STRUCTURE AND CONFORMATION OF SAFRAMYCIN R DETERMINED BY HIGH FIELD ^{1}H AND ^{13}C NMR AND ITS INTERACTIONS WITH DNA IN SOLUTION

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The chemical structure and conformation of the new antitumor antibiotic saframycin R have been determined by high field ^{1}H and ^{13}C NMR as well as FAB mass spectrometry. Unlike other members of the saframycin family, saframycin R contains a reduced quinone ring bearing a glycolic ester moiety. Saframycin R exhibits acid promoted equilibrium and reversible covalent binding to DNA templates and, in the presence of a reducing agent, oxygen dependent single strand scission of supercoiled DNA. The extent of DNA scission is enhanced by *in situ* porcine carboxyl esterase or base catalyzed cleavage of the glycolic ester function plausibly by the release of the more reactive reduced saframycin A. This suggests that saframycin R may be regarded as a less toxic pro-drug for the active forms of saframycins A or S.

The saframycin antibiotics which are isolated from the streptothricin-producing strain of *Streptomyces lavendulae* No. 314 have unique dimeric quinonoid structures.[1,2] Several members of this family of agents including saframycins A and S exhibit extreme cytotoxicity towards cultured cells and show promising antitumor activity against a range of experimental tumors.[3] Several lines of evidence suggest a key role for the quinone moiety in the *in vivo* activation and cytotoxic action in common with other quinonoid antitumor antibiotics.[4,5] Recently a new component of *Streptomyces lavendulae* No. 314 was isolated namely saframycin R which while showing activity against L1210 cultured cells and murine tumors comparable with saframycin A, apparently contains a reduced quinone moiety, but other structural features common to the saframycin group.[6]

We report a determination of the structure of saframycin R and its conformation by high field ^{1}H and ^{13}C techniques and an examination of its reactivity towards DNA, (which several lines of evidence indicate is a principal cell target for saframycins[4,5]) as it relates to the mechanism of antitumor action of the saframycins. We also report the action of saframycin R on DNA templates in the presence and in the absence of porcine liver carboxyl esterase or comparable hydrolytic conditions which cleaves the unique glycolic ester linkage in the antibiotic and indicates saframycin R may possess a unique pro-drug character among the saframycins.

Materials and Methods

Saframycin R was obtained from a culture of *Streptomyces lavendulae* No. 314 as described previously[3] and was purified by column chromatography on Florisil with elution by ethyl acetate - petroleum ether (bp 30~60°C) (2: 1) immediately before use. Ethidium bromide was obtained from Sigma Chemical Co.

PM2 covalently closed circular (ccc) DNA (92% ccc) was prepared as described previously.[7] Calf thymus DNA was a product of Worthington while λ DNA was purchased from Miles Biochemicals. Porcine liver carboxyl esterase (EC 3.1.1.1) was obtained from Sigma as a suspension in 3.2 M ammonium sulfate in 10 mM sodium borate buffer at pH 8.0.

Nuclear Magnetic Resonance Spectroscopy

The ^1H NMR measurements were carried out in the Fourier transform mode using the Bruker WH-400/DS and WH-200 NMR spectrometers and the Aspect 2000 Data System utilizing 16 or 32K of data memory. The spectra were measured in appropriate deuterated solvents at ambient temperatures (298K). The deuterium resonance of the solvent was used for the lock signal. The homonuclear decoupling experiments were also performed in the pulse mode. The ^1H chemical shifts and coupling constants were calculated to first order at 400 MHz and are reported with an accuracy of ± 0.01 ppm and ± 0.20 Hz respectively.

The ^{13}C and spin-echo experiments[8,9] were carried out on the Bruker WH200 spectrometer at 50 MHz while the broad-band and continuous wave ^1H decoupled spectra were obtained using both WH 200 and WH400 instruments at 50 MHz and 100 MHz respectively. Acetone was used as ^{13}C reference at δ 29.8 and δ 206.0. The relaxation times T_1 were measured using a micro-programme which places a variable delay (0.01 ~ 10 seconds) prior to acquisition so that T_1 is indicated when a signal is of zero intensity at the mid-point of negative to positive signal inversion for the particular delay time used. The values thus obtained were employed in the nuclear Overhauser effect (nOe) experiments[10] in which the delay must be at least five times the longest T_1 involved.

Mass spectral determinations were carried out at the Middle Atlantic Mass Spectrometry Laboratory, a National Science Foundation Shared Instrument Facility, on an MS-50 high resolution mass spectrometer operating at 4 KV.

Mass Spectra Data

(a) Positive ion FAB, sample in glycerol (*m/z*, %), 625 ($MH^+ + 2$, 12.19), 598 ($MH^+ + 2 - HCN$, 9.02), 583 ($MH^+ + 2 - HCN - CH_3$, 0.63), 540 (1.37), 524 (2.72), 461 (2.04), 425 (0.58), 409 (0.57), 369 (6.25), 317 (2.06), 277 (29.81), 220 (8.91), 186 (25.27), 185 (100), 149 (26.46), 133 (1.44), 117 (9.05), 93 (96.20).

(b) Electron impact (ST=185°C, T/C=4526) (*m/z*, %) 622 (M^+, 5.0), 524 (10.3), 522 (11.5), 318 (43.9), 279 (53.1), 278 (96.6), 220 (63.7), 205 (17.3), 205 (15.6), 78 (100), 77 (17.6), 57 (11.7), 44 (56.7).

Electrochemistry

Polarographic studies on the saframycin antibiotics were carried out with a Princeton Applied Research Model 9300–9301 polarographic cell employed in a three electrode configuration as described previously.[5,11,12] The temperature was 37.5°C throughout and all potentials were measured with respect to the aqueous SCE but are reported with respect to the hydrogen electrode at pH 7. The drop time in the polarographic runs was 2 seconds, all solutions were deaerated with purified nitrogen for at least 10 minutes prior to study. Cyclic voltammetry was done using the PAR Model 173–175–176 configuration. The resulting curves were recorded on an X-Y recorder or photographed on an oscilloscope as required by the scan rate, v, which was varied from 20 mV/second to 500 mV/second.

Antibiotic-DNA Binding Studies

The extent of equilibrium and reversible covalent binding of saframycin R to DNA was determined as a function of pH by the degree to which intercalative binding of ethidium bromide was suppressed.[13] As described previously this method shows a linear relationship between the amount of drug bound (determined by radioactive counting), and the corresponding suppression of the fluorescence of the ethidium-DNA complex determined before heat denaturation.[14]

Reactions were carried out on a 600 μl scale. Reaction solutions contained 1.27 OD units of DNA, 50 mM potassium phosphate buffer, 44 μM saframycin R and other components as indicated in the legends to the figures. The fluorescence values were read at intervals by adding 20 μl aliquots to 2 ml of the ethidium bromide[15] assay solution which was 20 mM potassium phosphate, pH 11.8, and 0.4 mM EDTA with 0.5 μg of ethidium bromide per milliliter as described previously.[13] The instrument was

blanked with the assay mixture.

Antibiotic Induced Scission of PM2-ccc-DNA in the Presence and Absence of Porcine Liver Carboxyl Esterase

The fluorometric method of measuring single strand breakage of PM2 ccc-DNA and its inhibition by cell protection enzymes and free radical scavengers has been described.[7,14,15] Ethidium bromide binds intercalatively to negatively supercoiled DNA and in consequence experiences an enhancement of fluorescence intensity which is recorded. The conversion of PM2-ccc-DNA to nicked or open-circular (oc) DNA results in the release of topological constraints allowing more ethidium to intercalate and consequently a characteristic 30% increase in fluorescence is observed in the pH 11.8 ethidium assay solution (see below). After heat denaturation (96°C, 4 minutes) and cooling to 22°C since the strands are now separable a loss of fluorescence is observed in contrast to that of the control ccc-DNA which returns to duplex register. Therefore at any given time the loss of fluorescence after the heating and cooling cycles compared to that of the control is proportional to the percentage of DNA experiencing single-strand scission.[14,15]

All measurements were performed on a Turner and Associates Model 430 spectrofluorometer equipped with a cooling fan to reduce fluctuations in the xenon lamp source. Wavelength calibration was performed as described in the manual for the instrument. One centimeter-square cuvettes were used. The excitation wavelength was 525 nm and the emission wavelength was 600 nm. The 30× and 100× scales of medium sensitivity were generally used and water was circulated between the cell compartment and a thermally regulated bath at 22°C.

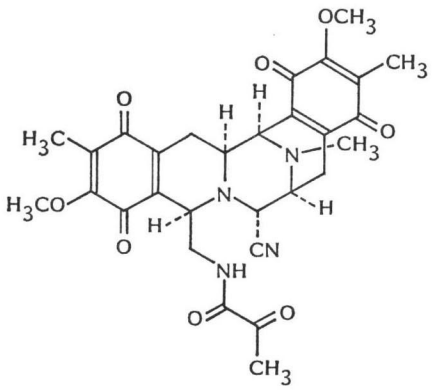
The reactions were carried out in a total volume of 100 μl at 37°C in deionized water and the reaction mixture contained 1.27 OD units/ml of PM2-ccc-DNA (92% ccc) 50 mM potassium phosphate buffer, appropriate concentrations of saframycin R, and sodium borohydride with or without porcine liver carboxyl esterase. The individual reaction conditions are given in the legends to the figures. At intervals 10 μl aliquots were withdrawn and added to 2 ml of assay solution described above. The fluorescence was measured using a blank without added sample. The solution was then heat denatured at 96°C on a Temp-Blok for 4 minutes and cooled rapidly in an ice-bath and then in a thermostatted water bath at 22°C for 5 minutes, and the fluorescence read again. In a control experiment it was shown that none of the components interfered with the ethidium fluorescence.

Results and Discussion

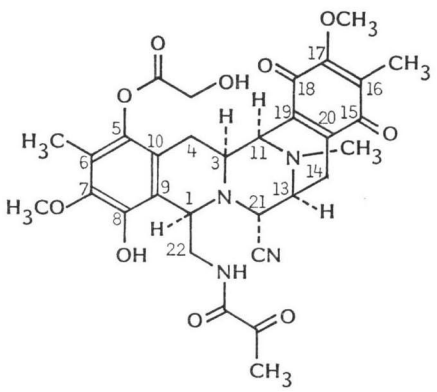
Mass Spectra

The electron impact mass spectrum showed a molecular ion at m/z 622 corresponding to the mole-

Fig. 1. Structural formulas of saframycins A and R.

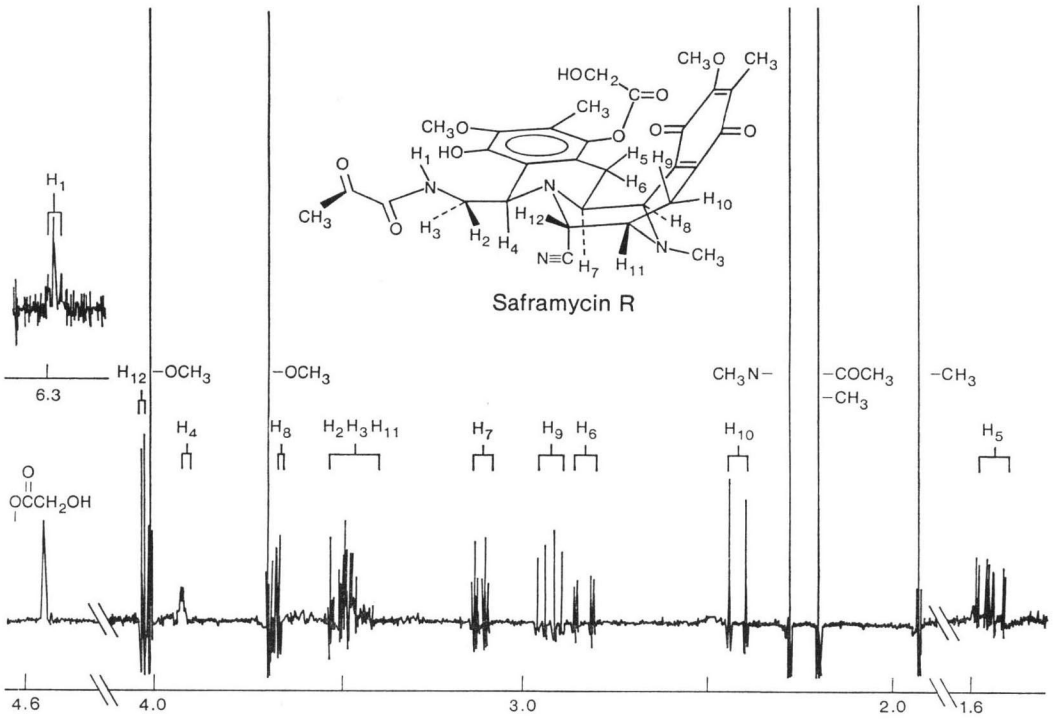


Saframycin A



Saframycin R

Fig. 2. High field (400 MHz) resolution enhanced ^1H NMR spectrum of saframycin R in $CDCl_3$ with TMS as internal reference.



cular formula of $C_{31}H_{34}N_4O_{10}$ previously established for saframycin R[6]. The positive ion FAB mass spectrum taken in glycerol showed an ion at m/z 625, equivalent to $MH^+ + 2$ and corresponding to the reduction of a quinone ring by the hydrogen donating solvent, as well as an ion at m/z 583 corresponding to loss of HCN which data are compatible with the assigned structure (Fig. 1). The presence of the quinone ring was confirmed by its characteristic electrochemical behavior $\nu_{1/2} = -0.240 \pm 0.005$ V (*vs.* SCE) and infrared spectrum.[6]

^1H NMR

For the purposes of discussion of the NMR spectra of saframycin R the fourteen protons (excluding the methyl groups) have been assigned as shown in Fig. 2.

Methine and Methylene Protons

Irradiation of the ddd at δ 6.23 (H-1) led to the collapse of the signals at δ 3.43 and δ 3.41 (H-2, H-3) from ddd to dd and collapse of the ddd δ 3.87 (H-4) to a dd. The same simplification was obtained by D_2O exchange of H-1. Complementary double irradiation experiments between H-1, H-2, H-3 and H-4 established their inter-relationship and permitted the determination of $J_{1,2}=6.4$ Hz, $J_{1,3}=6.4$ Hz, $J_{1,4}=1.1$ Hz. Values for $J_{H-C-N-H}$ have been observed to be in the range from 5 to 9 Hz.[17] $J_{2,3}$ could not be determined owing to overlapping of the signal and a second order component. For similar reasons the values of $J_{2,4}$ and $J_{3,4}$ determined by double irradiation as 3.0 and 2.6 Hz cannot be assigned unambiguously.

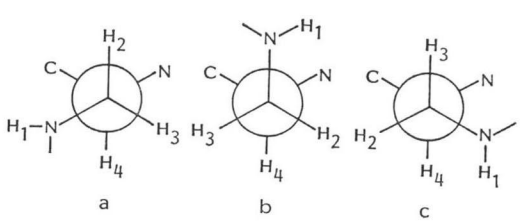
The stereochemical relationships among H-1, H-2, H-3 and H-4 were analyzed by considering the relative proportions of the three staggered rotamers a, b and c. The observed comparable values of

Table 1. ^{1}H NMR chemical shifts and coupling constants and nOe for saframycin R in $CDCl_3$.

Protons	Chemical shifts[a] δ (ppm)	Multiplicity	Coupling constants[b] (Hz)
1	6.23 (−0.16)[c]	ddd	$J_{1,2}$=6.4
			$J_{1,3}$=6.4
			$J_{1,4}$=1.1
2, 3	3.43[d] (−0.11)	ddd	$J_{1,2}$=6.4
	3.41 (−0.07)	ddd	$J_{1,3}$=6.4
			$J_{2,4}$=3.0
			$J_{3,4}$=2.6
4	3.88 (−0.16)	dddd	$J_{4,5}$=3.0
			$J_{4,2}$=3.0
			$J_{4,3}$=2.6
			$J_{4,1}$=1.1
			$J_{4,12}$=0.0
5	1.48[e] (−0.16)	ddd	$J_{5,6}$=17.7
			$J_{5,7}$=11.2
			$J_{5,4}$=3.0
6	2.78[e] (−0.28)	dd	$J_{6,5}$=17.7
			$J_{6,7}$=3.3
7	3.07 (−0.43)	ddd	$J_{7,8}$=3.2
			$J_{7,6}$=3.3
			$J_{7,5}$=11.2
8	3.64 (−0.82)	d	$J_{8,7}$=3.2
9	2.88[f] (−0.82)	dd	$J_{9,10}$=18.0
			$J_{9,11}$=8.0
10	2.38[f] (−0.75)	d	$J_{10,9}$=18.0
11	3.44 (−1.12)	ddd	$J_{11,9}$=8.0
			$J_{11,10}$=0.0
			$J_{11,12}$=2.7
12	3.88 (−0.08)	d	$J_{12,11}$=2.7
CH_3[g]	1.87 (−0.11)	s	
CH_3; $COCOCH_3$[g]	2.14 (−0.11)	2s	
NCH_3[g]	2.23 (−0.65)	s	
OCH_3 (red. quinone)[g]	3.65 (−0.16)	s	
OCH_3 (quinone)[g]	3.96 (0.10)	s	
$OCOCH_2OH$[h]	4.49 (−0.22)	s	$^5J_{HF}$=18.0 (q)

[a] The chemical shifts were obtained at 400 MHz and are given relative to internal TMS as reference.
[b] Assigned by double resonance experiments, however the numerical values of coupling constants were taken from the non-decoupled spectrum.
[c] Relative chemical shift observed upon addition of CF_3COOH.
[d] Overlapping signals with second order components.
[e] nOe of *ca.* 15% in accord with geminal protons. The nOe was unchanged at 200 MHz.
[f] nOe of *ca.* 5% in accord with geminal protons. The nOe was unchanged at 200 MHz.
[g] Relaxation times (T_1) methyls≈0.30~0.35 second.
Methylene and methine protons≈0.15~0.20 second.
[h] The corresponding methylene signal in glycolic acid in $(CD_3)_2CO$ appears at δ 4.20.

$J_{2,4}$ and $J_{3,4}$ of 3.0 and 2.6 Hz would seem to preclude a high population of either rotamer a or c but suggest rather an average conformation closer to that of conformer b (see Fig. 2) as was found in the case of saframycin A[18] suggesting that the glycolic ester group is not at position 8 of the



reduced quinone ring (*vide infra*).

Double irradiation of the ddd signal at δ 1.47 (H-5) led to the collapse of the signal at δ 2.78 (H-6) from a dd to a d which is partially overlapped by H-9 at δ 2.88 and also caused collapse of the H-7 absorption at δ 3.07 from a ddd to a dd. Protons H-5 and H-6 show typical $J_{5,6}=17.7$ Hz and strong nOe difference characteristic of geminal protons.[10] It was confirmed by ^{13}C NMR that there was only one quinone group present in the antibiotic. The coupling constants among protons 4, 5, 6 and 7 were determined as $J_{4,5}=3.0$, $J_{4,6}=0.0$, $J_{5,6}=17.7$, $J_{5,7}=11.2$ and $J_{6,7}=3.3$ Hz. As in the case of saframycin A the magnitude of the coupling between H-4 and H-5 over five bonds is consistent with such long range coupling over a W path[17] and requires that H-4 and H-5 be *trans* to each other permitting unambiguous stereochemical assignment of H-4 and H-5. The chemical shifts and coupling constants and therefore the appearance of that portion of the spectrum provided by protons H-8, H-9, H-10 and H-11 are closely similar to the corresponding signals in the NMR spectrum of saframycin A. Protons 9 and 10 show the large coupling of 18.0 Hz and nOe difference characteristic of geminal protons.[10] Relaxation time measurements (T_1 values) for the methyl groups in saframycin R were $0.3\sim0.35$ second while those of the methine and methylene protons were $0.15\sim0.20$ second which values were used to adopt optimum conditions for the nOe experiments. The additional methylene signal at δ 4.49 together with two exchangeable OH protons (contrasted with saframycin A) and the molecular formula of $C_{31}H_{34}N_4O_{10}$ point to the existence of a glycolic ester moiety ($OCOCH_2OH$) attached to the reduced quinone ring. The existence of a primary alcohol in this side chain was confirmed by addition of CF_3COOH which resulted in 0.22 ppm downfield shift typical of a primary alcohol[19] together with a long range $^5J_{HF}=18.0$ Hz causing the $-OCOCH_2OCOCF_3$ to appear as a quartet.

^{13}C NMR

Determination of the spin-echo ^{13}C effects[8,9] permitted determination of the multiplicities of the carbons *i.e.* the numbers of protons bound from individual carbon. A positive spin-echo result signifies bonding to 0 or 2 protons while a negative spin-echo effect indicates attachment to 1 or 3 protons.[8,9] Broad-band ^1H decoupled ^{13}C NMR spectral determinations were used for confirmatory purposes in those cases where the signal to noise ratio proved unsuitable for spin-echo studies. It may be seen from Table 2 that the assignments of multiplicities of individual carbons in this way are in accord with the ascribed structure. The group of lowest field ^{13}C signals were ascribable to carbonyl carbons *viz* the δ 171.7 was due to the glycolic ester carbonyl,[20] those at δ 196.8 and δ 161.4 were assigned to the COCO amide dione side-chain with the higher field CO probably that adjacent to the terminal CH_3.[20] Only two quinone carbonyls appear in the spectrum at δ 182.1 and δ 186.5 which are attributed to the C_{15} and C_{18} carbonyls respectively[20] on the basis that since C_{18} is adjacent to a $C-OCH_3$ and C_{15} is adjacent to $C-CH_3$ in the ring then C_{18} is assigned the lower field position of 186.5 ppm and C_{15} to 182.1 ppm.

The isolated lower intensity signal at δ 118.4 was ascribed to the nitrile group.[20] Assignment of the ring methyl groups at δ 8.5 and δ 9.5, the *N*-methyl signal at δ 41.4 and the ring methoxyls at δ 55.4 and δ 59.5 were made by comparison with the spectrum of saframycin A.[18]

Positions of Hydroquinone Ring and Glycolic Ester Substituent

A comparison of the chemical shift differences between the groups of protons adjacent to ring (C_5–C_{10}) *i.e.* H-4, H-5, H-6 and those immediately adjacent to ring (C_{15}–C_{20}) *i.e.* H-9, H-10, and H-11 in saframycins R and A show that the former experience shifts of 0.2 to 0.10 ppm whereas the latter group experience smaller shifts of 0.08 to 0.00 ppm. This suggests that it is the (C_5–C_{10}) ring which is in the

Table 2. Assignment of ^{13}C NMR signals of saframycin R in acetone-d_6 by spin-echo and broad-band ^1H decoupled spectra and determination of numbers of protons attached to different carbon atoms.[a]

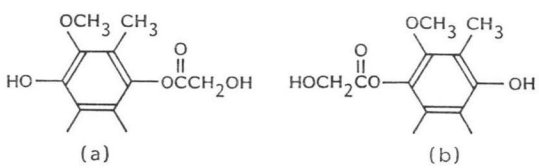
Carbon	δ (ppm)	Spin-echo (±)	Inference (number of protons)	Carbon	δ (ppm)	Spin-echo (±)	Inference (number of protons)
21	55.4	−	1	16	128.3[b]	+	0
3, 13	57.3	−	1	17	156.8	+	0
	57.5	−	1	20	142.4	+	0
1, 11	60.8	−	1	22	40.8	+	2
	60.9	−	1	$-C\equiv N$	118.4	+	0
4	25.1	+	2	$NCOCOCH_3$	196.8	+	0
5, 7	149.1	+	0	$NCOCOCH_3$	161.4	+	0
	150.4	+	0	$OCOCH_2OH$	171.7	+	0
6	116.8	+	0	CH_3	8.5	−	3
8, 19	136.7	+	0	CH_3	9.5	−	3
	137.1	+	0	$COCOCH_3$	24.2	−	3
9, 10	123.2	+	0	NCH_3	41.4	−	3
	124.9	+	0	OCH_3	55.4	−	3
14	21.3	+	2	OCH_3	59.5	−	3
15	182.1	+	0	$OCOCH_2OH$	61.0	+	2
18	186.5	+	0				

[a] A positive spin-echo effect signifies bonding of the carbon in question to 0 or 2 protons, while a negative effect signifies bonding of the carbon to 1 or 3 protons

[b] Obtained from broad-band decoupled spectrum in those cases where the signal to noise ratio was too small for the absorption to be detected by spin-echo.

hydroquinone form. The chemical shift difference δ (5–6) = 1.30 ppm is smaller in saframycin R than in saframycin A[15] (1.6 ppm) in accord with the removal of the diamagnetic anisotropy effect of the C_5 quinone carbonyl in the former case which moves H-5 to higher field.

Additional evidence is provided by inspection of the methyl group signals. The quinone CH_3's appear at δ 1.91 and δ 1.98 in saframycin A. The comparable CH_3's signals in saframycin R are assigned δ 1.87 to quinone and δ 2.14 to the hydroquinone ring. The N–CH_3 resonance at δ 2.28 in saframycin R is closely similar to that at δ 2.24 in saframycin A[15] indicating that, like protons H-9, H-10 and H-11 it is in a similar environment *i.e.* adjacent to a quinone moiety in ring C_{15}–C_{20}. Confirmation of the N-CH_3 assignment is provided by its selective shift to δ 2.88 resulting from protonation by CF_3COOH in $CDCl_3$ (Table 1). The comparable shift in the N–CH_3 signal in saframycin A in the presence of CF_3COOH is to δ 2.84. Protons H-8, H-9, H-10 and H-11 in saframycin R experience downfield shifts of 0.6 ~ 0.8 ppm upon addition of CF_3COOH indicating proximity to the site of protonation of N–CH_3 in contrast to the other methine and methylene protons in the molecule that experience an average shift of 0.4 ppm. There are two possible orientations of the glycolic ester substituents in the C_5–C_{10} hydroquinone ring. The observation that the average conformation of the amide-dione side chain is closely similar in saframycin R and in saframycin A[15] argues against any steric effects due to a large group at C_8 favoring orientation (a). Computations of the net ^{13}C chemical shifts of individual carbons in the two carbocyclic rings[20] are not sufficiently diagnostic to discriminate between orientations (a) and (b). The assigned orientation should therefore be regarded as tentative and awaits X-ray diffraction



data for confirmation.

Chemical Interconversion of Saframycin R to Saframycin A

Further evidence in support of the suggested structure of saframycin R is provided by its oxidative conversion to saframycin A upon treatment with FREMY's salt.[6]

Interactions of Saframycin R with DNA Binding

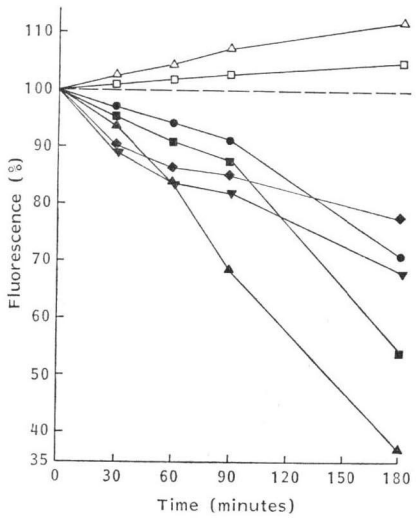
Saframycin R binds to DNA covalently and reversibly at a concentration of 44 μM at 37°C and pH 5.0. The ethidium fluorescence of PM2-ccc-DNA is suppressed by 5% in 3 hours. Saframycin A under comparable conditions causes a 12% suppression of fluorescence.[5]

Single Strand Scission of DNA

Saframycin R at a concentration of 44 μM with free exposure to atmospheric oxygen nicks PM2-ccc-DNA to the extent of 7.5% at pH 7.0 and 37°C in 2.5 hours (Fig. 3). This may be due to a slow hydrolysis of the ester moiety to generate reduced saframycin A.

Fig. 3. Single strand scission of PM2-ccc-DNA by reduced saframycin R and its pH dependence.

Reactions were performed at 37°C in 50 mM potassium phosphate buffer at different pH values and contained 1.27 OD units/ml PM2-ccc-DNA (92% ccc), 44 μM of saframycin R in 10% acetonitrile and 2.6 mM sodium borohydride. The before heat denaturation fluorescence readings are shown as open symbols, and the closed symbols are fluorescence readings after heat denaturation of the DNA at 96°C for 4 minutes followed by rapid cooling to 22°C. Some of the before heat fluorescent readings have been omitted for the sake of clarity. Reaction conditions pH: (○) 5; (□) 6; (△) 7; (▽) 8; (◇), 9.



In the presence of 2.6 mM sodium borohydride (which itself has no effect on DNA[5]) the extent of DNA cleavage is increased to 50% under identical conditions in 2.5 hours. Under these conditions the redox reaction of the second (C_{15}–C_{18}) quinone moiety may be involved in the reaction. The rate and extent of DNA cleavage is considerably lower than for saframycins A and C (85% and 90% respectively[5]) under closely similar conditions suggesting that the ester moiety in saframycin R is still substantially intact.

Saframycin R in the presence of sodium borohydride shows a marked pH dependence in its oxidative scission of PM2-ccc-DNA in the range 5~9 (Fig. 3). The extent of scission is maximal at pH 7.0 and decreases on either side of the pH scale. This phenomenon may suggest a compromise in the conditions permitting ester hydrolysis, equilibrium binding and redox generation of reactive oxygen species.

Scission of DNA in the Presence of Porcine Liver Carboxyl Esterase

Saframycin R (44 μM) and 2.6 mM sodium borohydride nicks PM2-ccc-DNA by 29% in 3 hours at 37°C and at pH 8.0. In the presence of 10 μl of porcine liver carboxyl esterase (EC 3.1.1.1, Type I *ca.* 17 units as a suspension in 3.2 M ammonium sulfate and 10 mM sodium borate pH 8.0) the extent of scission is increased to 45% (Fig. 4). The same quantity of esterase alone or with 44 μM saframycin R nicks DNA by <6% in 3 hours under similar conditions. Control experiments on saframycin R with ammonium sulfate and sodium borate at comparable concentrations show that this increase in scission may be partially due to base-catalyzed hydrolysis of the ester moiety of saframycin R due to the NH_3

Fig. 4. Single strand scission of PM2-ccc-DNA by saframycin R in the presence of porcine liver carboxyl esterase.

Reactions were performed at 37°C in 50 mM potassium phosphate buffer, pH 8.0 and contained 1.27 OD units/ml of PM2-ccc-DNA (92% ccc), 44 μM saframycin R in 10% acetonitrile. The before heat-denaturation fluorescence readings are shown as open symbols, and the closed symbols are fluorescence readings after heat denaturation of the DNA at 90°C for 4 minutes followed by rapid cooling to 22°C. Some before heat fluorescence readings have been omitted for the sake of clarity. Additional components were: (△) 17 units of esterase alone or 17 units of esterase and 44 μM saframycin R; (□) 2.6 mM sodium borohydride; (○) 17 units of esterase and 2.6 mM sodium borohydride; (◇) 0.32 M ammonium sulfate, 1 mM sodium borate and 2.6 mM sodium borohydride; (▽) 0.32 M ammonium sulfate and 1 mM sodium borate.

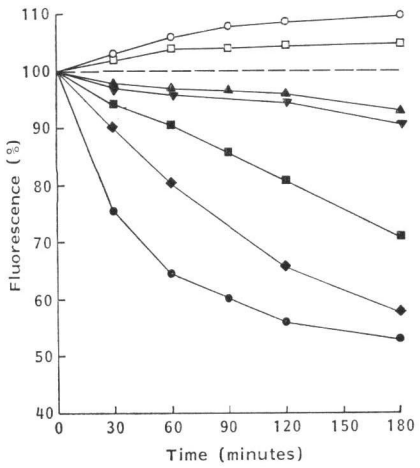
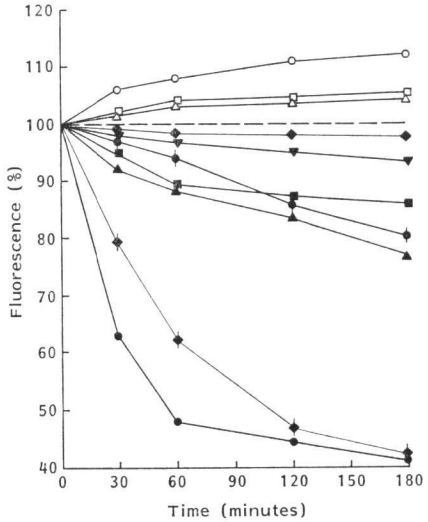


Fig. 5. Single strand scission of PM2-ccc-DNA by saframycin R after preincubation with porcine liver carboxyl esterase.

Reactions were performed at 37°C in 50 mM potassium phosphate buffer pH 8.0 and contained 1.27 OD units/ml of PM2-ccc-DNA (92% ccc), 44 μM saframycin R in 10% acetonitrile. The before heat fluorescence readings are shown as open symbols and the closed symbols are fluorescence readings after heat denaturation of the DNA at 96°C for 4 minutes followed by rapid cooling to 22°C. Some of the before heat fluorescence readings have been omitted for the sake of clarity. Additional components were: (◇) 17 units of esterase without drug; (▽) none; (□) 17 units of esterase with drug; (△) 2.6 mM sodium borohydride; (○) 17 units of esterase with drug and 2.6 mM sodium borohydride; (◊) 0.32 M ammonium sulfate, 1 mM sodium borate and 2.6 mM sodium borohydride; (◊) 0.32 M ammonium sulfate and 1 mM sodium borate.



present in the buffer. Thus 44 μM saframycin R and 2.6 mM sodium borohydride in the presence of 0.32 M ammonium sulfate and 1 mM sodium borate nicks PM2-ccc-DNA by 42% in 3 hours at 37°C and pH 8.0 whereas in the absence of the reducing agent the extent of scission is only ~10%.

Effect of Pre-incubation of Saframycin R with Porcine Liver Carboxyl Esterase

Treatment of PM2-ccc-DNA with saframycin R at 37°C, pH 8.0, pre-incubated with the esterase for 2 hours at 37°C and pH 8.0 increases the scission to 60% from 45% (for the unincubated sample) in the presence of sodium borohydride (Fig. 5). Under comparable conditions saframycin R, esterase, saframycin R and sodium borohydride, and saframycin R and esterase cleave PM2-ccc-DNA by 10%, 2%, 23% and 14% respectively. A control experiment in the presence of ammonium sulfate and sodium borate under comparable conditions gave 19.5% scission. This again may indicate a contribution due to hydrolysis of the ester group in saframycin R to reduced saframycin A by ammonia or other bases present in the buffer.

Conclusions

The spectroscopic and physical evidence are in accord with a structure for saframycin R in which the C_5–C_{10} aromatic ring is in the hydroquinone form and bears a glycolic ester group most probably at position 5. Saframycin R in common with other members of this antibiotic group saframycins A and S interacts with cellular DNA templates in reactions which include oxygen dependent DNA single strand scission. The extent of the reaction is increased by *in situ* esterase or base catalyzed cleavage of the glycolic ester group and at a pH optimum of 7.0 releasing reduced saframycin A which has previously been shown to both bind to DNA templates and effect oxygen-dependent scission. The results suggest that saframycin R may be regarded as a substantially less toxic[8] pro-drug for the active forms of saframycin A or S. It has been pointed out that the glycolic ester moiety offers the opportunity for derivatization to exploit the transport advantages of this potentially useful antitumor agent.[8]

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