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Synthesis and in vitro evaluation of taxol oxetane ring D precursors

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ABSTRACT

A series of potential taxoid substrates was prepared in radiolabeled form to probe in vitro for the oxirane formation step and subsequent ring expansion step to the oxetane (ring D) presumably involved in the biosynthesis of the anticancer agent Taxol. None of the taxoid test substrates underwent transformation in cell-free systems from *Taxus* suggesting that these surrogates bore substitution patterns inappropriate for recognition or catalysis by the target enzymes, or that taxoid oxiranes and oxetanes arise by independent biosynthetic pathways.

Taxol is an established antineoplastic agent that represents a platform for the development of new, second generation drugs for the treatment of cancers and other diseases.[1,2] The supply of Taxol and its immediate precursors, by isolation from *Taxus* tissues[3] or cell cultures,[4] is expected to be replaced by synthetic biology approaches[5,6] necessitating knowledge of the underlying biosynthetic pathway. Whereas many of the biosynthetic steps *en route* to Taxol have been characterized,[7] the biochemistry of formation of the oxetane D-ring is still uncertain.

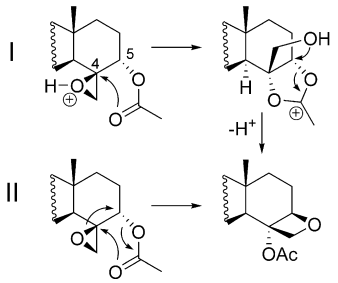
The complexity of the Taxol biosynthetic pathway has impeded the determination of the timing of oxetane ring formation. Within the proposed 19 distinct enzymatic steps leading to Taxol, oxetane ring formation is anticipated to occur in mid-pathway, presumably following the formation of an acetylated taxadien-2,5,7,9,10,13-hexaol but before the formation of baccatin III, to which the C13-side chain is appended in three steps to complete the pathway to Taxol.[7,8]

Theoretically feasible reaction mechanisms to account for the formation of the Taxol oxetane ring D have been proposed by several groups.[9–14] Based on an evaluation of known structures of naturally occurring taxoid derivatives, a simple and plausible mechanism leading to the oxetane D-ring of Taxol was first proposed by Potier and co-workers.[12] The co-occurrence of epoxy and oxetanyl ester taxoids in *Taxus* species led the authors to propose an enzyme-mediated acid-catalyzed epoxyester/oxetaneester rearrangement mechanism involving protonation of a proposed β4,20-epoxide intermediate, backside attack of a C5-acetate moiety onto C4, and rearrangement to the expanded oxetane ring with the formal migration of the secondary 5α-acetoxy group to the tertiary C4α position of the taxane core (Scheme 1, I).

This mechanism is thought to proceed via a reactive 1,3-dioxolan-2-ylium cation formed by acetate-assisted opening of the protonated β4,20-oxirane ring,[15] but it could also be formulated as a concerted reaction[7] (Scheme 1, II).

From a biochemical perspective, the intermediate 4β,20-function (e.g., **4**, Scheme 2) could be formed from the corresponding double bond by a cytochrome P450 oxygenase or a flavin-dependent monooxygenase, since double bond epoxidations involving these enzyme types have been observed previously.[16,17] The subsequent ring expansion from epoxide to oxetane with acetate migration could involve a transferase-type enzyme or a mutase of unknown type; this transformation might also be mediated by a cytochrome P450 oxygenase, as somewhat related rearrangements to cyclic ethers catalyzed by P450-enzymes have been recently reported.[18,19]

To explore the enzymology of the presumed epoxidation and oxetane formation reactions in vitro, a series of accessible substrate surrogates was synthesized in radiolabeled form (Scheme 2). Although the precise nature (oxidation and acylation state) of the taxoid substrates involved in these reactions are yet to be



Scheme 1. Proposed pathway to taxol oxetane ring D.

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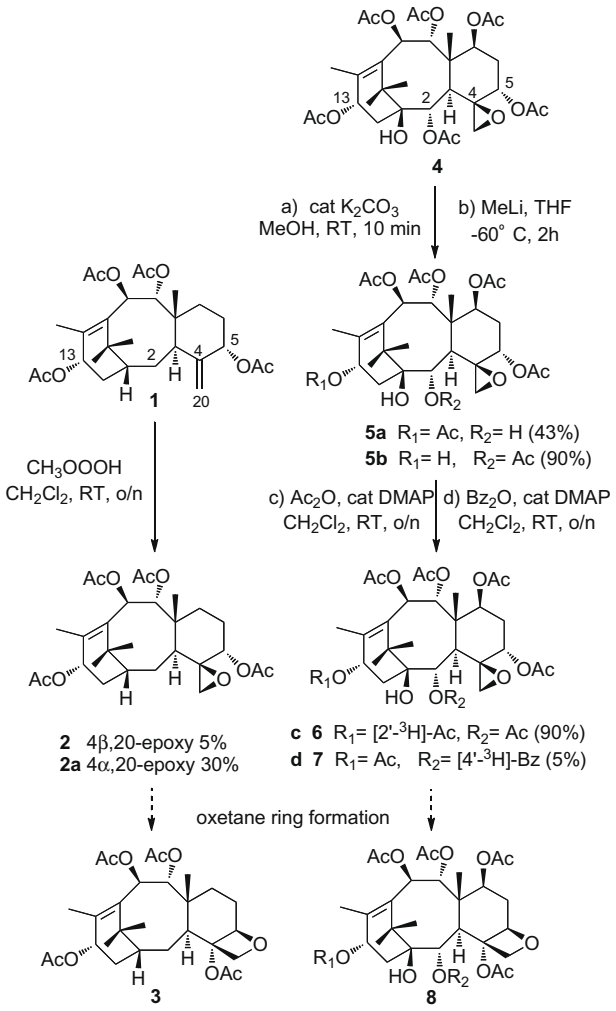

Scheme 2. Synthesis of radiolabeled surrogates.

discovered, we reasoned that allylic ester **1** could serve as a probe to study the initial oxirane ring formation, and the possibility that the resulting epoxide **2** might be processed further to oxetanyl taxoid **3** by subsequent ester-assisted ring expansion. In addition, the epoxy ester surrogates **6** and **7**, displaying a more advance oxidation–acylation state, were thought to be good candidates to test the ester-assisted ring expansion reaction outlined in Scheme 1, since oxetanyl taxoids with similar functionalities are known in nature.[20] Exhaustive literature searches to evaluate the relative abundances of the several hundred defined taxoids from *Taxus* species,[20–23] in combination with a wide range of biochemical studies,[7,9,11] have shown that none of the known 4β,20-epoxy taxoids bears a benzoate ester at the C2 position (C2-acetates are common), whereas over three quarters of the characterized oxetane derivatives do bear a benzoate group at the C2 position.[20,21] Based on these structural observations, it is tempting to suggest that 4β,20-epoxy taxoids α-benzoylated at C2 (such as synthesized **7**) might be transient intermediates of oxetane ring formation.

Labeled taxusin **1** was prepared by regioselective deacetylation at C13 of **1** using MeLi,[24] followed by re-acetylation with tritiated Ac_2O.[25] Taxusin-β4,20-epoxide **2** was obtained in low yield by peracetic acid epoxidation.[26] Commercially available 1-hydroxy baccatin I **4** was prepared, via diol **5b**, in radiolabeled form (**6**) following the C13 deacetylation[24]/reacetylation[25] procedure described for allylic alcohol **1**. Benzoate **7** was synthesized by regioselective hydrolysis (K_2CO_3/MeOH)[27] at C2 of **4** affording diol **5a**,[28] followed by benzoylation of the latter with Bz_2O.[29] After structural confirmation of the unlabeled compounds, the syntheses of **2** and **7** were carried out with tritiated Ac_2O and Bz_2O, respectively.

Surrogates (**1**, **2**, **6**, and **7**) were evaluated as potential substrates for the proposed oxirane and oxetane ring formation reactions in crude, soluble, and membranous enzyme preparations from *Taxus* cells under a broad range of redox-type[17,28] and transferase-type[29,30] reaction conditions. To rule out any possible artefacts from non-enzymatic reactions, incubations of compounds **2**, **6**, and **7** were initially conducted without or with boiled *Taxus* cell preparations; both experiments revealed that these epoxy ester surrogates were stable under incubation conditions. Since the oxetane (ring D) formation step en route to Taxol is thought to occur at mid biosynthetic pathway,[7,8] the *Taxus* cell-free preparations were tested for taxoid hydroxylase[30] and taxoid acetyl transferase[31] activities, confirming their catalytic competency for these early and intermediate pathway transformations. Radio-HPLC or HPLC–MS analysis of the reaction mixtures using authentic standards (e.g., baccatin IV and VI, **8**) showed that none of the surrogates employed in the present study was converted to the expected epoxide or oxetane derivatives, hence demonstrating that compounds **1**, **2**, **6**, and **7** did not act as functional substrates under the conditions used herein.

In contrast to previous Taxol biosynthetic studies, in which surrogate substrates were of value in defining the target reaction,[32,33] the present case did not yield useful information regarding precursors of taxoid oxiranyl or oxetanyl esters. These negative results suggest that the presumed epoxidation (**1**→**2**) and oxetane formation (**2**→**3** and **6**/**7**→**8**) reactions may require different conditions, or substrates with different substitution patterns to permit recognition and catalysis by the relevant enzymes. Alternatively, these results may indicate that 4β,20-epoxy ester and oxetanyl esters represent two distinct types of advanced taxoids formed by separate biosynthetic routes.

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Supplementary data

Supplementary data (additional NMR data and methods for enzyme preparation) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.02.033.

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26. *Compound* **2**: Peracetic acid (10 μL, 50 μmol) was added to radiolabeled **1** (5 mg, 10 μmol) dissolved in dry CH_2Cl_2 (1 mL). The reaction mixture was stirred over night at 6–10 °C. Purification by LC gave epoxide **2** (250 μg, 5%). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 6.02 (1H, d, J = 10.69 Hz, H-10), 5.83 (1H, d, J = 10.69 Hz, H-9), 5.81 (1H, m, overlapping H-13), 4.38 (1H, t, J = 2.57 Hz, H-5), 2.79 (1H, dd, J = 4.92 Hz, 2.13 Hz, H-3), 2.72 (1H, d, J = 3.86 Hz, H-20a), 2.77–2.68 (1H, m, H-14β), 2.42 (1H, d, J = 3.86 Hz, H-20b), 2.16 (3H, s, C(O)CH_3 at C5), 2.09 (3H, d, J = 0.95 Hz, CH_3-18), 2.08 (3H, s, C(O)CH_3 at C13), 2.06 (3H, s, C(O)CH_3 at C9), 2.00 (3H, s, C(O)CH_3 at C10), 1.95 (1H, m, H-6β), 1.84 (1H, m, H-7β), 1.76 (1H, m, H-1), 1.74 (1H, m, H-6α), 1.71 (1H, m, H-7α), 1.58 (3H, s, CH_3-17), 1.52 (1H, m, H-2β), 1.07 (3H, s, CH_3-16, overlapping H-14α, through HMQC), 0.97 (3H, s, CH_3-19), 0.88 (1H, m, H-2α); HRMS m/z found 543.25572 [M^++Na], $C_{28}H_{40}O_9Na$ requires 543.25699.
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29. *Compound* **7**: Bz_2O was prepared as follows: A 200 μL portion of dry benzene containing dry pyridine (16 μL) was supplemented with benzoyl chloride (7.2 μL) and stirred for 10 min. 7.5 mg benzoic acid (8.0 mg, 62.5 μmol) or 4-[^{3}H]-benzoic acid [450 μCi] for radiolabeling) in benzene was added in portions over 5 min period. After 10 min, the reaction mixture was diluted with CH_2Cl_2 (1 mL) and filtered through a SiO_2-column to elute pure benzoic anhydride. A stoichiometric amount of Bz_2O in CH_2Cl_2 (0.5 mL) and DMAP (1 mg) in CH_2Cl_2 (0.5 mL) were added to **5a** (2.0 mg, 3.3 μmol). The resulting solution was stirred at room temperature over night. H_2O was added, and the mixture was extracted with EtOAc. Purification by LC gives benzoate **7** in 5%. ^{1}H NMR ($CDCl_3$, 600 MHz), δ 7.95 (2H, d, J = 8.5 Hz, benzoyl *ortho*-H), 7.58 (1H t, J = 7.34 Hz, benzoyl *para*-H), 7.46 (2H, t, J = 7.8 Hz, benzoyl *meta*-H), 6.26 (1H, d, J = 10.9 Hz, H-10), 6.14 (1H, d, J=11.3 Hz, H-9), 6.13 (1H, m, H-13, overlapping with C9), 5.79 (1H, d, J=3.4 Hz, H-2), 5.53 (1H, dd, J = 4.4, 12.0 Hz, H-7), 4.20 (1H, t, J = 3.1 Hz, H-5), 3.59 (1H, d, J = 4.7 Hz, H-20a), 3.32 (1H, d, J = 3.6 Hz, H-3), 2.65 (1H, dd, J = 9.8, 14.6 Hz, Hβ-14), 2.30 (1H, d, J = 5.0 Hz, H-20b), 2.26 (3H, s, CH_3-18), 2.22 (3H, s, C(O)CH_3 at C-10), 2.14 (3H, s, C(O)CH_3 at C-13), 2.14 (Hβ-6, through HMQC, covered in ^{1}H NMR), 2.10 (3H, s, C(O)CH_3 at C7), 2.08 (3H, s, C(O)CH_3 at C9), 2.07 (m, Hα-14, through HMQC), 2.02 (3H, s, C(O)CH_3 at C5), (1H, ddd, J = 3.1, 3.9, 14.8, Hα-6), 1.716 (3H, s, CH_3-16), 1.36 (3H, s, CH_3-19), 1.26 (3H, s, CH_3-17); HRMS m/z found 737.27884 [M+Na], $C_{37}H_{46}O_{14}Na$ requires 737.27853.
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