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SYNTHESIS OF A [^{3}H]-LABELLED DERIVATIVE OF THE MICROTUBULAR POISON TAXOL

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SUMMARY

A labelled derivative of the microtubule poison taxol has been developed on acetylation of the 7-OH function of taxol with [^{3}H] acetic anhydride. The [^{3}H] 7-acetyl taxol synthesized was chemically and radiochemically pure and has not undergone radiolysis during 1.5 years. This compound was also stable in the presence of microtubule proteins. The ID_{50} of 7-acetyl taxol for stabilization of microtubules was two times the ID_{50} of taxol. Hence it can be used as a probe for biological studies. The acetylation of the hydroxyl group at position C-7 is a general method applicable to other taxol derivatives as starting compounds.

Keywords : taxol, baccatine, microtubules, tubulin, tritium labelling.

INTRODUCTION

Taxol is a diterpenoid (1) that stabilizes microtubules both *in vivo* (2) and *in vitro* (3). Taxol and some derivatives, recently prepared by hemisynthesis, show promising antitumor properties, as indicated by their action on various murine cancers (1,4-6). Moreover, the differential sensitivity, to taxol and baccatine derivatives, of microtubules assembled from

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mammalian brain tubulin and from tubulin derived from lower eucaryotes suggests that some microtubule poisons might be useful as antiparasitic agents (7). Although the mode of action of taxol has been studied *in vivo* (8) and *in vitro* (9, 10), using tritiated taxol labelled by a catalytic exchange method (9), many questions are still unanswered. For example, Manfredi and Horwitz (11) raised the possibility that taxol does not bind to free tubulin either *in vivo* or *in vitro*, while Lataste *et al* (7) have stressed the discrepancy between the *in vitro* and *in vivo* effects of baccatine derivatives on lower eucaryotes. Moreover, the recent completion of a human toxicity trial with taxol (12) requires future pharmacokinetic studies with this drug and its tumoristatic derivatives. Although the synthesis of a simplified taxane ring has been recently achieved (13), taxol and its derivatives are presently only available by extraction from the bark of several species of the genus *Taxus* (1,4,14,15). In order to label the molecule of taxol, we have developed a new hemisynthetic procedure for the acetylation of the 7-OH function with tritiated acetic anhydride (16). Briefly, the procedure involved protection of the hydroxyl group of the side chain attached at C-13 with a 2-2-2 trichloroethyloxy-carbonyl group, the acetylation of the 7-OH function with [^{3}H] acetic anhydride and the removal of the protective group by reduction with zinc powder in acetic acid.

EXPERIMENTAL

Reagents (Fig. 1) : Taxol (1) was a gift of the Natural Product Branch of the National Cancer Institut. Cephalomannine (6) and 10-deacetyl baccatine III (7) were isolated from the bark and the leaves respectively of *Taxus baccata* (14).

Synthesis of tritiated 7-acetyl taxol (Fig. 2) : Protection of the hydroxyl group in position 2' of the side chain attached at C-13 was performed in a micro-reactor (Pierce) with 195 mg (228 µmoles) of taxol (1), which was dissolved in 6 ml of methylene chloride containing 86 µl (1066 µmoles) of pyridine. The solution was cooled to -30°C using an acetone/dry ice mixture. Then 225 mg (1066 µmoles) of 2,2,2 trichloroethyl chloroformate dissolved in 2 ml of methylene chloride at room temperature was added slowly into the taxol

solution and the mixture was stirred gently for 1 hr at -30°C. Water (5 ml) was added and the mixture was allowed to warm to room temperature. The addition of water before the temperature increase was performed in order to keep the formation of 2',7-di(2,2,2-trichloroethyloxycarbonyl) taxol (3) to a minimum. The crude product (Fig. 3A) was extracted into methylene chloride and the

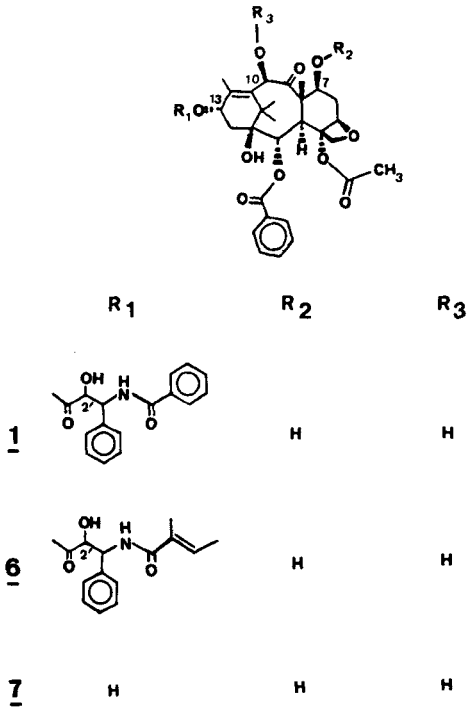

FIG. 1 : Formulae of taxol and some of its derivatives used as starting compounds. 1 : taxol ; 6 : cephalomannine and 7 : 10-deacetyl baccatine III.

mixture of unreacted taxol (1), 2'(2,2,2-trichloroethyloxycarbonyl) taxol (2) and 2',7-di(2,2,2-trichloroethyloxycarbonyl) taxol (3) was applied to a silicagel column (100 x 1.2 cm) equilibrated and eluted with methylene chloride/methanol (98/2). The recovery of 180 mg pure 2'(2,2,2-trichloroethyloxycarbonyl) taxol (2) represented a 76 % yield. In order to acetylate the hydroxyl group in position C-7,33 µmoles of [^{3}H] sodium acetate (3 Ci per mmole, 100 mCi, Amersham TRK 12) were dissolved in 0.2 ml of water, which was

then placed in a micro-reactor (Pierce). Water was evaporated, the micro-reactor heated at 190°C for one hr under reduced pressure (0.01 mm mercury) and then cooled to room temperature. Then 40 µl of tetrahydrofurane and 2.4 µl of acetyl chloride were added and the mixture was stirred overnight at room temperature. To the milky suspension that resulted, 6.8 mg (6.6 µmoles) of 2'(2,2,2-trichloroethyloxycarbonyl) taxol (2) dissolved in

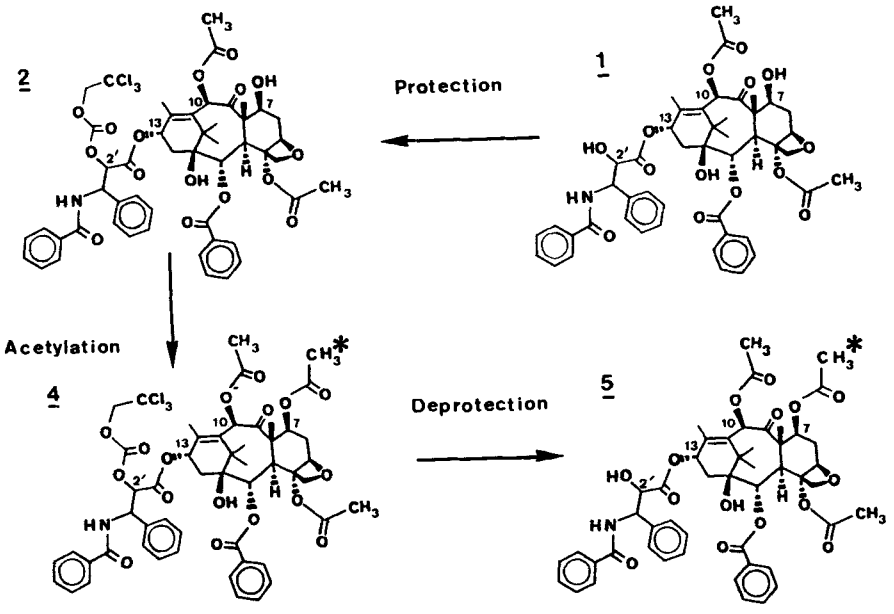

FIG. 2 : Scheme for synthesis of [^{3}H] 7-acetyl taxol. 1 : taxol ; 2 : 2'(2,2,2-trichloroethyloxycarbonyl)taxol ; 4 : 2'(2,2,2-trichloroethyl-oxycarbonyl) 7-acetyl taxol and 5 : 7-acetyl taxol. The asterisk indicates the labelling with tritium.

40 µl pyridine was added and the mixture was heated to 80°C for five hrs, allowing the formation of 2'(2,2,2-trichloroethyloxycarbonyl)7-acetyl taxol (4) (Fig. 3B). Then solvents were removed by flushing with a stream of nitrogen. In order to remove the protecting group 200 µl of acetic acid and 5 mg of zinc powder were added and the mixture was stirred for one hr in an oil bath held at 40°C. The suspension was poured into 2 ml of methylene chloride and filtered

through glass wool in order to eliminate particulates. The solvent was then removed by flushing with a stream of nitrogen. The residue (Fig. 3C) was chromatographed on a semi-preparative HPLC column (Micro-bondapak C18, Waters), eluted with a mixture of methanol/water (80/20). The amount of tritiated 7-acetyl taxol which was recovered accounted for 61% of the 2' (2,2,2-trichloroethyloxycarbonyl) taxol used as a starting compound. The structure of the 7-acetyl taxol (5) was confirmed by proton magnetic resonance and mass spectrometry, using unlabelled 7-acetyl taxol, prepared under the same conditions. The mass spectrometry was performed using a Ribermag 10-10A

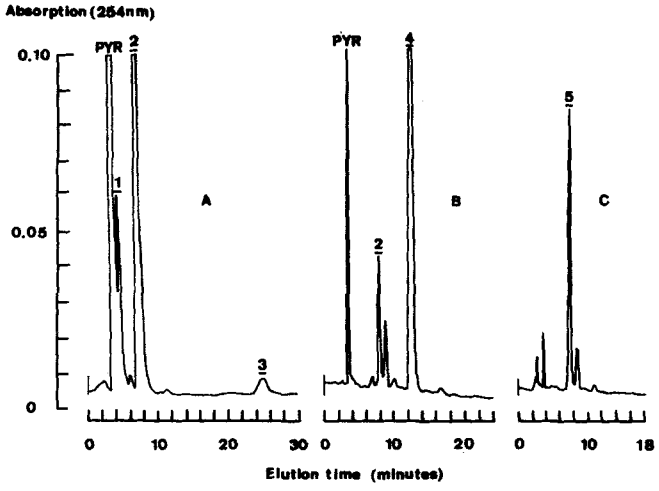

FIG. 3 : HPLC *analysis of the reaction products at various stages in the* synthesis of [^{3}H] 7-acetyl taxol. The HPLC was performed with a Micro-bondapak C18 column (Waters) eluted, with a mixture of methanol/water (80/20), at a flow rate of 1 ml per min. 1 : taxol ; 2 : 2'(2,2,2-trichloroethyloxycarbonyl) taxol ; 3 : 2',7-di(2,2,2-trichloroethyloxycarbonyl) taxol ; 4 : 7-acetyl 2'(2,2,2-trichloroethyloxycarbonyl) taxol ; 5 : 7-acetyl taxol and PYR : pyridine. A : protection of the hydroxyl group at position 2' of the chain in C-13 of taxol. B : acetylation of the hydroxyl group at position 7 of the 2' (2,2,2-trichloroethyloxycarbonyl) taxol and C : removal of the protective group at position 2' of the side chain attached at C-13 of the 7-acetyl 2'(2,2,2-trichloroethyloxycarbonyl) taxol.

spectrometer in order to obtain a desorption chemical ionisation mass spectrum, with ammonia as the reagent gas, showing the following M/Z peaks : 913 $(MNH4)^+$, 896 $(MH)^+$, 878 $(MH-H_2O)^+$. The spectrum obtained using proton magnetic resonance in $CDCl_3$ (IEF 400 MHz) was the following : C_2H : 5,68 d (J = 7) ; C_3H : 3.90 d (J = 7) ; C_5H : 4.95 d (J = 9) ; 2 x C_6H : 1.88 m - 2.62 m ; C_7H : 5.65 m ; $C_{10}H$: 6,27 s ; $C_{13}H$: 6.20 t (J = 9) ; $C_{14}H_2$: 2.33 m ; $C_{16}H_3$: 1.23 s ; $C_{17}H_3$: 1.16 s ; $C_{18}H_3$: 1.82 s ; $C_{19}H_3$: 1.82 s ; 2 x $C_{20}H$: 4.26 d - 4.32 d (J = 9) ; $COCH_3$: 2.02, 2.16 and 2.39 ; $C_{2'}H$: 4.82 d (J = 3) ; $C_{3'}H$: 5.82 dd (J = 3 & 9) ; $C_{3'}Ph$: 7.36 and 7.41 and NH : 7.08 d (J = 9).

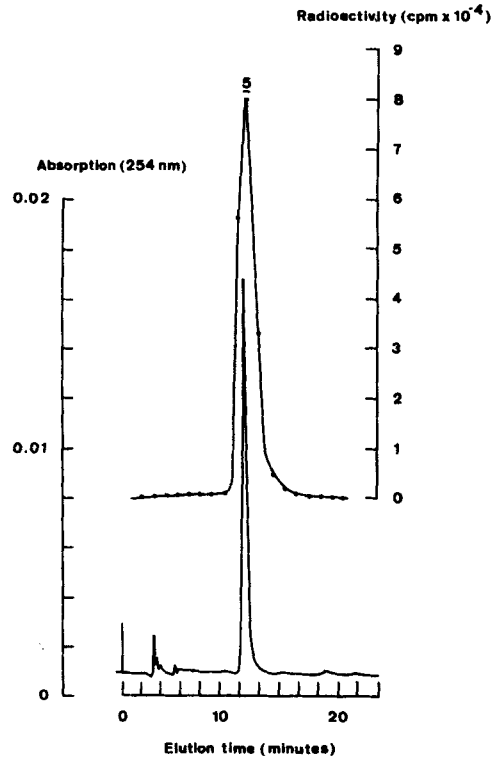

FIG. 4 : HPLC analysis of the [^{3}H] 7-acetyl taxol obtained after purification on a semi-preparative column. HPLC was performed as described in Fig. 3 except that the elution was made with methanol/water (70/30). Radioactivity was measured by scintillation counting (4 µl aliquots in 2 ml of Aquasol II, New England Nuclear).

The two preparations of [^{3}H] acetyl taxol which have been synthesized were pure as judged by HPLC analysis (Micro-bondapak C18 column, Waters, elution with methanol/water, 70/30), the radioactivity present in the peak of 7-acetyl taxol accounting for 99.5 % of the total radioactivity recovered (Fig.4). The specific activity of the 7-acetyl taxol (1.4 Ci per mmole) was half the specific activity of the tritiated acetic acid which was used, in agreement with the synthesis of tritiated acetic anhydride. [^{3}H]-acetyl taxol (0.17 mM) remained stable for more than 17 months when kept in methanol/water (80/20) at -20°C, since only 4% of the total radioactivity did not cochromatograph with 7-acetyl taxol after this period of storage.

Synthesis of 7-acetyl cephalomannine : The procedure of synthesis was the same as the one used for the synthesis of 7-acetyl taxol except that cephalomannine (6) served as the starting compound instead of taxol. The structure of 7-acetyl cephalomannine was confirmed by proton magnetic resonance and mass spectrometry. The mass spectrometry (chemical ionization) of 7-acetyl cephalomannine showed the following M/Z peak : 874 $(MH)^+$, 611, 551, 491, 429, 369, 309, 264, 246 and 123. The spectrum obtained using proton nuclear magnetic resonance was the following in $CDCl_3$: C_2H : 5,68 d (J = 7) ; C_3H : 3.90 d (J = 7) ; C_5H : 4.95 d (J = 9) ; 2 x C_6H : 1.88 m - 2.62 m ; C_7H : 5.65 m ; $C_{10}H$: 6,27 s ; $C_{13}H$: 6.20 t (J = 9) ; $C_{14}H_2$: 2.33 m ; $C_{16}H_3$: 1.23 s ; $C_{17}H_3$: 1.16 s ; $C_{18}H_3$: 1.82 s ; $C_{19}H_3$: 1.82 s ; 2 x $C_{20}H$: 4.26 d - 4.32 d (J = 9) ; $COCH_3$: 2.02, 2.16 and 2.36 ; C_2,H : 4.74 d ; C_3,H : 5.67 m ; C_3,Ph : 7.36 and 7.41 ; NH : 6.58 d (J = 9) ; amides : C_5,H_3 : 1.82 s ; C_6,H_3 : 1.74 d (J = 7) and C_6,H : 6.47 q (J = 7).

Synthesis of 7-acetyl baccatine III : Acetic anhydride was prepared as described for the synthesis of 7-acetyl taxol. Then 10-deacetyl baccatine III (7), in pyridine was added and the mixture heated to 60°C for 4 hrs. The products of the reaction (Fig. 5) were chromatographed on a semi-preparative HPLC column (Micro-bondapak C18, Waters) and eluted with a mixture of methanol/water (50/50). The amount of tritiated 7-acetyl baccatine III which

was recovered accounted for 24% of the 10-deacetyl baccatine III used as a starting compound. The structure of 7-acetyl baccatine III (9) was confirmed by proton magnetic resonance and mass spectrometry (17). The mass spectometry (chemical ionisation) of 7-acetyl baccatine III showed the following M/Z peaks : 629 (MH^+), 611, 569, 551, 509, 491, 387, 327, 309 and 123. The spectrum obtained using proton nuclear magnetic resonance was the following in $COCl_3$: C_2H : 5.62 d (J=7) , C_3H : 4.00 d (J=7) , C_5H : 4.97 d (J=9) , $2xC_6H$: 1.80-2.60 m , C_7H : 5.59 m , $C_{10}H$: 6.26 s , $C_{13}H$: 4.86 t (J=8) , $C_{14}H_2$: 2.28 m , $C_{16}H_3$: 1.13 s , $C_{17}H_3$: 1.08 s , $C_{18}H_3$: 2.03 s , $C_{19}H_3$: 1.79 s , $2xC_{20}H$: 4.14 d - 4.31 d (J=9) , $COCH_3$: 2.10, 2.16, 2.29 , Ph : 7.46, 7.58, 8.08.

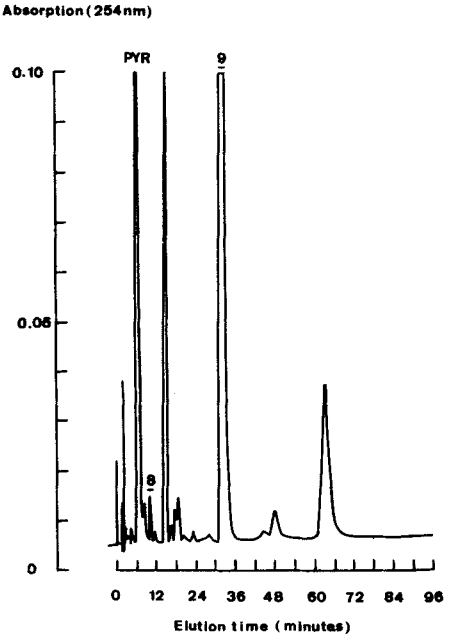

FIG. 5 : HPLC analysis of the reaction products in the synthesis of [^{3}H]7-acetyl baccatine III. The HPLC was performed as described in Fig. 3 except that the elution was made with methanol/water (50/50). PYR : pyridine , 8 : baccatine III and 9 : 7-acetyl baccatine III.

BIOLOGICAL INTEREST

[^{3}H] 7-acetyl taxol (5) has been synthesized from taxol (1) by a hemisynthetic procedure involving three steps. As judged by HPLC analysis this tritiated taxol derivative appears stable under the experimental conditions used to assemble tubulin into microtubules *in vitro* : 37°C, 0.1M piperazine-N,N'-bis (2-ethanesulfonic acid) (Pipes) pH 6.9, 1mM EGTA, 0.5 mM $MgCl_2$ and 1mM GTP (18). Neither the addition of 1.5 % dimethyl sulfoxide, a solvent used to solubilize many microtubular poisons (3,19,20), nor the presence of 1.2 mg per ml of two-cycle sheep brain microtubule protein (18) had an effect on the stability of [^{3}H] 7-acetyl taxol, even after a 6h incubation at 37°C. 7-acetyl taxol was only two times less potent than taxol in preventing microtubule disassembly. Using two-cycle pig brain tubulin (18), the ID_{50} of 7-acetyl taxol and taxol were 1.0 and 0.5 µM respectively at 37°C. These results indicate that [^{3}H] 7-acetyl taxol will be a valuable tool to probe the taxol binding site on the tubulin molecule (16). Using the same conditions of synthesis with cephalomannine (6) (ID_{50} : 0.7 µM) we obtained 7-acetyl cephalomannine (ID_{50} : 1.0 µM). Moreover, taxol derivatives lacking the side chain attached at C-13 like 10-deacetyl baccatine III (7), which are inactive on mammalian brain microtubules (10) but are as active (7) as taxol on *Physarum* microtubules (21) should be readily labelled by acetylation of the 7-OH function. Despite the small losses of activity which seem to result from the acetylation of the 7-OH function of various taxol derivatives, the acetylation process with acetic anhydride is simple and provides a convenient method for preparing various radioactive analogs of taxol and its derivatives.

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