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Biosynthesis of Camptothecin. 3. Definition of Strictosamide as the Penultimate Biosynthetic Precursor Assisted by ^{13}C and ^{2}H NMR Spectroscopy

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Abstract: The biosynthesis of camptothecin (**1**) is shown to involve tryptamine (**2**) and secologanin (**3**) by radioactive precursor feeding experiments with *Camptotheca acuminata* apical cuttings. The incorporation of radioactive strictosidine (**4**), but not vincoside (**16**), into **1** is consistent with the results of other studies that have proven **4** to be the key biogenetic precursor of monoterpene indole alkaloids found in other higher plants. Strictosamide (**5**) is the penultimate biosynthetic precursor of **1** in vivo as demonstrated by validation of the stereo- and regiospecific incorporation of [5-^{13}C]- and [14-^{2}H]**5a** with ^{13}C and ^{2}H NMR spectroscopic analysis. These two experiments serve to define the lower limits for the successful use of stable isotopes in biosynthetic studies in plants. Three possible explanations for the unexpected low loss of ^{3}H during the incorporation of [14-^{3}H,5-^{14}C]**5a** into **1** in vivo are examined experimentally. Since **1** is shown to be labeled only at C-14 by [14-^{2}H,^{3}H]**5a**, and since only a 5–9% loss of ^{3}H from C-14 of **5a** is observed regardless of the labeling stereochemistry of the C-14 diastereotopic hydrogens, it is concluded that the loss of ^{3}H from C-14 is by a nonstereospecific process. Several literature precedents and the results of oxidation of [14-^{3}H,5-^{14}C]**5** with DDQ to the [^{3}H,^{14}C]indolopyranoquinolizinones, **25** and **26**, in vitro are consistent with the low tritium loss being due to a significant isotope effect associated with a nonenzymatic step during the D ring aromatization of **5** in vivo.

A study of the biosynthesis of camptothecin (**1**), a pyrrolo[3,4-*b*]quinoline alkaloid found originally in the mainland China tree, *Camptotheca acuminata* Decne, and later in *Mappia foetida* Miers[4] and *Ophiorrhiza mungos* L.,[5] has been underway in our laboratory since 1971. The results of several initial feeding experiments with isotopically labeled precursors

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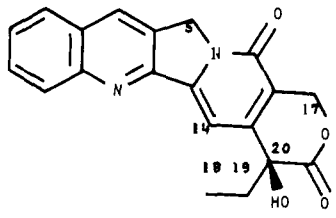

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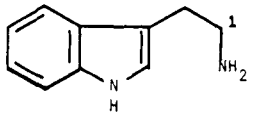

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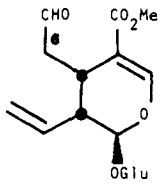

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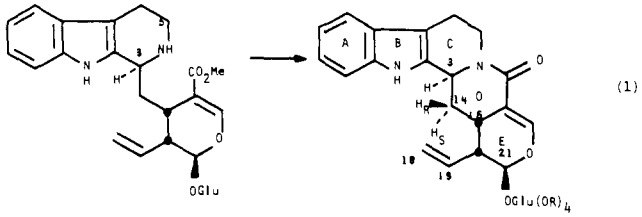
and apical cuttings of *C. acuminata* have been summarized.[6] These experiments and those of Sheriha and Rapoport[7] support our proposal[6] that **1** is derived from tryptamine (**2**) and secologanin (**3**), established biosynthetic precursors of monoterpene indole alkaloids found in several other higher plants.[8] A formal Pictet-Spengler condensation of **2** and **3** in vivo gives strictosidine (**4**),[9] whose intramolecular cyclization yields strictosamide[10] (**5**), eq 1. The latter neutral glucoside is the



(1)

4

16, 3(R) epimer

5a, R = H

5b, R = CO_2Me

15, 3(R) epimer

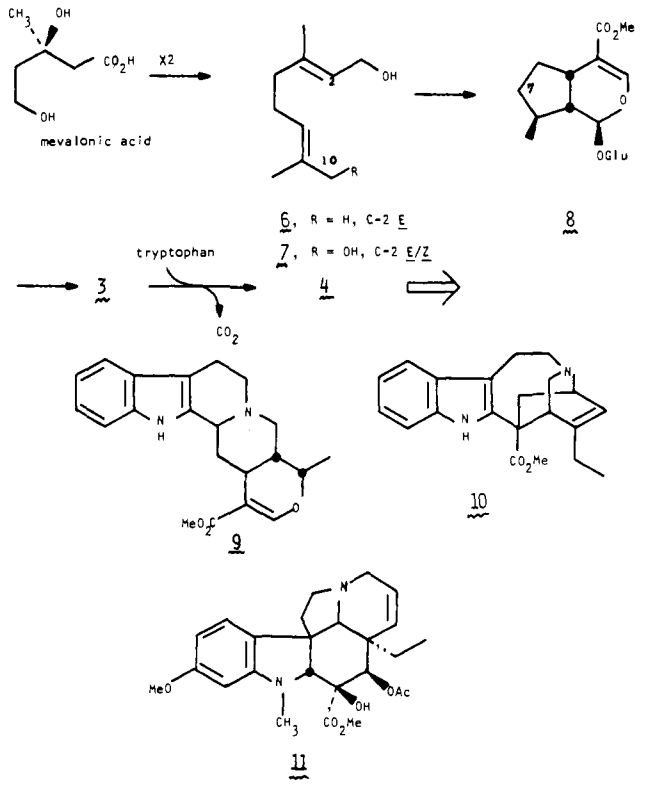
penultimate precursor of **1** in *C. acuminata*, as shown by its regiospecific and stereospecific incorporation into **1** in singly (^2H;^{13}C) or doubly (^3H,^{14}C) labeled form.[6,11]

The incorporation of (−)-[14-^{3}H,5-^{14}C]**5** into **1** in vivo is attended by only a 5-9% loss of tritium relative to the carbon-14 reference label[6,11] despite the apparent requirement that one hydrogen label be removed from C-14 of **5** during formation of the pyridone ring of **1**. Several explanations of this observation are conceivable, but our original data did not permit a distinction to be made. The purpose of the present account is to detail our initial[6] and subsequent[11] observations about the precursor-product relationships for the early stages of camptothecin's biosynthetic pathway. The incorporation of [14-^{2}H,14-^{3}H]**5** into **1** in vivo has been reanalyzed with the aid of ^{2}H NMR spectroscopy, which enables us to propose an explanation for the low loss of isotopic hydrogen label from C-14.

Prestrictosamide Biosynthetic Precursors of 1

Investigations of monoterpene indole alkaloid biosynthesis in higher plants, carried out primarily by the research groups of D. Arigoni, A. R. Battersby, and A. I. Scott, have led to a detailed picture for the formation of alkaloids such as ajmalicine (**9**), catharanthine (**10**), and vindoline (**11**) in *Catharanthus roseus* G. Don.[8] The primary metabolite, tryptophan,[12] and secondary monoterpene metabolites such as geraniol (**6**),[13] 10-hydroxygeraniol/nerol (**7**),[14] 7-deoxyloganin (**8**),[15] and secologanin (**3**)[16] were shown to be involved in the early stages of the biosynthetic pathway leading to **9**–**11** in vivo (Scheme

Scheme I



I). Most importantly, strictosidine (**4**) was proven to be the penultimate biosynthetic intermediate from which the structurally more complex *Catharanthus* alkaloids, **9**–**11**, are derived by a fascinating series of chemical and biochemical transformations.[8,17]

The results of some of the aforementioned experiments have been confirmed quite recently in vitro using cell-free systems obtained from tissue cultures[18] and mature plants[19] of *C. roseus*. The latter experiments clarified the role of strictosidine (**4**) in the biosynthesis of **9**[20] and other indole alkaloids.[9,11,21]

Although it is not immediately obvious from the structure of camptothecin that it could be derived biosynthetically from tryptophan and a monoterpene, Wenkert and co-workers speculated in 1967[22] that **1** in fact might be a monoterpene indole alkaloid using plausible chemical transformations of isositsirikine (**12**) as the basis from which to formulate a biogenetic scheme for **1**. Winterfeldt[23] later expanded on this idea based on his own finding that **9** underwent facile autoxidation to **13** in vitro, and proposed that geissoschizine (**14**) was a plausible biogenetic precursor of **1**. His concept eventually was applied in the execution of a novel and efficient biomimetic total synthesis of **1**.[24]

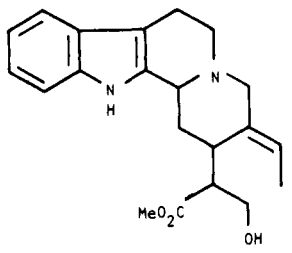
Our biogenetic reasoning took these two ideas into account, but also recognized the clear structural relationship between **1** and strictosamide (**5**). The latter neutral glucoside was known as a transformation product of strictosidine (**4**) under basic conditions.[9b] [At the time, **4** and **5** were named isovincoside and isovincoside lactam, respectively.[9b] In view of more recent developments,[20,21] **4** and **5** should now be named as originally chosen by Smith.[9a,10]]. Thus, transformation of **5** into **1** was considered by us to be possible via three basic transformations: ring BC oxidation-recyclization, ring D oxidation, and removal of the C-21 glucose moiety followed by ring E oxidation.[6] This biogenetic hypothesis has also been proposed independently by Cordell.[25]

We established in initial trial feeding experiments that radioactively labeled tryptophan was incorporated into **1** in apical

Table I. Incorporation of Radioactively Labeled Precursors into Camptothecin (**1**) in Vivo

expt	precursor (^3H:^{14}C ratio)	radioact. fed[a]	radioact. of isolated[b] **1**	total incorp[c], %
1	[5-^{3}H]**5**	1.66×10^7 (6.27×10^8)	8040 (3.5×10^5)	1.3
2	[14-^{3}H]**5**	5.27×10^7 (2.30×10^9)	1.9×10^5 (9.3×10^6)	1.9
3	[14-^{3}H,5-^{14}C]**5** (11.51)	3.73×10^7 (^{3}H) (1.11×10^9)	1.6×10^5 (2.6×10^6, ^{3}H)	2.0
4	[14-^{3}H;5-^{14}C]**5** (2.55)[e]	3.36×10^7 (^{14}C) (9.31×10^8)	4.8×10^5 (9.8×10^6, ^{14}C)	1.1
5	[Ar-^{3}H]**14**	4.84×10^7	(2.35×10^4)	0.002[d]
6	[Ar-^{3}H]**14**	$1.51' \times 10^7$	(2.4×10^4)	0.012[d]
7	[5-^{3}H]**15**	1.24×10^8	(2.5×10^4)	0.006[d]
8	[5-^{3}H]**15**	7.71×10^7	7320 (1.9×10^5)	0.006
9	[14-^{3}H,5-^{14}C]**15** (10.2)	1.97×10^6 (^{14}C) (1.01×10^8, ^{3}H)	(9.4×10^4, ^{3}H)	0.05[d]
10	[14-^{3}H]**4**	2.01×10^8	2.4×10^5 (2.9×10^6)	0.12
11	[14-^{3}H]**16**	2.26×10^8	3.0×10^4) (4.2×10^5)	0.013[f]

[a] Disintegrations per minute; values in parentheses are dpm/mmol. [b] Disintegrations per minute; values in parentheses are average dpm/mmol of three crystallizations. [c] Total dpm of ^{3}H (or ^{14}C) of **1** isolated divided by total dpm of precursor fed times 100. [d] Crystalline **1** did not have a constantly reproducible specific radioactivity. [e] This ^{3}H:^{14}C ratio was secured by conversion of an aliquot sample of [14-^{3}H,5-^{14}C]**5** to its crystalline tetraacetylquinolol derivative.[43] [f] This apparent radioactivity incorporation was due to contamination by **4** (Experimental Section).



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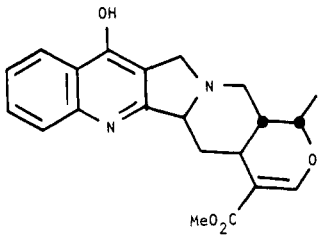

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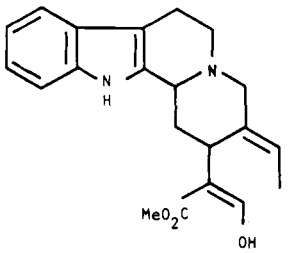


14

cuttings of young seedlings of *C. acuminata* to the extent of 4×10^{-4} to 2.6×10^{-2}%.[26] Similarly, radioactively labeled mevalonic acid and [6-^{3}H]secologanin (**3**) were found to give rise to radioactive **1** in vivo.[26] These incorporations of radioactivity into **1** were rather low and could not be confirmed as regiospecific because of a lack of suitable degradative chemistry for radioactive label localization. Sheriha and Rapoport[7] subsequently confirmed our initial observations using 8-month old *C. acuminata* seedlings. They found that singly and doubly labeled radioactive precursors gave the following total incorporations into **1**: tryptophan (1.9%), tryptamine (0.02%), mevalonate (0.2%), and a geraniol/nerol isomeric mixture (0.08%). Again, the lack of a suitable degradative chemistry prevented rigorous validation of these radioactivity incorporations, although it is very likely that their results reflect true biosynthetic precursor-product relationships.

Since our initial results strongly indicated that **1** was a monoterpene indole alkaloid, the C-3 epimeric mixture of strictosidine and vincoside ((3*R*)-**4**), tritium labeled at C-5 by synthesis from [1-^{3}H]tryptamine, was then tested as a precursor of **1**. The observed total radioactivity incorporation into **1** of 0.24% supported the implications of our initial results.

At this juncture three possibilities for the conversion of **4** into **1** in vivo had to be considered: via **5**, via **12**, or via **14** (vide supra). Since radiochemically labeled **5** and **14** (the latter a generous gift from Professor A. I. Scott) were available, two of these possibilities could be tested. In the event, it was immediately clear from its efficient incorporation into **1** (Table I, experiments 1 and 2) that only **5** need be considered for further experimentation in the elucidation of camptothecin's biosynthetic pathway.

When these feeding experiments were being designed, the available literature data[8,9b] indicated that vincoside was the precursor of monoterpene indole alkaloids. Based on this biogenetic analogy, vincoside ((3*R*)-**4**, denominated as **16** in Table I) and thus vincoside lactam, (3*R*)-**5** (**15** in Table I), would have been expected to be better precursors of camptothecin than are either **4** or **5**. However, the incorporation of **15** into **1** was quite low relative to that of **5** despite repeated experimentation (Table I, experiments 7-9), and a subsequent single experiment[11] showed that **16** also was a poorer precursor of **1** than is **4**. At the time[6] these observations were not felt to be remarkable, since *C. acuminata* (Nyssaceae) is not related taxonomically to *C. roseus* (Apocynaceae),[27] and thus complete stereochemical homogeneity between biosynthetic pathways in the two organisms need not be expected. Recent events, however, established that strictosidine (isovincoside) is the key precursor of monoterpene indole alkaloids in *C. roseus* and other plant genera.[20,21] Our results therefore are seen to be completely consistent with the developing picture of monoterpene indole alkaloid biosynthesis among those higher plants which have been studied experimentally.[8]

Strictosamide, the Penultimate Biosynthetic Precursor of 1

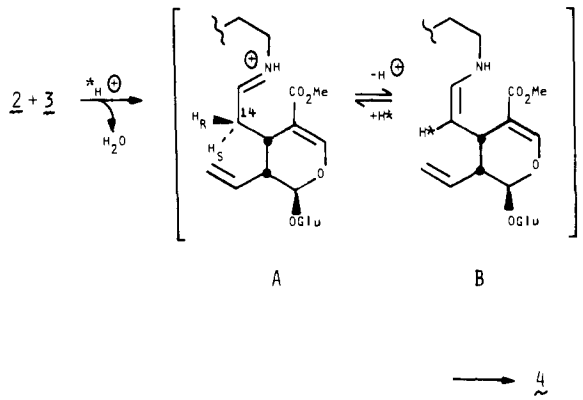
Although the results of the feeding experiments discussed above strongly support the role of strictosamide (**5**) as the key biosynthetic precursor of **1**, the regiospecificity of its incorporation into **1** had to be ascertained.[28] In spite of the lack of suitable degradative chemistry, the efficient incorporation of radioactive **5** into **1** suggested that the labeling regiochemistry could be determined directly by NMR spectroscopic analysis. Since the specific incorporation of **5** into **1** was between 1 and 2% (Table I, experiments 1-3), we felt that a ^{13}C-labeled **5** containing ≥85 mol % ^{13}C enrichment could result in the minimally permissable 50% peak height enhancement of an appropriate carbon signal in the ^{13}C NMR spectrum of **1**. [1-^{13}C]Tryptamine was synthesized by reaction of [^{13}C]cyanide with gramine to give [1'-^{13}C](3-indolyl)acetonitrile. Reduction with the $CoCl_2$–$NaBH_4$ catalyst of Satoh et al.[32] gave a 53% yield of [1-^{13}C]tryptamine (**2**), isolated as its hydrochloride salt.[33] [5-^{13}C]Strictosamide (**5a**) was prepared from this amine salt by literature procedures.[9b] Mass spectral analysis of the tetraacetate of [5-^{13}C]**5a** showed that it contained 83.9 mol% excess ^{13}C; ^{13}C NMR analysis of the same tetraacetate sample located the ^{13}C label at C-5 (δ_C 43.4) of **5**, as expected.[34] The remaining [5-^{13}C]**5a** (38 mg) was fed by the wick technique as a water suspension (Tween 20) to seven intact *C. acuminata* plants growing under artificial illumination in a glasshouse. After 19 days of further metabolism, **1** (21 mg) was isolated and purified by standard procedures.[26] The proton noise-decoupled ^{13}C NMR spectrum of labeled **1** showed that only the resonance corresponding to C-5[6] had been significantly enhanced (55%) using the height of the C-17 methylene signal as the internal reference. This observed enhancement corresponds to a specific ^{13}C incorporation of ca. 0.9%, in good agreement with the specific incorporations observed for [5-^{14}C]**5a** in separate radioactive feeding experiments.[35] Consequently, the requisite certification of the role **5** plays as a specific biosynthetic precursor of **1** was firmly established.

Earlier incorporations of [14-^3H,5-^{14}C]**5a** into **1** had been attended by only a 5-9% decrease in the $^3H/^{14}C$ ratio (Table I, experiments 3 and 4). This was a surprising finding, since **5** was expected to lose ca. one-half its C-14 ^3H labeling during oxidative formation of the pyridone ring of **1**.[30]

Three explanations for this low percentage ^3H loss were considered: (1) **5** might fortuitously have been stereospecifically labeled with ^3H at C-14 and oxidation to **1** might remove hydrogen from only the unlabeled diastereotopic position; (2) conversion of **5** into **1** might involve an intramolecular migration of one of the two C-14 tritium atoms to another site in some biosynthetic intermediate leading to **1**, resulting in retention of both labels; or (3) loss of hydrogen from C-14 during oxidation of the D ring of "**5**"[31] to the pyridone ring of **1** might be both nonstereospecific (and therefore nonenzymatic) and subject to a significant kinetic isotope effect discriminating against tritium removal. (Stereospecific enzymatic hydrogen removal would result in a 50% loss of ^3H in spite of any kinetic isotope effect.[30])

Each of the above alternatives was examined in turn. Stereospecific labeling of **5** at either the H-14 *pro-R* or H-14 *pro-S* diastereotopic positions would have had to occur when ^3H label was introduced during the synthesis of **4**. Since the labeling methodology introduced ^3H by C-14 protonation of an intermediate enamine (B), formed under weakly acidic conditions from tryptamine (**2**) and secologanin (**3**), eq 2, it is conceivable that a highly ordered transition state for the A ⇆ B process might result in stereospecific labeling of either H-14 *pro-R* or H-14 *pro-S* of **4**. This possibility was eliminated from further consideration by the following spectroscopic data.

(a) A sample of [14-^2H]**5a** was prepared by carrying out the



condensation of **2** and **3** in acetic acid-d_1, followed by conversion of the crude mixture of **4** and **16** to tetraacetyl-**5a** and -**15a**. Chromatographic separation gave deuterated **5a** tetraacetate (28.5% d_1, 53.2% d_2), whose proton noise-decoupled ^{13}C NMR spectrum showed a weak multiplet resonance ca. 10 Hz upfield of the small C-14 singlet resonance at δ_C 26.3.[34] Since the signals of all other carbons were undiminished in intensity, only the H-14 protons of **5** were significantly replaced by deuterium by the labeling methodology used.

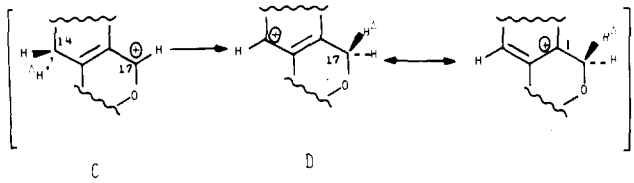
(b) The 270-MHz ^1H NMR spectra of tetramethoxycarbonyl strictosamide (**5b**) and tetramethoxycarbonyl vincoside lactam (**15b**) were recorded in $CDCl_3$ and fully assigned by appropriate double irradiation experiments (Experimental Section). The 14 *pro-R* hydrogen of **15b** appears as an apparent six-line multiplet at δ_H 2.22, and its 14 *pro-S* hydrogen as an apparent quartet at δ_H 1.62. Since the solution conformation of the corresponding tetraacetyl-**15a** is well defined,[34] the coupling relationships of these two diastereotopic hydrogens with H-3 and H-15 of **15b** can be interpreted clearly, thus permitting their secure assignments. The diastereotopic C-14 hydrogens of **5b**, however, while nearly resolved at high spectrometer field, appear as a 16-line multiplet with two approximate centers at 2.14 and 2.21 ppm. We interpret this multiplet as an ABXY system, in which H-3 at δ_H 4.98 and H-15 at δ_H 2.74 are the Y and X components, respectively. From the results of decoupling experiments (Experimental Section), $^3J_{14,15} \cong 4$ Hz and $^3J_{3,14} = 2.5$ and 4.7 Hz, leaving ~11 Hz for the geminal coupling constant of the hydrogens attached to C-14 of **5b**. These vicinal coupling constants are consistent with a slightly flattened chair conformation of the D ring in **5b**, as deduced earlier from the ^{13}C NMR data of the corresponding tetraacetyl-**5a** and -**15a**.[34] The 14 *pro-S* hydrogen of **5b** thus is axial, gauche to H-3 and approximately anti to H-15; the 14 *pro-R* hydrogen is equatorial, almost eclipsed by H-3 and gauche to H-15. In **15b** a similar relationship obtains, which is why the axial 14 *pro-S* hydrogen (δ 1.62) is shifted far upfield relative to the equatorial 14 *pro-R* hydrogen (δ 2.22). Since the C–H bonding orbital of the 14 *pro-S* hydrogen is nearly parallel with the π orbitals of the C-16a carbonyl, this proton experiences a strong shielding effect from these electrons. ^1H NMR spectral data[36] for the corresponding positions in a structurally related indole alkaloid, vallesiachotamine, can be referred to for additional support of our interpretation.

Samples of [14-^2H]**5b** and -**15b** were prepared from a C-3 epimeric mixture of **4** (vide supra) and their ^1H NMR and electron-impact mass spectra determined. Using the appropriate unlabeled compounds for reference standards as necessary, it could be shown by mass spectrometry that the M^+· ions of the sample of [14-^2H]**5b** consisted of 31 mol % d_0, 31 mol % d_1, and 38 mol % d_2 species, corresponding to an average of 53 mol % ^2H per labeled site (Experimental Section). Similarly, [14-^2H]**15b** contained 38 mol % d_0, 27 mol % d_1, and 35 mol % d_2 species, or 49 mol % ^2H per labeled site. Integra-

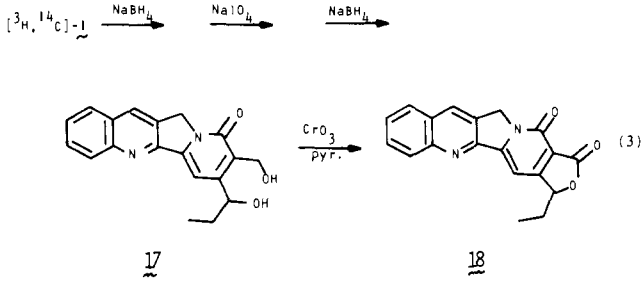
tion of the appropriate proton resonances of **5b**, using the averaged signal area of H-15 and H-20 as an internal reference, showed signals corresponding to ca. 0.38 H and 0.43 H in the upfield and downfield portions, respectively, of the δ_H 2.1–2.2 region. The averaged ^2H labeling at C-14 must therefore be 100 − 40 = 60% per potentially labeled site. Similarly, **15b** contained ca. 0.44 H at δ_H 1.62 and 0.38 H at δ_H 2.22, or 59% ^2H per potentially labeled site at C-14. Both of these values for ^2H enrichment are in acceptable agreement with the ^2H enrichment data obtained from the mass spectral analysis. Moreover, it is clear that the diastereotopic hydrogens attached to C-14 of **5** or **15** have been equally labeled within experimental error by the labeling methodology used. Assuming that the distribution of ^3H label at these two positions will parallel the established ^2H labeling, when both isotopes of hydrogen are present in the labeling reaction milieu, we conclude that the samples of [14-^{3}H,5-^{14}C]**5a** used in the biosynthetic feeding experiments were equally ^3H labeled intermolecularly at the diastereotopic hydrogens attached to C-14.

(c) ^2H NMR analysis confirmed directly that deuterium label was present only at C-14 of **5**. The 41.44-MHz ^2H NMR spectrum of a sample of tetraacetyl-**5a**, derived from the [14-^{2}H,14-^{3}H]**5a** used in the final feeding experiment described below, clearly showed only one signal corresponding to the H-14 deuteriums as an unresolved broad resonance at δ_D 2.1 relative to the methylene deuterons of CD_2Cl_2 at 5.35 ppm.

The results of the above ^{13}C, ^1H, and ^2H NMR studies and mass spectral analyses clearly show the nonstereospecific labeling of **5** at C-14. We next examined the possibility that the retention of ^3H in the conversion of **5** to **1** was due to an intramolecular tritium migration. To this end radioactive camptothecin, obtained from experiment 4, Table I, was subjected to chemical degradation. We reasoned that the only position to which a ^3H label originating at C-14 of some biosynthetic intermediate between **5** and **1** might migrate would be C-17. For example, this might have occurred by a hydride shift of one of the C-14 protons via some charged intermediate (e.g., C → D), although we recognize that the molecular geometry of C and D depicted would mitigate against the desirable cisoid transition state for a concerted, intramolecular [1,4] sigmatropic shift.[37] Such a possibility was ruled out by degradation of the sample of [^{3}H,^{14}C]**1** to **18** (eq 3). Since **18**



C D



17 18

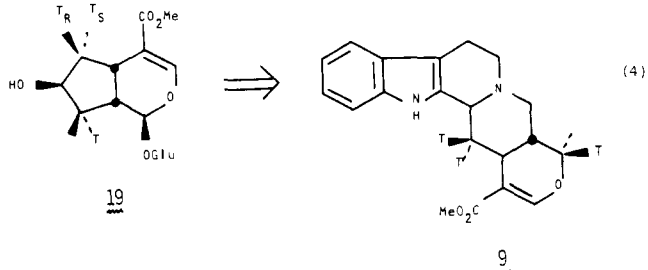
(3)

contained 97% of the molar ^3H content of **1**, it is clear that ^3H migration from C-14 to C-17 does not occur during the conversion of **5** into **1** in vivo.

The site of labeling in camptothecin derived from H-14 labeled **5** was established unambiguously by the use of ^2H NMR. A sample of [14-^{2}H,14-^{3}H]**5a** (5.3×10^7 dpm ^3H/mmol) was prepared as before. Mass spectral analysis of its tetraacetate showed the labeled material to consist of a mixture of 55 mol % d_0, 30 mol % d_1, and 15 mol % d_2 species. A quantity of this [14-^{2}H,14-^{3}H]**5a** (100 mg, ca. 5 μCi) was fed to 72 apical cuttings of *C. acuminata*, from which [^{2}H,^{3}H]**1** (ca. 100 mg) was isolated after a 14-day metabolism period. The isolated **1** was converted to its 20-methylthiomethylene derivative (20-MTM) for ease of recrystallization and ^2H NMR analysis. By radioactivity measurements, the specific incorporation of ^3H into **1** was found to be 0.57%, significantly lower than the analogous earlier incorporation of [5-^{13}C]**5a**. The broad-band proton-decoupled ^2H NMR spectrum of 20-MTM-[^{2}H,^{3}H]**1** showed only one significant signal at δ_D 7.48 (internal standard, methylene-d_1 chloride, δ_D 5.35). This resonance corresponds to that of H-14 in 20-MTM-**1**. Comparison of the signal area of the peak at δ 7.48 with that due to natural abundance deuterium in the solvent (CH_2Cl_2) indicated the presence of 0.24% deuterium at C-14 of [^{2}H,^{3}H]-20-MTM-**1**. Since the precursor [14-^{2}H,^{3}H]**5a** had been shown to contain 45% deuterium, the specific incorporation of **5** and **1** corresponds to 0.53%. This value is close to that calculated from the tritium enrichment. These results establish that [14-^{2}H,14-^{3}H]strictosamide labels *only* H-14 of camptothecin in vivo.

Although the deuterium enrichment achieved was significantly lower than the corresponding level in the experiment with the ^{13}C-labeled precursor, nonetheless the ^2H NMR technique was sufficiently sensitive to detect the resultant signal unambiguously. For example, the 35 mg of 0.24% enriched [14-^{2}H]-20-MTM-**1** (0.2 M in CH_2Cl_2) gave rise to a S/N ratio of ca. 6 to 1 after 35 000 transients. This signal, while near the lower limit for detection, is adequate for meaningful biosynthetic deductions, particularly when interpreted in conjunction with data obtained from parallel radioisotopic feeding experiments. Whereas biosynthetic experiments involving ^{13}C NMR are limited by the necessity of overcoming a natural abundance of 1.1%, no such problem exists with ^2H NMR (^2H natural abundance, 0.015%). The major limitation for the latter technique is the ability to detect signals with acceptable S/N ratios. The current lower limit would appear to be 0.1 to 0.2% deuterium enrichments for biosynthetically derived natural products. Such experiments as those described above illustrate the value of stable isotopes for biosynthetic investigations in higher plants as well as in microorganisms.

We had therefore established that the precursor [14-^{2}H or ^{3}H]-**5** was nonstereospecifically labeled at C-14, and at no other position, and that the product **1** was labeled only at C-14. These conclusions were further corroborated by an independent series of feeding experiments. Although stereospecifically C-14 labeled strictosamide (**5**) was not available, an indirect route to its partially stereospecific C-14 labeling was possible. Prior work carried out in A. R. Battersby's laboratory at Cambridge[38] has proven that [6,8-$^3H_3$,OCH_3-^{14}C]loganin (**19**) is incorporated into ajmalicine (**9**) in vivo (apical *C. roseus* cuttings) without significant loss of ^3H labeling relative to the ^{14}C reference label (eq 4). Since **19** must be converted to



19 9

(4)

strictosidine (**4**) in vivo during its incorporation into monoterpene indole alkaloids,[8] we reasoned that [6,8-$^3H_3$]**19** should label camptothecin (**1**) in *C. acuminata* via **4** and **5**. Hence, feeding of [6,8-$^3H_3$]**19** to the apical cuttings was equivalent

to feeding [14,19-$^3H_3$]**5a**. Furthermore, the 6 *pro-R* position of **19** could be shown by careful chemical degradation to contain about the same amount of ^3H label as at the C-8 position, but ca. four times the amount of ^3H label at the 6 *pro-S* position.[38,39] This corresponds to a ca. 4:1 ratio of ^3H labeling at the 14 *pro-R* and *pro-S* positions, respectively, in **5a** produced from **19** in vivo. In the event, feeding of [6,8-$^3H_3$]**19** (437 μCi) to *C. acuminata* gave radioactive **1** (0.011% absolute incorporation), which was subjected to a modified Kuhn-Roth oxidation to obtain carbons 18-20 as propionic acid, and carbons 18-19 as acetic acid. Since [6,8-$^3H_3$]**19** should have led to ^3H labeling at C-14 and C-19 of **1** by analogy to its demonstrated labeling regiochemistry in other monoterpene indole alkaloids,[38,39] the propionic acid (isolated as its *p*-bromophenacyl ester) could have represented ca. 45% of the relative molar ^3H radioactivity of **1**. That is, the C-8 labeling of **19** was expected to reside at C-19 of **1** and *if* no loss of ^3H label from C-14 of biosynthetic intermediates between **5** and **1** had occurred (vide supra), the molar ^3H labeling ratio of H-14 to H-19 of **1** should have been identical with that of H-6 to H-8 (5.5:4.5) in **19**. Since the isolated propionic acid ester contained 43 ± 0.8% of the molar radioactivity of **1**, whereas the acetic acid ester contained only 8 ± 0.7%, our biogenetic presumption is proven to be valid. Consequently, we had gained indirect evidence that the mechanism of D ring oxidation of some biosynthetic intermediate between strictosamide and camptothecin in vivo does not involve significant stereospecific loss of hydrogen from the C-14 position.

An attempt was made to corroborate the above experimental observations through synthesis and separate feedings of (3*RS*)-, (5*R*)-, and (5*S*)-[5-^3H,2-^{14}C]mevalonic acid to *C. acuminata*.[26,39] Although radioactive camptothecin could be obtained from these experiments, the correlation between the $^3H/^{14}C$ ratio of the administered mevalonate and the isolated camptothecin did not permit clear biosynthetic deductions to be made. Since the samples of strictosamide also isolated in these feeding experiments were almost radioinactive, the observed radiochemical labeling of **1** by the labeled mevalonate probably was not regiospecific, i.e., random.

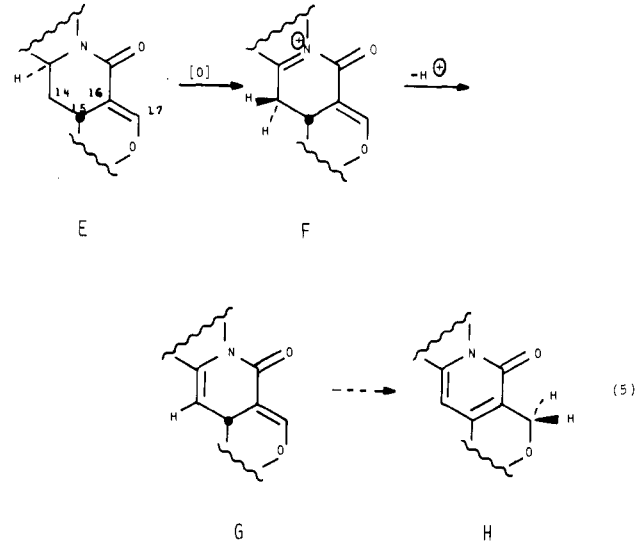
Discussion

We have demonstrated by a series of in vivo feeding experiments that camptothecin (**1**) is a monoterpene indole alkaloid in the biogenetic sense. The results of our experimental investigations thus validate the earlier speculations of Wenkert and Winterfeldt in principle and our own biogenetic hypothesis in detail. The precursor-product relationships between **1** and its "early" biosynthetic precursors, e.g., tryptamine (**2**) and secologanin (**3**), paralleling those established for other monoterpene indole alkaloids by the studies of Arigoni, Battersby, Leete, Scott, and others,[8] require no further comment here.

The biogenesis of the monoterpene alkaloids found in *C. roseus* has been established experimentally to involve strictosidine (**4**) as the penultimate biosynthetic intermediate both in vivo[9b,20,21b] and in vitro.[18] In the latter instance the "strictosidine synthetase" has been shown to have high stereoselectivity for formation of only the C-3 *S* epimer (**4**), which is the sole intermediate used in the subsequent steps of the alkaloid biosynthetic pathway(s). This observation seems to be at variance with the reports that both **4** and its C-3 epimer, **16**, are found in mature *C. roseus* plants[9b] (**16** also as its *N*-acetate[9b]), and both **5** and **15** in *C. acuminata*.[6] An important biochemical question is thus raised by these observations: are some steps of secondary natural product biosynthetic pathways more the result of chemical reactivity under optimal (or permissable) conditions and less the outcome of stereoselective, enzymatic control? This feeling has also been expressed by Scott and Wei.[40] While an answer to this question is not readily demonstrable, the following observation may be pertinent.

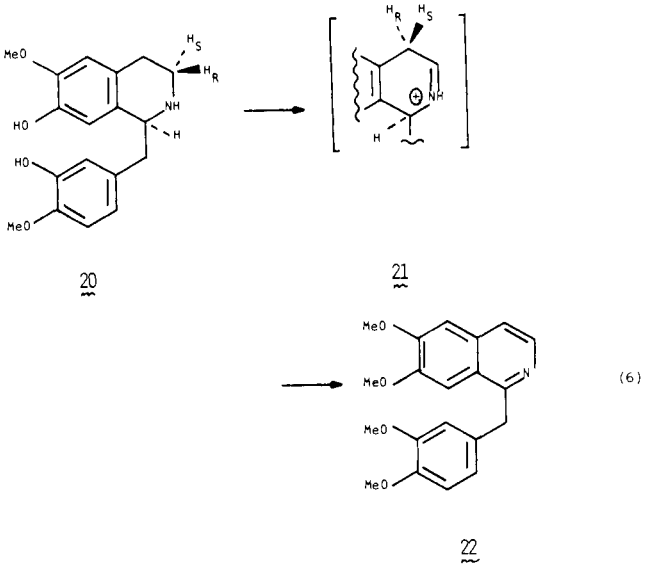
The common honeysuckle, *Lonicera morrowii* A. Gray, and its numerous cross-hybrids (*L. tataricae*, *L. bella*, etc.) produce large quantities of secologanin (**3**)[41] but no alkaloids, or at least not any monoterpene indole alkaloids.[42] Samples of [1-^{14}C]-tryptamine bissuccinate were administered to apical cuttings of Madison native honeysuckle plants. At the end of a 3-day metabolism period, the plants were extracted and carrier **4** and **16** were added. In a control experiment [1-^{14}C]tryptamine was added just prior to workup of identical cuttings. The radioactivity of the isolated tetraacetyl-**5a** and -**15a** from the 3-day feeding was verified by conversion to their respective tetraacetylquinolols[43] for recrystallization to a constant specific ^{14}C radioactivity. The control experiment[44] gave radioinactive **5** and **15** as expected, whereas the 3-day metabolism experiment gave *both* radioactive **5** (4×10^5 dpm/mmol) and **15** (4.8×10^5 dpm/mmol), whose total radioactivity taken together represents a 0.02% absolute incorporation of the [1-^{14}C]**2**. Although chemical degradations of the labeled **5** and **15** were not carried out (nor could be by reasonable degradative routes), it is unlikely that these two neutral alkaloids were not regiospecifically labeled at C-5. The observed formation of such compounds in a system that normally does not produce them supports the possibility that the formation of **4** and **16** in *C. roseus* and *C. acuminata*, as viewed through isolation or precursor labeling experiments, may not be under strict enzymatic control. Thus the presence of the requisite precursors (**2** and **3**) may permit the formation of **4** and **16** as a function of these plants' internal environment (pH, etc.). In the case examined above, the formation of the secondary natural products is unexceptional; in fact, it is to be expected based on the reaction conditions appropriate for the laboratory preparation of **4** and **16**. Yet the corollary to this observation that the spectrum of secondary natural products isolated from the parent living organisms may include compounds whose formation is in part chemically, not enzymatically, mediated seems to us to be very probable and perhaps more general than presently suspected. We refer to this possibility again below.

The results of the feeding experiments with loganin and strictosamide (**5**) detailed in this paper demonstrate that regardless of the stereochemistry of hydrogen isotope labeling at C-14 of **5**, an almost insignificant loss of isotopic hydrogen occurs from this position during the conversion of **5** into camptothecin in vivo. These results may be rationalized by postulating that the aromatization of the D ring involves both stereospecific, enzymatic oxidation processes, and chemical (nonstereospecific) tautomerization processes. The overall transformation can be depicted as proceeding from partial structure E to G (eq 5) for the first oxidation, then in an un-

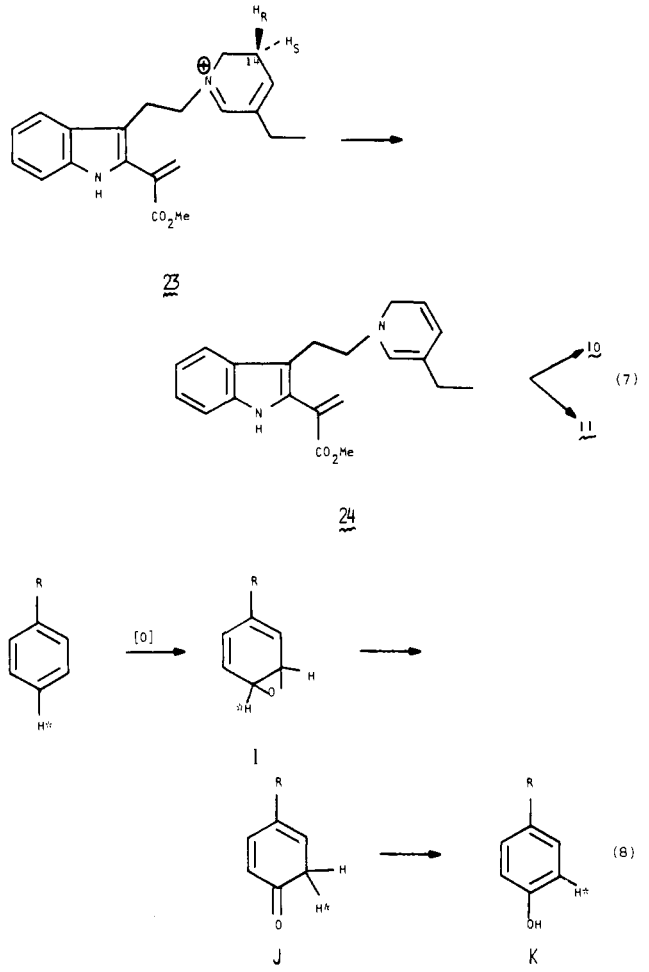


specified manner from G to H for the second oxidation. The oxidation of E to F must obviously be highly stereospecific since only strictosamide (**5**), not its 3*R* epimer, **15**, is utilized in the biosynthetic pathway. The conversion of F to G, a formal imine to enamine tautomerization, would be expected to involve stereospecific loss of one of the diastereotopic C-14 hydrogens *if* enzymatically mediated. For example, the analogous hydrolysis and tautomerization of phosphoenoylpyruvate to pyruvate are known to occur by stereoselective addition of a proton to C-3 on the *si* face of the intermediate enol.[45] Nonetheless our data prove that the F → G transformation is not stereospecific for either of the diastereotopic C-14 hydrogens. These results are consistent with a nonenzymatically catalyzed tautomerization. If this conclusion is valid, the insignificant loss of ^3H label from C-14 of "**5**" must be due to the expression of an isotope effect that discriminated heavily against loss of tritium relative to that of hydrogen.

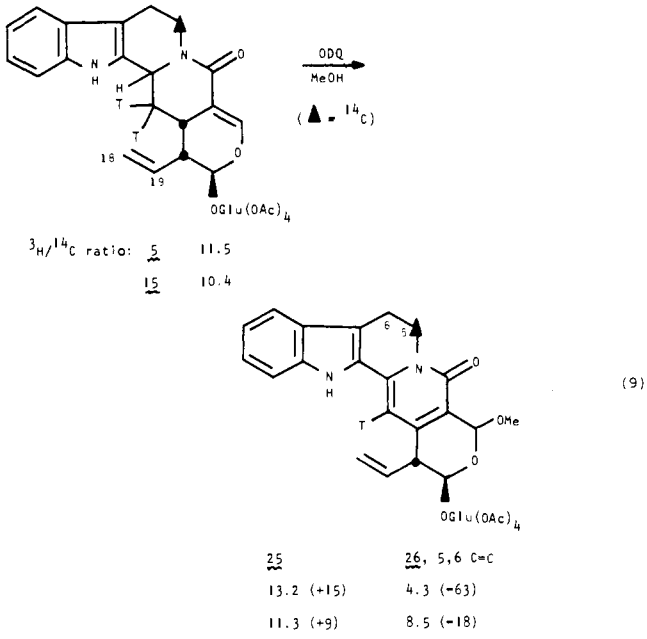
Although we cannot presently segregate the F → G transformation by suitable in vitro experiments for a quantitative validation of the rationalization proposed above, we note that several literature precedents are consistent with that conclusion. Battersby et al.[46] have observed a lack of complete stereospecificity in removal of one diastereotopic hydrogen from C-4 of norreticuline (**20**) during its oxidative transformation into papaverine (**22**) in *Papaver somniferum* L. (eq 6). Al-



though the C-3 *pro-S* hydrogen of **20** was clearly lost stereospecifically in forming the presumed imine intermediate **21**, the conversion of **21** to **22** occurred by loss of 38% of a 4 *pro-R* ^3H label, 25% loss of a 4 *RS* ^3H label, and 12% loss of a 4 *pro-S* ^3H label. Thus, the formal imine to enamine tautomerization does not appear to have been under tight enzymatic control, as the authors concluded.[46] Three laboratories[38,47,48] have independently found that in the conversion of the putative dehydrosecodine (**23**) intermediate into vindoline (**11**) and catharanthine (**10**) in *C. roseus*, during which a formal tautomization of **23** to **24** must occur (eq 7), the loss of ^3H label from C-14 of **23** is 10–22% less than the theoretically expected amount for a completely stereospecific process. Finally, the well-known NIH shift mechanism is considered to involve a keto-enol tautomerization (J → K), eq 8, in which the loss of isotopic hydrogen label (*H) from C-3 of J is subject to isotopic discrimination between the C^3-H and C^3-*H bond cleavage.[49] Since retentions of ^3H in K as high as 94% have been observed, depending on the nature of R,[49n] this enolization may well be a nonenzymatically controlled process. Regardless, the latter example supports our rationalization (eq 5) of one aspect of camptothecin's biosynthesis.



Valuable insight into the mechanism of individual steps of alkaloid biosynthesis often can be gained by studies of model systems in vitro.[17] Although the results of such studies at times have led to controversy about their validity and significance,[50] we have carried out one model study of a step in camptothecin's biosynthetic pathway whose results may be relevant to the in vivo mechanism of D ring aromatization of "**5**". Samples of [14-^3H,5-^{14}C]strictosamide (**5a**) and -18,19-dihydrovincoside lactam (**15a**) tetraacetates were individually oxidized by DDQ in methanolic solution to give the [^3H,^{14}C]indolopyranoquinolizinones, **25** and **26** (eq 9). The formation and structures of these two oxidation products have been detailed elsewhere.[51] Although the positions of ^3H and ^{14}C in the products were not established by chemical degradation, the changes in the $^3H/^{14}C$ ratio were revealing. (The values enclosed in parentheses in eq 9 indicate the percentage increase or decrease in the corresponding $^3H/^{14}C$ ratios.) It is seen that the formation of **26** involved loss of ^3H label from C-14 of either **5** or **15**, although clearly in unequal amounts. However, the formation of **25** occurred with an apparent ^3H enrichment relative to **5** or **15**. Further oxidation of isolated **25** under the same experimental conditions gave **26** without change in the $^3H/^{14}C$ ratio of **25**, as expected since ^3H was not attached to C-5 or C-6 of **25**. The apparent ^3H enrichment of **25** is the result of low chemical yields and formation of several products in addition to **25** and **26**. Tritium-labeled **5** or **15** will be protected by an isotope effect against reactions involving loss of hydrogen, whereas ^{14}C-labeled substrates will react at a faster (normal) rate. Thus, true ^3H enrichment of **5** or **15** is not occurring, only ^{14}C depletion (which of course is quite possible since no ^{14}C-labeled molecules also contained ^3H). The differential retention of ^3H in **25** vs. **26** indicates that **25** is not the sole precursor of **26**. For example, **26** may result by initial C^5N^4 oxidation



(9)

(or C^5C^6), followed by C^3N^4 and C^{15} oxidation; **25** by C^3N^4, then C^{15} oxidation.

The results of the above model experiment of D ring aromatization of "**5**" cannot be interpreted quantitatively nor in greater detail. However, they suggest that an in vivo aromatization process, *if* partially nonenzymatic, could result in high tritium retention during the incorporation of [14-^{3}H,5-^{14}C]**5** into **1**.

Experimental Section

General. Solvents were glass distilled before use. Solvent removal was done on a rotary evaporator at ca. 14 mmHg, or less than 1 mmHg at 40 °C. Chemicals and reagents were of commercially available quality unless otherwise specified. Anhydrous solvents or reagents were prepared by distillation from active metal hydrides under a dry nitrogen atmosphere, or as directed by Vogel. [52]Nitrogen gas was dried by passing through a Drierite tower and then deoxygenated by passage through a BASF R-31 catalyst prior to use. Tritiated water (1–5 Ci/g) and other radiochemicals were used as purchased from New England Nuclear or Amersham-Searle. The potassium [^{13}C]cyanide was obtained from Merck and Co. Liquid scintillation cocktails were either toluene-based, containing PPO (5 g/L) and POPOP (0.3 g/L), or dioxane-based, containing PPO (7 g/L), POPOP (0.5 g/L), and naphthalene (100 g/L). Samples were counted in duplicate three times for 10^4 counts each time on a Packard Tri-Carb 3255, 3330, or 3375 liquid scintillation spectrometer. Sample size was adjusted where possible to permit counting at a rate of at least 10 × background. Determination of counting efficiency was by means of internal standards of either tritiated or carbon-14 labeled *n*-hexadecane of known specific radioactivity. Sufficient standard was added to each counting vial to double the observed counts per minute. Constant specific radioactivity was taken as agreement to within ±5% of three successive recrystallizations. Noncrystalline substances were rechromatographed to a constant specific radioactivity and single radioscan peak on a Vanguard Systems, Inc., Model 930 TLC plate scanner. Chromatographic separations were done using Brinkmann adsorbents: for thin-layer (TLC) and thick-layer (PLC), silica gel PF_{254}; for columns, silica gel 60 for column chromatography. TLC and PLC plates were dried at 110 °C for at least 4 h before use. IR spectra were determined on a Perkin-Elmer Model 257 spectrophotometer. Electron-impact mass spectra were determined at 70 eV on an AEI MS-9 mass spectrometer interfaced with a Nova 2 data system, or on a Finnegan 1015 quadrapole mass spectrometer interfaced to a Finnegan M-6000 data system. ^{1}H and ^{13}C NMR spectra were determined on a Varian EM 390 or Bruker HX-90E nuclear magnetic resonance spectrometer at 90 or 22.63 MHz. Chemical shifts are given relative to Me_4Si as internal standard, or adjusted to Me_4Si by reference to the $CHCl_3$ resonance at δ_H 7.26 or δ_C 76.9. Broad-band proton decoupling was employed for ^{13}C spectra. High-field ^{1}H NMR spectra were determined at 270 MHz on a Bruker Super-Con nuclear magnetic resonance spectrometer; ^{2}H NMR spectra were determined similarly at 41.44 MHz using broad-band irradiation for proton decoupling and quadrature detection for improved sensitivity. Melting points were determined on a Koefler micro hot stage and are uncorrected.

Preliminary Feeding Experiments. The details of the feeding experiments using radioactively labeled tryptophan, mevalonic acid, secologanin, strictosidine/vincoside, and [5-^{3}H]- or [14-^{3}H]strictosamide (**5**) are available in ref 26.

[1-^{13}C]Tryptamine (2). To a RBF containing gramine methiodide (632 mg, 2.0 mmol) in EtOH (25 mL) was added [^{13}C]KCN (130 mg, 2.0 mmol, 90 atom % ^{13}C, lot no. E-214). The mixture was magnetically stirred at reflux for 18 h under N_2 in the dark. The mixture then was concentrated, and the residue was passed through alumina (Woelm Activity Grade III) using benzene–EtOAc (1:1) as solvent. Concentration of the eluate under high vacuum gave [1′-^{13}C](3-indolyl)acetonitrile (338 mg, 108%) as slightly colored needles, pure by TLC analysis using benzene–EtOAc (2:1) as solvent.

The nitrile was dissolved in EtOH (6 mL) and added to a solution of $CoCl_2 \cdot 6H_2O$ (953 mg, 4.0 mmol) in EtOH (10 mL) at ca. 15 °C. To this mixture was added, in three portions, at 20-min intervals, at 25 °C, $NaBH_4$ (722 mg, 19.8 mmol). The solution turned from a blue color to black, and effervesced. Magnetic stirring was continued for 16 h and then, after addition of 3 N HCl (11.4 mL), until the solution turned from black to pink (ca. 20 min.). The solution was extracted with Et_2O (3 × 20 mL), and the ethereal extract was back extracted once with H_2O (5 mL). The two aqueous fractions were combined, and N_2 bubbled through them for 20 min prior to extracting them with CH_2Cl_2 (4 × 20 mL). Nitrogen gas purging was continued between extractions to exclude O_2. The combined organic extract was dried over $MgSO_4$ (anhydrous), filtered, and concentrated under high vacuum to an amber gum (172 mg, 53.7%). To the entire residue dissolved in EtOH (3 mL) was added 0.2 N HCl (5.0 mL). Concentration to dryness gave [1-^{13}C]tryptamine hydrochloride (**2**) (196.5 mg, 1.0 mmol, 50% overall), mp 245.5–246.0 °C [lit.[53] 248 °C]), as amber crystals.

[5-^{13}C]Strictosamide (5a). To [1-^{13}C]**2** hydrochloride (196.5 mg, 1.0 mmol) suspended in phosphate buffer (2.0 mL, 0.2 M, pH 4.5, deoxygenated by warming under an N_2 flow) was added secologanin (**3**) (390 mg, 1.0 mmol). The mixture was flushed with N_2 and warmed to 37 °C for 48 h. The resultant solution was worked up exactly as for the radioactive synthesis of strictosidine/vincoside (below). The resultant lyophilized solid then was dissolved in MeOH (5 mL) and 15% Na_2CO_3 (10 mL), flushed with N_2, and heated to 70 °C for 90 min. Workup was exactly as for the radioactive synthesis of strictosamide (below) to give, after PLC purification, [5-^{13}C]**15a** (57 mg, 0.11 mmol, 11% overall), mp 200.5–202 °C, and [5-^{13}C]**5a** (38 mg, 0.076 mmol, 8% overall). A sample of the [5-^{13}C]**5a** was converted to its tetraacetate by acetylation with acetic anhydride and dry pyridine for 18 h at 25 °C. Excess reagents were removed in vacuo by means of a toluene–methanol azeotrope. Mass spectral analysis of tetraacetyl-[5-^{13}C]**5a** and data analysis according to Biemann[54] showed it to contain 83.9 mol % excess ^{13}C. ^{13}C NMR analysis of a 5-mg sample of tetraacetyl-[5-^{13}C]**5a** in $CDCl_3$ showed only one signal at δ 43.4 (tetraacetyl-[5-^{13}C]**15b**, δ_C 40.9).

[5-^{3}H]- or [14-^{3}H]Strictosamide (5a) and -Vincoside Lactam (15a). This synthesis followed the method of Battersby et al.[9b] To a flame-dried, N_2-flushed flask was added tryptamine hydrochloride (219 mg, 1.1 mmol), **3** (96 mg, 1.02 mmol), and KH_2PO_4 (13.5 mg). The flask was serum capped and flushed with N_2, and then tritiated water (0.2 mL, 1 Ci/g) and H_2O (0.3 mL) were added via a syringe. The suspension was magnetically stirred at 37 °C for 3 days under a N_2 atmosphere. The resulting brown solution was passed through a column of Bio-Rad AG 1-X8 anion exchange resin (Cl^- form, 90% equivalent excess) and eluted with 50–70 mL of H_2O. The effluent was lyophilized to give a pale brown powder. The specific radioactivity of this solid, which was shown by acetylation to consist of 50–65% **4** and **16**, was equivalent to ca. 4.5 × 10^9 dpm ^{3}H/mmol as **4** or **16** (as their hydrochloride salts).

A suitable quantity (ca. 200 mg) of this crude solid mixture was dissolved in MeOH (0.5 mL) and 15% aqueous Na_2CO_3 (10 mL) was added. This mixture was heated at 70 °C for 90 min under a N_2 atmosphere, cooled, and saturated with solid NaCl, and the reaction mixture was extracted with EtOAc (6 × 40 mL). The combined organic extracts were dried (Na_2SO_4) and the crude mixture of stric-

tosamide (**5a**)/vincoside lactam (**15a**) separated and purified by PLC in $CHCl_3$–MeOH (8:2), multiply developed. The UV-absorbing bands of **5a** (R_f 0.6) and **15a** (R_f 0.5) were eluted with CH_2Cl_2–MeOH (8:2) to give [14-^{3}H]**5a** (23 mg, ca. 3×10^9 dpm ^{3}H/mmol) and **15a** (37 mg, ca. 2.3×10^9 dpm ^{3}H/mmol). The radioactive **15a** was further purified by crystallization from aqueous methanol (mp 200–201 °C; lit.[9b] 201–202 °C). The radioactive, noncrystalline **5a** was rechromatographed two-dimensionally using $CHCl_3$–MeOH (8:2) and EtOAc–MeOH (95:5), and then the material eluted from the adsorbent was acetylated. The resulting tetraacetyl-[14-^{3}H]**5a** was purified by PLC in C_6H_6–EtOAc (2:1), and the resulting chromatographically pure tetraacetate deacetylated to [14-^{3}H]**5a** (2.30×10^9 dpm ^{3}H/mmol) by the Zemplen method[9b] prior to use in plant feeding experiments.

The synthesis of [5-^{3}H]**5a** and **-15a** was carried out in an analogous manner using [1-^{3}H]tryptamine hydrochloride (1.4×10^7 dpm ^{3}H/mmol) prepared as for [1-^{13}C]tryptamine, but replacing part of the $NaBH_4$ with [^{3}H]$NaBH_4$ (100 mCi, 3.6 Ci of ^{3}H/mmol, Amersham). The purified samples of [5-^{3}H]**5a** and **-15a** exhibited the following specific ^{3}H radioactivities: **5a**, 6.27×10^8 dpm/mmol; **15a**, 5.40×10^9 dpm/mmol.

[14-^{3}H,5-^{14}C]Strictosamide (5a). These doubly labeled samples of **5a** and **15a** were prepared by admixture of a crude mixture of [5-^{14}C]**4/-16** hydrochlorides, prepared from [1-^{14}C]tryptamine hydrochloride (2.9×10^9 dpm ^{14}C/mmol, from [^{14}C]KCN as described above for the synthesis of [1-^{13}C]**2**), with the crude [14-^{3}H]**4/-16** hydrochlorides prepared as described above, or as follows. A solution of tritiated acetic acid (9.9 mmol) was obtained by cautiously reacting acetic anhydride (0.42 mL, 4.45 mmol) with tritiated water (88 μL, 4.9 mmol, 1 Ci/g) and acetyl chloride (1 μL) at −10 to 25 °C. To this was added **3** (46 mg, 0.12 mmol), **2** (23.3 mg, as its hydrochloride, 0.12 mmol), and sodium acetate (10.8 mg, 0.13 mmol). After stirring for 66 h at 60 °C, MeOH (1 mL) and 10% aqueous Na_2CO_3 (2 mL) were added and the cyclization reaction was carried out as above to give, after workup, [14-^{3}H]**15a** (3.45 mg, 14.6%) and [14-^{3}H]**5a** (14.37 mg, 24%) as PLC pure material.

To a [5-^{14}C]**4/-16** hydrochloride mixture (15.5 mg, ca. 3×10^9 dpm/mmol) was added a [14-^{3}H]-**4/-16** hydrochloride mixture (171.8 mg, ca. 4×10^9 dpm/mmol) and a radioinactive **4/16** hydrochloride mixture (221 mg, 0.39 mmol). The mixture was treated with aqueous base as described above to give, after workup, crystalline [14-^{3}H,5-^{14}C]**15a** (80 mg, 22%, 1.03×10^9 dpm ^{3}H/mmol, ^{3}H/^{14}C = 10.20) and PLC pure [14-^{3}H,5-^{14}C]**5a** (34 mg, 9%, 1.22×10^9 dpm ^{3}H/mmol, ^{3}H/^{14}C = 11.51).

The second sample of [14-^{3}H,5-^{14}C]**5a** (17.93 mg, ^{3}H/^{14}C = 2.58) was prepared by admixture of [14-^{3}H]**5a** (14.37 mg), prepared by the acetic acid condensation method, with [5-^{14}C]**5a** (5.56 mg, 9.31×10^8 dpm ^{14}C/mmol) and PLC purifying in the usual manner. The ^{3}H/^{14}C ratio was determined by removal of 0.18 mg, dilution with radioinactive **5a** (45.5 mg, 0.068 mmol), and acetylation of the mixture. The resultant [14-^{3}H,5-^{14}C]**5a**-$(OAc)_4$ (30.7 mg, 0.046 mmol) was then converted to its [14-^{3}H,5-^{14}C]quinolol tetraacetate (30 mg, 97%, mp 231–232 °C, 1.20×10^7 dpm ^{3}H/mmol, ^{3}H/^{14}C = 2.55) by reaction with $NaIO_4$ (0.14 mmol) in aqueous methanol (ca. 3 mL), followed by treatment of the resulting crude reaction product with Et_3N (0.1 mL) in EtOH (3 mL) for 18 h at 25 °C.[43]

[14-^{2}H,14-^{3}H]Strictosamide. The following experimental procedures were used for preparation of the [14-^{2}H,^{3}H]**5a** used in the feeding experiment from which the [^{2}H,^{3}H]**1** was obtained for ^{2}H NMR analysis. Other preparations of [14-^{2}H,^{3}H]**5a** and **-15a** were carried out similarly. A solution of [^{2}H,^{3}H]acetic acid was made from Ac_2O, tritiated water (10 μL, 5 Ci/g), and D_2O (4 mL) as described directly above for the preparation of [14-^{3}H]**5a**. This solution was cooled to −70 °C, then **2** (295 mg, 1.5 mmol, hydrochloride), **3** (582 mg, 1.5 mmol), and anhydrous NaOAc (130 mg, 1.5 mmol) were added. The reaction mixture was flushed with N_2 and heated closed at 60 °C in an oil bath for 3 days with magnetic stirring. A precipitate of NaCl forms during this period. The reaction mixture was cooled to room temperature, the clear brown solution decanted from the solid material, and the solid residue washed with glacial acetic acid (3 × 10 mL). The combined reaction decantate and washings were treated with 0.05 N HCl (30 mL), to exchange OAc^- for Cl^-, and lyophilized. The lyophilized solid residue was treated with aqueous base as described above[35] to give [14-^{2}H,^{3}H]strictosamide (**5a**), 170 mg, 23%, 5.3×10^7 dpm ^{3}H/mmol. The tetraacetate of **5a** was prepared as above for mass spectral analysis and determination of its specific ^{3}H radioactivity.

Tetramethoxycarbonyl Strictosamide (5b) and Vincoside Lactam (15b). Strictosamide (**5a**, 20 mg, 0.04 mmol) was dissolved in a mixture of $CHCl_3$ (1 mL) and dry pyridine (0.1 mL) at ice bath temperatures under a N_2 atmosphere. Methyl chloroformate (0.08 mL, 1 mmol) was added dropwise to this mixture with magnetic stirring. A transient red-orange coloration appeared at the site of mixing, which became permanent toward the end of the reagent's addition. The reaction mixture was allowed to warm to room temperature and, after a total of 2 h, was diluted with a small volume of EtOAc and poured onto crushed ice. The two-phase mixture was washed with NaCl-saturated 1 N HCl, and the organic layer back-washed with saturated aqueous $NaHCO_3$, then dried (Na_2SO_4). The solid residue obtained from removal of the solvents in vacuo was purified by PLC in hexane–EtOAc (1:2) to give tetramethoxycarbonyl strictosamide (**5b**): 17 mg, 58%; mp 137–139 °C (EtOH–H_2O); IR ($CHCl_3$) ν 1760, 1660, 1595, 1445, 1300, and 1265 cm^{-1}; MS, *m/e* (rel intensity) 730 ($M^{+\cdot}$, 6), 654.2060 [$C_{32}H_{34}N_2O_{13}$ calcd for 654.2060; $M^{+\cdot} - HOCO_2CH_3$, 10], 610.2116 [$C_{31}H_{34}N_2O_{11}$ calcd for 610.2162; $M^{+\cdot} - (HOCO_2CH_3 + CO_2)$, 30], 596.1976 [$C_{30}H_{32}N_2O_{11}$ calcd for 596.2005; $M^{+\cdot} - (HOCO_2CH_3 + CO_2 + CH_2O)$, 17], 377 (13), 349 (21), 333 (31), 279 (51), 261 (56), 157 (72), 81 (80), 59 (100).

Tetramethoxycarbonyl vincoside lactam (**15b**) was prepared identically: 65%; mp 142–145 °C (EtOH–H_2O); IR ($CHCl_3$) ν 1760, 1660, 1595, 1445, 1300, and 1265 cm^{-1}; MS, *m/e* (rel intensity, 730 ($M^{+\cdot}$, 4) 654.2062 (27), 610.2192 (28), 596.2024 (16), 393 (16), 377 (27), 349 (23) 333 (36), 279 (30), 261 (94), 169 (79), 149 (66), 109 (96), 81 (96), 59 (100).

The relevant ^{1}H NMR data for tetraacetyl-**5a**, **5b**, and **15b** are shown in Table II. The following data from double irradiation experiments were used to confirm the δ_Hs assigned to the C-14 hydrogens. **5b**: {H-14 *pro-S*} caused the δ 2.21 signal to become an unsymmetrical quartet with apparent *J*s of 4 and 12.3 Hz, the $\Delta\nu_{1/2}$ of H-15 to be decreased by 4 Hz, and the $\Delta\nu_{1/2}$ of H-3 to be decreased by 3.5 Hz; {H-14 *pro-R*} caused the δ 2.14 signal to collapse to a d of d (J = 6, 7.5 Hz), the H-15 $\Delta\nu_{1/2}$ to decrease by 4 Hz, and the H-3 $\Delta\nu_{1/2}$ to decrease by 3.5 Hz; {H-15} caused the H-14 resonances to collapse to the AB portion of an ABX spin system (with H-3), H-20 to become a d of d (J = 2, 9 Hz), and H-17 to become a singlet; {H-3} was not done. **15b**: {H-14 *pro-S*} caused the δ_H 2.22 multiplet to simplify to a 4-line resonance with J = 4, 10 Hz, H-15 to lose its 13-Hz coupling, and H-3 to appear as a quartet with J = 1, 2.5 Hz; {H-14 *pro-R*} collapsed the δ_H 1.62 resonance to a triplet (J = 12 Hz), H-15 to a d of d of d with J = 2.5, 5, 13 Hz, and decreased the $\Delta\nu_{1/2}$ of H-3 by 3 Hz; {H-15} resulted in the δ_H 2.22 resonance appearing as a d of d with J = 1.5, 9.5 Hz, the δ_H 1.62 resonance appearing as a triplet with J = 12.5 Hz, and H-17 collapsing to a singlet.

Isotopic Enrichment of [14-^{2}H]5b and -15b. Samples of [14-^{2}H]**5b** and **-15b** were prepared as described above for [14-^{2}H,14-^{3}H]**5a** and **-5b/-15b**. Their 270-MHz ^{1}H NMR spectra were determined in $CDCl_3$ and the relative peak areas were measured by the cutting-and-weighing method. The isotopic distributions of the molecular ions at *m/e* 730 of their low-resolution mass spectra were analyzed in the following manner. Let M_{+i} and M'_{+i} be the measured heights of the *m/e* 730 + *i* signal of the undeuterated and deuterated compounds, respectively; d_i is the relative amount of non-, mono-, and dideuterated species reflected in the isotopic peaks of the compound's molecular ion. Thus it can be expressed that:

$$M' = d_0M + d_1M_{-1} + d_2M_{-2}$$

$$M'_{+1} = d_0M_{+1} + d_1M + d_2M_{-1}$$

$$M'_{+2} = d_0M_{+2} + d_1M_{+1} + d_2M$$

The M_{-i} values are insignificant and the above three equations can be solved for the d_i values by matrix algebra. Thereby, $d_0 = 1$, $d_1 = M'_{+1} - M_{+1}$, and $d_2 = (M'_{+2} - M_{+2}) - M_{+1}(M'_{+1} - M_{+1})$, if all isotopic peak heights are divided by the height of the *m/e* 730 peak ($M = M' = 1.0$) and the d_i values are normalized by dividing by ($d_0 + d_1 + d_2$).

The overall deuterium enrichment of [14-^{2}H]**5b** and **-15b** is a weighted average of the contributing deuterium-labeled species. This can be expressed by [(0 ^{2}H/molecule × % d_0) + (1 ^{2}H/molecule × % d_1) + (2 ^{2}H/molecule × % d_2)]/2 potentially labeled sites per molecule, which simplifies to (0.5% d_1 + % d_2)^{2}H/potentially labeled site.

Table II. ^{1}H NMR Data for **5a**-$(OAc)_4$, **5b**, and **15b**

proton[a]	chem shift (multiplicity, *J*, in Hz)		
	5a-$(OAc)_4$	**5b**	**15b**
N-1	8.12 (s)	8.08 (s)	7.91 (s)
9	7.48 (d, 7.7)	7.41 (d, 8.5)	7.51 (d, 7.5)
12	7.41 (d, 7.7)	7.38 (d, 8.5)	7.34 (d, 7.5)
17	7.37 (d, 1.9)	7.39 (d, 2.2)	7.46 (d, 2.3)
11	7.21 (dt, 1.3, 7.7)	7.16 (t, 7.5)	7.17 (ddd, 1, 7, 7.5)
10	7.11 (dt, 1.3, 7.2)	7.07 (t, 7.5)	7.11 (dt, 1, 7.8)
19	5.65 (m, 13)[c]	5.61 (m, 11)[c]	5.48 (m, 17)[c]
18	5.38, 5.31 (m, 9.6, 1.9, 9.6)[d]	5.37, 5.30 (7.5, 2, 9.0)[d]	5.26, 5.18 (9.8, 2, 9.5)[d]
21	4.99[f]	5.05 (d, 1.5)[e]	5.30 (d, 1.7)[e]
1′	5.30 (s)	5.28 (s)	5.13 (s)
5	4.99[f]	5.05 (m)[f]	5.17 (m)
3	4.99[f]	4.98 (ddd, 1.5, 2.5, 4.7)[e]	4.88 (ddd, 1, 2.5, 12)[e]
2′	4.85 (d, 8.1)	4.87 (d, 8.0)	5.02 (d, 7.4)
3′	4.83 (t, 8.1)	4.85 (t, 9)	4.94 (t, 9.5)
4′	4.72 (dd, 8.1, 9.6)	4.65 (dd, 8.5, 9.5)	4.85 (dd, 7.8, 9.6)
6′	4.26, 4.09 (m, 12.3)[g]	4.36, 4.29 (m, 12)[g]	4.35 (m, 12)[g]
5′	3.75 (m, 5.0, 2.6, 10)[h]	3.84 (m, 6, 3.2, 9.5)[h]	3.87 (m, 5, 3, 7.8)[h]
6	3.08, 2.94 (m)	3.09, 2.98 (m)	2.85 (m).
15	2.63 (m)[i]	2.74 (m)[i]	3.03 (m)[i]
20	2.63 (m)[i]	2.67 (ddd, 1.5, 5.5, 9)[e]	2.70 (ddd, 1.5, 5.5, 9.5)[e]
14 *pro-R*	2.14[j]	2.21[e]	2.22 (ddd, 3, 4, 12.5)[e]
14 *pro-S*	2.14[j]	2.14[e]	1.62 (ddd, 12.5, 12.5, 13)[e]
CH_3- or CH_3O-	2.06, 1.98, 1.88, 1.23	3.82, 3.79, 3.71, 2.92	3.83, 3.82, 3.73

[a] Numbered according to ref 34. [b] Determined in $CDCl_3$ at 270 MHz relative to the $CHCl_3$ at δ_H 7.26. [c] AMX system; J_{AM} given. [d] AMX system; J_{AX}, J_{MX}, $J_{19,20}$ given in that order. [e] *J* determined indirectly by decoupling experiments. [f] Complex signal consisting of H-3, -5, and -21. [g] AA′M system; $J_{AA'}$ given. [h] AA′M system; J_{AM}, J_{AM}, $J_{4'5'}$ given in that order. [i] Unresolved octet, whose δ_H is assigned by decoupling. [j] Signal partially overlapped by acetate methyl protons.

Table III

	5b	**15b**		**5b**	**15b**		**5b**	**15b**
M	1	1	M′	1	1	% d_0	31.5	37.6
M_{+1}	0.404	0.452	M'_{+1}	1.375	1.175	% d_1	30.6	27.2
M_{+2}	0.121	0.182	M'_{+2}	1.715	1.443	% d_2	37.9	35.1

The data shown in Table III were measured from the mass spectrum. Therefore, **5b** contained 53.2% ^{2}H and **15b**, 48.7% ^{2}H per potentially labeled site at C-14.

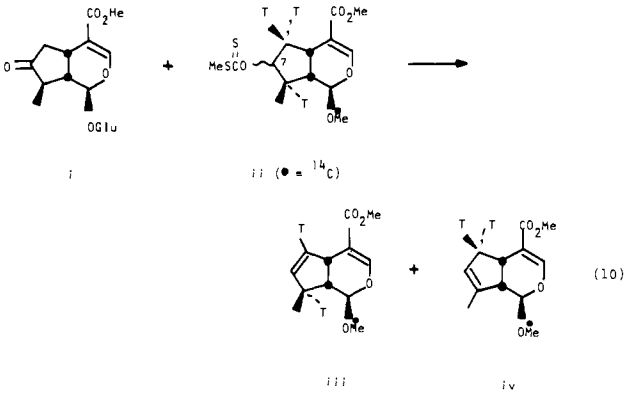
[14-^{3}H]Strictosidine (4) and Vincoside (6). The procedure of Mattes et al.[56] was used for the preparation of the penta-2,2,2-trichloroethoxycarbonyl derivatives of [14-^{3}H]**4** and **-16**. (The latter were prepared as described above for [14-^{3}H]**5a**.) The derivatives of **4** and **16** were purified by PLC and determined to contain ca. 5.64×10^9 dpm ^{3}H/mmol.

The original method of deprotection[56] was used (Zn, AcOH) to provide the crude hydrochlorides of [14-^{3}H]**4** and **-16** as lyophilized solids after removal of undissolved solids from the reaction mixture by filtration through Celite. The $ZnCl_2$ present in the lyophilate was only partially removed by passage of an aqueous solution of the lyophilate through a narrow Sephadex G-25 column. The use of ion exchange or chelation resins for Zn ion removal was less satisfactory due to decomposition of **4** and **16**. These and other difficulties caused us to modify the deprotection procedures by (a) use of the Zn(Ag) couple in THF–AcOH to reductively cleave the 2,2,2-trichloroethyl esters,[57] and (b) removal of soluble Zn ions with H_2S. These latter procedures gave nearly colorless samples of the hydrochlorides of **4** and **16** after lyophilization (cf. ref 20b).

Although it was stated in our earlier communication that the sample of [14-^{3}H]**16** used in the feeding experiment was not contaminated by **4**,[11] subsequent reanalysis by TLC of the pentaacetates and lactam derivatives of the [14-^{3}H]**16** showed it to also contain at least 5% of [14-^{3}H]**4**. This would account for the apparent small incorporation of radioactivity into **1** from [14-^{3}H]**16** (Table I, expt 11).

[6,8-$^3H_3$]Loganin (19). The preparation of this labeled compound was developed in Battersby's laboratory, whose prerogative it is to publish the experimental details. Briefly, it was made by base-catalyzed exchange labeling of 7-ketologanin (i), which can be converted to [6,8-$^3H_3$]loganin in a few steps according to literature methods.[58]

The determination of the relative amount of ^{3}H labeling at the C-6 diastereotopic hydrogens and H-8 of [6,8-$^3H_3$]**19** was done in our laboratories by pyrolysis of the two C-7 epimers of ii to iii and/or iv (eq 10).[39]



(10)

Feeding of Labeled Precursors to *C. acuminata*. A typical experimental procedure was as follows. Standard conditions for feeding experiments were defined for cut shoot feedings. Fresh apical shoot growth (ca. 6-in. long) was cut under the surface of distilled water and rinsed with distilled water and the cut stem immersed in a vial (2 dram) containing a solution of the precursor (ca. 0.01–0.02% of weight of plant's fresh weight). The plants were maintained in an enclosed Plexiglas box (2 ft × 4 ft × 2.5 ft) under 100–350 μEinsteins of fluorescent light (Westinghouse Agro Lites, F40) with a photoperiod of 14 h. The box was opened every 24 h to allow a change of air and to replenish the water in the vials. A layer of absorbent paper lining the bottom of the box was kept moist to maintain a high relative hu-

midity. The plants were harvested after 10–14 days and extracted as described below. Usually, not less than 85% of the precursor was taken up by the plants under these conditions.

A standard workup was followed for each feeding experiment. The plant material was removed from the feeding vials, the vials were rinsed with MeOH (100 mL total), and an aliquot of this solution was counted by liquid scintillation to determine the amount of precursor absorbed by the plants.

The fresh plant material was placed in a Mason jar (1 quart), EtOH (250–500 mL) was added, and the material was ground to a uniform pulp using a Sorvall Omni-Mixer. The marc was extracted by slow filtration using hot MeOH (ca. 500–1000 mL) until a colorless filtrate was obtained. The extract was concentrated to a thick syrup and then redissolved in MeOH–H_2O (1:9), 100 mL. An aliquot of this solution was usually taken to crudely determine the total radioactivity directly, or by combustion in a Packard sample oxidizer prior to liquid scintillation counting.

The aqueous methanolic solution was extracted with petroleum ether (bp 30–60 °C) or Skellysolv B (ca. 10 × 100 mL) until the organic layer was nearly colorless. The organic layer was concentrated, and an aliquot removed for radioactivity measurement.

The remaining brown-yellow aqueous layer was then extracted with $CHCl_3$ (ca. 10 × 100 mL) until the organic layer showed no fluorescence under long-wave UV. The combined organic extracts, which contained most of the alkaloids, were concentrated and an aliquot removed for radioactivity measurement and also from the remaining aqueous phase.

The desired alkaloid was obtainable from the $CHCl_3$ extract by PLC purification on silica gel using the mixed solvent of $CHCl_3$–Me_2CO–MeOH (85:12.5:2.5). The resulting camptothecin (**1**) was recrystallized to constant specific radioactivity from hot $CHCl_3$–MeOH (8:2) by addition of EtOAc, or from MeOH–CH_3CN.

20-Methylthiomethylene Camptothecin. Camptothecin (20 mg, 0.057 mmol) was dissolved in Me_2SO (2 mL) at 25 °C and AcOH (0.1 mL) and Ac_2O (0.25 mL) were added. After 24 h at 25 °C, the reaction was seen to be complete by TLC. Water was added to precipitate the product, which was then crystallized from EtOH to give 20-MTM-**1** (21 mg, 90%) as a colorless microcrystalline solid: mp 221–225 °C; IR ($CHCl_3$) ν 1746, 1663, 1608 cm^{-1}; ^1H NMR (CD_2Cl_2) δ 8.44 (s, H-7), 8.23 (d, J = 8.6 Hz, H-9), 8.00 (d, J = 8.6 Hz, H-12), 7.86 (dt, J = 1.7, 7.9 Hz, H-11), 7.70 (dt, J = 1.3, 7.5 Hz, H-10), 7.42 (s, H-14), 5.58, 5.36 (q, J_{AB} = 17 Hz, H-17), 5.30 (s, H-5), 4.57 (q, J_{AB} = 11 Hz, SCH_2O), 2.29 (s, SCH_3), 2.16 (m, H-19), and 0.93 (t, J = 8 Hz, H-18); MS, *m/e* (rel intensity) 408 (M^+, 10), 360 ($M^+ - HSCH_3$, 23), 348 ($M^+ - CHSCH_3$, 12), 330 ($M^+ - (HSCH_3 + CH_2O)$, 100), 287 (19) Anal. ($C_{22}H_{20}O_4N_2S$) C, H, N.

Degradation of [^{3}H,^{14}C]1. A degradation of the [^{3}H,^{14}C]**1** obtained from expt 4, Table I (125 mg, 0.36 mmol, mp 259–262 °C, 2.25 × 10^5 dpm $^3H/mmol$, $^3H/^{14}C$ = 2.29), was carried out by dissolving **1** in MeOH–$CHCl_3$ (2:8), 30 mL, and adding $NaBH_4$ (130 mg, 3.4 mmol). The mixture immediately effervesced and was magnetically stirred for 3 h. The mixture was acidified with 1 N HCl, saturated brine (10 mL) was added, and the mixture was extracted with $CHCl_3$ for 12 h. The organic extract was passed through silica gel (1-cm pad) in a 14-mL fritted funnel using MeOH–$CHCl_3$ (2:8) as solvent (50 mL), which removed most of the colored impurities. Concentration of eluate gave a pale yellow solid in quantitative yield, which was pure by TLC having an R_f identical with the reference standard of camptothecin hemilactol.

This compound was dissolved in 60 mL of MeOH–$CHCl_3$–THF (reagent grade, nondistilled, 1:1:1) at 45 °C. Then with rapid magnetic stirring, $NaIO_4$ (320 mg, 1.5 mmol) was added and stirring was continued (12 h) until TLC [using MeOH–acetone–$CHCl_3$ (1:1:8)] showed no starting material remained and only one DNP (red-orange) UV adsorbing spot at R_f 0.8 was present. To this mixture was added $NaBH_4$ (305 mg, 7.9 mmol). The solution turned dark yellow and effervesced, and soon formed a flocculant precipitate. A TLC analysis after 30 min indicated the formation of **17** had gone nearly to completion. Stirring was continued for 3 h and the solution was acidified with 1 N HCl, diluted with 20 mL of saturated brine solution, and extracted with MeOH–$CHCl_3$ (2:8, 7 × 40 mL). The iodine in the organic layer was removed by extracting with 20% $Na_2S_2O_3$ (5 mL). The organic extract was then dried over Na_2SO_4, filtered, and concentrated to a pale yellow solid. Purification by PLC using MeOH–acetone–$CHCl_3$ (1:1:8) gave pure [^{3}H,^{14}C]**17** (98 mg, 0.31 mmol, 93% $^3H/^{14}C$ = 2.33) corresponding in R_f to authentic material.

A portion of [^{3}H,^{14}C]**17** (88 mg, 0.27 mmol) was suspended in dry pyridine–CH_2Cl_2 (6:5, 11 mL) and, while being magnetically stirred, 1.5 equiv of CrO_3 (0.77 mmol) was added. The mixture was stirred 3 h, then another 0.77 mmol of CrO_3 was added, and the mixture was stirred another 3 h. The reaction mixture was concentrated under high vacuum and PLC purified using MeOH–acetone–$CHCl_3$ (5:10:80). The major UV fluorescent band, R_f 0.75, corresponding to the reference standard of **18** was scraped and eluted. Concentration of the eluate gave [^{3}H,^{14}C]**18** (35.5 mg, 41%, mp (sealed capillary) 287.5–290 °C dec, 2.46 × 10^5 dpm $^3H/mmol$, $^3H/^{14}C$ = 2.22). It was crystallized to constant specific radioactivity from MeOH–$CHCl_3$ and shown to be spectroscopically identical with authentic **18**.

DDQ Oxidation of [5-^{14}C,14-^{3}H]5a and -18,19-Dihydro-15a Tetraacetates. Acetylation of [14-^{3}H,5-^{14}C]**5a** (ca. 1 mg, 1.22 × 10^9 dpm $^3H/mmol$, $^3H/^{14}C$ = 11.51) by the usual method was followed by addition of radioinactive **5a**-$(OAc)_4$ (105 mg, 0.16 mmol) to the crude acetylation residue. PLC purification gave [14-^{3}H,5-^{14}C]**5a**-$(OAc)_4$, which was reacted directly with DDQ (72.2 mg, 0.32 mmol) in MeOH in the usual manner[51] to give [14-^{3}H,5-^{14}C]**26** (30.1 mg, 27%, mp 151.5–154 °C, 1.97 × 10^6 dpm $^3H/mmol$, $^3H/^{14}C$ = 4.32) and [14-^{3}H,5-^{14}C]**25** (24.9 mg, 23%, mp 140–143 °C, 6.13 × 10^6 dpm $^3H/mmol$, $^3H/^{14}C$ = 13.23) after workup. The partially oxidized [14-^{3}H,5-^{14}C]**25** (20 mg, 0.029 mmol) was resubjected to DDQ (8.2 mg, 0.036 mmol) in benzene to give [14-^{3}H,5-^{14}C]**26** (8.14 mg, 41%, mp 151.5–154 °C, 5.86 × 10^6 dpm $^3H/mmol$, $^3H/^{14}C$ = 13.20) after workup.

A portion of [14-^{3}H,5-^{14}C]**15a** (11.9 mg, 0.024 mmol, $^3H/^{14}C$ = 10.2) was converted to PLC-pure 18,19-H_2-[14-^{3}H,5-^{14}C]**15a** (1.06 × 10^9 dpm $^3H/mmol$, $^3H/^{14}C$ = 10.39) by catalytic hydrogenation over 10% Pd/C in EtOH (2 mL). A portion (0.1 mg) was diluted with radioinactive 18,19-H_2-**15a** (34.2 mg, 0.051 mmol), and the mixture was converted to 18,19-H_2-[14-^{3}H,5-^{14}C]**26** (10.5 mg, 29%, mp 155–157 °C, 2.32 × 10^6 dpm $^3H/mmol$, $^3H/^{14}C$ = 8.50) and 18,19-H_2-[14-^{3}H,5-^{14}C]**25** (10.2 mg, 28%, $^3H/^{14}C$ = 11.28) by reaction with DDQ in MeOH by procedures outlined elsewhere.[51]

Crystallization to constant specific radioactivity of the fully oxidized **26** was possible. The noncrystalline radiolabeled **25** was repeatedly purified by PLC using EtOAc–benzene (2:1) and eluting the UV fluorescent band corresponding in R_f to authentic **25**.

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