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Penicimutamides D–E: two new prenylated indole alkaloids from a mutant of the marine-derived *Penicillium purpurogenum* G59†

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Three prenylated indole alkaloids (**1–3**), including two new penicimutamides D–E (**1–2**), were isolated from a diethyl sulfate mutant of the marine-derived fungus *Penicillium purpurogenum* G59. The structures of **1–3**, and their absolute configurations, were determined by spectroscopic methods, including X-ray crystallography and CD analyses. HPLC-UV and HPLC-MS analyses showing that **1–3** were only produced in the mutant evidenced that the silent biosynthetic pathways that produce **1–3** in the parental strain are activated by DES mutagenesis.

Prenylated indole alkaloids (PIAs) are a broad class of secondary fungal metabolites with a bicyclo[2.2.2]diazaoctane core ring system.[1] Included in this group are brevianamides,[2] notoamides,[3] stephacidins,[4] versicolamides,[5] paraherquamides,[6] malbrancheamides[7] and marcfortines.[8] Total syntheses,[9] biomimetic syntheses[10] and biosyntheses[11] of PIAs, focusing on formation of the core ring system, have been extensively investigated because of the interesting structures of the PIAs.

In our previous work,[12] three rare carbamate-containing PIAs, penicimutamides A–C, were isolated from a fungal mutant AD-2-1 of a marine-derived *Penicillium purpurogenum* G59 *via* diethyl sulfate (DES) mutagenesis. As a continuation of this, we herein report on three other PIAs, including two new penicimutamides D–E (**1–2** in Fig. 1) and a known one (**3**). These compounds were produced in the same solid culture by the mutant AD-2-1 by activating silent pathways in parent G59 strain. The mutant AD-2-1 was selected by treating *Penicillium purpurogenum* G59 spores with 1% (v/v) DES in 50% (v/v) DMSO at 4 °C for 1 d. This produced a series of novel lipopeptides in liquid culture *via* the activation of pathways that were silent in the G59 strain.[13]

To obtain new fungal secondary metabolites, the one-strain-many-compounds approach,[14] chemical epigenetics[15] and co-cultivation[16] have been used to activate silent pathways by varying environmental factors for growth of the producing strains. In our previous work,[17] a series of new methods[17a–c] based on ribosome engineering[18] were developed for fungi, and several new secondary metabolites were isolated from the mutants.[17c–e] During this study, a practical mutagenesis strategy using DES was developed to activate silent fungal pathways.[13,19] A diverse range of secondary metabolites were isolated from the DES mutant of *Penicillium purpurogenum* G59 by activating pathways that were silent in the parent strain *via* DES mutagenisis.[19a]

As reported in our previous work, the mutant AD-2-1 and parental G59 strains were fermented concurrently under the same conditions at 28 °C for 50 d using rice as a solid substrate fermentation medium to obtain methanol (MeOH) extracts of their cultures. These cultures inhibited K562 cells with inhibition rates of 62.5% and 6.1% at 100 μg mL^{-1}, respectively. Production of new metabolites was tracked to guide separation of the mutant extract, and resulted in the isolation of **1–3** (Table 1).

After analysis by HR-ESI-MS, penicimutamide D (**1**) was assigned the molecular formula $C_{21}H_{25}N_3O_2$ (*m*/*z* 352.2025 [M +

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† Electronic supplementary information (ESI) available: Experimental procedures, NMR data for **1–3**, DFT-optimized structures of the low-energy conformers for **1** and **2**, figures for the HPLC-UV and HPLC-MS analyses, various spectra for **1–3**, and X-ray data of **1** (CIF file). CCDC 1532020. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c7ra02446k

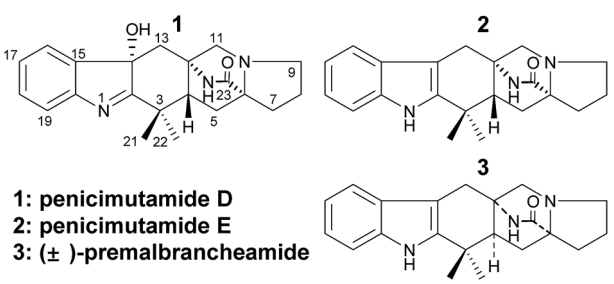

Fig. 1 Structures of compounds **1–3**.

Table 1 600 MHz ^{1}H and 150 MHz ^{13}C NMR data for **1–3** in CD_3OD[a]

No.		1 δ_H (J in Hz)	1 δ_C[b]		2 δ_H (J in Hz)	2 δ_C[b]		3 δ_H (J in Hz)	3 δ_C[b]
2		—	192.6 s		—	142.7 s		—	142.2 s
3		—	41.6 s		—	29.1 s		—	35.4 s
4		2.00 dd (9.0, 5.4)	51.6 d		2.19 dd (10.1, 3.7)	47.6 d		2.00 dd (10.8, 5.4)	48.5 d
5	Hβ	2.10 dd (12.6, 9.0)	32.9 t	Hβ	2.14 dd (13.4,10.1)	31.9 t	Hα	1.99 dd (13.2, 10.8)	32.5 t
	Hα	1.97 dd (12.6, 5.4)		Hα	1.94 dd (13.4,3.7)		Hβ	1.94 dd (13.2, 5.4)	
6		—	67.2 d		—	66.5 d		—	66.2 d
7	Hα	2.57 dt (12.6, 6.6)	28.1 t	Hα	2.52 ddd (12.6, 9.0, 4.8)	28.09 t	Hα	2.51 ddd (13.2, 9.0, 6.0)	28.2 t
	Hβ	1.47 td (12.6, 7.8)		Hβ	1.47 ddd (12.6, 10.8, 7.2)		Hβ	1.44 ddd (13.2, 10.8, 7.2)	
8		1.95–1.89 2H, m	23.4 t		1.96–1.89 2H, m	23.4 t		1.89–1.82 2H, m	23.6 t
9	Hα	3.10 dt (9.0, 5.4)	54.7 t	Hβ	3.115 td (9.0, 4.2)	54.3 t	Hβ	3.04 ddd (9.0, 7.2, 3.6)	55.4 t
	Hβ	2.31 q (9.0)		Hα	2.39 q (9.0)		Hα	2.15 br q (9.0)	
11	Hβ	2.89 d (10.2)	62.4 t	Hβ	3.110 d (10.2)	62.4 t	Hβ	3.45 d (10.2)	59.5 t
	Hα	2.49 d (10.2)		Hα	2.67 d (10.2)		Hα	2.24 dd (10.2, 1.8)	
12		—	58.0 s		—	56.8 s		—	57.7 s
13	Hα	2.68 d (14.7)	43.1 t	Hα	2.99 d (16.8)	29.1 t	Hβ	2.88 d (15.0)	30.5 t
	Hβ	1.49 d (14.7)		Hβ	2.85 d (16.8)		Hα	2.85 d (15.0)	
14		—	83.7 s		—	103.7 s		—	104.5 s
15		—	143.0 s		—	128.4 s		—	128.2 s
16		7.45 br d (7.8)	123.3 d		7.38 br d (7.7)	118.5 d		7.33 br d (8.0)	118.3 d
17		7.26 td (7.8, 0.8)	127.6 d		6.96 ddd (7.7, 7.1, 0.8)	119.5 d		6.93 td (8.0, 0.9)	119.5 d
18		7.37 td (7.8, 1.2)	130.6 d		7.04 ddd (8.1, 7.1, 1.2)	122.0 d		7.02 td (8.0, 1.1)	122.0 d
19		7.48 br d (7.8)	121.2 d		7.27 br d (8.1)	111.7 d		7.25 br d (8.0)	111.6 d
20		—	152.9 s		—	138.4 s		—	138.5 s
21		1.30 3H, s	26.9 q		1.31 3H, s	28.06 q		1.42 3H, s	24.4 q
22		1.41 3H, s	19.9 q		1.20 3H, s	24.6 q		1.32 3H, s	30.9 q
23		—	175.2 s		—	175.9 s		—	176.8 s

[a] Chemical shift (δ_H and δ_C) were recorded using the solvent signals of CD_3OD (δ_H 3.31/δ_C 49.00) as references. Signals assignments were based on the results of DEPT, ^{1}H–^{1}H COSY, HMQC and HMBC experiments. [b] Multiplicities of the carbon signals were determined by DEPT experiments and are indicated by s (singlet), d (doublet), t (triplet) and q (quartet).

H]$^+$, calcd for $C_{21}H_{26}N_3O_2$ 352.2025), which has one more oxygen atom than the known compound **3**. UV absorptions at 220 and 264 nm showed it contained the indole structure. The ^{1}H NMR data for H-16 (δ 7.53, br d, J = 7.8 Hz), H-19 (δ 7.52, br d, J = 7.8 Hz), H-18 (δ 7.46, td, J = 7.8, 1.2 Hz) and H-17 (δ 7.31, td, J = 7.8, 1.2 Hz) supported the presence of a disubstituted phenyl group in the indole structure. The ^{1}H NMR, HMQC and ^{1}H–^{1}H COSY spectra showed five spin systems of H_3-21–C-3–H_3-22, –H_2-13–, –H_2-11–, –H-4–H_2-5–, and –H_2-7–H_2-8–H_2-9– (Fig. 2). All of the above spin systems were the same as those for the known compound **3**, which suggested **1** had the same skeleton as **3**. When the ^{13}C NMR spectrum of **1** was compared with that of **3**, the biggest differences were the absence of a sp^2 carbon signal (C-14 of **3**) and appearance of an oxygenated sp^3 carbon signal (C-14 of **1**). So, it could be deduced that **1** is an oxidative product of **3** with a hydroxyl group at C-14. In addition the double bond was transferred from C_2=C_{14} to C_1=C_2, which resulted in a high-field shift of C-2 from δ_C 142.2 (in **3**) to 192.6 (in **1**). This gave a planar structure (Fig. 1), which was also supported by further NMR data (Fig. 2).

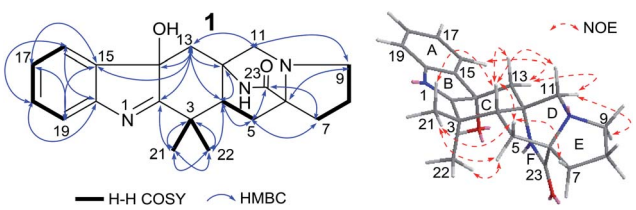

Fig. 2 Structures and key NMR data for **1**.

The NOEs observed in the NOESY of **1** between H-4/Hβ-11, H-4/Hβ-13, H-4/Hβ-5, H-4/H_3-21 and H_3-21/Hα,β-5 indicated that ring C had a chair conformation and ring D had a boat conformation with CH_3-22, HO-C_{14} groups and the lactam group located on the same face. The NOEs between Hβ-5/Hβ-7 and Hα-9/Hα-11 showed that the E ring had an envelope conformation with N-10 upwarped as shown in Fig. 2. Thus, the relative configuration was established.

To determine the absolute configuration of **1**, single-crystal X-ray diffraction using Cu Kα radiation for **1** was performed. An ORTEP drawing of the crystallographically determined structure of **1** is depicted in Fig. 3. The relative configuration of **1** determined was in accordance with the result based on NOEs. But the Flack parameter was −0.2 (2), which was not perfect enough to determine the absolute configuration.

To confirm the absolute configuration of **1**, TDDFT electronic CD (ECD) calculations[20,21] for **1** and its enantiomer were performed. The calculated ECD of **1** agreed with the measured

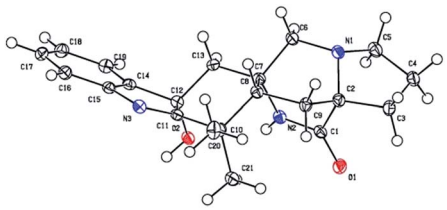

Fig. 3 The crystal structure of **1**.

CD data (Fig. 4), and showed that the absolute configuration was 4*R*, 6*S*, 12*S*, 14*S*.

Penicimutamide E (**2**) was assigned the molecular formula $C_{21}H_{25}N_3O_1$ by HR-ESI-MS (*m*/*z* 336.2070 $[M + H]^+$, calcd for $C_{21}H_{26}N_3O_2$ 336.2072), which was the same as that of the known compound **3**. The UV and IR spectra were also identical to those of **3**. The similar ^1H and almost identical ^{13}C NMR data indicated that **2** had the same planar structure as **3**, which was also supported by other NMR data.

The NOEs observed in the NOESY of **2** between H-4/Hβ-11, H-4/Hβ-13, H-4/Hβ-5, H-4/H_3-21, H_3-21/Hα,β-5, Hβ-5/Hβ-7 and Hα-9/Hα-11 established the relative configuration of **2** as shown in Fig. 5. To determine the absolute configuration of **2**, TDDFT ECD calculations[20,21] for **2** and its enantiomer were performed. The calculated ECD of **2** agreed with the measured CD data (Fig. 6), and the absolute configuration of **2** was determined as 4*R*, 6*S*, 12*S*.

Compound **3** was obtained as a crystalline powder. The ^1H and ^{13}C NMR data were almost identical to the published data of (+)-premalbrancheamide,[22] which indicated that **3** had the same planar structure as (+)-premalbrancheamide. The NOEs observed in the NOESY spectrum (Fig. 7) also indicated that **3** had the same relative stereochemistry as (+)-premalbrancheamide (Fig. 6). The $[\alpha]_D$ of **3** (+3.2°) was smaller than that of (+)-premalbrancheamide (+15°),[23] and there was no Cotton effect

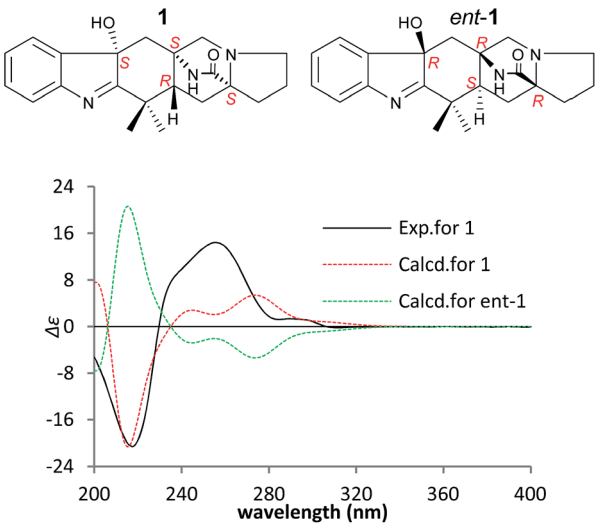

Fig. 4 Measured and calculated ECD spectra of **1** and its enantiomer in MeOH.

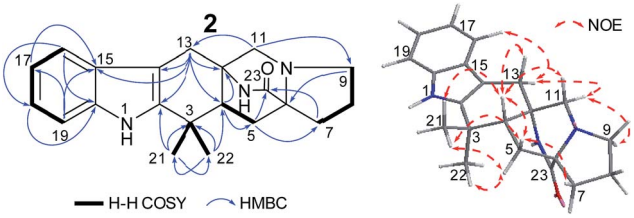

Fig. 5 Structures and key NMR data for **2**.

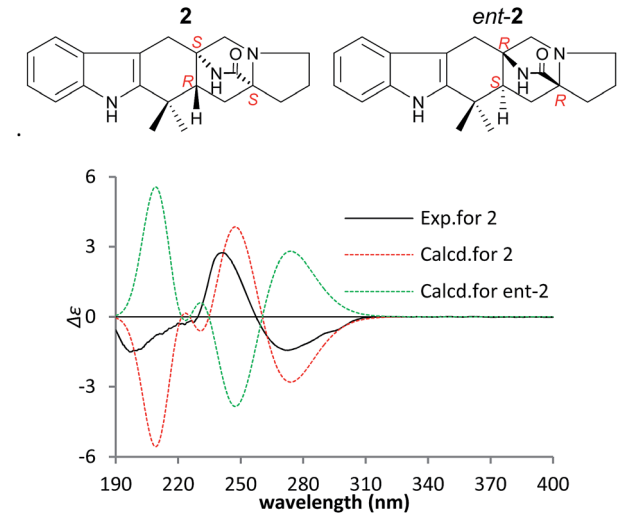

Fig. 6 Measured and calculated ECD spectra of **2** and its enantiomer in MeOH.

evident in the CD spectrum of **3**. This indicates that **3** is a racemic mixture of (±)-premalbrancheamide. According to the HPLC analysis of compound **3** on a CHIRALPAK IE column with 65% MeOH, the (+)-premalbrancheamide content was about 56%, and the (−)-premalbrancheamide content was about 44% (Fig. 8). The racemic mixture was not separated because we only had a small quantity of **3**.

The MeOH extracts from the mutant AD-2-1 and parent G59 strains were analyzed by HPLC with a photodiode array detector and HPLC-ESI-MS using **1**–**3** as reference standards. The retention times and the UV and MS spectra (Fig. S2 and S3 in the ESI†) showed that **1**–**3** were only present in the mutant extract and not in the parent extract. This is evidence that **1**–**3** are produced in mutant AD-2-1 following activation of biosynthetic pathways that are silent in the parent G59 strain and subsequently activated by the DES mutagenesis process in the mutant.

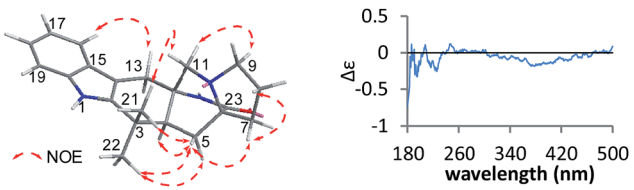

Fig. 7 Key NOE data and the CD spectrum for **3**.

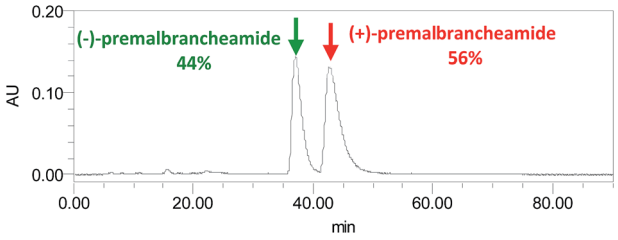

Fig. 8 The HPLC chromatograph of **3** on a CHIRALPAK IE column with 65% MeOH.

As reported in our previous work,[12] **1** and **2** were both intermediates in plausible biosynthetic pathways for penicimutamides A–C. Plausible biosynthetic pathways for **3** have been reported in earlier studies.[22,23] (+)-Premalbrancheamide was detected in one study,[22] and was subsequently isolated from the fermentation product of *Malbranchea aurantiaca*.[24] (−)-Premalbrancheamide was isolated for the first time in the present study.

To evaluate the inhibitory effect on human cancer cell lines, **1–3** and 5-fluorouracil were tested against human K562, HL-60, HeLa and BGC-823 cell lines at 100 μg mL^{-1} by the MTT assay. Compounds **1–3** only showed weak inhibition of the above four cell lines with inhibition rates ranging from 2% to 27%.

Conclusions

In our previous work, three rare carbamate-containing alkaloids, penicimutamides A–C, were isolated from the DES mutant AD-2-1 obtained from the marine-derived fungus *Penicillium purpurogenum* G59, and plausible biosynthetic pathways from the precursor deoxybrevianamide E were reported.[12] In the present work, two new prenylated indole alkaloids, penicimutamides D–E, which might be intermediates in the biosynthesis of penicimutamides A–C, were isolated from the mutant AD-2-1. The discovery of penicimutamides A–E from the mutant AD-2-1 strain demonstrates the effectiveness of our previously reported DES mutagenesis strategy[19] for obtaining new bioactive compounds from silenced fungal pathways. The present work confirms the proposed biosynthetic pathway of penicimutamides A–C.

Conflicts of interest

The authors declare no conflict interest.

Acknowledgements

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