STRUCTURE OF FREDERICAMYCIN A, AN ANTITUMOR ANTIBIOTIC OF A NOVEL SKELETAL TYPE; SPECTROSCOPIC AND MASS SPECTRAL CHARACTERIZATION

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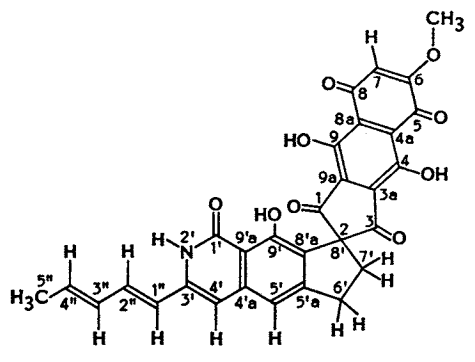
(Received for publication February 12, 1987)

IR, UV-visible spectroscopy, circular dichroism, ^{1}H and ^{13}C NMR studies, high resolution electron impact, field desorption, and fast atom bombardment mass spectral studies are reported for fredericamycin A (NSC-305263), a novel antitumor antibiotic of acid-base indicator type produced by *Streptomyces griseus* (FCRC-48). The spectral data are correlated with the structure obtained by X-ray crystallography as (*E,E*)-6′,7′-dihydro-4,9,9′-trihydroxy-6-methoxy-3′-(1,3-pentadienyl)-spiro-[2*H*-benz[*f*]indene-2,8′-[8*H*]-cyclopent-[*g*]-isoquinoline]-1,1′,3,5,8(2′*H*)-pentone. The novel spiro ring antibiotic exhibits unusual ^{1}H and ^{13}C NMR spectroscopic and chemical behavior, not previously observed in other antibiotic structures.

We reported earlier the isolation,[1~5] physico-chemical and biological properties[5,6] of three new antitumor antibiotics of acid-base indicator type—fredericamycins A, B and C, produced by *Streptomyces griseus* (FCRC-48), (**1**). Fredericamycin A (FM-A, NSC-305263), the major active component of the fermentation broth, possesses *in vitro* activity against Gram-positive bacteria and fungi and is highly cytotoxic, with ED_{50} values against murine leukemia KB, P388 and L1210 cell lines of 7×10^{-1}, 5×10^{-4} and 2×10^{-4} μg/ml, respectively. Human stem cell assays have also shown activities against ovarian and Hodgkin's tumor cell lines.[7,8] *In vivo* studies have shown it to be active against transplantable



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CD8F mouse mammary tumor (T/C 7 at 1.25 mg/kg) and P388 leukemic cells (T/C 200 at 0.5 mg/kg, 166 at 1.0 mg/kg).† Its mode of action appears to be through inhibition of RNA and protein biosynthesis.[6]

Subsequently we reported the X-ray crystal structure of FM-A,[8] discussed its biosynthesis[10] and reported the results of electron spin resonance studies.[11] The novel structure and unusual spectroscopic and chemical behavior of FM-A have aroused considerable interest. The unique spiro ring system places FM-A in a new antibiotic structural class and has encouraged several groups to attempt its synthesis.[12~19] Recently a complete synthesis[20] and the preparation of some derivatives[21] have been reported. In the present paper we wish to report in detail on the spectroscopic and mass spectral characterization of FM-A and its derivatives.

Materials and Methods

General

Melting points, determined on a Kofler micro hot stage apparatus, are uncorrected. IR spectra were recorded on a Perkin-Elmer model-180 grating spectrophotometer in KBr pellets. UV-visible (UV-VIS) spectra were taken in MeOH - DMF (8 : 2) or $CHCl_3$ on a Beckman-5230 spectrophotometer, on a Perkin-Elmer Lambda-5 spectrophotometer or on a Cary-17 UV spectrophotometer. High performance liquid chromatogram-UV (HPLC-UV) spectra were recorded on a Hewlett-Packard HP-1040A spectrophotometer with a diode array detector. The circular dichroism spectrum was taken on a Jasco J-500 CD spectropolarimeter in DMF - MeOH (2 : 8) solution, in a 10-mm sample cell at a scanning rate of 20 nm/minute with a time constant of 4 seconds.

Thin-layer Chromatography (TLC) Studies

Two types of TLC plates and solvent systems were used to check the purity of FM-A and its derivatives.

(a) Silica Gel TLC Plates with Fluorescent Indicator: FM-A and its derivatives were dissolved in $CHCl_3$ - MeOH - AcOH (CMA; 87 : 3 : 3) to give a 0.1% solution. Solutions were spotted on precoated Silica gel 60 F_{254} TLC plates (E. Merck, Darmstadt; 5×20 cm×0.25 mm thickness), using 3 μl disposable micropipettes and developed in the above solvent system. The spots were visualized under short and long wavelength UV light and/or by spraying with methanolic H_2SO_4 (10%) and heating at 110°C for 5 minutes.

(b) Reverse-phase TLC Plates: The TLC plates used were KC_{18}-silica gel plates, 20×20 cm× 0.25 mm (Whatman), and were developed in a solvent system CH_3CN - H_2O - AcOH (60 : 40 : 1 or 50 : 50 : 1). Visualization methods were the same as above.

High performance Liquid Chromatography (HPLC) Studies

HPLC was performed using a Waters Associates Model 6000A solvent delivery system, a U6K septumless injector (Waters Associates) and a Schoeffel Model SF770 variable wavelength detector. The detector was set at 254 nm and 0.1 absorbance unit full scale unless otherwise stated. Reverse-phase separations were carried out on (a) a C_{18} μBondapak column (3.9 mm×30 cm; 10 μm particle size; Waters Associates Millipore) using CH_3CN - H_2O - AcOH (70 : 30 : 1) or MeOH - H_2O - AcOH (70 : 30 : 1) as a solvent system, with a flow rate of 1 ml/minute; or (b) a CN-bonded C_{18} μBondapak column (3.9 mm×30 cm; 10 μm particle size; Millipore) and MeOH - 2% aq triethylaminophosphate, pH 2.35 (TEAP) (62 : 38) or CH_3CN - H_2O - AcOH (70 : 30 : 1) as solvent with a flow rate of 1.0 ml/minute. We also used a μPorasil column (3.9 mm×30 cm 10 μm particle size, Waters Associates) for which CMA (87 : 3 : 3) was used as the solvent (flow, 1.0 ml/minute) FM-A was injected as a 0.1% solution in CMA (87 : 3 : 3).

† T/C (test *versus* control): End point for P388 leukemia=medium survival time; T/C end point for CD8F mouse mammary tumor=median tumor weight.

^{1}H NMR Spectra

^{1}H NMR spectra were obtained at 100 MHz on a Varian XL-100 spectrometer with a Nicolet (TT-100) data system and at 300 MHz on a Nicolet NT-300 wide-bore spectrometer using a 5 mm fixed-tune probe. Spectra were recorded at ambient temperature. Nuclear Overhauser enhancement (NOE) experiments were performed using flip angles of 45°, a pulse width of *ca.* 1 second for selective presaturation of selected resonances, and an 8 seconds recycle time. All lines to be irradiated were assembled into a list of offset frequencies, and then data were acquired by interweaving frequencies. Difference spectra were then obtained to check for an NOE effect. 200 MHz spectra were obtained on a Varian XL-200 spectrometer equipped with the Advance data system and a 5 mm carbon-proton switchable probe. All deuterated solvents were of high quality (99.7~99.9% deuterium; E. Merck). ^{1}H NMR spectra were obtained while locked on deuterium of the solvent and referenced by Me_4Si (0.00 ppm). Chemical shifts are reported in ppm.

^{13}C NMR Spectra

^{13}C NMR spectra were obtained at 75.4 MHz on a Nicolet NT-300 wide-bore spectrometer, using either 12 mm (variable frequence probe) or 5 mm (fixed-tune probe) spinning tubes. The central resonance of the solvent was used as an internal reference with shifts reported relative to Me_4Si using δ (Me_4Si)=δ ($CDCl_3$)+76.90 or δ (Me_4Si)=δ (DMSO)+39.50 or δ (Me_4Si)=δ (pyridine)+135.50. All spectra were recorded at ambient temperature with an internal deuterium lock. Proton coupling was suppressed with 1.5 W (59 dB) of decoupler power by utilizing the MLEV-16 pulse sequence.[22] Typical acquisition parameters were 25,000 scans, 1.0 second acquisition time, 0.5 second delay (1.5 seconds recycle time), and 40° flip angle. For off-resonance-decoupled spectra, 2.0 W of continuous wave (CW) decoupling power was used.

Mass Spectrometry

Electron impact mass spectra (EI-MS) were measured with a Jeol Model JMS-01SG-2 high resolution instrument at 70 eV ionization voltage. Samples were introduced through the solid probe inlet. Spectra were taken at a sample probe temperature of 310~330°C. Low resolution spectra were taken at 10 kV accelerating voltage and high resolution (HF) data were obtained using Ionomet photographic plates, accelerating voltage of 8 kV, and magnet current of 4.3 amperes.

Field desorption (FD) and fast atom bombardment mass spectra (FAB-MS) were measured on a reversed geometry VG Micromass ZAB-2F (VG Analytical, Altrincham, UK) mass spectrometer, equipped with a combination electron impact (EI)-chemical ionization (CI)-field ionization (FI)-FD and FAB ion source and a VG 2035 data system. A xenon fast atom beam (8 keV, 1 mA plasma discharge current) was used to bombard the sample (1~2 μl) dissolved in a mixture of glycerol - DMF (1 : 1) on a stainless steel probe target introduced into a commercial FAB source (8 kV accelerating voltage). HR FAB-MS were measured on a Varian MAT mass spectrometer Model 731 equipped with a combination EI-FD-FI-FAB ion source.

Antibiotic and Reagents

All structural elucidation studies of FM-A were carried out using one preparation (FCRC-A48, NSC-305263) isolated from the strain of *Streptomyces griseus* (FCRC-48).[5] Crude FM-A was purified on a Waters Prep LC-500 using silica cartridges and CMA (87 : 3 : 3). Appropriate fractions were combined (TLC/HPLC), evaporated to dryness and the residue repeatedly washed with acetonitrile to yield pure FM-A which was crystallized from CH_3CN - H_2O (4 : 1).

Ag_2O was dried under high vacuum overnight and CH_3I was dried over fresh molecular sieve. $CHCl_3$ was made alcohol free and dried by passing through an Al_2O_3 grade I column. Acetic anhydride was freshly distilled, pyridine was dried over KOH pellets and 4-dimethylaminopyridine (DMAP) was from a fresh bottle (Eastman Kodak Co.).

Methylation of FM-A

FM-A (100 mg), Ag_2O (100 mg), CH_3I (2.0 ml) and $CHCl_3$ (50 ml) under N_2 at room temp in the dark were stirred for 6 hours and then more Ag_2O (50 mg) and CH_3I (2.0 ml) were added and stirring continued for 47 hours until TLC indicated that the reaction was complete. The

mixture was then filtered and the filtrate evaporated to dryness. Silica gel TLC in CMA (95:5:1) showed at least four spots. These were separated by preparative TLC on silica gel to afford the permethylate of FM-A as a gold brown, highly viscous mass (10.4 mg); HPLC [solvent system, MeOH - H_2O - AcOH (70:30:1); flow rate 1 ml/minute] showed one major peak at 6.6 minutes, (~72%) and minor peaks at 7.2 minutes, (~20%), 8.4 minutes (~6%) and 9.5 minutes (~2%).

^1H NMR ($CDCl_3$) δ 1.76 (3H, d, J=7.1 Hz, CH_3), 2.40 (2H, t, J=7.5 Hz, CH_2), 3.25 (2H, t, J=7.5 Hz, CH_2), 3.80 (s, OCH_3), 3.85 (s, OCH_3), 4.00 (s, OCH_3), 4.05 (s, OCH_3), 4.1 (s, OCH_3), 5.99~6.95 (6H, =CH), 6.1 (s, =CH); EI-MS (70 eV) m/z (relative intensity, %) 597 (16), 595 (14, tetramethylate), 583 (27), 581 (32, trimethylate), 569 (71), 567 (100, dimethylate), 555 (36), 553 (61, monomethylate).

Acetylation of FM-A

(a) Pyridine/Acetic Anhydride Method: To a stirred solution of FM-A (225 mg) in dry pyridine (1.0 ml), acetic anhydride (1.0 ml) was added under N_2 in the dark. The reaction was stirred under anhydrous conditions at room temp for 147 hours and the course of the reaction monitored by HPLC (C_{18} μBondapak column, MeOH - H_2O - AcOH, 70:30:1) and TLC (KC_{18} plates, MeOH - H_2O - AcOH, 70:30:1). After disappearance of FM-A, the reaction mixture was cooled (0°C) and deionized water was added followed by stirring for 15 minutes. The reaction mixture was evaporated to dryness after the addition of a little BuOH. The brownish residue showed three spots on TLC (silica gel plates, CMA, 87:3:3), and one major peak and two minor peaks in HPLC. The residue was purified on a C_{18} reverse-phase column using MeOH - H_2O - AcOH (70:30:1) as the eluent to afford one major fraction. This was evaporated to dryness after the addition of BuOH, 60.6 mg, brown solid: MP >350°C; IR ν_{max} (KBr) cm^{-1} 1775, 1705, 1648, 1625, 1610, 1450, 1370, 1200, 1110, 1045, 930; ^1H NMR ($CDCl_3$) δ 1.75 (3H, d, CH_3), 2.56 (2H, t, CH_2), 2.51 (3H, s, OAc), 2.61 (3H, s, OAc), 3.32 (2H, t, CH_2), 4.00 (3H, s, OCH_3), 5.9~6.91 (complex, 7H, br m, =C*H*), 8.9 (1H, br s, NH); UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$) 196 (746), 233 (644), 248 (656), 300 (437.5), 320 (490), 334 (504), 348 (468), 366 (441.5), 384 (361); EI-MS (70 eV) m/z (relative intensity, %) 667 (2, M^+, triacetate), 625 (20, M^+, diacetate), 583 (47, M^+, monoacetate), 541 (100, M^+, reduced form FM-A).

(b) Acetic Anhydride/Dimethylaminopyridine Method: FM-A (50 mg) was dissolved in acetic anhydride (10.0 ml, freshly distilled) and the red translucent solution made oxygen free by bubbling N_2 through it (15 minutes). The mixture was stirred for 5 minutes and then DMAP (5 mg) was added and the solution was stirred at room temp under N_2 and anhydrous conditions in the dark for 56 hours. The course of reaction was monitored by HPLC (CN-C_{18} μBondapak column, 3.9 mm×30 cm, 10 μm; solvent, CH_3CN - H_2O - AcOH, 75:25:1; flow rate, 1.0 ml/minute). The contents were evaporated to dryness and the residue was precipitated with hexane - ethyl acetate (5:3) to yield a maroon-brown solid, which was dried at 40°C under high vacuum overnight. This still showed DMAP contamination in ^1H NMR (signals at δ 2.73, 6.53, 8.46 for DMAP). DMAP was completely removed by centrifuging with hexane - benzene (20:80, 2.5 ml×3). The product was dried under high vacuum for 48 hours to yield FM-A acetate (52 mg) as a dark maroon-brown solid (NSC-601618), mp >350°C (dec), soluble in DMF, DMSO, pyridine, DMA, MeOH, EtOH and insoluble in H_2O, C_6H_6, hexane and petroleum ether. *In vivo* activity against P388 leukemic cells (T/C 138 at 4 mg/kg 141 and 133 at 2 mg/kg): IR ν_{max} (KBr) cm^{-1} 2930, 1765, 1710, 1650, 1640, 1610, 1601 (s), 1595, 1440, 1351, 1170, (s, CO), 1005; UV $\lambda_{max}^{CHCl_3}$ nm ($E_{1cm}^{1\%}$) 204 (720), 209 (690), 232 (645), 262 (650), 320 (520), 333 (509), 376 (400), 396 (350); ^1H NMR (pyridine-d_5) δ 1.69 (3H, d, J=6.41 Hz, CH_3), 2.64 (2H, t, J=7.5 Hz, CH_2), 2.40, 2.45, 2.50, 2.56, 2.59 and 2.60 (6H, all s, OAc), 3.30 (2H, t, J=7.5 Hz, CH_2), 3.72, 3.80 and 3.83 (3H, all s, OCH_3), 5.88 (1H, dq, =CH), 6.20 (1H, dd, =CH), 6.32 (1H, d, =CH), 6.55 (1H, s, =CH), 6.64 (1H, br s, =CH), 6.89 (1H, s, =CH), 7.37 (1H, d, =CH, partially embodied in C_5D_5N signal) and 12.23 (1H); FAB-MS (negative) m/z 624 [$(M-H)^-$, diacetoxy FM-A, 100%], 666 [$(M-H)^-$, triacetoxy FM-A, 5%].

Reductive Acetylation of FM-A

To a dark-maroon colloidal solution of FM-A (100 mg) in acetic anhydride (3 ml), zinc metal (200 mg) was added in one lot, followed by anhydrous sodium acetate (109 mg). The mixture was

heated with stirring at 75~85°C in an oil bath for 2 hours. The color slowly changed to blue-green and became light yellow after the oil bath temperature was increased to 155~160°C and the contents of the flask were boiled for 1 hour. Glacial AcOH (3.0 ml) was added and the mixture was refluxed at 160°C for 20 minutes. Heating and stirring were then stopped, and the reaction mixture was left in the dark at room temp overnight. The color changed to whitish yellow. The contents were heated to boiling (145°C oil bath) for 30 minutes. The reaction mixture was filtered through a cotton plug (previously washed with hot glacial AcOH); the flask and the contents of the plug were washed with hot glacial AcOH (1 ml×8). The total filtrate was boiled again for 10 minutes (oil bath temperature 150~160°C) and the solvent was removed under high vacuum to yield a brownish yellow semi-solid mass. The residue was taken up in $CHCl_3$ and washed with water, 5% sodium bicarbonate solution, brine and then dried over magnesium sulfate. Solvent was removed under vacuum to yield 104 mg of golden yellow solid. TLC in $CHCl_3$ - MeOH (95 : 5) showed it to be a mixture of at least three compounds. HPLC on a RCSS-CN column showed three peaks (solvent system MeOH - TEAP, 62 : 38; flow rate, 4.0 ml/minute) at 4.1, 5.1 major and 7.0 with a shoulder at 5.95 minutes. The HPLC-UV of all the peaks were only slightly different and it appears that the three compounds are closely related. The major compound was isolated by passing the mixture through a column of TLC grade silica gel (17.5×1.0 cm) from $CHCl_3$ - MeOH [(95 : 5), 2.0 ml]. Separation was monitored by TLC and HPLC. Pure fractions were pooled based on HPLC and analyzed by ^1H NMR and FAB-MS. ^1H NMR ($CDCl_3$) δ 1.82 (3H, d, J=6.2 Hz, CH_3), 2.50 (2H, t, J=7.5 Hz, CH_2), 2.43, 2.45, 2.48, 2.51 (OAc), 3.27 (2H, t, J=7.5 Hz, CH_2), 3.97 (s, OCH_3), 5.95 (1H, dq), 6.15 (1H, dd), 6.20 (1H, d), 6.33 (1H, s), 6.86 (1H, s), 6.9 (1H, s), 7.3 (d, 1H embodied in $CDCl_3$ signal) for seven =CH, 12.05 (s); FAB-MS (negative ion mode) m/z 750 (58%, pentaacetate), 708 (85%, tetraacetate), 666 (100%, triacetate), 624 (63%, diacetate) and 582 (8%, monoacetate).

Results and Discussion

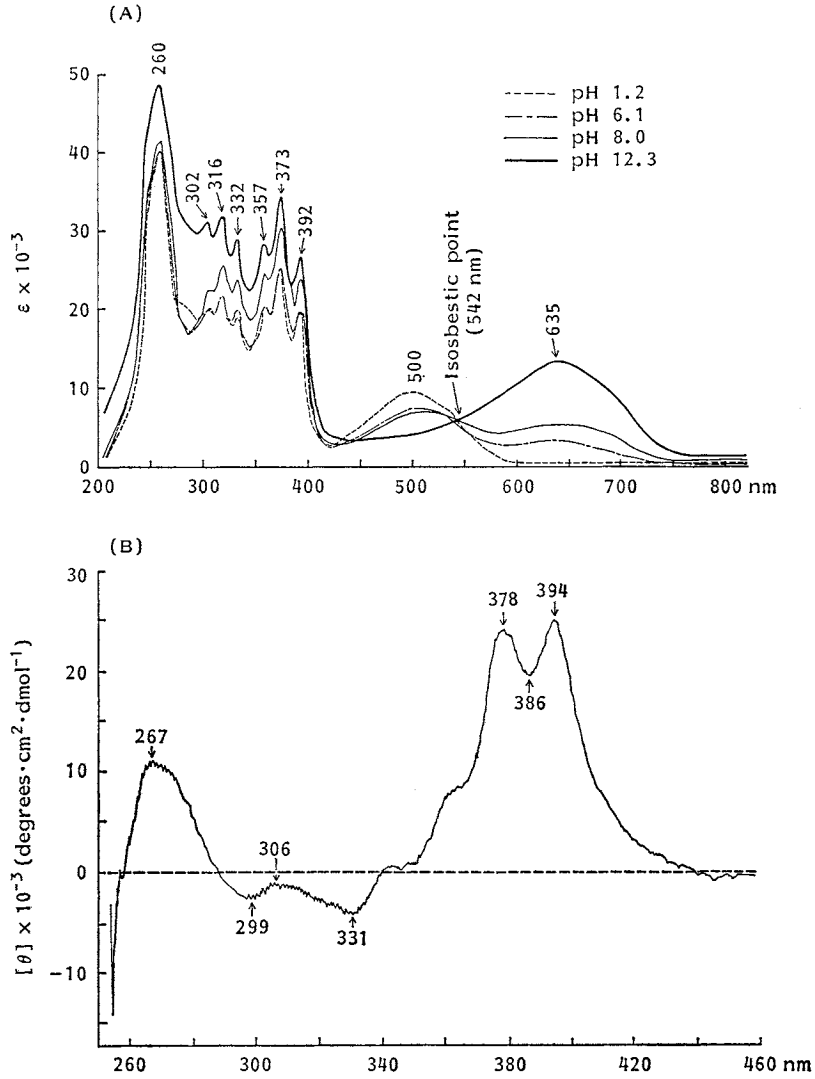
Crystallization, UV-visible and IR Spectral Studies

FM-A is stable at room temperature under anhydrous conditions. It can be heated to 70°C under vacuum without any signs of decomposition. Pure FM-A is quite insoluble in most common organic solvents and sparingly soluble in most polar solvents. *N,N*-Dimethylformamide, dimethylsulfoxide, dimethylacetamide and pyridine, offer the best solubilities. After several attempts, FM-A was crystallized as thin, highly fragile red platelets by slow evaporation from acetonitrile - water. The crystals readily acquire an electrostatic charge. Crystalline material did not show the absorption bands at 784 and 756 nm, in contrast to our earlier preliminary report,[5] in methanol - dimethylformamide (80 : 20) at various pHs (Fig. 1A). The antibiotic exhibits a color change from red to blue on elevation of pH from acidic to basic conditions in the presence of air, with the appearance of an optical absorption band at 635 nm. On addition of acid the changes are reversed. This behavior could be explained by FM-A existing in solution in at least two distinct reversible forms. FM-A shows two *pKa* values at 6.80 and 8.88[5] in DMF which suggests it to be a dibasic acid. The UV spectrum (Fig. 1A) shows an equilibrium between at least two species with an isosbestic point at 542 mm. Only the spectra at pH 1.2 and 12.3 are essentially of pure species which could be consistent with hydroquinone and quinone forms of FM-A, respectively, that are in redox equilibrium, depending on the pH. On elevation of pH of the solution to 12.3, marked changes are observed in the visible region, with a decrease in the 500 nm peak and the appearance of an absorption band centered at 635 nm. On acidification with HCl these changes are reversed (Fig. 1A). The red form obtained at an acidic pH of 1.2 is slightly more soluble in all solvents than the blue form obtained at a basic pH of 12.3 or on exposure to O_2 (an intermediate could be produced at pH 6.1 and 8.0). The absorption maxima in the UV-visible

Fig. 1.

(A) Electronic spectra of fredericamycin A (FM-A) in methanol - dimethylformamide (80 : 20) at various pHs.

(B) Circular dichroism spectrum of FM-A in methanol - dimethylformamide (80 : 20) at pH 8.0 in the 250～460 nm range.

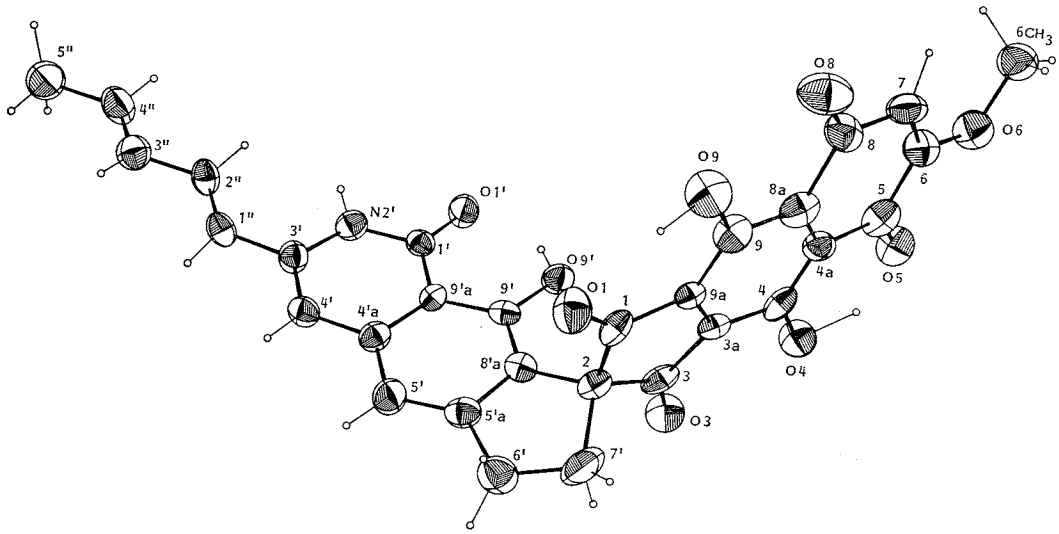


spectrum of FM-A are at 260, 302, 316, 332, 357, 373, 392 and 635 nm with ε of 43,489, 22,991, 25,152, 23,323, 24,044, 29,971, 23,711 and 8,033, respectively, which are consistent with the aromatic, conjugated system and quinone carbonyls.

The circular dichroism spectrum (Fig. 1B) demonstrates the optically active state of the natural product, with the highest molar ellipticity maximum at 394 nm and a $[\theta]$ value of $+2.53\times10^4$ degrees·cm²·dmol^{-1}.

FM-A displays strong characteristic bands in the IR spectrum at 1720 (C=O), 1647 (quinonoid C=O, NH-C=O), 1618 and 1562 (C=C) cm^{-1}. Very weak CH stretching vibrations around 2900～

Fig. 2. ORTEP[23] drawing of one of the two independent molecules of fredericamycin A in the crystal showing nomenclature and conformation.



3000 cm^{-1} show the highly conjugated nature of FM-A.

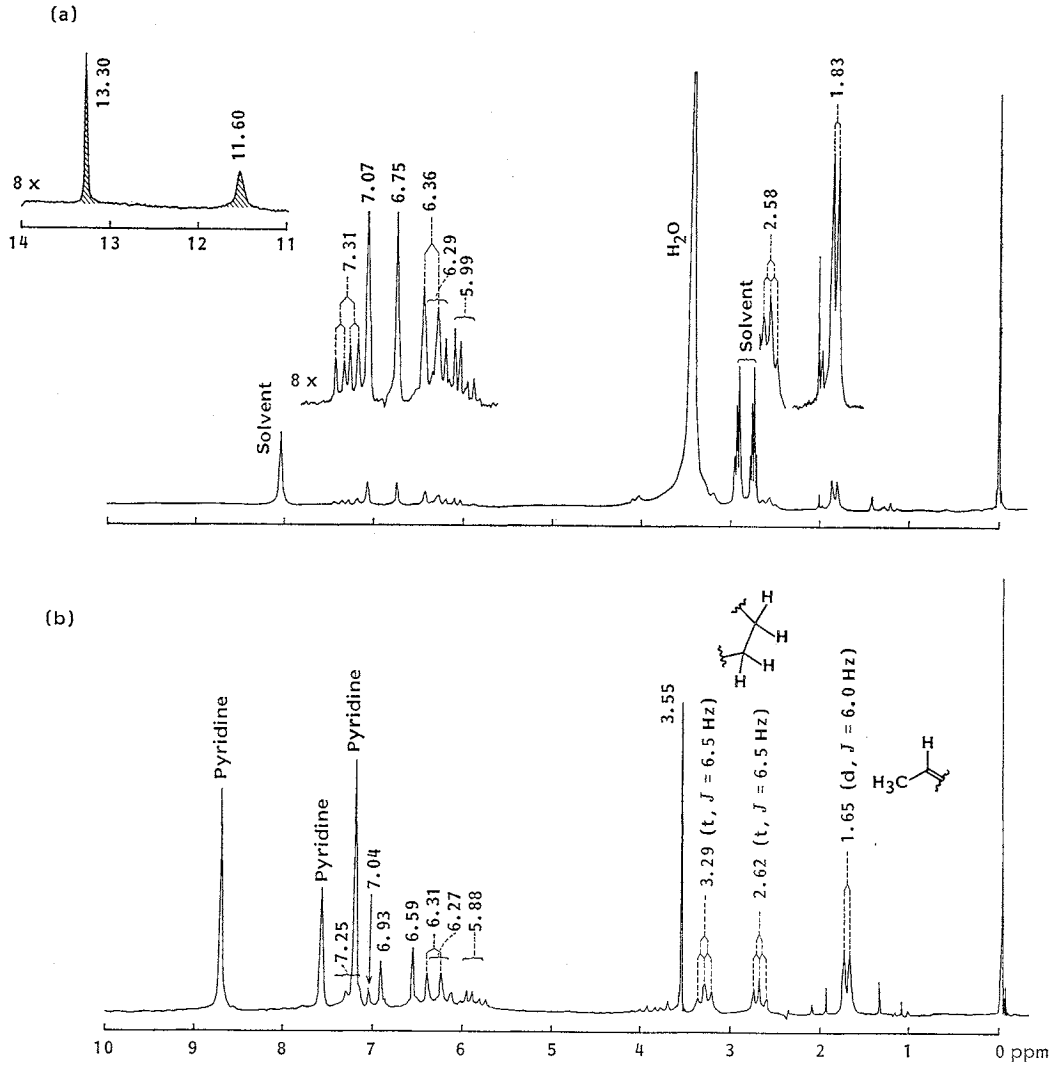
Crystal Structure and Conformation

There are two independent molecules in the asymmetric unit of the crystal and one of them is shown in Fig. 2.[23] The independent molecules are chemically identical but have different conformations and patterns of intramolecular hydrogen bonding. In both cases hydrogen bonds hold the H atoms in positions which could facilitate tautomerism although in the second molecule O9 is bonded to O8 and O4 to O3. The chemical formula was originally deduced from the crystal structure and bond orders were assigned on the bases of bond lengths and found H atoms. The single bond distances are C4-O4: 1.34, 1.34; C6-O6: 1.46, 1.47; O6-C6CH_3: 1.46, 1.47; C9-O9: 1.35, 1.33; C9′-O9′: 1.34, 1.37 and the double bond distances C1=O1: 1.22, 1.20; C3=O3: 1.20, 1.21; C5=O5: 1.21, 1.22; C8=O8: 1.23, 1.25 and C1′=O1′: 1.22, 1.20 Å, respectively. The pairs of values refer to the two crystallographically independent molecules and estimated standard deviations are of the order of 0.01 Å.

^{1}H NMR Studies

The 100 MHz ^{1}H NMR spectra of FM-A in DMF-d_7 and pyridine-d_5 - D_2O are shown in Fig. 3. These spectra are typical of the results obtained in various solvents including DMSO. Fig. 3 shows the presence of an olefinic methyl doublet (δ 1.83, J=6.3 Hz), two coupled noticeably broadened methylene triplets centered at δ 2.58 and 3.29 (J=7.5 Hz), a highly coupled vinylic system equivalent to four olefinic protons (δ 5.8~7.3), two aromatic singlets (δ 6.75 and 7.07), and from two to three downfield exchangeable resonances, the most easily observed being those at δ 11.60 and 13.30. The structure associated with the vinylic system is readily determined by inspection of the resonance frequencies and coupling pattern as shown in the expansion in Fig. 4. The set of shifts and coupling constants is virtually identical to the spectra of sorbic acid or ethyl sorbate.[24] The assignments were confirmed by single-frequency decoupling experiments at 300 MHz. These experiments also demonstrated a long range coupling between the resonance at δ 6.75 and the exchangeable resonance at δ 11.6 (NH). Re-

Fig. 3. ^{1}H NMR spectra of fredericamycin A at 45°C in DMF-d_7 (a) and pyridine-d_5 - D_2O (b) using a 100 MHz instrument.

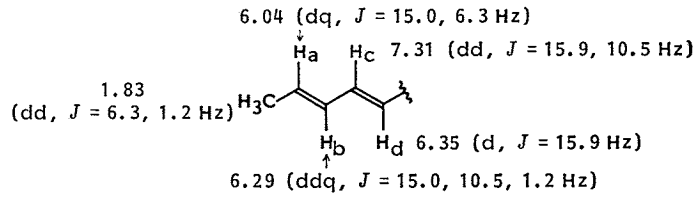


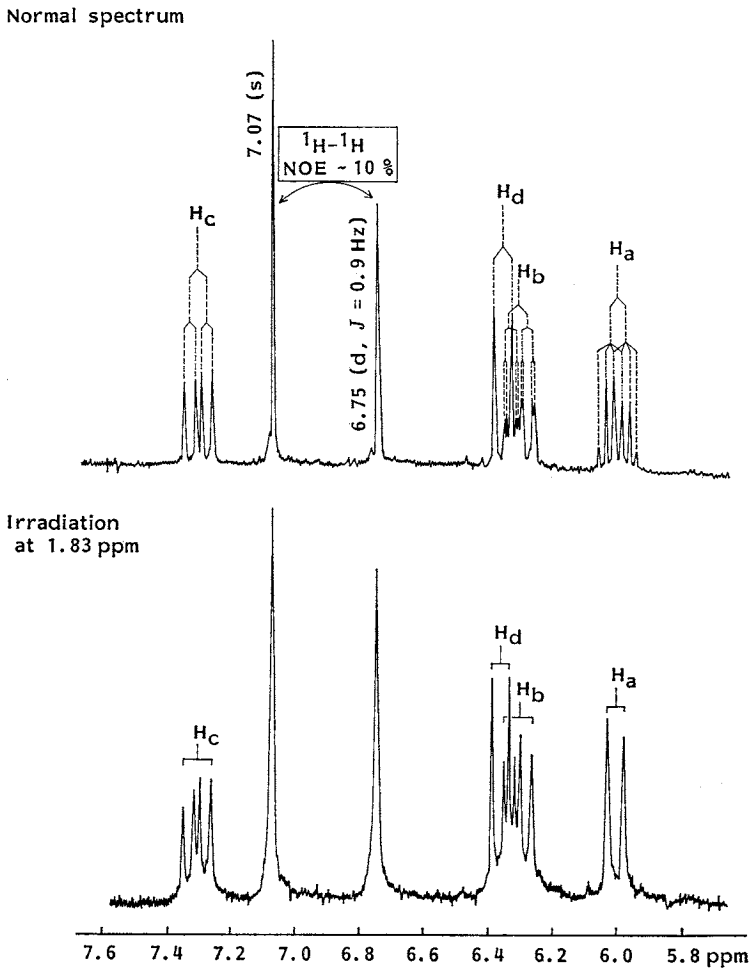
solution enhancement by sine bell apodization revealed a 0.9 Hz splitting present on the resonance signal at δ 6.75. This long range "W" coupling is between the NH proton and proton on C-4′. The signals at δ 6.75 (d, J=0.9 Hz) and 7.07 (s) show a positive NOE (~10%), indicating that the two protons are in the *peri* position. Part structure **2** illustrates the results of these experiments.

These findings are consistent with the crystal structure data for the aromatic half of the molecule but, in contrast to the structure of FM-A (**1**) as indicated by X-ray crystallography, the ^{1}H NMR data in DMF-d_7 did not reveal the presence of the methoxy (OCH_3) group and the methine proton adjacent to the methoxy group.

The discrepancy between the NMR and crystal structure data for the quinonoid half of the molecule is striking. The resonances missing in the proton spectra appear when the sample is treated with traces of TFA or if the FM-A is completely deoxygenated. A comparison of chemical shifts of FM-A in DMF-d_7, pyridine-d_5, DMSO-d_6 and $CDCl_3$ is shown in Table 1. The missing protons of the

Fig. 4. Olefinic region of fredericamycin A (300 MHz, DMF-d_7) in the ^{1}H NMR spectrum.





methoxy group and of the 7-H methine proton can be observed in all the solvents on addition of trace amounts of TFA-*d*. However, the chemical shifts of other protons remain almost unaffected after addition of TFA-*d* in solvents.

In very dilute solutions of FM-A in $CDCl_3$, the methoxy signal is seen without the addition of TFA-*d*, as a singlet at δ 4.00 and the methine

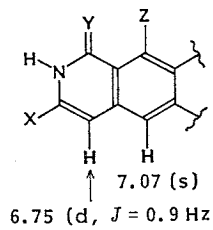


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Table 1. ^{1}H NMR chemical shifts[a] of fredericamycin A in various solvents.

Signal No.	Solvents				Assignment and multiplicity		
	DMF-d_7	Pyridine-d_5	$CDCl_3$	DMSO-d_6			
1	1.83	1.70	1.84	1.83	5″	dd	(J=6.3, 1.2 Hz)
2	2.58	2.73	2.55	2.46	7′	t	(J=7.5 Hz)
3	3.29	3.32	3.32	3.20	6′	t	(J=7.5 Hz)
4	4.07[b]	3.84[c]	4.00	3.97[b]	6-OCH_3	s	
5	5.99	5.88	5.98	5.93	4″	dq	(J=6.0, 15.0 Hz)
6	6.29	6.27	6.14	6.21	3″	ddq	(J=15.0, 10.5, 1.2 Hz)
7	6.36	6.36	6.20	6.26	1″	d	(J=15.9 Hz)
8	6.63[b]	6.85[c]	6.30	6.58[b]	7	s	
9	6.75	6.59	6.39	6.71	4′	d	(J=0.9 Hz)
10	7.07	6.93	6.90	7.03	5′	s	
11	7.31	7.25[d]	6.70	7.15	2″	dd	(J=15.9, 10.5 Hz)
12	11.60	—[d]	9.47	11.60	2′-NH	d	(J=0.9 Hz)
13	12.48[b]	12.40[c]	12.12	12.60[b]	4,9-OH	s	
			12.56				
14	13.30	13.85 (br)	13.19	13.15	9′-OH		

[a] In parts per million from Me_4Si (internal standard), coupling constants are measured at 300 MHz.
[b] Signals observed only on addition of traces of TFA-*d*.
[c] Broad signals; addition of traces of TFA-*d* sharpens these signals.
[d] Superimposed on solvent signal.

Fig. 5. Structures relevant to the discussion of the spectroscopic behavior of fredericamycin A.

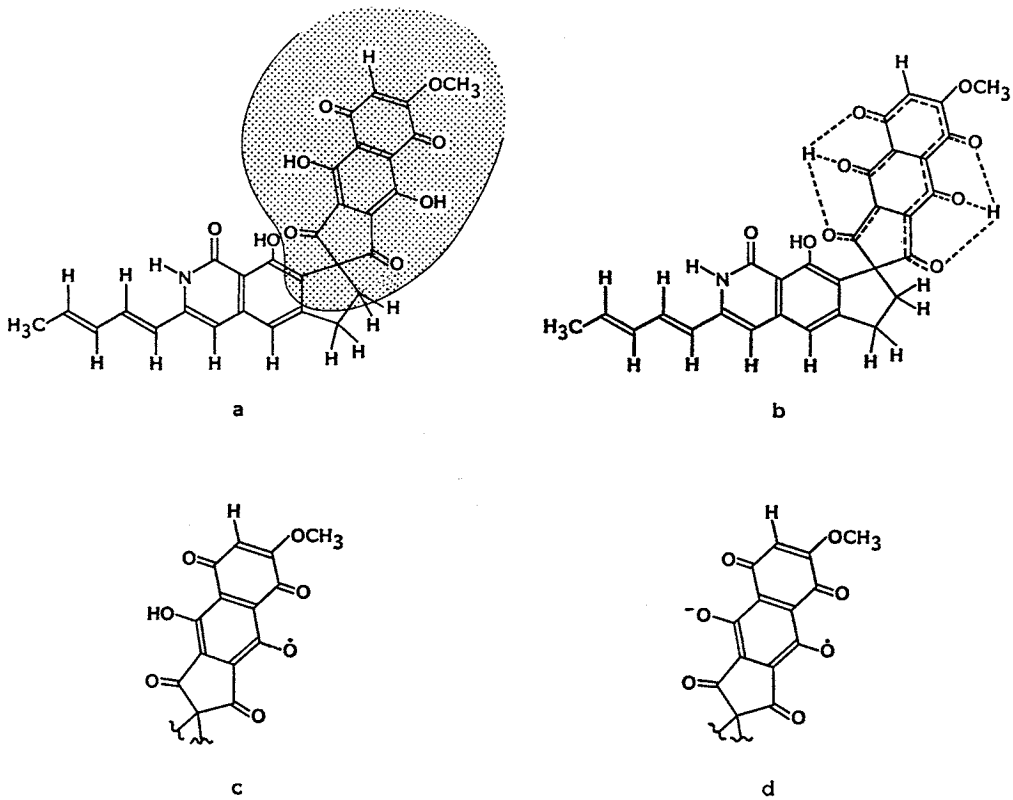
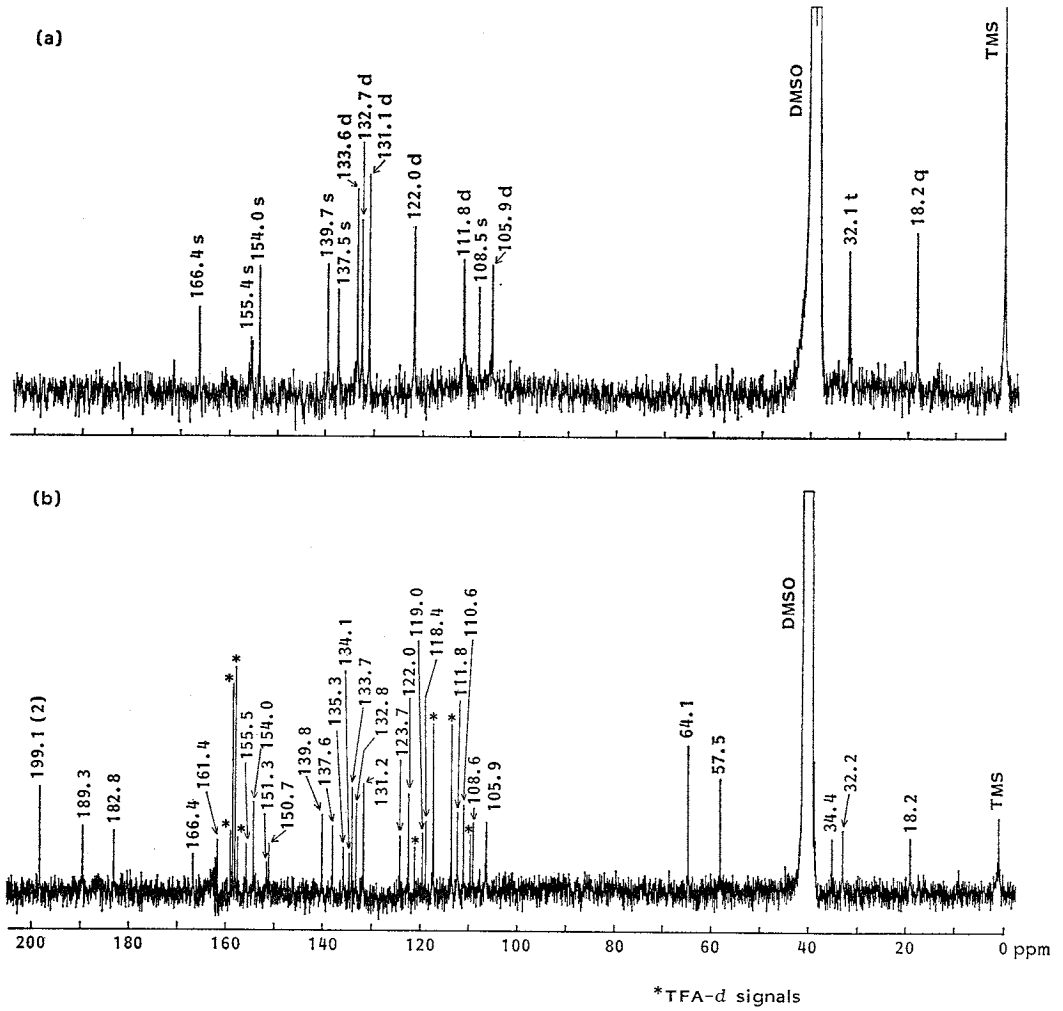


Fig. 6. Proton decoupled ^{13}C NMR spectra (75.4 MHz) of fredericamycin A in DMSO-d_6 (a) and after addition of traces of TFA-*d* (b).



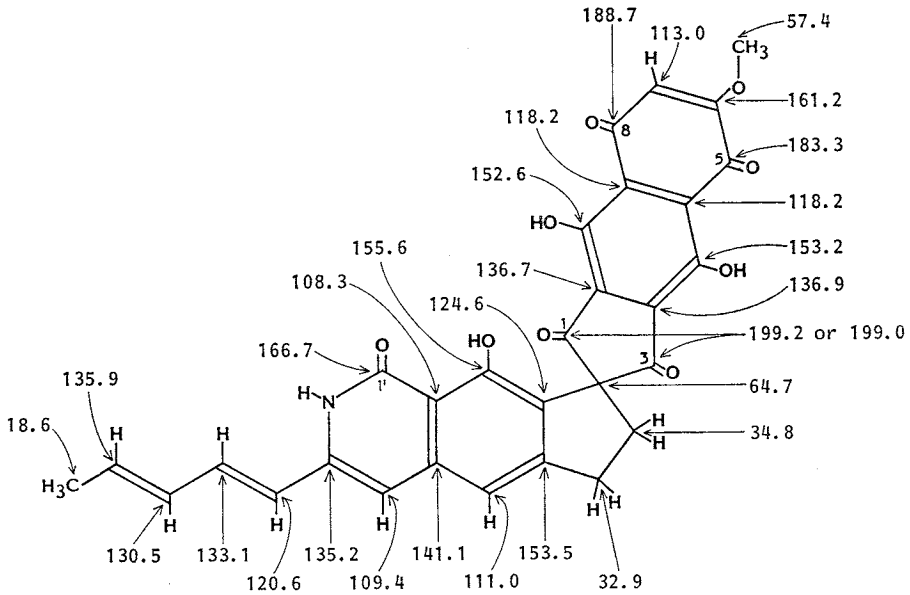
proton adjacent to the methoxy group is visible at δ 6.30 as a singlet. Also, NH and the three hydroxyl protons are seen clearly at δ 9.47, 12.12, 12.56 and 13.19, respectively. In other solvents only two exchangeable protons (9′-OH and 2′-NH) are observed in the spectra. The other two hydroxyl protons (4 and 9-OH) are observed as one signal at δ 12.40$\pm$0.2 only on addition of trace TFA-*d* (Table 1).

A likely explanation for this unusual behavior is the ability of FM-A to spontaneously form a semiquinone free radical in the presence of oxidising species as we reported earlier,[11] and will further discuss in the next section.

^{13}C NMR Studies

The ^{13}C NMR spectrum of FM-A in DMSO-d_6 (Fig. 6a) displayed only 14 carbon signals instead of the expected 30 (Fig. 6b). These are assigned to a methyl (δ 18.2, q), a methylene (δ 32.1, t), six olefinic methine (δ 105.9, 111.8, 122.0, 131.1, 132.7 and 133.6, all doublets) and six olefinic and/or carbonyl carbons (δ 108.5, 137.5, 139.7, 154.0, 155.4 and 166.4, all singlets). The ^1H NMR spectra indicate two vicinal methylene groups (positions 6′ and 7′), but one of the methylenic carbons assigned

Fig. 7. Assignments of carbon chemical shifts in the structure of fredericamycin A in $CDCl_3$ in presence of traces of TFA-*d*.



to C-7′ does not appear in the ^{13}C NMR spectrum. This discrepancy is removed when FM-A is dissolved in DMSO-d_6 with a trace of TFA-*d* when all 30 carbons are observed (Fig. 6b). The addition of a trace amount of TFA-*d* not only made it possible to record all FM-A carbon signals in $CDCl_3$ (Fig. 7), but also increased the solubility of FM-A in this nonpolar solvent. In the ^{13}C NMR spectrum FM-A showed two quartets (CH_3 and OCH_3), two triplets (CH_2), seven doublets (CH), and 19 singlets (>C=). The signal assignments were confirmed by analysis of the proton noise decoupled and off-resonance decoupled spectra taken in $CDCl_3$ containing a trace of TFA-*d*. Chemical shift assignments were also assisted by analysis of the ^{13}C-enriched FM-A obtained during biosynthetic studies.[10] Some ^{13}C NMR spectra showed several broad signals in the areas of the spectrum where the "missing" resonances were expected to appear. All 14 signals that appear in the spectra in polar nonacidic solvents are assignable to carbons in the isoquinoline portion of the molecule. Carbon signals that do not appear under these conditions are illustrated by the shaded areas in Fig. 5a.

As discussed above, FM-A displays unusual ^1H NMR and ^{13}C NMR behavior not previously observed with other antibiotics. The methoxyl and 7-H methine protons and 16 carbon resonances of the benzindane part of the molecule are not observable or considerably broadened in the spectra taken in polar non-acidic solvents but can be observed on addition of acid. Two possible explanations are proposed to explain this phenomenon. One is based on the tautomerization of the quinonehydroquinone-indanedione moiety, where the quinone-hydroquinone-indanedione part of the molecule may exist in a number of tautomeric forms with protonation sites on any one of the 6 oxygen atoms (Fig. 5b), and each tautomeric form could contribute a set of NMR signals at different chemical shift positions. The net effect may be extensive line broadening in the spectra.

Most probably the above phenomenon can be explained entirely by the facile generation of semiquinone free radicals stabilized by the fused quinone-indanedione conjugated system in non-acidic

Table 2. Mass spectral data on fredericamycin A.

EI-MS	*m/z* 539 (M^+), *m/z* 539/541 ratio, 2.6.[a,b] See Fig. 8 for spectrum.
HREI-MS	*m/z* 539.1218 ($C_{30}H_{21}NO_9$, Δ m 0.2 mmu). See Fig. 8 for additional data.
FD-MS[c]	*m/z* 539 (oxidized or quinone form) *m/z* 541 (reduced or hydroquinone form) } *m/z* 539/541 ratio 1.6~2.0.
FAB-MS[d]	Positive ion mode, *m/z* 542 $(M+H)^+$ (hydroquinone form) Negative ion mode, *m/z* 540 $(M-H)^-$ (hydroquinone form)
HRFAB-MS	*m/z* 542.1441 ($C_{30}H_{24}NO_9$) $(M+H)^+$ (hydroquinone form)

[a] *m/z* 539/541 ratios reported are as observed and are uncorrected for isotopic contributions.
[b] On an LKB instrument, *m/z* 539/541 was found to be 0.8.
[c] FD-MS were recorded on a VG ZAB instrument at 8 KV with 22~30 mA filament current.
[d] FAB-MS were recorded on a VG ZAB instrument at 8 KV with a sample matrix of glycerol-DMF.

media. One of the many contributing resonance forms is depicted in (Fig. 5c). Our earlier studies demonstrated the presence of acid-quenchable free radicals in DMSO solutions of FM-A.[11] Precedents exist to support these observations in ^1H NMR studies of 2-substituted naphthoquinones,[25,26] where traces of base cause broadening and even disappearance of NMR signals by the generation of naphthosemiquinone radical anions, which are in fast charge exchange with the large excess of uncharged naphthoquinone. This phenomenon was shown to be acid-quenchable. In the case of FM-A, either the semiquinone radical (Fig. 5c) or its radical anion (Fig. 5d) could be the species in neutral solvent. Three of the carbons (methoxy carbon C-7′ and C-8′) that are not directly part of the conjugated system are also affected. A radical species within a molecule can affect more distant sites since it is known,[26,27] that the extent of broadening of an NMR signal is dependent on the 1/6-th power of the distance between the observed nucleus and the paramagnetic center or unpaired electron and on the spatial distribution of that spin density in the conjugated system. A spiro carbon acts as a barrier to the delocalization of an unpaired electron,[28] thus the protons and carbons of the isoquinoline portion of the molecules are observable even in the presence of the free radical. On this basis it might be possible to map the radical distribution sites in FM-A using NMR methods similar to ref 26 in future work.

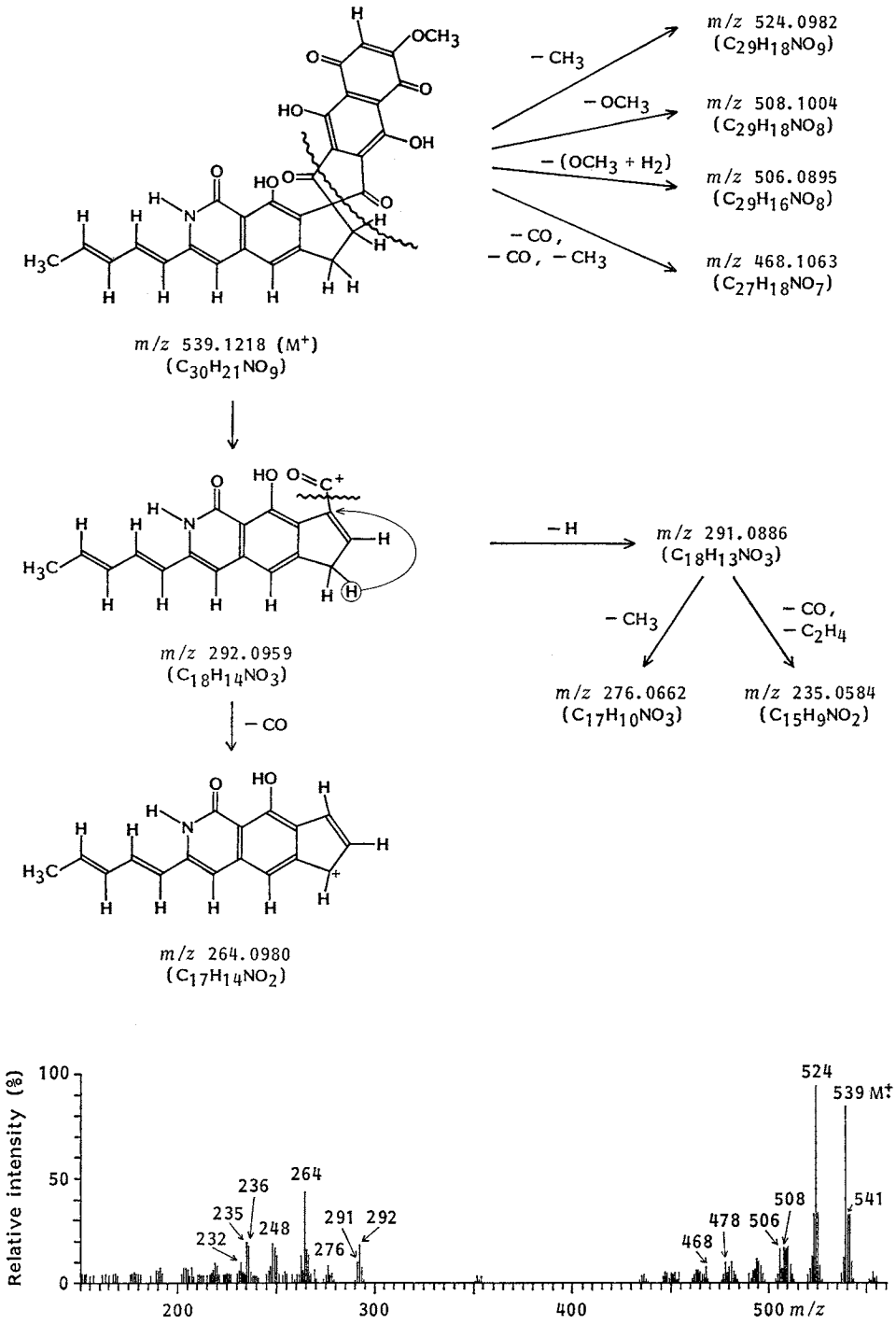
The propensity for radical formation in FM-A may be facilitated by the perpendicular spatial orientation of the quinonoid part of the molecule relative to the isoquinoline part. In this configuration, according to the X-ray structure, the hydroxyl group at C-9′ is positioned directly above the quinonoid ring system and may help to stabilize the free radical further.

Mass Spectrometric Studies

The data obtained with various mass spectral techniques are summarized in Table 2. The positive ion EI-MS showed the molecular ion for FM-A at *m/z* 539, which under HR conditions, provided an elemental formula of $C_{30}H_{21}NO_9$.[†] In addition to the abundant molecular ion, the EI-MS (Fig. 8) indicates a number of diagnostic ions. Several of these ions were also analyzed under HR conditions, and the results of this analysis are shown in Fig. 8. The high mass region is dominated by neutral fragment losses from the methoxyorthoquinone region of the molecule, such as the loss of a methyl group giving the base peak at *m/z* 524, the loss of a methoxy group and of two accompanying hydrogens to ions *m/z* 508 and 506, respectively, and the loss of various carbon monoxides. The

[†] The earlier reported highest mass ion for fredericamycin at *m/z* 554 presumably arose from the monomethyl derivative, formed during the earlier isolation procedure of the antibiotic in acidified methanol.[5] The elemental analysis of a sample dried at 56°C for 12 hours agrees with HREI-MS data.

Fig. 8. EI-MS fragmentation of fredericamycin A analyzed by HR.



concomitant loss of two carbon monoxides and a methyl group, giving in this case the ion at *m/z* 468, was shown earlier to be characteristic of methoxy substituted benzoquinones.[29] The spiro ring system fragments to give medium mass range ions, with all retained charges identified on the penta-

dienylisoquinoline half of the molecule. The highest mass of this series was found at *m/z* 292. This fragment can formally yield the major ion at *m/z* 264 on loss of carbon monoxide. Other ions in this series include *m/z* 291, 276 and 235. These five ions are potentially useful for diagnostic purposes when structural alterations have taken place in the molecule, either through synthesis, degradation, or occurring naturally.

The molecular ion region of FM-A also shows an ion at two mass units above *m/z* 539. This ion at *m/z* 541 is assigned to the reduced or hydroquinone form. The *m/z* 539/541 ratio can vary from 2.6 to 0.8 depending on the source temperature and the type of instrument used. The interconversion of quinone and hydroquinone forms in the ion source of the mass spectrometer is a known phenomenon,[30~33] which is catalyzed by traces of water or hydroxylated solvents.

Other ionization modes provided molecular weight information but very little fragmentation (Table 2). The quinone form was also predominant in the FD spectrum, giving an *m/z* 539/541 ratio of 1.6~2.0. On the other hand, in the FAB-MS,[34~36] the hydroquinone form predominates, the liquid matrix presumably facilitating the reduction. Thus, in the positive ion spectrum the protonated molecular ion appeared at *m/z* 542, whereas in the negative ion mode, hydride ion loss from the hydroquinone form provided *m/z* 540 as the molecular weight indicating ion. As reported earlier[9] HR positive ion FAB-MS also provided elemental composition data on the protonated molecular ion for the hydroquinone form at *m/z* 542.1441 [$C_{30}H_{24}NO_9$, $(M+H)^+$].†

FM-A Derivatives

FM-A was derivatized by acetylation with acetic anhydride and methylation with silver oxide/methyl iodide. Derivatization is very much dependent on the reagents and conditions used and, as the quinone and hydroquinone forms of the antibiotic have different numbers of hydroxyls, various products are formed. On acetylation with acetic anhydride - pyridine, FM-A gives mainly the monoacetate (*m/z* 583, M^+) in addition to the diacetate, (*m/z* 625, M^+) and traces of triacetate (*m/z* 667, M^+) whereas acetylation with dimethylaminopyridine - acetic anhydride yields a diacetate *m/z* 624 $[M-H]^-$. Though the negative ionization mode FAB-MS shows one major $[M-H]^-$ ion, six acetoxy methyl signals at δ 2.40, 2.45, 2.50, 2.56, 2.59 and 2.60 and three methoxy methyl signals at δ 3.73, 3.80 and 3.83 are seen in its ^1H NMR spectrum. The six acetoxy methyl signals integrate for a total of two acetate substituents. The chemical shifts of methoxy methyls indicate that the diacetoxy derivative of FM-A formed by the second procedure (DMAP - Ac_2O) is a mixture of various diacetylated FM-A's. There are six potential acetylation sites on the benzindane moiety of FM-A. For diacetate formation any of these sites may be accessible for acetylation through tautomerization of the four carbonyls and two phenolic groups. The diacetyl derivative shows good *in vivo* activity against P388 leukemic cells (T/C 138 at 4 mg/kg, 141 and 131 at 2 mg/kg).

Reductive acetylation of FM-A with metallic zinc, sodium acetate, and acetic anhydride yields a mixture of at least four acetates which was analyzed after purification by negative ion FAB-MS and ^1H NMR spectroscopy. The FAB-MS analysis clearly showed ions for pentaacetate (*m/z* 750, 58%), tetraacetate (*m/z* 708, 85%), triacetate (*m/z* 666, 100%), diacetate (*m/z* 624, 63%) and monoacetate (*m/z* 582, 8%). The ^1H NMR spectrum showed acetoxy methyl groups at δ 2.43, 2.45, 2.48 and 2.51.

All the resonances of carbon and hydrogen are seen in ^{13}C NMR and ^1H NMR of the derivatives

† Analyzed by HRFAB-MS.

and thus esterification or methylation of the phenolic groups appears to prevent further tautomerization or free radical formation.

Acknowledgments

We thank Dr. MATTHEW SUFFNESS of the National Cancer Institute for his interest and encouragement during the progress of this work. Our special thanks are due to Professor K. L. RINEHART, Jr. and Mr. J. C. COOK, Jr., University of Illinois, Urbana, IL, for FD-MS, FAB-MS and HRFAB-MS analyses and Dr. H. M. FALES of the NHLBI-LC Bethesda, MD for EI mass spectrometry, and J. M. ROMAN of NCI-FCRF for FAB-MS analysis of acetate derivatives. We would also like to thank Drs. J. DOUROS, M. G. HANNA, K. BYRNE, M. C. FLICKINGER, R. D. STROSHANE, J. C. CHAN and R. J. WHITE, who were at one time associated with NCI-Frederick Cancer Research Facility, Frederick, MD, for their interest, helpful discussions and encouragement of this work. Our thanks are also due to Drs. C. J. MICHEJDA and G. MUSCHIK of the NCI-Frederick Cancer Research Facility for discussions and suggestions at various times. Support of this research by the National Cancer Institute, Contracts NO-1-CO-75380 with Litton Bionetics, Inc., and NO-1-CO-23910 with Program Resources, Inc., is greatfully acknowledged. This project has been funded in part with Federal funds from the Department of Health and Human Services. The contents of this publication do not necessarily reflect the views of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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