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

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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.
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<td>AcO</td>
<td>Acetate</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon and dioxin receptor</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BaP</td>
<td>Benzo[a]pyrene</td>
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<tr>
<td>BRCA</td>
<td>Breast cancer gene</td>
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<td>Cat.</td>
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<td>Cyclin Dependant Kinase</td>
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<td>CDK Inhibitor</td>
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<td>CoA</td>
<td>4-Coumarate:coenzyme A</td>
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<td>COMT</td>
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<td>DMSO</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>ED&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Effective Dose at 50%</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>EI</td>
<td>Electronic impact</td>
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<td>ESI</td>
<td>Electrospray ionisation</td>
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<td>EWG</td>
<td>Electron withdrawing group</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
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<td>FCS</td>
<td>Foetal Calf Serum</td>
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<td>F.G.I.</td>
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<td>GF</td>
<td>Growth Factor</td>
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<td>HOBr</td>
<td>Hydroxybenzotriazole</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<td>IAP</td>
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<td>Inhibitory Concentration at 50%</td>
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<td>Inhibitor of NF-κB</td>
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<tr>
<td>IR</td>
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<td>INK 4</td>
<td>Inhibitor of the cyclin dependant Kinase 4</td>
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<tr>
<td>i.v.</td>
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<td>KITC</td>
<td>N-Hydroxy-N'- (3,4,5-trimethoxyphenyl)-3,4,5-trimethoxybenzamidine</td>
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<td>LAH</td>
<td>Lithium Aluminium Hydride</td>
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<td>LC</td>
<td>Liquid Chromatography</td>
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<td>Definition</td>
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<td>LDL</td>
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<td>LRP</td>
<td>LDL Related protein</td>
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<tr>
<td>Mad</td>
<td>Mitotic arrest deficient</td>
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<td>MAO</td>
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<td>Matrix Metalloproteinase</td>
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<td>mp</td>
<td>Melting point</td>
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<td>Multiple Reaction Monitoring</td>
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<td>Microwave</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate-oxidase</td>
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<td>NF-κB</td>
<td>Nuclear Factor κ-B</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<td>NSAID</td>
<td>Non steroidal anti-inflammatory drug</td>
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<td>PAPS</td>
<td>3’-Phosphoadenosine-5’-phosphosulfate</td>
</tr>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PCC</td>
<td>Pyridinium chlorochromate</td>
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<td>PCD</td>
<td>Programmed Cell Death</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
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<td>PG</td>
<td>Prostaglandin / Protecting group</td>
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<td>Definition</td>
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<td>PGE</td>
<td>Prostaglandin E</td>
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<td>pRb</td>
<td>Protein Retinoblastoma</td>
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<td>PS</td>
<td>Phosphatidyl serine</td>
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<td>Py</td>
<td>Pyridine</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>Standard deviation</td>
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<td>SN</td>
<td>Nucleophilic Substitution</td>
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<td>Sulfotransferase</td>
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<td>Triflate</td>
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<td>Tetrahydrofuran</td>
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<td>THP</td>
<td>Tetrahydropyranyl</td>
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<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of MMP</td>
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<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<tr>
<td>TMS</td>
<td>Tetramethylsilyle</td>
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</table>
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 haemapoetic 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 lifestyle 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).\textsuperscript{3,4}

1.2. The hallmarks of cancer

Weinberg \textit{et al.}\textsuperscript{5} defined six hallmarks of cancer which are the phenotype of the disease (Figure 1). Cancer can be described by these six main characteristics.

- \textit{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.

- \textit{Insensitivity to anti-growth signals}: the cells evade the quiescent state (G\textsubscript{0}) and continue to grow without differentiation, even if receiving signals telling them not to grow.

- \textit{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.\textsuperscript{6,7}

- \textit{Limitless replication potential}: tumour cells do not stop replicating compared to normal cells, which stop and eventually die after a certain number of replications.

- \textit{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, angiopoietin) and negative (angiostatin, MMP inhibitors) regulators of angiogenesis.  

- **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](image)

Recently, Luo *et al.* 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. (Figure 2)
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![Figure 2: Hallmarks of cancer by Luo et al.]

- **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.\(^\text{12}\)

- **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\(^*\) 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 environments.

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* Aneuploidy: an abnormal number of chromosomes in the cell.


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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). Oncogenes are genes which encode for a protein whose increased activity or expression can promote oncogenesis such as Src, Myc and Ras. 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). The activation of an oncogene can be due to:

- **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.
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 3: Roles of oncogene and tumour suppressor genes in cancer**

**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.
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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.
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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. 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\textsuperscript{29} or analogues (oxaliplatin)\textsuperscript{30} or DNA replication enzyme inhibitors such as doxorubicin\textsuperscript{31} or teniposide.\textsuperscript{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.

\begin{center}
\includegraphics[width=\textwidth]{chemcompounds.png}
\end{center}

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.\textsuperscript{33,34}

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

\begin{center}
\begin{tabular}{ccc}
\textbf{Methotrexate} & \textbf{Paclitaxel} & \textbf{Vinblastine} \\
\includegraphics[width=0.3\textwidth]{methotrexate.png} & \includegraphics[width=0.3\textwidth]{paclitaxel.png} & \includegraphics[width=0.3\textwidth]{vinblastine.png}
\end{tabular}
\end{center}
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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.

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\textsuperscript{35}) have also been developed and research is underway on finding new specific treatments for a wider range of cancers.\textsuperscript{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. Two other broad spectrum MMP inhibitors, marimastat and cipemastat, gave good results during the pre-clinical studies but are not used as drugs because of their toxicity.

Angiogenesis is naturally inhibited by angiopoietins, and angiostatins (cf. 1.2.). It can also be inhibited by biological analogues of human anti-bodies (benvacizumab), interferons (INF-α Human Type I interferon), platelet factors (Platelet factor-4) and by synthetic small molecules like Thalidomide or a less toxic analogue lenalidomide or by 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). It can also be induced by inhibition of pro-apoptotic enzymes or by modifying the balance of pro-/anti- apoptotic enzymes. For
example, Bortezomib\textsuperscript{46} is used to block proteosome 26S avoiding the degradation of pro-apoptotic enzymes.

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\begin{tabular}{c}
\includegraphics[width=\textwidth]{chem.png}
\end{tabular}
\end{center}

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.
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2. Chemoprevention of cancer

2.1. Definition

Chemoprevention of cancer can be defined as the use of natural products such as vitamins or phytochemicals, or synthetic drugs to prevent, retard or stop the process of carcinogenesis at an early stage. 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:

- **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. 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

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b Phytochemical: naturally occurring compound that is considered beneficial to human health.
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differences such as air and water quality, diet and lifestyle (Table 1). 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). It has been suggested that diet could be responsible of around 35% of cancer mortality, which is the highest cause of the disease after smoking. Epidemiological data and clinical trials also suggest that diet can be a source of compounds that protect against cancer.

<table>
<thead>
<tr>
<th>Site of origin of cancer</th>
<th>High-Incidence Population</th>
<th>Low-Incidence Population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Location</td>
<td>Incidence</td>
</tr>
<tr>
<td>Lung</td>
<td>USA (New-Orleans)</td>
<td>110</td>
</tr>
<tr>
<td>Breast</td>
<td>Hawaii</td>
<td>94</td>
</tr>
<tr>
<td>Prostate</td>
<td>USA (Atlanta)</td>
<td>91</td>
</tr>
<tr>
<td>Stomach</td>
<td>Japan (Nagasaki)</td>
<td>82</td>
</tr>
<tr>
<td>Liver</td>
<td>China (Shanghai)</td>
<td>34</td>
</tr>
<tr>
<td>Colon</td>
<td>USA (Connecticut)</td>
<td>34</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Australia (Queensland)</td>
<td>31</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>France (Calvados)</td>
<td>30</td>
</tr>
<tr>
<td>Bladder</td>
<td>Switzerland (Basel)</td>
<td>28</td>
</tr>
<tr>
<td>Uterus</td>
<td>USA (San Francisco)</td>
<td>26</td>
</tr>
<tr>
<td>Ovary</td>
<td>New-Zealand (Polynesia)</td>
<td>26</td>
</tr>
<tr>
<td>Rectum</td>
<td>Israel (US and European)</td>
<td>23</td>
</tr>
<tr>
<td>Pancreas</td>
<td>USA (Los Angeles)</td>
<td>16</td>
</tr>
<tr>
<td>Kidney</td>
<td>Canada (NWT and Yukon)</td>
<td>15</td>
</tr>
</tbody>
</table>

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. 

28
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. 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
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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.\textsuperscript{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.\textsuperscript{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
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natural phytoalexin found in red grape skin (Vitis vinifera), 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. 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.

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*Phytoalexin: antibiotic produced by plants that are under attack.*
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-hydroxystilbene $1^a$ is a natural occurring phytochemical of the family of the phytoalexins produced by spermatophytes$^{d}$ such as grapes ($vitis$ 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 $2^a$.

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

$^{a}$Spermatophyte: Plant which produces seeds
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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).\(^{70,71}\)

<table>
<thead>
<tr>
<th>Region of origin of the grapes</th>
<th>Europe</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>France</td>
<td>0.3–7.1</td>
<td>0.09-3</td>
</tr>
<tr>
<td>Italy</td>
<td>0.05–0.8</td>
<td>Up to 42</td>
</tr>
<tr>
<td>Spain</td>
<td>2.5–13.8(^{a})</td>
<td>~4.4(^{b})</td>
</tr>
<tr>
<td>Portugal</td>
<td>2.8</td>
<td>~2.5(^{b})</td>
</tr>
<tr>
<td>Central Europe</td>
<td>3.1</td>
<td>~1.8(^{a})</td>
</tr>
<tr>
<td>Switzerland</td>
<td>6.9</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) 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.\(^{69}\)

Besides its antibiotic properties in plants, resveratrol has shown several interesting biological properties, which have been reviewed at different occasions and recently by Brown \textit{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
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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). 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. 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 and has been shown to increase the life-span of some species including fish and flies, varying from species to species.

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

3.2. Anti-initiation activity

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) 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. 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. 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: PGH₂ 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% (ED₅₀) of 15 µM and the hydroperoxidase activity with an
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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.
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U937 cells. 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 G1/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/G2 transition arrest is due to an increase of p53 and p21 levels (subsequently to p53 up-regulation) as shown by Hsieh et al., 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. 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.\textsuperscript{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.\textsuperscript{77} Resveratrol has activity against NF-κB by diminishing the nuclear level of NF-κB and decreasing IκB (inhibitor of NF-κB) proteosomal degradation.\textsuperscript{80} It induces apoptosis in human breast cancer MCF-7 cells\textsuperscript{85} and in both rat and human pancreatic carcinoma cell lines.\textsuperscript{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
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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. The main metabolites found come from phase II metabolism. Two glucuronide forms exists, the resveratrol-3-\(O\)-\(\beta\)-glucuronide 2a and the resveratrol-4\(^{\prime}\)-\(O\)-\(\beta\)-glucuronide 2b. There are also two sulfate metabolites of resveratrol, resveratrol-3-\(O\)-sulfate 2c and resveratrol-4\(^{\prime}\)-\(O\)-sulfate 2d. Very recently our laboratory identified a glucuronide-sulfate metabolite and a disulfate in human colorectal tissues.

No phase I metabolites resulting from oxidation, reduction or hydrolysis are generally found in the different systems studied. Walle et al. 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. MS detection of the \([M - 1]\) 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.
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A very recent study of Burkon\textsuperscript{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 \textit{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-\(\alpha\)-D-glucuronic acid methyl ester following Wenkel's \textit{et al.}\textsuperscript{95} method.

\[\text{trans-resveratrol-2-C-\(\beta\)/5-\(O\)-\(\beta\)-diglucuronide} \]
\[\text{trans-resveratrol-2-C-\(\beta\)/4'-\(O\)-\(\beta\)-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.\textsuperscript{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 \textit{et al.}\textsuperscript{91} showed that resveratrol-3-\(O\)-sulfate \textit{2e} 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 \textit{2a} and \textit{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.](image-url)

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. Volunteers took orally one dose of resveratrol at
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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).  

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 \(\mu\)M) and peak plasma concentration (1135-4294 ng/mL or 3.7-14 \(\mu\)M) nearly 3-fold the two glucuronide metabolites concentrations.  

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.
Figure 10: UV-HPLC chromatograms of human plasma samples with or without resveratrol. Taken from Boocock et al.\textsuperscript{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.\textsuperscript{97}

Vitrac et al.\textsuperscript{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 \(\mu\)M) compared to 21.0 ± 0.46 \(\mu\)M for resveratrol as well as a slightly better inhibition of COX-1 (IC\(_{50}\) of 3.60 ± 0.8 \(\mu\)M vs. 6.65 ± 2.5 \(\mu\)M) 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, COX-1 and COX-2, NF-κB, COX-1 and COX-2, NF-κB, and tubulins, as well as enhancing the cytotoxicity and anti-proliferative effects on various cancer cell lines. Different approaches have been taken, either by starting with resveratrol itself and its natural analogues, like combrestatin A and piceatannol, or by developing new strategies to synthesise new series of analogues. 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 and methoxy groups on the different rings (Scheme 4).
Scheme 4: Wittig-Horner-Emmons reaction by Murias et al.¹⁰³

Other groups used either a Wittig/Wittig-Horner-Emmons reaction or a Heck coupling¹¹²,¹²¹ to add different rings such as pyridines, pyrroles, thiophenes (Scheme 5)¹⁰⁰ or benzene rings with different substituents including fluorine,¹¹² nitro groups or anilines¹¹⁴ by “normal” or by solid-phase synthesis (Scheme 6).¹⁰⁷,¹⁰⁸

Scheme 5: Wittig-Horner-Emmons reaction by Kim et al.¹⁰⁰
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**Scheme 6:** Solid-phase synthesis leading to 54 analogues by Kal *et al.*

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

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

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).
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\[
\begin{align*}
\text{R}_1, \text{R}_2, \text{R}_3 &= \text{OMe, OiPr, OH, H} \\
\text{X} &= -\text{m, p-H, OMe, OiPr, OH}
\end{align*}
\]

a) TEA, Ac₂O, reflux, 15h; b) Deprotection: BCl₃, DCM, -78°C; c) CuCr₂O – BaCr₂O/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

\[
\begin{align*}
\text{Scheme 9: Ramberg-Bäcklung reaction by Robinson et al.}^{123}
\end{align*}
\]

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).
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.
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Increased levels of COX-2 and prostaglandin E-2 have been implicated in the development of cancer.\textsuperscript{124} Murias \textit{et al.}\textsuperscript{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 hexahydroxy analogue 1c have better activity against COX-2 ($IC_{50}$ of 0.0113 $\mu$M and 0.00104 $\mu$M respectively) than resveratrol ($IC_{50}$ 0.996 $\mu$M) 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.

The absence of activity of the methoxy analogues such as \textit{trans}-tetramethoxy-3,4,4',5-stilbene DMU-212 1d against COX-2 has also been shown by Sale \textit{et al.}\textsuperscript{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 $\mu$M.
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Zykova et al.\textsuperscript{102} compared resveratrol with pentahydroxyresveratrol 1e and trimethoxyresveratrol 1f in different binding assay as well as their effects on COX-2\textsuperscript{+/+} and COX-2\textsuperscript{−/−} 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.\textsuperscript{107} who synthesised a combinatorial library of analogues of 1a. Their most active analogues (with similar IC\textsubscript{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.

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.\textsuperscript{55} Some of the enzymes studied with resveratrol and its analogues are CYP 1A1, 1A2, 1B1 and 2E1.
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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. 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. They synthesised a library of analogues of resveratrol and raphontigenin, 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. Heynekamp et al. 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. 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
analogs of Combrestatin A4, a very good inhibitor of this biological process. Simoni et al.\textsuperscript{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.\textsuperscript{111} synthesised $\beta$-$(E)$-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\textsuperscript{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 ($IC_{50} \geq 10 \text{ nM}$) and
the mode of action is not clear from the results.

Surprisingly, a study by Sale et al.\textsuperscript{104} showed that DMU-212 \textbf{1d} (p.54) was less active than resveratrol at inhibiting intestinal adenomas formation in Apc\textsuperscript{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 \(\mu\text{M}\). Zykova\textsuperscript{102} illustrated some similar results. The team evaluated the effect on the growth of colon cancer cell lines of a pentahydroxyresveratrol \textbf{1e} and of trimethoxyresveratrol \textbf{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 \textbf{1f} did not have any activity. In the same study they showed that resveratrol activity and of its analogue \textbf{1e} may be linked to the inhibition of COX-2.

In contrast, the methoxylated analogues of \textit{cis}-resveratrol and of combrestatins seem better anti-proliferative agents than \textit{trans}-resveratrol. Simoni found that \textit{cis}-trimethoxyresveratrol was a 33-fold better inhibitor of the proliferation of leukaemia cells HL-60.\textsuperscript{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\textsuperscript{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 11 which is 5-fold better on HT-29 (IC$_{50} = 21 \pm 1.3$ 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 dependent 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.\textsuperscript{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 nasopharyngeal carcinoma CNE-1 and CNE-2 cell lines and four analogues 1m-p stood out with better anti-proliferative properties ($IC_{50} \sim 6-8 \, \mu M$ on CNE-1 and $3-6 \, \mu M$ on CNE-2) than resveratrol ($IC_{50} = 68 \, \mu M$ on CNE-1 and $53 \, \mu M$ 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.
The methoxy analogues of resveratrol, especially trimethoxy-resveratrol 1f, tend to have a good pro-apoptotic effect. Minutolo et al.\textsuperscript{116} found out that 1f is a 20-fold better proliferation inhibitor (IC\textsubscript{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\textsuperscript{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\textsubscript{0}/G\textsubscript{1} phase (apoptotic) pancreatic cancer cells (AsPC-1 and BxPC-3).
In conclusion, a number of analogues, both natural and synthetic, have been shown to inhibit cancer cells \textit{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 \textbf{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.
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CHAPTER 2 – SYNTHESIS OF RESVERATROL ANALOGUES

6. Design of the library

As described previously (cf. Chapter 1) resveratrol 1a, curcumin 3\textsuperscript{127-130} and tricin 4\textsuperscript{67,131-133} are three naturally occurring polyphenols which show very good anticancer activity \textit{in vitro} but whose activity drops dramatically in \textit{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.

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.

\[ \text{General Structure} \]

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 \( \beta_2 \)-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.

\[ \text{Salbutamol 5a} \quad \text{Noradrenaline 6} \quad \text{Salbutamol sulfate 5b} \]
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.\textsuperscript{103,136} We decided to follow Murias \textit{et al.}\textsuperscript{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).\textsuperscript{137,138}

\[ \text{Scheme 13: Arbuzov reaction}\textsuperscript{139} \]

The Wittig-Horner\textsuperscript{137} type reaction occurs between a phosphonate anion and an aldehyde. The phosphonates 8 can be obtained by a Michaelis-Arbuzov\textsuperscript{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).

\[ X= \text{Leaving group (Cl, I, etc.)} \]
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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.\textsuperscript{103,138,140} It is possible to make a phosphonate by a Michaelis-Arbuzov reaction between a halogenated compound and a trialkylphosphite (Scheme 13).\textsuperscript{139} Table 3 summarises the phosphonates 8a-f which have been synthesised.

![Scheme 13: Wittig-Horner-Emmons reaction]

<table>
<thead>
<tr>
<th>Phosphonate</th>
<th>R&lt;sub&gt;3&lt;/sub&gt;</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>8a</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;OTBDMS</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>8b</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;OTHP</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>8c</td>
<td>OTBDMS</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>8d</td>
<td>OTHP</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>8e</td>
<td>OMe</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>8f</td>
<td>OH</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

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

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.

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
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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.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Base</th>
<th>Catalyst</th>
<th>Solvent</th>
<th>Time / T°C</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pyridine$^{\text{III}}$ (2mL)</td>
<td>-</td>
<td>-</td>
<td>1h30/0°C</td>
<td>SM consumed no product recovered</td>
</tr>
<tr>
<td>2</td>
<td>KOH pellets</td>
<td>-</td>
<td>-</td>
<td>18h/rt.</td>
<td>No reaction</td>
</tr>
<tr>
<td>3</td>
<td>Et$_3$N$^{\text{I}}$(2 eq.)</td>
<td>DMAP(0.02 eq.)</td>
<td>THF</td>
<td>2h/0-5°C then 18h/rt.</td>
<td>Chlorinated product</td>
</tr>
<tr>
<td>4</td>
<td>NaH$^{\text{I}}$(1.5 eq.)</td>
<td>-</td>
<td>THF</td>
<td>18h/rt.</td>
<td>No reaction</td>
</tr>
</tbody>
</table>

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
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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).

![Chemical structure diagram]

<table>
<thead>
<tr>
<th>Entry</th>
<th>TBDMSCl</th>
<th>Base</th>
<th>Solvent</th>
<th>Time / °C</th>
<th>Yield after purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 eq.</td>
<td>NaH(1.0 eq.)</td>
<td>THF</td>
<td>18h / rt.</td>
<td>16%</td>
</tr>
<tr>
<td>2</td>
<td>1 eq.</td>
<td>Et₃N(1.4 eq.)/ DMAP(0.02 eq.)</td>
<td>THF</td>
<td>18h / rt.</td>
<td>54%</td>
</tr>
<tr>
<td>3</td>
<td>1.1 eq.</td>
<td>Imidazole(0.75 eq.)</td>
<td>THF</td>
<td>18h / rt.</td>
<td>19%</td>
</tr>
</tbody>
</table>

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

The reaction in the presence of NaH as the base according to McDougal¹⁴⁴ 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.\textsuperscript{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).

<table>
<thead>
<tr>
<th>Entry</th>
<th>Solvent</th>
<th>Time/T°C</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Toluene</td>
<td>48h/70°C</td>
<td>Decomposition</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>MW,300W,110°C,45 min</td>
<td>16% yield</td>
</tr>
</tbody>
</table>

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

![Scheme 16: Proposed synthesis of phosphonate 8a]

8.1.2.1. Use of acetate as activation group

According to F. Tamaddon et al.\textsuperscript{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

![Scheme 18: Tosylation and phosphorylation sequence](image)

Lai et al.\textsuperscript{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\textsuperscript{−} (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](image)
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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.\textsuperscript{148} converted a benzylic alcohol into an iodide with I\textsubscript{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.\textsuperscript{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 TBDMS\textsubscript{Cl} 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).
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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).
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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\(^\text{103}\) 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.,\(^\text{150}\) 2-(4-(chloromethyl)benzyl)oxy)tetrahydro-2H-pyran 14b was obtained in 74% yield. Reacting 14b with trimethyl phosphite under microwave activation did not give the desired phosphonate 8b.

To facilitate the Arbuzov reaction, we substituted the chloride with an iodide according to published work\(^\text{151}\). Chloride 14b was reacted with sodium iodide in dry acetone to give the iodinated analogue 14c in 79% yield. (\textit{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

\[
\begin{align*}
\text{PG} &= \text{THP, TBDMS} \\
8c-d
\end{align*}
\]

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\textsuperscript{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.\textsuperscript{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
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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](image)

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).

<table>
<thead>
<tr>
<th>Aldehyde</th>
<th>R₁</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>9a</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>9b</td>
<td>H</td>
<td>OMe</td>
</tr>
<tr>
<td>9c</td>
<td>OMe</td>
<td>OMe</td>
</tr>
<tr>
<td>9d</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>9e</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>9f</td>
<td>CH₃OH</td>
<td>CH₃OH</td>
</tr>
<tr>
<td>9g</td>
<td>CH₃OTHP</td>
<td>CH₃OTHP</td>
</tr>
<tr>
<td>9h</td>
<td>OTHP</td>
<td>H</td>
</tr>
<tr>
<td>9i</td>
<td>OTHP</td>
<td>OTHP</td>
</tr>
</tbody>
</table>

Table 7: Aldehydes needed

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

Li et al.\textsuperscript{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.\textsuperscript{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).\textsuperscript{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-
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1,3,5-tricarboxylic acid was studied.

\[
\begin{align*}
\text{O} & \quad \text{O} & \quad \text{O} \\
\text{O} & \quad \text{H}_2/\text{Pd, MeOH, 1 atm, 47\%} & \quad \text{b) LAH, THF, 0\degree C \rightarrow r.t., 27\%} \\
\end{align*}
\]

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-pyran (THP) was chosen as the protecting group as with phosphonate 8b.

\[
\begin{align*}
\text{THPO} & \quad \text{F.G.I} & \quad \text{THPO} & \quad \text{F.G.I} \\
\text{O} & \quad \text{OTHP} & \quad \text{OH} & \quad \text{OH} \\
\text{9g} & \quad \text{12} & \quad \text{13a} \\
\end{align*}
\]

Scheme 25: Retrosynthetic analysis of 9g
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9.2.2. Synthesis of 3,5-bis((tetrahydro-2H-pyran-2-yl oxy)methyl)benzaldehyde 9g

3,5-Bis-((tetrahydro-2H-pyran-2-yl oxy)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.\textsuperscript{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)\textsuperscript{158-160} or manganese dioxide (MnO\textsubscript{2})\textsuperscript{138,161-163} A first attempt of oxidation to the aldehyde was realised following Nordlander’s et al. method\textsuperscript{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\textsuperscript{158} and the desired aldehyde was recovered with a good yield of 66% after 2h at r.t. and chromatographic purification.
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-
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hydroxybenzaldehyde 9d following Severi et al.\textsuperscript{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.\textsuperscript{164,165} Following Pyun et al. method,\textsuperscript{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.\textsuperscript{166,167} Aldehyde 9i was obtained by a subsequent oxidation of the alcohol 12b with PCC in dichloromethane\textsuperscript{158} in 81% yield (Scheme 27).
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Scheme 27: Synthesis of aldehyde 9i

a) DHP, PPTS, DCM, r.t., 92% ; b) LAH, dry THF, 0°C → r.t., 79% ; c) PCC, DCM, r.t., 81%
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 \(\alpha\)-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 \(\text{trans}\) 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 \(\text{trans}\)-double bond and the water soluble dialkyl phosphate salt 8j as a side product (Scheme 28).

![Scheme 28: Wittig-Horner-Emmons reaction](image-url)
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Synthesis of the THP-protected final products 21a-h were performed following work done on resveratrol analogues by Murias et al. using sodium methoxide or potassium \textit{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. 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.

![Reaction Scheme](image)

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).
### 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 $^3J_{trans} \sim 16-17$ Hz (Figure 13-Figure 15).

*Cis*-resveratrol has a coupling constant $^3J_{cis} \sim 12.5$ 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.
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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
Chapter 2 – Synthesis of resveratrol analogues

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. BBr₃ in dichloromethane is one of the most used reagents for methyl ether deprotection on aromatic methyl ethers¹⁷⁰-¹⁷² or primary alcohols.¹⁷³,¹⁷⁴ BCl₃ in dichloromethane has the same properties as BBr₃ 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 AlI₃ as a good deprotecting reagent¹⁷⁹ 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 resveratrol¹⁰³ contain a BBr₃ 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 BBr₃. 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.
Chapter 2 – Synthesis of resveratrol analogues

THP deprotection was achieved with pyridinium para-toluene sulfonate (PPTS) according to published work. 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 BBr₃ was tried on the resulting compounds 7a and 7b, under published conditions 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.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>Final compound</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21a</td>
<td>H</td>
<td>H</td>
<td>7h</td>
<td>59</td>
</tr>
<tr>
<td>2</td>
<td>21b</td>
<td>OMe</td>
<td>H</td>
<td>7b</td>
<td>89</td>
</tr>
<tr>
<td>3</td>
<td>21c</td>
<td>OMe</td>
<td>OMe</td>
<td>7c</td>
<td>44</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Entry</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>Stilbene-OH</th>
<th>%yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H</td>
<td>OMe</td>
<td>CH₂OH</td>
<td>7a</td>
<td>46</td>
</tr>
<tr>
<td>2</td>
<td>OMe</td>
<td>OMe</td>
<td>CH₂OH</td>
<td>7b</td>
<td>89</td>
</tr>
<tr>
<td>3</td>
<td>H</td>
<td>OH</td>
<td>CH₂OH</td>
<td>7c</td>
<td>52</td>
</tr>
<tr>
<td>4</td>
<td>OH</td>
<td>OH</td>
<td>CH₂OH</td>
<td>7d</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>CH₂OH</td>
<td>CH₂OH</td>
<td>CH₂OH</td>
<td>7e</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>CH₂OH</td>
<td>CH₂OH</td>
<td>OH</td>
<td>7f</td>
<td>67</td>
</tr>
<tr>
<td>7</td>
<td>CH₂OH</td>
<td>CH₂OH</td>
<td>OMe</td>
<td>7g</td>
<td>39</td>
</tr>
</tbody>
</table>

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 \textit{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 \textit{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.

![Chemical Structure](image)

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.\textsuperscript{91,96,97} The sulfation occurs by metabolic transformation of the alcoholic residue with cytosolic sulfotransferases SULTs.\textsuperscript{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,\textsuperscript{185,186} chlorosulfonic acid\textsuperscript{187-189} or sulphuric acid.
Chapter 2 – Synthesis of resveratrol analogues

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](image)

<table>
<thead>
<tr>
<th>Analogue</th>
<th>(R_1)</th>
<th>(R_2)</th>
<th>Equiv. of SO(_3)-DMF</th>
<th>Equiv. of Na(_2)CO(_3)</th>
<th>Time of reaction (h)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>7a</td>
<td>H</td>
<td>CH(_3)O</td>
<td>2.1</td>
<td>1.1</td>
<td>4</td>
<td>Mass seen in MS</td>
</tr>
<tr>
<td>7b</td>
<td>CH(_3)O</td>
<td>CH(_3)O</td>
<td>1.1</td>
<td>2.7</td>
<td>4</td>
<td>Mass seen in MS</td>
</tr>
</tbody>
</table>

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
Chapter 2 – Synthesis of resveratrol analogues

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

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

Figure 17: LC/MS of crude sulfation of analogue 7\text{b}. (A) Time of flight spectra of crude mixture after ESI. (B) Mass spectrograph of peak at 2.01 min corresponding to the mass of 22\text{b}.
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, Et₂O and H₂O) 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 Yu⁹¹, Hoshino⁹⁹ 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 ¹H 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-H = 5.6 Hz corresponding to the benzylic alcohol (Figure 18). In the case of the sulfation of 7a leading to 22a, the crude ¹H-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.
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.\textsuperscript{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.

![Diagram of the reaction](image)

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equivalents of 7c</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Equivalents of base</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Equivalents of DMF-SO$_3$</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Reaction solvent</td>
<td>Dry THF</td>
<td>Dry THF</td>
</tr>
<tr>
<td>Reaction temperature</td>
<td>R.T.</td>
<td>80°C for 1 hour</td>
</tr>
<tr>
<td>Reaction duration</td>
<td>o/n</td>
<td>65 hours</td>
</tr>
</tbody>
</table>

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).
$^1$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.
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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.\textsuperscript{191} Chemical addition of sugar moieties to an alcohol is quite well described and has been reviewed by Stachulski.\textsuperscript{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\textsubscript{2}CO\textsubscript{3}, Ag\textsubscript{2}O, 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\textsubscript{3}.OEt\textsubscript{2}\textsuperscript{193} or TMSOTf.\textsuperscript{194}

![Scheme 31: Synthesis of 1α-bromosugar 23 and of trichloroacetimidate-sugar 25](image)

a) NaOMe, MeOH then Ac\textsubscript{2}O, Pyridine; b) HBr-AcOH; c) n-Bu\textsubscript{3}SnOMe; d) Ag\textsubscript{2}CO\textsubscript{3}, H\textsubscript{2}O; e) Cl\textsubscript{3}CCN, base

Before doing the coupling reaction on the final analogues 7a-h, we decided to investigate different reaction conditions following published methods and techniques.
Chapter 2 – Synthesis of resveratrol analogues

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](image)

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>Sugar</th>
<th>Base</th>
<th>Coupling Agent</th>
<th>Solvent</th>
<th>T(°C)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CH₃</td>
<td>1α-bromo 23</td>
<td>(CH₃)₂N</td>
<td>Ag₂O</td>
<td>MeCN</td>
<td>rt.</td>
<td>Acetate</td>
</tr>
<tr>
<td>2</td>
<td>CH₃</td>
<td>ClCCNH₂₅</td>
<td>-</td>
<td>BF₃·OEt₂</td>
<td>DCM</td>
<td>-15</td>
<td>Complex mixture</td>
</tr>
<tr>
<td>3</td>
<td>CH₃</td>
<td>1α-bromo 23</td>
<td>TMU</td>
<td>AgOTf</td>
<td>DCM</td>
<td>-78 → -20</td>
<td>Complex mixture</td>
</tr>
<tr>
<td>4</td>
<td>H</td>
<td>1α-bromo 23</td>
<td>(CH₃)₂N</td>
<td>Ag₂O</td>
<td>MeCN</td>
<td>rt.</td>
<td>No more SM but complex mixture</td>
</tr>
</tbody>
</table>

Table 13: Glucuronidation conditions

Direct glucuronidation of analogue 7b was attempted by a Koenigs-Knorr method with the 1α-bromosugar, Ag₂CO₃, 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
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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,\textsuperscript{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.\textsuperscript{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 \textit{in vitro} systems compared to resveratrol.

![Figure 22: Schematic representation of \textit{in vitro} metabolism of resveratrol and of the analogues](image-url)
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).

*In vitro* metabolism of the synthetic resveratrol analogues was carried out according to the method developed in our laboratory by H. Cai *et al.* 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=0 min. 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=0 min. 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.
Chapter 3 – Pharmacological Studies

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 tribenzyl 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.

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

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 ($1.6 \pm 0.4\%$) 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 7e 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.

**Sulfation in mouse liver cytosol**

![Graph A](image1)

![Graph B](image2)

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-monophenol 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.

**Sulfation in human liver cytosol**

*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.*

**Comparison of sulfation in mouse and human liver cytosol**

*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.\textsuperscript{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 ($V_{\text{max}}$ 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 33-38.

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.
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 whereas 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.

<table>
<thead>
<tr>
<th>Method #</th>
<th>Method 1</th>
<th>Method 2</th>
<th>Method 3</th>
<th>Method 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elution</td>
<td>Time</td>
<td>%A</td>
<td>Time</td>
<td>%A</td>
</tr>
<tr>
<td>0</td>
<td>55</td>
<td>0</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>10</td>
<td>12</td>
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</tr>
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<td>18</td>
<td>10</td>
<td>18</td>
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<td>20</td>
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<td>30</td>
<td>55</td>
<td>30</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Flow rate (μL/min)</td>
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<td>150</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Compound analysed</td>
<td>1a, 7a,c</td>
<td>7b</td>
<td>7e</td>
<td>7d</td>
</tr>
</tbody>
</table>

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
### 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.\textsuperscript{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).

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

\textsuperscript{a} Retention times in the on-line LC/MS/MS system. See Experimental part for details. \textsuperscript{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](image)

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
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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).

<table>
<thead>
<tr>
<th>Compound</th>
<th>7a</th>
<th>7b</th>
<th>7c</th>
<th>7d</th>
<th>7e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation time (min)</td>
<td>30</td>
<td>30</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

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.

![Comparison of synthetic 22a with metabolic sulfate](image)

*Figure 47: Comparison of synthetic 22a with metabolic sulfate*
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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 in 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. 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/cytostatic 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.

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$c$ Any agent or process that kills cells
$f$ Inhibiting or suppressing cellular growth and multiplication
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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. 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.

![Graph showing effect of resveratrol on cell proliferation](image)

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≤0.01)

![Graph showing percentage decrease in cell number](image)
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.](image)

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-benzylc analogue 7b on HCA-7 cells

The dimethoxy-benzylc 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 $30 \pm 18\%$. However, the cell proliferation is significantly inhibited by $50 \pm 11\%$ at a concentration of 50 μM ($P \leq 0.01$).

![Image of compound 7b and bar chart]

**Figure 53:** Effect of dimethoxy-mono-benzylc 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. Growth 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 $20 \pm 10\%$ 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. 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.\textsuperscript{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.\textsuperscript{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 \textit{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 \textit{in vitro} metabolism.
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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\textsubscript{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\textsubscript{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\textsubscript{1}, S and G\textsubscript{2}/M phases of the cell cycle. During the transition from G\textsubscript{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.\textsuperscript{83,84} It was decided to treat the cells with a dose of 10 μM, a slightly higher dose than the IC\textsubscript{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\textsuperscript{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$^\text{C}$ solution)\textsuperscript{208} may be used to unstick the cells from the plates without degrading the cell membranes and so maybe avoid the false positives.\textsuperscript{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 \textit{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 \textit{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\textsubscript{50} \textasciitilde50 \(\mu\)M after three or six days incubation with a single dose treatment whereas resveratrol inhibits the proliferation with an IC\textsubscript{50} \textasciitilde6 \(\mu\)M.

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 \textit{in vivo} metabolism and pharmacokinetics of the lead compound 7a and assess its efficacy in a mouse model of colorectal cancer.
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 $\delta$ 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, acetronitriile 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

\[
\text{HO} - \text{Cl} \\
\text{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\(_2\)SO\(_4\)), 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 °C, mp lit.\(^{149}\) 58-60 °C).

IR ν/cm\(^{-1}\): 3332, 1421, 1261; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 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); \(^1\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 141.23 (C), 136.78 (C), 128.84 (CH\(_{Ar}\)), 127.31 (CH\(_{Ar}\)), 64.58 (CH\(_2\)), 46.14 (CH\(_2\)); m/z (El) 156.03402 ([M-H]\(^+\) C\(_8\)H\(_9\)ClO requires 156.03419)

*Synthesis of 2-(4-(chloromethyl)benzyloxy)tetrahydro-2H-pyran*\(^{150}\) 14b

\[
\text{Cl-} \text{O} \text{O} \\
\text{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 NaHCO₃ (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 (Na₂SO₄) 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; ¹H NMR (300 MHz, CDCl₃) δ 7.27 (s, 4H, CHAr), 4.81 (d, J = 12.3 Hz, 1H, Ar-CH₂-HTHP), 4.73 (t, J = 3.5 Hz, 1H, O-CH₂), 4.59 (s, 2H, CH₂Cl), 4.52 (d, J = 12.3 Hz, 1H, Ar-CH₂-HTHP), 3.97-3.89 (m, 1H, CH₂-HCH-CH₂-O), 3.60-3.53 (m, 1H, CH₂-CH₂-HCH₂-CH₂-O), 1.94-1.52 (m, 6H, 3 x CH₂(THP)); ¹³C NMR (75 MHz, CDCl₃) δ 138.73 (C), 136.50 (C), 128.50 (2 x CHAr), 128.07 (2 x CHAr), 97.80 (O-CH₂-O), 68.36 (CH₂-OTHP), 62.12 (Ar-CH₂O), 46.07 (CH₂Cl), 30.57 (CH₂), 25.48 (CH₂), 19.23 (CH₂); m/z (EI) 240.09126 ([M-H]+

C₁₃H₁₇ClO₂ 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$_2$SO$_4$) 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 (Na₂SO₄), 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.

¹H NMR (300 MHz, CDCl₃) δ 7.27-7.19 (m, 4H, 4 x CH₆Ar), 4.64 (s, 2H, CH₂O), 4.47 (s, 2H, CH₂Cl), 0.85 (s, 9H, 3 x CH₃), 0.00 (s, 6H, 2x CH₃) ; ¹³C NMR (75 MHz, CDCl₃) δ 141.81 (C), 136.03 (C), 128.49 (2 x CH₆Ar), 126.29 (2 x CH₆Ar), 64.57 (CH₂-O), 46.16 (CH₂-Cl), 25.93 (3 x CH₃), 18.39 (C), -5.29 (2 x CH₃).

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

New compound

![Image of chemical structure](Attachment)

**General procedure of phosphorylation 1:** 2-(4-(iodomethyl)benzyl)oxy)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.
**Chapter 4 – Experimental Part**

IR ν/cm\(^{-1}\): 2949, 2852, 1695, 1516, 1455, 1391, 1365, 1249; \(^1^H\) NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.26-7.18 (m, 4H, CH\(_Ar\)), 4.68 (d, \(J = 12.0\) Hz, 1H, Ar-CHH-OTHP), 4.62 (t, \(J = 3.5\) Hz, 1H, O-CH-O), 4.40 (d, \(J = 12.0\) Hz, 1H, Ar-CHH-THP), 3.86-3.79 (m, 1H, CH\(_2\)-CH\(_2\)-O), 3.59 (d, \(J = 10.8\) Hz, 6H, CH\(_3\)-O-P), 3.48-3.37 (m, 1H, CH\(_2\)-CHH-CH-O), 3.08 (d, \(J = 21.6\) Hz, 2H, Ar-CH\(_2\)-P), 1.79-1.44 (m, 6H, 3 x CH\(_2\) (THP)); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 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-CH-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, \(J = 138.21\) Hz, CH\(_2\)-P), 30.37 (CH\(_2\)), 25.28 (CH\(_2\)), 19.16 (CH\(_2\)); \(^{31}\)P NMR (400 MHz, CDCl\(_3\)) \(\delta\) 29.6; m/z (ESI) 315.1370 ([M-H]\(^+\) C\(_{15}\)H\(_{23}\)O\(_5\)P 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 trimethylphosphate 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**

[Chemical structure image]

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.
**Chapter 4 – Experimental Part**

IR $\nu_{\text{max}}/\text{cm}^{-1}$ 2909, 1512, 1244; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 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$_3$O), 3.65 (d, $J = 10.8$ Hz, CH$_3$-O-P), 3.49 (d, $J = 21.0$ Hz, Ar-CH$_2$P); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 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$_3$O), 52.62 (d, $J = 6.8$ Hz, 2 x CH$_3$OP), 31.53 (d, $J = 138.8$ Hz, CH$_2$P); $^{31}$P (300 MHz, CDCl$_3$) $\delta$ 29.34; m/z (ESI) 231.0781 ([$\text{M-H}^+]^+$ C$_{10}$H$_{16}$O$_4$P 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$_3$) $\delta$ 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$_3$) $\delta$ 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$_3$O), 31.69 (d, $J = 138.9$ Hz, CH$_2$-P); $^{31}$P (300 MHz, CDCl$_3$) $\delta$ 30.11; m/z (ESI) 301.1212 ([$\text{M-H}^+]^+$ C$_{14}$H$_{22}$O$_5$P requires 301.1205)
Chapter 4 – Experimental Part

*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 NaHCO₃ 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 (MgSO₄), 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, CDCl₃) δ 7.17 (dd, $J = 8.4, 2.4$ Hz, 2H, 2 x CH₂Ar), 6.97 (d, $J = 8.4$ Hz, 2H, 2 x CH₂Ar), 5.35 (t, $J =3.3$ Hz, Ar-O-CH-O), 3.91-3.83 (m, 1H, (THP)-CH₂O), 3.65-3.53 (dd, $J = 10.8, 1.2$ Hz, CH₃-O-P and m, 1H, (THP)-CHH-O), 3.08 (d, $J = 21.0$ Hz, Ar-CH₂P), 2.03-1.52 (m, 6H, 3 x CH₂ (THP)) ; $^{13}$C NMR (75 MHz, CDCl₃) δ 156.16 (d, $J = 3.5$ Hz, C), 130.59 (d, $J = 6.6$ Hz, 2 x CH₂Ar), 124.00 (d, $J = 9.3$ Hz, C), 116.67 (d, $J = 2.8$ Hz, 2 x CH₂Ar), 96.43 (O-CH-O), 62.05 (CH₂O (THP)), 52.88 (d, $J = 6.8$ Hz, 2 x CH₂OP), 31.93 (d, $J = 138.2$ Hz, CH₂P), 30.37 (CH₂), 25.19 (CH₂), 18.18 (CH₂) ; $^{31}$P (300 MHz, CDCl₃) δ 29.27.
Chapter 4 – Experimental Part

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 (MgSO₄), 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, CDCl₃) δ 7.14 (dd, J = 8.4, 2.7 Hz, 2H, 2 x CH₂Ar), 6.77 (d, J = 8.4 Hz, 2H, 2 x CH₂Ar), 3.64 (d, J = 10.8 Hz, CH₃-O-P), 3.09 (d, J = 21.0 Hz, Ar-CH₂P), 0.96 (s, 9H, 3 x CH₃), 0.17 (s, 6H, 2x CH₃) ; $^{13}$C NMR (75 MHz, CDCl₃) δ 154.70 (C), 130.70 (2 x CH₂Ar), 123.64 (C), 120.23 (2 x CH₂Ar), 52.86 (CH₃), 52.77 (CH₃), 32.00 (d, 2 x C, CH₂-P), 25.62 (3 x CH₃), 18.14 (C), -4.48 (2 x CH₃) ; $^{31}$P (300 MHz, CDCl₃) δ 29.27 (s).
Chapter 4 – Experimental Part

**Synthesis of dimethyl 4-((tert-butyl(dimethyl)silyloxy)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)benzyl)oxy)dimethylsilane 10f (5 g, 18.5 mmol, 1 equiv.) in dry THF (40 mL) was added dropwise with a canula at 0°C, N₂. 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 (MgSO₄), 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, CDCl₃) δ 7.17 (m, 4H, CH₆Ar), 4.62 (s, CH₂O), 3.58 (d, J = 10.8 Hz, CH₃-O-P), 3.09 (d, J = 21.0 Hz, Ar-CH₂P), 0.84 (s, 9H, 3 x CH₃), 0.00 (s, 6H, 2x CH₃); $^{13}$C NMR (100 MHz, CDCl₃) δ 140.24 (d, J = 3.6 Hz, C), 129.69 (d, J = 9.3 Hz, C), 129.53 (2 x CH₆Ar), 126.28 (2 x CH₆Ar), 64.67 (CH₂), 52.86 (d, J = 6.8 Hz, 2 x CH₃O-P), 32.57 (d, J = 137.6, CH₂-P), 25.96 (3 x CH₃), 18.41 (C), -5.24 (2 x CH₃); $^{31}$P (300 MHz, CDCl₃) δ 29.04 (s).
17. Aldehydes

Synthesis of methyl 3,5-bis(tetrahydro-2H-pyran-2-yl)benzoate\textsuperscript{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\textsubscript{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 then dried (MgSO\textsubscript{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 \(\nu_{\text{max}}/\text{cm}^{-1}\) 3457, 2970, 1725, 1439, 1365, 1216; \(^1\text{H} \)NMR (300 MHz, CDCl\textsubscript{3}) \(\delta\) 7.38 (t, \(J= 2.1\) Hz, 2H, 2 x CH\textsubscript{Ar}), 6.98 (q, \(J= 2.1\) Hz, 1H, CH\textsubscript{Ar}), 5.47 (q, \(J= 3.0\) Hz, 2H, 2 x O-CH-O), 3.93-3.85 (m, 2H, CH\textsubscript{2}-CHH-CHO and s, 3H, CH\textsubscript{3}O), 3.64-3.61 (m, 2H, CH\textsubscript{2}-CHH-CHO), 2.07-1.56 (m, 12H, 6 x CH\textsubscript{2} (THP)); \(^{13}\text{C} \)NMR (75 MHz, CDCl\textsubscript{3}) \(\delta\) 166.42 (C), 157.71 (C), 157.65 (C), 131.61 (C), 110.54 (CH\textsubscript{Ar}), 109.79 (CH\textsubscript{Ar}), 109.63 (CH\textsubscript{Ar}), 96.17 (CH-O), 96.03 (CH-O), 61.70 (CH\textsubscript{2}O), 61.66 (CH\textsubscript{2}O), 51.83 (CH\textsubscript{3}O), 29.98 (2 x CH\textsubscript{2}), 24.92 (2 x CH\textsubscript{2}), 18.38 (2 x CH\textsubscript{2}); \(m/z\) (ESI) 337.1657 ([M-H]\textsuperscript{+} C\textsubscript{18}H\textsubscript{25}O\textsubscript{6} requires 337.1651)
General procedure for LiAlH₄ reduction GP-4: In a dry three-neck round bottom flask under nitrogen equipped with a water condenser, a suspension of LiAlH₄ (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.

¹H NMR (300 MHz, CDCl₃) δ 6.70 (s, 3H, 3 x CHAr), 5.41 (q, J = 3.4 Hz, 2H, 2 x O-CH-O), 4.61 (d, J = 3.4 Hz, 2H, CH₂OH), 3.94-3.86 (m, 2H, CH₂-OH), 3.63-3.56 (m, 2H, CH₂-C₃H₄-CHO), 3.63-3.56 (m, 2H, CH₂-C₃H₄-CHO), 2.04-1.54 (m, 12H, 6 x CH₂ (THP)) ; ¹³C NMR (75 MHz, CDCl₃) δ 158.29 (C), 158.23 (C), 143.19 (C), 108.09 (CHAr), 104.56 (CHAr), 104.39 (CHAr), 96.41 (O-CH-O), 96.25 (O-CH-O), 65.32 (CH₂OH), 62.02 (2 x CH₂O), 30.32 (2 x CH₂), 25.20 (2 x CH₂), 18.73 (2 x CH₂) ; m/z (ESI) 309.1704 ([M-H]⁺

C₁₇H₂₅O₅ requires 309.1702)
**Chapter 4 – Experimental Part**

*Synthesis of 3,5-bis(tetrahydro-2H-pyran-2-yloxy)benzaldehyde*\(^{165,211}\) \(^9\)i

**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 \(12\)b (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 \(9\)i (1.40 g, 78%) as a pale yellow oil.

IR \(\nu_{\text{max}}/\text{cm}^{-1}\) 2941, 1697, 1589, 1017; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 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-CH-O), 3.85-3.77 (m, 2H, CH\(_2\)-CHH-CHO), 3.59-3.52 (m, 2H, CH\(_2\)-CHH-CHO), 3.00-1.49 (m, 12H, 6 x CH\(_2\) (THP)); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 191.69 (HC=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-CH-O), 61.90 (2 x CH\(_2\)O), 30.06 (2 x CH\(_2\)), 24.98 (2 x CH\(_2\)), 18.47 (2 x CH\(_2\))
Chapter 4 – Experimental Part

Synthesis of 3-(tetrahydro-2H-pyran-2-yl)oxy)benzaldehyde$^{164}$ 9h

Synthesised according to Severi et al.$^{164}$

3-(Tetrahydro-2H-pyran-2-yl)oxy)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 $v_{\text{max}}$/cm$^{-1}$ 2943.90, 1697, 1584, 1248.22; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 9.96 (s, H$\text{C}=\text{O}$), 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)CH$_2$O), 3.64-3.58 (m, 1H, (THP)CHH-O), 2.09-1.52 (m, 6H, 3 x CH$_2$); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 191.99 (HC=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$_2$O), 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 °C, mp lit.$^{212}$
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145-147°C).

IR $\nu_{\text{max}}$/cm$^{-1}$ 3546, 2970, 1737; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.80 (s, 3H, CH$_{Ar}$), 3.99 (s, 9H, CH$_3$O); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 165.39 (3 x C=O), 134.54 (3 x CH$_{Ar}$), 131.20 (3 x C), 52.67 (3 x CH$_3$O); m/z (ESI) 253.0721 ([M-H]$^+$ C$_{12}$H$_{12}$O$_6$ requires 252.0634)

*Synthesis of benzene-1,3,5-triytrimethanol*$_{212}$ *20a*

Benzene-1,3,5-triytrimethanol 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 °C mp litt.$^{212}$ 75-76°C)

IR $\nu_{\text{max}}$/cm$^{-1}$ 3211, 2871, 1446; $^1$H NMR (300 MHz, MeOD) $\delta$ 7.14 (s, 3H, H$_{Ar}$), 4.47 (s, 6H, 3 x CH$_2$OH); $^{13}$C NMR (75 MHz, MeOD) $\delta$143.0 (3 x C), 125.5 (3 x CH$_{Ar}$), 65.2 (3 x CH$_2$); m/z (ESI) 169.0863 ([M-H]$^+$ C$_9$H$_{13}$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-triytrimethanol 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.

\[ \text{IR } \nu_{\text{max}}/\text{cm}^{-1} \quad 3432, 2940 \]

\[ ^1\text{H NMR} \quad (300 \text{ MHz, CDCl}_3) \]

\begin{align*}
4.70 & (d, J = 12.0 \text{ Hz}, 2\text{H, } 2 \times \text{Ar-CH-OTHP}),
4.63 & (t, J = 3.3 \text{ Hz}, 2\text{H, } 2 \times \text{O-CH-O}),
4.59 & (s, 2\text{H, } CH_2\text{OH}),
4.41 & (d, J = 12.3 \text{ Hz}, 2\text{H, } 2 \times \text{Ar-CH-OTHP}),
3.88-3.80 & (m, 2\text{H, THP-CH}_2\text{O}),
3.51-3.44 & (m, 2\text{H, THP-CH}_2\text{O}),
2.30 & (br s, 1\text{H, OH}),
1.84-1.39 & (m, 12\text{H, } 6 \times \text{CH}_2 \text{(THP)}).
\end{align*}

\[ ^{13}\text{C NMR} \quad (75 \text{ MHz, CDCl}_3) \delta \]

\begin{align*}
141.30 & (\text{C}),
138.63 & (2 \times \text{C}),
126.34 & (\text{CH}_2\text{Ar}),
125.48 & (2 \times \text{CH}_2\text{Ar}),
97.76 & (2 \times \text{(THP)CH}_2\text{-CH-O}),
68.62 & (2 \times \text{Ar-CH-OTHP}),
64.96 & (\text{CH}_2\text{OH}),
62.04 & (2 \times \text{THP-CH}_2\text{O}),
30.45 & (2 \times \text{CH}_2),
25.37 & (2 \times \text{CH}_2),
19.24 & (2 \times \text{CH}_2).
\end{align*}

**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 MgSO₄ 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 ; ¹H NMR (300 MHz, CDCl₃) δ 9.56 (s, 1H, CHO), 7.74 (s, 2H, 2 x CH₆), 7.56 (s, 1H, CH₆), 4.79 (d, J = 12.3 Hz, 2H, 2 x Ar-CHH-OTHP), 4.67 (t, J = 3.6 Hz, 2H, 2 x O-CH-O), 4.50 (d, J = 12.3 Hz, 2H, 2 x Ar-CHH-OTHP), 3.88-3.80 (m, 2H, THP-CH₂O), 3.53-3.46 (m, 2H, THP-CH₂O), 1.86-1.46 (m, 12H, 6 x CH₂ (THP)) ; ¹³C NMR (75 MHz, CDCl₃) δ 192.21 (HC=O), 139.70 (2 x C), 136.66 (C), 132.64 (CH₆), 127.86 (2 x CH₆), 98.06 (2 x (THP)CH₂-CH-O), 68.03 (2 x Ar-CH-OTHP), 62.16 (2 x THP-CH₂O), 30.44 (2 x CH₂), 25.35 (2 x CH₂), 19.25 (2 x CH₂).
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 (MgSO₄), 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<sub>max</sub>/cm<sup>-1</sup>:** 3026, 2940, 1596, 1578, 1514, 1488, 1464, 1453, 1433; **¹H NMR (300 MHz, CDCl₃)** δ 7.49 (d, J = 8.1 Hz, 2H, CH<sub>Ar</sub>), 7.36 (d, J = 8.2 Hz, 2H, CH<sub>Ar</sub>), 7.26 (d, AB system, J = 16.0 Hz, 1H, trans CH=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<sub>Ar</sub>), 4.79 (d, J = 12.0 Hz, 1H, Ar-CH/THO-THP), 4.72 (t, J = 3.5 Hz, 1H, O-CH-O), 4.51 (d, J = 12.0 Hz, 1H, Ar-CH/HO-THP), 3.97-3.89 (m, 1H, CH₂-HCH-CHO), 3.82 (s, 3H, CH₃O), 3.59-3.52 (m, 1H, CH₂-HCH-CHO), 1.52-1.48 (m, 6H, 3 x CH₂ (THP)); **¹³C NMR (75 MHz, CDCl₃)** δ 159.71 (C), 138.58 (C), 137.65 (C), 136.33 (C), 129.42 (HC=CH), 128.49
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(HC=CH), 128.28 (CH₆), 127.97 (2 x CH₆), 126.35 (2 x CH₆), 119.02 (CH₂), 113.08 (CH₂H), 111.56 (CH₂), 97.53 (O-CH-O), 68.35 (Ar-CH₂O), 61.91 (CH₂O (THP)), 54.97 (CH₃), 30.42(CH₂), 25.33 (CH₂), 19.21 (CH₂).

Synthesis of (E)-2-(4-(3,5-dimethoxystyril)benzyl)oxy)tetrahydro-2H-pyran 21c

New compound

(E)-2-(4-(3,5-Dimethoxy styryl)benzyl)oxy)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.

¹H NMR (300 MHz, CDCl₃) δ 7.30 (d, J = 8.1 Hz, 2H, CH₆), 7.18 (d, J = 8.2 Hz, 2H, CH₆), 6.93 and 6.82 (2 x d, AB system, J = 16.0 Hz, 2H, trans CH=CH), 6.51 (d, J = 2.1 Hz, 2H, CH₆), 6.23 (t, J = 2.1 Hz, 1H, CH₆), 4.61 (d, J = 12.3 Hz, 1H, Ar-CHHO-THP), 4.55 (t, J = 3.3 Hz, 1H, O-CH-O), 4.32 (d, J = 12.3 Hz, 1H, Ar-CHHO-THP), 3.80-3.72 (m, 1H, CH₂-HCH-CHO), 3.61 (s, 6H, 2 x CH₃O), 3.44-3.36 (m, 1H, CH₂-HCH-CHO ), 1.78-1.34 (m, 6H, 3 x CH₂ (THP)); ¹³C NMR (75 MHz, CDCl₃) δ 160.66 (C), 139.00 (C), 137.59 (C), 136.09 (C), 128.49 (HC=CH), 128.24 (HC=CH), 127.80 (2 x CH₆), 126.24 (2 x CH₆), 104.24 (2 x CH₆), 99.63 (CH₆), 97.41 (O-CH-O), 68.20 (Ar-CH₂O), 61.73 (CH₂O (THP)), 54.87 (2 x CH₃), 30.30 (CH₂), 25.21
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(CH₂), 19.09 (CH₂) ; m/z (ESI) 355.1901 ([M-H]+ C₂₂H₂₇O₄ 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, CDCl₃) δ 7.42 (d, J = 8.1 Hz, 2H, CH₃), 7.37 (s, 2H, CH₂Ar), 7.28 (d, J = 8.1 Hz, 2H, CH₂Ar), 7.19 (s, 1H, CH₂A), 7.05 and 7.04 (2 x d, AB system, J = 16.8 Hz, CH=CH), 4.74 (d, J = 12.0 Hz, 2H, Ar-CHHO-THP), 4.72 (d, J = 12.0 Hz, 1H, Ar-CHHO-THP), 4.66 (t, J = 3.9 Hz, 3H, O-CH-O), 4.46 (d, J = 12.0 Hz, 1H, Ar-CHHO-THP), 4.44 (d, J = 12.0 Hz, 1H, Ar-CHHO-THP), 3.91-3.82 (m, 3H, CH₂HCH-CHO), 3.53-3.45 (m, 3H, CH₂HCH-CHO), 1.84-1.46 (m, 18H, 6 x CH₂ (THP)) ; ^1^C NMR (75 MHz, CDCl₃) δ 138.80 (2 x C), 137.74 (C), 137.57 (C), 136.59 (C), 128.67 (HC=CH), 128.36 (HC=CH), 128.17 (2 x CH₂Ar), 126.57 (CH₂A), 126.49 (2 x CH₂A), 125.13 (2 x CH₂A), 97.78 (2 x CHO), 97.69 (CHO), 68.65 (2 x CH₂O), 68.53
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(CHO), 62.17 (2 x CH₂O), 62.13 (CH₂O), 30.57 (3 x CH₂), 25.46 (3 x CH₂), 19.38 (3 x CH₂).

Synthesis of \((E)-2-\text{(3-((tetrahydro-2H-pyran-2-yloxy)methyl)styryl)phenoxy)}\text{tetrahydro-2H-pyran 21e}\)

New compound \((E)-2-\text{(3-((Tetrahydro-2H-pyran-2-yloxy)methyl)styryl)phenoxy)}\text{tetrahydro-2H-pyran 21e}\) 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 °C)

IR \(\nu_{\text{max}}/\text{cm}^{-1}\) 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, CDCl₃) \(\delta\) 7.42 (d, \(J = 8.1\) Hz, 2H, CH₆), 7.29 (d, \(J = 8.2\) Hz, 2H, CH₆), 7.22-7.07 (m, 3H includes AB system of \text{trans}-double bond), 7.01 (d, \(J = 2.0\) Hz, 2H, CH₆), 6.89 (dd, \(J = 8.1, 2.0\) Hz, 1H, CH₆), 5.40 (t, \(J = 3.3\) Hz, 1H, ArO-CH-O), 4.72 (d, \(J = 12.0\) Hz, 1H, Ar-CHH/O-THP), 4.65 (t, \(J = 3.3\) Hz, 1H, O-CH-O), 4.44 (d, \(J = 12.0\) Hz, 1H, Ar-CHH/O-THP), 3.91-3.83 (m, 2H, CH₂-CHO), 3.60-3.45 (m, 2H, CH₂-CH-
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CHO), 2.02-1.46 (m, 12H, 6 x CH₂ (THP)); ¹³C NMR (75 MHz, CDCl₃) δ 157.41 (C), 138.72 (C), 137.73 (C), 136.60 (C), 129.53 (HC=CH), 128.60 (HC=CH), 128.49 (CH₃), 128.18 (2 x CH₃), 126.51 (2 x CH₃), 120.11 (CH₃), 115.77 (CH₃), 114.30 (CH₃), 97.71 (ArO-CH-O), 96.32 (O-CH-O), 68.56 (Ar-CH₂O), 62.14 (CH₂O (THP)), 61.99 (CH₂O (THP)), 30.59 (CH₂), 30.41 (CH₂), 25.49 (CH₂), 25.24 (CH₂), 19.37 (CH₂), 18.74 (CH₂).

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.

¹H NMR (300 MHz, CDCl₃) δ 7.48 (d, J = 8.3 Hz, 2H, CH₃), 7.35 (d, J = 8.3 Hz, 2H, CH₃), 7.10 and 7.00 (2 x d, AB system, J = 16.4 Hz, CH=CH), 6.89 (t, J = 1.8 Hz, 2H, CH₃), 6.71 (dd, J = 3.3, 2.0 Hz, 1H, CH₃), 5.46 (t, J = 3.3 Hz, 2H, ArO-CH-O), 4.79 (d, J = 12.0 Hz, 1H, Ar-CHO-THP), 4.72 (t, J = 3.3 Hz, 1H, O-CH-O), 4.51
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(\textit{d}, J = 12.0 \text{ Hz}, 1\text{H}, \text{Ar-CH}2\text{HO-THP})$, 3.98-3.90 (m, 3\text{H}, \text{CH}2\text{-HCH-CHO}), 3.66-3.52 (m, 3\text{H}, \text{CH}2\text{-HCH-CHO }), 2.10-1.53 (m, 18\text{H}, 9 \times \text{CH}2 \text{(THP)}) ; ^{13}\text{C NMR (75 MHz, CDCl}3 \) \delta 158.29 (C), 158.23 (C), 139.14 (C), 137.68 (C), 136.54 (C), 128.67 (HC=CH), 128.56 (HC=CH), 128.13 (2 \times \text{CH}Ar), 126.49 (2 \times \text{CH}Ar), 107.99 (CHAr), 107.93 (CHAr), 104.81 (CH), 97.67 (O-CH-O), 96.39 (ArO-CH-O), 96.23 (ArO-CH-O), 68.52 (Ar-CH2O), 62.09 (CH2O (THP)), 61.93 (CH2O (THP)), 61.90 (CH2O (THP)), 30.55 (CH2), 30.33 (2 \times \text{CH}2), 25.45 (CH2), 25.21 (2 \times \text{CH}2), 19.33 (CH2), 18.68 (CH2), 18.64 (CH2).

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

New compound

\begin{center}
  \includegraphics[width=0.5\textwidth]{image}
\end{center}

\textit{(E)-2,2'-\textit{(5-(4-\textit{(Tetrahydro-2H-pyran-2-yloxy)styryl)-1,3-(phenylene)bis(methylene)bis(oxy)bis(tetrahydro-2H-pyran) 21g}}\textit{}}\textit{}}\textit{ 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 \textit{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 \rightarrow 80:20) as a colorless oil.

\textit{H NMR (300 MHz, CDCl}3 \) \delta 7.37 (d, J = 8.8 \text{ Hz}, 2\text{H}, 2 \times \text{CH}Ar), 7.33 (s, 2\text{H}, 2 \times \text{CH}Ar), 7.16 (s, 1\text{H}, \text{CHAr}), 7.01 and 6.91 (2 \times d, \text{AB system}, J = 16.8 \text{ Hz}, \text{CH}=\text{CH}),
6.96 (d, J = 8.8 Hz, 2H, 2 x CH\textsubscript{Ar}), 5.35 (t, J = 3.3 Hz, 1H, ArO-CH-O), 4.72 (d, J = 12.1 Hz, 2H, 2 x Ar-CH\textsubscript{H}HO-THP), 4.72 (t, J = 3.3 Hz, 2H, 2 x O-CH-O), 4.51 (d, J = 12.1 Hz, 2H, 2 x Ar-CH\textsubscript{H}HO-THP), 3.89-3.78 (m, 3H, 3 x CH\textsubscript{2}-HCH-CHO), 3.55-3.44 (m, 3H, 3 x CH\textsubscript{2}-HC\textsubscript{H}-CHO ), 1.95-1.44 (m, 18H, 9 x C\textsubscript{H} \textsubscript{2}(THP)); \textsuperscript{13}C NMR (75 MHz, CD\textsubscript{3}Cl) δ 156.72 (C), 138.71 (2 x C), 137.85 (C), 130.86 (C), 128.50 (HC=CH), 127.57 (2 x CH\textsubscript{Ar}), 126.57 (HC=CH), 126.20 (CH\textsubscript{Ar}), 124.91 (2 x CH\textsubscript{Ar}), 116.59 (2 x CH\textsubscript{Ar}), 97.73 (2 x O-CH-O), 96.27 (O-CH-O), 68.66 (2 x Ar-CH\textsubscript{2}-OTHP), 62.33 (2 x (THP) CH\textsubscript{2}O), 61.98 ((THP) CH\textsubscript{2}O), 30.67 (2 x CH\textsubscript{2}), 30.28 (CH\textsubscript{2}), 25.41 (3 x CH\textsubscript{2}), 19.60 (2 x CH\textsubscript{2}), 18.70 (CH\textsubscript{2}) ; m/z (ESI) 509.2903 ([M-H]\textsuperscript{+} C\textsubscript{31}H\textsubscript{41}O\textsubscript{6} requires 509.2902).

**Synthesis of (E)-2,2’-(5-(4-methoxystyryl)-1,3-phenylene)bis(methylene)bis(oxy)bis(tetrahydro-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.

\textsuperscript{1}H NMR (300 MHz, CD\textsubscript{3}Cl) δ 7.36 (d, J = 8.7 Hz, 2H, 2 x CH\textsubscript{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, CH=CH), 6.81 (d, $J = 8.7$ Hz, 2H, 2 x CH$_{Ar}$), 4.72 (d, $J = 12$ Hz, 2H, 2 x Ar-CHH-OTHP), 4.65 (t, $J = 3.6$ Hz, 2H, 2 x O-CH-O), 4.44 (d, $J = 12$ Hz, 2 x Ar-CHH-OTHP), 3.90-3.82 (m, 2H, (THP)CH$_2$O), 3.73 (s, 3H, CH$_3$O), 3.52-3.45 (m, 2H, (THP)CH$_2$O), 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=CH), 127.65 (2 x CH$_{Ar}$), 126.30 (HC=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-CH$_2$-OTHP), 62.10 (2 x (THP) CH$_2$O), 55.22 (CH$_3$O), 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

![Chemical structure](image)

**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 $\nu_{\max}$/cm$^{-1}$ 3728, 2970, 1584; UV $\lambda_{\max}$ (MeOH)/nm 286.50 ($\varepsilon$ 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 CH=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$_{Ar}$), 203
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4.59 (s, 2H, CH$_2$O), 3.81 (s, 3H, CH$_3$O); $^{13}$C NMR (75 MHz, MeOD) δ 211.50 (C), 142.20 (C), 140.30 (C), 137.86 (C), 130.65 (CH$_{Ar}$), 129.69 (CH=CH), 129.49 (CH=CH), 128.41 (2 x CH$_{Ar}$), 127.76 (2 x CH$_{Ar}$), 114.31 (CH$_{Ar}$), 112.75 (CH$_{Ar}$), 65.03 (CH$_2$), 55.20 (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 $\nu_{max}$/cm$^{-1}$: 3346, 2834, 1584, 1454; $^1$H 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$_2$O), 3.79 (s, 6H, 2 x CH$_3$O); $^{13}$C (300 MHz, MeOD) δ 162.53 (2 x C), 142.22 (C), 140.86 (C), 137.89 (C), 129.85 (CH=CH), 129.60 (CH=CH), 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-hydroxystyril)phenyl)methanol 7c

New compound

(E)-(4-(3-Hydroxystyril)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 $\nu_{\text{max}}$/cm$^{-1}$ 3393, 3137, 1588, 1445; $^1$H (300 MHz, MeOD) $\delta$ 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- CH=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$_2$O); $^{13}$C (300 MHz, MeOD) $\delta$ 159.78 (2 x C), 142.10 (C), 140.86 (C), 137.94 (C), 129.83 (CH=CH), 129.23 (CH=CH), 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

Chapter 4 – Experimental Part
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 $\nu_{\text{max}}$/cm$^{-1}$: 3223, 2970, 1584; $^1$H NMR (300 MHz, MeOD) $\delta$: 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-CH=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$_2$O); $^{13}$C NMR (300 MHz, MeOD) $\delta$: 159.78 (2 x C), 142.10 (C), 140.86 (C), 137.94 (C), 129.83 (CH=CH), 129.23 (CH=CH), 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$_{21}$O$_3$ requires 242.09429).

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

(E)-(5-(4-Hydroxymethylstyril)-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 $\nu_{\text{max}}$/cm$^{-1}$ 3201, 2867, 1597, 1453; $^1$H NMR (300 MHz, MeOD) $\delta$ 7.51 (d, $J$ = 8.1 Hz, 2H, CH$_{Ar}$), 7.44 (s, 2H, 2 x CH$_{Ar}$), 7.35 (d, $J$ = 8.1 Hz, 2H, CH$_{Ar}$), 7.24 (s, 1H, CH$_{Ar}$), 7.16 (br s, 2H, CH=CH), 4.62 (s, 4H, 2 x CH$_2$O), 4.59 (s, 2H, CH$_2$O); $^{13}$C (75 MHz, MeOD) $\delta$ 143.26 (2 x C), 142.16 (C), 139.05 (C), 137.83 (C), 129.64 (CH=CH), 129.38 (C=CH), 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 (El) 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) $\delta$ 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) $\delta$ 158.50 (C), 143.21 (2 x C), 139.60 (C), 130.45 (C), 129.88 (HC=CH), 128.91 (2 x CH$_{Ar}$), 129.65 (HC=CH), 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.
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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) $\delta$ 7.48 (d, $J = 8.7$ Hz, 2H, 2 x CH$_{\text{Ar}}$), 4.42 (s, 2H, 2 x CH$_2$), 7.22 (s, 1H, CH$_{\text{Ar}}$), 7.13 and 7.03 (2 x d, AB system, $J = 16.4$ Hz, trans-CH=CH), 6.91 (d, $J = 8.8$ Hz, 2H, 2 x CH$_{\text{Ar}}$), 4.63 (s, 4H, 2 x CH$_2$), 3.80 (s, 3H, CH$_3$O);

$^{13}$C NMR (300 MHz, MeOD) $\delta$ 160.95 (C), 143.27 (2 x C), 139.45 (C), 131.55 (C), 129.57 (HC=CH), 128.82 (2 x CH$_{\text{Ar}}$), 127.42 (HC=CH), 125.50 (CH$_{\text{Ar}}$), 124.81 (2 x CH$_{\text{Ar}}$), 115.18 (2 x CH$_{\text{Ar}}$), 65.21 (2 x CH$_2$), 55.78 (CH$_3$O); 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 MgCl₂ (5 μM), mercaptoethanol (3 mM) and KHPO₄ 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
Chapter 4 – Experimental Part

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.\textsuperscript{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\textsubscript{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.

<table>
<thead>
<tr>
<th>time (min)</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>12.5</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>16.5</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 18: Gradient of Elution

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\textsubscript{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\textsuperscript{®} 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.\textsuperscript{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\textsubscript{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\textsuperscript{®} Particle Count and Size Analyser (Beckman Coulter, High Wycombe, UK).

<table>
<thead>
<tr>
<th>Control DMSO</th>
<th>0.5 μM</th>
<th>2 μM</th>
<th>5 μM</th>
<th>10 μM</th>
<th>50 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control DMSO</td>
<td>0.5 μM</td>
<td>2 μM</td>
<td>5 μM</td>
<td>10 μM</td>
<td>50 μM</td>
</tr>
<tr>
<td>Control DMSO</td>
<td>0.5 μM</td>
<td>2 μM</td>
<td>5 μM</td>
<td>10 μM</td>
<td>50 μM</td>
</tr>
<tr>
<td>Control DMSO</td>
<td>0.5 μM</td>
<td>2 μM</td>
<td>5 μM</td>
<td>10 μM</td>
<td>50 μM</td>
</tr>
</tbody>
</table>

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.
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APPENDICES
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.


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 2\textsuperscript{nd} year organic chemistry practicals.
- Demonstration to 3\textsuperscript{rd} year MChem and MSc organic chemistry practicals.
- Supervision of 3\textsuperscript{rd} year BSc projects, 4\textsuperscript{th} 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

**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:
  - 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 of cancer
- Slows cancer progression
- Reduces the size of a tumour
- Kills cancer cells/tumour

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
- 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'? Yes, Environmental factors: Air, Water, Diet, Lifestyle... play a big role on cancer

Rice Bran / Tricin

Turmeric / Curcumin

Grapes / Resveratrol

Studies on Resveratrol
- Metabolised in Glucuronide 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

References
1. D. Hanahan, R. A. Weinreb, Cell, 2000, 100, 57-70
Can your Diet Prevent Cancer?

Chemoprevention of Cancer from Natural Dietary Phytochemicals

C. Simon, Dr R. G. Britton, Dr P. R. Jenkins, ca234@le.ac.uk, Department of Chemistry, University of Leicester, Leicester, UK

Introduction

Diet is the second most preventable cause of cancer after smoking. 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 (found in grape skin, red wine, nuts oils and berries), curcumin (found in turmeric-curry) and thion (found 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

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 of cancer
- Slows cancer progression
- Reduces the size of a tumour
- Kills cancer cells/tumour

Very fast metabolism and elimination

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

Resveratrol

It is a natural product which occurs in grape skin, red wine, nuts oils and berries. It is a polyphenol (antioxidant produced by plants) which has 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.

- Anti-tumour initiation: Inhibition of free radical formation
- TP induced E50 = 27 μM
- Anti-tumour promotion: Inhibition of COX-1 and COX-2
- COX-1: E50 = 3.7 μM, IC50 COX-1 = 0.535 μM
- COX-2: E50 = 85 μM, IC50 COX-2 = 0.996 μM
- Anti-tumour progression: HL-60 cells treated with resveratrol do not proliferate

Drug Design

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

References:
Chemoprevention of Cancer
Synthesis and Metabolism of Resveratrol Analgeses

A. Introduction

Diet is the second most preventable cause of cancer after smoking. Populations show that cancer has various incidences in different parts of the world. Studies 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 concurrent with the prevention of cancer.

B. Chemoprevention

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 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 aging
- 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.

2a

Glucuronides

2b

Sulfates

3a

3b

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.

Gluconoridation

Incubation of parent product with mouse microsomes and UDPGA as a cofactor for 0, 5, 15, 30 and 60 min.

Sulfation

Incubation of parent product with mouse liver cytosol and PAPS as a cofactor for 0, 15, 30, 60 and 120 min.

E. Synthesis


F. Results

HPLC

Gluconoridation

Compound 5

Compound 8

0 min

5 min

15 min

Time rates

Gluconoridation

Sulfation

Conclusions

- Two of the analogues have a similar glucuronidation rate than resveratrol, but the “best” ones are not metabolised in 3h.
- 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

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

A. Introduction

Diet is the second most preventable cause of cancer after smoking. 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 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.

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

**Resveratrol**
- Anti-Cancer
- Anti-oxidant

**Resveratrol Metabolites**
Metabolism occurs within 1h in humans.
- Sulfates
- Glucuronides

**B. Results and Discussion**

**Metabolism assays**
Incorporating resveratrol analogues with mouse liver cytosol or microsomes with a cofactor and following the disappearance of the parent product over time by HPLC.

**Cell proliferation assays**
Aim: Monitoring 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 analogues in 0.3% DMSO. Concentrations studied: Control (DMSO), 0.5 mM, 2 mM, 5 mM, 10 mM, 50 mM.

**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 (IC50 ~ 6 mM at day 6).


References: