Notes

WATER SOLUBLE SALTS OF FREDERICAMYCIN A: PREPARATION AND BIOLOGICAL ACTIVITY

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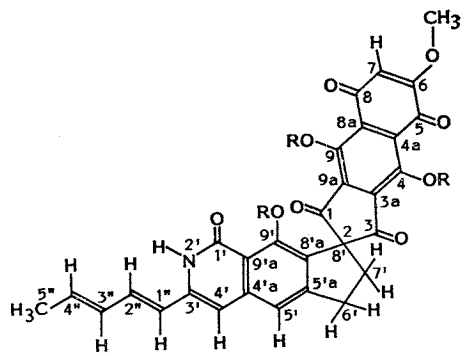
(Received for publication March 14, 1988)

The isolation,[1] physico-chemical and biological[2] properties of fredericamycin A (FM-A, NSC-305263) a novel antitumor antibiotic produced by *Streptomyces griseus* (FCRC-48) were reported earlier. The structure (**1**) was elucidated by spectroscopic and X-ray techniques[1,3,4] followed by its biosynthesis,[5] electron spin resonance,[6] and synthetic studies.†† FM-A exhibited good *in vitro* activity against Gram-positive bacteria, fungi and was found to be highly cytotoxic in *in vitro* and *in vivo* screening.[2] Further developmental work could not be carried out to broaden the utility of FM-A due to its poor solubility in water and common organic solvents.

Chemical modifications of FM-A (acetyl, lauroyl and hydrogenated acetyl derivatives[3,7,8] although slightly improved the solubility in organic solvents, their biological activity was reduced to a great extent compared to FM-A, making them less useful for exploration purposes. More importantly, these derivatives were not water soluble which is an important consideration in preparing formulations for toxicological and clinical studies. The present paper describes the preparation, spectroscopic characterization and the preliminary biological activity of the potassium and sodium salts of FM-A. This is the first report of a water soluble FM-A derivative which retains the high *in vitro* and *in vivo* antitumor activity of the parent compound.

A critical look at the functional groups of

Fig. 1. Structure of FM-A (**1**, R=H) and KFM-A (**2**, where one of the R's=K, other R's=K or H).



FM-A (**1**, Fig. 1) indicates that the molecule *inter alia* has phenolic hydroxyls and quinone moieties. Based on these potential salt-forming sites on the benzindane moiety of FM-A, it seemed attractive to convert it into alkali metal salt derivatives which should be water soluble.

Several experiments were tried using simple and modified addition of alkali metal hydroxides to FM-A solution without any success. Complex decomposition products of FM-A were obtained which could not be identified (TLC, HPLC, ^{1}H NMR, UV-Vis and fast atom bombardment mass spectra (FAB-MS)). The reaction in the presence of air lead to a complex unidentifiable mixture. This could be explained based on tautomerization or by the facile generation of semiquinone free radicals in FM-A solutions by oxygen, stabilized by the fused quinone-indene dione conjugated system in non-acidic media,[3] thereby interfering in the formation of the salt derivative. Further, the addition of metal hydroxide solution to FM-A solution doesn't yield the salt instantaneously. Perhaps the reaction is dependent upon the availability of the anion.

It is important to note that simple addition of alkali metal hydroxide solution to FM-A doesn't yield a detectable FM-A salt. Certain critical conditions must be met: (a) The reaction must be carried out strictly under deoxygenated conditions; (b) the reagents, solvents and the apparatus should be completely free from oxygen; (c) the reaction temperature should be 0~4°C;

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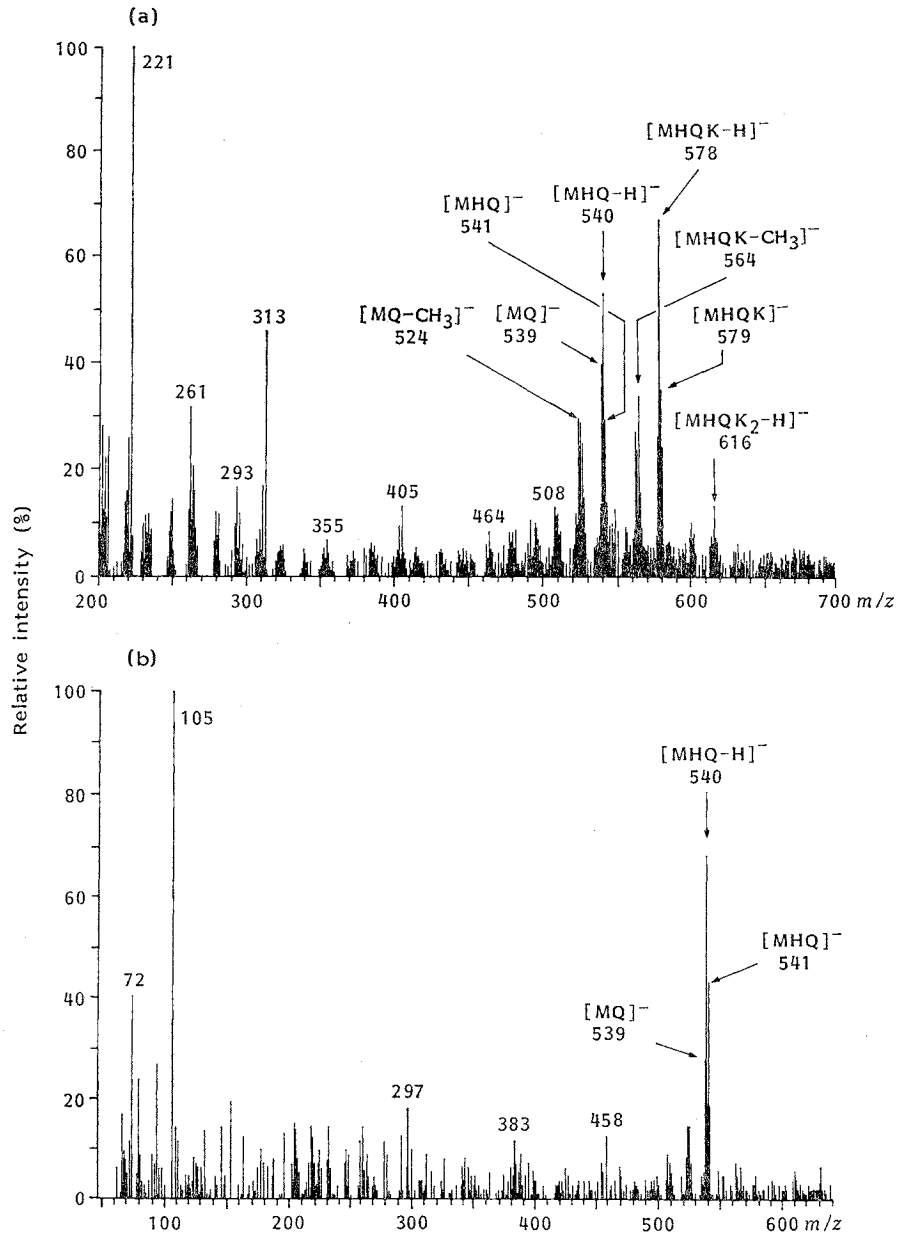
†† Refer to refs 12~19 cited in ref 3.

Fig. 2. Comparison of FAB-MS of KFM-A (a) with FM-A (b) in negative ion mode using glycerol-DMF (1:1).



MHQK and $MHQK_2$ are mono- and dipotassium salts of FM-A, respectively; MHQ and MQ represent the hydroquinone and quinone forms of FM-A, respectively.

(d) the alkali metal salt formed in the reaction mixture should be lyophilized.

Using the above criteria, potassium salt of FM-A (KFM-A, NSC No. 601617) was prepared as follows: FM-A (51.7 mg; 0.0959 mmol) was dissolved in degassed pyridine (5.0 ml) and nitrogen was bubbled through the clear purplish-red solution. The solution was cooled (0~4°C) with stirring and a precooled degassed KOH solution (26.64 ml (0.2781 mmol); 0.0108 N) was added. The solution changed to a bluish-green color (pH 9.54). The reaction was stirred

Table 1. Comparison of chemical shifts of protons in KFM-A with FM-A in DMSO-d_6 and in DMSO-d_6+TFA-d at 200 MHz.

Signal No.	KFM-A[a]		Standard FM-A	Multiplicity[b]	Assignments
	DMSO-d_6	DMSO-d_6+TFA-d	DMSO-d_6+TFA-d		
1	1.79	1.81	1.81	3H, dd (J=6.3, 1.2 Hz)	5″-CH_3
2	2.15	2.50	2.50	2H, t (J=7.5 Hz)	7′-CH_2
3	3.00	3.20	3.20	2H, t (J=7.5 Hz)	6′-CH_2
4	3.84	3.98	3.93	3H, s[c]	6-OCH_3
5	5.84	5.96	5.95	1H, dq (J=15.9, 6.0 Hz)	4″-H
6	6.16	6.20	6.22	1H, ddq (J=15.9, 11.0, 1.2 Hz)	3″-H
7	6.20	6.24	6.24	1H, d (J=15.9 Hz)	1″-H
8	6.20	6.59	6.59	1H, s[c]	7-H
9	6.42	6.71	6.71	1H, br s	4′-H
10	6.50	7.03	7.07	1H, s	5′-H
11	7.02	7.15	7.15	1H, dd (J=15.9, 10.5 Hz)	2″-H
12	—	11.60	11.59	1H, br s	2′-NH (conc depend)
13	—	12.25	12.19	1H, br s[c,d]	9-OH (conc depend)
14	—	13.14	13.09[c,d]	2H, br s	4-OH (conc depend)
15	—	13.16	13.10		9′-OH (conc depend)

[a] Note the upfield proton shift of signals (*ca.* 0.02~0.53 ppm) on addition of TFA-*d*.

[b] s: Singlet, br s: broad singlet, d: doublet, dd: doublet of doublets, dq: doublet of quartet, ddq: doublets of doublet of quartet, t: triplet.

[c] These protons are not observed in FM-A standard in DMSO-d_6 alone. They appear only after addition of trace amount of TFA-*d* to the DMSO-d_6 solution.

[d] Assignments may be interchangeable.

for ~44 minutes at 0°C and monitored by TLC and HPLC. The reaction mixture (pH 9.3) was lyophilized to yield 61.6 mg of KFM-A (98.4%) as a dark blue-green highly hygroscopic solid, mp >350°C (dec); pH (aqueous) 8.65±0.2 (26°C); potassium (atomic absorption) 7.13%; MW (*m/z*) 579, 617 $(C_{30}H_{23-n}NO_9)^-K_n$ where n=1 or 2 for mono- and dipotassium salts respectively, as determined by negative ion FAB-MS; IR ν_{max} (KBr) cm^{-1} 3400, 1648, 1600, 1560, 1490, 1350, 1228, 1115, 1038, 962, 920, 850; UV-Vis λ_{max} (DMF - MeOH, 2:8, pH 9.06) nm ($E^{1\%}_{1cm}$) 227 (418), 260 (665), 306 (377), 318 (398), 333 (359), 374 (431), 393 (369), 627 (118); ^1H NMR (DMSO-d_6) See Table 1; FAB-MS (negative ion mode) reported in Fig. 2. TLC† and HPLC††, after hydrolysis, showed a single spot (Rf 0.53) and a single peak (retention time 6.5 minutes) respectively, which were identical with standard FM-A. KFM-A is readily soluble in water (1.0 mg/ml), DMSO (8 mg/1.5 ml), DMSO - H_2O (1:1 and 5:95, 3.0 mg/ml and 1.5 mg/ml, respectively), DMF, dimethylacetamide and pyridine. It is sparingly soluble in EtOAc, acetonitrile, MeOH, $CHCl_3$ and insoluble in hexanes, benzene, acetone and ether.

The sodium salt of fredericamycin A (NaFM-A) was also prepared by the above method replacing KOH solution with NaOH solution to yield amorphous, highly hygroscopic dull blue-green sodium salt (yield 98%). Its UV, IR, ^1H NMR were similar to KFM-A; FAB-MS M^- at *m/z* 562 and 584 for mono- and disodium salts of FM-A. Solubility in water 0.5 mg/ml, pH 9.2±0.05 (26°C).

The solubility of KFM-A in water is better than the other salt derivatives. KFM-A was identified by the atomic absorption analysis where a potassium value of 7.13% was in agree-

† Plates; Silica gel 60 F254, solvent; $CHCl_3$ - MeOH - AcOH (87:3:3).

†† Column; C_{18} μbondapak-CN (3.9 mm×15 cm×10 μm), solvent; CH_3CN - H_2O - AcOH (40:60:1), Flow; 1.0 ml/minute, UV 254 nm.

ment with its being monopotassium salt of FM-A (calculated value 6.75%) as the major component. However, some lots of KFM-A analyzed for 10.1% potassium which indicated that the KFM-A is a mixture of mono- and dipotassium salts. This observation was also supported by FAB-MS of KFM-A in negative ion mode (Fig. 2) where *m/z* 578 $(MHQK-H)^-$ is the major molecular ion for monopotassium salt; and *m/z* 616 $(MHQK_2-H)^-$ is the minor molecular ion for dipotassium salt of FM-A. Other ions, including methyl radical losses[9] are shown in Fig. 2. The sodium content, analyzed by atomic absorption, in KFM-A was found to be 0.09%.

The ^1H NMR spectrum of KFM-A in DMSO-d_6 clearly showed all the expected proton signals not otherwise observed in the spectrum of FM-A.[3] A comparison of the two spectra in DMSO-d_6 and DMSO-d_6+trace amount of TFA-*d* is shown in Table 1. The missing protons of the methoxy group and of the 7-H methine proton of FM-A in DMSO-d_6 solution are observed clearly in potassium salt of FM-A at δ 3.84 and 6.20 as singlets. FM-A does not display these signals in DMSO-d_6 solution due to free radical and/or tautomeric effect in the quinone system[3] whereas salt formation of the phenolic groups appear to prevent further tautomerization or free radical formation. On addition of trace amount of TFA-*d* to the DMSO-d_6 solution of the KFM-A all the hydroxyls and NH protons appear and the spectrum is superimposed on FM-A spectrum in DMSO-d_6+trace amount of TFA-*d*. This also supports the salt formation. As expected, there is an upfield shift of 0.02~0.53 ppm observed in KFM-A spectrum compared to FM-A spectrum in DMSO-d_6 (Table 1).

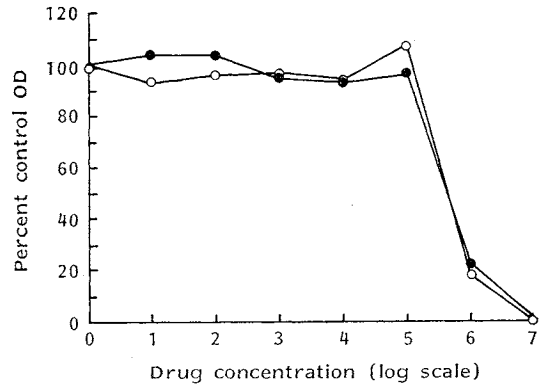
The UV-Vis spectrum of the salt does not exhibit any maximum at 500 nm, the spectrum resembled closely with the spectrum of FM-A at pH 12.3.[3] As expected, the spectrum is reversible on addition of acid and/or base.

KFM-A in water gives a transparent blue solution (1.0 mg/ml, pH 8.9±0.2) which is stable at room temperature for 1/2~1.0 hour. On standing for longer periods, the solution becomes turbid and the salt slowly hydrolyzes to FM-A. Anhydrous DMSO solution is fairly stable at room temperature (4.0 hours) and for several months at 4°C. Anhydrous salt is fairly stable if stored over silica gel under N_2 at 0~4°C.

KFM-A is active against Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and fungi (*Penicillium notatum*) at a concentration of 0.025 to 5.0 μg/ml (MIC). KFM-A shows equal or better *in vitro* cytotoxic activity as compared to that of FM-A. Assessment of KFM-A and FM-A against P388 ascites murine leukemia in an *in vitro* colorimetric assay[10] showed comparable drug sensitivity profiles as shown in Fig. 3. Both compounds exhibit profound drug inhibitory activity over a 100-fold drug concentrations with IC_{50} values of 0.423 and 0.441 μg/ml, respectively. Extensive work on further *in vitro* testing of KFM-A against several established human tumor cell lines is in progress.† Significant *in vivo* antitumor activity of KFM-A has been observed against human as well as murine cancer cell lines. As shown in Table 2, KFM-A exhibits significant activity against P388 leukemia cell line with T/C value of 178% at 1 mg/kg level and against LOX-human melanoma with T/C 148~177% at 1~5 mg/kg. The detailed biological activities will be published in a separate paper.†

The availability of a water soluble form of

Fig. 3. Assessment of KFM-A (●) and FM-A (○) growth inhibitory activities against P388 cells using a microculture tetrazolium assay.



Inoculation: 1,000 cells/well (growth control group, n=6; each treatment group, n=3) on day 1. Continuous drug exposure on days 2~5 (7=10 μg/ml, 1=0.00001 μg/ml). For clarity, error bars are not shown: The SD of replicate wells/group were generally less than 10% of the mean value.

† ALLEY, M. C.; M. SELBY and R. MISRA; unpublished results.

Table 2. *In vivo* activity of KFM-A and FM-A.

Tumor tested	KFM-A (water, ip)[a]			FM-A (DMSO, ip)	
	Drug dose (mg/kg)	T/C (%)[b]		Drug dose (mg/kg)	T/C (%)[b]
		Expt No. 1	Expt No. 2		
P388 lymphocytic	0.063	110	101	0.06	122
murine	0.125	119	112	0.12	146
leukemia	0.25	119	120	0.25	134
	0.50	153	132	0.50	200
	1.00	178	160	1.00	160
	2.00	Toxic	175	>1.00	Toxic
LOX human	0.50	128	142		
malignant	1.00/1.80	155	155		
melanoma	3.00	169	177		
	4.00/5.00	148	177		
	8.00/8.30	Toxic	189		

[a] KFM-A was dissolved in water only.

[b] T/C is the ratio expressed in percent of the median survival time of the treated group divided by the median survival time of the control group. NCI criteria of positive (moderate) activity in P388 leukemia is indicated by a T/C value of 130% and in LOX human melanoma is 140%. A T/C value of >175% is indicative of significant activity.

FM-A makes it possible for the first time to prepare pharmaceutical compositions in sterilized aqueous inert medium without organic solvents for further future developmental work on FM-A.

Acknowledgments

I am indebted to Dr. MATTHEW SUFFNESS of the National Cancer Institute for his keen interest and encouragement in this work. I acknowledge Drs. P. P. ROLLER of NCI/NIH, H. M. FALES of NHLBI-LC/NIH Bethesda, MD, and R. C. PANDEY of Xechem Inc., Rosemont, IL, for their help, Dr. G. CHURNEY and Mr. J. M. ROMAN of NCI-FCRF for recording ^{1}H NMR spectra and mass spectral analysis, respectively, and Dr. M. C. ALLEY and Mr. M. SELBY of the Cell Systems' R&D Lab., NCI-FCRF for *in vitro* biological assays. Support of this research by the National Cancer Institute, Contract NO-1-CO-2390 with Program Resources Inc. is gratefully acknowledged. This project has been funded in part with Federal funds from the Department of Health & Human Services. The contents of this publication do not necessarily reflect the views of the Department of Health & Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U. S. Government.

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