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Metabolite Structure Elucidation Using NMR Micro-/Nano-detection

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Abstract: The structure of the epoxide of 1-ethyl-phenoxathiin 10,10-dioxide, a potential monoamine oxidase-A inhibitor, was elucidated using a combination of 500 MHz homo- and heteronuclear NMR techniques. The proton reference spectrum and a COSY spectrum were obtained on a 30 µg sample (0.07 µMoles) of the metabolite in 35 µl of d_5-DMSO using a Varian homonuclear Nano-probe™. The ^{13}C reference spectrum was acquired on the same sample using a Varian heteronuclear Nano-probe™. An inverse-detected (HMQC) heteronuclear shift correlation spectrum was obtained on the 30 µg sample of the metabolite in 120 µl of d_5-DMSO using a Nalorac Z•SPEC™ 3 mm micro inverse probe.

Nuclear magnetic resonance (NMR) spectroscopy offers a powerful means of differentiating isomers and determining molecular structures. The sensitivity of ^1H, relative to low abundance/low γ nuclides, e.g. ^{13}C, has made ^1H the NMR nuclide of choice for structure elucidation of samples with limited availability. Under favorable circumstances, it is possible, due to the recent development of micro inverse detection probes,[1,2] to acquire both direct (HMQC)[3] and long-range (HMBC)[4] heteronuclear shift correlation data on samples as small as 35 µg (mw 232 Da[1]). Complimentary development of micro dual probes for direct ^{13}C observation have made it possible to acquire ^{13}C reference spectra on samples of several hundred micrograms in reasonable times.[5] The recent development of a magic angle sideways spinning heteronuclear Varian Nano-probe™ has allowed us to record a ^{13}C reference spectrum, albeit with relatively low signal:to:noise (s/n), on the 30 µg sample available in the present study. The new NMR probe technologies used in this study are, in our view, complementary rather than competitive. This hardware offers the spectrocopist a powerful new tool with vastly improved sensitivity. When coupled with new mass spectrometric techniques, detailed analysis of metabolites, natural products, synthetic impurities, and other precious samples, in very limited quantities, can now be accomplished in a fraction of the time.

The compound 1-ethyl-phenoxathiin 10,10-dioxide (**1**) (1370U87) is a novel, selective monoamine oxidase-A inhibitor which has been under development as an antidepressant agent. The metabolism of 1370U87 was investigated using *in vitro* systems. In cultured precision-cut human liver slices and human liver microsomes, 1370U87 was extensively metabolized. The major metabolites identified were the 1-(1-hydroxyethyl)-phenoxathiin 10,10-dioxide (**4**) and 1-(1',2'-dihydroxyethyl)-phenoxathiin 10,10-dioxide (**3**). Further investigations using epoxide hydrolase inhibitor 1,2-epoxy-3,3,3-trichloropropane (TCPO) have

indicated the presence of another compound, which was initially identified by mass spectrometry as 1-(2'-oxiranyl)-phenoxathiin 10,10-dioxide (**2**); an intermediate presumably further metabolized to **3**.

The ^{1}H and ^{13}C reference spectra of **2** are shown in Figure 1. These data were acquired by dissolving the isolated metabolite sample in 35 µl of d_6-DMSO (Cambridge) which was transferred via a 100 µl Hamilton syringe to a 40 µl Varian Nano-probe™ cell. The proton spectrum[6] (Figure 1) and the COSY spectrum (Figure 2) were acquired in 128 transients and in 13 minutes, respectively; the water and protiosolvent resonances in both spectra were suppressed. The ^{1}H data were acquired using a $^{1}H/^{19}F$ homonuclear Varian Nano-probe™. The ^{13}C reference spectrum was acquired overnight as 27,900 transients using a Varian heteronuclear Nano-probe™.[7] The COSY spectrum established the two aromatic spin systems and identified the three coupled spins of the epoxidized side chain. The protonated aromatic resonances were observed at 135.3, 134.8, 125.6, 123.0, 121.4, 119.1, and 118.8 ppm. The aliphatic carbons resonated at 51.0 and 48.7 ppm, consistent with an epoxide. Quaternary carbons resonated at 148.6, 148.2, 138.0, 136.9, and 131.0 ppm. The ^{13}C resonances were consistent with the substitution and the oxidation state of sulfur.[8]

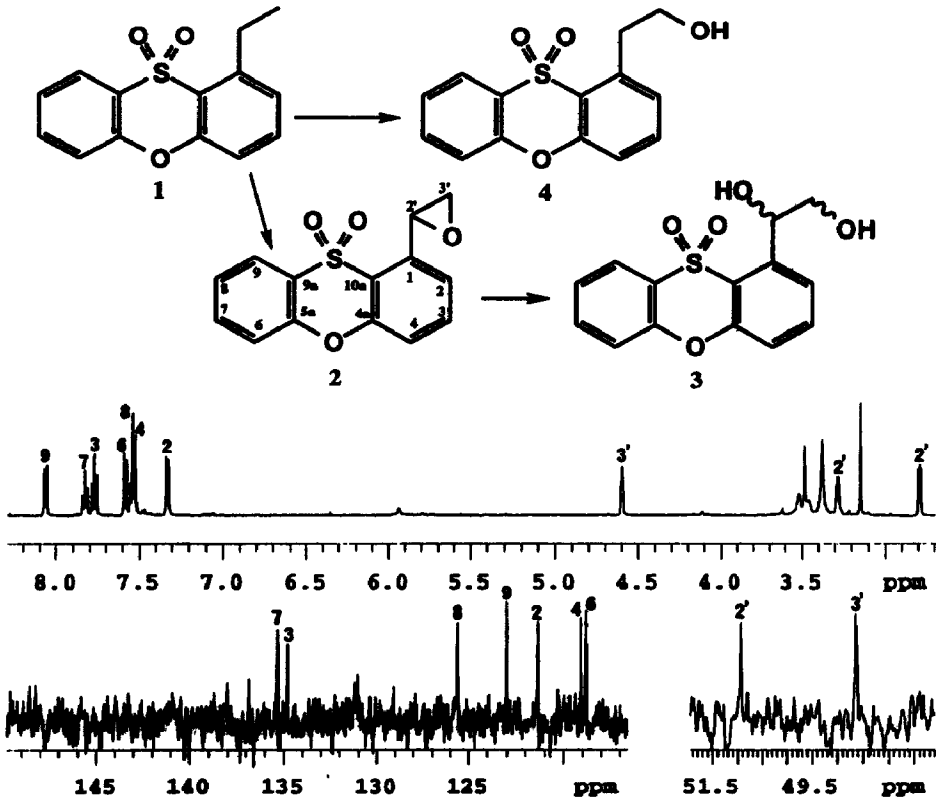

Fig. 1. ^{1}H and ^{13}C reference spectra of 30 µg (0.07 µMoles) of 1-(2'-oxiranyl)-phenoxathiin 10,10-dioxide (**2**) in 35 µl of d_6-DMSO. The proton spectrum was acquired[6] using a Varian homonuclear Nano-probe™; the ^{13}C reference spectrum was acquired[7] using a heteronuclear Nano-probe™.

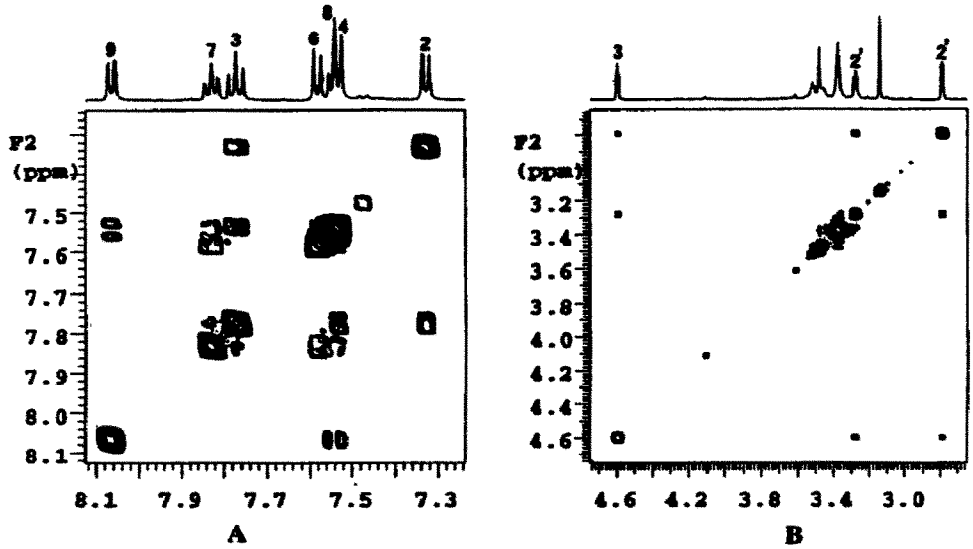

Fig. 2. COSY spectrum of 30 µg (0.07 µMoles) of 1-(2'-oxiranyl)-phenoxathiin 10,10-dioxide (2) in 35 µl of d_6-DMSO. The data were acquired as 2K points with 8 transients in each of 256 files; the data were zero-filled to 2K x 2K points, transformed, and symmetrized prior to plotting. The aromatic three and four proton spin-systems are shown in A; the correlations of the three aliphatic resonances associated with the epoxidized side-chain are shown in B.

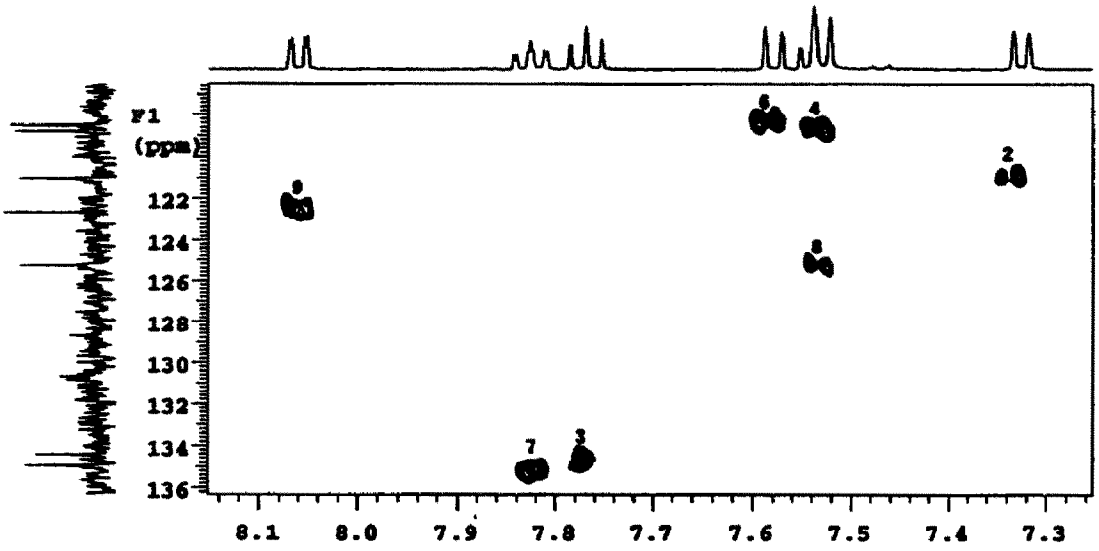

Fig. 3. HMQC spectrum of 30 µg (0.07 µMoles) of 1-(2-oxiranyl)-phenoxathiin 10, 10-dioxide (2) dissolved in 120 µl of d_6-DMSO. The data were acquired using a Unity 500 MHz spectrometer equipped with a Nalorac Z•SPEC™ micro inverse detection (MID-500-3) probe in an overnight acquisition.

While chemical shift arguments allow resonances in the ^{13}C spectrum to be subgrouped, definitive assignments are not possible. The HMQC spectrum shown in Figure 3 was acquired using 30 μg (0.07 μMoles) of **2** dissolved in 120 μl of d_6-DMSO using a Nalorac Z•SPEC™ micro inverse (MID-500-3) probe.[9] These data, interpreted in concert with the COSY data shown in Fig. 2, allowed the unequivocal assignment of all of the proton and protonated carbon resonances of **2**.

Resonance assignments for **2** derived from the two-dimensional NMR data shown in Figs. 2 and 3 are presented in Table 1.

Position	^{13}C Chemical Shift (ppm)	^{1}H Chemical Shift (ppm)
2	121.4	7.33
3	134.8	7.77
4	119.1	7.53
6	118.8	7.58
7	135.3	7.83
8	125.6	7.54
9	123.0	8.06
3'	48.7	4.60
2'	51.0	3.28, 2.79

References and Notes

1. R. C. Crouch and G. E. Martin, *J. Nat. Prod.*, **1992**, 55, 1343-7.
2. R. C. Crouch and G. E. Martin, *Magn. Reson. Chem.*, **1992**, 30, S66-S70.
3. A. Bax and S. Subramanian, *J. Magn. Reson.*, **1986**, 67, 565-569.
4. A. Bax and M. F. Summers, J. Am. Chem. Soc., **1986**, 108, 2093-4.
5. R. C. Crouch and G. E. Martin, unpublished data. As an example, a ^{13}C spectrum of 225 μg (0.58 μMoles) of cholesterol, in 120 μl of $CDCL_3$ can be acquired in 3 hr at 125 MHz using a Nalorac Z•SPEC™ carbon-optimized (MC-500-3) micro dual probe in a 500 MHz instrument.
6. The 90° proton pulse was 4.8 μsec. An acquisition time of 2.577 sec was employed with an interpulse delay of 1.5 sec. Spin rate was 2.5 KHz.
7. The 90° ^{13}C pulse was calibrated at 6.6 μsec at a transmitter power of 55 dB (max = 63 dB); the 90° pulse on the decoupler coil was 27 μsec at 61 dB (max = 63 dB). A 1.3 sec acquisition time was employed with a 1.0 sec interpulse delay. A spectral width of 25 KHz was used, digitized with 64K points. Waltz decoupling was employed with $\gamma H_2/2\pi$ = 2.85 KHz. Spin rate was 2.5 KHz. The data were zero-filled to 128K points and subjected to a 4.0 Hz exponential broadening to yield the data presented in Figure 1.
8. M.S. Cahuhan and I.W.J. Still, *Can . J. Chem.*, **1975**, 53, 2880.
9. The HMQC data were acquired using 30 μg of **2** dissolved in 120 μl of d_6-DMSO in a sealed 3 mm NMR tube. The pulse sequence of Bax and Subramanian (cited as ref. 3) was used for data acquisition. The data were acquired as 2048 x (82 x 2 hypercomplex files). A total of 176 transients was accumulated for each t_1 increment with an interpulse delay of 1.2 sec between acquisitions. The null interval between the BIRD pulse and the creation of heteronuclear multiple quantum coherence was 0.5 sec. Delays for the one-bond ($^{1}J_{CH}$) couplings were optimized as a function of an assumed average coupling of 150 Hz.. Pulse widths for ^{1}H and ^{13}C (X) were 6.9 and 14.0 μsec, respectively. Spectral widths were 4370 Hz for ^{1}H and 13198 Hz for ^{13}C. Garp1 decoupling was applied to ^{13}C during acquisition with $\gamma H_2/2\pi$ = 4.63 KHz. The data were zero-filled to 2048 x 256 points during processing and were subjected to Gaussian and cosine multiplication prior to the first and second fourier transforms, respectively.

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