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BIOSYNTHESIS OF *CAMPTOTHECA ACUMINATA* ALKALOIDS

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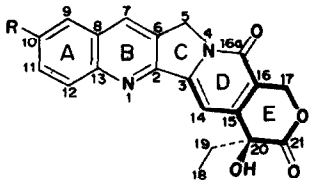
Key Word Index—*Camptotheca acuminate*; Nyssaceae; camptothecin biosynthesis; tryptophan; tryptamine; mevalonate; geraniol-nerol.

Abstract—Tracer feeding experiments with *Camptotheca acuminata* plants show that [1′-^{14}C]L-tryptophan, [Ar-$^{3}H_4$]L-tryptophan, [Ar-$^{3}H_4$,1′-^{14}C]tryptophan, [1′-^{14}C]-tryptamine, [2-^{14}C]DL-mevalonate, and [2-^{14}C]geraniol-[2-^{14}C]nerol are incorporated into camptothecin. Direct stem injection of the labeled precursors into *C. acuminata* plants resulted in a substantial increase in the activity of isolated Camptotheca alkaloids as compared to root feeding of the same tracer.

INTRODUCTION

Establishment of the structure **1** [1–3] for the initially reported promising antineoplastic pentacyclic alkaloids isolated from the tree *Camptotheca acuminata* Decne, native to China, has stimulated intensive investigation of the synthesis



(**1**)

(+)-Camptothecin, R=H
10-Hydroxycamptothecin, R=OH
10-Methoxycamptothecin, R=OMe

and chemotherapeutic properties of Camptothecin [4]. Our own interest was directed to possible biosynthetic precursors of camptotheca alkaloids, including the suggestion [5] that camptothecin may be biogenetically derived from an indole precursor which is converted to a pyrroloquinoline derivative. This system might also provide another example of indole alkaloids biosynthesized from a combination of indolic and monoterpenoid units [6]. Tryptophan [7–9], tryptamine [8,10,11], mevalonate [12–17], geraniol [16–21], loganin [22,23], secologanin [24], and vincoside [10,11], have already been established as precursors of indole alkaloids, and recently isovincoside (strictosidine) [10,11] was isolated from *Vinca rosea*, 5-α-carboxystrictosidine (5-α-carboxyisovincoside) [25] from *R. orientalis*, and vincosidelactam [26] from *Adina rubescens*.

RESULTS AND DISCUSSION

Our investigation of the biosynthesis of the *C. acuminata* alkaloid was undertaken by feeding probable labeled precursors. When [1′-^{14}C]L-tryptophan, [Ar-$^{3}H_4$]L-tryptophan, [Ar-$^{3}H_4$,1′-^{14}C]L-tryptophan, [1′-^{14}C]-tryptamine, [2-^{14}C]DL-mevalonate and [2-^{14}C]-geraniol[2-^{14}C]nerol were administered in nine separate feeding experiments (Table 1) to *Camptotheca acuminata* plants, after 3–7 days, camptothecin was isolated and was found to be active (*ca* 0·005–2·000% incorporation). These findings are consistent with the hypothesis that the biosynthesis of this novel type quinoline alkaloid is biogenetically linked to that of the indole alkaloid group, and like indole alkaloids, camptothecin is of tryptophan–monoterpenoid origin. This rationalization is supported by the complementary reported results [27] wherein satisfactory incorporation into camptothecin was found for tryptophan, tryptamine, mevalonate, loganin, secalaganin, isovincoside (strictosidine), isovincosidelactam (strictosamide) and 18,19-dihydroisovincosidelactam. Therefore one can postulate that in *C. acuminata* plants, the sequence of intermediates in the biosynthetic pathway leading to camptothecin, from mevalonate, geraniol-nerol, and tryptophan, is as summarized in the biosynthetic scheme (Fig. 1). Mevalonate is converted by the way of geraniol and loganin into secologanin (**2**) which combines with tryptophan to form isovincoside (strictosidine, **3**). Isovincoside (**3**) is then lactamized to the lactam (strictosamide, **4**) which via reduction to the corresponding 18,19-dihydro derivative and oxidative cleavage by molecular oxygen or hydrogen peroxide yields ketolactam **5**. The intramolecular cyclization of **5** produces a pyrroloquinoline derivative which in turn through a sequence of oxidation-reduction steps is transformed into camptothecin (**1**).

The results of our investigations of the possible precursors in the early stages of the biosynthetic pathway leading to camptothecin are presented in Table 1. These results demonstrate that when radioactively labeled precursors were administered by allowing the roots of the intact *C. acuminata* plants to grow in an aerated nutrient solution containing the tracer, the activity of the camptothecin isolated from these plants was always low. The

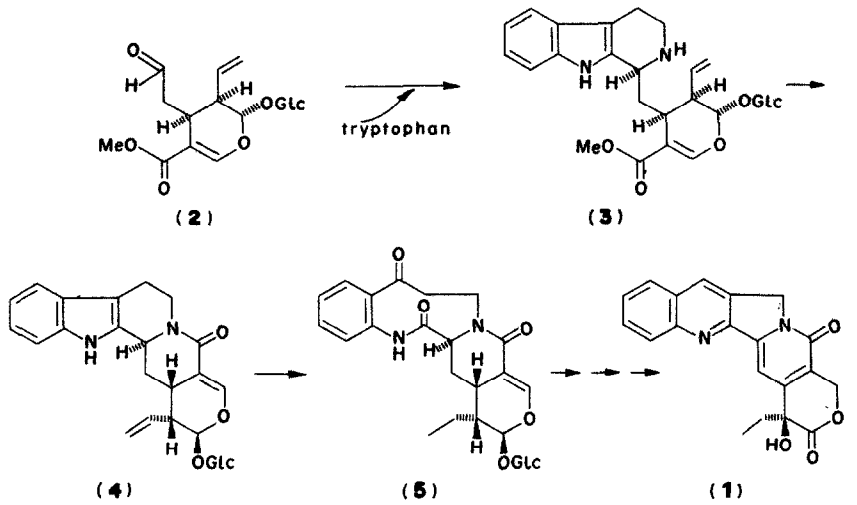


Fig. 1. Biosynthesis of Camptothecin.

approximately 4-fold higher activity of alkaloid isolated from 4-month old plants (expt. 2) as compared to 7–8-month old plants (expt. 1,3) may reflect the greater speed with which the tracer, after being absorbed by the much shorter roots of the 4-month old plants, reaches the site or sites of alkaloid biosynthesis (leaves and/or stems) in the plants.

This interpretation was supported by the finding that when the radioactively labeled precursors were injected directly into the stems of plants (expts. 5–9) a much greater amount of activity was found in the isolated alkaloid. From 25–100 fold increases in the incorporation of tryptophan into camptothecin were obtained when this precursor was administered to the plans via direct stem injection (expts. 6,7) as compared to root feeding.

The 2-fold greater incorporation of tryptophan into alkaloid in the experiments of 7-day duration (expts. 6,7) as compared to that of 3-day duration (expr. 5) may indicate that the incorporation of tryptophan into camptothecin is time-dependent. The equal % incorporation of tryptophan into alkaloid in both 11-month old plants (expt. 6) and 4-month old plants (expt. 7) may indicate that incorporation of tryptophan into camptothecin also is age-independent at least in the range of 4–11 months. Forty-three fold higher incorporation of mevalonate into alkaloid was observed when feeding was by direct stem injection in the experiment of 3-day duration (expt. 8) as compared to root feeding in that of 7-day duration (expt. 4). Experiment 9 indicates that the direct injection of [2-^{14}C]-geraniol[2-^{14}C]nerol into the stems of the plants still resulted in significant incorporation of these precursors into camptothecin despite the symptoms of severe toxicity which appeared in the plants.

Experiments 5, 6, and 7 show that when [Ar-4,5,6,7-$^{3}H_4$; 1′-^{14}C]-tryptophan was administered to *C. acuminata* plants, the radioactive alkaloid subsequently isolated contained virtually the same $^{3}H/^{14}C$ ratio as the tryptophan fed. These results indicate that there is no exchange or loss of tritium in the indole ring of [Ar-$^{3}H_4$; 1-^{14}C]tryptophan during its biosynthetic conversion to camptothecin. Furthermore the retention of the $^{3}H/^{14}C$ ratio in the isolated alkaloid is compatible only with the hypothesis that the entire [Ar-$^{3}H_4$; 1-^{14}C]tryptophan molecule, before or after decarboxylation to tryptamine, is incorporated intact during its bioconversion to the *C. acuminata* quinoline alkaloids. Thus these three separate feeding experiments demonstrate that tryptamine from fed tryptophan is specifically and completely incorporated into alkaloid, and together with the mevalonic acid and geraniol–nerol feeding experiments, support the proposal of the biogenesis of *C. acuminata* pentacyclic quinoline alkaloids as proposed in

Table 1. Incorporation of precursors into camptothecin isolated from *C. acuminata* plants, to which tracers were administered via hydroponic solution (HPS) or direct stem injection (DSI)

Expt. No./ duration (days)	No. of plants/ age (mo)/wet wt, g/ feeding method	Precursor fed*	Activity fed (dpm × 10^6) ^{3}H	^{14}C	Camptothecin isolated (mg)	Incorporation into camptothecin: dpm × 10^5/mM ^{3}H	^{14}C	% Incorpn ^{3}H	^{14}C
1/7	4/7/165/HPS	[1′-^{14}C]-Try	—	150	44·1	—	2·18	—	0·018
2/7	6/4/95/HPS	[Ar-$^{3}H_4$]-Try	430.	—	18·8	62	—	0·077	—
3/7	3/8/133/HPS	[1′-^{14}C]-Tre	—	282	31 3	—	6 25	—	0·020
4/7	3/8/72/HPS	[2-^{14}C]-Mev	—	163	16 7	—	1 70	—	0·005
5/3	1/9/31/DSI	[Ar-$^{3}H_4$, 1′-^{14}C]-Try	44 6	3·65†	6·5	246	20‡	1·034	1·030
6/7	1/11/135/DSI	[Ar-$^{3}H_4$, 1′-^{14}C]-Try	357	29·2†	47·5	508	41‡	1·941	1·914
7/7	6/4/90/DSI	[Ar-$^{3}H_4$, 1′-^{14}C]-Try	536	43 8†	25·1	1480	120‡	1·988	1·973
8/3	1/10/64/DSI	[2-^{14}C]-Mev	—	15·0	15 7	—	7 14	—	0·214
9/6	2/9/86/DSI	[2-^{14}C]Ger/ner	—	1 5	25 1	—	0·18	—	0·085

* Try = L-tryptophan; Tre = tryptamine; Mev = mevalonate; ger/ner = geraniol/nerol.
† Ratio $^{3}H/^{14}C \sim 12·2$.
‡ Ratio $^{3}H/^{14}C \sim 12·4$.

Fig. 1. If [Ar-$^3H_4$; 1'-^{14}C]tryptophan had been utilized in accordance with this biogenetic postulate (Fig. 1) the sites of labeling would be expected to be C-6-^{14}C and C-9, 10, 11, and 12-$^3H_4$ in camptothecin. The incorporation of [2-^{14}C]DL-mevalonic acid would lead to labeling at C-3,16a, and 17 while that of [2-^{14}C]geraniol/nerol would give alkaloid labeled at C-20.

EXPERIMENTAL

Administation of radioactive precursors to C. acuminata *plants and isolation of camptothecin. C. acuminata* seedlings, 6 months old, which had been dormant state for 3 months, were regrown in aerated hydroponic soln. Seeds were planted in soil and grown for 2 months, then transferred to the hydroponic soln. In expts 1–4 the radioactive precursors were administered to 4–8 month old plants hydroponically and the absorption of the radioactivity by the roots was followed by its disappearance from the solution at intervals of *ca* 12 hr during the exposure periods of 30–72 hr (Table 1). In experiments 5–9, the precursor was injected into the stems of the plants. After 3–7 days the plants were harvested and macerated in a Waring blendor with 95% EtOH (3·3 ml/g). Residue was Soxhlet extracted (95% EtOH) for about 48–72 hr. The combined extracts were evaporated and residue partitioned 4–5× between $CHCl_3$ and H_2O (3:1). Combined $CHCl_3$ extracts were evaporated and the residue dissolved in 20% MeOH–$CHCl_3$ and fractionated by preparative TLC on Si gel G in $CHCl_3$–Me_2CO–MeOH (34:5:1). Camptothecin (R_f 0·4–0·5), located by its UV fluorescence, was eluted with 20% MeOH–$CHCl_3$. The camptothecin was purified to constant specific activity by repeated crystallization from EtOAc and 20% MeOH–$CHCl_3$; the sp. act. being determined by a combination of UV absorption and liquid scintillation.

Preparation of [*Ar-4,5,6,7-$^3H_4$*]L-*tryptophan.* The aromatic hydrogens of tryptophan were exchanged with deuterium to establish both the synthetic and analytical method, using NMR absorption to monitor the reaction. In trifluoroacetic acid (0·1 g L-tryptophan + 1 ml TFA) tryptophan gives rise to three multiplets; one at δ 6·8–8·4, which integrates for 9H's; 6H's from the indole ring and the other from the $-NH_3$ group; the other two multiplets are at δ 4·58 and δ 3·63, integrating for 1H and 2H's respectively, and corresponding to methine (C-2') and methylene (C-1') hydrogens of the side chain. To 5 ml of TFA anhydride and 0·67 ml of ^2H_2O was added 0·5 g of L-tryptophan. Exchange with deuterium was followed by NMR for 73 hr at room temp. and at 8, 20, 29 and 53 hr the TFA-^{2}H was removed and fresh TFA-^{2}H was added. 45 Min after the first addition, the multiplet at δ 6·8–8·4 integrated only for 4H's [Ar-4,5,6,7-H], hence the 5 more labile hydrogens, [Ar-1,2-H, and $-\overline{NH}_3$) are exchanged very rapidly. At 8, 20, 29, 53 and 73 hr this aromatic multiplet integrated for 2·1H, 1H, 0·62H, 0·34H, and 0·3H respectively. Therefore, after 73 hr of exchange >95% of the starting material was converted to [Ar-^{2}H]L-try. Methine and methylene hydrogens of the side persisted unchanged during the 73 hr, and they were used as a reference for the estimation of the unexchanged hydrogens in the aromatic region. To exchange the labile deuterium [Ar-1,2-^{2}H, $-^2H_3N^+$, $-CO_2{}^2H$], the TFA-^{2}H was evaporated and 5 ml of TFA was added; this treatment was repeated at 55 min. NMR examination at 45 min, 1:10 hr, and 1:30 hr showed that the aromatic region multiplet integrated for 4H's, 4·67H's and 5·57H's, respectively which is interpreted to indicate that all 5 labile deuteriums [Ar-1,2-^{2}H and $-^2H_3N^+$] were exchanged and the desired [Ar-4,5,6,7-^{2}H]L-tryptophan was obtained. The rapid conversion of L-try to [Ar-2-^{2}H]L-try and its reversal was followed by the disappearance and appearance of the singlet at δ 7·21. Residue, after evaporation of TFA, was dissolved in 3 ml of 10% NaOH, and the cooled alkaline soln acidified to pH 5 with glacial HOAc. [Ar-4,5,6,7-^{2}H]L-tryptophan which separated from the soln was filtered and recrystallized from 35% EtOH; recovery, 30 mg, 6%, mp 278–282°. In a similar way [Ar-4,5,6,7-^{3}H]L-tryptophan was obtained by treating 5 ml frozen TFA anhydride with 0·67 ml of [^{3}H]H_2O (40 Ci/ml). The mixture was shaken for 10 min and 0·5 g L-tryptophan was then added and the soln stored at room temp. for 48 hrs. At the end of this period, the solution was frozen and the [^{3}H]TFA was evaporated in high vaçuum and trapped (liquid N_2). Inactive TFA was added to exchange out the labile ^{3}H [Ar-1,2-^{3}H, $-^3H_3N^+$ and $-CO_2{}^3H$] of tryptophan and after 30 min the soln was frozen and TFA was evaporated as before. The exchange was repeated 3×, the residue of [Ar-4,5,6,7-$^3H_4$]L-tryptophan was dissolved in 3 ml of 10% NaOH, shaken thoroughly then filtered, and filtrate adjusted to pH 2·5 with HCl and purified on Dowex 50W-X8 resin (25 g, H^+ form). The labeled tryptophan was crystallized from 35% EtOH; recovery, 25 mg, 5%; mp 279–282°; sp. act. 3·4 mCi/mg. For further purification, radioactive L-tryptophan (25 mg) was dil with an equal amount of inactive L-try and rechromatographed and purified as above. The final product was recrystallized from 50% EtOH; (10 mg), mp 281–284°; sp. act. 1·8 mCi/mg.

Preparation and administration of [*2-^{14}C*]*geraniol–nerol.* A crude mixture of [2-^{14}C]geraniol–nerol (1 g, $6·3 \times 10^4$ dpm/mg) was prepared [17,28] from methyl [2-^{14}C]bromoacetate (0·25 mCi) and 6-methyl-5-hepten-2-one. The pure mixture of geraniol and nerol (195 mg, 1:1, 1×10^5 dpm/mg) was obtained from the crude mixture of these *cis* and *trans* isomers by preparative GLC (5% QF-1 on 80–100 Chromosorb, W, 139°). A mixture of 15 mg of pure geraniol–nerol was emulsified in H_2O (60 mg) with Tween 80 and administered via stem injection to two 9-month-old *C. acuminata* plants. The toxicity of these monoterpenoids was clearly apparent as the plants died slowly during a period of 6 days, after which the plants were harvested and alkaloid was isolated (expt. 9, Table 1).

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