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Note

Synthesis of the 3″-sulfate ester of β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*

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Green *et al.*[1–3] have reported that the asparagine-linked carbohydrate chains of bovine lutropin (luteinizing hormone) contain both D-GalNAc and sulfate groups, and that the major ionic oligosaccharide has structure **1**. The position of esterification of the D-Gal*p*NAc groups by a sulfate group could not be unequivocally established in these studies but could be restricted to OH-3 or -4.

To aid in the structural elucidation of the lutropin carbohydrate chains, and to provide chemically well-defined structures for use in a study of the significance of hormonal glycosylation, we have prepared a number of synthetic oligosaccharides corresponding to partial structures of **1**. We have recently reported[4] the preparation of both the trisaccharide **2** and its 4″-sulfate ester **3** which were used to demonstrate that the major bovine lutropin oligosaccharides are in fact sulfated exclusively at C-4 of the D-Gal*p*NAc groups. We simultaneously prepared the 3″-sulfated trisaccharide **4**, whose synthesis is presented herein. This trisaccharide served as a ^{1}H-n.m.r.-reference standard to demonstrate the absence of 3″-sulfated D-Gal*p*NAc groups in lutropin[4]. Structures **2–4** are further being used in the production and screening of monoclonal antibodies specifically directed against the carbohydrate chains of the glycoprotein hormones.

Glycosylation of disaccharide **6** (ref. 4) with the D-*galacto*-phthalimido[5,6] bromide **5** gave the protected β-D-linked trisaccharide **7** in 85% yield. Removal of phthalimido groups by treatment with hydrazine acetate[7] in refluxing methanol, followed by acetylation then gave the di-*N*-acetyl derivative **8** (79%) from which

β-D-Gal*p*NAc-3- or 4-SO_3^--(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→)-Asn
6
↑
1
β-D-Gal*p*NAc-3- or -4-SO_3^--(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*

1

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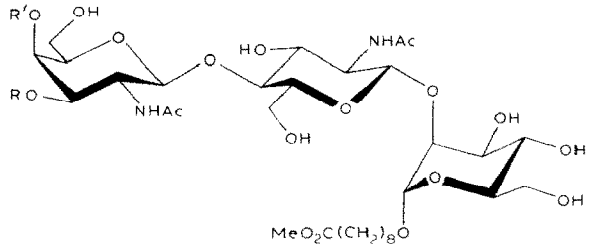
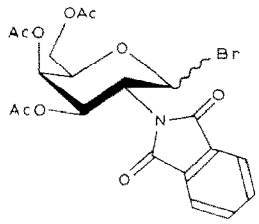


2 R = R′ = H
3 R = H, R′ = SO_3Na
4 R = SO_3Na, R′ = H



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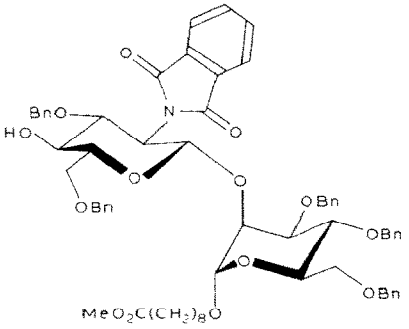
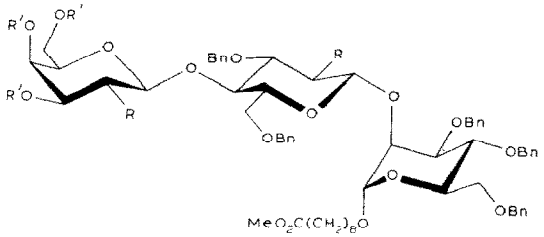
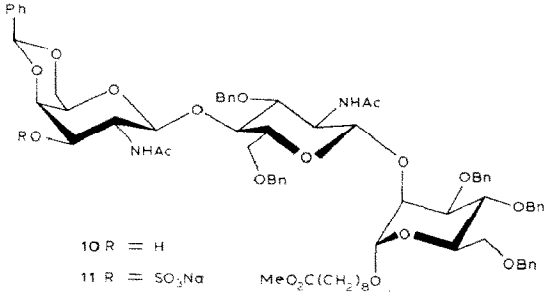


6



7 R = NPhth, R′ = Ac
8 R = NHAc, R′ = Ac
9 R = NHAc, R′ = H



10 R = H
11 R = SO_3Na

TABLE I

CHARACTERISTIC ^{1}H-N.M.R. CHEMICAL SHIFTS (δ) AND COUPLING CONSTANTS (Hz)[a] FOR TRISACCHARIDES **2**, **3**, AND **4**[b]

Atom	*Compound*		
	2	**3** *(4''-sulfate)*	**4** *(3''-sulfate)*
H-1 ($J_{1,2}$)	4.854 (1.6)	4.856 (1.5)	4.855 (1.6)
H-1' ($J_{1',2'}$)	4.559 (8.2)[c]	4.558 (8.3)[c]	4.561 (8.3)[c]
H-1'' ($J_{1'',2''}$)	4.514 (8.4)	4.587 (8.2)[c]	4.672 (8.5)
H-2 ($J_{2,3}$)	4.032 (3.4)	4.032 (3.4)	4.034 (3.4)
H-2' ($J_{2',3'}$)	3.73 (—)[d]	3.76 (—)[d]	3.75 (– ·)[d]
H-2'' ($J_{2'',3''}$)	3.94 (—)[d]	3.81 (—)[d]	4.066 (11.0)
H-3'' ($J_{3'',4''}$)	<4.0[d]	3.81 (<1)	4.254 (<1.0)
H-4'' ($J_{4'',5''}$)	<4.0[d]	4.692 (<1)	4.254 (<1.0)

[a]In parentheses. [b]Recorded at 360 MHz for solutions in deuterium oxide at 296 K and acetone (0.01%, δ 2.225) as internal standard. [c]Multiplets due to virtual coupling[11]. [d]Were not determined because of spectral overlap.

the *O*-acetyl protecting groups were removed with sodium methoxide in methanol to provide triol **9** (91%).

Selective benzylidenation[8] of **9** gave the 4,6-*O*-benzylidene derivative **10** (80%), which was sulfated with sulfur trioxide–pyridine complex in *N,N*-dimethylformamide to provide **11** (94%). Hydrogenolysis of the benzylidene and benzyl groups in **11** then provided, after ion-exchange, the required sodium salt **4** (81%).

Table I summarizes the most readily measured ^{1}H-n.m.r. parameters for trisaccharides **2–4**. Sulfation of **2** to produce either **3** or **4** can be seen to cause a large downfield shift in the signal for the proton attached to the ring carbon atom carrying the sulfate ester. The signal for this proton moves into a region of the spectrum, normally reserved for anomeric protons, where it is no longer obscured by the ring-proton "envelope". This characteristic deshielding has long been known[9] and allowed a definitive assignment of the position of sulfation as recently noted by Jacquinet and Sinaÿ[10] in synthetic dermatan sulfate fragments. The chemical shifts of H-3 and H-4 of the β-D-Gal*p*NAc groups in **3** and **4**, readily identified by homonuclear decoupling, can therefore be considered diagnostic of the position of sulfation in terminally sulfated β-D-Gal*p*NAc-containing oligosaccharides.

EXPERIMENTAL

General methods. — Optical rotations were measured with a Perkin–Elmer 241 polarimeter at ambient temperatures (22 ±2°). ^{1}H-N.m.r. spectra were recorded at either 360 MHz (Bruker WM-360) or 300 MHz (Bruker AM-300) with either tetramethylsilane (δ 0 in $CDCl_3$ and CD_3OD) or acetone (δ 2.225 in D_2O) as internal standard at ambient temperature. ^{13}C-N.m.r. spectra were recorded at 75.5 MHz (Bruker AM-300) with either internal tetramethylsilane (δ 0 in $CDCl_3$ or

CD_3OD) or external 1% 1,4-dioxane (δ 67.4 in D_2O) as reference standards. Only partial n.m.r. data are reported. Other spectra features were in accord with the proposed structures. The ^{1}H-n.m.r. chemical shifts and coupling constants are reported as though they were first order. Assignments of ^{13}C resonances are tentative. T.l.c. was performed on precoated plates of Silica Gel 60-F_{254} (E. Merck, Darmstadt) with detection by quenching of fluorescence, or by charring, or both, after spraying with 5% H_2SO_4 in ethanol. Unless otherwise noted, column chromatography was performed on Silica Gel Merck 60 (30–63 μM). Iatrobeads refers to a silica gel (product 6RS-8060) produced by Iatron Laboratories (Tokyo, Japan). For gel filtration, Bio-Gel P-2 (200–400 mesh) (Bio-Rad Laboratories, Richmond, CA) was used. Unless otherwise noted, all reactions were carried out at ambient temperature and, in the processing of reaction mixtures, solutions of organic solvents were washed with equal volumes of aqueous solutions. SO_3–pyridine complex was from Aldrich. Organic solutions were dried over Na_2SO_4 prior to solvent removal on a rotary evaporator under the vacuum of a water aspirator with bath temperature of 40° or lower. The microanalyses were carried out by the Analytical Services Laboratory of this department.

8-Methoxycarbonyloctyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl)-(1→4)-O-(3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→2)-3,4,6-tri-O-benzyl-α-D-mannopyranoside (**7**). — A mixture of **6** (ref. 4; 110 mg, 0.10 mmol), silver trifluoromethanesulfonate (57 mg, 0.22 mmol), 2,4,6-trimethylpyridine (30 μL, 0.23 mmol) and 4A molecular sieves (0.2 g) in dichloromethane (0.5 mL) was stirred for 1 h, and a solution of **5** (refs. 5 and 6; 100 mg, 0.20 mmol) in dichloromethane (1.0 mL) was then added dropwise. After 4 h, the mixture was diluted with dichloromethane (50 mL), solids were removed by filtration and the filtrate was washed sequentially with water, 5% HCl, saturated $NaHCO_3$, and water. After solvent evaporation, the residue was purified by chromatography on Iatrobeads using 1:1 ethyl acetate–hexane as eluent to provide **7** as a white foam (130 mg, 85.5%); $[\alpha]_D^{22}$ −15.7° (*c* 1.7, chloroform); R_f 0.34 (1:1 ethyl acetate–hexane); ^{1}H-n.m.r. ($CDCl_3$): δ 5.791 (dd, 1 H, $J_{2'',3''}$ 11.5, $J_{3'',4''}$ 3.2 Hz, H-3''), 5.495 (d, 1 H, $J_{1'',2''}$ 8.5 Hz, H-1''), 5.378 (dd, 1 H, $J_{4'',5''}$ <1.0 Hz, H-4''), 5.124 (d, 1 H, $J_{1',2}$ 8.0 Hz, H-1'), 4.531 (dd, H-2''), 4.403 (d, 1 H, $J_{1,2}$ 1.5 Hz, H-1), 3.945 (dd, 1 H, $J_{2,3}$ 3.5 Hz, H-2), 3.648 (s, 3 H, OCH_3), 3.093 (dt, 2 H, OCH_2CH_2), 2.263 (t, 2 H, *J* 7.5 Hz, $CH_2CO_2CH_3$), 2.083, 2.035, and 1.833 (each s, 3 H, $OCOCH_3$); ^{13}C-n.m.r. ($CDCl_3$): δ 174.24 (CO_2CH_3), 170.30, 170.19 and 169.70 ($OCOCH_3$), 168.32 and 167.48 (NC=O), 97.42 and 96.82 (2 C) (C-1,1',1''), 61.02 (C-6''), 55.47 (C-2'), 52.10 (C-2''), 51.41 (OCH_3), 34.05 (CH_2CO_2), 20.70, 20.62, and 20.48 ($OCOCH_3$).

Anal. Calc. for $C_{85}H_{92}N_2O_{23}$: C, 67.62; H, 6.14; N, 1.86. Found: C, 67.31; H, 6.07; N, 1.66.

8-Methoxycarbonyloctyl O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranosyl)-(1→4)-O-(2-acetamido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-3,4,6-tri-O-benzyl-α-D-mannopyranoside (**8**). — A mixture of **7**

(150 mg, 0.10 mmol) and hydrazine acetate (275 mg, 3.0 mmol) was refluxed in dry methanol (15 mL) for 3 h; more hydrazine acetate (275 mg, 3.0 mmol) was then added and the refluxing was continued for a further 20 h. The mixture was then taken to dryness, the residue was dissolved in pyridine (3.0 mL), and acetic anhydride (2.0 mL) was added. After stirring for 20 h, excess acetic anhydride was decomposed by dropwise addition of ethanol to the mixture at 0°. Solvent was evaporated and toluene (3 × 5 mL) was added to and evaporated from the residue, which was then dissolved in dichloromethane. The solution was washed with 5% HCl, saturated $NaHCO_3$ and cold water. Evaporation of the solvent left a white solid which was purified by chromatography on Iatrobeads using 1:1 acetone–hexane as eluent to give **8**, a white foam (105 mg, 79%); $[\alpha]_D^{22}$ −13.2° (*c* 0.82, chloroform); R_F 0.22 (4:1 ethyl acetate–hexane); ^{1}H-n.m.r. ($CDCl_3$): δ 5.748 (d, 1 H, D_2O exchangeable, $J_{NH',2'}$ 7.2 Hz, NH′), 5.249 (dd, 1 H, $J_{3'',4''}$ 3.0, $J_{4'',5''}$ <1.0 Hz, H-4″), 5.053 (d, 1 H, $J_{1',2'}$ 7.2 Hz, H-1′), 4.752 (d, 1 H, $J_{1,2}$ 1.5 Hz, H-1), 3.655 (s, 3 H, OCH_3), 3.267 (ddd, $J_{2',3'}$ 9.3 Hz, H-2′), 2.296 (t, 2 H, *J* 7.5 Hz, CH_2CO_2), 2.075, 2.018 and 1.978 (each s, 3 H, $OCOCH_3$), 1.767 and 1.741 (each s, 3 H, $NHCOCH_3$); ^{13}C-n.m.r. ($CDCl_3$): δ 174.30 (CO_2CH_3), 171.58, 171.19 and 170.58 ($OCOCH_3$), 170.41 and 170.20 ($NHCOCH_3$), 100.15 (C-1″), 98.00 and 97.50 (C-1,1′), 61.00 (C-6″), 55.74 (C-2′), 51.33 (C-2″), 51.10 (OCH_3), 33.96 (CH_2CO_2), 24.77 (2 C) and 23.16 ($OCOCH_3$), 20.62 and 20.53 ($NHCOCH_3$).

Anal. Calc. for $C_{73}H_{92}N_2O_{21}$: C, 65.74; H, 6.95; N, 2.10. Found: C, 65.12; H, 6.96; N, 2.26.

O-*Deacetylation of compound* **8**. — Compound **8** (100 mg, 0.075 mmol) was dissolved in dry methanol (2.0 mL) containing sodium methoxide (5 mg). After 1 h, the solution was made neutral by addition of Amberlite IR-120 (H^+) cation-exchange resin, which was removed by filtration, and the solvent was evaporated to provide 8-methoxycarbonyloctyl *O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1→4)-*O*-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (**9**) as a clear syrup (80 mg, 91%); R_F 0.30 (9:1 dichloromethane–methanol). This material was not further characterized but was used directly for the preparation of **10**.

8-Methoxycarbonyloctyl O-(2-acetamido-4,6-O-benzylidene-2-deoxy-β-D-galactopyranosyl)-(1→4)-O-(2-acetamido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranosyl)-(1→2)-3,4,6-tri-O-benzyl-α-D-mannopyranoside (**10**). — A mixture of **9** (80 mg, 0.066 mmol) and *p*-toluenesulfonic acid (5 mg, 0.026 mmol) in *N,N*-dimethylformamide (2.0 mL) was stirred for 1 h at 0°. α,α-Dimethoxytoluene (50 μL, 0.33 mmol) was then added dropwise and stirring was continued for 2 h at 0°. The reaction was allowed to warm to room temperature and stirring was continued for a further 4 h. After neutralization with triethylamine and solvent evaporation, the residue was dissolved in dichloromethane and washed with water. Solvent evaporation left a residue which was purified by chromatography using 19:1 dichloromethane–methanol as eluent to provide **10** as a hygroscopic white solid (68.4 mg, 80%); $[\alpha]_D^{22}$ −5.7° (*c* 0.97, chloroform); R_F 0.41 (9:1 dichloromethane–methanol); ^{1}H-n.m.r. ($CDCl_3$): δ 6.086 (d, 1 H, D_2O exchangeable, $J_{NH',2'}$ 6.2 Hz,

NH′), 5.653 (d, 1 H, D_2O exchangeable, $J_{NH'',2''}$ 7.0 Hz, NH″), 5.513 (s, 1 H, C_6H_5CHOO), 5.107 (d, 1 H, $J_{1',2'}$ 8.0 Hz, H-1′), 4.734 (d, 1 H, $J_{1,2}$ 1.5 Hz, H-1), 3.658 (s, OCH_3), 3.324 (dt, 2 H, OCH_2CH_2), 3.163 (ddd, $J_{4'',5''}$ <1, $J_{5'',6a''}$ <2, $J_{5'',6b''}$ <2 Hz, H-5″), 3.157 (ddd, $J_{2',3'}$ 10.0 Hz, H-2′), 2.294 (t, 2 H, J 7.5 Hz, $CH_2CO_2CH_3$), 1.846 and 1.689 (each s, 3 H, $NHCOCH_3$), and 1.773 (bs, 1 H, D_2O exchangeable, OH); ^{13}C-n.m.r. ($CDCl_3$): δ 174.31 (CO_2CH_3), 172.38 and 171.32 ($NHCOCH_3$), 101.30 (C_6H_5CHOO), 100.27 (C-1″), 97.75 and 97.59 (C-1,1′), 57.50 (C-2′), 55.19 (C-2″), 51.46 (OCH_3), 34.10 ($CH_2CO_2CH_3$), 23.38 and 23.22 ($NHCOCH_3$).

Anal. Calc. for $c_{74}H_{90}N_2O_{18}$: C, 68.60; H, 7.00; N, 2.16. Found: C, 68.11; H, 6.81; N, 2.17.

8-Methoxycarbonyloctyl O-*(sodium 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-*D*-galactopyranosyl 3-sulfate)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-benzyl-β-*D*-gluco-pyranosyl)-(1→2)-3,4,6-tri-*O*-benzyl-α-*D*-mannopyranoside* (**11**). — Compound **10** (64 mg, 0.049 mmol) was dissolved in dry *N,N*-dimethylformamide (2.0 mL) and SO_3–pyridine complex (40 mg, 0.25 mmol) in dry *N,N*-dimethylformamide (1.0 mL) was added dropwise. After 1 h, excess reagent was destroyed by addition of methanol (1.0 mL). Solvent was evaporated (<35°) and the residue was dissolved in chloroform and washed with cold water. Chloroform evaporation left a white solid which was dissolved in methanol and passed through Dowex 50-X8 (Na^+) cation-exchange resin (10 mL) in methanol. Solvent removal provided **11** as a hygroscopic white solid (65 mg, 94%); R_F 0.30 (9:1 dichloromethane–methanol); ^{1}H-n.m.r. (CD_3OD): δ 5.585 (s, 1 H, C_6H_5CHOO), 4.507 (dd, $J_{3'',4''}$ 3.2, $J_{4'',5''}$ <1.0 Hz, H-4″), 4.391 (dd, $J_{2'',3''}$ 11.0 Hz, H-3″), 3.649 (s, 3 H, OCH_3), 3.394 (dt, 2 H, OCH_2CH_2), 3.209 (ddd, 1 H, $J_{5'',6a''}$ <2.0, $J_{5'',6b''}$ <2.0, $J_{4'',5''}$ <1.0 Hz, H-5″), 2.290 (t, 2 H, J 7.5 Hz, $CH_2CO_2CH_3$), 1.973 and 1.834 (each s, 3 H, $NHCOCH_3$); ^{13}C-n.m.r. (CD_3OD): δ 174.21 (CO_2CH_3), 171.43 (2 C, 2 $NHCOCH_3$), 100.68, 100.21, 98.77 and 96.66 (C_6H_5CHOO, C-1,1′,1″), 54.56 (C-2′), 50.50 and 50.39 (C-2″ and OCH_3), 33.10 ($CH_2CO_2CH_3$), 21.86 and 21.73 ($NHCOCH_3$).

8-Methoxycarbonyloctyl O-*(sodium 2-acetamido-2-deoxy-β-*D*-galacto-pyranosyl 3-sulfate)-(1→4)-*O*-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1→2)-α-*D*-mannopyranoside* (**4**). — Compound **11** (64 mg, 0.046 mmol) was dissolved in 95% ethanol (10 mL) containing 5% Pd–C (65 mg) and was stirred under H_2 (0.1 MPa) for 20 h. Catalyst was removed by filtration, washed with 95% ethanol, and the solvent was evaporated to dryness. The residue was then passed through a column (2.5 × 50 cm) of Bio-Gel P-2 (200–400 mesh) with 10% aqueous ethanol as eluent. The carbohydrate-containing fractions were pooled, concentrated, and the residue was dissolved in water and passed through Dowex 50-X8 (Na^+) (10 mL). Lyophilization of the eluent gave a hygroscopic white powder (32 mg, 81%); $[\alpha]_D^{22}$ −2.5° (*c* 0.48, chloroform); R_F 0.20 (60:35:6 chloroform–methanol–water); ^{1}H-n.m.r. (D_2O): δ 4.855 (d, 1 H, $J_{1,2}$ 1.6 Hz, H-1), 4.672 (d, 1 H, $J_{1'',2''}$ 8.5 Hz, H-1″), 4.561 (m, 1 H, $J_{1',2'}$ 8.3 Hz, virtual coupling to H-3′, H-1′), 4.434 (dd, 1 H, $J_{3'',4''}$ 3.1, $J_{2'',3''}$ 11.0 Hz, H-3″), 4.254 (dd, 1 H, $J_{4'',5''}$ <1.0 Hz, H-4″), 4.066 (dd, 1 H, H-2″),

4.034 (dd, 1 H, $J_{2,3}$ 3.4 Hz, H-2), 3.80 (dd, $J_{3,4}$ 9.5 Hz, H-3), 3.75 (dd, $J_{2',3'}$ 10.0 Hz, H-2'), 3.687 (s, OCH_3), 2.387 (t, 2 H, J 7.5 Hz, $CH_2CO_2CH_3$), 2.059 and 2.047 (each s, 3 H, $NHCOCH_3$); ^{13}C-n.m.r. (D_2O): δ 178.72 (CO_2CH_3), 175.59 (2 C, 2 $NHCOCH_3$), 102.10, 100.31 and 97.60 (C-1,1',1''), 80.08, 78.30, 77.38, 75.78, 75.35, 73.70, 72.96, 70.57, 68.14 and 66.94 (C-2,3,4,5,3',4',5',3'',4'',5''), 68.94 (OCH_2CH_2), 62.38, 61.71 and 60.92 (C-6,6',6''), 55.56 (C-2'), 52.93 (OCH_3), 51.68 (C-2''), 34.58 (CH_2CO_2), 23.20 and 23.09, $NHCOCH_3$).

Anal. Calc. for $C_{32}H_{55}N_2NaO_{21}S \cdot 2\ H_2O$: C, 42.95; H, 6.20; N, 3.13. Found: C, 43.08; H, 6.24; N, 2.78.

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