Notes

Roquefortine E, a Diketopiperazine from an Australian Isolate of *Gymnoascus reessii*

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The new isoprenylated diketopiperazine roquefortine E (**6**) has been isolated from an Australian soil isolate of the ascomycete *Gymnoascus reessii*. The known fungal metabolite roquefortine C (**1**) was also recovered as the major antibacterial principle, and all structures were assigned by detailed spectroscopic analysis.

Roquefortine C (**1**), also known as roquefortine, is a modified diketopiperizine mycotoxin produced by a number of *Penicillium* species. First isolated in 1975[1] by Ohmomo et al. from a *Penicillium roqueforti* strain, its structure was not elucidated until its reisolation by Scott et al. the following year.[2] Roquefortine C (**1**) is a relatively common fungal metabolite, with over 130 published papers on its occurrence, biosynthesis, and biological activity. Early studies found **1** to possess neurotoxic properties in mice;[2] however subsequent studies failed to replicate these findings.[3] Nevertheless, given that **1** is widely distributed in a range of blue cheeses, the health implications must be of note.[4]

While there exists a vast array of naturally occurring diketopiperizines,[5,6] in practice few are closely related to roquefortine C (**1**), which is derived from condensation of tryptophan and histidine residues and is further modified by heterocyclization and isoprenyl (dimethylallyl) addition. Tryptophan is commonly incorporated into diketopiperazine natural products, with hundreds of examples in the literature: however, those containing a histidine moiety are very rare indeed (<15 published analogues).[7] Although recovered from the original isolate of *P. roqueforti* that yielded **1** and bearing similar trivial names, roquefortines A and B belong to an entirely different structural class, the isofumigaclavines.[2,8] Only a limited number of compounds (**2**−**5**) possessing the same core structure as **1** have been reported as natural products, all from *Penicillium* species.[9−12] Of these analogues, only roquefortine D (**2**) is relatively common, with >40 reports in the scientific literature, while the remaining metabolites have been reported only a handful of times. As can be seen from the analysis presented above, the roquefortine molecular motif occupies a rare niche within the larger family of diketopiperazines. The discovery of new roquefortine analogues would further enhance our understanding of this structure class.

Prior to our investigation the published occurrence of roquefortine diketopiperazines was restricted to *Penicillium* species. In this report we describe the isolation and structure elucidation of a new member of this structure class, roquefortine E (**6**), from an Australian strain of *Gymnoascus reessii* Baranetzki, together with the known co-metabolite roquefortine C (**1**). In prior studies on the same culture we reported on a group of new and unrelated antifungal butenolides, gymnoascolides A−C.[13]

A MeOH extract of *G. reessii* (MST-F9977) was found to possess activity against *Bacillus subtilis* such that the strain was recultured on a larger scale with a view to isolating and identifying the active agent(s). The resulting culture broth was concentrated in vacuo and fractionated by repeated C_{18} solid-phase extraction (SPE) and HPLC. The fractions obtained from this process were analyzed by ^{1}H NMR, analytical HPLC, and ESI(±)MS, as well as in an antibacterial bioassay. Both biologically active and chemically interesting fractions were selected for further investigation. Semipreparative C_{18} SPE and HPLC of the antibacterially active fractions yielded the known compound roquefortine C (**1**) as the active agent, while a new analogue, designated roquefortine E (**6**), was isolated from one of the nonactive fractions. The identity of roquefortine C (**1**) was confirmed by comparison of ^{1}H and ^{13}C NMR, $[\alpha]_D$, and ESI(+)MS data with those reported in the literature.[10] The structure of **6** was determined by comparison with **1** and by detailed spectroscopic analysis.

High-resolution ESIMS analysis of roquefortine E (**6**) returned a pseudomolecular ion ($[M + H]^+$, *m/z* 458.2545) consistent with a molecular formula of $C_{27}H_{31}N_5O_2$, representing the addition of C_5H_8 to roquefortine C (**1**). Examination of the ^{1}H and ^{13}C NMR data for **6** (see Table 1) clearly revealed its structural similarity to roquefortine C (**1**) and confirmed the presence of a second 1,1-dimethylallyl substituent. Strong HMBC correlations from both C-28 methyls and H-17 to a common carbon (δ_C 141.2) suggested that the attachment was at C-22, an observation that was also supported by the presence of only one histidine proton (δ_H 8.75) in the ^{1}H NMR spectrum. The ^{13}C NMR chemical shift for C-22 in **6** was deshielded relative to that of C-22 in **1** (δ_C 141.2 vs 135.1), consistent with attachment of the dimethylallyl substituent at this location. The substitution pattern was confirmed by the observation of an NOE enhancement of H-17 (2%) upon

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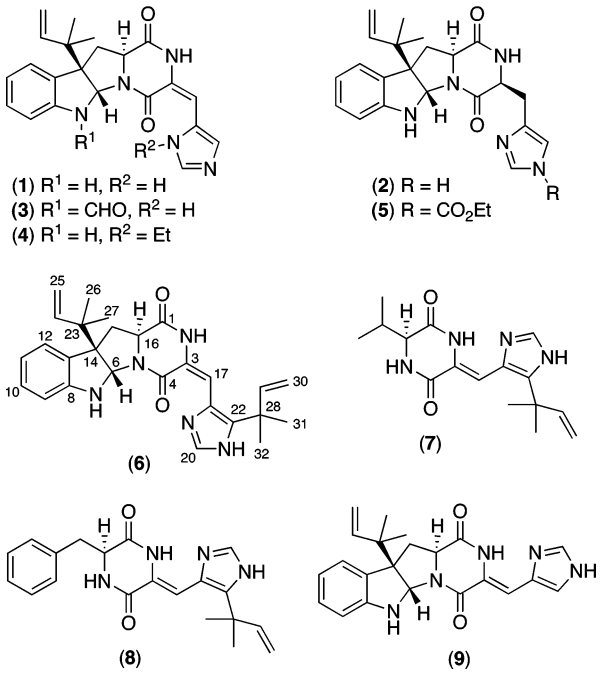
Table 1. NMR Data (400 MHz, $CDCl_3$) for Roquefortine E (**6**)

position[a]	δ_C[b]	δ_H (m, *J* (Hz))	COSY	HMBC
C-1	166.2			
N-2		9.21 (brs)		
C-3	125.3			
C-4	158.9			
C-6	78.9	5.67 (s)		C-8, C-13, C-16, C-23
C-8	149.5			
C-9	109.3	6.63 (d, 7.3)	H-10	C-11, C-13
C-10	129.4	7.13 (dd, 7.3, 7.5)	H-9, H-11	C-8, C-12
C-11	119.4	6.80 (dd, 7.5, 7.7)	H-10, H-12	C-9, C-13
C-12	125.0	7.18 (d, 7.7)	H-11	C-8, C-10, C-14
C-13	128.0			
C-14	61.5			
C-15	36.5	2.60 (dd, 6.2, 12.5)	H-16	C-6, C-13, C-14
		2.48 (dd, 11.0, 12.5)	H-16	C-1, C-13, C-14, C-16, C-23
C-16	58.8	4.11 (dd, 6.2, 11.0)	H-15	C-1, C-15
C-17	108.2	6.60 (s)		C-3, C-4, C-22
C-18	121.7			
C-20	132.3	8.75 (brs)		C-18,[c] C-22[c]
C-22	141.2			
C-23	40.9			
C-24	143.0	5.97 (dd, 10.6, 17.2)	H-25	C-23, C-26/27
C-25	113.8	5.17 (dd, 10.6, 1.1)	H-24	C-23, C-24
		5.14 (dd, 17.2, 1.1)	H-24	C-23, C-24
C-26	22.4	1.16 (s)		C-14, C-23, C-24, C-27
C-27	22.7	1.03 (s)		C-14, C-23, C-24, C-26
C-28	38.3			
C-29	144.0	6.01 (dd, 10.6, 17.2)	H-30	C-22, C-28, C-31/32
C-30	115.2	5.20 (m)	H-29	C-28, C-29
C-31	27.7	1.57 (s)		C-22, C-28, C-29, C-32
C-32	28.0	1.53 (s)		C-22, C-28, C-29, C-31

[a] The numbering scheme of Vleggaar et al.[17] has been preferred to that of Scott et al.[2] [b]Assignments supported by HMQC, DEPT, and HMBC data. [c]Correlations only observed with multiple bond coupling optimized for 11 Hz.



irradiation of the C-28 methyl resonances. The fungal diketopiperazines aurantiamine (**7**) and phenylahistin (**8**)[14,15] are also known to possess a similar isoprenylated histidine moiety.

A deshielded exchangeable proton resonance (δ_H 9.21) in the ^1H NMR ($CDCl_3$) spectrum of **6** was attributed to the amide proton H-2, an assignment that was supported by reacquisition in d_6-DMSO to reveal the same resonance, but somewhat shifted (δ_H 10.21) and much sharper, with HMBC correlations to carbonyls at C-1 and C-4 now visible. Although the analogues **1** and **3** both possess *E* stereochemistry about $\Delta^{3,17}$, the precedent for *Z* stereochemistry has been set by the previously mentioned aurantiamine (**7**) and phenylahistin (**8**),[14,15] in addition to a number of other fungal metabolites. The *Z*-isomer of **1**, isoroquefortine C (**9**), is also known to be formed by photoisomerization of **1**.[16] Isoroquefortine C (**9**) possesses a number of characteristic differences in the ^{13}C NMR spectrum relative to **1**,[17] including a significant downfield shift of C-18 (δ_C 125.5 to 136.8). The chemical shift for C-18 of **6** (δ_C 121.7) was much closer to that of **1**, suggesting a common $\Delta^{3,17}$ *E* stereochemistry. Confirmation was provided by a ROESY (d_6-DMSO, 600 MHz) experiment in which a correlation was observed between H-17 and the amide proton H-2. An unsuccessful attempt was made to isomerize **6** by exposure to a UV light source (λ 360 nm) in a manner similar to that demonstrated for **1**.

Excellent ^1H NMR comparisons between **6** and **1** about the remaining residues supported a common relative stereochemistry at these stereocenters. Support for the relative stereochemistry was provided by an NOE difference experiment, which revealed an enhancement (2%) to H-6 on irradiation of H_3-26 and H_3-27, requiring that all these protons occupy the same face of the roquefortine E (**6**) ring system. Also of note were the very similar ^1H NMR characteristics for H-16 in **6** (δ_H 4.11, dd, *J* 6.0 and 11.1 Hz) and **1** (δ_H 4.07, dd, *J* 6.1 and 11.5 Hz),[18] respectively, which strongly supported a common relative stereochemistry. As the absolute stereochemistry of **1** is known,[18] and **6** is a co-metabolite, on biogenetic grounds we propose a common absolute stereochemistry. It should also be noted that the optical rotations of **1** (−806°, $CHCl_3$; lit. −703°, $CHCl_3$[2]) and **6** (−223°, $CHCl_3$) were also of the same sign, supporting this conclusion, although without a thorough knowledge of the effects of the additional dimethylallyl moiety on the configuration of **6**, such correlations should be treated with caution.

Roquefortine E (**6**) is noteworthy in that it is the first roquefortine to be isolated from a fungus other than *Penicillium*. It also combines the main structural features of two structure classes: the cyclized isoprenylated tryptophan of the roquefortines, and the isoprenylated dehydrohistidine of the phenylahistins. The biosynthesis of **1** has been studied in some detail:[19] its biosynthesis from histidine, tryptophan, and mevalonic acid has been confirmed by isotopic labeling studies,[20] and it is known to be a precursor in the biosynthesis of several other mycotoxins.[21] One of the most contentious issues in the biosynthesis of **1** has been the mechanism of isoprenylation at C-14:[19] the currently favored theory is a direct attack by isoprenyl pyrophosphate.[22] While no similar studies have been conducted on the isoprenylation of histidine in **6**−**8**, it seems that a similar mechanism could also be involved in these cases.

In addition to the toxic properties discussed earlier, roquefortine C (**1**) reportedly possesses bacteriostatic activity against Gram-positive bacteria[23] and has been shown to interact with cytochrome P450 by binding to heme.[24] (−)-Phenylahistin (**8**) also possesses interesting biological properties: it was reported to be a cell-cycle inhibitor at the time of its first isolation,[15] and several patents[25] and

further publications[26] have pursued this property. Further studies have indicated that **8** inhibits tubulin polymerization.[27]

With a ready supply of roquefortine E (**6**) at hand, we investigated this analogue for both antimicrobial and cell-cycle inhibitory activity. Unlike roquefortine C (**1**) (*B. subtilis* MIC 6.3 μg/mL), **6** did not possess any discernible antibacterial activity, but did display weak cytotoxic activity to mammalian cells (murine NS-1, LD_{99} 24 μg/mL) comparable to that displayed by **1** (LD_{99} 50 μg/mL). Roquefortine E (**6**) was also submitted to a range of cell-cycle inhibition assays, using a mouse bone marrow macrophage cell line, without evidence of noteworthy activity. These results add to current SAR knowledge of, and help further define the pharmacophore for, the roquefortine and phenylahistin structure classes.

Experimental Section

General Experimental Procedures. The procedures were as previously reported,[28] except for the following: preparative HPLC work was carried out on a system consisting of two Shimadzu LC-8A preparative liquid chromatographs with static mixer, a Shimadzu SPD-M10AVP diode array detector, and a Shimadzu SCL-10AVP system controller. UV−vis absorption spectra were obtained using a Shimadzu UV-1650PC spectrophotometer, while infrared (IR) spectra were acquired using a Shimadzu FTIR-8400 spectrometer. For roquefortine C (**1**), the optical rotation was obtained using a JASCO P-1010 intelligent remote module type polarimeter, and ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance 600 spectrometer. Low-resolution ESI(±)MS data was obtained using a Agilent 1100 Series separations module equipped with a Agilent 1100 Series LC/MSD mass detector, while high-resolution (HR) ESIMS measurements were obtained on a Finnigan MAT 900 XL-Trap instrument with a Finnigan API III source.

Biological Material. The fungal strain (MST-F9977) was isolated from a roadside soil sample collected in July 1995 near Sussex Inlet on the southern coast of New South Wales, Australia, in an area regenerating from a recent bushfire. The isolate was identified as an ascomycete, *Gymnoascus reessii* Baranetzki, on morphological grounds. On malt extract agar it is characterized by orange hyphae and a pinkish reverse.

Bioassays. Antibacterial and cytotoxicity assays were carried out as described previously.[29] For cell-cycle studies, murine bone marrow-derived macrophages (BMM) from C57Bl6 mice were cultivated as outlined previously.[30] Day 6 BMM were plated in 60 mm bacteriological Petri dishes at either 0.7×10^6 with colony stimulating factor 1 (CSF-1, a gift from Chiron, Emeryville, CA) added at 104 units/mL or at 1×10^6 in the absence of CSF-1 in order to arrest cells in G_0. The following day, cells were treated with either no additive (control), with DMSO (vehicle control), or with **6** at 0.4 mM, 2 mM, or 10 mM (in DMSO). The CSF-1 starved cells were treated in the same manner, with CSF-1 (104 units/mL) added 1 h after the aforementioned treatments. All cells were then incubated for a further 24 h, after which time cells were harvested and processed for cell cycle analysis as previously described.[31]

Extraction and Isolation. A solid fermentation (100 g wheat, 21 days 28 °C) was extracted with MeOH. This extract was concentrated in vacuo to an aqueous residue that was diluted with H_2O and passed through two parallel C_{18} SPE cartridges (2 × 10 g, Varian HF C_{18}), eluting with MeOH (2 × 40 mL each). Concentration of the aqueous MeOH fractions in vacuo yielded a combined residue that was subjected to preparative HPLC (60 mL/min with a gradient elution of 70% to 10% H_2O/MeCN (0.01% TFA) over 20 min followed by MeCN (0.01% TFA) for 10 min, through a Platinum EPS C_{18} 5 μm 50 × 100 mm column). One hundred fractions were collected, concentrated, and combined into pools on the basis of analytical HPLC analysis. One of these fractions was further fractionated by C_{18} SPE (10% stepwise gradient elution from 60% H_2O/MeOH to 100% MeOH) to yield pure roquefortine E (**6**) (15.1 mg).

A subsequent fermentation of the same organism, optimized for roquefortine C (**1**) production (malt extract agar (16%) containing peptone (0.1%) and glucose (2%), 200 × 15 g Petri plates, 21 days, 28 °C) was found to possess higher antibacterial activity. Initial C_{18} SPE and HPLC fractionations were carried out in a manner similar to those described above, followed by repeated preparative HPLC (first at 10 mL/min isocratic 71% H_2O/MeCN (0.01% TFA) for 20 min and then 10 mL/min isocratic 75% H_2O/MeCN (0.01% TFA) for 20 min, both through a Luna C_{18} 5 μm 21 × 100 mm column), to afford roquefortine C (**1**) (17 mg). Roquefortine C (**1**) was also detected in the initial culture but at levels below practical isolation.

Roquefortine C (1): white solid; HRESI(+)MS *m/z* 390.1933 ($[M + H]^+$, $C_{22}H_{24}N_5O_2$ requires 390.1930); $[\alpha]_D$, ESI(+)MS, and ^{1}H and ^{13}C NMR data matched well with literature values.[2,10]

Roquefortine E (6): white solid; $[\alpha]_D$ −223° (*c* 0.060, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3375, 1693, 1664, 1608, 1468, 1439, 1418, 1381, 1180, 1148 cm^{-1}; UV−vis (MeOH) λ_{max} (ϵ) 228 (13 600), 342 (11 100) nm; ^{1}H NMR data ($CDCl_3$, 400 MHz), see Table 1; ^{13}C NMR data ($CDCl_3$, 100 MHz), see Table 1; ESI(+)MS (30 kV) *m/z* 458 $[M + H]^+$; HRESI(+)MS *m/z* 458.2545 ($[M + H]^+$, $C_{27}H_{32}N_5O_2$ requires 458.2556).

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