Citriperazines A-D produced by a marine algae-derived fungus *Penicillium* sp. KMM 4672

Anton Nikolaevich Yurchenko, Dmitry V. Berdyshev, Olga F. Smetanina, Elena V. Ivanets, Olesya I. Zhuravleva, Anton B. Rasina, Yuliya V. Khudyakova, Roman S. Popov, Sergey A. Dyshlovo, Gunhild von Amsberg, and Shamil Sh. Afiyatullova

G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia; Laboratory of Biologically Active Compounds, Far Eastern Federal University, Vladivostok, Russia; Laboratory of Experimental Oncology, Department of Oncology, Haematology and Bone Marrow Transplantation, Section Pneumology, Hubertus Wald-Tumorzentrum, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

ABSTRACT

Four new diketopiperazine alkaloids, citriperazines A-D were isolated from algae-derived *Penicillium* sp. KMM 4672. The structures of compounds 1-4 were determined using spectroscopic methods. The absolute configurations of compounds 1 and 4 were established by comparison of calculated and experimental ECD spectra. The cytotoxicity of compounds 1-4 against several human prostate cell lines was evaluated.

1. Introduction

Marine-derived fungi are a prolific source of chemically diverse bioactive metabolites (Blunt et al. 2018). 2,5-Diketopiperazines (DKPs) are one of the most common groups of fungal metabolites (Borthwick 2012; Huang et al. 2014). Many of them exhibited various bioactivities, such as antibiotic (Song et al. 2012), antiviral (Cai et al. 2012), antitumor (Wang et al. 2012) and some others. Remarkably, plinabulin, a synthetic...
derivative of marine fungal 2,5-DKP phenylahistin, now is under phase III of clinical trials.

During our ongoing search for structurally novel and bioactive metabolites from marine-derived fungi we have investigated the fungus *Penicillium* sp. KMM 4672 isolated from Vietnamese marine brown algae *Padina* sp. Recently, we have reported the isolation of several new epidithiodiketopiperazines together with the known alkaloids and polyketides from this strain (Smetanina et al. 2016; Yurchenko et al. 2016; Smetanina et al. 2017). Thorough investigation of low- and medium-polar fractions from EtOAc extract allow to isolate some new compounds. Herein, we report the isolation, structure elucidation and biological activity of the new 2,5-DKP alkaloids 1–4 produced by the marine-derived fungus *Penicillium* sp.

2. Results and discussion

The EtOAc extract of the fungal culture was suspended in H2O-EtOH (4:1) and partitioned successively with hexane, EtOAc, and n-BuOH. The EtOAc fraction was subjected to repeated column chromatography over silica gel and then separated by normal and reverse phase HPLC to yield individual compounds 1–4 as white solids (Figure 1).

The molecular formula of compound 1 was determined as C_{13}H_{16}N_{2}O_{2}S_{2} from a HRESIMS peak at *m/z* 319.0551 [M + Na]⁺ which was in accordance with ¹³C NMR data. A close inspection of the ¹H and ¹³C NMR data (Supplementary material, Table S1, Figures S22–S25) of 1 by DEPT and HSQC techniques and revealed the presence of two amide protons (δH 8.97, 8.73), two S-methyls (δC 15.0, 13.3, δH 2.21, 2.27), one methylene (δC 42.9, δH 3.50, 2.96), five olefinic methines (δC 130.5, 2C, 127.8, 2C, 126.8, δH 7.26, 2H, 7.23, 3H), and sp³-methine (δC 57.9, δH 4.52). The remaining functional groups corresponding to the carbon signals at δC 165.0 (C), 164.4 (C), 135.2 (C) and 65.3 (C) suggested the presence of two amide groups, one substituted sp²-carbon and one quaternary sp³-carbon.

A direct comparison of ¹H and ¹³C NMR spectra of 1 with those of known diketopiperazine alkaloid fusaperazine A (Usami et al. 2002) showed similarities with the exception of aromatic ring signals. The molecular mass difference of 16 mass units between 1 and fusaperazine A and the chemical shifts values in the aromatic ring...
suggested that 1 is the dehydroxy derivative of fusaperazine A. Compound 1 was named citriperazine A.

The absolute configuration of known fusaperazine A was reported earlier as 3S, 6S (Usami et al. 2002). The similarity of NMR data of 1 and fusaperazine A together with optical rotation values differences (+47.1 for 1 and −110.8 for fusaperazine A) suggested the 3R, 6R configurations for 1. This was proved by ECD spectroscopy using methodology described in Experimental section (Supplementary material, p. 25). First, thermodynamically most stable conformations of 1 in methanol were selected based on conformational analysis, performed at B3LYP/6-311G(d,p)_PCM level of theory (Figure S1).

Then, the ECD spectra for each of these conformations were calculated using TDDFT approach and B3LYP/6-311G(d,p)_PCM level of theory. The rotatory strengths of 65 lowest electronic transitions were calculated and accounted for. The bandwidth was chosen to be Δ = 0.34 eV. The value of the UV shift Δλ = +3 nm was chosen to reproduce well the position of the intensive band in the UV spectrum in the long-wave region (λ_{exp} = 281 nm).

The comparison of the experimental and statistically averaged theoretical ECD spectra of 1 is presented on Figure S2.

The theory reproduces well all main qualitative features of the experimental ECD spectrum. Since ECD spectrum for the enantiomer of 1 must be mirror-imaged to ECD spectrum, presented on Figure S2, we could undoubtedly conclude, that the stereoconfigurations of chiral centers of 1 are 3R, 6R.

The molecular formula of citriperazine B (2) was determined as C_{13}H_{16}N_{2}O_{2}S_{2}, the same as 1, based on a HRESIMS and 13C NMR analysis. The NMR data (Table S1, Figures S26–S29) for 2 were very similar to those obtained for citriperazine A (1) with maximal differences at CH-3 (δC 57.3, δH 5.02) and CH-15 (δC 9.2, δH 1.13). These data together with significant 3J_{H3-H4} coupling constants differences between 2 (1.7 Hz) and 1 (3.4 Hz) proposed that 2 was a C-3 epimer of 1 according to published data for fusaperazines A and B and related compounds (Usami et al. 2002). Thus, the stereoconfigurations of chiral centers of 2 are 3S, 6R.

Citriperazine C (3) was isolated as colorless amorphous solid. The molecular formula of 3 was determined to be C_{19}H_{18}N_{2}O_{5}S from a HRESIMS peak at m/z 409.0832 [M + Na]^+ which was supported by the 13C NMR spectrum.

The 1H and 13C NMR spectra of 3 (Table S2, Figures S30–S35) showed the presence of two amide protons (δH 9.32, 8.18), one hydroxy group proton (δH 5.50), one S-methyl (δC 13.1, δH 2.32), one methylene (δC 43.1, δH 3.54, 3.03), one oxygen-bearing sp3-methine (δC 73.5, δH 4.88), two amide carbonyls (δC 165.2, 164.3) and two quaternary sp3-carbons (δC 90.7, 66.1). The remaining signals were assigned to monosubstituted benzene ring (δC 130.3, 2C, 127.9, 2C, 126.9; δH 7.20-7.29, 5H-multiplet) and trisubstituted benzene ring (δC 121.6, 116.7, 115.4; δH 6.65-6.71, 3H-multiplet). The HMBC correlations (Figure S3, S33) from H2-3 (δH 3.54, 3.03) to C-7 (δC 126.9) and C-5/9, from H-6/8 (δH 7.24) to C-4 (δC 134.7) and C-8/6 (δC 127.9), and from H3-10 (δH 2.32) to C-2 established the β-phenylalanine residue with S-methyl as α-substituent. Another part of 2,5-DKP ring was established by HMBC correlations from 2-NH (δH 9.32) to C-1 (δC 165.2) and C-2′ (δC 90.7), and from 2′-NH (δH 8.18) to C-2 (δC 66.1) and C-1′
The HMBC correlations from H-3’ (δ_H 4.88) to C-1’, C-2’ (δ_C 90.7), C-4’ (δ_C 129.3), C-5’ (δ_C 115.4) and C-9’ (δ_C 144.5), from 2’-NH to C-3’ (δ_C 73.5), from H-5’ (δ_H 6.67) to C-3’, C-7’ (δ_C 116.7) and C-9’, together with downfield chemical shifts of C-2’, C-6’, C-7’, C-8’ and C-9’ suggested substituted dihydrobenzofuran moiety with spiro fusion to diketopiperazine ring at C-2’. Two hydroxy groups were proposed at C-3’ and at the aromatic atom at δ_C 141.1 based on its characteristic chemical shifts values. Moreover, the location of C-3’ alcohol group was proved by COSY correlation (Figure S34) between 3’-OH (δ_H 5.51) and H-3’ and 3_J_HH coupling constants (8.0 Hz). Unfortunately, a mutual overlapping of two aromatic protons in the benzofuran part did not allow exactly assign the aromatic protons and carbons at the C-6’, C-7’ and C-8’ position.

The ROESY spectrum (Figure S35) did not include any correlations that could be used for stereochemistry establishing. Nevertheless, we have tried to apply the modified Mosher’s method for determination of absolute configuration of C-3’. Thus, compound 3 was treated with (R)- and (S)-MTPA-Cl to yield 3’,8’-diesters (Table S3, Figure S4). The structure of benzofuran moiety was exactly established by the coupling constants (for (S)-MTPA-ester) of H-5’ (δ_H 7.30, brd, 7.7 Hz), H-6’ (δ_H 7.06, t, 7.7 Hz) and H-7’ (δ_H 7.21, d, 7.7 Hz) together with the HMBC correlations from H-5’ to C-7’ (δ_C 124.1), C-9’ (δ_C 148.2), from H-7’ to C-9’, and from H-6’ to C-4’ (δ_C 124.9) and C-8’ (δ_C 132.1). Unfortunately, the chemical shifts differences indicated inapplicability the Mosher’s method to this compound.

The molecular formula of citriperazine D (4) was determined as C18H16N2O6 by a HRESIMS peak at m/z 355.0936 [M–H]− and by 13C NMR analysis. The general features of the 1H and 13C NMR spectra (Table S2, Figures S36–S42) of 4 resembled those of 3 with the lacking of S-methyl signals and chemical shifts differences at C-2 and its close surrounding (2-NH, C-1 and C-3). The HMBC correlations from both H-3 (δ_H 3.41, 2.91) to C-1 (δ_C 167.1), C-2 (δ_C 81.9), C-4 (δ_C 134.7) and C-5/9 (δ_C 130.4), from 2-NH (δ_H 9.13) to C-1 and C-2’ (δ_C 90.8), and from 2’-NH (δ_H 8.00) to C-2 and C-1’ (δ_C 164.8) (Figures S36–S42) were identical with those of 3 and suggested the planar structure of 4 as 2-hydroxy-2-dethiomethyl derivative of citriperazine C (3).

All possible combinations of three stereocenters were investigated in silico, and the obtained results were compared with the experimental data. The total conformational analysis was carried out using B3LYP/6-31G(d)_PCM, B3LYP/6-311++G(d,p)_PCM and B3LYP/cc-pVTZ_PCM methods and following calculations of ECD spectra for individual conformers of all possible stereoisomers of 4 were performed using TDDFT approach at the same levels of theory (Supplementary material, pages 10–22). The data for RRR stereoisomer are presented on Figure S11.

Furthermore, the modelling of real solvation shells for RRR stereoisomer was performed using B3LYP/6-311++G(d,p)_PCM method. As a result the qualitative agreement in the short wave region (200 nm < λ < 240 nm) between calculated and experimental ECD spectra was achieved.

Additionally the optical rotation values ([α]D<sup>25</sup>) were calculated for all stereoisomers (Table S4). The [α]D<sup>25</sup> –85.7 obtained for RRR stereoisomer was in accordance with experimental [α]D<sup>20</sup> –65.6.

Thus, the obtained data undoubtedly confirmed the 2R2′R3′R absolute configurations for 4.
Based on similar optical rotation values and CD data (Figures S17, S18) of citriperazines C (\([\alpha]_D^{20} - 59.6\)) and D (\([\alpha]_D^{20} - 65.6\)), we suggested the identical absolute configurations for 3 and 4.

The effect of the compounds 1–4 on viability and apoptosis induction of human prostate cancer cells was investigated. All isolated compounds did not exhibit cytotoxic activity against human prostate cancer cells at the concentrations up to 100 \(\mu\)M. No significant effect on cell cycle progression was observed for any compound at the concentrations up to 100 \(\mu\)M.

3. Conclusion

A spirobenzofurandiketopiperazine scaffold is rare for natural compounds and was described only for three metabolites of *Aspergillus* spp (Berg et al. 1976; Sakata et al. 1987; Guo et al. 2013). Two more compounds with this moiety were isolated as artificial products formed by cyclization of hydroxylated bis-N-norgliovictin from *Aspergillus fumigatus* (Forseth et al. 2011). Finally, recent studies described similar moiety in the metabolites of sponge-derived *Penicillium adametzioides* (Liu et al. 2015) and mangrove-derived *P. brocae* (Meng et al. 2016). To the best of our knowledge, this report is the third case of isolation spirobenzofurandiketopiperazine alkaloids from *Penicillium* species.

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Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Anton Nikolaevich Yurchenko (http://orcid.org/0000-0001-6816-3605)
Elena V. Ivanets (http://orcid.org/0000-0002-4610-1718)

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