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Hirsutellone F, a Dimer of Antitubercular Alkaloids from the Seed Fungus *Trichoderma* Species BCC 7579

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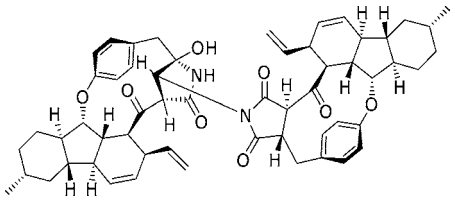
ABSTRACT



Hirsutellone F (7), a novel alkaloid dimer, was isolated together with known monomers, hirsutellones A (1), B (2), and C (3), from the seed fungus *Trichoderma* sp. BCC 7579. The structure of 7 was elucidated by spectroscopic analysis. Studies on biomimetic chemistry, using the dimer 7, suggested that compound 8 (17,1′-dehydrohirsutellone B) should be the precursor for all hirsutellones.

As part of an ongoing research program to search for biologically active substances from local fungi in Thailand,[1] we recently reported the isolation and structure elucidation of novel antitubercular alkaloids, hirsutellones A−E (e.g., **1**−**3**), from an insect pathogenic fungus *Hirsutella nivea* BCC 2594.[2] The unique chemical skeleton is shared only with GKK1032A$_2$ (**4**), GKK1032B (**5**),[3,4] and pyrrocidines (e.g., pyrrocidine A, **6**).[5] In a further search for antitubercular fungal metabolites, we came across another hirsutellone-producing seed fungus, *Trichoderma* sp. BCC 7579.[6] Although *H. nivea* BCC 2594 predominantly produced hirsutellone B (**2**), the mycelia of cultured *Trichoderma* sp. BCC 7579 contained hirsutellone A (**1**) as the most abundant metabolite along with hirsutellone B (**2**) and hirsutellone C (**3**). When applied to incubation of BCC 7579 using a bioreactor, hirsutellones were produced more efficiently than with incubation in Erlenmeyer flasks, wherein a novel dimer, hirsutellone F (**7**), was also present. We report herein the structural elucidation of **7** by combination of spectroscopic analyses and chemical means; the latter study provided unique observations related to final-stage biosynthesis of hirsutellones.

The fungus *Trichoderma* sp. BCC 7594 was fermented in potato dextrose broth medium (PDB, 4 L) using a stirred-tank bioreactor. Methanolic extract from mycelia was subjected to chromatographic fractionation using Sephadex LH20, silica gel, and preparative HPLC (ODS column) to furnish hirsutellones A (**1**; 1890 mg), B (**2**; 575 mg), C (**3**; 169 mg), and F (**7**; 642 mg). The molecular formula of hirsutellone F (**7**)[7] was determined by HRMS (ESI-TOF) as $C_{56}H_{62}N_2O_8$; therefore, it was a dimer of hirsutellones. The

(1) (a) Isaka, M.; Kittakoop, P.; Kirtikara, K.; Hywel-Jones, N. L.; Thebtaranonth, Y. *Acc. Chem. Res.* **2005**, *38*, 813−823. (b) Isaka, M.; Palasarn, S.; Rachtawee, P.; Vimuttipong, S.; Kongsaeree, P. *Org. Lett.* **2005**, *7*, 2257−2260.

(2) Isaka, M.; Rugseree, N.; Maithip, P.; Kongsaeree, P.; Prabpai, S.; Thebtaranonth, Y. *Tetrahedron* **2005**, *61*, 5577−5583.

(3) Koizumi, F.; Hasegawa, A.; Ando, K.; Ogawa, T.; Hara, M. *Jpn. Kokai Tokkyo Koho* **2001**, JP 2001247574.

(4) Hasegawa, A.; Koizumi, F.; Takahashi, Y.; Ando, K.; Ogawa, T.; Hara, M.; Yoshida, M. *43rd Symposium on the Chemistry of Natural Products, Symposium Papers*; Osaka, 2001; pp 467−472.

(5) He, H.; Yang, H. Y.; Bigelis, R.; Solum, E. H.; Greenstein, M.; Carter, G. Y. *Tetrahedron Lett.* **2002**, *43*, 1633−1636.

(6) The *Trichoderma* sp. was isolated from a decaying pod of *Entada perseatha* (Leguminosae) collected in Khao Yai National Park, Central Thailand, by Dr. Sayanh Somrithipol, and it is deposited in the BIOTEC Culture Collection (BCC) as BCC 7579.

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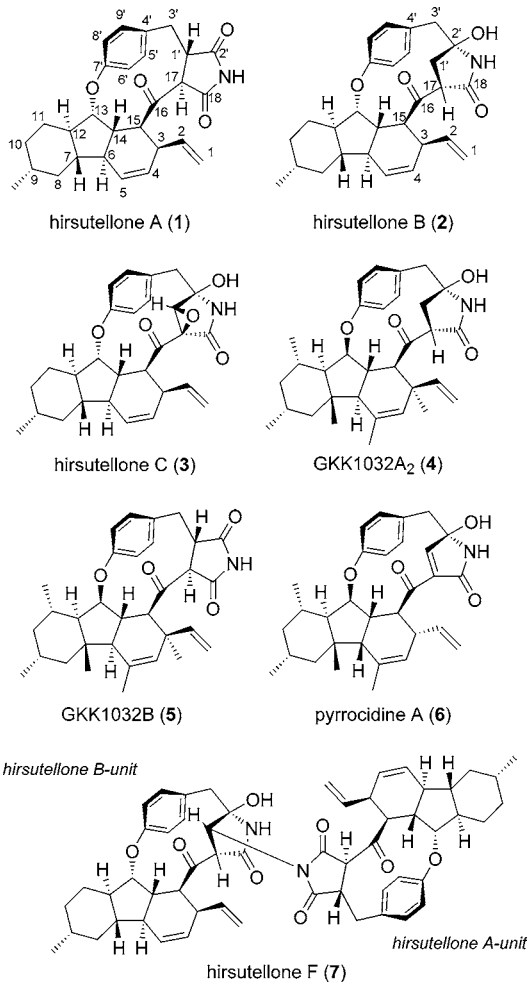


^{1}H and ^{13}C NMR spectra indicated that hirsutellone F (**7**) existed as a mixture of two rotamers. The ratio was estimated to be 2.5:1 in $CDCl_3$, 1.5:1 in acetone-d_6, and 1.3:1 in DMSO-d_6. For clarity, NMR analyses for the major rotamer in $CDCl_3$ are described below. The NMR data (^{1}H, ^{13}C, DEPTs, COSY, NOESY, HMQC, HMBC) for one-half of the molecule were closely related to those of **1** (hirsutellone A-unit). The other half was similar to **2** (hirsutellone B-unit) except for the replacement of C-1′ methylene in **2** with a methine in **7**, situated at δ_H 3.54 (d, J = 6.1 Hz, H-1′), δ_C 58.8 (d, C-1′).

The stereochemistry of the macrocyclic ring moiety of the hirsutellone B-unit was addressed on the basis of NOESY correlations as shown in Figure 1. HMBC correlation from H-1′ of the hirsutellone B-unit to succinimide carbonyls (C-18, δ_C 169.5, and C-2′, δ_C 179.2) of the hirsutellone A-unit indicated the linkage of the two units. The configuration of C-1′ (hirsutellone B-unit) was deduced from intense NOESY cross signals from H-1′ (δ_H 4.91, d, J = 4.6 Hz) to H-9′ (δ_H 7.31, dd, J = 8.3, 1.7 Hz) and one of the H-3′ methylene protons (δ_H 2.93, d, J = 13.0 Hz). Similar HMBC and NOESY correlations were observed for the minor rotamer. The conformation due to the rotation of the C-1′ nitrogen bond was not addressed.

Biosynthesis of GKK1032A$_2$ (**4**) has recently been studied by administration of isotopically labeled (^{13}C and ^{2}H) precursors to *Penicillium* sp. GKK1032.[8] The backbone of

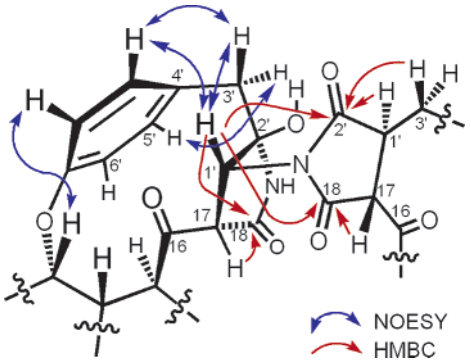


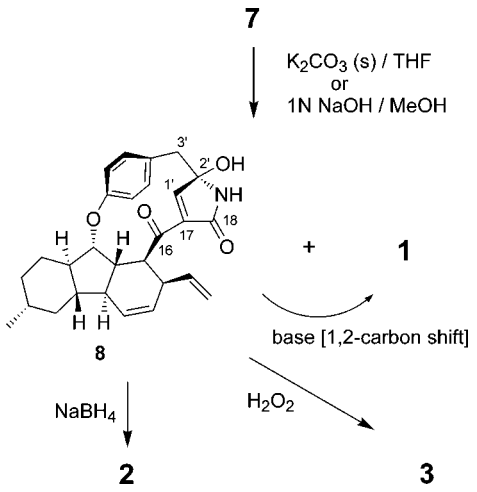
Figure 1. Local structure and selected NOESY and HMBC correlations for hirsutellone F (**7**).

4 and **5** is constructed from L-tyrosine and a nonaketide chain flanked by five methyl groups (attached to C-3, -5, -7, -9, and -11), probably by a polyketide synthase and a nonribosomal peptide synthase hybrid. The proposed biosynthetic pathway could be applied to hirsutellone production by BCC 7579 and BCC 2594. Final transformations may also be similar to those speculatively proposed for **4** and **5**.[8] Thus, hirsutellones A (**1**) and B (**2**) will be produced, respectively, by a 1,2-shift of C-3′ from C-2′ to C-1′ and hydrogenation of the intermediate **8** (Scheme 1). Formation of the epoxide (**3**) and the dimer (**7**), which are known only for the hirsutellone series, can be accounted for by epoxidation of

(7) Hirsutellone F (**7**): colorless solid; mp 235−237 °C; $[\alpha]^{25}_D$ +169° (c 0.25, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 203 (4.60), 228 sh (4.03), 285 sh (3.41) nm; IR (KBr) ν_{max} 3357, 2920, 1775, 1716, 1702, 1682, 1507, 1367, 1243, 1199, 909, 734 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ hirsutellone A-unit, 7.16 (1H, m, H-9′), 7.04 (1H, m, H-8′), 7.03 (1H, m, H-6′), 6.99 (1H, m, H-5′), 5.96 (1H, br d, J = 10.1 Hz, H-5), 5.57 (1H, ddd, J = 9.7, 5.3, 2.2 Hz, H-4), 5.22 (1H, m, H-2), 5.03 (1H, br d, J = 10.5 Hz, Ha-1), 4.93 (1H, br d, J = 16.0 Hz, Hb-1), 4.47 (1H, t, J = 3.2 Hz, H-13), 4.30 (1H, m, H-3), 3.64 (1H, dd, J = 12.5, 5.4 Hz, Ha-3′), 3.60 (1H, d, J = 6.1 Hz, H-17), 3.22 (1H, m, H-15), 3.21 (1H, m, H-1′), 2.39 (1H, t, J = 12.3 Hz, Hb-3′), 2.25 (1H, m, H-6), 2.13 (1H, m, Ha-11), 1.99 (1H, m, Ha-8), 1.85 (1H, m, Ha-10), 1.70 (1H, m, H-12), 1.51 (1H, m, H-14), 1.43 (1H, m, H-9), 1.36 (1H, m, Hb-11), 1.13 (1H, m, Hb-10), 0.98 (3H, d, J = 6.6 Hz, 9-CH_3), 0.96 (1H, m, Hb-8), 0.77 (1H, m, H-7); hirsutellone B-unit, 7.32 (1H, br s, NH), 7.31 (1H, m, H-9′), 7.08 (1H, m, H-5′), 7.07 (1H, m, H-8′), 6.92 (1H, dd, J = 8.4, 2.3 Hz, H-6′), 5.79 (1H, br d, J = 9.9 Hz, H-5), 5.32 (1H, m, H-4), 5.14 (1H, m, H-2), 4.91 (1H, d, J = 4.6 Hz, H-1′), 4.88 (1H, m, Ha-1), 4.84 (1H, m, H-13), 4.83 (1H, m, Hb-1), 3.54 (1H, d, J = 6.1 Hz, H-17), 3.51 (1H, m, H-3), 3.44 (1H, m, H-15), 3.14 (1H, d, J = 12.7 Hz, Ha-3′), 2.93 (1H, d, J = 13.0 Hz, Hb-3′), 2.14 (1H, m, Ha-11), 2.08 (1H, br t, J = 11.2 Hz, H-6), 1.96 (1H, m, Ha-8), 1.85 (1H, m, Ha-10), 1.62 (1H, m, H-12), 1.47 (1H, m, H-14), 1.42 (1H, m, H-9), 1.40 (1H, m, Hb-11), 1.13 (1H, m, Hb-10), 0.95 (3H, d, J = 6.8 Hz, 9-CH_3), 0.92 (1H, m, Hb-8), 0.83 (1H, m, H-7); ^{13}C NMR ($CDCl_3$, 125 MHz) δ hirsutellone A-unit, 198.4 (s, C-16), 179.2 (s, C-2′), 169.5 (s, C-18), 158.8 (s, C-7′), 136.7 (d, C-2), 132.5 (s, C-4′), 130.8 (d, C-9′), 130.4 (d, C-5′), 127.7 (d, C-5), 127.6 (d, C-4), 123.7 (d, C-8′), 123.3 (d, C-6′), 119.1 (t, C-1), 87.1 (d, C-13), 58.9 (d, C-17), 56.0 (d, C-12), 54.4 (d, C-15), 50.5 (d, C-7), 48.0 (d, C-14), 47.0 (d, C-1′), 43.1 (d, C-6), 38.5 (d, C-3), 38.0 (t, C-8), 36.6 (t, C-10), 36.0 (t, C-3′), 33.2 (d, C-9), 29.5 (t, C-11), 22.6 (q, 9-CH_3); hirsutellone B-unit, 198.4 (s, C-16), 171.0 (s, C-18), 158.5 (s, C-7′), 137.4 (d, C-2), 132.2 (d, C-9′), 132.1 (d, C-5′), 128.4 (d, C-4), 127.5 (d, C-5), 126.5 (s, C-4′), 121.8 (d, C-6′), 121.5 (d, C-8′), 116.6 (t, C-1), 87.3 (s, C-2′), 84.4 (d, C-13), 56.8 (d, C-17), 55.6 (d, C-12), 52.7 (d, C-1′), 49.8 (d, C-7), 48.7 (d, C-15), 47.5 (d, C-14), 46.6 (t, C-3′), 43.9 (d, C-3), 42.4 (d, C-6), 38.0 (t, C-8), 36.5 (t, C-10), 33.1 (d, C-9), 29.5 (t, C-11), 22.6 (q, 9-CH_3); HRMS (ESI-TOF) m/z 913.4408 (calcd for $C_{56}H_{62}N_2O_8Na$, 913.4404) [M + Na]$^+$.

(8) Oikawa, H. *J. Org. Chem.* **2003**, *68*, 3552−3557.

Scheme 1. Degradation of Hirsutellone F (**7**) with Base and Biomimetic in Situ Transformations to Hirsutellones A (**1**), B (**2**), and C (**3**)



8 and by heteroconjugate addition of **1** to **8**, respectively. On the basis of common reactivity of succinimides, intermolecular dimerization during the isolation process (extraction, chromatography) is unlikely. All hirsutellones (**1**, **2**, **3**, and **7**) were isolated from the mycelia extract of BCC 7579, but they very poorly present in the liquid medium (culture filtrate). Therefore, it will be more reasonable to assume that "intracellular" dimer formation occurred under the artificial and optimal fermentation conditions.

To confirm the proposed structure and stereochemistry of hirsutellone F (**7**), its degradation in base was undertaken. When compound **7** (20.0 mg) was treated with K_2CO_3(s) in THF, slow transformation (rt, 48 h) to hirsutellone A (**1**) occurred. The crude reaction product (19.6 mg) was exclusively composed of **1**, which was further purified by silica gel column chromatography to obtain 18.7 mg of **1**. This result indicated that the dimer **7** degraded (β-elimination) to afford **1** and the intermediate, **8**, and the latter subsequently transformed to **1** via a 1,2-carbon shift under the reaction conditions. The hypothetical intermediate, **8**, was not detected when the reaction was terminated before completion of conversion. The same reaction occurred when **7** was treated with 1 N NaOH in MeOH/THF (rt, 20 min), but the degradation did not occur in Et_3N/THF or pyridine (rt, 3−20 h). These results strongly supported the proposed stereochemistry of both hirsutellone units in **7**. Compound **7** was stable under acidic conditions, for example, in 1 N HCl/THF, rt, 2 h or in cat. *p*-TsOH/$CDCl_3$, rt, 24 h.

Following these unique observations, we then examined the in situ conversion of **8** to other final natural products, **2** and **3** (Scheme 1). Treatment of **7** (20.0 mg) with $NaBH_4$/1 N NaOH in THF/MeOH (rt, 20 min) gave hirsutellones A (**1**; 12.3 mg) and B (**2**; 6.6 mg). The uneven product composition (**1**, >10 mg) indicated competition between the 1,2-carbon shift (**8** to **1**) and hydride reduction (**8** to **2**). Likewise, reaction of **7** (20.0 mg) with 30% H_2O_2/2 N NaOH (1:1, v/v) in MeOH (rt, 20 min) afforded hirsutellones A (**1**; 5.9 mg) and C (**3**; 8.2 mg) as predominant products. Compounds **1**−**3**, produced in these reactions, possessed the same stereochemistries as those of the natural products.

On the basis of these experiments on biomimetic chemistry, it was strongly suggested that compound **8** is the precursor for all final natural products, **1**−**3** and **7**. It is interesting to note that although pyrrocidine A (**6**) was isolated as a natural product,[5] corresponding compounds with an unsaturated bond (e.g., **8**) have not been found in the hirsutellone and GKK1032 series. In contrast, the succinimide derivative has not been reported in pyrrocidine series. Presumably, the stereochemistry of the tricyclic ring system is closely related to the ring strain of the macrocycle and, hence, the stability/reactivity of the enone intermediate.

Hirsutellone F (**7**) displayed antitubercular activity against *Mycobacterium tuberculosis* H_{37}Ra with a MIC value of 3.12 μg/mL, which was weaker compared to those for hirsutellones A and B (both, MIC 0.78 μg/mL).[9] Instead, compound **7** was found to exhibit moderate activity against the malarial parasite *Plasmodium falciparum* K1 with an IC_{50} value of 4.2 μg/mL (hirsutellones A and B; IC_{50} >20 μg/mL).[10]

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

Supporting Information Available: Experimental procedures and spectral data of **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Growth inhibitory activity against *Mycobacterium tuberculosis* H_{37}Ra was tested employing the Microplate Alamar Blue Assay (MABA): Collins, L.; Franzblau, S. G. *Antimicrob. Agents Chemother*. **1997**, *41*, 1004−1009.

(10) The assay for activity against *Plasmodium falciparum* K1 was performed using the microculture radioisotope technique as described by Desjardins et al.: Desjardins, R. E.; Canfield, C. J.; Chulay, J. D. *Antimicrob. Agents Chemother*. **1979**, *16*, 710−718.