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# A82775B AND A82775C, NOVEL METABOLITES OF AN UNKNOWN FUNGUS OF THE ORDER SPHAEROPSIDALES

Dale R. Sanson[+], Hanna Gracz[+], Michael S. Tempesta[+*], David S. Fukuda, Walter M. Nakatsukasa, Thomas H. Sands, Patrick J. Baker, Jon S. Mynderse*

[+]Department of Chemistry, University of Missouri, Columbia, Missouri 65211, U.S.A.
The Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, Indiana 46285, U.S.A.

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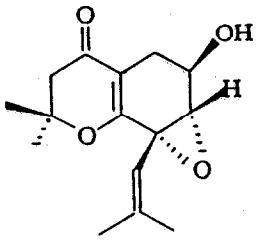
Abstract-Compounds A82775B (**1**) and A82775C (**2**) were isolated as non-antimicrobial by-products during attempts to isolate an elusive G+, G- antibiotic from culture A82775, an unidentified mold. A82775 factors B and C are present in both the mycelium and filtered broth. The metabolites were recovered from the filtered broth by adsorption on Diaion HP-20 nonfunctionalized macroreticular resin and purified by preparative reverse phase HPLC on C18 bonded phase supports. The structure elucidation of A82775 factors B (**1**) and C (**2**) has been accomplished by spectroscopic means including COSY, NOESY, INAPT, HMQC and 2D-INADEQUATE NMR experiments.

During our attempts to isolate an elusive antibacterial from culture A82775, an unidentified mold, we have discovered a novel allene-containing compound, A82775C (**2**), and related cyclization product, A82775B (**1**). The unique structural features of these compounds warranted full characterization. This paper describes the producing organism, fermentation conditions, purification, physico-chemical properties, and structural elucidation of A82775B (**1**) and A82775C (**2**) using extensive NMR as well as other spectral techniques.



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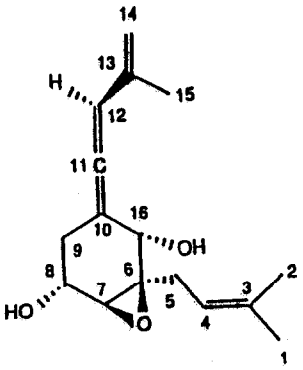

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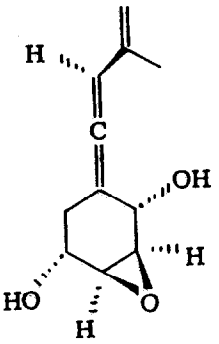

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## MATERIALS and METHODS

**Identification of Culture A82775.** Culture A82775 was isolated from a soil sample collected in Egypt. The culture is an unknown genus in the order Sphaeropsidales. It resembles *Phyllosticta* sp., the producer of phyllostinol and phyllostine,[1] but it differs significantly in spore and conidia shape. A82775 also differs significantly from *Eutypa lata*, a plant pathogenic fungus recently reported to produce structures similar to A82775C.[2]

**Fermentation.** A liquid nitrogen ampoule of culture A82775.1, a natural variant of the parent culture, was used to inoculate 50 ml of GSPM medium in a 250 ml Erlenmeyer flask and the culture was incubated for 48 h on a rotary shaker at 25°C (250 rpm, 6.4 cm throw). A 1 or 2 % (v/v) inoculum was made from the above vegetative medium to 50 ml production medium in 250 ml Erlenmeyer flasks and incubated at 25°C on a rotary shaker. The flasks were harvested by centrifugation and the supernatant and the MeOH extract of the biomass were assayed on seeded agar plates of *Micrococcus luteus* ATCC 9341 and *Escherichia coli* ESS-22-31. GSPM vegetative medium consists of potato dextrin 2.0%, corn steep liquor 0.7%, peanut meal 2.0%, $CaCO_3$ 0.3%, $MgSO_4 \cdot 7H_2O$ 0.05%, $KH_2PO_4$ 0.3%, $ZnSO_4 \cdot 7H_2O$ 0.005%, $FeSO_4 \cdot 7H_2O$ 0.01%, $MnCl_2 \cdot 4H_2O$ in deionized water. The pH of the medium was adjusted to 5.5 with NaOH. For inoculation of approximately 100-110 l of SGSM production medium, 10 ml of the vegetative culture was used to inoculate 2 l Erlenmeyer flasks containing 400 ml vegetative medium. This second stage vegetative medium was incubated at 25°C on a rotary shaker for 48 h and a 2 % inoculum was normally used for inoculation of the production medium. The fermentation was terminated at or near 65 h and the pH of the medium increased from an adjusted initial pH of 6.5 to around 6.9 - 7.3. The 165 l vessel was maintained at 0.5 volume of air per volume of medium per minute. Dissolved oxygen was controlled at greater than 30% of air saturation and aeration at 150 rpm. SGSM production medium consists of glucose 4.0%, corn starch 1.0%, soybean grits 1.0%, $CaCO_3$ 0.5%, Tween 80 0.1% in tap water. The pH was adjusted to 6.5 with $H_2SO_4$. The 165 l stirred fermentors also contained antifoaming agents SAG 471 (0.02%) and P-2000 (0.01%).

**Isolation of A82775B(1).** Whole broth (220 l, pH 7.0) was filtered with 3% Hyflo filter aid. Although A82775B was present in both the mycelium and the filtrate, we chose to proceed with isolation of the compound from the filtrate. Following adjustment of the filtered broth (200 l) to pH 6.5, Diaion HP-20 macroreticular resin (10 l) was added and stirred for 2 h. After removal of the effluent, the resin was washed with water (50 l). The resin was then eluted batchwise with $CH_3CN$-$H_2O$ (8:2, 30 l), followed by $CH_3CN$ (12 l). The $CH_3CN$ eluate was concentrated to approximately 300 ml, then extracted with EtOAc (3 x 1 l). The EtOAc extract was concentrated under reduced pressure to give an oil (18.4 g) which was slurried in hexanes. The hexane-insoluble material (4.0 g) was subjected to reversed phase high performance liquid chromatography (HPLC) on a steel column (1 in. dia. x 16 in.) packed with Du Pont Zorbax $C_8$ resin (12μ). The column was eluted with $CH_3CN$ - $H_2O$ (1:3, 670 ml), $CH_3CN$ - $H_2O$ (1:1, 740 ml), then $CH_3CN$ (510 ml), at a flow rate of 8 ml/min., collecting 16 ml fractions. Fractions 44 - 54 were combined, concentrated under reduced pressure, and lyophilized to give impure A82775B (139.9 mg). The impure A82775B was subjected to a second RPHPLC step on a steel column (1 in. dia x 12 in.) packed with Amicon Matrex silica LC resin ($C_{18}$, 100Å, 10μ). The column was eluted with $CH_3CN$ - $H_2O$ (1:3, 1130 ml), $CH_3CN$ - $H_2O$ (27.5:75.5 1 liter), $CH_3CN$ - $H_2O$ (1:1, 140 ml), then $CH_3CN$ (500 ml), at a flow rate of 7 ml/min., collecting 14 ml fractions. Fractions 134 - 140 were combined, concentrated under reduced pressure, and lyophilized to give A82775B (**1**) (22.3 mg).

**Isolation of A82775C(2).** Filtered broth (95 liters, pH 6.5) from whole broth (100 L, filtered as above), was extracted with EtOAc (65, 40 L) and the extract concentrated to give an oil (31.6 g). Dried extract (5.0 g) was subjected to a RPHPLC step on a steel column (1 in. dia. x 12 in.) packed with Amicon Matrex silica LC resin ($C_{18}$, 100Å, 10μ). The column was eluted with $CH_3CN$ - $H_2O$ (1:3, 600 ml), $CH_3CN$ - $H_2O$ (35:65, 1360 ml), $CH_3CN$ - $H_2O$ (8:2, 500 ml), then $CH_3CN$ (54 ml), at a flow rate of 7.5 ml/min., collecting 15 ml fractions. Fractions 100 - 137 were combined and concentrated under reduced pressure, from which A82775C (**2**) (407 mg) precipitated as a white amorphous solid.

**Physical Analyses.** Optical rotations were measured on a Perkin Elmer 241 polarimeter. Ir and UV spectra were recorded on Beckman Model 4210, Nicolet 10DX FTIR, Cary 219 and Pye Unicam Sp 8-100 UV/VIS spectrophotometers, respectively. MS of the samples were obtained as follows: FD measurements were made with a Varian MAT 731, and FAB data were collected on VG Analytical ZAB2-SE and ZAB3 instruments.

**NMR Experiments.** NMR experiments were performed on a Nicolet NT-300 WB spectrometer equipped with 5-mm $^1H$ and $^{13}C$-NMR single frequency probes operating at 300 and 75.5 MHz, respectively. All $^1H$-NMR chemical shifts were referenced to internal TMS (0.00 ppm). The homonuclear 2-D COSY and NOESY experiments[3] used a delay of 2 s and a 90-deg pulse of 9.0 μs, block size of 512, 128 increments and 32 transients. All $^{13}C$ NMR chemical shifts were referenced to the deuterated solvent (acetone-$d_6$ = 29.8 ppm). All one-dimensional $^1H$ and $^{13}C$ NMR experiments were run with a 16K block size using a spectral window of 2,400 Hz (0-8 ppm) and 17,000 Hz (0-225 ppm), respectively. The pulse sequences BILEV and DEPT were used to obtain $^{13}C$ NMR spectra.[4] The θ values were varied from $\pi/4$, $\pi/2$ and $3\pi/4$. The sensitive 1-D long-range heteronuclear chemical shift correlation experiment INAPT[5] was performed on A82775B and A82775C with a 90-deg carbon pulse of 11 μs and a 90-deg proton soft pulse of 10 ms. The polarization transfer delays Δ1 and Δ2 were optimized for $J_{CH}$ = 6 Hz. Due to a sample size of 200 mg of (**2**), only 500 transients per irradiation were needed to observe INAPT spectra. In the reverse detection experiments heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC), both short and long range $^1H$ detected heteronuclear chemical shifts were correlated.[6,7] HMQC was run with a 1K block size, using 128 increments and 128 transients. A $\pi/2$ $^{13}C$ pulse of 11.0 μs was used

with a $\pi 2^1H$ pulse of 9.0 μs. Acquisition time for the one-bond $^1H$-$^{13}C$ HMQC experiment was 10 h, while the long-range HMBC experiment required 14 h. Homonuclear $^{13}C$-$^{13}C$ couplings used to confirm the novel carbon skeleton of (2) were found using the 2D-INADEQUATE experiment,[8] with a block size of 1K, 256 increments, 256 transients and a sample size of 200 mg (acetone-$d_6$) in a 5 mm tube. Acquisition time was 24 h. Quaternary carbons were distinguished from protonated carbons by generation of $^{13}C$ subspectra via the heteronuclear spin-echo pulse sequence QUAT.[9] A $\pi/2$ $^{13}C$ pulse of 9.90 μsec with $^1J_{CH}$ coupling of 135 Hz was used to optimize experimental conditions, with a delay time of 6 sec and 800 total transients. One bond $^1H$-$^{13}C$ connectivities were found using the 2-D heteronuclear correlation experiment HETCOR.[10] A $\pi/2$ pulse of 9.0 μsec, 128 increments, 128 acquisitions and a block size of 1K were used. A delay time of 1.72 msec (1/2 $J_{CH}$) was used for observing one-bond $^1H$-$^{13}C$ couplings.

Table 1. Physico-chemical properties of A82775B and A8775C

| | | A82775B(1) | A82775C(2) |
|---|---|---|---|
| Appearance | | Pale yellow solid | White amorphous solid |
| $[\alpha]_D$ | | -204 (c 0.05, MeOH) | +175 (c 0.10, MeOH) |
| UV $\lambda_{max}^{EtOH}$ nm (ε) | | 288 (9,444) | 220 (26,356) |
| IR (KBr) cm-1 | | 3450, 2989,1649<br>1612,1405, 1259<br>1176 | 3250, 2971, 2917<br>1955, 1500, 1455<br>1048,1029, 905 |
| Molecular formula | | $C_{16}H_{22}O_4$ | $C_{16}H_{22}O_3$ |
| Anal. | Calcd | C 69.04, H 7.97 | C 73.25, H 8.45 |
| | Found | C 68.75, H 7.93 | C 73.53, H 8.50 |
| Mass spectrometry | | FD-MS m/z 278 (M) | FD-MS m/z 262 (M), 245 |
| HRFABMS | Calcd | 279.1596 (M+H) | 399.1664 (M+H-$H_2O$+magic bullet) |
| | Found | 279.1634 | 399.1663 |

## RESULTS AND DISCUSSION

**Physico-chemical Properties and Structure Elucidation.** A82775B (1) and A82775C (2) are insoluble in water, slightly soluble in hexanes, and freely soluble in MeOH, EtOAc, and $CHCl_3$. Additional physico-chemical properties are listed in Table 1. Our attention was first attracted to A82775C (2) by the sharp absorption at 1955 $cm^{-1}$ in the IR spectrum. This feature, accompanied by a peak at δ 204.3 in the $^{13}C$ NMR, without any evidence for carbonyl in the IR suggested the presence of an allene functionality. The UV absorption at 220 nm suggested that the allene is conjugated. The molecular ion was observed in the FD-MS at *m/z* 262. The elemental analysis is consistent with a molecular formula of $C_{16}H_{22}O_3$, which requires six degrees of unsaturation. The carbon multiplicities were obtained by the NMR experiments DEPT[4] and QUAT[9] as shown in Figure 1. The central sp carbon of the allene (δ 204.3, s) as well as the six remaining $sp^2$ carbons (δ 140.1, s;

135.0, s; 118.6, d; 102.0, s; 97.9, d) accounted for four degrees of unsaturation, with the remaining two due to rings. One of the two rings was assigned as an epoxide on the basis of chemical shifts and multiplicities (δ 63.6, s; 62.3, d). The 2-D INADEQUATE experiment[8] in Figure 2 allowed one-bond $^{13}C$-$^{13}C$ couplings to be determined, except to the sp carbon of the allene at 204.3 ppm, giving the gross structure of **2** as depicted (sans stereochemistry). The 2-D Heteronuclear $^{1}H$-$^{13}C$ Chemical Shift Correlation (HETCOR) experiment[10] depicted in Figure 3 allowed complete assignment of protons and their

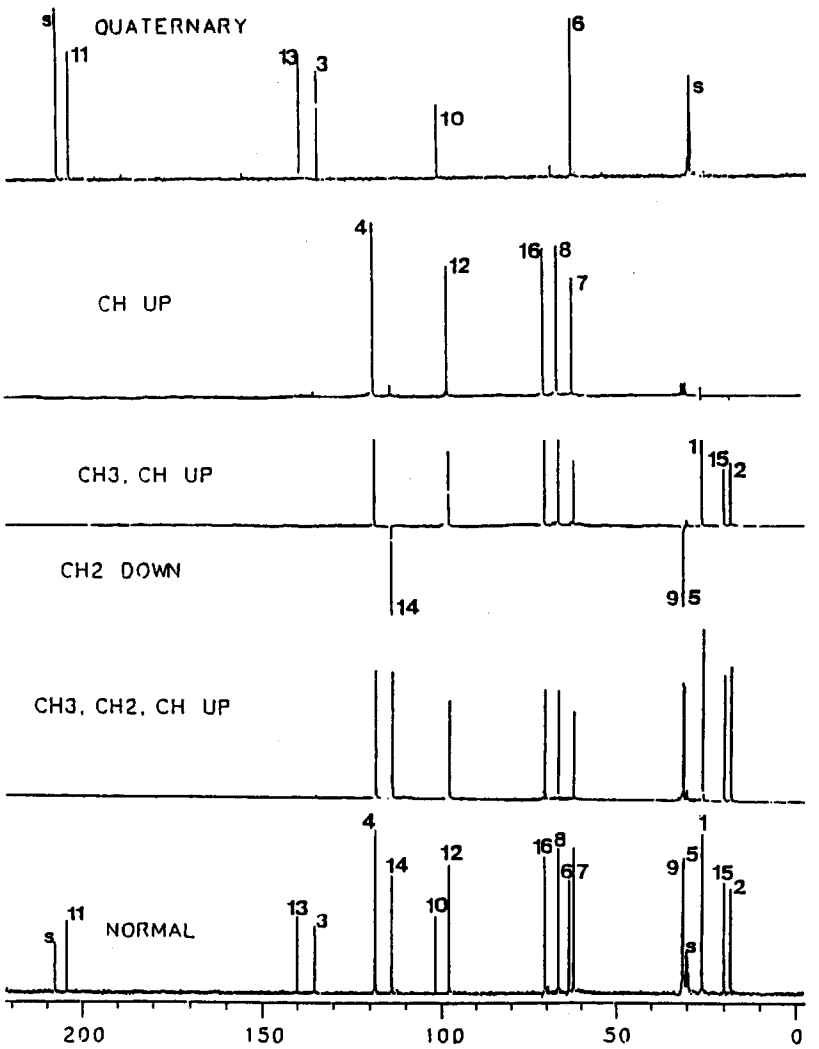


Figure 1. Broad-band decoupled, DEPT and QUAT $^{13}C$ NMR spectra of 2 at 75.5 MHz.

carbons to be made. The 2-D one-bond heteronuclear reverse-detection HMQC experiment[6] shown in Figure 4 gave the same assignments as in Figure 3. Completion of the gross structure of **2** was carried out using the 1-D long range heteronuclear experiment INAPT.[5] In particular, when 12-H (δ 5.90) was irradiated in the INAPT experiment, polarization transfer was observed to C-11 (δ 204.3, the central allene sp carbon), as well as C-9, C-10, C-13, C-14 and C-16 as shown in Figure 5. The 2-D long-range heteronuclear reverse-detection experiment HMBC[7] given in Figure 6 verified the NMR assignments obtained by the above methods. The relative stereochemistry of **2** except between the allene and ring centers was determined through the use of the 2-D homonuclear $^{1}H$-$^{1}H$ chemical shift correlation experiment COSY (Figure 7), NOESY and J values. The nOe's obtained from the NOESY experiment are reflected in the proton assignments shown in Figure 8. The observance of nOe's from the epoxide methine (7-H,

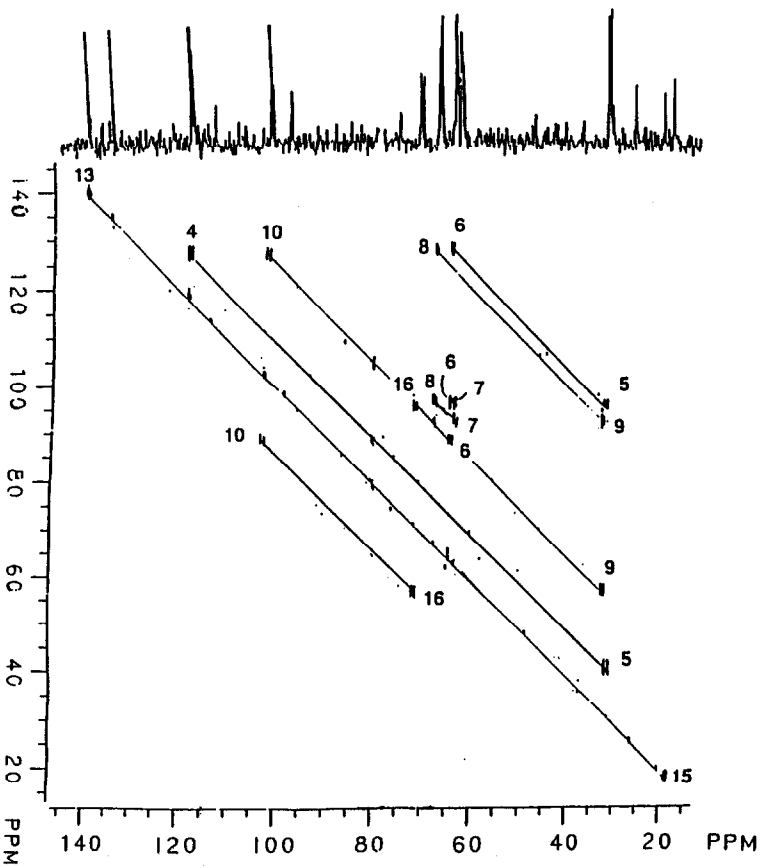


Figure 2. Homonuclear $^{13}C$-$^{13}C$ 2-D INADEQUATE plot of **2** in acetone-$d_6$ at 75 MHz.

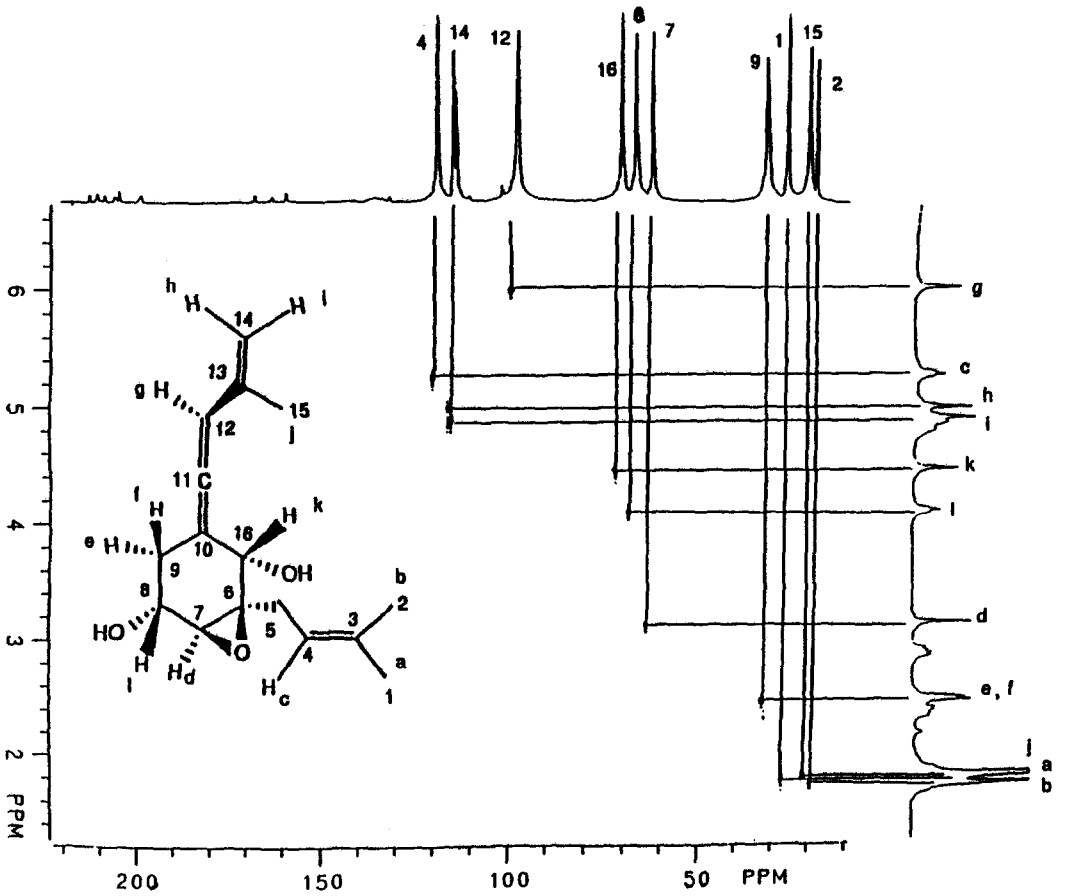


Figure 3. 2-D Heteronuclear $^{1}H$-$^{13}C$ Chemical Shift Correlation plot of 2 in acetone-$d_6$.

δ 3.00) to the methine attached to OH at δ 3.97 (8H), and to the isoprenyl methylene proton to the adjacent methine bearing OH at δ 4.34 (16H) suggested the relative stereochemistries of the four contiguous centers at C-16, C-6, C-7, C-8 are as depicted. The relative stereochemistry between the allene and the ring centers was assumed to be the same as found in **3**, which was derived from an X-ray study.[2] It should also be noted that the ring stereochemistries of **2** and **3** are similar.

Elemental analysis of **1** indicated a molecular formula of $C_{16}H_{22}O_4$, which is appropriate for 6 degrees of unsaturation. The $^{13}C$ NMR data ($Me_2CO$-$d_6$) of **1** require three degrees of unsaturation for a ketone and two enes (δ 190.2, s, C=O; δ 106.2, s, 118.4, d, 133.7, s, 163.9, s). The remaining degrees of unsaturation must therefore be due to three rings. One of the rings was assigned as an epoxide ($^{13}C$ NMR: δ 58.7, s; 62.1, d) using similar arguments to those given for **2**. Isolated proton spin systems were found using both COSY and NOESY and are depicted in the proton assignments shown in Figure 9. INAPT

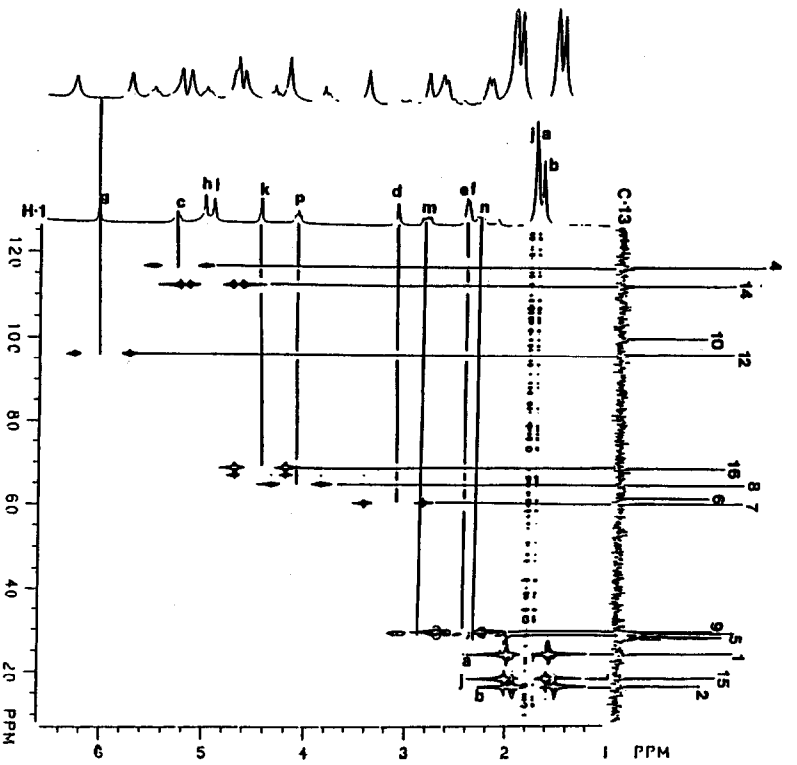


Figure 4. 2-D one bond reverse detection heteronuclear multiple bond correlation (HMBC) plot of **2** in acetone-$d_6$.

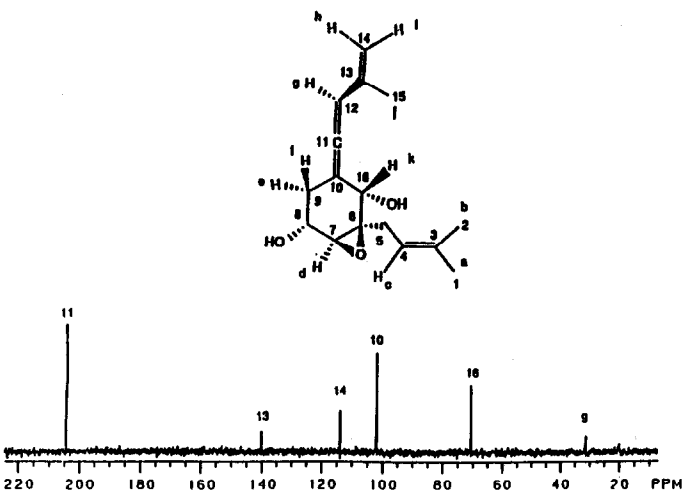


Figure 5. 1-D long range heteronuclear $^{1}H$-$^{13}C$ INAPT spectrum of **2** in acetone-$d_6$ showing polarization transfer from allenylic proton at 5.90 ppm to C-9, C-10, C-11, C-13, C-14 and C-16.

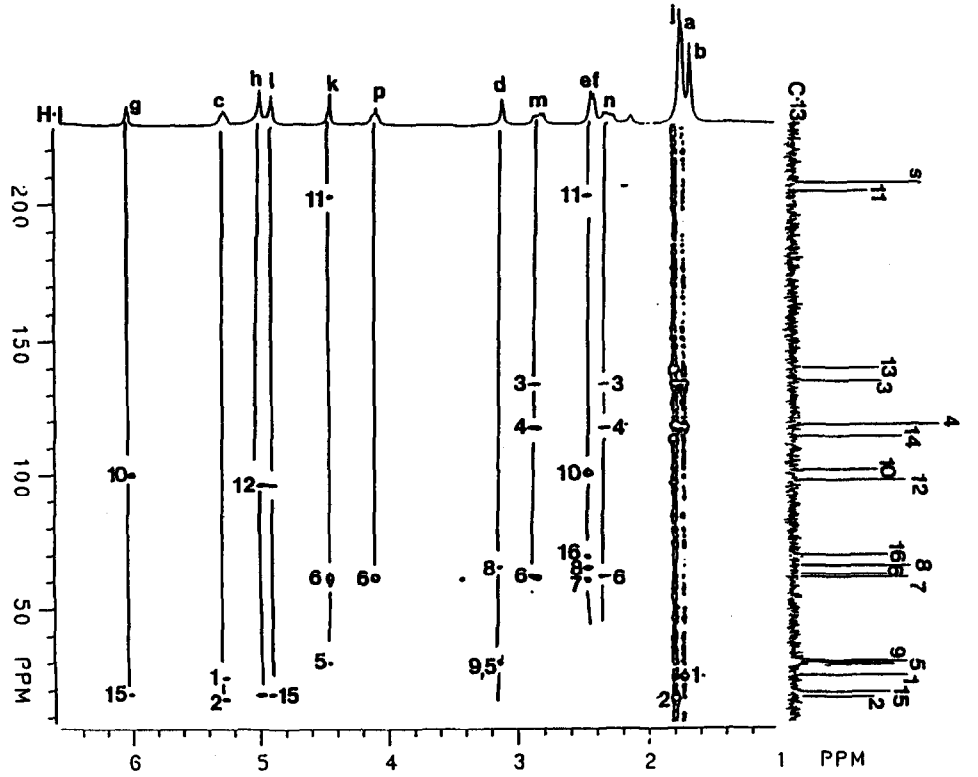


Figure 6. 2-D long-range reverse detection heteronuclear multiple quantum coherence (HMQC) plot of **2** in acetone-$d_6$.

experiments on **1** allowed the gross structure to be as depicted, as well as assigning the $^{13}C$ NMR peaks unambiguously. The relative stereochemistry of **1** was derived from NOESY. The absolute stereochemistry of **1** was determined using the chiral O-methyl mandelate ester method of Mosher-Trost.[11] The results from the Mosher-Trost method applied to **1** are summarized in Figure 10. The $^1H$ NMR $\delta$ values of the S- and R-mandelate ester derivatives of **1** given in Figure 10 (R-mandelate in **bold**) are consistent with the absolute stereochemistry depicted. By biosynthetic analogy, the absolute stereochemistry of **2** is expected to be the same as that of **1** and **3**.

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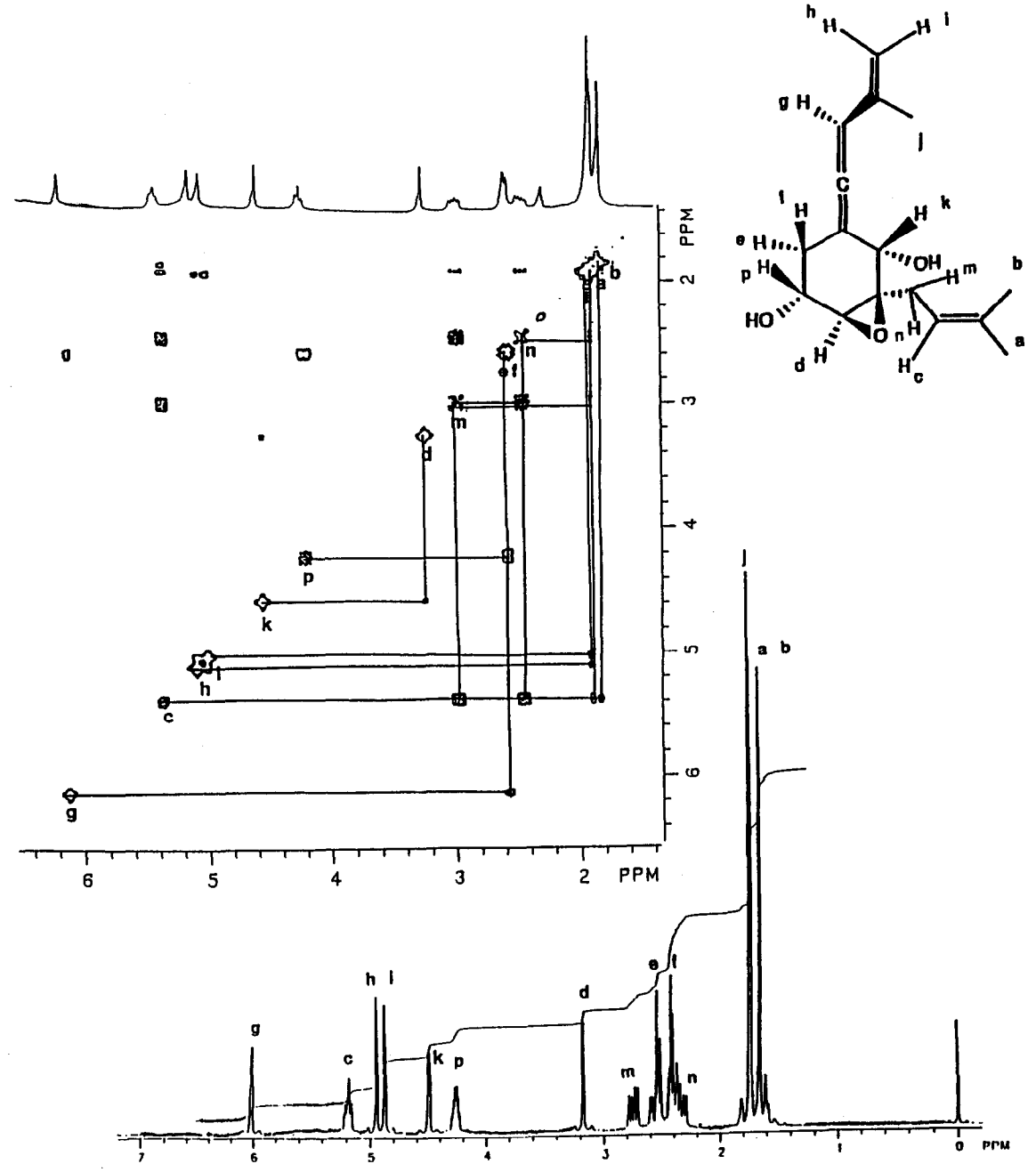


Figure 7. 1-D $^1H$ NMR spectrum and 2-D homonuclear $^1H$-$^1H$ chemical shift correlation spectroscopy (COSY) plot of 2 in acetone-$d_6$ at 300 MHz.

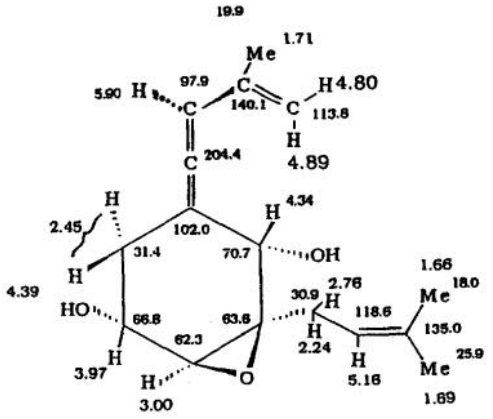


Figure 8. Proton assignments of **2** in acetone-$d_6$ derived from COSY and NOESY spectral data at 300 MHz, as well as $^{13}C$ NMR assignments based on 2-D INADEQUATE data.

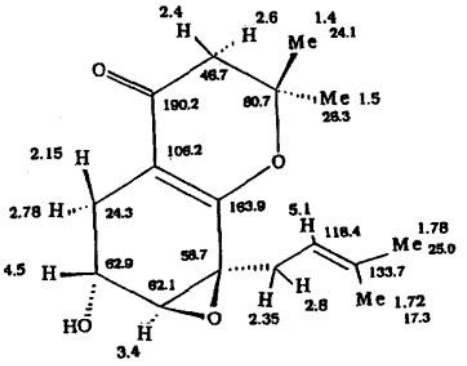


Figure 9. Proton assignments of **1** in $CDCl_3$ derived from COSY and NOESY spectral data at 300 MHz, as well as $^{13}C$ NMR assignments at 75 MHz in acetone-$d_6$ based on INAPT data.

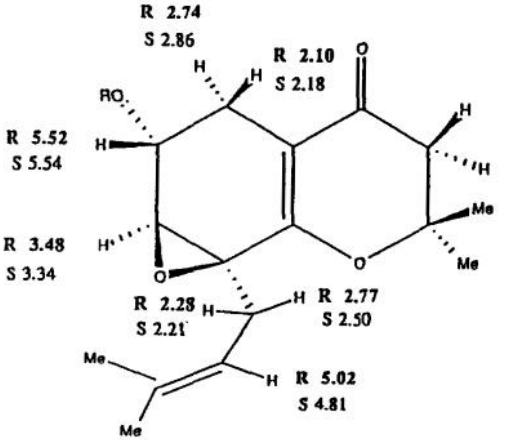


Figure 10. $^1H$ NMR values of the R- and S-mandelate ester derivatives of **1** at 300 MHz with the R-mandelate in **bold**.

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