Two New Verticillane-Type Diterpenoids from the Formosan Soft Coral *Cespitularia hypotentaculata*

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Chemical investigations of the Formosan soft coral *Cespitularia hypotentaculata* Roxas led to the isolation of two new verticillane diterpenoids, cespitularins R and S (1, 2), along with seven known compounds (3—9). The structures of these isolated compounds were elucidated on the basis of extensive spectroscopic analysis and by comparison with those of reported in literature. The anti-inflammatory activity using RAW 264.7 macrophages of compounds 1—9 were evaluated *in vitro*.

Key words *Cespitularia hypotentaculata*; verticillane diterpenoid; anti-inflammatory activity

Earlier studies of the genus *Cespitularia* (Xeniidae) led to the isolation of a diverse array of diterpenoids including alcyonolides, caryophyllanoids, cembranolides, cespitularanoids, dolabellanoids, norverticillanoids, verticillanoids, and xenicanoids.[1—9] Previous bioassay results of some verticillane-type, norverticillane-type, and cespitularane-type diterpenoids have demonstrated remarkable pharmacological activity such as *in vitro* cytotoxicity against various cancer cell lines.[1,6—9] The ongoing researches for bioactive constituents prompted us to investigate the Formosan soft coral *Cespitularia hypotentaculata* Roxas. We have previously obtained verticillanes, norverticillanes, and cespitularanes, cespitularins A—Q,[6,7] from the organic-soluble of the organism.

Our continuing chemical examinations on the secondary metabolites of this soft coral have resulted in the purification of two new verticillane diterpenoids, cespitularins R and S (**1**, **2**), and seven known compounds (**3**—**9**)[6—8] (Fig. 1). The structures of **1** and **2** were determined by detailed 1D and 2D NMR techniques, especially employing correlation spectroscopy (COSY), distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum correlation (HSQC), and nuclear Overhauser effect spectroscopy (NOESY) experiments. Furthermore, compound **2** (10 μM) was evaluated for the ability to inhibit the expression of the pro-inflammatory inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) proteins. Compounds **7** and **8** did not inhibit the COX-2 protein expression, but significantly inhibited iNOS protein expression. With the exception of the above findings, the obtained negative results indicated that **1**, **3**—**6**, and **9** exhibited insignificantly anti-inflammatory activity against lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.

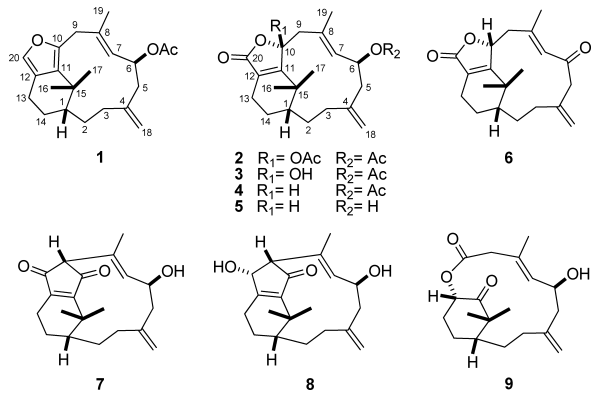

Fig. 1. Structures of Compounds **1**—**9**

Results and Discussion

Specimens of *C. hypotentaculata* were frozen immediately after collection. Conventional extraction procedures were used, and the acetone extract was exhaustively partitioned between EtOAc and H_2O to afford the EtOAc-soluble fraction, which was evaporated under vacuum to yield a dark brown gum (15 g). Then, the concentrated residue was successively subjected to column chromatography and high-performance liquid chromatography (HPLC), leading to the isolation of two new verticillane diterpenoids, cespitularins R and S (**1**, **2**), and seven previously characterized compounds **3**—**9** (see Experimental).

Cespitularin R (**1**) was isolated as a colorless, viscous oil. High resolution electrospray ionization mass spectrometry (HR-ESI-MS) of **1** exhibited a pseudomolecular ion peak at *m*/*z* 365.2095 $[M+Na]^+$, consistent with the molecular formula of $C_{22}H_{30}O_3$, requiring eight degrees of unsaturation. The IR spectrum of **1** at 1735 cm^{-1} demonstrated absorption band diagnostic of an acetoxy functionality, which was further supported by the ^{1}H-NMR signal at δ_H 2.04 (3H, s) and ^{13}C-NMR signals at δ_C 170.1 (qC) and 21.3 (CH_3). A careful analysis of the NMR data (Table 1) coupled with COSY, HSQC, and HMBC correlations proved that the structure of **1** was identical to that of cespitularin A[6] except for the presence of a hydroxyl instead of an acetoxy group. The carbonyl signal at δ_C 170.1 was attributed to the acetate moiety linked to C-6, as further confirmed through the crucial HMBC correlations from H-6 to the carbonyl carbon of 6-OAc. Therefore, the structure of cespitularin R was defined as **1**.

The relative stereochemistry of **1** assigned by the NOESY spectrum was compatible with those of cespitularin A offered by computer-generated perspective model using Molecular Mechanics (MM2) force field calculations, in which the close contacts of atoms calculated in space were consistent

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Table 1. ^{1}H- and ^{13}C-NMR Spectroscopic Data of Compounds **1** and **2**

C/H	**1**[a]		**2**[b]	
	^{13}C	^{1}H	^{13}C	^{1}H
1	43.2 (CH)[c]	1.84 m	44.1 (CH)[c]	1.64 m
2	32.1 (CH_2)	a: 1.39 m; b: 1.69 m	17.7 (CH_2)	a: 2.18 m; b: 2.38 m
3	30.0 (CH_2)	a: 1.65 m; b: 1.79 m	33.3 (CH_2)	1.50 m
4	147.1 (qC)		145.1 (qC)	
5	41.0 (CH_2)	a: 2.16 m b: 2.35 m	40.7 (CH_2)	a: 2.33 dd (2.5, 13.5)[d] b: 2.45 dd (8.0, 13.5)
6	72.1 (CH)	5.38 m	71.2 (CH)	5.35 td (8.0, 2.5)
7	125.6 (CH)	5.20 d (8.3)[d]	132.1 (CH)	5.50 d (8.0)
8	136.2 (qC)		132.7 (qC)	
9	38.0 (CH_2)	a: 3.23 d (14.4); b: 3.51 d (14.4)	46.8 (CH_2)	a: 2.87 d (14.5); b: 3.29 d (14.5)
10	144.0 (qC)		108.2 (qC)	
11	126.5 (qC)		164.3 (qC)	
12	124.1 (qC)		130.6 (qC)	
13	17.6 (CH_2)	2.55 m	32.2 (CH_2)	α: 2.05 m; β: 2.20 m
14	26.0 (CH_2)	α: 1.40 m; β: 2.23 m	24.1 (CH_2)	α: 1.69 m; β: 2.18 m
15	34.4 (qC)		37.3 (qC)	
16	33.6 (CH_3)	1.20 s	33.9 (CH_3)	1.18 s
17	26.4 (CH_3)	1.49 s	24.8 (CH_3)	1.47 s
18	113.4 (CH_2)	a: 4.74 s; b: 4.76 s	114.9 (CH_2)	a: 4.81 s; b: 4.82 s
19	17.5 (CH_3)	1.63 s	17.1 (CH_3)	1.65 s
20	134.7 (CH)	7.04 s	169.6 (qC)	
6-OAc	21.3 (CH_3)	2.04 s	22.2 (CH_3)	2.06 s
	170.1 (qC)		167.8 (qC)	
10-OAc			21.2 (CH_3)	2.03 s
			169.9 (qC)	

a) Spectra were measured in $CDCl_3$ (300 MHz). *b*) Spectra were measured in $CDCl_3$ (500 MHz). *c*) Multiplicities are deduced by HSQC and DEPT experiments. *d*) *J* values (in Hz) are in parentheses.

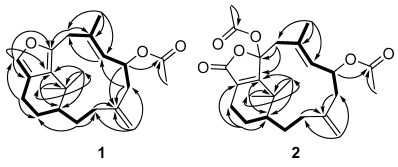

Fig. 2. Selected ^{1}H–^{1}H COSY (—) and HMBC (→) Correlations of **1** and **2**

with the NOESY correlations (Fig. 3). The NOESY spectrum and the conformer generated by Chem3D Pro 11.0 indicated that **1** possessed the same configuration as cespitularin A at the C-1 stereocenter and the *E* geometry of the trisubstituted double bond at C-7/C-8 in the molecule. From the NOESY spectrum of compound **1**, H_3-19 was found to show the NOE correlations with both H-6 and H-9a (δ_H 3.23), together with H_3-17 was found to show the NOE correlations with both H-7 and H-9b (δ_H 3.51), indicating the *S**-configuration of the acetoxy group attached at C-6. On the basis of the aforementioned observations, cespitularin R (**1**) was established definitely.

The positive HR-ESI-MS of compound **2** exhibited a pseudomolecular ion peak at *m/z* 439.2093 $[M+Na]^+$, corresponding to the molecular formula $C_{24}H_{32}O_6$ and nine degrees of unsaturation. Analysis of the COSY and HMBC correlations (Fig. 2) were diagnostic in determining that the gross framework of cespitularin S, having a verticillane skeleton, was assigned as **2**. The structure of **2** was identical to that of **3**[8] with exception that the hydroxy group was replaced by an acetoxy group. Although there were no direct HMBC correlations available, the acetoxy group attached to C-10 was supported by the key NOESY correlation from OAc-10 (δ_H 2.03) to H_3-16 and H_3-17. The relative stereo-

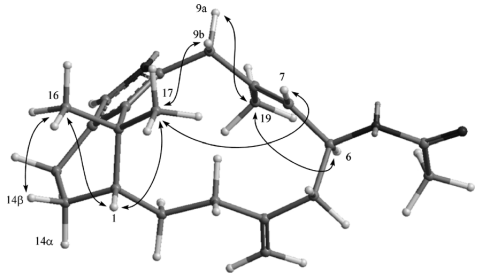

Fig. 3. Key NOESY Correlations of **1**

chemistry of **2** assigned by NOESY spectrum was compatible with those of **2** offered by computer modeling, in which the close contacts of atoms calculated in space were consistent with the NOESY correlations. The crucial NOESY correlations between H_3-16/OAc-10, H_3-17/OAc-10, H_3-16/H-1, H_3-17/H-1, H_3-16/H-14β (δ_H 2.18), H-14β/H-1, H-9b (δ_H 2.87)/H-7, H-9a (δ_H 3.29)/H_3-19, H_3-19/H-6, and H_3-17/H-7 indicated that H-1, H-7, H-9b, H-14β, H_3-16, H_3-17, OAc-6, and OAc-10 were on the same side of the molecule, whereas H-6, H-9a and H_3-19 are oriented toward the opposite side. This assumption was further identical to the X-ray diffraction data of compound **3** (Fig. 4). Moreover, compound **3** was acetylated with Ac_2O in pyridine at room temperature to afford the acetylated product. The structure of **2** was elucidated by comparison of spectroscopic data with that of the product derived from **3**, whose relative configurations have been unambiguously determined, further supporting the 1*R**, 6*S**, and 10*S** configurations for **2**. Accordingly, the structure of cespitularin S (**2**) was proposed unambiguously.

The anti-inflammatory activities of compounds **1**—**9** were

tested using LPS-stimulated cells. Stimulation of RAW 264.7 macrophages with LPS resulted in up-regulation of the pro-inflammatory iNOS and COX-2 proteins (Fig. 5). Compound **2** (10 μM) reduced the levels of the iNOS protein (52.7±3.2%) and COX-2 protein (78.3±0.6%) compared with the control cells (LPS alone). However, compounds **7** and **8** did not inhibit the COX-2 protein expression, but significantly inhibited iNOS protein expression (86.7±8.4% and 72.0±15.1%). With the exception of the above findings, the obtained negative results indicated that **1**, **3**—**6**, and **9** exhibited insignificantly activity against LPS-stimulated RAW 264.7 macrophages. Furthermore, the housekeeping protein β-actin was not changed by the presence of these tested compounds. Under the same experimental conditions, 10 μM caffeic acid phenylthyl ester (CAPE) reduced the levels of the iNOS and COX-2 proteins to 1.5±2.1% and 70.2±11.5%, respectively, relative to the control cells stimulated with LPS.

As compounds **2**, **7**, and **8** significantly inhibited iNOS protein expression, the dose-dependent relationship for its activity was evaluated *in vitro*. The resulting effects on LPS-induced inflammatory response exhibited the inhibition on iNOS protein expression followed a dose-dependent pattern with stronger inhibition accompanied at higher concentration (Fig. 6). Indeed, compounds **2**, **7**, and **8** clearly showed suppressive effects on iNOS protein expression in a dose-dependent manner, with the effective concentrations 50 (EC_{50}s) of 7.8±0.7, 18.8±3.7, and 11.5±1.9 μM, respectively.

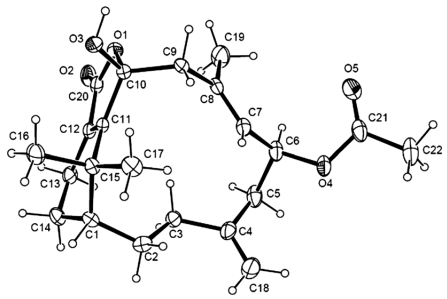

Fig. 4. X-Ray ORTEP Diagram of **3**

Experimental

General Experimental Procedures Optical rotations were determined with a JASCO P1020 digital polarimeter. Ultraviolet (UV) and infrared (IR) spectra were obtained on a JASCO V-650 and JASCO FT/IR-4100 spectrometer, respectively. The NMR spectra were recorded on a Bruker Advance 300 NMR spectrometer at 300 MHz for ^{1}H and 75 MHz for ^{13}C or on a Varian Unity INOVA 500 FT-NMR spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C, respectively, using $CDCl_3$ with tetramethylsilane (TMS) as internal standard. Chemical shifts are given in δ (ppm) and coupling constants in Hz. ESI-MS were recorded by ESI FT-MS on a Bruker APEX II mass spectrometer. Silica gel 60 (Merck, Germany, 230—400 mesh) and Sephadex LH-20 (Pharmacia, Sweden) were used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) were used for thin-layer chromatography (TLC) analysis. High-performance liquid chromatography (HPLC) was carried out using a Hitachi L-7100 pump equipped with a Hitachi L-7400 UV detector at 220 nm and a semi-preparative reversed-phase column (Merck, Hibar Purospher RP-18e, 5 μm, 250×10 mm).

Animal Material The Formosan soft coral *C. hypotentaculata* was collected by hand using scuba at the Green Islands located in the southeast coast of Taiwan, in March 2004, at a depth of 6 m, and was stored in a freezer until extraction. Identification was kindly verified by Prof. Chang-Feng Dai, Institute of Oceanography, National Taiwan University, Taipei, Taiwan. A voucher specimen (GN-67) was deposited in the Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Taiwan.

Extraction and Isolation The frozen soft coral *C. hypotentaculata* was chopped into small pieces and extracted with acetone for 24 h at room temperature. The quantity of solvent used for each extraction (2.0 l) was at least

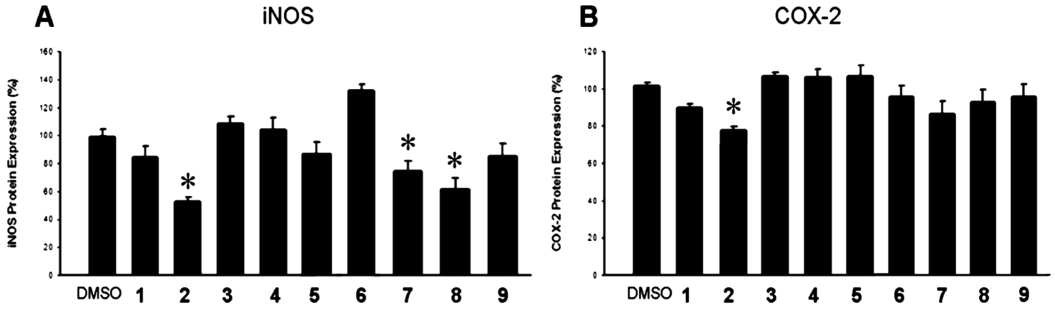

Fig. 5. Effect of Compounds **1**—**9** at 10 μM on the LPS-Induced Pro-inflammatory iNOS and COX-2 Proteins Expression of RAW 264.7 Macrophage Cells by Immunoblot Analysis

(A) Immunoblot of iNOS; (B) immunoblot of COX-2. The values are mean±S.E.M. (n=5). Relative intensity of the LPS alone stimulated group was taken as 100%. * Significantly different from LPS-stimulated (control) group (p<0.05).

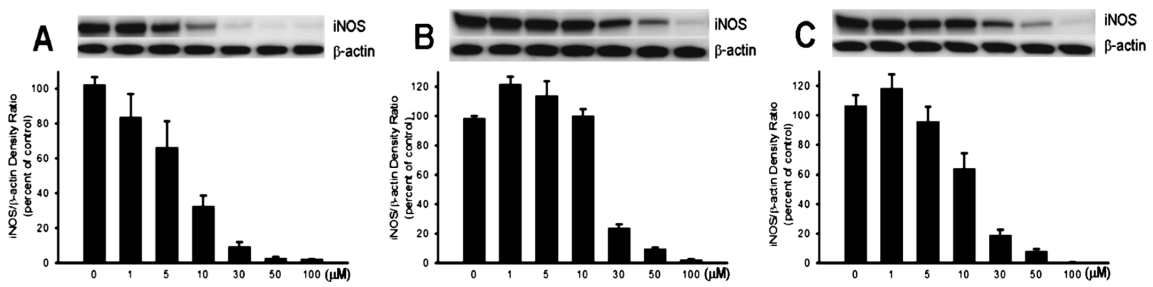

Fig. 6. Effects of **2**, **7**, and **8** on the LPS-Induced Pro-inflammatory iNOS Protein Expression in a Dose-Dependent Manner by Immunoblot Analysis

The cells were activated by incubation in medium containing *Escherichia coli* LPS (0.01 μg/ml) for 16 h in the presence or absence of **2**, **7**, and **8** (0—100 μM). (A) Immunoblot of iNOS/β-actin density ratio for **2**; (B) immunoblot of iNOS/β-actin density ratio for **7**; (C) immunoblot of iNOS/β-actin density ratio for **8**. The values are mean±S.E.M. (n=5). Relative intensity of the LPS alone stimulated group was taken as 100%.

three times the amount of the soft coral material used (1.5 kg). The combined extracts were concentrated *in vacuo* (under 35 °C) to obtain 20 g of dry extract, which was suspended in water and extracted with EtOAc and *n*-BuOH (saturated with H_2O) sequentially. The EtOAc phase was evaporated to dryness *in vacuo* to give a dark brown residue (15 g). The resulting EtOAc residue was subjected to a silica gel chromatography using a stepwise gradient mixture of *n*-hexane–EtOAc–MeOH as elution and separated into 38 fractions on the basis of TLC and ^{1}H-NMR analyses. Fraction 12 (1.2 g) was submitted to repeated chromatography over silica gel using *n*-hexane–acetone mixtures of increasing polarity as eluent to yield **6** (2 mg) and a mixture (124 mg). In turn, the mixture was further purified by RP-18 HPLC eluting with 90% MeOH in H_2O (flow rate 5.0 ml/min) to afford **1** (4 mg), **2** (3 mg), **4** (5 mg), and **5** (10 mg). Fraction 20 (0.7 g) was fractionated over Sephadex LH-20 eluting CH_2Cl_2–MeOH (3 : 1) to produce a mixture (78 mg) that was further purified by RP-18 HPLC using 85% MeOH in H_2O to give **3** (7 mg). Compound **9** (20 mg) was obtained by separation of fraction 23 (1.5 g) on a Sephadex LH-20 column eluting with CH_2Cl_2–MeOH (1 : 1). Fraction 28 (0.8 g) was submitted to repeated chromatography over silica gel using *n*-hexane–acetone (2 : 1) to afford **7** (30 mg). Similarly, compound **8** (40 mg) was obtained by separation of fraction 23 (1.5 g) on a silica gel column eluting with *n*-hexane–acetone (1 : 1).

Cespitularin R (**1**): Colorless, viscous oil; $[\alpha]_D^{25}$ +35 (c=0.2, CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 233 (4.80) nm; IR (KBr) ν_{max} 2934, 1735, 1640, 1020 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) and ^{13}C-NMR ($CDCl_3$, 75 MHz) spectroscopic data, see Table 1; ESI-MS *m/z*: 365 $[M+Na]^+$; HR-ESI-MS *m/z*: 365.2095 $[M+Na]^+$ (Calcd for $C_{22}H_{30}O_3Na$, 365.2093).

Cespitularin S (**2**): Colorless, viscous oil; $[\alpha]_D^{25}$ +116 (c=0.2, CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 228 (4.80) nm; IR (KBr) ν_{max} 2925, 1775, 1734, 1236, 1211 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 125 MHz) spectroscopic data, see Table 1; ESI-MS *m/z*: 439 $[M+Na]^+$; HR-ESI-MS *m/z*: 439.2093 $[M+Na]^+$ (Calcd for $C_{24}H_{32}O_6Na$, 439.2096).

Crystallographic Data and X-Ray Structure Analysis of 3[10] A suitable colorless crystal of compound **3** was grown by slow evaporation of the acetone containing few drop of *n*-hexane at room temperature. Diffraction intensity data were acquired with a Rigaku AFC7S single-crystal X-ray diffractometer with graphite-monochromated Mo$K\alpha$ radiation (λ=0.71073 Å). Crystal data for **3**: $C_{22}H_{30}O_5$ (formula weight 374.46), approximate crystal size, 0.7×0.7×0.2 mm^3, monoclinic, space group, $P2_12_12_1$, T=298(2) K, a=10.391(2) Å, α=90°, b=8.9666(18) Å, β=113.69(3)°, c=11.953(2) Å, γ=90°, V=1019.8(4) Å^3, D_c=1.219 Mg/m^3, Z=2, F(000)=404, $\mu_{(MoK\alpha)}$=0.085 mm^{-1}. A total of 2238 reflections were collected in the range 1.86°<θ<25.99°, with 2136 independent reflections [$R_{(int)}$=0.0106], completeness to θ_{max} was 100.0%; psi-scan absorption correction applied; full-matrix least-squares refinement on F^2, the number of data/restraints/parameters were 2136/1/248; goodness-of-fit on F^2=1.067; final R indices [I>2σ(I)], R_1=0.0378, wR_2=0.1007; R indices (all data), R_1=0.0471, wR_2=0.1069, largest difference peak and hole, 0.182 and −0.172 e/Å^3.

Acetylation of 3 A mixture of **3** (1.0 mg), Ac_2O (a drop) and pyridine (1.0 ml) stood at room temperature overnight and then diluted with 1.0 ml H_2O. The crude residue was suspended in H_2O and extracted with EtOAc. The EtOAc extract was purified by a short silica gel column using *n*-hexane–EtOAc (3 : 1) to afford an acetylated product (0.9 mg). The ^{1}H-NMR spectroscopic data of the product were in good agreement with those of **2**.

***In Vitro* Anti-inflammatory Assay** Murine RAW 264.7 macrophages were obtained from the American Type Culture Collection (ATCC, No. TIB-71) and cultured in Dulbecco's modified essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum, at 37 °C in a humidified 5% CO_2–95% air incubator under standard conditions. For statistical analysis, all the data were analyzed by a one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls *post hoc* test for multiple comparisons. A significant difference was defined as a p value of <0.05. The anti-inflammatory assay was carried out according to the procedures described previously.[11—14]

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10) Crystallographic data for **3** have been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 756969). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).
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