GYPSETIN, A NEW INHIBITOR OF ACYL-CoA: CHOLESTEROL ACYLTRANSFERASE PRODUCED BY *Nannizzia gypsea* var. *incurvata* IFO 9228

II. STRUCTURE DETERMINATION

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(Received for publication August 19, 1993)

The elucidation of the structure of gypsetin, a new inhibitor of acyl-CoA: cholesterol acyltransferase, is described in this paper. By spectroscopic and X-ray crystallographic analyses, the structure of gypsetin has been determined to be 8a,16a-dihydroxy-5a,13a-bis[1,1-dimethylallyl]-[1]benzazolidine[3‴,2‴:4″,5″]azolidino[1″,2″:4′,5′][1,4]perhydrodiazin[1′,2′:1,5]azolidino[2,3-*b*]-[1]benzazolidine-7,15-dione.

In the preceding paper, we have described the fermentation, isolation, physico-chemical properties and biological activity of gypsetin, a new competitive inhibitor of acyl-CoA: cholesterol acyltransferase produced by *Nannizzia gypsea* var. *incurvata* IFO 9228[1]. In this paper, we present the structure determination of gypsetin.

Spectroscopic Studies

The molecular formula of gypsetin was elucidated to be $C_{32}H_{36}N_4O_4$ (MW 540) from SI-MS (*m/z* 563 $(M+Na)^+$ and 541 $(M+H)^+$), HREI-MS (found: *m/z* 540.2728, calcd: *m/z* 540.2722 M^+) and ^1H and ^{13}C NMR spectra of gypsetin.

The ^1H NMR spectrum (Fig. 1) showed eight aromatic protons, six olefinic protons, eighteen aliphatic protons including four methyl groups, and four exchangeable protons at δ_H 6.36, 6.05 and ~2.3. Although only 31 carbon signals were observed in the ^{13}C NMR spectrum of gypsetin in $CDCl_3$ (Fig. 2), 32 carbon signals were found in the spectrum determined in $CDCl_3$-CD_3OD (1:9) as well as in DMSO-d_6. The number of protons attached to each carbon was determined by recording the DEPT and ^1H-^{13}C COSY spectra.

From the HMBC (heteronuclear multiple bond connectivity), COLOC and ^1H-^{13}C long-range COSY spectra of gypsetin, it was revealed that olefinic protons at δ_H 4.87 (24-H) coupled to two carbons at δ_C 43.9 (C-22) and 144.2 (C-23), and protons at δ_H 5.03 and 5.09 (19-H) correlated with carbons at δ_C 44.7

Fig. 1. ^{1}H NMR spectrum of gypsetin ($CDCl_3$, 270 MHz).

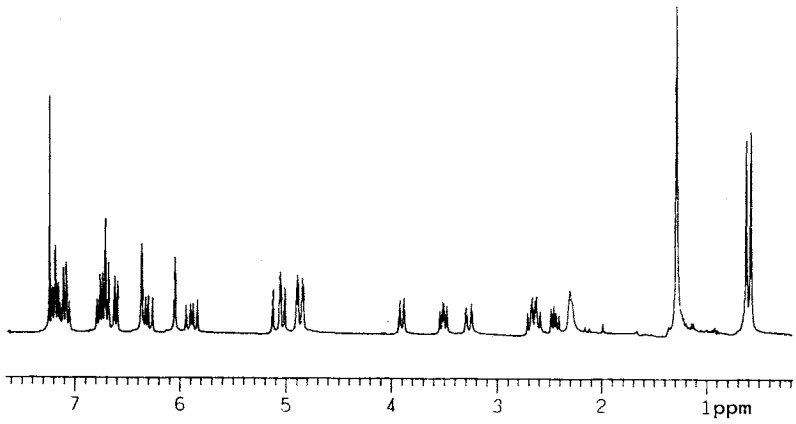
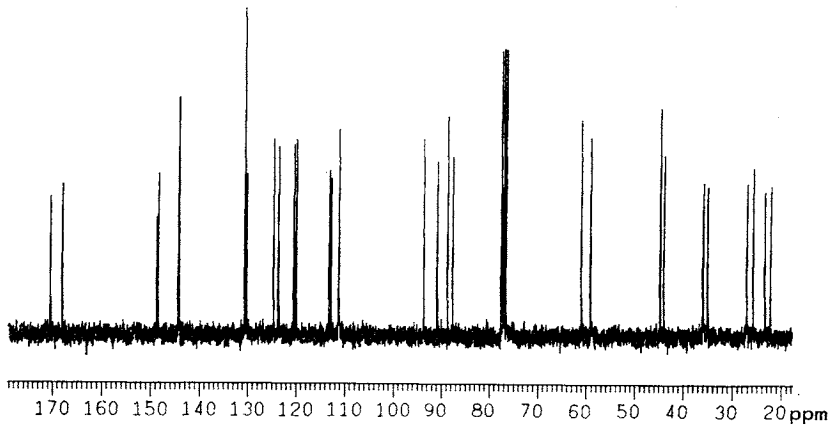


Fig. 2. ^{13}C NMR spectrum of gypsetin ($CDCl_3$, 67.9 MHz).



(C-17) and 144.1 (C-18). The signals of methyl protons at δ_H 0.58 (26-H) and 0.63 (25-H) showed cross peaks with the signals at δ_C 91.0 (C-13a), 43.9 (C-22) and 144.2 (C-23), and methyl protons at δ_H 1.29 (20-H, 21-H) correlated with three carbons at δ_C 93.7 (C-5a), 44.7 (C-17) and 144.1 (C-18). From these results, along with ^{1}H-^{1}H COSY data, it was suggested that two 1,1-dimethylallyl groups bind to the quaternary carbons at δ_C 91.0 (C-13a) and 93.7 (C-5a) (Fig. 3).

The exchangeable protons at δ_H 6.05 (13-H) coupled to the signals at δ_C 88.7 (C-8a), 130.2 (C-8b) and 91.0 (C-13a), and the signal of δ_H 6.36 (5-H) showed cross peaks with the signals at δ_C 93.7 (C-5a), 87.7 (C-16a) and 130.2 (C-16b). Methine proton at δ_H 3.51 (7a-H) coupled to carbons at δ_C 167.9 (C-7), 35.0 (C-8) and 170.4 (C-15), and methine proton at δ_H 3.90 (15a-H) correlated with carbons at δ_C 170.4 (C-15) and 35.8 (C-16). Methylene protons at δ_H 2.42 and 2.70 (8-H) correlated with the signals at δ_C 167.9 (C-7), 59.1 (C-7a), 88.7 (C-8a), 130.2 (C-8b) and 91.0 (C-13a), and methylene protons at δ_H 2.63 and 3.27 (16-H) coupled to the signals at δ_C 93.7 (C-5a), 170.4 (C-15), 61.0 (C-15a), 87.7 (C-16a) and 130.2 (C-16b). The exchangeable protons at δ_H 2.31 (8a-OH) coupled to carbons at δ_C 35.0 (C-8), 88.7 (C-8a) and 130.2 (C-8b), and the signal of δ_H 2.31 (16a-OH) showed cross peaks with the signals at δ_C 35.8 (C-16), 87.7 (C-16a)

Fig. 3. Long-range couplings observed in the HMBC, COLOC and/or ^{1}H-^{13}C long-range COSY spectra of gypsetin.

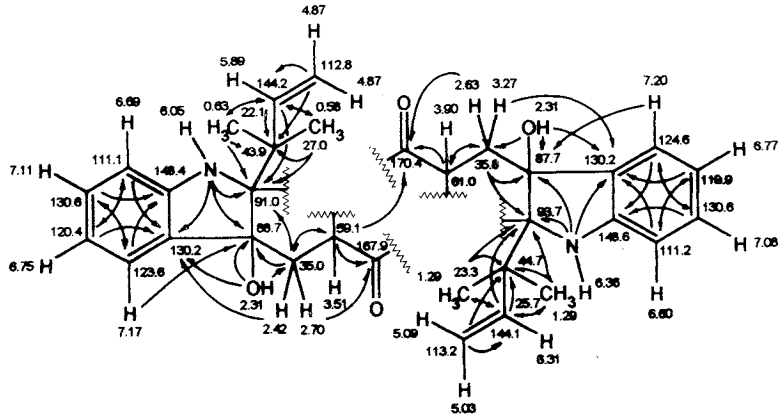
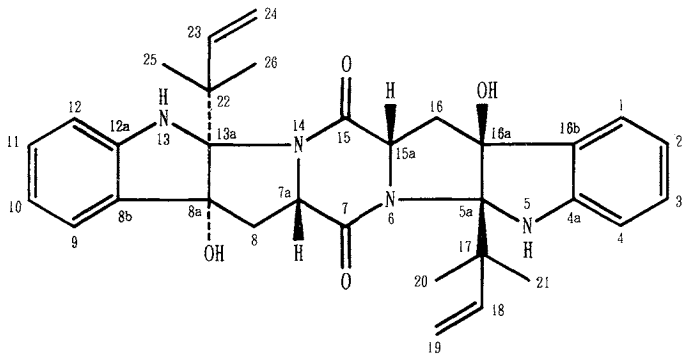


Fig. 4. The structure of gypsetin.

The configuration shown is relative one.



and 130.2 (C-16b). The aromatic protons showed long-range couplings as shown in Fig. 3. From these results, the presence of two identical partial structures was revealed.

When ^{13}C NMR spectrum was measured in $CDCl_3$ after the addition of a drop of D_2O, the carbon signals at δ_C 148.6 (C-4a), 93.7 (C-5a), 88.7 (C-8a), 148.4 (C-12a), 91.0 (C-13a) and 87.7 (C-16a) were upfield-shifted by 0.06, 0.08, 0.14, 0.08, 0.08 and 0.14 ppm, respectively. Gypsetin showed positive reaction with nitroprusside-acetaldehyde reagent[2] but not with ninhydrin reagent[2] suggesting the presence of secondary amine(s). These results are consistent with the presence of the structure shown in Fig. 3.

By means of an X-ray crystallographic analysis, structure (relative configuration) of gypsetin (Fig. 4) was determined as described below. Since NOE was observed between the olefinic methylene protons (δ_H 5.03 and 5.09) (19-H) and the methine proton at δ_H 3.90 (15a-H) but not between the methylene protons at δ_H 4.87 (24-H) and the methine proton at δ_H 3.51 (7a-H) or 3.90 (15a-H), ^{1}H and ^{13}C NMR signals of gypsetin were assigned as shown in Table 1. However, the assignments of the positions 2 and 4 are concomitantly interchangeable with those of the positions 10 and 12, respectively, as carbon signals of C-8b and C-16b (both at δ_C 130.2), to which 2-H, 4-H, 10-H and 12-H signals correlate, have been

Table 1. ^{13}C and ^{1}H NMR data for gypsetin in $CDCl_3$.

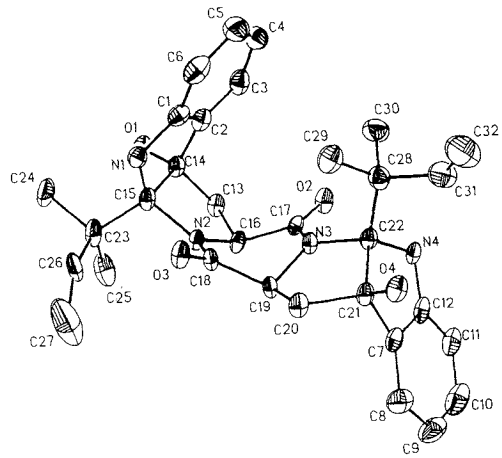
Position	δ_C (67.9 MHz)	δ_H (270 MHz) (J in Hz)	Position	δ_C (67.9 MHz)	δ_H (270 MHz) (J in Hz)
1	124.6	7.20 (1H, m)	13a	91.0	—
2*	119.9	6.77 (1H, m)	15	170.4	—
3	130.6	7.08 (1H, m)	15a	61.0	3.90 (1H, dd, J=2.5, 11.1)
4**	111.2	6.60 (1H, m)	16	35.8	2.63 (1H, dd, J=11.1, 13.6),
4a	148.6	—			3.27 (1H, dd, J=2.5, 13.6)
5	—	6.36 (1H, s)	16a	87.7	—
5a	93.7	—	16a-OH	—	2.31 (1H, br s)
7	167.9	—	16b	130.2	—
7a	59.1	3.51 (1H, dd, J=7.3, 11.0)	17	44.7	—
8	35.0	2.42 (1H, dd, J=7.3, 13.2),	18	144.1	6.31 (1H, dd, J=11.0, 17.6)
		2.70 (1H, dd, J=11.0, 13.2)	19	113.2	5.03 (1H, dd, J=1.5, 11.0),
8a	88.7	—			5.09 (1H, dd, J=1.5, 17.6)
8a-OH	—	2.31 (1H, br s)	20	23.3	1.29 (3H, s)
8b	130.2	—	21	25.7	1.29 (3H, s)
9	123.6	7.17 (1H, m)	22	43.9	—
10*	120.4	6.75 (1H, m)	23	144.2	5.89 (1H, dd, J=10.6, 18.0)
11	130.6	7.11 (1H, m)	24	112.8	4.87 (1H, dd, J=1.5, 10.6),
12**	111.1	6.69 (1H, m)			4.87 (1H, dd, J=1.5, 18.0)
12a	148.4	—	25	22.1	0.63 (3H, s)
13	—	6.05 (1H, s)	26	27.0	0.58 (3H, s)

*,** The assignments of the positions 2* and 4** are concomitantly interchangeable with the assignments of positions 10* and 12**, respectively.

Table 2. The crystal data for gypsetin.

Formula	$C_{32}H_{36}N_4O_4$
Crystal system	Monoclinic
Space group	C2/2; P2.1, (4)
Cell dimensions	
a (Å)	11.190 (4)
b (Å)	12.480 (4)
c (Å)	12.575 (4)
β (°)	109.63 (2)
Z	2
d, calcd (g/cm^3)	1.32
Linear abs. μ (mm^{-1})	0.32

Fig. 5. The molecular structure of gypsetin as determined by X-ray crystallography.



indistinguishable.

X-Ray Crystallographic Analysis

The structure of gypsetin was determined by single crystal X-ray analysis using MoKα radiation. The crystal data are shown in Table 2. The structure was solved by direct methods and the relative molecular structure is shown in Fig. 5.

Discussion

In the present study, we determined the structure of gypsetin, a new metabolite belonging to complex diketopiperazine compounds. A variety of diketopiperazine compounds of microbial origin has been identified so far. Amauromine[3], a hypotensive vasodilator, is one of these diketopiperazines and is

structurally related to gypsetin in that it consists of bis(1,1-dimethylallyl)-substituted [1]benzazolidine[3‴,2‴:4″,5″]azolidino[1″,2″:4′,5′][1,4]perhydrodiazin[1′,2′:1,5]azolidino[2,3-*b*][1]benzazolidine structure. The positions of 1,1-dimethylallyl substitution are 5a and 13a in gypsetin, whereas these are 8a and 16a in amauromine. In gypsetin the positions 8a and 16a are substituted with hydroxyl groups. Due to a difference in the configuration of the substituents, the two compounds showed distinct nuclear magnetical characteristics; amauromine but not gypsetin possesses a symmetrical feature as a dimmer in ^{1}H and ^{13}C NMR spectra. It is an intriguing subject to determine biosynthetic mechanisms leading to the formation of these compounds.

Experimental

Spectroscopic Studies

Mass spectra were obtained on a Hitachi M-80B mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL GX 270 (270 MHz for ^{1}H and 67.9 MHz for ^{13}C) spectrometer.

X-Ray Crystallography

Single crystal X-ray analysis was carried out with a SYNTEX R3 using MoKα radiation ($\lambda=0.71073$ Å). Crystallographic calculations were performed with the SHELXTL PLUS program package on a MICROVAX II computer. Empirical absorption corrections were carried out. The structure was solved by direct methods and refined with the SHELXTL system. The refinement converged with $R=7.7\%$; $wR=7.1\%$. Hydrogen atoms were located by option HFIX of the SHELXTL program.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Science Research from the Ministry of Education, Science and Culture and a Research Grant for Cardiovascular Diseases from the Ministry of Health and Welfare, Japan.

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