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Clethramycin, a New Inhibitor of Pollen Tube Growth with Antifungal Activity from *Streptomyces hygroscopicus* TP-A0623

II. Physico-chemical Properties and Structure Determination

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Clethramycin is a novel linear polyene polyketide produced by a plant-associated actinomycete *Streptomyces hygroscopicus* TP-A0623. It possesses a guanidino and carboxyl residue at each end and an *O*-sulfate group and a hexaene moiety in the molecule, and its molecular formula is $C_{63}H_{99}N_3O_{18}S$. The structure was determined by analyzing 2D-NMR and FAB-MS data, using ^{13}C-enriched compounds. Clethramycin is structurally similar to linearmycin, an inhibitor of spheroplast regeneration in *Candida albicans*. Clethramycin shows potent antifungal and pollen tube growth inhibitory activity.

Plant-associated actinomycetes have been recognized as an additional unexplored source of new bioactive compounds in our screening program[1~7]. In the screening of pollen tube growth inhibitors from the metabolites of plant-associated actinomycetes, clethramycin was found from the fermentation broth of *Streptomyces hygroscopicus* TP-A0623. This strain was isolated from a root of *Clethra barbinervis* collected in Toyama, Japan. Clethramycin (Fig. 1) is a novel polyene antibiotic, resembling the linearmycins[8]. The screening, taxonomy and fermentation of the producing strain and isolation and biological properties of clethramycin have been described in the preceding paper[9]. We herein report on the physico-chemical properties and structure determination of clethramycin.

Results and Discussion

Physico-chemical Properties

The physico-chemical properties of clethramycin are summarized in Table 1. Clethramycin was obtained as a yellow powder, soluble in methanol and insoluble in chloroform and ethyl acetate. Its UV-vis spectrum showed a typical pattern of polyene antibiotics with the absorption maxima at 337, 356 and 378 nm, suggesting the presence of a conjugated hexaene moiety. The FAB-MS measurement of clethramycin gave the parent ion peak $[M-H]^-$ at *m/z* 1216.7 in negative mode and $[M+Li]^+$ at *m/z* 1224.6 in the presence of lithium chloride in positive mode. In the negative mode FAB-MS/MS spectrum, distinctive fragment ions were observed at *m/z* 97 and 80, indicating the presence of a sulfate or phosphate group.

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Fig. 1. Structure of clethramycin.

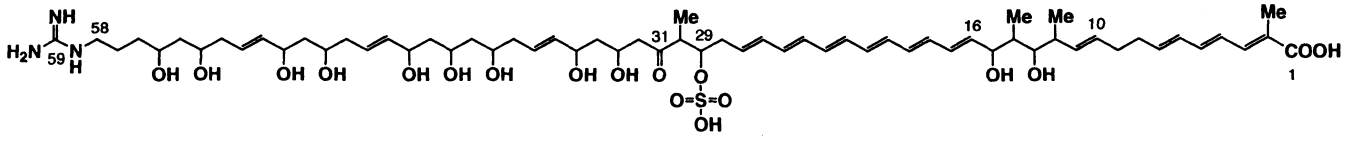
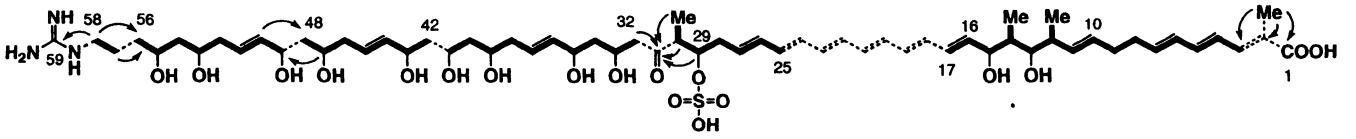


Fig. 2. NMR analysis of clethramycin.

1) DQF-COSY (bold lines) and HMBC (arrows) correlations



2) TOCSY (bold lines) and ROESY (arrows) correlations

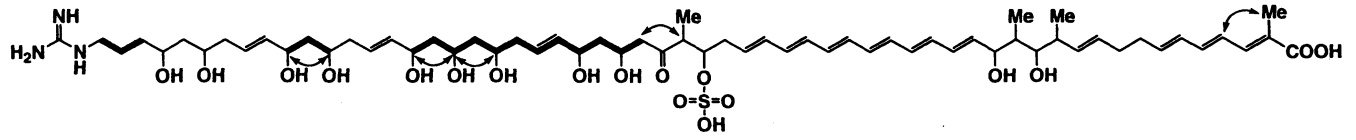


Table 1. Physico-chemical properties of clethramycin.

Appearance	Yellow amorphous
Melting point	>200°C (dec)
$[\alpha]_D^{20}$	-11.4 (*c* 0.4, methanol)
HRFAB-MS	
Found:	1216.6473 $[M-H]^-$
Calcd:	1216.6566 (for $C_{63}H_{98}N_3O_{18}S$)
Molecular formula	$C_{63}H_{99}N_3O_{18}S$
UV λ_{max}^{MeOH} nm (ε)	305 (36,800), 315 (sh), 337 (38,400), 356 (51,800), 378 (48,100)
IR ν_{max} (cm^{-1})	3400, 2930, 1670, 1380
HPLC (Rt)	6.2 min

HPLC conditions: Cosmosil AR-II (250 × 4.6 mm, i.d.), CH_3CN-0.15% KH_2PO_4 (pH 3.5), flow rate: 0.7 ml/min.

Structure Determination

The ^1H and ^{13}C NMR spectra of clethramycin showed a characteristic feature of the reduced-type polyolefinic polyketide. Olefinic carbons and protons were observed in the narrow spectral regions and the peaks for more than twenty carbons were detected. In addition, fifteen methylene and twelve hydroxylated methine groups were detected. One singlet and three doublet methyl groups, presumably derived from the methylmalonate, were easily recognized in the spectrum. The singlet methyl had HMBC correlations to three sp^2 carbons at 135.0, 134.5 and 177.6 ppm, and these carbons were speculated to constitute the biosynthetic ending unit of this polyketide. The three doublet methyls had HMBC correlations to three carbons including the methine carbon respectively, and two partial structures composed of the carbons from C-11 to C-16 and C-29 to C-31 were established. Since it was considered that most of the remaining carbons were derived from the malonate, ^{13}C-labeled clethramycin was prepared by the fermentation fed with 1,2-$^{13}C_2$-acetate. The 2D-INADEQUATE experiments in combination with HMQC and DQF-COSY confirmed the presence of the following acetate units: $-CH_2-C(=O)-\times 1$, $-CH_2-CH=$ (or $=CH-CH_2-)\times 6$, $=CH-CH=\times 7$, $=CH-CH(OH)-\times 4$, $-CH_2-CH(OH)-\times 6$. The remaining units of which INADEQUATE correlation were not detected were three methyl-containing units from the methylmalonate, and a unit of $-CH_2-CH(OH)-$ and an sp^2 carbon at 158.7 ppm from unknown precursors. Combined with these preliminary data, the molecular formula of clethramycin was determined to be $C_{63}H_{99}N_3O_{18}S$ on the basis of the high

resolution FAB-MS spectrum (1216.6473, Δ −9.3 mmu).

By precisely analyzing DQF-COSY, TOCSY, HMBC and ROESY, the overall carbon skeleton of clethramycin was determined as shown in Fig. 2. The partial structure from C-3 to C-17 was confirmed by the DQF-COSY correlations with the aid of HMBC analysis. Therefore the substructure containing the ending unit from C-1 to C-17 was established. Connectivities from C-25 to C-30 was confirmed by DQF-COSY and the connection from C-30 to the ketone carbonyl carbon (C-31) at 212.8 ppm was indicated by the HMBC correlations from the neighboring protons, H-29, H-30 and 30-Me. Another substructure from C-32 to C-41 was confirmed by the DQF-COSY correlations, and the HMBC correlation from H-32 to C-31 established the connectivity from C-25 to C-41. DQF-COSY analysis provided three more substructures, from C-42 to C-48, C-49 to C-56 and C-57 to C-58, and the linkage of these substructures in the order of the carbon number as C-42 to C-58 was determined by HMBC correlations. In consideration of the regularity of polyketide biosynthesis, the direction of chain-elongation from C-58 to C-42 was dictated from the alignment of the double bond. The direction from C-41 to C-25 and from C-17 to C-1 was also dictated by the same reason. The connectivity between C-41 and C-42 was confirmed by TOCSY and ROESY correlations and thus the substructure from C-58 to C-25 was established. The remaining three =CH–CH= units must be located between C-17 and C-25 to make the hexaene moiety predicted from the UV-vis spectrum.

The sulfate was positioned at the hydroxyl group at C-29 from the following observations: 1) the carbon and proton signals at C-29, δ_C 79.9 ppm and δ_H 4.67 ppm, were observed in the lower field than other hydroxylated methines; 2) the C-29 carbon was a singlet, not coupled with ^{31}P; 3) formation of a macrolactone at C-1 and C-29 requires high constraint because of the rigid polyolefinic structure. Furthermore, the guanidino residue was positioned at C-58 by the following reasons: 1) an HMBC correlation was detected from H-58 to C-59 (δ_C 158.7 ppm); 2) the proton and carbon chemical shifts at C-58 are downfield shifted compared with the α-methylene of a primary amine; 3) the molecular formula dictated by FABMS requires three nitrogen atoms in the molecule. The geometry of carbon-carbon double bonds was determined to be *E* from the coupling constants, except for the bonds between C-17 and C-26 of which geometry could not be confirmed due to the signal overlapping.

Perhaps the closest known structural analogs of clethramycin are linearmycins. The major differences between clethramycin and linearmycins are the presence of

Table 2. ^1H (750 MHz) and ^{13}C (189 MHz) NMR data for clethramycin in methanol-d_4.

Position	^{13}C	^1H
1	177.56	
2	134.50	
2-Me	14.24	1.92 (3H, s)
3	134.97[a]	6.96 (1H, dd, 10.5, 15)
4	128.28	6.40 (1H, dd, 9.8, 15)
5	137.58	6.37 (dd, 9.8, 15)
6	132.42	6.19 (dd, 9.8, 15)
7	137.09	5.80 (dt, 15, 6.8)
8	33.95	2.21 (2H)
9	33.44	2.15 (2H, 1, 6.8)
10	131.36	5.49 (1H, dt, 15 6.8)
11	134.54[b]	5.43 (1H, dd, 8, 15)
12-Me	18.38	0.96 (3H, d, 6.8)
13	77.83	3.39 (1H, dd, 5.3, 6)
14	42.34	1.65 (1H)
14-Me	8.59	0.94 (3H, d, 7.5)
15	75.34	4.25 (1H)
16	137.00	5.72 (1H)
17	131.94	6.28 (1H)
18	133.29[b]	6.28 (1H)
19	133.77[b]	6.28 (1H)
20	134.03[b]	6.28 (1H)
21	134.12[b]	6.28 (1H)
22	134.23[b]	6.28 (1H)
23	134.31[b]	6.28 (1H)
24	134.38[b]	6.28 (1H)
25	134.46[b]	6.23 (1H)
26	135.23[a]	6.17 (1H, dd, 10 15)
27	130.75	5.73 (1H, dt, 15 7.5)
28	37.02	2.54 (1H, dt, 15.8, 7)
		2.58 (1H, dt, 15.8, 7)
29	79.91	4.67 (1H, q, 6)
30	51.00	2.88 (1H, quint, 6.7)
30-Me	11.88	1.12 (3H, d, 6.8)
31	212.81	
32	50.84	2.71 (1H, dd, 3.8, 17.3)
		2.77 (1H, dd, 8.3, 17.3)
33	66.69	4.14 (1H)
34	45.00	1.70 (2H)
35	71.80	4.21 (1H, q, 6.8)
36	136.52	5.50 (1H, dd, 10 15)
37	129.04	5.70 (1H)
38	41.74	2.21 (2H)
39	71.17	3.83 (1H)
40	44.78[c]	1.56 (2H)
41	68.43	3.99 (1H)
42	45.64	1.60
43	70.21	4.27
44	137.17[d]	5.57 (dd, 7, 15)
45	127.99[e]	5.69
46	41.55	2.21
47	68.93	3.86
48	44.64[c]	1.52 (2H)
49	70.21	4.28
50	137.38[d]	5.56 (dd, 6.8, 15)
51	127.65[e]	5.69
52	41.55	2.21 (2H)
53	71.17	3.80
54	44.22	1.55 (2H)
55	70.94	3.78
56	35.12	1.44, 1.57
57	26.05	1.64, 1.73
58	42.45	3.19
59	158.66	

a-e: interchangeable

O-sulfate and guanidino functionalities in clethramycin. These structural differences seem to reflect on the biological properties. Linearmycin A shows more potent antimicrobial activity against bacteria than yeasts, whereas the trend in clethramycin is adverse[9].

A couple of groups of polyketides from *Streptomyces* have a guanidino residue in the biosynthetic starter unit, but the origin of the guanidino group is obscure. Azalomycin F and the relating antibiotics such as copiamycin and niphimycin have a guanidino-containing starter unit. The biosynthetic study using ^{13}C-labeled precursors demonstrated that the carbon signals of the α-, β-, γ- and δ-positions of the guanidino residue were not enhanced by the incorporation of 1-^{13}C-acetate, and it was suggested that the origin of the guanidino residue of azalomycin F was guanidine[10]. Lydicamycin[11] is another polyketide which has a guanidino functionality in the starter unit. In our study of the biosynthesis of lydicamycin, $^{13}C_6$-DL-guanidine was not incorporated into the expected carbons (unpublished result). In the biosynthesis of linearmycin that has an amino residue in the starter unit, it was suggested that 4-aminobutyric acid is the biosynthetic precursor of this unit on the basis of the incorporation pattern of ^{13}C-labeled acetate. The α-carbon comes from the carboxyl carbon of acetate and β, γ, δ-carbons from the methyl carbon of acetate, and the INADEQUATE correlation is observed between α- and β-carbons, but not between γ- and δ-carbons. Our incorporation experiments of 1,2-$^{13}C_2$-acetate with clethramycin, the α- and β-carbons of the guanidino residue showed the INADEQUATE correlation, but not with the γ- and δ-carbons. We therefore speculate that the guanidino-containing starter unit of polyketide originates from 4-aminobutyric acid.

Experimental

Instrumental Analysis

Melting point was determined on a Yanagimoto apparatus and is uncorrected. NMR experiments were performed on a Bruker DMX-750 NMR spectrometer in methanol-d_4. The FABMS spectra were measured on a JEOL JMS-HX110A spectrometer. UV spectrum was recorded on a Hitachi U-3210 spectrophotometer. IR spectrum was recorded on a Shimadzu FT IR-300 spectrophotometer. Optical rotation was recorded on a Jasco P-1030 polarimeter.

Isotope Labeling

The seed fermentation of *Streptomyces hygroscopicus* TP-A0623 was carried out as described in the preceding paper[9]. The seed culture was inoculated into fifty 500-ml K-1 flasks each containing 100 ml of the production medium and the fermentation was carried out at 30°C on a rotary shaker (200 rpm). After 1 and 3 days of fermentation, a sterilized aqueous solution of sodium 1,2-$^{13}C_2$-acetate (Isotec Inc.) was added to each flask (0.01% w/v, 10 mg per flask). After 5 days, the fermentation was finished, and 50.3 mg of ^{13}C-labeled clethramycin was obtained by chromatographic purification.

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