Natural Product Discovery: Studies on the Phenolic Antioxidants from *Smilax Glyciphylla* and the Synthesis and Formation of Guaiane Sesquiterpenoids.

A thesis presented in fulfilment
of the requirements for the degree of

Doctor of Philosophy

by

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Abstract

The work within this thesis is positioned in the field of natural product (NP) chemistry and covers three main integrated studies along with some additional explorations. These studies not only included the isolation and characterisation of NP but also involved total syntheses of various NP and related derivatives and detailed mechanistic studies into potential routes of formation in nature. Given my naturally emerging zest for natural products, I have begun this thesis with a detailed discussion of the numerous syntheses of Taxol. This exemplar highlights not only why the field of natural products is so important, but also highlights the ever growing significance of total and semi-syntheses.

The first major study investigated the phenolic profile and antioxidant activity of the leaves of the Australian native plant *Smilax glyciphylla*. Along with the sweet principle glycyphyllin A, seven phenolic compounds including two new dihydrochalcone rhamnosides, glycyphyllin B and C, and five known flavonoids were isolated from the ethanolic extract of the leaves of *Smilax glyciphylla* for the first time. The structures of these compounds were characterised by spectroscopic methods including UV, HRMS, 1D and 2D NMR. *In vitro* antioxidant capacity tests employing the FRAP and DPPH assays indicated that three of the isolated compounds exhibited potent antioxidant activity and are the key phenolics responsible for the high antioxidant activity of the leaf extract of *S. glyciphylla*.

The second major study focused on the synthesis of guaiane type sesquiterpenoids via the diastereoselective epoxidation of guaiol and realized by manipulating the types of remote protecting groups on the isopropanoyl side chain, choice of solvent and epoxidising reagent. This stragety allowed for a concise stereoselective synthesis of a range of guaiane-type sesquiterpenoids including the natural products guaia-4(5)-en-11-ol, guaia-5(6)-en-11-ol, and aciphyllene and epimers of the recently isolated natural products, 1-*epi*-guaia-4(5)-en-11-ol, 1-*epi*-aciphyllene and 1-*epi*-melicodenone C and E in up to 31% yield within 11 steps.

The third study explored the autoxidation of α-guaiene and the mechanisms involved. Over a dozen sesquiterpenoids including natural rotundone, corymbolone and the C7 epimers of natural chabrolidione A and several unstable hydroperoxide intermediates were isolated from the autoxidation products of α-guaiene. Their structures were elucidated on the basis of spectroscopic data along with the synthesis of authentic compounds. Detailed mechanistic

studies have allowed many of the mechanisms involved in the formation of these downstream oxidation products to be elucidated.

Together with the above main studies, several deuterium labelled precursors including d_7-α-guaiene, d_5-(2*R*/2*S*)-rotundols, d_5-α-bulnesone, d_7-α-bulnesene and d_5-2*R*-bulnesol were synthesised and used as internal standards to develop a robust analytical method (SIDA) to monitor the transformation of certain precursors to the sesquiterpneoid fragrances rotundone and 2*R*-bulnesol.

A total of five publications support my research works herein and are included as the main research chapters of this thesis.

List of Publications

1. Huang, A.-C.; Wilde, A.; Ebmeyer, J.; Skouroumounis, G. K.; Taylor, D. K. Examination of the Phenolic Profile and Antioxidant Activity of the Leaves of the Australian Native Plant *Smilax glyciphylla*. *J. Nat. Prod*. 2013, *76* (10), 1930-1936.

2. Huang, A.-C.; Sumby, C. J.; Tiekink, E. R.T.; Taylor, D. K. Synthesis of Guaia-4(5)-en-11-ol, Guaia-5(6)-en-11-ol, Aciphyllene, 1-*ep*i-Melicodenone C and E and other Guaiane-type Sesquiterpenoids via the Diastereoselective Epoxidation of Guaiol. *J. Nat. Prod*. 2014, DOI: 10.1021/np500611z.

3. Huang, A.-C.; Burrett, S.; Sefton, M. A.; Taylor, D. K. Production of the Pepper Aroma Compound, (-)-Rotundone by Aerial Oxidation of α-Guaiene. *J. Agric. Food Chem*, 2014, *62* (44), 10809–10815.

4. Huang, A.-C.; Sefton, M. A.; Sumby, C. J.; Tiekink, E. R.T.; Taylor, D. K. Mechanistic Studies on the Autoxidation of α-Guaiene: The Structural Diversity of the Sesquiterpenoid Downstream Products. *J. Nat. Prod.* 2014, submitted.

5. Huang, A.-C.; Sefton, M. A.; Taylor, D. K. Rationalizing the Formation of Peppery and Woody Sesquiterpenes Derived from α-Guaiene and α-Bulnesene under Aerial Oxidative Conditions: Synthesis of Deuterium Analogues and SIDA/GC-MS Studies. *J. Agric. Food Chem*. 2014, submited.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Ancheng Huang

Date: 13-11-2014

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The second biggest thank you goes to my co-supervisor Dr. Mark A. Sefton. Thanks for mentoring a young strayed chemistry laymen through the door of chemistry and being a beacon when I was fumbling in darkness. Your 'prophet' status in my heart has never been shaken and your scientific calibre, humour, and the scientific skills and fun stories you shared with me will be carried throughout my whole life.

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Abbreviations

Å	Angstroms
ABS	Absorbance
Ac	Acetyl
app. d	Apparent doublet
Ar	Aromatic
Bn	Benzyl
br	Broad
COSY	Correlation spectroscopy
cm	Centimetres
d	Doublet
DCM	Dichloromethane
dd	Doublet of doublets
ddd	Doublet of doublet of doublets
DEAD	Diethyl azocaboxylate
DFT	Density function theory
DMSO	Dimethylsulfoxide
Et	Ethyl
Et_2O	Diethyl ether
EtOAc	Ethyl acetate
EtOH	Ethanol
FRAP	Ferric cyanide reducing antioxidant power assay
g	Gram
GC	Gas chromatography
GCMS	Gas chromatography mass spectrometry
h	Hour
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Coherence
HPLC-DAD	High performance liquid chromatography- diode array detector
HRMS	High resolution mass spectrometry
Hz	Hertz
Hν	Light/irradiation
J	Coupling constant

L	Litre
LCMS	Liquid chromatography mass spectrometry
Lit.	Literature
LTMP	Lithium tetramethyl piperidide
m	Multiplet
M	Molar (moles/litre)
min.	Minute
m/z	Mass to charge ratio
MEOH	Methanol
Me	Methyl
mg	Milligram
$MgSO_4$	Magnesium sulphate
MHz	Megahertz
mL	Millilitre
mmol	Millimole
mol	Mole
mp	Melting point
MS	Mass spectrometry
nm	Nanometre
NMR	Nuclear magnetic resonance
Ph	Phenyl
ppm	Parts per million
PTSP	Pyridinium paratoluenesulfonate
psi	Pounds per square inch
q	Quartet
quint	Quintet
R_f	Retention factor
ROESY	Rotating frame overhauser effect spectroscopy
RT	Room temperature
s	Singlet
sext	Sextet
t	Triplet
TBS	*tert*-Butyldimethylsilyl
THF	Tetrahydrofuran

TLC	Thin layer chromatography
TPTZ	2,4,6-Tripyridyl-1,3,5-triazine
UV	Ultra-violet
μ	Micro

Chapter 1

Introduction to Natural Products

1.0 The emergence of natural products.

As one of the species on this planet, human beings have been seeking inarguably everything from our Mother Nature. We have progressed through the years and have gained immense inspiration from nature in order to feed and cloth ourselves, develop flavours and fragrances and gained an understanding of natures traditional medicines in order to cure our ailments. Natural products (NP) are small molecules that are produced by biological sources[1] including plants, bacteria, fungi, marine organisms and animals (e.g. insects). The seemingly unlimited structural diversity of NP that has been accumulated and selected by evolution since the beginning of earth, display miscellaneous bioactivities of which only a relatively small fraction have thus far been discovered. Screening for bioactive NP as lead compounds is an enormous task and has found application in various industries such as the health and food,[2-4] flavour and fragrance,[5, 6] and pharmaceutical industries[7-13] with the latter enjoying most of the cynosure. About 47% of the newly approved drugs over the past decades from 1981 to 2010 are either NP or derivatives of NP.[14] Even though newly emerging methodologies such as high-throughput screening (HTS),[15-18] combinatorial chemistry,[19-21] genetic-, bio- and metabolic engineering[15, 22-24] have certainly shortened the turn-around time and have provided more potential lead candidates and hopes for better production in drug discovery,[25] the low success rate of finding promising leads with these 'modern' methodologies has renewed focus on NP and reconsolidated the role of NP as the pool for inspiration in the pharmaceutical industry.[12, 26]

The tracked record of NP in drug discovery dates back to the usage of herbs and plant materials as folk medicine and led to the first isolation of the analgesic natural plant alkaloid morphine (**1**) from opium and later on aspirin (**2**) from willow bark in the early 1800's.[7, 26] The serendipitous discovery of penicillin (**3a**, **b**) by Fleming in 1928 then ushered in the golden age of antibiotics from the 1940-1970's.[7, 26] During the same period, numerous NP

lead compounds have been discovered and developed into drugs targeting a variety of diseases.[7] Notable examples that marked the progress of NP in drug discovery include the antifungal amphotericin B (**4**) from *Streptomyces nodosus* obtained from the soil adjacent to the Oronoco River in Venezulea in 1955,[27] the anticancer drug paclitaxel (Taxol, **5**) from the

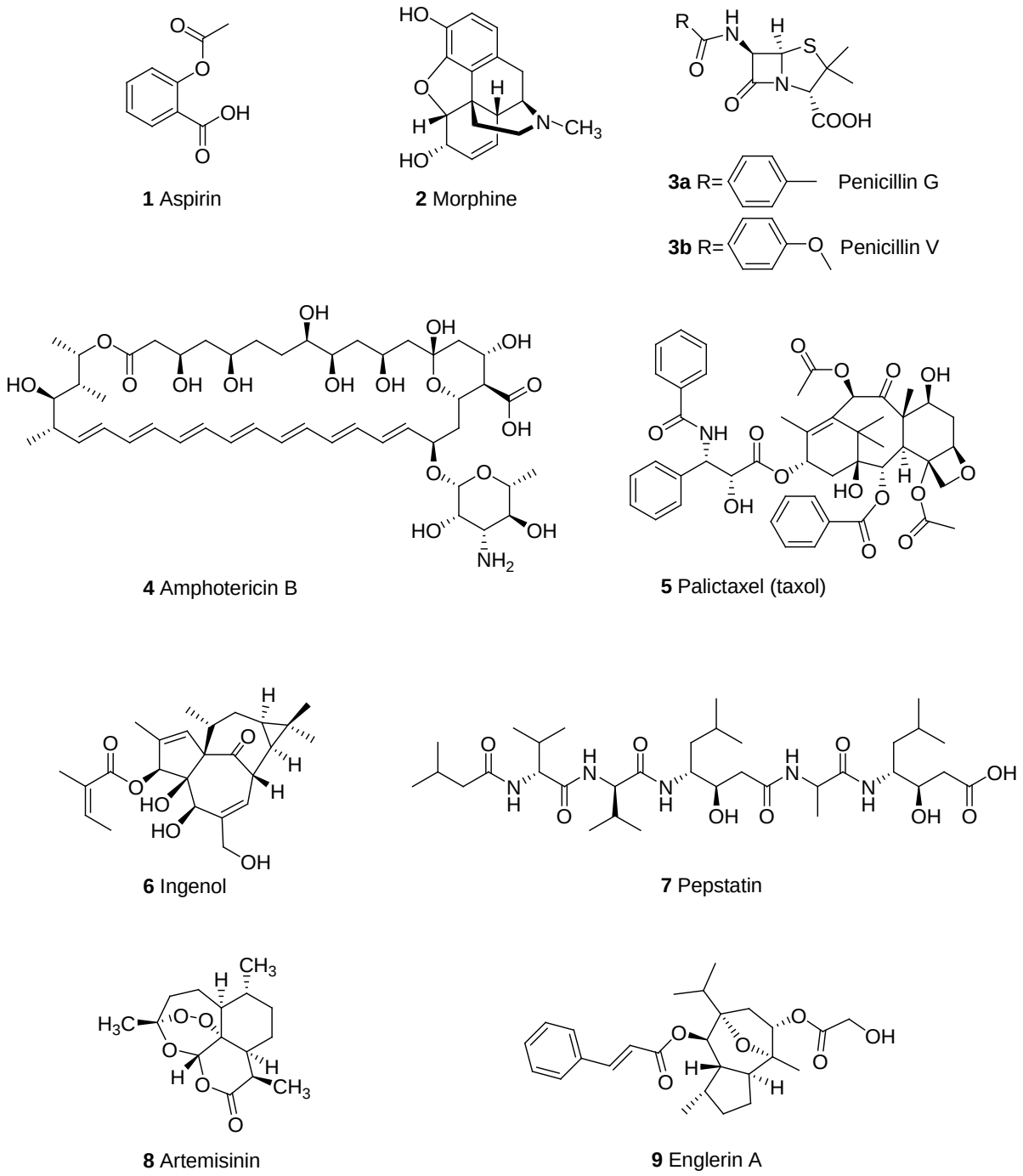


Figure 1. Structures of some typical natural product drugs.

bark of pacific yew tree T*axus brevifolia* in the 1960's,[28, 29] the pre-skin cancer actinic keratosis drug ingenol (**6**) from euphorbiaceae plants in 1968,[30-32] the antiviral and anti-HIV pepstatin (**7**) from *Actinomyces* in 1970,[33] the antimalarial drug artemisinin (**8**) from the *Artemisia annua* in China in 1971,[34] and the recent discovery of the selective inhibitor of renal cancer englerin A (**9**)[35] from *Phyllanthus engleri* in 2009, Figure 1.

Although a reasonable number of NP drugs have been developed, we are still at 'the tip of the iceberg' due to the vast biological diversity that Nature embraces.[1] The majority of the natural product research in the past was based on plant materials[36, 37] and microbes,[38-41] and other sources such as marine organisms,[42-45] animals including insects[46] and venoms.[47-51] Other unconventional sources[52] are still far from being explored and are considered to be the targets of the next wave of drug discovery. Together with the fact that many diseases tend to develop resistance to drugs, the continuing demand for new drugs based on NP is substantial.

1.1 The discovery process of NP and novel techniques developed for NP research.

Conventional NP screening processes begin with the selection of potential candidate sources based on available records of their biological properties followed either by isolation and characterisation of the bioactive compounds and sequential bioactivity testing or may be driven by bioassay guided fractionation to identify and characterise the compounds responsible for the assayed bioactivity.[53, 54] Whilst this strategy has been very successful in discovering bioactive compounds from natural sources, the time-consuming and labour-intensive nature of the discovery process posts a significant challenge in its commercial application in pharmaceutical industry. As illustrated in figure 1; in the pilot trial phase, potential bioactive samples are extracted using methods suitable for the target bioactive compounds and the chromatographic traces of the extract (TLC, GC-MS or HPLC-DAD/MS/FTIR/NMR) obtained to generate an overall picture of all potential compound identities. Based on the profiles of the extract, a dereplication process is followed to narrow down the target bioactive molecules. This is normally done by fractionation of the extract employing various preparative chromatography techniques including solid-liquid chromatography such as silica, sephadex LH-20, size-exclusion (for large molecules like peptides and proteins) and affinity chromatography, liquid-liquid counter-current chromatography and preparative gas chromatography depending on the nature of the target

compounds. After dereplication and concentration of the bioactive compounds, further purification will be performed to isolate pure target compounds whose biological activity will be validated in the pure form. Due to the complexity of the matrices of extracts and the potential decomposition of labile compounds during purification, the extraction and dereplication processes may often need repeating several times to optimise the isolation methods for the desired pure bioactive compounds. Once novel compounds with promising biological activities are identified and their structures fully characterised, scale-up extraction and isolation will have to be carried out to obtain lager amounts of the pure compounds for further biological activity tests.

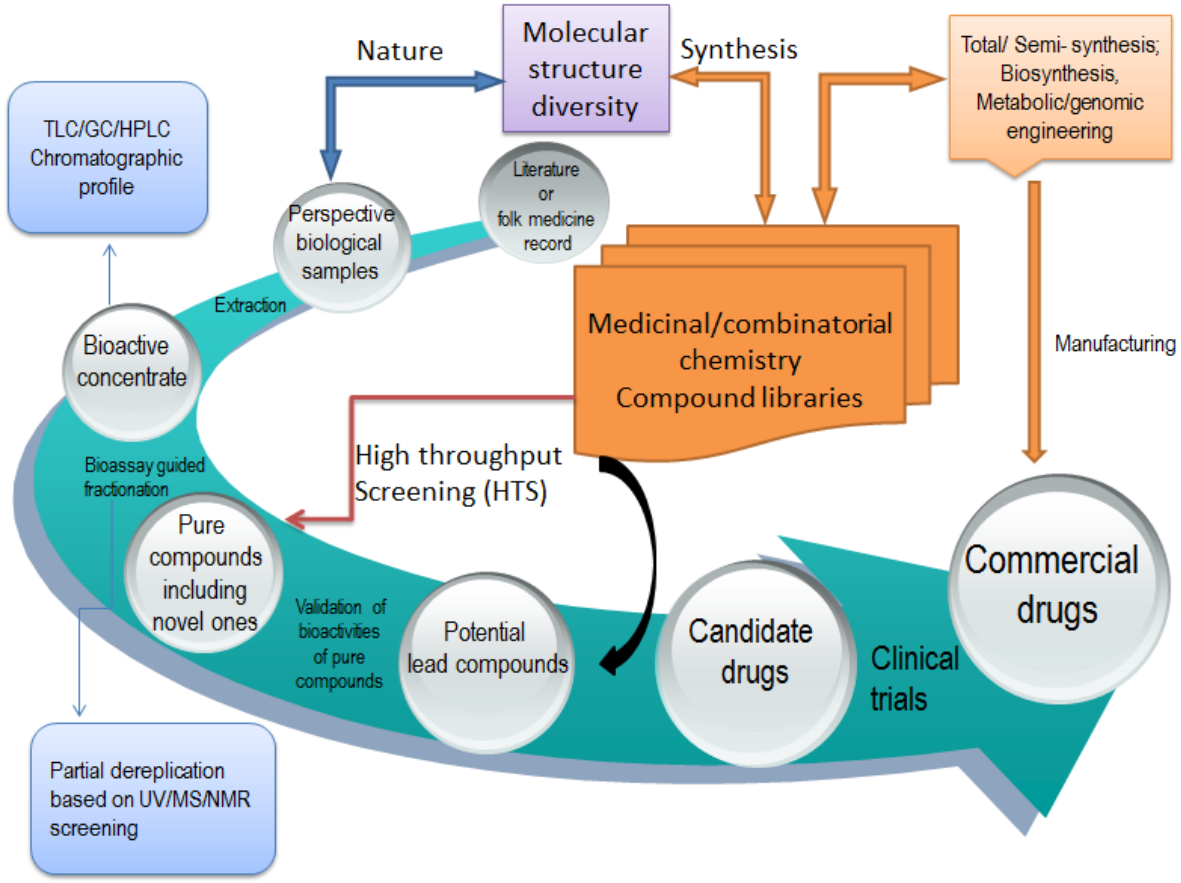


Figure 2. Generic NP based drug discovery process.

This conventional process is well illustrated by the discovery of the anticancer drug Taxol.[29] Plant materials (Pacific yew) containing Taxol were first collected by the United States Department of Agriculture in 1962 and the cytotoxicity of its extract confirmed within two

years by 1964. After recollection and fractionation which took another five years, Taxol was isolated as the active constituent in 1969. Its structure was fully established in 1971 by x-ray diffraction analysis of several crystal structures from derivatives of its two fragments obtained from based-catalysed methanolysis of Taxol itself.[28] However, it wasn't until 1983 that the phase I trials (human safety and evaluation of alternative doses and regimens) were approved to be conducted. In 1993, Taxol was approved by the Food and Drug Administration (FDA) for use in the U.S. for treatment of breast and ovarian cancer. The long discovery process in turn showcases the urgent need for developing more efficient extraction, purification and characterisation techniques.

The difficulty in natural product isolation arises from the poor understanding of the nature of the extracts. If the majority of constituents of the extracts could be easily identified in a pilot scale trial, good extraction, dereplication and isolation schemes could be easily developed. The key to achieving efficient identification of compounds from NP extracts is chromatographic resolution and structural information assembly. While traditional one dimensional chromatography tandem UV/MS has been serving the role in a satisfactory manner in the past, new two dimensional chromatography techniques (e.g. GC x GC, LC x LC) in tandem with more powerful detectors such as HR(ESI/FT)MS and 1D/2D NMR and other hyphenation techniques could greatly enhance chromatographic resolution and structural information output.[55] Many reviews on different aspects of the recent development of hyphenation techniques have underscored the great prospects of these developing techniques and their self-explicit advantages over the older techniques.

Starting with TLC, the simplest tool that will provide the first-hand information of an extract, its two dimensional application has shown much greater resolution than normal one dimensional TLC in both separation and analysis of complex plant extracts.[56] Especially with more sorbents or coating methods being developed, the role of 2D-TLC in NP discovery is becoming more important. The emergence of 2D GC including comprehensive GC x GC and heart-cutting 1D/2D selectable GC coupled with mass spectrometry offers great solutions to resolving the overlapping problems of volatiles that have very similar vapour pressure, polarity and other physical properties.[57-60] This is realised by employing a modulator and deans switch installed in between different dimensions of columns to retain periodically eluted analytes and control the flow and temperature of the system so that analytes eluted from the first dimension could be further separated on the second column.[59] Unlike the

thermal modulator and micro flow deans switch used in 2D GC, 2D LC including comprehensive LC x LC and heart-cutting LC uses high pressure switching valves as interfaces for different dimensions and requires relatively more mobile phase compatibility between column dimensions, which is a hurdle that comprehensive GC doesn't encounter.[61]

Mass spectrometry (MS) is the most often used detector that is coupled with chromatography instruments including multidimensional instruments that are employed for routine analyses.[57, 62, 63] However, NMR is gradually becoming a popular detector and is able to hyphenate capillary separation[64] thanks to the advances in high-field and small volume NMR[65, 66] and the development of stop-flow,[67-69] continuous flow techniques[70] and other interfacing techniques.[71] The high structural information output of NMR renders it unsurmountable advantages when hyphenated with chromatography techniques and can reshape the landscape of the whole field of analytical chemistry in NP research when the development approaches maturity. Another very important separation technique is the liquid-liquid counter current chromatography (CCC) which is very useful in preparative scale separation and analysis.[72] Recent advance in hyphenating HSCCC to ESIMS demonstrated the possibility of coupling CCC with other high-end detectors such as NMR.[73] Together with the development of other hyphenation techniques such as LC-GC,[74] supercritical fluid tandem 2D GC,[75] and TLC-PAMLDI-MS (TLC-Plasmer Assisted Multiwavelength Laser Desorption Ionisation Mass Spectrometry),[76] their application in NP research will simplify the complexity of NP isolation and identification and shorten the dereplication process thus improving the efficiency of NP discovery.

1.2 Natural product synthesis.

Isolation and structural characterisation by chromatographic and spectroscopic techniques are certainly two solid cornerstones in the field of NP research as they lead to the discovery of novel molecular structures and also offer straightforward approaches for NP production. NP synthesis, be it chemical or biological synthesis, provides means for confirming the complex structures of NP, constructing libraries of new analogues and allows for potential faster and more sustainable production of NP on large scale at relatively low cost, while in the meantime aids advances the field of synthetic and biological chemistry.

Over the last century, organic synthesis and especially total syntheses of NP has developed from an art into a science[77] thanks to the continuously growth of synthetic methods and strategies, reagents and catalysts, mechanisms and theories and improved synthetic, separating and analytical tools. Semi-synthesis based on more abundant NP precursors as starting materials is still one of the best options when it comes to manufacturing NP of practical interest due to the often higher yields and lower cost as compared to total synthesis. Biosynthesis of NP has also been a tremendous field that is marching forward and aided by organic synthesis. Understanding the biosynthesis of NP means potential harnessing of a sustainable NP manufacturing tool which is extremely desirable and has been a theme in NP research. Whilst powered by synthetic chemistry, biosynthesis of NP in turn enlightens chemists to construct NP in more efficient manners by revealing how NP are built *in vivo*. The advances in all these fields have been comprehensive, but will only be illustrated by the landmark synthesis of terpenoid Taxol in the following text.

1.3 The total synthesis of Taxol as an exemplar.

Total synthesis of Taxol is a landmark in organic synthesis due to its structural complexity and potent antitumor activity. The Taxol scaffold consists of A (six-member), B (eight-member), C (six-member), D (four-member) four fused rings and a total number of 11 stereogenic centres including two on the side chain, Figure 3.

Construction of the ABCD ring itself is very challenging, let alone the number of stereochemistry that need to be correctly installed on the corresponding carbons. Although difficult as it is, the total synthesis of Taxol has been achieved by seven different groups, Robert A. Holton,[78, 79] K. C. Nicolaou,[80] Samuel Danishefsky,[81, 82] Paul Wender,[83] Isao Kuwajima,[84, 85] Teruaki Mukaiyama,[86] Takashi Takahashi[87]. All total syntheses of Taxol start with the construction of ABCD ring with quite different strategies and starting materials as illustrated in Figure 3.

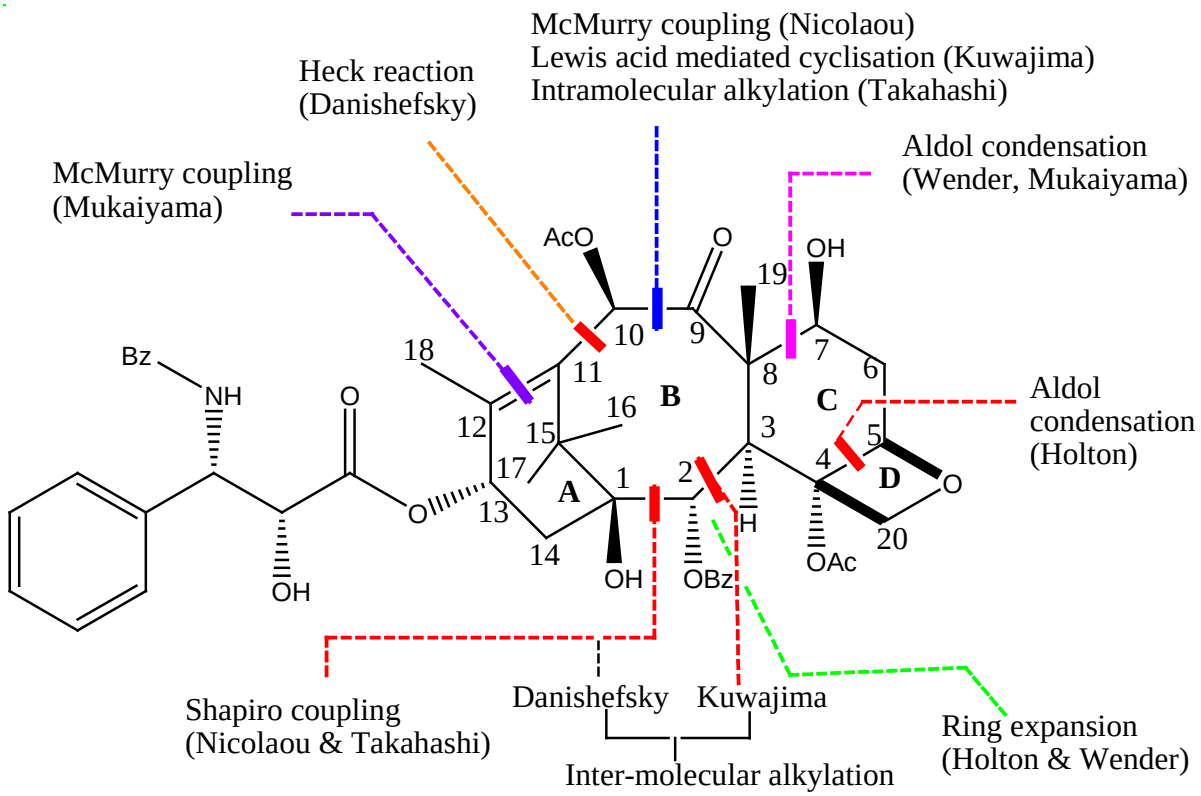
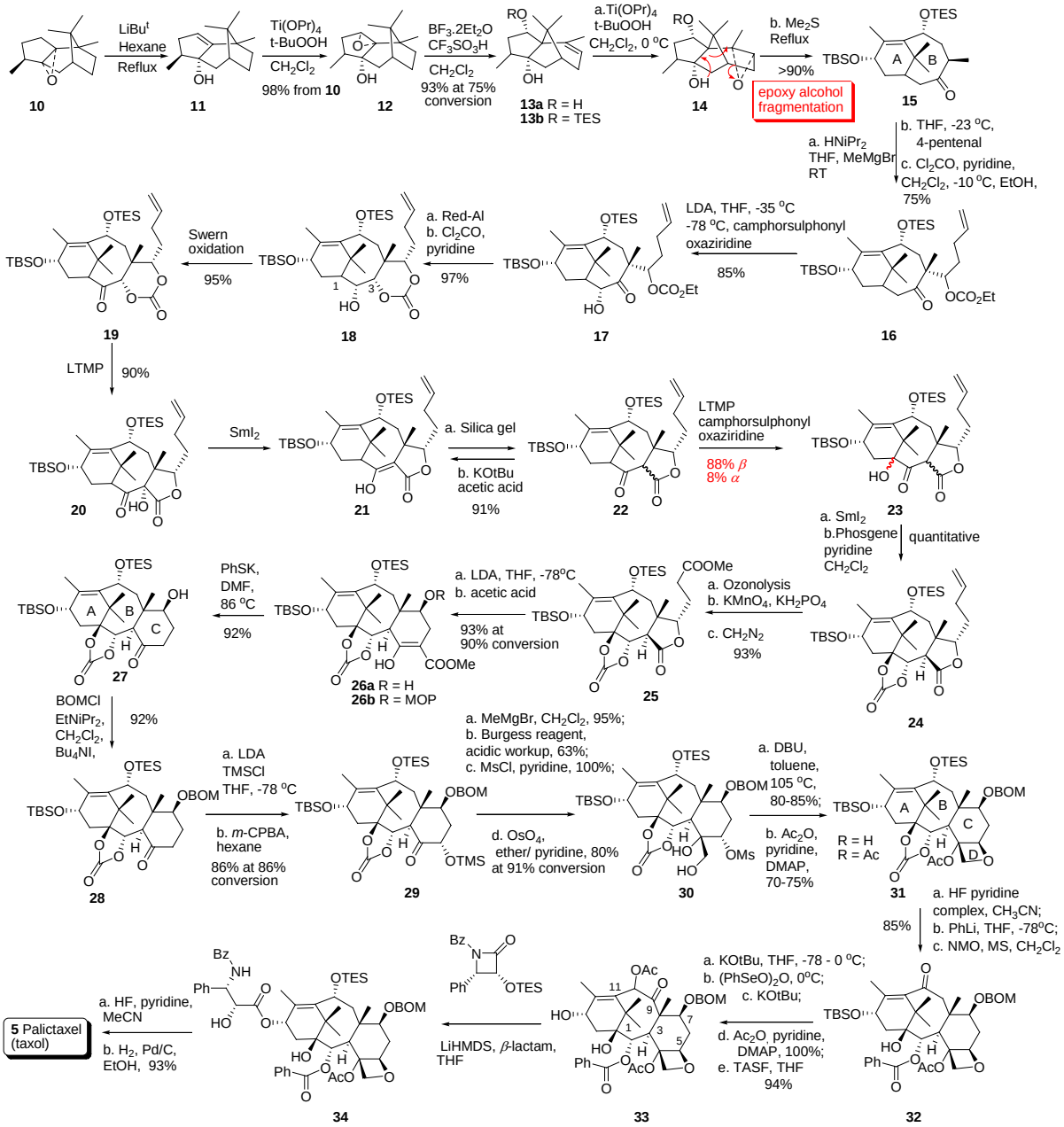


Figure 3. Different synthetic strategies in the total synthesis of Taxol.

1.3.1 Holton Taxol total synthesis.

The first total synthesis of Taxol was accomplished by the Holton group in 1994, Scheme 1. The total synthesis began with patchouli alcohol which was readily synthesised from camphor and was chosen as the best starting material to achieve relatively facile access to the AB ring skeleton.[88, 89] Further functionalisation of the B ring enabled construction of the CD ring.[78, 79] One key step in Holton's synthesis was the utilisation of 'epoxy alcohol fragmentation' to expand the [5,3,1] bicyclic core of patchouline oxide (**10**) into a [6,4,0]-bicyclic skeleton as the AB ring core of Taxol. Due to the great flexibility of the eight membered B ring, the readily synthesised AB ring system displayed four conformations and required the introduction of conformational control elements to maintain the favoured conformation for certain reactions. For example, the TES protecting group was introduced to narrow the energy difference between the chair-chair and boat-chair conformers so that

deprotonation of α carbonyl hydrogen of **15**, a step which could not take place without TES protection, could occur to furnish **16**.



Scheme 1. The Holton Taxol total synthesis.

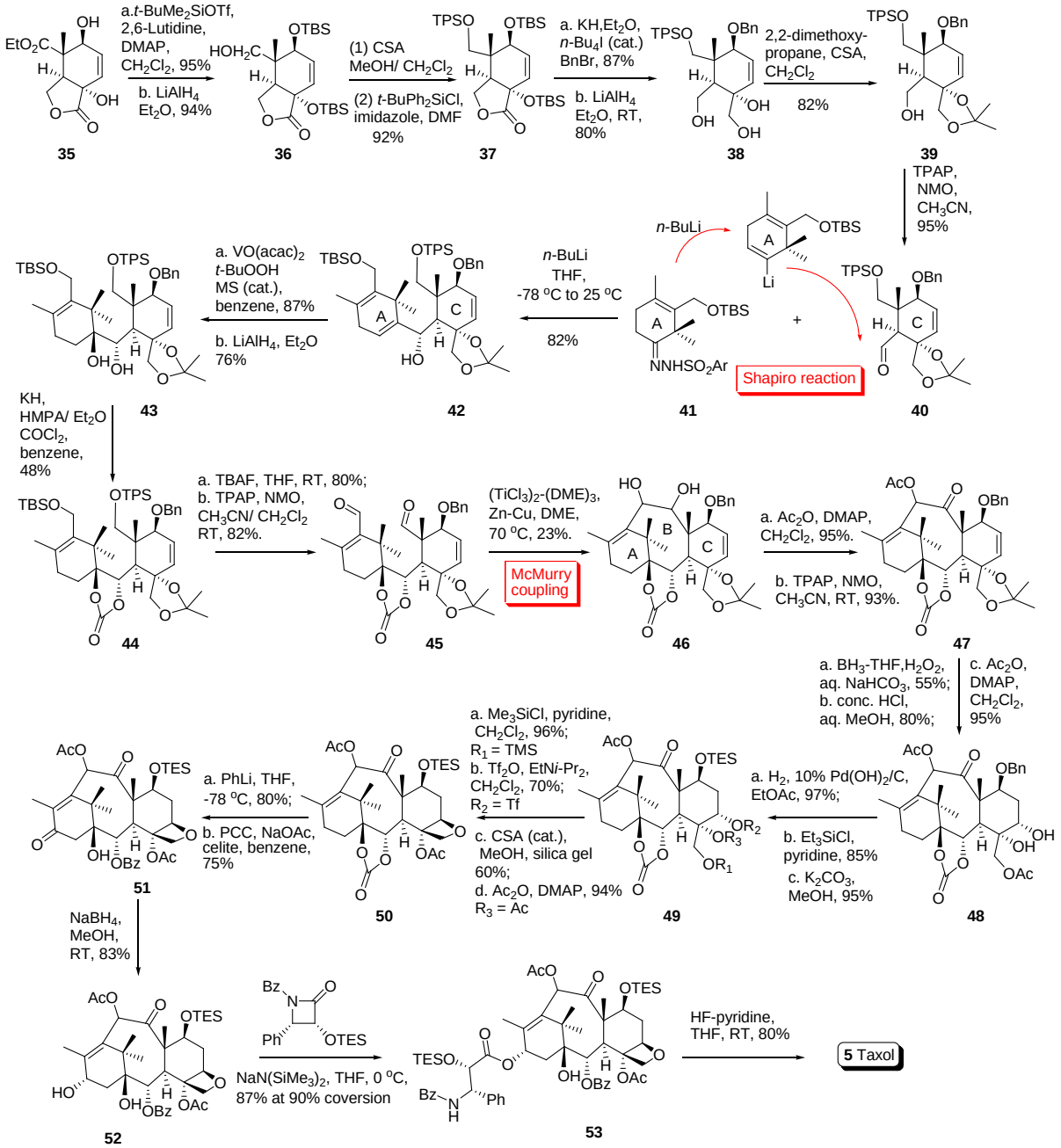
Moreover, Holton's synthesis exploited deprotonation of the α carbonyl hydrogen followed by hydroxylation as an important tool to achieve step-wise functionalisation of the B ring. After formation of the carbonate **18** by reduction and carbonation, the C2 alcohol was oxidised to generate the ketone (**19**) which activates the C1 hydrogen after some conformational adjustments and thus allows installation of the C1 hydroxyl functionality on

the bridged carbon C1 (**23**). The readily formed vicinal keto alcohol was protected as a carbonate (**24**) to avoid further depronation at undesired sites during the cyclisation process to build the C ring. Construction of the C ring (**27**) was achieved by a novel lactone ring opening with the *in situ* lactone and ester Aldol condensation followed by decarbomethoxylation. Before the construction of the D ring, the vulnerable hydroxyl moiety at C7 was protected as a BOM ether **28** due to the subsequent hostile reaction conditions. Moreover, the carbonyl moiety of **28** was again used to generate a vicinal protected alcohol **29** via an interesting enolate epoxidation and epoxy ring opening procedure. Methylation of **29** gave a tertiary methyl alcohol that was dehydrated and then dihydroxylated with OsO_4 to form diol **30**. Upon replacement of the protecting group of the C5 alcohol as a mesylate or tosylate, cyclisation took place to complete the construction of the D ring. Further functionalisation of the B ring, side chain esterification with *β*-lactam and deprotection furnished Taxol (**5**) and completed its assembly.

1.3.2 Nicolaou Taxol total synthesis.

Almost published at the same time in 1994, Nicolaou took a different approach by starting with the construction of the two flanked A and C rings which were then joined by Shapiro and McMurry coupling reactions to build the central flexible B ring and subsequently complete the synthesis with the installation of the four membered D ring and side chain, Scheme 2.[90-93] The Nicolaou Taxol total synthesis used a CD ring synthetic intermediate **35**[94] as the starting material to synthesise the flanked C ring aldehyde **40** for the Shapiro reaction. Lactone diol **35** was first protected with the bulky TBS group and its ester moiety further reduced with $LiAlH_4$ to form a primary alcohol. The C9 primary alcohol is the key building block for the late-stage McMurry coupling reaction and was first protected as a *tert* butyl diphenyl silyl ether **37**. Replacement of the TBS protecting group of the C7 alcohol with a benzyl grouping followed by lactone ring opening afforded triol **38**. Protection of the D ring diol as a ketal **39** exposes the C2 alcohol as the only non-protected functional group for oxidation and further coupling while in the meantime saves the diol for late stage D ring construction. Shapiro reaction of the C ring aldehyde **40** and the engineered A ring hydrazone connects the AC ring fragments to form **42**. Following an asymmetric epoxidation and ring opening procedure, the C1 alcohol functionality was successfully installed. Before exposing

the two C9 and C10 silyl ether protected alcohols for McMurry coupling, the C1 and C2 diol was converted into carbonate **44**. Oxidation of the deprotected side chain diol into a di-aldehyde enables McMurry coupling to take place to build the Taxol B ring **46**. Upon the complete assembly of ABC ring skeleton, B ring was further functionalised to form the keto acetate **47**.



CSA = (±)-camphorsulfonic acid; TRAP= tetra propylammonium perruthenate; NMO= *N*-methylmorpholine *N*-oxide; MS = Molecular sieves; TBAF = *tert* butylammonium fluoride; PCC = pyridinium chlorochromate; Tf_2O = triflate anhydride.

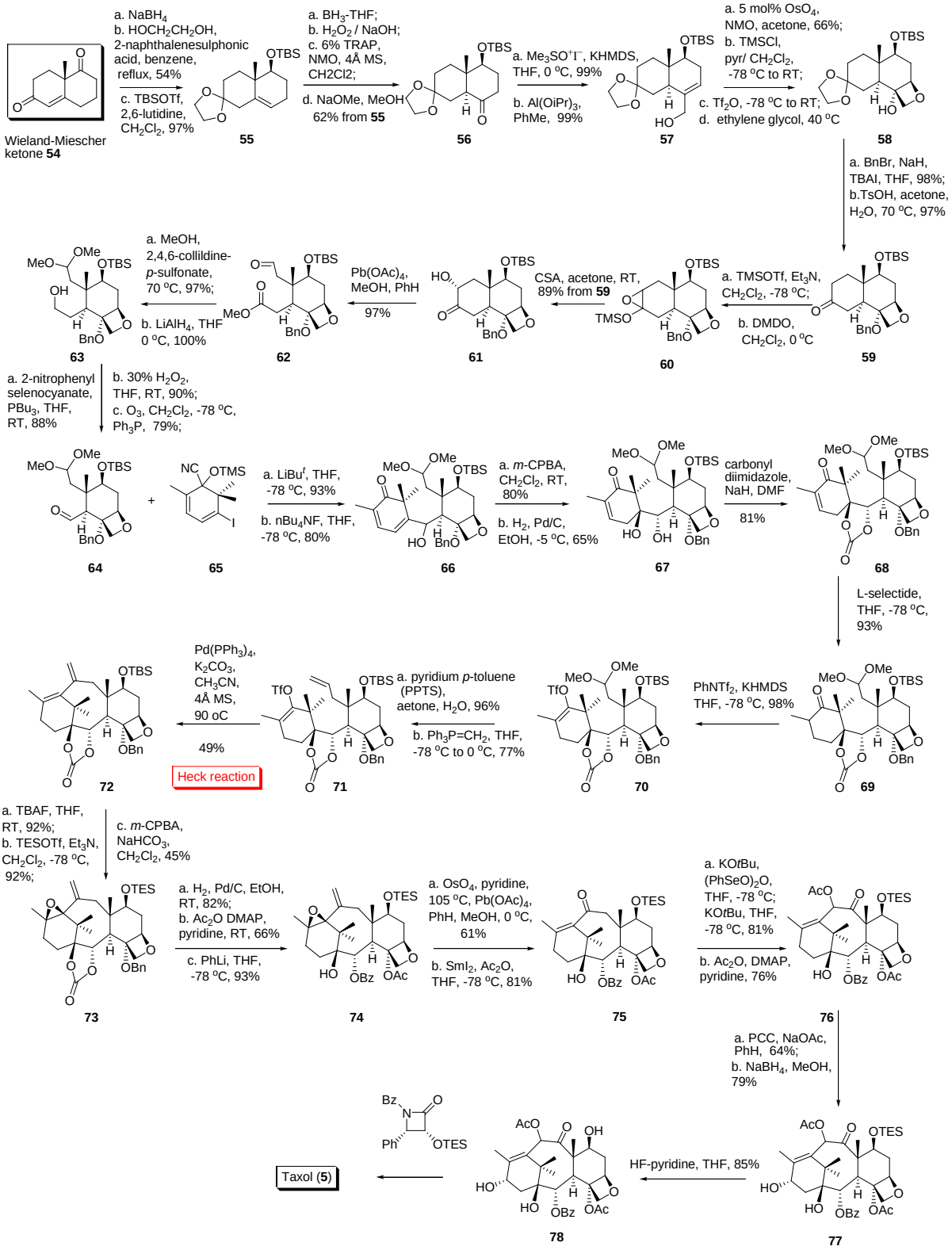
Scheme 2. The Nicolaou Taxol total synthesis.

Construction of the Taxol D ring proved to be rather difficult as different protecting groups of the C7 hydroxyl influenced the ring conformations of the AB ring as noted in the Holton synthesis and would result various oxetane products when constructing the D ring. Henceforth, the C7 benzyl ether was replaced with a triethylsilyl ether **49.** Acid catalysed intramolecular cyclisation of the triflate ester and TMS ether **49** furnished the Taxol D ring **50** in 60% yield. Since the ABCD ring skeleton had been successfully assembled, the C1 and C2 carbonate could be converted into the tertiary alcohol **51** due to the less severe conditions used in the subsequent reactions. Installation of the hydroxyl in the A ring enables coupling with β-lactam installs the side chain and final acid catalysed desilylation completed the total synthesis of Taxol (**5**).

1.3.3 Danishefsky's Taxol total synthesis.

Danishefsky published the total synthesis of Taxol in 1996 as the third group to accomplish the total synthesis of Taxol, Scheme 3.[81, 82] The synthetic strategy employed by Danishefsky somewhat resembles Nicolaou's in that they both assembled the baccatin ABCD ring via coupling reactions of the readily built flanked AC rings. Differing from Nicolaou, Danishefsky built the CD rings prior to its coupling to the A ring fragment.[95, 96] In comparison to the Shapiro reaction and McMurry coupling utilised in Nicolaou's total synthesis, Danishefsky exploited an alkyl lithium aldehyde coupling and a Heck reaction to connect the AC ring fragment and form the B ring. Starting with the commercially available Wieland-Miescher ketone **54**, its 6,6-bicyclic ring structure was used to build the CD ring backbone of Taxol and also offers the C ring side chains necessary for the coupling reactions to construct the eight-membered B ring. Ketalisation of **54** resulted in the double bond migration to give **55**[97] which underwent hydroboration and oxidation to generate the C4 ketone **56**. In order to construct the D ring, the C4 carbonyl was converted into the primary alcohol **57** employing an improved Corey's oxirane synthesis[98] which transferred the methylene group via sulfonium iodide addition to carbonyls and which was followed by epoxy ring opening with $Al(O\textit{i}\text{-}Pr)_3$ under reflux. Upon the completion of the D ring oxetane synthesis, the 6,6-bicyclic ring was ring-opened by oxidative cleavage of the vicinal keto alcohol **61** to form the keto ester **62**. Protection of the aldehyde **62** as a dimethyl acetal followed by dehydration of Grieco's selenide[99] and ozonolysis afforded the C2 aldehyde **64** and allows for the Shapiro coupling reaction with the diene A ring fragment **65**. Subsequent

functionalisation of the A ring enables the installation of the C1 hydoxyl moiety which was protected as a carbonate **68**.



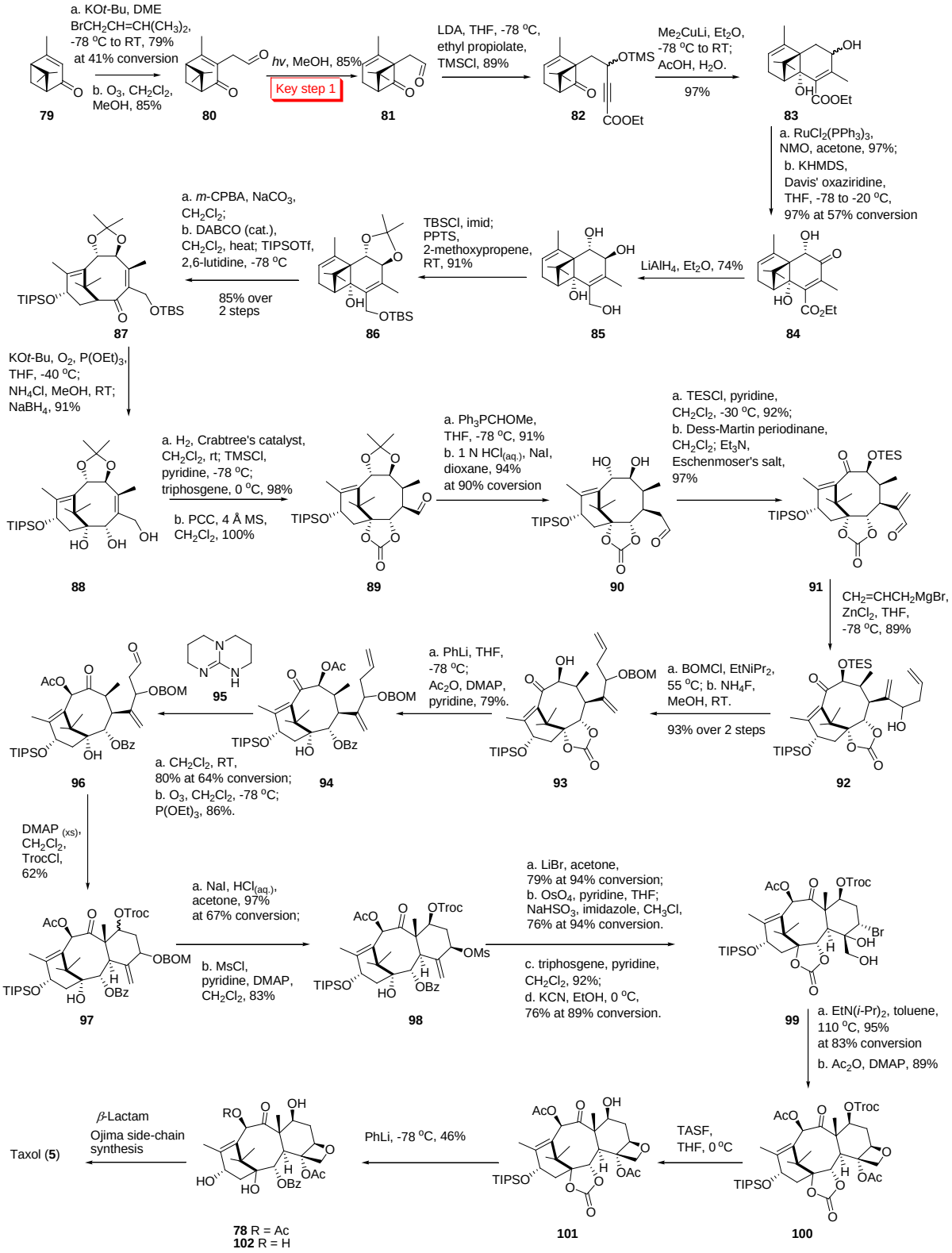
Scheme 3. The Danishefsky Taxol total synthesis.

Danishefsky's synthesis utilised the C11 carbonyl moiety in the A ring to generate an enol triflate **71** instead of a halide so that Heck coupling with the C ring side chain olefin could take place smoothly. At this stage the assembly of the Taxol ABCD ring skeleton had been completed. The remaining task was to further functionalise the B ring and install the C13 allylic alcohol for side chain coupling. Before the oxidative cleavage of the B ring olefin to form ketone **75**, the C ring TBS protecting group was replaced with TES due to the difficulty of removal of TBS once the ABCD ring core was fully functionalised, The double bond in the A ring was masked as an epoxide **73**, the carbonate ring opened and the D ring benzyl ether was replaced by acetate **74**. Oxidative cleavage product **75** was converted to the vicinal keto selenyl ester whose selenyl moiety was replaced by acetyl to furnish **76**. Allylic oxidation of **76** followed by desiylation with HF-pyridine complex furnished baccatin (**78**). Coupling of **78** with β-lactam using Ojima's method[100] furnish Taxol **1**.

1.3.4 Wender Taxol total synthesis-the pinene path.

Five years after their first discovery of using pinene to access the ABC tricyclic taxane core in 1992,[101] Paul Wender's group completed the pinene path to the Taxol total synthesis.[83, 102] Similar to Holton's Taxol total synthesis, Wender's synthesis assembled the taxane ABCD ring in a liner order. The synthesis began with the commercially available cyclohexyl verbenone (**79**), an oxidation product of the pine tree principle pinene. The advantage of using verbenone as the starting material is its inbuilt gem dimethyl on the four-membered ring within a six-member ring and a ketone moiety already installed for the attachment of a C ring precursor. Moreover, after adding the vinyl substituted side chain (**80**), the relocation (1,3-alkyl shift) of the gem dimethyl substituted C15 from C13 to C11 (**81**) could be realised by simple photochemical rearrangement.[101] An important theme in the Wender's synthesis is the Aldol type cyclisation as the ring construction tool. The first cyclisation was the intramolecular alkynic carbanion nucleophilic addition onto the carbonyl carbocation effected by Me_2CuLi to form a [4.4.2.0] tricyclic **83**. Oxidation of the C9 hydroxyl into a ketone allows for vicinal C10 hydroxylation with Davis' oxiziridine to furnish **84**. Its carbonyl moiety was reduced into a hydroxyl and together with the C10 hydroxyl protected as a ketal **86**. Ring expansion of the B ring precursor via epoxy ring fragmentation induced by DABCO was succeeded with *in situ* TBS protection of the C4 alcohol and furnished the AB ring core

87. The C1 hydroxyl moiety was then installed via an enolate hydroxylation to generate a diol **88** and protected as a carbonate **89** before further construction of the C ring.



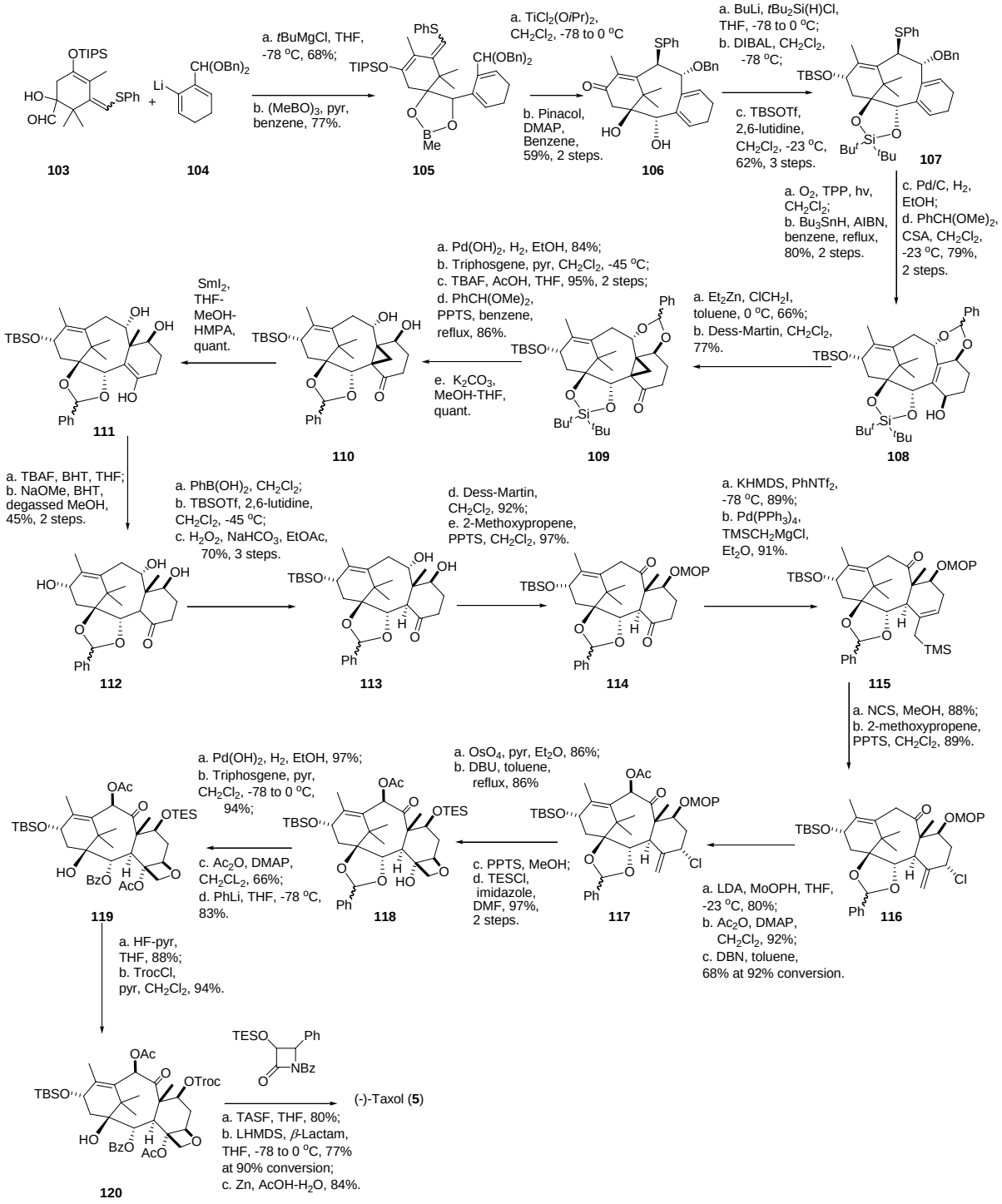
Scheme 4. The Wender Taxol total synthesis.

Wender's strategy in attaching the C ring onto the readily formed AB ring was to build a C ring Aldol cyclisation precursor that also has the necessary branches to enable D ring oxetane construction after C ring closure. Therefore, the C4-C20 olefin (**91**) essential for D ring construction was introduced effectively with Eschenmoser's salt based on aldehyde **90** formed by elongation of the B ring side chain aldehyde with a methoxy methyl ylide. Further elongation of the B ring side chain by Grignard reaction to afford **92** was followed by the ring opening of cyclic carbonate (**94**) to resettle the C8 hydrogen in a better spacial orientation and the reorganisation of the protecting group of the C9 hydroxyl to allow an intriguing guanidinium base **95** induced acetoxyketone transposition to relocate the carbonyl moiety from C10 to C9 (**96**) and set up for Aldol condensation ring closure. C ring formation by Aldol cyclisation (**97**) was accompanied with the *in situ* C7 hydroxy protection with TrocCl. Formation of the D ring oxetane would require the installation of the C20 hydroxyl and a good leaving group at C5 for nucleophilic ring closure. Moreover, the C2 benzoate was found to migrate onto the C20 hydroxyl during the D ring closure reaction. Therefore, the C1 and C2 oxygenated functionality was protected as a carbonate **98** using triphosgene before oxetane formation (**100**) between the C20 hydroxyl and bromine bearing C5 could take place. At this stage, the ABCD ring skeleton had been assembled. Removal of the protecting groups on the ABC ring yielded baccatin which was coupled with *β*-Lactam using Ojima's method to finalise the Wender Taxol total synthesis.

1.3.5 Kuwajima Taxol total synthesis.

Kuwajima completed the total synthesis of Taxol in 1998 and became the fifth group to overcome this challenging task, Scheme 5. Kuwajima's synthesis constructed the ABDC ring in the same order (AC-ABC-ABCD) as Nicolaou. In connecting the AC fragments, Kuwajima utilised a Mg^{2+} mediated nucleophilic reaction between the C ring alkyl lithium carbanion and the A ring aldehyde carbenium, which is similar to the alkyl lithium coupling reaction used in the Danishefsky's Taxol synthesis, whereas the Lewis acid induced cyclisation of the vinyl phenyl thiol ether with a dibenzyl ketal to close the B ring was secured with $TiCl_2(O\textit{i}Pr)_2$. Instead of constructing the bridged head C19 methyl on the C ring before B ring closure, Kuwajima exploited a ring cleavage of the keto cyclopropane which was preceded by cyclopropanation of the bridged double bond introduced from peroxidation

of conjugative diene **107** and subsequent reduction. In the course of constructing the C4 and C20 diols, a fragment commonly used for the D ring oxetane formation, it was found that the C10 acetate group was essential in creating a crowded environment surrounding the C11 and C12 double bond to subject the C4 and C20 double bond to prioritised dihydroxylation and cyclisation to reveal **118**. Further deprotection and side chain coupling furnished Taxol (**5**).



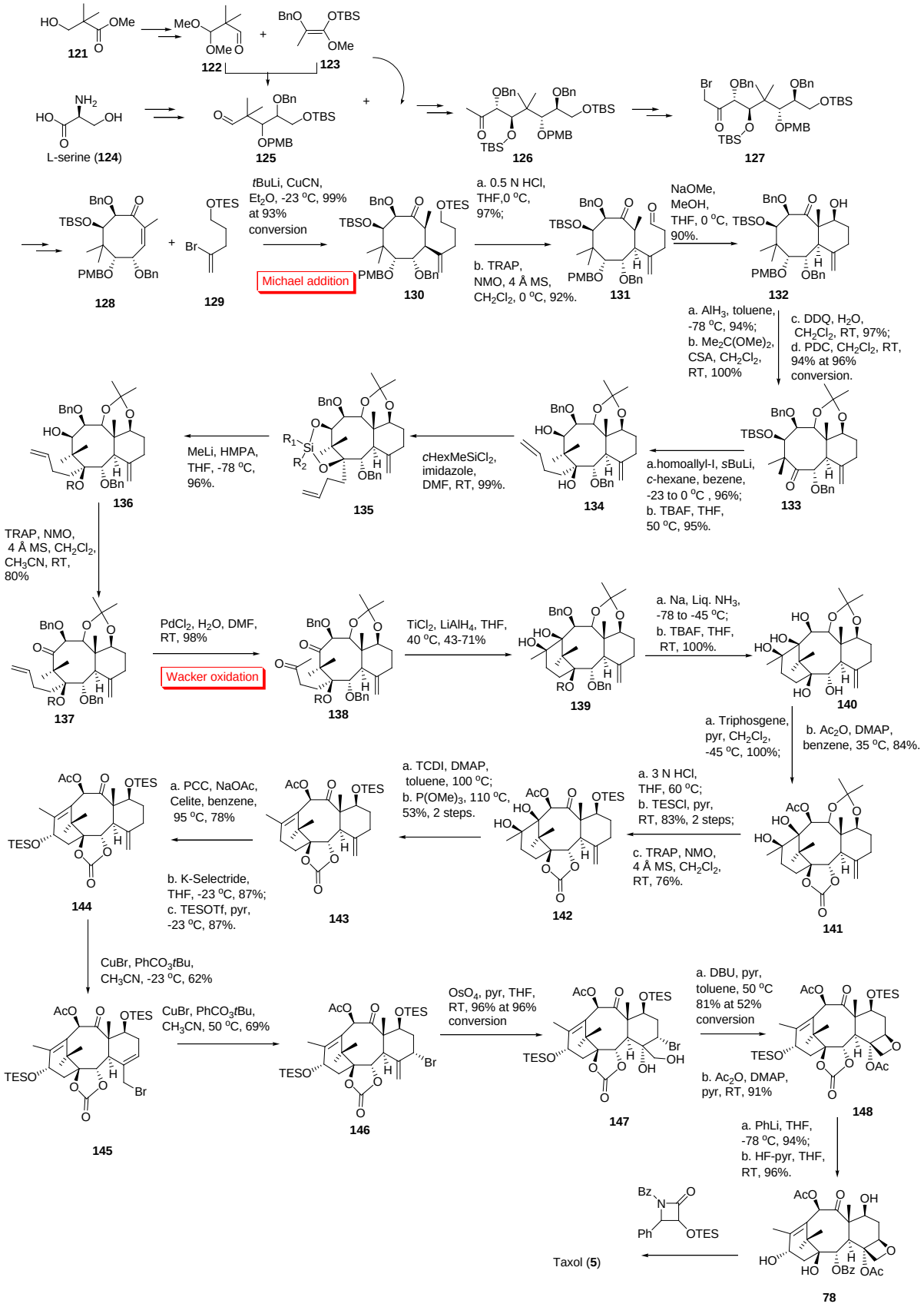
Scheme 5. The Kuwajima Taxol total synthesis.

1.3.6 Mukaiyama Taxol total synthesis.

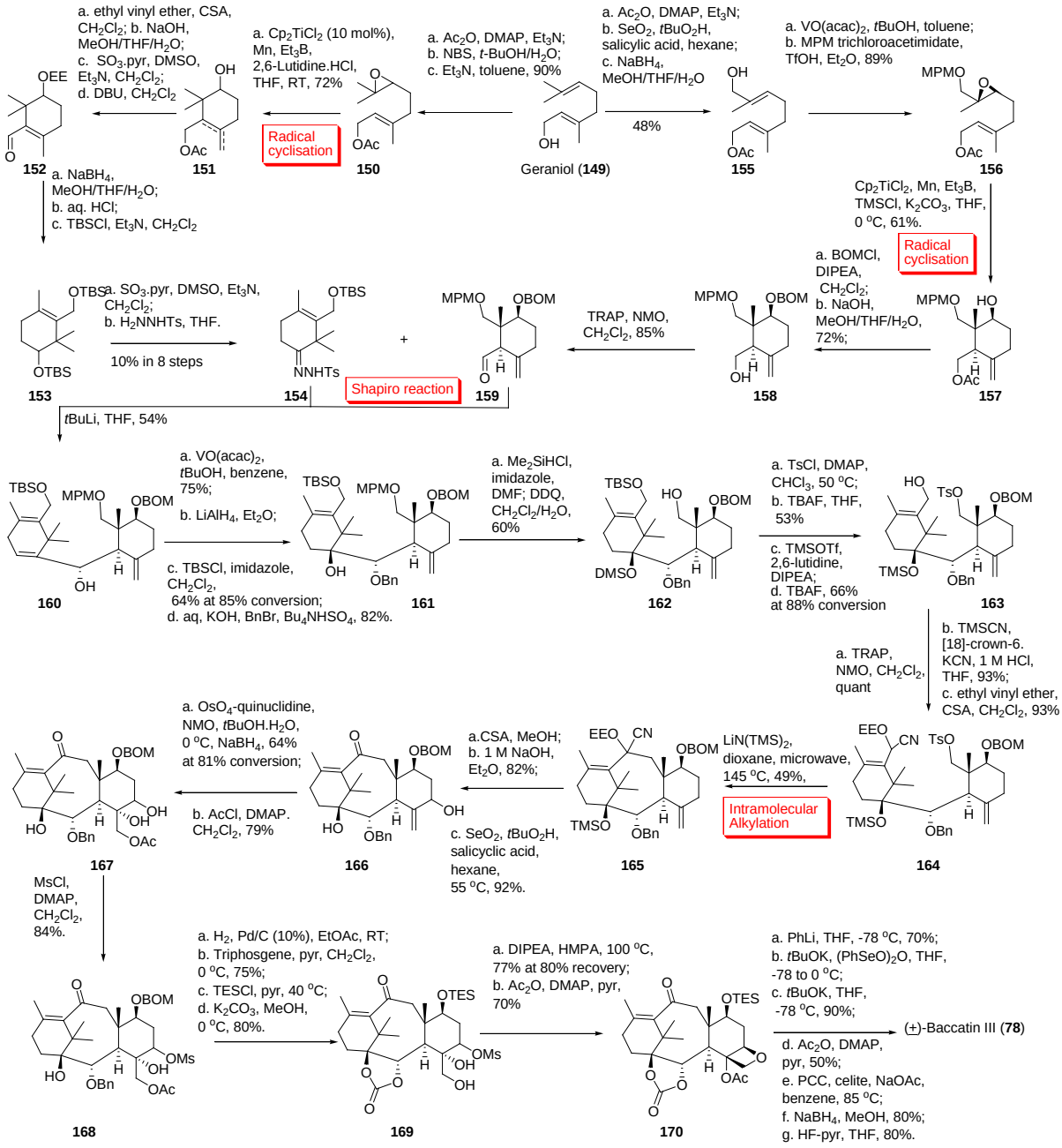
The Mukaiyama Taxol total synthesis was completed in 1999. Adopting a strategy with B ring construction as the starting point followed by A, C and D ring elaboration, Mukaiyama's synthesis is distinctive among all Taxol total syntheses, Scheme 6.[86] Starting with L-serine, Mukaiyama built a fully functionalised and well protected eight membered ring **128** in 23 steps. Ring **128** has the C1 and C2 hydroxyl protected, the C9 and C10 oxygenated moieties readily installed and protected, and the C11 oxygenated functionality and C3-C8 double bond available for further A, C ring construction. The C ring backbone **129** was attached onto the B ring via Michael addition and the ring closure (**132**) imposed by Aldol condensation. The A ring backbone was attached onto the B ring C1 (**133)** via Grignard reaction and ring closure effected via McMurry coupling. Further functional group manipulation enables the introduction of the D ring diol and C5 bromine leaving group for nucleophilic cyclisation to form the D ring oxetane. Deprotection of carbonate **148** revealed baccatin and which was readily transformed into Taxol (**5**).

1.3.7 Takahashi Taxol total synthesis.

Takahashi's group completed a formal synthesis of Taxol in 2006 using geraniol as the starting material.[87] The synthesis features radical cyclisation as a key step for both A and C ring closure, Shapiro reaction and intramolecular alkylation for B ring construction and an automated synthesiser for multi-step large scale synthesis and auto-purification. Both the A and C ring fragments could be synthesised from geraniol via an Cp_2TiCl_2 induced intra-molecular cyclisation of the epoxy olefins **150** and **156** to form the desired six-membered ring secondary and primary alcohols **151** and **157**, respectively. Secondary alcohol **151** was transformed into a hydrazone **154** after protection, deprotection and oxidation, whereas primary alcohol **157** was oxidised into aldehyde **159** for subsequent Shapiro reaction to connect the A and C ring fragments. In closing the B ring, Takahashi exploited an interesting intramolecular alkylation of cyanohydrin effected by $LiN(SiMe_3)_2$ at elevated temperatures.[103, 104] (±)-Baccatin was synthesised following further functional group modifications and D ring construction in a similar manner as detailed for the other total syntheses described above.



Scheme 6. The Mukaiyama Taxol total synthesis.



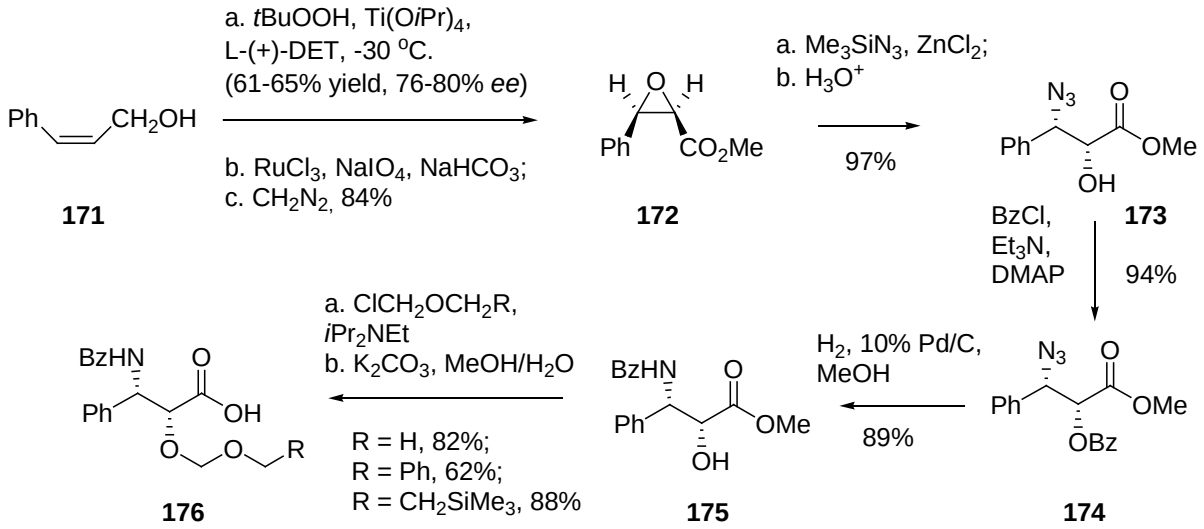
Scheme 7. The Takahashi Taxol total synthesis.

1.4 Semi-synthesis of Taxol and side chain strategies.

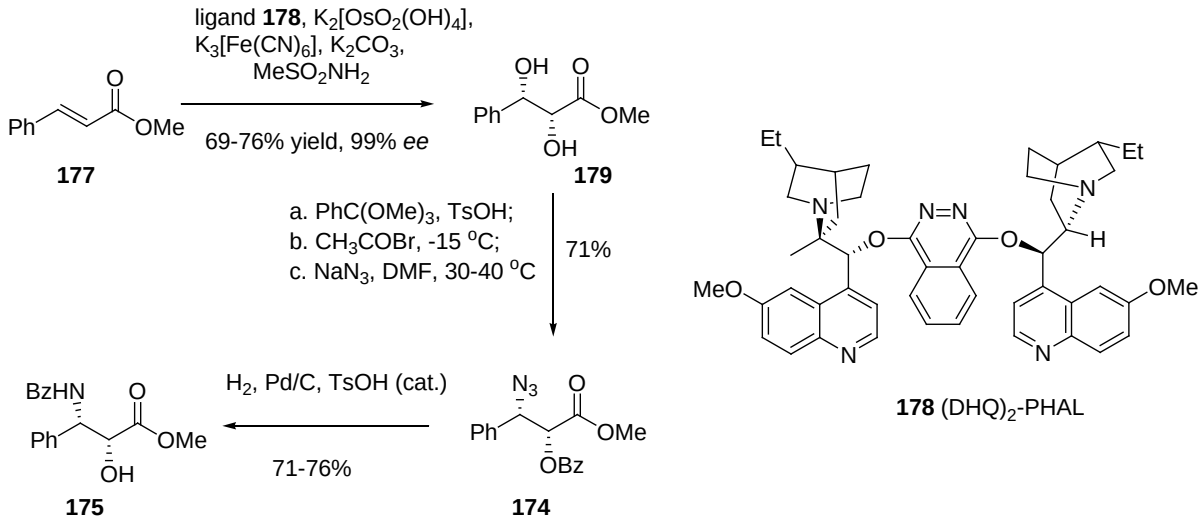
The total syntheses of Taxol summerised above showcase the power of synthetic organic chemistry and have generated a pool of synthetic tools along the way. However, total syntheses simply can not meet the demand of the drug market owing to the low yields (up to 0.4%), multiple steps (35 – 51 steps) and numerous reagents required in completing these total syntheses. Semi-synthesis from more naturally abundant taxane precursors such as 10-deacetyl baccatin III (**102**) and baccatin III (**78**) serves as a practical alternative for the

production of Taxol. Among the nine species of the *Taxus* family, over 550 taxanes with various ring scaffolds have been isolated to date.[105] 10-Deacetyl baccatin III (**102**) preserves the core ABCD ring structures of Taxol itself and is more abundant and can be isolated from the leaves, needles and twigs of yew trees in a more sustainable fashion in yields of ca. 1 g/kg for leaves.[106-109] The abundance of this precursor **102** has led to the development of a number of semisynthetic protocols to access Taxol, many of which have focused on the efficient synthesis of the side chain for final coupling and are summarised below.

In 1986, Greene was the first to synthesise the side chain utilising Sharpless's asymmetric epoxidation of olefinic alcohol **171** followed by ring opening with Me_3SiN_3 and hydrogenation to introduce the amine group (**175**), Scheme 8. Protection of the C2' alcohol and C3' amine furnished the side chain precursor **176**.[110] Whilst Greene only achieved 76-80% *ee* in the epoxidation step, Sharpless realised the asymmetric dihydroxylation of olefinic ester **177** with 99% *ee* by employing a chiral metal complex to direct the installation of the hydroxyl moiety, Scheme 9. The installed C3' hydroxyl was converted to an azide and which was hydrogenated to furnish the side chain precursor **175** in 35-41% yields.[111]

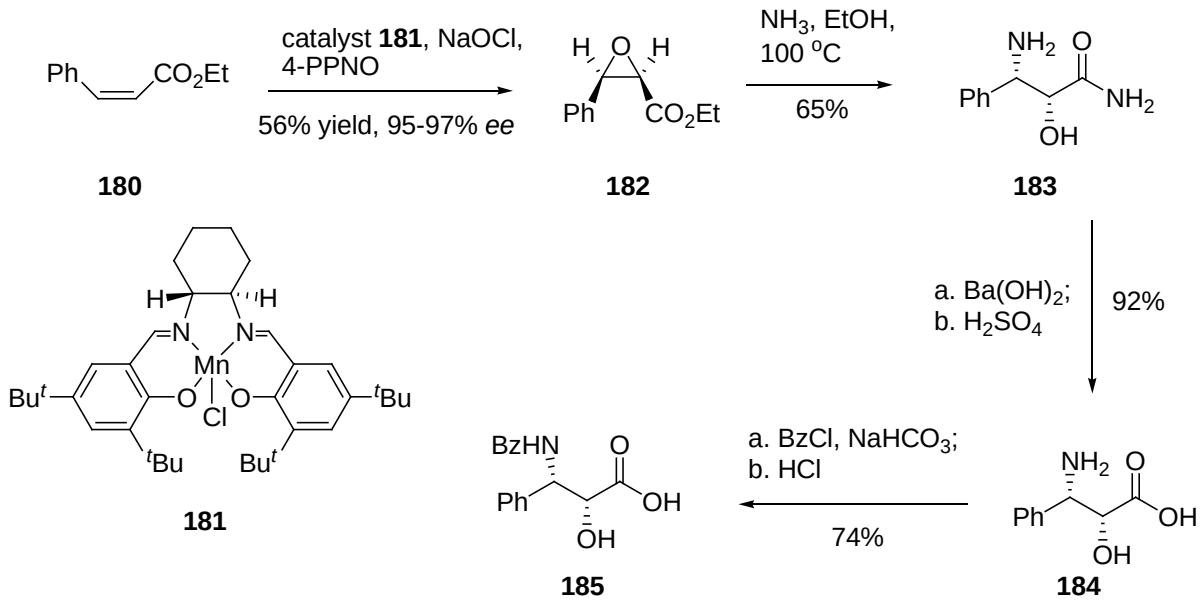


Scheme 8. Greene's side chain synthesis via azide-induced epoxy ring opening in 1986.



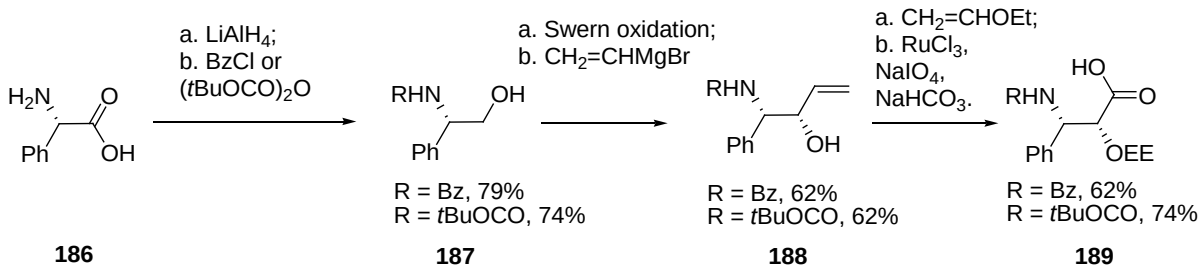
Scheme 9. Sharpless's side chain synthesis through asymmetric dihydroxylation in 1994.

Jacobsen also succeeded in obtaining high *ee* in the epoxidation of olefinic aryl ester **180** via the use of a chiral manganese metal complex **181**, Scheme 10. Unlike Greene and Sharpless who used azide for introducing the amino group, Jacobsen exploited ammonia as the nucleophile at high temperature to open the epoxide ring and enable the installation of the C3' amino group (**183**). The terminal amide was then converted into an acid **184** and subsequent benzoylation furnished the side chain precursor **185**.

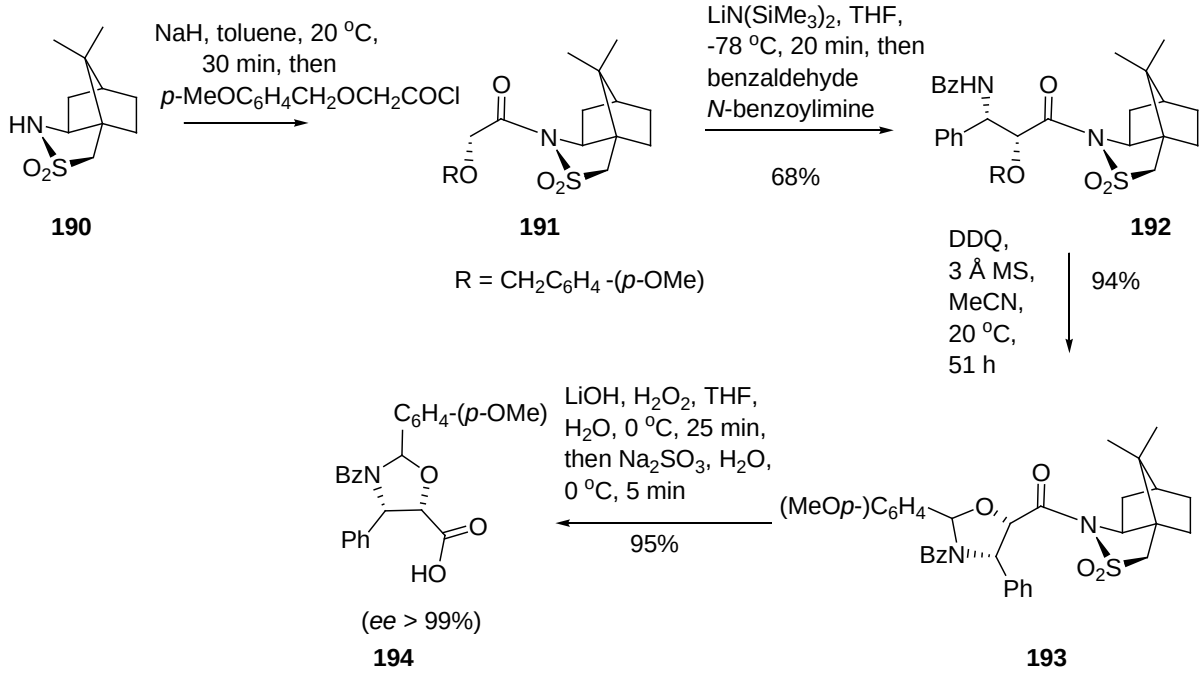


Scheme 10. Jacobsen's Taxol semisynthesis through asymmetric epoxidation in 1992.

Apart from the epoxidation and ring opening sequence, Greene also utilised phenylglycine as a starting material for the side chain synthesis, Scheme 11. Phenylglycine was first benzoylated and the acid moiety reduced to the corresponding alcohol **187**. Swern oxidation of the alcohol to the aldehyde allows for a Grignard reaction to form the secondary alcohol **188** which was protected as an OEE ether for further coupling. Moreover, Greene also developed a protocol to synthesise the side chain protected precursor in an oxazolidine form for better coupling with higher *ee* in 1994, Scheme 12. The synthesis began with the commercially available Oppozler's camphorsultam[112] and emploed an enolate-imine and Aldol condensation as key steps for elongating the carbon chain. DDQ induced intramolecular cyclisation generated oxazolidine **193** which was hydrolysed to afford the oxazolidine **194** with 99% *ee*.[113]

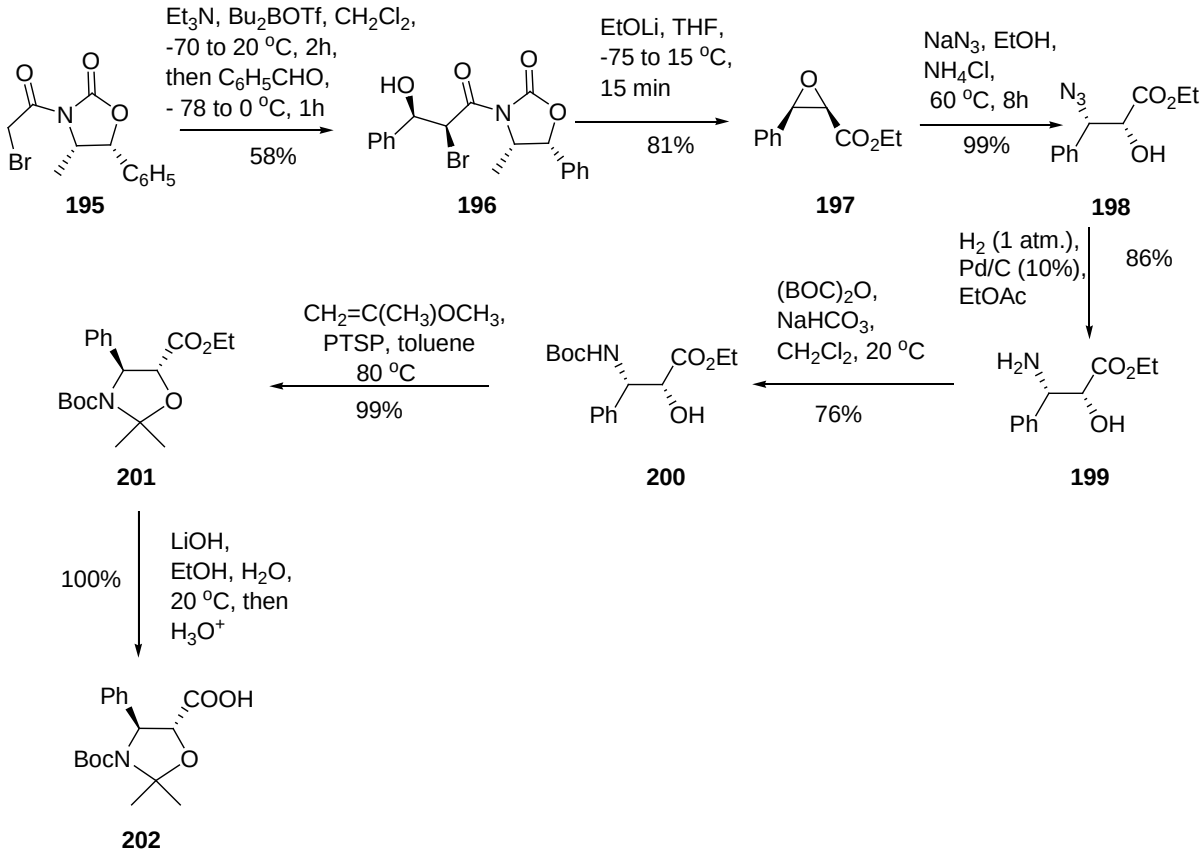


Scheme 11. Greene's side chain synthesis from phenylglycine in 1992.



Scheme 12. Greene's Taxol side chain synthesis from Oppozler's camphorsultam in 1994.

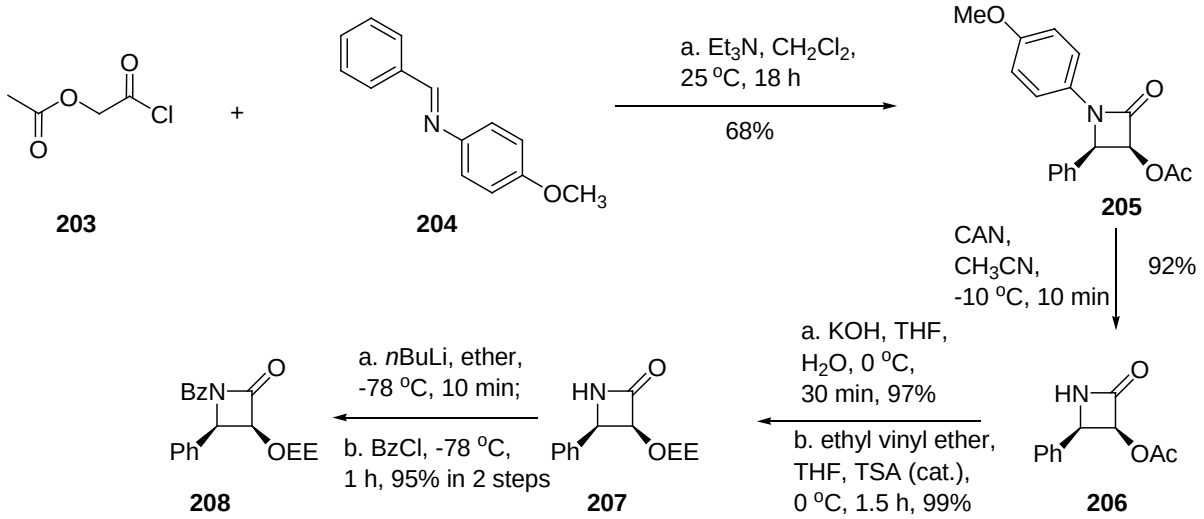
Similar to Greene in choice of using an oxazolidine as the coupling side chain precursor, Commerçon prepared the *N*,*O*-protected *β*-phenylisoserine **202** with very high stereoselectivity from the oxazolidine **195** synthesised using Evan's method, Scheme 13.[114, 115] Aldol condensation of the boronic enolate of **195** with benzaldehyde gave bromohydrin **196** which underwent cyclisation and simultaneous esterification to afford epoxide **197** in 81% yield. Epoxy ring opening with NaN_3 followed by hydrogenation and amine protection yielded a Boc protected amino alcohol. PTSP catalysed cyclic protection with methoxypropene generated the *N*,*O*-protected oxazolidine **201** which was then hydrolysed to furnish the free acid **202** for coupling.



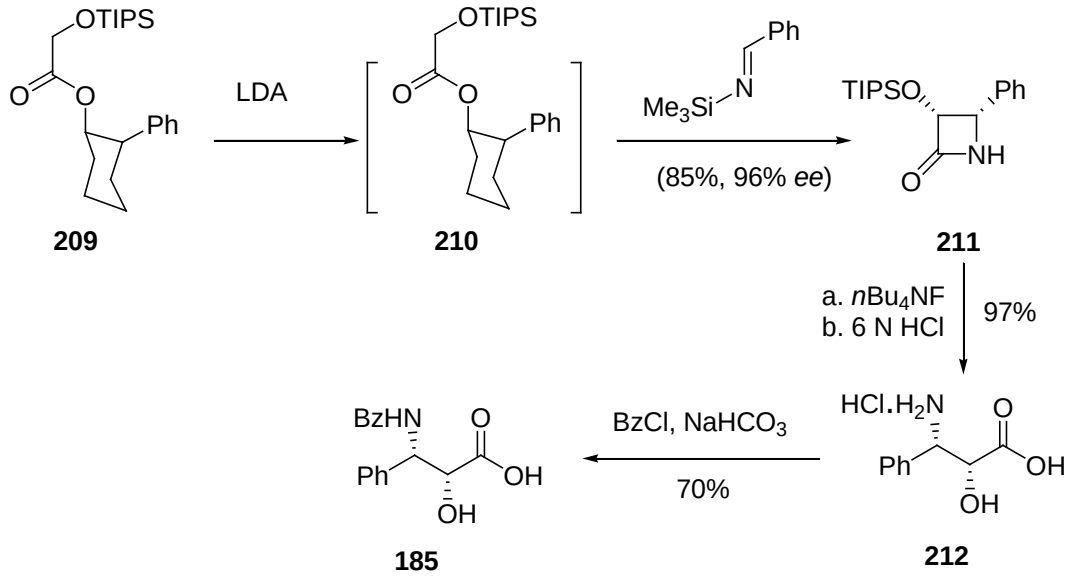
Scheme 13. Commerçon's side chain synthesis in 1992.

Holton[116] and Ojima[100, 117] chose to develop a *β*-lactam as the side chain coupling precursor to reserve the stereochemistry of the C2' alcohol and reduce epimerisation. Lactam **205** was easily prepared by base induced lactamisation of imine **204** with acetoxyacetyl chloride in 68% yield under mild conditions, Scheme 14. Oxidative cleavage of the *N*-methoxyphenyl grouping with CAN, replacement of the hydroxyl protecting group with vinyl ethoxy ether

and final benzoylation furnished *β* lactam **208** in a very high 87% yield over 4 steps. This efficient side chain synthesis allowed for the commercial production of Taxol via semi-synthesis in bulk and aided the supply shortage. In comparison to Holton's *β*-lactam synthesis, Ojima achieved better enantioselectivity and shortened the procedure by exploiting a chiral auxillary and bulky *O*-protecting groups, Scheme 15. The phenylcyclohexyl acts as the chiral auxillary together with the *O*-TIPS protecting group on **209** directing the enolate-imine cyclocondensation in an enantioselective manner to give the *O*-protected lactam in 85% yield with 96% *ee*. Further deprotection and hydrolysis furnished **185**.

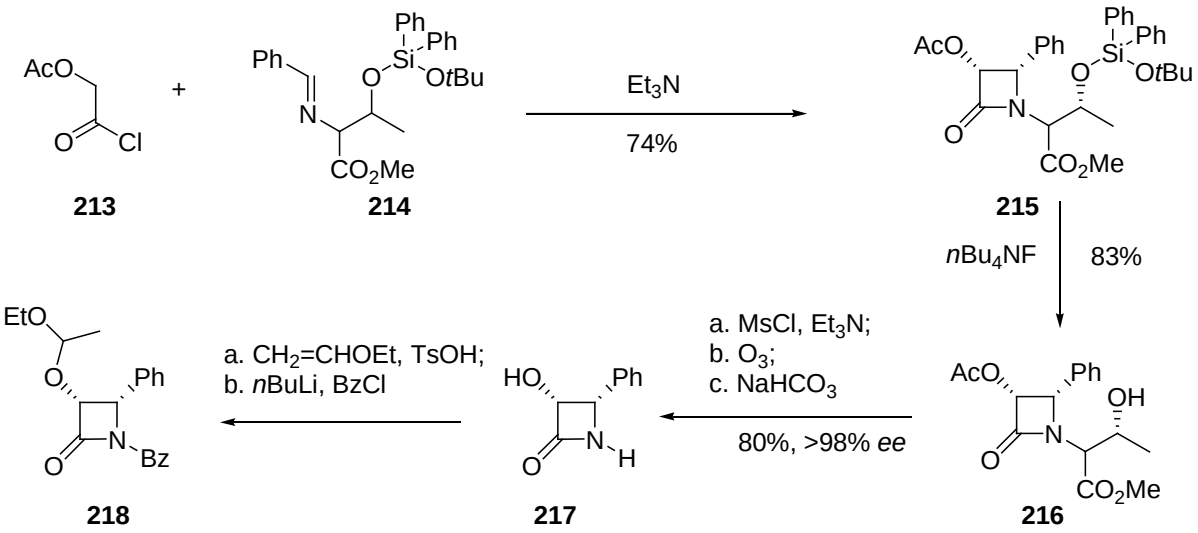


Scheme 14. Holton *β*-lactam synthesis in 1990.

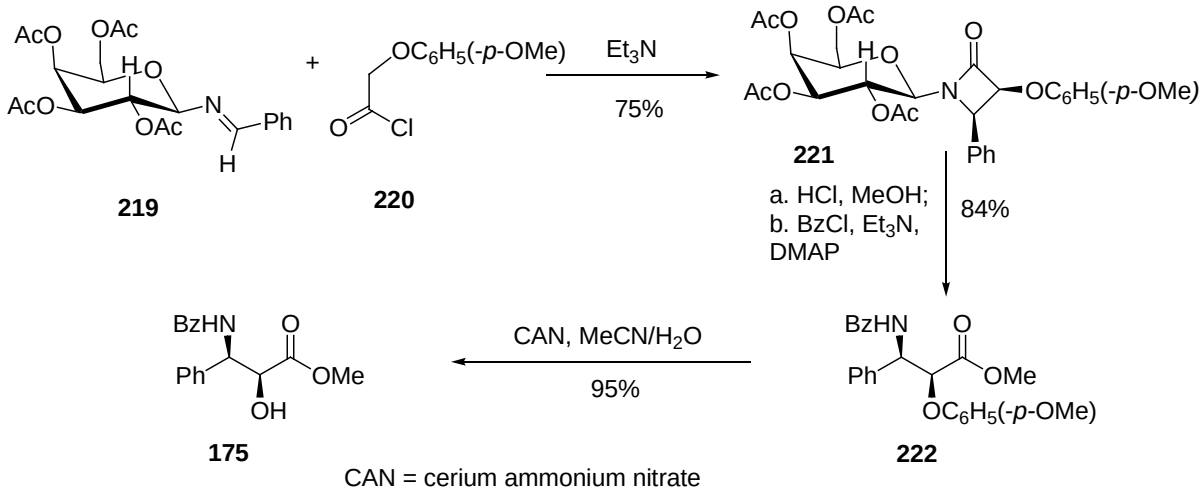


Scheme 15. Ojima Taxol side chain synthesis via *β*-lactam in 1992.

The approach of utilising bulky protecting groups as the chiral auxillary was also demonstrated in the *β*-lactam synthesis by Farina[118] and Georg.[119-122] Farina achieved high enantioselectivity (98% *ee*) in the enolate-imine cyclocondensation by applying a bulky protecting *O*-silyl group. Removal of chiral auxiliaries followed by dehydration and ozonolysis and subsequent hydrolysis and benzoylation yielded the suitable lactam **218** for coupling with baccatin III. Similar to the phenylcyclohexyl chiral auxiliary in Ojima's *β*-lactam synthesis, Georg employed a carbohydrate as the chiral auxiliary in constructing the *β*-lactam but this resulted in the formation of non-natural enantiomer in 60% yield and high *ee*.

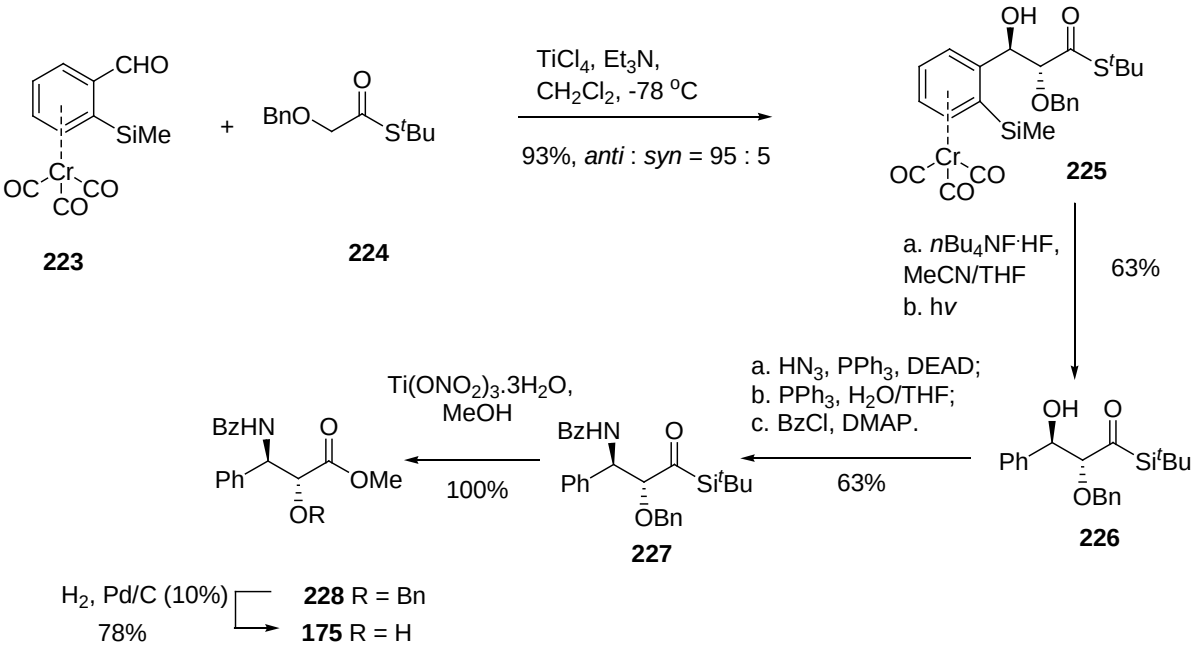


Scheme 16. Farina's taxol side chain synthesis in 1992.

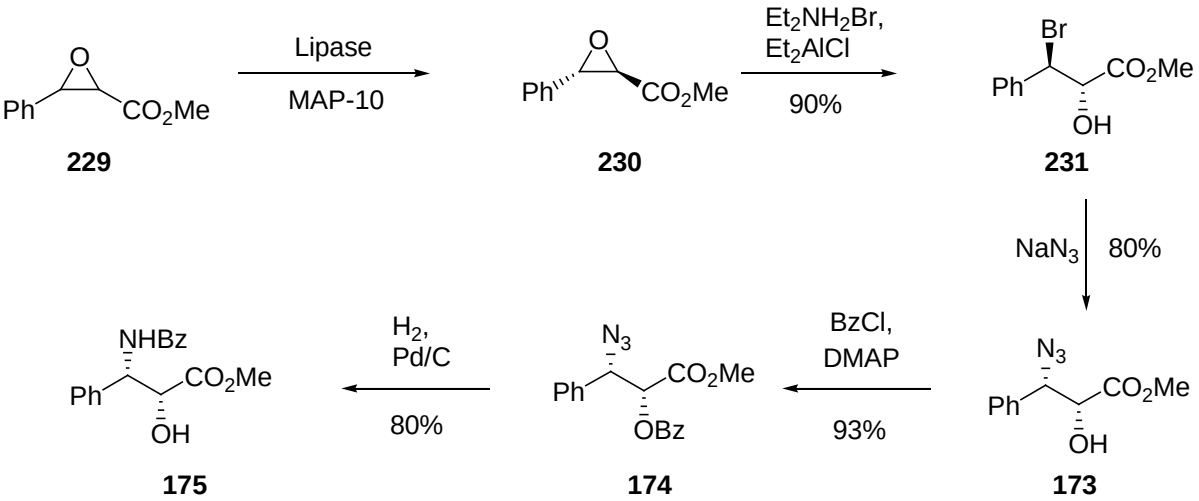


Scheme 17. Georg's taxol side chain synthesis in 1992.

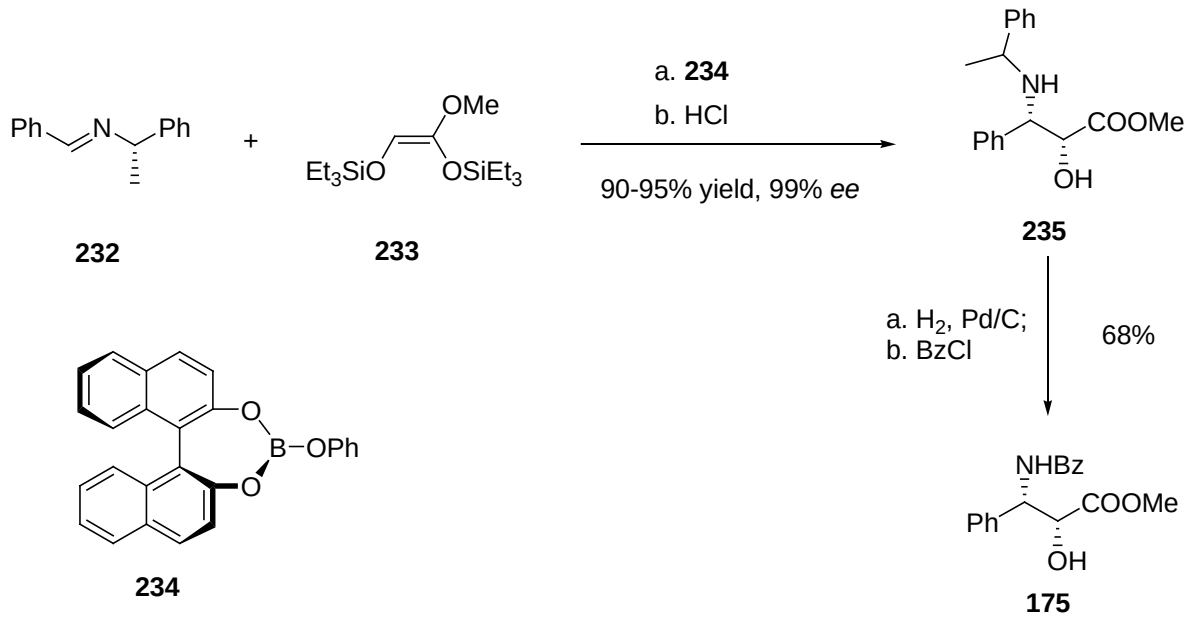
Besides utilising bulky protecting groups as a chiral auxiliary, metal complexes such as the chromium benzaldehyde complex **223** were found to also aid to enrich the *ee* of the Aldol condensation product **225** as illustrated by Hanaoka's side chain synthesis, Scheme 18.[123] Moreover, lipase was employed by Chen et al. to resolve racemic starting epoxide for the synthesis of the optically active Taxol C13 side chain, Scheme 19.[124] Not only can enantioselectivity be exerted by chiral auxiliaries on substrates, but the bulky chiral reagents that participated in the reaction could enhance selectivity as demonstrated by the chiral boron reagent **234** utilised in Yamamoto's Taxol side chain synthesis, Scheme 20.[125] The binaphthol phenyl borate serves as a Lewis acid in triggering the imine-enolate condensation and resulted in the formation of adduct **235** with very high 99% *ee*.



Scheme 18. Hanaoka's Taxol side chain synthesis via aldol condensation in 1992.

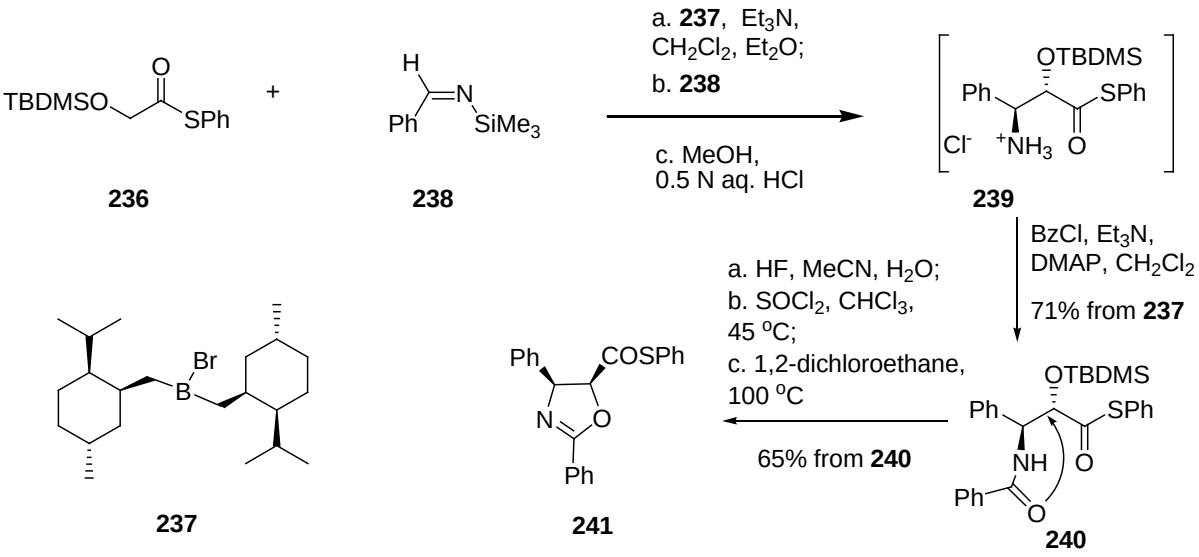


Scheme 19. Chen's side chain synthesis in 1993.



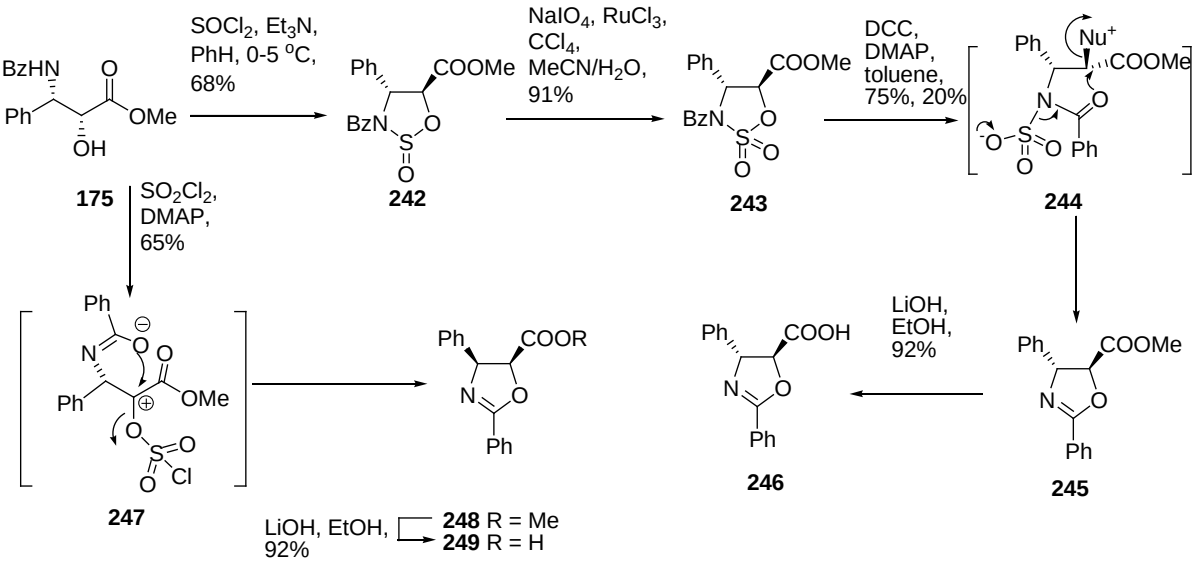
Scheme 20. Yamamoto's side chain synthesis in 1993.

In contrast to the conventional methods using side chain carboxylic acids or their alkyl esters for coupling with baccatin III, Gennari developed a new coupling protocol in 1996 exploiting the thioester as the side chain fragment for coupling, Scheme 21.[126, 127] The synthesis featured an enantio- and diastereoselective enolate-imine condensation reaction effeced by the (+)-methone-boron complex **237** to furnish the acyclic thioester **240** with > 96% *ee* and 96% *syn* diastereomer, a step that somewhat resembles Yamamoto's binaphthol boron complex induced condensation. Further treatment of thioester **240** with 2-methoxyproene under the catalysis of PPTS furnished the oxazolidine thioester as a side chain precursor for final coupling.



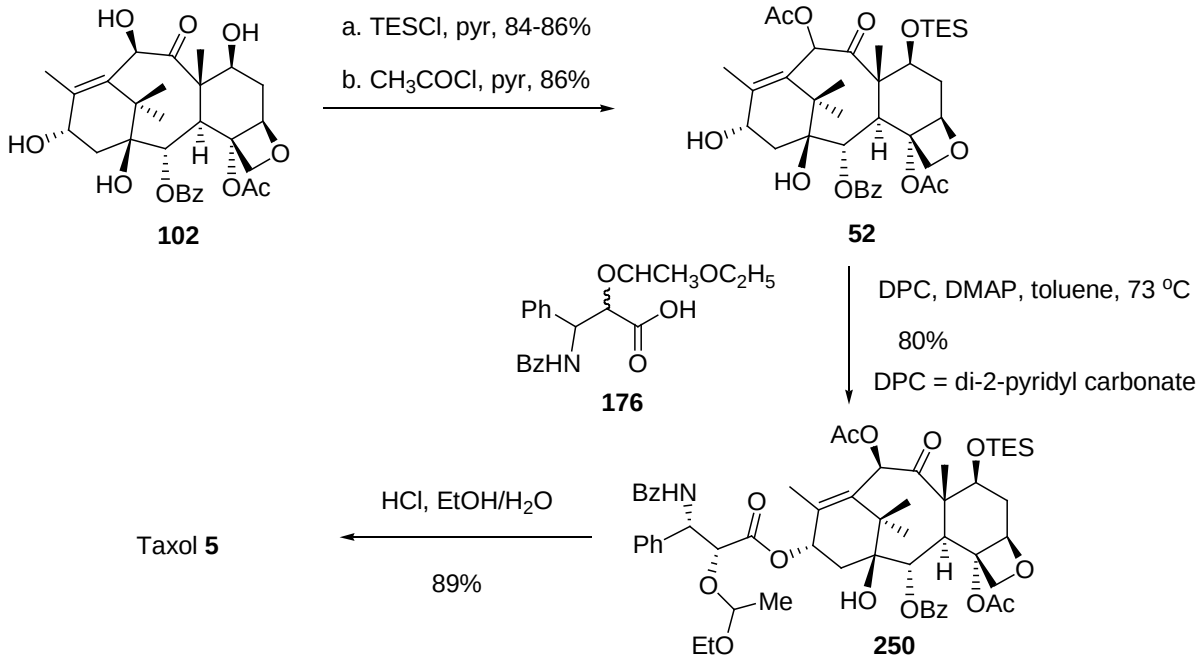
Scheme 21. Gennari's side chain thioester oxazolidine synthesis in 1996.

Kingston discovered a new method for preparing oxalidine diastereomers for the Taxol side chain synthesis in 1999 in several serendipitous trials to synthesise cyclic side chain precursors with a view to improving yields and reducing C2' epimerisation.[128] When acyclic methylester **175** was treated with thionyl chloride ($SOCl_2$) and Et_3N in benzene it generated a mixture of 2-oxo-oxathiazolidines with **242** as the major product in 68% yield, Scheme 22. Further oxidation of the sulfoxide into a sulfonyl **243** with $RuCl_3$-$NaIO_4$ increased the inductive effect and exposed the ester bearing cationic carbon to nucleophilic attack with simultaneous desulfonylation and oxazolidine formation to furnish **245** which still reserved its *anti* diastereomeric distribution. Intriguingly, treating **175** with electrophile sulfuryl chloride (SO_2Cl_2) and the nucleophile DMAP simultaneously induced cyclisation in a stereoselective manner to furnish *syn* oxazolidine **248** in 65% yield. This synthesis enabled access to several diastereomeric oxazolidines.

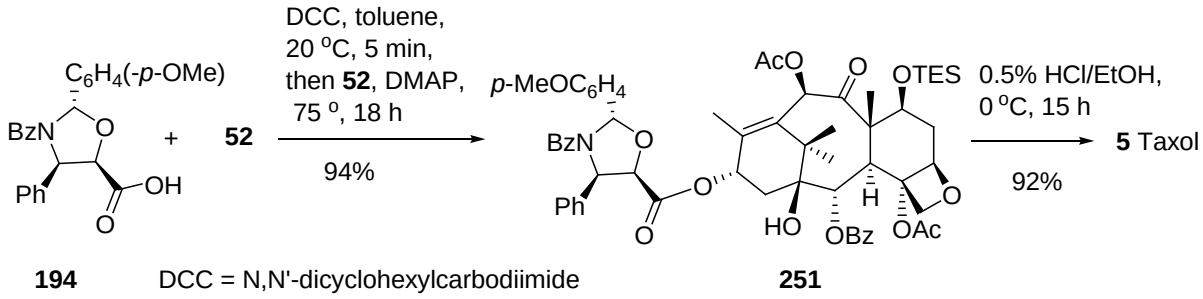


Scheme 22. Kingston's oxazolidine synthesis in 1999.

Apart from the high enantio- and diastereoselective syntheses of the side chain precursors, the synthesis of Taxol also requires efficient esterification methods for coupling various baccatin III derivatives. It was found that the C7 hydroxy grouping was more susceptible to undergo esterification than the C13 counterpart owning to less space hindrance and would thus necessitate the regioselective protection of the C7 hydroxy moiety before coupling the taxane



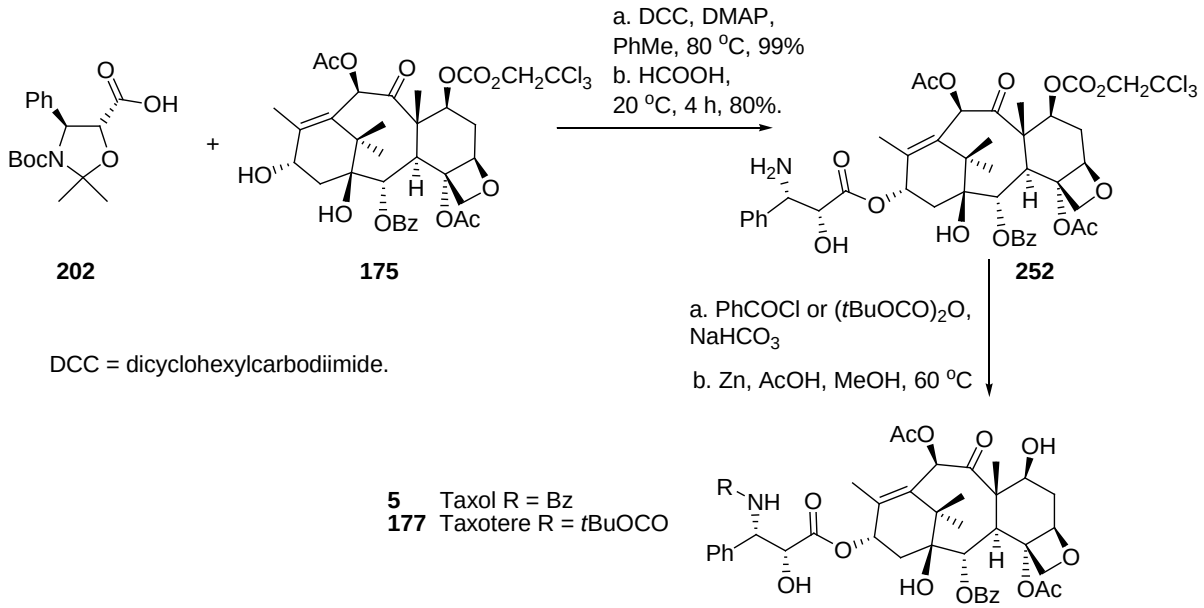
Scheme 23. Greene and Portier's Taxol semisynthesis via direct esterification in 1988.



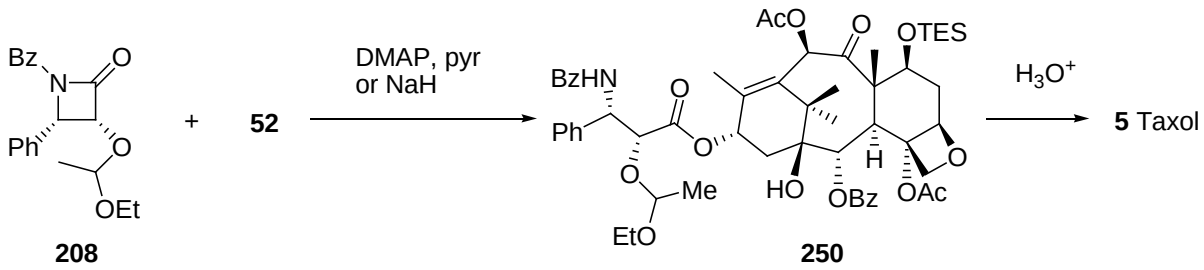
Scheme 24. Greene's improved Taxol semisynthesis via oxazolidine coupling in 1994.

core **102** or **78** with the side chain. After some initial trials,[129] Greene and Portier[106] found that the bulkier TES group was superior for regioselective protection of the C7 hydroxy group and succeeded in coupling the side chain[110] with the C7 hydroxy TES protected baccatin III **52** in high yields to furnish Taxol, Scheme 23. This C7 hydroxy TES protecting procedure later became a standard procedure and is reflected in the late-stage coupling reactions in Nicolaou, Danishefsky, Mukaiyama and Takahashi's Taxol total syntheses. The C7 hydroxy protected baccatin III and its derivatives are ready for coupling with the side chain precursors prepared by the methods outlined above. Most esterification methods are

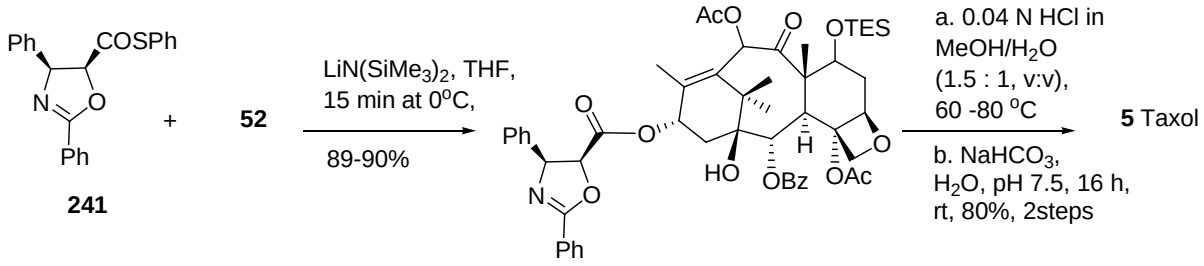
based on base-induced esterification of a carboxylic acid and the C13 alcohol regardless of what form of side chain precursors are in. Various examples of coupling are summarised in schemes 24-27.[100, 106, 113, 115-117, 121, 122] Standard protocols for coupling involve the use of bases such as di-2-pyridyl carbonate (DPC), *N*,*N*-dicyclohexylcarbodiimide (DCC), or stronger bases such as NaH and $LiN(SiMe_3)_2$ depending on the nature (e.g. sterics and sensitivity) of the side chain precursors. In constructing the side chain precursors, many approaches were pursued with some quite different side chain precursor designs. Greene and Portier initially used a C2' hydroxyl protected acyclic chain for direct coupling effected by bases DPC, DMAP in pyridine but later in 1994 Greene developed an improved protocol employing an oxazolidine as the side chain precursor for coupling followed by simultaneous ring opening and C7 deprotection by acid cleavage to give Taxol in 86% yield over 2 steps from **173 and** with very high enantiomeric excess (*ee*), Scheme 24. In fact, the exploitation of an oxazolidine as the side chain precursor had been demonstrated in Commerçon's Taxol semisynthesis in 1992 but by a different means of oxazolidine preparation, Scheme 25. In comparison to the oxazolidine approach, Holton, Ojima and Georg employed a *β*-lactam as the coupling side chain precursor in the semisynthesis of Taxol with various preparation strategies, Scheme 26.



Scheme 25. Commerçon's Taxol semisynthesis via oxazolidine coupling in 1992.

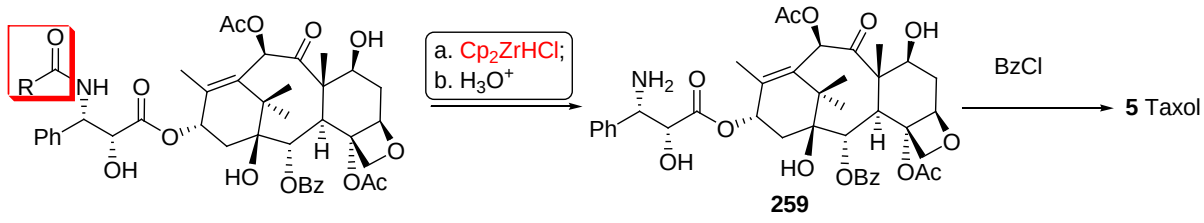


Scheme 26. Holton, Ojima and Georg's Taxol semisynthesis via *β*-lactam coupling.



Scheme 27. Gennari's Taxol semisynthesis via thioester coupling.

More recently, National Pharmaceuticals, Inc. (NPI) developed and commercialised a concise and efficient new semisynthesis of Taxol from the six primary taxanes (**5** and **254**-**258**) that possess the core structures of Taxol and differ only in the side chain amides and are naturally abundant in ornamental yew trees.[130] The side chain amide could be easily reduced by Schwartz's reagent followed by hydrolysis to give the free primary amine which can be protected with benzoyl chloride to yield Taxol **5**, Scheme 28. It's noteworthy that the plant materials from the ornamental yew trees can be harvested in an sustainable manner by pruning the tops of the trees. This semisynthesis approach realises the production of Taxol in bulk at very competitive costs and highlights again how natural products aid in drug discovery.



5 R = Ph
254 R = (*E*)-$CH_3CH{=}C(CH_3)$
255 R = *n*-C_5H_{11}
256 R = *n*-C_3H_7
257 R = $CH_3CH_3CH(CH_3)$
258 R = $PhCH_2$

Scheme 28. NPI's commercialised Taxol semi-synthesis.

1.5 Biosynthesis of Taxol.

The biosynthesis of the diterpenoid Taxol is known to proceed via three phases: 1. The biosynthesis of universal precursor of diterpene geranygeranyl pyrophosphate (GGPP); 2. Intramolecular cyclisation of GGPP catalysed by taxadiene synthases to form the tricyclic six-eight-six membered ring taxadiene scaffolds and 3. Post-cyclisation modifications including hydroxylation, epoxidation, acylation and side chain coupling of the taxadiene by various enzymes (e.g. oxygenases, and acyltransferases).[131, 132] The universal diterpene precursor GGPP is assembled by the most basic isoprenoid units isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) biosynthesised via two upstream pathways These being the cytosolic movalonic acid (MVA) and the plastidial 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (also known as movalonate-independent pathway discovered in 1993[133, 134]). In the case of the biosynthesis of Taxol, the GGPP precursor supplied for the biosynthetic pool is actually produced via the plasidial MEP pathway.[135] The first committed step which is also the beginning of the second phase in the Taxol biosynthesis is the taxadiene synthase (TS) catalysed cyclisation of GGPP.[136-140] This process proceeds via the intramolecular proton transfer from the C12 carbocation generated after C1-C2 and C11-C15 ring closure to C8 where both the C ring and C3-C8 ring closure is triggered. The resulting C4 carbocation undergoes elimination to form taxa-4,11-diene **273**. The cyclisation process has been rationalised by deuterium labelling experiments which supported the proposed mechanism outlined in Figure 4.[137]

Enzymatic oxidation is the main theme in the third phase. There are a total of eight oxidation sites on the taxadiene scaffold along the way to the formation of Taxol. P450 cytochrome monooxygenases are believed to be heavily involved in the oxidation of taxadiene and are estimated to mediate nine oxidation steps out of the approximately 19 enzymatic steps for Taxol biosynthesis.[141] Early-stage hydroxylation is thought to be in the order of C5, C10, C2, C9, C13, C7 and C1.[142] The D oxetane ring is believed to be constructed at late stage oxidation following the functionalisation of the ABC ring by epoxidation of the C4-C20 double bond and intramolecular epoxy fragmentation.[142] The first P450 mediated oxidation of taxadiene occurrs at the C5 position with the installation of hydroxyl moiety with α orientation.[143] The C5 α hydroxyl moiety can then be acylated by *O*-acetyl transferase which has been partially purified and characterised.[144] Following the C5 hydroxylation is the hydroxylation at C13 and C10 which require C5α-hydroxy and C5α-acetate taxadiene as

substrates and are both P450 cytochrome monooxygenase catalysed reactions.[145, 146] Moreover, the taxoid 7β-hydroxylase and 2α-hydroxylase that catalysed the hydroxylation of a taxusin surrogate at the C7 and C2 positions has been successfully isolated and characterised with their cDNA successfully cloned.[147, 148]

More recently, the gene encoding the taxoid C9α hydroxylase that possessed potential C9α hydroxylation capacity has also been identified from *Ginkgo biloba* suspension cells.[149] All of these P450 cytochrome oxygenase catalysed hydroxylations displayed substrate specificity and suggest that the functionalisation process of the taxadiene core is indeed a step-wise process and there is more than one route to the biosynthesis of Taxol.[145] Even with all of these enzymes in hand, it's still difficult to clarify the order of oxidation of taxadiene and more specific substrates are needed for addressing these problems. D ring formation is speculated to be via the epoxy fragmentation but no evidence thus far has supported this hypothesis, Figure 5.[131] A study using various surrogates containing the C4-C20 epoxy moiety failed to produce any oxetane ring suggesting that the proposed epoxy intermediate may not be the real intermediate in the D ring formation process.[131, 150]

The last step in the biosynthesis of taxol is the appending of the side chain amide to baccatin III. Phenylalanine **279** has been proven to be the precursor of the side chain amide by feeding experiments using deuterium labelled substrates.[151] The pathway proceeds via the amino intermediate **280** before coupling with baccatin III, Figure 4. Hydroxylation at C2' and benzoylation of the side chain amine occurrs sequentially after the coupling reaction under the catalysis of hydroxylase and a co-enzyme to furnish Taxol.[142]

Understanding the biosynthesis of bioactive NP provides opportunities for possible manipulation of certain biosynthetic steps to achieve the regulation of certain intermediates, and in turn, yields the target NP or their abundant precursors. Such findings will have immense impact in industries such as the food and pharmaceutical industries if it can be practically and industrially realised as it reduces the use of hazardous chemicals during production and thus reduces potential exposure to humans. Moreover, the biosynthesis of NP also inspires chemists to pursue more efficient synthetic protocols by showing us how these substrates are constructed so easily in nature.

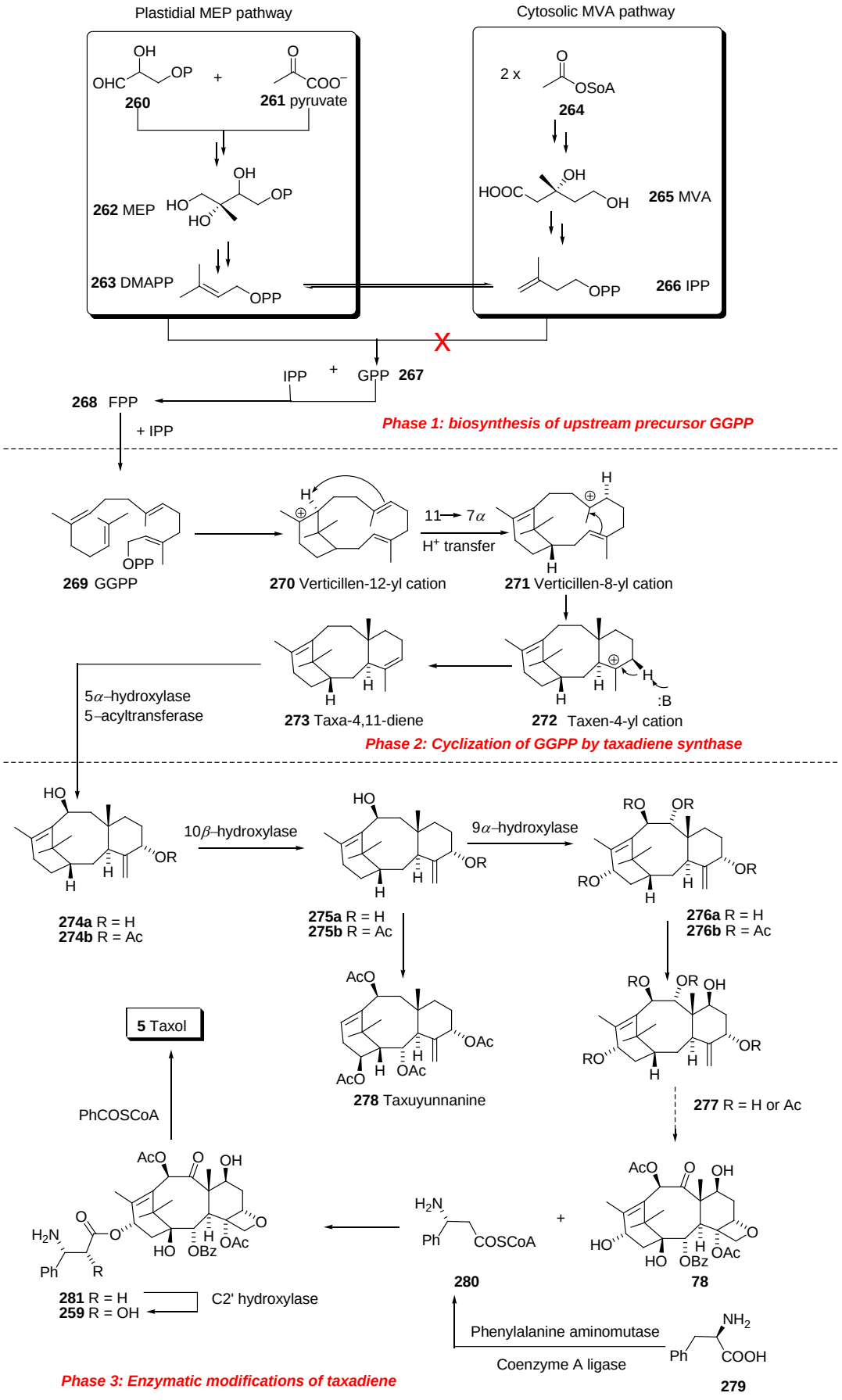


Figure 4. Putative Taxol biosynthetic pathway.

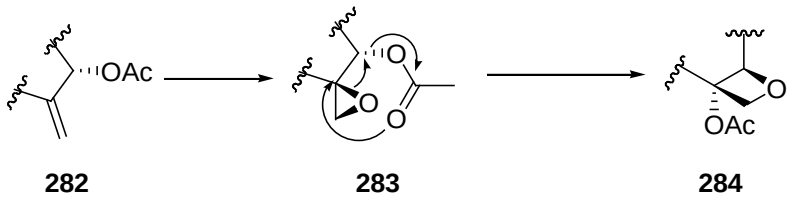


Figure 5. Plausible D ring formation pathway via epoxy fragmentation.

1.6 Summary.

NP research is centred on the discovery, chemistry and biochemistry of NP. It is well illustrated by the scope of the '*Journal of Natural Products*' reading that 'Specifically, there may be articles that describe secondary metabolites of microorganisms, including antibiotics and mycotoxins; physiologically active compounds from terrestrial and marine plants and animals; biochemical studies, including biosynthesis and microbiological transformations; fermentation and plant tissue culture; the isolation, structure elucidation, and chemical synthesis of novel compounds from nature; and the pharmacology of compounds of natural origin'. In the discovery phase, isolation, structure elucidation and chemical synthesis are of particular importance. Chemical synthesis and biosynthesis are crucial approaches leading to the production of important NP. These two phases of NP research are also the themes in the thesis presented herein and underpin my thirst for becoming a natural product chemist.

1.7 Aims of this thesis.

Keeping to the theme of the discovery of bioactive natural products, three main integrated studies together with several side explorations have been performed in this thesis aimed to increase our knowledge of certain areas of natural product chemistry and to grasp the techniques and skills required for future independent research.

The first study tackles the isolation, characterisation and antioxidant activity testing of phenolics from the leaves of the Australian native plant *Smilax glyciphylla* (*S. glyciphylla*). This study was launched based on a previous study[152] showing that the leaf extracts of *S. glyciphylla* exhibited significant antioxidant activity, however, they did not identify the main contributor(s), coupled with the fact that only one principle compound glycyphyllin A[153, 154] had been isolated and characterised from this plant. Plant material collected from New South Wales was analysed by TLC and HPLC traces of the butanol fraction of the ethanolic extract suggested numerous compounds are present, Figure 6.

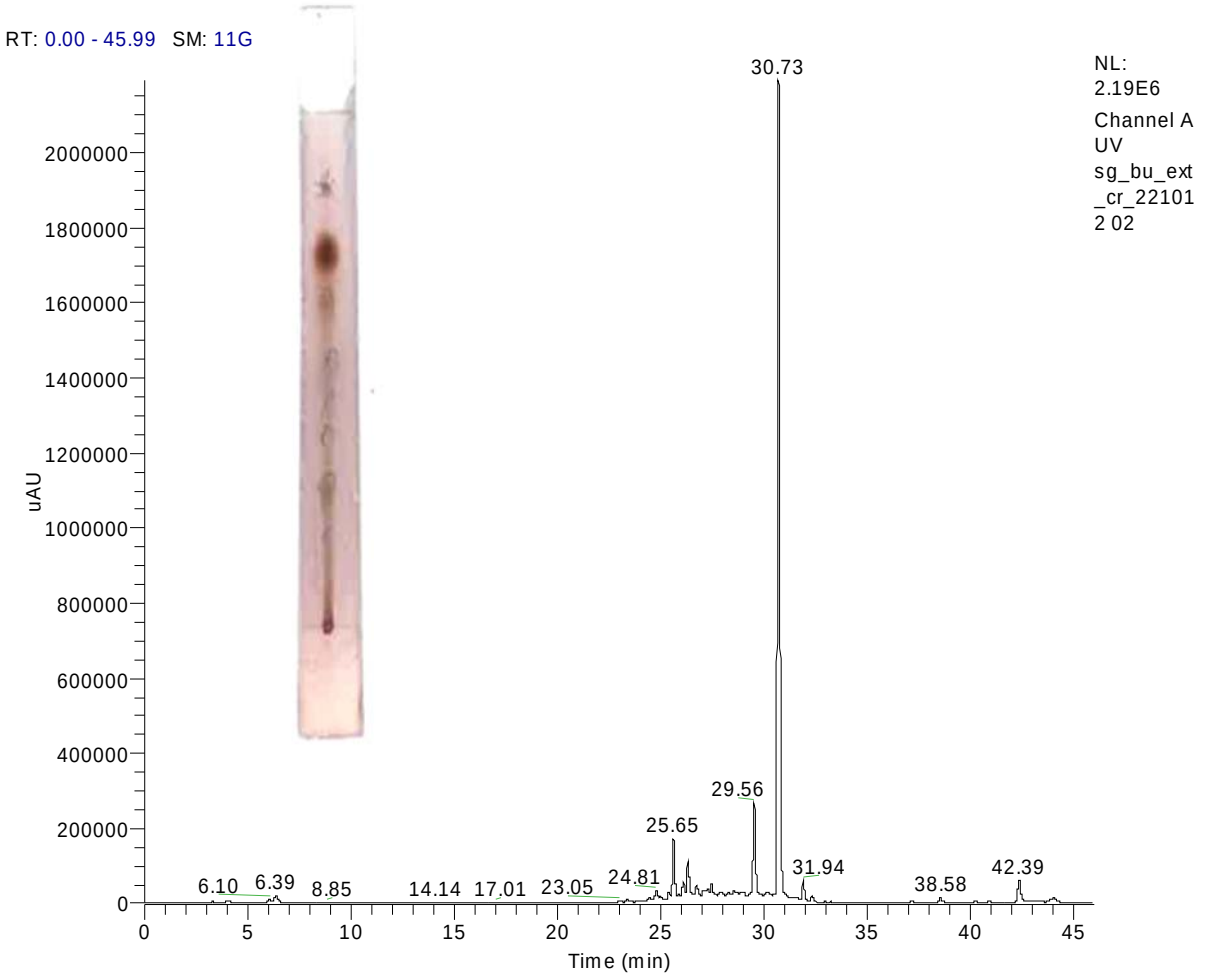


Figure 6. TLC and HPLC trace of the butanol fraction of the ethanolic extract of the leaves of *Smilax glyciphylla*.

The notable bitter-sweetness of the leaves of *S. glyciphylla*, its documented medicinal use by indigenous Australians and the confirmed anticancer activity of the hydrolysed aglycone of

glycyphyllin A, phloretin[155, 156] rendered it an appealing subject for further exploration. Besides the common chromatographic and spectroscopic techniques including solid phase chromatography (e.g. TLC, silica chromatography, HPLC), HRMS and NMR employed for isolation and characterisation, this study also features the use of Multi-Layer Coil Counter-current Chromatography (MLCCC) as the main tool for large scale isolation of natural products. The skills of harnessing these techniques would be developed in the course of this study. Moreover, the successful accomplishment of this project would provide a better picture of the phytochemical composition and the origin of the antioxidant activity of the leaf extract of the plant. Compounds isolated and identified with significant antioxidant activity could be potential candidates as synthetic/biosynthetic targets and their values could be further exploited for the food and pharmaceutical industries.

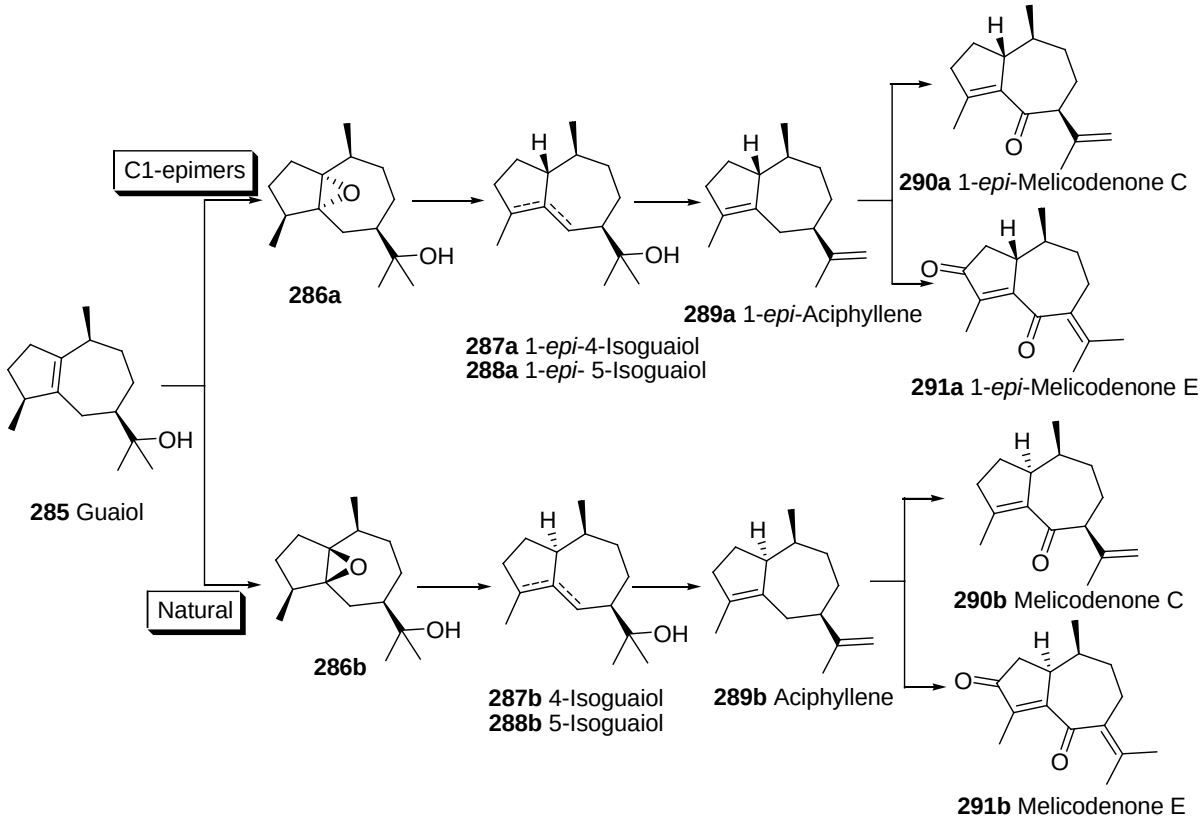


Figure 7. Proposed synthetic route to guaiol isomers, natural melicodenones C and E and their C1 epimers via the diastereoselective epoxidation of guaiol.

On the basis of the skills developed in isolating and characterising natural products employing comprehensive chromatographic and spectroscopic methods, the focus of the second study was placed on the synthesis of guaiane-type sesquiterpenoids including the

natural product aciphyllene, anti-inflammatory isoguaiols, two epimers of the recently isolated sesquiterpenoids, 1-epimelicodenone C and E and their derivatives via a diastereoselective epoxidation, ring opening and functional group manipulation sequence. Just like the importance of the terpenes that make up the Taxol class of analogues, guaiane-type sesquiterpenoids are an important subclass of sesquiterpenes with numerous molecular structures that display a wide spectrum of biological activities ranging from the anti-inflammatory (isoguaiols) to anticancer (englerin A) to olfactorily potent (rotundone). Total synthesis of these types of compounds often suffers from low overall yield and difficulty in installing the correct stereochemistry when constructing the bicyclic core. Starting with the inexpensive chiral natural product (-)-guaiol we hypothesised a simple new synthetic route to a host of guaiane natural products, Figure 7.

The planed sequence would allow for tight control over the stereochemistry of the bridged carbons via a diastereoselective epoxidation and ring opening sequence, which in turn would provide facile access to numerous guaiol analogues including two known anti-inflammatory isoguaiols. Further manipulation of various functional groups based on the readily synthesised aciphyllene and 1-epiaciphyllene should lead to the synthesis of melicodenone C and E for the first time, Figure 7. The synthesis of melicodenone C and E, would also confirm the assignments of isolated natural melicodenone C and E in the original paper. This sequence could also be further explored to synthesise other sesquiterpenoids. Substantial knowledge in the practices and methodologies in organic synthesis would be gained along with a greater understanding of the associated mechanisms of these types of reactions. Moreover, planed exposure to the theoretical study of reaction mechanisms using quantum computational chemistry would further shape our understanding of the theoretical bases of these organic syntheses.

With the synthetic methods developed in the second study in hand, it would be possible to access a wide range of relevant sesquiterpenoids for studying the formation of rotundone, an important sesquiterpenoid with a strong peppery note which is found as a key aroma compound in wines, grapes and a wide range of herbs and spices. Based on the observation from a former group member that rotundone may be formed as a major product after exposing α-guaiene to air, the deliberate autoxidation of α-guaiene will be carried out in the third study to elucidate its downstream oxidation products and to characterise any unstable

peroxide intermediates and to further shed light on the mechanisms involved in the formation of these downstream products including rotundone itself, Figure 8.

Chemical syntheses would be also employed to establish and confirm the molecular structures of some of the products of the autoxidation of *α*-guaiene. Based on the products isolated from the autoxidation of *α*-guaiene itself, plausible mechanisms of formation would be proposed and rationalised with the aid of *ab initio* quantum calculations which allows for an understanding of many intermediates that are experimentally difficult or impossible to be obtained. By gaining an understanding of the intermediates and products formed from the oxidation of precursor *α*-guaiene, this study will build the foundation for further investigation into the biosynthesis of rotundone *in vivo*. It could also provide implications of free radical mechanisms for the biosynthesis of postcyclisation sesquiterpenoids catalysed by oxidases such as cytochrome P450s. In-depth knowledge of free radical chemistry will be gained upon completion of this study.

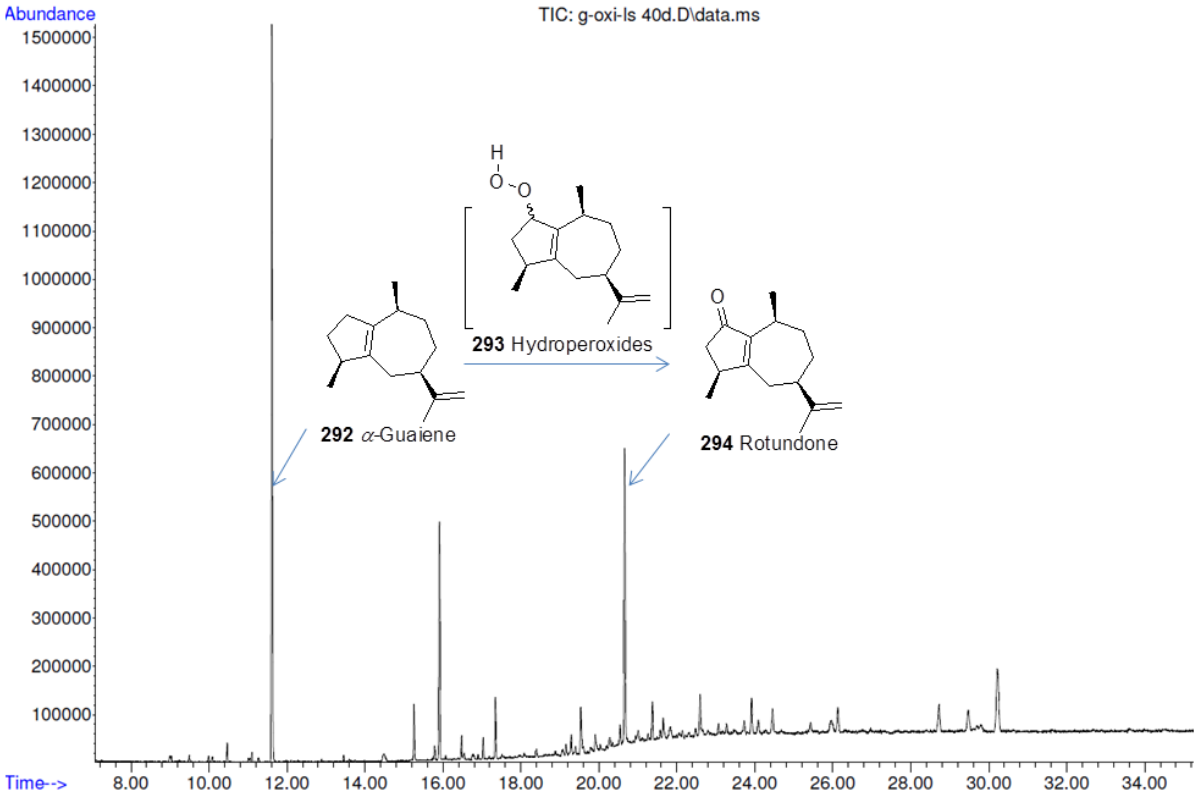


Figure 8. GC trace of products of oxidation of neat α-guaiene with pure O_2 at room temperature for 40 days.

With a view to understanding the biosynthesis of some important aroma compounds derived from the two important sesquiterpene precursors α-guaiene and α-bulnesene, attempts would also be made to develop quantification methods for these two precursors by stable isotope labelling coupled with GC-MS. Sesquiterpenoids widely exist in plants but often at low concentrations. To probe the biotransformation of these trace compounds requires reliable quantification methods with high sensitivities. Stable isotope dilution analysis (SIDA) utilises deuterium labelled analytes as internal standards to alleviate the inaccuracy arising from the different properties between analytes and internal standards and thus realises accurate and reliable quantification. This final study aimed to develop robust quantification methods for the aroma precursors, α-guaiene and α-bulnesene employing 1D/2D selectable GC-MS and would be initiated by the synthesis of deuterium labelled α-guaiene and α-bulnesene themselves.

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Chapter 2

Examination of the Phenolic Profile and Antioxidant Activity of the Leaves of the Australian Native Plant *Smilax glyciphylla*

Sweetness is one of the most important tastes and is often imparted by sugar. Being over indulged by the sweetness imposed by sugar has created many sugar-associated health concerns such as obesity, metabolic syndrome and chronic kidney disease. Natural sweetening agents that are low in calories could be beneficial to human health and are the best alternatives for food developers. *Smilax glyciphylla* is a plant with a sweet taste and its sweet principle glycyphyllin A has been isolated and characterised. Moreover, the hot water extract of the leaves of *Smilax glyciphylla* was found to possess significant antioxidant activity but does not specifically originate from the sweet principle glycyphyllin A.

This paper examined the phenolic composition of the leaves of the Australian native plant *Smilax glyciphylla* and their asociated antioxidant activities. Together with glycyphyllin A, seven phenolic compounds including two new dihydrochalcone rhamnopyranosides, glycyphyllin B and glycyphyllin C, and five known flavonoids, were isolated from the ethanolic extract of the leaves of *Smilax glyciphylla* for the first time employing various chromatography techniques including repeated MLCCC, silica and LH-20 column chromatography. The structures of these compounds were characterised by spectroscopic methods including UV, MS, 1D and 2D NMR. *In vitro* antioxidant capacity tests of the isolated compounds employing FRAP and DPPH assays revealed that the major component glycyphyllin B exhibited potent antioxidant activity and was the key phenolic compound responsible for the antioxidant activity of the leaf extract of *S. glyciphylla*.

This study provided a much better picture of the phenolic composition of the leaves of *Smilax glyciphylla* and their corresponding antioxidant activities. The two new compounds discovered herein, especially glycyphyllin B could be potential candidates for further sensory and application studies. Skills in extraction, isolation and structural elucidation of isolated compounds using various chromatographic and spectroscopic techniques have been developed in the course of this work.

Presented in the following publication is the detailed description of the extraction, isolation, structural elucidation and antioxidant activity assays that were performed. Original 1D and 2D NMR spectra of isolated new compounds, tabled ^{1}H and ^{13}C NMR data of isolated compounds and raw data of the antioxidant activity assays are available as supporting information free of charge via the internet at http://pubs.acs.org.

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis

Name of Principle Author (Candidate)	Ancheng Huang		
Contribution to the Paper	Designed experiments, performed sample extraction, compound isolation and characterization, antioxidant activity assays and structure elucidation of all isolated compounds. analysed and interpreted data, drafted/constructed manuscript.		
Signature		Date	27/08/14

Name of Co- Author	Amelie Wilde		
Contribution to the Paper	Helped with initial small-scale isolation trials and solvent system optimization.		
Signature		Date	02.08.2014

Name of Co- Author	Johanna Ebmeyer		
Contribution to the Paper	Helped with initial small-scale isolation trials and solvent system optimization.		
Signature		Date	02.08.14

Name of Co- Author	George K. Skouroumounis		
Contribution to the Paper	Aided in sourcing plant materials. Introduced the key instrument MLCCC to Ancheng Huang and provided technical support.		
Signature		Date	27/08/14

Name of Co- Author	Dennis K Taylor		
Contribution to the Paper	Supervised the work, conceived project, assisted in the preparation and editing of the manuscript and acted as corresponding author.		
Signature		Date	27/8/14

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Chapter 3

Synthesis of Guaia-4(5)-en-11-ol, Guaia-5(6)-en-11-ol, Aciphyllene, 1-*epi*-Melicodenone C and E and other Guaiane-type Sesquiterpenoids via the Diastereoselective Epoxidation of Guaiol.

Based on the diastereoselectivity observed in the epoxidation of guaiol and its derivatives, a facile synthesis of guaiane sesquiterpenes including aciphyllene, two anti-inflammatory isoguaiols, and their C1 epimers via an epoxidation ring opening sequence was developed. Moreover, the synthesis of recently isolated sesquiterpenoids, melicodenones from aciphyllene and 1-epi-aciphyllene by simple functional group manipulation was also explored, which sucessfully resulted in the synthesis of their C1 epimers, 1-*epi*-melicodenone C and 1-*epi*-melicodenone E for the first time.

It was found that the diastereoselectivity of epoxidation of the centrally bridged double bond of bicyclic guaiol could be readily controlled by modifying the protecting groups of the side chain isopropanoyl, reaction solvents and epoxidising reagents, with the latter having the largest impact. Theoretical calculations of guaiol and epoxy guaiols showed that the β face of guaiol was much more hindered by the two methyl moieties and would hamper the approach of the spiro shaped DMDO while allowed access for the more linear MCPBA. Control over on which face the epoxide would form, was thus able to be achieved. Ring opening and further manniputation lead to a host of natural products and their epimers in a facile manner.

This simple new synthetic approach also allows access to a range of other guaiane sesquiterpenoids for structural confirmation and mechanistic studies obtained from the autoxidation of α-guaiene which is presented in the following chapters. Skills in synthesis and theoretical chemistry were developed after extensive synthetic practice and mechanistic studies on the diastereoselective epoxidation of guaiol. Presented in the following publication is the detailed description of the syntheses that have been performed. All 1D and 2D NMR spectra of the compounds synthesised, details regarding theoretical calculations including DFT and semi-empirical calculations of related compounds, and x-ray diffraction data of five sesquiterpnoid compounds synthesised are also available as supporting information free of charge via the internet at http://pubs.acs.org.

Statement of Authorship

Title of Paper	**Synthesis of Guaia-4(5)-en-11-ol, Guaia-5(6)-en-11-ol, Aciphyllene, 1-*epi*-Melicodenone C and E and other Guaiane-type Sesquiterpenoids via the Diastereoselective Epoxidation of Guaiol**
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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis

Name of Principle Author (Candidate)	Ancheng Huang		
Contribution to the Paper	Designed synthetic schemes, performed all syntheses and theoretical calculations and grew crystals, wrote the manuscript in collaboration with Prof. Dennis Taylor.		
Signature		Date	10/09/14

Name of Co- Author	Christopher Sumby		
Contribution to the Paper	Collected crystal data and solved crystal structures and aided in the writing up of the data.		
Signature		Date	10/09/14

Name of Co- Author	Edward R.T. Tiekink		
Contribution to the Paper	Collected crystal data and solved crystal structures and aided in the writing up of the data.		
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Name of Co- Author	Dennis K. Taylor		
Contribution to the Paper	Supervised Ancheng Huang and aided in experimental design and interpretation of data, wrote the manuscript in collaboration with Ancheng Huang.		
Signature		Date	10/9/14

Huang, A., Sumby, C.J., Tiekink, E.R.T. & Taylor, D.K. (2014) Synthesis of Guaia-4(5)-en-11-ol, Guaia-5(6)-en-11-ol, Aciphyllene, 1-epi-Melicodenones C and E, and other Guaiane-type sesquiterpenoids via the diastereoselective epoxidation of Guaiol.
Journal of Natural Products, v. 77(11), pp. 2522-2536

Chapter 4

Production of the Pepper Aroma Compound, (-)-Rotundone, by Aerial Oxidation of α-Guaiene.

The discovery of rotundone as a potent aroma compound responsible for the black pepper note from peppercorns and a wide range of herbs, spices, and even wines in 2008 has captured great attention from organic and analytical chemists and biochemists. Rotundone is a sesquiterpene ketone that shares the same guaiane scaffold with α-guaiene. α-Guaiene was found to be biosynthesised together with δ-guaiene (also known as α-bulnesene) by δ-guaiene synthases isolated from agarwood cell cultures. However, the biosynthesis of rotundone is still currently unknown. Cytochrome P450 monooxygenases could be the key enzymes responsible for the oxidation of α-guaiene into rotundone *in vivo*.

Apart from enzymatic pathways towards the formation of rotundone, it was recently found within our group that rotundone was easily produced during simple aerial oxidation of α-guaiene which allowed for the identification of α-guaiene as a precursor of rotundone for the first time. Consequently, this important pepper aroma impact compound may be produced in foodstuffs and beverages upon heating and exposure to air, which results in the conversion of α-guaiene into rotundone. Together with rotundone, three additional main products, two mono epoxy guaienes and one diketone have also been isolated and characterised by chromatographic and spectroscopic techniques with the structures of two mono epoxides confirmed by synthesis. The formation of rotundone from α-guaiene is believed to be via the generation and subsequent decomposition of the 2-hydroperoxy guaiane.

In order to better mimic the formation of rotundone in real plant materials, we coated neat α-guaiene onto cellulose filter paper, which was then subjected to aerial oxidation at different temperatures. It was found that the formation rate of rotundone from α-guaiene coated on filter paper was significantly faster than those without filter paper. Moreover, the formation rate increased dramatically with temperature. The temperature dependent nature of this reaction supported our postulation that it proceeds via a free radical mechanism. This study demonstrates that aerial oxidation is an alternative pathway to the formation of rotundone from the precursor α-guaiene.

Statement of Authorship

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Ancheng Huang		
Contribution to the Paper	Designed and performed oxidation of guaiene on filter paper experiments; Isolated and characterised some of the key compounds including the diketone in pure form, contributed to the writing of the paper in collaboration with Taylor.		
Signature		Date	1/9/14

Name of Co-Author	Stacey Burrett		
Contribution to the Paper	Aided in carrying out some of the experiments, running of GCMS and interpreting data.		
Signature		Date	9/9/14

Name of Co-Author	Mark A. Sefton		
Contribution to the Paper	Co-supervised Ancheng Huang and Stacey Burrett. Conceived filter paper for mimicking plant cell wall and provided constructive comments throughout the project.		
Signature		Date	1/9/14

Name of Co-Author	Dennis K. Taylor		
Contribution to the Paper	Acted as principle supervisor supervising Ancheng Huang and Stacey Burrett. Conceived the formation of rotundone by autoxidation of α-guaiene; Wrote the paper in collaboration with Ancheng Huang and acted as corresponding author.		
Signature		Date	1/9/14

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Chapter 5

Mechanistic Studies on the Autoxidation of α-Guaiene: The Structural Diversity of the Sesquiterpenoid Downstream Products.

Based on the discovery in the previous chapter, further investigation into the mechanism of formation of rotundone and other downstream products by autoxidation of α-guaiene was deemed appropriate. This study confirmed our previous hypothesis that hydroperoxides were generated and decomposed to furnish rotundone and other compounds by successfully isolating and characterising the unstable hydroperoxide intermediates and other oxidation products. A total of thirteen downstream sesquiterpenoids and two unstable hydroperoxides were isolated from the autoxidation products of α-guaiene.

One of the natural products isolated in significance was rotundone, which is the only known impact odorant displaying a peppery aroma. Other products included corymbolone and its C6 epimer, the 2*R*- and 2*S*-rotundols and several hitherto unknown epimers of natural Chabrolidione A, namely 7-*epi*-chabrolidione A and 1,7-*epi*-chabrolidione A. Several C4 hydroxy rotundones and a range of epoxides were also found in significant amounts after autoxidation. Their structures were elucidated on the basis of spectroscopic data, HRMS and X-ray crystallography and a number of them confirmed through total synthesis. The mechanisms of formation of many of the products may be accounted for by initial formation of the 2- and 4-hydroperoxy guaienes followed by various fragmentation or degradation pathways. Given that α-guaiene is well know to exist in the essential oils of numerous plants, coupled with the fact that aerial oxidation to form this myriad of downstream oxidation products occurs so readily at ambient temperature suggests that many of them have been missed being identified during past isolation studies from natural sources. This study highlights the structural diversity of sesquiterpenoids that could possibly originate from the autoxidation of sesquiterpene precursors in nature.

All 1D and 2D NMR spectra of the compounds isolated and synthesised, and x-ray diffraction data of four sesquiterpnoid crystals obtained are available as supporting information free of charge via the internet at http://pubs.acs.org.

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Contribution to the Paper	Isolated and characterised all autoxidation products, designed all synthetic schemes, performed all syntheses and theoretical calculations and grew crystals. wrote the manuscript in collaboration with Prof. Dennis Taylor.		
Signature		Date	10/09/14

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Contribution to the Paper	Co-supervised Ancheng Huang. Provided constructive suggestions and ideas on both experiments and manuscript writing.		
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Contribution to the Paper	Supervised Ancheng Huang and aided in the interpretation of results/data, wrote the manuscript in collaboration with Ancheng Huang		
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Chapter 6

Rationalizing the Formation of Peppery and Woody Sesquiterpenes Derived from α-Guaiene and α-Bulnesene under Aerial Oxidative Conditions: Synthesis of Deuterium Labelled Analogues and SIDA/GC-MS Studies.

After extensive *in vitro* chemical studies on the formation of sesquiterpenoids during the autoxidation of α-guaiene, we shifted our focus towards the development of methods for quantifying various peppery and woody aroma compounds generated during aerial oxidation of α-guaiene and α-bulnesene. A robust quantification method would allow us to identify and determine these compounds at trace levels from complex natural product matrices and monitor their evolution over time. Moreover, it would also make studying the subtle changes in the levels of these trace compounds and the location of them within plant tissues from biological systems possible.

This publication describes the synthesis of deuterium labelled sesquiterpene fragrances and their precursors as internal standards for the development of stable isotope dilution analysis (SIDA). Along with α-guaiene and α-bulnesene, the peppery and woody sesquiterpenoids generated from the autoxidation of the first two compounds were identified and quantified. α-Bulnesene was identified for the first time as a precursor to two bulnesols with woody aroma. The analytical methods developed herein could also be modified and applied to more complex matrices.

Statement of Authorship

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Name of Co- Author	Mark Sefton		
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Contribution to the Paper	Supervised Ancheng Huang and the work; aided in data interpretation, revised and edited the manuscript.		
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Rationalizing the Formation of Peppery and Woody Sesquiterpenes Derived from α-Guaiene and α-Bulnesene under Aerial Oxidative Conditions: Synthesis of Deuterium Labelled Analogues and SIDA/GC-MS Studies.

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ABSTRACT: Deuterium labelled guaiane derivatives and their precursors, namely d_5-2*R*-rotundol (**11a**), d_5-2*S*-rotundol (**11b**), d_5-bulnesone (**14**), d_5-2*R*-bulnesol (**16**), d_7-α-guaiene (**10**) and d_7-α-bulnesene (**15**) have been synthesized in good yields as internal standards (IS) for stable isotope dilution analysis (SIDA). Employing the SIDA methods developed herein, the evolution of the potent peppery compound rotundone (**3**), the related rotund-2-ols (**2a/b**) and the woody aroma compounds bulnes-2-ols (**6a/b**) and α-bulnesone (**7**) from precursors α-guaiene (**1**) and α-bulnesene (**5**) was monitored by GC-MS. It was found that up to 7% of **3** and 0.6% of **2a/b** were formed at the consumption of 380 μg of α-guaiene after exposing α-guaiene coated filter paper to air at ambient temperature for 2 days, whereas **5** yielded less than 0.3% of **6a**, 0.9% of **6b** and 1.5% of α-bulnesone (**7**) with 75% conversion of **5** in 5 days. All concentrations of products decreased after reaching peak concentrations, suggesting the occurrence of further downstream oxidation and implying possible changes of aroma profiles of herbs and other plant materials overtime when exposed to air.

KEYWORDS: *Sesquiterpene fragrances, rotundone, rotund-2-ol, bulnes-2-ol, α-guaiene, α-bulnesene, peppery, woody, aerial oxidation, stable isotope dilution analysis (SIDA), GC-MS*

INTRODUCTION

Sesquiterpenes are an important aroma source and have long been used as fragrances in the perfumery and cosmetic industry.[1] The discovery of rotundone, which has a potent peppery note and an orthonasal detection threshold down to 8 ng/L (in water), from a wide range of herbs, spices and wines[2] has encouraged further research on guaiane sesquiterpenoids in the hope of finding new potent aroma compounds. We have recently uncovered α-guaiene (**1**) as a universal precursor to many fragrances including 1,5-epoxy-guaiene (minty), the rotund-2-ols (**2a/b**, peppery, woody, rosy) and rotundone (**3**, peppery) under simple aerial oxidation conditions.[3] Other isomeric guaiane sesquiterpenoids such as bulnes-2-ols (**6a/b**) have also been found to possess a woody note and are used as flavour and fragrance ingredients for newly developed formulae.[4]

The mechanism of formation of the rotund-2-ols (**2a/b**) and rotundone (**3**) from α-guaiene (**1**) has recently been found to proceed via the intermediacy and decomposition of the unstable hydroperoxides (**4a/b**) generated by autoxidation of **1**.[5] However, the mechanism of formation of the woody bulnes-2-ols (**6a/b**) has not been investigated. It's feasible that **6a/b** are produced from α-bulnesene (**5**) via the same pathway as **2a**/**2b** from **1**, Figure 1. In nature, sesquiterpenes α-guaiene (**1)** and α-bulnesene (**5**) may be biosynthesized by the same δ-synthase from the universal sesquiterpene precursor farnesyl pyrophosphate (FPP).[6] Moreover, the wide occurrence of rotundone in plant products has suggested that its precursor α-guaiene, the isomer α-bulnesene and other related oxygenated guaiane derivatives might also be common metabolites widely present in plants. The often low natural abundance of these metabolites in nature coupled with the complexity of the plant matrices are the main obstacles in identifying their occurrence and understanding their biotransformations *in vivo* and necessitates the development of robust analytical methods such as stable isotope dilution analysis which enjoys high sensitivity, accuracy and traceability. We therefore have synthesized a series of deuterium labelled standards and report herein the determination of the evolution of several guaiane fragrances during *in vitro* aerial oxidation of their precursors over time by means of SIDA using GC-MS.

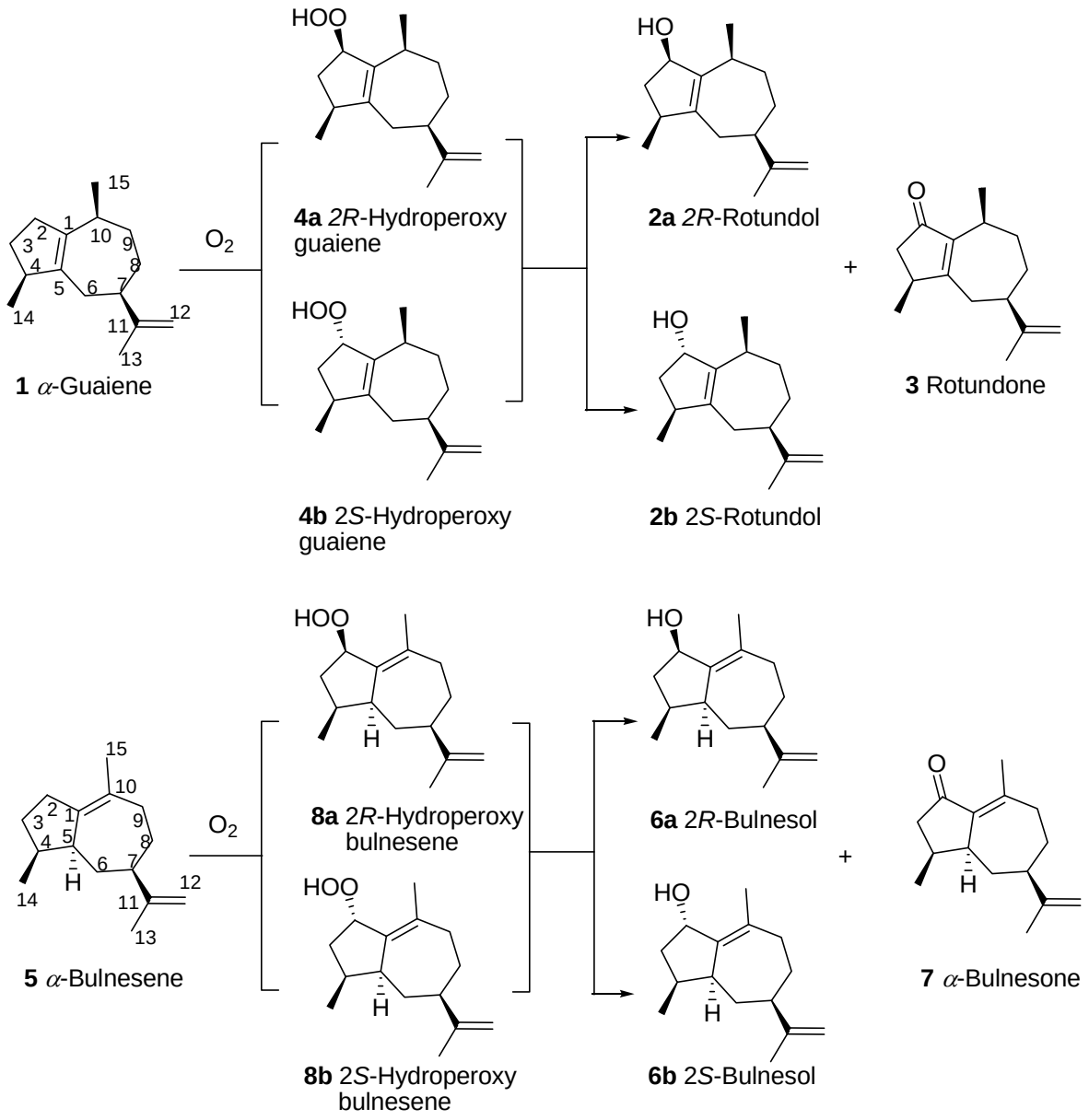


Figure 1. Formation of the peppery and woody compounds rotund-2-ol (**2a/b**), rotundone (**3**) and bulnes-2-ol (**6a/b**) from α-guaiene (**1**) and α-bulnesene (**5**).

MATERIALS AND METHODS

General Experimental Procedures. All reagents were purchased from commercial sources and used directly unless otherwise stated. Solvents for synthesis were purified according to known procedures wherever needed.[7] Solvents for general chromatography were AR grade except those used for GC-MS and HRMS analysis which were HPLC grade. NMR data were recorded on Varian-Inova 500/600 MHz spectrometers. $CDCl_3$ or C_6D_6 were used as solvents for NMR experiments. All ^1H and ^{13}C NMR spectra were calibrated utilizing residual ^1H and

^{13}C signals of deuterated solvents set at 7.26 ppm and 77.0 ppm for $CDCl_3$, and 7.16 ppm and 128.06 ppm for C_6D_6 respectively. GC-MS quantitative analysis was performed with an Agilent 6890 GC- 5973N MSD equipped with a DB-Wax capillary column (30 m x 250 μm I.D. x 0.25 μm film thickness, Agilent technologies). GC-MS analysis was performed with the following acquisition parameters: oven temperature was initially set at 100 ^oC and was increased to 200 ^oC at 8 ^oC/min and then to 240 ^oC at 20 ^oC/min and held at 240 ^oC for a further 3 min; injection was in splitless mode with a purge flow of 6 mL/min in 0.05 min; front inlet temperature was set at 240 ^oC with a pressure of 12.92 psi; injection volume was 2 μL and gas flow rate 1.2 mL/min; MSD was in selective ion monitoring (SIM) mode. The ions 204 (for **1**, **5**), 220 (for **2a**, **2b**, **6a**, **6b**), 211 (for **10**, **15**), 218 (for **3**, **7**), 225 (for **11a, 11b**, **16**), and 223 (for **9**, **14**) were selected as quantifiers. Qualifier ions selected for the corresponding quantifiers were 105, 147, 161 for **1**; 105, 163 and 205 for **2a** and **2b;** 105, 147 and 203 for **3**; 107, 147 and 161 for **4**; 147, 163 and 204 for **6a** and **6b;** 147, 161 and 203 for **7**; 108, 165 and 208 for **9**; 108, 165 and 196 for **10**; 108, 165 and 208 for **11a** and **11b**; 111, 163 and 208 for **14**; 112, 150, 166 for **15**; and 111, 165, 208 for **16.** The dwell times for all selected ions were set at 100 ms; gain factor was 1 and EI 70 eV; transfer line temperature was 240 ^oC, the MS source was at 230 ^oC and the MS quadrupler was at 150 ^oC. Whatman grade 1 filter paper was the support medium for oxidation of **1** and **5**. Silica column chromatography (SCC) was performed using either LC60A 40-63 micron silica (Grace Davison) or Silica gel 60 (0.015-0.040 mm, Merck). TLC was conducted with TLC silica gel 60 F_{254} plates (Merck KGaA) using standard vanillin stain for visualization. Silver nitrate impregnated silica (SNIS) was prepared according to the literature.[8] Rotundone (**3**) and the rotund-2-ols (**2a/b**) were prepared as described previously. [3,5]

Synthesis of standards for SIDA.

***d*$_5$-Rotundone (9).** Rotundone (**3**) (180 mg, 0.83 mmol) was dissolved in a 1 M solution of NaOEt in ethanol-d_6 (1 mL) in a vial (4 mL), which was capped under N_2 and left at room temperature overnight (ca 20 h). Brine (2 mL) was added and the resulting mixture extracted with Et_2O (4 x 5 mL). The combined ether layers were concentrated, dried over anhydrous $MgSO_4$, filtered and the filtrate concentrated *in vacuo*. The residue was purified with silica (15-40 μm) column chromatography (Et_2O / hexanes, 8%) affording **9** (75 mg, 42%) as a pale

yellow oil. Spectroscopic data of **9** including NMR and MS was in accord with those reported in the literature.[9]

***d*$_7$-*α*-Guaiene (10).** $LiAlD_4$ (42 mg, 1 mmol) was suspended in dry ether (1 mL) at room temperature under N_2. To the above solution was added dropwise a solution of anhydrous $AlCl_3$ (159 mg, 1.19 mmol) in dry ether (1 mL) with stirring. After 10 min, a premixed solution of d_5-rotundone **9** (32.1 mg, 0.14 mmol) in dry ether (0.5 mL) and anhydrous $AlCl_3$ (133.5 mg, 1 mmol) in dry ether (0.5 mL) was added dropwise. After stirring for 1 h, the reaction mixture was chilled in an ice-bath before being quenched by the slow addition of D_2O (2 mL) followed by 6 N H_2SO_4 (2 mL). The resulting mixture was extracted with ether (3 x 20 mL) and the combined ether layers washed with brine (10 mL), dried over anhydrous $MgSO_4$ and filtered. The filtrate was concentrated *in vacuo* and the residue purified by SNIS chromatography (hexanes) to yield **10** (27.2 mg, 88%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.68 (br s, 1H, H_{12a}), 4.62 (dq, 1H, J = 2.4, 1.5 Hz, H_{12b}), 2.34 (m, 1H, H_{10}), 2.11 (m, 1H, H_7), 1.72 (s, 3H, H_{13}), 1.71-1.67 (m, 3H, $H_{8a, 8b, 9a}$), 1.59 (m, 1H, H_{9b}), 1.01 (d, 3H, J = 6.6 Hz, H_{15}), 0.92 (s, 3H, H_{14}); ^{13}C NMR (150 MHz, $CDCl_3$) δ 152.4 (C_{11}), 140.5 (C_1), 138.5 (C_5), 107.9 (C_{12}), 46.4 (C_7), 33.8 (C_9), 33.7 (C_{10}), 31.0 (C_8), 20.4 (C_{13}), 19.7 (C_{14}), 18.6 (C_{15}); see Supporting Information for the mass spectrum of **10**.

***d*$_5$-2*R*-Rotundol** (**11a**) and ***d*$_5$-2*S*-rotundol** (**11b**) were synthesized from d_5-rotundone using the same protocol as described for preparing d_0-rotundols (**2a/b**)[5] except that d_5-rotundone (**5**, 40 mg, 0.18 mmol), $CeCl_3.7H_2O$ (70 mg, 0.19 mmol), MeOH (5 mL) and $NaBH_4$ (10 mg, 0.27 mmol) were used. d_5-2*R*-rotundol (**11a**, 23 mg, 65%, based on 87% conversion) and d_5-2*S*-rotundol (**11b**, 4 mg, 11% based on 87% conversion) were obtained as a white solid and a colorless liquid respectively, with d_5-rotundone (5 mg, 13%) recovered after purification by silica (15-40 μm) column chromatography (Et_2O / hexanes, 8%-12%). **11a**: ^{1}H NMR (600 MHz, C_6D_6): δ 4.79 (dq, 1H, J = 1.8, 1.5 Hz, H_{12a}), 4.73 (dq, 1H, J = 2.0, 1.4 Hz, H_{12b}), 4.38 (s, 1H, H_2), 2.50 (qdd, 1H, J = 7.2, 4.2, 3.6 Hz, H_{10}), 1.95 (m, 1H, H_7), 1.84 (dddd, 1H, J = 13.8, 12.0, 11.4, 2.4 Hz, H_{8a}), 1.73 (dddd, 1H, J = 13.8, 6.0, 2.7, 2.3 Hz, H_{8b}), 1.65 (s, 3H, H_{13}), 1.60 (dddd, 1H, J = 13.8, 6.0, 4.2, 2.4 Hz, H_{9a}), 1.56 (m, 1H, H_{9b}), 1.21 (d, 3H, J = 7.2 Hz, H_{15}), 0.96 (s, 3H, H_{14}); ^{13}C NMR (150 MHz, C_6D_6): δ 151.7 (C_{11}), 144.1 (C_1), 142.4 (C_5), 108.3 (C_{12}), 80.5 (C_2), 46.5 (C_7), 33.6 (C_9), 31.7 (C_{10}), 31.1 (C_8), 20.7 (C_{14}), 20.0 (C_{13}), 18.2 (C_{15}); EI-MS *m/z* (rel. intensity): 225 ($[M]^+$, 30), 206 (14), 191 (15), 179 (19), 167 (100), 148 (26), 135 (21), 121 (36), 108 (42), 95 (45), 81 (30), 67 (24), 55 (21). **11b**: ^{1}H NMR (600

MHz, C_6D_6): δ 4.80 (br s, 1H, H_{12a}), 4.74 (dq, 1H, J = 2.4, 1.8 Hz, H_{12b}), 4.57 (br s, 1H, H_2), 2.65 (qdd, 1H, J = 7.2, 4.2, 3.6 Hz, H_{10}), 2.14 (m, 1H, H_7), 1.75-1.71 (m, 2H, $H_{8a, 8b}$), 1.70-1.69 (m, 1H, H_{9a}), 1.64 (s, 3H, H_{13}), 1.61 (m, 1H, H_{9b}), 1.01 (d, 3H, J = 7.2 Hz, H_{15}), 0.83 (s, 3H, H_{14}); ^{13}C NMR (150 MHz, C_6D_6): δ 152.2 (C_{11}), 144.5 (C_5), 143.2 (C_1), 109.0 (C_{12}), 79.0 (C_2), 47.1 (C_7), 34.8 (C_9), 31.7 (C_8), 30.8 (C_{10}), 20.8 (C_{13}), 20.6 (C_{14}), 18.4 (C_{15}); see Supporting Information for the mass spectra of **11a/b**.

47% α-Bulnesene hydrocarbons. The guaiacwood oil mother liquor containing ca 47% α-bulnesol (**12**) was obtained after repeated recrystallization of guaiol with MeCN and was used for the synthesis of bulnesene enriched hydrocarbons employing acylation followed by pyrolysis as described for the synthesis of **1**.[10] To the guaiacwood oil mother liquor (33 g) was added acetic anhydride (100 mL) and 4-dimethylaminopyridine (DMAP, 330 mg). The resulting mixture was heated under reflux at 150 ^{o}C under N_2 for 6 h. After being cooled to room temperature, the reaction products were extracted with hexanes (4 x 200 mL) and the combined hexane layers washed with substantial water, brine and dried over anhydrous $MgSO_4$. The hexane extract was filtered through filter paper and solvent removal gave a crude product (40 g) containing 47% bulnesyl acetate (**13**, GC-MS). Crude bulnesyl acetate (23 g) was heated to ~220 ^{o}C under N_2 for 3.5 h whilst the acetic acid generated was condensed and collected in a receiving flask. The reaction was terminated and its temperature lowered to room temperature when no more acetic acid was being generated. To this reaction mixture was added hexanes (100 mL) and silica (ca 30 g). The resulting suspension was filtered through a bed of silica (ca 100 g) and further eluted with hexanes (3 x 100 mL). The combined filtrates were concentrated *in vacuo* to afford the bulnesene rich hydrocarbons (47% by GC-MS, 15.6 g, 90% from **12**). Pure α-bulnesene (**5**) was obtained as a colorless oil after repeated SNIS chromatography (hexanes) of the 47% bulnesene hydrocarbon mixture. α-bulnesene (**5**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.66 (br s, 1H, H_{12a}), 4.64 (dq, 1H, J = 2.0, 1.5 Hz, H_{12b}), 2.47 (dd, 1H, J = 12.0, 7.2 Hz, H_5), 2.33 (m, 1H, H_{2a}), 2.23-2.15 (m, 2H, $H_{9a, 2b}$), 2.13-2.08 (m, 2H, $H_{4, 7}$), 2.01 (br dd, 1H, J = 15.0, 7.2 Hz, H_{9b}), 1.73-1.69 (m, 1H, H_{8a}), 1.71 (br s, 3H, H_{13}), 1.67 (br s, 3H, H_{15}), 1.65 (m, 1H, H_{3a}), 1.62 (m, 1H, H_{6a}), 1.35 (dq, 1H, J = 12.0, 7.8 Hz, H_{3b}), 1.26 (dddd, 1H, J = 13.2, 11.4, 11.4, 1.5 Hz, H_{8b}), 1.03 (ddd, 1H, J = 12.6, 12.0, 12.0 Hz, H_{6b}), 0.90 (d, 3H, J = 7.2 Hz, H_{14}). ^{13}C NMR (150 MHz, $CDCl_3$): δ 152.4 (C_{11}), 142.0 (C_1), 128.8 (C_{10}), 108.0 (C_{12}), 50.8 (C_7), 46.8 (C_5), 38.8 (C_4), 34.6 (C_9), 33.1 (C_3), 32.6 (C_6), 31.7 (C_8), 30.4 (C_2), 22.2 (C_{15}), 20.9 (C_{13}), 15.4 (C_{14}). EI-MS *m/z* (rel. intensity):

204 ($[M]^+$, 27), 189 (55), 161 (18), 147 (36), 133 (35), 119 (35), 107 (100), 93 (86), 79 (55), 67 (28), 55 (28).

2*S*-Bulnesol (**6b**). SeO_2 (1.38 g, 0.5 equiv, 12.5 mmol) was suspended in CH_2Cl_2 (100 mL) under N_2 and the suspension cooled down to 0 ^oC. To the stirred suspension at 0 ^oC was added dropwise a solution of TBHP (5-6 M in decane, 10 mL, 50 – 60 mmol) in CH_2Cl_2 (15 mL) followed by a solution of α-bulnesene (47% by GC-MS, 5.1 g, 25 mmol) in CH_2Cl_2 (10 mL). After stirring at 0 ^oC for 3.5 h, the reaction was quenched with saturated aq Na_2SO_3 (50 mL). The resulting mixture was allowed to stir for an additional 0.5 h before being extracted with CH_2Cl_2 (3 x 100 mL) and the combined organic layers further washed with brine, dried over anhydrous $MgSO_4$ and filtered. The filtrate was concentrated *in vacuo* and the residue purified by SCC (Et_2O / hexanes, from 4% to 12%) to furnish **6b** (1.54 g, 57%) as a white solid. Spectroscopic data was in agreement with those in literature.[11]

***α*-Bulnesone (7)**. α-Bulnesone was synthesized by oxidation of **6b** using palladium catalysed oxidation with molecular oxygen.[12] To a stirred solution of **6b** (660 mg, 3 mmol) in THF/Toluene (15%, 30 mL) was added $Pd(OAc)_2$ (20.2 mg, 90 μmol) and Et_3N (20 μL, 0.14 mmol). The solution was stirred under an O_2 atmosphere at 45 ^oC after being evacuated and refilled with O_2 three times. The oxidation was terminated after 52 hours by filtration of the reaction mixture through a silica plug (ca 10 g), which was further rinsed with Et_2O (3 x 20 mL). The combined filtrates were concentrated *in vacuo* and the residue purified by SCC (Et_2O / hexanes, from 4% to 16%) to yield **7** (347 mg, 53%) as a colorless oil. ^1H NMR (600 MHz, $CDCl_3$): δ 4.66 (m, 2H, $H_{12a,b}$), 2.84 (dd, 1H, J = 12.0, 7.2 Hz, H_5), 2.36 (dd, 1H, J = 16.8, 7.8 Hz, H_{3a}), 2.30 (dd, 1H, J = 14.4, 10.8 Hz, H_{9a}), 2.26 (ddq, 1H, J = 7.8, 7.8, 7.2 Hz, H_4), 2.22 (d, 3H, J = 2.4 Hz, H_{15}), 2.25 (ddd, 1H, J = 14.4, 9.6, 7.8 Hz, H_{9b}), 2.18 (m, 1H, H_7), 2.01 (dd, 1H, J = 16.8, 7.8 Hz, H_{3b}), 1.78 (m, 1H, H_{8a}) , 1.72 (ddd, 1H, J = 13.2, 3.6, 1.8 Hz, H_{6a}), 1.68 (br s, 3H, H_{13}), 1.31 (dddd, 1H, J =13.8, 10.8, 9.6, 1.8 Hz, H_{8b}), 1.20 (ddd, 1H, J = 13.2, 12.0, 12.0 Hz, H_{6b}), 0.93 (d, 3H, J = 7.2 Hz, H_{14}). ^{13}C NMR (150 MHz, $CDCl_3$): δ 207.1 (C_2), 155.0 (C_1), 150.8 (C_{11}), 137.0 (C_{10}), 108.8 (C_{12}), 49.4 (C_7), 48.0 (C_3), 44.7 (C_5), 37.2 (C_9), 32.5 (C_6), 31.6 (C_4), 29.8 (C_8), 21.4 (C_{15}), 20.9 (C_{13}), 16.0 (C_{14}). EI-MS *m/z* (rel. intensity): 218 ($[M]^+$, 64), 203 (83), 185 (10), 175 (29), 161 (55), 147 (30), 133 (50), 122 (90), 108 (100), 91 (69), 79 (58), 67 (32), 55 (30).

***d_5*-Bulnesone (14).** Ketone **7** (326 mg, 1.5 mmol) was dissolved in a 1 M solution of NaOEt (102 mg, 1.5 mmol) in d_6-ethanol (1.5 mL) and the resulting mixture left at room temperature overnight. After ca 24 h brine was added (5 mL), the aqueous solution was extracted with Et_2O (3 x 15 mL) and the organic layers combined, dried over anhydrous $MgSO_4$ and filtered. The filtrate was concentrated *in vacuo* and purified by SCC (Et_2O / hexanes, 8%) to give **14** (293 mg, 88%) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.68 (br s, 2H, $H_{12a,b}$), 2.86 (dd, 1H, J = 12.0, 7.2 Hz, H_5), 2.30 (m, 0.6H, H_{9a}), 2.28 (q, 1H, J = 7.2 Hz, H_4), 2.25 (ddd, 0.9H, J = 14.4, 9.6, 7.8 Hz, H_{9b}), 2.21-2.18 (m, 1H, H_7), 1.80 (m, 1H, H_{8a}), 1.74 (dd, 1H, J = 13.2, 3.6 Hz, H_{6a}), 1.70 (br s, 3H, H_{13}), 1.33 (m, 1H, H_{8b}), 1.22 (m, 1H, H_{6b}), 0.94 (d, 3H, J = 7.2 Hz, H_{14}). ^{13}C NMR (150 MHz, $CDCl_3$): δ 207.2 (C_2), 154.9 (C_1), 150.8 (C_{11}), 137.0 (C_{10}), 108.8 (C_{12}), 49.3 (C_7), 44.6 (C_5), 36.6 (C_9), 32.5 (C_6), 31.6 (C_4), 29.8 (C_8), 20.9 (C_{13}), 16.0 (C_{14}). see Supporting Information for the mass spectrum of **14**.

***d_7*-α-Bulnesene (15).** d_7-Bulnesene was synthesized using the same procedure as that for d_7-α-guaiene except that $LiAlD_4$ (48 mg, 1.12 mmol), anhydrous $AlCl_3$ (159 mg 1.19 mmol) and the mixture of d_5-bulnesone (80 mg, 0.36 mmol) and $AlCl_3$ (133.5 mg, 1 mmol) were utilized. The crude reaction products were purified by repeated SNIS chromatography to yield d_7-bulnesene (33 mg, 44%) as a colorless oil. **15**: ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.66 (br s, 1H, H_{12a}), 4.64 (br s, 1H, H_{12b}), 2.45 (dd, 1H, J = 12.0, 7.2 Hz, H_5), 2.19 (br dd, 0.6H, J = 15.0, 11.4 Hz, H_{9a}), 2.11-2.06 (m, 2H, $H_{4,7}$), 2.01 (m, 0.9H, H_{9b}), 1.71 (br s, 3H, H_{13}), 1.70 (m, 1H, H_{8a}), 1.62 (ddd, 1H, J = 13.2, 1.8, 1.5 Hz, H_{6a}), 1.26 (m, 1H, H_{8b}), 1.03 (ddd, 1H, J = 13.2, 12.0, 10.8 Hz, H_{6b}), 0.90 (d, 3H, J = 7.2 Hz, H_{14}). ^{13}C NMR (150 MHz, $CDCl_3$): δ 152.4 (C_{11}), 141.9 (C_1), 128.8 (C_{10}), 108.0 (C_{12}), 50.8 (C_7), 46.1 (C_5), 38.7 (C_4), 34.4 (C_9), 32.7 (C_6), 31.8 (C_8), 20.9 (C_{13}), 15.4 (C_{14}). see Supporting Information for the mass spectrum of **15**.

2*R*-Bulnesol (6a). Compound **6a** was synthesized employing Luche reduction as used for the synthesis of 2*R*-rotundol (**4a**)[5] except that α-bulnesone (**7**, 14 mg, 64 μmol), MeOH (3 mL), $CeCl_3.7H_2O$ (13 mg, 35 μmol) and $NaBH_4$ (9 mg, 243 μmol) were used and the reaction was conducted at 0 ^{o}C. Purification of the reaction mixture by SCC (Et_2O / hexanes, 10%) yielded 2*R*-bulnesol (6.8 mg, 48%) as a colorless oil. ^{1}H NMR (500 MHz, C_6D_6) δ 4.81 (dq, 1H, J = 3.0, 0.8 Hz, H_{12a}), 4.78 (dq, 1H, J = 2.0, 1.5 Hz, H_{12b}), 4.53 (dd, 1H, J = 6.5, 4.5 Hz, H_2), 2.35 (br dd, 1H, J = 12.0, 7.5 Hz, H_5), 2.12 (br dd, 1H, J = 15.0, 11.5 Hz, H_{9a}), 2.02 (dddd, 1H, J = 12.0, 10.5, 3.0, 2.0 Hz, H_7), 1.94 (qd, 1H, J = 7.0, 6.5Hz, H_4), 1.91 (ddd, 1H, J = 15.0, 7.0,

1.5 Hz, H_{9b}), 1.81 (dd, 1H, *J* = 13.0, 6.5 Hz, H_{3a}), 1.79 (ddd, 3H, *J* = 1.5, 1.0, 0.5 Hz, H_{15}), 1.75 (m, 1H, H_{6a}), 1.71 (m, 1H, H_{8a}), 1.68 (dd, 3H, *J* = 1.5, 0.8 Hz, H_{13}), 1.46 (dddd, 1H, *J* = 13.0, 6.5, 4.5, 0.5 Hz, H_{3b}), 1.28 (dddd, 1H, *J* = 13.5, 11.5, 10.5, 1.5 Hz, H_{8b}), 1.22 (ddd, 1H, *J* = 13.0, 12.0, 12.0 Hz, H_{6b}), 1.02 (d, 3H, *J* = 7.0 Hz, H_{14}). ^{13}C NMR (125 MHz, C_6D_6): δ 151.9 (C_{11}), 145.3 (C_1), 135.5 (C_{10}), 108.8 (C_{12}), 74.0 (C_2), 51.3 (C_7), 46.6 (C_5), 43.5 (C_3), 36.3 (C_4), 35.7 (C_9), 34.0 (C_6), 32.1 (C_8), 22.1 (C_{15}), 21.0 (C_{13}), 17.0 (C_{14}). EI-MS *m/z* (rel. intensity): 220 ([M]$^+$, 23), 205 (16), 187 (21), 177 (13), 163 (42), 138 (28), 119 (47), 108 (100), 95 (64), 79 (42), 67 (37), 55 (38).

***d*$_5$-2*R*-Bulnesol (16)**. *d*$_5$-Bulnesone was synthesized using the same procedure as that for 2*R*-bulnesol except that *d*$_5$-bulnesone (16.7 mg, 75 μmol), $CeCl_3.7H_2O$ (16 mg, 43 μmol) and $NaBH_4$ (8 mg, 216 μmol) were used. Purification of the reaction products by SCC (Et_2O / hexanes, 10%) gave *d*$_5$-2*R*-bulnesol (10.2 mg, 61%) as a colorless oil. ^{1}H NMR (500 MHz, C_6D_6) δ 4.81 (dq, 1H, *J* = 3.0, 0.8 Hz, H_{12a}), 4.78 (dq, 1H, *J* = 2.0, 1.5 Hz, H_{12b}), 4.52 (s, 1H, H_2), 2.35 (br dd, 1H, *J* = 12.0, 7.5 Hz, H_5), 2.12 (br dd, 0.6H, *J* = 15.0, 11.5 Hz, H_{9a}), 2.02 (dddd, 1H, *J* = 12.0, 10.5, 3.0, 2.0 Hz, H_7), 1.94 (dq, 1H, *J* = 7.5, 7.0 Hz, H_4), 1.91 (m, 0.9H, H_{9b}), 1.75 (m, 1H, H_{6a}), 1.71 (m, 1H, H_{8a}), 1.68 (dd, 3H, *J* = 1.5, 0.8 Hz, H_{13}), 1.28 (m, 1H, H_{8b}), 1.22 (ddd, 1H, *J* = 13.0, 12.0, 12.0 Hz, H_{6b}), 1.02 (d, 3H, *J* = 7.0 Hz, H_{14}). ^{13}C NMR (125 MHz, C_6D_6): δ 151.9 (C_{11}), 108.8 (C_{12}), 73.9 (C_2), 51.2 (C_7), 46.5 (C_5), 43.5 (C_3), 36.3 (C_4), 35.7 (C_9), 34.0 (C_6), 32.1 (C_8), 21.0 (C_{13}), 17.0 (C_{14}). see Supporting Information for the mass spectrum of **16**.

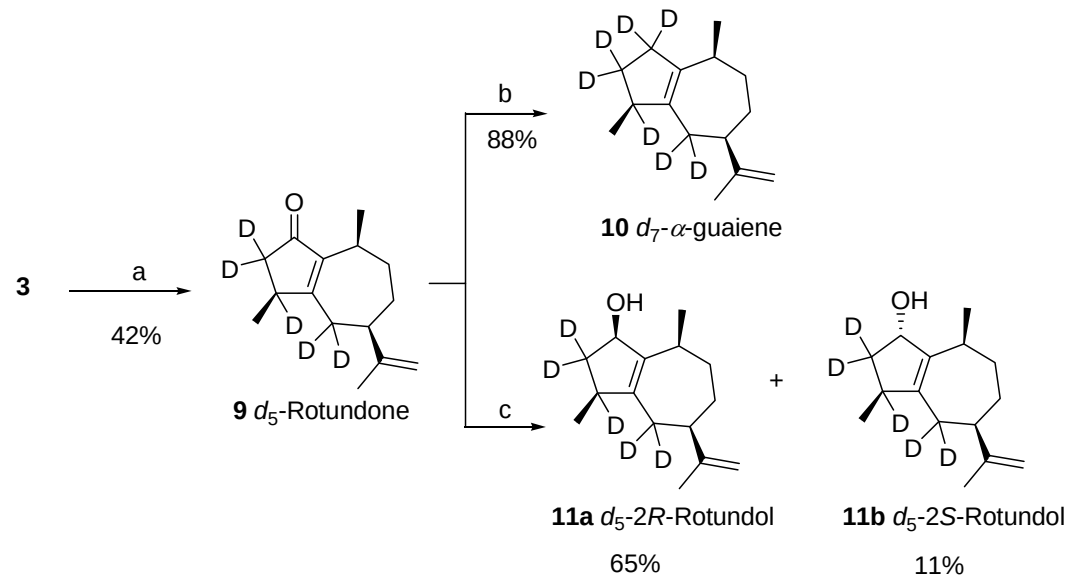
Preparation and validation of calibration curves. Stock standard solutions in EtOAc were prepared from more concentrated solutions of synthetic standards as follows: Solution A contained **1** (160 μg/mL) and **2a**, **2b**, and **3** (each 80 μg/mL); solution B contained **5**, **6a**, **6b** and **7** (each 160 μg/mL); solution C contained **9** and **10** (each 80 μg/mL)**, 11a** (32 μg/mL) and **11b** (128 μg/mL); solution D contained **14**, **15** and **16** (each 80 μg/mL). Solution A was diluted with EtOAc to a scale of eight concentration levels and each spiked with an equal volume (250 μL) of diluted solution C (1 : 10 dilution) to make up the final concentrations of 0, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, and 40.0 μg/mL for **2a**, **2b** and **3** and 0, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, and 80.0 μg/mL for **1** with corresponding labeled IS at 4.0 μg/mL for **9** and **10**, 1.6 μg/mL for **11a** and 6.4 μg/mL for **11b**. In a similar fashion, solution B was diluted with EtOAc to a scale of eight concentration levels and each spiked with equal volume (250 μL) of

diluted solution D (1 : 10 dilution) to make up the final concentrations of 0, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, and 80.0 μg/mL for **5**, **6a**, **6b** and **7** with corresponding labeled IS at 4.0 μg/mL for **14**, **15** and **16**. The above concentrations of analytes were prepared in duplicate and analyzed by the GC-MS method outlined in the General Experimental Section. For generating the calibration curves, the ratios of response areas of selected ions at their corresponding retention times at each concentration [204/211 (**1/10**), 220/225 (**2a/11a**), 220/225 (**2b/11a**), 218/223 (**3a/9**) and 204/211 (**5/15**), 220/225 (**6a/16**), 220/225 (**6b/16**), and 218/223 (**7/14**)] were plotted against the ratios of concentrations of analytes versus those of labeled IS [c(**1/8**), c(**2a/11a**), c(**2b/11b**), c(**3/9**), c(**5/15**), c(**6a/16**), c(**6b/16**), c(**7/14**)]. Samples for quantitative analysis were each spiked with deuterium labeled IS mixed solutions C or D to make up the final concentrations as those of IS in the preparation of the calibration curves. The concentrations of analytes in the samples were calculated based on calibration equations listed in Table S1, Supporting Information. The method was verified by linearity, relative standard deviation of quantitative analysis, limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ are defined as the concentration giving a signal to noise (S/N) ratio of 3:1 and 10:1, respectively.

Oxidation of α-guaiene (1) and α-bulnesene (5). An α-guaiene solution (ca 92 mg/mL) was prepared by dissolving **1** (ca 92 mg) with EtOAc to a total volume of 1.0 mL. An aliquot (5 μL) of the above solution was added to a 1 cm^2 filter paper (FP) each in an empty vial (SAMCO specimen tubes 50 x 19 mm). The vials were left uncapped to allow contact with air at room temperature and the changes of concentrations of **1**, **2a**, **2b** and **3** at different time points were quantified for duplicate samples at each time point by SIDA with GC-MS. For determining analyte concentrations, EtOAc (1 mL) was added to each sample vial. An aliquot of the analyte solution (80 μL) was diluted with EtOAc and spiked with solution of labeled IS mixture to make up a total volume of 0.5 mL solution containing 4 μg/mL labeled IS with a dilution factor of 6.25 for analytes. This solution was analyzed by GC-MS using the parameters described in the general experimental section and concentrations of analytes were obtained based on the calibration curves generated above. α-Bulnesene solution was prepared by dissolving α-bulnesene enriched essential oil containing no **6a**, **6b**, or **7** (79% bulnesene, 4% β-guaiene, 2% aciphyllene, 14% selinene isomers and 1% others by GC-MS, ca 150 mg) in EtOAc (1 mL). Oxidation of α-bulnesene (**5**) and determination of analytes were performed using the same procedure as that described for α-guaiene (**1**).

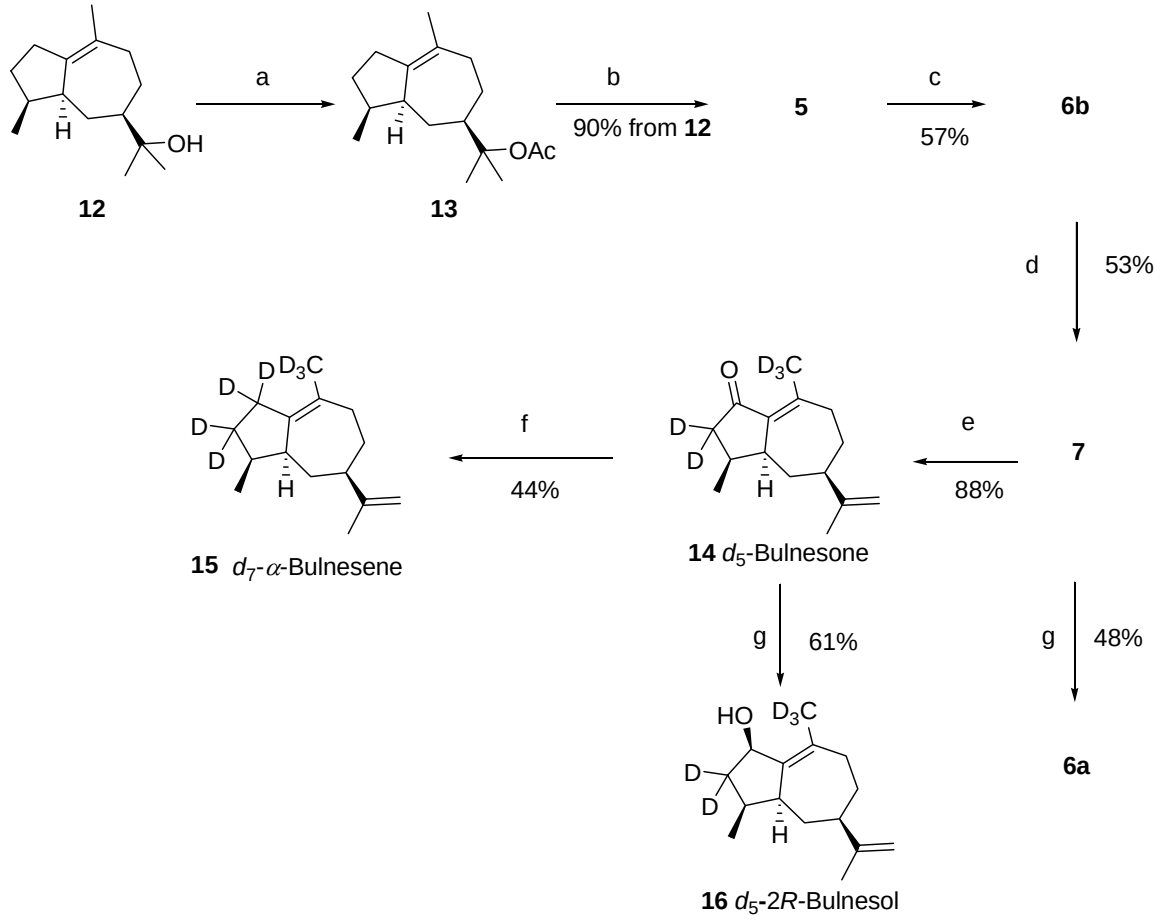
RESULTS AND DISCUSSION

Synthesis of deuterium labelled internal standards (IS). Based on the readily synthesized d_5-rotundone (**9**), the d_5-rotund-2-ols (**11a/b**) could be easily prepared by Luche reduction of **9**. Employing the stronger reducing reagent AlD_3 generated *in situ* by $LiAlD_4$ and anhydrous $AlCl_3$ realized the reduction of the enone carbonyl to furnish desired **10** in an excellent 88% yield, Figure 2. Initial attempts to access deuterated α-bulnesene **15** via simple isomerization of d_7-α-guaiene **10** under various group VIII metal catalyzed (e.g. $PdCl_2$, $Pd(OAc)_2$, $RhCl_3$), strongly acidic or basic conditions proved to be ineffective. Therefore, focus was shifted onto constructing a carbonyl moiety at C2 within α-bulnesene **5** itself to enable initial deuterium exchange followed by removal of the carbonyl moiety by reduction to allow for the preparation of d_7-α-bulnesene (1**5**), Figure 3. Direct attempts at allylic oxidation of **5** into α-bulnesone (**7**) in a single step exploiting chromium and cobalt based oxidants coupled with TBHP were unsuccessful, however allylic oxidation of **5** with SeO_2 and TBHP furnished 2*S*-bulnesol (**6b**) in a moderate 57% yield. Palladium catalysed oxidation of **6b** afforded ketone **7** again in moderate yield which was subjected to deuterium exchange under basic conditions to furnish the desired d_5-bulnesone (**14**) in an excellent yield of 88% yield. d_5-2*R*-Bulnesol (**16**) and d_7-α-bulnesene (**15**) were successfully synthesized by reduction of **14** in similar fashions as those utilized for the synthesis of the d_5-rotund-2-ols (**11a/b**) and d_7-α-guaiene (**10**). Naturally deuterium incorporation by deuterium exchange is an equilibrium and will always yield a mixture of deuterium labelled compounds with various degrees of deuterium incorporation at different sites of the substrates depending on the ease of exchange of the hydrogen atoms. For example, deuterium exchange of α-bulnesone **7** was found to be not thoroughly complete after the exchange equilibrium was reached, with a very small percentage of α-bulnesone **7** still existing in the labelled d_5-bulnesone **14** mixture. Consequently, this undeuterated analogue was carried through to the d_5-2*R*-bulnesol (**16**) after direct Luche reduction with $NaBH_4$. The nomenclature used here for the deuterium standards **9**, **10**, **11a/b**, **14**, **15**, and **16** and their structures are depicted in Figures 2 and 3 and represent the number of exchangeable hydrogens that are mainly substituted with deuterium. A low incorporation of deuterium onto C_9 was apparent for **14** – **16**. The determined deuterium incorporation percentages for all the synthetic standards are presented in Table 1.



a. NaOEt, CD_3CD_2OD, rt, 24 h; b. $LiAlH_4$, $AlCl_3$, Et_2O, rt, 10 min;c. $CeCl_3{\cdot}7H_2O$, $NaBH_4$, MeOH, -78 ^oC, 30 min.

Figure 2. Synthesis of d_7-α-guaiene (**10**) and d_5-rotund-2-ols (**11a/b**).



a. Ac_2O, DMAP, 150 ^oC, 6h; b. 220 ^oC, 3.5 h; c. SeO_2, TBHP, CH_2Cl_2, 0 ^oC, 3.5 h; d. $Pd(OAc)_2$, O_2, Et_3N, THF/toluene(15%), 45 ^oC, 52 h; e. NaOEt, CD_3CD_2OD, 24 h; f. $LiAlD_4$, $AlCl_3$, Et_2O; g. $CeCl_3{\cdot}7H_2O$, $NaBH_4$, MeOH, 0 ^oC, 30 min.

Figure 3. Synthesis of d_5-α-bulnesone (**14**), d_7-α-bulnesene (**15**), and d_5-2R-bulnesol (**16**).

Table 1. Percentage of deuterium incorporation of deuterium labelled IS.*

IS	d_0	d_1	d_2	d_3	d_4	d_5	d_6	d_7	d_8	d_9
9	nd	1.1%	5.6%	21.3%	39.5%	28.6%	2.4%	1.6%	nd	nd
10	nd	nd	0.6%	4.4%	14.3%	28.0%	29.4%	16.3%	5.1%	2.1%
11a	nd	0.4%	9.5%	23.2%	35.3%	27.6%	2.8%	1.3%	nd	nd
11b	nd	nd	4.8%	23.3%	38.0%	29.3%	22.0%	1.9%	0.5%	nd
14	0.6%	4.1%	13.4%	25.3%	28.7%	19.7%	7.2%	1.0%	nd	nd
15	nd	nd	nd	3.5%	12.1%	25.0%	30.2%	21%	7.6%	0.8%
16	0.7%	4.2%	13.4%	24.6%	28.7%	20.0%	7.4%	1.0%	nd	nd

* signals arising from the ^{13}C signals of the parent ions have been excluded; nd: not detected

Quantification method development. The deuterium labeled compounds prepared as described above were used as IS for the determination of their corresponding analytes. d_5-2*R*-Bulnesol (**16**) was also used as an IS for quantifying 2*S*-bulnesol (**6b**). Approximately 0.6% of **14** and **16** had no deuterium incorporation when equilibrium of exchange had been reached. The d_0 analytes from spiked IS were negligible compared to those for the analytes. All calibration curves displayed great linearity with correlation coefficient (R^2) values ranging from 0.9942 to 0.9997. LOD and LOQ were determined to be as low as 1.7 μg/L and 5.5 μg/L, respectively. The precision of the method was also verified by relative standard deviation (RSD) of five replicate quantifications of control samples at two different concentration levels. The RSD for all replicate analyses ranged from 0.8% to 3.9%. (Table S1, Supporting Information) The labeled standard solutions for analysis prepared at room temperature, stored under N_2, and kept in a freezer before and after each usage, however were allowed to attain ambient temperature before usage. These standard solutions were found to be stable under the analysis conditions.

Evolution of sesquiterpene products under aerial oxidation conditions. Employing the quantification methods developed herein, we were able to monitor the evolution of key aroma

compounds generated from the aerial oxidation of α-guaiene **1** and α-bulnesene **5** by determining their concentration changes over time. To better mimic the oxidation of **1** and **5** on dry herbs and plant materials, we used cellulose filter paper (FP) as the supporting medium by coating **1** (ca 380 μg) and **5** (ca 600 μg) onto FP (1 cm^2), respectively. Aerial oxidation of both **1** and **5** produced the corresponding C2 alcohols and ketones, suggesting that oxidation of **5** could follow a similar oxidative pathway as that of **1** via the generation and decomposition of the C2 hydroperoxide epimers as recently described.[3,5]

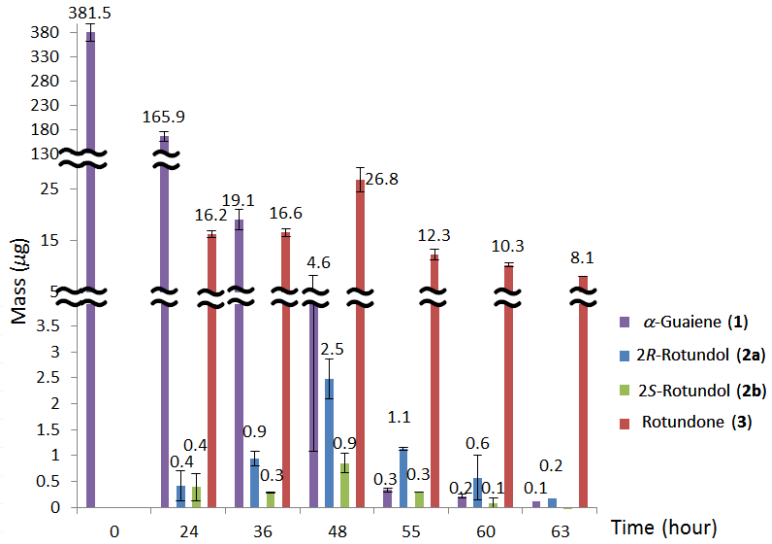


Figure 4. Formation of products from the aerial oxidation of α–guaiene (**1**).

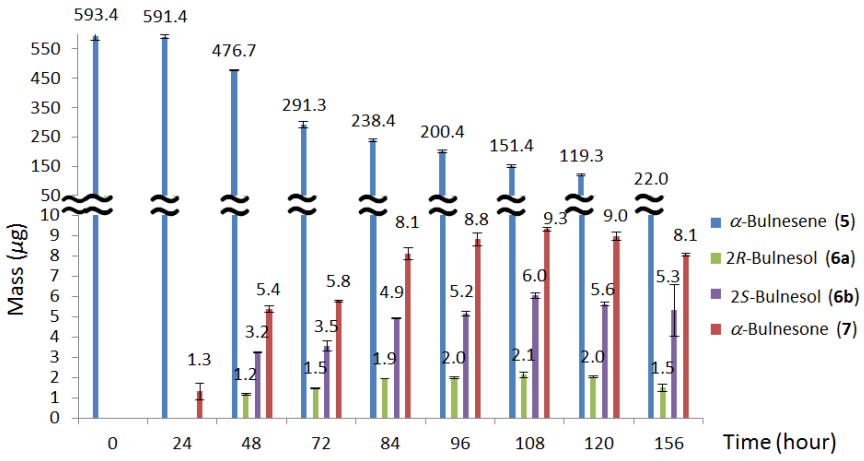


Figure 5. Formation of produts from the aerial oxidation of α–bulnesene (**5**).

As shown in Figure 4, 95% of α-guaiene **1** (ca 360 μg) was consumed in 36 h with 16.6 μg of rotundone **3** generated. Small amounts of **2a** and **2b** were generated and their maximum concentrations were reached after 48 h. As the formation of **2a/b** and **3** is determined by the decomposition of C2 hydroperoxides (**4a/b**), the above mass changes suggested that a significant amount of **4a/b** was being formed in ca 48 h since the onset of oxidation. After 48 h when the peak masses were recorded, the mass for **2a/b** and **3** started declining, indicative of the occurrence of further oxidation degradation.

In contrast to the autoxidation of **1**, autoxidation of α-bulnesene **5** proceeded more slowly with only half of precursor **5** consumed in 84 h and the peak masses for **6a/b** and **7** reaching a maximum after 108 h. The overall pattern of autoxidation of **5** resembles that of **1** but at a lower overall reaction rate. A very small quantity of α-bulnesene **5** (ca 2 μg) was lost during aerial oxidation over the first 24 h, suggesting that evaporation was not significant under the autoxidation conditions and indicates that the loss of α-guaiene **1** during oxidation was due to facile chemical reactions rather than evaporation based on their similar physical properties. Interestingly, the formation of the rotund-2-ols (**2a/b**) and bulnes-2-ols (**6a/b**) over time displayed discrepant stereochemical preferences with the formation of the rotund-2-ols favouring the β orientation (**2a**) over α-(**2b**) and the bulnes-2-ols favouring the formation of the opposite α orientation (**6b**) over β-(**6a**). The formation ratio of **2a** vs **2b** was ca 1:1 in 24 h, increased to ca 3:1 at 36 h through 48 h and further rose to ca 4:1 and 6:1 at 55 h and 60 h respectively, whereas that of **6a** vs **6b** stayed at approximately 1:3. Consequently, it appears that the 2*S*-rotundol **2b** undergoes further oxidation into rotundone **3** in a much more facile fashion when compared to its 2*R*-counterpart **2a** under the specific oxidative conditions outlined herein.

This study has demonstrated that in a similar fashion to the formation of rotundone (**3**) from α-guaiene (**1**), α-bulnesene (**5**) will also generate the aroma compounds 2*R*/2*S*-bulnesol (**6a** and **6b**) under simple aerial oxidation condition. Furthermore, it was found that the formation of 2*R*/2*S*-rotundols (**2a** and **2b**) and 2*R*/2*S*-bulnesols (**6a** and **6b**) displayed stereochemical preferences, which is undoubtedly related to a) the initial stereochemical preference for formation of the C2-hydroperoxide intermediates from the C2 requisite free radicals followed by decomposition into the observed C2-alcohols with retention of stereochemistry and b) the unequal consumption of these C2-alcohols by downstream oxidative processes. This study

also provides further evidence on how various oxygenated sesquiterpenes may be formed from their precursor hydrocarbons under simple aerial oxidative conditions and may have implications on the storage and processing of foodstuffs and beverages originating from plant materials that contain these sesquiterpene hydrocarbons. Finally, the deuterium labelled analogues prepared herein may be used to develop more sensitive quantification methods for the analysis of more complex plant based matrices.

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Notes

The authors declare no competing financial interest.

ABREVIATIONS AND NOMENCLATURE

TBHP, *tert*-butyl hydroperoxide; FP: filter paper; SNIS: silver nitrite impregnated silica; SCC: silica column chromatography; LOQ: limit of quantitation; LOD: limit of detection.

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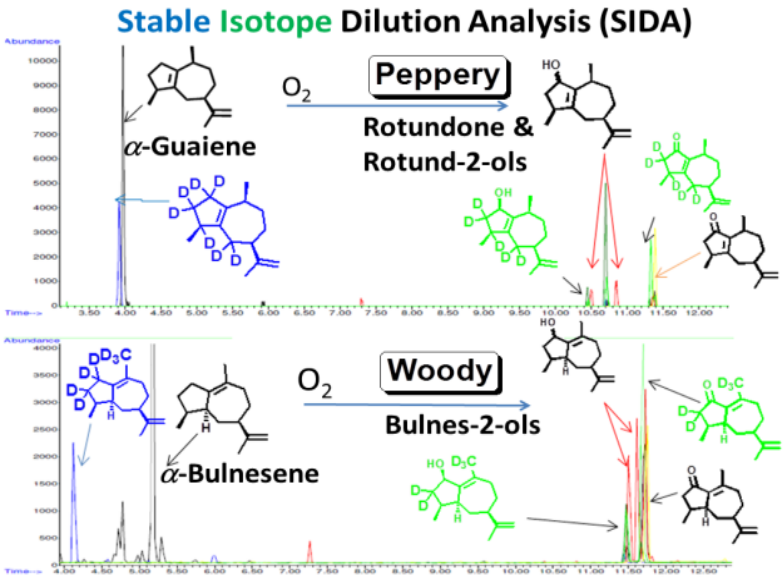
TABLE OF CONTENTS GRAPHICS



SUPPORTING INFORMATION

Mass spectra of deuterium labelled internal standards **9**, **10**, **11a/b**, **14-16**. Calibration curves for quantitative analysis of analytes **1**, **2**, **3a/b**, **5**, **6a/b** and **7.** This materials is available free of charge via the Internet at http://pub.acs.org.

Chapter 7

Conclusions

Our Mother Nature presents countless rewarding puzzles for people to solve and we learn and grow in the course of finding these answers. Natural product discovery emphasises on discovering novel natural products, searching for their potential biological activities that may be utilised to benefit human beings and also on understanding the mechanisms behind their formation in the hope of reproducing those natural products of interest employing chemical and biochemical approaches.

Extraction and isolation of novel compounds has long been an indispensable primitive approach in natural product discovery. Among the seven phenolic compounds that were isolated for the first time from the Australian native *Smilax glyciphylla* in our study, two of them are novel compounds, three of them including one new phenolic glycoside exhibited high levels of antioxidant activity. This has provided a much better picture of the origin of the antioxidant activities of the leaves of *Smilax glyciphylla* reported previously. The sensory property of the two new phenolic glycosides is yet to be determined due to the small quantity of pure compounds available. Consequently they could be potential candidates to be used as functional food additives if further investigation reveals that they are perceptually pleasant. Larger scale isolation or chemical synthesis may be carried out to obtain larger quantities of these two new compounds for other biological activity assays.

Chemical synthesis has also been an indispensable tool to access natural products of low natural abundance and their analogues while at the same time allowing us to unveil their hidden chemical and physical properties. Our studies on the synthesis of newly isolated anti-inflammatory guaiol isomers have provided facile access to both 4-isoguaiol and 5-isoguaiol in a single sequence in 26% and 31% yields respectively. It was also found that the diastereomeric ratio of epoxidation of these sterically biased substrates could be impacted by choice of solvents, the nature of epoxidation reagents, and interactions between the epoxidation reagents and remote side chain protecting groups of the [5,7] bicyclic structures such as guaiol. This demonstrates the possibility of achieving diastereoselective epoxidation by choosing appropriate epoxidising reagents and by simple functional group derivatisation without having to resort to ligated metal catalysts. The mechanism behind this selectivity has

yet to be precisely determined although our theoretical calculations have shed light into the conformations and their relative energies. Further theoretical calculations on the transition states of the MCPBA and DMDO epoxidation of various functional group protected substrates would yield more accurate structural information and is being performed. The potential of using aciphyllene and 1-*epi*-aciphyllene to synthesise guaiane sesquiterpenoids of different oxygenated levels was explored with difficulties encountered in attempting the epimerisation of the C1 epimers of melicodenones C and E. However, by exploiting other synthetic strategies, synthesis of other sesquiterpenoids via these two precursors may be of significance in the future.

One of the very obvious advantages of being able to access natural products of low abundance either via synthesis or isolation is that the biochemical properties of these natural products and other related compounds of relevance could be investigated *in vitro*. With the large amount of α-guaiene in hand, a wide range of guaiane sesquiterpenoids were synthesised. Careful analysis of the structural features of α-guaiene and the peppery aroma compound rotundone allowed us to postulate that intermediate unstable hydroperoxides would be involved during autoxidation, which in turn led us to the isolation and characterisation of two hitherto unknown guaiene hydroperoxides. This finding demonstrated that the formation mechanism of rotundone from its precursor α-guaiene under aerial oxidation is via the generation of hydroperoxide intermediates followed by decomposition. Moreover, oxidation of α-guaiene coated on filter paper was found to proceed much faster than that of neat α-guaiene in terms of the formation of rotundone. Elevated temperature would enhance the reaction rate exponentially. This has significant implication within the food industry considering the ubiquitous availability of the precursor α-guaiene in plant materials and the amount of rotundone that may be formed during food processing and storage. It also paved the way for studying the biosynthesis of this important aroma compound *in vivo*.

After this initial preliminary study a further detailed investigation into the autoxidation of α-guaiene allowed for the isolation and characterisation of a total of thirteen downstream sesquiterpenoids and two unstable hydroperoxides from the autoxidation products of α-guaiene. One of the natural products isolated in significance was rotundone, which is the only known impact odorant displaying a peppery aroma. Other products included corymbolone

and its C6 epimer, the 2*R*- and 2*S*-rotundols and several hitherto unknown epimers of natural chabrolidione A, namely 7-*epi*-chabrolidione A and 1,7-*epi*-chabrolidione A. Several C4 hydroxy rotundones and a range of epoxides were also found in significant amounts after autoxidation. Their structures were elucidated on the basis of spectroscopic data, HRMS and X-ray crystallography and a number of them confirmed through total synthesis. The mechanisms of formation of many of the products may be accounted for by initial formation of the 2- and 4-hydroperoxy guaienes followed by various fragmentation or degradation pathways. Given that α-guaiene is well know to exist in the essential oils of numerous plants, coupled with the fact that aerial oxidation to form this myriad of downstream oxidation products occurs so readily at ambient temperature suggests that many of them have been missed being identified during past isolation studies from natural sources. This study highlights the structural diversity of sesquiterpenoids that could possibly originate from the autoxidation of sesquiterpene precursors in nature. The sensory properties of the newly isolated compounds from the autoxidation of α-guaiene is largely unknown except for the two peppery and woody rotund-2-ols. Knowing the aroma profiles of these compounds and their derivatives would benefit the perfumery industry in providing more aroma substances.

Besides chemical synthesis, biosynthesis of natural products has been deemed as a more sustainable approach to produce bioactive targets for the pharmaceutical industry. The prerequisite of utilising biosynthesis for bio-production is the understanding of the biosynthetic pathways, for which feeding experiments with isotope labelled analytes is a common approach. In order to study the formation and occurrence of rotundone and other guaiane fragrances in nature, deuterium labelled standards including d_7-α-guaiene, d_5-2-rotundols, d_9-α-bulnesene, d_7-2*R*-bulnesol and d_7-α-bulnesone were synthesised for developing quantification methods and feeding experiments. With the readily available quantification methods developed herein, woody 2*R*-bulnesol and 2*S*-bulnesol were identified as the oxidation products of α-bulnesene with their concentrations determined. The methods developed herein could be further refined and used to determine these aroma compounds in living plants, plant materials, and processed food and beverages that exhibit these aroma profiles.

Overall, the development of more sophisticated preparative and analytical instruments, high throughput screening assays, synthetic and biosynthetic methodologies are shortening the

natural product discovery period with more hidden biological activities of various natural products being discovered on a continual basis. The focus of natural product discovery is gradually shifting towards those less commonly available sources such as marine creatures, insects and microbes. Despite the continuing effort that is put into natural product research, the natural cosmos is still far from being explored and will continue to offer the largest and most reliable source of lead compounds and inspiration into the future.