Isolation and Structure Assignment of an Iminotetrasaccharide from a Cultured Filamentous Cyanobacterium *Anabaena* sp.†

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In search of bioactive metabolites in cultured microalgae, a novel acylated iminopseudotetrasaccharide was isolated from a filamentous cyanobacterium *Anabaena* sp., and its structure was established by NMR, chemical degradation, and X-ray crystallography. The compound consists of glucose, glucuronic acid, arabinose, and 1,4,6-trideoxy-1,4-imino-D-allitol, which is closely related to potent glucosidase inhibitors such as nojirimycin. The tetrasaccharide itself showed moderate inhibitory activity against β-D-glucuronidase.

Cyanobacteria, also known as blue-green algae, have yielded a great number of secondary metabolites with interesting bioactivity.[1−7] As part of a consorted effort to discover anticancer therapeutic agents in aquatic organisms,[8] we have been screening cultured microalgae of both marine and freshwater origins. Our major targets were the lower eukaryotic algae such as dinoflagellates, but we also examined the blue-green algal strains, which had been collected and maintained over the years in our laboratory for the study of algal toxins. Our particular interest was in the water-soluble compounds, which had been studied little in the past. In this paper, we report the isolation of an unusual modified tetrasaccharide derivative containing a cyclic iminohexose alcohol.

Results and Discussion

The freeze-dried cells of the cyanobacterium *Anabaena* sp. were extracted with aqueous acetic acid. The extract was subjected to solid-phase extraction using Diaion HP-20. The Diaion HP-20 column was washed with water and extracted with methanol/water. The methanol/water eluate was chromatographed on a C-18 silica gel column using mixtures of methanol/water. The combined fractions were subjected to further fractionation on a Whatman ODS-3 column to yield pure compound **1**.

Compound **1** was freely soluble in water and sparingly in alcohols. It showed an $(M + H)^+$ ion at *m*/*z* 716 by positive FABMS and an $(M - H)^-$ ion at *m*/*z* 714 by negative FABMS. The HRFABMS suggested that **1** had the molecular formula $C_{28}H_{45}NO_{20}$. The UV spectrum of the compound showed only an end absorption, indicating the absence of any conjugated system.

The ^{1}H NMR spectrum of **1** in D_2O showed one secondary methyl doublet at δ 1.34 (3H, d, 6.5 Hz), two acetoxy methyl singlets at δ 2.10 (3H, s) and 2.16 (3H, s), one methoxy methyl singlet at δ 3.62 (3H, s), three signals at δ 5.15 (1H, d, 3.6 Hz), 5.17 (1H, d, 3.5 Hz), and 5.62 (1H, d, 3.2 Hz), which appeared to be the anomeric protons of sugars, two protons at δ 5.28 (1H, dd, 6.5, 3.5 Hz) and 5.37 (1H, br s), and signals between δ 3.40 and 4.60 for 19 protons (Table 1).

Upon acetylation with acetic anhydride/pyridine (1:1), **1** gave a peracetylated product, **2**, which had 10 acetate groups as confirmed by HRFABMS and NMR. Since two acetyl groups already existed in the compound, **1** must contain eight open primary/secondary OH/NH groups.

Table 1. Assignment of ^{1}H and ^{13}C NMR Signals for Compound **1**[a] in D_2O

position	δ H	δ C
A-1	5.15	97.7
A-2	3.57	71.4
A-3	3.63	73.4
A-4	3.48	72.5
A-5	4.00	71.9
A-6		176.7
B-1	5.62	96.7
B-2	4.27	71.9
B-3	5.28	68.8
B-4	5.37	69.6
B-5	3.80, 4.08	61.3
B-3 OAc	2.10	20.8, 173.6
B-4 OAc	2.16	20.6, 173.7
C-1	5.17	98.6
C-2	3.76	70.6
C-3	3.83	82.6
C-4	3.85	72.1
C-5	3.85	72.1
C-6	3.89	60.5
OCH3	3.62	58.9
D-1	3.45	50.0
D-2	4.59	68.7
D-3	4.51	76.3
D-4	3.79	65.0
D-5	4.24	65.2
D-6	1.34	18.8

[a] 400 MHz for ^{1}H and 100.6 MHz for ^{13}C NMR; assignments by COSY, HMQC, and DEPT.

The proton noise-decoupled ^{13}C NMR spectrum of **1** in D_2O showed 25 signals when it was taken at $D_1 = 200$ ms. However, at $D_1 = 2.00$ s the spectrum showed 26 signals. When the spectrum was taken in pyridine containing D_2O, the spectrum was further resolved into 28 carbon signals. The analysis of DEPT-135° and DEPT-90° spectra indicated the presence of four methyl groups (δ 18.8, 20.6, 20.8, 58.9), three methylenes (δ 50.0, 60.5, 61.3), 18 methines, and three quaternary carbons (δ 173.6, 173.7, 176.7). There were three sp^2 carbon signals, and their chemical shifts suggested that all of them belong to carbonyl or carboxyl carbons. Since the molecular formula suggested the presence of a total of 7 degrees of unsaturation in the molecule, the remaining 4 degrees of unsaturation must belong to rings.

The analysis of the ^{1}H−^{1}H COSY spectra of **1** led to four separate spin-systems, **A**−**D** (Figure 1), which are essentially sugar skeletons. The assignment of protons and corresponding carbon

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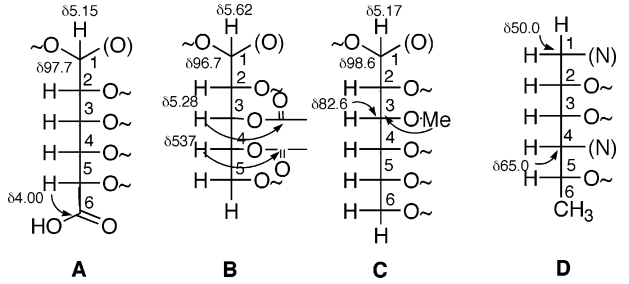


Figure 1. Four separate spin-systems of **1** obtained by ^{1}H COSY, HMQC, DEPT, HMBC, and ^{13}C NMR analysis.

signals of the fragments was made by HMQC and further confirmed by TOCSY and HMQC-TOCSY spectra.

The carboxyl carbon at δ 176.7 had a cross-peak with the methine proton at δ 4.00, suggesting that the carboxyl group is in fragment **A**. The two methine protons at δ 5.28 and 5.37 in fragment **B** were assigned to acetoxyl-bearing methines. In the HMBC spectrum, the carbon at δ 82.6 had a cross-peak with the methoxy methyl at δ 3.62, suggesting that the methoxy group is in fragment **C**. The presence of one methylene carbon at δ 50.0 and the corresponding methylene protons at δ 3.45 suggested that the nitrogen atom was attached to the methylene in fragment **D**. These results are summarized in Figure 1. Thus, fragment **D** is essentially an iminohexose alcohol.[9]

The linkage of fragments **A**−**D** was established by the HMBC and ROESY correlations, which are shown in Figure 2. The pyranose ring structures of the three sugar units, **A**, **B**, and **C** and the five-membered iminocyclic form were confirmed by HMBC correlations (Figure 2). Comparing the proton coupling constants and carbon chemical shifts of known hexo- and pentopyranoses,[10−13] we concluded that the three sugar units are glucuronic acid, arabinose, and glucose derivatives. Thus, **A**, **B**, and **C** are glucuronic acid, 3,4-di-*O*-acetylarabinopyranose, and 3-*O*-methylglucopyranose,[10] respectively, which were later confirmed by hydrolysis/analysis and X-ray crystallography. The coupling constants of the three anomeric protons at δ 5.15 (1H, d, 3.6 Hz), 5.17 (1H, d, 3.5 Hz), and 5.62 (1H, d, 3.2 Hz) and the corresponding carbon signals at δ 97.7, 96.7, and 98.6 indicated that the sugar units were all connected by α-linkages.[10,13]

When compound **1** was subjected to hydrolysis in 4 N trifluoroacetic acid at 100 °C, the reaction mixture contained three major compounds; two of them were identified as glucuronic acid and arabinose. The third major spot was a disaccharide derivative according to its R_f values on TLC. After acetylation of the hydrolysis mixture and normal-phase chromatography on silica gel, the acetylated compound, **3**, was obtained (Scheme 1). The ^{1}H NMR spectrum of **3** showed signals matching fragments **C** and **D** in **1**. Further hydrolysis of **3** with 4 N TFA at 120 °C for 2 h yielded two major compounds. The mixture was reacetylated and chromatographed on silica gel to give the acetylated cyclic iminosugar alcohol **4**, whose ^{1}H NMR spectrum showed the attachment of four acetate groups. The proton and COSY spectral data of **4** are in good agreement with partial structure **D** in the original compound **1**.

It was assumed that methylglucose, glucuronic acid, and arabinose moieties are the normal naturally occurring forms, i.e., D-, D-, and L-forms, respectively, and this was confirmed by X-ray crystallography.

Not many structures of oligosaccharides have been determined by X-ray crystallography, but we succeeded in forming suitable crystals of **1** itself from a mixture of water and ethanol. The structure obtained by X-ray crytallography confirmed the previously assigned sugar components and linkage, and unequivocally established its stereochemistry. Assuming glucose as the normal D-form, the absolute configurations of the other two sugars are D-glucuronic

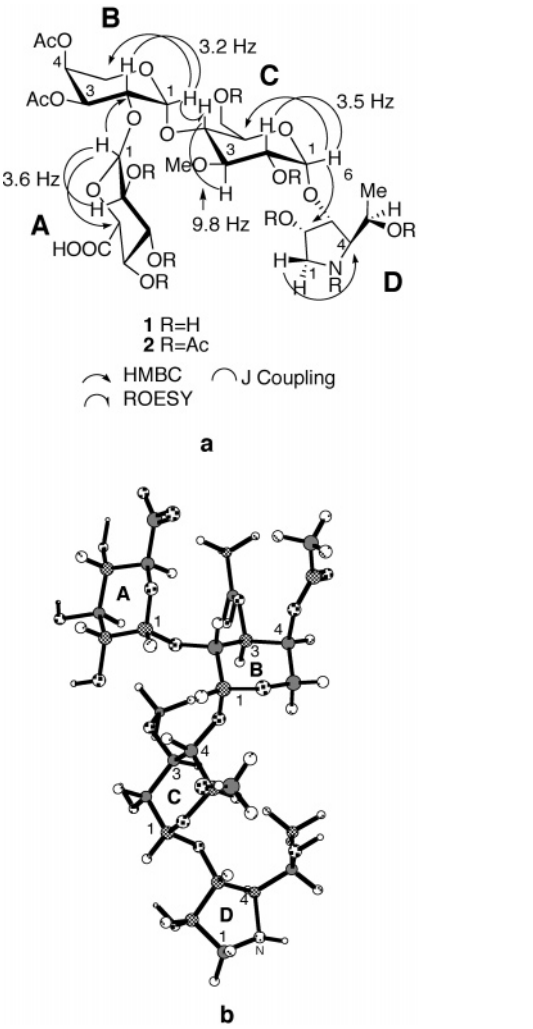
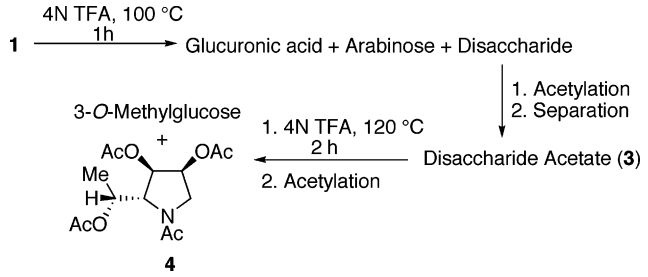


Figure 2. Structure and ChemDraw3D plotting of the X-ray structure of iminotetrasaccharide **1**. Arrows show important *J* couplings and correlations observed by HMBC and ROESY.

Scheme 1. TFA Hydrolysis of Tetrasaccharide **1**



acid, L-arabinose, and 1,4,6-trideoxy-1,4-imino-D-allitol. Before the X-ray work, we unsuccessfully attempted to establish the stereochemistry of the fragment **D** by chemical correlation with known 6-deoxysugars. In retrospect, the relative stereochemistry speculated from the proton coupling constants of the five-membered ring, though it was energy-minimized, was quite misleading.

Sugar modification of proteins and other cell components plays important roles in various biological processes. For that reason, cyclic azasugar derivatives or iminosugars such as the nojirimycins have been attracting enormous attention as selective glycosidase inhibitors.[14,15] A great number of natural and synthetic compounds have been tested against different types of glycosidases as potential anticancer drugs and other therapeutic agents.[15,16] We tested compound **1** with several glycosidases, but **1** demonstrated only moderate inhibitory activity against *E. coli* glucuronidase. The

structures isomeric or very similar to the iminosugar component **D** are all known to have potent inhibitory activity against rhamnosidase and other glycosidic enzymes.[17−24] Among them, 1,4,6-tridexoy-1,4-imino-D-mannitol[20] and 1,4-dideoxy-1,4-imino-L-allitol[21,22] are potent α-mannosidase inhibitors, while 1,4-dideoxy-1,4-imino-L-rhamnitol is a potent naringinase inhibitor.[23]

Blue-green algae are a rich source of various peptidase and other enzyme inhibitors.[25] Although the significance of those inhibitors to the producing organisms is not fully understood, defense against predators is the most discussed function.[26,27] In that context, it is quite possible that **1** is a precursor, which can release the active inhibitor after facile hydrolysis in the digestive system of predators. Iminosugars have been found in many plants and microorganisms, but so far there is only one report of di(hydroxymethyl)dihydroxy-pyrrolidine (DMDP; 2(*R*),5(*R*)-bis(hydroxymethyl)-3(*R*),4(*R*)-dihydroxylpyrrolidine) from a blue-green alga, *Cylindrospermum* sp.[26] As to oligosaccharides, there are a couple of reports of compounds such as cyclodextrin carbamate from cyanobacteria.[28,29] In general, the water-soluble compounds in microalgae have not been well studied; the current study shows the high likelihood of finding many interesting carbohydrates and other classes of compounds in blue-green algae.

Experimental Section

General Experimental Procedures. Optical rotation measurements were obtained on an AUTOPOL III automatic polarimeter (Rudolf Research, Flanders, NJ). UV spectra were measured on a Milton Roy Spectronic 1201. The NMR spectra were recorded on a Bruker Avance DPX-400 spectrometer. The 1D and 2D NMR spectra were analyzed using Bruker's WIN NMR (V. 6.0, 6.01) software. The high-resolution FABMS was taken in M.B. at the University of Illinois, Urbana−Champaign, mass spectrometry laboratory. Diaion HP-20 resin was purchased from Supelco (Supelco Park, Bellefonte, PA) and washed with methanol followed by deionized water prior to use. C18 silica gel (Bakerbond octadecyl, 40 uM for preparative LC packing) and silica gel (Baker, 40 μM for flash chromatography) were purchased from J. T. Baker Inc. (Phillipsburg, NJ).

Solvents used in this work were of HPLC grade and/or freshly distilled.

Culturing of Organism. The cyanobacterium *Anabaena* sp., which was originally isolated as a toxin-producing organism from Burringjuck Dam, NSW, Australia, in 1993 and in maintenance at the University of Rhode Island for 11 years (URI stock number, S2-217-2), was cultured at 25−27 °C in sterilized Fitzgerald medium under fluorescent lighting with a 16 h/8 h light and dark cycle. The large-scale culture in 5 gallon glass carboys was maintained for four to five weeks under aeration. The stationary phase culture was harvested by adding 5% alum solution. After settling the cells at the bottom, the supernatant was siphoned out and the cell suspension was centrifuged at 5000 rpm for 10 min using a Sorvall RC5B superspeed refrigerated centrifuge. The cells were frozen at −20 °C and then lyophilized.

Isolation of Iminotetrasaccharide 1. The freeze-dried cells (10 g) were extracted with 1% aqueous acetic acid (3 × 150 mL) in a microwave oven for 2 min. The larger scale extraction was carried out at 60 °C by stirring. The cell suspension was centrifuged at 5000 rpm for 10 min. The supernatant aqueous acetic acid extract was diluted with an equal volume of deionized water and subjected to solid-phase extraction using a nonionic resin Diaion HP-20 column (11 × 3 cm), which was previously equilibrated with deionized water. After eluting the extract, the column was washed with deionized water (200 mL) and was extracted with methanol (500 mL). The saccharides were eluted immediately, and the aqueous methanol extract was evaporated to dryness under reduced pressure at ∼50 °C. The extract (0.20 g) was subjected to reversed-phase chromatography on C18 silica gel column (15 × 2 cm). The column was eluted with water followed by 20%, 50%, and 70% aqueous methanol and methanol. The 20% aqueous methanol fraction (37 mg), which contained compound **1**, was further purified on HPLC using a Whatman Partisil 10 ODS-3 column (250 × 10 mm) with methanol/water (1:9). Compound **1** eluted at 27 min as a pure compound (13 mg) [R_f 0.76, Merck RPC_{18} silica gel, thickness 0.25 mm, methanol/water (1:1)].

Crystallization of 1 for X-ray Crystallography. Compound **1** was dissolved in 80% aqueous ethanol at 70 °C, and the solution was left standing at 5 °C. Hexagonal crystals were collected and used for crystallography.

Compound 1: colorless solid or plates; mp 211−213 °C (dec); $[\alpha]^{26}_D$ +156.2 (*c* 1.0, methanol/water, 1:1); ^{1}H and ^{13}C NMR, see Table 1; COSY; H-A1/H-A2, H-A2/H-A1, -A3, H-A3/H-A2, -A4, H-A4/H-A3, -A5, H-B1/H-B2, H-B2/H-B1, -B3, H-B3/H-B2, -B4, H-B4/H−B3, -B5a, B5b, H-B5a/H-B4, H-B5b/H-B4, H-C1/H-C2, H-C2/H-C1, -C3, H-C3/H-C2, -C4, H-C4/H-C3, -C5, H-C5/H-C4, H-D1/H-D2, H-D2/H-D1, -D3, H-D3 /H-D2, -D4, H-D4/H-D3, -D5, H-D5/H-D4, -D6, H-D6/H-D5; FABMS (positive mode) *m*/*z* 716 $[M + H]^+$; FABMS (negative mode) *m*/*z* 714 $[M - H]^-$; HRFABMS *m*/*z* $[M + H]^+$ 716.2616 (calcd for $C_{28}H_{46}NO_{20}$, 716.2613).

Acetylation of Compound 1. To 9.0 mg of compound **1** were added 0.5 mL of pyridine and 0.5 mL of acetic anhydride. The contents were kept at RT for 15 h. The solvent was evaporated to dryness under a stream of N_2 gas. The residue (14.7 mg) was purified on a silica gel column (10 × 1 cm) using the solvent chloroform/methanol (10:1). The pure acetylated compound **2** was obtained as a viscous oil (9.2 mg) [R_f 0.32, Merck RPC_{18} silica gel, thickness 0.25 mm, methanol/water (1:1)].

Compound 2: colorless viscous oil; $[\alpha]^{26}_D$ +103.4 (*c* 0.85, $CHCl_3$); ^{1}H NMR (pyridine-d_5, 400 MHz) δ 1.39 (3H, d, 6.6.Hz), 1.95 (3H, s), 2.04 (3H, s), 2.07 (3H, s), 2.08 (3H, s), 2.09 (3H, s), 2.14 (3H, s), 2.22 (3H, s), 2.26 (3H, s), 2.29 (3H, s), 2.30 (3H, s), 3.66 (3H, s), 3.60−6.12 (24 H); ^{13}CNMR (pyridine-d_5, 100.6 MHz) δ 17.6, 21.0, 21.0, 21.0, 21.0, 21.1, 21.1, 21.4, 21.4, 22.8, 49.4, 59.7, 62.1, 63.7, 67.3, 69.3, 69.3, 70.0, 70.1, 70.6, 70.9, 71.2, 71.6, 72.7, 73.7, 74.2, 75.2, 80.2, 81.2, 95.0, 97.0, 97.6, 170.2, 170.3, 170.3, 170.3, 170.4, 170.5, 170.6, 170.9, 171.0, 171.0, 171.0, 171.1; HRFABMS *m*/*z* $[M + H]^+$ 1052.3477 (calcd for $C_{44}H_{62}NO_{28}$, 1052.3458).

Trifluoroacetic Acid Hydrolysis of Compound 1. Compound **1** (10.5 mg) was hydrolyzed in 4 N trifluoroacetic acid at 100 °C in a Pierce Reactvial for 1 h. The reaction mixture was evaporated to dryness under N_2 gas. The residue was dissolved in distilled water. The reaction mixture contained three major spots; two were comparable to glucuronic acid [Whatman K6F silica gel (60 Å, thickness 250um), R_f 0.69, 1-PrOH/8% NH_4OH (1:1)] and arabinose [Whatman K6F silica gel (60 Å, thickness 250 μm) impregnated with 0.02 M sodium acetate (R_f 0.38, acetone/water (9:1)]. The hydrolyzed reaction mixture was dried and acetylated with pyridine/acetic anhydride (2:1) at 60 °C for 3 h. The acetylated reaction mixture (9.8 mg) was chromatographed on silica gel to give the disaccharide compound **3** as a hexaacetate (1.8 mg): ^{1}H NMR of **3** ($CDCl_3$, 400 MHz) δ 1.32 (3H, d, 6.5 Hz), 2.04 (3H, s), 2.06 (3H, s), 2.08 (3H, s), 2.10(6H, s), 2.13 (3H, s), 3.36 (1H, t, 9.2 Hz), 3.49 (3H, s), 3.73 (1H, t, 9.7 Hz), 3.82 (1H, m), 3.90 (1H, t, 8.5 Hz), 4.05 (1H, dd, 12.3, 1.6 Hz), 4.14 (1H, br s), 4.21 (1H, dd, 12.4, 4.9 Hz), 4.52 (1H, d, 4.2 Hz), 4.75 (1H, dd, 10.0, 3.7 Hz), 5.00 (1H, t, 9.6 Hz), 5.35 (1H, d, 3.5 Hz), 5.37 (2H, m). The acetylated disaccharide compound (1.7 mg) was further hydrolyzed using 4 N trifluoroacetic acid (500 μL) at 120 °C for 2 h. The solvent was evaporated to dryness, and the residue (1.5 mg) was acetylated with pyridine/acetic anhydride (1:1) at 60 °C for 3 h. The acetylated reaction mixture was poured on ice and extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with 0.5 N HCl followed by 2% sodium bicarbonate and water, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue (1.2 mg) was chromatograhed on a silica gel column (7.5 × 0.5 cm) using benzene/ethyl acetate (4:1) to yield pure cyclic iminosugar alcohol acetate **4** (0.8 mg): ^{1}HNMR ($CDCl_3$, 400 MHz) δ 1.31 (3H, d, 6.5 Hz), 2.07 (3H, s), 2.08 (6H, s), 2.10 (3H, s), 3.49 (1H, dd, 10.1, 7.0 Hz), 3.83 (1H, dd, 10.1, 7.5 Hz), 4.14 (1H, br s), 5.38 (1H, dq, 2.01, 6.53 Hz), 5.48 (1H, td, 7.0, 4.5 Hz), 5.57 (1H, dd, 4.5, 1.5 Hz).

Glycosidase Inhibition Assay.[18] The enzymes α-D-glucosidase from *Bacillus stearothermophilus*, β-D-glucosidase from almonds, β-mannosidase from snail, α-fucosidase from bovine kidney, α-galactosidase from coffee beans, β-galactosidase from bovine liver, neuraminidase from *Clostridium perfringens*, β-glucuronidase from *E. coli*, and rhamnosidase from *Penicillium decumbens* and its substrates *p*-nitrophenyl glycosides were purchased from Sigma Chemical Co. (St. Louis, MO). The glycosidase inhibition assay was done at pH 6.8 for glucosidase, pH 4.0 for mannosidase, rhamnosidase, and fucosidase, pH 5.0 for glucuronidase and fucosidase, and pH 7.3 for β-galactosidase and *p*-nitrophenyl α/β-glycosides as substrate. The mixture was

incubated for 30 min at 37 °C. The reaction was terminated by adding 0.1 N NaOH. The released *p*-nitrophenol was measured at 400 nm. Compound **1** showed 50% inhibition at 560 μM against β-glucuronidase.

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Supporting Information Available: NMR spectra and crytallographic data of compound **1**.[30] This material is available free of charge via the Internet at http://pubs.acs.org.

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(30) Crystallographic data for the structure **1** reported in this paper have been deposited with the Cambridge Crystallographic Data Center (deposition number CCDC 298737). 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-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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