Isolation of some New 3,6-Dialkyl-1,4-dihydroxypiperazine-2,5-diones from *Aspergillus terreus*

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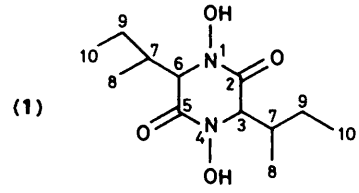
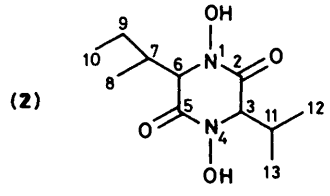
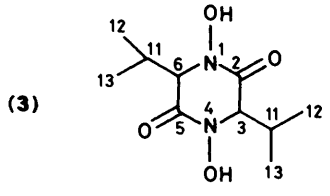
A series of piperazine-2,5-diones in which both amino acid constituents are hydroxamic acid residues has been isolated from *Aspergillus terreus* Thom. The metabolites were identified by i.r., ^{1}H n.m.r., and mass spectroscopy as (3*S*,6*S*)-1,4-dihydroxy-3,6-bis[(1*R*)-1-methylpropyl]piperazine-2,5-dione (terramide A) (**1**), (3*S*,6*S*)-1,4-dihydroxy-3-isopropyl-6-[(1*R*)-1-methylpropyl]piperazine-2,5-dione (terramide B) (**2**), and (3*S*,6*S*)-1,4-dihydroxy-3,6-bisisopropylpiperazine-2,5-dione (terramide C) (**3**).

Separation of the three components in the mixture was achieved by reverse-phase high pressure liquid chromatography (h.p.l.c.). The *S* configuration of the chiral centres was established by reduction and hydrolysis of the terramide mixture to the component amino acids and analysis by g.l.c. using a chiral column.

N-Hydroxylation of amides is an important biological process. It has been suggested that *N*-hydroxypeptides may play a role in the biosynthesis of β-lactam antibiotics,[1] dehydro or functionalised α-amino acids,[2–4] and some mould metabolites, such as gliotoxin.[5] A variety of compounds containing the hydroxamic acid moiety, CON(OH), have been isolated, mainly from microbial sources, which act as growth factors, antibiotics, antibiotic antagonists, cell-division factors, or iron-chelating agents.[6] In the course of biosynthetic studies on metabolites of *Aspergillus terreus* Thom., we isolated three cyclic bishydroxamic acids whose structures and spectroscopic properties are the subject of this paper. The new metabolites differ in oxidation level from the other naturally occurring cyclic hydroxamic acids, mycelianamide,[7] pulcherriminic acid,[8,9] and the aspergillic acid family,[10,11] and thus represent a new class of compound.







The numbering shown is that adopted to describe the n.m.r. results in the Experimental Section and the Tables.

Results and Discussion

Aspergillus terreus (CMI 44339) was grown on a sucrose based Czapek-Dox medium. Extraction of the culture with ethyl acetate and subsequent chromatography and recrystallisation gave a buff powder. This isolate, to which we assign the name terramide, contains approximately equal amounts of two bishydroxamic acids, designated terramide A and terramide B, together with traces of a third compound terramide C.

The terramide mixture is soluble in polar organic solvents such as acetone or methanol. It dissolves in hot aqueous sodium carbonate, but not in aqueous sodium hydrogen carbonate. It gives a blood red colour with alcoholic ferric chloride solution and a white precipitate with alcoholic copper(II) acetate. These tests together indicate the presence of a hydroxamic acid group,[9,11] and the i.r. spectrum of terramide reveals strong peaks at 3 400 and 1 620 cm^{-1} as required for a hydroxamic acid.[11]

The electron impact mass spectrum shows a weak molecular ion at *m/z* 258. The chemical ionisation mass spectrum of terramide run in the presence of methane contains strong peaks at *m/z* 287, 273, 259, and 245. Under these conditions, peaks corresponding to (M + $C_2H_5^+$) and (M + H^+) are routinely observed. The f.a.b. mass spectrum gives three peaks at *m/z* 259, 245, and 231. Thus these data support the presence of three molecular species with molecular weight 258 ($C_{12}H_{22}N_2O_4$), 244 ($C_{11}H_{20}N_2O_4$), and 230 ($C_{10}H_{18}N_2O_4$). The terramide mixture shows no strong u.v. absorption maximum above 254 nm. In preliminary attempts to obtain individual components of terramide, the ditosylate, diacetate, dibenzoate, and dimethyl ether of the mixture were prepared. While these confirmed the bishydroxamic acid nature of terramide, they could not be separated and characterised.

Eventually the terramide mixture was separated by h.p.l.c. using acetonitrile–water (7:3) as eluant and a reversed phase Spherisorb S5 ODS 30-cm column. Three compounds were eluted and the remaining structural details (except the absolute configuration) elucidated by n.m.r. spectroscopy, demonstrated that the compounds are: terramide A (**1**) (3*S*,6*S*)-1,4-dihydroxy-3,6-bis[(1*R*)-1-methylpropyl]piperazine-2,5-dione, terramide B (**2**) (3*S*,6*S*)-1,4-dihydroxy-3-isopropyl-6-[(1*R*)-1-

Table 1. ^{1}H N.m.r. data for terramide A

Hydrogen atom irradiated	δ (multiplicity)	Response (observation) δ
6-H	4.26 (d, *J* 2 Hz)	2.31 (simplified)
7-H	2.31 (m, *J* 7.2 Hz)	4.26 (s), 1.71 and 1.53 (simplified) 0.99 (s)
9-H	1.71 (m)	2.31 (simplified), 1.53 (simplified) 0.96 (d)
9-H	1.53 (m)	2.31 (simplified), 1.71 (simplified) 0.96 (d)
8-H	0.99 (d, *J* 7 Hz)	2.31 (simplified), 1.71 (dd,) 1.53 (dd)
10-H	0.96 (t, *J* 7 Hz)	1.71 (dd), 1.53 (dd)

Table 2. ^{1}H N.m.r. data for terramide B

Hydrogen atom irradiated	δ (multiplicity)	Response (observation) δ
6-H	4.26 (dd, *J* 2.4, 1.4 Hz)	4.16 (d), 2.30 (simplified)
3-H	4.16 (dd, *J* 2.4, 1.4 Hz)	4.26 (d), 2.57 (septet)
11-H	2.57 (br m)	4.16 (d), 1.13 (s), 1.07 (s)
7-H	2.30 (br m)	4.26 (d), 1.71 (sextet), 1.53 (sextet), 0.99 (s)

Table 3. ^{1}H N.m.r. data for terramide C

Hydrogen atom irradiated	δ (multiplicity)	Response (observation) δ
3-H	4.15 (d, *J* 2.4 Hz)	2.55 (simplified)
11-H	2.55 (br m)	4.15 (s), 1.13 (s), 1.06 (s)
12-H, 13-H	1.13 (d), 1.06 (d, 7 Hz)	2.55(d)

methylpropyl]piperazine-2,5-dione, and terramide C (**3**) (3*S*,6*S*)-1,4-dihydroxy-3,6-bisisopropylpiperazine-2,5-dione.

Proton decoupling experiments were performed on terramides A, B, and C and the results are summarised in Tables 1—3.

The broad signal observed at δ 9.4—10.1, which disappeared on exchange with D_2O, is assigned to the hydroxy group proton. The ^{1}H n.m.r. spectrum of terramide A (**1**) has signals at δ 0.96, 0.99, 1.53, 1.71, 2.31, 4.26, and 9.4—10.1, and that of terramide C (**3**) has signals at δ 1.06, 1.13, 2.55, 4.15, and 9.4—10.1. The small number of signals for both these compounds implies a high degree of symmetry, and therefore the stereochemistry of both chiral centres in terramide A (**1**) and in terramide C (**3**) must be the same.

The ^{1}H n.m.r. spectrum of terramide B (**2**) has signals at δ 0.93, 0.99, 1.07, 1.13, 1.52, 1.71, 2.30, 2.57, 4.16, 4.26, and 9.4—10.1. A proton–proton correlation experiment (COSY)[12] was performed on terramide B, in order to confirm that the protons giving rise to the double doublets at δ 4.26 and 4.16 are coupled across the ring. The presence of off-diagonal peaks shows clearly that this coupling is occurring.

The absolute configurations were determined by hydrolysis of the piperazine-2,5-diones formed by reduction of the terramide mixture with zinc and acetic acid. The resulting amino acids were subjected to chiral assay using a Chirasil-Val g.l.c. column and compared with reference samples. The results showed that the mixture contained (2*S*,3*R*)-isoleucine and (*S*)-valine in a ratio of 3:1 with only slight traces of the *R*-amino acids, which could be accounted for by partial racemisation during acid hydrolysis.

Thus the configuration at the 3- and 6-positions of terramide is *S* (as found in the natural amino acids); moreover this experiment also shows that the methylpropyl side chain has the same stereochemistry as that found in natural isoleucine.

Experimental

M.p.s were determined on a Reichert hot-stage apparatus and are uncorrected. Thin layer chromatography and preparative chromatography were carried out on Merck Kieselgel. Solvents were distilled before use and organic extracts were dried over anhydrous sodium sulphate. I.r. spectra were recorded on a Perkin-Elmer 297 spectrophotometer in solutions in chloroform unless otherwise stated. U.v. spectra were recorded for solutions in chloroform on a Pye-Unicam SP8-400 spectrophotometer.

^{1}H N.m.r. spectra were recorded on Bruker WH-400 or WP-80 instruments; chemical shifts are in p.p.m. (δ) from tetramethylsilane. ^{13}C N.m.r. spectra were recorded on a Bruker WM-250 instrument. Mass spectra were recorded on an A.E.I. MS30 instrument. Optical rotations were measured for solutions in MeOH or EtOH on a Perkin-Elmer SP241 polarimeter using 1 dm path length.

Isolation of Terramide.—Aspergillus terreus Thom. (CMI 44339) was grown in still culture at 28 °C on a solution of sucrose (50 g), sodium nitrate (2 g), potassium hydrogen phosphate (1 g), potassium chloride (0.5 g), magnesium sulphate heptahydrate (0.5 g), and iron(II) sulphate (0.01 g) in glass distilled water (1 l) containing oxoid yeast powder (5 g). After 14 days growth the fungal mat (16 g dry weight) was filtered off and the culture fluid (1 l) was then extracted with ethyl acetate (4 × 500 ml). The combined organic solutions were dried and concentrated to a brown semi-solid (*ca.* 1 g) which was purified using flash column chromatography. The semi-solid was pre-adsorbed onto silica, placed on a silica column (30 mm × 300 mm), and eluted with diethyl ether (1 l). The resulting eluate was evaporated under reduced pressure to give a beige solid which was recrystallised from methanol to give *ca.* 400 mg of buff crystals, m.p. 196—199 °C $[\alpha]_D^{22}$ −108.2° (*c* 0.178 in EtOH) (Found: C, 54.5; H, 8.2; N, 11.0. $C_{12}H_{22}N_2O_4 + C_{11}H_{20}N_2O_4$ requires C, 54.9; H, 8.4; N, 11.15%); $\nu_{max.}$ (KBr disc) 3 400b (N–OH), 3 230 (OH), 2 960 (CH), 1 620s (C=O), and 1 390 cm^{-1} (OH); δ_H (400 MHz, $CDCl_3$) 0.96 (9 H, t, *J* 7 Hz, 10-H), 0.99 (9 H, d, *J* 7 Hz, 8-H), 1.07 and 1.13 (each 3 H, d, *J* 7 Hz, 12-, 13-H), 1.52 and 1.71 (each 3 H, m, 9-H), 2.30 (3 H, br m, *J* 7, 2 Hz, 7-H), 2.57 (1 H, br m, *J* 7, 2.5 Hz, 11-H), 4.16 (1 H, dd, *J* 2.5, 1.4 Hz, 3-H), 4.26 (3 H, dd, *J* 2.4, 1.4 Hz, 6-H), and 9.4—10.1 (4 H, br s, OH); *m/z* (e.i.) 258 (M_A^+, 52%), 244 (M_B^+, 41), 230 (M_C^+, 5) 187 ($M_B^+ - C_4H_9^+$, 100), and 57 ($C_4H_9^+$, 100).

Separation of the Terramide Mixture.—Separation of terramides A, B, and C was achieved with an LDC h.p.l.c. instrument using an 8 mm × 25 cm column packed with Spherisorb S5 ODS reversed-phase silica equilibrated in acetonitrile–water (7:3), and eluted using the same solvent system at a flow rate of 3.4 ml min^{-1}. The terramide sample (250 mg) in ethanol (5 ml) was applied to the column in 50 μl aliquots. Fractions were monitored by u.v. detection at 254 nm. The retention times and yields of the three compounds were as follows: terramide C, 4.6 min, 10%; terramide B, 6.4 min, 40%; terramide A, 9.5 min, 41%.

The relative amounts of the terramides A, B, and C in the mixture remained the same throughout the period of culture (2 years).

The mixture gave *terramide A* {(3*S*,6*S*)-1,4-dihydroxy-3,6-bis[(1*R*)-1-methylpropyl]piperazine-2,5-dione}, m.p. 193—196 °C (from MeOH), $[\alpha]_D^{22}$ −101° (*c* 0.16 in EtOH) and −110° (*c* 0.17 in MeOH) (Found: C, 55.75; H, 8.6; N, 10.9%;

M^+, 258.1554. $C_{12}H_{22}N_2O_4$ requires C, 55.8; H, 8.6; N, 10.85%; M^+, 258.1579); $\nu_{max.}$ as for terramide mixture; δ_H (400 MHz, $CDCl_3$) 0.96 (6 H, t, *J* 7 Hz, 10-H), 0.99 (6 H, d, *J* 7 Hz, 8-H), 1.53 and 1.71 (each 2 H, m, 9-H), 2.31 (2 H, m, *J* 7, 2 Hz, 7-H), 4.26 (2 H, d, *J* 2 Hz, 6-H), and 9.4—10.1 (2 H, br s, OH); δ_C (62 MHz, $CDCl_3$) 12.1 (q, C-10), 15.1 (q, C-8), 25.7 (t, C-9), 37.0 (d, C-7), 65.1 (d, C-6), and 160.1 (s, C-5); *terramide B* [(3*S*,6*S*)-1,4-dihydroxy-3-isopropyl-6-[(1*R*)-1-methylpropyl]piperazine-2,5-dione, m.p. 179—184 °C (from MeOH), $[\alpha]_D^{22}$ −137° (*c* 0.13 in EtOH) and −223° (*c* 0.30 in MeOH) (Found: C, 54.2; H, 8.3; N, 11.3%; M^+, 244.1423. $C_{11}H_{20}N_2O_4$ requires C, 54.1; H, 8.25; N, 11.5%; *M*, 244.1423); $\nu_{max.}$ as for terramide mixture; δ_H (400 MHz, $CDCl_3$) 0.93 (3 H, t, *J* 7 Hz, 10-H), 0.99 (3 H, d, *J* 7 Hz, 8-H), 1.07 and 1.13 (each 3 H, d, *J* 7 Hz, 12-, 13-H), 1.52 and 1.71 (each 1 H, m, 9-H), 2.30 (1 H, br m, 7-H), 2.57 (1 H, br m, 11-H), 4.16 (1 H, dd, *J* 2.4, 1.4 Hz, 3-H), 4.26 (1 H, dd, *J* 2.4, 1.4 Hz, 6-H), and 9.4—10.1 (2 H, br s, OH); δ_C (62 MHz, $CDCl_3$) 12.1 (q, C-10), 15.1 (q, C-8), 17.5 and 17.7 (both q, C-12, -13), 25.7 (t, C-9), 31.2 (d, C-11), 37.1 (d, C-7), 65.1 (d, C-6), 67.1 (d, C-3), 159.9 (s, C-2), and 160.1 (s, C-5); and *terramide C* [(3*S*,6*S*)-1,4-dihydroxy-3,6-bisisopropylpiperazine-2,5-dione], m.p. 155—159 °C (from MeOH), $[\alpha]_D^{22}$ −62° (*c* 0.05 in EtOH) and −130° (*c* 0.02 in MeOH) (Found: C, 52.2; H, 7.8; N, 12.0%; M^+, 230.1258. $C_{10}H_{18}N_2O_4$ requires: C, 52.2; H, 7.9; N, 12.2%; *M*, 230.1266); $\nu_{max.}$ as for terramide mixture; δ_H (400 MHz, $CDCl_3$) 1.06 and 1.13 (each 6 H, d, *J* 7 Hz, 12-, 13-H), 2.55 (2 H, br m, 11-H), 4.15 (2 H, d, *J* 2.4 Hz, 3-H), and 9.4—10.1 (2 H, br s, OH); δ_C (62 MHz, $CDCl_3$) 17.5 and 17.7 (both q, C-12, C-13), 31.2 (d, C-11), 67.1 (d, C-3), and 159.9 (s, C-2).

Determination of Absolute Configuration.—Reduction of the terramide mixture to piperazinedione. The terramide mixture (0.1 g, 0.40 mmol), zinc powder (0.8 g, 0.012 mol), and glacial acetic acid (12 ml, 12.59 g, 0.21 mol) were refluxed for 24 h. The mixture was evaporated to dryness under reduced pressure, and dissolved in boiling ethanol (10 ml). The solution was filtered whilst hot and, on cooling, crystals of the piperazinedione mixture formed. These were filtered off under suction (0.087 g, 93.0%), m.p. 241—243 °C, $\nu_{max.}$ 3 380b (NH) and 1 670s cm^{-1} (C=O); δ_H (100 MHz, CF_3CO_2D) 0.91 (9 H, t, *J* 7.1 Hz, Ileu-Me), 0.93 (3 H, d, *J* 6.6 Hz, Val-Me), 1.06 (12 H, d, *J* 7 Hz, Val-Me and Ile-Me), 1.15 and 1.32 (each 3 H, m), 2.10 (3 H, m), 2.42 (1 H, m), and 4.34 (4 H, dd, *J* 3 Hz); *m/z* (f.a.b.) 227 $(M + H)^+$.

Hydrolysis of dioxopiperazine mixture to amino acids. The piperazinedione mixture (0.11 mg) was placed in a thick walled glass tube with 6M-HCl (1 ml). The tube was sealed *in vacuo*, heated at 110 °C for 96 h, and the mixture freeze dried.

Derivatisation of the peptide hydrolysate. The above material was dissolved in isopropyl alcohol (2 ml) containing dry HCl so that the solution was 1—5 M in HCl, in a screw top vial. The vial was sealed and heated at 110 °C for 30 min. After opening, the solvent was removed using a stream of nitrogen, and the last traces were removed *in vacuo*. The residue was then dissolved in CH_2Cl_2 (2 ml) and trifluoroacetic anhydride (0.2 ml) added. The vial was resealed and heated to 110 °C for 15—20 min. The solvent and excess reagent were removed using a gentle stream of nitrogen at room temperature [care is required since the derivative is volatile]. In addition to the peptide hydrolysate described above, a mixture of (*S*)-valine and (2*S*,3*R*)-isoleucine, and a mixture of (*R*,*S*)-valine, (2*S*,3*R*)-isoleucine and (2*R*,3*R*)-alloisoleucine were derivatised. These samples in CH_2Cl_2 (200 μl) were then analysed using a Chirasil-Val column (25 m × 0.24 mm i.d.) in an Erba Science Fractovap 4130 capillary gas chromatograph programmed for a temperature gradient of 4 °C min^{-1} from 70 to 180 °C with 2 min holds at 80 and 180 °C. The above solutions (0.7 μl) were used in a split injection. The carrier gas was hydrogen at a flow-rate of 1.5 ml min^{-1} and the injector temperature was 250 °C. The retention times of the sample peaks from the hydrolysate were 6.9 and 8.9 min and the peak areas had an approximate ratio of 1:3.

For comparison, the retention time for (*R*)-valine is 6.2 min, for (*S*)-valine is 6.9 min, for (2*R*,3*R*)-alloisoleucine is 7.8 min, and for (2*S*,3*R*)-isoleucine is 8.9 min.

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