Highly Conjugated Ergostane-Type Steroids and Aranotin-Type Diketopiperazines from the Fungus *Aspergillus terreus* BCC 4651

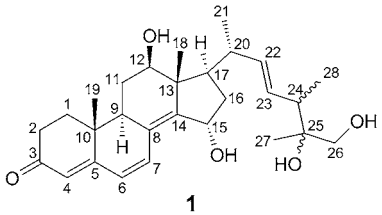
by **Rachada Haritakun**, **Pranee Rachtawee**, **Somjit Komwijit**, **Sutichai Nithithanasilp**, and **Masahiko Isaka***

National Center for Genetic Engineering and Biotechnology (BIOTEC), 113 Thailand Science Park, Phaholyothin Road, Klong Luang, Pathumthani 12120, Thailand (phone: +66-25646700 ext 3554; fax: +66-25646707; e-mail: isaka@biotec.or.th)

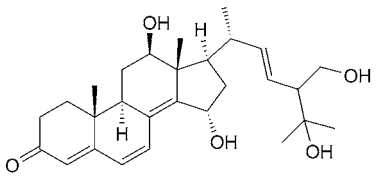
Two new ergostane derivatives, 12β,15α,25,26-tetrahydroxyergosta-4,6,8(14),22-tetraen-3-one (**1**) and 12β,15α,25,28-tetrahydroxyergosta-4,6,8(14),22-tetraen-3-one (**2**), and a new aranotin-type diketopiperazine, bisdethiobis(methylsulfanyl)apoaranotin (**3**), were isolated from the fungus *Aspergillus terreus* BCC 4651. The structures of the new compounds were elucidated by means of NMR spectroscopic and MS analyses.

Introduction. – The filamentous fungus *Aspergillus terreus* is a prolific producer of biologically active secondary metabolites such as terreic acid [1][2], terrain [2], citrinin [3], lovastatin [4], gliotoxin [5], acetylaranotin [6], and butyrolactone I [7] and its derivatives [8][9]. As part of our research program on the utilization of fungal sources in Thailand, we recently investigated the *Aspergillus terreus* strain BCC 4651, which led to the isolation of butyrolactone I and its derivatives, including two new butenolides, butyrolactones VI and VII, together with the common metabolites of *A. terreus*, bisdethiobis(methylsulfanyl)acetylaranotin and terrain [10]. Since this fungal strain proved to be a unique source of secondary metabolites, it has been further chemically explored under different fermentation conditions. Herein, we report the isolation and structure elucidation of two new steroids, **1** and **2**, and a new diketopiperazine, bisdethiobis(methylsulfanyl)apoaranotin (**3**), along with the three known compounds bisdethiobis(methylsulfanyl)acetylapoaranotin (**4**) [11], bisdethiobis(methylsulfanyl)-aranotin (**5**, alternarosin A) [12], and bisdethiobis(methylsulfanyl)acetylaranotin (**6**) [13–15], which were produced by extending the duration of incubation (42 d).

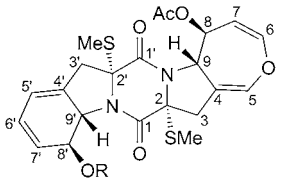
Results and Discussion. – Compound **1** was isolated as a pale yellow solid, and the molecular formula was determined as $C_{28}H_{40}O_5$, from the sodiated *quasi*-molecular ion peak at *m*/*z* 479.2769 (calc. for $C_{28}H_{40}NaO_5^+$, 479.2773) in the HR-ESI-MS. The IR spectrum showed broad and intense absorption bands at $\tilde{\nu}_{max}$ 3372 (broad) and 1640 cm^{-1}. The ^1H- and ^{13}C-NMR, DEPT-135, and HMQC data for **1** revealed the presence of a conjugated ketone (δ(C) 197.4), three olefinic quaternary C-atoms, five olefinic CH groups, an O-bearing quaternary C-atom, two HO–CH groups, a HO–CH_2 group, two sp^3 quaternary C-atoms, four CH groups, four CH_2 groups, and five Me groups. The tetracyclic skeleton with a highly conjugated trienone and the ergostane-type side chain were deduced by analyses of COSY and HMBC data (*Table 1*). The



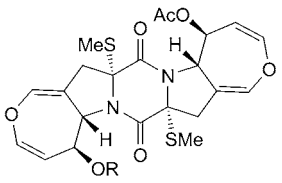
1



2



3 R = H
4 R = Ac



5 R = H
6 R = Ac

location of the C=O group was indicated by the HMBCs from H_β–C(1), H_α–C(2), and H_β–C(2) to C(3) (δ(C) 197.4), while the olefinic H–C(4) (δ(H) 5.65 (*s*)) exhibited correlations to C(2), C(6), and C(10). The (22*E*)-configuration of the side chain was apparent from the vicinal ^{1}H,^{1}H coupling constant of 15.4 Hz. The relative configuration of **1** was assigned on the basis of NOESY correlations (*Fig.*). A pseudoaxial (α) orientation of H–C(9) was indicated by the NOESY correlations of this H-atom with H_α–C(1) and H_α–C(11), and by the absence of a cross-peak with Me(19). The O-bearing CH group H–C(12) exhibited intense NOESY correlations with H–C(10), H_α–C(11), and H–C(17), which demonstrated the coplanar (α-face) relation of these H-atoms. In addition, both H–C(12) and H–C(17) lacked a NOESY correlation to Me(18). On the other hand, O-bearing H–C(15) showed NOESY correlations with Me(18) and H_β–C(16). The NOESY correlations from Me(18) to H–C(20), and from H–C(17) to Me(21) provided the relative configurations at C(13), C(17), and C(20). Therefore, compound **1** was assigned as 12β,15α,25,26-tetrahydroxyergosta-4,6,8(14),22-tetraen-3-one. The configurations of C(24) and C(25) remain unassigned.

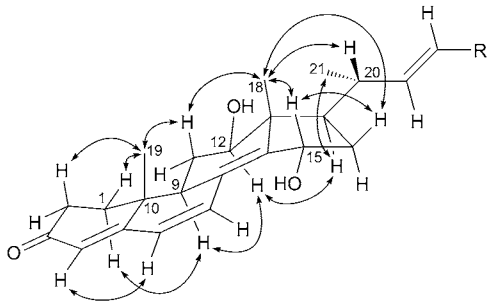


Figure. *Selected NOESY correlations for* **1**

Table 1. *^{1}H- and ^{13}C-NMR Data* (500 and 125 MHz, resp.; in (D_6)acetone) *of Compounds* **1** *and* **2**. δ in ppm, J in Hz.

	1			**2**	
	δ(H)	δ(C)	HMBC	δ(H)	δ(C)
CH_2(1)	1.78–1.82 (m, H_α)	33.9	2, 9, 10, 19	1.79–1.84 (m, H_α)	33.9
	1.99–2.02 (m, H_β)		2, 3, 5, 10	1.99–2.03 (m, H_β)	
CH_2(2)	2.29–2.32 (m, H_α)	33.8	3, 10	2.29–2.33 (m, H_α)	33.8
	2.46–2.52 (m, H_β)		1, 3	2.47–2.53 (m, H_β)	
C(3)		197.4			197.2
H–C(4)	5.65 (s)	123.1	2, 6, 10	5.64 (s)	123.1
C(5)		162.9			162.7
H–C(6)	6.10 (d, J = 9.7)	124.8	4, 5, 8, 10	6.11 (d, J = 9.7)	124.9
H–C(7)	7.26 (d, J = 9.7)	134.4	5, 8, 9, 14	7.27 (d, J = 9.7)	134.3
C(8)		128.4			128.5
H–C(9)	2.40 (dd, J = 10.3, 7.6)	45.5	8, 10, 11, 14, 19	2.40 (dd, J = 10.3, 7.8)	45.6
C(10)		36.7			36.7
CH_2(11)	1.72–1.74 (m, H_α)	28.5	8, 9, 12, 13	1.73–1.77 (m, H_α)	28.5
	1.68–1.71 (m, H_β)		10, 12, 13	1.68–1.71 (m, H_β)	
H–C(12)	3.66–3.70 (m)	75.4		3.66–3.70 (m)	75.4
C(13)		49.3			49.3
C(14)		156.0			155.8
H–C(15)	4.86 (br. t, J = 6.2)	68.9	8, 13, 14, 17	4.87 (br. t, J = 6.4)	68.9
CH_2(16)	1.71–1.73 (m, H_α)	35.5	14, 15	1.70–1.75 (m, H_α)	35.6
	2.04–2.08 (m, H_β)		15, 20	2.04–2.08 (m, H_β)	
H–C(17)	1.88–1.92 (m)	53.5	12, 13, 18	1.88–1.92 (m)	53.4
Me(18)	0.96 (s)	15.5	12, 13, 14, 17	0.96 (s)	15.4
Me(19)	1.05 (s)	16.4	1, 5, 9, 10	1.06 (s)	16.4
H–C(20)	2.98–3.03 (m)	35.8	13, 16, 17, 21, 22, 23	3.02–3.07 (m)	36.0
Me(21)	1.07 (d, J = 7.0)	22.8	17, 20, 22	1.09 (d, J = 7.0)	22.6
H–C(22)	5.44 (dd, J = 15.4, 8.6)	135.1	20, 21, 23, 24	5.56 (dd, J = 15.3, 9.0)	138.2
H–C(23)	5.51 (dd, J = 15.4, 7.8)	131.1	20, 22, 24, 28	5.29 (dd, J = 15.3, 9.5)	127.2
H–C(24)	2.28–2.31 (m)	43.7	22, 23, 25, 26, 27, 28	2.23–2.27 (m)	55.1
C(25)		73.6			72.3
CH_2(26) or Me(26)	3.44 (dd, J = 10.7, 5.2)	68.0	25, 27	1.17 (s)	29.4
	3.34 (dd, J = 10.7, 5.7)		27		
Me(27)	1.06 (s)	21.2	24, 25, 26	1.16 (s)	25.2
Me(28) or CH_2(28)	0.98 (d, J = 7.0)	14.3	23, 24, 25	3.80–3.84 (m)	63.6
				3.63–3.67 (m)	
HO–C(12)	3.91 (d, J = 5.2)		12, 13	3.86 (d, J = 5.3)	
HO–C(15)	3.94 (d, J = 6.5)		14, 15	3.88 (d, J = 6.7)	
HO–C(25)	3.13 (s)		24, 25, 26, 27	4.08 (s)	
HO–C(26)	3.59 (br. t, J = 5.5)				
HO–C(28)				3.95 (br. t, J = 4.6)	

The molecular formula of compound **2** was the same as **1**, $C_{28}H_{40}O_5$ (HR-ESI-MS). The NMR data showed very close resemblance to those of **1** for the tetracyclic ring moiety, but were different for the C(20)–C(28) side chain. The structure of the side chain was addressed on the basis of COSY and HMBC data. The connections C(21)–C(20)–C(22)=C(23)–C(24)–C(28) were accomplished by COSY correlations. Two Me signals at δ(H) 1.17 (Me(26)) and 1.16 (Me(27)) showed HMBCs HO–*C*H

signals at δ(C) 72.3 (C(25)) and to C(24), and also correlated to each other. Therefore, compound **2** was elucidated as 12β,15α,25,28-tetrahydroxyergosta-4,6,8(14),22-tetraen-3-one.

Compounds **1** and **2** are highly OH-substituted analogs of ergosta-4,6,8(14),22-tetraen-3-one, which was previously isolated from several fungi and plants. Derivatives of ergosta-4,6,8(14),22-tetraen-3-one with less OH groups were also reported as fungal secondary metabolites: ganodermanosides A and B (15α- and 15β-hydroxyergosta-4,6,8(14),22-tetraen-3-ones) from *Ganoderma lucidum* [16], 25-hydroxyergosta-4,6,8(14),22-tetraen-3-one from *Zopfiella longicaudata* [17], gymnasterone D (ergosta-4,6,8(14),22-tetraene-3,15-dione) from *Gymnascella dankaliensis* OUPS-N134 [18], and d1067331 (25,28-dihydroxyergosta-4,6,8(14),22-tetraen-3-one; undefined configuration) from *Aspergillus terreus* SANK22295 [19].

Compound **3** was obtained as a yellow powder, and the molecular formula was determined by HR-ESI-MS as $C_{22}H_{24}N_2O_6S_2$. The ^{1}H- and ^{13}C-NMR data (*Table 2*) were similar to those of the known co-metabolite **4** [11]. The significant differences were the absence of one of the two AcO groups and the upfield shift of H–C(8′) signal (δ(H) 4.75) when compared to **4** (H–C(8′), δ(H) 6.09 (*d*, *J* = 13.9)). In addition, a OH group assignable to HO–C(8′) exhibited HMBCs to C(7′), C(8′), and C(9′). The large coupling constant for H–C(8′)/H–C(9′) (*J* = 13.4) was consistent with an antiperiplanar relation of these H-atoms, similar to **4**. The similarity of the NMR spectroscopic data with those of the known compounds **4**–**6** and their co-occurrence suggested that **3** should possess the same relative and absolute configuration. Therefore, compound **3** was assigned as bisdethiobis(methylsulfanyl)apoaranotin.

Table 2. *^{1}H- and ^{13}C-NMR Data* (500 and 125 MHz, resp.; in (D_6)acetone) *of Compound* **3**. δ in ppm, *J* in Hz.

	δ(H)	δ(C)		δ(H)	δ(C)
C(1)		167.9	C(1′)		164.0
C(2)		70.2	C(2′)		73.7
CH_2(3)	3.18 (br. *s*)	39.0	CH_2(3′)	3.05 (br. *s*)	37.9
C(4)		111.0	C(4′)		133.4
H–C(5)	6.76 (*q*, *J* = 2.1)	137.5	H–C(5′)	5.97–5.99 (*m*)	119.1
H–C(6)	6.39 (*dd*, *J* = 8.3, 2.3)	139.6	H–C(6′)	5.87–5.91 (*m*)	123.0
H–C(7)	4.70 (*dd*, *J* = 8.3, 1.8)	106.0	H–C(7′)	5.64 (br. *d*, *J* = 9.7)	130.5
H–C(8)	5.73 (*dt*, *J* = 8.1, 2.1)	71.6	H–C(8′)	4.75 (br. *d*, *J* = 13.4)	74.3
H–C(9)	5.05–5.07 (*m*)	60.1	H–C(9′)	4.85 (br. *d*, *J* = 13.4)	68.9
*Me*S–C(2)	2.27 (*s*)	13.9	*Me*S–C(2′)	2.23 (*s*)	13.8
*Ac*O–C(8)	2.00 (*s*)	20.2	HO–C(8′)	5.35 (*s*)	
		169.2			

As a part of the search for drug leads from fungal metabolites, the new compounds **1**–**3** were subjected to our biological assay protocols to investigate antitubercular (*Mycobacterium tuberculosis* H37Ra) and antimalarial (*Plasmodium falciparum* K1) activities, and cytotoxicities against three cancer cell lines (KB, MCF-7, and NCI-H187). Compound **3** exhibited weak antimycobacterial activity with a *MIC* of 25 μg/

ml, while it was inactive against the malarial parasite and cancer cells at 10 μg/ml and 50 μg/ml, respectively. Steroids **1** and **2** were inactive in these assays.

Financial support from the *Bioresources Research Network, National Center for Genetic Engineering and Biotechnology* (BIOTEC), is gratefully acknowledged.

Experimental Part

General. M.p.: *Electrothermal IA9100* digital melting-point apparatus. Optical rotations: *JASCO P-1030* digital polarimeter. UV Spectra: *analytikjena SPEKOL 1200* spectrophotometer. IR Spectra: *Bruker ALPHA* spectrometer. NMR Spectra: *Bruker AV500D* spectrometer; at 500 and 125 MHz for ^{1}H and ^{13}C, rsp.; in (D_6)acetone. HR-ESI-MS: *Bruker micrOTOF* mass spectrometer.

Fermentation, Extraction, and Isolation. The profile of *Aspergillus terreus* BCC 4651 was previously reported [10]. This fungus was maintained on potato dextrose agar at 25°. The agar was cut into small plugs and inoculated into 4 × 250-ml *Erlenmeyer* flasks containing 25 ml of potato dextrose broth (PDB; potato starch 4.0 g/l, dextrose 20.0 g/l). After incubation at 25° for 6 d on a rotary shaker (200 rpm), each primary culture was transferred into a 1000-ml *Erlenmeyer* flask containing 250 ml of the same liquid medium (PDB), and incubated at 25° for 6 d on a rotary shaker (200 rpm). These secondary cultures were transferred into 40 × 1000-ml *Erlenmeyer* flasks containing 250 ml of *Czapek–Dox* broth (sucrose 30.0 g/l, $NaNO_3$ 3.0 g/l, K_2HPO_4 1.0 g/l, $MgSO_4 \cdot 7\ H_2O$ 0.5 g/l, KCl 0.5 g/l, $FeSO_4 \cdot 7\ H_2O$ 0.1 g/l), and the final fermentation was carried out at 25° for 42 d under static conditions. The cultures were filtered to separate broth (filtrate) and mycelia (residual cakes). The filtrate was extracted with AcOEt (3 × 15 l) to give a brown gum (1.84 g). This extract was fractionated by column chromatography (CC) on *Sephadex LH-20* (60 × 4.0 cm i.d., MeOH) to obtain eight pooled fractions. *Fr. 2* (139 mg) was subjected to CC on SiO_2 (MeOH/$CHCl_3$, step gradient elution from 5 : 95 to 100 : 0) followed by prep. HPLC using a reverse phase column (*Dionex SunFire*, 10 μm, 150 mm × 19 mm i.d.; MeCN/H_2O, gradient from 20 : 80 to 100 : 0 over 30 min, flow rate 10 ml/min) to furnish **1** (4.5 mg) and **2** (8.5 mg). *Fr. 3* (922 mg) was fractionated by CC on SiO_2 and prep. HPLC (MeCN/H_2O) to yield **3** (4.7 mg), **4** (33.7 mg), **5** (22.6 mg), and **6** (64.9 mg). Butyrolactone I (23.2 mg) was isolated from *Fr. 5* (201 mg).

*(12β,15α,22*E*)-12,15,25,26-Tetrahydroxyergosta-4,6,8(14),22-tetraen-3-one* (**1**). Pale yellow solid. M.p. 118–119°. $[\alpha]_D^{25} = +519$ ($c = 0.067$, MeOH). UV (MeOH): 338 (4.56). IR (ATR): 3372, 2964, 1640, 1583, 1196, 1019, 876. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS: 479.2769 ($[M+Na]^+$, $C_{28}H_{40}NaO_5^+$; calc. 479.2773).

*(12β,15α,22*E*)-12,15,25,28-Tetrahydroxyergosta-4,6,8(14),22-tetraen-3-one* (**2**). Pale yellow solid. M.p. 124–125°. $[\alpha]_D^{25} = +556$ ($c = 0.097$, MeOH). UV (MeOH): 339 (4.71). IR (ATR): 3350, 2962, 1735, 1644, 1586, 1374, 1228, 1022, 872. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS: 479.2773 ($[M+Na]^+$, $C_{28}H_{40}NaO_5^+$; calc. 479.2773).

Bisdethiobis(methylsulfanyl)apoaranotin (=*(5*S*,5a*S*,7a*R*,12*S*,12a*S*,14a*R*)-5,5a,7a,8,12,12a,14a,15-Octahydro-12-hydroxy-7a,14a-bis(methylsulfanyl)-7,14-dioxo-7 H,14 H-oxepino[3'',4'':4',5']pyrrolo-[1',2':4,5]pyrazino[1,2-*a*]indol-5-yl Acetate*; **3**). Yellow solid. M.p. 193–194°. $[\alpha]_D^{24} = -152$ ($c = 0.05$, MeOH). UV (MeOH): 224 (4.04), 261 sh (3.85), 341 sh (3.36). IR (ATR): 3336, 1734, 1665, 1638, 1382, 1233, 1026. ^{1}H- and ^{13}C-NMR: see *Table 2*. HR-ESI-MS: 499.0969 ($[M+Na]^+$, $C_{22}H_{24}N_2NaO_6S_2^+$; calc. 499.0973).

Biological Assays. Growth inhibitory activity against *Mycobacterium tuberculosis* H37Ra (African green monkey kidney fibroblasts) was performed in triplicate using the green fluorescent protein microplate assay (GFPMA) [20]. The standard anti-TB drug, isoniazid, showed *MIC* values of 0.0234–0.0468 μg/ml. The assay to test the activity against *Plasmodium falciparum* (K1, multi-drug resistant strain) was performed using the microculture radioisotope technique [21]. Cytotoxic activities against human cancer cell lines (KB, MCF-7, and NCI-H187) were evaluated using the resazurin microplate assay [22].

REFERENCES

[1] J. C. Sheehan, W. B. Lawson, R. J. Gaul, *J. Am. Chem. Soc*. **1958**, *80*, 5536.
[2] R. J. Cole, R. H. Cox, in ' Handbook of Toxic Fungal Metabolites', Academic Press, New York, 1981, Chapt. 18, pp. 753 – 822.
[3] W. Sankawa, Y. Ebizuka, H. Noguchi, Y. Ishikawa, S. Kitaghawa, Y. Yamamoto, T. Kobayashi, Y. Iitak, H. Seto, *Tetrahedron* **1983**, *39*, 3583.
[4] R. L. Monaghan, A. W. Alberts, C. H. Hoffman, G. Albers-Schonberg, U.S. Patent, 4,231,938, 1980.
[5] R. E. Lewis, N. P. Wiederhold, M. S. Lionakis, R. A. Prince, D. P. Kontoyiannis, *J. Clin. Microbiol*. **2005**, *43*, 6120.
[6] P. A. Miller, P. W. Trown, W. Fulmor, G. O. Morton, J. Karliner, *Biochem. Biophys. Res. Commun*. **1968**, *33*, 219.
[7] N. Kiriyama, K. Nitta, Y. Sakaguchi, Y. Taguchi, Y. Yamamoto, *Chem. Pharm. Bull*. **1977**, *25*, 2593.
[8] K. Nitta, N. Fujita, T. Yoshimura, K. Arai, Y. Yamamoto, *Chem. Pharm. Bull*. **1983**, *31*, 1528.
[9] K. V. Rao, A. K. Sadhukhan, M. Veerender, V. Ravikumar, E. V. S. Mohan, S. D. Dhanvantri, M. Sitaramkumar, J. M. Babu, K. Vyas, G. O. Reddy, *Chem. Pharm. Bull*. **2000**, *48*, 559.
[10] R. Haritakun, P. Rachtawee, R. Chanthaket, N. Boonyuen, M. Isaka, *Chem. Pharm. Bull*. **2010**, *58*, 1545.
[11] N. Neuss, R. Nagarajan, B. B. Molloy, L. L. Huckstep, *Tetrahedron Lett.* **1968**, *9*, 4467.
[12] W. Wang, Y. Wang, H. Tao, X. Peng, P. Liu, W. Zhu, *J. Nat. Prod*. **2009**, *72*, 1695.
[13] R. Nagarajan, L. L. Huckstep, D. H. Lively, D. C. DeLong, M. M. Marsh, N. Neuss, *J. Am. Chem. Soc*. **1968**, *90*, 2980.
[14] J. W. Moncrief, *J. Am. Chem. Soc*. **1968**, *90*, 6517.
[15] S. Kamata, H. Sakai, A. Hirota, *Agric. Biol. Chem*. **1983**, *47*, 2637.
[16] Y. Weng, L. Xiang, A. Matsuura, Y. Zhang, Q. Huang, J. Qi, *Bioorg. Med. Chem*. **2010**, *18*, 999.
[17] H. Fujimoto, E. Nakamura, E. Okuyama, M. Ishibashi, *Chem. Pharm. Bull*. **2004**, *52*, 1005.
[18] T. Amagata, M. Tanaka, T. Yamada, M. Doi, K. Minoura, H. Ohishi, T. Yamori, A. Numata, *J. Nat. Prod*. **2007**, *70*, 1731.
[19] M. Sugano, Y. Ogura, E. Hatano, T. Hamada, T. Hosoya, Japanese Patent, JP9202797, 1997.
[20] C. Changsen, S. G. Franzblau, P. Palittapongarnpim, *Antimicrob. Agents Chemother*. **2003**, *47*, 3682.
[21] R. E. Desjardins, C. J. Canfield, J. D. Haynes, J. D. Chulay, *Antimicrob. Agents Chemother.* **1979**, *16*, 710.
[22] J. O'Brien, I. Wilson, T. Orton, F. Pognan, *Eur. J. Biochem*. **2000**, *267*, 5421.

Received August 22, 2011