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Novel Steroids from *Trichilia Hirta* as Identified by Nanoprobe INADEQUATE 2D-NMR Spectroscopy.[1]

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Abstract. Two novel steroids, 3-hydroxypregnane-2,16-dione [**1**] and 2-hydroxyandrosta-1,4-diene-3,16 dione [**2**], were isolated from ethanolic extracts of the wood and bark of *Trichilia hirta*. The structure of **1** was rigorously determined from 2D-INADEQUATE NMR data that was: 1) acquired with a new higher-sensitivity ^{13}C probe called a Nano•nmr probe, and; 2) processed with a new NMR-analysis program called FRED, which automatically generated the complete carbon structure (as shown). In this first known application of both a Nanoprobe and FRED software to an unknown compound, the complete carbon skeleton was easily determined using only 11 mg (30 μmoles) of sample.
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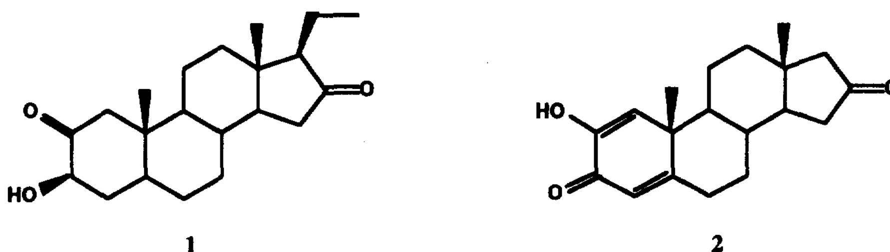
The mahogany family (Meliaceae) is a family of woody tropical plants, characterized by its synthesis of tetranortriterpenoids, also known as limonoid compounds; the family's well-known bioactivity against insects is generally attributed to these compounds.[2-5] As part of a program to develop new "green" insecticides, we have screened a large number of species of Meliaceae from Central America for biological activity.[6] Ethanolic extracts of *Trichilia hirta* proved to be among the most active in a study of the growth and development of the second instar European corn borer, *Ostrinia nubilalis*, and the neonate variegated cutworm, *Peridroma saucia*. The active extracts were thus further fractionated in order to identify the compounds responsible for the observed activity.

T. hirta wood and bark were collected in bulk from Costa Rica. The biologically-active ethanol extracts were partitioned into hexane, methylene chloride, butanol-ethyl acetate, and water-soluble fractions. The highest growth-reducing activity resided in the methylene-chloride fraction, whose freeze-dried residues were subjected to silica gel column chromatography, followed by preparatory reverse-phase HPLC. Two pure compounds were isolated, affording approximately 11 and 9 mg of each (ca. 0.005% yield from the original ethanol extract). High resolution mass spectral analysis provided the molecular formulae, $C_{21}H_{32}O_3$ [**1**] and $C_{16}H_{24}O_3$ [**2**]. The ^{1}H NMR spectra (500 MHz) were exceptionally well resolved, but did not contain the expected furan-ring resonances in the aromatic region (identified by their characteristic 2-Hz couplings) as would be observed for any typical limonoid-type structures.

^{13}C NMR spectra revealed the presence of two carbonyl functionalities on each compound (at 218.95 and 211.11 ppm for **1** and at 217.19 and 181.46 ppm for **2**), and IR analysis suggested that one carbonyl in each compound (at 1734 cm^{-1}) could reasonably be placed in a five-membered ring. Two isolated methyl groups on each compound exhibited unusually low chemical shifts, both by ^{1}H and ^{13}C NMR (at 0.75 / 13.39

ppm and 0.67 / 12.65 ppm for **1** and at 1.26 / 19.74 ppm and 0.94 / 18.09 ppm for **2**). The presence of a hydroxy substituent a to one carbonyl in each compound was easily determined, along with the a,b-unsaturation in compound **2**. Despite a detailed 2D ^{1}H NMR analysis of the extensive ^{1}H-^{1}H scalar couplings in both the A- and D-rings of each compound, the two sets of spin systems exhibited no inter-ring couplings that could substantiate any proposed structures, causing the assembly of the carbon skeletons to be a formidable task. While DEPT, HMQC, and HMBC NMR experiments suggested, rather inconclusively, that compound **1** was 3-hydroxypregnane-2,16-dione and compound **2** was 2-hydroxyandrosta-1,4-diene-3,16-dione, neither of these structures had previously been described in the chemical literature. Additional efforts to confirm these structures were constrained by the limited amount of pure isolated compound (~11 mg of **1** and ~ 9 mg of **2**).



One powerful strategy for elucidating organic structures is the ^{13}C-^{13}C INADEQUATE experiment.[7-9] INADEQUATE can unambiguously determine the carbon backbone of a structure; it does this by detecting carbon-carbon bonds directly, as opposed to merely inferring their presence from long-range ^{1}H-^{13}C coupling data as it is typically obtained in HMBC- and COLOC-style experiments. While INADEQUATE can typically provide a more rigorously-determined structure, the weakness of the technique is its low sensitivity.[10] Since INADEQUATE is a ^{13}C-detection experiment which detects only adjacent pairs of ^{13}C atoms, any given atom in a normal unlabeled sample (having 1.1% natural-abundance ^{13}C) may statistically have only a 1.1% x 1.1% = 0.012% chance of being detected. This is 100 times less sensitive than conventional ^{13}C NMR detection, and therefore usually requires much larger quantities of sample (often $\geq$ 100 mg) than can typically be obtained in natural-product isolation studies.

Two kinds of tools have recently become available which can radically improve the detection sensitivity of NMR in general, and of INADEQUATE in particular. The first are the Nano•nmr™ probes (Nanoprobes), which are high-resolution NMR probes developed specifically to improve the spectral resolution, sensitivity, and detection efficiency of very small (< 40 μL) liquid samples.[11] The second is FRED™, a spectral-interpretation software package for NMR, which can more thoroughly analyze a INADEQUATE data set, and thereby achieve effectively higher sensitivity.[12-15] After an automated FRED analysis, the software provides either the ^{13}C-^{13}C coupling data in a correlation-table format, or more importantly, a graphics drawing of the chemical structure complete with ^{13}C NMR assignments (mimicked in Figure 1).

We describe here the first known use of a *combination* of both a ^{13}C Nanoprobe and the FRED software *on an unknown compound* to obtain not only a complete 2D-NMR INADEQUATE spectrum, but also *the complete structure, using only 11 mg* (30 μmoles) of compound **1**.[16] These data, acquired in only 62 hours on a UNITY*plus* 500 MHz spectrometer, demonstrate a dramatic improvement in INADEQUATE detection limits by using almost ten times less material than conventionally required.[17] We also found the FRED data analysis to

be rapid (1.5 h), easy, objective, and virtually automated; this eliminates the subjective and tedious data analysis usually required when organic structures are culled from long-range ^{1}H-^{13}C coupling (HMBC) data.

Once FRED had determined the carbon backbone, and automatically assigned the ^{13}C chemical shifts of each atom (Figure 1), the ^{1}H chemical-shift assignments were straightforward with the NMR data obtained from the other homo- and heteronuclear 2D experiments (HMQC, COSY, TOCSY, and HMBC). After the structure of **1** was rigorously determined, the structure of **2** was assigned by analogy (without INADEQUATE data).[18]

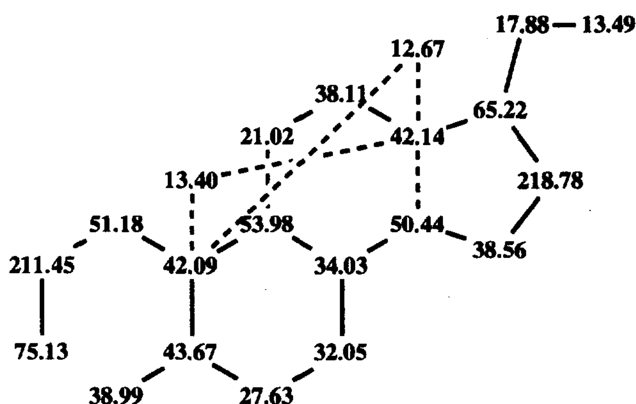


Figure 1. FRED structure output for compound **1** (redrawn; NMR solvent was CD_2Cl_2).

Stereochemical assignments at C3 and C17 of compound **1** were made in accordance with literature precedent. All naturally-occurring pregnane skeletons have their ethyl group oriented ß at C17. The hydroxy substituent at C3 was shown to be ß through analysis of the H3-H4 coupling patterns (11.6 Hz (ax-ax) and 7.7 Hz (ax-eq)), which were in agreement with values observed on other 3-hydroxylated steroidal A-rings (10.5 Hz (ax-ax) and 5 Hz (ax-eq)).[19]

While steroidal structures of the type isolated here are most commonly found in animals, they have been isolated from plants, although this is rather unusual.[20] Due to the small quantity of **1** and **2** available, the pure compounds have not yet been tested for insect antifeedant activity. We do anticipate, however, that larger quantities of **1** and **2** might be obtained more easily via direct synthesis, perhaps starting with testosterone derivatives.

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16. The combined use of a ^{13}C Nanoprobe and FRED was recently used to provide ^{13}C NMR assignments for a known compound whose structure had previously been determined by X-ray crystallography (Harper, J. K.; Dunkel, R.; Wood, S. G.; Owen, N. L.; Li, D.; Cates, R. G.; Grant, D. M. *J. Chem. Soc., Perkin Trans. 2* **1996**, 91-100). In other work, spectra obtained with a ^{1}H Nanoprobe were used to determine the structures of microgram quantities of novel carbohydrates isolated from tissues (Manzi, A.; Salimath, P. V.; Spiro, R. C.; Keifer, P. A.; Freeze, H. H. *J. Biol. Chem.* **1995**, *270*, 9154-9163).
17. The data were acquired over a weekend (62 hr) using a UNITY*plus* 500-MHz spectrometer. The ^{13}C T_1s are the critical parameters in INADEQUATE; our longest (isolated ^{13}C atom) T_1 was 6.2 sec for C-16. The repetition rate was 9.0 sec (including a 136 msec acquisition time) for 128 scans for each of 92 x 2 increments (States-TPPI). Apodizations and zero-filling do not influence the FRED analysis (output shown in Fig. 1) and were not used. The C3-C4 bond was presumably below the detection limit, while the near-coincidental C-10 and C-13 resonances caused methyl-group assignments to be ambiguous.
18. Compound **1**: **^{1}H NMR** (500 MHz, $CDCl_3$) d 4.13 (1H, ddd, J = 11.6, 7.7, and 1.2 Hz, H-3), 5.07 (1H, d, J = 13.0 Hz, H-1b), 2.21 (1H, dd, J = 18.3 and 7.1 Hz, H-15b), 2.16 (1H, ddd, J = 13.0, 7.7, and 3.2 Hz, H-4b), 2.07 (1H, dd, J = 13.0 and 0.7 Hz, H-1a), 1.91 (1H, dt, J = 11.6 and 3.0 Hz, H-12*), 1.75 (1H, dd, J = 18.3 and 12.8 Hz, H-15a), 1.74-1.63 (3H, m, H-5,7,17), 1.61 (1H, dq, J = 14.3 and 7.4 Hz, H-20), 1.55-1.20 (9H, m, H_2-6,11, H-4a,8,12,14,20), 1.14-1.02 (2H, m, H-7,9), 1.00 (3H, t, J = 7.4 Hz, H_3-21), 0.75 (3H, d, J = 0.7 Hz, H_3-19), and 0.67 (3H, s, H_3-18); **^{13}C NMR** (125 MHz, $CDCl_3$) d 218.95 (C-16), 211.11 (C-2), 74.92 (C-3), 65.23 (C-17), 53.91 (C-9), 50.96 (C-1), 50.31 (C-14), 43.54 (C-5), 42.01 (C-10,13), 38.68 (C-4), 38.41 (C-15), 37.93 (C-12), 33.87 (C-8), 31.83 (C-7), 27.35 (C-6), 20.77 (C-11), 17.60 (C-20), 13.39 (C-18,21), and 12.65 (C-19). Compound **2**: **^{1}H NMR** (500 MHz, $CDCl_3$) d 6.29 (1H, s, H-1), 6.17 (1H, d, J = 1.4 Hz, H-4), 2.50 (1H, ddt, J = 13.4, 5.1, and 1.4 Hz, H-6a), 2.43 (1H, ddd, J = 13.4, 4.6, and 2.6 Hz, H-6b), 2.19 (1H, dd, J = 18.1 and 7.6 Hz, H-15b), 2.13 (1H, d, J = 16.8 Hz, H-17), 1.97 (1H, dd, J = 18.1 and 13.5 Hz, H-15a), 1.94 (1H, d, J = 16.8 Hz, H-17), 1.93-1.83 (3H, m, H-7b,11,12), 1.80-1.70 (2H, m, H-9,12), 1.48 (1 H, ddd, J = 13.5, 10.9, and 7.6 Hz, H-14), 1.39 (1H, dt, J = 12.9 and 4.4 Hz, H-11), 1.26 (3H, s, H_3-19), 1.17 (1H, ddd, J = 12.2 , 10.9, and 3.8 Hz, H-8), 1.12 (1H, dd, J = 12.2 and 4.6 Hz, H-7a), and 0.94 (3H, s, H_3-18); **^{13}C NMR** (125 MHz, $CDCl_3$) d 217.19 (C-16), 181.46 (C-3), 172.06 (C-5), 146.23 (C-2), 123.66 (C-1), 121.30 (C-4), 55.46 (C-17), 53.38 (C-8), 50.68 (C-14), 44.04 (C-10*), 39.29 (C-13*), 39.25 (C-15), 37.76 (C-11), 34.84 (C-9), 34.05 (C-7), 32.62 (C-6), 22.84 (C-12), 19.74 (C-19), and 18.09 (C-18). *Assignment tentative.
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