ORGANIC LETTERS

2010
Vol. 12, No. 19
4356−4359

Isolation of Putative Biosynthetic Intermediates of Prenylated Indole Alkaloids from a Thermophilic Fungus *Talaromyces thermophilus*

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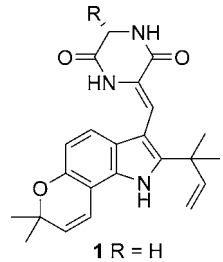
Received August 4, 2010

ABSTRACT



1 R = H
2 R = CH_3

The putative key biosynthetic intermediates of prenylated indole alkaloids have long been proposed but never isolated. Two such alkaloids, named talathermophilins A and B (1 and 2), were isolated from a thermophilic fungus *Talaromyces thermophilus* strain YM1−3 and were identified by NMR and MS spectroscopic analyses. The ratio of 1 and 2 in the culture broths was unexpectedly rather constant (about 2:3), which even remained unchanged despite the addition of exogenous 1 or 2, suggesting that talathermophilins might be of special function for the extremophilic fungus.

The family of prenylated indole alkaloids including aspergamides, norgeamides, notoamides, avrainvillamide, stephacidins, and sclerotiamide is a well-known group of secondary metabolites mainly produced by *Aspergillus* and *Penicillium* spp.[1] They have aroused great fascination for their manifold structural architectures and promising biological activities. Up to now, nearly 100 members of this family of secondary metabolites have been discovered. Three hallmark features of prenylated indole alkaloids are their common biogenesis from tryptophan, one to three isoprene units and a 2,5-diketopiperazine. Interestingly, only a very small group of amino acids (glycine, alanine, proline, and its derivatives) could be naturally chosen as a starting building block to form the 2,5-diketopiperazine with tryptophan.

From a biosynthetic perspective, a significant gap has existed among these interesting metabolites, namely, a scaffold as possessed by intermediates **I**−**III** (Figure 1) has never been obtained. Among them, intermediate **III**, named as notoamide E, has long been proposed as a key versatile precursor in the biosynthetic pathway for most of the

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10.1021/ol101817g
Published on Web 09/02/2010

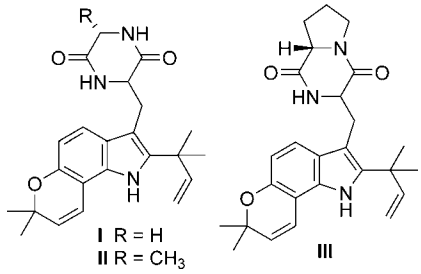

Figure 1. Three putative intermediates of prenylated tryptophan alkaloids.

prenylated indole alkaloids derived from proline.[2] Recent investigation with a synthetic notoamide E as standard found that notoamide E could be detected only in the early stage of fungal growth and immediately disappeared, indicating that notoamide E was a very short-lived intermediate.[2h] To date, no example of the biogenesis of the congeners of the above intermediates has been truly isolated.

In our screening experiments for metabolite profiles of thermophilic microorganisms, one fungal strain *Talaromyces thermophilus* YM1−3 attracted our attention because it could produce yellow pigments. The compounds showed dark purple fluorescence on TLC under UV 365 nm and red color after being treated with 20% H_2SO_4 in ethanol followed by heating at 120 °C. Chemical investigation on these pigments led to the isolation of two yellowish compounds (**1** and **2**). Through extensive NMR and MS analyses, their stuctrures were elucidated as two novel prenylated tryptophan alkaloids with the long proposed scaffold.

The EIMS of **1** showed a strong molecular ion peak at *m/z* 391 ($[M]^+$) and a base peak of a fragmental ion at *m/z* 376 ($[M - CH_3]^-$). The molecular formula of **1** was determined to be $C_{23}H_{25}N_3O_3$ on the basis of its positive high-resolution ESI mass spectrum, indicating 13 degrees of unsaturation and an odd number of nitrogen atoms. Strong UV absorptions[3] at 212.0 242.0, 312.0, 347.0 nm were indicative of the presence of an extended conjugation system in **1**. The IR spectrum[3] of **1** showed absorption bands for NH (3354 cm^{-1}), C═O (1678 cm^{-1}), and aromatic functionalities (1638 and 1441 cm^{-1}) in the molecules. The ^1H NMR spectrum of **1** recorded in acetone-d_6 exhibited signals attributable to three NH groups (δ_H 9.85, 7.97, and 7.23), six olefinic methines (δ_H 7.07, 6.98, 6.94, 6.05, 6.13, 5.69), one olefinic methylene (δ_H 5.06 and 5.03), and four methyl groups (δ_H 1.53 and 1.40, each 6H). The ^{13}C NMR and DEPT spectra of **1** (see Table 1) contained signals for 23 carbon atoms. The NMR data revealed the presence of two pairs of *gem*-dimethyl groups, a methylene, a quaternary carbon, an oxygenated quaternary carbon, six aromatic methines, an olefinic methylene, seven aromatic quaternary carbons, two conjugated amide carbonyl groups. The HSQC spectrum of **1** enabled the assignment of all protons to the directly bonded carbons. All of the above data indicated the presences of a tetrasubstituted indole core, a diketopiperazine moiety, and two isoprenyl groups.

Table 1. NMR Spectral Data for Compounds **1** and **2**[a]

	1		**2**	
no.	^{13}C	^1H	^{13}C	^1H
1		9.85 brs		9.88 brs
2	143.45 s		143.53 s	
3	104.87 s		104.86 s	
4	119.94 d	7.07 d (8.5)	119.81 d	7.04 d (8.5)
5	111.33 d	6.55 d (8.5)	111.33 d	6.56 d (8.5)
6	149.49 s		149.45 s	
7	106.29 s		106.30 s	
8	132.59 s		132.57 s	
9	121.97 s		121.98 s	
10	40.11 s		40.08 s	
11	146.24 d	6.13 dd (10.5, 17.3)	146.20 d	6.13 dd (10.5, 17.4)
12	112.01 t	5.06 d (17.3)	111.99 t	5.07 d (17.4)
		5.03 d (10.5)		5.03 d (10.5)
13	27.98 q	1.53 s	27.96 q	1.53 s
14	27.98 q	1.53 s	27.96 s	1.53 s
15	110.59 d	6.98 s	110.71 d	6.99 s
16	126.56 s		126.65 s	
17	159.99 s		160.18 s	
18		7.23 brs		7.42 brs
19	46.07 t	4.14 s	52.12 d	4.27 q (6.9)
20	163.53 s		166.85 s	
21		7.97 brs		7.95 brs
22	118.47 d	6.94 d (9.8)	118.45 d	6.95 d (9.8)
23	130.08 d	5.69 d (9.8)	130.08 d	5.70 d (9.8)
24	76.04 s		76.03 s	
25	27.71 q	1.40 s	27.70 q	1.40 s
26	27.71 q	1.40 s	27.70 q	1.40 s
27			20.61 q	1.50 d (6.9)

[a] Recorded in acetone-d_6 at 500 MHz for ^1H and 125 Hz for ^{13}C.

The connectivity of these partial structures and the positions of the substituents were determined by $^1H-^{13}C$ long-range correlations detected in the HMBC spectrum (Figure 2). The correlations of H-22 with C-24, C-23, C-6, C-7, and C-8, together with the correlations of two methyls at δ_H 1.40 (6H, s) with C-23 and C-24, suggested the existence of a 1,7-dihydro-7,7-dimethylpyrano[2,3-*g*]indole. Additional evidence was provided by a NOE correlation between the H-22 and NH-1 in the NOESY spectrum (Figure 2). The attachment of a 2-methylbut-3-en-2-yl moiety to C-2 of the pyranoindole was assigned by the HMBC couplings of the methyls at δ_H 1.53 (6H, s) with C-2, C-10, and C-11 and of H-11 with C-10 and C-2. The linkage of this prenylated pyranoindole substructure with the diketopiperazine moiety through an olefinic methine bridge was revealed by three $^1H-^{13}C$ couplings of the olefinic

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(3) Talathermophilin A (**1**): yellow powder; $[\alpha]^{23.5}_D$ −15.67 (*c* 0.1, MeOH); UV (MeOH) λmax (log ε) 212.0 (4.07), 242.0 (4.15), 312.0 (3.67), 347.0 (3.63); IR (KBr) ν_{max} 3354, 2971, 2925, 2872, 2854, 1678, 1638, 1441, 1433, 1392, 1361, 1330, 1256, 1216, 1195, 1162, 1119, 1079, 1060, 995, 937, 924, 900, 873, 845, 806, 781, 729, 671, 497, 428 cm^{-1}; EI-MS *m/z* 391 $[M]^+$ (47), 376 (100), 322 (18), 248 (38), 222 (18); HRESI-MS (positive) 414.1791 $[M + Na]^+$ (calcd for $C_{23}H_{25}N_3O_3Na$, 414.1793).

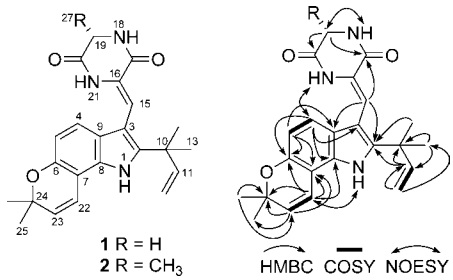

Figure 2. Chemical structures of compounds **1** and **2** with HMBC, ^{1}H−^{1}H COSY, and NOESY correlations (indicated by arrows).

proton at δ_H 6.98 (H-15) with C-2, C-9, and C-16 in the HMBC spectrum (Figure 2).

Careful inspection of the ^{13}C NMR data of **1** indicated that its C-15- and C-16 were similar to those of neoechulin A and isoechulin A,[4] suggesting that the configuration of the double bond between C-15 and C-16 in **1** was also *Z* (cis). This was further confirmed by a NOE correlation of H-4 (δ_H 7.07) with NH-21 (δ_H 7.97) in the NOESY spectrum of **1**, which also indicated the conformation of the diketopiperazine as shown in Figure 2. The conformation of the diketopiperazine in **1** described above is opposite to that of dipodazine,[5] which was determined by a cross peak between H-4 and the vinyllic H-15 of the conjugated side chain in the NOESY spectrum and the fact that decoupling of H-1 resulted in enhancement of H-2 (10%). Thus, compound **1** was characterized as (3*Z*)-3-((1,7-dihydro-7,7-dimethyl-2-(2-methylbut-3-en-2-yl)pyrano[2,3-*g*]indol-3-yl) methylene)piperazine-2,5-dione and named talathermophilin A.

The EIMS of **2** gave a strong molecular ion peak at *m/z* 405 $[M]^+$ and a base fragmental ion peak at *m/z* 391 ($[M - CH_3]^-$), which revealed a molecular formula of $C_{24}H_{27}N_3O_3$ for **2**, indicating that **2** possessed one more CH_2 unit than **1**. The UV and IR spectra[6] of **2** were almost identical to those of **1**, suggesting similar structural properties to **1**. Comparison of the ^{1}H and ^{13}C NMR data of **2** with those of **1** demonstrated that **2** was very similar to **1**. The only difference between them was that the methylene group (δ_C 46.07, δ_H 4.14 (s)) in **1** was replaced by a methine group (δ_C 52.12, δ_H 4.27 (q, J = 6.9 Hz)) and a methyl group (δ_C 20.61, δ_H 1.50 (d, J = 6.9 Hz)) in **2** (see Table 1). All the above data suggested that compound **2** was C-19 methylated analogue of compound **1**. The configuration of $\Delta^{15,16}$ and the conformation of the diketopiperazine in **2** were determined to be identical to those in **1**, since the NOESY spectrum of **2** was shown to be the same as that of **1**.

Acid hydrolysis of **2**, followed by analysis by TLC on a chiral stationary phase (CHIRALPLATE),[7] showed the presence of L-alanine in the hydrolysate. Thus, the structure of **2** was established as (*S*,3*Z*)-3-((1,7-dihydro-7,7-dimethyl-2-(2-methylbut-3-en-2-yl)pyrano[2,3-*g*]indol-3-yl)methylene)-6-methyl piperazine-2,5-dione and named talathermophilin B.

Compounds **1** and **2** are the first natural pyranoindole alkaloids bearing the striking structural features of the key putative intermediates **I**−**III**. In addtion, this is the first time that pyranoindol alkaloids were isolated from a thermophilic fungus which do not belong to the genus *Aspergillus* or *Penicillium*.

A time−course experiment in which the metabolite profile was analyzed daily by HPLC and TLC indicated that **1** and **2** appeared in the culture broths of *T. thermophilus* YM1−3 incubated for 4 days and did not disappear for one month. Further temperature experiments demonstrated that both **1** and **2** existed in the fermentation broths incubated at temperatures ranging from 35 to 65 °C (the fungus could not grow under 35 °C). These experiments indicated that **1** and **2** are heat-stable metabolites.

It is most interesting that the content ratio of **1** and **2** in the culture broth of *T. thermophilus* YM1−3 was rather constant (about 2:3) under different culture conditions. Addition of exogenic **1** to the medium at concentrations ranging from 0.05

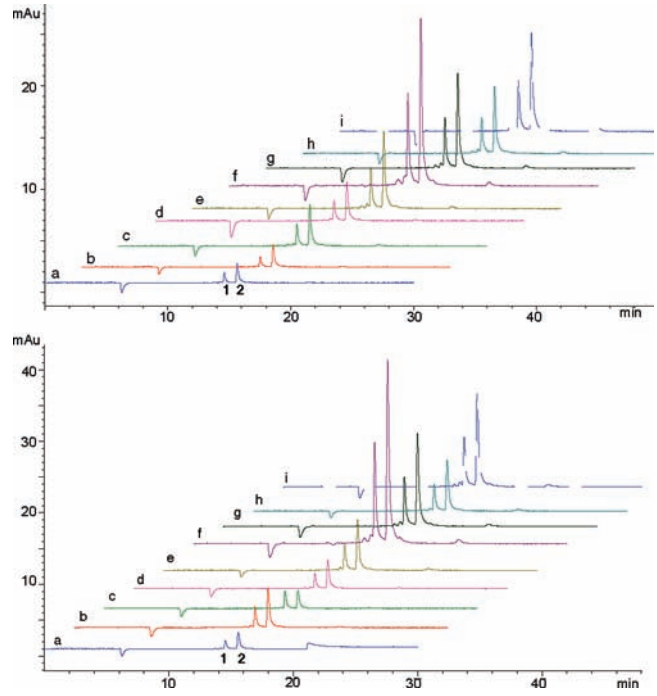

Figure 3. HPLC profiles of culture extracts of *T. thermophilus* YM1−3 monitored at 347 nm for the detection of talathermophilins: (top) *T. thermophilus* YM1−3 treated with **1**; (bottom) *T. thermophilus* YM1−3 treated with **2**. Key: (a) *T. thermophilus* YM1−3 without treatment containing talathermophilins (**1**, 84 μg/mL; **2**, 125 μg/mL); (b−i) *T. thermophilus* YM1−3 treated with **1** or **2** (0.025, 0.05, 0.1, 0.5, 5, 10, 100, 200 μg/mL). The peaks marked with **1** and **2** represent compounds **1** and **2** respectively. mAU, milliabsorbance units.

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(6) Talathermophilin B (**2**): yellow powder; $[\alpha]^{23.5}_D$ −13.33 (*c* 0.3, MeOH); UV (MeOH) λmax (log ε) 211.5 (4.06), 241.5 (4.13), 310.0 (3.66), 346 (3.62); IR (KBr) ν_{max} 3354, 2969, 2926, 2870, 2855, 1679, 1638, 1441, 1363, 1327, 1258, 1216, 1195, 1162, 1119, 1060, 1041, 995, 921, 902, 804, 780, 761, 730, 687, 620, 499 cm $^{-1}$; EI-MS *m/z* 405 $[M]^+$ (71), 390 (100), 336 (18), 280 (20), 248 (20); HRESI-MS (negative) *m/z* 404.1978 $[M - H]^+$ (calcd for $C_{24}H_{26}N_3O_3$, 404.1974).

to 10 μg/mL could induce the production of **2**, and vice versa, thus maintaining the ratio of **1** and **2** surprisingly unchanged (Figure 3). Meanwhile, the contents of both **1** and **2** increased largely in the fermentation cultures of the thermophilic fungus. The exogenic talathermophilins at a concentration of 5 μg/mL in the media could induce the content of both metabolites in the fermentation broths as high as about 1000 μg/mL for **1** and 1500 μg/mL for **2**. Additionally, the *T. thermophilus* strain YM1−3 cultivated in the media containing talathermophilin, appeared to grow better than those in the media without any treatment. Our results suggested that talathermophilins might be stimulators of their own and partner productions at certain concentrations. They might be also of other special physiological or ecological functions for the fungus, which warrant further investigation.

We have also attempted to obtain 15,16-dehydro-**III** employing thermophilic fungus YM1−3 cultivated with varying media containing peptone, proline, and glutamic acid. It was unfortunately not successful though **1** and **2** were found in all the fermentation broths of this organism as major components. It seemed that this thermophilic fungus could only use glycine and alanine as starting materials for the biosynthesis of this type of alkaloids.

In our general bioactivity profiling programs, e.g., antimicrobial, cytotoxic, and nematicidal testing,[8] both compounds showed nematicidal toxicity (ca. 38% and 44% inhibition, respectively) toward the worms of the free-living nematode *Panagrellus redivevus* at a concentration of 400 μg/mL for 72 h. However, talathermophilins were not active in other assays. This result would account for the absence of the oxidative proline ring system, which is presumably vital for biological activity of this family of alkaloids.[1]

In conclusion, our findings first confirmed the existence of the putative key biosynthetic intermediates of prenylated indole alkaloids and should provide insight into the biosynthetic relationships of the important family of secondary metabolites. The study also indicated that thermophilic fungi could be a potential source with natural products, which could complement the metabolite libraries of the fungi which live at a common temperature.

Acknowledgment. This work was sponsored by projects from Major State Basic Research Development Program (2009CB125905), National Natural Science Foundation of China (30870083 and 31070051), the Department of Science and Technology of Yunnan Province (2008CD068), the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry ([2009]1341), and the 12th Young Academic and Technical Leader Raising Foundation of Yunnan Province (2009CI051).

Supporting Information Available: ^{1}H and ^{13}C NMR spectra of compounds **1** and **2** and 2D NMR spectra of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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