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APPLICATIONS OF 2D-NMR SPECTROSCOPY TO PHYTOCHEMICAL STUDIES: CYPERENOL AND CYPERENOIC ACID

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ABSTRACT.—Initial phytochemical investigation of the previously unstudied plant species *Sandwithia guyanensis* yielded two crystalline compounds that were unambiguously identified by 2D-nmr spectroscopy as cyperenol and cyperenoic acid. The former compound has been reported only once previously, while the latter has never previously been isolated as a natural product. While the two structural characterizations required ca. 40 mg of sample and 16-24 h of spectrometer time, very recent advances should make it possible to carry out similar characterizations with as little as 1-2 mg of sample. The implications of this for strategies of phytochemical research are discussed.

The traditional approach to the chemical investigation of a natural product is to gather chemical and physical data for the compound at hand, and then search the literature to see whether these characteristics are consistent with a compound of established structure. The phytochemistry of taxa related to that under study provides a useful guide to the classes of compounds to be expected. It is only when the compound cannot be identified after exhaustive literature search that further structural work is warranted. The advent of various spectroscopic techniques generally increased the number of data available for comparison with published values but did not fundamentally alter the approach.

However, modern developments of nmr spectroscopy now offer the possibility of a radically different approach. They can be used to assign structures rapidly, non-destructively, and unambiguously to complex organic molecules, even without any prior structural information other than the molecular formula. The approach then becomes, if the structure of the compound cannot be immediately identified, to carry out a rapid structure assignment followed by a search of the literature to determine whether the structure has been assigned previously. In this paper we illustrate this approach for the case of two natural products isolated from a plant with no closely related species to provide phytochemical guidance, and for which very few structural clues were available at the start. The two compounds are the rare sesquiterpenoid alcohol cyperenol and the related cyperenoic acid, which has not previously been isolated from plant sources.

RESULTS AND DISCUSSION

Sandwithia guyanensis Lanj., an endemic plant of Guyana, was classified in 1932 as the first and, so far, only species of a new genus in the Euphorbiaceae (1). From this tree we isolated a white cystalline compound, mp 96-97°. Routine spectroscopic techniques provided evidence that its molecular weight was 220 (lrms) and that it was an alcohol (ir). Normal and DEPT-edited (2) ^{13}C-nmr spectra (in C_6D_6 inasmuch as two pairs of carbons almost overlapped in $CDCl_3$) indicated the presence of three CH_3 groups, six CH_2 groups, two CH groups, and four non-protonated carbons (two of them olefinic). These data are in accord with a molecular formual $C_{15}H_{24}O$, assuming the presence of one OH group. The ^{1}H-nmr spectrum revealed two CH_3 singlets and one CH_3 doublet; of the other signals, the most significant was at δ 4.07, attributed to a CH_2 group with a small difference in the proton chemical shifts. This was assigned as part of a CH_2OH

group. All chemical shift evidence from ^{13}C- and ^{1}H-nmr spectra were in accord with the assumption that this identified the only heteratom in the molecule, and the $C_{15}H_{24}O$ formula was considered established.

The number of known $C_{15}H_{24}O$ isomers is vast,[1] and many are incompletely characterized. Even if the reasonable assumption is made that the compound is a sesquiterpene (although these are not common in the Euphorbiaceae), comparison of our data with values in the literature presents a formidable task. It was soon obvious that we did not have one of the common sesquiterpenoids. Consequently, we undertook a structural assignment utilizing 2D-nmr spectroscopy.

The first two spectra obtained were a heteronuclear (^{13}C-^{1}H) shift-correlated spectrum optimized for one-bond ^{13}C-^{1}H coupling (3) and a homonuclear (^{1}H) COSY-45 spectrum (4). The former established direct ^{13}C-^{1}H connectivities and identified individual ^{1}H multiplets as due to methylene or methine protons, while the latter established coupling networks between the different ^{1}H multiplets. These two experiments allowed definite identification of a two carbon fragment, **1**, and probable identification of a six-carbon fragment, **2**. There was a slight uncertainty concerning the first three

δ_H	2.45 2.66	1.43 1.67		1.77 2.25	1.77	1.21 1.77	1.08 1.36	1.89	0.865		
	$-CH_2-CH_2-$		**1**	$-CH_2-CH-CH_2-CH_2-CH-CH_3$							**2**
δ_C	38.2	26.4		27.8	48.8	27.9	28.4	35.7	18.2		

carbons of **2** since the ^{13}C-^{1}H shift-correlated experiment showed that, by coincidence, two methylene carbons and one methine carbon all had an attached proton appearing at δ 1.77. The COSY-45 spectrum seemed to be most consistent with **2**, because no cross-peak was observed between the protons at δ 2.25 and δ 1.21 (see Figure 1). To confirm this fragment, a homonuclear relayed coherence transfer experiment (5) was carried out. This experiment involves transfers of magnetization between protons in a sequence of protonated carbons, aiding the assignment of three-carbon sequences. A cross-section through the multiplet at δ 2.25 revealed the expected relayed connectivity to the proton at δ 1.21, while the cross-section through δ 1.21 revealed relayed peaks to δ 2.25 and δ 1.89, as expected from structure **2**.

The COSY-45 spectrum also showed weaker connectivities between the pair of protons at δ 2.66 and 2.45 and the pair at δ 2.25 and 1.77. The chemical shifts of both sets of methylene protons were consistent with their being allylic to a double bond, suggesting that these connectivities arose from long range [homoallylic (6)] coupling.

In order to complete the structures, a second heteronuclear (^{1}H-^{13}C) shift-correlated experiment was carried out using delay times optimized for two- and three-bond ^{13}C-^{1}H coupling (7). This used a modified version of the standard pulse sequence (3) that incorporated a BIRD (bilinear rotation decoupling) pulse sandwich at the midpoint of the final delay before acquisition (8). This pulse sandwich serves not only to suppress spurious peaks corresponding to directly bonded ^{13}C-^{1}H pairs (8-11) but also to reduce the sensitivity of the experiment to the exact choice of delay (8-10).

Some typical results are illustrated in Figure 2 with other observed connectivities summarized in Table 1. One key observation was that the quaternary carbon at δ 66.2 showed cross-peaks with all three methyl ^{1}H signals (Figure 2a). The two methyl groups which gave ^{1}H singlets (δ_C 26.4, δ_H 0.81 and δ_C 19.5, δ_H 0.91) showed cross-peaks with each other, indicating that they were a *gem*-dimethyl pair (12). Both methyl ^{1}H singlets also showed cross-peaks with a second quaternary carbon (δ 41.8) and the

[1]The 1976-1981 cumulative index of *Chemical Abstracts* shows more than 1000 entries for $C_{15}H_{24}O$.

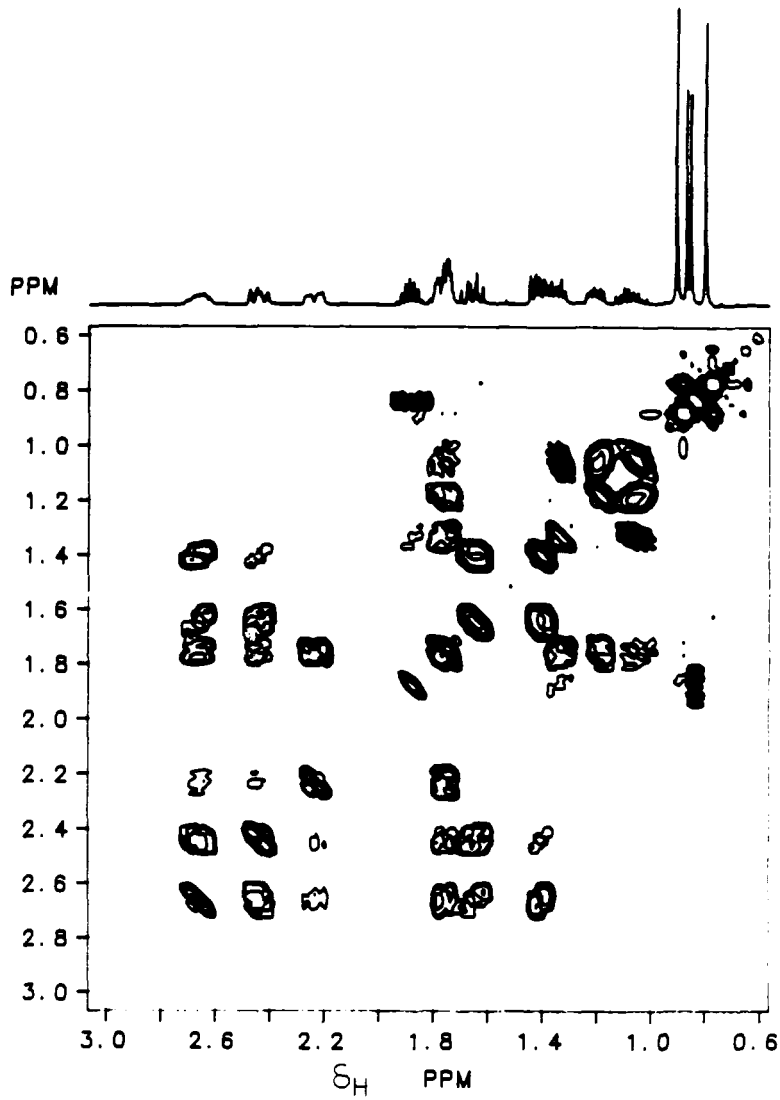


FIGURE 1. COSY-45 spectrum for **6**. Normal ^{1}H-nmr spectrum lies along the diagonal from lower left to upper right with off-diagonal peaks indicating coupling between pairs of protons.

methine carbon at δ 48.8. The methyl ^{1}H doublet (δ 0.87) also gave cross-peaks with a methine carbon (δ 35.7, Figure 2b) and a methylene carbon (δ 28.4). The only part structure which is consistent with all of these two-bond and three-bond connectivities is **3**. Combining this segment with the carbons in common for the previously assigned

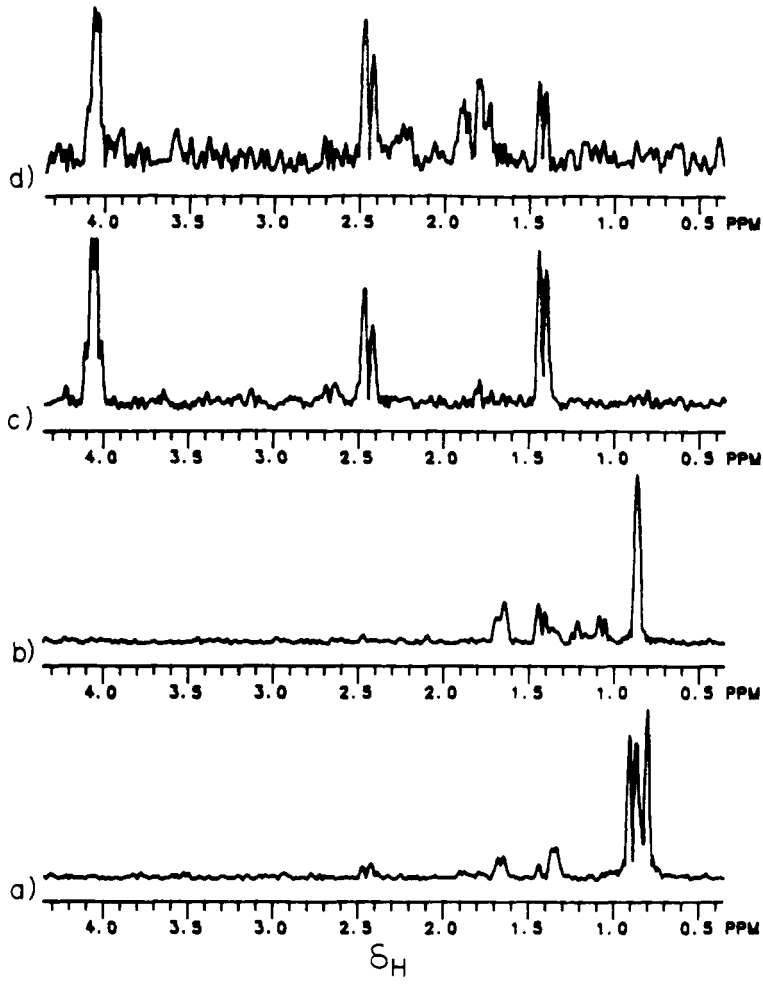
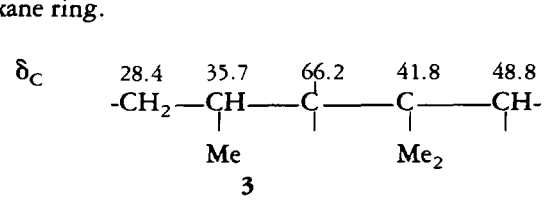


FIGURE 2. Long-range ^{13}C-^{1}H shift correlation spectrum of **6**: f_1 (^{1}H cross-sections) through specific ^{13}C (f_2) frequencies (a) δ 66.0 (C-3a), (b) δ 35.7 (C-4), (c) δ 132.2 (C-1), (d) δ 145.2 (C-8a).

segment **2** gave a more complete part structure **4**, involving a hexa-substituted cyclohexane ring.



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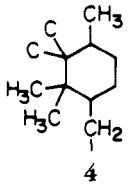

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The rest of the structure was deduced from observed two-bond and three-bond connectivities involving methylene or methine protons. For example, the quaternary car-

TABLE 1. Assigned ^{13}C and ^{1}H Chemical Shifts for Cyperenol [**6**] and Cyperenoic Acid [**7**]

Carbon	**6**				**7**	
	δ_C[a]		δ_H[b]	^{1}H-^{13}C Connectivities[c]	δ_C[d]	δ_H[d]
1	132.20	(131.09)		(2a), 2b, 3b, (8b), 10a, 10b	123.09	
2	38.17	(37.80)	2.66(2.63), 2.45(2.39)	3a, 3b, 10a, 10b	36.29	2.79, 2.69
3	26.42	(26.13)	1.67(1.65), 1.43(1.44)	2b	25.72	1.75, 1.53
3a	66.02	(65.88)		2b, 3a, 3b, 7, 11, 12, 13	68.19	
4	35.66	(35.28)	189(1.96)	3a, 3b, 5b, 6b, 11	35.97	2.07
5	28.43	(28.09)	1.36(1.42), 1.08(1.06)	6b, 11	27.86	1.51, 1.11
6	27.94	(27.57)	1.77(1.82), 1.21(1.28)	8a, 8b, 7, 5b	26.92	1.88, 1.36
7	48.83	(48.49)	1.77(1.86)	5a, 6, 12, 13	48.13	1.95
8	27.84	(27.54)	2.25(2.29), 1.77(1.86)	6a, 7	31.32	2.76, 2.25
8a	145.21	(146.26)		2b, 3b, 4, (8a), 8b, 10a, 10b	173.19	
9	41.29	(41.13)		3a, (6b), 12, 13	41.71	
10	60.34	(60.61)	4.97(4.17), 4.04(4.11)		170.92	
11	18.22	(17.93)	0.86_5(0.80)	4,5a	17.98	0.86
12	19.42	(19.29)	0.91(0.94)	13	19.28	0.99
13	26.36	(26.11)	0.80_5(0.77)	12	26.21	0.82

[a] ^{13}C chemical shifts of **6** in C_6D_6 and (in parentheses) in $CDCl_3$

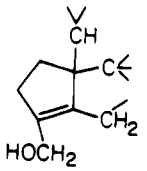
[b] ^{1}H chemical shifts of **6** in C_6D_6 and (in parentheses) in $CDCl_3$.

[c] ^{1}H-^{13}C cross-peaks corresponding to 2-bond or 3 bond C-H connectivities. The number refers to the proton giving a cross-peak with a particular carbon; (a) refers to the high-field proton of a pair of methylene protons. Weak cross-peaks are listed in parentheses.

[d] ^{13}C chemical shifts and ^{1}H chemical shifts of **7** in $CDCl_3$.

bon at δ 66.0 (Figure 2a) and the two low-field carbons at δ 132.2 (Figure 2c) and δ 145.2 (Figure 2d) all showed cross-peaks with protons bonded to both carbons in segment **1**. This is consistent only with a five-membered ring structure. Both the methine carbon at δ 35.7 (Figure 2b) and the quaternary carbon at δ 41.3 also showed cross-peaks with the δ 1.67 proton. Because all other possible two-bond and three-bond C-H connectivities involving these carbons have been established [see Bax and Freeman (4)], these must represent three-bond connectivities through the quaternary carbon at δ 66.0. Similarly, the methine proton at δ 1.89 shows a cross-peak with the low field olefinic carbon (Figure 2d), which must also represent a three-bond connectivity through the δ 66.0 carbon. These results establish that the remaining two bonds to this carbon involve the δ 26.4 and the δ 145.2 carbons. The latter carbon also shows cross-peaks with protons at δ 1.77 and δ 2.25, indicating that it is bonded to the methylene carbon at δ 28.4. Finally, the low-field methylene protons centered at δ 4.07 show cross-peaks with both olefinic carbons and with the δ 38.2 methylene carbon. The latter connectivity indicates a geminal arrangement for the two methylene groups which must involve both being attached to the δ 132.2 carbon. This wealth of connectivity data is consistent only with part structure **5**.

Combining **5** with **4** yielded the final structure **6** (without stereochemical information). Assigned ^{13}C- and ^{1}H-nmr spectral data in C_6D_6 and $CDCl_3$ are given in Table 1. A search of the literature revealed that **6** (with the illustrated stereochemistry) had been assigned in 1967 as the structure of cyperenol, a sesquiterpenoid isolated from *Cyperus scariosus* (Cyperaceae) (13). There have been no subsequent reports of the isolation of this



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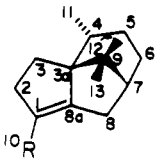

6 R=CH_2OH
7 R=COOH

compound, and only limited ^{1}H-nmr spectral data and no ^{13}C data were reported in the original communication. However, based on the limited previous available data, it appears that the two compounds are identical. Furthermore, complete ^{1}H- and ^{13}C-nmr spectral data have been relatively recently reported for the parent hydrocarbon, cyperene (14). With the exception of the expected short-range effects of replacing CH_2OH with CH_3, there is excellent agreement between our data (Table 1) and those for cyperene (14), indicating that the two compounds have the same molecular skeleton.

A more polar fraction of the *S. guyanensis* extract yielded a second crystalline component, mp 162-164°, which, on the basis of hrms, had an empirical formula $C_{15}H_{22}O_2$. Thus, the presence of an ir band at 1673 cm^{-1} and close similarities with many of the ^{1}H- and ^{13}C-nmr spectral features of cyperenol all suggested that the second compound was cyperenoic acid [7].

This was confirmed by a set of three 2D spectra (^{1}H COSY-45, and ^{13}C-^{1}H shift correlated spectra for directly and indirectly bonded carbons and hydrogens) that established all of the expected connectivities for cyperenoic acid and led to the ^{13}C- and ^{1}H-nmr spectral assignments given in Table 1. A literature search revealed that **7** had not previously been isolated from plant sources. As part of the earlier investigation (13) of the structure of cyperenol, it was oxidized to a $C_{15}H_{22}O_2$ compound, considered to be an αβ-unsaturated carboxylic acid. This compound was only partially characterized, but the published data show no correspondence with our values for cyperenoic acid.

In order to provide chemical verification for our assignments, cyperenoic acid was reduced to cyperenol by first converting it to its methyl ester and then reducing the ester with $LiAlH_4$. The carbonyl stretching frequency of the ester (1698 cm^{-1}) is unusually low, as is the corresponding value for the acid (1673 cm^{-}). Furthermore, the uv absorption of cyperenoic acid is at an unusually long wavelength (λmax 239 nm) for an αβ-unsaturated carboxylic acid. These features appear to be associated with a high degree of strain in the conjugated carbon-carbon double bond (15). In our hands, the Jones oxidation product of cyperenol was identical with our cyperenoic acid.

The results presented above clearly demonstrate how the skeletal structures of complex natural products can be unequivocally determined and ^{1}H- and ^{13}C-nmr spectra unambiguously assigned by 2D-nmr techniques. The main stumbling block is the relatively low sensitivity of the ^{13}C-^{1}H shift-correlated spectra requiring the use of relatively large amounts of sample. For example, the total time for the set of experiments used to deduce the structure of **6** was 23 h, using 42 mg of sample, while the spectra for **7** were accumulated in only 16 h, using 45 mg of sample.[2]

However, very recent improvements should largely overcome this problem. For example, microtube technology specially designed for high-field spectrometers should reduce the amount of sample required for compounds in this molecular weight range to

[2]Note, however, that the original structural assignment of **6** involved a number of chemical interconversions and required far more compound (13).

ca. 5 mg,[3] while another order of magnitude increase in sensitivity can be obtained using one of the new "reverse" ^{13}C-^{1}H shift-correlated experiments involving ^{1}H detection (16). Thus, in the near future it should be possible to carry out a similar structure determination by 2D nmr (including ^{1}H nOe spectra to obtain stereochemical information) in an overnight run using as little as 1-2 mg of sample. This capability could dramatically alter how one carries out phytochemical research, particularly if used in conjunction with a computerized data base of spectroscopic characteristics of known and well-characterized natural products. An obvious approach would be to carry out a set of routine spectroscopic measurements on each isolated compound (preferably including an edited ^{13}C-nmr spectrum). These could be checked against the data base to identify known compounds, while 2D nmr could be used to identify non-destructively new compounds or compounds which had previously been incompletely characterized. This would allow very thorough and efficient phytochemical investigations of individual plant species. With this in mind, we would encourage workers in the field of natural products chemistry to use 2D nmr to totally assign ^{1}H- and ^{13}C-nmr spectra of even known compounds whose spectra have not previously been assigned so that a comprehensive data base for the rapid identification of known compounds can be assembled.

EXPERIMENTAL

Melting points were determined on a Thomas-Kofler micro hot stage. A Nicolet 5DX ftir spectrometer (samples as KBr discs), a Cary 14 uv spectrometer (samples in 95% EtOH), a Varian XL-400 nmr spectrometer, Bell and Howell CEC 21-490 (lrms) and AEI MS30 (hrms) mass spectrometers, and a Perkin-Elmer 243B polarimeter were used.

2D-NMR SPECTRA.—COSY-45 spectra were obtained using a 1600 Hz ^{1}H spectral window, 512 data points, 256 time increments (zero-filled to 512), and 16 transients per time increment with a relaxation delay of 0.8 s. The data were processed using pseudo-echo weighting followed by symmetrization. Similar procedures were used for the homonuclear relayed coherence transfer experiments except that 48 transients per time increment were used while the relayed coherence transfer delay was 0.05 s. Direct ^{1}H-^{13}C shift-correlated spectra were obtained using a ^{13}C spectral width of 5000 Hz, with 2048 data points, a ^{1}H spectral width of 1000 Hz with 128 time increments (zero filled to 512), 128 transients per time increment, a relaxation delay of 0.5 s and fixed delays $\Delta_1=(2J_{CH})^{-1}$ and $\Delta_2=(3J_{CH})^{-1}$, assuming $J_{CH}=125$ Hz. The indirect ^{13}C shift correlated spectra were obtained using a ^{13}C spectral window of 16000 Hz with 4096 points, a ^{1}H spectral window of 1000 Hz with 256 time increments (zero-filled to 512), 640 transients per time interval for **6** and 480 transients per time interval for **7**, a relaxation delay of 0.5 s, $\Delta_1=0.064$ s and $\Delta_2=0.032$ s.

PLANT MATERIAL.—Material was collected in February 1985, at Bartica, Essequibo, Guyana. Voucher specimens are deposited at the Herbarium of the University of Guyana and at the Institute of Systematic Botany, Utrecht.

ISOLATION PROCEDURES.—Root wood and bark were air dried, ground, and extracted by cold percolation with MeOH. The semi-solid obtained by evaporation was partitioned between hexane and MeOH-H_2O (9:1). The hexane-soluble fraction was chromatographed (SiO_2, gradient elution). The fractions eluted with EtOAc-hexane (1:10) yielded cyperenol (0.025% of plant material), which, recrystallized from hexane, was obtained as white crystals, mp 96-97°; $[\alpha]D^{25}-14.4°$ (*c*, 2.34 in $CHCl_3$); ir 3388, 3325 cm^{-1}; ms M^+ 220. Later fractions (20-40% EtOAc) afforded cyperenoic acid (0.056% of plant material), which, recrystallized from Me_2CO/hexane, formed white crystals, mp 162-164°; $[\alpha]D^{25}-18.8°$ (*c*, 0.08 in $CHCl_3$); ir ~3000 (broad), 1673 cm^{-1}; uv λmax 239 nm (ε 11,950); ms exact mass 234.1618 (calcd. for $C_{15}H_{22}O_2$; 234.1620).

CONVERSION OF CYPERENOIC ACID TO CYPERENOL.—Cyperenoic acid on treatment with CH_2N_2 in Et_2O/MeOH formed its methyl ester, ir 1698 cm^{-1}, ^{1}H nmr δ 3.70 (s, 3H), which was reduced with $LiAlH_4$ in THF to provide cyperenol identical with material above (mp, tlc, ir, ^{1}H nmr).

[3]Personal communication to W.F. Reynolds from J.N. Shoolery, Varian Associates.

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LITERATURE CITED

1. J. Lanjouw, *Kew Bull.*, 183 (1932).
2. D.M. Doddrell, D.T. Pegg, and M.R. Bendall, *J. Magn. Reson.*, **48**, 323 (1982).
3. A. Bax and G.A. Morris, *J. Magn. Reson.*, **42**, 501 (1981).
4. A. Bax and R. Freeman, *J. Magn. Reson.*, **44**, 542 (1981).
5. P.H. Bolton and G. Bodenhausen, *Chem. Phys. Lett.*, **89**, 139 (1982).
6. M. Barfield and B. Chakrabarti, *Chem. Rev.*, **69**, 757 (1969).
7. K. Hallenga and G. van Binst, *Bull. Magn. Reson.*, **2**, 343 (1980).
8. M. Perpick-Dumont, M.Sc. Thesis, University of Toronto, 1986.
9. M. Perpick-Dumont, S. McLean, R.G. Enriquez, and W.F. Reynolds, *J. Magn. Reson.*, in press.
10. C. Bauer, R. Freeman, and S. Wimperis, *J. Magn. Reson.*, **58**, 526 (1984).
11. W.F. Reynolds, D.W. Hughes, M. Perpick-Dumont, and R.G. Enriquez, *J. Magn. Reson.*, **63**, 413 (1985).
12. W.F. Reynolds, S. McLean, J. Poplawski, R.G. Enriquez, L.I. Escobar, and I. Leon, *Tetrahedron*, **42**, 3419 (1986).
13. S.B. Nerali and K.K. Chakravarti, *Tetrahedron Lett.*, 2447 (1967).
14. P. Joseph-Nathan, E. Martinez, R.L. Santillon, J.R. Wesener, and H. Gunther, *Org. Magn. Reson.*, **22**, 308 (1984).
15. E.R.H. Jones, G.H. Mansfield, and M.C. Whiting, *J. Chem. Soc.*, 4073 (1956).
16. A. Bax and M.F. Summers, *J. Am. Chem. Soc.*, **108**, 2093 (1986).

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