Application of INADEQUATE NMR techniques for directly tracing out the carbon skeleton of a natural product

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Short Abstract

^{13}C NMR (DEPTQ) offers a powerful probe of natural products skeletal. NMR measurement of $^{1}J_{CC}$ coupling by 2D INADEQUATE is especially suited to solving structures rich in quaternary carbons and poor in hydrogen content (Crews rule). It continues to solve “intractable” problems in natural product chemistry, and using mg quantities with cryoprobe techniques combined with CASE/PANACEA experiments provides an increase in machine time efficiency. ^{13}C-^{13}C-based structural elucidation by dissolution single-scan DNP NMR can overcome disadvantages of ^{13}C insensitivity at natural abundance. Selected examples are highlighted to map the trajectory of INADEQUATE spectroscopy from structural determination to the clarification of metabolomics analysis and use of DFT and coupling constants to clarify the connectivity, hybridisation and stereochemistry within natural products.

Abstract

Introduction:

NMR measurement of $^{1}J_{CC}$ coupling by 2D INADEQUATE (Incredible Natural Abundance DoublE QUAntum Transfer Experiment), which is a special case of double-quantum (DQ) spectroscopy that offers unambiguous determination of ^{13}C-^{13}C spin-spin connectivities through the DQ transitions of the spin system, is especially suited to solving structures rich in quaternary carbons and poor in hydrogen content (Crews rule).

Objective:

To review published literature on the application of NMR methods to determine structure in the liquid-state, which specifically considers the interaction of a pair of ^{13}C nuclei adjacent to one another, to allow direct tracing out of contiguous carbon connectivity using 2D INADEQUATE.

Methodology:

A comprehensive literature search was implemented with various databases: Web of Knowledge, PubMed and SciFinder, and other relevant published materials including published monographs. The keywords used, in various combinations, with INADEQUATE being present in all combinations, in the search were 2D NMR, $^{1}J_{CC}$ coupling, natural product, structure elucidation, ^{13}C-^{13}C connectivity, cryoprobe and CASE (Computer-Assisted Structure Elucidation) /PANACEA (Protons And Nitrogen And Carbon *Et Alia*).

Results:

2D INADEQUATE continues to solve “intractable” problems in natural product chemistry, and using mg quantities with cryoprobe techniques combined with CASE/PANACEA experiments can increase machine time efficiency. ^{13}C-^{13}C-based structural elucidation by dissolution single-scan DNP NMR can overcome disadvantages of ^{13}C insensitivity at natural abundance. Selected examples have demonstrated the trajectory of INADEQUATE spectroscopy from structural determination to clarification of metabolomics analysis and use of DFT (Density Functional Theory) and coupling constants to clarify the connectivity, hybridisation and stereochemistry within natural products.

Conclusions:

Somewhat neglected over the years because of perceived lack of sensitivity, the 2D INADEQUATE NMR technique has re-emerged as a useful tool for solving natural products structures, which are rich in quaternary carbons and poor in hydrogen content.

Keywords:

INADEQUATE; 2D NMR; $^{1}J_{CC}$ coupling; structure elucidation; ^{13}C-^{13}C connectivity; cryoprobe; CASE/PANACEA; natural product

1 INTRODUCTION

Historically, a proof of chemical structure (including that of natural products) relied on their controlled reagent-based degradation to known compounds, analysis of which then suggested suitable synthetic targets[1]. Preferably made through an unambiguous route, a synthetic campaign was synonymous with ultimate proof of atomic hybridisation, connectivity and stereochemistry. Consequently, if physical properties matched those of its synthetic counterpart, it was considered sufficient proof of structure. Even highly complex molecules such as hemin[2,3] and strychnine[4-6] succumbed to this rather laborious but necessary endeavour by teams of chemists led by such luminaries as Fischer and Woodward[7-10].

X-ray crystallography proved to be a more general technique, providing also the shape and stereochemsitry, but has limitations not often appreciated since hydrogen atoms are assigned and inferred_unless structures are determined using neutron diffraction[11]. If a suitable crystal cannot be obtained, cryo-electron microscopy (cryo-EM) in the form of Micro Electron Diffraction (MicroED), is an alternative approach that has now been extended to small molecules, including natural products[12,13] Classical degradation studies can still prove useful in the modern age to selectively deconstruct large, complex molecules into manageable fragments before conducting spectroscopy, e.g., palytoxin[14]

For X-ray diffraction, a suitable crystal must be grown but all good institutions possess such instrumentation. The disadvantages of using Cryo-Electron Microscopy is the difficulty in accessing instrumentation. Also, samples can suffer from a very low signal to noise ratio (the electron absorption of some molecules can be very low). Consequently, image contrast is also poor and it is hard to detect features when dealing with just a few images. In addition, it is difficult to obtain images from tilted specimens all of which have to be prepared using liquid ethane. In contrast, the advantage of NMR is that sample preparation is relatively straightforward and the instrumentation is widely available. Hence, in most natural product research, acquisition of NMR data is still the preferred analytical technique for structural elucidation, especially the combination of ^{1}H-DEPTQ-HSQC (Distortionless Enhancement by Polarisation Transfer with retention of Quaternaries-Heteronuclear Single Quantum Correlation) and HMBC (Heteronuclear Multiple-Bond Correlation) experiments, which are considered sufficient to assign/deduce the structure of a compound, but occasionally, inaccurate assignments come to light mostly when investigators attempt to make the

postulated structure only to find the synthetic product fails to match the spectroscopic and physical properties of the isolated natural product[15,16]. In all cases, support is always sought from both nominal and high resolution mass spectrometry to determine the molecular weight of the compounds.

In this brief review, we consider the application of NMR methods to determine structure in the liquid-state, which specifically considers the interaction of a pair of ^{13}C nuclei adjacent to one another, to allow direct tracing out of contiguous carbon connectivity. The technique is a powerful but under-utilized aid to structural elucidation does not require specific isotopic enrichment, which is often impossible, impractical or simply unaffordable. An important limitation is that at the natural abundance of 1.1%, the ^{13}C signal from molecules containing an isolated ^{13}C is at least 200 times as intense as the signal arising from a pair of interacting ^{13}C-^{13}C nuclei. Conversely, the proportion of an isotopomer with a ^{l3}C atom in a specific position is 0.011^{1} x 0.989^{n-1} (n is the number of carbon atoms in the molecule), that of an isotopomer with two ^{l3}C atoms in defined positions (irrespective of occurring in directly adjacent positions or not) is $0.01\ 1^{1}$ x $0.989^{\ n-2}$, and so on and so forth[17]. Consequently, when a compound cannot be crystallized but is available in sufficient quantity (10-300 mg), this solution state technique, if applied correctly, can provide outstanding information for natural product structural elucidation.

During the last 40 years, INADEQUATE experiments have appeared in various guises and have been reviewed[17-22] Here selected seminal examples are highlighted focusing on natural products and xenobiotics but excludes a detailed discussion of pulse sequences.

2 IN THE BEGINING

The liquid state incredible natural abundance double quantum transfer experiment (INADEQUATE) was first proposed in 1980 by Ray Freeman[23,24] 23 years after the first report of ^{13}C spectroscopy by Lauterbur[25]. ^{13}C NMR has several advantages over ^{1}H spectroscopy, specifically, a larger chemical shift dispersion, direct detection of carbons multiplicity, and can outline the scaffold, including quaternary carbons within the natural product. When a number of contiguous quaternary centres are present, ^{1}H spectra alone cannot provide the complete range of probable structures. The development of new NMR methodologies continues to play a seminal role in ensuring NMR spectroscopy remains an essential and routine tool for

structure determination of natural products. Any given pulse sequence must satisfy various criteria to ensure routine and long term adoption. Factors considered important are: (i) sensitivity and/or resolution must be enhanced, (ii) clean non-speckled spectra with minimal artefacts must be obtained in minimal acquisition times, (iii) wide applicability on a wide range of compounds, (iv) high accuracy and reproducibility, (v) good tolerance to miscalibrated parameters, (vi) ease of analysis and logical interpretation, and (viii) produce graphic-based output employing such as a Cartesian coordinate system (2D)[26]. Notably, for the time, a suitably complex natural product was selected to showcase the technique: 5α-androstane (Figure 1).

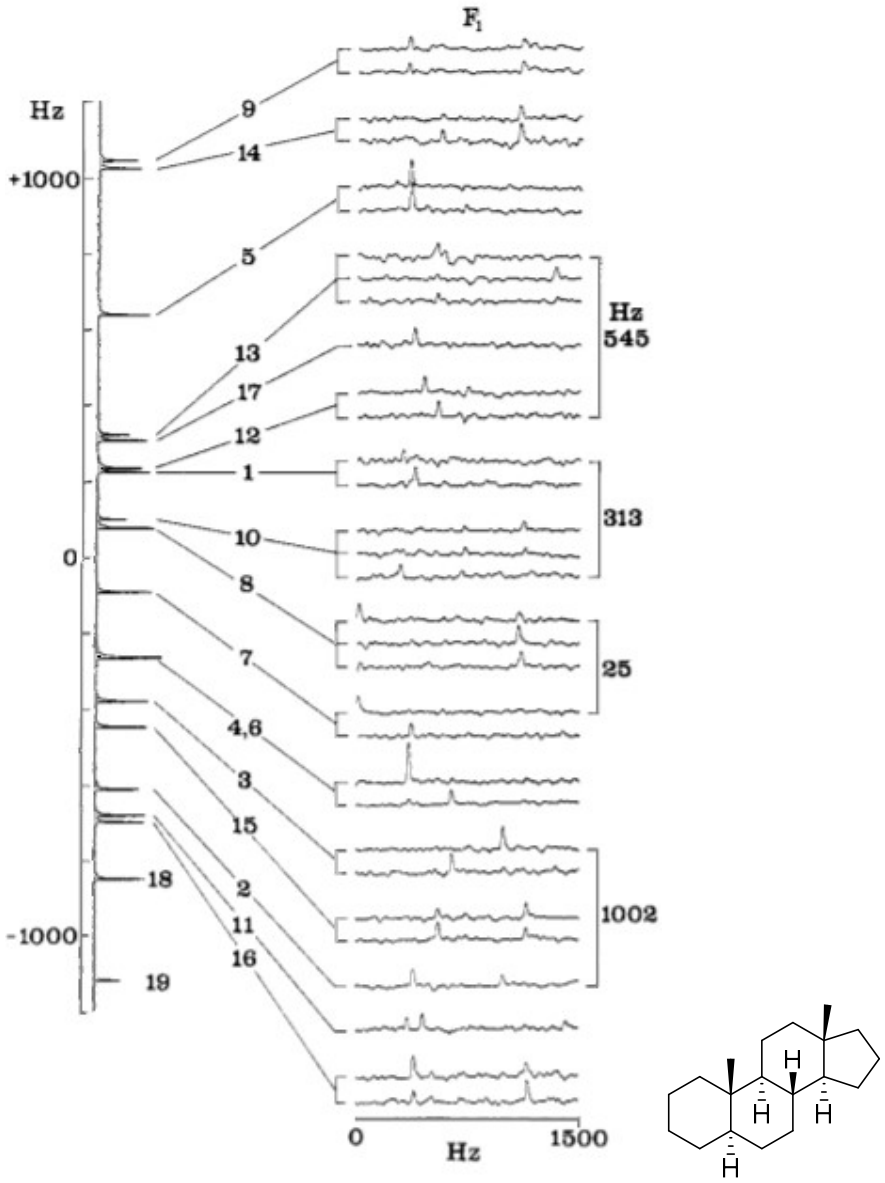


Figure 1: Showcasing the 1D-INADEQUATE experiment using 5α-androstane[24].

The technique was based on NMR experiments for elucidating ^{13}C-^{13}C spin-spin coupling, which employed the transient creation of double-quantum coherence[27-29] to suppress the strong signals from isolated ^{13}C spins, which then uncovered the weak ^{13}C satellite spectrum[24] based on earlier experiments[23,30]. Of the 44 possible double-quantum frequencies, 37 were detected unequivocally, demonstrating the power of the approach despite the inherently low sensitivity of such experiments.

As a powerful illustration of the method, the ^{13}C satellites of the ^{13}C spectrum of sucrose were acquired using a Varian XL-200 operating at 50 MHz with a 16-mm sample diameter tube (70^{o}C, 11 h). Interestingly, the two-dimensional spectrum was displayed in the form of a now familiar intensity contour plot (Figure 2a)[31]. This can be compared with an experiment conducted at 125 Mz at 25 ^{o}C using a Helium cooled probe

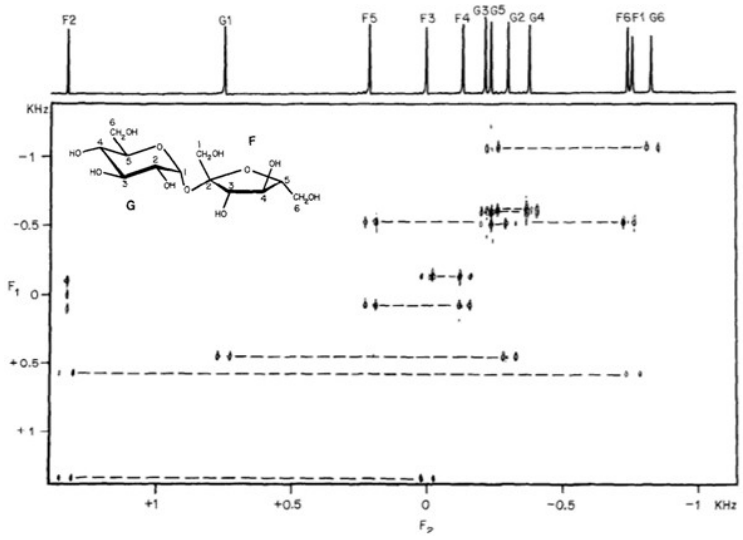


Figure 2a: The 2-dimensional FT ^{13}C spectrum of sucrose (adapted from Bax et al[32])

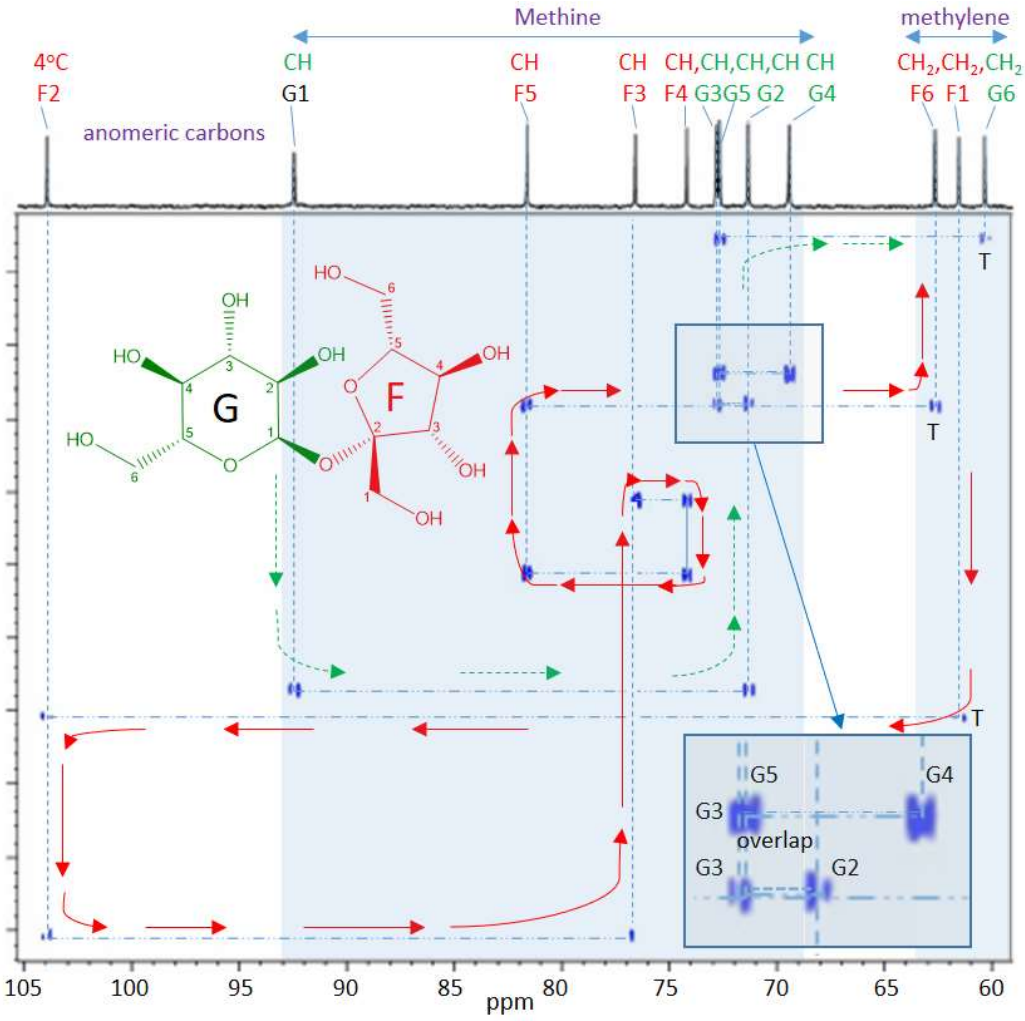


Figure 2b: INADEQUATE spectrum (125 MHz) 75 mM sucrose solution (6.4mg in D_2O using a Shigemi tube; (helium-cooled Cryoprobe, Bruker Prodigy 500MHz, DUL CHD cryoprobe, total experiment time: 19h). At lower concentrations of sucrose, the doublet deduced as F1 was weak and only detected when slicing at lower contour levels. T indicates the terminal portion of the molecule which as only one connection. By following the arrows, it is possible to determine all adjacent ^{13}C-^{13}C nuclei.

The AX- or AB-type satellite spectra are the four-line patterns joined by broken lines; their centres of gravity lie on a line with $\Delta F_1/\Delta F_2 = 2$. The conventional ^{13}C spectrum running along the top of the diagram were assigned by noting which resonances possess a direct ^{13}C-^{13}C spin coupling. The double quantum frequencies appear in the F_1 dimension (Y axis). The F_2 dimension corresponds to the conventional ^{13}C spectrum and notably, that the strong lines

from isolated nuclei are suppressed, revealing weak satellite signals. Since the proton resonances were broadband decoupled throughout, these show four-line spectra of the AX or AB type. Ten such spectra are displayed in Figure 2a and 2b, (denoted by broken lines); four of them reveal the characteristic AB intensity pattern.

The F_1 dimension separates these spectra according to their individual double quantum frequencies, unequivocally identifying them. Since the double quantum frequency is equal to the sum of the appropriate ^{13}C shifts, the centres of gravity, of all AX or AB spectra appear on a diagonal with $\Delta F_1/\Delta F_2 = 2$. This additional constraint was suggested to be useful for identifying incompletely suppressed "single-quantum" signals.

Notably, the resonance of the quaternary carbon of sucrose (F_2) was identified by its long spin-lattice relaxation time. The chain of linkages G6-G5-G4-G3-G2-G1 was deduced from Figure 2 by inspection, and the deductions agreed with that of Pfeffer *et al.*[33] Conversely in Figure 2b, the multiplicity of each carbon was readily assigned using a DEPTQ experiment.

3 ASSIGNMENT OF CARBON-13 NMR SPECTRA

Assignments of the ^{I3}C-NMR signals of known compounds are critical both for the determination of the structures of "unknowns" and importantly to map biosynthetic pathways in primary and secondary metabolism. Consequently, assignments on the basis of chemical shifts and CH couplings contain uncertainties, particularly for complex scaffolds. Since evaluation of the ^{I3}C, ^{I3}C-couplings can remove such uncertainties, the 2D INADEQUATE pulse sequence giving the most unambiguous results. An early list of such missassigned structures are given in by Buddrus & Bauer 1987[17].

The sensitivity of cryoprobes, allows measurement of coupling constants involving ^{13}C-^{13}C pairs at the natural abundance of ^{13}C using tens (rather than hundreds) of milligrams of compounds. Jin and Uhrın described a novel INADEQUATE experiment for simultaneous correlation of one-bond and long-range ^{13}C–^{13}C pairs and the measurement of both types of coupling constants in ^{13}C natural abundance samples[34]. It was claimed to yield accurate values of one-bond and long-range coupling constants by manipulation of pure phase in-phase (IP) and antiphase (AP) doublets, and was referred to as ^{13}C detected IPAP-INADEQUATE. Measurement of inter-glycosidic ^3J CCOC coupling constants in a disaccharide molecule providing important information about the conformation of the glycosidic linkage[34]. A recent

study[35] explored the feasibility of using a combination of both experimental and theoretical one-bond $^{13}C^{13}C$ scalar couplings ($^1J_{CC}$) to establish structures of various organic compounds, including unknowns. Historically, $^nJ_{CC}$ and $^nJ_{CH}$ studies focus exclusively on both two and three-bond couplings, whereas $^1J_{CC}$ couplings exhibit significantly larger variations. Comparison of computed $^1J_{CC}$ values to experimental data allowed comparison of theoretical methods, thus the B3LYP, B3PW91, and PBE0 functionals were evaluated by comparing to 27 experimental values from INADEQUATE and testing values for 5-methylmellein, a dehydro-isocoumarin isolated from *Fusicoccum amygdali* Del.[36]. The presence of a very strong, low-barrier hydrogen bond in 5-methylmellein presents a challenge to theoretical methods absent in the original investigation[37]. In that case, only a single candidate matched experimental data with high statistical confidence. This analysis also established the preferred intramolecular hydrogen-bonding arrangement, ring heteroatom identity, and conformation at one position. Extension to hydroheptelidic acid, a natural product not fully characterized in previous investigations, identified a single best-fit structure from among 26 candidates and established, for the first time, one configuration and contributions from three conformations to complete the characterization. These results suggest that accurate and complete structural characterizations of many moderately sized organic structures (<800 Da) may be possible focusing on $^1J_{CC}$ data coupled with INADEQUATE type experiments.

3.1 Practical considerations

3.1.1 Delta value

In the 2D INADEQUATE pulse sequence, DELTA should be set to 1/(4*J*), where *J* is the one-bond ^{13}C-^{13}C coupling. The one-bond coupling constants are used to estimate carbon-carbon bond order. The constant range between 35 to 45 Hz for a single bond ca. 65 Hz for a double bond which are further increased by electronegative substituents. The solution should be sufficiently concentrated to produce a DEPT-Q spectrum in which all the quaternary carbons are detectable and S/N≥25[17]. 2D-INADEQUATE spectroscopy can show vertical streaks of T_1 noise. Gradient selected (GS) 2D INADEQUATE, when applied to uniformly labelled glucose and then compared with a phase-cycled spectrum, revealed that the signal-to-noise ratio is approximately the same in both cases. However, the GS spectrum was free from artefacts such as t_1 noise[38].

If sample enrichment is possible by 10% of ^{13}C content, then the INADEQUATE experiment can be acquired at normal sample concentrations. However, at natural abundance, it is recommended to use between 100 and 500 mg of sample. If it fails to dissolve in 0.8 mL of solvent then use a 10 mm NMR tube and 2.5 mL of solvent, alternatively a 25 mg of sample dissolved in 350 μL of D_2O (Shigemi tube, 200 mM sample; compare Figure 2b). Warming can help and appears to remain in solution within the first hour or so but may begin to slowly precipitate or crystalize or even form a gel, compromising acquisition. Occasionally, some natural products cannot be freed from a minor impurity. However, the presence of up to 20% of impurities can be endured, when employing INADEQUATE spectroscopy[17]. From a number of investigations of routinely running INADEQUATE spectra, it has been noted that if a single scan DEPTQ spectrum successfully shows all peaks (including quaternary carbons) then the concentration is sufficient for finding most, if not all pairs of peaks adjacent ^{13}C peaks at natural abundance[21,22].

3.1.2 Addition of paramagnetic substances

Another possibility to reduce the spin lattice relaxation time T_1[39,40] by adding a paramagnetic substances such as Cr(acac) (around 0.03 M), but this then makes the compound unusable for further investigations (often pharmacological). An alternative approach is to pressurize the tube with oxygen[41], provided the compound is stable to auto-oxidation. An example is the 125-MHz spectrum of methyl salicylate. The addition of perfluoro-*t*-butanol (related to various artificial blood substitutes) increased the amount of oxygen that can be dissolved was such that that similar reductions in the relaxation times were achieved thereby avoiding pressurization by oxygen,. The nuclear Overhauser enhancements (nOe) were only slightly depressed by addition of oxygen.

3.2 Custom designed probes for natural product experiments

Ramaswamy et al.[42] reported a 1.5-mm NMR probe based on high-temperature superconductors operating at 14.1 T optimized for ^{13}C detection is particularly worth mentioning. The probe has a total sample volume of about 35 µL with an active volume of 20 µL and provided exceptional mass sensitivity for ^{13}C detection, excellent 1H sensitivity and employed a ^{2}H lock. The coils were cooled to about 20 K using a standard Agilent cryogenic refrigeration system, whereas the temperature of the sample is regulated near room

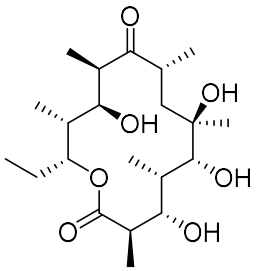
temperature. Specifically, this probe proved ideal for directly detected ^{13}C NMR experiments for natural products chemistry and metabolomics applications, for which 35 µL was an optimal sample volume. The outstanding ^{13}C sensitivity of this probe allowed direct determination of the ^{13}C connectivity on a 1.1 mg in 35 µL of D_2O (200 mM solution) of natural abundance histidine using an INADEQUATE experiment. The spectrum was collected using adiabatic ^{13}C 180 pulses with the Agilent INADEQUATEAD experiment. The acquisition time was 0.134 s using 4096 complex points and a spectral width of 30487.8 Hz, collected with 256 t_1 increments with a double quantum spectral width of 60975.6 Hz, and 108 scans for a total experiment time of 48 h and 24 m. Based on comparisons of sensitivity with a 5-mm XSens probe at the same field strength, this same experiment would require nearly 2 weeks with the same sample on the most sensitive ^{13}C -optimized commercial probes. Furthermore, they demonstrated the utility of this probe for ^{13}C -based metabolomics using a synthetic mixture of common natural abundance metabolites whose concentrations ranged from 1 to 5 mM (40–200 nmol). Notably, this probe is available to external users through the National High Magnetic Field Laboratory at Florida.

4 SELECTED EXAMPLES OF STRUCTURES ELUCIDATED WITH INADEQUATE EXPERIMENTS DURING THE LAST FOUR DECADES

Since earlier spectral assignments were ambiguous, both one- and a two-dimensional INADEQUATE NMR approaches afforded one-bond ^{13}C-^{13}C coupling constants (at natural abundance) and total unambiguous signal assignment for erythronolide-B (**1**) (Figure 3) demonstrating the potential of the technique[43], which is one of the early examples of application of INADEQUATE technique in structure elucidation of natural products.

A bisnorditerpene, (4aR,4bR,7S,8aR,10aR)-7-hydroxy-1,1,7-trimethyldecahydro-2H,6H-8a,4a-(epoxymethano)phenanthren-12-one (HTEP) (**2**), was isolated from *Vellozia bicolor* L. B. Smith and its structure was determined by a combination of spectral methods, especially, by ^{13}C NMR, including the natural abundance ^{13}C-^{13}C coupling constants observed via double quantum coherence[44]. A solution of **2** was prepared in [$^2H_5$] pyridine [470 mg of in 2 mL of solvent; 1.6 M concentration] and the spectrum, optimized for the 2D experiment at $^1J_{CC}$ = 40 Hz (J_τ = 1/4), was accumulated at 60˚C (at just over half the boiling point of the solvent) overnight using a relaxation delay of 3.0 s. Quadrature detection in both directions

was employed. Early experiments often employed higher temperatures than those used currently to improve solubility allowing an increase in signal strength.



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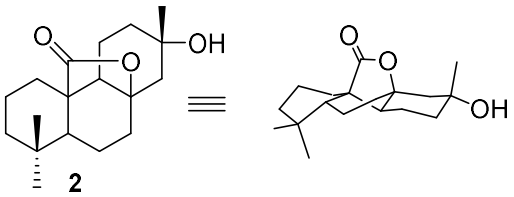

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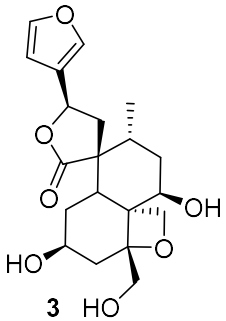

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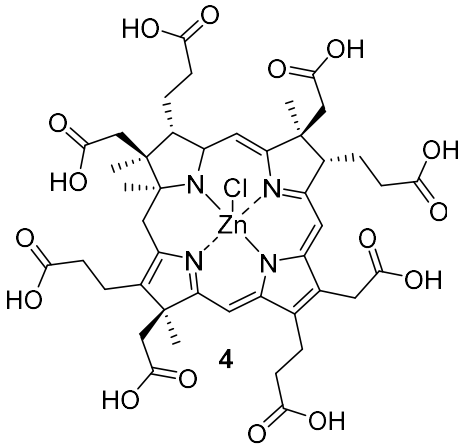

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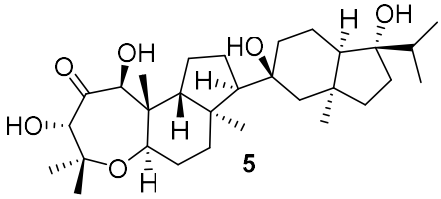

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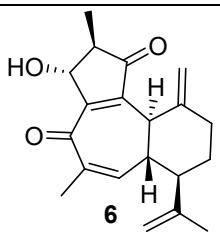

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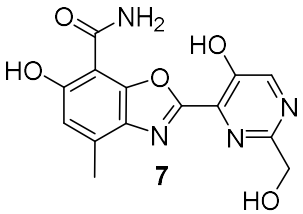

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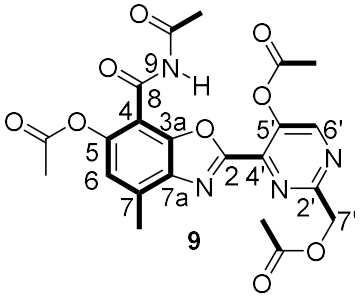

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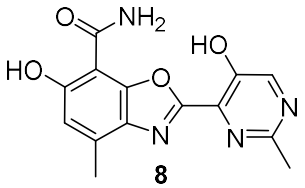

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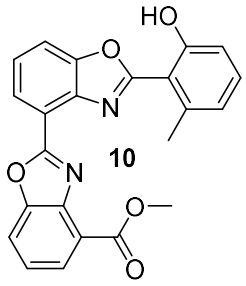

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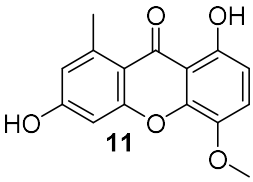

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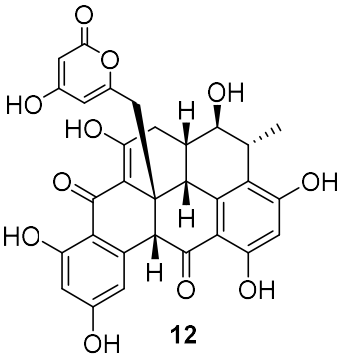

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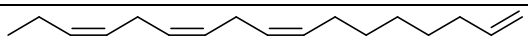

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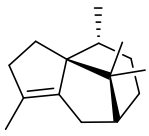

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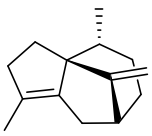

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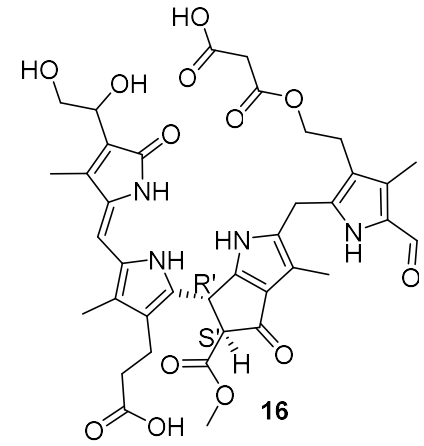

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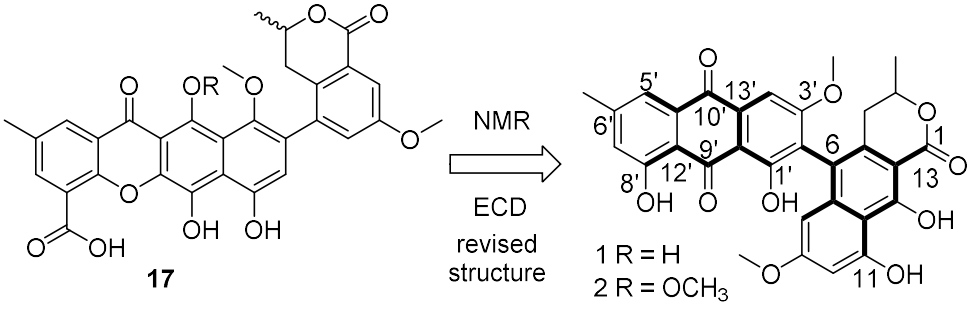

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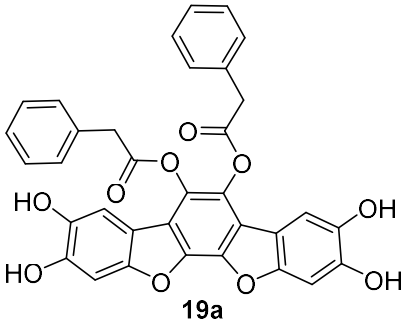

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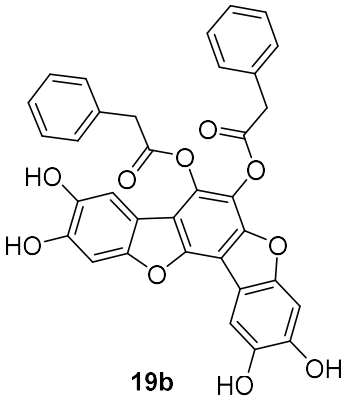
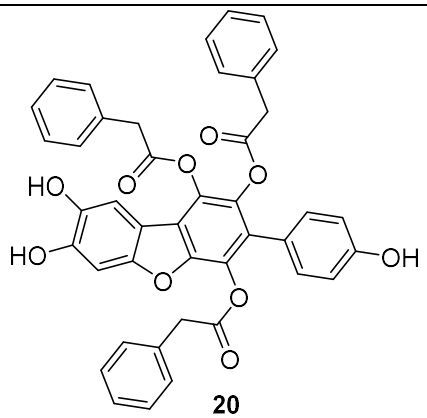
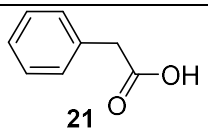
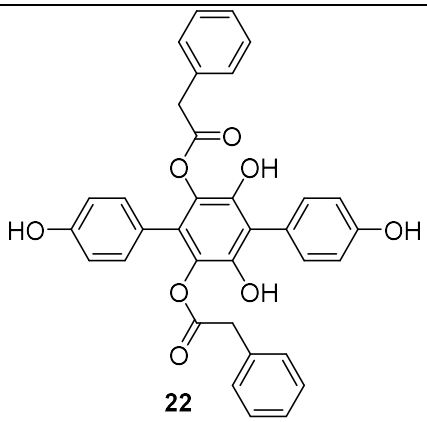
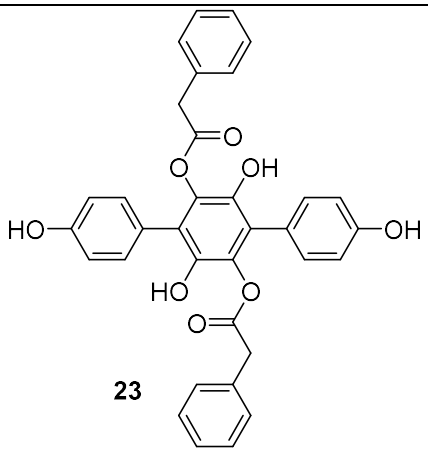
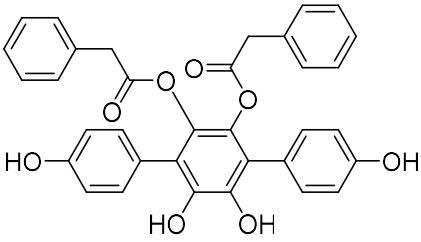
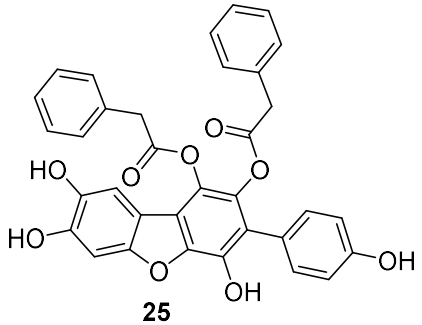
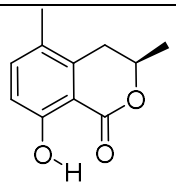
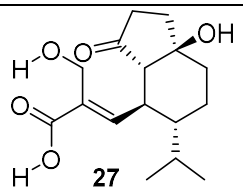
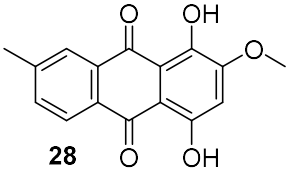
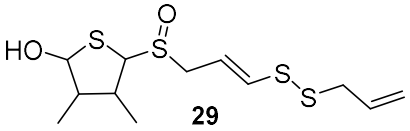


19b

 **20**	 **21**
 **22**	 **23**
 **24**	 **25**
 **26** s	 **27**
 **28**	 **29**

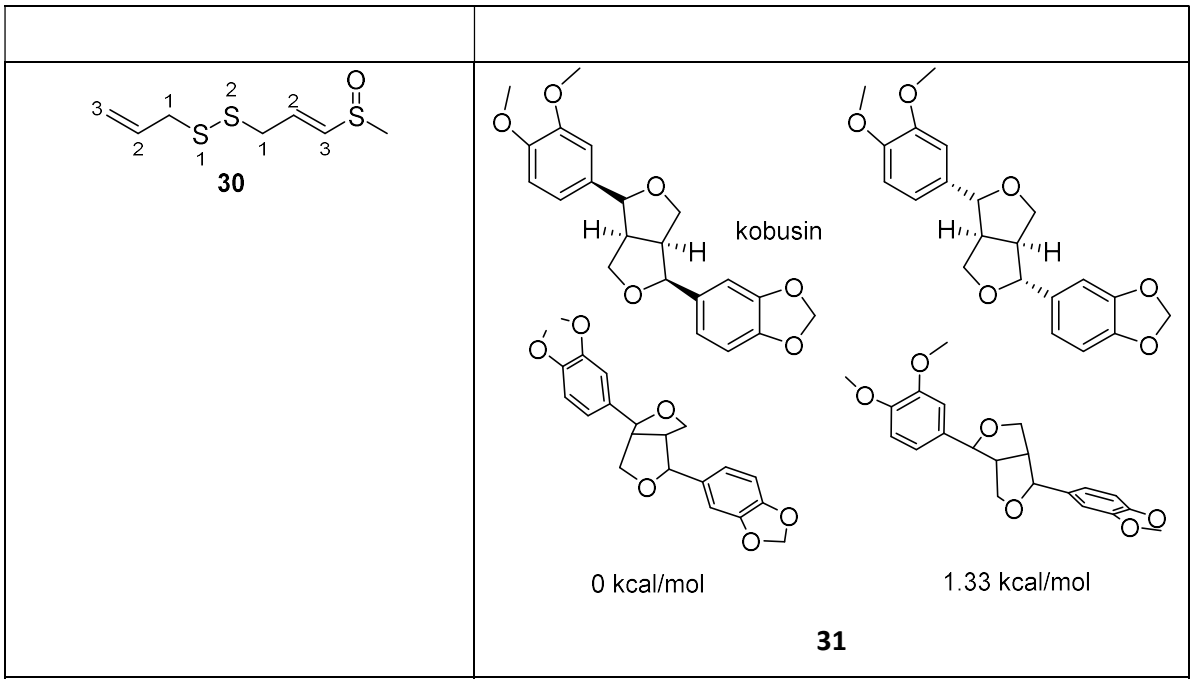


Figure 3. Structures of selected natural products deduced using INADEQUATE experiments

The genus *Teucrium* of the Labiatae family is represented by 300 globally distributed species. One of its species, *T. chamaedrys* L. is an aromatic and medicinal plant used in traditional medicine and known to produce neoclerodane diterpenoids[45,46]. Teucroxide (**3**) (Figure 3), one of its neoclerodane diterpenoids, contains an oxetane and two five- and six-membered rings. It was investigated using the triacetyl derivative (TAT) and 1-D INADEQUATE experiment (50.31 MHz with a Bruker WM-200 spectrometer in xylene-d10 (800 mg of TTA in 2 mL of solvent) with a pulse sequence was optimized for Vcc = 60 Hz (*J*r = 1/4) and data were accumulated at 110 °C, overnight, using a relaxation delay of 3.0 s. However, this experiment detected only $^{1}J_{CC}$ involving sp^2 carbon atoms. Further experiments used a Bruker WM-400 spectrometer operating at 100.62 MHz. A solution of TAT was prepared in pyridine-d_6 (800 mg of TTA in 2 mL of solvent) with a pulse sequence optimized for Vcc 45 (*J*r = ¼; relaxation delay of 3.0, overnight at 60°C in order to shorten spin-lattice relaxation times relative to the previous experiment). The small $^{1}J_{CC}$ coupling constants within the oxetane ring were consistent with previously published data on related heterocyclic systems. The absolute configuration of teucroxide (**3**) was not ascertained but was considered synonymous with neoclerodane series co-occurring within the same plant determined by X-ray crystallography.

At the time, the oxetane ring moiety was a rare feature in natural products, and only a handful of compounds possessing this function had been described[47] Recently, analysis of published NMR data for natural products containing the oxetane moiety, in conjunction with a recently developed parametric/DFT hybrid computational method DU8+, has revealed that oxetanes (as well as related compounds) also offer significant challenge in structure elucidation[48]. Specifically, the stereochemical assignment, since more than 30 structures identified so far, required revision. It discussed the most common pitfall and revised structures were suggested for 26 previously reported natural products.

The structure of Factor S3 (**4**) (Figure 3), a Zn corphinate isomer, was assigned to a metabolite of *Propionibacterium shermanii.* The molecule is derived from the Type I rather than the normal Type III uroporphyrinogen and is formed by the insertion of four methyl groups from *S*-adenosylmethionine (SAM), three at the β and one at the α-pyrrolic positions of uroporphyrinogen S3. A biosynthetically enriched sample (with ^{13}C) allowed structural assignment using an INADEQUATE experiment on a 70-100 μg of sample[49]. It also revealed that only one SAM-derived methyl group was coupled to the quaternary carbon at 79 ppm. At the time Factor S3 was the first natural product derived biosynthetically from the Type I porphyrin template, and its structure prompted important questions about the evolution of the Type III system as the building block for vitamin B_{12}[50].

The structure of neviotine A (**5**), a triterpene isolated from the Red Sea sponge *Siphonochalina siphonella,* was elucidated by chemical transformations and principally on the basis of a 2D INADEQUATE NMR set of experiments[51] (Figure 3). Neviotine-A (**3**) was shown to possess a novel tetracyclic skeleton related to the sipholanes and siphonellanes (previously isolated from the same sponge). Recently neviotine A has been shown to be a potent inhibitor of RANKL induced osteoclastogenesis in RAW264 macrophages (32.8 μM)[52].

The genus *Jatropha* belongs to the Euphorbiaceae family and comprises around 175 species[53]. Originally from tropical America, the *Jatropha* genus can now be found all over the tropics and subtropics of both Asia and Africa. Investigations on the chemical aspects of the genus *Jatropha* identified cyclic peptides, lignans, flavonoids, coumarins, alkaloids, eudesmenoic acids, and mainly terpenes. Jaherin (**6**) (Figure 3), a rare daphnane type diterpene possessing activity against the microbes, *Streptococcus pyogenes*, *Microsporum canis*, *Absidia corymbifera* and *Trichophyton rubrum*[53], was isolated in 0.01 % yield from (6.3 kg) of *Jatropha zeyheri* Sond. var. *zeyheri,* a well-known South African folk medicine for the

treatment of open sores and bums[54]. The structure of **6** was determined by 2D INADEQUATE experiments[55]. It is noteworthy that chromium tris-acetylacetonate was added to shorten the relaxation times facilitating the acquisition revealing all 22 carbon-carbon bonds. However, some ambiguity was found for both vinylic methyl carbon atoms possessing similar chemical shifts (18.4 and 18.5 ppm), which the authors suggested was structurally related to helanin[56], a compound used in American Indian medicine.

It may seem counter-intuitive, but some very simple ^{1}H spectra can prove especially challenging. An example is that of the boxazomycins[57]. Boxazomycins A (**7**) and B (**8**) (Figure 3) are antibiotics produced by *Actinomycetes* strain No. G495-11, which was originally isolated from a Taiwanese soil sample. Boxazomycins are insoluble in ordinary organic solvents and had to be converted into tetra-acetate (BAT) to impart solubility in $CDCl_3$. INADEQUATE spectroscopy of BAT (**9**) (600 mg) in $CDCl_3$ (10-mm tube at 318 K) proved successful in obtaining a suitable spectrum. The 2D-INADEQUATE spectrum of BAT (**9**) (Figure 3) derivative showed almost all of the double quantum coherent peaks, from which the C-C connectivities (depicted by thick lines in the structure) were obtained. However, C-4' and -5' (Figure 3) were not observed within the INADEQUATE spectrum because of their similar chemical shifts (143.5 and 142.7 ppm, respectively). The related antitumour natural product nataxazole (**10**)[58] (Figure 3) is a model for a large class of benzoxazole-containing molecules that are synthesised by a pathway that is not so far been characterized. Recently, structural, biochemical, and chemical evidence that benzoxazole biosynthesis proceeds through an ester generated by an ATP-dependent adenylating enzyme. These insights have allowed biosynthesis of a series of novel halogenated benzoxazoles[59] showing excellent antibacterial activity against strains of *E. coli* and *S aureus*.

Structures of six xanthones, drimiopsins A-F, isolated from the South African *Drimiopsis maculate*, proved difficult to elucidate due to absence of correlating protons within the NMR spectra, yet another example where the Crew's rule becomes apparent[60,61]. Consequently, INADEQUATE spectra were used to confirm the structures of these previously unreported xanthones, e.g., drimiopsin A (**11**) (Figure 3) within the family Hyacinthaceae. Specifically, the INADEQUATE NMR experiment was used to place the remaining two methoxy and two hydroxy groups on the ring and to confirm the xanthone structure[62].

A-74528 (**12**) is a metabolite of *Streptomyces* sp. discovered during the screening for 2',5'-oligoadenylate phosphodiesterase inhibitors[63]. The planar structure of A-74528 was

principally elucidated by NMR techniques including natural abundance INADEQUATE, and the relative configuration and the conformation were elucidated by the analyses of NOEs and assessment of dihedral angles predicted by QUANTA/CHARMm computations and coupling constants. It was proved that A-74528 possessed a highly fused polyketide with a side-chain branching site that had not been previously recognised within natural product extracts.

A detailed analysis of *Rhaponticum carthamoides* (Willd.) Iljin root essential oil afforded 30 compounds, amongst which the most interesting were norsesquiterpene 13-norcypera-1(5),11(12)-diene (**13**), aplotaxene (**14**) and cyperene (**15**) (Figure 3)[64]. Their structures were confirmed by 1D and 2D-NMR spectra (Correlation spectroscopy: COSY, (Rotating frame Overhause Effect SpectroscopY (ROESY), HSQC, HMBC and INADEQUATE. Notably, independent evidence for the suggested structure of 13- norcypera-1(5),11(12)-diene (**13**) was provided by DFT B3LYP calculations with basis set 6-311G+(2d,p) which provided an excellent agreement with experimentally observed NMR data. Additionally, nOe correlations observed in 2D-H, H-ROESY spectrum proved relative configurations at C4, C7 and C10 (identical with those in cyperene) and stereochemical assignment of hydrogen atoms of the methylene groups.

Autumnal leaf pigmentation of deciduous tree plants is one of the representative events in plant senescence[65,66]. During this process, degradation of chlorophyll occurs by environmental changes including lower temperature and shortening of daylight, and then leaves turn brown, yellow, and red. Reddish colouring of autumn leaves is caused by synthesis and accumulation of anthocyanins coincident with the degradation of chlorophyll. A yellow chlorophyll catabolite, Ed-YCC (**16**) (Figure 3), was isolated from leaves detached from *E. densa* shoots, in which both chlorophyll degradation and anthocyanin synthesis were induced in 0.1M fructose soln. under light illumination as a plant senescence process, a model of autumnal leaf colouration. Various NMR techniques were used to provide structural identity including 2D-INADEQUATE[67].

Two heterodimers comprising an anthraquinone moiety linked to a 3-methylbenzodihydro isocoumarin (**17**) unit were isolated from *Pyrenacantha kaurabassana* tubers, together with emodin and physcion (**18**) (Figure 3)[68].The structures of all compounds were established by NMR spectroscopy, including the analysis of a 2D INADEQUATE spectrum (identifying the pairs of bond emboldened). The structure of the second compound was

defined as the 5'-methoxy derivative of the first. On the basis of the data obtained, the structures that were previously proposed in the literature for these compounds were revised.

Vialisyl A (**19**), ganbajunin B (**20**), phenylacetic acid (**21**), a mixture of ganbajunins D (**22**) and E (**23**), and vialinins A (**24**) and B (**25**) (Figure 3) were isolated from the fruiting bodies of *Thelephora vialis*, a Chinese medicinal plant from the family, Thelephoraceae, is mainly distributed in Yunnan Province[69]. Their structures were established by extensive analysis of spectroscopic data (including a combination of ^{1}H- and ^{13}C -NMR, HSQC, HMBC, 2D-INADEQUATE) as well as by comparison with previous literature reports. As the HMBC experiment could not distinguish **19a** from **19b**, a 2D-INADEQUATE experiment was applied to elucidate substituent positions. If the structure was **19a**, a correlation between δ_C 130.6 (C(2',5')) and 137.0 (C(3',6')) would be expected. A cross-peak was absent so **19a** was excluded. The correlations between δ(C) 116.6 (C(1',4')) and 130.6 (C(2',3')), between δ_C 116.6 (C(1',4')) and 137.0 (C(6',5')), between δ_C 116.6 (C(1',4')) and 112.7 (C(1',1'')), and a lack of correlations between δ(δ_C C) 137.0 and 130.6 confirmed that the correct structure must be **19a** rather than **19b**[69].

By combining theoretical predictions with experimental INADEQUATE $^{1}J_{CC}$ coupling values, challenging structural problems can be solved. 5-methylmellein (**26**) (Figure 3), a DNA polymerase l inhibitor, hydroheptelidic acid (**27**), which displays antitumor and antimalarial activity, and austrocortinin (**28**), an anthraquininoid pigment, are three natural products extracted from endophytic fungi found in the Central Florida area with potential medical application were all subjected to structural elucidation. This group set out to test challenging structural motifs that included: a) the presence of strong intramolecular hydrogen bonding; b) verifying heteroatom identity; c) assigning double bond configuration; and d) assigning the correct tautomeric form. Interestingly, all ambiguities were resolved and single best-fit structures were uniquely identified; verifying that such motifs can be accurately modelled[70].

Stereoisomers of 5-(2-allylsulfinyl)-3,4-dimethylthiolane-2-ol, a family of 3,4-dimethylthiolanes have been named, ajothiolanes, which were isolated from garlic (*Allium sativum*) macerates and characterized by a variety of analytical and spectroscopic techniques, as well as LC-MS/MS. Ajothiolanes were found to be spectroscopically identical to a family of known compounds named garlicnins B1-4. Notably, it was shown they were previously misassigned. 2D INADEQUATE disproved that nine contiguous carbons existed in these

compounds. Also, X-ray absorption spectroscopy (XAS) along with computational modelling further disproved their previous identification as thiolanesulfenic acids. On the basis of the similarity of their NMR spectra to those of the ajothiolanes, they suggested that so-called onionins from extracts of onion (*Allium cepa*) and *Allium fistulosum*, and garlicnin A (**29**), from garlic extracts were reassigned, in each case as isomeric mixtures of 5-substituted-3,4-dimethylthiolane-2-ols. Importantly, they concluded that 3,4-dimethylthiolanes might be a common motif in *Allium* chemistry. Also, another garlic component, garlicnin D (**30**), claimed previously to have an unprecedented structure, is synonymous with a compound from garlic with a structure different from that suggested, namely, 2(*E*)-3-(methylsulfinyl)-2-propenyl 2-propenyl disulfide[71].

A furofuran lignan, (1*S*,3a*S*,4*S*,6a*S*)-1-(3',4'-dimethoxyphenyl)-4-(3'',4'' methylenedioxyphenyl) hexahydrofuro [3,4-c]furan (**31**), was isolated from Colombian *Piper jericoense* leaf[72]. Its relative configuration at the stereogenic centres was established on the basis of various spectroscopic analyses, including 1D-1H, ^{13}C, and DEPT) and 2D-NMR (COSY, NOESY, HMQC and HMBC) and, importantly, a 2D INADEQUATE NMR experiment. Nevertheless, comparison of their spectral data with those of related compounds such as (+)-kobusin still proved necessary.

5 APPLICATION OF INADEQUATE IN STUDYING BIOSYNTHETIC PATHWAYS OF NATURAL PRODUCTS

2D INADEQUATE can come extremely handy while studying biosynthetic pathways of natural products, as exemplified in this section. Unarmored dinoflagellates of the *Karenia* (previously *Gymnodinium*) genus are able to form blooms, most commonly red tides, and produce toxins affecting human health, fishes, and less frequently, other marine animals. To date, 10 species are considered potentially toxic: *K. bidigitata* (synonym of *K. bicuneiformis*), *K. brevis* (formerly *Ptychodiscus brevis* and *Gymnodinium breve*), *K. brevisulcata, K. concordia, K. cristata, K. digitata, K. mikimotoi, K. papilionaceae, K. selliformis,* and *K. umbella*[73]. Blooms of the dinoflagellate *Karenia* breve are responsible for the Florida red tide which causes massive fish kills as well as birds. The causative agents for this poisoning are the neurotoxic brevetoxins (Figure 4), which possess unique structures consisting of 10-11 *trans*-fused 5-9-membered oxacyclic rings.

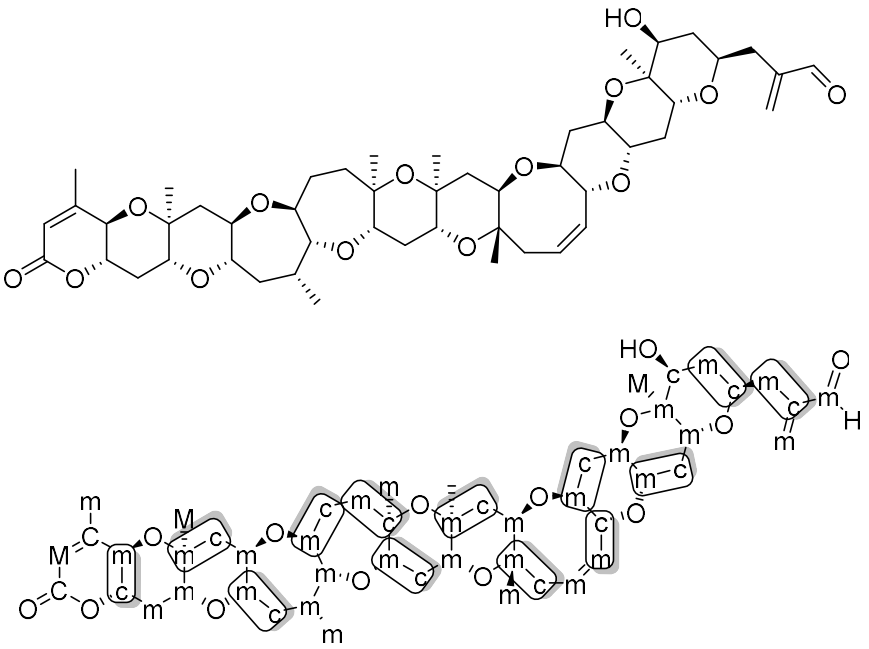


Figure 4: Biogenesis of brevetoxin B (shown on top) and (below) the C2 units that originate from the same acetates are enclosed in squares; m and c denote, respectively, the carbons derived from the methyl and carboxy carbons of acetic acid

The biosynthetic origin and NMR assignments of all the ^{13}C atoms in brevetoxin B (BVTB) were elucidated by NMR measurements of BVTB enriched with [1-^{13}C]-, [2-^{13}C]-, and [1,2-^{13}C]-acetates and Me-^{13}C-methionine[74]. 2D INADEQUATE ^{13}C-NMR sequence clarified the ^{13}C - ^{13}C connectivities of acetate-derived units by measurements using only 1.5 mg BVTB (*inter alia* [1,2-^{13}C2] acetate. The results revealed an unprecedented biosynthetic pattern in which the single C-chain contains 6 m-m moieties (m represents the methyl group of an acetate unit) and even 2 contiguous m-m-m moieties, 1 of which is extended further by an additional sec-Me group. The ultimate proof of structure and validation of structural elucidation was the total synthesis of this molecule which first took twelve years to achieve. It was synthesized in 1995 by K. C. Nicolaou and coworkers in 123 steps with 91% average yield (final yield ~$9\cdot10^{-6}$). In a subsequent review, the ^{13}C -NMR spectrum placed above that of the natural isolate over that of the synthetic compound is a superlative example of how INADEQUATE spectroscopy aided this 20th century Odyssey[75].

This type of approach continues to the modern day. Using feeding experiments to solid callus cultures of *T. peltatum*, the isolation of the alkaloid 5'-*O*-methyldioncophylline D revealed successful incorporation of the isotopic precursor[76]. Both ^{13}C NMR and 2D INADEQUATE spectra revealed the successful incorporation of the applied precursor [$^{13}C2$]-4 into 5 by 8%, consistent to the labelling content in the purified alkaloids habropetaline A and dioncophylline A. The INADEQUATE spectra of [$^{13}C2$]- 5'-*O*-methyldioncophylline D and [$^{13}C2$]-habropetaline A revealed the two specific doublets for both C-1 and Me-1 (at 50.0 and 18.2 ppm, respectively) exceeding peak heights of corresponding peaks of the non-labelled compounds 5'-*O*-methyldioncophylline and habropetaline A proving successful biosynthetic incorporation of the advanced precursor, *trans*-configured tetrahydroisoquinoline, phylline, into the complete alkaloids.

6 APPLICATION OF INADEQUATE IN METABOLOMICS

Two-dimensional ^{13}C–^{13}C correlation experiments like INADEQUATE also has great but untapped potential for metabolomics analysis. Clendinen et al.[77] demonstrated a new and semi-automated approach called INETA (INADEQUATE network analysis) for the untargeted analysis of INADEQUATE data sets using an *in silico* INADEQUATE database. They demonstrated this approach using isotopically labelled *Caenorhabditis elegans* mixtures. Mixture analysis was further simplified with the use of INETA that allowed them to semiautomate the analysis of complex labelled mixtures by matching to a database library. The INETA approach was able to identify nine other metabolites not found in the previous studies. Several strategies could be developed to identify metabolites from these networks. They suggested that algorithms could be developed to match the INADEQUATE single quantum chemical shifts of unassigned metabolomics networks to database entries and thereby identify unassigned spectra. Secondly, algorithms could be developed to automatically adjust INETA thresholds to increase the number of full networks for database matching. This may be especially useful in the detection of peaks in heavily overlapped regions together with Quantum mechanical calculations of ^{13}C chemical shifts using large computer clusters. Using this approach, they predict INADEQUATE data on metabolomics mixtures would be an outstanding way to discover unknown molecules.

6 CURRENT DEVELOPMENTS

Two-dimensional (2D) experiments remain a cornerstone of current structure determinations. On a cost basis as well as flexibility and adaptability in terms of programing new experiments, as well as ease of sample preparation, NMR has the capability of deducing atom-to-atom connectivities superior to all other spectroscopic techniques or for that matter X-ray crystallography or MicroED.

The difficulty of differentiating between 2*J*CH from 3*J*CH correlations within HMBC spectra and obtaining 4*J*CH correlation information remains challenging. Differentiating between 2*J*CH from 3*J*CH correlations was first established using ^{13}C-detected XCORFE experiment reported in 1987 by Reynolds and co-workers as reiterated by senior et al. [61]. Historically, subsequent 2*J*,3*J*-HMBC[78] experiment was followed by H2BC and variants[79-81]. These experiments only proposed a partial solution to the problem by identifying adjacent protonated carbons (via 2*J*CH), since they could not identify adjacent quaternary carbon resonances until a combination of HMBC and 1,1-ADEQUATE spectra were used. This solved the problem of differentiating between $^{2}J_{CH}$ from $^{3}J_{CH}$ correlations using the proton-deficient staurosporine molecule as an example[61,82-86] The latest advance includes developing non-uniform sampling strategies to improve sensitivity and resolution in 1,1-ADEQUATE experiments and previously described programs such as COCON (now available on a web server: https://cocon-nmr.de/) have been supplanted by DFT methods[87].

Familiarity with natural products databases can be used to eliminate improbable structures (to a certain extent), but could exclude novel skeleta. If strongly coupled AB systems are present, there is a loss in intensity of the satellite signals due to inappropriately chosen delay times[17]. Ambiguities with regard to connectivity arises if NMR signals from isolated ^{13}C nuclei overlap one another[88]. The determination of configurations with the help of the Karplus equation is only possible in exceptional cases. However, when the constitution is known and the proton NMR signals can be correctly assigned (via a C,H-correlation), the configuration can generally also be determined by using trusted methods such as proton-proton coupling and nuclear Overhauser effects. The approach involving DFT, considering $^{1}J_{CC}$ constants and allowing all feasible arrangements of these structural fragments to be considered, including all variations of heteroatoms. The results obtained herein suggest that only the correct structure will fit the experimental $^{1}J_{CC}$ values at all positions[35].

An increase in proton deficiency, structure elucidation becomes progressively difficult; that is when the ratio of ^{1}H to ^{13}C and the sum of other heavy atoms decreases below a value of two, an axiom designated as the "Crews rule" becomes relevant. Consequently, some structures become increasingly difficult if not impossible to solve using a conventional strategy of NMR experiments. The strategy always used in the laboratory is to acquire both proton and DEPT-Q spectra and cross correlate them through a multiplicity edited HSQC to ensure concurrent information is obtained and thereby identifying any symmetry elements. Addition of DQF-COSY and COSY followed by HMBC (^{1}H - ^{13}C and ^{1}H–^{15}N). When available, cryogenic probes and micro-cyroprobes, can be used to investigate inverted $^{1}J_{CC}$ 1, n-ADEQUATE becomes feasible[61] with quantity-limited sized samples, especially if a reduced volume Shigemi tube is used. Martin and Colleagues[82] have used a cogent exemplar, the highly proton-deficient alkaloid staurosporine as a model proton-deficient compound for their investigation promoting the combination of inverted $^{1}J_{CC}$ 1,n-ADEQUATE with 1.7 mm cryoprobe technology.

An impressive increase in the spectral information content of NMR can be achieved with experimental pulse schemes with direct observation of multiple free induction decays (FIDs). In addition, substantial improvement in measurement sensitivity per unit time can be observed, reducing the cost of NMR interpretation. This has become possible due to the design and construction of multiple receivers involving current, state-of-the-art commercial spectrometers. This allows spectra from different nuclear species to be recorded concurrently and in a parallel mode. Notably, Kupce *et al*[89] have reviewed this area and is essential reading for those considering the purchase of an efficient spectrometer. Experiments with multi-FID detection are available with both, homo-nuclear and multinuclear acquisition. Such Probes with multiple RF micro-coils routed to multiple NMR receivers provide a useful method of increasing rapid throughput of samples. The possibility of structure elucidation of small molecules from a single measurement was first demonstrated by the PANACEA experiment in 2008[90,91]. The MMFD (Multinuclear Many multi-FID detection) based PANACEA pulse sequence relies on the ^{13}C –^{13}C INADEQUATE experiment that gives unambiguous knowledge about the C-C connectivities in the carbon framework of organic molecules, and indicated earlier, requires high sensitivity/high sample concentration and/or protracted acquisition times. In INADEQUATE studies, the final goal focuses on determining the entire, carbon-carbon skeletal arrangement. Since the presence of heteroatoms within a molecular skeleton

induces disconnectivity, it prevents unambiguous determination of the entire structure. However, the resulting collection of molecular fragments or spin systems viz: C-X-C (X = O, S, N), can be stitched together fragments together using both the chemical shifts of the I3C nuclei as well as three bond connectivity (using HMBC). While the PANACEA experiment is specifically designed to analyse proton-deficient molecules, as is current practice, the structure of small natural products deduced from three basic 1H-detected 2D NMR experiments – HSQC, HMBC and COSY. The HSQC, HMBC and COSY modules can be linked into several NOAH super sequences, such as NOAH-3 SBC, NOAH-3 BSC, NOAH-4 SBCN, NOAH-4 BSCN, NOAH-5 MSBCN and so on and so forth[89].Moreover, the NOAH super sequences can also incorporate NOESY (N) and/or a ROESY (R) "module" providing essential stereospecific information[92]. Since such super sequences provide all the essential information required for the structure elucidation of small molecules in a single measurement application of automated CASE systems[93-96] will allow routine deduction of even challenging proton deficient molecules and should be considered essential in all natural product elucidation campaigns. However, the versatility of the technique is such that it has been applied to challenging material such as fullerenes[22] but in this lab, it has been established as a routine technique to solve the unambiguous solution state structures of semisynthetic and synthetic analogues of natural products antimalarials (such as quinine derivatives, chloroquine, amodiaquine), antioxidants (Trolox C) and other compounds. Consequently, INADEQUATE experiments deserve wider utilisation in organic, physical organic, medicinal and as well as natural product drug discovery programs incorporated into a strategy that avoids structural elucidation by overreliance on HMBC which can occasionally lead to missassignments[15,16] but is still needed to stitch together isolated spin systems where there are heteroatomic discontinuities in the framework.

Finally, in order to overcome the insensitivities arising from the probability of required spin pairs present at natural abundance, dissolution dynamic nuclear polarization (dDNP) offers a potential solution[97]. It has sufficient sensitivity to acquire 1D ^{13}C INADEQUATE spectra in a single scan at natural abundance. Moreover, by adopted a strategy that endows sub-Hertz precision to such measurements, carbon-carbon *J* couplings over both one and multiple bonds for each chemical site can be achieved. As mentioned previously, such $^{1}J_{CC}$ couplings are usually sufficiently distinct from one another to enable the univocal pairing of the nuclei under consideration. This provides an equivalent to a 2D INADEQUATE experiment. Although

relatively simple compounds were considered α-pinene, menthol and limonene, this approach is currently limited by the high cost of DNP-NMR instrumentation and operating costs but when combined with a combination of aforementioned approaches[98] and machine learning and Artificial intelligence approaches, first implemented as DENDRAL[99,100], may one day make INADEQUATE as routine as ^{1}H NMR.

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