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THE ISOLATION OF MAJUSCULAMIDE C FROM THE SPONGE *PTILOCAULIS TRACHYS* COLLECTED IN ENEWETAK AND DETERMINATION OF THE ABSOLUTE CONFIGURATION OF THE 2-METHYL-3-AMINOPENTANOIC ACID RESIDUE

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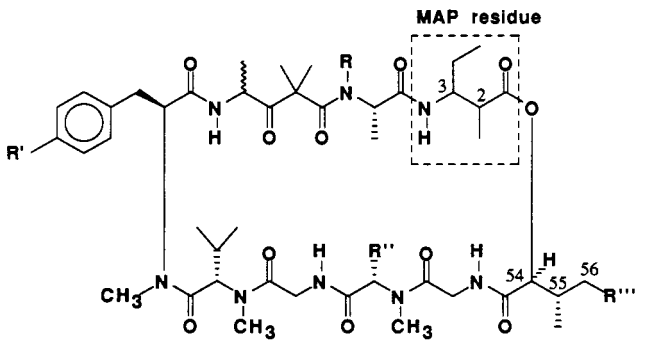
ABSTRACT.—Majusculamide C [**1**], a cyclic depsipeptide metabolite of the blue-green alga *Lyngbya majuscula*, has been isolated from the sponge *Ptilocaulis trachys* collected at Enewetak Atoll in the Marshall Islands. The absolute configuration of the novel β amino acid 2-methyl-3-aminopentanoic acid (MAP), a component of the structure of **1**, has been determined to be 2*S*,3*R*.

Complex cyclic peptides and depsipeptides have emerged as an important new class of metabolites present in extracts of marine sponges (1–13). Many of these sponge peptides have been found to be extremely potent cytotoxins (5–9) and/or enzyme inhibitors (10–13). The apparent structural relationships between some of the sponge peptides and peptides isolated from blue-green algae and other microbial sources has prompted several authors to raise the possibility that peptides which they have isolated from sponges are actually microbial products (2,4,10,12). As part of an ongoing study of biologically active metabolites from tropical marine invertebrates (14,15), we have isolated the cyclic depsipeptide majusculamide C [**1**] from the sponge *Ptilocaulis trachys* DeLaubenfels (class Demospongiae, order Axinellida, family Axinellidae) collected at Enewetak Atoll in the Marshall Islands. Majusculamide C [**1**] (16) and 57-normajusculamide C [**2**] (17) were first reported from *Lyngbya majuscula*, a toxic blue-green alga also collected at Enewetak Atoll. Both **1** and **2** have been shown to exhibit antifungal activity against pathogens of commercially important plants. Dolastatins 11 [**3**] and 12 [**4**], two closely related cyclic depsipeptides isolated from the Indo-Pacific sea hare *Dolabella auricularia*, were found to exhibit promising cytotoxic activity (murine leukemia P388 ED_{50} 2.7×10^{-3} and 7.5×10^{-2} μg/ml, respectively) (18). The



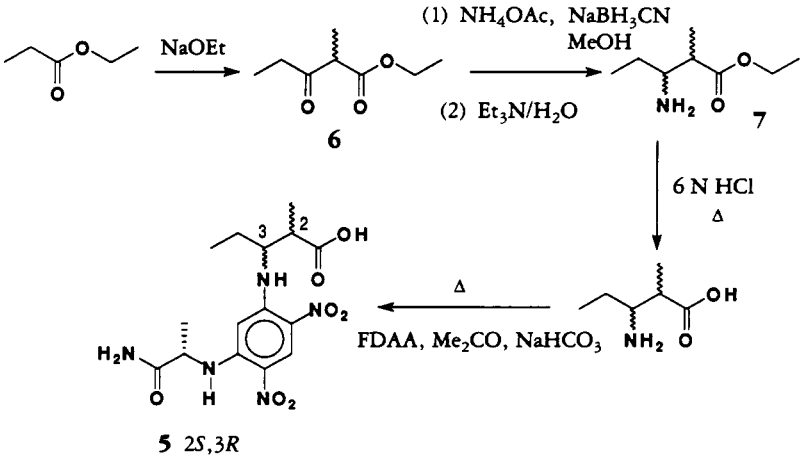
1 R=H, R′=OMe, R″=CH(Me)CH_2Me, R‴=Me (2*S*,3*R*)
2 R=H, R′=OMe, R″=CH(Me)CH_2Me, R‴=H
3 R=H, R′=OMe, R″=$CH_2CH(Me)_2$, R‴=Me
4 R=Me, R′=H, R″=$CH_2CH(Me)_2$, R‴=Me

biological activities of the majusculamides and the dolastatins make them attractive synthetic targets. In order to facilitate the synthesis of these peptides, we have determined the absolute configuration of the novel β amino acid 2-methyl-3-aminopentanoic acid (MAP) in majusculamide C [**1**]. The configuration of the MAP residue was not elucidated in the original investigations of the majusculamides (16,17) or the dolastatins (18).

RESULTS AND DISCUSSION

Specimens of *P. trachys* were collected by hand using scuba. The sponge tissue was frozen on site and maintained frozen until workup. EtOH extraction of the sponge gave a crude extract that showed in vitro cytotoxicity against the murine leukemia P-388 cell line. Bioassay-guided fractionation of the crude extract using Si gel flash and Sephadex LH 20 chromatography resulted in the isolation of majusculamide C [**1**] as the major cytotoxic component in the extract. The majusculamide C sample from *P. trachys* gave a parent ion in the hrfabms at *m/z* 985.5974 $[M+H]^+$, appropriate for the required molecular formula of $C_{50}H_{80}N_8O_{12}$ (ΔM −0.1 mmu). Detailed analysis of COSY, HOHAHA, HMQC, HMBC, and ROESY data collected on the sample confirmed that it was identical to the previously reported majusculamide C [**1**] (16).

The configurations of the α-amino and *N*-methyl-α-amino acids in the *P. trachys* sample of **1** were determined by acid hydrolysis followed by derivatization with Marfey's reagent and hplc analysis of the derivatives (19). Authentic samples of the methylated amino acids were synthesized for comparison purposes (20,21). The absolute configurations of the alanine, *N*-methylvaline, *N*-methylisoleucine, and *N,O*-dimethyltyrosine were all found to be L in agreement with Carter *et al.* (16). An unidentified component in the hplc trace, which gave an $[M+H]^+$ ion at *m/z* 384.1520 in the hrdcims corresponding to a molecular formula of $C_{15}H_{22}N_5O_7$ (ΔM +0.1 mmu), was assumed to be the Marfey's reagent derivative of MAP. In order to confirm this assumption, a mixture of all four stereoisomers of MAP was synthesized using literature procedures (22–24) as shown in Scheme 1. The mixture was derivatized with Marfey's reagent (19), and the four derivatized MAP stereoisomers were separated by hplc and characterized by ms and nmr analysis. One of the synthetic derivatized MAP stereoisomers (i.e., **5**) had an hplc retention time corresponding to the unidentified peak in the hplc trace of the Marfey's reagent derivatives formed from the



SCHEME 1. Synthesis of the derivatized MAP stereoisomers.

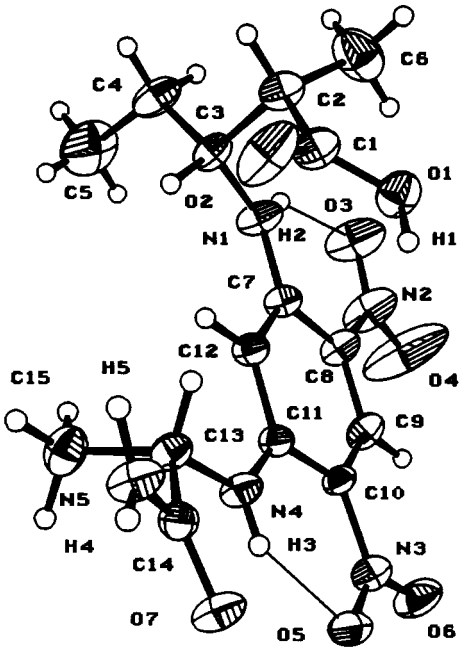


FIGURE 1. Perspective view of **5**; 33% probability thermal elipsoids shown for the non-hydrogen atoms.

majusculamide C hydrolysate. This synthetic MAP derivative **5** was crystallized, and its relative configuration was determined by single crystal X-ray diffraction analysis (Figure 1). Since Marfey's reagent contains an L-alanine residue, it was apparent that the MAP residue in majusculamide C [**1**] has the 2*S*,3*R* configuration.

The isolation of majusculamide C [**1**], a metabolite of *L. majuscula*, from the sponge *P. trachys* provides the first direct evidence that a cyclic depsipeptide present in a sponge extract is actually a blue-green algal metabolite. How the peptide gets from the alga into the sponge tissue remains to be determined. It has frequently been suggested that there is a symbiotic relationship between sponges and the microbes that are the putative producers of metabolites in the sponge extracts; however, there is little hard evidence to support that possibility. It may be that in some instances sponges simply concentrate microbial metabolites during the course of their normal filter feeding. In the present case, such a scenario is a distinct possibility since there were extensive beds of *L. majuscula* in the vicinity of the sites where specimens of *P. trachys* were collected.

EXPERIMENTAL

GENERAL PROCEDURES.—The ^{1}H- and ^{13}C-nmr spectra were recorded on a Bruker AMX-500 spectrometer. ^{1}H chemical shifts are referenced to the residual DMSO-d_6 signal (2.49 ppm), and ^{13}C chemical shifts are referenced to the DMSO-d_6 solvent peak (39.5 ppm). Low resolution and high resolution eims were recorded on Kratos AEI MS-59 and AEI MS-50 mass spectrometers. Chemical ionization mass spectra were recorded on Kratos MS80 and Delsi-Nermag R-10-10 quadrupole mass spectrometers using either methane or NH_3 as the ionization gas. High resolution fabms were recorded on a Kratos Concept HQ mass spectrometer. Optical rotations were measured using a Jasco J-710 spectrophotometer. Melting points were taken using a Fisher-Johns apparatus.

Merck Type 5554 Si gel plates and Whatman MKC18F plates were used for analytical tlc. Normal phase flash chromatography was conducted on Merck Si gel G60 (230–400).

Reversed-phase hplc purifications were performed on a Perkin-Elmer Series 2 liquid chromatograph attached to a Perkin-Elmer Spectrophotometer LC-55 using a Whatman Magnum-9 Partisal 10 ODS-3

column. Hplc analysis of amino acid derivatives employed a Waters 600E System Controller and a Waters 994 Programmable Photodiode Array Detector using a 100 mm × 4.6 mm (5 μ) C18 column. All solvents used for hplc were BDH Omnisolve grade.

ISOLATION OF MAJUSCULAMIDE C FROM *PTILOCAULIS TRACHYS*.—Specimens of *P. trachys* were collected by hand using scuba at 15 m depth on patch reefs and pinnacles in and around Medren, Japtan, and Sand Islands north of Enewetak Atoll. A voucher sample of *P. trachys* is deposited at the Zoological Museum of Amsterdam. The sponge tissue (450 g wet wt), which was free of epiphytes, was frozen on site and transported to Edmonton over dry ice. Thawed sponge was homogenized and extracted with 95% EtOH. Fractionation of the concentrated crude EtOH extract via repeated application of Sephadex LH 20 (eluent 95% EtOH) and Si gel flash chromatographies yielded pure majusculamide C [**1**] (ca. 2 mg). COSY, HOHAHA, HMQC, HMBC, and ROESY data collected on the sample confirmed that it was identical to the previously reported majusculamide C [**1**] (16).

PREPARATION OF ETHYL 3-OXO-2-METHYLPENTANOATE [**6**].—The β-keto ester **6** was prepared by the Claisen condensation from ethyl propionate (20.00 g, 0.196 mol) and alcohol-free freshly prepared sodium ethoxide (2.22 g, 0.033 mol), according to the general procedure given by Hauser and Hudson (22). Distillation of the combined ester layer and Et_2O extracts, after drying over anhydrous Na_2SO_4, removed volatiles with boiling points below 133°. The product was purified from the residue by flash chromatography on Si gel 60 (230–400 mesh, 2.5 × 17 cm column) using EtOAc as eluent to yield 2.2 g (0.014 mol, 7.1%) of pure β-keto ester **6** as a clear oil. Spectral data included here are additional to those reported previously (23): ^{13}C nmr (125 MHz, $CDCl_3$) δ 7.4, 12.6, 13.8, 34.4, 52.3, 61.0, 170.4, 206.1; eims *m/z* (rel. int.) 158 (7), 129 (19), 113 (30.9), 102 (97), 83 (16), 74 (50), 57 (100); hreims *m/z* 158.0944 $[M]^+$ ($C_8H_{14}O_3$, calcd 158.0943).

PREPARATION OF ETHYL 3-AMINO-2-METHYLPENTANOATE [**7**].—A solution of ethyl 3-oxo-2-methylpentanoate [**6**] (506.1 mg, 3.2 mmol), ammonium acetate (2.48 g, 32.0 mmol), and $NaBH_3CN$ (149.0 mg, 2.3 mmol) in 10 ml of absolute MeOH was stirred for 67 h at 25° (24). Concentrated HCl was added until pH <2, and the MeOH was removed in vacuo. The residue was taken up in 10 ml of H_2O and extracted with three 25-ml portions of EtOAc. The aqueous solution was brought to pH 6–7 with solid NaOH and then to pH 10 with triethylamine and extracted with three 10 ml portions of EtOAc. The combined EtOAc extracts were dried over Na_2SO_4, filtered, and evaporated in vacuo to give 241 mg (1.5 mmol, 47.4%) of a stereoisomeric mixture of ethyl 3-amino-2-methylpentanoates [**7**] (3:2 mixture by ^1H nmr) as a clear oil. Spectral data: ^1H nmr (500 MHz, MeOH-d_4) δ 0.95 and 0.96 (t, J = 7.5 Hz, 3H), 1.14 and 1.16 (d, J = 7 Hz, 3H), 1.25 (t, J = 7 Hz, 3H), 1.38, 1.48, and 1.53 (m, 2H), 2.53 (m, 1H), 2.85 and 2.93 (m, 1H), 4.14 (m, 2H); ^{13}C nmr (125 MHz, MeOH-d_4) δ 10.6 and 11.0, 11.9 and 12.0, 14.0 and 14.6, 27.6 and 28.1, 45.2 and 45.7, 55.5 and 56.1, 61.5 and 61.6, 176.7 and 176.9; cims (NH_3) *m/z* $[M + 1]^+$ 160.

PREPARATION OF THE MAP STEREOISOMERS.—The mixture of ethyl 3-amino-2-methylpentanoate stereoisomers **7** (154.5 mg, 0.97 mmol) in 1 ml 6 N HCl (freshly distilled, constant boiling HCl) was heated at 108° with stirring for 16 h in a threaded Pyrex tube sealed with a Teflon screw cap. The cooled reaction mixture was evaporated to dryness, and traces of HCl were removed from the residual hydrolyzate by repeated evaporation from H_2O (3 × 4 ml).

DERIVATIZATION OF THE MAP STEREOISOMERS WITH MARFEY'S REAGENT.—To a 50-ml round bottom flask containing 40.9 mg (0.26 mmol) of residual MAP hydrolyzate in 5.1 ml of H_2O was added 100 mg (0.36 mmol) of 5-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA or Marfey's reagent) in 10.3 ml Me_2CO followed by 2.6 ml of 1 N $NaHCO_3$. The mixture was heated for 1 h at 40°. After cooling to room temperature, 1.3 ml of 2 N HCl was added and the Me_2CO was removed in vacuo. The residue was taken up in 30 ml of H_2O and extracted with three 15-ml portions of EtOAc. The combined EtOAc extract was evaporated in vacuo and then fractionated via reversed-phase hplc [63% triethylammonium phosphate (50 mM, pH 3.0)/MeCN, detected by uv at 340 nm] to yield, eluting sequentially, fractions A, B, and C. Fractions A, B, and C were evaporated in vacuo, and the yellow residue was taken up in 15 ml of H_2O and extracted with three 7-ml portions of EtOAc. In each case the combined EtOAc extracts were dried over Na_2SO_4, filtered, and evaporated in vacuo to yield single derivatized MAP stereoisomer for fractions B (3.2 mg) and C [**5**] (7.5 mg), as yellow crystalline solids, and a mixture of two MAP derivatives for fraction A (11.1 mg) (total yield 21.8 mg, 0.06 mmol, 21.9%). Fraction A was further chromatographed via reversed-phase hplc (50% MeOH/H_2O adjusted to pH 3 with HOAc) to yield single MAP derivatives, fractions A-1 (8.1 mg) and A-2 (2.9 mg), as yellow crystalline solids. Physical and spectral data for fraction C [**5**]: mp 214–216°; $[\alpha]^{27}D$ −35.6° (c = 0.70, MeOH); ^1H nmr (500 MHz, DMSO-d_6) δ 0.93 (t, J = 7.5 Hz, 3H), 1.14 (d, J = 7.0 Hz, 3H), 1.43 (d, J = 6.5 Hz, 3H), 1.59 (m, 1H), 1.72 (m, 1H), 2.78 (m, 1H), 3.85 (ddt, J = 9, 9, 5 Hz, 1H), 4.30 (qd, J = 6.5, 6.5 Hz, 1H), 5.84 (s, 1H), 7.40 (s, 1H), 7.71 (s,

1H), 8.45 (d, $J=9$ Hz, 1H), 8.70 (d, $J=6.5$ Hz, 1H), 8.98 (s, 1H); ^{13}C nmr (125 MHz, DMSO-d_6) δ 10.6, 13.0, 18.1, 24.4, 42.1, 51.2, 56.0, 91.4, 123.5, 123.7, 128.9, 146.5, 148.1, 173.0, 175.2; hreims *m/z* $[M]^+$ 383.1443 ($C_{15}H_{21}N_5O_7$) (calcd 383.1442); cims (NH_3) *m/z* $[M+1]^+$ 384.

HYDROLYSIS OF MAJUSCULAMIDE C [1] WITH 6 N HCl.—Majusculamide C [**1**] (0.15–0.20 mg, 0.16–0.21 μmol) in 0.4 ml 6 N HCl (freshly distilled, constant boiling HCl) was heated at 108° with stirring for 16 h in a threaded Pyrex tube sealed with a Teflon screw cap. The cooled reaction mixture was evaporated to dryness, and traces of HCl were removed from the residual hydrolyzate by repeated evaporation from H_2O (3 × 0.4 ml).

DERIVATIZATION OF AMINO ACIDS WITH MARFEY'S REAGENT AND HPLC ANALYSIS [**19**].—To a 0.5 ml vial containing 2.0 μmol of the pure amino acid standard in 40 μl of H_2O was added 2.8 μmol of FDAA in 80.0 μl Me_2CO followed by 20.0 μl of 1 N $NaHCO_3$. The mixture was heated for 1 h at 40°. After cooling to room temperature, 10 μl of 2 N HCl was added and the resulting solution was filtered through a 4.5 μ filter and stored in the dark until hplc analysis.

The majusculamide C [**1**] residual hydrolyzate in 34 μl of H_2O was reacted with 2.35 μmol of FDAA in 67 μl of Me_2CO as described above. A 5 μl aliquot of the resulting mixture of FDAA derivatives was analyzed by reversed-phase hplc. A linear gradient of (A) triethylammonium phosphate (50 mM, pH 3.0) MeCN (9:1) and (B) MeCN with 0% B at start→40% B over 55 min (flow rate 1 ml/min) was used to separate the FDAA derivatives which were detected by uv at 340 nm. Each peak in the chromatographic trace was identified by comparing its retention time and uv spectrum with that of the FDAA derivative of the pure amino acid standard and by coinjection. The majusculamide C [**1**] Marfey's derivatized hydrolyzate showed peaks at 36.09, 37.43, 51.58 (area of peak approximately twice that of others), 52.26, and 56.18 min. The amino acid standards gave the following retention times in minutes: 36.04 for glycine; 37.37 for L- and 46.89 for D-alanine; 48.12 for A-1 and A-2, 51.12 for B, and 52.27 for the derivatized MAP fraction C (i.e., **5**); 51.65 for L- and 52.77 for *N,O*-dimethyl-D-tyrosine; 51.65 for L- and 54.73 for *N*-methyl-D-valine; and 56.17 for L- and 59.64 for *N*-methyl-D-isoleucine. In all cases a peak at 40.89 min was observed, which was attributed to excess FDAA.

TABLE 1. Final Atomic Coordinates (fractional) and B_{eq} ($Å^2$)[a] for Compound **5**.

Atom	*x*	*y*	*z*	B_{eq}
O-1	0.7163(5)	0.7185(3)	0.5988(1)	4.7(2)
O-2	0.9319(4)	0.6340(4)	0.5953(1)	7.1(2)
O-3	0.3732(5)	0.6295(4)	0.7005(1)	7.8(3)
O-4	0.1749(5)	0.5952(7)	0.6718(1)	15.2(5)
O-5	0.3130(4)	0.3897(3)	0.5162(1)	5.0(2)
O-6	0.1303(4)	0.4503(3)	0.5500(1)	5.8(2)
O-7	0.6764(4)	0.3371(3)	0.47527(9)	4.3(2)
N-1	0.6253(5)	0.5602(5)	0.6713(1)	4.2(2)
N-2	0.3069(6)	0.5934(5)	0.6724(1)	6.6(3)
N-3	0.2635(5)	0.4326(4)	0.5458(1)	4.4(3)
N-4	0.5856(5)	0.3918(4)	0.5458(1)	3.6(2)
N-5	0.9177(5)	0.3650(5)	0.4890(1)	4.2(3)
C-1	0.8349(7)	0.6750(5)	0.6143(1)	4.4(3)
C-2	0.8419(6)	0.6775(5)	0.6586(1)	3.9(3)
C-3	0.7865(6)	0.5666(5)	0.6733(1)	3.6(3)
C-4	0.8338(6)	0.5443(5)	0.7154(1)	5.2(3)
C-5	0.798(1)	0.4337(7)	0.7292(2)	9.5(5)
C-6	0.7604(9)	0.7716(5)	0.6776(2)	7.1(4)
C-7	0.5416(5)	0.5267(4)	0.6409(1)	3.3(2)
C-8	0.3837(6)	0.5438(5)	0.6406(1)	3.8(3)
C-9	0.3010(5)	0.5114(4)	0.6091(2)	3.9(3)
C-10	0.3622(5)	0.4631(4)	0.5773(1)	3.0(2)
C-11	0.5188(5)	0.4405(4)	0.5763(1)	3.2(2)
C-12	0.6010(5)	0.4738(4)	0.6087(1)	3.2(2)
C-13	0.7438(5)	0.3699(4)	0.5422(1)	3.3(2)
C-14	0.7764(5)	0.3566(4)	0.4990(1)	3.2(2)
C-15	0.7888(6)	0.2705(5)	0.5651(1)	4.9(3)

[a] $B_{eq} = (8/3)\pi^2\Sigma\Sigma U_{ij}a_i^*a_j^*(a_i \cdot a_j)$.

The total eluent corresponding to the peak eluting at 52.26 min in the hplc separation of the Marfey's reagent derivatives formed from the majusculamide C [**1**] hydrolysate was collected and extracted exhaustively with EtOAc. The combined EtOAc layers were back-washed with H_2O and then concentrated to dryness in vacuo to give the pure MAP derivative **5**. High resolution desorption chemical ionization mass spectrometric (NH_3) analysis of this sample of **5** showed an $[M+H]^+$ ion (100%) at *m/z* 384.1520 corresponding a molecular formula of $C_{15}H_{22}N_5O_7$ (ΔM +0.1 mmu).

X-RAY CRYSTALLOGRAPHIC ANALYSIS OF THE MAP DERIVATIVE **5**[1].—Crystals of $C_{15}H_{21}N_5O_7$, **5**, fw = 383.36, are orthorhombic, space group $C222_1$, $a = 8.999$ (2), $b = 12.464$ (3), $c = 34.054$ (2) Å, $Z = 8$, $D_c = 1.333$ g·cm^{-3}. A total of 2185 reflections ($+h, +k, +l$, 2θ max = 155°) was collected at 21° on a Rigaku AFC6S diffractometer using CuK_α radiation ($\lambda = 1.54178$ Å). The data were corrected for Lorentz and polarization effects and absorption (empirical, based on azimuthal scans for three reflections, relative transmission factors 0.81–1.00). The structure was solved by direct methods and was refined by full-matrix least-squares procedures to $R = 0.040$ and $R_w = 0.032$ for 1157 reflections with $I \geq 2\sigma(I)$. All non-hydrogen atoms were refined with anisotropic thermal parameters. The O-H and N-H hydrogen atoms were refined with isotropic thermal parameters, and all other hydrogen atoms were fixed in idealized positions (staggered methyl groups, C-H = 0.98 Å, $B_H = 1.2\ B_{\text{bonded atom}}$). A parallel refinement of the mirror-image structure gave marginally higher residuals, the R and R_w factor ratios both being 1.002. Final atomic coordinates and equivalent isotropic thermal parameters appear in Table 1.

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[1]Atomic coordinates for this compound have been deposited with the Cambridge Crystallographic Data Centre and can be obtained on request from Dr. Olga Kennard, University Chemical Laboratory, 12 Union Road, Cambridge CB2 1EZ, UK.

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