Journal of Natural Products
Vol. 58, No. 1, pp. 93-99, January 1995

TEREZINES A–D: NEW AMINO ACID-DERIVED BIOACTIVE METABOLITES FROM THE COPROPHILOUS FUNGUS *SPORORMIELLA TERETISPORA*

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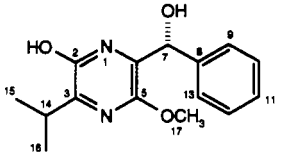
ABSTRACT.—Four new antifungal compounds, terezines A–D [**1–4**], have been isolated from liquid cultures of the coprophilous fungus *Sporormiella teretispora* by preparative tlc and hplc. The structures of these compounds were determined on the basis of nmr and ms data. The known fungal metabolite hyalopyrone [**6**] was also isolated from *S. teretispora*.

Interspecies antagonism within fungal communities has often been observed and reported (1,2). Chemical investigation of secondary metabolites responsible for antagonistic effects provides an approach to the discovery of new natural products with antifungal activity. We have previously reported the isolation of a number of bioactive compounds from antagonistic coprophilous fungi (3–8). During our continuing search for new antifungal compounds from these sources, organic extracts from liquid cultures of *Sporormiella teretispora* Ahmed and Cain (JS 148) were found to have antifungal and antibacterial activities. Studies of these extracts afforded four new compounds with derivatized pyrazine, dihydropyrazinone, and diketopiperazine structures. We wish to report here details of the isolation, structure determination, and bioactivity of these new compounds, which we have named terezines A–D [**1–4**].

RESULTS AND DISCUSSION

S. teretispora was fermented in liquid shake culture using a soy flour medium. Filtration and EtOAc extraction of the culture filtrate afforded a crude extract with antifungal and antibacterial activity. Fractionation of the extract by prep. tlc and further purification by reversed-phase hplc afforded terezines A–C [**1–3**].

The hreims of the major component, terezine A [**1**], suggested the formula $C_{15}H_{18}N_2O_3$. DEPT, ^{13}C- (Table 1), and ^1H-nmr (Table 2) data confirmed the presence of fifteen carbons, and revealed the presence of phenyl, MeO, oxygenated methine, and allylic isopropyl groups, as well as two exchangeable protons. The four carbon signals not directly accounted for by these units were all downfield-shifted singlets located at 154.1, 150.4, 148.2, and 135.7 ppm. Several selective INEPT nmr experiments were conducted to provide long-range CH correlations useful in determining the structure of **1**. Irradiation of the oxygenated methine singlet at 5.88 ppm (H-7) established its position adjacent to the phenyl group (127.8 and 143.7 ppm) and its proximity to two other sp^2 carbons (C-5 and C-6; 150.4 and 135.7 ppm). The MeO group (3.84, 54.1 ppm) was found to be linked to one of these sp^2 carbons (150.4 ppm). Irradiation of the isopropyl methine resonance at 3.27 ppm resulted in polarization transfer to C-2 and C-3 (154.1 and 148.2 ppm), as well as the isopropyl Me groups (21.0 ppm). Both isopropyl Me groups showed correlations with C-3 (148.2 ppm), indicating direct connection of the isopropyl group to C-3. These data accounted for all of the carbons and non-exchangeable hydrogens in the molecule. Treatment of **1** with Ac_2O/pyridine afforded a diacetate, as indicated by the presence of two new acetyl Me signals in the ^1H-nmr spectrum. A significant downfield shift of the oxygenated methine proton signal (H-7) from 5.88 to 6.84 ppm indicated that C-7 bears a free OH group in the natural product.



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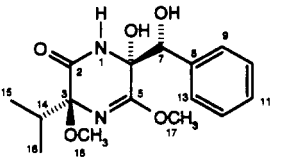

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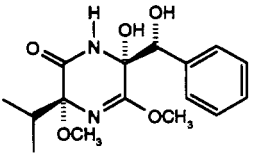

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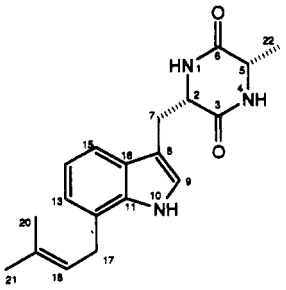

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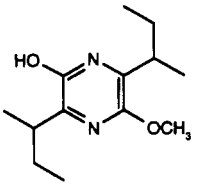

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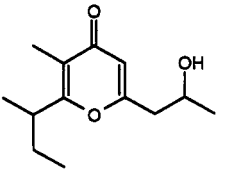

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Based on these results, the number of unsaturations, the presence of two nitrogen atoms, and the chemical shifts of the sp^2 carbons compared to those of model compounds (9,10), the remaining atoms must be arranged to form a pyrazine ring, leading to assignment of the structure of terezine A as **1**. The absence of a strong carbonyl absorption in the ir spectrum, chemical shift considerations, and comparison of the nmr and ir data with data published for model compounds [e.g., **5**] (9,10), suggest that terezine A [**1**] exists predominantly in the hydroxypyrazine tautomeric form rather than in the more common pyrazinone (amide) form.

Treatment of **1** with racemic 2-phenylbutyryl chloride afforded a pair of diastereomeric monoacylated products as indicated by the significant (>1 ppm) downfield shift of the H-7 signals, nmr integrations, and eims data. Two separate H_3-17 proton signals were observed for the two diastereomeric products at 3.92 ppm and 3.83 ppm. Treatment of **1** with (*R*)-2-phenylbutyryl chloride afforded a single diastereomeric monoacylated product with an H_3-17 signal at 3.83 ppm. Based on Helmchen's rules (11–13), the absolute stereochemistry at C-7 of **1** is proposed to be *R*. The predominance of monoacylation over diacylation in this reaction was presumably due to steric factors and the use of a much smaller excess of reagent than was employed in the acetylation reaction.

The nmr data for terezine B [**2**] were similar to those of **1**. Fabms and ^{13}C-nmr analysis indicated that **2** has the formula $C_{16}H_{22}N_2O_5$. ^{1}H-Nmr, ^{13}C-nmr, HMQC, and DEPT data again indicated the existence of phenyl, MeO (two), oxygenated methine, and isopropyl groups. In addition, two of the sp^2 ring-carbons of **1** were found to be replaced by oxygenated quaternary sp^3 carbons, and the ir and ^{13}C-nmr spectra revealed the presence of an amide carbonyl group. One of the MeO group proton signals for **2** was shifted dramatically upfield (to 1.97 ppm; ^{13}C-nmr signal at 50.7 ppm), presumably requiring its orientation into the shielding region of the phenyl group. The isopropyl Me group signal at 0.90 ppm and the MeO group resonance at 1.97 ppm each showed selective INEPT correlations with C-3 (91.0 ppm), indicating that the isopropyl and MeO groups are both attached to C-3. The MeO signal at 3.77 ppm showed a selective INEPT correlation with C-5 (161.0 ppm). Irradiation of the methine resonance at 5.13 ppm optimizing for different $^nJ_{CH}$ values caused polarization transfer only to phenyl carbons (128.9 and 142.3 ppm) and an oxygenated quaternary carbon (C-6, 84.2 ppm). The isopropyl methine proton showed selective INEPT correlations to C-3 and the

amide carbonyl signal (C-2; 172.5 ppm). HMBC data obtained for compound **2** gave the same set of correlations. NOESY data indicated that the methine and one Me of the isopropyl group were close in space to the downfield MeO group resonating at 3.77 ppm. The unusual chemical shift of the upfield MeO proton signal (H_3-18) was consistent with strong NOESY correlations between the protons of the phenyl group and those of the upfield MeO group. On the basis of these results, the structure of this component, including the relative stereochemistry at positions 3 and 6, was assigned as shown in **2**. This assignment is consistent with a close biogenetic relationship between compounds **1** and **2**. The configuration at C-7 was proposed to be *R* by analogy to structure **1**, but the stereochemistry at C-3 and C-6 could not be related to that at C-7, so the absolute stereochemistry at those positions was not assigned.

Nmr and fabms data for terezine C [**3**] clearly indicated a stereoisomeric relationship between **2** and **3**, and the difference was immediately suggested by significant upfield shifts of the isopropyl group protons (e.g., one Me at −0.19 ppm), along with a more typical shift for the C-18 MeO group proton signal. As expected, a strong NOESY correlation was observed between the upfield Me of the isopropyl group and the phenyl ring protons, confirming that the isopropyl group and the phenyl group are on the same face of the ring in **3**. HMBC, HMQC, and selective INEPT experiments gave results analogous to those for compound **2**. Based on these observations, the structure of this metabolite was assigned as **3**. As is the case for **2**, the relative stereochemistry at C-3 and C-6 can be assigned, and the configuration at C-7 is presumed to be *R*, but the absolute stereochemistry at C-3 and C-6 could not be assigned with the available data.

Terezines A–C [**1–3**] appear to be derived through biogenetic modification of a diketopiperazine formed from the amino acids valine and phenylalanine. These compounds bear resemblance to several diketopiperazine-derived fungal metabolites that have been reported previously (9, 14–18), including the aspergillic acids (14), a series of *N*-hydroxypyrazinones from *Aspergillus* spp.; septorine (9), a phytotoxin from the wheat parasite *Septoria nodorum*; and emeheterone, a bis-benzyl pyrazine from another fungal source (18).

When potato dextrose broth (PDB) was used as a growth medium for *S. teretispora*, an additional metabolite was obtained. In this instance, prep. tlc of the EtOAc extract afforded terezine D [**4**]. Fabms data suggested the formula $C_{19}H_{23}N_3O_2$, and the uv spectrum was characteristic of an indole. DEPT, COSY, ^{13}C-, and ^1H-nmr data provided evidence for two secondary amide groups, a disubstituted indole moiety, a 3-methyl-2-butenyl group, a CH-CH_3 unit, and a CH-CH_2 unit. These substructures were linked with the aid of long-range C-H correlations obtained through selective INEPT experiments. Selective INEPT irradiation of the Me doublet (H_3-22; 0.35 ppm) resulted in correlations to the neighboring methine (C-5; 51.7 ppm) and an amide carbon (C-6; 170.6 ppm), while the H-5 methine signal (3.67 ppm) showed correlations to both amide carbons (C-3 and C-6; 169.5 and 170.6 ppm), as well as to C-22 (19.9 ppm). These results, and the chemical shifts of C-5 and H-5, indicated that C-5 is bound to the carbonyl of one amide and the nitrogen atom of the other. Similarly, selective INEPT irradiation of the second methine proton signal (4.24 ppm) and the neighboring methylene protons (3.13 and 3.45 ppm) indicated that the second methine was also connected to one amide carbonyl carbon and to the nitrogen atom of the other amide group. These data indicated that **4** must be a diketopiperazine. The methylene protons also showed correlations to three sp^2 carbons of the indole group (109.7, 125.6, and 129.2 ppm). Irradiation of the vinylic proton signal of the prenyl group (H-18; 7.06 ppm) resulted in polarization transfer to one of the aromatic carbons (125.7 ppm). Irradiation of the allylic methylene proton signal (3.51 ppm) provided correlations to three of the

indole ring carbon signals (136.5, 125.7, and 121.7 ppm). These data and chemical shift considerations located the prenyl group at position 12 (the signal at 136.5 must correspond to C-11), and permitted the assignment of the structure of terezine D as **4**. Acid hydrolysis of compound **4** afforded alanine. Preparation of the pentafluoropropionyl isopropyl ester derivative, followed by chiral gc analysis, indicated that the alanine unit in **4** has the L-configuration. The configuration of the prenylated Trp was not determined, but is presumed to be L. Diketopiperazines are relatively common as fungal metabolites, and an isomer of **4** with a prenyl group at the indole 2-position has been described as a metabolite of *Aspergillus chevalieri* (19). However, to our knowledge, compound **4** has not been previously reported.

The known compound hyalopyrone [**6**] was also isolated as a minor constituent of the PDB cultures of *S. teretispora*. Compound **6** has been previously reported as an antimicrobial metabolite of the fungus *Ascochyta hyalospora* (20). Analysis of selective INEPT experiments confirmed that the regiochemistry of the sample isolated from *S. teretispora* matched that of the compound reported previously.

Terezines A–C [**1–3**] showed activity in standard disk assays (21) against *Bacillus subtilis* (ATCC 6051) with zones of inhibition ranging from 8 to 14 mm at 100 μg/disk. Terezines A [**1**] and C [**3**] displayed activity against *Staphylococcus aureus* (ATCC 29213) with zones of inhibition from 8 to 12 mm at 100 μg/disk. Terezine A [**1**] was also slightly active against *Candida albicans* (ATCC 14053), causing an 8-mm zone of inhibition at 200 μg/disk. In centerpoint inoculation disk assays against two coprophilous fungal species, terezines A [**1**], C [**3**], and D [**4**] showed activity against *Sordaria fimicola* (NRRL 6459), causing 95%, 41%, and 50% reductions in radial growth rate at 200 μg/disk, respectively, while terezine A [**1**] also caused a 95% reduction in radial growth rate of *Ascobolus furfuraceus* (NRRL 6460) at 200 μg/disk.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All nmr spectra were recorded in CD_3OD, and chemical shifts were referenced relative to the corresponding solvent signals (3.30 ppm/49.0 ppm). ^{1}H-Nmr, ^{13}C-nmr, COSY, NOESY, and selective INEPT experiments were performed on a Bruker AC-300 spectrometer operating at 300 and 75 MHz for ^{1}H and ^{13}C, respectively. HMBC and HMQC data were obtained on a Bruker AMX-600 instrument operating at 600 MHz (^{1}H dimension). All carbon assignments (Table 1) are consistent with multiplicities established by DEPT experiments. Selective INEPT experiments were performed with $^nJ_{CH}$ optimized for values of 4 or 7 Hz. Eims data were recorded at 70 eV using a VG Trio 1 quadrupole mass spectrometer. Hreims and fabms data were recorded on a VG ZAB-HF mass spectrometer. Uv spectra were measured with a Gilford Response uv-vis spectrometer. Optical rotations were determined using a Perkin-Elmer 141 polarimeter. Chiral gc employed a Hewlett-Packard 5890A gas chromatograph with an Alltech Heliflex Chirasil-Val capillary column (0.32 mm×25 m; He carrier gas; 35–185° at 5°/min). Procedures for the antifungal assays have been described previously (5).

CULTIVATION OF *S. TERETISPORA*.—The culture of *S. teretispora* employed in this work was a subculture of an isolate originally obtained from a sample of rabbit dung collected by D.M. in a location east of Soquel, Santa Cruz, California. Thirty 2-liter Erlenmeyer flasks containing soy flour medium (4 g Sigma type I defatted soy flour, 4 g glucose, 1 g NaCl, 0.5 g $CaCO_3$, and 400 ml H_2O) were individually inoculated with one-cm^2 agar plugs of *S. teretispora* from stock cultures maintained on potato dextrose agar. The cultures were aerated by agitation on an orbital shaker at 150 rpm for 30 days. These conditions were used to produce compounds **1–3**. When potato dextrose broth (PDB; Difco, 400 ml each) was employed, compounds **1–2** were not isolated, but **3** and **4** were obtained.

ISOLATION AND CHARACTERIZATION OF TEREZINES A–D [**1–4**].—The filtered broth (12 liters) was extracted with an equivalent amount of EtOAc, which was then dried ($MgSO_4$) and evaporated to afford 1.5 g of crude extract. Portions of the extract (3×200 mg) were chromatographed on Si gel tlc plates (20×20×0.1 cm) developing with $CHCl_3$-MeOH (9:1). Two fractions (44.5 mg, R_f 0.55; 125 mg, R_f 0.40) with antifungal activity were further purified by reversed-phase hplc using MeOH/H_2O mixtures (Beckman Ultrasphere C_{18} column, 1×25 cm, 5 μm particles, 2.0 ml/min, monitored by uv absorption at 215 nm). Terezine A [**1**; 9 mg, R_t 16.4 min] was obtained by hplc from one fraction (R_f 0.55) using 75% MeOH.

TABLE 1. ^{13}C-Nmr Data (75 MHz) for Terezines A–D [**1–4**] in CD_3OD.

Carbon	Compound			
	1	**2**	**3**	**4**
2	154.1	172.5	171.9	57.5
3	148.2	91.0	91.0	169.5
5	150.4	161.0	161.9	51.7
6	135.7	84.2	84.0	170.6
7	70.5	77.8	77.0	30.9
8	143.7	142.3	142.1	109.7
9	127.8	128.9	128.7	125.6
10	129.2	129.1	129.2	
11	128.4	129.3	129.3	136.5
12	129.2	129.1	129.2	125.7
13	127.8	128.9	128.7	121.7
14	30.5	38.4	37.0	117.8
15	21.0	16.3	15.9	120.5
16	21.0	17.5	16.1	129.2
17	54.1	53.7	53.7	30.5
18		50.7	52.0	123.4
19				133.7
20				17.9
21				25.9
22				19.9

Terezines B [**2**; 6 mg, R_t 22.8 min] and C [**3**; 5 mg, R_t 24.8 min] were isolated from another fraction (R_f 0.40) using 57% MeOH.

EtOAc extracts (250 mg) obtained from 4 liters of PDB culture broth were fractionated by prep. tlc (20×20×0.1 cm, $CHCl_3$-MeOH, 9:1). Further purification of the antifungal fraction (83 mg; R_f 0.37) by prep. tlc ($CHCl_3$-MeOH, 85:15) afforded terezine D [**4**; 9 mg]. Purification of another bioactive fraction (60 mg; R_f 0.25) by prep. tlc [$(CH_3)_2CO$-$CHCl_3$-MeOH, 3:6:1] afforded hyalopyrone [**6**; 4.3 mg] and terezine C [**3**; 2 mg].

Terezine A [**1**].—Yellow oil: $[\alpha]_D$ −20.2° (c=0.21, MeOH); uv λ max (MeOH) 204 (ε 15000), 230 sh (8000), 325 (7100), 362 sh (2000) nm; ir ν max (KBr) 3219, 2969, 2934, 1593, 1457, 1405, 1164, 1027 cm^{-1}; ^{1}H- and ^{13}C-nmr data, see Tables 1 and 2; selective INEPT correlations (H-#=>C-#) H-7=>C-5, 6, 8, 9/13; H-9 =>C-7, 13; H-10 =>C-8, 9, 12; H-12 =>C-8, 10, 13; H-13 =>C-7, 9; H-14 =>C-2, 3, 15, 16; H-15 =>C-3, 14, 16; H-17 =>C-5; eims *m/z* 274 $[M]^+$ (22), 259 (5), 245 (23), 241 (6), 229 (2), 213 (5), 197 (9), 169 (100), 153 (9), 105 (49), 91 (11), 79 (29), 77 (61); hreims *m/z* 274.1308, calcd for $C_{15}H_{18}N_2O_3$, 274.1317.

Terezine B [**2**].—Yellow oil: $[\alpha]_D$ −14.4° (c=0.083, MeOH); uv λ max (MeOH) 204 (ε 11000) nm; ir ν max (KBr) 3327, 2969, 2934, 1671, 1456, 1387, 1088 cm^{-1}; ^{1}H- and ^{13}C-nmr data, see Tables 1 and 2; selective INEPT and HMBC correlations (H-#=>C-#) H-7 =>C-6, 8, 9/13; H-9 =>C-7, 10, 13; H-13 =>C-7, 9, 12; H-14 =>2, 3, 15, 16; H-15 =>C-3, 14, 16; H-17 =>C-5, H-18 =>C-3; fabms (glycerol) *m/z* 323 $[M+H]^+$ (12), 305 (2), 279 (6), 261 (4), 245 (3), 218 (5), 107 (29), 89 (20), 77 (19), 65 (7).

Terezine C [**3**].—Yellow oil: $[\alpha]_D$ −63.1° (c=0.59, MeOH); uv λ max (MeOH) 205 (ε 13000) nm; ir ν max (KBr) 3342, 2970, 2945, 1670, 1457, 1364, 1294, 1153, 1093 cm^{-1}; ^{1}H- and ^{13}C-nmr data, see Tables 1 and 2; selective INEPT and HMBC correlations (H-#=>C-#) H-7 =>C-6, 8, 9/13; H-9 =>C-7, 10, 13; H-13 =>C-7, 9, 12; H-14 =>C-2, 3, 15, 16; H-15 =>C-3, 14, 16; H-17 =>C-5; H-18 =>C-3; fabms (glycerol) *m/z* 323 $[M+H]^+$ (74), 305 (4), 291 (8), 261 (4), 257 (11), 169 (13), 155 (8), 128 (13), 106 (21); hrfabms *m/z* 323.1610, calcd for $C_{16}H_{22}N_2O_5$+H, 323.1606.

Terezine D [**4**].—White powder: mp 192–194°; $[\alpha]_D$ +7.0° (c=0.58, MeOH); uv λ max (MeOH) 204 (ε 24000), 221 (24000), 279 (3800) nm; ir ν max (KBr) 3400, 3294, 2914, 1669, 1647, 1457, 1101 cm^{-1}; ^{1}H- and ^{13}C-nmr data, see Tables 1 and 2; selective INEPT correlations (H-#=>C-#) H-2 =>C-3, 6, 7, 8; H-5 =>C-3, 6, 22; H-7 =>C-2, 3, 8, 9, 16; H-9 =>C-8, 11, 16; H-15 =>C-8, 11, 13, 16; H-17 =>C-11, 12, 13, 18, 19; H-18 =>C-12; H-20 =>C-18, 19; fabms (glycerol)

TABLE 2. ^{1}H-Nmr Data (300 MHz) for Terezines A–D [**1–4**] in CD_3OD.

Protons	Compound			
	1	**2**	**3**	**4**
2				4.24 (m)
5				3.67 (q, 5.3)
7	5.88 (s)	5.13 (s)	5.12 (s)	3.13 (dd, 4.4, 14.6)
				3.45 (dd, 3.9, 14.6)
9	7.41 (d, 7.2)	7.38 (d, 7.3)	7.35 (d, 6.6)	7.06 (s)
10	7.28 (m)	7.26 (m)	7.27 (m)	
11	7.21 (m)	7.20 (m)	7.22 (m)	
12	7.28 (m)	7.26 (m)	7.27 (m)	
13	7.39 (d, 7.2)	7.38 (d, 7.3)	7.35 (d, 6.6)	6.85 (br d, 6.7)
14	3.27 (m)	2.00 (m)	1.43 (m)	6.92 (dd, 7.2, 7.7)
15	1.20 (d, 5.6)	0.74 (d, 7.7)	−0.19 (d, 6.9)	7.44 (br d, 7.0)
16	1.22 (d, 5.6)	0.90 (d, 6.8)	0.68 (d, 6.8)	
17	3.84 (s)	3.77 (s)	3.81 (s)	3.51 (d, 7.4)
18		1.97 (s)	3.05 (s)	5.21 (t, 7.2)
20				1.71 (s)
21				1.71 (s)
22				0.35 (d, 7.1)

m/z 326 $[M+H]^+$ (22), 301 (3), 270 (2), 258 (3), 242 (1), 152 (8), 151 (3), 142 (11), 139 (10), 107 (20), 89 (19), 77 (19).

Hyalopyrone [**6**].—Colorless oil: $[\alpha]_D$ −15.9° (*c*=0.22, MeOH). The identity of this compound was verified by selective INEPT experiments and by comparison of its properties with literature values (eims, ^{1}H-nmr, ^{13}C-nmr, ir) (20).

ACETYLATION OF TEREZINE A [**1**].—A sample of terezine A (**1**; 1 mg) was dissolved in 0.2 ml of pyridine-Ac_2O (1:1), and the solution was allowed to stir at room temperature for 24 h. H_2O (1 ml) was added, and the solution was then extracted with $CHCl_3$ (2×2 ml). The $CHCl_3$ solution was dried and evaporated to afford a yellow oil which was further purified by reversed-phase hplc to afford the diacetate (1 mg) as a colorless oil: ^{1}H nmr (CD_3OD) δ 7.42–7.30 (5H, m, Ar-H), 6.84 (1H, s, H-7), 3.98 (3H, s, OMe), 3.05 (1H, septet, *J*=7 Hz, H-14), 2.34 (3H, s, OAc), 2.11 (3H, s, OAc), 1.22 (3H, d, *J*=7 Hz, H_3-15), 1.20 (3H, d, *J*=7 Hz, H_3-16).

FORMATION OF THE 2-PHENYLBUTYRYL DERIVATIVE OF TEREZINE A [**1**].—To a solution of **1** (1.3 mg) in pyridine (32 μl) was added (*R/S*) or (*R*)-2-phenylbutyryl chloride (9.8 mg) in 0.3 ml of freshly distilled THF, and the solution was allowed to stir at room temperature for 24 h. 3-[(Dimethylamino)propyl]amine (3 μl) was added, and after standing for 10 min, the solvent was evaporated under N_2. The residue was subjected to prep. tlc (Merck, Kieselgel 60, F_{254}) developing with $(CH_3)_2CO$-$CHCl_3$ (1:9), affording pure (^{1}H nmr) monoacylated product (1.2 mg). ^{1}H-Nmr data (for the product obtained using the *R*-isomer): δ 7.45 (2H, dd, *J*=1.6 and 7.6 Hz), 7.31–7.22 (8H, m), 6.92 (1H, s), 3.83 (3H, s), 3.61 (1H, t, *J*=7.5 Hz), 3.22 (1H, m), 1.83 (2H, m), 1.20 (3H, d, *J*=6.6 Hz), 1.18 (3H, d, *J*=6.6 Hz), 0.85 (3H, t, *J*=7.4 Hz); eims *m/z* 442 $[M^+]$ (2), 274 (14), 273 (14), 257 (29), 243 (14), 213 (4), 187 (6), 164 (12), 119 (43), 91 (100), 77 (12), 43 (10).

CHIRAL AMINO ACID ANALYSIS OF TEREZINE D [**4**].—The hydrolysate of **4** (1 mg) was placed in a 5-ml reaction vial with 3 ml of 0.2 N HCl and heated at 110° for 5 min. The solution was dried under N_2. A solution of HCl in *i*-PrOH was prepared by slowly adding acetyl chloride to *i*-PrOH (1:4). A 2.8-ml portion of this mixture was added to the reaction vial and the vial was heated at 100° for 45 min. After the reaction mixture was dried under N_2, CH_2Cl_2 (1.5 ml) and pentafluoropropionic anhydride (1 ml) were added, and the solution was heated at 100° for 15 min. The resulting solution was evaporated under N_2 and the residue was dissolved in 0.4 ml of CH_2Cl_2 for chiral gc analysis. The absolute configuration of the alanine derivative was determined by co-injection with authentic L- and D,L-standards.

ACKNOWLEDGMENTS

Support for this work from the National Institutes of Health (R01 AI27436 and K04 CA01571) and the Alfred P. Sloan Foundation is gratefully acknowledged.

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Received 12 July 1994