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Microbial Transformation of Taxanes and Steroids

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To My Family

Résumé

Le Paclitaxel, (Taxol®), qui a été découvert en 1971 et approuvé par la FDA pour la mise en marché en décembre 1992, est devenu l'un des médicaments anti-cancéreux disponible le plus utilisé et ayant le plus de succès. Contrairement aux autres agents anti-tumoraux qui agissent en prévenant la polymérisation de la tubuline en microtubules, le Paclitaxel empêche la division cellulaire en promouvant l'assemblage de la tubuline et en stabilisant le complexe. Le Paclitaxel a eu beaucoup de succès comme traitement contre le cancer du sein et des ovaires. Ce médicament est également prometteur comme traitement contre une variété d'autres tumeurs solides telles que des tumeurs au cerveau, au cou, aux poumons, aux voies gastro-intestinales et à la vessie. Des nouveaux taxanes sont hautement désirables.

Les champignons filamenteux sont reconnus pour avoir la capacité d'hydrolyser une grande variété de composés organiques d'une façon régio- et stéréo-sélective. Il y a des avantages à la transformation microbienne. On pourrait avoir des réactions rares qui pourraient donner des nouveaux produits, difficiles à obtenir par synthèses chimiques. Par contre, il y a aussi des désavantages à cette transformation. Généralement on ne peut pas prédire les résultats et le rendement normalement est très bas. Le but de ce projet est d'utiliser cette caractéristique enzymatique des champignons filamenteux afin de modifier les taxanes et ainsi obtenir de nouveaux taxanes.

Pendant notre recherche, nous avons trouvé que quelques champignons utilisés pouvaient hydroxyler des taxanes. Nous voulions aussi essayer de les utiliser pour transformer d'autres composés organiques, tels que l'androst-4-ene-3,17-dione. Ce composé est disponible commercialement et est aussi un produit de départ pour la synthèse commerciale de la testostérone et d'autres nouveaux stéroïdes. Notre but est d'hydroxyler l'androst-4-ène-3,17-dione à différents positions pour fournir des intermédiaires qui pourraient stimuler la découverte de nouveaux médicaments.

La transformation microbienne de taxanes (le chapitre 2-6) et de stéroïdes (le chapitre 7) est présentée dans cette thèse.

Vingt-sept taxanes naturels ou modifiés chimiquement ont été incubés avec 16 espèces de champignons filamenteux. (chapitre 6, **Figure 6.1** et **Figure 6.2**). Six d'entre eux ont été métabolisés (**4, 50, 52, 56, 57,** and **61**). Leurs produits ont été purifiés à l'aide de plusieurs méthodes de chromatographie et leurs structures ont été déterminées par Resonance Magnéfique Nucléaive (RMN) et par spectrométrie de masse à haute résolution (HRMS).

Le composé **4** (9-dihydro-13-acetylbaccatin III) (substrat #1 au chapitre 5) peut être métabolisé par *Absidia coerulea* ATCC 10738a pour donner 4 produits (**Figure 6.3**). En plus des produits de déacétylation **71** et **72**, les produits **73** et **74** sont des abéo-taxanes. Les abéo-taxanes provenant du substrat avec 1-groupement hydroxylé par transformation microbienne ont été découverts pour la première fois dans cette thèse.

Le composé **50** (2-déacetoxyltaxinine J) (substrat #2 au chapitre 4) peut être transformé par le champignon *Absidia coerulea* ATCC 10738a pour donner 4 produits, **75** à **78** (**Figure 6.4**).

Le composé **52** (5α, 7β, 9α, 10β, 13α-pentahydroxy-4(20),11(12)-taxadiene) (substrat #1 au chapitre 3) a été converti en un dérivé de taxane, **79** (32.4%) avec un C10-C11 double liaison et deux 1(15→11) abeo-taxanes, **80** (42.3%) et **81** (3.7%) par le champignon *Absidia coerulea* ATCC 10738a. Contrairement aux 11(15→11) abéo-taxanes plus communs, les produits **80** et **81** ont un squelette de 1 (15→11) abéo-taxane non décrit dans la littérature. Ce squelette n'a jamais été trouvé dans la nature ou par modification chimique des taxanes.

Le composé **56** (substrat #1 au chapitre 4) fut auparavant utilisé pour l'étude de la transformation microbienne. Le champignon *Absidia coerulea* ATCC 10738a ainsi que le milieu utilisé pour ce projet sont quelque peu différents. Les produits majeurs pour l'hydroxylation en C1, C-14 et des abéo-taxanes (**82**, **84** et **90** respectivement) sont identiques. Les produits secondaires par contre sont différents. Il y a six produits de déacétylation (**83, 85, 86, 87, 91, 92** et **93**) qui n'ont jamais été isolés auparavant. Le produit **88** est un produit secondaire ayant été isolé et possédant un groupe $OCOH_2COCH_3$ à la position C-5. Ce genre de modification n'a jamais été identifié chez les taxanes naturels. Le produit **89** possédant une hydroxylation à la position C-16 est rarement observé dans les taxanes naturels. Deux autres taxanes, **94** et **95**, ont un système annulaire 6-7-6. Cette modification est identifiée pour la première fois chez les taxanes naturels et également chez les taxanes modifiés chimiquement. Cependant, leur rendement est très faible.

Le composé **57** (substrat #1 au chapitre 2) peut être métabolisé par *Cunninghamella elegans* AS3.2033 pour donner 5 nouveaux taxanes (**Figure 6.7**). Le produit **96** résultant d'une hydroxylation en C-1 a le plus haut rendement soit de 43.0%, tandis que le produit **97** résultant d'une hydroxylation en C-1, a un isomère *cis* sur la chaîne secondaire cinnamoyl du produit **96** ainsi qu'un rendement de 1.6%. L'abéo-taxane **101** a un rendement de 16.0%. Les produits **98** et **99** résultants d'une hydroxylation en C-14 sont des *trans-cis* isomères sur la chaîne secondaire cinnamoyl et ont un rendement de 1.0% chacun. Le composé **57** peut également être métabolisé par *Cunninghamella elegans* var chibaensis ATCC 20230 pour donner 3 taxanes. En plus du produit **96** d'hydroxylation C-1 (20.2%) et de l'abéotaxane **101** (6.8%), il y a le produit **100** d'hydroxylation C-17 (5.4%) qui est très rare chez les taxanes naturels.

Le Composé **61** (substrat #2 au chapitre 5) peut être métabolisé par le champignon *Cunninghamella echinulata* AS 3.1990 pour donner six produits **102-107** (**Figure 6.8**). Les produits **102**, **103** et **104** ont échangé leur groupe benzoyle avec le groupe acétyle à la position 2 et la position 9 comparé au matériel de départ.

Les produits **105-107**, avec le groupe de 2 Bz aussi déplacé à la position 9, a un anneau oxétane ouvert et un nouvel anneau de cinq membres formé avec -C2-C3-C4-C20-O-. Tout ces trois abéo-taxanes ont un -OAc à C-15 très rares dans les taxanes naturels.

La relation entre la structure du taxane et la réactivité du champignon décrite ci-dessous est également représentée dans le chapitre 6 de cette thèse.

1. le groupe 2-OR peut bloquer l'hydroxylation de C-1 et C-14.
2. le groupe 5-OH a un impact négatif sur l'hydroxylation de C-1 et C-14.
3. en enlevant les trois groupes acetyle en position 7,9,10, il est possible d'augmenter le rendement de l'hydroxylation en C-1 et à la fois diminuer le rendement de l'hydroxylation en C-14.
4. 1-OH et 14-OH peuvent se bloquer eux-mêmes.
5. 13-cétone n'est pas éssentiel pour la transformation microbienne.

Les résultats présentés dans cette thèse démontrent que la transformation microbienne peut être un moyen unique afin d'obtenir de nouveaux taxanes qui peuvent être utilisés pour développer de nouveaux médicaments. La relation entre la structure du taxane et la réactivité du champignon démontrée dans cette thèse aidera à choisir ou à modifier des substrats afin d'obtenir les produits désirés pour des recherches plus poussées dans ce domaine.

Puisque nous avons utilisé les taxanes comme les substrats de la transformation microbienne et nous avons découvert que quelques champignons peuvent modifier les taxanes, nous pensions que le stéroïde androst-4-ène-3,17-dione (**108**) qui un produit de départ pour la synthèse commerciale de la testostérone et d'autres nouveaux stéroïdes, est un substrat parfait pour la transformation microbienne grâce à son faible taux de substitution par des functions hydroxyles d'oxygénation. En effet, le stéroïde androst-4-ène-3, 17-dione (**108**) avec la purité de 99.9% a été transformé par *Absidia coerulea* ATCC 10738a et *Rhizopus coeryzae* Went et ATCC 11145 en seize métabolites (**109-124**) (**Figure 7.1**). La

biotransformation de l'androst-4-ène-3,17-dione (**108**) par *Absidia coerulea* ATCC 10738a a donné 14 dérivés, (**109-112**, **114-123**). La biotransformation de l'androst-4-ène-3,17-dione (**108**) par *Rhizopus coeryzae* Went et ATCC 11145 a donné 7 dérivés, **110-113**, **122-124**.

Ces sept dérivés, tels ques la 7α-hydroxyandro-4-ène-3,17-dione (**109**), la 11α-hydroxyandro-4-ène-3,17-dione (**111**), la 6β-hydroxyandro-4-ène-3,17-dione (**112**), la 14α-hydroxyandro-4-ène-3,17-dione (**114**), la 6β-hydroxytestostérone (**115**), la 7α-hydroxytestostérone (**116**), et la 6β, 11α-dihydroxyandrost-4-ène-3,17-dione (**122**) ont été trouvés dans la transformation microbienne de l'androst-4-ène-3,17-dione en utilisant différents types de microorganismes auparavant. Ces sept composés étaient les premiers trouvés dans la biotransformation de l'androst-4-ène-3,17-dione (**108**) par *Absidia coerulea* ATCC 10738a. Le composé **122** a été aussi trouvé pour la première fois dans la transformation microbienne de l'androst-4-ène-3,17-dione (**108**) en utilisant *Rhizopus oryzae* Went et ATCC 11145.

Neuf des métabolites, tels ques la 7β-hydroxyandrost-4-ène-3,17-dione (**110**), la 16β-hydroxyandrost-4-ène-3,17-dione (**113**), la 2α,7α-dihydroandrost-4-ène-3,17-dione (**117**), la 6β,12β -dihydroandrost-4-ène-3,17-dione (**118**), la 1β,6β-dihydroandrost-4-ène-3,17-dione(**119**), la 11α,16β-dihydroandrost-4-ène-3,17-dione (**120**), la 14α,16α-dihydroandrost-4-ène-3,17-dione (**121**), la 6β,16β-dihydroandrost-4-ène-3,17-dione (**123**), la 6β,7α-dihydroandrost-4-ène-3,17-dione (**124**) étaient les premiers trouvés dans la transformation microbienne de l'androst-4-ène-3,17-dione. La structure de ces produits a été déterminée par les données de RMN et de HR FAB MS.

Nos résultats ont démontré que les nouveaux champignons et les nouveaux metabolites pouvaient toujours être trouvés dans la transformation microbienne de stéroïdes. Ces nouveaux métabolites qui sont difficiles à obtenir par la modification chimique peuvent fournir la nouvelle route vers la découverte de nouveaux médicaments.

Table of Contents

List of Tables

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

Chapter 7:

List of Figures

Chapter 7:

List of Schemes

List of Abbreviations

TLC:	Thin Layer Chromatography
PTLC:	Preparative Thin Layer Chromatography
HPLC:	High Performance Liquid Chromatography
NMR:	Nuclear Magnetic Resonance
MS:	Mass Spectrum
HRMS:	High Resolution Mass Spectrum
FABMS:	Fast Atom Bombardment Mass Spectrum
FAB-HRMS:	Fast Atom Bombardment High Resolution Mass Spectrum
DMSO:	Dimethyl Sulfoxide
ATCC:	American Type Culture Collection
HMQC:	Heteronuclear Multiple Quantum Correlation
HMBC:	Heteronuclear Multiple Bond Correlation
NOESY:	Nuclear Overhauser Enhancement Spectroscopy
COSY:	Correlated Spectroscopy
10-DAB:	10-Deacetylbaccatin III
Ac	Acetyl
Bz	Benzoyl
La	Lanthanum
Ce	Cerium

Abstract

This thesis focuses on microbial transformations of two major groups of natural products: taxanes and steroids. Microbial transformation of taxanes (chapters 2-6) and steroids (chapter 7) are described in this thesis.

In an effort to find new poly-oxygenated taxanes, 27 natural or chemically modified taxanes (chapter 6, **Figure 6.1** and **Figure 6.2**) were incubated with 16 filamentous fungi, which are known to carry out regio- and stereoselective hydroxylation of a wide range of organic compounds to find useful biotransformations. Six of these taxanes (**4, 50, 52, 56, 57,** and **61**) could be metabolized. The products were purified by several chromatographic methods and their structures were elucidated from NMR and high resolution FAB-MS data.

Compound **4** (9-dihydro-13-acetylbaccatin III) (substrate #1 in chapter 5) can be metabolized by *Absidia coerulea* ATCC 10738a to give four products (**Figure 6.3**). Besides the deacetylation products **71** and **72**, product **73** and **74** are abeo-taxanes. The substrate with 1-OH can be metabolized by fungus to give abeo-taxanes was reported in this thesis for the first time.

Compound **50** (2-deacetoxyltaxinine J) (substrate #2 in chapter 4,) can be transformed by fungus *Absidia coerulea* ATCC 10738a to give four products, **75-78,** in moderate yields (**Figure 6.4**).

Compound **52** (5α,7β,9α,10β,13α-pentahydroxy-4(20),11(12)-taxadiene) (substrate #1 in chapter 3) was converted to three taxane derivatives, **79-81**, by fungus *Absidia coerulea* ATCC 10738a (**Figure 6.5**). Compound **79** (32.4%) has a C10-C11 double bond. Unlike the usual 11(15→1) abeo-taxanes, compounds **80** (42.3%), and **81** (3.7%) have an unexpected 1(15→11) abeo-taxanes skeleton. This skeleton has not previously been found in natural or chemically modified taxanes.

The biotransformation of compound **56** (substrate #1 in chapter 4) was previously investigated. The fungus, *Absidia coerulea* ATCC 10738a, and the medium used in this project are slightly different from that previously reported. The results are summarized in **Figure 6.6**. The major products, the hydroxylation of C-1, C-14 and abeo-taxanes (**82, 84,** and **90** respectively), are the same. The minor products are different. There are seven deacetylation products (**83, 85-87, 91-93**) that were not found before. Besides the major ones and the deacetylation products, there is a minor product, **88**, with an $OCOCH_2COCH_3$ group induced at C-5. This kind of substitution is not found in natural taxanes. The product **89** with C-16 hydroxylation is also rare in natural taxanes. Another two taxanes, **94** and **94,** which have a 6-7-6-ring system that is totally new, were also obtained from this reaction, albeit in very low yield.

Compound **57** (substrate #1 in chapter 2) can be metabolized by *Cunninghamella elegans* AS 3.2033 to give 5 new taxanes (**Figure 6.7**). The C-1 hydroxylation product, **96**, has the highest yield of 43.0%, while the C-1 hydroxylation product, **97**, a *cis* isomer at cinnamoyl side chain of **96**, has a yield of 1.6%. The abeo-taxane, **101**, has a yield of 16.0%. A pair of C-14 hydroxylation products **98** and **99**, which were *trans-cis* isomers at the cinnamoyl side-chain, have a yield of 1% each. Compound **57** can also be metabolized by *Cunninghamella elegans var chibaensis* ATCC 20230 to give 3 taxanes. Besides the C-1 hydroxylation product, **96** (20.2%) and the abeo-taxane, **101** (6.8%), there is a C-17 hydroxylation product, **100** (5.4%), which is very rare among the natural taxanes.

Compound **61** (substrate #2 in chapter 5) can be metabolized by fungus *Cunninghamella echinulata* AS 3.1990 to give six products **102-107** (**Figure 6.8**). Products **102**, **103** and **104** have exchanged the benzoyl group and acetyl group at position 2 and position 9 compared to the starting material. Products **105-107**, with 2-Bz group also shifted to position 9, have an opened oxetane-ring and a new-formed 5 member-ring involving -C2-C3-C4-C20-O-. All of these three abeo-taxanes have a –OAc at C-15, which is very rare in natural taxanes.

The results presented in this thesis showed that microbial transformation could be a unique way to provide new taxanes, which might be important in drug design. The relationship between the taxanes structure and fungi reactivity is also described in this thesis.

Since we have used taxanes as substrates for microbial transformation and have found that some fungi can hydroxylate taxanes, we think androst-4-ene-3,17-dione (**108**), an important raw material for the commercial synthesis of testosterone and other new steroids, may be a good substrate for microbial transformation because of its low oxygenation. Indeed, Androst-4-ene-3,17-dione (**108**) could be transformed, by *Absidia coerulea* ATCC 10738a and *Rhizopus oryzae* Went et ATCC 11145, into sixteen metabolites (**109-124**) (**Figure 7.1**). Biotransformation of androst-4-ene-3,17-dione (**108**) by *Absidia coerulea* ATCC 10738a gave 14 derivatives, **109-112**, **114-123**. Biotransformation of androst-4-ene-3,17-dione by *Rhizopus oryzae* Went et ATCC 11145 gave 7 derivatives, **110-113**, **122-124.**

Seven derivatives, 7α-hydroxyandro-4-ene-3,17-dione (**109**), 11α-hydroxyandro-4-ene-3,17-dione (**111**), 6β-hydroxyandro-4-ene-3,17-dione (**112**) 14α-hydroxyandro-4-ene-3,17-dione (**114**), 6β-hydroxytestosterone(**115**), 7α-hydroxytestosterone(**116**), and 6β, 11α-dihydroxyandrost-4-ene-3,17-dione (**122**) were found previously in the microbial transformation of androst-4-ene-3,17-dione (**108**). These seven compounds were first found in the biotransformation of androst-4-ene-3,17-dione by *Absidia coerulea* ATCC 10738a. Compound **122** was also found for the first time from microbial transformation of androst-4-ene-3,17-dione using *Rhizopus oryzae* Went et ATCC 11145.

Nine of the metabolites [7β-hydroxyandrost-4-ene-3,17-dione (**110**), 16β-hydroxyandrost-4-ene-3,17-dione (**113**), 2α,7α-dihydroandrost-4-ene-3,17-dione (**117**), 6β,12β-dihydroandrost-4-ene-3,17-dione (**118**), 1β,6β-dihydroandrost-4-ene-3,17-dione (**119**), 11α,16α-dihydroandrost-4-ene-3,17-dione (**120**), 14α,16α-dihydroandrost-4-ene-3,17-dione (**121**), 6β,16β–dihydroandrost-4-ene-3,17-dione (**123**), and 6β,7α–dihydroandrost-4-ene-3,17-dione (**124**)] were first found in the

microbial transformation of androst-4-ene-3,17-dione (**108**). The structures of these products were elucidated by NMR and high resolution FAB-MS data.

This project focused on the microbial transformations of two major groups of natural products: taxanes and steroids.

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General Introduction

Paclitaxel, (Taxol®), which was discovered in 1971 (Wani et al., 1971) and was approved by FDA for marketing in December 1992, has become one of the most successful and useful anticancer drugs now available. Unlike usual anticancer agents that act by preventing the polymerization of tubulin into microtubles, paclitaxel prevents cancer cell division by promoting assembly of tubulin into microtubules and stabilizing it (Schiff et al., 1979; Horwitz, 1992). Paclitaxel has been successfully used for the treatment of ovarian and breast cancer. It also shows promising for the treatment of a variety of other solid tumors, such as head, neck, lung, gastrointestinal, and bladder (Suffness and Wall, 1995a; Holmes et al., 1995). In the last two decades, great efforts have been made to find new taxanes from natural sources or by chemical modification of natural taxanes (Suffness, 1995b).

Since the early 1990's, the laboratory of Dr. Zamir has investigated the constituents of the Canadian yew, *Taxus canadensis* (Zamir et al., 1992a; 1992b; 1995a; 1995b; 1996a; 1998; 1999a. 1999b; Zhang et al., 2000a; 2000b; 2001). Zamir's group has published a series of papers using chemical methods to modify the compounds obtained from *Taxus canadensis* (Zamir et al., 1996b; 1997; Nikolakakis et al., 2000) and has used molecular modeling to study and design taxanes (Boulanger et al., 1996; Wu and Zamir, 2000) in an effort to find new taxanes with increased anti-cancer activity and water-solubility.

Filamentous fungi are known to carry out regio- and stereo-selective hydroxylations of a wide range of organic compounds (Zhang et al., 1996; 1998; Hu et al., 1996a; 1996b; 1997a; 1997b; 2000; Dai et al., 2001; Xu et al., 1997; Chen et al., 2001; Patel et al., 1995). Our research goal is to try to use the power of the filamentous fungi enzyme system to modify taxanes and thus to obtain new taxanes that are difficult to get from chemical modification.

The availability of large amounts of 9-dihydro-13-acetylbaccatin III from *Taxus canadensis* and 2-deacetoxytaxinine J from *Taxus yunnanensis* meant that

these compounds may be used as starting materials; we can chemically modify them and feed the modified compounds to the fungi culture in the hope of discovering some interesting reactions and new compounds.

During our investigation, we found that some of the fungi used could hydroxylate taxanes as well as steroids, such as androst-4-ene-3,17-dione. This compound is commercially available and is an important raw material for the commercial synthesis of testosterone and other new steroids. A second research goal was to hydroxylated androst-4-ene-3,17-dione at different sites.

Our department has all the necessary facilities for the culture of microorganisms. We also have the equipment and reagents for chemical modification and the purification of taxanes (analytical and preparative HPLC). A 500 MHz NMR instrument is available on campus. Some of the fungal strains used in our investigations were purchased from ATCC or from China, and others were strains previously cultured in Dr. Zamir's laboratory.

Our general procedure is the following: The microorganism is cultured in Potato Dextrose broth for 3 days. To each cultivar the substrate was added (1 mg in 0.1 ml DMSO) except for one used as control and culturing was continued for another 10 days. This cultivar was extracted with CH_2Cl_2, and the CH_2Cl_2 fraction was checked by TLC and HPLC. If a new compound is detected, this procedure is repeated with a parallel control experiment run (with no microorganism) to see if this compound is formed due to a microbial or non-microbial process. If there is no chemical reaction in the control during the course of culturing, the procedure will be scaled up to obtain enough material to do structure elucidation.

When the reaction is scaled up, the culture is first homogenized and then extracted with CH_2Cl_2. The extract will then be separated on a silica gel column for a first purification. The fractions containing the biotransformation products will be then separated by HPLC or preparative TLC. The structures of the resulting pure compounds will be elucidated from NMR and HRMS data.

This thesis is divided into seven chapters.

Chapter one: Literature review of Taxol and taxanes.

Chapter two to six: Microbial transformation of taxanes. Chapters two to five include four scientific articles, published or in preparation. The style of each chapter is in accordance with the Journal, to which the work will be submitted. Chapter 6 is a general discussion of the results of microbial transformation of taxanes, and taxane structure–fungi reaction activity relationship.

Chapter seven deals with the microbial transformation of steroids.

Since the results are presented in five articles involving over one hundred compounds, the compound numbering system is extremely difficult to merge into the same format throughout the thesis. I will leave the compound numbering system and the references of chapters 2 to 5 independently. The numbering system of the compounds in chapter 1, 6, and 7 will be in the same format. The references of the general introduction and chapters 1, 6, and 7 will be in the same format in the last part of the thesis.

Chapter 1

Literature Review of Taxol and Taxanes

1.1. Taxol and Taxanes: History, Discovery and Development

Since paclitaxel (Taxol®, **1**, **Figure 1.1,** Taxol is a trademark of Bristol-Myers Squibb Company, its generic name is paclitaxel) was approved by FDA for marketing in December 1992, it has become one of the most successful and useful anticancer drugs now available. It is a remarkable success story, which spans 30 years.

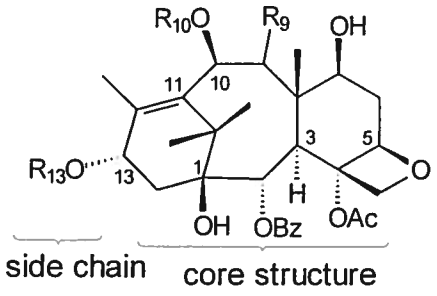
The first taxoid isolated from yew dates back to 1856, when Lucas isolated a toxic material, which he named Taxine (Lucas, 1856). This material turned out to be a mixture of compounds, whose structures were only published in the 1960s (Kurono et al., 1963; Lythgoe, 1968).

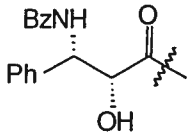
The massive investigation of taxanes did not begin with *Taxus* but was associated with cancer research. In the 1950s deaths from infectious diseases were on the decline duo to penicillin and other antibiotics, and cancer had become a major killer.

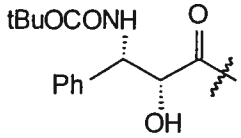
In 1962, samples of the pacific yew (*Taxus brevifolia* Nutt.), collected in Washington State, USA, were found to be active in KB cell cytotoxicity tests and to have *in vivo* antitumor activity in 1964 (Wall et al., 1976). It took 2 years to purify 0.5 g of pure Taxol (**1**) from the *Taxus brevifolia* bark, with an isolated yield of about 0.02%. The structure of Taxol was first published in 1971 (Wani et al., 1971).

Research on Taxol (**1**) was at a standstill in the early 1970s because it didn't have adequate activity for development in any of the models then used in NCI. Later, the activity of Taxol against B16 melanoma led NCI to select Taxol as a development candidate (Venditti et al 1984). In 1979, Horwitz's group found that unlike usual anticancer agents that act by preventing the polymerization of tubulin into microtubules, Taxol promoted the assembly of tubulin into microtubules, stabilized it and suppressed depolymerization (Schiff et al., 1979).

This study generated great interest in Taxol and taxanes. Taxol (**1**) has a very poor water-solubility and the yield from pacific yew is very low. A massive research effort into formulation studies and bulk production was launched in order to address these significant obstacles to development.



1. Paclitaxel R_9= =O R_{10}=Ac R_{13} = 

2. Docetaxel R_9= =O R_{10}=Ac R_{13} = 

3. 10-Deacetylbaccatin III R_9= =O R_{10}=H R_{13} =H

4. 9-dihydro-13-acetylbaccatin III R_9= α-OH R_{10}=H R_{13} =Ac

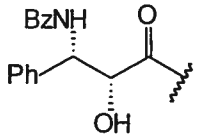
5. 9-dihydrotaxol R_9= α-OH R_{10}=Ac R_{13} = 

Figure 1.1 Structures of Paclitaxel, Docetaxel, 10-Deacetylbaccatin III, 9-dihydro-13-acetylbaccatin III, and 9-dihydrotaxol

1.2. Formulation studies

1.2.1 Formulation and Administration

Two problems are associated with the clinical use of Taxol: 1. The extremely low water solubility of Taxol (**1**) (with a reported data vary from 0.7 to 35 μg/ml) (Swindell and Krauss, 1991; Ringel and Horwitz, 1991; Mathew et al., 1992) and 2. The relative high dose needed. The formulation of Taxol in a biocompatible carrier or solvent has been a challenge throughout the process of therapeutic development. The formulation selected for clinical development consists of Taxol dissolved at a concentration of 6 mg/ml in polyethoxylated castor oil (Cremophor EL®) containing 50% anhydrous EtOH. The most common packaging of formulated Taxol is in vials containing 5 ml of Taxol/EtOH:cremophor solution. When administered by the intravenous route, the drug is diluted 5 to 20-fold in normal saline or 5% dextrose to a final concentration of 0.3 to 1.2 mg/ml. An in-line filter is required during administration as a safeguard against the infusion of particulate (NCI, 1990).

Although Taxol (**1**) solutions in the range of 0.3 to 1.2 mg/ml were physically and chemically stable for at least 24h, (Waugh et al., 1991), it is recommended to be used within 12h of dilution in aqueous media (NCI, 1990).

1.2.2. Adverse Pharmacological Effects

The ethanol:cremophor (1:1) solution has been used to administer other drugs, such as cyclosporine (Howrie et al, 1985). The amount of Cremophor needed to deliver the required doses of Taxol is higher (Rowinsky et al., 1992). This solution has been shown to cause serious or fatal hypersensitivity attacks during preclinical and clinical testing (Rowinsky et al., 1990; Lorenz et al., 1977; Weiss et al., 1990).

Hypersensitivity reactions are most common in bolus administration and shorter infusion schedules (Weiss et al., 1990; Rowinsky et al., 1993). Thus, many Phase II and Phase III trials have used 6-24 h infusions (Rowinsky et al., 1992). Also premedication with corticosteriods (dexamethasone) and antihistamines is used with Taxol in order to reduce the intensity and incidence of side effects with Taxol (**1**) administration in Cremophor (Weiss et al., 1990).

The premedication approach has reduced the incidence of hypersensitivity reaction to 2.2% in Phase II and Phase III studies of breast and ovarian cancer (Arbuck et al., 1993), milder reactions still occur in approximately 30% of patients (Weiss et al., 1990; Runowicz et al., 1993).

1.2.3. Alternative Formulation of Taxol

Clinically, pharmacological intervention may be less desirable than a safer, better-tolerated formulation. Alternative formulations of Taxol (**1**) were also studied. The drug delivery system of liposomes represents a relatively mature and versatile technology (Filding, 1991). A great effort has been made on the design and synthesis of more water-soluble derivatives or prodrugs that retain their pharmacological activity (Mathew et al., 1992; Deutsch et al., 1989; Zhao et al., 1991; Nicolaou et al., 1993).

Some prodrugs are sufficiently water soluble to allow administration without excipents and are unmasked at a rate appropriate for therapy. New water-soluble drugs would be very useful.

1.3. Supply of Taxol

Since Taxol (**1**) was first isolated from *Taxus brevifolia* at about 0.02% yield (Wani et al., 1971) and the harvest of the yew bark is devastating to yew trees, the supply issue of Taxol (1) was a tremendous obstacle that slowed the development of taxol into Market. A lot of efforts have made to increase the supply of Taxol (**1**). These efforts include: Isolation from the natural sources, total synthesis; semi-synthesis from 10-DAB (**3**), which also led to the synthesis of Docetaxel (Taxotere®, **2**. Taxotere is a trademark of Rhone Poulenc Rorer. Docetaxel is its generic name); cell culture; biosynthesis studies; biotransformation.

1.3.1. From Natural Products

Since Taxol (**1**) was first isolated from *Taxus brevifolia* stem bark, it is natural to investigate other parts of *Taxus brevifolia* and other species of *Taxus* and other parts of these species. *Taxus* species are members of the *Taxaceae*. *Taxus* species are widely distributed throughout the Far East, Northern and Central American and Europe. Taxol was found in every species of Taxus, although the yields vary with the species, different part (needles, bark etc), the collection site and the time of the year the sample was collected (Croom Jr, 1995).

During the course of our investigations, a series of reviews have appeared to summarize the natural products work in this area (Kingston, 1991; 1993; Parmar and Jha, 1998; Appendino, 1995; Khan and Parveen, 1987). The two most recent reviews, which included the literature up until 1998, reported over 350 taxane diterpenoids from Taxus species (Baloglu and Kingston, 1999; Parmar et al., 1999). To our knowledge, there are more than 400 natural taxanes reported as of May 2002. At present, *Taxus baccata* (European yew), and *Taxus wallichiana* (Himalayan yew) are the major commercial sources of 10-deacetylbaccatin III (**3**, 10-DAB), the starting material for Taxol (**1**) and Taxotere (**2**, Docetaxel) semi-syntheses. *Taxus canadensis*, a small bush abundant in Quebec, has needles that contain 9-dihydro-13-acetylbaccatin III (**4**) in high concentration and moderate concentration of Taxol (**1**) and 10-DAB (**3**). This bush can be used as a source of Taxol (**1**) and 10-DAB (**3**). In addition, isolated 9-dihydro-13-acetylbaccatin III

(**4**) can be transformed into 10-DAB (**3**) and finally to Taxol (**1**) and Taxotere (**2**) (Nikolakakis et al., 2000). 9-dihydro-13-acetylbaccatin III can also be used as starting material to synthesize the new taxane 9-dihydrotaxol (**5**), which shows an increased water solubility and activity (Klein et al., 1995).

1.3.2. Semi-synthesis

The potential of semi-synthesis was first recognized by Potier, who found that 10-deacetyl baccatin III (**3**, 10-DAB) was a relatively abundant taxane in the needles of *Taxus baccata* L., the European yew, with a yield of 0.02% (Chauviere et al., 1981; Senilh et al., 1984). The isolated yield could be improved to 0.1% (Denis et al., 1988). More significantly, 10-DAB (**3**) is easier to isolate from natural sources than Taxol and the needles are easily regenerated. 10-DAB (**3**) seems to be an easily and permanently accessible Taxol precursor. The semi-synthesis of Taxol (**1**) from 10-DAB (**3**) mainly involves the synthesis of the side chain and the attachment of the side chain to the protected 10-DAB (**3**). The first semi-synthesis of Taxol (**1**) from 10-deacetyl baccatin III (**3**), and so far the only successful esterification of a baccatin III derivative with an intact side chain, was achieved by Greene and Potier in 1988 (Denis et al., 1988). The semi-synthesis also led to the discovery of Taxotere (**2**), which showed increased water-solubility and anti-cancer activity (Gueritte-Voegelein et al., 1986; Mangatal et al., 1989). Since then, numerous methods to synthesize the side chain of taxol have been developed, and right now all Taxotere and mostly Taxol was provided through semi-synthesis from 10-DAB (**3**) (Holton et al., 1995).

1.3.3. Total Synthesis

Taxol (**1**) is one of the most challenging molecules that have been synthesized by chemists to date (Nicolaou and Sorensen, 1996). The great effort towards achieving of total synthesis of Taxol (**1**) before 1994 was well documented (Wender et al., 1995). In 1994, Holton's group (Holton et al., 1994a; 1994b) and Nicolaou's group (Nicolaou et al., 1994; 1995a; 1995b; 1995c; 1995d) (almost at the same time) published the total synthesis of taxol. Danishefsky group's (Masters et al., 1995; Danishefsky et al., 1996), Wender's group (Wender et al., 1997a; 1997b), Kuwajima's group (Morihira et al., 1998),

and Makaiyama's group (Shiina et al., 1998a; 1998b; Makaiyama et al., 1999) have also achieved the total synthesis of Taxol (**1**). Although these approaches provided excellent understanding of the chemistry of taxol and taxanes, the multi-step reactions, the low yields, and the high cost make these approaches to produce Taxol (**1**) and Taxotere (**2**) commercially unfeasible.

1.3.4. Cell culture for Taxane Production

Plant cell culture is expected to be a major contributor to Taxol (**1**) supply. Cell culture could eliminate the need to use the limited natural resource of Taxus species worldwide.

1.3.4.1. The Establishment of Cell Culture

Callus (an unorganized, proliferating mass of undifferentiated cells) cultures of *T. cuspidata* and *T. canadensis* were induced using different tissue explants (a living plant tissue) including green and red arils, seed contents, young stems and needles. Callus derived from stem segments displayed the best growth in totally defined media. The presence of taxol in callus tissues of *T. cuspidata* and suspension cultures established from the callus tissues were at levels up to 0.002% and 0.012% of the extracted dry weight, respectively (Fett-Neto et al., 1992).

Tissue of *Taxus brevifolia* has been successfully cultured to produce taxol and taxanes from both callus and suspension culture (Cristen et al., 1991).

One thing common to all of the *Taxus* callus cultures is that the slow growth rates and low frequency of callus formation from explants. There is strong need to optimize media to lead to faster growth rates and higher fraction of successful initiation callus from explanted tissues. Plant cell cultures are usually induced from established callus cultures by transferring callus into one of a number of liquid media favoring suspension culture growth. An advantage of suspension cultures is that this system is amenable to scale-up production. Most of the cultures of *Taxus* belong to cell suspension cultures.

The successful culture of embryo was first obtained from *T. baccata* mature seeds (Le Page, 1968; 1973), and from *T. brevifolia* and *T. × media* mature seeds (Flores and Scrignoli, 1991; Flores et al., 1993).

Since the root has the second highest concentration of Taxol (**1**) next to the bark *in T. brevifolia* (Vidensek et al., 1990), root cultures were used to produce Taxanes (Chee, 1995).

Nodule cultures were also used for the production of Taxol (**1**) (Ellis et al., 1996).

1.3.4.2. Optimization of the parameters affecting growth rate and production of taxanes

The goal for production of Taxol (**1**) and taxanes using plant cell cultures is to produce significant quantities of product in a short period of time. In order to achieve the goal, several options are possible. A slow-growing culture that produces a large amount of taxol may yield equivalent amounts of product as a fast-growing culture that produces small amounts of taxol. The most attractive option is a fast-growing culture that produces large amount of taxol. An extension of the last option is to first produce significant amount of biomass quickly with a fast growing culture, which is then stimulated to produce taxol. A combination of strategies is key to the development of a plant cell culture production system for taxanes. The culture system must reproduce the environment that allows production of taxanes under defined, controlled conditions.

1. Light

A study showed that Taxol (**1**) and baccatin III accumulation in callus and cell suspension cultures were of approximately three times higher in dark-grown environment than in light-grown environment (Fett-Neto et al., 1995).

2. Nutrient

Media control can either increase or inhibit the production of Taxane. The chemically defined media for optimal cell growth is very important to Taxus cell culture (Fett-Neto et al., 1993; Monacelli et al., 1995; Kim et al., 1995; Hirasuna et al., 1996).

3. Elicitor Treatments

Elicitors are compounds added to the medium, which stimulate secondary metabolite production. Fungal elicitor preparations from *Cytospora abietis* and *Penicillium minioluteum* (Cristen et al., 1991), *Pencillium minioluiteum*, *Botrytis cinerea*, *Verticillium dahliae*, and *Gilocladium deliquescens* (Ciddi et al., 1995), aromatic carboxylic acid, amino acid (Fett-Neto et al., 1994b), Arachidonic acid (Jha et al., 1998), IAA-phenylalanine (Mirjalili et al., 1996), ethylene and methyl jasmonate (Yukimune et al., 1996; Ketchum et al., 1999) can all stimulate the production of taxol in cell culture.

Taxol production rates of up to 23.4 mg/L. day after elicitation of the cell culture with methyl jasmonate were reported with Taxol (**1**) comprising 13-20% of total taxoid fraction (Ketchum et al., 1999). Such production rates demonstrate the impressive biosynthetic capacity of *Taxus* cell cultures.

4. Product Removal

Since Taxol/taxanes may act as a feedback inhibitor in the biosynthetic pathway or a transport process, removal of Taxol/taxanes can increase their overall production (Collins-Pavao et al., 1996; Hoffman et al., 1996; Kwon et al., 1998).

5. Kinetics Studies

Kinetic studies can lead to a better understanding of taxane production, and of growth, and nutrient uptake in the cell suspensions. They are useful in determining the optimal condition for *taxus* cell culture (Fett-Neto et al., 1994a; Pestchanker et al., 1996; Srinivasan et al., 1996; Mei et al., 1996).

6. Immobilization

Total Taxol yield of the self-immobilized aggregate culture was 4.9 mg/L, 5 times as much as that of the dispersed cell culture (Seki et al., 1997; Xu et al., 1998).

7. Precursor feeding

Precursor feeding of (phenylalanine, benzamide and sodium acetate) to *Taxus chinensis var. mairei* resulted in significant increase of taxol production (Wu et al., 1999).

8. Rare earth

Yuan et al (Yuan et al., 1998) found that adding of a high concentration of La^{3+} and Ce^{4+} can alter the growth pattern of *T. cuspidata* cell culture and increase the taxol production.

1.3.4.3. Selection and Preservation of Cell Lines

Since *Taxus* cell lines vary in their ability to produce taxanes, cell line selection is a key strategy to increase production levels. An efficient way for the selection of high taxane-production cell lines was developed recently (Kawamura et al., 1998).

Repeated subcultures have been shown to lead to increased ploidy; spontaneous mutations; loss in biosynthetic capacity, yield, and morphogenetic potential; and changes in phenotype (Kartha, 1987). Selection methods attempt to control these changes by continually selecting for the desired cell type, while the preservation methods hold the most potential for effectively controlling desirable lines. Wickremesinhe and Arteca (Wickremesinhe and Arteca, 1994) found that culturing suspension cultures at 12°C slowed the doubling times 3-fold, thereby providing an effective temporary alternative for long-term storage of suspension cultures.

Despite the intense effort and the fact that some of the results are promising, taxus plant cell cultures have not yet proven to be commercially viable, due to unstable production yields, especially at large scale.

1.3.5. Biosynthesis of Taxanes

In the long term, it would be desirable to obtain Taxol (**1**) and its analogs under controlled conditions, either by cell-fermentation or a microbial process. The use of this process largely depends on the understanding of the biosynthesis of Taxol in plants, by characterization of the enzymes catalyzing the key reactions, and on the identification of the genes coding the key enzymes.

The first biosynthetic experiments done by the groups of Leete (Leete and Bodem, 1966) and Haslam (Platt et al., 1984) focused mainly on the origin of the Winterstern acid part of the side chain. In 1992, using radiolabelling feeding experiments, Zamir's group demonstrated that acetate, mavalonate, and phenylalanine represented the biosynthetic building blocks of *Taxus canadensis* taxanes (Zamir et al., 1992b). From then on, several groups published a series of papers on the biosynthesis of Taxol and taxanes.

As shown in **Scheme 1.1**, the taxane skeleton comes from the cyclization of the universal diterpenoid precursor geranylgeranyl diphosphate (**6**) to taxa-4(5),11(12)-diene (**7**) catalyzed by the taxadiene synthase (Eisenreich et al., 1996; Koepp et al., 1995; Lin et al., 1996), which was purified and characterized from *Taxus brevifolia* (Hezari et al., 1995) and *Taxus canadensis* (Hezari et al., 1997a).

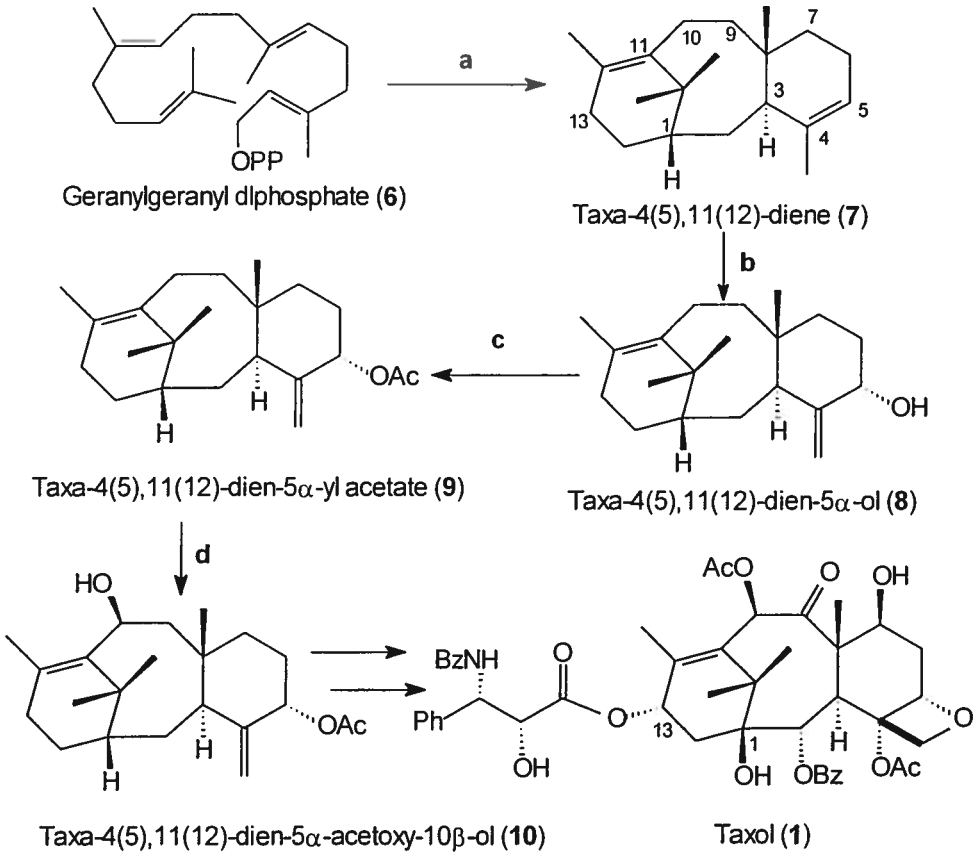
Whereas many terpene synthases of plant secondary metabolism produce multiple products, taxadiene synthase produces almost exclusively taxa-4(5),11(12)-diene(**7**) with very small amounts of other taxadiene isomers (Williams et al., 2000). A cDNA clone for this enzyme was obtained (Wildung and Croteau, 1996), and the taxadiene synthase was overproduced in *Escherichia coli* (Huang et al., 1998).

The key intermediate taxa-4(5),11(12)-diene(**7**) and its isomer taxa-4(20),11(12)-diene, which was supposed to be the intermediate in *Taxus* genus, were synthesized (Rubenstein and Willams, 1995).

The next oxygenation step is the oxidation of taxa-4(5),11(12)-diene(**7**) at C-5 to taxa-4(20),11(12)-dien-5α-ol (**8**) by a mixed function cytochrome P450 dependent hydroxylase with the use of molecular oxygen as substrate (Hefner et al., 1996).

The acylation of 5-hydroxy group producing taxa-4(20),11(12)-dien-5α-yl acetate (**9**) is the subsequent step in the taxoid pathway, which may be responsible for the

formation of the oxetane ring of Taxol. A mono-oxygenase catalyzed the hydroxylation of taxa-4(20),11(12)-dien-5α-yl acetate(**9**) at the C-10 position to afford taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol (**10**), which was further transformed into Paclitaxel (**1**)(Schoendorf et al., 2001).



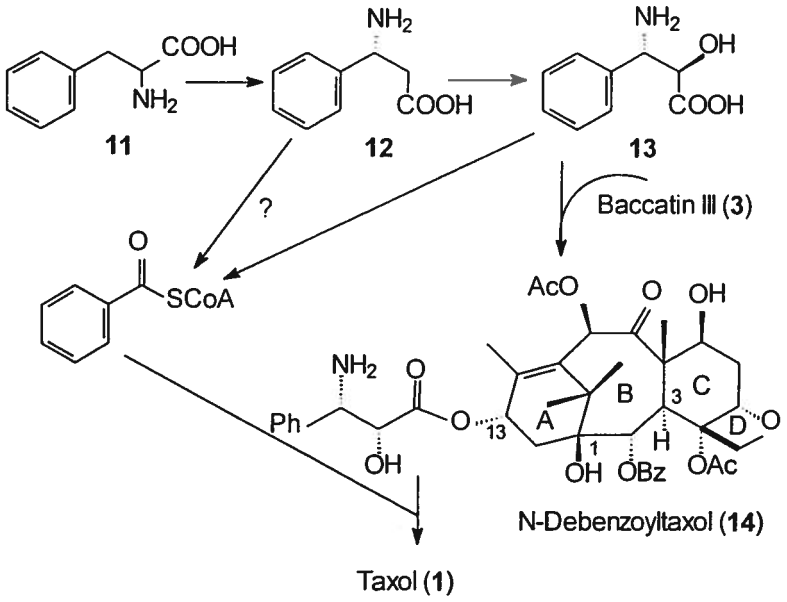
Scheme 1.1. Biosynthetic pathway of Taxol and the key enzymes. **a**. Taxadiene synthase, **b**. cytochrome P450 taxadiene 5α-hydroxylase (involving allylic rearrangement). **c**. taxa-4(20),11(12)-dien-5α-*o*-acetyl transferase. **d**. cytochrome P450 taxane-10β-hydroxylase.

Based on the relatively abundance of oxygen function at each carbon at the taxane ring system, it is suggested that the order of oxygenation might be C-5, C-10, then C-2 and C-9 (Hezari and Croteau, 1997b). The oxygenation at C-2, C-10, and C-14 are also introduced by molecular oxygen catalyzed by monooxygenase (Eisenreich et al., 1998).

Yet the order of oxygenation, hydroxyl group acylation, oxidation to ketone, formation of oxetane ring system in the skeleton as well as the mechanisms and the

enzymes catalyzing these reactions are still unknown. The less common 5/7/6, 6/5/5/6 ring systems were proposed to origin from taxa-4(5), 11(12)-diene (Kobayashi and Shigemori, 1998).

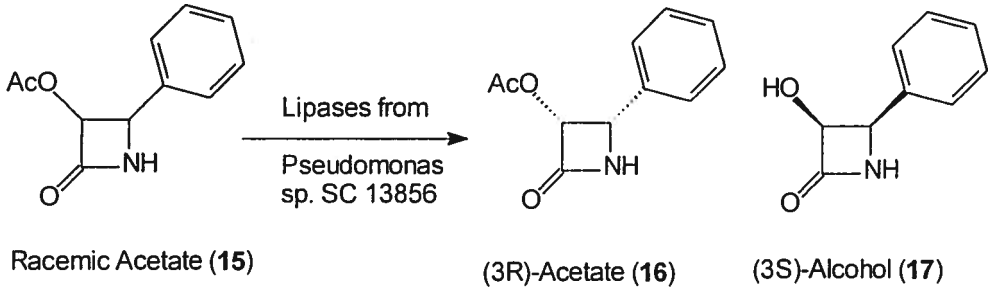
The side chain of Taxol originates from phenylalanine (**11**) (Zamir et al., 1992b; Fleming et al., 1993), which was catalyzed by phenylalanine aminomutase (Walker and Floss, 1998) to form β-phenylalanine (**12**). The β-phenylalanine was most likely transformed to phenylisoserine (**13**), which was attached to baccatin III to form N-debenzyoltaxol (**14**). The final step was benzoylation of the side chain. The benzoate moiety is also formed from phenylalanine and phenylisoserine (Fleming et al., 1994a, 1994b), as shown in **Scheme 1.2**.



Scheme 1.2 The Formation and attachment of Taxol side chai

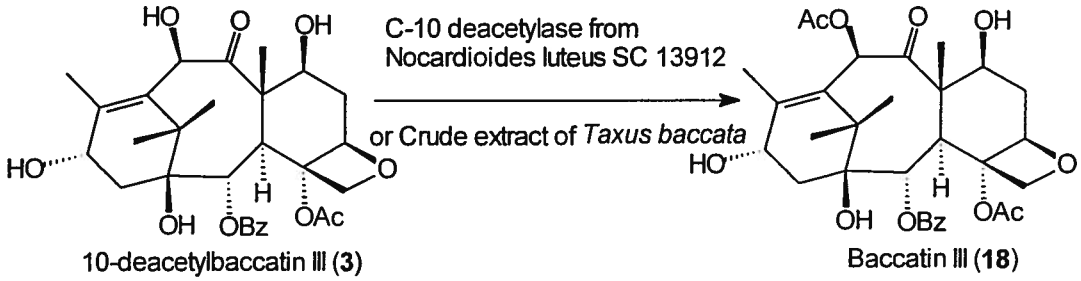
1.3.6. Enzymatic reactions in Taxanes

During the preparation of the side chains of Taxol and Taxotere, lipases were used for the resolution of racemic acetate (**15**) to afford optically active 3-hydroxy-4-phenyl β-lactam derivatives (**16**) and (**17**) (**Scheme 1.3**) (Patel et al., 1994; Gou et al., 1993; Hamamoto et al., 2000). Compound **16** was further used for the semi-synthesis of Taxol and Taxotere.



Scheme **1.3**. Resolution of racemic lactam derivatives by lipases

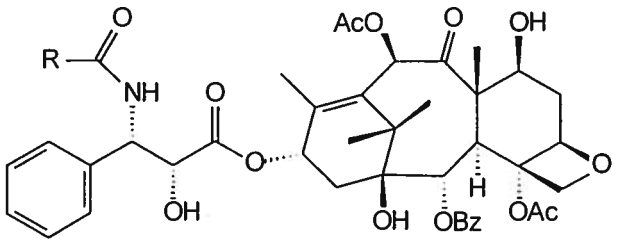
10-Deacetylbaccatin III (**3**) can be selectively acetylated to baccatin III (**18**) by the crude extracts from the roots of *Taxus baccata* (Zocher et al., 1996) and by the C-10 deacetylase from *Nocardioides luteus* SC 13913 (**Scheme 1.4**) (Patel et al., 2000).



Scheme **1.4**. Acetylation of 10-DAB (**3**) to Baccatin III (**18**) by enzymes

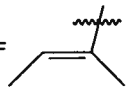
The selective acylation of the less active 13-hydroxyl group is chemically difficult to achieve. 10-deacetylbaccatin III can be selectively acylated by the *Pseudomonas cepacia* lipase (PCL) either at 7- or 10- or 13-hydroxyl groups (Lee et al., 1998).

By using selective enrichment techniques, three novel enzymes were isolated. The C-13 taxolase, which was isolated from *Nocardioides albus* SC 13911, can selectively cleave the C-13 side chain of Taxol (**1**), Cephalomannine (**19**), and Taxol C (**20**) to afford baccatin III (**18**). The C-10 deacetylase, which was isolated from *Nocardioides albus* SC 13912, can selectively cleave the 10-acetyl of baccatin III to afford 10-deacetylbaccatin III (**3**) (**Scheme 1.5**). The 7-xylosidase, which was isolated from a strain of *Morexella* sp. SC 13963, can cleave the 7-xylose of 7-xylosetaxol (**21**) and 7-xylose-10-deacetyltaxol (**22**) to afford Taxol (**1**) or 10-deacetyltaxol (**23**) (**Scheme 1.6**)(Hanson et al., 1994; 1996a; 1996b; 1997; Nanduri et al., 1995; Patel, 1997).



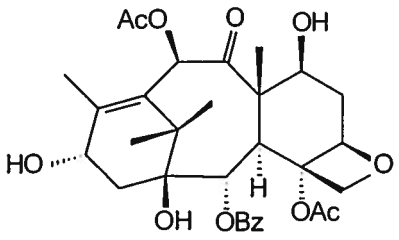
(**1**) Taxol R = Ph

(**19**) Cephalomannine R=



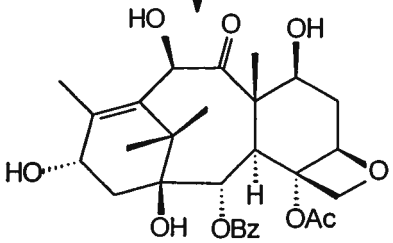
(**20**) Taxol C R= C_5H_8

C-13 taxolase from
Nocardioides albus SC 13911



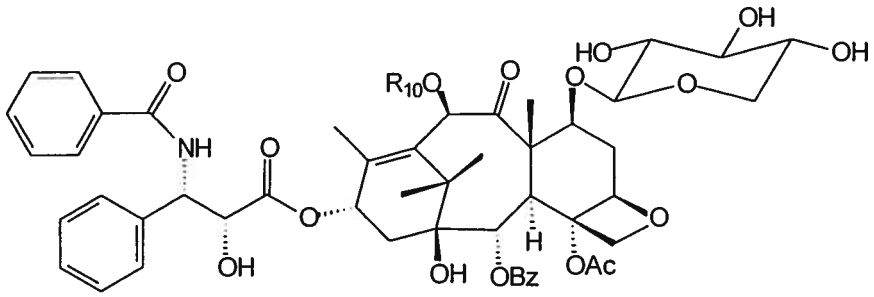
(**18**) Baccatin III

C-10 deacetylase from
Nocardioides luteus SC 13912



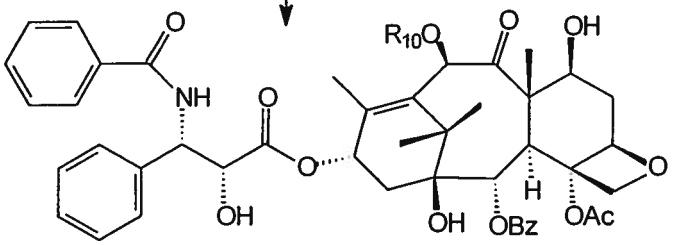
(**3**) 10-deacetylbaccatin III

Scheme **1.5**. Function of C-13 taxolase and C-10 deacetylase



(**21**) 7-xylosetaxol R_{10}=Ac
(**22**) 7-xylose-10-deacetyltaxol R_{10}=H

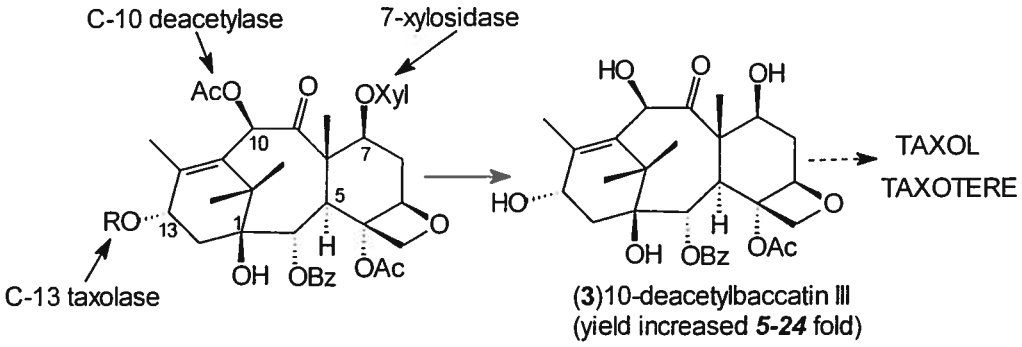
C-7 xylosidase from
Moraxella sp. SC 13963



(**1**) Taxol R_{10}=Ac
(**23**) 10-Deacetyltaxol R_{10}=H

Scheme **1.6**. Function of C-7 xylosidase

By treatment of these three enzymes, the concentration of 10-deacetylbaccatin III, the starting material for the semi-synthesis of Taxol and Taxotere, in extracts of a variety of *Taxus* cultivars was increased by 5.5- to 24-fold (**Scheme 1.7**) (Patel, 1998). This approach is really economically attractive.



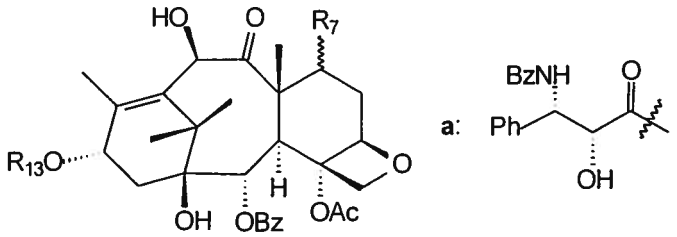
Scheme 1.7 Function of 7-xylosidase, C-10 deacetylase, and C-13 taxolase

1.3.7. Microbial biotransformation of taxanes

In an attempt to search for new potent anticancer agents, various microorganisms were used for the transformation of taxanes.

The endophytic fungi *Aspergillus niger* 3.4523 (Zhang et al., 1996), and *Alternaria alternata*, isolated from the inner bark of *Taxus yunnanensis*, were found to be able to selectively hydrolyze 1β-hydroxy-baccatin I (Zhang et al., 1998).

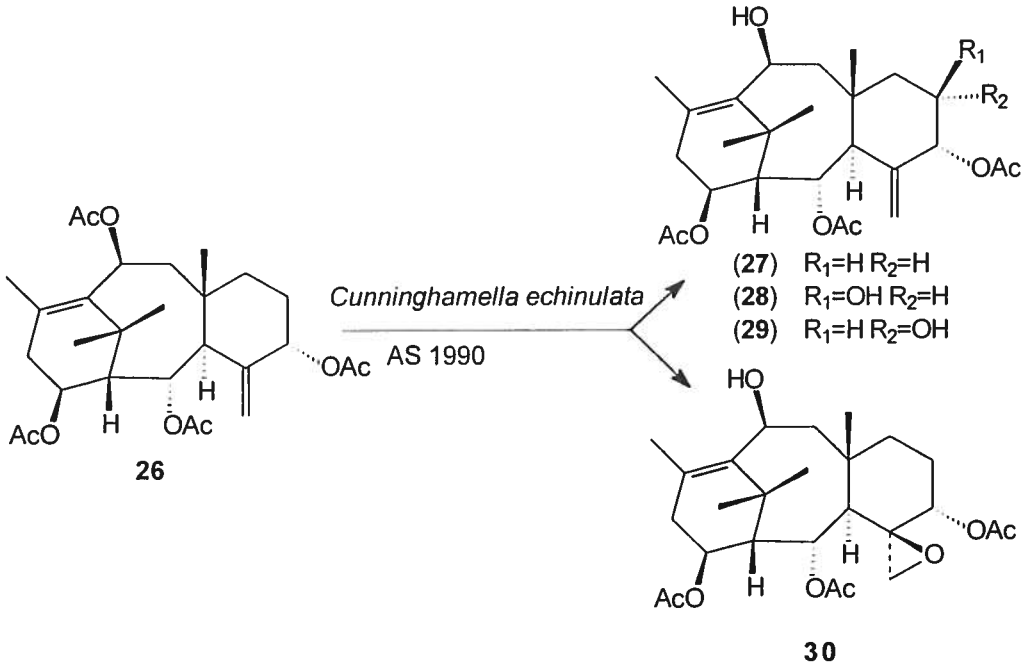
Incubation of the endophytic fungi *Microsphaeropsis onychiuri*, and *Mucor* sp., with 10-deacetyl-7-epitaxol (**24**) can afford 10-deacetylbaccatin V (**25**), 10-deacetyltaxol (**23**) and 10-deacetylbaccatin III (**3**) (**Figure 1.2**) (Zhang et al., 1998).



(**24**) 10-deacetyl-7-epitaxol R_7=α–OH R_{13} = **a**
(**25**) 10-deacetylbaccatin V R_7=α–OH R_{13} = H
(**23**) 10-deacetyltaxol R_7=β–OH R_{13} = **a**
(**3**) 10-deacetylbaccatin III R_7=β–OH R_{13} = H

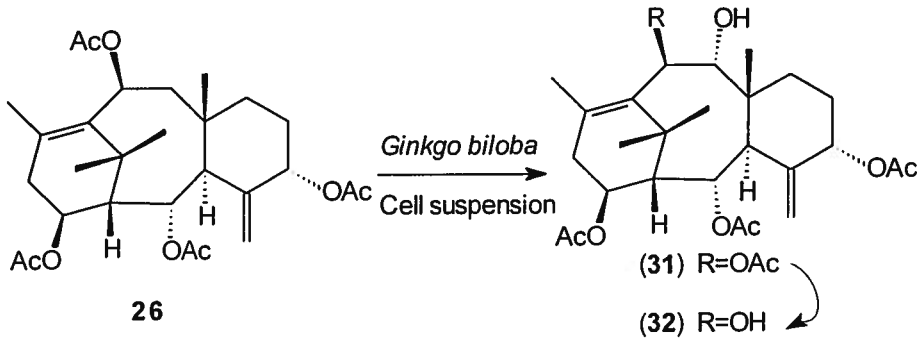
Figure **1.2**. Biotransformation of 10-deacetyl-7-epitaxol by *Microsphaeropsis onychiuri* and *Mucor sp*

2α,5α,10β,14β-tetra-acetoxy-4(20),11-taxadiene (**26**), a compound from the cell culture of *Taxus yunnanensis*, can be selectively hydrolyzed at position 10 and was further hydroxylated at position 6 or epoxidized by *Cunninghamella echinulata* AS 1990 to give out products **28** to **30** (**Scheme 1.8**) (Hu et al., 1996a; 1996b).



Scheme **1.8**. Microbial transformation of **26** by *Cunninghamella echinulata* AS 1990

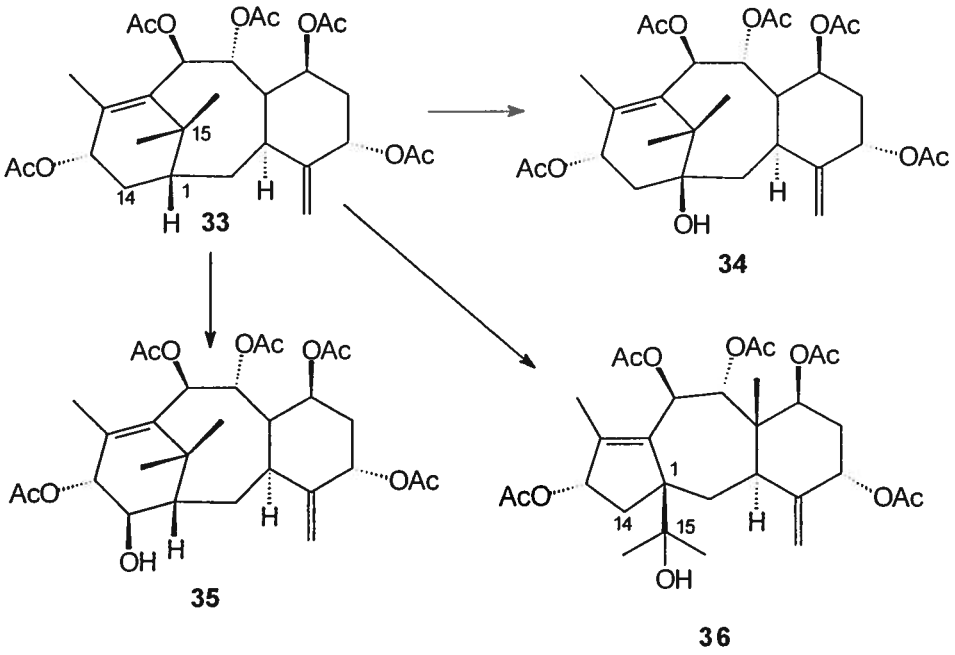
The *Ginkgo biloba* cell suspension cultures can selectively hydroxylate 2α,5α,10β,14β-tetra-acetoxy-4(20),11-taxadiene (**26**) at position 9 to afford compound **31**. Taxane **31** was further hydrolyzed, to remove the 10-acetyl group, to give taxane **32**. (**Scheme 1.9**) (Dai et al., 2001).



Scheme **1.9**. Biotransformation of **26** by *Ginkgo biloba* Cell suspension

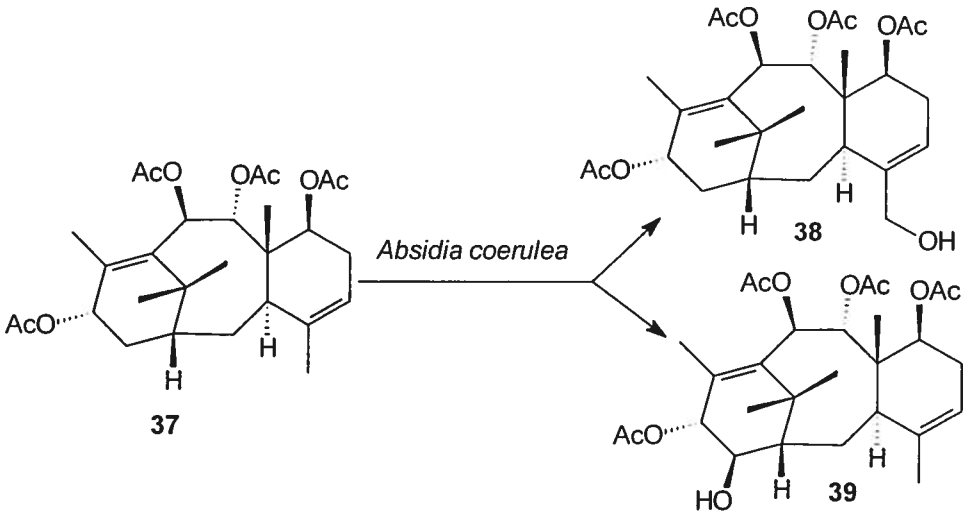
Cunninghamella elegans AS 3.2033 (Hu et al., 1996b), and an endophytic fungus from *Taxus yunnanensis* (Xu et al., 1997). *Cunninghamella echinulata* AS 1990 , can also selectively hydroxylate and deacetylate some other taxanes (Hu et al., 1997a).

5α,7β,9α,10β,13α,-pentaacetoxy-4(20),11-taxadiene (**33**) can be easily oxidized into three more polar metabolites (**34-36**) by several filamentous fungi (Hu et al., 1997b). The oxidation can even occur at C-1, which is impossible for chemical reagents. This compound can be changed from a 6/8/6 ring system into a 5/7/6 ring system (**Scheme 1.10**).



Scheme 1.10 Microbial transformation of Taxane **33**.

Absidia coerulea can hydroxylate a 4(5), 11(12)-taxadiene (**37**) derivative at position 20 and position 14 (Scheme **1.11**) to afford taxanes **38** and **39** (Hu et al., 2000).



Scheme **1.11**. Biotransformation of taxane **37** by *Absidia coerulea*

Bioconversion of Taxol/cephalomannine by *Streptomyces* sp. MA 7065 resulted in hydroxylation of Taxol on the 10-acetyl methyl group (**40**) in 60% yield and at the *para* position of the aromatic ring of the phenylisoserine side chain (**41**) in 10% yield. This culture could also hydroxylate the allylic methyl group of the phenylisoserine side chain of cephalomannine quantitatively (**42**) (**Figure 1.3**) (Chen et al., 2001).

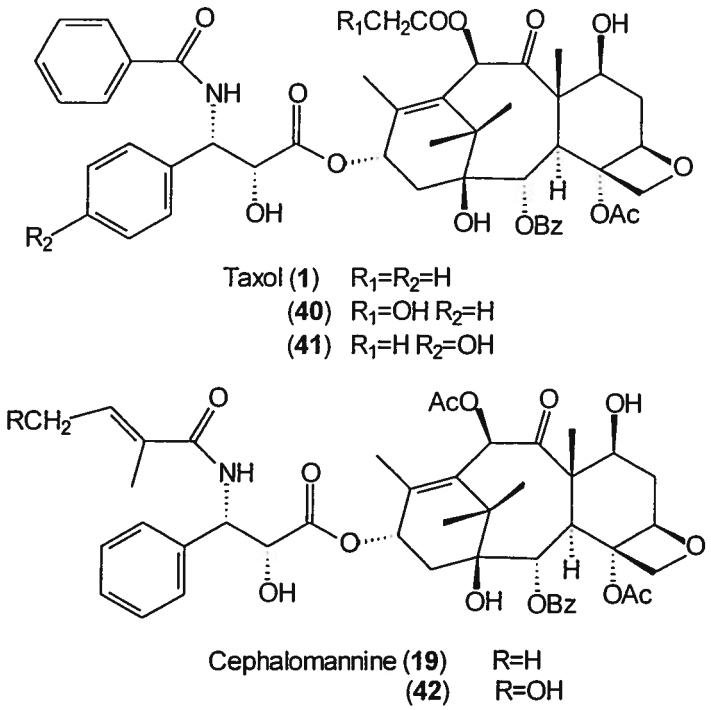
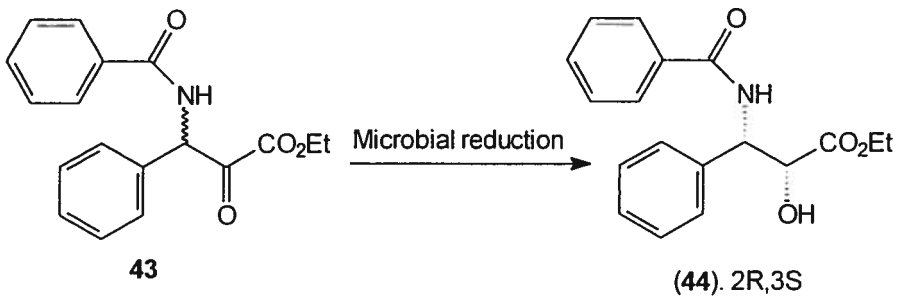


Figure **1.3** Biotransformation of Taxol (**1**) and Cephalomannine (**19**) by *Streptomyces sp* MA7065

Microbial reduction was useful during the preparation of a Taxol side-chain synthon, several microorganisms can reduce the 2-keto-3-(N-benzoylamino)-3-phenyl propionic acid ethyl ester (**43**) to (2R. 3S)-(-)-N-benzoyl-3-phenyl isoserine ethyl ester (**44**) in yields as high as 85% and with an optical purity of 99% (Scheme **1.12**)(Patel et al.,1995).



43 (**44**). 2R,3S

Scheme **1.12**. Microbial reduction of Taxol side chain synthon

1.4. Conclusion

Although the semi-synthesis of Taxol and Taxotere from 10-DAB has partially solved the supply problem, 10-DAB still has to be obtained from natural sources. Total synthesis involves too many steps and cell culture still faces some difficulties. Enzymes and biotransformation could therefore be useful.

Chapter 2

Biotransformation of a 4(20),11(12)-Taxadiene Derivative

Publication l

Cet article a dû être retiré en raison de restrictions liées au droit d'auteur.

Biotransformation of a 4(20),11(12)-Taxadicne Derivative

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Published in *Bioorganic & Medicinal Chemistry* **2001,** *9,* 793-800.

Résumé

Le dérivé 4(20), Il (12)-taxadiène a été converti en composés hydroxylés utilisant les champignons *cunninghamella elegans* AS32033 et *cuninghamel/a e/egans var chibaensis* ATCC 20230. Ces deux microorganismes ont mené à l'hydroxylation du C-1 et à la convertion en un composé aheo-taxane hydroxylé en C-15. De plus, les composés dérivant de ces deux champignons. présentent une oxidation en C-14, une isomérisation *trans-ci.*\· du cinnamoyl pour l'un ct une hydroxylation en C-17 pour l'autre.

Chaptcr 3

Microbial and Reducing Agents Catalyze the Rcarrangcment ofTaxancs

Publication 2

Cet article a dû être retiré en raison de restrictions liées au droit d'auteur.

Microbial and Reducing Agents Catalyze the Rearrangement of Taxanes

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Published in *Bioorganic & Medicinal Chemist1y* 2001, *9,* 1985-1992.

Résumé

Le dérivé 1: 5a, 7p, 9a, 1op, 13a-pentahydroxy-4(20),**11**(12)-taxadiène, fut converti par *Absidia coerula* ATCC 10738a, en deux nouvelles abeo-taxanes **1(15**11) et en un dérivé de taxane présentant une double liaison en C10-C11. Un composé simlaire fut obtenu en traitant au zinc le dérivé triacetoxy-4(20),11 (12)-taxadiène.

Chapter 4

Reanalysis of the Biotransformation of 4(20),11(12)-Taxadiene Derivatives

Publication 3

Cet article a dû être retiré en raison de restrictions liées au droit d'auteur.

Reanalysis of the Biotransformation of 4(20), J J(J 2)-Taxadiene Derivatives

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Chapter 5

Microbial Transformation of 9-Dihydro-13-acetylbaccatin III by *Absidia coerulea* ATCC 10738a and 10,13-Diacetyltaxayuntin E by *Cunninghamella echinulata* AS 3.1990

Microbial Transformation of 9-Dihydro-13-acetylbaccatin III by *Absidia coerulea* ATCC 10738a and 10,13-Diacetyltaxayuntin E by *Cunninghamella echinulata* AS 3.1990

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Manuscript prepared for ***Journal of Natural Products***

Résumé

La transformation microbienne du 9-dihydro-13-acetylbaccatin (**1**) et de 10,13-diacetyltaxayuntin E (**2**) a été étudiée. 9-dihydro-13-acetylbaccatin III (**1**) peut être métabolisé par le champignon *Absidia coerulea* ATCC 10738a pour donner les produits **3-6**. Les composés **5** et **6** ont été les premiers abéo-taxanes issus d'un substrat 1-OH par transformation microbienne. Le 10,13-Diacetyltaxayuntin E (**2**) peut être métabolisé par le champignon *Cunninghamella echinulata* AS 3,1990 pour donner six produits **7-12**. Les groupes 2-Bz et 9-Ac retrouvés dans le substrat ont échangé leur position dans les produits **7-9**. Les produits **10-12**, dont le groupe 2-Bz est relocalisé à la position 9, ont un anneau oxetane ouvert et un nouvel anneau formé de cinq membres. Ces derniers sont très rares chez les taxanes naturels.

Abstract

Microbial transformation of 9-dihydro-13-acetylbaccatin III (**1**) and 10,13-diacetyltaxayuntin E (**2**) was studied. 9-Dihydro-13-acetylbaccatin III (**1**) can be metabolized by the fungus *Absidia coerulea* ATCC 10738a to give products **3-6**. Compound **5** and **6** were the first abeo-taxanes derived from a 1-OH substrate by microbial transformation. 10,13-Diacetyltaxayuntin E (**2**) can be metabolized by the fungus *Cunninghamella echinulata* AS 3.1990 to give six products **7-12**. The 2-Bz and 9-Ac groups in the substrate have exchanged their position in products **7-9**. Products **10-12**, with 2-Bz group also shifted to C-9, have an opened oxetane-ring and a new formed five membered-ring. All of these three abeo-taxanes have a –OAc at C-15, a very rare feature in natural taxanes.

Introduction

Paclitaxel, (Taxol®), which was discovered in 1971[1] and was approved by FDA for marketing in December 1992, has become one of the most successful and useful anticancer drugs now available. Paclitaxel prevents cancer cell division by promoting assembly of tubulin into microtubules and stabilized it.[2-3] Paclitaxel has been successfully used for the treatment of ovarian and breast cancer. It also shows promising for the treatment of a variety of other solid tumors, such as head, neck, lung, gastrointestinal, and bladder.[4-5]

In the last two decades, great efforts have been made for the search of new taxanes from natural sources and chemical modification of natural taxanes.[6] Filamentous fungi have been used to transform taxanes and to obtain new structures.[7-16] We have previously shown that fungi could provide new taxanes not obtained from nature or from chemical modification.[17-19]

9-Dihydro-13-acetylbaccatin III, **1**, and 10,13-diacetyltaxayuntin E, **2**, were used in this work as substrates for microbial transformation. 9-Dihydro-13-acetylbaccatin III, **1**, is the most abundant taxanes from *Taxus canadensis*. 10,13-Diacetyltaxayuntin E, **2**, was obtained from taxayuntin E[20] by standard acetylation procedure. Taxayuntin E is a generous gift from Dr. Zhichao Li [Zhongling (Huizhou) High Science and Technology Co. Ltd. P.R.China]

In this publication, we were able to transform **1** into four taxanes **3-6** using the fungus *Absidia coerulea* ATCC 10738a. Compounds **3** and **4** are deacetylation products of **1**. Although abeo-taxanes could be obtained from microbial transformation, they all were obtained from substrates without 1-OH. Products **5** and **6** are abeo-taxanes first obtained from a substrate that has a 1-OH by microbial transformation. Substrate **2** could be metabolized by fungus *Cunninghamella echinulata* AS 3.1990 to give products **7-12**. All of these products have a 9-Bz group shifted from position 2. Products **10-12** have an opened oxetane-ring and a new formed five member-ring and a –OAc at C-15, which are rare in natural taxanes.

Results and Discussion

Incubation of 1 with *Absidia coerulea* ATCC 10738a (Figure 1).

Characterization of taxanes 3-6.

The major difference between compound **3** and the substrate **1**[21] in proton NMR is the chemical shift of H-7 (5.45 ppm in **3** vs 4.45 ppm in substrate **1**) and H-10 (4.74 ppm in **3** vs 6.20 ppm in substrate **1**). Clearly the 10-Ac has shifted to position 7. FABHRMS showed that compound **3** has the same MW and MF as that of substrate **1**. The structure of **3** was 7-acetyl-9-dihydro-13-acetyl-10-deacetylbaccatin II.

Compound **4** has previously been isolated from *Taxaus canadensis,*[22] and compound **5** was isolated from the needles of *Taxus baccata.*[23]

Compound **6** has a very similar ^{1}H NMR data as that of compound **5** except for the chemical shift of H-7 [5.34 ppm in **6** vs 4.24 ppm in **5**]. This data suggested a 7-OAc in compound **6.** FABMS showed it has 42 units more than that of compound **5,** which also suggested the presence of an acetyl group. Thus, the structure of **6** was 9,10-dideacetyl-abeo-baccatin VI as indicated in **Figure 1**.

Incubation of 10,13-Diacetyltaxayuntin E, 2, with Fungus *Cunninghamella echinulata* AS 3.1990 (Figure 2).

Characterization of Taxanes 7-12.

Although taxayuntin E is a known natural products, 10,13-Diacetyltaxayuntin E (**2**), the substrate obtained from taxayuntin E, is new. we therefore reported the ^{1}H and ^{13}C NMR data (from HSQC or HMBC) in this publication (**Table 1**).

The ^{1}H NMR of **7** (**Table 2**) is very similar to that of **2** (**Table 2**), except for the H-10 (6.43 ppm in **2** vs 4.80 ppm in **7**). This suggested a 10–OH group in **7** instead of 10-OAc. As expected, the ^{13}C NMR data of C-9, C-10, C-11, C-12 are more influenced than other carbons. The ^{1}H NMR signal of H-2 overlaps with H-9 in compound **7**. An acetyl (170.8 ppm) and a benzoyl carbonyl (166.7 ppm) have HMBC with these protons. The benzoyl group was put on position 9 was because of the longer cross peak in the HMBC. FABHRMS also support the structure.

In ^{1}H NMR, compound **8** (**Table 3**) has a very similar chemical shift and coupling patern to that of the substrate **2**, except the chemical shift of H-13 (5.61 ppm in **2** vs 4.59 ppm in **8**). It suggests the 13-OH in compound **8**. H-9 has corelation with OBz group in HMBC, which confirmed the OBz group had shifted to position 9 from position 2. FABHRMS data also support the fact that the compound **8** has 42 unit less than substrate . The structure of **8** was assignated as in **Figure 2**.

Compound **9** (**Table 4**) has a very similar ^{1}H NMR to that of **8**. The only exceptions is the chemical shift of H-7 (5.55 ppm in **8** vs 4.42 ppm in **9**), and H-13 (4.59 ppm in **8** vs 5.67 ppm in **9**). It shows that there is a 7-OH and 13-OAc in **9**. FABHRMS also suggested that **8** and **9** had the same MW and MF. The ^{13}C NMR are very similar for these two compounds. That the OBz group also was at position 9 is based on the fact that H-9 has corelation with OBz group in HMBC.

Compound **10** (**Table 5**) has a very different kind of ^{1}H NMR from the substrate or compounds **7-9**. The coupling constants between H-9 and H-10 (3.9 Hz) in **10** is significantly smaller than that in the substrate **2** (10.8 Hz). Also the J_{20ab} in **10** (11.2 Hz) is significantly bigger than that in the substrate **2** (6.9 Hz). The H-20ab in **10** is more shielded (4.17 and 3.90 ppm vs 4.49 and 4.40 ppm in the substrate **2**) and has a bigger shift difference (0.27 ppm vs 0.09 ppm in the substrate **2**). The ^{13}C NMR data of **10** are different from that of the substrate **2**, especially for C-2, C-3, C-4, C-5 and C-15 (See **Table 1** and **Table 4**). The chemical shift of C-5 (70.1 ppm) are

typically carbon attached with –OH, not with a oxetane ring (normally at 85 ppm). It may suggest the opening of the oxetane ring. The chemical shifts of C-2, C-3 and C-4 are more deshielded than usual. H-20a has HMBC with C-2, which suggested the forming of a new five member ring involving –C2-C3-C4-C20-O-. Indeed, we were able to find one natural abe-taxane, taxuyunnanine E, with a similar five member ring.[24] Compound **10** has very similar ^{1}H NMR pattern as that of taxuyunnanine E. The C-2, C-3, C-4 and C-5 chemical shifts of these two compounds are also very similar. From the above discussion, we suggested that compound **10** has an opened oxetane ring and a new formed five member ring. The OBz was put on position 9 based on the HMBC between H-9 and Bz group. In ^{1}H and ^{13}C NMR, there are five OAc. Three of them were attached to C-7, C-10, C-13 based on the ^{1}H NMR data. The other two –OAc groups must be put on C-4, and C-15, since C-5 must attached to a -OH (H-5 at 4.61 ppm). The attachment of -OAc to C-15, which is unusual in taxanes, is based on the fact that compound **10** has a more deshielded C-15 (87.7 ppm) than usual C-15 in abeo-taxanes (around 76 ppm). Indeed, the chemical shift of C-15 (87.7 ppm) is comparable to that of 15-benzoyl-10-deacetyl-2-debenzoyl-10-dehydro-abeo-baccatin III, 15-benzoyl-2-debenzoyl-7,9-dideacetyl-abeo-baccatin VI (89.9 and 91.0 ppm respectively)[25] and that of wallifoliol (90.4 ppm).[26] The 5-α–OH is deduced from the fact that H-20ab all have NOSEY with H-5, which has a small couplings with surrounding protons. Considering all above evidences, we assignated structure of compound **10** as in **Figure 2**. The FAB HRMS also support the above structure.

The ^{1}H NMR of compound **11** is very similar to that of **10** except for H-13 (5.72 ppm in **10** vs 4.64 ppm in **11**). It suggested 13-OH in **11** instead of 13-OAc. FAB HRMS indicated that compound **11** has 42 unit less than that of **10**, which also supports the above deduction.

The ^{1}H NMR of compound **12** was similar to that of **10** except for H-7 (5.54 ppm in **10** vs 4.19 ppm in **12**), which clearly suggested the 7-OH in **12**. FAB HRMS

indicated that **12** has 42 unit less than that of **10**, which also supports the above deductions. The structure are presented in **Figure 2**.

Conclusion

Although the fungi used in this publication are known to hydroxylate organic compounds, no hydroxylation was found in these two substrates. However, this publication has found some interesting reactions like the shift of 2-OBz to position 9, the opening of oxetane-ring and the formation of a new five-membered ring. The formation of 15-OAc in abeo-taxanes is new.

Experimental

Instrumentation

Flash chromatography was performed on Silica gel 60 (230-400 mesh EM Science). Thin layer chromatography was conducted on Silica Gel 60 F254 pre-coated TLC plates (0.25 mm, EM Science). The compounds were visualized on TLC plates with 10% sulfuric acid in ethanol and heating on a hot plate. Na_2SO_4 was the drying agent used in all work up procedures. Analytical HPLC was performed on a Waters 600 FHU delivery system coupled to a PDA 996 detector. Preparative and semi-preparative HPLC were carried out on a Waters Delta Prep 3000 instrument coupled to a UV 486 Tunable Absorbance detector set at 227 nm (Waters, Montreal, Quebec, Canada). Analytical HPLC was performed with two Whatman partisil 10 ODS-2 analytical columns (4.6 x 250 mm) in series. Semi-preparative HPLC was performed with two Whatman partisil 10 ODS-2 Mag-9 semi-preparative columns (9.4 x 250 mm) in series. Preparative HPLC was performed with one partisil 10 ODS-2 MAG-20 preparative column (22 x 500 mm). The products were eluted with a 50 min linear gradient of acetonitrile (25 to 100 %) in water at a flow rate of 18 mL/min (preparative HPLC) and 3mL/min (semi-preparative HPLC). All the reagents and solvents were of the best available commercial quality and were used without further purification.

NMR and Mass Spectrometry Measurement.

Except for compound **7**, which is recorded at $-30^{\circ}C$, All the NMR data were obtained at room temperature on a Bruker Avance-500 spectrometer operating at 500.13 MHz for proton and at 125.77 MHz for carbon-13. The solvent was used as an internal reference (in $CDCl_3$, 7.25 ppm for proton and 77.0 ppm for carbon-13; in Acetone-d6, 2.06 ppm for proton and 206.7 ppm for carbon). The various 2D spectra were acquired and processed using standard procedures. For phase sensitive 2D experiments (NOESY and HMQC), the data were acquired using the TPPI phase mode. The NOESY experiment was obtained using a mixing time of 0.3 s and a

relaxation delay of 1 s. The intensity of the cross-peaks in the NOESY experiment is designated as strong (s), medium (m) and weak (w). Positive ion Fast Atom Bombardment Mass Spectra (FAB-MS) were obtained with a Vacuum Generators ZAB-HS double-focussing instrument using a xenon beam having 8 kV energy at 1 mA equivalent neutral current. Low resolution mass spectra were obtained in glycerol. Samples were dissolved in 0.2 μl DMSO before addition of 0.5 μl glycerol. FABHRMS was similarly obtained in glycerol-DMSO at a resolving power of 12,000.

Substrates

Our 9-dihydro-13-diacetylbaccatin III, **1**, was isolated from the Canadian yew by our group member Dr. Junzeng Zhang. Taxayuntin E (obtained from *Taxus yunnanensis*) is a generous gift from Dr. Li Zhicao, Zhongling (Huizhou) High Science and Technology, Co. Ltd, P.R. China. 10,13-diacetyltaxayuntin E, **2**, was prepared from taxayuntin E through standard acetylation procedure: To a 0.6 mL solution of acetic anhydride and pyridine (1:1), was added taxayuntin E (22.5 mg). The mixture was stirred at room temperature for 24 hours. After the reaction reached completion (indicated by TLC), to the resulting mixture was added 10 mL heptane. The solvent was removed under decreased pressure. The resulting product is pure and the yield is quantitative.

Microorganism:

Absidia coerulea ATCC 10738a was purchased from American Type Culture Collection (ATCC). *Cunninghamella echinulata* AS 3.1990 was purchased from the Institute of Microbiology, Chinese Academy of Science, P.R. China,

Incubation, Biotransformation procedure

General: Cultures were grown in Potato Dextrose (24g/L, DIFCO laboratories) broth, and was incubated at 25°C and 125 rpm, unless otherwise indicated. The *Absidia coerulea ATCC 10738a* was first preserved on silica gel and kept at 4°C cold room to prevent mutation.[27] The seed culture was prepared by the addition of several grains of silica gel that absorbed the fungus to 30 mL of medium in a 125 mL Erlermeyer flask. The culture was incubated for 3 days. At preparative scale, to a 2000 ml flask, containing 1000 ml Potato Dextrose broth, was added a small fraction of the above fungus seed (homogenized before use). The flasks were at first cultured for 2 days. Then to the flasks was added substrate (dissolved in DMSO). Continued culturing for another 12 days. At the end of incubation, the culture was first homogenized and then was extract with CH_2Cl_2. And finally, the CH_2Cl_2 extract was obtained for further purification. Culture controls with the same medium and substrate but without fungi were performed at the same condition. The exact procedure was used for *Cunninghamella echinulata* AS 3.1990.

For 9-dihydro-13-diacetylbaccatin III, **1**. To each of the two 2L flasks containing 1L of medium, was added 50 mg substrate (dissolved in 2 mL DMSO). After 12 days incubation, the culture was extracted with CH_2Cl_2 (500 mL×3 for 1L culture). The CH_2Cl_2 crude residue was 159 mg. The extract was first applied to a silica gel flash chromatography column (16g), eluted with CH_2Cl_2 (250 mL), CH_2Cl_2/MeOH (300:1, 200:1, 150:1; 100:1, 100:2, 100:3, 100:10 each 250 mL) and sixty-five 30 mL fractions were obtained. The fractions 37-40, 41-46, 47-55, 56-63, containing relevant products, were combined based on the TLC. Each of the above fractions were separated by preparative HPLC and finally the following compounds were obtained: **3** (2.2 mg 2.2%), **4** (2.5 mg, 2.5%), **5** (0.8 mg, 0.9%), **6** (0.5 mg, 0.5%). 74 mg (74%) of the initial substrate was recovered.

For 10,13-diacetyltaxayuntin E, **2**. To each of the four 4L flasks containing 2L of medium, was added 200 mg substrate (dissolved in 4 mL DMSO). After 12 days incubation, the culture was extracted with CH_2Cl_2 (1000 mL×3 for 2L culture). The

CH_2Cl_2 crude residue was 1150 mg. The extract was first applied to a silica gel flash chromatography column (44 g), eluted with CH_2Cl_2 (200 mL), CH_2Cl_2/Acetone (40:1, 15:1; 100:1, 5:1, 1:1 each 300 mL) and forty-one 40 mL fractions were obtained. The fractions 3-9, 10-13, 14-33, 34-41, containing relevant products, were combined based on the TLC. Each of the above fractions was separated by preparative HPLC and the following compounds were obtained: **7** (29.2 mg, 3.7%), **8** (14.4 mg, 1.9%), **9** (6.6 mg, 1.0%), **10** (32 mg, 3.9%), **11+12** (1:1, 2.6 mg, 0.4%), 443 mg (55.4%) of the initial substrate, **2**, was recovered.

Compound Data

Compound **3** was obtained as white powder. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.09 (d, J = 8.0 Hz, 2H, Bz-*o*), 7.62 (t, J = 7.5 Hz, 1H, Bz-*p*), 7.49 (t, J = 7.5 Hz, 2H, Bz-*m*), 6.21 (br.t, 1H, H-13), 5.77 (d, J = 5.8 Hz, 1H, H-2), 5.45 (dd, J = 9.9, 7.5 Hz, 1H, H-7), 4.97 (d, J = 8.2 Hz, 1H, H-5), 4.74 (d, J = 10.6 Hz, 1H, H-10), 4.34 (d, J = 8.0 Hz 1H, H-20a), 4.27 (br.m, 1H, H-9), 4.18 (d, J = 8.0 Hz 1H, H-20b), 3.19 (d, J = 5.8 Hz, 1H, H-3), 2.64 (o.m, 1H, H-6a), 2.30 (s, 3H, OAc), 2.20 (o.m, 1H, H-14), 2.20 (s, 3H, OAc), 2.15 (s, 3H, OAc),1.95 (o.m, 1H, H-6b), 1.91 (s, 3H, H-19), 1.84 (d, J = 1.3 Hz 3H, H-18), 1.72 (s, 3H, H-17), 1.32 (s, 3H, H-16); FAB HRMS for $C_{33}H_{42}O_{12}K$ $[M+K]^+$requires 669.2313, found 669.2312.

Compound **4** was obtained as white powder. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.09 (d, J = 8.0 Hz, 2H, Bz-*o*), 7.61 (t, J = 7.5 Hz, 1H, Bz-*p*), 7.48 (t, J = 7.6 Hz, 2H, Bz-*m*), 6.20 (t, J = 9.0 Hz, 1H, H-13), 5.76 (d, J = 6.1 Hz, 1H, H-2), 4.59 (d, J = 10.5 Hz, 1H, H-10), 4.59 (o.d, J = 8.6 Hz, 1H, H-5), 4.39 (dd, J = 9.7, 7.5 Hz, 1H, H-7), 4.34 (o.d, J = 10.5 Hz, 1H, H-9), 4.32 (o.d, 1H, H-20a), 4.18 (d, J = 8.2 Hz 1H, H-20b), 3.07 (d, J = 6.1 Hz, 1H, H-3), 2.60 (o.m, 1H, H-6a), 2.29 (s, 3H, OAc), 2.20 (s, 3H, OAc), 2.19 (o.m, 1H, H-14), 1.94 (o.m, 1H, H-6b), 1.85 (s, 3H, H-18), 1.83 (s, 3H, H-19), 1.72 (s, 3H, H-16/17), 1.32 (s, 3H, H-17/16); FAB HRMS for $C_{31}H_{40}O_{11}K$ $[M+K]^+$requires 627.2208; found 627.2211.

Compound **5** FAB HRMS for $C_{31}H_{40}O_{11}K$ $[M+K]^+$requires 627.2208; found 627.2211.

Compound **6** was obtained as white powder. ^1H NMR ($CDCl_3$, 500 MHz) δ 7.97 (d, *J* = 7.5 Hz, 2H, Bz-*o*), 7.60 (t, *J* = 7.2 Hz, 1H, Bz-*p*), 7.46 (t, *J* = 7.4 Hz, 2H, Bz-*m*), 6.14 (d, *J* = 7.4 Hz, 1H, H-2), 5.72 (br.t, *J* = 7.2 Hz, 1H, H-13), 5.34 (t, *J* = 8.3 Hz, 1H, H-7), 4.93 (d, *J* = 8.3 Hz, 1H, H-5), 4.60 (d, *J* = 10.3 Hz, 1H, H-10), 4.46 (d, *J* = 8.0 Hz 1H, H-20a), 4.35 (br.d, *J* = 10.3 Hz, 1H, H-9), 4.11 (d, *J* = 8.0 Hz 1H, H-20b), 2.95 (d, *J* = 7.6 Hz, 1H, H-3), 2.61 (m, 1H, H-6a), 2.29 (dd, *J* = 14.2, 6.7 Hz 1H, H-14), 2.15 (s, 6H, 2xOAc), 2.06 (s, 3H, OAc), 1.93 (s, 3H, H-18), 1.88 (o.m, 1H, H-6b), 1.88 (o.m, 1H, H-14b), 1.87 (s, 3H, H-19), 1.12 (s, 3H, H-16/17), 1.09 (s, 3H, H-17/16); FABMS found its MW is 630.

Compound **7** was obtained as white powder. ^1H NMR (Acetone-d_6, 500 MHz) and ^{13}CNMR (Acetone-d_6, 125 MHz) at –30°C see **table 1**. FAB HRMS for $C_{35}H_{44}O_{13}K$ $[M+K]^+$requires 711.2419; found 711.2419.

Compound **8** was obtained as white powder. ^1H NMR (Acetone-d_6, 500 MHz) and ^{13}CNMR (Acetone-d_6, 125 MHz) see **table 2**. FAB HRMS for $C_{35}H_{44}O_{13}K$ $[M+K]^+$requires 711.2419; found 711.2419.

Compound **9** was obtained as white powder. ^1H NMR (Acetone-d_6, 500 MHz) and ^{13}CNMR (Acetone-d_6, 125 MHz) see **table 3**. FAB HRMS for $C_{35}H_{44}O_{13}K$ $[M+K]^+$requires 711.2419; found 711.2419.

Compound **10** was obtained as white powder. ^1H NMR (Acetone-d_6, 500 MHz) and ^{13}CNMR (Acetone-d_6, 125 MHz) see **table 4**. FAB HRMS for $C_{37}H_{46}O_{14}K$ $[M+K]^+$requires 753.2525; found 753.2526.

Compound **11** was obtained as white powder. ^{1}H NMR (Acetone-d_6, 500 MHz) δ 8.15 (d, J = 7.4 Hz, 2H, Bz-*o*), 7.66 (t, J = 7.5 Hz, 1H, Bz-*p*), 7.55 (t, J = 7.4 Hz, 2H, Bz-*m*), 6.11 (d, J = 3.6 Hz, 1H, H-10), 5.37 (dd, J = 12.1, 3.8 Hz, 1H, H-7), 5.12 (d, J = 3.6Hz, 1H, H-9), 5.03 (d, J = 8.5 Hz, 1H, H-2), 4.65 (o.m, 1H, H-5), 4.64 (o.m, 1H, H-13), 4.16 (o.d, 1H, H-20a), 3.94 (d, J = 10.9 Hz 1H, H-20b), 3.04 (d, J = 8.5 Hz, 1H, H-3), 2.41 (dd, J = 14.3, 6.8 Hz 1H, H-14a), 2.12 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.07 (o.m, 1H, H-6a), 2.03 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.83 (o.m, 1H, H-6b), 1.88 (o.m, 1H, H-14b), 1.69 (s, 3H, H-17), 1.61 (s, 3H, H-19), 1.52 (s, 3H, H-18), 1.42 (s, 3H, H-16); ^{13}CNMR (Acetone-d_6, 125 MHz) δ 152.2 (C-12), 134.3 (C-11), 93.3 (C-4), 81.0 (C-2), 87.9 (C-15), 77.6 (C-13), 75.5 (C-9), 75.2 (C-20), 71.2 (C-10), 70.9 (C-7), 67.1 (C-1), 69.6 (C-5), 49.8 (C-3), 44.1 (C-8), 39.4 (C-14), 32.7 (C-6), 24.4 (C-16), 23.8 (C-17), 15.0 (C-19), 13.2 (C-18); FAB HRMS for $C_{35}H_{44}O_{13}K$ [M+K] $^{+}$requires 711.2419; found 711.2419.

Compound **12** was obtained as white powder. ^{1}H NMR (Acetone-d_6, 500 MHz) δ8.14 (d, J = 7.4 Hz, 2H, Bz-*o*), 7.64 (t, J = 7.5 Hz, 1H, Bz-*p*), 7.56 (t, J = 7.4 Hz, 2H, Bz-*m*), 6.26 (d, J = 4.0 Hz, 1H, H-10), 5.72 (t, J = 7.2 Hz, 1H, H-13), 5.21 (d, J = 4.0 Hz, 1H, H-9), 4.98 (d, J = 8.1 Hz, 1H, H-2), 4.19 (o.m, 1H, H-7), 4.14 (o.d, 1H, H-20a), 3.83 (d, J = 11.0 Hz 1H, H-20b), 2.92 (d, J = 7.8 Hz, 1H, H-3), 2.60 (dd, J = 14.6, 7.0 Hz 1H, H-14a), 2.11 (s, 3H, OAc), 2.09 (s, 3H, OAc), 1.94 (o.m, 1H, H-6a), 1.94 (o.dd, 1H, H-14b), 2.03 (s, 3H, OAc), 1.89 (s, 3H, OAc), 1.85 (o.m, 1H, H-6b), 1.69 (s, 3H, H-17), 1.54 (s, 3H, H-16), 1.48 (s, 3H, H-19), 1.45 (s, 3H, H-18); ^{13}CNMR (Acetone-d_6, 125 MHz) δ 146.8 (C-12), 136.5 (C-11), 93.8 (C-4), 81.0 (C-2), 87.8 (C-15), 81.7 (C-13), 76.1 (C-9), 75.2 (C-20), 70.4 (C-10), 67.8 (C-7), 67.7 (C-1), 70.8 (C-5), 50.2 (C-3), 45.3 (C-8), 36.6 (C-14), 32.7 (C-6), 24.7 (C-16), 24.2 (C-17), 13.6 (C-19), 13.2 (C-18); FAB HRMS for $C_{35}H_{44}O_{13}K$ [M+K] $^{+}$requires 711.2419; found 711.2419.

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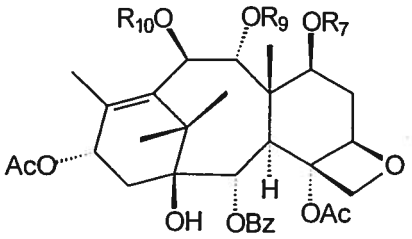
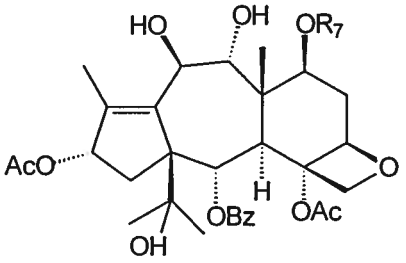
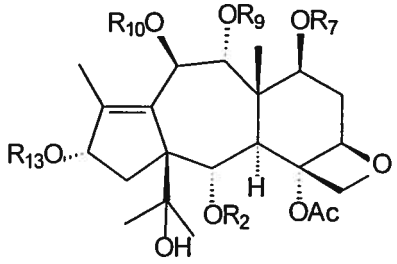


	R_7	R_9	R_{10}
1.	H	H	Ac
3.	Ac	H	H
4.	H	H	H

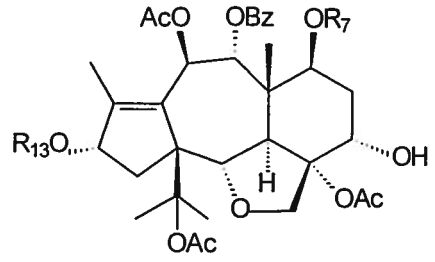


5. R_7 = H
6. R_7 = Ac

Figure 1. Microbial transformation products of 9-dihydro-13-acetylbaccatin III (**1**) by fungus *Absidia coerulea* Bainier ATCC 10738a



	R_2	R_7	R_9	R_{10}	R_{13}
2.	Bz	Ac	Ac	Ac	Ac
7.	Ac	Ac	Bz	H	Ac
8.	Ac	Ac	Bz	Ac	H
9.	Ac	H	Bz	Ac	Ac



	R_7	R_{13}
10.	Ac	Ac
11.	Ac	H
12.	H	Ac

igure 2. Microbial transformation products of 10,13-diacetyltaxayuntin E (**2**) by fungus *Cunninghamella echinulata* AS 3.1990.

Table 1. ^{1}H NMR and ^{13}CNMR data for compound **2** in acetone-d_6

Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C
1	--	67.5
2	6.15 br.d (*6.8*)	68.0
3	2.97 br.d (*6.8*)	43.8
4	--	78.9
5	4.96 br.d (*7.5*)	84.8
6a	2.70 m	34.6
6b	1.85 m	
7	5.54 br.t (*7.5*)	70.3
8	--	43.7
9	6.32 br.d (*10.8*)	77.2
10	6.43 br.d (*10.8*)	67.7
11	--	135.9
12	--	147.0
13	5.61 br.t (*7.3*)	78.5
14a	2.32 m	36.6
14b	1.74 m	
15	--	75.4
16/17	1.18 s	24.8
17/16	1.15 br.s	27.4
18	1.85 br.s	11.6
19	1.75 s	13.0
20a	4.49 br.d (*6.9*)	74.4
20b	4.40 d (6.9)	
OAc	2.10x2 s	21.7
	2.01 s	21.4
	1.81 s	21.4
	1.60	20.5
OBz	--	
o	7.92 br.d (*≈7*)	129.7
m	7.43 t (*7.4*)	128.3
p	7.55 t (*7.4*)	133.2

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b] The ^{13}C chemical shifts were extracted from the HSQC and HMBC (for quaternary carbons) experiments (±0.2 ppm).

Table 2. ^{1}H NMR and ^{13}CNMR data for compound **7** (at –30°C) in acetone-d_6

Position	$^{a}\delta_H$-mult-*J* (Hz)	$^{b}\delta_C$	HMBC
1	--	67.0	
2	6.07 o.d	67.7	1, 8, 14, 15, 170.8 (see 9)
3	3.01 br.d (*7.6*)	44.6	2, 7, 8, 19
4	--	78.0	
5	4.95 d (*7.9*)	84.3	3, 4, 7
6a	2.45 br.m	34.6	Not sure
6b	1.70 o.m		
7	5.45 o.t (*8.4*)	70.6	6, 8, 19, 169.7
8	--	42.8	
9	6.07 o.d (*10.1*)	80.3	7, 8, 10, 19, 166.7 (see 2)
10	4.80 o.m	65.9	
11	--	139.9	
12	--	141.5	
13	5.64 br.t (*7.4*)	78.8	
14a	2.24 o.m	36.2	
14b	1.75 o.m		
15	--	75.2	
16/17	1.07 s	27.4	1, 15, Me
17/16	1.19 s	24.5	1, 15, Me
18	1.77 o.s	10.7	11, 12, 13
19	1.68 s	12.8	3, 7, 8, 9
20a	4.41 d (*7.4*)	73.8	3, 4
20b	4.31 d (*7.4*)		3, 4, 5
OAc	Not sure		
OBz			
o	7.96 br.d (*7*)	129.5	
m	7.51 broad	128.2	
p	7.61 t (*7*)	132.7	

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b] The ^{13}C chemical shifts were extracted from the HSQC and HMBC (for quaternary carbons) experiments (±0.2 ppm),

Table 3. ^{1}H NMR and ^{13}CNMR data for compound **8** in acetone-d_6

Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC	[c]NOESY
1	--	68.6		
2	6.21 d (*7.4*)	69.0	1, 3/8, 14, 15, OAc	
3	3.11 br.d (*7.4*)	45.0	2, 8	2^m, 7^s, 10^m, $14b^m$
4	--	80.0		
5	4.95 d (*7.9*)	85.3	3, 4, 7	
6a	2.61 dt (*15.9, 8.0*)	35.7		5^s, $6b^s$, 7^s
6b	1.68 dd (*15.9, 7.3*)			5^m, $6a^s$
7	5.55 t (*7.8*)	71.5		
8	--	45.2		
9	6.34 br.d (*10.2*)	78.9	3/8, 4, 7, 11, Bz	
10	6.39 d (*10.2*)	69.1	1, 9, 11, 12, OAc	
11	--	135.4		
12	--	152.7		
13	4.59 br.q (*6.7*)	76.6	18, OAc	
OH-13	4.29 br.d (*5.0*)			13^w
14a	2.26 dd (*14.9, 7.3*)	40.1		13^s, $14b^s$, 16^s, 17^s
14b	1.81 dd (*14.7, 7.5*)			3^s, $14a^s$
15	--	76.6		
OH-15	2.99 br.s			9^m, 16^s, 17^s
16	1.16 s	26.2		2^s, 13^s, $14a^s$, OH-1^s
17	1.13 s	28.1		2^s, 9^m, 13^s, $14a^s$, OH-15^s
18	1.81 s	11.9		10^s, 13^m
19	1.74 s	13.8		2^s, 9^s, $20b^s$
20a	4.44 d (*7.3*)	74.9	3, 4	
20b	4.37 br.d (*7.3*)		3, 5	
OAc	2.14 s	22.1	171.4	
	2.03 s	21.8	172.3	
	1.75 s	21.8	170.9	
	1.61 s	20.7	169.9	
OBz			167.6	
o	7.94 d (*7.2*)	130.5		19^m, Bz-m^s
m	7.54 t (*7.2*)	129.4		
p	7.67 t (*7.2*)	134.2		

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b] The ^{13}C chemical shifts were extracted from the HSQC and HMBC (for quaternary carbons) experiments (±0.2 ppm), [c]NOESY intensities are marked as strong (s), medium (m), or weak (w).

Table 4. ^{1}H NMR and ^{13}CNMR data for compound **9** in acetone-d_6

Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC	[c]NOESY
1	--	69.1		
2	6.19 d (*7.7*)	69.1		9^s, $16/17^s$, 19^s
3	2.93 br.d (*7.2*)	45.2		7^s, 18^m
4	--			
5	4.95 d (*8.6*)	85.3		$6a^w$
6a	2.54 dt (*15.8, 8.1*)	38.2		5^s, $6b^s$, 7^m
6b	1.72 dd (*15.8, 8.6*)			5^w, $6a^s$
7	4.42 o.m	71.7		3^s, 5^w, $6a^w$, 10^s, OH^m -7
OH-7	3.72 br.s			7^w
8	--	45.2		
9	6.48 d (*10.4*)	79.4	Bz-CO	2^s, 19^s, $16/17^w$
10	6.36 d (*10.4*)	67.9		3^w, 7^m, 18^s
11	--	138.6		
12	--	147.9		
13	5.67 br.t (≈ *7.2*)	79.1		$16/17^s$, 18^w
14a	2.40 dd (*14.0, 7.3*)	37.4		13^m, $14b^s$, $16/17^s$
14b	1.80 dd (*14.2, 7.6*)			3^s, $14a^s$
15	--	76.7		
OH-15	3.23 br.s			$16/17^s$
16	1.23 o.s	26.5	Me, 1, 15	2^s, 9^w, 13^s, $14a^s$, OH^s-15
17	1.23 o.s	28.0	Me, 1, 15	See 16
18	1.75 s	11.7	11, 12, 13	10^s, 13^w
19	1.62 s	12.1	3/8, 7, 9	2^s, 9^s, $20b^s$
20a	4.39 br.d (*7.5*)	74.7		19^s
20b	4.34 br.d (*7.5*)			
OAc	2.15 s	22.1	170.9	
	2.09 s	21.0	171.8	
	2.03 s	21.6	172.1	
	1.67 s	20.6	170.4	
OBz				
o	8.03 d (*8.0*)	130.4		
m	7.54 t (*7.7*)	129.4		
p	7.67 t (*7.3*)	134.2		

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b] The ^{13}C chemical shifts were extracted from the HSQC and HMBC (for quaternary carbons) experiments (±0.2 ppm), [c]NOESY intensities are marked as strong (s), medium (m), or weak (w).

Table 5. ^{1}H NMR and ^{13}CNMR data for compound **10** in acetone-d_6

Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC	[c]NOESY
1	--	66.9		
2	4.98 d (*7.9*)	80.7	1, 3, 8, 14, 15	3^m, 10^w, 16^s, 17^s, 19^s, $20b^s$
3	3.01 d (*7.9*)	50.0	1, 2, 4,	2, $6b^s$, 7^s, Bz-o^m
4	--	93.8		
5	4.61 o.m	70.1	4, 5, 6, 7	$6a^s$, 7^s,
6a	2.01 o.m	32.5	4, 8, 5/7	5^s, $6b^s$, 7^s
6b	1.82 o.m	32.5	5/7	$6a^s$, $20b^m$
7	5.32 dd (*12.2, 3.6*)	70.8	5, 6, 8, 9, 19, Ac	3^s, 5^s, $6a^s$, Bz-o^m
8	--	44.3		
9	5.15 d (*3.9*)	74.9	3, 4!!, 7/10, 8, 11, 19, Bz	10^s, 19^s
10	6.13 d (*3.9*)	70.4	1, 8, 9, 11, 12, Ac	2^w, 9^s, 16^w
11	--	136.0		
12	--	147.3		
13	5.72 t (*7.2*)	81.5	11, 12, Ac	$14a^s$, *16*^{w}, 17^s, 18^s
14a	2.61 dd (*14.7, 7.0*)	36.8	1, 2, 11, 12, 15	13^s, $14b^s$, 16^s, 17^s
14b	1.92 o.dd (*14.7, 7.2*)		1, 2, 15	3^s, $14a^s$,
15	--	87.7		
16	1.69 s	24.2	1, 15, Me	2^s, 10^w, $14a^s$, 17^s
17	1.53 s	24.7	1, 15, Me	2, 13^s, $14a^s$, 16^m
18	1.43 s	13.2	11, 12, 13	10^s, 13^s
19	1.60 s	15.0	3, 7, 8, 9	2^s, $6b^s$, 9^s, $20b^s$, Bz-o^m
20a	4.17 dd (*11.2, 1.5*)	75.1	2, 3, 4	5^w, $20b^s$
20b	3.90 d (*11.2*)		4, 4, 5, Ac	2^m, 5^w, 19^s, $20a^s$
OAc	2.16 s	21.5	170.08	
	2.12 s	22.5	171.	
	2.07 s	21.2	170.07	
	2.00 s	23.5	169.9	
	1.90 s	21.0	170.02	
OBz		130.4	165.4	
o	8.17 d (*7.2*)	131.0		
m	7.54 t (*7.7*)	129.4		
p	7.67 t (*7.2*)	134.4		

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b] The ^{13}C chemical shifts were extracted from the HSQC and HMBC (for quaternary carbons) experiments (±0.2 ppm), [c]NOESY intensities are marked as strong (s), medium (m), or weak (w).

Chapter 6

General Discussion, Conclusion and Perspective for the Microbial Transformation of Taxanes

This chapter will discuss the results of microbial transformation of taxanes, including the negative ones, in an overview manner with unified substrate and products numbering system and integrate them in order to draw some general conclusions that were not presented in previous chapters.

6.1. Fungi Used in the Project

Although in previous chapters, we reported only the fungi that gave positive results with taxanes, sixteen fungi were investigated. Ten of them were purchased from China (Institute of Microbiology, Chinese Academy of Sciences, P.R. China) and the others were purchased from ATCC (American Type Culture Collection). All of them are known to promote regio- or stereo-selective hydroxylation.

The following is the fungi list:

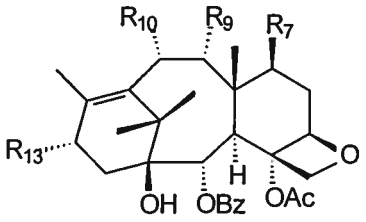
1. *Absidia coerulea* AS 3.2462
2. *Cunninghamella blakesleana* AS 3.910
3. *Cunninghamella echinulata* AS 3.953
4. *Cunninghamella echinulata* AS 3.2000
5. *Cunninghamella echinulata* AS 3.1990
6. *Cunninghamella echinulata* AS 3.2474
7. *Cunninghamella elegans* AS 3.1207
8. *Cunninghamella elegans* AS 3.2477
9. *Cunninghamella elegans* AS 3.2033
10. *Rhizopus arrhizus* AS 3.2744
11. *Absidia coerulea* Bainier ATCC 6647
12. *Absidia coerulea* Bainier ATCC 10738a
13. *Cunninghamella echinulata* ATCC 8987
14. *Cunninghamella elegans var. chibaensis* ATCC 20230
15. *Nocardia corallina* ATCC 19070
16. *Rhizopus oryzae* Went et ATCC 11145

6.2. The Substrates Used in the Project

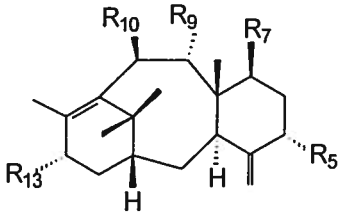
One of the most important and critical aspects to the success of the project is the availability of the taxanes used for substrates. We need enough material to screen and to scale up, and we need to take into account unavoidable losses during the extraction, separation, and purification process. The substrate used cannot be less than 50 mg. Although our group has obtained nearly 100 natural taxanes from *Taxus canadensis*, the yields are normally very low. Fortunately, we were able to get enough **4** (9-dihydro-13-acetylbaccatin III), **68** (Taxinine), and **70** from *Taxus canadensis* and were able to chemically modify **4** (to obtain **45**, **46**, **47**, **48**, and **49**), and **68** (to obtain **69**).

We also were able to obtain large amount of natural **50** (2-deacetoxyltaxinine J), **58** (Taxinine J), **60** (Taxayuntine E), and **62** (1β-hydroxybaccatin I) from *Taxus yunnanensis*. These compounds were generous gifts from Dr. Li Zhichao [Zhongling (Huizhou) High Science and Technology Co. Ltd. P.R.China].

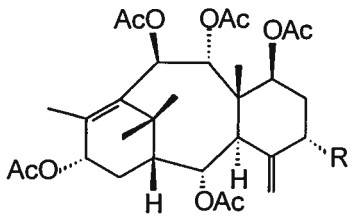
From **50**, we were able to get compounds **51-57** by chemical modification. From **58**, we were able to get **59**. From **60**, we were able to get **61**. From **62**, we were able to get compounds **63-67**. Altogether, we were able to obtain 27 taxanes for the project. Their structures are shown in **Figure 6.1** and **Figure 6.2.**



	R_7	R_9	R_{10}	R_{13}
4*	OH	OH	OAc	OAc
45	OH	OH	OH	OAc
46	OH	OH	OAc	OH
47	OH	OH	OH	OH
48	OAc	OAc	OAc	OAc
49	=O	=O	OAc	OAc



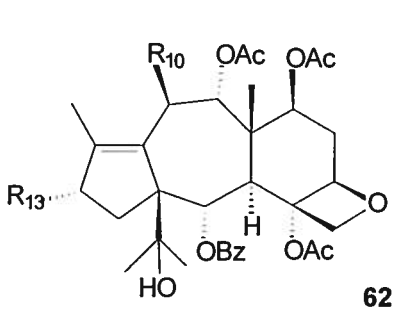
	R_5	R_7	R_9	R_{10}	R_{13}
50*	$OCOCH=CHC_6H_5$	OAc	OAc	OAc	OAc
51	OH	OAC	OAc	OAc	OAc
52*	OH	OH	OH	OH	OH
53	OH	OAc	OAc	OAc	OH
54	OH	OAc	OAc	OAc	=O
55	OH	OH	OH	OH	=O
56*	OAc	OAc	OAc	OAc	OAc
57*	$OCOCH=CHC_6H_5$	OH	OH	OH	OAc



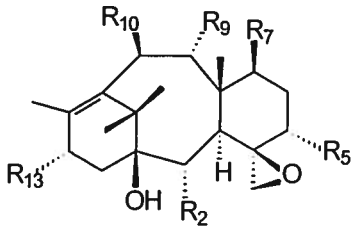
58 R=$OCOCH=CHC_6H_5$
59 R=OH

★ Can be metabolized

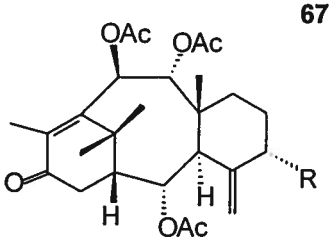
Figure 6.1. Structures of the Substrates (1)



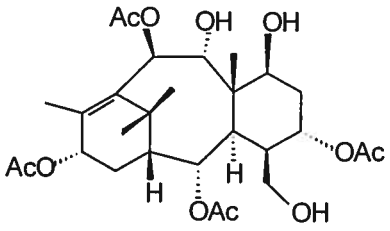
	R_{10}	R_{13}
60	OH	OH
61*	OAc	OAc



	R_2	R_5	R_7	R_9	R_{10}	R_{13}	
62	OAc	OAc	OAc	OAc	OAc	OAc	
63	OH	OAc	OAc	OAc	OAc	OAc	
64	OH	OH	OAc	OAc	OAc	OAc	
65	OH	OH	OAc	OAc	OAc	OH	
66	OH	OH	OAc	OAc	OAc	OH	β-4(20)
67	OH	OAc	OH	OH	OH	OAc	



68 R=OCOCH=CHC$_6$H$_5$
69 R=OH



70

★ Can be metabolized

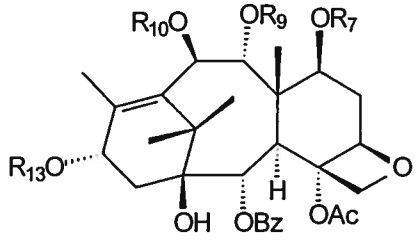
Figure 6.2. Structure of the Substrates (2)

6.3. Microbial Transformation of Taxanes

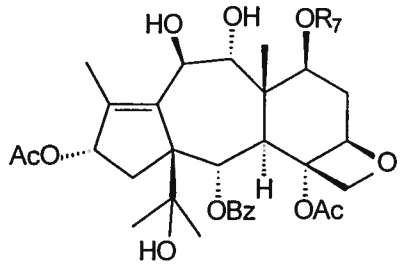
Each of the 27 taxanes was first screened using all of the 16 fungi and the procedure that was described in chapter 2-5. When biotransformation was detected, as observed by TLC and HPLC, the screening was repeated to confirm. Then the process was scaled up. After incubation, products were extracted with an organic solvent, separated by flash column chromatography, PTLC, or HPLC chromatography (for details see individual chapters) and the structures were elucidated from NMR and FAB HRMS data. Of the 27 compounds, only 6 of them (**4, 50, 52, 56, 57,** and **61**) can be metabolized as indicated in **Figure 6.1** and **Figure 6.2**.

Compound **4** (9-dihydro-13-acetylbaccatin III) (chapter 5) can be metabolized by *Absidia coerulea* ATCC 10738a to give four products (**Figure 6.3**). In addition to the deacetylation products **71** and **72**, other products include abeo-taxane **73** and **74**. Although the isolation of abeo-taxanes from microbial transformations was previously reported (Hu et al., 1997b) and abeo-taxanes could also be obtained from substrate **50**, **56**, and **57**, the result is still interesting, since compound **4** (9-dihydro-13-acetylbaccatin III) has a –OH at position 1, while the substrates used in other reactions have only a hydrogen at position 1. The mechanisms must be a little different.

Compound **50** (2-deacetoxyltaxinine J), could not be metabolized by 25 collected fungi including *Absidia coerula* (Hu et al., 1997b). In this project, the fungus and medium used for **50** were slightly different from those described in the literature, and the results are different. We were able to get four products, **75-78**, from this reaction (**Figure 6.4**). The compound types are similar to the products reported. This suggested that control of the medium and selection of the source of a strain could lead to the control of products and yields.

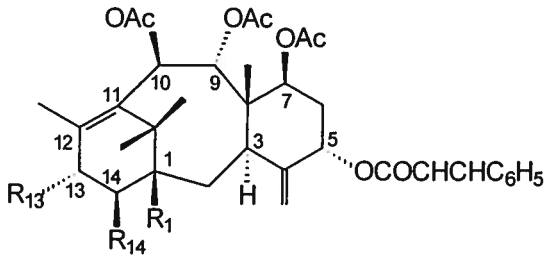


	R_7	R_9	R_{10}	R_{13}	
4.	H	H	Ac	Ac	
71.	Ac	H	H	Ac	2.2%
72.	H	H	H	Ac	2.5%

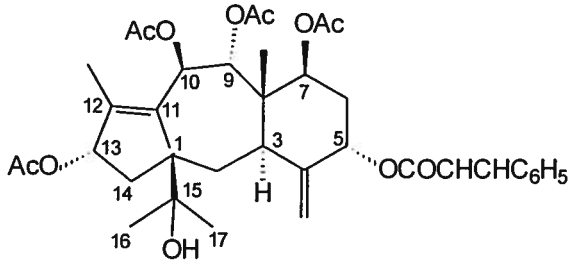


73. R_7 = H 0.9%
74. R_7 = Ac 0.5%

Figure **6.3**. Microbial transformation products of 9-dihydro-13-acetylbaccatin III (**4**) by fungus *Absidia coerulea* Bainier ATCC 10738a and their yields.



	R_1	R_{13}	R_{14}	
50.	H	OAc	H	
75.	OH	OAc	H	6.9%
76.	H	OAc	OH	11.5%
77.	H	OH	H	0.5%



78. 3.3%

Figure **6.4.** Microbial transformation products of taxane **50** by fungus *Absidia coerulea* Bainier ATCC 10738a and their yields.

Compound **52** (5α,7β,9α,10β,13α-pentahydroxy-4(20),11(12)-taxadiene) was converted to a taxane derivative, **79** (32.4%) with a C10-C11 double bond and two unprecedented 1(15→11) abeo-taxanes, **80** (42.3%), and **81** (3.7%) by fungus *Absidia coerulea* ATCC 10738a (**Figure 6.5**).

Unlike the usual 11(15→1)abeo-taxanes, products **80**, and **81** have a unprecedented 1(15→11)abeo-taxanes skeleton. This skeleton has never been found in natural or chemically modified taxanes. The significance of this reaction is that not only it provided some taxanes with a new skeleton, but also it provided the products with the highest yields (**79** and **80**) in the project.

This reaction showed that through chemoenzymatic reactions some unusual products, which were difficult to obtain from a natural source or from chemical modification, could be obtained. Microbial transformation could be an excellent tool that complements to chemical reactions or under certain conditions, is superior to chemical reactions.

Compound **56** was investigated before (Hu et al., 1997b). The fungus and medium used in this project is slightly different. The major products are the same, that is the hydroxylation of C-1, C-14 and abeo-taxane (**82, 84**, and **90**, respectively), the minor products were not found before. There are seven deacetylation products (**83, 85-87, 91-93**) that were not found before.

In addition to the major products (**82, 84**, and **90**) and the deacetylation products (**83, 85-87, 91-93**), there is a minor product, **88**, with an $OCOCH_2COCH_3$ group induced at C-5. This kind of substitution was not reported for natural taxanes. The product, **89**, has a C-16 hydroxylation, which is rare in natural taxanes.

Another two taxanes, **94** and **95**, having a 6-7-6-ring system that was new and was not found in natural taxanes or from the chemically modified taxanes, were also found in this reaction, but yields were very low. The results are summarized in **Figure 6.6.**

Microbial transformation can be a unique way to provide new taxanes. Medium and/or fungus manipulation could improve the yields of the products.

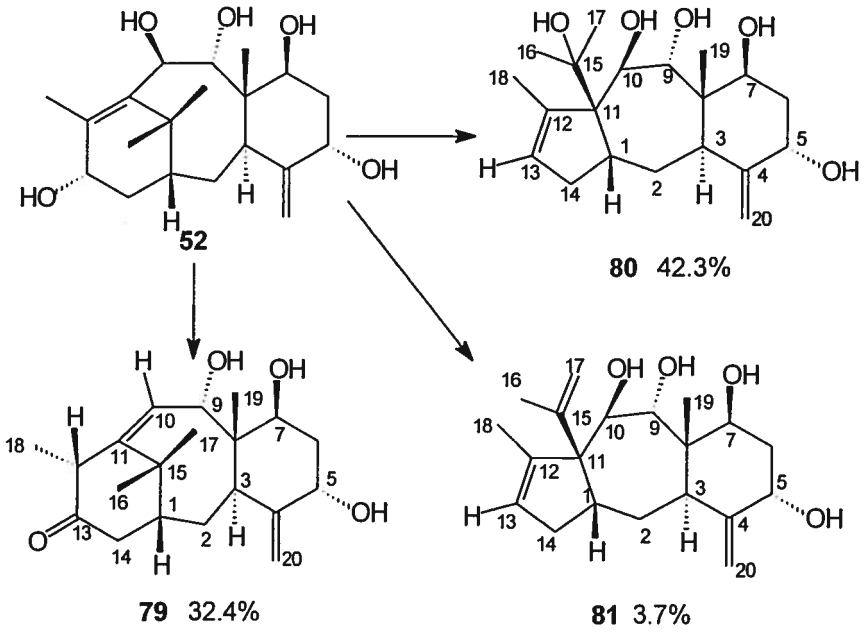
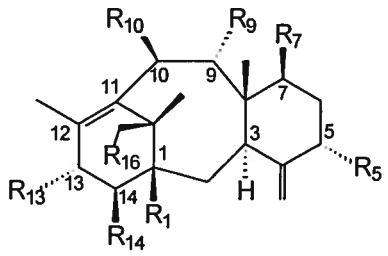
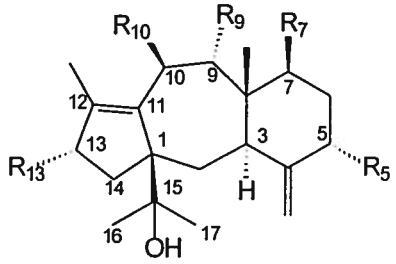


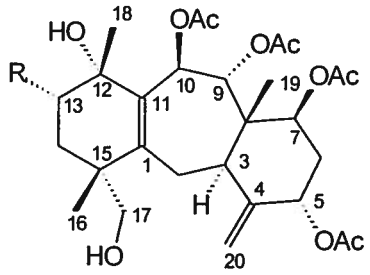
Figure **6.5** Microbial transformation products of taxane **52** by fungus *Absidia coerulea* Bainier ATCC 10738a and their yields.



	R_1	R_5	R_7	R_9	R_{10}	R_{13}	R_{14}	R_{16}	
56.	H	OAc	OAc	OAc	OAc	OAc	H	H	
82.	H	OAc	OAc	OAc	OAc	OAc	OH	H	7.0%
83.	H	OAc	OAc	OAc	OAc	OH	OH	H	0.2%
84.	OH	OAc	OAc	OAc	OAc	OAc	H	H	9.9%
85.	OH	OAc	OAc	OAc	OAc	OH	H	H	0.3%
86.	OH	OH	OAc	OAc	OAc	OAc	H	H	1.4%
87.	H	OAc	OAc	OAc	OAc	OH	H	H	0.2%
88.	H	$OCOCH_2COCH_3$	OAc	OAc	OAc	OAc	H	H	0.8%
89.	H	OAc	OAc	OAc	OAc	OAc	H	OH	0.2%

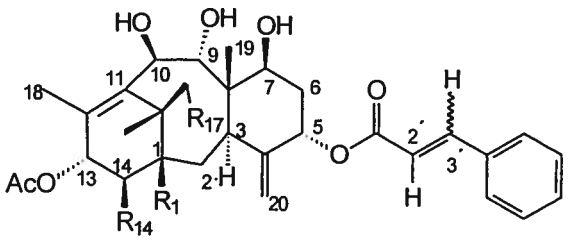


	R_5	R_7	R_9	R_{10}	R_{13}	
90.	OAc	OAc	OAc	OAc	OAc	3.1%
91.	OH	OAc	OAc	OAc	OAc	0.1%
92.	OAc	OAc	OAc	OAc	OH	0.2%
93.	OH	OAc	OH	OAc	OAc	0.3%

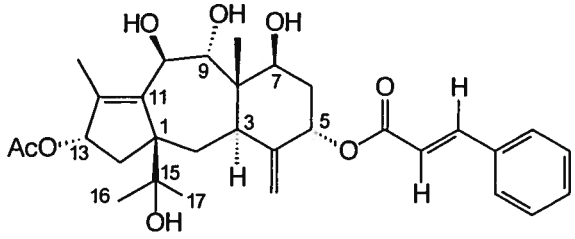


	R	
94.	OAc	0.4%
95.	OH	0.1%

Figure **6.6**. Microbial transformation products of taxane **56** by fungus *Absidia coerulea* Bainier ATCC 10738a and their yields.

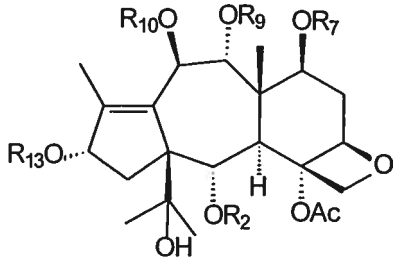


	R_1	R_{14}	R_{17}	$H_{2'-3'}$	A%	B%
57.	H	H	H	*trans*		
96.	OH	H	H	*trans*	43.0	20.2
97.	OH	H	H	*cis*	1.6	–
98.	H	OH	H	*trans*	1.0	–
99.	H	OH	H	*cis*	1.0	–
100.	H	H	OH	*trans*	–	5.4

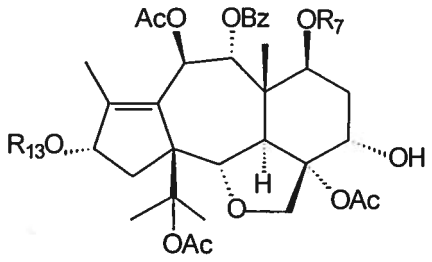


101. A% 16.0 B% 6.8

Figure **6.7.** Microbial transformation products of taxane **57** by fungi *Cunninghamella elegans* AS3.2033(A) and ATCC 20230(B) and their yields.



	R_2	R_7	R_9	R_{10}	R_{13}	
61.	Bz	Ac	Ac	Ac	Ac	
102.	Ac	Ac	Bz	H	Ac	3.7%
103.	Ac	Ac	Bz	Ac	H	1.9%
104.	Ac	H	Bz	Ac	Ac	1.0%



	R_7	R_{13}	
105.	Ac	Ac	3.9%
106.	Ac	H	0.4%
107.	H	Ac	0.4%

Figure 6.8. Microbial transformation products of 10,13-diacetyltaxayuntin E (**61**) by fungus *Cunninghamella echinulata* AS 3.1990 and their yields.

Compound **57** can be metabolized by *Cunninghamella elegans* AS 3.2033 to give five new taxanes. The C-1 hydroxylation product, **96**, has the highest yield of 43.0%, while the C-1 hydroxylation product, **97**, a *cis* isomer at cinnamoyl side chain of **96**, has a yield of 1.6%. The abeo-taxane, **101**, has a yield of 16.0%. A pair of C-14 hydroxylation products **98** and **99**, which were *trans-cis* isomers at the cinnamoyl side-chain, have a yield of 1% each. The *cis* isomers at the cinnamoyl side-chain have not been observed among the natural taxanes when the result was published. Only recently, our group has found one from natural source (Zamir et al., unpublished results).

Compound **57** can also be metabolized by *Cunninghamella elegans var chibaensis* ATCC 20230 to give three taxanes. Besides the C-1 hydroxylation product, **96** (20.2%) and the abeo-taxane, **101** (6.8%), there is a C-17 hydroxylation product, **100** (5.4%) that is very rare in the natural taxanes. Unlike *Cunninghamella elegans* AS 3.2033, *Cunninghamella elegans var chibaensis* ATCC 20230 cannot metabolize compound **57** to give the *cis* isomers at the cinnamoyl side chain. Clearly, the small variation of fungi can lead to a big difference in the microbial transformation. The results are in **Figure 6.7**

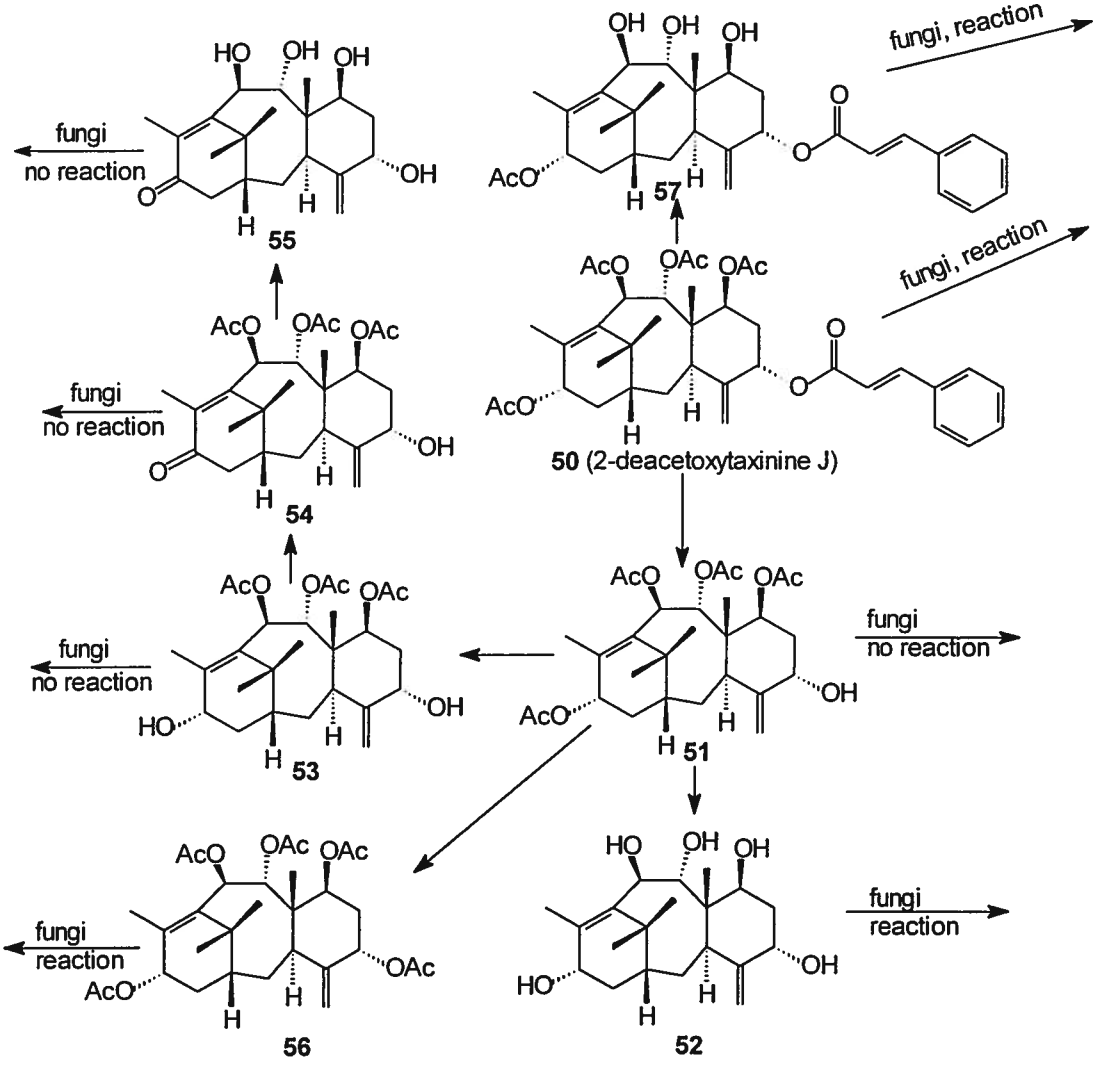
Compound **61** could be metabolized by fungus *Cunninghamella echinulata* AS 3.1990 to give six products **102-107** (**Figure 6.8**). The 2-Bz and 9-Ac groups in the substrate have exchanged their positions in products **102-104.** Products **105-107**, with 2-Bz group also shifted to position 9, have an opened oxetane-ring and a new formed five-member-ring and a –OAc at C-15. The new-formed five-member ring is very rare in natural taxanes. So far only two natural taxanes were found to have this kind of five-member ring (Baloglu et al., 1999). There are no natural abeo-taxanes reported so far with a 15-OAc. This reaction further showed that microbial transformation could provide some taxanes that are not easy to obtain from natural source or from chemical modification.

6.4. Structure-Biotransformation Reactions

6.4.1. 2-OR group can block the hydroxylation of C-1 and C-14.

When we go back to the different compounds shown in **Figure 6.1** and **Figure 6.2**, we find that the structures can be divided into two categories. The first category is the one without a 2-OR (OH, OAc, or OBz) group. This category includes substrate **50** (2-deacetoxytaxinine J) and its derivatives (**51-57**). The second category is the one with a 2-OR (OH, OAc, or OBz) group. This category includes 19 taxanes (**4, 45-49, 58-69**).

One of the very interesting reactions found in this project is the hydroxylation of taxanes at C-1 and C-14. These reactions happened to the substrates in the first category. Although two compounds in the second category (**4** and **61**) could also be metabolized by fungi, none of them was hydroxylated at C-1 or C-14. Because of the U-shape of the taxane skeleton (Wu and Zamir, 2000), the 2-OR (OH, OAc, or OBz) group is very close to position 1 and 14. The rationale is that the 2-OR (OH, OAc, or OBz) can block the enzyme's access to the position 1 or position 14, thus blocking the hydroxylation of those substrates at C-1 and C-14.



Scheme 6.1. 2-Deacetoxytaxinine J and its derivatives: structure and fungi activity

6.4.2. 5-OH has a negative impact on C-1 and C-14 hydroxylation

Although compound **50** and its derivatives are more easily metabolized by the fungi (4/8) than the other substrates (2/19), the structure variation of the substrates can significantly influence the reaction and the products and their yields.

Scheme 6.1 showed the reactivity of compound **50** and its derivatives. Compounds **50, 52, 56**, and **57** can be metabolized by the fungi, while compounds **51, 53, 54,** and **55** cannot.

Compounds **50**, **56**, and **57**, which have 5-OAc or 5-cinnamoyl substitution, could be hydroxylated by fungi at position 1 and 14. Compound **51**, **53, 54,** and **55,** which have 5-OH, could not be metabolized by fungi. Although **52** can be metabolized by fungi, its reaction doesn't involve C-1 or C-14 hydroxylation. It clearly showed that 5-OH has a negative impact on the hydroxylation of taxanes at C-1 or C-14.

6.4.3. Removal of 7,9,10-triacetyl can increase the yield of C-1 hydroxylation while decreasing the yield of C-14 hydroxylation

Compounds **50**, **56**, and **57**, all can be hydroxylated at C-1 or C-14. The yields of the products are different.

For compounds **50**, and **56**, there are not big differences between the yields of C-1 hydroxylation (6.9% for **50**, and 9.9% for **56**) and C-14 hydroxylation (11.5% for **50**, and 7.0% for **56**). For compound **57**, the ratio between C-1 and C-14 hydroxylation is very big (44.6% to 2% for one fungus, 20.2% to 0% for another fungus).

The above facts showed that the removal of 7,9,10-triacetyl could increase the yield of C-1 hydroxylation while decreasing the yield of C-14 hydroxylation. The rationale is that when the 7,9,10-triacetyls were removed, there is more space for the enzyme to access position 1, while the space for the access of position 14

remains the same. This observation would help us select a specific type of taxane substrate for fungal hydroxylation.

6.4.4. 1-OH and 14-OH can block each other

Also, during the project, we did not find any products with C-1 and C-14 hydroxylated at the same time. This showed that 1-OH or 14-OH can block each other during microbial transformation. Indeed, molecular modeling shows that C-1 and C-14 are very close to one another.

6.4.5. 13-Keto is not suit for microbial transformation

Compounds **50** and **55** differ in that compound **50** has a 13-OH, while **55** has a 13-keto group. The results from microbial transformation are totally different. **55** cannot be metabolized by any of the fungi we used, while **50** can be metabolized by fungus *Absidia coerulea* ATCC 10738a to give three taxanes (**Figure 6.5**) with two of them having very good yields. These results clearly demonstrated that 13-keto group can block this microbial reaction. This fact also supports our putative mechanism for the formation of the 1(15→11)abeo-taxanes (chapter 3).

6.5. Conclusion

In an effort to find new taxanes, we have used some fungi to do biotransformations using natural or chemically modified taxanes as substrates. Indeed, we were able to find some interesting compounds and our results are summarized below:

1. In addition to the C-1 or C-14 hydroxylation products, and the abeo-taxanes, we were able to get C-16, C-17 hydroxylation products, which were very rare in natural taxanes.
2. The abeo-taxanes from the substrate with 1-OH were first reported in this thesis.
3. We were able to find a reaction to shift 2-OBz to position 9.
4. We were able to open the oxetane-ring and form a $-C_2$-C_3-C_4-C_{20}-O- five member-ring.
5. 15-OAc substitution in abeo-taxanes was first reported in this thesis.
6. A product with a β-keto-butyrate substitution at position 5 was found. This kind of product has not previously been found in natural taxanes.
7. We were able to obtain *trans-cis* isomers at the cinnamoyl side chain, a similar compound was found only recently in our lab from natural source.
8. Two compounds with a new 6-7-6-ring skeleton were found.
9. Two taxanes with a unprecedented *abeo*-1(15→11) skeleton were isolated in high yields.

Our studies showed that microbial transformation could be an excellent tool to obtain new taxanes.

In this project, from the results we have obtained, we were able to find some substrates structure and fungi activity relationships

1. 2-OR group can block the hydroxylation of C-1 and C-14.
2. 5-OH has a negative impact to C-1 and C-14 hydroxylation
3. Removal of 7,9,10-triacetyl can increase the yield of C-1 hydroxylation while decrease the yield of C-14 hydroxylation

4. 1-OH and 14-OH can block each other
5. 13-Keto is not suit for microbial transformation

6.6. Perspective

Our comprehension of the biotransformation process is still empirical. To understand the whole process, the enzymes from these fungi should be purified.

Our microbial hydroxylation transformation at different sites shows some similarity between enzyme system in yew plant and fungi.

For future work, we should choose less oxygenated taxanes.

One of the problems connected to microbial transformation is the low yield of the products. However, conditions can be selected to increase the product yields.

Chapter 7

Microbial Transformation of Androst-4-ene-3,17-dione by *Absidia coerulea* ATCC 10738a and *Rhizopus oryzae* Went et ATCC 11145.

Microbial Transformation of Androst-4-ene-3,17-dione by *Absidia coerulea* ATCC 10738a and *Rhizopus oryzae* Went et ATCC 11145.

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Résumé

La transformation microbienne de l'androst-4-ène-3,17-dione (**108**) par *Absidia coerulea* ATCC 10738a et *Rhizopus coeryzae* Went et ATCC 11145 a été obtenus. Seize métabolites (**109-124**) ont été trouvés. La biotransformation de l'androst-4-ène-3,17-dione (**108**) par *Absidia coerulea* ATCC 10738a a produit 14 dérivés, **109-112, 114-123**. La biotransformation de l'androst-4-ène-3,17-dione (**108**) par *Rhizopus coeryzae* Went et ATCC 11145 a donné 7 dérivés, **110-113, 122-124**. Ces sept dérivés **109, 111-112, 114-116, 122** ne sont pas nouveaux. Ils ont été obtenu en utilisant d'autres microorganismes. Ces sept composés étaient découverts par la biotransformation de l'androst-4-ène-3,17-dione (**108**) par *Absidia coerulea* ATCC 10738a. Le composé **122** a été aussi trouvé pour la première fois par la transformation microbienne de l'androst-4-ène-3,17-dione (**108**) avec *Rhizopus oryzae* Went et ATCC 11145. Neuf des métabolites, tels ques la 7β-hydroxyandrost-4-ène-3,17-dione (**110**), la 16β-hydroxyandrost-4-ène-3,17-dione (**113**), la 2α,7α-dihydroandrost-4-ène-3,17-dione (**117**), la 6β,12β-dihydroandrost-4-ène-3,17-dione (**118**), la 1β,6β-dihydroandrost-4-ène-3,17-dione(**119**), la 11α,16α-dihydroandrost-4-ène-3,17-dione (**120**), la 14α,16α-dihydroandrost-4-ène-3,17-dione (**121**), la 6β,16β-dihydroandrost-4-ène-3,17-dione (**123**), la 6β,7α-dihydroandrost-4-ène-3,17-dione (**124**) étaient les premiers trouvés dans la transformation microbienne de l'androst-4-ène-3,17-dione. La structure de ces produits a été déterminée par RMN et HRFAB MS.

Abstract

Microbial transformation of androst-4-ene-3,17-dione (**108**) by *Absidia coerulea* ATCC 10738a and *Rhizopus oryzae* Went et ATCC 11145 was investigated. Sixteen metabolites (**109-124**) were found. Microbial transformation of androst-4-ene-3,17-dione (**108**) by *Absidia coerulea* ATCC 10738a gave 14 derivatives, **109-112, 114-123**. Microbial transformation of androst-4-ene-3,17-dione (**108**) by *Rhizopus oryzae* Went et ATCC 11145 gave 7 derivatives, **110-113, 122-124.** Seven derivatives **109, 111-112, 114-116, 122** were found previously in the microbial transformation of androst-4-ene-3,17-dione (**108**) using other microorganisms. These seven compounds were first found in the biotransformation of androst-4-ene-3,17-dione (**108**) by *Absidia coerulea* ATCC 10738a. Compound **122** was also found for the first time by microbial transformation of androst-4-ene-3,17-dione (**108**) using *Rhizopus oryzae* Went et ATCC 11145. Nine of the metabolites 7β-hydroxyandrost-4-ene-3,17-dione (**110**), 16β-hydroxyandrost-4-ene-3,17-dione (**113**), 2α,7α-dihydroandrost-4-ene-3,17-dione (**117**), 6β,12β-dihydroandrost-4-ene-3,17-dione (**118**), 1β,6β-dihydroandrost-4-ene-3,17-dione (**119**), 11α,16α-dihydroandrost-4-ene-3,17-dione (**120**), 14α,16α-dihydroandrost-4-ene-3,17-dione (**121**), 6β,16β–dihydroandrost-4-ene-3,17-dione (**123**), 6β,7α–dihydroandrost-4-ene-3,17-dione (**124**) were first found in the microbial transformation of androst-4-ene-3,17-dione. The structures of these products were elucidated by NMR and HR FABMS data.

Introduction

Microbial transformation of steroids could be traced back to late 1930s, when Italian scientist Mamoli first realized the microbial transformation of dehydroepiandrosterone into testosterone (Mamoli, 1940). But only after 1952, when American scientists Murry and Peterson patented the process of 11α-hydroxylation of progesterone by a *Rhizopus* species (Murray and Peterson, 1952), was the importance of producing steroids hormones by microbial transformation realized.

Since then, numerous papers have been published involving different steroids as substrates and different microorganisms. Some excellent monographs (Capek et al., 1966; Charney and Herzog, 1967; Akhrem and Titov, 1970; Fronken and Johnson, 1972; Iizuka and Naito, 1967; 1981) and reviews (Mahato and Mukherjee, 1984; Mahato and Banerjee. 1985; Mahato et al., 1989; Mahato and Mazumder, 1995; Mahato and Subhadra, 1996; Holland, 1999) have covered the literatures of this area.

Androst-4-ene-3,17-dione (**108**) is sold in health food stores and vitamin shops as a dietary supplement. It is made commercially by direct microbial oxidation of cholesterol or phytosterols (Petrow, 1980; Kutney et al., 2000). It is also an important raw material for the commercial synthesis of testosterone and other new steroids.

The microbial transformation of androst-4-ene-3,17-dione (**108**) has been well documented (Iizuka and Naito, 1967; 1981; Mahato and Mukherjee, 1984; Mahato and Banerjee. 1985; Mahato et al., 1989; Mahato and Mazumder, 1995; Mahato and Subhadra, 1996; Holland, 1999). Since we have used taxanes as substrates for microbial transformation and have found that some of the fungi have a hydroxylating ability (results in chapter 2-6 of this thesis), we think the steroid androst-4-ene-3,17-dione (**108**) may be a good substrate for microbial transformation because of its low oxygenation. Thanks to Dr. Jinqiang Xia for his generous gift of androst-4-ene-3,17-dione (**108**), we were able to screen this compound with our fungi (names of the fungi in chapter 6 of this thesis) and found that *Absidia coerulea* ATCC 10738a and *Rhizopus oryzae* Went et ATCC 11145 were able to transform androst-4-ene-3,17-dione (**108**).

Biotransformation of androst-4-ene-3,17-dione (**108**) by *Absidia coerulea* ATCC 10738a and *Rhizopus oryzae* Went et ATCC 11145 resulted in sixteen metabolites (**109-**

124). Biotransformation of androst-4-ene-3,17-dione (**108**) by *Absidia coerulea* ATCC 10738a gave 14 derivatives, **109-112, 118-123**. Biotransformation of androst-4-ene-3,17-dione (**108**) by *Rhizopus oryzae* Went et ATCC 11145 gave 7 derivatives, **110-113, 122-124.**

Seven derivatives: 7α-hydroxyandro-4-ene-3,17-dione (**109**) (Madyasha, 1994; Fujiwara et al., 1982; Hu et al., 1995), 11α-hydroxyandro-4-ene-3,17-dione(**111**) (Yoshihama, 1993), 6β-hydroxyandro-4-ene-3,17-dione(**112**) (Abul-Hajj and Qian, 1986),. 14α-hydroxyandro-4-ene-3,17-dione(**114**) (Yoshioka and Asada, 1994; Asada, 1994; Nakakoshi et al., 1993; Weber and Kewnnecke, 1993), 6β-hydroxytestosterone(**115**) (Hu et al., 1995), 7α-hydroxytestosterone(**116**) (Fujiwara et al., 1982), and 6β, 11α-dihydroxyandrost-4-ene-3,17-dione (**122**) (Yoshihama, 1993) were found previously in the microbial transformation of androst-4-ene-3,17-dione (**108**). These seven compounds were first found in the biotransformation of androst-4-ene-3,17-dione (**108**) by *Absidia coerulea* ATCC 10738a. 6β, 11α-dihydroxyandrost-4-ene-3,17-dione (**122**) (Banerjee et al., 1993; Yoshihama, 1993) was also found for the first time from microbial transformation of androst-4-ene-3,17-dione using *Rhizopus oryzae* Went et ATCC 11145.

Nine of the metabolites 7β-hydroxyandrost-4-ene-3,17-dione (**110**), 16β-hydroxyandrost-4-ene-3,17-dione (**113**), 2α,7α-dihydroandrost-4-ene-3,17-dione (**117**), 6β,12β-dihydroandrost-4-ene-3,17-dione (**118**), 1β,6β-dihydroandrost-4-ene-3,17-dione (**119**), 11α,16α-dihydroandrost-4-ene-3,17-dione (**120**), 14α,16α-dihydroandrost-4-ene-3,17-dione (**121**), 6β,16β–dihydroandrost-4-ene-3,17-dione (**123**), and 6β,7α–dihydroandrost-4-ene-3,17-dione (**124**) were first found in the microbial transformation of androst-4-ene-3,17-dione. Two of these nine products have single hydroxylation. Seven of these nine products have double hydroxylation.

Results and Discussion

All of the following discussions concerning the stereochemistry are referred to **Figure 7.2**. Stereochemistry of androst-4-ene-3,17-dione and its derivatives.

Compound **110**. This compound has a single hydroxylation as can be seen from the ^{1}H (3.58 ppm, dt, *10.6, 10.6, 5.3* Hz) and ^{13}C (74.3 ppm) NMR data (**Table 7.2**). As that of **109**, this compound has –OH at position 7 as can be deduced from COSY and HMBC. The big difference between these two compounds in ^{1}H NMR is H-7. The H-7 of **109** (4.07 ppm) is a broad singlet, which means H-7 has small coupling constants with H-6a and H-6e, and H-8a, thus H-7 must be equatorial (**Figure 7.2**). The H-7 (3.58 ppm, dt, 10.6, 10.6, 5.3 Hz) of **110** appeared as a double triplet ($J_{7a.6a}=_{7a.8a}=10.4$ Hz and $J_{7a.6e}=$ 5.3 Hz), which indicated that H-7 is axial (**Figure 7.2**). The difference of the ^{13}C NMR data between these two compounds is mainly in C-6, C-7, C-8, and C-9 (**Table 7.1** and **Table 7.2**). Thus, the structure of **110** was determined as 7β-hydroxyandrost-4-ene-3,17-dione. This compound has not been found in the microbial transformation of androst-4-ene-3,17-dione before.

Compound **113**. From the ^{1}H (3.96 ppm, t, 8.6 Hz) and ^{13}C (74.2 ppm) NMR data (**Table 7.5**), this compound has only one hydroxy group. Combined with COSY and HMBC data (**Table 7.5**), the –OH group is at position 16. Since H-16 has strong NOE with the axial H-14, H-16 must be down (α) (**Figure 7.2**). Thus, the structure of **113** is 16β-hydroxyandrost-4-ene-3,17-dione. This compound is one of the major metabolites from microbial transformation of androst-4-ene-3,17-dione using *Rhizopus oryzae* Went et ATCC 11145. It has not been found in the microbial transformation of androst-4-ene-3,17-dione before.

Compound **117**. From the ^{1}H (4.22 ppm, dd, *13.7, 5.5* Hz, and 4.17 ppm, br.q, *~2.9* Hz) and ^{13}C (68.4 and 68.7 ppm) NMR data (**Table 7.9**), this compound has two hydroxy groups. From COSY and HMBC data, these two –OH groups were attached to position 2 and position 7, respectively. Since the coupling constants of H-2 (4.22 ppm, dd) with H-1a and H-1e is very different (*13.7,* and *5.5* Hz, respectively), it must be axial (β), thus 2-OH must be equatorial (α). Since the coupling constants of H-7 (4.17 ppm, br.q) with H-6a, H-6e, and H-8a is very small (*~2.9* Hz), it must be equatorial (β). The 7-OH must be axial (α) (**Figure 7.2**). The NOE between H-7e and the two protons at

position 6 also supports the above orientation. Thus, **117** was elucidated as 2α,7α-dihydroandrost-4-ene-3,17-dione.

Compound **118**. From the ^{1}H (3.80 ppm, dd, *11.1, 4.6* Hz, and 4.40 ppm, t, *2.8* Hz) and ^{13}C (72.4 and 72.8 ppm) NMR data (**Table 7.10**), this compound has two hydroxy groups. That the two –OH groups were put on position 6 and position 12 was based on the COSY and HMBC data (**Table 7.10**). Because H-6 (4.40 ppm) has a relatively small coupling constant of 2.8 Hz with H-7a and H-7e in the six-member ring, it must be equatorial (α), which means 6-OH must be axial (β). In the mean time, H-12 (3.80 ppm), which is also in the six-member ring, appears to be a double doublet and has two different coupling constants with the H-11a and H-11e (11.1 and 4.6 Hz, respectively.). This indicated that H-12 is axial (α), while 12-OH must be equatorial (β) (**Figure 7.2**). Thus, Compound **118** was elucidated as 6β,12β-dihydroandrost-4-ene-3,17-dione.

Compound **119**. This compound also has two hydroxy groups as can be seen from the ^{1}H (4.10 ppm, dd, *11.2, 5.0* Hz, and 4.47 ppm, t, *2.8* Hz) and ^{13}C (75.0 and 73.6 ppm) NMR data (**Table 7.11**). That the double doublet was attributed to H-1 was deduced from the COSY and HMBC data (**Table 7.11**). Since H-1 has different coupling constants with H-2a and H-2e (11.2 and 5.0 Hz, respectively) in a six-member ring, H-1 must be axial (α), thus 1-OH must be equatorial (β). Also from COSY and HMBC data, the other –OH was at position 6. Since H-6 has the same coupling constants with H-7a and H-7e, it must be equatorial (α), thus 6-OH must be axial (β) (**Figure 7.2**). Based on the above discussion, compound **119** was elucidated as 1β,6β-dihydroandrost-4-ene-3,17-dione.

Compound **120**. From the ^{1}H (4.05 ppm, br.td, *10.3, 10.3, 5.5* Hz, and 4.41 ppm, o.d, *~7.2* Hz) and ^{13}C (68.5 and 71.0 ppm) NMR data (**Table 7.12**), this compound has two hydroxy groups. From COSY and HMBC data, these two –OH groups were attached to position 11 and position 16 (**Table 7.12**). Since H-11 is in a six member ring, it is easy to deduce that it is axial (β), since it has relatively bigger J value at 10.3, 10.3 and 5.5 Hz,

which are the coupling constants with H-9a, H-12a and H-12e respectively (**Figure 7.2**). The above assignment was also support by the fact that H-11 has NOE with H-12e, H-18, and H-19. For H-16, it is at a five-member ring, which is not as stable as a six-member ring. The orientation of H-16 cannot be deducted from its coupling constants with other protons. It was put at UP (β) position because H-16 has NOE with 18-CH_3 (axial, β)(**Figure 7.2**). 16-OH thus must be down (α). Thus, compound **120** was elucidated as 11α,16α-dihydroandrost-4-ene-3,17-dione.

Compound **121**. That this compound has two hydroxy groups was deduced from ^{13}C (78.8 and 72.7 ppm) NMR data (**Table 7.13**). From ^{1}H NMR (**Table 7.13**), only one proton (4.24 ppm, t, 7.4 Hz) that was connected to a hydroxylated carbon can be found. This suggested that one of the –OH groups was attached to a quaternary carbon. From COSY and HMBC data, the two –OH groups were attached to C-14 and C-16 (**Table 7.13**). We put 14-OH at DOWN position (α), since the starting material has an H-14α. There is no convincing NOE to determine the orientation of H-16. However, the ^{1}H and ^{13}C NMR data of **121** at position 16 (H-16, 4.26 ppm, t, 7.4 Hz, C-16, 72.7 ppm) was more similar to that of **120** (H-16β, 4.41 ppm, o.d, 7.2 Hz, C-16, 71.0 ppm) than that of **113** (H-16α, 3.96 ppm, t, 8.6 Hz, C-16, 74.2 ppm) and **123** (H-16α, 3.96 ppm, t, 8.5 Hz, C-16, 74.9 ppm). We put H-16 in a up (β) configuration, thus 16-OH of **121** at down (α) orientation (**Figure 7.2**). Thus compound **121** was tentatively elucidated as 14α,16α-dihydroandrost-4-ene-3,17-dione.

Compound **123**. From the ^{1}H (4.40 ppm, t, *2.5* Hz, and 3.96 ppm, t, *8.5* Hz) and ^{13}C (72.5 and 74.9 ppm) NMR data (**Table 7.15**), this compound has two hydroxy groups. From COSY and HMBC data, these two –OH groups were attached to position 6 and position 16. That the 6-OH was axial (β) while H-6 was equatorial (α) was deduced from the *J* value of H-6 (2.5 Hz) and NOE between H-6 and H-7a and H-7e. The down (α) orientation of H-16 is deducted from the fact that H-16 has medium NOE with H-14 that is also down (α). Thus compound **123** was elucidated as 6β,16β–dihydroandrost-4-ene-3,17-dione.

Compound **124**. From the ^{1}H (4.20 ppm, d, *2.7* Hz, and 4.00 ppm, t, *2.7* Hz) and ^{13}C (77.1 and 70.1 ppm) NMR data (**Table 7.16**), this compound has two –OH groups. From COSY and HMBC data (**Table 7.16**), these two –OH groups were attached to position 6 and position 7. Since H-7 is a triplet and has a J value at 2.7 Hz ($J_{H7e.8a}=_{7e.6e}=2.7$ Hz) it must be equatorial (β). Thus 7-OH must be axial (α). In the mean time, H-6 showed to be a doublet that had a coupling constant at 2.7 Hz. It must be equatorial (α), thus 6-OH must be axial (β). Based on the above discussion, compound **124** was elucidated as 6β,7α–dihydroandrost-4-ene-3,17-dione.

Conclusion

Although a lot of articles and patents, which involved the use of a large number of microorganisms, were published on the biotransformation of androst-4-ene-3,17-dione, *Absidia coerulea* ATCC 10738a had not been used previously. This fungus indeed transformed androst-4-ene-3,17-dione into 14 metabolites. Seven of them were not reported before by biotransformation. *Rhizopus oryzae* Went et ATCC 11145 transformed androst-4-ene-3,17-dione into seven compounds. Two of them are new.

Experimental

Instrumentation.

Flash chromatography was performed on Silica gel 60 (230-400 mesh, EM Science). Thin layer chromatography was conducted on Silica Gel 60 F254 pre-coated TLC plates (0.25 mm, EM Science). The compounds were visualized on TLC plates with 10% sulfuric acid in ethanol and heating on a hot plate. Na_2SO_4 was the drying agent used in all work up procedures. Analytical HPLC was performed on a Waters 600 FHU delivery system coupled to a PDA 996 detector. Preparative and semi-preparative HPLC were carried out on a Waters Delta Prep 3000 instrument coupled to a UV 486 Tunable Absorbance detector set at 227 nm (Waters, Montreal, Quebec, Canada). Analytical HPLC was performed with two Whatman partisil 10 ODS-2 analytical columns (4.6 x 250 mm) in series. Semi-preparative HPLC was performed with two Whatman partisil 10 ODS-2 Mag-9 semi-preparative columns (9.4 x 250 mm) in series. Preparative HPLC was performed with one partisil 10 ODS-2 MAG-20 preparative column (22 x 500 mm). The products were eluted with a 50 min linear gradient of acetonitrile (25 to 100 %) in water at a flow rate of 18 mL/min (preparative HPLC) and 3mL/min (semi-preparative HPLC). All the reagents and solvents were of the best available commercial quality and were used without further purification.

NMR and Mass Spectrometry Measurement.

All the NMR data were obtained at room temperature on a Bruker Avance-500 spectrometer operating at 500.13 MHz for proton and at 125.77 MHz for carbon-13. The solvent was used as an internal reference (in $CDCl_3$, 7.25 ppm for proton and 77.0 ppm for carbon-13; in Acetone-d6, 2.06 ppm for proton and 206.7 ppm for carbon). The various 2D spectra were acquired and processed using standard procedures. For phase sensitive 2D experiments (NOESY and HMQC), the data were acquired using the TPPI phase mode. The NOESY experiment was obtained using a mixing time of 0.3 s and a relaxation delay of 1 s. The intensity of the cross-peaks in the NOESY experiment is designated as strong (s), medium (m) and weak (w). Positive ion Fast Atom

Bombardment Mass Spectra (FAB-MS) were obtained with a Vacuum Generators ZAB-HS double-focussing instrument using a xenon beam having 8 kV energy at 1 mA equivalent neutral current. Low resolution mass spectra were obtained in glycerol. Samples were dissolved in 0.2 μl DMSO before addition of 0.5 μl glycerol. FAB HRMS was similarly obtained in glycerol-DMSO at a resolving power of 12,000.

Substrates

The androst-4-ene-3,17-dione was a generous gift from Dr. Jinqiang Xia, Ace Chemicals and Pharmaceuticals, Inc.

Microorganism:

Absidia coerulea ATCC 10738a and *Rhizopus oryzae* Went et ATCC 11145 were purchased from American Type Culture Collection (ATCC).

Incubation, Biotransformation Procedure

General: Cultures were grown in Potato Dextrose broth (24g/L, DIFCO laboratories), and was incubated at 25°C and 125 rpm, unless otherwise indicated. The *Absidia coerulea ATCC 10738a* was first preserved on silica gel and kept at 4°C cold room to prevent mutation (Perkins, 1962). The seed culture was prepared by the addition of several grains of silica gel that absorbed the fungus to 30 mL of medium in a 125 mL Erlermeyer flask. The culture was incubated for 3 days. At preparative scale, to a 2000 ml flask, containing 1000 ml Potato Dextrose broth, was added a small fraction of the above fungus seed (homogenized before use). The flasks were at first cultured for 2 days. Then to the flasks was added substrate (dissolved in acetone). Continued culturing for another 12 days. At the end of incubation, the culture was homogenized and was extract with CH_2Cl_2. And finally, the CH_2Cl_2 extract was obtained for further purification. Culture controls with the same medium and substrate but without fungi were performed at

the same condition. The exact procedure was used for *Rhizopus oryzae* Went et ATCC 11145.

For *Absidia coerulea* ATCC 10738a, to each of the two 2L flasks containing 1L of medium and the fungus seeds, was added the substrate androst-4-ene-3,17-dione (100 mg and 400 mg, dissolved in 1 mL and 4 ml acetone, respectively). After 8 days incubation, the culture was extracted with CH_2Cl_2 (500 mL×3 for 1L culture). The CH_2Cl_2 crude residue was 110 mg and 430 mg. The extracts were checked with TLC and HPLC and found to be almost the same and thus were combined. To the combined extract were added CH_3CN/MeOH, and 102 mg of needles were obtained (**109**). The remained mother liquor was then applied to a silica gel flash chromatography column (8 g silica gel, 230-400 mesh), eluted with Hexanes (200 mL), Hexanes/EtOAc (10:1, 5:1, 3:1; 3:2, 1:1, each 110 mL, 2:3, 300 mL, 1:4, 100 mL) and forty-seven 25 mL fractions were obtained. The fractions 8-20, 21-25, 26-28, 29-30, 31-33, 34-41, 42-47, containing relevant products, were combined based on the TLC. Each of the above fractions was separated by preparative HPLC or PTLC. The following compounds were obtained: **109** (50.9 mg, plus 102 mg from crystallization, altogether 152.9 mg, 29.0%), **110** (13.2 mg, 2.5%), **111** (74.4 mg, 14.1 %), **112** (53.9 mg, 10.2%), **114** (12.7 mg, 2.4%), **115** (31.9 mg, 6%), **116** (4.8 mg, 0.9%), **117** (1.7 mg, 0.3%), **118** (1.7 mg, 0.3%), **119** (1.7 mg, 0.3%), **120** (3.3 mg, 0.6%), **121** (0.5 mg, 0.1%), **122** (13.9 mg, 2.5%), **123** (8.9 mg, 1.6%), No Substrate was recovered.

For *Rhizopus oryzae* Went et ATCC 11145, to the two 2L flasks containing 1L of medium and the fungus seeds, was added the substrate androst-4-ene-3,17-dione (400 mg, dissolved in 4 ml acetone). After 8 days incubation, the culture was extracted with CH_2Cl_2 (500 mL×3 for 1L culture). The CH_2Cl_2 crude residue was 366 mg.

The extract was first applied to a silica gel flash chromatography column (4 g silica gel, 230-400 mesh), eluted with CH_2Cl_2/EtOAc (20:1, 10:1; 22 mL each; 5:1, 2:1, 35 mL each), and Acetone (10 mL), and twenty 6 mL fractions were obtained. The fractions 3, 4-5, 6-8, 9-19, 20, containing relevant products, were combined based on the TLC. Each of the above fractions was separated by preparative HPLC or PTLC, and the

following compounds were obtained: **110** (3.4 mg, 0.8%), **111** (22.1 mg, 5.2%), **112** (53.8 mg, 12.7%), **113** (51.4 mg, 12.2%), **122** (1 mg, 0.2%), **123+124** (2:1, 3.1 mg, 0.7%). 99 mg (25%) of the substrate, androst-4-ene-3,17-dione, was recovered.

Compounds Data

Compound **109**. Colorless needles. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.1**). FAB HRMS for $C_{19}H_{26}O_3$+H^+[M+H^+] required 303.1961; found 303.1961.

Compound **110**. White powder. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.2**). FAB HRMS for $C_{19}H_{26}O_3$+H^+[M+H^+] required 303.1961; found 303.1961.

Compound **111**. White powder. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.3**). FAB HRMS for $C_{19}H_{26}O_3$+H^+[M+H^+] required 303.1961; found 303.1961.

Compound **112**. White powder. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.4**). FAB HRMS for $C_{19}H_{26}O_3$+H^+[M+H^+] required 303.1961; found 303.1961.

Compound **113**. White powder. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.5**). FAB HRMS for $C_{19}H_{26}O_3$+H^+[M+H^+] required 303.1961; found 303.1961.

Compound **114**. White powder. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.6**). FAB HRMS for $C_{19}H_{26}O_3$+H^+[M+H^+] required 303.1961; found 303.1961.

Compound **115**. White powder. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.7**). FAB HRMS for $C_{19}H_{28}O_3$+H^+[M+H^+] required 305.2117; found 305.2116.

Compound **116**. White powder. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.8**). FAB HRMS for $C_{19}H_{28}O_3$+H^+[M+H^+] required 305.2117; found 305.2116.

Compound **117**. White powder. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.9**.). FAB HRMS for $C_{19}H_{26}O_4$+H^+[M+H^+] required 319.1909; found 319.1911.

Compound **118**. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.10**). $C_{19}H_{26}O_4$+H^+[M+H^+] required 319.1909; found 319.1911.

Compound **119**. White powder. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.11**). $C_{19}H_{26}O_4$+H^+[M+H^+] required 319.1909; found 319.1911.

Compound **120**. White powder. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.12**). $C_{19}H_{26}O_4$+H^+[M+H^+] required 319.1909; found 319.1911.

Compound **121**. White powder. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.13**). $C_{19}H_{26}O_4$+H^+[M+H^+] required 319.1909; found 319.1911.

Compound **122**. White powder. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.14**). $C_{19}H_{26}O_4$+H^+[M+H^+] required 319.1909; found 319.1911.

Compound **123**. White powder. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.15**). $C_{19}H_{26}O_4+H^+[M+H^+]$ required 319.1909; found 319.1911.

Compound **124**. White powder. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}CNMR ($CDCl_3$, 125 MHz) (See **table 7.16**). $C_{19}H_{26}O_4+H^+[M+H^+]$ required 319.1909; found 319.1911.

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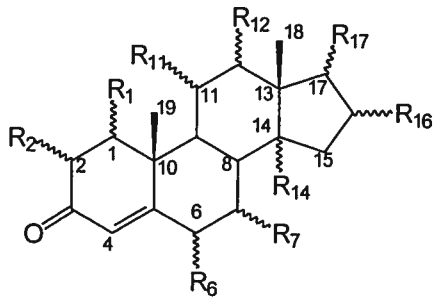

	R_1	R_2	R_6	R_7	R_{11}	R_{12}	R_{14}	R_{16}	R_{17}	(%, A)	(%, B)
108.	H	H	H	H	H	H	H	H	=O	—	—
109.	H	H	H	α-OH	H	H	H	H	=O	29.0	—
110.	H	H	H	β-OH	H	H	H	H	=O	2.5	0.8
111.	H	H	H	H	α-OH	H	H	H	=O	14.1	5.2
112.	H	H	β-OH	H	H	H	H	H	=O	10.2	12.7
113.	H	H	H	H	H	H	H	β-OH	=O	—	12.2
114.	H	H	H	H	H	H	α-OH	H	=O	2.4	—
115.	H	H	β-OH	H	H	H	H	H	β-OH	6.0	—
116.	H	H	H	α-OH	H	H	H	H	β-OH	0.9	—
117.	H	α-OH	H	α-OH	H	H	H	H	=O	0.3	—
118.	H	H	β-OH	H	H	β-OH	H	H	=O	0.3	—
119.	β-OH	H	β-OH	H	H	H	H	H	=O	0.2	—
120.	H	H	H	H	α-OH	H	H	α-OH	=O	0.6	—
121.	H	H	H	H	H	H	α–OH	α-OH	=O	0.1	—
122.	H	H	β-OH	H	α-OH	H	H	H	=O	2.5	0.2
123.	H	H	β-OH	H	H	H	H	β-OH	=O	1.6	0.5
124.	H	H	β-OH	α-OH	H	H	H	H	=O	—	0.3

Figure 7.1. Microbial transformation products of andro-4-ene-3,17-dione (**108**) by *Absidia coerula* ATCC 10738a (A) and *Rhizopus oryzae* Went et ATCC 11145 (B) and their yields.

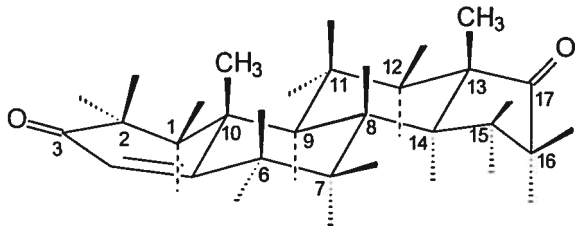

Figure 7.2. Stereochemistry of androst-4-ene-3,17-dione (**108**) and its derivatives

Table 7.1. ^{1}H NMR and ^{13}C NMR data for compound **109** in $CDCl_3$

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC
1e	2.05 o.m	35.4	2, 5, 10, 19
1a	1.78 o.m		3
2	2.48-2.35 o.m	33.9	1, 3, 4, 10
3	--	198.7	
4	5.80 s	127.2	2, 6, 10, (19)
5	--	166.8	
6	2.67 dt (*15.0, 2.3, 2.3*)	41.0	4, 5, 7
6	2.44 o.m		
7e	4.09 br.s	67.1	9/14
8a	1.77 o.m	39.3	
9a	1.55 o.m	45.4	1, 8, 11, 14, 19
10	--	38.5	
11e	1.73 o.m	20.1	
11a	1.46 qd (*12.7, 4.6*)		9, 10, 12, 13
12e	1.83 dt (*12.8, 3.0, 3.0*)	31.0	11, 13, 17, 18
12a	1.27 dt (*13.0, 13.0, 4.2*)		
13	--	47.3	
14a	1.72 o.m	45.6	
15e	2.08 o.m	21.2	
15a	1.58 o.m		
16'	2.47 o.m	35.7	14, 15, 17
16"	2.13 o.m		
17	--	220.2	
18	0.90 s	13.5	12, 13, 14, 17
19	1.21 s	17.0	1, 5, 9, 10

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b]The ^{13}C chemical shifts were from real ^{13}C NMR. *Letters a/e mean axial/equatorial

Table 7.2. ^{1}H NMR and ^{13}C NMR data for compound **110** in $CDCl_3$

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC
1e	2.05 o.dt	35.6	2, 5, 10, 19
1a	1.66 td (*14.2, 14.2, 5.1*)		2, 3, 9, 10, 19
2	2.39 o.m	33.9	1, 3, 10
3	--	199.0	
4	5.77 br.s	125.1	
5	--	166.3	
6e	2.57 dd (*13.9, 5.2*)	42.6	4, 5, 7, 8, 10
6a	2.48 o.m		
7a	3.58 dt (*10.6, 10.6, 5.3*)	74.3	
8a	1.76 o.q (*10.6*)	42.7	6, 7, 9/14
9	1.00 br.td (*11.7, 11.7, 4.1*)	50.8	1, 7, 6/8, 10, 11, 19
10	--	38.0	
11e	1.74 o.m	20.4	
11a	1.48 o.m		
12e	1.87 dt (*13.0, 3.0, 3.0*)	31.2	
12a	1.25 o.td (*13.4, 13.4, 4.1*)		11, 13, 17, 18
13	--	48.0	
14	1.47 o.m	50.5	
15'	2.31 o.m	25.0	14, 17
15"	1.93 o.m		8, 14, 16
16'	2.47 o.m	35.9	17
16"	2.10 o.m		17
17	--	220.4	
18	0.94 s	13.9	12, 13, 14, 17
19	1.23 s	17.4	1, 5, 9, 10
OH	1.57 br		

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b]The ^{13}C chemical shifts were from real ^{13}C NMR except for C-5 and C-17, which were extracted from HMBC. *Letters a/e mean axial/equatorial

Table 7.3. ^{1}H NMR and ^{13}C NMR data for compound **111** in $CDCl_3$

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC
1e	2.66 dt (*14.0, 4.6, 4.6*)	37.4	2, 3, 5, 9, 10, 19
1a	2.02 o.td (*13.9, 13.9, 4.5*)		2, 3, 5, 9, 10, 19
2a	2.43 o.m	34.1	1, 3, 4, 5, 10
2e	2.33 o.m		1, 3, 4, 5, 10
3	--	199.9	
4	5.75 s	124.8	10
5	--	170.0	
6	2.43-2.33 m	33.3	
7	1.97 o.m	30.3	
	1.15 o.m		
8	1.71 qt (*10.8, 3.6*)	34.6	6, 7, 9, 13, 14
9	1.16 o.m	59.2	
10	--	40.0	
11a	4.06 td (*10.6, 10.6, 5.0*)	68.7	9, 10, 12
12e	2.15 o.m	43.0	9, 11, 13, 14, 18
12a	1.32 o.t (*11.7*)		9, 11, 13, 18
13	--	48.0	
14	1.40 m	50.1	
15'	1.98 o.m	21.7	
15"	1.55 m		14
16'	2.49 dd (*19.5, 8.7*)	35.7	14, 17
16"	2.14 o.m		
17	--	218.4	
18	0.94 s	14.7	12, 13, 14, 17
19	1.33 s	18.4	1, 5, 9, 10

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b] The ^{13}C chemical shifts were from real ^{13}C NMR. *Letters a/e mean axial/equatorial

Table 7.4. ^{1}H NMR and ^{13}C NMR data for compound **112** in $CDCl_3$

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC
1e	2.05 o.m	(37.1)	
1a	1.72 o.m		2, 9, 10, 19
2a	2.52 o.m	34.2	1, 3
2e	2.39 dt (*17.3 3.1*)		1, 3
3	--	200.1	
4	5.83 s	126.6	2, 6, 10, 19
5	--	167.6	
6e	4.40 t (*2.7*)	72.7	
7e	2.13 o.m	(37.1)	
7a	1.32 o.m		
8a	2.17 o.m	29.4	7, 9, 14
9a	0.97 o.m	53.6	
10	--	38.0	
11e	1.68 o.m	20.3	
11a	1.51 qd (*13.5, 4.3*)		9, 10, 12, 13
12e	1.87 dt (*13.3, ~3.4*)	31.3	11, 13, 14, 18
12a	1.28 o.m		
13	--	47.6	
14a	1.29 o.m	50.9	
15'	1.99 m	21.7	13, 14, 16, 17
15"	1.62 o.m		17
16'	2.48 o.m	35.8	14, 15, 17
16"	2.10 o.m		
17	--	220.4	
18	0.94 s	13.8	12, 13, 14, 17
19	1.40 s	19.6	1, 5, 9, 10
OH	1.80 br.s		

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b] The ^{13}C chemical shifts were from real C-13. C-1 (37.18 ppm) and C-7 (37.08 ppm) cannot be assigned unambiguously. *Letters a/e mean axial/equatorial

Table 7.5. ^{1}H NMR and ^{13}C NMR data for compound **113** in $CDCl_3$.

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC	[c]NOESY
1e	2.05 o.m	35.6	2, 10, 19	
1a	1.71 o.m		2, 3, 10, 19	
2	2.35-2.42 o.m	33.9	1, 3, 4	
3	--	199.3		
4	5.75 s	124.2	2, 6, 10	
5	--	169.9		
6	2.43-2.39 o.m	32.5		
7e	1.98 o.m	31.0		
7a	1.14 o.m			
8a	1.78 o.m	34.2		
9a	1.03 o.m	54.0		
10	--	38.6		
11e	1.70 o.m	20.1		
11a	1.50 o.m		9	
12e	1.92 o.m	31.5		
12a	1.34 o.m		13	
13	--	46.6		
14a	1.26 o.m	45.2		
15'	2.41 o.m	30.4	13, 14, 16	
15"	1.55 o.m		14	
16	3.96 t (*8.6*)	74.2	15, 17	$14a^{s}$, $15'^{s}$, $15''^{s}$
17	--	219.5		
18	0.99 s	14.8	12, 13, 14, 17	
19	1.21 s	17.3	1, 5, 9, 10	

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b]The ^{13}C chemical shifts of quaternary carbons were extracted from HMBC experiments (±0.2 ppm).
[c]NOESY intensities are marked as strong (s), medium (m) or weak (w). *Letters a/e mean axial/equatorial

Table 7.6. ^{1}H NMR and ^{13}C NMR data for compound **114** in $CDCl_3$

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC
1e	2.05 m	35.7	2, 10, 19
1a	1.77 o.m		
2	2.39 o.m	33.8	
3	--	199.3	
4	5.75 s	124.1	
5	--	169.4	
6	2.39 o.m	32.9	
7'	1.84 o.m	25.6	
7"	1.40 o.m		
8a	1.93 o.m	37.9	
9a	1.52 o.m	46.9	10, 19
10	--	38.5	
11'	1.65 o.m	19.1	
11"	1.42 o.m		
12a	1.80 o.m	24.5	
12e	1.60 o.m		
13	--	52.5	
14	--	80.8	
15	1.94 o.m	30.2	
16'	2.44 o.m	32.3	
16"	2.37 o.m		
17	--	218.0	
18	1.04 s	17.9	12, 13, 14, 17
19	1.22 s	17.4	1, 5, 9, 10

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b] The ^{13}C chemical shifts were extracted from the HSQC and HMBC (for quaternary carbons) experiments (±0.2 ppm). *Letters a/e mean axial/equatorial

Table 7.7. ^{1}H NMR and ^{13}C NMR data for compound **115** in $CDCl_3$

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC	[c]NOESY
1e	2.04 o.m	37.1		
1a	1.70 o.td (*14.3, ~14.3, 4.2*)		2, 9, 10, 19	
2a	2.52 ddd (*17.3, 15.2, 5.1*)	34.2	3, 10	$1e^{m}$, $2e^{s}$, 19^{m}
2e	2.38 dt (*17.2, 3.2*)		3, 10	$1a^{m}$, $1e^{m}$, $2a^{s}$
3	--	200.3		
4	5.81 s	126.1	2, 6, 10, 19	6^{s}
5	--	168.2		
6e	4.34 t (*2.6*)	72.9	8, 7/10	4^{s}, $7e^{s}$, $7a^{s}$
7e	2.00 o.m	38.0		
7a	1.22 o.m		8	6^{s}, $7e^{s}$
8a	2.00 o.m	29.8		
9a	0.91 o.m	53.7	8, 10, 11, 14	
10	--	38.2		
11e	1.60 o.m	20.5		
11a	1.48 o.m		9, 12	
12e	1.87 dt (*12.7, 3.1, 3.1*)	36.4	11, 13	$11e^{m}$, $11a^{m}$, $12a^{s}$
12a	1.09 td (*12.8, 12.8, 4.1*)		11, 13, 17, 18	$12e^{s}$, 17^{s}
13	--	43.0		
14a	0.97 o.m	50.5	8, 13, 18	
15'	1.63 o.m	23.2		
15"	1.36 o.m			
16'	2.09 o.m	30.5	13, 17	
16"	1.46 o.m			
17	3.65 t (*8.5*)	81.5	12, 13, 18	$12a^{s}$, $14a^{s}$, $16'^{w}$, $16"^{m}$
18	0.81 s	11.0	12, 13, 14, 17	8^{s}, $12e^{m}$, $16"^{s}$
19	1.38 s	19.4	1, 5, 9, 10	$1e^{m}$, $2a^{s}$, 8^{s}

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b] The ^{13}C chemical shifts were extracted from the HSQC and HMBC (for quaternary carbons) experiments (±0.2 ppm). [c] NOESY intensities are marked as strong (s), medium (m) or weak (w).

*Letters a/e mean axial/equatorial

Table 7.8. ^{1}H NMR and ^{13}C NMR data for compound **116** in $CDCl_3$

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC	[c]NOESY
1e	2.05 ddd (*13.5, 4.9, 3.3*)	35.5	2, 5, 10, 19	
1a	1.77 td (*13.8, 13.5, 5.0*)		2, 3, 9, 10, 19	
2	2.40-2.38 m	33.9	1, 3, 4, 5,	
3	--	198.6		
4	5.80 br.s	127.0		
5	--	167.2		
6e	2.63 ddd (*15.1, 3.0, 2.2*)	40.9	4, 5	
6a	2.40 o.m		see 2, 7, 10	
7e	3.96 q (*~2.8*)	67.9		$6e^{s}$, $6a^{s}$, $8/11e^{s}$, $15'^{w}$, $14a/15''^{s}$
8a	1.64 o.m	39.8		
9a	1.45 o.m	45.4		
10	--	38.5		
11e	1.64 o.m	20.5		
11a	1.45 o.m			
12e	1.83 dt (*12.6, 2.9, 2.9*)	36.0	11, 13, 18	
12a	1.09 o.m		11, 13, 17	
13	--	42.8		
14a	1.35 o.m	45.1		
15'	1.71 m	22.8	13, 17	
15"	1.35 o.m			
16'	2.11 m	30.4	13, 17	
16"	1.46 o.m			
17	3.70 t (*8.4*)	81.5		$12a^{s}$, $16'^{w}$, $14a/15''^{s}$
18	0.79 s	10.9	12, 13, 14, 17	
19	1.20 s	17.0	1, 5, 9, 10	

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b]The ^{13}C chemical shifts were extracted from the HSQC and HMBC (for quaternary carbons) experiments (±0.2 ppm). [c]NOESY intensities are marked as strong (s), medium (m) or weak (w).
*Letters a/e mean axial/equatorial

Table 7.9. ^{1}H NMR and ^{13}C NMR data for compound **117** in $CDCl_3$

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC	[c]NOESY
1e	2.49 o.m	39.0	2, 3, 5, 10	
1a	1.59 o.m		2, 3, 10, 19	
2	4.22 dd (*13.7, 5.5*)	68.4	1	
3	--	198.8		
4	5.87 d (*1.2*)	122.2	2, 10	$6"^{w}$
5	--	168.3		
6'	2.88 ddd (*13.4,3.0, 1.4*)	41.0		
6"	2.34 dd (*13.4, 3.2*)		4, 5, 7, 10	
7e	4.17 br.q (*~2.9*)	68.7		$6'^{w}$, $6"^{m}$
8a	1.86 o.m	39.6		
9a	1.88 o.m	42.7		
10	--	41.3		
11e	1.91 o.m	21.9		
11a	1.55 o.m			
12e	1.87 o.m	30.9		
12a	1.32 o.m			
13	--	47.6		
14a	1.70 o.m	45.8		
15e	2.08 o.m	21.3	13, 14, 16	
15a	1.58 o.m			
16'	2.48 o.m	35.6	17	
16"	2.15 dt (*19.5, 9.1, 9.1*)			
17	--	219.3		
18	0.91 s	13.7	12, 13, 14, 17	
19	1.20 s	22.1	1, 5, 9, 10	

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b]The ^{13}C chemical shifts were extracted from the HSQC and HMBC (for quaternary carbons) experiments (±0.2 ppm). [c]NOESY intensities are marked as strong (s), medium (m) or weak (w).
*Letters a/e mean axial/equatorial

Table 7.10. ^{1}H NMR and ^{13}C NMR data for compound **118** in $CDCl_3$

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC
1e	2.04 o.m	36.9	
1a	1.72 o.m		3
2a	2.52 o.m	34.2	1, 3/10
2e	2.42 o.m		1, 3/10
3	--	199.7	
4	5.84 s	126.8	2, 6, 10
5	--	6.7	
6e	4.40 t (*2.8*)	72.8	4, 10
7e	2.13 o.m	36.6	6, 8
7a	1.29 o.m		
8a	2.17 o.m	28.5	
9a	1.07 o.m	52.1	
10	--	37.7	
11e	1.81 o.m	27.9	12, 13
11a	1.48 o.m		12, 13
12a	3.80 dd (*11.1, 4.6*)	72.4	11, 17, 18
13	--	51.6	
14a	1.27 o.m	48.7	
15e	2.02 o.m	21.6	
15a	1.75 o.m		
16'	2.48 o.m	35.5	15, 17
16"	2.08 o.m		14, 15, 17
17	--	222.2	
18	1.02 s	8.3	12, 13, 14, 17
19	1.41 s	19.5	1, 5, 9, 10

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b]The ^{13}C chemical shifts were from real C-13 and extracted from the HSQC and HMBC experiments (±0.2 ppm). *Letters a/e mean axial/equatorial

Table 7.11. ^{1}H NMR and ^{13}C NMR data for compound **119** in $CDCl_3$

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC
a	4.10 dd (*11.2, 5.0*)	75.0	9, 19
2	2.58 o.m	44.2	1, 3, 10
3	--	197.7	
4	5.90 s	126.9	6, 2/10
5	--	166.7	
6	4.47 t (*2.8*)	73.6	4, 8, 10
7e	2.12 o.m	37.3	
7a	1.32 o.m		
8a	2.21 o.m	30.0	
9a	1.17 o.m	54.2	
10	--	44.2	
11e	2.25 o.m	23.1	
11a	1.63 o.m		
12e	1.86 o.m	31.5	
12a	1.30 o.m		
13	--	47.3	
14a	1.29 o.m	50.9	
15e	1.97 o.m	21.6	
15a	1.61 o.m		
16'	2.48 o.m	35.5	14, 17
16"	2.10 o.m		11, 17
17	--	220.4	
18	0.94 s	13.8	12, 13, 14, 17
19	1.43 s	13.3	1, 5, 9, 10

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b]The ^{13}C chemical shifts were from real C-13 and extracted from the HSQC and HMBC experiments (±0.2 ppm). *Letters a/e mean axial/equatorial

Table 7.12. ^{1}H NMR and ^{13}C NMR data for compound **120** in $CDCl_3$

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC	[c]NOESY
1e	2.67 dt (*9.5, 4.5, 4.5*)	37.3	2, 5, 10, 19	
1a	2.01 o.td (*14.1, 14.1, 4.0*)		2, 3, 9, 10, 19	
2	2.41-2.30 m	33.9	1, 3, 4, 10	
3	--	200.0		
4	5.75 s	124.7	2/6, 10	6^{s}
5	--	169.8		
6	2.41-2.30 o.m	33.1		
7e	1.92 o.m	29.7		
7a	1.15 o.m			
8	1.70 o.m	34.4		
9	1.15 o.m	59.2	1, 10, 11, 19	
10	--	40.0		
11	4.05 br.td (*10.3, 10.3, 5.5*)	68.5		$12e^{m}$, 18^{s}, 19^{s}
OH-11	1.89 o.m			
12e	2.14 o.m	42.6	9, 11, 13, 18	
12a	1.42 o.m		11, 13, 18	
13	--	47.6		
14	1.64 o.m	47.0		
15'	1.97 o.m	30.4	14, 16	
15"	1.92 o.m		14, 16	
16	4.41 o.d (*~7.2*)	71.0	13/14	15^{s}, 18^{m}
17	--	217.0		
18	1.03 s	14.8	12, 13, 14, 17	
19	1.33 s	18.2	1, 5, 9, 10	

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b]The ^{13}C chemical shifts were from real C-13. [c]NOESY intensities are marked as strong (s), medium (m) or weak (w). *Letters a/e mean axial/equatorial

Table 7.13. ^{1}H NMR and ^{13}C NMR data for compound **121** in $CDCl_3$

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC	[c]NOESY
1e	2.05 ddd (*13.2, 4.7, 3.4*)	35.6	2, 5, 10, 19	
1a	1.75 o.td (*13.0, 13.0, 4.8*)		2, 3, 9, 10, 19	
2	2.40 o.m	33.8	1, 3, 10	
3	--	199.0		
4	5.75 s	124.3	2, 6, 10	
5	--	169.0		
6'	2.48 o.m	32.1	10	
6"	2.39 o.m			
7e	1.86 o.m	25.8		
7a	1.39 o.m		6, 8	
8a	1.98 o.m	37.4		
9a	1.49 o.m	46.7		
10	--	38.6		
11e	1.65 o.m	18.8		
11a	1.48 o.m			
12e	1.81 o.m	24.8	18	
12a	1.68 o.m			
13	--	51.8		
14	--	78.8		
15'	2.45 o.m	38.8	13, 14, 16, 17	
15"	1.85 o.m		16	
16	4.26 t (*7.4*)	72.7	17	$15'^{s}$
17	--	217.6		
18	1.15 s	18.2	12, 13, 14, 17	8^{s}, $11a^{s}$
19	1.23 s	17.3	1, 5, 9, 10	8^{s}, $H11a^{s}$

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b]The ^{13}C chemical shifts were extracted from the HSQC and HMBC (for quaternary carbons) experiments (±0.2 ppm). [c]NOESY intensities are marked as strong (s), medium (m) or weak (w).
*Letters a/e mean axial/equatorial.

Table 7.14. ^{1}H NMR and ^{13}C NMR data for compound **122** in $CDCl_3$

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC
1e	2.80 ddd (*13.9, 4.8, 3.4*)	38.9	2, 10, 19
1a	1.91 td (*13.9, 13.9, 4.4*)		
2a	2.57 o.td (*17.6, 4.8*)	34.4	
2e	2.37 dt (*17.6, 3.7, 3.7*)		
3	--	200.4	
4	5.83 s	127.3	2, 6, 10, 19
5	--	167.3	
6e	4.40 t (*2.5*)	73.0	4, 10
7e	2.11 o.m	36.2	
7a	1.38 o.m		
8a	2.23 m	28.1	
9a	1.11 t (*10.2*)	59.2	
10	--	39.5	
11a	4.13 m	68.8	
12e	2.16 o.m	43.0	
12a	1.34 o.m		
13	--	48.2	
14a	1.41 o.m	50.1	
15'	1.99 o.m	21.7	
15"	1.61 o.m		
16'	2.52 o.m	35.7	
16"	2.15 o.m		
17	--	218.4	
18	0.97 s	14.7	12, 13, 14, 17
19	1.53 s	20.3	1, 5, 9, 10
OH-11	1.06 br.d (*5.4*)	--	
OH-6	1.65 br.s		

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b] The ^{13}C chemical shifts were from real C-13 and extracted from HMBC (for quaternary carbons) experiments (±0.2 ppm). *Letters a/e mean axial/equatorial.

Table 7.15. ^{1}H NMR and ^{13}C NMR data for compound **123** in $CDCl_3$

*Position	[a]δ_H-mult-*J* (Hz)	[b]δ_C	HMBC	[c]NOESY
1e	2.04 ddd (*13.3, 5.0, 2.8*)	37.0	2, 10, 19	$1a^s$, $2a^s$, $2e^s$, 19^w
1a	1.72 o.m			
2a	2.51 m	34.0	1, 3	$1e^s$, $1a^m$, $2e^s$, 19^s
2e	2.40 o.m		1, 3	
3	--	200.0		
4	5.83 s	126.3	2, 6, 10, (*19*)	$6e^s$
5	--	167.3		
6e	4.40 t (*2.5*)	72.5	4, 8, 10	4, $7e^s$, $7a^s$
7e	2.14 dt (*13.9, 3.1, 3.1*)	37.3	8	$6e^s$, $7a^s$, $15'^s$
7a	1.33 o.m			
8a	2.23 qd (*11.2, 3.3*)	28.4	7, 9, 14	18^s, 19^s
9a	1.01 o.m	53.9	8, 10, 19/11	$1a^s$, 7a/$12a^s$, $14a^s$
10	--	38.0		
11e	1.69 o.m	19.9	9, 12	
11a	1.57 o.m			
12e	1.93 ddd (*13.0, 4.0, 2.7*)	31.5	11, 13, 18	$12a^s$, 18^s
12a	1.34 o.m			
13	--	46.7		
14a	1.26 o.m	45.3		9^s, $15'^s$, $15''^w$, 16^m
15'	2.42 o.m	30.4	13, 14, 16	
15"	1.34 o.m		8, 14, 16	
16	3.96 t (*8.5*)	74.9	15	$15'^s$, $14a^m$, $15''^w$
17	--	218.8		
18	1.02 s	14.7	12, 13, 14, 17	$8a^s$, 11a/$15''^s$, $12e^w$
19	1.40 s	19.5	1, 5, 9, 10	$1e^s$, $2a^s$, $8a^s$, $11a^s$

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b]The ^{13}C chemical shifts were extracted from the HSQC and HMBC (for quaternary carbons) experiments (±0.2 ppm). [c]NOESY intensities are marked as strong (s), medium (m) or weak (w). *Letters a/e mean axial/equatorial.

Table 7.16. ^{1}H NMR and ^{13}C NMR data for compound **124** in $CDCl_3$

*Position	δ (H) -mult	δ (C)	HMBC
1	2.06 o.m	36.9	
	1.75 o.m		
2	2.50-2.46 o.m	34.0	
3	--	199.6	
4	5.91 s	129.7	2, 6, 10, *19*
5	--	165.9	
6e	4.20 d (*~2.7*)	77.1	4, 7, 8, 10
7e	3.40 t (*~2.7*)	70.1	
8a	2.22 o.m	34.0	
9a	1.53 o.m	44.5	
10	--	37.8	
11	1.71-1.57 o.m	19.9	
12e	1.86 o.m	30.8	
12a	1.29 o.m		
13	--	47.2	
14a	1.76 o.m	45.0	
15'	2.05 o.m	21.2	
15"	1.65 o.m		
16'	2.49 o.m	35.7	17
16"	2.13 o.m		17
17	--	220.3	
18	0.94 s	13.6	12, 13, 14, 17
19	1.38 s	19.8	1, 5, 9, 10

[a]Mult., Multiplicity: br., broad; s, singlet; d, doublet; t, triplet; m, multiplet; o, overlapping. The precision of the coupling constants is ±0.25 Hz. [b] The ^{13}C chemical shifts were extracted from the HSQC and HMBC (for quaternary carbons) experiments (±0.2 ppm). *Letters a/e mean axial/equatorial.

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