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# *neo*-Dicitrinols A–C: Unprecedented PKS-NRPS hybrid citrinin dimers with ferroptosis inhibitory activity from the deep-sea-derived *Penicillium citrinum* W22

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ABSTRACT

The chemical investigation into the EtOAc extract of the deep-sea-derived fungus *Penicillium citrinum* W22 yielded three unprecedented citrinin dimers, *neo*-Dicitrinols A–C (**1**–**3**) and a known one, penicitrinone A (**4**). Their structures were elucidated by extensive analysis of spectroscopic data, electronic circular dichroism (ECD) calculation, X-ray diffraction, and biogenetic consideration. *neo*-Dicitrinols A–C (**1**–**3**), bearing a tetramic acid unit, represent the first example of citrinin analogues as hybrid polyketide synthase-nonribosomal peptide synthase (PKS-NRPS) products. *neo*-Dicitrinol C (**3**) significantly inhibited renin-angiotensin system-selective lethal 3 (RSL3)-induced ferroptosis with a half maximal effective concentration ($EC_{50}$) value of 21.6 μmol/L.



*Penicillium citrinum* are important medicinal fungi that biosynthesized a broad spectrum of bioactive compounds, such as alkaloids [1], polyketides [2,3], sterols [4] and terpenes [5,6]. In particular, this species of *Penicillium* is characterized to produce citrinin, a well-known mycotoxin featuring an 8–hydroxy-3,4,5-trimethyl-6-oxo-4,6-dihydro-3*H*-isochromene core [7]. The related analogues from the same species usually derived from the degradation, dimerization or trimerization of citrinin through different pathways, thereby leading to a vast range of structure diversity, complexity and biological activity of this polyketides family [8–10]. For example, citrifelin B, possessing a unique tetracyclic framework, showed strong inhibitory activity against the human pathogens *Escherichia coli* and *Staphylococcus aureus* with minimum inhibitory concentration (MIC) values of 2.0 and 4.0 μg/mL, respectively [11]. Tricitrinols A and B, isolated from *Penicillium citrinum* HGY1–5, consisting of two coupled citrinin monomers as well as a third moiety linked through a single bond between C-7′ and C-10′ display a DNA topoisomerase II*α* inhibitory activity [12]. In a few cases, citrinin analogues incorporate some unusual substructures like pyrrolidine [13], furanone [14] or isoquinolinone [15], furtherly extend the understanding about the chemical structures of this polyketide family.

In the course of discovering structurally new and bioactive secondary metabolites from deep-sea derived fungi [16–21], the *Penicillium citrinin* W22 isolated from a sediment sample at a depth of 5278 m from the Western Pacific was subjected to a systematic chemical investigation. The subsequent series of purifications finally afforded three unprecedented citrinin dimers (**1**–**3**, Fig. 1) along with a known derivative, penicitrinone A (**4**). We report herein the fermentation, isolation, structure, and bioactivities of these isolates.

The EtOAc extract of the rice culture of *Penicillium citrinum* W22 was subjected to extensive column chromatography (CC) over silica gel, octadecyl silane (ODS), Sephadex LH-20, and preparative high-performance liquid chromatography (HPLC) to yield compounds **1**–**4**. Compound **4** was identified as a known compound, penicitrinone A, by comparing the nuclear magnetic resonance (NMR), mass spectra (MS), and optical rotation (OR) data with those reported in the reference [8].

The positive high-resolution electrospray ionization-mass spectrometry (HRESIMS) analysis of compound **1** returned a molecu-

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**Table 1**
The $^{1}H$ and $^{13}C$ NMR spectroscopic data for *neo*-dicitrinols A–C (**1**–**3**) (*J* in Hz).

| Pos. | **1**[a] | | **2**[b] | | **3**[b] | |
|---|---|---|---|---|---|---|
| | $\delta_C$, type | $\delta_H$ (mult, *J* in Hz) | $\delta_C$, type | $\delta_H$ (mult, *J* in Hz) | $\delta_C$, type | $\delta_H$ (mult, *J* in Hz) |
| 2 | 87.8 d | 4.67 (dq, 1.9, 6.2) | 88.9 d | 4.51 (dq, 1.6, 6.0) | 89.0 d | 4.57 (dq, 2.2, 6.2) |
| 3 | 44.0 d | 3.29 (m) | 45.3 d | 3.10 (dq, 2.4, 6.8) | 45.3 d | 3.12 (dq, 3.0, 7.0) |
| 4 | 140.9 s | | 133.8 s | | 132.9 s | |
| 5 | 118.4 s | | 116.8 s | | 117.1 s | |
| 6 | 147.7 s | | 149.5 s | | 148.5 s | |
| 7 | 102.7 s | | 107.1 s | | 108.8 s | |
| 8 | 137.0 s | | 134.0 s | | 134.1 s | |
| 9 | 134.5 s | | 138.8 s | | 138.8 s | |
| 10 | 20.8 q | 1.35 (d, 6.3) | 21.0 q | 1.37 (d, 6.3) | 21.2 q | 1.39 (d, 6.8) |
| 11 | 18.6 q | 1.26 (d, 6.9) | 19.7 q | 1.30 (d, 6.8) | 19.7 q | 1.30 (d, 6.9) |
| 12 | 11.8 q | 2.17 (s) | 11.7 q | 2.14 (s) | 11.6 q | 2.11 (s) |
| 2′ | 82.2 d | 5.16 (q, 6.3) | 80.0 d | 4.13 (m) | 76.9 d | 4.40 (dq, 5.1, 6.8) |
| 3′ | 33.6 d | 3.29 (m) | 38.7 d | 2.97 (m) | 35.8 d | 2.82 (m) |
| 4′ | 133.1 s | | 142.0 s | | 139.7 s | |
| 5′ | 129.3 s | | 114.7 s | | 114.7 s | |
| 6′ | 185.1 s | | 158.8 s | | 158.2 s | |
| 7′ | 103.6 s | | 109.9 s | | 111.0 s | |
| 8′ | 153.3 s | | 145.8 s | | 147.3 s | |
| 9′ | 97.6 s | | 113.2 s | | 111.9 s | |
| 10′ | 160.2 s | | 67.2 d | 5.69 (s) | 63.4 d | 5.92 (s) |
| 11′ | 18.1 q | 1.31 (d, 6.6) | 22.4 q | 1.36 (d, 6.2) | 22.3 q | 1.43 (d, 6.9) |
| 12′ | 18.7 q | 1.21 (d, 6.7) | 20.3 q | 1.26 (d, 7.2) | 18.1 q | 1.22 (d, 7.1) |
| 13′ | 10.1 q | 2.01 (s) | 11.0 q | 2.13 (s) | 10.2 q | 2.11 (s) |
| 2″ | 171.3 s | | 173.2 s | | 173.1 s | |
| 3″ | 75.8 s | | 82.4 s | | 82.3 s | |
| 4″ | 206.4 s | | 110.9 s | | 110.6 s | |
| 5″ | 71.9 d | 4.17(d, 6.6) | 72.0 d | 3.69 (d, 4.4) | 72.2 d | 3.77 (d, 4.2) |
| 6″ | 67.9 d | 4.04 (m) | 67.8 d | 4.27 (dq, 1.6, 6.0) | 67.8 d | 4.27 (dq, 2.4, 6.6) |
| 7″ | 20.3 q | 1.21 (d, 6.7) | 20.9 q | 1.43 (d, 6.6) | 20.6 q | 1.41 (d, 6.8) |
| *N*-Me | 30.3 q | 3.06 (s) | 31.6 q | 2.98 (s) | 31.6 q | 2.94 (s) |
| 6-OH | | 9.15 (s) | | | | |
| 6″-OH | | 5.30 (d, 5.9) | | | | |

[a] Acquired in DMSO–$d_6$;
[b] Acquired in $CD_3OD$.

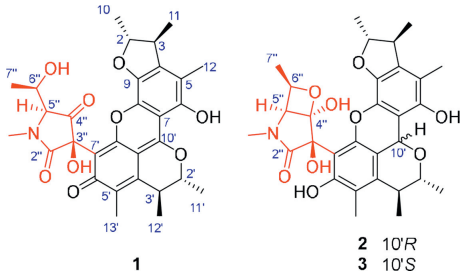



**Fig. 1.** Chemical structures of compounds **1**–**3**.

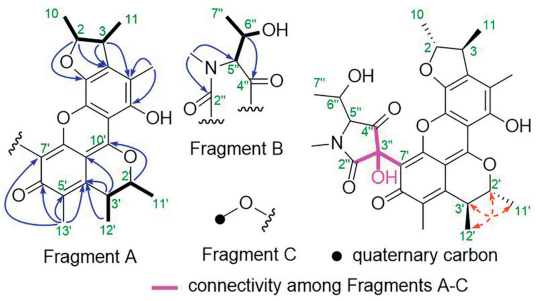



**Fig. 2.** The key COSY (▬), HMBC (⌒), and NOESY (⌒) NMR correlations for compound **1**.

lar formula $C_{30}H_{33}NO_9$, requiring for 15° of unsaturation. The $^{1}H$, $^{13}C$ NMR together with heteronuclear single quantum coherence (HSQC) spectra of **1** (Table 1) revealed resonances for eight methyl groups including two singlets, five doublets, and one *N*-methyl; six $sp^3$ methine; and 16 non-hydrogenated carbons consisting of one $sp^3$, 12 olefinic, and three carbonyl carbons. Therefore, **1** incorporated six rings. In the $^{1}H$–$^{1}H$ correlation spectroscopy (COSY) spectrum, two suites of correlations of $H_3$–10/H-2/H-3/$H_3$–11 and $H_3$–11′/H-2′/H-3′/$H_3$–12′ confirmed that the four methyl doublets and four $sp^3$ methine were mutually coupled in two pairs, which much resembled resonances in the spectra of penicitrinone A (**4**). An in-depth comparison of the 1D and 2D NMR data of **1** with those of **4** indicated **1** incorporated a C-7-substituted citrinin dimer moiety (Fragment A). The assumption was supported by the diagnostic heteronuclear multiple bond correlation (HMBC) correlations of H-2 to C-9, H-3 to C-4/9, $H_3$–11 to C-4, $H_3$–12 to C-4/5/6, H-2′ to C-10′, H-3′ to C-9′, $H_3$–12′ to C-4′, $H_3$–13′ to C-4′/5′/6′, and a long-range correlation of $H_3$–13′ to C-7′ (Fig. 2).

Apart from the dimeric citrinin substructure, the rest of 2D NMR signals were attributed to a *N*-methyl-containing fragment B, evident by the HMBC correlations from the *N*-methyl to C-2″/5″ and COSY correlation of H-6″/6″-OH, as well as an oxygenated non-hydrogenated carbon ($\delta_C$ 82.2, Fragment C). Considering the molecular formula and the ketonic nature of C-4″ ($\delta_C$ 206.4), the planar structure of **1** was assigned as shown in Fig. 2. The nuclear Overhauser effect spectroscopy (NOESY) correlations of H-2′/$H_3$–12′, H-3′/$H_3$–11′ permitted a *cis* relationship of the two methyl, which could be applied to $H_3$–10/$H_3$–11 due to a biogenetic ground [8]. However, the relative configuration of C-3″, C-4″, and C-5″ remains unclear since no useful NOESY correlations could be observed on these chiral centres. Fortunately, a crystal of **1** was obtained by slowly evaporating in MeOH. Therefore, the X-ray diffraction analysis was conducted, which eventually established the absolute configuration of **1** (Fig. 3). Accordingly, compound **1** was

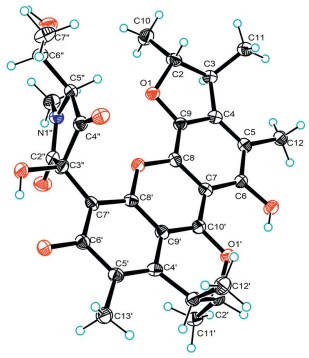


**Fig. 3.** The single X-ray crystallography structure of **1**.

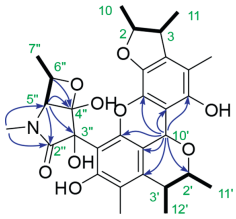


**Fig. 4.** Selected COSY (bold) and HMBC (arrow) correlations of **2** and **3**.

identified as a novel dimeric citrinin analogue, and named *neo*-dicitrinol A.

Compound **2** was assigned the molecular formula $C_{30}H_{35}NO_9$ based on its positive HRESIMS spectrum. Comparison of the NMR spectra of **2** with those of **1** (Table 1) revealed a high degree of similarities with the principal differences as (i) the presence of a $sp^3$ hybrid oxymethine ($\delta_H$ 5.69, $\delta_C$ 67.2; CH-10′) in **2**; (ii) the replacement of two ketone in **1** with a deshielded aromatic carbon ($\delta_C$ 158.8; C-6′) and one hemiketal carbon ($\delta_C$ 110.9; C-4″); (iii) the deshielding of C-3″ ($\Delta\delta_C$ +6.6). Diagnostic HMBC correlations observed from H-10′ to C-6/7/8/2′/4′/8′/9′ and from $H_3$–13′ to C-6′ confirmed a reduction at C-10′ and an enol-ketol transformation at C-6′. Additionally, the COSY correlation of H-5″/H-6″ together with HMBC cross-peaks from H-5″ to C-3″/4″ and H-6″ to C-4″ established that the hemiketal was formed through C-6″-O-C-4″ (Fig. 4). Therefore, the planar structure of **2** was assigned. The NOESY cross-peaks of H-2′/$H_3$-12′, H-10′/H-2′, H-10′/$H_3$-12′, and *N*-methyl/$H_3$-7″ indicated a co-facial relationship of those protons, thus established the relative configuration among C-10′/2′/3′ as well as C-4″/5″/6″ (Fig. 5). This along with a biogenetic consideration with **1** permitted the assignment for the absolute configurations of all those chiral centres. Finally, the configuration of **2** was well supported by a DP4+ probability analysis (Figs. S26 and S28 in Supporting information) and the agreement between the predicted and experimental electronic circular dichroism (ECD)

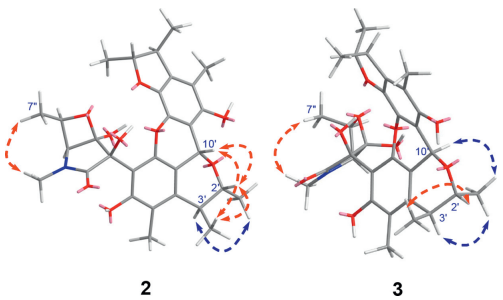



**Fig. 5.** Selected NOESY correlations of compounds **2** and **3**.

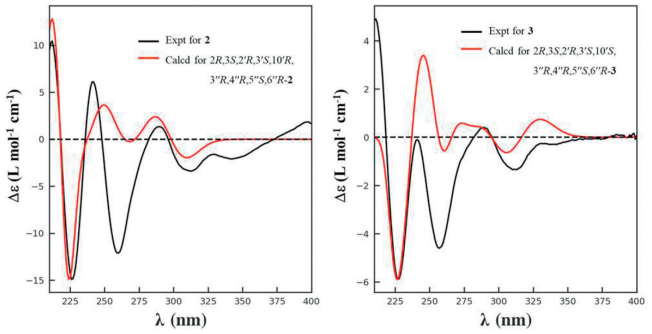


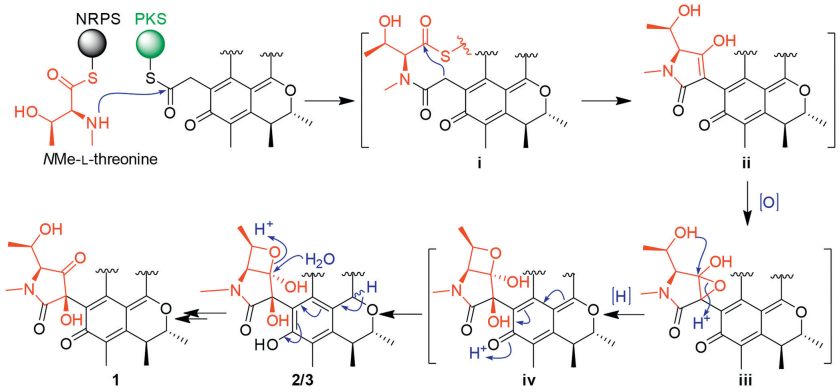

**Fig. 6.** The experimental and calculated ECD spectra of **2** and **3**.

spectra (Fig. 6). On the basis of the above evidence, the complete structure of **2** was then assigned, and was given a trivial name *neo*-dicitrinol B.

Analysis of the positive HRESIMS of **3** returned the identical molecular formula $C_{30}H_{35}NO_9$ as **2**. Interpretation of the 1D and 2D NMR data of **3** (Table 1) revealed the same planar structure as **2**. Comparison of the data of both compounds disclosed principal differences in the chemical shifts of carbons around the chiral center C-10′, including the shielding of C-10′ ($\Delta\delta_C$ −3.8), C-2′ ($\Delta\delta_C$ −3.1), C-3′ ($\Delta\delta_C$ −2.9), C-4′ ($\Delta\delta_C$ −2.3), C-9′ ($\Delta\delta_C$ −1.3) as well as the deshielding of C-7 ($\Delta\delta_C$ +1.7), C-8′ ($\Delta\delta_C$ +1.5), implying a probable inversion of the configuration at C-10′ in **3**. This was evidenced by the observation of NOESY correlation between H-10′ and $H_3$–11′ instead of H-10′ and H-2′/$H_3$–12′ in **3**. For a further confirmation, a theoretical ECD spectra of (2*R*,3*S*,2′*R*,3′*S*,10′*S*,3″*R*,4″*R*,5″*S*,6″*R*)–**3** and the enantiomer was calculated, in which the tendency of the experimental curve matched well with that of 10′-*S* (Fig. 5). Thus, compound **3** was elucidated as 10′-*S* epimer of **2**, and named *neo*-dicitrinol C.

Citrinin and the related analogues have been documented as polyketide synthase (PKS) products in previous studies [22]. However, the 5–hydroxy-ethyl-*N*-methyl-tetramic acid moiety in **1**–**3** was not induced by such enzyme to best of our knowledge. This droves us to seek for the origin of it in literature. Being rarely involved in citrinin-analogous molecules, the 5-hydroxyethyl-*N*-methyl-tetramic acid substructure was found common in fungal metabolites like penicillenols [23], epicoccarines [24] and methiosetin [25], derived from the amino acid precursors *via* mixed polyketides synthase and non-ribosomal peptide synthase (PKS-NRPS) pathways [26]. On the basis of this fact, a plausible biosynthetic transformation was proposed for compounds **1**–**3** (Scheme 1), which started from the condensation between a NRPS module-carrying *N*-methyl-L-threonine and PKS catalysed homocitrinin precursors. Then the intermediate **i** that underwent a Dickmann condensation-alike reaction [27] formed the tetramic acid moiety **ii** and the subsequent epoxide **iii**. The addition from the free hydroxy of the amino acid to the epoxy with a concomitant hydrogenation on the aromatic ring afforded the 10′-epimers **2** and **3**. Compounds **2** and **3** went through a hydrolysis on the oxetane moiety and a subsequent oxidation-dehydration on the aromatic ring to deliver **1**. Of particular note, *neo*-dicitrinols A-C represent the first example of citrinin analogues as hybrid PKS-NRPS products and furtherly enrich the chemical diversity of this class of compounds.

Ferroptosis is a novel type of regulated cell death, which has been implicated in multiple diseases including ischemic organ damage, neurodegeneration, and liver and lung fibrosis [28]. Intervention of ferroptosis would be beneficial to the treatment of these diseases. As shown in Fig. 7, ferroptosis-inducing compound renin-angiotensin system-selective lethal 3 (RSL3) dramatically re-




**Scheme 1.** Plausible biosynthetic pathway linking compounds **1**–**3**.

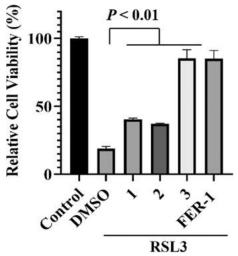



**Fig. 7.** Compounds **1**–**3** inhibited RSL3-induced ferroptosis in A375 cells. Results are represented as means ± SD ($n = 4$). Two-tailed Student's *t*-test was used to compare differences between two groups. Statistical significance was defined as $P < 0.01$.

duced cell viability in human melanoma A375 cells, which can be reversed by ferroptosis inhibitor ferrostatin-1 (Fer-1, 10 μmol/L). Interestingly, compounds **1**–**3** were effective in inhibiting RSL3-induced ferroptosis at a concentration of 40 μmol/L, of which, *neo*-dicitrinols C (**3**) exhibited the most potency with a half maximal effective concentration ($EC_{50}$) value of 21.6 μmol/L (Fig. S32 in Supporting information).

In conclusion, *neo*-dicitrinols A–C (**1**–**3**), three unprecedented PKS-NRPS hybrid citrinin dimers were discovered from the deep-sea-derived fungus *Penicillium citrinum* W22. Compound **3** exhibited potent inhibition against RSL3-induced ferroptosis.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2024.109723.

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