A-94964, Novel Inhibitor of Bacterial Translocase I, Produced by *Streptomyces* sp. SANK 60404

II. Physico-chemical Properties and Structure Elucidation

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Abstract In our screening for translocase I inhibitors, we found the novel nucleoside antibiotic, A-94964 in the culture broth of *Streptomyces* sp. SANK 60404. The structure of A-94964 was elucidated primarily by various NMR studies, including a ^1H - ^{31}P HMBC experiment. A-94964 has a unique structure which possesses a nucleoside moiety and an *N*-acylglucosamine moiety connected via a phosphate.

Keywords A-94964, translocase I, physico-chemical properties, structure, nucleoside antibiotics, NMR, phosphate

Introduction

Bacterial translocase I is known as one of the prime targets for anti-microbial chemotherapy. To date, a number of compounds have been reported to be translocase I inhibitors [1], including mureidomycins [2], pacidamycin [3], napsamycin [4], liposidomycins [5], tunicamycin [6], capuramycins [7–9], muraymycins [10] and caprazamycins [11, 12]. Though none of these are in clinical use, bacterial translocase I is still an attractive target for discovering

novel antibiotics. Translocase I is the first enzyme of the bacterial lipid cycle and it is essential for the survival of both Gram-positive and negative bacteria. A novel antibiotic that has a unique mechanism of action without cross-resistance with known antibiotics is currently in demand due to the recent marked increase of multi-drug-resistant bacteria. Based on the background described above, we screened for bacterial translocase I inhibitors in microbial culture extracts and discovered a novel nucleoside antibiotic with a phosphoric ester, A-94964 (Fig. 1) was isolated from the culture broth of *Streptomyces* sp. SANK 60404. In our preceding paper [13], we described the taxonomy of the producing strain and the fermentation, isolation and biological activity of A-94964. Here we report the structural elucidation of A-94964.

Materials and Methods

NMR spectra were recorded at 298 K on an AVANCE 500 spectrometer (Bruker) operating at 500 and 125 MHz. ^{31}P -NMR spectra were obtained on a DMX 600 spectrometer (Bruker) at 243 MHz. The samples for NMR characterization were dissolved in dimethylsulfoxide- d_6 .

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Fig. 1 Structure of A-94964.

The optical rotation and IR spectra were measured with a DIP-370 (JASCO) and FT-IR 8300 (Shimadzu), respectively. The high-resolution FAB-MS spectra were recorded on a JMS-700QQ mass spectrometer (JEOL). The UV spectra were obtained on a UV-265FW spectrometer (Shimadzu).

Results

Physico-chemical Properties

The physico-chemical properties of A-94964 are summarized in Table 1. This compound was obtained as white powder. The molecular formula of A-94964 was determined by high-resolution FAB-MS analysis to be $C_{45}H_{73}N_4O_{23}P$. IR absorptions of A-94964 implied the presence of a phosphate group (1233 cm^{-1}). The UV spectrum showed a maximum at 267 nm in MeOH. This compound is soluble in organic solvents such as EtOAc, MeOH, MeCN, dimethylsulfoxide and Me_2CO .

Structure Elucidation

The ^1H - and ^{13}C -NMR assignments of A-94964 in DMSO- d_6 are listed in Table 2. The ^1H -NMR spectrum of A-94964 contained 60 proton resonances. A pair of signals at δ_{H} 5.62 (1H, d, $J=8.0\text{ Hz}$) and δ_{H} 7.54 (1H, d, $J=8.0\text{ Hz}$) suggested the presence of a uracil moiety, the same as the known translocase inhibitors mentioned above. The exchangeable proton of δ_{H} 8.16 was expected to be an amide NH proton. In the ^{13}C -NMR, 45 carbon signals were classified into 4 methyl groups, 12 methylenes, 23 methines (sp^2 and sp^3), a quaternary olefin and 5 carbonyl carbons were observed by an analysis of the DEPT spectra. The degree of unsaturation calculated from the molecular formula was 13. Five of them were assigned to the carbonyl groups, one was assigned to phosphate group and three were assigned to double bonds. So the remainders were considered to be due to four ring systems. The structure elucidation of A-94964 was established through a ^1H - ^1H

Table 1 Physico-chemical properties of A-94964

Appearance	White powder
Molecular formula	$C_{45}H_{73}N_4O_{23}P$
HRFAB-MS (m/z) Found	1091.4328 (M+Na) $^+$
Calcd	1091.4301
UV λ_{max} nm in MeOH (ϵ)	267 nm (26400)
IR ν_{max} cm^{-1} (KBr)	3328, 2925, 1686, 1613, 1259, 1233, 1053, 963
$[\alpha]_{\text{D}}^{25}$ (50% MeOH in water)	+60.0 $^\circ$ (c 0.5)

COSY, HSQC, HSQC-HOHAHA and HMBC experiment. The results of the NMR studies of each partial structure (Fig. 2) are described as follows.

Partial Structure A: The presence of uracil was clarified by a pair of characteristically coupled ^1H -NMR signals, δ_{H} 5.62 and δ_{H} 7.54 ($J=8.0\text{ Hz}$) in DQF-COSY spectrum, followed by an analysis of the HMBC correlations between H-6 (δ_{H} 7.54) and C-2 (δ_{C} 150.7) and C-4 (δ_{C} 162.0), between H-5 (δ_{H} 5.62) and C-4.

Next, a set of carbon chain fragments, $\text{C}_1'\text{-C}_2'\text{-C}_3'\text{-C}_4'\text{-C}_5'\text{-C}_6'\text{-C}_7'$ was revealed by DQF-COSY and HSQC analysis. Two oxymethine carbons C-1' (δ_{C} 87.7) and C-4' (δ_{C} 81.8) were determined to be connected via an oxygen atom based on an HMBC correlation between H-1' (δ_{H} 5.70) and C-4'. The linkage between positions 1 and 1' was established by an HMBC correlation: H-6/C-1' and H-1'/C-2 and C-6. The carbonyl carbon C-8' (δ_{C} 172.3) was assigned next to oxymethine carbon C-7' (δ_{C} 77.0) by an HMBC correlation between H-7' (δ_{H} 4.94) and C-8'.

The oxymethine carbon linkage, $\text{C}_1''\text{-C}_2''\text{-C}_3''\text{-C}_4''\text{-C}_5''$, was assigned to a pyranose ring system by the analysis of the DQF-COSY and HSQC spectra. Finally, the pyranose was disclosed by HMBC correlations between H-5'' (δ_{H} 3.89) and oxymethine carbon C-6'' (δ_{C} 62.3) and between anomeric proton H-1'' (δ_{H} 4.12) and C-5'' (δ_{C} 73.5). Connectivity to the nucleoside moiety was revealed by long-range couplings between H-6' and anomeric carbon

Table 2 NMR data of A-94964 in DMSO- d_6

No.	δ_C^a	δ_H^b
2	150.7 (s)	
4	163.0 (s)	
5	102.1 (d)	5.62 (1H, d, $J=8.0$ Hz)
6	140.6 (d)	7.54 (1H, d, $J=8.0$ Hz)
1'	87.7 (d)	5.70 (1H, d, $J=6.0$ Hz)
2'	72.6 (d)	3.99 (1H, dd, $J=5.0, 6.0$ Hz)
3'	72.8 (d)	3.8 (1H, m)
4'	81.8 (d)	3.81 (1H, m)
5'	34.5 (t)	1.68 (1H, m)
		1.86 (1H, m)
6'	78.9 (d, br)	4.13 (1H, m)
7'	77.0 (d, br)	4.98 (1H, m)
8'	172.3 (s)	
1''	102.4 (d)	4.72 (1H, br, s)
2''	70.6 (d)	3.57 (1H, m)
3''	70.8 (d)	3.45 (1H, m)
4''	68.0 (d)	3.23 (1H, m)
5''	73.5 (d)	3.89 (1H, m)
6''	62.3 (t)	3.20 (1H, m)
		3.74 (1H, m)
1'''	94.4 (d)	5.36 (1H, dd, $J=2.7, 6.4$ Hz)
2'''	54.0 (d, $J_{C-P}=6.1$ Hz)	3.67 (1H, dd, $J=9.0, 9.0$ Hz)
3'''	70.5 (d)	3.51 (1H, m)
4'''	69.9 (d)	3.29 (1H, m)
5'''	73.3 (d)	3.56 (1H, m)
6'''	60.2 (t)	3.5 (2H, m)
1a	169.1 (s)	
2a	42.0 (t)	3.8 (1H, m)
		3.9 (1H, m)
1b	165.6 (s)	
2b	119.6 (d)	6.04 (1H, d, $J=15.4$ Hz)
3b	144.0 (d)	7.01 (1H, d, $J=15.4$ Hz)
4b	131.0 (s)	
5b	145.4 (s)	5.59 (1H, d, $J=10.0$ Hz)
6b	32.4 (d)	2.48 (1H, m)
7b	36.7 (t)	1.3 (2H, m)
8b	27.0 (t)	1.0~1.3 (2H, m)*
9b	28.9 (t)**	1.0~1.3 (2H, m)*
10b	29.0 (t)**	1.0~1.3 (2H, m)*
11b	29.4 (t)**	1.0~1.3 (2H, m)*
12b	26.5 (t)	1.0~1.3 (2H, m)*
13b	36.0 (t)	1.0~1.3 (2H, m)*
14b	33.8 (d)	1.0~1.3 (1H, m)*
15b	29.1 (t)	1.0~1.3 (2H, m)*
16b	11.3 (q)	0.81 (3H, t, $J=7.0$ Hz)
17b	12.4 (q)	1.71 (3H, s,)
18b	20.5 (q)	0.93 (1H, d, $J=6.7$ Hz)
19b	19.1 (q)	0.80 (3H, d, $J=6.4$ Hz)

^a 125 MHz, ^b 500 MHz, * signal overlapping, ** indistinguishable.

C-1'' (δ_C 102.4), H-1'' and C-6' (δ_C 78.9), and thus partial structure A was established.

Partial structure B: By the analysis of DQF-COSY and HSQC-HOHAHA, the carbon linkage C₁'''-C₂'''-C₃'''-C₄'''-C₅'''-C₆''' was determined. The oxymethine carbon C-5''' (δ_C 73.3) turned out to be connected with anomeric carbon C-1''' (δ_C 94.4) via oxygen by the observation of an HMBC correlation from H-1''' (δ_H 5.36) to C-5''' (δ_C 73.3). The chemical shift value of C-2''' (δ_C 54.0) showed an upfield shift comparable to those of other oxymethine carbons, suggesting that C-2''' bears a nitrogen atom to form amino sugar.

Next, the structure of 4,6,14-trimethyl-2,4-hexadecenoyl moiety was established as follows. The two olefinic protons H-2b and H-3b showed ¹H-¹³C long-range couplings to carbonyl carbon C-1b and olefinic carbon C-4b in the HMBC spectrum. The olefinic proton H-5b and methyl proton H-17b showed ¹H-¹³C long-range couplings to C-3b, and additional HMBC correlations from H-17b to C-4b and C-5b were observed. These observations indicated a γ -methyl- $\alpha\beta\gamma\delta$ -unsaturated ketone moiety. The geometries of both Δ^{2b} and Δ^{4b} were determined to be *E* by the observed proton coupling constants ($J_{H_{2b}-3b}=15.4$ Hz) and NOE correlations between H-6b and H-17b and between H-3b and H-5b, respectively. Although the fatty acid chain of C-5b-C-6b (C-18b)-C-7b-C-8b could be assigned by analysis of DQF-COSY, the remaining part of the chain from C-8b to C-12b could not be determined by the couplings due to a severe overlap. However, the chain was estimated to be linear because of the equivalence of these protons. The tail structure of fatty acid was elucidated to be *anteiso* by the observed coupling patterns of H-16b and H-19b, and the following HMBC and HSQC-HOHAHA analyses. The ¹H-¹³C long-range couplings from two methyl protons, H-19b (δ_H 0.80) and H-16b (δ_H 0.81), to methylene carbon C-15b (δ_C 29.1) and methine carbon C-14b (δ_C 33.8) were observed in the HMBC spectrum. In the HSQC-HOHAHA spectrum, correlations from both methyl proton signals to the δ_C 36.0 methylene carbon signal were observed and only H-19b showed a correlation with the δ_C 26.5 methylene carbon. As a result of these observations, the methylene carbon signals at δ_C 36.0 and δ_C 26.5 were assigned to C-13b and C-12b, respectively.

The chemical shift value of the carbonyl carbon signal (C-1b, δ_C 165.6) of this fatty acid moiety suggested that it formed an amide bond and this connection was confirmed by the ¹H-¹³C long-range coupling between H-2a and C-1b in the HMBC spectrum. Subsequently, this methylene carbon C-2a turned out to be connected to C-2''' of the amino sugar moiety via an amide bond by ¹H-¹³C long-range couplings from H-2a to the carbonyl carbon (C-1a,

Fig. 2 Partial structures of A-94964.

δ_C 169.1) and from H-2''' to C-1a. Thus, partial structure B was elucidated as shown in Fig. 2.

The existence of a phosphate group was indicated by the molecular formula. To confirm the carbon substitution with the phosphate, a ^1H - ^{31}P HMBC experiment was performed. ^1H - ^{31}P long-range couplings were observed from each of the H-7' and H-1''' protons. Furthermore, a two-bond ^{13}C - ^{31}P coupling was also observed with the anomeric carbon of the amino sugar (C-1''') with a J value of 6.1 Hz in the ^{13}C -NMR spectrum. By these observations, it was proved that partial structures A and B were connected through the phosphate group. Consequently, the whole structure of A-94964 was elucidated as shown in Fig. 1.

Discussion

In the course of screening for bacterial translocase I inhibitors, we discovered the novel nucleoside antibiotic A-94964 from the culture broth of the strain identified as *Streptomyces* sp. SANK 60404. The structure of A-94964 elucidated by 2D-NMR and mass spectrometry was found to have a unique structure with a phosphoric acid diester.

Among other phosphate-containing antibiotics, the moenomycin group [14, 15] was considered related because of its diester moiety. Similarly as A-94964, the moenomycin group of antibiotics also inhibits bacterial peptidoglycan biosynthesis. However, the target molecule of moenomycin is penicillin binding protein (PBP1b) which is distinct from the target of A-94964. Surprisingly, during the process of A-94964 isolation, we found that

Streptomyces sp. SANK 60404 also produced antibiotics of the moenomycin group. In addition, although the structure of A-94964 is not similar to that of moenomycin, their components are similar in that they have unsaturated fatty acid, phosphoric acid diester and amino sugar. Based on the above observations, it is possible that the biosynthesis of the A-94964 shares common pathways with the biosynthesis of the moenomycin group of antibiotics.

Interestingly, A-94964 has a uronic acid moiety consisting of eight carbons, in contrast to moieties of five carbons in natural uridine, six carbons in capuramycins and seven carbons in liposidomycins and caprazamycins. To the best of our knowledge, the only other example for nucleoside compound having such long chain uronic acid is sinefungin [16], but it consists of ten carbons. No such structure has been reported so far among natural products. The nucleoside structure of A-94964 is expected to be biosynthesized by condensing uridine and 2,3-dihydroxy propanoic acid, as in the case of sinefungin [17].

As a result of its interesting structural profile of A-94964, studies on the biosynthesis of A-94964 may be of assistance in the creation of novel antibiotics with unique structures.

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