HETEROCYCLES, Vol. 94, No. 2, 2017, pp. 326 - 333. © 2017 The Japan Institute of Heterocyclic Chemistry
Received, 21st November, 2017, Accepted, 10th January, 2017, Published online, 24th January, 2017
DOI: 10.3987/COM-16-13624

NEW TWO PEBROLIDE DERIVATIVES, 14-DEACETOXY-1-DEOXYPEBROLIDE AND 7'-HYDROXYASPERPHENAMATE ISOLATED FROM *PENICILLIUM* sp. IFM62525

Syuhei Arai,[a] Daigo Wakana,[a] Takeshi Itabashi,[a] Hisashi Takeda,[a] Takashi Yaguchi,[b] Galba Maria de Campos Takaki,[c] and Tomoo Hosoe[a*]

[a]Department of Organic Chemistry, Faculty of Pharmaceutical Sciences, Hoshi university, 2-4-41 Ebara, Shinagawa-ku, Tokyo, 142-8501, Japan; [b]Medical mycology research center Chiba university, 1-8-1 Inohana, Chuo-ku, chiba city, Chiba, 260-8673, Japan; [c]Núcleo de Pesquisas em Ciências Ambientais Universidade Católica de Pernambuco, Rua Nunes Machado, 42 Boa Vista, Bloco J, Recife, 50050-590, Brasil; E-mail: hosoe@hoshi.ac.jp

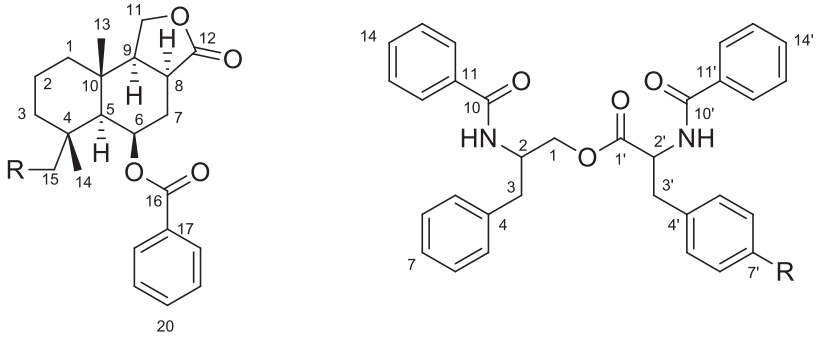
Abstract – New two pebrolide type sesquiterpenoids, deacetoxy-1-deoxypebrolide (**1**) and 7'-hydroxyisoasperphenamate (**2**) along with asperphenamate (**3**), mycophenolic acid (**4**) and 1-deoxypebloride (**5**) were isolated from *Penicillium* sp. IFM 62525. Their structures were established from spectroscopic and chemical method. The absolute stereochemistry of **2** was confirmed by Marfey's method. Antifungal activities of these compounds were tested and **4** showed strong activity.

The fungi belong to genus *Penicillium* were one of the most important sources of new and bioactive conpounds. Our previous study searching the new and biological compounds from genus *Penicillium* have been reported three citrinin dimers penicitrinone A, B and penicitrinol A from *P. citrinum* IFM53298,[1] an indolediterpene penijanthine A from *P. janthinellum* IFM55557,[2] two meroterpenoids penisimplicin A and B from *P. simplicissimum* IFM53375.[3]

In our continuous searching for new antifungal compounds, we focused the fungus *Penicillium* sp. IFM62525, because the methanol extract of this fungus showed antifungal activities against *Aspergillus fumigatus* IFM41243, *A. niger* IFM41398, *Candida albicans* IFM40009 and *Cryptococcus neoformans* ATCC90112 by paper disk method.[1-3] This strain was classified into *Penicillium* sp. based on morphology, but its sequences of the ribosomal internal transcribed spacer (ITS) region and the β-tubulin gene did not

show high similarities with those registered as the species of genus *Penicillium* on the GenBank. Therefore, the isolate have been considered as a new species of genus *Penicillium*. In this paper, we have reported that the study of its secondary metabolites lead to two new compounds, deacetoxy-1-deoxypebrolide (**1**), 7'-hydroxyisoasperphenamate (**2**), along with asperphenamate (**3**),[4,5] mycophenolic acid (**4**)[6] and 1-deoxypebloride (**5**).[7]

The methanol extract of *Penicillium* sp. IFM62525 cultured on rice medium was separated and purified by HPLC to afford deacetoxy-1-deoxypebrolide (**1**), 7'-hydroxyisoasperphenamate (**2**), asperphenamate (**3**),[4,5] mycophenolic acid (**4**)[6] and 1-deoxypebloride (**5**)[7] (Figure 1).



deacetoxy-1-deoxypebrolide (**1**): R=H
1-deoxypebrolide (**5**): R=OAc

7'-hydroxyasperphenamate (**2**): R=OH
asperphenamate (**3**): R=H

Figure 1. The structures of the compounds isolated from *Penicillium* sp. IFM62525

Deacetoxy-1-deoxypebrolide (**1**) was obtained as colorless amorphous powder and the molecular formula was confirmed to $C_{22}H_{28}O_4$ by HR-EI-MS. The IR spectrum (1716 and 1782 cm^{-1}) indicated the presence of two ester group in **1**. The ^{1}H-NMR spectrum showed the mono substituted phenyl moiety [δ 7.90 (2H, brd, J = 7.4 Hz), δ 7.41 (2H, d, J = 7.4 Hz), δ 7.52 (1H, t, J = 7.4 Hz)], an oxygenated methine proton (δ 5.77), and a pair of methylene protons [δ 4.38 (d, J = 9.7 Hz), δ 4.19 (dd, J = 9.7, 5.5 Hz), three singlet methyl protons (δ 1.39, δ 1.04, and δ 0.90) and other eleven aliphatic protons (Table 1). ^{13}C-NMR spectrum showed eight sp^2 carbons including two carbonyl carbons (δ 178.0, δ 166.8), four sp^3 methine carbons including an oxygenated one (δ 67.4), five sp^3 methylene carbons including an oxygenated one (δ 67.8), three methyl carbons (δ 33.9, δ 23.7, and δ 17.8) and two sp^3 quaternary carbons (Table 1). The ^{1}H- and ^{13}C-NMR spectra of **1** were similar to that of **5**, which was isolated from this fungus, except for the disappearance of acetoxy (δ_H 2.08, δ_C 20.9 and δ_C 170.9) and methylene (δ_H 3.78 and δ_H 3.99, δ_C 72.5) moiety of **5** and appearance of methyl group (δ_H 1.04, δ_C 33.9) of **1**. Therefore, **1** was estimated to deacetoxy derivative of 1-deoxypebrolide (**5**).

Table 1. ^{1}H- and ^{13}C-NMR data of deacetoxy-1-deoxypebrolide (**1**) in chloroform-d_1

deacetoxy-1-deoxypebrolide (**1**)		
Position	δ_C	δ_H (J in Hz)
1	43.5	1.76 (ddd, 12.8, 4.8, 2.9), 1.09 m
2	18.3	1.64 m, 1.49 m
3	44.0	1.43 m, 1.25 s
4	33.8	
5	53.0	1.18 brs
6	67.4	5.77 (ddd, 4.5, 2.3, 1.9)
7	28.4	2.02 (ddd, 15.7, 8.4, 4.5), 2.61 (brd, 15.7)
8	36.1	2.66 (t, 8.4)
9	49.6	2.25 (dd, 8.4, 5.5)
10	35.0	
11	67.8	4.38 (d, 9.7), 4.19 (dd, 9.7, 5.5)
12	178.0	
13	17.8	1.39 s
14	23.7	0.90 s
15	33.9	1.04 s
16	166.8	
17	130.7	
18/22	129.8	7.90 (brd, 7.4)
19/21	128.5	7.41 (t, 7.4)
20	132.9	7.52 (t, 7.4)

The detail analysis of ^{1}H-^{1}H COSY and HMBC spectra showed in Figure 2A. Three spin systems (H_2-1 to H_2-3, H-5 to H_2-11 and H-18 to H-22) were confirmed by the correlations of ^{1}H-^{1}H COSY correlations. The HMBC cross correlations between methyl group at C-14 and C-15 showed a presence of dimethylated moiety. The decalin moiety was suggested by the correlations from two methyl group at H_3-14 and H_3-15 to C-3, C-4 and C-5 and from other methyl proton H_3-13 to C-1, C-5, C-9 and C-10 and from H_2-7 to C-5. The correlations from H-7, H-8, H-9 and H-11 to the carbonyl carbon at C-12 suggested to the presence of γ-butyrolactone moiety connected to decalin moiety. It was confirmed that the phenyl moiety connected to C-6 through ester linkage at C-16 because of the correlations from H-6 and H-18/22 to C-16. Therefore, the structure of **1** was established to the deacetoxy compound of 1-deoxypebloride (**5**).

The relative configuration of **1** was confirmed by the detail analysis of NOESY spectrum (Figure 2B). The correlations between H-2ax and H_3-14, H_3-13 and H_3-14, H-5 and H-7ax and H-9 suggested that the two cyclohexane rings was chair form. Phenyl moiety was axial by the small coupling constant between H-5 and H-6 and the NOESY correlation between H_3-15 and H-6eq. The absolute stereochemistry of **1** was confirmed same configuration to **5**, which isolated from this fungus, by the comparison of CD spectra (Supporting Information S5). Therefore, the structure of **1** was elucidated as shown in Figure 1.

The molecular formula of **2** was established to $C_{32}H_{20}N_2O_5$ by HR-ESI-MS spectrum and the appearance was white amorphous powder. The IR spectrum (1602, 1631 and 1736 cm^{-1}) indicated the presence of two amide and an ester group in **2**.

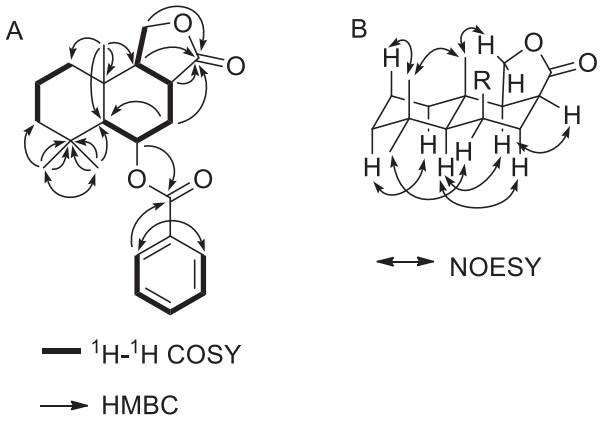


Figure 2. The detail 2D-NMR spectral analysis of deacetoxy-1-deoxypebrolide (**1**)
A: ^{1}H-^{1}H COSY correlation and selected HMBC correlation, B: selected NOESY correlation

Table 2. ^{1}H- and ^{13}C-NMR data of 7'-hydroxyisoasperphenamate (**2**)

hydroxyasperphenamate (**2**)		
Position	δ_C	δ_H (J in Hz)
1	65.7	4.50 (dd, 11.3, 3.6), 4.05 (dd, 11.3, 4.6)
2	50.7	4.60 m
3	37.6	3.00 (dd, 13.8, 6.3), 2.90 (dd, 13.8, 8.2)
4	137.4	
5/9	129.7	7.22 (d, 6.9)
6/8	129.1[b]	7.30 (t, 6.9)
7	127.2	7.23 (t, 6.9)
10	166.4	
11	133.7	
12/16	127.4[a]	7.66 m
13/15	128.8[b]	7.38 (t, 7.5)
14	132.4	7.50 (t, 7.5)
1'	172.4	
2'	54.9	4.87 (dt, 6.7, 6.6)
3'	37.2	3.20 (dd, 13.9, 6.6), 3.13 (dd, 13.9, 6.7)
4'	127.9	
5'/9'	130.7	7.05 (d, 8.5)
6'/8'	116.2	6.75 (d, 8.5)
7'	155.5	
10'	167.3	
11'	134.5	
12'/16'	127.5[a]	7.69 m
13'/15'	129.0[b]	7.32 (t, 7.5)
14'	131.8	7.42 (t, 7.5)
2-NH		6.66 (d, 8.4)
2'-NH		6.57 (d, 6.7)

[a, b] These chemical shifts may be exchanged.

The ^{1}H-NMR spectrum showed 19 aromatic protons including 1,4-disubstituted benzene moiety [δ 7.05 (2H, d, *J* = 8.5 Hz), δ 6.75 (2H, d, *J* = 8.5 Hz)], three pairs of methylene protons [δ 4.50 (dd, *J* = 11.3, 3.6 Hz) and δ 4.05 (dd, *J* = 11.3, 4.6 Hz), δ 3.20 (dd, *J* = 13.9, 6.6 Hz) and δ 3.13 (dd, *J* = 13.9, 6.7 Hz), δ 3.00 (dd, *J* = 13.8, 6.3 Hz) and δ 2.90 (dd, *J* = 13.8, 8.2 Hz)], two methine protons (δ 4.87, δ 4.60) and other two protons (δ 6.66, δ 6.57) in Table 2. The ^{13}C-NMR spectrum showed a carbonyl carbon (δ 172.4), two amide carbons (δ 167.3 and δ 166.4), 24 sp^2 carbons including an oxygenated one (δ 155.5), two sp^3 methine carbons and three sp^3 methylene carbons (Table 2). Comparison of NMR data between **2** and **3** suggested that **2** was monohydroxylated compound at the aromatic moieties of **3**. The detail analysis of ^{1}H-^{1}H COSY and HMBC spectra were shown in Figure 3.

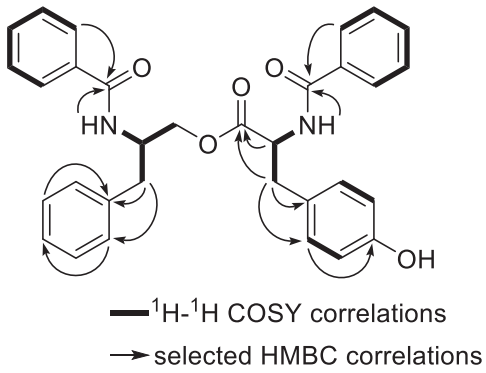


Figure 3. Selected ^{1}H-^{1}H COSY and HMBC correlations of 7'-hydroxyisoasperphenamate (**2**)

Two monosubstituted benzene moieties (C11-C16 and C-11'-C-16'), a 1,4-disubstituted benzene ring (C-4'-C-9'), an ethylamine (C-2'-C-3') and isopropylamine (C-1-C-3) moieties were confirmed by the correlations of ^{1}H-^{1}H COSY (Figure 3). The connection of these partial structures was confirmed by correlations of HMBC spectra (Figure 3). The HMBC correlations from 2-NH (2-NH') to C-10 (C-10') and from H-12 (H-12') to C-10 (C-10') showed that the isopropylamine moiety conjugated to a monosubstituted benzene ring (C11-C16) and ethylamine moiety connected to another monosubstituted benzene ring (C-11'-C-16') through amide linkage. The 1,4-disubstituted benzene ring linked to C-3' of ethylamine moiety because of the correlations from H-3' to C-4' and C-5'. This benzene ring was confirmed to have hydroxy group by the correlation from H-5' to C-7'(δ 155.5). The remained benzene ring was verified by the correlations from H_2-3 to C-4 and C-5/9, from H-5/9 to C-7 and from H-6/8 to C-4. The HMBC correlations from H_2-3 to C-4 and C-5/9 showed that the benzene ring connected to C-3 position of isopropylamine moiety. Unfortunately, the correlation proved the connection between isopropylamine moiety and ethylamine moiety was not observed. But the molecular formula and 1736

cm^{-1} of IR absorption suggested that these moieties were connected through ester linkage.

Compound **2** was derived from two benzoic acid, a tyrosine and a phenylalaninol moiety. Therefore, the absolute stereochemistry of **2** was confirmed by Marfey's method.[8] Compound **2** was hydrolyzed by 6M HCl. The reactant was treated Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine: FDAA) and the LC chromatogram was measured at the reaction mixture and compared with the FDAA derivatives of D- or L-tyrosine and phenylalaninol. As the results, HPLC analysis revealed the presence of D-tyrosine and L-phenylalaninol moiety in **2**. Therefore, the structure of **2** was estimated to the stereoisomer of asperphenamate (**3**) at C-2' shown in Figure 1.

The antifungal activities of **1** and **3**-**5** against *Aspergillus fumigatus*, *A. niger*, *Candida albicans* and *Cryptococcus neoformans* were tested by paper disk method using an amphotericin B as a positive control. Mycophenolic acid (**4**) showed inhibitory zone at 42, 28, 37, and 46 mm for *A. fumigatus*, *A. niger*, *C. albicans,* and *C. neoformans*, respectively.[9]

EXPERIMENTAL

General procedure

EI-MS were taken with a JEOL JMS-MS600W spectrometer and ESI-MS were JEOL JMS-T100LP spectrometer. UV and IR spectra were recorded on a Amersham Biosciences Ultrospec 2100 spectroplarimeter and a JASCO IR-810 spectrometer, respectively. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AvanceII (^{1}H: 400 MHz, ^{13}C: 100 MHz) spectrometer using tetramethylsilane as an internal standard. CD spectra were measured on a JASCO J-600 spectropolarimeter. Column chromatography was performed using GE healthcare Japan Sephadex LH-20 resin. Middle pressure liquid chromatography was performed using a Yamazen Ultrapack SI-40B (26 x 300 mm). HPLC was performed with a GL sciences Inertsil ODS-3 column equipped with a Shimamura YRD-883 RI recorder. Linear gradient mode was performed using 20-100% MeCN (40 min) and the flow rate was 0.1 mL/min.

Fungal Material

The studied strain was isolated from a soil in Recife, Pernambuco, Brazil, identified as *Penicillium* sp. based on morphology and its sequences of the ITS region and the β-tubulin gene and deposited at the Medical Mycology Research Center, Chiba University, under the accession number IFM62525.

Fermentation and Isolation

Rice (dry weight: 960 g) was soaked in water at an hour and the moisture rice (dry volume: 120 g) was put into each Roux flasks. These Roux flasks were sterilized at 120 °C for 20 min. *Penicillium* sp. IFM 62525 was precultured on PDA, and this fungus was put into each Roux flasks together with agar medium, and cultured at 25 °C for 3 weeks. After cultivation, methanol was poured into each Roux flasks and extracted at room temperature for overnight. The extracted solution was filtered and evaporated in *vacuo*.

The MeOH extract was suspended by water and extracted twice by EtOAc. The EtOAc extract was filtered and mycophenolic acid (**4**: 7.0 g) was obtained. The filtrate of EtOAc extract was suspended by MeCN and extracted at hexane. The MeCN extract was chromatographed on 100 g of sephadex LH-20 resin using stepwise method which mobile phase were hexane-$CHCl_3$ (1:4), $CHCl_3$-acetone (4:1), $CHCl_3$-acetone (2:3), acetone and MeOH. The second fraction (160.7 mg) was filtered and asperphenamate (**3**: 20.5 mg) was isolated. The filtrate of second fraction was separated by HPLC on ODS with linear gradient mode to give 1-deoxypebrolide (**5**: 4.8 mg) and **1** (2.6 mg). The forth fraction (140.2 mg) was purified by HPLC on ODS with linear gradient mode to yield **2** (1.3 mg).

Deacetoxy-1-deoxypebrolide (1): white amorphous powder; $[\alpha]_D$ -10.4 (c 0.26, MeOH); UV (MeOH) λ_{max} (logε) 229.3 (1.61) nm; IR (KBr) ν_{max} 3394, 1782, 1716 cm^{-1}; CD (MeOH) $\Delta\varepsilon$ (λ_{max}) +4.56 (221), -2.58 (243), +1.09 (275); HR-EI-MS *m/z* 356.1977 (calcd. 356.1987 for $C_{22}H_{28}O_4$); ^{1}H- and ^{13}C-NMR data were summerlized in Table 1.

7'-Hydroxyisoasperphenamate (2): white amorphous powder; $[\alpha]_D$ -92.7 (c 0.05, MeOH); UV (MeOH) λ_{max} (logε) 204.3 (4.47), 225.2 (4.40) nm; IR (KBr) ν_{max} 3427, 1736, 1631, 1602 cm^{-1}; CD (MeOH) $\Delta\varepsilon$ (λ_{max}) -20.9 (222), -50.2 (234); HR-ESI-MS *m/z* 545.2049 (calcd. 545.2052 for $C_{32}H_{30}N_2O_5Na$); ^{1}H- and ^{13}C-NMR data were summarized in Table 2.

Determination of absolute configurations of 2 by Marfey's method

A solution of **2** (1 mg) in 6M HCl was stirred at 110 ºC for 12 h. After removal the solvent, L-FDAA (2 mg) and 1M $NaHCO_3$ solution were added to this hydrolysate of **2**. The solution was stirred at 40 ºC for 1 h. After the reaction was quenched by addition of 1M HCl solution, the reaction mixture was diluted with MeOH. In a similar fashion, D- and L-tyrosine and D- and L-phenylalaninol were derivatized with FDAA. The FDAA derivatives of the reactant, tyrosine and phenylalaninol were analyzed by PDA-HPLC (GLscience ODS-3 column; 5 μm, 4.6 x 250 mm), using the following program: Analysis of phenylalaninol-FDAA, solvent A (H_2O), solvent B (MeCN), linear gradient: 10 – 100% of B (30 min). Analysis of tyrosine, isochratic 40% MeCN. The retention times (t_R) for the FDAA-D-phenylalaninol, FDAA-L-phenylalaninol, FDAA-D-tyrosine and FDAA-L-tyrosine were 15.73, 14.98, 7.85 and 7.60 min, respectively, and the t_R of the FDAA derivative of hydrolysates of **2** was 15.73 and 7.85 min.

Antifungal assay by paper disc method

Antifungal assay was performed by the paper disc method against pathogenic filamentous fungi, *Aspergillus fumigatus* IFM41243 and *A. niger* IFM41398, and pathogenic yeasts, *Candida albicans* IFM 40009 and *Cryptococcus neoformans* ATCC90112. Test substances were dissolve in $CHCl_3$ and added to paper disc (8 mm) at 100 μg/disc of density, dried, and then placed on the assay plates. The plates were incubated at 25 ºC for 24-48 h and measured the inhibitory zone. Amphotericine B was used as active

control at the density was 5 μg/disk (inhibitory zone; *A. fumigatus*: 21 mm, *A. niger*: 24 mm, *C. albicans*: 23 mm and *C. neoformans*: 24 mm).

REFERENCES

1. D. Wakana, T. Hosoe, T. Itabashi, K. Okada, G. M. C. Takaki, K. Fukushima, and K. Kawai, *J. Nat. Prod.*, 2006, **60**, 279.
2. T. Itabashi, T. Hosoe, D. Wakana, K. Fukushima, K. Takizawa, T. Yaguchi, K. Okada, G. M. C. Takaki, and K. Kawai, *J. Nat. Med.*, 2009, **63**, 96.
3. S. Komai, T. Hosoe, T. Itabashi, K. Nozawa, K. Okada, G. M. C. Takaki, T. Yaguchi, K. Takizawa, K. Fukushima, and K. Kawai, *Chem. Pharm. Bull.*, 2005, **9**, 1114.
4. A. M. Clark, C. D. Hufford, and L. W. Robertson, *Lloydia*, 1977, **40**, 146.
5. L. Yuan, J. H. Wang, and T. M. Sun, *Chin. Chem. Lett.*, 2010, **21**, 155.
6. Y. Lee, Y. Fujisawa, K. Ujita, M. Nagamoto, H. Ohta, and I. Shimidzu, *Bull. Chem. Soc. Jpn.*, 2001, **74**, 1437.
7. N. J. Mccorkindale, C. H. Calzadilla, S. A. Hutchinson, and D. H. Kitson, *Tetrahedron*, 1981, **37**, 649.
8. T. Itabashi, N. Matsuishi, T. Hosoe, N. Toyazaki, S. Udagawa, T. Imai, M. Adachi, and K. Kawai, *Chem. Pharm. Bull.*, 2006, **54**, 1639.
9. T. Noto, M. Sawada, K. Ando, and K. Koyama, *J. Antibiot*., 1969, **22**, 165.