ISOLATION AND STRUCTURAL ELUCIDATION OF NEW SAFRAMYCINS Y3, Yd-1, Yd-2, Ad-1, Y2b AND Y2b-d

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A new series of saframycins Y3, Yd-1, Yd-2, Ad-1, Y2b and Y2b-d was produced by directed biosynthesis employing resting cells of saframycin producer, *Streptomyces lavendulae* No. 314. Their structures were determined by comparison with the spectral data of UV, IR, and ^{1}H and ^{13}C NMR of saframycin A, whose structure has already been established. Saframycin Y3 is 25-deoxy-25-aminosaframycin A, while saframycin Yd-1 is 26-homosaframycin Y3, and saframycin Y2b is a dimer of saframycin Y3. Saframycin Ad-1 is 26-homosaframycin A. Saframycin Yd-2 is 26-demethylsaframycin Y3, and saframycin Y2b-d is a dimer of saframycin Yd-1.

Nine saframycin components have been isolated from the culture filtrates of *Streptomyces lavendulae* No. 314 and their structures have been determined[1]. In order to prepare new saframycin derivatives with different antitumor spectra, chemical and biological modification of saframycin A (SA) (Fig. 1a), which is the most active saframycin component hitherto reported, was attempted, but without much success (unpublished data).

Recently a method for preparing novel saframycins with an amino functional group in their side chain was developed based on studies of SA biosynthesis[2,3].

In this paper, isolation of new saframycins and their structural elucidation are described.

Biological activity of these newly prepared saframycins will be reported in a separate publication[4].

Isolation of New Saframycins

New saframycins were prepared by the directed biosynthetic method described previously[3]. Briefly, in addition to methionine and tyrosine, saframycins Y3 and Y2b were produced by supplementation of alanine and glycine or alanylglycine to saframycin-producing *S. lavendulae* resting cells. Supplementation of tyrosine, methionine, 2-amino-*n*-butyric acid and glycine or 2-amino-*n*-butyrylglycine produced saframycins Yd-1, Ad-1 and Y2b-d. Saframycin Yd-2 was produced by the addition of tyrosine, methionine and glycylglycine. All these new saframycins were extracted with dichloromethane from the reaction mixture of a resting cell system[3], and were removed to an acidic solution. The acidic solution containing the saframycins was adjusted to pH 9.0 and extracted with ethyl acetate. This fraction, designated as crude basic fraction, was purified by silica gel column chromatography followed by preparative thin-layer chromatography. Only saframycin Ad-1 was pre-

Table 1. ^{13}C NMR spectral data of newly prepared saframycins in comparison with that of saframycin A (SA).

Carbon No.	SA	Y3	Yd-1	Ad-1	Y2b	Y2b-d
15	186.59 (s)	186.4 (s)	186.4 (s)	186.6 (s)	186.3 (s), 187.2 (s)	186.2 (s), 187.2 (s)
5	185.28 (s)	185.7 (s)	185.5 (s)	185.3 (s)	185.6 (s), 185.8 (s)	185.6 (s), 185.8 (s)
18	182.44 (s)	182.5 (s)	182.5 (s)	182.5 (s)	182.4 (s), 182.4 (s)	181.9 (s), 182.4 (s)
8	180.81 (s)	180.7 (s)	180.8 (s)	180.8 (s)	180.4 (s), 181.9 (s)	180.5 (s), 181.8 (s)
7	155.90 (s)	156.7 (s)	156.6 (s)	156.0 (s)	156.6 (s), 157.0 (s)	156.8 (s), 156.9 (s)
17	155.56 (s)	155.3 (s)	155.3 (s)	155.7 (s)	155.3 (s), 155.4 (s)	155.3 (s), 155.6 (s)
20	141.55 (s)	141.7 (s)	141.7 (s)	141.5 (s)	141.2 (s), 141.8 (s)	141.2 (s), 141.5 (s)
10	141.22 (s)	139.7 (s)	139.7 (s)	141.2 (s)	139.1 (s), 139.3 (s)	139.2 (s), 139.7 (s)
9	135.59 (s)	136.4 (s)	136.5 (s)	135.6 (s)	138.4 (s)	138.3 (s), 138.8 (s)
19	135.53 (s)	135.9 (s)	135.9 (s)		136.8 (s), 136.8 (s)	135.6 (s), 136.8 (s)
16	129.21 (s)	128.4 (s)	128.3 (s)	129.3 (s)	128.5 (s), 129.4 (s)	128.2 (s), 129.4 (s)
6	128.30 (s)	127.0 (s)	127.2 (s)	128.4 (s)	126.2 (s)	126.5 (s), 128.0 (s)
CN	116.67 (s)	116.8 (s)	116.8 (s)	116.6 (s)	117.1 (s), 117.3 (s)	117.1 (s), 117.3 (s)
7,17-OCH_3	61.10 (q)	61.1 (q)	61.0 (q)	61.1 (q)	60.8 (q), 61.1 (q)	61.0 (q), 61.1 (q)
	60.96 (q)	61.0 (q)		60.9 (q)	60.7 (q), 60.7 (q)	61.1 (q),
6,16-CH_3	8.72 (q)	8.6 (q)	8.7 (q)	8.7 (q)	9.0 (q),	8.9 (q), 9.2 (q)
		8.5 (q)	8.5 (q)		8.4 (q), 8.6 (q)	8.4 (q), 8.6 (q)
NCH_3	41.58 (q)	41.7 (q)	41.6 (q)	41.6 (q)	41.6 (q), 42.9 (q)	41.7 (q), 42.7 (q)
21	58.22 (d)	58.2 (d)	58.1 (d)	58.2 (d)	58.5 (d), 58.7 (d)	58.3 (d)
1	56.25 (d)	56.8 (d)	56.8 (d)	56.2 (d)	56.4 (d), 57.7 (d)	56.4 (d), 57.7 (d)
13	54.46 (d)	54.5 (d)	54.5 (d)	54.5 (d)	54.9 (d), 55.8 (d)	54.6 (d), 55.8 (d)
11	54.18 (d)	54.3 (d)	54.2 (d)	54.2 (d)	54.4 (d), 54.6 (d)	54.2 (d), 54.5 (d)
3	53.93 (d)	53.6 (d)	53.6 (d)	53.9 (d)	53.9 (d), 54.2 (d)	53.7 (d), 53.9 (d)
4	24.28 (t)	25.4 (t)	25.4 (t)	25.0 (t)	25.5 (t), 25.6 (t)	25.5 (t), 25.5 (t)
14	21.48 (t)	21.6 (t)	21.5 (t)	21.6 (t)	21.6 (t)	21.6 (t)
14	—	—	—	—	—	62.2 (d)
22	40.62 (t)	39.9 (t)	40.5 (t)	40.5 (t)	39.6 (t)	39.7 (t), 42.9 (t)
NHCO	160.17 (s)	174.5 (s)	174.7 (s)	160.1 (s)	174.1 (s), 175.8 (s)	173.7 (s), 175.1 (s)
COCO	196.68 (s)	—	—	199.4 (s)	— —	— —
NH_2CH	—	50.1 (d)	56.0 (d)	—	49.8 (d), 50.1 (d)	49.9 (d), 50.2 (d)
CH_2	—	—	27.9 (t)	30.2 (t)	— —	27.5 (t), 27.9 (t)
CH_3	24.28 (q)	21.3 (q)	10.3 (q)	6.9 (q)	20.5 (q), 21.2 (q)	10.4 (q), 10.7 (q)

Chemical shift: in ppm downfield from internal TMS standard, measured in $CDCl_3$.
Multiplicity under off-resonance condition; s=singlet, d=doublet, t=triplet, q=quartet.

pared from neutral fraction. Further purification of basic and neutral components and the procedure for the isolation of each new saframycin is described in following experimental section.

Structure of Saframycins

Saframycin Y3 (Y3)

Y3 is a basic, amorphous yellow powder which decomposes at 143~146°C. The UV and IR spectra are similar to those of SA[5], indicating that the structure of Y3 resembles, but somewhat differs from, that of SA.

A major ion peak of m/z 563 (M^+) in a field desorption (FD) mass spectrum of Y3 was consistent with the molecular formula $C_{29}H_{33}N_5O_7$, which agreed well with the elemental analysis. The molecular formula indicated that Y3 has one oxygen atom less than SA, while it has three hydrogen and one nitrogen atoms more than SA. These data also indicate the presence of an amino group in the structure of Y3. The fact that the signal at 1.62 ppm (br s) is exchangeable with D_2O and that quan-

Table 2. ^{1}H NMR spectral data of newly prepared saframycins in comparison with that of saframycin A (SA).

Proton No.	SA[6,7]		Y3		Yd-1		Ad-1		Yd-2	
	δ	J	δ	J	δ	J	δ	J	δ	J
H-1	6.70	9.5 (1–3) 4.0 (1–2)	7.23	10.0 (1–3) 4.0 (1–2)	7.25	9.2 (1–3) 3.9 (1–2)	6.73	8.5 (1–3) 2.8 (1–2)	7.23	8.6 (1–3) 3.4 (1–2)
H-2	3.26	14.0 (2–3) 4.0 (2–1) 4.0 (2–4)	3.00	14.5 (2–3) 4.0 (2–1) 4.0 (2–4)	3.06	13.1 (2–3) 3.9 (2–1) 3.9 (2–4)	3.23	14.9 (2–3) 2.8 (2–1) 2.8 (2–4)	3.10	15.8 (2–3) 3.4 (2–1) 3.4 (2–4)
H-3	3.84	14.0 (3–2) 9.5 (3–1) 1.5 (3–4)	3.83	14.5 (3–2) 10.0 (3–1) 2.0 (3–4)	3.83	13.1 (3–2) 9.2 (3–1) 2.3 (3–4)	3.76	14.9 (3–2) 8.5 (3–1)	3.82	15.8 (3–2) 8.6 (3–1) 2.4 (3–4)
H-4	3.98	4.0 (4–2) 3.0 (4–5) 1.5 (4–3)	3.94 (br s)		3.91 (br s)		3.99	2.8 (4–3)	3.93 (br s)	
H-5	1.28	17.0 (5–6) 11.0 (5–7) 3.0 (5–4)	1.37	17.0 (5–6) 14.0 (5–7) 3.0 (5–4)	1.35	17.3 (5–6) 12.0 (5–7) 3.9 (5–4)	1.25 (m)		1.38	16.4 (5–6) 12.1 (5–7) 2.5 (5–4)
H-6	2.87	17.0 (6–5) 3.0 (6–7)	2.88	17.0 (6–5) 4.0 (6–7)	2.86	17.3 (6–5) 3.1 (6–7)	2.88	16.3 (6–5) 2.0 (6–7)	2.89	16.4 (6–5) 3.6 (6–7)
H-7	3.14	11.0 (7–5) 3.0 (7–6) 3.0 (7–8)	3.15	14.0 (7–5) 4.0 (7–6) 4.0 (7–8)	3.14	12.0 (7–5) 3.1 (7–6) 3.1 (7–8)	3.15	11.5 (7–5) 2.0 (7–6) 2.0 (7–8)	3.14	12.1 (7–5) 3.6 (7–6)
H-8	4.06	3.0 (8–7) 2.5 (8–11)	4.07 (br s)		4.05～4.03*		4.07	2.0 (8–7)	4.05 (br s)	
H-9	2.30	20.5 (9–10) <0.5 (9–11)	2.27	20.0 (9–10)	2.32	20.0 (9–10)	2.23	19.5 (9–10)	2.31	21.9 (9–10)
H-10	2.83	20.5 (10–9) 4.5 (10–11)	2.82	20.0 (10–9) 8.0 (10–11)	2.81	20.0 (10–9) 7.3 (10–11)	2.76	19.5 (10–9) 6.8 (10–11)	2.82	21.9 (10–9) 7.3 (10–11)
H-11	3.44	7.5 (11–10) 2.5 (11–12) <0.5 (11–9)	3.45	8.0 (11–10) 3.0 (11–12)	3.45	7.3 (11–10) 2.6 (11–12)	3.44	6.8 (11–10) 2.0 (11–12)	3.44	7.3 (11–10) 3.6 (11–12)
H-12	3.99	2.5 (12–11)	4.00	3.0 (12–11)	4.01	2.6 (12–11)	3.99	2.0 (12–11)	4.00	3.6 (12–11)
OCH_3		—	4.06 (s) 4.03 (s)		4.03 (s) 4.05 (s)		4.03 (s)×2		4.03 (s)×2	
NCH_3		—	2.33 (s)		2.32 (s)		2.31 (s)		2.32 (s)	
CH_3		—	1.88 (s) 1.92 (s)		1.89 (s) 1.93 (s)		1.91 (s) 1.99 (s)		1.90 (s) 1.93 (s)	
NH_2		—	1.62 (br s)		1.45 (br s)		—		2.05 (br s)	
$CH(NH_2)$		—	3.27 (q) 8.0		3.05 (t) 8.5		—		2.97, 3.11 (d) 17.0	
CH_2		—	—		1.06 (m), 1.48 (m)		2.69 (q) 7.1		—	
CH_3	2.25 (s)		0.92 (d) 8.0		0.74 (t) 7.7		0.94 (t) 7.1		—	

Chemical shift: in ppm downfield from internal TMS standard, measured in $CDCl_3$ J: coupling constant in Hz. Multiplicity under off-resonance condition; s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br s=broad signal.

* Overlapping with OCH_3 signals.

Fig. 1. Structures of saframycins SA, Y3, Yd-1, Ad-1 and Yd-2.

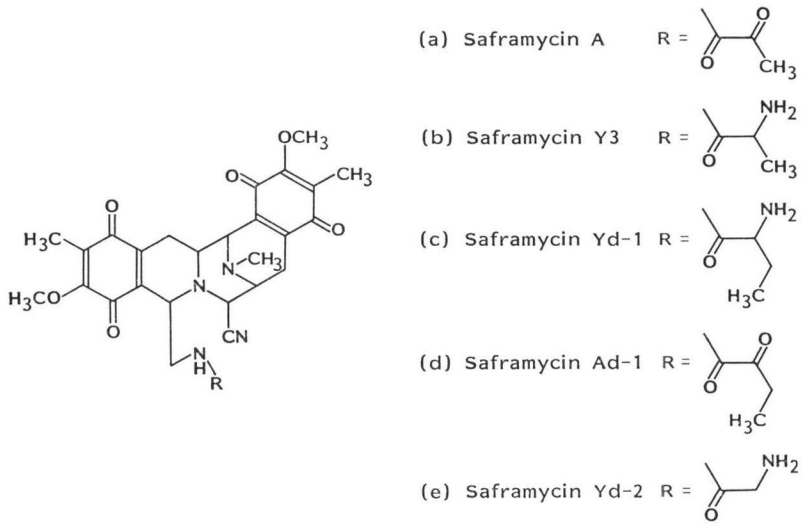


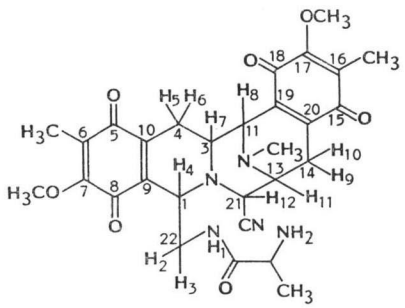
Table 3. Comparison of ^{13}C NMR spectral data of the side chain of *N*-acetylsafracin A (**I**), *N*-acetylsaframycin Y3 (**II**) and saframycin Y3 (**III**).

$$-\overset{1}{C}H_2NH\overset{2}{C}O\overset{3}{C}H\overset{4}{C}H_3$$
$$\qquad\qquad\quad |$$
$$\qquad\quad NH\overset{5}{C}O\overset{6}{C}H_3$$

	I[8]	II	III
1	41.2	40.3 (t)*	39.9 (t)*
2	172.5	171.6 (s)	174.5 (s)
3	58.0	48.5 (d)	50.1 (d)
4	19.1	21.2 (q)	21.3 (q)
5	168.9	169.3 (s)	—
6	23.0	17.3 (q)	—

* Multiplicity under off-resonance condition; s=singlet, d=doublet, t=triplet, q=quartet.

Fig. 2. NMR assignments of saframycin Y3.
The numbering system shown here is arbitrary and serves only as an aid in NMR spectral identification.



titative production of monoacetate (*m/z* 605 (M^+); 1.80 ppm (3H, s), 169.3 ppm (s), 17.3 ppm (q)) is done by acetylation with sodium acetate in acetic anhydride indicates that the amino group is a primary amine.

The ^{13}C NMR spectrum of Y3 is compared with that of SA† in Table 1. The basic carbon skeleton of Y3 is clearly shown to be identical with that of SA. The signal displaying characteristic of pyruvoylamide α-carbonyl carbon (196.68 ppm) of SA is not observed in Y3. The signals of amide and methyl carbon are shifted from 160.17 ppm to 174.5 ppm and from 24.28 ppm to 21.3 ppm, respectively. Furthermore, a new signal at 50.1 ppm (d) is observed in Y3. The ^1H NMR spectral data are also compared with those of SA[6,7] as shown in Table 2. The signal of carbonylmethyl at 2.25 ppm (3H, s), which is observed in SA, disappeared in Y3, however, two new signals were observed, at 3.27 ppm (1H, q, J=8.0 Hz) and 0.92 ppm (3H, d, J=8.0 Hz). The two signals were ascribed to

† ARAI, T.; K. KISHI, Y. MIKAMI, M. NAMIKOSHI, S. IWASAKI & S. OKUDA: in preparation.

the structure of the 2-aminopropionyl group based on their chemical shifts. Presence of this side chain structure was further confirmed by comparison with ^{13}C NMR spectral data of safracin A, which is produced by *Pseudomonas* sp. and has been reported to have a 2-aminopropionyl group in the side chain[8] (Table 3).

Finally the structure of Y3 was determined as shown in Fig. 1b and Fig. 2. Y3 has the same stereochemical configuration as SA, because the latter was produced from Y3 enzymatically by treatment with the homogenate of saframycin producer[2].

Saframycin Yd-1 (Yd-1)

Yd-1 is a basic, amorphous yellow powder which decomposes at 124~127°C. The UV and IR spectra closely resemble those of Y3. Yd-1 gave a major ion peak at *m/z* 577 (M^+) in an FD mass spectrum. The molecular formula was determined as $C_{30}H_{35}N_5O_7$ based on elemental analysis. This formula shows that Yd-1 possesses one carbon and two hydrogen atoms more than Y3. A comparison of the ^{13}C and ^1H NMR spectra of Yd-1 with those of Y3 shows that their basic carbon skeletons are identical (Tables 1 and 2). But in the ^{13}C NMR spectrum of Yd-1, the methyl signal at 21.3 ppm (q) was shifted to high-field at 10.3 ppm (q) and a new methylene signal appeared at 27.9 ppm (t). In addition, in the ^1H NMR spectrum of Yd-1, new signals at 1.06 and 1.48 ppm (2H, m) due to methylene proton and 0.74 ppm (3H, t, J=7.7 Hz) due to methyl proton were observed. The proton signal due to the carbon attached to the amino group was also observed at 3.05 ppm as a triplet (8.5 Hz).

These results clearly indicate that an aminopropyl group exists as a partial structure of Yd-1 and that the C-1 side chain of Yd-1 is the 2-aminobutyloylamidemethylene group. The structure of Yd-1 is shown in Fig. 1c.

Saframycin Ad-1 (Ad-1)

Ad-1 is a weakly basic, amorphous yellow powder which melts at 124~128°C. The UV and IR spectra closely resemble to those of SA, suggesting its structural similarity to SA. A major ion peak in an electron impact (EI) mass spectrum (*m/z* 576, M^+) suggests that its molecular formula is $C_{30}H_{32}N_4O_8$. This formula shows that Ad-1 has one carbon and two hydrogen atoms more than SA. The ^{13}C NMR spectrum of Ad-1 is compared with that of SA in Table 1. These data suggest that the basic carbon skeleton of Ad-1 is identical with that of SA. A new signal appeared in Ad-1 at 30.2 ppm (t) and the methyl signal of SA (24.28 ppm, q) was shifted to high-field (6.9 ppm, q). In the ^1H NMR spectrum of Ad-1, a triplet methyl signal was observed at 0.94 ppm (3H t, J=7.1 Hz), but the pyruvoylmethyl signal observed in SA at 2.25 ppm disappeared (Table 2). EI mass fragment pattern of Ad-1 was identical with that of SA above for a major ion peak. These observations suggest that the pyruvoylamidemethylene group of SA is changed to the 2-oxobutylylamidemethylene group in Ad-1.

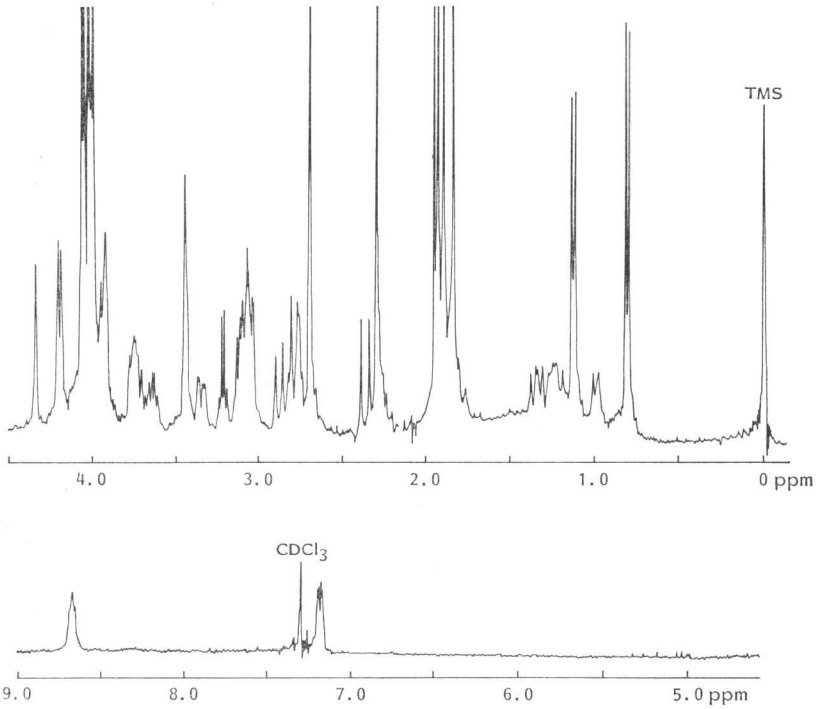
Therefore the structure of Ad-1 was determined as shown in Fig. 1d.

Saframycin Yd-2 (Yd-2)

Yd-2 is a basic amorphous dark yellow powder which melts and decomposes at 144~148°C. The UV and IR spectra closely resemble to those of Y3 and Yd-1, suggesting its structural similarity to these of saframycins.

The major ion peak in an FD mass spectrum (*m/z* 549, M^+) indicates that the molecular formula of Yd-2 is $C_{28}H_{31}N_5O_7$. This formula is one carbon and two hydrogen atoms less than that of Y3.

Fig. 3. ^{1}H NMR spectrum of saframycin Y2b.

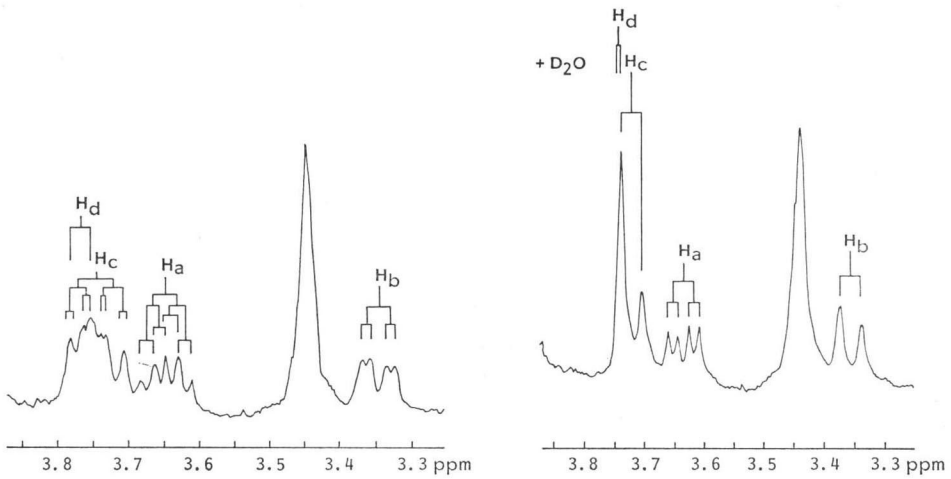


The ^{1}H NMR spectral data of Yd-2 is compared with that of Y3 in Table 2. New signals appeared at 2.97 ppm (1H, J=17 Hz) and 3.11 ppm (1H, J=17 Hz) as AB doublets in Yd-2, and the signals at 3.27 ppm (1H, q, J=8.0 Hz) and 0.92 ppm (3H, d, J=8.0 Hz) due to the aminoethyl group of Y3 disappeared. Other signals of Yd-2 are consistent with those of Y3. These data suggest that the C-1 side chain of Yd-2 is of the aminoacetamidomethylene group, and that the other part of Yd-2 is the same as the structure of Y3. On the basis of this data, the structure of Yd-2 was determined and is shown in Fig. 1e.

Saframycin Y2b (Y2b)

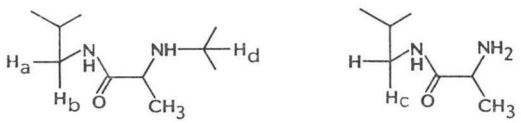
Y2b is a basic amorphous orange yellow powder which decomposes at above 200°C and does not show a clear melting point. The UV and IR spectra are closely related to those of Y3. The major ion peak is not seen clearly in an FD mass spectrum, but in a fast atom bombardment (FAB) mass spectrum a major ion peak of m/z 1,133 ($MH^+ + 8$) and in a secondary ion mass spectrum (SI-MS) one of m/z 1,127 ($MH^+ + 2$) were observed. In an FAB mass spectrum of saframycins, a major peak of ($MH^+ + 2$) or ($MH^+ + 4$) was often observed, due to the fact that one or two quinone rings of saframycins are reduced to hydroquinone depending on the experimental conditions used[9]. Those data indicate that the molecular weight of Y2b is 1,124. The molecular formula of Y2b is determined as $C_{58}H_{64}N_{10}O_{14}$ based on the elemental analysis. This formula indicates that Y2b corresponds to the dimer of Y3 being a dehydrogenation product of two molecules of Y3. In addition, in the ^{1}H NMR spectrum of Y2b, 4 singlet signals (4.09, 4.08, 4.06 and 4.00 ppm) caused by *O*-methyl groups, 4 singlet signals (1.95, 1.93, 1.90 and 1.84 ppm) from methyl groups which are attached to the quinone ring, and 2 singlet signals (2.70 and 2.30 ppm) from *N*-methyl group are observed. Signals

Fig. 4. ^{1}H NMR spectrum of saframycin Y2b and in D_2O.



resulting from the methyl group at 0.80 ppm (3H, d, J=7.0 Hz) and 1.13 ppm (3H, d, J=7.0 Hz) are also observed and show chemical shifts and coupling constants similar to those of the methyl group of Y3. The number of methyl groups of Y2b is twice that of Y3, and therefore Y2b was considered a dimer of Y3.

Fig. 5. Partial structure of saframycin Y2b.



The ^{13}C NMR spectral data of Y2b is compared with those of Y3 in Table 1. The numbers of most signals of Y2b are twice those of Y3 except for the single signals assigned to C-6, 9, 14, 22 and the C-6 or -16 methyl group. Therefore, it was suggested that the binding of the two molecules is at one of the above exceptional positions. Because the UV spectrum of Y2b is the same as that of Y3 and all methyl groups are observed in the ^{1}H NMR spectrum of Y2b, the binding at C-6, C-9 and C-6 or C-16 methyl group might be altered. The disappearance of these singlet signals was thus ascribed to the overlapping. Therefore, a binding position at C-14 or C-22 was suggested.

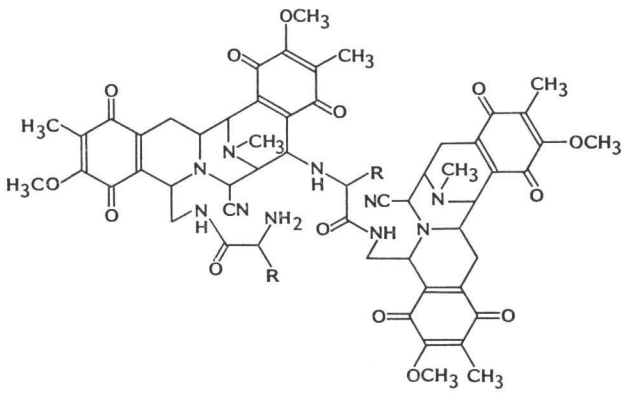
^{1}H NMR spectrum of Y2b is shown in Fig. 3. When D_2O was added, the signals at 8.68, 7.19 and 1.00 ppm disappeared and the signal pattern at the 3.3~3.8 ppm region was simultaneously changed (Fig. 4). Irradiation of the amide proton at 8.68 ppm caused two signals at 3.65 ppm (H_a, J=14.3, 7.0, 7.0 Hz) and 3.35 ppm (H_b, J=14.3, 4.8, 1.0 Hz) to change from triple doublets to double doublets (J=14.3, 7.0 Hz), (J=14.3, 1.0 Hz), respectively. And irradiation of another amide proton at 7.19 ppm caused one signal at 3.74 ppm (H_c, J=17.2, 9.7, 2.0 Hz) to change from a triple doublet to a double doublet (J=17.2, 2.0 Hz). In addition, irradiation at 3.05 ppm caused the H_c signal to change from a triple doublet to a double doublet (J=9.7, 2.0 Hz). Therefore these three signals (H_a, H_b, H_c) are assigned to amide-methylene protons. Irradiation of an amine proton at 1.00 ppm led to the collapse of one doublet signal at 3.77 ppm (H_d, d, J=12.0 Hz) to a singlet-like signal (J<2.0 Hz). The binding at C-22 can be altered due to the above data and the partial structure of the Y2b molecule is shown in Fig. 5. Determination of other signals of Y2b in the ^{1}H NMR spectrum (400 MHz) after the irradiation experiment was also attempted. The results of assignment of the signals are shown in Table 4. Low-field shifts of the signal of H-9 (C-14, axial) to 3.77 ppm seems reasonable

Table 4. Chemical shift and coupling constant of ^1H NMR spectrum of saframycin Y2b (400 MHz).

Proton No.	Chemical shift (ppm) (coupling constant Hz)
H-1 (amide proton)	7.19 (J_{1-3}=9.7, J_{1-2}=3.5), 8.68 (triplet like)
H-2 (amide-methylene)	3.05~3.09, 3.35 (J_{2-3}=14.3, J_{2-1}=4.8, J_{2-4}<1.0)
H-3 (amide-methylene)	3.74 (J_{3-2}=17.2, J_{3-1}=9.7, J_{3-4}=2.0), 3.65 (J_{3-2}=14.3, J_{3-1}=7.0, J_{3-4}=7.0)
H-4 (C-1 ax proton)	3.94 (br s), 3.96 (br s)
H-5 (C-4 ax proton)	1.35 (J_{5-6}=17.2, J_{5-7}=11.6, J_{5-4}=2.1), 1.25~1.30
H-6 (C-4 ex proton)	2.88 (J_{6-5}=17.2, J_{6-7}=3.3), 2.79 (J_{6-5}=16.8, J_{6-7}=3.3)
H-7 (C-3 ax proton)	3.05 (J_{7-5}=11.6, J_{7-6}=3.3, J_{7-8}=3.3), 3.09 (J_{7-5}=12.0, J_{7-6}=3.3, J_{7-8}=2.7)
H-8 (C-11 eq proton)	3.93 (br s), 4.20 (J_{8-7}=2.7)
H-9 (C-14 ax proton)	2.37 (J_{9-10}=20.8, J_{9-11}<2.0), 3.77 (J_{9-11}<2.0, J_{9-NH}=12.0)
H-10 (C-14 eq proton)	2.79 (J_{10-9}=20.8, J_{10-11}=8.0)
H-11 (C-13 eq proton)	3.45 (J_{11-10}=8.0, J_{11-12}=2.0), 3.45 (br s)
H-12 (C-21 eq proton)	4.22 (J_{12-11}=2.0), 4.36 (br s)
NH	1.00 (J_{NH-9}=12.0)
Methyl group	
$=CCH_3$	1.84 (s), 1.90 (s), 1.93 (s), 1.95 (s)
NCH_3	2.30 (s), 2.70 (s)
$=COCH_3$	4.00 (s), 4.06 (s), 4.08 (s), 4.09 (s)
Alanyl group	0.80 (J_{CH_3-CH}=7.0), 1.13 (J_{CH_3-CH}=6.8)
	3.22 (J_{CH-CH_3}=7.0×3), 3.13 (J_{CH-CH_3}=6.8×3)

Chemical shift: in ppm downfield from internal TMS standard, measured in $CDCl_3$.

Fig. 6. Structures of saframycins Y2b and Y2b-d.



(a) Saframycin Y2b R = CH_3

(b) Saframycin Y2b-d R = CH_2CH_3

due to the anisotropy of the amino group.

Finally, it was determined that Y2b possesses the structure given in Fig. 6a. The stereochemistry of the amino group (Y3) at C-14 is equatorial because it shows the same coupling constant (H10-H11, J<2.0 Hz) as that of saframycin C which has an equatorial OCH_3 group at C-14[10].

Saframycin Y2b-d (Y2b-d)

Y2b-d is a basic amorphous orange yellow powder which decomposes at above 200°C and does not show a clear melting point. The UV and IR spectra resemble to those of Y2b, suggesting its structural similarity to Y2b.

The major ion peak in an FAB mass spectrum shows m/z 1,161 ($MH^+ + 8$) and in an SI-MS, 1,155 ($MH^+ + 2$). These data, together with the data of elemental analysis, indicate that the molecular weight of Y2b-d is 1,152 and its molecular formula is $C_{60}H_{68}N_{10}O_{14}$. In the ^1H NMR spectrum of Y2b-d, existence of 4 singlet signals of OCH_3 groups (4.08, 4.07, 4.06 and 4.03 ppm), 4 singlet signals of methyl groups (2.02, 1.94, 1.86 and 1.80 ppm) and two singlet signals of N-CH_3 groups (2.74 and 2.29 ppm) suggest that Y2b-d is a dimer of saframycin, indicating a similarity to Y2b. But Y2b-d is somewhat different from Y2b in the followings; signals due to the methyl group in Y2b-d at 0.83 and 0.73 ppm are observed as triplet (J=7.2, 7.0 Hz), showing a similarity to that of Yd-1 (0.74 ppm, t, 7.7 Hz), while the signals in Y2b are doublet and similar to that of Y3. These observations indicate that Y2b-d is a dimer of Yd-1.

^{13}C NMR spectral data of Y2b-d are compared with those of Yd-1 in Table 2. Signals observed in Y2b-d are twice those of Yd-1, except for the signals of C-14, C-21 and OCH_3. A new signal appeared at 62.2 ppm. The chemical shift value of this signal was assumed to be a methylene carbon attached to a hetero atom. It is reasonable that the signal of C-14 carbon is shifted to low-field due to the effect of the attached amino group as was observed in Y2b.

Assignments of signals in ^{13}C NMR data of Y2b-d have not yet been completed due to the complex overlapping. The characteristic signal observed was 3.71 ppm (d, 10.7 Hz) and addition of D_2O lead to the collapse of the double signal to a singlet, which is seen in the spectrum of Y2b. These observations suggested that Y2b-d is a dimer of Yd-1, while Y2b is a dimer of Y3. The structure of Y2b-d is shown in Fig. 6b.

Experimental

General

IR spectra in $CHCl_3$ solution were recorded using an IRA-2 spectrometer (Nihonbunko, Japan), and UV spectra were determined using a model 323 spectrophotometer (Hitachi, Japan), FD mass spectrum using a JMS-O1SG-2 (Jeol, Japan), and EI mass spectrum using an LKB 9000 (Shimadzu, Japan) [under a direct probe insert at 70 eV]. ^1H NMR spectra (270 MHz) and ^{13}C NMR spectra were determined with a Jeol-GX270 (Jeol, Japan) and ^1H NMR spectrum (400 MHz) was determined with a JNM-GX400 (Jeol, Japan). Respective chemical shifts (in ppm) are recorded relative to internal TMS. Optical rotations were taken with a DIP-4 polarimeter (Nihonbunko, Japan). Melting points were established using an MP-J2 (Yanagimoto, Japan) and are uncorrected. Thin-layer chromatography (TLC) by precoated silica gel of 0.5 mm thickness (Merck, Germany) was used. Silica gel of 70~230 mesh (Merck, Germany) was also used for column chromatography.

Isolation of Y3 and Y2b

New saframycins were prepared by the method described by ARAI *et al.*[3] using resting-cells from a 100 liters fermentor. The filtrate was extracted twice with dichloromethane, and the extracts were concentrated *in vacuo* to a small volume. The orange crude extract was solved in 500 ml of EtOAc, and the EtOAc solution was extracted twice with 250 ml of 10% AcOH. The organic solvent layer was reextracted twice with 150 ml of 1 N HCl. The organic layer was washed with 1 N Na_2CO_3 and water, and was evaporated *in vacuo*. Neutral orange oily fraction was obtained. The 10% AcOH layer was adjusted to pH 9.0 using ammonia water and was extracted with 250 ml of EtOAc three times. The extract was washed with water and dried. Two grams of yellow amorphous basic fraction (Base-1) were obtained. The 1 N HCl solution was processed with the same method of 10% AcOH solution. One gram of yellow amorphous basic fraction (Base-2) was obtained from the HCl solution.

Base-1 fraction was chromatographed on a column of 60 g silica gel with benzene - EtOAc (1 : 4) and EtOAc as the eluents. The fraction containing Y2b and Y3 was eluted with benzene - EtOAc

(1: 4), and Y3 was eluted with EtOAc. Each fraction was purified by preparative TLC (silica gel) with $CHCl_3$ - EtOH (10: 1 or 20: 1) as the eluent. Thirty-two mg of Y2b and 920 mg of Y3 were obtained.

Base-2 fraction was chromatographed on a column of 30 g silica gel with benzene - EtOAc (1: 1 and 1: 4) and EtOAc as the eluents. Firstly, the fraction containing a small amount of SA was eluted by benzene - EtOAc (1: 1) and, secondly the fraction containing Y2b and Y3 was eluted by benzene - EtOAc (1: 4). The final fraction containing Y3 was eluted by EtOAc. Each fraction was further purified by preparative TLC (silica gel) with $CHCl_3$ - EtOH (10: 1) or benzene - EtOAc (1: 2) as the eluent. Finally, 61 mg of Y2b and 121 mg of Y3 were obtained.

Isolation of Yd-1, Ad-1 and Y2b-d

Similar isolation procedures with Y3 and Y2b were carried out using the reaction mixture from directed biosynthesis. Neutral (3.5 g), Base-1 (2.6 g) and Base-2 (0.34 g) fractions were obtained. Base-1 fraction was poured onto a column of 75 g silica gel and developed with benzene - EtOAc (1: 2 and 1: 3) and EtOAc as the eluent. The fraction containing a small amount of Y2b-d and Y2b was eluted with benzene - EtOAc (1: 2) and that of Yd-1 and Y3 by benzene - EtOAc (1: 3). Base-2 fraction was chromatographed on a column of 10 g silica gel with benzene - EtOAc (1: 3) and EtOAc as the eluents. The first fraction which was eluted by benzene - EtOAc (1: 3) contained Y2b-d and the second fraction which was eluted by EtOAc contained Yd-1. Each fraction was further purified by preparative TLC with $CHCl_3$ - EtOH (10: 1 or 20: 1) as the eluent. The neutral fraction was chromatographed on a column of 30 g silica gel with benzene - EtOAc (4: 1) as the eluent. A fraction containing a small amount of Ad-1 was obtained. This fraction was further purified by preparative TLC with benzene - EtOAc (1: 2) as the eluent.

The yields of saframycins were as follows; from Base-1 fraction, 654 mg of Yd-1, from Base-2 fraction, 60 mg of Y2b-d, and from neutral fraction, 9 mg of Ad-1.

Isolation of Yd-2

Yd-2 was isolated from the resting-cell system by the same method as described in the section on isolation of Y3. Base-1 (1.8 g) and Base-2 (1.4 g) were obtained from a resting-cell system of 100 liters fermentation.

Base-1 fraction was chromatographed on a column of 60 g silica gel with benzene - EtOAc (1: 4), EtOAc and 2% MeOH - EtOAc as the eluents. The fraction containing Yd-2 was eluated with 2% MeOH - EtOAc. The Yd-2 fraction was further purified by preparative TLC (silica gel) with $CHCl_3$ - EtOH (10: 1) as the eluent. Forty-six mg of Yd-2 were obtained. From Base-2 fraction, 24 mg of Yd-2 was obtained by the same processing method as Base-1.

Physico-chemical Properties of Saframycins

Y3: Basic amorphous yellow powder; mp 143~146°C (dec); $[\alpha]_D^{22}$ −46.1 (*c* 0.7, MeOH); elemental analysis, found C 61.65, H 5.99, N 11.91, calcd for $C_{29}H_{33}N_5O_7$: C 61.80, H 5.90, N 12.43; UV λ_{max}^{MeOH} nm (log ε) 268 (4.27), 3.40 (sh); IR ν^{CHCl_3} cm^{-1} 3380, 1655, 1615, 1515; ^1H NMR see Table 2; ^{13}C NMR see Table 1; FD-MS *m/z* 563 (M^+); Rf values (silica gel TLC) 0.35 ($CHCl_3$ - EtOH, 10: 1); positive color reaction is Dragendorf and ninhydrin.

Yd-1: Basic amorphous yellow powder; mp 124~127°C (dec); $[\alpha]_D^{22}$ −43.5 (*c* 1.0, MeOH); elemental analysis, found C 60.78, H 5.83, N 11.78, calcd for $C_{30}H_{35}N_5O_7 \cdot H_2O$: C 60.49, H 6.26, N 11.76; UV λ_{max}^{MeOH} nm (log ε) 269 (4.26); IR ν^{CHCl_3} cm^{-1} 3380, 1660, 1615, 1515; ^1H NMR see Table 2; ^{13}C NMR see Table 1; FD-MS *m/z* 577 (M^+); Rf values (silica gel TLC) 0.41 ($CHCl_3$ - EtOH, 10: 1); positive color reaction is same as Y3.

Yd-2: Basic amorphous dark yellow powder; mp 144~148°C (dec); elemental analysis, found C 60.35, H 5.70, N 11.98, calcd for $C_{28}H_{31}N_5O_7 \cdot \frac{1}{2}H_2O$: C 60.20, H 5.77, N 12.53; UV λ_{max}^{MeOH} nm (log ε) 268 (4.23); IR ν^{CHCl_3} cm^{-1} 3380, 1660, 1615, 1515; ^1H NMR see Table 1; FD-MS *m/z* 549 (M^+); positive color reaction is same as Y3.

Ad-1: Amorphous yellow powder; mp 124~128°C; elemental analysis, found C 62.59, H 5.89, N 9.64, calcd for $C_{30}H_{32}N_4O_8$: C 62.49, H 5.59, N 9.72; UV λ_{max}^{MeOH} nm (log ε) 268 (4.30); IR ν^{CHCl_3} cm^{-1} 3400, 1710, 1685, 1660, 1615; ^1H NMR see Table 2; ^{13}C NMR see Table 1; EI-MS *m/z* 578 ($M^+ + 2$),

576 (M^+), 464 ($M^+ +2-114$), 462 ($M^+ -114$), 243, 220, 218; Rf values (silica gel TLC) 0.53 (benzene - EtOAc, 1: 1); positive color reaction is Dragendorf.

Y2b: Basic amorphous orange yellow powder; mp >200°C (dec); $[\alpha]_D^{22}$ −42.2 (*c* 0.1, MeOH); elemental analysis, found C 61.05, H 5.72, N 11.82, calcd for $C_{58}H_{64}N_{10}O_{14}\cdot H_2O$: C 60.39, H 5.82, N 12.25; UV λ_{max}^{MeOH} nm (log ε) 268 (4.66); IR ν^{CHCl_3} cm^{-1} 3360, 1655, 1615, 1450; ^1H NMR see Table 2 and Fig. 3; ^{13}C NMR see Table 1; FAB-MS *m/z* 1,133 ($MH^+ +8$); SI-MS *m/z* 1,127 ($MH^+ +2$); Rf values (silica gel TLC), 0.56 ($CHCl_3$ - EtOH, 10: 1); positive color reaction is same as Y3.

Y2b-d: Basic amorphous orange yellow powder; $[\alpha]_D^{22}$ −41.9 (*c* 0.1, MeOH); elemental analysis, found C 61.43, H 5.62, N 11.52, calcd for $C_{60}H_{68}N_{10}O_{14}\cdot H_2O$: C 61.52, H 6.02, N 11.96; UV λ_{max}^{MeOH} nm (log ε) 269 (4.64); IR ν^{CHCl_3} cm^{-1} 3360, 1655, 1615, 1450; ^1H NMR ($CDCl_3$) δ 8.51 (1H, t), 7.24 (1H, q), 4.21 (1H, s), 4.18 (1H, s), 4.17 (1H, s), 4.08 (3H, s), 4.03 (3H, s), 3.95 (1H, d), 3.92 (1H, s), 3.72 (1H, dd), 3.71 (1H, d), 3.57 (1H, m), 3.48~3.41 (3H, m), 3.12~3.04 (3H, m), 3.00 (1H, dd), 2.80 (1H, s), 2.74 (3H, s), 2.70 (1H, s), 2.29 (3H, s), 2.28 (1H, d), 2.20 (3H, s), 1.94 (3H, s), 1.86 (3H, s), 1.29 (1H, m), 1.18 (1H, m), 0.83 (3H, t), 0.37 (3H, t); ^{13}C NMR see Table 1; FAB-MS *m/z* 1,161 ($MH^+ +8$), SI-MS *m/z* 1,155 ($MH^+ +2$); Rf values (silica gel TLC), 0.63 ($CHCl_3$ - EtOH, 10: 1); positive color reaction is same as Y3.

Acetylation of Y3

Y3 (280 mg, 0.5 mM) was dissolved in 40 ml of acetic anhydride and then 100 mg of sodium acetate was added to the solution. After stirring at room temp for 3 hours, the reaction mixture was evaporated *in vacuo*. The residue was dissolved in 100 ml of EtOAc and was washed with 0.5 N Na_2CO_3 and water. The organic layer was dried up. The yield was 320 mg. The residue was subjected to column chromatography on 10 g silica gel using an eluent system consisting of benzene and EtOAc. The main fraction containing acetylsaframycin Y3 was collected. The product was purified by preparative TLC using benzene - EtOAc (1: 2) as a solvent system. The yield of acetylsaframycin Y3 was 245 mg (81.7%).

Physico-chemical Properties of Acetyl-Y3: Amorphous yellow powder; mp 131~135°C (dec); UV λ_{max}^{MeOH} nm (log ε) 264 (4.26), 340 (sh); IR ν^{CHCl_3} cm^{-1} 3380, 1660, 1615, 1515; ^1H NMR ($CDCl_3$) δ 6.15 (1H, dd), 5.95 (1H, d), 4.16 (1H, d), 4.05 (3H, s), 4.02 (3H, s), 3.90 (1H, d), 3.65 (1H, ddd), 3.42 (1H, dd), 3.26 (1H, ddd), 3.05 (1H, ddd), 2.79 (1H, dd), 2.77 (1H, dd), 2.34 (1H, d), 2.30 (3H, s), 1.93 (3H, s), 1.90 (3H, s), 1.80 (3H, s), 1.62 (1H, ddd), 1.10 (3H, d); ^{13}C NMR δ 8.6, 17.3, 21.2, 22.6, 25.1, 40.3, 41.4, 48.5, 54.0, 54.0, 54.3, 56.8, 58.2, 116.1, 127.2, 127.5, 134.9, 135.4, 141.0, 141.2, 155.1, 155.5, 169.3, 171.6, 180.4, 182.0, 184.9, 186.3; FD-MS *m/z* 605 (M^+).

Acknowledgments

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