Nocardimicins A, B, C, D, E, and F, Siderophores with Muscarinic M3 Receptor Inhibiting Activity from *Nocardia* sp. TP-A0674

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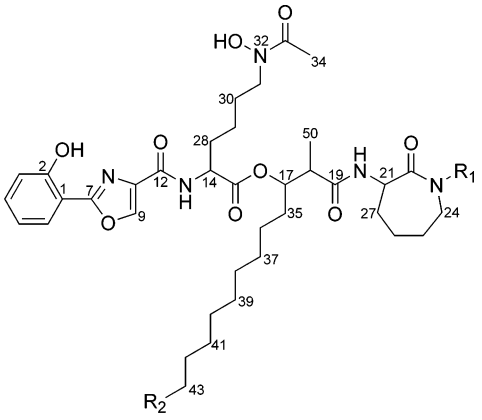
In the screening for muscarinic M3 receptor binding inhibitors from microbial secondary metabolites, the extract of *Nocardia* sp. TP-A0674 was found to be highly active. Bioassay-guided fractionation of it led to the isolation of six new siderophores, nocardimicins A (**1**), B (**2**), C (**3**), D (**4**), E (**5**), and F (**6**), as active principles. Their chemical structures were determined by spectroscopic and degradation analysis. Of these congeners, nocardimicin B (**2**) inhibited the binding of tritium-labeled *N*-methylscopolamine to the muscarinic M3 receptor most potently with a K_i value of 0.13 μM. Compound **2** showed more selective activity to M3 and M4 receptors than other subtypes.

Acetylcholine was first identified in 1914 by Henry Hallett Dale, then confirmed as a neurotransmitter by Otto Loewi.[1] It plays many pharmacological roles that are mediated by muscarinic and nicotinic receptors in peripheral and central nervous systems. Five muscarinic receptors, M1−M5, have been identified and cloned to date.[2] Of these, we are interested in the M3 receptor because it can be a therapeutically important target for the treatment of respiratory disorders such as chronic obstructive pulmonary disease, gastrointestinal disorders such as irritable bowel syndrome, and urinary tract disorders such as urinary incontinence.[3]

In the course of our screening for muscarinic M3 receptor binding inhibitors from natural sources, a new pyrrolizidine alkaloid, cremastrine, was found to inhibit selectively the muscarinic M3 receptor binding from *Cremastra appendiculata* (Orchidaceae).[4] In our ongoing search for muscarinic M3 inhibitors from natural products, six new siderophores, nocardimicins A (**1**), B (**2**), C (**3**), D (**4**), E (**5**), and F (**6**), were isolated from the culture of *Nocardia* sp. TP-A0674 as active principles. This paper describes the taxonomy of the producing strain, fermentation, isolation, structure determination, and biological properties of **1**−**6**.



Nocardimicin A (**1**):R_1=-OH, R_2=-H
B (**2**):R_1=-OH, R_2=-C_2H_5
C (**3**):R_1=-H, R_2=-C_2H_5
D (**4**):R_1=-OH, R_2=-C_4H_9
E (**5**):R_1=-H, R_2=-C_4H_9
F (**6**):R_1=-OH, R_2=-C_6H_{13}

Results and Discussion

The mycelial cake of *Nocardia* sp. TP-A0674 was extracted with acetone, and the acetone solution was concentrated. The remaining aqueous solution was chromatographed on a porous-polymer resin Diaion HP-20 column with a gradient of aqueous acetone. The active fractions were concentrated and fractionated by reversed-phase preparative HPLC. On the basis of the muscarinic M3 receptor binding assay, the active fractions were further purified using preparative HPLC to afford six new siderophores, nocardimicins A−F (**1**−**6**), as active principles.

Nocardimicin B (**2**) was obtained as a pale yellow amorphous solid with a melting point of 133−137 °C. The molecular formula of **2** was established as $C_{39}H_{59}O_{10}N_5$ on the basis of the high-resolution ESI-LC-MS and ^{13}C NMR data. The ^1H NMR spectrum of **2** indicated the presence of two amide protons (δ 9.46 and 8.67), a 1,2-disubstituted benzene moiety (δ 7.79, 7.35, 7.06, and 6.95), and three methyl groups (δ 2.32, 1.30, and 0.87). The ^{13}C NMR and DEPT spectra revealed the presence of 39 carbon signals comprising five carbonyl carbons (δ 173.3, 171.8, 171.7, 169.6, and 160.7), nine aromatic carbons (δ 161.0, 157.4, 141.3, 137.0, 133.2, 127.0, 119.9, 117.7, and 111.2), one oxymethine carbon (δ 76.7), two nitrogen-bearing methylene carbons (δ 53.1 and 47.9), two nitrogen-bearing methine carbons (δ 53.0 and 51.8), and one acetyl carbon (δ 20.8). The remaining carbon signals were assigned to two methyl, 16 methylene, and one methine carbon (Table 1).

Detailed analysis of 2D-NMR spectra revealed that **2** was comprised of four partial structures, A−D: a hydroxyphenyloxazoline moiety, a fatty acid moiety, and two lysine-derived moieties (Figure 1). The partial structure A was confirmed by HMBC correlations from H-3 to C-1, C-2, C-4, and C-5, from H-6 to C-1, C-2, C-5, and C-7, and from H-9 to C-7, C-10, and C-12. The proton spin network from H-13 to H-31 was confirmed by COSY and TOCSY correlations. HMBC correlations from ϵ-methylene proton H-31 and a singlet methyl proton H-34 to C-33 indicated the attachment of an acetyl group at N-32. The partial structure B

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Table 1. ^{13}C NMR Data (δ) for **1**–**6** in Pyridine-d_5 (27 °C)

position	**1**	**2**	**4**	**6**	**3**	**5**
1	111.2 (s)	111.2 (s)	111.2 (s)	111.2 (s)	111.2 (s)	111.2 (s)
2	157.4 (s)	157.4 (s)	157.4 (s)	157.4 (s)	157.5 (s)	157.5 (s)
3	117.7 (d)	117.7 (d)	117.7 (d)	117.7 (d)	117.8 (d)	117.8 (d)
4	133.2 (d)	133.2 (d)	133.2 (d)	133.2 (d)	133.2 (d)	133.2 (d)
5	119.9 (d)	119.9 (d)	119.9 (d)	119.9 (d)	119.8 (d)	119.8 (d)
6	127.0 (d)	127.0 (d)	127.0 (d)	127.0 (d)	126.9 (d)	126.9 (d)
7	161.0 (s)	161.0 (s)	161.0 (s)	161.0 (s)	160.9 (s)	160.9 (s)
9	141.3 (d)	141.3 (d)	141.3 (d)	141.3 (d)	141.2 (d)	141.3 (d)
10	137.0 (s)	137.0 (s)	136.9 (s)	137.0 (s)	137.1 (s)	137.1 (s)
12	160.7 (s)	160.7 (s)	160.7 (s)	160.7 (s)	160.7 (s)	160.7 (s)
14	53.1 (d)	53.0 (d)	53.1 (d)	53.1 (d)	53.0 (d)	53.0 (d)
15	171.7 (s)	171.7 (s)	171.7 (s)	171.7 (s)	171.7 (s)	171.7 (s)
17	76.7 (d)	76.7 (d)	76.7 (d)	76.7 (d)	76.8 (d)	76.8 (d)
18	44.9 (d)	44.9 (d)	44.9 (d)	44.9 (d)	44.9 (d)	44.9 (d)
19	173.3 (s)	173.3 (s)	173.4 (s)	173.4 (s)	173.3 (s)	173.3 (s)
21	51.8 (d)	51.8 (d)	51.8 (d)	51.8 (d)	52.3 (d)	52.3 (d)
22	169.6 (s)	169.6 (s)	169.6 (s)	169.6 (s)	176.4 (s)	176.4 (s)
24	53.1 (t)	53.1 (t)	53.1 (t)	53.1 (t)	41.7 (t)	41.7 (t)
25	26.4 (t)	26.4 (t)	26.4 (t)	26.4 (t)	29.3 (t)	29.3 (t)
26	28.1 (t)	28.1 (t)	28.1 (t)	28.1 (t)	28.5 (t)	28.6 (t)
27	31.1 (t)[a]	31.1 (t)[a]	31.1 (t)[a]	31.1 (t)[a]	31.4 (t)[a]	31.4 (t)[a]
28	31.6 (t)[a]	31.6 (t)[a]	31.6 (t)[a]	31.6 (t)[a]	31.7 (t)[a]	31.7 (t)[a]
29	23.8 (t)	23.8 (t)	23.8 (t)	23.8 (t)	23.8 (t)	23.8 (t)
30	27.0 (t)	27.0 (t)	27.0 (t)	27.0 (t)	27.0 (t)	27.0 (t)
31	48.0 (t)	47.9 (t)	47.9 (t)	47.9 (t)	48.1 (t)	48.1 (t)
33	171.8 (s)	171.8 (s)	171.8 (s)	171.8 (s)	171.8 (s)	171.8 (s)
34	20.8 (q)	20.8 (q)	20.8 (q)	20.8 (q)	20.8 (q)	20.8 (q)
35	31.8 (t)[a]	31.8 (t)[a]	31.8 (t)[a]	31.8 (t)	31.9 (t)[a]	31.9 (t)[a]
36	24.9 (t)	24.9 (t)	25.0 (t)	25.0 (t)	24.8 (t)	24.8 (t)
37	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]
38	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]
39	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]
40	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]
41	32.1 (t)[a]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]
42	22.9 (t)	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]
43	14.3 (q)	32.1 (t)[a]	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	32.1 (t)[a]	30.0–29.6 (t)[b]
44		22.9 (t)	30.0–29.6 (t)[b]	30.0–29.6 (t)[b]	22.9 (t)	30.0–29.6 (t)[b]
45		14.3 (q)	32.2 (t)[a]	30.0–29.6 (t)[b]	14.3 (q)	32.2 (t)[a]
46			23.0 (t)	30.0–29.6 (t)[b]		23.0 (t)
47			14.3 (q)	32.2 (t)[a]		14.3 (q)
48				23.0 (t)		
49				14.3 (q)		
50	14.5 (q)	14.5 (q)	14.6 (q)	14.6 (q)	14.7 (q)	14.7 (q)

[a] Assignments may be interchangeable. [b] Overlapped each other.

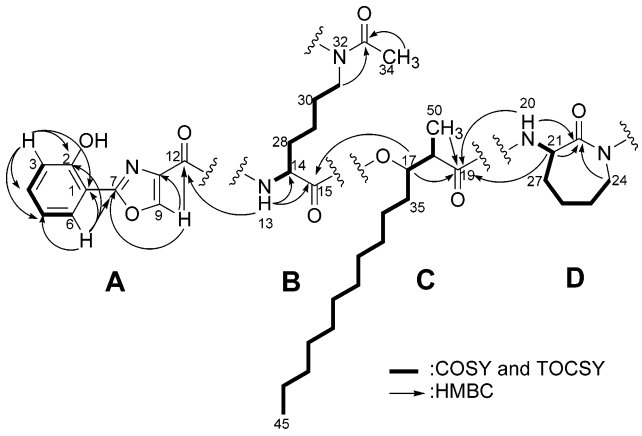

Figure 1. Key COSY and HMBC correlations for **2**.

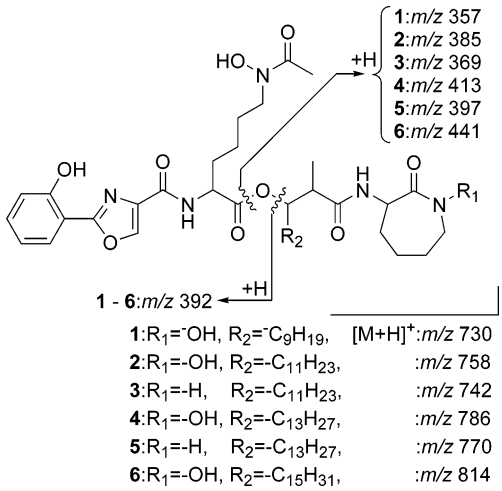

Figure 2. Fragment ions of **1**–**6** in LC-MS/MS (positive mode).

was thus determined as N_ϵ-acetyllysine. The partial structure C was deduced as 2-methyl-3-hydroxytetradecanoic acid on the basis of the analysis of COSY and TOCSY spectra, together with the HMBC correlations from H-17 and H-50 to C-19. The partial structure D was confirmed as ϵ-aminocaprolactam by the long-range couplings from the α-proton H-21, amide proton H-20, and the ϵ-methylene proton H-24 to the carbonyl carbon C-22.

The connectivities of partial structures were elucidated by the HMBC experiment (Figure 1). The connectivity between the partial structures A and B was confirmed by the long-range coupling from H-13 to C-12. The HMBC correlation from H-17 to C-15 established the linkage between partial structures B and C through an ester bond, and that from H-20 and H-21 to C-19 revealed the linkage of the partial structures C and D through an amide bond. Taking the molecular formula of **2** into consideration, the remaining two hydroxyl groups were assigned to substituents at the nitrogen atoms N-23 and N-32.

In the positive LC-MS/MS experiment of **2**, fragment ions corresponding to a mycobactic acid moiety (**7**) and a cobactine moiety (**8**)[5] were observed at *m/z* 392 and 385, respectively (Figure 2). In addition, alkaline hydrolysis of

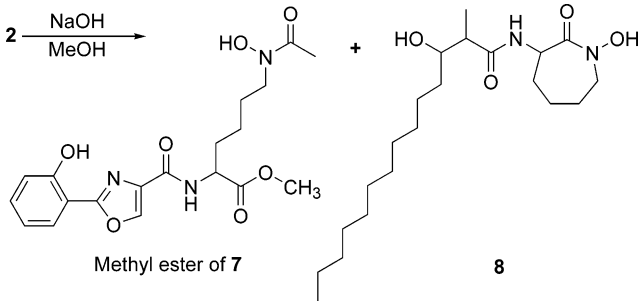

Figure 3. Alkaline hydrolysis of **2**.

Table 2. K_i Values (μM) of **1**−**6** to Muscarinic M3 Receptor

compound	K_i[a]
1	1.25 (5.89)
2	0.13 (0.63)
3	1.00 (4.70)
4	0.23 (1.10)
5	0.97 (4.56)
6	1.33 (6.29)

[a] IC_{50} values (μM) are in parentheses.

2 gave the methyl ester of **7** and **8** (Figure 3). These data supported the structure assigned to nocardimicin B (**2**).

The molecular formulas of nocardimicins A (**1**), D (**4**), and F (**6**) were established as $C_{37}H_{55}O_{10}N_5$, $C_{41}H_{63}O_{10}N_5$, and $C_{43}H_{67}O_{10}N_5$, respectively, on the basis of the HR-LC-MS and ^{13}C NMR data. These compounds showed ^1H and ^{13}C NMR spectra quite similar to those of nocardimicin B (**2**). In the LC-MS/MS spectra, the fragment ion derived from the mycobactic acid moiety was detected at *m/z* 392, as in the case of **2**. On the other hand, the fragment mass derived from the cobactin moiety of **1** was 28 mass units smaller (*m/z* 357), that of **4** was 28 mass units larger (*m/z* 413), and that of **6** was 56 mass units larger (*m/z* 441), indicating that these compounds are the homologues of **2** differing in the fatty acid chain length.

In the ^1H NMR spectrum of nocardimicin C (**3**), an amide proton (δ 8.86, H-23) was observed as a triplet peak instead of an *N*-hydroxyl proton in nocardimicin B (**2**) and was coupled to the ϵ-methylene H-24. Chemical shifts of H-24 (δ 3.30) and C-24 (δ 41.7) were high-field shifted, whereas the carbonyl carbon C-22 of **3** was low-field shifted to δ 176.4 in comparison to compound **2**. In the LC-MS/MS spectrum of **3**, the fragment ion for the cobactin moiety was detected at *m/z* 369, 16 mass units smaller than that of **2**, while the fragment mass (*m/z* 392) for the mycobactic acid moiety was identical to that of **2**. In addition, the molecular formula was calculated as $C_{39}H_{59}O_9N_5$, indicating the lack of an oxygen atom from **2**, on the basis of the HR-LC-MS. These observations confirmed that the hydroxyl group at N-23 of **2** is replaced with a hydrogen atom in **3**.

The NMR spectra of nocardimicin E (**5**) were very close to those of nocardimicin C (**3**). The molecular formula was determined as $C_{41}H_{63}O_9N_5$ on the basis of the HR-LC-MS and ^{13}C NMR data. In the LC-MS/MS spectrum of **5**, the fragment ion for the mycobactic acid moiety was identical to that for other congeners (*m/z* 392), but the fragment ion derived from the cobactin moiety appeared at *m/z* 397, 28 mass units larger than that of **3**. Therefore, **5** was determined to be a homologue of **3**, in which the fatty acid chain length was two carbons longer than **3**.

The inhibition activities of **1**−**6** to the muscarinic M3 receptor are summarized in Table 2. Compound **2** showed the most potent activity among the congeners. It inhibited the binding of tritium-labeled *N*-methylscopolamine ([^3H]-NMS) to the muscarinic M3 receptor with a K_i value of 0.13 μM (IC_{50} = 0.63 μM). Compound **4**, which has a two carbons longer alkyl chain than **2**, was about two times weaker than **2**. In addition, compounds **1** and **6**, the alkyl chains of which are two carbons shorter and four carbons longer, respectively, showed about 10 times weaker activity than **2**. Compound **3** was about seven times less potent than **2**, indicating that the loss of an *N*-hydroxyl group in the caprolactam moiety lessened the activity. A similar tendency was observed in the case of **4** and **5**. These findings suggest that the fatty acid chain moiety and the *N*-hydroxyl group of the caprolactam moiety play a role in the interaction of these compounds with the muscarinic M3 receptor.

Table 3. K_i Values (μM) of **2** to Five Subtypes: M1−M5

subtype	K_i[a]
M1	0.49 (2.03)
M2	0.63 (1.78)
M3	0.13 (0.63)
M4	0.14 (1.04)
M5	1.18 (1.64)

[a] IC_{50} values (μM) are in parentheses.

In a comparative study of five subtypes of muscarinic receptors, compound **2** showed more potent inhibition against M3 and M4 receptors, with K_i values of 0.13 and 0.14 μM, respectively, than other subtypes (Table 3).

Nocardimicins A−F (**1**−**6**) are classified as siderophores, defined as relatively low molecular weight compounds with high affinity for ferric ion.[6] A large number of siderophores with different types of structures have been isolated from microorganisms.[7a,b] The chemical structures of **1**−**6** are closely related to those of nocobactin NA,[8] formobactin,[9] and amamistatins.[10] Although a variety of pharmacological activities of siderophores, such as iron-binding ability,[8] free radical scavenging,[9] protection of neuronal cells,[9] growth inhibition of human tumor cells,[10,11,12a,b,13] immunosuppressive activity,[14] antibiotic activity,[13,15a,b] and inhibition of HIV proliferation,[12b] are known, their inhibition activity of the muscarinic receptors has not been reported so far. Nocardimicins are the first example of siderophores that have demonstrated inhibition activity to the muscarinic M3 receptor. Further studies on the stereochemistry and the pharmacological functions of **1**−**6** are in progress.

Experimental Section

General Experimental Procedures. Melting points were determined on a Yanaco micro melting point apparatus. Optical rotations were measured on a JASCO P-1020 digital polarimeter. NMR spectra were measured on a Bruker AMX-500 spectrometer using standard Bruker pulse programs. Chemical shifts are given in δ values with reference to tetramethylsilane as an internal standard. IR spectra were recorded on a Perkin-Elmer 1725X FT-TR spectrophotometer. LC-MS spectra were measured on an Agilent MSD spectrometer, and LC-MS/MS spectra were measured on a Finnigan TSQ QUANTUM Ultra spectrometer and a Finnigan TSQ 7000 spectrometer. High-resolution MS spectra were measured on a JEOL JMS-700 spectrometer. UV spectra were recorded on a Shimadzu UV-240 and a Hitachi U-3010 spectrophotometer.

***Nocardia* sp. TP-A0674.** The producing microorganism, strain TP-A0674, was isolated from a soil sample collected at Keta, Ishikawa, Japan. A pure culture of strain TP-A0674 was preserved in 20% glycerol at −80 °C. It was also maintained on an ISP medium No. 5 slant at 10 °C for laboratory use.

By scanning electron microscope, strain TP-A0674 formed spiral type spore chains and the spores were cylindrical with a warty surface. The aerial mass color was grayish olive, and the color of the reverse side was pale yellow. Diffusible pigments were not formed. Strain TP-A0674 utilized D-

glucose, sucrose, inositol, D-mannitol, D-raffinose, and L-arabinose for growth. Whole cell hydrolysates contained *meso*-diaminopimelic acid, glycine, ribose, arabinose, and galactose. In addition, sequencing analysis[16] revealed that 16S ribosomal DNA from the strain had 99.11% identity with *Nocardia niigatensis* IFM0833.[17] Thus the strain was identified as *Nocardia* sp. TP-A0674. The strain is currently on deposit in the International Patent Organism Depository (IPOD, Tsukuba, Japan).

Fermentation of *Nocardia* sp. TP-A0674. A loopful of a mature slant culture of *Nocardia* sp. TP-A0674 was inoculated into five 500 mL K-1 flasks containing 100 mL of the seed medium consisting of soluble starch 1%, glucose 0.5%, NZ-case 0.3%, yeast extract 0.2%, tryptone 0.5%, K_2HPO_4 0.1%, $MgSO_4$ 0.05%, and $CaCO_3$ 0.3% (pH 7.0). The flask was incubated at 32 °C for 5 days on a rotary shaker (200 rpm). Three-milliliter aliquots of the seed culture were transferred into fifty 500 mL K-1 flasks each containing 100 mL of the production medium consisting of glucose 0.5%, glycerol 2%, soluble starch 2.0%, Pharmamedia 1.5%, yeast extract 0.3%, and Diaion HP-20 1% (pH 7.0). Fermentation was carried out at 32 °C for 5 days on a rotary shaker (200 rpm).

Extraction and Separation. The fermented whole broth (5 L) was centrifuged (5000 rpm, 10 min) to separate the mycelium. The supernatant was discarded, and the mycelium was extracted by agitating in acetone (4 L). The mycelium was removed by centrifugation, and acetone was removed by evaporation. The resultant aqueous solution was applied to a column of Diaion HP-20 (700 mL), and the column was eluted with a stepwise gradient of aqueous acetone (0, 20, 40, 60, 80, and 100%). The active fractions (80 and 100% acetone eluents) were combined and evaporated in vacuo to dryness. The active portion (1.5 g) was fractionated into four active fractions (fractions A−D) by preparative HPLC using an ODS column (Delta-Pak C_{18}, 40 mm i.d. × 100 mm, Waters, MA) with CH_3CN−0.2% HOAc (a linear gradient from 50 to 100% of CH_3CN over 20 min) at a flow rate of 80 mL/min. Fraction A (25.3 mg) was subjected to preparative HPLC using an ODS column (XTerra Prep RP18 C_{18}, 19 mm i.d. × 150 mm, Waters, MA) with the eluent of CH_3CN−10 mM ammonium acetate (pH 9.0, isocratic elution, 50:50) at a flow rate of 20 mL/min. On the basis of results of bioassay, active fractions were combined and evaporated in vacuo to give **1** (4.9 mg) as active principle. Similarly, **2** (8.0 mg) and **3** (3.2 mg), **4** (20.1 mg) and **5** (3.4 mg), and **6** (5.8 mg) were isolated from fraction B (31.8 mg), fraction C (42.9 mg), and fraction D (33.5 mg), respectively.

Nocardimicin A (1): pale yellow amorphous solid; mp 115−118 °C; $[\alpha]^{25}_D$ −5.1° (*c* 0.45, MeOH); UV (MeOH) λ_{max} (log ϵ) 263 (3.91), 275 (3.77), 308 (3.59); IR (film) ν_{max} 2928, 2360, 1739, 1645, 1488, 1256, 754 cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz) δ 11.67 (1H, br s, −O*H*), 11.5−10.5 (2H, br s, −O*H*), 9.46 (1H, d, J = 8.1 Hz, −N*H*, H-13), 8.79 (1H, s, H-9), 8.67 (1H, d, J = 6.7 Hz, −N*H*, H-20), 7.79 (1H, dd, J = 7.9, 1.2 Hz, H-6), 7.35 (1H, dt, J = 7.7, 1.3 Hz, H-4), 7.06 (1H, d, J = 8.3 Hz, H-3), 6.95 (1H, br d, J = 7.5 Hz, H-5), 5.60 (1H, dt, J = 8.2, 3.3 Hz, H-17), 5.23 (1H, m, H-14), 5.05 (1H, br dd, J = 9.8, 7.5 Hz, H-21), 3.94 (2H, m, H-24), 3.83 (2H, m, H-31), 3.05 (1H, dq, J = 8.5, 7.2 Hz, H-18), 2.32 (3H, s, H-34), 2.22−2.14 (1H, m, H-28a), 2.11−2.00 (2H, m, H-27a, H-28b), 1.92−1.77 (5H, m, H-26a, H-27b, H-30, H-35a), 1.77−1.70 (1H, m, H-29a), 1.70−1.57 (5H, m, H-25, H-26b, H-29b, H-35b), 1.52−1.36 (2H, m, H-36), 1.30 (3H, d, J = 7.1 Hz, H-50), 1.30−1.15 (8H, m, H-37−H-42), 0.87 (3H, br t, J = 7.1 Hz, H-45); ^{13}C NMR, see Table 1; HR-ESI-MS *m/z* 730.3999 $[M + H]^+$ (calcd for $C_{37}H_{56}O_{10}N_5$, 730.4027).

Nocardimicin B (2): pale yellow amorphous solid; mp 133−137 °C; $[\alpha]^{25}_D$ −5.9° (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ϵ) 262 (3.91), 274 (3.78), 309 (3.58); IR (film) ν_{max} 2929, 2361, 1739, 1645, 1489, 1256, 751 cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz) δ 11.67 (1H, br s, −O*H*), 11.5−10.5 (2H, br s, −O*H*), 9.46 (1H, d, J = 8.3 Hz, −N*H*, H-13), 8.79 (1H, s, H-9), 8.67 (1H, d, J = 7.0 Hz, −N*H*, H-20), 7.79 (1H, dd, J = 7.6, 1.9 Hz, H-6), 7.35 (1H, dt, J = 7.8, 1.6 Hz, H-4), 7.06 (1H, d, J = 8.3 Hz, H-3), 6.95 (1H, dt, J = 7.5, 1.0 Hz, H-5), 5.60 (1H, dt, J = 8.2, 3.3 Hz, H-17), 5.23 (1H, m, H-14), 5.04 (1H, br dd, J = 9.6, 7.3 Hz, H-21), 3.94 (2H, m, H-24), 3.83 (2H, m, H-31), 3.04 (1H, dq, J = 8.7, 7.0 Hz, H-18), 2.32 (3H, s, H-34), 2.22−2.14 (1H, m, H-28a), 2.11−2.00 (2H, m, H-27a, H-28b), 2.00−1.82 (5H, m, H-26a, H-27b, H-30, H-36a), 1.78−1.57 (6H, m, H-25, H-26b, H-29, H-36b), 1.52−1.36 (2H, m, H-35), 1.30 (3H, d, J = 7.0 Hz, H-50), 1.30−1.15 (12H, m, H-37−H-44), 0.87 (3H, br t, J = 7.0 Hz, H-45); ^{13}C NMR, see Table 1; HR-ESI-MS *m/z* 758.4348 $[M + H]^+$ (calcd for $C_{39}H_{60}O_{10}N_5$, 758.4340).

Nocardimicin C (3): pale yellow amorphous solid; mp 46−49 °C; $[\alpha]^{25}_D$ −13.3° (*c* 0.29, MeOH); UV (MeOH) λ_{max} (log ϵ) 263 (3.91), 275 (3.79), 309 (3.58); IR (film) ν_{max} 2928, 2360, 1739, 1645, 1489, 1256, 751 cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz) δ 11.71 (1H, br s, −O*H*), 10.98 (1H, br s, −O*H*), 9.59 (1H, d, J = 8.7 Hz, −N*H*, H-13), 8.86 (1H, t, J = 6.1 Hz, −N*H*, H-23), 8.79 (1H, s, H-9), 8.72 (1H, overlapped with pyridine, −N*H*, H-20), 7.79 (1H, br d, J = 7.9 Hz, H-6), 7.36 (1H, br t, J = 8.0 Hz, H-4), 7.09 (1H, d, J = 8.3 Hz, H-3), 6.95 (1H, br t, J = 7.6 Hz, H-5), 5.58 (1H, dt, J = 8.2, 3.1 Hz, H-17), 5.25 (1H, dt, J = 9.3, 4.1 Hz, H-14), 5.04 (1H, br t, J = 9.2 Hz, H-21), 3.85 (2H, m, H-31), 3.30 (2H, m, H-24), 3.03 (1H, dq, J = 9.0, 7.0 Hz, H-18), 2.32 (3H, s, H-34), 2.20−2.13 (1H, m, H-28a), 2.11−2.00 (2H, m, H-27a, H-28b), 2.00−1.85 (4H, m, H-26a, H-27b, H-30a, H-36a), 1.75−1.62 (6H, m, H-25a, H-26b, H-29, H-30b, H-36b), 1.50−1.35 (3H, m, H-25b, H-35), 1.30 (3H, d, J = 6.9 Hz, H-50), 1.30−1.15 (12H, m, H-37−H-44), 0.87 (3H, br t, J = 6.9 Hz, H-45); ^{13}C NMR, see Table 1; HR-ESI-MS *m/z* 742.4387 $[M + H]^+$ (calcd for $C_{39}H_{60}O_9N_5$, 742.4391).

Nocardimicin D (4): pale yellow amorphous solid; mp 126−129 °C; $[\alpha]^{25}_D$ −4.8° (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 262 (3.90), 274 (3.78), 309 (3.59); IR (film) ν_{max} 2925, 2361, 1739, 1645, 1489, 1256, 751 cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz) δ 11.67 (1H, br s, −O*H*), 11.5−10.5 (2H, br s, −O*H*), 9.45 (1H, d, J = 8.2 Hz, −N*H*, H-13), 8.78 (1H, s, H-9), 8.66 (1H, d, J = 6.9 Hz, −N*H*, H-20), 7.79 (1H, br d, J = 7.8 Hz, H-6), 7.35 (1H, br t, J = 7.2 Hz, H-4), 7.06 (1H, d, J = 8.3 Hz, H-3), 6.95 (1H, br t, J = 7.5 Hz, H-5), 5.59 (1H, dt, J = 8.1, 2.9 Hz, H-17), 5.22 (1H, m, H-14), 5.04 (1H, br dd, J = 9.6, 7.5 Hz, H-21), 3.94 (2H, m, H-24), 3.83 (2H, m, H-31), 3.03 (1H, dq, J = 7.6, 7.0 Hz, H-18), 2.31 (3H, s, H-34), 2.22−2.14 (1H, m, H-28a), 2.11−2.00 (2H, m, H-27a, H-28b), 1.92−1.77 (5H, m, H-26a, H-27b, H-30, H-35a), 1.77−1.70 (1H, m, H-29a), 1.70−1.57 (5H, m, H-25, H-26b, H-29b, H-35b), 1.52−1.36 (2H, m, H-36), 1.29 (3H, d, J = 6.9 Hz, H-50), 1.30−1.15 (16H, m, H-37-H-46), 0.87 (3H, br t, J = 6.8 Hz, H-45); ^{13}C NMR, see Table 1; HR-ESI-MS *m/z* 786.4635 $[M + H]^+$ (calcd for $C_{41}H_{64}O_{10}N_5$, 786.4653).

Nocardimicin E (5): pale yellow amorphous; mp 48−52 °C; $[\alpha]^{25}_D$ −15.2° (*c* 0.25, MeOH); UV (MeOH) λ_{max} (log ϵ) 263 (3.90), 275 (3.77), 308 (3.58); IR (film) ν_{max} 2924, 2360, 1731, 1645, 1488, 1256, 751 cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz) δ 11.72 (1H, br s, −O*H*), 10.75 (1H, br s, −O*H*), 9.59 (1H, d, J = 8.7 Hz, −N*H*, H-13), 8.86 (1H, t, J = 6.1 Hz, −N*H*, H-23), 8.81 (1H, s, H-9), 8.72 (1H, overlapped with pyridine, −N*H*, H-20), 7.79 (1H, dd, J = 8.0, 1.6 Hz, H-6), 7.36 (1H, dt, J = 7.8, 1.6 Hz, H-4), 7.09 (1H, d, J = 7.9 Hz, H-3), 6.95 (1H, dt, J = 7.5, 0.7 Hz, H-5), 5.57 (1H, dt, J = 8.3, 3.3 Hz, H-17), 5.24 (1H, dt, J = 9.5, 4.3 Hz, H-14), 5.03 (1H, br t, J = 8.7 Hz, H-21), 3.84 (2H, m, H-31), 3.30 (2H, m, H-24), 3.02 (1H, dq, J = 9.2, 7.0 Hz, H-18), 2.31 (3H, s, H-34), 2.20−2.13 (1H, m, H-28a), 2.11−2.00 (2H, m, H-27a, H-28b), 2.00−1.85 (4H, m, H-26a, H-27b, H-30a, H-35a), 1.75−1.62 (6H, m, H-25a, H-26b, H-29, H-30b, H-35b), 1.50−1.35 (3H, m, H-25b, H-36), 1.30 (3H, d, J = 6.9 Hz, H-50), 1.30−1.15 (16H, m, H-37-H-46), 0.88 (3H, br t, J = 6.9 Hz, H-45); ^{13}C NMR, see Table 1; HR-ESI-MS *m/z* 770.4682 $[M + H]^+$ (calcd for $C_{41}H_{64}O_9N_5$, 770.4704).

Nocardimicin F (6): pale yellow amorphous; mp 109−113 °C; $[\alpha]^{25}_D$ −9.7° (*c* 0.29, MeOH); UV (MeOH) λ_{max} (log ϵ) 262 (3.91), 274 (3.78), 309 (3.58); IR (film) ν_{max} 2925, 2360, 1731, 1645, 1489, 1256, 751 cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz) δ 11.67 (1H, br s, −O*H*), 11.5−10.5 (2H, br s, −O*H*), 9.46 (1H, d, J = 8.3 Hz, −N*H*, H-13), 8.78 (1H, s, H-9), 8.67 (1H, d, J = 6.9 Hz, −N*H*, H-20), 7.79 (1H, dd, J = 7.9, 1.4 Hz, H-6), 7.35 (1H, dt, J = 7.8, 1.6 Hz, H-4), 7.06 (1H, d, J = 8.3 Hz, H-3), 6.95 (1H, br t, J = 7.4 Hz, H-5), 5.59 (1H, dt, J = 8.3, 3.3 Hz,

H-17), 5.22 (1H, m, H-14), 5.04 (1H, br dd, J = 9.2, 7.4 Hz, H-21), 3.94 (2H, m, H-24), 3.83 (2H, m, H-31), 3.04 (1H, dq, J = 8.7, 7.0 Hz, H-18), 2.31 (3H, s, H-34), 2.22−2.14 (1H, m, H-28a), 2.11−2.00 (2H, m, H-27a, H-28b), 1.92−1.77 (5H, m, H-26a, H-27b, H-30, H-35a), 1.77−1.70 (1H, m, H-29a), 1.70−1.57 (5H, m, H-25, H-26b, H-29b, H-35b), 1.52−1.36 (2H, m, H-36), 1.30 (3H, d, J = 7.0 Hz, H-50), 1.30−1.15 (20H, m, H-37−H-48), 0.87 (3H, br t, J = 7.0 Hz, H-45); ^{13}C NMR, see Table 1; HR-ESI-MS *m/z* 814.4987 $[M + H]^+$ (calcd for $C_{43}H_{68}O_{10}N_5$, 814.4966).

Alkaline Hydrolysis of 2. Fifty microliters of 1 N NaOH (MeOH) was added to a solution of **2** (1.0 mg) in MeOH (1.0 mL), and the solution was allowed to stand for 22 h at ambient temperature. The reaction mixture was neutralized with 1 N HCl and purified to afford the methyl ester of **7** (0.2 mg) and **8** (0.3 mg) by preparative HPLC under the following conditions: column, XTerra Prep RP18 column (7.8 mm i.d. × 150 mm, Waters, MA); eluent, CH_3CN−10 mM ammonium acetate (pH 9.0, a linear gradient from 5% to 100% of CH_3CN over 20 min); flow rate, 3.0 mL/min; oven temperature, ambient; detection, UV at 220 nm. Methyl ester of **7**: ^{1}H NMR (pyridine-d_5, 500 MHz) δ 11.81 (1H, br s), 11.0−10.5 (1H, br s), 9.48 (1H, d, J = 7.9 Hz), 8.82 (1H, s), 7.82 (1H, dd, J = 7.9, 1.6 Hz), 7.34 (1H, dt, J = 7.4, 1.7 Hz), 7.09 (1H, d, J = 8.7 Hz), 6.94 (1H, t, J = 7.1 Hz), 3.99 (1H, m), 3.86 (1H, m), 3.65 (3H, s), 2.35 (3H, br s), 2.2−1.6 (6H, m); LC-MS *m/z* 406 $[M + H]^+$, *m/z* 404 $[M - H]^-$. **8**: ^{1}H NMR (pyridine-d_5, 500 MHz) δ 8.83 (1H, d, J = 6.6 Hz), 4.05 (1H, m), 3.86 (2H, m), 2.78 (1H, m), 2.2−1.5 (10H, m), 1.41 (3H, d, J = 7.0 Hz), 1.3−1.2 (16H, m), 0.86 (3H, t, J = 7.1 Hz); LC-MS *m/z* 385 $[M + H]^+$, *m/z* 383 $[M - H]^-$.

Muscarinic Receptor Binding Assay. According to the method previously described,[4] the binding affinities (K_i) to five subtypes were determined by inhibition of specific binding of [^{3}H]-NMS using the human receptor membranes at MDS Pharma Services (Taiwan). In competitive experiments, membranes from insect Sf9 cells stably expressing human M1−M5 were incubated with 0.29 nM [^{3}H]-NMS in the medium consisting of a buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM EDTA at pH 7.4 and 25 °C for 1 h. Nonspecific binding was determined in the presence of 1 μM atropine. IC_{50} values was determined from competition binding curves and converted to apparent K_i values using the Cheng−Prusoff equation.[18] The dissociation constant (K_d) and maximal number of binding sites (B_{max}) of the muscarinic M3 receptor binding assay were 0.078 nM and 3.2 pmol/mg protein, respectively. K_d values for the other receptor binding assay (M1, M2, M4, and M5) were 0.092, 0.16, 0.047, and 0.74 nM, respectively. B_{max} values for the other receptor binding assay (M1, M2, M4, and M5) were 2.1, 4.9, 1.9, and 2.5 pmol/mg protein, respectively.

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