GLYCOCINNASPERIMICIN D, A NEW MEMBER OF GLYCOCINNAMOYLSPERMIDINE ANTIBIOTIC

KAZUYUKI DOBASHI, KATSUHIKO NAGAOKA, YOSHINORI WATANABE, MAKOTO NISHIDA, MASA HAMADA, HIROSHI NAGANAWA, TOMOHISA TAKITA, TOMIO TAKEUCHI and HAMAO UMEZAWA

Institute of Microbial Chemistry
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

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Glycocinnasperimicin D was isolated from the fermentation filtrate of a strain of *Nocardia* sp. using various procedures of column chromatography. Glycocinnasperimicin D exhibited broad antibacterial spectrum. Its chemical structure was determined by NMR spectrometric analyses.

In the course of our screening for antibiotics, a new member of the glycocinnamoylspermidine group was found in the culture filtrate of *Nocardia* sp. strain MG615-7F6, and named glycocinnasperimicin D. This antibiotic has broad antibacterial spectrum. In this paper, we report on its fermentation, isolation, physico-chemical and biological properties. The strain also produced LL-BM123β, γ_1 and γ_2[1].

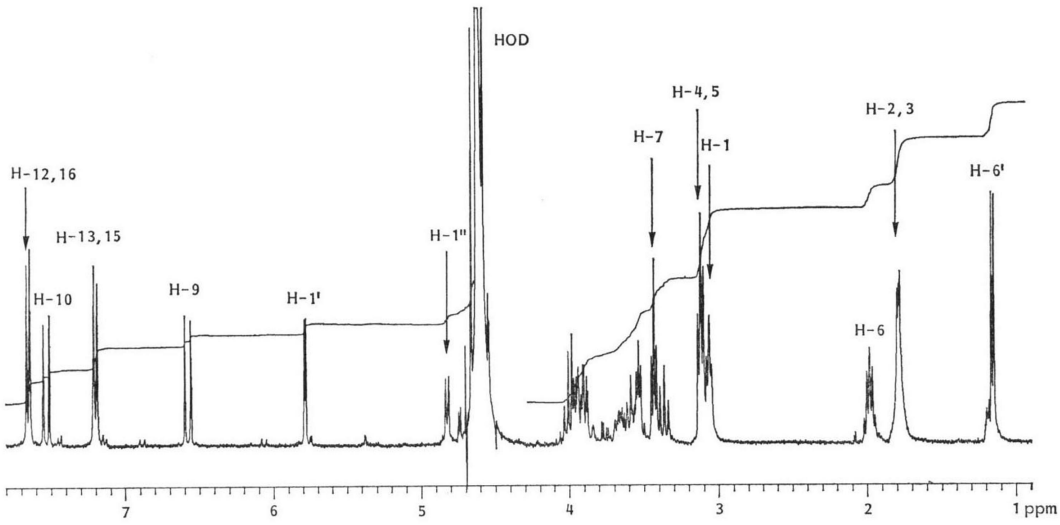
Microorganism

The strain MG615-7F6 was isolated from a soil sample collected in Fuchu City, Tokyo, Japan. Cultural characteristics of the strain were related to those of BM123, NRRL 5646[1].

Fermentation and Isolation

A loopful of a mycelial suspension from a slant culture of strain MG615-7F6 was transferred into a seed medium containing glucose 1.0%, glycerol 1.0%, sucrose 1.0%, oat meal 0.5%, soy bean meal 2.0%, pressed yeast 1.0%, Casamino Acids 0.5%, $CaCO_3$ 1.0% (pH 7.0), and incubated with rotary shaking (180 rpm) for 3 days at 30°C. The seed culture (600 ml) was inoculated into 15 liters of a medium composed of glucose 1%, starch 1%, glycerol 1%, Polypeptone 0.5%, meat extract 0.5%, NaCl 0.5%, and $CaCO_3$ 0.32% (pH 7.4), in a 30-liter jar-fermentor. The fermentation was carried out at 28°C for 72 hours under an agitation of 350 rpm, aeration of 15 liters per minute. The cultured broth was adjusted to pH 5.0 with 1 M HCl and filtered. The filtrate (14.5 liters) thus obtained was passed through a column of Amberlite IRC-50 (Na^+-, H^+-form, 1: 1, 2.0 liters). After washing with water and 50% aqueous acetone, the adsorbed material was eluted with 1 M HCl - acetone (1: 1). The active fractions (assayed by a disk agar diffusion method using *Bacillus subtilis* PCI219) were collected and neutralized with 0.5 M NaOH. After evaporation of acetone, the solution was passed through a column of Amberlite XT-2 (80 ml) followed by elution with water. The active eluate was concentrated and lyophilized to give a pale yellow crude powder (124 mg). The powder was further purified by column chromatography on CM-Sephadex C-25 (150 ml) with linear gradient elution (NaCl, from 0.1 M to 1.2 M). The bioactive fraction was divided into two parts (fractions A and B);

Fig. 1. ^{1}H NMR spectrum of glycocinnasperimicin D (400 MHz, 40.5°C, D_2O).



the bioactive agent present in fractions A and B was adsorbed on a small column of CHP-20P (37~75 μm) (Mitsubishi Chemical Industries Limited) and desorbed with water. The activity in fraction A was separated further into two fractions. The early fraction of fraction A was glycocinnasperimicin D (7.1 mg) and the late one was identified as LL-BM123γ complex (8.5 mg). The antibiotic from fraction B was identified as LL-BM123β (10 mg) by direct comparison with authentic sample. These antibiotics could be distinguished by cellulose TLC: Eastman chromagram sheet No. 6065, 1-propanol - pyridine - acetic acid - water (20: 10: 3: 12); glycocinnasperimicin D, Rf 0.28; LL-BM123γ, Rf 0.24; LL-BM123β, Rf 0.13. LL-BM123α was not found in our culture broth.

Physico-chemical Properties and Structure

Glycocinnasperimicin D is obtained as a colorless powder, $[\alpha]_D^{18}$ +88° (c 0.5, H_2O); mp >300°C (dec); it is a basic substance, easily soluble in water and very hygroscopic. It shows positive reaction to ninhydrin, Rydon-Smith, and Sakaguchi reagents. Elemental analysis did not give a satisfactory value; a characteristic of LL-BM123 antibiotics[2].

The secondary ion mass spectrum [SIMS, m/z 695 $(M+H)^+$] and the ^{13}C NMR spectrum indicated the molecular formula $C_{30}H_{50}N_{10}O_8$. The antibiotic showed UV maxima at 218 (log ε 4.13) and 287 nm (log ε 4.34) in water. In the ^{1}H NMR spectrum in D_2O (Fig. 1), two olefinic protons at δ 6.59 (1H, d, J=16.0 Hz), 7.53 (1H, d, J=16.0 Hz) and four aromatic protons at δ 7.20 (2H, d, J=9.0 Hz), 7.66 (2H, d, J=9.0 Hz) suggested the presence of a p-hydroxycinnamoyl moiety which is also present in LL-BM123β and γ. The presence of a spermidine moiety was shown by methylene signals at δ 1.80 (4H, m), 1.99 (2H, m), 3.07 (2H, t), 3.13 (4H, t×2), and 3.43 (2H, t). Spin decoupling experiments indicated that the triplet signal at δ 3.43 was H-7 of spermidine; it is linked to the carbonyl group of cinnamoic acid through an amide bond. Known glycocinnamoylspermidine antibiotics are composed of a cinnamoylspermidine aglycone and a trisaccharide moiety[3]. However, glycocinnasperimicin D has a disaccharide moiety, as suggested by two anomeric protons at δ 5.79 (d, J=3.3 Hz) and 4.83 (d, J=9.1 Hz). A doublet signal at δ 1.06 (3H, d, J=6.2 Hz) and nine protons from δ 3.3 to 4.1 were attributed to the signals of methylenes and methines in the disaccharide moiety.

Table 1. ^{13}C NMR spectrum of glycocinnasperimicin D and LL-BM123β, γ_1 (25 MHz, D_2O internal dioxane δ=67.4).

Position	Glycocinnasperimicin D·HCl	LL-BM123β·H_2SO_4[a]	LL-BM123γ_1·H_2SO_4[a]
1	37.1 (t)[b]		37.2
2	24.7 (t)		24.8
3	23.6 (t)		23.6
4	47.7 (t)		47.8
5	45.9 (t)		46.1
6	26.5 (t)		26.6
7	39.6 (t)		39.7
8	170.1 (s)		169.9
9	119.5 (d)		119.7
10	141.5 (d)		141.4
11	130.2 (s)		130.2
12, 16	130.6 (d)		130.6
13, 15	118.0 (d)		118.2
14	157.8 (s)	157.9[c]	158.0[c]
1′	96.0 (d)	96.0	96.4 (d)
2′	58.2 (d)	58.2	58.3 (d)
3′	69.2 (d)	69.2	69.2 (d)
4′	57.1 (d)	57.1	57.3 (d)
5′	70.8 (d)	70.8	71.0 (d)
6′	17.5 (q)	17.5	17.7 (q)
1″	82.5 (d)	82.2	82.4 (d)
2″	55.8 (d)	55.4	55.8 (d)
3″	75.4 (d)	73.2	73.4 (d)
4″	70.3 (d)	78.0	75.3 (d)
5″	67.2 (t)	64.6	64.6 (t)
1‴		101.8	98.2 (d)
2‴		53.2	56.1 (d)
3‴		57.0	60.8 (d)
4‴		66.9	70.0 (d)
5‴		66.7	66.9 (t)
2′ C=N	158.2 (s)	158.3	158.3 (s)
4′ C=O	159.8 (s)	159.7[c]	159.7 (s)[c]
2″ C=O	162.1 (s)	161.7	162.1 (s)[c]
2‴ C=O		162.1[c]	160.8 (s)
			156.5 (s)

[a] Taken from ref 3.
[b] Multiplicity in off-resonance spectrum.
[c] These assignments were referred to the result of ref 4.

The ^{13}C NMR spectrum of glycocinnasperimicin D showed six aromatic carbons, two olefinic carbons, and four carbonyl and/or imino carbons. The presence of two anomeric carbons at δ 96.0 and 82.5 and nine carbons in the disaccharide moiety was in accord with the result of the ^1H NMR analysis.

Comparison of ^{13}C NMR spectrum of glycocinnasperimicin D with those of LL-BM123β and γ_1 (Table 1)[3,4] indicated that the chemical shifts for the relevant carbons of these antibiotics, except for a substituent effect at C-4″, were very similar. The observed downfield shifts of C-3″, C-5″ and the upfield shift of C-4″ in glycocinnasperimicin D indicated a free hydroxyl group at C-4″. Therefore, the disaccharide moiety should correspond to that of LL-BM123β without the distal sugar part.

Fig. 2. Structure of glycocinnasperimicin D.

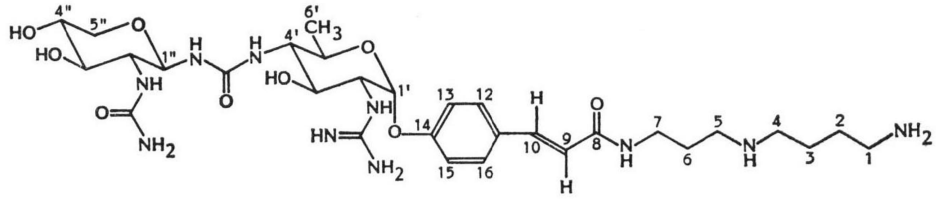


Fig. 3. IR spectrum of glycocinnasperimicin D (KBr).

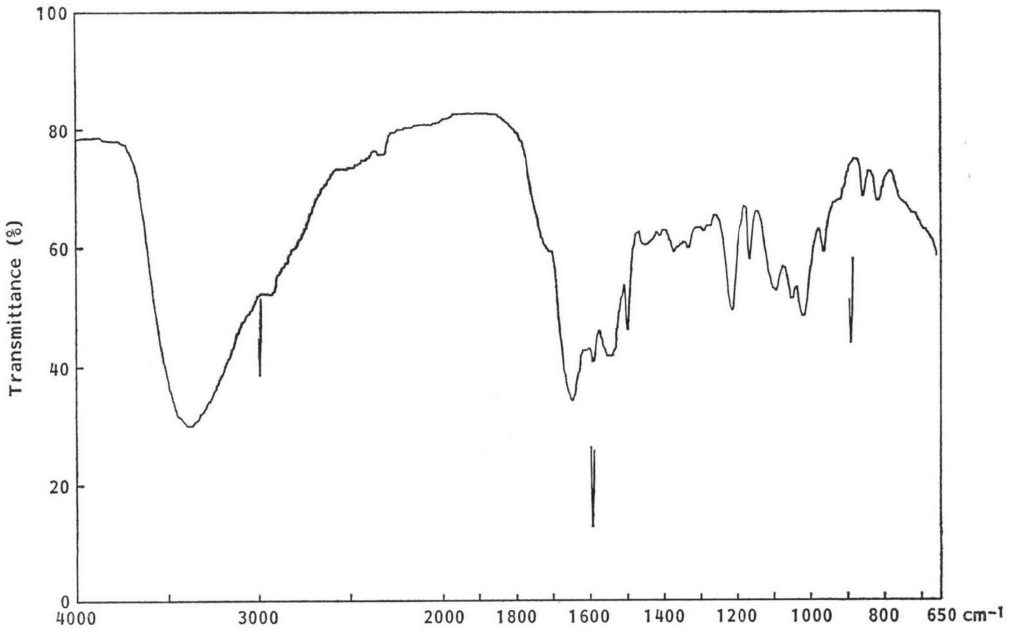


Table 2. Antimicrobial activity of glycocinnasperimicin D.

Organism	MIC (μg/ml)	Organism	MIC (μg/ml)
Staphylococcus aureus 209P	1.56	*Shigella dysenteriae* JS11910	1.56
S. aureus Smith	0.78	*S. flexneri* 4b JS11811	1.56
S. aureus Ap01	6.25	*S. sonnei* JS11746	3.12
S. epidermidis 109	3.12	*Salmonella typhi* T-63	0.39
Corynebacterium bovis 1810	3.12	*Proteus vulgaris* OX19	0.39
Escherichia coli NIHJ	0.39	*P. rettgeri* GN311	1.56
E. coli K-12	0.39	*P. rettgeri* GN466	0.78
E. coli JR66/W677	0.78	*Serratia marcescens*	6.25
Mycobacterium smegmatis ATCC 607	6.25	*Pseudomonas aeruginosa* A3	12.5
Klebsiella pneumoniae PC1602	1.56	*P. aeruginosa* GN-315	50

The C-1″ position linked to ureido nitrogen atom was reported by ELLESTAD *et al.*[3] to be an α-linkage in LL-BM123β, γ_1, and γ_2. However, the signal of H-1″ was covered by water signals when ^{1}H NMR was taken at 23°C. The 400 MHz ^{1}H NMR spectra at 40°C (D_2O) of glycocinnasperimicin D and LL-BM123β clearly showed the proton signal of H-1″; the doublet at δ 4.83 with J of 9.1 Hz,

δ 4.84 with J of 9.0 Hz, respectively. These data indicated that the linkage at C-1'' of both antibiotics should be β. From these results, we propose the structure of glycocinnasperimicin D as shown in Fig. 2.

Biological Effects

The antibacterial spectrum of glycocinnasperimicin D tested by serial agar dilution method is shown in Table 2. The 50% growth inhibitory concentration (IC_{50}) against Leukemia L-1210 cells was 2.0 μg/ml.

LD_{100} (iv) of glycocinnasperimicin D in mice was 50 mg/kg and LD_0 (iv) 25 mg/kg.

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