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Targeted Isolation of *N*-Acetylcysteine-Containing Angucycline Derivatives from *Streptomyces* sp. MC16 and Their Antiproliferative Effects

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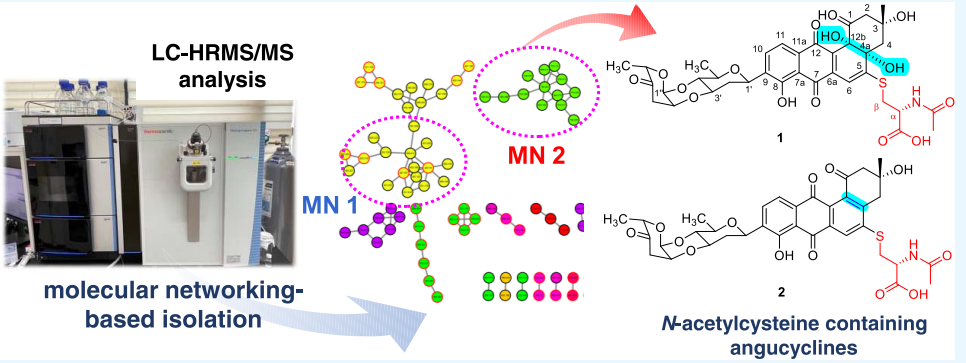

ABSTRACT: Liquid chromatography-mass spectrometry (LC-MS/MS)-based molecular networking analysis was applied to *Streptomyces* sp. MC16. The automatic classification of the MolNetEnhancer module revealed that its major constituent was an angucycline derivative. By targeted isolation of unique clusters in the molecular network, which showed different patterns from typical angucycline compounds, two new *N*-acetylcysteine-attached angucycline derivatives (**1** and **2**) were isolated. The structures were elucidated based on intensive NMR analysis and high-resolution electrospray ionization mass spectrometry (HR-ESI-MS). All isolated compounds (**1**−**4**) were tested for their inhibitory effects on the proliferation of A431, A549, and HeLa cell lines. Antibiotics 100-1 (**3**) and vineomycinone B_2 (**4**) showed moderate inhibitory effects on these three cell lines with IC_{50} values ranging from 18.5 to 59.0 μM, while compounds **1** and **2** with an additional *N*-acetylcysteine residue showed weak inhibitory effects only on the HeLa cell line with IC_{50} values of 54.7 and 65.2 μM, respectively.

INTRODUCTION

Angucycline is the largest group of polycyclic aromatic polyketides, produced mainly by various species of *Streptomyces*, and has various chemical structures of angularly assembled tetracyclic scaffolds and rearranged linear forms.[1,2] Additionally, many kinds of deoxy sugars, such as D-olivose, L-cinerulose, L-rhodinose, and certain amino sugars like L-rednose, could attach to the aglycone of angucycline via a *C*- or *O*-glycosidic bond.[3,4] With this diversity of the chemical structure, angucycline derivatives showed a broad spectrum of biological activities, such as antibacterial, antiviral, and especially anticancer activities for many cancer cell lines.[1,2,5] As part of the ongoing research to find antiproliferative compounds from natural sources, *Streptomyces* sp. MC16 was selected for further investigation after the detection of various angucycline derivative peaks using the liquid chromatography-mass spectrometry (LC-MS/MS) dereplication method.

Molecular networking as a dereplication strategy effectively and efficiently isolates bioactive constituents from natural sources. This method is easily accessible through the web-based "Global Natural Products Social (GNPS)" platform, which has a rapidly growing social library.[6] In cases where the GNPS social library lacks the necessary spectra, open-source spectrum libraries, such as the Human Metabolome Database (HMDB), SUPER NATURAL 2 (SUPNAT), FooDB, and the Natural Product Atlas (NPAtlas), can be utilized for *in silico* analysis.[7,8] The NPAtlas is especially useful as it contains

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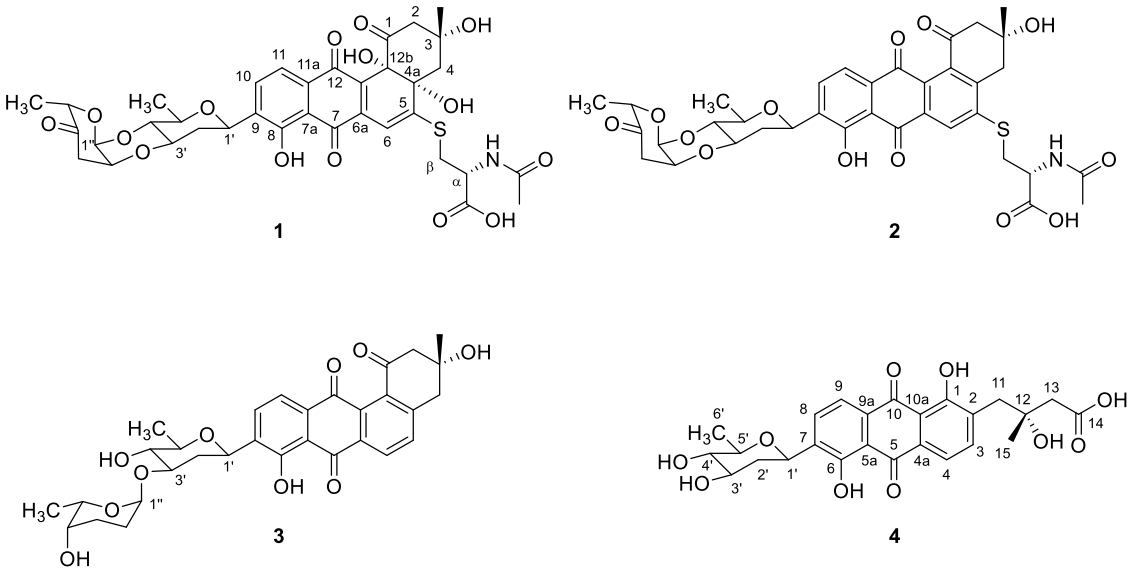

Figure 1. Chemical structures of compounds **1**−**4**.

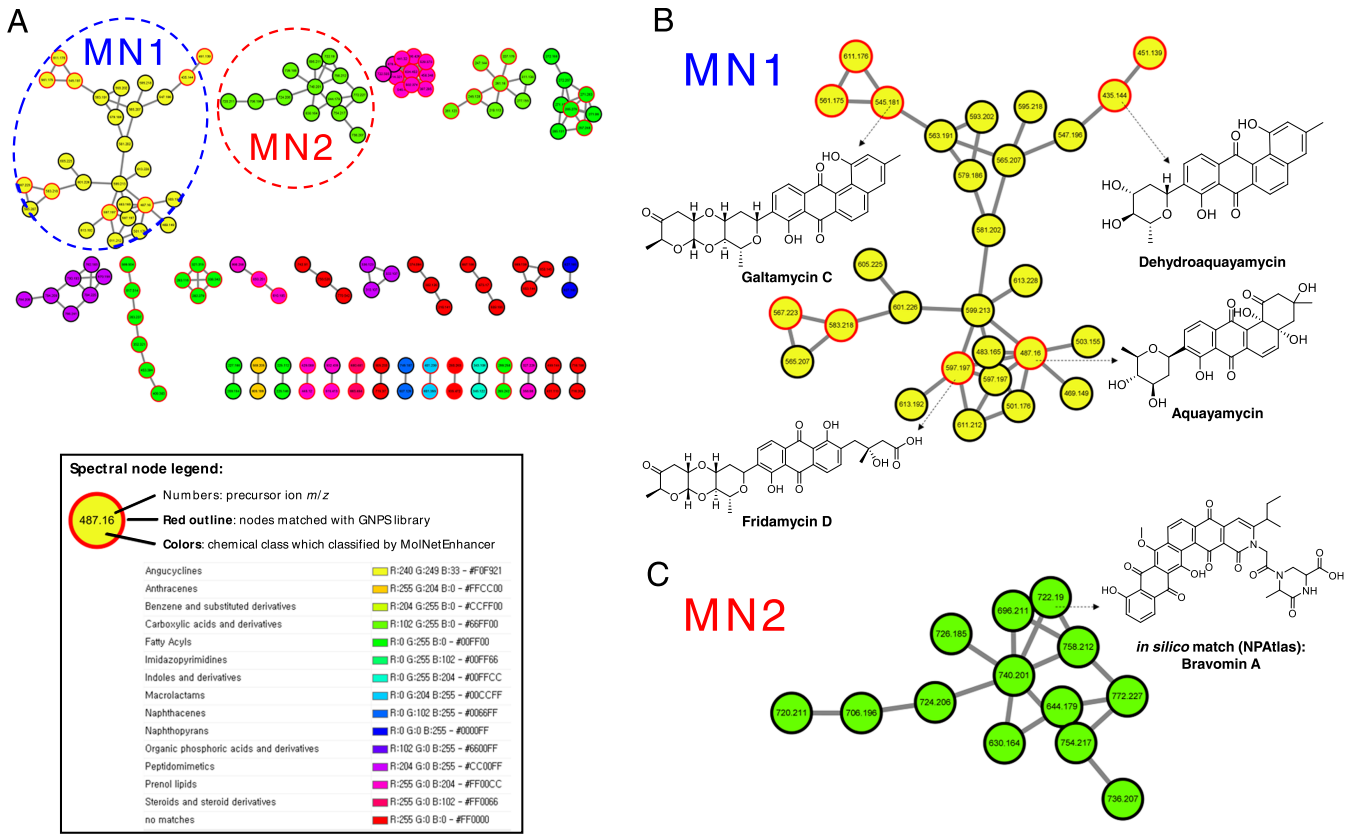

Figure 2. Molecular networking analysis of *Streptomyces* sp. MC16 extract. (A) The molecular network is automatically classified by MolNetEnhancer (the singleton node is excluded in this figure). (B) The GNPS library matched the structure of the representative node in MN1, which is classified as angucycline. (C) The candidate structure by *in silico* fragment analysis using NPAtlas in MN2.

information on about 30,000 compounds from bacterial and fungal sources[9] and can be coupled with *in silico* analysis, such as network annotation propagation (NAP), to aid in dereplication. The MolNetEnhancer module of GNPS can automatically classify molecular network clusters based on outputs from *in silico* tools (NAP or DEREPLICATOR) and provide visualization of the chemical classes of major natural source constituents.[10]

This study applied molecular networking analysis with *in silico* analysis (NAP and MolNetEnhancer) to the *Streptomyces* sp. MC16 extract. Among the molecular networks, one cluster showed a pattern different from that of a typical angucycline and was selected for further isolation. As a result, two new *N*-acetylcysteine-containing angucycline derivatives (**1**−**2**) were isolated. Herein, we describe the molecular networking-guided isolation and structure determination of compounds **1** and **2**

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Table 1. ^{1}H NMR Data for Compounds 1−3

position	1[a]	2[b]	3[b]		1	2	3
	Aglycone				Sugar		
1				1′	5.10, d (10.3)	5.15 (m)	4.92 (m)
2	3.45, d (12.6) 3.24, d (12.8)	3.23 (br s)	3.11, dd (1.6, 14.3) 2.91, dd (1.2, 14.2)	2′	2.60 (m) 1.81 (m)	2.60 (m) 1.81 (m)	2.46 (m) 1.42 (m)
3				3′	3.87 (m)	3.86 (m)	3.23 (m)
4	2.68 (br s)	3.58 (m) 3.04 (m)	3.27, d (16.6) 3.17, d (16.6)	4′	3.89 (m)	3.87 (m)	3.12 (m)
4a				5′	3.68 (m)	3.66 (m)	3.56 (m)
5			7.70, d (7.6)	6′	1.50, d (6.1)	1.49, d (5.9)	1.38, d (6.0)
6	7.03 (s)	8.50 (br s)	8.36, d (7.4)	1″	5.47, d (2.8)		4.93 (m)
6a				2″	4.45 (m)	4.42 (br s)	1.85 (m) 1.77 (m)
7				3″	2.96, dd (2.6, 17.3) 2.85, dd (3.5, 17.2)	2.92, d (16.2) 2.82, d (16.2)	1.93 (m) 1.83 (m)
7a				4″			3.82 (m)
8				5″	5.12 (m)	5.10 (m)	3.93 (m)
9				6″	1.46, d (6.7)	1.46, d (6.6)	1.25, d (6.3)
10	7.92, d (7.4)	8.02, d (7.4)	7.90, d (7.7)	*N*-acetylcysteine			
11	7.73, d (7.4)	7.86, d (7.4)	7.63, d (7.7)	C═O			
11a				*α*	5.71 (m)	5.59 (m)	
12				*β*	4.14, dd (4.2, 12.9) 3.77, dd (8.8, 12.8)	4.22 (m) 3.97 (m)	
12a				*N*-acetyl C═O			
12b				*N*-acetyl CH_3	2.20 (s)	2.21 (s)	
13	1.52 (s)	1.56 (s)	1.45 (s)	NH	9.52, d (8.0)	9.37 (br s)	

[a]Recorded at 400 MHz. [b]Recorded at 900 MHz.

Table 2. ^{13}C NMR Data for Compounds 1−3

position	1[a]	2[b]	3[b]		1	2	3
	Aglycone				Sugar		
1	205.9	197.5	199.7	1′	71.8	71.8	72.5
2	53.7	53.7	54.4	2′	36.9	36.8	41.1
3	76.6	71.8	73.1	3′	77.1	77.1	72.7
4	46.6	42.3	44.9	4′	74.7	74.7	88.5
4a	84.3	146.1	150.4	5′	74.8	74.8	76.5
5	163.6	164.0	135.5	6′	17.8	17.8	18.8
6	106.6	124.5	130.4	1″	91.8	91.9	99.8
6a	138.4	132.7	134.9	2″	71.9	71.9	28.1
7	189.3	188.2	189.2	3″	40.5	40.4	31.0
7a	114.6	115.5	116.2	4″	208.4	208.3	72.7
8	157.7	158.4	159.2	5″	78.0	78.0	72.1
9	136.9	136.4	138.1	6″	16.6	16.5	18.2
10	133.7	134.2	134.7	*N*-acetylcysteine			
11	118.7	119.0	119.0	C═O	173.8	173.8	
11a	131.7	134.9	134.9	*α*	51.6	53.1	
12	182.4	182.8	184.5	*β*	34.1	35.4	
12a	135.4	133.2	137.4	*N*-acetyl C═O	170.5	170.5	
12b	79.6	137.2	136.9	*N*-acetyl CH_3	23.0	23.0	
13	30.5	31.0	29.9				

[a]Recorded at 100 MHz. [b]Recorded at 225 MHz.

along with two known angucycline derivatives (**3** and **4**) (Figure 1) and their antiproliferative activity against A431, A549, and HeLa cell lines.

RESULTS AND DISCUSSION

The LC-HRMS/MS data for *Streptomyces* sp. MC16 extract was analyzed using classical molecular networking via the GNPS web platform (https://gnps.ucsd.edu) and an *in silico* fragment analysis tool, NAP, combined with the structure libraries of GNPS and NPAtlas. The results of classical molecular networking and the output from NAP were integrated by using the MolNetEnhancer module, which afforded automatic classification via the ClassyFire algorithm. The largest cluster (MN1) was classified as angucycline. The results of the GNPS library matching and *in silico* analysis using NAP revealed the structure of each node as various types of

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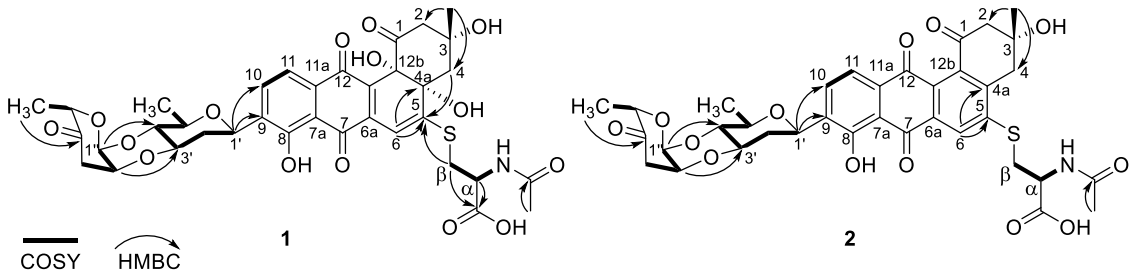

Figure 3. Key HMBC and correlated spectroscopy (COSY) correlations of compounds **1** and **2**.

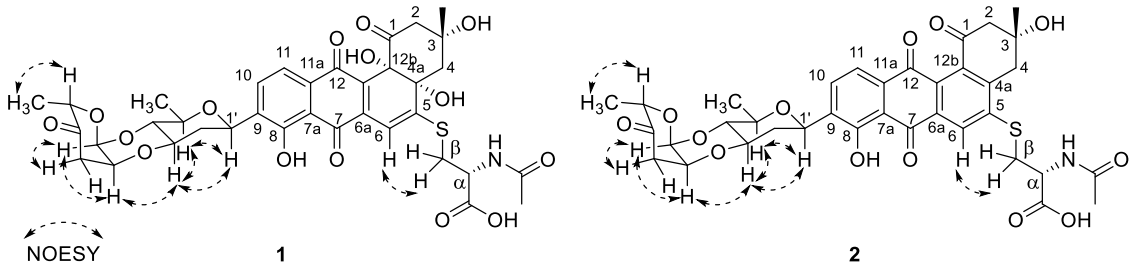

Figure 4. Key NOESY correlations of compounds **1** and **2**.

angucycline compounds (Figure 2). In contrast to MN1, the next cluster (MN2) did not match any spectrum in the GNPS library. Only a few nodes matched the *in silico* analysis using NPAtlas.

The proposed structure of the node with m/z 722.1900, determined by *in silico* fragmentation analysis, has a tetracyclic ring core. However, its entire structure shows a different pattern from typical angucyclines. The element composition analysis by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) data revealed its molecular formula as $C_{36}H_{36}NO_{13}S$. Therefore, the node in cluster MN2 was assumed to be an unusual angucycline derivative containing a sulfur atom. Using the retention time at each node, peaks in the total ion chromatogram were identified and selected for targeted isolation.

Compound **1** was isolated as a reddish amorphous powder, and its molecular formula was assigned as $C_{36}H_{39}NO_{15}S$ from the HR-ESI-MS data (m/z 758.2111 $[M + H]^+$; calcd 758.2113). The ^{1}H NMR data for **1** closed to saquayamycin B_1[3] and displayed three olefinic protons (δ_H 7.92, 7.73, and 7.03), seven oxymethine protons (δ_H 5.47, 5.12, 5.10, 4.45, 3.89, 3.87, and 3.68), four methylene protons (δ_H 3.45, 3.24, 2.96, 2.85, 2.68, 2.60, and 1.81), two secondary methyl protons (δ_H 1.50 and 1.46), and a tertiary methyl proton (δ_H 1.52) (Table 1). The ^{13}C NMR and HSQC spectra exhibited 31 carbon signals, similar to those of saquayamycin B_1, including 4 carbonyls, 3 olefinic carbons, 10 quaternary carbons, 4 methylenes, 7 methines, and 3 methyl groups (Table 2). The molecular formula of the remaining moiety corresponded to $C_5H_7NO_3S$, which was identified as *N*-acetylcysteine from the ^{1}H and ^{13}C NMR data. The proton signals at δ_H 5.71 (m), δ_H 4.14 (dd, J = 4.2, 12.9 Hz), and δ_H 3.77 (dd, J = 8.8, 12.8 Hz) were revealed as H-α and H_2-β of a cysteine. The protons at δ_H 9.52 (d, J = 8.0 Hz) and δ_H 2.20 (s) were revealed as a secondary amine proton and methyl proton of an *N*-acetyl moiety, respectively. Through the heteronuclear multiple bond correlation (HMBC) of H_2-β (δ_H 4.14 and 3.77), H-4 (δ_H 2.68), and H-6 (δ_H 7.03) with C-5 (δ_C 163.6), the position of *N*-acetylcysteine and the attachment through the sulfur bridge at C-5 were determined (Figure 3). The nuclear overhauser effect spectroscopy (NOESY) correlation between H-6 (δ_H 7.03) and H_2-β (δ_H 4.14 and 3.77) supports the attachment of *N*-acetylcysteine at the C-5 position (Figure 4).

In addition, by the HMBC correlations between H-1′ (δ_H 5.10) and C-9 (δ_C 136.9), C-10 (δ_C 133.7), the attachment of the sugar moiety at C-9 was determined. The relative configuration of the sugar moiety was determined by the NOESY experiment along with the coupling constants of anomeric protons ($J_{H\text{-}1'}$ = 10.3 Hz and $J_{H\text{-}1''}$ = 2.8 Hz) (Figure 4). Thus, the structure of **1** was elucidated as shown in Figure 1, and it was named 5-*N*-acetylcysteinylsaquayamycin B_1.

Compound **2** was obtained as a reddish amorphous powder, and its molecular formula was determined as $C_{36}H_{37}NO_{13}S$ from the HR-ESI-MS data (m/z 724.2054 $[M + H]^+$; calcd 724.2059), which was 34 Da less than that of **1**. The mass difference between **1** and **2**, along with the disappearance of two oxymethine carbons around 80 ppm, revealed that the aglycone of **2** is a moromycin-type angucycline.[3,11] The NMR signals of *N*-acetylcysteine were also observed in ^{1}H and ^{13}C NMR spectra [H-α (δ_H 5.59), H_2-β (δ_H 4.22 and 3.97), NH (δ_H 9.37), *N*-acetyl CH_3 (δ_H 2.21); C═O (δ_C 173.8), C-α (δ_C 53.1), C-β (δ_C 35.4), *N*-acetyl C═O (δ_C 170.5), and *N*-acetyl CH_3 (δ_C 23.0)]. To determine the position of *N*-acetylcysteine, an HMBC experiment was performed. However, the HMBC correlations of these two protons were not observed because of the broad peaks of H-α and H_2-β of cysteine. The NOE correlations between H_2-β (δ_H 4.22 and 3.97) and H-6 (δ_H 8.50) were observed in the ROESY spectrum. As a result, the attachment of *N*-acetylcysteine at C-5 via a sulfur bridge was determined (Figure 4). In addition, the ^{1}H and ^{13}C NMR signals of sugar were closed to the α-L-cinerulosyl-(1 → 4, 2 → 3)-β-D-olivosyl chain of **1**, and its position and relative configuration were determined by the HMBC and ROESY experiments, respectively (Figures 3 and 4). Thus, the structure of **2** was elucidated, as shown in Figure 1, and the compound was named 5-*N*-acetylcysteinylmoromycin B.

To determine the absolute configuration of the *N*-acetylcysteine moiety, compounds **1** and **2** were defined by

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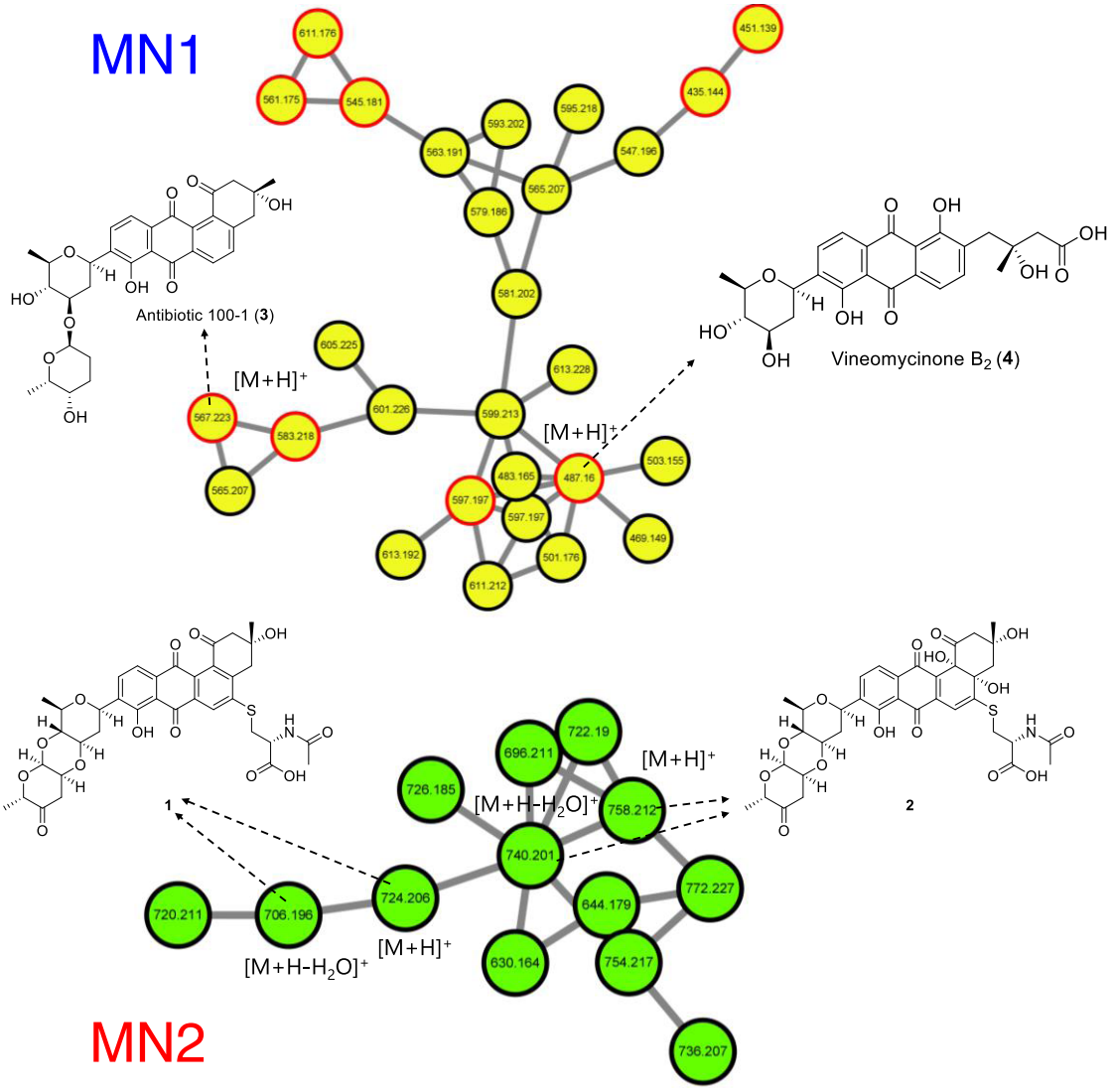

Figure 5. Annotation of isolated compounds **1**−**4** on the clusters.

advanced Marfey's analysis of the hydrolysate of **1** and **2** after desulfurization with Raney nickel as a catalyst.[12] The advanced Marfey's method led to identification of the absolute configuration of the alanine as the L-form (Figure S16).

Compound **3** was obtained as an amorphous yellow powder, and its molecular formula was assigned as $C_{31}H_{34}O_{10}$ from the HR-ESI-MS data (m/z 567.2225 $[M + H]^+$; calcd 567.2225). In the ^1H and ^{13}C NMR data, the signals corresponding to the aglycone moiety were close to those of compound **2**, except for the disappearance of the *N*-acetylcysteine moiety and slight differences in the sugar signals (Tables 1 and 2). The NMR data of the sugar moieties corresponded to that of D-olivose and L-rhodinose, compared to the previous reports.[3,13] The arrangement of the α-L-rhodinosyl-(1 → 3)-β-D-olivose and its attachment to C-9 of aglycone were determined by HMBC correlations (Figures S17−S22). These assignments and configurations were consistent with antibiotic 100-1, an urdamycin derivative isolated from a mutant strain of *Streptomyces fradiae*.[14]

Compound **4** was obtained as a yellow amorphous powder, and its molecular formula was determined as $C_{25}H_{26}O_{10}$ from the HR-ESI-MS data (m/z 487.1596 $[M + H]^+$, calcd 487.1599), with 13 hydrogen deficiency indices. The ^1H and ^{13}C NMR data of **4** were similar to those of compounds **1**−**3**. However, the signal of the carboxylic acid (δ_C 176.1) was observed instead of the ketone carbon near 200 ppm, implying the rearrangement of the tetracyclic core into a linear tricyclic form (Table S1, Supporting Information). The full NMR assignment using 2D NMR corresponded to the reported data for vineomycinone B_2 (also known as fridamycin A),[15,16] and the relative configuration determined by the ROESY experiment was consistent (Figures S24−S29).

After the isolation and structure determination of compounds **1**−**4**, they were all annotated to the molecular network (Figure 5). First, the node with m/z = 487.1600 in MN1 was matched to aquayamycin by comparing its spectrum to that of the GNPS library. However, the structure of this node, as elucidated by NMR, was vineomycinone B_2 (**4**), a rearranged isomer of aquayamycin. Next, the isolated antibiotic 100-1 (**3**) was annotated with a node of m/z = 567.2230 and was close to the candidate structure (sakyomycin A) by the GNPS library matching. However, the predicted structure had only one sugar and different positions. In MN2, the node with m/z = 722.1900, which matched that of bravomicin A by *in silico* analysis with the NPAtlas library, could not be isolated because of its small amount in the extract. However, compounds **1** and

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Table 3. Inhibitory Effects (IC_{50}) of Proliferation Activities on Various Cell Lines[a]

cell lines	compound 1	compound 2	antibiotic 100-1 (3)	vineomycinone B_2 (4)	doxorubicin[b]
A431	>100	>100	38.6	38.5	1.1
A549	>100	>100	27.8	59.0	1.1
HeLa	54.7	65.2	20.3	18.5	2.1

[a]Cells were exposed to varying concentrations of compounds for 72 h, and the IC_{50} values (μM) were calculated. [b]Doxorubicin was used as the positive control.

2 were annotated to the connected nodes with m/z = 724.2060 and 758.2120, respectively. Their structure showed an unusual attachment of the *N*-acetylcysteine moiety to angucycline, as predicted by the *in silico* analysis of MN2. Assuming a cosine score and m/z difference between the nodes in MN2, the other nodes seem to be derivatives of the *N*-acetylcysteine-attached angucycline. Sulfur-containing angucyclines such as WS009A and B (from *Streptomyces* sp. no. 89009)[17] and nocardiopsistin E and F (from *Nocardiopsis* sp. HB-J378)[18] are rare compounds observed in Actinomycetes. Therefore, the biosynthetic gene cluster (BGC) responsible for angucycline synthesis was investigated; however, the specific gene involved in the addition of *N*-acetylcysteine was not identified (Tables S2 and S3, Supporting Information). Rohr observed the nonenzymatic formation of methanethiol on angucycline urdamycin E through a reaction with methionine.[19] More recently, Terenzi et al. demonstrated that Michael adducts of landomycin derivatives with biothiols, such as reduced glutathione and cysteine, can form spontaneously both inside and outside cells.[20]

The inhibitory effects of all of the isolated compounds on the proliferation of A431, A549, and HeLa cell lines were evaluated. Antibiotics 100-1 (**3**) and vineomycinone B_2 (**4**) exhibited moderate inhibitory effects on all three cell lines, with IC_{50} values ranging from 18.5 to 59.0 μM. In contrast, compounds **1** and **2**, which possessed an additional *N*-acetylcysteine moiety, showed inhibitory effects only on the HeLa cell line (Table 3). These results suggest that the attachment of *N*-acetylcysteine reduces cytotoxicity, consistent with a previous report indicating a decrease in cytotoxicity of extracellular *N*-acetylcysteine adducts.[19]

CONCLUSIONS

Two new *N*-acetylcysteine-attached angucyclines (**1**−**2**) were isolated from *Streptomyces* sp. MC16 using molecular networking and element composition analysis based on UPLC-HR-MS/MS. Annotation of the isolated compounds in the molecular network revealed that MN2 was a cluster of *N*-acetylcysteine-attached angucycline derivatives formed by the Michael-type addition of biothiol groups. The biological activities of *N*-acetylcysteine-attached angucycline derivatives have been reported to go beyond anticancer, such as WS009A and B, which have been shown to exhibit antagonistic binding activity on endothelin receptors.[17] The results of this study suggest that *Streptomyces* sp. MC16 may be a promising source for large-scale fermentation and targeted isolation of *N*-acetylcysteine-attached angucyclines, which are presented as clustered nodes in the molecular network.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations and UV and IR spectra were obtained on a JASCO DIP-1000 polarimeter, a JASCO UV-550 spectrophotometer, and a JASCO FT-IR 4100 spectrometer, respectively. NMR spectra were recorded on Bruker AVANCE 400 and 900 MHz spectrometers using CD_3OD and pyridine-d_5 as solvents. HR-ESI-MS and UPLC-MS/MS analyses were performed by using an Orbitrap Exploris 120 mass spectrometer coupled with a Vanquish UHPLC system (ThermoFisher Scientific). Column chromatography was performed by using a silica gel column (Merck, 70−230 mesh). Medium-pressure liquid chromatography (MPLC) system was performed using a CombiFlash RF (Teledyne ISCO) system with a Redisep RF C18 reverse-phase column 43 g (Teledyne ISCO). Preparative HPLC was performed using a Waters HPLC system equipped with two Waters 515 pumps, a 2996 photodiode-array detector, and a YMC J'sphere ODS H-80 column (4 μm, 150 × 20 mm, i.d., flow rate 6.0 mL/min). Thin-layer chromatography was performed using precoated silica gel 60 F_{254} (0.25 mm, Merck) plates, and spots were detected using a 10% vanillin-H_2SO_4 aqueous spray reagent. *Streptomyces* sp. MC16 was isolated from a soil sample collected at Ochang, South Korea. The nucleotide sequence, obtained through 16S rRNA sequencing, shared a 98% homology with *Streptomyces leeuwenhoekii*.

LC-MS/MS-Based Molecular Networking Analysis. The LC-MS/MS data were acquired by a Vanquish UHPLC-Orbitrap Exploris 120 system by the following conditions. Chromatographic separation was conducted with a YMC Triart C18 (100 × 2.1 mm, 1.9 μm, 0.3 mL/min) column at a temperature of 30 °C, using a mobile phase of water +0.1% formic acid (A) and CH_3CN + 0.1% formic acid (B), and the gradient consisted of a linear gradient of 10−100% B (0−10 min). The mass detection range was set as m/z 200−2000, and the resolution for the full MS scan was fixed at 60,000 for the full MS scan and 15,000 for the data-dependent MS^n scan. The following parameters were used for the tuning parameter for MS measurements: a spray voltage of 3.5/2.5 kV for the positive/negative modes, an ion transfer tube temperature of 320 °C, a HESI probe vaporizer temperature of 275 °C, and RF lens 70 (%). Ultrapure nitrogen (>99.999%) was used as both sheath and auxiliary gases of HESI probe and set to 50 arb and 15 arb, respectively. A normalized higher-energy collision dissociation (HCD) energy of 30% was used for the collision of ions in the Orbitrap detector. The four most intense ions in the full MS spectrum were selected for MS/MS fragmentation by the data-dependent MS^n mode, and a dynamic exclusion filter was used to exclude the repeated fragmentation of ions within 2.5 s after acquiring the MS^2 spectrum. The acquired MS/MS data were uploaded to the GNPS (http://gnps.ucsd.edu) for classical molecular networking. In addition, the network annotation propagation (NAP) tool in the GNPS web platform was used for *in silico* analysis with the following parameters: 10 first candidates, mass tolerance of 5 ppm, database of GNPS and NPAtlas (Natural Products atlas). The result of the classical molecular networking and NAP was integrated by the MolNetEnhancer module, which was conducted via the "Advanced Views-

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Experimental Views" tab of the result of classical networking with the job ID of NAP above. After analysis, the resulting file was exported to Cytoscape 3.9.1, and the structures were visualized using the ChemViz2 plug-in. The detailed method and result of molecular networking analysis could be browsed on the following GNPS website links: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=8d0bc780cdf44c62a556966d63b8ba14 (classical molecular networking) https://proteomics2.ucsd.edu/ProteoSAFe/status.jsp?task=522525e2910143c2bae7864553c85f35 (NAP).

Fermentation, Extraction, and Isolation. The *Streptomyces* sp. MC16 was cultured in M2 media (2.0 g/L yeast extract, 5.0 g/L glucose, 25.0 mL/L glycerol, 4.0 g/L soytone, and 0.03 g/L $CaCO_3$) for 7 days at 28 °C on a rotary shaker operating at 165 rpm in a baffled Erlenmeyer flask. The cultured broth (8 L) was extracted with an equal volume of ethyl acetate (EtOAc) three times. The EtOAc-soluble extract (3.1 g) was fractionated by a CombiFlash RF (Teledyne ISCO) MPLC system on a Redisep RF C18 reverse-phase column 43 g (Teledyne ISCO) using a stepwise gradient of MeOH-H_2O (from 20:80, 30:70, 40:60, 60:40, 80:20 to 100:0; 200 mL for each step). The 60% MeOH MPLC fraction (213.5 mg) was further purified by preparative HPLC (MeCN-H_2O with 0.1% formic acid, 40:60, isocratic) to yield vineomycinone B_2 (**4**) (t_R = 12.6 min, 4.0 mg), antibiotic 100-1 (**3**) (t_R = 19.1 min, 1.8 mg), and compound **2** (t_R = 24.7 min, 3.6 mg). The 40% MeOH MPLC fraction (156 mg) was isolated by preparative HPLC (MeCN-H_2O with 0.1% formic acid, 40:60, isocratic) to obtain compounds **1** (t_R = 18.1 min, 5.8 mg).

*5-N-Acetylcysteinylsaquayamycin B_1 (**1**).* Reddish amorphous powder; $[\alpha]_D^{25}$ −61.1 (*c* 0.02, MeOH); UV (MeOH) λ_{max} 236.1, 294.1 nm; IR ν_{max} (film) 3410, 2990, 2933, 2362, 2336, 1732, 1634, 1559, 1430, 1379, 1282, 1102, 1079, 1028, 898, 714, 613 cm^{-1}; ^1H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5); see Tables 1 and 2; HR-ESI-MS *m*/*z* 758.2111 $[M + H]^+$ (calcd for $C_{36}H_{40}NO_{15}S$, 758.2113).

*5-N-Acetylcysteinylmoromycin B (**2**).* Reddish amorphous powder; $[\alpha]_D^{25}$ +2.3 (*c* 0.01, MeOH); UV (MeOH) λ_{max} 217.3, 291.7 nm; IR ν_{max} (film) 3395, 2979, 2935, 2362, 2337, 1728, 1633, 1517, 1431, 1375, 1286, 1103, 1079, 1058, 1029, 900, 850, 753 cm^{-1}; ^1H NMR (900 MHz, pyridine-d_5) and ^{13}C NMR (225 MHz, pyridine-d_5); see Tables 1 and 2; HR-ESI-MS *m*/*z* 724.2054 $[M + H]^+$ (calcd for $C_{36}H_{38}NO_{13}S$, 724.2059).

*Antibiotic 100-1 (**3**).* Yellow amorphous powder; $[\alpha]_D^{25}$ +2.9 (*c* 0.01, MeOH); UV (MeOH) λ_{max} 211.4, 270.4 nm^1; IR ν_{max} (film) 3402, 2969, 2362, 2336, 1700, 1670, 1633, 1592, 1429, 1372, 1278, 1120, 1054, 982, 863 cm^{-1}; ^1H NMR (900 MHz, CD_3OD) and ^{13}C NMR (225 MHz, CD_3OD); see Tables 1 and 2; HR-ESI-MS *m*/*z* 567.2225 $[M + H]^+$ (calcd for $C_{31}H_{35}O_{10}$, 567.2225).

*Vineomycinone B_2 (**4**).* Yellow amorphous powder; $[\alpha]_D^{25}$ +2.1 (*c* 0.01, MeOH); UV (MeOH) λ_{max} 229.1, 258.5, 294.1 nm; IR ν_{max} (film) 3398, 2931, 2362, 2337, 1724, 1628, 1431, 1375, 1260, 1071, 899, 853, 793 cm^{-1}; ^1H NMR (900 MHz, CD_3OD) and ^{13}C NMR (225 MHz, CD_3OD); see Table S1 (Supporting Information); HR-ESI-MS *m*/*z* 487.1596 $[M + H]^+$ (calcd for $C_{25}H_{27}O_{10}$, 487.1599).

Absolute Configuration of the *N*-Acetylcysteine Moiety of Compounds 1−4. Advanced Marfey's analyses for acid hydrolysis of desulfurized **1** and **2** with Raney nickel were used to determine the absolute configurations of the *N*-acetylcysteine moiety of both. Raney nickel (10.0 mg) was added to a solution of **1** (1.0 mg) and **2** (0.8 mg) in MeOH (1.0 mL), and the mixture was stirred at room temperature. The suspensions were purged with a H_2 atmosphere under 1 atm. After being stirred for 18 h at room temperature, the suspensions were filtered through a filter pad and washed with MeOH (1.0 mL × 3) and H_2O (1.0 mL × 3), respectively. MeOH and H_2O filtrates were concentrated in vacuo. To hydrolyze the acetyl moiety of *N*-acetylcysteine, the H_2O filtrates, processed as above, were dissolved in 500 μL of 6 M hydrochloric acid, followed by heat treatment at 100 °C for 12 h. The product of each sample was concentrated to dryness in vacuo, and the residue was dissolved in 500 μL of H_2O. Twenty microliters of 1 M $NaHCO_3$ and 50 μL of N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (L-FDLA) were added to the 50 μL of hydrolysates, followed by incubation at 37 °C for 1 h. The mixtures were neutralized by the addition of 20 μL of 1 M HCl and then concentrated to dryness in vacuo. The resulting dried residue was dissolved in 1 mL of acetonitrile, followed by passage through a filter. Similarly, the standard L- and D-alanine were derivatized according to the method described above. The L-FDLA derivatives of the hydrolysate and the standard amino acids were subjected to a Micromass ZQ (Waters) mass spectrometer coupled with a 1260 Infinity HPLC (Agilent technology) with a Phenomenex Luna C18 (100 × 2.0 mm, 3.0 μm, 0.4 mL/min) column at 40 °C using the following gradient program: solvent A, H_2O with 0.1% formic acid; solvent B, CH_3CN with 0.1% formic acid; linear gradient 10−100% of B (0−15 min).

Measurement of Cytotoxicity of Compounds 1−4. A431 (epidermoid carcinoma of the human epidermis), A549 (carcinoma of the human lung), and HeLa (adenocarcinoma of the human cervix) cells were purchased from the American Type Culture Collection (ATCC). The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, 11965092) containing 10% fetal bovine serum (FBS; Gibco, Australia, 10099141), 100 units of penicillin, and 100 μg/mL of streptomycin (Gibco, 15140-122) at 37 °C in a humidified atmosphere containing 5% CO_2. Cell viability activities were determined using the Cyto X colorimetric assay (LPSsolution, Korea, CYT3000). For cytotoxicity, cells were cultured in 96-well plates (0.7×10^4 cells/well) for 12 h. The cells were treated with various concentrations of compounds for 24, 48, and 72 h. The cells were replaced with a Cyto X solution for 1 h. The cells were measured by using a microplate reader (Molecular Devices, Spectra Max 190) at 450 nm. Cell viability was normalized to the DMSO control group.

Genome Analysis of *Streptomyces* sp. MC16. The sequenced genome of the *Streptomyces* sp. MC16 strain was analyzed for the prediction of secondary metabolites and biosynthetic gene clusters (BGCs) using the genome mining tool of the antibiotics & secondary metabolite shell (antiSMASH).[21] A total of 17 putative BGCs were annotated (Table S2, Supporting Information), including two type II polyketide synthases BGCs. Region 5 in the assembly displayed a high similarity (>54%) to that of BGCs linked to several angucycline compounds, such as saprolmycin E (91%), grincamycin (88%), saquayamycin A (85%), saquayamycin Z (76%), and landomycin A (71%). Since the *Streptomyces* sp. MC16 produces primarily tricyclic glycosylated structures, vineomycinone B_2, antibiotic 100-1, and new *N*-acetylcysteine derivatives (**1**−**2**), we were interested in possible enzymatic

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processes that could lead to the biosynthesis of these *N*-acetylcysteine derivatives. The *N*-acetylcysteine moiety has been rarely found in *Streptomyces*-produced polyketides but is also found in the angucyclinones, seongomycin, homoseongomycin, and cysfluoretin. On the other hand, the seongomycins are readily formed from diazofluorenes, suggesting that they are shunt or detoxification metabolites in the biosynthesis of the kinamycins and lomaiviticins.[22] Recently, it has been reported that a flavin-dependent monooxygenase AlpJ in kinamycin BGC can generate the addition of *N*-acetyl-L-cysteine during diazofuran ring formation by cleaving the C-ring of the angucyclinone skeleton.[23] However, we were unable to identify AlpJ homogeneous gene in our BGC. However, there are several monooxygenase genes found in the BGC, including the UrdM homologous gene, which is involved with D-ring opening for the production of fridamycins (Table S3, Supporting Information). Perhaps, the process of the addition of *N*-acetyl-L-cysteine to the benzene structure of compounds **1** and **2** is assumed to be bound by a mechanism different from the previously reported process in a diazofuran ring.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c04647.

1D NMR, 2D NMR, and HRESIMS of compounds **1**−**4** (PDF)

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Notes

The authors declare no competing financial interest.

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