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. Included in this group are brevianamides, notoamides, stephacidins, versicolamides, paraherquamides, malbrancheamides and marcfortines. Total syntheses, biomimetic syntheses and biosyntheses 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, 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.

To obtain new fungal secondary metabolites, the one-strain-many-compounds approach, chemical epigenetics and co-cultivation have been used to activate silent pathways by varying environmental factors for growth of the producing strains. In our previous work, a series of new methods based on ribosome engineering were developed for fungi, and several new secondary metabolites were isolated from the mutants. During this study, a practical mutagenesis strategy using DES was developed to activate silent fungal pathways. 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 mutagenesis.

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 \(\text{C}_{21}\text{H}_{24}\text{N}_{3}\text{O}_{2}~(m/z~352.2025)\) [M + H⁻].

![Fig. 1 Structures of compounds 1–3.](image-url)
Table 1 600 MHz $^{1}$H and 150 MHz $^{13}$C NMR data for 1-3 in CD$_{3}$OD$^{a}$

| No. | $\delta_{H}$ (J in Hz) | $\delta_{C}$ | $\delta_{H}$ (J in Hz) | $\delta_{C}$ | $\delta_{H}$ (J in Hz) | $\delta_{C}$ |
|-----|------------------------|------------|------------------------|------------|------------------------|------------|
| 2   | 192.6 s                | 142.7 s    | 2.00 dd (9.0, 5.4)     | 51.6 s     | 147.7 s                | 2.19 dd (10.1, 3.7) |
| 3   | 41.6 s                 | 29.1 s     | 2.00 dd (10.8, 5.4)    | 48.5 s     | 2.19 dd (10.8, 5.4)    | 48.5 s     |
| 4   | 2.10 dd (12.6, 9.0)    | 32.9 t     | 2.14 dd (13.4,10.1)    | 31.9 t     | 2.19 dd (13.2,10.8)    | 32.5 t     |
| 5   | 19.97 dd (12.6, 5.4)   | Hz         | 19.94 dd (13.4,3.7)    | Hz         | 19.94 dd (13.2, 5.4)   | 35.4 s     |
| 6   | 67.2 d                 | 66.5 d     |                       | 25.3 t     | 35.4 s                 | 66.2 d     |
| 7   | 2.57 dt (12.6, 6.6)    | 28.1 t     | 2.52 dddd (12.6, 9.0, 4.8) | 28.09 t  | 2.51 dddd (13.2, 9.0, 6.0) | 28.2 t     |
| 8   | 1.47 td (12.6, 7.8)    | Hz         | 1.47 ddd (12.6, 10.8, 7.2) | Hz         | 1.44 dddd (13.2, 10.8, 7.2) | Hz         |
| 9   | 1.30 dt (9.0, 5.4)     | Hz         | 1.15 td (9.0, 4.2)     | Hz         | 3.04 dddd (9.0, 7.2, 3.6) | 55.4 t     |
| 10  | 2.31 q (9.0)           | Hz         | 2.39 q (9.0)           | Hz         | 2.15 br q (9.0)        | 59.5 t     |
| 11  | 2.89 d (10.2)          | Hz         | 2.76 d (10.2)          | Hz         | 2.24 dd (10.2, 1.8)    | Hz         |
| 12  | 1.38 d (14.7)          | Hz         | 2.99 d (16.8)          | Hz         | 2.88 d (15.0)          | 30.5 t     |
| 13  | 1.49 d (14.7)          | Hz         | 2.85 d (16.8)          | Hz         | 2.85 d (15.0)          |           |

$^{a}$ Chemical shift ($\delta_{H}$ and $\delta_{C}$) were recorded using the solvent signals of CD$_{3}$OD ($\delta_{H}$ 3.31/ $\delta_{C}$ 49.00) as references. Signals assignments were based on the results of DEPT, $^{1}$H, $^{13}$C COSY, HMOC 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).
CD data (Fig. 4), and showed that the absolute configuration was 4R, 6S, 12S, 14S.

Penicimutamide E (2) was assigned the molecular formula C_{21}H_{25}N_{3}O_{1} by HR-ESI-MS (m/z 336.2070 [M + H]^+), calculated for C_{21}H_{26}N_{3}O_{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 ^13C 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/Hb-11, H-4/Hb-13, H-4/H-21, H-21/Hz-5, Hb-5/Hb-7 and Hz-9/Hz-11 established the relative configuration of 2 as shown in Fig. 5. To determine the absolute configuration of 2, TDDFT ECD calculations 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 4R, 6S, 12S.

Compound 3 was obtained as a crystalline powder. The ^1H and ^13C NMR data were almost identical to the published data of (+)-premalbrancheamide, 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 [a]_D of 3 (+3.2°) was smaller than that of (+)-premalbrancheamide (+15°), and there was no Cotton effect 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.
As reported in our previous work,\textsuperscript{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.\textsuperscript{22,23} (+)-Premalbrancheamide was detected in one study,\textsuperscript{22} and was subsequently isolated from the fermentation product of Malbranchea aurantiaca.\textsuperscript{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.\textsuperscript{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\textsuperscript{18} 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.

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