STRUCTURE-ACTIVITY RELATIONSHIPS OF SAFRAMYCINS

Koichiro Kishi, Katsukiyo Yazawa, Katsuhiro Takahashi,
Yuzuru Mikami and Tadashi Arai

Department of Antibiotics, Research Institute for Chemobiodynamics, Chiba University,
Chiba, Japan

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In vitro antitumor activities of 13 saframycins, including the potent antitumor component, saframycin A, were determined with the highly sensitive established cell line of L1210 mouse leukemia to investigate structure-activity relationships. Saframycins which lack the α-cyanoamine group or the α-carbinolamine group exhibited much lower cytotoxic activity than saframycin A. The modification of active saframycins either at the C-14 position on the basic skeleton or at the C-25 position on the side chain with bulky substituents resulted in a decrease in cytotoxic activity. These structure-activity relationships corroborated the proposed major mechanism of action for the antitumor activity of saframycin A and supported our proposed model for the saframycin A-DNA adduct.

Saframycins are satellite antibiotics which are co-produced in trace quantities with streptothricin by *Streptomyces lavendulae* No. 314[1]. Saframycins A, B, C, D and S were the first members of this complex to be isolated[1~4].

Recently, saframycins F, G and H were also isolated. The structures of saframycins D, F, G and H were elucidated on the basis of spectroscopic data (Figs. 1A & 1B) (unpublished data by Arai, T.; K. Takahashi, K. Yazawa & A. Kubo).

Saframycins AR_1 and AR_3 were produced by microbial conversion from saframycin A and their structures elucidated (Fig. 1A)[5,6]. Saframycins AH_1 and AH_2 were produced by the chemical reduction of saframycin A, and shown to be stereoisomers at C-25 (Fig. 1A)[5]. Furthermore, saframycin AH_2 was shown to be identical with saframycin AR_1[5].

Among these components, saframycin A had already been shown to be a potent antitumor agent that binds to duplex DNA in a unique manner, inhibiting its template activity[7,8].

The structure-activity relationships for saframycins A, C, AR_1 and AR_3 have been discussed elsewhere[5,9]. This paper reports structure-activity relationships for the 11 saframycins isolated to date and saframycins AH_1Ac and AH_2Ac which were newly obtained by the acetylation of saframycins AH_1 and AH_2, respectively (Fig. 1A). The results obtained allow discussions of the proposed major mechanism of action for the antitumor activity of saframycin A and our proposed model for the saframycin A-DNA adduct.

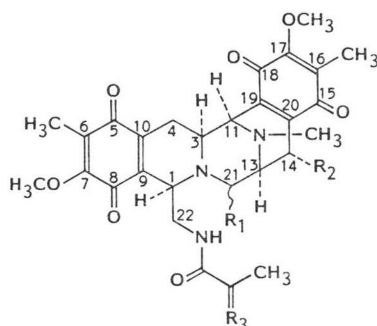
Materials and Methods

Chemicals

Saframycins A, B, C, D, F, G, H, S, AH_1, AH_2(=AR_1) and AR_3 were prepared in our laboratory as described in previous papers[1~6].

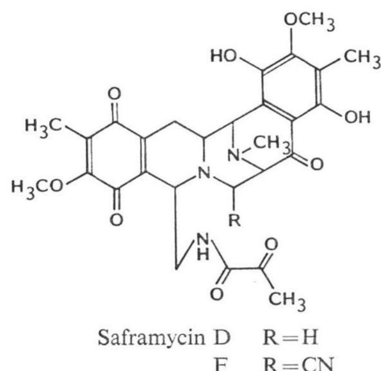
Saframycins AH_1Ac and AH_2Ac were prepared by the acetylation of saframycins AH_1 and AH_2, respectively. Saframycins AH_1 or AH_2 was dissolved in pyridine, then acetic anhydride was added to the solution. After stirring at room temperature for 24 hours, the solvent was evaporated. The residue

Fig. 1A. Structures of saframycins A, B, C, G, H, S, AH_1, AH_2 (=AR_1), AH_1Ac, AH_2Ac and AR_3.



Saframycin	R_1	R_2	R_3
A	CN	H	O
B	H	H	O
C	H	OCH_3	O
G	CN	OH	O
H	CN	H	OH, CH_2COCH_3
S	OH	H	O
AH_1 AH_2 (=AR_1)	CN	H	H, OH
AH_1Ac AH_2Ac	CN	H	H, $OCOCH_3$
AR_3	H	H	H, OH

Fig. 1B. Structures of saframycins D and F.



Saframycin D R=H
F R=CN

was dissolved in ethyl acetate and washed with distilled water. Further purification by preparative TLC using ethyl acetate - benzene (2: 1) as a solvent system gave saframycins AH_1Ac and AH_2Ac, respectively.

All the saframycins were dissolved in ethanol at a concentration of 10 mg/ml. In the cytotoxicity assays, the final concentration of the solvent in the culture medium was lower than 0.1%, at which concentration no effect of the solvent was observed.

Spectroscopic Measurements

IR absorption spectra were taken on an IRA-2 spectrometer (Jasco, Japan). NMR spectra were determined on a JNM-PFT-100 (Jeol, Japan) with tetramethylsilane as an internal standard.

In Vitro Cytotoxicity Assays

In vitro antitumor activities were determined by using a cultured cell line of L1210 mouse leukemia. Cells were maintained in suspension culture using EAGLE's minimum essential medium with HANK's salts (Gibco Laboratories, New York) supplemented with 10% fetal calf serum (Gibco Laboratories, New York)[10].

Cells were suspended at a concentration of 3×10^4 cells/ml and incubated at 37°C for 24 hours to initiate exponential growth. These cultures were exposed to a series of 10-fold dilutions of saframycins. After incubation for 3 days, the cell concentrations were determined by electronic counting with a Toa Micro Cell Counter (Toa Electronics Ltd., Tokyo).

The percent growth-inhibition (I) at each dose level was calculated according to the formula,

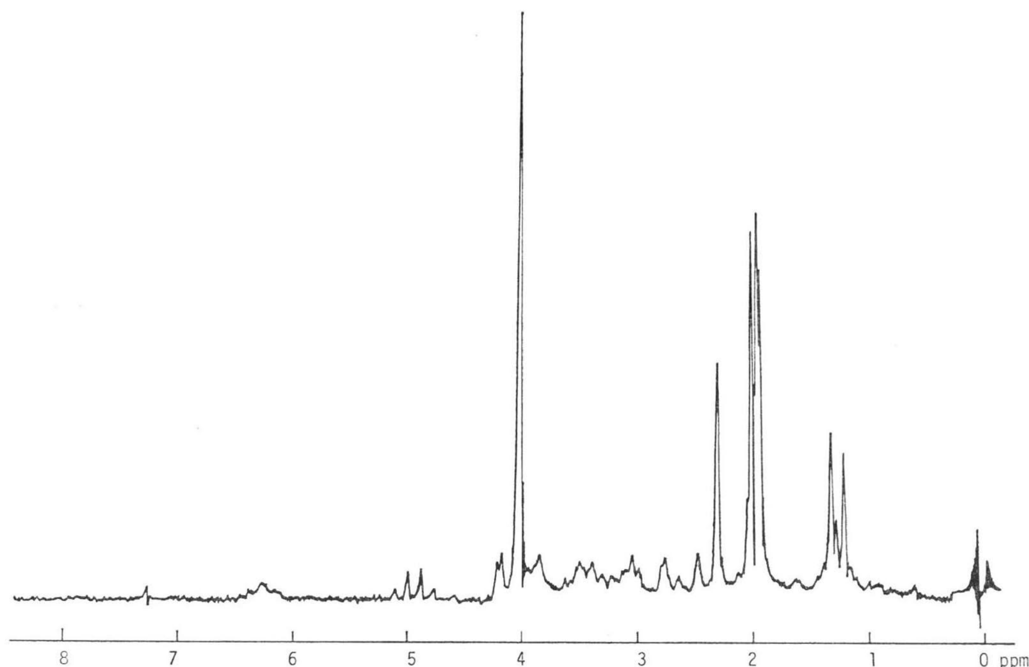
$$I=(C_3-T_3)/(C_3-C_0)\times100,$$

where T_3 is the cell concentration in the culture tube with each dilution of drugs after 3-day-long incubation, C_3 is the cell concentration in the corresponding control, and C_0 is the cell concentration in the culture tube before 3-day-long incubation. C_0 was within the range of $0.80\times10^5\sim1.04\times10^5$ cells/ml. C_3 was within the range of $1.04\times10^6\sim1.18\times10^6$ cells/ml. ID_{50} (50% inhibition dose) was determined by interpolation.

Results and Discussion

The IR spectra of saframycins AH_1Ac and AH_2Ac exhibited characteristic bands at 1735 cm^{-1} (-CO-O-), which were not observed in the spectra of saframycins AH_1 and AH_2. The ^1H NMR spectrum of saframycin AH_1Ac gave characteristic signals at 1.26 ppm (3H, d, J=6 Hz, $-CO-CH-CH_3$) and

Fig. 2. ^{1}H NMR spectrum of saframycin AH_1Ac (100 MHz, $CDCl_3$, 25°C).



4.95 ppm (1H, q, $J=6$ Hz, $-CO-CH(CH_3)-O-CO-$) (Fig. 2).

In the ^{1}H NMR spectrum of saframycin AH_2Ac, characteristic signals at 1.20 ppm (3H, d, $J=6$ Hz, $-CO-CH-CH_3$) and 4.75 ppm (1H, q, $J=6$ Hz, $-CO-CH(CH_3)-O-CO-$) were observed. These spectral data indicated that saframycins AH_1 and AH_2 were acetylated at the C-25 carbinol group. Therefore, the structures of saframycins AH_1Ac and AH_2Ac were elucidated as 25-*O*-acetylsaframycin A (Fig. 1A). These two compounds are stereoisomers at C-25, because saframycins AH_1 and AH_2 are stereoisomers at C-25[5].

Figs. 3A and 3B show the percent growth-inhibition against the cultured cell line of L1210 mouse leukemia at each dose level of saframycins.

Saframycins A, S, AH_1 and AH_2, which possess the α-cyanoamine group or the α-carbinolamine group at the C-21 position, exhibited potent cytotoxicity. ID_{50} values for saframycins A, S, AH_1 and AH_2 were 0.0056, 0.0053, 0.0080 and 0.0061 μM, respectively. However, among saframycins which possess the α-cyanoamine group, saframycins G, H, F, AH_1Ac and AH_2Ac which have bulky substituents either at the C-14 position or at the C-25 position were shown to exhibit lower cytotoxic activities than saframycin A. ID_{50} values for saframycins G, H, F, AH_1Ac and AH_2Ac were 0.030, 0.033, 0.59, 0.025 and 0.027 μM, respectively. The cytotoxic activities of saframycins which possess neither the α-cyanoamine group nor the α-carbinolamine group were much lower. ID_{50} values for saframycins B, C, D and AR_3 were 0.80, 3.9, 4.8 and 0.65 μM, respectively.

The mode of action of saframycin A, which possesses the highest cytotoxic activity, has been extensively studied[7,8]. All the data so far obtained suggest that the major antitumor activity of saframycin A is caused by covalent binding to duplex DNA and inhibition of its template activity[7,8].

It was revealed that saframycin A binds to duplex DNA covalently in the presence of reducing agents

Fig. 3A. Cytotoxicity assays for saframycins A (●), B (■), C (▲), G (○), H (□) and S (△) with L1210 cells *in vitro*.

Details were described in Materials and Methods.

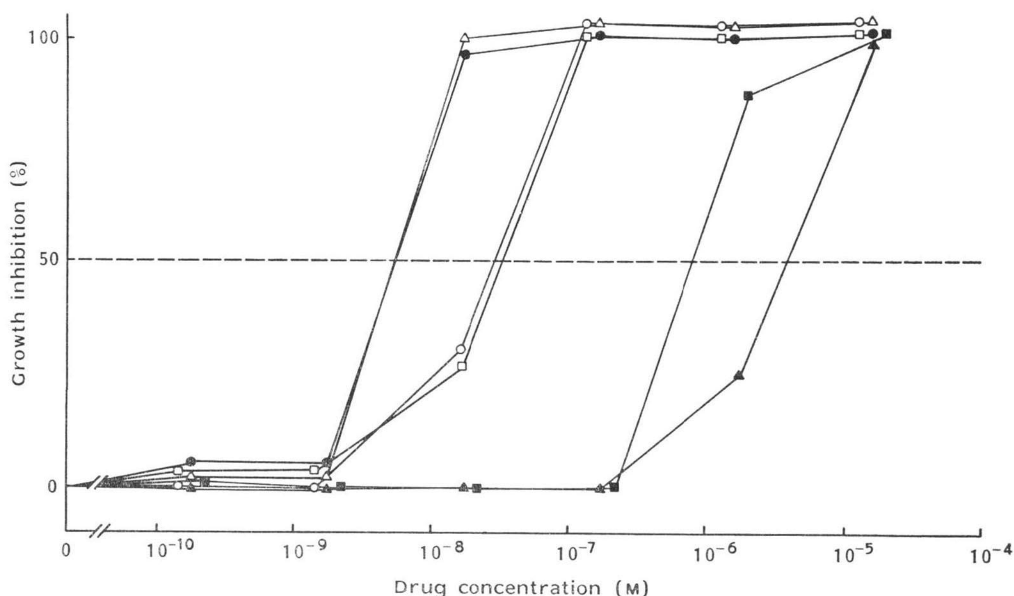
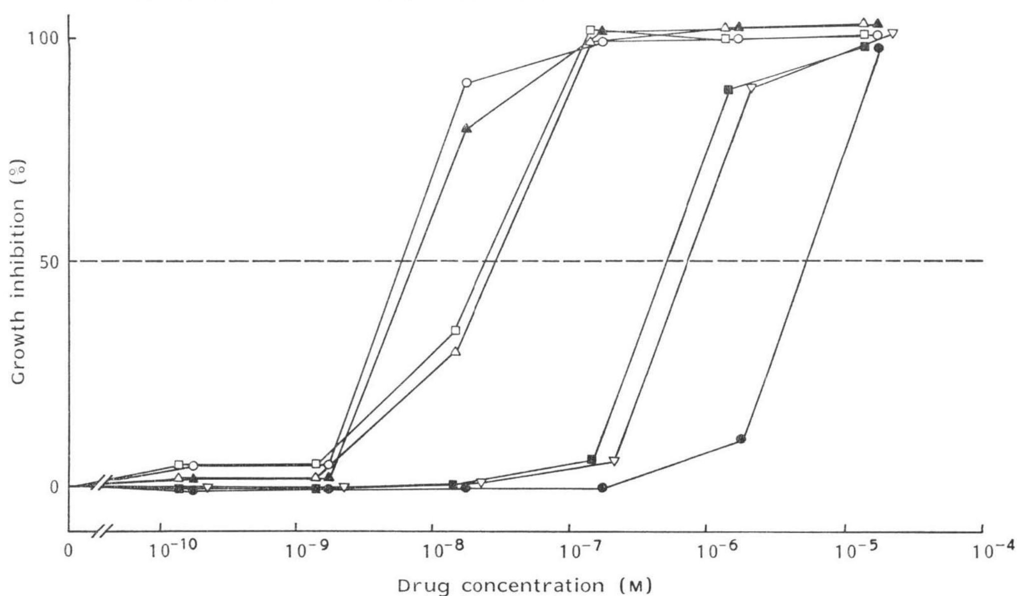


Fig. 3B. Cytotoxicity assays for saframycins D (●), F (■), AH_1 (▲), AH_2 (○), AH_1Ac (□), AH_2Ac (△) and AR_3 (▽) with L1210 cells *in vitro*.

Details were described in Materials and Methods.



such as dithiothreitol, *in vitro*[7,8]. It was also suggested that release of the cyano group from the antibiotic was triggered by this reduction[8]. Therefore, 21-decyano-21-hydroxysaframycin A, which was identical with saframycin S and proved to be the unstable biosynthetic precursor of saframycin A, was regarded as the active intermediate[8]. Saframycin S, indeed, bound to duplex DNA without reducing

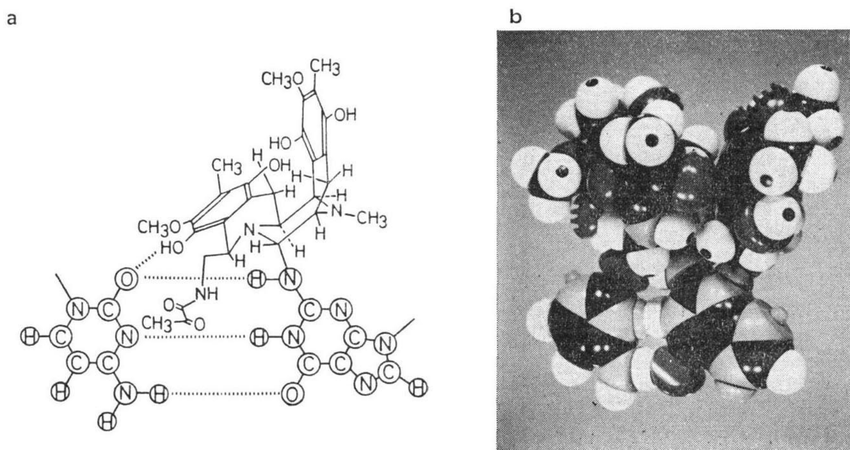
agents[8]. However, addition of reducing agents enhanced its binding activity up to that of saframycin A in the reduced form[8]. These observations indicated that the hydroquinone form plays an additional role in enhancing the binding activity. The fact that saframycin A binds covalently to duplex DNA and G : C containing complementary polydeoxyribonucleotides but not to poly dI : dC strongly suggested that the 2-amino group of guanine should directly be involved in the adduct formation[8].

Based on these informations and the determined structure of saframycin A, we have proposed the structure for the saframycin A-DNA adduct in which the active form of saframycin A is linked at C-21 to N-2 of guanine, and forms hydrogen bonding between the 8-hydroxyl group of the hydroquinone ring and the 2-keto group of cytosine as the secondary stabilizing force (unpublished data, in preparation by ARAI, T.; K. KISHI, Y. MIKAMI, M. NAMIKOSHI, S. IWASAKI & S. OKUDA). The Corey-Pauling-Koltun (CPK) space filling model for the saframycin A-DNA adduct demonstrated that the activated form of saframycin A molecule was able to fit the minor groove of duplex DNA. Furthermore, NMR studies of the saframycin A-DNA adduct supported this model (unpublished data, in preparation by KISHI, K.; K. YAZAWA, K. TAKAHASHI, Y. MIKAMI, T. ARAI, M. NAMIKOSHI, S. IWASAKI & S. OKUDA). Fig. 4 shows the outline of the model.

According to this model for the saframycin A-DNA adduct, saframycins which lack the α-cyanoamine group or the α-carbinolamine group, such as saframycins B, C, D and AR_3, should exhibit no cytotoxicity, because these groups should be essential for the activity. Modification at the C-14 position on the basic skeleton of saframycin A with bulky substituents should interfere with the binding of the antibiotic to DNA and result in a decrease in cytotoxic activity, because the C-14 position has to be brought into contact with the minor groove of duplex DNA in the model for the saframycin A-DNA adduct. Similarly, modification at the C-25 position on the side chain of saframycin A with bulky substituents should also result in a decrease in cytotoxic activity, because the side chain carbonyl group should fit along the minor groove of duplex DNA in the model.

The results of the cytotoxicity assays for saframycins corroborated these predictions except that saframycins which lack the α-cyanoamine group or the α-carbinolamine group exhibited much lower

Fig. 4. Proposed schematic structure (a) and CPK space filling model (b) of the saframycin A-DNA adduct. The hydrogen atoms of saframycin A in the CPK model were labeled with black spots. The skeletal conformation of saframycin A was assumed to be identical with the crystal conformation of saframycin C.



but detectable cytotoxic activities. However, this lower level of cytotoxicity might be explained by the minor mechanism of action for saframycins such as the DNA single-strand scission described by LOWN *et al.*[11]. Therefore, the structure-activity relationships for the 13 saframycins studied in this paper support the proposed major mechanism of action for the antitumor activity of saframycin A and our proposed model for the saframycin A-DNA adduct.

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