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NMR Techniques for the Structure Elucidation and Conformational Analysis of Natural Products

Geoffrey A. Cordell
Program for Collaborative Research in the Pharmaceutical Sciences,
College of Pharmacy, University of Illinois at Chicago, Chicago, IL 60612

Abstract—The combined use of the *J*-modulated selective INEPT and CSCM 1D NMR techniques is described for the structure elucidation of several new classes of compound including prionitin, the loureirins and larreantin, and for the regiosubstitution of the furanonaphthoquinones. Spectroscopic studies on the conformation of the cytotoxic agent savinin are also described, together with the NMR assignments and preliminary biosynthetic experiments on the antitumor antibiotic staurosporine.

Keywords—Structure elucidation • conformational analysis • biosynthesis • NMR techniques • lignans • alkaloids • diterpenes

Techniques for the structure determination and detailed spectroscopic analysis of biologically active natural products have advanced dramatically in the past few years. Several years ago it would not have been reasonable to unambiguously determine the complete proton and carbon assignments for a complex, new natural product. Currently, the available techniques are such that it is possible to **prove** these assignments, without relyng on prior information or using chemical shift theory to assign carbon chemical shifts. Philosophically, this is an important distinction to make, for relatively few of the carbon-13 attributions in the literature could be regarded as rigorously proven.

Perhaps the most important single development in the past ten years has been the ability to conduct correlation spectroscopy. Of the many variants of this technique that are available, three in particular have been of importance to the natural product chemist. These are, a) ^{1}H-^{1}H COSY in which either two bond or long range couplings may be emphasized, b) nOe COSY (NOESY) spectra in which proximate proton-proton relationships are displayed, and c) ^{1}H-^{13}C COSY (hetcor) spectra in which either one-bond or three-bond couplings can be shown.

The latter technique unfortunately has two substantial disadvantages, i) significant amounts of material (at least 30 mg) are normally required, which may be impossible for a new natural product, and ii) assignment of carbon signals with close chemical shifts may be difficult because the digital resolution required cannot normally be achieved in a hetcor experiment. In addition, unless the hetcor experiment can be run under conditions where the *J* value emphasized is 4~8 Hz, no information concerning the assignment of quaternary carbons is possible.

Several pulse programming sequences have recently been developed which overcome some of these disadvantages and are extremely useful.

Two of these, the CSCM 1D[1] and the selective INEPT[2] techniques have proven to be invaluable in our structure determination and spectral assignment studies where sample size was a limiting factor.[3~13] In both cases, a well resolved, unambiguously assigned proton NMR spectrum obtained at moderate to high field (300 to 500 MHz) is of crucial importance for their success.

In the CSCM 1D technique,[1] a carbon satellite either upfield or downfield of the proton is irradiated, magnetization is transfered to the attached carbon and a signal, either positive (upfield irradiation) or negative (downfield irradiation), is then observed. This might therefore be interpreted as a one dimensional technique corresponding to a ^{1}H-^{13}C hetcor experiment. The major limitation to the technique is that it is not useful in the instance of substantial proton overlap, but the substantial advantages of sensitivity and digital resolution typically outweigh this consideration.

In the selective INEPT technique,[2] the proton is irradiated with a soft pulse and the dwell time prior to observation is varied depending on the anticipated (or preferably known) coupling constant. Typically, the experiment is utilized for the selective enhancement of carbons three bonds away from the irradiated proton where J values in the range 6~8 Hz are used. The technique can also be effectively used to examine the three-bond coupling through a heteroatom, either O or N. Judicious interpretation of selective INEPT experiments therefore corresponds to a carbon-carbon connectivity study and thus the skeletal framework of the molecule under investigation.

In this brief review, the importance of these NMR techniques, particularly the selective INEPT method, in making rigorous ^{13}C-NMR assignments and in elucidating structures will be presented. Strategies involving the use of the selective INEPT technique will be described for the structure elucidation of a new diterpene skeleton, for the loureirins, for larreantin, a biogenetically novel naphthoquinone, and for furanonaphthoquinones where the ability of the technique to distinguish between the regioisomers was critical. Also described will be preliminary work on the biosynthesis of the antitumor antibiotic staurosporine and studies on the conformation of the cytotoxic lignan, savinin.

1. Prionitin

Salvia prionitis Hance (Labiatae) is native to the Southern Provinces of the People's Republic of China and is used as an antibacterial, antitubercular and antiphlogistic drug in traditional Chinese medicine. Previous phytochemical studies[14,15] on this plant have reported the isolation of several abietane and two 4, 5-seco-5, 10-*friedo*-abietane diterpenoids. The latter compounds can be formed from quinone methide abietane diterpenes through acid catalyzed migration of the 10-methyl group to C-5 accompanied by fission of ring A.[16]

From the dried and powdered roots of *Salvia prionitis* a new compound, prionitin, was isolated, mp: 98~100°, $[\alpha]_D$ −11.9° (0.042, MeOH). It exhibited a molecular ion in the high resolution mass spectrum at 310.1922 corresponding to $C_{21}H_{26}O_2$ (calcd. 310.1952), and intense absorptions in the UV spectrum (MeOH) λ_{max} (log ϵ): 254 (4.41), 296 (3.56) nm, and the IR spectrum (KBr) ν_{max}: 2975, 2955, 1645, 1575, 1470, 1370, 1300 and 1100cm^{-1} suggested the presence of a naphthalene chromophore. The ^{1}H-NMR spectrum indicated the presence of an isopropyl group (δ 1.31 d, J=6.7 Hz; δ 1.38 d, J=6.7 Hz; δ 3.52 sep., J=6.7 Hz), an aromatic methyl (δ 2.37 s), an aromatic methoxyl (δ 3.88 s) and two non-equivalent aliphatic methyl groups (δ 1.17 s and 1.71 s) which suggested the presence of an abietane

type diterpene skeleton. The two *ortho* coupled aromatic protons at δ 7.08 (d, J=8.8 Hz) and δ 7.68 (d, J=8.8 Hz) were in good agreement with this hypothesis, however, the degree of unsaturation indicated that the molecule had four condensed rings instead of the usual three. The homonuclear COSY spectrum of prionitin displayed an unusual coupling pattern for the 1-H_2, 2-H_2 and 3-H protons suggesting that ring A was five-membered instead of the normal six-membered of the abietane diterpenes. The non-equivalent methylene protons appeared as geminal coupled pairs of signals at δ 3.16 and 2.80, and at δ 2.16 and 1.66. The latter two signals showed further coupling with the dd of 3-H at δ 3.34 permitting their assignment as 2-H_2 absorptions. The multiplet (dddd) at δ 2.16 exerted two small couplings (4.4 Hz and 2.5 Hz) toward 3-H (δ 3.34) and one of the 1-H_2 signals at δ 3.16, indicating the pseudo-equatorial (α) orientation of these two hydrogens.

The ^{13}C-NMR and APT spectra of prionitin exhibited six methyl carbons, two methylene carbons, two aliphatic and two aromatic methine carbons. One aliphatic quaternary carbon appeared in the APT spectrum at δ 93.58 together with eight quaternary aromatic carbons of which two could be assigned as oxygen-bearing quaternary carbons. The unusual down-field chemical shift of the aliphatic quaternary carbon (δ 93.58) could be explained by the presence of a highly substituted oxygen-bearing carbon. Unambiguous assignment of the ^{13}C-NMR spectrum of prionitin and the unequivocal determination of its skeleton and substitution pattern were established by series of CSCM 1D,[1] selective INEPT[2] and nOe experiments. Due to the limited amount of prionitin available (ca. 4 mg), the use of one-bond or long-range HECTOR spectroscopic techniques were precluded.

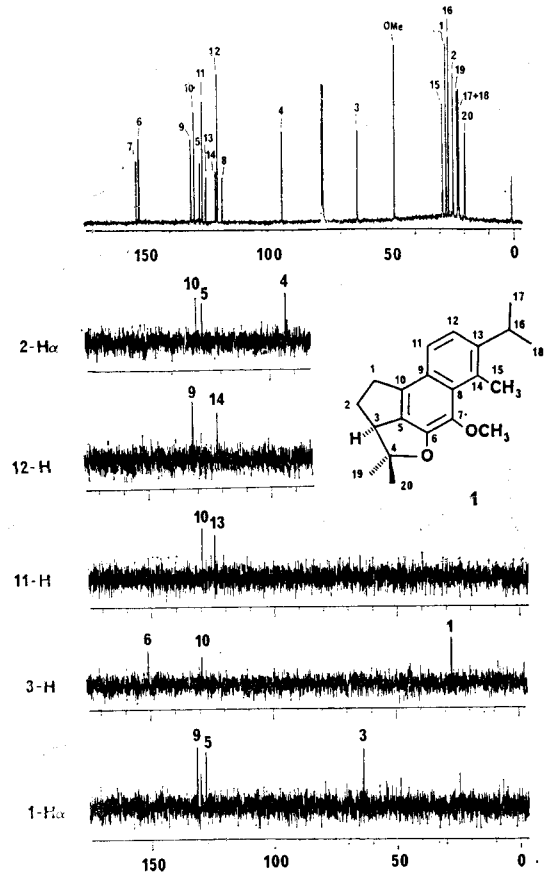


Fig. 1. Selective INEPT spectra of prionitin (1).

CSCM 1D irradiation of the ^{13}C satellite of 12-H enhanced the signal at δ 119.98 thereby permitting the assignment of C-12, and consequently the other aromatic methine carbon, C-11 at δ 126.07. Polarization transfer *via* irradiation of 3-H resulted in the enhancement of the signals at δ 26.64, 129.46 and 152.12, which could be assigned as C-1, C-10 and C-6, respectively (Fig. 1). Irradiation of 1-Hα enhanced the aliphatic methine carbon at δ 62.87, which should be C-3, and the aromatic quaternary carbons at δ 127.33 and 130.81. The latter two signals, C-5 and C-9, were distinguished through the irradiation of 2-Hα resulting in enhancements at δ 93.58 (C-4), 127.33 (C-5) and 129.46 (C-10). Selective INEPT irradiation of 12-H enhanced the signals

Table I. Proton and carbon-13 NMR assignments of prionitin (1)[a)]

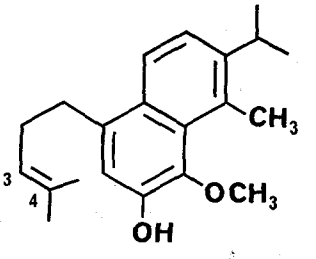
Carbon	δH	Multiplicity, J(Hz)	δC
1-Hα	3.16	ddd, 16.8, 4.2, 2.6	26.64
1-Hβ	2.80	ddd, 17.0, 12.3, 4.4	
2-Hα	2.16	dddd, 17.4, 12.1, 4.4, 2.5	23.71
2-Hβ	1.66	ddd, 17.4, 12.3, 4.5	
3	3.34	dd, 12.2, 4.6	62.87
4		—	93.58
5		—	127.33
6		—	152.12
7		—	153.22
8		—	118.07
9		—	130.81
10		—	129.46
11	7.08	d, 8.8	126.07
12	7.68	d, 8.8	119.98
13		—	124.87
14		—	120.81
15	2.37	s	28.07
16	3.52	sep., 6.7	25.68
17	1.31*	d, 6.7	21.33+
18	1.38*	d, 6.7	21.53+
19	1.71	s	21.95
20	1.17	s	18.89
OCH_3	3.88	s	47.74

[a)] Spectra were recorded in $CDCl_3$. Proton chemical shifts are reported as δ values (ppm) from internal TMS at 360 MHz. Carbon chemical shifts are reported as δ values (ppm) at 90.8 MHz.

*,+ Interchangeable.

at δ 130.81 (C-9) and 120.81, assigned as C-14, indicating that this latter carbon was substituted by an alkyl group. Magnetization transfer from 11-H led to the assignment of C-10 (δ 129.46) and C-13 (δ 124.87). The complete assignment of the ^{13}C-NMR spectra of prionitin together with its ^{1}H-NMR spectra are shown in Table I. The relative locations of the isopropyl, methyl and methoxy group were firmly established by an nOe experiment. Irradiation of the methyl singlet at δ 2.37 resulted in enhancement of the methoxyl (δ 3.88) and isopropyl methyl groups (δ 1.31 and 1.38) thereby placing the aromatic methyl group at C-14.

Prionitin (1) appears to be a novel diterpenoid skeleton biogenetically derived from a rearranged 4,5-seco-abietane intermediate (2) *via* oxidation, possible epoxidation of the $\Delta^{3,4}$ double bond, and recyclization to the tetracyclic ring system.



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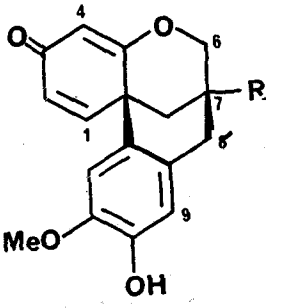
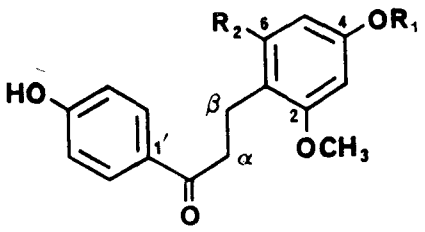
2. Loureirins—A new chalcone system

The Thai medicinal plant, *Dracaena loureiri* Gagnep (Agavaceae) was evaluated for its antibacterial constituents through an activity-directed fractionation procedure. During the course of the work, we obtained four classes of compound,[13)] namely flavonoids, chalcones, two cyclized homoisoflavan derivatives (3 and 4), to which we gave the name dracaenones, and the first four members, the loureirins (5-8),



3 R=H

4 R=OH



	R_1	R_2
5	CH_3	H
6	CH_3	OCH_3
7	H	H
8	H	OH

of another new class of natural product, the retrodihydrochalcones.[17] We have reported previously on the structure elucidation[18] and biomimetic synthesis[19] of the dracaenones.

The molecular formula of $C_{17}H_{18}O_4$ for loureirin A (5) was deduced from the EIMS in combination with the ^1H and ^{13}C-NMR spectra. A UV maximum at 279 nm, showing a bathochromic shift when NaOAc was added, suggested a free 4'-hydroxyl group, and the absence of a phenolic hydroxyl group *ortho* to the carbonyl group was demonstrated from the lack of a hypsochromic shift on the addition of $AlCl_3$. The ^1H-NMR and APT spectra displayed two sets of methylene, two methoxyl, and seven aromatic protons, in addition to a carbonyl resonance in the latter spectrum. In the proton spectrum, three of the aromatic protons appeared as an ABX pattern at 6.40 (dd, J=8.1, 2.1 Hz), 6.43 (d, J=2.1 Hz) and 7.05 ppm (d, J=8.1 Hz) assigned to H-5, H-3 and H-6, respectively, with the remaining four protons as an AA'XX' pattern at 6.92 (d, J=8.5 Hz) and 7.91 ppm (d, J=8.5 Hz), assigned to H-3'/5' and H-2'/6', respectively. The downfield resonance of the latter protons implied that they were attached *ortho* to the carbonyl group, in agreement with a 4-hydroxyacetophenone (7.9 ppm) derivative. The dominant peaks in the EIMS spectrum, at m/z 121 and 151 from α- and β-cleavage, respectively, revealed the existence in loureirin A of a monohydroxybenzoyl group, in which ring A bore no oxygen function at C-2' or C-6', and an oxygenated benzyl group.

From the available literature data on related compounds,[20~22] it appeared that assignment of the ^1H and ^{13}C-NMR chemical shifts of the aliphatic α and β positions was ambiguous. In our studies, evidence to assign H-α and H-β was obtained from the homonuclear COSY spectrum which revealed the presence of long range coupling between the signals at 7.05 (H-6) and 2.96 ppm. The latter resonance could therefore be assigned to H-β, leaving the methylene triplet at 3.19 ppm to be assigned to H-α. In addition, the long range coupling and nOe effects between the methoxy group singlet at 3.77 ppm and H-3, H-5, as well as between the singlet at 3.76 ppm and H-3 were observed in the COSY and NOESY spectra. Consequently, these methoxy group singlets were assigned to positions 4 and 2, respectively. A nOe effect between the 2-OCH_3 and H-α was also observed, in substantiation of these attributions. Thus, loureirin A was deduced to be 4'-hydroxy-2,4-dimethoxydihydrochalcone (5).

The position of the carbonyl group in the dihydrochalcone moiety of 5 was independently established with the selective INEPT technique. Polarization transfer from H-2'/6' (J=9 Hz) enhanced a carbonyl resonance at 200.86 ppm, an oxygenated carbon at 161.33 ppm (C-4'), and a protonated resonance at 130.96 ppm (C-2'/6') (Fig. 2). Transfer from H-β (J=4 Hz) enhanced the **same** carbonyl resonance at 200.86 ppm, a protonated carbon at 130.20 ppm (C-6), as well as two quaternary carbons at 158.21 (C-2) and 121.57 (C-1) ppm. Hence the car-

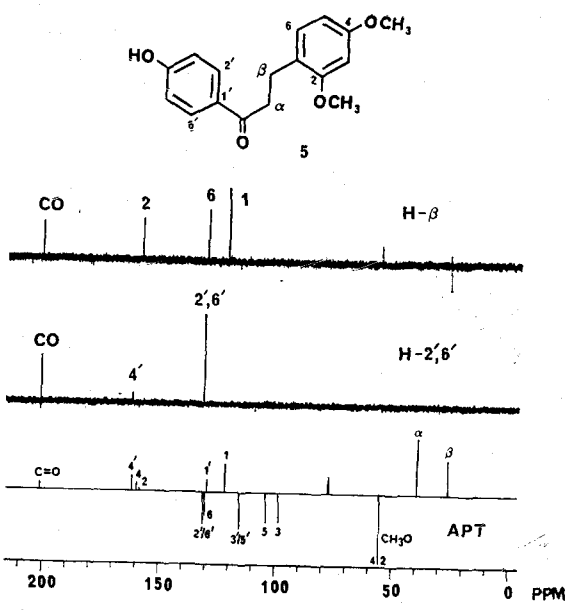


Fig. 2. Selective INEPT spectra of loureirin A(5).

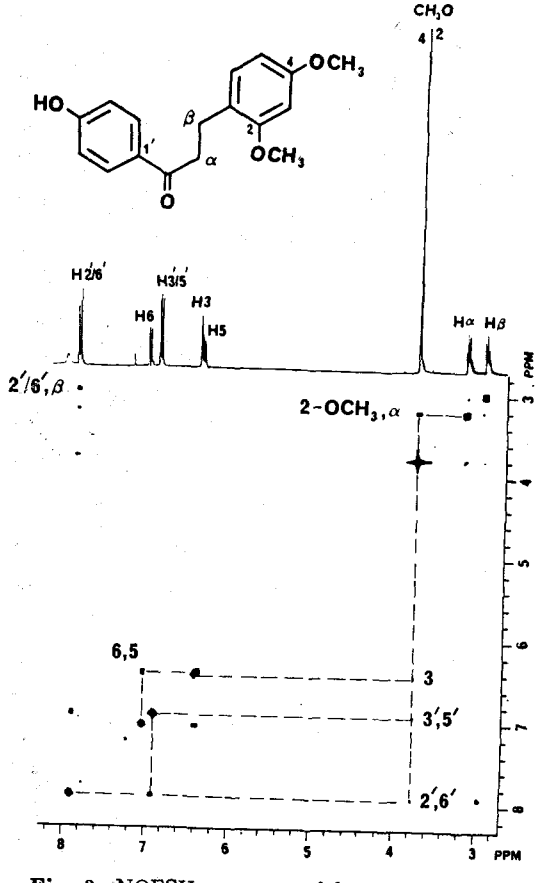


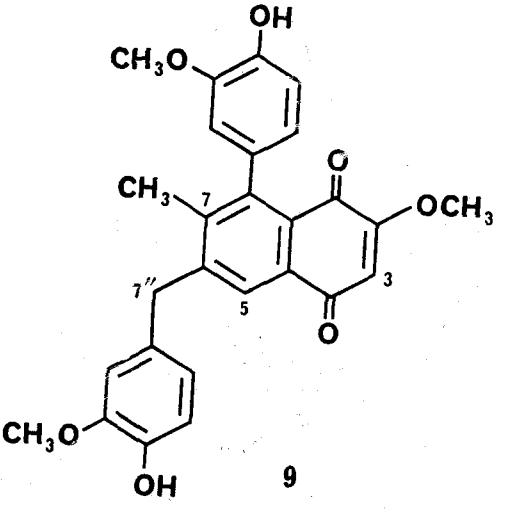
Fig. 3. NOESY spectrum of loureirin A (5).

bonyl is attached to the *para*-hydroxy benzoyl moiety, and the ethylene bridge is connected to the 2, 4-dimethoxy benzene unit. Assignment of the protonated carbons was confirmed by two-dimensional heteronuclear ^{1}H-^{13}C shift correlation.

Conformational information regarding the retrodihydrochalcones was achieved through the aid of a NOESY experiment (Fig. 3). NOE effects were observed between H-β and H-2′/6′ and between the 2-OCH_3 and H-α, H-3, H-3′/5′, H-2′/6′ in loureirin A. It was therefore concluded that the solution conformation of the retrodihydrochalcones was probably a highly distorted, bent form.

3. Larreantin

We have previously described the isolation of several new triterpenes[23] and lignans[24,25] from the stems and leaves and the roots of the creosote bush, *Larrea tridentata* (DC) Coville (Zygophyllaceae). Bioactivity-directed fractionation of the cytotoxic methanol extract afforded larreantin (9) showing, in the P-388 assay, an ED_{50} value of 0.38 μg/ml. Larreantin displayed a molecular ion at m/z 460 analyzing for $C_{27}H_{24}O_7$, and in the IR spectrum hydroxyl group absorption at 3400 cm^{-1}, and with carbonyl



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bands at 1687 and 1649 cm^{-1}.

The ^1H-NMR spectrum indicated the presence of an aromatic methyl group (δ 2.024), a benzylic methylene (δ 4.057), three aromatic methoxy groups at δ 3.792, 3.818 and 3.837, and eight aromatic protons. Six of these protons were observed in two 1,2,4- (or 1,3,4-) trisubstituted aromatic systems, with two singlet aromatic protons at δ 6.083 and 7.983. The final two protons were observed as exchangeable phenolic protons at δ 5.697 and 5.781. The interrelationships of the aromatic protons were determined through a homonuclear COSY experiment and a COSY spectrum enhancing the long range couplings established that the three aromatic methoxyl groups were each coupled to *different*, single aromatic protons.

In the ^{13}C-NMR spectrum all of the individual resonances were revealed and the APT spectrum permitted the observation of fourteen quaternary aromatic carbons, in addition to the protonated carbons anticipated from the ^1H-NMR spectrum. Two carbonyl carbons were observed at δ 185.28 and 179.93, and this information, together with an analysis of the carbon framework requirements, suggested that the central nucleus was a naphthoquinone, substituted by a methoxyl, a methyl, a 4-hydroxy-2 (or 3)-methoxyphenyl and a 4-hydroxy-2 (or 3)-methoxyphenylmethyl group.

In determining the structure, the only assumption that was made, and later confirmed, was that the singlet aromatic proton at δ 7.983 was *peri* to one of the carbonyl groups. The overall substitution on the naphthaquinone ring was deduced through the selective INEPT irradiation of the two singlet aromatic protons examining which carbonyl groups were enhanced. Selective INEPT irradiation (Fig. 4) of the *peri* proton (Fig. 4a) with $^3J_{CH}$=6 Hz enhanced three quaternary carbons (δ 185.28, 143.49 and 127.09), which should be C-4, C-8a and C-7. The latter two signals could not be further assigned at this time. Irradiation

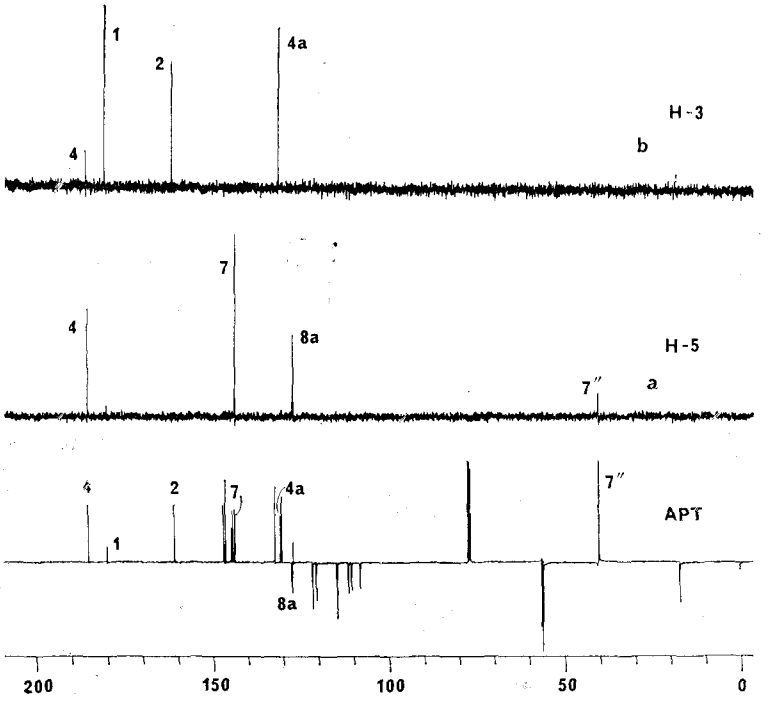


Fig. 4. Selective INEPT spectra of larreantin (9).

of the methylene group at δ 4.057 led to the enhancement of six signals, δ 146.20, 143.49, 130.33, 127.23, 121.50 and 111.37. The signal at δ 127.23 was assigned to C-5, and the latter two to C-6″ and C-2″, respectively through appropriate CSCM 1D irradiations.

The irradiation of H-3 provided extremely valuable structure information. Under nOe conditions one of the methoxyl groups (δ 3.792) was enhanced as expected, and under selective INEPT conditions (Fig. 4b) it was apparent that a **different** carbonyl carbon was also enhanced, thereby placing the methoxyl group at C-2. The COSY spectrum enhancing the long range couplings also revealed the coupling of the aromatic methyl group with both the benzylic methylene protons and H-5, indicating that the methyl group was at C-7 and the benzylic group was at C-6 or C-8. A distinction between the latter two possibilities was achieved when H-5 was irradiated under selective INEPT conditions (Fig. 4a), where it was observed that the signal at δ 40.93 was also enhanced. The benzylic group is therefore at C-6. The relationship of the hydroxy and methoxyl groups on the two phenolic rings were defined through nOe difference experiments. For example, when the 7-CH_3 was irradiated, nOe effects were observed for the 2′- and the 6′- protons, indicating the presence of a 3,4-substituted system in ring C where the 3-substituent is a methoxyl group. Larreantin therefore has the structure **9**.

Selective INEPT experiments provided internally consistent confirmatory evidence for this structure proposal. Thus irradiation of the 7-CH_3 led to the enhancement of the quaternary carbon signals at δ 146.20, 143.57 and 143.49. Irradiation of the 2′- or 6′- protons enhanced the **same** resonance at δ 143.57 indicating that it should be C-8. Irradiation of H-5 (Fig. 4a) enhanced the signals at δ 143.49 and 127.09 (C-8a), and consequently the signal at δ 143.49 could be attributed to C-7, whilst the signal at δ 146.20 should be C-6. The resonance for C-7 was also enhanced through the irradiation of the 7″-H_2.

All of the protonated carbons, including the three methoxyl carbons, could be assigned through the use of the CSCM 1D technique and the remaining quaternary carbons in the

Table II. Proton and carbon-13 NMR assignments for larreantin (**9**)

Carbon	δC[a]	δH[b]	Multiplicity, *J*(Hz)
1	179.93	—	
2	160.75	—	
3	108.04	6.083	s
4	185.28	—	
4a	130.63	—	
5	127.23	7.983	s
6	146.20	—	
7	143.49	—	
8	143.57	—	
8a	127.09	—	
1′	132.12	—	
2′	110.46	6.539	d 2.1
3′	146.86	—	
4′	144.62	—	
5′	114.52	6.954	d 7.7
6′	120.33	6.499	dd 2.1, 7.7
1″	130.33	—	
2″	111.37	6.680	d 2.1
3″	146.74	—	
4″	144.29	—	
5″	114.59	6.839	d 7.7
6″	121.50	6.620	dd 2.1, 7.7
7″	40.35	4.057	s
2 -OCH_3	55.90	3.792	s
3′ -OCH_3	55.95	3.816	s
3″-OCH_3	56.34	3.837	s
7 -CH_3	17.29	2.024	s
4′ -OH	—	5.781	s
4″-OH	—	5.697	s

[a] Recorded at 90.8 MHz in $CDCl_3$, δ TMS=0 ppm.
[b] Recorded at 360 MHz in $CDCl_3$, δTMS=0 ppm.

phenyl rings were assigned through the selective INEPT technique. In this way all of the carbon atoms in larreantin could be attributed unambiguously (Table II).

Larreantin represents a new class of natural product in which it would appear biogenetically that two isomeric phenylpropene units have combined with a preformed benzoquinone to afford an intermediate which can undergo oxidation to the naphthoquinone nucleus.

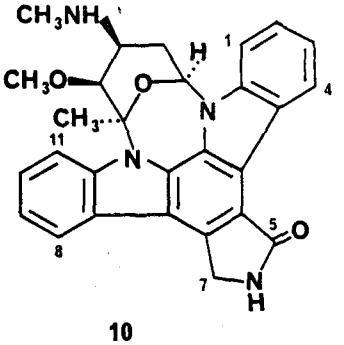
4. Biosynthesis of staurosporine

Staurosporine (**10**), an indolocarbazole alkaloid, was first isolated from *Streptomyces staurosporeus* Awaya, by Omura and co-workers[26] and subsequently from several other actinomycetes.[27,28] The structure and stereochemistry were deduced by an X-ray crystallographic analysis,[29,30] and preliminary ^{1}H and ^{13}C-NMR spectral data of staurosporine aglycone have been reported.[31] Syntheses of staurosporine aglycone based on its biogenesis have also been reported.[32,33]

Staurosporine possesses inhibitory activity against fungi and yeasts, but has no significant effects on bacteria. Preliminary evidence has also shown that staurosporine possesses strong antihypertensive activity,[34,35] pronounced *in vitro* activity against numerous experimental tumors, e.g., a human neuroblastoma cell line (NB-1),[27] HeLa S3 cells, B16 melanoma cells, and P-388 leukemia cells,[27,36,37] is a potent inhibitior of protein kinase C[37] and platelet aggregation.[26] In our laboratories, staurosporine was evaluated for cytotoxicity in both the murine P-388 lymphocytic leukemia and human carcinoma KB test systems *in vitro* according to established protocols.[38] Potent cytotoxic activity, i.e. $ED_{50}=0.0024\ \mu g/ml$ for the KB system and $ED<0.08\ \mu g/ml$ for the P-388 system was observed. However, it had no ability to bind to assembled steady-state microtubules or disrupt microtubular function. Evaluation against a variety of human cancer cell lines is presently underway.

A biogenetic pathway for staurosporine has been proposed,[33] but to date no reports on its biosynthesis have appeared. As a working hypothesis, staurosporine was viewed as being constructed from tryptophan and an amino sugar moiety which is connected by an unusual double N-glycosidic linkage. Prior to initiation of the labeling studies, conditions for the production and isolation of stauroporine were investigated in some detail.

Preliminary investigation of the ^{1}H-NMR spectrum, in different NMR solvents, of staurosporine aglycone (**11**) and of K-252a,[31,39] had previously shown that there was a chemical shift difference between the two parts of the indolocarbazole nucleus. The ^{1}H-NMR spectrum of staurosporine in $CDCl_3$ was partially assigned using homonuclear correlation spectroscopy (COSY), in combination with NOESY experiments. Remaining doubts about the assignments,



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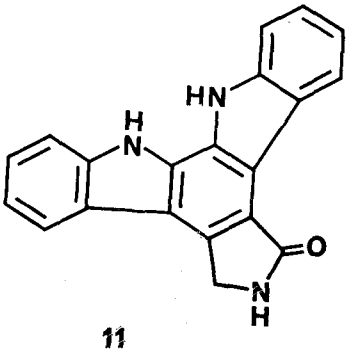

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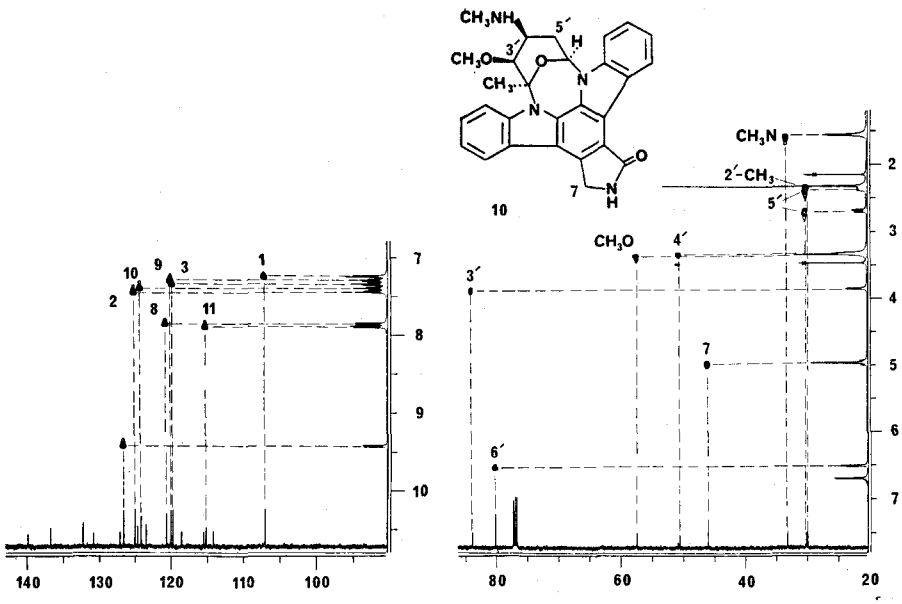


Fig. 5. HMQC spectrum of staurosporine (**10**), a) Aliphatic region, b) Aromatic region.

namely, H-1, H-9, H-10 and H-3, were resolved on the basis of a long-range ^{1}H-^{13}C connectivity study.

Subsequent examination of both broad band proton-decoupled and attached proton test (APT) ^{13}C-NMR spectra did not permit complete and unambiguous resonance identification. But, the quaternary carbon signals could not be assigned through the selective INEPT technique because of the overlap of the aromatic proton resonances, and the low solubility of staurosporine in $CDCl_3$ made the use of regular heteronuclear ^{1}H-^{13}C and 2D INADEQUATE experiments difficult. These problems were overcome by the application of sensitivity enhanced ^{1}H-detected heteronuclear multiple-quantum coherence *via* direct coupling (HMQC) [40~44] for protonated carbons and *via* multiple-bond coupling NMR spectroscopy (HMBC)[45,46] for the determination of carbon connectivity bridging of heteroatom and nonprotonated carbons, which then permitted complete ^{13}C-NMR assignment.

With the proton assignments tentatively completed, assignment of protonated carbons was obtained straightforwardly by using the ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) for correlation of ^{1}H and ^{13}C chemical shifts (Fig. 5). The use of $^{2}J_{CH}$ and $^{3}J_{CH}$ to generate multiple-bond heteronuclear multiple quantum coherence (HMBC) provided a more sensitive alternative equivalent of a heteronuclear shift correlation spectrum *via* long-range couplings (COLOC)[45,47] which had been previously used to assign the quaternary carbons in K-252a.[31] Since two-bond J_{CH} couplings in aromatic systems are relatively small, whereas three-bond couplings are usually 4~6Hz,[48] it was assumed that the correlation between indolocarbazole protons was principally due to three-bond connectivity (Fig. 6). Connectivity of H-11 with C-9 and C-7c was observed, and H-8 showed three-bond connectivity to C-7b, C-10 and C-11a and two-bond connectivity to C-7c. Distinction between C-7b and C-11a became apparent from the observation of the connectivity of H-10 with C-8 and C-11a, thereby also removing the ambiguity

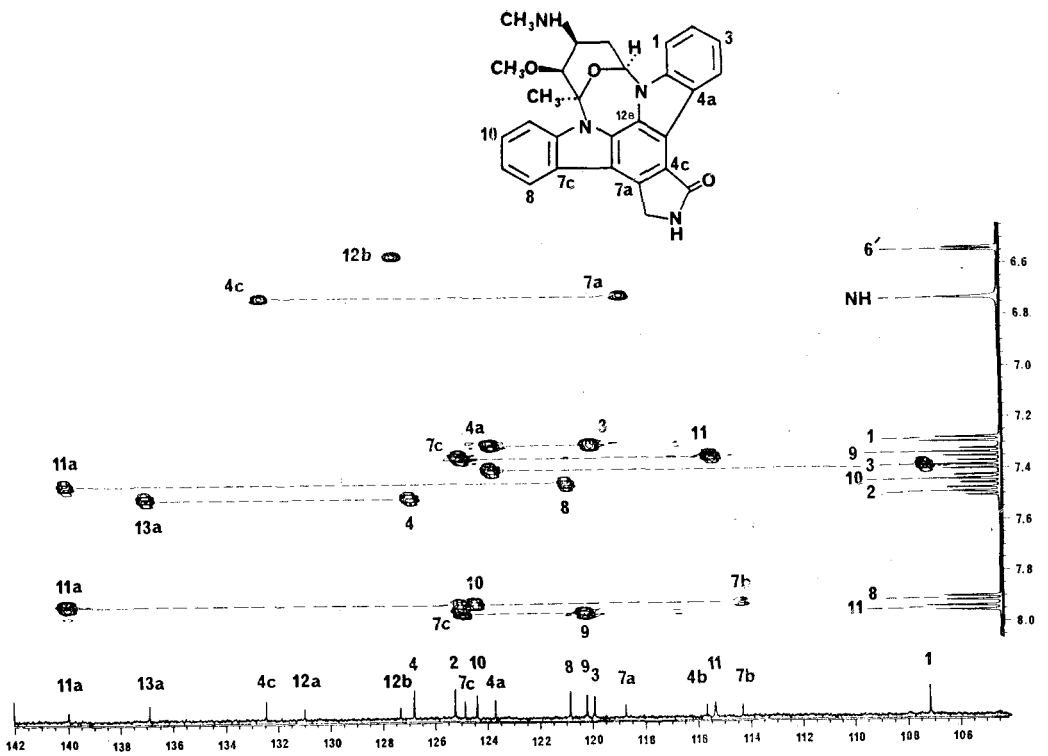


Fig. 6. HMBC spectrum of staurosporine (**10**).

between H-9 and H-10 mentioned earlier. Connectivity of the remaining indolocarbazole carbons was easily established, i.e. H-2 was coupled to C-4 and C-13a, H-3 coupled to C-1 and C-4a, H-9 coupled to C-11 and C-7c, H-1 coupled to C-3 and C-4a, and H-6′ coupled to C-12b. Interestingly, HMBC can utilize the full proton magnetization of NH-6[46,49] providing the connectivity of C-7a and C-4c. The latter assignment was confirmed by the connectivity of H-7 with this resonance, and the attribution of the remaining quaternary carbons C-4b and C-12a was made unambiguous by the observation of the connectivity of H-4 to C-2, C-13a, and C-4b. No other unidentified resonances remained, and the complete proton and carbon assignments are summarized in Table III.

Experiments designed to look at various growth media suggested that staurosporine production was at least pertly under the control of endogenous tryptophan, i.e. that feedback inhibition was in operation.[50,51] Based on a time course study of staurosporine production, the optimum time for precursor addition was found to be at the onset of significant antibiotic production,[52,53] i.e., after 24 h of the fermentation. After feeding L-[side chain-3-^{14}C] tryptophan to 24-hour old cultures of *S. staurosporeus*, staurosporine was isolated 41 hours later and purified to constant specific radioactivity. The 1.4% incorporation, indicated an effective precursor role of tryptophan in staurosporine biosynthesis.

Since tryptophan was indeed a primary precursor in staurosporine biosynthesis, it was necessary to establish that either one or two units of tryptophan was incorporated intact. This was achieved using a mixture of L-[5-^{3}H] tryptophan and L-[side chain-3-^{14}C] tryptophan

Table III. Proton and carbon-13 NMR assignments of staurosporine (**10**)

Carbon	$\delta H^{a)}$	Multiplicity, J(Hz)	$\delta C^{b)}$
1	7.26	t, 7.6	106.90
2	7.46	t, 7.6	124.96
3	7.35	t, 7.6	119.66
4	9.42	t, 7.6	127.06
4a			123.38
4b			115.34
4c			132.17
5			173.62
7	4.99	AB	45.95
7a			118.43
7b			114.00
7c			124.57
8	7.87	d, 7.8	120.57
9	7.30	t, 7.8	119.94
10	7.41	t, 7.8	124.12
11	7.91	d, 7.8	115.13
11a			139.67
12a			130.69
12b			128.26
13a			136.58
2′			91.09
3′	3.86	d, 3.6	84.05
4′	3.33	t, 3.6	50.35
5′	2.71	dd, 14.7, 3.6	30.14
	2.39	ddd, 14.7. 5.2, 3.6	
6′	6.52	d, 5.2	80.10
CH_3	2.33	s	30.00
CH_3O	3.37	s	57.27
CH_3N	1.54	s	33.27

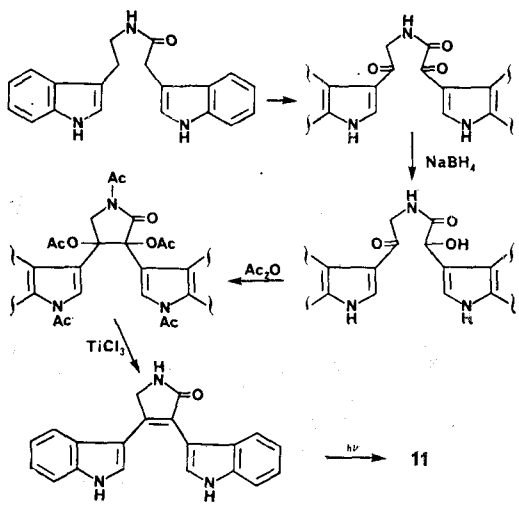
[a] Obtained at 360 MHz in $CDCl_3$, δ TMS=0 ppm.
[b] Obtained at 90.8 MHz in $CDCl_3$, δ TMS=0 ppm.

($^3H/^{14}C$=11.93) was fed. The results revealed that the ratio of ^3H and ^{14}C found in the isolated staurosporine remained the same ($^3H/^{14}C$=11.17) (Table II) as that in the precursor, thereby indicating that essentially no loss of tritium or carbon-14 had occurred in the formation of staurosporine. Hence either one or two units of tryptophan was incorporated intact.

In order to distinguish between these two alternatives, DL-[side chain-2-^{13}C] tryptophan was fed. Based on earlier studies of amino acid regulation, it was anticipated that the addition of a mixture of the two enantioners would have no effect on staurosporine production. The proton decoupled ^{13}C-NMR spectrum was compared to the reference spectrum of staurosporine under identical conditions. At 173.62 and 45.95 ppm, enhanced signals were present which, according to the carbon-13 assignments established for stauropsorine, were C-5 and C-7 of the aglycone moiety, the atoms corresponding to the β-carbons of the tryptophan precursor units. Enrichment factors of C-5 and C-7 over natural abundance (1.11%) were 17.7 and 9.2, respectively. The close agreement of the enrichment confirmed that two units of tryptophan were, indeed, incorporated with the two carbon side-chain intact into the staurosporine aglycone moiety. Although the possibility remains that there is a chiral recognition difference between the two indole moieties.

To obtain more conclusive evidence for the potential participation of acetate in the biosynthetic route, feeding experiments with [1,2-$^{13}C_2$] sodium acetate and [1-^{13}C] sodium acetate were investigated in a similar manner. The results indicated that no incorporation had occurred.

In the metabolic pathway from tryptophan to staurosporine, at least two substantially different pathways, i.e. unsymmetrical or symmetrical modification of tryptophan, are possible. One would involve carbocyclic ring formation according to the biogensis proposed by Winterfeldt.[32] In that route it was suggested that by acylation of tryptamine by indole acetic acid was followed by intramolecular carbon-carbon formation between C-4c and C-7a and subsequent cyclization between the two nucleophilic α-positions of the indole rings. This was the

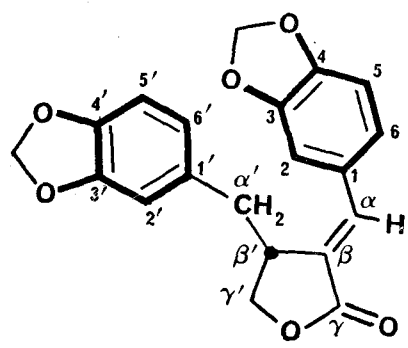


Scheme 1. Biomimetic synthesis of staurosporine aglycone (**11**).

route used for biomimetic synthesis (Scheme 1). As an alternative, in Weinreb's biogenetic hypothesis,[33] the carbocyclic ring formation of staurosporine aglycone proceeds symmetrically by condensation of two units of indole glycolic acid with ammonia. More detailed experiments are planned to delineate whether there is symmetrical or unsymmetrical incorporation of two tryptophan units, as well as to establish the precursor units of the amino sugar moiety of staurosporine.

5. Carbon-13 assignments and conformation of savinin

The conformational analysis of small flexible molecules with biological activity is presently an area of great interest. For our initial studies in this area we chose savinin (**12**), an α-arylidene-γ-lactone lignan found in several plant extracts,[54~61] and isolated by us as a cytotoxic constituent of *Aristolochia indica* roots.[62] The present study of savinin was initiated to i) establish the proton and carbon-13 nmr assignments, and ii) provide information regarding the solution conformation. In conducting this study a broad combination of complimentary 1D and 2D NMR techniques, including a variety of homonuclear shift correlations (COSY, Relayed COSY), two dimensional nuclear Overhauser experiments(NOESY), heteronuclear shift correlation (hetcor) emphasizing both one-bond and long-range coupling were employed. As a result complete and unambiguous assignments of both the carbon and proton chemical shifts of savinin including several revisions to our previous assignments have been achieved.



The NOESY spectrum, using a mix time of 3 sec revealed that the α'-protons were not proximate to the β'- or γ'-protons. But that the β'-H was proximate to the 2-and 6-protons, and the 2-H also showed a nOe cross peak with the 2'-H (Fig. 7). Since the γ'-proton

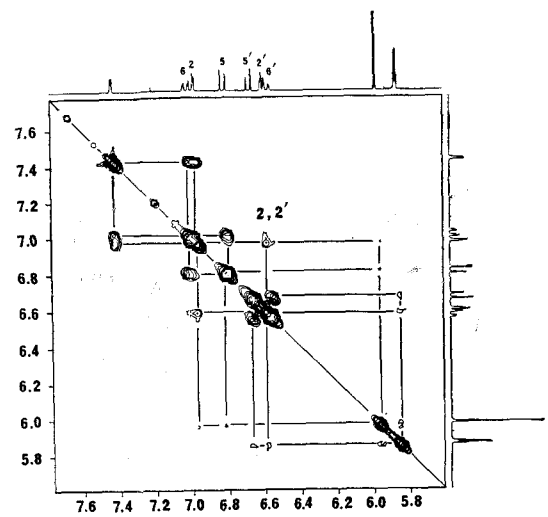
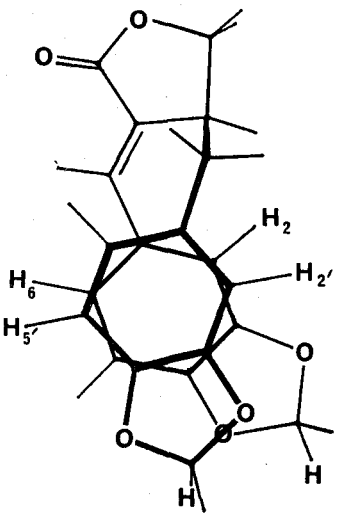


Fig. 7. NOESY spectrum of savinin (**12**).



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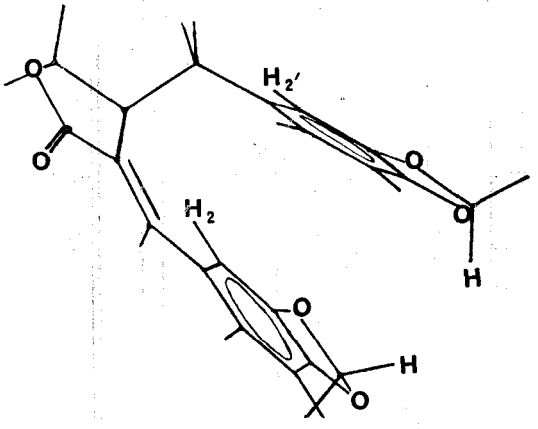
was not coupled to one of the α'-H, these can be attributed stereotopically. A weak nOe was also observed between the pairs of methylenedioxy protons, but this still has not yet afforded a precise conformation of savinin in solution. Our next step will be to conduct quantitative nOe experiments in order to establish interproton distance ranges. We will then be able to compare those data with the results of molecular force field calculations which have led to two energy-minimzed conformations shown in **13** and **14**. In these conformers, the 2'-H to 2-H interproton distance is 2.734Å in **13** and is 2.959Å in **14**, where the intermethylenedioxy proton distances are 2.736Å and 2.468A, respectively. The energy minimized calculations for **13** and **14** are 46.128 Kcal/mol and 45.820 Kcal/mol, respectively.



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6. Furanonaphthoquinones

Finally, we would like to discuss a classic example of a structure elucidation where the selective INEPT technique provided rapid resolution to an otherwise difficult problem.[62~66] Wagner and co-workers[67] isolated a mixture of the furanonaphthoquinones **15** and **16** from *Tabebuia avellandeae* (Bignoniaceae), and succeeded in separating one of the isomers by HPLC only with great difficulty. It was felt that a distinction between the two possible structures could be made on the basis of the irradiation of the furan 3-proton and the aromatic proton *peri* to the carbonyl group. The theory was that in isomer **15** irradiation of these two protons would lead to the same carbonyl carbons being enhanced, whereas in isomer **16** two different carbonyl carbons would be enhanced. In practice, the experiment proved somewhat more difficult than anticipated because only 4.5 mg of the compound was available and because although the coupling constant between the *peri* proton and the carbonyl was correctly estimated as about 4 Hz, we were surprised to find that the coupling constant between the furan 3-proton and the carbonyl carbon was only 1.5 Hz.[68]

Thus initially we estimated the ^{1}H-^{13}C coupling constant between H-3 and the carbonyl to be of the order 6 Hz. However, this gave rise to enhancement of C-9a and when the delay time was adjusted to correspond to a J value of 4 Hz, C-3a was enhanced. A similar result was obtained using J=3 Hz. It was only when the delay was set for J=1.5 Hz that the carbonyl group at δ 187.01 was selectively enhanced. In the case of the irradiation of

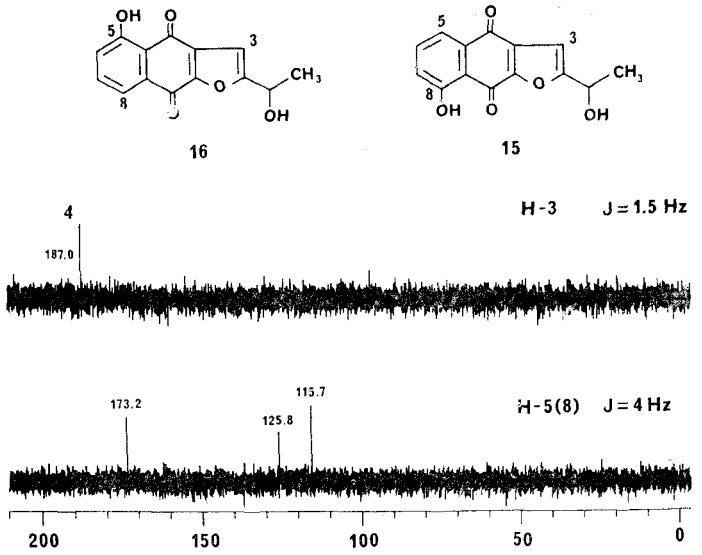

Fig. 8. Selective INEPT spectra on the furanonaphthoquinone **15.**

H-5(8), the delay was set for $J=4$ Hz with the result that three carbons were enhanced, C-6, C-8a and C-9 (Fig. 8).

The results of the irradiation experiments indicated that the two protons were three-bond coupled to two **different** carbonyl groups. Consequently, the pure isolate has the structure **16** and its isomer **15**. The complete carbon-13 assignments of the isolate were determined through the concurrent use of the CSCM 1D technique.

Summary—The selective INEPT technique provides a facile solution to what would otherwise be difficult or intractable problems of structure elucidation and, in combination with the CSCM 1D technique permits unambiguous carbon assignment when sample size is limited. Some examples have included prionitin,[69] larreantin,[70] the loureirins[17] and the furanonaphthoquinones.[71] We have also described efforts to examine the conformation of savinin in solution[72] and the biosynthesis of staurosporine.[73]

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Literature Cited

1. Sarkar, S.K. and Bax, A.: *J. Magn. Reson.* **62**, 109 (1985).
2. Bax. A.: *J. Magn. Reson.* **57**, 314 (1984).
3. Lin, L.-J. and Cordell, G.A.: *J. Chem. Soc., Chem. Commun.* 377 (1986).
4. Schun, Y. and Cordell, G.A.: *J. Nat. Prod.* **49**, 483 (1986).
5. Schun, Y. and Cordell, G.A.: *J. Nat. Prod.* **49**, 806 (1986).

6. Schun, Y. and Cordell, G.A.: *J. Nat. Prod.* **50**, 195 (1987).
7. Lin, L.-J., Meksuriyen, D., Cordell, G.A., Woo, W.S., and Lee, C.K.: *Kor. J. Pharmacogn.* **18**, 94 (1987).
8. Blasko, G., Xun, L., and Cordell, G.A.: *J. Nat. Prod.* **51**, 60 (1988).
9. Hamburger, M.O., Cordell, G.A.. Tantivatana, P., and Ruangrungsi, N.: *J. Nat. Prod.* **50**, 696 (1987).
10. Meksuriyen, D., Lin, L.-J., Cordell, G.A., Mukhopadhyay, S., and Banerjee, S.K.: *J. Nat. Prod.* **51**, 88 (1988).
11. Blasko, G., Cordell, G.A., Bhamarapravati, S., and Beecher, C.W.W.: *Heterocycles* **27**, 911 (1988).
12. Topcu, G., Cordell, G.A., Farnsworth, N.R., and Fong, H.H.S.: *J. Pharm. Sci.*, **77**, 553 (1988).
13. Meksuriyen, D. and Cordell, G.A.: *J. Sci. Soc. Thailand*, **14**, 3 (1988).
14. Lin, L.-Z., Wang, X.-M., Huang, X.-L., Huang, Y., and Yang B.-J.: *Planta Medica*, in press.
15. Lin, L.-Z., Blaskó, G., and Cordell, G.A.: *Phytochemistry*, in press.
16. Lin, L.-Z., Blaskó, G., and Cordell, G.A.: *Phytochemistry*, in press.
17. Meksuriyen, D, and Cordell, G.A.: *J. Nat. Prod.*, in press.
18. Meksuriyen, D., Cordell, G.A., Ruangrungsi, N., and Tantivatana, P.: *J. Nat Prod.*, **50**, 1118 (1987).
19. Blaskó, G. and Cordell, G.A.: *Heterocycles* **27**, 445 (1988).
20. Jensen, S.R., Nielsen, B.J., and Norn, V.: *Phytochemistry* **16**, 2036 (1977).
21. Tanaka, T., Kawamura, K., Kohda, H., Yamasaki, K., and Tanaka, O.: *Chem. Pharm. Bull.* **30**, 2421 (1982).
22. Gonzalez, A.G., Darias, V., Suarez, M.C., and Janssen, K.: *Farmaco. Ed. Sci.* **38**, 3 (1983).
23. Konno, C., Xue, H.-Z., Lu, Z.-Z., Soejarto, D.D., Cordell, G.A., Fong, H.H.S., and Hodgson, W.: *Phytochemistry* **27**, 233 (1988).
24. Konno, C., Xue, H.-Z., Lu, Z.-Z., Ma, B.-X., Erdelmeier, C.A.J., Cordell, G.A., Soejarto, D.D., Waller, D.P., and Fong, H.H.S.: *J. Nat. Prod.*. Submitted for publication.
25. Konno, C., Xue, H.-Z., Lu, Z.-Z., Ma, B.-X., Erdelmeier, C.A.J., Cordell, G.A., Soejarto, D.D., Waller, D.P., and Fong, H.H.S.: Unpublished results.
26. Omura, S., Iwai, Y., Hirano, A., Nakagawa, A., Awaya, J., Tsuchiya, H., Takahashi, Y., and Masuma, R.: *J. Antibiot.* **30**, 275 (1977).
27. Morioka, H., Ishihara, M., Shibai, H., and Suzuki, T.: *Agric. Biol. Chem.* **49**, 1959 (1985).
28. Oka, S., Kodama, M., Takeda, H., Tomizuka, N., and Suzuki, T.: *Agric. Biol. Chem.* **50**, 2723 (1986).
29. Furusaki, A., Hashiba, N., Matsumoto, T., Hirano, A., Iwai, Y., and Omura, S.: *J. Chem. Soc. Chem. Commun.* 800 (1978).
30. Furusaki, A., Hashiba, N., Matsumoto, T., Hirano, A., Iwai, Y., and Omura, S.: *Bull. Chem. Soc. Japan* **55**, 3681 (1982).
31. Yasuzawa, T., Iida, T., Yoshida, M., Hirayama, N., Takahashi, M., Shirahata, K., and Sano, H.: *J. Antibiot.* **39**, 1072 (1986).
32. Sarstedt, B. and Winterfeldt, E.: *Heterocycles* **20**, 469 (1983).
33. Weinreb, S.M., Garigipati, R.S., and Gainor, J.S.: *Heterocycles* **21**, 309 (1984).
34. Omura, S., Iwai, Y., and Hirano, A.: Ger. Offen. 2,754,326; *CA* **89**, 58348y (1978).
35. Omura, S., Iwai, Y., and Hirano, A.:, Japan. Kokai 78 73,501; *CA* **89**, 178086h (1978).
36. Morioka, H., Shibai, H., Yokogawa, Y., Ishihara, M., Kida, T., and Suzuki, T.: Japan. Kokai 85 185,719; *CA* **104**, 18649u (1986).
37. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F.: *Biochem. Biophys. Res. Commun.* **135**, 397 (1986).
38. Geran, R.I., Greenberg, N.H., MacDonald, M.M., Schumacher, A.M., and Abbott, B.J.: *Cancer Chemother. Rep.* **3**(1), 1 (1972).
39. Kase, H., Iwahashi, K., and Matsuda, Y.: *J. Antibiot.* **39**, 1059 (1986).
40. Muller, L.: *J. Am. Chem. Soc.* **103**, 4481 (1981).
41. Bendall, M.R., Pegg, D.T., and Doddrell, D.M.:

J. Magn. Reson. **52**, 81 (1983).
42. Bax, A., Griffey, R.H., and Hawkins, B.L.: *J. Magn. Reson.* **55**, 301 (1983).
43. Bax, A., Griffey, R.H., and Hawkins, B.L.: *J. Am. Chem. Soc.* **105**, 7188 (1983).
44. Live, D.H., Davis, D.G., Agosta, W.C., and Cowburn, D.: *J. Am. Chem. Soc.* **106**, 6104 (1984).
45. Kessler, H., Bermel, W., and Griesinger, C.: *J. Am. Chem. Soc.* **107**, 1083 (1985).
46. Summers, M.F., Marzilli, L.G., and Bax, A.: *J. Am. Chem. Soc.* **108**, 4285 (1986).
47. Kessler, H., Griesinger, C., Zarbock, J., and Loosli, H.R.: *J. Magn. Reson.* **57**, 331 (1984).
48. Hansen, P.E.: *Prog. NMR Spectrosc.* **14**, 175 (1981).
49. Bax, A., Aszalos, A., Dinya Z., and Sudo, K.: *J. Am. Chem. Soc.* **108**, 8056 (1986).
50. Alim, S. and Ring, K.: *Arch. Microbiol.* **111**, 105 (1976).
51. Ring, K., Gross, W., and Heinz, E.: *Arch. Biochem. Biophys.* **137**, 243 (1970).
52. Chiu, S.H.L., Fiala, R., Kennett, R., Wozniak, L., and Bullock, M.W.: *J. Antibiot.* **37**, 1000 (1984).
53. Beale, J.M., Jr., Chapman, R.L., and Rosazza, J.P.: *J. Antibiot.* **37**, 1376 (1984).
54. Matsumura, M. and Okumura, F.S.: *J. Am. Chem. Soc.* **77**, 1906 (1955).
55. Lin, Y.T., Wang, K.T., and Weinstein, B.: *Chem. Commun.* 592 (1965).
56. Reisch, J., Novak, I., Szendrei, K., and Minker, E.: *Die Pharmazie* **22**, 220 (1967).
57. Martinez, E.A., Funes, J.L.B., Gonzalez, A.G., and Luis, F.R.: *Anal. Real Soc. Esp. Fis. Quim.* **65**, 809 (1969).
58. Reisch, J., Szendrei, K., Novak, I., and Minker, E.: *Die Pharmazie* **25**, 435 (1970).
59. Corrie, J.E.T., Green, G.H., Ritchie, E., and Taylor, W.C.: *Aust. J. Chem.* **23**, 133 (1970).
60. Badawi, M.M., Seida, A.A., Kinghorn, A.D., Cordell, G.A., and Farnsworth, N.R. *J. Nat. Prod.* **44**, 331 (1981).
61. Ozaki, N., Hasegawa, S., and Hirose, Y.: *Phytochemistry* **22**, 1771 (1983).
62. Che, C.-T., Ahmed, M.S., Kang, S.S., Waller, D.P., Bingel, A.S., Martin, A., Rajamehendran, P., Bunypraphatsara, N., Cordell, G.A. Soejarto, D.D., Wijesekera, R.O.B., and Fong, H.H.S.: *J. Nat. Prod.* **47**, 331 (1984).
63. Inoue, K., Inouye, H., and Chen, C.C.: *Phytochemistry*, **20**, 2271 (1981).
64. Rao, M. and Kingston, D.G.I.: *J. Nat. Prod.* **45**, 600 (1982).
65. Dominguez, X.A., Franco, R., Cano, G., Consuelo Garcia, M., Dominguez, X.A., and de la Pena, L.: *Planta Med.* **49**, 63 (1983).
66. Ueda, S., Inoue, K., Shiobara, Y., Kimura, I., and Inouye, H.: *Planta Med.* **40**, 168 (1980).
67. Wagner, H., Kreher, B., and Seligmann, O.: Unpublished results.
68. Benassi, R., Folli, U, Mucci, A., Schnetti, L., and Taddei, F.: *Magn. Reson. Chem.* **25**, 804 (1987).
69. Blaskó, G., Lin, L.-Z., and Cordell, G.A.: *J. Org. Chem.*, Submitted for publication.
70. Luo, Z., Meksuriyen, D., Fong, H.H.S., and Cordell, G.A.: *J. Org. Chem.* **53**, 2183 (1988).
71. Hamburger, M.O., Cordell, G.A., Kreher, B., and Wagner, H.: *Helv. Chim. Acta*, in preparation.
72. Shieh, H., Lankin, D.C., and Cordell, G.A.: *Magn. Reson. Chem.*, in preparation.
73. Meksuriyen, D. and Cordell, G.A.: *J. Nat. Prod.*, in press.