Isolation of Novel Saquayamycins as Inhibitors of Farnesyl-protein Transferase

RYUICHI SEKIZAWA[†], HIRONOBU IINUMA[††], HIROSHI NAGANAWA[††], MASA HAMADA[††], TOMIO TAKEUCHI[††], JIRO YAMAIZUMI[†††] and KAZUO UMEZAWA[†,*]

[†]Department of Applied Chemistry, Faculty of Science and Technology, Keio University 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223, Japan
[††]Institute of Microbial Chemistry 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan
[†††]National Cancer Center Research Institute 5-1-1 Tsukiji, Chuo-ku, Tokyo 104, Japan

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All *ras* proteins require membrane localization for their functions through the C-terminal farnesyl group[1], and farnesyl-protein transferase (FPTase) inhibitors would thus be expected to suppress the *ras* functions selectively by inhibiting their posttranslational modifications[2~4]. Therefore, we screened microbial culture broths for FPTase inhibitors, and isolated two novel compounds belonging to the saquayamycin group, and named them saquayamycins E and F, as they are closely related to saquayamycins A and C.

For the production of saquayamycin E and F, growth of the *Actinomycetes* strain MK290-AF1 on an agar slant were inoculated into 500-ml Erlenmeyer flasks containing 110 ml of medium consisted of glycerol 2.5%, meat extract 0.5%, peptone 0.5%, yeast extract 1.0%, NaCl 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.05%, and $CaCO_3$ 0.32%, pH 7.4. The fermentation was carried out for four days at 27°C on a rotary shaker (180 rpm). After filtration, 4.0 liters of filtered broth was extracted with the same volume of BuOAc, and the extract was concentrated to dryness to give 826 mg of the crude material. The active compound was applied to a silica gel column and eluted with $CHCl_3$. Further purification of the active compound (65 mg) was carried out by HPLC using a Capcell Pak C_{18} column (20 × 250 mm) with 50% CH_3CN to give 4.9 mg of saquayamycin E and 3.8 mg of saquayamycin F. Though some minor related compounds were contained in the broth, they were not identified.

Saquayamycins E and F were obtained as orange

Fig. 1. Structures of saquayamycins and aquayamycin.

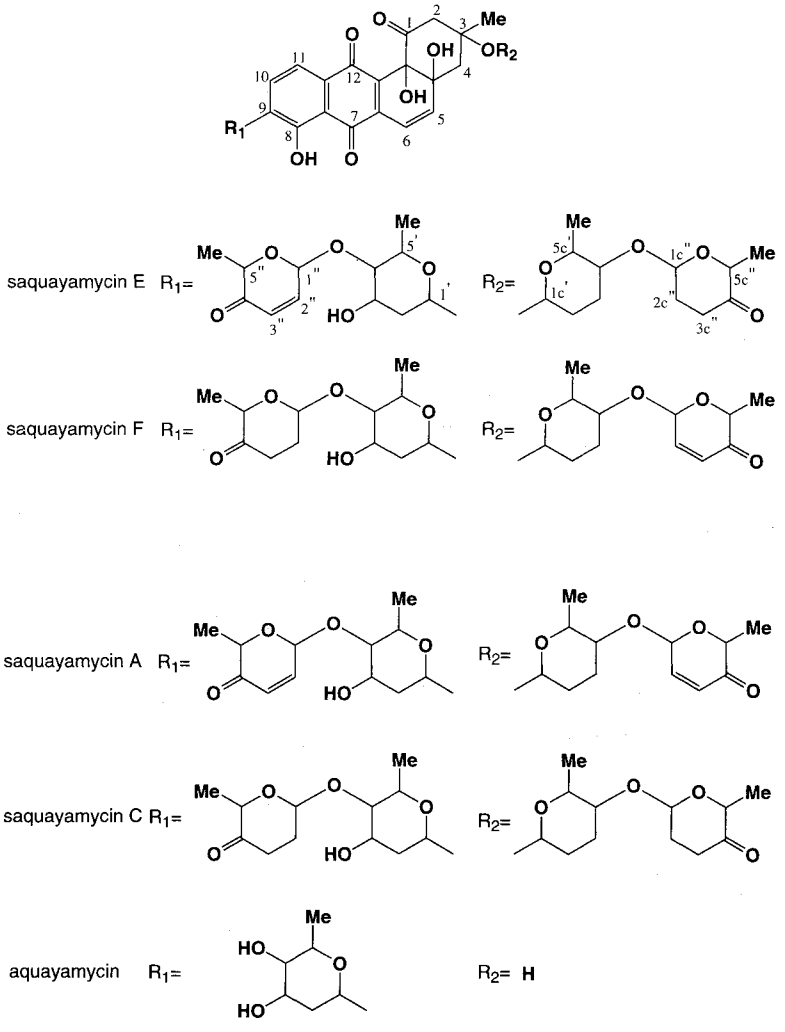

Table 1. Physico-chemical properties of saquayamycins E and F.

	Saquayamycin E	Saquayamycin F
Appearance	Orange powder	Orange powder
Melting point	150~153°C (decomp.)	148~152°C (decomp.)
Optical rotation	$[\alpha]_D^{23}$ −11.5° (*c* 0.2 $CHCl_3$)	$[\alpha]_D^{25}$ −120° (*c* 0.2 $CHCl_3$)
Molecular formula	$C_{43}H_{50}O_{16}$	$C_{43}H_{50}O_{16}$
FAB-MS (*m/z*)	823 $(M+H)^+$	823 $(M+H)^+$
HRFAB-MS (*m/z*)		
Calcd	845.2997	845.2997
Found	845.2999 $(M+Na)^+$	845.3019 $(M+Na)^+$
UV $\lambda_{max}^{MeOH-HCl}$ nm (log ε)	218 (4.45), 318 (3.66), 425 (3.76)	218 (4.49), 318 (3.66), 425 (3.76)
IR ν_{max} (cm^{-1}) KBr	1728, 1699, 1639	1730, 1700, 1639
Retention time[a] (minute)	10.5	11.7

[a] Capcell pak UG120, 4.6 mm i.d. × 150 mm (Shiseido), CH_3CN-H_2O (20:80).

powders, and were soluble in MeOH, $CHCl_3$, and EtOAc, but insoluble in water or in *n*-hexane. The physico-chemical properties of saquayamycin E and F are listed in Table 1. From The UV spectrum of these compounds, it was suggested that they have the same chromophore with saquayamycins or aquayamycin. The molecular formula of saquayamycin E was determined to be $C_{43}H_{50}O_{16}$ from HRMS and ^{13}C NMR data, and that of saquayamycin F was the same with that of saquayamycin E. The ^1H and the ^{13}C NMR data of saquayamycins E and F are shown in Table 2.

The ^1H and ^{13}C NMR spectra indicated that the structures of these compounds are closely related to those of saquayamycins A and C (Fig. 1), reported by UCHIDA *et al.*[5]. The structures of R_1 and R_2 residues of saquayamycins E and F are elucidated as shown in Fig. 1 by comparison of chemical shifts in the ^1H and the ^{13}C NMR spectra with those of saquayamycins A and C.

Compared with those of saquayamycin A, the chemical shifts of C-2c″ (28.4 ppm) and C-3c″ (33.6 ppm) carbons in saquayamycin E indicated that they are aliphatic rather than olefinic carbons. These structures were supported by the HMBC and ^1H-^1H COSY spectra. Since long-range couplings between 1′-H (4.89 ppm) and C-8 (158.1 ppm) and between 10-H (7.88 ppm) and C-1′ (71.1 ppm) were observed in the HMBC spectrum, the R_1 residue of saquayamycin E is suggested to be attached to the 9-position. And since long-range coupling between C-3 (82.5 ppm) and 1c′-H (5.27 ppm) was detected in the HMBC experiment and from the chemical shifts of C-3 (82.5 ppm), C-1c′ (92.6 ppm), and 1c′-H (5.27 ppm), the R_2 residue of saquayamycin E is suggested to be attached to the 3-position through a glycosidic linkage. Thus we have elucidated the structure of saquayamycin E as shown in Fig. 1.

The HMBC data of saquayamycin F indicated ^1H-^{13}C long-range couplings between 1′-H (4.89 ppm) and C-8 (158.1 ppm) and between 10-H (7.88 ppm) and C-1′ (71.1 ppm) as in saquayamycin E. Moreover, the long-range coupling between C-3 (82.5 ppm) and 1c′-H (5.26 ppm) indicated the R_2 residue was linked to the 3-position through a glycosidic linkage. Compared with those of saquayamycin A, the chemical shifts of C-2″ (28.1 ppm) and C-3″ (33.4 ppm) indicated that they are aliphatic rather than olefinic carbons. Moreover, the hydrogenation of saquayamycin F was carried out in EtOAc-MeOH (1:1) with 6% $Pd/BaSO_4$ at room temperature for 15 minutes, and the product was purified by silica gel TLC with $CHCl_3$-EtOAc-AcOH (10:10:0.1). Then, the ^1H NMR data of this compound was completely identical with saquayamycin C[5]. Thus, the structure of saquayamycin F was elucidated and is also shown in Fig. 1.

FPTase activity was assayed with partially purified FPTase from bovine brain and purified human c-Ha-*ras* protein prepared in a bacterial expression system. For preparation of the enzyme, bovine brain (50 g) was homogenized at 4°C in 50 ml of a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonylfluoride (PMSF), and 0.1 mM leupeptin, and the homogenate was ultracentrifuged at 100,000 × *g* for 60 minutes. Then, the supernatant was added with ammonium sulfate to 30% saturation. The supernatant was further added with ammunium sulfate to 50% saturation, then the precipitate was collected. The active fraction was dissolved in 20 ml of dialyzing buffer (20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 20 μM $ZnCl_2$). The protein solution was dialyzed against 4 liters of the same buffer for 4 hours and then against another 4 liters for 15 hours. Human recombinant c-Ha-*ras* protein was produced in *E. coli* and purified as described before[6,7].

The FPTase reaction was carried out as follows: For the FPTase reaction, the mixture containing 2 μl of enzyme preparation (17 mg protein/ml), 15 μM c-Ha-*ras* protein, 0.2 μCi farnesylpyrophosphate (22.5 Ci/mmol, Du Pont/NEN Research Products), and 3 μl of test sample solution in 30 μl of reaction buffer (50 mM Tris-HCl (pH 7.5), 100 μM $ZnCl_2$, 10 mM $MgCl_2$, 40 mM KCl and 1 mM PMSF) was incubated for 1 hour at 37°C. To stop the reaction, a 20-μl amount of the reaction mixture was withdrawn and added to 50 μl of 15% TCA

Table 2. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR data of saquayamycins E and F in $CDCl_3$.

Position	Saquayamycin E		Saquayamycin F	
	δ_C	δ_H	δ_C	δ_H
1	204.8	—	204.9	—
2	50.2	2.51 d, 3.18 dd	50.2	2.51 d, 3.18 dd
3	82.5	—	82.5	—
3-CH_3	25.5	1.41 s	25.5	1.41 s
4	44.6	1.84 d, 2.28 dd	44.6	1.84 d, 2.28 dd
4a	80.0	—	80.0	—
4a-OH	—	4.35 s	—	4.32 s
5	145.6	6.45 d	145.5	6.45 d
6	117.5	6.92 d	117.5	6.92 d
6a	138.8	—	138.8	—
7	188.2	—	188.2	—
7a	114.0	—	114.0	—
8	158.1	—	158.1	—
8-OH	—	12.29 s	—	12.30 s
9	138.2	—	138.4	—
10	133.6	7.88 d	133.6	7.88 d
11	119.7	7.62 d	119.7	7.62 d
11a	130.5	—	130.5	—
12	182.2	—	182.3	—
12a	138.9	—	138.8	—
12b	77.5	—	77.5	—
12b-OH	—	4.59 s	—	4.59 s
1′	71.1	4.89 dd	71.1	4.89 dd
2′	38.9	1.39~1.45 m, 2.57 ddd	38.8	1.35~1.45 m, 2.55 ddd
3′	71.3	3.91 m	71.2	3.86 m
3′-OH	—	4.26 d	—	4.52 d
4′	89.4	3.21 br dd	88.7	3.15 dd
5′	74.4	3.58 dq	74.5	3.57 dq
6′	18.4	1.39 d	18.3	1.38 d
1″	95.2	5.38 d	99.7	5.18 t
2″	142.1	6.84 dd	28.1	2.10 m, 2.40~2.51 m
3″	127.3	6.15 d	33.4	2.40~2.57 m
4″	195.2	—	209.3	—
5″	71.6	4.76 q	72.0	4.50 q
6″	15.2	1.45 d	14.8	1.36 d
1c′	92.6	5.27 br d	92.5	5.26 br d
2c′	24.8	2.04 m, 1.49 m	24.7	2.03 m, 1.49 m
3c′	24.7	1.88 m	24.6	1.87~1.93 m
4c′	74.7	3.66 br s	76.2	3.69 br s
5c′	67.2	4.22 dq	67.0	4.25 dq
6c′	17.2	1.28 d	17.2	1.29 d
1c″	99.1	5.09 t	95.3	5.27 d
2c″	28.4	2.10 m, 2.36 m	143.1	6.89 dd
3c″	33.6	2.47 dt	127.3	6.10 d
4c″	211.0	—	196.8	—
5c″	71.1	4.29 q	70.7	4.54 q
6c″	14.9	1.27 d	15.2	1.37 d

Chemical shifts were determined in ppm based on TMS as an internal standard.

containing 2% SDS in a 96-well microplate, and the plate was incubated for 1 hour on ice. Then, the insoluble fraction was trapped on a filter paper and washed with 6% TCA in a cell harvester. The filter was dried and counted for the radioactivity by a Matrix 9600 beta counter (Packard). In this assay system, the tetrapeptide CVIM inhibited the reaction with an IC_{50} of 1.0 μM.

Fig. 2. Inhibition of FPTase by saquayamycins E and F.

Partially purified bovine brain FPTase was incubated with c-Ha-*ras* protein and [^{3}H]-farnesylpyrophosphate in the presence of saquayamycin E (○) or saquayamycin F (●) for 60 minutes. Values are means of triplicate determinations.

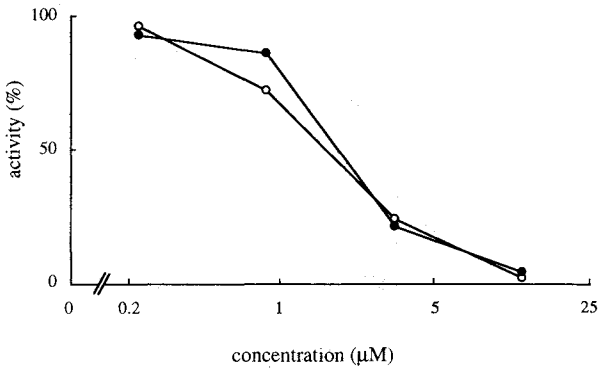

Table 3. Inhibition of farnesyl-protein transferase by saquayamycins.

Compounds	IC_{50} (μM)
Saquayamycin E	1.8
Saquayamycin F	2.0
Saquayamycin A	1.8
Saquayamycin B	1.8
Saquayamycin C	1.4
Saquayamycin D	1.5
Aquayamycin	1.3
Adriamycin	>60
CVIM	1.0

As shown in Fig. 2, saquayamycins E and F inhibited the FPTase from bovine brain with IC_{50} values of 1.8 and 2.0 μM, respectively. Several related compounds such as saquayamycins A, B, C, D or aquayamycin[8] also inhibited the enzyme, whereas adriamycin had no inhibitory activity, as shown in Table 3. Kinetic analysis with Lineweaver-Burk plotting suggested that the saquayamycins non-competitively inhibit the enzyme with respect to *ras* proteins.

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