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Coupling of Amino Acids and Amino Sugars with Cyanuric Chloride (2,4,6-Trichloro-*s*-triazine)[1]

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2,4,6-Trichloro-*s*-triazine (cyanuric chloride) has been used to couple glycine ethyl ester with seven carbohydrate derivatives that contain amino groups. The reactions proceed in high yield under mild conditions. The compounds serve as models for the coupling of carbohydrates to proteins by well-defined linkages through amino groups.

La 2,4,6-trichloro-*s*-triazine (chlorure cyanurique) a été utilisée pour coupler l'ester éthylique de la glycine avec sept dérivés de carbohydrates contenant des groupes amines. Les réactions se font avec d'excellent rendement et sous des conditions douces. Les composés sont utilisés en tant que modèles pour les couplages de carbohydrates avec des protéines au moyen de liaisons bien définies par l'intermédiaire de groupes amines.

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A recent preliminary communication (1) described the preparation of an antigenic polysaccharide–protein conjugate by the use of 2,4,6-trichloro-*s*-triazine (cyanuric chloride) as a coupling reagent. Gel electrophoresis and the production of antibody to the carbohydrate moiety showed that the polysaccharide and the protein were covalently linked. The linkage must have occurred through hydroxyl groups in the nitrogen-free polysaccharide and amino groups in the protein, but the extent and location of the cross-linkages were not determined. In the absence of amino groups in the polysaccharide, the cyanuric chloride must have reacted with hydroxyl groups at random. These factors are not important for the simple preparation of polysaccharide antigens. However, the introduction of specific determinant groups into a peptide or protein requires conjugates in which linkages are more closely defined. The present paper describes the preparation of some model compounds representing carbohydrate–protein conjugates in which the linkages are well-characterized.

It is known that replacement of one chlorine atom in 2,4,6-trichloro-*s*-triazine by an amino, or substituted amino, group deactivates the remaining two so that stepwise reactions can be obtained by temperature control (2–5). However, the deactivating effects of alkoxy- or aryloxy-groups are weaker and the reaction of chlorotriazines with hydroxylated compounds yields mixtures from which it is difficult to isolate and characterize any product (4). This observation was confirmed during the present work by unsuccessful attempts to isolate characterizable products from the reaction of 2,4,6-trichloro-*s*-triazine with 2,3,4,6-tetra-*O*-acetyl-β-D-glucose or with methyl α-D-glucopyranoside. Furthermore, it has been established that 2,4,6-trichloro-*s*-triazine reacts preferentially with amino groups when both amino groups and hydroxyl groups are present in the same substrate molecule (4, 5).

From these results it was clear that successive replacement of two chlorine atoms from 2,4,6-trichloro-*s*-triazine by amino substituents offered the best possibilities for preparing well-defined, carbohydrate–protein conjugates. As there are several ways to introduce amino groups into

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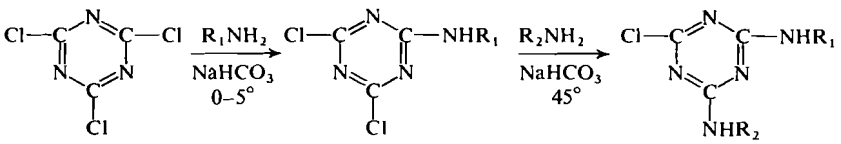

A B

Compounds type **A**

1	NHR_1 = ethoxycarbonylmethylamino
5	= 2-amino-2-deoxy-D-glucopyranose
6	= 2-amino-2-deoxy-D-galactopyranose
7	= 2-hydroxyethylamino

Compounds type **B**

	NHR_1 = ethoxycarbonylmethylamino
2	NHR_2 = amino
3	= 2-hydroxyethylamino
4	= 2-ethoxycarbonylmethylamino
8	= 2-amino-2-deoxy-D-glucopyranose
9	= 2-amino-2-deoxy-D-galactopyranose
10	= 2-amino-2-deoxy-D-mannopyranose
11	= 1-amino-1-deoxy-D-glucitol
12	= 1-amino-1-deoxy-D-mannitol
13	= 1-amino-1-deoxy-D-galactitol
14	= 2-amino-2-deoxy-D-glucosediethyl-dithioacetal

SCHEME 1

carbohydrates the method should be of general applicability.

The coupling of amino acids and amino sugars with cyanuric chloride according to Scheme 1, can be achieved in two ways by using either the amino sugar or the amino acid derivative in the first step, *i.e.* the introduction of NHR_1. Thus, replacement at 0 °C of one of the chlorine atoms of cyanuric chloride by the amino groups of glucosamine and galactosamine gave compounds **5** and **6** respectively. Further reaction of these compounds at 45 °C with glycine ethyl ester gave the disubstituted products **8** and **9**. The same compounds could also be prepared by the initial reaction of cyanuric chloride with the amino acid derivative to give compound **1**, and subsequent reaction of this at 45 °C with the amino sugars to give compounds **8** and **9**. Overall yields of these compounds were better by the latter route, probably because of the ease with which compound **1** can be made in high yield.

Compounds **2**, **3**, and **7** were prepared with the intention of having amino or hydroxyl groups available for subsequent reaction with acetobromo sugars. However, the difficulty with which these compounds (**2**, **3** and **7**) dissolve in organic solvents precluded attempts to extend the reaction sequence in that direction.

The 1-amino-1-deoxy alditols (NHR_2 on compounds **11**, **12**, and **13**) were prepared by the method of Kagan *et al.* (6) in which *N*-benzyl aminitols were formed by the reductive alkylation of benzylamine with the appropriate sugar. The *N*-benzyl-1-amino-1-deoxy-alditols were then hydrogenolyzed to the 1-amino-1-deoxy alditols which, on reaction with compound **1** yielded compounds **11**, **12**, and **13**. The *N*-benzyl-1-amino-1-deoxy-D-mannitol that was prepared for this series of reactions does not seem to have been reported previously; it was therefore characterized further as a crystalline acetate. The 2-amino-2-deoxy-D-glucose diethyldithioacetal was prepared by methods described (7); condensation of this derivative with compound **1** gave compound **14**. The other starting compounds were available commercially and were used without further purification.

The structures of these compounds follow from the method of synthesis because it is known that 2,4,6-trichloro-*s*-triazine or a monosubstituted dichloro-*s*-triazine reacts preferen-

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tially with the amino group of hydroxylamines (4, 5). However, some of the compounds were examined in further detail to confirm that this generalization held for the carbohydrate derivatives used in the present study.

The structure of compound **1** was confirmed by the analysis that showed two atoms of chlorine remaining in the molecule and by n.m.r. spectroscopy that accounted for all of the protons as follows: N—H (singlet; one proton; δ, 7.2), C—CH_3 (triplet; three protons; δ, 1.32), 2 × CH_2 (quartet; four protons; δ, 4.30). Compound **4**, the disubstituted analogue of compound **1**, gave a similar n.m.r. spectrum with two nitrogen protons, eight methylene protons, and six C—methyl protons accounted for by integration.

With the structure of compound **1** established it was clear from the method of synthesis and from the chlorine analyses that the compounds of type B had to be disubstituted monochloro-*s*-triazine derivatives. The location of the linkage through the amino group in the carbohydrate moiety was confirmed for **8** and **14** as representative examples of these compounds. Thus, the n.m.r. spectrum of **14** showed nine C—CH_3 protons (δ, 1.3), five C—H protons (δ, 2.5–2.8), four OH protons (δ, 3.77), ten CH_2 protons (δ, 4.0–4.5), and two NH protons (δ, 7.2). The presence of four hydroxyl groups showed that the linkage must have been through the amino group of the diethyldithioacetal. Acetylation of **8** and **14** introduced four *O*-acetyl groups into each of them as determined by analysis and by the ratio of *C*-methyl to acetyl protons in the n.m.r. spectra. The method of acetyl analysis (8) and the absence of any absorption at 1618–1665 cm^{-1} in the i.r. established that all of the acetyl groups were *O*-acetyl and that no *N*-acetyls were present. These results prove that all of the hydroxyl groups in **8** and **14** were free and that the linkages were through the amino groups of the carbohydrate moieties. Compounds **8**, **9**, and **10**, were probably anomeric mixtures and it is likely that they were in the pyranose ring form.

The present results show that cyanuric chloride (2,4,6-trichloro-*s*-triazine) can be used for the selective coupling of compounds that contain amino groups. The reaction takes place preferentially with amino groups and any hydroxyl groups present need not be blocked. The condensations proceed in high yield (66–88%) and under mild conditions that makes the method amenable for use with sensitive compounds. In particular, this method of coupling would seem to offer an attractive alternative to diazotization (9–11) for introduction of determinant groups into peptides or proteins for use as artificial antigens or as immunoabsorbents.

Experimental

Solutions were evaporated under reduced pressure at bath temperatures not exceeding 40°. A Perkin–Elmer 140 polarimeter was used to measure optical rotations which are equilibrium values unless otherwise specified. Melting points were determined on a Fisher–Johns hot stage and are uncorrected. The t.l.c. was done on glass plates coated with silica gel G using the following solvent mixtures: (A) chloroform:ethanol, 3:1; (B) ethyl acetate:acetic acid:water, 9:2:2. Unless stated otherwise, compounds containing amino sugars were homogeneous by t.l.c. in both solvent systems. The u.v. spectra were recorded on aqueous solutions using a Cary Recording spectrophotometer. All of the compounds showed the same spectral characteristics due to the triazine absorption; λ_{max} 261 mμ, $\varepsilon = 3150 \rightarrow 3400$. The i.r. spectra were recorded with a Perkin–Elmer Infracord spectrophotometer using chloroform solutions of the compounds. The n.m.r. spectra were obtained in deuterochloroform solution using a Varian HA-60 spectrometer with tetramethylsilane as a standard.

*N-Benzyl-1-amino-1-deoxy-*D*-mannitol*

This compound does not seem to have been reported before. It was prepared in 76% yield by the method of Kagan *et al.* (5). The crystalline product, m.p. 162–163 °C, was recrystallized from methanol to a constant melting point of 164–165 °C; $[\alpha]_D^{22}$ +9.0° (*c*, 1.0 in water).

Anal. Calcd. for $C_{13}H_{21}O_5N$: C, 57.55; H, 7.80; N, 5.16. Found: C, 57.39; H, 7.90; N, 5.33.

*N-Benzyl-1-amino-1-deoxy-2,3,4,5,6-penta-O-acetyl-*D*-mannitol*

The *N*-benzyl-1-amino-1-deoxy-D-mannitol (1 g) was acetylated in acetic anhydride:pyridine (1:1, 20 ml) at room temperature. The product was recrystallized from ethanol: m.p. 76–77 °C, $[\alpha]_D^{22}$ +20° (*c*, 1.0 in chloroform).

Anal. Calcd. for $C_{23}H_{31}O_{10}N_1$: C, 57.38; H, 6.44; N, 2.91. Found: C, 57.73; H, 6.73; N, 2.65.

2,4-Dichloro-6-(ethoxycarbonylmethylamino)-s-triazine (1)

Cyanuric chloride (2,4,6-trichloro-*s*-triazine) (5.3 g) was dissolved in hot acetone (10–12 ml) and poured into well-stirred ice water (20 ml). To this stirred solution was added a cold (0–5 °C) aqueous solution (20 ml) of glycine hydrochloride ethyl ester (4.0 g) and sodium bicarbonate (4.82 g). The mixture was stirred at 0 °C for 1 h. The white, crystalline product was filtered, washed with ice-cold water, and dried. The compound (85% yield) required no further purification because recrystallization from acetone did not change its properties: m.p. 85–86 °C.

Anal. Calcd. for $C_7H_8O_2N_4Cl_2$: C, 33.48; H, 3.21; N,

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22.32; Cl, 28.24. Found: C, 33.20; H, 3.04; N, 22.10; Cl, 28.44.

2-Chloro-4-amino-6-(ethoxycarbonylmethylamino)-s-triazine (2)

Compound **1** (12 g) was dissolved in hot acetone (50 ml) and this solution was added with stirring to water (75 ml) through which ammonia gas was passed continuously. The temperature of the reaction was maintained at 45 °C. After 15–20 min the white, crystalline product was filtered off and dried; yield 10.2 g, 88%. Recrystallized from acetone: nitromethane (1:1), the product had m.p. 235–237 °C.

Anal. Calcd. for $C_7H_{10}O_2N_5Cl_1$: C, 36.28; H, 4.31; N, 30.23; Cl, 15.33. Found: C, 35.91; H, 4.57; N, 30.5; Cl, 15.18.

2-Chloro-4-(2-hydroxyethylamino)-6-(ethoxycarbonylmethylamino)-s-triazine (3)

Compound **1** (3 g) in hot acetone (20 ml) was added with stirring to water (10 ml) at 0–5 °C. A solution of ethanolamine (0.75 ml) in acetone (10 ml) was then added and the reaction mixture was allowed to warm up to room temperature (22 °C). An aqueous solution (5 ml) of sodium bicarbonate (1 g) was then added slowly to the stirred reaction mixture over a period of 30 min. The reaction was stirred at 45 °C for 1.5 h after which the product was filtered and dried; yield, 2.2 g, 85%. Recrystallized from acetone:ethanol (1:1), the compound had m.p. 197–199 °C.

Anal. Calcd. for $C_9H_{14}O_3N_5Cl$: C, 39.20; H, 5.08; N, 25.40; Cl, 12.88. Found: C, 39.08; H, 5.04; N, 25.18; Cl, 12.91.

6-Chloro-(2,4-di-ethoxycarbonylmethylamino)-s-triazine (4)

Cyanuric chloride (2,4,6-trichloro-*s*-triazine) (1.84 g) was condensed with glycine hydrochloride ethyl ester (2.8 g) in the presence of sodium bicarbonate (3.36 g) as described for compound **1** except that the temperature of the reaction was maintained at 45 °C instead of 0–5 °C. The disubstituted product which precipitated out in the reaction was filtered and dried; yield 2.3 g, 72%. When recrystallized from acetone it had m.p. 182–183 °C.

Anal. Calcd. for $C_{11}H_{16}O_4N_5Cl$: C, 41.58; H, 5.04; N, 22.04; Cl, 11.18. Found: C, 41.48; H, 5.08; N, 22.2; Cl, 11.27.

2-(2,4-Dichloro-s-triazin-6-yl)amino-2-deoxy-D-glucopyranose (5)

Cyanuric chloride (2,4,6-trichloro-*s*-triazine) (1.84 g) was condensed with 2-amino-2-deoxy-D-glucopyranose hydrochloride (2.16 g) in the presence of sodium bicarbonate (1.68 g) at 0–5 °C for 1.5 h as described for compound **1**. The solid product which precipitated out in the reaction was filtered, dried, and recrystallized from acetone; yield, 2.2 g, 67%; the compound decomposed with charring above 200 °C; $[\alpha]_D^{22}$ +45° (*c*, 1.4 in methanol).

Anal. Calcd. for $C_9H_{12}O_5N_4Cl_2$: C, 33.02; H, 3.67; N, 17.12; Cl, 21.71. Found: C, 32.60; H, 3.74; N, 17.50; Cl, 21.39.

2-(2,4-Dichloro-s-triazin-6-yl)amino-2-deoxy-D-galactopyranose (6)

The condensation of cyanuric chloride (2,4,6-trichloro-*s*-triazine) (0.925 g) and 2-amino-2-deoxy-D-galactopyranose hydrochloride (1.08 g) in the presence of sodium bicarbonate (0.84 g) was performed in the same way as for compound **5**. The product was recrystallized from acetone; yield, 1.03 g, 62%; the compound decomposed with charring above 200 °C; $[\alpha]_D^{22}$ +56.5° (*c*, 1.2 in methanol).

Anal. Calcd. for $C_9H_{12}O_5N_4Cl_2$: C, 33.02; H, 3.67; N, 17.12; Cl, 21.71. Found: C, 32.76; H, 3.44; N, 17.37; Cl, 21.42.

2,4-Dichloro-6-(2-hydroxyethylamino)-s-triazine (7)

Cyanuric chloride (2,4,6-trichloro-*s*-triazine) (1.84 g), ethanolamine (0.61 ml), and sodium bicarbonate (0.84 g) were stirred in acetone:water at 0–5 °C as described for compound **1**. The reaction mixture was clarified by filtration and the filtrate was evaporated leaving a solid residue. Recrystallization from methanol yielded compound **7**, 1.12 g, 53%; m.p. 113–115 °C.

Anal. Calcd. for $C_5H_6ON_4Cl_2$: C, 28.70; H, 2.86; N, 26.79; Cl, 33.97. Found: C, 28.84; H, 2.99; N, 26.64; Cl, 33.59.

2-(4-Chloro-2-ethoxycarbonylmethylamino-s-triazin-6-yl)-amino-2-deoxy-D-glucopyranose (8)

Compound **1** (3.23 g) in hot acetone (15 ml) and 2-amino-2-deoxy-D-glucopyranose hydrochloride (3.76 g) in water (10 ml) were added to well-stirred ice water at 0.5 °C. The temperature of the reaction mixture was allowed to rise to room temperature and then an aqueous solution (15 ml) of sodium bicarbonate (2.52 g) was added slowly over a period of 30 min. The reaction was stirred continuously for 1 h at 45 °C and was then clarified by filtration. The filtrate was evaporated to dryness and extracted with methanol. Evaporation of the methanol extract yielded compound **8** which was recrystallized from water, yield, 4.2 g, 71%; m.p. 125–127°; $[\alpha]_D^{22}$ +24° (*c*, 1.0 in ethanol).

Anal. Calcd. for $C_{13}H_{20}O_7N_5Cl$: C, 39.64; H, 5.08; N, 17.78; Cl, 9.02. Found: C, 39.46; H, 5.04; N, 17.66; Cl, 8.83.

2-(4-Chloro-2-ethoxycarbonylmethylamino-s-triazin-6-yl)-amino-2-deoxy-D-galactopyranose (9)

This compound was prepared from compound **1** (3 g), 2-amino-2-deoxy-D-galactopyranose hydrochloride (2.59 g), and sodium bicarbonate (2 g) as described for compound **8**. The product (4.8 g) was not homogeneous on t.l.c. and required purification by column chromatography on silica gel using solvent A; a large portion of the major product was lost due to overlap with the impurity. The purified compound, 2.5 g, 51%, was recrystallized from ethanol:water (1:1); m.p. 118-120 °C, $[\alpha]_D^{22}$ +25° (*c*, 1.0 in ethanol).

Anal. Calcd. for $C_{13}H_{20}O_7N_5Cl$: C, 39.64; H, 5.08; N, 17.78; Cl, 9.02. Found: C, 39.51; H, 5.01; N, 17.89; Cl, 8.92.

2-(4-Chloro-2-ethoxycarbonylmethylamino-s-triazin-6-yl)-amino-2-deoxy-D-mannopyranose (10)

Compound **1** (2.1 g), 2-amino-2-deoxy-D-mannopyranose hydrochloride (1.8 g), and sodium bicarbonate (1.4 g) were reacted as described for compound **8**. The reaction product (2.8 g) was purified on silica gel as for compound **9** to yield compound **10**, 2.4 g, 73%. Crystallized from ethanol this compound had m.p. 108–111 °C and $[\alpha]_D^{22}$ −2° (*c*, 1.0 in ethanol).

Anal. Calcd. for $C_{13}H_{20}O_7N_5Cl$: C, 39.64; H, 5.08; N, 17.78; Cl, 9.02. Found: C, 39.79; H, 5.14; N, 17.58; Cl, 8.88.

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*1-(4-Chloro-2-ethoxycarbonylmethylamino-s-triazin-6-yl)-amino-1-deoxy-D-glucitol (**11**)*

Compound **1** (2.51 g) in acetone (15 ml) and 1-amino-1-deoxy-D-glucitol (6) (1.81 g) in water (12 ml) were mixed together with continuous stirring in a further 10 ml of water. An aqueous solution (10 ml) of sodium bicarbonate (0.84 g) was added slowly during 30 min. The reaction mixture was stirred at 45 °C for 1 h. The insoluble product was filtered and washed with cold water and cold acetone; yield, 2.5 g, 63%; m.p. 192–194 °C.; $[\alpha]_D^{22}$ −21° (*c*, 1.0 in ethanol:water (1:1)).

Anal. Calcd. for $C_{13}H_{22}O_7N_5Cl$: C, 39.44; H, 5.56; N, 17.69; Cl, 8.98. Found: C, 39.12; H, 5.84; N, 17.44; Cl, 9.4.

*1-(4-Chloro-2-ethoxycarbonylmethylamino-s-triazin-6-yl)-amino-1-deoxy-D-mannitol (**12**)*

Compound **1**, 1-amino-1-deoxy-D-mannitol, and sodium bicarbonate were reacted in the same quantities and under the same conditions as for compound **11**. However, in this instance the product was soluble so the reaction mixture was filtered and concentrated to a syrup which then crystallized. The crystalline product was filtered, washed with cold ethanol, and recrystallized from methanol; yield 2.68 g, 67%; m.p. 174–175 °C; $[\alpha]_D^{22}$ +15° (*c*, 1.2 in ethanol:water (1:1)).

Anal. Calcd. for $C_{13}H_{22}O_7N_5Cl$: C, 39.44; H, 5.56; N, 17.69; Cl, 8.98. Found: C, 39.41; H, 5.74; N, 17.69; Cl, 9.27.

*1-(4-Chloro-2-ethoxycarbonylmethylamino-s-triazin-6-yl)-amino-1-deoxy-D-galactitol (**13**)*

Compound **1** (2.51 g) in acetone (15 ml), 1-amino-1-deoxy-D-galactitol (6) (1.81 g) in water (15 ml), and sodium bicarbonate (0.84 g) in water (12 ml) were reacted as described for **11**. The reactants were dissolved by addition of ethanol (30–40 ml) and the solution was heated at 50° for 1.5 h. The solution was concentrated and the product crystallized when most of the acetone and ethanol had been removed. It was recrystallized from acetone:water (1:3); yield 3.1 g, 78%; m.p. 187–188 °C; $[\alpha]_D^{22}$ −18.5° (*c*, in ethanol:water (1:1)).

Anal. Calcd. for $C_{13}H_{22}O_7N_5Cl$: C, 39.44; H, 5.56; N, 17.69; Cl, 8.98. Found: C, 39.19; H, 5.68; N, 17.68; Cl, 8.81.

*2-(4-Chloro-2-ethoxycarbonylmethylamino-s-triazin-6-yl)-amino-2-deoxy-D-glucose Diethyl Dithioacetal (**14**)*

Compound **1** (3 g) in acetone (15 ml), 2-amino-2-deoxy-D-glucose diethyl dithioacetal hydrochloride (7) (3.8 g) in water (15 ml), and sodium bicarbonate (1 g) in water (10 ml) were reacted as described for compound **11**. The mixture was clarified by filtration and the filtrate was evaporated to dryness. The residue was extracted with ethanol and evaporation of the extract yielded a syrupy product that was purified by chromatography on silica gel in solvent A. The pure compound was crystallized from ether:light petroleum (1:1); yield, 3.5 g, 58%; m.p. 56–58 °C; $[\alpha]_D^{22}$ −25° (*c*, 1.0 in ethanol).

Anal. Calcd. for $C_{17}H_{30}O_6N_5ClS_2$: C, 40.84; H, 6.00; N, 14.01; Cl, 7.10; S, 12.81. Found: C, 40.98; H, 5.87; N, 13.69; Cl, 7.13; S, 12.61.

*Acetylation of Compounds **8** and **14***

The compound (100 mg) was added slowly to a heated mixture of acetic anhydride (5 ml) and anhydrous sodium acetate (75 mg). The mixture was heated under reflux for 1 h and was then poured into ice water (50 ml). The product was extracted into chloroform (2 × 25 ml); the extract was washed successively with saturated aqueous sodium bicarbonate and water, dried (anhydrous sodium sulfate), and evaporated. The acetates of both compounds crystallized spontaneously and were analyzed without recrystallization.

The acetate of compound **8** had m.p. 90–93 °C, $[\alpha]_D^{22}$ +29° (*c*, 2% in chloroform), and *O*-acetyl, 30.10%; calcd. *O*-acetyl, 30.66%. Integration of the well-resolved signals for C—CH_3 and C(=O)—CH_3 protons at 1.32 and 2.0–2.3 δ respectively in the n.m.r. spectrum gave a ratio of 3:12 in agreement with the proposed structure. There was strong absorption at 1740 cm^{-1} in the i.r. spectrum of this compound indicative of *O*-acetyl group; no absorption attributable to acetamido groups could be detected in the range 1618–1665 cm^{-1}.

The acetate of compound **14** had m.p. 52–54 °C and $[\alpha]_D^{22}$ 0° (*c*, 2% in chloroform). Integration of the signals for the C—CH_3 and C(=O)—CH_3 protons as before gave a ratio of 9:12 in agreement with the proposed structure.

Anal. Calcd. for $C_{25}H_{38}N_5O_{10}ClS_2$: C, 44.98; H, 5.70; N, 10.50; *O*-acetyl, 25.8. Found: C, 45.04; H, 5.86; N, 10.47; *O*-acetyl, 25.48.

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