Journal of Natural Products
Vol. 48, No. 5, pp. 823-825, Sep-Oct 1985

UNAMBIGUOUS ^{1}H-NMR ASSIGNMENT OF PHORBOL TRIACETATE USING A TWO-DIMENSIONAL NMR TECHNIQUE

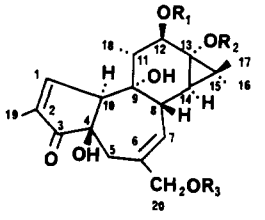
GARY T. MARSHALL, LEE-JUIAN LIN, and A. DOUGLAS KINGHORN*

Program for Collaborative Research in the Pharmaceutical Sciences and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, Health Sciences Center, University of Illinois at Chicago, Chicago, Illinois 60612

Croton oil, the seed oil of *Croton tiglium* L., has proven to be a valuable source of diesters of the diterpene alcohol, phorbol (**1**). Such compounds exhibit a variety of acids esterified at positions C-12 and C-13 and include the potent tumor-promoting agent, 12-*O*-tetradecanoylphorbol-13-acetate (**2**) (1). As a result of recent investigations in this laboratory, a number of new short-chain phorbol monoesters and diesters have been isolated from croton oil and identified by spectroscopic and chemical means (2,3).

Previously published ^{1}H-nmr spectral studies of phorbol derivatives have usually been reported with the assignment of all protons of the diterpene parent alcohol, with the exception of the C-11 methine proton signal, which is a complex multiplet of low intensity that usually overlaps more intense signals. In addition, the C-16 and C-17 methyl signals are left as interchangeable (4,5) or have been inconsistently assigned (2,6-9). With the increasing use of highfield nmr spectrometers, the C-16 and C-17 methyl group signals are well defined, especially in the case of phorbol esters with short-chain acyl units, and preferably should not be left unassigned. We detail here the assignment of the C-16 and C-17 methyl signals in the ^{1}H-nmr spectrum of phorbol-12,13,20-triacetate (**3**) and correct a previous assignment of the C-11 methine signal (10) using a two-dimensional (2-D) nmr technique.

The problem of correctly assigning the C-16 and C-17 methyl group ^{1}H-nmr chemical shifts of the phorbol esters was addressed using a 2-D ^{1}H-^{13}C-



	R_1	R_2	R_3
1	H	H	H
2	$CO(CH_2)_{12}CH_3$	$COCH_3$	H
3	$COCH_3$	$COCH_3$	$COCH_3$

heteronuclear shift correlation experiment (11). This experiment uses polarization transfer via *J*-coupling of protons and carbons to give a correlation map of carbon resonances on one axis and the directly bonded proton resonances on the other axis (11-13). Thus, only carbons bearing a proton are apparent in the carbon spectrum. Providing sufficient resolution can be achieved in the proton domain of this experiment, the clear separation of the C-16 and C-17 carbons in the carbon domain (10,14,15) might therefore provide an unambiguous assignment of these proton signals. The 2-D heteronuclear shift correlation spectrum of phorbol-12,13,20-triacetate (**3**) was obtained in the usual way. Although slightly overlapping, the correlation signals were found to be discrete enough to assign the methyl groups. The carbon resonating at 17.1 ppm, C-17, correlates with the further-downfield methyl ^{1}H-nmr signal, while the carbon signal at 24.2 ppm, C-16, correlates with the more upfield methyl ^{1}H-nmr chemical shift. Cross-sections of the 2-D heteronuclear shift correlation matrix, parallel to the proton spectrum through specific carbons, allowed for the repre-

sentation of the signals of the attached protons and their chemical shifts (16). In this manner, quantitative assessment of the chemical shifts of the C-16 and C-17 methyl protons was made. The C-17 methyl signal at 1.250 ppm appeared downfield from that of the C-16 methyl at 1.232 ppm. This heteronuclear chemical shift correlation experiment, therefore, indicated that the more downfield methyl group of the geminal dimethyl system is the C-17 methyl group.

Confirmation of the relative assignments of the C-17 and C-16 ^{1}H-nmr signals of phorbol-12,13,20-triacetate (**3**) was obtained using the nOe difference spectroscopy. Irradiation of the methyl signal at 1.250 ppm (C-17) resulted in the expected nOe enhancement of about 5% in the C-8 proton signal at 3.273 ppm, as well as a somewhat smaller enhancement of the C-11 proton signal at 2.206 ppm (see below for a discussion of this assignment). In contrast, while an obvious nOe enhancement was apparent for the C-14 proton signal when the C-16 methyl signal was irradiated at 1.232 ppm, no substantial nOe enhancements for the C-8 and C-11 protons were observed.

In addition, the 2-D ^{1}H-^{13}C-shift correlation experiment provided the correct assignment of the C-11 proton resonance in the ^{1}H-nmr spectrum of phorbol-12,13,20-triacetate (**3**). The chemical shift of C-11 is at 43.3 ppm (10,14,15). The peak in the contour plot corresponding to this carbon lies under the downfield edge or shoulder of the acetate signals at about 2.2 ppm.

TABLE 1. ^{1}H-nmr and ^{13}C-nmr Spectral Data for Phorbol-12,13,20-triacetate (**3**)[a]

Carbon Number	^{1}H nmr (δ,M,*J*)[b]	^{13}C nmr (δ)[c]
C-1	7.589, bs	161.1
C-2		136.0
C-3		209.2
C-4		73.9
C-5	2.423, 2.603, AB, 19.0 Hz	39.1
C-6		133.2
C-7	5.716, m	132.9
C-8	3.273, m	39.6
C-9		78.4
C-10	5.233, m	56.4
C-11	2.206, m	43.3
C-12	5.360, d, 10.3 Hz	77.2
C-13		65.9
C-14	1.085, d, 5.1 Hz	36.4
C-15		26.1
C-16	1.232, s	24.2
C-17	1.250, s	17.1
C-18	0.897, m	14.8
C-19	1.753, m	10.5
C-20	4.450, 4.473, AB, 12.8 Hz	69.7
C-9-OH[d]	5.544, bs	
Acetates	2.096, 2.127, 2.139, s	21.3, 21.4, 21.4 171.1, 171.4, 174.1

[a]The spectra were run in $CDCl_3$. Abbreviations: δ chemical shift in ppm downfield from TMS; M, multiplicity; s, singlet; d, doublet; AB, AB system; m, multiplet; b, broad; *J*, coupling constant.

[b]Recorded at 360 MHz.

[c]Recorded at 90.8 MHz.

[d]The C-4 hydroxyl proton was not observed in the ^{1}H-nmr spectrum.

This value differs substantially from that made in a previous report (10). Comparison of other phorbol carbon shifts and their correlations with their attached protons, as summarized in Table 1, is in agreement with those previously published (10,14,15).

EXPERIMENTAL

MATERIALS.—Phorbol-12,13,20-triacetate (**3**) was prepared from phorbol, obtained from croton oil (17), by acetylation following procedures outlined previously (18).

GENERAL EXPERIMENTAL PROCEDURES.—The 2-D ^{1}H-^{13}C-shift-correlated spectrum of phorbol-12,13,20-triacetate (**3**, 0.36 M in $CDCl_3$), was obtained using a Nicolet NT-360 nmr spectrometer operating at 90.8 MHz with a 5 mm ^{13}C tuned probe. The pulse sequence was that of Bax and Morris (11). The initial data matrix consisted of 256 spectra of 2048 points each, with 800 transients per spectrum. Zero-filling in the second dimension gave a final 512 by 1024 data matrix. The delay times used during the pulse sequence were, for Δ_1 and Δ_2, 3.8 msec and 2.3 msec, respectively. The spectral width in the first dimension is 8620 Hz and the accompanying ^{13}C-spectrum is a projection of the F_1 domain of the data matrix. The proton spectrum in the F_2 dimension has a 1502 Hz spectral width. However, the proton spectrum along the axis of the contour plot is a 360 MHz one-dimensional spectrum.

The nOe difference experiments performed on phorbol-12,13,20-triacetate (**3**) were run with a modified FALNOE pulse sequence (19), using a long, low-power decoupler pulse (10 sec). Since the ^{1}H-nmr signals of the C-16 and C-17 methyl groups of this compound are so close to one another, we decided to use subsaturation power to achieve frequency selectivity in this study. The sample (ca. 40 mg in 0.5 ml $CDCl_3$) was degassed using repeated freezing and thawing before being finally flushed with N_2. Since enhancements and saturation transfers associated with hydroxyl resonances could have created problems, preliminary deuterium exchange was conducted with D_2O to avoid such possible difficulties. Two sets of data, with and without nOe, were alternatively collected in adjacent blocks of memory for subsequent comparison of nOe effects. A total of 2496 transients was accumulated for each irradiation, with the same number of transients also being acquired for a control (without nOe) experiment. The accumulated free induction decays were then processed with identical line broadening (1 Hz) and phase correction. The nOe difference spectra were obtained on an NT-360 nmr spectrometer at ambient temperature.

LITERATURE CITED

1. E. Hecker and R. Schmidt, *Fortschr. Chem. Org. Naturst.*, **31**, 377 (1974).
2. G.T. Marshall and A.D. Kinghorn, *J. Am. Oil Chem. Soc.*, **61**, 1220 (1984).
3. G.T. Marshall, J.A. Klocke, L.J. Lin, and A.D. Kinghorn, *J. Chem. Ecol.*, **11**, 191 (1985).
4. G. Fürstenberger and E. Hecker, *Experientia*, **33**, 986 (1977).
5. B. Sorg, G. Fürstenberger, D.L. Berry, E. Hecker, and F. Marks, *J. Lipid Res.*, **23**, 443 (1982).
6. M. Ogura, K. Koike, G.A. Cordell, and N.R. Farnsworth, *Planta Med.*, **33**, 128 (1978).
7. S.E. Taylor, M. Edwards, E. Williamson, and F.J. Evans, *J. Pharm. Pharmacol.*, **33**, 54P (1981).
8. S.E. Taylor, M.A. Gafur, A.K. Choudhury, and F.J. Evans, *Tetrahedron Lett.*, **22**, 3321 (1981).
9. S.E. Taylor, M.A. Gafur, A.K. Choudhury, and F.J. Evans, *Phytochemistry*, **21**, 405 (1982).
10. F.J. Evans and S.E. Taylor, *Fortschr. Chem. Org. Naturst.*, **44**, 1 (1983).
11. A. Bax and G.A. Morris, *J. Magn. Reson.*, **42**, 501 (1981).
12. R. Benn and H. Günther, *Angew. Chem. Int. Ed. Engl.*, **22**, 350 (1983).
13. B.A. Blackwell, R. Greenhalgh, and A.D. Bain, *J. Agric. Food Chem.*, **32**, 1078 (1984).
14. M. Neeman and O.D. Simmons, *Can. J. Chem.*, **57**, 2071 (1979).
15. S.E. Taylor, A. Scutt, and F.J. Evans, *J. Pharm. Pharmacol.*, **34**, 109P (1982).
16. K. Nagayama, P. Bachmann, K. Wüthrich, and R.R. Ernst, *J. Magn. Reson.*, **31**, 133 (1978).
17. A.D. Kinghorn and G.T. Marshall, U.S. Patent 4,468,328, 28 August 1984.
18. G.T. Marshall and A.D. Kinghorn, *J. Chromatogr.*, **206**, 421 (1981).
19. "Nicolet NMC-1280 Manual," Nicolet Magnetics Corporation, Fremont, California, 1982, p. 125.

Received 4 February 1985