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Studies on Taxol® biosynthesis. Preparation and tritium labeling of biosynthetic intermediates by deoxygenation of a taxadiene tetra-acetate obtained from Japanese yew

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Taxa-4(20),11(12)-diene-5α-acetate 5 and taxa-4(20),11(12)-diene-5α-acetate, 10β-ol 6, have been identified as early stage intermediates involved in the biosynthesis of Taxol® (paclitaxel). Tritium-labeled 5 and 6 were successfully prepared by Barton deoxygenation using tri-*n*-butyltintritiide of the C-14-hydroxyl group of a taxoid obtained from Japanese yew.

Keywords: Taxol®; biosynthesis; Japanese yew; tritiation; tritium; deoxygenation

Introduction

Taxol® (**1**), first isolated from the bark of the Pacific yew tree *Taxus brevifolia* Nutt.,[1] is a powerful therapeutic drug for cancer chemotherapy.[2] It exhibits remarkably high cytotoxicity and strong antitumor activity against a range of cancer cell lines that are resistant to existing anticancer drugs.[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. Alternative sources of the drug have therefore become an important objective. A method that has proven viable is the semi-synthesis of Taxol® from 10-deacetylbaccatin III that can be isolated from the needles, a renewable source, of the European yew, *T. baccata*.[4] This has attenuated this problem somewhat, but as the drug becomes more widely adopted, particularly for use earlier in the course of cancer intervention and for new therapeutic applications, pressure on the yew population is likely to increase worldwide. Alternative means of Taxol® production are therefore being vigorously pursued since cost and availability will continue to be significant issues. The commercial supply of Taxol® and semi-synthetic precursors will have to increasingly rely on biological methods of production: (1) extraction from intact *Taxus* plants, (2) *Taxus* cell culture,[5] or (3) genetically engineered microbial systems.[6] In order to produce large quantities of Taxol® or a pharmacophoric equivalent by semi-synthetic or, perhaps, by genetically engineered biosynthetic methods, it is essential to gain a better understanding of the detailed biosynthetic pathways in *T. brevifolia* and other related Taxol®-producing species.

We have shown that the early steps in Taxol® biosynthesis involve: (1) the cyclization of geranylgeranyl pyrophosphate (**2**) to afford taxa-4(5),11(12)-diene (**3**) catalyzed by taxadiene synthase[7]; (2) the cytochrome P450 hydroxylation of taxa-4(5),11(12)-diene (**3**) to taxa-4(20),11(12)-diene-5α-ol (**4**) catalyzed by taxadiene hydroxylase[8]; (3) the acetylation of **4** to taxa-4(20),11(12)-diene-5α-acetate (**5**) catalyzed by taxadienol acetyltransferase[9]; (4) the cytochrome P450 hydroxylation of **5** to taxa-4(20),11(12)-diene-5α-acetate-10β-ol (**6**) catalyzed by a 10β-hydroxylase followed by a series of complex and as yet not fully defined oxygenations (Scheme 1).[10,11]

Owing to the very low yield of the intermediate metabolites that may be obtained from natural sources, we have relied heavily on synthetic **3**,[12] **4**,[8] **5**,[8] and **6**[13] as substrates from which *in vivo* and *in vitro* bioconversion strategies have been utilized to identify lightly oxygenated taxoids downstream of these substances. In this paper, we report the procedure for the preparation of radioisotopically labeled **5** and **6** that are useful substrates to probe further intermediate steps in the biosynthesis of Taxol®.

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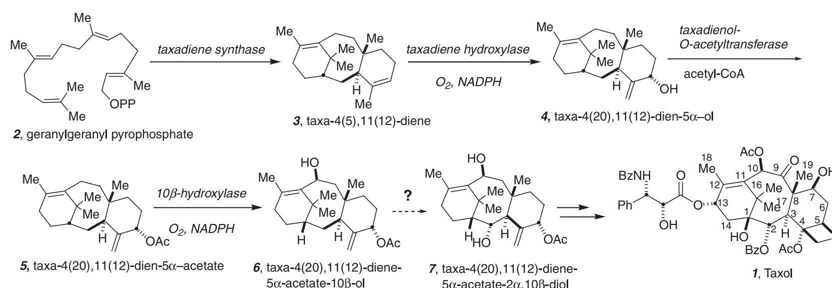
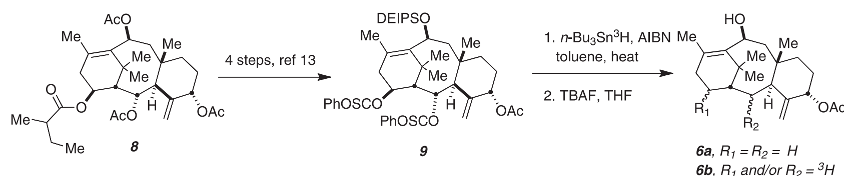
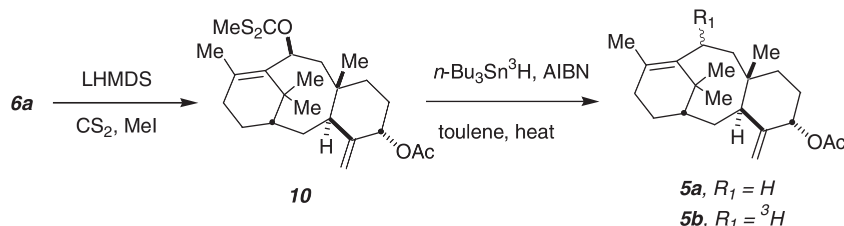


Scheme 1. Early stages of Taxol® biosynthesis.



Scheme 2. Synthesis of tritium-labeled **6**.



Scheme 3. Preparation of tritium-labeled **5**.

Results and discussion

We have previously reported the preparation of taxa-4(20),11(12)-diene-5α-acetate-10β-ol (**6**) by Barton deoxygenation of the C-14-hydroxyl group of a differentially protected derivative of natural 2α, 5α, 10β-triacetoxy-14β-(2-methyl)-butyryloxytaxa-4(20),11(12)-diene (**8**), a major taxoid metabolite isolated from Japanese yew heartwood.[13a] The synthetic protocol devised was successfully optimized and applied to the preparation of ^{3}H-labeled **6** (1.49 mCi, 0.75% radiochemical yield) by the treatment of **9** with a mixture of *n*-Bu_3Sn^3H (0.0056–0.11 eq. 50 mCi) and *n*-Bu_3SnH (2.0 eq.) (Scheme 2).

Next, the conversion of **6a** to **5** was investigated as illustrated in Scheme 3. Attempted acylation of the 10β-hydroxy group of **6a** by treatment with PhOCSCl and various bases including triethylamine, 2,6-lutidine, and lithium hexamethyldisilazide (LHMDS) proved unsuccessful. Fortunately, the conversion of **6a** to the corresponding xanthate ester (**10**) proceeded in a good yield (68%) and a Barton deoxygenation of **10** with a mixture of *n*-Bu_3Sn^3H (0.064–0.13 eq. 50 mCi) and *n*-Bu_3SnH (1.0 eq.) successfully gave the desired isotopically labeled substrate **5** (0.53 mCi, 0.27% as radiochemical yield) as the sole product.

In summary, we have successfully prepared selectively tritium-labeled taxoids **5** and **6**. Despite the relatively low radiochemical yields, the specific activities of these substrates are more than sufficient for conducting many bioconversion experiments. Utilization of these labeled taxoids is presently being explored to probe downstream steps in the biosynthesis of Taxol® and will be reported on in due course.

Experimental

Materials and methods

n-Bu_3Sn^3H (100 mCi in 100 μl hexane, 15–30 Ci/mmol 0.00333–0.00666 mmol) was purchased from American Radiolabeled Chemicals, Inc. Other chemicals were purchased from commercial suppliers. The identity of intermediates and precursors was identified by comparison with unlabeled, authentic standards either by thin layer chromatography (TLC) and/or spectroscopic methods. Since the tritiation by *n*-Bu_3Sn^3H produces an unknown amount of tritium gas, the head space of two reaction flasks for the two Barton deoxygenation runs was flushed gently with nitrogen gas through a series of five traps made of Erlenmeyer flasks containing excess cinnamic acid, Pd/Carbon in

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100 ml of ethanol at 0°C. A stopcock was attached between the second reaction flask and the first Erlenmeyer flask; in addition, a balloon was attached at the end of the series of traps, as a safety precaution. The reactions were run for 3 h, after which the nitrogen gas flow was stopped and the stopcock was shut. The mixtures in the trap flasks were stirred at room temperature (rt) for 12 h and any evolved tritium gas was trapped by catalytic hydrogenation of cinnamic acid. The gross activity of tritium gas trapped for two runs of Barton deoxygenation was calculated as 0.76 mCi.

*[2α, 14β-^{3}H]-5α-acetoxy-10β-hydroxytaxa-4(20),11(12)-diene (**6b**)*

To a solution of **9** (23.3 mg, 0.0299 mmol) in toluene (1 ml), azobis-isobutyronitrile (AIBN) (cat.), *n*-Bu_3SnH (16 μl, 0.0598 mmol), and *n*-Bu_3Sn^3H (50 mCi, 0.00167–0.00333 mmol in 50 μl hexane) were added. The mixture was stirred at reflux temperature for 3 h. The mixture was condensed and purified by chromatography (hexane 2 ml, hexane/EtOAc 15:1, 15 ml) yielding a crude compound (10.7 mg, 0.0225 mmol, 75%). The crude material was analyzed by TLC and immediately used for the next reaction without spectroscopic identification.

To a solution of the crude product obtained above (10.7 mg, 0.0225 mmol,) in tetrahydrofuran (THF) (1 ml) were added tetrabutylammonium fluoride (25 μL, 0.25 mmol as 1 M THF) and AcOH (1 drop). The mixture was stirred for 24 h. The mixture was diluted with EtOAc (20 ml) and washed with NH_4Claq (10 ml) and brine (5 ml). The EtOAc layer was dried over anhydrous Na_2SO_4, filtered, and the organic layer was condensed and purified by silica gel chromatography (hexane/EtOAc 4:1, 15 ml) yielding ^{3}H-labeled **6b** (7.2 mg, 0.0208 mmol, 92%) with a calculated activity of 72 mCi/mmol. $[\alpha]_D^{20} = +62$ (*c* 0.0019, $CHCl_3$); IR (NaCl): 3420, 2932, 1734, 1647, 1448, 1369, 1240, 1200, 1140, 1106, 1000, 959, 944, 893, 756; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.48 (1H, dd, *J* = 2.4, 2.9 Hz, H-5), 5.88 (1H, s, H-20), 4.93 (1H, dd, *J* = 5.4, 11.7 Hz, H-10), 4.69 (1H, s, H-20), 2.87 (1H, d, *J* = 5.4 Hz, H-3), 2.29 (1H, ddd, *J* = 4.4, 12.2, 18.6 Hz, H-13), 2.23 (1H, dd, *J* = 11.7, 14.7 Hz, H-9), 1.99 (1H, ddt, *J* = 2.9, 9.3, 14.6 Hz, H-14), 1.88 (1H, dt, *J* = 4.4, 13.7 Hz, H-7), 1.84 (1H, ddd, *J* = 2.9, 10.3, 18.6 Hz, H-13), 1.73 (3H, s, Me-18), 1.71 (3H, s, Ac), 1.66 (2H, m, H-6), 1.62 (1H, m, H-1), 1.60 (3H, s, Me-17), 1.56 (1H, m, H-2), 1.48 (1H, dd, *J* = 3.9, 11.7 Hz, H-2), 1.44 (1H, dd, *J* = 5.9, 14.6 Hz, H-9), 1.28 (1H, ddd, *J* = 4.9, 10.3, 14.2 Hz, H-14), 1.16 (3H, s, Me-16), 0.93 (1H, ddd, *J* = 1.5, 4.4, 12.7 Hz, H-7), 0.80 (1H, br. OH), 0.59 (3H, s, Me-19); ^{13}C NMR (100 MHz, C_6D_6) δ: 169.20 (Ac), 151.36 (C4), 140.19 (C11), 133.73 (C12), 112.36 (C20), 76.76 (C5), 68.11 (C10), 48.53 (C9), 44.83 (C1), 39.42 (C8), 38.84 (C15), 36.80 (C3), 34.60 (C7), 32.74 (C16), 31.21 (C13), 29.02 (C2), 28.86 (C6), 26.27 (C17), 23.61 (C14), 22.30 (C19), 21.74 (Ac), 21.59 (C18).

*Taxa-4(20),11(12)-diene-10β-S-methyl dithiocarboxy-5α-acetate (**10**)*

To a solution of **6a** (59.4 mg, 0.171 mmol) in THF (2 ml), LHMDS (188 μl, 0.188 mmol, as 1 M THF solution) was added at 0°C. After being stirred for 10 min, CS_2 (0.25 ml) was added and the mixture was stirred for 2 h at rt. Then MeI (13 μl, 0.21 mmol) was added and the mixture was stirred for 24 h at rt. The mixture was diluted into CH_2Cl_2 (15 ml) and washed with water. The organic layer was dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by chromatography ($CHCl_3$/MeOH 20:1) yielding **10** (50.7 mg, 0.116 mmol, 68%).

$[\alpha]_D^{20} = +2.0$ (*c* 1.9, $CHCl_3$); ^{1}H NMR (400 MHz, CD_3CN) δ: 5.40 (dd, *J* = 4.7, 13.2 Hz, 1H, H-10), 5.27 (dd, *J* = 2.8, 3.0 Hz, 1H, H-5), 5.08 (s, 1H, H-20), 4.79 (s, 1H, H-20), 2.93 (d, *J* = 5.8 Hz, 1H, H-3), 2.41 (s, 3H, $MeCS_2$), 2.45–2.36 (m, 2H, H-9, H-13), 2.18 (m, 1H, H-7), 2.06 (m, 1H, H-14), 2.04 (s, 3H, MeCO), 1.99 (s, 3H, Me-18), 1.96 (m, 1H, H-13), 1.82–1.70 (m, 3H, H-1, H-6), 1.68 (ddd, *J* = 2.3, 6.0, 15.8 Hz, 1H, H-2), 1.60 (ddd, *J* = 1.9, 4.9, 15.8 Hz, 1H, H-2), 1.54 (dd, *J* = 4.7, 14.9 Hz, H-9), 1.39 (s, 3H, Me-16), 1.20 (m, 2H, H-7, H-14), 1.05 (s, 3H, Me-17), 0.68 (ddd, *J* = 1.9, 4.9, 13.2 Hz, 1H, H-7), 0.59 (s, 3H, Me-19); ^{13}C NMR (100 MHz, CD_3CN) δ: 190.87 ($\underline{C}S_2Me$), 170.96 (Me$\underline{C}$O), 152.13 (C4), 137. 21 (C11), 136.90 (C12), 112.92 (C20), 77.44 (C5), 48.68 (C9), 45.59 (C1), 44.33 (C15), 41.63 (C8), 40.50 (C10), 37.90 (C3), 34.59 (C7), 32.29 (C17), 31.82 (C13), 29.54 (C2), 29.19 (C6), 27.04 (C16), 23.91 (C14), 22.47 ($\underline{Me}$CO), 22.22 (C19 and C18), 13.84 ($CS_2\underline{Me}$); HR-FABMAS (M^+) calculated for $C_{24}H_{36}O_3S_2$ 436.2096, found 436.2106.

*[10-^{3}H]-taxa-4(20),11(12)-diene-5α-acetate (**5b**)*

To a solution of **10** (11.3 mg, 0.0259 mmol) in toluene (1 ml), AIBN (cat.), *n*-Bu_3SnH (7 μl, 0.025 mmol), and *n*-Bu_3Sn^3H (50 mCi, 0.00167–0.00333 mmol in 50 μl hexane) were added. The mixture was stirred at reflux temperature for 3 h. The mixture was condensed and purified by chromatography (hexane/EtOAc 25:1, 26 ml) yielding **5b** (1.9 mg, 0.0057 mmol, 22%) with a calculated activity of 93 mCi/mmol.

$[\alpha]_D^{20} = +71$ (*c* 1.1, $CHCl_3$); ^{1}H NMR (400 MHz, C_6D_6) δ: 5.52 (dd, *J* = 2.8, 3.0 Hz, 1H, H-5), 5.10 (s, 1H, H-20), 4.71 (s, 1H, H-20), 3.17 (m, 1H, H-3), 2.79 (dt, *J* = 5.3, 13.6 Hz, 1H, H-10), 2.35 (m, 1H, H-13), 2.10 (dt, *J* = 4.9, 13.4 Hz, 1H, H-7), 2.08 (m, 1H, H-10), 2.00 (m, 1H, H-14), 1.96 (m, 1H, H-9), 1.87 (m, 1H, H-13), 1.81 (s, 3H, Me-18), 1.75 (s, 3H, MeCO), 1.75 (m, 1H, H-6), 1.65 (m, 2H, H-6, H-1), 1.54 (dd, *J* = 3.6, 4.0 Hz, 2H, H-2), 1.31 (s, 3H, Me-16), 1.31 (m, 1H, H-14), 1.12 (s, 3H, Me-17), 1.11 (m, 1H, H-9), 0.87 (ddd, *J* = 1.9, 4.9, 13.2 Hz, 1H, H-7), 0.59 (s, 3H, Me-19); ^{13}C NMR (100 MHz, C_6D_6) δ: 169.33 (Ac), 151.47 (C4), 137. 75 (C11), 130.50 (C12), 111.91 (C20), 76.83 (C5), 44.19 (C1), 40.71 (C9), 40.19 (C8), 39.79 (C15), 37.73 (C3), 33.94 (C7), 31.39 (C16), 30.95 (C13), 28.97 (C6), 28.79 (C2), 25.96 (C17), 25.47 (C10), 23.50 (C14), 22.79 (C19), 21.81 (Ac), 21.77 (C18); HR-FABMAS (M^+) calculated for $C_{22}H_{34}O_2$ 330.2549, found 330.2559.

Note: We observed that the radiochemical yields in these experiments were unexpectedly low. The gross activity of tritium gas trapped for two kinds of Barton deoxygenation protocols (see Experimental; **9** to **6b** and **10** to **5b**) was calculated as 0.76 mCi, which is 0.76% of the activity of the 100 mCi *n*-Bu_3Sn^3H reagent employed. The gross radiochemical activity for the liquid waste was determined to be 26 mCi and the remainder of the theoretical 71 mCi resided in the solid waste. The reason for the low radiochemical yields may be rationalized by the slower reaction rate of the Sn–T bond cleavage relative to that of Sn–H due to a primary kinetic isotopic effect.

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[3] Paclitaxel is the generic name for Taxol®, a registered trademark of Bristol-Myers Squibb; because of its greater familiarity, the term Taxol® is used herein throughout.

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