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^{13}C-NMR SPECTRAL ASSIGNMENT AND EVALUATION OF THE CYTOTOXIC POTENTIAL OF ROTENONE

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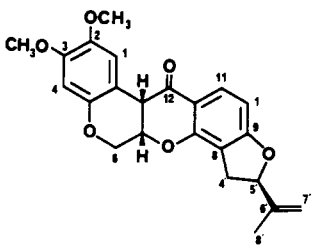
ABSTRACT.—Unambiguous ^{13}C-nmr assignments for the widely used pesticide rotenone [**1**] have been made through the judicious use of APT, CSCM 1D, and selective INEPT spectroscopy. Also, in order to more fully characterize the biologic potential of rotenone [**1**], studies were performed with cultured cells. Intense, but nonspecific, activity was observed in the P-388 lymphocytic leukemia, KB carcinoma of the nasopharynx, and a number of human cancer cell types: e.g., HT-1080 human fibrosarcoma, LU-1 lung cancer, COL-2 colon cancer, MEL-2 melanoma, and BC-1 breast cancer cell lines in vitro.

Rotenone [**1**] is a naturally occurring flavonoid-based compound isolated from *Derris elliptica* Benth. (Leguminosae). Currently, the substance is widely used as an agricultural and horticultural insecticide and as a piscicide (1), but historically the use of rotenone in establishing molecular aspects of the respiratory chain is of paramount importance. Rotenone [**1**] has also been evaluated as a potential antitumor agent; thus the growth-inhibitory effect of rotenone [**1**] has been demonstrated both with cultured cells and experimental tumors (2–6). As described here, rotenone [**1**] is broadly cytotoxic against cultured P-388 and KB cells, as well as a number of solid human tumor types (fibrosarcoma, lung, colon, breast cancer, and melanoma). However, no cell-type specificity was discernable.



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The structure of rotenone [**1**] was established by chemical (7,8) and ^{1}H-nmr spectroscopic investigations (9–11) and finally by X-ray analysis (12). In 1975 and 1983, two research groups reported (13,14) on the ^{13}C-nmr analysis of rotenone [**1**]. However, the two sets of assignments showed significant differences, and the magnetic field strength applied (25.15 or 22.5 MHz) could not resolve some signals. Because of the industrial importance of rotenone [**1**] as a pesticide, the substantial interest concerning its degradation products in the environment and interest in its biosynthesis, we decided to clarify this confusion and hereby provide unambiguous assignments for the ^{13}C-nmr spectra of **1** in several important solvents.

The unambiguous assignments of the ^{13}C-nmr spectra of rotenone [**1**] were based on two recently developed pulse programming sequences: the CSCM 1D (15) and selective INEPT (16) techniques that we have used extensively in previous work on other significant, biologically active natural products (17–21). For maximum effectiveness, both the CSCM 1D and selective INEPT experiments require a well-resolved and unambiguously assigned ^{1}H-nmr spectrum. The latter was already reported for rotenone [**1**] (11), and our initial studies

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independently verified these assignments (Table 1).

The APT spectrum of rotenone [**1**] in $CDCl_3$ showed three methyl, three methylene, seven methine, and ten quaternary carbon atoms. The three methylene signals at δ 31.12, 66.10, and 112.40 were assigned to C-4′, C-6, and C-7′, respectively. CSCM 1D irradiation of the ^{13}C satellites of H-1, H-4, H-6a, H-10, H-11, H-12a, and H-5′ consequently, from the last two experiments, the signal at δ 112.80 could be unambiguously assigned to C-8. The signals at δ 112.40 and 113.14 are therefore C-7′ and C-11a, respectively. Polarization transfer from H-4 resulted in the enhancement of δ 143.61 and 104.63, which should be the signals of C-2 and C-12b, respectively. Irradiation of H-1 enhanced carbon atoms at δ 44.41 (C-12a), 147.19, and 149.23.

TABLE 1. ^1H-Nmr Spectral Assignments[a] of Rotenone [**1**] in Various Solvents.

Proton	Solvent		
	$CDCl_3$	C_6D_6	C_5D_5N
H-1	6.71 (s)	7.18 (s)	7.18 (s)
H-4	6.38 (s)	6.53 (s)	6.72 (s)
H-6	4.61 (dd, 12.2,2.5)	4.29 (dd, 12.0,2.7)	4.80 (dd, 12.2,2.9)
	4.17 (bd, 12.2)	3.55 (bd, 12.0)	4.31 (dd, 12.2)
H-6a	4.86 (bs)	4.14 (t, 2.8)	5.11 (bs)
H-12a	3.76 (d, 3.5)	3.61 (d, 3.7)	4.17 (d, 3.7)
H-10	6.43 (d, 8.5)	6.41 (d, 8.5)	6.61 (d, 8.5)
H-11	7.76 (d, 8.5)	8.14 (d, 8.5)	8.11 (d, 8.5)
H-4′	3.30 (dd, 15.5,8.2)	2.73 (d, 7.3)	3.12 (dd, 15.7,9.7)
	2.92 (dd, 15.5,8.0)	2.71 (d, 6.4)	2.92 (dd, 15.7,8.2)
H-5′	5.21 (t, 9.2)	4.74 (t, 9.0)	5.16 (t, 8.8)
H-7′	5.01 (s)	4.94 (s)	5.08 (s)
	4.88 (s)	4.70 (s)	4.88 (s)
8-Me	1.70 (s)	1.48 (s)	1.67 (s)
OMe	3.73 (s)	3.40 (s)	3.65 (s)
OMe	3.70 (s)	3.27 (s)	3.62 (s)

[a]Measured at 360.1 MHz, $\delta_{TMS} = 0$ ppm.

resulted in magnetization transfer to their carbon atoms resonating at δ 110.09, 100.70, 72.04, 104.68, 129.77, 44.41, and 87.66, respectively. Selective INEPT irradiation of H-11 enhanced the carbon signals at δ 157.73, 167.14, and 188.73, of which the last could be assigned to C-12. The two other quaternary carbon enhancements, C-7a and C-9, were distinguished when H-5′ was irradiated, resulting in enhancements at δ 17.00 (C-8′), 112.40, 112.80, and 167.14. This permitted assignment of the last signal at C-9. Irradiation of H-10 enhanced the carbon atoms at δ 112.80 and 113.14; The last two signals were unambiguously assigned as C-4a and C-3, respectively, when H-12a was irradiated, and enhancement of the oxygenated carbon atom C-4a at δ 147.19 was observed. Similar experiments were performed on rotenone [**1**] in C_6D_6 and C_5D_5N, and the complete sets of unambiguous ^{13}C-nmr assignments are shown in Table 2.

In summary, all of the ^{13}C-nmr assignments of rotenone [**1**] have now been finally established, and the data reported by Crombie *et al.* (13) were confirmed in general and refined by the resolution of the signals previously reported as overlapping. However, revision of the

TABLE 2. ^{13}C-Nmr Spectral Assignments[a] of Rotenone [1] in Various Solvents.

Carbon	Solvent		
	$CDCl_3$	C_6D_6	C_5D_5N
C-1	110.09	111.90	112.19
C-2	143.61	145.19	144.69
C-3	149.23	150.87	150.72
C-4	100.70	102.01	102.23
C-4a	147.19	148.14	148.65
C-4′	31.12	31.56	31.50
C-5′	87.66	87.68	87.99
C-6	66.10	66.24	66.70
C-6a	72.04	72.45	72.98
C-6′	142.83	143.45	143.75
C-7a	157.73	158.19	158.48
C-7′	112.40	111.92	112.29
C-8	112.80	113.28	113.58
C-8′	17.00	17.14	17.24
C-9	167.14	167.41	167.45
C-10	104.68	104.98	105.01
C-11	129.77	129.68	130.14
C-11a	113.14	114.03	114.12
C-12	188.73	188.55	189.28
C-12a	44.41	45.00	44.81
C-12b	104.63	105.41	105.99
OMe	56.13	56.16	56.61
OMe	55.69	55.37	55.84

[a]Measured at 90.8 MHz, $\delta_{TMS} = 0$ ppm.

^{13}C-nmr assignments for C-2, C-3, C-4a, C-7a, and C-9 is necessary for the data appearing in Abidi and Abidi (14).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Rotenone [1] was purchased from Aldrich Chemical Company, and its purity was established through tlc analysis. ^{1}H-Nmr spectra were obtained on a Nicolet NMC-360 spectrometer operating at 360.1 MHz. Chemical shift values are reported in ppm (δ) using TMS as internal standard. The ^{13}C-nmr measurements, including the CSCM 1D and selective INEPT spectra, were obtained on a Nicolet NMC-360 spectrometer operating at 90.8 MHz. Data sets of 16K covering a spectral width of 10,000 Hz were acquired. Proton pulse widths were calibrated by using a sample of HOAc in 10% C_6D_6 ($^{tr}J = 6.7$ Hz) in a 5-mm nmr tube. The radio frequency field strength for the soft proton pulse was on the order of 25 Hz in these experiments. For aromatic and aliphatic methine protons 8 Hz was used as the ^3J value. Four thousand acquisitions were accumulated in each irradiation.

CYTOTOXICITY TESTING.—P-388, KB, and HT-1080 (human fibrosarcoma) were purchased from ATCC. LU-1 (lung cancer), COL-2 (colon cancer), MEL-2 (melanoma), and BC-1 (breast cancer) were established from primary human tumors in the University of Illinois at Chicago, College of Medicine, Division of Surgical Oncology.

The P-388 cells were cultured in Fisher's medium supplemented with 10% heat-inactivated (56° for 30 min) fetal bovine serum (FBS). The KB cells were maintained in BME containing 10% heat-inactivated FBS. The HT-1080 and LU-1 cell lines were cultured in minimal essential medium (MEM) containing Earle's salts and supplemented with 1% of nonessential amino acids (NAA) and 10% heat-inactivated FBS. The COL-2 and BC-1 cell lines were maintained in MEM containing Earle's salts, supplemented with 1% NAA and 15% heat-inactivated FBS, and the MEL-2 cell line was grown in MEM containing Hank's salts supplemented with 1% NAA and 15% heat-inactivated FBS. All the cell lines, except MEL-2, were cultured at 37° in a humidified atmosphere of 5% CO_2 in air. The MEL-2 cell line was maintained at 37° in closed tissue culture flasks.

Rotenone [1] was evaluated for cytotoxicity basically by the procedures established by the National Cancer Institute (22) as described previously (23–25). The cultured cells (at log growth-phase) were treated, in duplicate, with five concentrations (0.0125–2.0 μg/ml) of rotenone [1] and incubated for periods of 48 (P-388, HT-1080) or 72 h (BC-1, COL-2, KB, and MEL-2) at 37° in a humidified atmosphere of 5% CO_2 in air, with the exception of MEL-2 which was incubated at 37° without CO_2. The quantity of cells in each tube was then determined by counting (P-388) or protein analysis (all other cell lines), and the averaged data were expressed as a percentage, relative to controls treated only with solvent (DMSO). The dose that inhibited cell growth by 50% (ED_{50}) was calculated. Results are given in Table 3.

TABLE 3. Cytotoxicity Data of Rotenone [1].

Cell line	ED_{50} (μg/ml)
BC-1 breast cancer	0.039
COL-2 colon cancer	0.15
HT-1080 human fibrosarcoma	0.047
LU-1 lung cancer	0.044
MEL-1 melanoma	0.092
KB carcinoma of the nasopharynx	0.067
P-388 lymphocytic leukemia	0.005

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