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Biosynthesis of Taxol: Enzymatic Acetylation of 10-Deacetylbaccatin-III to Baccatin-III in Crude Extracts from Roots of *Taxus baccata*

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The biosynthesis of taxol is a multistep process. One intermediate reaction is the acetylation of 10-deacetylbaccatin-III (10-DAB) to baccatin-III, an assumed precursor of taxol. Here we describe the cell free acetylation of 10-DAB in crude extracts from roots of *Taxus baccata* saplings using ^{14}C- or ^{3}H-labeled acetyl-coenzyme A as the acetyl donor. The reaction is strictly dependent on the addition of 10-DAB and is specific for the 10-hydroxyl group of the taxane ring. Formation of radiolabeled baccatin-III was confirmed by co-chromatography of the labeled product with authentic baccatin-III in different TLC-systems and HPLC. Furthermore, the acetylation product showed an identical UV spectrum as authentic baccatin-III. Crude extracts from cambium of stems yielded three- to fivefold lower activity. This is in agreement with our finding that the taxol titer in roots was considerably higher than that in cambium.

Taxol is a potent diterpenoid anticancer agent isolated from the inner bark of the Pacific yew tree *Taxus brevifolia* and is also present in other species of the genus *Taxus* (1). Due to the limitation of this drug from its natural source alternative methods for the production of this compound have to be developed. Therefore it is of great interest to study the biosynthetic pathways leading to this complex diterpenoid compound. Reports on the biosynthesis of taxol are mainly based on *in vivo* studies (2). The first cell free experiments that have recently been described concerned taxadiene synthase from *Taxus brevifolia* (3,4,5,6) which catalyses the cyclisation of geranylgeranyl pyrophosphate, the commited step in taxol biosynthesis. Furthermore, the first oxygenation step in taxol biosynthesis has been described (7). Here we report on the cell free acetylation of 10-DAB in crude extracts of *Taxus baccata.*

MATERIALS AND METHODS

Plants, substrates and standards. Ten month old *T. baccata* saplings in active growth maintained in a green house were used as a starting material.

Taxol, 10-deacetylbaccatin-III and baccatin-III standards were purchased from Sigma, Germany. HPLC grade

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Abbreviations used: HPLC, high performance liquid chromatography; TLC, thin layer chromatography; 10-DAB, 10-deacetyl baccatin-III.

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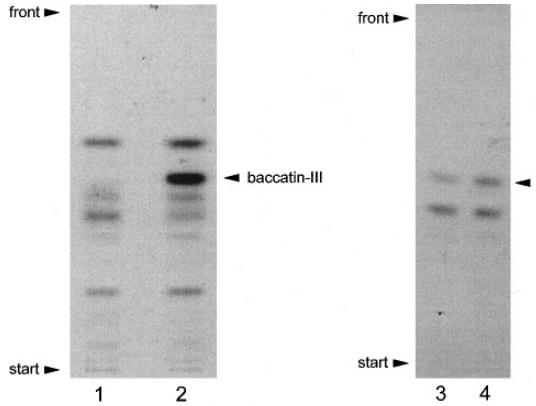

FIG. 1. TLC-separation of acetylation products (autoradiograph). Acetylation experiments were performed as described under Materials and Methods. TLC solvent system I was used. Lane 1: addition of 1 μl EtOH, lane 2: addition of 1 μl 10-DAB (1 mg/ml in EtOH). Time dependence of the acetylation reaction. Lane 3: 5 min incubation, lane 4: 30 min incubation.

acetonitril and water were obtained from Merck, Germany. [1-^{14}C]-acetyl-CoA (spec. act. 1.85 GBq/mmol) and [^{3}H]-acetyl-CoA (spec. act. 148 GBq/mmol) were purchased from Amersham International.

Preparation of cell free extracts. Roots from *T. baccata* saplings were frozen in liquid N_2 and pulverized in a mortar. The resulting powder was extracted with buffer containing 100 mM Tris/HCl pH 7.8, 10 mM $Na_2S_2O_5$, 10 mM sodium ascorbate, 5 mM dithiothreitol, 1% (w/v) polyvinylpyrrolidone ($M_r \approx$ 10,000), 10% (v/v) glycerol. The homogenate was centrifuged at 15,000 rpm in a Sorvall RC 2-B for 60 min. The resulting supernatant was desalted by passage through a Sephadex G 25 column which was previously equilibrated with the same buffer (without dithiothreitol) and used as crude extract for the experiments.

Acetylation experiments. For cell free acetylation experiments 100 μl of crude extract were mixed with 5 mM $MgCl_2$, 20 μM DAB, 0.5 μCi [1-^{14}C]-acetyl-CoA. 10-DAB was previously purified by HPLC to remove traces of baccatin-III (see Methods of analysis) and added dissolved in EtOH (1 mg/ml). The final EtOH concentration in the assay was less than 1%. For tritium labeling 1 μCi of [^{3}H]-acetyl-CoA per assay was used in the presence of 100 μM unlabeled acetyl-CoA. After incubation for one hour at 26°C 2 ml of water were added, and the mixture was extracted with 2 ml of chloroform. The organic layer was evaporated and the residue was dissolved in 50μl EtOAc and subjected to TLC.

Methods of analysis. Thin layer chromatography (TLC): Baccatin-III was separated on silica gel plates (Merck, Darmstadt). Solvent systems used were water saturated ethyl acetate (solvent system I), chloroform/methanol (95:5, v/v) (solvent system II) and EtOAc/MeOH/water (100:5:1, v/v/v) (solvent system III).

High performance liquid chromatography (HPLC): Further investigations of the samples were performed (after purification by TLC) on a Waters HPLC system consisting of two W 510 pumps, a W717 autosampler, a W996 photodiode array detector and an analytical RP18 column (4 μm, 250 $\times$ 4.6 mm) from Merck. A linear gradient ranging from 20% to 100% acetonitril in 15 minutes, followed by 5 minutes with 100% acetonitril (flow rate 1ml/min) was used. Taxanes were detected at 230 nm.

RESULTS AND DISCUSSION

During an investigation on the taxol content of the European yew *Taxus baccata* we found rather high amounts of taxol (up to 0.05% per dry weight) in roots of the trees in contrast to trace amounts of this compound in needles and cambium of stems (these results will be published elsewhere). Therefore we chose roots of *Taxus baccata* saplings as a starting material for our enzymatic studies on the biosynthesis of taxol. In figure 1 (lanes 1 and 2) is shown the enzymatic formation of radiolabeled baccatin-III from 10-DAB and

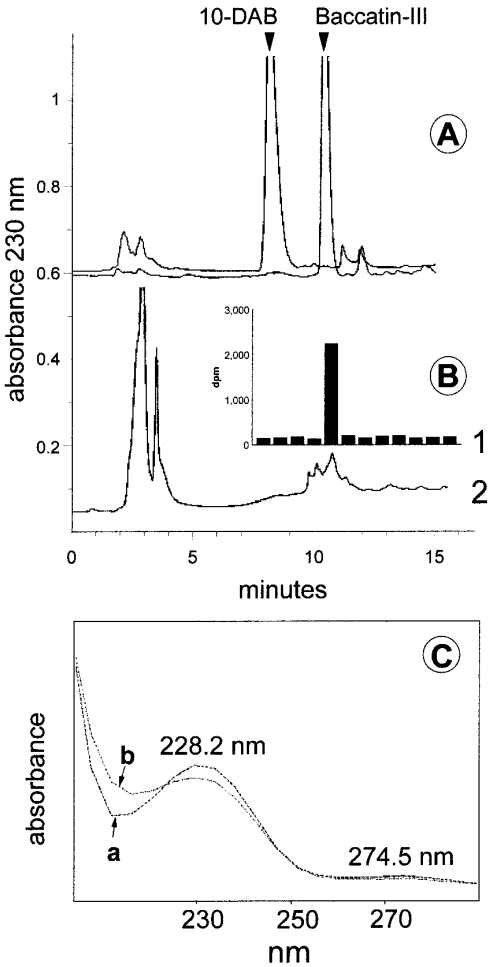

FIG. 2. HPLC-analysis of acetylation products (conditions, see Materials and Methods). (A) Authentic baccatin-III and 10-DAB; (B, 1) radioactive acetylation product and (B, 2) unlabeled acetylation product eluting with baccatin-III. (C) Curve a: UV spectrum of authentic baccatin-III; curve b: UV spectrum of unlabeled acetylation product. Maxima for both substances were 228 and 274 nm, respectively.

[1-^{14}C]-acetyl coenzyme A in crude extracts, separated by TLC. As can be seen, the formation of a radioactive compound is observed which is strictly dependent on the addition of 10-DAB and which has the same Rf-value as authentic baccatin-III in the solvent system I and in two further solvent systems (system II and III, results not shown). Lanes 3 and 4 show the time dependence of the acetylation reaction. Radioactive bands were isolated from the plates by extraction with EtOH and subjected to HPLC analysis using authentic baccatin-III as a marker. The result is shown in figure 2 (B). As demonstrated in the different TLC systems also in HPLC separation the radioactive compound comigrated with authentic baccatin-III. Further evidence for the authenticity of the enzymatic product comes from the HPLC experiment shown in figure 2 (B). We were able to synthesize several microgramms of the nonlabeled acetylation product which was purified by TLC. It behaved like baccatin-III in the HPLC system and yielded an authentic UV spectrum (see Figure

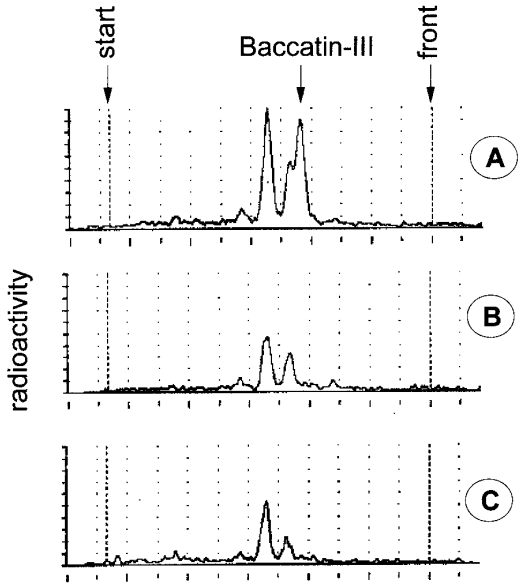

FIG. 3. Specificity of acetylation reaction. Experiments were carried out as described under Fig. 1, however baccatin-III and taxol were used as possible substrates and [^{3}H]-acetyl-CoA was used as the radiolabel. (A) Control experiment using 10-DAB as a substrate. (B) Addition of baccatin-III instead of 10-DAB. (C) Addition of taxol instead of 10-DAB. Radioactivity was detected by radioscanning.

2 C). In the control experiment (omission of acetyl coenzyme A) no baccatin-III formation was observed (not shown). Taxol and related taxanes are sensitive towards mild alkaline hydrolysis (8). The same holds true for our radioactive acetylation product. When treated with $NaHCO_3$ in aqueous methanol the labeled acetyl group could easily be released indicating the presence of an ester bond (results not shown).

To demonstrate, that the acetylation of 10-DAB indeed occurs at position 10 of the taxane ring, we performed the same experiments as shown in Figure 1, however with baccatin-III and taxol as possible substrates instead of 10-DAB. The result is shown in Fig. 3. Only in the control reaction with 10-DAB a new radioactive compound (baccatin-III) appeared, indicating that the free OH-groups in positions 1, 7,13 (baccatin-III) and 1 and 7 (taxol) were not recognized by the enzyme as a target.

From the results described above we conclude that an enzymatic activity in crude extracts of *T. baccata* is present, which is able to convert 10-DAB into baccatin-III. The latter compound has been assumed to be the direct precursor of the taxol molecule (2). To our knowledge this is the first cell free acetylation reaction involved in taxol biosynthesis that has been described. Work is in progress to isolate and characterize the enzyme.

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