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Thermolides, Potent Nematocidal PKS-NRPS Hybrid Metabolites from Thermophilic Fungus *Talaromyces thermophilus*

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Supporting Information

ABSTRACT: Macrocyclic PKS-NRPS hybrid metabolites represent a unique family of natural products mainly from bacteria with broad and outstanding biological activities. However, their distribution in fungi has rarely been reported, and little has been reported regarding their nematocidal activity. Here we describe an unprecedented class of PKS-NRPS hybrid metabolites possessing a 13-membered lactam-bearing macrolactone, thermolides A–F (**1**–**6**) from a thermophilic fungus *Talaromyces thermophilus*. We showed that **1** and **2** displayed potent inhibitory activity against three notorious nematodes with LC_{50} values of 0.5–1 μg/mL, as active as commercial avermectins. This work provided a new class of promising lead compounds for nematocide discovery.

Nematodes are among the most abundant multicellular animals on earth, and phytoparasitic nematodes are among the most notoriously difficult crop and wood pests to control.[1] Due to concerns about public health and environmental safety, chemical nematocides such as organophosphate and carbamate insecticides are being withdrawn from the market. In the past 20 years, only a small repertoire of agents, mainly avermectins, have arisen to address the growing need for nematode control.[2,3] Nematodes also are distinguished as useful tools for the discovery of novel drugs because several human disease models have been built in the *Caenorhabditis elegans*.[4]

Macrocyclic PKS-NRPS hybrid metabolites are a unique family of natural products from microorganisms, mainly from bacteria.[5] They display a wide range of impressive biological activities and include the immunosuppressant rapamycin, the anticancer agent epothilone, the pristinamycin components of the antibiotic synercid, the ansa antibiotics including the antitubercular drugs of the rifamycin group, the Hsp90 inhibitor geldanamycin, and the maytansine family antitumor agent ansamitocin.[6,7] Moreover, the products of the PKS-NRPS pathways present a large pool of novel chemical entities that have been selected during evolution by providing an advantage to the producer/host.[8] Nevertheless, their distribution in fungi has not been well investigated, and no work has been reported about their application as nematocidal agents.[9]

Thermophilic fungi are eukaryotes that thrive at high temperatures. They represent a potential reservoir of thermostable enzymes for industrial applications and could be developed into cell factories to support the production of chemicals and materials at elevated temperatures.[10] However, very few of them have been screened for their production of structurally and biologically novel secondary metabolites.[11] Recently, we found that a thermophilic fungus *Talaromyces thermophilus*, collected from the Tengchong hotspring of Yunnan, China, could produce two putative key biosynthetic intermediates, talathermophilins A and B,[12] which had long been proposed for the biosynthesis of crenulated tryptophan alkaloids but never really isolated and identified. Continuing work on the fungus resulted in the discovery of the precursors for the postulated biosynthetic pathways for talathermophilins.[13]

In our further investigations on the metabolic profiles of thermophilic fungi collected from the same habitat, a novel class of PKS-NRPS hybrid molecules was obtained from the thermophilic fungus *T. thermophilus* YM 3-4. These metabolites possess a 13-membered lactam-bearing macrolactone and are shown as thermolides A–F (**1**–**6**) in Figure 1. Interestingly, compounds **1** and **2** were found to show potent inhibitory activity against three notorious nematodes.

Thermolides A–C (**1**–**3**) were obtained as major constituents among these metabolites from the culture broths of *T. thermophilus*. They all exhibited a quasi-molecular ion peak at *m*/*z* 544 $[M-H]^-$ in their negative ESI spectra and were

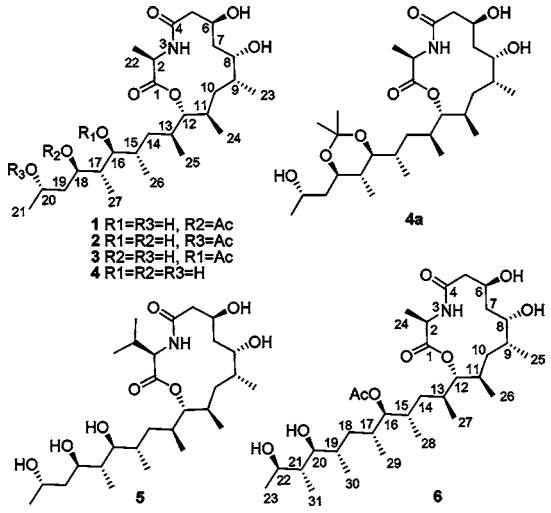

Figure 1. Structures of **1**–**6** and **4a**.

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assigned to a molecular formula of $C_{28}H_{51}NO_9$, on the basis of HRESIMS and NMR data (Table S1).

The ^{1}H NMR spectrum of **1** displayed obvious signals for seven secondary methyl groups (δ_H 1.28, 1.11, 0.98, 0.95, 0.87, 0.85, and 0.79), one tertiary methyl of an acetoxyl group (δ_H 1.96), six O-bearing methines, and one N-bearing methine (δ_H 5.46, 4.75, 4.57, 4.23, 3.73, 3.55, and 3.25). The ^{13}C NMR and DEPT spectra of **1** indicated signals attributable to three carbonyl groups (δ_C 169.8, 171.1, and 172.6), twelve methines (including six O-bearing ones), five methylenes, and eight methyl groups. The presence of the three carbonyl groups accounted for 3 of 4 sites of unsaturation, suggesting the existence of one ring in **1**. The HSQC spectrum of **1** enabled the assignment of all H-atoms to the directly bonded C-atoms.

Interpretation of the ^{1}H−^{1}H COSY spectrum of **1** led to the unambiguous establishment of three segments, the moieties H2/Me22 (A), $H_2$5/H6/$H_2$7/H8 (B), and H16/H17(Me27)/H18/$H_2$19/$H_2$20/Me21 (C) (Figure 2) due to the congested proton

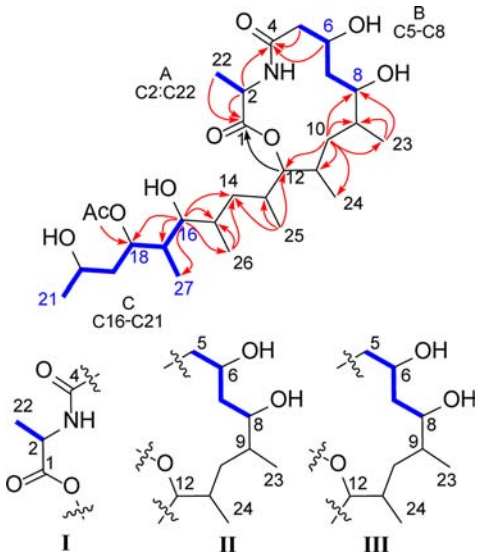

Figure 2. Key correlations in ^{1}H−^{1}H COSY (bold blue bonds) and HMBC spectra (red curves) leading to partial chemical substructures and eventually the planar structure of **1**.

signals ranging from δ_H 1.80−1.65 ascribable to three methines (δ_C 34.9, 33.1, and 32.1). The HMBC correlations of H2 at δ_H 4.75 with two carbonyl carbons at δ_C 172.6 (C1) and 169.8 (C4), and the methyl carbon at δ_C 16.7 (C22), and of Me22 at δ_H 1.28 with C1 and one methine carbon at δ_C 47.4 (C2) (Figure 2), together with one nitrogen in the molecular formula, led to elucidation of moiety I derived from segment A. Similarly, moiety II derived from segment B were deduced by the HMBC correlations of H10 at δ_H 1.38 with two methine carbons at δ_C 34.9 (C9) and δ_C 31.0 (C11), two methyl carbons at δ_C 17.1 (C23) and 17.4 (C24), two oxymethine carbons at δ_C 75.9 (C8) and at 84.4 (C12), and of Me23 at δ_H 0.98 with C8 and C9, combining with the above segment B. The strong HMBC correlations of H12 at δ_H 4.58 with C1 and of H5 at δ_H 2.57 and H6 at δ_H 4.23 with C4 were the key correlations to determine the linkage of moieties I and II to complete an unprecedented 13-membered macrocyclic core bearing one lactam, accounting for the remaining site of unsaturation. The downfield shifted chemical value of H18 at δ_H 5.46 suggested that the only acetoxyl group was attached to C18 at δ_C 73.4, which was further supported by a strong correlation between H18 and the acetyl carbonyl carbon at δ_C 171.1 in the HMBC spectrum. The HMBC correlations of H16 with two methine carbons at δ_C 33.1 (C15) and 39.9 (C17), two methyl carbons at δ_C 17.5 (C26) and 12.6 (C27), one methylene carbon at δ_C 33.6 (C14). and C18 allowed us to establish partial substructure III derived from segment C. This assignment was supported by the ^{1}H−^{13}C long-range correlations from Me26 to C14/C15/C16. The ^{1}H−^{1}H correlation of the methyl at δ_H 0.79 (Me25) with H13 at δ_H 1.78 and HMBC correlations from Me25 to C13/C12/C14 allowed for incorporation of the last methine C13 into the structure, which served as a bridge to connect the macrocyclic core and the partial substructure III to form the whole planar structure of **1**.

Compounds **2**−**3** shared the same 13-membered macrocyclic core as **1** deduced by the analysis of NMR spectrometric data (Table S1). The chemical values of H20 in **2** and H16 in **3** were downshifted to δ_C 5.09 and 4.76, respectively, and revealed that the acetyl group was linked to C20 in **2** and C16 in **3**, respectively. Thermolide D (**4**) was assigned to a molecular formula of $C_{26}H_{49}NO_8$ on the basis of HRESI spectra. Analysis of NMR spectrometric data of **4** (Table S1) revealed that **4** was just a deacetyl derivative of **1**−**3**.

Thus, **1**−**4** feature a novel class of hybrid metabolites of an unprecedented 13-membered lactam-bearing macrolide linked to a saturated side chain with an ordered arrangement of methyls and hydroxyls, which together incorporate seven secondary methyl groups and five oxygenated groups at C6, C8, C16, C18, and C20. The stereochemistry of these metabolites was segregated into two distinct macrocyclic and side chain clusters.

Only one acetonide derivative (**4a**) of **4** was obtained in this study, which was assigned to a molecular formula of $C_{29}H_{53}NO_8$. The 1D and 2D NMR spectrometric data (Table S2) revealed that **4a** was a 16,18 acetonide derivative of **4**. The fact that acetonide derivatization occurred at C16/C18 in **4**, where the steric hindrance was comparatively larger than that at C18/C20 and C6/C8, led to the arbitrary deduction of two moieties of 6,8-*anti*-diols and 16,18,20-*syn-syn-anti* triols in **1**−**4**. The *anti* configuration of the 6,8-diol moiety was in agreement with the absence of a NOE contact between H8 and H6 in the ROESY spectra of **1**−**4**.

The ROESY experiments for the side chain in **4a** displayed a strong 1,3-diaxial NOE correlation between H16 and H18, illustrating a preferable chair conformation of the six-membered ring where the hydroxyl groups at C16 and C18 were both in equatorial positions. The NOE contacts from Me27 to H16 and H18 suggested that H17 was also axial, which was confirmed by the vicinal coupling constant (J = 8.8 Hz) between H16 and H17. The NOE contacts from H14a to H17, H16 to Me26, and H15 to Me27, indicated the stereochemistry of the C15 stereocenter was the same as that of C16. The small coupling constant (J = 2.0 Hz) between H16 and H15 supported this deduction. The NOE correlations of OH20 to H18, and Me21 to H19b, together with a strong NOE contact between H20 and H19a, displayed that the stereochemistry of the C20 stereocenter was opposite to that of C18 (Figure 3).

Due to rotameric restrictions imposed by the 13-membered macrocyclic core and the 6-membered ring, a 1,3-*anti* relationship between C13 and C15 could be assigned on the basis of the strong NOE correlations of H13 to Me26, H14b to Me25, H14a to Me26, and H15 to Me25 in **4a** (Figure 3). Further evidence came from the large coupling constants (J = 13.0 Hz) for H14a/H13 and H14b/H15 in the ^{1}H NMR spectrum of the 6,20-*bis*-

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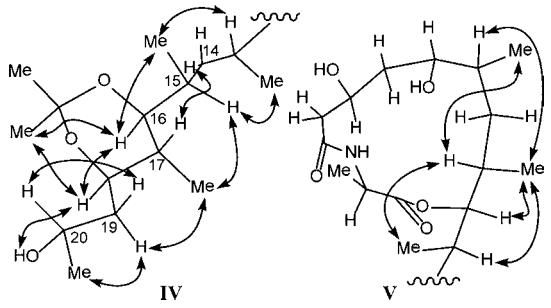

Figure 3. Selected key NOE contacts for the C13−C19 side chain (**IV**) and the C1−C13 macrolactone region (**V**).

MTPA derivative (**4R2**) of **4** (Table S4), while the signals for $H_2$14 in **1**−**4** and **4a** were overlapped.

NMR methods alone are insufficient to unambiguously define the remaining stereochemical permutations. We attempted to solve the configuration of the thermolides through preparation and ^{1}H NMR analysis of the corresponding −OH (*R*)- and (*S*)-MTPA ester derivatives of **4**.

The *R* and *S penta*-MTPA esters (**4R5** and **4S5**) were prepared from **4**, and the respective ^{1}H NMR signals (Table S3) were assigned from HSQC spectra. Mosher's analysis of negative $\Delta\delta^{SR}$ (δS-δR) of the *R* and *S penta*-MTPA esters in the region of H2−H11 conformed with the 1,3-*anti*-diols model[14,15] and allowed assignment of 6*S* and 8*S*. The analysis of the 1,3,5-triol moiety was carried out by examination of pairwise additive anisotropic shifts in MTPA esters of 1,3-*syn*-diols and 3,5-*anti*-diols in accordance with the interpretation of Riguera et al.[14,15] for MTPA esters of diols and of Molinski et al.[16,17] for MTPA esters of *syn*-*syn*-*anti* triols (Table S3; Figures S1 and S2). The characteristic positive $\Delta\delta^{SR}$ (δS-δR) values for H13−H21 supported the 16*S*,18*R*,20*S* configurations.

The stereochemistry of C9−C12 in the macrocyclic cores of **1**−**4** was established with the help of the ^{1}H NMR and ROESY experiments for **4R5** due to its rotameric restrictions imposed by large MTPA groups. The broad singlet for H8 in **4R5**, in combination with the absence of an NOE contact between H8 and Me23 in the ROESY spectrum of **4R5**, indicated a *syn* configuration at C8/C9. A partial MTPA derivatization experiment in which MTPA esterification of the macrocyclic core in **4** occurred initially at C6 (6,20-*bis*-MTPA esters of **4**, **4R2**) further implied the existence of a steric-hindrance effect at C8 caused by Me23 residing on the same side as the OH8 (Figure 3).

The strong NOE contacts between H9 and Me24 and between Me23 and H11 in the ROESY spectrum of **4R5** indicated a 1,3 *trans* configuration at C9/C11 (Figure 3). The observation of a strong NOE correlation between H12 and Me24 suggested a *tran*-configuration for C11/C12 with the two H-atoms H11/H12 in a quasibisaxial conformation. The vicinal coupling constants (J = 7.6−8.3 and 3.2−3.8 Hz) of H12 signals in the ^{1}H NMR spectra of **1**−**4**, together with the key NOE correlations of Me24 to H13, Me25 to H11, and H14 to H11, in combination with the absence of NOE contacts of Me24 to Me25 in the ROESY spectrum of **4R5**, confirmed the stereochemistry of C13 deduced from a 1,3-*anti* relationship between C13 and C15.

Acidic hydrolysis of **4**, followed by treatment with Marfey's regent and analysis by HPLC,[18,19] showed the presence of D-alanine in **4**. The configurations of the C1−C12 macrocyclic core and C13−C21 chains have thus been established as shown in **4a** (Figure 1). The energy-minimized conformer **4aa** provided by molecular modeling for **4a** (Supporting Information)[20,21] was in good agreement with the above deduction.

For the first congener of **4**, thermolide E (**5**) was assigned a molecular formula of $C_{28}H_{53}NO_8$ according to HRESIMS and NMR spectroscopy (Table S2). The signal characteristic for Me22 at δ_H 1.29 in the ^{1}H NMR spectrum of **4** was missing while two geminal secondary methyls existed in the higher field region of the ^{1}H NMR spectrum of **5**. The diagnostic C2 at δ_H 48.4 in **4** appeared downfield at δ_C 58.5 in **5**. The correlations in ^{1}H−^{1}H COSY, HSQC, and HMBC spectra suggested that D-alanine in **4** was replaced by D-valine in **5**.

The HRESIMS analysis of thermolide F (**6**) established a molecular formula of $C_{32}H_{59}NO_9$. The NMR data (Table S2) displayed that the carbon scaffold of **6** shared the same macrolactone core as **1**−**4** but possessed a C_{11} side chain with penta-methyl groups and similar oxygenation pattern to **3**. The absolute configurations at the stereogenic centers in the C1−C13 macrocyclic core in **6** were established to be identical to the corresponding parts in **1**−**5**, as similar NOE correlations in the regions of H2−H13 were observed in the ROESY spectra. However, the assignment of the stereochemistry of the side chain in **6** was tricky due to the overlap of key signals. As derivatization experiments were hampered by the minute yields of **6** (0.7 mg), we sought to obtain clues with respect to its cometabolites **1**−**5**. It is interesting to note that not only the C1−C17 substructure in **6** was the same as that in **3** (Blue square in Figure 4) but also the

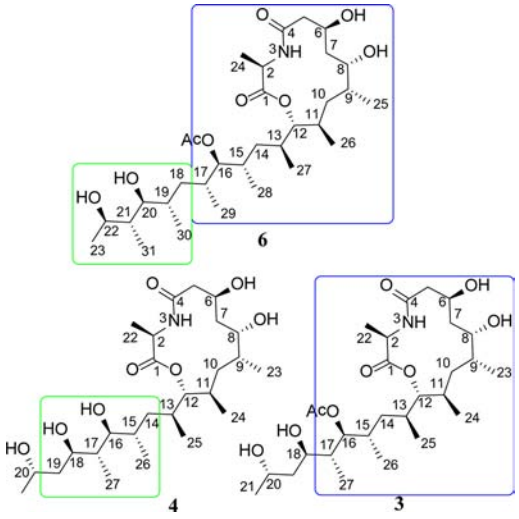

Figure 4. Analysis for the structural similarity of **3**, **4**, and **6**.

C18−C23 moiety with a 1,3-dihydroxy-2,4-dimethyl substitution pattern in **6** was identical to the C14−C19 regions in **4** (green square in Figure 4). Thus, the configurations of the C18−C23 regions in **6** are tentatively determined to be identical to those of the C14−C19 regions in **4**.

From the biosynthetic point of view, the macrolide cores in **1**−**6** could be formed by the linkage of one polyketide chain and one amino acid. The macrolide core consisting of one lactone and one lactam could be also found in the structures of unusual metabolites including rapamycin, an unusual PKS-NRPS hybrid metabolite bearing a 31-membered macrolide core, isolated from the bacterium *Streptomyces hygroscopicus*,[6,7] divergolides from an endophytic *Streptomyces* sp. HKI0576 of the mangrove tree *Bruguiera gymnorrhiza*,[22] and myxovirescins from gliding bacterium *Myxococcus xanthus* DK1622.[23] However, one amino acid involved in the formation of both the lactone and lactam is

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not observed in these macrolide-core bearing compounds. To the best of our knowledge, this is the first report on the discovery of hybrid macrolides from a fungus origin. This finding may provide a new impetus for delineating the regulatory mechanism governing the variability in fungal metabolites and shed new light onto the unexplored biosynthetic abilities of unprecedented secondary metabolites in extremophilic fungi.

Compounds **1**−**4** were evaluated for their nematodetoxic activities against three types of nematodes including the root knot nematode *Meloidogyne incognita*, pine-wood nematode *Bursaphelenches siylopilus*, and free-living nematode *Panagrellus redivevus*.[12] **1** and **2** showed the strongest activities against all the worms with LC_{50} values 0.5−1.0 μg/mL, similar to those of avermectins. **3** displayed moderate activity, and a weak inhibitory effect on the worms was observed for **4** (Table 1). Taken together, **1**−**2** covered a range of nematode toxic activities. Investigation on the biosynthesis of the unique class of metabolites in the extremophilic fungus is currently underway in our laboratory.

Table 1. Nematodetoxic Activities (LC_{50}, μg/mL) of Thermolides A−D (1−4)

	test nematode		
compd	*M. incognita*	*B. siylopilus*	*P. redivevus*
1	0.8	1.0	0.6
2	0.7	0.9	0.5
3	30.5	25.6	40.8
4	55.6	48.4	56.9
avermectins	0.7	0.5	0.8

In conclusion, we have isolated and characterized a novel class of potent nematocidal thermolides from a thermophilic fungus *T. thermophilus*. Thermolides A−F feature unprecedented PKS-NRPS hybrid metabolites containing a 13-membered lactam-bearing macrolactone and represent an exciting new class of promising leads for nematocidal agent discovery.

■ ASSOCIATED CONTENT

Supporting Information

A description of experimental procedures, materials, extraction and isolation of seven compounds, bioassays of thermolides, crucial data (ORD, UV, IR, MS, ^{1}H, and ^{13}C NMR) and ^{1}H and ^{13}C NMR, COSY, HSQC, HMBC, and ROESY spectra of **1**−**6** and **4a**, and ^{1}H, ^{13}C spectra of the R and S MTPA esters of **4** were included. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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