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ONE-DIMENSIONAL PROTON-CARBON CORRELATIONS FOR THE STRUCTURE DETERMINATION OF NATURAL PRODUCTS

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ABSTRACT.- The selective INEPT technique, a one-dimensional, *J*-modulated, proton-carbon correlation technique, has proved to be a very powerful tool for both unambiguous carbon and proton spectral assignment, as well as providing critical carbon framework information. In this brief review we offer some examples of recent work in our laboratories which demonstrates the power of the technique, particularly when sample size is limited.

INTRODUCTION

NMR techniques for the detailed spectroscopic analysis and structure elucidation of biologically active natural products have resulted in a bewildering array of experiments which, given appropriate software and instrumentation, one might potentially apply to solve a given problem[1,2]. For the natural product chemist, who is typically faced with the enigma of spectral and/or structure assignment vs. biological evaluation, optimum use of analytical technologies is essential. It was relatively recently that it became possible to unambiguously determine the complete proton and carbon assignments for a complex, new natural product. Currently, a variety of correlation shift experiments is available to prove these assignments, without relying on prior data or using chemical shift theory to assign particular carbon chemical shifts. The reasons to firmly establish assignments are two-fold: firstly, as a matter of scientific precision, and secondly, as an essential adjunct to studies aimed at examining either the ways in which molecules interact with enzymes during the course of biological reactions, or to monitor biological processes.

Undoubtedly, the most important single development in NMR spectroscopy in the past ten years has been the ability to conduct correlation spectroscopy[2] and many variants of this technique are available. Three of these in particular have been of importance to the natural product chemist: a) ^{1}H-^{1}H COSY, in which either two bond or long range couplings may be emphasized, b) nOe COSY (NOESY), in which proximate proton-proton relationships are displayed, and c) ^{1}H-^{13}C COSY (HETCOR), in which either one-bond or three-bond couplings can be emphasized.

Unfortunately, the HETCOR technique, although exceptionally powerful and widely used, 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. 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. Inverse detection may provide some opportunities to address some of these issues, but sample size is still limited for HMBC and HMQC experiments, and the difficulties of assigning carbons with close chemical shifts cannot be ignored.

In addressing these issues, we have resorted to the use of two powerful NMR pulse programming sequences originally developed by Bax, the CSCM 1D[3] and the selective INEPT[4]. These techniques have been invaluable for both spectral assignment and structure determination studies where sample size was a limiting factor for the use of two-dimensional techniques. It cannot be emphasized too strongly that for both techniques, a well resolved, unambiguously assigned proton nmr spectrum obtained at moderate to high field (300 to 500 MHz) is of critical importance. We have tried to develop these techniques for natural product chemists, and aspects of this work have been reviewed[5,6] and described in the proceedings of various meetings[7-13]. Table 1 indicates some of the broad range of natural products that we have studied, and in this brief review we offer some specific examples that have involved the use of the selective INEPT technique.

In the selective INEPT technique[4], the proton is irradiated with a soft pulse and the delay 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 6-10 Hz may be used. Importantly, the technique can also be effectively used to examine the three-bond coupling through a heteroatom, either O or N. A well-constructed series of selective INEPT experiments therefore corresponds to a carbon-carbon connectivity study, and often reveals the skeletal framework of the compound under investigation. The importance of the selective INEPT method in making rigorous carbon-13 nmr assignments and in the structure elucidation of natural products will be illustrated using a variety of alkaloid, terpenoid and flavonoid skeleta as examples. Throughout the course of this work, and particularly with small samples, judicious choice of both the experiments and the conditions was essential in order to minimize spectrometer time. Since the selective INEPT technique is J-modulated, some information about three-bond couplings is necessary. This was obtained in some cases from literature values, in some cases through determination from the coupled carbon spectrum, while in other cases an empirical approach was necessary, and the more that we study the technique, the more surprised we are about the magnitude of $^3J_{CH}$ values. Simplicity in the resulting enhanced spectrum was typically sought in order to avoid, as far as possible, the enhancement of carbons two and four carbons away from the irradiated proton. When several protons in a molecule are successively irradiated and the resulting spectra analyzed, an essential element in assignment is internal consistency of the data.

From a historical perspective, our first foray into this area involved the coumarinolignans and resulted in the revision and/or resolution of several structures[44,45,84]. The strategy for the structure elucidation is shown conceptually in Fig. 1 and is described in detail elsewhere[45]. Fundamentally, the question is whether in an individual regioisomer the protons at C-5 and C-7′ are correlated to the **same** or **different** carbon atoms.

The strategy of whether the same or a different carbon was enhanced during the selective INEPT experiments[85] was one which became, and continues to be, a recurring theme[5,6]. One of the prime examples of such a strategy provided a unique and rapid solution to an old and difficult dilemma involving the structure elucidation of a family of immunostimulating furanonaphthoquinones[54] isolated from a traditional anticancer remedy from Argentina, *Tabebuia avellandeae* (Bignoniaceae).

Table 1. Examples of the Selective INEPT Technique used for the Spectral Assignment and Structure Elucidation of Natural Products[a].

Spectral Assignment	Structure Elucidation
Acronycine oxime (14)	Abrusoside A (38,39)
Akuammidine (15)	Abrusosides B-D (39)
Americanin A (16)	(+)-Afzelechin-7-*O*-β-D-apioside (32)
Ancistrocladidine (17)	Agrostistachin (40)
Artemisinin (18)	Agroskerin (41)
Berberine (19)	Budmunchiamines (42)
Bruceantin (20)	Casuarinondiol (29)
Camptothecine (21)	Chrysoeriol-6-*C*-boivinoside (43)
Colchicine (22)	Coumarinolignans (44,45)
Didrovaltrate (23)	14-Dehydroagrostistachin (41)
Flavone (24)	10-Demethoxykopsidasinine (46)
Fulvoplumierin (25)	15-Demethylplumieride (25)
Holacanthone (20)	Dracaenones (47-50)
10-Hydroxycamptothecine (21)	Enkleine (51)
22-Hydroxytingenone (26)	Eupatorenone (52)
Hypophyllanthin (27)	Flavan/flavone derivatives (53)
Jatrorrhizine (28)	Furanonaphthoquinones (54)
Lumicolchicines (22)	*Gelsemium* alkaloids (55-64)
Lyoniresinol (29)	*Gelsemium* steroids (65)
Nimbolide (30)	Hortensin (66)
Palmatine (28)	22-Hydroxyacuminatine (67)
Phyllanthin (27)	Hydroxyborneol, 5-Exo- (68)
Plumericin (31)	2'-Hydroxyflavone (24)
Polydin (32)	4-Hydroxysapriparaquinone (69)
Polyneuridine (15)	Javanicin (70)
Pseudolaric Acid B (33)	Koumidine (71)
Pterocarptriol (34)	Larreantin (72)
Rotenone (35)	Loureirins (50,73)
Sanguinarine (19)	Marsdekoiside A (74)
Steviol 16,17α-epoxide methyl ester (36)	19-*O*-Methylangustoline (75)
Tetrahydroberberine (28)	Nimbolide, 28-Deoxo- (30)
Tetrahydrojatrorrhizine (28)	Plumerubroside (76)
Tetrahydropalmatine (28)	Polypodoside A (77)
Valtrate (23)	Polypodosides B & C (78)
Voacarpine, 16-Epi (15)	Prionitin (79)
Yenhusomine, 13-Epi (37)	Pyramidatine (80)
	Salvonitin (81)
	Salvinolone (69)
	Sapriolactone (82)
	Selina-3β,4α,11-triol (34)
	Swertiabisxanthone-I (83)
	Valepotriate derivatives (23)

[a] Reference Numbers are in parentheses.

A distinction between the two possible structures **1** and **2** was made on the basis of the irradiation of the furan 3-proton and the aromatic proton peri to the carbonyl group. In isomer **2** irradiation of these two protons would lead to the **same** carbonyl carbons being enhanced, whereas in isomer **1** two different carbonyl carbons would be enhanced. Although the experiments proved more difficult than expected, because of an unusually small $^3J_{CH}$ (1.5 Hz) for the furan 3-H and the small sample size

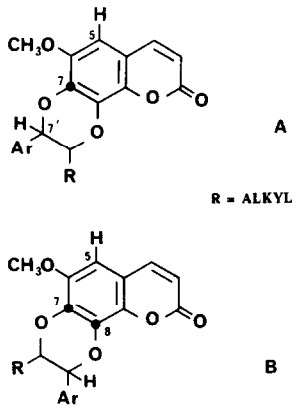


Figure 1. Structure Elucidation Strategy for Coumarinolignans.

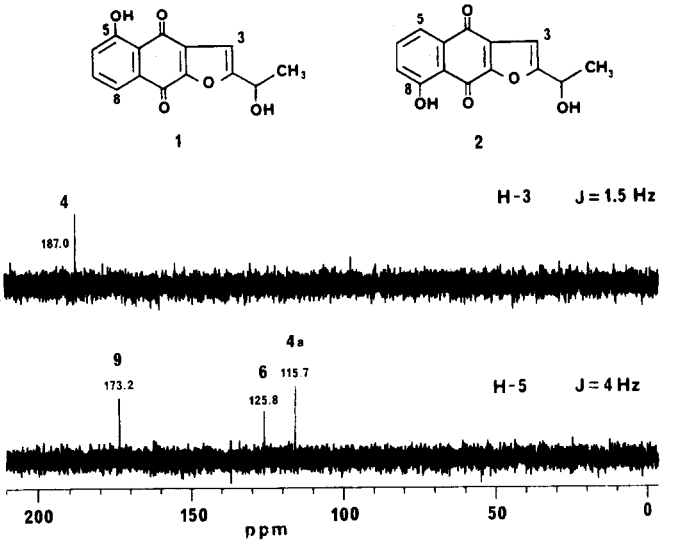
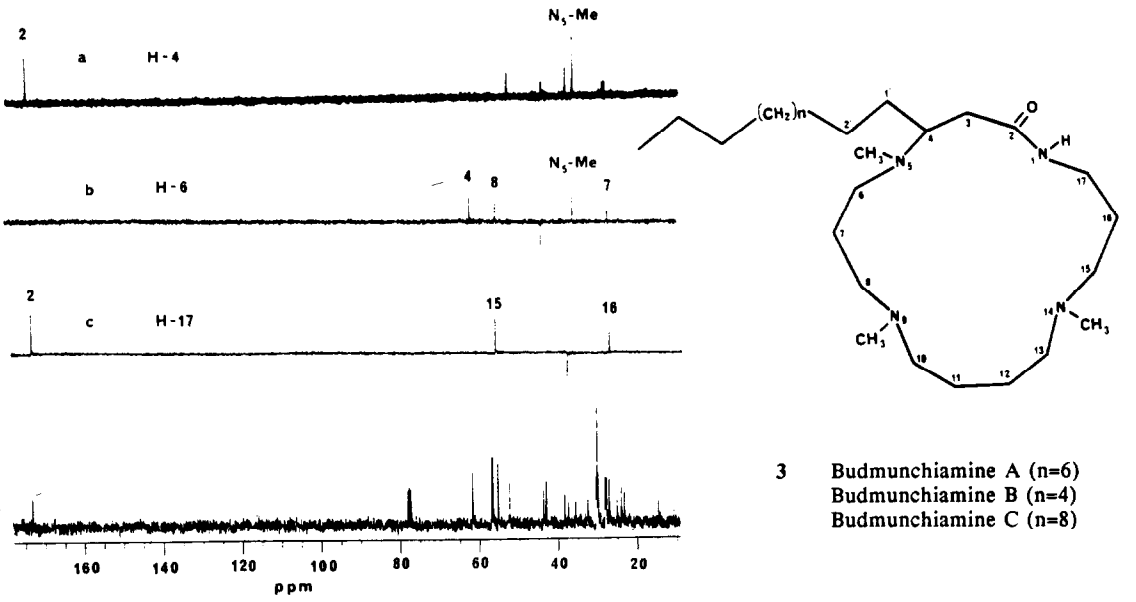


Figure 2. Selective INEPT Experiments on Furanonaphthoquinone (1).

(4.5 mg), the successful result is shown in Fig. 2 permitting the structure to be determined as 1.

As a part of our effort to find new approaches to the discovery of biologically active natural products, we have recently described the use of DNA affinity chromatography on plant extracts in order to identify potential sources of novel anticancer agents[86]. Through this technique[87], an isolate was obtained[88] as a yellow oil from an extract prepared from the seeds of *Albizia amara* Bolv. (Leguminosae)[42]. The isolate ($C_{27}H_{56}N_4O$) demonstrated no UV absorption spectrum, but did show amide ir absorption. The ^{13}C-NMR, APT and DEPT spectra displayed 24 distinct resonances, the intensity of one of these (at δ 29.6 ppm) corresponding to four carbon atoms. Consideration of chemical shift correlation values led to the conclusion that the molecule contained one C-methyl [(δ 13.5) in an aliphatic chain], three N-methyl (δ 35.3, 42.5 and 43.1), 14 C-CH_2 groups (mostly in a side chain), 5 N-CH_2 groups (δ 51.9, 55.0, 56.2, 56.6 and 56.8), one N-$CHCH_2$ (δ 37.5), one CONH-CH_2 (δ 38.0), one N-CH (δ 61.4), and one amide carbonyl carbon atom (δ 172.5). These data, in conjunction with the COSY spectrum, demonstrated the presence of two chains each containing three methylene groups, and one chain of four methylene groups in a macrocycle component together with an aliphatic side chain. The locations of these with respect to the side chain were established using the selective INEPT technique.

Selective INEPT irradiation (Fig. 3) of H-4 (δ 2.83) enhanced C-2 (δ 172.5), the N_5-methyl (δ 35.3) and C-2′ (δ 27.3) (Fig. 3a), indicating that the side chain should be located at position C-4, and the amide group located at position C-3. Irradiation of H-6 (δ 2.62) enhanced C-4 (δ 61.2), the N_5-methyl and C-8 (δ 55.0) through three bonds, and also C-7 (δ 26.8) through two bonds (Fig. 3b); these data position one of the chains containing three methylene groups between N_5 and N_9. Similarly, irradiation of H-17 (δ 3.32) enhanced the signals corresponding to C-2 and C-15 (δ 56.2), and also C-16 (δ 27.4) through two bonds (Fig. 3c). In this way, a second chain containing three methylene



3 Budmunchiamine A (n=6)
Budmunchiamine B (n=4)
Budmunchiamine C (n=8)

Figure 3. Selective INEPT Experiments on Budmunchiamines (3).

groups between N_1 and N_{14} was found. The remaining chain containing four methylene groups must be located between N_9 and N_{14}.

Thus, the *Albizia* isolate has the macrocyclic structure 3, and belongs to the class of pithecolobine alkaloids reported by Wiesner *et al.*[89-91] from *Pithecolobium saman*. Observation of the temperature-dependence of the mass spectrum revealed that peaks observed at m/z 424 and 480 belonged to minor components of the isolate with an elemental composition corresponding to $\pm C_2H_4$. The side chain in the constituents, named budmunchiamines A, B and C[92], therefore varies in length and contains 9, 11 or 13 carbon atoms in an approximate ratio of 1:4:1, respectively. Preliminary studies conducted with *in vitro* model systems have established that the isolate interacts with DNA, mediates a significant cytotoxic response with a number of cultured mammalian cells, and is bactericidal, but not mutagenic; further biological studies are in progress.

Quite a different use of the selective INEPT technique is evidenced by some recent work on the alkaloid pyramidatine (4), a new bisamide derivative of spermidine, from the Thai medicinal plant *Aglaia pyramidata* Hance (Meliaceae)[80]. The basic structure was readily determined through hrms (m/z 322.1677, for $C_{20}H_{22}N_2O_2$), UV, IR and NMR. Selective INEPT (Fig. 4), APT and HETCOR experiments led to the unequivocal assignments for all of the quaternary carbon resonances, as well as a distinction between the two amide protons. Polarization transfer following irradiation of H-3(7) at 7.84 ppm resulted in enhancements of the C-1 and C-5 resonances at 166.2 and 131.0 ppm, respectively. The amide proton at 8.48 ppm was assigned to 6'-NH since it displayed long-range coupling with C-1 (166.2 ppm) on selective INEPT irradiation emphasizing two-bond coupling. Analogous enhancement of C-1" (164.9 ppm) was observed when the 1'-NH (8.13 ppm) was selectively irradiated. Magnetization transfer via irradiation of H-2" at 6.63 ppm enhanced the C-1" (164.9 ppm)

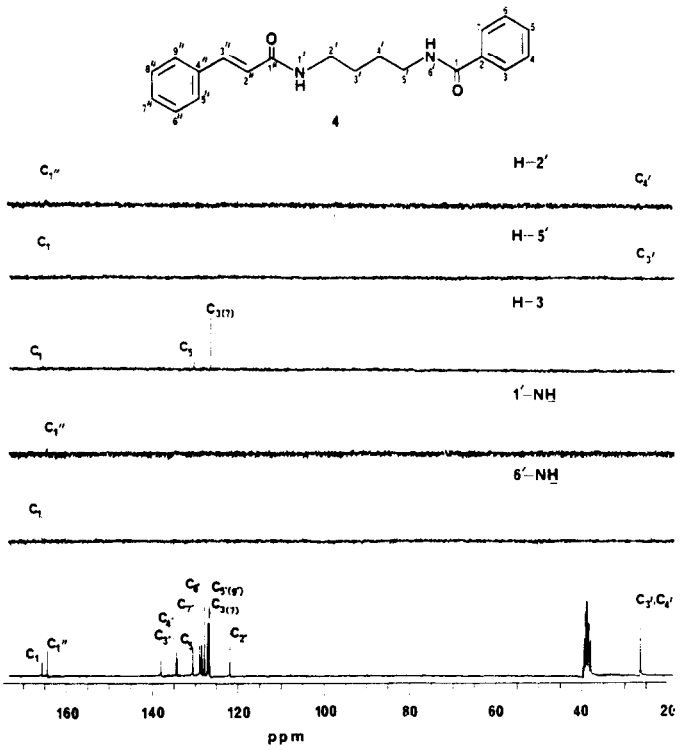
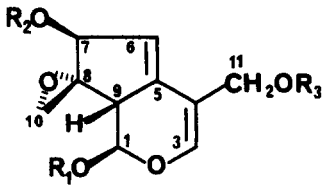


Figure 4. Selective INEPT Experiments on Pyramidatine (4).

and C-4″ (134.9 ppm) resonances. Enhancements of the C-3″ and C-7″ signals were observed through polarization transfer by irradiation of H-5″(9″). Successive selective INEPT irradiation of H-2′ and H-5′ led to the enhancement of C-1″ and C-4′, and of C-1 and C-3′, respectively, as expected. Cinnamic acid-derived bisamides are a unique group of alkaloids found in plants of the genus *Aglaia* (Meliaceae)[93-96], and are reported to possess interesting antileukemic properties *in vivo*[95]. We are presently evaluating pyramidatine (4) for its antineoplastic potential.

Valmane^R is one of more than eighty valerian preparations which are widely used in Europe as mild sedative agents[97,98]. Valmane^R is reported[98,99] to contain three active valepotriates (valtrate, didrovaltrate and "acevaltrate"). These valepotriates are chemically labile, highly cytotoxic iridoid triesters in which the hydroxy groups at C-1, C-7 and C-11 are esterified with acetic, isovaleric, hydroxyisovaleric, β-methylvaleric, and related acids[98,100-102]. Application of the selective INEPT technique has now provided the first direct and unambiguous evidence for the location of the ester groups. Thus separate irradiation of H-1, H-7 and H-11 will specifically enhance the corresponding carbonyl carbon (three-bond ^1H-^{13}C couplings) to which the various ester groups are attached, leading to the unambiguous assignment of these carbonyl carbon resonances. Furthermore, irradiation of the acetate methyl group protons will selectively enhance only the carbonyl carbon (two-bond couplings) to which the acetate methyl group is attached. Therefore, by approaching each ester carbonyl group from two bonding directions, the location of the ester groups can be assigned unambiguously.

The selective INEPT experiments carried out for valtrate (5) (Fig. 5) revealed that irradiation of H-1 resulted in the enhancement of the carbonyl signal at 170.19 ppm (Fig. 5b). Irradiation of H-7 selectively enhanced the carbonyl signal at 172.31 ppm (Fig. 5c), and irradiation of H-11 enhanced the carbonyl signal at 170.72 ppm (Fig. 5d). When the methyl of the acetate group was irradiated, using a



	R_1	R_2	R_3
5	isovaleryl	isovaleryl	acetyl
6	α-acetoxyisovaleryl	isovaleryl	acetyl
7a	isovaleryl	isovaleryl	β-hydroxyisovaleryl
7b	isovaleryl	β-methylvaleryl	β-hydroxyisovaleryl

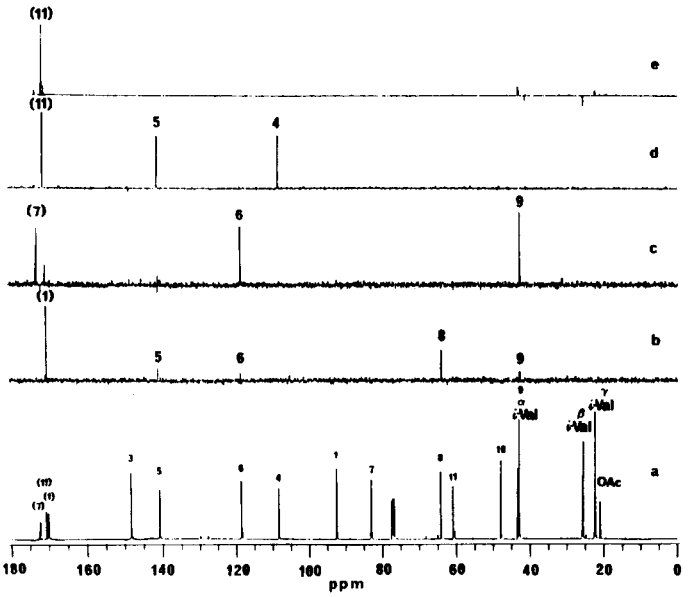


Figure 5. Selective INEPT Experiments on Valtrate (5).

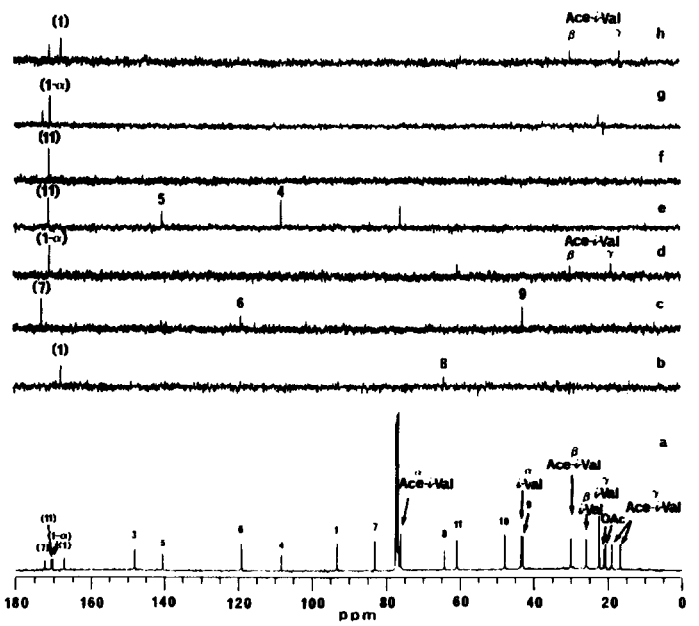


Figure 6. Selective INEPT Experiments on 1α-Acevaltrate (6).

delay corresponding to J=7 Hz, enhancement of the carbonyl signal at 170.72 ppm was observed (Fig. 5e). Thus, the acetoxy group could be placed at C-11, leaving the two isovaleryl groups to be located at C-1 and C-7. This conclusion is in agreement with the published structure **5** of valtrate[100].

The presence of three different ester groups (acetate, isovalerate and acetoxyisovalerate) was evident in Valmane^R isolate **6**. Separate irradiation (Fig. 6) of H-1 (Fig. 6b) and the doublet at 4.93 ppm (Fig. 6h) resulted in the enhancement of the same carbonyl carbon at 167.44 ppm, indicating that the acetoxyisovalerate residue was at C-1. When the downfield acetoxy signal at 2.15 ppm (Fig. 6g) and the doublet at 4.93 ppm (Fig. 6d) were irradiated the same carbonyl carbon at 170.51 ppm was enhanced; the acetoxy group could therefore be placed at the α-position of this isovalerate. By irradiating H-11 (Fig. 6e) and the upfield acetoxy signal at 2.03 ppm (Fig. 6f) it was observed that the same carbonyl carbon at 170.83 ppm was enhanced, revealing that the upfield acetoxy group was at C-11. The remaining ester group (isovalerate) was confirmed to be at C-7 by irradiation of H-7, resulting in the enhancement of the carbonyl carbon at 172.42 ppm (Fig. 6c). The structure of Valmane^R isolate **6** was therefore established to be 1-α-acevaltrate[100].

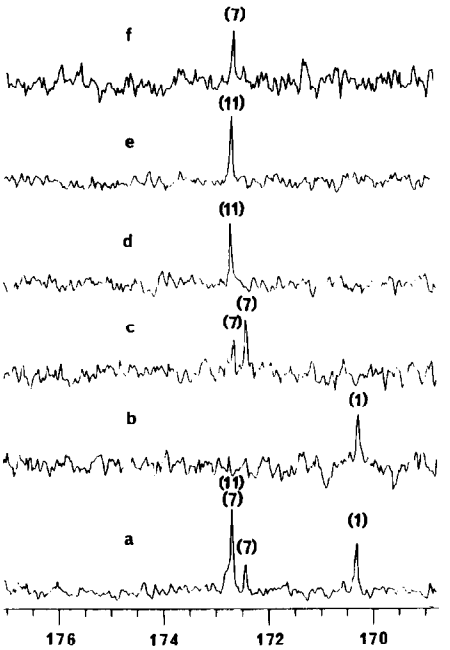


Figure 7. Selective INEPT Experiments on Valtrate Derivatives **7a** and **7b**.

Valmane^R isolate **7** was determined spectroscopically to be an unresolvable mixture of two constituents; one contained a β-hydroxyisovaleryl and two isovaleryl groups, whereas the other contained an isovaleryl, β-methylvaleryl and β-hydroxyisovaleryl group on the valtrate nucleus. Selective INEPT experiments were performed on the mixture. Irradiation of the methylene singlet at 2.47 ppm resulted in the enhancement of the carbonyl carbon at 172.68 ppm (Fig. 7e) which was the same carbonyl carbon enhanced when H-11 was irradiated (Fig. 7d), demonstrating that the β-hydroxyisovalerate was at C-11 in both compounds. For compound **7a**, which has one β-hydroxyisovaleryl and two isovaleryl groups, these two isovaleryl groups can therefore be assigned to C-1 and C-7.

For the structure determination of compound **7b**, carrying three different esterifying substituents (isovalerate, β-methylvalerate and β-hydroxyisovalerate), additional selective INEPT experiments were required. Since irradiation of H-7 resulted in the enhancement of two carbonyl carbons at 172.68 and 172.43 ppm (Fig. 7c), and irradiation of H-1 only enhanced the carbonyl carbon at 170.31 ppm (Fig. 7b), an isovalerate group must be located at C-1 in both compounds, and a β-methylvalerate at C-7 in the compound **7b**. Due to the coincidental chemical shift equivalence of the two carbonyl carbons which are attached to C-7 in **7b** and C-11, it was observed that separate

irradiation of H-7 (Fig. 7c) and H-11 (Fig. 7d) resulted in the enhancement of the same carbon signal. It was thus rationalized that in compound **7a**, which bears an isovalerate at C-7, this carbonyl carbon must exhibit a different chemical shift from that which bears a β-methylvalerate at the same position. This explains why when H-7 was irradiated, two carbonyl signals were enhanced. These two carbonyl carbons were assigned when further irradiation of the methylene protons of the β-hydroxyisovalerate protons at 2.47 ppm (Fig. 7e) or the methine proton of the β-methylvalerate at 1.84 ppm (Fig. 7f) showed that the same carbonyl carbon at 172.68 ppm was enhanced significantly. This result demonstrated that the carbonyl carbon of the compound bearing the β-methylvaleryl group at C-7 was more downfield than that of the compound bearing the isovaleryl group at C-7. The structures of these isolates in the mixture were thus determined to be **7a** and **7b**.

A chloroform extract of the twigs of *Agrostistachys hookeri* Benth. & Hook. f. (Euphorbiaceae), collected in Sri Lanka, was found to display significant inhibitory activity against P-388 lymphocytic leukemia cells. As a result of activity-guided chromatographic fractionation using this same bioassay system, four novel casbane-type diterpenoids were obtained as cytotoxic constituents from this extract[40,41]. The most abundant of these analogs, and the first to be characterized, was accorded the trivial name agrostistachin (**8**). At the time of its initial purification, agrostistachin (**8**) was only the second oxygenated casbane diterpene that had been discovered.[40]

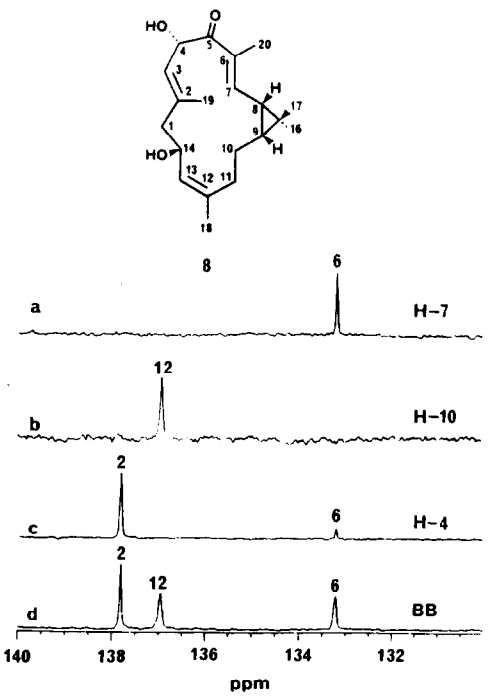
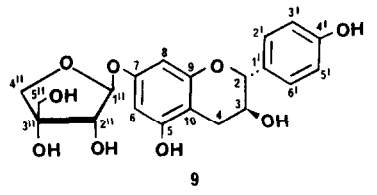


Figure 8. Selective INEPT Experiments on Agrostistachin (**8**).

Preliminary analysis of its spectroscopic parameters indicated that compound **8** was a macrocyclic diterpene possessing a cyclopropyl ring, with the presence of three double bonds, five methyl groups, two secondary hydroxyl groups and an α,β-unsaturated keto group. The compound was tentatively assigned as having a casbane skeleton by consideration of the isoprene rule and the allylic coupling evident in the ^{1}H-^{1}H COSY NMR spectra of this compound and its triacetate. In order to determine unambiguously the chemical shifts of the quaternary carbons of **8** at C-2, C-6, and C-12, recourse was taken to the selective INEPT technique. Irradiations at the 7-H (δ 6.25, J = 2 Hz), 10-H (δ 0.83, J = 8 Hz) and 4-H positions (δ 5.22, J = 6 Hz) led to enhancements of C-6, C-12, and C-2 and C-6, respectively, among the quaternary carbons of **8** (Fig. 8a-c). The geometry of the double bonds of agrostistachin (**8**) between positions C-2 and C-3, C-6 and C-7, and C-12 and C-13 was proposed as *E*, *E*, and *Z*, respectively, on the basis of comparison of ^{13}C-NMR chemical shift

values with model compounds. Confirmation of the structure and stereochemistry of this cytotoxic substance was conducted using single-crystal X-ray crystallography, employing a crystal obtained from diisopropyl ether.[40]

Conventional methods such as methylation which are used to determine the position of attachment of a sugar unit of a flavonoid glycoside are often quite time-consuming and may lead to ambiguous results. The application of the selective INEPT technique on intact heterosides has proven useful for the determination of the placement of sugars. As an initial example, (+)-afzelechin-7-*O*-β-D-apioside (9), which was isolated as a novel bitter-tasting flavonoid glycoside constituent of the rhizomes of the fern *Polypodium glycyrrhiza* D.C. Eaton (Polypodiaceae), was investigated. Preliminary spectroscopic observations on this glycoside and its products obtained by hydrolysis with 5% acetic acid indicated that the two components of the molecule were (+)-afzelechin and D-apiose.



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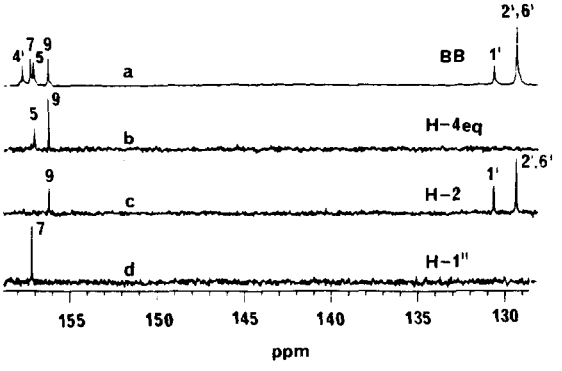

Figure 9. Selective INEPT Experiments on (+)-Afzelechin-7-*O*-β-D-apioside (9).

The position of attachment of the sugar was established as C-7 of the aglycone after three selective INEPT experiments (Fig. 9) on 40 mg of compound 9, in a total of 2 hr. When the anomeric proton (H-1″) was irradiated (δ 5.34, *J* = 6 Hz), only the C-7 carbon atom was enhanced in the downfield region of the ^{13}C-NMR spectrum of 9 (Fig. 9d). The closely related C-5 and C-7 resonances of this glycoside were differentiated by the irradiation of its 4_{eq}- (δ 2.71, *J*=6 Hz) (Fig. 9b) and 2β-protons (δ 4.60, *J*=6 Hz) (Fig. 9c), which resulted, in turn, in enhancements of C-5 and C-9, and C-9, C-1′ and C-2′,C-6′. The sugar attachment in the molecule of 9 was determined as β- by application of Klyne's rule. The validity of using the selective INEPT NMR technique in this manner was confirmed by the repetition of similar experiments on a related flavonoid heteroside of known structure, polydin [(+)-catechin-7-*O*-α-L-rhamnoside], which was also isolated by us from *P. glycyrrhiza* rhizomes.[32]

A somewhat more involved application of the utilization of the selective INEPT technique to discern the nature of saccharide substitution in a glycoside is exhibited by the potently sweet compound, abrusoside D (10). Among four novel sweet-tasting triterpene glycosides purified from *Abrus precatorius* L. (Leguminosae) leaves collected in Florida, abrusoside D (10) was present at the highest concentration levels. The aglycone (abrusogenin) obtained on the acid hydrolysis of compound

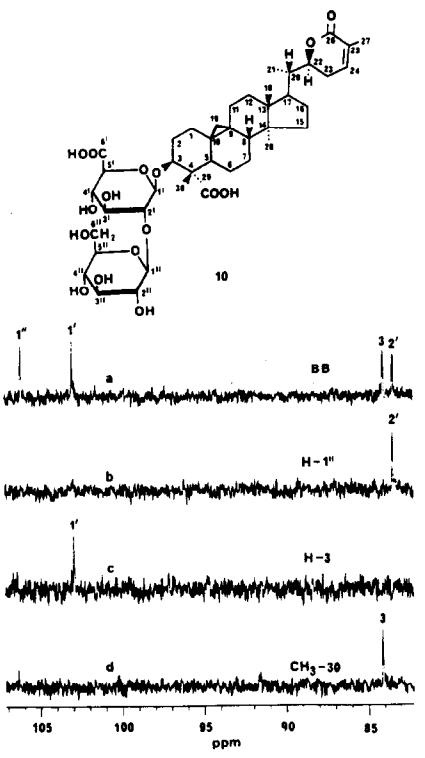

Figure 10. Selective INEPT Experiments on Abrusoside D (10).

10 and its three analogs was found after spectral data interpretation to be a new cycloartane derivative with a δ-lactone ring, and its structure was confirmed by X-ray crystallography.[38] Abrusoside D (10) was the most polar of the four sweet abrusoside derivatives obtained, and produced on hydrolysis both D-glucose and D-glucuronic acid. Interpretation of the ^{1}H- and ^{13}C-NMR spectral data of compound 10 suggested that a disaccharide unit was affixed to the C-3 position of the aglycone. By comparison with ^{13}C-NMR chemical shifts for model sugars, and from analysis of the coupling constants and chemical shifts at the anomeric sites, the sugar unit of compound 10 was tentatively assigned as β-D-glucuronic acid-(1→2)-β-D-glucose. The selective INEPT technique applied on the intact molecule of abrusoside (10) (Fig. 10) proved to be useful in confirming the order of saccharide substitution. Thus, irradiation of H-1″ (on the terminal D-glucuronic unit) at δ 5.22 (J = 6 Hz) (Fig. 10b) selectively enhanced C-2′ of the D-glucose, and similar irradiation of H-3 (δ 4.83) (Fig. 10c) on the aglycone enhanced the D-glucose C-1′ carbon. The chemical shift of the C-3 carbon on the aglycone was confirmed by irradiation of the C-30 methyl protons at δ 1.70 (J = 8 Hz) (Fig. 10d).

Abrusoside D and its three sweet analogs were shown to be not acutely toxic for mice and nonmutagenic for bacteria in preliminary toxicity tests. The ammonium salts of these compounds are water-soluble, stable towards heat, and were rated as between 30-100 times sweeter than sucrose by a human taste panel.[39]

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