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Synthesis of Stable and Radioisotopomers of Taxa-4(5),11(12)-diene, Taxa-4(20), 11(12)-diene and Taxa-4(20), 11(12)-dien-5-α-ol, Early Intermediates in Taxol® Biosynthesis

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SUMMARY

The synthesis of [20-C^2H_3]-Taxa-4(5), 11(12)-diene (**3b**), [20-C^2H_2]-Taxa-4(20), 11(12)-diene (**7b**), [20-$^3H_3$]-Taxa-4(5), 11(12)-diene (**3c**), [20-$^3H_2$]-Taxa-4(20), 11(12)-diene (**7c**), [20-C^3H_2]-Taxa-4(20), 11(12)-diene-5-α-ol (**4b**), [20-$^{13}CH_2$]-Taxa-4(20), 11(12)-diene (**7d**) and [20-$^{13}CH_3$]-taxa-4(5), 11(12)-diene (**3d**) from the previously reported ketone **6** is described. Grignard addition to ketone provides the tertiary carbinols **8** which were dehydrated to a mixture of **3** and **7**. Alternatively, **7** can be prepared directly from ketone **6** by Wittig olefination.

Keywords: [[20-C^2H_3]-Taxa-4(5), 11(12)-diene, [20-C^2H_2]-Taxa-4(20), 11(12)-diene, [20-$^3H_3$]-Taxa-4(5), 11(12)-diene, [20-$^3H_2$]-Taxa-4(20), 11(12)-diene, [20-C^3H_2]-Taxa-4(20), 11(12)-diene-5-α-ol, [20-$^{13}CH_2$]-Taxa-4(20), 11(12)-diene and [20-$^{13}CH_3$]-Taxa-4(5), 11(12)-diene.

INTRODUCTION

Taxol® (**1**, paclitaxel) was first isolated from the bark of the pacific yew, *Taxus brevifolia*.[1] This complex diterpenoid has potent antimitotic activity and has been in clinical use for the treatment of several cancers, most notably breast and ovarian tumors.[2,3] Unfortunately the pacific yew is slow growing and is primarily found in environmentally sensitive areas of the Pacific Northwest and stripping the tree of its bark kills the yew and alternative sources of the drug have therefore become an important objective.[4-6]

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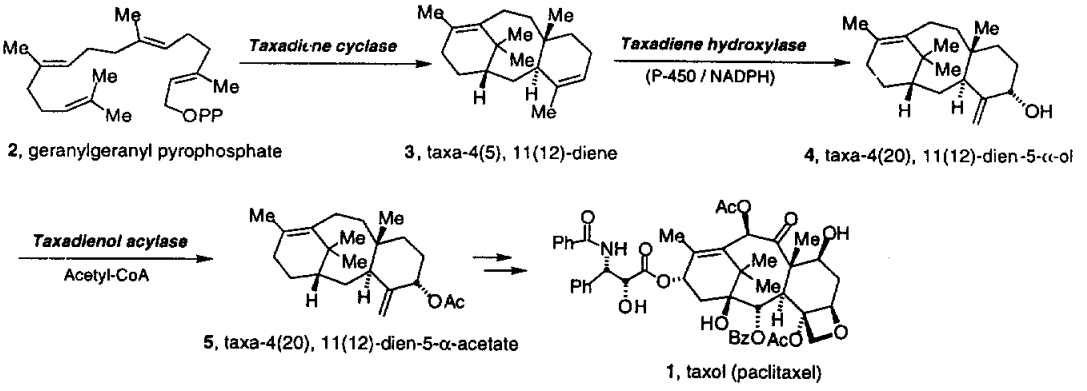
Collection of renewable *Taxus* sp. needles and clippings has attenuated the supply problem somewhat, but as the drug becomes more widely adopted, pressure on the yew population is likely to increase worldwide. Taxol® has subsequently been detected as a metabolite of various *Taxus* sp. and most recently from the endophytic fungus *Taxomyces andreanae*,[7] *Fusarium, Penicillium, Phoma* and *Alternaria alternata* ssp. *Taxus hicksii*. It has been reported that fermentation of *Alternaria alternata* yields about 116 mg of taxol® per liter and tissue culture yields of *Taxus* sp. is rapidly approaching economically viable levels. It is clear that taxol® production will continue to be developed *via* biological vectors and there has recently been a sharp increase in interest in the biosynthesis of taxol® as a means of improving yield from cell culture and other emerging technologies including cell-free biosynthesis.[8] Most significant in this regard, is the identification of the rate-limiting steps in taxol® biosynthesis that might be amenable to manipulation by genetic and other means.

It has recently shown that the early steps in taxol® biosynthesis involve: (1) the cyclization of geranylgeranyl pyrophosphate (**2**, GGPP) to afford taxa-4(5), 11(12)-diene (**3**) catalyzed by taxadiene cyclase;[9] (2) the cytochrome P450 hydroxylation of taxa-4(5), 11(12)-diene (**3**) to taxa-4(20), 11(12)-dien-5-α-ol (**4**) catalyzed by taxadiene hydroxylase;[10] (3) the acetylation of **4** to the corresponding acetate (**5**) followed by a series of as yet undefined oxygenations (Scheme 1).

It has been suggested that the first two steps in taxol® biosynthesis may be slow and perhaps limiting the net carbon flux to a complex matrix of taxoids.[9,10] Due to recent mechanistic interest in the initial cyclase and cytochrome P450 hydroxylation reactions, there has emerged a need for the synthesis of various stable- and radioisotopomers of these early biosynthetic intermediates upon which downstream metabolite elucidation, manipulation and mechanistic studies will be based. In this paper, we report the procedures for the synthesis of various stable- and radioisotopomers of these

natural biosynthetic intermediates that should be of use to laboratories examining the taxol® biosynthetic pathway.



2, geranylgeranyl pyrophosphate
3, taxa-4(5), 11(12)-diene
4, taxa-4(20), 11(12)-dien-5-α-ol
5, taxa-4(20), 11(12)-dien-5-α-acetate
1, taxol (paclitaxel)

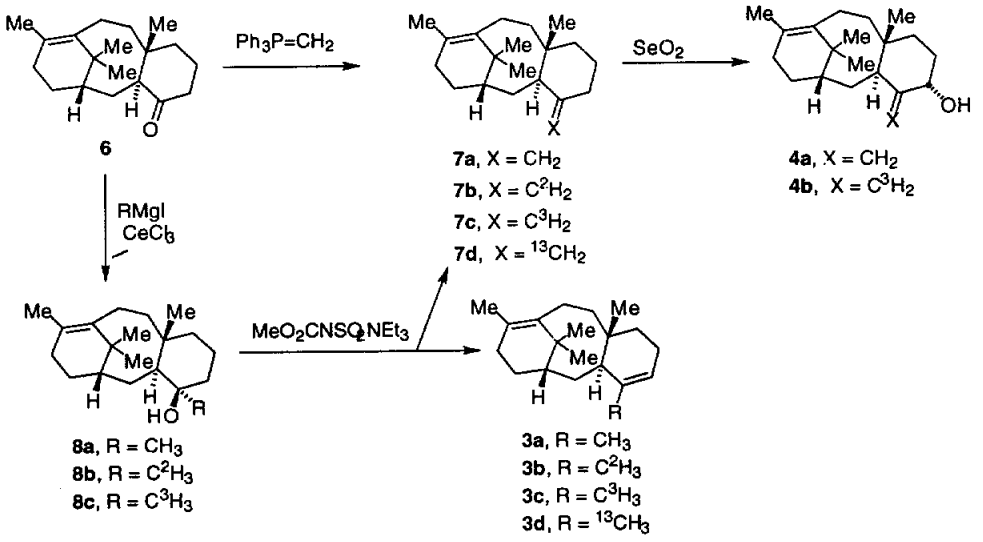
Scheme 1

RESULTS and DISCUSSION

We have previously reported the synthesis of the tricyclic ketone **6** (Scheme 2) which served as the key substrate from which all of the isotopomers described here were prepared.[11] For the preparation of taxa-4(5), 11(12)-diene (**3**), methyl Grignard addition furnishes the tertiary carbinol **8a** in high yield which, upon treatment with the Burgess reagent[12] gives a mixture of taxa-4(5), 11(12)-diene (**3a**), and taxa-4(20), 11(12)-diene (**7a**) which can be separated by argentation TLC. We applied this reaction to the synthesis of the deuterium- and tritium-labeled isotopomers in this series as shown in Scheme 2. In the unlabeled reaction, we previously reported a 1 : 1 ratio of **3a** : **7a** when **8a** was treated with the Burgess reagent.[11] However, when the same reaction was carried out on the deuterium-labeled substrate **8b**, we observed a 3 : 1 ratio of **3b** : **7b**. This interesting result merits some comment.

When acyclic alcohols having more than one kind of β-proton were dehydrated using the Burgess reagent, the more substituted (Saytzeff)

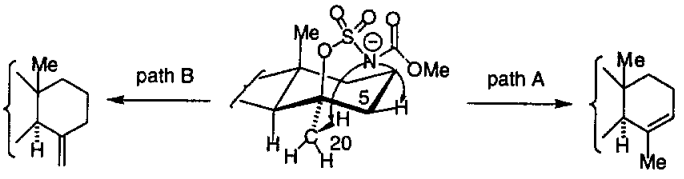
alkene was produced as the major product, assuming no rearrangements can take place. However, when 2-endo-methylbicyclo[2.21]heptan-2-ol was allowed to react with the Burgess reagent the Saytzeff product (tri-substituted *endo*-olefin) and Hofmann product (disubstituted *exo*-methylene) were produced in a 1:1 ratio.[12]



Scheme 2

The unexpected faster rate of formation of the disubstituted *exo*-methylene relative to the Saytzeff product was attributed to greater strain energy in the transition state for the formation of the tri-substituted *endo*-olefin. Also, it was hypothesized that the C-5 endo hydrogen sterically interfered with the ion pair transition state geometry necessary for the C-3 endo deprotonation thus leading to the tri-substituted *endo*-olefin. The same kind of rationale might also be used to explain the observed ratio of regioisomers **3a** : **7a** when **8a** is subjected to elimination with the Burgess reagent as outlined in Scheme 3. Thus, the Saytzeff product (**3**) was not formed in greater amounts because of the possible increase of strain energy in the transition state upon deprotonating the C-5 exo-hydrogen (path A)

relative to the C-20 hydrogen (path B). In addition, models suggest that in order for the C-5 deprotonation to occur the C-19 methyl group will sterically interfere with one of the lone pair orbitals on the C-4 oxygen of the incipient sulfamate ester anion while, no such steric interaction exists for the intramolecular C-20 hydrogen abstraction. The dramatic increase in the ratio of **3b** relative to **7b** is a manifestation of the sum of the primary deuterium isotope effect plus the square of the secondary deuterium isotope effect at the C-20 methyl residue. In the case of the tritium-labeled substrate **8c** which, is actually a mixture of tritium-labeled substrate **8c** and unlabeled substrate **8a**, we observed a 1.4 : 1 ratio of **3c** : **7c**.



Scheme 3

In practice, we initially found it convenient and economical to prepare both of the tritium-labeled taxoids **3c** and **4b** *via* the single radio-Grignard addition to **6** followed by dehydration to **3c** and **7c**. The mixture of **3c** and **7c** could be separated by argentation TLC to give analytically pure **3c** while **7c** proved difficult to separate from traces of **3c**. The crude **7c** was therefore carried on to **4b** where, the significant change in polarity allowed for a clean separation of **4b** from other reaction by-products. For the synthesis of pure ^{13}C-labeled **7d**, or, for a more direct synthesis of **4b**, the Wittig olefination as described for the preparation of **7a** was employed (see Experimental Section).

The versatility of ketone **6** for the synthesis of various isotopomers of the early natural taxoids **3** and **4** should be applicable to the synthesis of single- and doubly labeled substrates that will constitute useful probes for

studying the biosynthesis of taxol® and the mechanisms of the early stages of taxoid elaboration to the more highly oxygenated and biologically important taxoids. Multi-gram amounts of ketone **6** can be prepared by the route previously described[11] although shorter and more efficient routes to this substrate are currently being pursued in these laboratories.

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METHODS

Chemicals. CD_3MgBr (99+ atom %D) was purchased from Aldrich Chemical Co. Burgess reagent ($MeO_2CNSO_2NEt_3$) was prepared as described in reference 12. 10 mCi of tritiated methyl iodide was purchased from Amersham Co. [^{13}C]-Methyltriphenyl phosphonium iodide (99 Atom %) was purchased from Aldrich Chemical Co.

Analytical Methods. ^{1}H NMR and ^{13}C NMR spectra were obtained on the Bruker AC 300 MHz spectrometer. Chemical shifts are reported in parts per million from $CHCl_3$ as the internal standard. Infrared spectra were recorded on Perkin-Elmer 1600 Series FTIR and are reported as λ_{max} in cm^{-1}. Melting points were determined in open-ended capillary tubes on a Mel-Temp apparatus and are uncorrected. Elemental analyses were performed by MHW laboratories, Phoenix, Az, and are accurate to within the calculated values by ±0.4%. High-resolution mass spectra were carried out by UCR Mass Spectrometry Facility, Department of Chemistry, University of California at Irvine, Irvine, Ca. and CSU Mass Spectrometry Facility, Department of Chemistry at Colorado State University, Fort Collins, Co. Thin layer chromatography (TLC) was performed on 0.25-mm E. Merck precoated silica gel glass plates. Visualization on TLC was achieved with ultraviolet light, an I_2 developing chamber, and/or heating of TLC plates submerged in a 7%

solution of phosphomolybdic acid in 95% ethanol. The ^3H analysis was based on liquid scintillation counting using 10 mL of a cocktail solution consisting of 0.4% (w/v) omnifluor (Dupont/New England Nuclear) dissolved in 30% ethanol in toluene (^3H efficiency ~42%).

[20-C^2H_3]-Taxa-4(5), 11(12)-diene (3b) and [20-C^2H_2]-Taxa-4(20), 11(12)-diene (7b) - To a suspension of ketone **6** (76 mg, 0.28 mmol) in dry THF (1.4 mL) was added anhydrous $CeCl_3$ (205 mg, 0.83 mmol); the resulting mixture was allowed to stir for 2h. This solution was cooled to 0 °C and a 3.1 M solution of (99+ atom %D purchased from Aldrich) C^2H_3MgBr (0.83 mL, 0.83 mmol) was added dropwise. The reaction mixture was allowed to stir for 30 min. at 0 °C. The reaction was quenched with saturated ammonium chloride solution (15 mL). The layers were separated and the aqueous layer was extracted twice with Et_2O (15 mL). The organic layer was washed once with brine solution (20 mL), dried over anhydrous Na_2SO_4, and concentrated to give 65 mg (80%) of the tertiary alcohol **8b** as a pale yellow oil. The crude, oily product was directly used for the next step without further purification. An analytical sample was prepared by column chromatography (silica gel, 32:1, hexanes/EtOAc). IR (neat) 3468, 2927, 1455, 1381 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 2.68 (apparent dt, J = 14.8, 6.8 Hz, 1H), 2.35-2.19 (m, 1H), 2.12-1.95 (m, 3H), 1.89 (dd, J = 5.4, 2.5 Hz, 1H), 1.8 (ddd, J = 13.4, 3.8, 3.8 Hz, 1H), 1.75-1.55 (m, 7H), 1.62 (s, 3H), 1.55-1.21 (m, 4H), 1.32 (s, 3H), 1.2-1.1 (ddd, J = 12.3, 4.9, 2.79 Hz, 1H), 1.0 (s, 3H), 0.95 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 138.3, 128.9, 43.5, 42.9, 41.4, 41.2, 39.4, 39.0, 38.9, 31.7, 29.7, 26.8, 25.5, 24.8, 24.4, 22.2, 21.5, 19.0. HRMS Calcd for $C_{20}H_{31}D_3O$: 293.2844, found: 293.2784.

To a 0.02M solution of alcohol **8b** (65 mg, 0.22 mmol) prepared as described above in toluene at reflux temperature was added Burgess reagent ($MeO_2CNSO_2NEt_3$) (106 mg, 0.44 mmol) and the resulting mixture was allowed to stir at reflux temperature for 10 min. The mixture was cooled to 25 °C and diluted with EtOAc (45 mL). The organic layer was washed once with brine (45 mL), dried over anhydrous Na_2SO_4, and concentrated under

reduced pressure. The crude oil was purified by using $AgNO_3$ impregnated silica gel and a 80:1, 40:1 (hexanes/ Et_2O) gradient elution to give 36 mg (60%) of [20-C^2H_3]-taxa-4(5),11(12)-diene (**3b**) and 12 mg (20%) of [20-C^2H_2]-taxa-4(20), 11(12)-diene (**7b**). Data for [C-20-C^2H_3]-taxa-4(5), 11(12)-diene (**3b**): IR (Neat) 2922, 1458, 1375 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 5.27 (m, 1H), 2.6 (ddd, J = 14.8, 10.2, 5.4 Hz, 1H), 2.5 (m, 1H), 2.38-2.21 (m, 1H), 2.2-1.95 (m, 3H), 1.95-1.48 (m, 8H), 1.64 (s, 3H), 1.4 (ddd, J = 11.8, 6.4, 6.4 Hz, 1H), 1.31 (s, 3H), 1.18 (dd, J = 12.6, 6.0 Hz, 1H), 1.0 (s, 3H), 0.8 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 138.5, 137.7, 129.6, 121.1, 44.3, 41.4, 39.7, 39.0, 38.5, 37.3, 30.7, 29.8, 28.4, 26.3, 24.5, 23.2, 22.6, 21.6, 21.5. HRMS Calcd for $C_{20}H_{27}D_3$: 275.2689, found: 275.2686. Due to difficulties in the separation of **3b** from **7b**, it was not possible to obtain an analytically pure sample of **7b**. Data for the unlabeled material have been reported previously.[11]

[20-$^3H_3$]-Taxa-4(5), 11(12)-diene (3c) and [20-$^3H_2$]-Taxa-4(20), 11(12)-diene (7c) - To a suspension of magnesium (29 mg, 1.2 mmol) in dry Et_2O (6 mL) was added a catalytic amount of iodine followed by methyl iodide (65 mL, 1 mmol) diluted with 10 mCi of tritiated methyl iodide (obtained from Amersham). The resulting solution was refluxed for 1h. To a 0.1M solution of ketone **6** (146 mg, 0.53 mmol) in THF was added anhydrous $CeCl_3$ (197 mg, 0.79 mmol); the resulting mixture was allowed to stir for 2.5 hr. This solution was cooled to 0 °C and the freshly made C^3H_3MgI was added dropwise. The reaction mixture was allowed to stir for 1 h at 0 °C. The reaction was quenched with saturated ammonium chloride solution (30 mL). The layers were separated and the aqueous layer was extracted twice with Et_2O (30 mL). The organic layer was washed once with brine solution (30 mL), dried over anhydrous Na_2SO_4, and concentrated to give 110 mg (71%) of the alcohol **8c**, with a calculated specific activity of 6.8 mCi/mmol. as a pale yellow oil. The crude, oily product was directly used for the next step without further purification.

To a 0.02M solution of alcohol **8c** (110 mg, 0.38 mmol) obtained as described above in toluene at reflux temperature was added Burgess reagent ($MeO_2CNSO_2NEt_3$) (181 mg, 0.76 mmol) and the resulting mixture was allowed to stir at reflux temperature for 10 min. The mixture was cooled to 25 °C and diluted with EtOAc (45 mL). The organic layer was washed once with brine (45 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude oil was purified by using $AgNO_3$ impregnated silica gel and a 80:1, 40:1 (hexanes/ Et_2O) gradient elution to give 57 mg (55%) (R_f = 0.21 (50:1, hexanes/Et_2O), of [20-$^3H_3$]-taxa-4(5),11(12)-diene (**3c**), with a calculated specific activity of 4.48 mCi/mmol, and 40 mg (39%) (R_f = 0.13 (50:1(hexanes/Et_2O), of [20-$^3H_2$]-taxa-4(20) ,11(12)-diene (**7c**); this material was contaminated with a small amount of **3c**.

[20-$^3H_2$]-Taxa-4(20), 11(12)-diene (7c) prepared directly from ketone 6 - To 315 mg of triphenylphosphine (1.20 mmol) a solution of 10 mCi of C^3H_3I (obtained from Amersham) in 1 mL of toluene was added, followed by 20 μL of unlabeled methyl iodide (170 mg, 1.20 mmol). 7 hours later, an additional 54.7 μL of unlabeled methyl iodide (which made a total of 74.7 μL, 170 mg, 1.20 mmol of unlabeled compound) was added. After stirring at room temperature for 16h more, the white precipitate that formed was decanted using a syringe, washed with 5 mL of absolute toluene in 1 mL portions, and dried under vacuum to give 394 mg of [^{3}H]-methyltriphenylphosphonium iodide (0.975 mmol, 81% yield). This salt was dissolved in 2.5 mL of dry THF, and 0.98 mL of a 1M solution of sodium bis(trimethylsilyl)amide in THF (0.98 mmol) was added. The resulting solution was taken to reflux for 1 h, and then cooled to room temperature. Then, a solution of 162 mg of ketone **6** (0.604 mmol) in dry THF (2 mL) was added to the phosphorus ylid solution, and the reaction mixture was taken to reflux for 18 h. The reaction was cooled to 25 °C and saturated aqueous NH_4Cl solution (15 mL) followed by hexanes (15 mL) were added. The phases were separated and the aqueous layer was extracted twice with hexanes (15 mL). The organic layer was

washed once with brine (15 mL), dried over Na_2SO_4, and concentrated. The crude oil was purified through column chromatography on silica gel (100 % hexanes) to give 160 mg (99.6% yield) of pure [20-$^{13}CH_2$]-taxa-4(20) ,11(12)-diene (**7d**) as a clear colorless oil that was used in the following reaction.

[20-C^3H_2]-Taxa-4(20), 11(12)-diene-5-α-ol (4b) - To a solution of SeO_2 (33.4 mg, 0.301 mmol) in CH_2Cl_2 (500 µl) was added *tert*-BuOOH (134 µL of 90% purity, 1.203 mmol) and the resulting mixture was allowed to stir for 0.5 h. To this solution was added a solution of [20-C^3H_2]-taxa-4(20) ,11(12)-diene (**7c**) (160 mg, 0.602 mmol) prepared as described above in CH_2Cl_2 (2 mL) and the mixture was allowed to stir for 6 h at room temperature. The solvent was removed under reduced pressure and the resulting crude oil was purified through column chromatography on silica gel. Hexanes eluted unreacted starting material (21.5 mg), while a mixture of hexanes-Et_2O 5:1 eluted 65.7 mg of [20-C^3H_2]-taxa-4(20) ,11(12)-diene -5α-ol (**4b**) (39% yield; 45% yield based on recovered starting material), with a specific activity of 8.03 mCi/mmol, as a colorless oil. This compound was stored as a solution, frozen in benzene at -80 °C.

[20-$^{13}CH_2$]-Taxa-4(20), 11(12)-diene (7d) - To a 0.07M solution of [^{13}C]-methyltriphenyl phosphonium iodide (99 Atom % purchased from Aldrich) (115 mg, 0.29 mmol) in THF at 25 °C was added a 1.4M solution of *n*-BuLi (0.2 mL, 0.29 mmol) in hexanes and the mixture was allowed to reflux for 30 min. To this solution was added a 0.04 M solution of ketone **6** (39 mg, 0.14 mmol) in THF and the mixture was allowed to reflux for 18.5 h. The reaction was cooled to 25 °C and saturated aqueous NH_4Cl solution (15 mL) followed by hexanes (15 mL) were added. The phases were separated and the aqueous layer was extracted twice with hexanes (15 mL). The organic layer was washed once with brine (15 mL), dried over Na_2SO_4, and concentrated. The crude oil was purified by preparative TLC (100 % hexanes) to give 27 mg (80% based on recovered starting material) of [20-$^{13}CH_2$]-taxa-4(20) ,11(12)-diene (**7d**) as a clear colorless oil. IR (Neat) 2928, 1620, 1457, 1376, 872 cm^{-1}.

^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.7 (dd, J = 154, 1.3 Hz, 1H), 4.5 (d, J = 154 Hz, 1H), 2.76 (m, 1H), 2.6 (br s, 1H), 2.4-2.2 (m, 2H), 2.1-1.7 (m, 6H), 1.75-1.69 (m, 1H), 1.75 (s, 3H), 1.65-1.5 (m, 4H), 1.3 (s, 3H), 1.3-1.1 (m, 3H), 1.1 (s, 3H), 0.6 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 153.8 (d, J = 73.5 Hz), 137.9, 129.7, 105.2, 43.7, 42.6, 40.24, 40.21, 39.4, 38.4 (d, J = 2.4 Hz), 38.0, 30.8, 30.2, 28.9 (d, J = 3.5 Hz), 25.5, 24.8, 24.1 (d, J = 1.9 Hz), 22.9, 22.7, 22.0.

[20-$^{13}CH_3$]-taxa-4(5), 11(12)-diene (3d). The same procedures were followed as described for the preparation of the tritium-labeled taxadienes except ^{13}C methyl iodide (99 Atom% purchased from Aldrich) was used instead of ^{3}H methyl iodide. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 138.5 (d, J = 45.5 Hz), 137.7, 129.6, 121.1, 44.3, 41.4, 39.8, 39.0, 38.5, 37.3, 30.7, 29.8, 28.4, 26.3, 24.5, 24.0, 23.3, 22.6, 21.7, 21.5.

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