Université du Québec
INRS
Institut Armand-Frappier

L'If Canadien: composition des boutures *versus* les plantes matures

Par
Tracy L. Petzke

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Examinateur externe	Dr Youla Tsantrizos R&D, Boehringer Ingleheim Canada Ltd.
Examinateur interne	Dr François Lepine, IAF
Directeur de recherche	Dr Lolita Zamir, IAF

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10-DAB-III: 10-deacetyl-baccatin III

Ac: -$COCH_3$

Bz: -COC_6H_5

C: (as in C-1, C-2, C-x…) carbon-#

CH_2Cl_2: Dichloromethane

CH_3CN: Acetonitrile

CoA: Coenzyme A

CPM: Counts per million

DMAPP: Dimethylallyl diphosphate

DPM : Disintegrations per minute

EtOAc: Ethyl acetate

FDA: Federal Drug Association

FPP: Farnesyl diphosphate

g: Gram(s)

G2/M (boundary): Gap 2 / Mitosis (boundary)

GAP: Glyceraldehyde-3-phosphate

GGPP: Geranylgeranyl diphosphate

GPP: Geranyl diphosphate

H_2O: Water

HMBC: Heteronuclear Multiple Bond Correlation

HMG: 3-hydroxy-3-methylglutaryl

HPLC: High Pressure Liquid Chromatography or High Performance Liquid Chromatography

HR FAB-MS: High resolution fast atom bombardment mass spectroscopy

IPP: Isopentenyl diphosphate

L: Litre(s)

MDR: Multidrug resistance

MECP: 2-C-Methyl-D-erythritol-2,4-cyclodiphosphate

MeOH: Methanol

Mg: Magnesium

mg: Milligram(s)

mL: Millilitre(s)

mM : Millimolar

Mn: Manganese

NaCl: Sodium chloride

NADPH: Nicotinamide adenine dinucleotide phosphate (reduced form)

Na_2SO_4: (Anhydrous) sodium sulphate

NCI: National Cancer Institute

NMR: Nuclear Magnetic Resonance

NOESY: Nuclear Overhauser Effect Spectroscopy

Ph: $-C_6H_5$

R_t: Retention time

Taxoid: Taxane diterpenoid

TLC: Thin Layer Chromatography

µCi: Microcurie(s)

µL: Microlitre(s)

v/v: Volume per volume

Summary

The isolation of paclitaxel (Taxol®, Bristol-Meyers Squibb) in 1971 by Wani *et al.* and the eventual approval of it's use as a treatment for breast, ovarian and lung cancer almost twenty years later has greatly increased the study of paclitaxel and it's analogues, the taxanes. There are over three hundred and fifty of these natural products already reported from various natural sources, largely from the many species of yew, each with varying structural characteristics and bioactivities. The taxanes are studied not only for their potential anti-cancerous properties but also for the information they may provide towards the elucidation of the biosynthetic pathway of paclitaxel.

The study of the Canadian Yew, *Taxus canadensis*, has shown that this species of yew differs from the other species of yew not only its appearance, which is smaller and rambling, but also in its composition of taxanes. While major taxanes such as paclitaxel and 10-deacetyl-baccatin III found in the numerous other species of yew are also found in the Canadian Yew, their proportions are different. Also different is the taxane 9-dihydro-13-acetyl-baccatin III, which has been reported as the major taxane of the Canadian Yew. Found only in minute quantities in other species of yew, this taxane is found in quantities three to seven times greater than the amount of paclitaxel in the Canadian Yew, regardless of the season of year or location of the plant (Zamir *et al.*, 1995:73:655). This difference in the composition of the Canadian Yew *versus* the other species of yew has spurred the continued study of the Canadian Yew in the laboratory of Dr Lolita Zamir.

In this study, the taxane content of rooted cuttings of *Taxus canadensis* was assessed and compared to the content of the previously studied needles and stems of the mature *Taxus canadensis* plant. The study of the composition of rooted cuttings was done in order to establish whether a difference in composition between the mature Canadian yew and its rooted cuttings existed, and to thereby lead to the possible establishment of rooted cuttings as a feasible model for future biosynthetic studies, as well as to possibly lead to the discovery of new taxanes.

Twenty-nine taxanes were isolated and characterized from the rooted cuttings of *Taxus canadensis* by a series of chromatography purifications, using column chromatography and high-pressure liquid chromatography (HPLC) techniques. The

resulting purified taxanes or mixtures of taxanes were analysed by NMR and mass spectroscopy. These analyses allowed the characterization of three new taxanes as well as the identification of twenty-six known taxanes, of which fourteen taxanes were isolated for the first time from *Taxus canadensis*. The three major taxanes isolated from the rooted cuttings were 5-decinnamoyl-taxagifine, 10-deacetyl-baccatin III and paclitaxel, compared to 9-dihydro-13-acetylbaccatin III, taxinine and taxinine E as already reported for the mature Canadian yew (Zamir *et al.*, 1992:33:36:5173, Zamir *et al.*, 1995:73:655).

These results show a difference in the taxane content of the rooted cuttings *versus* the mature *Taxus canadensis* and suggest a change in the taxane content of the plant may be due to the preparation of the rooted cuttings from the mature trees. The previously reported difference in taxane content of the Canadian yew compared to the other yew species, combined with the difference in taxane content of the rooted cuttings compared to the mature Canadian Yew encourages the continued study of the rooted cuttings of the Canadian Yew in our laboratory. This may lead to the possible future use of these plants as models for biosynthetic studies of paclitaxel in the Canadian yew.

Tracy L. Petzke

Dr Lolita Zamir

Résumé

- **Revue de littérature**

Le cancer est une des causes majeures de mortalité en Amérique du Nord et dans le monde (Nicolaou, Dai et Guy, 1994:33:15). Malgré les nombreuses drogues et les formes de traitements déjà découverts et couramment utilisés, la découverte de nouveaux traitements avec moins d'effets secondaires est toujours nécessaire et importante.

Le paclitaxel, un taxane isolé d'abord de l'if du pacifique *Taxus brevifolia* Nutt. (Wani *et al.*, 1971 :93 :2325), est une drogue antimitotique qui est couramment utilisée pour combattre le cancer du sein, des ovaires et des poumons entre autres. La découverte du paclitaxel (Taxol®, Bristol-Meyers Squibb) a eu un impact sur la communauté scientifique et a donné un espoir dans la lutte contre le cancer grâce à ses propriétés anticancéragènes uniques. Les autres drogues antimitotiques connues avant la découverte du paclitaxel, par exemple les vinca alcaloïdes, avaient des modes d'actions différents du paclitaxel. Le paclitaxel agit en stabilisant la tubuline et alors promouvant la formation de microtubules (Rowinsky et Donehower, 1995:332:15: 1004). Les vinca alcaloïdes agissent de façon inverse, c'est à dire qu'ils promeuvent la dépolymérisation des microtubules (Hezari et Croteau, 1997: 63: 291). Cette variation dans le mode d'action du paclitaxel suggère une efficacité supérieure aux autres drogues, pour lesquelles le cancer pourrait déjà être résistant (Nicolaou, Guy et Potier, 1996:274:94).

Le paclitaxel et ses analogues ont des actions inhibitrices contre des lignées de cellules humaines résistant à de multiples drogues, MDR (Distefano *et al.*, 1997 :72 :5 :844, Hosoyama *et al.*, 1999 :9 :389). Le paclitaxel est actif contre le cancer du sein, des ovaires et des poumons ainsi que contre la leucémie et il est couramment approuvé pour le traitement des cancers du sein et ovaires par la FDA aux États-Unis. Pour être un agent thérapeutique idéal, il y a de nombreux problèmes et effets secondaires du paclitaxel qui doivent toutefois être résolus.

Le problème le plus grave associé au paclitaxel est qu'il ne peut être obtenu qu'en petites quantités, d'où le prix élevé du traitement. Isolé en premier de l'écorce de l'if du Pacifique, le paclitaxel est aussi retrouvé dans les aiguilles de l'arbre et dans d'autres espèces d'ifs et aussi chez certains champignons (Baloglu et Kingston, 1999:62:1448,

Kingston, 2001:10:867). Plus récemment, de petites quantités ont été isolées de la noisette (Hoffman *et al.*, 2000). La quantité isolée des sources naturelles est faible. La croissance lente et ardue des ifs augmente le problème, et la quantité isolée est souvent limitante pour l'utilisation commerciale (Wheeler *et al.*, 1992: 55:4:430, Vance *et al.*, 1994: 36:5:1241). Plusieurs productions chimiques ont été réalisées mais leurs complexités, leurs rendements faibles et leurs coûts élevés rendent leur production commerciale difficile (Masters *et al.*, 1995: 34:16:1723, Holton *et al.*, 1994: 116:1597, Nicolaou *et al.*, 1994: 367:6464:630, Nicolaou *et al.*, 1995: 117:2:624). Présentement, une forme semi-synthétique de la drogue est distribuée mais cette forme requiert également une source naturelle. Elle est formée par l'addition de la chaîne latérale du paclitaxel sur le squelette de la 10-deacétyle-baccatin III (10-DAB-III), qui est isolée de l'if européen, *Taxus baccata* (Guenard, Guéritte-Voegelein, et Potier, 1993: 26:4:160, Rohr, 1997:36:20:2190). Malgré cette source de paclitaxel, le prix s'élève à quelques centaines de dollars américains par gramme de drogue. Il est alors essentiel de trouver d'autres moyens de se procurer la drogue, c'est à dire soit par des lignées de cellules de l'if modifiées génétiquement (Rohr, 1997: 36:20:2190, Vaněk *et al.*, 1999: 65:275). Ceci demande une connaissance avancée de la biosynthèse du paclitaxel. Cette connaissance pourra permettre le développement d'une production à grande échelle et c'est pour cette raison que l'étude et l'élucidation de la biosynthèse du paclitaxel est impérative (Kingston, 2001,10:861, Patel, 1998:98:361).

Il y a deux types d'approches qui sont utilisées pour l'étude de la biosynthèse du paclitaxel. Premièrement, il y a les expériences de nutrition avec des précurseurs radioactifs. Ce type d'étude permet de voir s'il y a une incorporation de précurseurs et si oui, si un motif est présent. Le deuxième type d'étude est l'étude de la phytochimie de plusieurs espèces d'ifs. Ceci permet non seulement la caractérisation de plusieurs types de structures de taxanes, mais aussi la comparaison de la composition d'une espèce d'if avec les autres (Baloglu et Kingston, 1999:62:1448, Parmar *et al.*, 1999:50:1267). La prédominance d'un type de taxane comparée aux autres peut suggérer la séquence d'étapes par laquelle se produit la biosynthèse (Kingston, 2001, 10:867). Avec l'isolement de nombreux taxanes vient non seulement l'analyse de la composition des ifs,

mais aussi la possibilité qu'un ou plusieurs de ces taxanes aient des propriétés anti-cancérigènes ou qu'ils puissent avoir moins d'effets secondaires.

Étant donnée que le paclitaxel est une des drogues anti-cancérigènes présentement disponible qui promet de bons résultats comme de nombreuses autres drogues provenant de sources naturelles, l'étude de la biosynthèse du paclitaxel et l'isolement de nouveaux taxanes est sans doute indispensable et très importante pour la lutte contre le cancer.

Plusieurs types de structures de taxanes existent et leur classification est basée sur des caractéristiques telles que la présence ou l'absence d'une chaîne latérale en C-13 comme celle du paclitaxel. L'organisation des taxanes comme telle permet l'étude des relations structure-activité et facilite la comparaison de leur structures chimiques.

Un taxane « régulier » peut être décrit comme un taxane où le squelette est similaire à celui du paclitaxel, avec un anneau de huit carbone entre deux anneaux de six carbones chacun. Ils peuvent être décrit avec plus de détail par l'absence ou la présence d'une chaîne latérale en C-13 ou d'un anneau oxetane. La classe de taxane avec ces deux caractéristiques est une des classes les plus étudiées car c'est la classe à laquelle paclitaxel appartient et qui a donc les caractéristiques possiblement nécessaires pour l'activité anti-cancérigène (Suffness et Wall, 1995:1:3, Parmar *et al.*, 1999:50:1267, Kingston *et al.*, 1993:61:1).

Un 3,11-cyclotaxane est comme un taxane régulier avec une liaison supplémentaire entre C-3 et C-11. Avec les taxanes qui ont un pont C-12(16)-oxido, ce type de taxane fait partie du groupe de taxanes qui ont des liaisons transannulaires (Suffness et Wall, 1995:1:3, Parmar *et al.*, 1999:50:1267, Kingston *et al.*, 1993:61:1).

Un 11(15→1) abeotaxane est un taxane où il y a une liaison entre C-11 et C-1 et non entre C-11 et C-15. Ceci change le squelette du taxane de la structure tricyclique de 6, 8, et 6 carbones à une structure tricyclique de 5, 7 et 6 carbones (Suffness et Wall, 1995:1:3, Parmar *et al.*, 1999:50:1267, Kingston *et al.*, 1993:61:1).

Un 2(3→ 20) abeotaxane est un taxane où une liaison entre C-2 et C-20 remplace la liaison entre C-2 et C-3. Ce réarrangement donne un taxane avec un squelette tricyclique de 6, 10 et 6 carbones (Suffness et Wall, 1995:1:3, Parmar *et al.*, 1999:50:1267, Kingston *et al.*, 1993:61:1).

Un type de structure très rare est celle d'un 11(15→ 1),11(10→9) bisabeotaxane, par exemple le Wallifoliol. Ce type de taxane a deux liaisons réarrangées: une liaison entre C-11 et C-1 et non entre C-11 et C-15, et une liaison entre C-11 et C-9 et non entre C-11 et C-10 (Suffness et Wall, 1995:1:3, Parmar *et al.*, 1999:50:1267, Kingston *et al.*, 1993:61:1).

Un dernier exemple d'un type de taxane est la classe de taxanes bicycliques, parfois nommées les pretaxanes. Comme indiqué par leurs noms, le squelette de ce type de taxane est formé de deux anneaux. Un exemple de ce type de taxane est le 5-epi-canadensène, isolée de l'if canadien (Suffness et Wall, 1995:1:3, Parmar *et al.*, 1999:50:1267, Kingston *et al.*, 1993:61:1).

Il y a plusieurs espèces d'ifs qui font tous partie du genre *Taxus*, de la famille *Taxaceae*. On nomme une espèce d'if selon sa localisation géographique, par exemple l'if canadien (*Taxus canadensis*).

Le contenu des ifs change avec l'espèce, la saison, et leur localisation géographique. L'if canadien est différent des autres espèces d'ifs non seulement par son profil rampant, mais aussi par sa composition (voir **tableau 1, page 15**). Alors que le paclitaxel, le cephalomanine, le 10-deacétyle-baccatin III et d'autres taxanes majeurs qui ont été isolées de l'if canadien, le taxane le plus prédominant est le 9-dihydro-13-acétyle-baccatin III (Zamir *et al.*, 1992:33:36:5235). Chez l'if canadien mature, ce composée est présent en quantités de trois à sept fois plus grandes que le paclitaxel, peu importe la saison. Un nouveau composé nommé le canadensène (Zamir *et al.*, 1995:529) a aussi été isolé de l'if canadien et a des caractéristiques structurales similaires à un des précurseurs de paclitaxel.

Cette différence dans la composition pourrait suggérer une voie biosynthétique unique pour le paclitaxel chez l'if canadien, différent de celle proposée pour les autres espèces d'ifs. Présentement, la voie acceptée pour la biosynthèse du paclitaxel est la voie de Rohmer où un des produits métaboliques du glucose, le glyceraldehyde-3-phosphate (GAP), est un précurseur du diterpènoïde. Avant l'élucidation de cette voie, la voie de biosynthèse suggérée du paclitaxel était la voie du mevalonate. Cette voie n'est plus la voie acceptée pour la biosynthèse du paclitaxel. Les deux voies se terminent par la

formation de geranylgeranyl phosphate (GGPP) qui est le précurseur universel des diterpènoïdes. La formation du paclitaxel est identique dans les deux voies et ce à partir de la formation de GGPP (Rohr, 1997:36:20:2190, Eisenreich *et al.*, 1996:93:6431, Rohmer, 1999:16:565, Hezari et Croteau, 1997:63:291, Bach, 1995:30:3:191, Floss et Mocek, 1996:7:191, Rohmer, 1998:50:136, Koepp *et al.*, 1995:270:15:8686).

L'élucidation d'une voie biosynthétique majeure est très complexe et nécessite de nombreuses années de recherche. L'avantage d'avoir de nombreux groupes de recherche qui travaillent pour l'élucider est que non seulement chacun contribue à la recherche, mais aussi que les différents groupes utilisent différentes espèces d'ifs des locations géographiques, et des temps de récolte différents ce qui donne de nombreux échantillons variés. Alors que plusieurs espèces d'ifs ont déjà été étudiées, les études utilisant l'if canadien sont rares (à l'exception du laboratoire de Dr L.O. Zamir) et ceci est la première étude qui étudie les boutures d'if.

Les boutures sont de jeunes branches desquelles on fait pousser des racines. La pousse des racines est stimulée en appliquant un mélange d'hormones spécifiques sous certaines conditions. La création des boutures est très ardue surtout chez l'if du Canada. Le protocole suivi pour créer ces boutures est gardé confidentiel par les pépiniéristes. Bien que les boutures seront intéressantes comme modèle pour étudier la biosynthèse, il est important d'abord d'établir si une différence entre la composition de ces boutures et la plante mature existe ou non. S'il y a une différence, la prochaine question qui se pose est que signifie la biosynthèse des taxanes chez les boutures d'ifs. Même si les boutures sont crées à partir de sections d'if mature qui devraient avoir le même contenu de taxanes et d'enzymes, la stimulation des racines avec les hormones peut changer l'équilibre de la plante, et peut-être la biosynthèse du paclitaxel. Grâce aux études précédentes chez l'if canadien mature montrant une composition différente de chez les autres ifs, l'étude de la composition des boutures de *Taxus canadensis* est encore plus intéressante. Le contenu des boutures de *Taxus canadensis* pourra permettre des études futures qui pourront aider à expliquer la voie biosynthétique. Avec ces types d'études, un protocole pour la nutrition des précurseurs radioactifs doit aussi être défini.

- **Matériel et méthodes : Étude de la composition des boutures de *Taxus canadensis***

Deux cent trente cinq boutures de *Taxus canadensis*, obtenues à la mi-septembre 2001, ont été lavées et séchées à l'air. Une fois séchées, les boutures ont été broyées en utilisant un robot culinaire (Robot Coupe USA Inc, BX3) et 583.93g de poudre fine a été produit (Mettler PC2000, Fisher Scientific). La poudre a été extraite avec du méthanol à 100% et ensuite avec un mélange de méthanol dichlorométhane (1 :1, vol/vol). Tous les extraits ont été combinés et séchés.

Une substance gluante a été obtenue après l'évaporation des extraits combinés. La viscosité est due à la concentration de composées et du contenu élevé en lipides et en cire de la plante. La première étape de l'extraction a alors été la séparation des composés les plus liposolubles. Ceci a été accompli par l'introduction d'hexane dans une ampoule d'extraction avec l'extrait de plante dissous dans l'eau distillée. La phase hexane a ensuite été extraite avec du méthanol pour enlever les composés les plus polaires de ce mélange. Après l'extraction à l'hexane, la phase aqueuse a été saturée avec du chlorure de sodium. Cette solution saturée a de nouveau été extraite, cette foi-ci avec du dichlorométhane. La solution de dichlorométhane a ensuite été séchée avec du sulfate de sodium. La phase organique extraite avec le dichlorométhane est la phase dans laquelle la plupart des taxanes ont ensuite été purifiés. En plus des taxanes isolés de la phase de dichlorométhane, des taxanes ont aussi été purifiés de la phase de méthanol (voir **figure 20, page 39**).

La solution de dichlorométhane a donné un extrait sec de 8.36g. Cet extrait brut a été purifié partiellement par chromatographie sur colonne. Les systèmes de solvants utilisés sont les suivants : 0.5L de 33% d'acétate d'éthyle dans du dichlorométhane (165mL EtOAc avec 335mL CH_2Cl_2) pour donner les fractions #1-5 de 100mL chacune, 0.5L de 50% acétate d'éthyle dans du dichlorométhane (250mL de EtOAc et de CH_2Cl_2) pour donner les fractions #6-15 de 50mL chacune, 0.2L de 66% acétate d'éthyle en dichlorométhane (132mL EtOAc avec 68mL CH_2Cl_2) pour donner les fractions # 16-19 de 50mL chacune, et 1.3L de 100% acétate d'éthyle pour donner les fractions #20-45 de 50mL chacune. Ces fractions ont été analysées par chromatographie liquide à haute pression (CLHP) à l'échelle analytique. Basée sur leurs contenus, les fractions obtenues

après passage dans la colonne de chromatographie ont été soit purifiées encore plus par CLHP à l'échelle préparative ou par chromatographie sur couche mince (CCM), soit mises de côté et gardées pour des expériences futures dans notre laboratoire.

Les temps de rétention de nombreux taxanes sont connus dans notre laboratoire. La comparaison de ces standards avec les spectres obtenus après CLHP des fractions à l'échelle analytique a permis une identification préliminaire de plusieurs taxanes majeurs. En se basant sur cette comparaison, plusieurs fractions ont été groupées et combinées pour former des mélanges qui contenaient le 10-déacétyle-baccatin III, le 9-dihydro-13-acétyle-baccatin III et le paclitaxel et le céphalomanine. Ces mélanges ont ensuite été purifiés par CLHP à l'échelle préparative.

Les fractions récoltées après CLHP à l'échelle préparative ont été analysées par CLHP à l'échelle analytique. Les spectres obtenues après ces injections de CLHP à l'échelle analytique ont été étudiées afin de choisir les fractions d'intérêt. Ces fractions ont été ensuite analysées par ^{1}H RMN (Bruker, AMX 500MHz). Ces échantillons ont tous été dissous dans du chloroforme deutéré (CDN-isotope, 99.8% atome % D). Les échantillons qui consistaient de mélanges de composés ont soit été purifiés de nouveau en utilisant la CLHP ou la CCM, soit mis de coté pendant que la purification des autres échantillons se poursuivait. Les échantillons qui ont été purifiées en premier étaient les échantillons qui avaient le 10-deacétyle-baccatin III, le 9-dihydro-13-acétyle-baccatin III, le paclitaxel et le céphalomanine. Les autres échantillons qui ont été purifiés étaient les échantillons qui avaient un ou plusieurs pics majeurs, ceux qui étaient moins compliqués à purifier, et ceux qui avaient des pics avec des temps de rétention différents pour assurer qu'une grande variété de composés serait purifiée et identifiée. Une fois les échantillons pures analysés par ^{1}H RMN (*analyses faites par le Dr Qing-Wen Shi*) et les structures des composés caractérisées, les échantillons ont été envoyés pour analyse par spectroscopie de masse à haute résolution pour confirmer leur poids moléculaire (*McGill Mass Spectroscopy Unit, Dr O. Mamer*). Les nouveaux composés qui n'ont jamais été rapportés dans la littérature ont été envoyés pour leur caractérisation par RMN COSY, HMQC, HMBC et soit par NOESY ou ROESY (*Université de Queens, Dr F. Sauriol*) en premier. Une fois les données de RMN 2D obtenues, les échantillons ont ensuite été envoyés pour

être analysés par spectroscopie de masse. Pour les nouveaux taxanes qui ont été isolés ici pour la première fois, l' $[\alpha]_D$ a aussi été mesuré (JASCO, DIP-370, Digital polarimeter).

Le contenu de l'extrait dans la phase de méthanol a été purifié par chromatographie sur colonne. Le même système a été utilisé que pour la purification de l'extrait de dichlorométhane. Trente quatre échantillons ont été récoltés et de ces fractions, quatre ont été combinées et purifiées par CLHP préparative.

- **Matériel et méthodes : Étude de la composition des boutures de *Taxus cuspidata capitata***

Soixante boutures de *Taxus cuspidata capitata*, obtenues à la mi-mai 2001, ont été lavées et séchées à l'air. Une foi séchées, les boutures ont été broyées en utilisant un robot culinaire (Robot Coupe USA inc, BX3) et 148.83g de poudre fine a été produite (Mettler PC2000, Fisher Scientific). Cette poudre a ensuite été déposée dans un flacon Erlenmeyer de 2L et extraite plusieurs fois avec du méthanol à 100% et ensuite avec un mélange de méthanol dichlorométhane (1:1, vol/vol). Les extraits ont été combinées et évaporés.

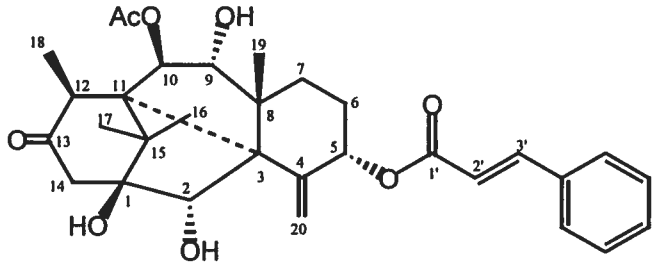
Ce mélange brut a ensuite été dissous dans de l'eau distillée et extrait avec de l'hexane. Après l'extraction avec l'hexane, la phase aqueuse a été saturée avec du chlorure de sodium et extrait avec du dichlorométhane. La phase organique de dichlorométhane a ensuite été séchée avec du sulfate de sodium. Un fois évaporé, l'extrait de dichlorométhane a donné un échantillon de 1097mg (Mettler H51AR, Fisher Scientific).

L'extrait de dichlorométhane a premièrement été purifié par colonne de chromatographie en utilisant le système suivant: hexane:acétone (1:1, vol/vol) pour donner les fractions #1 à 7 de 100mL chacune, hexane:acétone (1:2, vol/vol) pour donner les fractions #8 à 15 de 100mL chacune, 100% acétone pour donner les fractions #16 à 27. Chacune de ces fractions a été analysée par CLHP à l'échelle analytique pour déterminer leur contenu. Les échantillons ont aussi été pesés et ont été donnés à un étudiant post-doctoral de notre laboratoire, Dr Qing-Wen Shi pour poursuivre leurs purifications par CLHP et CCM.

- **Résultats: Étude de la composition des boutures de *Taxus canadensis***

Plusieurs taxanes aux caractéristiques structurales différentes ont été isolés de l'extrait des boutures de *Taxus canadensis*. Le contenu des boutures d'if n'a jamais été étudié auparavant et cette étude en est le premier résultat. Des taxanes isolés, **trois** étaient des taxanes inconnus, jamais publiés dans la littérature (voir **figure A-D**). **Quatorze** autres taxanes ont été isolés pour la première fois de l'if canadien (voir **figure 31, page 68**). Les **douze** derniers taxanes isolés sont des taxanes connus, et qui ont déjà été identifiés chez l'if du Canada (voir **figure 32, page 85**).

Figure A. Structures des nouveaux taxanes isolés des boutures de *Taxus canadensis*, avec leurs données de HR FAB-MS et de RMN (RMN ^{1}H et ^{13}C pour les taxanes en $CDCl_3$)



1β,2α,9α-trihydroxy-10β-acétoxy-5α-cinnamoyloxy-3-11-cyclictaxa-4(20)-ene-13-one

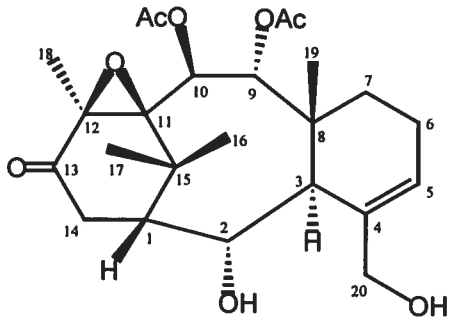
(Nouveau taxane #1)

HR FAB-MS: *m/z* 577.22016 ($M+K^+$)

Confirme une structure avec un poids moléculaire: 538.63 g/mol, $C_{31}H_{38}O_8$

$[\alpha]_D^{24} = 7.3394°$, où c = 0.109 $CHCl_3$

2-3 mg au total.



2α,9α,10β-triacétoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one

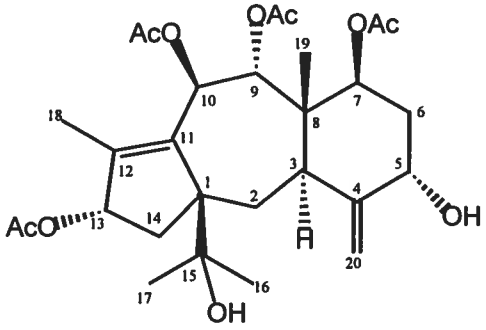
(Nouveau taxane #2)

HR FAB-MS: *m/z* 531.19967 ($M+K^+$)

Confirme une structure avec un poids moléculaire: 492.56 g/mol, $C_{26}H_{36}O_9$

$[\alpha]_D^{24}$ = -19.580°, où c = 0.0715 $CHCl_3$

0.5-1 mg au total.



7β,9α,10β,13α-tetraacétoxy-11(15→1)*abeo*taxa-4(20),11-diene-5α,15-diol

(Nouveau taxane #3)

HR FAB-MS: *m/z* 575.22600 (for $M+K^+$)

Confirme structure avec un poids moléculaire: 536.26 g/mol, $C_{28}H_{40}O_{10}$

$[\alpha]_D^{22}$ = -50°, où c = 0.02 $CHCl_3$

8-9 mg au total.

Données de RMN pour le nouveau taxane #1, 1β,2α,9α-trihydroxy-10β-acétoxy-5α-cinnamoyloxy-3-11-cyclictaxa-4(20)-ene-13-one

Position	δ (H) mult	J (Hz)	δ (C)	HMBC	NOESY
1	--		**78.3**		
2	4.780 (br.s)	(COSY 14b)	78.6	1, 4, 8	**17/19**
3	--		**62.3**		
4	--		**142.5**		

5	5.594 (o.t)	8.8	75.8	**6, 166.2**	**6a, 6b**
6a 6b	2.20 (o.m) 1.70 (o.m)		26.0	 7, 8	**5**, **6b**, **7a**, 7b/17/19 **6a**, **10**
7a 7b	1.93 (o.m) 1.34 (o.m)		29.2		**7b**, **10** **7a**
8	--		**45.8**		
9	4.395 (d)	9.8	82.5	7, 8, 10, 19	2, **17/19**
10	5.389 (d)	9.8	84.0	9, 11, 12, 15, 172.5	**6b**, **7a**, **12**, **18**
11	--		**56.8**		
12	3.494 (q)	7.3	50.9	3, 13	**10**, **18**, **20a**
13	--		**213.9**		
14a 14b	3.048 (d) 2.357 (br.d)	19.8 19.8	46.4	1, 13, 15	**14b**, **20a** **14a**, **16**
15	--		**45.0**		
16	1.117 (s)		23.0	1, 11, 15, Me17	**14b**, **17/19**, **18**
17	1.396 (o.s)		23.3	1, 11, 15, Me16	**2**, 5, **9**, **16**, (overlap 19)
18	1.307 (d)	7.3	15.8	11, 12, 13	**12**, **10**, **16**
19	1.396 (o.s)		25.2	3, 7, 8, 9	Voir 17
20a 20b	5.804 (s) 5.594 (o.s)		126.1	3 3, 4, 5	**12**, **14a**, **20b** **20a**
OAc	2.157 (s)		21.2	**172.4**	
1'	--		**166.2**		
2'	6.386 (d)	16.1	117.4	Ph-C1, 1'	
3'	7.664 (d)	16.1	145.3	1', 2', Ph-o	
Ph 3' o m, p	 7.55 (m) 7.38 (m)		**134.4** 128.2 129.0 130.4		

Données de RMN pour le nouveau taxane #2, 2α,9α,10β-triacétoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one

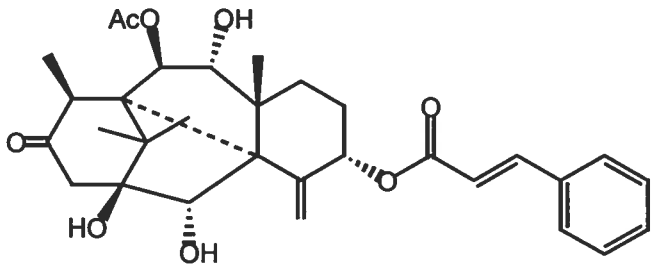
Position	δ (H) mult	J (Hz)	δ (C)	HMBC	NOESY
1	1.94 (o.m)		48.6		
2	5.825 (br.m)	~2.4	71.4		1, 3, **9**, **17**, **19**
3	3.225 (br.m)		40.9		*2*, **18**
4	--		**138.0**		
5	5.927 (m)		131.2		**20b**
6	2.10 (m)		21.9		
7a 7b	1.96 (o.m) 1.389 (td)	 12.2, 12.2, 6.1	27.2		
8	--		**39.6**		
9	6.116 (d)	11.1	76.5	7, 8, 10, 19, 169.3	**2**, **17**, **19**
10	5.201 (s)	11.2	71.4	9, 11, 12, 15, 169.0	*3*, **18**
11	--		**65.1**		

12	--		**59.6**		
13	--		**208.1**		
14a 14b	3.039 (d) 2.626 (o.dd)	20.1 20.1, 8.4	37.6	1, 2, 13, 15	3, **14b**, **20a** **1**, **14a**, 16
15	--		**39.1**		
16	0.841 (s)		28.7	1, 11, 15, Me	**1**, **17**
17	1.896 (s)		25.1	1, 11, 15, Me	**2**, **9**, **16**
18	1.585 (s)		15.0	11, 12, 13	**3**, **10**
19	0.999 (s)		18.0	3, 7, 8, 9	**2**, **6**, 7a, **9**, **20b**
20a 20b	4.618 (br.d) 4.117 (d)	~11.9 11.7	68.0	 3, *4*, 5	**14a**, **20b** **5**, **20a**
OAc	Pas assignée				

Données de RMN pour le nouveau taxane #3, 7β,9α,10β,13α-tetraacétoxy-11(15→1)*abeo*taxa-4(20),11-diene-5α,15-diol

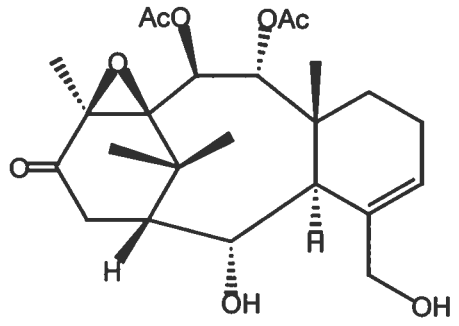
Position	δ (H) - mult	J (Hz)	δ (C)	HMBC	NOESY
1	--		**62.8**		
2a 2b	2.352 (dd) 1.431 (br.d)	14.8, 9.6 14	29.1	8, 15	**2b**, **9**, **19** **2a**, 17, **20b**
3	2.819 (d)	9.4	37.4	2, 7	**7**, **14b**
4	--				
5	4.334 (br.dd)	~2.6, ~1.9	72.5		**6a**, **6b**, **20a**
6a 6b	1.97 (o.m) 1.79 (m)		35.7		**5**, **6b**, 7 **5**, **6a**, **19**
7	5.562 (dd)	11.1, 5.2	69.5	8, 19, **169.7**	**3**, **6a**, **10**
8	--		**44.9**		
9	5.80 (br.)		**77.1**		**2a**, **19**
10	6.321 (br.d)	~8.9	69.5		**7**, **18**
11	--		**136.5**		
12	--		**146.4**		
13	5.460 (br.t)	~7	79.5		**14a**, **17**, **18**
14a 14b	2.480 (dd) 1.30 (o.m)	13.9, 7.5	43.9	15	**13**, **14b**, 16 voir 17
15	--		**75.3**		
16	1.301 (s)		24.8	11, 15, Me17	**17**, **13**
17	1.097 (s)		26.8	11, 15, Me17	**2b**, **3**, **14a** (overlap 14b)
18	1.899 (s)		11.7	11, 12, 13	**10**, *13*
19	0.845 (br.d)		12.6	3, 7, 8, 9	**2a**, **6b**, 20b, **9**
20a 20b	5.111 (s) 4.764 (br.s)		111.4	5 *5*	**5**, **20b** **2b**, **20a**
OAc	2.045 (s) 2.028 (s) 1.984 (s) 1.961 (s)		21.0 21.0 20.6 20.6	169.8 171.0 169.7 167.7	
OH	2.73 (br.)				

Figure B. (Nouveau taxane #1) 1β,2α,9α-trihydroxy-10β-acétoxy-5α-cinnamoyloxy-3-11-cyclictaxa-4(20)-ene-13-one



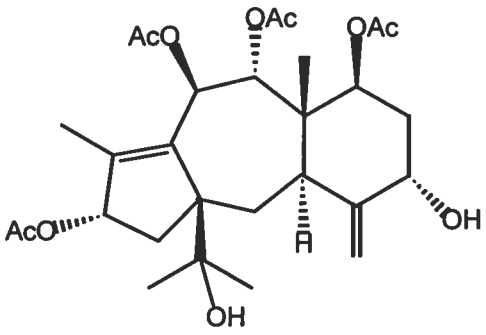
1β,2α,9α-trihydroxy-10β-acétoxy-5α-cinnamoyloxy-3-11-cyclictaxa-4(20)-ene-13-one (nouveau taxane #1) a été identifiée à partir d'un échantillon. Entre 2 et 3 mg au total ont été isolés. Le spectre de RMN ^{1}H de ce nouveau taxane a démontré les signaux caractéristiques d'un taxane (Appendino, 1995 :22 :55). La présence d'un groupe cinnamique a été démontrée par les signaux à δ6.39 (1H, *d*, *J*=16.1 Hz), 7.66 (1H, *d*, *J*=16.1, *trans*-orientation), 7.55 (2H, m), et 7.38 (3H, m) et par le fragment à *m/z* 131 (C_9H_7O) et $(M\text{-cinn})^+$ au spectre de HR FAB-MS correspondant à la fission du groupe cinnamique de la molécule. L'analyse du spectre de RMN ^{1}H a démontré que le nouveau taxane avait quelques caractéristiques spectrales différentes des taxanes normaux : le signal de H-3α, qui est normalement à δ3.2-3.6 avec un valeur de J environ de 5.0-6.0 Hz, était absent; un des groupes méthyle a donné un doublet à δ1.31 (d, *J*=7.3 Hz, 3H), qui était couplé à un signal quartet à δ3.49 (1H, *q*, *J*=7.3 Hz) au spectre de ^{1}H-^{1}H COSY. Ces signaux ont été assignés au Me-18 et H-12 respectivement. La structure du nouveau taxane a donc été établie comme étant un 3,11-cyclotaxane. Les corrélations à longue portée de C-H ont permis l'identification du composé tel que démontré et la stéréochimie a été déterminée par NOESY.

Figure C. (Nouveau taxane #2) 2α,9α,10β-triacétoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one



2α,9α,10β-triacétoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one (Nouveau taxane #2) a été identifié à partir d'un échantillon; un mélange de deux composés avec une proportion de 2 :1, c'est à dire de 1-2 mg et de 0.5-1 mg respectivement. Le composé majeur a été identifié comme étant le **5-decinnamoyl-taxinine B-11,12-oxide**, un taxane inconnu chez l'if du Canada. Le composé mineur était le **2α,9α,10β-triacetoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one** (nouveau taxane #2). Chacun de ces composés a été identifié par RMN et par spectroscopie de masse. Le spectre de RMN ^{1}H de ce nouveau taxane a démontré des signaux caractéristiques d'un taxane (Appendino, 1995 :22 :55). L'absence des signaux caractéristiques d'un groupe méthylène exocyclique et la présence des signaux de deux carbones dans la région oléfinique a démontré que la double liaison en C-4 est endocyclique et non exocyclique. Le déplacement du signal de C-13 vers le bas-champs et le déplacement du signal de Me-18 vers les hauts champs, ainsi que celle de H-10 et de Me-16, et le manque d'autres carbones oléfinique dans le spectre de RMN ^{13}C a indiqué la présence d'un époxyde d'un taxane avec un 11,12-époxide au lieu d'une double liaison au C-11,12 (δ65.1 et δ59.6). Cette conclusion a été supportée par le spectre HMBC et la composition moléculaire. Le H-10 du nouveau taxane était en résonance à δ5.20, qui est situé plus dans le domaine des hauts champs que normalement à cause de l'effet d'anisotropie de l'anneau d'époxyde C-11,12. La stéréochimie relative du nouveau taxane #2 a été établie selon l'analyse des données de NOESY, les déplacements chimiques et leurs constantes de couplage. En se basant sur ces données, la structure du nouveau taxane #2 a été établie comme étant celle de 2α,9α,10β-triacetoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one.

Figure D. (Nouveau taxane #3) 7β,9α,10β,13α-tetraacetoxy-11(15→1)*abeo*taxa-4(20),11-diene-5α,15-diol



7β,9α,10β,13α-tetraacetoxy-11(15→1)*abeo*taxa-4(20),11-diene-5α,15-diol (**Nouveau taxane #3**) a été identifié à partir d'un échantillon. Un poids total de 8 à 9 mg a été isolé. Le spectre de RMN ^{1}H de ce nouveau taxane a démontré les signaux caractéristiques d'un taxane (Appendino, 1995 :22 :55). Au spectre de HMBC, le signal à δ62.8 ppm a été attribué au C-1, mais était situé plus vers le domaine des bas champs que d'habitude pour un taxane avec un squelette tricyclique de 6/8/6 carbones qui résonnent à l'entours de δ43-46 ppm (Appendino, 1995: 22: 55). Ceci suggère que le nouveau taxane #3 avait un squelette de 5/7/6 carbones (Shen, Chen et Hung, 2000: 48(9): 1344). L'absence de pics croisés entre H_3-16, H_3-17 et les carbones oléfiniques C-11 au spectre HMBC supporte aussi l'élucidation d'une structure 5/7/6.

Le **7β-Acetoxytaxinine A** a été identifié de trois échantillons pour un poids total de 4-5 mg. Le premier échantillon était un mélange de trois composés, le **7β-acetoxytaxinine A**, le **5-decinnamoyl-taxuspine D** et le **baccatin III,** avec un ratio de 1 :1 :1. Le deuxième échantillon était un mélange de deux composés avec une proportion de 65 :35. Ceci correspond à **4-5 mg** de **5-decinnamoyl-taxuspine D** et **2-3 mg** de **7β-acetoxytaxinine A**. Le dernier échantillon, ayant la même composition que le premier échantillon, a donné **1-2 mg** de **7β-acetoxytaxinine A**. Ce composé a déjà été isolé des tiges et aiguilles de *Taxus mairei.*

Le **Baccatin III** a été obtenu de deux échantillons qui contiennent le **7β-acetoxytaxinine A**, pour un poids total de **1-2mg**. Ce composé a déjà été isolé de l'écorce, des tiges, des racines et des aiguilles de *Taxus baccata*, *Taxus brevifolia*, *Taxus cuspidata*, *Taxus wallichiana*, *Taxus yunnanensis* et *Taxus mairei.*

Le **5-DecinnamoyI-taxinineB-11,12-oxide** a été identifié du même échantillon que le **2α,9α,10β-triacetoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one** (nouveau taxane #2), pour un poids total de **1-2 mg**. Ce composée a déjà été isolé des aiguilles et tiges de *Taxus yunnanensis*.

Le **5-Decinnamoyl-taxinine J** a été obtenu de trois échantillons. Le premier échantillon consiste en **3-4 mg** de **5-decinnamoyl-taxinine J** pur, tandis que le deuxième échantillon consiste en un mélange de **taxayuntin** et **5-decinnamoyl-taxinine J** avec une proportion de 2:1. Ce mélange a donné un autre **1-2 mg** de **5-decinnamoyl-taxinine J**. Le dernier échantillon consiste en un mélange de trois composés, **5-decinnamoyl-taxinine J**, **taxayuntin** et **taxinine A**, du quel le **5-decinnamoyl-taxinine J** est majoritaire avec un poids de **6-7 mg**. Ce composé a déjà été isolé de l'écorce de *Taxus brevifolia*, *Taxus yunnanensis* et *Taxus mairei*.

Taxayuntin a été obtenu de deux échantillons avec le **5-decinnamoyl-taxinine J**. Un poids total de **7-8 mg** a été isolé. Ce composé a déjà été isolé des aiguilles de *Taxus baccata*, *Taxus cuspidata*, *Taxus wallichiana* et *Taxus yunnanensis*.

Taxuspine W a été obtenu d'un échantillon pour un poids total de **3-4 mg**. Ce composé a déjà été isolé des aiguilles et tiges de *Taxus cuspidata*.

Le **baccatin IV** a été obtenu de deux échantillons. Le premier échantillon a donné **4 mg** de baccatin IV alors que le deuxième a donné **1-2 mg**. Ce composé a déjà été isolé de *Taxus baccata, Taxus brevifolia, Taxus mairei* et *Taxus wallichiana.*

Taxinine M a été obtenu d'un échantillon pour un poids total de **6-7 mg**. Ce composé a déjà été isolé de l'écorce de *Taxus brevifolia* et *Taxus cuspidata.*

1β-Hydroxy-2,7,9-trideacétyle-baccatin I a été obtenu d'un échantillon pour un poids total de **3-4 mg**. Ce composé a déjà été isolé de l'écorce de *Taxus yunnanensis.*

Baccatin VI a été obtenu de trois fractions pour un poids total de **8-9 mg**. Ce composé a déjà été isolé de l'écorce de *Taxus baccata.*

13-Acétyle-13-decinnamoyltaxchinin B a été obtenu d'un échantillon pour un poids de **1-2 mg**. Ce composé a déjà été isolé des aiguilles de *Taxus baccata.*

Taxuspine L a été obtenu d'un échantillon pour un total de **3-4 mg.** Ce composé a déjà été isolé des tiges de *Taxus chinensis.*

7,13-Deacétyle-9,10-debenzoyltaxchinin C a été identifié d'un échantillon pour un poids de **2-3 mg**. Ce composé a déjà été isolé de *Taxus brevifolia.*

Taxinine NN-3, aussi connue comme le **9-deacétyletaxinine**, a été obtenu d'un échantillon pour un poids total de **0.5-1.0 mg**. Ce composé a récemment été isolé des aiguilles et jeunes tiges de *Taxus cuspidata.*

Le **Paclitaxel** été obtenu de quatre échantillons. Un total de **31-32 mg** a été isolé. Ce composé a déjà été isolé de l'écorce, des tiges, racines et aiguilles de *Taxus baccata, Taxus brevifolia, Taxus canadensis, Taxus chinensis, Taxus cuspidata, Taxus media, Taxus wallichiana* et *Taxus yunnanensis.*

Le **10-deacétyle-céphalomanine** a été obtenu de deux échantillons pour un poids total de**11-12 mg**. Ce composé a déjà été isolé des racines, de l'écorce, des tiges et des aiguilles de *Taxus baccata, Taxus brevifolia, Taxus cuspidata, Taxus wallichiana* et *Taxus yunnanensis.*

Le **10-deacétyle-baccatin III** a été obtenu de trois échantillons pour un poids total de **38-39 mg.** Ce composé a déjà été isolé des aiguilles, tiges et de l'écorce de *Taxus baccata, Taxus brevifolia, Taxus cuspidata, Taxus wallichiana, Taxus canadensis, Taxus yunnanensis* et *Taxus mairei.*

Le **9-dihydro-13-acétyle-baccatin III** a été obtenu de quatre échantillons pour un poids total de **26-27 mg**. Ce composé a déjà été isolé des aiguilles de *Taxus canadensis, Taxus brevifolia* et *Taxus chinensis.*

Taxinine et **2α-deacetoxytaxinine J** ont été obtenus d'un échantillon. Chaque composé est present dans une proportion de 1:1 pour un total de **19-20 mg** de chacun. Le **taxinine** a déjà été isolé des aiguilles de *Taxus canadensis, Taxus chinensis, Taxus cuspidata* et *Taxus mairei.* Le **2α-deacetoxytaxinine J** a déjà été isolé de l'écorce de *Taxus baccata, Taxus chinensis, Taxus cuspidata, Taxus mairei, Taxus wallichiana* et *Taxus yunnanensis.*

Le **céphalomanine** a été obtenue d'un échantillon pour un poids total de **7-8 mg**. Ce composé a déjà été isolé de l'écorce, des aiguilles, tiges et racines de *Taxus baccata, Taxus brevifolia, Taxus canadensis, Taxus cuspidata, Taxus media, Taxus wallichiana* et *Taxus yunnanensis.*

Le **5-decinnamoyl-taxuspine D** a été obtenu de quatre échantillons pour un poids total de **12-13 mg**. Ce composé a déjà été isolé des aiguilles de *Taxus canadensis.*

Le **taxinine A** a été obtenu de trois échantillons pour un poids total de **15-16 mg**. Ce composé a déjà été isolé des aiguilles de *Taxus chinensis, Taxus cuspidata, Taxus media* et *Taxus canadensis.*

Le **5-*epi*-canadensène** a été obtenu d'un échantillon avec un poids de **2-3 mg**. Ce composé a déjà été isolé des aiguilles de *Taxus canadensis.*

Le **2-deacétyle-5-decinnamoyltaxinine J** a été obtenu de deux échantillons pour un poids total de **7-8 mg**. Ce composé a déjà été isolé des aiguilles et/ou l'écorce de *Taxus canadensis, Taxus chinensis, Taxus wallichiana* et *Taxus yunnanensis.*

Le **5-decinnamoyltaxagifine** a été obtenu de quatre échantillons pour un poids total de **52-53 mg**. Ce composée a déjà été isolé des aiguilles et tiges de *Taxus canadensis* et *Taxus chinensis.*

- **Résultats : Étude de la composition des boutures de *Taxus cuspidata capitata***

Trois nouveaux taxanes et trente-neuf taxanes connus ont été identifiés de l'extrait de dichlorométhane des boutures de *Taxus cuspidata* par Dr Qing-Wen Shi. La publication de ce travail est en progression.

- **Discussion : Étude de la composition des boutures de *Taxus canadensis***

Vingt neuf taxanes ont été identifiés de l'extrait de bouture de *Taxus canadensis*. De ces taxanes, trois étaient des nouveaux taxanes, quatorze étaient des taxanes jamais purifiés auparavant chez l'if canadien, et douze étaient des taxanes déjà connus chez l'if canadien. Cette étude était la première étude où la composition des boutures d'ifs a été étudiée.

La comparaison de la composition des boutures de *Taxus canadensis*, décrite en haut, avec la composition du *Taxus canadensis* mature, déjà connue dans notre laboratoire, a démontré quelques divergences importantes. Premièrement, seize des vingt neuf taxanes isolés des boutures n'ont jamais été isolés chez l'if canadien mature. À noter que plus de soixante-dix taxanes ont déjà été isolées chez l'if canadien. Ces résultats

suggèrent qu'une différence existe dans la composition des boutures *versus* la composition des plantes matures. En plus, les taxanes isolés en majorité des boutures d'if canadien diffèrent des taxanes majeures d'if canadien mature. Le 9-dihydro-13-acétyle-baccatin III, le taxinine et le taxinine E sont les trois taxanes majeures chez l'if canadien mature (Zamir *et al.*, 1992:33:36:5173, Zamir *et al.*, 1995:73:655), tandis que les taxanes majeurs chez les boutures de l'if canadien sont le 5-decinnamoyl-taxagifine, 10-deacétyle-baccatin III et le paclitaxel. Le plus significatif de ces résultats est la différence en quantité du 9-dihydro-13-acétyle-baccatin III isolée des boutures. Le 9-dihydro-13-acétyle-baccatin III se retrouve normalement en quantités de trois à sept fois plus abondantes que le paclitaxel chez l'if canadien mature, peu importe la saison ou la provenance de l'if (Zamir *et al.*, 1995:73:655). Chez les boutures étudiées, le 9-dihydro-13-acétyle-baccatin III a été isolé en quantité plus petite que le paclitaxel. Ceci est intéressant car le 9-dihydro-13-acétyle-baccatin III est retrouvé presque exclusivement chez l'if canadien, avec seulement de petites quantités retrouvées chez d'autres espèces d'ifs (Zhang, Chen et Chen, 1992:27:4:268). L'absence de taxinine E, un des composés majeurs chez l'if canadien mature ainsi que chez les boutures est aussi intéressante. Il est important de noter que les mélanges obtenus à partir de l'extrait des boutures n'ont pas tous été purifiés, et alors il demeure possible que le taxinine E reste dans une de ces fractions impures. Le 9-dihydro-13-acétyle-baccatin III, le 10-deacétyle-baccatin III, le paclitaxel et le céphalomanine ont été purifiés entièrement et c'est donc avec certitude que la comparaison de ces composées est rapportée. La présence d'une grande quantité de 5-decinnamoyl-taxagifine isolée des boutures est aussi intéressante puisque ce n'est pas un composé majeur chez l'if canadien mature. Même si ce composé a déjà été isolée de l'if du Canada, il n'était pas isolé en quantité plus grande que le 9-dihydro-13-acétyle-baccatin III, le taxinine ou le taxinine E. Le **tableau A** suit avec les vingt-neuf taxanes isolés des boutures de *Taxus canadensis* et listés par l'ordre des quantités isolées.

Tableau A. Liste des taxanes isolés des boutures de *Taxus canadensis* et leurs quantités.

nt : nouveau taxane, **ntic** : nouveau taxane chez l'if canadien, **cic** : connu chez l'if canadien

Échantillon	**Quantité**
5-decinnamoyl-taxagifine **cic**	52-53 mg
10-deacétyle-baccatin III **cic**	38-39 mg
Paclitaxel **cic**	31-32 mg
9-dihydro-13-acétyle-baccatin III **cic**	26-27
Taxinine **cic**	19-20
2α-deacetoxytaxinine J **cic**	19-20
Taxinine A **cic**	15-16 mg
5-decinnamoyl-taxuspine D **cic**	12-13 mg
10-deacétyle-cephalomanine **cic**	11-12 mg
5-decinnamoyl-taxinine J **ntic**	11-12 mg
Baccatin VI **ntic**	8-9 mg
Nouveau taxane #3 **nt** 7β,9α,10β,13α-tetraacetoxy-11(15→1)*abeo*taxa-4(20),11-diene-5α,15-diol	8-9 mg
Taxayuntin **ntic**	7-8 mg
Cephalomanine **cic**	7-8 mg
2-deacétyle-5-decinnamoyl-taxinine J **cic**	7-8 mg
Taxinine M **ntic**	6-7 mg
Baccatin IV **ntic**	5-6 mg
7β-acetoxy-taxinine A **ntic**	4-5 mg
Taxuspine W **ntic**	3-4 mg
Taxuspine L **ntic**	3-4 mg
1β-hydroxy-2,7,9-trideacétyle-baccatin I **ntic**	3-4 mg
Nouveau taxane #1 **nt** 1β,2α,9α-trihydroxy-10β-acetoxy-5α-cinnamoyloxy-3-11-cyclictaxa-4(20)-ene-13-one	2-3 mg
Baccatin III **ntic**	2-3 mg
7,13-deacétyle-9,10-debenzoyltaxchinin C **ntic**	2-3 mg
5-*epi*-canadensène **cic**	2-3 mg
5-decinamoyl-taxinineB-11,12-oxide **ntic**	1-2 mg

13-acétyle-13-decinnamoyl-taxchinin B **ntic**	1-2 mg
Nouveau taxane #2 **nt** 2α,9α,10β-triacetoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one	0.5-1 mg
Taxinine NN-3 **ntic**	0.5-1 mg

Les différences observées entre le contenu de taxanes chez l'if canadien mature et chez les boutures de l'if canadien suggèrent qu'une étude plus détaillée de la composition des boutures devra être réalisée et que l'utilisation et l'étude des boutures de *Taxus canadensis* dans notre laboratoire devra être continuée. La différence en contenu des taxanes suggère que l'équilibre de la plante est changé avec la création des boutures de l'if mature, et qu'une différence dans la biosynthèse des taxanes chez les boutures pourrait aussi exister. Si des protocoles efficaces sont établis pour faire des nutritions d'homogénats de plante, de sections de plantes ou des boutures avec des précurseurs radioactifs, les différences possibles entre la biosynthèse des taxanes chez les boutures ou chez les plantes matures encourage ces types d'expériences. Les différences déjà démontrées entre l'if canadien et les autres espèces d'ifs rendent l'étude des boutures de l'if canadien encore plus excitante. Ce type d'étude pourra donner des résultats importants pour mieux permettre l'étude de la biosynthèse des taxanes, leur fonction dans la plante et aussi la découverte de nouveaux taxanes.

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Merci, Thank you,

Tracy

1. Introduction

While great progress has been made in the quest to find a cure for cancer, it still remains one of the primary causes of death in North America and throughout much of the world (Nicolaou, Dai and Guy, 1994:33:15). Despite the many drugs and forms of treatments which have been developed and are currently in use, the identification of novel treatments with fewer side effects is still needed.

Anti-cancer drugs unfortunately come with many severe side-effects as the drugs not only target and kill the cancerous cells, but also kill the rapidly dividing healthy cells of the body. To compound the problem, many cancers are developing a resistance to the drugs currently used for treatment. This further emphasises the need for the development of drugs with less side effects and which have a unique mode of action to which resistant cancers may respond (Nicolaou, Guy and Potier, 1996:274:96).

The discovery of paclitaxel (Taxol®, Bristol-Myers Squibb) raised hope because of its novel anticancer properties. Paclitaxel, a taxane isolated originally from the Pacific Yew tree, *Taxus brevifolia* Nutt. (Wani *et al.*, 1971:93:2325). Paclitaxel is an antimitotic drug similar to the vinca alkaloids, which are also natural products isolated instead from the periwinkle. Although paclitaxel and the vinca alkaloids both target the spindle assembly of the mitotic cell, they differ in their specific mode of action. Whereas the vinca alkaloids promote depolymerisation of the tubulin assembly (Hezari and Croteau, 1997: 63: 291), taxanes such as paclitaxel stabilize the tubulin assembly (Fuchs *et al.*, 1978: 62: 1219, Schiff, Fant, and Horwitz, 1979, 277:5698:665). Stabilization is achieved by the binding of paclitaxel to the N-terminal 31 amino acids of the β-tubulin subunit in the microtubule and thereby blocking mitosis at the G2/M boundary (Rowinsky and Donehower, 1995:332:15: 1004). This variation in the mode of action gives hope that paclitaxel will be more effective and used to treat cancers which have developed resistance to other conventionally used drugs (Nicolaou, Guy and Potier, 1996:274:96). Since the discovery of paclitaxel, other natural products have shown a similar mode of action, such as the epothilones which were first extracted from the bacterium *Sorangium* and identified by X-Ray crystallography (He *et al.*, 2001:6:22:1153). Further studies however, suggest that in addition to its microtubule

stabilizing action, paclitaxel also acts by other means. These include interacting directly with a suppressor of apoptosis Bcl-2 (Rodi *et al.*, 1999: 285: 1: 197), by stimulating the release of tumour necrosis factor (TNF-α) and Interleukine-I (IL-I) as well as down-regulating the number of tumour necrosis factor receptors (TNF-2) (Heinstein and Chang, 1994:45:663, Burkhart *et al.*, 1994: 54:22:5779, O'Brien *et al.*, 1995: 154:8:4113). While these effects have not been shown to increase the cytotoxicity of paclitaxel, they may influence its ability to treat certain resistant cancers. Paclitaxel-like compounds have already been shown to improve the efficiency of drug delivery to tumours by altering the vascular surface and thus the interstitial fluid pressure within the tumour (Griffon-Etienne *et al.*, 1999:59:3776). Studies also show that these compounds have an inhibitory action on human cancer cell lines expressing classic multidrug resistance, MDR (Distefano *et al.*, 1997:72:5:844, Hosoyama *et al.*, 1999:9:389). Paclitaxel has already been shown to be active against a variety of solid tumours such as breast, ovarian and lung tumours as well as some leukemias and is currently approved by the FDA for treatment of both ovarian and breast cancers. For paclitaxel to become an ideal chemotherapeutic agent however, there are many side effects and problems which need to be overcome.

Paclitaxel has been shown to often lower the baseline neutrophil count of the patient (Nicolaou, Dai and Guy, 1994:33:15). Another problem is the low solubility of paclitaxel in water (0.03 mg/mL) thus making it difficult to effectively and comfortably administer the drug to the patient. The resulting formulation of paclitaxel in Cremophor EL causes the additional problem of sometimes-severe allergic reactions (Nicolaou, Dai and Guy, 1994:33:15). While these problems are without doubt unfavourable, progress has been made to overcome them. Supplementing with granulocyte colony stimulating factor may lessen the negative effects on the bone marrow (Nicolaou, Dai and Guy, 1994:33:15). By modifying the structure of paclitaxel its bioactivity is retained while its solubility in water is increased. An analogue of paclitaxel, named docetaxel or Taxotère® (Rhone-Poulenc Rorer) retains the bioactivity of paclitaxel, but is more water-soluble due to a modified side chain on C-13 (see **figure 1**). Co-administration of drugs to counteract the allergic reactions sometimes observed with the formulated paclitaxel is common (Nicolaou, Dai and Guy, 1994:33:15), and interestingly, it has been demonstrated that the

Cremophor EL itself may play an important role in the active properties of the paclitaxel formulation (Suffness and Wall, 1995:1:3).

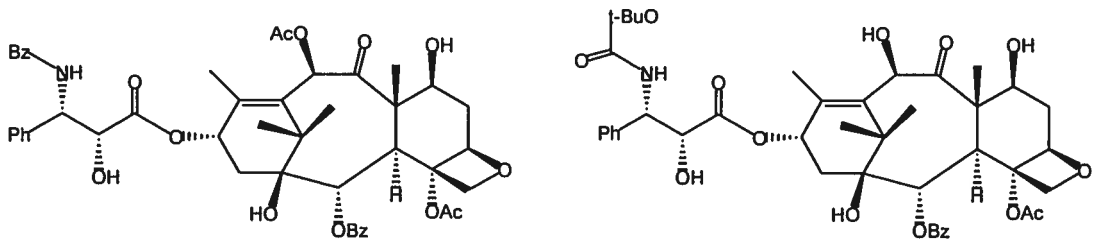


Figure 1. Left to right: Paclitaxel (Taxol®, Bristol-Meyers Squibb) and Docetaxel (Taxotère®, Rhone-Poulenc Rorer)

One of the gravest problems surrounding paclitaxel however is its limited supply and the inferred high cost of treatment due to the drug's scarcity. Although originally purified from the bark of the Pacific Yew tree, paclitaxel is also found in the needles of the tree as well as in other yew species and in some fungi (Baloglu and Kingston, 1999:62:1448, Kingston, 2001:10:867). More recently, it has been reported that small quantities of paclitaxel have been isolated from the Hazelnut and its associated fungal endophytes (Hoffman *et al.*, 2000). The quantity of the compound available from all natural sources however is low. With a drug content of approximately 0.01% per dry weight of bark, the bark of three yew trees is needed to treat one patient (Witherup *et al.*, 1990:53:5:1249, Rohr *et al.*, 1997:36:20:2190). The slow and difficult growth of these trees further complicates this problem. The amount of paclitaxel and the other taxanes varies with the physiology of the plant as well as with the season, however never reaches concentrations great enough for commercial use (Wheeler *et al.*, 1992: 55:4:430, Vance *et al.*, 1994: 36:5:1241). Although several successful and elegant syntheses of paclitaxel have been reported, synthesis of the compound for distribution is not feasible due to the complexity of the reactions involved, their low yields and their high cost (Masters *et al.*, 1995: 34:16:1723, Holton *et al.*, 1994: 116:1597, Nicolaou *et al.*, 1994: 367:6464:630, Nicolaou *et al.*, 1995: 117:2:624). At present, a semi-synthetic version of the drug is distributed, however even this is dependant on a natural source as it involves the modification of an intermediate, 10-deacetyl-baccatin III (10-DAB-III), which is isolated from the European Yew, *Taxus baccata* (Guenard, Guéritte-Voegelein, and Potier, 1993:

26:4:160, Rohr, 1997:36:20:2190). Although this provides a source of paclitaxel for current treatment, the cost is still high at several hundred US dollars per gram of the substance. It is therefore imperative to find a new means of production of the drug, perhaps via genetically engineered cell lines (Rohr, 1997: 36:20:2190, Vaněk *et al.*, 1999: 65:275). This requires advanced knowledge of the biosynthesis of paclitaxel, a process that is not fully understood. Information on the conditions of its biosynthesis would allow a large-scale production to be developed. For this reason, research on the biosynthesis of paclitaxel is very important. The study of the biosynthesis of paclitaxel may lead to not only the eventual increase in production of paclitaxel and its derivatives, but may also allow the discovery of new taxanes. These new taxanes may themselves be of great importance in the fight against cancer (Kingston, 2001,10:861, Patel, 1998:98:361).

Understanding the biosynthesis of paclitaxel involves elucidating numerous multi-step pathways. Although understanding the key steps in the biosynthesis of paclitaxel is paramount, one must not ignore the associated shunt or dead-end pathways. The compounds from these associated pathways may influence the biosynthesis of paclitaxel or even prove to be more active than paclitaxel itself. It should also be noted that paclitaxel might not be the final product of the biosynthetic route within the plant, but rather a precursor to other taxanes.

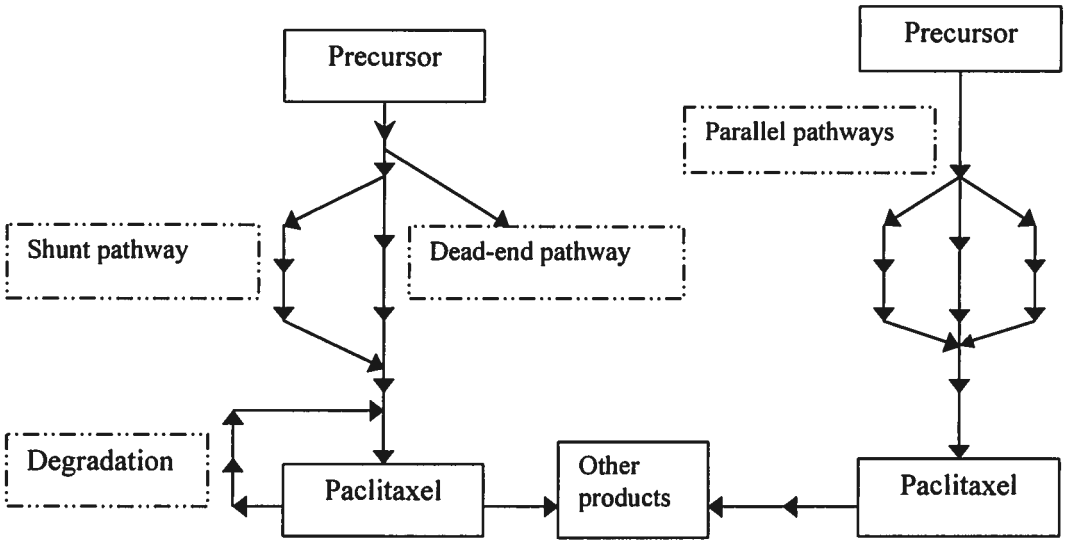


Figure 2. Schematic representation of dead-end, shunt pathways, and parallel running pathways.

Two types of studies are typically used to help elucidate the biosynthesis of taxanes. The first method consists of feeding radioactively labelled taxane precursors to an intact or prepared fragment of a yew tree, or to cultured cells of *Taxus* (Zamir, Nedea and Garneau, 1992:33:36:5235, Fleming *et al.* 1994:116:4137). NMR spectroscopy or chemical degradation techniques are then used to observe incorporation and more importantly the pattern of incorporation of radiolabelled precursors into paclitaxel and other taxanes.

The second type of study involves examining the taxane content of the numerous species of yew. Taxanes are isolated from the trees and classified based on structural differences. This classification later facilitates the comparison of taxane content between species of yew (Baloglu and Kingston, 1999:62:1448, Parmar *et al.*, 1999:50:1267). Studying the structure types and relative quantities of taxanes may help elucidate a biosynthetic pathway. For example, the predominance of a certain taxane may suggest its importance in a possible biosynthetic route or that it is the result of a dead-end pathway. Also, determination of structural similarities between the various taxane classes may help to understand the structure-activity relationships (Kingston, 2001:10:867). With the isolation of such taxanes also comes the potential that one of these taxanes may itself have anti-cancer activity equal to or greater than paclitaxel or that it might prove to have less side-effects if used therapeutically.

With paclitaxel being one of the most promising anti-cancer drugs available and given the huge proportion of drugs which are derived from natural sources, the investigation of the biosynthesis of paclitaxel and the isolation of new taxanes is most certainly warranted and of great importance in the fight against cancer.

2. Literature revue

2.1 The discovery of paclitaxel and its importance.

Paclitaxel is the largest selling anticancer drug of all time (Kingston, 2001:10:867). A discovery which was plagued with bad politics due to environmental issues as well as a delayed entrance into clinical trials nonetheless produced a drug of great interest to not only the research community but also to the public. Bioactivity was first observed in 1964 in sample extracts of *Taxus brevifolia* Nutt. that were originally collected in 1962 as a part of a huge initiative taken by the National Cancer Institute (NCI) in collaboration with the US Department of Agriculture, to explore natural products as the source of novel anti-cancer drugs (Kingston, 2001:10:867). The identity of paclitaxel as the compound responsible for the activity observed in the extract was unknown until the diterpene was isolated in 1967 by Dr M. Wall, which was followed by its structure characterization in 1971.

Figure 3. The Pacific Yew, *Taxus brevifolia*

While paclitaxel showed anti-cancer activity in the various bioassays used by the NCI at that time, the results were not overly impressive and this combined with the many other problems surrounding paclitaxel, created a rocky start for the compound. One of the gravest problems surrounding paclitaxel was that it was only isolated in low yield from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. This was further complicated by the slow growth of this uncommon tree in the old growth forests of the Pacific Northwest, the habitat of the endangered spotted owl. The harvesting of the bark of these trees ultimately results in the death of the tree and therefore the depletion of this natural source. This was a scenario that pitted cancer patients and the pharmaceutical industry against environmental conservationists and stirred the public interest in this compound (Kingston,

2001:10:867). The weak solubility of paclitaxel in water and its therefore difficult formulation was an added predicament. Fortunately, paclitaxel was screened again using bioassays newly employed to the NCI in the 1970s and the resulting strong activity observed in one of the models was greatly encouraging and bought much support for further studies (Kingston, 2001:10:867). Its subsequent development as a drug candidate resulted in paclitaxel's formulation in Cremophor EL which itself created both a new problem as well as a possible new advantage. The problem with this formulation is a severe allergic response to the Cremophor EL observed in many patients, which can thankfully be counteracted by the co-administration of other drugs. Studies however showed that Cremophor EL is perhaps capable of reversing multidrug resistance, suggesting that its use in the formulation of the drug may be beneficial (Suffness and Wall, 1995:1:3).

The elucidation of paclitaxel's mode of action in 1979 by Susan Horwitz was key in further promoting interest in this potential anti-cancer drug. It was demonstrated that paclitaxel acts by promoting the assembly of tubulin proteins and stabilizing the microtubules (Fuchs *et al.*, 1978: 62: 8: 1219, Schiff, Fant and Horwitz, 1979: 277: 5698: 665). While other compounds act on tubulin, they differ in their action by preventing the assembly of microtubules. Paclitaxel's novel mode of action was therefore of great interest and suggested a benefit over other drugs to which some cancers had already developed resistance. The disruption of the equilibrium between microtubules and tubulin protein by paclitaxel disrupts cell division by blocking the cell cycle at the G2/M boundary and leads to cell death by apoptosis (Rowinsky and Donehower, 1995:332:15:1004). Although other compounds have since been found which act by the same microtubule stabilizing action, paclitaxel was the first in this category.

Limited by the quantity of the compound available, paclitaxel nonetheless went into Phase I and Phase II trials and showed activity against breast and ovarian cancers significant enough to warrant their future use therapeutically (Kingston, 2001:10:867). The demand created by this success in turn caused a strain on the supply of paclitaxel. An alternate source of paclitaxel was required to afford its production of a scale large enough to provide the market with the quantities needed. This was accomplished when a semi-synthetic route of production was established and paclitaxel was formed through the

intermediate 10-deacetyl-baccatin III (Chauvière *et al.*, 1981:2:293:501, Holton *et al.*, 1995:288). Although this allowed the supply of paclitaxel to meet its rising demand, it still remains dependant on a natural source and thus a more reliable source is still ideal. While several successful syntheses of paclitaxel have been reported, they remain impractical for distribution due to the complexity of the reactions involved and their high cost (Masters *et al.*, 1995:34:16:1723, Holton *et al.*, 1994:116:4:1597, Nicolaou *et al.*, 1994:367:6464:630, Nicolaou *et al.*, 1995:117:2:624). Conversely, much progress has been made using cell culture and this remains a viable possibility for future supply of paclitaxel. Paclitaxel and its analog docetaxel are currently used separately or in combination for the treatment of breast, ovarian, bladder, and cervix cancers, as well as non-small-cell lung carcinoma and the AIDS-related Karposi's sarcoma (Kingston, 2001:10:867).

Paclitaxel has more recently been shown to bind another target, the anti-apoptotic protein Bcl-2 (Rodi *et al.*, 1999:285:1:197) and to induce its phosphorylation. Phosphorylation of this anti-apoptotic protein induces apoptosis although it is unknown if this second mechanism for inducing cell death is related or not to paclitaxel's effect on tubulin assembly. The many possible anti-cancerous properties that have been associated with paclitaxel, its mode of action and its highly complex structure make it of great interest to the research community. Despite its isolation three decades ago and many years of work devoted to this compound, there are still many mysteries surrounding it, in particular its biosynthetic pathway. Elucidation of this pathway will without doubt aid in the study of paclitaxel production using cell culture and in achieving an increased yield of the compound by these means (Kingston, 2001:10:867). This may ultimately lower the cost of paclitaxel. Just as importantly, investigation into the biosynthesis of paclitaxel and advances made in the field of yew tree phytochemistry will allow the discovery of novel taxanes which may themselves prove to be of great importance in the fight against cancer.

Although paclitaxel is by far the best know taxane, it is only one of hundreds of taxoids already isolated from the many species of yew (Parmar *et al.*, 1999:50:1267). These taxanes are important not only because they may also prove to be active against cancer or assist in the semi-synthetic production of paclitaxel, but also because their different structures and their relative quantities in the different species of yew may assist

in the elucidation of the biosynthetic pathway of paclitaxel. The distinct structures, along with their different oxygenation patterns may suggest the order of steps involved in the biosynthetic pathway and whether these structures are formed as the result of a shunt pathway, a dead-end pathway or if they are actually a precursor of paclitaxel. The variety of compounds and their relative quantities may also show a significant difference between the phytochemistry of the different species of yew.

2.2 Taxanes

Taxanes may be subdivided into several classes based on their differing characteristics, such as the presence or absence of a paclitaxel-like side chain at C-13. The classification of the taxanes as such facilitates the comparison of structures, and the study of the structure activity relationships.

A "normal" taxane, can be described as one in which the core of the molecule is similar to paclitaxel, with an eight-membered ring sandwiched between two six-membered rings. They may be further classified as to whether this cyclic core has a side chain at C-13 as well as whether an oxetane ring is present. The class of taxanes possessing a side chain and an oxetane ring has been the most studied since it is the class to which paclitaxel belongs and which therefore possesses the characteristics thought to be imperative to anti-cancer activity. If an oxetane ring is absent, the taxane may instead hold a C-4(20) exocyclic double bond, a C-4(20) epoxide or an opened oxetane ring. If a C-4(20) exocyclic double bond is present, an additional characteristic that allows for further differentiation is C-5 and whether it has an acetyl or hydroxyl group, a basic group, or a cinnamoyl group. Another area for distinction is the double bond usually present between C-11 and C-12 of Ring A of the diterpene, but sometimes shared instead between C-12 and C-13. The oxygenation pattern at the various other carbons, in particular at C-9 may differ as well, and may be used as a means for comparison between taxoids present within any of the many different subclasses described (Suffness and Wall, 1995:1:3, Parmar *et al.*, 1999:50:1267, Kingston *et al.*, 1993:61:1).

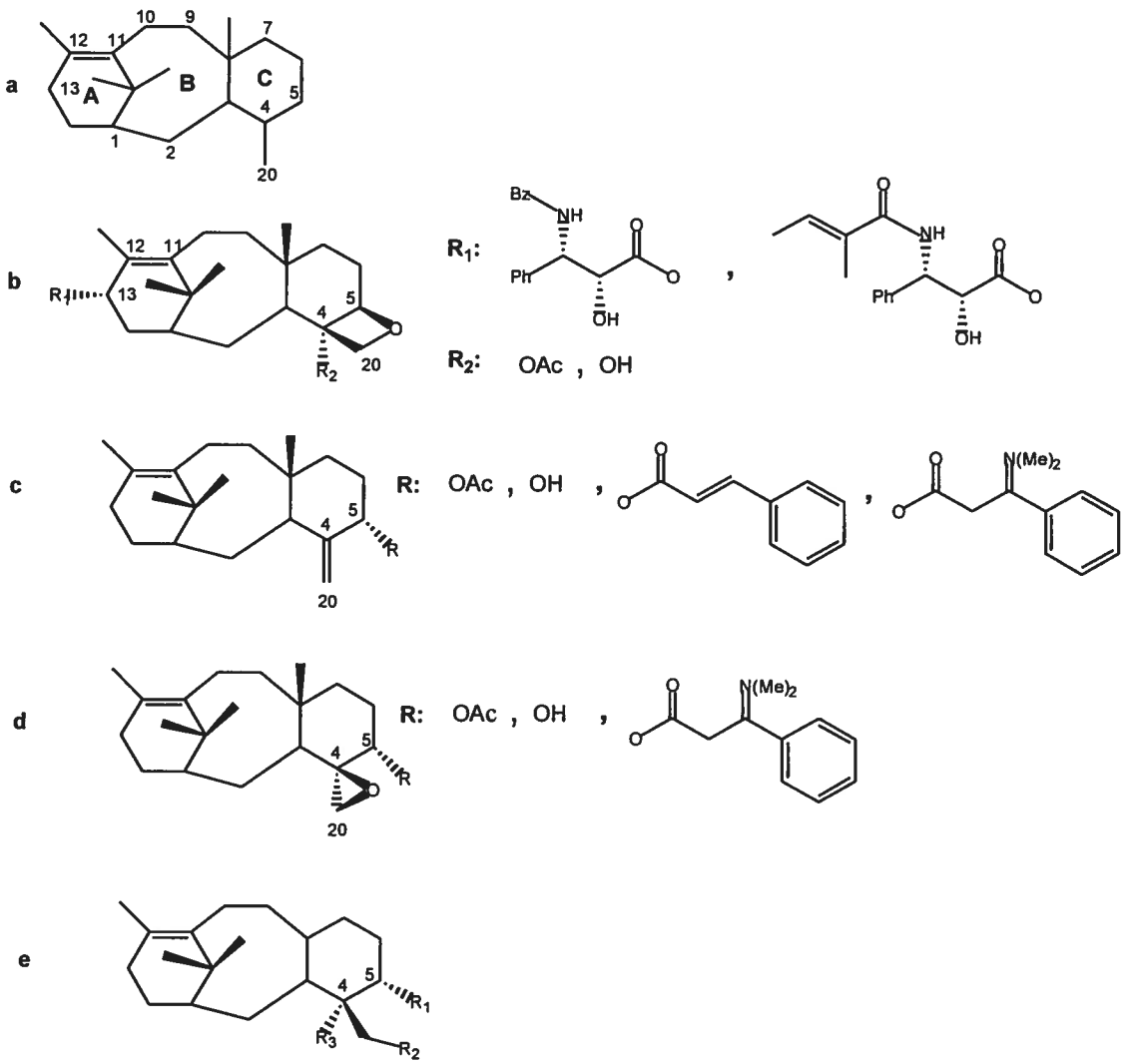


Figure 4. Normal taxanes: **(a)** skeleton structure, **(b)** with oxetane ring and side chain at C-13, **(c)** with C-4(20) exocyclic double bond and acetyl, hydroxyl, basic or cinnamoyl group at C-5, **(d)** with C-4(20) epoxide and acetyl, hydroxyl or basic group at C-5, **(e)** with opened oxetane ring.

A 3,11-cyclotaxane is a taxane with a normal taxane core but with an extra bond between C-3 and C-11. Along with taxanes that possess a C-12(16)-Oxido bridge, this taxane belongs to the group of taxoids that possess a transannular bond (Suffness and Wall, 1995:1:3, Parmar *et al.*, 1999:50:1267, Kingston *et al.*, 1993:61:1).

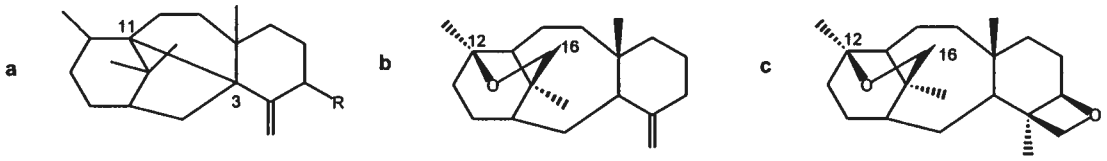


Figure 5. Taxanes with a transannular bond: **(a)** skeleton of a 3,11-cyclotaxane, **(b)** skeleton of a taxane with a C-12(16)-Oxido bridge and a 4(20) exocyclic double bond, **(c)** skeleton of a taxane with a C-12(16)-Oxido bridge and an oxetane ring.

An 11(15→1) abeotaxane is a taxane where the bond normally present between C-11 and C-15 is instead shared between C-11 and C-1, transforming the cyclic core to one which consists of linearly fused rings of five, seven and six carbons. The elucidation of this structure type was not immediate, as many these compounds were first described as having a normal taxane skeleton. When the abeotaxane skeleton was correctly identified, it was proposed that these compounds were perhaps artefacts. The isolation of 11(15→1) abeotaxanes by various research groups and from many different species of yew has since contradicted this proposal and suggested that they are indeed legitimate natural products. 11(15→1) abeotaxanes can be classified under the category of A-Nor taxoids as well, a group to which other compounds that possess an extra oxido bridge also belong. This group of compounds all have five membered "A" rings (Suffness and Wall, 1995:1:3, Parmar *et al.*, 1999:50:1267, Kingston *et al.*, 1993:61:1).

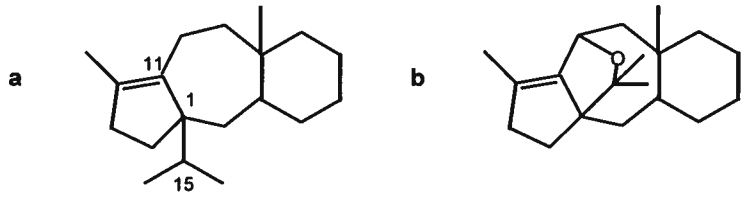


Figure 6. A-Nor Taxoids: **(a)** skeleton of a 11(15→ 1) abeotaxane, **(b)** skeleton of a taxane with a five carbon A ring and an extra oxido bridge.

A 2(3→ 20) abeotaxane is a taxane where the bond normally shared between C-2 and C-3 is instead between C-2 and C-20. This rearrangement results with a ten membered ring sandwiched between two six membered rings (Suffness and Wall, 1995:1:3, Parmar *et al.*, 1999:50:1267, Kingston *et al.*, 1993:61:1).

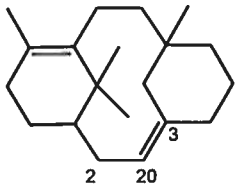


Figure 7. Skeleton of a 2(3→20) abeotaxane.

A very uncommon structure is that of an 11(15→ 1),11(10→9) bisabeotaxane, of which Wallifoliol was the first isolated compound. This rare type of compound has two rearranged bonds; the C-11/C-15 bond is instead shared between C-11 and C-1, and the C-11/C-10 bond is instead shared between C-11 and C-9 (Suffness and Wall, 1995:1:3, Parmar *et al.*, 1999:50:1267, Kingston *et al.*, 1993:61:1).

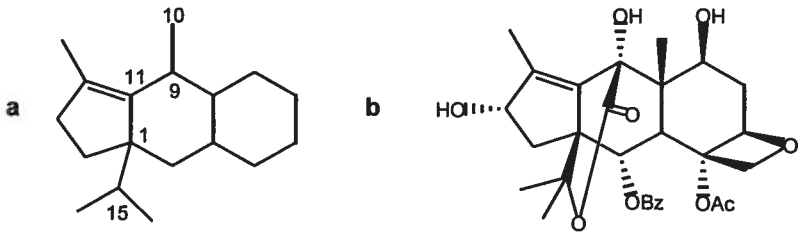


Figure 8. 11(15→1),11(10→9) bisabeotaxane: (a) skeleton, (b) Wallifoliol

One last class of taxanes are the bicyclic taxanes, often refered to as the pretaxoids. Compounds belonging to this group are compounds in which the four rings present in paclitaxel and other normal taxanes have not yet been formed. They therefore show similarity to the verticillyl intermediate proposed as the biogenetic precursor of paclitaxel. 5-Epi-canadensene, isolated from *Taxus canadensis*, is an example of such a type of compound. While these compounds show similarity to proposed intermediates of paclitaxel and are therefore often refered to as pretaxanes, there is no evidence that they are in fact precursors of paclitaxel. Such compounds have an A ring, but the bond normally shared between C-3 and C-8 of the diterpene core is missing and thus the B and C rings normally observed in the taxane skeleton are absent (Suffness and Wall, 1995:1:3, Parmar *et al.*, 1999:50:1267, Kingston *et al.*, 1993:61:1).

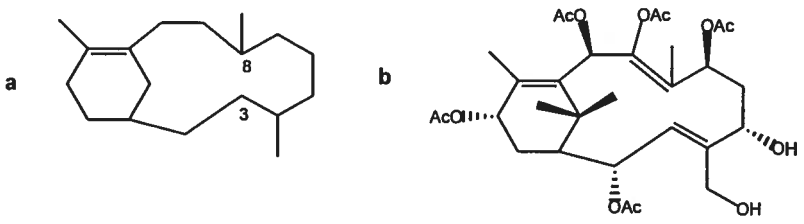
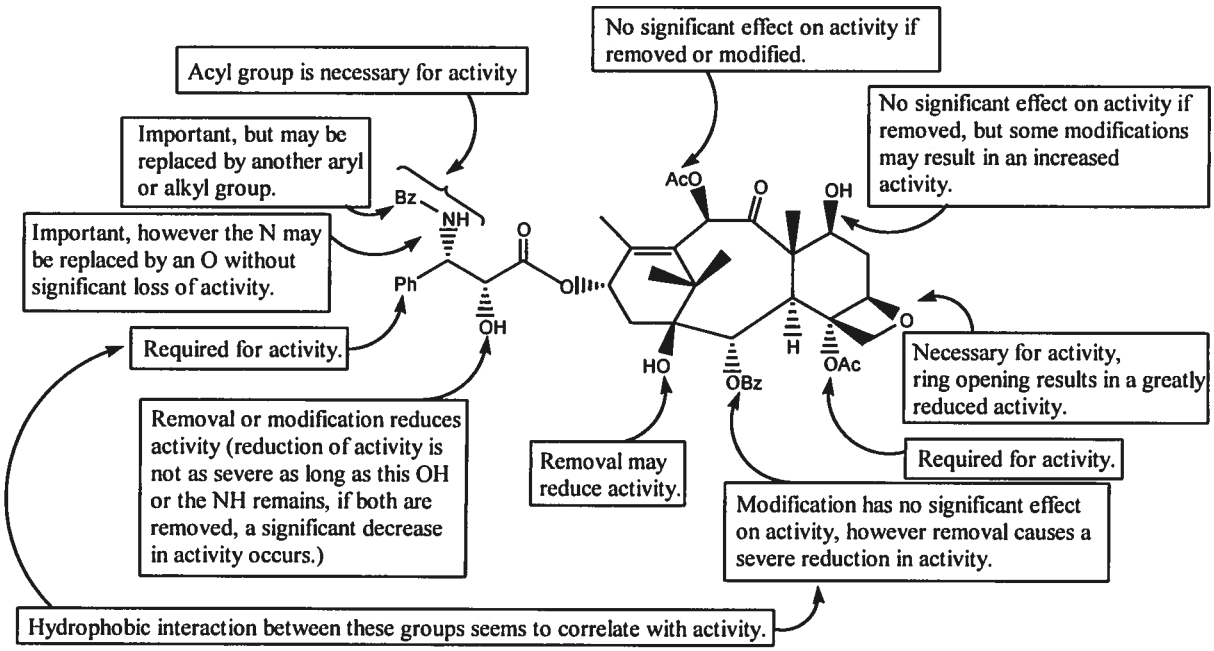


Figure 9. Pretaxanes: (a) skeleton, (b) 5-epi-canadensene.



Kingston, 2001, 10:867.

Figure 10. Structure activity relationship of paclitaxel.

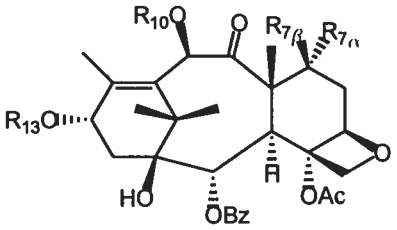
2.3 Yews: differences between *Taxus canadensis* and the other species

Many species of yew exist, each belonging to the genus *Taxus*, of the family *Taxaceae*. The species of yew is normally named after its geographical location, for example the Canadian Yew (*Taxus canadensis*). Due to the multitude of studies done on the phytochemistry of yew trees and the many species of yews examined, it is important to understand the different names used to represent the various species. There are many

Latin and common names which are used interchangeably, however with some discrepancies. There are also many species names that are supposedly synonymous, although this still remains unclear. A fine example of this is the Chinese yew, which is referred to as *Taxus chinensis*, *Taxus yunnanensis* and *Taxus mairei*. The Himalayan yew is also represented by two botanical names, *Taxus wallichiana* and *Taxus baccata*, although *Taxus baccata* is also referred to as the European or English yew. *Taxus baccata* seems to be used on occasion to represent a single collective species of yew. It is clear that this is an area for significant confusion (Baloglu and Kingston, 1999:62:1448, Parmar *et al.*, 1999:50:1267). A large problem lies with the methods used for identification of yew species, and whether the classification is based on the morphology of the plant (macroscopic features) or rather on the more microscopic characteristics of the plants anatomy. Classification based solely on the appearance of the plant such as the length and shape of the needles and branches is more difficult, particularly between certain species that are very similar in appearance (Parmar *et al.*, 1999:50:1267). Upon comparing the taxane content of the various yew species, it is important to take these discrepancies into account. Lastly, while doing research in this field, it is important to catalogue a sample of the plant material for future reference and to try to minimize future confusion in the area of identification and nomenclature of the various yew species.

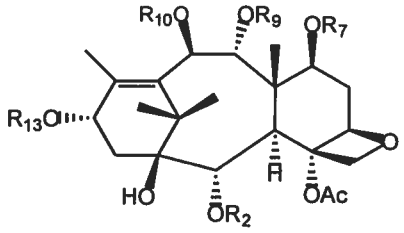
The taxane content of yews varies with their geographical location, with the season of the year, as well as with each species. The Canadian Yew, *Taxus canadensis*, differs greatly from other yew trees in its small and ramping nature. It diverges from the other species not only in its appearance however, but also in its composition (see **table 1**). While paclitaxel, cephalomanine, 10-deacetyl-baccatin III, and other major taxanes identified in other yew species have also been identified in the Canadian yew, its most predominant taxane is 9-dihydro-13-acetyl-baccatin III (Zamir *et al.*, 1992:33:36:5235). In the mature Canadian yew tree, this compound is present in quantities three to seven times greater than paclitaxel regardless of the time of year. In addition, a novel bicyclic taxane was also found in *Taxus canadensis*, named canadensene (Zamir *et al.*, 1995:529), which has significant structural similarity with the core ring of paclitaxel. This structure may suggest that canadensene is a precursor to paclitaxel.

Table 1. Taxanes isolated from the Canadian Yew, *Taxus canadensis*.



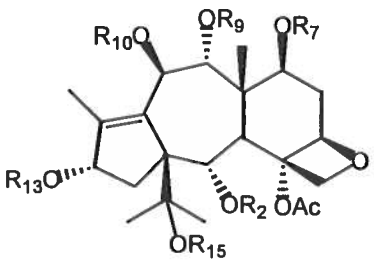
Compound name	R group	Structure
Paclitaxel [B]	$R_{7\alpha}$	H
	$R_{7\beta}$	OH
	R_{10}	Ac
	R_{13}	PhCONHCH(Ph)CH(OH)CO-
10-deacetyltaxol [B]	$R_{7\alpha}$	H
	$R_{7\beta}$	OH
	R_{10}	H
	R_{13}	PhCONHCH(Ph)CH(OH)CO-
7-epitaxol [B]	$R_{7\alpha}$	OH
	$R_{7\beta}$	H
	R_{10}	Ac
	R_{13}	PhCONHCH(Ph)CH(OH)CO-
Cephalomanine [B]	$R_{7\alpha}$	H
	$R_{7\beta}$	OH
	R_{10}	Ac
	R_{13}	$CH_3CH=C(CH_3)CONHCH(Ph)CH(OH)CO-$
10-deacetyl-cephalomanine [I]	$R_{7\alpha}$	H
	$R_{7\beta}$	OH
	R_{10}	H
	R_{13}	$CH_3CH=C(CH_3)CONHCH(Ph)CH(OH)CO-$
10-deacetyl-baccatin III [B]	$R_{7\alpha}$	H
	$R_{7\beta}$	OH
	R_{10}	H
	R_{13}	H
7-epi-10-deacetyl-baccatin III [B]	$R_{7\alpha}$	OH
	$R_{7\beta}$	H
	R_{10}	H
	R_{13}	H
Taxcultine [D]	$R_{7\alpha}$	H
	$R_{7\beta}$	OH
	R_{10}	Ac
	R_{13}	$CH_3CH_2CH_2CONHCH(Ph)CH(OH)CO-$
N-acetyl-N-debenzoyltaxol [H]	$R_{7\alpha}$	H
	$R_{7\beta}$	OH
	R_{10}	Ac
	R_{13}	$CH_3CONHCH(Ph)CH(OH)CO-$
2'-acetyl-7-epi-taxol [G]	$R_{7\alpha}$	OH
	$R_{7\beta}$	H
	R_{10}	Ac
	R_{13}	PhCONHCH(Ph)CH(OAc)CO-
2'-acetyl-7-epi-cephalomanine [I]	$R_{7\alpha}$	OH
	$R_{7\beta}$	H

	R_{10}	Ac
	R_{13}	$CH_3CH=C(CH_3)CONHCH(Ph)CH(OAc)CO-$
N-debenzoyl-N-hexanoyl-7-epi-taxol [I]	$R_{7\alpha}$	OH
	$R_{7\beta}$	H
	R_{10}	Ac
	R_{13}	$CH_3(CH_2)_5CONHCH(Ph)CH(OAc)CO-$
7-acetyl-10-deacetyltaxol [I]	$R_{7\alpha}$	H
	$R_{7\beta}$	OAc
	R_{10}	H
	R_{13}	PhCONHCH(Ph)CH(OH)CO-

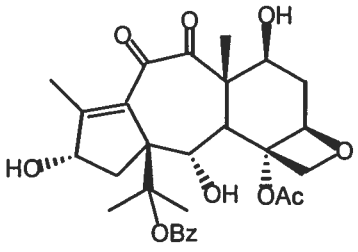


Compound name	R group	Structure
9-dihydro-13-acetyl-baccatin III [A]	R_2	Bz
	R_7	H
	R_9	H
	R_{10}	Ac
	R_{13}	Ac
7,9-deacetyl-baccatin IV [B]	R_2	Ac
	R_7	H
	R_9	H
	R_{10}	Ac
	R_{13}	Ac
7,9,10-deacetyl-baccatin VI [B]	R_2	Bz
	R_7	H
	R_9	H
	R_{10}	H
	R_{13}	Ac
10-hydroxyacetyl-baccatin VI [B]	R_2	Bz
	R_7	H
	R_9	Ac
	R_{10}	$COCH_2OH$
	R_{13}	Ac
1β-hydroxy-10-deacetyl-10-glycolyl-baccatin I [H]	R_2	Ac
	R_7	H
	R_9	Ac
	R_{10}	$COCH_2OH$
	R_{13}	Ac
7,9,13-trideacetyl-baccatin VI [H]	R_2	Bz
	R_7	H
	R_9	H
	R_{10}	Ac
	R_{13}	H
10-acetylglycollyl-baccatin VI [I]	R_2	Bz
	R_7	Ac
	R_9	Ac

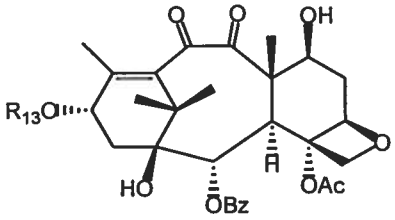
	R_{10}	$AcOCH_2CO$-
	R_{13}	Ac



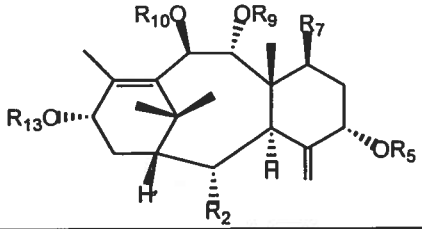
Compound name	R group	Structure
Taxacustin [H]	R_2	Ac
	R_7	Ac
	R_9	Ac
	R_{10}	H
	R_{13}	H
	R_{15}	H
15-benzoyl-2-debenzoyl-7,9-dideacetyl-abeo-baccatin VI [H]	R_2	H
	R_7	H
	R_9	H
	R_{10}	Ac
	R_{13}	Ac
	R_{15}	Bz
9,10,13-trideacetyl-abeo-bacctinVI [F]	R_2	Bz
	R_7	Ac
	R_9	H
	R_{10}	H
	R_{13}	H
	R_{15}	H
2-acetyl-13-deacetyl-2-debenzoyl-abeo-baccatin VI [F]	R_2	Ac
	R_7	Ac
	R_9	Ac
	R_{10}	Ac
	R_{13}	H
	R_{15}	H



15-Benzoyl-10-deacetyl-2-debenzoyl-10-dehydro-abeo-baccatin III [H]



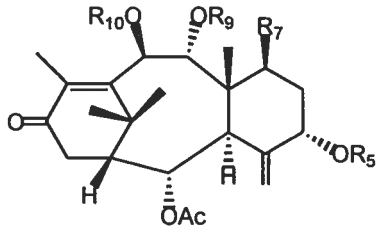
Compound name	R group	Structure
10-deacetyl-10-oxo-baccatin V [I]	R_{13}	H
10-deacetyl-10-oxo-7-epi-taxol [I]	R_{13}	PhCONHCH(Ph)CH(OH)CO-
10-deacetyl-10-oxo-7-epi-cephalomanine [I]	R_{13}	CH3CH=C(CH_3)CONHCH(Ph)CH(OH)CO-



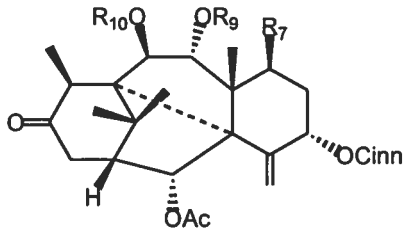
Compound name	R group	Structure
2-deacetyl-5-decinnamoyltaxinine J [B]	R_2	OH
	R_5	H
	R_7	OAc
	R_9	Ac
	R_{10}	Ac
	R_{13}	Ac
2-deacetoxytaxinine J [C]	R_2	H
	R_5	Cinn
	R_7	OAc
	R_9	Ac
	R_{10}	Ac
	R_{13}	Ac
Taxinine E [J]	R_2	OAc
	R_5	Cinn
	R_7	H
	R_9	Ac
	R_{10}	Ac
	R_{13}	Ac
Taxinine J [K]	R_2	OAc
	R_5	Cinn
	R_7	OAc
	R_9	Ac
	R_{10}	Ac
	R_{13}	Ac
10-deacetyltaxinine E	R_2	OAc
	R_5	Cinn
	R_7	H
	R_9	Ac
	R_{10}	H
	R_{13}	Ac

2-deaceoxy-7-deacetylyaxinine J	R_2	H
	R_5	Cinn
	R_7	OH
	R_9	Ac
	R_{10}	Ac
	R_{13}	Ac
2,7-deacetoxytaxinine J	R_2	H
	R_5	Cinn
	R_7	H
	R_9	Ac
	R_{10}	Ac
	R_{13}	Ac
5-decinnamoyltaxinine E [L]	R_2	OAc
	R_5	H
	R_7	H
	R_9	Ac
	R_{10}	Ac
	R_{13}	Ac
Taxezopidine G [M]	R_2	OH
	R_5	Cinn
	R_7	H
	R_9	Ac
	R_{10}	Ac
	R_{13}	Ac
2-deacetyl-5-decinnamoyltaxinine E [G]	R_2	OH
	R_5	H
	R_7	H
	R_9	Ac
	R_{10}	Ac
	R_{13}	Ac
2-deacetyl-taxinine J [G]	R_2	OH
	R_5	Cinn
	R_7	OAc
	R_9	Ac
	R_{10}	Ac
	R_{13}	Ac
7,9-deacetyl-5-decinnamoyl-taxinine J [F]	R_2	OAc
	R_5	H
	R_7	H
	R_9	OH
	R_{10}	Ac
	R_{13}	Ac
2-deacetoxy-9-deacetyl-taxinine E	R_2	H
	R_5	Cinn
	R_7	H
	R_9	H
	R_{10}	Ac
	R_{13}	Ac
2-deacetoxy-10-deacetyl-taxinine E	R_2	H
	R_5	Cinn
	R_7	H
	R_9	Ac
	R_{10}	H

	R_{13}	Ac
9-deacetyl-taxinine E [C]	R_2	OAc
	R_5	Cinn
	R_7	H
	R_9	H
	R_{10}	Ac
	R_{13}	Ac

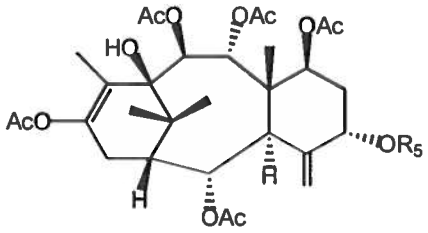


Compound name	R group	Structure
Taxinine	R_5	Cinn
	R_7	H
	R_9	Ac
	R_{10}	Ac
Taxinine A [N]	R_5	H
	R_7	H
	R_9	Ac
	R_{10}	Ac
Taxinine B	R_5	Cinn
	R_7	OAc
	R_9	Ac
	R_{10}	Ac
10-deacetyltaxinine B	R_5	Cinn
	R_7	OAc
	R_9	Ac
	R_{10}	H
7,9-deacetyltaxinine B	R_5	Cinn
	R_7	OH
	R_9	H
	R_{10}	Ac
9,10-di-O-deacetyltaxinine A	R_5	H
	R_7	H
	R_9	H
	R_{10}	H

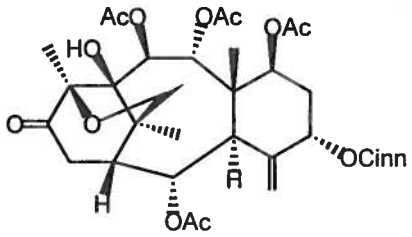


Compound name	R group	Structure
Taxuspine C [O]	R_7	H

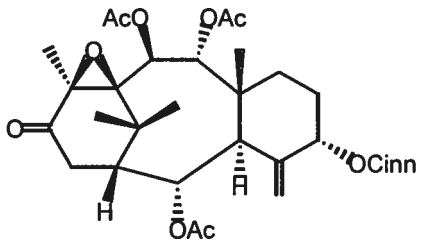
	R_9	Ac
	R_{10}	Ac
7-acetoxytaxuspine C	R_7	OAc
	R_9	Ac
	R_{10}	Ac
2,10-diacetyl-5-cinnamoyl-7β-hydroxyphototaxicin II[E]	R_7	OH
	R_9	H
	R_{10}	Ac
2,9-diacetyl-5-cinnamoyl-phototaxicin II[E]	R_7	H
	R_9	Ac
	R_{10}	H
2,10-diacetyl-5-cinnamoyl-phototaxicin II[E]	R_7	H
	R_9	H
	R_{10}	Ac



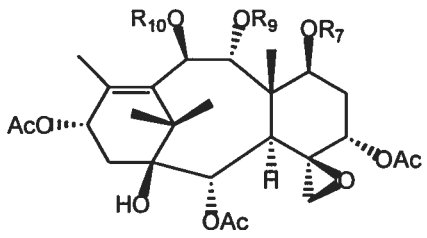
Compound name	R group	Structure
Taxuspine D [F]	R_5	Cinn
5-decinnamoyltaxuspine D [F]	R_5	H



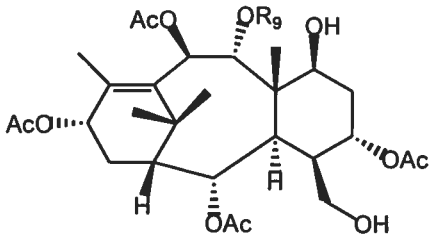
Taxagifine [H]



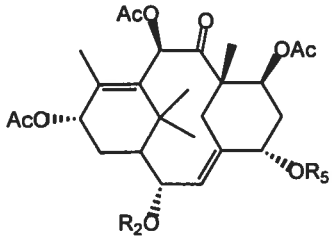
Taxinine-11,12-oxide



Compound name	R group	Structure
1β-hydroxy-7,9-deacetyl-baccatin I [B]	R_7	H
	R_9	H
	R_{10}	Ac
1β,7β-dihydroxy-4β,20-epoxy-2α,5α,9α,10β,13α-pentaacetoxytax-11-ene [G]	R_7	H
	R_9	Ac
	R_{10}	Ac
1β,9α-dihydroxy-4β,20-epoxy-2α,5α,7β,10β,13α-pentaacetoxytax-11-ene [G]	R_7	Ac
	R_9	H
	R_{10}	Ac
1β-hydroxy-10-deacetyl-baccatin I [C]	R_7	Ac
	R_9	Ac
	R_{10}	H
1β-hydroxy-10-deacetyl-10-glycolyl-baccatin I [I]	R_7	Ac
	R_9	Ac
	R_{10}	$COCH_2OH$

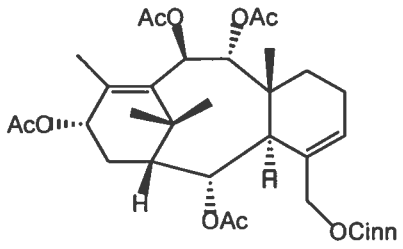


Compound name	R group	Structure
7,9-deacetyl-taxuspine L [F]	R_9	H
7-deacetyl-taxuspine L [H]	R_9	Ac

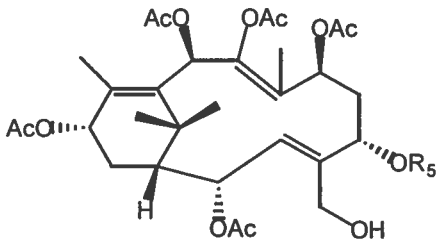


Compound name	R group	Structure
Taxin B [Q]	R2	Ac

	R5	H
2-deacetyl-7,10-diacetyl-5-deaminoacyltaxine A [H]	R2	H
	R5	H
5-cinnamoyltaxin B [H]	R2	Ac
	R5	Cinn



2,9,10,13-tetracetoxy-20-cinnamoxy-taxa-4(5),11(12)-diene [G]



Compound name	R group	Structure
5-epi-canadensene [E]	R_5	H
5-epi-cinnamoyl- canadensene [G]	R_5	Cinn

Refs.: [A]: Zamir *et al.*, 1992, 33:36:5173, [B]: Zamir *et al.*, 1995, 73:655, [C]: Zamir *et al.*, 1995, 529, [D]: Zamir *et al.*, 1996, 41:3:803, [E] Zamir *et al.*, 1998, 54:15845, [F]: Zamir *et al.*, 1999, 62:1268, [G]: Zhang *et al.*, 2000, 63:929, [H]: Zhang *et al.*, 2000, 54:2:22, [I] Zhang *et al.*, 2001, 64:450. [J] Yeh *et al.*, 1988: 35:309, [K] Liang *et al.*, 1988, 46:1053, [L] Chen *et al.*, 1999, 62(1):149, [M] Wang *et al.*, 1998, 61:474, [N] Chiang *et al.*, 1967, 1201, [O] Kobayashi *et al.*, 1994, 50(25):7401, [P] Kobayashi *et al.*, 1995, 51:592, [Q] Yue *et al.*, 1995, 61(4):375,

This difference in composition could possibly suggest a unique biosynthetic route for paclitaxel in the Canadian Yew, different from that proposed for the other yew species. The accepted biosynthetic pathway of paclitaxel is the Rohmer pathway (Rohmer, 1999:16:565) in which one of the metabolic products of glucose, glyceraldehyde-3-phosphate (GAP), is the early precursor of the diterpenoid. Before the elucidation of this pathway, the biosynthesis of paclitaxel was thought to follow the

previously accepted mevalonate pathway. While all components of the mevalonate pathway are present in the cytoplasm and the biosyntheses of other compounds indeed follow this route, it is no longer accepted as the route followed for the formation of paclitaxel or other diterpenes. Should the biosynthesis of paclitaxel follow a different route in the Canadian yew, it could then corroborate the early evidence found by Zamir *et al.* (1992:33:36:5235) that mevalonate is indeed a precursor of paclitaxel in *Taxus canadensis*. To better understand the possible differences between the biosynthesis of paclitaxel in the Canadian Yew versus other species of the yew, a brief literature review of the work done on the biosynthesis of paclitaxel follows.

2.4 Biosynthesis of paclitaxel

The biosynthesis of paclitaxel can be divided into three categories: (1) the formation of the core ring structure, (2) the formation of the side chain, and (3) the combination of these two components to form paclitaxel.

2.4.1 Formation of the diterpene core (core ring structure)

The diterpene moiety of paclitaxel, the core ring structure, was originally thought to follow the mevalonate pathway which was the accepted pathway for the formation of all isoprene unit derived compounds such as sterols and carotenoids. This was an area of great controversy as conflicting results have been obtained from many research groups (Rohr, 1997:36:20:2190, Zamir *et al.*, 1992:33:36:5235, Eisenreich *et al.*, 1996:93:6431, Rohmer, 1999:16:565, Hezari and Croteau, 1997:63:291, Bach, 1995:30:3:191, Floss and Mocek, 1996:7:191, Rohmer, 1998:50:136). More recently, these results have been re-examined and have led to the elucidation of a novel pathway. This second pathway, already known to bacteria is mevalonate-independent and is often referred to as the Rohmer pathway (Rohmer, 1999:16:565), and has been suggested for the synthesis of isoprenoids in plants as well. It appears that while all components of the mevalonate pathway are present in the cytoplasm and that sterol biosynthesis indeed follows this route, the isoprenoid structures formed in the plastids including carotenoids and

diterpenes, are all formed via the non-mevalonate pathway. Both of these pathways lead to the formation of geranylgeranyl diphosphate (GGPP), the universal precursor of diterpenoids. Isopentenyl diphosphate (IPP), geranyl diphosphate (GPP) and farnesyl diphosphate (FPP) are all precursors to GGPP and are present in both pathways and thus in both the cytoplasm and the plastids. There has been evidence that there is an exchange of these earlier precursors, between the cytoplasm and the organelle (Rohmer, 1999:16:565). It is only the formation of the early precursor isopentenyl diphosphate that varies between pathways and thus formation of the isoprenoid end structure from geranylgeranyl diphosphate does not differ.

The mevalonate pathway consists of two successive condensations of acetyl CoA to yield acetoacetyl CoA (catalyzed by acetoacetyl-CoA thiolase) and 3-hydroxy-3-methylglutaryl coenzyme A (catalyzed by HMG CoA synthase) respectfully. HMG CoA is then reduced to mevalonate in a reaction that is catalyzed by HMG CoA reductase and is the first committed step of the pathway. Two successive phosphorylations of this mevalonate by mevalonate kinase and phosphomevalonate kinase as well as a decarboxylation by mevalonate decarboxylase give rise to IPP. IPP is a five-carbon skeleton isoprene unit, the building block of all isoprenoids. Isopentenyl diphosphate isomerase allows an equilibrium to be obtained between IPP and dimethylallyl diphosphate (DMAPP) and the two of them combine through a reaction catalyzed by prenyl transferase to form GPP and FPP to ultimately give rise to GGPP (Rohr, 1997:36:20:2190, Rohmer, 1999:16:565, Hezari and Croteau, 1997:63:291, Bach, 1995:30:3:191, Floss and Mocek, 1996:7:191, Rohmer, 1998:50:136). Geranylgeranyl diphosphate synthase, which is responsible for the formation of the diterpene precursor, has been isolated from *Taxus baccata* and partially characterized (Laskaris *et al.*, 1999:50:939). GGPP is the universal precursor of diterpenes, and it undergoes cyclisation via a verticillyl intermediate to form taxa-4(5),11(12)-diene, which subsequently goes on to form the skeleton structure of paclitaxel after several oxygenations (Koepp *et al.*, 1995:270:15:8686, Hezari *et al.*, 1995:332:2:437, Lin *et al.*, 1996:35:2968, Williams *et al.*, 2000:379:1:137).

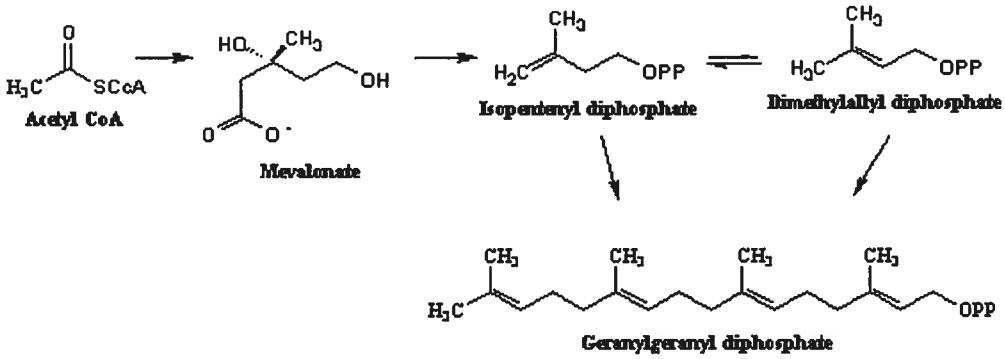


Figure 11. Formation of IPP, DMAPP and GGPP by the mevalonate pathway.

The mevalonate-independent pathway, also known as the deoxyxylulose 5-phosphate pathway, differs in its formation of IPP. In this model, IPP is formed by the condensation of the two-carbon product formed from the decarboxylation of pyruvate, with glyceraldehyde-3-phosphate (GAP). 1-Deoxy-D-xylulose 5-phosphate results and is reduced by deoxyxylulose 5-phosphate reductoisomerase to form 2-C-methyl-D-erythritol 4-phosphate. The reductoisomerase requires Mn^{2+} as a cofactor and is NADPH dependent. A phospho-cytidine moiety is added to the 4-phosphate to yield 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol, which is in turn phosphorylated by its kinase on the hydroxyl group of C-2. 2-C-Methyl-D-erythritol-2,4-cyclodiphosphate is formed by MECP synthase in the last identified step before the formation of IPP. Formation of GGPP and ultimately paclitaxel from IPP and DMAPP does not differ between the two the pathways (Rohr, 1997:36:20:2190, Eisenreich *et al.*, 1996:93:6431, Rohmer, 1999:16:565, Hezari and Croteau, 1997:63:291, Bach, 1995:30:3:191, Floss and Mocek, 1996:7:191, Rohmer, 1998:50:136, Koepp *et al.*, 1995:270:15:8686).

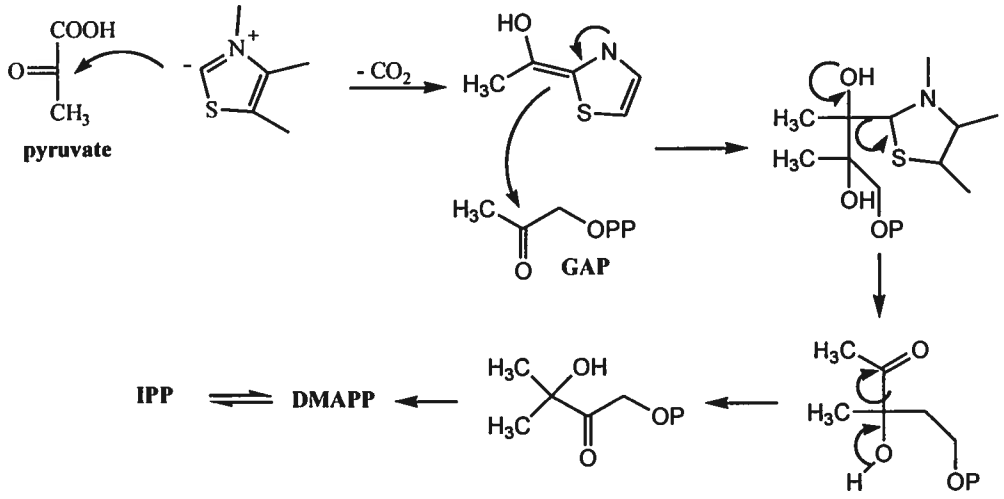


Figure 12. Formation of IPP and DMAPP by the Rohmer or non-mevalonate pathway.

Proof that the mevalonate pathway was perhaps not the route followed in the biosynthesis of isoprenoids emerged early on. While incorporation of mevalonate and acetate was observed in carotenoids and mono and diterpenes, the percent incorporation was very poor and often not observed at all. The same precursors in contrast, were efficiently incorporated in sterols and triterpenoids. Difficulty in obtaining incorporation could be tentatively explained by the fact that sterols were synthesised in the cytosol and diterpenes were synthesised in the plastids and thus perhaps did have the same access to the precursors fed. The reason why a potent inhibitor of HMG CoA reductase was able to inhibit the synthesis of sterols but not that of carotenoids or chlorophylls containing a diterpene side chain could not be explained however. It therefore seemed unlikely that these compounds were all synthesised by the plant using the same pathway. It became more apparent that while sterols were perhaps synthesised by the mevalonate pathway and thus observed good incorporation of the precursors and were inhibited by the inactivation of a key enzyme in that pathway, the mono and diterpenes were most likely formed via a different route. It seems that the diterpenes, including paclitaxel, are most likely formed in the plastids via a mevalonate independent route, with a small contribution from the mevalonate pathway in the form of IPP, FPP, and GPP (Rohmer, 1999:16:565).

Once geranylgeranyl diphosphate is obtained, it undergoes several reactions to ultimately form the paclitaxel core structure. The first of these steps is the cyclization of

geranylgeranyl diphosphate to form taxa-4(5),11(12)diene (Hezari and Croteau, 1997:63:291, Hezari *et al.*, 1995:332:2:437, Lin *et al.*, 1996:35:2968, Williams *et al.*, 2000:379:1:137). This was demonstrated by the efficient incorporation of the labelled olefin into paclitaxel and related taxoids as well as by its isolation from the *Taxus brevifolia* bark. A cell-free preparation from *T. brevifolia* showed that taxa-4(5),11(12)-dien-5a-ol was essentially the only olefin produced from geranylgeranyl diphosphate in a reaction catalyzed by taxadiene synthase, a reaction that is Mg^{2+} dependant. The reaction involves the formation of a short-lived verticillyl cation intermediate, and the initiation of the transannular B/C-ring closure by the transfer of a hydrogen from C-11 of the intermediate to C-7. Deprotonation at C-5 follows and results in the formation of taxadiene (Koepp *et al.*, 1995:270:15:8686). Formation of taxa-4(20),11(12)-dien-5a-ol follows with the hydroxylation of taxadiene at C-5 and the subsequent migration of the double bond. This reaction is catalyzed by a NADPH-dependant cytochrome p450 oxygenase, taxadiene-5-hydroxylase (Rohr, 1997:36:20:2190, Hefner *et al.*, 1996:3:479). It was previously thought that taxa-4(20),11(12)-dien-5a-ol was formed directly from geranylgeranyl diphosphate due to the significant presence of taxoids with a 4(20)-ene-5-oxy group (Rohr, 1997:36:20:2190, Hezari and Croteau, 1997:63:291). However, it was unable to be isolated from *T. brevifolia* bark whereas taxadiene was isolated in minute amounts and showed a good incorporation into paclitaxel. Its formation is instead the second step in the development of the diterpene core of paclitaxel. The presence of taxa-4(20),11(12)-dien-5a-ol as an intermediate in the biosynthesis pathway was confirmed by studies in *T. brevifolia* (Koepp *et al.*, 1995:270:15:8686, Hefner *et al.*, 1996:3:479).

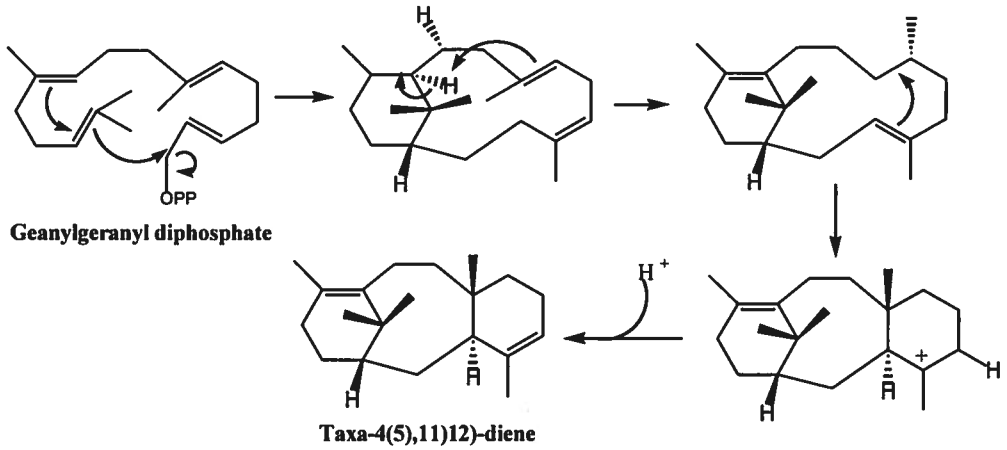


Figure 13. Cyclisation of GGPP to taxa-4(5),11(12)-diene via a verticyllyl intermediate.

Acetylation at the C-5 hydroxyl group of the taxadienol is the third step in the biosynthesis of the core of paclitaxel and is dependant on the presence of acetyl CoA. An Acetyl CoA:Taxa-4(20),11(12)-dien-5a-ol O-acetyl transferase has been characterized after a partial purification from both *Taxus canadensis* and *Taxus cuspidata* (Hezari and Croteau, 1997: 63:291, Walker and Croteau, 2000: 97:2:583, Walker *et al.*, 1999:364:2:273). This acetylation is thought to be the next step in the biosynthesis of the core structure of paclitaxel due to the isolation of many related taxoids which are acetylated at this position, as well due to the functional groups possible role in the subsequent formation of the oxetane ring. It is proposed that an epoxide is formed from the 4(20)-double bond and that this in conjunction with the acetate group at C-5 allows an intramolecular acetate migration and oxirane ring opening, resulting in the oxetane ring. The acetylation at C-5 is followed by further oxygenations of the taxane core, with the addition of the hydroxyl groups seemingly proceeding in the order of C-5, C-10, C-2, C-9, C-13, C-7, and C-1 (Wheeler *et al.*, 2001:390:2:265). The sequence of these oxidation steps is based on the numerous known taxane structures and is therefore an educated guess rather than a certainty. With over three hundred and fifty known taxanes already isolated from yew trees, it is important to remember that many of these taxanes may not play a part in the direct biosynthetic pathway of paclitaxel, but rather may be the product of a shunt or dead-end pathway. More recently, the discovery of a 13α-hydroxylase which catalyses the formation of polyoxygenated taxanes via taxa-4(20),11(12)-dien-5α-13α-ol suggested that the order of oxygenation steps might not be as previously hypothesised (Jennewein *et al.*, 2001, 98:24:13595). This recent evidence could suggest that two parallel pathways may exist within the biosynthesis of paclitaxel, and further studies may help elucidate the presence of both shunt and dead-end pathways present within the scheme. The exact timing of the oxetane ring formation is unclear, although again, based on the predominance of taxanes with or without an oxetane ring and oxygenated at various carbons, oxetane ring formation most likely occurs before oxygenation at C-9 and before acetylation at C-13 (Hezari and Croteau, 1997:63:291).

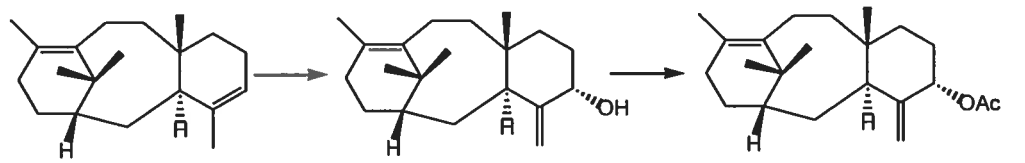


Figure 14. Formation of taxa-4(20),11(12)-dien-5α-yl acetate from taxadiene.

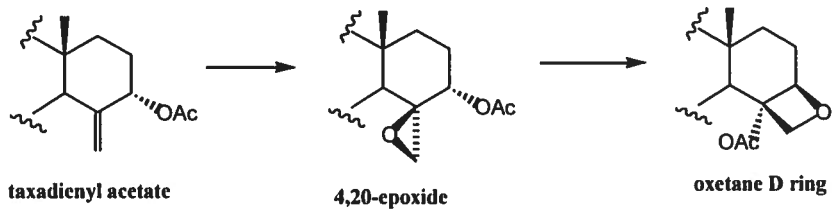


Figure 15. Formation of the oxetane ring (D ring of paclitaxel)

2.4.2 Side chain formation

It has previously been shown that the side chain of the paclitaxel analogue Taxine is derived from β-phenylalanine and as such it is probable that the side chain of paclitaxel, *N*-benzoylphenylisoserine, is also formed from β-phenylalanine. It was originally thought to be formed from cinnamic acid and its epoxide (Flemming *et al.*, 1993:115:805). Using feeding experiments in *T. brevifolia*, incorporation of phenylalanine and phenylisoserine was observed, while incorporation of cinnamic acid was not (Floss and Mocek, 1996:7:191). Previous work on the biosynthesis of benzoic acid in higher plants has been shown to proceed via cinnamic acid using phenylalanine. It has since been shown that paclitaxel's side chain is generated from phenylalanine and that its benzoyl moiety is most likely formed by a hydroxylation and an acylation (Rohr, 1997:36:20:2190, Flemming *et al.*, 1993:115:805, Walker and Floss, 1998:120:5333, Flemming *et al.*, 1994:116:4137). While the side chain of paclitaxel is thought to be one of the elements necessary for the molecules bioactivity, certain molecules lacking such components but retaining anti-cancer properties have been found. It is hypothesised that other portions of these molecules mimic the side chain of paclitaxel and as such are still able to interact at the subcellular level as paclitaxel and its active derivatives do.

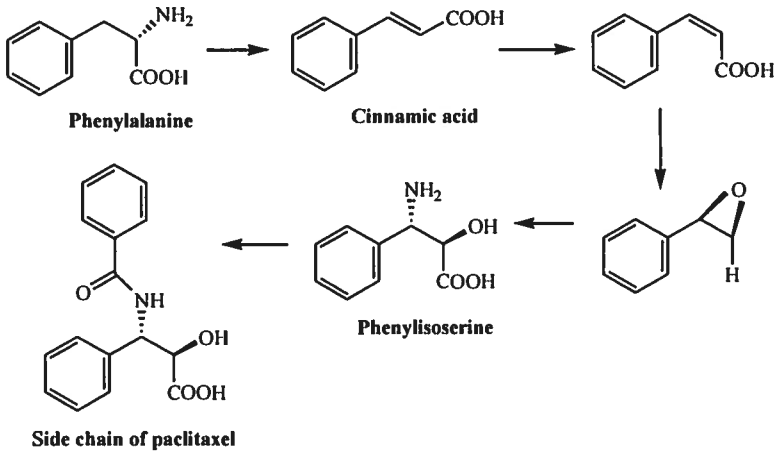


Figure 16. Formation of the paclitaxel side chain via cinnamic acid and its epoxide.

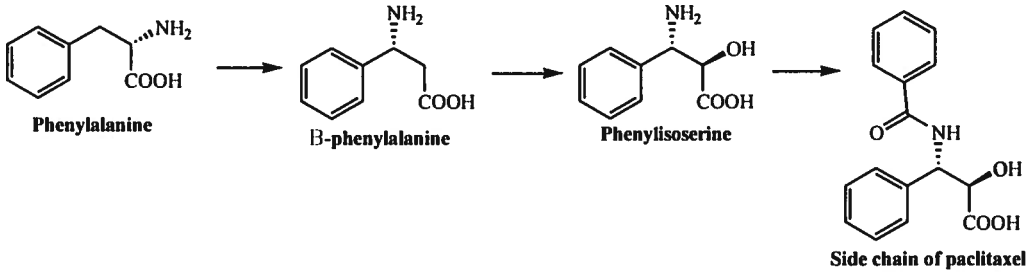


Figure 17. Formation of the paclitaxel side chain via β-phenylalanine.

2.4.3 Assembly of the diterpene core and the side chain to form paclitaxel

It had been hypothesised by Potier and colleagues that due to the three dimensional U-form of the paclitaxel molecule, the side chain which is attached at C-13 of the molecule was first attached to C-4 or C-5 and then transferred through an intramolecular transesterification (Flemming *et al.*, 1994:116:4137). Potier's hypothesis was proven unlikely due to the discovery that the oxetane ring and acetyl groups on C-5 and C-4 are formed prior to the addition of the side chain and therefore does not concur with these results (Flemming *et al.*, 1994:116:4137). It was also unknown whether the side chain was attached as one unit or whether it is assembled from different components on the diterpene core. It has been shown in *T. brevifolia* that the side chain is not attached intact, but rather that it is most likely attached as phenylisoserine and that the addition of

the benzoyl group is the last step in the biosynthesis of paclitaxel (Floss and Mocek, 1996:7:191, Flemming *et al.*, 1994:116:4137).

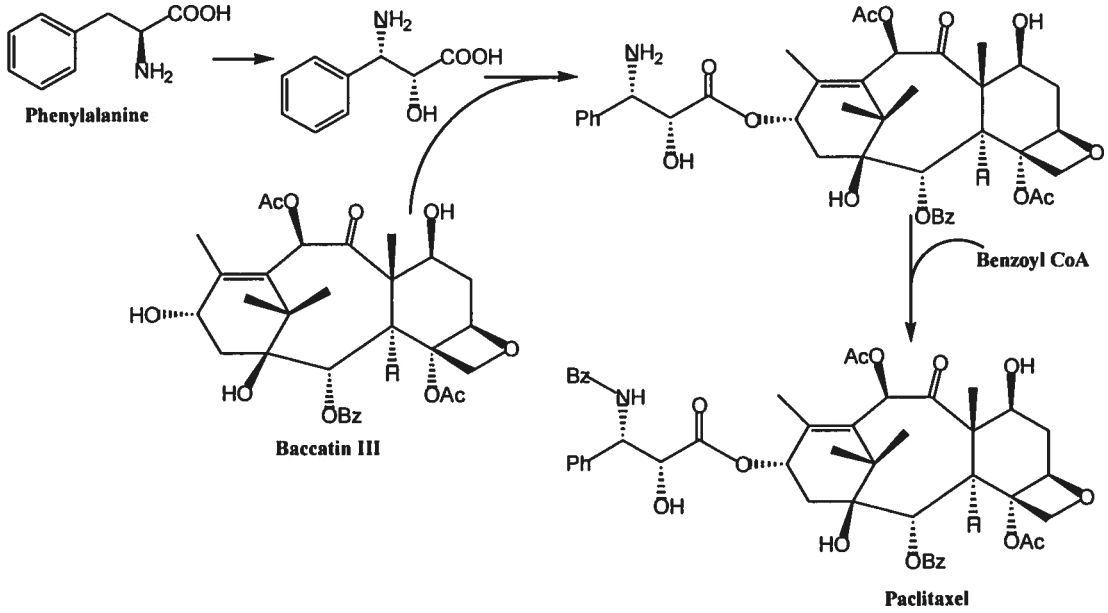


Figure 18. Formation and attachment of side chain giving paclitaxel.

2.4.4 Possible differences between biosynthesis of paclitaxel in the Canadian yew and the other species

The difference in taxane content between *Taxus canadensis* and the other species of yew could possibly suggest a unique biosynthetic route for paclitaxel in the Canadian yew. This pathway could deviate from the accepted Rohmer pathway in which one of the metabolic products of glucose, glyceraldehyde-3-phosphate, is the early precursor of the paclitaxel. Before the elucidation of this pathway, the biosynthesis of paclitaxel was thought to follow the mevalonate pathway. While all components of the mevalonate pathway are present in the cytoplasm and the biosyntheses of other compounds indeed follow this route, it is no longer accepted as the route followed for the formation of paclitaxel or other diterpenes as was described above. Should the biosynthesis of paclitaxel follow a different route in the Canadian yew, it could then corroborate the early

evidence found by Zamir *et al.* that mevalonate is indeed a precursor of paclitaxel in *Taxus canadensis*. While this is a possibility, the incorporation of radiolabelled mevalonate observed by Zamir *et al.* was in fact very low and can perhaps be explained by the contribution of the mevalonate pathway via precursors from the cytosol. The early precursors are free to cross the plastid membranes to where paclitaxel synthesis occurs and could thus be incorporated into the non-mevalonate pathway. This would allow a minute incorporation of the radiolabelled mevalonate into paclitaxel. Due to feeding experiments in *T. canadensis* cell culture where no evidence of the mevalonate pathway was observed (Ketchum *et al.*, 1999:62:1395), the latter seems the most viable of hypotheses. A significant difference between the side pathways or shunt pathways present within the Canadian yew and the other species of yew seems possible, in this way regulating the accumulation of compounds or conversion of one compound to another. This could be a means of altering the biosynthesis of compounds or their storage within the plant until they are later needed. It is also possible that several alternate reactions within the pathways run side by side and later coincide further down the pathway. These alternate pathways could each be present within the yew however it is also possible that amongst these pathways certain ones may be more predominant in certain species of yew. This could contribute to the variety amongst the species. It is important to nevertheless retain the view of the biosynthetic pathway as a complex one with many purposes and interconnections, such that the sole objective is not only the production of paclitaxel, but also the production of the other taxanes. The purpose of these compounds in the plant is also important. While the benefits of paclitaxel are evident in its human application, the role it plays in the life and survival of the plant are uncertain. It has been proposed that paclitaxel is involved in the pest control and the stress response of the plant, but until its role and its regulation are better understood, the intricacies of the biosynthesis are also poorly appreciated.

Although many steps in the biosynthetic pathway of paclitaxel have been elucidated and many of the enzymes responsible have been characterized, it is important to continue more in-depth studies. Ultimately, understanding the biosynthesis of paclitaxel will allow an increase in the production of the antineoplastic agent or the production of its derivatives. This will most likely be done using genetically engineered

cell lines where the rate limiting enzymes have been altered and by providing the cells with the optimal conditions for the synthesis of paclitaxel. If the tertiary structures of the enzyme are known, as well as their active sites and how they interact with their substrate, it is then possible to try to improve the kinetics of the system. This information, combined with *in vivo* studies to define slow steps can be used to then target these steps in the pathway. Genes for enzymes catalyzing such reactions can be over expressed. The contribution of different reactions to pathway flux can also be determined, and all these factors may be combined to ultimately improve paclitaxel production. This in turn will not only relieve the demand for the natural source, but it will also reduce the price of the drug dramatically making it more affordable and allows it use for treating cancer without prejudice based on price and availability (Jennewein and Croteau, 2001:57:13).

The elucidation of such a major biosynthetic pathway is by no means a small task, and one that will take many years of study. One of the advantages of having many research groups working in this field is not only their individual contribution to this worldwide effort, but also their use of different species of yews, with differences in geographical location, collection time and therefore a large sample variety. This scenario, although increasing the difficulty in organizing the obtained data, also increases the variety of compounds isolated. While many species of yews have been studied before, as well as the different parts of the tree, studies using the Canadian Yew are few (restricted mainly to our lab) and there are no published studies using rooted cuttings of yews.

Rooted cuttings are cuttings of yew trees which have been stimulated to grow roots at their base. This is done by applying a specific hormone mixture under the appropriate conditions. The creation of rooted cuttings is very difficult and is especially challenging using the Canadian yew. Whilst it is possible that rooted cuttings may be an interesting possibility for biosynthetic studies, it is firstly important to characterize the content of these yews in order to establish whether a significant difference in taxane content exists. If one does exist, the next important question is why and what does this infer to the biosynthesis of taxanes in rooted cuttings. Although rooted cuttings do originate from mature trees and should thus contain approximately the same content and the same enzymes as they do, the hormone stimulated root growth will also change the many biological pathways within the plant, possibly including the biosynthesis of

paclitaxel. Depending on the needs of the rooted cutting, taxanes already present within it may be further modified or may decompose and form new taxanes, establishing a new equilibrium within the plant. Due to the previously determined variation in taxane content between the Canadian yew and the other yew species, the use of rooted cuttings of *Taxus canadensis* described in the following experimental section is even more interesting. The determined taxane content of the rooted cuttings may determine whether there is a significant difference in taxane content, and will also allow the groundwork to be laid for future biosynthetic studies using these plants. Before these rooted cuttings may be used for biosynthetic feeding experiments however, a protocol for such feeding experiments must also be developed.

3. Experimental *(see Appendix 3 for list of products used)*

3.1 *Taxus canadensis* rooted cuttings: taxane content

3.1.1 Extraction of *Taxus canadensis* rooted cuttings

Two hundred and thirty five *Taxus canadensis* rooted cuttings (see **figure 19**), obtained in mid September 2001, were washed free of dirt and allowed to air dry. Once dried, the rooted cuttings were cut into small pieces averaging 1cm apiece. These were then ground using a commercial food processor (Robot Coupe USA inc, model BX3) to produce 583.93g of fine powder (Mettler PC2000, Fisher Scientific). This powder was divided into four portions of approximately equal value, and each was placed in a 2L flask. To each flask, 750 mL of 100% methanol was added. The flasks were stoppered with foam/cheesecloth stoppers and covered with aluminium foil. The flasks were then placed on a shaker and left to extract for a period of twenty-four hours at room temperature in the absence of light (see **figure 20**). The methanol extract was then removed and replaced by another 750 mL of fresh 100% methanol. The decanted methanol extract was placed in a Pyrex baking tray and left to evaporate under the fume hood. The flasks were once again placed on the shaker and the plant material was left to extract for another 24 hours. This process was repeated for a third time with 100% methanol, and an additional four times where the 100% methanol was replaced by a mixture of MeOH:CH_2Cl_2 (50:50 vol/vol). Each time the extraction mixture was decanted, it was added to the Pyrex baking tray and left to evaporate. Evaporation was done in a large dish rather than in a round bottom flask under reduced pressure to avoid unnecessary loss of material, which is very viscous in nature and difficult to redissolve in the following step. Taxanes are very stable compounds and therefore limiting their exposure to oxygen throughout the many purification steps is not necessary. This extraction protocol is similar to that established by previous students in Dr Zamir's laboratory.

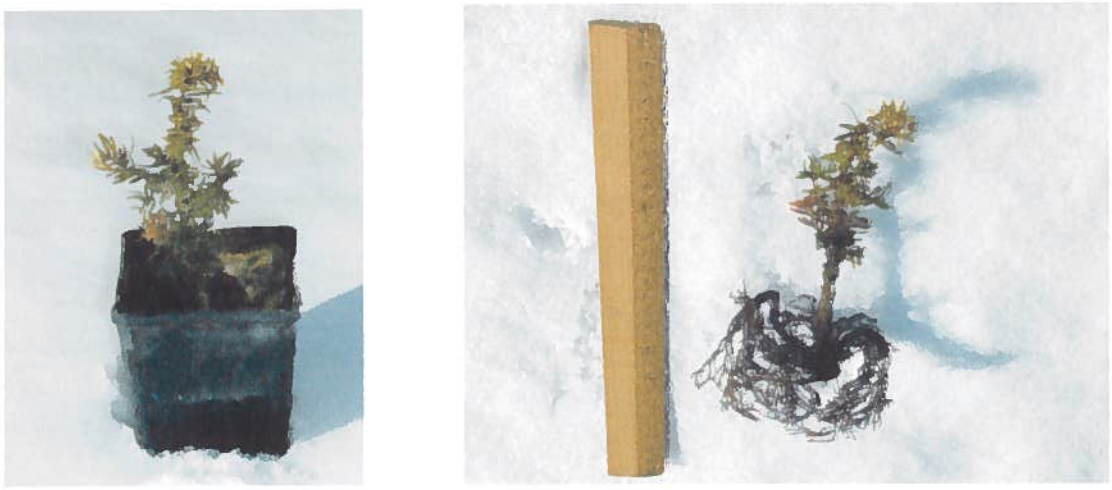


Figure 19. Rooted cuttings of *Taxus canadensis* as obtained from Cramer Nurseries.

Due to the high content of lipids and waxes of the extract, as well as the concentration of compounds, a molasses-like consistency was obtained after evaporation of the extract rather than a solid dry substance. The first step in the extraction therefore involved the removal of the more lipophyllic compounds. In order to do this, a third of the extract at a time was dissolved in approximately 400 mL of warm distilled water ensuring that a good suspension was achieved. This aqueous mixture was then extracted three times with 750-900 mL of hexane in a separation funnel. Since the concentration of the layers varied between extractions, the volume of solvent used to extract the aqueous layer was altered slightly as needed to improve the degree of separation of the layers. The volume of solvent used for extraction therefore fell into the range of 750-900 mL, which allowed an optimized separation particularly when an emulsion layer was present. The volume of water used was equally adjusted as needed to optimize the separation. The extraction of the crude mixture was done in thirds due to the high concentration and extremely sticky consistency of the extract, which made it difficult to dissolve the extract in its entirety in a volume of water small enough to allow extraction in a 2L separation funnel. For this reason, the extraction was divided up and the final layers obtained were later combined: all the hexane layers (upper layers) together and all the aqueous layers (lower layers) together.

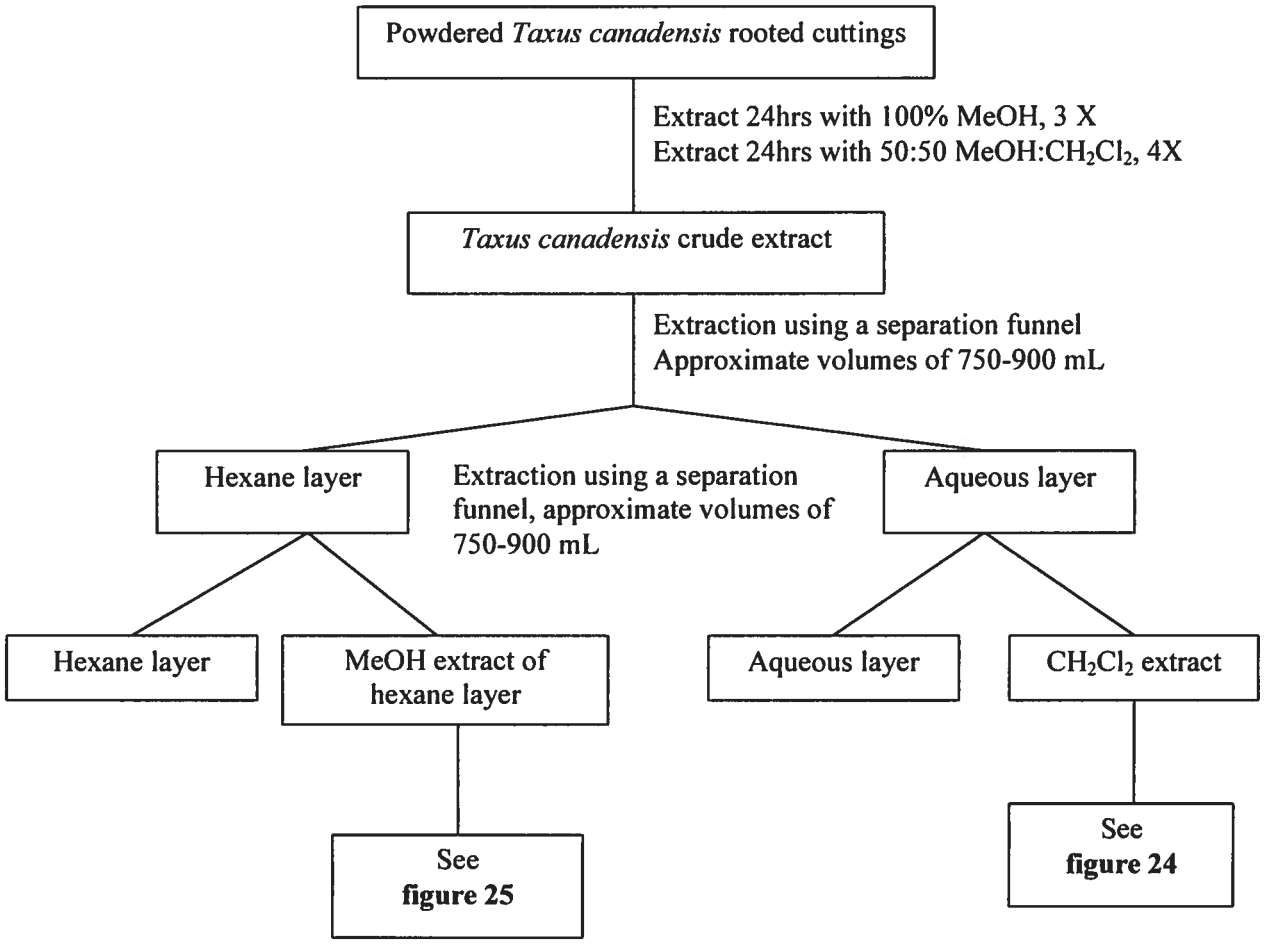
The analysis of the content of *Taxus canadensis* rooted cuttings was semi-quantitative and therefore the major taxanes that were being considered needed be

purified in their entirety. Since we have previously recovered taxanes from the hexane layer, the hexane layer was extracted three times with 750-900 mL of methanol in order to remove the most polar compounds from the typically non-polar mixture. Once again due to the large quantity of extract being handled, the extraction was done in fractions and the final layers obtained were later combined: all the hexane layers (upper layers) together and all the methanolic layers (lower layers) together.

Once the aqueous layer was extracted three times with hexane, it was saturated with sodium chloride (NaCl) and extracted three times with 750-900 mL of dichloromethane. Again, due to the large quantity of extract being handled, the extraction was done in fractions and the final layers obtained were later combined: all the aqueous layers (upper layers) together and all the dichloromethane layers (lower layers) together. The combined dichloromethane layer was the layer from which the majority of the taxanes were purified from, as described in the subsequent steps. In order to remove any remaining water from this organic layer, anhydrous sodium sulphate (Na_2SO_4) was added to the dichloromethane layer a bit at a time with continuous swirling of the dichloromethane solution. The anhydrous sodium sulphate was added until it ceased to clump together, indicating that drying of the extract was achieved, and it was then removed from the solution by gravity filtration through a Whatman (No. 4) filter paper. The dried dichloromethane layer was then evaporated down under reduced pressure using a rotary evaporator (Büchi, Rotavapor R120) coupled to a temperature controlled water bath (Brinkmann).

The same extraction procedure was repeated until all parts of the initial plant extract had been extracted and all the dichloromethane layers were combined, as were the aqueous layers, the hexane layers and the methanolic extracts of the hexane layers.

Figure 20: Extraction of rooted cuttings of *Taxus canadensis*



3.1.2 Initial purification of the dichloromethane extract of *Taxus canadensis* rooted cuttings by flash column chromatography

The purification of taxanes began with the dichloromethane layer (see **figure 20** and **24**), which weighed a total of 8.36g. This crude mixture partially composed of taxanes was first purified by flash column chromatography. 200g of silica (Silica gel 60, 230-400 mesh, Sili Cycle) was mixed with 100% dichloromethane, ensuring that all bubbles were removed. This slurry was poured into a 5.5 cm diameter column with a fritted glass bed support. The solvent was allowed to run through until the solvent level was just above the silica, therefore avoiding drying of the column but allowing the silica to be packed. Once packed, the column was 22 cm in height. The sample was applied to

the top of the column, dissolved in a minimal volume of 100% dichloromethane, and was subsequently eluted with a series of solvent mixtures. Firstly, 0.5L of 33% ethyl acetate in dichloromethane (165mL EtOAc with 335mL CH_2Cl_2) was eluted to give fractions #1-5 of 100 mL each. 0.5L of 50% ethyl acetate in dichloromethane (250mL each of EtOAc and CH_2Cl_2) gave fractions #6-15 of 50 mL each, 0.2L of 66% ethyl acetate in dichloromethane (132mL EtOAc with 68mL CH_2Cl_2) gave fractions # 16-19 of 50 mL each, and 1.3L of 100% ethyl acetate gave fractions #20-45 of 50 mL each. Each solvent mixture was allowed to elute until the solvent line was just above the level of the silica to avoid mixing of solvent systems. Once all fractions had been collected, the column was washed with 100% methanol. This fraction was also collected and retained. Throughout the running of the column, a small pressure was applied to the top of the column to speed the elution of solvents and keep the flow rate constant. Each of the fractions collected from the column was evaporated and every second one was analysed by analytical HPLC, using **program #1** (see **Appendix 1**).

3.1.3 Analytical scale high-pressure liquid chromatography system used throughout experimental

The analytical HPLC system referred to throughout this experimental consisted of a Waters 600 Controller, coupled to a Waters 996 Photodiode Array detector and a Waters 717plus Autosampler. Two C18-partisil (4.6mm x 500mm) columns connected in series preceded by a Pelicular-ODS pre-column were used. The flow rate used with this HPLC system was fixed at 1mL per minute. The analytical scale HPLC injections cited throughout the experimental always refer to the above-described system and the different programs used, **programs #'s1-4**, are listed in **Appendix 1**. Several sample spectra are included to illustrate the general appearance of the spectra (see **figures 21, 22, 23**), since it was impractical to include all of the spectra obtained throughout this experimental due to the large number of analyses done.

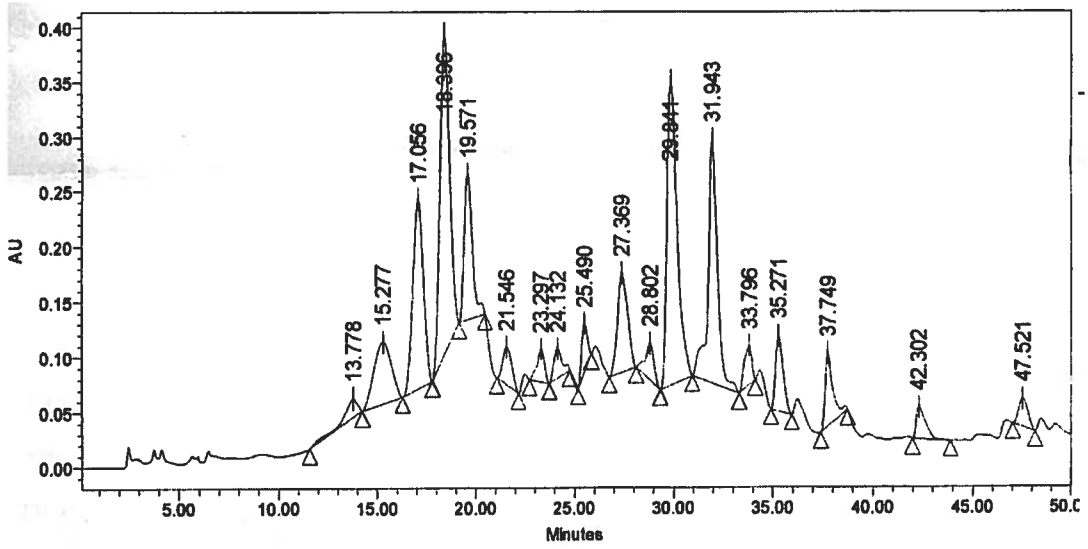


Figure 21: Spectra of 9-dihydro-13-acetyl-baccatin III containing sample, analytical scale HPLC, using program #1.

3.1.4 Combination of samples based on content, determined after analysis by analytical HPLC

Each of the fractions collected from the column was evaporated and every second one was analysed by analytical HPLC, using **program #1** (see **Appendix 1**). This programmed run was the most commonly used, as the retention times of many taxanes are known in these conditions, and this therefore allowed an estimation of what taxanes may be present within the analysed mixture. The comparison of the different retention times present within a mixture with the retention times of standards known to our lab allowed fractions containing major taxanes such as paclitaxel to be identified rather reliably. Although a simple comparison of retention times is not sufficient to identify a compound with 100% accuracy, it was sufficient to guide the preliminary purification steps involved in this experimental. For the fractions thought to contain 10-deacetyl-baccatin III (10-DAB-III), **program #3** (see **Appendix 1**) was used for analysis by analytical HPLC. This afforded a better separation of 10-DAB-III from compounds which had otherwise similar retention times when **program #1** (see **Appendix 1**) was instead used. Once analysis of

the samples was complete, the fractions found to contain 10-DAB-III were pooled together, as were those found to contain 9-dihydro-13-acetyl-baccatin III, and those which contained paclitaxel and cephalomanine.

Fractions numbered 10, 11, 12, 13, and 14 were combined to form the mixture containing paclitaxel and cephalomanine. Fractions numbered 15, 16, 17, 18, 19, 20, 21, 22, and 23 were combined to form the mixture with 9-dihydro-13-acetyl-baccatin III. Fractions numbered 27, 28, 29, 30, 31, 32, and 33 were combined to form the mixture containing 10-deacetyl-baccatin III (see **figure 24**).

A sample of each of these mixtures was re-injected and run at the analytical scale using the program that was intended for the purification at the preparative scale, in order to visualise the separation that would be achieved. **Program #3** and **Program #1** (see **Appendix 1**) were used for the separation of 10-DAB-III and 9-dihydro-13-acetyl-baccatin III respectively. **Program #4** (see **Appendix 1**) was used to obtain a suitable separation of paclitaxel and cephalomanine.

While separation of compounds within the 9-dihydro-13-acetyl-baccatin III mixture was observed with the analytical scale HPLC injection, the closeness of the retention times of the compounds resulted with **program #3** (see **Appendix 1**) being used for the preparative scale injections instead. This allowed the same type of separation of compounds, but allowed a greater difference in retention times to be achieved. The 10-DAB-III mixture was also injected and purified using **program #3** (see **Appendix 1**) as previously described. A good separation of paclitaxel and cephalomanine was observed with the multi-step **program #4**.

The weights of the mixtures containing 10-DAB-III, paclitaxel and cephalomanine, and 9-dihydro-13-acetyl-baccatin III were determined to be 319mg, 2.03g and 515mg respectively (Mettler H51AR, Fisher scientific). Due to the size of the samples, the purification of each mixture by preparative HPLC could not be achieved in one injection. In the case of the paclitaxel and cephalomanine mixture, to avoid wasting too much solvent due to repetitive injections using the time-consuming **Program #4** (see **Appendix 1**), an intermediate purification step using **Program #1** (see **Appendix 1**) was introduced. This allowed fewer injections to be made using the multi-step program and thus saved much solvent and time. **Program #1** (see **Appendix 1**) was used to separate

the paclitaxel and cephalomanine from the other compounds within the mixture. The longer multi-step program was then used on the samples containing mainly paclitaxel and cephalomanine purified from the previous mixture, which were therefore smaller in size and required fewer injections to be done. The 9-dihydro-13-acetyl-baccatin III and 10-deacetyl-baccatin III samples were purified by multiple injections of **program #3** (see **Appendix 1**) as described above.

3.1.5 Preparative scale high-pressure liquid chromatography system used throughout experimental

For all of the preparative HPLC injections made throughout this experimental, the same protocol was followed. The preparative HPLC system used was a Waters Delta Prep 3000 coupled to a Waters 486 Tuneable Absorbance Detector, adjusted to measure absorbance at 227nm. A Whatman partisil 10 ODS-2 (22mm x 500mm) column was used preceded by a Pelicular-ODS pre-column. Flow rate was fixed at 18 mL per minute. See **Appendix 1** for a list of the programs used for the preparative scale injections. All peaks, representative of an increase in absorption and corresponding to the detection of a compound or compounds, were collected in individual flasks. In cases where the peak visualised was a large one or a broad one, the collection of the sample was divided up. The dividing up of single peaks into three sections, for example two shoulders and a middle portion, allowed for a more efficient removal of impurities. Once a programmed run was completed, the samples collected were evaporated to dryness. These samples, with emphasis placed on those corresponding to the major peaks observed from the spectra, were analysed by analytical HPLC using **program #1** (see **Appendix 1**). A sample spectrum is included to illustrate the general appearance of the spectra obtained during a preparative scale injection (see **figure 22**). A sample spectrum of a fraction obtained after a preparative scale injection is also included (see **figure 23**).

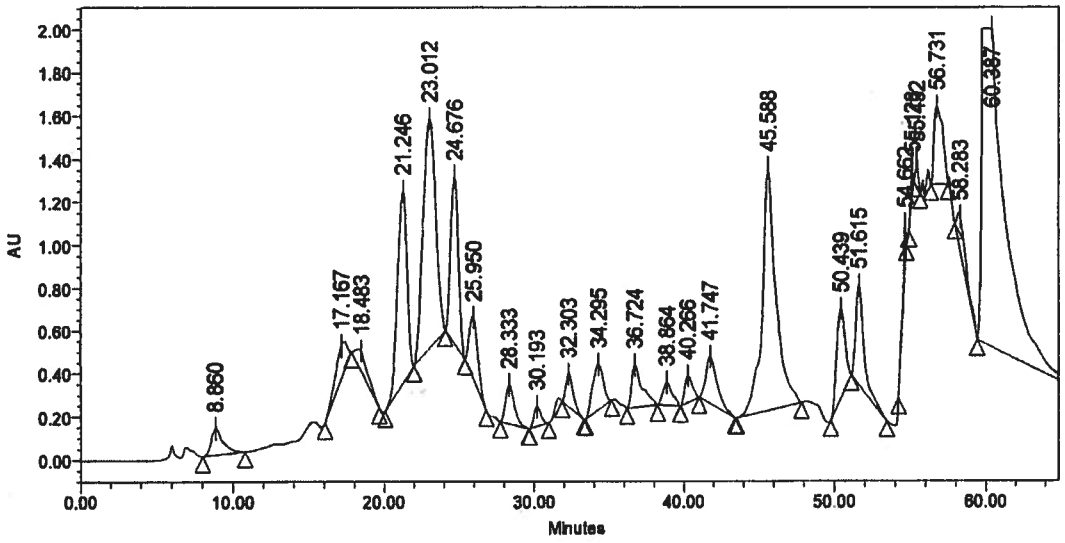


Figure 22: Spectra of a preparative scale injection of the 9-dihydro-13-acetyl-baccatin III containing sample, using program #3.

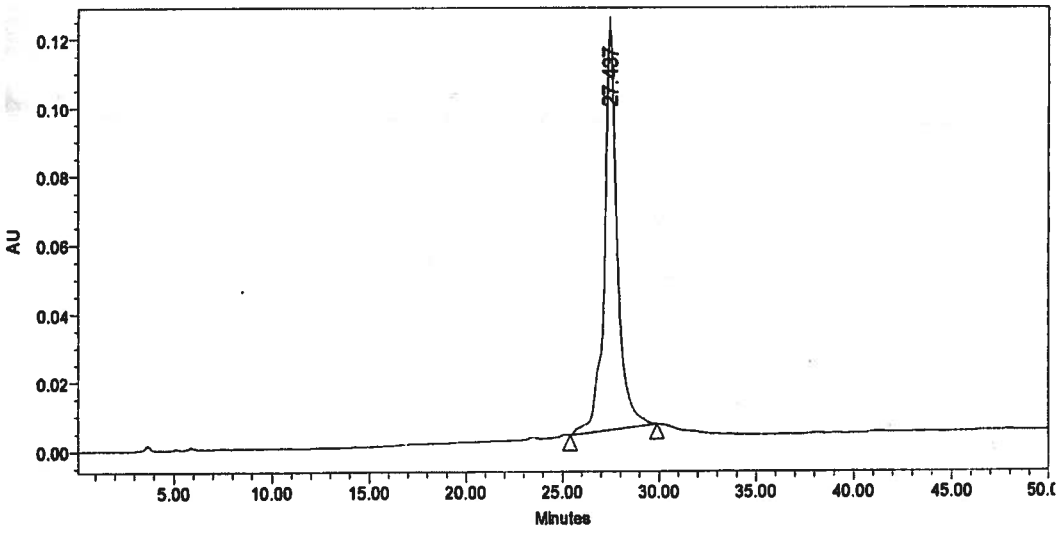
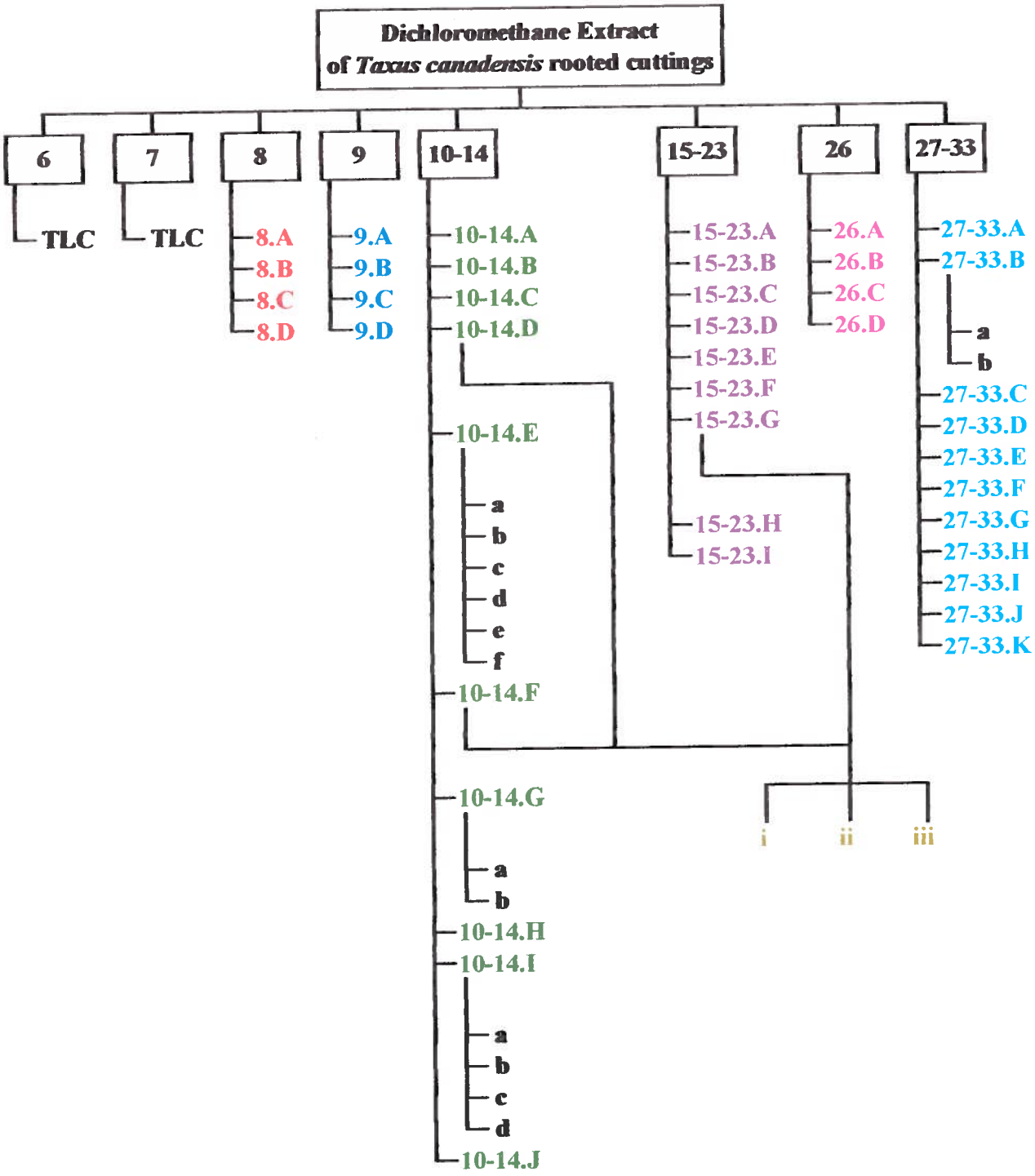


Figure 23: Spectra of a fraction collected after preparative scale injections of the 9-dihydro-13-acetyl-baccatin III containing sample, analysed using analytical scale HPLC, using program #1.

Figure 24: Purification of dichloromethane extract by chromatography (column, HPLC, TLC).



3.1.6 General protocol for purification of samples and characterization of compounds

Upon consultation of the many analytical HPLC absorption spectra obtained after the initial purification steps, samples that appeared to be pure were then analysed by ^{1}H NMR (Bruker, AMX 500MHz). These samples were all prepared in deuterated chloroform (CDN-isotope, 99.8% atom % D). The first of such samples that were characterized by NMR showed the presence of a small amount water and solvent in their spectra. All future samples where therefore placed on a vacuum oil pump (Precision Vacuum Pump, model D75, GCA corporation) for between 30 minutes and an hour to rid the samples of the remaining moisture. Samples that held mixtures of compounds were either further purified by HPLC or by TLC, or where put to one side while purification of other samples progressed. The order of samples to be purified was determined by several factors. Firstly, samples containing 10-deacetyl-baccatin III, 9-dihydro-13-acetyl-baccatin III, paclitaxel and cephalomanine were given priority. Further purification was done on samples that held one or several large peaks, which were straightforward to purify, and which held compounds of different retention times such that a wide range of compounds would be isolated. After samples were analysed by ^{1}H NMR (Bruker, AMX 500MHz) and compounds were identified (*spectra interpretation performed by Dr Qing-Wen Shi*), they were sent for analysis by high-resolution fast-atom-bombardment mass spectroscopy (HR FAB-MS) for confirmation by their molecular weight (*McGill Mass Spectroscopy Unit, Dr. O. Mamer*). This was done for all compounds that were already known in the literature. Compounds whose structures remained unreported were instead first sent for characterization by COSY, HSQC, HMBC, and either NOESY or ROESY NMR (*Queens University, Dr. F. Sauriol*). Once the 2D NMR data was obtained, the samples were then sent for HR FAB-MS. For the samples which were identified as novel taxanes, $[\alpha]_D$ was also measured (JASCO, DIP-370, Digital polarimeter).

3.1.7 Purification of 10-deacetyl-baccatin III containing samples

The 10-deacetyl-baccatin III containing mixture, formed from the combination of fractions # 27, 28, 29, 30, 31, 32, and 33 of the original flash column chromatography

described above (total weight: 319mg), was partially purified by two duplicate preparative HPLC injections. Both of these injections were run using **program #3** (see **Appendix 1**) and samples 27-33.A through 27-33.K were obtained (see **figure 24**). Fraction 27-33.C was re-injected using the same **program #3** as the previous two preparative HPLC injections and samples 27-33.C-**a** and 27-33.C-**b** were obtained. Other fractions collected from either set of injections, which were mixtures of compounds and which were not further purified, were also retained but are not listed in **figure 24**.

3.1.8 Purification of paclitaxel and cephalomanine containing samples

The paclitaxel and cephalomanine containing mixture, formed from the combination of fractions 10, 11, 12, 13, and 14 (total weight: 2.026g) was partially purified by five duplicate preparative HPLC injections. Each of these injections was run using **program #1** (see **Appendix 1**) and samples 10-14.A through 10-14.J were obtained (see **figure 24**). Other fractions collected, which were mixtures of compounds and which were not further purified, were also retained but are not listed in **figure 24**. Sample 10-14.E was further purified by an additional preparative HPLC injection, run using **program #2** (see **Appendix 1**). Fractions 10-14.E-**a** through 10-14.E-**f** were obtained (see **figure 24**). Sample 10-14.G was further purified by an additional preparative HPLC injection, run using **program #1** (see **Appendix 1**) and fractions 10-14.G-**a** and 10-14.G-**b** were obtained (see **figure 24**). Sample 10-14.I (see **figure 24**) was collected in three vials with weights of 37.1 mg, 55.9 mg and 30.1 mg (Mettler H51AR, Fisher scientific), and was further purified by three preparative HPLC injections using **program #4** (see **Appendix 1**). Fractions 10-14.I-**a** through 10-14.I-**d** were obtained. Samples 10-14.D and 10-14.F were combined with another sample obtained from the 9-dihydro-13-acetyl-baccatin III mixture, (15-23.G), described also in the next section of the experimental. This mixture was further purified by a preparative HPLC injection, run using **program #2** (see **Appendix 1**), and fractions i, ii and iii were obtained (see **figure 24**). Samples 10-14.A, 10-14.B and 10-14.C were also further purified by preparative HPLC using **program #1** (see **Appendix 1**), but the fractions obtained as a result of these injections are not listed as their purification and characterization was not pursued any further.

3.1.9 Purification of 9-dihydro-13-acetyl-baccatin III containing samples

The 9-dihydro-13-acetyl-baccatin III containing mixture (see **figure 21**), formed from the combination of fractions 15, 16, 17, 18, 19, 20, 21, 22, and 23 of the original flash column chromatography (total weight: 515mg), was partially purified by three duplicate preparative HPLC injections (see **figure 22**). Each of these injections was run using **program #3** (see **Appendix 1**), and fractions **15-23.A** through **15-23.I** were obtained (see **figure 24**). Sample **15-23.G** was combined with samples **10-14.D** and **10-14.F** from the paclitaxel and cephalomanine-containing mixture described previously. These combined fractions were further purified by a preparative HPLC injection run using **program #2** (see **Appendix 1**) and gave samples **i**, **ii** and **iii**. Other fractions collected throughout these purification steps, which were mixtures of compounds and which were not further purified, were also retained but are not listed in **figure 24**.

3.1.10 Purification of fraction 8 by preparative HPLC

Fraction 8, collected from the initial flash column chromatography, was also further purified by a preparative HPLC injection using **program #1** (see **Appendix 1**). Samples **8.A** through **8.D** were obtained (see **figure 24**), along with other samples which contained mixtures of compounds and were not pursued any further. These untouched samples are not listed.

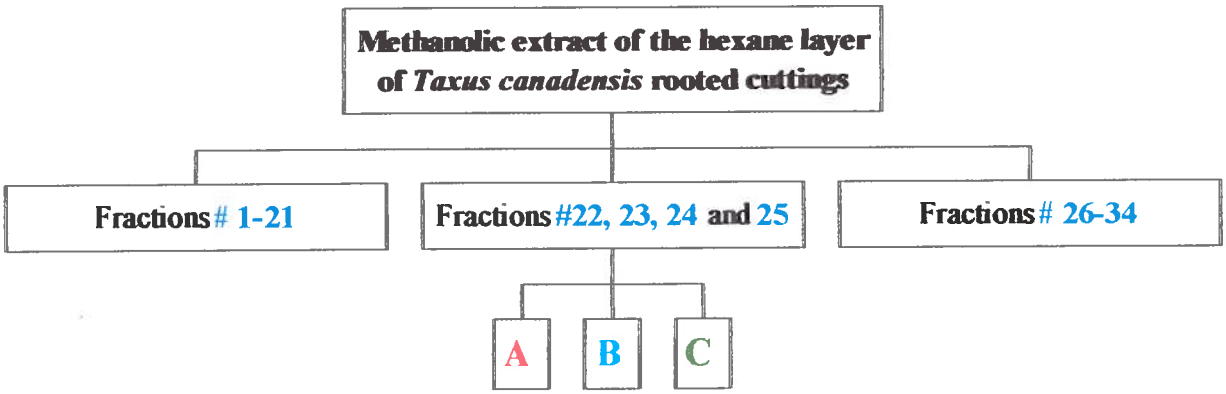
3.1.11 Purification of the methanolic extract of the hexane layer by flash column chromatography

Upon analysis of the methanolic extract of the hexane layer (total weight: 14.74g) by analytical HPLC, compounds with retention times similar to those of the taxanes being studied where observed. In order to avoid the loss of these possible taxanes, especially those being studied quantitatively, further purification of the methanol extract of hexanes was done. The same column chromatography system as that described in *section 3.1.2* was used to further purify this mixture since the compounds of interest were suspected to be the same as those purified from the dichloromethane layer earlier. It was noted during

the previous flash column chromatography that, due to the viscosity of the extract being purified, the sample applied directly to the top of the packed silica made it more difficult for the solvents to pass through the column. For this reason the method of applying the sample to the column was altered for its use in this next purification step. The methanolic extract of the hexane layer was re-dissolved in a minimal volume of methanol and was added to 28g of silica gel (Silica gel 60, 230-400 mesh, Sili Cycle). The methanol was allowed to evaporate off under the fume hood and the mixture was periodically stirred to ensure equal mixing. The final product was the sample absorbed onto the silica. This dry sample-silica mixture was then ground with a mortar and pestle to ensure that no lumps were present. The column was prepared as before: 200g of silica (Silica gel 60, 230-400 mesh, Sili Cycle) were mixed with 100% dichloromethane, ensuring that all bubbles were removed. This slurry was poured into a 5.5 cm diameter column with a fritted glass bed support. The solvent was allowed to run through until the solvent level was just above the silica, therefore avoiding drying of the column but allowing the silica to be packed. Once packed, the column was 22 cm in height. Rather than pipette the sample onto the top of the column as done previously, the dry silica-sample mixture was slowly added and allowed to settle atop the packed silica. Care was taken to ensure that the powder was evenly distributed. The sides of the column were then rinsed down with dichloromethane such that the sample-silica mixture atop the previously paced silica was level and just under the solvent line. The column was subsequently eluted with 0.5L of 33% ethyl acetate in dichloromethane (165mL EtOAc with 335mL CH_2Cl_2) to give fractions #1-5 of 100 mL each. 0.5L of 50% ethyl acetate in dichloromethane (250mL each of EtOAc and CH_2Cl_2) was added next and gave fractions #6-15 of 50 mL each. 0.2L of 66% ethyl acetate in dichloromethane (132mL EtOAc with 68mL CH_2Cl_2) was passed through the column next and gave fractions # 16-19 of 50 mL each, followed by 0.4L of 100% ethyl acetate which gave fractions #20-27 of 50 mL each. Finally, 400mL of 100% ethyl acetate was passed through the column followed by 450 mL of methanol to wash the column and samples 28 to 34 were collected. These last fractions were 50mL each in size, with the exception of the last two, which were slightly larger than this amount in order to dry the column. Each solvent mixture used was allowed to elute until the solvent line was just above the level of the silica to avoid mixing of solvent systems. Throughout the

running of the column, a small pressure was applied to the top of the column to speed the elution of solvents and keep the flow rate constant.

Figure 25. Purification by chromatography (column and HPLC) of the methanolic extract of the hexane layer



3.1.12 Purification of samples from the methanol extract of the hexane layer by preparative HPLC

Fractions 22, 23, 24, and 25 from the above flash column chromatography (of the methanolic extract of the hexane layer) were combined due to their similar content of a peak perhaps corresponding to paclitaxel. The resulting sample formed from the combined fractions was further purified by a preparative HPLC injection (see **figure 25**), **using program #1 (see Appendix 1), yielding samples A, B and C. Other samples which were also obtained from this preparative HPLC injection but whose purification was not** further pursued are not listed.

3.1.13 Purification of fraction 9 by preparative HPLC

Fraction 9 from the flash column chromatography of the dichloromethane extract was further purified by a preparative HPLC injection using **program #1** (see **Appendix 1). Samples 9.A through 9.D were obtained (see figure 24), along with other samples**

which contained mixtures of compounds and were not pursued any further. These untouched samples are not listed.

3.1.14 Purification of fraction 26 by preparative HPLC

Fraction 26 from the flash column chromatography of the dichloromethane extract was further purified by a preparative HPLC injection using **program #1** (see **Appendix 1**). **Samples 26.A through 26.D were obtained (see figure 24), along with other samples which contained mixtures of compounds and were not pursued any further. These** untouched samples are not listed.

3.1.15 Partial purification of fractions 6 and 7 by thin-layer chromatography (TLC)

Fractions **6** and **7** from the initial flash column chromatography of the dichloromethane layer were further purified by preparative thin-layer chromatography (TLC). TLC was chosen as the means of further purification due to the low polarity of these mixtures and thus the difficulty in dissolving them in their entirety in a solvent suitable for injection into the HPLC system with reverse-phase columns. The samples were instead dissolved in 100% dichloromethane and applied to TLC plates. To find the optimal solvent system for the elution of the TLC plates, samples were spotted on small plates of approximately 5cm in height and a variety of solvents mixtures were tested. The plates were first observed under UV light (UV lamp, Ultra-Violet Products Inc.) and then sprayed with a 10% sulphuric acid solution in ethanol. Once treated with this mixture, the plates were heated. This treatment allowed the spots to be visualized. A solvent system of hexane:acetone (3:2 vol/vol) was chosen for both fraction 6 and fraction 7. The samples were then spotted on four 20x20 cm TLC plates each (Silica Gel F_{254}, 250µm thickness) and allowed to elute to a level of 1cm below the top of the plate. Each TLC plate was prepared by marking off this 1cm boundary below the top level, as well as by marking a similar line on the bottom of the plate. It was on this bottom line that the sample was spotted evenly using thin capillary tubes. The capillary tubes were made by heating Pasteur pipettes above a Bunsen burner and pulling the glass. These thin capillaries of glass were then cut to size, discarding the ends or parts of the tube that were too thick.

Solvent was added to the developing tank such that it did not go above the marked 1cm line where the sample was applied, but that enough was present to allow it to travel to the desired level on the TLC plate. As such approximately 50 mL of solvent was used per developing tank. Once the solvent reached the marked level at the top of the plate, the plates were removed from the tank and allowed to dry, and were then observed under UV light (Ultra-Violet Products Inc.). Since the compounds separated on the plate were to be isolated and recuperated, it was important to not develop the plates using the same 10% sulphuric acid solution described earlier, as isolation of the compounds would otherwise no longer be possible. The plates were instead observed only under a UV lamp (Ultra-Violet Products Inc.) and the bands were marked in pencil. Bands 0 through 14 were marked for fraction 6 and the Rfs were measured as 0.167, 0.206, 0.250, 0.306, 0.333, 0.361, 0.389, 0.416, 0.431, 0.444, 0.472, 0.500, 0.528, 0.556, and 0.639 respectively. Bands 1 through 12 were marked for fraction 7 and the Rfs were measured as 0.250, 0.306, 0.333, 0.389, 0.417, 0.444, 0.472, 0.500, 0.528, 0.556, 0.611, and 0.750 respectively. The silica from the bands circled was scraped off the glass plate and collected. The separated compounds were removed from the silica by rinsing the silica several times each with dichloromethane and then ethyl acetate. The collected solvents were then evaporated to dryness, leaving only the dried isolated compounds. Each of the samples corresponding to a band originating from either fraction 6 or 7 was analyzed by analytical HPLC using **program #1** (see **Appendix 1**). Due to a time restraint, further purification of these samples was not continued, and the samples were kept for possible future use in our lab.

3.2 Extraction of *Taxus cuspidata capitata* rooted cuttings

Sixty *Taxus cuspidata capitata* rooted cuttings, obtaineed in mid May 2001, were washed free of dirt and allowed to air dry. Once dried, the rooted cuttings were cut into small pieces averaging 1cm in size. These were then ground using a commercial food processor (Robot Coupe USA inc, model BX3) to produce 148.83g of fine powder (Mettler PC2000, Fisher Scientific). This powder was placed in a 2L flask to which 900 mL of 100% methanol was added. The flask was then stoppered with a foam/cheese cloth

stopper and covered with aluminium foil. The flask was placed on a shaker and left to extract for a period of 24 hours at room temperature in the absence of light. The methanolic extract was then removed and replaced by another 900 mL of fresh 100% methanol. The decanted methanolic extract was placed in a Pyrex baking tray and left to evaporate under the fume hood. The flasks were once again placed on the shaker and the plant material left to extract for another 24 hours. This process was repeated for a third time with 100% methanol, and an additional four times where the 100% methanol was replaced by a mixture of MeOH:CH_2Cl_2 (50:50 vol/vol). Each time the extraction mixture was decanted, it was added to the Pyrex baking tray and left to evaporate.

The crude extract was dissolved in approximately 400 mL of warm distilled water and this aqueous mixture was then extracted with hexane three times (approximately 750mL each time) to remove the lipids. The aqueous layer was then saturated with sodium chloride and extracted three times with dichloromethane (approximately 750mL each time). The dichloromethane layers were combined and dried over anhydrous sodium sulphate. The dried dichloromethane was then evaporated down to dryness using a rotary evaporator (Büchi, Rotavapor R120) coupled to a temperature controlled water bath (Brinkmann), yielding an extract weighing 1097mg (Mettler H51AR, Fisher scientific).

3.2.1 Purification of the dichloromethane extract of *Taxus cuspidata capitata* rooted cuttings by flash column chromatography

The crude dichloromethane extract was dissolved in a minimal volume of solvent and transferred to a beaker containing 2g of silica (silica gel 60, particle size: 230-400 Mesh, Sili Cycle). This was mixed and allowed to evaporate to dryness, resulting in a dry silica-sample mixture. The mixture was then ground with a mortar and pestle to remove any lumps. 75g of silica (silica gel 60, particle size: 230-400 Mesh, Sili Cycle) were mixed with a mixture of hexane:acetone (1:1, vol/vol). This slurry was poured into a 3.3cm diameter column and the solvent was eluted until the solvent line was just above the silica. Once packed, the silica was 20cm in height. The sample-silica mixture was added next, slowly and evenly to the top of the packed silica. The sides of the column were then rinsed down with more of the hexane:acetone (1:1, vol/vol) mixture such that

the solvent level was just above the sample-silica level. The column was then eluted with hexane:acetone (1:1, vol/vol) and fractions 1 through 7 of 100mL each were collected. The solvent was then changed to hexane:acetone (1:2, vol/vol) and fractions 8 through 15 of 100mL each were collected. 100% acetone was used next and fractions 16 through 27 were collected. Each of these samples were analysed by analytical HPLC using **program #1** (see **Appendix 1**). Each of the fractions were weighed and a table of the sample names and their weights follows. At this time, the twenty-seven partially purified samples obtained from the flash column chromatography were given to Dr Qing-Wen Shi, a post-doctorate fellow in our laboratory, for continued purification. The ensuing work was done by Dr Shi, and consisted of a series of purifications by TLC and HPLC.

Table 2. Weights of samples obtained from the flash column chromatography separation of the *Taxus cuspidata capitata* rooted cuttings extract
Note (*) that the weights of fractions 2 and 3 are not accurate as the samples were not completely dry. They were later re-weighed by Dr Shi.

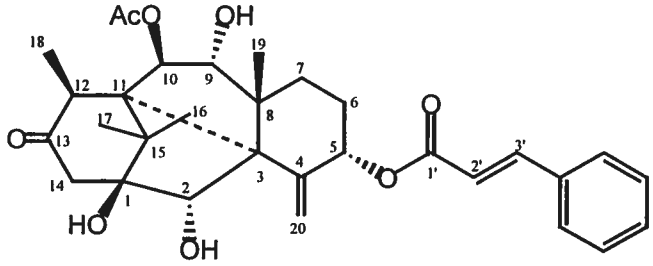
Samples from flash column chromatography of *T. cusp* extract	
Sample name	**Sample weight**
TP-tcusp-f1	32.42mg
TP-tcusp-f2	122.94mg *
TP-tcusp-f3	119.45mg *
TP-tcusp-f4	92.38mg
TP-tcusp-f5	45.96mg
TP-tcusp-f6	31.02mg
TP-tcusp-f7	29.77mg
TP-tcusp-f8	28.91mg
TP-tcusp-f9	13.00mg
TP-tcusp-f10	26.82mg
TP-tcusp-f11	19.62mg
TP-tcusp-f12	17.79mg
TP-tcusp-f13	18.02mg
TP-tcusp-f14	20.15mg
TP-tcusp-f15	10.34mg
TP-tcusp-f16	10.44mg
TP-tcusp-f17	9.90mg
TP-tcusp-f18	14.43mg
TP-tcusp-f19	8.07mg

4. Results

4.1 Results from the *Taxus canadensis* rooted cuttings taxane content study

Several different taxanes, with varying structural characteristics, were identified from the extract of the *Taxus canadensis* rooted cuttings. Since the taxane content of rooted cuttings of yews has never before been studied, these findings mark the first of such results. Of these taxanes characterized, **three** were novel taxanes, never before reported in the literature (see **figures 26-30**). Another **fourteen** taxanes isolated from the extract of *Taxus canadensis* rooted cuttings were found for the first time in *Taxus canadensis* (see **figure 31**). The remaining **twelve** taxanes were known compounds, which have previously been isolated from the Canadian Yew (see **figure 32**).

Figure 26. Structures of novel taxanes isolated from *Taxus canadensis* rooted cuttings, with their HR FAB-MS and NMR data (^{1}H and ^{13}C NMR spectral data for the novel taxanes in $CDCl_3$)



1β,2α,9α-trihydroxy-10β-acetoxy-5α-cinnamoyloxy-3-11-cyclictaxa-4(20)-ene-13-one

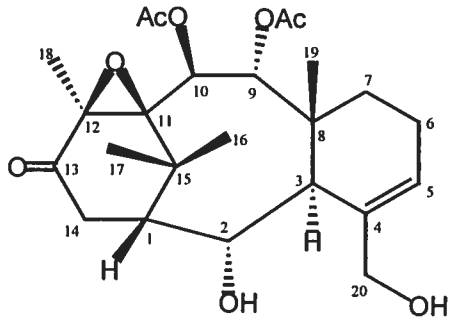
(Novel taxane #1)

HR FAB-MS: *m/z* 577.22016 ($M+K^+$)

Confirms structure with MW: 538.63 g/mol, $C_{31}H_{38}O_8$

$[\alpha]_D^{24} = 7.3394°$, where c = 0.109 $CHCl_3$

2-3 mg in total.



2α,9α,10β-triacetoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one

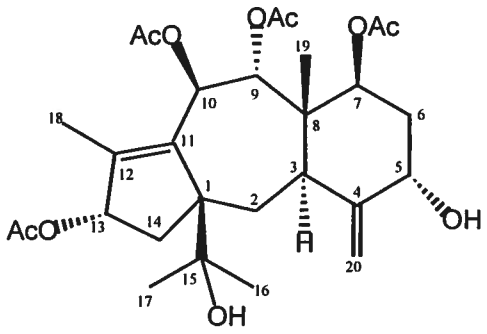
(Novel taxane #2)

HR FAB-MS: *m/z* 531.19967 ($M+K^+$)

Confirms structure with MW: 492.56 g/mol, $C_{26}H_{36}O_9$

$[\alpha]_D^{24}$ = -19.580°, where c = 0.0715 $CHCl_3$

0.5-1 mg in total.



7β,9α,10β,13α-tetraacetoxy-11(15→1)*abeo*taxa-4(20),11-diene-5α,15-diol

(Novel taxane #3)

HR FAB-MS: *m/z* 575.22600 (for $M+K^+$)

Confirms structure with MW: 536.26 g/mol, $C_{28}H_{40}O_{10}$

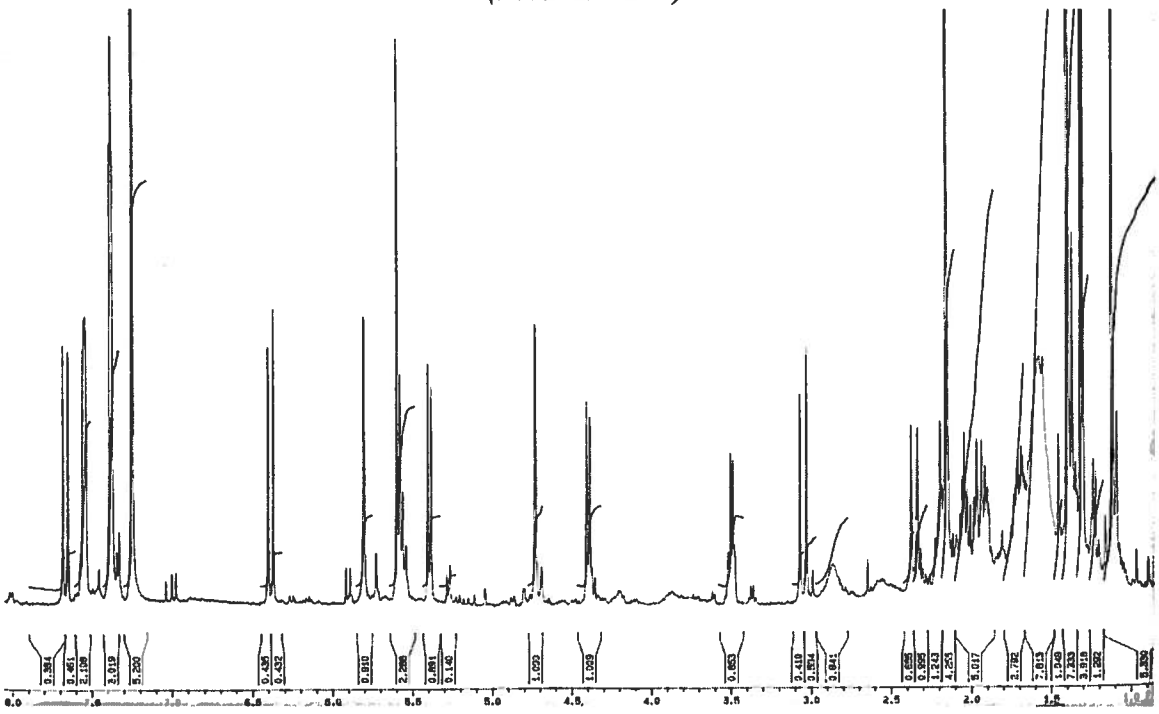
$[\alpha]_D^{22}$ = -50°, where c = 0.02 $CHCl_3$

8-9 mg in total.

NMR data for novel taxane #1, 1β,2α,9α-trihydroxy-10β-acetoxy-5α-cinnamoyloxy-3-11-cyclictaxa-4(20)-ene-13-one

Position	δ (H) mult	J (Hz)	δ (C)	HMBC	NOESY
1	--		**78.3**		
2	4.780 (br.s)	(COSY 14b)	78.6	1, 4, 8	**17/19**
3	--		**62.3**		
4	--		**142.5**		
5	5.594 (o.t)	8.8	75.8	**6, 166.2**	**6a, 6b**
6a 6b	2.20 (o.m) 1.70 (o.m)		26.0	 7, 8	**5**, **6b**, **7a**, 7b/17/19 **6a**, **10**
7a 7b	1.93 (o.m) 1.34 (o.m)		29.2		**7b**, **10** **7a**
8	--		**45.8**		
9	4.395 (d)	9.8	82.5	7, 8, 10, 19	2, **17/19**
10	5.389 (d)	9.8	84.0	9, 11, 12, 15, 172.5	**6b**, **7a**, **12**, **18**
11	--		**56.8**		
12	3.494 (q)	7.3	50.9	3, 13	**10**, **18**, **20a**
13	--		**213.9**		
14a 14b	3.048 (d) 2.357 (br.d)	19.8 19.8	46.4	1, 13, 15	**14b**, **20a** **14a**, **16**
15	--		**45.0**		
16	1.117 (s)		23.0	1, 11, 15, Me17	**14b**, **17/19**, **18**
17	1.396 (o.s)		23.3	1, 11, 15, Me16	**2**, 5, **9**, **16**, (overlap 19)
18	1.307 (d)	7.3	15.8	11, 12, 13	**12**, **10**, **16**
19	1.396 (o.s)		25.2	3, 7, 8, 9	See 17
20a 20b	5.804 (s) 5.594 (o.s)		126.1	3 3, 4, 5	**12**, **14a**, **20b** **20a**
OAc	2.157 (s)		21.2	**172.4**	
1'	--		**166.2**		
2'	6.386 (d)	16.1	117.4	Ph-C1, 1'	
3'	7.664 (d)	16.1	145.3	1', 2', Ph-o	
Ph 3' o m, p	 7.55 (m) 7.38 (m)		**134.4** 128.2 129.0 130.4		

1β,2α,9α-trihydroxy-10β-acetoxy-5α-cinnamoyloxy-3-11-cyclictaxa-4(20)-ene-13-one
(Novel taxane #1)

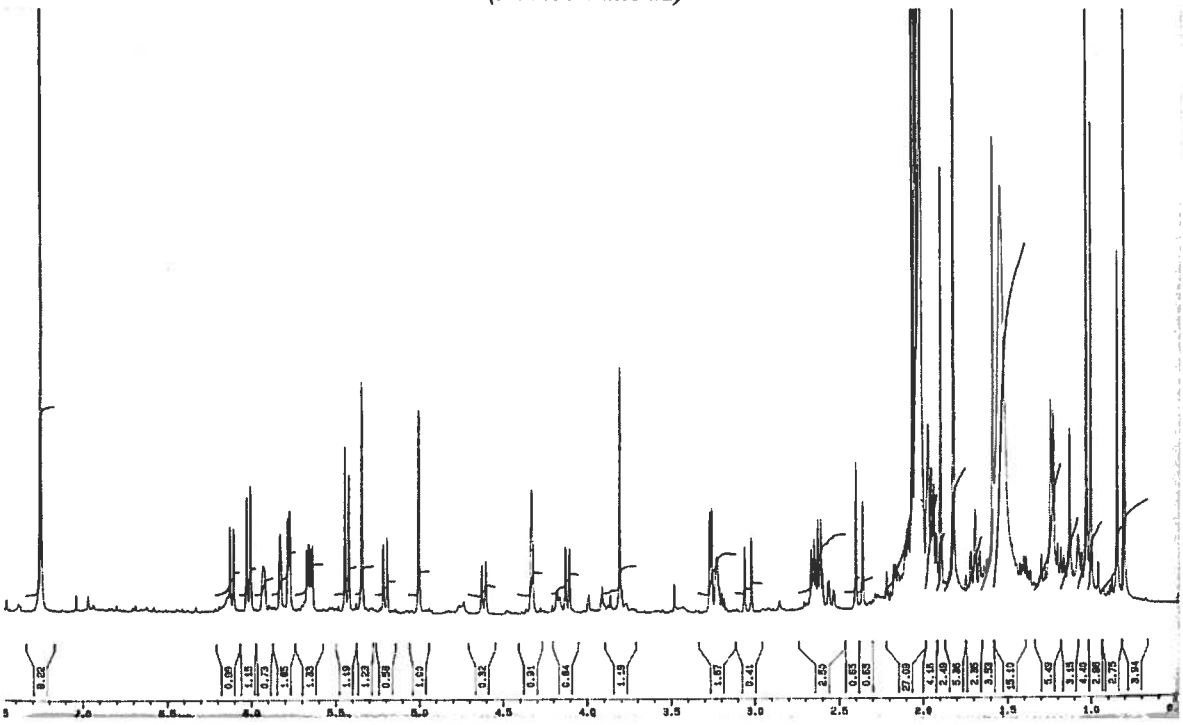


NMR data for novel taxane #2, 2α,9α,10β-triacetoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one

Position	δ (H) mult	J (Hz)	δ (C)	HMBC	NOESY
1	1.94 (o.m)		48.6		
2	5.825 (br.m)	~2.4	71.4		**1**, 3, **9**, **17**, **19**
3	3.225 (br.m)		40.9		*2*, **18**
4	--		**138.0**		
5	5.927 (m)		131.2		**20b**
6	2.10 (m)		21.9		
7a 7b	1.96 (o.m) 1.389 (td)	 12.2, 12.2, 6.1	27.2		
8	--		**39.6**		
9	6.116 (d)	11.1	76.5	7, 8, 10, 19, 169.3	**2**, **17**, **19**
10	5.201 (s)	11.2	71.4	9, 11, 12, 15, 169.0	*3*, **18**
11	--		**65.1**		
12	--		**59.6**		
13	--		**208.1**		
14a 14b	3.039 (d) 2.626 (o.dd)	20.1 20.1, 8.4	37.6	1, 2, 13, 15	**3**, **14b**, **20a** **1**, **14a**, 16

15	--		**39.1**		
16	0.841 (s)		28.7	1, 11, 15, Me	**1, 17**
17	1.896 (s)		25.1	1, 11, 15, Me	**2, 9, 16**
18	1.585 (s)		15.0	11, 12, 13	**3, 10**
19	0.999 (s)		18.0	3, 7, 8, 9	**2, 6, 7a, 9, 20b**
20a 20b	4.618 (br.d) 4.117 (d)	~11.9 11.7	68.0	 3, *4*, 5	**14a, 20b** **5, 20a**
OAc	Not-assign too much overlap				

2α,9α,10β-triacetoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one
(Novel taxane #2)



NMR data for novel taxane #3, 7β,9α,10β,13α-tetraacetoxy-11(15→1)*abeo*taxa-4(20),11-diene-5α,15-diol

Position	δ (H) - mult	J (Hz)	δ (C)	HMBC	NOESY
1	--		**62.8**		
2a 2b	2.352 (dd) 1.431 (br.d)	14.8, 9.6 14	29.1	8, 15	**2b, 9, 19** **2a, 17, 20b**
3	2.819 (d)	9.4	37.4	2, 7	**7, 14b**
4	--				
5	4.334 (br.dd)	~2.6, ~1.9	72.5		**6a, 6b, 20a**
6a 6b	1.97 (o.m) 1.79 (m)		35.7		**5, 6b, 7** **5, 6a, 19**

7	5.562 (dd)	11.1, 5.2	69.5	8, 19, **169.7**	**3**, **6a**, **10**
8	--		**44.9**		
9	5.80 (broad)		**77.1**		**2a**, **19**
10	6.321 (br.d)	~8.9	69.5		**7**, **18**
11	--		**136.5**		
12	--		**146.4**		
13	5.460 (br.t)	~7	79.5		**14a**, **17**, **18**
14a 14b	2.480 (dd) 1.30 (o.m)	13.9, 7.5	43.9	15	**13**, **14b**, 16 see 17
15	--		**75.3**		
16	1.301 (s)		24.8	11, 15, Me17	**17**, **13**
17	1.097 (s)		26.8	11, 15, Me17	**2b**, **3**, **14a** (overlap 14b)
18	1.899 (s)		11.7	11, 12, 13	**10**, *13*
19	0.845 (br.d)		12.6	3, 7, 8, 9	**2a**, **6b**, 20b, **9**
20a 20b	5.111 (s) 4.764 (br.s)		111.4	5 *5*	**5**, **20b** **2b**, **20a**
OAc	2.045 (s) 2.028 (s) 1.984 (s) 1.961 (s)		21.0 21.0 20.6 20.6	169.8 171.0 169.7 167.7	
OH	2.73 (br.)				

7β,9α,10β,13α-tetraacetoxy-11(15→1)*abeo*taxa-4(20),11-diene-5α,15-diol
(Novel taxane #3)

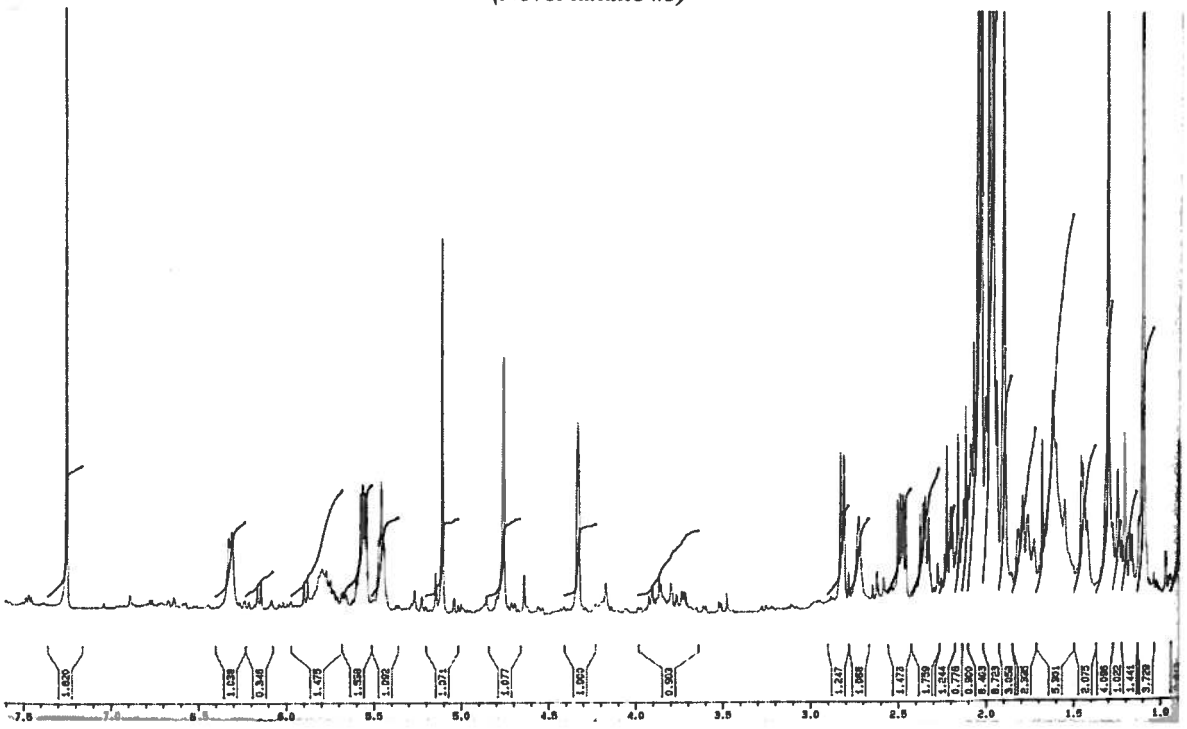
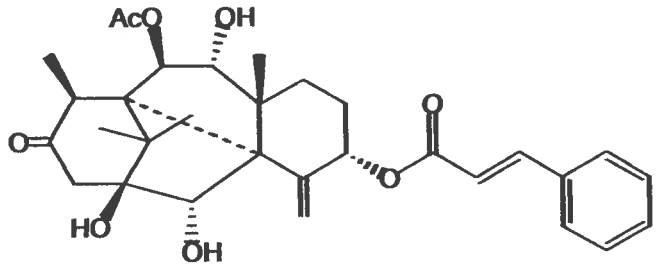


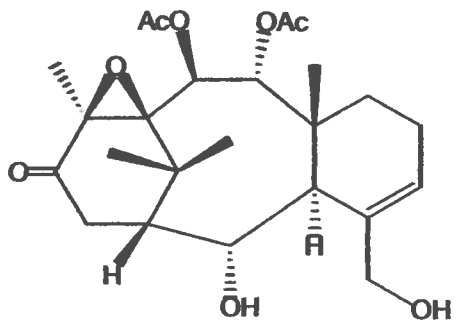
Figure 27: (Novel taxane #1)1β,2α,9α-trihydroxy-10β-acetoxy-5α-cinnamoyloxy-3-11-cyclictaxa-4(20)-ene-13-one



1β,2α,9α-trihydroxy-10β-acetoxy-5α-cinnamoyloxy-3-11-cyclictaxa-4(20)-ene-13-one (Novel taxane #1) was identified from sample 27-33.1 **(see figure 24) and therefore originated from the mixture of taxanes containing 10-deacetyl-baccatin III. As such, this** novel compound was purified from the mixture formed from the combined fractions 27 to 33 obtained from the flash column chromatography purification of the original dichloromethane extract. Two preparative scale HPLC injections using **program #3** (see **Appendix 1**) further purified this 10-deacetyl-baccatin III containing mixture and gave many fractions. The fraction collected during time **44 minutes** and **44.8 minutes** gave the purified compound as identified by NMR and mass spectroscopy. Between **2** and **3 mg** in total were isolated. The ^{1}H NMR spectrum of this novel taxane (#1), shown above in **figure 26**, exhibited the characteristic signals of taxoids (Appendino, 1995: 22: 55). The chemical shifts of the proton resonances characteristic of an exomethylene moiety were observed at δ5.94 and δ5.80 (each 1H, *s*). The presence of a cinnamoyl moiety was revealed in the ^{1}H NMR spectrum by the signals at δ6.39 (1H, *d*, *J*=16.1 Hz), 7.66 (1H, *d*, *J*=16.1, *trans*-orientation), 7.55 (2H, m), and 7.38 (3H, m) and by the fragment at *m/z* 131 (C_9H_7O) and $(M\text{-cinn})^+$ in the FABMS spectrum corresponding to the fission of a cinnamoyl group from the molecule. In addition, the presence of one acetyl group and one ketone group were implied by the resonances at δ2.16, 21.2, 172.4 and δ213.9 in the ^{1}H NMR and/or ^{13}C NMR spectra. Since 12 out of a total of 13 unsaturation degrees deduced from the molecular formula were thus accounted for, either an additional double bond or a saturated ring were required in this compound. Detailed examination of the ^{1}H NMR spectrum demonstrated that this novel taxane (#1) exhibited different spectral features compared with normal taxanes: the signal of H-3α, which usually appears at δ3.2-3.6

with a coupling constant in the range of *ca* *J*=5.0-6.0 Hz, disappeared; one of the methyl groups gave rise to a doublet at δ1.31 (d, *J*=7.3 Hz, 3H), which showed a coupling with a quartet signal at δ3.49 (1H, *q*, *J*=7.3 Hz) in the ^{1}H-^{1}H COSY spectrum. These characteristic signals were assigned as Me-18 and H-12, respectively. On analysis of these spectral features and available data, it is clear that the novel taxane (#1) was a 3,11-cyclotaxane. The identification of the compound as such was further confirmed by the long-range C-H correlation. H-12 showed three-bond correlation with C-3 in the HMBC spectrum. Combined analysis of ^{1}H-^{1}H COSY, HMQS, HMBC spectra, together with chemical shifts and coupling constants permitted assignments of all the functional group on the taxane skeleton. The acetyl group was located at C-10 as shown by correlation between H-10 and the carbonyl carbon of acetate (δ172.5) in the HMBC spectrum; H-5 showed three-bond correlation with the carbonyl of cinnamate (δ166.2) in the HMBC experiment. The hydroxyl groups were connected to C-2 and C-9 as indicated by the chemical shifts of H-2 and H-9. The remaining hydroxyl group was attached to C-1 as indicated by the spin pattern of H-14a, H-14b and H-2β. This assigment was verified by the chemical shift of C-1 (δ78.3). In order to determine the relative stereochemistry of the compound, a NOESY experiment was performed (see **figure 26**). Both H-2 and H-9 showed NOE correlations with Me-17 and Me-19 indicated that both H-2 and H-9 adopt β-oritations. The protons of Me-18 showed a cross peak with the protons of Me-16 and suggested that Me-18 was β-orientated; accordingly, H-12 was in an α-orientation. H-10 was in an α-orientation deduced from its NOE correlation with H-12 and H-6b. Thus, the structure of the novel taxane (#1) was characterized as 1β,2α,9α-trihydroxy-10β-acetoxy-5α-cinnamoyloxy-3-11-cyclictaxa-4(20)-ene-13-one.

Figure 28: (Novel taxane #2) 2α,9α,10β-triacetoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one



2α,9α,10β-triacetoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one (Novel taxane #2) was identified from sample 9.A (see **figure 24**) and therefore originated from fraction #9 collected from the flash column chromatography purification of the original dichloromethane extract. Fraction #9 was further purified by a preparative HPLC injection using **program #1** (see **Appendix 1**). The fraction collected between time **31.2 minutes** and **32.3 minutes** of this injection gave a mixture of two compounds with a ratio of 2:1, corresponding to **1-2 mg** and **0.5-1 mg** respectively. The major compound was identified as **5-decinnamoyl-taxinine B-11,12-oxide**, a taxane that has not previously been identified in the Canadian Yew and is shown in **figure 31**. The minor compound was **2α,9α,10β-triacetoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one** (novel taxane #2). Both of these compounds were identified by NMR and mass spectroscopy. Due to the small size of this mixture and the ability to nonetheless identify the two structures by NMR and HR FAB-MS, the compounds were not separated since this risked a loss of compound. The ^{1}H-NMR spectrum, shown above in **figure 26**, exhibited the characteristic signals of the taxane skeleton (Appendino, 1995: 22: 55), including well-dispersed signals due to protons connected to oxygenated carbons, four tertiary methyl groups at δ0.84, 1.90, 1.59, and δ1.00. The connectivities of the protons on the taxane skeleton this novel taxane (#2) were determined by analysis of the ^{1}H-^{1}H COSY spectrum. Interpretation of the ^{1}H-, ^{13}C-NMR and HMBC spectra permitted the positional assignment of functional groups. A pair of signals as an AB system which resonated at δ6.12 (1H) and 5.20 (1H) with a large coupling constant (J=11.2 Hz) were assigned as H-9 and H-10, and two acetoxy groups were attached to C-9 and C-10, as indicated by the

fact that H-9 correlated with C-7, C-8, C-10, C-19 and a carbonyl signal at δ169.3, while H-10 correlated with C-9, C-11, C-12, C-15 and a carbonyl signal at δ169.0 in the HMBC spectra. The ^{13}C-NMR signal at δ208.1 suggested the presence of C-13 ketone moiety. In accordance with this, H_2-14 displayed a large coupling constant J_{gem}=20.1 Hz. Using H-14 as a starting point, the connectivities from C-14 to C-1 to C-2 to C-3 were deduced from the ^{1}H-^{1}H COSY spectrum. The absence of the characteristic signals of an exocyclic methylene and the presence of two carbon signals in the olefinic region revealed that the C-4 double bond was endocyclic instead of exocyclic. The signal at δ4.97 (br.d, J = 11.8 Hz,1H) and δ4.12 (d, J = 11.8 Hz, 1H), which correlated with C-3, -4, C-5, was attributed to the methylene of H-20a and H-20b, respectively. The downfield chemical shift of C-13 and upfield chemical shift of Me-18, and especially H-10 and Me-16, as well as the lack of further olefinic carbons from the ^{13}C-NMR spectrum indicated that the endocyclic double bond at C-11,12 was instead an epoxide of a 11,12-epoxide taxoid (δ65.1 and δ59.6). This conclusion was supported by HMBC spectrum and molecular composition. H-10 of the novel taxane (#2) resonated at δ5.20, this unusual up-field shift of H-10 caused by the magnetic anisotropy effect of 11,12-epoxy ring. H-10 in 11,12-epoxide taxoids is similar to H-5 in the taxoids with a C-4(20) oxirane ring, both of them dramatically shifted up-field compared with corresponding protons in the taxoids with C-4(20),11-didouble bonds. The relative stereochemistry of the novel taxane (#2) was established from analysis of the NOESY data, chemical shifts and their coupling constants (see **figure 29**). The coupling constant between H-9 and H-10 (J=11.2 Hz) and observed NOESY correlations of H-2/H-9, H-9/H_3-17 established a chair-boat conformation for ring-B, which is the typical conformation of natural taxoids. The β-orientation of H-2 and H-9 were assigned by NOESY correlation of H-2/H-1, H-2/H_3-17, H-2/H_3-19, and H-9/H-2, H-9/H_3-17, H-9/H_3-19. The α-orientation of H-10 was determined by the observation of NOESY correlation of H-10/H-3 and H-10/H_3-18. The β-orientation of the epoxide group at C-11 and C-12 was established by the NOESY correlation of H_3-18/H-3 and H_3-18/H-10. The up-field chemical shift of H_3-16 due to the presence of C-11,12 epoxide near H_3-16 and γ-effect between C-16 methyl group and 11,12-epoxide also suggested that the epoxide group had a β-orientation. From this data,

the structure of the novel taxane (#2) was established as 2α,9α,10β-triacetoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one.

Figure 29. The relative stereochemistries of 2α,9α,10β-triacetoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one (novel taxane #2), the arrows show selected NOE correlations

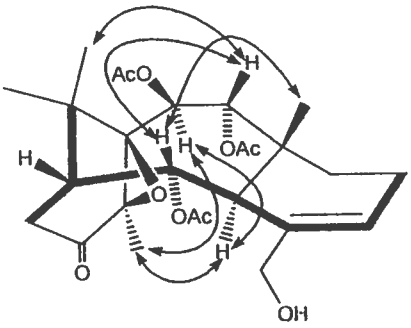
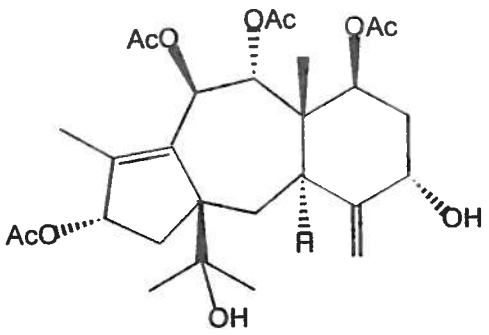


Figure 30. (Novel taxane #3) 7β,9α,10β,13α-tetraacetoxy-11(15→1)*abeo*taxa-4(20),11-diene-5α,15-diol



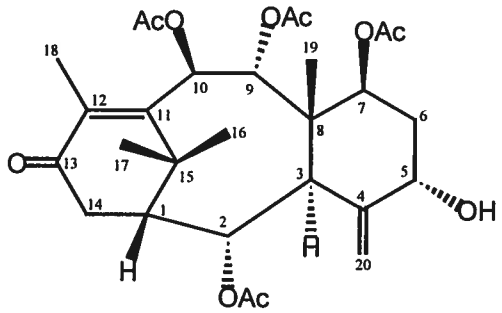
7β,9α,10β,13α-tetraacetoxy-11(15→1)*abeo*taxa-4(20),11-diene-5α,15-diol

(**Novel taxane #3**) was identified from sample **10-14.E-b** (see **figure 24**). This sample originated from the paclitaxel and cephalomanine mixture obtained from fractions numbered 10 to 14 obtained from the flash column chromatography purification of the original dichloromethane extract. This mixture containing paclitaxel and cephalomanine was subsequently further purified by five preparative HPLC injections using **program #1** (see **Appendix 1**). The fractions collected from times **28.9 minutes** to **30.5 minutes** of all five injections were combined to give sample **10-14.E** (see **figure 24**) which was further purified by another preparative HPLC injection, using **program #2** (see **Appendix 1**).

7β,9α,10β,13α-tetraacetoxy-11(15→1)*abeo*taxa-4(20),11-diene-5α,15-diol (Novel taxane #3) was identified from the fraction collected between times **27.4 minutes** and **29.3 minutes** of this last purification step, with a total weight of **8** to **9 mg**. The ^{1}H NMR spectrum of the novel taxane, shown above in **figure 26**, exhibited three-proton signals due to the four tertiary methyl groups at δ0.85, 1.10, 1.30, and 1.90 ppm, and four acetyl groups at relatively lower field (δ1.96, 1.98,2.03, and 2.05 ppm). This was verified by the observation of ^{13}C NMR signals at δ20.6, 167.7; 20.6, 169.7; 21.0, 169.8; and 21.0, 171.0 ppm. These signals suggested that the novel taxane (#3) had a taxane-type skeleton (Appendino, 1995: 22: 55). The connectivities of the protons on the taxane skeleton were determined by analysis of the ^{1}H-^{1}H COSY spectrum. Interpretation of ^{1}H, ^{13}C NMR and HMBC spectra permitted the positional assignment of functional groups. The ^{1}H NMR signals at δ5.11 (1H, *br.s*), 4.76 (1H,.*br.s*) together with the signals at δ111.4 in the ^{13}C NMR spectrum and δ2.82 ppm (1H, *d*, *J*=9.4 Hz)) in the ^{1}H NMR spectrum are the characteristic of an exocyclic methylene and C-3 ring junction proton in a taxane with a 4(20)-double bond, respectively (Appendino, 1995: 22: 55). The one proton doublet of doublets at δ2.35 ppm (1H, *dd*, *J*=14.8, 9.4 Hz) and one-proton doublet at δ1.43 ppm (1H, *br.d*, *J*=14.8 Hz) were assigned to the C-2 methylene protons, H-2α and H-2β, respectively, based on their geminal coupling ($J_{2\alpha,2\beta}$=14.8 Hz), and coupling to the H-3α. The signal at δ4.33 ppm (1H, *br.dd*, *J*=2.6, 1.9 Hz) was assigned to H-5β. Similarly, H-5β was correlated with the two one-proton multiplets at δ1.97 and δ1.79 ppm, which were assigned to H-6α and H-6β, respectively. Both H-6α and H-6β shared cross-peaks with the multiplets at δ5.56 ppm (1H, *dd*, *J*=11.1, 5.2 Hz), which was assigned to H-7. A pare of isolated broad and broad doublet signals at δ5.80 and δ 6.32 ppm (1H, *br.d*, *J*=8.9 Hz) were attributed to H-9β and H-10α, respectively. The broad triplet signal of one-proton which resonated at 5.46 ppm was assigned to H-13β; a pair of doublet of doublets at 2.48 ppm (1H, *dd*, *J*=13.9, 7.5 Hz) and one-proton multiplet signal at 1.30 ppm (1H, *m*) were assigned to H-14α and H-14β, respectively, according to their germinal coupling and coupling with H-13β in the ^{1}H-^{1}H COSY. All of the proton-bearing carbons were assigned by analysis of the HMQC spectrum. The chemical shifts of H-7α, 9β, 10α and H-13β indicated that there were four acetyl groups attached to these positions and these

assignments were confirmed by observing the long-range correlations of these protons to corresponding carbonyl signals in the HMBC experiment. C-5 had a free hydroxyl group as suggested by its chemical shift in the ^{1}H NMR spectrum. There are two signals relatively downfield (δ75.3 and 62.8 ppm) which remained in the ^{13}C NMR spectrum, but only one bears a hydroxyl group as required by the molecular composition. The signal at δ75.3 ppm, which showed cross peaks with H_3-16 and H_3-17 in the HMBC spectrum, was assigned to C-15 and one hydroxyl group attached to C-15. A remaining signal at δ62.8 was attributed to C-1, but was an unusually downfield chemical shift for a quaternary carbon which usually resonates around δ43-46 ppm in the normal 6/8/6-membered ring system skeleton (Appendino, 1995: 22: 55). This suggested that the novel taxane (#3) possessed a 5/7/6 ring system skeleton (Shen, Chen and Hung, 2000: 48(9): 1344). Since no cross-peaks were observed between H_3-16, H_3-17 and C-11 olefinic carbons in the HMBC spectrum, this further supported the 5/7/6 ring system skeleton, although we did not observe three-bond correlations between H-2 and H-14 with C-11 in the HMBC spectrum. From the above results, the structure of the novel taxane (#3) was established to be 7β,9α,10β,13α-tetraacetoxy-11(15→1)*abeo*taxa-4(20),11-diene-5α,15-diol.

Figure 31. Structures of taxanes isolated for the first time in *T. canadensis* from extract of *T. canadensis* rooted cuttings, with their HR FAB-MS and NMR data (^{1}H and some ^{13}C NMR spectral data for the taxanes in $CDCl_3$)



7β-Acetoxy-taxinine A

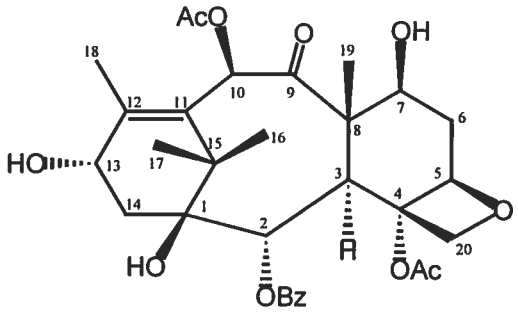
(Yeh *et al.*, 1988:35:309)

HR FAB-MS: *m/z* 573.21021 ($M+K^{+}$)

Confirms structure with MW 534.60 g/mol, $C_{28}H_{38}O_{10}$

4-5 mg in total.

Position	δ (H) mult	J (Hz)	δ (C)	HMBC	NOESY
1	2.18 (o.m)		48.7		**2**, **14a**, **16**, **17**
2	5.586 (dd)	6.1, 1.6	68.7	8, 14, 169.2	**1**, **9**, **17**, **19**, *20b*
3	3.528 (d)	6.3	39.7	2, 5, 8, 19, 20	**7**, **14b**
4	--		**144.4**		
5	4.198 (t)	3.2	74.4		**6b**, **20b**
6a 6b	1.93 (o.m) 1.61 (o.m)		37.4		
7	5.516 (dd)	11.3, 5.3	69.2	3, 19, 169.5	**3**, **6a**, **10**, **18**
8	--		**47.7**		
9	5.891 (d)	10.7	74.9	7, 8, 10, 19, 169.6	**2**, **17**, **19**
10	6.258 (d)	10.7	72.6	9, 11, 12, 15, 168.8	**7**, **18**
11	--		**149.6**		
12	--		**139.0**		
13	--		**199.7**		
14a 14b	2.761 (dd) 2.255 (d)	19.7, 6.9 19.6	36.0	2, 13 1, 2, 13, 15	**1**, **14b**, 16 **3**, **7**, **10**, **14a**
15	--		**37.6**		
16	1.109 (s)		37.3	1, 11, 15, Me	**1**, 14a, **17**
17	1.732 (s)		25.3	1, 11, 15, Me	**1**, **2**, **9**, **16**
18	2.316 (s)		14.4	11, 12, 13	**3**, **7**, **10**
19	0.963 (s)		12.8	3, 7, 8, 9	**2**, **6b**, **9**, **20b**
20a 20b	5.208 (s) 4.799 (s)		115.4	3 3, 4, 5	**5**, **20b** 2, **19**, **20a**
OAc	2.060 (s) 2.046 (s) 2.043 (s)		21.2 21.6 21.2	169.1 169.5 169.0	



Baccatin III

(Chan *et al.*, 1966:24:923, Della Casa de Marcano *et al.*, 1975:365)

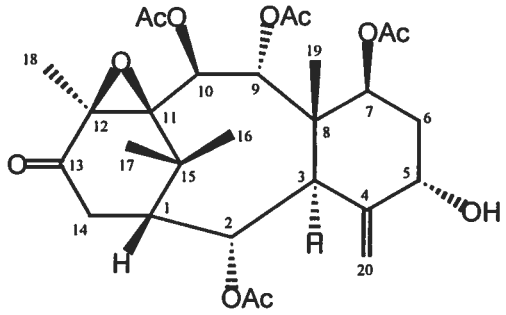
HR FAB-MS: *m/z* 625.20512 ($M+K^+$)

Confirms structure with MW 586.63 g/mol, $C_{31}H_{38}O_{11}$

2-3 mg in total.

Position	δ (H) mult	J (Hz)	δ (C)	HMBC	NOESY
1			**78.9**		
2	5.613 (d)	6.9	74.7	1, 3, 8, 14, 167.0	

3	3.88 (o.d)		45.9	1, 2, 8, 19	
4	--		**80.8**		
5	4.976 (br.d)	9.8	84.3		
6	2.55 (o.m) 1.86 (o.m)		35.6		
7	4.46 (br.m)		72.1		
8	--		**58.5**		
9	--		**203.9**		
10	6.312 (s)		76.0	9, 11, 12, 15, 171.2	
11	--		**131.5**		
12	--		**146.2**		
13	4.887 (br. t)	≈8.3	67.7		
14	2.30 (o.m)		38.5		
15	--		**42.5**		
16	1.097 (o.s)		26.9	1, 11, 15, Me	
17	1.097 (o.s)		20.9	1, 11, 15, Me	
18	2.040 (s)		15.4	11, 12, 13	
19	1.656 (s)		9.4	3, 7, 8, 9	
20	4.299 (d) 4.144 (d)	8.3 8.4	76.2	3, 4 5	
OAc	Not sure too many overlaps				
Bz-2	-- -- 8.095 (d) 7.47 (t) 7.60 (t)		167.0		



5-Decinnamoyl-taxinineB-11,12-oxide

(Yue, Fang and Liang, 1996:43:3:639)

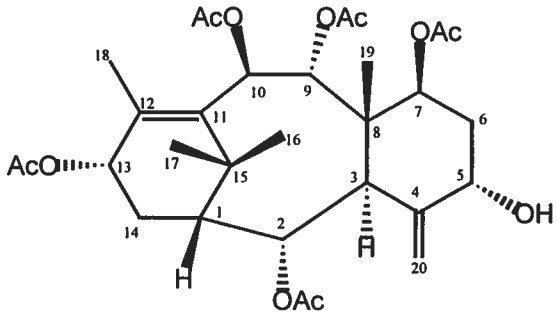
HR FAB-MS: *m/z* 589.20499 ($M+K^+$)

Confirms structure with MW: 550.59 g/mol, $C_{28}H_{38}O_{11}$

1-2 mg in total.

Position	δ (H) mult	J (Hz)	δ (C)	HMBC	NOESY
1	1.97 (o.m)		51.6	2, 3, 13, 15	
2	5.771 (dd)	5.7, 1.5	68.7	3, 8, 14, 168.6	1, 3, 9, 17, 19

3	3.261 (d)	5.7	40.3	1, 2/7, 5, 8, 19	**7, 14b, 18**
4	--		**142.8**		
5	4.332 (t)	2.8	75.0		**6a, 6b, 20a**
6a 6b	1.99 (o.m) 1.69 (m)		37.1		
7	5.648 (dd)	11.4, 5.2	68.9	5, 6, 8, 19, 169.1	**3, 6a, 10, 18**
8	--		**46.8**		
9	6.013 (d)	11.2	75.5	7, 8, 10, 19, 169.4	**2, 17, 19**
10	5.431 (d)	11.2	71.2	9, 11, 12, 15, 168.4	**7, 18**
11	--		**64.3**		
12	--		**59.6**		
13	--		**208.0**		
14a 14b	2.638 (o.dd) 2.380 (d)	20.1, 8.9 20.1	38.0	 1, 2, 13, 15	**1, 14b**, 16 **3, 14a**
15	--		**38.2**		
16	0.799 (s)		28.9	1, 11, 15, Me	**1, 17**
17	1.820 (s)		25.4	1, 11, 15, Me	**2, 9, 16**
18	2.03 (o.s)		14.4	11, 12, 13	
19	1.026 (s)		13.1	3, 7, 8, 9	**2, 6b, 9, 20b**
20a 20b	5.345 (s) 4.998 (s)		117.3	3, 5, *8* 3, 4, 5	**5, 20b** **18**, *19*, **20a**
OAc	Not-assign too much overlap				



5-decinnamoyl-taxinine J

(Kingston, Hawkins and Ovington, 1982:45:4:466)

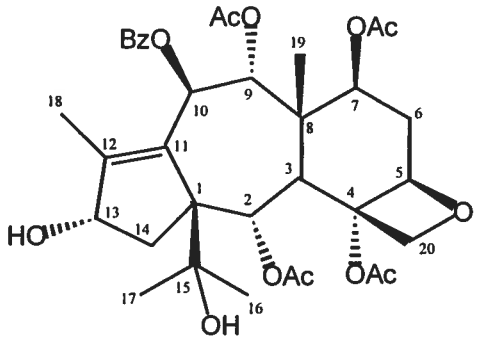
HR FAB-MS: *m/z* 617.23642 ($M+K^+$)

Confirms structure with MW: 578.65g/mol, $C_{30}H_{42}O_{11}$

11-12 mg in total.

Position	δ (H) mult	J (Hz)	δ (C)	HMBC	NOESY
1	1.850 (dd)	8.7, 1.5	48.0		
2	5.500 (o.dd)	~6.5, 1.7	70.3	3, 8, 14, 169.2	
3	6.483 (d)	6.0	40.4	4, 5/9, 2/7, 19, 20	
4			**145.0**		
5	4.230 (t)	2.7	75.1	3	

6a 6b	1.98 (o.m) 1.60 (o.m)		37.1		
7	5.602 (dd)	11.4, 5.3	69.4	6, 8, 9, 19, 169.6	
8			**48.1**		
9	5.839 (d)	10.8	75.8	7, 8, 10, 11, 19, 169.9	
10	6.202 (d)	10.8	71.8	9, 11, 12, 15, 169.3	
11	--		**134.1**		
12	--		**138.6**		
13	5.755 (dd)	10.5, 5.7	69.4	11, 12, 14, 170.0	
14a 14b	2.64 (o.m) 1.448 (dd)	 15.8, 5.3	28.4		
15	--		**37.4**		
16	1.016 (s)		31.7	1, 11, 15, Me	
17	1.699 (s)		25.8	1, 11, 15, Me	
18	2.218 (br.o.s)		15.7	11, 12, 13	
19	0.940 (s)		13.0	3, 7, 8, 9	
20a 20b	5.278 (s) 4.837 (s)		116.5	4, 3, 5, 8 4, 3, 5	
OAc	Too many overlaps				



Taxayuntin

(Chen *et al.*, 1993:4:8:695)

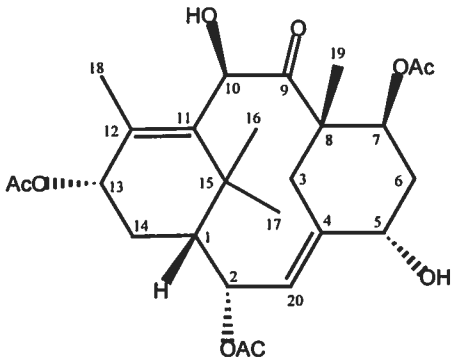
HR FAB-MS: *m/z* 711.24190 (M+ K^+), 695.26796 (M+Na^+)

Confirms structure with MW: 672.72g/mol, $C_{35}H_{44}O_{13}$

7-8 mg in total.

Position	**δ (H) mult**	**J (Hz)**	**δ (C)**	**HMBC**	**NOESY**
1	--		**68.0**		
2	6.11 (o.m)		67.8		
3	3.142 (d)	7.7	43.8	2, 7, 8, **19**, 20	
4	--		**80.3**		
5	4.936 (d)	7.8	84.9	4, 7	
6a 6b	2.55 (o.m) 1.89 (o.m)		34.6		
7	5.55 (o.m)		~70.7		

8	--		**44.1**		
9	6.15 (o.d)		76.4	8, 10, 19	
10	6.523 (d)	10.8	69.2	1, 9, 11, 12, **164.2**	
11/12			**151.8**		
12/11			**134.5**		
13	4.46 (br.m)		77.1		
14a 14b	2.24 (m) 1.65 (o.m)		39.5		
15	--		**76.0**		
16	1.15 (s)		25.2		
17	1.05 (s)		27.3		
18	2.03 (o.)		11.7	11, 12, 13	
19	1.67 (o.s)		**12.6**	3/8, 7, 9	
20a 20b	4.504 (d) 4.390 (d)	7.8 7.8	74.9	5 3, 4	
OAc	Too many overlaps				
CO	--		**164.2**		
Ph o m p	 7.84 (d) 7.41 (t) 7.54 (t)	 7.5 7.9 7.4	 129.4 128.7 133.2		



Taxuspine W

(Hosoyama *et al.*, 1996:52:13145)

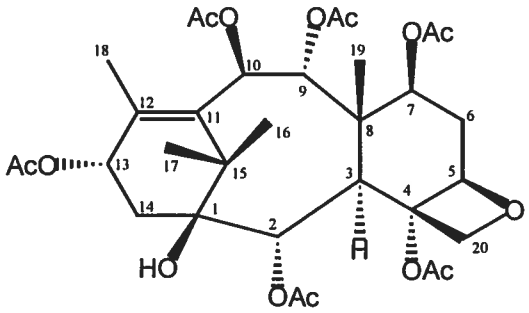
HR FAB-MS: *m/z* 531.19964 (M+ K^+)

Confirms structure with: MW 492.56g/mol, $C_{26}H_{36}O_9$

3-4 mg in total.

Position	**δ (H) mult**	**J (Hz)**
1	1.65 (dd)	6.3, 1.6
2	5.71 (dd)	9.6, 1.6
3a 3b	2.70 (d) 2.00 (d)	15.8 15.8
5	4.49 (brt)	7.1
6	2.09 (m)	

7	5.07 (dd)	12.0, 3.8
10	5.45 (d)	2.5
13	5.35 (d)	10.0
14a 14b	2.70 (m) 1.95 (d)	 16.6
20	5.65 (d)	9.6
Me-16	1.21 (s)	
Me-17	1.19 (s)	
Me-18	1.94 (s)	
Me-19	1.31 (s)	
OH-5	2.61 (d)	7.1
OH-10	4.20 (d)	2.5
CH_3OH	2.19 (s) 2.04 (s) 2.04 (s)	



Baccatin IV

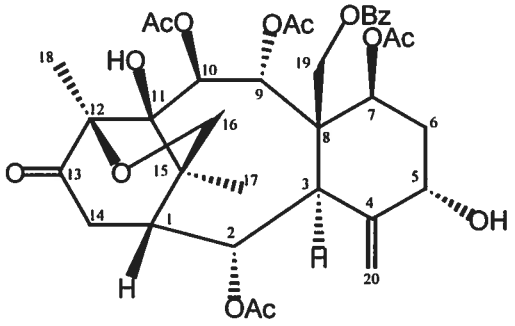
(Chan *et al.*, 1966:24:923, Della Casa de Marcano *et al.*, 1975:365)

HR FAB-MS: *m/z* 691.23682 ($M+ K^+$)

Confirms structure with: MW 652.68g/mol, $C_{32}H_{44}O_{14}$

5-6 mg in total.

Position	**δ (H) mult**	**J (Hz)**
2	5.62 (d)	6
3	3.06 (d)	6
5	5.00 (d)	10
7	5.52 (q)	7, 10
9	5.95 (d)	11
10	6.22 (d)	11
13	6.20 (m)	
16	1.21	
17	1.70	
18	2.00	
19	1.55	
20	4.55, 4.14	8



Taxinine M

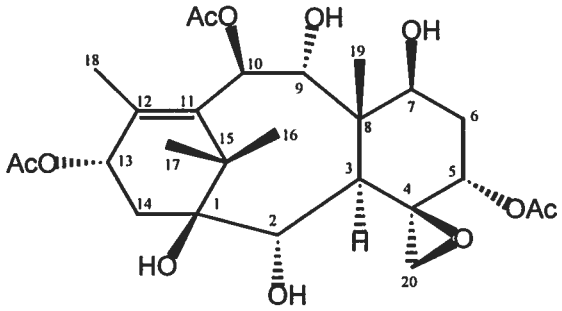
(Beutler *et al.*, 1991:54:893)

HR FAB-MS: *m/z* 725.22117 ($M+K^+$)

Confirms structure with: MW 686.70g/mol, $C_{35}H_{42}O_{14}$

6-7 mg in total.

Position	δ (H) mult	J (Hz)
2	6.14 (dd)	10.4, 2.4
4a	3.00 (dd)	11.6, 19.2
4b	2.75 (d)	19.2
	2.48 (ddd)	0.7, 2.4, 11.6
	2.23 (ddd)	2.1, 6.1, 14.2
6a	4.08 (d)	8.1
	3.71 (d)	10.4
6b	3.63 (d)	8.1
7	5.51 (dd)	10.7, 6.2
9	5.36 (d)	3.0
10	5.31 (d)	3.0
19a	5.14 (d)	12.2
19b	4.42 (d)	12.2
20-Z	5.41 (s)	
20-E	4.68 (s)	
	4.45 (brt)	2-3
Bz	8.16	
Bz	7.61	
Bz	7.51	
OH	4.10 (brs)	
OAc	2.15 (s)	
OAc	2.11 (s)	
OAc	2.03 (s) 1.70 (ddd) 1.29 (s) 1.17 (s)	3.6, 10.7, 14.2



1β-Hydroxy-2,7,9-trideacetyl-baccatin I

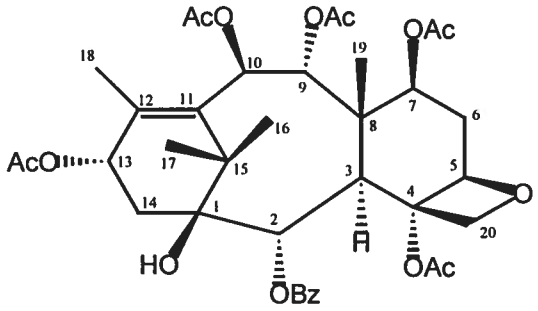
(Chen, Zhang, and Zhou, 1994:29:207)

HR FAB-MS: *m/z* 565.20512 ($M+K^+$)

Confirms structure with: MW 526.57g/mol, $C_{26}H_{38}O_{11}$

3-4 mg in total.

Position	δ (H) mult	J (Hz)
2	4.00 (d)	3.5
3	3.12 (d)	3.5
5	4.26 (t)	3.0
6	1.92 (ddd)	2.8, 4.4, 14.8
6'	2.07 (m)	
7	4.26 (dd)	4.4, 11.5
9	4.49 (d)	10.5
10	5.99 (d)	10.5
13	6.04 (dd)	6.3, 9.7
14	1.84 (dd)	6.3, 15.0
14'	2.56 (dd)	9.7, 15.0
16-Me	1.46 (s)	
17-Me	1.23 (s)	
18-Me	2.12 (d)	1.5
19-Me	1.45 (s)	
20	2.44 (d)	4.7
20'	3.71 (d)	4.7
OAc	2.08 (s) 2.13 (s) 2.17 (s)	



Baccatin VI

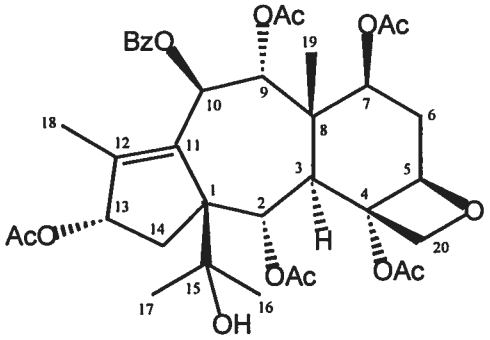
(Della Casa de Marcano *et al.*, 1975:365)

HR FAB-MS: *m/z* 753.25247 ($M+K^+$)

Confirms structure with: MW 714.29 g/mol, $C_{37}H_{46}O_{14}$

8-9 mg in total.

Position	δ (H) mult	J (Hz)
2	5.90 (d)	6
3	3.19 (d)	6
5	4.98 (d)	10
7	5.57 (bt)	8
9	6.05 (d)	10
10	6.22 (d)	10
13	6.20 (m)	
16	1.22	
17	1.78	
18	2.12	
19	1.54	
20	4.35 (d), 4.13 (d)	8



13-Acetyl-13-decinnamoyltaxchinin B

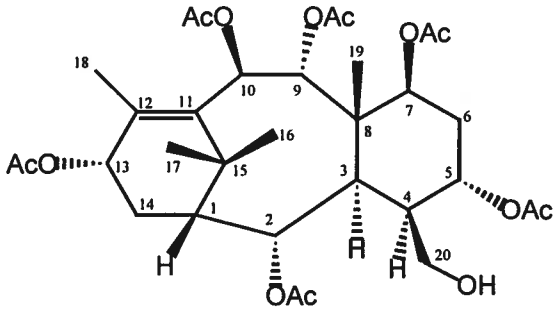
(Das *et al.*, 1995:38:3:671)

HR FAB-MS: *m/z* 753.25247 ($M+K^+$)

Confirms structure with MW 714.29 g/mol, $C_{37}H_{46}O_{14}$

1-2 mg in total.

Position	δ (H) mult	J (Hz)
2	6.17 (d)	7.9
3	3.01 (d)	7.9
5	4.99 (d)	7.6
6a 6b	2.60 (m) 1.91 (dd)	 15.4, 8.3
7	5.59 (t)	8.2
9	6.21 (d)	10.9
10	6.58 (d)	10.9
13	5.62 (t)	7.7
14a 14b	2.50 (dd) 2.00 (m)	15.0, 7.5
16	1.15 (s)	
17	1.07 (s)	
18	2.01 (s)	
19	1.68 (s)	
20a 20b	4.50 (d) 4.41 (d)	7.7 7.7
Me-2	2.02 (s)	
Me-4	2.02 (s)	
Me-7	2.08 (s)	
Me-9	1.74 (s)	
Me-13	2.12 (s)	
Bz-2 and Bz-6	7.86 (d)	7.8
Bz-3 and Bz-5	7.43 (t)	7.8
Bz-4	7.55 (t)	7.8



Taxuspine L

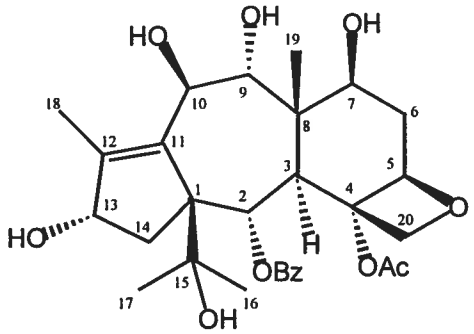
(Wang, Shigemori, and Kobayashi, 1996:52:7:2337)

HR FAB-MS: *m/z* 677.25247 ($M+K^+$)

Confirms structure with: MW 638.29 g/mol, $C_{32}H_{46}O_{13}$

3-4 mg in total.

Position	δ (H) mult	J (Hz)
1	1.98 (m)	
2	5.44 (dd)	5.4, 2.1
3	2.75 (t)	5.4
5	5.11 (d)	2.4
6a 6b	1.85 (dd) 2.66 (m)	5.0, 2.4
7	5.40 (dd)	5.0, 5.8
9	5.86 (d)	11.0
10	6.17 (d)	11.0
13	5.93 (t)	8.3
14a 14b	2.61 (m) 1.46 (m)	
16	1.74 (s)	
17	1.14 (s)	
18	2.23 (s)	
19	0.85 (s)	
20a 20b	3.48 (d) 3.41 (dd)	11.4 11.4, 7.4
OAc	2.24 (s) 2.11 (s) 2.09 (s) 2.04 (s) 2.02 (s) 1.97 (s)	



7,13-Deacetyl-9,10-didebenzoyltaxchinin C

(Chen and Kingston, 1994:57:7:1017)

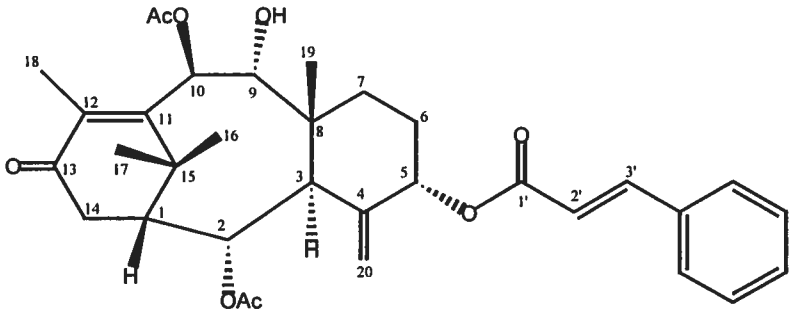
HR FAB-MS: *m/z* 585.21021 ($M+K^+$)

Confirms structure with: MS 546.25 g/mol, $C_{29}H_{38}O_{10}$

2-3 mg in total.

Position	δ (H) mult	J (Hz)
2	6.04 (d)	7.4
3	3.00 (d)	7.4

5	4.89 (br d)	8.5
6 α	1.80 (m)	
β	2.55 (ddd)	16.0, 8.2, 1.5
7	4.20 (br t)	8.2
9	4.30 (d)	10.4
10	4.55 (d)	10.4
13	4.58 (br t)	
14 α	1.75 (dd)	15, 7.4
β	2.25 (dd)	15, 7.4
16	1.08 (s)	
17	1.05 (s)	
18	1.93 (s)	
19	1.84 (s)	
20 α	4.43 (d)	7.9
β	4.08 (d)	7.9
$OCOCH_3$	2.20 (s)	
Ar o	7.99 (d)	7.2
m	7.46 (m)	
p	7.60 (m)	



Taxinine NN-3 (9-deacetyl taxinine)

(Sakai *et al.*, 2001:54:2:999)

HR FAB-MS: *m/z* 603.23603 ($M+K^+$)

Confirms sample with: MW 564.27 g/mol, $C_{33}H_{40}O_8$

0.5-1 mg in total.

Position	**δ (H) mult**	**J (Hz)**
1	2.16 (m)	
2	5.49 (dd)	6.2, 2.0
3	3.39 (br d)	6.2
5	5.35 (br t)	2.7
6 α	1.99 (br ddd)	13.7, 2.7, 2.7
β	1.75 (br dddd)	13.7, 11.0, 4.9, 2.7
7 α	1.56 (m)	
β	1.88 (br ddd)	13.3, 4.9, 2.7
9	4.32 (br d)	9.6

10	5.83 (d)	9.6
14 α β	2.43 (d) 2.83 (dd)	19.8 19.8, 7.1
16	1.15 (s)	
17	1.65 (s)	
18	2.29 (s)	
19	1.14 (s)	
20a b	5.33 (br s) 4.87 (br s)	
OAc	2.16 (s) 2.07 (s)	
2'	6.44 (d)	15.9
3'	7.66 (d)	15.9
o-Ph	7.76 (m)	
m-	7.43 (m)	
p-	7.43 (m)	

7β-Acetoxytaxinine A was identified in three samples, i (from 10-14.D&F + 15-23.G), 27-33.H and 10-14.E-a (see **figure 24**), for a combined weight of **4-5 mg**. Sample i (from 10-14.D&F + 15-23.G) originated from both the 9-dihydro-13-acetyl-baccatin III and paclitaxel-cephalomanine containing samples, and thus from fractions 10 to 14 and 15 to 23 originating from the column chromatography of the dichloromethane extract. Samples 10-14.D and 10-14.F (see **figure 24**), which originated from the five preparative HPLC injections used to further purify the paclitaxel and cephalomanine mixture, were combined with sample 15-23.G (see **figure 24**) from the three preparative HPLC injections used to further purify the 9-dihydro-13-acetyl-baccatin III mixture. These combined samples were further purified by another preparative HPLC injection using **program #2** (see **Appendix 1**). Sample i (from 10-14.D&F + 15-23.G) was the fraction collected between times **26.9 minutes** and **27.5 minutes** of this injection and was shown to contain a mixture of three compounds in a ratio of 1:1:1 of **7β-acetoxytaxinine A**, **5-decinnamoyl-taxuspine D** and **baccatin III**. Again, due to the small size of the sample and the ability to identify the mixture of compounds by NMR and HR FAB-MS, the mixture was not further purified to avoid loss of compound. Sample 27-33.H (see **figure 24**) originated from the mixture of taxanes containing 10-deacetyl-baccatin III. As such, this compound was purified from the mixture formed from combined fractions 27 to 33

obtained from the flash column chromatography purification of the original dichloromethane extract. Two preparative scale HPLC injections using **program #3** (see **Appendix 1**) further purified this mixture and gave many fractions. The fraction collected during time **40 minutes** and **41.5 minutes** of the HPLC injection gave a mixture of two compounds with a ratio of 65:35 corresponding to **4-5 mg** of **5-decinnamoyl-taxuspine D** and **2-3 mg** of **7β-acetoxytaxinine A**. Finally the last sample containing **7β-acetoxytaxinine A**, **10-14.E-a** (see **figure 24**), originated from the paclitaxel and cephalomanine mixture. Fractions collected during times **28.9 minutes** and **30.5 minutes** of all five preparative injections of this mixture were combined to give sample **10-14.E**, which was further purified by another preparative HPLC injection using **program #2** (see **Appendix 1**). The sample collected between times **26.9 minutes** and **27.4 minutes** of this injection was **10-14.E-a** and had the same composition as sample **i** (from **10-14.D&F** + **15-23.G**) described earlier, giving another **1-2 mg** of **7β-acetoxytaxinine A**. This compound was previously isolated from the stems and leaves of *Taxus mairei* and *Taxus cuspidata*.

Baccatin III was obtained from two samples already described above that contain 7β-acetoxytaxinine A, **i** (from **10-14.D&F** + **15-23.G**) and **10-14.E-a** (see **figure 24**), for a total weight of **1-2mg**. This compound was previously isolated from the bark, stems, roots, and leaves of *Taxus baccata*, *Taxus brevifolia*, *Taxus cuspidata*, *Taxus wallichiana*, *Taxus yunnanensis* and *Taxus mairei*.

5-DecinnamoyI-taxinineB-11,12-oxide was identified in the same sample as **2α,9α,10β-triacetoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one** (novel taxane #2), **9.A** (see **figure 24**) as described above, with a total weight of **1-2 mg**. This compound was previsouly isolated from the leaves and stems of *Taxus yunnanensis*.

5-Decinnamoyl-taxinine J was identified in three samples, **10-14.I-a**, **10-14.I-b** and **10-14.H** (see **figure 24**). All three samples originated from the paclitaxel mixture formed after the column chromatography of the dichloromethane extract. Both **10-14.I-a** and **10-14.I-b** were fractions collected from the preparative HPLC injection of sample **10-14.I** using multi-step **program #4** (see **Appendix 1**) and were collected during times **34.0 minutes** and **34.3 minutes**, and **34.3 minutes** and **34.6 minutes** respectively. Sample **10-14.I-a** consists of 3-4 mg of pure **5-decinnamoyl-taxinine J**, while sample **10-14.I-b**

consists of a 2:1 mixture of **taxayuntin** and **5-decinnamoyl-taxinine J** respectively. This 2:1 mixture yields another **1-2 mg** of **5-decinnamoyl-taxinine J**. Sample **10-14.H** was a fraction collected between times **32.7 minutes** and **33.7 minutes** of the initial purification of the paclitaxel mixture by preparative HPLC using **program #1** (see **Appendix 1**). This sample was a mixture of three compounds, **5-decinnamoyl-taxinine J**, **taxayuntin** and **taxinine A**, of which **5-decinnamoyl-taxinine J** was the major one with a weight of **6-7 mg**. This compound was previously isolated from the bark of *Taxus brevifolia*, *Taxus yunnanensis*, and *Taxus mairei*.

Taxayuntin was identified along with **5-decinnamoyl-taxinine J** in two samples described above, **10-14.H** and **10-14.I-b** (see **figure 24**) for a total of **7-8 mg**. This compound was previously isolated from the leaves of *Taxus baccata*, *Taxus cuspidata*, *Taxus wallichiana* and *Taxus yunnanensis*.

Taxuspine W was identified in the sample **8.A** (see **figure 24**). This sample originated from the purification of fraction #8, collected from the flash column chromatography of the dichloromethane extract, by preparative HPLC using **program #1** (see **Appendix 1**). The fraction was collected during times **31.0 minutes** and **31.3 minutes** of this preparative HPLC injection. A total of **3-4 mg** of taxuspine W was purified. This compound was previously isolated from the leaves and stems of *Taxus cuspidata*.

Baccatin IV was identified from two samples, **10-14.E-d** and **9.B** (see **figure 24**). Sample **10-14.E-d** was obtained from fraction **10-14.E** which was further purified by a preparative HPLC injection, using **program #2** (see **Appendix 1**), giving sample **10-14.E-d** of **4 mg** of **baccatin IV**. The sample was collected between times **29.9 minutes** and **30.5 minutes** of the preparative HPLC injection. Sample **9.B** was obtained from fraction #9 obtained from the column chromatography of the dichloromethane extract. Fraction #9 was further purified by a preparative HPLC injection using **program #1** (see **Appendix 1**). The fraction collected between time **34.7 minutes** and **35.7 minutes** of this injection gave sample **9.B** with **1-2 mg** of **baccatin IV**. This compound was previously isolated from *Taxus baccata*, *Taxus brevifolia*, *Taxus mairei* and *Taxus wallichiana*.

Taxinine M was identified from sample **15-23.I** (see **figure 24**). This sample was obtained from the combined fractions collected from time **47.6 minutes** to the initial

wash steps (just after **50 minutes**) of the three preparative HPLC injections of the 9-dihydro-13-acetyl-baccatin III mixture. A total of **6-7 mg** of **taxinine M** was obtained. This compound was previously isolated from the bark of *Taxus brevifolia* and *Taxus cuspidata*.

1β-Hydroxy-2,7,9-trideacetyl-baccatin I was identified from sample 27-33.A (see **figure 24**). This sample was obtained from the combined fractions collected between time **26 minutes** and **27 minutes** of both preparative HPLC injections of the 10-deacetyl-baccatin III containing mixture. A total of **3-4 mg** of **1β-Hydroxy-2,7,9-trideacetyl-baccatin I** was obtained. This compound was previously isolated from the bark of *Taxus yunnanensis*.

Baccatin VI was identified from three fractions, 8.C, 8.D and 9.C (see **figure 24**). Both samples 8.C and 9.C contained only **baccatin VI** and came from the further purification of fractions #8 and #9 respectively, as already described above. They were collected during times **39.1 minutes** and **39.3 minutes**, and during times **37.3 minutes** and **37.8 minutes** of their respective preparative HPLC injections. Sample 8.D, also originating from fraction #8, contained a 2:1 mixture of **baccatin VI** to **13-acetyl-13-decinnamoyltaxchinin B** with weights of **2-3 mg** and **1-2 mg** respectively and was collected during times **39.3 minutes** and **39.6 minutes** of it's preparative HPLC injection. This compound was previously isolated from the bark of *Taxus baccata* and the heartwood of *Taxus mairei*.

13-Acetyl-13-decinnamoyltaxchinin B was identified from 8.D (see **figure 24**) as described above, in a quantity of **1-2 mg**. This compound was previously isolated from the leaves of *Taxus baccata*.

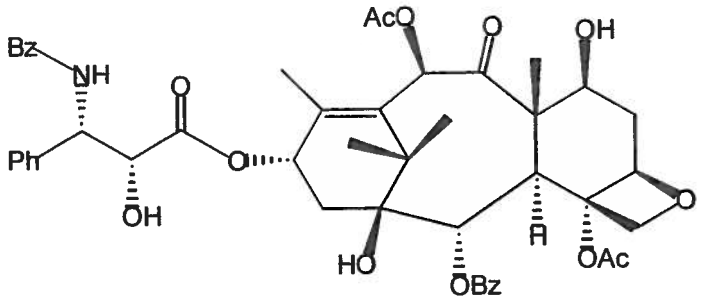
Taxuspine L was identified from sample 10-14.E-e (see **figure 24**), obtained from fraction 10-14.E described earlier above. This sample was further purified by a preparative HPLC injection, using **program #2** (see **Appendix 1**), and sample 10-14.E-e was collected between times **30.9 minutes** and **31.2 minutes** giving **3-4 mg** of **Taxuspine L**. This compound was previously isolated from the stems of *Taxus chinensis*.

7,13-Deacetyl-9,10-didebenzoyltaxchinin C was identified from sample 27-33.D (see **figure 24**), obtained from the 10-deacetyl-baccatin III containing mixture formed from the combination of fractions 27 to 33 of the original column chromatography of the

dichloromethane extract. As previously described, this mixture was further purified by preparative HPLC using **program #3** (see **Annexe 1**). The fraction collected during times **31.0 minutes** and **31.9 minutes** of this preparative HPLC injection gave **2-3 mg** of 7,13-deacetyl-9,10-didebenzoyltaxchinin C. This compound was previously isolated from *Taxus brevifolia*.

Taxinine NN-3, or **9-deacetyltaxinine** as it is also known, was identified from sample 26.D (see **figure 24**). This sample was obtained from fraction 26 of the flash column chromatography of the dichloromethane extract, after further purification by a preparative HPLC injection using **program #1** (see **Appendix 1**). The fraction collected during times **45.1 minutes** and **45.8 minutes** of this preparative HPLC injection gave **0.5-1.0 mg** of taxinine NN-3. This compound was recently isolated from the needles and young stems of *Taxus cuspidata*.

Figure 32. Structures of taxanes already known to *T. canadensis*, isolated from extract of *Taxus canadensis* rooted cuttings, and their HR FAB-MS data



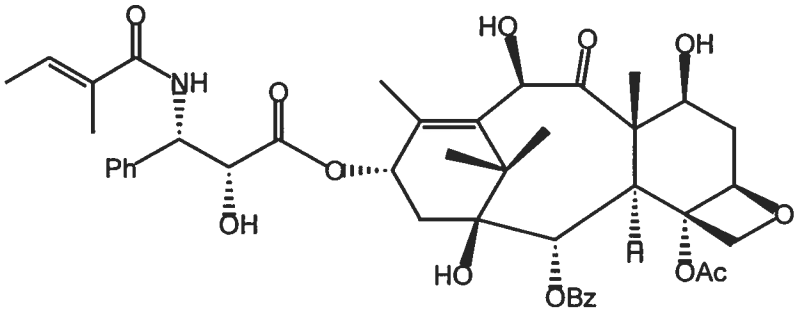
Paclitaxel

(Wani *et al.*, 1971:93:2325, Zamir *et al.*, 1992:33:36:5173)

HR FAB-MS: *m/z* 892.29430 ($M+K^+$)

Confirms sample with: MW 853.33 g/mol, $C_{47}H_{51}NO_{14}$

31-32 mg in total.



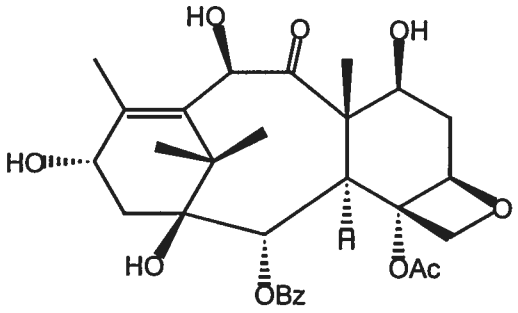
10-deacetyl-cephalomanine

(Chauvière *et al.*, 1981:2:293:501, McLaughlin *et al.*, 1981:44:3:312, Zhang *et al.*, 2001:64:450)

HR FAB-MS: *m/z* 812.32553 ($M+Na^+$)

Confirms sample with: MW 789.86 g/mol, $C_{43}H_{51}NO_{13}$

11-12 mg in total.



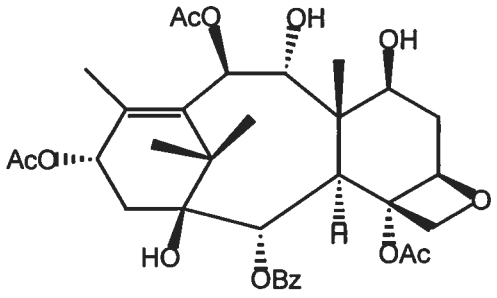
10-deacetyl-baccatin III

(Senilh *et al.*, 1984:2:299(15):1039, Zamir *et al.*, 1992:33:36:5173)

HR FAB-MS: *m/z* 583.19456 ($M+K^+$)

Confirms structure with MW 544.59 g/mol, $C_{29}H_{36}O_{10}$

38-39 mg in total.



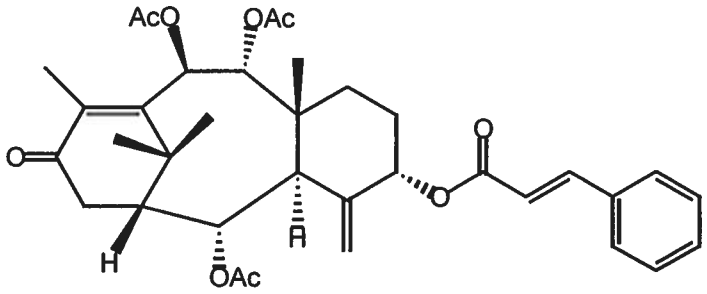
9-dihydro-13-acetyl-baccatin III

(Zamir *et al.* 1992:33:36:5173, Gunawardana *et al.*, 1992:55:11:1686)

HR FAB-MS: *m/z* 669.23133 ($M+K^+$)

Confirms structure with MW 630.68 g/mol, $C_{33}H_{42}O_{12}$

26-27 mg in total.



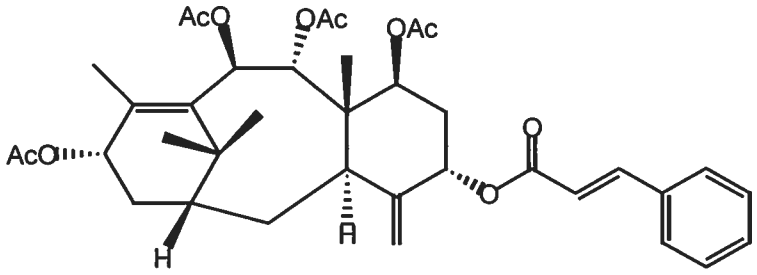
Taxinine

(Uyeo *et al.*, 1965:85:5:401, Zamir *et al.*, 1999:62:1268)

HR FAB-MS: *m/z* 645.24659 (M+K+)

Confirms a structure with MW 606.70 g/mol, $C_{35}H_{42}O_9$

19-20 mg in total.



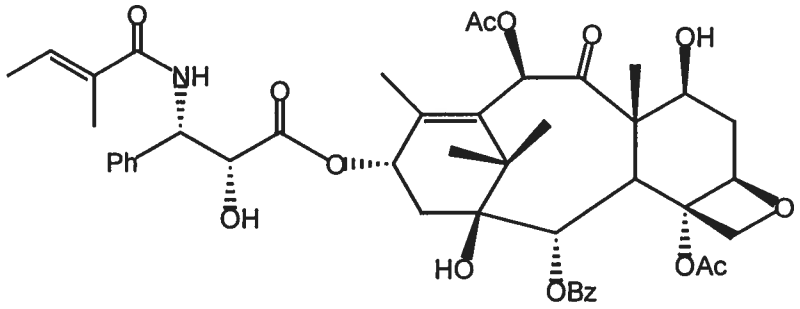
2α-deacetoxytaxinine J

(Liang, Min, and Niwa, 1988:46:1053, Zamir *et al.*, 1999:62:1268)

HR FAB-MS: *m/z* 689.27281 ($M+K^+$)

Confirms structure with MW 650.76 g/mol, $C_{37}H_{46}O_{10}$

19-20 mg in total.



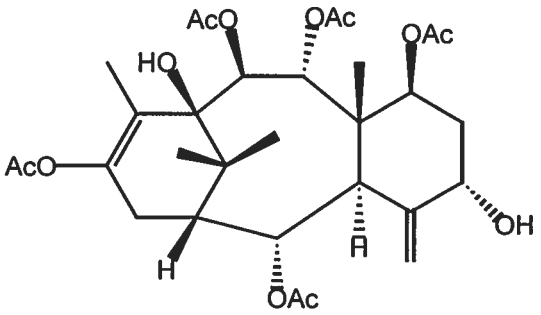
Cephalomanine

(Chauvière *et al.*, 1981:2:293:501, Zamir *et al.*, 1992:33:36:5173)

HR FAB-MS: *m/z* 870.31031 ($M+K^+$)

Confirms structure with MW 831.35 g/mol, $C_{45}H_{53}NO_{14}$

7-8 mg in total.



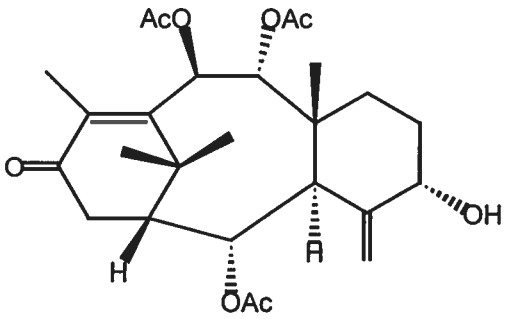
5-Decinnamoyl-taxuspine D

(Zamir et al., 1999:62:1268)

HR FAB-MS: *m/z* 633.23133 ($M+K^+$)

Confirms structure with MW 594.65 g/mol, $C_{30}H_{42}O_{12}$

12-13 mg in total.



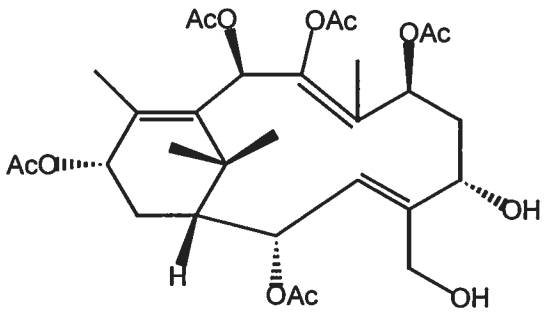
Taxinine A

(Chiang *et al.*, 1967:1201, Zamir *et al.*, 1999:62:1268)

HR FAB-MS: *m/z* 515.20473 (M+K+)

Confirms structure with MW 476.56 g/mol, $C_{26}H_{36}O_8$

15-16 mg in total.



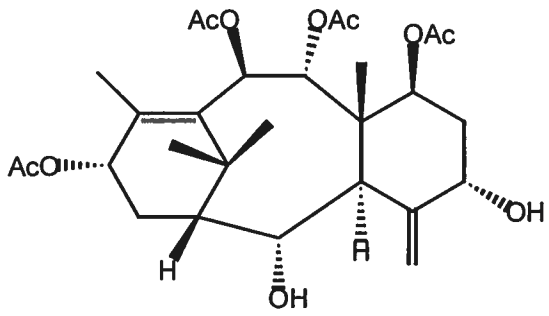
5-epi-canadensene

(Zamir *et al.*, 1995:529)

HR FAB-MS: *m/z* 633.23133 ($M+K^+$)

Confirms structure with: MW 594.27 g/mol, $C_{30}H_{42}O_{12}$

2-3 mg in total.



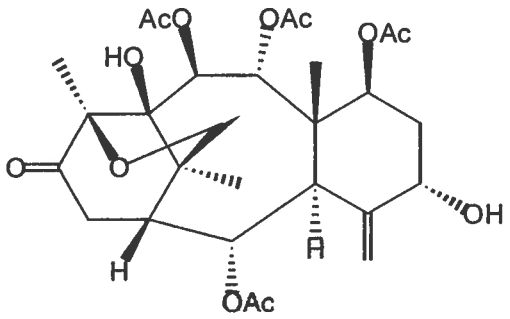
2-Deacetyl-5-decinnamoyltaxinine J

(Zamir *et al.*, 1992:33:36:5173)

HR FAB-MS: *m/z* 575.22586 ($M+K^+$)

Confirms structure with: MW 536.61 g/mol, $C_{28}H_{40}O_{10}$

7-8 mg in total.



5-Decinnamoyltaxagifine

(Zhang *et al.*, 1990:29:11:3673, Zamir *et al.*, 1992:33:36:5173)

HR FAB-MS: *m/z* 605.20003 ($M+K^+$)

Confirms structure with: MW 566.59 g/mol, $C_{28}H_{38}O_{12}$

52-53 mg in total.

Paclitaxel was isolated from four samples, one of which was **10-14.I-d** (see **figure 24**) originating from the original paclitaxel-containing mixture formed from fractions 10 through 14 of the original column chromatography of the dichloromethane extract. This paclitaxel mixture was purified by preparative HPLC using **program #1** (see **Appendix 1**) and gave sample **10-14.I** (see **figure 24**) which was itself further purified by preparative HPLC using **program #4** (see **Appendix 1**). The fraction collected during times **36.3 minutes** and **36.4 minutes** of this injection gave sample **10-14.I-d**. The remaining three samples came from the HPLC purification of fractions 22 to 25 of the column chromatography of the methanolic extract of the hexane layer. These three fractions were collected during times **34.0 minutes** and **34.5 minutes**, **34.5 minutes** and **35.0 minutes**, and **35.0 minutes** and **35.9 minutes** respectively of the preparative HPLC injection. They are shown in **figure 25** and were named **A**, **B** and **C**. In total, **31-32 mg** were obtained from the four samples. This compound was previously isolated from the bark, stems, roots and leaves of *Taxus baccata*, *Taxus brevifolia*, *Taxus canadensis*, *Taxus chinensis*, *Taxus cuspidata*, *Taxus media*, *Taxus wallichiana* and *Taxus yunnanensis*.

10-deacetyl-cephalmonanine was identified in two samples, 27-33.J and 26.B (see **figure 24**), originating from the 10-deacetyl-baccatin III mixture and fraction 26 from the dichloromethane extract column chromatography. Each of these was purified by preparative HPLC injection(s) using **program #3** and **program #1** (see **Appendix 1**) and collected during times **48.0 minutes** and **50 minutes**, and **28.6 minutes** and **29.5 minutes** respectively. In total, **11-12 mg** of 10-deacetyl-cephalomanine was obtained. This compound was previously isolated from roots, bark, stems and leaves of *Taxus baccata*, *Taxus brevifolia*, *Taxus cuspidata*, *Taxus canadensis*, *Taxus wallichiana* and *Taxus yunnanensis*.

10-deacetyl-baccatin III was identified in three samples, 27-33.B, 27-33.C-**a** and 27-33.C-**b** (see **figure 24**), each originating from the 10-deacetyl-baccatin III containing mixture. After further purification of this 10-DAB III containing mixture by preparative HPLC using **program #3** (see **Appendix 1**), samples 27-33.B and 27-33.C were obtained. These samples represent the fractions collected between times **27 minutes** and **29 minutes**, and **29 minutes** and **31 minutes** respectively. Sample 27-33.C (see **figure 24**) was further purified by another preparative HPLC, again using **program #3**, and subfractions 27-33.C-**a** and 27-33.C-**b** were obtained, collected between times **25.4 minutes** and **25.5 minutes**, and **25.5 minutes** and **25.6 minutes** respectively. In total **38-39 mg** were obtained. This compound was previously isolated from the leaves, stems and bark of *Taxus baccata*, *Taxus brevifolia*, *Taxus cuspidata*, *Taxus wallichiana*, *Taxus canadensis*, *Taxus yunnanensis* and *Taxus mairei*.

9-dihydro-13-acetyl-baccatin III was identified in four samples (see **figure 24**), 15-23.H, 10-14.E-**c**, 10-14.G-**a** and ii (from 10-14.D&F and 15-23.G). All of these samples originated from either the paclitaxel containing mixture or the 9-dihydro-13-acetyl-baccatin III mixture, and sample ii (from 10-14.D&F and 15-23.G) originated from both. The paclitaxel containing mixture was partially purified by preparative HPLC using **program #1**, as was the 9-dihydro-13-acetyl-baccatin III mixture using **program #3** (see **Appendix 1**) instead. Samples 10-14.D, 10-14.E, 10-14.F and 10-14.G were obtained from the paclitaxel injections, and samples 15-23.H and 15-23.G were obtained from the 9-dihdro-13-acetyl-baccatin III injections. Sample 15-23.H was formed from the fraction collected during times **45.6 minutes** and **47.6 minutes** of the preparative HPLC injection.

10-14.E and 10-14.G were further purified by preparative HPLC injections using **program #2** and **#1** respectively, giving sample 10-14.E-**c** collected during times **29.7 minutes** and **29.9 minutes**, and sample 10-14.G-**a** collected during times **27.9 minutes** and **28.6 minutes**. Samples 10-14.D, 10-14.F and 15-23.G were combined and further purified by a preparative HPLC injection using **program #2** and gave sample ii (from 10-14.D&F and 15-23.G), collected during times **29.4 minutes** and **29.8 minutes**. In total **26-27 mg** of 9-dihydro-13-acetyl-baccatin was obtained. This compound was previously isolated from the leaves of *Taxus canadensis*, as well as small quantities from *Taxus brevifolia* and *Taxus chinensis*.

Taxinine and **2α-deacetoxytaxinine J** were identified in one sample, 10-14.J, which originated from the paclitaxel containing mixture. After purification of the paclitaxel containing mixture by preparative HPLC using **program #1** (see **Appendix 1**), sample 10-14.J was obtained from the fraction collected between times **50.0 minutes** and **53.4 minutes**, and was characterized as a 1:1 mixture of taxinine and 2α-deacetoxytaxinine J with **19-20 mg** of each. **Taxinine** was previously isolated from leaves and heartwood of *Taxus canadensis*, *Taxus chinensis*, *Taxus cuspidata* and *Taxus mairei*. **2α-deacetoxytaxinine J** was previously isolated from the bark of *Taxus baccata*, *Taxus chinensis*, *Taxus cuspidata*, *Taxus mairei*, *Taxus wallichiana* and *Taxus yunnanensis*.

Cephalomanine was identified in one sample, 10-14.I-**c**, which originated from the paclitaxel containing mixture. Sample 10-14.I was obtained after purification of this mixture by preparative HPLC using **program #1**, and was further purified by preparative HPLC itself using **program #4** (see **Appendix 1**). Sample 10-14.I-**c** resulted from the fraction collected during times **35.2 minutes** and **35.3 minutes** of this last HPLC injection. **7-8 mg** of cephalomanine in total was obtained. This compound was previously isolated from the bark, leaves, stems and roots of *Taxus baccata*, *Taxus brevifolia*, *Taxus canadensis*, *Taxus cuspidata*, *Taxus media*, *Taxus wallichiana* and *Taxus yunnanensis*.

5-decinnamoyl-taxuspine D was identified in four samples: i (from 10-14.D&F and 15-23.G), 15-23.E, 27-33.H and 10-14.E-**a** (see **Figure 24**). Samples 10-14.D, 10-14.E and 10-14.F were obtained from the paclitaxel containing mixture after partial purification by preparative HPLC using **program #1** (see **Appendix 1**). Samples 15-23.E

and 15-23.G were obtained from the 9-dihydro-13-acetyl-baccatin III containing mixture after partial purification by preparative HPLC using **program #3** (see **Appendix 1**). Sample 15-23.E was obtained from the fraction collected during times **40.4 minutes** and **41.6 minutes** of this injection. Sample 27-33.H was obtained from the 10-deacetyl-baccatin III containing sample after partial purification by preparative HPLC also using **program #3** and was collected during times **40.0 minutes** and **41.5 minutes**. Sample 10-14.E-a was obtained after even further purification of sample 10-14.E by preparative HPLC using **program #2** (see **Appendix 1**) and was collected during times **26.9 minutes** and **27.4 minutes**, while samples 10-14.D, 10-14.F and 15-23.G were combined. Once combined, these fractions were together further purified by preparative HPLC also using **program #2** to give sample i (from 10-14.D&F and 15-23.G), collected during times **26.9 minutes** and **27.5 minutes**. In total, **12-13 mg** were obtained. This compound was previously isolated from the leaves of *Taxus canadensis*.

Taxinine A was identified in three samples, iii (from 10-14.D&F and 15-23.G), 8.B and 10-14.H (see **figure 24**). Sample 8.B was obtained after further purification of fraction 8, from the column chromatography purification of the dichloromethane extract, by preparative HPLC using **program #1** (see **Appendix 1**) and was collected during times **34.3 minutes** and **34.4 minutes**. Samples 10-14.D, 10-14.F and 10-14.H were obtained after further purification of the paclitaxel containing mixture by preparative HPLC also using **program #1**. Sample 10-14.H was obtained from the fraction collected during times **32.7 minutes** and **33.7 minutes** of this HPLC injection. Sample 15-23.G, obtained after purification of the 9-dihydro-13-acetyl-baccatin III containing mixture by preparative HPLC using **program #3** (see **Appendix 1**), was combined with samples 10-14.D and 10-14.F. These combined samples were then also further purified by preparative HPLC using **program #2** (see **Appendix 1**) and the fraction collected during times **32.3 minutes** and **32.5 minutes** gave sample iii. In total, **15-16 mg** of taxinine A was obtained. This compound was previously isolated from the leaves of *Taxus chinensis*, *Taxus cuspidata*, *Taxus media* and *Taxus canadensis*.

5-*epi*-canadensene was identified in one sample, 15-23.F (see **figure 24**). This sample was obtained after further purification of the 9-dihydro-13-acetyl-baccatin III containing mixture by preparative HPLC using **program #3** (see **Appendix 1**) and

corresponded to the fraction collected between times **44 minutes** and **45 minutes** of this injection. In total, **2-3 mg** were obtained. This compound was previously isolated from the leaves of *Taxus canadensis*.

2-deacetyl-5-decinnamoyltaxinine J was identified in two samples, 15-23.D and 27-33.G (see **figure 24**), from the 9-dihydro-13-acetyl-baccatin III and 10-deacetyl-baccatin III containing mixtures respectively. Each of these mixtures, providing the two samples in question, was purified by preparative HPLC using **program #3** (see **Appendix 1**). The samples correspond to the fraction collected between times **38.8 minutes** and **40.4 minutes** of the injection for sample 15-23.D, and the fraction collected during times **38.6 minutes** and **40.0 minutes** of the injection for sample 27-33.G. In total, **7-8 mg** were obtained. This compound was previously isolated from the bark and/or leaves of *Taxus canadensis*, *Taxus chinensis*, *Taxus wallichiana* and *Taxus yunnanensis*.

5-decinnamoyltaxagifine was identified in four samples, 15-23.C, 26.A, 27-33.E and 27-33.C-**b** (see **figure 24**). Sample 15-23.C was obtained after purification of the 9-dihydro-13-acetyl-baccatin III mixture by preparative HPLC using **program #3** (see **Appendix 1**) and was collected during times **33.9 minutes** and **37.0 minutes** of this injection. Sample 26.A was obtained after purification of fraction 26, obtained after the column chromatography of the dichloromethane extract, by preparative HPLC using **program #1** (see **Appendix 1**) and was collected during time **24.1 minutes** and **24.7 minutes** of this injection. Samples 27-33.C and 27-33.E were both obtained after purification of the 10-deacetyl-baccatin III containing mixture by preparative HPLC also using **program #3**, where sample 27-33.E was formed from the fraction collected during times **34.0 minutes** and **36.0 minutes**. Sample 27-33.C was purified still further by another preparative HPLC injection again using **program #3** and the fraction collected during times **22.3 minutes** and **22.4 minutes** gave sample 27-33.C-**b**. In total, **52-53 mg** of 5-decinnamoyltaxagifine was obtained. This compound was previously isolated from the leaves and stems of *Taxus canadensis* and *Taxus chinensis*.

4.2 Results from the *Taxus cuspidata* rooted cuttings taxane content study

Three novel taxanes and thirty-nine known taxanes were ultimately isolated from the dichloromethane extract of *Taxus cuspidata capitata* rooted cuttings by Dr Qing-Wen Shi. The publication of this work is in progress.

5. Discussion

5.1 *Taxus canadensis* rooted cuttings taxane content study

In total, 29 taxanes were identified from the extract of *Taxus canadensis* rooted cuttings. Of these taxanes, three were novel taxanes, fourteen were taxanes never before reported in *Taxus canadensis* and twelve were taxanes already known to *Taxus canadensis*. This study marks the first of its kind, as the taxane content of rooted cuttings of any species of Yew has never before been reported.

The comparison of the taxane content of the rooted cuttings of *Taxus canadensis*, studied above, with the taxane content of the mature plant of *Taxus canadensis*, already known to our lab, revealed several significant differences. Firstly, seventeen out of the twenty-nine taxanes identified from the rooted cuttings were unknown to the mature *Taxus canadensis* plant, where over seventy taxanes have already been reported. Such a great proportion of the taxanes identified from the rooted cuttings being previously unreported in the Canadian yew suggests that a noteworthy difference in taxane content between rooted cuttings and the mature tree of *Taxus canadensis* is possible. In addition, the major taxanes isolated from the rooted cuttings of the Canadian yew differed from the major taxanes previously known to the mature Canadian yew. While 9-dihydro-13-acetyl-baccatin III, taxinine and taxinine E have been reported as the major taxanes of the mature plant (Zamir *et al.*, 1992:33:36:5173, Zamir *et al.*, 1995:73:655), the major taxanes isolated from the rooted cuttings were 5-decinnamoyl-taxagifine, 10-deacetyl-baccatin III and paclitaxel. Most important in this difference is the quantity of 9-dihydro-13-acetyl-baccatin III recovered from the rooted cuttings. While 9-dihydro-13-acetyl-baccatin III is normally present in quantities three to seven times greater than paclitaxel in the mature plant regardless of the season or source of Canadian yew (Zamir *et al.*, 1995:73:655), it was found in quantities smaller than that of paclitaxel in the rooted cuttings of the Canadian yew. This is especially intriguing since 9-dihydro-13-acetyl-baccatin III is nearly unique to *Taxus canadensis*, with only minute quantities found in other yew species (Zhang, Chen and Chen, 1992:27:4:268). Also, one of the major taxanes of the mature Canadian yew, taxinine E, was not even identified in minute quantities from the extract of rooted cuttings. While this is true, it is important to note that

not all of the mixtures of compounds retrieved from the extract of the *Taxus canadensis* rooted cuttings were purified, and so it remains possible that taxinine E remains in the un-purified portions of the extract. Great effort however was made to purify 9-dihydro-13-acetyl-baccatin III, 10-deacetyl-baccatin III, paclitaxel and cephalomanine in their entirety to ensure that a quantitative comparison could be done. It is for this reason, as described in the experimental section, that the hexane layer obtained during the extraction of the rooted cuttings was extracted with methanol and that this methanolic extract was then partially purified to recover any taxanes which may have remained in the normally discarded hexane layer. It is therefore with much certainty that a significant difference in the proportion of 9-dihydro-13-acetyl-baccatin III and paclitaxel is reported. Also interesting in this study is the large amount of 5-decinnamoyl-taxagifine isolated from the rooted cuttings extract, which when compared with the quantities of the other taxanes isolated, becomes the major taxane isolated from the rooted cuttings of the Canadian yew. While this compound was previously known to *Taxus canadensis*, it was not present in quantities greater than that of 9-dihydro-13-acetyl-baccatin III, taxinine or taxinine E. **Table 3** follows with the twenty-nine taxanes identified from the rooted cuttings of *Taxus canadensis*, as well as their quantities, listed in decreasing order.

Table 3. List of taxanes isolated from *Taxus canadensis* rooted cuttings and their quantities

nt: novel taxane, **ntc**: new to Canadian yew, **kc**: Known to Canadian yew

Sample	**Quantity**
5-decinnamoyl-taxagifine [kc]	52-53 mg
10-deacetyl-baccatin III [kc]	38-39 mg
Paclitaxel [kc]	31-32 mg
9-dihydro-13-acetyl-baccatin III [kc]	26-27
Taxinine [kc]	19-20
2α-deacetoxytaxinine J [kc]	19-20
Taxinine A [kc]	15-16 mg
5-decinnamoyl-taxuspine D [kc]	12-13 mg
10-deacetyl-cephalomanine [kc]	11-12 mg

5-decinnamoyl-taxinine J [ntc]	11-12 mg
Baccatin VI [ntc]	8-9 mg
Novel taxane #3 [nt] 7β,9α,10β,13α-tetraacetoxy-11(15→1)*abeo*taxa-4(20),11-diene-5α,15-diol	8-9 mg
Taxayuntin [ntc]	7-8 mg
Cephalomanine [kc]	7-8 mg
2-deacetyl-5-decinnamoyl-taxinine J [kc]	7-8 mg
Taxinine M [ntc]	6-7 mg
Baccatin IV [ntc]	5-6 mg
7β-acetoxy-taxinine A [ntc]	4-5 mg
Taxuspine W [ntc]	3-4 mg
Taxuspine L [ntc]	3-4 mg
1β-hydroxy-2,7,9-trideacetyl-baccatin I [ntc]	3-4 mg
Novel taxane #1 [nt] 1β,2α,9α-trihydroxy-10β-acetoxy-5α-cinnamoyloxy-3-11-cyclictaxa-4(20)-ene-13-one	2-3 mg
Baccatin III [ntc]	2-3 mg
7,13-deacetyl-9,10-debenzoyltaxchinin C [ntc]	2-3 mg
5-*epi*-canadensene [kc]	2-3 mg
5-decinamoyl-taxinineB-11,12-oxide [ntc]	1-2 mg
13-acetyl-13-decinnamoyl-taxchinin B [ntc]	1-2 mg
Novel taxane #2 [nt] 2α,9α,10β-triacetoxy-20-hydroxy-11,12-epoxy-taxa-4-en-13-one	0.5-1 mg
Taxinine NN-3 [ntc]	0.5-1 mg

The differences noted between the content of the rooted cuttings and the mature tree of the Canadian yew suggest that a more in depth study of the content of rooted cuttings should be done in the future, and that the use of rooted cuttings in our lab should be continued. The difference in taxane content suggests that a change in equilibrium within the plant occurs with the creation of the rooted cuttings from the mature tree, and that a corresponding difference in the biosynthesis of taxanes within the rooted cutting might also exist. This could be due to the hormone-stimulated growth of roots on the cuttings of the mature tree. Unfortunately since the hormones used for the creation of the

rooted cuttings are kept confidential by Cramer nurseries, we will be unable to study in more detail the effect such specific hormones may have on the biosynthesis of taxanes unless a new agreement can be made with the nursery.

If efficient feeding protocols are established such that radioactive precursors may be fed to plant homogenates, live plant sections or rooted cuttings, the possible differences within the rooted cuttings greatly encourage such work to be pursued. Given the already intriguing differences observed between the Canadian yew and other species, the use of rooted cuttings of the Candian yew in our laboratorie's future research is doubly exciting and may provide important results in the future towards better understanding the biosynthesis of taxanes, their function within the plant, or also towards the discovery of new important taxanes (see **Appendix 4**).

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Appendix 1

Programs used for all analytical and preparative scale HPLC injections listed throughout the experimental.

Program #	Conditions
Program #1	Linear gradient of 25% to 100% CH_3CN in H_2O in 50 minutes.
Program #2	Linear gradient of 25% to 100% CH_3CN in H_2O in 70 minutes.
Program #3	Linear gradient of 25% to 100% CH_3CN in H_2O in 120 minutes.
Program #4	*Multi-step*: **(a)** isocratic 25% CH3CN for 50 minutes, **(b)** linear gradient 25% to 40% CH_3CN in H_2O in 20 minutes, **(c)** isocratic 40% for 170 minutes.
Program #5	isocratic 50% CH_3CN in H_2O.
Program #6	isocratic 55% CH_3CN in H_2O.
Program #7	isocratic 46% CH_3CN in H_2O.
Program #8	isocratic 27% CH_3CN in H_2O.
Program #9	isocratic 35% CH_3CN in H_2O.

Appendix 2

Hoagland's nutritive solution

⇒ Prepare both the macronutrients and micronutrients solutions as shown below.

1. Macronutrients solution

4.0 mM Calcium nitrate	$Ca(NO_3)_2 \cdot 4H_2O$
2.0 mM Magnesium sulphate	$MgSO_4 \cdot 7H_2O$
6.0 mM Potassium nitrate	KNO_3
1.0 mM Ammonium phosphate	$NH_4H_2PO_4$

2. Micronutrients solution

28 g/L Boric acid	H_3BO_3
34 g/L Manganese sulphate	$MnSO_4 \cdot H_2O$
1.0 g/L Copper Sulphate	$CuSO_4 \cdot 5H_2O$
2.2 g/L Zinc sulphate	$ZnSO_4 \cdot 7H_2O$
1.0 g/L Ammonium molybdate	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$
5.0 mL Sulphuric acid (concentrated)	H_2SO_4

⇒ Prepare the iron stock solution as described below.

- Dissolve the KOH in 286 mL of water, and then add the EDTA.
- In a separate flask, dissolve the iron sulphate in 500 mL of water and slowly add the first mixture (of EDTA and KOH) while stirring. Make the volume up to 1 L.

3. Iron stock solution

26.1 g Ethylene-diamine-tetraacetic acid	EDTA
19.0 g Potassium hydroxide	KOH
24.9 g Iron sulphate	$FeSO_4 \cdot 7H_2O$
786 mL water	H_2O

⇒ Make Hoagland's Nutritive Solution by doing as listed below.

- Add 2.5 mL of the iron stock solution to 1L of micronutrients solution.
- Add 1.0 mL of the micronutrients solution (with the 2.5 mL of iron stock solution) to 1L of macronutrients solution.
- Adjust the pH to 6.7 using a pH-meter.
- Enrich with Plantex 10-52-10 starter fertiliser

Appendix 3

Products used:

[1,2-^{14}C]-Acetic acid, sodium salt (Amersham-Pharmacia Biotech)

Acetone (EM Science, HPLC grade)

Acetonitrile: CH_3CN (EM Science, HPLC grade)

Ammonium molybdate: $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (Merck)

Ammonium phosphate: $NH_4H_2PO_4$ (Fisher)

Boric acid (J.T. Baker Chemical Co.)

Calcium nitrate: $Ca(NO_3)_2\cdot 4H_2O$ (Fisher)

Copper sulphate: $CuSO_4\cdot 5H_2O$ (Fisher)

D-[U-^{14}C]-glucose (ICN)

Dichloromethane: CH_2Cl_2 (EM Science, HPLC grade)

Ethyl Acetate: EtOAc (EM Science, high purity grade)

Ethylene-diamine-tetraacetic acid: EDTA (Sigma)

Fertiliser 10-52-10 (Plantex)

Hexane (EM Science, high purity grade, non UV)

Hydrochloric acid: HCl (Fisher)

Iron sulphate: $FeSO_4\cdot 7H_2O$ (Fisher)

Isopropyl Alcohol (EM Science, HPLC grade)

Magnesium sulphate (anhydrous): $MgSO_4$ anhydrous (Fisher)

Magnesium sulphate: $MgSO_4\cdot 7H_2O$ (Fisher)

Manganese sulphate: $MnSO_4\cdot H_2O$ (Fisher)

Methanol: MeOH (EM Science, HPLC grade)

Milli-Q filtered water (Waters)

Potassium hydroxide: KOH (Anachemia)

Potassium nitrate: KNO_3 (Fisher)

Scintillation liquid (Packard)

Sodium chloride: NaCl (table salt)

Sulphuric acid: H_2SO_4 (Allied Chemical Canada Ltd.)

Zinc sulphate: $ZnSO_4\cdot 7H_2O$ (Fisher)

Appendix 4

Experimental: Feeding of radioactively labelled precursors to rooted cuttings of *Taxus canadensis* and *Taxus cuspidata capitata*

- **Two protocols used for the feeding experiments**

Two different feeding protocols were used for the biosynthetic studies of taxanes in rooted cuttings of *Taxus canadensis* and *Taxus cuspidata*. The objective of this study was to establish the groundwork for a feeding protocol, where radiolabelled compounds could be fed and hopefully incorporated into the rooted cuttings of yew trees and their taxanes. Since the feeding protocol was not yet established, two methods were used in order to compare the results and determine not only if one or both of these protocols would work, but also if they should be pursued in future studies in our lab.

The primary goal of this experiment was not yet the study of the biosynthesis of paclitaxel and other taxanes in the rooted cuttings, but rather the elucidation of a feeding protocol. For this purpose, the radioactively labelled compounds chosen to be fed to the rooted cuttings where simple so as to not complicate the interpretation of results or lessen the chances of any incorporation occurring. Glucose [U-^{14}C] and acetate [1,2-^{14}C] were chosen as the two compounds to be fed since the incorporation of glucose and acetate into taxanes has already been observed (Eisenreich *et al.*, 1996: 93: 13: 6431). Once a feeding protocol is established, the precursors fed may be altered such that the purpose of future experiments will then be the study of the biosynthetic steps involved in the production of the taxanes in rooted cuttings of yew trees.

The only difference between the two feeding protocols employed is the treatment of the rooted cuttings and the way in which these plants were exposed to the feeding solution enriched with the radioactively labelled compounds. In the first protocol, the rooted cuttings were cut into small pieces of 0.5-1.0 cm in size and the plant material was added directly to the enriched feeding solution. In the second protocol, an intact rooted cutting was fixed above a glass vial, and the roots were suspended in the enriched feeding

solution. While it was hypothesised that the large root volume of the rooted cuttings would perhaps allow an increased uptake of the fed material and thereby perhaps an increased incorporation of these compounds into the plants taxanes, feeding experiments using rooted cuttings have never before been reported in the literature. Conversely, feeding experiments using cut-up or ground segments of yews are more customary and have been reported with some success (Zamir, Nedea and Garneau, 1992:33:36:5235).

The rooted cuttings to be used for the radioactively labelled precursor feeding experiments were all obtained in October 2000 from Cramer Nurseries. The yews were therefore more yellow or grey in colour due to the seasonal changes that they had undergone. It is normal for yews to undergo such seasonal changes and the needles of the yews were checked in order to verify that they still retained moisture and were in fact alive. All rooted cuttings were removed from their soil and were washed gently under water to remove all dirt and debris. The excess water was then removed by drying under paper towels.

Protocol 1: Three *Taxus canadensis* and three *Taxus cuspidata capitata* rooted cuttings were prepared and cut up into small pieces ranging in size from 0.5 cm to 1 cm. The combined pieces of *Taxus canadensis* rooted cuttings weighed a total of 13.16g (Mettler PC 2000), whereas the combined pieces of *Taxus cuspidata capitata* rooted cuttings weighed a total of 10.28g (Mettler PC 2000). The experiment was set up such that there were six 125mL Erlenmeyer flasks for each species of yew and the plant tissue was divided up accordingly. In this way, there was 2.19g (Mettler PC 2000) of *Taxus canadensis* tissue per flask, and 1.71g (Mettler PC 2000) of *Taxus cuspidata capitata* per flask. To each flask, 15mL of Hoagland's Nutritive Solution (see **Appendix 2**) was added. See **figure 33**.

Protocol 2: Another six *Taxus canadensis* and six *Taxus cuspidata capitata* rooted cuttings were washed and prepared for the second protocol. Each rooted cutting was suspended above a vial by a copper wire that was affixed around the mouth of the vial and allowed the root bundle to be positioned halfway into the vial. Each vial had a small magnetic stirring bar and 15mL of Hoagland's Nutritive Solution (see **Appendix 2**) added. Before suspending the rooted cuttings above the vial, the roots of each rooted cutting were bundled in cheesecloth which was previously soaked in Hoagland's

Nutritive Solution. The roots were bundled in this fashion so that they would not impede the stirring of the feeding solution by the magnetic stirring bar placed at the base of the vial. The cheesecloth was soaked previous to its use to avoid it absorbing any of the nutritive solution enriched in radioactively labelled compounds, which would make a portion of the solution and its contents unavailable for absorption by the plantlet. See **figure 33**.

In each protocol, the experiment was done in duplicate such that for each radioactively labelled compound fed (glucose and acetate) as well as a control where no radioactive compounds were included, there were two samples each for *Taxus canadensis* and *Taxus cuspidata capitata* rooted cuttings. This gave six *Taxus canadensis* and six *Taxus cuspidata capitata* samples for each protocol. This was particularly important in case one of the vials or flasks should brake while on the shaker or stirring plate and the contents be lost.

For each of the samples where acetate was the radiolabelled compound fed, in either of the two protocols, 5μL of the acetate [1,2-^{14}C] solution was added to the 15mL Hoagland's Nutritive Solution in either the flask or the vial. This corresponded to 2,687,173 dpm, calculated as follows: 5μL of the stock [1,2-^{14}C] acetate solution was diluted to 1mL in distilled water. 10μL of this diluted solution was counted using a scintillation counter (Liquid Scintillation System, Tracor Analytic, Delta 300) with 2mL of scintillation liquid for 2 minutes. Subtracting the background control reading of 35.21 dpm from the measured 26,906.94 dpm of the sample, a total reading of 26,871.73 dpm was obtained. With a dilution factor of 100, this corresponded to 2,687,173 dpm per 5μL of the stock solution. For each of the samples where glucose was the radiolabelled compound fed, 5μL of the glucose [U-^{14}C] solution was added to the 15mL Hoagland's Nutritive Solution in either the flask or the vial. This corresponded to 15,191,167 dpm, calculated as follows: 5μL of the stock solution was diluted to 1mL in distilled water. 10μL of this diluted glucose [U-^{14}C] solution was counted in a scintillation counter (Liquid Scintillation System, Tracor Analytic, Delta 300) with 2mL of scintillation liquid for 2 minutes. Subtracting the background control reading of 35.21 dpm from the measured 151,946.88 dpm of the sample, a total reading of 151,911.67 dpm was obtained.

With a dilution factor of 100, this corresponded to 15,191,167 dpm per 5μL of the stock solution.

Once the radioactively labelled compounds were added to the Hoagland's Nutritive Solution, the flasks containing the solution and the cut up rooted cuttings were stoppered with foam/cheesecloth stoppers and were placed on a shaker and left overnight at room temperature in the absence of light. The vials containing the root bundles, magnetic stirring bars and solution were placed inside empty beakers, which were used to secure and prevent these vials from overturning. These beakers were in turn placed on stirring plates and left at room temperature in the absence of light. A humidifier was left running near the intact rooted cuttings to discourage the evaporation of the feeding solution from the vials, while a flask containing water was left open in the shaker to also increase the humidity within the shaker container.

The level of the solution was marked on each flask and vial to allow monitoring of the solution level and thus determine if evaporation was occurring. A decrease in the volume of the feeding solution would have caused an increase the concentration of radioactively labelled compounds and would have prevented the concentration of precursors available to each sample from remaining constant. For this reason, as the volumes of the feeding solutions were noted to fluctuate slightly due to some evaporation, additional Hoagland's Nutritive Solution was added to maintain the desired level.

As described above, 15,191,167 dpm of glucose [U-^{14}C] versus 2,687,173 dpm of acetate [1,2-^{14}C] was added to each sample. Due to this difference in the amount of radioactivity added between the glucose and acetate samples, an additional volume of acetate [1,2-^{14}C] was added to each of the acetate-fed samples three days after the feeding experiments were begun. In order to have the same number of counts of radioactively labelled acetate as of glucose and to facilitate a comparison, an additional 12,503,994 dpm of labelled acetate were added per sample. This corresponded to an additional 23μL of the acetate solution, using the same conversion factors used above for the previous calculations.

The feeding experiments were continued for a total of 15 days. During this time, the volumes of the feeding solutions were constantly monitored as described above. All samples were labelled as radioactive, and a large area of absorptive paper was laid down

to avoid contamination of the surrounding area should any spilling or splashing of the radioactive solution have occurred. One problem that was observed was that the electric current fluctuated slightly during the night and thereby changed the speed of the magnetic stirring bars previously adjusted. This in some cases led to an overly vigorous stirring and the loss of a small amount of feeding solution in the form of a mist expelled from the vial.

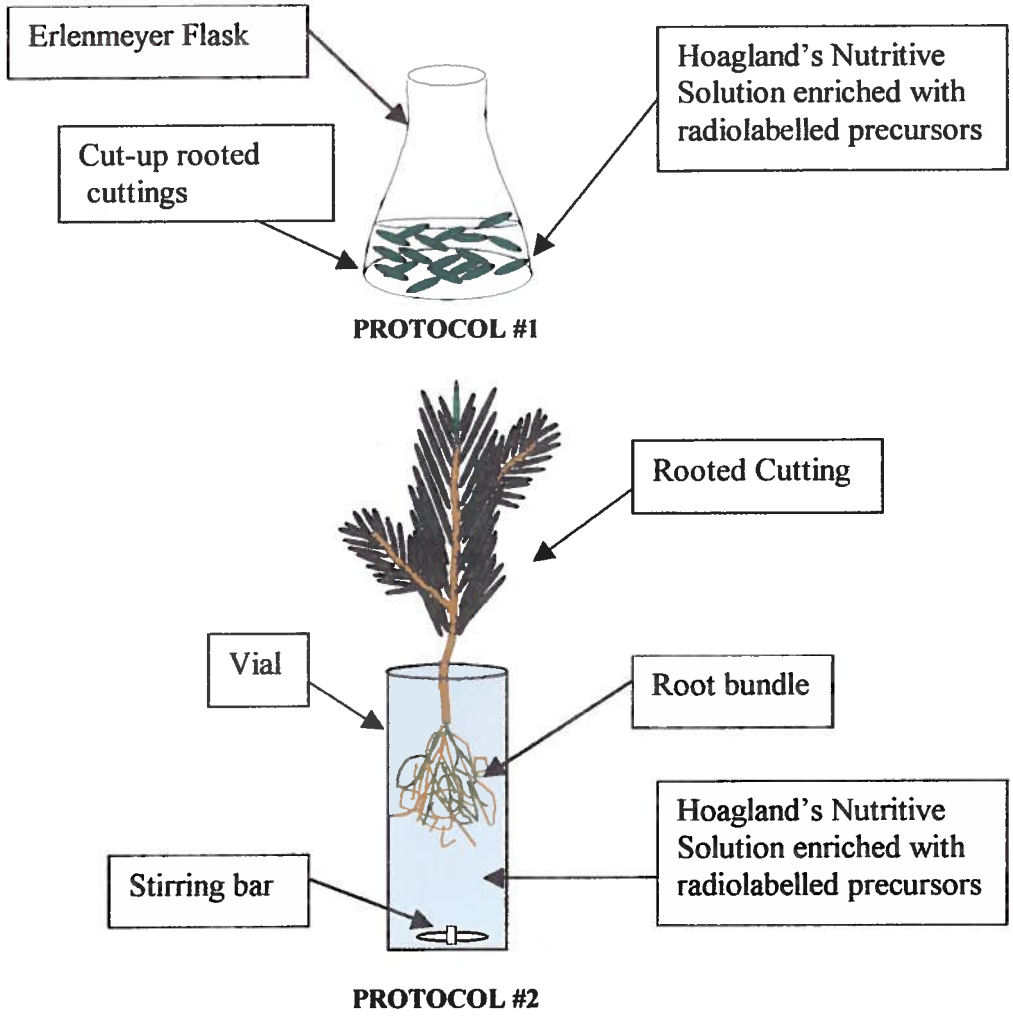

Figure 33. Setup of feeding experiments for protocol #1 and protocol #2

- **Extraction of rooted cuttings after feeding with radioactive precursors**

Once the feeding experiments were finished, the intact rooted cuttings from protocol #2 were removed from the vials containing the feeding solution, and were rinsed

repeatedly with water to remove all traces of the radioactive feeding solution. The water used to wash the plantlets was counted from time to time using the scintillation counter (Liquid Scintillation System, Tracor Analytic, Delta 300) to monitor radioactive content and to determine when the rooted cuttings were sufficiently clean. The water was sampled after several washings and counted with scintillation liquid in order to evaluate whether the washing of the plantlets should be continued or were devoid of excess feeding liquid. Once the plantlets were clean, they were cut into small pieces to facilitate extraction. Care was taken to clean the scissors or shears such that samples were not contaminated with compounds or radioactivity present from one of the other plantlets. Once washed and cut up, the plant material was weighed (Mettler PC 2000) such that approximate weight of the plant tissue per flask would be known. The *Taxus cuspidata* glucose sample weighed 5.48g and therefore there was approximately 2.74g per flask. The *Taxus cuspidata* acetate sample weighed 6.0g and there was therefore 3.04g per flask. The *Taxus cuspidata* control sample weighed 4.11g and there was therefore 2.06g per flask. The *Taxus canadensis* glucose sample weighed 2.37g and there was therefore 1.19g per flask. The *Taxus canadensis* acetate sample weighed 2.86g and there was therefore 1.43g per flask. The *Taxus canadensis* control sample weighed 2.93g and there was therefore 1.47g per flask. 5μL of the feeding solution from each sample was taken before the remaining feeding solution was discarded. This 5μL sample was made up to 1mL in water and 30μL of this diluted sample was counted along with 2mL of scintillation liquid using the scintillation counter (Liquid Scintillation System, Tracor Analytic, Delta 300). The resulting counts obtained from these readings allowed the degree of uptake of radioactive compounds by the rooted cuttings to be estimated. A noteworthy difference in the counts read from the feeding solution before and after the feeding experiments would signify that an uptake of the compounds into the rooted cuttings did occur. A similar radioactive content would conversely signify that no uptake and no incorporation occurred.

If the plantlet was cut up and in the feeding solution, as in protocol #1, the plant tissue was separated from the feeding solution by straining through cheesecloth once the feeding experiments were finished. The feeding solution itself was be conserved in case there was any leaking of compounds into the solution. While filtering the feeding solution

from the cut up rooted cuttings, the plant material was also rinsed with distilled water. The plant tissue was conserved separately.

The plant tissue obtained from either of these two feeding protocols was placed in a flask with a determined volume of CH_2Cl_2:MeOH (1:1, vol/vol) and left on the shaker (room temperature in darkness) overnight. The amount of solvent used was dependant on the amount of plant material to be extracted (for a half to a whole plantlet, 15 to 20mL of solvent was used). The solvent was removed the following day and new solvent was added. This step was repeated approximately four times (more if needed) with the four volumes of solvents later combined. The solvent was evaporated off and a residue was obtained.

The plantlet residue obtained after four consecutive overnight extractions with CH_2Cl_2:MeOH (1:1, vol/vol) was further extracted to obtain a mixture of taxanes, from which the desired compounds were then isolated. The residue was dissolved (or suspended) in warm distilled water. This aqueous solution was then extracted with a volume of hexanes in order to remove any lipids (80-100mL each time). All extractions were repeated three times. The aqueous layer was retained and saturated with NaCl. This saturated aqueous solution was then extracted with dichloromethane (100-120mL each time). The dichloromethane layer was then dried over anhydrous sodium sulphate. The dried dichloromethane layer was then allowed to evaporate to dryness. The resulting crude mixture of compounds was then injected and partially purified by analytical scale HPLC (*described in the following section*) using a variety of programs with the goal of eventually purifying the desired taxanes such as paclitaxel, 10-DAB-III and 9-dihydro,13-deacetyl-baccatin III. If protocol #1 was followed, the feeding solution that was also retained was saturated with sodium chloride and extracted with dichloromethane (approximately 100mL each time, with an additional 30mL of water added). It was not necessary to extract with hexanes as not enough lipids were present in the solution to warrant the added step. As a safety measure, all the layers (even the ones not needed) were conserved in case of error. In this way, no compounds were lost and steps could have easily been repeated or adapted to recover desired compounds.

- **Purification of taxanes by HPLC, from the extract of rooted cuttings used in radioactive feeding experiments**

The crude extract was dissolved into a small volume of solvent and injected into the HPLC. The HPLC system used for purification of all radioactive mixtures consisted of a Perkin Elmer Binary LC pump 250 with a Waters 990 Photodiode Array Detector and a LKB Bromma 2111 Multirac Auto-collector. The columns used were two Whatman Partisil 10-ODS-2 columns connected in series, preceded by a pre-column. If particles in suspension were observed upon dissolution of the extract in solvent, the solution was then filtered through a 0.45 μm filter attached to a 20cc syringe. The filter was rinsed several times to avoid loss of compound. The solvent system generally used to prepare the sample was CH_3CN:MeOH (1:1, vol/vol), however this depended on the compounds present in the sample and was at times modified to make it more or less polar as needed. All crude samples were first injected (100 μL per injection) using **program #1** (see **Appendix 1**) with fractions collected every minute. These fractions were evaporated down and combined such that all undesired compounds were pooled together and conserved in case of error, and fractions containing the desired compounds such as paclitaxel, 10-deacetyl-baccatin III, and 9-dihydro-13-acetyl-baccatin III, were kept separate. The fractions were sorted based on retention times from standards previously injected under the same conditions. Based on these standards, fractions collected from 1-18 minutes were pooled and retained in case of future use, fractions collected from 18-22 minutes were pooled together to form the sample containing 10-deacteyl-baccatin III, fractions collected from 22-26 minutes were pooled together and retained in case of future use, fractions collected from 26-29 minutes were combined to form the sample containing 9-dihydro-13-acetyl-baccatin III, fractions collected from 29-36 were pooled together to form the sample containing paclitaxel and cephalomanine, and finally samples collected from 36 minutes to the end of the programmed run were pooled and also retained in case of future use.

The selected samples resulting from the injections using **program #1** (see **Appendix 1**) were then ready to be injected using a series of other programs in which the retention times of the various taxanes were also already known. In using a series of other

programs in which the retention time of the compound of interest varies between each program, the taxane is retained while the various other compounds are progressively removed from the mixture. The sequence of programs to be used was based on work done previously by another Masters student from our lab, Isabelle Caron. For each of the programs described, fractions must be collected every minute. Based on known retention times, the many fractions are combined to give samples containing the compounds of interest. These samples are then evaporated to dryness and injected again using the next program.

For the purification of paclitaxel and cephalomanine, the series of programs are as follows: (1) **program #5**, (2) **program #6**, and (3) **program #4** (see **Appendix 1**).

For the purification of 9-dihydro-13-acetyl-baccatin III, the series of programs are as follows: (1) **program #7**, (2) **program #5** (see **Appendix 1**), followed by (3) a preparative TLC eluted with 100% ethyl acetate.

For the purification of 10-DAB-III, the series of programs are as follows: (1) **program #8**, (2) **program #9** (see **Appendix 1**) followed by (3) a preparative TLC eluted with CH_2Cl_2:MeOH (95:5).

Since only 0.5 to 1 plantlet was used per sample, the amount of taxanes that could be purified was extremely small. Once the initial purification steps using **program #1** (see **Appendix 1**) were completed, the retained fractions that contained the desired taxanes were very small in size. In order to facilitate the further purification of these taxanes, a small measured amount of the relevant un-labelled standard taxane in the range of 2mg (Cahn electrobalance, model 4400) was added to each sample. This was done to allow visualisation of the peak corresponding to the taxane during running of the HPLC and help avoid the loss of the taxane purified from the fed rooted cuttings during the subsequent purifications. Since this added taxane was not radiolabelled, it would not contribute to the radioactivity counted by the scintillation counter (Liquid Scintillation System, Tracor Analytic, Delta 300) and would still allow the incorporation of radioactivity into the taxanes to be measured. The weight of the taxane purified from the rooted cutting would be too little to be accurately weighed, so adding the standard taxane would be of no consequence. As such, once purification was complete, the samples could be divided in two and half could be counted using the scintillation counter and the other

half sent for analysis by HR FAB-MS. By counting the radioactivity, the amount of incorporation, or lack of it, may be calculated for each purified taxane.

As described in the results section, due to other data obtained which showed the results of this experiment to be unreliable (see next section, and **tables 4 & 5**), the purification of these samples was not continued or completed. This was due largely to the radioactivity measured in the non-fed controls and thus the suspicion that cotamination of samples occurred.

Results from radioactive feeding experiments to *Taxus canadensis* and *Taxus cuspidata* rooted cuttings

The feeding experiments did not present results that were adequately reliable to warrant completion of the study. As mentioned in the experimental section, 5μL samles of the feeding solution (from protocol #2) were taken from each sample vial for counting. The radioactivity of each of these samples at the beginning and end of the feeding experiments are listed in **table 4**, and the corresponding changes in radioactivity are listed in **table 5**. Upon consultation of this data, it is evident that in most cases either no significant change in radioactivity occurred or that the duplicate samples gave conflicting results. While several of the average changes listed in **table 5** show a decrease in radioactivity at the end of the feeding experiment and thus suggest that uptake of the radioactive compounds may have occurred, it is most important to note the conflicting results obtained between each of the individual samples. Also of the greatest importance is the elevated radioactivity of two of the control samples. As also noted in the experimental section, a variation in the speed of the stirring bars might have caused some of the feeding solution enriched with radioactive compounds to have been expelled from the vials of *protocol #2* in the form of a fine mist. It is suspected that this mist may have contaminated adjacent samples thus leaving the results unreliable. Since it appears then that the samples contaminated each other, the purification of the taxanes and the counting of any incorporation was not continued as the data obtained would be unreliable.

Table 4. List of samples and their radioactive counts, at the beginning of and the end of the feeding experiments.

Sample	Total DPMs added to feeding solution	DPMs at t =15 days	Average at t=15 between #1 & #2
***T.can* Glu #1**	1.519×10^7 - *background* = 1.167×10^7	7.190×10^6 - *control t.can* = 3.719×10^6	1.111×10^7
***T.can* Glu #2**	1.519×10^7 - *background* = 1.167×10^7	2.198×10^7 - *control t.can* = 1.851×10^7	
***T.can* Ace #1**	1.505×10^7 - *background* = 1.153×10^7	1.232×10^7 - *control t.can* = 8.849×10^6	5.271×10^6
***T.can* Ace #2**	1.505×10^7 - *background* = 1.153×10^7	5.163×10^6 - *control t.can* = 1.692×10^6	
***T.cusp* Glu #1**	1.519×10^7 - *background* = 1.167×10^7	2.22×10^7 - *control t.cusp* = 1.738×10^7	9.157×10^6
***T.cusp* Glu #2**	1.519×10^7 - *background* = 1.167×10^7	5.758×10^6 - *control t.cusp* = 9.33×10^5	
***T.cusp* Ace #1**	1.505×10^7 - *background* = 1.153×10^7	2.658×10^8 - *control t.cusp* = 2.610×10^8	1.317×10^8
***T.cusp* Ace #2**	1.505×10^7 - *background* = 1.153×10^7	7.306×10^6 - *control t.cusp* = 2.481×10^6	
***T.can* control#1**	0 + *background* = 3.521×10^6	8.490×10^6 contaminated	3.471×10^6 Use control#2 only
***T.can* control#2**	0 + *background* = 3.521×10^6	3.471×10^6	
***T.cusp* control#1**	0 + *background* = 3.521×10^6	2.772×10^7 contaminated	4.825×10^6 Use control#2 only
***T.cusp* control#2**	0 + *background* = 3.521×10^6	4.825×10^6	
Background noise	3.521×10^6	See controls *t.can* or *t.cusp*	

Table 5. Change in radioactivity over time of feeding experiments

Samples	Change in DPMs over time	Average change in DPMs over time	% increase or decrease of DPMs from t = 0 to t = 15 days
***T.can* Glu #1**	-7.951×10^6	-5.555×10^5	Decrease of 4.76%
***T.can* Glu #2**	$+6.840 \times 10^6$		
***T.can* Ace #1**	-2.681×10^6	-6.260×10^6	Decrease of 54.29%
***T.can* Ace #2**	-9.838×10^6		
***T.cusp* Glu #1**	$+5.710 \times 10^6$	-2.515×10^6	Decrease of 21.55%
***T.cusp* Glu #2**	-1.074×10^7		
***T.cusp* Ace #1**	$+2.495 \times 10^8$	$+1.202 \times 10^8$	Increase of 1042.50%
***T.cusp* Ace #2**	-9.049×10^6		

***T.can* control#1**	$+ 4.969 \times 10^6$	Use control #2 only $- 5.00 \times 10^4$	Decrease of 1.42%
***T.can* control#2**	$- 5.00 \times 10^4$		
***T.cusp* control#1**	$+ 2.420 \times 10^7$	Use control #2 only $+ 1.304 \times 10^6$	Increase of 37.03%
***T.cusp* control#2**	$+ 1.304 \times 10^6$		

***Discussion*: Radioactive feeding experiments to *Taxus canadensis* and *Taxus cuspidata* rooted cuttings**

Due to the contamination of the samples and the therefore unreliable data obtained, the two feeding protocols are unable to be evaluated. Several important observations may be made however: (1) It is important to assure that the stirring of the feeding solution is more carefully monitored in futures attempts of protocol #2 to avoid contamination, (2) It is important to attempt to have more exchange between the feeding solution and the air as it was noted that at the end of the 15 day feeding period the solution was becoming more viscous, appeared to have some contaminants growing in it, and portions of the plant material were starting to degrade slightly.

The establishment of a feeding protocol for rooted cuttings would allow the differences observed between the rooted cuttings and the mature tree of the Canadian yew to be better studied and understood. While this is true, it appears that obtaining incorporation of compounds using plants or plant segments is much more difficult that using cell culture, which is the method of choice as demonsatretd by most recent publications in this field. It may therefore also be interesting for our lab to do biosynthetic studies using cell culture, with the rooted cuttings as the starting material and provider of these cells.