DOI: 10.1002/chem.200700038

Cytotoxic Polyketides Containing Tetramic Acid Moieties Isolated from the Fungus *Myceliophthora Thermophila*: Elucidation of the Relationship between Cytotoxicity and Stereoconfiguration

Yu-Liang Yang,[a] Chun-Ping Lu,[a, b] Mao-Yen Chen,[a] Kuei-Yu Chen,[c] Yang-Chang Wu,[d] and Shih-Hsiung Wu*[a, b]

Abstract: Five new polyketides that contain tetramic acids, myceliothermophins A–E, were isolated from the thermophilic fungus *Myceliophthora thermophila*. Two sets of 5-alkyl-5-hydroxyl (or 5-methoxyl)-1*H*-pyrrol-2(5*H*)-one diastereomers, myceliothermophins A/B and C/D, were separated as pure compounds by using silica-gel column chromatography and recycling reverse-phase high-performance liquid chromatography (RP-HPLC). The relative configurations of the chiral centers in 5-alkyl-5-hydroxyl (or 5-methoxyl)-1*H*-pyrrol-2(5*H*)-one moieties were deduced from NOESY correlations. In the cytotoxic assay, the 5-(2-methylpropyldiene)-1*H*-pyrrol-2(5*H*)-one analogue (myceliothermophin E) exhibited inhibition against four cancer cell lines. In addition, the significant inhibitory effect of myceliothermophins A and C and the inactivity of myceliothermophins B and D revealed the importance of the relative configurations of 5-alkyl-5-hydroxyl (or 5-methoxyl)-1*H*-pyrrol-2(5*H*)-one moieties on their cytotoxicity potency against cancer cells.

Keywords: configuration determination · cytotoxicity · myceliothermophins · polyketides · structure elucidation

Introduction

Secondary metabolites that contain the tetramic acid (2,4-pyrrolidine-2,4-dione) ring system (parent system) have been known for almost half a century,[1,2] well before the parent system was synthesized and have been isolated from marine mollusks, sponges, cyanobacteria and terrestrial and marine microorganisms, particularly endophytic fungi. From a biosynthetic perspective, natural tetramic acids are regarded as having arisen from the assembly of an amino acid and an activated acyl entity derived from an acetyl group or a more complex activated ester. Recent studies have confirmed that these metabolites have a wide distribution and play a significant role in ecological interactions.[2] Compounds that contain this structural unit exhibit a diverse range of biological activities and have attracted the interest not only of natural product chemists, but also of chemical ecologists and synthetic chemists. Among them, dolastatin 15, isolated from 1600 kg of sea hare (yield 1.8×10^{-7}%), is the most famous.[3,4] Now dolastatin 15 and its analogue have entered phase I and II clinical trials for patients with various cancers.[5]

Recently, we investigated the thermophilic fungus, *Myceliophthora thermophila*, and found that the methanol extract of *M. thermophila* showed potent cytotoxic activity against a Chinese hamster ovary cell line. Based on the purification experiments and a bioassay, five polyketides that contain tetramic acid moieties, myceliothermophins A–E (**1**–**5**) were isolated. The structures were elucidated on the basis of spectroscopic data. The cytotoxicity assays for myceliothermophins A–E (**1**–**5**) against four types of cancer cell, human

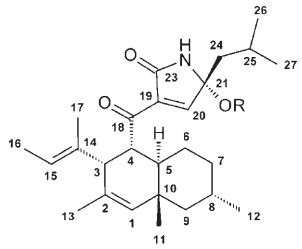
[a] Dr. Y.-L. Yang, C.-P. Lu, Dr. M.-Y. Chen, Prof. Dr. S.-H. Wu
Institute of Biological Chemistry
Academia Sinica, Taipei 115 (Taiwan)
Fax: (+886) 2-2653-9142
E-mail: shwu@gate.sinica.edu.tw

[b] C.-P. Lu, Prof. Dr. S.-H. Wu
Institute of Biochemical Sciences, National Taiwan University
Taipei 106 (Taiwan)

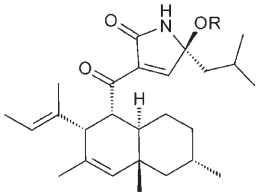
[c] Prof. Dr. K.-Y. Chen
Department of Biology, Chinese Culture University
Taipei 108 (Taiwan)

[d] Prof. Dr. Y.-C. Wu
Graduate Institute of Natural Products
Kaohsiung Medical University, Kaohsiung 807 (Taiwan)

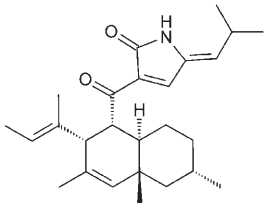
Supporting information for this article is available on the WWW under http://www.chemeurj.org or from the author



1: R = H
3: R = CH_3



2: R = H
4: R = CH_3



5

hepatoblastoma (HepG2), human hepatocellular carcinoma (Hep3B), human lung carcinoma (A-549), and human breast adenocarcinoma (MCF-7) are reported herein. The relationship between the configurations of **1**–**4** and their cytotoxicities are also revealed.

Results and Discussion

Myceliothermophins A–E (**1**–**5**) were isolated from the methanolic extract of *M. thermophila* by way of general partition and repeated chromatographic methods. The ^{1}H NMR spectra of **1**, **2**, and the mixture of **3** and **4**, showed similar features with slight differences in chemical shifts, this indicated that there were two sets of stereoisomers. The mixture of **3** and **4** was difficult to separate by using normal-phase and reverse-phase chromatographic methods. Thus, recycling reverse-phase high-performance liquid chromatography (RP-HPLC) was applied and **3** and **4** were obtained as pure compounds at the 20th cycle (eluted with CH_3CN/H_2O, 90:10).

The high-resolution electron spray ionization mass spectroscopy (HR-ESIMS) data of **1** and **2** indicated that their molecular formula is $C_{26}H_{39}NO_3$ and that they contain eight degrees of unsaturation. The formula was supported by the ^{13}C NMR data, which showed seven CH_3, four CH_2, eight CH, and seven quaternary carbon atoms. Eight downfield ^{13}C signals in the NMR data (Table 1), including one ketone, one amide or ester carbonyl, and three double bonds, revealed the three-ring structures of **1** and **2**.

The ^{1}H NMR spectra (Table 2) of **1** and **2** displayed one quaternary methyl singlet, three doublets for secondary methyl groups, two olefinic methyl singlets, and one olefinic methyl quartet of doublets. Based upon the analysis of the above methyl groups in the ^{1}H–^{1}H COSY and HMBC spectra (Figure 1a,c), the presence of one isopropyl moiety and one isoprenyl moiety was deduced. The highly deshielded doublet ($\delta_{H\text{-}20}$ = 7.49 and 7.45 ppm in **1** and **2**, respectively), assignable to a β proton of an α,β-unsaturated carbonyl, together with NH and OH signals were connected together by the HMBC correlations to reveal a 5-hydroxyl-1*H*-pyrrol-2(5*H*)-one moiety (Figure 1a). The long-range correlations also revealed that the isopropyl group and one ketone carbonyl (C-18) were linked, respectively, to the 5- and 3-positions of the 5-hydroxyl-1*H*-pyrrol-2(5*H*)-one moiety. Consequently, the deoxy-tetramic acid moieties were proposed for compounds **1** and **2**.

The proposal for the decalin ring can be supported by other NMR characteristics (Figure 1c). The connectivity of four methine groups (H-3, H-4, H-5, and H-8), including one allylic methine group ($\delta_{H\text{-}3}$ = 3.24 and 3.13 ppm in **1** and **2**, respectively), three methylene groups (H-6, H-7, and H-9), together with one doublet methyl group (H-12) are indicated by the cross peaks in the ^{1}H–^{1}H COSY spectrum. The isoprenyl group was found to be coupled with the allylic methine by means of the HMBC correlations (H-3 to C-14, C-15, C-16, and H-4 to C-14). Diagnostic HMBC correlations from the resonances for one double bond (C-1 and C-2) and one quaternary carbon (C-10) to those for the above-mentioned moieties determined by ^{1}H–^{1}H COSY permitted the assignments of the decalin ring to be completed. In addition, C-2 and C-10 were substituted with methyl groups (C-13 and C-11, respectively), which were also deduced from long-range HMBC correlations (H-11 to C-5, and H-3 to C-2/C-13). HMBC correlations (H-4 to C-18) show that it is possi-

Table 1. The ^{13}C NMR data[a] of myceliothermophins A–E (**1**–**5**).

Carbon	1	2	3	4	5
1	136.9	136.9	137.0	137.1	136.9
2	131.1	131.1	130.9	131.0	131.2
3	51.6	51.9	51.7	51.9	51.7
4	50.1	50.3	50.2	50.3	50.0
5	40.8	40.9	41.0	41.0	40.8
6	24.9	24.8	24.9	24.9	24.8
7	36.7	36.7	36.7	36.7	36.7
8	28.2	28.2	28.3	28.3	28.5
9	49.3	49.3	49.4	49.4	49.3
10	35.9	36.0	36.0	36.0	35.9
11	20.9	20.9	21.0	21.0	20.9
12	23.2	23.2	23.2	23.2	23.1
13	22.5	22.5	22.5	22.5	22.4
14	136.8	136.2	137.2	137.2	136.7
15	123.8	124.4	123.8	123.9	123.7
16	13.3	13.4	13.2	13.3	13.3
17	14.1	14.3	14.3	14.0	14.3
18	197.5	198.0	196.6	196.7	197.0
19	137.1	137.2	139.4	139.5	135.8
20	156.9	156.4	155.4	155.9	143.3
21	87.0	87.1	91.2	91.4	134.7
23	168.7	168.7	168.7	168.8	169.3
24	47.4	47.5	47.2	47.2	130.2
25	25.2	25.2	25.2	25.1	28.5
26	24.5	24.5	24.5	24.5	22.7
27	24.9	24.8	24.7	24.7	22.6
OCH_3	–	–	50.5	50.6	–

[a] Chemical shifts [ppm] (150 MHz, [D_6]acetone).

Table 2. The ^{1}H NMR data of myceliothermophins A–E (**1**–**5**).[a]

Hydrogen	1	2	3	4	5
1	5.37, s	5.36, s	5.39, s	5.39, s	5.38, s
3	3.24, d (7.2)	3.13, d (7.3)	3.26, d (7.6)	3.26, d (7.6)	3.23, d (7.3)
4	4.00, dd (12.5, 7.2)	3.94, dd (12.3, 7.3)	4.01, dd (12.3, 7.6)	4.01, dd (12.3, 7.6)	4.04, dd (12.4, 7.2)
5	1.81, m	1.81, m	1.83, m	1.83, m	1.83, td (11.7, 2.4)
6	0.95, m	0.96, m	0.96, m	0.97, m	0.97, m
	1.66, m	1.66, m	1.63, m	1.63, m	1.69, m
7	0.91, m	0.91, m	0.94, m	0.94, m	0.95, m
	1.69, m	1.70, m	1.74, m	1.74, m	1.74, m
8	1.70, m	1.71, m	1.70, m	1.70, m	1.70, m
9	0.92, m	0.93, m	0.92, m	0.92, m	0.92, m
	1.48, m	1.50, m	1.49, m	1.49, m	1.50, m
11	0.92, s	0.92, s	0.92, s	0.92, s	0.95, s
12	0.85, d (6.2)	0.85, d (6.1)	0.85, d (6.1)	0.85, d (6.1)	0.86, d (6.1)
13	1.45, s	1.46, s	1.46, s	1.46, s	1.46, s
15	5.13, qd (6.7, 0.8)	5.19, qd (6.7, 1.0)	5.18, q (6.2)	5.15, q (6.2)	5.06, q (6.9)
16	1.47, d (6.7)	1.47, d (6.7)	1.46, d (6.2)	1.46, d (6.2)	1.41, d (6.9)
17	1.43, d (0.8)	1.43, d (1.1)	1.43, s	1.43, s	1.43, s
20	7.49, d (1.7)	7.45, d (1.6)	7.50, d (1.0)	7.49, d (1.0)	7.55, d (1.9)
24	1.81, m	1.84, m	1.80, m	1.80, m	–
25	1.71, m	1.82, m	1.77, m	1.77, m	2.96, m
26	0.96, d (6.4)	0.93, d (6.3)	0.91, d (6.5)	0.91, d (6.5)	1.10, d (6.7)
27	0.98, d (6.4)	0.96, d (6.3)	0.93, d (6.5)	0.93, d (6.5)	1.12, d (6.7)
NH	7.83, brs	7.79, brs	7.61, brs	7.62, brs	9.57, s
OH	5.07, brs	5.06, brs	–	–	–
OCH_3	–	–	3.13, s	3.09, s	–

[a] Chemical shifts [ppm], multiplicity J in parentheses [Hz] (600 MHz, [D_6]acetone).

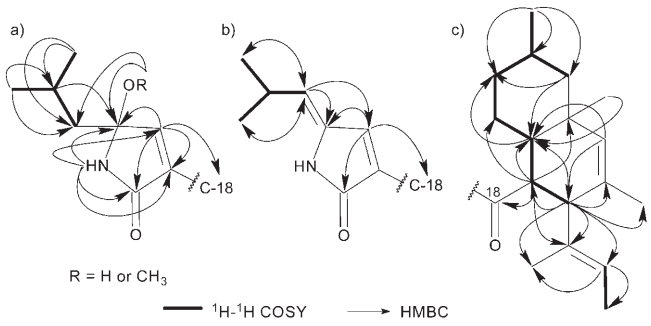


Figure 1. a)–c) ^{1}H–^{1}H COSY and HMBC correlations of **1**–**5**.

ble that the deoxy-tetramic acid moiety couples to the decalin moiety to establish the core structures of **1** and **2**.

The core structure of **1** may be supported by the electron ionization mass spectroscopy (EIMS) data. The retro-Diels–Alder (rDA) fragmentation of the decalin ring of **1** forms as a deoxy-tetramic acid that has a branched, long chain, of which the stable fragments, *a*, *b*, *d*, and *e* ions, were produced in the EIMS spectrum (Table 3 and Figure 2, top). The other important fragmentations, such as the double bond migration (*y* ion), allylic cleavage (*c* ion), and the loss of methyl, methylene, carbonyl, and hydroxyl groups, are also listed in Table 3. Compound **1** was dissolved in 1,3-dimercaptobutane to give compound **1a** and the solution was also subjected to EIMS. The fragments of **1a** are similar to those of **1** and are accompanied by the vital ion peaks *z* that reveal the core structure of **1** (Table 3 and Figure 2, bottom).

Table 3. EIMS data and fragmentations of **1** and **1a**.[a]

Fragmentations	1		1a	
	m/z	%	*m/z*	%
M	413	100	–	–
$[M-CH_3]^+$	398	16	–	–
$[M-CH_3-CH_2]^+$	384	45	–	–
$[M-S(CH_2)_3SH]^+$	–	–	414	6
$[a]^+$	55	69	55	73
$[b]^+$	135	81	135	83
$[b-H-CH_3]^+$	119	50	119	88
$[b-2CH_3]^+$	105	48	105	76
$[c-H-CH_3]^+$	175	40	175	38
$[c-2CH_3]^+$	161	18	161	33
$[c-2CH_3-CH_2]^+$	147	18	147	26
$[d]^+$	231	31	231	18
$[d-H-CH_3]^+$	215	10	–	–
$[d-2CH_3]^+$	201	50	–	–
$[d-2CH_3-CH_2]^+$	187	38	187	86
$[e]^+$	259	67	259	24
$[e-H-CH_3]^+$	243	46	–	–
$[e-2CH_3]^+$	229	31	–	–
$[x-OH]^+$	341	12	–	–
$[y-H-C=O]^+$	316	19	–	–
$[z-H-CH_3]^+$	–	–	274	100
$[z-C=O-OH]^+$	–	–	245	89
$[z-CH(CH_3)_2]^+$	–	–	219	35
$[z-CH(CH_3)_2-OH]^+$	–	–	202	21
$[z-(CH_2)_3SH]^+$	–	–	187	86

[a] See Figure 2 for an explanation of *a*–*e* and *x*–*z*.

A NOESY experiment resulted in two 1,3-diaxial correlations (H-4/H-11 and H-5/H-9) that indicate that both **1** and **2** have *trans*-decalin rings (Figure 3). The other 1,3-diaxial NOE correlation, H-6/H-8, suggests that the orientation of

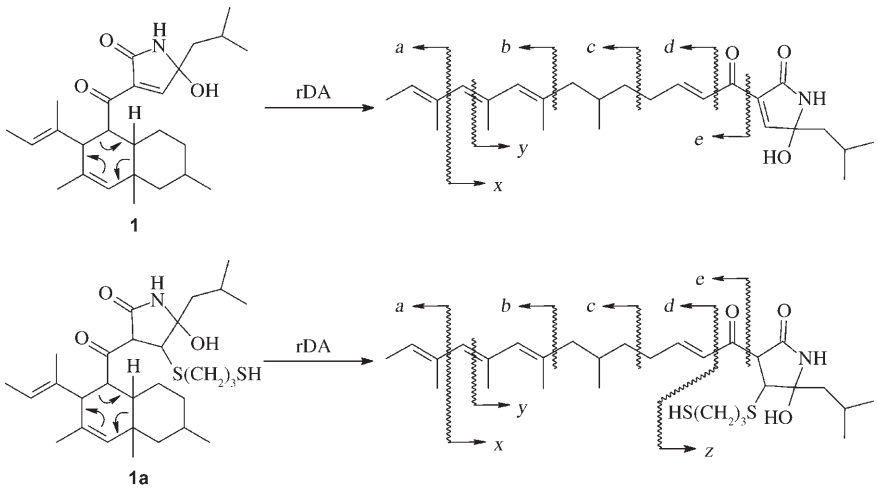


Figure 2. EIMS fragmentations of **1** (top) and **1a** (bottom).

groups in compounds **3** and **4**. The assignments of ^{1}H and ^{13}C signals are summarized in Tables 1 and 2.

After stereochemical analysis, the relative configurations of five of the stereocenters of **3** and **4** were confirmed to be identical, 3*S**4*R**5*R**8*R**10*S**, based on NOESY experiments. The key NOE correlation between the methoxyl group and H-15 in **4** (Figure 4, bottom) and the absence of correlation between the two protons in **3**, shown in Figure 4 (top), revealed that the relative configurations of C-21 are *R** in **3** and *S** in **4**. By application of the same method, the chirality of C-21 was identified as *R** in **1** and *S** in **2**.

the C-12 methyl group is equatorial. The NOE cross peak between H-3 and H-4 indicates that H-3 must be equatorially oriented in a pseudochair conformation, thus forcing the isoprenyl group into an axial position.

The strong NOE effect between H-4 and H-20 suggests that the two dihedral angles, H-4/C-4/C18/C-19 and C-18/C-19/C-20/H-20, of compound **1** should be close to those of compound **2**. The observations also suggest that the core structures and conformations of **1** and **2** are very similar. However, based upon the different chemical shifts of H-3, H-4, and the deoxy-tetramic acid moieties proposed, the configuration of C-21 in **1** and **2** is reversed. To our knowledge, there has been no previous report about the purification of 5-alkyl-5-hydroxyl-1*H*-pyrrol-2(5*H*)-one diastereomers, and no suitable methodology to elucidate the absolute configurations of the diastereomers even though several 5-alkyl-5-hydroxyl-1*H*-pyrrol-2(5*H*)-one derivatives from natural sources have been isolated. In our case, the NOE correlation between H-15 and H-26 in **1** (Figure 3, top) and the absence of a correlation between H-15 and H-26 in **2** (Figure 3, bottom) suggest the relative configuration of C-21. Thus, the relative configurations of **1** and **2** were determined as 3*S**4*R**5*R**8*R**10*S**21*R** and 3*S**4*R**5*R**8*R**10*S**21*S**, respectively.

The molecular formulas of **3** and **4** were determined to be $C_{27}H_{41}NO_3$ and to contain eight degrees of unsaturation by analysis of the HR-ESIMS data. The ^{1}H NMR data (Table 2) of **3** and **4** are similar to that of **1** and **2**, except for the additional methoxyl groups and the absence of the OH signals. The ^{13}C signals (Table 1) of **3** and **4** are highly superimposable over those of **1** and **2**, except for C-18, C-19, C-20, C-21, and the methoxyl carbon. Careful analyses of HMQC, ^{1}H–^{1}H COSY, and HMBC spectra (Figure 1a,c) of **3** and **4** revealed that they have the same core structures, which are proposed as diastereomers. The difference between diastereomers **3**/**4**, and **1**/**2**, is that the hydroxyl groups at C-21 of **1** and **2** are replaced with methoxyl

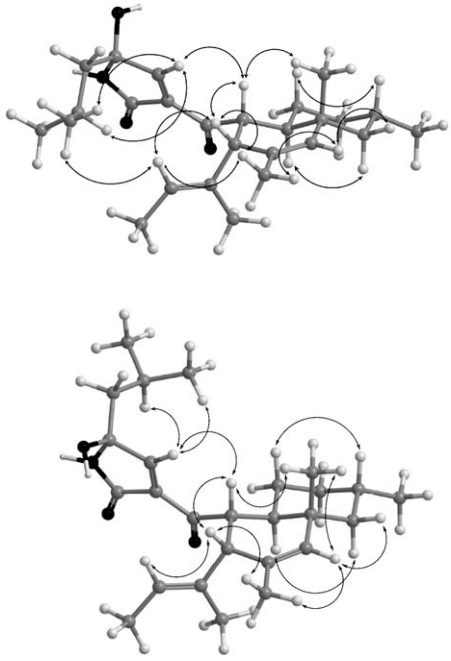


Figure 3. Key NOESY correlations of **1** (top) and **2** (bottom).

The molecular formula of **5** was confirmed (by using HR-ESIMS) as $C_{26}H_{37}NO_2$, which corresponds to the addition of one degree of unsaturation to **1**. The ^{13}C NMR spectrum (Table 1) was very similar to that of **1**, except for the appearance of upfield peaks at $\delta = 135.8$ and 130.2 ppm in **5**, rather than $\delta = 87.0$ and 47.4 ppm in **1**. The combination of different ^{13}C signals, an additional degree of unsaturation, and more upfield ^{13}C signals of C-19 and C-20 in **5** than in **1** (**1**: $\delta = 137.1$ (C-19), 156.9 ppm (C-20); **5**: $\delta = 134.7$ (C-19), 143.3 ppm (C-20)) indicated that the corresponding 5-alkyl-5-hydroxyl-1*H*-pyrrol-2(5*H*)-one moiety, found in **1**, was a 5-

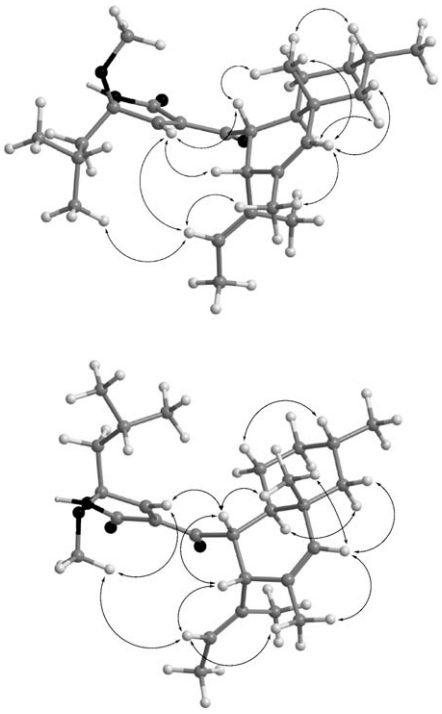


Figure 4. Key NOESY correlations of **3** (top) and **4** (bottom).

(2-methylpropyldiene)-1*H*-pyrrol-2(5*H*)-one moiety in **5**. The deshielded proton H-25 ($\delta = 2.96$ ppm), NH ($\delta =$ 9.57 ppm), doublet olefinic proton H-24 ($\delta = 5.53$ ppm), and HMBC correlations (H-26 and H-27 to C-24; H-24 to C-20, C-21, C-26, and C-27; H-20 to C-18, C-21 and C-23, Figure 1b) also confirmed the structure of this moiety. The core structure and configuration of the decalin moiety, deduced from ^{1}H–^{1}H COSY, HMBC (Figure 1b,c) and NOESY experiments (Figure 5), proved to be the same as those in compound **1**. The stereochemistry of the double bond between C-21 and C-24 was determined to be in the *Z* configuration based on the key correlations, H-20/H-24 and H-25/NH, in the NOESY spectrum (Figure 5).

The ^{1}H NMR spectra of **5** showed signals from a tiny amount of impurity. Based on the different ^{1}H signals of H-24 ($\delta = 5.70$ ppm, d, $J = 10.4$ Hz), H-25 ($\delta = 3.02$ ppm, m), and NH ($\delta = 9.15$ ppm, s) the impurity could be the diastereomer of compound **5** that has the *Z* configuration in the 5-(2-methylpropyldiene)-1*H*-pyrrol-2(5*H*)-one moiety. Compound **5** could be purified by using RP-HPLC to remove the diastereomer, which could not be collected in sufficient quantity for further structural elucidation studies and biological assay.

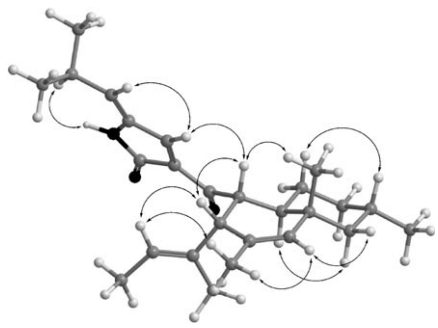


Figure 5. Key NOESY correlations of **5**.

Some secondary metabolites that contain the deoxy-tetramic acid ring system have been reported, such as oteromycin,[6] ZG-1494α, USC1025A and B,[8,9] pyrrocidines A and B,[10] ascosalipyrrolidinones A and B,[11] and talaroconvolutins A–D.[12] Although the biogenetic pathways of **1**–**5** and the deoxy-tetramic acid related compounds mentioned above remain unclear, a plausible pathway can be proposed. This pathway is based on the biosynthetic route of the tetramic acid analogue, trichosetin, and involves a polyketide reacting with one amino acid.[13] In **1**–**5**, the decalin rings were derived from an octaketide intermediate and five *S*-adenosylmethionines (see the Supporting Information). The deoxy-tetramic acid moieties of **1** and **2** were proposed following the fusarin C[14] and lucilactaene[15] modes. The octaketide intermediate reacts with homoleucine aldehyde to form 1,5-dihydronpyrrol-2-one, which may undergo tautomerization, epoxidation, and isomerization to produce the racemic 5-alkyl-5-hydroxyl-1*H*-pyrrol-2(5*H*)-ones. The methoxyl groups in **3** and **4** may be introduced during the extraction or isolation procedures, similar to the cases reported for ascosalipyrrolidinones[11] and talaroconvolutins.[12] Compound **5**, the 5-(2-methylpropyldiene)-1*H*-pyrrol-2(5*H*)-one derivative, may be produced from **1** or **2** by dehydration.

Owing to the cytotoxic nature of *M. thermophila*, the inhibitory effect of compounds **1**–**5** against cancer cells, HepG2, Hep3B, A-549, and MCF-7 was investigated. According to the results shown in Table 4, compound **5** exhibited significant inhibitory effects against all four cancer cells.

Table 4. Cytotoxicity data (IC_{50}, $\mu g\,mL^{-1}$) of myceliothermophins A–E (**1**–**5**) against cancer cells.

	A549	Hep3B	MCF-7	HepG2
1	1.12	0.91	1.03	1.30
2[a]	>4.00	>4.00	>4.00	>4.00
3	1.05	0.51	0.52	0.62
4[a]	>4.00	>4.00	>4.00	>4.00
5	0.26	0.41	0.25	0.28
paclitaxel	0.0124	>8.00[a]	0.001	0.012

[a] The cell survival ratio is >90% at that concentration.

Furthermore, the other two sets of diastereomers, **1**/**2** and **3**/**4**, revealed an interesting result: compounds **1** and **3**, which have the same relative configuration, showed potent inhibitory effects against four cancer cells but **2** and **4** did not. These results indicate that the configuration of C-21 in the two sets of diastereomers plays a critical role in the biological toxicities toward cancer cells. In addition, the cytotoxic data showed little difference in potency between **1** and **3**. Unlike the 5-(2-methylpropyldiene)-1*H*-pyrrol-2(5*H*)-one

moiety in **5**, the 5-alkyl-5-hydroxyl (or 5-methoxyl)-1*H*-pyrrol-2(5*H*)-one moieties in **1**–**4** have a hydrophobic (isopropyl group) part and a hydrophilic (hydroxyl or methoxyl group) part on both sides of the pyrrolone ring. The correlation between the configuration of C-21 in **1**–**4** and their cytotoxicity implies that the stereoconfiguration of the hydrophobic (isopropyl group) or hydrophilic (hydroxyl or methoxyl group) moieties may influence the interaction of **1**–**4** with target biomolecules in a way that would result in the cytotoxicity of **1**–**4**.

Although the detailed mechanism of the inhibitory action remains unclear, we assume that compounds **1**, **3**, and **5** may react with nucleophiles of the biomolecule to covalently form adducts at C-20 to inhibit the function of the biomolecule. For instance, some natural products, such as lipstatin, lactacystin, and some L-*trans*-(*S*,*S*)-epoxysuccinic acids actively react with target enzymes to form covalent complexes.[16] Some reports of deoxy-tetramic acids[12,15] suggest that an intramolecular OH group or extraction and isolation solvent molecules can attack the β position of an α,β-unsaturated ketone by way of a Michael reaction during the biogenetic route or in the processes of isolation and purification to form artificial products. The mechanism of action of **1**–**5** is currently under investigation.

Experimental Section

General: The optical rotations were measured by using a PerkinElmer 341 digital polarimeter. The UV spectra were obtained by using a Hitachi 200-20 spectrophotometer. IR spectra were recorded by using a Mattson Genesis II spectrophotometer. ^{1}H NMR (400 and 600 MHz, using [D$_6$]acetone as the solvent for measurements), ^{13}C NMR, HMQC, HMBC, and NOESY spectra were obtained on Bruker Avance NMR spectrometers. GC–MS data were obtained by using a Hewlett–Packard HP6890 gas chromatograph connected to an HP5973 mass-selective detector. An HP-5MS fused silica capillary column (30 m × 0.25 mm i.d., Hewlett–Packard) was used. High-resolution ESIMS data were measured on a Bruker Bio-TOF III electrospray ionization mass spectrometer. Silica gel 60 (Merck, 230–400 mesh) was used for column chromatography. A preparative column (Discovery, C18, 5 μm, 25 cm × 10.0 mm) was used for preparative HPLC (Shimadzu LC-10 AT VP with the recycling system). A diode array detector (Shimadzu SPD-M10AVP) was used for HPLC. The TLC spots were detected by spraying the TLC plates with a molybdate solution (0.02 M ammonium cerium sulfate dehydrate/ammonium molybdate tetrahydrate in aqueous 10% (w/v) H_2SO_4) and then heating them on a hot plate.

Fungal material: Thermophilic fungal strain *M. thermophila* was isolated from the soil of fumaroles in the Hsiaoyoukeng area of Yangmingshan National Park, Taipei, Taiwan. The fungal isolate was identified by Professor Chen according to various morphological, biochemical, and physiological characteristics described previously.[17] The fungal strain was cultured on potato dextrose agar (PDA) plates at 45 °C for 14 days. Mass mycelium and mature spores were harvested and washed with distilled water. All samples underwent freeze-drying for further extraction. A voucher specimen (F-15) has been deposited at the Institute of Biological Chemistry, Academia Sinica, Taiwan.

Extraction and isolation: The freeze-dried mycelium and spores of *M. thermophila* were extracted with CH_3OH (500 mL) three times. The methanolic extract was separated by using 20% aqueous CH_3OH and EtOAc. The organic phase was dried and treated with Sephadex LH-20 (eluting with CH_3OH/EtOAc, 1:1) to give five fractions. Fractions 3 and 4 were combined and passed through a silica-gel column (eluting with EtOAc/*n*-hexane, 1:4) and separated into seven subfractions. Subfraction 2 was purified by using RP-HPLC (CH_3CN/H_2O, 90:10) to obtain Myceliothermophin E (**5**) and a mixture of Myceliothermophins C (**3**) and D (**4**). The mixture of **3** and **4** was further separated by using recycling RP-HPLC (CH_3CN/H_2O, 90:10). Myceliothermophins A (**1**) and B (**2**) were isolated from subfraction 2 by using a silica-gel column (eluting with EtOAc/*n*-hexane, 1:4).

Myceliothermophin A (1): White powder; $[\alpha]_D^{20} = -135$ ($c = 0.2$ in $CHCl_3$); ^{1}H NMR (600 MHz, [D$_6$]acetone) and ^{13}C NMR (150 MHz, [D$_6$]acetone): see Tables 1 and 2; IR ($CHCl_3$): $\nu = 3270$, 2950, 2923, 2870, 1698 cm^{-1}; HR-ESIMS: m/z: calcd for $C_{26}H_{40}NO_3$: 414.3008; found: 414.3003 $[M+H]^+$; calcd for $C_{26}H_{39}NO_3Na$: 436.2828; found: 436.2822 $[M+Na]^+$.

Myceliothermophin B (2): White powder; $[\alpha]_D^{20} = -100$ ($c = 0.4$ in $CHCl_3$); ^{1}H NMR (600 MHz, [D$_6$]acetone) and ^{13}C NMR (150 MHz, [D$_6$]acetone): see Tables 1 and 2; IR ($CHCl_3$): $\nu = 3272$, 2952, 2922, 2868, 1698 cm^{-1}; HR-ESIMS: m/z: calcd for $C_{26}H_{40}NO_3$: 414.3008; found: 414.3003 $[M+H]^+$; calcd for $C_{26}H_{39}NO_3Na$: 436.2828; found: 436.2822 $[M+Na]^+$.

Myceliothermophin C (3): White powder; $[\alpha]_D^{20} = -120$ ($c = 0.1$ in $CHCl_3$); ^{1}H NMR (600 MHz, [D$_6$]acetone) and ^{13}C NMR (150 MHz, [D$_6$]acetone): see Tables 1 and 2; IR ($CHCl_3$): $\nu = 3310$, 2953, 2922, 2868, 1703 cm^{-1}; HR-ESIMS: m/z: calcd for $C_{27}H_{42}NO_3$: 428.3165; found: 428.3159 $[M+H]^+$; calcd for $C_{27}H_{41}NO_3Na$: 450.2984; found: 450.2979 $[M+Na]^+$.

Myceliothermophin D (4): White powder; $[\alpha]_D^{20} = -55$ ($c = 0.2$ in $CHCl_3$); ^{1}H NMR (600 MHz, [D$_6$]acetone) and ^{13}C NMR (150 MHz, [D$_6$]acetone): see Tables 1 and 2; IR ($CHCl_3$): $\nu = 3310$, 2950, 2920, 2870, 1705 cm^{-1}; HR-ESIMS: m/z: calcd for $C_{27}H_{42}NO_3$: 428.3165; found: 428.3159 $[M+H]^+$; calcd for $C_{27}H_{41}NO_3Na$: 450.2984; found: 450.2979 $[M+Na]^+$.

Myceliothermophin E (5): White powder; $[\alpha]_D^{20} = -40$ ($c = 0.2$ in $CHCl_3$); ^{1}H NMR (600 MHz, [D$_6$]acetone) and ^{13}C NMR (150 MHz, [D$_6$]acetone): see Tables 1 and 2; IR ($CHCl_3$): $\nu = 3410$, 2960, 2930, 2880, 2400, 1710 cm^{-1} HR-ESIMS: m/z: calcd for $C_{26}H_{38}NO_2$: 396.2903 $[M+H]^+$; found: 396.2897; calcd for $C_{26}H_{37}NO_2Na$: 418.2722; found: 418.2717 $[M+Na]^+$.

Human cell lines: Human hepatoblastoma HepG2 (BCRC 60025), human hepatocellular carcinoma Hep3B (BCRC 60434), human lung carcinoma A-549 (BCRC 60074), and human breast adenocarcinoma MCF-7 (BCRC 60436) were used. HepG2 and Hep3B were cultured in minimum essential medium (MEM, Gibco) supplemented with Earle's salts, nonessential amino acid, and L-glutamine (2 mM), 10% fetal bovine serum (Hyclone), and sodium pyruvate (1 mM, Gibco). A-549 was cultured in Ham's F-12 medium with L-glutamine (1 mM) and 10% fetal bovine serum (Hyclone). MCF-7 was cultured in MEM as described above and further supplemented with 0.01 mg mL^{-1} bovine insulin (Sigma). All of these culture media contain 100 units of penicillin, 100 μg of streptomycin, and 0.25 μg of amphotericin B per milliliter. The cells were incubated at 37 °C in a 5% CO_2 humidified incubator. The optimal plating densities of each cell line were determined from their growth profiles (6.4×10^4 cells mL^{-1} for Hep 3B, Hep G2, and A-549, 3.2×10^4 cells mL^{-1} for MCF-7).

Cytotoxicity assay: Cell suspensions (100 μL per well) were seeded in 96-well microtiter plates (Nunc) and incubated for 24 h for cell attachment. After 24 h, the cells were treated with the pure compounds (**1**–**5**) and paclitaxel (Sigma) was used as a positive control. The final concentrations of the tested compounds ranged between 0.25 and 8 μg mL^{-1} (twofold dilution). The concentration of paclitaxel was between 0.085 ng mL^{-1} and 8.5 μg mL^{-1} (tenfold dilution). Four replicate wells for each concentration were used. Cytotoxicity was evaluated 48 h later by way of a sulforhodamine B assay. Dose versus response curves were plotted and IC_{50} values were determined by sigmoidal fit analysis by using Microcal Origin Software.

Sulforhodamine B assay (SRB): The SRB assay was completed according to previous reports.[18,19] The protocol was divided into two parts: one was cell fixation and the second was an SRB assay. For the fixation procedure, the cultures were fixed with cold 50% trichloroacetic acid (TCA; 100 μL) and incubated at 4 °C for 1 h and then washed five times with de-

ionized water. Plates were dried and then stored at 4 °C before use. For the SRB assay, 100 μL of a solution of 0.4 % SRB in 1 % acetic acid were added to each well and left at room temperature for 30 min. The SRB was removed and the plates were washed five times with 1 % acetic acid. Bound SRB was solubilized by using 100 μL of unbuffered Tris-based (10 mM, pH 10.5) solution for 5 min and the absorbance was read at 570 nm by using an MRX II Absorbance Reader (DYNEX Technologies).

Acknowledgements

The authors would like to thank Dr. Shu-Chuan Jao, of the Institute of Biological Chemistry, Academia Sinica, Taiwan, for NMR technological support. The work was supported in part by the National Science Council of Taiwan.

[1] B. J. L. Royles, *Chem. Rev.* **1995**, *95*, 1981–2001.
[2] E. L. Ghisalberti in *Studies in Natural Product Chemistry, Vol. 28* (Ed.: A.-u. Rahman), Elsevier, Amsterdam, **2003**, pp. 109–163.
[3] G. R. Pettit, Y. Kamano, C. Dufresne, R. L. Cerny, C. L. Herald, J. M. Schmidt, *J. Org. Chem.* **1989**, *54*, 6005–6006.
[4] G. R. Pettit, T. J. Thornton, J. T. Mullaney, M. R. Boyd, D. L. Herald, S. B. Singh, E. J. Flahive, *Tetrahedron* **1994**, *50*, 12097–12108.
[5] a) R. S. Marks, D. L. Graham, J. A. Sloan, S. Hillman, S. Fishkoff, J. E. Krook, S. H. Okuno, J. A. Mailliard, T. R. Fitch, F. Addo, *Am. J. Clin. Oncol.* **2003**, *26*, 336–337; b) S. Ebbinghaus, E. Rubin, E. Hersh, L. D. Cranmer, P. L. Bonate, R. J. Fram, A. Jekunen, S. Weitman, L. A. Hammond, *Clin. Cancer Res.* **2005**, *11*, 7807–7816; c) C. Cunningham, L. J. Appleman, M. Kirvan-Visovatti, D. P. Ryan, E. Regan, S. Vukelja, P. L. Bonate, F. Ruvuna, R. J. Fram, A. Jekunen, S. Weitman, L. A. Hammond, J. P. Eder, Jr., *Clin. Cancer Res.* **2005**, *11*, 7825–7833; d) A. C. Mita, L. A. Hammond, P. L. Bonate, G. Weiss, H. McCreery, S. Syed, M. Garrison, Q. S. Chu, J. S. DeBono, C. B. Jones, S. Weitman, E. K. Rowinsky, *Clin. Cancer Res.* **2006**, *12*, 5207–5215.
[6] S. B. Singh, M. A. Goetz, E. T. Jones, G. F. Bills, R. A. Giacobbe, L. Herranz, S. Stevens-Miles, D. L. Williams, *J. Org. Chem.* **1995**, *60*, 7040–7042.
[7] R. R. West, J. VanNess, A. M. Varming, B. Rassing, S. Biggs, S. Gasper, P. A. McKernan, J. Piggott, *J. Antibiot.* **1996**, *49*, 967–973.
[8] R. Nakai, H. Ogawa, A. Asai, K. Ando, T. Agatsuma, S. Matsumiya, S. Akinaga, Y. Yamashita, T. Mizukami, *J. Antibiot.* **2000**, *53*, 294–296.
[9] T. Agatsuma, T. Akama, S. Nara, S. Matsumiya, R. Nakai, H. Ogawa, S. Otaki, S. Ikeda, Y. Saitoh, Y. Kanda, *Org. Lett.* **2002**, *4*, 4387–4390.
[10] H. Y. He, H. Y. Yang, R. Bigelis, E. H. Solum, M. Greenstein, G. T. Carter, *Tetrahedron Lett.* **2002**, *43*, 1633–1636.
[11] C. Osterhage, R. Kaminsky, G. M. König, A. D. Wright, *J. Org. Chem.* **2000**, *65*, 6412–6417.
[12] S. Suzuki, T. Hosoe, K. Nozawa, K. Kawai, T. Yaguchi, S. Udagawa, *J. Nat. Prod.* **2000**, *63*, 768–772.
[13] E. C. Marfori, T. Bamba, S. Kajiyama, E. Fukusaki, A. Kobayashi, *Tetrahedron* **2002**, *58*, 6655–6658.
[14] Z. S. Song, R. J. Cox, C. M. Lazarus, T. J. Simpson, *Chembiochem* **2004**, *5*, 1196–1203.
[15] J. Yamaguchi, H. Kakeya, T. Uno, M. Shoji, H. Osada, Y. Hayashi, *Angew. Chem.* **2005**, *117*, 3170–3175; *Angew. Chem. Int. Ed.* **2005**, *44*, 3110–3115.
[16] C. Drahl, B. F. Cravatt, E. J. Sorensen, *Angew. Chem.* **2005**, *117*, 5936–5958; *Angew. Chem. Int. Ed.* **2005**, *44*, 5788–5809.
[17] K.-Y. Chen, PhD thesis, National Taiwan University, **1991**.
[18] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney, M. R. Boyd, *J. Natl. Cancer Inst.* **1990**, *82*, 1107–1112.
[19] W. Voigt, *Methods Mol. Med.* **2005**, *110*, 39–48.

Received: January 10, 2007
Published online: May 14, 2007

