Synthetic and Biosynthetic Studies of Natural Products

by Eduardo Alberto Véliz Chanis

B.S., University of Panama, 1981

M.S., University of Kansas, 1986

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SYNTHETIC AND BIOSYNTHETIC STUDIES OF NATURAL PRODUCTS

PART I. STUDIES DIRECTED TOWARD THE TOTAL SYNTHESIS OF 4-DEMETHOXY -A-HOMODAUNORUBICIN ANALOGS

PART II. BIOSYNTHESIS OF COLORADOCIN. ORIGIN OF THE CARBONS

PART III. TOTAL SYNTHESIS OF THE NATURALLY OCCURRING PRENYLATED BIBENZYL AMORFRUTIN A

BY E. VÉLIZ CHANIS

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Part I. Studies Directed Toward the Total Synthesis of 4-Demethoxy -A-Homodaunorubicin Analogs

Part II. Biosynthesis of Coloradocin. Origin of the Carbons

Part III. Total Synthesis of the Naturally Occurring Prenylated Bibenzyl Amorfrutin A

by

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Submitted to the Department of Medicinal Chemistry and the Faculty of the Graduate School of the University of Kansas in partial fulfillment for the degree of Doctor of Philosophy.

Dissertation Committee:

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To my mother, my father,

and those who always believed in me

Synthetic and Biosynthetic Studies of Natural Products

Eduardo Alberto Véliz Ch., Ph.D.

University of Kansas, 1992

Part I describes the design and efforts toward the total synthesis of novel analogs of 4-demethoxydaunorubicin. The design of the analogs (**24** and **25**) was based on the ability of these compounds to intercalate into DNA demonstrated by computer graphics studies. The synthesis of the 9a-homo analog started from known 1,4-dimethoxy-2,3-dimethylbenzene. Friedel-Crafts acylation of **27** with phthalic anhydride afforded **28** in 76% yield. Introduction of the benzylic hydroxyl group at carbon 7 of **32** failed during the hydrolysis step. Standard and/or alternative hydrolysis procedures gave dehydration product **50**, decomposition or starting material.

1,2-Keto transposition of **26** gave ketone **38**, which gave us an entry into the synthesis of the 8a-homo analog. Standard transposition procedures proved to be more difficult than anticipated. The transformation, however, was accomplished in six steps in 43% yield. A direct route for analog **25** started from 1,4-dihydronaphthalene derivative **39**. A ring expansion reaction performed on tetralone **42** gave **38** in 75% yield. Friedel-Crafts acylation of **44** gave **45** in 85% yield. Functionalization of the benzylic position of compound **46** failed during the bromination step.

The syntheses of analogs **24** and **25** could not be finished due to the intractable behavior of some of the intermediates.

Part II describes the elucidation of the biosynthesis of coloradocin by the use of stable isotopes. The major carbon chain is polyketide and derived from acetate-propionate and methionine as shown by feeding experiments with [1-^{13}C]-, [2-^{13}C]-, [1,2-^{13}C]-acetate, [1-^{13}C]-propionate and L-[*methyl*-^{13}C]-methionine.

The bioorigin of the minor carbon chain was examined using the above mentioned isotopes as well as with L-[1-^{13}C]-glutamate, [2,3-^{13}C]-succinate, [3-^{13}C]-oxaloacetate and [U_6]-glucose. Based on the results, the chain is not a polyketide and its bioorigin remains to be elucidated.

Part III describes the total synthesis of the antimicrobial agent amorfrutin A. The synthesis was accomplished in 12 steps from known 3,5-dimethoxybenzaldehyde in 11% yield. The synthetic route employed was efficient, apparently flexible, and regiospecific for 2-carboxylated bibenzyls and related molecules.

Acknowledgements

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Part I

Studies Directed Toward the Total Synthesis of 4-Demethoxy-A-Homodaunorubicin Analogs

A. Introduction

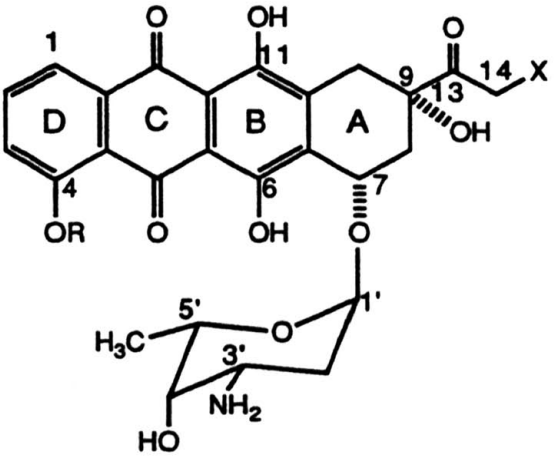
Although surgery is the primary method for treatment of solid tumors, and radiation is used for skin cancer and deeper localized tumors, the effective treatment of occult micrometastases not accessible to surgery or localized radiotherapy requires chemotherapy. It is clear that the most effective role of chemotherapeutic agents against solid tumors is as an adjuvant to these initial modes of therapy.

1. Anthracycline Antibiotics: Classification, Isolation, and Biological Properties of Doxorubicin and Daunorubicin.

The anthracyclines, including doxorubicin (**1**, adriamycin) and daunorubicin (**2**, daunomycin), are a large and important family of antitumor antibiotics. Within this class, doxorubicin is the leading drug in present cancer chemotherapy.

The anthracyclines are divided into two classes, in accordance with the selective effects on the inhibition of nucleic acid synthesis: Type I anthracyclines, exemplified by doxorubicin and carminomycin (Figure 1), inhibit DNA, whole cell RNA, and nucleolar RNA synthesis at comparable concentrations. Type II anthracyclines, exemplified by aclacinomycin and marcellomycin (Figure 2), inhibit whole cellular RNA synthesis and nucleolar preribosomal synthesis at six- or seven-fold lower and at 170-1250-fold lower concentrations, respectively, than those required for the inhibition of DNA synthesis.[1]

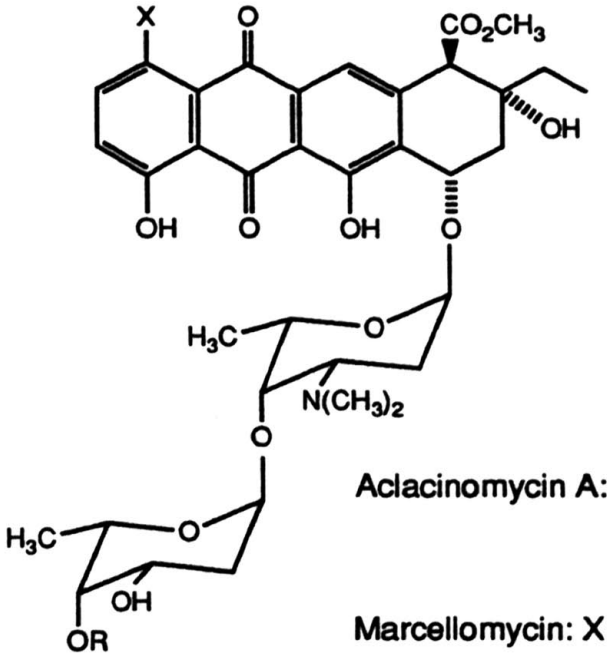
Type-I Anthracyclines

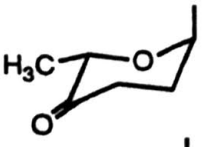


Doxorubicin: R = CH_3, X = OH (**1**, Adriamycin)
Daunorubicin: R = CH_3, X = H (**2**, Daunomycin)
Carminomycin: R = H, X = H

Figure 1

Type-II Anthracyclines



Aclacinomycin A: X = H, R = 

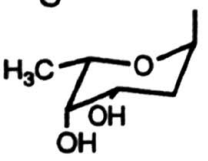
Marcellomycin: X = OH, R = 

Figure 2

Daunorubicin was isolated in 1963 from *Streptomyces peucetius* by Farmitalia in Italy,[2] and independently at Rhône-Poulenc in France[3] from *S. coeruleorubidis*. Six years later, doxorubicin was isolated from a mutant strain of *S. peucetius* by the Farmitalia group.[4]

In vivo and *in vitro* studies[5-8] of these two compounds showed significant inhibition of different experimental tumors such as Erlich carcinoma, L1210 leukemia, and sarcoma 180. Doxorubicin was superior to daunorubicin in most of these cases.

After clinical trials, daunorubicin was one of the first succesful and efective drugs for the treatment of acute leukemia.[9] Doxorubicin, on the other hand, became the leading drug in cancer chemotherapy, as a result of its broad spectrum against solid tumors and malignant lymphomas.[8, 10]

Unfortunately, these agents exhibit undesired side effects, such as stomatitis, alopecia, myelodepression, and gastrointestinal disturbances. However, the most serious dose-limiting side effect is the development of cumulative dose-dependent cardiotoxicity.[10] The clinical importance of these agents, together with the serious side effects, have provided a powerful stimulus to the search for better analogs.

2. Mechanism of Action.

Several mechanistic pathways have been proposed for the antineoplastic activity of these agents, including:

a) inhibition of DNA and RNA biosynthesis by intercalation into the DNA double helix

b) alkylation of macromolecules by bioreductive activation

c) generation of highly active superoxides by bioreductive activation

d) interaction with topoisomerase II/DNA complexes

e) cell membrane surface interaction.

Each mechanistic pathway will be discussed later in this section.

a. Inhibition of DNA and RNA Biosynthesis by Intercalation into the DNA Double Helix.

The effects of daunorubicin and doxorubicin on DNA synthesis have been studied in detail. It was found that DNA polymerase α, the putative replicative polymerase, was inhibited in preference to DNA polymerase β, the repair polymerase.[11] Furthermore, the observation that daunorubicin was more lethal to cells in the late S-phase, than in G_1 or G_2, prompted the suggestion that the drug can find access to double stranded helical DNA during the replication process.[12] Matsuzawa and co-workers,[13] using 92 anthracyclines, showed a correlation between cytotoxicity and inhibition of nucleic acid synthesis. Studies which show the correlation between the degree of inhibition of DNA polymerases and the ability of the drugs to bind to DNA, demonstrated that the inhibition was a direct consequence of DNA binding.[14-16] Stutter and co-workers[17] showed that the formation constant of the daunorubicin- and doxorubicin-DNA complexes were approximately 7×10^5 and 2×10^6 M^{-1}, respectively. These studies as well as the elevation of DNA melting temperatures indicated that intercalation is the mechanism by which daunorubicin and related anthracyclines inhibit nucleic acid biosynthesis. Spectroscopic studies on daunorubicin, such as hypsochromic and bathochromic changes in the visible spectrum, as well as physical characteristics including increased viscosity and decreased buoyant density

of DNA,[18] uncoiling of supercoiled closed circular DNA,[19] and changes in the fluorescence emission spectrum,[20] showed that this drug satisfied the criteria for an intercalating agent.

Analysis of NMR data of aromatic and alicyclic protons of daunorubicin followed by complexation with different deoxydinucleotides indicated differences in affinity and suggested that ring A was in the half-chair conformation with the acetyl side chain equatorially oriented, both in solution and in the complexes (Figure 3).[21] The analysis also suggested that there were intermolecular hydrogen-bonds between the axial 9-OH with either water molecules or the phosphate anion at the intercalation site.[21] Downfield shifts and line broadening of DNA ^{31}P signals suggested unwinding of the DNA double helix as a consequence of daunorubicin intercalation.[22]

Solution and Solid State Conformation of Daunorubicin

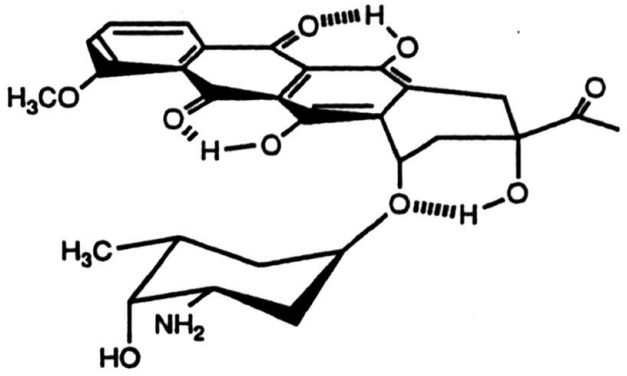


Figure 3

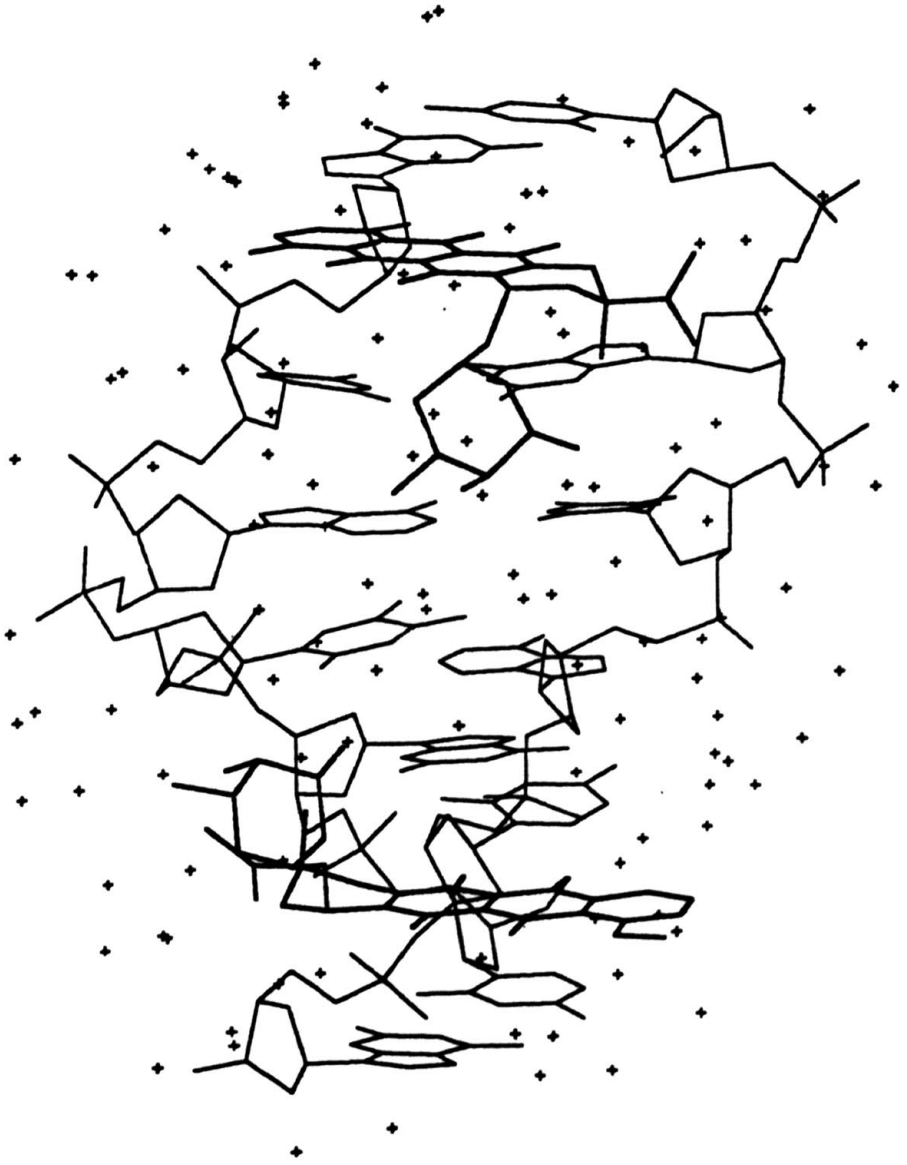
All of these suggestions were corroborated when Quigley and co-workers[23] solved the crystal structure of daunorubicin-d(CpGpTpApCpG) complex by X-ray diffraction analysis at 1.54Å resolution. Several years

later, this group reported another molecular structure of this complex at 1.2Å resolution, where the six base pair fragment of double-helical DNA had two molecules of daunorubicin, 166 water molecules, and two sodium ions bound to it.[24] These studies allowed the researchers to identify three principal functional components of the anthracycline antibiotics: a) the intercalator portion (rings B-D), b) the anchoring functions associated with ring A, and c) the amino sugar.

The aglycone chromophore of daunorubicin intercalates at the CpG sequences of the d(CGTACG) duplex by skewering the double helix, and is oriented at right angles to the long dimension of the DNA base pairs (Figures 4a and 4b). Ring D protrudes out along the major groove and ring A, along the minor groove. The methoxy group of ring D, found within the major groove, participates in the coordination with the sodium ion, and this coordination provides some stability for the complex. Substituents on ring A have hydrogen-bonding interactions with the base pairs above and below the intercalation site.

The functional groups at C-9 seem to anchor the molecule into the DNA, by stabilizing the complex *via* three hydrogen bonds. The O-9 hydroxyl group forms two hydrogen bonds with a guanine residue adjacent to the chromophore, donating one to N-3 and receiving one from N-2. The other hydrogen-bonding interaction is seen on the other side of the aglycone ring, which involves the C-13 carbonyl oxygen. This carbonyl oxygen is within hydrogen-bonding distance to a bridging water molecule, which itself is hydrogen-bonded to carbonyl O-2 of the cytosine ring C-1 in the base pair above the intercalator.

X-Ray Crystal Structure of
Daunorubicin-d(CGTACG) Complex



Crystal structure of daunorubicin intercalated into the d-(CGTACG) hexamer viewed perpendicular to the 2-fold axis. The + marks represent water molecules.

Figure 4a

Diagram of Daunorubicin Intercalated into the d-(CGTACG) Hexamer

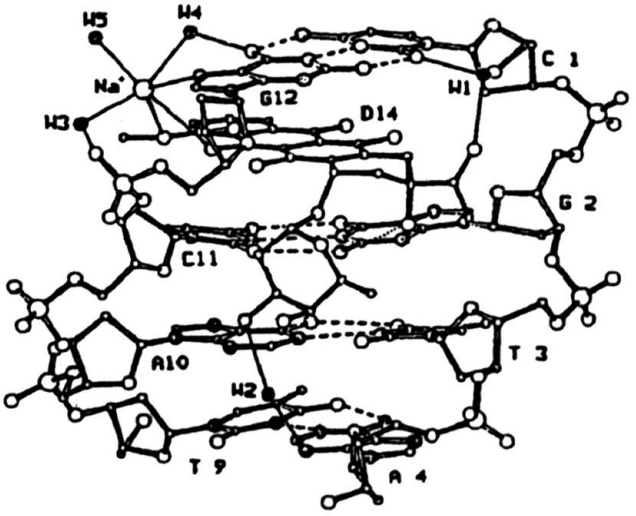


Figure 4b shows a diagram of daunorubicin (D14) intercalated into the DNA hexamer. Four base pairs of the hexamer are shown. Hydrogen bonds in the base pairs are represented by dashed lines. There are two bridging waters (W1 and W2) that form hydrogen bonds between O13 of daunorubicin and O2 of C1 and between N3' of daunorubicin and N3 of A4, respectively. A sodium ion is found to coordinate to N7 of G12, O4 and O5 of daunorubicin, and to three water molecules to form a distorted octahedral arrangement in the major groove.

Figure 4b

The amino group on the amino sugar ring has been implicated in electrostatic interactions between daunorubicin and the phosphate group of the nucleic acid;[25] however, direct interactions are not observed in this complex. The sugar N-3' atom is strongly hydrogen-bonded to two water molecules and possibly to the O-2 of residue C-5 (C-11).

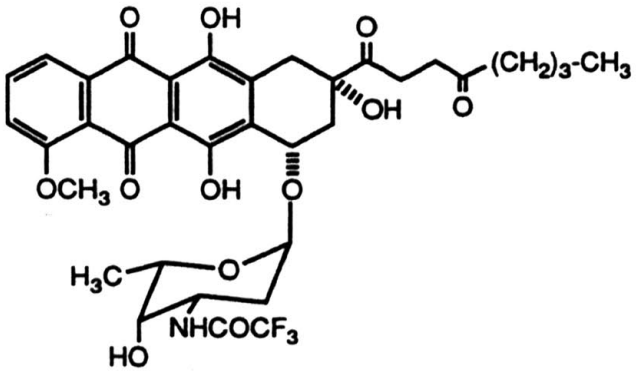
Another important interaction in the major groove involves a hydrated sodium ion, which is coordinated to the N-7 position of G-12 and the O-4 and O-5 of daunorubicin in a distorted octahedral geometry.

The intercalation model described above indicates that the antitumor properties exhibited by most of the active anthracyclines is proportional to the extent of its binding to DNA. However, N-trifluoroacetyl-doxorubicin-14-valerate (**3**),[26] N,N-dibenzyldaunorubicin (**4**),[27] and carminomycin-11-methyl ether (**5**),[28] shown in Figure 5, have impressive antitumor activity in mice, but little or no affinity for DNA. These results suggest that intercalation might not be the only mechanism for expression of antineoplastic activity of the anthracyclines.

b. Alkylation of Macromolecules by Bioreductive Activation (Two-electron Reduction Process).

Anaerobic reduction of daunorubicin and doxorubicin in microsomes by NADPH and in solution by dithionite gives 7-deoxydaunomycinone (**6**) and 7-deoxyadriamycinone (**7**), respectively[29-32] and/or covalent binding of the drugs to biological macromolecules.[33-35] On the basis of these findings, Moore and co-workers[36, 37] proposed a bioreductive activation mechanism by which these drugs are activated by reductive elimination of the sugar.

N-Trifluoroacetyldoxorubicin-14-valerate (3), N,N-dibenzyldaunorubicin (4), and carminomycin-11-methyl ether (5)



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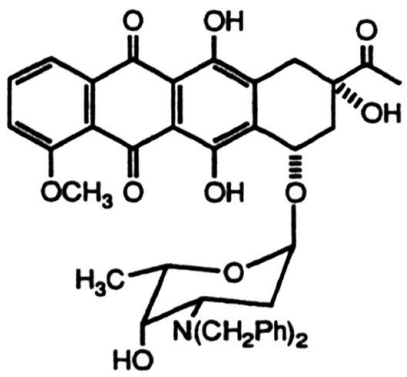

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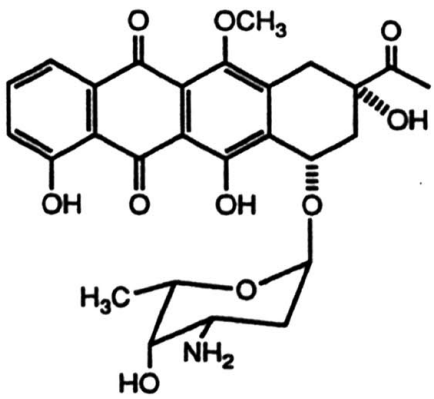


5

Figure 5

Koch and Kleyer[38] demonstrated that anaerobic reduction of daunorubicin with the reducing agent 3,5,5-trimethyl-2-oxo-morpholin-3-yl (**8**) yields the deoxyaglycone **6** *via* two sequential one-electron reduction steps to give the hydroquinone **9**, which rapidly eliminates daunosamine to give quinone methide **10**. Protonation of **10** leads to 7-deoxydaunomycinone (Figure 6). Tautomer **10** was characterized *via* UV-visible spectroscopy and is proposed as the biologically active form of daunorubicin which reacts with nucleophilic sites in DNA or other macromolecules.

Examination of the structure of quinone methide **10** as a possible intermediate, which could lead to covalent binding to biological macromolecules, reveals two possible pathways. In the first pathway, the 7 position of **10** serves as an electrophilic site for reaction with nucleophilic sites in the macromolecules. In the second pathway, the 7 position serves as a nucleophilic site for reaction with electrophilic sites in the macromolecules.

The reactivity of the quinone methide intermediate as an electrophile has been investigated by Fisher and co-workers.[39, 40] They demonstrated that the quinone methide intermediate can undergo nucleophilic trapping with thiol and thiolate groups such as ethyl xanthate and N-acetylcysteine. In the nucleophilic trapping experiment of the quinone methide derived from 11-deoxydaunorubicin with ethyl xanthate, two diastereomeric adducts, **11** and **12**, were isolated in an 85:15 ratio (Figure 7).

Mechanism for the Reductive Glycosidic Cleavage of Daunorubicin and Doxorubicin

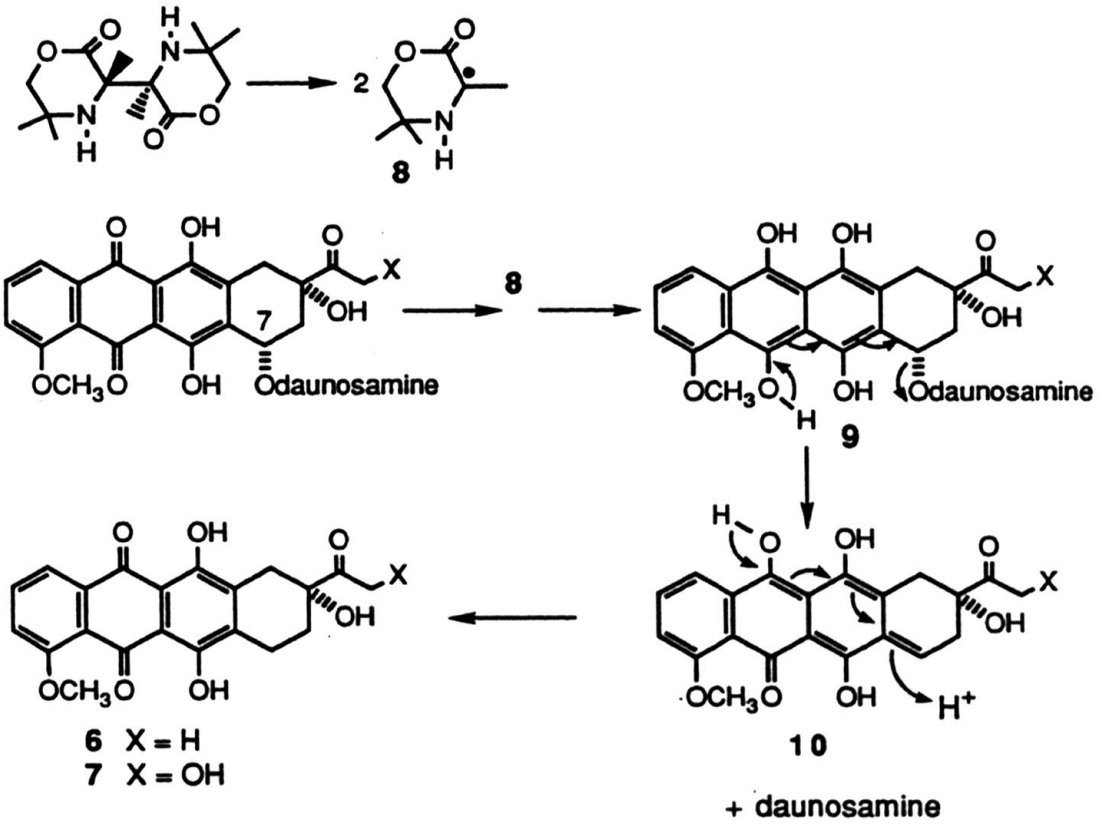


Figure 6

Diastereomeric Adducts from Nucleophilic Trapping Experiment

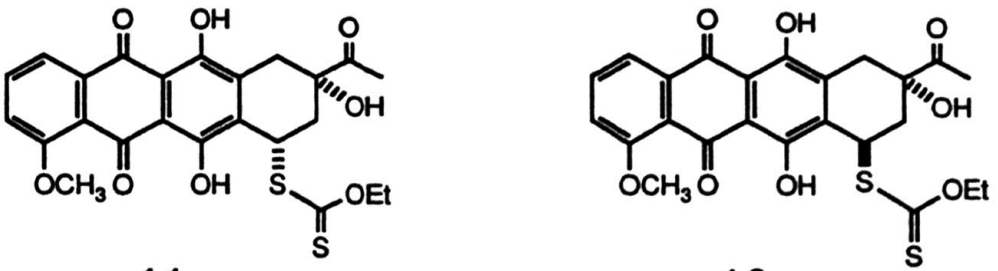
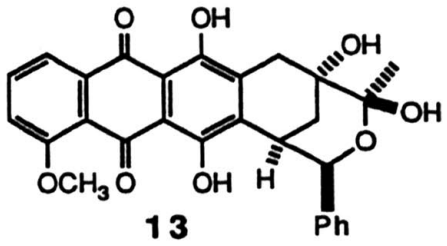


Figure 7

On the other hand, Koch and Kleyer[41] isolated, for the first time, the adduct formed from the electrophilic reaction of quinone methide **10** with benzaldehyde. Benzaldehyde reacted in what is nominally an aldol condensation to afford adduct **13** (Figure 8).

Adduct from Electrophilic Trapping Experiment



13

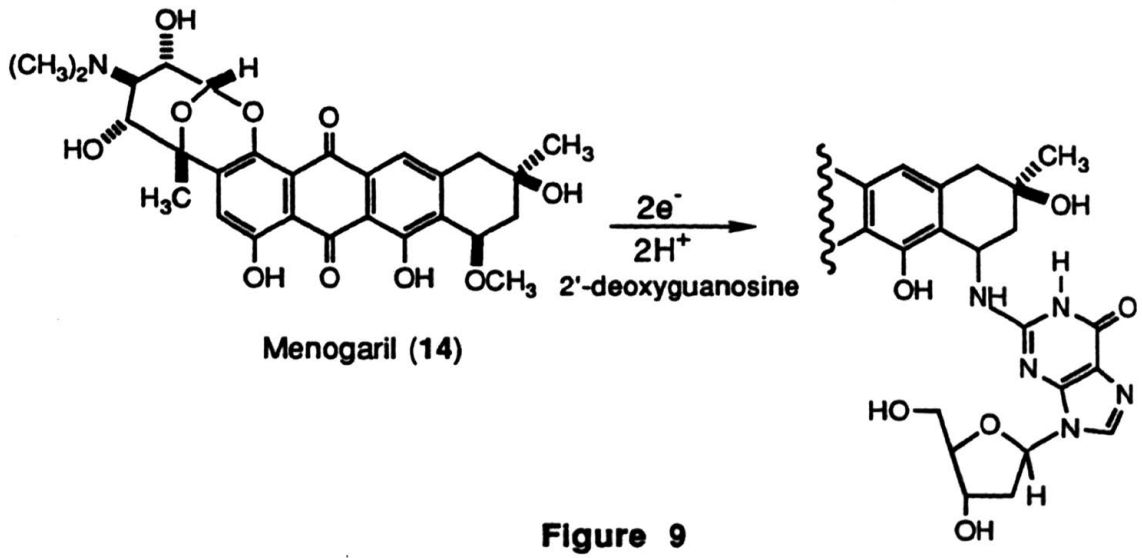
Figure 8

The first report of the coupling of an anthracycline antitumor drug to a nucleophilic site in a nucleic acid was done by Egholm and Koch.[42] They described the successful coupling of menogaril (**14**) at the 7 position to 2'-deoxyguanosine at the 2-amino position through a reductive activation (Figure 9). However, similar reduction of menogaril in the presence of a large excess of 2'-deoxyadenosine did not result in adduct formation.

Continuing with these trapping experiments, Koch and co-workers[43] were able to isolate, for the first time, adducts from the intramolecular trapping of the quinone methide derived from daunorubicin with oxygen and nitrogen nucleophilic sites. The oxygen and nitrogen nucleophiles were located at the 13 position of daunorubicin in oxime and semicarbazone derivatives. Thus, anaerobic reduction of daunorubicin oxime (**15**) and semicarbazone (**16**) with the one-electron reducing agent bi(3,5,5-trimethyl-

2-oxomorpholin-3-yl) afforded cyclooxime **17** and cyclosemicarbazone **18**, respectively (Figure 10).

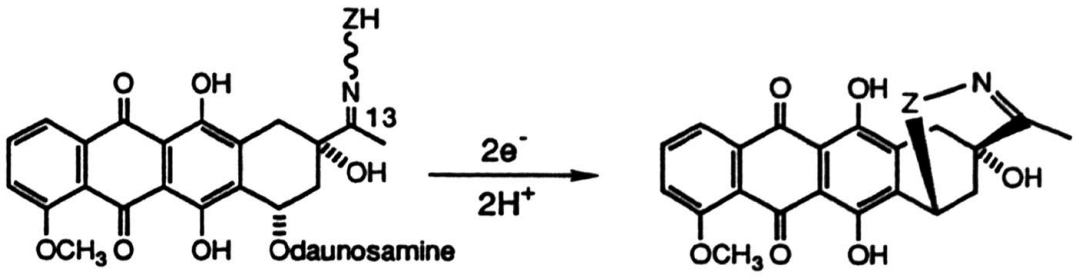
Reductive Coupling of Menogaril to 2'-Deoxyguanosine



Menogaril (**14**)

Figure 9

Intramolecular Trapping Experiment with O and N Nucleophiles



15 Z = O
16 Z = -N(I)-CONH$_2$

17 Z = O cyclooxime
18 Z = -NHCONH- cyclosemicarbazone

Figure 10

The importance of the reactivity of the quinone methide state with nucleophilic sites in nucleic acids remains obscure.[44] Although daunorubicin intercalates in duplex DNA,[24, 45] once intercalated, its reduction is sterically inhibited.[46-49] This, at least, explains in part the lack of correlation between covalent binding and antitumor activity.

c. Generation of Highly Reactive Superoxides by Bioreductive Activation (One-electron Reduction Process).

It has been demonstrated that rapid electron transfer from reduced anthracyclines to oxygen takes place under aerobic conditions.[50] Goodman and Hochstein[51] proposed that the observed lipid peroxidation caused by these agents is due to the generation of oxygen radicals *via* this redox process. Furthermore, Lown and co-workers[52] and Winterbourn and co-workers[53, 54] proposed that the oxygen radicals generated by the redox process may lead to oxygen-dependent DNA scission (Figure 11).

Lown's group[52] added superoxide dismutase, catalase, and radical scavengers (i.e. sodium benzoate) to an incubation of covalently closed circular DNA and anthracyclines reduced *in situ*, in an attempt to identify the plausible oxygen radical involved in this process. Inhibition of the oxygen-dependent DNA scission was observed and this group identified superoxide ion and hydrogen peroxide radical as the active oxygen species. Lown's group strongly implicated the presence of hydroxide radicals as active oxygen species.

Schreiber and co-workers[55] detected, by high resolution electron spin resonance (ESR), the presence of anthracycline semiquinones in samples of both enzymatically and chemically reduced anthracyclines. This group

postulated the semiquinone form as an obligatory intermediate in the radical process.

Mechanism for Oxygen-dependent DNA Scission

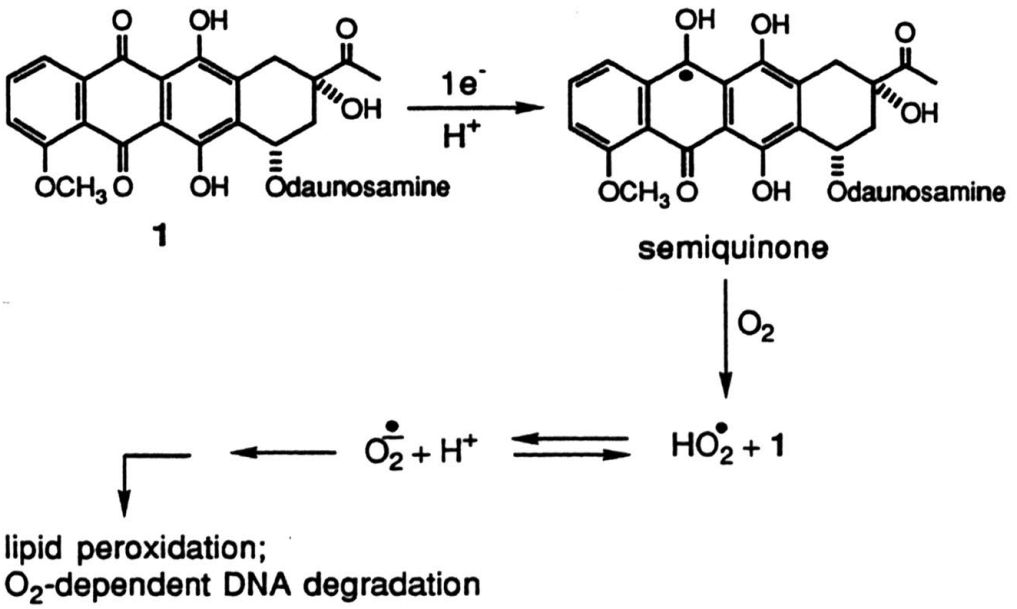
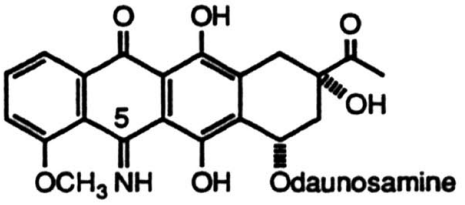


Figure 11

Lown and co-workers[56] postulated that the mechanism involved in the generation of oxygen radicals was linked to the dose-dependent cardiotoxicity of anthracyclines. This is because the heart tissue contains both mitochondrial and microsomal activating systems and is deficient in the protective enzymes superoxide dismutase, glutathione peroxidase, and catalase.[29, 57, 58] This provides the conditions which may account for the preferential susceptibility of this tissue to daunorubicin and doxorubicin. Evidence necessary to support this hypothesis was provided by Acton and co-workers[59, 60] and Lown and co-workers.[61-63] These two groups synthesized several glycosides modified at the anthraquinone chromophore,

the redox center of anthracyclines, with reduced or suppressed ability to undergo redox activation. Unfortunately, an undesirable concomitant reduction in the antitumor activity was also observed. Only the imino analog **19** (Figure 12) showed comparable activity to daunorubicin at higher optimal doses.

5-Iminodaunorubicin



19

Figure 12

This approach has showed some moderate success; however, there is no clear evidence that cytotoxicity can be dissociated from cardiotoxicity.

d. Interaction with Topoisomerase II/DNA Complex.

DNA topoisomerases are a unique class of enzymes which alter the topological state of DNA by breaking and rejoining the phosphodiester backbone of DNA.[64-68] These enzymes catalyze DNA relaxation/supercoiling, catenation/decatenation, and knotting/unknotting reactions.[66] Based on fundamental differences in their reaction mechanisms, DNA topoisomerases are classified into two types. Type I DNA topoisomerases alter the topological state of DNA by transiently breaking one strand of the DNA double helix and allowing the adjacent DNA strands to rotate around the phosphodiester bond opposite the nick before

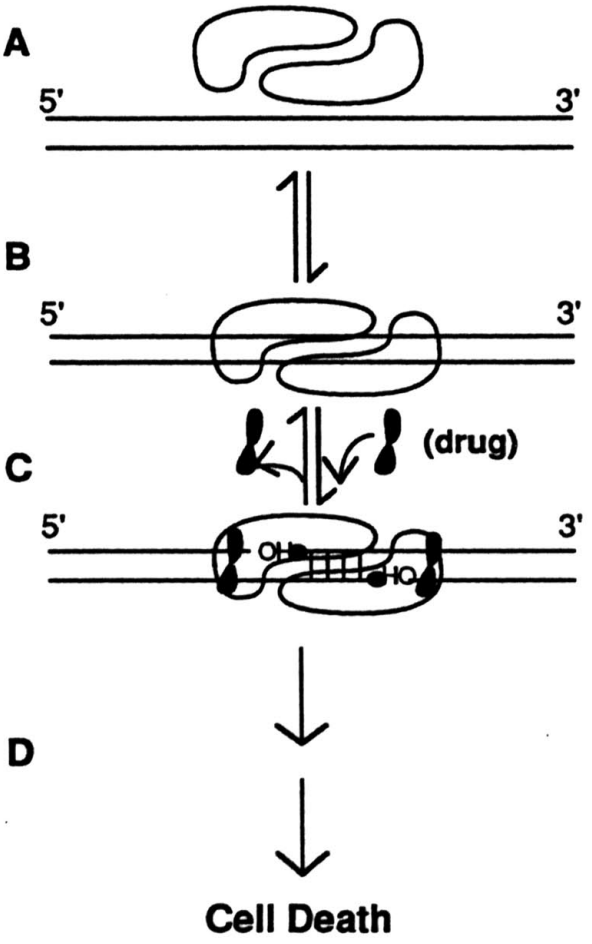
resealing. Type II DNA topoisomerases break both strands of the helix, with a four base pair stagger between the cuts, and allow a second DNA duplex to pass through the break prior to resealing. This reaction requires ATP hydrolysis.

Topoisomerases form a covalent protein-DNA complex during their reactions. In the case of topoisomerase I, this covalent complex involves a phosphotyrosyl bond between the enzyme and the 3' terminus of the break site.[69, 70] On the other hand, topoisomerase II forms a phosphotyrosyl bond with each 5' terminus.[71, 72]

DNA topoisomerase II has recently been identified as the primary intracellular target for a number of antitumor drugs currently being used in cancer chemotherapy,[73-76] including the anthracyclines. The interactions of these drugs with topoisomerase II and cellular DNA produces the primary lesion involved in cytotoxicity and other cellular responses such as sister chromatid exchanges and chromosomal aberrations.

Studies in both purified systems using DNA topoisomerase II and cultured mammalian cells have shown that these antitumor drugs interfere with the breakage-reunion reaction of DNA topoisomerases II. These drugs trap a key enzyme-DNA intermediate, termed the "cleavable complex" (Figure 13).[72, 73, 75-77] It has been proposed that these topoisomerase II targeted drugs alter the equilibrium between noncovalent topoisomerase II-DNA complex and the cleavable complex, which results in an increased concentration of the latter.[73]

Model for Topoisomerase II Mediated DNA Cleavage and Cell Death



(**A**) Unbound DNA topoisomerase II and substrate DNA. (**B**) Reversible formation of a noncleavable complex. (**C**) Reversible formation of a drug-induced cleavable complex containing topoisomerase II, DNA, and intercalative topoisomerase II-targeting antitumor drug. (**D**) Processing of the cleavable complex by cellular functions resulting in cell death.

Figure 13

The rapid cell killing caused by these agents is believed to be caused by induction and stabilization of the cleavable complex, rather than the inhibition of the catalytic activity of topoisomerase II. However, the cellular mechanism which translates the rapid formation of reversible complexes into a sequence of events that ultimately causes cell death is unclear.

Structure-activity studies of drugs from the same chemical class showed a strong correlation between cytotoxicity and the ability of the drug to induce cleavable complexes.[72] Anthracycline analogs that intercalate into DNA, and do not induce cleavable complex formation, do not cause rapid cell killing.[73] This fact strengthens belief in the interaction of anthracyclines with topoisomerases, rather than the intercalative proporties of these agents, as the mechanism of action of these antitumor drugs. However, they may both contribute.

e. Cell Membrane Surface Interaction.

The anthracycline drug doxorubicin has been shown to affect a number of membrane activities, including lectin interaction,[78] glycoprotein biosynthesis,[79] phospholipid structure and organization,[80] and transportation of small molecules and ions.[81] Based on these findings, the surface of the cell membrane was proposed as a possible extracellular target of the anthracyclines, and this interaction could lead to cytotoxicity. Tritton and co-workers[82-84] carried out a study which involved doxorubicin and other anthracyclines covalently attached to insoluble polymeric matrixes that were larger than L1210 leukemia cells (Figure 14). Doxorubicin was coupled to cross-linked agarose beads, presumably by the amino group of the sugar, through carbamate bonds[82] and to cross-linked polyvinyl alcohol

supports activated with cyanuric chloride.[84] Carminomycin was presumably anchored through ring D to cross-linked polyvinyl alcohol supports activated with diazonium salts.[84]

Polymer-bound Anthracyclines

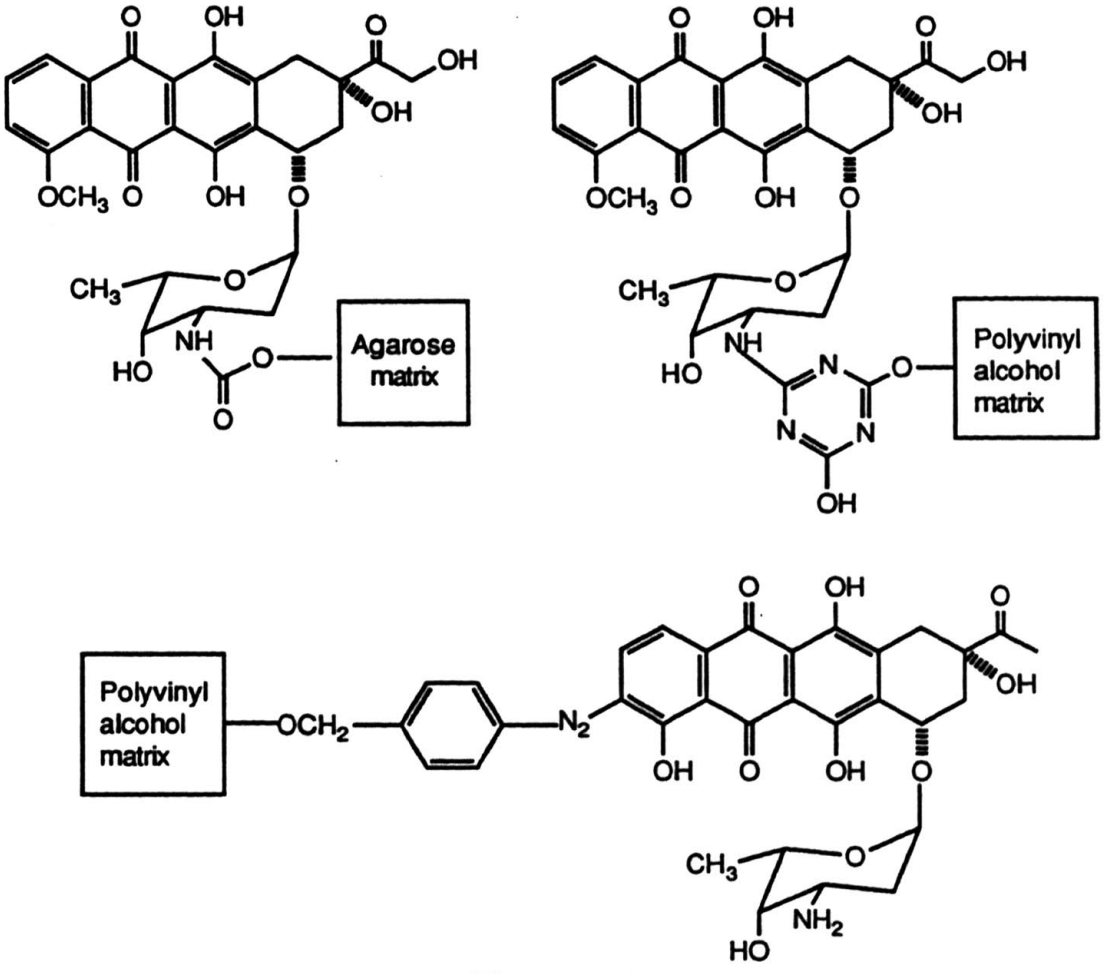


Figure 14

These preparations produced cytotoxicity to cells under conditions where no anthracycline drug entered them. This group concluded from this

study that anthracyclines can exert a cytotoxic effect solely by interaction at the cell surface.

The mechanism by which the polymer-bound anthracyclines may ultimately lead to cell death is not clearly understood.

In summary, the biologically active anthracyclines may exert a cytotoxic effect by a combination of different and parallel modes of action. Evidence has been accumulated which supports each of the mechanistic pathways, but additional studies must be performed to choose between them.

3. Structure-Activity Relationships.

The clinical importance of doxorubicin and daunorubicin, together with its serious side effects, has provided a powerful stimulus for the search for superior analogs. In order to find the most improved analog, the most potent and least cardiotoxic, hundreds of analogs have been prepared to establish the minimal structural requirements for biological activity. Some of these analogs have been prepared semi-synthetically, while others by total synthesis. Numerous modifications have been performed to find this improved analog,[85] as a consequence, a great number of analogs have been synthesized and the field is rich in extensive authoritative reviews.[86-89]

Because of the plethora of examples found in the literature, only those analogs considered pertinent to this work, in addition to the most successful derivatives, will be discussed.

a. Modifications in the Alicyclic Ring A.

The 13-dihydroanthracyclines (see Figure 15 for modifications) displayed substantial antitumor activity similar to the parent agents in

different test systems (P388; Gross leukemia; sarcoma 180).[90, 91] However, higher doses were necessary to achieve comparable cytotoxic potency. These derivatives were made by reduction of the carbonyl moiety at carbon 13 *via* microorganisms[92] or chemical reagents.[93] These 13-dihydroderivatives are also found to be the major metabolic products of daunorubicin and doxorubicin.[94]

Modifications in the Alicyclic Ring A

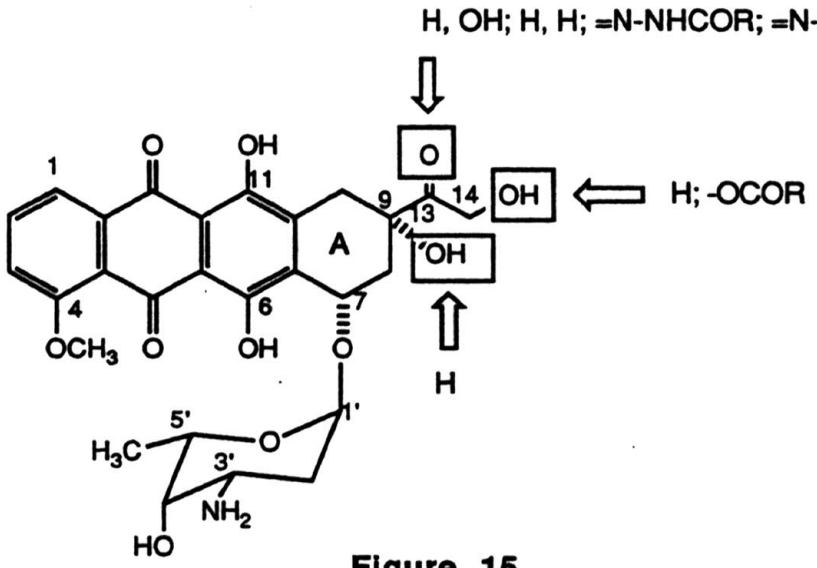


Figure 15

The presence of an ethyl side chain in a large number of naturally occurring anthracyclines such as aclacinomycin A (Figure 2), led to the complete reduction of the C-13 carbonyl group in daunorubicin and doxorubicin. These 13-deoxy derivatives showed comparable activity to that of the parent agents in the P388 screen, but optimal doses were higher.[95]

Derivatization at the C-13 carbonyl (semicarbazone and oxime derivatives) generally resulted in analogs with reduced antitumor activity.[90, 96]

Formation of ester derivatives at the C-14 hydroxyl group of doxorubicin gave analogs which retained significant levels of antitumor activity against Gross leukemia, P388, and L1210 tumor cells.[97] These derivatives may be acting as pro-drugs of doxorubicin, which is released after enzymic hydrolysis.

Substitution of the C-9 hydroxyl group by a hydrogen atom gave analogs which showed similar activity to that of daunorubicin, but 6-fold higher doses were required.[98, 99]

b. Modifications in the Anthraquinone Chromophore.

Figure 16 shows the modifications which led to retention or increase antitumor activity.

Modifications in the Anthraquinone Chromophore

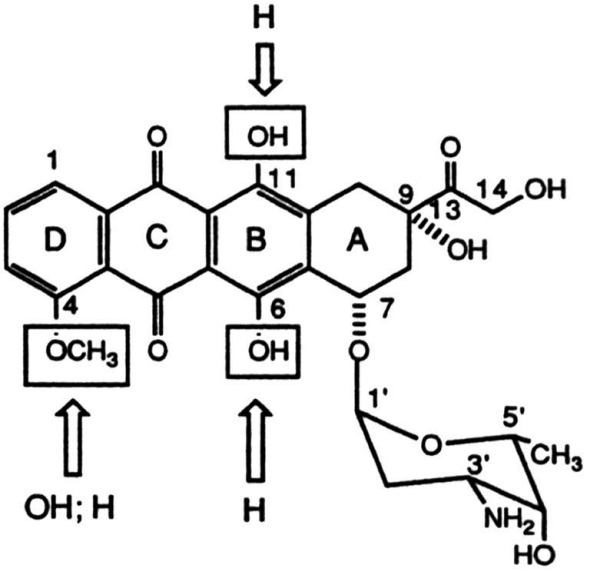


Figure 16

Analogs with substantial retention of antitumor activity were obtained after modifications in the phenolic substitution pattern of the anthraquinone nucleus, primarily at positions 4, 6, and 11.

The 4-demethoxy series of analogs was produced when the 4-methoxy group was substituted by a hydrogen atom. This series was achieved exclusively by total chemical synthesis,[100-102] since carbon 4 is hydroxylated in all known biosynthetic anthracyclines. 4-Demethoxydaunorubicin (4-DMDA) and 4-demethoxydoxorubicin (4-DMDX) were equally or more effective than the parent anthracyclines when tested in different cultured cells, leukemia, and sarcomas.[100, 103] 4-DMDA showed remarkable activity *via* oral administration.[104] In addition, this analog showed less severe cardiotoxic effects than doxorubicin at equiactive doses in mice and rabbits.[105] This 4-demethoxy analog is currently available on the market as idarubicin and is indicated for acute non-lymphocytic and acute lymphocytic leukemias, and acute myeloid leukemia.[106, 107] This drug is also reportedly active in daunorubicin-resistant patients, breast cancer, Hodgkin's and non-Hodgkin's lymphoma.[106, 107]

The 11-deoxyanthracyclines were first isolated from a mutant strain of *S. peucetius* and these analogs showed significant biological activity, although higher optimal doses were required to achieve the same magnitude of antitumor activity.[108] Finally, a few derivatives where the phenolic moiety at C-6 has been replaced by a hydrogen atom have also been prepared.[109] 6-Deoxycarminomycin has shown promising antitumor activity within this new series.[110]

c. Modifications in the Sugar Residue.

Modifications in the aminosugar moiety which led to retention or increase in antitumor activity are shown in Figure 17.

Modifications in the Aminosugar Moiety

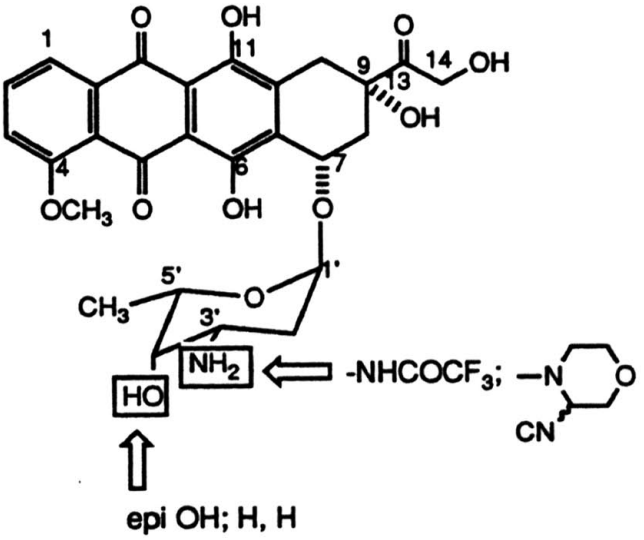


Figure 17

Removal of the aminosugar moiety by acid hydrolysis[111] leaves the isolated aglycone portion, which is completely devoid of antitumor activity. This indicates that the presence of a sugar is a *sine qua non* structural feature for the expression of activity.

New analogs were prepared by the coupling of the aglycone portions of daunorubicin and doxorubicin with configurational isomers of daunosamine at the C-3' and C-4' positions.[112, 113] The L-xylo (C-3' epimer) derivatives were completely devoid of antitumor activity on the L1210 experimental leukemia in mice at the highest dose tested, while the L-ribo (C-3', C-4' di-epimer) analogs showed decreased activity when compared to the parent glycosides. However, the L-arabino (C-4' epimer) analogs fully retained the cytotoxic activity of the parent compounds at comparable doses.[112-115]

Because these preliminary results were promising, the 4'-epidoxorubicin analog was subjected to additional studies against experimental tumors, and after significant clinical evaluation, this derivative seems to be a better tolerated and less toxic drug.[110]

Little or no antitumor activity was displayed by anthracycline analogs with the unnatural β-glycosidic bond (C-1' epimer).[116] However, the β-anomer of 4-DMDA showed antitumor activity similar to that of daunorubicin, but the optimal doses were higher.[116]

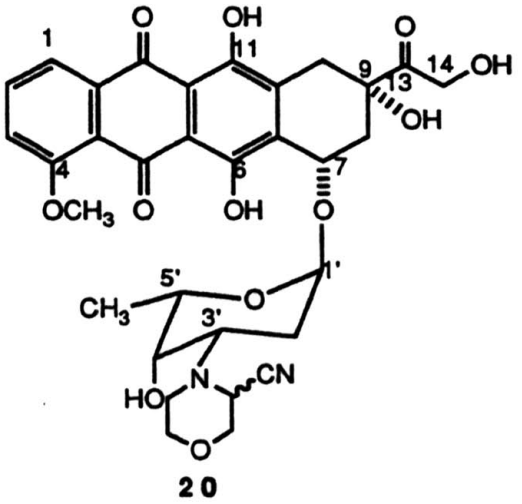
Remarkably active analogs were obtained when the C-4' hydroxyl group was substituted by a hydrogen atom.[117] 4'-Deoxydaunorubicin and 4'-deoxydoxorubicin analogs showed antitumor activity equal to that of the parent drugs on the L1210 leukemia in mice. The results against solid tumor (sarcoma 180) were more promising and exciting because 4'-deoxydoxorubicin displayed similar activity to that of doxorubicin at lower optimal doses. The clinical evaluation of this analog has been reviewed.[118] Furthermore, these studies also indicated that new promising drugs with full retention or increase biological activity might be obtained by modification at the 4'-position.

Numerous modifications have been performed at the amino functionality of the sugar, modifications which include amide formation,[96, 119] and derivatization to secondary and tertiary amines.[120, 121] The N-trifluoroacetyl analogs have shown antitumor activity, but significantly higher doses were required to obtain the same level of potency as the parent drugs.

The 3-cyanomorpholino analog **20** (Figure 18) showed, in both cell cultures and murine experimental leukemias, to be 100-1000 fold more

potent than doxorubicin.[121] This analog is the most potent anthracycline known to date and **20** may be less cardiotoxic than doxorubicin according to preliminary *in vivo* data. This agent is undergoing exhaustive clinical evaluation.

3'-Deamino-3'-(3-cyano-4-morpholinyl)doxorubicin



20

Figure 18

In summary, biological activity is lost when the sugar residue is removed by hydrolysis. The alicyclic ring A can be modified without significant loss in activity, although the C-9 carbonyl and hydroxyl groups are required for maximal activity. Modifications of the phenolic substitution pattern of the anthraquinone chromophore and at the C-3' and C-4' positions of the aminosugar residue give analogs which display the same, or even greater, antitumor activity.

B. Design of A-Homoanthracycline Analogs of 4-Demethoxydaunorubicin (Idarubicin)

The anthracycline field has received a great deal of interest and has been the focus of intense research since the isolation of daunorubicin and doxorubicin. The discovery of these compounds as remarkable cytotoxic agents and the development of dose-dependent cardiotoxicity stimulated researchers to perform extensive systematic modifications and derivatizations of these drugs in order to obtain new analogs with greater antitumor activity and lower cardiotoxicity.

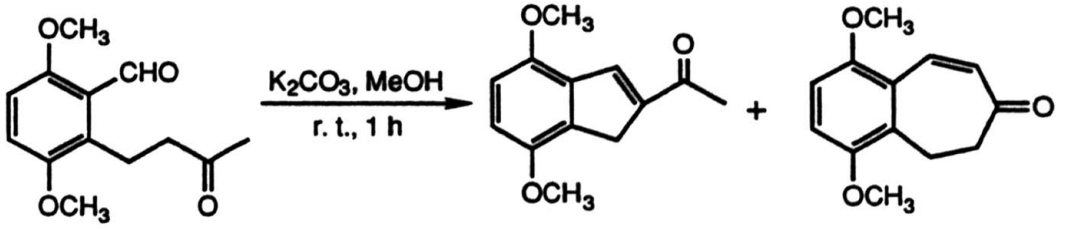
As described in the previous section, these modifications afforded a plethora of new analogs, among which idarubicin, 4'-epidoxorubicin, and 3'-deamino-3-(cyano-2-morpholinyl)doxorubicin emerged on top because of the remarkable antitumor activity these analogs possess. Unfortunately, every class of drugs reaches a plateau or saturation level and this one is not an exception. The synthesis and discovery of new anthracyclines has declined during the last few years; therefore, it is necessary to design and synthesize novel analogs of doxorubicin and daunorubicin which may help us to find new or underexplored directions in this area. Thus, our objective is the design and synthesis of A-homo analogs of idarubicin (4-DMDA), which, if successful, will open new trends for the underexplored ring A of the anthracyclines.

We chose to make derivatives of idarubicin (4-demethoxydaunorubicin) because of the low cardiotoxicity and high potency of this compound in comparison with the parent drugs. Furthermore, from a synthetic point of

view, the absence of the methoxy group makes the synthetic approach more facile.

Our group has been engaged in the total synthesis of new analogs of 4-DMDA modified at the alicyclic ring A.[122-124] Among our earlier studies, we succeded in the synthesis of the 8-noraglycone of 4-DMDA.[123] During the course of this synthesis an intramolecular Claisen-Schmidt condensation which gave unexpected results was encountered.

The Claisen-Schmidt condensation of benzaldehyde derivative **21** in the presence of K_2CO_3 in methanol afforded a mixture of the desired five-membered ring enone **22** and the seven-membered ring isomer **23** in 31 and 51% yield, respectively (Scheme 1). The mechanism and the electronic factors which control this reaction have been studied and explained.[125]



21 **22** **23**

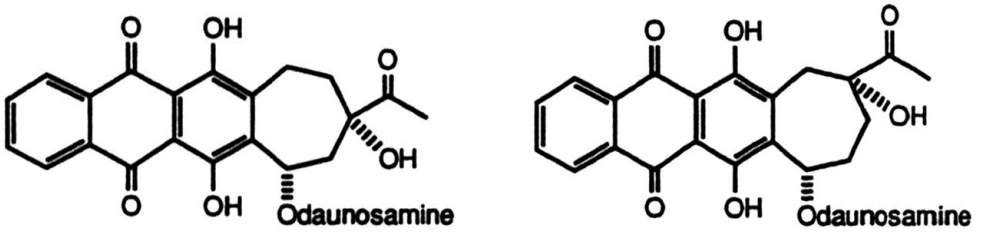
31% 51%

Scheme 1

This unprecedented and unexpected outcome opened a new direction in the design and synthesis of novel anthracycline analogs, mainly, the 9a-homo derivatives of 4-DMDA (**24**, Figure 19).

To expand the scope of this reaction and increase the number of A-homo analogs, a derivative with an 8a-homo methylene insertion was also designed (**25**, Figure 19).

A-Homo Analogs of Idarubicin (4-DMDA)



24 **25**

Figure 19

Synthetic analysis of these compounds indicated that the 9a-homo derivative was the intuitive analog to make as a consequence of the presence of a plane of symmetry. This element of symmetry would make the introduction of the hydroxyl group at the 7 position relatively simple. That is, this transformation could be carried out at either benzylic carbon because these positions were equivalent. On the other hand, the 8a-homo derivative was the counter-intuitive analog to make.

Before we began the synthesis of these analogs, we utilized a series of molecular graphic modeling techniques (MACROMODEL[126] v. 2.3, SYBYL[127] v. 5.41) to study the ability of these compounds to intercalate into double helical DNA.

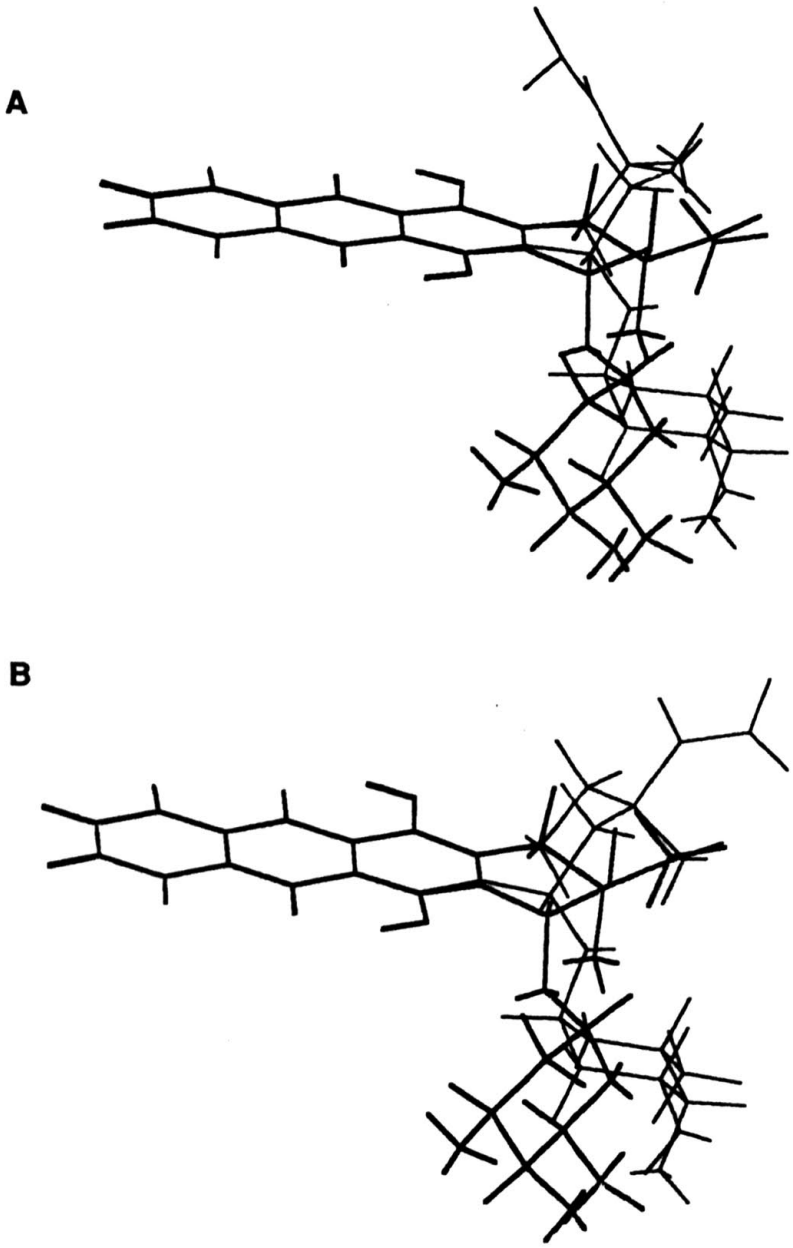
Comparison of the MM2-minimized structures of these analogs with that of 4-DMDA showed that the aminosugar and the side chain moved significantly to a new area in these new analogs (Figure 20). As can be

seen, the extra methylene group in these A-homo analogs moved the side chain above the plane of the aromatic portion of these molecules.

Modeling was caried out with a d(CpGpTpApCpG) hexamer of double-helixcal DNA obtained from the X-ray crystal structure of the daunorubicin-DNA complex.[24] The intercalation was performed in the following manner: the daunorubicin structures were deleted from this complex and the MM2-minimized analogs were intercalated in lieu. An overlay of the minimized analogs with daunorubicin was done prior to the deletion, so as to obtain the same orientation that daunorubicin had in the DNA complex. In this manner, we assured almost the same spatial orientation of the intercalation portion, the side chain, and the sugar of the proposed analogs as that of daunorubicin (Figures 21 and 22).

While conducting these computer graphics studies, it was found that the counter-intuitive analog **25** actually fit the intercalative model better than the 9a-homo analog **24**. As can be seen in Figures 21 and 22, the methyl ketone group of **25** is closer to the DNA than that of the 9a-homo analog. Thus, the carbonyl oxygen of the former can form hydrogen-bonding interactions with the base pair above the intercalator *via* a bridging water molecule. However, the methyl group of **25** is positioned so close to the base pair that might give rise to steric interactions not present in **24**.

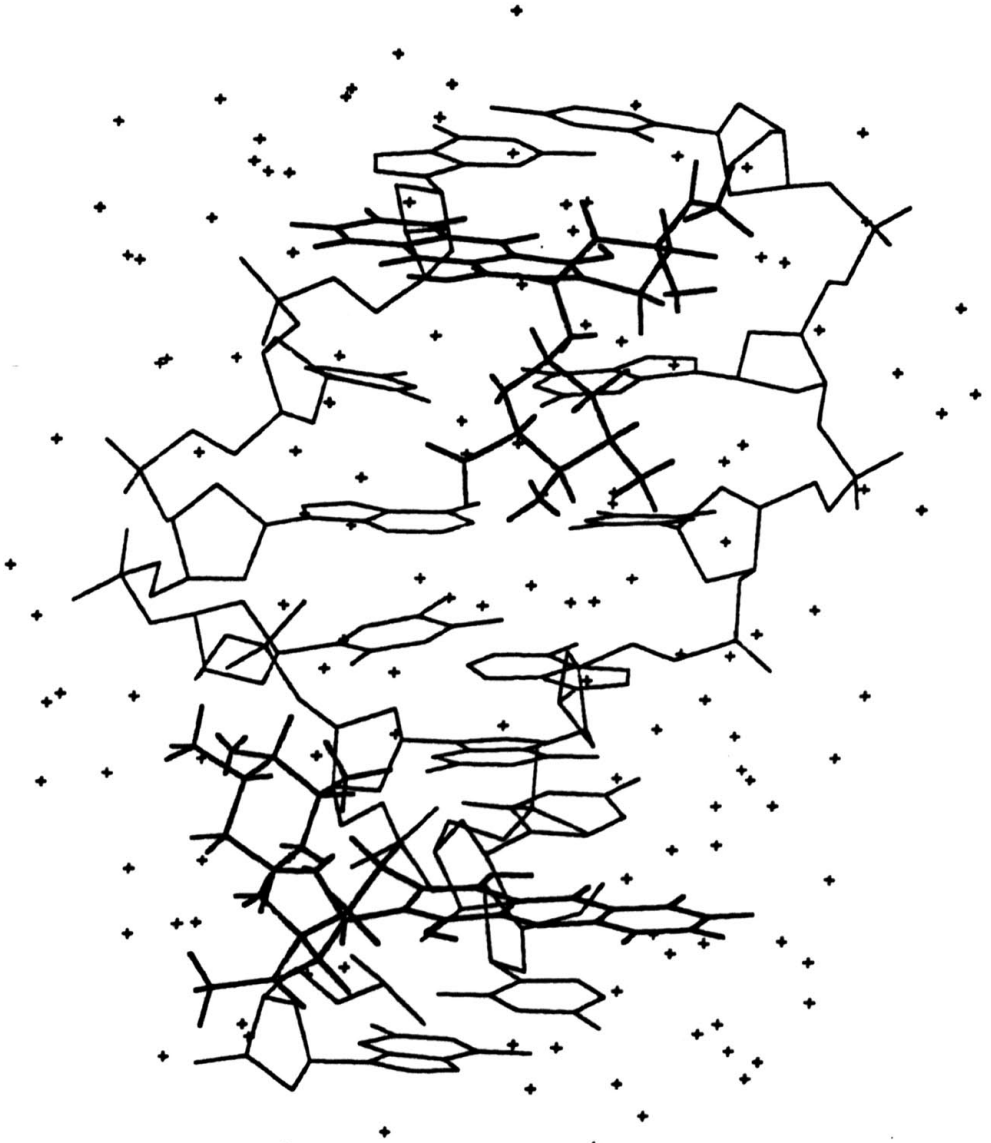
Overlay of the A-Homo Analogs with 4-DMDAU



(A) Overlay of 4-DMDAU and the 8a-homo analog. (B) Overlay of 4-DMDAU and the 9a-homo analog. 4-DMDAU in thick lines and A-homo anlogs in thin lines.

Figure 20

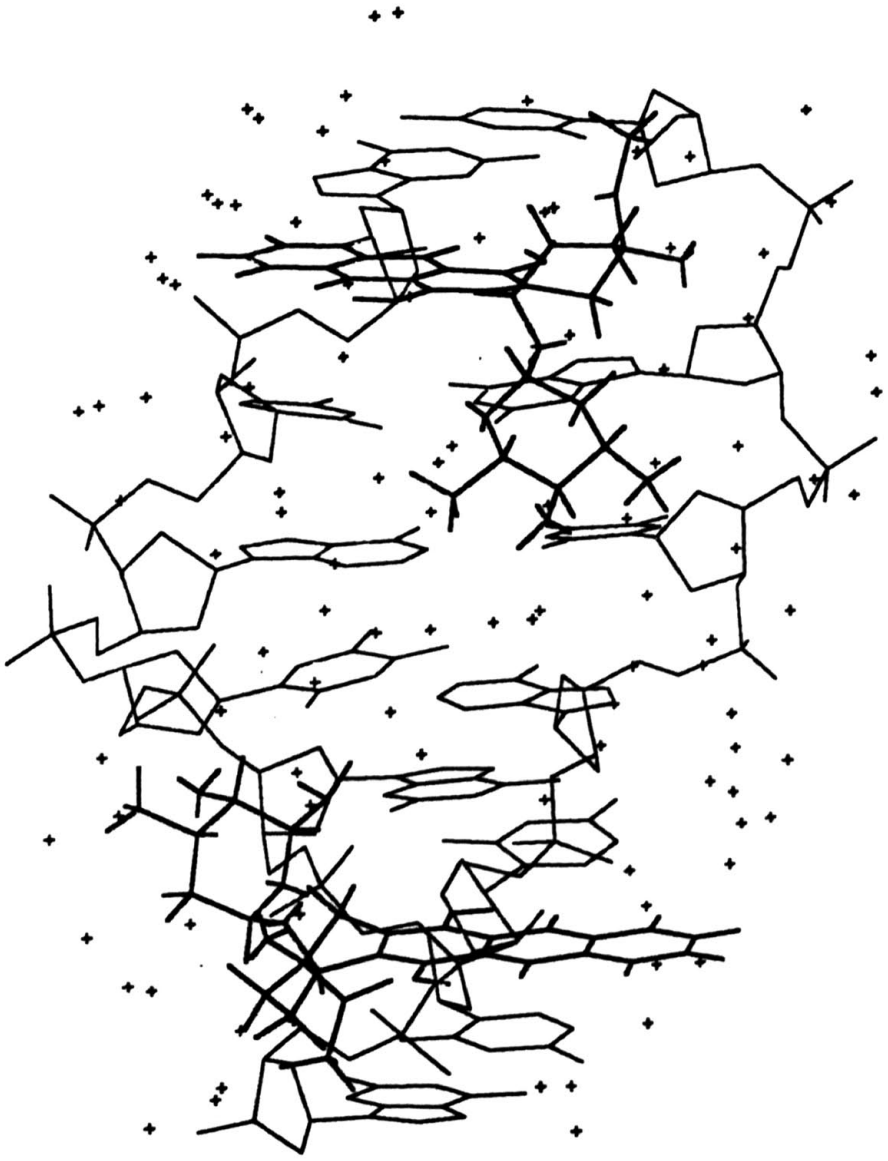
Intercalative Model of the 9a-Homo Analog 24



9a-Homo analog intercalated into the d(CGTACG) hexamer obtained from the X-ray crystal structure of daunorubicin-DNA complex. An overlay of the MM2-minimized analog with daunorubicin was done to obtain the same spatial orientation of **24** as that of daunorubicin in the complex.

Figure 21

Intercalative Model of the 8a-Homo Analog 25



8a-Homo analog intercalated into the d(CGTACG) hexamer. The same spatial orientation of daunorubicin was obtained by an overlay of the MM2-minimized analog **25** with daunorubicin.

Figure 22

Furthermore, these studies also showed that the conformation of the 8a-homo analog is closer to that of the 6-membered analog. Thus, both series became our synthetic targets.

Because of the intercalative property of these A-homo analogs, antitumor activity or cytotoxicity might be expressed *via* this property. These analogs could also express cytotoxicity by any of the other mechanisms mentioned before, that is, one- and two-electron bioreductive activation, inhibition of DNA topoisomerase, and/or interaction with the surface of cell membranes.

C. Synthetic Analysis

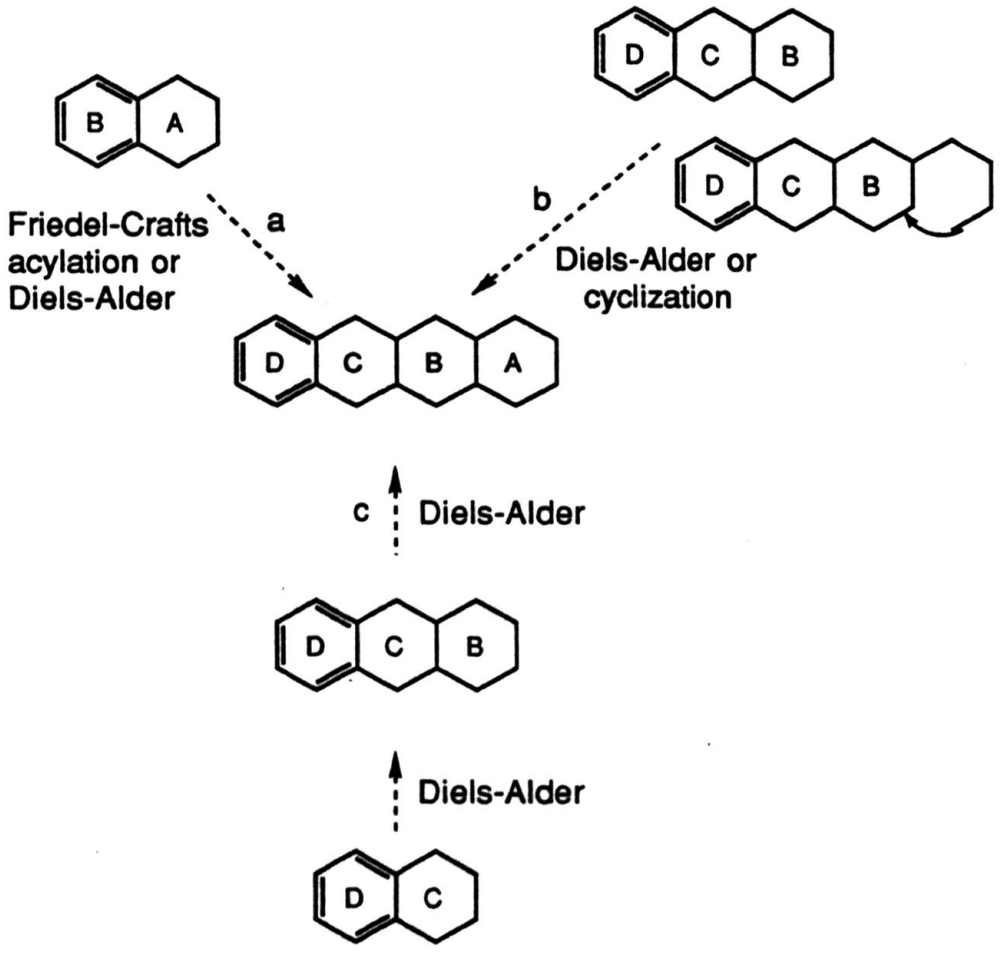
Scheme 2 shows the basic synthetic strategies employed in the preparation of racemic and optically active 4-demethoxy anthracyclines. The strategies employed can be classified in three basic groups:

1. Preparation of a properly functionalized tetralin derivative which corresponds to rings AB, and subsequent annulation of rings CD by Friedel-Crafts acylation or Diels-Alder reactions (pathway a in Scheme 2).

2. Synthesis of tricyclic intermediates (BCD) and construction of ring A by cyclization of a suitably functionalized side chain, or by Diels-Alder condensation with the appropriate diene (pathway b).

3. Construction of bicyclic BC intermediates and synthesis of rings A and D by Diels-Alder processes (pathway c).

Synthetic Strategies for the Preparation of 4-Demethoxy Anthracyclines



Scheme 2

The literature is filled with numerous representative examples of each of the above synthetic strategies, and extensive reviews have been published.[86, 89, 128] Analysis of the existing synthetic strategies indicates that the sensitive benzylic aminosugar moiety needs to be coupled to the aglycone portion during the final stages of the synthesis. This glycosidation

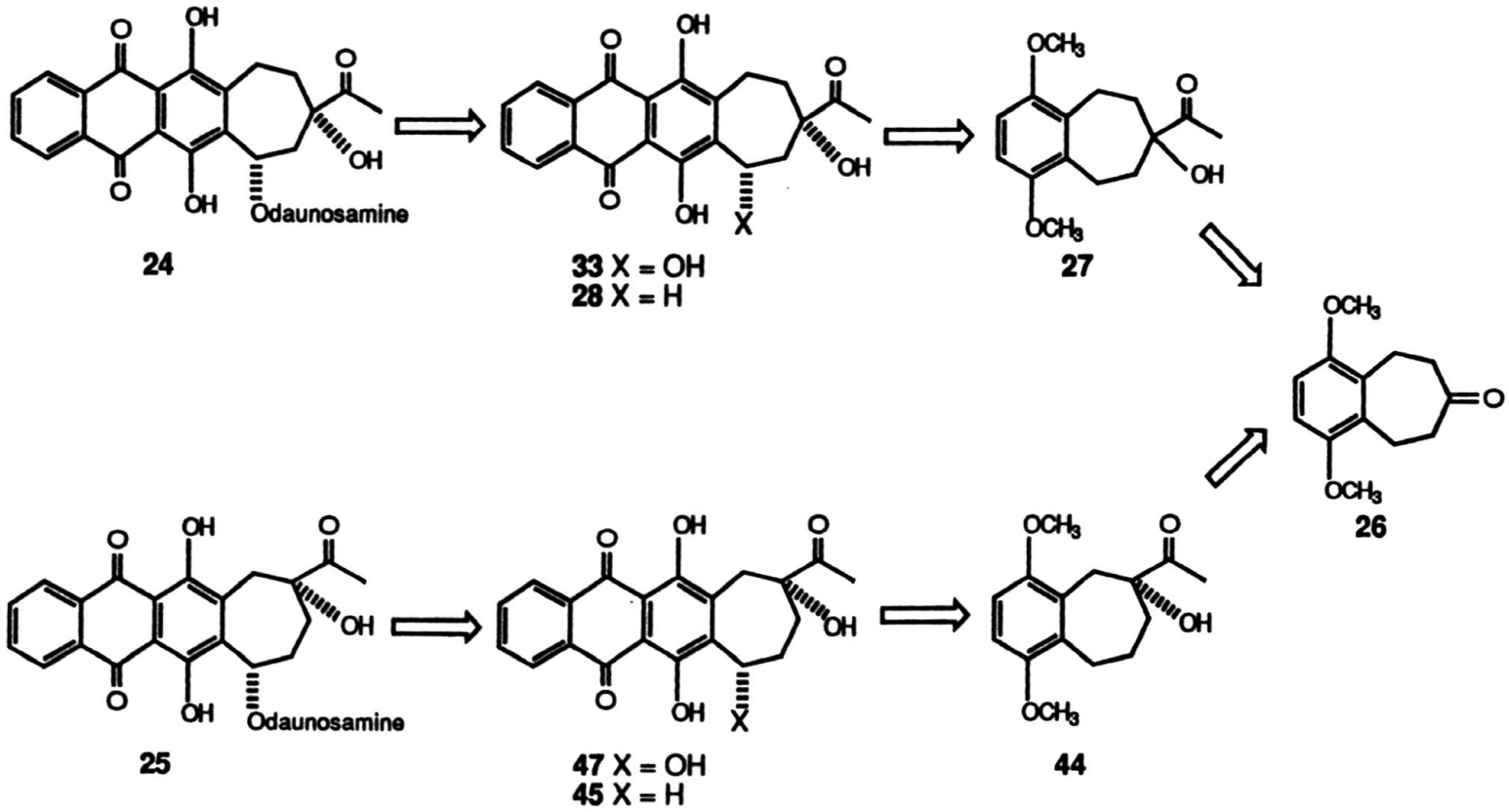
step has been performed by variations of the Koenigs-Knorr method with the suitably protected α-daunosamyl chloride or bromide in the presence of mercury (II)[32] or silver (I)[102, 129, 130] salts.

The syntheses of analogs **24** and **25** could be achieved employing any of the synthetic strategies described in the beginning of this section. Thus, compounds **24** and **25** were envisioned to be obtainable from the procedure which involves annulation of rings CD to the appropriately derivatized rings AB *via* a Friedel-Crafts acylation reaction. The retrosynthetic analysis is shown in Scheme 3.

Based on the reviews of previous syntheses of anthracyclines, the first disconnection would take place at the glycosidic linkage. Because of the anticipated sensitivity of the sugar to a variety of acidic or basic conditions, the coupling of this moiety with aglycones **33** and **47** was planned late in the syntheses.

Stereocontrolled introduction of the benzylic hydroxyl group at the 7 position of deoxyaglycone **28** could afford the aglycone **33**. Because of the plane of symmetry present in deoxyaglycone **28**, we did not have to be concerned about regiochemistry. That is, introduction of the hydroxyl group could be performed at either benzylic position of **28**. According to the literature, this transformation is achieved by radical bromination followed by solvolysis or its equivalent. Deoxyaglycone **28**, in turn, would be synthesized *via* Friedel-Crafts acylation of the electron-rich benzocyclo-heptenol derivative **27** with phthalic anhydride. Derivative **27** would be prepared from the benzocycloheptenone derivative **26**.

Retrosynthetic Analysis



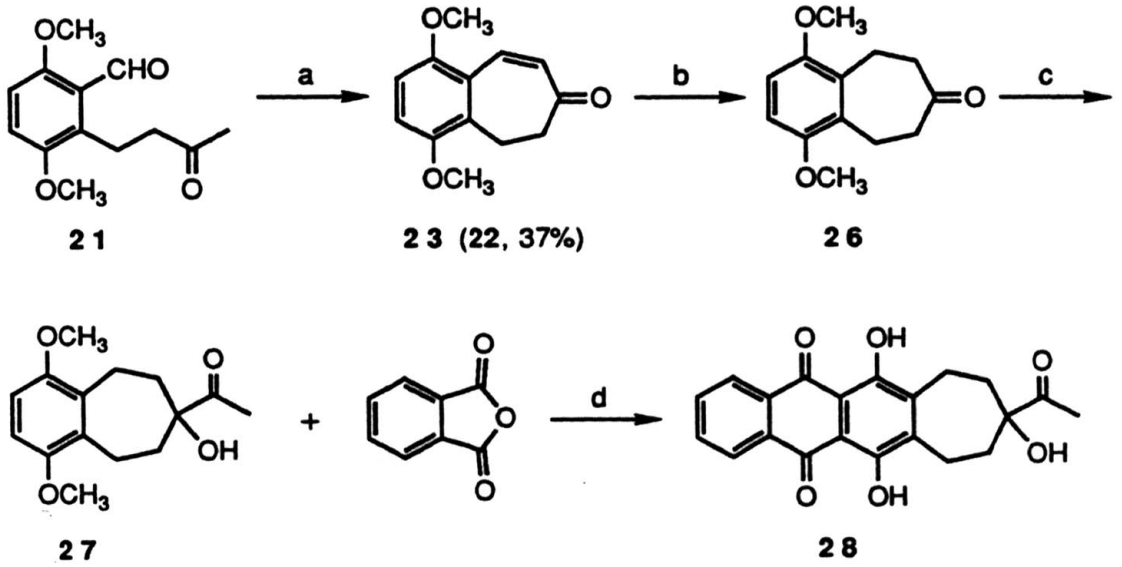
Scheme 3

Similar disconnections could be performed for analog **25**. In this case, deoxyaglycone **45** would be obtained *via* Friedel-Crafts acylation of the benzocycloheptene derivative **44**, which in turn, would be derived from the benzocycloheptenone derivative **26** followed by a 1,2-keto transposition.

D. Synthesis of the 9a-Homo Analog

Two different syntheses were devised for the preparation of analog **24** (symmetric analog). The first route is based on the unexpected results previously obtained during the intramolecular Claisen-Schmidt cyclization. This synthesis was performed in collaboration with Professor Noboru Motohashi (Meiji College, Japan). The second route is a completely different synthesis, which includes a double alkylation reaction followed by acid hydrolysis and decarboxylation to obtain the common intermediate **26**.

The first synthesis (Scheme 4) started with the intramolecular Claisen-Schmidt condensation of the benzaldehyde derivative **21** under basic conditions to afford the seven-membered ring enone in 55% yield (the five-membered isomer was obtained in 37%). Catalytic hydrogenation over Pd/C gave the benzocycloheptenone derivative **26** in quantitative yield. Reaction of **26** with Baldwin's reagent at -78 °C afforded the bicyclic ketone **27** in 56%. Friedel-Crafts acylation and demethylation of the phenolic hydroxyl groups, with the same reaction conditions, afforded the tetracyclic intermediate **28** in 50% overall yield. Currently, the synthesis of analog **24** is under its way.



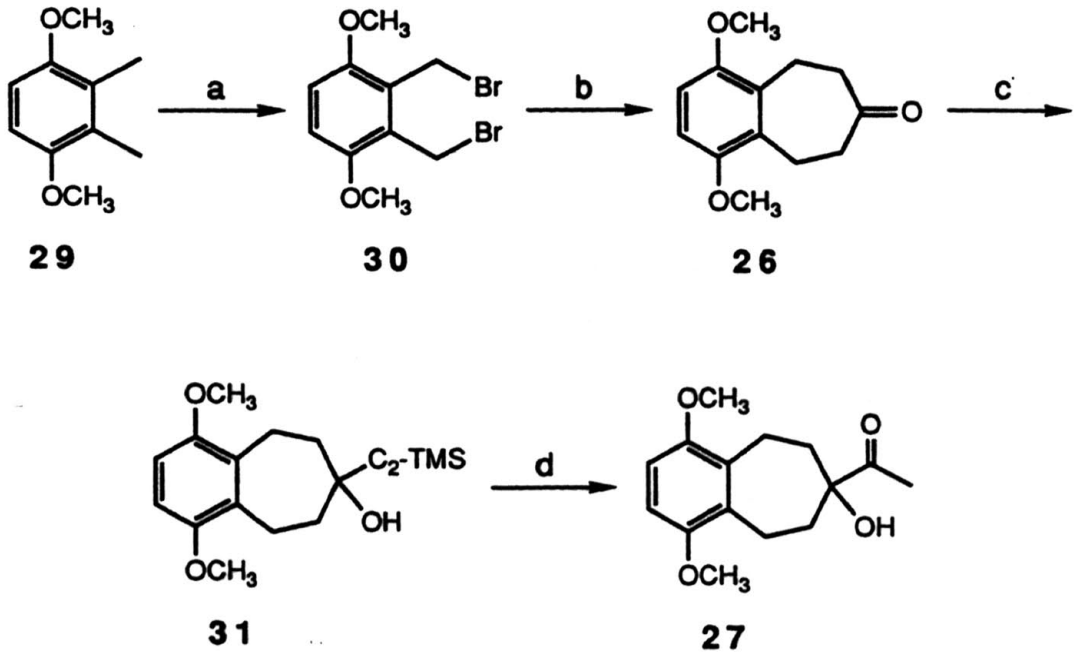
a. K_2CO_3, MeOH, r.t. 1h, 55%
b. H_2, Pd/C, 100%
c. Baldwin's reagent*, -78 °C, acid-catalyzed hydrolysis, 56%
d. $AlCl_3$, NaCl, heat, 50% overall

*Baldwin's reagent: $CH_2=C(Li)OCH_3$

Scheme 4

The second approach (Scheme 5) started with 2,3-dimethyl-1,4-dimethoxybenzene (**29**), which was prepared according to a literature procedure from 2,3-dimethyl *p*-hydroxyquinone.[131] Benzylic bromination of **29,** under free radical conditions (NBS, hv), afforded the dibromo compound **30** in quantitative yield. The benzocycloheptenone derivative **26** was obtained *via* a two-step reaction sequence. Reaction of **30** with dimethyl 1,3-acetonedicarboxylate under basic conditions in acetone followed by acid-catalyzed hydrolysis and decarboxylation gave **26** in quantitative yield.

Synthesis of Rings AB of 9a-Homo Analog 24

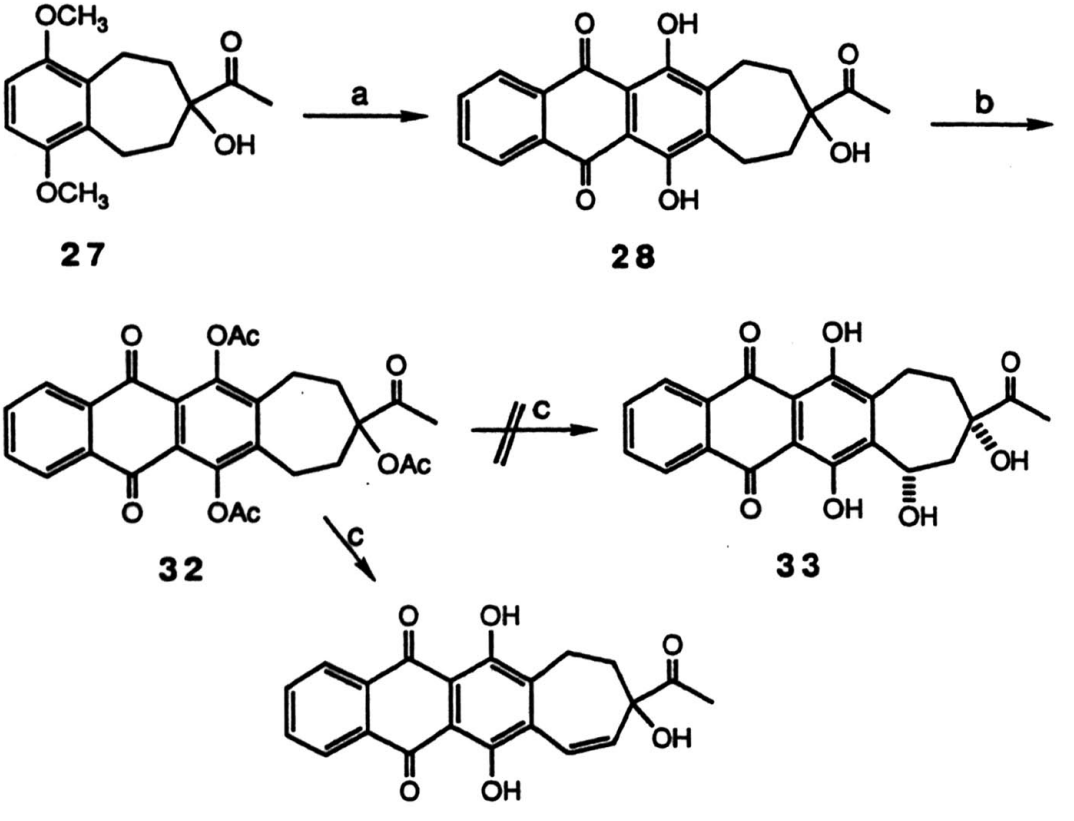


a. NBS, hv, benzoyl peroxide, 100%
b. i. dimethyl 1,3-acetonedicarboxylate, K_2CO_3, acetone
   ii. 4*M* H_2SO_4/MeOH, reflux, 100% overall
c. LiC_2-TMS/THF, -78 °C, 95%
d. cat. HgO (yellow), 3*M* H_2SO_4/THF, dark, 94%

Scheme 5

Treatment of **26** with (1-trimethylsilyl)ethynyl lithium in THF afforded alcohol **31** in 95%. Conversion of the acetylenic unit into a methyl ketone group was accomplished *via* a mercury(II)-catalyzed hydration. Thus, treatment of **31** with yellow HgO catalyst and 3*M* H_2SO_4/THF gave compound **27** in 94%. In this manner, compound **27** completes the synthesis of rings AB of our anthracycline analog **24**.

Next in our synthesis was the annulation of rings CD onto benzocycloheptenol derivative **27** to obtain deoxyaglycone **28** (Scheme 6). In this manner, double acylation and demethylation of the phenolic hydroxyl groups were achieved, in one-pot reaction, *via* Friedel-Crafts acylation of **27** with phthalic anhydride in 76% overall yield.



a. phthalic anhydride, $AlCl_3$, NaCl, 185-187 °C, 76% overall
b. acetic anhydride, pyridine, cat. DMAP, 90%
c. i. DBDMH, hν, benzoyl peroxide; ii. AgOAc/HOAc/H_2O, dark; iii. see Table 1 for hydrolysis conditions

Scheme 6

Samples of **28** from both routes were analyzed by HPLC, ^1H NMR, and IR for comparison purposes. The results indicated that both samples were identical, and these results, therefore, confirmed both synthetic approaches.

During the synthesis of 4-demethoxy-8-nordaunomycinone,[123] it was found that acetylation of the phenolic hydroxyl groups facilitated the introduction of the benzylic hydroxyl group. A rationale for this decisively favorable effect on the reactivity invoked participation by the neighboring acetate moiety (anchimeric assistance). Backside displacement of the β-bromo group by the acetyl group, followed by solvolysis gave the desired *cis*-glycol (Figure 23). Evidence for this explanation came from the observed migration of the phenolic acetate group involved in the neighboring group participation.

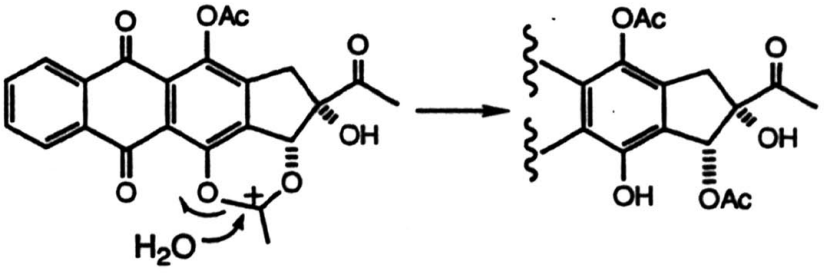


Figure 23

Based on the favorable effect on stability and reactivity induced by the acetate esters, we decided to protect the phenolic hydroxyl groups as acetates instead of methyl ethers. Thus, reaction of deoxyaglycone **28** with acetic anhydride in pyridine and DMAP catalytic afforded the triacetate **32** in 90% (Scheme 6). Besides the neighboring group participation of the acetate functionality, the triacetate derivative was much more soluble in CCl_4 than

the deoxyaglycone. When compounds with free phenolic hydroxyl groups were used as starting materials, their utility was limited to small-scale experiments as a consequence of their low solubility in CCl_4.

Procedures for the introduction of the benzylic hydroxyl group at the 7 position of the anthracyclines are well established and have excellent precedents.[86, 132-135] However, transformation of **28** to the aglycone **33** proved to be impossible in our hands. We explored all known standard procedures, but the results were always unsatisfactory.

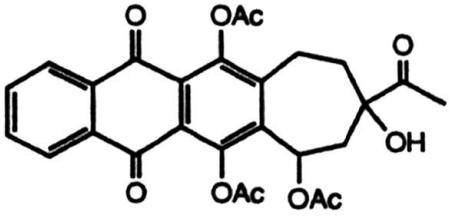
Introduction of the benzylic hydroxyl group is generally done *via* a three-step process, without purification of the intermediates due to the instability of these compounds. Benzylic bromination of **28** with 1,3-dibromo-5,5-dimethylhydantoin (DBDMH) and light (hv) gave the bromo derivative, which was carried directly onto the next step without further purification. In this manner, the reaction mixture was reacted with silver acetate in HOAc and H_2O to give the substitution product. This latter was also taken onto the next step without further purification. The acetate was hydrolyzed under acidic or basic conditions to give the dehydrated product **50**, decomposition, or starting material (Scheme 6). The desired aglycone **33** was never obtained.

It is worth mentioning that from all the syntheses of DA, DOX, and 4-DMDA reported in the literature, few of them have reached the final compound. Generally, these syntheses were stopped at the deoxyaglycone intermediate. The authors[136-140] claimed that their work constituted a total formal synthesis of the target molecules because the subsequent

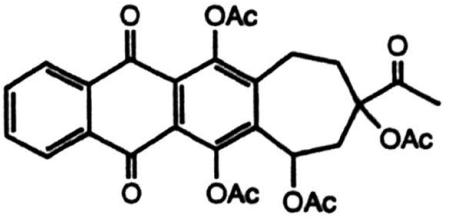
transformations had already been carried out and there was enough documentation in the literature to perform this sequence of reactions.

Although these transformations have already been performed, these researches did not bridge the gap. The reason for this is that this series of reactions is not so trivial as reported in the literature. We know by our own experience and by findings of others[141] that this series of reactions often do not work on big-scale experiments. Furthermore, in the synthesis of DA by Swenton and Raynolds,[137] the authors stated that they were not able to functionalize the 7 position on a scale needed for subsequent work.

In an effort to pin-point the problem, the unstable substitution product was isolated and partially characterized (^{1}H NMR and MS). The spectroscopic data indicated that the triacetate **48** was formed instead of the tetraacetate **49** (Figure 24). These findings indicated that the β–bromo group was displaced by the neighboring acetate group, followed by solvolysis to give the isolated product **48**. This process was similar to that of the 8-nor series with the exception that the 9 acetate group was the participating group in the anchimeric assistance instead of the phenolic acetate.



48



49

Figure 24

Triacetate **48** was subjected to different hydrolysis conditions as depicted in Table 1. Acid-catalyzed hydrolysis afforded the dehydrated product in entry # 2 and decomposition product(s) in entry # 3. Basic hydrolysis, on the other hand, afforded dehydration product, decomposition, or starting material.

Table 1. Hydrolysis Conditions for the Introduction of the Benzylic Hydroxyl Group.

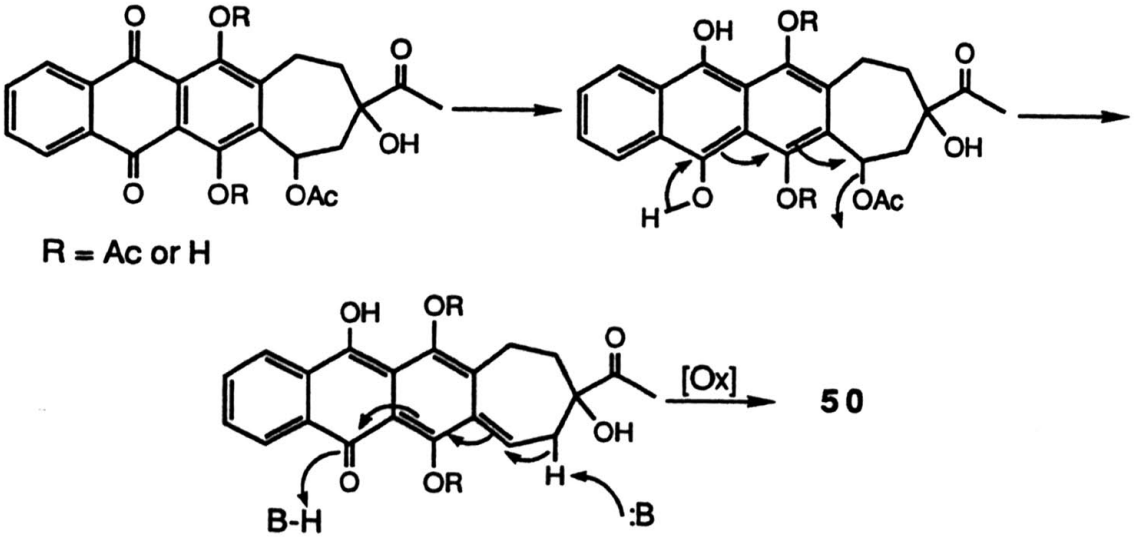
Entry	Reaction Conditions	Results
1	0.5*M* K_2CO_3/acetone[125]	Decomposition
2	3*N* HCl/MeOH[142]	Dehydration
3	0.5*N* HCl/HOAc/THF[143]	Decomposition
4	5% K_2CO_3 in MeOH/H_2O/THF[144]	Decomposition
5	0.1*N* LiOH in THF/H_2O/i-PrOH[145]	Dehydration
6	MeOH/NaOMe[146]	Starting Material
7	NH_3/MeOH[147]	Decomposition
8	anh. MeOH/pTsOH or PPTS[148]	St. Mat. & Dec.
9	#5 + air	Dehydration
10	#5 + argon	Dehydration
11	0.5*M* K_2CO_3 /PCC	15 min St. Mat. 30 min Dehyd.
12	TPFPFeCl/PhI$(OAc)_2$[149]	Decomposition

A previous report[141] indicated that the benzylic position of the anthraquinone system was surprisingly inert to direct oxidation. Different oxidizing reagents were employed, ie lead tetraacetate, ceric ammonium nitrate, and SeO_2, but all of them gave only recovered starting material. However, Holland and Viski[149] reported a direct oxidation of the benzylic position of the intermediate 2-acetyl-5,8-dimethoxy-2-hydroxytetrahydro-naphthalene by the use of a porphyrine derivative and $PhI(OAc)_2$.

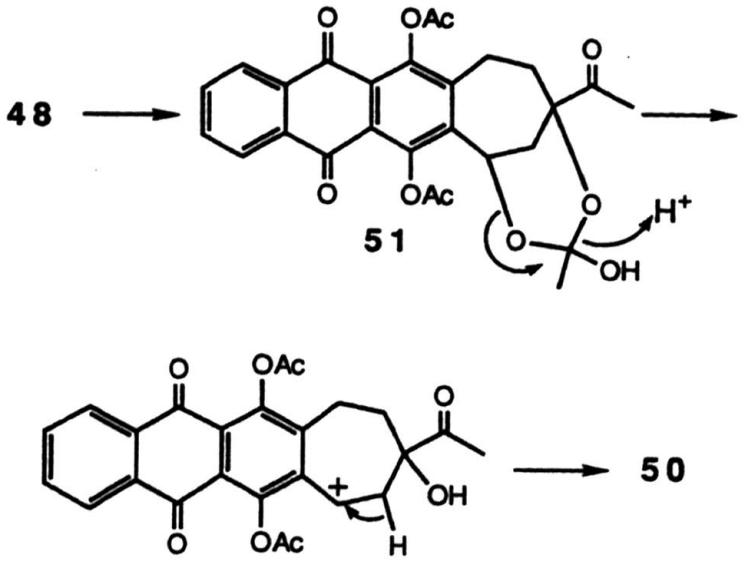
Thus, treatment of compound **32** under conditions similar to those reported by Holland and Viski gave decomposition products and starting material (entry # 12).

Formation of compound **50** could be rationalized by invoking a mechanism similar to that of the bioreductive activation of anthracyclines. Formation of the corresponding quinone methide followed by proton abstraction could have led to the formation of the dehydrated product (Scheme 7). To examine this hypothesis, we added PCC (oxidizing agent) or passed air into the solution in order to stop the formation of **50** (entries 5 and 10). This was not observed, however.

An alternative explanation for the formation of compound **50** could be through the putative intermediate orthoester **51** (Scheme 8). Under acidic conditions, **48** could form orthoester **51** (1,3-diaxial relationship of the hydroxy and acetate groups) which, in turn, eliminates (*via* an E_1 process) the orthoacetate group to generate the double bond.



Scheme 7



Scheme 8

At this point, we do not have a conclusive explanation for the formation of **50**. More work is necessary for the better understanding of this series of reactions which, in turn, could lead to a better functionalization of the benzylic position.

Because of the great amount of effort already spent in these transformations and lack of a better understanding of the mechanistic details occurring in this series of reactions, we decided to leave the synthesis at this stage and continue our work with the preparation of the 8a-homo analog in the hope of achieving a better result.

E. 1,2-Keto Transposition

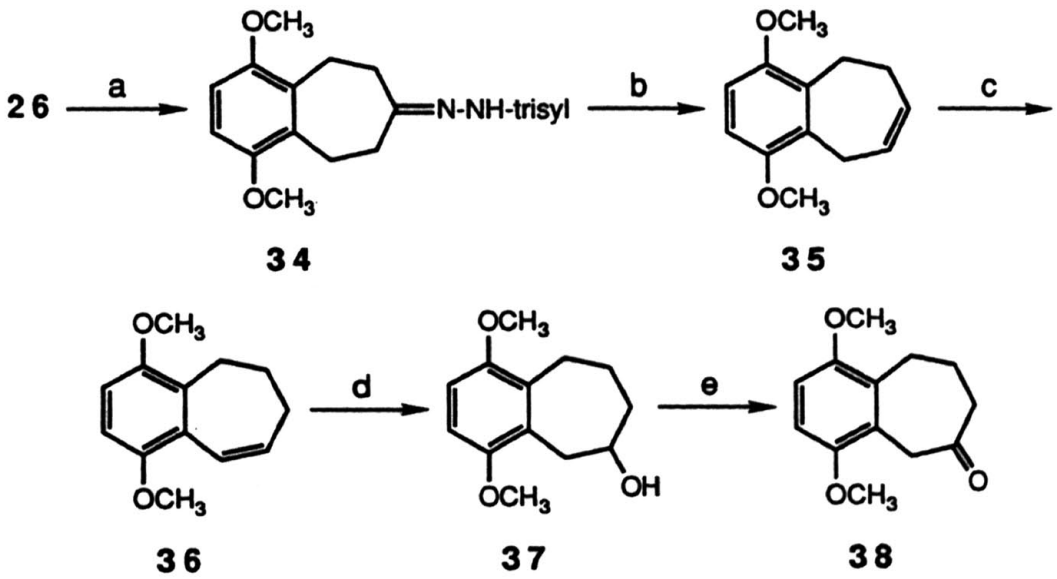
In the retrosynthetic analysis of the 8a-homo analog, the benzocycloheptenol derivative **44** was envisioned to be derived from benzocycloheptenone **26** followed by a 1,2-keto transposition.

The 1,2-keto transposition of **26** proved to be more difficult than anticipated. Standard procedures, such as enolsilylation,[150, 151] 1,2-(alkylative) carbonyl transposition,[152] and Nakai's method,[153] failed in our hands. This transformation, however, was ultimately accomplished in six steps with an overall yield of 43% from **26**. The key step was the regioselective opening of an epoxide (Scheme 9).

Treatment of **26** with trisylhydrazide gave the corresponded hydrazone **34** in 84%. Reaction of **34** with n-BuLi (Shapiro reaction) afforded the benzocycloheptene derivative **35** in 86%. Alkene isomerization was performed with $RhCl_3 \cdot 3H_2O$ in refluxing ethanol[154] to give **36** (88%).

Epoxidation was accomplished with dimethyldioxirane (0.1*M* in acetone) in CH_2Cl_2.[155, 156] The epoxide was used without further purification and treatment with LAH followed by basic work-up afforded the benzocycloheptenol derivative **37** in 75% overall yield. Sometimes, alkene **36** was also formed during the work-up, reducing the yield to 60%. Oxidation with PCC afforded, in 89% yield, the desired transposed ketone **38**.

1,2-Keto Transposition



a. NH_2-NH-trisyl/HCl, 84%
b. n-BuLi/THF, -78 °C, 86%
c. $RhCl_3 \cdot 3H_2O/CHCl_3$/EtOH, reflux, 88%
d. i. dimethyldioxirane/CH_2Cl_2; ii. LAH/THF, 75% overall
e. PCC/acetone, 89%

Scheme 9

F. Synthesis of the 8a-Homo Analog

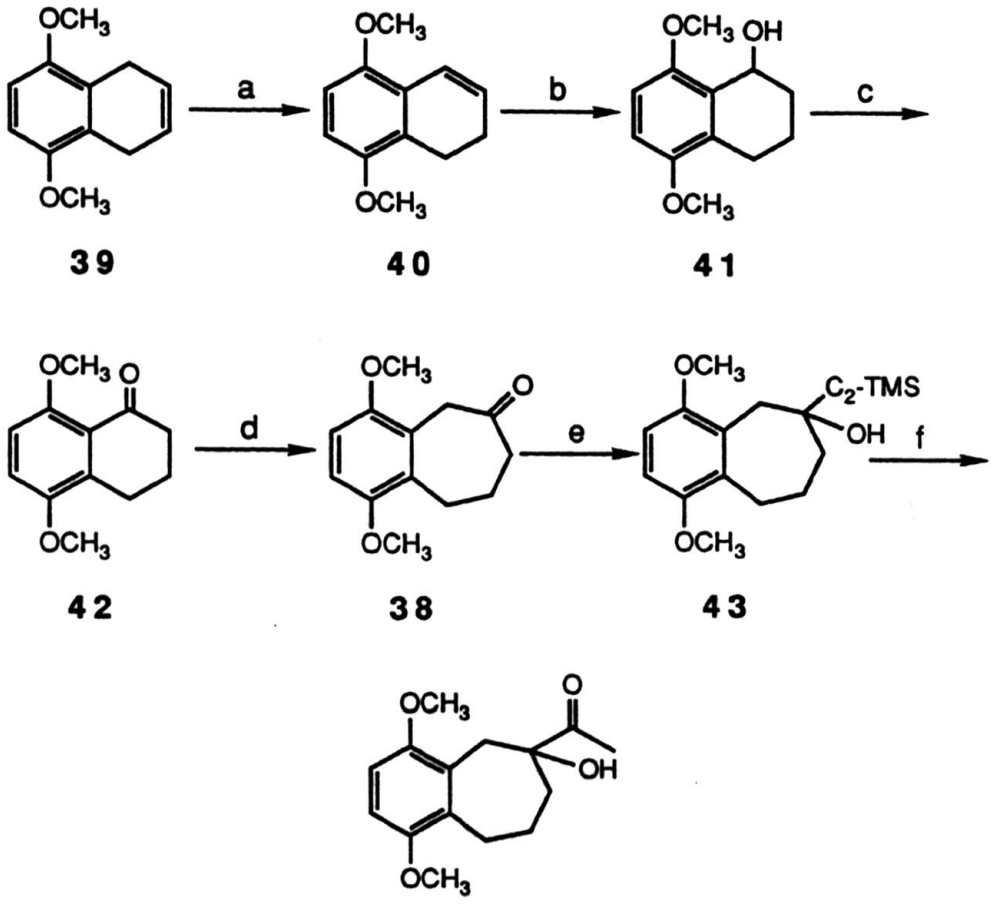
Once the 1,2-keto transposition was accomplished, we decided to find a more direct alternative route toward the ketone **38**. This direct route utilizes a ring expansion reaction for the preparation of **38**.

The synthesis (Scheme 10) starts with compound **39** which was previously synthesized in our laboratories.[157] Alkene isomerization of **39** with $RhCl_3 \cdot 3H_2O$ afforded the 1,2-dihydronaphthalene derivative **40** (90%). Formation of the α-tetralol derivative **41** was accomplished in 77% when **40** reacted with diborane followed by oxidation with H_2O_2/NaOH. The β-tetralol isomer was also formed in a 10:1 α:β selectivity ratio. Oxidation of **41** with PCC gave the corresponding ketone **42** in 87%.

The ring expansion reaction[158, 159] was performed in a two-reaction process. First, reaction of **42** with methyltriphenylphosphonium bromide and NaH in DMSO afforded an exo-methylene intermediate. Without purification, this intermediate was treated with $Tl(NO_3)_3$ in MeOH to afford **38** in 75% overall yield.

At this point, we had to transform **38** into rings AB. To do this, a similar transformation used for the symmetric analog could be employed. However, **38** is an easily enolizable ketone and, therefore, a less basic reagent must be employed. To avoid enolization by the lithium reagent, a cerium reagent[160, 161] was used instead.

Synthesis of Rings AB of 8a-Homo Analog 25



a. $RhCl_3 \cdot 3H_2O$, $CHCl_3$/EtOH, reflux, 90%
b. i. B_2H_6/THF; ii. H_2O_2/NaOH, 77%
c. PCC/acetone, 87%
d. i. $MePh_3PBr$, NaH, DMSO; ii. $Tl(NO_3)_3$/MeOH, 75% overall
e. $Ce(C_2\text{-TMS})Cl_2$/THF, -78 °C, 100%
f. cat. HgO (yellow), 3*M* H_2SO_4/THF

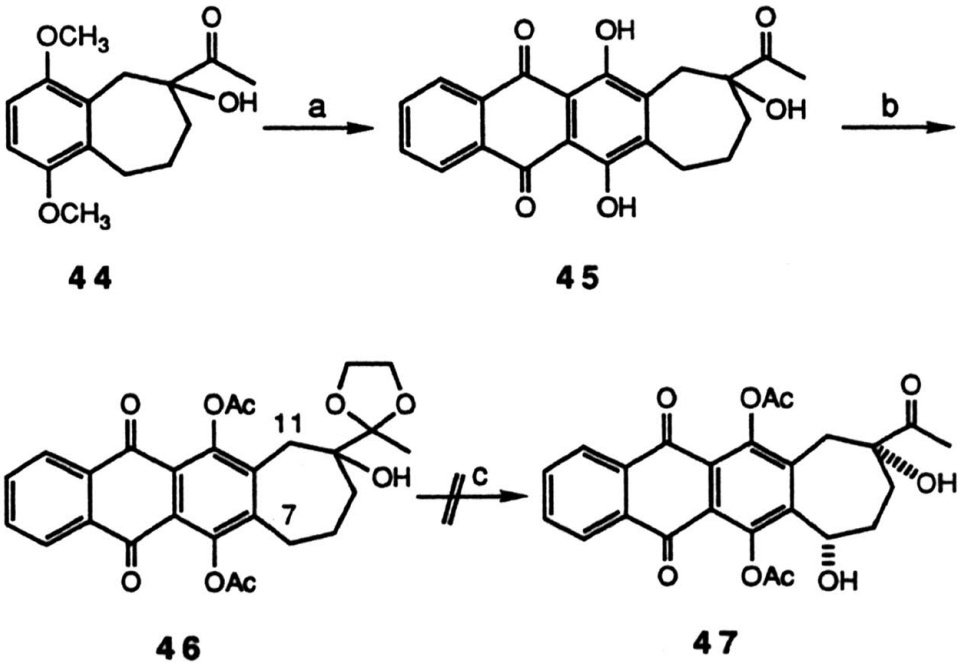
Scheme 10

Thus, transformation of **38** to **44** was accomplished in the following manner (Scheme 10). Reaction of the seven-membered ring ketone with cerium [(1-trimethylsilyl)ethynyl]Cl_2[162] at -78 °C in THF gave the acetylenic alcohol **43** in 100% yield. When an enolizable reagent is used instead (Li and Mg reagents), this reaction works in 40% yield and one half of the starting material is recovered. Hydration of **43** was accomplished with yellow HgO catalytic in 3*M* H_2SO_4/THF to give rings AB in 87% yield.

At this stage, we just needed to annulate rings CD onto rings AB to obtain the deoxyaglycone **45**. This was accomplished using the same procedure employed in the conversion of **27** to **28**. Thus, Friedel-Crafts acylation of **44** with phthalic anhydride gave **45** in 85% overall yield (Scheme 11).

Next in our synthesis was the regio- and stereocontrolled introduction of the benzylic hydroxyl group at the 7 position. To control the regiochemistry, the methyl ketone group was protected as the ethylene ketal (Scheme 11), in order to increase its steric bulk. In this way, the ketal group is expected to hinder bromination at the 11 position, and at the same time to favor bromination at the desired 7 position. We anticipated stereocontrolled introduction of the hydroxyl group by the anchimeric assistance of the phenolic acetate group as described in the preceding discussion.[123]

In this manner, deoxyaglycone **45** was treated with ethylene glycol and *p*-TsOH catalytic and azeotropic removal of water to give the protected ketone. Because of the high insolubility of this compound, the ketal was not purified and treatment with acetic anhydride, pyridine, and DMAP catalytic afforded the diacetate **46** in 90% overall yield (Scheme 11).



a. phthalic anhydride, $AlCl_3$, NaCl, 180-182 °C, 85% overall
b. i. $HO(CH_2)_2OH$, cat. *p*-TsOH, PhH; ii. Ac_2O, Pyr, cat. DMAP, 90% overall
c. see Table 1 for hydrolysis conditions

Scheme 11

Once this point was reached in our synthesis, we only needed one more intermediate before the final compound. However, formation of aglycone **47** was exhaustively pursued without success. Again, we explored all the known procedures for this transformation (see Table 1), but all of our efforts did not help us to achieve our goal. It seems that the intermediates were much more labile even than those of the symmetric counterpart. In this case, no dehydrated product was formed, only decomposition products were obtained.

Isolation of the substitution product was impossible. This indicated that in this series of reactions the problem resided in the bromination step.

Perhaps, the bromo derivative is much more unstable in the 8a-homo series than in the 9a series.

Because of the great deal of effort, the amount of time consumed, and the idiosyncratic reactivity or unstability of the bromo compound intermediate, we decided to leave this synthesis at this stage, until more studies about this series of reactions are peformed to shed more light on the mechanisms of these reactions.

G. Conclusions

Computer graphic analyses indicated that the non-intuitive analog **25** might be more active than the intuitive analog **24**. Unfortunately, the power of computer graphic studies could not be corroborated, since the synthesis of these analogs could not be completed.

Standard 1,2-keto transposition procedures proved to be more difficult than anticipated. At this stage of the synthesis, the compounds already started to show some idiosyncratic behavior. The route employed in our case had a regioselective opening of an epoxide as the key step. This transformation was accomplished in six steps with an overall yield of 43%.

The syntheses of analogs **24** and **25** could not be finished due to the intractible behavior of some of the intermediates. Unfortunately, this intractible behavior could not have been anticipated during the design of the syntheses since no complications have been reported for the introduction of this functionality in the six-membered series.

These syntheses were left at the stage of advanced intermediates. So, if new strategies for the introduction of the benzylic hydroxyl group are discovered, hopefully these new procedures could be tried on these advanced intermediates for the completion of the syntheses.

More studies must be conducted in order to understand and shed more light on the reactions needed for the functionalization of the 7 position.

EXPERIMENTAL

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared (IR) spectra were obtained on an IBM FTIR 32 or a Perkin Elmer 1420 spectrophotometer. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Varian XL-300 (300 MHz) and Bruker AM-500 (500 MHz) spectrometers in the indicated solvents using tetramethylsilane as internal reference, unless otherwise specified. All chemical shifts are expressed in parts per million (δ). Electron impact mass spectra (EIMS), chemical ionization mass spectra (CIMS), and high resolution mass spectra (HRMS) were obtained on a Varian CH-5 or Ribermag R10-10 mass spectrometer by Dr. Charles Judson, Dr. Todd Williams, Dr. Tho I. Nguyen, and Robert Drake. Microanalyses were performed by Dr. Tho I. Nguyen on a Hewlett-Packard Model 185 CHN analyzer at the University of Kansas, or by Midwest Microlab, Ltd., Indianapolis, IN. Ultraviolet (UV) spectra were recorded on a Hewlett-Packard Diode Array 8450A. Flash chromatography and medium pressure liquid chromatography (MPLC) were performed on Merck silica gel (230–400 mesh), and gravity column chromatography on Merck silica gel (70–270 mesh). Preparative centrifugal thin-layer chromatography (PCTLC) was performed on a Harrison Model 7924 Chromatotron using Merck silica gel 60 PF-254 containing $CaSO_4 \cdot 1/2\ H_2O$ binder. "Workup" indicates drying over anhydrous Na_2SO_4, filtration, and concentration *in vacuo*.

5,8-Dimethoxy-1,4-dihydronaphthalene[157] (**39**) and 1,4-dimethoxy-2,3-dimethylbenzene[131] (**29**) were prepared *via* literature procedures. Reagents

and solvents were used as obtained without further purification unless otherwise indicated. Bulk grade solvents: diethyl ether, hexanes, acetone, and methanol were distilled before use. Tetrahydrofuran (THF) and diethyl ether (Et_2O) were distilled immediately before use from sodium benzophenone ketyl. Methylene chloride (CH_2Cl_2) was distilled immediately before use from phosphorus pentoxide. Benzene was distilled from powdered calcium hydride. Methanol (MeOH) and ethanol (EtOH) were distilled from Mg turnings. Anhydrous carbon tetrachloride (CCl_4) and anhydrous pyridine were purchased from Aldrich Chemical Co. N-bromosuccinimide (NBS) was recrystallized from ten parts of water.

All reactions involving moisture- or oxygen-sensitive materials were performed in flame- or oven-dried glassware under a positive pressure of argon.

2,3-Bis(bromomethyl)-1,4-dimethoxybenzene (30). A mixture of 2,3-dimethyl-1,4-dimethoxybenzene (5.00 g, 30.1 mmol), NBS (11.24 g, 63.1 mmol), and benzoyl peroxide (50 mg) in anhydrous CCl_4 (150 mL) was refluxed with irradiation, using a sunlamp for 8 h. After the mixture cooled, the succinimide was filtered off and the solvent was evaporated under reduced pressure. The residue, upon crystallization from CH_2Cl_2-hexanes, furnished the dibromide **30** as off-white needles (9.68 g, 99%): mp 153.5-154 °C (lit.[163] 149 °C; lit.[164] 147-149 °C). ^1H NMR and IR data agree with reported values.[163, 164]

1,4-Dimethoxy-5,6,8,9-tetrahydro-7*H*-benzocyclohepten-7-one (26). A mixture of **30** (9.35 g, 28.9 mmol), dimethyl 1,3-acetonedicarboxylate (5.02 g, 28.9 mmol), and K_2CO_3 (8.37 g, 60.6 mmol) in

dry acetone (100 mL) was stirred at room temperature under Ar for 8 h. The mixture was filtered and concentrated under reduced pressure. The solid obtained was redissolved in MeOH (120 mL), 4*M* H_2SO_4 solution (15 mL) was added and the reaction mixture was refluxed overnight. After the mixture cooled, it was diluted with water (100 mL) and the solid obtained was filtered off and dried under high vacuum at 35 °C to afford **26** in quantitative yield. Analytical sample was obtained by recrystallization from hexanes to give pale yellow flakes: mp 123.5-124 °C (lit.[165] 124-125.5 °C). ^{1}H NMR and IR data agree with reported values.[165]

1,4-Dimethoxy-7-(trimethylsilyl)ethynyl-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-7-ol.(31). To a cold solution (-20 °C) of trimethylsilylacetylene (267.6 mg, 2.72 mmol) in THF (20 mL) was added dropwise n-BuLi (1.6*M* in hexanes, 1.6 mL 2.50 mmol) and the mixture was stirred at this temperature for 15 min. After this, it was cooled to -65 °C and a solution of ketone **26** (500 mg, 2.27 mmol) in THF (20 mL) was added dropwise. After the mixture was stirred for 45 min at this temperature and for another 15 min at room temperature, it was poured into sat. aq. NH_4Cl (20 mL). The layers were separated, the aqueous layer was extracted with ether (2 x 10 mL), and the combined organic extracts were worked up to afford **31** as a yellowish white solid (684.9 mg, 95%). Analytical sample was obtained by recrystallization from ether-hexanes: mp 121-121.5 °C; UV (CH_3CN), λ_{max} (ε) 224 (7 560, sh), 289 (3 630) nm; IR ($CHCl_3$) 3580 (sharp), 3000, 2940, 2810, 2130, 1580, 1480, 1460, 1340, 1250, 1080, 1040, 900, 850 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.690 (s, 2H), 3.761 (s, 6H), 3.234 (br s, 2H), 2.668 (br s, 4H), 1.961 (br s, 1H, D_2O exch.), 0.169 (s, 9H); EIMS *m/z*

(relative intensity) 319 (M^+ + 1, 19), 318 (M^+, 93), 300 (15), 285 (14), 269 (18), 227 (13), 163 (19), 99 (29), 73 (100), 45 (37).

Anal. Calcd for $C_{18}H_{26}O_3Si$: C, 67.88; H, 8.23. Found: C, 67.78; H, 8.40.

7-Acetyl-1,4-dimethoxy-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-7-ol (27). A solution of **31** (250.0 mg, 0.78 mmol) in THF (10 mL) was added dropwise to a solution of yellow HgO in 3*M* H_2SO_4 (6 mL). The mixture was stirred at room temperature in the dark under Ar for 5 h. After this time, the mixture was diluted with water (50 mL), extracted with CH_2Cl_2 (3 x 100 mL), and the combined organic layers were worked up. Purification by MPLC (EtOAc-hexanes 1:4) afforded **27** as a yellowish white solid (195.5 mg, 94%). Analytical sample was obtained by recrystallization from ether-hexanes: mp 117-117.5 °C; UV (CH_3CN), λ_{max} (ε) 208 (7 350), 225 (3 110, sh), 290 (1 380) nm; IR ($CHCl_3$) 3470 (sharp), 3000, 2940, 2830, 1700, 1600, 1480, 1340, 1240, 1170, 1080, 1035, 950, 900, 800 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 6.690 (s, 2H), 3.888 (s, 1H, D_2O exch.) 3.755 (s, 6H), 3.268 (ddd, J = 14.8, 6.7, 2.5 Hz, 2H), 2.912 (ddd, J = 14.8, 10.4, 2.9 Hz, 2H), 2.117 (s, 3H), 1.742-1.682 (m, 4H); EIMS *m/z* (relative intensity) 264 (M^+, 15), 221 (33), 203 (7), 191 (9), 177 (10), 151 (33), 91 (14), 77 (13), 43 (100).

Anal. Calcd for $C_{15}H_{20}O_4$: C, 68.16; H, 7.63. Found: C, 68.50; H, 7.68.

4-Demethoxy-7-deoxy-9a-homodaunomycinone (28). An intimate mixture of **27** (1.50 g, 5.68 mmol), phthalic anhydride (2.52 g, 17.03 mmol), $AlCl_3$ (16.65 g, 124.85 mmol), and NaCl (3.65 g, 62.43 mmol) was placed in a beaker and immersed in a sand bath pre-heated at 185-187 °C. The mixture became a dark red melt instantly and it was stirred continously with a rod glass for 6 min. After the reaction mixture was allowed to cool, a

sat. aq. solution of oxalic acid (10 mL) was added cautiously until no HCl gas evolved. Next, 50 mL of the sat. solution were added and the resulting mixture was heated for 10 min at 80 °C. The red precipitate was filtered off, redissolved in $CHCl_3$ (250 mL), filtered through celite, and concentrated under reduced pressure. The purple solid obtained was purified by MPLC (EtOAc-hexanes 1:2) to afford **28** as a red-orange solid (1.59 g, 76%). Analytical sample was obtained by recrystallization from $CHCl_3$ to give an orange solid: mp 238.5 °C dec.; UV (CH_3CN), λ_{max} (ε) 205 (28 080), 230 (13 480), 252 (41 160 sh), 257 (46 080), 292 (7 440) nm; IR ($CHCl_3$) 3350 (br), 2940, 1700, 1615, 1585, 1405, 1375, 1345, 1255, 1090, 905 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) 13.667 (2H, D_2O exch.), 8.352 (dd, J = 5.9, 3.3 Hz, 2H), 7.815 (dd, J = 5.9, 3.4 Hz, 2H), 3.980 (s, 1H, D_2O exch.), 3.496 (t; dd, J = 4.3; 5.4, 2.6 Hz, 2H), 3.050 (dt, J = 14.1, 6.8 Hz, 2H), 2.161 (s, 3H), 1.770 (br t, J = 4.3 Hz, 4H); EIMS *m/z* (relative intensity) 367 (M^+ + 1, 3), 366 (M^+, 11), 324 (21), 323 (98), 305 (14), 281 (14), 187 (14), 77 (12), 43 (100).

Anal. Calcd for $C_{21}H_{18}O_6 \cdot 1/2H_2O$: C, 67.20; H, 5.10. Found: C, 67.40; H, 4.80.

4-Demethoxy-7-deoxy-6,9,12-triacetyl-9a-homodaunomycinone (32). To a solution of **28** (370.0 mg, 1.01 mmol) and DMAP (2-3 mg) in pyridine (12 mL) was added acetic anhydride (3.43 mL, 36.4 mmol). The reaction mixture was stirred for 1 h at room temperature and then concentrated under high vacuum to afford a dark yellow solid. Purification by MPLC (EtOAc-hexanes 1:1) afforded **32** as a yellow solid (445.3 mg, 90%): mp 243-244.5 °C dec.; UV (CH_3CN), λ_{max} (ε) 258 (30 370), 280 (10 180), 344 (5 425) nm; IR ($CHCl_3$) 2940, 1760, 1730, 1710, 1670, 1580, 1430,

1370, 1330, 1290, 1240, 1180, 1060, 1010, 940 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 8.127 (dd, *J* = 5.8, 3.3 Hz, 2H), 7.716 (dd, *J* = 5.8, 3.3 Hz, 2H), 3.035 (dd, *J* = 15.0, 7.7 Hz, 2H), 2.898 (app. t, *J* = 14.6 Hz, 2H), 2.505 (s, 6H), 2.368 (br s, 2H), 2.174 (s, 3H), 2.092 (s, 3H), 1.819 (br t, *J* = 13.0 Hz, 2H); CIMS (NH_3) *m/z* (relative intensity) 510 (M^+ + 18, 21), 493 (M^+ + 1, 19), 451 (26), 408 (11), 390 (18), 348 (47), 306 (22), 43 (100).

Anal. Calcd for $C_{27}H_{24}O_9$: C, 65.85; H, 4.91. Found: C, 65.96; H, 5.13.

4-Demethoxy-7,8-dehydro-9a-homodaunomycinone (50). A mixture of **32** (50.0 mg, 0.102 mmol), 1,3-dibromo-5,5-dimethylhydantoin (29.0 mg, 0.102 mmol), and benzoyl peroxide (10 mg) in anhydrous CCl_4 (12 mL) was refluxed with irradiation, using a sunlamp for 2 h. After the mixture cooled, the solvent was removed under reduced pressure and the solid obtained was re-dissolved in HOAc (5 mL). Silver acetate (42.4 mg, 0.254 mmol) and H_2O (0.21 mL) were added and the mixture was stirred in the dark for 12 h. After this period, the reaction mixture was concentrated under high vacuum and the solid obtained was purified by MPLC (EtOAc-hexanes 1:2) to afford the unstable intermediate **48** (17.7 mg, 34%). After partial characterization (^1H NMR, MS), compound **48** was suspended in MeOH-H_2O (2:1, 12 mL), 1 mL of 6*N* HCl was added and the reaction mixture was refluxed under Ar for 72 h. After this time, the mixture was diluted with H_2O (20 mL) and extracted with $CHCl_3$ (3 x 15 mL). The combined organic layers were washed with brine (15 mL) and worked-up. Purification by MPLC (EtOAc-hexanes 1:4) afforded **50** as an orange-red solid (6.2 mg, 58%).

48: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.147-8.121 (m, 2H), 7.732-7.713 (m, 2H), 6.805 (br s, 1H), 2.851 (br s, 1H), 2.527 (s, 6H), 2.482 (s, 3H), 2.276-2.151 (m, 2H), 2.082 (s, 3H), 1.927 (br s, 4H); CIMS (NH_3) *m/z* (relative intensity) 508 (M^+, 6), 450 (1), 415 (2), 389 (5), 347 (4), 187 (2), 105 (3), 91 (2), 43 (100).

50: mp 215 °C dec.; UV (EtOH), λ_{max} (ε) 209 (19 840), 266 (41 700) nm; IR ($CHCl_3$) 3360-3500 (br), 2920, 2840, 2360, 2340, 1715, 1620, 1590, 1455, 1395, 1345, 1320, 1290, 1245, 1125, 1085, 965 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 13.904 (s, 1H, D_2O exch.), 13.637 (s, 1H, D_2O exch.), 8.354-8.336 (m, 2H), 7.833-7.815 (m, 2H), 7.260 (d, J = 12.6 Hz, 1H), 5.944 (dd, J = 12.6, 2.3 Hz, 1H), 4.520 (s, 1H, D_2O exch.), 3.764 (dd, J = 15.1, 7.7 Hz, 1H), 2.538 (dd, J = 15.2, 11.1 Hz, 1H), 2.149 (s, 3H), 2.084 (ddd, J = 14.2, 7.8, 2.2 Hz, 1H), 1.975 (dd, J = 14.3, 11.3 Hz, 1H); EIMS *m/z* (relative intensity) 364 (M^+, 4), 346 (4), 321 (100), 303 (17), 187 (15), 105 (11), 43 (96).

HRMS Calcd for $C_{21}H_{16}O_6$: 364.0947. Found: 364.0944.

1,4-Dimethoxy-5,6,8,9-tetrahydro-7*H*-benzocyclohepten-7-one trisylsulfonylhydrazone (34). To a stirred suspension of finely ground trisylhydrazide (6.77 g, 22.7 mmol) in 30 mL of methanol was added the ketone **26** (5.00 g, 22.7 mmol). The addition of 0.5 mL of conc. HCl caused the mixture to clear rapidly, after which a fine granular product began to crystallize. The reaction mixture was chilled overnight and filtered. The product was washed with cold methanol and dried under vacuum at room temperature to afford **34** as a white solid (9.49 g, 84%). Analytical sample was obtained by recrystallization from CH_2Cl_2-hexanes to give white crystals: mp 163 °C dec.; UV (CH_3CN), λ_{max} (ε) 260 (6 1250), 286 (5 150)

nm; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.185 (s 1H, D_2O exch.), 7.133 (s, 2H), 6.662 (s, 2H), 4.235-4.181 (m, 2H), 3.737 (s, 3H), 3.707 (s, 3H), 2.933-2.874 (m, 4H), 2.480-2.455 (m, 2H), 2.372-2.348 (m, 2H), 1.238 (d, *J* = 2.9 Hz, 9H), 1.224 (d, *J* = 2.5 Hz, 9H); IR ($CHCl_3$) 3280 (br), 3000, 2950, 1600, 1480, 1460, 1325, 1220, 1165, 1075, 1030, 940, 885, 800, 660 cm^{-1}; EIMS *m/z* (relative intensity) 501 (M^+ + 1, 4), 500 (M^+, 5), 421 (1), 267 (4), 251 (11), 204 (51), 189 (53), 173 (36), 91 (47), 43 (100).

Anal. Cald for $C_{28}H_{40}N_2O_4S$: C, 67.16; H, 8.05; N, 5.60. Found: C, 67.10; H, 8.20; N, 5.71.

1,4-Dimethoxy-6,7-dihydro-5*H*-benzocycloheptene (35). n-BuLi (1.6 *M* in hexanes, 3.9 mL, 3.00 mmol) was added to a cold (-65 °C) solution of **34** (3.00 g, 5.99 mmol) in THF (40 mL). After the reaction mixture was stirred at this temperature for 15 min, the cold bath was removed and the mixture was stirred at room temperature for another 15 min, after which the mixture was poured into sat. NH_4Cl (20 mL). The layers were separated, the aq. layer was extracted with ether (2 x 15 mL), and the combined organic extracts were worked up. Purification by PCTLC (EtOAc-hexanes 1:4) afforded **35** as a clear yellow liquid (1.19 g, 98%). UV (CH_3CN), λ_{max} (ε) 206 (16 880), 225 (7 230, sh), 289 (2 610) nm; IR (neat) 3000, 2910, 2880, 2810, 1590 (vw), 1480, 1255, 1220, 1205, 1080, 790, 715 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.712-6.667 (m, 2H), 5.727-5.705 (m, 1H), 5.520-5.495 (m, 1H), 3.771 (s, 3H), 3.756 (s, 3H), 3.528-3.513 (br m, 2H), 3.118-3.093 (m, 2H), 2.288-2.276 (br m, 2H); EIMS *m/z* (relative intensity) 205 (M^+ + 1, 14), 204 (M^+, 100), 189 (47), 174 (19), 129 (20), 115 (28), 91 (31).

Anal. Calcd for $C_{13}H_{18}O_2$: C, 76.44; H, 7.90. Found: C, 76.37; H, 8.00.

1,4-Dimethoxy-8,9-dihydro-5*H*-benzocycloheptene (36). Rhodium trichloride trihydrate (10 mg) was added to a solution of diene **35** (1.00 g, 4.90 mmol) in $CHCl_3$-EtOH (1:1, 30 mL) and the reaction mixture was heated at reflux for 8 h. After the mixture cooled, it was diluted with water (75 mL), extracted with CH_2Cl_2 (3 x 15 mL), and worked up. Purification by PCTLC (EtOAc-hexane 1:4) afforded **36** as a clear yellow liquid in quantitative yield. UV (CH_3CN), λ_{max} (ε) 209 (22 550), 255 (8 310), 312 (3 980) nm; IR (neat) 3020, 2920, 2820, 1590, 1470, 1250, 1210, 1080, 780, 710 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 6.768 (app. t, J = 1.4 Hz, 1H), 6.735 (d, J = 9.0 Hz, 1H), 6.665 (d, J = 9.0 Hz, 1H), 6.125-6.080 (m, 1H), 3.793 (s, 3H), 3.780 (s, 3H), 2.793-2.769 (m, 2H), 2.262-2.220 (m, 2H), 2.033-1.981 (m, 2H); EIMS *m/z* (relative intensity) 205 (M^+ + 1, 16), 204 (M^+, 100), 189 (53), 174 (21), 129 (26), 115 (33), 91 (34).

Anal. Calcd for $C_{13}H_{18}O_2$: C, 76.44; H, 7.90. Found: C, 76.32; H, 8.18.

1,4-Dimethoxy-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-6-ol (37). Alkene **36** (514.7 mg, 2.52 mmol) was dissolved in dry CH_2Cl_2 (20 mL), and the resulting solution was cooled to 0 °C. A solution of freshly prepared dimethyldioxirane in acetone (1.1 eq, *ca* 0.1*M*) was added dropwise. The reaction mixture was stirred at this temperature for 1 h. Then, the solution was dried over Na_2SO_4 and concentrated under reduced pressure to afford a light yellow solid. The crude product was dissolved in anhydrous THF (20 mL) and added to a cold (0 °C) suspension of LAH in THF (1*M* solution, 2.77 mL, 2.77 mmol) under Ar. The mixture was stirred at this temperature for 15 min and then allowed to warm to room temperature with continued stirring for 5 h. The mixture was cooled to 0 °C and quenched

with a sat. Na^+/K^+ tartrate solution. When the solids turned completely white, the mixture was filtered through a silica gel plug, washed with ether (3 x 15 mL), and the filtrate was worked up. Purification by MPLC (EtOAc-hexanes 1:4) afforded **37** as a white solid (499.7 mg, 89% overall). Analytical sample was obtained by recrystallization from CH_2Cl_2-hexanes: mp 100-101 °C; UV (CH_3CN), λ_{max} (ε) 204 (28 300), 226 (7 375, sh), 289 (3 165) nm; IR ($CHCl_3$) 3600 (sharp), 3000, 2920, 1595, 1480, 1245, 1215, 1090, 795 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 6.693 (d, J = 8.9 Hz, 1H), 6.666 (d, J = 8.9 Hz, 1H), 3.750 (s, 3H), 3.735 (s,3H), 3.270 (d, J = 13.8 Hz, 1H), 3.043 (dd, J = 14.0, 8.5 Hz, 1H), 2.950 (dd, J = 13.6, 9.4 Hz, 1H), 2.604 (dd, J = 13.8, 10.7 Hz, 1H), 2.126-2.039 (br m, 1H), 1.835-1.763 (m, 2H), 1.487 (br s, 1H, D_2O exch.), 1.448-1.397 (m, 2H); EIMS *m/z* (relative intensity) 223 (M^+ + 1, 17), 222 (M^+, 100), 204 (9), 189 (29), 178 (31), 151 (37), 121 (34), 105 (34), 91 (52).

Anal. Calcd for $C_{13}H_{18}O_3$: C, 70.24; H, 8.16. Found: C, 70.00; H, 8.10.

1,4-Dimethoxy-5,7,8,9-tetrahydro-6*H*-benzocyclohepten-6-one (38). To a suspension of PCC (252.1 mg, 1.17 mmol) in CH_2Cl_2 (20 mL) was added dropwise a solution of **37** in CH_2Cl_2 (5 mL) at room temperature. The mixture was stirred at this temperature for 1.5 h. Then, it was filtered through a silica gel plug and the gummy residue was washed thoroughly with CH_2Cl_2 (2 x 15 mL) and EtOAc (3 x 15 mL). Evaporation afforded a pale yellow solid which was purified by PCTLC (EtOAc-hexanes 1:2) to afford **38** as a white solid (114.0 mg, 89%); mp 87-87.5 °C; UV (CH_3CN), λ_{max} (ε) 206 (25 010), 292 (3 500) nm; IR ($CHCl_3$) 3000, 2930, 1700, 1480, 1200, 1160, 1090 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 6.760 (d, J

= 9.0 Hz, 1H), 6.711 (d, J = 8.9 Hz, 1H), 3.773 (s, 2H), 3.766 (s, 3H), 3.756 (s, 3H), 2.927 (t, J = 6.7 Hz, 2H), 2.467 (t, J = 7.0 Hz, 2H), 1.932 (quintet, J = 7.0 Hz, 2H); EIMS *m/z* (relative intensity) 221 (M^+ + 1, 11), 220 (M^+, 100), 191 (14), 177 (34), 165 (76), 149 (35), 105 (21), 91 (30).

Anal. Calcd for $C_{13}H_{16}O_3$: C, 70.89; H, 7.32. Found: C, 70.82; H, 7.29.

5,8-Dimethoxy-1,2-dihydronaphthalene (40). Rhodium trichloride trihydrate (100 mg) was added to a solution of **39** (21.90 g, 0.115 mol) in $CHCl_3$-EtOH (1:1, 200 mL) and the mixture was refluxed overnight. During this time, the mixture turned black. Purification by column chromatography (EtOAc-hexanes 1:4) after usual work up afforded **40** as a white solid (20.63 g, 94%): mp 62-62.5 °C; UV (CH_3CN), λ_{max} (ϵ) 211 (19 400), 214 (19 225), 268 (7 400), 283 (3 920, sh), 317 (4 165) nm; IR ($CHCl_3$) 3010, 2940, 1595, 1480, 1345, 1255, 1100, 950 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 6.793 (dt, J = 10.0, 2.0 Hz, 1H), 6.661 (q, J = 9.0 Hz, 2H), 6.048-6.010 (m, 1H), 3.769 (s, 6H), 2.743 (t, J = 8.6 Hz, 2H), 2.260-2.214 (br m, 2H); EIMS *m/z* (relative intensity) 191 (M^+ + 1, 14), 190 (M^+, 100), 175 (61), 159 (34), 144 (20), 131 (14), 115 (74), 91 (19).

Anal. Calcd for $C_{12}H_{14}O_2$: C, 75.76; H, 7.42. Found: C, 75.48; H, 7.60.

5,8-Dimethoxy-1,2,3,4-tetrahydronaphthalen-1-ol (41). To a cold solution (ice bath temperature) of **40** (10.92 g, 57.4 mmol) in THF (75 mL) was added slowly *via* syringe 57.4 mL of borane-THF complex (1*M* solution). The reaction mixture was stirred at this temperature for 15 min and at room temperature for 36 h. After this time, the mixture was cooled down (ice bath temperature) and water (60 mL) was added dropwise, followed by a solution of 3*M* NaOH (48 mL) and 30% H_2O_2 (24 mL). The ice bath was

removed and the mixture was stirred at room temperature for 1 h. Then, the mixture was diluted with brine and extracted with ether (3 x 50 mL). The combined organic extracts were worked up. Purification by MPLC (EtOAc-hexanes 1:4) afforded **41** as a white solid (9.24 g, 77%): mp 62-63 °C (lit.[139] 65 °C). Spectroscopic data agree with reported values.[139]

5,8-Dimethoxy-1-tetralone (42). To a suspension of PCC (8.70 g, 40.4 mmol) in CH_2Cl_2 (25 mL) was added dropwise a solution of **41** (4.20 g, 20.2 mmol) in CH_2Cl_2 (10 mL). The mixture was stirred at room temperature for 1 h, then diluted with CH_2Cl_2 (15 mL), filtered through a silica gel plug, and concentrated under reduced pressure. Purification by MPLC (EtOAc-hexanes 1:2) afforded **42** as a yellowish white solid (3.60 g, 87%): mp 59.5-60 °C (lit.[139] 60-62 °C; lit.[166] 58-62 °C). Spectroscopic data agree with reported values.[139, 166]

1,4-Dimethoxy-5,7,8,9-tetrahydro-6*H*-benzocyclohepten-6-one (38). A slurry of 50% NaH (609.5 mg, 12.7 mmol) in dry DMSO (40 mL) was stirred at room temperature for 1 h or until the NaH was completely dissolved. Methyltriphenylphosphonium bromide (4.52 g, 12.7 mmol) was added in portions over a period of 5 min and the mixture was stirred for an hour. After this time, ketone **42** (1.31 g, 6.33 mmol) was added in one portion. After 2 h of stirring, the mixture was poured into ice-water (100 mL). The aqueous phase was extracted with ether (3 x 50 mL) and the combined organic extracts were worked up to afford a yellow solid. The crude product was dissolved in MeOH (25 mL) and added in one portion to a freshly prepared solution of thallium (III) nitrate trihydrate (4.74 g, 10.7 mmol) in MeOH (30 mL). The mixture was stirred vigorously for 1 min and $CHCl_3$ (100

mL) was added. The mixture was filtered, washed with brine, and worked up. Purification by MPLC (EtOAc-hexanes 1:2) afforded **38** as a white solid (1.043 g, 75% overall). Spectroscopic data agree with those of the compound obtained by the keto transposition.

1,4-Dimethoxy-6-(2-trimethylsilyl)ethynyl-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-6-ol (43). Anhydrous $CeCl_3$ (942.1 mg, 3.82 mmol) was dried *in vacuo* at 140 °C for 1 h with stirring. After cooling to room temperature, it was suspended in THF (10 mL) and stirred overnight at this temperature under Ar. A solution of (2-trimethyl-silyl)ethynyl lithium, prepared by adding n-BuLi (1.56*M* in hexanes, 2.1 mL, 3.28 mmol) to a solution of trimethylsilylacetylene (398.6 mg, 4.06 mmol) in THF (10 mL) at -78 °C, was added to the suspension of $CeCl_3$ at -78 °C. The white mixture was stirred at this temperature for 1 h. Ketone **38** (400.0 mg, 1.82 mmol) was added in one portion and the reaction mixture was stirred at -78 °C for 1 h and at room temperature for 2 h. Then the reaction mixture was quenched with sat. NH_4Cl (30 mL), diluted with ether (50 mL) and the organic layer was washed with brine (2 x 25 mL), and worked-up. Purification by column chromatography (EtOAc-hexanes 1:2) afforded **43** in quantitative yield (580 mg): mp 66-67 °C, UV (EtOH), λ_{max} (ε) 204 (30 605), 226 (7 125, sh), 291 (3 140) nm; IR ($CHCl_3$) 3580 (sharp), 3000, 2930, 2820, 2160, 1590, 1480, 1250, 1100, 860, 840 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 6.703 (d, J = 8.9 Hz, 1H), 6.654 (d, J = 8.9 Hz, 1H), 3.761 (s, 3H), 3.732 (s, 3H), 3.426 (d, J = 13.9 Hz, 1H), 3.135 (d, J = 13.4 Hz, 1H), 3.064 (br t, J = 12 Hz, 1H), 2.576 (br t, J = 11.5 Hz, 1H), 2.152-2.110 (m, 1H), 2.044 (s, 1H, D_2O exch.), 2.205-1.961 (m, 1H), 1.774 (br s, 1H), 1.553 (br s, 1H); EIMS *m/z* (relative

intensity) 319 (M^+ + 1, 15), 318 (M^+, 66), 301 (10), 178 (100), 165 (32), 105 (15), 91 (24).

Anal. Calcd for $C_{18}H_{26}O_3Si$: C, 67.88; H, 8.23. Found: C, 67.49; H, 8.60.

6-Acetyl-1,4-dimethoxy-6,7,8,9-tetrahydro-5*H*-benzocyclo-hepten-6-ol (44). A solution of **43** (1.31 g, 4.11 mmol) in THF (20 mL) was added dropwise to a solution of yellow HgO (240.4 mg, 1.11 mmol) in 3*M* H_2SO_4 (12 mL). The mixture was stirred at room temperature in the dark under Ar for 2h. Then, the mixture was diluted with H_2O (50 mL), extracted with CH_2Cl_2 (3 x 75 mL), and the combined organic layers were washed with brine (30 mL) and worked-up. Purification by MPLC (EtOAc-hexanes 1:2) afforded **44** as a yellowish white solid (942.9 mg, 87%): mp 91.5-92 °C; UV (EtOH), λ_{max} (ε) 204 (26 355), 226 (7 280, sh), 291 (3 185) nm; IR ($CHCl_3$) 3580 (sharp), 3000, 2940, 2830, 1700, 1590 (vw), 1480, 1255, 1100, 900 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 6.737 (d, J = 8.9 Hz, 1H), 6.691 (d, J = 8.9 Hz, 1H), 3.752 (s, 3H), 3.724 (s, 3H), 3.337 (ddd, J = 14.4, 7.8, 2.6 Hz, 1H), 3.294 (dd, J = 14.2, 1.2 Hz, 1H), 3.104 (s, 1H, D_2O exch.), 2.968 (d, J = 14.2 Hz, 1H), 2.531 (ddd, J = 14.2, 9.8, 2.6 Hz, 1H), 1.977-1.915 (m, 1H), 1.848-1.810 (m, 1H), 1.680-1.617 (m, 2H); EIMS *m/z* (relative intensity) 265 (M^+ + 1, 7), 264 (M^+, 38), 222 (13), 221 (60), 204 (15), 203 (98), 191 (32), 177 (25), 161 (33), 121 (23), 91 (36), 77 (25), 44 (100).

Anal. Calcd for $C_{15}H_{20}O_4$: C, 68.16; H, 7.63. Found: C, 68.00; H, 8.00.

4-Demethoxy-7-deoxy-8a-homodaunomycinone (45). An intimate mixture of **44** (2.10 g, 7.94 mmol), phthalic anhydride (3.53 g, 23.8 mmol), $AlCl_3$ (23.31 g, 174.8 mmol), and NaCl (5.11 g, 87.4 mmol) was placed in a beaker and immersed in a sand bath pre-heated at 180-182 °C.

The mixture became a dark red melt in approximately 1 min and it was stirred continuously with a glass rod for another 6 min. After the reaction mixture was allowed to cool, a sat. aq. solution of oxalic acid (150 mL) was added and the red aq. solution was poured into an Erlenmeyer. $CHCl_3$ (250 mL) was added and the resulting mixture was stirred until the aqueous layer became pale yellow. The organic layer was separated and filtered through a celite plug. TLC (EtOAc-hexanes 1:2) indicated the presence of the product and baseline impurities. Concentration and recrystallization from $CHCl_3$ gave **45** as a red-orange solid (2.47 g, 85%): mp 223-224 °C; UV (EtOH), λ_{max} (ε) 204 (25 310), 228 (14 005, sh), 253 (40 255), 257 (40 835), 292 (8 070) nm; IR ($CHCl_3$) 3590 (sharp), 2930, 2850, 2360, 1700, 1620, 1585, 1455, 1400, 1370, 1340, 1260, 970 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 13.706 (s, 1H, D_2O exch.), 13.465 (s, 1H, D_2O exch.), 8.345-8.308 (m, 2H), 7.819-7.801 (m, 2H), 4.147 (br s, 1H, D_2O exch.), 3.629 (app. dd, J = 14.2, 6.1 Hz, 1H), 3.533 (dd, J = 14.1, 1.4 Hz, 1H), 3.032 (d, J = 14.0 Hz, 1H), 2.619-2.563 (m, 2H), 2.364 (s, 3H), 2.103-2.041 (m, 1H), 1.780-1.732 (m, 1H); EIMS *m/z* (relative intensity) 366 (M^+, 10), 323 (17), 305 (2), 268 (13), 167 (5), 149 (59), 91 (16), 43 (100).

Anal. Calcd for $C_{21}H_{18}O_6$: C, 68.84; H, 4.95. Found: C, 68.68; H, 5.01.

4-Demethoxy-7-deoxy-6,12-diacetyl-8a-homodaunomycinone ketal (46). A mixture of ketone **45** (105.0 mg, 0.287 mmol), ethyleneglycol (355.8 mg, 5.73 mmol), and *p*TsOH (10 mg) in dry benzene (25 mL) was heated to reflux under Ar for 12 h with azeotropic removal of H_2O (Dean-Stark apparatus). The reaction mixture was allowed to cool and the benzene was decanted. The red solid was dissolved in pyridine (8 mL) and

acetic anhydride (1.05 g, 10.3 mmol) and DMAP (2-3 mg) were added to the solution. The mixture was stirred for 19 h at room temperature under Ar. Then the solution was concentrated under high vacuum and the brownish-yellow solid obtained was purified by MPLC (EtOAc-hexanes 1:2) to afford **46** as a yellow solid (127.9 mg, 90% overall): mp 252 °C dec.; UV (EtOH), λ_{max} (ε) 209 (27 920), 238 (14 715, sh), 260 (43 700), 281 (11 460, sh), 346 (6 015) nm; IR ($CHCl_3$) 3580 (sharp), 2990, 1770, 1670, 1590, 1570, 1450, 1360, 1330, 1280, 1250, 1180, 1100, 970, 950 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 8.134-8.109 (m, 2H), 7.710-7.690 (m, 2H), 4.030 (s, 4H), 3.575 (d, J = 14.0 Hz, 1H), 3.223 (dd, J = 14.1, 6.6 Hz, 1H), 2.810 (d, J = 14.2 Hz, 1H), 2.556 (s, 3H), 2.513 (s, 3H), 2.290 (dt, J = 5.8, 1.5 Hz, 1H), 2.166-2.114 (br m, 1H), 1.885-1.822 (br m, 1H), 1.754 (app. t, J = 8.4 Hz, 1H), 1.357 (s, 3H); CIMS (NH_3) m/z (relative intensity) 512 (M^+ + 18, 30), 495 (M^+ + 1, 4), 453 (3), 435 (100), 99 (58), 87 (22), 43 (16).

Anal. Calcd for $C_{27}H_{26}O_9 \cdot 1/2H_2O$: C, 64.40; H, 5.41. Found: C, 64.20; H, 5.40.

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Part II

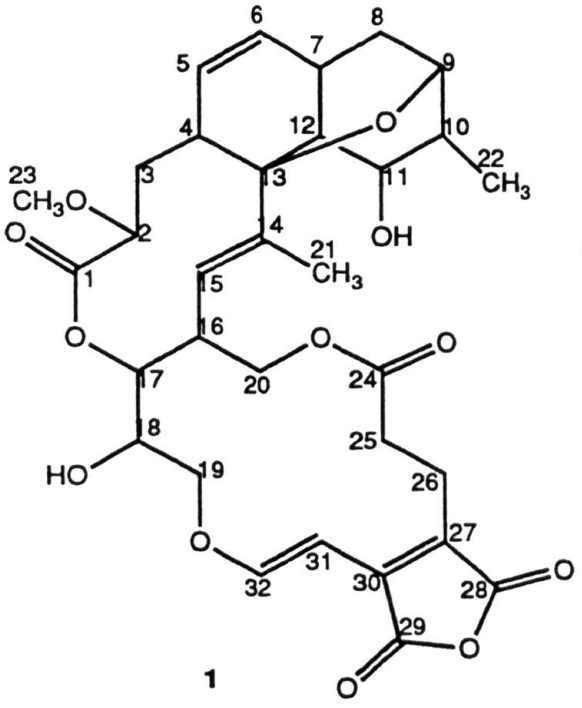
Biosynthesis of Coloradocin. Origin of the Carbons

A. Introduction

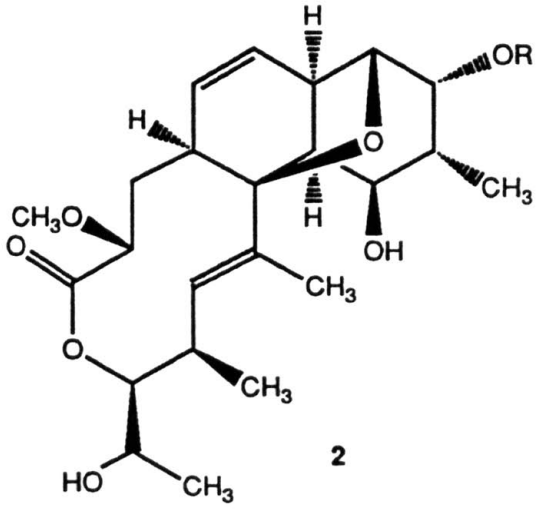
The macrolide antibiotic coloradocin (luminamicin, **1**) is an antianaerobic antibiotic which was first reported by Omura and co-workers[1] in 1985 by isolation from a fermentation broth of soil microorganisms belonging to the genus *Nocardiodes*. In 1987, Jackson and co-workers[2] reported finding it in a new species of *Actinoplanes*, designated *Actinoplanes coloradoensis*.

Coloradocin has good to moderate activity against a broad range of anaerobic organisms, such as *Clostridium* sp. and *Bacteroides* sp. It is most potent against microaerophilic bacteria, such as *Neisseria gonorrhoeae* and *Haemophilus influenzae*, including ampicillin-resistant strains. Coloradocin does not inhibit most aerobic microorganisms.

The stereochemical details of the structure of coloradocin have not been elucidated. The carbon framework, however, has been established[3] and reveals an unusual octahydronaphthalene moiety apparently related to a small family of antibiotics comprising the nargenicins[4] and nodusmicins (**2**).[5] Distinctive structural features of coloradocin, including a) the different placement of the oxygen bridge across the octalin ring system in comparison with nargenicin and nodusmicin; b) the presence of the maleic anhydride moiety, quite rare in natural products; and c) the non-apparent biosynthetic origin of the second carbon chain forming the 14-membered oxamacrolide ring, prompted us to pursue a biosynthetic study of this antibiotic.

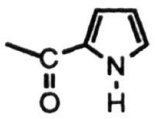


1



2

R = H Nodusmicin

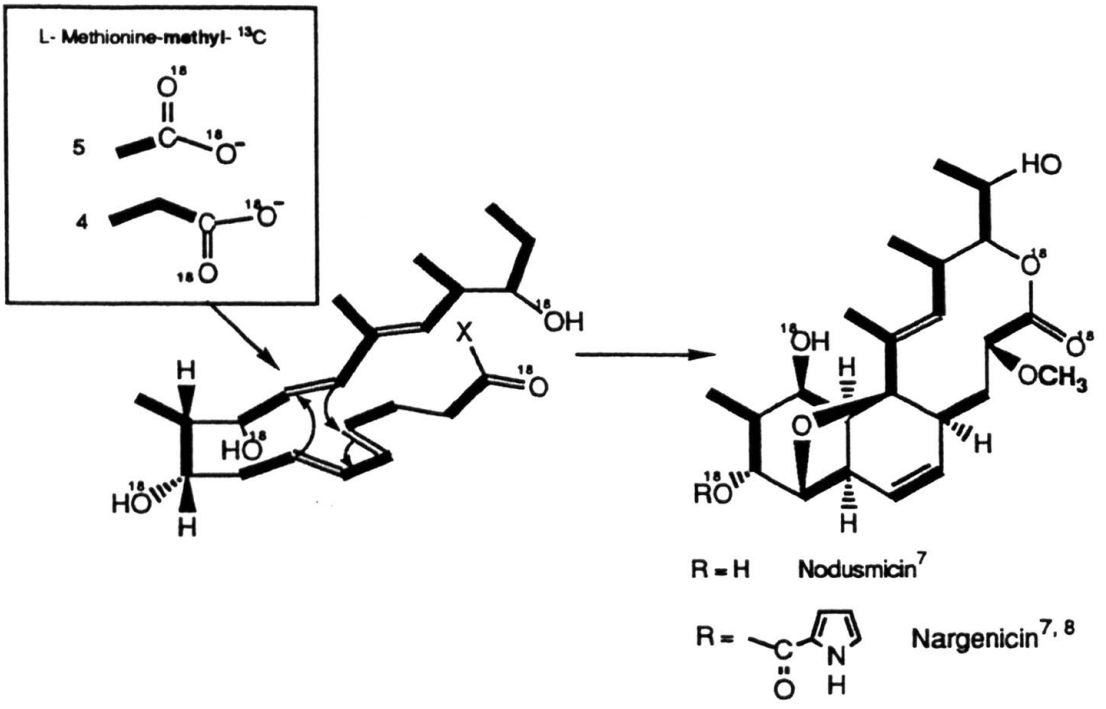
R =  Nargenicin

B. Biosynthetic Origin of the Carbon Skeleton of Nargenicin and Nodusmicin

The macrolide ring of nargenicin and nodusmicin is derived from the typical *Actinomycete* acetate-propionate pathway,[6, 7] which incorporates four propionate and five acetate units. This pathway involves an intramolecular Diels-Alder reaction at a late stage of the biosynthesis[8, 9] (Scheme 1). Chlorothricin,[10-12] kijanimicin,[13] and tetrocarcin[14] are other examples of natural products that exhibit an intramolecular Diels-Alder reaction in their biosyntheses, a process necessary for the construction of the octalin ring system. This Diels-Alder reaction is believed to be a nonenzymic-catalyzed process. It was also found that the *S*-methyl of methionine labeled the

O-methyl carbon of these antibiotics. Furthermore, molecular oxygen was found to be the biosynthetic source of the 2, 18 and 8-13 bridge oxygens.[8]

Biosynthetic Origin of Nargenicin and Nodusmicin



Scheme 1

C. NMR Studies of Coloradocin

Prior to the biosynthetic studies of coloradocin, structure proof and essential elements of the structure were confirmed using ^{1}H-, ^{13}C-, and 2D NMR techniques. Structure proof was relevant and mandatory for the correct interpretation of the results. If the structure proposed by McAlpine and co-workers[3] had been wrong, this would have led us, consequently, to a wrong biosynthetic derivation. Furthermore, the results from the feeding

experiments would have been inconsistent with the proposed derivation and lack any logical significance.

From a structural point of view, we were concerned about the point of attachment of the minor carbon chain to the major chain, that is, whether or not the molecule is tethered as proposed by McAlpine and co-workers. We were also concerned about the possibility of solvent induced chemical shift (SICS) when the NMR solvent was changed from DMSO-d_6 (used by McAlpine) to CD_2Cl_2 (used in our NMR studies); therefore, all ^{13}C-signals were re-assigned using CD_2Cl_2 as solvent.

Figures 1 and 2 show ^1H- (300 MHz) and ^{13}C NMRs (125 MHz) of coloradocin. In these spectra, most signals are well resolved and separated, facilitating proper assignment and correlation, with the exception of C17 and C19, C4 and C12, and C3 and C25.

Simple inspection of the ^1H NMR indicated the presence of a polarized double bond (protons at 7.86 and 5.65 ppm), and other protons on unsaturated centers as well (5.44 to 6.06 ppm). Resonances from 3.27 to 4.90 ppm indicated the presence of several protons on carbons bearing oxygen. The resonance at 3.27 ppm was indicative of a methoxy group. Protons on saturated carbons resonated from 0.99 to 2.87 ppm. Two methyl groups were found in this area: one at 0.99 ppm and the other at 1.75.

A ^1H-^{13}C correlation spectrum (500 MHz) in CD_2Cl_2 (Figure 3) helped us with the assignment of each proton to its carbon. The assignment was facilitated by running a DEPT ^{13}C NMR experiment (result not shown) which revealed the carbon multiplicities.

This correlation experiment also helped us to solve the problem related to SICS. All ^{13}C NMR assignments were identical to those obtained in DMSO-d_6, with the exception of C11 and C13 that were reversed: DMSO-d_6: C11, 75.3 ppm; C13, 75.9 ppm. In CD_2Cl_2: C11, 77.7 ppm; C13, 76.8 ppm.

The results from a long-range ^1H-^{13}C correlation NMR (FUCOP, Figure 4) in conjunction with those of the ^1H-^{13}C correlation NMR spectrum provided enough evidence to build a partial structure for coloradocin. The complete structure of this molecule was obtained after examination of the IR spectrum (not shown). The spectrum indicated the presence of two bands at 1835 cm^{-1} (weak) and 1760 cm^{-1} (strong), due to symmetrical and antisymmetrical stretching frequencies, respectively, of a substituted maleic anhydride functionality.

The FUCOP experiment showed long-range correlation between C29 and H31 and also between C28 and H26. These findings allowed us to formulate a structure in which the olefinic anhydride carbons were connected to carbons 26 and 31.

The attachment of the minor carbon chain to the major one was indicated by a long-range correlation between H32 and C19. Unfortunately, no long-range correlation was seen between H22 and C24. Examining regions deeper in the contour plot increased the noise level such that it was difficult to differentiate signal from noise.

A ^1H-^1H correlation (90-90 COSY) spectrum was also run to verify proton connectivity. ^1H-^1H Connectivities are shown on the spectrum (Figure 5). No discrepancies in the signals were found from those obtained by McAlpine and co-workers.

^{1}H NMR Spectrum of Coloradocin (300 MHz)

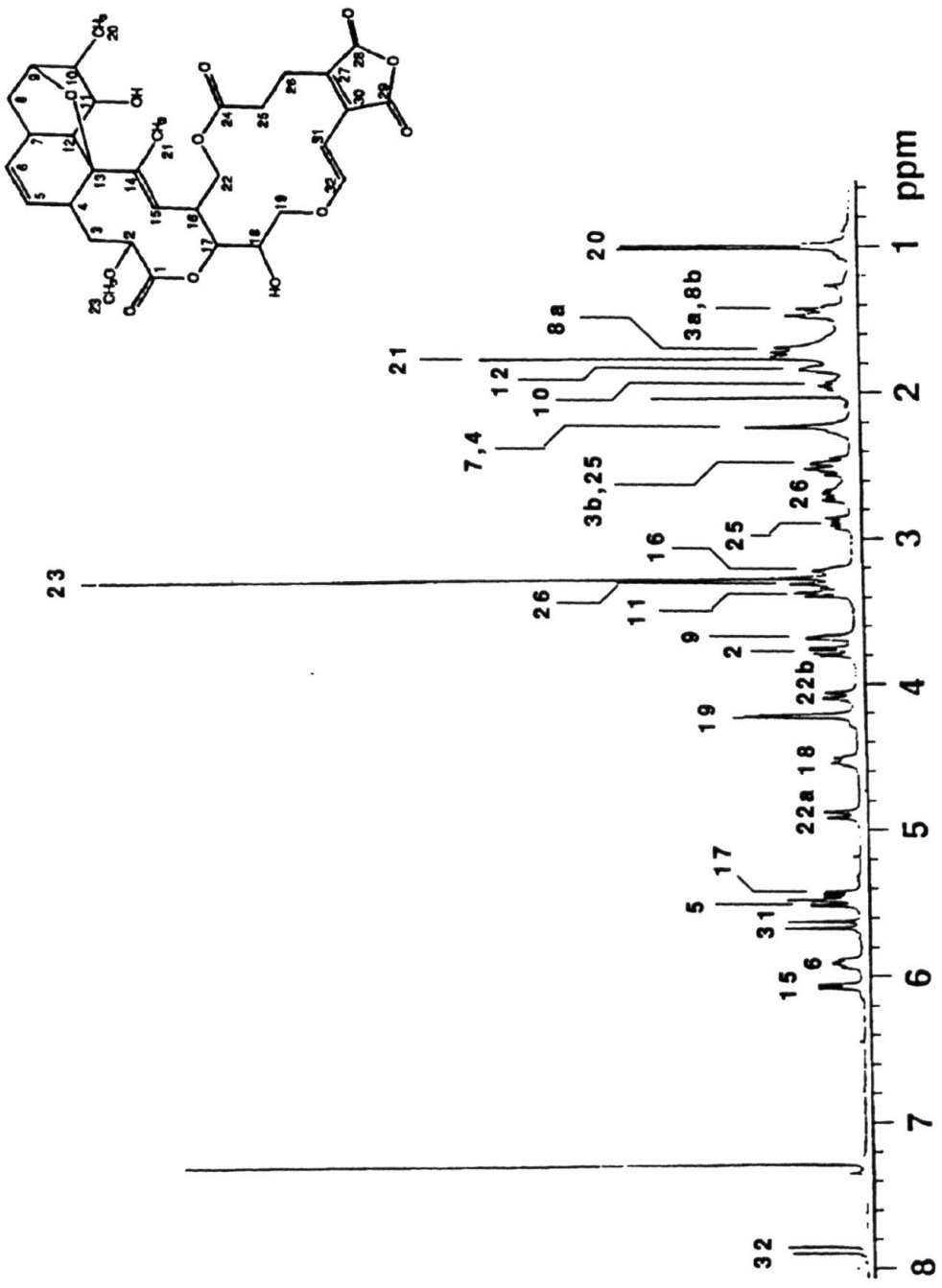


Figure 1

^{13}C NMR Spectrum of Coloradocin (125 MHz)

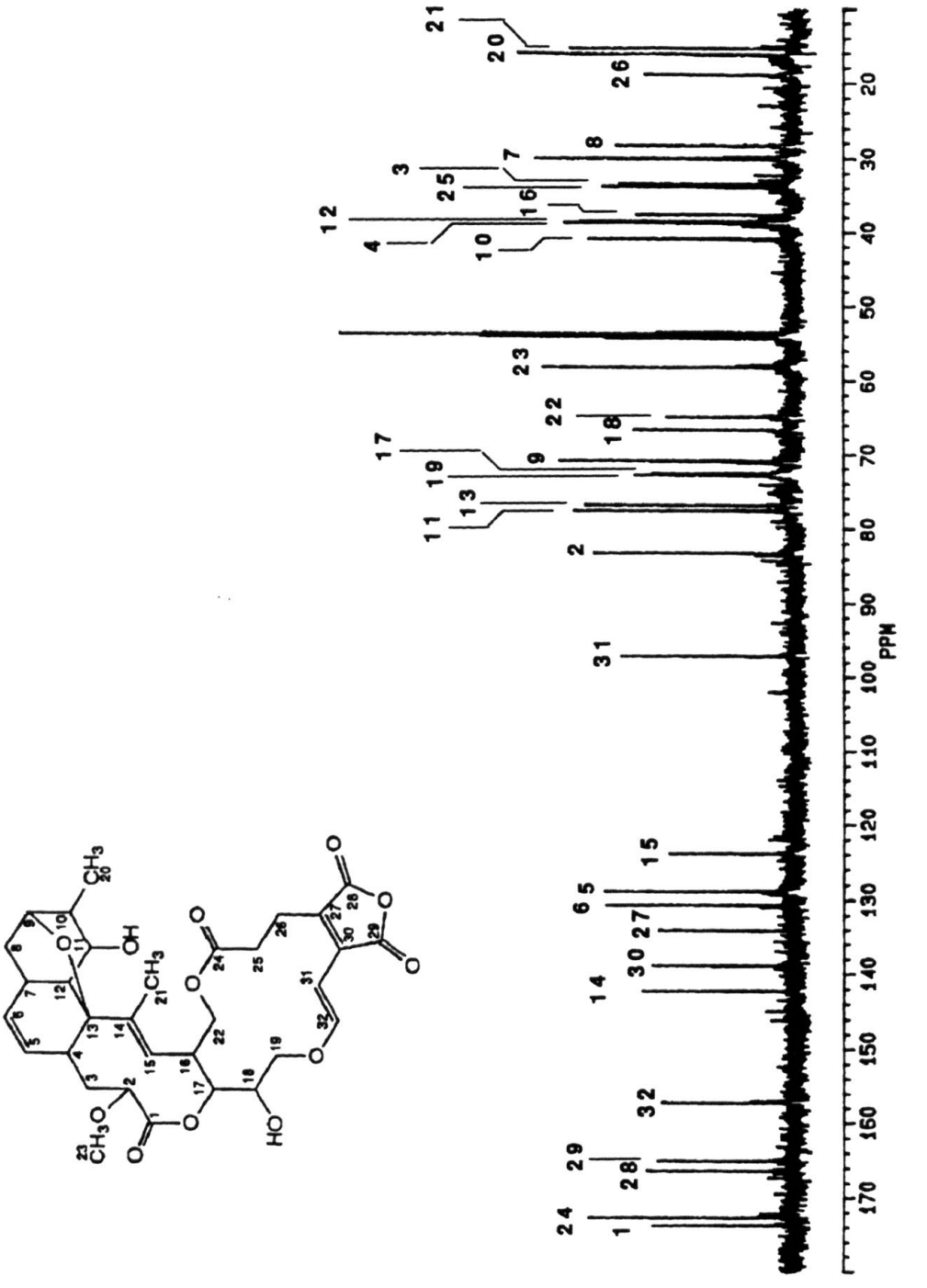


Figure 2

^{1}H-^{13}C Correlation Spectrum of Coloradocin

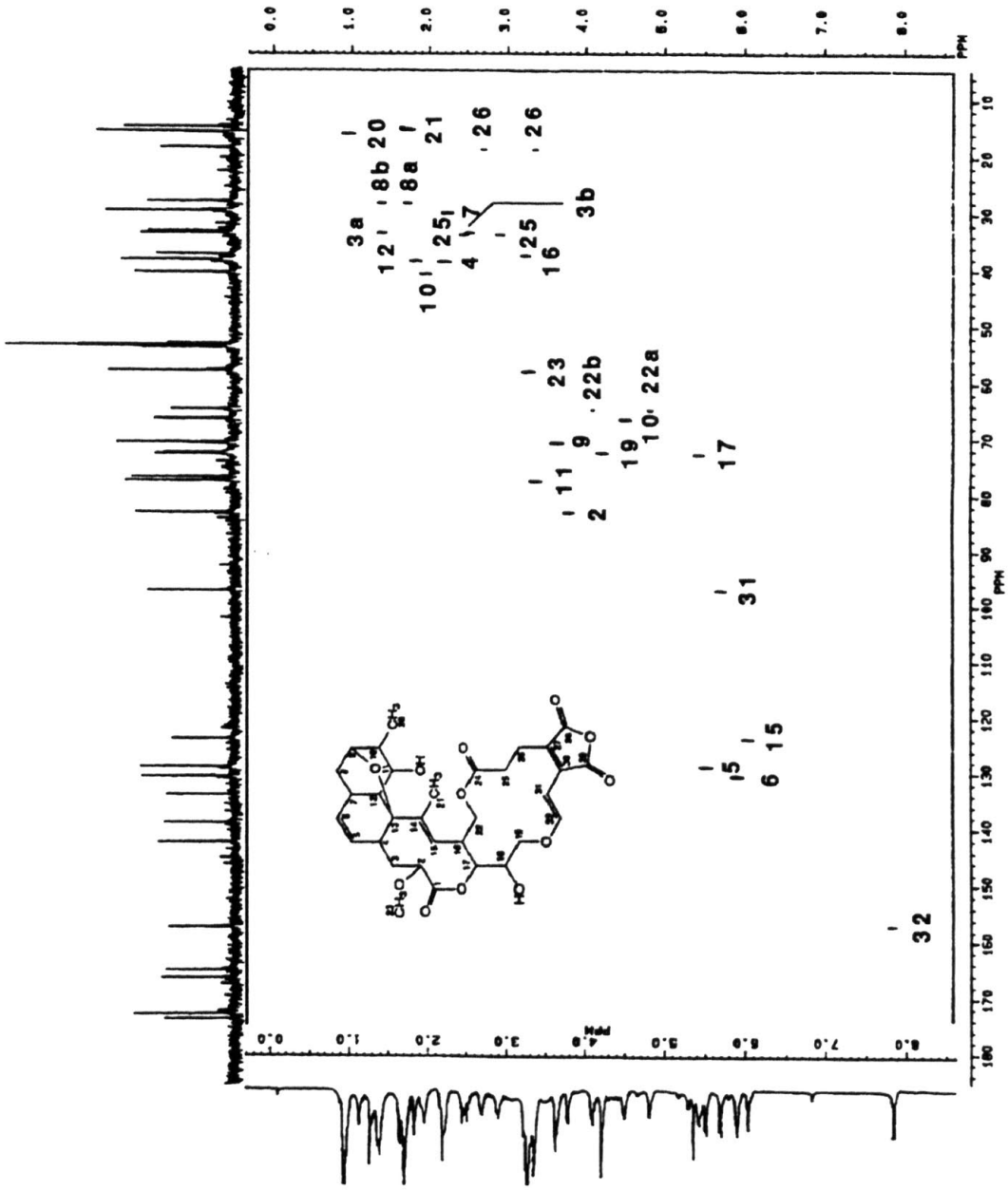


Figure 3

Long-Range ^{1}H-^{13}C Correlation Spectrum of Coloradocin

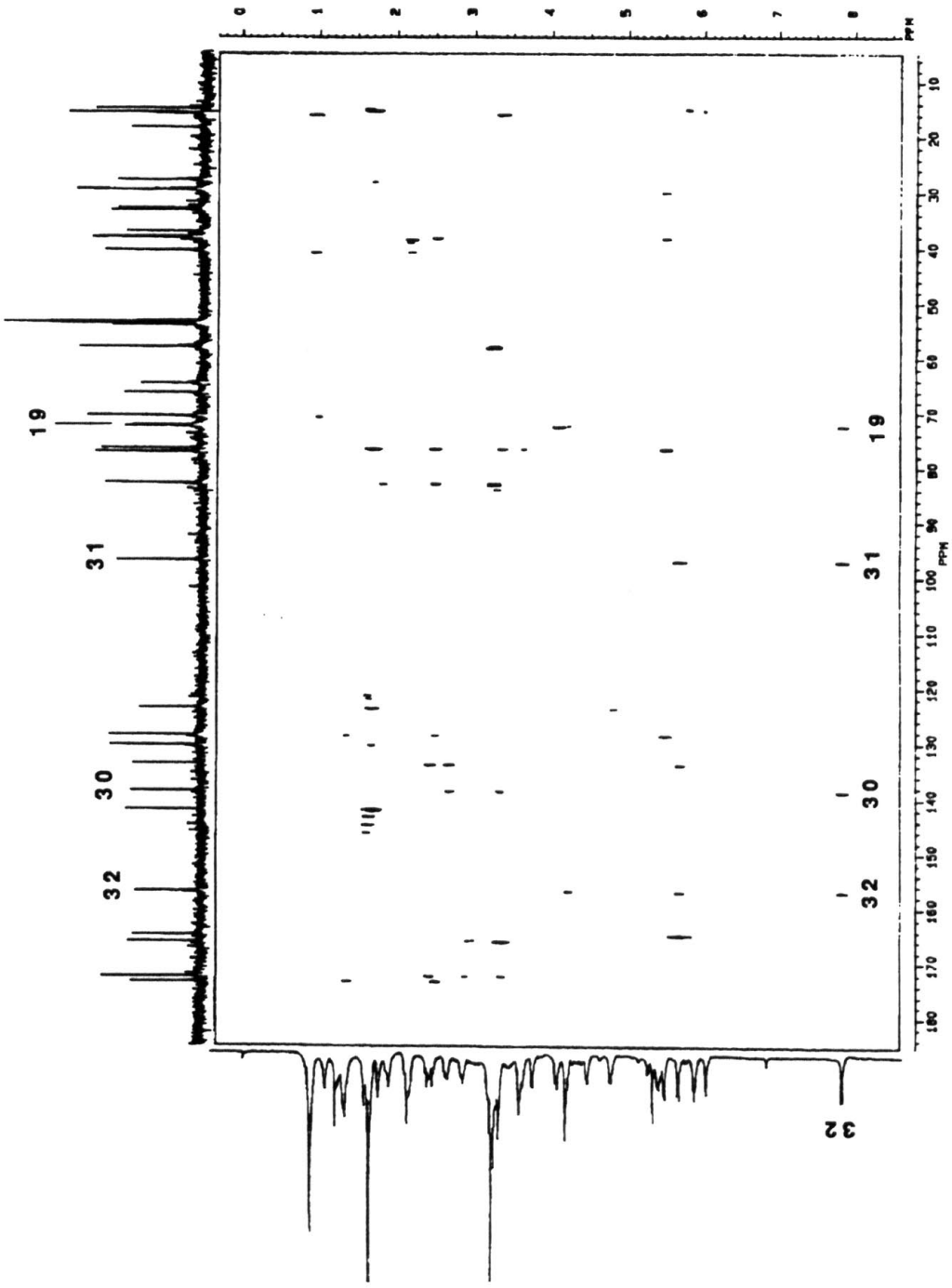


Figure 4

^{1}H-^{1}H Correlation Spectrum of Coloradocin

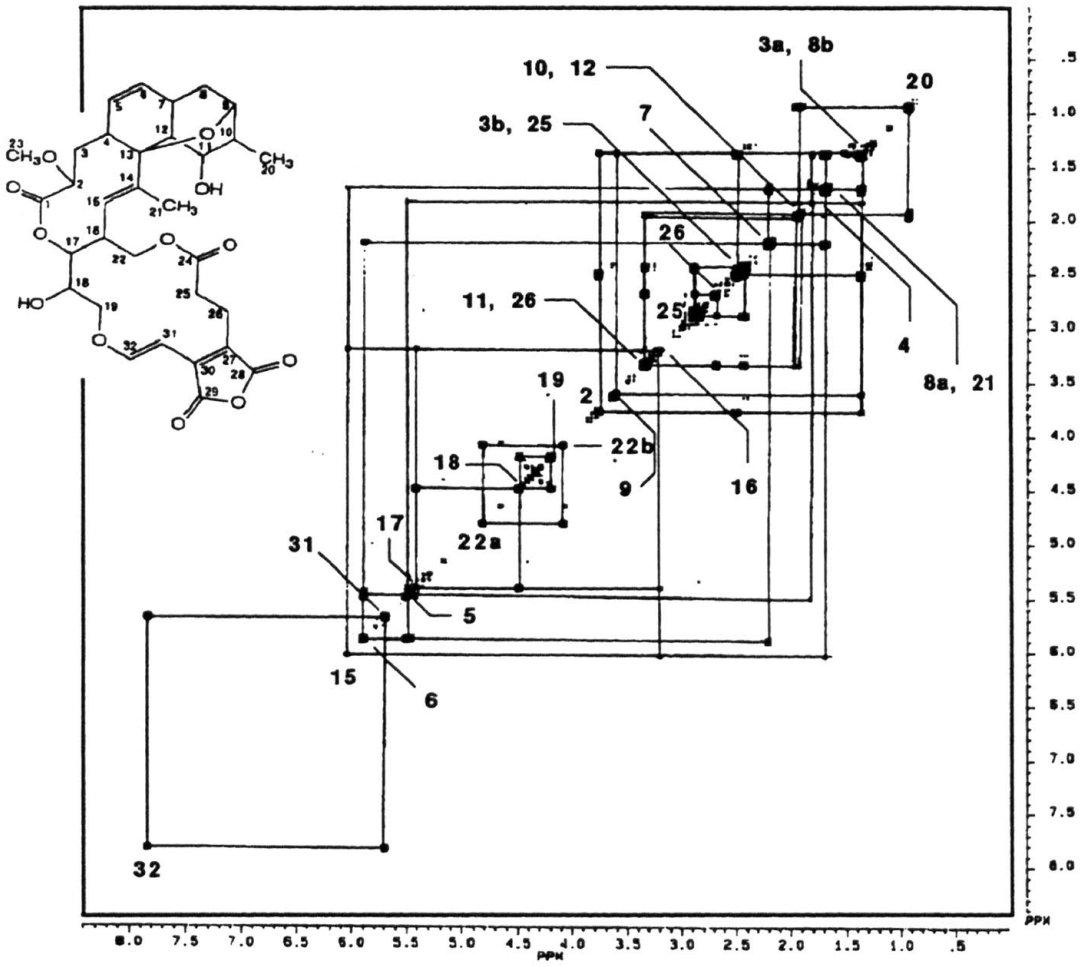


Figure 5

2D INADEQUATE NMR Spectrum of Double Labeled Coloradocin

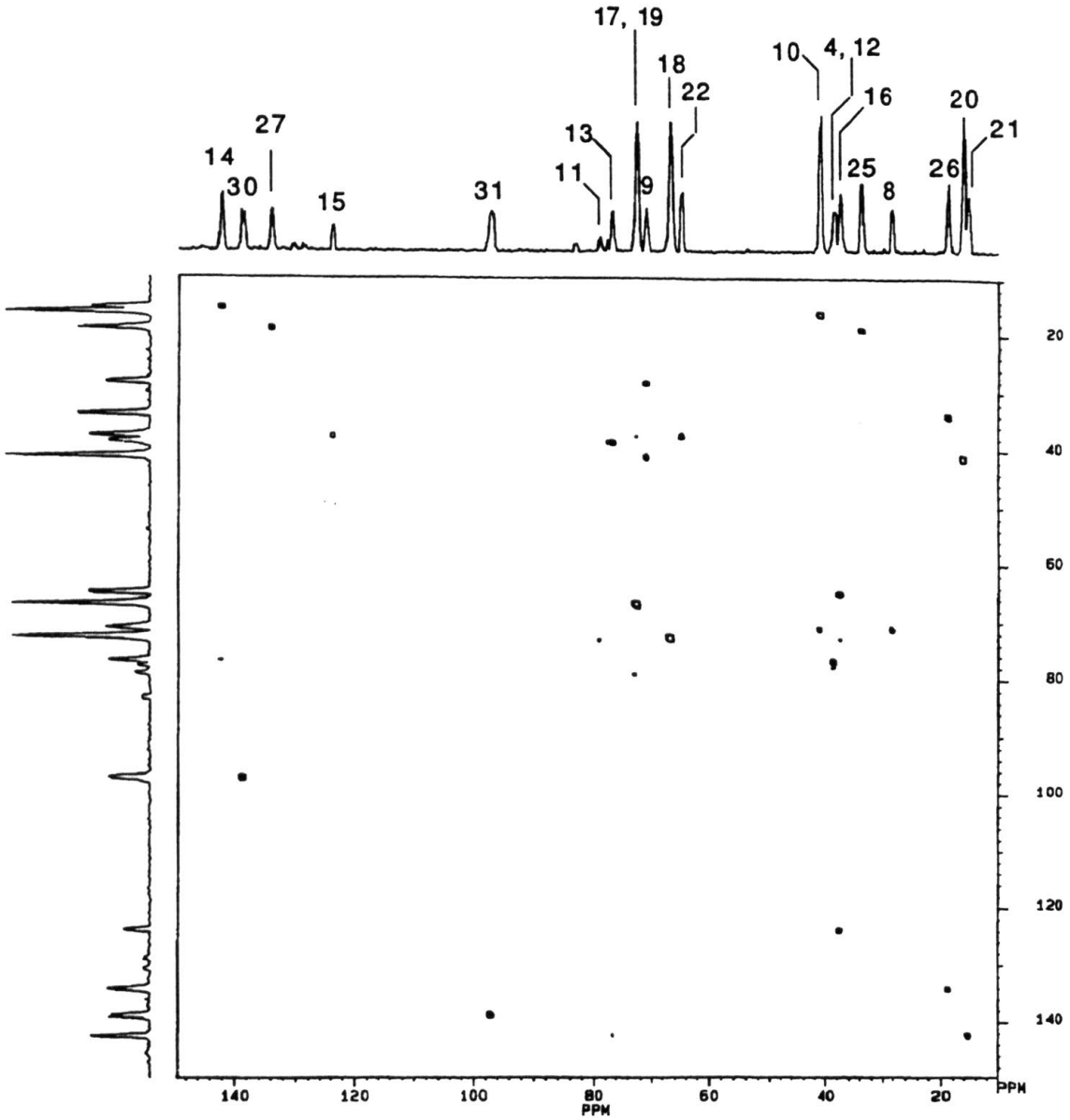


Figure 6

Once we confirmed the structure proposed, we proceeded with the feeding experiments using ^{13}C-labeled precursors.

Further evidence of the carbon skeleton connectivity was obtained by running a 2D INADEQUATE experiment of coloradocin obtained from the [2-^{13}C]-acetate labeling experiment (Figure 6). See details in Results and Discussion section.

D. Fermentation of *Actinoplanes coloradoensis* and Incorporation of ^{13}C-Labeled Precursors

Fermentation experiments and incorporation of labeled precursors were done at Abbott Laboratories according to Dr. Jackson's procedure described in reference 2 (see Experimental Section for details). Because of the slow growth of the microorganism, a complex medium was necessary to assure full development and antibiotic production. The medium contained energy sources, such as glucose for rapid growth, and starch to sustain the growth of the microorganism. Amino acids were added in the form of partially digested peptides (peptone) and amino acid mixtures (NZ amine). Yeast extract provided the co-factors necessary for the biochemical transformations. Ions necessary as catalysts for the biochemical reactions were administered as salts.

Coloradocin was produced by submerged fermentations of *A. coloradoensis*. The precursors were administered to second passage seed on days 3, 4, and 5 according to Table 1. The fermentation was harvested on day 6 and the pH adjusted to 4. The precursors were added in portions to

avoid an overload of the Kreb's cycle, in that way, the precursors were not used as energy source and their utilization in secondary metabolism was assured.

The antibiotic was extracted by passage over a column of XAD-2 resin, which was rinsed well with water and stripped with methanol. The crude material was purified by reabsorption onto XAD-2 and elution with a gradient from water to methanol. Coloradocin-containing fractions were combined and purified by chromatography on low pressure columns of C18 RP silica gel in CH_3CN-H_2O to obtain spectroscopically pure coloradocin (Scheme 2). The resulting labeled samples were analyzed by 125 MHz ^{13}C NMR.

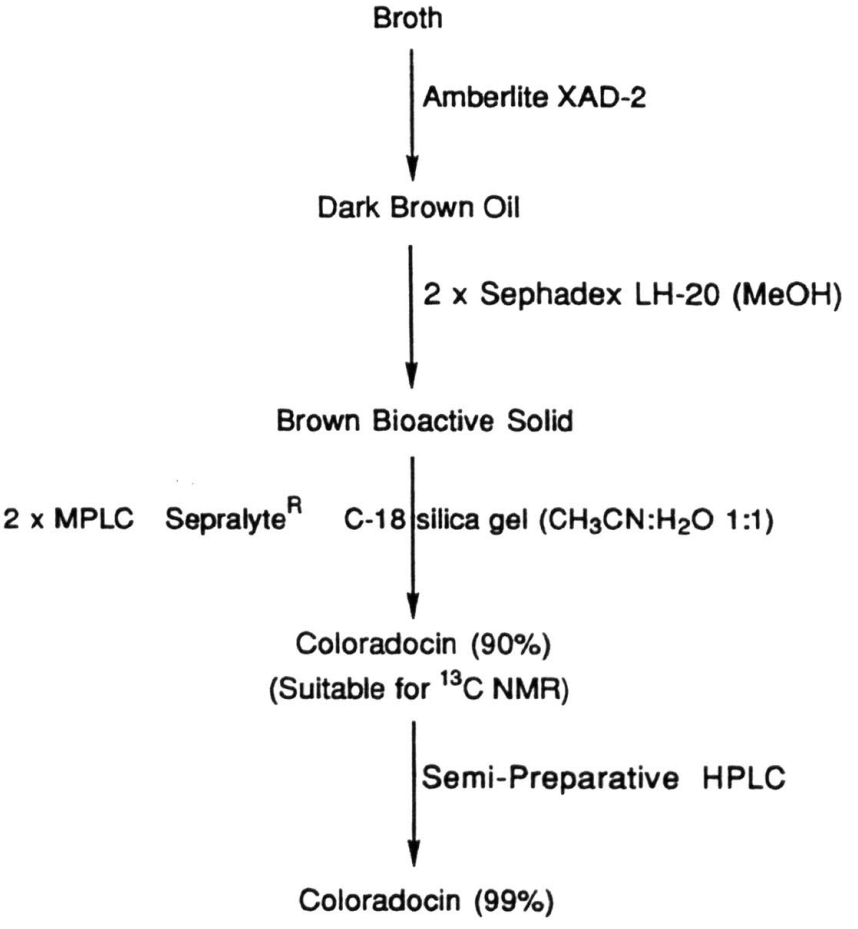
The general instability of coloradocin in hydroxylic solvents was demonstrated by its loss of activity upon exposure to hydroxide ion and also by changes in its ^1H NMR spectrum after standing in MeOH. This instability made the isolation procedure cumbersome because the crude material had to be worked quickly and carefully to avoid decomposition. The fact that coloradocin was isolated in small amounts also made its isolation formidable.

Table 1

Substrate	Addition
sodium [1-^{13}C]-acetate	0.02%
sodium [2-^{13}C]-acetate	0.02%
sodium [1,2-$^{13}C_2$]-acetate	0.02%
sodium [1-^{13}C]-propionate	0.01%
L-[*methyl*-^{13}C]-methionine	0.01%
sodium [2,3-$^{13}C_2$]-succinate	0.007%

L-[1-^{13}C]-glutamic acid	0.003%
[3-^{13}C]-oxaloacetic acid	0.003%

Isolation of Labeled Coloradocin



Scheme 2

E. Results and Discussion

Enrichment for single labeled substrates were calculated by comparing peak heights of labeled samples relative to that of the same carbon from

unlabeled coloradocin spectrum measured under identical parameters. The relative peak heights for experiments with substrates other than methionine were normalized to the ratio of the C23 signals at 58.2 ppm. For double labeled substrates, the enrichment was presented as the ratio of the sum of the peak height of the satellites to the height of the center peak.

In principle, the major macrolide ring could be derived from the typical *Actinomycete* acetate-propionate pathway or from the acetate-methionine pathway of fungi. The oxygens at C11, C13, and C17 are appropiately placed for either pattern.

Major carbon chain. Acetate Feeding. The results (Table 2), which were fully in accord with a polyketide derivation for the major carbon chain portion of coloradocin, showed that carbons 1, 3, 5, 7, and 11 were derived from C-1 of acetate and that carbons 2, 4, 6, 8, and 12 from C-2 of acetate (Figure 7). The results with respect to the minor carbon chain will be discussed later.

Those carbons directly derived from acetate showed a relative enrichment of approximately 3-4 fold. It is noteworthy that incorporation of [1-^{13}C]- and [2-^{13}C]-acetate led to secondary enrichment of propionate-derived carbons (see Table 2 and 2D INADEQUATE spectrum of [2-^{13}C]-acetate labeled coloradocin, Figure 6). These secondary enrichments were approximately 2 fold, and these carbons are starred in the table. This enrichment pattern indicates an active tricarboxylic acid (TCA) cycle,[15] which transforms acetate to succinate, which then is converted into methylmalonate (and propionate) by an active methylmalonyl-CoA mutase (Figure 8).[16] This interpretation is supported by the high number of

secondary enrichments obtained from the succinate feeding experiment (Table 3). To avoid this excess intramolecular multiple labeling effect, unlabeled propionate was added to the medium. This is different from the procedure of Cane and co-workers,[8] which added the corresponding unlabeled acetate and propionate precursors.

Our strategy was based on the equilibrium of the biotransformation of acetyl-CoA to propionyl-CoA. Being the latter at one end of the process, it was obvious that adding unlabeled propionate would shift the equilibrium process to its other end (acetyl-CoA), and this shift in the equilibrium would diminish the excess intramolecular multiple effect.

The incorporation experiment using [1-^{13}C]-propionate, on the other hand, did not result in the enrichment of carbons originating from [1-^{13}C]- nor [2-^{13}C]-acetate. We postulate that this is due to acetyl-CoA being found in much higher concentration in the cells (bigger pool) than propionyl-CoA; therefore, addition of more propionate does shift the equilibrium to the acetyl-CoA end, but the dilution factor brings this to a barely detectable incorporation level.

Table 2. ^{13}C Enrichment of Coloradocin Carbons by Precursors

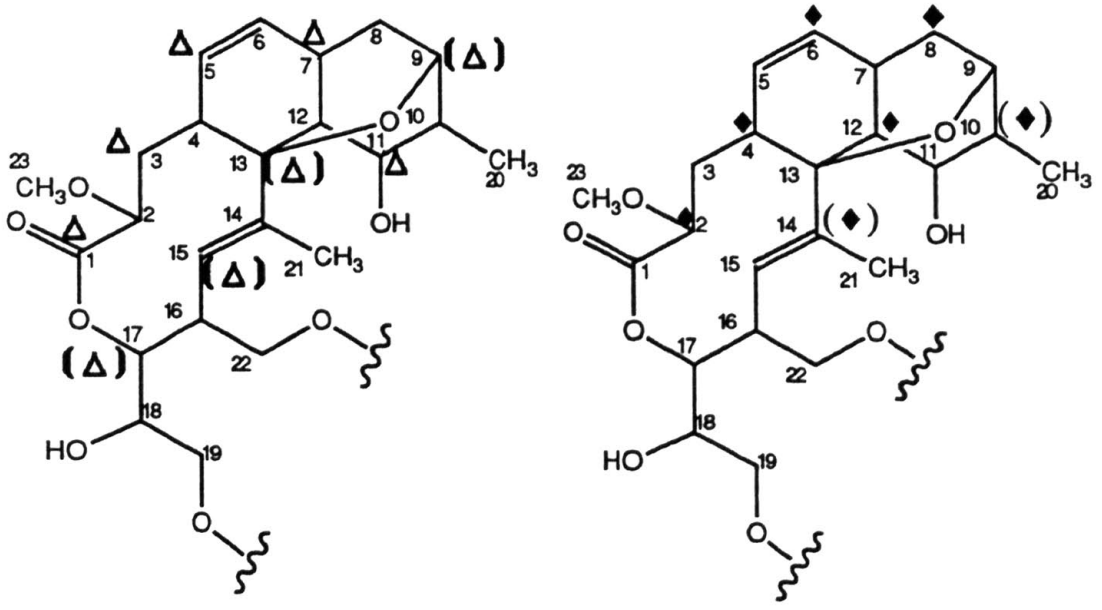
	enrichment			
carbon	[1-^{13}C]-acetate	[2-^{13}C]-acetate	[1,2-$^{13}C_2$]-acetate	[1-^{13}C]-propionate
1	4.1	1.3	1.4	
2	1.0	4.3	1.4	1.2
3	4.5	1.1	1.1	1.1

4	1.1	3.7	1.1	1.0
5	4.5	1.1	1.0	1.0
6	1.0	3.7	1.0	1.0
7	3.6	1.0	1.0	1.2
8	1.2	3.9	1.0	1.0
9	1.8*	0.9	0.4	6.9
10	1.1	1.6*	0.4	1.0
11	4.5	1.1	0.6	1.2
12	1.0	3.7	0.6	1.0
13	1.7*	0.8	0.2	5.7
14	0.9	1.2*	0.2	1.0
15	2.7*	1.0	0.2	5.8
16	1.3*	1.1	0.2	1.1
17	2.0*	1.2*	0.2	4.9
18	1.4*	1.1	0.2	0.9
19	2.0*	1.1		4.9
20	1.0	1.0		1.2
21	1.0	1.1		0.9
22	1.4*	1.1		1.1
23	1.0	1.0		1.0
24	4.2	1.3	2.2	0.8
25	1.3	3.3	2.2	0.8
26	1.2	1.7		1.0
27	1.0	1.9	0.6	1.2
28	1.9	1.2	0.6	2.1

29	2.5	0.9	0.6	
30	0.9	1.1	0.6	1.0
31	1.1	1.2	0.9	1.2
32	3.9	0.9	0.9	0.9

* secondary enrichment.

Incorporation of [1-^{13}C]- and [2-^{13}C]-Acetate



( ) Secondary enrichment

Figure 7

Biotransformation of Acetate to Propionate

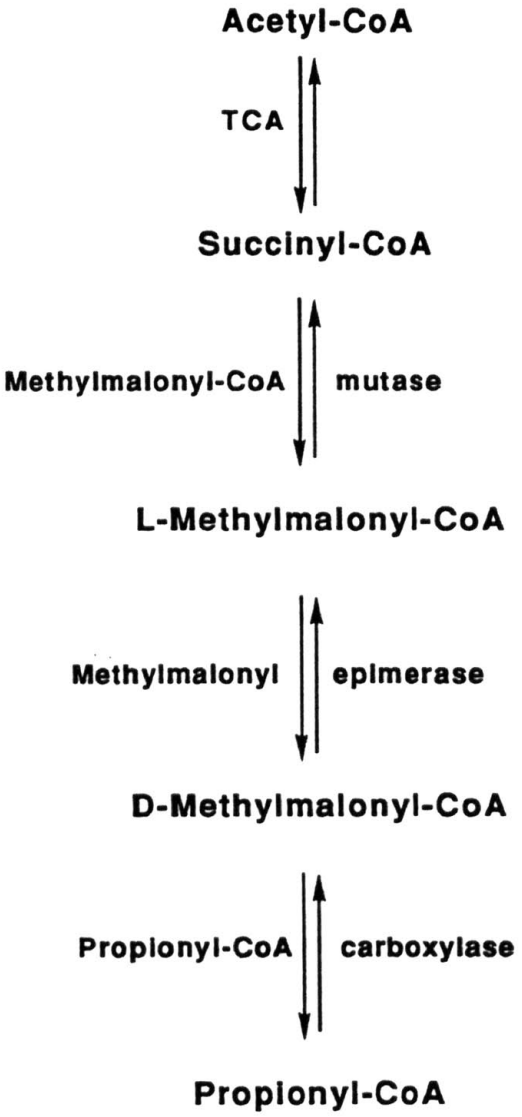


Figure 8

Propionate Feeding. The [1-^{13}C]-propionate experiment (Table 2, Figure 9) enriched carbons 9, 13, 15, 17-19 (poorly resolved in CD_2Cl_2). These carbons showed relative enrichment of 5-7 fold. This relative

enrichment is consistent with a smaller supply of propionate in the medium and a smaller pool in the microorganism.

These results, in conjunction with those obtained from the acetate-labeling experiments, indicated that the major carbon chain of coloradocin is of polyketide origin. It is derived from five acetate and four propionate units. This incorporation pattern is analogous to that of the nargenicin/nodusmicin antibiotics.

Incorporation of [1-^{13}C]-Propionate

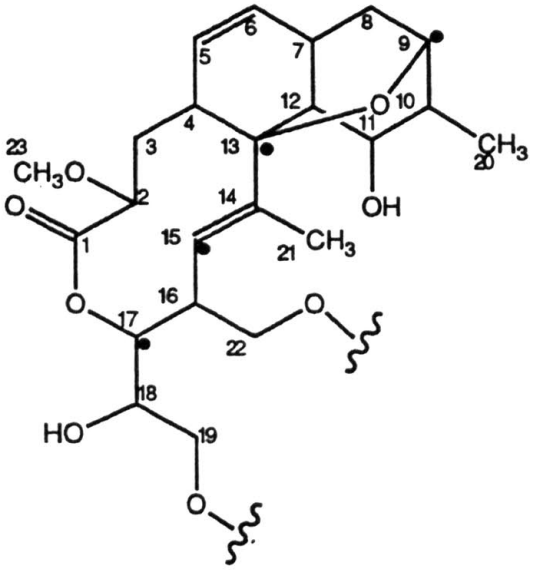


Figure 9

Methionine Feeding. The L-[*methyl*-^{13}C]-methionine experiment indicated that only the *O*-methyl carbon of the major carbon chain showed enrichment (greater than 15 fold). This experiment also proved that the major carbon chain was not derived from the acetate-methionine pathway.

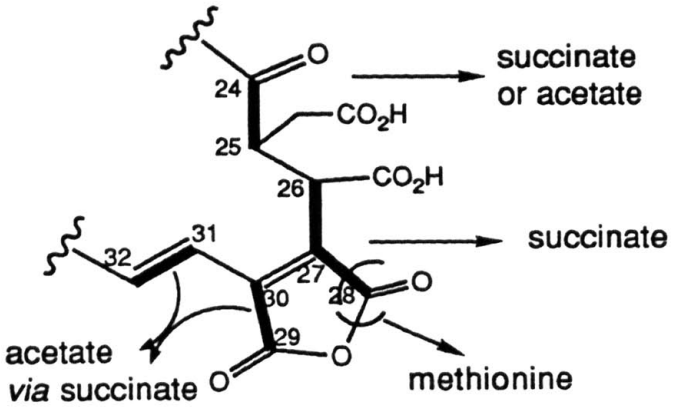
Double Labeled Feeding. The incorporation results (Table 2) and a double quantum filtered INADEQUATE experiment of coloradocin obtained from the [1,2-$^{13}C_2$]-acetate feeding experiment (conducted at Abbott Labs.) showed intact incorporation of acetate and a realtive enrichment of 2-10 fold higher than those obtained by secondary enrichment.

Summary. The conducted experiments accounted for the bioorigin of all the carbons of the major carbon chain and there were no unexpected results.

Minor carbon chain. With the fundamental precursors of the major carbon chain firmly established, we directed our efforts to the determination of the biosynthetic origin of the minor carbon chain.

Derivation analysis (Scheme 3) of the minor carbon chain indicated that a polyketide pathway was not apparent. Various alternatives were plausible, including acetate or acetate *via* succinate, methionine, and succinate.

Minor Carbon Chain Derivation



Scheme 3

Acetate Feeding. The [1-^{13}C]-acetate experiment indicated that carbons 24, 28, 29, and 32 were enriched, whereas carbons 25, 26, and 27 were enriched when [2-^{13}C]-acetate was the precursor (Table 2, Figure 10). The former experiment showed relative enrichment of 3-4 fold, and the latter, of 1.5-3 fold. Incorporation results (Figure 10) and a double quantum filtered INADEQUATE experiment of coloradocin obtained from the [1,2-$^{13}C_2$]-acetate feeding experiment (conducted at Abbott Labs.) indicated that this label went intact into C24-C25, C27-C28, C29-C30, and C31-C32. Altogether, these results indicate that the methyl group of acetate could be the biosource for C25, C26, C27, C30, and C31. Surprisingly, these are five contiguous carbons. The direction of the acetate incorporation indicates that this unit was not derived from the typical *Actinomycete* acetate-propionate pathway. The double labeled experiment confirmed that the C29-C32 segment was of polyketide origin but *via* succinate (low incorporation, Scheme 4). Carbons 24 and 25 were derived from acetate (polyketide origin), and the high relative enrichment, especially in the double labeled experiment (10 fold over secondary enrichment), confirmed their derivation. The low incorporation of the precursors could indicate that this minor chain was synthesized prior to the upper unit and therefore was diluted in the pool before coupling.

Incorporation of [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]-Acetate

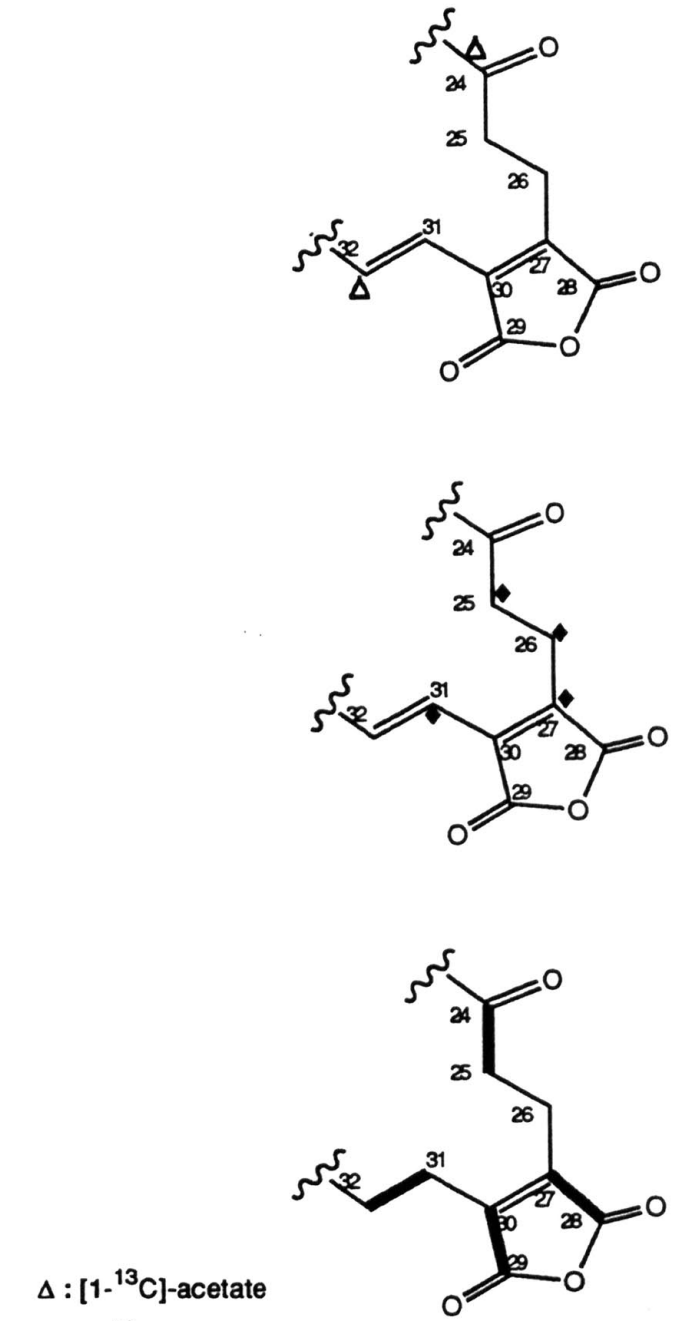
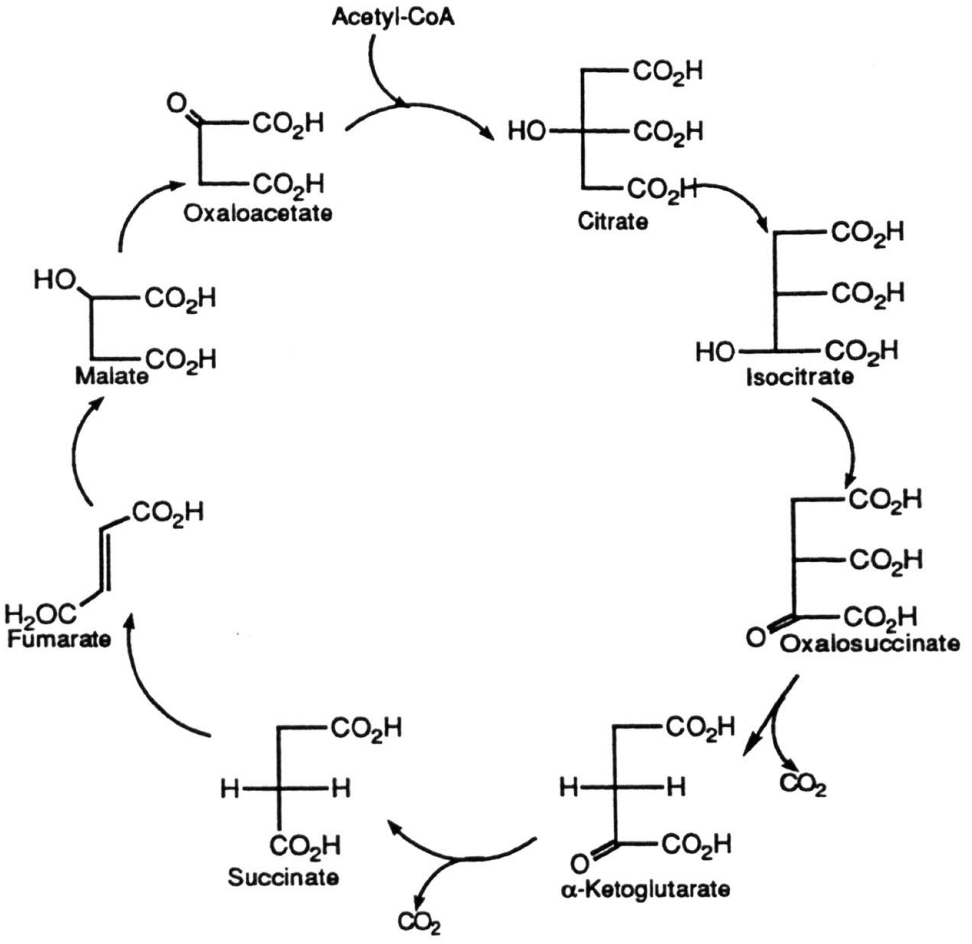


Figure 10

Tricarboxylic Acid Cycle



Scheme 4

Propionate Feeding. The [1-^{13}C]-propionate experiment (Table 2, Figure 11) showed enrichment in C28. The results indicated that the C26-C28 segment might be derived from this precursor, but these results were not in agreement with the results obtained in the acetate feeeding experiments.

Incorporation of [1-^{13}C]-Propionate

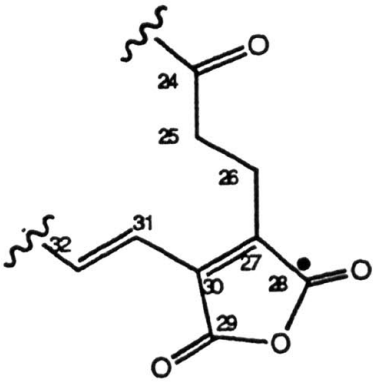


Figure 11

Methionine Feeding. The next precursor examined for the elucidation of the biosynthetic origin of the minor carbon chain was L-[*methyl*-^{13}C]-methionine. We proposed that the anhydride moiety could come from the oxidation of 2 methyl groups which, in turn, might be derived from L-methionine. Feeding experiments indicated that only the *O*-methyl carbon of the major carbon chain showed enrichment, thereby disproving this hypothesis.

At this point, we turned our attention to TCA precursors. [2,3-$^{13}C_2$]-Succinate, [3-^{13}C]-oxaloacetate, and L-[1-^{13}C]-glutamate were the TCA intermediates used in the experiments that followed. TCA precursors were chosen in part because of the low acetate incorporation into the minor carbon chain.

Table 3. ^{13}C Enrichment of Coloradocin Carbons by TCA Precursors

	enrichment			
carbon	[2,3-$^{13}C_2$]-succinate	[2,3-$^{13}C_2$]-succinate[a]	L-[1-^{13}C]-glutamate	[3-^{13}C]-oxaloacetate
1	1.6*	1.5*	1.1	1.0
2	1.5*	1.5*	1.1	1.3
3	3.3*	1.2	1.1	1.2
4	1.1	1.2	1.1	1.2
5	1.1	1.3*	1.2	1.2
6	1.1	1.2	1.1	1.3
7	1.3*	2.0*	1.0	1.1
8	2.3*	1.4*	1.1	1.3
9	1.3*	1.0	1.1	1.2
10	3.3*	1.3*	1.3	1.2
11	1.1	1.2	1.0	1.0
12	1.1	1.2	1.1	1.3
13	1.1	0.9	1.0	1.1
14	1.8*	1.3*	1.0	1.2
15	1.3*	1.1	1.1	1.3
16	1.9*	1.1	1.1	1.1
17	1.8*	1.1	1.2	1.3
18	2.0*	1.0	1.1	1.1
19	1.8*	1.2	1.2	1.2
20	2.3*	0.9	1.0	1.2
21	1.2	0.9	1.1	1.2

22	3.9*	1.1	1.1	1.3
23	1.0	1.0	1.0	1.0
24	0.6	1.1	1.2	1.0
25	1.0	1.1	1.1	1.2
26	2.0	1.9	1.1	1.2
27	1.9	1.7	1.0	1.4
28	1.2	0.9	1.3	1.0
29	0.9	0.8	0.9	0.8
30	0.9	0.8	1.0	1.0
31	0.9	1.2	1.1	1.2
32	0.9	0.9	1.1	1.0

* secondary enrichment.
[a] succinate + unlabeled propionate.

Succinate Feeding. The [2,3-$^{13}C_2$]-succinate experiment was based on the premise that C30 and C27 or the C29-C32 segment might come from this precursor. In fact, the results did not support this hypothesis (Table 3). Carbons 24, 25, 26, and 27 were the only carbons of the minor carbon chain showing enrichment. This finding suggested that C2 and C3 of succinate might have been incorporated intact into C26 and C27 (Figure 12). Incorporation into C24 and C25 (carbons of acetate origin) was probably due to the intramolecular multiple labeling effect (secondary enrichment). To avoid this secondary enrichment, unlabeled acetate was added to the fermentation medium. Unfortunately, no suppression was obtained (results not shown). The secondary enrichment was similar to that obtained without the co-substrate. When unlabeled propionate, on the other hand, was added

to the medium, excess intramolecular multiple labeling was diminished considerably, especially for propionate-derived carbons(see Table 3). These results created a great deal of concern. It seemed that succinate was transformed to acetate, *via* TCA, before incorporation. Neither the acetate nor the propionate shunting gave support to the succinate hypothesis. It was shown that propionate did not exert a back control on succinate. The relative enrichments reinforced a succinate bioorigin for C26-C27.

Glutamate Feeding. The next precursor examined was L-[1-^{13}C]-glutamate. It was fed at low levels because of the very high cost of this material. The results (Table 3) indicated that it was a very poor substrate at low concentration. A possible explanation could be the utilization of glutamate in protein biosynthesis, decreasing the bioavailability of this precursor for secondary metabolism. The experiment showed enrichment in C28, consistent with the incorporation of this unit into the C24-C28 segment. The results also showed enrichment in C10 (Figure 13).

Incorporation of [2,3-$^{13}C_2$]-Succinate

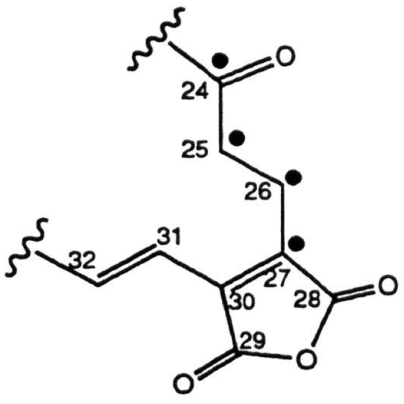


Figure 12

Incorporation of L-[1-^{13}C]-Glutamate

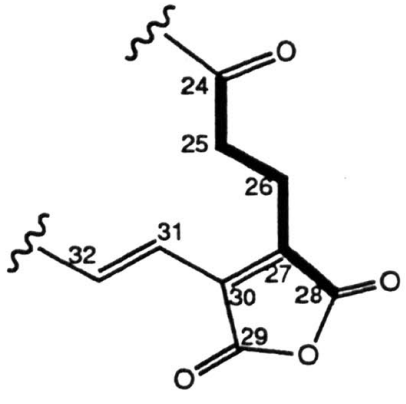


Figure 13

Oxaloacetate Feeding. The feeding experiment with [3-^{13}C]-oxaloacetate indicated that this precursor was also a very poor substrate. The explanation is similar to that of L-[1-^{13}C]-glutamate. Excess oxaloacetate could be pulled out of the pool by transaminases, enzymes that convert oxaloacetate to aspartate. The latter is needed in protein biosynthesis and it is also a precursor for the biosynthesis of asparagine, threonine, isoleucine, methionine, and lysine. Excess oxaloacetate beyond the amount required in biosyntheses is reduced to malate.[17] The experiment showed enrichment in carbon 27 (Table 3, Figure 14), consistent with the incorporation of C2, C3, and C4 of the oxaloacetate unit into the C26-C28 segment of coloradocin. Unfortunately, several other carbons of other biosynthetic origin (C2, C6, C8, C15, and C22) showed enrichment as well, rendering the conclusions hazardous.

Incorporation of [3-^{13}C]-Oxaloacetate

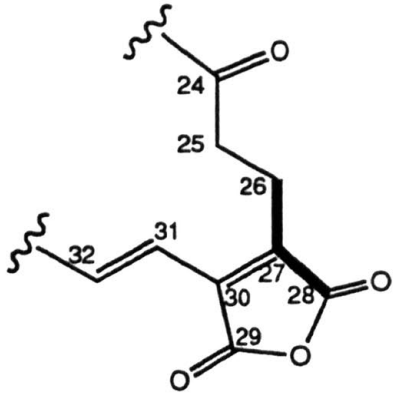


Figure 14

A plausible explanation for the incorporation of carbons of other biosynthetic origin by these TCA precursors could be the high activity of the

Kreb's cycle, transforming these TCA intermediates into propionate and/or acetate.

Glucose Feeding. The [U_6-^{13}C]-glucose feeding experiment was conducted with the hypothesis that C24-C28 and C29-C32 segments might be derived from a pentose and a tetrose, respectively. A double quantum filtered INADEQUATE experiment of the labeled sample showed enrichments in carbons of different bioorigin. This result indicated that the labeled glucose was used as an energy source by the microorganism. The results were not surprising or unexpected because glucose belongs to a big pool and it is not a specific precursor.

F. Conclusions

The major carbon chain of coloradocin incorporates five acetate, four propionate units and one methionine carbon in a typical *Actinomycete* acetate-propionate pathway. This incorporation pattern is analogous to patterns for the nargenicin/nodusmicin antibiotics.

The minor chain, however, incorporates acetate *via* an undefined route. This fact indicates that the minor chain is not of polyketide origin. Carbons 24 and 25 are clearly acetate-derived. They may also be derived as part of a glutamate residue. Carbon 26 is a mystery unless it comes as part of glutamate or succinate. The acetate experiments indicate that the C29-C32 segment is derived from succinate but a direct feeding experiment failed to support this. None of these carbons come from methionine.

Based on the results of the glucose experiment, no evidence was found for the involvement of the shikimic pathway in the biosynthesis of the minor chain. L-Glutamic acid could be the precursor for the C24-C28 segment based on the incorporation results.

We also showed the importance of metabolic shunting when co-substrate was added in the feeding experiments to avoid excess intramolecular multiple labeling effect, especially when TCA precursors were used as substrates.

Clearly, more experimental work is called for in setting the remaining issues raised by this work.

Experimental

Feeding experiments were conducted at Abbott Laboratories by Dr. Marianna Jackson and co-workers. ^{13}C NMR and 2D INADEQUATE experiments of the [1,2-$^{13}C_2$]-acetate labeling experiment were run at Abbott Laboratories. NMR spectra were obtained with either a General Electric GN 300 or GN 500 spectrometer using 5mm probes.

Organism

Actinoplanes coloradoensis sp. nov. strain AB 921J-26 was used for this work.

Fermentation

The fermentation medium consisted of glucose monohydrate 1%, starch (Staley's Staclipse JUB) 0.4%, molasses (Del Monte's Brer Rabbit green label) 0.5%, Lexein F-152 liquid peptone (Inolex) 0.4%, soybean flour 0.5%, NZ amine type A (Sheffield) 0.1%, yeast extract (Difco) 0.1%, $MgSO_4$ (anhydrous) 0.02%, $CaCO_3$ 0.1%, and antifoam XFO-371 (Ivanhoe Chemical Co.) 0.01%. The fermentation was performed in a New Brunswick 150-liter fermentor charged to a volume of 80 liters. Incubation was at 30 °C for 162 hours with an agitation rate of 200 rpm, an aeration rate of 0.7 vvm and a head pressure of 0.35 kg/cm^2.

Inoculum for Fermentation

Vegetative seed growth was frozen at -75 °C and used to inoculate the seed tubes of subsequent fermentations. The seed medium consisted of glucose monohydrate 0.1%, starch (Staley's Staclipse JUB) 2.4%, yeast extract (Difco) 0.1%, Tryptone (Difco) 0.5%, beef extract (Scott) 0.3%, and

$CaCO_3$ 0.4%. The medium was prepared in distilled water and adjusted to pH 7 prior to sterilization. Ten mL of medium in 25 x 150 mm culture tubes, covered with Bellco stainless caps, were inoculated with 0.5 mL of frozen seed stock. The tubes were incubated for 96 hours. Two-liter Erlenmeyer flasks, containing 600 mL medium, were inoculated with the tube growth at 5% and incubated for 72 hours. The second passage flask growth was used to inoculate the fermentation al 5%. Both seed steps were incubated at 30°C on a rotary shaker at 250 rpm (3.2 cm stroke).

Labeled substrate (99% ^{13}C-enriched) was added to the second passage seed on each of days 3, 4, and 5. The fermentation was harvested on day 6 and the pH adjusted to 4.

Isolation of Labeled Coloradocin

The labeled sample was extracted by passage over a column of XAD-2 resin, which was rinsed well with water and stripped with methanol. The crude material was purified by reabsorption onto XAD-2 and eluted with a gradient of water to methanol. Coloradocin-containing fractions were combined and repurified on low pressure columns of C18 RP silica gel in CH_3CN-H_2O (1:1) to obtain spectroscopically pure coloradocin.

13C NMR Spectra

NMRs were obtained on a Bruker AM-500 (500 MHz) spectrometer using a 5 mm probe. Samples were run in CD_2Cl_2 in which solvent the C17 and C19 carbon signals were poorly resolved. Signals were reported in parts per million (ppm) using the CD_2Cl_2 signal as reference peak. Enrichment for single labeled substrates were calculated by comparing peak heights of labeled samples relative to that of the same carbon from

unlabeled coloradocin spectrum measured under identical parameters. The relative peak heights for experiments with substrate other than methionine were normalized to the ratio of the C23 signals at 58.2 ppm. For double labeled substrates, the enrichment was presented as the ratio of the sum of the peak height of the satellites to the height of the center peak.

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12. Mascaretti, O.; Chang, C.-J. ; Floss, H. G. *J. Nat. Prod.* **1979**, *42*, 455.

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Part III

Total Synthesis of the Naturally Occurring Prenylated Bibenzyl Amorfrutin A

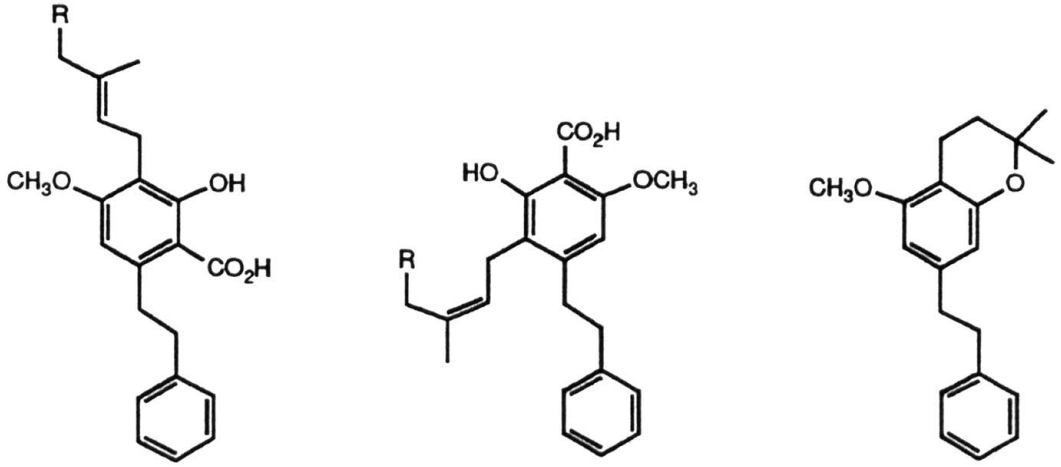
A. Introduction

Amorpha fruticosa is an indigenous American shrub used by Amerindians for bedding and as a mat for keeping freshly butchered meat clean. In a previous study, ethanolic extracts derived from the powdered fruits, stems, and leaves of *Amorpha fruticosa* showed reproducible antibacterial activity *in vitro* against *Mycobacterium smegmatis* (ATCC 607) and *Staphylococcus aureus* (ATCC 13709).[1] Systematic bioassay-directed fractionation led to the isolation of the new rotenoid 11-hydroxytephrosin,[2] and two new bioactive carboxylated bibenzyl metabolites, amorfrutin A (**1**) and B (**2**).[3]

Two possible structures (**1** and **3**) for amorfrutins A and B were consistent with the spectral data (Figure 1). Although biogenetic considerations[4] favored structure **1**, unambiguous proof of the structure ultimately came from the synthesis of degradation product **4**.[2] This synthesis (Scheme 1) could have been modified for the production of the natural products themselves, however, the possibility of complications during the demethylation step, i.e. regioselective control of the cleavage and Lewis acid-catalyzed cyclization of the prenyl group with the newly formed hydroxyl group, would have reduced the unambiguity of the synthesis and made it more cumbersome.

Because of the increasing number of bioactive carboxylated bibenzyls being reported in the literature,[5-11] an efficient and flexible synthetic route to these compounds was desired in order to allow for convenient structure proof and to make them available in quantity for biological evaluation.

Structures of Amorfrutin A and B and of Degradation Product 4

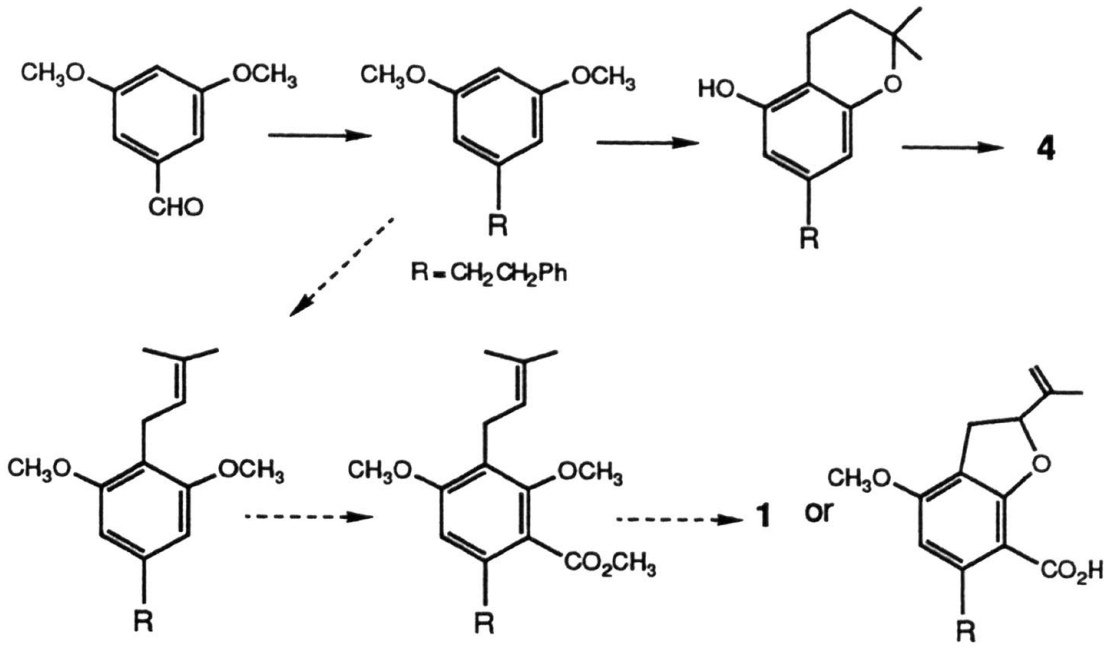


1 R = H
2 R = $CH{=}C(CH_3)_2$

3

4

Figure 1



Scheme 1

Curiously, no synthesis is yet available. One notes that, in addition to their antimicrobial properties, these compounds also show insect repellent properties[12] and are endogenous plant growth regulators.[12-14] Some examples of carboxylated stilbenoids are shown in Figure 2.

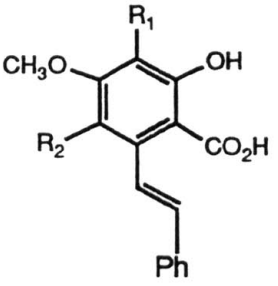
Because of the reasons mentioned above, our objective was the design of a flexible and unambiguous route to 2-carboxylated bibenzyls. The key intermediate **10** (Scheme 4), in principle, can also provide convenient access to analogously substituted 3,4-dihydroisocoumarin,[15] isocoumarin,[15] and stilbene derivatives. The synthesis of these natural products is both important and desirable due to their interesting biological properties as antimicrobial agents (phytoalexins) and plant endogenous growth regulatory substances.

B. Results and Discussion

The synthesis of amorfrutin A presented two problems dealing with regiospecificity: the introduction of the carboxyl and the prenyl groups. The regiospecific introduction of the prenyl group was solved as a consequence of the solution found for installing the carboxyl group. The introduction of the carboxyl group was conceived as occurring *via* an intramolecular Friedel-Crafts acylation of intermediate **7**, and with the appropriate catalyst and reaction conditions, selective monodemethylation would also be accomplished during the process of the reaction (see retrosynthetic analysis, Scheme 2). Further manipulation of **8** would generate the carboxyl group at the 2 position.

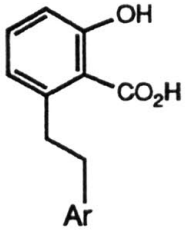
2-Carboxylated Stilbenoids and their Sources

from *Cajanus cajan* [11]



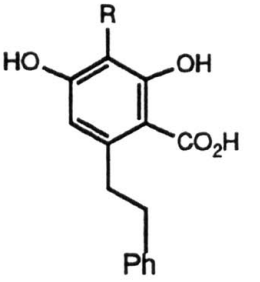
R_1 : H R_2 : $Me_2C{=}CHCH_2$
R_1 : $Me_2C{=}CHCH_2$ R_2 : H

from *L. cruciata*,[5] *F.dilatata*,[6] and *F. tamarisci* [10]



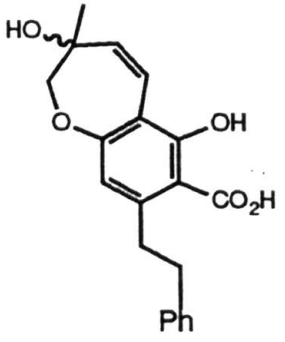
Ar : 4-OH-Ph
Lunularic Acid

from *H.umbraculigerum* [9] and *R. complanata* [7]

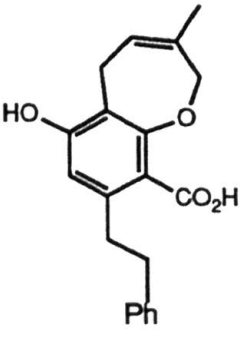


R : $Me_2C{=}CHCH_2$

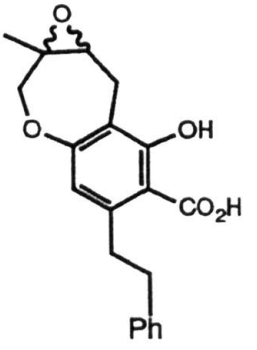
from *Radula variabilis* [8]



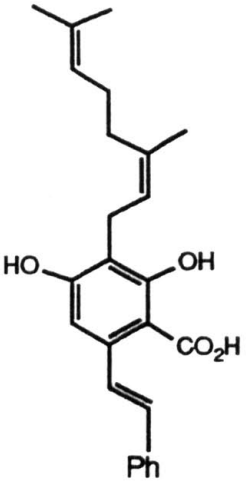
from *R. variabilis* [8] & *R. complanata* [7]



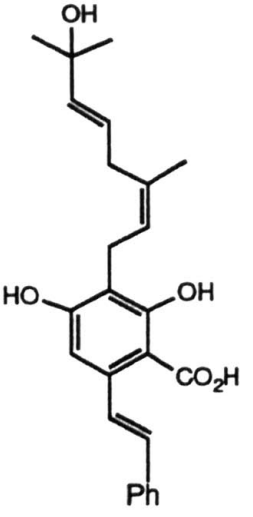
from *R. complanata* [7]



from *Helichrysum umbraculigerum* [9]



from *Helichrysum umbraculigerum* [9]



from *Helichrysum umbraculigerum* [9]

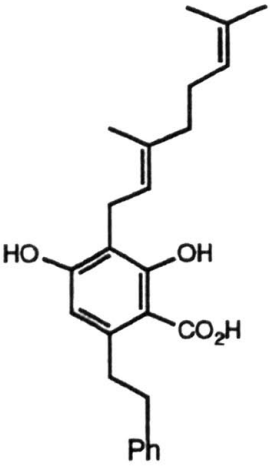
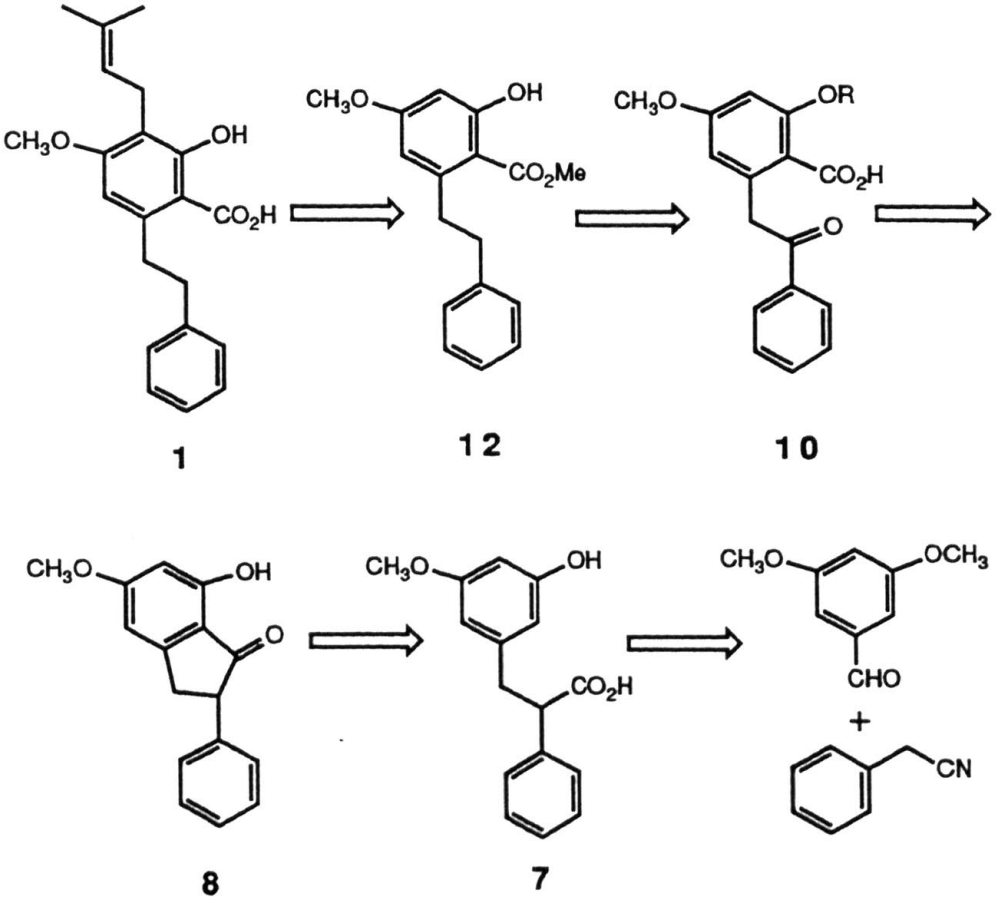


Figure 2

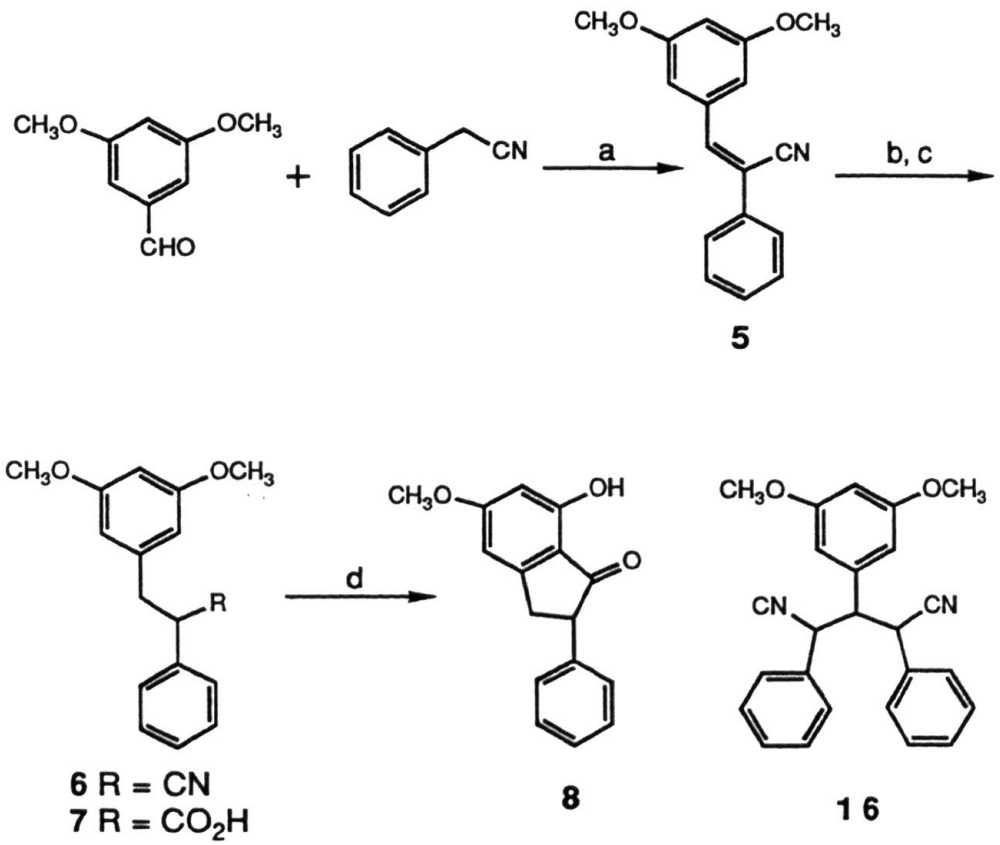
Retrosynthetic Analysis



Scheme 2

The synthesis began with the Knoevenagel condensation of benzyl cyanide with 3,5-dimethoxybenzaldehyde under basic conditions to afford the stilbene derivative **5** in 94% yield. Occasionally, under the same apparent conditions, bisadduct **16** was obtained as the major product. Alkene reduction of **5** was performed with 5% Na(Hg) to afford the bibenzyl intermediate **6** in 99% yield. Acid hydrolysis of the cyano group gave carboxylic acid **7** (80%). As planned, intramolecular Friedel-Crafts acylation

and selective monodemethylation were accomplished in the same step by the use of BCl_3 at -20 °C to produce the indanone derivative **8** in 97% yield (Scheme 3).



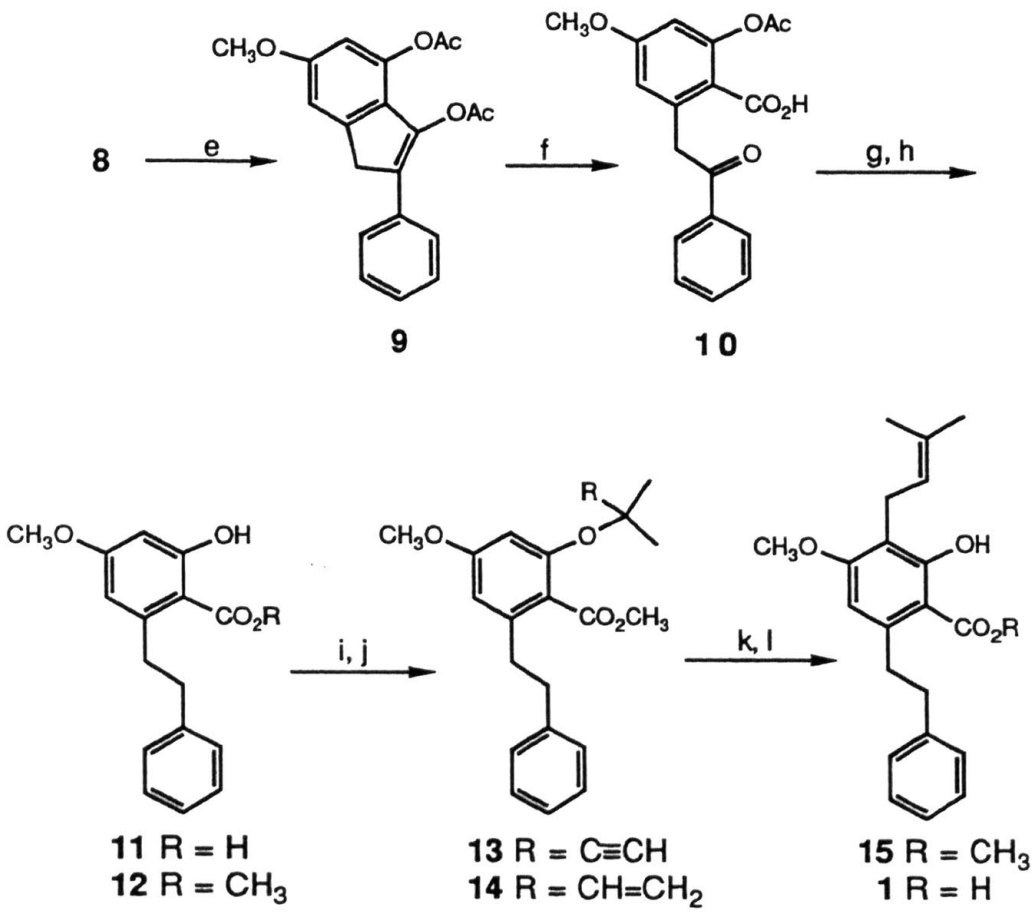
a) 6*N* NaOH/EtOH, 94%; b) 2.5% Na(Hg)/EtOH, 99%
c) 2*N* NaOH/90% EtOH, 80%; d) BCl_3, -20 °C/CH_2Cl_2, 97%

Scheme 3

At this point, the regiospecific introduction of the carboxyl group was essentially solved and the appropriate hydroxyl group of amorfrutin A was freed to serve as a conductor for installation of the prenyl or geranyl units. At this stage, it was necessary to transform the cyclic ketone into a carboxylic

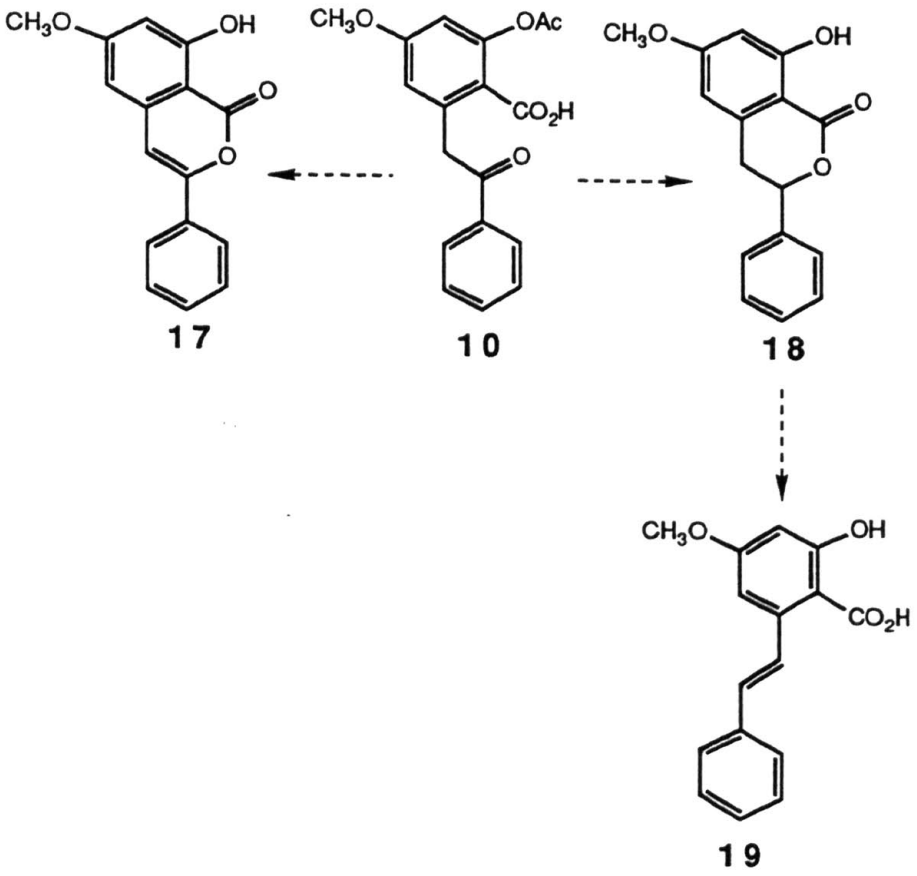
group through cleavage on the appropriate side. This transformation could have been performed by a Baeyer-Villager rearrangement of compound **8**, but this process would have cleaved the cyclic ketone on the wrong side. Cleavage on the appropiate side was accomplished by reacting **8** with acetic anhydride and H_2SO_4 catalyst to give enol acetate **9** in 97% yield. Under the same conditions, the phenolic hydroxyl group was also acetylated. Ozonolysis of **9** afforded the desired keto-acid intermediate **10** in 97% yield. For production of amorfrutin A, removal of the keto group was accomplished in quantitative yield *via* hydrogenolysis over Pd-C to afford carboxylic acid **11**. For the introduction of the prenyl group, methyl ester **12** was prepared with diazomethane in 95% yield from **11**. Compound **12** was then reacted with 3-chloro-3-methylbut-1-yne and KI under basic conditions (K_2CO_3) to afford dimethylprop-2-ynyl ether **13** in 30% yield. Partial hydrogenation over $BaSO_4$/Pd-C poisoned with quinoline gave ether **14** (91%), which was rearranged in N,N-dimethylaniline under reflux to give amorfrutin A methyl ester (**15**) in 82% yield. Basic hydrolysis afforded amorfrutin A in 75% yield (Scheme 4).

Intermediate keto-acid **10** could, if desired, afford isocoumarin **17** by enol-lactonization, and 3,4-dihydroisocoumarin **18** by reduction followed by lactonization. Base-promoted elimination of **18** could produce stilbene **19** (Scheme 5).



e) Ac_2O/cat. H_2SO_4, 97%; f) O_3/MeOH-CH_2Cl_2, 97%; g) H_2/Pd-C, 100%; h) CH_2N_2/Et_2O, 95%; i) 3-chloro-3-methylbut-1-yne, KI, K_2CO_3, 30%; j) $H_2/BaSO_4$/10% Pd-C/quinoline, 91%; k) N,N-dimethylaniline/reflux, 82%; l) 0.1 *N* NaOH/H_2O, 75%.

Scheme 4



Scheme 5

C. Conclusions

The total synthesis of the plant antimicrobial agent amorfrutin A (**1**) was accomplished in 12 steps from known 3,5-dimethoxybenzaldehyde and benzyl cyanide in 11% overall yield. The synthetic route employed was efficient, apparently flexible, and regiospecific for 2-carboxylated bibenzyls and related molecules. It should make this and related molecules conveniently available in reasonable quantity.

EXPERIMENTAL

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared (IR) spectra were obtained on an IBM FTIR 32 or Perkin Elmer 1420 spectrophotometer. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian FT-80 (80 MHz) spectrometer in the indicated solvents using tetramethylsilane as internal reference, unless otherwise specified. All chemical shifts are expressed in parts per million (δ). Electron impact mass spectra (EIMS), chemical ionization mass spectra (CIMS), and high resolution mass spectra (HRMS) were obtained on a Varian CH-5 or Ribermag R10-10 mass spectrometer by Dr. Charles Judson and Robert Drake. Microanalyses were performed by Dr. Tho I. Nguyen on a Hewlett-Packard Model 185 CHN analyzer at the University of Kansas. Ultraviolet (UV) spectra were recorded on a Hewlett-Packard Diode Array 8450A. Ozonolyzes were carried out on a Welsbach T-23 ozonator. Flash chromatography and medium pressure liquid chromatography (MPLC) were performed on Merck silica gel (230-400 mesh), and gravity column chromatography on Merck silica gel (70-270 mesh).

Reagents and solvents were used as obtained without further purification, unless otherwise indicated. Bulk grade solvents: Et_2O, hexanes, acetone, and MeOH were distilled before use. Methylene chloride (CH_2Cl_2) was distilled immediately before use from P_2O_5. MeOH and EtOH were distilled from Mg turnings. Benzene was distilled from powdered CaH_2. Acetic anhydride was distilled from NaOAc.

All reactions involving moisture- or oxygen-sensitive materials were performed in flame- or oven-dried glassware under a positive pressure of argon.

2-Phenyl-3,5-dimethoxycinnamonitrile (5). 3,5-Dimethoxy benzaldehyde (5.0 g, 30.09 mmol), benzyl cyanide (5.5 g, 46.95 mmol) in ethanol (40 mL), and sodium hydroxide (0.3 mL, 6*N*) were stirred at room temperature for 24 h. The precipitate obtained was filtered, washed with cold ethanol, and crystallized from CH_2Cl_2 to give **5** (7.5g, 94%) as white crystals: mp 69-70 °C; UV (CH_3CN), λ_{max} (ε) 314 (2 215), 228 (16 410) nm; IR (neat) 3100, 2950, 2900, 2800, 2250 (CN), 1587, 1456, 1155, 837, 762 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.68-7.25 (m, 7H), 7.05 (d, J = 2 Hz, 1H), 6.54 (s, 1H), 3.84 (s, 6H); EIMS *m/z* (relative intensity) 266 (M^+ + 1, 19.8), 265 (M^+, 100), 250 (15), 234 (12.5), 219 (9.6), 190 (28.5), 151 (8), 76 (10.5), 191 (11.6).

Anal. Calcd for $C_{17}H_{15}NO_2$: C, 76.98; H, 5.66; N, 5.28. Found: C, 76.80; H, 6.00; N, 5.28.

1,3-Dicyano-1,3-diphenyl-2-(3,5-dimethoxyphenyl)propane (16). To a solution of 3,5-dimethoxybenzaldehyde (12.5 g, 75.4 mmol), benzyl cyanide (9.7 g, 82.9 mmol), and ethanol (100%, 30 mL) was added dropwise 6*N* NaOH (0.2 mL, 1.2 mmol). The reaction mixture was stirred overnight at room temperature. A precipitate was seen after 30 minutes of stirring which was removed by filtration, washed with cold water-methanol solution (1:1, 3 x 25 mL), and then with cold water (3 x 25 mL). The resulting solid was recrystallized from CH_2Cl_2-hexanes (1:3) to yield **16** (17.04 g, 59%) as a pure white solid: mp 203.5-204 °C; UV (CH_3CN), λ_{max} (ε) 204 (29

450), 231 (6 000, sh), 285 (2 260) nm; IR (KBr) 2961, 2938, 2250, 1587, 1354, 1315, 1064, 837, 762, 690 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.37 (s, 10H), 6.40 (s, 1H), 6.24 (s, 2H), 4.1 (d, *J* = 8 Hz, 2H), 3.69 (s, 6H), 3.29 (t, *J* = 8 Hz, 1H); EIMS *m/z* (relative intensity) 382 (M^+), 265 (100), 250 (20), 190 (40).

Anal. Calcd for $C_{25}H_{22}N_2O_2$: C, 78.51; H, 5.80; N, 7.33. Found: C, 78.49; H, 5.81; N, 7.30.

2-Phenyl-3-(3,5-dimethoxyphenyl)propionitrile (6). A suspension of **5** (4.5 g, 16.96 mmol) and 2.5% sodium amalgam (4.5 g) in anhydrous ethanol (50 mL) was kept at 60 °C for 2 h. The reaction was filtered and the solvent was removed under reduced pressure. The residue was redissolved in CH_2Cl_2 (50 mL) and washed with dil. HCl (3 x 20 mL) and water (4 x 25 mL), dried (Na_2SO_4), and concentrated under reduced pressure to give **6** (4.5 g, 99%) as a thick colorless oil: UV (CH_3CN), λ_{max} (ε) 298 (17 850), 292 (17 850) nm; IR (neat) 3003, 2959, 2939, 2250, 1599, 1464, 1153, 837, 756, 736 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.22 (s, 5H), 6.62 (d, *J* =2 Hz, 1H), 6.18 (d, *J* = 2 Hz, 1H), 3.90 (t, *J* = 7 Hz, 1H), 3.63 (s, 6H), 3.0 (d, *J* = 7 Hz, 2H); EIMS, *m/z* (relative intensity) 268 (M^+ + 1, 5.2), 267 (M^+, 30.8), 151 (100), 116 (5.4), 91 (11.6), 78 (22.4), 77 (36.7).

Anal. Calcd for $C_{17}H_{17}NO_2$: C, 76.38; H, 6.41; N, 5.24. Found: C, 76.10; H, 6.30; N, 4.90.

2-Phenyl-3-(3,5-dimethoxyphenyl)propanoic acid (7). A solution of **6** (4.0 g, 14.96 mmol) in 90% ethanol (50 mL) containing sodium hydroxide (4.0 g, 100.01 mmol) was refluxed for 24 h. The mixture was diluted with water (50 mL), concentrated to 40 mL, acidified with dil. HCl (2*M*, 60 mL), and extracted with ethyl acetate (3 x 25 mL). The organic layer was

washed with water (4 x 50 mL), dried (Na_2SO_4), and concentrated under reduced pressure to afford **7** (3.43 g, 80%): mp 70-72 °C; UV (CH_3CN), λ_{max} (ε) 285 (1 850), 282 (1 830) nm; IR (neat) 3386 (br), 3165, 3088, 3063, 3003, 2961, 1708, 1606, 1597, 1330, 1195, 1067, 835, 735, 698 cm^{-1}; ^1H NMR ($CDCl_3$) δ 10.48 (s, 1H), 7.28 (s, 5H), 6.24 (s, 2H), 3.65 (s, 6H), 3.62 (d, J = 7 Hz, 2H), 3.58 (q, J = 7 Hz, 1H); EIMS *m/z* (relative intensity) 287 (M^+ + 1, 5.7), 286 (M^+, 27.3), 152 (15.9), 151 (100), 78 (8.0), 77 (17.0).

Anal. Calcd for $C_{17}H_{18}O_4$: C, 71.31; H, 6.34. Found: C, 71.20; H, 6.20.

7-Hydroxy-5-methoxy-2-phenylindan-1-one (8). To a solution of **7** (2.93 g, 10.23 mmol) in CH_2Cl_2 (40 mL) at -20 °C, boron trichloride (5mL, 1 *M* solution in CH_2Cl_2) was added. After 3 h, the reaction was quenched by adding 95% ethanol (1.5 mL). The reaction mixture was reduced to about half the volume, washed with 5% sodium bicarbonate (3 x 10 mL) and water (3 x 10 mL). The solvent was removed and the residue was crystallized from ether to give **8** (2.53 g, 97%): mp 92-93 °C; UV (CH_3CN), λ_{max} (ε) 272 (21 385), 221 (26 315) nm; IR (neat) 3333, 2950, 2850, 1668, 1630, 1599, 1495, 1367, 1234, 1197, 1153, 841, 700, 677 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.1 (br, s, 1H), 7.24 (s, 5H), 6.49 (br, 1H), 6.32 (br, 1H), 3.86 (s, 3H), 3.75 (m, 1H), 3.25 (m, 2H); EIMS *m/z* (relative intensity) 254 (M^+, 100), 253 (M^+ - 1, 21.5), 225 (11.4), 211 (10.4), 177 (19.3), 165 (14.8), 152 (9.1), 127 (10.2), 91 (7.4).

Anal. Calcd for $C_{16}H_{14}O_3$: C, 75.57; H, 5.55. Found: C, 75.20; H, 5.50.

5-Methoxy-1,7-diacetatoxy-2-phenylindene (9). To a cold (0 °C) solution of **8** (2.03 g, 7.56 mmol) in acetic anhydride (20 mL), conc. H_2SO_4 (1 mL) was added and the reaction mixture was allowed to warm up to room temperature. After stirring for 12 h, the reaction mixture was poured over

3.85 (s, 3H), 2.20 (s, 3H); EIMS *m/z* (relative intensity) 328 (M^+, 5.4), 311 (57), 287 (19.4), 233 (12), 206 (5.4), 164 (30.1), 105 (100), 86 (20.4), 77 (26.9).

Anal. Calcd for $C_{18}H_{16}O_6$: C, 65.85; H, 4.91. Found: C, 65.60; H, 5.10.

3-Hydroxy-5-methoxybibenzyl-2-carboxylic acid (11). To a solution of **10** (1.5 g, 4.57 mmol) in 100% ethanol (70 mL), water (25 mL), and HCl (25 mL) was added 5% palladium charcoal (2.0 g) and the mixture was hydrogenated for 7 h at room temperature and 760 mm/Hg. The reaction mixture was filtered, concentrated to 50 mL, and diluted with cold water (50 mL). The mixture was extracted with ethyl acetate (3 x 50 mL) and the combined organic layer washed well with brine (3 x 25 mL), dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The solid obtained was crystallized from ethyl acetate-hexanes to afford **11** (1.19 g, 100%): mp 128-131 °C (softens at 110 °C); UV (CH_3CN), λ_{max} (ε) 302 (6 770), 262 (15 960), 218 (36 220) nm; IR (KBr) 3433 (br), 3082, 2980, 2851, 2685, 1799, 1628, 1616, 1581, 1473, 1367, 1261, 1205, 1122, 1005, 956 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.24 (s, 5H), 6.33 (s, 2H), 5.20 (br, 1H), 3.80 (s, 3H), 3.25 (m, 2H), 2.80 (m, 2H); EIMS, *m/z* (relative intensity) 272 (M^+, 47.7), 254 (100), 239 (3.4), 225 (10.2), 181 (14.8), 135 (13.6), 91 (13.6).

Anal. Calcd for $C_{16}H_{16}O_4$: C, 70.57; H, 5.92. Found: C, 70.50; H, 5.80.

3-Hydroxy-5-methoxy-2-carbomethoxybibenzyl (12). To a cold (5 °C) solution of **11** (1.0 g, 3.67 mmol) in ether (25 mL) was added excess of diazomethane in ether. After concentration, the residual solid which recrystallized from ether-hexanes to give **12** (1.0 g, 95%): mp 48.5-49.5 °C; UV (CH_3CN), λ_{max} (ε) 302 (5 330), 262 (12 545), 218 (28 530) nm; IR (neat)

3084, 3026, 3003, 2953, 1724, 1653, 1616, 1496, 1435, 1381, 1259, 1159, 1047, 956, 750 cm^{-1}; ^1H NMR (acetone-d_6) δ 12.42 (s, 1H), 7.22 (br, 5H), 6.37 (d, J = 2.5 Hz, 1H), 6.27 (d, J = 2.5 Hz, 1H), 3.95 (s, 3H), 3.80 (s, 3H), 3.15 (m, 2H), 2.90 (m, 2H); EIMS *m/z* (relative intensity) 286 (M^+, 17.6), 254 (49.2), 195 (13.9), 165 (9.1), 135 (12.6), 91 (100).

Anal. Calcd for $C_{17}H_{18}O_4$: C, 71.31; H, 6.34. Found: C, 71.02; H, 6.30.

3-O-(α, α-Dimethylpropargyl)-5-methoxybibenzyl (13). A suspension of **12** (400 mg, 1.40 mmol), K_2CO_3 (900 mg, 6.51 mmol), KI (1.2 g, 7.23 mmol), and 3-chloro-3-methylbut-1-yne (160 mg, 1.56 mmol) in acetone (20 mL) was refluxed for 24 h. The reaction mixture was filtered and the residue washed with acetone (20 mL). The filtrate and washings were combined and the solvent removed under reduced pressure. The residue was purified by PCTLC (hexanes-ether 9:1) to obtain **13** as a colorless liquid (150 mg, 30%): UV (CH_3CN), λ_{max} (ε) 302 (5 850), 262 (13 735) nm; IR (neat) 3063, 3001, 2953, 2847, 1653, 1616, 1578, 1435, 1381, 1306, 1215, 1159, 1047, 700 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.18 (m, 5H), 7.10 (d, J = 2 Hz, 1H), 6.32 (d, J = 2 Hz, 1H), 3.82 (s, 3H), 3.72 (s, 3H), 3.87 (s, 4H), 2.60 (s, 1H), 1.64 (s, 6H); EIMS *m/z* (relative intensity) 352 (M^+, 0.5) 321 (0.9), 286 (2.8), 254 (19), 165 (6), 105 (2.6), 91 (100), 77 (16.1), 67 (23.5); EIHRMS calcd for $C_{20}H_{17}O_7$: 352.16732; found: 352.16729.

3-O-(α, α-Dimethylallyl)-5-methoxybibenzyl (14). To a solution of **13** (100 mg, 0.28 mmol) in methanol (25 mL) was added $BaSO_4$/Pd-C (10%) poisoned by quinoline (9 mg) and hydrogenated for 2 h at room temperature and 760 mm/Hg. The reaction mixture was filtered through celite and the residue washed with methanol (4 x 5 mL). The solvent was

evaporated and the residue was purified by PCTLC (hexanes-ether 3:1) to give **14** as a colorless liquid (91 mg, 91%): UV (CH_3CN), λ_{max} (ε) 229 (1435), 283 (365, sh), 305 (130, sh) nm; IR (neat) 2982, 2932, 1728, 1601, 1581, 1454, 1431, 1325, 1261, 1219, 1157, 1132, 1099, 1049 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.20 (s, 5H), 6.56 (d, J = 2 Hz, 1H), 6.28 (d, J = 2 Hz, 1H), 6.20 (m, 1H), 3.84 (s, 3H), 3.66 (s, 3H), 2.84 (s, 4H), 1.44 (s, 6H); EIMS *m/z* (relative intensity) 354 (M^+, 32), 337 (48), 322 (23), 307 (85), 279 (38), 267 (50), 238 (29), 226 (100), 207 (18.4).

Anal. Calcd for $C_{22}H_{26}O_4$: C, 74.55; H, 7.39. Found: C, 74.40; H, 7.30.

Amorfrutin A Methyl Ester (15). A solution of **14** (85 mg, 0.24 mmol) in N,N-dimethylaniline (3 mL) was refluxed for 2 h. The reaction mixture was cooled and ether was added (30 mL). The mixture was transfered to a separatory funnel and washed with 2*N* sulfuric acid (2 x 10 mL) and brine (2 x 15 mL). The organic layer was dried (Na_2SO_4) and the solvent removed. The residue was purified by PCTLC (hexanes-ether 5:1) to give **15** (70 mg, 82%) which was identical (^1H NMR, IR, MS) to a sample prepared from natural amorfrutin A.

Amorfrutin A (1). A solution of **15** (37 mg, 0.11 mmol) and NaOH (40 mg, 1.00 mmol) in 10 mL of water was refluxed for 4 h. The reaction mixture was cooled and acidified with dil. HCl and extracted with ethyl acetate (3 x 10 mL). The combined organic layer was washed with water (2 x 10 mL) and brine (2 x 10 mL), dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was crystallized from ether-hexanes to give pure amorfrutin A (27 mg, 75%): mp 145-146 °C, which was identical (^1H NMR, IR, MS) with an authentic sample.

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