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Note

Synthesis of analogs of *N*-acetylneuraminic acid and their effect on CMP-sialate synthase*

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The diverse functions of cell surface sialic acid suggest that chemotherapeutic intervention in the biosynthesis of sialic acid could result in significant changes in the membranes properties of tumor cells. We have attempted, in the past, to manipulate the biosynthesis of sialic acid by use of synthetic analogs of D-glucosamine and D-mannosamine[1], the biosynthetic precursors to sialic acid, and also by the inhibition of sialyltransferases[2]. This paper describes yet another approach, namely the development of inhibitors and substrates of CMP-sialate synthase (EC 2.7.7.43). This enzyme catalyzes the transfer to CMP from CTP to O-2 of sialic acid, thus providing two opportunities for intervention in the incorporation of sialic acid into cell-membrane glycoconjugates. Sialic acid analogs that inhibit the enzyme would presumably reduce the amount of sialic acid incorporated into cell-surface glycoconjugates; and analogs that are substrates for the synthetase could produce either substrates or inhibitors of the sialyltransferase and, thus, affect the incorporation of sialic acid onto the cell surface.

Most sialic acid analogs synthesized to date have been ketosides or various *N*-acetyl derivatives[3,4]. A few analogs have been synthesized from *N*-acetylglucosamine and *N*-acetylmannosamine, such as *N*-acetyl-3-deoxy-3-fluoroneuraminic acid[5] and *N*-acetyl-3-hydroxyneuraminic acid[6]. In both cases, the configuration at C-3 was not determined and the yields were poor. More recently, the synthesis of the α- and β-glycosyl fluorides of *N*-acetylneuraminic acid was

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reported[7]. The 4-*O*-methyl derivative[8] was synthesized, and was later shown to be a substrate for CMP-sialate synthase from equine submandibular glands[9].

The *N*-acetylneuraminic acid (**1**) used in our synthetic studies was isolated from edible birds nest substance by the procedure of Czarnecki and Thornton[10]. Methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosyl chloride)onate[11] (**3**) and 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid[12] were synthesized by modifications of published procedures. Compound **1** was esterified with methanol in the presence of Dowex 50 (H^+) resin to give **2**, which was treated with acetyl chloride saturated with hydrogen chloride to give an excellent yield of the glycosyl chloride **3**. Treatment of **3** with triethylamine gave the desired 2,3-unsaturated derivative **7** in 62% yield, and this was deprotected to give **8** (90%). Treatment of **3** with tributyltin hydride and azobis(isobutyryl)nitrile in benzene[13] gave the 2,6-anhydro compound **4** in 82% yield. The orientation and configuration of H-2 were determined by ^{1}H-n.m.r. spectroscopy (Table I). The proton resonances of **4** were assigned by several decoupling experiments after D_2O exchange. This resulted in the loss of the NH resonance at δ 5.48 and the collapse of the quartet at δ 4.00 into a triplet, indicating that the signal for H-5 is at δ 4.00. Irradiation of the multiplet at δ 5.05 resulted in the collapse of the quartet at δ 2.40 into a doublet. The upfield position of the quartet at δ 2.40 suggested that it corresponds to one of the H-3 protons; therefore, the multiplet at δ 5.05 corresponds to H-4. Irradiation of the quartet at δ 2.40 simplified the multiplet at δ 5.05, and collapsed the quartet at δ 4.06 into a doublet, indicating that this resonance corresponds to H-2. Irradiation of the quartet at δ 4.65 collapsed the quartet at δ 4.14 into a doublet, and simplified the multiplet at δ 5.25. Irradiation of the quartet at δ 4.14 collapsed the quartet at 4.65 and simplified the multiplet at δ 5.25. These observations showed that the resonances at δ 4.14 and 4.65 correspond to H-9 and H-9b, respectively. Therefore, the resonance at δ 5.25 corresponds to H-8. Irradiation of the quartet at δ 3.70 collapsed δ 4.00 resonance into a doublet and the quartet at δ 5.37 into a doublet. Therefore, the

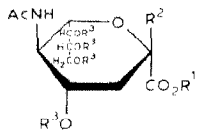
TABLE I

^{1}H-N.M.R. DATA[a] FOR COMPOUND **4**

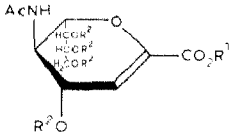
Chemical shifts (δ)										
H-2	*H-3*e	*H-3*a	*H-4*	*H-5*	*H-6*	*H-7*	*H-8*	*H-9*a	*H-9*b	*NH*
4.06	2.40	[a]	5.05	4.00	3.70	5.37	5.25	4.14	4.65	5.48

Coupling constants (J in Hz)											
*2,3*e	*2,3*a	*3*e,*3*a	*3*e,*4*	*3*a,*4*	*4,5*	*5,6*	*5,NH*	*6,7*	*7,8*	*8,9*a	*8,9*b	*9*a,*9*b
2.15	11.9	12.9	5.00	11.0	10.2	10.2	10.2	2.02	4.70	7.24	2.36	12.2

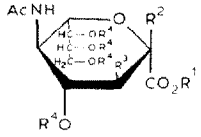
[a]Not determined.



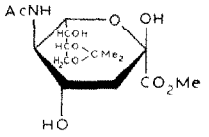
1 $R^1 = H, R^2 = OH, R^3 = H$
2 $R^1 = Me, R^2 = OH, R^3 = H$
3 $R^1 = Me, R^2 = Cl, R^3 = Ac$
4 $R^1 = Me, R^2 = H, R^3 = Ac$
5 $R^1 = R^2 = R^3 = H$
6 $R^1 = H, R^2 = OMe, R^3 = H$



7 $R^1 = Me, R^2 = Ac$
8 $R^1 = R^2 = H$



9 $R^1 = Me, R^2 = R^3 = F, R^4 = Ac$
10 $R^1 = H, R^2 = R^3 = F, R^4 = H$
11 $R^1 = H, R^2 = OH, R^3 = F, R^4 = H$
12 $R^1 = Me, R^2 = R^3 = Br, R^4 = H$



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resonance at δ 3.70 corresponds to H-6 and that at δ 5.37 to H-7. The coupling constant between the signal for H-4 and that at δ 2.40 was smaller than the coupling constant between the signal for H-4 and the hidden signal for H-3, which indicated a diaxial interaction. Therefore, the resonance at δ 2.40 is due to H-3*e*. The large coupling constants, $J_{4,5}$ 10.2 and $J_{3a,4}$ 11 Hz, indicated diaxial interactions, which are possible only if the molecule is in the $^2C_5$(D) conformation. The magnitudes of $J_{3a,2}$ of 11.9 and $J_{3e,2}$ of 2.15 Hz are indicative of an axial orientation for H-2. The assignment of the proton resonances and the determination of the coupling constants were based on a first-order interpretation of the spectrum. However, the ν/J for H-7 and H-8 was only 5, and hence the coupling constants could be in slight error.

Reduction of optically active halides with tributyltin hydride normally gives racemic mixtures[14,15]. However, in the case of **3**, only one product was formed, because the sugar radical may exist only in one configuration owing to the anomeric effect and, in the β configuration, the underside attack would be hindered by the methyl ester group. Compound **4** was deblocked to give the free acid **5**.

Reaction of the unsaturated derivative **7** with xenon difluoride in dichloromethane with BF_3·etherate as catalyst in an oxygen atmosphere gave the 2,3-difluoro compound **9**. When the reaction was performed without oxygen, the reaction failed to go to completion and gave considerable decomposition. The configuration

TABLE II

^{19}F-N.M.R. DATA FOR COMPOUNDS **9**, **10**, AND **11**

Cpd	*Solvent*	*Chemical shifts (δ)*		*J values (Hz)*			
		F-2	*F-3*	*F-2,F-3*	*F-2,H-3*	*F-3,H-3*	*F-3,H-4*
9[a]	$CDCl_3$	139.1	202.7	−19.5	19.5	47.0	13.5
10[b]	D_2O	56.7	124.7	−20.4	20.4	47.2	12.1
12[b]	D_2O		121.7			48.6	11.6

[a]From the signal of internal trichlorofluoromethane. [b]From the signal of external trifluoroacetic acid.

of the fluorine atoms of **9** were determined by ^{19}F-n.m.r. spectroscopy (Table II). The magnitude of $J_{F\text{-}2,F\text{-}3}$ (−19.5 Hz) indicated an axial–equatorial arrangement, and that of $J_{F\text{-}2,H\text{-}3}$ (19.5 Hz) a diaxial interaction. These coupling constants and the absence of any long-range coupling (*i.e.*, $J_{F\text{-}2,H\text{-}4}$, $J_{F\text{-}3,H\text{-}5}$, and $J_{F\text{-}2,H\text{-}6}$) suggested a $^2C_5$(D) conformation with F-3 equatorial and F-2 axial. The 2,3-difluoro compound **9** was deblocked to give **10** in 96% yield. Defluorination at C-2 was accomplished by heating **10** in 0.05M hydrochloric acid to give **11** (89%).

Addition of bromine to the double bond of **7** gave, in quantitative yield, the 2,3-dibromo derivative **12**. The configuration of Br-3 could be determined from the ^{1}H-n.m.r. spectrum. The magnitude of $J_{3,4}$ (10.4 Hz) was indicative of diaxial orientation for H-3 and H-4. Thus, Br-3 is in equatorial orientation and the molecule has the $^2C_5$(D) conformation. The configuration of Br-2 was not determined.

The methyl ester **2** was readily isopropylidenated in dry acetone with anhydrous copper sulfate as catalyst to give, in 91% yield, the crystalline 8,9-*O*-isopropylidene derivative **13**. Mass spectrometry (Table III) and ^{13}C-n.m.r. spectroscopy (Table IV) located the position of the isopropylidene group. The mass spectra of the methyl ester **2**, the 7,9-di-*O*-acetyl methyl ester[16], and the 8,9-isopropylidene

TABLE III

CHARACTERISTIC FRAGMENTATIONS A–G OF COMPOUNDS **2**, **13**, AND METHYL 7,9-DI-*O*-ACETYL-*N*-ACETYL-NEURAMINATE

Compound	*Fragment*						
	A	*B*	*C*	*D(D')*	*E*	*F*	*G*
2	668	624	478	298	317	205	173
13	564	520	478	298(388)	317	101	173
Neu 5,7,9Ac	608	564			317	175	173

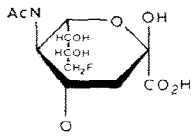
TABLE IV

^{13}C-CHEMICAL SHIFTS FOR COMPOUNDS **3, 4, 5, 7, 8, 12,** AND **13**

Carbon atom	*Chemical shifts (δ) of compound*						
	3	**4**	**5**	**7**	**8**	**12**	**13**
1	165.4	168.9	175.8	161.5	166.2	[a]	171.5
2	96.6	77.5	76.7	145.0	142.8	91.7	96.5
3	40.7	33.5	37.0	108.0	113.6	53.8	40.8
4	67.0	68.2	69.3	67.7	68.1	67.0	70.9
5	48.7	49.4	53.1	49.4	50.7	45.4	53.1
6	73.9	74.3	[a]	76.6	77.0	76.0	76.6
7	68.8	71.5	[a]	68.1	69.0	68.6	72.6
8	70.2	71.9	[a]	70.9	70.9	69.8	67.7
9	62.1	62.5	64.2	62.0	64.1	62.0	62.6
$NHCOCH_3$	23.1	23.1	23.2	23.1	23.3	23.2	22.7
OCH_3	53.7	52.4		53.0		53.0	54.2
$OCOCH_3$	20.5	20.5		20.5		20.5	
$C(CH_3)_2$							27.1
$C(CH_3)_2$							25.7
$C(CH_3)_2$							110.0

[a]Not assigned.

methyl ester derivative **13** were compared as per-*O*-(trimethylsilyl) derivatives (Table III). Fragments A and B indicated the number of substituents present. Fragment A ($M^+ - CH_3$) is formed by elimination of a methyl unit from a trimethylsilyl group. Fragment B ($M^+ - CO_2CH_3$) is formed by elimination of the carboxyl group at C-2. Fragments C, D, E, F, and G may be used for the determination of the positions of the various substituents. Thus, fragment C ($M^+ - CHOR_8CH_2OR_9$) is formed by elimination of the C-8,9 part of the molecule and does not occur when the C-7 position is blocked. In **13,** its presence indicated that the isopropylidene group is at O-8,9. Fragment D forms fragment C by elimination of R_2OH and R_4OH. In **13**, the elimination of one ROH group could be seen at *m/z* 388 (D′). Elimination of the side-chain and the substituent at C-5 from the parent ion yielded fragment E ($M^+ - CHOR_7CHOR_8CH_2OR_9$ and $M^+ - R_5CONH_2$). Fragment F ($CHOR_8CH_2OR_9$) contains the C-8,9 part of the molecule. In **13**, this fragment was at *m/z* 101. Fragment G contains the C-4,5 part of the molecule.



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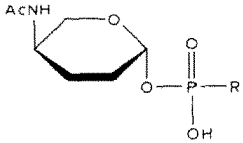

15 R = OH

16 R = F

TABLE V

ENZYMIC ACTIVITY OF *N*-ACETYLNEURAMINIC ACID ANALOGS WITH CMP-SIALATE SYNTHASE

Compound	K_m *(mM)*	V_{max} *(nmol·min^{-1})*	K_i *(mM)*	*Type of inhibition*
1	1.84 ±0.028	3.43 ±0.022		
5				None
6[a]			15.0 ±1.22	Competitive
8				None
10				None
11				None
14	9.40 ±0.283	10.2 ±0.780		Competitive
15			29.4 ±2.20	Competitive
16			6.36 ±0.650	Competitive
CMP				None
CTP	1.35 ±0.179	2.64 ±0.173		

[a]Ref. 11.

The ^{13}C-n.m.r. spectrum of **13** (Table IV) showed a large upfield shift of the signal for C-8 when compared to the spectrum of **2**. The C-8 signal of **13** overlaps the C-9 signal.

Compounds **6** (ref. 11), **14** (ref. 17), **15** (ref. 2), and **16** (ref. 2) were prepared as previously described in the literature.

Several of these *N*-acetylneuraminic acid derivatives were tested for activity in our CMP-sialate synthase system[17], and the results are shown in Table V. *N*-Acetyl-9-deoxy-9-fluoroneuraminic acid was found to be the only alternative substrate for the enzyme. This was not unexpected considering that many of the naturally occurring sialic acids are substituted at these positions with acetyl groups and are substrates for the CMP-sialate synthase. The methyl glycoside **6** was the only Neu5Ac derivative to show any enzyme inhibition. It is interesting that a minor modification at C-3 (**10** and **11**) or C-2 (**5** and **10**) completely prevented enzyme binding. On the other hand, compounds **15** and **16**, which have structures radically different from that of NeuAc, were capable of binding and showed significant inhibition of the enzyme. The unusual activity of **15** and **16** is difficult to explain. It is possible that these compounds are binding as product analogs or that the phosphate group is capable of mimicking both the carboxyl function as well as the anomeric hydroxyl group simultaneously.

On the basis of our results and those of Beau and Schauer[9], it would appear that the likely sites for modification of NeuAc are the side chain and C-4 in order to allow CMP-sialate synthase activity.

EXPERIMENTAL

General methods. — Melting points (uncorrected) were determined by the

capillary method. Optical rotations were measured with a 10-cm cell in a Perkin–Elmer 141 polarimeter. ^{1}H-, ^{13}C-, and ^{19}F-n.m.r. spectra were recorded with a Varian XL100 instrument. Mass spectra were obtained with a Finnigan Model 4021 gas chromatograph–quadrupole mass spectrometer, equipped with a glass column (2 mm i.d. × 1.8 m) containing 3% OV-1 on Supelcoport. T.l.c. was conducted on uniplate Silica Gel GF-250 (Analtech) glass plates; detection was with H_2SO_4 spray and heating. Flash evaporations were performed in a rotary evaporator *in vacuo* at a bath temperature <40°. Flash chromatography was used for all separations on silica gel (Bio-sil 100–200 mesh). Elemental analyses were performed by Robertson Laboratory, 72 West End Ave., Florham Park, NJ. Enzyme analyses were performed as previously described[17].

*Methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-*D-glycero-α-D-galacto-*nonulopyranosyl chloride)onate* **3**. — The methyl ester **2** (600 mg) in acetyl chloride (25 mL), saturated with HCl gas, was stirred for 48 h at 4°. The solution was evaporated to dryness with a bath temperature kept at 10–15°. The residue was chromatographed on silica gel in 14:6:1 ether–dichloromethane–methanol as the eluent to give **3** (852 mg, 90%) as a chromatographically pure light-yellow foam, $[\alpha]_D^{22}$ −65° (*c* 1, chloroform); lit.[11] $[\alpha]_D^{22}$ −63° (*c* 1, chloroform); ^{13}C-n.m.r., see Table IV.

*Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,6-anhydro-3,5-dideoxy-*D-erythro-L-manno-*nononate* (**4**). — Tributyltin hydride (170 μL) and azobis(isobutyryl)nitrile (1 mg) were added to a benzene solution (50 mL) of **3** (275 mg) and boiled at reflux for 1 h. The solution was evaporated to dryness and the residue chromatographed on a silica gel column with 1:1 ether–ethyl acetate as the eluent. Fractions containing **4** were pooled and evaporated to dryness to give 200 mg (82%) as a dry foam, m.p. ~183–187°, $[\alpha]_D^{22}$ −11.2° (*c* 1, chloroform); ^{13}C-n.m.r., see Table III.

Anal. Calc. for $C_{20}H_{29}NO_{12}$: C, 50.42; H, 6.15; N, 2.95. Found: C, 50.42; H, 6.18; N, 2.80.

*5-Acetamido-2,6-anhydro-3,5-dideoxy-*D-erythro-L-manno-*nononic acid* (**5**). — To a solution of **4** (90 mg) in methanol (15 mL) was added M sodium methoxide until pH 7. After stirring for 2 h, 0.1M NaOH was added, and the solution was stirred for an additional 3 h. Dowex 50 (H^+) cation-exchange resin was added, and the solution was stirred, filtered, and then evaporated to dryness; yield 54 mg (98%), m.p. 216° (dec.), $[\alpha]_D^{22}$ −1.6° (*c* 0.5, water); ^{13}C-n.m.r., see Table III.

Anal. Calc. for $C_{11}H_{19}NO_8 \cdot 0.5\ H_2O$: C, 43.70; H, 6.67; N, 4.63. Found: C, 43.60; H, 6.57; N, 4.40.

*Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,6-anhydro-3,5-dideoxy-*D-glycero-D-galacto-*non-2-enonate* (**7**). — To a solution of **3** (500 mg) in dry dichloromethane (100 mL) was added triethylamine (5 mL) with stirring. After 1 h, the solution was evaporated to dryness and the residue chromatographed in a silica gel column with solvent C as eluent. Fractions containing **7** were pooled and evaporated to dryness to give 290 mg (62%) as a dry foam, m.p. 228° (dec.); lit.[23] m.p. 225–227°; ^{13}C-n.m.r., see Table III.

5-Acetamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-*non-2-enonic acid* (**8**). — The pH of a solution of **7** (100 mg) in methanol (15 mL) was adjusted to 7 with M sodium methoxide. After stirring for 1.5 h, 0.1M NaOH (15 mL) was added. After stirring for an additional 1 h, Dowex 50 (H^+) cation-exchange resin was added. The solution was evaporated to dryness to give 55 mg (90%) of **8** as a tan solid, m.p. 134–136°; lit.[12] m.p. 137–140°; ^{13}C-n.m.r., see Table III.

Methyl 5-acetamido-4,7,8,9-tetra-O-*acetyl-2,6-anhydro-3,5-dideoxy-2,3-difluoro*-D-arabino-L-talo-*nononate* (**9**). — To a solution of **7** (700 mg) in dichloromethane (25 mL) at −50° and purged with O_2 was added XeF_2 (450 mg). A solution of BF_3·etherate (1 mL) in ether (10 mL) was added dropwise over 15 min. The solution was allowed to warm to room temperature and, after 15 h, the reaction was completed. The mixture was stirred with 5% aqueous $NaHCO_3$ (25 mL) for 30 min, and then extracted three times with dichloromethane. The extracts were pooled, dried (Na_2SO_4), and evaporated to dryness. The residue was chromatographed in a silica gel column with 2.5% methanol in diethyl ether as the eluent. Fractions containing **9** were pooled and evaporated to dryness to give 470 mg (62%) as a foam, m.p. 66–69° (dec.), $[\alpha]_D^{22}$ 145° (*c* 0.5, chloroform); ^{19}F-n.m.r., see Table II.

Anal. Calc. for $C_{20}H_{27}F_2NO_{12}\cdot H_2O$: C, 45.36; H, 5.25; N, 2.65; F, 7.18. Found: C, 45.53; H, 5.33; N, 2.58; F, 7.41.

5-Acetamido-2,6-anhydro-3,5-dideoxy-2,3-difluoro-D-arabino-L-talo-*nononic acid* (**10**). — The pH of solution of **9** (470 mg) in methanol (25 mL) was adjusted to 7 with 0.5M sodium methoxide. After 4 h, a 0.1M NaOH solution (10 mL) was added, and the solution was stirred for an additional 30 min. The mixture was treated with Dowex 50 (H^+) cation-exchange resin, filtered, and evaporated to dryness; yield 280 mg (69%), m.p. 148° (dec.), $[\alpha]_D^{22}$ −35° (*c* 0.55, water); ^{19}F-n.m.r., see Table II.

Anal. Calc. for $C_{11}H_{17}F_2NO_8\cdot 0.5\ H_2O$: C, 39.05; H, 5.36; N, 4.14; F, 11.23. Found: C, 38.96; H, 5.56; N, 3.79; F, 11.30.

5-Acetamido-3,5-dideoxy-D-erythro-L-manno-*nonulosonic acid* (**11**). — A solution of **10** (147 mg) in 50mM HCl (50 mL) was heated on a steam bath. The course of the reaction was monitored by ^{19}F-n.m.r. spectroscopy. After 2.5 h, the solution was cooled and repeatedly evaporated after additions of water until free of HCl. The residue was chromatographed in a Dowex 1 (HCO_2^-) column with M formic acid as the eluent. Fractions containing **11** were pooled and lyophilized to give 130 mg (89%) of a white foam, m.p. 180° (dec.), $[\alpha]_D^{22}$ −36° (*c* 0.35, water); ^{19}F-n.m.r., see Table II.

Anal. Calc. for $C_{11}H_{18}FNO_8\cdot H_2O$: C, 38.26; H, 5.83; N, 4.06; F, 5.50. Found: C, 38.27; H, 5.74; N, 3.90; F, 5.75.

Methyl 5-acetamido-4,7,8,9-tetra-O-*acetyl-2,6-anhydro-2,3-dibromo-3,5-dideoxy*-D-arabino-L-talo-*nononate* (**12**). — A solution of **7** (25 mg) in tetrachloromethane (50 mL) was titrated with 10% Br_2 in tetrachloromethane (~1 mL) to give a light orange tint to the solution. The solution was stirred for 2 h and

evaporated several times after additions of dichloromethane, and then taken to dryness. The residue was chromatographed on a preparative layer plate in solvent *C*. The desired band was eluted from the plate, the solution evaporated to dryness, and the residue crystallized from ether, yield 32 mg (96%), m.p. 139–140°, $[\alpha]_D^{22}$ −29.5° (*c* 0.5, chloroform); ^{13}C-n.m.r., see Table III.

Anal. Calc. for $C_{20}H_{27}Br_2NO_{12}$: C, 37.93; H, 4.30; N, 2.21; Br, 25.24. Found: C, 38.21; H, 4.42; N, 2.22; Br, 25.51.

Methyl 5-acetamido-3,5-dideoxy-8,9-O-isopropylidene-D-glycero-D-galacto-*nonulosonic acid* (**13**). — A suspension of **2** (200 mg) and anhydrous $CuSO_4$ (1.2 g) in dry acetone (30 mL) was shaken for 36 h at room temperature. The suspension was filtered and the solution evaporated to dryness. The residue was dissolved in hot dichloromethane and, after being kept overnight, the crystals were filtered off and dried to give 215 mg (91%) of **13**, m.p. 108–110°, $[\alpha]_D^{22}$ −23° (*c* 0.5, methanol); ^{13}C-n.m.r., see Table III.

Anal. Calc. for $C_{15}H_{25}NO_9 \cdot H_2O$: C, 47.24; H, 7.14; N, 3.67. Found: C, 46.89; H, 6.89; N, 3.49.

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