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Naphthomycins L–N, Ansamycin Antibiotics from *Streptomyces* sp. CS

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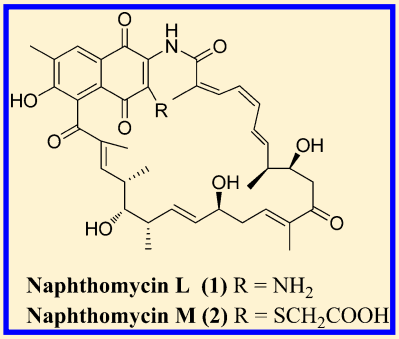
Supporting Information

ABSTRACT: Previous analyses of the naphthomycin biosynthetic gene cluster and a comparison with known naphthomycin-type products from *Streptomyces* sp. CS have suggested that new products can be found from this strain. In this study, screening by LC-MS of *Streptomyces* sp. CS products formed under different culture conditions revealed several unknown peaks in the product spectra of extracts derived from oatmeal medium cultures. Three new naphthomycins, naphthomycins L (**1**), M (**2**), and N (**3**), and the known naphthomycins A (**4**), E (**5**), and D (**6**) were obtained. The structures were elucidated using spectroscopic data from 1D and 2D NMR and HRESIMS experiments.



Ansamycins are an important class of antibiotics that are characterized by a cyclic structure consisting of an aromatic moiety (naphthalene or a benzene derivative) and an aliphatic chain that forms a bridge linking two nonadjacent positions on the aromatic moiety.[1] The biosynthesis of ansamycin antibiotics involves the assembly of a polyketide from the starter unit 3-amino-5-hydroxybenzoic acid by chain extension with several acetate and propionate units catalyzed by a type I polyketide synthase.[2] Naphthalenoid ansamycins with a C23 ansa chain include naphthomycins,[3−5] diastrovaricins,[6] naphthoquinomycins,[7] naphthostatin,[8] naphthomycinol, and actamycin.[9,10] These ansamycins all display significant antimicrobial and antineoplastic activity.[7,11] In the course of our study of the chemical constituents of endophytic microorganisms on plants, the commensal microorganism *Streptomyces* sp. CS was isolated from tissue cultures of *Maytenus hookeri*. In previous chemical studies, naphthomycins A, E, and K and new macrolide antibiotics were identified from extracts of the *Streptomyces* sp. CS strain cultivated on YMG agar medium.[4,12,13] According to previous reports,[6−8] the naphthomycin-type compounds formed in most *Streptomyces* species have different substituents, such as −OH, −Cl, −H, −SR, or −NR groups at position 30.[6−8] However, only two types of substituents (−Cl and −H) at position 30 have been isolated from *Streptomyces* sp. CS.[4,12]

The naphthomycin biosynthetic gene cluster was cloned from *Streptomyces* sp. CS,[14] and several ORFs were found to encode putative modification enzymes (Figure 1, modified from Figure 1 in Bai.[14]). Nat1 is a putative halogenase, and its corresponding product is naphthomycin A. Nat2 was predicted to be a naphthalene ring hydroxylase, but no corresponding product was obtained from *Streptomyces* sp. CS.[4,12,13] Several unknown ORFs were also found in the naphthomycin biosynthetic gene cluster.[14] In this study, *Streptomyces* sp. CS was grown in five types of media, and LC-MS analyses of the extracts were used to reveal evidence of the potential of *Streptomyces* sp. CS to produce new naphthomycins, as suggested by the unknown ORFs in the gene clusters.

Several unknown peaks were present in the product spectra of extracts of *Streptomyces* sp. CS cultured on different media. The key ESIMS peaks for naphthomycins A and E are *m*/*z* 742 $[M + Na]^+$ (718 $[M - H]^-$) and *m*/*z* 686 $[M + H]^+$ (684 $[M - H]^-$), respectively. Thus, in the LC-MS spectra generated in this study, we focused on the peaks corresponding to molecular weights greater than *m*/*z* 684 $[M - H]^-$ (686 $[M + H]^+$). The products of the strain cultivated on oatmeal medium were more diverse and abundant than those of strains grown on the other culture media (Figure S1, Supporting Information). The products from the YMG-, YEME-, and ISP4-cultured strains were almost the same, and no obvious products were found from the TSBY medium. A selected ion monitoring (SIM) peak at *m*/*z* 699 $[M - H]^-$ was present in the product spectra (Figures S1-A and S4) of the strain cultured on the YMG medium and the oatmeal medium, but the strength of the peak was weaker in the former than in the latter (Figure S1-B). Other SIM peaks at *m*/*z* 700, 774, and 1442 (Figures S5−7) were also observed in the LC-MS profiles.

Given these results, the oatmeal medium was selected for the fermentation of *Streptomyces* sp. CS to search for naphthomycin analogues. Three simple methods can be used to identify

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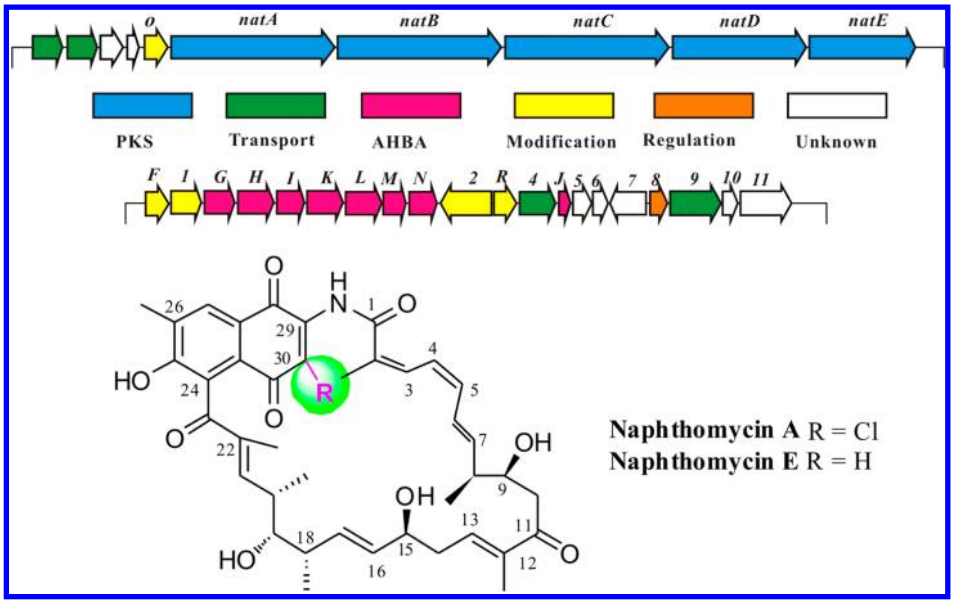
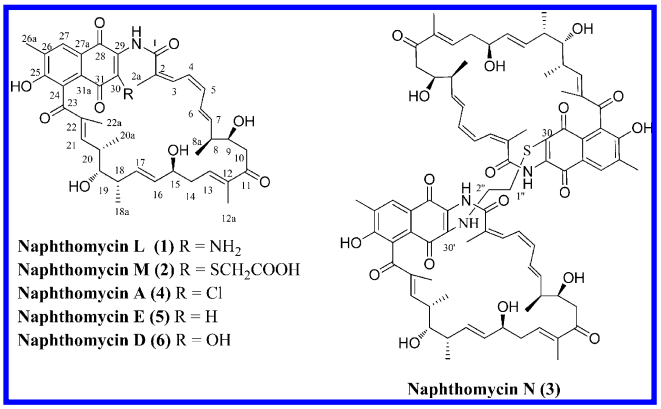


Figure 1. Naphthomycin biosynthetic gene cluster from *Streptomyces* sp. CS. Several unknown ORFs were found in the naphthomycin biosynthetic gene cluster.

naphthomycin analogues: (i) search for visibly red or yellow compounds; (ii) view the compounds under a UV lamp at 254 nm; (iii) cleave the macrolactam amide bonds by heating with sulfuric acid to produce an amine, which is then treated with the Dragendorff reagent to produce an orange product on a TLC plate. Only naphthomycin analogues were selected and purified in the isolation process. Six naphthomycins (**1**–**6**) including three new ones (**1**–**3**) were isolated and identified from *Streptomyces* sp. CS. Compounds **4**–**6** were determined to be naphthomycin A (**4**),[4] naphthomycin E (**5**),[4] and naphthomycin D (**6**)[5] on the basis of their NMR data and comparison with published data.



Naphthomycin L (**1**) was obtained as a red powder. It had the molecular formula $C_{40}H_{48}N_2O_9$, as deduced from HRESIMS (*m*/*z* 701.3427 $[M + H]^+$). In accordance with the molecular formula, 40 carbon resonances were resolved in the ^{13}C NMR spectrum (Table 1) and were further classified by DEPT experiment as seven methyls, two methylenes, 16 methines, and 15 quaternary carbons. The ^1H and ^{13}C NMR spectra showed that compound **1** closely resembles naphthomycin A (**4**),[4] except that the substituent at position 30 in **1** appears to be NH_2, whereas in naphthomycin A it is a −Cl group. The COSY, HSQC, and HMBC spectroscopic data (Table 1) revealed the detailed structure of **1**. An NH_2 group was placed at C-30 (δ_C 138.7) on the basis of HMBC correlations from the protons of 30-NH_2 (δ_H 5.47) to C-29 (δ_C 113.0) and C-31 (δ_C 182.3). Naphthomycin L (**1**) and naphthomycin A (**4**) share the same general macrocyclic structures, and because they co-occur in the extract, the relative and absolute configurations **1** are proposed to be the same as that of naphthomycin A.[5] The specific rotation value of **1** [+1010 (*c*, 0.15)] was larger than typically observed for naphthomycins,[4,5] possibly due to the dark red color of **1** and a UV−vis absorbance that encroaches on the sodium D line (589 nm). The specific rotation values can be affected by the concentration for some natural products.[15] Interestingly, the specific rotation value of **1** was +350 at 0.01 g/100 mL. An aminotransferase gene (*orf*9) was reported in the gene cluster for rifamycin biosynthesis in *Amycolatopsis mediterranei*,[16] and the alignment of *nat8* with *orf*9 showed 31% identity. We thus speculate that *nat8* possesses an aminotransferase function.

Naphthomycin M (**2**) was obtained as a red powder. It had the molecular formula $C_{42}H_{49}NO_{11}S$, as deduced by HRESIMS (*m*/*z* 798.2937 $[M + Na]^+$). The molecular formula and comparison with naphthomycin E (**5**)[4,5] showed **2** to contain an additional $C_2H_2O_2S$ (Table 1). The HMBC data (Table 1) showed correlations from new methylene protons at δ_H 4.02 and 3.50 (2H, m, H-1′) to C-2′ (δ_C 172.3) and C-30 (δ_C 133.7), indicating a structural modification at C-30. In addition to the methylene and the carbonyl (δ_C 172.3), a sulfur and an oxygen still remain to be placed. The chemical shift of C-30 is at δ_C 147 in naphthomycin D (**6**), a compound with a C-30 OH, so the C-30 shift in **2** (δ_C 133.7) suggests a sulfur bridge rather than an oxygen bridge. These findings support the presence of a thioglycolic acid group that is connected to C-30 of the naphthomycin skeleton via sulfur.[4,5] The relative and absolute configurations of naphthomycin M (**2**) are proposed to be the same as that of co-isolated naphthomycin A.[5] According to a previous report, similar thioansamycins were obtained from other *Streptomyces* strains.[17] Those compounds derived from reactions of chloroansamycins and thiols *in vivo* and are presumed to be nonenzymic products, as similar conversions can be effected *in vitro*.[17] In the present work, *nat5* (693 bp) and *nat6* (564 bp) were postulated to encode an alpha/beta

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Table 1. NMR Spectroscopic Data (^{1}H 600 MHz, ^{13}C 150 MHz) for Compounds 1 and 2

	1			2		
no.	δ_C, type	δ_H (J, Hz)	HMBC[a]	δ_C, type	δ_H (J, Hz)	HMBC[a]
1	168.9, C			170.0, C		
2	133.3, C			131.06, C		
2a	21.2, CH_3	2.19, s	1, 3	20.5, CH_3	2.08, s	1, 2
3	131.36, CH	6.66, d (10.1)	1, 2a, 5	129.6, CH	6.53, d (11.1)	5(w), 2a
4	124.1, CH	6.06, dd (10.1, 21.0)	6	123.7, CH	6.18, t (11.1)	2
5	132.7, CH	6.10, dd (10.3, 21.0)	3, 7	132.1, CH	6.02, t (11.1)	6, 7
6	127.0, CH	6.46, dd (10.3, 14.9)	4, 5, 8	126.9, CH	6.39, dd (11.6, 14.8)	4, 5, 8
7	140.4, CH	5.50, m	5, 8, 9, 8a	139.7, CH	5.40, dd (10.2, 14.8)	5, 8, 9, 8a
8	45.1, CH	2.30−2.33, m		44.8, CH	2.23, m	
8a	17.6, CH_3	1.21, d (6.5)	7, 8, 9	17.3, CH_3	1.12, d (6.4)	7, 8, 9
9	73.5, CH	3.57, m	8, 8a, 11	73.1, CH	3.49, m	
10	40.5, CH_2	3.14, dd (2.9, 17.2)	8, 9, 11	40.3, CH_2	3.13, dd (5.2, 19.7)	8, 9, 11
		2.69, m			2.56, m	8, 9, 11
11	204.2, C			204.0, C		
12	138.0, C			137.8, C		
12a	11.4, CH_3	1.70, s	11, 12, 13	11.1, CH_3	1.63, s	11, 12, 13
13	143.2, CH	6.79, t (5.8)	11, 12a, 14 (w), 15	143.0, CH	6.72, t (6.6)	11, 15, 12a
14	36.8, CH_2	2.24, m		36.2, CH_2	2.23, m	13, 15
15	71.6, CH	4.00, m	13, 14, 17	71.9, CH	3.97, m	13, 14
16	137.7, CH	5.65, dd (6.9, 15.1)	15, 18, 18a	136.1, CH	5.52, m	15, 18
17	133.1, CH	5.38, dd (9.7, 15.1)	15, 18, 18a, 19	134.4, CH	5.48, m	15, 16, 18, 18a
18	41.8, CH	2.20, m	16, 19	41.7, CH	2.18, m	17, 19
18a	16.4, CH_3	0.96, d (6.7)	17, 18, 19	16.3, CH_3	0.89, d (6.6)	17, 18, 19
19	77.7, CH	2.98, d (9.8)	21, 17, 20, 20a	76.9, CH	3.18, d (9.9)	17, 20, 20a
20	33.9, CH	2.69, m		33.6, CH	2.65, m	21,22, 20a
20a	10.9, CH_3	0.84, d (6.6)	19, 20, 21	10.6, CH_3	0.76, d (6.6)	19, 20, 21
21	147.0, CH	5.95, d (10.1)	22a, 23	146.9, CH	5.87,d (10.0)	23, 20a
22	137.9, C			137.6, C		
22a	12.8, CH_3	2.00, s	21, 22, 23	12.5, CH_3	1.93, s	21, 22, 23
23	202.4, C			202.2, C		
24	119.3, C			120.3, C		
25	159.8, C			160.9, C		
26	133.7, C			133.3, C		
26a	16.9, CH_3	2.38, s	25, 26, 27	16.5, CH_3	2.30, s	25, 26, 27
27	131.32, CH	7.93, s	25, 26, 26a, 28	131.09, CH	7.85, s	25, 26a, 28, 31a
27a	123.3, C			122.1, C		
28	178.9, C			178.6, C		
29	113.0, C			139.5, C		
30	138.7, C			133.7, C		
31	182.3, C			182.1, C		
31a	130.6, C			135.6, C		
25-OH		9.50, s	24, 25, 26			
29-NH		8.27, s	1, 28, 29		8.54, s	1, 28, 30
15-OH		4.08, s	14, 15			
30-NH_2		5.47, s	29, 31			
9-OH		3.83, d (5.8)	8, 9, 10			
1′				36.1, CH_2	4.02, m	2′, 30
					3.50, m	2′, 30
2′				172.3, C		

[a]HMBC correlations are from the proton(s) stated to the indicated carbon.

fold family protein and an activator of Hsp90 ATPase in *Streptomyces* sp. CS, respectively.[14] However, when *nat5*, the intervening sequence, and *nat6* were joined, a reversed ORF (795 bp, *nat5/6*) was found. Alignment of *nat5/6* with three identified sulfurtransferase genes (*Uba4* (GenBank accession no. NC_001140), *SCO4164* (GenBank accession no. NC_003888), and *SseA* (GenBank accession no. NC_000962)) showed identities from 31% to 34%. This may suggest that sulfur introduction into the new naphthomycin could be enzyme mediated. More experiments are needed to confirm the function of *nat5/6*. Similarly, the source of sulfur at the C-30 position of naphthomycin M needs further experimental evidence.

Naphthomycin N (**3**) was also obtained as a red powder. It had the molecular formula $C_{82}H_{97}N_3O_{18}S$, as deduced from HRESIMS (*m/z* 1466.6385 $[M + Na]^+$). The NMR data

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suggested the planar structure of **3** to be that of a naphthomycin, except that the carbon signals were present in couples in the ^{13}C NMR and DEPT spectra (Table S1), which is consistent with a dimer-like structure suggested by the MS data. The substituent groups at the 2 position in both monomers in compound **3** were methyls rather than one methyl group and one hydrogen as in the related pseudodimer naphthostatin C.[8] The HMBC data (Table S1) showed correlations between the methyl protons at δ_H 2.03 (s, H-2a) and C-1 (δ_C 171.6), C-2 (δ_C 132.1), and C-3 (δ_C 128.3) and between the methyl protons at δ_H 2.06 (s, H-2a′) and C-1′ (δ_C 169.6), C-2′ (δ_C 133.6), and C-3′ (δ_C 130.0). The HMBC data (Table S1) showed correlations between the methylene protons at δ_H 4.03 and 2.67 (H-1″) and C-30 (δ_C 132.1) and C-2″ (δ_C 44.4) and between the one proton of the methylene at δ_H 3.37 (H-2″) and C-30′ (δ_C 138.7) and C-1″ (δ_C 35.5). The monomers were therefore connected with 2-aminoethanethiol, and the N and S groups were attached at C-30 of the individual naphthomycin units. The relative and absolute configurations of naphthomycin N (**3**) are proposed to be the same as that of co-isolated naphthomycin A.[5]

Antifungal Activities. Compounds **1**−**6** were assayed for antifungal activity, but only **1** and **4** showed any evident inhibition of phytopathogenic fungi. The minimal inhibitory concentrations (MICs) of compounds **1** and **4** were 300 and 100 μg/mL, respectively, against *Fusarium moniliforme* and 600 and 400 μg/mL, respectively, against several other phytopathogenic fungi (*Fusarium oxysporum*, *Fusarium moniliforme*, *Gaeumannomyces graminis*, *Verticillium cinnabarium*, and *Phyricularia oryzae*). The experiments were repeated in triplicate, and consistent results were obtained. Compounds **2**, **3**, **5**, and **6** did not inhibit the tested phytopathogenic fungi at 800 μg/mL. Surprisingly, although **1** and **4** are very similar to **2**, **5**, and **6**, they displayed different antifungal activity. It is clear that the substituent groups 30-NH_2 and 30-Cl are the key groups in antifungal activity. These results concur with those of a previous study.[18] None of the compounds showed obvious cytotoxicity against various human tumor cell lines.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotations were measured with a Jasco DIP-370 digital polarimeter. UV spectra were recorded on a Shimadzu UV-2401PC spectrophotometer, λ_{max} (log ε) in nm. The NMR spectra were obtained with a Bruker AVANCE III-600 spectrometer with TMS as the internal standard. The ESIMS and HRESIMS were recorded on a Finnigan LCQ-Advantage and VG Auto-Spec-3000 mass spectrometer, respectively. Column chromatography was performed on silica gel G (200−300 mesh, Qingdao Marine Chemical Factory) and Sephadex LH-20 (Amersham Pharmacia). The LC-MS was performed on a Waters series HPLC 2695 (Waters Corporation) with a Thermo Finnigan LCQ Advantage (Thermo Finnigan) mass detector (ion trap). The samples were separated by an Xterra MS C_{18} with a 3.5 μm pore size and a 3.0 mm × 150 mm column (Waters Corporation) with isocratic elution (75:25 MeOH/0.1% formic acid in H_2O) at a flow rate of 300 μL/min at room temperature.

Bacterial Material. *Streptomyces* sp. CS was isolated from tissue cultures of *M. hookeri*.[4,12,14] The 16S partial sequence of CS was submitted to GenBank under accession number JX011639. A BLAST search result showed the sequence of CS to be highly homologous to other *Streptomyces* species, confirming that the strain is a member of the genus *Streptomyces*. The sample was deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences. Five pathogenic fungi (*G. graminis*, *F. oxysporum*, *V. cinnabarium*, and *P. oryzae*) provided by Dr. L. M. Fan at Yunnan Agricultural University were used to assay the antifungal activity of the sample.

Selection of Fermentation Conditions. Five media were selected to reveal evidence for the potential production of new naphthomycins. The strain was initially cultured on YMG medium at 26 °C for 10 days as a seed plate. Subsequently, 200 mL of the strain was cultured on oatmeal solid medium, YEME, IPS4, TSBY, and YMG. After cultivation for two weeks at 26 °C, the cultures were extracted exhaustively three times by EtOAc/MeOH/AcOH (80:15:5, v/v/v) for LC-MS analysis.

Isolation of the Naphthomycin Analogues. *Streptomyces* sp. CS was cultured on oatmeal solid medium (20 L, which was distributed into about 700 90-mm Petri dishes) and cultivated for two weeks at 26 °C. The culture was then extracted exhaustively three times by EtOAc/MeOH/AcOH (80:15:5, v/v/v) to obtain the extract. The extract (31.50 g) was placed on a silica gel column (silica gel G, 150 g) and eluted with petroleum ether/acetone (10:1 to 7:3) and then $CHCl_3$/MeOH (20:1 to 1:1), which gave six fractions (Fr-1 to Fr-6). Fr-2 (5.36 g) was placed in a silica gel column (silica gel G, 100 g) and eluted with petroleum ether/acetone with an increasing polarity that ranged from 9:1 to 1:1 to yield fractions Fr2-1 to Fr2-6. Fr2-1(1.10 g) was purified with Sephadex LH-20, eluted with $CHCl_3$/MeOH (1:1), and then subjected to further elution on a silica gel G column with $CHCl_3$/acetone (10:1−9:1−8:2) to give compound **1** (280 mg). Fr2-2 (1.3 g) was chromatographed over a silica gel G column (100 g) and eluted with petroleum ether/EtOAc with increasing polarity from 9:1 to 6:4 to yield fractions Fr2-2-1 to Fr2-2-5. Fr2-2-1 (365 mg) was purified over a silica gel H column (8.0 g) and eluted with $CHCl_3$/MeOH, 100:0.5, to obtain compound **4** (180 mg). Fr2-2-4 (630 mg) was chromatographed over a silica gel G column (120 g, $CHCl_3$), eluted with petroleum ether/EtOAc with increasing polarity, ranging from 9:1 to 6:4, and then chromatographed further over a silica gel H column (6 g, $CHCl_3$/acetone/formic acid, 200:1:0.5) to obtain compounds **5** (86 mg) and **6** (18 mg). Fr2-4 (384 mg) was chromatographed over a silica gel H column (8.0 g), loaded onto a Sephadex LH-20 (60 g) column, and eluted with acetone to yield fractions Fr2-4-1 to Fr2-4-4. Fr2-4-4 (98 mg) was purified on a silica gel H column (5.0 g, $CHCl_3$/MeOH, 100:0.5) to obtain compound **3** (15 mg). Fr4 (264 mg) was chromatographed over a silica gel H column (7.0 g, petroleum ether/acetone (9:1 to 7:3), applied to a Sephadex LH-20 (60 g) column, and eluted with MeOH to obtain compound **2** (8 mg).

Naphthomycin L (1): red powder; $[\alpha]_D^{16}$ +1010 (*c* 0.15, $CHCl_3$), $[\alpha]_D^{24}$ +350 (*c* 0.01, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 241 (4.68), 301 (4.59), 485 (3.67) nm; NMR data, see Table 1; HRESIMS *m*/*z* 701.3427 $[M + H]^+$ (cacld for $C_{40}H_{48}N_2O_9$, 701.3438).

Naphthomycin M (2): red powder; $[\alpha]_D^{18}$ +20 (*c* 0.74, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 241 (4.33), 295 (4.29), 485 (3.09) nm; NMR data, see Table 1; ESIMS *m*/*z* 776 $[M + H]^+$; HRESIMS *m*/*z* 798.2937 $[M + Na]^+$ (cacld for $C_{42}H_{49}NO_{11}SNa$, 798.2924).

Naphthomycin N (3): red powder; $[\alpha]_D^{18}$ +389 (*c* 0.55, $CHCl_3$), $[\alpha]_D^{24}$+250 (*c* 0.01, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 241 (5.45), 291 (5.37), 486 (3.87) nm; NMR data, see Table S1; ESIMS *m*/*z* 1444 $[M + H]^+$; 1442 $[M - H]^-$; HRESIMS *m*/*z* 1466.6385 $[M + Na]^+$ (cacld for $C_{82}H_{97}N_3O_{18}SNa$, 1466.6385).

Naphthomycin A (4): yellow powder; $[\alpha]_D^{18}$ +190 (*c* 0.68, $CHCl_3$), literature[5] $[\alpha]_D^{24}$ +432 ($CHCl_3$); ESIMS *m*/*z* 742 $[M + Na]^+$.

Naphthomycin E (5): yellow powder; $[\alpha]_D^{18}$ +321 (*c* 0.18, $CHCl_3$), literature[5] $[\alpha]_D^{24}$ +135 ($CHCl_3$); ESIMS *m*/*z* 686 [M + H.

Naphthomycin D (6): yellow powder; $[\alpha]_D^{18}$ +44 (*c* 0.37, $CHCl_3$), literature[5] $[\alpha]_D^{24}$ +323 ($CHCl_3$); ESIMS *m*/*z* 702 $[M + H]^+$.

Antifungal Activity Assay. The six naphthomycins were assayed to determine their antifungal activity. The MICs for antifungal activity were determined by a method modified from the standardized microdilution method.[19] Five phytopathogenic fungi were incubated on PDA at 22 °C for 7 to 10 days. The hyphae (fresh weight 600 μg) were ground and suspended in 10 mL of SD broth (0.67% yeast nitrogen base, 2.0% glucose). The fungal solutions were then diluted with SD broth (1:5000) for use. All of the tested compounds were dissolved in DMSO to make stock solutions of 20 mg/mL. Different

doses of the test compounds (ranging from 50 to 800 μg/mL) were added to each well (Deepwell Plates 96, Eppendorf) containing a fungal solution and maintained at a final volume of 0.2 mL (the final concentration of DMSO in each medium was not more than 3%). DMSO was used as a negative control, and the fungal solution without the compound was used as a positive control. Deepwell plates containing the various components were incubated at 22 °C for 48 h. The MIC is the lowest concentration of the test compound at which no visible growth occurs. All of the experiments were performed in triplicate.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental data on the methods, NMR data for naphthomycin N, effect of compounds on the growth of human tumor cell lines, LC-MS spectra of products cultivated on four different media, LC-MS profiles of the various naphthomycins, and ^{1}H and ^{13}C NMR, HSQC, HMBC, COSY, and ROESY spectra of naphthomycins L–N are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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