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CAMPTOTHECIN: CHEMISTRY, BIOGENESIS AND MEDICINAL CHEMISTRY

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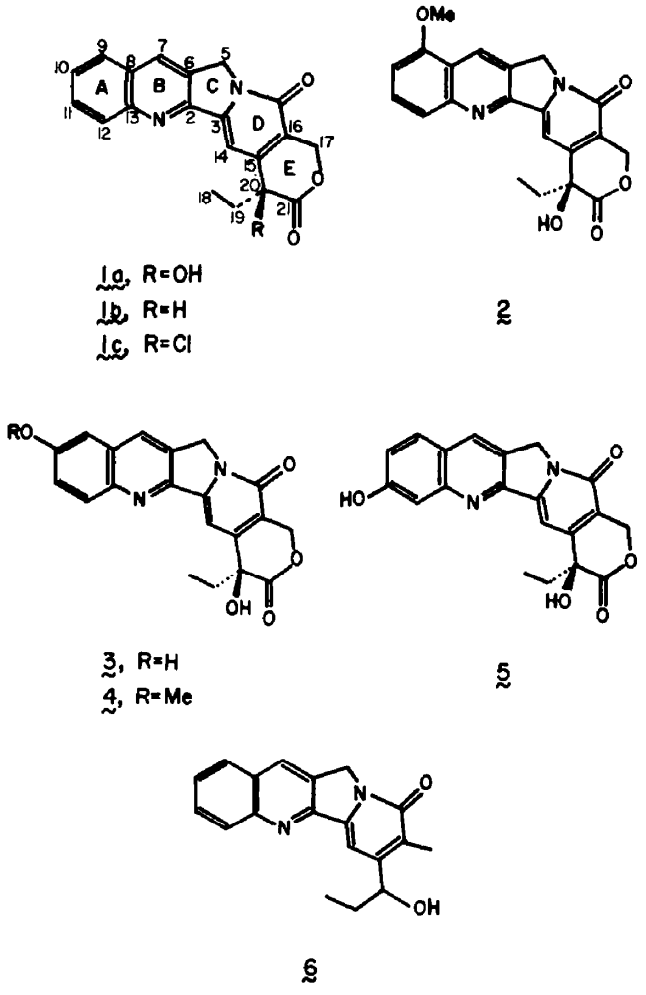
CONTENTS

1. ISOLATION, CHARACTERIZATION AND DISTRIBUTION

Wall *et al.*[1] isolated the novel pyrrolo[3,4-*b*]quinoline alkaloid, camptothecin (**1a**), (Fig. 1), from *Camptotheca acuminata* Decne (Nyssaceae) in 1966 as part of an antitumor agent screening program carried out under the auspices of the National Cancer Institute of the National Institutes of Health, U.S.A. The structure of **1a** was deduced from its spectral properties (UV, IR, ^{1}H NMR, MS), certain chemical properties (formation of mono-O-acetate, reaction with $SOCl_2$ and pyridine to give 20-chlorocamptothecin, rapid saponification to a sodium salt that gave **1a** on acidification, and reduction with $NaBH_4$ to a lactol at room temperature[1,2]), and the X-ray crystallographic analysis of its 20-iodoacetate derivative.[1,3] The latter technique established that **1a** is 4(*S*)-4-ethyl-4-hydroxy-1H-pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-3,14[4H,12H]-dione. Subsequently, camptothecin has been found in *Nothapodytes foetida* (Wight) Sleumer (Icacinaceae) [formerly, *Mappia foetida* Miers],[4] in *Ophiorrhiza mungos* Linn. (Rubiaceae),[5] and in *Ervatamia heyneana* (Wall.) T. Cooke (Apocynaceae).[6] Some of these plants also contain the oxygenated camptothecin analogues **2**, **3**,[7,8] **4**[9] and **5**,[10] mappicine (**6**),[11] and 20-deoxycamptothecin (**1b**).[10,12]

Camptothecin has two notable chemical properties. Its lack of significant bascity causes it to behave as a neutral molecule, i.e. it does not form stable salts with mineral acids[1] and thus it is not an alkaloid in the usual sense of the definition. The presence of the C-20 tertiary alcohol imparts an unusual electrophilicity to the lactone carbonyl of **1a**, perhaps via a strong intramolecular H- bond. This structural feature explains the behaviour of **1a** towards aqueous alkali and amine nucleophiles[2] and $NaBH_4$.[1] It also justifies the inability of preparing stable C-21 ester or amide derivatives of **1a**: rapid reversion of such derivatives to **1a** occurs by intramolecular attack of the C-17 primary alcohol at the electrophilic C-21 carbonyl.



1a, R=OH
1b, R=H
1c, R=Cl

2

3, R=H
4, R=Me

5

6

Fig. 1. Naturally occurring pyrrolo[3,4-*b*]quinoline alkaloids

2. TOTAL SYNTHESIS

(1) *The need*

The announcement of camptothecin's structure in 1966 caused considerable excitement in the scientific community for two reasons. One, the molecule represented a new heterocyclic ring system and, two, it exhibited excellent biological activity in the *in vivo* rodent assays for antitumor activity.[1,2] There was an evident need for the development of practical synthetic routes to **1a** since *C. acuminata* bark contains only about 0.012% by weight[12] of **1a**. Furthermore, the challenge to devise a general synthesis of the pyrrolo[3,4-*b*]quinoline ring system was not ignored by many research groups.

Since two comprehensive reviews of camptothecin's total synthesis are available in the earlier literature,[13,14] I discuss only six syntheses in this review. Each of these synthetic routes represents either a pioneering synthetic development, a unique synthetic approach, or a particularly efficient synthetic route to **1a** and/or its structural analogues.

(2) *Stork synthesis*

Stork and Schultz achieved the first total synthesis of camptothecin in 1971.[15] A base-catalyzed Friedländer condensation of pyrrolidone **8** (Fig. 2) with *o*-aminobenzaldehyde (**7**) gave the pyrrolo[3,4-*b*]quinoline acid **9**, which was converted in *ca* 35% overall yield to the tetracyclic β-ketoester **10** using a Dieckmann cyclization. Hydrolysis and decarboxylation of **10**, followed by reduction with $NaBH_4$ and elimination of water from the resulting β-hydroxylactam gave the desired dihydropyridone **11**. The latter compound reacted efficiently at low temperature with the lithium

anion of a protected α-hydroxybutyric acid ester to give the key pentacyclic compound **12** via intermediate **11A**. This transformation represented a new annulation method of α,β-unsaturated carbonyl compounds.[13] Finally, hydrolysis of the ethyl ester of **12** and reduction of the resulting amino acid hydrochloride salt with $NaBH_4$ gave hemiacetal-acid **13**, whose conversion to (±)-**1a** occurred in good yield by a five-step reaction sequence involving dehydrogenation of the C-17 acetate of **13** with DDQ, acetate hydrolysis, hemiacetal reduction with $NaBH_4$, and acidification to form the α-hydroxy lactone ring. The overall yield of racemic **1a** was 1–2%.

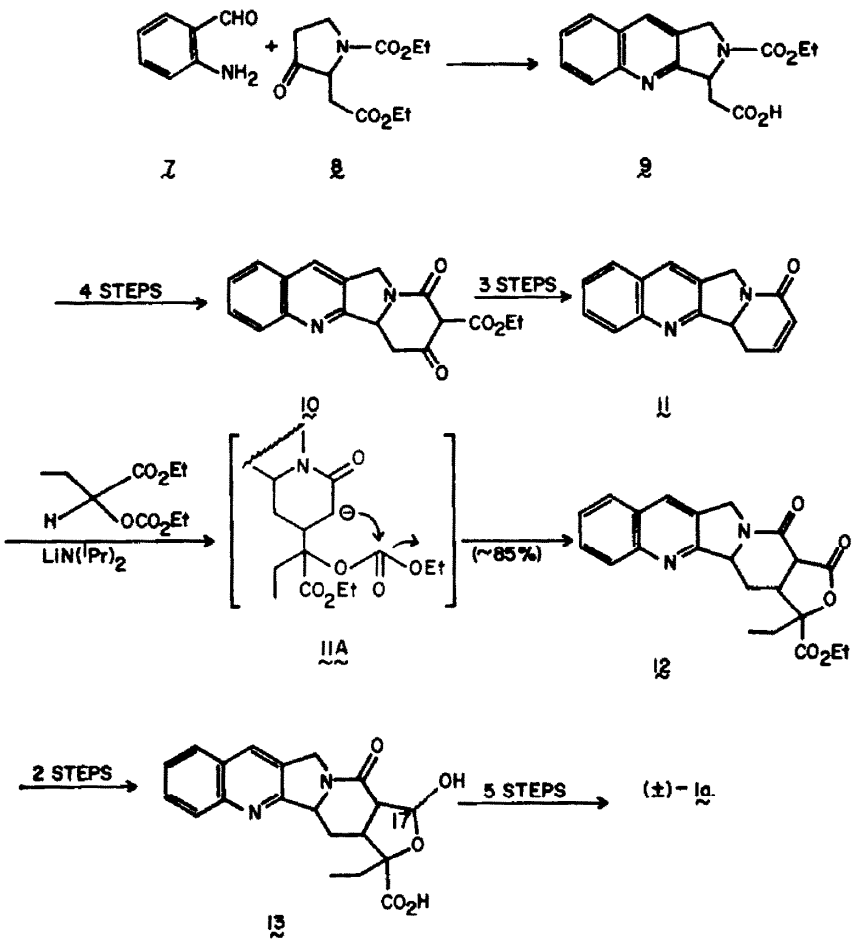


Fig. 2. Camptothecin synthesis of Stork and Schultz.

(3) *Danishefsky synthesis*

The report of the first synthesis of camptothecin preceded the description of the second total synthesis of (±)-**1a** by only 9 weeks. Danishefsky *et al.*[16] assembled the alkaloid by a Friedländer condensation approach (Fig. 3) in which the key pyridone **16** was prepared by a novel synthetic route to 4,6-disubstituted pyridones.[16b] The latter utilized the Michael addition of enamine **14** to dicarbethoxyallene to yield an N,3,4,5-tetrasubstituted pyridone (**15**) via intermediates **14A** and **14B**. Transformation of **15** to **16** through Dieckmann cyclization of an intermediate tetramethyl ester (C-3, C-20 = CO_2Me) was followed by hydrolysis-decarboxylation in aqueous acid, then a Friedländer condensation to give the tetracyclic diacid **17**. Unfortunately, decarboxylation of the C-20 methyl ester of **17** proved difficult, giving only a 23% yield of the desired tetracyclic monomethyl ester by pyrolysis over Cu'O. The latter compound was monoethylated to **18** in 20% yield. Treatment of **18** with paraformaldehyde in acidic solution resulted in C-16 alkylation and lactonization to give (±)-**1b** (35%) along with a structurally isomeric minor product (isodeoxycamptothecin due to C-14

alkylation[17]). Synthetic 20-deoxycamptothecin (**1b**) spontaneously oxidized to (±)-**1a** on exposure of its solutions to air; on a preparative scale, Danishefsky *et al.* carried out this oxidation by treating a solution of the C-20 anion of **1b** with H_2O_2. The ease by which **1b** gives **1a** may have biogenetic significance.

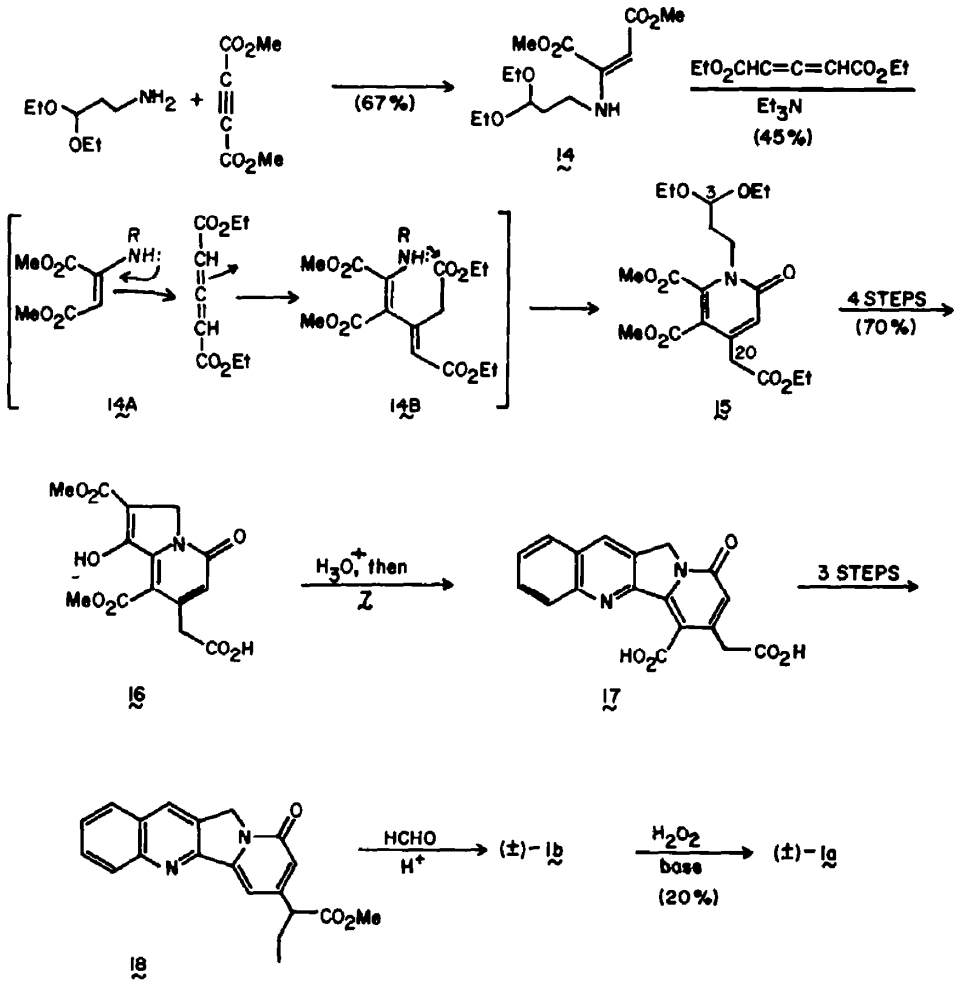


Fig. 3. Camptothecin synthesis of Danishefsky *et al.*

(4) *Winterfeldt biomimetic synthesis*

Winterfeldt *et al.* discovered that certain tetrahydro-β-carboline alkaloids could be autoxidized to pyrrolo[3,4-*b*]quinolones in strongly basic DMF solutions.[18] Thi observation led them to develop an imaginative total synthesis of camptothecin,[19] whose strategy in part may parallel the alkaloid's biogenesis.

The Winterfeldt synthesis of (±)-**1a** employed the tetracyclic lactam **21** (Fig. 4) as the keystone for formation of the pyrrolo[3,4-*b*]quinoline ring system. Lactam **21** was available from **19** by unexceptional chemistry, which utilized a Dieckmann cyclization to construct the tetracyclic ring system. The 1,4-addition-elimination of sodio di-*t*-butylmalonate to **21** followed by autoxidation of the tetrahydro-β-carboline **22** and treatment of quinolone **23** with $SOCl_2$ in DMF gave the 12-chloro-9-oxo-9,11-dihydroindolizino[1,2-*b*]quinoline **24** in good overall yield. Introduction of the 10a,6-3,14-double bond in **24** was postulated to involve a novel oxidative elimination of SOCl.[18b] After hydrogenolytic removal of the chlorine atom, the C-17 carbethoxy group in **24** enabled its chemoselective reduction to a primary alcohol with, first, diisobutylaluminum hydride at low temperature, then KBH_4, from which **25** resulted by treatment with CF_3COOH. Regiospecific

ethylation at C-20 of **25** proceeded poorly because of concurrent dialkylation at C-5 and C-20, but the resulting deoxycamptothecin was oxidized to (±)-**1a** quantitatively by O_2, $Cu^{II}Cl_2$ in aqueous dimethylamine.

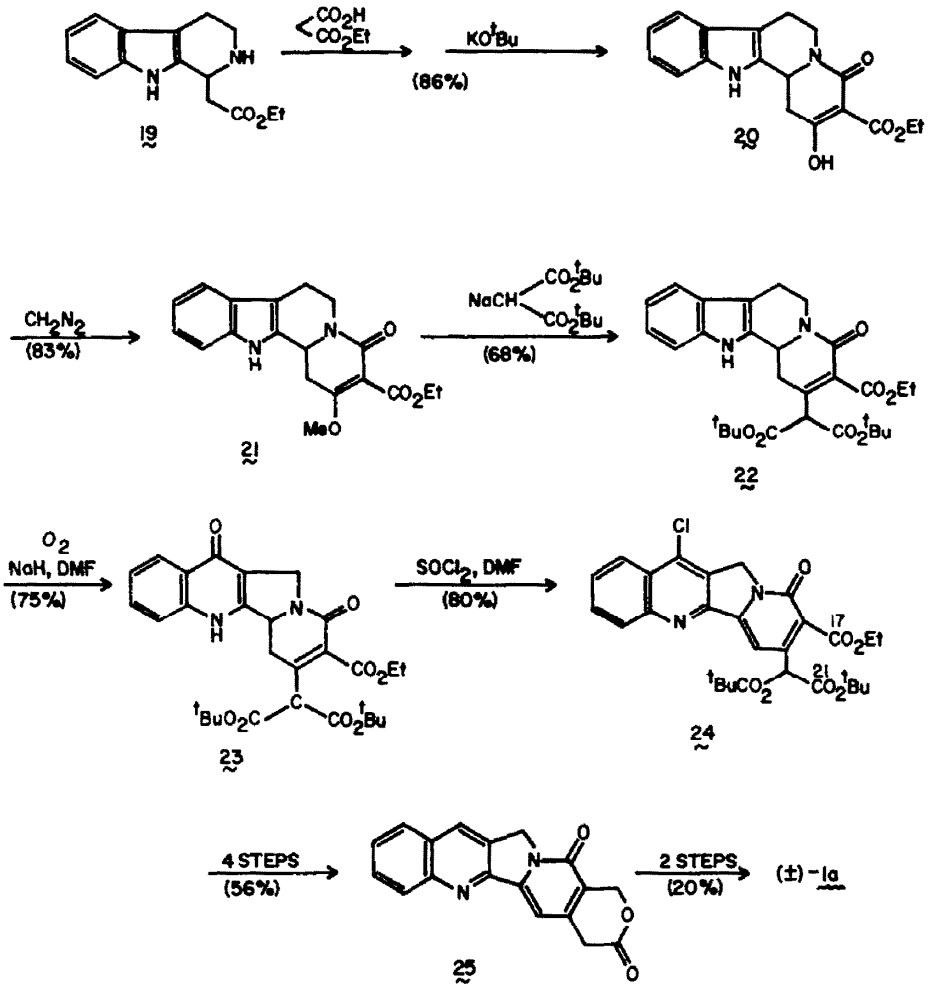


Fig. 4. Winterfeldt biomimetic camptothecin synthesis.

Winterfeldt's group later reported the results of further developments in the synthetic chemistry of camptothecin, which markedly improved the overall yield of their total synthesis. The problem of C-5 ethylation was circumvented[21] by ethylation of an open-ring C-21 monoisopropyl ester analogue of **24**, from which 20-deoxycamptothecin could be prepared as before in 86% overall yield. They also investigated the remarkable ease by which 20-deoxycamptothecin is autoxidized to **1a**, finding that this reaction has strict structural limitations.[22] Only **1b** and its rings DE analogue (as the N–CH_3 pyridone) could be oxidized at C-20 using their experimental conditions. Three other compounds (dimethyl 2-ethyl malonate, methyl 2-ethyl phenylacetate, and the benzene ring analogue of rings DE of camptothecin) examined as models for this oxidation were completely unreactive. Clearly, the pyridone ring of **1b** plays a vital role in the mechanism of C-20 autoxidation.

(5) *Rapoport synthesis*

The total synthesis of (±)-**1a** by Rapoport *et al.*[23] is notable because of the impressive 15% overall yield from the starting material, pyridine-2,5-dicarboxylic acid (isocinchomeronic acid). In addition, these workers employed a novel rearrangement of a nipecotic acid to an α-methylene lactam[24] in one key synthetic step.

The Rapoport camptothecin synthesis (Fig. 5) used the nipecotic acid **27**, prepared from **26** by unexceptional chemistry, for rearrangement to the α-methylene lactam **28** in refluxing Ac_2O. They had planned to carry out this rearrangement at the tetracyclic stage after Friedländer condensation of **27** with **7**. Although the rearrangement could be done in 60% yield, the subsequent oxidation with SeO_2 to the tetracyclic analogue of **29** was not possible (complete aromatization occurred). Consequently, they oxidized **28** with SeO_2, hydrolyzed the intermediate to **29**, then employed a Claisen orthoester rearrangement and Pfitzner–Moffatt oxidation to obtain the α-methylene lactam **30**. Friedländer condensation of **30** with **7** gave the expected tetracyclic material (**31**), whose oxidation with SeO_2 simultaneously aromatized the D ring and introduced a C-17 acetoxy substituent. The acetoxy group probably was introduced by [3,3]sigmatropic rearrangement of a 3,14-dehydro-15-acetoxy derivative of **31**, because SeO_2 oxidation (and also NBS) of a C-17 desacetoxy analogue of **32** failed to occur. Finally, treatment of **32** first with acid then with O_2, $Cu^{II}Cl_2$, and aqueous dimethylamine in DMF[22] gave (±)-**1a** in excellent overall yield.

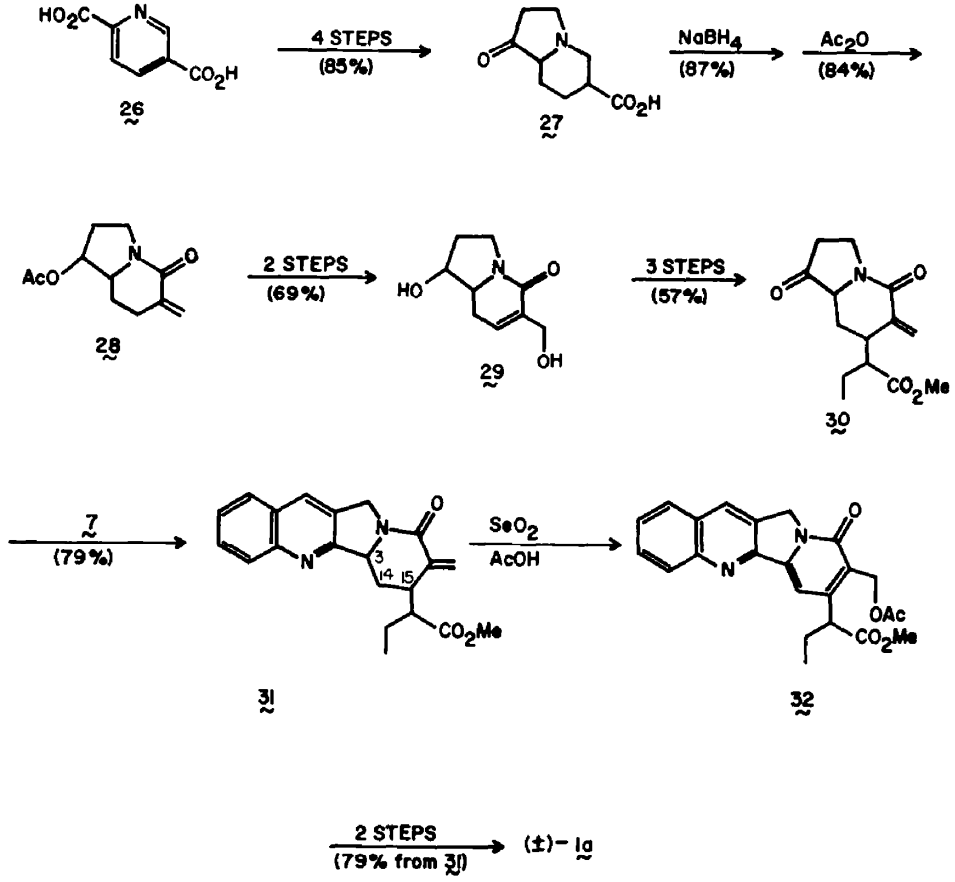


Fig. 5. Camptothecin synthesis of Rapoport *et al.*

(6) *Corey synthesis of* (+)*-camptothecin*

The only synthesis of optically active 20(*S*)-camptothecin reported to date is that of Corey *et al.* Although the overall yield of the Harvard group's synthesis was low, their strategy is typically novel.

Their convergent synthetic approach brought together a chiral pseudoacid chloride (ring E of **1a**) with a tricyclic diamine (rings ABC) through which the D ring was formed by cyclization of an intermediate γ-aldehydo-*t*-amide (Fig. 6).

Resolution of the 3,4-disubstituted furan α-hydroxy acid **37**—prepared from 3,4-dicarboxy furan by standard, but delicate, synthetic transformations in 10% overall yield—*via* its diastereomeric quinine salts and protection of the tertiary OH in the lactonized form of (+)-**37** gave (+)-**38** in good

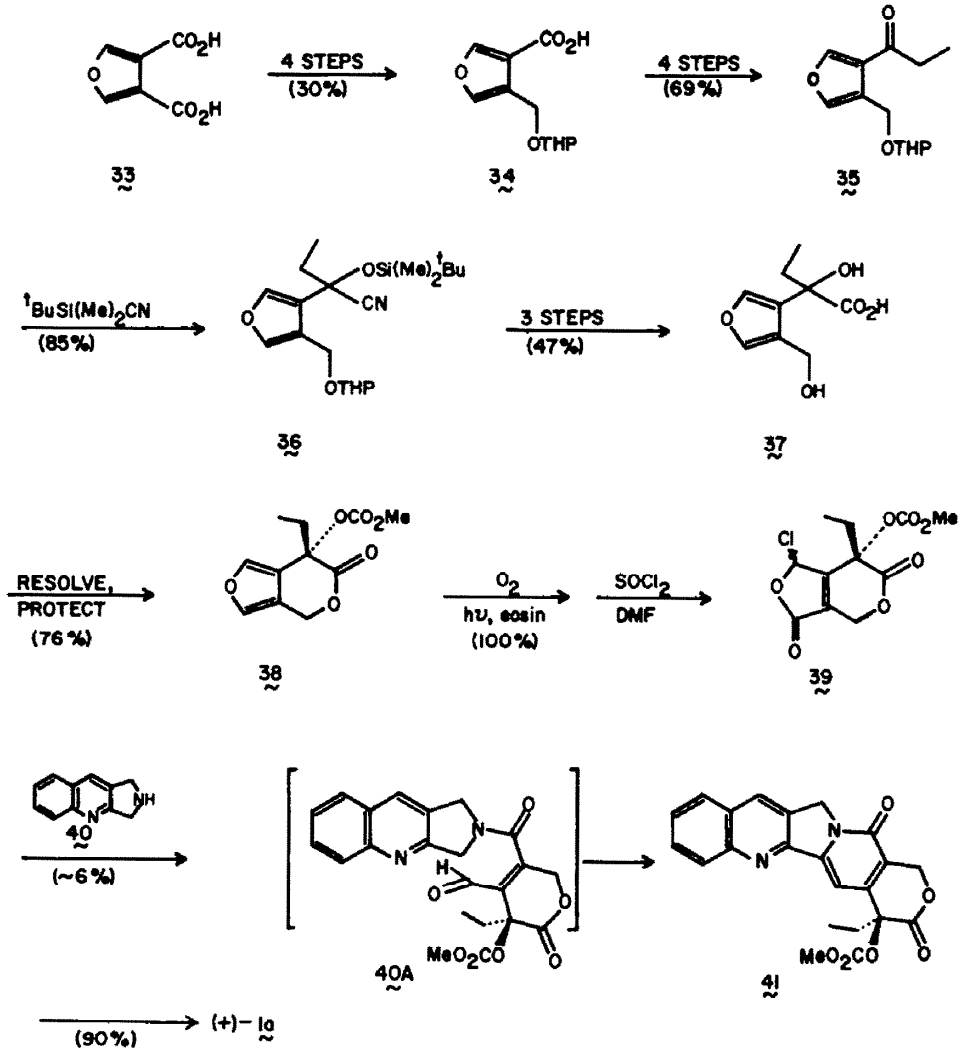


Fig. 6. Synthesis of 20(*S*)-camptothecin by Corey *et al.*

yield. Photooxidation of (+)-**38** to the hemiacylal followed by its treatment with $SOCl_2$ in a catalytic amount of DMF gave a 2.5:1 mixture of pseudo-acid chloride **39** and its undesired regioisomer. Condensation of this mixture with tricyclic amine **40**, prepared from acridine in three steps (18%), in pyridine-acetonitrile (to **40A**) followed by base-catalyzed cyclization gave 20(*S*)-20-methoxycarbonyl-**1a** (**41**) in low yield. Time and material limitations did not permit the Harvard group to develop a means for improving the yield of this condensation-cyclization reaction, although it could be done quantitatively in a model system. Deprotection of **41** by treatment with lithium mercaptide in HMPA gave (+)-**1a** cleanly.

(7) *Chinese/Wall synthesis*

Chemists at the Shanghai Institute of Materia Medica, Shanghai Nos. 5 and 12 Pharmaceutical Plants and Shanghai Institute of Pharmaceutical Industrial Research developed an efficient, practical synthesis of (±)-camptothecin in 1976,[26] but the full details of their work did not appear in the easily available English literature until 1978.[27] Since Wall *et al.* recently have used a slightly modified version of the Chinese synthesis for the efficient preparation of (±)-**1a** and several of its analogues,[29] I discuss these two syntheses together. The new chemistry developed in these two laboratories would enable the total synthesis of racemic **1a** on a large scale, if this becomes advisable in the future.

The Chinese/Wall camptothecin synthesis employed pyridone **42** (Fig. 7), available from the condensation of cyanoacetamide and the *O*-ethyl ether of ethyl acetopyruvate,[28] to make pyridone **43**

by a three-step sequence involving Michael addition to methyl acrylate, hydrolysis-decarboxylation in aqueous acid, and ketalization. The C-4 methyl of **43** was sufficiently acidic to permit its carbethoxylation by reaction with NaH in diethyl carbonate. α-Ethylation of the resulting intermediate ester gave **44** which underwent simultaneous deketalization and Friedländer condensation with **7** in acidic media. Following reductive acetylation of the cyano group of **45** (→**46**), formation of the N-nitroso acetamide of **46** *in situ* in the acid filtrate obtained from the hydrogenation reaction resulted in its rearrangement to its C-17 acetoxy analogue. The researchers then cyclized the latter intermediate without isolation to (±)-**1b** in dilute acid from which (±)-**1a** resulted by Winterfeldt's method.[19] The overall yield of this Chinese chemists' 10-step synthesis was an impressive 18%.

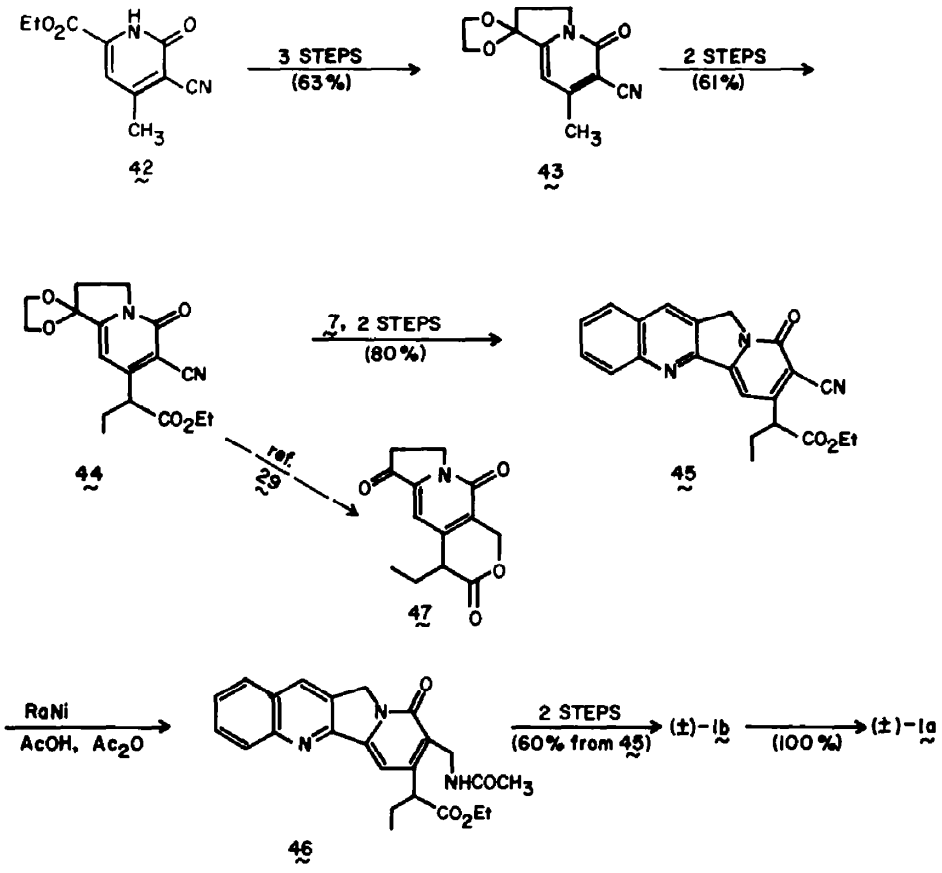


Fig. 7. Camptothecin synthesis of mainland Chinese and Wall.

Wall's group at the Research Triangle Institute modified the Chinese group's camptothecin synthesis by first converting **44** to **47**, the complete CDE rings portion of **1b**, from which they could prepare (±)-**1a** (28% overall) and (±)-10-hydroxy-**1a** (20% overall) by suitable Friedländer condensations and subsequent standard transformations.[29] Their synthetic modification, which the Chinese workers had attempted but could not execute successfully, improved the overall yield of (±)-**1a** significantly. (Wall *et al.* earlier had prepared (±)-**1a** by a completely different, but less efficient, synthetic approach.[30])

My review of the total syntheses of camptothecin is eclectic because of space limitations. Readers who wish to see a more extensive review of this expansive subject may consult the other reviews cited,[13,14] as well as more recent descriptions of camptothecin syntheses.[31-33]

3. BIOGENESIS

(1) *Biogenetic speculations*

Although it is not immediately obvious from the structure of camptothecin that it could be derived biosynthetically from tryptophan and a monoterpene, Wenkert *et al.* speculated in 1967[34] that **1a** in fact might be a monoterpene indole alkaloid using plausible chemical transformations of isositsirikine (**48**, Fig. 8) as the basis from which to formulate a biogenetic scheme for **1a**. Winterfeldt[18] later expanded on this idea based on his own finding that **49** underwent facile autoxidation to **50** *in vitro*, and proposed that geissoschizine (**51**) was a plausible biogenetic precursor of **1a**.

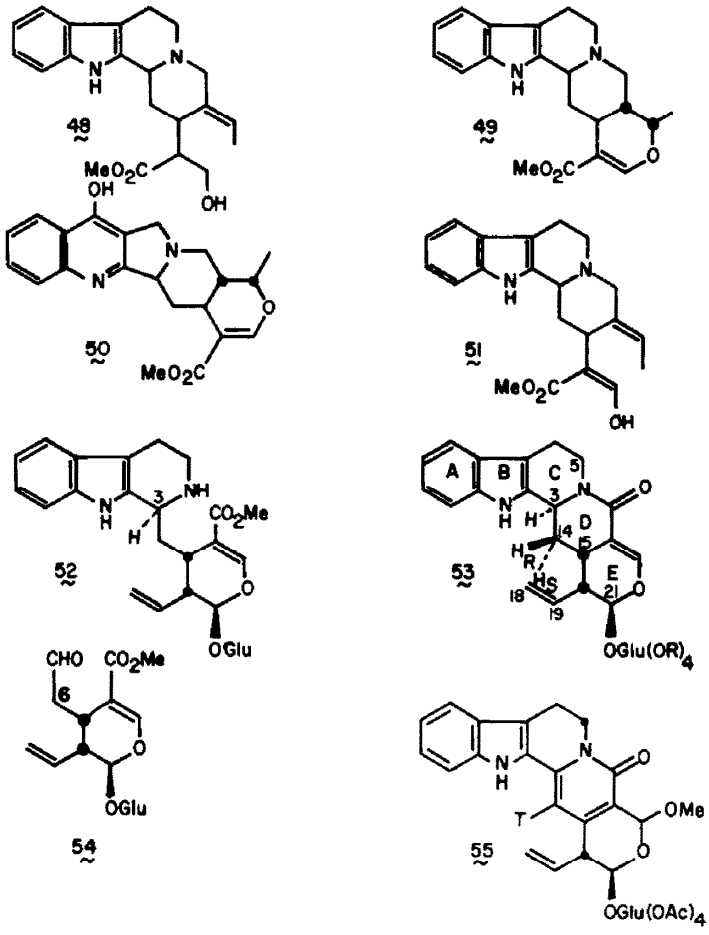


Fig. 8. Hypothetical and actual intermediates of camptothecin biosynthesis.

Our biogenetic reasoning took these two ideas into account, but also recognized the clear structural relationship between **1a** and strictosamide (**53**). The latter neutral glucoside was known as a transformation product of strictosidine (**52**) under basic conditions.[35] Thus, transformation of **53** into **1a** 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.[36] This biogenetic hypothesis also was proposed independently by Cordell.[37]

(2) *Preliminary biosynthetic studies*

We established in initial trial feeding experiments that radioactively labeled tryptophan was incorporated into **1a** in apical cuttings of young seedlings of *C. acuminata* to the extent of 4×10^{-4} to 2.6×10^{-2}%. Similarly, radioactively labeled mevalonic acid and [6-^{3}H]-secologanin (**54**) were found to give rise to radioactive **1a** *in vivo*.[38] These incorporations of radioactivity into **1a** 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[39] 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 **1a**: 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 **1a** was a monoterpene indole alkaloid, the C-3 epimeric mixture of strictosidine/vincoside ((3*R*)-**52**), tritium labeled at C-5 by synthesis from [1-^{3}H]tryptamine, was then tested as a precursor of **1a**. The observed total radioactivity incorporation into **1a** of 0.24% supported the implications of our initial results.

At this juncture three possibilities for the conversion of **52** into **1a** *in vivo* had to be considered: via **53**, via **48** or via **51**. Since radiochemically labeled **53** and **51** were available, two of these possibilities could be tested. In the event, it was immediately clear from its efficient incorporation into **1a** (1-4%) that only **53** need be considered for further experimentation in the elucidation of camptothecin's biosynthetic pathway.[36]

When these feeding experiments were being designed, the available literature data,[35,37] indicated that vincoside was the precursor of monoterpene indole alkaloids. Based on this biogenetic analogy, vincoside,(3*R*)-**52**, and thus vincoside lactam,(3*R*)-**53**, would have been expected to be better precursors of camptothecin that are either **52** or **53**. However, the incorporation of vincoside lactam into **1a** was quite low relative to that of **53** despite repeated experimentation and a subsequent single experiment[42] showed that vincoside also was a poorer precursor of **1a** than is **52**. At the time[36] these observations were not felt to be remarkable, since *C. acuminata* (Nyssaceae) is not related taxonomically to *C. roseus* (Apocynaceae), and thus complete stereochemical homogeneity between biosynthetic pathways in the two organisms need not be expected. Recent events, however, have established that strictosidine (isovincoside) is the key precursor of monoterpene indole alkaloids in *C. roseus* and other plant genera.[43,44] Our results therefore are seen to be completely consistent with the developing picture of monoterpene indole and alkaloid biosynthesis among those higher plants which have been studied experimentally.[37]

(3) *Strictosamide, the penultimate biosynthetic precursor*

Although the results of the feeding experiments discussed above strongly support the role of strictosamide (**53**) as the key biosynthetic precursor of **1a**, we had to ascertain the regiospecificity of its incorporation into **1a**. In spite of the lack of suitable degradative chemistry, the efficient incorporation of radioactive **53** into **1a** suggested that the labeling regiochemistry could be determined directly by NMR spectroscopic analysis.[36] Since the specific incorporation of **53** into **1a** was between 1 and 2%, we felt that a ^{13}C-labeled **53** containing $\geq$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**. A suitable quantity of [5-^{13}C]-**53** containing 84 mole % ^{13}C was synthesized and fed to *C. acuminata* plants growing in a glasshouse. The proton noise-decoupled ^{13}C NMR spectrum of the resulting labeled **1a** showed that only the resonance corresponding to C-5[36] 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]-**53** in separate radioactive feeding experiments. Consequently, the requisite certification of the role **53** plays as a specific biosynthetic precursor of **1a** was firmly established.

Earlier incorporations of [14-^{3}H,5-^{14}C]-**53** into **1a** had been attended by only a 5–9% decrease in the ^{3}H:^{14}C ratio. This was a surprising finding, since **53** was expected to lose *ca* one-half its C-14 ^{3}H labeling during oxidative formation of the pyridone ring of **1a**.[45]

We considered three explanations for this low percentage ^{3}H loss: (1) **53** might fortuitously have been stereospecifically labeled with ^{3}H at C-14 and oxidation to **1a** might remove hydrogen from only the unlabeled diastereotopic position; (2) conversion of **53** into **1a** might involve an intramolecular migration of one of the two C-14 tritium atoms to another site in some biosynthetic intermediate leading to **1a**, resulting in retention of both labels, or (3) loss of hydrogen from C-14 during oxidation

of the D ring of **53** to the pyridone ring of **1a** 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.[45])

We examined each of the above alternatives in turn.[40] Analysis of the ^1H, ^2H and ^{13}C NMR spectra of samples of [14-^2H]**53**, which had been prepared in the same manner as for [14-^3H]**53**, clearly showed that the C-14 diastereotopic positions of **53** were ^2H labeled equally. Furthermore, these NMR spectra showed that ^2H was not incorporated into any other position in **53** during the isotopic labeling reactions. We concluded that the samples of [14-^3H, 5-^{14}C]**53** used in the biosynthetic feeding experiments were equally ^3H labeled intermolecularly at the diastereotopic hydrogens attached to C-14, assuming that the distribution of ^3H label at these two positions will parallel the established ^2H labeling stereoselectivity.

We next examined the possibility that the high retention of ^3H in the conversion of **53** to **1a** *in vivo* was due to an intramolecular ^3H migration. For example, we considered one likely possibility to be a [1,4] migration of ^3H from C-14 to C-17 via some ionically charged intermediate.[40] Chemical degradation of a sample of radioactive **1a**, labeled by the incorporation of [14-^3H, 5-^{14}C]**53**, to a C-17 lactone (see **12**, Fig. 2) eliminated this possibility since the lactone degradation product contained 97% of the molar ^3H content of **1a**. A second and more conclusive result (than the latter) was the finding that ^2H NMR analysis of the 20-methylthiomethylene derivative of **1a** labeled by [14-^2H, ^3H]**53** showed ^2H to reside only at C-14 of **1a**.[40] The specific incorporation of ^2H (0.53% by NMR analysis) agreed closely with the same value calculated from the ^3H radioactivity (0.57%). These data established that [14-^2H, ^3H]strictosamide labels only H-14 of camptothecin *in vivo*.

We had therefore established that the precursor [14-^2H or ^3H]**53** was nonstereospecifically labeled at C-14, and at no other position, and that the product **1a** was labeled only at H-14. The results of an independent feeding experiment with [6,8-^3H]loganin further corroborated these observations, and also revealed indirectly that the mechanism of D ring oxidation of the unknown biosynthetic intermediate laying between strictosamide and camptothecin does not involve significant stereospecific loss of hydrogen (as ^3H) from the C-14 diastereotopic positions.[40] Consequently, we believe that presumption (3) of our three possible explanations (*vide supra*) of the low ^3H loss attending the biosynthetic incorporation of [14-^3H, 5-^{14}C]**53** into **1a** is correct.

Verification that presumption (3) explains our observations will be difficult until we are able to examine the D ring oxidation of post-strictosamide biosynthetic intermediates as a discrete event, i.e., by cell-free or purified enzyme experiments. Since experiments of this type are not yet feasible, we have presented data from four other literature sources that support the sensibility of our rationalization.[40] Furthermore, we observed that the D ring oxidation of tetraacetyl [14-^3H, 5-^{14}C]**53** to **55** with DDQ *in vitro* was attended by a strikingly high retention of ^3H (115%) relative to the intermolecular ^{14}C reference label.[40]

(4) *Poststrictosamide biosynthetic events*

Our original biogenetic hypothesis for camptothecin[36] thus is valid overall as far as the above results support it. However, the exact sequence of biosynthetic transformations between strictosamide (**53**) and **1a** remains unclear. Removal of glucose from **53** is, intuitively, likely to be the step immediately following the formation of **53** by analogy with the biosynthetic fate of strictosidine (**52**) in other higher plants.[37,43,44] We also believe that formation of the pyrrolo[3,4-*b*]quinoline ring system should precede the oxidation of the D ring to a pyridone. This presumption is supported by the fact that **55** and related model compounds are inert to laboratory reagents and conditions known to transform **53** and other tetrahydro-β-carbolines smoothly into 12-hydroxy-9-oxo-9,11-dihydroindolizino[1,2-*b*]quinolines (J. L. Straughn and C. R. Hutchinson, unpublished observations). However, the observation that neither radioactive strictosamide aglucon (**56**) nor **57**, the quinolone analogue of **53** (Fig. 9), were incorporated into **1a** in *C. acuminata* cuttings[42] does not support our presumptions.

In our current biosynthetic study of camptothecin we are thus investigating the sequential relationships of the pathway intermediates and the mechanism of quinoline and pyridone ring formation. For example, we will test our idea that the mechanism of formation of the pyrrolo[3,4-*b*]quinoline ring system *in vivo* could proceed by reduction of the ketolactam **58**, derived from

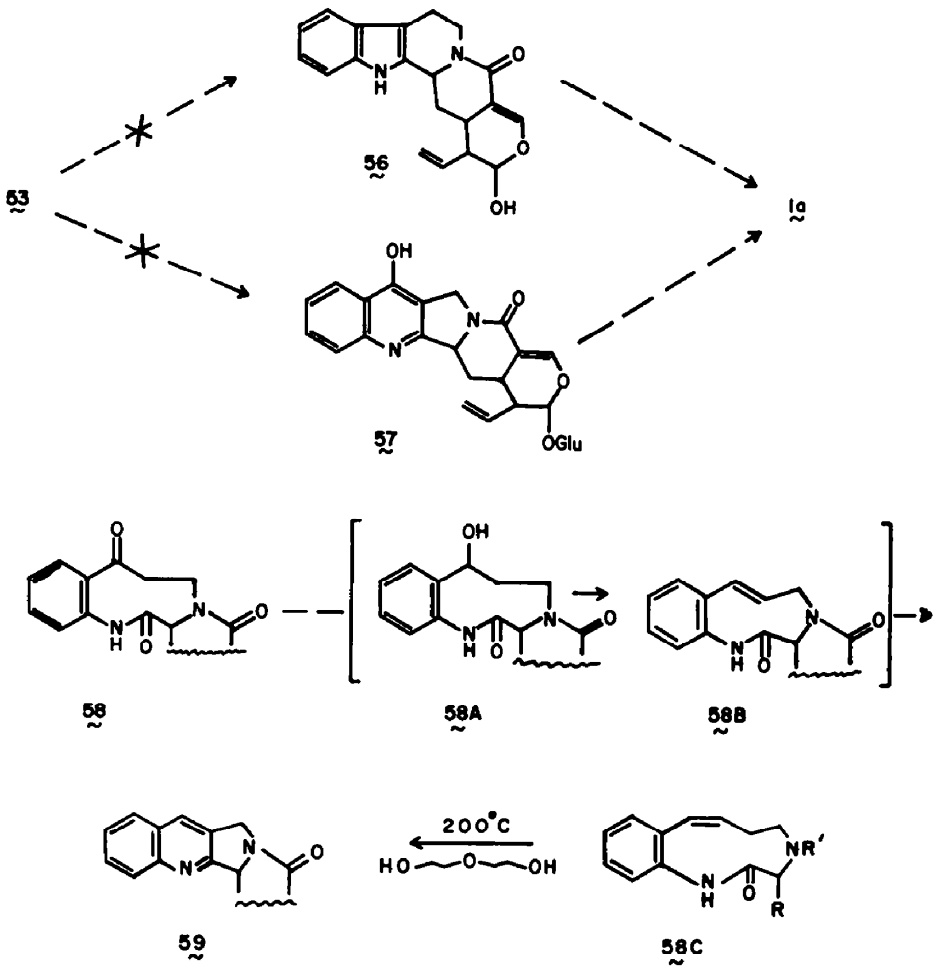


Fig. 9. Hypothetical steps in the latter stage of camptothecin's biosynthesis

stictosamide, to **58A**, followed by ring closure to quinoline **59** via stepwise ionic or concerted electrocyclic processes. The thermal cyclization of **58C** to the corresponding analogue of **59** supports the latter biosynthetic concept (J. L. Straughn and C. R. Hutchinson, unpublished observations). Reductive removal of the C-7 hydroxy of **57**, which also could form from **58** *in vivo* as it does *in vitro*,[48] of course is an alternate biosynthetic possibility. We also are developing the reported tissue cultures of *C. acuminata*[46] for possible use in cell-free and other biosynthetic experiments. This approach promises to be exciting and fruitful in view of the superb biosynthetic results obtained by Zenk *et al.* with experimental systems derived from *Catharanthus roseus* tissue cultures.[47]

(5) *Biogenesis of Mappicine*

The co-occurrence of camptothecin and mappicine (**6**) in *N. joetida* suggests that these two alkaloids have common biosynthetic precursors. Moreover, it is quite plausible that **1a** is the precursor of **6** by the mechanism proposed in Fig. 10. This mechanism is operative in the fragmentation of molecular ions produced from **1a** in the electron impact mass spectrometer,[46] and is the most reasonable explanation of the apparent electron deficiency at C-17 of synthetic intermediates prepared during the total synthesis of **1a**[21] and the partial synthesis of **6**.[11] Of course, alternative biogenetic hypotheses are conceivable, e.g. formation of **6** from an iridoid with a C-3 Me rather than an enolized aldehyde [C-3 of the iridoid becomes C-17 of **6**]. We shall be testing these ideas experimentally in future work.

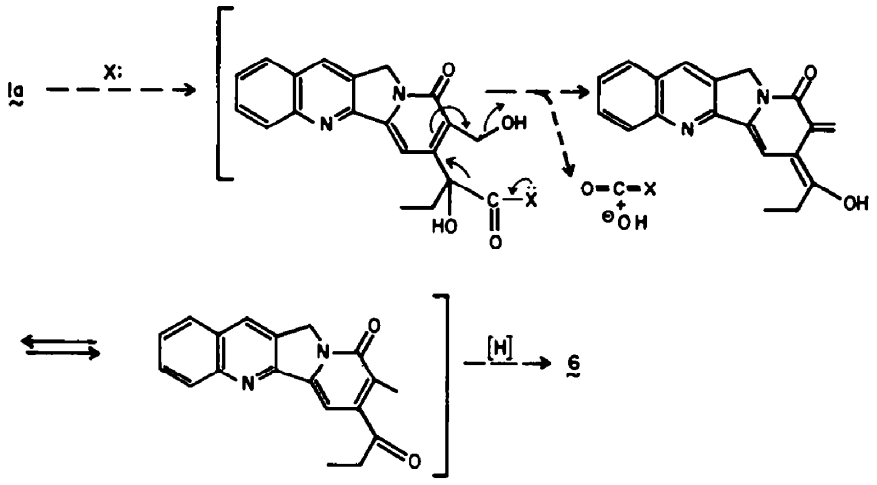


Fig. 10. Hypothetical biogenesis of mappicine.

4. MEDICINAL CHEMISTRY

(1) *Structure-activity relationships*

Camptothecin is a potent inhibitor of the growth of leukemia cells *in vitro* and shows good antitumor activity against murine L1210 and P388 leukemia and B16 melanocarcinoma *in vivo*.[1,4,9,50] Unfortunately, despite an initial encouraging report that the sodium salt (**60**) of **1a** (Fig. 11) was active clinically against solid tumors of the gi tract,[51] subsequent clinical evaluation of this drug led to discouraging results.[50,52] Today it and 10-hydroxycamptothecin (**3**) are used in the clinical treatment of cancer only in the People's Republic of China with apparent success against liver carcinoma and tumors of the head and neck.[10b,53] Since only **60** was used in the clinical evaluation of camptothecin in the United States, the potential of the compound as a cancer chemotherapeutic drug may have been misconstrued, for Wall *et al.* have reported quite recently that **60** has only about one-tenth the potency of **1a** in one antitumor assay (P388 rodent leukemia).[29]

Wall *et al.* established early in the chemical investigation of camptothecin that the ring E α-hydroxy lactone of **1a** is the most critical structural feature with respect to the alkaloid's antitumor activity *in vitro* and against L1210 and P388 *in vivo* assays: 20-deoxycamptothecin (**1b**), 20-chlorocamptothecin (**1c**), and camptothecin hemilactol (**61**) were completely inactive in such assays.[2,54,55] Although the chemistry of **1a** (*vide supra*) supports an alkylating role for the α-hydroxy lactone of **1a** as the chief determinant of its molecular mechanism of action, the remaining portion of the molecule also must play an important role. The fact that a a large number of rings DE and CDE analogues of **1a** are inactive as antitumor agents[56] supports this conclusion.[55]

Five research groups have prepared camptothecin analogues (Fig. 11) that show significant antitumor activity *in vivo*. Sugasawa *et al.*[57] reported that analogues having the C-20 Et group of **1a** replaced by either $-CH_2CH{=}CH_2$, $-CH_2C{\equiv}CH$, $-CH_2C_6H_5$, or $CH_2COC_6H_5$ groups showed good increases in life span for L1210 leukemic mice. The 20-allyl analogue (**62**) was slightly more active than **1a**, and the 17-ethyl ether-20-carbethoxy analogue, **63**, surprisingly retained antitumor activity similar to **1a**. P. Pei-chuan *et al.* at the Shanghai Institute of Materia Medica reported the synthesis of eleven 12-substituted analogues of **1a**; preliminary pharmacological tests revealed that the 12-chloro, -hydroxy and -methoxy analogues exhibited greater potency *in vivo* than **1a** against one leukemia assay and Ehrlich ascites carcinoma.[58] These researchers also stated that 10-hydroxycamptothecin (**3**) was more effective and less toxic than **1a** towards a variety of animal tumors.[10] Adamovics and Hutchinson described the preparation and evaluation of five derivatives of camptothecin's (+)-21-isopropylamide analogue (**64a**).[50] The rationale for preparation of these derivatives was based on the observation that the 21-methylamide analogue of **1a** had *ca* three-fifths the activity of **1a** in the L1210 antitumor assay.[54] Analogues **64b** and **64d** showed activity *ca.* 85% that of **1a** in the P388 antileukemic assay, whereas the basic analogues **64e** and **64f** were inactive *in vivo*.

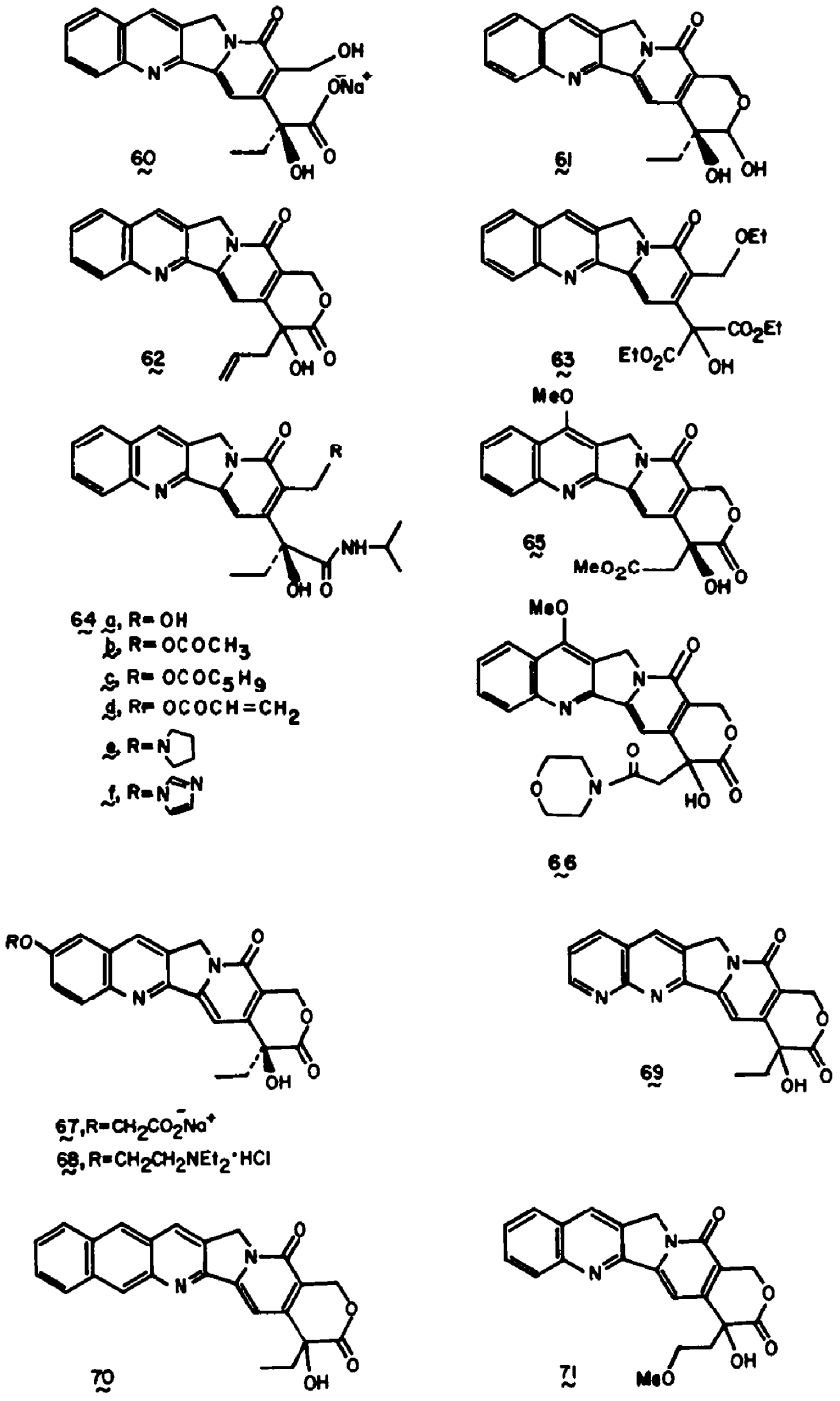


Fig. 11. Camptothecin analogues evaluated for their antitumor activity *in vivo*.

Winterfeldt and co-workers synthesized 7-chloro-[19] and 7-methoxy-[60] **1a** during their development of the total synthesis of **1a**. Subsequent work led to the synthesis of camptothecin analogues **65** and **66**.[60] Although compounds **65** and **66** showed moderate antitumor activity (T/C ~ 169–184 @ 100 mg/kg), and 7-chloro-**1a** good activity, 7-methoxy-**1a** was inactive *in vivo*.[61] The recent paper of Wani *et al.*[29] is the most comprehensive study of camptothecin analogue synthesis and biological evaluation. These workers prepared the water soluble derivatives **67** and **68** of natural 10-hydroxy camptothecin (**3**), the novel (±)-12-aza (**69**) and (±)-benz[j]camptothecin (**70**) analogues, and (±)-18-methoxycamptothecin (**71**) by means of an efficient camptothecin total synthesis (*vide supra*) As

shown in Table 1, several of these new analogues exhibited good antitumor activity in the P388 antileukemic assay. In particular, the water soluble amine salt derivative **68** of **3** had a 400% better therapeutic index than **3**, and 100% better than **1a**. The authors of this study believe that the activity of **68** may be due to its conversion to **3** *in vivo*;[29] the similar possibility also exists for compounds **64b-d**.[59]

Table 1. Antileukemic activity against P388 system of camptothecin and its analogues[a]

Compound	Dose Range (mg/kg)	Optimal T/C	Optimal Dose (mg/kg)	Lowest Toxic Dose (mg/kg)	Therapeutic[b] Index
1a	8.0-0.5	197	4.0	8.0	8
60	80.0-2.5	212	40.0	80.0	4
3	8.0-0.5	314	4.0	8.0	8
67	32.0-4.0	Inactive	--	--	-
68	32.0-2.0	234	32.0	--	16
69	32.0-2.0	175	32.0	--	2
70	32.0-1.0	198	16.0	--	4
71	8.0-0.5	160	4.0	8.0	2

[a]Adapted from ref. 29. [b]Lowest toxic dose divided by lowest effective dose.

(2) *Mechanism of action*

The novel structure and significant antitumor activity of camptothecin stimulated several groups of researchers to investigate its affect on whole animals and isolated mammalian cells in attempts to understand its molecular mechanism of action. Horwitz has reviewed the outcome of the studies completed during the period 1966–75[62] in detail; thus, I shall only summarize this information and then comment on more recent developments in this interesting story.

The principal effect of camptothecin on cultured mammalian cells is the potent inhibition of polynucleic acid biosynthesis. This apparently is not due to inhibition of nucleotide biosynthesis or of the enzymatic activity of DNA and RNA polymerases, and the effect on RNA formation is easily reversible on removal of the drug. The drug affects the biosynthesis of ribosomal RNA more than other types of cellular RNA. It does not significantly inhibit protein biosynthesis.

Camptothecin induces single strand breaks in cellular DNA in intact HeLa cells as viewed by alkaline sucrose density gradient analysis. This effect is reversible. Since **1a** does not affect the enzymes involved in DNA biosynthesis, its inhibition of DNA formation appears to be a result of some effect on the template function of DNA. Most investigators have concluded that the latter event is the primary determinant of camptothecin's cytotoxicity.

Camptothecin is an effective inhibitor of the replication of DNA containing viruses, but not those containing primarily RNA. Its effect here again is on DNA biosynthesis and is reversible on the drug's removal. Thus, the observations with viral systems corroborate the conclusion that **1a** is cytotoxic because of a disruption of DNA's normal function in cellular ontogenesis.

The information I summarize above points to a rationale for the molecular mechanism of action of camptothecin that should include a DNA binding component and a mechanism for covalent bond breakage in polydeoxyribonucleotides. These processes must be readily reversible with or without the intervention of DNA repair enzymes. Despite the attractiveness of such a rationale, the available data[62] do not support it unambiguously. For example, two research groups reported that the binding of **1a** to isolated DNA was very weak[63,64]—too weak, in fact, for analysis by conventional methods (except for the effect on the hyperchromicity that occurs when DNA strands separate[63]). Furthermore, **1a** had no significant effect on DNA integrity in isolated rat liver mitochondria,[65] nor in isolated cell nuclei from rat liver or Morris hepatoma (a slight amount of single-strand DNA breakage was seen by alkaline sucrose density gradient analysis only).[66] Since **1a** did not alter the integrity of superhelical plasmid Col EI DNA (H. Gamper, University of California, Berkeley, unpublished

results communicated to C. R. Hutchinson, 1978) nor PM2-ccc-DNA[67] *in vitro*, the collective data suggest that camptothecin must be "activated" *in vivo* to become cytotoxic.

The results of recent studies support the latter conclusion that some altered form of camptothecin is the actual cytotoxic agent *in vivo*. Sartiano *et al.* reported that **1a** could stimulate the ability of bleomycin to stimulate the incorporation of thymidine triphosphate into DNA of isolated rat liver and Morris hepatoma cell nuclei.[66] Camptothecin could replace the normal requirement of a reducing agent in this process, which probably is the result of repair of scissions induced in DNA by the action of bleomycin. [Bleomycin binds strongly to DNA and induces single- and double-strand breaks in DNA in the presence of metal ions and reducing agents *in vitro*.[68] O_2^-, H_2O_2 or ·OH are implicated to be part of this process.] These data suggest that **1a** can participate in a redox cycle with bleomycin-complexed Fe(II), which results in DNA breakage due to the production of O_2^-, H_2O_2 or ·OH in its immediate vicinity.[68b,c] However, Bachur *et al.*[69] noted that **1a** did not stimulate O_2 consumption by mammalian microsomes in the presence of NADPH whereas several quinone-containing anticancer drugs that did are easily reduced to semiquinone free radicals. Camptothecin apparently has too high of a redox potential compared with quinones to participate directly in a redox cycle catalyzed by the microsomal enzyme system, which could lead to the production of DNA damaging free radicals.

Recently Lown and Chen, with the author's collaboration, described the results of a novel model system for studying the effects of **1a** and several of its analogues on DNA *in vitro*.[67] Although **1a** had no effect on PM2-ccc-DNA *in vitro* in the dark, the following observations were noted upon photoactivation of **1a**. Aqueous solutions of **1a**, or its sodium salt (**60**), or a number of its derivatives when irradiated with 360 nm light in the presence of PM2-ccc-DNA produced single strand breaks in the latter. The chromophore of **1a** essential for the scission reaction consisted of intact rings A, B, C and D. The overall DNA breakage showed an inverse dependence on oxygen and there was evidence for at least two reaction mechanisms. Photosensitization of **1a** may generate radicals to attack DNA or, in the presence of oxygen, generate hydroperoxy radicals. The photolytic reaction of camptothecin itself proceeded via formation of racemized photolabile camptothecin hemiacetal **74** (Fig. 12), which suggests an alternative mechanism. In the anaerobic pathway photodecarbonylation of the alkaloid may generate a diradical which can collapse to **74** or abstract H atoms from DNA leading to strand scission. In the presence of oxygen the alternative aerobic pathway can supervene in which hydroperoxy radicals are generated leading to the generation of hydrogen peroxide and then the principal reactive species, OH radicals, which can then attack DNA. The intermediacy of these three species was proven unambiguously by (i) selective inhibition of scission with superoxide dismutase (ii) selective inhibition with catalase and (iii) spin-trapping and esr spectroscopy, respectively. Sequence specific DNA binding agents in conjunction with a topoisomerase relaxed PM2-ccc-DNA demonstrated a preferential photoinitiated camptothecin breakage of supercoiled DNA compared with relaxed. This may indicate a weak intercalative interaction of the alkaloid.

The authors of the latter study rationalized their results to be supportive of the molecular mechanism shown in Fig. 12. The important point is that photoactivation of camptothecin can cause the generation of free radicals (**72**, **73** or **75**) that, in turn, can lead to DNA strand scission by two alternative pathways through either direct interaction (**72**, **73**) or indirectly via HO·.[70] Although scission of DNA in this way by photoactivated **1a** is probably abiological, this study is the first successful duplication *in vitro* of the effects that **1a** has on DNA *in vivo* (intact cells). Thus, we believe that a detailed search for "activated" forms of camptothecin in other *in vitro* studies, and, especially, in *in vivo* experiments is now justified.

It is not at all clear if the results (and their interpretation) of structure-activity relationship studies of camptothecin and its analogues carried out *in vitro* are meaningful for the understanding of its molecular mechanism of action *in vivo* as an antitumor agent. Several camptothecin analogues, which are capable of inhibiting polynucleic acid biosynthesis and causing the fragmentation of DNA *in vitro*,[62,67] are completely inactive as antitumor agents in the animal assays.[55,62] Consequently, at this moment it is not certain that the antitumor activity of **1a** is due to its potential ability to act as an alkylating agent,[55] or as a source of DNA-damaging free radicals.[67] The fact that 5-hydroxy- and 5-acetoxy-**1a** (C. R. Hutchinson and J. A. Adamovics, unpublished results), as well as 7-methoxy-**1a**,[61] are completely inactive in the animal antitumor assays supports a free radical hypothesis more than an alkylating hypothesis. That is, the latter three analogues of **1a** should be as effective alkylating agents as **1a** because the crucial α-hydroxy lactone is still intact in their structures, but could be less

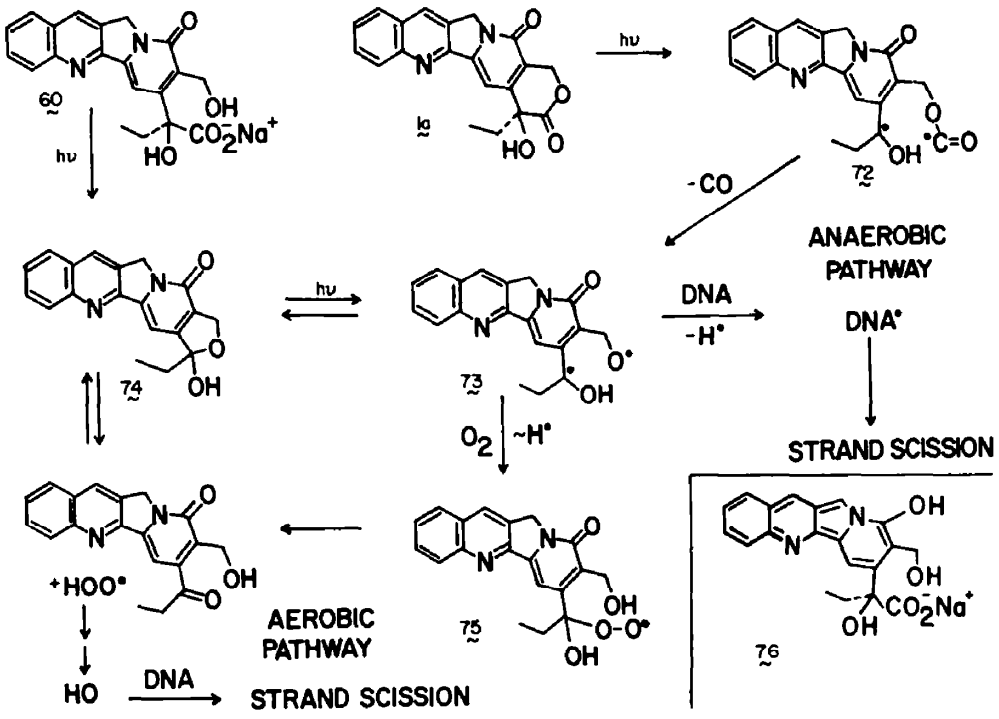


Fig. 12. Proposed molecular mechanism of action of photoactivated camptothecin, which causes the strand scission of DNA *in vitro*.

prone than **1a** to redox reactions occurring at C-5[22] or at C-7.[71] [Lown *et al.* found that 5-hydroxy-**1a** was nearly inactive in their model study.[67]] I believe that the free radical hypothesis for the molecular mechanism of action of **1a** *in vivo* is the best interpretation of the available data pertaining to camptothecin's interaction with cellular DNA as the primary determinant of the drug's cytotoxicity. It even is possible to accommodate a crucial role for the α-hydroxy lactone in this hypothesis, e.g. by suitable analogy to the mechanistic rationale drawn in Fig. 12, or that of Moore.[71]

Some very recent results from Lown's laboratory strongly support the above concept that camptothecin "catalyzes" the formation of DNA damaging free radicals *in vivo*.[72] These researchers investigated in detail the reported ability of **1a** to potentiate the cleavage of DNA by bleomycin.[66] Although under non-photoactivated conditions either component separately produced relatively lower levels of single strand scission of PM2-ccc-DNA *in vitro*, they found that a mixture of the glycopeptide antibiotic bleomycin, or its (1:1) complex with iron, together with sodium camptothecin (**60**) at comparable concentration—in place of the normal reducing agent NADPH—produced substantially increased extents of DNA breakage as determined by an ethidium fluorescence assay. A similar enhancement by **60** was observed with the antibiotics tallysomycin and tallysomycin E_{1a}, which are analogues of bleomycin, and in each case the characteristic pH profile for strand scission was maintained.

Since the latter observations support the contention that the sodium salt of camptothecin can act as a reducing agent in solution, thus substituting for the reducing requirement for the antibiotics' effects on DNA *in vitro*,[68] Lown *et al.* then investigated the ability of **1a** and **60** to undergo oxidation or reduction in solution. They found that **60** easily was oxidized to the known hemiacetal **74** by dilute aqueous H_2O_2 (25°C, 64 hr, dark) and by the (1:1) bleomycin-iron complex. In contrast, we had found that under much more vigorous reaction conditions than used by the Alberta group—SeO_2 in glacial acetic acid —**1a** was oxidized to a mixture of its 5-hydroxy and 5-acetoxy derivatives (J. A. Adamovics and C. R. Hutchinson, unpublished observations). Both types of behavior of **1a** and **60** with oxidizing agents can be explained mechanistically by proposing that a tautomeric form of the parent alkaloid (compound **76** in Fig. 12) is the key intermediate in the oxidative transformations. In fact Lown *et al.* give acceptable spectral evidence for the presence of **76** in alkaline solutions of **60**.[72]

These researchers found subsequently that **1a** and related compounds also readily undergo electrochemical reduction in solution.[72] The least negative reduction potential was at -0.75 ± 0.01 V relative to an aqueous S.C.E., which the authors convincingly ascribe to one-electron reduction of the α-pyridone ring D of the compounds. This process is compatible with a coupled reduction of the sequestered Fe(III) to Fe(II) in the glycopeptide antibiotics that is necessary for the full expression of their antitumor properties.[68] Consequently, instead of supplying electrons directly to bleomycin or to tallysomycin, camptothecin and its biologically active analogues conceivably could act only as intermediary electron carriers in the reductive activation process. Further work is necessary before a distinction can be made between these two possible redox mechanisms for the reductive activation of the glycopeptide antibiotics. Nevertheless, the recent data from Lown's laboratory establishing that camptothecin may serve as a reducing agent for bleomycin and tallysomycin *in vitro*, together with their supporting chemical and electroanalytical evidence, provide a plausible mechanistic rationale for the observed enhancement of the antitumor activity of bleomycin by camptothecin *in vivo*.[66]

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