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PENIPHILONES A AND B: AZAPHILONE ALKALOIDS FROM THE ENDOPHYTIC FUNGUS *PENICILLIUM MAXIMAE*

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Abstract – Two new azaphilone alkaloids, peniphilones A and B (**1** and **2**), and a known congener, (+)-sclerotiorin (**3**), were isolated from the endophytic fungus *Penicillium maximae* by screening based on chemical analysis by UPLC-HRMS with our in-house extract library of natural sources. The structures of **1** and **2** were elucidated on the basis of NMR and HRESI-MS spectroscopic data.

Azaphilones are polyketides with a bicyclic core and a large conjugated chromophore in their structures, produced by fungi. So far, over 170 azaphilone derivatives have been isolated from fungi[1] belonging to *Aspergillus*,[2] *Penicillium*,[3–9] *Chaetominum*,[10] and *Talaromyces*[11] species. Azaphilone derivatives are reported to show various biological activities,[12] including antimalarial,[10] cytotoxic,[8,10] anti-inflammatory,[9] topoisomerase IIα inhibitory,[8] cholesteryl ester transfer protein inhibitory,[13] monoamine oxidase inhibitory,[11] gp120-CD4 binding inhibitory,[3] acyl-CoA:cholesterol acyltransferase inhibitory,[4] endothelin receptor binding antagonistic,[5] aldose reductase inhibitory,[6] and lipoxygenase inhibitory[7] activities. In the course of our screening based on chemical analysis by UPLC-UV/vis-HRMS with our in-house extract library of natural sources,[14] we detected an unidentified azaphilone derivative that showed a molecular ion peak at *m/z* 519.2223 and a characteristic UV spectrum with longer wavelength absorption, in the culture extract of the fungus *P. maximae* 17F04201. In this paper, the isolation, structure elucidation, and biological activities of two new azaphilone alkaloids, peniphilones A and B (**1** and **2**), and a known congener, (+)-sclerotiorin[15] (**3**), are described (Figure 1).

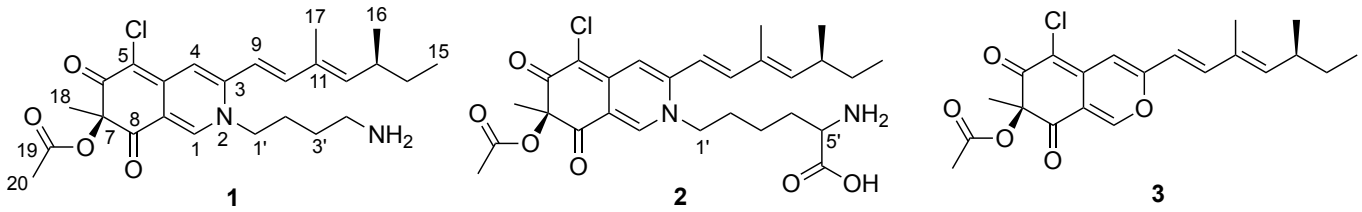

Figure 1. Structures of **1**–**3**

The fungus was cultured on rice medium and extracted with MeOH. Compound **2** having the unidentified molecular ion peak at *m/z* 519.2223 was purified by a LC-UV/vis-MS-guided fractionation along with two congeners (**1** and **3**) showing similar UV spectra. Compound **3** was identified to be (+)-sclerotiorin, which was first isolated from the fungus *Penicillium sclerotiorum.*[15,16]

Peniphilone A (**1**) had the molecular formula $C_{25}H_{33}ClN_2O_4$, which was determined by HRESI-MS. The presence of a chlorine atom was indicated by isotope peaks at *m*/*z* 461/463 (intensity, 3:1). The ^{1}H NMR (Table 1) and HSQC data revealed five olefin protons [δ_H 8.21 (H-1), 7.16 (H-10), 6.97 (H-4), 6.43 (H-9), and 5.85 (H-12)], a methine proton [δ_H 2.49 (H-13)], five methylene protons [δ_H 4.17 (H-1'), 2.73 (H-4'), 1.52/1.69 (H-2'a/2'b), 1.53 (H-3'), and 1.29/1.38 (H-14a/14b)], and five methyl protons [δ_H 2.05 (H_3-20), 1.86 (H_3-17), 1.39 (H_3-18), 0.97 (H_3-16), and 0.83 (H_3-15)]. Similarly, 25 carbon signals in the ^{13}C NMR spectrum (Table 1) were classified into three carbonyl carbons [δ_C 192.9 (C-8), 182.3 (C-6), and 169.1 (C-19)], ten olefinic carbons [δ_C 148.6 (C-3), 146.7 (C-12), 144.5 (C-4a), 143.9 (C-10), 142.7 (C-1), 132.4 (C-11), 115.8 (C-9), 113.8 (C-8a), 109.6 (C-4), and 99.1 (C-5)], an oxygenated quaternary carbon [δ_C 84.8 (C-7)], a methine carbon [δ_C 34.2 (C-13)], five methylene carbons [δ_C 52.9 (C-1'), 38.6 (C-4'), 29.5 (C-14), 26.6 (C-2'), and 25.0 (C-3')], and five methyl carbons [δ_C 23.0 (C-18), 20.1 (C-16), 20.0 (C-20), 12.4 (C-17), and 11.8 (C-15)]. The ^{1}H-^{1}H COSY experiment revealed the presence of three spin-spin systems, H-9/H-10, H_3-17/H-12/H-13(H_3-16)/H_2-14/H-15, and H_2-1'/H_2-2'/H_2-3'/H_2-4' (Figure 2). The HMBC correlations from H-10 to C-17 and from H_3-17 to C-10 and C-11 indicated the presence of a 3,5-dimethyl-1,3-heptadiene moiety. A large coupling constant (15.4 Hz) between H-9 and H-10 and a NOE correlation between H-10 and H-12 clearly showed a 9*E*,11*E* configuration. The HMBC correlations from H-1 to C-3, C-4a, C-8, and C-8a, from H-4 to C-3, C-5, and C-8a, from H_3-18 to C-6, C-7, and C-8, and from H_3-20 to C-19 together with their ^{13}C chemical shifts indicated the presence of an isoquinoline-6,8(2*H*,7*H*)-dione moiety, which is a typical core structure in azaphilone derivatives. The HMBC correlations, H-1/C-1', H-4/C-9, and H-10/C-3, showed that the 3,5-dimethyl-1,3-heptadiene moiety was attached to C-3. The HMBC correlation from H-1 to C-1' showed that the butyl moiety (C-1'−C-4') was bonded to N-2. The chemical shifts, H_2-4' (δ_H 2.73)/C-4' (δ_C 38.6), indicated that an amino group was attached at C-4'. Thus, the planar structure of **1** was determined (Figure 1).

Table 1. ^{1}H and ^{13}C NMR data for **1** and **2** in DMSO-d_6

	1		**2**	
No.	δ_C, type	δ_H, mult. (*J* in Hz)	δ_C, type	δ_H, mult. (*J* in Hz)
1	142.7, CH	8.21, s	142.7, CH	8.18, s
3	148.6, C		148.6, C	
4	109.6, CH	6.97, s	109.7, CH	6.95, s
4a	144.5, C		144.6, C	
5	99.1, C		99.0, C	
6	182.3, C		182.2, C	
7	84.8, C		84.8, C	
8	192.9, C		192.9, C	
8a	113.8, C		113.8, C	
9	115.8, CH	6.43, d (15.4)	115.8, CH	6.43, d (15.4)
10	143.9, CH	7.16, d (15.4)	143.8, CH	7.14, d (15.4)
11	132.4, C		132.3, C	
12	146.7, CH	5.85, d (9.5)	146.6, CH	5.84, d (9.5)
13	34.2, CH	2.49, m	34.4, CH	2.48, m
14	29.5, CH_2	1.29, m	29.5, CH_2	1.29, m
		1.38, m		1.38, m
15	11.8, Me	0.83, t (7.4)	11.8, Me	0.83, t (7.3)
16	20.1, Me	0.97, d (6.7)	20.1, Me	0.97, d (6.7)
17	12.4, Me	1.86, s	12.4, Me	1.85, s
18	23.0, Me	1.39, s	23.1, Me	1.39, s
19	169.1, C		169.1, C	
20	20.0, Me	2.05, s	20.0, Me	2.05, s
1’	52.9, CH_2	4.17, t (7.0)	53.4, CH_2	4.12, m
2’	26.6, CH_2	1.52, m	29.3, CH_2	1.65, m
		1.69, m		
3’	25.0, CH_2	1.53, m	22.0, CH_2	1.37, m
4’	38.6, CH_2	2.73, m	30.4, CH_2	1.61, m
				1.73, m
5’			53.8, CH	3.14, m
6’			169.8, C	

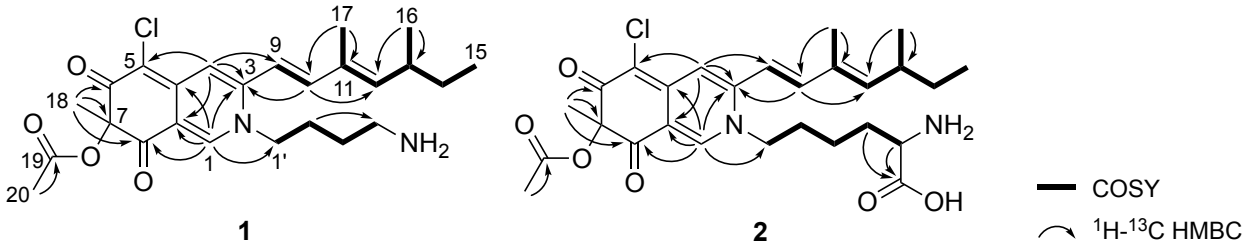

Figure 2. ^{1}H-^{1}H COSY and key HMBC correlations of **1** and **2**

The molecular formula of peniphilone B (**2**) was determined to be $C_{27}H_{35}ClN_2O_6$, having one more $C_2H_2O_2$ unit than **1**, by HRESI-MS. The ^{1}H and ^{13}C NMR spectra (Table 1) were almost superimposable on those of **1** except for the presence of a carbonyl carbon [δ_C 169.8 (C-6’)] and a methine group [δ_H 3.14

(H-5')/δ_C 53.8 (C-5')], and the absence of an amino group. The HMBC correlation from H_2-4' and H-5' to C-6' showed that 2-aminohexanoic acid was attached to N-2 instead of the aminobutyl moiety in **1**.

It is known that **3** reacts with primary amines to afford amino-derivatives,[13,17] and **1** and **2** were considered to be formed from **3** with 1,4-diaminobutane and lysine, respectively, in the fungus. To confirm the absolute configuration of **1**, **3** was reacted with 1,4-diaminobutane. The synthetic and natural **1** showed the same ^{1}H NMR spectra (Figures S1 and S12), specific rotation, and retention times of the LC-MS analysis (Figure 3 (a-2 and a-3), and therefore **1** may have the 7*R*,13*S*-configuration. To determine the absolute configuration of **2**, **3** was reacted with L- and D-lysine to afford **2a** and **2b**, respectively.[13] Although **2a** and **2b** were diastereomers, they showed the same ^{1}H NMR spectra (Figures S13 and S14) and specific rotations (**2a**: $[\alpha]^{25}_D$ +272 (*c* 0.100, EtOH) and **2b**: $[\alpha]^{25}_D$ +280 (*c* 0.100, EtOH)). The diastereomers **2a** and **2b** were analyzed by LC-MS using various C18 and chiral HPLC columns (b-2 and b-3, respectively)), but their retention times could not be separated. The absolute configurations of C-7 and C-13 of **2** are probably *R* and *S*, respectively, but that of C-5' remains unknown.

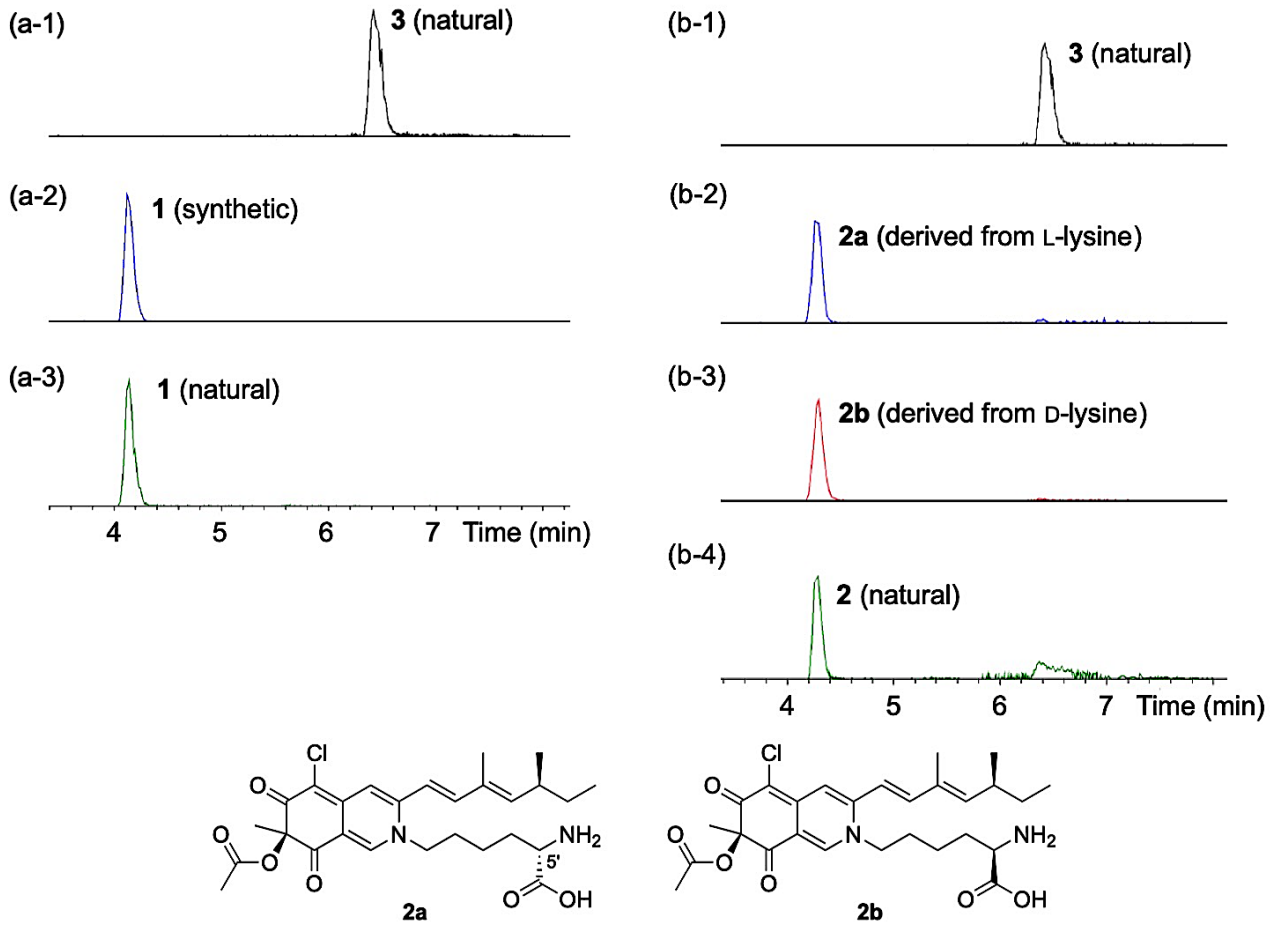

Figure 3. LC-ESIMS profiles of natural **3** (a-1 and b-1), **1** (a-3), and **2** (b-4). LC-ESIMS profiles of the reaction product of **3** and 1,4-diaminobutane extracted with *m/z* 461 $[M+H]^+$ (a-2) and those of **3** and L- and D-lysine (**2a** and **2b**) extracted with *m/z* 519 $[M+H]^+$ (b-2 and b-3, respectively).

BIOLOGICAL ACTIVITIES

The biological activities of **1** and **2** were tested, but they did not show cytotoxicity against HeLa cells and antimicrobial activities against *Bacillus cereus*, *Escherichia coli*, and *Candida albicans* at 50 μM.

EXPERIMENTAL

General Experimental Procedures.

Optical rotation was measured on a JASCO DIP-1000 polarimeter in EtOH. UV spectra were measured on a JASCO V-550 spectrophotometer in MeOH. IR spectra were recorded on a Perkin Elmer Frontier FT-IR spectrophotometer. NMR spectra were measured on a Bruker Avance I 600 NMR spectrometer. Chemical shifts were referenced to the residual solvent peaks (δ_H 2.49 and δ_C 39.5 for DMSO-d_6). ESI-HRMS data were recorded on a Waters Xevo G2-XS QTof mass spectrometer. The preparative MPLC was performed on a Biotage Isolera I. LC-MS experiments were performed on a Shimadzu LC-20AD solvent delivery system and interfaced to a Bruker amaZon Speed mass spectrometer. The preparative HPLC system comprised a Waters 515 HPLC pump, Waters 2489 UV/visible detector, and Pantos Unicorder U-228.

Fungal Strain.

The fungal strain 17F4201 was isolated from a leaf of an unidentified plant at Hyuga, Miyazaki Prefecture, Japan, in 2017, and deposited at the Graduate School of Pharmaceutical Sciences, Kumamoto University. The strain was identified from its ITS sequences. A 253 base pair ITS sequence had 100% sequence identity to that of *Penicillium maximae* (MN737732). The sequence data of this strain had been deposited to GenBank with accession number MW142485.

Culture Conditions.

The fungus was cultured on solid rice medium in two plastic containers (W17 cm × D22 cm × H9 cm). To prepare rice medium, 100 g of rice was added to each container with 100 mL of deionized water. After autoclaving at 121 °C for 20 min, the rice medium was inoculated with the fungus and cultured at 27 °C for 14 days.

Extraction and Isolation.

The fungal culture on rice medium (200 g) was extracted with MeOH (1 L × 3), and the solution was concentrated. The red extract (15.9 g) was absorbed on Diaion HP-20 resin and eluted with H_2O, MeOH, and acetone. Analysis by UPLC-UV/vis-HRMS showed that the MeOH eluent contained an unidentified ion peak at *m*/*z* 519 and its congeners. The MeOH eluent (9.56 g) was subject to reversed-phase MPLC (Purif-Pack®-EX ODS-25μm, Shoko Science Co., Ltd., size: 60) with 30, 40, 50, and 100% MeCN−H_2O. The fraction eluted by 40% MeCN−H_2O (602.4 mg) was fractionated by reversed-phase MPLC (Purif-Pack®-EX ODS-25μm, size: 60) with 40, 60, 80, and 100% MeOH−H_2O. The fraction eluted by

60% MeOH−H_2O (154 mg) was purified by ODS HPLC (CAPCELLPAK C_{18} UG120Å 5 μm column, Osaka Soda Inc., 20 × 250 mm; linear gradient elution using 30−70% MeCN−H_2O containing 0.2% formic acid in 70 min; flow rate, 6 mL/min) to yield a fraction (t_R 28 min) containing **2** (*m/z* 519 $[M + H]^+$; 10.6 mg). This fraction was purified by gel filtration HPLC (Asahipak GS-310P 20G, Shodex, 20 x 500 mm; MeOH containing 0.2% formic acid; flow rate, 2 mL/min) to afford **2** (1.85 mg). The fraction (t_R 21 min, 11.1 mg) obtained by the initial ODS HPLC was further purified by ODS HPLC (Luna 5u Phenyl-Hexyl column, Phenomenex Inc., 21.2 × 250 mm; 30% MeCN−H_2O containing 0.2% formic acid; flow rate, 6 mL/min) to yield **1** (1.7 mg). The MeCN eluent (1.68 g) from the first reversed-phase MPLC was subjected to SiO_2 MPLC (Purif-Pak®-EX SI-25 μm, size: 60) and eluted with a linear gradient system of 0−15% EtOAc−*n*-hexane and then a stepwise gradient system of 0, 1, 2, 5, 10, 30, and 100% MeOH-CH_2Cl_2. The fractions eluted with 0−15% EtOAc−*n*-hexane (381.6 mg) were combined and fractionated by SiO_2 MPLC (Purif-Pak®-EX SI-25 μm, size: 60) with 0, 5, 10, 15, 20, 50, and 100% EtOAc−*n*-hexane. The fraction (42.6 mg) eluted with 5−10% EtOAc−*n*-hexane was purified by SiO_2 HPLC (YMC-Pack R&D D-SIL-5, 20 x 250 mm; flow rate, 6 mL/min) with 15% EtOAc−*n*-hexane to yield **3** (20.6 mg; t_R 28.2 min).

Peniphilone A (1): red powder; $[\alpha]^{25}_D$ +228 (*c* 0.100, EtOH); UV λ_{max} (MeOH) nm (log ε): 233 (4.31), 339 (4.38), 370 (4.39), 491 (3.74); IR (film) ν_{max} cm^{-1}: 3384, 2961, 2927, 2864, 1737, 1702, 1584, 1492, 1370, 1348; ^1H and ^{13}C NMR data, see Table 1; ESI-HRMS *m/z* 461.2191 $[M + H]^+$ (calcd for $C_{25}H_{34}ClN_2O_4$, 461.2207).

Peniphilone B (2): red powder; $[\alpha]^{25}_D$ +298 (*c* 0.100, EtOH); UV λ_{max} (MeOH) nm (log ε): 236 (4.30), 314 (4.18), 374 (4.29), 490 (3.65); IR (film) ν_{max} cm^{-1}: 3375, 2965, 2930, 2860, 1733, 1702, 1588, 1492, 1370; ^1H and ^{13}C NMR data, see Table 1; ESI-HRMS *m/z* 519.2223 $[M + H]^+$ (calcd for $C_{27}H_{36}ClN_2O_6$, 519.2262).

Reaction of 3 with 1,4-diaminobutane, and L- and D-lysines.

A mixture of **3** (2.0 mg) and 1,4-diaminobutane (50 μL) in MeOH (600 μL) was adjusted to pH 8 with 1 M HCl, and the mixture was kept at 25 °C for 1 h. The reaction mixture was purified by HPLC (CAPCELLPAK C_{18} UG120Å 5 μm column, 20 × 250 mm; 45% MeCN containing 0.2% formic acid; flow rate, 6 mL/min) followed by gel filtration HPLC (Asahipak GS-310P 20G, Shodex, 20 x 500 mm; MeOH containing 0.2% formic acid; flow rate, 2 mL/min) to yield **1** (0.58 mg; $[\alpha]^{25}_D$ +214 (*c* 0.100, EtOH)). To **3** (2.0 mg) in MeOH (600 μL) was added L-lysine (5.0 mg) in H_2O (100 μL), and the solution was adjusted to pH 8 with 0.2 M NaOH and kept at 25 °C for 1 h. The reaction mixture was purified by HPLC (CAPCELLPAK C_{18} UG120Å 5 μm column, 20 × 250 mm; 45% MeCN containing 0.2% formic

acid; flow rate, 6 mL/min) to afford **2a** (1.18 mg; $[\alpha]^{25}_{D}$ +272 (*c* 0.100, EtOH)). In the same way, **2b** (0.59 mg) was obtained from **3** (2.0 mg) and D-lysine (5.0 mg; $[\alpha]^{25}_{D}$ +280 (*c* 0.100, EtOH)).

LC-MS Analysis of Reaction Mixtures.

Reaction mixtures were analyzed by LC-HR-ESIMS on a COSMOSIL 2.5C_{18}-MS-II column (Nacalai Tesque Inc., 2.5 × 100 mm) at 40 °C, eluting with a gradient of two solvents, 0.1% acetic acid in H_2O (solvent A) and 0.1% acetic acid in MeCN (solvent B), at 0.30 mL/min. The gradient program was 10–100% B from 0 to 5 min. Mass spectra were detected in positive and negative ion modes, and data were analyzed using DataAnalysis software (Bruker).

Cytotoxicity and Antimicrobial Assay.

The details of the biological assay performed in this experiment were previously described.[18,19]

ACKNOWLEDGEMENTS

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REFERENCES

1. N. Osmanova, W. Schultze, and N. Ayoub, *Phytochem. Rev*., 2010, **9**, 315.
2. A. A. Stierle, D. B. Stierle, and T. Bugni, *J. Nat. Prod.*, 2001, **64**, 1350.
3. S. Ōmura, H. Tanaka, K. Matsuzaki, H. Ikeda, and R. Masuma, *J. Antibiot.*, 1993, **46**, 1908.
4. N. Arai, K. Shiomi, H. Tomoda, N. Tabata, D. J. Yang, R. Masuma, T. Kawakubo, and S. Ōmura, *J. Antibiot*., 1995, **48**, 696.
5. L. Pairet, S. K. Wrigley, I. Chetland, E. E. Reynolds, M. A. Hayes, J. Holloway, A. M. Ainsworth, W. Katzer, X.-M. Cheng, D. J. Hupe, P. Charlton, and A. M. Doherty, *J. Antibiot.*, 1995, **48**, 913.
6. C. Chidananda, L. Jagan Mohan Rao, and A. P. Sattur, *Biotechnol. Lett*., 2006, **28**, 1633.
7. C. Chidananda and A. P. Sattur, *J. Agric. Food Chem*., 2007, **55**, 2879.
8. L. Du, H.-C. Liu, W. Fu, D.-H. Li, Q.-M. Pan, T.-J. Zhu, M.-Y. Geng, and Q.-Q. Gu, *J. Med. Chem.*, 2011, **54**, 5796.
9. J.-L. Tang, Z.-Y. Zhou, T. Yang, C. Yao, L.-W. Wu, and G.-Y. Li, *J. Agric. Food Chem.*, 2019, **67**, 2175.
10. N. Panthama, S. Kanokmedhakul, K. Kanokmedhakul, and K. Soytong, *J. Nat. Prod.*, 2011, **74**, 2395.
11. E. Yoshida, H. Fujimoto, M. Baba, and M. Yamazaki, *Chem. Pharm. Bull*., 1995, **43**, 1307.
12. N. Khurana, S. B. Sharma, A. A. Jafri, and A. M. Sharma, *Asian J. Biomed. Pharm. Sci*., 2017, **7**, 34.

13. H. Tomoda, C. Matsushima, N. Tabata, I. Namatame, H. Tanaka, M. J. Bamberger, H. Arai, M. Fukazawa, K. Inoue, and S. Ōmura, *J. Antibiot*., 1999, **52**, 160.
14. Y. Koyanagi, T. Kawahara, Y. Hitora, and S. Tsukamoto, *Heterocycles*, 2020, **100**, 1686.
15. T. MacCurtin and J. Reilly, *Nature*, 1940, **146**, 335.
16. A. R. Germain, D. M. Bruggemeyer, J. Zhu, C. Genet, P. O'Brien, and J. A. Porco, Jr., *J. Org. Chem.*, 2011, **76**, 2577.
17. R. A. Eade, H. Page, A. Robertson, K. Turner, and W. B. Whalley. *J. Chem. Soc.*, 1957, 4913.
18. A. H. Afifi, A. H. El-Desoky, H. Kato, R. E. P. Mangindaan, N. J. de Voogd, N. M. Ammar, M. S. Hifnawy, and S. Tsukamoto, *Tetrahedron Lett*., 2016, **57**, 1285.
19. M. Gushiken, I. Kagiyama, H. Kato, T. Kuwana, F. Losung, R. E. P. Mangindaan, N. J. de Voogd, and S. Tsukamoto, *J. Nat. Med*., 2015, **69**, 595.