ORIGINAL ARTICLE

Nocardithiocin, a novel thiopeptide antibiotic, produced by pathogenic *Nocardia pseudobrasiliensis* IFM 0757

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Nocardithiocin is a novel thiopeptide compound produced by the pathogenic *Nocardia pseudobrasiliensis* strain IFM 0757. It shows a strong activity against acid-fast bacilli such as the *Mycobacterium* and *Gordonia* species. Nocardithiocin was highly active against rifampicin-resistant as well as -sensitive *Mycobacterium tuberculosis* strains, and most of the resistant strains were inhibited at concentrations ranging from 0.025 to 1.56 $\mu\text{g ml}^{-1}$. The structure of the thiopeptide antibiotic containing thiazole, natural and unnatural amino acids was elucidated by MS and NMR spectral analyses.

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INTRODUCTION

Actinomycetes produce numerous secondary metabolites including various biologically active compounds such as antimicrobial, cytotoxic, immunosuppressive and antifungal agents.¹ As the potential to isolate useful new antibiotics from *Streptomyces* sp. is now low and diminishing all the time, we have focused our attention on other sources. Pathogenic actinomycetes are distributed on a limited basis in the natural environment and are difficult to isolate. They can be considered among the rare actinomycetes and represent a relatively unexplored resource for the discovery of new bioactive compounds. We have collected many pathogenic actinomycetes from clinical materials, which mainly belong to the genus *Nocardia* and may cause human infections called nocardiosis.^{2,3} They have produced several biologically active compounds such as antitumor and immunosuppressive ones.^{4,5}

During our ongoing research for new metabolites from a *Nocardia* strain (IFM 0757 strain) obtained from a patient at the Health Center, University of Texas, we isolated a new cyclic thiopeptide antibiotic with potent antibacterial activity against rifampicin-resistant *Mycobacterium tuberculosis*, called nocardithiocin (Figure 1), which was classified as a 'series d' group peptide antibiotic based on the definition by Bagley *et al.*⁶ Cyclic thiopeptides are known as activators of thioestrepton-induced protein A, which is a member of the mercury resistance regulator family, and are active against Gram-positive bacteria.^{3,7} In this paper, we describe the fermentation, isolation, structure elucidation and antimicrobial activities of the antibiotic.

RESULTS

Isolation

The separation of the active compounds was monitored by the antimicrobial activity against *Corynebacterium xerosis* IFM 2057.⁸ After incubation, the culture broth was centrifuged at 3000 r.p.m. for 10 min. Six hundred milliliters of MeOH was then added to the mycelial cake and stirred for 3 hours. The MeOH fraction was filtered, evaporated to dryness and suspended in distilled water. The active compound was then extracted with EtOAc. The compound was further purified by HPLC. The isolation was carried out under dark conditions because the antibiotic was not stable in the light.

Structure elucidation

The physicochemical data for nocardithiocin are shown in Table 1. The positive ion FAB mass spectrum (JMS-AX500) of nocardithiocin showed a protonated molecule $[M+H]^+$ at m/z 1161. HRFAB-MS (JMS-HX110) displayed the $[M+K]^+$ ion at m/z 1199.1469 (calcd. 1199.1527). The molecular formula was determined to be $C_{48}H_{48}N_{12}O_{11}S_6$ based on the HRFAB-MS data by considering the number of protons and carbons from the NMR spectra. In the ^1H -NMR spectrum, one singlet and four doublet signals assigned to five methyl groups were observed between δ 0.86–1.78, and two singlet signals (δ 3.70 and 3.97) were assigned to methoxyl groups. Amino acid moiety α -proton signals were also observed at δ 4.52 (H-46), 5.11 (H-32) and 5.56 (H-22). Thirteen sp^2 proton signals and amido proton signals were detected between δ 8.06–9.52. The ^{13}C -NMR spectrum showed 48 signals that were assigned to eight methyl, 15

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Figure 1 Structure of nocardithiocin.

Table 1 The physicochemical data of nocardithiocin

Appearance	White powder
Molecular weight	1160
Molecular formula	C ₄₈ H ₄₈ N ₁₂ O ₁₁ S ₆
<i>HRFAB-MS</i> (<i>m/z</i>)	
Found:	1199.1469 [M+K] ⁺
Calcd.:	1199.1527
IR $\lambda_{\max}$ (CHCl ₃)	3728, 3699, 3628, 3596, 3383, 3113, 2961, 2269, 1665, 1533, 1477, 1273, 1236, 1273, 1236, 1148, 1067, 743, 675
[α] _D (23.2 °C, MeOH)	+34.0° (c 1 mg ml ⁻¹ , MeOH)
UV $\lambda_{\max}$ (MeOH) nm (log ϵ)	344.5 (3.89), 339.5 (3.97), 232.0 (4.25)

methine, and 25 quaternary carbons by heteronuclear multiple quantum coherence and distortionless enhancement by polarization transfer experiments (Table 2). The C–H connectivity and assignment of the signals were confirmed by 2D NMR experiments using ¹H–¹H COSY and heteronuclear multiple bond coherence (HMBC) techniques as follows:

Thiazole units

The thiazole units were contained in substructures B, C and D (Figure 2), and the whole structure was deduced by comparison with those of the corresponding ¹H and ¹³C chemical shifts with

those reported for promoinducin,⁹ nocardiacins¹⁰ and radamycin.¹¹ As a result, six thiazole units were determined by the following long-range correlations; in the HMBC spectrum, the thiazole ring proton signal at δ 8.43 (H-9) showed long-range couplings to carbons at δ 149.8 (C-10) and 161.5 (C-8), H-12 signal at δ 8.56 to δ 149.2 (C-11) and 168.2 (C-13), H-20 signal at δ 8.15 to δ 151.6 (C-19) and 169.0 (C-21), H-30 signal at δ 8.22 to δ 149.1 (C-29) and δ 170.3 (C-31), H-39 signal at δ 8.24 to 146.7 (C-38) and 160.3 (C-40), and H-52 signal at δ 8.14 to δ 149.2 (C-51) and 163.5 (C-53).

Substructure A

Substructure A was a terminal moiety of the side chain (Figure 2). In the COSY spectrum, CH₃-5 (δ 1.78) correlated with the olefinic proton at δ 6.76 (H-4) and showed a long-range correlation to the quaternary carbon at δ 127.3 (C-3). Furthermore, in the HMBC spectrum, the H-4 proton showed a long-range correlation to the carbonyl carbon at δ 164.3 (C-2), which showed a long-range correlation from the *O*-methyl proton at δ 3.70 (CH₃-1). An amide proton (disappearing with the addition of CD₃OD) at δ 9.52 (H-6) was correlated to C-2 and C-4, indicating the presence of substructure A.

Substructure B

Substructure B contained a pyridine ring and four thiazole rings. The *ortho*-coupled aromatic protons at δ 8.34 (H-15) and 8.53 (H-16), respectively, were connected by the COSY spectrum. H-16 showed long-range correlations to δ 149.6 (C-14) and 150.9 (C-18), and a long-range coupling from H-15 to δ 128.6 (C-17) was observed. These data revealed the presence of a pyridine ring. The methyl proton signal at δ 1.00 (H-26) showed long-range couplings to the oxygenated

Table 2 ^1H and ^{13}C NMR spectral data of nocardithiocin in $\text{DMSO}-d_6$

Position	δ_{C}	$\delta_{\text{H}}, J (\text{Hz})$	Position	δ_{C}	$\delta_{\text{H}}, J (\text{Hz})$
1	51.7	3.70 (s)	27		8.17 (d, 9.5)
2	164.3		28	159.9	
3	127.3		29	149.1	
4	134.0	6.76 (q, 7)	30	124.2	8.22 (s)
5	13.6	1.78 (s)	31	170.3	
6		9.52 (s)	32	55.5	5.11 (dd, 8, 0.5)
7	158.9		33	31.0	2.65 (m)
8	161.5		34	17.8	0.88 (d, 7)
9	125.4	8.43 (s)	35	19.6	0.86 (d, 7)
10	149.8		36		8.48 (d, 9.5)
11	149.2		37	160.1	
12	121.5	8.56 (s)	38	146.7	
13	168.2		39	124.0	8.24 (s)
14	149.6		40	160.3	
15	118.5	8.34 (d, 8)	41	111.5	
16	140.1	8.53 (d, 8)	42	152.0	7.30 (s)
17	128.6		43	61.6	3.97 (s)
18	150.9		44		9.33 (s)
19	151.6		45	169.8	
20	121.7	8.15 (s)	46	58.7	4.52
21	169.0		47	66.9	4.36 (m)
22	54.9	5.56 (d, 9.5)	OH		5.21 (d, 6)
23	75.2		48	20.0	1.27 (d, 6)
OH		4.37 (s)	49		8.06 (d, 8)
24	69.5	3.66 (m, 6)	50	160.1	
OH		4.52 (d, 5.5)	51	149.2	
25	19.5	0.90 (s)	52	125.9	8.14 (s)
26	17.2	1.00 (d, 6.5)	53	163.5	

methine carbon at δ 69.5 (C-24) and oxygenated quaternary carbon at δ 75.2 (C-23). Moreover, long-range correlations from the methyl proton signal at δ 0.90 (CH_3 -25) to C-23, C-24 and C-22 (δ 54.9) were observed. The methine proton at δ 5.56 (H-22) was coupled to an amide proton at δ 8.17 (H-27) and showed a long-range correlation to δ 169.0 (C-21), which was a part of the thiazole unit, indicating the presence of the thiostreptin unit.¹² The proton signal of the thiazole unit at δ 8.15 (H-20) showed long-range coupling to δ 150.9 (C-18), which was C-2 of the pyridine unit. Furthermore, long-range coupling from the proton signal of the pyridine unit at δ 8.53 (H-16) to the quaternary carbon of the thiazole unit at δ 163.5 (C-53), and from H-15 at δ 8.34 to δ 168.2 (C-13) was observed. The presence of the long-range couplings from the proton signal of the third thiazole unit at δ 8.56 (H-12) to δ 149.8 (C-10), H-9 (δ 8.43) to the carbonyl carbon at δ 158.9 (C-7), and from the olefinic proton of the fourth thiazole unit at δ 8.14 (H-52) to the carbonyl carbon at δ 160.1 (C-50) indicated the presence of substructure B.

Substructure C

In the COSY spectrum, vicinal coupling signals were detected between the methyl protons at δ 0.86 (CH_3 -35)/ δ 0.88 (CH_3 -34) and the methine proton at δ 2.65 (H-33), H-33 and the methine proton at δ 5.11 (H-32), and the H-32 and the amide proton at δ 8.48 (H-36). Furthermore, the HMBC spectrum revealed the presence of substructure C by the long-range correlations from CH_3 -35 to the methyl carbon at δ 17.8 (C-34), H-32 to a quaternary carbon of the thiazole ring at δ 170.3 (C-31), and H-30 to the carbonyl carbon at δ 159.9 (C-28).

Substructure D

Substructure D was determined by the HMBC spectrum, which showed long-range coupling from an oxygenated methyl proton at δ 3.97 (H₃-43) to an olefinic carbon at δ 152.0 (C-42), and long-range correlations from the olefinic proton at δ 7.30 (H-42) to a quaternary carbon of the thiazole ring at δ 160.3 (C-40) and an olefinic carbon at δ 111.5 (C-41), an amide proton at δ 9.33 (H-44) to C-42, and the olefinic proton of the thiazole ring at δ 8.24 (H-39) to δ 160.1 (C-37).

Substructure E

Vicinal coupling signals in the COSY spectrum were observed between the methyl group at δ 1.27 (H-48) and the methine proton at δ 4.36 (H-47), H-47 and the hydroxy proton at δ 5.21, the methine proton at δ 4.52 (H-46) and H-46 and an amide proton at δ 8.06 (H-49). The long-range couplings in the HMBC spectrum from CH_3 -48 to C-46 and H-46 to a carbonyl carbon at δ 169.8 (C-45) revealed the presence of the threonine moiety in Substructure E.

Finally, each substructure was connected by the long-range correlations of the HMBC technique from the amide proton (H-6) to the carbonyl carbon (C-7), H-22 to C-28, H-32 to C-37, H-44 to C-45 and H-49 to C-50. Thus, the planar structure of nocardithiocin was determined as shown in Figure 1 and the ^1H and ^{13}C -NMR spectral data of nocardithiocin are summarized in Table 2.

Stereochemistry

To determine the absolute configurations of substructures C and E, the advanced Marfy method^{13,14} was performed. Nocardithiocin was first hydrolyzed with 6 M HCl and the liberated threonine (substructure E) and the valine-derived thiazole moiety (substructure C) were derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-leucineamide (L-FDLA) and D-FDLA. Figure 3 shows the HPLC chromatograms of the 2,4-dinitrophenyl-5-L-leucineamide (DLA) derivatives ((A) with L-FDLA and (B) with D-FDLA) of the hydrolyzate. Two desired peaks were observed together with large amounts of the residual reagent and related compounds (*). Furthermore, an intense peak was also found at around 4 min. These derivatives were also analyzed by LC/MS at m/z 414 and 459 in the positive ion mode (Figure 4).¹⁵ Using the DL-FDLA method,¹⁶ it was possible to conclude that the two amino acids have the L configuration. It was also found that the intense peak observed at around 4 min shown in Figure 3 has a molecular weight of 616. The geometries of the double bonds in substructures A and D and the absolute configuration of substructure B could not be determined in this study.

Antimicrobial activity

Nocardithiocin showed a strong antimicrobial activity against acid-fast bacteria but its activity against other Gram-positive microorganisms was moderate. The MIC values of the antibiotic against *Corynebacterium xerosis* and *Gordonia bronchialis* were <0.0078 and $0.039 \mu\text{g ml}^{-1}$, respectively (Table 3). As shown in Table 4, nocardithiocin showed a strong antimicrobial activity against the rifampicin-resistant strains of *M. tuberculosis* as well as the susceptible ones, and their MIC values were 0.025 – $6.25 \mu\text{g ml}^{-1}$ and 0.025 – $>6.25 \mu\text{g ml}^{-1}$, respectively. In the comparison of the antimicrobial activity of nocardithiocin with the reference drug of levofloxacin against rifampicin-susceptible as well as rifampicin-resistant mycobacteria, nocardithiocin was found to show an activity higher than that of levofloxacin. Nocardithiocin did not show any antimicrobial activities against gram-negative bacteria and fungi (Table 3). The cytotoxicity of nocardithiocin against LH-60 was $64 \mu\text{g ml}^{-1}$ (data not shown).

Figure 2 COSY and HMBC correlations of nocardithiocin.

Figure 3 HPLC chromatograms of the DLA (2,4-dinitrophenyl-5-L-leucineamide) derivatives of the reaction mixture with (a) L-FDLA and (b) D-FDLA. Asterisks indicate residual reagent and related compounds.

DISCUSSION

During our continuing studies of biologically active compounds from pathogenic actinomycetes, we have isolated many biologically active compounds from the *Nocardia* species. According to a review by Bagley *et al.*,⁶ the first thiopeptides produced by the *Nocardia* species to be identified were the nocardiacins, which belonged to series *e*

because they possessed a tetrasubstituted hydroxypyridine as the central heterocyclic domain. In this study, *Nocardia pseudobrasiliensis* (IFM 0757) obtained from a patient at the Health Center, University of Texas, was studied. The strain produced several active compounds, of which nocardicyclin A was characterized as a broth metabolite¹⁷ and nocardithiocin as a metabolite associated with the mycelium.

Figure 4 LC/MS data of the DLA derivatives of the reaction mixture with (A) L-FDLA and (B) L- + D-FDLA: (a) Total ion current chromatogram (TIC); (b) mass chromatogram at m/z 414; (c) mass chromatogram at m/z 459; (d) mass chromatogram at m/z 617.

Table 3 *In vitro* antimicrobial activity of nocardithiocin

Test organisms	MIC values ($\mu\text{g ml}^{-1}$)
<i>Micrococcus luteus</i>	>133
<i>Bacillus subtilis</i>	>133
<i>Gordonia bronchialis</i>	0.03
<i>Corynebacterium xerosis</i>	<0.0078
<i>Mycobacterium smegmatis</i>	0.062
<i>Nocardia asteroides</i>	0.062
<i>Escherichia coli</i>	>133
<i>Candida albicans</i>	>133
<i>Cryptococcus neoformans</i>	>133
<i>Aspergillus niger</i>	>133
<i>Paecilomyces variotii</i>	>133
<i>Trichophyton mentagrophytes</i>	>133

2% MeOH was added in broth to increase solubility of nocardithiocin.

The latter structurally belongs to the thiopeptide group of antibiotics and was classified into series *d*, because it has a 2,3,6-trisubstituted pyridine domain as shown in Figure 1. The identification of nocardithiocin was the second example of a thiopeptide from a *Nocardia* species. Nocardithiocin has a very similar thiopeptide nucleus to micrococins P1 and P2 but differs in having a side chain terminating in an *O*-methyl moiety (substructure A) and subtle differences to the thiopeptide ring substructures termed substructures B and D.⁶ Although the absolute configurations of C-32, -46 and -47 were firmly determined to be *S*, *S* and *R*, respectively, by advanced Marfey's method, those of C-23 and C-24 still remained unsolved. As men-

tioned above, the compound was slightly sensitive to light and the cause of this instability is currently under investigation.

During our screening program of antimicrobial compounds, we have found and reported a new anthracycline type antibiotic called nocardicyclin. The structures were characterized by 1- and 8-methyl groups, a 10-carbonyl group and novel carbon-methylated amino sugar constituent.¹⁷ Although the antibiotics exerted a cytotoxic activity against leukemia in mice, they showed anti-Gram-positive bacteria, especially a strong antimycobacterial activity. We also reported a new siderophore and a benzonoid compound called asterobactin and nocaracins, respectively.^{18,19} Interestingly, both the siderophore and benzonoid compounds also showed strong antimycobacterial activity. These studies suggested to us that the *Nocardia* strains are useful sources for new antimycobacterial compounds. Indeed, this study showed that a pathogenic *Nocardia* strain produced a unique antibiotic, nocardithiocin. Although the reason for higher productivity of the antimycobacterial substances in *Nocardia* strains is not clear, it may be because of the high similarity of *Nocardia* to *Mycobacterium* strains regarding their physiological characteristics such as cell membrane or cell wall permeability. Both genera belong to a special bacterial group of acid-fast bacteria.

METHODS

Instrumentation

The NMR experiments were performed using JEOL-A400, -EXP-500 and -A600 spectrometers (Tokyo, Japan) in DMSO- d_6 or CD₃OD at 40 °C. The MS spectra were measured by JEOL-HX110 and JEOL-JMS-AX500 spectrometers for the FAB/MS and a Finnigan MAT TSQ700 (San Jose, CA, USA) for the ESI LC/MS. High-resolution MS spectra were obtained using a JEOL-HX110 under FAB

Table 4 Antimicrobial activities of nocardithiocin against rifampicin-susceptible and -resistant *Mycobacterium tuberculosis*, and *M. avium* and *M. intracellulare*

Test organisms	Strain no.	MIC ($\mu\text{g ml}^{-1}$)		
		Nocardithiocin	LVFX	RFP
<i>M. tuberculosis</i>	H37Rv	0.78	0.39	0.39
Rifampicin-susceptible	40	0.78	0.2	0.1
	41	0.025	0.1	≤ 0.006
	43	0.78	3.13	6.25
	47	0.78	3.13	25
	68	1.56	0.39	3.13
	2	1.56	0.2	0.2
	3	1.56	0.39	0.2
	4	1.56	0.39	0.2
	5	6.25	0.2	0.39
	6	6.25	0.39	0.39
	7	6.25	0.39	0.39
	8	1.56	0.2	0.1
	9	3.13	0.39	0.2
Rifampicin-resistant	45	0.78	6.25	>100
	50	0.78	0.39	>100
	51	1.56	3.13	100
	56	0.025	6.25	>100
	71	>6.25	3.13	>100
	97	0.39	6.25	>100
	1	0.78	25	50
	3	1.56	3.13	>100
	4	0.39	6.25	>100
	5	0.025	25	>100
	7	>6.25	3.13	>100
	9	0.39	3.13	>100
	12	1.56	3.13	>100
<i>M. avium</i>	N-357	>6.25	3.13	50
	N-458	>6.25	1.56	>100
	N-444	>6.25	12.5	12.5
	N-472	>6.25	50	25
<i>M. intracellulare</i>	N-294	>6.25	12.5	3.13
	N-313	1.56	6.25	0.78
	N-338	>6.25	12.5	3.13
	N-345	>6.25	25	3.13

Abbreviations: LVFX, levofloxacin; MIC, minimum inhibitory concentration.

conditions. The UV spectra were recorded on a Shimadzu UV-2100 (Kyoto, Japan). The optical rotations were measured by a Jasco DIP-370 polarimeter (Tokyo, Japan). HPLC was carried out using the following two systems: pump; Shimadzu LC-9A, detector, Shimadzu SPD-10A (UV-VIS) or Shimadzu SPD-M10A (diode array) and pump; TOSOH CCPS, detector; TOSOH UV-8020 (Tokyo, Japan).

Fermentation

The seed broth was prepared by inoculating mycelial elements of *Nocardia pseudobrasiliensis* IFM 0757 grown on the Brain Heart Infusion broth (BHI; Difco Laboratories, Detroit, MI, USA) in 10 ml with 2% glucose in a 50 ml Erlenmeyer shake flask. The culture was incubated on a rotary shaker at 250 r.p.m. for 4 days. Ten percent of the inoculum was transferred to a 500 ml

Erlenmeyer shake flask containing 100 ml of the production medium (two times concentrated nutrient broth medium, Difco Laboratories) and the culture was incubated on a rotary shaker at 250 r.p.m. for 7 days.

Isolation

After centrifugation of the culture broth (21), the mycelia were subjected to extraction by MeOH with stirring. The extract was evaporated and the residue was subjected to liquid-liquid extraction with EtOAc and water. The EtOAc fraction was evaporated and the residue was separated by the following HPLC procedure under dark conditions to provide nocardithiocin in a pure state (10.5 mg): column, Cosmosil 5C18-ARII (250×10 mm ID, Nacalai Tesque, Kyoto, Japan); mobile phase, MeOH: 0.01% TFA=52:48; flow rate, 1.0 ml min⁻¹; detection; 254 nm.

Advanced Marfey's method

One milligram of nocardithiocin was dissolved in 500 μl of 6 M HCl, and then heated at 110 °C for 1 h under nitrogen. The reaction solution was evaporated to dryness and 150 μl of water and 50 μl of MeOH were added. The solution was divided into two portions and 20 μl of 1 M NaHCO₃ and 100 μl of 1% L-FDLA (1-fluoro-2,4-dinitrophenyl-5-L-leucineamide) or D-FDLA acetone solution were added to each portion. The solution was heated at 40 °C for 1 h and the reaction was quenched by the addition of 20 μl of 1 M HCl. After dilution with 220 μl of acetonitrile, 0.1 and 1 μl of the DLA derivatives of all the samples were analyzed by the following HPLC and ESILC/MS, respectively: column; TSK gel ODS-80Ts (150×4.6 mm ID, TOSOH), mobile phase; acetonitrile:0.05% TFA, gradient condition; acetonitrile 30→70%, flow rate; 1.0 ml min⁻¹, detection; 340 nm, column; TSK gel ODS-80Ts (150×2 mm ID, TOSOH), mobile phase; acetonitrile:0.05% TFA, gradient condition; acetonitrile 30→70%, flow rate; 0.2 ml min⁻¹, split ratio; 1:40, detection; MS (positive ion mode).

Antimicrobial activity measurement

The antimicrobial activities were determined by a method described earlier²⁰ using the microbroth dilution method, except for the determination of MIC values against the slowly growing mycobacteria. The antimicrobial activities against the slowly growing mycobacteria were determined by the agar dilution method using the MIDDLEBROOK 7H10 agar medium (BD, Difco). Inocula were prepared by incubation of the *Mycobacterium* species in MIDDLEBROOK (7H9) liquid medium at 37 °C for 2–3 weeks in a 5% CO₂ incubator. After adjustment of the cell concentration by the optical density measurement, the diluted cell suspensions were inoculated onto the MIDDLEBROOK 7H10 agar medium containing various drug concentrations. After incubation at 37 °C for 14 days (for *Mycobacterium avium* and *Mycobacterium intracellulare*) and 21 days (for *M. tuberculosis*), the MIC values were determined. The *M. tuberculosis* H37Rv strain was used as the standard strain. Rifampicin (REF; Sigma-Aldrich Co., Tokyo, Japan) and levofloxacin (Daiichi Seiyaku Co., Tokyo, Japan) were used as the reference drugs in this study.

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