Biosynthesis of Taxoids. Mode of Attachment of the Taxol Side Chain

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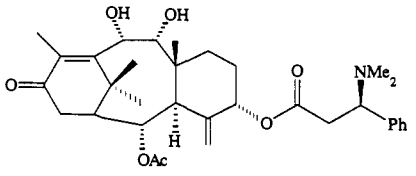
Taxol (**1**) and taxotere have continued to attract considerable attention because of their promise as agents against various types of cancer (Figure 1). While the threat to the original source of taxol, the Pacific yew, has been diminished as new semisynthetic avenues[1] to supply (based upon the availability of 10-deacetylbaccatin III from renewable sources[2]) have been developed, supply and cost are still major issues. It is anticipated that biosynthetic studies might provide insights which could help alleviate these problems.

Early work on taxanes, cf.[3,4] for example, taxine B (**4**), showed that there are several natural taxanes in which side chains structurally analogous to the taxol C-13 side chain are esterified to the 5-hydroxyl group of the diterpene moiety. This, together

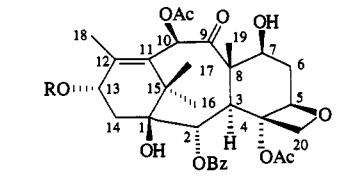


Taxine-B (4)

with the fact that curvature of the taxane framework brings the C-13 hydroxyl group into close proximity with the C-5 position, led Potier and colleagues[5] to hypothesize that the side chain is first attached to the 5 (or 4) position and then transferred to the C-13 oxygen by an intramolecular transesterification.

In previous work, we[6] reported that the C-13 side chain is derived from phenylalanine by way of β-phenylalanine and phenylisoserine. Feeding of side chain labeled in the *N*-benzoyl group also resulted in significant incorporation of label into taxol, suggesting, although not proving, that the side chain is attached as an intact unit.

The present work addresses the issues of the timing and mode of attachment of the side chain to the diterpene moiety, specifically the question of whether the side chain is attached as an intact unit to baccatin III (**3**).



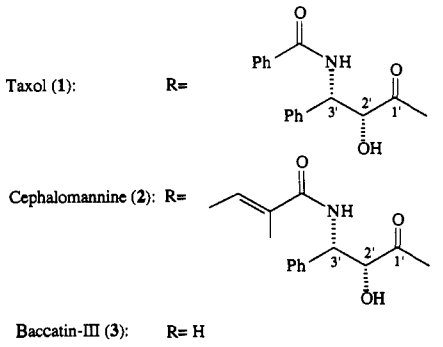


Figure 1.

In order to ascertain whether the side chain is attached to **3** or to an earlier diterpene precursor, we prepared both [13-^{3}H]- and [10-acetyl-$^2H_3$,13-$^2H_1$]baccatin III by adaptations of procedures in the literature.[7] These compounds were fed to cuttings and cambial tissue of *Taxus brevifolia* by methods described before.[6] Incorporation of label into taxol was seen in both experiments. In the feeding experiment with tritiated baccatin III (52.2 μCi), the isolated taxol was purified to constant specific radioactivity by cocrystallization with unlabeled taxol from methanol/water. An absolute incorporation of 0.1% was seen. Chemical degradation[9] proved that the label remained in the 13 position. In the experiment with deuterated baccatin III, electrospray MS/MS of the M + 4 isotopomer of taxol (carried out as described before[6]) showed that the labels were in the expected positions, as shown in Table 1. The three deuterium atoms seen in the 10-acetyl group of the [$^2H_4$]taxol isolated indicate that the material fed was not hydrolyzed to 10-deacetylbaccatin III prior to conversion into taxol.

As alluded to above, previous feeding experiments had shown that *N*-benzoyl-$^2H_5$-labeled side chain was incorporated into taxol. We thought it prudent, however, to seek additional evidence that the side chain was incorporated intact, rather than by way of hydrolysis and reattachment of the *N*-benzoyl group. To this end, doubly labeled side chain containing five deuterium atoms in each of the two aromatic rings was prepared[11] and fed as a 1:1 mixture with unlabeled side chain to cambial tissue of *T. brevifolia*. The taxol isolated contained 1.7% of [$^2H_5$]taxol, with only a small amount of [$^2H_{10}$]taxol evident. This indicated that extensive

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(1) DeFuria, M. D., Commercial Supply Strategy: Taxol. Presented at Stony Brook Symposium on Taxol and Taxotere. New Hope for Breast Cancer Chemotherapy, SUNY Stony Brook, May 14–15, 1993. Fabre, J. J. Taxotere Supply Strategy. Presented at Stony Brook Symposium on Taxol and Taxotere. New Hope for Breast Cancer Chemotherapy, SUNY Stony Brook, May 14–15, 1993.
(2) Denis, J. N.; Greene, A. E.; Guenard, D.; Gueritte-Voegelein, F.; Mangatal, L.; Potier, P. *J. Am. Chem. Soc.* **1988**, *110*, 5917–5919.
(3) Miller, R. *J. Nat. Prod.* **1980**, *43*, 425–437.
(4) Kingston, D. G. I.; Molinero, A. A.; Rimoldi, J. M. *Prog. Chem. Org. Nat. Prod.* **1993**, *61*, 1–206.
(5) Gueritte-Voegelein, F.; Guenard, D.; Potier, P. *J. Nat. Prod.* **1987**, *50*, 9–18.
(6) (a) Fleming, P. E.; Mocek, U.; Floss, H. G., *J. Am. Chem. Soc.* **1993**, *115*, 805–807. (b) Fleming, P. E.; Mocek, U.; Floss, H. G. *Proceedings of the International Yew Resources Council*, Berkeley, CA, March 12–13, 1993, in press.
(7) For the preparation of tritium-labeled baccatin III, the unlabeled compound was protected with TESCl[2], the 13-OH group oxidized with MnO_2,[8a] and the resulting ketone reduced with sodium borotritiide.[8b] Deprotection gave the desired compound. For the synthesis of the deuterium-labeled compound, 7-(triethylsilyl)-10-deacetylbaccatin III was acetylated with CD_3COCl and deuterium introduced at the 13 position by oxidation and reduction as for the tritiated compound.
(8) (a) Della Casa de Marcano, D. P.; Halsall, T. G. *J. Chem. Soc., Chem. Commun.* **1975**, 365–366. (b) Senilh, V.; Gueritte, F.; Guenard, D.; Colin, M.; Potier, P. *C. R. Acad. Sci., Ser. 2* **1984**, *299*, 1039–1043.
(9) Reductive cleavage of the side chain[10] yielded **3** and the side chain diol, which was converted to its diacetate to facilitate purification. Very little radioactivity was evident in the side chain (288 dpm, 5.4×10^4 dpm/mmol). TES protection of the 7-hydroxyl group of **3** gave a product which had the same specific radioactivity as the starting taxol (1.06×10^6 dpm/mmol vs 1.09×10^6 dpm/mmol). Manganese dioxide oxidation of the 13-hydroxyl group gave 7-(triethylsilyl)-13-oxobaccatin III which was shown, as expected, to contain essentially none (1.9×10^3 dpm/mmol, <0.2%) of the tritium label.
(10) Magri, N. F.; Kingston. D. G. I.; Jitrangsri, C.; Piccariello, T. *J. Org. Chem.* **1986**, *51*, 3239–3242.
(11) By benzoylation of [$^2H_5$]phenylisoserine produced as described before in ref 6.

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Table 1. Electrospray MS/MS Analysis of **1** and **2** from Feeding Experiments with Deuterated Precursors[a]

molecule analyzed	$(M + MeNH_3)^+$	fragment A[b]	fragment B[c]	fragment C[d]
standard **1**	885	569	509	286
1 from [10-acetyl-$^2H_3$,13-$^2H_1$]-**3**	885			
	889 (1.0%)[e]	573	510	286
1 from [ring-$^2H_{10}$]-*N*-benzoylphenylisoserine	885			
	890 (1.7%)	569	509	291
	895 (low)			
1 from [side-chain-ring-$^2H_5$,10-acetyl-$^2H_3$]-**5**	885			
	888 (<1%)	572	509	286
	890 (<1%)	569	509	291
	893 (5.9%)	572	509	291
standard **2**	863	569	509	264
2 from [side-chain-ring-$^2H_5$,10-acetyl-$^2H_3$]-**5**	863			
	866 (<1%)	572	509	264
	868 (<1%)	568	509	269
	871 (5.0%)	572	509	269

[a] Spectra were run in the presence of CH_3NH_2·HCl to shift the molecular ion beyond interfering ions derived from the sodiated species. Fragment ions A, B, and C are observed in the daughter ion spectra of the respective isotopomers of the molecular ion; relative intensities of the isotopomers of the molecular ion were determined from parent ion scans. [b] Taxol or cephalomannine minus the side chain. [c] Fragment A minus 10-acetyl group. [d] *N*-Acylphenylisoserine side chain. [e] Note that substantial seasonal variation in incorporations has been seen, so levels of incorporation are not directly comparable. For example, a feeding of [$^2H_5$]-β-phenylalanine done at the same time as the baccatin III feeding showed only 0.5% incorporation, whereas it showed 2.8% incorporation in earlier feedings.

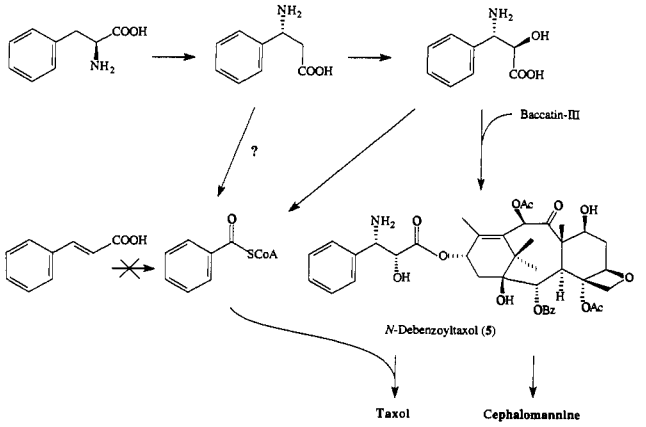

Figure 2.

hydrolysis of the *N*-benzoyl group of the precursor was occurring and suggested that phenylisoserine was the actual biosynthetic intermediate being attached to baccatin III. In order to confirm that the hydrolysis of the *N*-benzoyl group was not occurring so rapidly that *Taxus brevifolia* was unable to utilize intact $^2H_{10}$-labeled side chain, a feeding experiment was carried out with *N*-benzoyl-7-^{14}C-labeled side chain and the extent of hydrolysis determined. Approximately 92% of the radioactivity recovered from the plant tissue after the feeding was found in the side chain, with only 8% recovered as benzoate. These two results combined make it clear that the side chain is not attached as an intact unit.

The above results led to a revised biosynthetic hypothesis as shown in Figure 2. To test this hypothesis, doubly labeled[12] [$^2H_8$]-*N*-debenzoyltaxol (**5**, Figure 2) was prepared by a modification of literature methods[13] for producing the unlabeled compound. Feeding of this compound resulted in high incorporation of eight atoms of deuterium (see Table 1) into both taxol and cephalomannine (**2**). A small amount of [$^2H_5$]- and [$^2H_3$]taxol was evident, indicating that some hydrolysis of the precursor had occurred. The majority of the label, however, clearly resulted from intact incorporation of the precursor, as predicted by the revised hypothesis.

In conclusion, the feeding experiments reported above indicate the following: (*1*) Baccatin III is a precursor of taxol. This casts serious doubt on Potier's biosynthetic hypothesis, because the oxetane ring precludes transesterification from the 5 position, and the acetyl group from the 4 position. (*2*) The side chain is not attached as an intact unit, but most likely as phenylisoserine.[14] (*3*) The final step in taxol (cephalomannine) biosynthesis is benzoylation (tigloylation) of *N*-debenzoyltaxol.

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Supplementary Material Available: ES-MS/MS spectra of **1** derived from deuterated **3** and **5**, of **2** derived from deuterated **5**, and of **1** and **2** standards (5 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(12) The compound carried three deuterium atoms in the 10-acetyl group and five deuterium atoms in the phenyl ring of the phenylisoserine side chain.

(13) Commercon, A.; Bezard, D.; Bourzat, J. D. *Tetrahedron Lett.* **1992**, 5185–5188.

(14) Given that earlier results had shown higher incorporation of β-phenylalanine than of phenylisoserine and that in some taxanes such as **4** the side chain which is structurally analagous to the C-13 side chain of taxol is lacking a hydroxyl group in the 2′ position, we cannot discount the possibility that the side chain is normally attached as β-phenylalanine and that phenylisoserine is incorporated only when administered externally, but is not a natural precursor. This possiblity will be explored when feeding experiments again become possible in the Spring.