **10b** was obtained in 94% yield by reacting benzene-1,4-dimethanol **11a** with an excess of hydrochloric acid in toluene at room temperature. An attempt of direct phosphorylation from **10b** by microwave activation[103] did not give **8b**, maybe because of the presence of the benzylic alcohol, which may be not a good enough leaving group for the Arbuzov reaction to occur.

Using the method by Puyn et al,[150] 2-(4-(chloromethyl)benzyloxy)tetrahydro-2H-pyran **14b** was obtained in 74% yield. Reacting **14b** with trimethyl phosphite under microwave activation did not give the desired phosphonate **8b**.



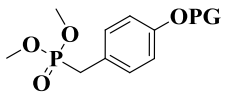
a) $P(OMe)_3$, MW, neat, 0%.

To facilitate the Arbuzov reaction, we substituted the chloride with an iodide according to published work[151]. Chloride **14b** was reacted with sodium iodide in dry acetone to give the iodinated analogue **14c** in 79% yield. (*NB: This intermediate is very*

light sensitive and degrades very easily. It might also degrade during the purification on chromatography column which may explain the reduced yield). The reaction of this iodinated product with trimethylphosphite with microwave activation gave us the desired Arbuzov phosphonate **8b** with 65% yield.

Heating the chlorinated intermediate **14b** under reflux (110°C) for 24h with trimethylphosphite in neat conditions did give us the desired phosphonate **8b** with a 65% yield. A lower yield of the phosphonate is obtained from the chlorinated heated in a sealed vessel at 120°C in neat conditions and traditional heating.

8.3. Synthesis of PG-*O*-phenol phosphonate 8c-d



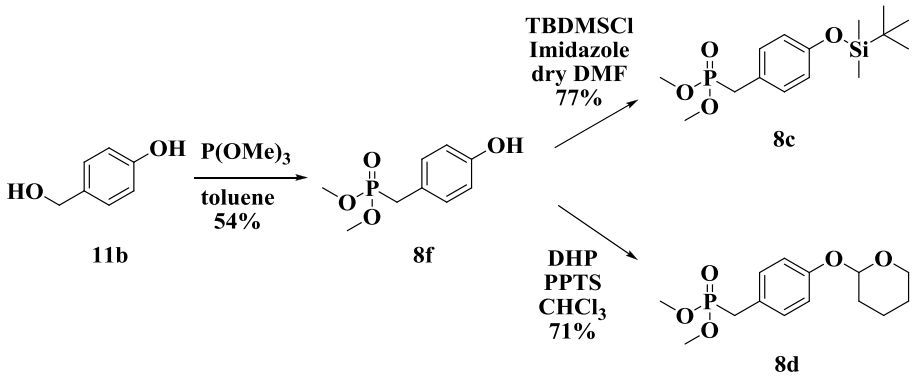
8c-d

PG= THP, TBDMS

Similarly to the "benzylic alcohol" phosphonates **8a-b** described previously, a protection is needed on the phenol to prevent the deprotonation during the Wittig-Horner-Emmons reaction. We attempted the mono-protection of **11b** on the phenol position with TBDMSCl and imidazole, but the diprotected intermediate was obtained instead. Deprotection of the benzylic alcohol according to published methods[152,153] did not yield any desired product. As an alternative, a direct phosphorylation of **11b** with trimethylphosphite in refluxing toluene following the Bohmer *et al.*[145] method was attempted giving dimethyl-4-hydroxybenzylphosphonate **8f** in 54% yield (Scheme 22).

Phenolic phosphonate **8f** was treated with TBDMSCl and imidazole in dry DMF giving **8c** in 77% yield after column purification. THP protected phosphonate **8d** was

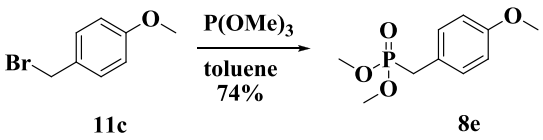
obtained in 71 % yield by treating phenol **8f** with 1.1 equivalent of DHP and a catalytic amount of *para*-toluene sulfonic acid (PPTS) in dry chloroform (Scheme 22).



Scheme 22: Synthesis of 8c-d

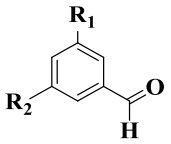
8.4. Synthesis of dimethyl *p*-methoxy phosphonate 8e

Diethyl 4-methoxybenzylphosphonate is commercially available and the dimethoxy phosphonate **8e** can be obtained in 74% yield from commercially available 4-methoxybenzylbromide **11c**.



9. Synthesis of the aldehydes

Aldehydes **9a-e** are commercially available but aldehydes **9d,e** require an *O*-protection prior to the Wittig-Horner-Emmons reaction. **9f** requires synthesis and **9d-f** requires THP-*O*-protection to form aldehydes **9g-i** in order to prevent the deprotonation during the Wittig-Horner-Emmons reaction and to perform only one deprotection step at the end of the synthesis (Table 7).



9a-f

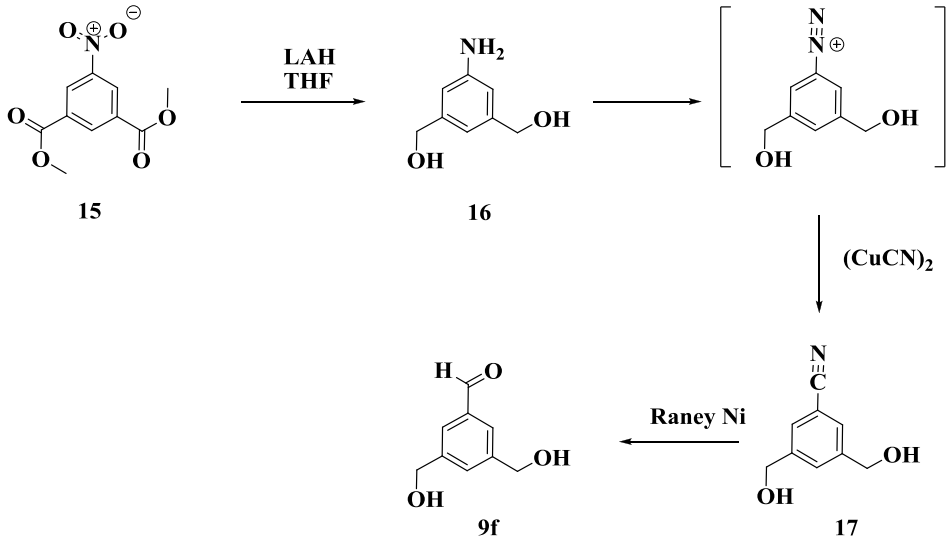
Aldehyde	R_1	R_2
9a	H	H
9b	H	OMe
9c	OMe	OMe
9d	OH	H
9e	OH	OH
9f	CH_2OH	CH_2OH
9g	CH_2OTHP	CH_2OTHP
9h	OTHP	H
9i	OTHP	OTHP

Table 7: Aldehydes needed

9.1. Synthesis of 3,5-bis(hydroxymethyl)benzaldehyde 9f

Li *et al.*[154] described a synthesis of unprotected 3,5-bis-(hydroxymethyl)benzaldehyde **9f** from dimethyl 5-nitroisophthalate **15** which was

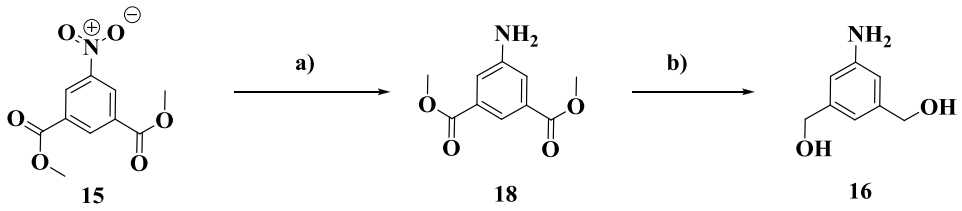
reduced to 5-amino-1,3-bis-(hydroxymethyl)benzene **16** with LAH in THF (Scheme 23). The aniline derivative **16** was then converted to the corresponding diazonium salt which was subsequently treated with copper (II) cyanide to afford 5-cyano-1,3-bis-(hydroxymethyl)benzene **17**. A final treatment with Raney Nickel in formic acid gave the desired aldehyde **6f** with an overall yield of 14.3% (Scheme 23).



Scheme 23: Synthesis of 3,5-bis(hydroxymethyl)benzaldehyde 9f by Li *et al.*[154]

The reduction step from the nitro diester **15** to the aniline-diol **16** was attempted with five equivalents of LAH in dry THF but the desired compound was never isolated. An alternative two-step approach to the amino-diol **16** was attempted *via* hydrogenolysis of the nitro-diester **15** followed by LAH reduction of the resulting amino-diester **18** (Scheme 24).[155,156] Hydrogenolysis of **15** gave the diester **18** in 47% yield. A subsequent reduction of both esters with LAH gave the amino diol **16** in only 27% yield. Low yields at this stage were deemed to be unacceptable; therefore this route was stopped and an alternative sequence from commercially available benzene-

1,3,5-tricarboxylic acid was studied.



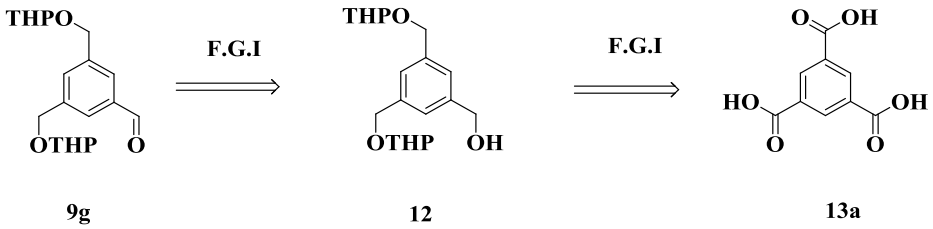
a) H_2/Pd, MeOH, 1atm, 47% ; b) LAH, THF, 0°C → r.t., 27%

Scheme 24: Alternative synthesis leading to 5-amino-1,3-bis(hydromethyl)benzene

9.2. Alternative synthesis of 9f in its protected form 9g

9.2.1. Retrosynthetic analysis of 9g

Aldehyde **9f** could not be obtained by the published method, so we decided to prepare it in a protected form **9g** from commercially available benzene-1,3,5-tricarboxylic acid. **9g** may be obtained through a succession of functional group interconversions (F.G.I.) including reduction, protection and oxidation reactions (Scheme 25). To utilise a single global deprotection step at the end of the synthesis tetrahydro-*2H*-pyranyl (THP) was chosen as the protecting group as with phosphonate **8b**.

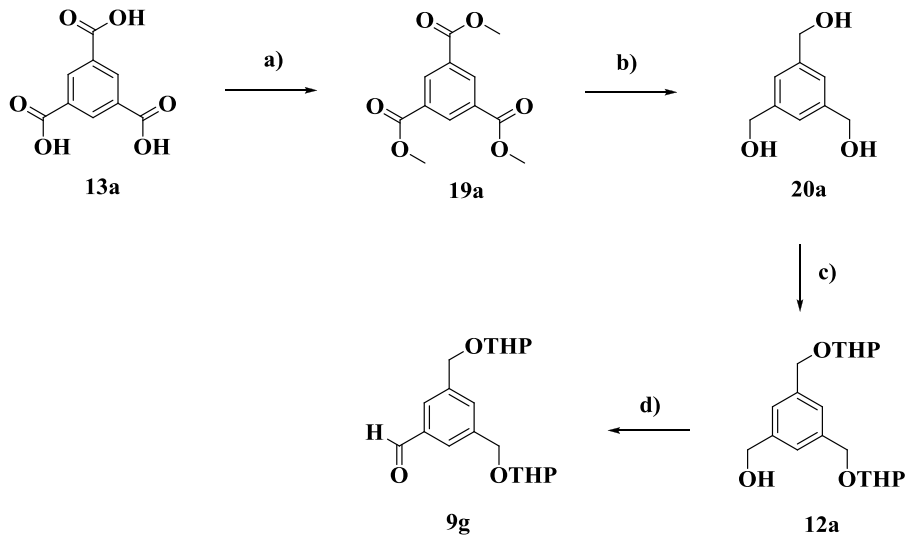


Scheme 25: Retrosynthetic analysis of 9g

9.2.2. Synthesis of 3,5-bis((tetrahydro-2H-pyran-2-yloxy) methyl)benzaldehyde 9g

3,5-Bis-((tetrahydro-*2H*-pyran-2-yloxy)methyl)benzaldehyde **9g** can be obtained from commercially available benzene-1,3,5-tricarboxylic acid **13a** in four steps with an overall yield of 24% (Scheme 26). **13a** was reacted with methanol and a catalytic amount of concentrated sulphuric acid to give quantitatively trimethoxybenzene-1,3,5-tricarboxylate ester **19a**. The triester was then reduced to the tri-alcohol **20a** with LAH in dry THF in 94% yield. The latter has to be selectively diprotected to stop the formation of a poly-aldehyde during oxidation and to prevent the deprotonation of the free benzylic alcohols during the Wittig-Horner reaction (Scheme 26). Using the method by Meric *et al.*[157], tri-alcohol **20a** was reacted with two equivalents of 3,4-dihydro-*2H*-pyran and a catalytic amount of aqueous HCl 1M in a mixture of dichloromethane-THF to produce the desired di-THP protected alcohol **12a** in a 43% yield.

The oxidation of a benzylic alcohol to the corresponding aldehyde is generally achieved using pyridinium chlorochromate (PCC)[158-160] or manganese dioxide (MnO_2).[138,161-163] A first attempt of oxidation to the aldehyde was realised following Nordlander's *et al.* method.[163] **12a** was dissolved in dry chloroform at r.t. and was reacted with 20 equivalents of oven activated manganese dioxide and **9g** was obtained with 36% after 48h reaction at r.t. The same reaction was then carried out using two equivalents of PCC as an oxidant in dry dichloromethane following Duhamel's *et al.* method[158] and the desired aldehyde was recovered with a good yield of 66% after 2h at r.t. and chromatographic purification.



a) MeOH, conc. H_2SO_4 cat., reflux, 96% ; b) LAH, dry THF, 0°C → r.t., 94% ; c) DHP, HCl 1M cat., DCM:THF 1:1, r.t., 43% ; d) PCC, $CHCl_3$, r.t., 66%

Scheme 26: Synthesis of 9g

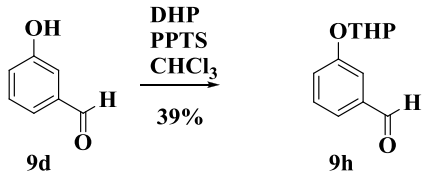
9.3. Synthesis of aldehydes 9h-i

As stated previously, the aldehydes **9d-e** are commercially available but cannot be used directly in the Wittig-Horner-Emmons reaction as they contain at least one free alcohol, which may be deprotonated during the W.H.E. reaction. Like **9g**, THP is going to be used as a protecting group on aldehydes **9d-e** so that only one deprotection step at the end of the synthesis. **9h-i**, the protected forms of aldehydes **9d-e** are going to be synthesised in a similar manner as **9g** *via* different functional groups interconversions.

9.3.1. Synthesis of 3-(tetrahydro-2H-pyran-2-yloxy) benzaldehyde 9h

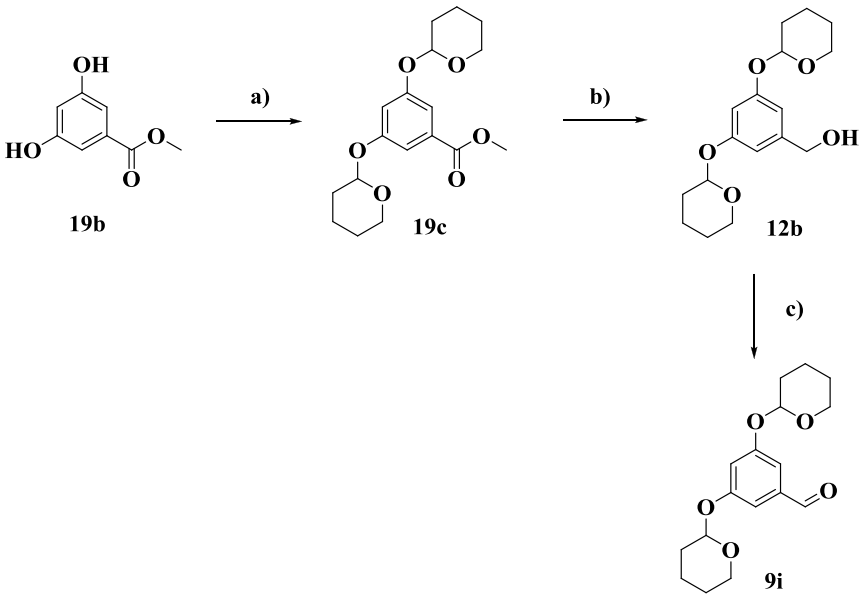
THP-protected aldehyde **9h** was synthesised from commercial 3-

hydroxybenzaldehyde **9d** following Severi *et al.*[164] method in 39% yield by reacting the aldehyde with 1.1 equivalent of 3,4-dihydro-*2H*-pyran and 0.1 equivalent of PPTS in dry chloroform.



9.3.2. Synthesis of 3,5-bis(tetrahydro-2H-pyran-2-yloxy) benzaldehyde 9i

3,5-Dihydroxybenzaldehyde **9e** is commercially available. However, we decided to synthesize **9e** as its THP-ether **9i** from the cheaper commercially available methyl 3,5-dihydroxybenzoate **19b**.[164,165] Following Pyun *et al.* method,[150] methyl 3,5-dihydroxybenzoate **19b** was reacted with DHP and 0.1 equivalent of PPTS in dry DCM under nitrogen to afford the corresponding di-THP protected compound **19c** in 92% yield. The ester **19c** was then reduced with LAH in dry THF to give the corresponding benzylic alcohol **12b** in 79% yield.[166,167] Aldehyde **9i** was obtained by a subsequent oxidation of the alcohol **12b** with PCC in dichloromethane[158] in 81% yield (Scheme 27).



a) DHP, PPTS, DCM, r.t., 92% ; b) LAH, dry THF, 0°C → r.t., 79% ; c) PCC, DCM, r.t., 81%

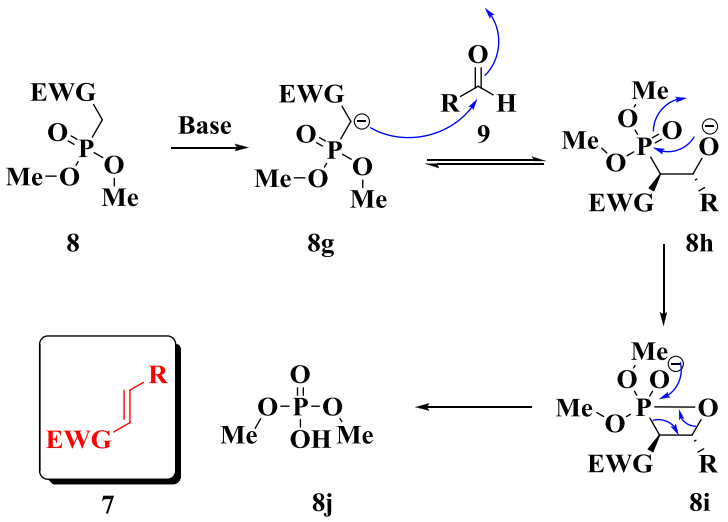
Scheme 27: Synthesis of aldehyde 9i

10. Wittig-Horner-Emmons couplings

10.1. Synthesis

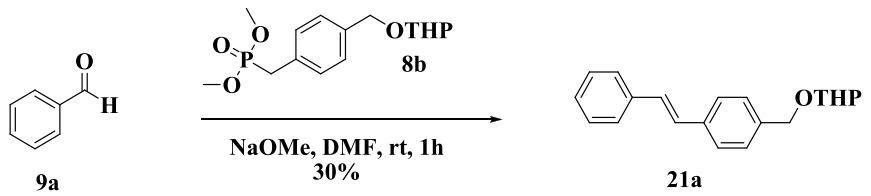
The Wittig-Horner-Emmons reaction is known to give, almost exclusively, the (*E*)-double bond product from the reaction between an aldehyde and a phosphonate if the group in α-position to the phosphonate is an electron-withdrawing group.[139,168] In contrast to phosphonium ylides used in the Wittig reaction, phosphonate-stabilized carbanions are more nucleophilic and more basic. Likewise, phosphonate-stabilized carbanions can be alkylated, unlike phosphonium ylides. The dialkylphosphate salt by-product is easily removed by aqueous extraction.[168]

Nucleophilic attack of the phosphorus carbanion **8g** on the aldehyde **9** forms the oxygen-anion **8h**. This addition step is reversible and allows the more thermodynamic stable product **8h** where the substituents are *trans* to each other to be formed. The oxygen-anion then attacks the phosphorous to form the oxaphosphetane **8i**. This ring then opens to give the product **7** with the *trans*-double bond and the water soluble dialkyl phosphate salt **8j** as a side product (Scheme 28).

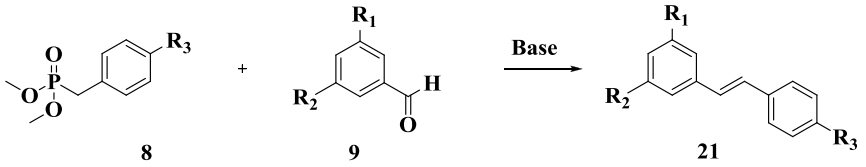


Scheme 28: Wittig-Horner-Emmons reaction

Synthesis of the THP-protected final products **21a-h** were performed following work done on resveratrol analogues by Murias *et al.*[103] using sodium methoxide or potassium *tert*-butoxide as the base in dry DMF under nitrogen between 0°C and r.t. This reaction is widely used in the synthesis of resveratrol or stilbene analogues.[100,109,122,169] The coupling reaction was first tried with silylated phosphonates **8a** and benzaldehyde but it did not give the desired product. Instead a mixture of the starting materials and of deprotected phosphonate was formed. Trying the equivalent reaction between THP-protected phosphonate **8b** with benzaldehyde **9a** on a small scale afforded the desired stilbenes **21a** with a 30% yield.



Even though the reaction yield was quite low, it showed that the reaction was taking place. The reaction was repeated with new sodium methoxide and on a larger scale and the desired product was obtained with a 70% yield. The reaction was carried out under the same conditions on the commercial aldehydes **9b-c** and on the synthesised aldehydes **9g-i** with the phosphonate **8b** and **8d-e** to give the desired coupling products **21b-h** with varying yields (Table 8).



Entry	Phosphonate	Aldehyde	Base	R_1	R_2	R_3	Stilbene	%yield
1	**8b**	**9a**	NaOMe	H	H	CH_2OTHP	**21a**	70
2	**8b**	**9b**	NaOMe	H	OMe	CH_2OTHP	**21b**	65
3	**8b**	**9c**	NaOMe	OMe	OMe	CH_2OTHP	**21c**	60
4	**8b**	**9g**	NaOMe	CH_2OTHP	CH_2OTHP	CH_2OTHP	**21d**	80
5	**8b**	**9h**	NaOMe	H	OTHP	CH_2OTHP	**21e**	50
6	**8b**	**9i**	NaOMe	OTHP	OTHP	CH_2OTHP	**21f**	73
7	**8d**	**9g**	tBuOK	CH_2OTHP	CH_2OTHP	OTHP	**21g**	58
8	**8e**	**9g**	tBuOK	CH_2OTHP	CH_2OTHP	OMe	**21h**	62

Table 8: Wittig-Horner-Emmons reaction results

10.2. NMR study: Proof of *trans*-double bond

The *trans*-double bond of resveratrol is represented by an A-B system in proton NMR at 300 MHz with a coupling constant $^3J_{trans}$ = 16.2 Hz at around 7 ppm (Figure 12). In the case of resveratrol, the double bond signal overlaps with one of the doublets corresponding to the *para*-system of the ***B*** ring. We observe the same A-B system for all the intermediates in the same area of the spectrum and similar coupling constant $^3J_{trans}$ ~ 16-17 Hz (Figure 13-Figure 15).

Cis-resveratrol has a coupling constant $^3J_{cis}$ ~ 12.5 Hz characteristic of the *cis*-systems. No such coupling constant is observed in our semi-final products, meaning that the *trans*-double bond is formed exclusively.

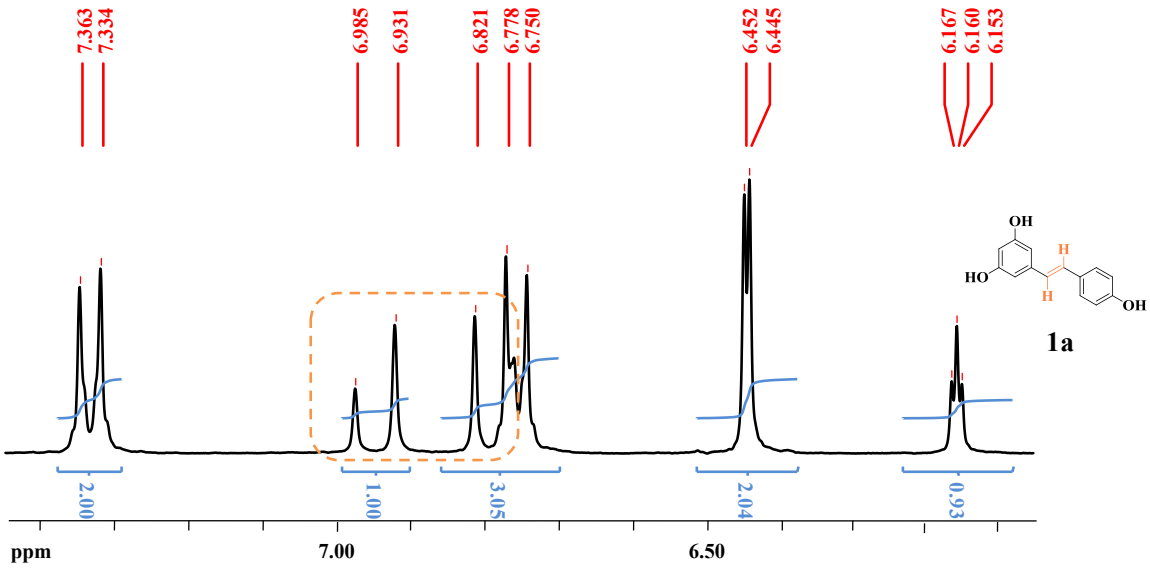


Figure 12: Expansion of aromatic region of ^{1}H-NMR of *trans*-resveratrol

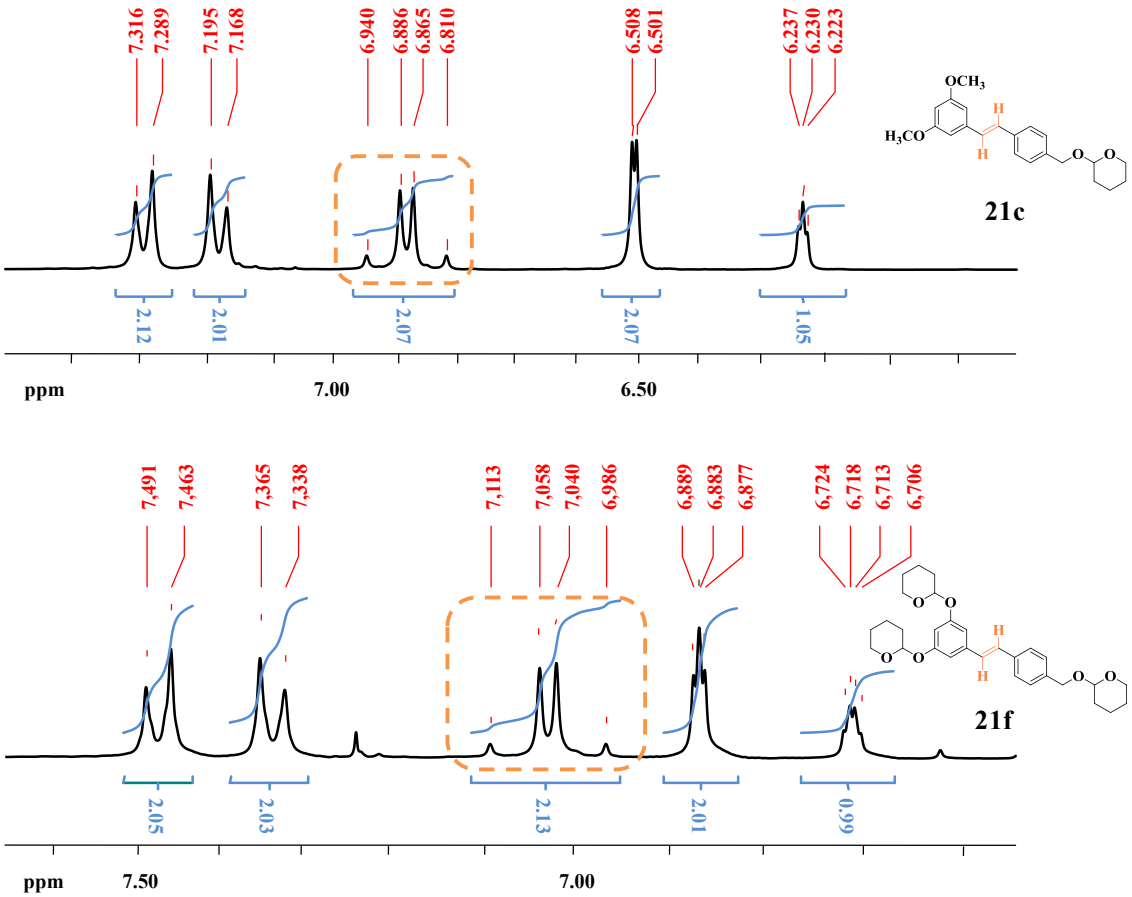


Figure 13: Expansion of aromatic region of ^{1}H-NMR of *trans*-resveratrol analogues 21c and 21f

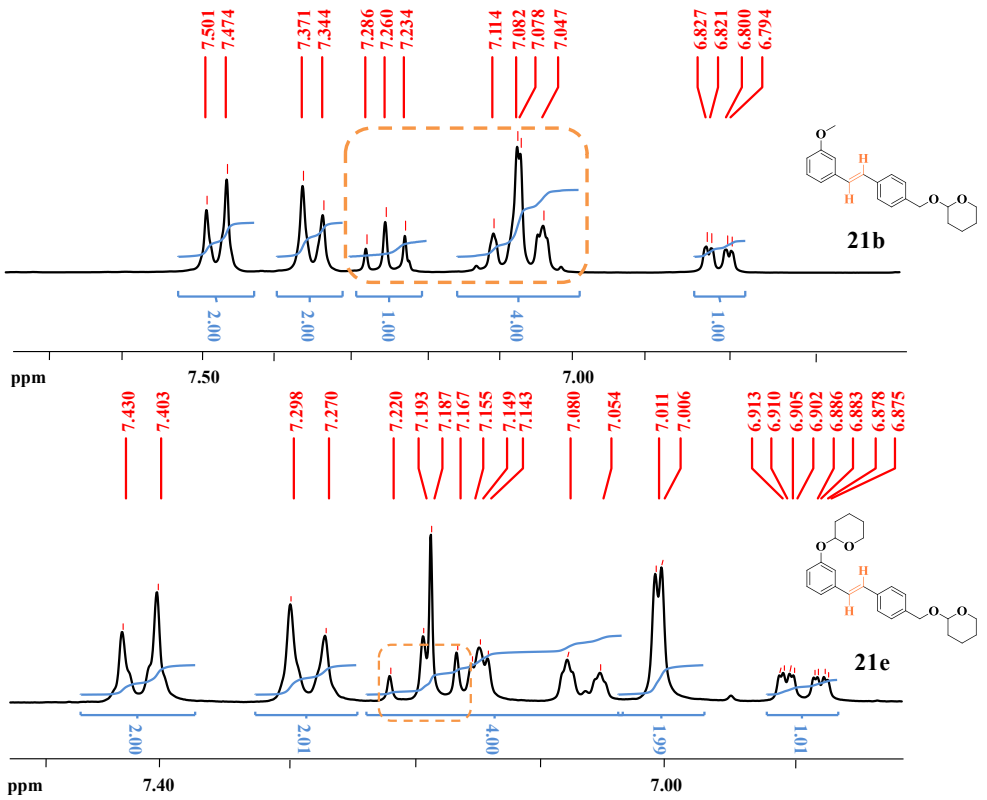


Figure 14: Expansion of aromatic region of ^{1}H-NMR of *trans*-resveratrol analogues 21b and 21e

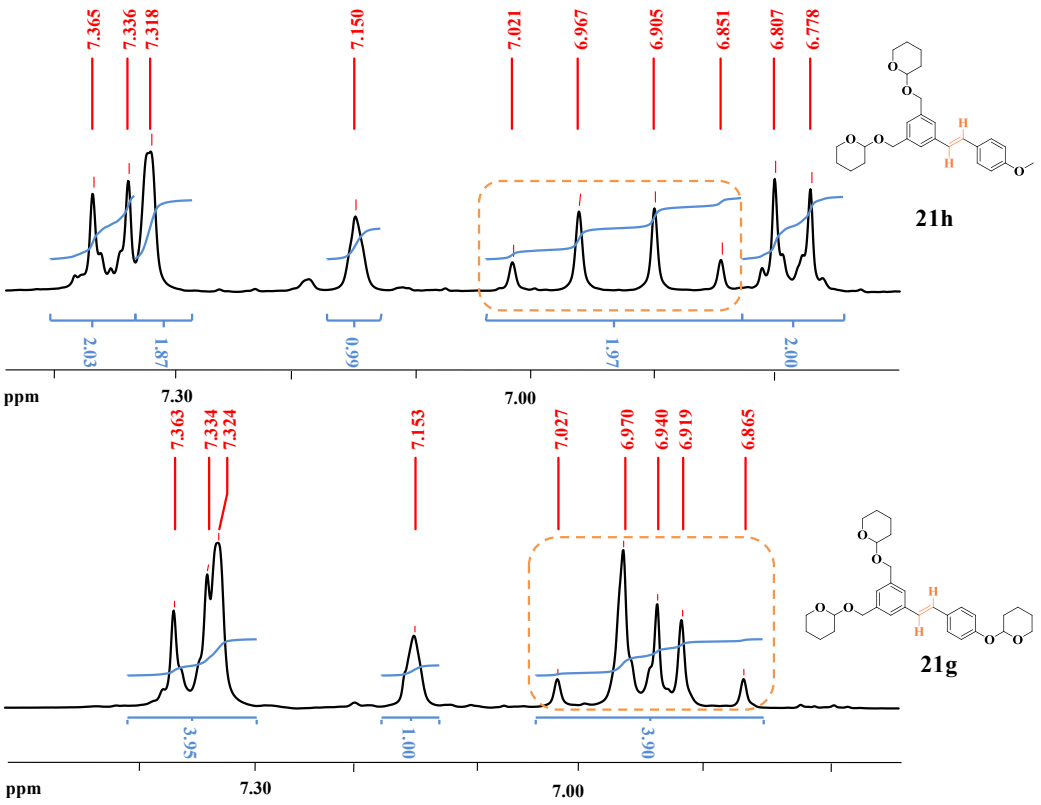
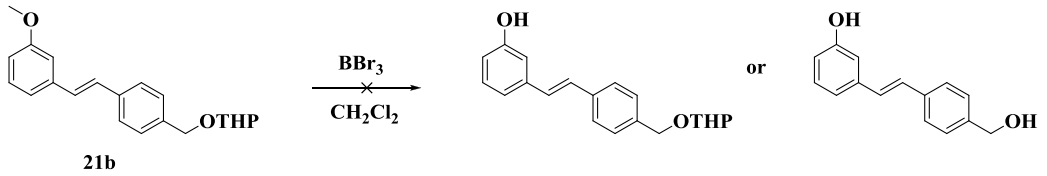


Figure 15: Expansion of aromatic region of ^{1}H-NMR of *trans*-resveratrol analogues 21g and 21h

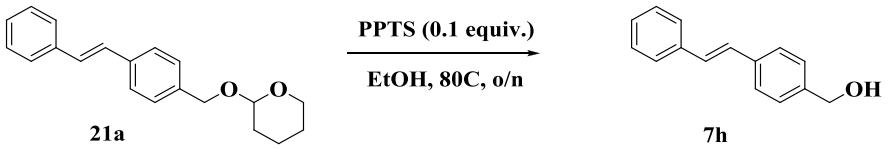
11. Deprotection of the alcohols

The desired resveratrol analogues **7a-e** were obtained after deprotection. Demethylation of THP-protected analogues **21b-c** was attempted prior to THP deprotection to give analogues **7c-d**. Deprotection of methyl-ethers is quite well studied. BBr_3 in dichloromethane is one of the most used reagents for methyl ether deprotection on aromatic methyl ethers[170-172] or primary alcohols.[173,174] BCl_3 in dichloromethane has the same properties as BBr_3 but has a lower reactivity. It can however deprotect aromatic methyl ethers in mild conditions very efficiently.[175-177] According to Dodge *et al.*[178] alkaline thiolate is a good regioselective reagent for aryl methyl ether deprotection; methyl ethers in *para*- position to a carbonyl are deprotected by ethane thiolate in hot DMF with high yields (54-89 %). Other studies have been conducted on aryl methyl ethers or aryl alkyl ethers using AlI_3 as a good deprotecting reagent[179] with or without a catalyst such as tetrabutylammonium iodide (TBAI) in many solvents, but higher yields are observed in benzene or cyclohexane.[180] Trimethylsilylchloride and silicon tetrachloride/sodium iodide had also been reported as highly regioselective ether cleaving reagents on aryl methyl ethers or alkyl methyl ethers with deprotections yielding up to 90%.

Murias *et al.* synthesis of analogues of resveratrol[103] contain a BBr_3 deprotection of the methyl ethers. As we were following this route we tried this deprotection on the methyl and THP protected stilbenes **21b**. **21b** was dissolved in dry dichloromethane at -5 °C under nitrogen and was treated with 1.5 equiv. of BBr_3. After work-up, no product was recovered as the brown solid obtained was not soluble in any solvent. We thought the reactivity could have been disturbed by the presence of the THP protecting group, so we decided to deprotect it before deprotecting the methyl ether.



THP deprotection was achieved with pyridinium para-toluene sulfonate (PPTS) according to published work.[181] The reaction was tried on the stilbene **21a** with 0.1 equivalent of PPTS in refluxing ethanol overnight. The desired deprotected benzylic alcohol **7h** was obtained with 59% yield.

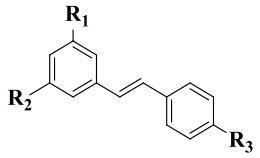


The same THP deprotection was carried out on analogues **21b-c** with average yields (Table 9). The methyl deprotection with BBr_3 was tried on the resulting compounds **7a** and **7b**, under published conditions[103] but the desired products were not obtained. This could be due to the free benzylic alcohol which interferes in the reaction. Interpretation of the product NMR was not possible in either case.

Entry	Compound	R1	R2	Final compound	Yield (%)
1	**21a**	H	H	**7h**	59
2	**21b**	OMe	H	**7b**	89
3	**21c**	OMe	OMe	**7c**	44

Table 9: THP deprotection results

Deprotection of the methyl ether was not achieved; therefore methoxy analogues were included in the final library following THP deprotection to afford the compounds **7a-g** (Table 10).



7a-g

Entry	R1	R2	R3	Stilbene-OH	%yield
1	H	OMe	CH_2OH	**7a**	46
2	OMe	OMe	CH_2OH	**7b**	89
3	H	OH	CH_2OH	**7c**	52
4	OH	OH	CH_2OH	**7d**	44
5	CH_2OH	CH_2OH	CH_2OH	**7e**	23
6	CH_2OH	CH_2OH	OH	**7f**	67
7	CH_2OH	CH_2OH	OMe	**7g**	39

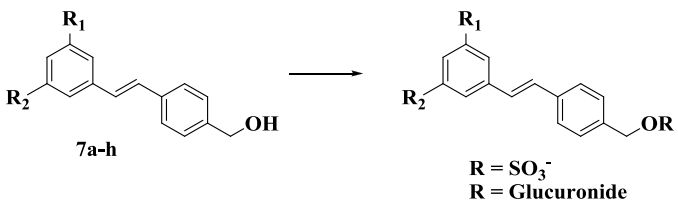
Table 10: THP-deprotection to the final analogues

In conclusion we successively synthesised in a convergent manner a library of seven analogues of resveratrol *via* a Wittig-Horner-Emmons reaction. The final steps need more optimisation to obtain the analogues in better yields. The quantities obtained were sufficient for full characterisation as well as for the biological evaluations in metabolism and cell proliferation assays.

12. Synthesis of the metabolites

The analogues made were going to be subjected to *in vitro* enzymatic metabolic conversion to be potentially transformed to the sulfates and glucuronide conjugates. To assist the HPLC and LC/MS/MS identification of the *in vitro* metabolites, standards of these possible metabolites were required. It will also give us a better understanding of the metabolic transformation process.

The proposed synthesis is based on the synthesis previously described using a Wittig-Horner type reaction to make the (*E*)-double bond. The regio-isomers of the metabolites (sulfates and glucuronides) may be made selectively by differential protection of the alcohols (phenols or benzylic). In the case of the methoxy analogues **7a-b**, the sulfate and glucuronide metabolites may be obtained directly from the parent product.



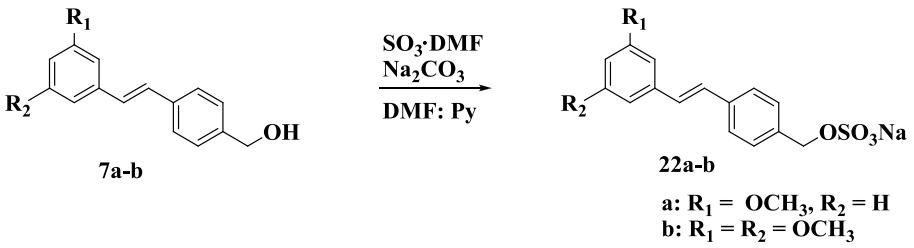
12.1. Sulfation

Sulfation is a common metabolism seen in biological systems generally leading to elimination of the compound. In the case of resveratrol, the 3-*O*-sulfate is the major metabolite found in rat and human plasma after administration.[91,96,97] The sulfation occurs by metabolic transformation of the alcoholic residue with cytosolic sulfotransferases SULTs. [182-184] Different methods are available in chemistry to synthesise sulfates from alkyl or aryl alcohols. Most of them involve the use of sulphur trioxide, sulphur trioxide complexes,[185,186] chlorosulfonic acid [187-189] or sulphuric acid.

The results below were obtained by Miss Zelina Philipou, a MSc student, who prepared the synthesis of the sulfates of the analogues **7a-c**.

12.1.1. Sulfation of analogues 7a-b

Initial attempts at sulfation were carried out on the dimethoxy analogues **7a-b** as there is only one possible position for the conjugation to occur. The reactions were based on Yu *et al.*[91] and on work previously done in the lab using SO_3·DMF complex as sulfation agent in DMF/pyridine with sodium carbonate as a base (Scheme 29, Table 11).



Scheme 29: Sulfation reaction

Analogue	R_1	R_2	Equiv. of SO_3·DMF	Equiv. of Na_2CO_3	Time of reaction (h)	Results
7a	H	CH_3O	2.1	1.1	4	Mass seen in MS
7b	CH_3O	CH_3O	1.1	2.7	4	Mass seen in MS

Table 11: Experimental conditions for sulfation of analogues 7a-b

The crude reaction mixtures were injected directly on LC/MS to monitor the formation of the sulfates as the reaction was difficult to monitor by TLC. Figure 16 and Figure 17 show the LC/MS results of the injections. The presence of peaks with a *m/z* of 319 in negative mode corresponding to **22a** less the sodium (Figure 16) and a *m/z* of

349 in negative mode corresponding to **22b** less sodium (Figure 17) indicate that the sulfation occurred in both cases.

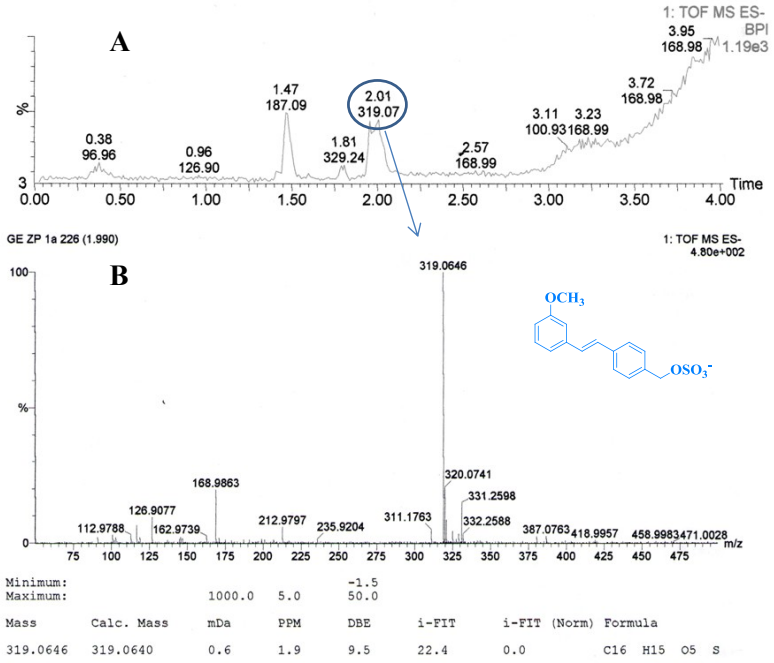


Figure 16: LC/MS of crude sulfation of analogue 7a. (A) Time of flight spectra of crude mixture after ESI. (B) Mass spectrograph of peak at 2.01 min corresponding to the mass of **22a**.

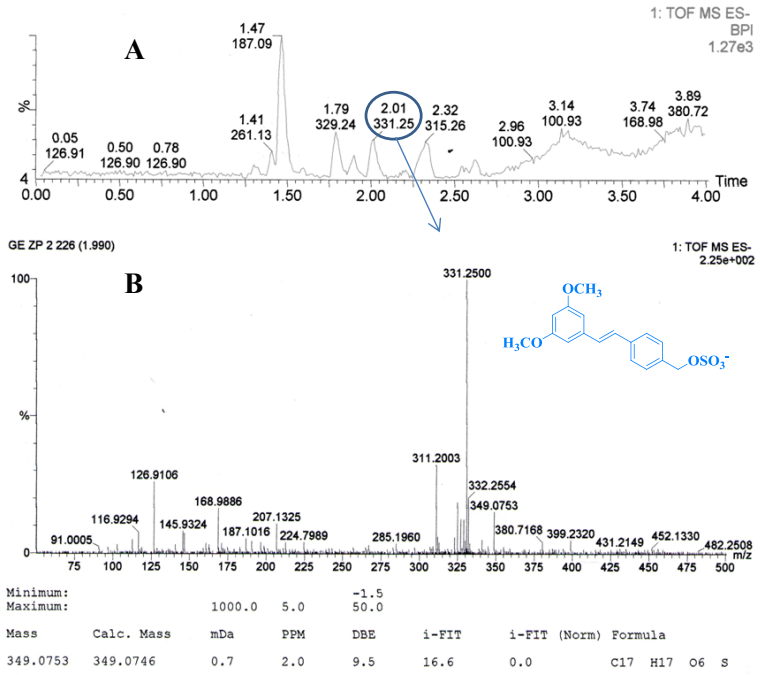


Figure 17: LC/MS of crude sulfation of analogue 7b. (A) Time of flight spectra of crude mixture after ESI. (B) Mass spectrograph of peak at 2.01 min corresponding to the mass of **22b**.

A further purification by preparative HPLC using a reverse phase column would have been considered if there were no problems of solubility. Indeed the two crude solids obtained after reaction were not soluble in any solvent tried (MeOH, EtOH, MeCN, DCM, Et_2O and H_2O) except DMSO, which made the HPLC purification impossible to perform. Preparative HPLC was indeed considered as a possible technique for the isolation of the pure sulfates because it has been used by both Yu[91], Hoshino[99] and during previous work in the lab. This method of purification of the synthetic mono- and di-sulfates of resveratrol was previously used applying a gradient of elution starting with 5 mM ammonium acetate with 2% propanol-2-ol and gradually increasing the concentration to 98% MeOH with 2% propan-2-ol. Even if obtaining pure monosulfates **22a** and **22b** would have been better, having a sample containing the sulfates and the parent compounds is enough for a comparison with the future potential enzymatically-produced-monosulfates.

The 400MHz ^{1}H NMR in DMSO of analogue **7a** shows a triplet (a) integrating for 1H at 5.18 ppm and a doublet (b) integrating for 2H at 4.50 ppm both with a coupling constant of $^3J_{H\text{-}H}$ = 5.6 Hz corresponding to the benzylic alcohol (Figure 18). In the case of the sulfation of **7a** leading to **22a**, the crude ^{1}H-NMR shows a singlet (c) at 4.77 ppm corresponding to the methylene of the benzylic alcohol. The coupling with the hydroxyl is lost which allows us to conclude that the sulfation occurred (Figure 19). We cannot conclude on the formation of **22b** as the NMR did not show the singlet corresponding to the methylene protons of the benzylic alcohol.

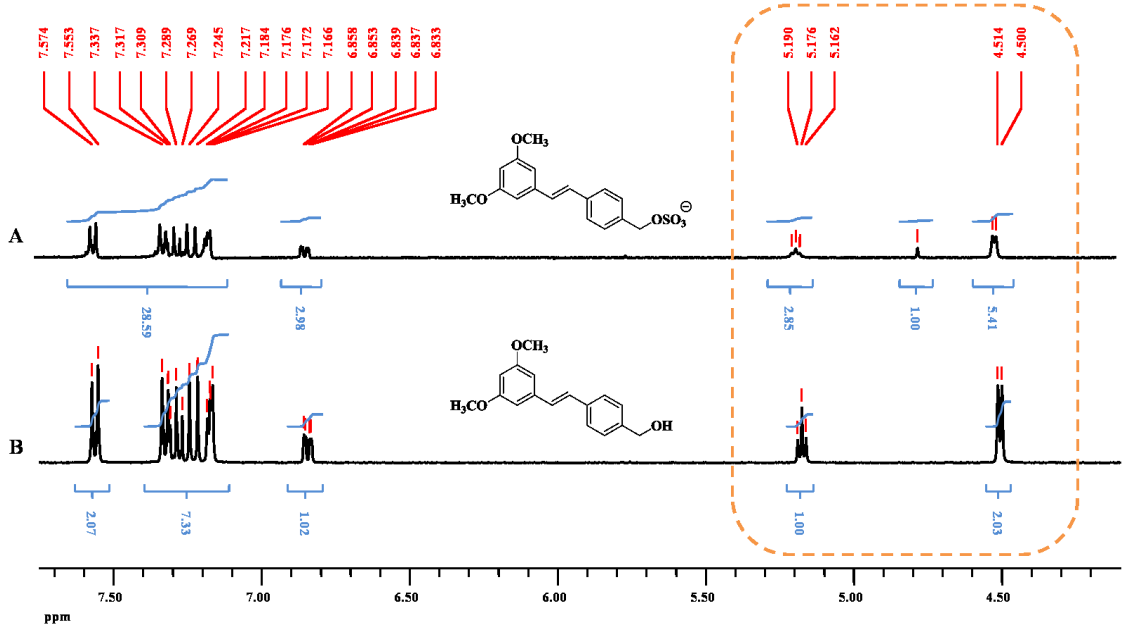


Figure 18: 400 MHz ^{1}H-NMR of crude sulfation of 7b (A) and of 7b alone (B) in DMSO

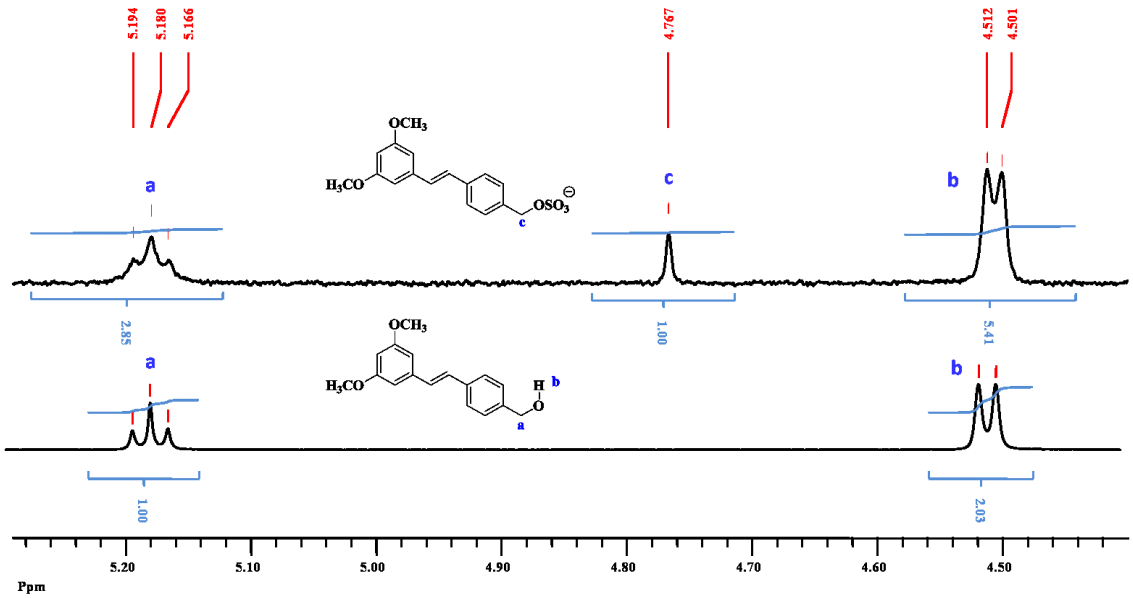


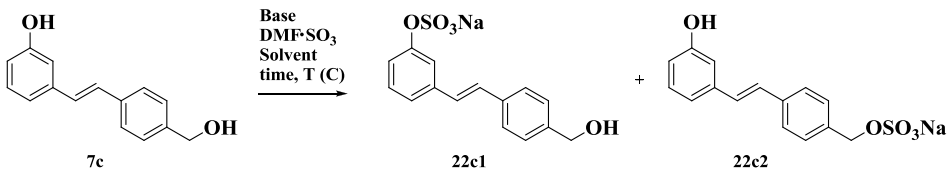
Figure 19: Expansion of the 4.40-5.30 ppm region of the crude sulfation of 7b and 7b alone

12.1.2. Sulfation of analogue 7c

Analogue **7c** has two hydroxyl groups which means that both of them can be sulfated. Initially it was decided to carry out a selective mono-sulfation on the phenolic

hydroxyl group by reacting **7c** first with a base and then with a sulfating agent to give **22c1**. Megati *et al.*[190] patented a method for selective sulfation of an aromatic hydroxyl group over an aliphatic hydroxyl group where both are present in the same molecule (estradiol and estradiol derivatives), without employment of protecting groups. They developed this method based on the pK_a difference between an aromatic hydroxyl which is lower than 14 (*e.g.* pK_a = 9.92 for phenol) and an aliphatic alcohol which pK_a is greater than 14 (pK_a = 15.9 for ethanol). They stated that by the use of a strong base such as sodium methoxide or a metal hydride in a polar aprotic solvent, the sulfation would occur exclusively on the aromatic hydroxyl and not on the aliphatic.

Based on these suggestions two experiments were designed for the monosulfation of **7c** using two different bases, NaOMe (Experiment 1) and NaH (Experiment 2) and DMF·SO_3 as a sulfating agent. Table 12 summarizes the conditions for the two experiments that have been performed.

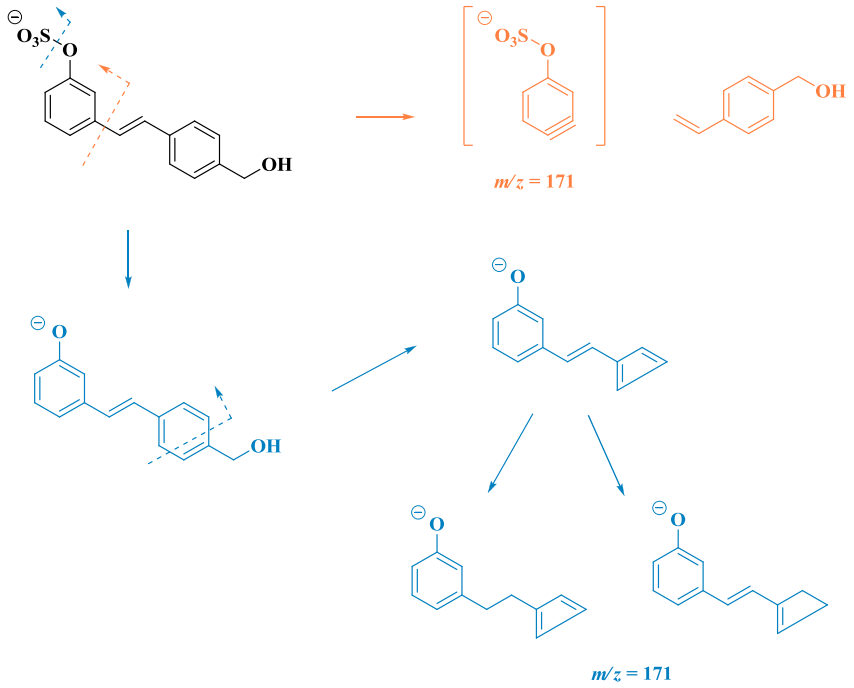


	Experiment 1	Experiment 2
Equivalents of 7c	1	1
Equivalents of base	1	1
Equivalents of DMF·SO_3	1	1
Reaction solvent	Dry THF	Dry THF
Reaction temperature	R.T.	80 °C for 1 hour
Reaction duration	o/n	65 hours

Table 12: Details and condition of the experiments performed for the selective sulfation of the aromatic hydroxyl group of 7c.

The ratio 1:1:1 **7c**: base: SO_3·DMF was chosen in order to make sure that only the aromatic hydroxyl group would be sulfated. For experiment 1 it was decided to leave it stirring overnight as the TLC monitoring of the reaction did not show completion of the reaction in the first 3 hours. For experiment 2 it was decided to heat the reaction for 1 hour in order to see if the reaction would go to completion. TLC monitoring again did not show the completion of the reaction thus it was decided to leave the reaction stirring at room temperature over the weekend. The same solubility limitations were observed for these crudes as for the **7b-c** sulfation crudes. Due to the fact that the reactions were performed on a small scale it was decided not to try any other methods (for example extraction or precipitation) to isolate the pure product. Instead it was chosen to analyze the crude with LC-MS.

The crude reaction mixtures were injected directly on LC/MS to monitor the formation of the sulfates as the reaction was difficult to monitor by TLC. Figure 20 and Figure 21 show the LC/MS results of the injections. The presence of peaks with a *m/z* of 305 in negative mode corresponding to **22c1** or **22c2** less the sodium indicates that the sulfation occurred in both reactions but that they did not go to completion as a major peak with a *m/z* of 225 in negative mode corresponds to **7c**. A peak corresponding to a disulfate is not seen in either case. However, we cannot really deduce from the LC/MS studies on which position the sulfation occurred, except maybe for the peak with a *m/z* of 171. This *m/z* value could correspond to a fragmentation between the two rings, the sulfate being attached to the phenol or of a fragmentation/ rearrangement sequence from the parent product (Scheme 30).



Scheme 30: Possible fragmentation leading to *m/z* 171 peak

^{1}H-NMR of the crude products **22c1** and **22c2** were not conclusive as we could not see much difference with the analogue **7c**. The NMR did not allow us either to measure a conversion level for the sulfation reaction.

Another sulfation reaction was attempted on **7c** in the same conditions as for analogues **7a-b** but it did not give any mono- or di-sulfate (LC/MS, results not shown). No further reactions were performed due to time limitations, but other solvents, temperature and base conditions should be tried to form the desired sulfates.

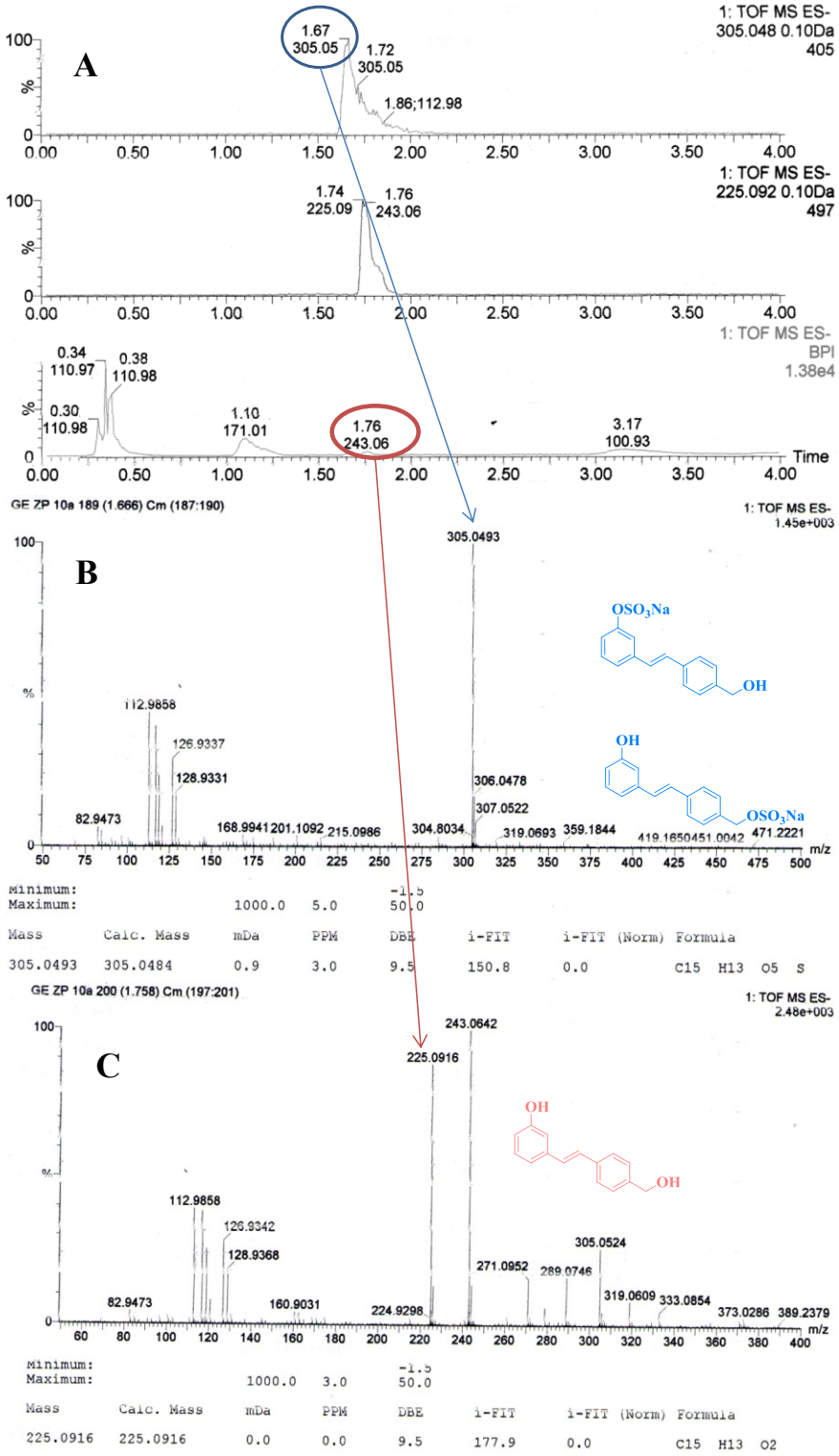


Figure 20: LC/MS of crude sulfation of analogue 7c using NaOMe as a base. (A) Time of flight after ESI. (B) Mass spectrograph of peak at 1.66 min. (C) Mass spectrograph of peak at 1.75 min.

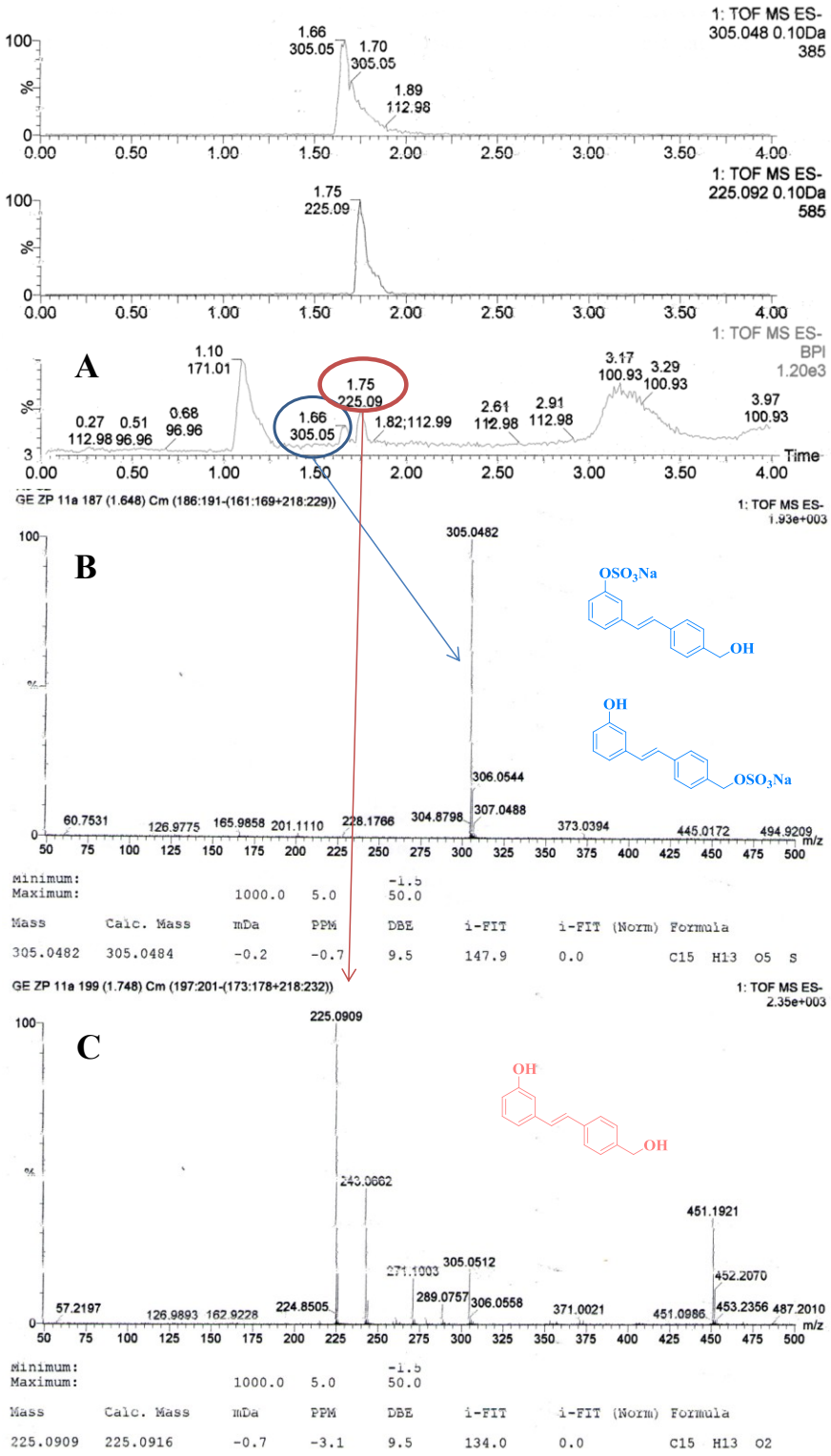
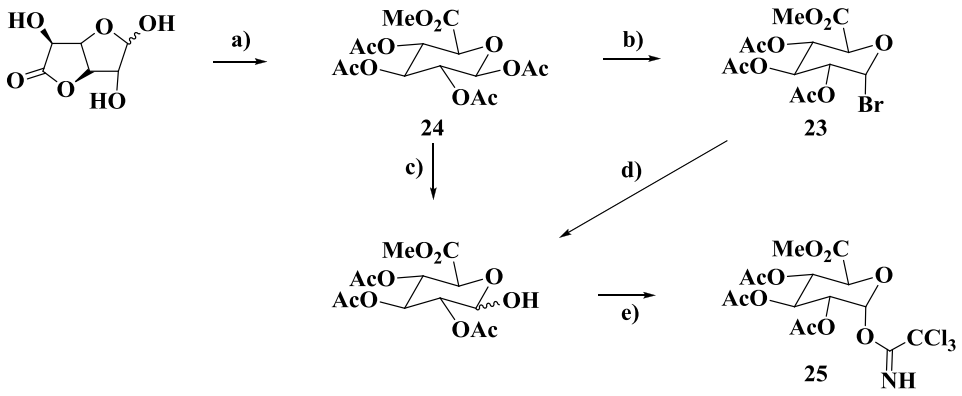


Figure 21: LC/MS of crude sulfation of analogue 7c using NaH as a base. (A) Time of flight after ESI. (B) Mass spectrograph of peak at 1.66 min. (C) Mass spectrograph of peak at 1.75 min.

12.2. Glucuronidation

Glucuronidation is a glycosylation reaction generally occurring in biological systems during a metabolic transformation of a substrate. Biologically, the substrate is attached to a glucuronide moiety by uridine 5'-diphosphoglucuronyl transferase.[191] Chemical addition of sugar moieties to an alcohol is quite well described and has been reviewed by Stachulski.[192] The most common and popular reaction of glucuronidation of aryl and alkyl alcohols is the Koenigs-Knorr method using 1α-bromo-sugar **23** and a catalyst which is generally a silver (I) salt (Ag_2CO_3, Ag_2O, AgOTf). The bromo-sugar **23** is commercially available but is not very stable and can be obtained from tetra-*O*-acetyl-(α or β)-D-glucopyranuronate **24** (Scheme 31). The other quite widely used glucuronide precursor is the 1α-trichloroacetimidate **25** which can be indirectly made from **23** (Scheme 31) and is generally used with Lewis acid $BF_3.OEt_2$[193] or TMSOTf.[194]



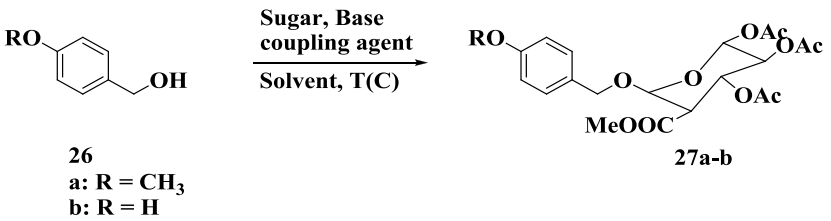
a) NaOMe, MeOH then Ac_2O, Pyridine; b) HBr-AcOH; c) *n*-Bu_3SnOMe; d) Ag_2CO_3, H_2O; e) Cl_3CCN, base

Scheme 31: Synthesis of 1α-bromosugar 23 and of trichloroacetimidate-sugar 25

Before doing the coupling reaction on the final analogues **7a-h**, we decided to investigate different reaction conditions following published methods and techniques

used in the lab on 4-methoxybenzylalcohol **26a** which would be a good model compound for the analogues **7a-b** containing both a benzylic alcohol and at least one methoxy group. The method was also attempted using 4-hydroxybenzylalcohol **26b**, a mimic of analogues **7c-d** (Scheme 32, Table 13).

The desired glucuronides were not obtained under any of our reaction conditions (Table 13). Using the bromo-sugar **23** with silver (I) oxide in acetonitrile at r.t. only gave the acetate protected benzylic alcohol (entry 1). In the other cases (entries 2-4) only complex mixtures were recovered not showing any sugar attachment (ESI-MS, results not shown).



Scheme 32: Test reaction of glucuronidation

Entry	R	Sugar	Base	Coupling Agent	Solvent	T(°C)	Result
1	CH_3	1α-bromo **23**	$(CH_3)_3N$	Ag_2O	MeCN	r.t.	Acetate
2[195]	CH_3	Cl_3CCNH **25**	-	$BF_3.OEt_2$	DCM	-15	Complex mixture
3[196]	CH_3	1α-bromo **23**	TMU	AgOTf	DCM	-78 → -20	Complex mixture
4	H	1α-bromo **23**	$(CH_3)_3N$	Ag_2O	MeCN	r.t.	No more SM but complex mixture

Table 13: Glucuronidation conditions

Direct glucuronidation of analogue **7b** was attempted by a Koenigs-Knorr method[197] with the 1α-bromosugar, Ag_2CO_3, molecular sieves in dry toluene at r.t. but

the reaction did not go to completion and the main product was the starting material **7b** in a complex mixture. Similar results were obtained when the reaction was tried on analogue **7d**.

No further work on glucuronidation was done because of time constraints but other published method may be attempted in the future. Stachulski[198] attached glucuronic acid to a benzylic position quantitatively with pivaloyl analogue of acetate-1α-bromo sugar **23**, with *N*-iodosuccinimide (NIS) as a coupling agent in 1,2-dichloroethane (DCE). Fischer used the trichloroacetimidate **25** with TMS-OTf and obtained the glucuronide of benzyl alcohol in average yield.[194]

In summary we managed to synthesise and identify sulfates **22a** and **22b**, and probably the formation of **22c1** or **22c2**. Further work is required on the synthesis of the sulfates to have them in better yields and fully characterised as well as the synthesis of the other possible sulfates. The glucuronidation attempts were unsuccessful, and further work is required to obtain the glucuronides of the analogues **7a-g**.

CHAPTER 3: PHARMACOLOGICAL STUDIES

CHAPTER 3 – PHARMACOLOGICAL STUDIES

13. Metabolism studies

Numerous metabolism studies have been carried out on resveratrol, but to our knowledge, none of the analogues synthesised have had their phase II conjugation investigated.

Methoxy substituents on an aromatic ring cannot be directly metabolised by phase II enzymes, but can be demethylated by phase I enzymes such as CYP450s with NADPH as a cofactor,[199,200] to the phenol metabolite. The latter can be metabolised further to the sulfates or glucuronides in presence of phase II enzymes. As explained earlier, substitution of one of the phenolic groups to a benzylic alcohol in noradrenaline led to salbutamol where metabolism by COMT was stopped.[135] Therefore, systematically replacing the phenols present on the resveratrol structure with benzylic alcohols and/or methoxy groups will allow us to assess whether the replacement of phenols can slow down or even stop the metabolism in *in vitro* systems compared to resveratrol.

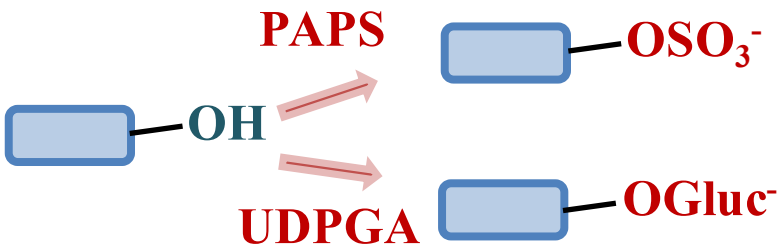
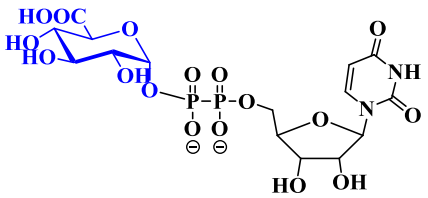
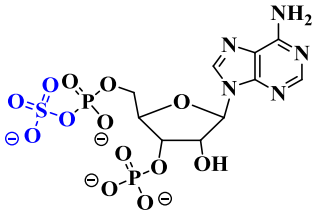


Figure 22: Schematic representation of *in vitro* metabolism of resveratrol and of the analogues

The metabolism assays were performed using both mouse and human liver fractions with the corresponding cofactor (Figure 22). Sulfation and glucuronidation were assessed separately. Sulfation was performed with mouse or human liver cytosol in presence of the cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS - Figure 23) and the glucuronidation was assessed with mouse or human liver microsomes in presence of the cofactor uridine 5'-diphosphate glucuronic acid (UDPGA - Figure 23).



UDPGA: Uridine 5'-diphosphate glucuronic acid



PAPS: 3'-phosphoadenosine-5'-phosphosulfate

Figure 23: Cofactors used for the sulfation and glucuronidation metabolism assays

13.1. Comparison of metabolism of resveratrol and resveratrol analogues with mouse and human liver fractions.

In vitro metabolism of the synthetic resveratrol analogues was carried out according to the method developed in our laboratory by H. Cai *et al.*[132] Each experiment was performed in duplicate and is described in the experimental section (cf. 20. Pharmacology experiments – Materials and Methods).

13.1.1. Sulfation

13.1.1.1. Chromatograms of the metabolic mixtures of sulfation of resveratrol and its analogues in mouse liver cytosol

In order to compare the sulfation of resveratrol analogues **7a-e** and resveratrol **1a**, the compounds (10 μM) were incubated with liver cytosol in the presence of PAPS as cofactor. The disappearance of the parent compound was quantitated by HPLC. The corresponding HPLC traces are shown in Figure 24-Figure 29.

With the HPLC system used, **1a** has a retention time of around 8.7 min and the peak completely disappeared within 30 min in the mouse metabolism system in favour of a more polar peak, with a retention time ~ 6.0 min (Figure 24). The area of the new metabolite peak increased with incubation time. This is consistent with the attachment of a sulfate moiety to produce a mono-sulfate. The area of this peak decreased after 30 min incubation time in favour of a more polar peak at 2.5 min, which could correspond to a disulfate. This peak is in the solvent front, so no conclusion on its

nature can be made.

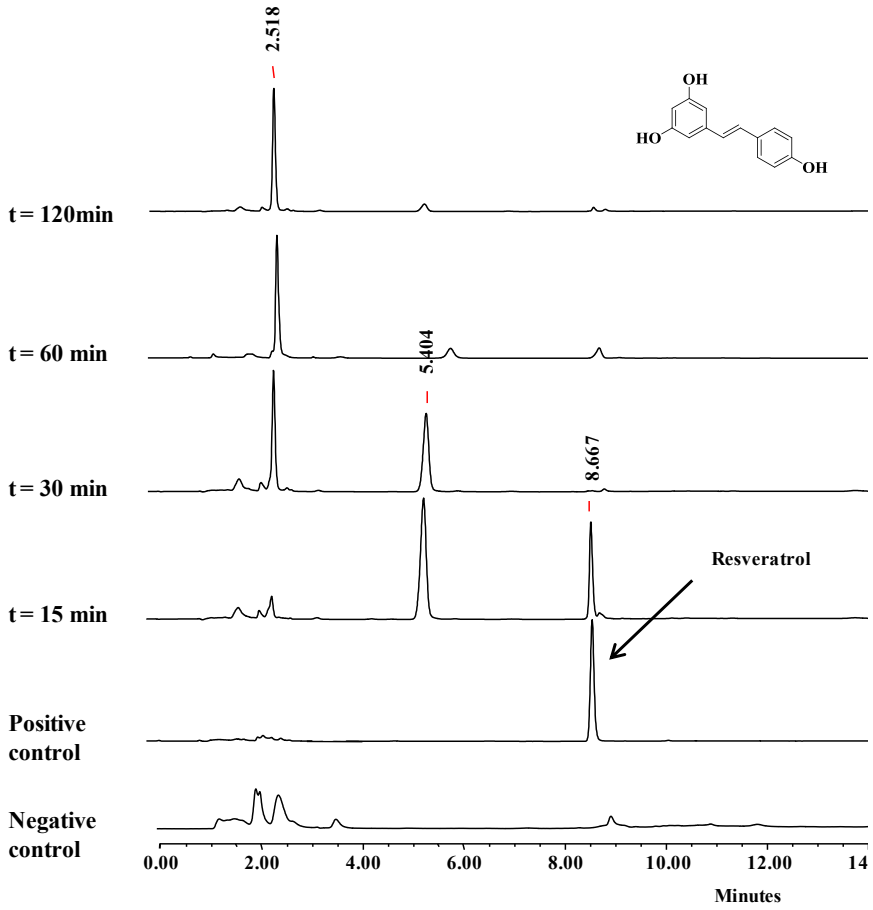


Figure 24: Representative chromatograms of incubation mixtures of resveratrol 1a with mouse liver cytosol in the presence of PAPS as cofactor for different incubation times. The negative control corresponds to the cytosol without any resveratrol. The positive control corresponds to incubation time at t=0min. For the HPLC conditions see Materials and Methods

The mono-methoxy-monobenzylic analogue **7a** has only one free hydroxyl group on the scaffold available for sulfation. **7a** has a retention time of around 11.5 min under the same HPLC conditions and the peak intensity decreased with increasing length of incubation until there was almost no more **7a** detected at 120 min (Figure 25). A new peak was eluted with a shorter retention time at 9.7 min. The increased polarity is consistent with the attachment of a sulfate to the hydroxyl group of the parent compound. The peak at 9.5 min could be a degradation product.

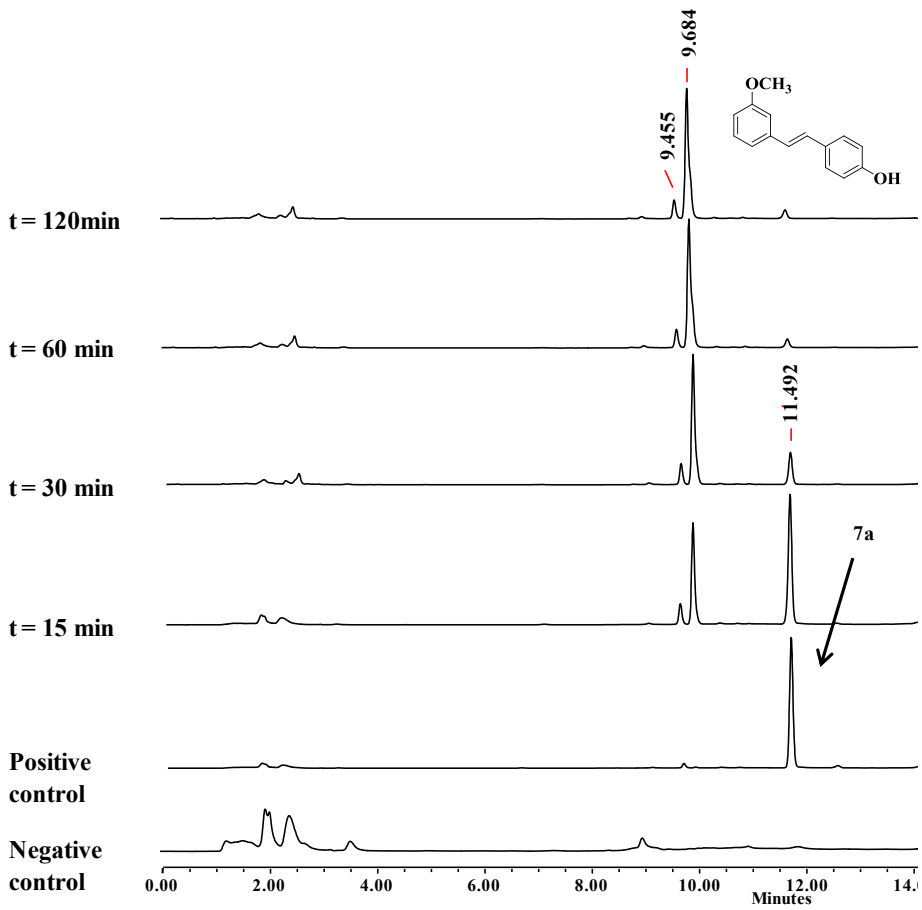


Figure 25: Representative chromatograms of incubation mixtures of resveratrol analogue 7a with mouse liver cytosol in the presence of PAPS as cofactor for different incubation times. The negative control corresponds to the cytosol without any **7a**. The positive control corresponds to incubation time at t=0min.For the HPLC conditions see Materials and Methods

The profile of sulfation of dimethoxy-monobenzylic analogue **7b** is very similar to analogue **7a**. **7b** has a retention time of around 11.9 min under the HPLC conditions, and the peak intensity decreased with incubation time (Figure 26). A peak was eluted in front of the parent compound with a retention time of 9.9 min, which is constituent with the increased polarity due to the attachment of a sulfate to the hydroxyl group of the parent compound.

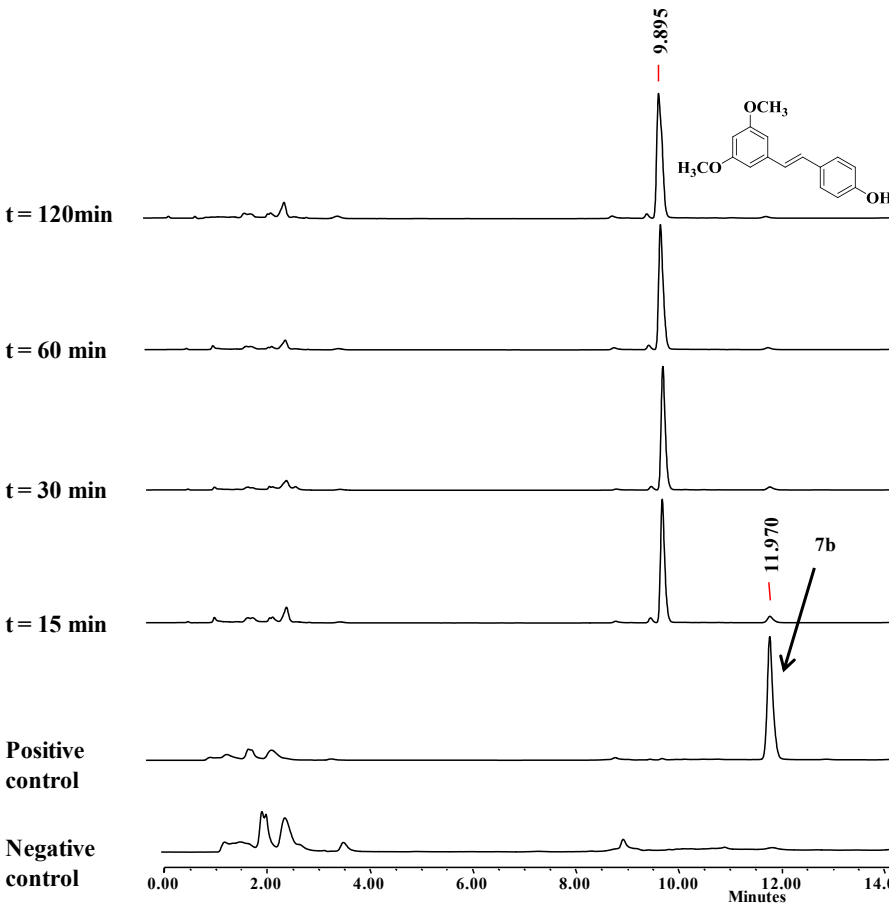


Figure 26: Representative chromatograms of incubation mixtures of resveratrol analogue 7b with mouse liver cytosol in the presence of PAPS as cofactor for different incubation times. The negative control corresponds to the cytosol without any **7b**. The positive control corresponds to incubation time at t=0min. For the HPLC conditions see Materials and Methods

The mono-phenol-mono-benzylic analogue **7c** has a retention time of 10.5 min in the HPLC system employed (Figure 27). The peak area of the parent compound decreased over time in favour of two major peaks at 4.1 and 8.4 min. The area of the peak at 8.4 min increased after 15 min incubation but decreased afterwards in favour of the peak at 4.1 min. This could be related to the formation of a mono-sulfate, with a retention time of 8.4 min, this mono-sulfate being the precursor to the possible disulfate eluting at 4.1 min. The peak at 9.0 min came from the negative control.

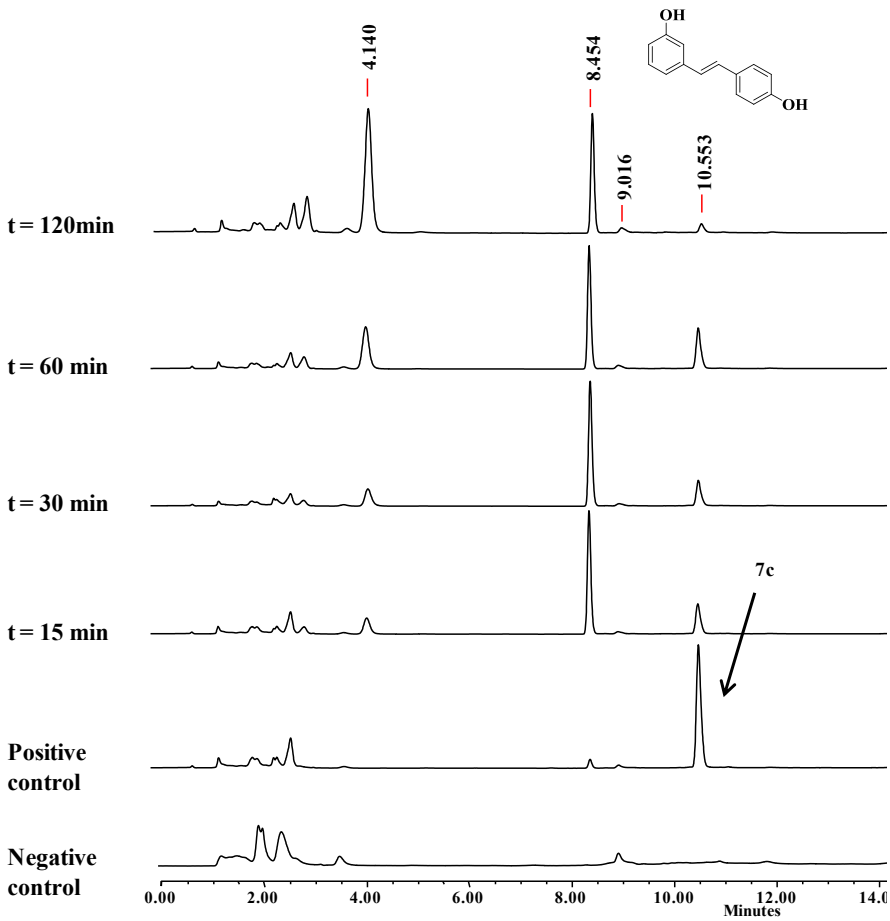


Figure 27: Representative chromatograms of incubation mixtures of resveratrol analogue 7c with mouse liver cytosol in the presence of PAPS as cofactor for different incubation times. The negative control corresponds to the cytosol without any **7c**. The positive control corresponds to incubation time at t=0min. For the HPLC conditions see Materials and Methods

The di-phenol-monobenzylic alcohol analogue **7d** has a similar pattern of disappearance as analogue **7c**. **7d** has a retention time of 9.2 min and its peak area diminished during the incubation time, this being accompanied by the appearance of a more polar peak at 6.0 min (Figure 28). The area of this peak was the greatest after 15 min incubation time and then decreased after longer reaction time (30 to 120 min). The disappearance of this peak coincided with an increase of the peak at 2.3 min, but this is near the solvent front so it is hard to make definite conclusions.

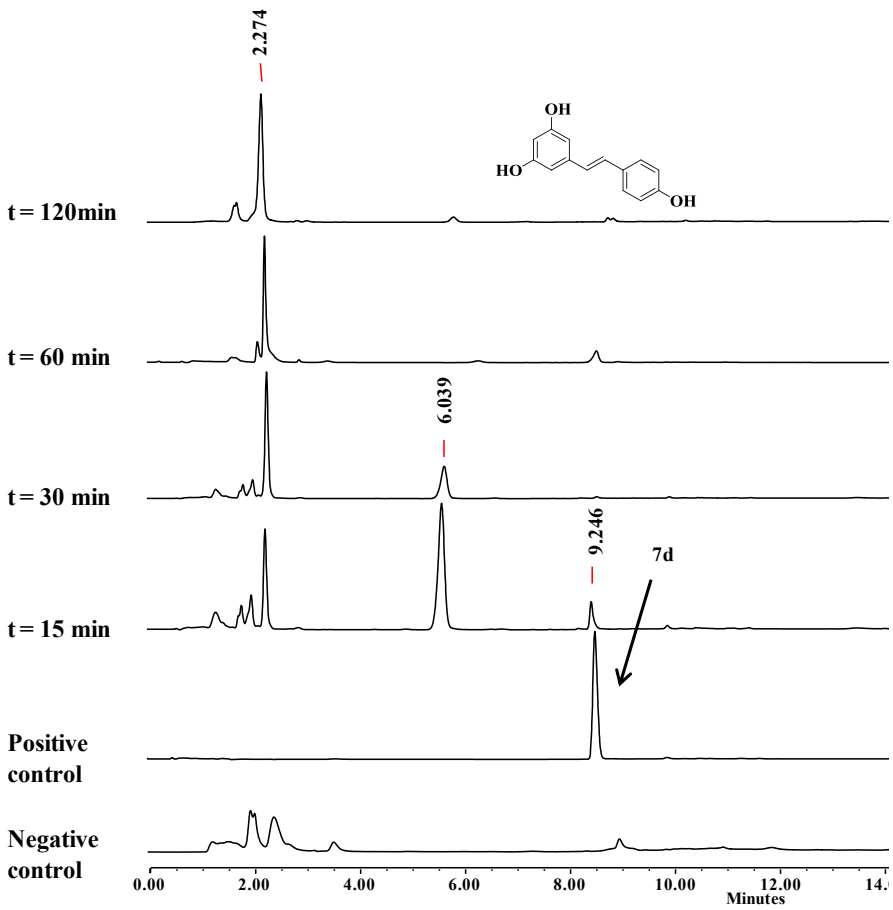


Figure 28: Representative chromatograms of incubation mixtures of resveratrol analogue 7d with mouse liver cytosol in the presence of PAPS as cofactor for different incubation times. The negative control corresponds to the cytosol without any **7d**. The positive control corresponds to incubation time at t=0min. For the HPLC conditions see Materials and Methods

In the case of the tribenzylic analogue **7e**, the HPLC traces obtained after the different incubation periods are more complicated as there are three possible positions for sulfation (Figure 29). **7e** has a retention time of around 9.1 min. The peak intensity decreased in favour of three major peaks at 2.4, 3.9 and 6.5 min which could be related to the tri-, di-, and mono-sulfate metabolites respectively. However, the peak at 2.4 min is in the solvent front so it is hard to draw conclusions as to the nature of this peak.

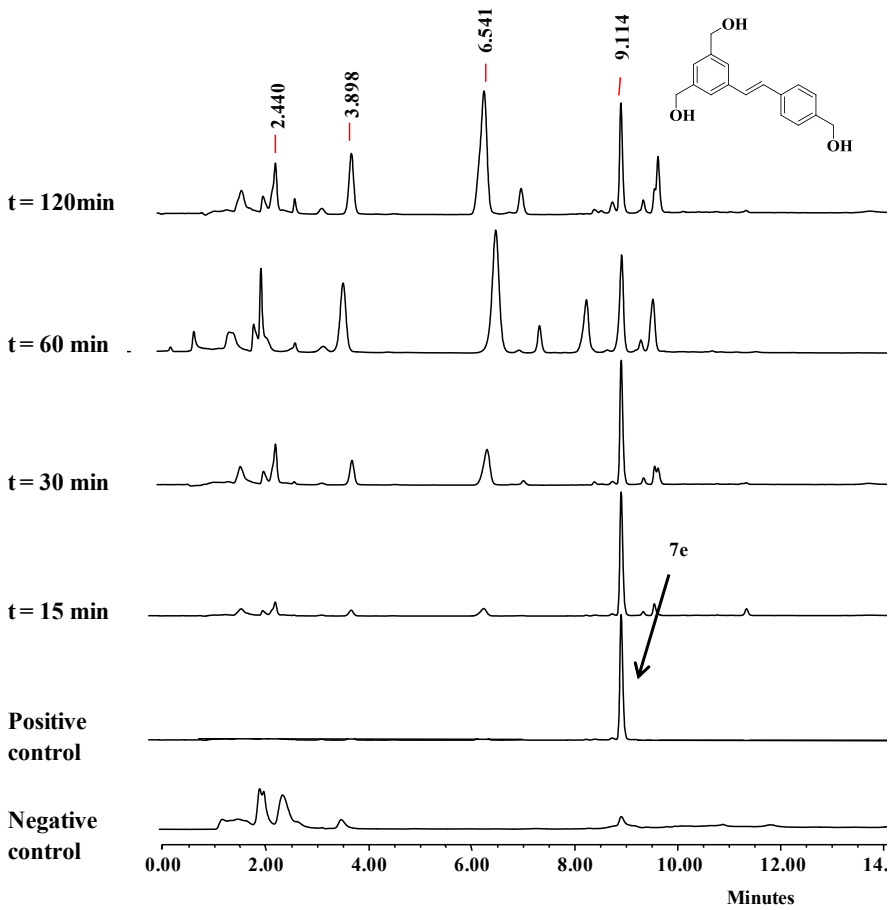


Figure 29: Representative chromatograms of incubation mixtures of resveratrol analogue 7e with mouse liver cytosol in the presence of PAPS as cofactor for different incubation times. The negative control corresponds to the cytosol without any **7e**. The positive control corresponds to incubation time at t=0min. For the HPLC conditions see Materials and Methods

13.1.1.2. Evaluation of the sulfation in mouse liver cytosol

The graphs below express the percentage of remaining parent compound at a time *t* as compared to the positive control (t = 0 min). The starting concentration of resveratrol, its analogues and cytosol was the same in each of the metabolic mixture. The volume injected on HPLC was the same for each metabolic mixture. The percentage of remaining parent compound in the incubation mixture at a time *t* can then be expressed as the ratio of the peak area at time *t* over the peak area at t = 0 min.

$$\% \text{ (Remaining of compound)} = (\text{peak area (t)})/(\text{peak area (0)}) \times 100$$

Figure 30 shows the remaining amount of resveratrol and its analogues over time compared to a positive control as 100%, obtained after incubation without the cofactor. Figure 30A shows the disappearance of each analogue **7a-b** and **7e** in comparison with resveratrol **1a**. These analogues do not contain any free phenol and have a rate of disappearance slower than resveratrol. Resveratrol disappears within 30 min, while traces of monomethoxy analogue **7a** can still be detected after 2h incubation. Dimethoxy analogue **7b** seems to have an initial metabolism rate faster than resveratrol because only 5-10% of it is detected after 15 min whereas 20% of resveratrol is still detected. However, **7b** is still detected at trace levels (1.6±0.4%) after 1h, which is not the case for resveratrol (0%). The tribenzylic analogue **7e** has a much slower metabolism rate than resveratrol after 30 min incubation, as around 50% of the analogue is still present, and after 2h incubation approximately 15% is still detected.

In contrast, the two analogues **7c** and **7d**, which contain one and two phenols on the left ring respectively, behave very similarly to resveratrol which is completely

metabolised after incubation longer than 30 min (Figure 30B). Surprisingly, the peak of analogue **7c** never disappears completely and its intensity slowly increases after 30 min to approximately 24% ± 20% of the parent compound being detected after 2h. This latter result with a large variation can be explained by a potential instability of the new potential sulfate product or because of a possible equilibrium between the different states of sulfation.

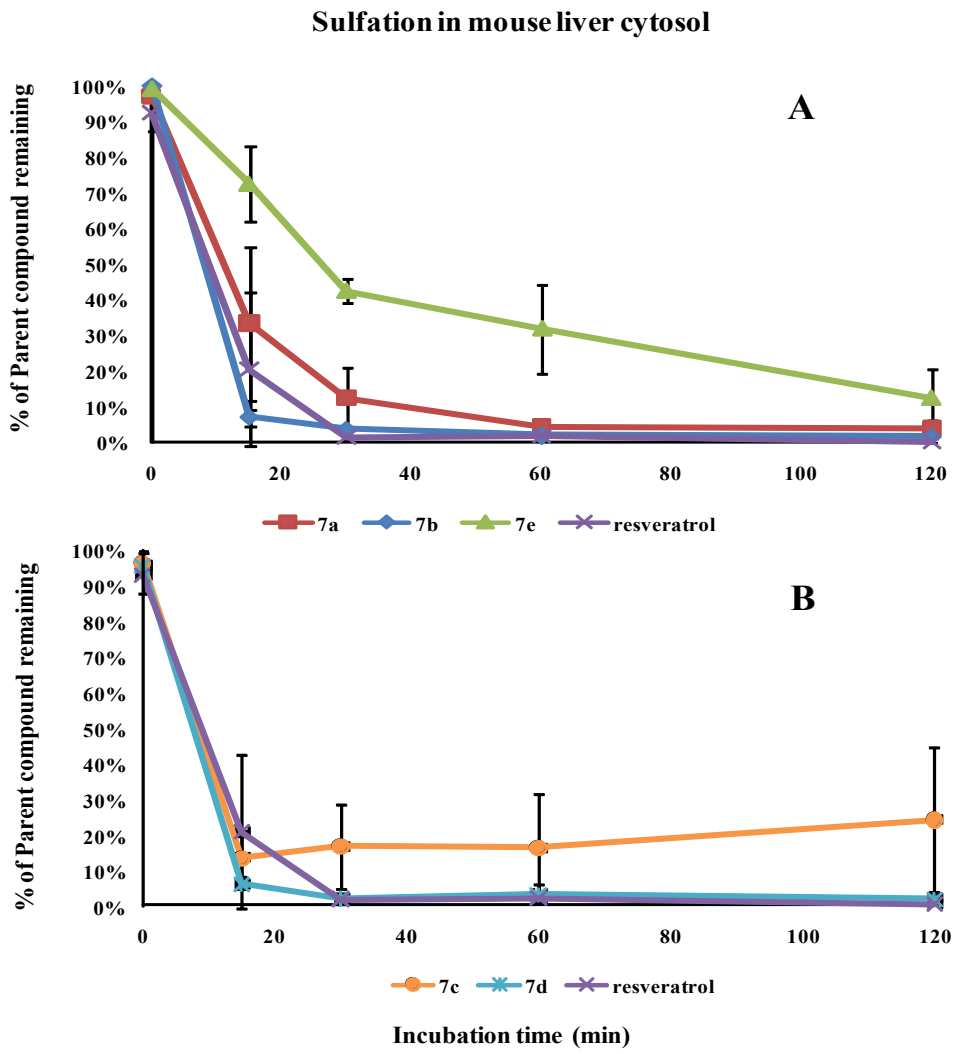


Figure 30: Removal of 7a (red line), 7b (blue line), 7e (green line) and resveratrol (purple line) (A) or 7c (orange line), 7d (cyan line) and resveratrol (purple line) (B) from incubations with mouse liver cytosol and PAPS. Substrate concentration was 10 µM. Values are expressed as percentage of control incubations without mouse liver cytosol and they represent mean ± SD of 6 separate incubations using pooled liver fraction from 6 mice (3 males and 3 females).

13.1.1.3. Evaluation of the sulfation in human liver cytosol

The sulfation assay with human liver cytosol was carried out in the same way as the one using the mouse cytosol with the same HPLC system. The disappearance of the 3-mono-phenol resveratrol analogue **7c** and of 3,5-diphenol analogue **7d** were similar to resveratrol in mouse liver cytosol during the time scale studied (Figure 30B). Mono-methoxy-benzylic analogue **7a** has a slower metabolism rate than resveratrol during the time frame 0-30 min, but does not have the same substitution pattern than resveratrol. So it was decided that only the dimethoxy analogue **7b** (disappearing in a similar manner as resveratrol) and the tribenzylic analogue **7e** (slower disappearance of the parent compound) would be compared to resveratrol in human liver cytosol and also because of the presence of no free phenol and the same substitution pattern of the stilbene.

The HPLC chromatograms of the analogues tested with human liver cytosol (not shown) are very similar to those obtained from the incubation with mouse liver cytosol (Figure 24, Figure 26 and Figure 29). Overall, the removal rate by sulfation during the time frame 0-120 min of resveratrol and its analogues **7b** and **7e** was remarkably slower than in the incubation mixture with an equivalent amount of mouse liver cytosol. Resveratrol **1a** disappeared completely within 60 min in the human system compared to 30 min in the mouse assay. Disappearance of analogue **7b** is similar to resveratrol in both mouse and human systems, but was still present in very low quantities (~5%) after 60 min incubation in human system. In contrast analogue **7e** was metabolised faster in this system than in the mouse assay. In the human system, **7e** completely disappeared after 2h and only 10% remained after 60 min, whereas around 35% was un-metabolised after the same incubation time in the presence of mouse liver cytosol (Figure 31). Figure 32 shows a direct comparison of

the disappearance of the analogues by sulfation in mouse cytosol *versus* in human models.

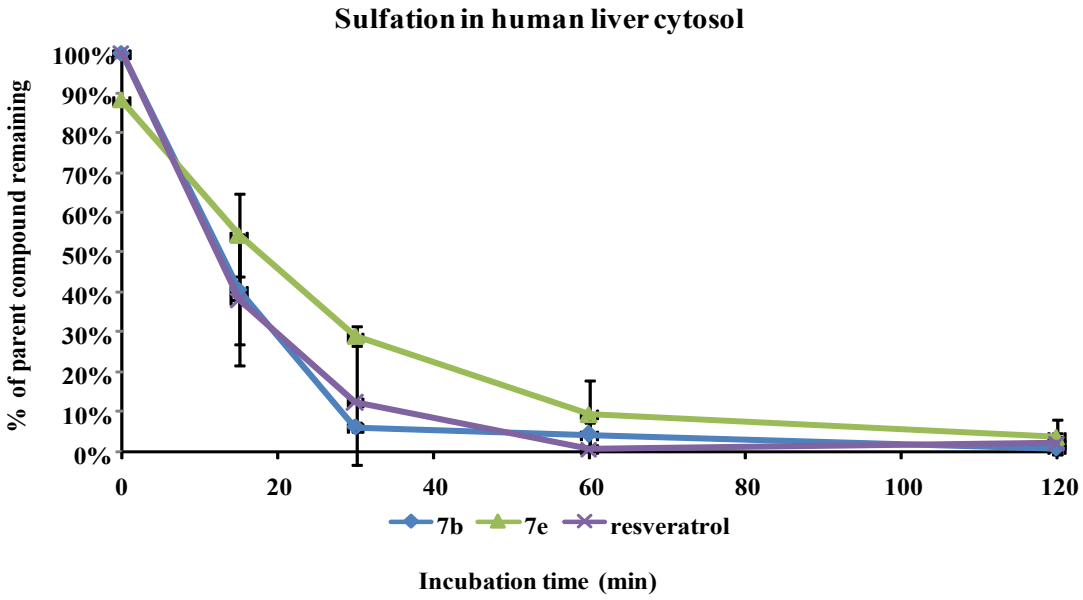


Figure 31: Removal of 7b (blue line), 7e (green line) and resveratrol (purple line) from incubations with human liver cytosol and PAPS. Substrate concentration was 10 µM. Values are expressed as percentage of control incubations without human liver cytosol and they represent the mean ± SD of 6 separate incubations using pooled liver cytosolic fractions from 23 humans.

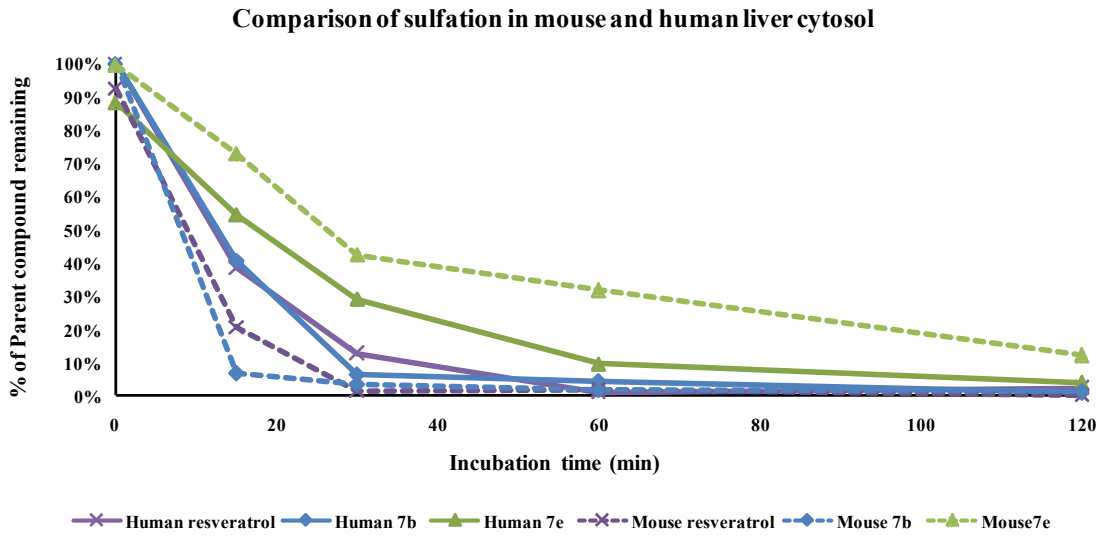


Figure 32: Removal of 7b (blue line), 7e (green line) and resveratrol (purple line) from incubations with human liver cytosol (bold lines) or mouse liver cytosol (dashed lines) and PAPS. Comparison of the values obtained for Figure 29 and 30.

These results suggest that there is an overall pattern in the structure of the analogues *versus* the rate of phase II metabolism. It seems that when there is no free phenol on the molecule, the rate of enzymatic transformation is slower in both human and mouse models. These results suggest as well that as soon as there is at least one free phenol on the stilbene, the sulfation occurs in a similar manner as resveratrol. According to Binder *et al.*[201], the lipophilicity of the molecule bearing a benzylic alcohol is a major factor in the catalytic efficiency of the rat arylsulfotransferase (AST) IV to sulfate the alcohol. They also showed that the sulfation of benzylalcohol was slower than the sulfation of phenol by this enzyme (V_{max} 22.5 and 89.9 nmol/min/mg AST IV respectively). These facts may partially explain the difference in the sulfation rates between resveratrol and the different analogues.

13.1.2. Glucuronidation

13.1.2.1. Chromatograms of the metabolic mixtures of glucuronidation of resveratrol and its analogues in mouse liver microsomes

In order to compare the glucuronidation of resveratrol analogues **7a-e** and resveratrol **1a**, the compounds (10 μM) were incubated with mouse liver microsomes in the presence of UDPGA as cofactor. The disappearance of the parent compound was measured by HPLC. The corresponding HPLC traces are shown in Figure 33-Figure 38.

In the HPLC system used, resveratrol **1a** peak at ~8.7 min completely disappeared within 15 min in the mouse system in favour of a more polar peak with a retention time of around 3.5 min (Figure 33). The retention time being shorter is

consistent with an increased polarity, which could be related to the attachment of a glucuronide moiety on the parent structure. A mixture containing all the reagents except the microsomes was used as a positive control (Figure 33). The new peak at 3.5 min appears in the positive control in a very small amount and increased immediately after addition of the microsomes at t = 0 min. This could be explained by a direct reaction of resveratrol with UDPGA in the incubation conditions. This shows that the glucuronidation may occur directly between resveratrol and UDPGA in the incubation conditions and may go faster immediately after addition of the microsomes.

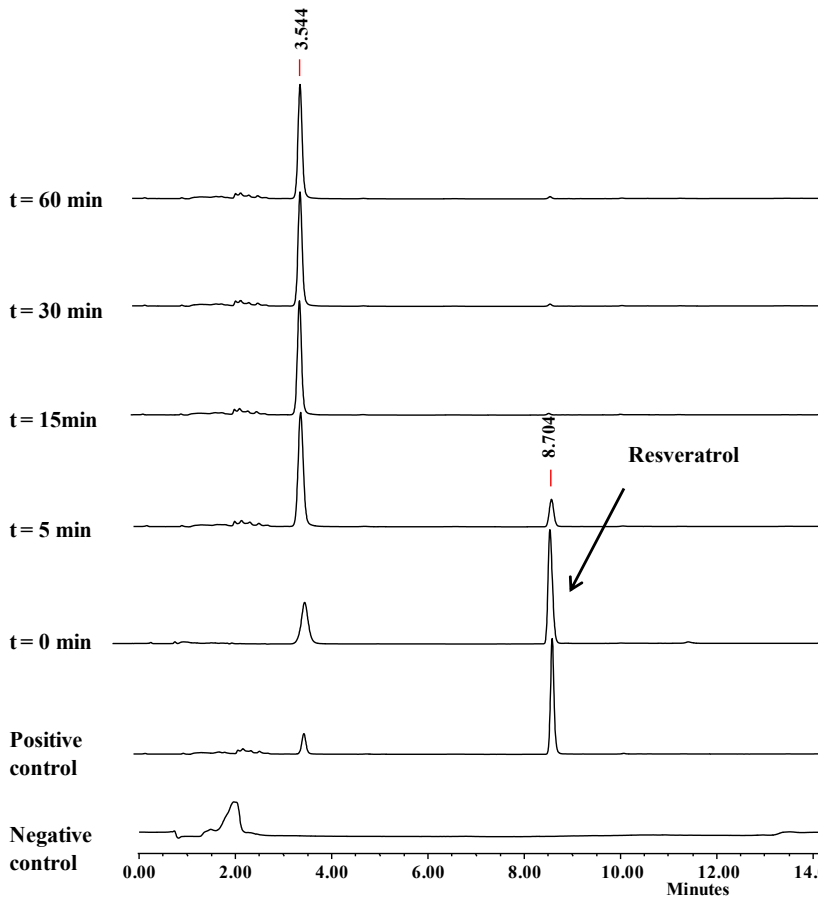


Figure 33: Representative chromatograms of incubation mixtures of resveratrol 1a with mouse liver microsomes in the presence of UDPGA as the cofactor for different incubation times. The negative control corresponds to the microsomes without any resveratrol. The positive control corresponds to the incubation mixture without microsomes. For the HPLC conditions see Materials and Methods

In the case of the mono-methoxy analogue **7a** and dimethoxy analogue **7b**, where there was only one possible position of conjugation possible, nearly no disappearance of the peaks at 11.1 and 12.0 min (for **7a** (Figure 34) and **7b** (Figure 35) respectively) was observed. A new peak at approximately 9.8 min for both analogues was detected but the peak area was very small compared to the parent compounds indicating only a small fraction is metabolised. The retention time being shorter is consistent with an increase in the polarity of the analogues which could be related to the addition of a glucuronide on the benzylic alcohol position.

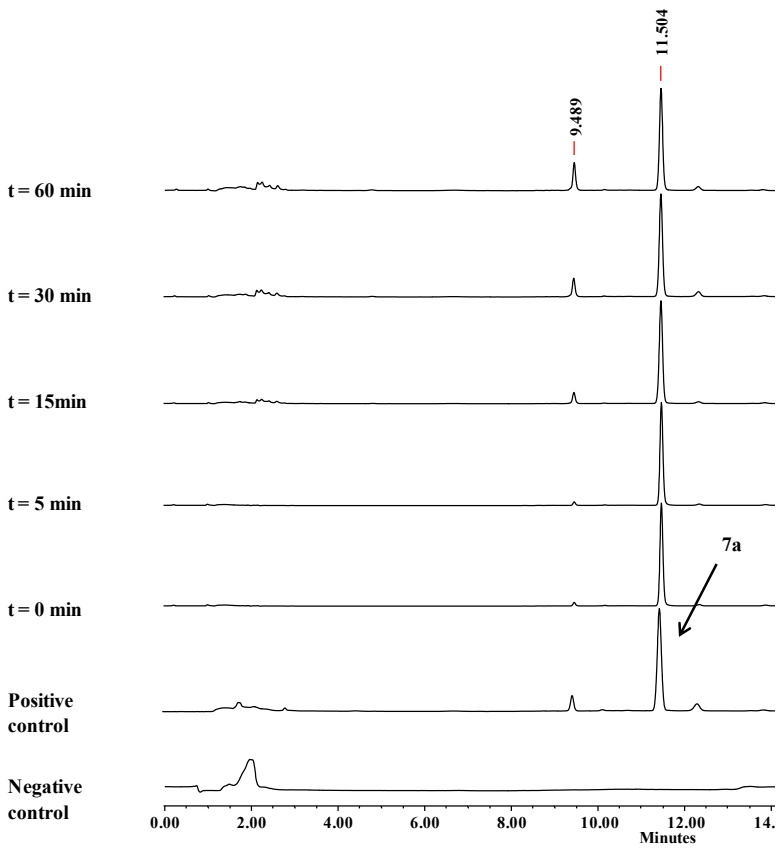


Figure 34: Representative chromatograms of incubation mixtures of resveratrol analogue 7a with mouse liver microsomes in the presence of UDPGA as the cofactor for different incubation times. The negative control corresponds to the microsomes without any **7a**. The positive control corresponds to the incubation mixture without microsomes. For the HPLC conditions see Materials and Methods

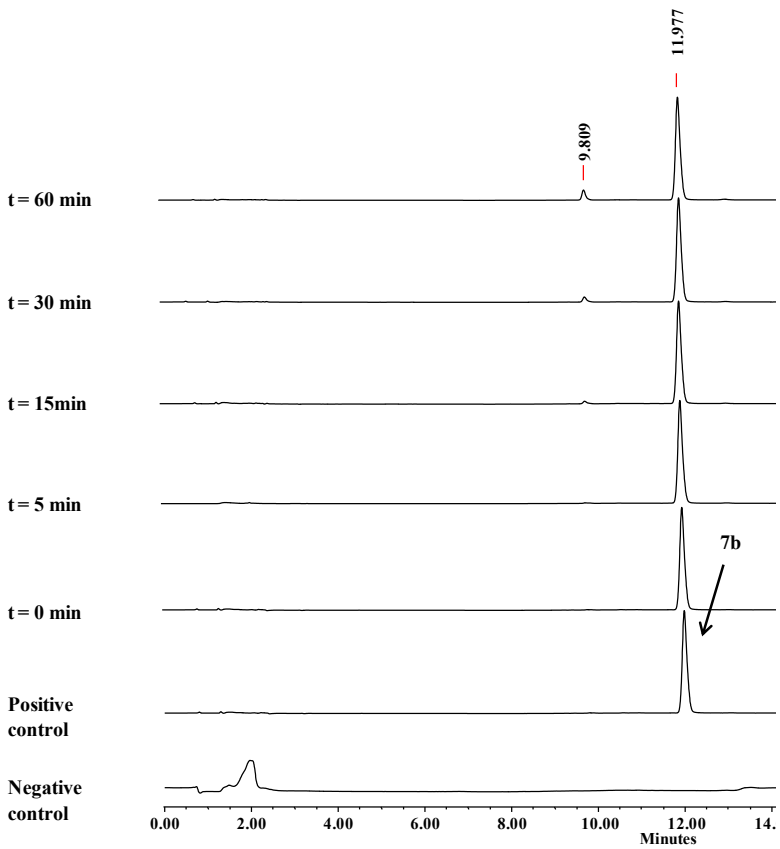


Figure 35: Representative chromatograms of incubation mixtures of resveratrol analogue 7b with mouse liver microsomes in the presence of UDPGA as the cofactor for different incubation times. The negative control corresponds to the microsomes without any **7b**. The positive control corresponds to the incubation mixture without microsomes. For the HPLC conditions see Materials and Methods

In the case of the mono-phenol analogue **7c**, a similar pattern as resveratrol is observed. The parent compound has a retention time of 10.6 min and there is a peak eluting at 7.8 min directly after addition of the microsomes (Figure 36). This is consistent with the addition of a sugar on the structure either on the phenol or on the benzylic alcohol. The peak corresponding to **7c** never disappeared completely and the minimum quantity observed is after 30 min incubation. The intensity of the peak corresponding to **7c** seems to be re-increasing slightly after 30 min incubation which could be explained by a possible instability of the possible metabolite.

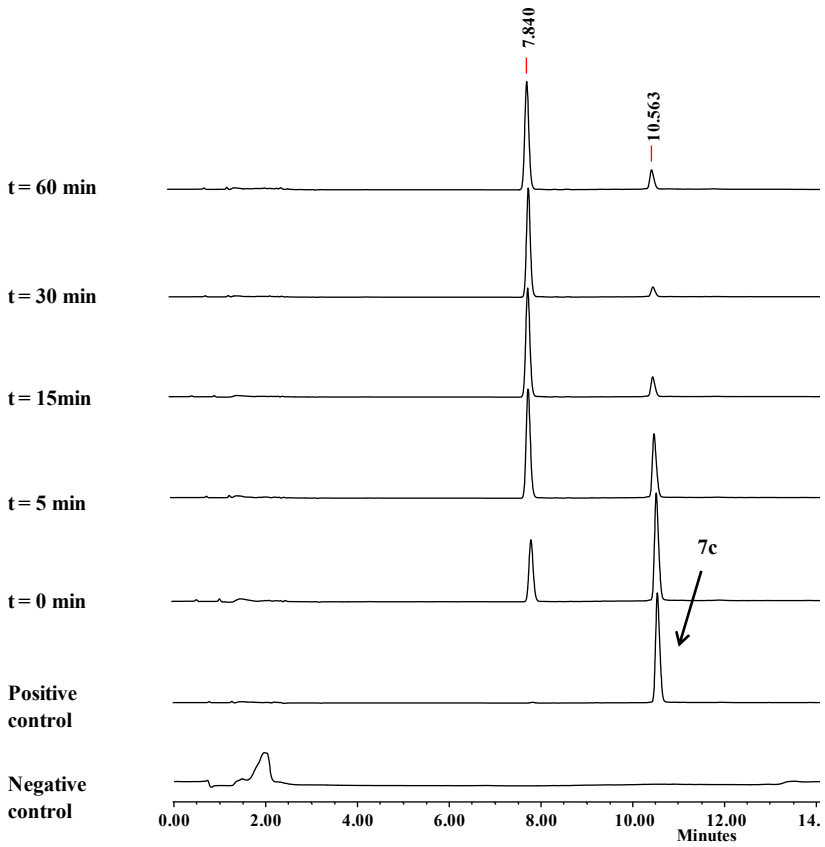


Figure 36: Representative chromatograms of incubation mixtures of resveratrol analogue 7c with mouse liver microsomes in the presence of UDPGA as the cofactor for different incubation times. The negative control corresponds to the microsomes without any **7c**. The positive control corresponds to the incubation mixture without microsomes. For the HPLC conditions see Materials and Methods

A similar pattern of disappearance was also observed with the di-phenol analogue **7d,** which has a retention time of 9.2 min (Figure 37). There are three possible positions of glucuronidation on **7d** (two phenols and one benzylic alcohol). The peak corresponding to the parent compound completely disappeared within 15 min generating a product eluting at 4.3 min, which is consistent with the increased polarity of a metabolite with a sugar attached.

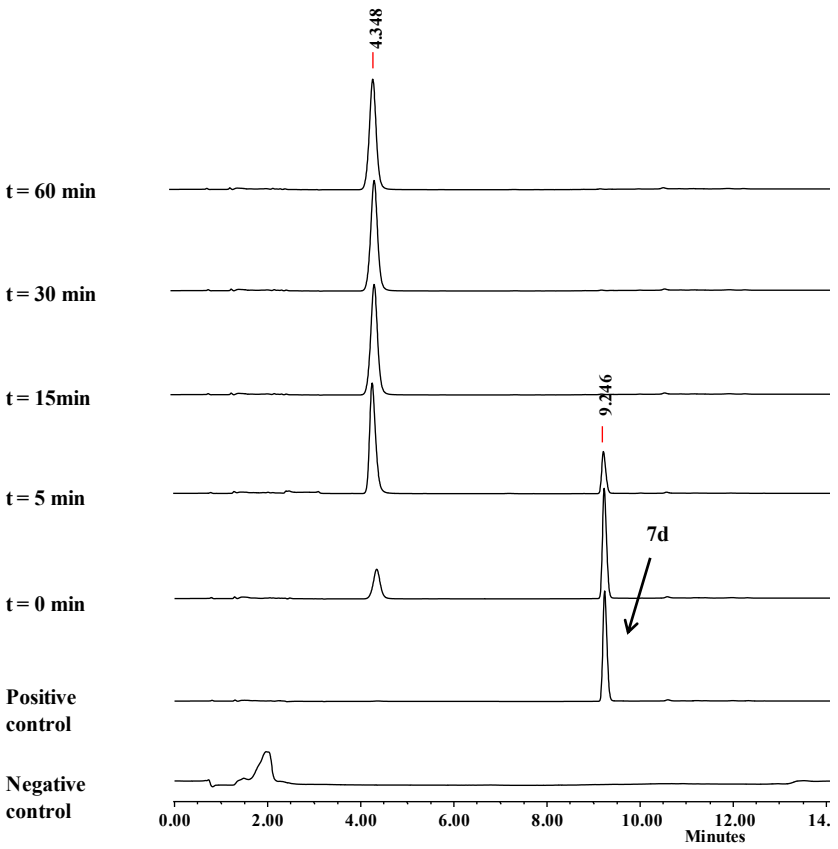


Figure 37: Representative chromatograms of incubation mixtures of resveratrol analogue 7d with mouse liver microsomes in the presence of UDPGA as the cofactor for different incubation times. The negative control corresponds to the microsomes without any **7d**. The positive control corresponds to the incubation mixture without microsomes. For the HPLC conditions see Materials and Methods

In the case of the tribenzylic analogue **7e**, no decrease of the parent compound peak at ~9.4 min was observed during the same period of time, nor was any appearance of other peaks (Figure 38).

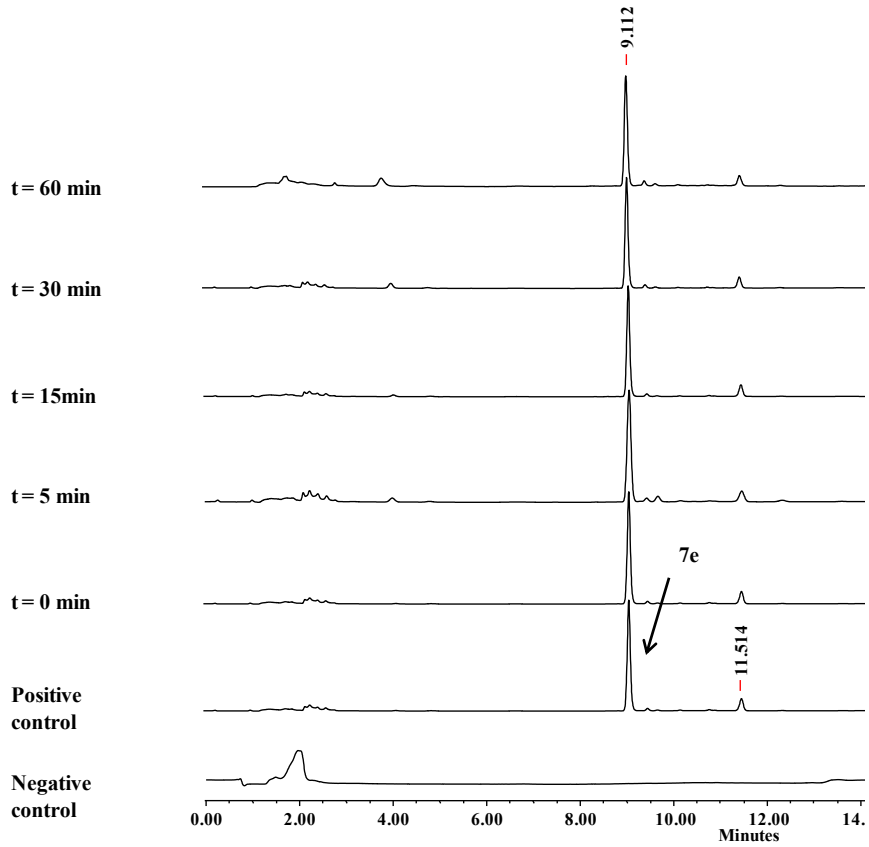


Figure 38: Representative chromatograms of incubation mixtures of resveratrol analogue 7e with mouse liver microsomes in the presence of UDPGA as cofactor for different incubation times. The negative control corresponds to the microsomes without any **7e**. The positive control corresponds to the incubation mixture without microsomes. For the HPLC conditions see Materials and Methods

13.1.2.2. Evaluation of the glucuronidation in mouse liver microsomes

Figure 39 illustrates the rate of metabolism of the analogues **7a-e** compared to resveratrol after incubation with mouse microsomes and UDPGA. Resveratrol **1a** disappeared immediately after the addition of the microsomes (0 min), and resveratrol was completely metabolised after 15 min. A similar decrease was observed for analogues **7c** and **7d**, with **7d** behaving exactly like resveratrol (Figure 39B). The amount of analogue **7c** peak intensity reduced by 40% just after addition of the microsomes, but the compound was never completely metabolised. Instead, an increase in the amount of the parent compound was observed after 30 min, similar to that occurring in the sulfation assay (c.f. p.122). The concentration of **7a,b** and **e** decreased just by ~10% after 1h incubation (Figure 39A).

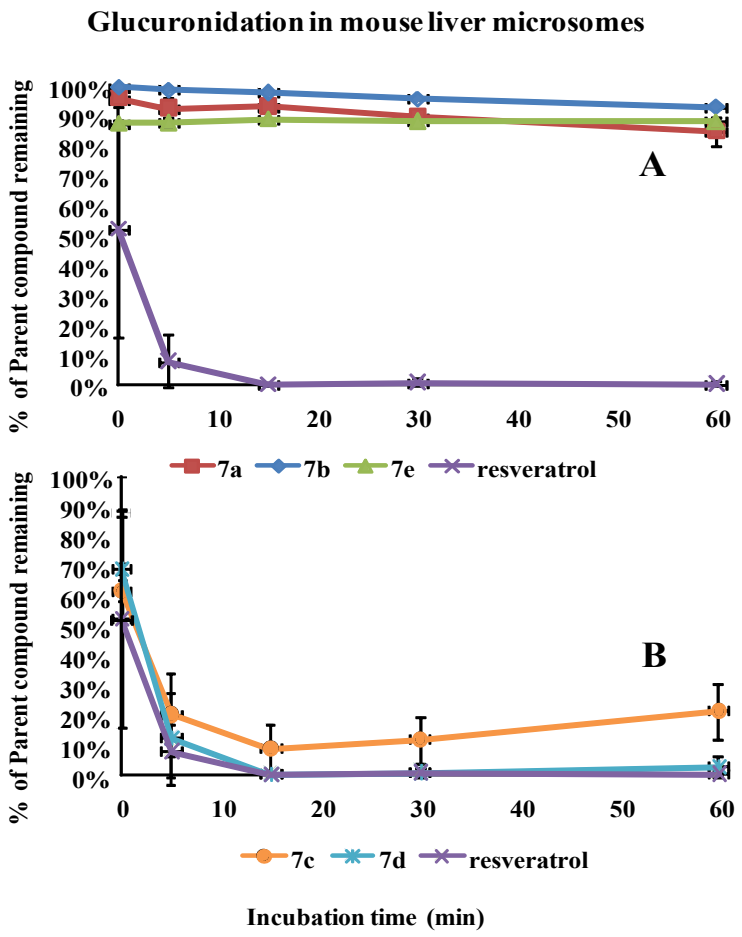


Figure 39: Removal of 7a (red line), 7b (blue line), 7e (green line) and resveratrol (purple line) (A) or 7c (orange line), 7d (cyan line) and resveratrol (purple line) (B) from incubations with mouse liver microsomes and UDPGA. Substrate concentration was 10 µM. Values are expressed as percentage of control incubations without mouse liver microsomes and they are the mean ± SD of 6 separate incubations using pooled liver microsomal fraction from 6 mice (3 males and 3 females).

The glucuronidation results with mouse microsomes show a significant difference between the analogues. The analogues are split into two groups, one where the products are not metabolised within 60 minutes (Figure 39A) and a second group where the molecules behave like resveratrol (Figure 39B). The first group of analogues which are not significantly metabolised are compounds on which there is no free phenol. The second group contains the analogues with at least one free phenol and

resveratrol which completely disappears after 15 min. The exception is **7c** which still has 20 ± 9.5% of the starting amount at 60 min. The slight increase of the concentration of **7c** over time after the initial rapid phase of metabolism suggests the glucuronide metabolite may be unstable. These results indicate that a benzylic alcohol is not as sensitive to metabolism by mouse microsomes to the corresponding glucuronide as a free phenol, which is transformed very rapidly under the same conditions.

13.1.2.3. Evaluation of the glucuronidation in human liver microsomes

The glucuronidation assay in human systems was performed under the same conditions as the mouse assay. Only **1a** and the products **7b** and **7e** were tested as only these ones were assayed in the sulfation assay. Resveratrol was completely metabolised within 60 min, and the quantity remaining after 15 min incubation was ~35% (Figure 40). The dimethoxy analogue **7b** was not significantly transformed within an hour as there was still ~90% of the product. The tribenzylic alcohol analogue **7e** had quite a slow metabolic transformation with 40% of the parent compound still detected after 2 hours of incubation.

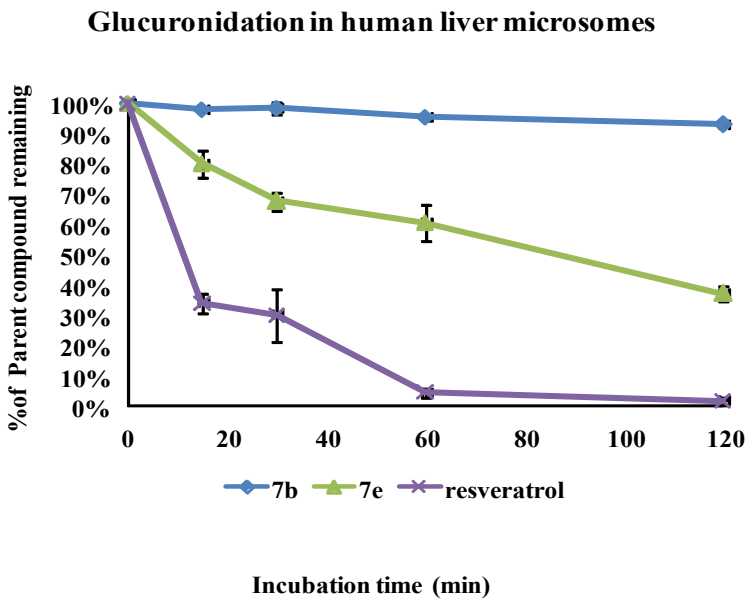


Figure 40: Removal of 7b (blue line), 7e (green line) and resveratrol (purple line) from incubations with human liver microsome and UDPGA. Substrate concentration was 10 µM. Values are expressed as a percentage of control incubations without human liver microsomes and are the mean ± SD of 6 separate incubates using pooled liver microsomal fraction from 23 human

13.1.2.4. Comparison of glucuronidation in mouse and human liver microsomes

Glucuronidation of resveratrol is much slower in human than in mouse system (Figure 41). About 35% of **1a** was still detectable after 15 min when incubated with human enzymes wherease none was detected after the same period in mouse system. In contrast, the glucuronidation rate of tribenzylic analogue **7e** was faster with human microsome; a similar effect was observed for this compound in the sulfation assays. The dimethoxy analogue **7b** behaved exactly the same way in both glucuronidation assays. Overall, the enzymatic process seems to be slower in the human system than the mouse one.

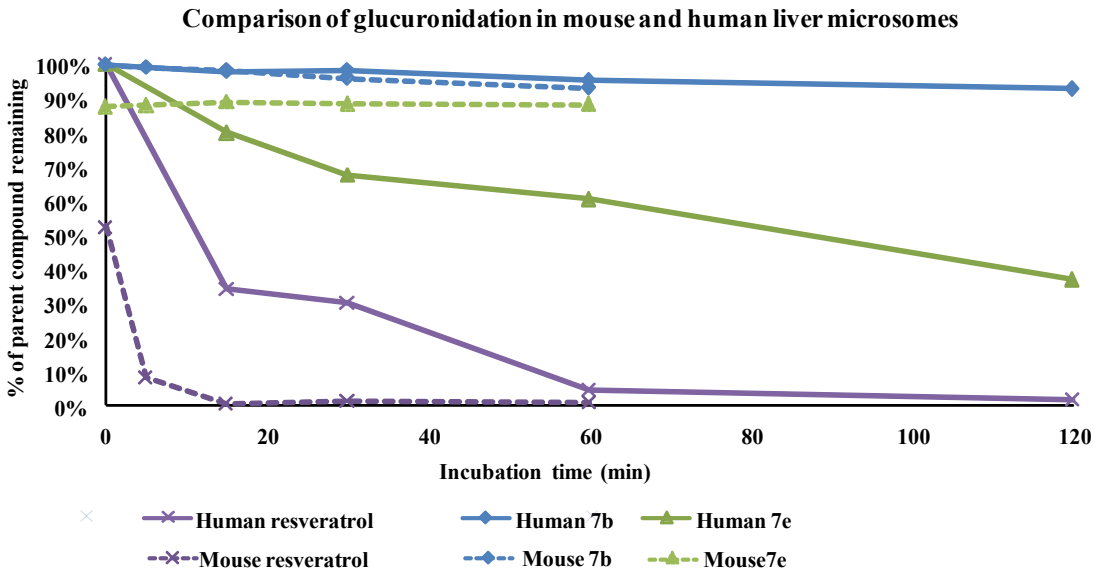


Figure 41: Removal of 7b (blue line), 7e (green line) and resveratrol (purple line) from incubations with human liver cytosol (bold lines) or mouse liver cytosol (dashed lines) and UDPGA. Substrate concentration was 10 µM. Comparison of the values obtained for Figures 38-39.

When there is no free phenol on the stilbene (analogues **7a,b** and **7e**), metabolic sulfation occurs but the rate of sulfation is slower than for resveratrol. On these same analogues, the rate of glucuronidation by both mouse and human microsomes is much slower than for resveratrol as nearly no disappearance is observed over time, except for **7e** in human system.

From both the sulfation and glucuronidation assays it can be concluded that the benzylic alcohols decrease the rate of phase II enzymatic transformations in both human and mouse systems. HPLC/MS/MS and hydrolysis studies were further carried out to confirm that the products of the incubations were actually the sulfate and glucuronide metabolites.

13.2. Identification of resveratrol analogue sulfates and glucuronides by on-line HPLC-ESI-MS/MS and enzymatic hydrolysis

In order to confirm the presence of sulfate and glucuronide metabolites in the incubations of resveratrol analogues with mouse or human liver fractions *in vitro*, on-line HPLC-ESI-MS/MS and enzymatic hydrolysis were carried out to identify the possible metabolites.

13.2.1. On-line HPLC-ESI-MS/MS analysis

Due to the lack of authentic resveratrol analogue sulfates and glucuronides, the parent analogues were used to optimise the mass spectrometric conditions. The optimised ESI-MS/MS conditions for the tested compounds are listed in Table 14 and Table 15.

Method #	**Method 1**		**Method 2**		**Method 3**		**Method 4**	
Elution	**Time**	**%A**	**Time**	**%A**	**Time**	**%A**	**Time**	**%A**
	0	55	0	40	0	60	0	65
	12	10	12	10	12	10	12	10
	18	10	18	10	15	10	15	10
	20	55	20	40	20	60	20	65
	30	55	30	40	30	60	30	65
Flow rate (µL/min)	200		150		150		150	
Compound analysed	**1a, 7a,c**		**7b**		**7e**		**7d**	

Table 14: HPLC methods for each compound analysed. HPLC system: phase A: aqueous ammonium acetate 5 mM; phase B: 5mM ammonium acetate HPLC-grade methanol. For complete HPLC/MS/MS conditions see Materials and Methods

Method #		MS1	MS2	MS3	MS4	MS5	MS6
Compound analysed		1a	7a	7b	7c	7d	7e
Ionisation Mode		Negative	Positive	Positive	Negative	Negative	Positive
MRM ion pair (parent)		227/143	271/165	241/165	225/193	241/131	253/165
Source/gas							
	Curtain gas	12	12	12	12	15	13
	Collision gas	3	4	4	4	4	3
	Ionspray voltage	-3000	4000	4500	-4500	-4500	4500
	Temp. (°C)	350	400	350	350	350	400
	Ion source gas 1	30	40	40	40	40	20
	Ion source gas 2	40	40	40	40	30	20
Compound							
	Declustering potential	-76	21	76	-106	-76	81
	Focusing potential	-240	370	330	-350	-310	370
	Entrance potential	-12	10	10	-10	-12	10
	Collision Energy	-33	61	51	-34	-44	49
	Collision cell exit potential	-30	24	20	-40	-30	28

Table 15: Optimised MS conditions for each compound tested.

As the metabolites fragmentations often contain the product ion relevant to the parent molecular ion, resveratrol analogues were studied to characterise the fragmentation of the parent compounds. The parent molecular ion and the dominant fragment ion were chosen for the selected/multiple reaction monitoring (SRM/MRM). For the compounds containing a free phenol residue, the ion pair of molecular ion $[M-H]^-$ and the dominant fragment were chosen to monitor the presence of the parent compound in negative ionisation mode. The pair of *m/z* $[M-H+80]^-$ and $[M-H]^-$ and pair of $[M-H+176]^-$ and $[M-H]^-$ were used to detect the sulfates and glucuronides respectively as shown in Table 16. Figure 42 shows the MRM chromatogram of a

mixture of resveratrol, 3-*O*-glucuronide, 4'-*O*-glucuronide, 3-*O*-sulfate and 4'-*O*-sulfate as an example. The resveratrol glucuronides and sulfates were synthesized and identified by Dr. Britton in the Cancer Studies and Molecular Medicine laboratory, University of Leicester.

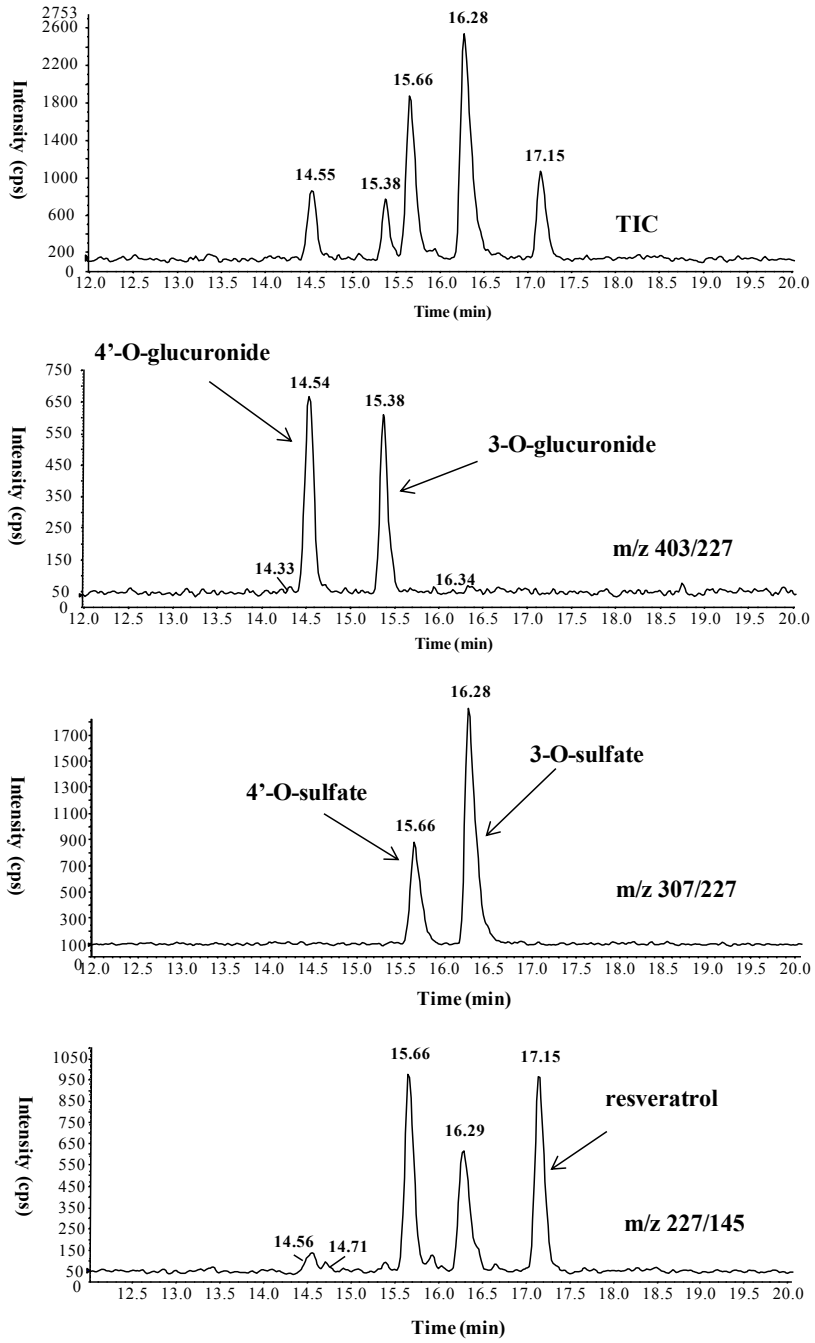


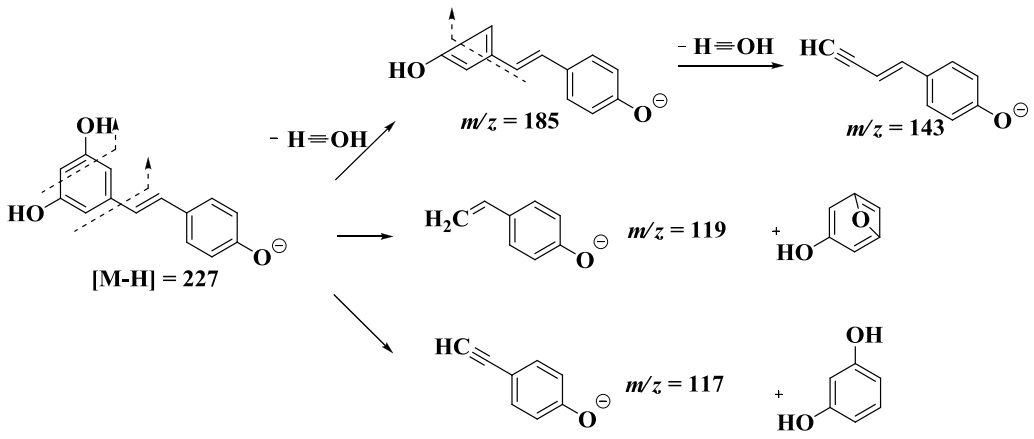
Figure 42: HPLC-ESI-MS/MS chromatograms of mixture of resveratrol, 3-glucuronide, 4'-glucuronide, 3-*O*-sulfate and 4'-*O*-sulfate synthetic standards

Selected/Multiple Reaction Monitoring (MRM) is the standard technique used for LC/MS/MS experiments. MRM allows the detection of transitions between a major product ion formed from a precursor ion, the latter being made by the first fragmentation. D. Wang *et al.*[202] identified the major metabolites of resveratrol in rat by using the LC/MS/MS-MRM, and proposed fragmentation mechanisms for resveratrol and the analogues related to the fragment ions they detected in LC/MS/MS (Scheme 33). The loss of the glucuronide moiety is seen by a loss of *m/z* 176, and the fragmentation of the sulfate moiety is characterised by a loss of *m/z* 80 (Scheme 34).

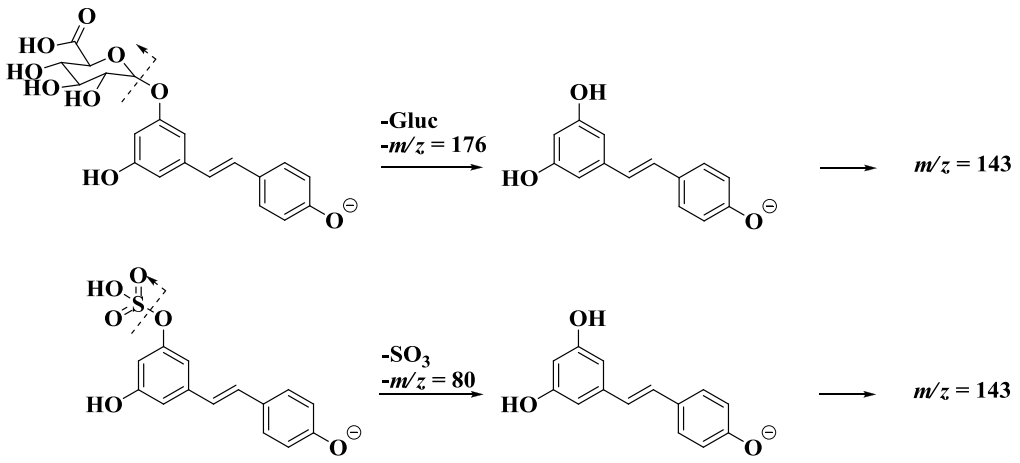
Entry	Compound	MRM (*m/z*)	R_t^a(min)
1	Resveratrol	227 > 143[b] (neg)	12.4
2	Resveratrol monosulfate	307 > 227 (neg)	11.2
3	Resveratrol monoglucuronide	403 > 227 (neg)	7.3
4	7a	271 > 165	12.1
5	7a monoglucuronide	447 > 165	10.3
6	7a monosulfate	351 > 165	10.1
7	7b	241 > 165 and 223>165	11.8
8	7b monoglucuronide	223 > 165	10.3
9	7b monosulfate	223 > 165	10.0
10	7c	225 > 193 (neg)	10.7
11	7c monoglucuronide	401 > 225 (neg)	8.6
12	7c monosulfate	305 > 225 (neg)	7.9
13	7d	241 > 131 (neg)	9.4
14	7d monoglucuronide	417 > 241 (neg)	7.6
15	7d monosulfate	312 > 241 (neg)	8.3
16	7e	253 > 189 and 271>178	9.7
17	7e monoglucuronide	253 > 189	7.8, 8.4
18	7e monosulfate	253>189	8.6

[a] Retention times in the on-line LC/MS/MS system. See Experimental part for details. [b] (>) represents the transition between the parent ion and the fragment.

Table 16: Identification of resveratrol analogues metabolites and resveratrol metabolites by LC/MS/MS using MRM transitions seen in incubation mixtures.



Scheme 33: Proposed mechanism for the decomposition of *m/z* 227 [M-H]⁻ ion of resveratrol by D. Wang *et al.*[202]

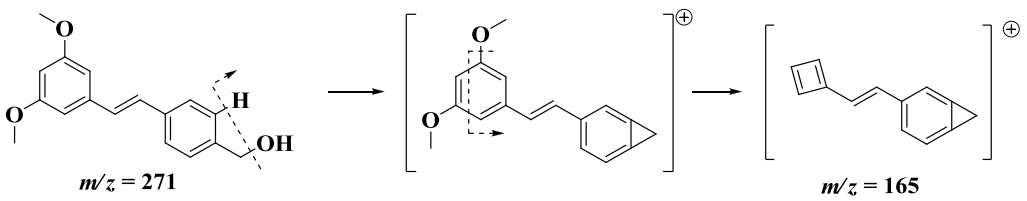


Scheme 34: Proposed mechanism for the decomposition of the 3-*O*-glucuronide and 3-*O*-sulfate of resveratrol by D. Wang *et al.*[202]

The mass spectrometric analysis of the sulfation and glucuronidation metabolic mixtures from resveratrol **1a** showed the MRM transitions in negative ionisation mode (Table 16, entries 1-3) for resveratrol (*m/z* 227 > 143) and its metabolites resveratrol mono-glucuronide (*m/z* 403 > 227) and resveratrol monosulfate (*m/z* 307 > 227), confirming that the metabolism occurs in these *in vitro* incubation systems. The MRM transitions in negative ionisation mode for **7c** (*m/z* 225 > 193) and **7d** (*m/z* 241 > 131)

and their metabolites are seen in the respective metabolic mixtures: the two possible mono-glucuronide metabolites for **7c** (positions 3 and 4', Table 16, entry 11) and the other fragmentations derived from the respective sulfates and glucuronides (Table 16, entries 10-15).

For the compounds without a free phenol, the benzylic alcohol analogues **7a,b** and **7e** were ionised in positive mode due to the difficulty for these compounds to lose a proton in negative ionisation mode. For this group of compounds, a water molecule was easily lost in the positive ionisation mode, and further fragmentation can lead to the most prominent fragment *m/z* 165 (Scheme 35). Therefore the transition $[M+H]^+$ to *m/z* 165 was chosen for the analyses of the metabolite incubations. As shown in Figure 43, *m/z* 271>165 responds to the $[M+H]^+$ and major fragment of compound **7b**. The peaks eluted before **7b** from the incubation mixtures gave *m/z* of 351>165 and 447>165 for **7b** mono-sulfate and **7b** mono-glucuronide respectively. These results showed that the molecular ion of the metabolite was not stable and it fragmented to *m/z* 165 ion in the mass spectrometer.



Scheme 35: Proposed mechanism for the decomposition of *m/z* 271 $[M+H]^+$ ion of 7b

MS analysis of **7a** showed a very weak peak corresponding to the protonation of molecular ion at *m/z* 241 and an abundant $[M+H-18]^+$ peak at *m/z* 223 (Figure 44A). Therefore both ion pairs of *m/z* 241>165 and 223>165 were chosen to identify the presence of **7a** and possible sulfate and glucuronide metabolites. As shown in

Figure 44B no molecular ions of **7a** sulfate (*m/z* 321) and glucuronide (*m/z* 417) were detected due to the instability of the metabolites in the ionisation source which was tuned for the parent compound **7a**. The fragmentation pattern in positive ionisation mode for **7e** is the same as **7a,b**. Table 16 indicated that only $[M+H-18]^+ > 189$ (major fragment) were detected for glucuronide and sulfate metabolites (MRM chromatograms not shown).

For this group of compounds the enzymatic hydrolysis provides an important way to prove the presence of the sulfates and glucuronides. MS alone cannot verify the position of the conjugation, other analytical technologies such as NMR can be applied for the further investigation.

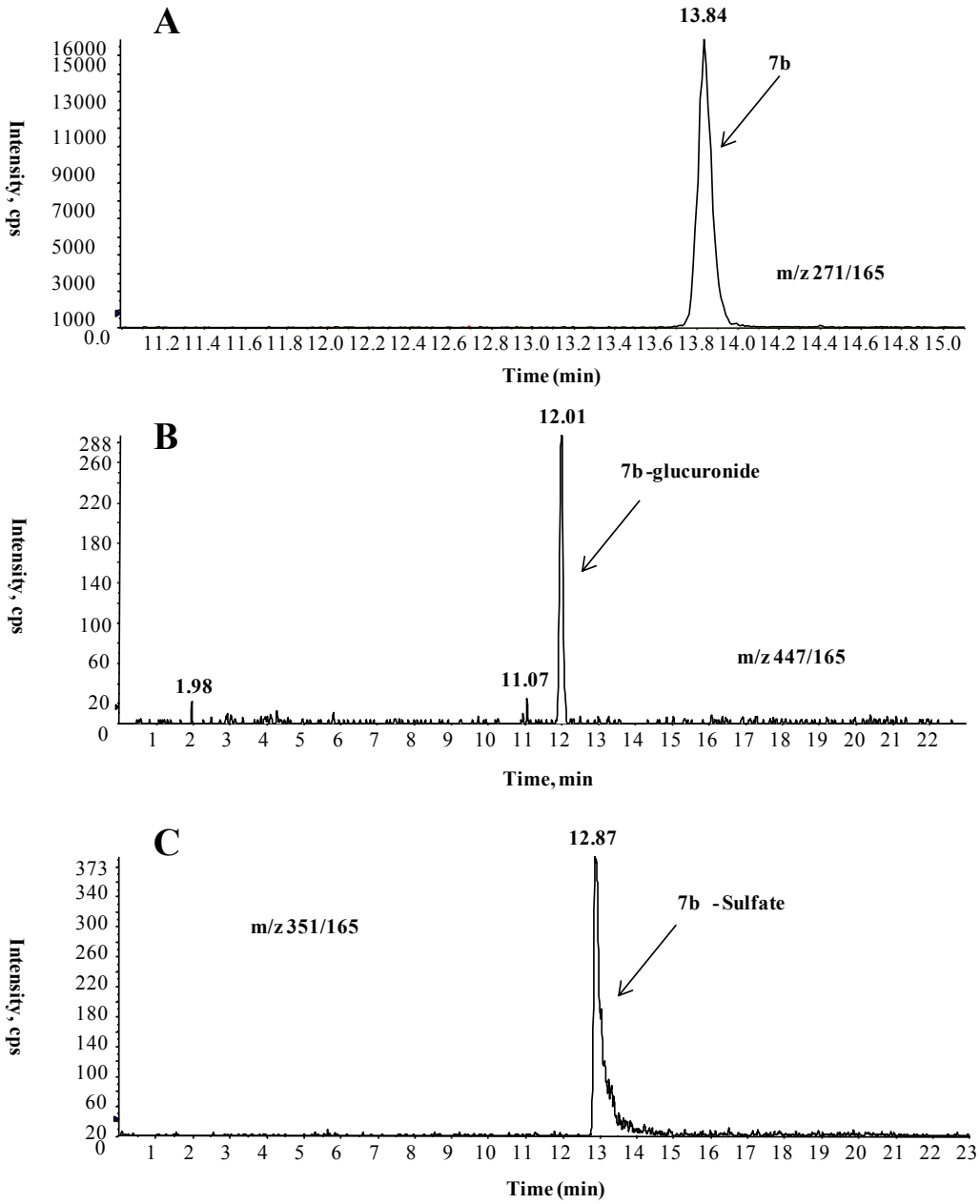


Figure 43: MRM chromatograms of 7b (A), 7b glucuronidation incubation mixture (B) and 7b sulfation incubation mixture (C) with mouse liver fractions. For experimental conditions see Material and Methods

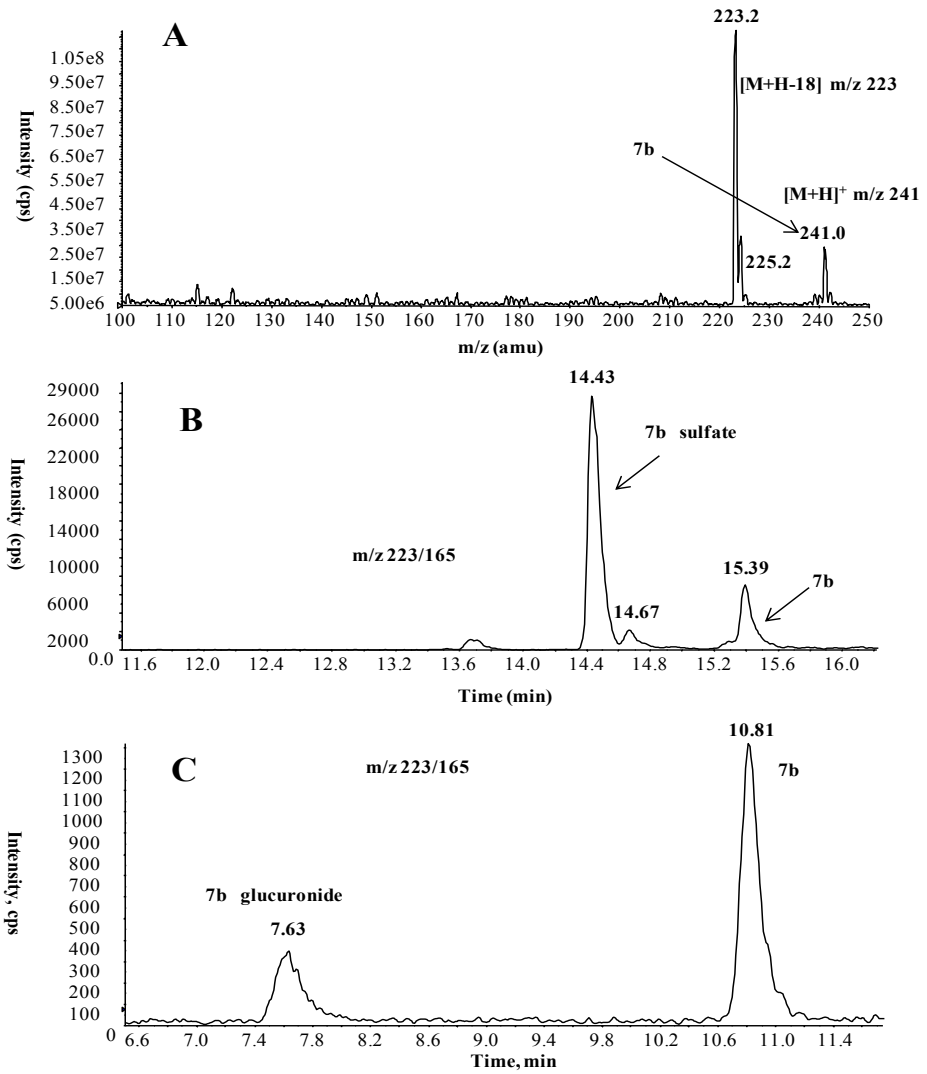


Figure 44: MS of 7b (10 µg/mL) with infusion rate of 10 µL/min (A). MRM chromatogram of 7b sulfation mixture (B) and glucuronidation mixture (C). For experimental conditions see Material and Methods.

13.2.2. Enzymatic hydrolysis of the metabolites of the analogues obtained after *in vitro* incubation

In addition to the identification of resveratrol analogue phase II conjugates by HPLC-ESI-MS/MS, enzymatic hydrolysis was carried out for the sulfation and glucuronidation products as another positive identification of resveratrol analogue sulfates and glucuronides.

13.2.2.1. Hydrolysis of the possible sulfates with sulfatases

Resveratrol and its analogues were incubated with mouse liver cytosol and PAPS as described previously for a determined length of time (Table 17). The incubation was then stopped and the incubation mixtures were hydrolysed overnight with sulfatases (see Experimental part).

Compound	7a	7b	7c	7d	7e
Incubation time (min)	30	30	15	15	15

Table 17: Incubation time for the indirect proof of sulfation

Figure 45 shows the comparative HPLC traces of the sulfation and hydrolysis reactions. In all the cases we observe a reappearance of the peak corresponding to the parent compound. **7a** and **7b** only have one free hydroxyl group for sulfation and as shown in Figure 45, the small peak corresponding to the parent compound appears after the incubation with the sulfatases, which confirm a sulfate on the benzylic alcohol. **7c** and **7d** have two and three possible sulfation positions (two and three hydroxyl respectively), a phenol and a benzylic alcohol, hence the possibility to

generate the mono- and di-sulfates. Two new products were created after the sulfation, and the intensity of both of their peaks decreases in favour of the parent compounds which suppose that they were both containing sulfate moieties. The different peaks could be representative of sulfation on two different positions, either on the phenol or on the benzylic alcohol. A phenol sulfate may have a different polarity than a benzylic one, which could explain the different retention times. The more polar peaks could also have been representative of disulfates, but none were seen in ESI-MS/MS studies. This pattern of decrease of the intensity of the "new" peak in favour of the parent compound is seen as well for the product **7e**.

These results confirm that the analogues **7a-e** are sensitive to enzyme mediated sulfation, although we cannot draw conclusions regarding the exact structure of the metabolites with respect to the position of sulfation from these results, especially in the case of **7c** and **7d**.

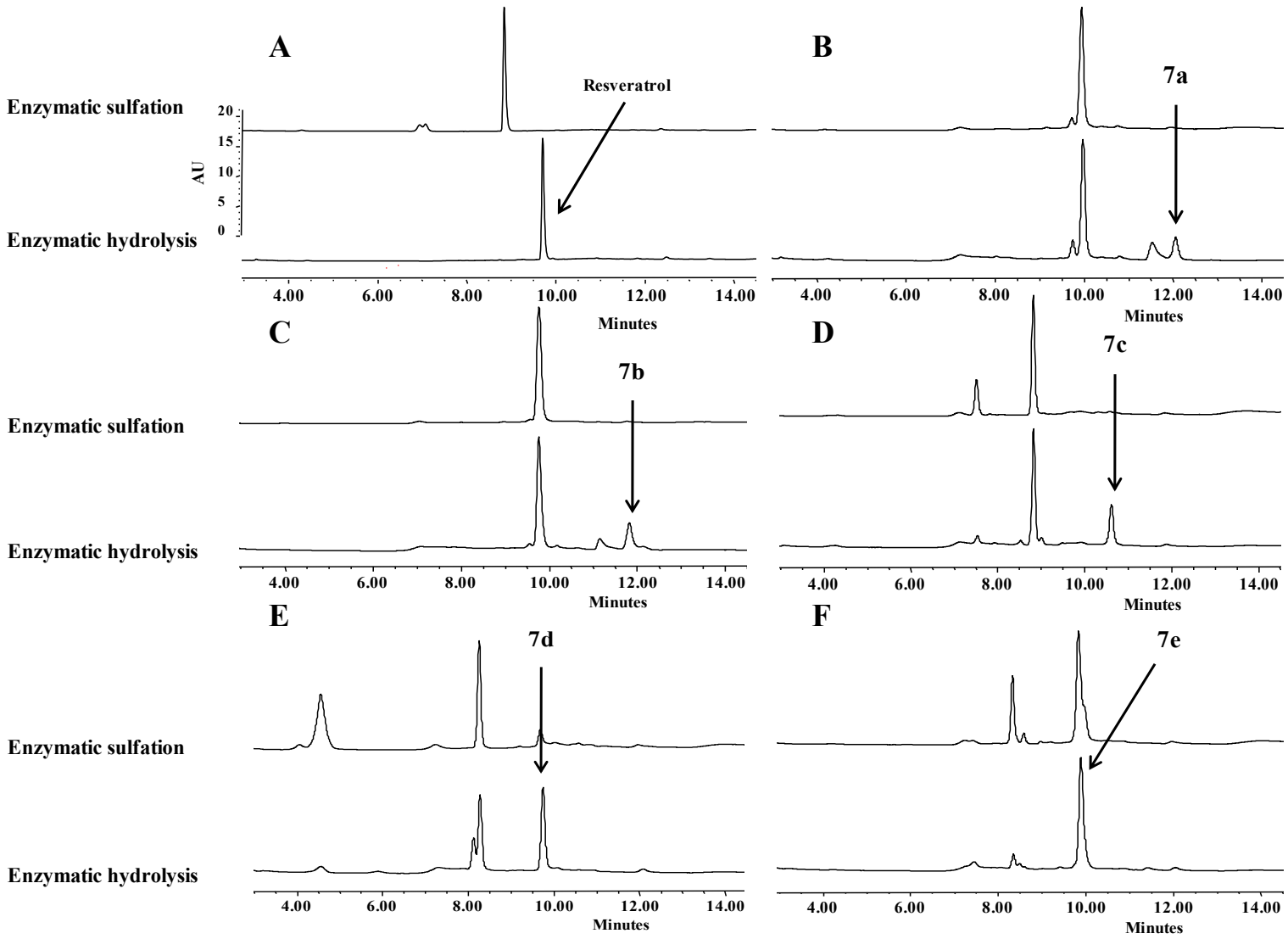


Figure 45: Comparison of HPLC traces of the enzymatic sulfation and enzymatic hydrolysis for resveratrol (A), analogues 7a (B), 7b (C), 7c (D), 7d (E) and 7e (F).

13.2.2.2. Hydrolysis of the possible glucuronides with glucuronidases

Hydrolysis reactions were carried out on the glucuronidation incubation mixtures of resveratrol and its analogues with glucuronidases (see Experimental part). Figure 46 shows the comparative HPLC traces of the glucuronidation and hydrolysis reactions. In the cases of resveratrol **1a** and its analogues **7c** and **7d**, we observe a complete disappearance of the peak corresponding to the glucuronide due to total hydrolysis. **7a** and **7b** only have one possible position for the glucuronidation and in both cases a small peak corresponding to the potential metabolite was detected after glucuronidation with the microsomes. The disappearance of these small peaks after incubation with glucuronidase did not occur, and for **7b**, the "glucuronide" peak area increased. A similar observation as **7a-b** was made from the traces corresponding to analogue **7e**. **7e** was metabolised to the glucuronide as there was a small new peak appearing after incubation with the microsomes, which did not disappear after hydrolysis with the glucuronidase but increased in peak area. These results for the hydrolysis of **7a,b** and **7e** may be explained by a lack of reactivity of the glucuronidase with the possible benzylic glucuronides.

On-line HPLC-ESI and enzymatic hydrolysis provided the positive identification of the resveratrol analogues sulfates and glucuronides in the *in vitro* systems as shown in Table 16 and Figure 45-Figure 46 except maybe for analogues **7a,b** and **7e** which will need further evaluations. All the results presented here suggest that the substitution of the phenols on resveratrol with a methoxy or a benzylic alcohol reduces the rate of phase II conjugation *in vitro*.

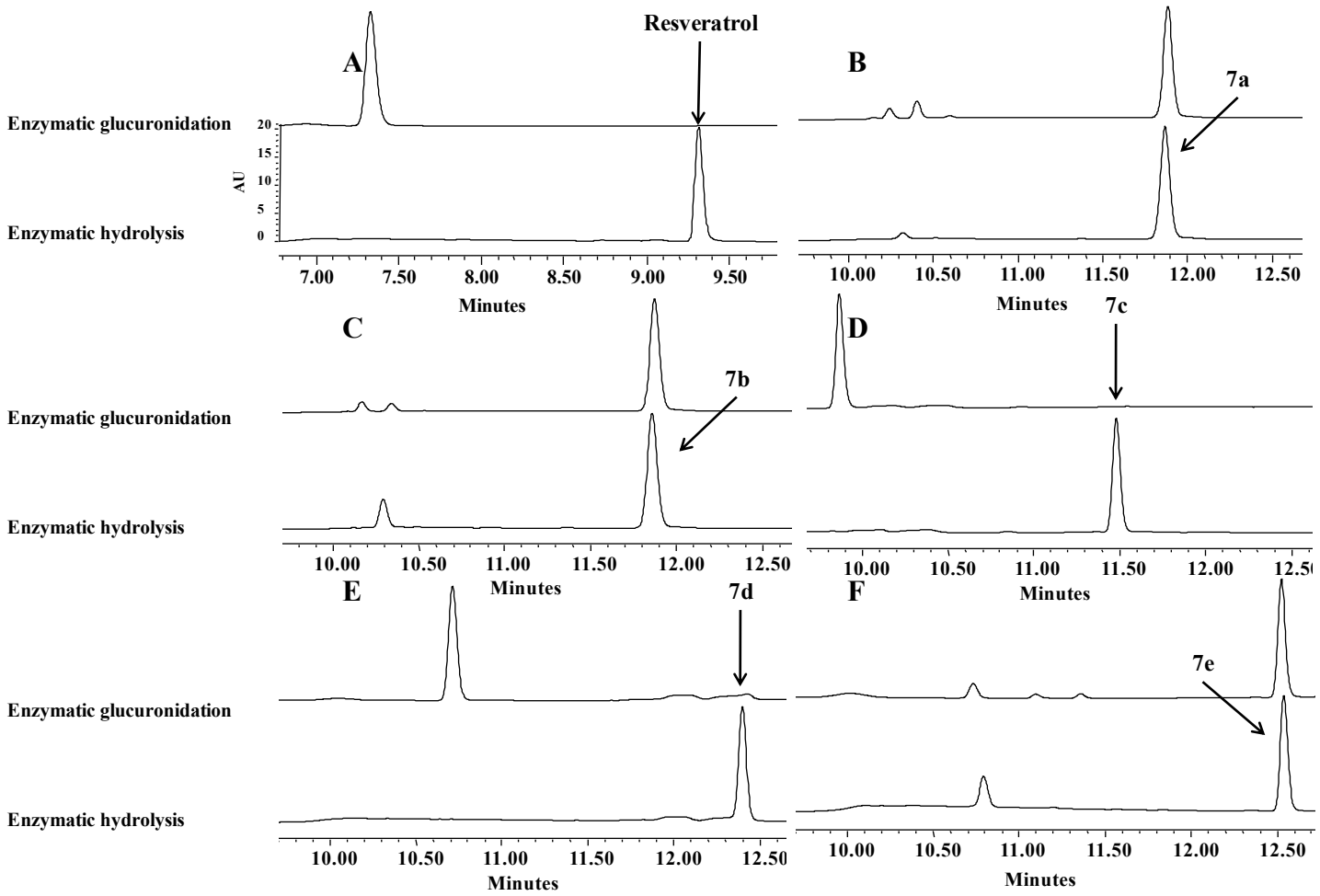


Figure 46: Comparison of HPLC traces of the enzymatic glucuronidation and enzymatic hydrolysis for resveratrol (A), analogues 7a (B), 7b (C), 7c (D), 7d (E) and 7e (F).

13.2.2.3. Comparison of metabolic and synthetic metabolites

Another possibility of identification of the position of sulfation and glucuronidation is by synthesising the different metabolites and comparing the HPLC retention times under identical chromatographic conditions. In Chapter 2, the synthesis of the sulfates **22a-c** from analogues **7a-c** was described. As it was pointed before, the metabolites could not be purified, but the presence of metabolites was detected by LC/MS analysis and NMR. It was decided to inject on the same HPLC system used for the monitoring of the metabolism the synthetic sulfates and then to compare them with the metabolite traces.

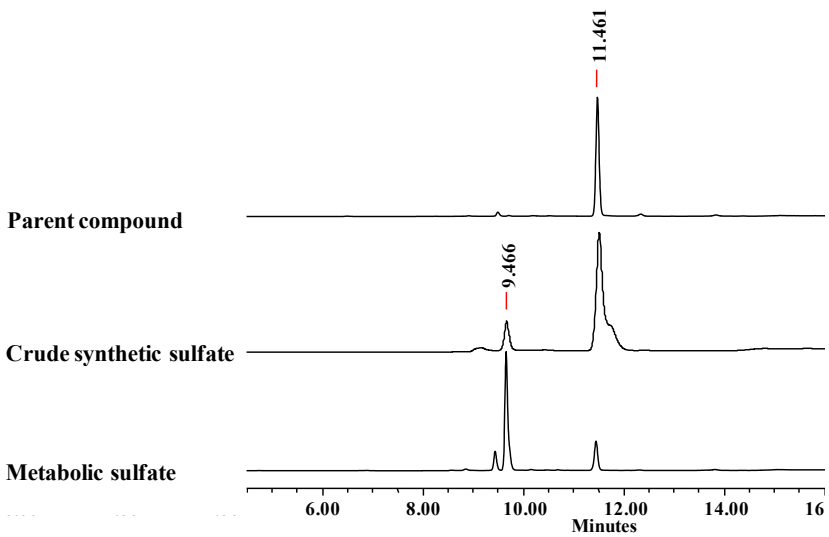


Figure 47: Comparison of synthetic 22a with metabolic sulfate

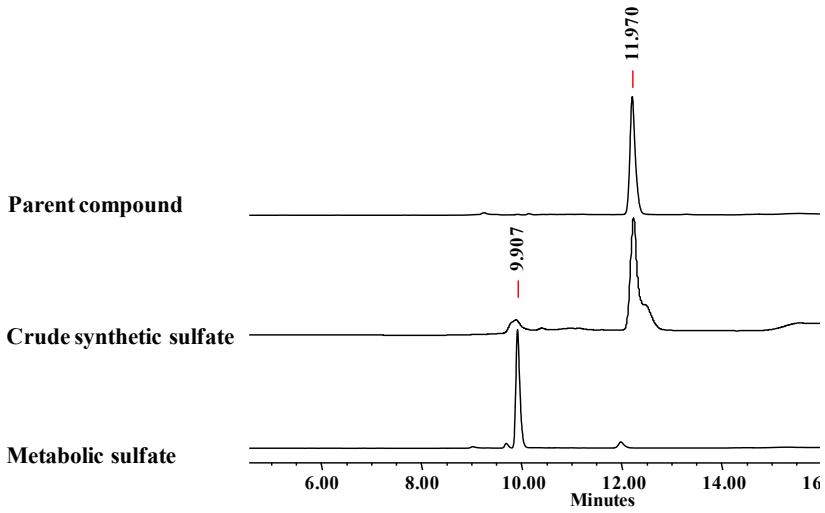


Figure 48: Comparison of synthetic 22b with metabolic sulfate

Figure 47 shows the comparison between the metabolic sulfate HPLC trace, the parent compound **7a** and the synthetic sulfate **22a** dissolved in DMSO:MeOH (100 ng/mL). It can be noticed that the peak at 9.5 min in the metabolic trace corresponds to the smaller peak in the synthetic crude. As the synthetic crude contains the mono-sulfate **22a**, it confirms that the peak at 9.5 min is the mono-sulfate. Similar results can be deduced from Figure 48 as the peak at 9.5 min in the synthetic crude trace corresponds to the peak in the metabolic mixture, confirming the sulfate **22b** as the metabolite after enzymatic sulfation. The injection of **22c1** and **22c2** did not give any usable result, possibly because of a very low conversion and solubility of the synthetic crude mixture.

As the other sulfates or glucuronides could not have been synthesised, the comparison with the metabolic crude mixtures could not be performed. However, these results combined with the HPLC/MS/MS analysis prove that the analogues **7a,b** are sensitive to the enzymatic sulfation with mouse and human cytosol. Further work on the specific synthesis of the sulfates and glucuronides of the other analogues may allow the determination of the exact structure of the different metabolites and the assignment

of each HPLC peak to a specific metabolite. However, to be absolutely sure of the structure of the metabolites, the use of multi-dimensional NMR would be recommended.

In summary, we assessed the metabolic removal of the analogues synthesised by enzymatic liver fractions from mouse and human systems. We can conclude from the results obtained that the sulfation occurs in a slower manner than resveratrol if there is no free phenol on the scaffold and that the sulfation rate was the same than resveratrol as soon as there is at least one free phenol. The same conclusion can be made for the glucuronidation of the phenolic analogues; indeed, the metabolism rate of analogues **7c,d** is similar to resveratrol. On the other hand, the glucuronidation is stopped the methoxy analogues **7a,b** and for tribenzylic analogue **7e**. The metabolism rate is slower in human than in mice systems, except for the tribenzylic analogue **7e** which is sulfated and glucuronidated more rapidly than in mouse. This may be explained by a potential better interaction between the analogue and the metabolic enzymes.

The confirmation of the nature of the new metabolic peaks was made by LC/MS/MS studies on the side of enzymatic hydrolysis, which proved that the sulfation and glucuronidation occurred. Comparison of the metabolic peaks with the synthetic standards **22a,b** further confirmed the nature of the enzymatic sulfate metabolites of **7a,b**. However, more work on the synthesis of the regio-specific metabolites (sulfates and glucuronides) of the other analogues will be required to confirm the structure of the other enzymatic metabolites.

14. Cell proliferation assays

Stopping growth and so the proliferation of cancer cells is a big asset for the treatment of cancer. Inhibiting their growth selectively is also a very big advantage as toxicity to normal cells would be reduced. The smaller the dose the product is active at is also very important as it may reduce the incidence of side effects.

The first part of our hypothesis was that by replacing the phenol moiety with a benzylic alcohol and/or a methoxy group, metabolism of the resveratrol analogues could be prevented and was demonstrated previously (cf. 13. Metabolism studies). The second part of our hypothesis is to see whether these modifications would enhance anti-proliferative efficacy of resveratrol in cancer cell populations. The synthesised analogues **7a-e** were investigated for their anti-proliferative effects in the HCA-7 colorectal cancer cell line (c29 clone), which has high COX-2 expression.[203] The concentration at which the analogues inhibit cellular proliferation by 50% (IC_{50}) was assessed over a period of three and six days, in conjunction with flow cytometric analysis for determination of cytotoxic[e]/cytostatic[f] effects. This approach, combined with *in vitro* metabolism data, may allow better understanding of possible structure-activity relationships, alluding to ways in which the structure of resveratrol could potentially be altered to improve likely chemopreventive and chemotherapeutic efficacy.

[e] Any agent or process that kills cells
[f] Inhibiting or suppressing cellular growth and multiplication

14.1. Characteristic growth curve of HCA-7 c29 cells

A characteristic growth curve of HCA-7 c29 cells (Figure 49) was obtained after cell culture according to the method described (See Experimental part) over a period of six days. Cell counts were undertaken on days 4, 5 and 6 post-treatment (with 0.1% DMSO only). Cell numbers were expressed as a mean ± SD of 6 values measured on the same day. The cell number increases by approximately 10-fold after 4 days and around 24-fold over 6 days, giving a cell doubling time of approximately 12h.

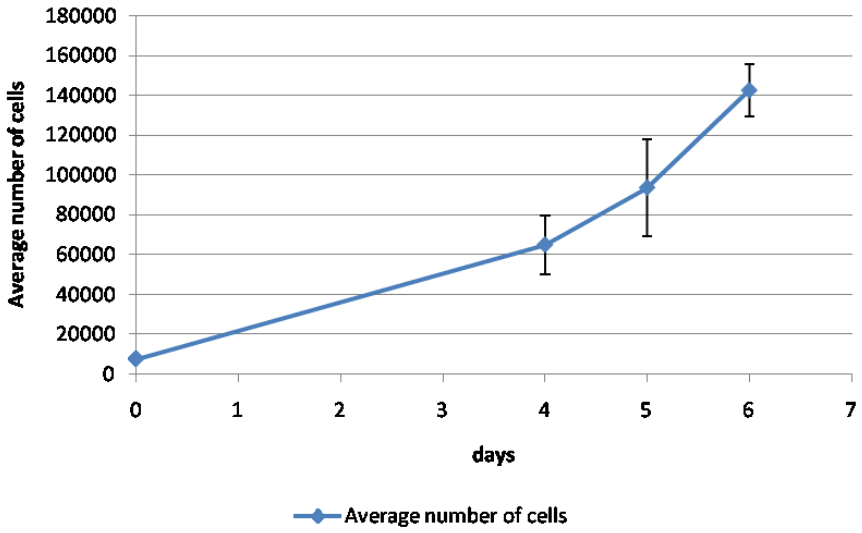


Figure 49: HCA-7 c29 characteristic growth curve

14.2. Treatments

All the analogues which have been tested were compared to resveratrol included as a positive control in each set of incubations and were tested according to the procedure stated in the experimental part (c.f. 20.7 Cell proliferation assay - Method). Cell numbers are expressed as a mean ± SD of three independent experiments where 3 or 4 replicate incubations were performed in each experiment for each concentration/time point.

14.2.1. Growth inhibitory of resveratrol 1a on HCA-7 cells

The incubation of cells with resveratrol at different concentrations showed the same characteristics obtained in previous studies done in the lab.[204] Figure 50 shows a distinct decrease in the cell number per well after 6 days incubation.

Significant growth inhibition by resveratrol **1a** was observed at 6 days with concentration of 0.5 μM and above; each concentration reduced proliferation by 30, 33, 44, 70 and 95% at 0.5, 2, 5, 10 and 50 μM respectively as seen in Figure 51 ($P \leq 0.01$ for each concentration). Resveratrol inhibited cell proliferation in a dose dependant manner with an IC_{50} of ~ 6 μM in the HCA-7 c29 after 6 days of treatment.

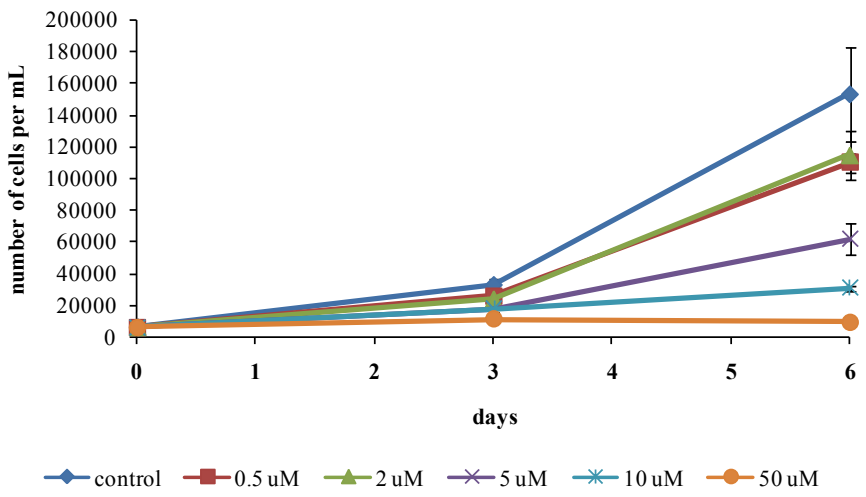


Figure 50: Effect of resveratrol on the proliferation of HCA-7 c29 human colon cancer cells. HCA-7 c29 cells were treated with resveratrol for 3 and 6 days at concentration of 0.5, 2, 5, 10 and 50 µM in 0.1% DMSO and a control DMSO (0.1%). Each curve represents the number of cells per mL of after treatment at a defined concentration and each time point is expressed as a mean ± SD of 4 replicate incubations performed at the same time.

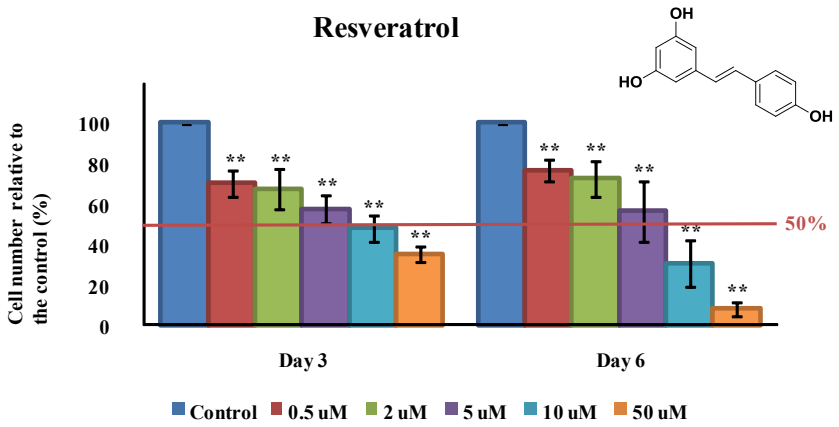


Figure 51: Percentage decrease in the number of cells/well after 3 and 6 days of treatment. Each bar represents the % of cells compared to the control considered at 100% at a defined concentration and is expressed as a mean± SD of 3 separate experiments with 3-4 incubations per experiment. *Asterisks* indicate that values are significantly different from the control (**$P \leq 0.01$)

14.2.2. Growth inhibitory effect of monomethoxy-monobenzylic analogue 7a on HCA-7 cells

The monomethoxy-monobenzylic alcohol analogue of resveratrol **7a** has a significant effect on cell number following three days of treatment (Figure 52). There is an overall dose-dependent reduction in the cell number, with a maximum decrease (50%) observed at 50 µM. However, this dose dependence is not observed after six days of treatment. The maximum decrease in the cell number observed at concentrations of 50 µM is 40 ± 11% at the latter time point.

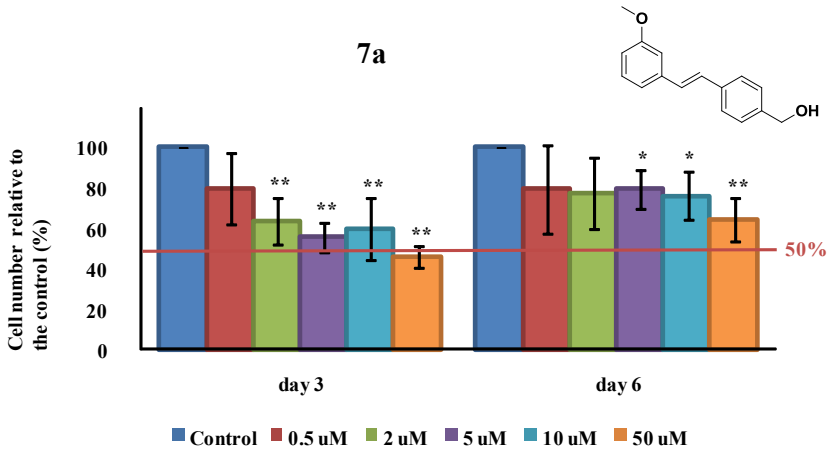


Figure 52: Effect of mono-methoxy-mono-benzylic alcohol analogue 7a on the proliferation of HCA-7 c29 human colon cancer cells. HCA-7 c29 cells were treated with **7a** for 3 and 6 days at concentration of 0.5, 2, 5, 10 and 50 µM in 0.1% DMSO and a control DMSO (0.1%). Each bar represents the % of cells compared to the control considered at 100% at a defined concentration and is expressed as a mean± SD of 3 separate experiments with 3-4 incubations per experiment. *Asterisks* indicate that values are significantly different from the control (**P*≤0.05, ***P*≤0.01)

14.2.3. Growth inhibitory effect of dimethoxy-benzylic analogue 7b on HCA-7 cells

The dimethoxy-benzylic alcohol analogue of resveratrol **7b** has an effect on the cell number after 3 and 6 days of treatment (Figure 53). After three days there is a significant decrease in the cell number with a mean reduction of 30% at 5, 10 and 50 µM ($P \leq 0.01$ at each concentration). A dose dependent reduction in cell number is more apparent following six days of treatment. The decrease in the cell number observed at the concentrations of 2, 5 and 10 µM is 30 ± 18%. However, the cell proliferation is significantly inhibited by 50 ± 11% at a concentration of 50 µM ($P \leq 0.01$).

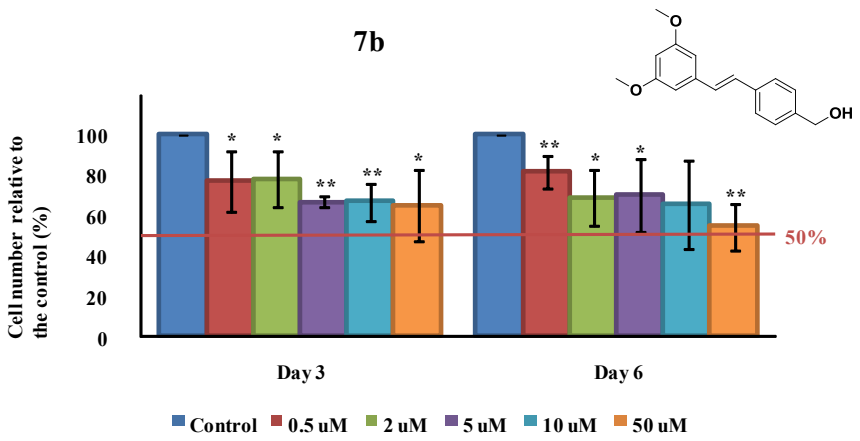


Figure 53: Effect of dimethoxy-mono-benzylic alcohol analogue 7b on the proliferation of HCA-7 c29 human colon cancer cells. HCA-7 c29 cells were treated with **7b** for 3 and 6 days at concentration of 0.5, 2, 5, 10 and 50 µM in 0.1% DMSO and a control DMSO (0.1%). Each bar represents the % of cells compared to the control considered at 100% at a defined concentration and is expressed as a mean± SD of 3 separate experiments with 3-4 incubations per experiment. *Asterisks* indicate that values are significantly different from the control (*$P \leq 0.05$, **$P \leq 0.01$)

14.2.4. Gowth inhibitory effect of mono-phenol-mono-benzylic alcohol analogue 7c on HCA-7 cells

The analogue of resveratrol **7c** has a significant effect on cell number following three days of treatment (Figure 54). There is some dose-dependent reduction in the cell number, with a maximum decrease (35%) observed at 5 and 50 µM. This remains similar after six days of treatment with a maximum decrease of ~50 ± 7.5% at the highest concentration.

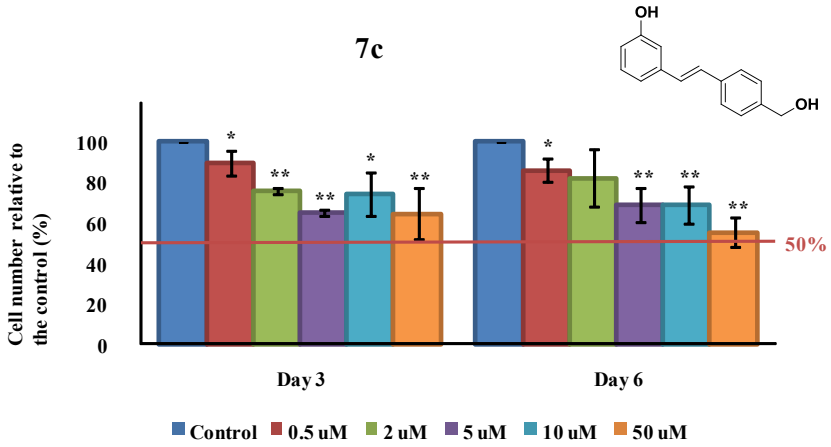


Figure 54: Effect of mono-phenol-mono-benzylic alcohol analogue 7c on the proliferation of HCA-7 c29 human colon cancer cells. HCA-7 c29 cells were treated with **7c** for 3 and 6 days at concentration of 0.5, 2, 5, 10 and 50 µM in 0.1% DMSO and a control DMSO (0.1%). Each bar represents the % of cells compared to the control considered at 100% at a defined concentration and is expressed as a mean± SD of 3 separate experiments with 3-4 incubations per experiment. *Asterisks* indicate that values are significantly different from the control (**P*≤0.05, ***P*≤0.01)

14.2.5. Growth inhibitory effect of diphenol-mono-benzylic analogue 7d on HCA-7 cells

The analogue **7d** inhibits cell proliferation at day 3 but not in a dose dependant manner. Cell number decreases by around 30% at concentrations of 0.5, 2 and 5 µM, whilst the 50 µM dose causes 50% inhibition (Figure 55). However, this relative activity is lost following 6 days incubation, with the mean decrease in cell number not exceeding 30% at any concentration.

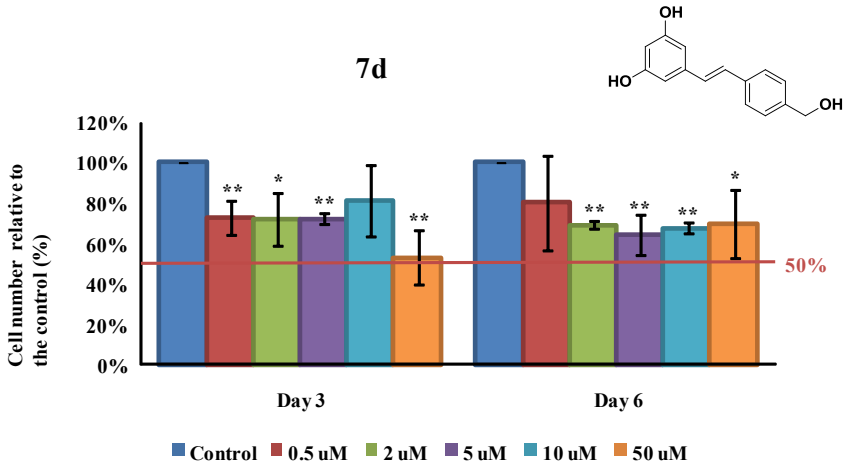


Figure 55: Effect of diphenol-mono-benzylic alcohol analogue 7d on the proliferation of HCA-7 c29 human colon cancer cells. HCA-7 c29 cells were treated with **7d** for 3 and 6 days at concentration of 0.5, 2, 5, 10 and 50 µM in 0.1% DMSO and a control DMSO (0.1%). Each bar represents the % of cells compared to the control considered at 100% at a defined concentration and is expressed as a mean± SD of 3 separate experiments with 3-4 incubations per experiment. *Asterisks* indicate that values are significantly different from the control (**P*≤0.05, ***P*≤0.01)

14.2.6. Growth inhibitory effect of tribenzylic analogue 7e on HCA-7 cells

The analogue **7e** seems not to have any real activity after three or six days treatment (Figure 56). The maximum decrease observed after three days treatment is around 20 ± 10% at the concentrations of 2 µM and 50 µM. At the same concentration of 2 µM there is no decrease in the cell number after 6 days. Looking at the pattern of cell concentration, we can see that there is no significant inhibition of the growth of the cells for concentrations below 50 µM for analogue **7e**.

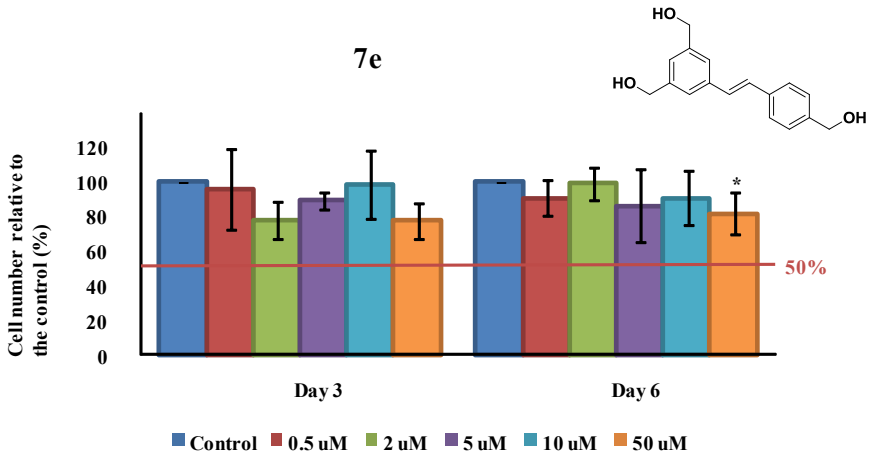


Figure 56: Effect of tribenzylic alcohol analogue 7e on the proliferation of HCA-7 c29 human colon cancer cells. HCA-7 c29 cells were treated with **7e** for 3 and 6 days at concentration of 0.5, 2, 5, 10 and 50 µM in 0.1% DMSO and a control DMSO (0.1%). Each bar represents the % of cells compared to the control considered at 100% at a defined concentration and is expressed as a mean± SD of 3 separate experiments with 3-4 incubations per experiment. *Asterisks* indicate that values are significantly different from the control (*$P \leq 0.05$)

14.3. Discussion

HCA-7 c29 cells underwent a single dose treatment of resveratrol **1a** and of the synthetic analogues **7a-e** at different concentrations for a six-day period. Resveratrol inhibits the proliferation of these cells in a dose dependant manner with an IC_{50} of around 6 μM, which is consistent with previous results obtained within our laboratory.[204] Amongst all the analogues, **7e** is the only one which presents absolutely no activity at the studied concentrations. The four other analogues **7a-d**, all containing at least a phenol or a methoxy moiety, all inhibit proliferation either on day 3 or day 6 with an IC_{50} of around 50 μM.

By comparing the "phenol analogues" to the "methoxy analogues", we can observe that their anti-proliferative efficacy is neither dependent upon the degree of substitution on the stilbene nor on the type of substituent. The dimethoxy analogue **7b** behaves in the same way as the monophenol analogue **7c**, and the mono-methoxy analogue **7a** acts comparably to the di-phenol analogue **7d** (Scheme 36). This renders it impossible to deduce a structure-activity relationship between the analogues and their anti-proliferative properties.

Resveratrol is a multi-targeted anti-cancer agent; it may activate one enzymatic pathway while deactivating another one at the same time. It is a possibility that the analogues **7a-e** are multi-targeted as well, which could maybe explain their lower activity against cell proliferation. These possible multiple interactions may lower the apparent **7a-e** activity by potential activation of growth/proliferation or survival pathways. Another possible explanation is a potential degradation in the media or in the cellular environment over different periods of time. This might explain why activity over a period of three days was observed, but not at day 6 for analogues **7b** and **7d**. The cells were treated with only one dose; it might be possible that the analogues have

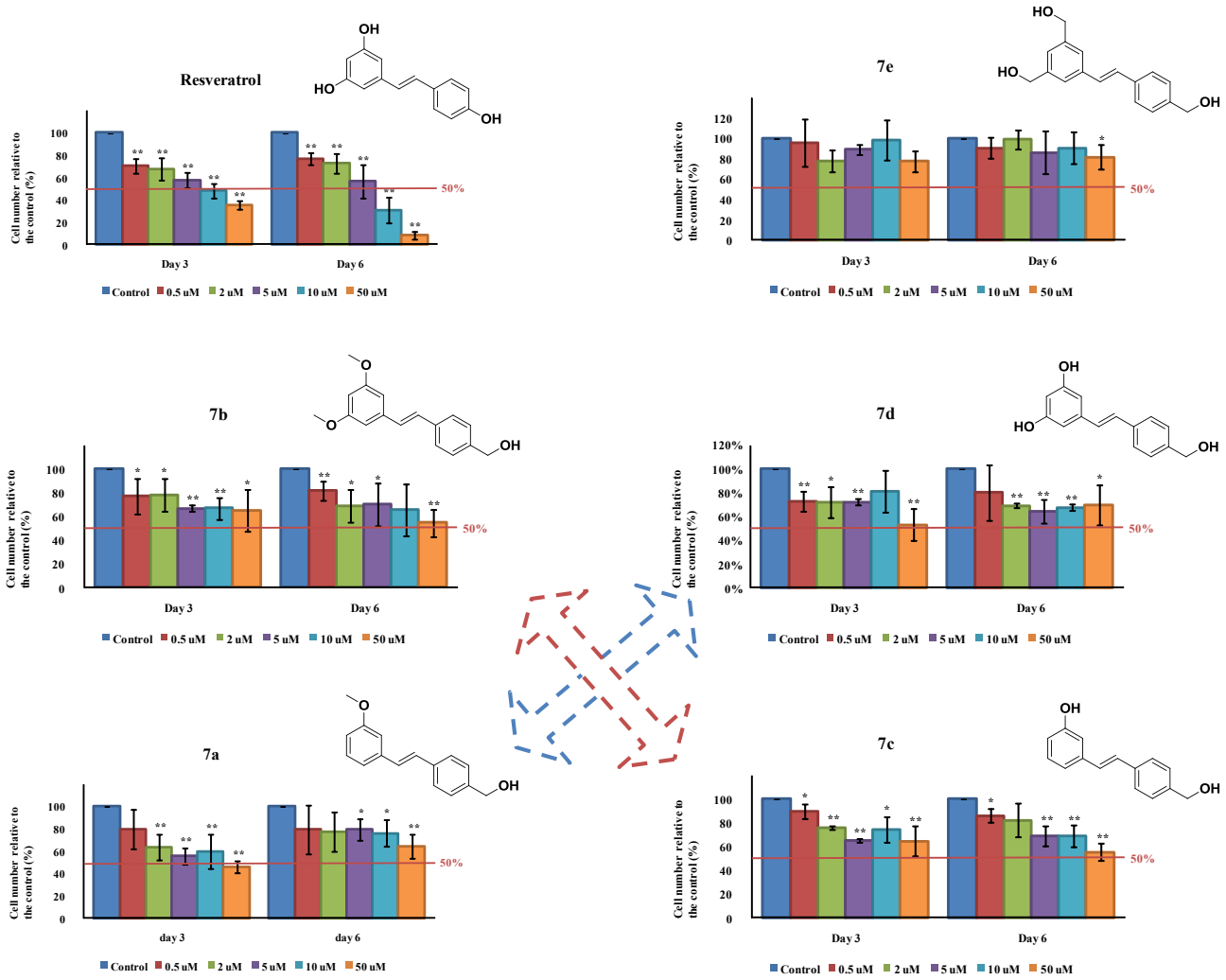
greater activity using a multi-dosing strategy. However, lack of time did not allow us to further investigate this possibility.

The chemopreventive activity of resveratrol may be due to its anti-oxidant properties coming from the phenolic moieties. Kelkel *et al*[205]. recently reviewed resveratrol anti-oxidant properties and its effect on leukaemia and they precise that the 4'-hydroxy position seems very important for its activity.[206] None of our analogues tested contain this 4'-hydroxy moiety but all have a 4'-benzylic alcohol moiety, which may be an explanation of the lower activity observed on the cell proliferation inhibition.

Finally, out of these five analogues, two are of particular interest. Mono-methoxy analogue **7a** and mono-phenol **7c** both induce a dose-response inhibition of HCA-7 c29 cells either on day 3 or day 6. In addition to this effect, their rate of metabolism is different to resveratrol. **7a** is not glucuronidated and disappears within one hour into the sulfate metabolite. **7c** behaves like resveratrol with respect to its sulfation and glucuronidation but is never completely conjugated, and the quantity of the parent product increases slightly over time after an initial reduction.

Modification of the substituents on the stilbene did not allow us to keep nor improve the activity against cell proliferation. When the three phenols are replaced by benzylic alcohols the activity is completely lost, whereas there is still some activity when there is at least one phenol or one methoxy. Some of the numerous analogues of resveratrol containing methoxy groups and/or phenols, natural or synthetic, have better activities on some cancer cell line or specific enzyme inhibition *in vitro*. So we can conclude that the addition of a benzylic alcohol does not allow the maintenance of activity against HCA-7 c29 cell proliferation, but it does slow down the *in vitro* metabolism.

Complementary flow cytometry studies to investigate the degree of apoptosis and cell cycle arrest have been undertaken on these two analogues **7a** and **7c** in order to better understand the anti-proliferative mechanisms of action in the HCA-7 c29.



Scheme 36: Comparison of the activities of resveratrol and its analogues 7a-e against HCA-7 c29 cell proliferation. The analogues are compared by the number and nature of their substituents. The red dashed arrow represents the similar activity between **7b** and **7c**. The blue dashed arrow represents the similar activity between **7a** and **7d**

15. Determination of the mechanism of growth inhibition by resveratrol analogues

Based on the results described above, the two most efficient anti-proliferative analogues of resveratrol, **7a** and **7c** (monomethoxy and monophenol respectively) were chosen for further investigation. **7a** is not metabolised *via* glucuronidation and gives a dose response regarding the reduction of number of HCA-7 c29 cells, with an IC_{50} of approximately 50 μM (3 days incubation). **7c** behaves like resveratrol in the metabolism process, but its quantity increases slightly over time in both assays. Compound **7c** similarly reduces the number of the HCA-7 c29 cells, and has an IC_{50} of around 50 μM after 6 days of incubation.

Flow cytometry analysis will give information on the effect of the two analogues on the cell cycle and apoptosis in the HCA-7 c29 cells following 3 and 6 days of treatment. Resveratrol at a concentration of 10 μM was used as a positive control and **7a** and **7c** will be tested at concentrations of 2 μM and 50 μM. These results may give an insight into the mechanisms of action of the analogues compared to resveratrol.

15.1. Analysis of the effect on 7a and 7c on the cell cycle

Evaluation of the cell cycle can be undertaken by flow cytometry, which can be used to measure the quantity of DNA in cells in the G_1, S and G_2/M phases of the cell cycle. During the transition from G_1 to S-phase, the DNA content of the cell will double prior to undergoing mitosis. To evaluate the DNA quantity, DNA-intercalating dyes such as propidium iodide (PI) can be used. However, most nuclear stains are not

permeable to the cell membrane, so that the cells must first be fixed and permeabilised to allow nuclear entry of the dye. Fluorescence intensity of emitted light will thus depend upon the amount of DNA present within a cell, which will alter according to which phase of the cell cycle it currently resides in.

HCA-7 c29 cells were incubated and treated according to the method described in the experimental part (cf. 20. Pharmacology experiments – Materials and Methods). Evaluation of PI-stained HCA-7 cells by flow cytometry showed the percentage of the cells in the different cell cycle phases (Figure 57). The control cells were mainly in the G1 phase (60%) and equally split between S and G2 phases (~22% and ~18% respectively) after three days (Figure 58). However, on day 6, the cells were mainly in G1 (~69%) and S phase (~29%). Following treatments with resveratrol (10 μM) or the analogues **7a** and **7c**, the distribution was always split between the G1 (45-60%) and S phase (40-55%), with very low amounts of cells in G2 (0-5%) at both day 3 and day 6. The distribution of the cells in each phase following treatments at the concentrations assessed was not significantly different from the control (Figure 58).

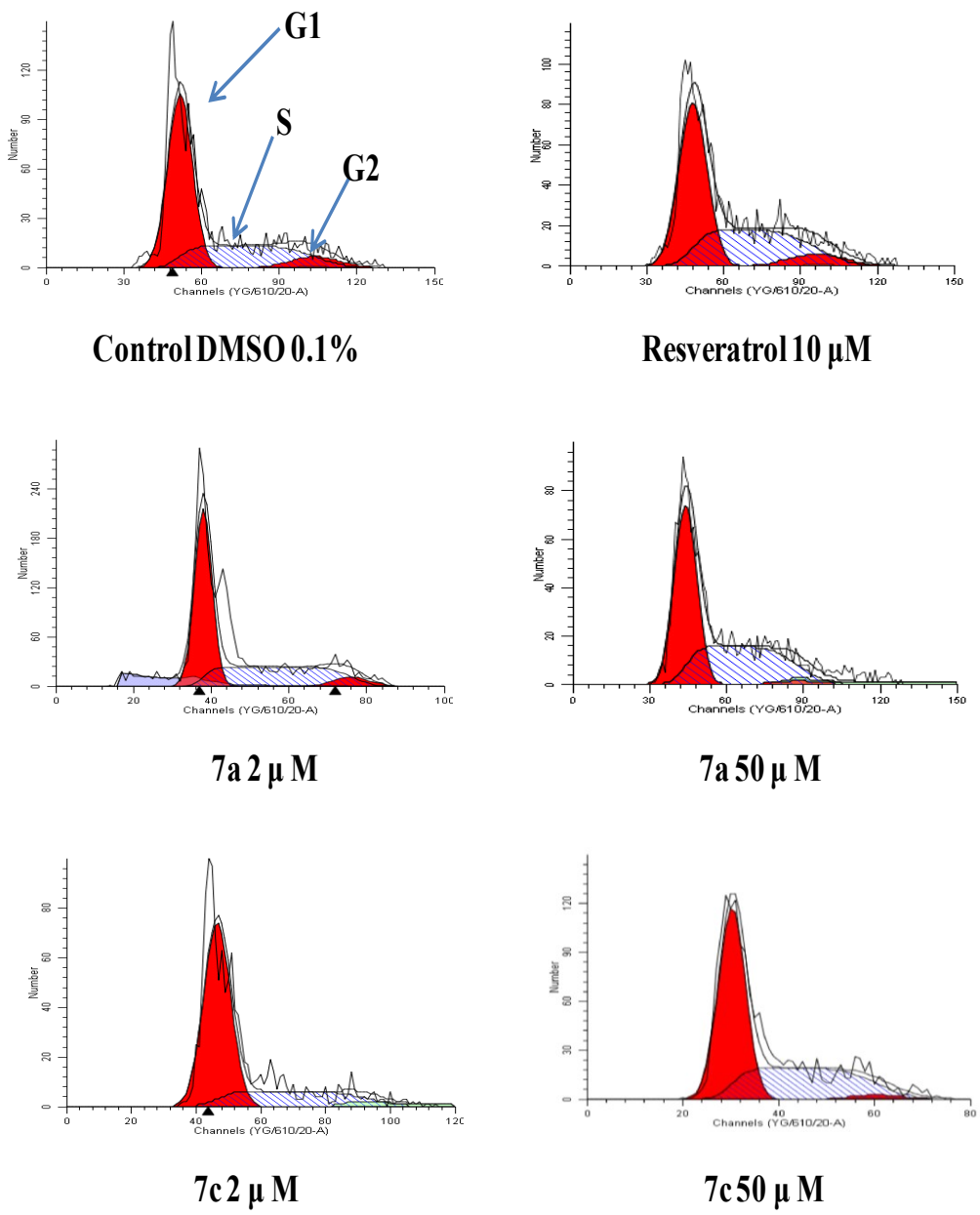


Figure 57: Flow cytometry graphs obtained after 3 days incubation representing the partition of HCA-7 c29 in the different phases of the cell cycle. HCA-7 c29 cells were treated with Resveratrol for 3 and 6 days at concentration of 10 µM in 0.1% DMSO, **7a** at 2 and 50 µM in 0.1% DMSO and **7c** at 2 and 50 µM in 0.1% DMSO and a were compared to a control DMSO (0.1%)

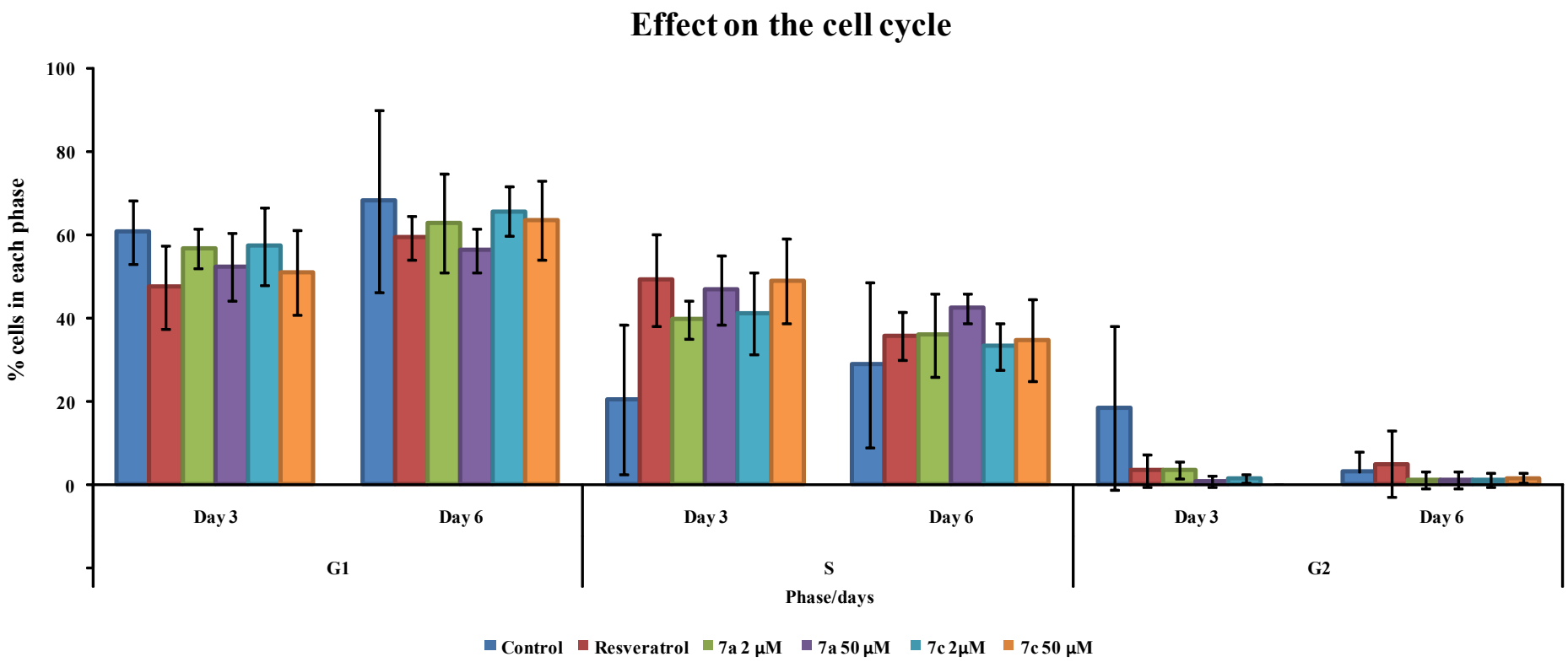


Figure 58: Effects of resveratrol 10 µM, 7a at 2 and 50 µM and 7c at 2 and 50 µM compared to a DMSO control on the cell cycle after 3 and 6 days treatment. Each bar represents the % cell number in each phase at a defined concentration and time and is expressed as a mean ± SD of 3 different experiments of 1 replicate. No result is significant compared to the control.

15.2. Analysis of the effect of 7a and 7c on the apoptosis

One of the characteristics of apoptotic cells is the translocation of phosphatidylserine from the inner to the outer cell membrane. To measure the number of apoptotic cells by flow cytometry, the annexin V assay was used. Annexin is a protein which has a high affinity with acidic phospholipids in a calcium-dependent manner and binds to phosphatidylserine in the apoptotic cells. Annexin V is linked to the fluorophore fluorescein isothiocyanate (FITC), and is used in conjunction with the nuclear dye, PI to differentiate between apoptotic and necrotic cells. Viable cells therefore have a low fluorescence in Annexin V-FITC and PI, early apoptotic cells will have a high fluorescence in FITC and low in PI and late apoptotic and necrotic cells will have a high fluorescence in FITC and PI.

HCA-7 c29 cells were incubated and treated according to the method described in the experimental part (cf. 20. Pharmacology experiments – Materials and Methods). A negative control was use to set the distribution gates allowing analysis of the cells within each quadrant depending on the relative FITC and PI fluorescence (Figure 59). No significant difference can be seen between the control and the different compounds tested on the distribution of cells between the different quadrants after three days of treatment. However, despite a high background in the control, we can see that incubation with 10 μM resveratrol and 50 μM of **7a** reduces the number of live cells (~10% less than the control in both cases) and increases the number of early apoptotic (~ 6% and 3% respectively) and late apoptotic cells (~3% increase in each case). There is no significant effect compared to the control after treating the cells with 2 μM of **7a** or 2 and 50 μM of **7c** (Figure 60).

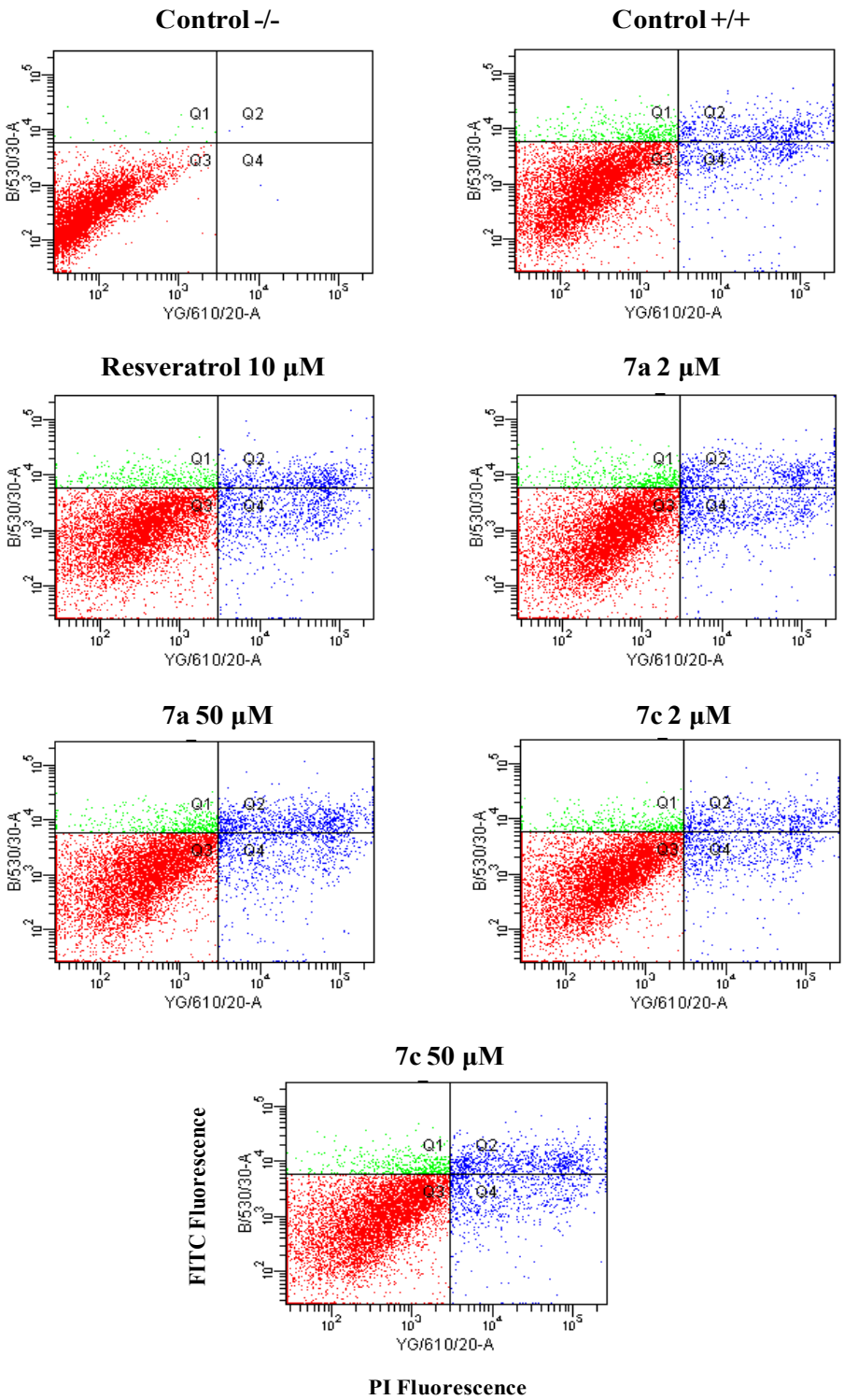


Figure 59: Quadrants obtained after flow cytometry measuring the fluorescence in PI (x axis) and in FITC (y axis). Control -/- represent the control DMSO with no stain. Control +/+ represent the control DMSO with PI and FITC stains. Q1 represents the early apoptotic cells, Q2 the late apoptotic cells, Q3 the alive cells and Q4 the necrotic cells.

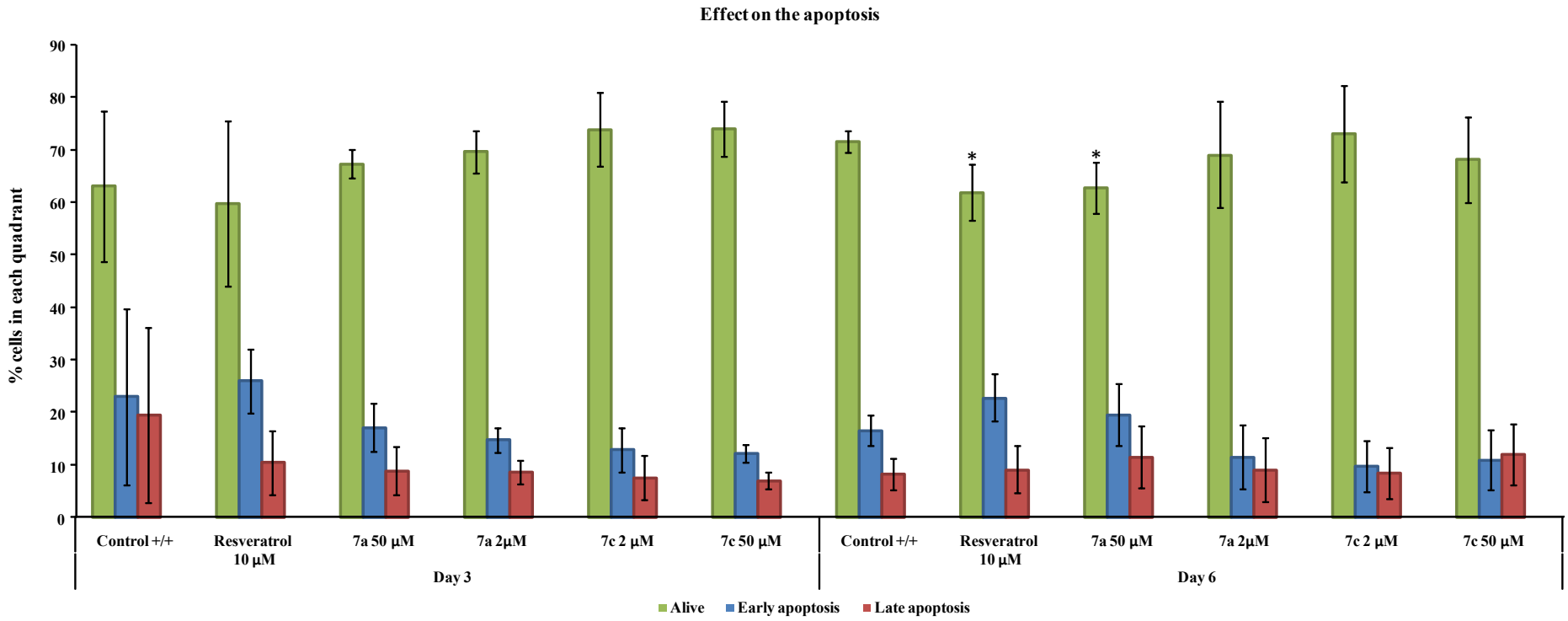


Figure 60: Effects of resveratrol 10 µM, 7a at 2 and 50 µM and 7c at 2 and 50 µM compared to a DMSO control on the apoptosis after 3 and 6 days treatment. Each bar represents the % cell number in each quadrant at a defined concentration and time and is expressed as a mean ± SD of 3 different experiments of 1 replicate. *Asterisks* indicate that the values are significantly different from the control (* $P \leq 0.05$)

15.3. Discussion

HCA-7 c29 cells were treated with a single dose of resveratrol 10 μM, **7a** and **7c** at doses of 2 and 50 μM following the results obtained in the metabolism and cell proliferation assays. Resveratrol is known to be an inducer of apoptosis in some cancer cell lines.[83,84] It was decided to treat the cells with a dose of 10 μM, a slightly higher dose than the IC_{50} of ~ 6μM found during the cell proliferation assay as positive control of apoptosis. The background obtained with the control shows a relatively high number of apoptotic cells, but in spite of this, an increase in the number of apoptotic cells ($P \leq 0.05$) was still observed following six days treatment with resveratrol. A similar effect is seen after six days following treatment with **7a** at 50 μM, although, overall there was no significant increase in apoptosis relative to the control. This high background may mask the effects of the treatments and could be due to an over-trypsinisation of the samples, which is a problem inherent with highly adherent cell lines such as HCA-7. Trypsin tends to degrade the cell membrane[207] resulting in a high phosphatidylserine exposure and thus producing a high level of annexin V binding as an artefact. Alternative commercially available agents (like Accutase® solution)[208] may be used to unstick the cells from the plates without degrading the cell membranes and so maybe avoid the false positives.[209]

In addition there was no significant effect on the cell cycle. After three and six days treatments the cells are mainly in G1 and S phases, and very low numbers are observed in the G2 phase. This may be explained by a possible cell cycle slow down in the S phase for DNA repair or a slow-down in DNA synthesis.

These results do not allow us to draw a definite conclusion regarding a possible mechanism of action of resveratrol analogues to explain whether the effects on cell

proliferation inhibition are due to cytostasis or cytotoxicity. However, further tests are required in both induction of the apoptosis and cell cycle arrest to be sure of the effects. Typically, Western-Blots can be undertaken to identify appearance of cleaved caspases (the effector proteins in the apoptotic cascade) or of other pro-apoptotic proteins to reveal whether apoptosis is induced by treatment with resveratrol analogues **7a** and **7c** at the concentration studied. Western blots may also be used as well to identify increases in the quantity of proteins linked with cell cycle arrest including the cyclin dependant kinase (CDK) inhibitors p21 and p27.

GENERAL CONCLUSION AND FUTURE WORK

In conclusion, we successfully designed, synthesised and analysed a library of seven new analogues of naturally occurring resveratrol using a Wittig-Horner-Emmons reaction. Resveratrol is a good cancer chemopreventive agent, but its metabolism to sulfates and glucuronides is very fast which does not permit a great *in vivo* activity. The library was designed on the supposition that by replacing systematically the phenols on the stilbene structure by benzylic alcohols, it would be possible to stop or at least slow down the metabolism whilst keeping the activity against cancer cell proliferation.

Synthetic analogues **7a-e** were examined for their effect towards sulfation and glucuronidation metabolism using *in vitro* enzymatic assays with mouse and human liver fractions plus the appropriate cofactor. It was concluded that as soon as there is no free phenol on the scaffold, the glucuronidation is stopped and the sulfation is slowed-down, whereas in the presence of at least one phenol, the analogue behaves in a similar manner as resveratrol. However, modifying the structure did not retain the activity of resveratrol against colon cancer cell proliferation. This loss of activity may be due to the absence of 4'-hydroxy group. Assessment of analogues **7f-g** would give us more information on the structure activity relationship, and would confirm the importance of the 4'-hydroxy group for the cell proliferation inhibition. Analogues **7a-d** inhibited HCA-7 colon cancer cell proliferation with an IC_{50} ~50 μM after three or six days incubation with a single dose treatment whereas resveratrol inhibits the proliferation with an IC_{50}~6 μM.

Out of these four relatively active analogues, **7a** and **7c** were the best ones,

being not metabolised or in a slower manner. **7a** does not contain any free phenol and does not behave like resveratrol whereas **7c** contain one phenol and is conjugated in a similar manner to resveratrol. These two analogues were chosen to do flow cytometry studies in order to have a possible insight of the mechanism of inhibition of cell proliferation. Unfortunately the results were inconclusive, except for analogue **7a** which might induce apoptosis at a concentration of 50μM.

Further work is required to optimise the synthesis of the library by using other routes to form the double bond such as the Heck reaction, or the palladium-cross-coupling reactions such as the Suzuki reaction. We may as well complete the series with other analogues where the linker could be modified to a single, *cis*-double or a triple bound. These latter linkers may be obtained after a Sonogashira cross-coupling reaction and specific reduction steps. Other protecting groups or hidden forms of benzylic alcohols or reaction conditions may be tried as well to optimise the reactions yields. Further work is also required to optimise the synthesis of the different sulfate and glucuronide metabolites in order to compare them with the enzymatic metabolites. The sulfates may be synthesised using chlorosulfonic acid as sulfation agent or any other agent which may lead to the desired regio-selective sulfates. Recently, all the resveratrol sulfates metabolites were synthesised specifically using the Heck reaction, starting from sulfated intermediates. An analogue route may be tried to synthesise the sulfate metabolites corresponding to **7a-g**. The glucuronide metabolites of **7a-g** may as well be synthesised specifically according to a similar route as well as by trying other sugar derivatives and coupling agents.

Additional testing such as western-blotting would be needed in cell cycle arrest and apoptosis assays to have a better insight of the mechanism of action of resveratrol and its analogues. Another possibility to find specific targets of resveratrol and compare

with the synthesised analogues would be to do proteomics studies. This would allow a more thorough evaluation and determination of the potential mechanisms of cell proliferation inhibition/survival in which resveratrol is involved in, and would allow potential rational drug designs. Some proteomics studies carried out by Dr Robert Britton are currently under investigation in the group.

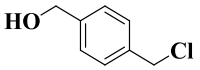
Cell proliferation assays with a daily dosage may be envisaged as well to see whether it induces a difference in the cell proliferation inhibition compared to a single dose treatment. It would also be interesting to investigate the *in vivo* metabolism and pharmacokinetics of the lead compound **7a** and assess its efficacy in a mouse model of colorectal cancer.

CHAPTER 4 – EXPERIMENTAL

Melting points were determined using Microscope "RCH" (Original KOFLER Micro heating and cooling stage). The proton ^{1}H, carbon ^{13}C and phosphorus ^{31}P Nuclear Magnetic Resonance spectra were recorded using Bruker 300 or 400 MHz spectrometers with NMR grade $CDCl_3$ as solvent unless otherwise stated. Chemical shifts δ were recorded in ppm (with tetramethylsilane as internal reference). FAB mass spectra were obtained on a Kratos concept mass spectrometer using NOBA as matrix. The electrospray (ES) mass spectra were recorded using a Micromass Quattro LC mass spectrometer with DCM, acetronitrile or NBA as solvent. FT-IR spectra were recorded using a Perkin Elmer Instruments Spectrum One FT-IR Spectrometer. The elemental analyses on the final analogues were performed by the Elemental Analysis Service at the London Metropolitan University. Flash and gravity chromatographic purification were performed using Silica Gel 60. Chemicals were all obtained from Sigma-Aldrich and were used without further purification. Solvents were reagent grade and purchased from Fluka. Dry solvents were taken from a Solvent Purification System Innovative Technologies, Inc. Microwave experiments were carried out on a CEM Explorer with an automatic sampler.

16. Phosphonates

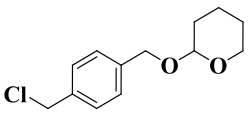
Synthesis of (4-(chloromethyl)phenyl)methanol[149] 10b



In a round bottom flask at r.t. conc. HCl (50 mL, 600 mmol, 5.6 equiv.) was added to a solution of benzene-1,4-dimethanol **11a** (14.7 g, 106.3 mmol, 1 equiv.) in toluene (300 mL). The reaction mixture was stirred vigorously o/n. Saturated solution of $NaHCO_3$ (200 mL) was poured onto the mixture and let to stir for 15 min. The toluene layer was removed and the aqueous phase was extracted with diethyl ether (2 x 50 mL). Brine (50 mL) was added and the combined aqueous layer was then extracted with chloroform (2 x 50 mL). The combined organic layers were dried (Na_2SO_4), the solvents were removed under reduced pressure and the title compound **10b** (15.98 g, 96%) was obtained as a white solid (mp 58-59 ºC, mp lit.[149] 58-60 ºC).

IR ν/cm^{-1} : 3332, 1421, 1261 ; ^{1}H NMR (300 MHz, *CDCl$_3$*) δ 7.23 (d, *J* = 8.1 Hz, 2H, CH_{Ar}), 7.17 (d, *J* = 8.1 Hz, 2H, CH_{Ar}), 4.46 (s, 2H, CH_2), 4.45 (s, 2H, CH_2), 2.83 (br s, 1H, OH) ; ^{13}C NMR (75 MHz, *CDCl$_3$*) δ 141.23 (C), 136.78 (C), 128.84 (CH_{Ar}), 127.31 (CH_{Ar}), 64.58 (CH_2), 46.14 (CH_2) ; *m/z* (EI) 156.03402 ($[M-H]^+$ C_8H_9ClO requires 156.03419)

Synthesis of 2-(4-(chloromethyl)benzyloxy)tetrahydro-2H-pyran[150] 14b



In a round bottomed flask at r.t. 10% aqueous HCl (15 mL, 15 mmol, 0.3 equiv.) and dihydropyran (8 ml, 87.7 mmol, 2

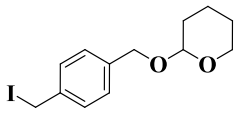
equiv.) were successively added to a solution of 4-(chloromethyl)phenyl)methanol **10b** (7g, 44.7 mmol, 1 equiv.) in dichloromethane (50 mL). The reaction mixture was stirred at r.t. overnight. Saturated solution of $NaHCO_3$ (60 mL) was added and the mixture was stirred at r.t. for 10 minutes. The aqueous layer was extracted with diethyl ether (2 x 30 mL). The combined organic layers were washed with brine (30 mL), dried (Na_2SO_4) and the solvent was removed under reduced pressure. Purification of the crude yellow oil by chromatography column (petrol 40-60:ethyl acetate 95:5) gave the title compound **14b** (7.34 g, 68%) as a pale slightly yellow oil.

IR ν/cm^{-1} 2942, 2870, 1610, 1516, 1421, 1350, 1265; ^{1}H NMR (300 MHz, *CDCl$_3$*) δ 7.27 (s, 4H, CH_{Ar}), 4.81 (d, *J* = 12.3 Hz, 1H, Ar-C*H*H-OTHP), 4.73 (t, *J* = 3.5 Hz, 1H, O-CH-O), 4.59 (s, 2H, CH_2Cl), 4.52 (d, *J* = 12.3 Hz, 1H, Ar-C*H*H-OTHP), 3.97-3.89 (m, 1H, CH_2-*H*CH-CH-O), 3.60-3.53 (m, 1H, CH_2-C*H*H-CH-O), 1.94-1.52 (m, 6H, 3 x C*H$_2$* (THP)) ; ^{13}C NMR (75 MHz, *CDCl$_3$*) δ 138.73 (C), 136.50 (C), 128.50 (2 x CH_{Ar}), 128.07 (2 x CH_{Ar}), 97.80 (O-*C*H-O), 68.36 (*C*H_2-OTHP), 62.12 (Ar-*C*H_2O), 46.07 (CH_2Cl), 30.57 (CH_2), 25.48 (CH_2), 19.23 (CH_2); *m/z* (EI) 240.09126 ([M-H]$^+$ $C_{13}H_{17}ClO_2$ requires 240.09171)

Synthesis of 2-(4-(iodomethyl)benzyloxy)tetrahydro-2H-pyran 14c

Method from Daub *et al.*[151]

New compound



2-(4-(Chloromethyl)benzyloxy)tetrahydro-2H-pyran **14b** (7.34 g, 30.5 mmol, 1 equiv.) was dissolved in dry acetone (40 mL). Sodium iodide (5.08 g, 33.9 mmol, 1.1 equiv.) was added and the solution was

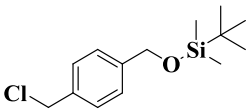
stirred at room temperature for 1h. A formation of a white precipitate was observed during the reaction and it was removed by further filtration and washed with acetone (2 x 10 mL). The acetone was removed under reduced pressure and the residue was taken up with brine (40 mL) and extracted with dichloromethane (3 x 40 mL). The combined organic layers were dried (Na_2SO_4) and the solvents were removed under reduced pressure to give a crude as an orange oil. The title compound **14c** (8.0 g, 79%) was obtained as light sensitive yellow oil after purification by column chromatography (chloroform).

^{1}H NMR (300 MHz, *CDCl$_3$*) δ 7.39 (dd, J = 9.6, 1.9 Hz, 4H), 4.83 (d, J = 12.2 Hz, 1H), 4.79 (t, J = 3.4 Hz, 1H), 4.54 (d, J = 12.2 Hz, 1H), 4.50 (s, 2H), 3.94-4.03 (m, 1H), 3.59-3.66 (m, 1H), 1.54-2.01 (m, 6H) ; ^{13}C NMR (300 MHz, *CDCl$_3$*) δ 5.92 (CH_2), 19.37 (CH_2), 25.52 (CH_2), 30.59 (CH_2), 62.00 (CH_2), 68.41 (CH_2), 99.78 (CH), 128.06 (C_{Ar}), 128.83 (C_{Ar}), 136.70 (C_{Ar}), 138.35 (C_{Ar}); MS: 332, 127, 105, 102, 85

Synthesis of tert-butyl(4-(chloromethyl)benzyloxy)dimethylsilane 14a

New compound

Method from Katz *et al.*[210]



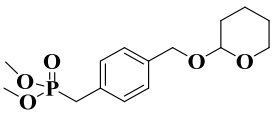
In a round bottom flask at r.t. under nitrogen, Imidazole (6.5 g, 95.7 mmol, 3 equiv.) and TBDMSCl (7.2 g, 47.9 mmol, 1.5 equiv.) were added to a solution of (4-(chloromethyl)phenyl)methanol **10b** (5 g, 31.9 mmol, 1.0 equiv.) in dry DMF (130 mL). The mixture was stirred with vigorous stirring o/n at r.t. Ice-water (80 mL) and diethyl ether (80 mL) were poured onto the mixture and let to stir for 15 min. The organic layer is removed and the aqueous layer is then

extracted with ethyl acetate (3 x 50 mL) and diethyl ether (3 x 50 mL) and the combined organic layers were washed with saturated LiCl solution (3 x 50 mL), dried (Na_2SO_4), filtered and the solvents were removed under reduced pressure. Purification of the crude pale yellow oil by chromatography column (Petroleum ether 40-60°C: ethyl acetate – 95:5) gave the title product **14a** (7.97 g, 92%) as a colorless oil.

^{1}H NMR (300 MHz, *CDCl$_3$*) δ 7.27-7.19 (m, 4H, 4 x CH_{Ar}), 4.64 (s, 2H, CH_2O), 4.47 (s, 2H, CH_2Cl), 0.85 (s, 9H, 3 x CH_3), 0.00 (s, 6H, 2x CH_3) ; ^{13}C NMR (75 MHz, *CDCl$_3$*) δ 141.81 (C), 136.03 (C), 128.49 (2 x CH_{Ar}), 126.29 (2 x CH_{Ar}), 64.57 (CH_2-O), 46.16 (CH_2-Cl), 25.93 (3 x CH_3), 18.39 (C), -5.29 (2 x CH_3).

Synthesis of dimethyl 4-((tetrahydro-2H-pyran-2-yloxy)methyl) benzylphosphonate 8b

New compound

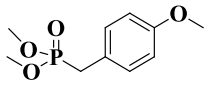


General procedure of phosphorylation 1: 2-(4-(iodomethyl)benzyloxy)tetrahydro-2H-pyran **14b** (429 mg, 1.29 mmol, 1 equiv.) and trimethyl phosphite (0.5 mL, 4.24 mmol, 3.3 equiv.) were mixed together in a microwave vessel. The mixture was heated under microwave irradiation (300W, 110 °C, 1h). The temperature increased very quickly to 135 °C which led to the arrest of the reaction. Monitoring by TLC showed the nearly completion of the reaction. The mixture was heated an additional 5 min to complete the reaction. The excess of trimethyl phosphite was evaporated with toluene (5 mL). Purification of the crude oil on chromatography column (dichloromethane:methanol 99:1 → 70:30) gave the title compound **8b** (264 mg, 65%) as a yellow oil.

IR ν/cm^{-1}2949, 2852, 1695, 1516, 1455, 1391, 1365, 1249; ^{1}H NMR (400 MHz, *CDCl$_3$*) δ 7.26-7.18 (m, 4H, CH_{Ar}), 4.68 (d, *J* = 12.0 Hz, 1H, Ar-C*H*H-OTHP), 4.62 (t, *J* = 3.5 Hz, 1H, O-CH-O), 4.40 (d, *J* = 12.0 Hz, 1H, Ar-C*H*H-THP), 3.86-3.79 (m, 1H, CH_2-C*H*H-CH-O), 3.59 (d, *J* = 10.8 Hz, 6H, CH_3-O-P), 3.48-3.37 (m, 1H, CH_2-C*H*H-CH-O), 3.08 (d, *J* = 21.6 Hz, 2H, Ar-CH_2-P), 1.79-1.44 (m, 6H, 3 x C*H$_2$* (THP)) ; ^{13}C NMR (100 MHz, *CDCl$_3$*) δ 136.92 (d, *J* = 3.6 Hz, C), 130.21 (d, *J* = 9.2 Hz, C), 129.47 (d, *J* = 6.6 Hz, 2 x CH_{Ar}), 127.84 (d, *J* = 2.6 Hz, 2 x CH_{Ar}), 97.55 (O-*C*H-O), 68.24 (CH_2-O), 61.90 (CH_2-O), 52.65 (d, *J* = 6.8 Hz, 2 x CH_3), 32.32 (d, *J* = 138.21, *C*H_2-P), 30.37 (CH_2), 25.28 (CH_2), 19.16 (CH_2) ; ^{31}P NMR (400 MHz, *CDCl$_3$*) δ 29.6; *m/z* (ESI) 315.1370 ([M-H]$^+$ $C_{15}H_{23}O_5P$ requires 314.1283)

General procedure of phosphorylation 2: In a sealed microwave flask the chlorinated compound (1 equiv.) and trimethylphosphite (2 equiv.) were added and the mixture was heated to 110°C o/n. The excess trimethylphosphite is then removed under reduced pressure. Purification of the crude product is done on chromatography column or by recrystallisation.

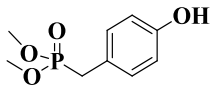
Synthesis of dimethyl 4-methoxybenzylphosphonate 8e



Dimethyl 4-methoxybenzylphosphonate **8e** was synthesised according to general procedure 1 from 4-methoxybenzyl bromide (0.36 mL, 2.5 mmol, 1 equiv.) and trimethyl phosphite (0.6 mL, 5 mmol, 2 equiv.). The title compound **8e** (428 mg, 74%) was obtained after work-up and purification on chromatography column (dichloromethane: methanol – 99:1) as a yellow oil.

IR ν_{max}/cm^{-1} 2909, 1512, 1244 ; ^{1}H NMR (300 MHz, *CDCl$_3$*) δ 7.21 (dd, *J* = 8.7, 4.8 Hz, 2H, 2 x CH_{Ar}), 6.85 (d, *J* = 8.7 Hz, 2H, 2 x CH_{Ar}), 3.77 (s, 3H, CH_3O), 3.65 (d, *J* = 10.8 Hz, CH_3-O-P), 3.49 (d, *J* = 21.0 Hz, Ar-CH_2P); ^{13}C NMR (75 MHz, *CDCl$_3$*) δ 158.07 (d, *J* = 3.4 Hz, C), 130.54 (d, *J* = 6.5 Hz, 2 x CH_{Ar}), 122.42 (d, *J* = 9.3 Hz, C), 113.95 (d, *J* = 2.6 Hz, 2 x CH_{Ar}), 54.98 (s, CH_3O), 52.62 (d, *J* = 6.8 Hz, 2 x CH_3OP), 31.53 (d, *J*=138.8 Hz, CH_2P); ^{31}P (300 MHz, *CDCl$_3$*) δ 29.34 ; *m/z* (ESI) 231.0781 ([M-H]$^+$ $C_{10}H_{16}O_4P$ requires 231.0786)

Synthesis of dimethyl 4-hydroxybenzylphosphonate 8f

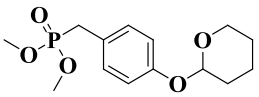


Dimethyl 4-hydroxybenzylphosphonate **8f** was synthesised according to general procedure 2 from 4-hydroxybenzylmethanol (5 g, 40.3 mmol, 1 equiv.) and trimethylphosphite (5.2 mL, 44.3 mmol, 1.1 equiv.). Recrystallisation from ethyl acetate gave the title compound **8f** (3.13 g, 36%) as a white solid (m.p. 119-121°C)

^{1}H NMR (300 MHz, *CDCl$_3$*) δ 8.06 (br s, 1H, OH), 7.04 (dd, *J* = 8.3, 2.4 Hz, 2H, 2 x CH_{Ar}), 6.65 (d, *J* = 8.6 Hz, 2H, 2 x CH_{Ar}), 3.70 (d, *J* = 11.1 Hz, CH_3-O-P), 3.08 (d, *J* = 21.0 Hz, Ar-CH_2-P) ; ^{13}C NMR (75 MHz, *CDCl$_3$*) δ 156.04 (d, *J* = 3.5 Hz, C), 130.61 (d, *J* = 6.5 Hz, 2 x CH_{Ar}), 120.82 (d, *J* = 9.3 Hz, C), 116.06 (d, *J* = 2.9 Hz, 2 x CH_{Ar}), 53.08 (d, *J* = 7.1 Hz, 2 x CH_3O), 31.69 (d, *J* = 138.9 Hz, CH_2-P) ; ^{31}P (300 MHz, *CDCl$_3$*) δ 30.11 ; *m/z* (ESI) 301.1212 ([M-H]$^+$ $C_{14}H_{22}O_5P$ requires 301.1205)

Synthesis of dimethyl 4-(tetrahydro-2H-pyran-2-yloxy)benzylphosphonate 8d

New Compound

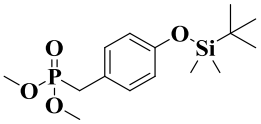


In a round bottom flask at r.t. under nitrogen, PPTS (116 mg, 0.46 mmol, 0.1 equiv.) and DHP (0.46 mL, 5.09 mmol, 1.1 equiv.) were added to a solution of phosphonate **8f** (1 g, 4.63 mmol, 1.0 equiv.) in dry chloroform (60 mL). The mixture was stirred with vigorous stirring o/n at r.t. Saturated $NaHCO_3$ solution (60 mL) is poured onto the reaction mixture and left to stir at r.t. for 10 min. The chloroform layer is removed and the aqueous layer is extracted with chloroform (2 x 30 mL). The combined organic layers are then washed with brine (2 x 30 mL), dried ($MgSO_4$), filtered and the solvents were removed under reduced pressure. Purification of the crude colorless oil by chromatography column (dichloromethane: methanol – 95:5) gave the title product **8d** (990 mg, 71%) as a colourless oil which crystallizes on standing.

^{1}H NMR (300 MHz, *CDCl$_3$*) δ 7.17 (dd, *J* = 8.4, 2.4 Hz, 2H, 2 x CH_{Ar}), 6.97 (d, *J* = 8.4 Hz, 2H, 2 x CH_{Ar}), 5.35 (t, *J* =3.3 Hz, Ar-O-CH-O), 3.91-3.83 (m, 1H, (THP)-C*H*H-O), 3.65-3.53 (dd, *J* = 10.8, 1.2 Hz, CH_3-O-P and m, 1H, (THP)-C*H*H-O), 3.08 (d, *J* = 21.0 Hz, Ar-CH_2P), 2.03-1.52 (m, 6H, 3 x C*H*$_2$ (THP)) ; ^{13}C NMR (75 MHz, *CDCl$_3$*) δ 156.16 (d, *J* = 3.5 Hz, C), 130.59 (d, *J* = 6.6 Hz, 2 x CH_{Ar}), 124.00 (d, J = 9.3 Hz, C), 116.67 (d, *J* = 2.8 Hz, 2 x CH_{Ar}), 96.43 (O-*C*H-O), 62.05 (*C*H$_2$O (THP)), 52.88 (d, *J* = 6.8 Hz, 2 x CH_3OP), 31.93 (d, *J* = 138.2 Hz, CH_2P), 30,37 (CH_2), 25.19 (CH_2), 18.18 (CH_2) ; ^{31}P (300 MHz, *CDCl$_3$*) δ 29.27.

Synthesis of dimethyl 4-(tert-butyldimethylsilyloxy)benzylphosphonate 8c

New compound

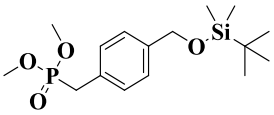


In a round bottom flask at r.t. under nitrogen, Imidazole (2.09 g, 30.74 mmol, 4.5 equiv.) and TBDMSCl (1.5 g, 10.24 mmol, 1.5 equiv.) were added to a solution of phosphonate **8f** (1.48 g, 6.83 mmol, 1.0 equiv.) in dry DMF (30 mL). The mixture was stirred with vigorous stirring o/n at r.t. Ice-water (20 mL) was poured onto the mixture and let to stir for 15 min. The mixture was then extracted by ethyl acetate (3 x 20 mL) and the combined organic layers were washed with saturated LiCl solution (3 x 30 mL), dried ($MgSO_4$), filtered and the solvents were removed under reduced pressure. Purification of the crude colorless oil by chromatography column (chloroform: ethanol – 95:5) gave the title product **8c** (1.32 g, 65%) as a colorless oil.

^{1}H NMR (300 MHz, *CDCl$_3$*) δ 7.14 (dd, *J* = 8.4, 2.7 Hz, 2H, 2 x CH_{Ar}), 6.77 (d, *J* = 8.4 Hz, 2H, 2 x CH_{Ar}), 3.64 (d, *J* = 10.8 Hz, CH_3-O-P), 3.09 (d, *J* = 21.0 Hz, Ar-CH_2P), 0.96 (s, 9H, 3 x CH_3), 0.17 (s, 6H, 2x CH_3) ; ^{13}C NMR (75 MHz, *CDCl$_3$*) δ 154.70 (C), 130.70 (2 x CH_{Ar}), 123.64 (C), 120.23 (2 x CH_{Ar}), 52.86 (CH_3), 52.77 (CH_3), 32.00 (d, 2 x C, CH_2-P), 25.62 (3 x CH_3), 18.14 (C), -4.48 (2 x CH_3) ; ^{31}P (300 MHz, *CDCl$_3$*) δ 29.27 (s).

Synthesis of dimethyl 4-((tert-butyldimethylsilyloxy)methyl) benzylphosphonate 8a

New compound

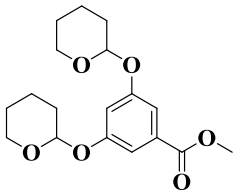


In a three-neck-round bottom flask at 0°C under nitrogen containing sodium hydride 60% dispersed in mineral oil (1.48 g, 37.0 mmol, 2 equiv.) washed with hexane, a solution of dimethyl phosphite (1.78 mL, 19.4 mmol, 1.05 equiv.) was added dropwise with a canula. After the bubbling stopped a solution of *tert*-butyl-(4-(chloromethyl)benzyloxy)dimethylsilane **10f** (5 g, 18.5 mmol, 1 equiv.) in dry THF (40 mL) was added dropwise with a canula at 0°C, N_2. The reaction mixture was stirred o/n from 0°C to r.t. Water (20 mL) was added slowly and after the bubbling stopped the aqueous layer was extracted with diethyl ether (3 x 30 mL) and the combined organic layers were washed with saturated brine (3 x 30 mL), dried ($MgSO_4$), filtered and the solvents were removed under reduced pressure. Purification of the crude off-white oil by chromatography column (dichloromethane: ethanol – 98:2 → 95:5) gave the title product **8a** (1.98 g, 30%) as a colorless oil.

^{1}H NMR (400 MHz, *CDCl$_3$*) δ 7.17 (m, 4H, CH_{Ar}), 4.62 (s, CH_2O), 3.58 (d, *J* = 10.8 Hz, CH_3-O-P), 3.09 (d, *J* = 21.0 Hz, Ar-CH_2P), 0.84 (s, 9H, 3 x CH_3), 0.00 (s, 6H, 2x CH_3); ^{13}C NMR (100 MHz, *CDCl$_3$*) δ 140.24 (d, J = 3.6 Hz, C), 129.69 (d, *J* = 9.3 Hz, C), 129.53 (2 x CH_{Ar}), 126.28 (2 x CH_{Ar}), 64.67 (CH_2), 52.86 (d, *J* = 6.8 Hz, 2 x CH_3O-P), 32.57 (d, *J* = 137.6, CH_2-P), 25.96 (3 x CH_3), 18.41 (C), -5.24 (2 x CH_3) ; ^{31}P (300 MHz, *CDCl$_3$*) δ 29.04 (s).

17. Aldehydes

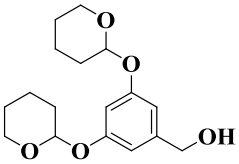
Synthesis of methyl 3,5-bis(tetrahydro-2H-pyran-2-yloxy)benzoate[167,166] 19c



General procedure of THP protection GP-3: In a round bottom flask at r.t. under nitrogen, PPTS (151 mg, 0.6 mmol, 0.2 equiv.) and 3,4-dihydro-2H-pyran (1.5 mL, 16.4 mmol, 6 equiv.) were added to a solution of methyl 3,5-dihydroxybenzoate **19b** (500 mg, 3.0 mmol, 1 equiv.) in dry dichloromethane (20 mL) and the reaction mixture was stirred o/n. $NaHCO_3$ saturated solution (30 mL) was poured onto the reaction mixture and left to stir for 10 min. The organic layer was removed and the aqueous layer was then extracted with ethyl acetate (3 x 30 mL). The combined organic layer were dried ($MgSO_4$), filtered and the solvents were removed under reduced pressure. Purification of the crude off-white oil by column chromatography (petroleum ether 40-60°C-ethyl acetate 95:5 → 90:10) gave the title product **19c** (986 mg, 92%) as a colorless oil which crystallizes on standing (mp 88-91°C).

IR ν_{max}/cm^{-1} 3457, 2970, 1725, 1439, 1365, 1216; ^{1}H NMR (300 MHz, *CDCl₃*) δ 7.38 (t, *J*= 2.1 Hz, 2H, 2 x CH_{Ar}), 6.98 (q, *J* = 2.1 Hz, 1H, CH_{Ar}), 5.47 (q, *J* = 3.0 Hz, 2H, 2 x O-C*H*-O), 3.93-3.85 (m, 2H, CH_2-CH*H*-CHO and s, 3H, CH_3O), 3.64-3.61 (m, 2H, CH_2-C*H*H-CHO), 2.07-1.56 (m, 12H, 6 x CH_2 (THP)) ; ^{13}C NMR (75 MHz, *CDCl₃*) δ 166.42 (C), 157.71 (C), 157.65 (C), 131.61 (C), 110.54 (CH_{Ar}), 109.79 (CH_{Ar}), 109.63 (CH_{Ar}), 96.17 (*C*H-O), 96.03 (*C*H-O), 61.70 (CH_2O), 61.66 (CH_2O), 51.83 (CH_3O), 29.98 (2 x CH_2), 24.92 (2 x CH_2), 18.38 (2 x CH_2) ; *m/z* (ESI) 337.1657 ($[M-H]^+$ $C_{18}H_{25}O_6$ requires 337.1651)

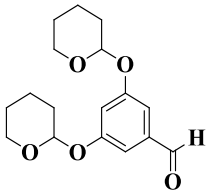
Synthesis of (3,5-bis(tetrahydro-2H-pyran-2-yloxy)phenyl)methanol[166,167] 12b



General procedure for $LiAlH_4$ reduction GP-4: In a dry three-neck round bottom flask under nitrogen equipped with a water condenser, a suspension of $LiAlH_4$ (1.70 g, 44.6 mmol, 1.5 equiv./ester) in dry THF (100 mL) was made. A solution of the ester **19c** (10.5 g, 29.8 mmol, 1 equiv.) in dry THF (100 mL) was added dropwise via an equalizing pressure dropping funnel and the reaction mixture was stirred at r.t. for 1h. Water (1 mL) was added slowly via the condenser (exothermic). When bubbling stopped 3M NaOH solution (1 mL) was added via the condenser and then water (3 mL). The mixture was filtered on Celite and the cake was washed with THF (3 x 50 mL). The solvents were then removed under reduced pressure. Purification of the crude colorless oil by chromatography column (petroleum ether 40-60°C: ethyl acetate – 90:10) gave the title product **12b** (6.25 g, 68%) as a pale yellow oil.

^{1}H NMR (300 MHz, *CDCl$_3$*) δ 6.70 (s, 3H, 3 x CH_{Ar}), 5.41 (q, *J* = 3.4 Hz, 2H, 2 x O-CH-O), 4.61 (d, *J* = 3.4 Hz, 2H, CH_2OH), 3.94-3.86 (m, 2H, CH_2-C*H*H-CHO), 3.63-3.56 (m, 2H, CH_2-C*H*H-CHO), 2.04-1.54 (m, 12H, 6 x C*H$_2$* (THP)) ; ^{13}C NMR (75 MHz, *CDCl$_3$*) δ 158.29 (C), 158.23 (C), 143.19 (C), 108.09 (CH_{Ar}), 104.56 (CH_{Ar}), 104.39 (CH_{Ar}), 96.41 (O-*C*H-O), 96.25 (O-*C*H-O), 65.32 (CH_2OH), 62.02 (2 x CH_2O), 30.32 (2 x CH_2), 25.20 (2 x CH_2), 18.73 (2 x CH_2) ; *m/z* (ESI) 309.1704 ($[M-H]^+$ $C_{17}H_{25}O_5$ requires 309.1702)

Synthesis of 3,5-bis(tetrahydro-2H-pyran-2-yloxy)benzaldehyde[165,211] 9i

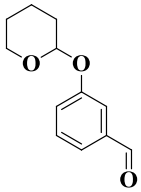


General procedure for PCC oxidation GP-5: Pyridinium chlorochromate (2.52 g, 11.6 mmol, 2.0 equiv.) was added to a solution of (3,5-bis(tetrahydro-2H-pyran-2-yloxy)phenyl)methanol **12b** (1.80 g, 5.8 mmol, 1 equiv.) in chloroform (60 mL). The brown mixture was stirred at r.t. for 18h. Diethyl ether (50 mL) was added to the mixture which was then vigorously stirred for 15 min. The mixture was then filtered on a pad of Celite and Silica and the cake was washed with diethyl ether (3 x 50 mL) and dichloromethane (2 x 50 mL). The solvents were removed under reduced pressure and purification of the light green oil on chromatography column (dichloromethane: methanol 99:1) gave the title compound **9i** (1.40 g, 78%) as a pale yellow oil.

IR ν_{max}/cm^{-1} 2941, 1697, 1589, 1017; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.83 (s, 1H, CHO), 7.14-7.13 (m, 2H, 2 x CH_{Ar}), 6.95 (s, 1H, CH_{Ar}), 5.40 (q, *J* = 3.0 Hz, 2H, 2 x O-C*H*-O), 3.85-3.77 (m, 2H, CH_2-C*H*H-CHO), 3.59-3.52 (m, 2H, CH_2-C*H*H-CHO), 3.00-1.49 (m, 12H, 6 x CH_2 (THP)) ; ^{13}C NMR (75 MHz, *$CDCl_3$*) δ 191.69 (H*C*=O), 158.42 (C), 158.38 (C), 138.16 (C), 111.40 (CH_{Ar}), 110.47 (2 x CH_{Ar}), 96.30 (2 x O-*C*H-O), 61.90 (2 x *C*H_2O), 30.06 (2 x CH_2), 24.98 (2 x CH_2), 18.47 (2 x CH_2)

Synthesis of 3-(tetrahydro-2H-pyran-2-yloxy)benzaldehyde[164] 9h

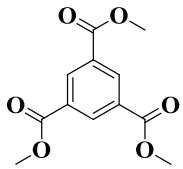
Synthesised according to Severi *et al.*[164]



3-(Tetrahydro-*2H*-pyran-2-yloxy)benzaldehyde **9h** was synthesised according to general procedure GP-3 from 3-hydroxybenzaldehyde (3 g, 24.6 mmol, 1 equiv.), PPTS (47 mg, 0.25 mmol, 0.01 equiv.) and DHP (11.2 mL, 123 mmol, 5 equiv.). The title compound **9h** was obtained (2 g, 39%) after work-up and purification on chromatography column (petroleum ether 40-60°C: ethyl acetate – 90:10) as a yellow oil.

IR ν_{max}/cm^{-1} 2943.90, 1697, 1584, 1248.22 ; ^{1}H NMR (300 MHz, *CDCl$_3$*) δ 9.96 (s, *H*C=O), 7.55-7.40 (m, 3H, 3 x CH_{Ar}), 7.30 (ddd, *J* = 8.7, 2.7, 1.5 Hz, 1H, CH_{Ar}), 5.48 (t, *J* = 2.7 Hz, 1H, O-CH-O), 3.91-3.83 (m, 1H, (THP)C*H*H-O), 3.64-3.58 (m, 1H, (THP)C*H*H-O), 2.09-1.52 (m, 6H, 3 x CH_2) ; ^{13}C NMR (75 MHz, *CDCl$_3$*) δ 191.99 (H*C*=O), 157.58 (C), 137.76 (C), 129.95 (CH_{Ar}), 123.35 (CH_{Ar}), 122.93 (CH_{Ar}), 116.40 (CH_{Ar}), 96.38 (O-CH-O), 62.03 ((THP)CH_2O), 30.17 (CH_2), 25.28 (CH_2), 18.62 (CH_2)

Synthesis of trimethyl benzene-1,3,5-tricarboxylate 19a

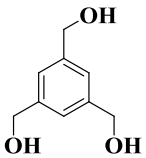


A catalytic amount of concentrated sulphuric acid (3 mL) was added to 1,3,5-tricarboxylic acid **13a** (14 g, 66.6 mmol, 1 equiv.) dissolved in methanol (200 mL) and the mixture was refluxed overnight. The reaction mixture was then let to cool down in an ice bath. The white solid which was formed was filtered and washed with cold methanol. The title product **19a** (15.43 g, 92%) was obtained as white fluffy crystals (mp 143-145 ºC, mp lit.[212]

145-147°C).

IR ν_{max}/cm^{-1} 3546, 2970, 1737; ^{1}H NMR (300 MHz, *CDCl$_3$*) δ 8.80 (s, 3H, CH_{Ar}), 3.99 (s, 9H, CH_3O) ; ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.39 (3 x C=O), 134.54 (3 x CH_{Ar}), 131.20 (3 x C), 52.67 (3 x CH_3O) ; *m/z* (ESI) 253.0721 ($[M-H]^+$ $C_{12}H_{12}O_6$ requires 252.0634)

Synthesis of benzene-1,3,5-triyltrimethanol[212] 20a

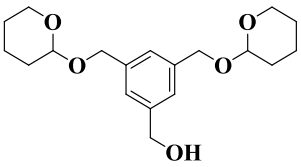


Benzene-1,3,5-triyltrimethanol **20a** was synthesised according to general procedure GP-4 from triester **19a** (2.19 g, 8.67 mmol, 1 equiv.), LAH (1.48 g, 39.04 mmol, 4.5 equiv.) in dry THF (90 mL mL). The title compound **20a** (1.38 g, 95 %) was obtained after work-up and purification. (m.p: 70-71 ºC mp litt.[212] 75-76°C)

IR ν_{max}/cm^{-1} 3211, 2871, 1446; ^{1}H NMR (300 MHz, *MeOD*) δ 7.14 (s, 3H, H_{Ar}), 4.47 (s, 6H, 3 x C*H*$_2$OH) ; ^{13}C NMR (75 MHz, *MeOD*) δ143.0 (3 x C), 125.5 (3 x CH_{Ar}), 65.2 (3 x CH_2); *m/z* (ESI) 169.0863 ($[M-H]^+$ $C_9H_{13}O_3$ requires 169.0865)

Synthesis of ((3,5-bis-(tetrahydro-2H-pyran-2-yloxy)methyl)phenyl)methanol 12a

Synthesised according to Meric *et al.*[157]



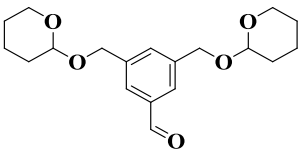
In a round bottomed flask at r.t. 10% aqueous HCl (3 mL) and dihydropyran (3.25 mL, 25.7 mmol, 2 equiv.) were

successively added to a solution of benzene-1,3,5-triyltrimethanol **20a** (3 g, 17.8 mmol, 1 equiv.) in dichloromethane:THF (10 mL: 10 mL). The reaction mixture was stirred at r.t. overnight. Saturated solution of $NaHCO_3$ (60 mL) was added and the mixture was stirred at r.t. for 10 minutes. The organic layer is removed and the aqueous layer was extracted with dichloromethane (2 x 50 mL). The combined organic layers were washed with brine (2 x 30 mL), dried ($MgSO_4$) and the solvents were removed under reduced pressure. Purification of the crude pale yellow oil by chromatography column (dichloromethane: methanol – 97:3) gave the title compound **12a** (1.94 g, 32 %) as a pale slightly yellow oil.

IR ν_{max}/cm^{-1} 3432, 2940 ; ^{1}H NMR (300 MHz, *CDCl$_3$*) 7.21 (s, 3H, 3 x CH_{Ar}), 4.70(d, *J* = 12.0 Hz, 2H, 2 x Ar-C*H*H-OTHP), 4.63 (t, *J* = 3.3 Hz, 2H, 2 x O-C*H*-O), 4.59 (s, 2H, C*H$_2$*OH), 4.41 (d, *J* = 12.3 Hz, 2H, 2 x Ar-C*H*H-OTHP), 3.88-3.80 (m, 2H, THP-C*H$_2$*O), 3.51-3.44 (m, 2H, THP-C*H$_2$*O), 2.30 (br s, 1H, OH), 1.84-1.39 (m, 12H, 6 x C*H$_2$* (THP)) ; ^{13}C NMR (75 MHz, *CDCl$_3$*) δ 141.30 (C), 138.63 (2 x C), 126.34 (CH_{Ar}), 125.48 (2 x CH_{Ar}), 97.76 (2 x (THP)*C*H$_2$-CH-O), 68.62 (2 x Ar-*C*H-OTHP), 64.96 (CH_2OH), 62.04 (2 x THP-CH_2O), 30.45 (2 x CH_2), 25.37 (2 x CH_2), 19.24 (2 x CH_2).

Synthesis of (3,5-bis-(tetrahydro-2H-pyran-2-yloxy)methyl)benzaldehyde 9g

New compound



(3,5-Bis-(tetrahydro-2H-pyran-2-yloxy)methyl) benzaldehyde **9g** was synthesised according to general procedure GP-5 from alcohol **12a** (1.90 g, 5.80 mmol, 1

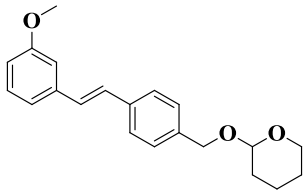
equiv.) and PCC (2.5 g, 11.60 mmol, 2 equiv.) in $MgSO_4$ dried chloroform (60 mL). The title compound **9g** (1.37 g, 71 %) was obtained after work up with diethyl ether (50 mL), washing of the cake with dichloromethane (2 x 40 mL) and ethyl acetate (2 x 40 mL) and purification on chromatography column (petroleum ether 40-60°C: ethyle acetate – 80:20) as a colorless oil.

IR ν_{max}/cm^{-1} 2941, 2850, 1698, 1118.65 ; ^{1}H NMR (300 MHz, *CDCl₃*) δ 9.56 (s, 1H, C*H*O), 7.74 (s, 2H, 2 x CH_{Ar}), 7.56 (s, 1H, CH_{Ar}), 4.79 (d, *J* = 12.3 Hz, 2H, 2 x Ar-C*H*H-OTHP), 4.67 (t, *J* = 3.6 Hz, 2H, 2 x O-C*H*-O), 4.50 (d, *J* = 12.3 Hz, 2H, 2 x Ar-CH*H*-OTHP), 3.88-3.80 (m, 2H, THP-CH_2O), 3.53-3.46 (m, 2H, THP-CH_2O), 1.86-1.46 (m, 12H, 6 x CH_2 (THP)) ; ^{13}C NMR (75 MHz, *CDCl₃*) δ 192.21 (*H*C=O), 139.70 (2 x C), 136.66 (C), 132.64 (CH_{Ar}), 127.86 (2 x CH_{Ar}), 98.06 (2 x (THP)CH_2-*C*H-O), 68.03 (2 x Ar-*C*H-OTHP), 62.16 (2 x THP-*C*H_2O), 30.44 (2 x CH_2), 25.35 (2 x CH_2), 19.25 (2 x CH_2).

18. Wittig-Horner-Emmons reaction

Synthesis of (E)-2-(4-(3-methoxystyryl)benzyloxy)tetrahydro-2H-pyran 21b

New compound



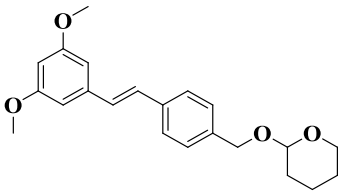
General procedure for the Wittig-Horner-Emmons GP-6: In a round bottom flask at 0°C under nitrogen sodium methoxide (690 mg, 12.73 mmol, 2 equiv.) was added in one portion to a solution of phosphonate **8a** (2 g, 6.36 mmol, 1 equiv.) in dry DMF (10 mL). A solution of 3-methoxybenzaldehyde **9b** (0.78 mL, 6.36 mmol, 1 equiv.) in dry DMF (10 mL) was added dropwise at 0°C with a canula and the reaction mixture was stirred o/n from 0°C to r.t. Ice-water (50 mL) was poured onto the thick orange mixture and let to stir for 15 min. The mixture was then extracted by ethyl acetate (3 x 50 mL) and the combined organic layers were washed with saturated LiCl solution (3 x 30 mL), dried ($MgSO_4$), filtered and the solvents were removed under reduced pressure. Purification of the crude oil by chromatography column (petroleum ether 40-60°C: ethyl acetate - 90:10) gave the title product **21b** (1.32 g, 65%) as a very thick yellow oil.

IR ν_{max}/cm^{-1} 3026, 2940, 1596, 1578, 1514, 1488, 1464, 1453, 1433; ^{1}H NMR (300 MHz, *CDCl₃*) δ 7.49 (d, *J* = 8.1 Hz, 2H, CH_{Ar}), 7.36 (d, *J* = 8.2 Hz, 2H, CH_{Ar}), 7.26 (d, AB system, *J* = 16.0 Hz, 1H, trans C*H*=CH), 7.11-7.05 (m, 4H includes AB system of *trans*-double bond), 6.81 (dd, *J* = 8.1, 1.8 Hz, 1H, CH_{Ar}), 4.79 (d, *J* = 12.0 Hz, 1H, Ar-C*H*HO-THP), 4.72 (t, *J* = 3.5 Hz, 1H, O-C*H*-O), 4.51 (d, *J* = 12.0 Hz, 1H, Ar-CH*H*O-THP), 3.97-3.89 (m, 1H, CH_2-HC*H*-CHO), 3.82 (s, 3H, CH_3O), 3.59-3.52 (m, 1H, CH_2-*H*CH-CHO), 1.52-1.48 (m, 6H, 3 x CH_2 (THP)) ; ^{13}C NMR (75 MHz, *CDCl₃*) δ 159.71 (C), 138.58 (C), 137.65 (C), 136.33 (C), 129.42 (H*C*=CH), 128.49

(HC=*C*H), 128.28 (CH_{Ar}), 127.97 (2 x CH_{Ar}), 126.35 (2 x CH_{Ar}), 119.02 (CH_{Ar}), 113.08 (CH_{Ar}), 111.56 (CH_{Ar}), 97.53 (O-*C*H-O), 68.35 (Ar-CH_2O), 61.91 (*C*H_2O (THP)), 54.97 (CH_3), 30.42(CH_2), 25.33 (CH_2), 19.21 (CH_2).

Synthesis of (E)-2-(4-(3,5-dimethoxystyryl)benzyloxy)tetrahydro-2H-pyran 21c

New compound



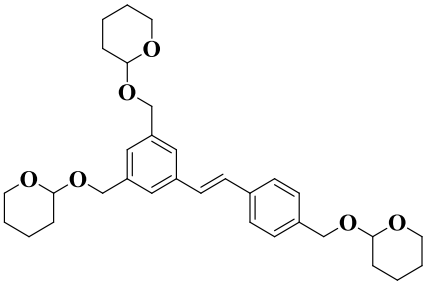
(*E*)-2-(4-(3,5-Dimethoxystyryl)benzyloxy)tetrahydro-*2H*-pyran **21c** was synthesised according to the general procedure GP-6 from phosphonate **8b** (2 g, 6.36 mmol, 1 equiv.) dissolved in dry DMF (10 mL), sodium methoxide (690 mg, 12.73 mmol, 2 equiv.) and 3,5-dimethoxybenzaldehyde **9c** (1.06 g, 6.36 mmol, 1 equiv.) dissolved in DMF (10 mL). The title compound **21c** (1.36, 60%) was obtained after work-up and purification on chromatography column (dichloromethane) as a yellow oil.

^{1}H NMR (300 MHz, *CDCl$_3$*) δ 7.30 (d, *J* = 8.1 Hz, 2H, CH_{Ar}), 7.18 (d, *J* = 8.2 Hz, 2H, CH_{Ar}), 6.93 and 6.82 (2 x d, AB system, *J* = 16.0 Hz, 2H, trans C*H*=CH), 6.51 (d, *J* = 2.1 Hz, 2H, CH_{Ar}), 6.23 (t, *J* = 2.1 Hz, 1H, CH_{Ar}), 4.61 (d, *J* = 12.3 Hz, 1H, Ar-C*H*HO-THP), 4.55 (t, *J* = 3.3 Hz, 1H, O-C*H*-O), 4.32 (d, *J* = 12.3 Hz, 1H, Ar-CH*H*O-THP), 3.80-3.72 (m, 1H, CH_2-HC*H*-CHO), 3.61 (s, 6H, 2 x CH_3O), 3.44-3.36 (m, 1H, CH_2-*H*CH-CHO), 1.78-1.34 (m, 6H, 3 x CH_2 (THP)) ; ^{13}C NMR (75 MHz, *CDCl$_3$*) δ 160.66 (C), 139.00 (C), 137.59 (C), 136.09 (C), 128.49 (HC=*C*H), 128.24 (H*C*=CH), 127.80 (2 x CH_{Ar}), 126.24 (2 x CH_{Ar}), 104.24 (2 x CH_{Ar}), 99.63 (CH_{Ar}), 97.41 (O-CH-O), 68.20 (Ar-CH_2O), 61.73 (CH_2O (THP)), 54.87 (2 x CH_3), 30.30 (CH_2), 25.21

(CH_2), 19.09 (CH_2) ; *m/z* (ESI) 355.1901 ($[M-H]^+$ $C_{22}H_{27}O_4$ requires 355.1909)

Synthesis of (E)-2,2'-(5-(4-((tetrahydro-2H-pyran-2-yloxy)methyl)styryl)-1,3-phenylene)bis (methylene)bis(oxy)bis(tetrahydro-2H-pyran) 21d

New compound



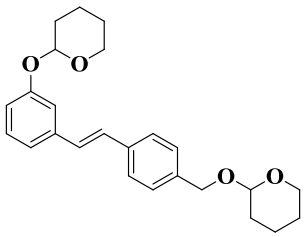
(*E*)-2,2'-(5-(4-((Tetrahydro-*2H*-pyran-2-yloxy)methyl)styryl)-1,3-phenylene)bis(methylene)-bis(oxy)bis(tetrahydro-2H-pyran) **21d** was synthesised according to the general procedure GP-6 from phosphonate **8b** (896 mg, 2.85 mmol, 1.13 equiv.) dissolved in dry DMF (4 mL), sodium methoxide (292 mg, 5.4 mmol, 2.13 equiv.) and the aldehyde **9g** (846 mg, 2.53 mmol, 1 equiv.) dissolved in DMF (4 mL). The title compound **21d** (1.06 g, 80%) was obtained after work-up and purification on chromatography column (hexane: ethyl acetate – 85:15 → 80:20) as a pale yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42 (d, *J* = 8.1Hz, 2H, CH_{Ar}), 7.37 (s, 2H, CH_{Ar}), 7.28 (d, *J* = 8.1Hz, 2H, CH_{Ar}), 7.19 (s, 1H, CH_{Ar}), 7.05 and 7.04 (2 x d, AB system, *J* = 16.8 Hz, C*H*=CH), 4.74 (d, *J* = 12.0 Hz, 2H, Ar-C*H*HO-THP), 4.72 (d, *J* = 12.0 Hz, 1H, Ar-C*H*HO-THP), 4.66 (t, *J* = 3.9 Hz, 3H, O-C*H*-O), 4.46 (d, *J* = 12.0 Hz, 1H, Ar-CH*H*O-THP), 4.44 (d, *J* = 12.0 Hz, 1H, Ar-CH*H*O-THP), 3.91-3.82 (m, 3H, CH_2-HC*H*-CHO), 3.53-3.45 (m, 3H, CH_2-*H*CH-CHO), 1.84-1.46 (m, 18H, 6 x CH_2 (THP)) ; ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.80 (2 x C), 137.74 (C), 137.57 (C), 136.59 (C), 128.67 (*H*C=CH), 128.36 (HC=C*H*), 128.17 (2 x CH_{Ar}), 126.57 (CH_{Ar}), 126.49 (2 x CH_{Ar}), 125.13 (2 x CH_{Ar}), 97.78 (2 x CHO), 97.69 (CHO), 68.65 (2 x CH_2O), 68.53

(CHO), 62.17 (2 x CH_2O), 62.13 (CH_2O), 30.57 (3 x CH_2), 25.46 (3 x CH_2), 19.38 (3 x CH_2).

Synthesis of (E)-2-(3-(4-((tetrahydro-2H-pyran-2-yloxy)methyl)styryl) phenoxy)tetrahydro-2H-pyran 21e

New compound



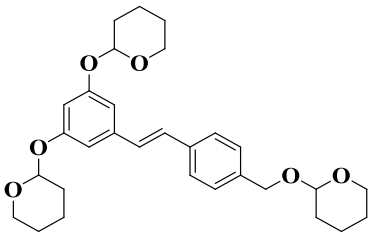
(*E*)-2-(3-(4-((Tetrahydro-*2H*-pyran-2-yloxy)methyl)-styryl)phenoxy)tetrahydro-2H-pyran **21e** was synthesised according to the general procedure GP-6 from phosphonate **8b** (762 mg, 2.4 mmol, 1 equiv.) dissolved in dry DMF (5 mL), sodium methoxide (260 mg, 4.8 mmol, 2 equiv.) and the aldehyde **9h** (500 mg, 2.4 mmol, 1 equiv.) dissolved in DMF (5 mL). The title compound **21e** (834 mg, 88%) was obtained after work-up and purification on chromatography column (petroleum ether 40-60°C: ethyl acetate – 90:10) as a thick colorless oil which crystallised on standing. Recrystallisation from petroleum ether 60-80°C gave **21e** as a white solid (466 mg, 50%, m.p: 65-67 ºC)

IR υ_{max}/cm^{-1} 3028, 2940, 2872, 1633, 1601, 1576, 1449, 1349, 1201, 1183, 1158, 1108, 1020, 1005, 967, 946, 905, 807, 779, 712 and 687 ^{1}H NMR (300 MHz, *CDCl$_3$*) δ 7.42 (d, *J* = 8.1 Hz, 2H, CH_{Ar}), 7.29 (d, *J* = 8.2 Hz, 2H, CH_{Ar}), 7.22-7.07 (m, 3H includes AB system of *trans*-double bond), 7.01 (d, *J* = 2.0 Hz, 2H, CH_{Ar}), 6.89 (dd, *J* = 8.1, 2.0 Hz, 1H, CH_{Ar}), 5.40 (t, *J* = 3.3 Hz, 1H, ArO-C*H*-O) 4.72 (d, *J* = 12.0 Hz, 1H, Ar-C*H*HO-THP), 4.65 (t, *J* = 3.3 Hz, 1H, O-C*H*-O), 4.44 (d, *J* = 12.0 Hz, 1H, Ar-CH*H*O-THP), 3.91-3.83 (m, 2H, CH_2-HC*H*-CHO), 3.60-3.45 (m, 2H, CH_2-*H*CH-

CHO), 2.02-1.46 (m, 12H, 6 x C*H*$_2$ (THP)) ; ^{13}C NMR (75 MHz, *CDCl$_3$*) δ 157.41 (C), 138.72 (C), 137.73 (C), 136.60 (C), 129.53 (H*C*=CH), 128.60 (HC=*C*H), 128.49 (CH$_{Ar}$), 128.18 (2 x CH$_{Ar}$), 126.51 (2 x CH$_{Ar}$), 120.11 (CH$_{Ar}$), 115.77 (CH$_{Ar}$), 114.30 (CH$_{Ar}$), 97.71 (ArO-*C*H-O), 96.32 (O-*C*H-O), 68.56 (Ar-CH$_2$O), 62.14 (*C*H$_2$O (THP)), 61.99 (*C*H$_2$O (THP)), 30.59 (CH$_2$), 30.41 (CH$_2$), 25.49 (CH$_2$), 25.24 (CH$_2$), 19.37 (CH$_2$), 18.74 (CH$_2$).

Synthesis of (E)-2,2'-(5-(4-((tetrahydro-2H-pyran-2-yloxy)methyl)styryl)-1,3-phenylene)bis-(oxy)bis(tetrahydro-2H-pyran) 21f

New compound



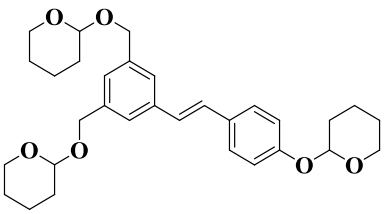
(I)-2,2'-(5-(4-((Tetrahydro-*2H*-pyran-2-yloxy) methyl)styryl)-1,3-phenylene)bis(oxy)bis(tetra-hydro-2H-pyran) **21f** was synthesised according to the general procedure GP-6 from phosphonate **8b** (2.24 g, 7.12 mmol, 1.05 equiv.) dissolved in dry DMF (12 mL), sodium methoxide (733 mg, 13.56 mmol, 2 equiv.) and the aldehyde **9i** (2.09 g, 6.78 mmol, 1 equiv.) dissolved in DMF (12 mL). The title compound **21f** (2.58 g, 73%) was obtained after work-up and purification on chromatography column (hexane: ethyl acetate – 85:15 → 80:20) as a pale yellow oil.

^{1}H NMR (300 MHz, *CDCl$_3$*) δ 7.48 (d, *J* = 8.3 Hz, 2H, CH$_{Ar}$), 7.35 (d, *J* = 8.3 Hz, 2H, CH$_{Ar}$), 7.10 and 7.00 (2 x d, AB system, *J* = 16.4 Hz, C*H*=CH), 6.89 (t, *J* = 1.8 Hz, 2H, CH$_{Ar}$), 6.71 (dd, *J* = 3.3, 2.0 Hz, 1H, CH$_{Ar}$), 5.46 (t, *J* = 3.3 Hz, 2H, ArO-C*H*-O), 4.79 (d, *J* = 12.0 Hz, 1H, Ar-C*H*HO-THP), 4.72 (t, *J* = 3.3 Hz, 1H, O-C*H*-O), 4.51

(d, J = 12.0 Hz, 1H, Ar-C*H*HO-THP), 3.98-3.90 (m, 3H, CH_2-HC*H*-CHO), 3.66-3.52 (m, 3H, CH_2-*H*CH-CHO), 2.10-1.53 (m, 18H, 9 x CH_2 (THP)) ; ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.29 (C), 158.23 (C), 139.14 (C), 137.68 (C), 136.54 (C), 128.67 (H*C*=CH), 128.56 (HC=*C*H), 128.13 (2 x CH_{Ar}), 126.49 (2 x CH_{Ar}), 107.99 (CH_{Ar}), 107.93 (CH_{Ar}), 104.81 (CH), 97.67 (O-*C*H-O), 96.39 (ArO-*C*H-O), 96.23 (ArO-*C*H-O), 68.52 (Ar-CH_2O), 62.09 (*C*H_2O (THP)), 61.93 (CH_2O (THP)), 61.90 (CH_2O (THP)), 30.55 (CH_2), 30.33 (2 x CH_2), 25.45 (CH_2), 25.21 (2 x CH_2), 19.33 (CH_2), 18.68 (CH_2), 18.64 (CH_2).

Synthesis of (E)-2,2'-(5-(4-(tetrahydro-2H-pyran-2-yloxy)styryl)-1,3-phenylene)bis-(methylene)bis(oxy)bis(tetrahydro-2H-pyran) 21g

New compound



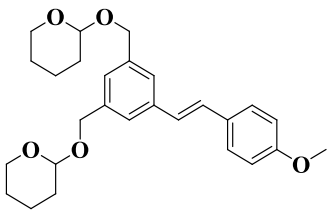
(*E*)-2,2'-(5-(4-(Tetrahydro-*2H*-pyran-2-yloxy) styryl)-1,3-(phenylene)bis(methylene)bis(oxy) bis(tetrahydro-2H-pyran) **21g** was synthesised according to the general procedure GP-6 from phosphonate **8d** (500 mg, 1.66 mmol, 1.1 equiv.) dissolved in dry DMF (10 mL), potassium *tert*-butoxide (373 mg, 3.22 mmol, 2.2 equiv.) and the aldehyde **9g** (504 mg, 1.51 mmol, 1 equiv.) dissolved in DMF (10 mL). The title compound **21g** (593 mg, 77%) was obtained after work-up and purification on chromatography column (hexane: ethyl acetate – 85:15 → 80:20) as a colorless oil.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37 (d, J = 8.8 Hz, 2H, 2 x CH_{Ar}), 7.33 (s, 2H, 2 x CH_{Ar}), 7.16 (s, 1H,CH_{Ar}), 7.01 and 6.91 (2 x d, AB system, J = 16.8 Hz, C*H*=CH),

6.96 (d, *J* = 8.8 Hz, 2H, 2 x CH_{Ar}), 5.35 (t, *J* = 3.3 Hz, 1H, ArO-C*H*-O), 4.72 (d, *J* = 12.1 Hz, 2H, 2 x Ar-CH*H*O-THP), 4.72 (t, *J* = 3.3 Hz, 2H, 2 x O-C*H*-O), 4.51 (d, *J* = 12.1 Hz, 2H, 2 x Ar-CH*H*O-THP), 3.89-3.78 (m, 3H, 3 x CH_2-*H*CH-CHO), 3.55-3.44 (m, 3H, 3 x CH_2-HC*H*-CHO), 1.95-1.44 (m, 18H, 9 x CH_2 (THP)); ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.72 (C), 138.71 (2 x C), 137.85 (C), 130.86 (C), 128.50 (HC=*C*H), 127.57 (2 x CH_{Ar}), 126.57 (H*C*=CH), 126.20 (CH_{Ar}), 124.91 (2 x CH_{Ar}), 116.59 (2 x CH_{Ar}), 97.73 (2 x O-CH-O), 96.27 (O-CH-O), 68.66 (2 x Ar-*C*H_2-OTHP), 62.33 (2 x (THP) *C*H_2O), 61.98 ((THP) *C*H_2O), 30.67 (2 x CH_2), 30.28 (CH_2), 25.41 (3 x CH_2), 19.60 (2 x CH_2), 18.70 (CH_2) ; *m/z* (ESI) 509.2903 ($[M-H]^+$ $C_{31}H_{41}O_6$ requires 509.2902).

Synthesis of (E)-2,2'-(5-(4-methoxystyryl)-1,3-phenylene)bis(methylene) bis(oxy)bis(tetra-hydro-2H-pyran)) 21h

New compound



(*E*)-2,2'-(5-(4-Methoxystyryl)-1,3-phenylene)bis (methylene)bis(oxy)bis(tetrahydro-*2H*-pyran)) **21h** was synthesised according to the general procedure GP-6 from phosphonate **8e** (316 mg, 1.05 mmol, 1.1 equiv.) dissolved in dry DMF (10 mL), potassium *tert*-butoxide (237 mg, 2.11 mmol, 2.2 equiv.) and the aldehyde **9g** (320 mg, 0.96 mmol, 1.00 equiv.) dissolved in DMF (10 mL). The title compound **21h** (211 mg, 55%) was obtained after work-up and purification on chromatography column (hexane: ethyl acetate – 90:10 → 85:15) as a colorless oil.

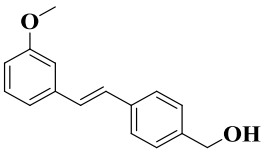
^{1}H NMR (300 MHz, *CDCl₃*) δ 7.36 (d, *J* = 8.7 Hz, 2H, 2 x CH_{Ar}), 7.33 (s, 2H, 2

x CH_{Ar}), 7.16 (s, 1H, CH_{Ar}), 7.00 and 6.90 (2 x d, AB system, *J* = 16.5 Hz, C*H*=CH), 6.81 (d, *J* = 8.7 Hz, 2H, 2 x CH_{Ar}), 4.72 (d, *J* = 12 Hz, 2H, 2 x Ar-CH*H*-OTHP), 4.65 (t, *J* = 3.6 Hz, 2H, 2 x O-C*H*-O), 4.44 (d, *J* = 12 Hz, 2 x Ar-C*H*H-OTHP), 3.90-3.82 (m, 2H, (THP)CH_2O), 3.73 (s, 3H, CH_3O), 3.52-3.45 (m, 2H, (THP)CH_2O), 1.84-1.44 (m, 12H, 6 x CH_2 (THP) ; ^{13}C NMR (75 MHz, *CDCl$_3$*) δ 159.25 (C), 138.68 (2 x C), 137.84 (C), 130.02 (C), 128.43 (HC=*C*H), 127.65 (2 x CH_{Ar}), 126.30 (H*C*=CH), 126.15 (CH_{Ar}), 124.86 (2 x CH_{Ar}), 114.05 (2 x CH_{Ar}), 97.81 (2 x O-CH-O), 68.64 (2 x Ar-*C*H_2-OTHP), 62.10 (2 x (THP) *C*H_2O), 55.22 (CH_3O), 30.52 (2 x CH_2), 25.42 (2 x CH_2), 19.33 (2 x CH_2).

19. THP Deprotection

Synthesis of (E)-(4-(3-methoxystyryl)phenyl)methanol 7a

New compound



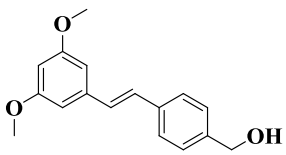
General procedure for THP deprotection GP-7: In a round bottom flask, THP protected alcohol **21b** (1.32 g, 4.12 mmol, 1 equiv.) and PPTS (104 mg, 0.41 mmol, 0.1 equiv.) were dissolved in ethanol (30 mL) at r.t. The reaction mixture was heated to 60°C for 6h. The solvent was then removed under reduced pressure to give a beige solid. Recrystallisation from petroleum ether 60-80°C gave the title compound **7a** (454 mg, 46%) as a white solid (m.p. 85-87°C).

IR ν_{max}/cm^{-1} 3728, 2970, 1584 ; UV λ_{max} (MeOH)/nm 286.50 (ε dm^{-3}.mol^{-1}.cm^{-1} 52517) ; ^{1}H NMR (300 MHz, *MeOD*) δ 7.51 (d, *J* = 8.4 Hz, 2 H, CH_{Ar}), 7.33 (d, *J* = 8.1 Hz, 2 H, CH_{Ar}), 7.24 (d, AB system, *J* = 16.0 Hz, *trans* C*H*=CH), 7.08-7.21 (m, 4H includes 1H AB system of *trans*-double bond), 6.80 (dd, *J* = 2.1, 8.0 Hz, 1 H, CH_{Ar}),

4.59 (s, 2H, CH_2O), 3.81 (s, 3H, CH_3O); ^{13}C NMR (75 MHz, *MeOD*) δ 211.50 (C), 142.20 (C), 140.30 (C), 137.86(C), 130.65 (CH_{Ar}), 129.69 (*C*H=CH), 129.49 (CH=*C*H), 128.41 (2 x CH_{Ar}), 127.62 (2 x CH_{Ar}), 120.76 (CH_{Ar}), 114.31 (CH_{Ar}), 112.75 (CH_{Ar}), 65.03 (CH_2), 55.70 (CH_3); *m/z* (EI) 240.11461 (M^+ $C_{16}H_{16}O_2$ requires 240.11503). Elem. Anal. calculated C, 80.08 ; H, 6.70. ($C_{16}H_{16}O_2$ requires C, 79.97 % ; H, 6.71 %).

Synthesis of (E)-(4-(3,5-dimethoxystyryl)phenyl)methanol 7b

New compound

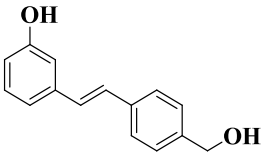


(*E*)-(4-(3,5-Dimethoxystyryl)phenyl)methanol **7b** was synthesised according to the general procedure GP-7 from THP protected alcohol **21c** (724 mg, 2.04 mmol, 1 equiv.) dissolved in ethanol (15 mL) and PPTS (63 mg, 0.25 mmol, 0.12 equiv.) The title compound **7b** (243 mg, 89%) was obtained after work-up and recrystallisation from petrol 60-80 as a white solid (m.p. 77-80°C).

IR ν_{max}/cm^{-1} 3346, 2834, 1584, 1454 ; ^1H NMR (300 MHz, *MeOD*) δ 7.50 (d, *J* = 8.4 Hz, 2 H, CH_{Ar}), 7.33 (d, *J* = 8.4 Hz, 2 H, CH_{Ar}), 7.06 and 7.11 (2 x d, AB system, *J* = 16.1 Hz, *trans*-CH=CH), 6.69 (d, *J* = 2.1 Hz, 2 H, CH_{Ar}), 6.38 (t, *J* = 2.1 Hz, 1 H, CH_{Ar}), 4.59 (s, 2H, CH_2O), 3.79 (s, 6H, 2 x CH_3O) ; ^{13}C (300 MHz, *MeOD*) δ 162.53 (2 x C), 142.22 (C), 140.86 (C), 137.89 (C), 129.85 (*C*H=CH), 129.60 (CH=*C*H), 128.41 (2 x CH_{Ar}), 127.65 (2 x CH_{Ar}), 105.58 (CH_{Ar}), 100.89 (CH_{Ar}), 65.03 (CH_2), 55.80 (2 x CH_3) ; *m/z* (EI) 270.12522 (M^+ $C_{17}H_{18}O_3$ requires 270.12559). Elem. Anal. calculated C, 75.53 ; H, 6.71. ($C_{17}H_{18}O_3$ requires C, 75.66% ; H, 6.60%)

Synthesis of (E)-(4-(3-hydroxystyryl)phenyl)methanol 7c

New compound

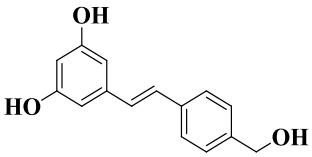


(*E*)-(4-(3-Hydroxystyryl)phenyl)methanol **7c** was synthesised according to the general procedure GP-7 from THP protected alcohol **21e** (706 mg, 1.79 mmol, 1 equiv.) dissolved in ethanol (20 mL) and PPTS (127 mg, 0.51 mmol, 0.29 equiv.) The title compound **7c** (210 mg, 52%) was obtained after work-up and recrystallisation from petrol 60-80 as a white solid (m.p. > 200°C).

IR ν_{max}/cm^{-1} 3393, 3137, 1588, 1445; ^{1}H (300 MHz, *MeOD*) δ 7.49 (d, *J* = 8.1 Hz, 2 H, CH_{Ar}), 7.33 (d, *J* = 8.1 Hz, 2 H, CH_{Ar}), 6.99 and 7.03 (2 x d, AB system, *J* = 16.2 Hz, *trans*- C*H*=CH), 6.49 (d, *J* = 2.1 Hz, 2 H, CH_{Ar}), 6.20 (t, *J* = 2.0 Hz, 1 H, CH_{Ar}), 4.60 (s, 2H, CH_2O); ^{13}C (300 MHz, *MeOD*) δ 159.78 (2 x C), 142.10 (C), 140.86 (C), 137.94 (C), 129.83 (*C*H=CH), 129.23 (CH=*C*H), 128.44 (2 x CH_{Ar}), 127.53 (2 x CH_{Ar}), 106.12 (2 x CH_{Ar}), 103.22 (CH_{Ar}), 65.04 (CH_2) ; *m/z* (EI) 225.0920 ([M-H]$^-$ $C_{15}H_{13}O_2^-$ requires 225.0921).

Synthesis of (E)-(5-(4-hydroxymethyl)styryl)benzene-1,3-diol 7d

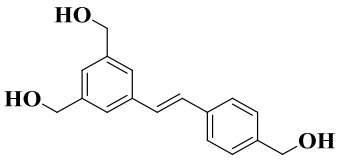
New compound



(*E*)-(5-(4-Hydroxymethyl)styryl)benzene-1,3-diol **7d** was synthesised according to the general procedure GP-7 from THP protected alcohol **21f** (724 mg, 2.04 mmol, 1 equiv.) dissolved in ethanol (15 mL) and PPTS (63 mg, 0.25 mmol, 0.12 equiv.) The title compound **7d** (243 mg, 44%) was obtained after work-up and recrystallisation from petrol 60-80 as a white solid (mp > 220°C).

IR ν_{max}/cm^{-1} 3223, 2970, 1584; ^{1}H NMR (300 MHz, *MeOD*) δ 7.49 (d, *J* = 8.1 Hz, 2 H, CH_{Ar}), 7.33 (d, *J* = 8.1 Hz, 2 H, CH_{Ar}), 6.99 and 7.03 (2 x d, AB system, *J* = 16.2 Hz, *trans*-C*H*=CH), 6.49 (d, *J* = 2.1 Hz, 2 H, CH_{Ar}), 6.20 (t, *J* = 2.0 Hz, 1 H, CH_{Ar}), 4.60 (s, 2H, CH_2O); ^{13}C NMR (300 MHz, *MeOD*) δ 159.78 (2 x C), 142.10 (C), 140.86 (C), 137.94 (C), 129.83 (*C*H=CH), 129.23 (CH=*C*H), 128.44 (2 x CH_{Ar}), 127.53 (2 x CH_{Ar}), 106.12 (2 x CH_{Ar}), 103.22 (CH_{Ar}), 65.04 (CH_2) ; *m/z* (EI) 242.09387 (M^+ $C_{14}H_{12}O_3$ requires 242.09429).

Synthesis of (E)-(5-(4-hydroxymethylstyryl)-1,3-phenylene)dimethanol 7e

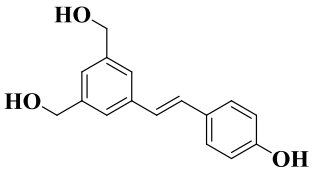


(*E*)-(5-(4-Hydroxymethylstyryl)-1,3-phenylene) dimethanol **7e** was synthesised according to the general procedure GP-7 from (E)-2,2'-(5-(4-((tetrahydro-*2H*-pyran-2-yloxy)methyl)styryl)-1,3-phenylene)bis(methylene)bis(oxy)bis(tetrahydro-*2H*-pyran) **21d** (1.0 g, 1.90 mmol, 1 equiv.) dissolved in ethanol (10 mL) and PPTS (143 mg, 0.57 mmol, 0.3 equiv.). Recrystallisation from petrol 60-80 gave the title

compound **7e** (120 mg, 23%) as a yellowish solid (m.p. 123-126°C).

IR ν_{max}/cm^{-1} 3201, 2867, 1597, 1453 ; ^{1}H NMR (300 MHz, *MeOD*) δ 7.51 (d, *J* = 8.1 Hz, 2 H, CH_{Ar}), 7.44 (s, 2 H, 2 x CH_{Ar}), 7.35 (d, *J* = 8.1 Hz, 2 H, CH_{Ar}), 7.24 (s, 1 H, CH_{Ar}), 7.16 (br s, 2 H, C*H*=CH), 4.62 (s, 4H, 2 x CH_2O), 4.59 (s, 2H, CH_2O) ; ^{13}C (75 MHz, *MeOD*) δ 143.26 (2 x C), 142.16 (C), 139.05 (C), 137.83 (C), 129.64 (*C*H=CH), 129.38 (CH=*C*H), 128.41 (2 x CH_{Ar}), 127.58 (2 x CH_{Ar}), 125.85 (CH_{Ar}), 125.02 (2 x CH_{Ar}), 65.13 (2 x CH_2), 65.01 (CH_2) ; *m/z* (EI) 270.12559 (M^+ $C_{17}H_{18}O_3$ requires 270.12504).

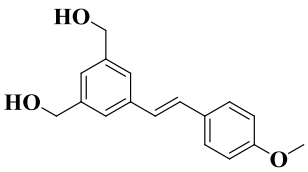
Synthesis of (E)-(5-(4-hydroxystyryl)-1,3-phenylene)dimethanol 7f



(*E*)-(5-(4-Hydroxystyryl)-1,3-phenylene)dimethanol **7f** was synthesised according to the general procedure GP-7 from (E)-2,2'-(5-(4-(tetrahydro-2H-pyran-2-yloxy)styryl)-1,3-phenylene)bis(methylene)bis (oxy)bis(tetrahydro-2H-pyran) **21g** (400 mg, 0.79 mmol, 1 equiv.) dissolved in ethanol (20 mL) and PPTS (55 mg, 0.22 mmol, 0.3 equiv.). Recrystallisation from dichloromethane: methanol gave the title compound **7f** (193 mg, 67%) as a white solid (m.p. 162-165°C).

^{1}H NMR (300 MHz, *MeOD*) δ 7.41 (s, 2H, 2 x CH_{Ar} and d, *J* = 8.7 Hz, 2H, 2 x CH_{Ar}), 7.21 (s, 1H, CH_{Ar}), 7.05 and 6.99 (2 x d, AB system, *J* = 16.2 Hz, *trans*-CH=CH), 6.78 (d, *J* = 8.7 Hz, 2H, 2 x CH_{Ar}), 4.62 (s, 4H, 2 x CH_2) ; ^{13} C NMR (300 MHz, *MeOD*) δ 158.50 (C), 143.21 (2 x C), 139.60 (C), 130.45 (C), 129.88 (H*C*=CH), 128.91 (2 x CH_{Ar}), 129.65 (HC=*C*H), 125.36 (CH_{Ar}), 124.74 (2 x CH_{Ar}), 116.55 (2 x CH_{Ar}), 65.23 (2 x CH_2). *m/z* not found.

Synthesis of (E)-(5-(4-methoxystyryl)-1,3-phenylene)dimethanol 7g



(*E*)-(5-(4-Methoxystyryl)-1,3-phenylene)dimethanol **7g** was synthesised according to the general procedure GP-7 from (*E*)-2,2'-(5-(4-methoxystyryl)-1,3-phenylene)bis-(methylene)bis(oxy)bis(tetra-hydro-*2H*-pyran)) **21h** (359 mg, 0.89 mmol, 1 equiv.) dissolved in ethanol (20 mL) and PPTS (50 mg, 0.20 mmol, 0.2 equiv.). Recrystallisation from dichloromethane: methanol gave the title compound **7g** (94 mg, 39%) as a white solid (m.p. 131-134°C).

^{1}H NMR (300 MHz, *MeOD*) δ 7.48 (d, *J* = 8.7 Hz, 2H, 2 x CH_{Ar}),4.42 (s, 2H, 2 x CH_{Ar}), 7.22 (s, 1H, CH_{Ar}), 7.13 and 7.03 (2 x d, AB system, *J* = 16.4 Hz, *trans*-C*H*=CH), 6.91 (d, *J* = 8.8 Hz, 2H, 2 x CH_{Ar}), 4.63(s, 4H, 2 x CH_2), 3.80 (s, 3H, CH_3O) ; ^{13} C NMR (300 MHz, *MeOD*) δ 160.95 (C), 143.27 (2 x C), 139.45 (C), 131.55 (C), 129.57 (H*C*=CH), 128.82 (2 x CH_{Ar}), 127.42 (HC=*C*H), 125.50 (CH_{Ar}), 124.81 (2 x CH_{Ar}), 115.18 (2 x CH_{Ar}), 65.21 (2 x CH_2), 55.78 (CH_3O); *m/z* (EI) 270.12513 (M^+ $C_{17}H_{18}O_3$ requires 270.12559).

20. Pharmacology experiments – Materials and Methods

20.1. Chemicals and tissue samples preparation.

Resveratrol was purchased from Novanat (Shanghai, China). Uridine 5'-diphosphate glucuronic acid (UDPGA) and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and reagents for HPLC analyses were purchased from Sigma Chemical Comp. (Poole, UK). HPLC-grade methanol was obtained from Fisher Chemicals (Loughborough, UK) and water was purified in our laboratory by a Nano-Pure water purification system (Barnstead, UK).

Mouse liver cytosol and microsomes were prepared in the laboratory by Dr H. Cai according to the published method.[213] Briefly, mouse liver tissues were excised from six C57BL/6J mice (three females, three males). An aliquot (3 g) of tissue was suspended in 27 mL buffered sucrose (sucrose 0.25 M, Tris 10 mM, EDTA 1 mM, pH 7.4), and homogenized under cooling with ice. Human liver microsomes and cytosols were obtained from Bioscience (Cambridge, UK) were pooled. Subcellular fractions of mouse liver were isolated and cytosolic and microsomal proteins were quantified in the usual way. Tissue preparations were kept at -80°C until analysis.

20.2. Sulfation

The analogues (10 μM) were incubated with mouse or human liver cytosol (1 mg/mL of protein) in solution (total incubation volume 200 μL) with $MgCl_2$ (5 μM), mercaptoethanol (3 mM) and $KHPO_4$ buffer (pH 7.4). The metabolic reaction was initiated by the addition of the cofactor PAPS (200 μM). The mixture was incubated at 37°C with shaking for 0, 15, 30, 60 and 120 min. After the incubation time ice-cold

MeOH (160 µL) was added to stop the reaction and the mixture was vortex-mixed (2 min) and centrifuged (13000 rpm, 15 min). The supernatant (10 µL) was then injected on the HPLC column for analysis.

20.3. Glucuronidation

The analogues (10 µM) were incubated with mouse or human liver microsomes (1 mg/mL of protein) in solution (total incubation volume 200 µL) with MgCl (8 mM), Alamethicin (25 µg/mL), Tris-HCl buffer (50 mM, pH 7.5). The metabolic reaction was initiated by the addition the cofactor UDPGA (2 mM). The reaction mixture was incubated at 37°C with shaking for 0, 5, 15, 30 and 60 min. After the incubation time, ice-cold methanol (160 µL) was added to stop the reaction. The incubation mixtures were vortex-mixed (2 min) and centrifuged (13000 rpm, 15 min). The supernatant (10 µL) was then injected on the HPLC column for analysis.

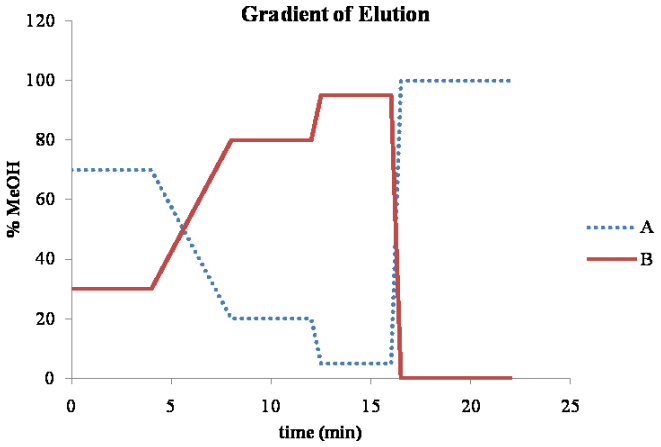
20.4. HPLC analysis of resveratrol, resveratrol analogues and their metabolites

The analysis of resveratrol **1a**, resveratrol analogues **7a-e** and their metabolites in the incubation mixtures were performed using a reverse phase HPLC-UV method from Cai *et al.*[132] with modifications. The HPLC system used was a HPLC/UV system Waters Alliance 2695 (Manchester, UK) comprising an auto-sampler and a UV detector. The separation was achieved on an Atlantis C_{18} reverse phase column (4.6 mm x 150 mm, 3 µm, Waters, UK). The gradient elution for the analogues and the metabolic mixtures consisted of a binary mobile phase system (phase A: aqueous ammonium

acetate 5 mM with 2% *iso*-propanol; phase B: HPLC-grade methanol with 2% *iso*-propanol) with a gradient from 70-30% to 5%-95% A-B (Scheme 37) as detailed in Table 18. The retention time for resveratrol and the analogues were first measured as a positive control and the appropriate peaks in metabolism mixtures were identified from these retention times.

time	*A*	*B*
0	70	30
4	70	30
8	20	80
12	20	80
12.5	5	95
16	5	95
16.5	100	0
22	100	0

Table 18: Gradient of Elution



Scheme 37: Gradient of elution HPLC system

20.5. HPLC/MS/MS analysis

HPLC-Electrospray ionization tandem mass spectrometric analysis of the parent compound references and the metabolites in the incubation mixtures was realised using an API2000 mass spectrometer (Applied Biosystems, Warrington, UK) attached to an HPLC system Agilent 1100 series (Agilent Technologies UK, South Queensferry, UK). The separation was achieved on an Atlantis dC18 column (2.1x150 mm, 3µM, Waters, UK) using a binary mobile phase system (phase A: aqueous ammonium acetate 5 mM; phase B: 5mM ammonium acetate HPLC-grade methanol). The flow rate and the elution conditions are described in Table 14 (c.f. p.137). Previous LC/MS Electrospray experiments showed that the analogues **7a-b,e** were detectable in positive mode and

that analogues **7c-d** and resveratrol **1a** were seen in negative mode. MS conditions are described in Table 15 (c.f. 138).

20.6. Enzymatic hydrolysis

20.6.1. Hydrolysis of the sulfates

The analogues (10 μM) were incubated with mouse or human liver cytosol in the same conditions as described before (c.f. 20.2. Sulfation) and the reactions were stopped by addition of ice-cold MeOH (200 μL) after a defined period of incubation (Table 17, c.f. p.146). The mixtures were then split in half, the supernatant of the first half being injected in HPLC after treatment as stated before and the second one being dried under a flow of nitrogen. The residues were then redissolved with 0.1 M phosphate buffer pH 5.5 (250 μL) containing 5 units of sulfatase. The mixture was incubated at 37°C overnight (18h) with shaking. After the incubation ice-cold MeOH (200 μL) was added and the mixture was vortex-mixed (2 min) and centrifuged (13000 rpm, 15 min). The supernatants were then injected on the HPLC column for analysis and comparison with the sulfation reaction

20.6.2. Hydrolysis of the glucuronides

The analogues (10 μM in MeOH) were incubated with mouse or human liver microsomes in the same conditions as described before (c.f. 20.3. Glucuronidation) and the reactions were stopped by addition of ice-cold MeOH (200 μL) after a defined period of incubation (Table 17, c.f. p.146). The mixtures were then split in half, the supernatant of the first half being injected in HPLC after treatment as stated before and

the second one being dried under a flow of nitrogen. The residues were then redissolved with 50 mM phosphate buffer pH 5.5 (200 μL) containing 2000 units of β-glucuronidase. The mixture was incubated at 37°C overnight (18h) with shaking. After the incubation, ice-cold MeOH (200 μL) was added and the mixtures were vortex-mixed (2 min) and centrifuged (13000 rpm, 15 min). The supernatants were then on the HPLC column for analysis.

20.7. Cell proliferation assay - Method

20.7.1. Cells and reagents

HCA-7 cells were purchased from HPA culture collections (Salisbury, UK). The stock solutions containing the analogues of resveratrol **7a-e** (100 mM) in DMSO were stored at -20°C. The solutions used for the treatments were prepared in sterile conditions just before use. The cells were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, Germany). The media contained 10 % of Foetal Calf Serum (FCS, Invitrogen, Paisley, UK), [+] 4500 mg/L of glucose, 110 mg/L of sodium pyruvate and L-glutamine. The cells were washed with Phosphate Buffered Saline (PBS, Invitrogen, Paisley, UK) and unstuck from the plates with Trypsin 5x (Invitrogen, Paisley, UK).

20.8. Cell culture and proliferation assay

20.8.1. Culture

Human colon cancer HCA-7 were grown in a 500 mL flask with media containing 10 % of Foetal Calf Serum (20 mL) and were left to incubate at 37 °C with

5% CO_2 until they were 70% confluent. The media was removed, the cells were washed with PBS (2 x 5 mL), Trypsin 5x (3 mL) was added and the cells were incubated for 5 min at 37°C. Media (7 mL) was added and the cells were transferred to a Corning® tube and vortex mixed for 5 min at 13000 rpm. The supernatant was removed and the cells were redissolved in fresh media containing 10% FCS (10 mL) and were ready for use.

20.8.2. Cell proliferation assay

The cell proliferation assay was performed in triplicate according to a slightly modified method by Sale *et al.*[204] Human colon cancer cells HCA-7 c29 were seeded onto 24-well plates at a concentration of 6000 cells/well in media (1 mL). After one day incubation at 37°C with 5% CO_2, the media was removed and the cells were treated with a single treatment of media (2 mL) containing the analogues in 0.1 % DMSO for further incubation (3 and 6 days) at concentrations of 0.5 µM, 2 µM, 5 µM, 10 µM and 50 µM. The controls were treated with 0.1 % DMSO alone (Figure 61). After incubation, the media was removed and the cells were washed with PBS buffer (2 x 0.5 mL), detached from the bottom of the plate by Trypsin 5x (0.5 mL) and resuspended with media (0.5 mL). A fraction of this solution (100 µL) was then dissolved in isotone (10 mL) and the cells were counted using a Z2 Coulter® Particle Count and Size Analyser (Beckman Coulter, High Wycombe, UK).

Control DMSO	0.5 µM	2 µM	5 µM	10 µM	50 µM
Control DMSO	0.5 µM	2 µM	5 µM	10 µM	50 µM
Control DMSO	0.5 µM	2 µM	5 µM	10 µM	50 µM
Control DMSO	0.5 µM	2 µM	5 µM	10 µM	50 µM

Figure 61: General Culture Plate

20.9. FACS Studies

20.9.1. Apoptosis evaluation: Annexin V assay

Human colon cancer cells HCA-7 c29 were grown in 500 mL as described before. HCA-7 c29 were seeded onto 6-well plates at a concentration of 75000 cells/well in media (2 mL) for a three-day treatment and at a density of 30000 cells/well for a six-day treatment. After one day incubation the media was removed and the cells were treated with a single treatment of media (2 mL) containing the analogues **7a** and **7c** in 0.1 % DMSO for further incubation (3 and 6 days) at a concentration of 2 and 50 μM. The control was treated with 0.1% DMSO alone and was used as a negative control. Treatment of the cells with resveratrol at a concentration of 10 μM was used as a positive control. After incubation, the media was removed and put in a tube and the cells were washed with PBS buffer (2 x 1 mL), detached from the bottom of the plate by Trypsin 5x (1 mL) for a minimum amount of time (3-5 min) and resuspended immediately with media (1 mL). The content of the wells were combined with incubation media and then centrifuged at 1300 rpm for 3 min. The supernatant was removed and the cells were resuspended in fresh media containing 10% FCS (10 mL) and left to incubate for 30 min at 37°C. The cells were then centrifuged at 13000 rpm for 3 min, the media was discarded and the cells were resuspended in Annexin 1X buffer (1mL). They were then stained with Annexin FITC (4 μL) and propidium iodide (1.5 μg/mL final concentration) and left to incubate for 10 min at r.t. The suspensions were then submitted to FACS study.

20.9.2. Cell Cycle

Human colon cancer cells HCA-7 c29 were grown in 500 mL as described before (cf. 20.8.). HCA-7 c29 were seeded onto 6-well plates at a concentration of 75000 cells/well in media (2 mL) for a three-day treatment and at a density of 30000 cells/well for a six-day treatment. After one day incubation the media was removed and the cells were treated with a single treatment of media (2 mL) containing the analogues **7a** and **7c** in 0.1 % DMSO for further incubation (3 and 6 days) at a concentration of 2 and 50 μM. The control was treated with 0.1 % DMSO alone and was used as a negative control. Treatment of the cells with resveratrol at a concentration of 10 μM was used as a positive control. After incubation, the media was removed and put in a tube and the cells were washed with PBS buffer (2 x 1 mL), detached from the bottom of the plate by Trypsin 5x (1 mL) for a minimum amount of time (3-5 min) and resuspended immediately with media (1 mL). The content of the wells were combined with incubation media and then centrifuged at 1300 rpm for 3 min. The supernatant was discarded and the cell pellet was resuspended in PBS (4 mL), centrifuged at 1300 rpm for 3 min. The supernatant was discarded and the cells were dissolved in 70% EtOH (4 mL) with vortex mix. The cells solutions were kept at 4°C for at least 30 min and up to 7 days.

REFERENCES

(1) Cancer Research UK **2008**, http://cancerresearchuk.org/.
(2) National Cancer Institute *Dictionary of Cancer Terms* **2007**, http://www.cancer.gov/dictionary/?CdrID=45333.
(3) Weinberg, R. A. *The biology of cancer*; Garland Science, 2007.
(4) Vogelstein, B.; Kinzler, K. W. *The genetic basis of human cancer*; 2nd ed.; McGraw-Hill Professional, 2002.
(5) Hanahan, D.; Weinberg, R. A. *Cell* **2000**, *100*, 57-70.
(6) Pantel, K.; Brakenhoff, R. H. *Nature Reviews Cancer* **2004**, *4*, 448-456.
(7) Yang, J.; Mani, S. A.; Weinberg, R. A. *Cancer Research* **2006**, *66*, 4549-4552.
(8) Tarui, T.; Miles, L. A.; Takada, Y. *Journal of Biological Chemistry* **2001**, *276*, 39562-39568.
(9) The angiogenesis foundation *Angiogenesis: a cascade of events* **2000**, http://www.angio.org/img/cascade_image.jpg.
(10) Troyanovsky, B.; Levchenko, T.; Mansson, G.; Matvijenko, O.; Holmgren, L. *Journal of Cell Biology* **2001**, *152*, 1247-1254.
(11) Luo, J.; Solimini, N. L.; Elledge, S. J. *Cell* **2009**, *136*, 823-837.
(12) Kroemer, G.; Pouyssegur, J. *Cancer Cell* **2008**, *13*, 472-482.
(13) Knudson, A. G. *Nature Reviews Cancer* **2001**, *1*, 157-162.
(14) Croce, C. M. *New England Journal of Medicine* **2008**, *358*, 502-511.
(15) Downward, J. *Nature Reviews - Cancer* **2003**, *3*, 11-22.
(16) Sherr, C. J. *Cell* **2004**, *116*, 235-246.
(17) Balmain, A. *Nature Reviews - Cancer* **2001**, *1*, 77-82.
(18) Elledge, S. J. *Science* **1996**, *274*, 1664-1672.
(19) Coleman, M. L.; Marshall, C. J.; Olson, M. F. *Nature Reviews Molecular Cell Biology* **2004**, *5*, 355-366.
(20) Bartek, J.; Lukas, J. *Febs Letters* **2001**, *490*, 117-122.
(21) Massague, J. *Nature* **2004**, *432*, 298-306.
(22) Kastan, M. B.; Bartek, J. *Nature* **2004**, *432*, 316-323.
(23) McGowan, C. H.; Russell, P. *Current Opinion in Cell Biology* **2004**, *16*, 629-633.
(24) Nigg, E. A. *Nature Reviews Molecular Cell Biology* **2001**, *2*, 21-32.
(25) Pardee, A. B. *Science* **1989**, *246*, 603-608.
(26) Thompson, C. B. *Science* **1995**, *267*, 1456-1462.
(27) Burlacu, A. *Journal of Cellular and Molecular Medicine* **2003**, *7*, 249-257.
(28) Ashkenazi, A.; Dixit, V. M. *Science* **1998**, *281*, 1305-1308.
(29) Richards, A. D.; Rodger, A. *Chemical Society Reviews* **2007**, *36*, 471-483.
(30) Stordal, B.; Pavlakis, N.; Davey, R. *Cancer Treatment Reviews* **2007**, *33*, 347-357.
(31) Lal, S.; Mahajan, A.; Chen, W. N.; Chowbay, B. *Current Drug Metabolism*, *11*, 115-128.
(32) Xu, H.; Lv, M.; Tian, X. *Current Medicinal Chemistry* **2009**, *16*, 327-349.
(33) Band Horwitz, S. *Trends in Pharmacological Sciences* **1992**, *13*, 134-136.
(34) Haldar, S.; Jena, N.; Croce, C. M. *Proceedings of the National Academy of Sciences of the United States of America* **1995**, *92*, 4507-4511.
(35) Garnock-Jones, K. P.; Keating, G. M.; Scott, L. J. *Drugs*, *70*, 215(25).
(36) Dursun, D.; Kim, M. C.; Solomon, A.; Pflugfelder, S. C. *American Journal of Ophthalmology* **2001**, *132*, 8-13.

(37) Millar, A. W.; Brown, P. D.; Moore, J.; Galloway, W. A.; Cornish, A. G.; Lenehan, T. J.; Lynch, K. P. *British Journal of Clinical Pharmacology* **1998**, *45*, 21-26.
(38) Hemmings, F. J.; Farhan, M.; Rowland, J.; Banken, L.; Jain, R. *Rheumatology* **2001**, *40*, 537-543.
(39) Escudier, B.; Cosaert, J.; Pisa, P. *Expert Review of Anticancer Therapy* **2008**, *8*, 1545-1557.
(40) Therapontos, C.; Erskine, L.; Gardner, E. R.; Figg, W. D.; Vargesson, N. *Proceedings of the National Academy of Sciences of the United States of America* **2009**, *106*, 8573-8578.
(41) Vallet, S.; Palumbo, A.; Raje, N.; Boccadoro, M.; Anderson, K. C. *Leukemia & Lymphoma* **2008**, *49*, 1238-1245.
(42) Armoiry, X.; Aulagner, G.; Facon, T. *Journal of Clinical Pharmacy & Therapeutics* **2008**, *33*, 219-226.
(43) Perabo, F. G. E.; Demant, A. W.; Wirger, A.; Schmidt, D. H.; Sitia, M.; Wardelmann, E.; Muller, S. C.; Kohn, E. C. *Anticancer Research* **2005**, *25*, 725-729.
(44) Pao, W.; Miller, V.; Zakowski, M.; Doherty, J.; Politi, K.; Sarkaria, I.; Singh, B.; Heelan, R.; Rusch, V.; Fulton, L.; Mardis, E.; Kupfer, D.; Wilson, R.; Kris, M.; Varmus, H. *Proceedings of the National Academy of Sciences of the United States of America* **2004**, *101*, 13306-13311.
(45) Sordella, R.; Bell, D. W.; Haber, D. A.; Settleman , J. *Science* **205**, *305*, 1163-1167.
(46) Nawrocki, S. T.; Carew, J. S.; Pino, M. S.; Highshaw, R. A.; Dunner, K. J.; Huang, P.; Abbruzzese, J. L.; McConkey, D. J. *Cancer Research* **2005**, *65*, 11658-11666.
(47) Surh, Y.-J. *Nature Reviews Cancer* **2003**, *3*, 768-780.
(48) Kellhoff, G. J.; t.;, H. E.; Sigman, C. S. *Cancer Chemoprevention, Volume 2: Strategies for cancer chemoprevention*; Humana Press, 2005; Vol. 2.
(49) Doll, R. *World Journal of Surgery* **1978**, *2*, 595-602.
(50) Rastogi, T.; Hildesheim, A.; Sinha, R. *Nature Reviews Cancer* **2004**, *4*, 909-917.
(51) World Health Organisation (WHO) **2008**, http://www.who.int/en/.
(52) Doll, R.; Petto, R. *Journal of National Cancer Institute* **1981**, *66*, 1191-1308.
(53) Peto, J. *Nature* **2001**, *411*, 390-395.
(54) Takata, Y.; Maskarinec, G.; Franke, A.; Nagata, C.; Shimizu, H. *Public Health Nutrition* **2004**, *7*, 319-326.
(55) Savouret, J. F.; Quesne, M. *Biomedicine & Pharmacotherapy* **2002**, *56*, 84-87.
(56) Aggarwal, B. B.; Kumar, A.; Bharti, A. C. *Anticancer Research* **2003**, *23*, 363-398.
(57) Jang, M. S.; Cai, E. N.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W. W.; Fong, H. H. S.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. *Science* **1997**, *275*, 218-220.
(58) Mori, A.; Lehmann, S.; O'Kelly, J.; Kumagai, T.; Desmond, J. C.; Pervan, M.; McBride, W. H.; Kizaki, M.; Koeffler, H. P. *Cancer Research* **2006**, *66*, 3222-3229.
(59) Grunberger, D.; Banerjee, R.; Eisinger, K.; Oltz, E. M.; Efros, L.; Caldwell, M.; Estevez, V.; Nakanishi, K. *Experientia* **1988**, *44*, 230-232.
(60) Katiyar, S. K.; Agarwal, R.; Mukhtar, H. *Cancer Research* **1996**, *56*, 1023-1030.
(61) Dausch, J. G.; Nixon, D. W. *Preventive Medicine* **1990**, *19*, 346-361.
(62) Jung, Y. D.; Kim, M. S.; Shin, B. A.; Chay, K. O.; Ahn, B. W.; Liu, W.; Bucana, C. D.; Gallick, G. E.; Ellis, L. M. *British Journal of Cancer* **2001**, *84*, 844-850.
(63) Newfield, L.; Goldsmith, A.; Bradlow, H. L.; Auborn, K. *Anticancer Research* **1993**, *13*, 337-341.
(64) Lamartiniere, C. A. *American Journal of Clinical Nutrition* **2000**, *71*, 1705S-

1707S.
(65) Zhang, Y. S.; Talalay, P.; Cho, C. G.; Posner, G. H. *Proceedings of the National Academy of Sciences of the United States of America* **1992**, *89*, 2399-2403.
(66) Rao, A. V. R.; Agarwal, S. *Journal of the American College of Nutrition* **2000**, *19*, 563-569.
(67) Hudson, E. A.; Dinh, P. A.; Kokubun, T.; Simmonds, M. S. J.; Gescher, A. *Cancer Epidemiology Biomarkers & Prevention* **2000**, *9*, 1163-1170.
(68) Arichi, H.; Kimura, Y.; Okuda, H.; Baba, K.; Kozawa, M.; Arichi, S. *Chemical & Pharmaceutical Bulletin* **1982**, *30*, 1766-1770.
(69) Fremont, L. *Life Sciences* **2000**, *66*, 663-673.
(70) Austin, M. B.; Bowman, M. E.; Ferrer, J. L.; Schroder, J.; Noel, J. P. *Chemistry & Biology* **2004**, *11*, 1179-1194.
(71) Beekwilder, J.; Wolswinkel, R.; Jonker, H.; Hall, R.; de Vos, C. H. R.; Bovy, A. *Applied and Environmental Microbiology* **2006**, *72*, 5670-5672.
(72) Brown, L.; Kroon, P. A.; Das, D. K.; Das, S.; Tosaki, A.; Chan, V.; Singer, M. V.; Feick, P. *Alcoholism-Clinical and Experimental Research* **2009**, *33*, 1513-1523.
(73) Das, D. K.; Maulik, N. *Molecular Interventions* **2006**, *6*, 36-47.
(74) Ferrieres, J. *Heart* **2004**, *90*, 107-111.
(75) Wang, L. X.; Heredia, A.; Song, H. J.; Zhang, Z. J.; Yu, B.; Davis, C.; Redfield, R. *Journal of Pharmaceutical Sciences* **2004**, *93*, 2448-2457.
(76) Baur, J. A.; Sinclair, D. A. *Nature Reviews Drug Discovery* **2006**, *5*, 493-506.
(77) Kundu, J. K.; Surh, Y.-J. *Cancer Letters* **2008**, *269*, 243-261.
(78) Fang, J. L.; Beland, F. A.; Doerge, D. R.; Wiener, D.; Guillemette, C.; Marques, M. M.; Lazarus, P. *Cancer Research* **2002**, *62*, 1978-1986.
(79) Perwez Hussain, S.; Harris, C. C. *International Journal of Cancer* **2007**, *121*, 2373-2380.
(80) Gusman, J.; Malonne, H.; Atassi, G. *Carcinogenesis* **2001**, *22*, 1111-1117.
(81) Park, J. W.; Choi, Y. J.; Jang, M. A.; Lee, Y. S.; Jun, D. Y.; Suh, S. I.; Baek, W. K.; Suh, M. H.; Jin, I. N.; Kwon, T. K. *Cancer Letters* **2001**, *163*, 43-49.
(82) Hsieh, T. C.; Juan, G.; Darzynkiewicz, Z.; Wu, J. M. *Cancer Research* **1999**, *59*, 2596-2601.
(83) Aziz, M. H.; Nihal, M.; Fu, V. X.; Jarrard, D. F.; Ahmad, N. *Molecular Cancer Therapeutics* **2006**, *5*, 1335-1341.
(84) Juan, M. E.; Wenzel, U.; Daniel, H.; Planas, J. M. *Journal of Agricultural and Food Chemistry* **2008**, *56*, 4813-4818.
(85) Banerjee, S.; Bueso-Ramos, C.; Aggarwal, B. B. *Cancer Research* **2002**, *62*, 4945-4954.
(86) Mouria, M.; Gukovskaya, A. S.; Jung, Y.; Buechler, P.; Hines, O. J.; Reber, H. A.; Pandol, S. J. *International Journal of Cancer* **2002**, *98*, 761-769.
(87) Lin, M. T.; Yen, M. L.; Lin, C. Y.; Kuo, M. L. *Molecular Pharmacology* **2003**, *64*, 1029-1036.
(88) Cao, Y.; Fu, Z. D.; Wang, F.; Liu, H. Y.; Han, R. *Journal of Asian Natural Products Research* **2005**, *7*, 205-213.
(89) Meng, X. F.; Maliakal, P.; Lu, H.; Lee, M. J.; Yang, C. S. *Journal of Agricultural and Food Chemistry* **2004**, *52*, 935-942.
(90) Wenzel, E.; Somoza, V. *Molecular Nutrition & Food Research* **2005**, *49*, 472-481.
(91) Yu, C. W.; Shin, Y. G.; Chow, A.; Li, Y. M.; Kosmeder, J. W.; Lee, Y. S.; Hirschelman, W. H.; Pezzuto, J. M.; Mehta, R. G.; van Breemen, R. B. *Pharmaceutical Research* **2002**, *19*, 1907-1914.

(92) Patel, K. R.; Brown, V. A.; Jones, D. J. L.; Britton, R. G.; Hemingway, D.; Miller, A. S.; West, K. P.; Booth, T. D.; Perloff, M.; Crowell, J. A.; Brenner, D. E.; Steward, W. P.; Gescher, A. J.; Brown, K. *Cancer Research*, *70*, 7392-7399.
(93) Walle, T.; Hsieh, F.; DeLegge, M. H.; Oatis, J. E.; Walle, U. K. *Drug Metabolism and Disposition* **2004**, *32*, 1377-1382.
(94) Burkon, A.; Somoza, V. *Molecular Nutrition & Food Research* **2008**, *52*, 549-557.
(95) Wenzel, E.; Soldo, T.; Erbersdobler, H.; Somoza, V. *Molecular Nutrition and Food Research.* **2005**, *49*, 482-494.
(96) Boocock, D. J.; Faust, G. E. S.; Patel, K. R.; Schinas, A. M.; Brown, V. A.; Ducharme, M. P.; Booth, T. D.; Crowell, J. A.; Perloff, M.; Gescher, A. J.; Steward, W. P.; Brenner, D. E. *Cancer Epidemiology Biomarkers & Prevention* **2007**, *16*, 1246-1252.
(97) Boocock, D. J.; Patel, K. R.; Faust, G. E. S.; Normolle, D. P.; Marczylo, T. H.; Crowell, J. A.; Brenner, D. E.; Booth, T. D.; Gescher, A.; Steward, W. P. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2007**, *848*, 182-187.
(98) Vitrac, X.; Desmouliere, A.; Brouillaud, B.; Krisa, S.; Deffieux, G.; Barthe, N.; Rosenbaum, J.; Merillon, J. M. *Life Sciences* **2003**, *72*, 2219-2233.
(99) Hoshino, J.; Park, E. J.; Kondratyuk, T. P.; Marler, L.; Pezzuto, J. M.; van Breemen, R. B.; Mo, S. Y.; Li, Y. C.; Cushman, M. *Journal of Medicinal Chemistry* **2010**, *53*, 5033-5043.
(100) Kim, S.; Ko, H.; Park, J. E.; Jung, S.; Lee, S. K.; Chun, Y. J. *Journal of Medicinal Chemistry* **2002**, *45*, 160-164.
(101) Mikstacka, R.; Rimando, A. M.; Szalaty, K.; Stasik, K.; Baer-Dubowska, W. *Xenobiotica* **2006**, *36*, 269-285.
(102) Zykova, T. A.; Zhu, F.; Zhai, X. H.; Ma, W. Y.; Ermakova, S. P.; Lee, K. W.; Bode, A. M.; Dong, Z. G. *Molecular Carcinogenesis* **2008**, *47*, 797-805.
(103) Murias, M.; Handler, N.; Erker, T.; Pleban, K.; Ecker, G.; Saiko, P.; Szekeres, T.; Jager, W. *Bioorganic and Medicinal Chemistry* **2004**, *12*, 5571-5578.
(104) Sale, S.; Tunstall, R. G.; Ruparelia, K. C.; Potter, G. A.; Steward, W. P.; Gescher, A. J. *International Journal of Cancer* **2005**, *115*, 194-201.
(105) Chun, Y. J.; Ryu, S. Y.; Jeong, T. C.; Kim, M. Y. *Drug Metabolism and Disposition* **2001**, *29*, 389-393.
(106) Chun, Y. J.; Lee, S. K.; Kim, M. Y. *Drug Metabolism and Disposition* **2005**, *33*, 1771-1776.
(107) Kang, S. S.; Cuendet, M.; Endringer, D. C.; Croy, V. L.; Pezzuto, J. M.; Lipton, M. A. *Bioorganic & Medicinal Chemistry* **2009**, *17*, 1044-1054.
(108) Heynekamp, J. J.; Weber, W. M.; Hunsaker, L. A.; Gonzales, A. M.; Orlando, R. A.; Deck, L. M.; Jagt, D. L. V. *Journal of Medicinal Chemistry* **2006**, *49*, 7182-7189.
(109) Thakkar, K.; Geahlen, R. L.; Cushman, M. *Journal of Medicinal Chemistry* **1993**, *36*, 2950-2955.
(110) Weng, C. J.; Yang, Y. T.; Ho, C. T.; Yen, G. C. *Journal of Agricultural and Food Chemistry* **2009**, *57*, 5235-5243.
(111) Pettit, R. K.; Pettit, G. R.; Hamel, E.; Hogan, F.; Moser, B. R.; Wolf, S.; Pon, S.; Chapuis, J. C.; Schmidt, J. M. *Bioorganic & Medicinal Chemistry* **2009**, *17*, 6606-6612.
(112) Moran, B. W.; Anderson, F. P.; Devery, A.; Cloonan, S.; Butler, W. E.; Varughese, S.; Draper, S. M.; Kenny, P. T. M. *Bioorganic & Medicinal Chemistry* **2009**, *17*, 4510-4522.
(113) Weng, C. J.; Wu, C. F.; Huang, H. W.; Wu, C. H.; Ho, C. T.; Yen, G. C. *Journal of Agricultural and Food Chemistry* **2010**, *58*, 2886-2894.

(114) Liu, H. C.; Dong, A. J.; Gao, C. M.; Tan, C. Y.; Liu, H. X.; Zu, X. Y.; Jiang, Y. Y. *Bioorganic & Medicinal Chemistry* **2008**, *16*, 10013-10021.
(115) Pagliai, F.; Pirali, T.; Del Grosso, E.; Di Brisco, R.; Tron, G. C.; Sorba, G.; Genazzani, A. A. *Journal of Medicinal Chemistry* **2006**, *49*, 467-470.
(116) Minutolo, F.; Sala, G.; Bagnacani, A.; Bertini, S.; Carboni, I.; Placanica, G.; Prota, G.; Rapposelli, S.; Sacchi, N.; Macchia, M.; Ghidoni, R. *Journal of Medicinal Chemistry* **2005**, *48*, 6783-6786.
(117) Simoni, D.; Roberti, M.; Invidiata, F. P.; Aiello, E.; Aiello, S.; Marchetti, P.; Baruchello, R.; Eleopra, M.; Di Cristina, A.; Grimaudo, S.; Gebbia, N.; Crosta, L.; Dieli, F.; Tolorneo, M. *Bioorganic & Medicinal Chemistry Letters* **2006**, *16*, 3245-3248.
(118) Simoni, D.; Romagnoli, R.; Baruchello, R.; Rondanin, R.; Grisolia, G.; Eleopra, M.; Rizzi, M.; Tolomeo, M.; Giannini, G.; Alloatti, D.; Castorina, M.; Marcellini, M.; Pisano, C. *Journal of Medicinal Chemistry* **2008**, *51*, 6211-6215.
(119) Murias, M.; Jager, W.; Handler, N.; Erker, T.; Horvath, Z.; Szekeres, T.; Nohl, H.; Gille, L. *Biochemical Pharmacology* **2005**, *69*, 903-912.
(120) Cheng, J. C.; Fang, J. G.; Chen, W. F.; Zhou, B.; Yang, L.; Liu, Z. L. *Bioorganic Chemistry* **2006**, *34*, 142-157.
(121) Botella, L.; Najera, C. *Tetrahedron* **2004**, *60*, 5563-5570.
(122) Solladie, G.; Pasturel-Jacope, Y.; Maignan, J. *Tetrahedron* **2003**, *59*, 3315-3321.
(123) Robinson, J. E.; Taylor, R. J. K. *Chemical Communications* **2007**, 1617-1619.
(124) Anderson, W. F.; Umar, A.; Hawk, E. T. *Expert Opinion on Pharmacotherapy* **2003**, *4*, 2193-2204.
(125) Liehr, J. G.; Ricci, M. J. *Proceedings of the National Academy of Sciences of the United States of America* **1996**, *93*, 3294-3296.
(126) Yang, Y. T.; Weng, C. J.; Ho, C. T.; Yen, G. C. *Molecular Nutrition & Food Research* **2009**, *53*, 407-416.
(127) Hanif, R.; Qiao, L.; Shiff, S. J.; Rigas, B. *Journal of Laboratory and Clinical Medicine* **1997**, *130*, 576-584.
(128) Thangapazham, R. L.; Sharma, A.; Maheshwari, R. K. *The APPS Journal* **2006**, *8*, 443-449.
(129) Johnson, J. J.; Mukhtar, H. *Cancer Letters* **2007**, *255*, 170-181.
(130) Rajasingh, J.; Raikwar, H. P.; Muthian, G.; Johnson, C.; Bright, J. J. *Biochemical and Biophysical Research Communications* **2006**, *340*, 359-368.
(131) Sale, S.; Fong, I. L.; de Giovanni, C.; Landuzzi, L.; Brown, K.; Steward, W. P.; Gescher, A. J. *European Journal of Cancer* **2009**, *45*, 2731-2735.
(132) Cai, H.; Boocock, D. J.; Steward, W. P.; Gescher, A. J. *Cancer Chemotherapy and Pharmacology* **2007**, *60*, 257-266.
(133) Cai, H.; Brown, K.; Steward, W. P.; Gescher, A. J. *Biomedical Chromatography* **2009**, *23*, 335-339.
(134) Collin, D. T.; Hartley, D.; Jack, D.; Lunts, L. H. C.; Press, J. C.; Ritchie, A. C.; Toon, P. *Journal of Medicinal Chemistry* **1970**, *13*, 674-680.
(135) Lunts, L. H. C. In *Medicinal Chemistry*; 2nd ed.; Ganellin, C. R., Roberts, S. M., Eds.; Academic Press Ltd: 1993, p 209-226.
(136) Lee, H. J.; Seo, J. W.; Lee, B. H.; Chung, K.-H.; Chi, D. Y. *Bioorganic and Medicinal Chemistry Letters* **2004**, *14*, 463-466.
(137) Wadsworth, W. S.; Emmons, W. D. *Journal of American Chemical Society* **1961**, *83*, 1733-1738.
(138) Lee, H. J.; Seo, J. W.; Lee, B. H.; Chung, K.-H.; Chi, D. Y. *Bioorganic & Medicinal Chemistry Letters* **2004**, *14*, 463-466.
(139) Arbusow, B. A. *Pure and Applied Chemistry* **1964**, *9*, 307-336.

(140) Wadsworth, W. R. J.; Emmons, W. D. *Journal of American Chemical Society* **1961**.
(141) Majumdar, K. C.; Chattopadhyay, S. K.; Mukhopadhyay, P. P. *Synthetic Communication* **2006**, *36*, 1291-1297.
(142) Lai, G.; Tan, P.-Z.; Ghoshal, P. *Synthetic Communication* **2003**, *33*, 1727-1732.
(143) Yu, B.; Hui, Y. *Synthetic Communication* **1995**, *25*, 2037-2042.
(144) McDougal, P. G.; Rico, J. G.; Oh, Y.-I.; Condon, B. D. *Journal of Organic Chemistry* **1986**, *51*, 3388-3390.
(145) Bohmer, V.; Vogt, W.; Chafaa, S.; Meullemeestre, J.; Schwing, M.-J.; Vierling, F. *Helvetica Chimica Act*
1993, *76*, 139-149.
(146) Klar, U.; Buchmann, B.; Schwede, W.; Skuballa, W.; Hoffinann, J.; Lichtner, R. B. *Angewandte Chemie-International Edition* **2006**, *45*, 7942-7948.
(147) Tamaddon, F.; Amrollahi, M. A.; Sharafat, L. *Tetrahedron Letters* **2005**, *46*, 7841-7844.
(148) Hajipour, A. R.; Falahati, A. R.; Ruoho, A. E. *Tetrahedron Letters* **2006**, *47*, 4191-4196.
(149) Breschi, M. C.; Calderone, V.; Digiacomo, M.; Macchia, M.; Martelli, A.; Martinotti, E.; Minutolo, F.; Rapposelli, S.; Rossello, A.; Testai, L.; Balsamo, A. *Journal of Medicinal Chemistry* **2006**, *49*, 2628-2639.
(150) Pyun, J.; Tang, C. B.; Kowalewski, T.; Frechet, J. M. J.; Hawker, C. J. *Macromolecules* **2005**, *38*, 2674-2685.
(151) Daub, G. H.; Castle, R. N. *Journal of Organic Chemistry* **1954**, *19*, 1571-1574.
(152) Prakash, C.; Saleh, S.; Blair, I. A. *Tetrahedron Letters* **1994**, *35*, 7565-7568.
(153) Das, B.; Reddy, K. R.; Thirupathi, P. *Tetrahedron Letters* **2006**, *47*, 5855-5857.
(154) Li, R. L.; Dietrich, S. W.; Hansch, C. *Journal of Medicinal Chemistry* **1981**, *24*, 538-544.
(155) Behrens, C.; Dahl, O. *Nucleosides & Nucleotides* **1999**, *18*, 291-305.
(156) Yu, K. Q.; Sommer, W.; Richardson, J. M.; Weck, M.; Jones, C. W. *Advanced Synthesis & Catalysis* **2005**, *347*, 161-171.
(157) Meric, R.; Lehn, J. M.; Vigneron, J. P. *Bulletin De La Societe Chimique De France* **1994**, *131*, 579-583.
(158) Duhamel, L.; Ancel, J. E. *Tetrahedron* **1992**, *48*, 9237-9250.
(159) Wessjohann, L. A.; Wild, H.; Schrekker, H. S. *Tetrahedron Letters* **2004**, *45*, 9073-9078.
(160) Hunsen, M. *Tetrahedron Letters* **2005**, *46*, 1651-1653.
(161) Xie, R. G.; Zhang, Z. J.; Yan, J. M.; Yuan, D. Q. *Synthetic Communications* **1994**, *24*, 53-58.
(162) Amato, M. E.; Ballistreri, F. P.; Pappalardo, A.; Sciotto, D.; Tomaselli, G. A.; Toscano, R. M. *Tetrahedron* **2007**, *63*, 9751-9757.
(163) Carlsson, H.; Haukka, M.; Nordlander, E. *Inorganic Chemistry* **2004**, *43*, 5681-5687.
(164) Severi, F.; Benvenuti, S.; Costantino, L.; Vampa, G.; Melegari, M.; Antolini, L. *European Journal of Medicinal Chemistry* **1998**, *33*, 859-866.
(165) Pouget, C.; Fagnere, C.; Basly, J.-P.; Besson, A.-E.; Champavier, Y.; Habrioux, G.; Chulia, A.-J. *Pharmaceutical Research* **2002**, *19*, 286-291.
(166) Pyun, J.; Rees, I.; Frechet, J. M. J.; Hawker, C. J. *Australian Journal of Chemistry* **2003**, *56*, 775-782.
(167) Zhang, Y.; Huang, J.; Chen, Y. *Macromolecules* **2005**, *38*, 5069-5077.
(168) Wadsworth, W. S. J.; Emmons, W. D. *Organic Syntheses* **1973**, *Coll. Vol. 5*, 547.

(169) Zeng, D.; Mi, Q.; Sun, H.; Wang, H. *Journal of Labelled Compounds and Radiopharmaceuticals* **2004**, *47*, 167-174.
(170) McOmie, J. F. W.; Watts, M. L.; West, D. E. *Tetrahedron* **1968**, *24*, 2289-2292.
(171) Taub, D.; Girotra, N. N.; Hoffsommer, R. D.; Kuo, C. H.; Slates, H. L.; Weber, S.; Wendler, N. L. *Tetrahedron* **1968**, *24*, 2443-2461.
(172) Williard, P. G.; Fryhle, C. B. *Tetrahedron Letters* **1980**, *21*, 3731-3734.
(173) Corey, E. J.; Weinshenker, N. M.; Schaaf, T. K.; Huber, W. *Journal of American Chemical Society* **1969**, *91*, 5675-5677.
(174) McOmie, J. F. W.; Watts, M. L. *Chemistry and Industry* **1963**, 1658.
(175) Dean, F. M.; Goodchild, J.; Houghton, L. E.; Martin, J. A.; Morton, R. B.; Parton, B.; Price, A. W.; Somvichien, N. *Tetrahedron Letters* **1966**, *7*, 4153-4159.
(176) Arkley, V.; Attenburrow, J.; Gregory, G. I.; Walker, T. *Journal of the chemical society* **1692**, 1260.
(177) Carvalho, C. F.; Sargent, M. V. *Journal of the Chemical Society, Chemical Communications* **1984**, 227-229.
(178) Dodge, J. A.; Stocksdale, M. G.; Fahey, K. J.; Jones, C. D. *Journal of Organic Chemistry* **1995**, *60*, 739-741.
(179) Bhatt, M. V.; Babu, J. R. *Tetrahedron Letters* **1984**, *25*, 3497-3500.
(180) Andersson, S. *Synthesis* **1985**, 437-439.
(181) Fukuda, H.; Kitazume, T. *Journal of Fluorine Chemistry* **1995**, *74*, 171-176.
(182) Glatt, H. *Faseb Journal* **1997**, *11*, 314-321.
(183) Chen, G. P.; Banoglu, E.; Duffel, M. W. *Chemical Research in Toxicology* **1996**, *9*, 67-74.
(184) Banoglu, E.; Duffel, M. W. *Drug Metabolism and Disposition* **1997**, *25*, 1304-1310.
(185) Learmonth, D. A.; Freitas, A. P. *Bioconjugate Chemistry* **2002**, *13*, 1112-1118.
(186) Manning, D. D.; Bertozzi, C. R.; Pohl, N. L.; Rosen, S. D.; Kiessling, L. L. *Journal of Organic Chemistry* **1995**, *60*, 6254-6255.
(187) Yoshiizumi, K.; Nakajima, F.; Dobashi, R.; Nishimura, N.; Ikeda, S. *Bioorganic & Medicinal Chemistry Letters* **2004**, *14*, 2791-2795.
(188) White, E. H.; Li, M.; Lu, S. *Journal of Organic Chemistry* **1992**, *57*, 1252-1258.
(189) Banks, G. R.; Cohen, D. *Journal of the Chemical Society (Resumed)* **1965**, 6209-6212.
(190) Megati, S.; Raveendranath, P.; Raveendranath, G.; Raveendranath, V.; Raveendranath, S.; A61K31/566; C07J1/00; A61P5/24; A61P19/08; A61P35/00; A61P9/00; C12N5/06 ed.; WYETH (Madison, NJ, US): United States, 2008; Vol. US2008/0194533 A1, p 18.
(191) Stevenson, D. E.; Hubl, U. *Enzyme and Microbial Technology* **1999**, *24*, 388-396.
(192) Stachulski, A. V.; Jenkins, G. N. *Natural Product Reports* **1998**, *15*, 173-186.
(193) T. Brown, R.; Scheinmann, F.; V. Stachulski, A. *Journal of Chemical Research, Synopses* **1997**, 370-371.
(194) Fischer, B.; Nudelman, A.; Ruse, M.; Herzig, J.; Gottlieb, H. E.; Keinan, E. *Journal of Organic Chemistry* **1984**, *49*, 4988-4993.
(195) Schmidt, R. R.; Grundler, G. *Synthesis* **1981**, 885-887.
(196) Kim, H.-J.; Ahn, K. C.; Ma, S. J.; Gee, S. J.; Hammock, B. D. *Journal of Agricultural and Food Chemistry* **2007**, *55*, 3750-3757.
(197) Madec-Lougerstay, R.; Florent, J.-C.; Monneret, C. *Journal of the Chemical Society, Perkin Transactions 1* **1999**, 1369-1376.
(198) Stachulski, A. V. *Tetrahedron Letters* **2001**, *42*, 6611-6613.

(199) Grothusen, A.; Hardt, J.; Brautigam, L.; Lang, D.; Bocker, R. *Archives of Toxicology* **1996**, *71*, 64-71.
(200) Wu, W. N.; McKown, L. A.; Codd, E. E.; Raffa, R. B. *European Journal of Drug Metabolism and Pharmacokinetics* **2002**, *27*, 193-197.
(201) Binder, T. P.; Duffel, M. W. *Molecular Pharmacology* **1987**, *33*, 477-479.
(202) Wang, D.; Hang, T.; Wu, C.; Liu, W. *Journal of Chromatography B* **2005**, *829*, 97-106.
(203) Marsh, K. A.; Stamp, G. W. H.; Kirkland, S. C. *Journal of Pathology* **1993**, *170*, 441-450.
(204) Sale, S.; Verschoyle, R. D.; Boocock, D.; Jones, D. J. L.; Wilsher, N.; Ruparelia, K. C.; Potter, G. A.; Farmer, P. B.; Steward, W. P.; Gescher, A. J. *British Journal of Cancer* **2004**, *90*, 736-744.
(205) Kelkel, M.; Jacob, C.; Dicato, M.; Diederich, M. *Molecules*, *15*, 7035-7074.
(206) Ovesna, Z.; Horvathova-Kozics, K. *Neoplasma* **2005**, *52*, 450-455.
(207) Wu, M.-M.; Chiou, H.-Y.; Lee, T.-C.; Chen, C.-L.; Hsu, L.-I.; Wang, Y.-H.; Huang, W.-L.; Hsieh, Y.-C.; Yang, T.-Y.; Lee, C.-Y.; Yip, P.-K.; Wang, C.-H.; Hsueh, Y.-M.; Chen, C.-J. *Journal of Biomedical Science* **2010**, *17*.
(208) Innovative cell tech *Accutase: The solution for cell detachment* **2010**, http://www.innovativecelltech.com/accutase.html.
(209) Wachs, F.-P.; Couillard-Despres, S.; Engelhardt, M.; Wilhelm, D.; Ploetz, S.; Vroemen, M.; Kaesbauer, J.; Uyanik, G.; Klucken, J.; Karl, C.; Tebbing, J.; Svendsen, C.; Weidner, N.; Kuhn, H.-G.; Winkler, J.; Aigner, L. *Lab Invest* **2003**, *83*, 949-962.
(210) Katz, T. J.; Sudhakar, A.; Teasley, M. F.; Gilbert, A. M.; Geiger, W. E.; Robben, M. P.; Wuensch, M.; Ward, M. D. *Journal of the American Chemical Society* **1993**, *115*, 3182-3198.
(211) Nicolaou, K. C.; Pfefferkorn, J. A.; Roecker, A. J.; Cao, G. Q.; Barluenga, S.; Mitchell, H. J. *Journal of the American Chemical Society* **2000**, *122*, 9939-9953.
(212) Castaldi, M. P.; Susan, E. G.; Matthew, R.; Andrew, J. P. W. *Chemistry - A European Journal* **2006**, *12*, 138-148.
(213) Gibson, G. G.; Sketts, P. e. *Introduction to drug metabolism*; 3rd ed.; Nelson Thornes Publishers: Cheltenham, 2001.

APPENDICES

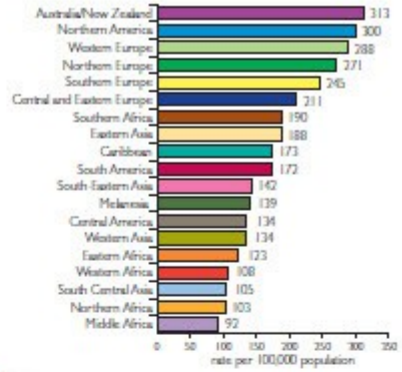
CANCER RESEARCH UK

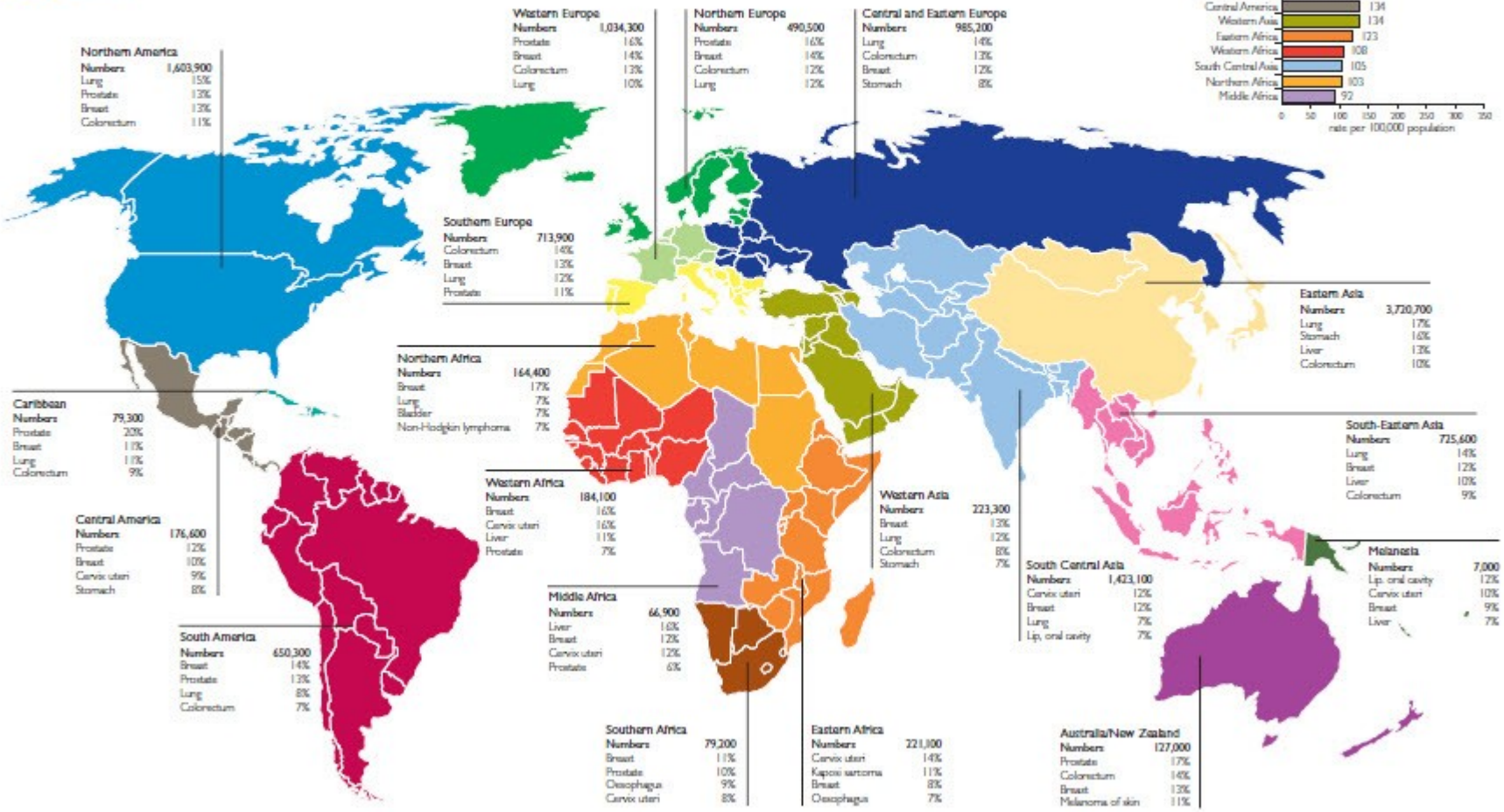
International Agency for Research on Cancer

World Health Organization

Cancer Incidence Worldwide

Breakdown of the estimated 12.7 million new cases, age standardised incidence rates and the most commonly diagnosed cancers by the different regions of the world, 2008.





Source: GLOBOCAN 2008, Cancer Incidence and Mortality Worldwide. IARC, 2010 (http://globocan.iarc.fr)

http://info.cancerresearchuk.org/cancerstats/

Additional training done during the PhD

- Attending to the postgraduate trainings organised by the University of Leicester and by the Chemistry Department.
- Validation of 20 credits in BS3003 course, *Cancer Cell and Molecular Biology*: attendance to the lectures, tutorials and exam, and validation of the 20 credits of the course by having 55% mark overall.
- Participation to the Thursdays Organic Group sessions: literature discussion, problem sessions, project presentations.

Teaching and supervision:

- Demonstration to 2nd year organic chemistry practicals.
- Demonstration to 3rd year MChem and MSc organic chemistry practicals.
- Supervision of 3rd year BSc projects, 4th year MChem, MSc projects and Erasmus students.

Poster Presentations:

- Presentation as a finalist of a poster entitled "*Can your Diet Prevent Cancer? Chemoprevention of cancer from natural phytochemicals*" during the fourth postgraduate festival in Leicester in June 2008.
- Presentation of a poster with the same title but different content at the BACR special conference on the "Advances in Cancer Drug Discovery" in Cambridge in September 2008.
- Presentation of the poster entitled "Chemoprevention of cancer: Synthesis and metabolism of resveratrol analogues":
- At the EACR conference in July 2009 at the University of Nottingham.
- Winner of runner-up poster presentation prize at RSC Midlands Organic section meeting 2010 at the University of Leicester.
- Presentation of a poster entitled "*Chemoprevention of Cancer: Synthesis and Biological Evaluation of New Resveratrol Analogues*" at the

International Symposia on Advancing the Chemical Sciences: Challenges in Organic Chemistry and Chemical Biology in San Francisco (USA) in July 2010.

Oral Presentations:

- Masters' degree presentation during a group meeting.
- Literature discussion in group meetings.
- Presentation of the PhD project at the "Postgraduate Annual Seminar" at the University of Leicester.

Conferences/Seminars attended:

- Attendance to Leicester Organic and Green Chemistry Seminars.
- Attendance to different RSC symposia in different universities like Nottingham, Loughborough, Sheffield, Leeds, Cambridge and Leicester.
- Attendance to the conferences:
- BACR special conference on the "Advances in Cancer Drug Discovery" in Cambridge in September 2008.
- EACR conference in July 2009 at the University of Nottingham.
- 42nd IUPAC Congress on Chemistry Solutions in Glasgow in August 2009.
- International Symposia on Advancing the Chemical Sciences: Challenges in Organic Chemistry and Chemical Biology (ISACS 1) in San Francisco (USA) in July 2010.

Can your Diet Prevent Cancer ?

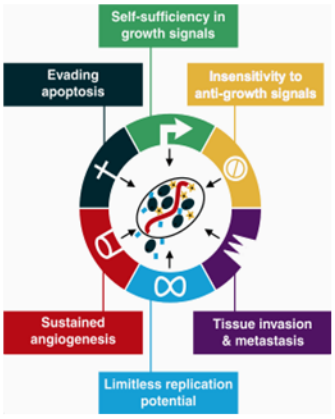
Chemoprevention of Cancer from Natural Dietary Phytochemicals

C. Simon, Dr R. G. Britton, Dr P. R. Jenkins*,
cs234@le.ac.uk, Department of Chemistry, University of Leicester, Leicester, UK

What is Chemoprevention?

The use of drugs, vitamins, or other agents to try to reduce the risk of, or delay the development or recurrence of, cancer

- **Prevents** cancer initiation
- **Delays** the onset on cancer
- **Slows** cancer progression
- **Reduces** the size of a tumour
- **Kills** cancer cells/ tumour



The Hallmarks of cancer[1]

What is Cancer ?

Any malignant growth or tumour caused by abnormal and uncontrolled cell division; it may spread to other parts of the body

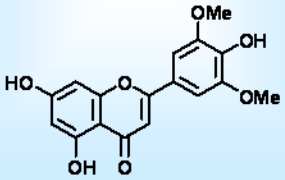
Characterized by 6 Hallmarks

Population studies[2]

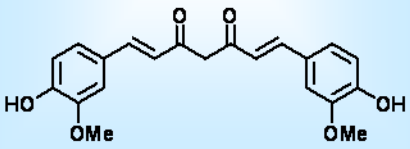
- French paradox : high levels of fat in the diet but low rate of heart diseases → *effect of **red wine** ?*
- Japanese in Hawaii : higher rate of breast cancer than in Japan → *effect of '**western lifestyle**' ?*

↳ ***Environmental factors : Air, Water, Diet, Lifestyle... play a big role on cancer***

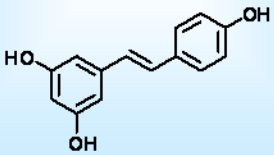
Rice Bran / Tricin



Turmeric / Curcumin



Grapes / Resveratrol



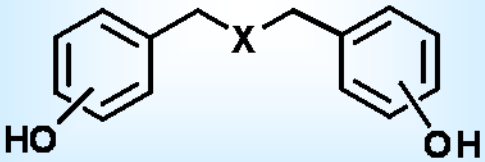
Studies on Resveratrol

- Metabolised in *Glucoronide* or *Sulfate* too quickly → No activity on cancer cells
- Good activity on **skin cancer** cell lines → size of tumours and number of tumours smaller than without treatment
- **Antioxidant** and **anti-inflammatory** activities

Further studies

Synthesis of a library of analogues

DRUG DESIGN



References

1. D. Hanahan, R. A. Weinreb, *Cell*, **2000**, 100, 57-70
2. http://www.who.int/en/

Can your Diet Prevent Cancer ?

Chemoprevention of Cancer from Natural Dietary Phytochemicals

C. Simon, **Dr R. G. Britton**, **Dr P. R. Jenkins***, *cs234@le.ac.uk, Department of Chemistry, University of Leicester, Leicester, UK*

Introduction

Diet is the second most preventable cause of cancer after smoking[1]. Population studies show that cancer has various incidences in different parts of the world. Studies of the diet in those areas highlighted different natural phytochemicals have chemopreventive properties against cancer. A drug design has been developed based on three of them, resveratrol (contained in grape skin, red wines, nuts oils and berries), curcumin (contained in turmeric-curry) and tricin (contained in rice). The aim of this design is to create a new cancer chemopreventive drug which will have a better bioavailability and which will give little or no side effects during the treatment.

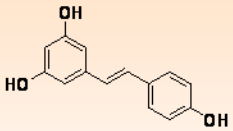
Chemoprevention[2]

The use of drugs, vitamins, or other agents to try to reduce the risk or delay the development or recurrence of cancer.

- **Prevents** cancer initiation
- **Delays** the onset on cancer
- **Slows** cancer progression
- **Reduces** the size of a tumour
- **Kills** cancer cells / tumour

Resveratrol

It is a natural product which occurs in ***grape skin***, ***red wine***, ***nuts oils*** and ***berries.*** It is a phytoalexin (antibiotic produced by plants) which had been shown to have different chemopreventive activities.



Resveratrol

3,5,4'-trihydroxy-trans-stilbene

Pezzuto et al. showed that the three stages of the carcinogenesis process were inhibited by resveratrol[3].

- **Anti-tumour initiation:** Inhibition of free radical formation

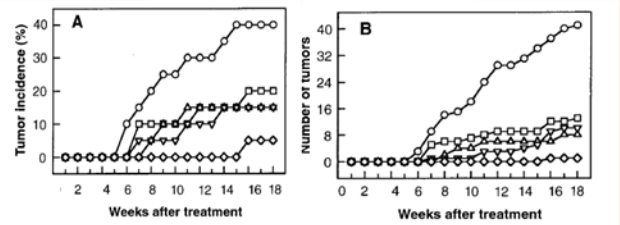
TPA induced: ED_{50} = 27 µM

- **Anti-tumour promotion:** Inhibition of COX-1 and COX-2

COX-1: ED_{50} = 3.7 µM; IC_{50} COX-1 = 0.535 µM

COX-2: ED_{50} = 85 µM; IC_{50} COX -2 = 0.996 µM

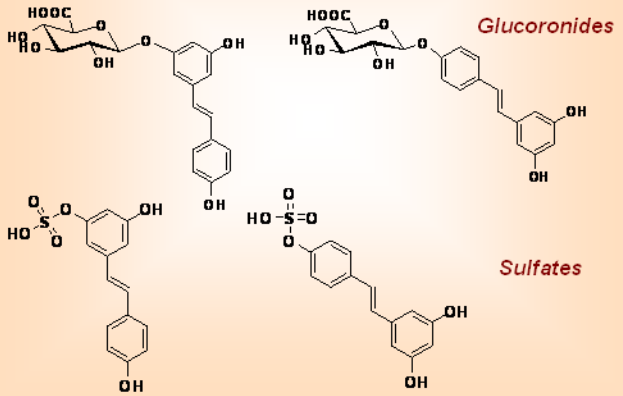
- **Anti-tumour progression:** HL-60 cells treated with resveratrol do not proliferate.



Figures A and B show the effect of resveratrol in a dose and time dependant manner on the tumour incidence and on the number of tumour cells. ○ No resveratrol; □ 1µmol or △ 5 µmol or ▽ 10 µmol or ◇ 25 µmol of resveratrol in 0.2 mL of acetone.

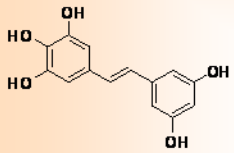
Very fast metabolism and elimination[4]

Resveratrol contained in wine is absorbed within 1h (maximum after 30 min) and excreted as metabolites (glucoronides and sulfates) between 4 and 10h.



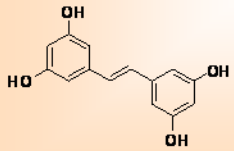
Resveratrol analogues anti COX-1/2[5]

The following molecules are the best resveratrol analogues currently synthesised against COX-1/2 activity.



IC_{50} COX-1 = 0.01027 µM

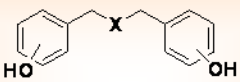
IC_{50} COX-2 = 0.00138 µM



IC_{50} COX-1 = 0.01027 µM

IC_{50} COX-2 = 0.00138 µM

Drug Design



Drug design based on the similar structures of Curcumin, Tricin and Resveratrol. Polyphenolic rings linked by a variable linker in length and functional groups.

References: [1] http://cancerresearchuk.com/ ; [2] Suh, Y.J. *Nature Reviews - Cancer*, 2003, *3*, 768-780 ; [3] Jang, M. S.; Cai, E. N.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W. W.; Fong, H. H. S.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. *Science* 1997, *275*, 218-220 ; [4] Wenzel, E.; Somoza, V. *Mol. Nutr. Food. Res.* 2005, *49*, 472-481 ; [5] Murias, M.; Handler, N.; Erker, T.; Pleban, K.; Ecker, G.; Saiko, P.; Szekeres, T.; Jager, W. *Bioorganic and Medicinal Chemistry* 2004, *12*, 5571-5578.

Chemoprevention of Cancer
Synthesis and Metabolism of Resveratrol Analogues

University of Leicester

Charles Simon*, Dr. Robert G. Britton*, Dr. Hong Cai#, Dr. Karen Brown# and Dr. Paul R. Jenkins*
*cs234@le.ac.uk, Department of Chemistry, University of Leicester, Leicester, UK
Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester, UK

A. Introduction

Diet is the second most preventable cause of cancer after smoking.[1] Population studies show that cancer has various incidences in different part of the world.[2] Studies of the diet in those areas highlighted different natural phytochemicals which have chemopreventive properties against cancer. **Resveratrol 1** is a constituent of grape skin, red wine, nuts and berries which engages multiple mechanisms constituent with the prevention of cancer.[3]

B. Chemoprevention

The use of drugs, vitamins, or other agents to try to reduce the risk or delay the development or recurrence of cancer.[4]

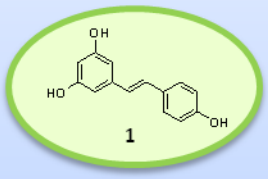
- **Prevents** cancer initiation
- **Delays** the onset of cancer
- **Slows** cancer progression
- **Reduces** the size of the tumour
- **Kills** cancer cells / tumours

C. Resveratrol: *trans*-3,5,4'-trihydroxystilbene

"French Paradox"

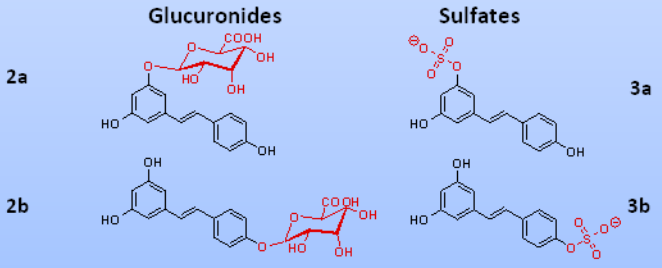
Prevention of cardiovascular diseases

Anti-Cancer
Anti-Initiation
Anti-Progression
Anti-Promotion
Pro-Apoptotic



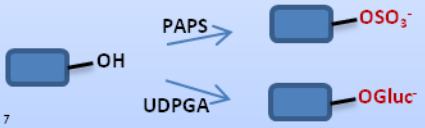
Anti-oxidant
Prevention of skin ageing
Radical and ROS scavenger

- Resveratrol *in vivo* metabolism occurs very fast : no more free resveratrol is found after 30 min.
- **Glucuronides 2a-b** and **Sulfates 3a-b** are the main metabolites found.[5]



D. Biology

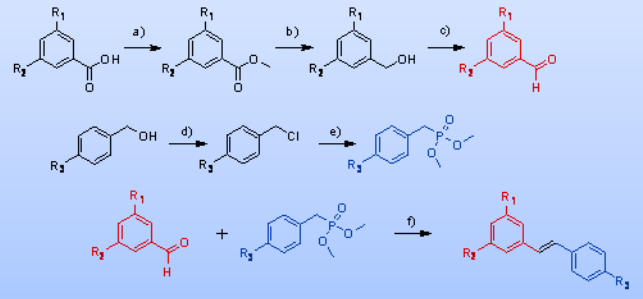
Aim
Incubating resveratrol analogues with mouse liver cytosol or microsomes with a cofactor and following the disappearance of the parent product over time by HPLC.



Glucuronidation [7]
Incubation of parent product with mouse **microsomes** and **UDPGA** as a cofactor for 0, 5, 15, 30 and 60 min.
Sulfation [7]
Incubation of parent product with mouse **liver cytosol** and **PAPS** as a cofactor for 0, 15, 30, 60 and 120 min.

E. Synthesis

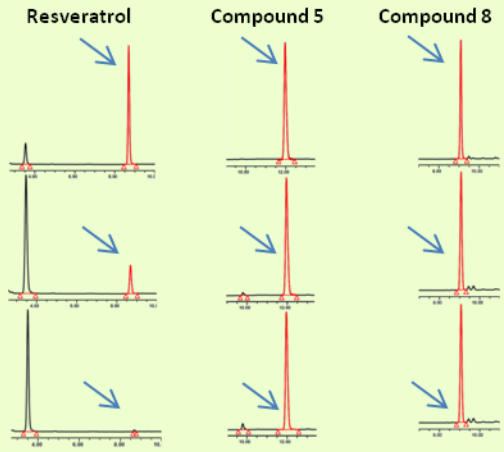
Aim: Synthesis of analogues of resveratrol with a slower metabolism via a Wittig-Horner-Emmons reaction.[6]



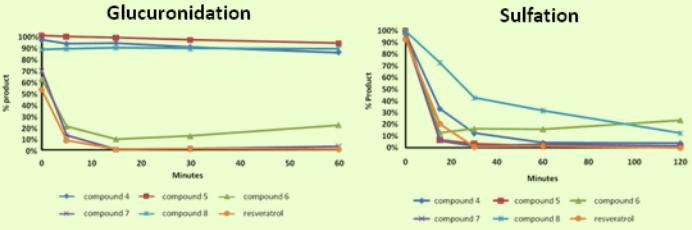
a) MeOH, H_2SO_4; b) $LiAlH_4$, dry THF ; c) PCC, dry DCM ; d) conc. HCl, Toluene ; e) $P(OMe)_3$, reflux ; f) NaOMe, dry DMF

F. Results

HPLC
Glucuronidation



Time rates



Conclusions

- Two of the analogues have a similar glucuronidation rate than resveratrol, but the "best" ones are **not metabolised** in 1h.
- All the analogues are metabolised to sulfates, but the "best" analogue is metabolised **much more slowly** in 2h.

Future work

Study of the compounds activity on cancer cell proliferation and their pro-apoptotic effect. The cancer cells will be incubated under controlled conditions and the evolution of the number of cells after 5-7 days incubation will be measured.

References

[1] Jang, M. S.; Cai, E. N.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W. W.; Fong, H. H. S.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. *Science* **1997**, *275*, 218-220; [2] Doll, R. *World Journal of Surgery* **1978**, *2*, 595-602; [3] Yu, C. W.; Shin, Y. G.; Chow, A.; Li, Y. M.; Kosmeder, J. W.; Lee, Y. S.; Hirschelman, W. H.; Pezzuto, J. M.; Mehta, R. G.; van Breemen, R. B. *Pharmaceutical Research* **2002**, *19*, 1907-1914 ; [4] Suh, Y.-J. *Nature Reviews - Cancer* **2003**, *3*, 768-780 ; [5] Wenzel, E.; Somoza, V. *Molecular Nutrition & Food Research* **2005**, *49*, 472-481 ; [6] Murias, M.; Handler, N.; Erker, T.; Pleban, K.; Ecker, G.; Saiko, P.; Szekeres, T.; Jager, W. *Bioorganic and Medicinal Chemistry* **2004**, *12*, 5571-5578; [7] Cai, H.; Boocock, D. J.; Steward, W. P.; Gescher, A. J. *Cancer Chemotherapy and Pharmacology* **2007**, *60*, 257-266.

CANCER RESEARCH UK

Chemoprevention of Cancer:
Synthesis and Biological Evaluation of New Resveratrol Analogues

Charles Simon[a,*], Dr. Robert G. Britton[a,b], Dr. Hong Cai[b], Dr. Karen Brown[b] and Dr. Paul R. Jenkins[a]
[*]cs234@le.ac.uk, kin@le.ac.uk, Department of Chemistry, [b] Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester, UK

A. Introduction

Diet is the second most preventable cause of cancer after smoking.[1] Population studies show that cancer has various incidences in different parts of the world.[2] Studies of the diet in those areas highlighted different natural phytochemicals which have chemopreventive properties against cancer. **Resveratrol** is a constituent of grape skin, red wine, nuts and berries which engages multiple mechanisms constituent with the prevention of cancer.[3]

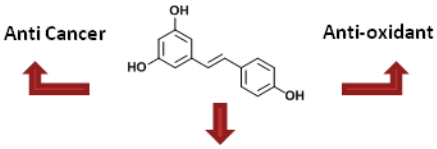
Project Aim: Synthesise a library of new analogues of Resveratrol to see whether we can stop or at least slow down the metabolism whilst keeping the activity against cancer cell proliferation.

Chemoprevention

The use of drugs, vitamins, or other agents to try to reduce the risk or delay the development or recurrence of cancer.[4]

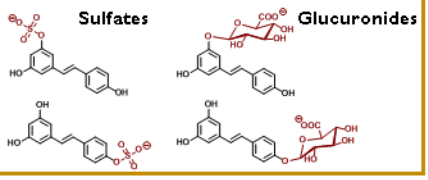
- Prevents cancer initiation
- Delays the onset of cancer
- Slows cancer progression
- Reduces the size of the tumour
- Kills cancer cells/tumours

Resveratrol

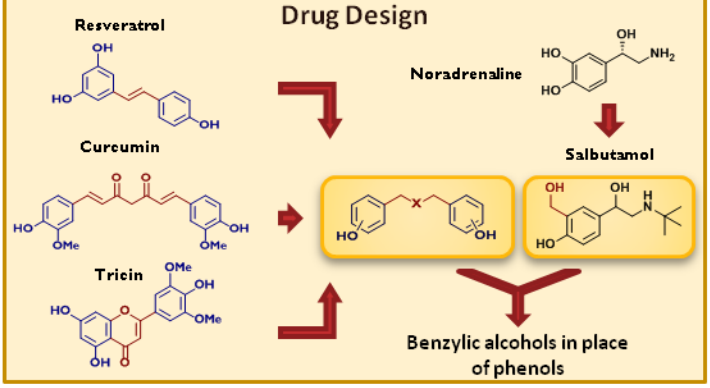


Resveratrol Metabolites

Metabolism occurs within **1h** in humans.[5]

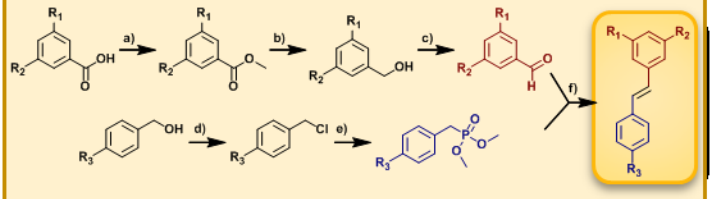


Drug Design



General Synthesis

Analogues made *via* a **Wittig-Horner-Emmons** type reaction between a phosphonate and an aldehyde.[6]



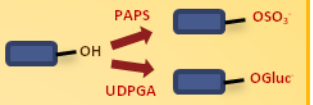
R_1,R_3 = CH_3O, OH, CH_2OH ; R_2 = H, CH_2OH, CH_3O, OH

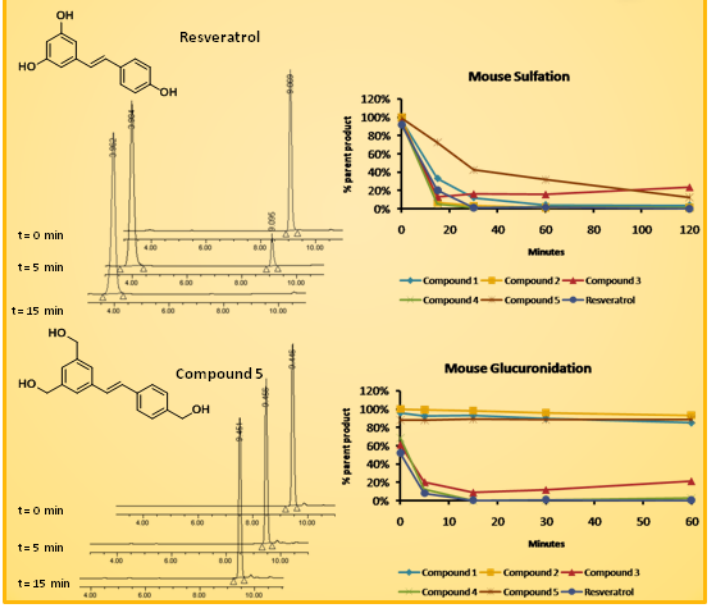
a) MeOH, H_2SO_4, 95%; b) $LiAlH_4$, dry THF, 65-95% ; c) PCC, dry DCM, 66-81% ; d) conc. HCl, Toluene, 96% ; e) $P(OMe)_3$, reflux, 65-74% ; f) NaOMe, dry DMF, 50-80%

B. Results and Discussion

Metabolism assays

Aim:
Incubating resveratrol analogues with mouse **liver cytosol or microsomes** with a cofactor and following the disappearance of the parent product over time by HPLC.[7]

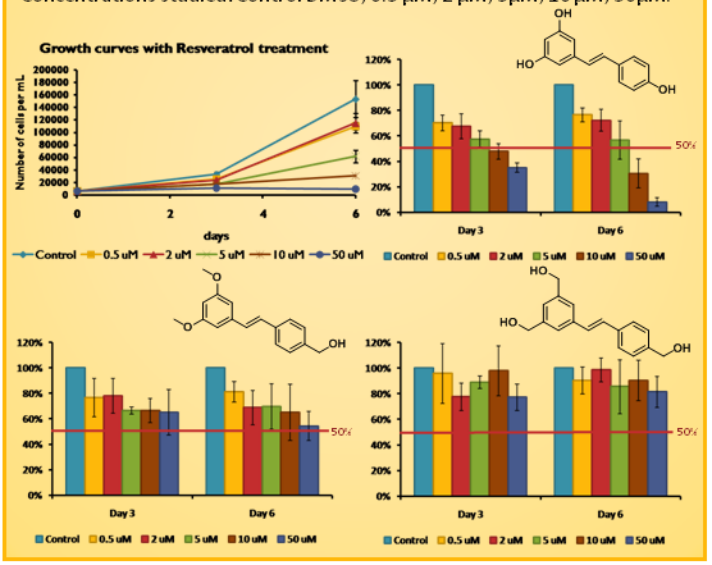




Cell proliferation assays

Aim:
Monitoring of the proliferation of **HCA-7 cells** after **72 h** and **144 h** of a **single dose** treatment.

Method:
6000 cells seeded. Treatment solutions: 2mL of media containing the analogue in 0.1% DMSO.
Concentrations studied: Control DMSO, 0.5 µM, 2 µM, 5µM, 10 µM, 50µM.



C. Conclusion and Future Work

Conclusions: Glucuronidation is prevented when there is **no free phenol** on the scaffold, and the sulfation is slowed down. But the activity is lost compared to resveratrol (IC_{50} ~ 6 µM at day 6).

Future Work: Investigate metabolism and pharmacokinetics of a lead compound in a mouse model. Assess efficacy in a mouse model of colorectal cancer.

References
[1] Jang et al. *Science* **1997**, *275*, 218-220; [2] Doll et al. *World Journal of Surgery* **1978**, *2*, 595-602; [3] Yu et al. *Pharmaceutical Research* **2002**, *19*, 1907-1914 ; [4] Surh et al. *Nature Reviews - Cancer* **2003**, *3*, 768-780 ; [5] Wenzel et al. *Molecular Nutrition & Food Research* **2005**, *49*, 472-481 ; [6] Murias et al. *Bioorganic and Medicinal Chemistry* **2004**, *12*, 5571-5578; [7] Cai et al. *Cancer Chemotherapy and Pharmacology* **2007**, *60*, 257-266.