SAFRAMYCIN Mx1, A NEW NATURAL SAFRAMYCIN ISOLATED FROM A MYXOBACTERIUM†

HERBERT IRSCHIK, WOLFRAM TROWITZSCH-KIENAST††, KLAUS GERTH, GERHARD HÖFLE†† and HANS REICHENBACH

GBF, Gesellschaft für Biotechnologische Forschung,
Arbeitsgruppe Mikrobielle Sekundärstoffe
and ††Abteilung Naturstoffchemie,
Mascheroder Weg 1, D-3300 Braunschweig, FRG

(Received for publication February 16, 1988)

A new natural saframycin was discovered in the culture broth of the myxobacterium, *Myxococcus xanthus* strain Mx x48. The fermentation and isolation of the antibiotic are described. The name, saframycin Mx1, is proposed. The compound appears to interact with cellular DNA.

In a screening for antibiotics from myxobacteria we discovered in the culture supernatant of *Myxococcus xanthus* strain Mx x48 an extremely strong activity against Gram-positive bacteria. Isolation and chemical characterization of the responsible substance showed that the antibiotic was closely related to saframycin from *Streptomyces lavendulae*[1]. We therefore named our antibiotic saframycin Mx1. Its structure is shown in Fig. 1. In this paper we describe the production and some physicochemical and biological properties of the compound, while the elucidation of the chemical structure is published elsewhere[2].

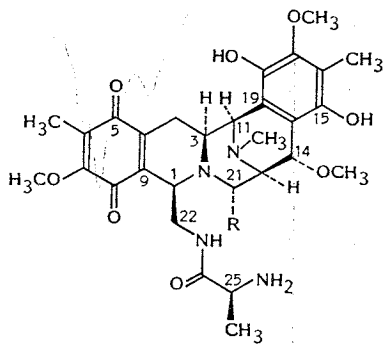
Production of the Antibiotic

The producing strain was isolated in 1980 from a soil sample from Gabès, Tunesia. The organism was cultivated on MD1 liquid medium[3]: Peptone from casein, tryptically digested 0.3%, $MgSO_4 \cdot 7H_2O$ 0.2%, $CaCl_2 \cdot 2H_2O$ 0.05%, pH 7.2. Laboratory cultures in shake flasks (50 ml in 250-ml Erlenmeyer flasks) were incubated at 30°C on a rotary shaker (160 rpm).

For antibiotic production the bacterium was cultivated in a Type b 500 fermentor from Giovanola Frères, Monthey, Switzerland, equipped with a turbine impeller and containing 700 liters of MD1 medium. The culture was inoculated from a seed fermentor with 10%. The aeration rate was 1 m^3 air/hour, and the stirring rate 150 rpm. The pO_2 was kept at or above 60% saturation by increasing the stirring rate when required.

The temperature was 30°C. During the

Fig. 1. The chemical structure of saframycin Mx1[2].



Saframycin Mx1 R = OH

† Article No. 34 on antibiotics from gliding bacteria. Article No. 33: KUNZE, B.; G. HÖFLE and H. REICHENBACH: J. Antibiotics 40: 258~265, 1987.

fermentation the pH rose from 7.2 to about 8. The antibiotic activity in the culture broth was estimated by the agar diffusion assay with *Staphylococcus aureus*. After about 50 hours the diameter of the inhibition zone reached 15~16 mm, and the culture was harvested.

Isolation of the Antibiotic

At the end of the fermentation 1% of the adsorber resin Amberlite XAD-1180 (from Rohm and Haas, Darmstadt) was added to the culture. The fermentation was continued for another 3 hours. Then the resin was separated from the culture by filtration and extracted with 2-propanol (1.5 times the bed volume of the column). The 2-propanol was evaporated from the eluate, and the remaining aqueous phase was chromatographed on CM-Sephadex-C-25 (Pharmacia). Saframycin Mx1 eluted with 0.07 M phosphate buffer pH 5+0.5 M NaCl. The salts were removed from the active fraction by chromatography on XAD-2 resin. Saframycin Mx1 eluted with methanol. After concentration of the methanolic phase the final purification was achieved by HPLC on DH-Sil 18 (Organogen).

Physico-chemical Properties

Pure saframycin Mx1 was obtained as a dark oil. The compound dissolved well in polar solvents like water, methanol or ethanol but little or not at all in non-polar solvents like benzene or hexane. It was very sensitive to light and to oxygen, and above pH 7 was quickly oxidized to the bisquinone. The UV, IR and ^{13}C NMR spectra of saframycin Mx1 are shown in Figs. 2, 3 and 4. The optical rotation was $[\alpha]_D^{20}$ $-70.7°$ (c 0.5, MeOH). The molecular formula, $C_{29}H_{38}N_4O_9$, could be deduced from a high resolution fast atom bombardment mass spec-

Fig. 2. UV spectrum of saframycin Mx1.
MeOH; log ε 3.95, 273 nm.

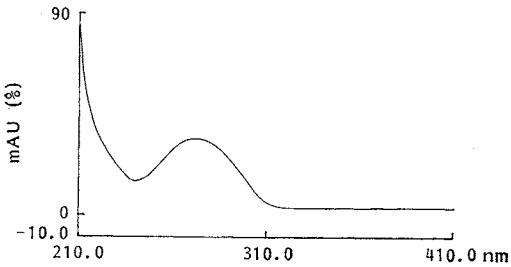


Fig. 3. IR spectrum of saframycin Mx1 in KBr.
Nicolet-20 DXB-FTIR spectrometer.

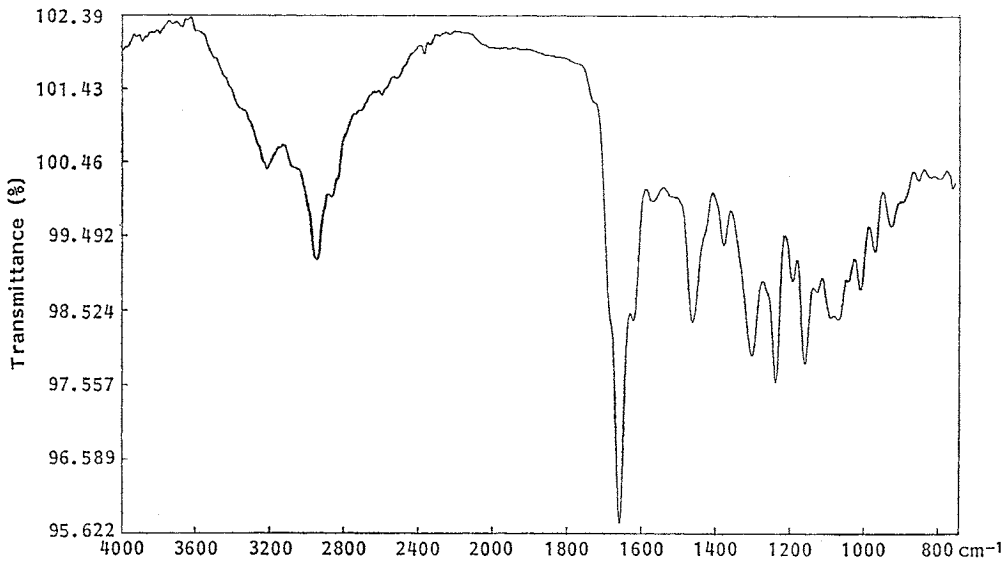


Fig. 4. 100 MHz ^{13}C NMR spectrum of saframycin Mx1.
In CD_3OD; Bruker W-400 spectrometer.

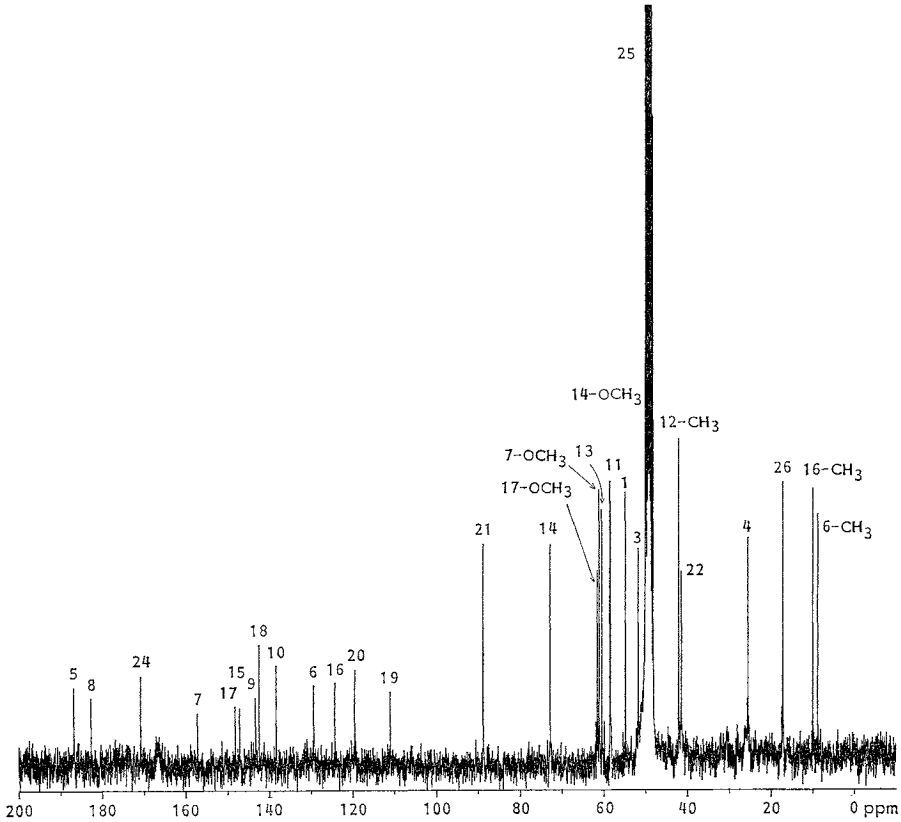
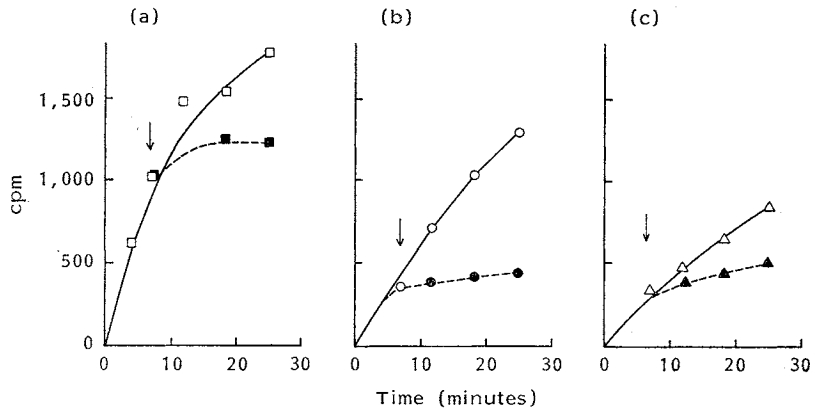


Fig. 5. The effect of saframycin Mx1 on various macromolecular syntheses in *Staphylococcus aureus*.



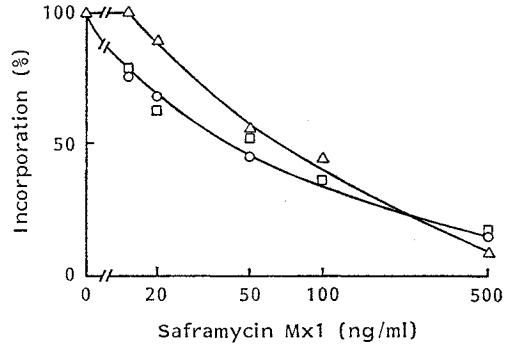
The antibiotic (0.5 μg/ml) was added to the culture at the time indicated by the arrow. The rate of synthesis was measured as incorporation of labeled specific precursors into perchloric acid insoluble material: (a) [^{14}C]Thymidine (0.1 μCi/ml) for DNA, (b) [^{14}C]uracil (0.05 μCi/ml) for RNA, and (c) [^{14}C]isoleucine (0.05 μCi/ml) for protein. The open symbols represent the control without the antibiotic.

Table 1. The antibiotic spectrum of saframycin Mx1.

Test organism	Diameter of inhibition zone (1 μg/disc)[a]	MIC (μg/ml)
Staphylococcus aureus	36	0.004
Bacillus subtilis	25	0.062
B. megaterium	18	0.5
B. polymyxa	17	
Mycobacterium phlei	15	
Nocardia corallina	22	
N. flava	25	
Corynebacterium mediolanum	20	
Arthrobacter rubellus	14	
A. simplex	15	
Micrococcus luteus	45	0.001
Brevibacterium ammoniagenes	32	0.002
Escherichia coli	21	0.32
E. coli, tol C[b]	22	0.16
Pseudomonas fluorescens	9	5
P. acidovorans	0	
Salmonella typhimurium	0	10
Proteus morganii	19	
Serratia marcescens	15	
Rhizobium meliloti	17	
Klebsiella sp.	12	
Myxococcus fulvus, Mx f50	9	
M. fulvus, Mx f65	15	
M. xanthus, Mx x48[c]		15
Sorangium cellulosum, So ce12	11	
Halobacterium halobium	30	
H. cutirubrum	35	
Candida albicans	0	
Nadsonia fulvescens	0	
Saccharomyces cerevisiae	0	
Torulopsis glabrata	0	
Hansenula anomala	0	
Mucor hiemalis	0	

[a] Diameter of paper disc was 6 mm.
[b] Mutant with increased membrane permeability.
[c] Producing strain.

Fig. 6. The inhibition of DNA (□), RNA (○) and protein (△) synthesis in *Staphylococcus aureus* in dependence of the dose of saframycin Mx1.



The same labeled precursors were used as in Fig. 5.

Table 2. Effect of saframycin Mx1 on RNA synthesis by *Escherichia coli* RNA polymerase *in vitro*[a].

Experimental conditions	Saframycin Mx1 (μg/ml)	Inhibition (%)
Preincubation of	0	0
DNA+antibiotic+2 mM	5	70
dithiothreitol, 15 minutes	28	81
No preincubation,	0	0
without dithiothreitol	5	70
	28	82

[a] Calf thymus DNA was used as the template.

trogram (HRFAB-MS) (which yielded, however, a molecular ion that derived from the bishydroquinone[2]). The calculated molecular weight is 586.6382.

Biological Activity

Table 1 shows that saframycin Mx1 is particularly active against Gram-positive bacteria, but also is a rather efficient inhibitor of several Gram-negative organisms and of halobacteria. Yeasts and the mold, *Mucor hiemalis*, did not respond to a concentration of 1 μg/disc.

The effects of saframycin Mx1 on the biosyntheses of various cellular macromolecules were tested with *S. aureus* as described recently[4]. The results for DNA, RNA and protein synthesis are shown in Fig. 5. At a concentration of 0.5 μg/ml all three syntheses were inhibited immediately and almost completely. While the dose-effect curves for DNA and RNA synthesis did not show significant differences, the dose response of protein synthesis was shifted to slightly higher concentrations (Fig. 6). On the other hand, in the cell-free system from *Escherichia coli* there was no inhibition of protein syn-

thesis by saframycin Mx1 up to at least 50 μg/ml (data not shown), whereas the activity of RNA-polymerase from *E. coli* (Boehringer Mannheim) was inhibited *in vitro* and showed a residual activity of 30% with 5 μg antibiotic per ml (Table 2). This effect was obtained with oxidized as well as with reduced saframycin Mx1.

Discussion

The saframycins were discovered in 1977 by ARAI *et al.* in *S. lavendulae* as satellite antibiotics[1]. Presently 9 natural saframycins are known from this source to which have been added several compounds modified by chemical or biological means[5,6]. Related substances have been described from other organisms, *viz.* the renieramycins from the marine sponge, *Reniera* sp.[7], and the safracins from *Pseudomonas aeruginosa*[8]. We add now a further natural variant, saframycin Mx1, which we isolated from a myxobacterium, *M. xanthus* strain Mx x48. The corresponding bisquinone was also obtained, which was to be expected as the two compounds interconvert readily[2]. The myxobacterial saframycin differs from the *Streptomyces* compounds in its side chain which ends with an amide bound alanine instead of a pyruvic acid. The alanine compound could also be obtained from *Streptomyces*, but only by mutasynthesis[6]. A second difference is the OH-group on C-21 which is only found in saframycin S[9] while in the other saframycins this position is occupied by H or, in the case of saframycin A, by CN. A third difference is the methoxy group on C-14 which is only known from saframycin C[1,5]. The safracins lack the OH-group on C-15 and the OCH_3 on C-14. The renieramycins have an H or ethoxy on C-14, either a carbonyl function or an H on C-21, and an ester bound *cis* α-methylcrotonic, or angelic, acid in the side chain. They also are supposed to differ in their stereochemistry.

While the saframycins are very strong growth inhibitors for certain Gram-positive bacteria, halobacteria and, less so, Gram-negative bacteria, yeasts and molds are insensitive (Table 1)[1]. The most interesting aspect of the saframycin-type compounds is their good antitumor activity[5,8,10,11]. The saframycins act by destroying the template function of the cellular DNA[5,12~14]. This effect apparently is produced by at least three different reactions which depend on the exact chemical structure of the specific saframycin applied. This explains the tremendous differences in the effective doses of the various saframycins in spite of relatively minor chemical modifications. Firstly, the antibiotic binds by electrostatic attraction in the minor groove of the DNA double helix (single strand DNA and RNA are not attacked). This reaction is possible for all saframycins unless they bear a bulky substituent[5]. Secondly, a covalent bond may be formed between C-21 of the saframycin and the NH_2-group of guanine (covalent saframycin-binding to DNA requires G-C pairs). This reaction is possible only for such saframycins that have a leaving group on C-21, either CN (saframycin A) or OH (saframycin S, and probably also saframycin Mx1)[5,12~14]. Finally, reduced saframycins, with or without CN or OH on C-21, induce single strand breaks in the DNA in the presence of oxygen, probably by creating oxygen and hydroxyl radicals which then lead to the strand scission[14].

The inhibitory effects described above for saframycin Mx1 essentially fit the proposed hypotheses for the mechanism of action of saframycins A and S: Fast and efficient inhibition of DNA, RNA and protein synthesis *in vivo*, inhibition of RNA, but not of protein synthesis *in vitro*. In contrast to saframycins S and A[12,13], however, saframycin Mx1 blocks *in vitro* RNA synthesis as efficiently in its oxidized form as under reducing conditions (Table 2).

Acknowledgments

We wish to thank Mrs. G. BADURA and Mrs. K. SCHOBER for skilful technical assistance, Eng. H. G. RENG and his team of the Fermentation Serive of the GBF for performing large scale fermentations, and Dr. V. WRAY of the spectroscopy group of the GBF for the ^{13}C NMR spectrogram.

References

1) ARAI, T.; K. TAKAHASHI & A. KUBO: New antibiotics, saframycins A, B, C, D and E. J. Antibiotics

30: 1015~1018, 1977

2) TROWITZSCH-KIENAST, W.; H. IRSCHIK, H. REICHENBACH, V. WRAY & G. HÖFLE: Isolierung und Strukturaufklärung der Saframycine Mx1 und Mx2, neue antitumor-aktive Antibiotika aus *Myxococcus xanthus*. Liebigs Ann. Chem. 1988: 475~481, 1988
3) REICHENBACH, H. & M. DWORKIN: The order Myxobacterales. *In* The Prokaryotes. *Ed.*, P. STARR *et al.*, pp. 328~355, Springer Verlag, Berlin, 1981
4) IRSCHIK, H.; K. GERTH, G. HÖFLE, W. KOHL & H. REICHENBACH: The myxopyronins, new inhibitors of bacterial RNA synthesis from *Myxococcus fulvus* (Myxobacterales). J. Antibiotics 36: 1651~1658, 1983
5) KISHI, K.; K. YAZAWA, K. TAKAHASHI, Y. MIKAMI & T. ARAI: Structure-activity relationships of saframycins. J. Antibiotics 37: 847~852, 1984
6) ARAI, T.; K. YAZAWA, K. TAKAHASHI, A. MAEDA & Y. MIKAMI: Directed biosynthesis of new saframycin derivatives with resting cells of *Streptomyces lavendulae*. Antimicrob. Agents Chemother. 28: 5~11, 1985
7) FRINCKE, J. M. & D. J. FAULKNER: Antimicrobial metabolites of the sponge *Reniera* sp. J. Am. Chem. Soc. 104: 265~269, 1982
8) IKEDA, Y.; H. IDEMOTO, F. HIRAYAMA, K. YAMAMOTO, K. IWAO, T. ASAO & T. MUNAKATA: Safracins, new antitumor antibiotics. I. Producing organism, fermentation and isolation. J. Antibiotics 36: 1279~1283, 1983
9) ARAI, T.; K. TAKAHASHI, K. ISHIGURO & K. YAZAWA: Increased production of saframycin A and isolation of saframycin S. J. Antibiotics 33: 951~960, 1980
10) ARAI, T.; K. TAKAHASHI, K. ISHIGURO & Y. MIKAMI: Some chemotherapeutic properties of two new antitumor antibiotics, saframycins A and C. Gann 71: 790~796, 1980
11) ASAOKA, T.; K. YAZAWA, Y. MIKAMI, T. ARAI & K. TAKAHASHI: A new saframycin, saframycin R. J. Antibiotics 35: 1708~1710, 1982
12) ISHIGURO, K.; S. SAKIYAMA, K. TAKAHASHI & T. ARAI: Mode of action of saframycin A, a novel heterocyclic quinone antibiotic. Inhibition of RNA synthesis in vivo and in vitro. Biochemistry 17: 2545~2550, 1978
13) ISHIGURO, K.; K. TAKAHASHI, K. YAZAWA, S. SAKIYAMA & T. ARAI: Binding of saframycin A, a heterocyclic quinone anti-tumor antibiotic to DNA as revealed by the use of the antibiotic labeled with [^{14}C]-tyrosine or [^{14}C]cyanide. J. Biol. Chem. 256: 2162~2167, 1981
14) LOWN, J. W.; A. V. JOSHUA & J. S. LEE: Molecular mechanisms of binding and single-strand scission of deoxyribonucleic acid by the antitumor antibiotics saframycins A and C. Biochemistry 21: 419~428, 1982