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PREPARATION OF 1-THIOGLYCOSIDES HAVING ω-ALDEHYDO AGLYCONS USEFUL FOR ATTACHMENT TO PROTEINS BY REDUCTIVE AMINATION*

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ABSTRACT

A series of 1-thioglycosides containing an ω-aldehydo group (as the dimethyl acetal) on the aglycon were prepared by reaction of *O*-acetyl-1-thioaldoses with *N*-(chloroacetyl)aminoacetaldehyde dimethyl acetal, a compound readily prepared by the action of chloroacetyl chloride or chloroacetic anhydride on 2-aminoacetaldehyde dimethyl acetal. *O*-Deacetylation of the 1-thioglycosides, followed by deacetalation, yielded the desired products. An analogous 1-thioglycoside having a longer aglycon was prepared by reaction of 1-thio-D-galactose with (6-aminohexanoyl)-aminoacetaldehyde dimethyl acetal, obtained by condensing 6-bromohexanoic acid and aminoacetaldehyde with 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide. These glycosides were found to be useful for modification of proteins to yield neoglycoproteins.

INTRODUCTION

Proteins chemically modified with carbohydrate derivatives (neoglycoproteins) have been found to be extremely useful in biological research for elucidation of possible roles of carbohydrates. Many methods are now available for attaching sugars to proteins[1]; the ideal methods therefor should be such that (*a*) the modified proteins can retain the original, overall charge-distribution, and (*b*) there is minimal introduction of hydrophobicity.

We have previously developed a new group of reagents, 2-imino-2-methoxyethyl 1-thioglycosides, that meet these requirements by forming an amidino linkage with proteins[2]. Although members of this group of reagents have produced fruitful results in the past[3,4], availability of an alternative procedure based on a different

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principle was considered desirable, for comparison of possible influence by the aglycon in biological experiments.

We report here synthetic procedures for two types of ω-aldehydo-aglycon 1-thioglycosides that can be attached to protein through reductive amination. These compounds have been attached to several proteins, and they promise to be a useful alternative to our earlier, imidate reagents[2].

$$(ClCH_2CO)_2O + NH_2CH_2(OMe)_2 \rightarrow ClCH_2CONHCH_2CH(OMe)_2$$
1

$$R\text{-}SH + \mathbf{1} \rightarrow R\text{-}SCH_2CONHCH_2CH(OMe)_2$$
2, 5, 7, 9

$$\xrightarrow{NaOMe} R'\text{-}SCH_2CONHCH_2CH(OMe)_2 \xrightarrow{H^+} R'\text{-}SCH_2CONHCH_2CHO$$
3, 6, 8, 10 **4, 11**

2 R = 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl
5 R = 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl
7 R = 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl
9 R = 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl
3 and **4** R′ = β-D-galactopyranosyl
6 R′ = β-D-glucopyranosyl
8 R′ = α-D-mannopyranosyl
10 and **11** R′ = 2-acetamido-2-deoxy-β-D-glucopyranosyl

$$Br(CH_2)_5CO_2H + NH_2CH_2CH(OMe)_2 \rightarrow Br(CH_2)_5CONHCH_2CH(OMe)_2$$
$$R\text{-}SH + Br(CH_2)_5CONHCH_2CH(OMe)_2 \rightarrow R\text{-}S(CH_2)_5CONHCH_2CH(OMe)_2$$
12

$$\xrightarrow{NaOMe} R'\text{-}S(CH_2)_5CONHCH_2CH(OMe)_2$$
13

12 R = 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl
13 R′ = β-D-galactopyranosyl

EXPERIMENTAL

Materials. — Chloroacetic anhydride and 6-bromohexanoic acid were obtained from Fluka (through Tridom, N.Y.), 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide (EDAC) from Story Chemical Corp. (Muskegon, Mich.), aminoacetaldehyde dimethyl acetal and $NaCNBH_3$ from Aldrich, $NaCNBH_3$ from Alfa-Ventron, and bovine serum albumin (BSA) from Sigma, and were used without purification.

2-*S*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-2-thiopseudourea hydrobromide and its β-D-glucopyranosyl[5], α-D-mannopyranosyl[2], and 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl analogs[6] were prepared from the corresponding per-*O*-acetylglycosyl bromide or chloride.

General methods. — All evaporations were performed under diminished

pressure at 25–40° in a rotary evaporator. Melting points (uncorrected) were measured with a Fisher–Johns apparatus. Proton magnetic resonance spectra were recorded with a JEOL NMH-100 spectrometer. Elemental analyses (single analyses) were performed by Galbraith Labs (Knoxville, TN). Thin-layer chromatography (t.l.c.) was performed with silica gel F-254 layers (0.25 mm) precoated on aluminum sheets (Merck), using the following solvent systems: (*A*) 1:4 (v/v) benzene–ethyl acetate, (*B*) 9:4:2 (v/v) ethyl acetate–isopropyl alcohol–water, and (*C*) 3:2:1 (v/v) ethyl acetate–acetic acid–water. For the detection of carbohydrates, t.l.c. sheets were sprayed with 15% sulfuric acid in 50% ethanol and heated for a few minutes at 140°. Aldehydes and aldehyde acetals were detected by spraying with a 0.4% solution[7] of (2,4-dinitrophenyl)hydrazine in 2M HCl, and warming the plates slightly. The presence of sulfhydryl group was detected by spraying with the Ellman reagent[8].

Neutral sugars were determined by a modified version[9] of the phenol–sulfuric acid method, and the aldehyde group was determined by the neocuproine (2,9-dimethyl-1,10-phenanthroline) method[10]. In the case of an aldehyde dimethyl acetal, the sample was heated in 0.05M HCl in a heating block for 15 min at 100° before the aldehyde determination. For determination of the sugar content of crystalline products, samples of known weight were hydrolyzed with mercuric acetate[11], and the sugar released was determined by means of an automated sugar-analyzer[12].

When necessary, derivatives soluble in organic solvents were purified either by gel filtration on a column (4 × 194 cm) of Sephadex LH-20, using 95% ethanol as the eluant, or by high-performance, liquid chromatography (h.p.l.c.) on silica gel 60H (for t.l.c.) with Chromatopac Prep 100 (Jobin–Yvon, Inc.). For the purification of water-soluble products, a column (5 × 215 cm) of Sephadex G-25 was used, using 0.1M acetic acid as the eluant and collecting 22-mL fractions. Fractions were analyzed by the phenol–sulfuric acid method, and examined by t.l.c. (solvent *B*) to locate the desired product and determine its purity.

(Chloroacetyl)aminoacetaldehyde dimethyl acetal (**1**). — Chloroacetic anhydride (7.52 g, 44 mmol) was added, in portions, to a solution of aminoacetaldehyde dimethyl acetal (4.32 mL, 40 mmol) in cold, dry methanol (200 mL) while the solution was stirred in an ice bath. After 30 min, the mixture was brought to neutral pH by addition of triethylamine, and evaporated to a syrup. This was dissolved in cold chloroform (100 mL), and the solution was washed successively with cold water (30 mL) and cold, saturated sodium hydrogencarbonate (30 mL), dried (anhydrous Na_2SO_4), and evaporated to a syrup. Aminoacetaldehyde dimethyl acetal and derivatives thereof can be detected on a t.l.c. sheet by charring with H_2SO_4; the syrup gave a single, charred spot in t.l.c. (solvent *A*); p.m.r. data ($CDCl_3$): δ 3.29 (s, 6, OCH_3), 3.33 (s, 2, NCH_2C), 3.93 (s, 2, $ClCH_2CO$) and 4.23–4.33 [t, 1, J 10 Hz, $CH(OMe)_2$]. This syrup was used directly in the next sequence of reactions.

[(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)thioglycolyl]aminoacetaldehyde dimethyl acetal (**2**). — Syrupy **1**, obtained from 40 mmol of aminoacetaldehyde dimethyl acetal, was dissolved in 1:1 (v/v) acetone–water (140 mL). In this solution were dissolved the pseudothiourea derivative of 2,3,4,6-tetra-*O*-acetyl-D-galacto-

pyranose (0.74 g, 20 mmol), K_2CO_3 (3.32 g, 24 mmol), and $NaHSO_3$ (2.08 g, 20 mmol), in that order, and the mixture was kept overnight at room temperature; t.l.c. in solvent *A* then showed that 1-thio-D-galactose (generated from the pseudothiourea derivative) was no longer present (Ellman reagent). The mixture was partitioned between chloroform (120 mL) and water (80 mL), the aqueous layer was extracted once with chloroform (60 mL), and the chloroform solutions were combined, washed with M NaCl (2 × 50 mL), dried (Na_2SO_4), and evaporated. The syrup obtained was routinely used without purification for the next step, namely, deacetylation.

Pure **2** could be obtained either by h.p.l.c. using solvent *A*, or by gel filtration on a column of Sephadex LH-20. Fractions that contained single, charring material (R_F 0.37 in solvent *A*) were combined and evaporated; the p.m.r. spectrum of this material showed *O*-acetyl (4 s, δ 1.98–2.15) and *O*-methyl (δ 3.35) groups in the correct ratio.

(β-D-Galactopyranosylthioglycolyl)aminoacetaldehyde dimethyl acetal (**3**). — Syrupy **2** obtained by evaporation of the foregoing chloroform solution thereof was deacetylated with 5mM barium methoxide in dry methanol (40 mL) overnight at room temperature. The turbid solution was brought to neutral pH with 60% acetic acid, and evaporated. The residue was dissolved in water (40 mL), and fractionated in two batches on a column of Sephadex G-25. Analysis of the fractions of effluent by the phenol–sulfuric acid method showed essentially a single peak; the trailing end of this peak showed, upon t.l.c. in solvent *B*, two other, minor, charring components. Fractions containing **3** only (R_F 0.25, solvent *B*) were combined and evaporated, and the residue was dried overnight in a vacuum desiccator over NaOH pellets. Recrystallization of the solid from warm isopropyl alcohol yielded 4.03 g (11.8 mmol) of **3**. Addition of ether to the filtrate produced more crystals, 0.62 g (1.8 mmol); the total yield of crystalline **3** was 68% (from the pseudothiourea derivative); m.p. 103–105°.

Analysis for the D-galactose content of **3**, as described in *General methods*, gave 102% of the theoretical value of D-galactose. After mild hydrolysis with acid, analysis for the aldehyde group by the neocuproine method gave a molar absorbance of 2.36×10^4 M^{-1}. The p.m.r. spectrum of ^2H-exchanged **3** in Me_2SO-d_6 showed δ 3.25 (s, 6, OCH_3), 3.50 (s, 4, CH_2C), 4.2–4.45 (m, ring protons), 4.62 [t, 1, *J* 10 Hz, $CH(OMe)_2$], and 4.8 (d, 1, *J* 6 Hz, anomeric H).

Anal. Calc. for $C_{12}H_{23}NO_8S$ (341.38): C, 42.22; H, 6.79; N, 4.10; S, 9.39. Found: C, 41.90; H, 6.92; N, 3.88; S, 9.50.

(β-D-Galactopyranosylthioglycolyl)aminoacetaldehyde (**4**). — A solution of **3** (1 g, 2.9 mmol) in 0.05M HCl (2 mL) was heated for 20 min in a boiling-water bath, cooled immediately, and made neutral with 2M NaOH (50 μL). Storage overnight in the cold yielded crystals which were filtered off, and washed thrice with cold water and then with 95% ethanol, yielding 0.4 g (1.35 mmol) of **4**. The crystals were homogeneous by t.l.c. in solvent *C* (R_F 0.44), m.p. 198–200°. The **4** remaining in the filtrate could be obtained by means of a column (2 × 140 cm) of Sephadex G-15, using 0.1M

acetic acid as the eluant, and collecting 5 mL per fraction. Fractions containing mainly **4** were combined, and concentrated to a small volume. Addition of 95% ethanol produced crystals (0.1 g, 0.34 mmol) of **4**; total yield: 0.59 g (1.69 mmol, 58%). These crystals were analyzed for D-galactose content as described earlier, giving 98.5% of the theoretical value of D-galactose. Compound **4** produced the expected brown color in the neocuproine assay without prior acid hydrolysis; the molar absorbance at 460 nm was $2.17 \times 10^4\ M^{-1}$. Due to its low solubility in water or Me_2SO, a p.m.r. spectrum was not obtained for **4**.

*[(2,3,4,6-Tetra-*O*-acetyl-β-*D*-glucopyranosyl)thioglycolyl]aminoacetaldehyde dimethyl acetal* (**5**) *and (β-*D*-glucopyranosylthioglycolyl)aminoacetaldehyde dimethyl acetal* (**6**). — The D-glucose analogs of **2** and **3** were prepared in exactly the same way. T.l.c. examination of the reaction mixtures indicated that both reactions proceeded in essentially the same way as that of the D-galactose derivatives. Purification of the reaction mixture by h.p.l.c. using solvent *A* produced homogeneous **5**, which crystallized from the solvent on cooling, m.p. 103–104°.

By chromatography on Sephadex G-25, compound **6** was obtained as an amorphous material (70% yield from the pseudothiourea) that was homogeneous in t.l.c. in solvent *B* (R_F 0.33). Aliquots of a solution of **6** were analyzed for D-glucose by the automated method after mercuric hydrolysis, and for aldehyde by the neocuproine after mild hydrolysis with acid. The ratio of D-glucose to the aldehyde group thus determined was 1.1 : 1.0, assuming the molar absorbance at 460 nm of **6** in the neocuproine assay to be $2.2 \times 10^4\ M^{-1}$.

*[(2,3,4,6-Tetra-*O*-acetyl-α-*D*-mannopyranosyl)thioglycolyl]aminoacetaldehyde dimethyl acetal* (**7**) *and (α-*D*-mannopyranosylthioglycolyl)aminoacetaldehyde dimethyl acetal* (**8**). — The mannose analogs of **2** and **3** were prepared in the same way. The first reaction (formation of **7**) proceeded as fast as that of **2**. However, t.l.c. examination (solvent *A*) showed formation of some of a byproduct of lower R_F not observed in the preparation of the D-galactose analog. Fractionation of the reaction mixture by h.p.l.c. using solvent *A* produced crystalline **7**, which was recrystallized from ethanol, m.p. 103–104°. The deacetylated product, **8**, did not crystallize, but was homogeneous by t.l.c. (solvent *B*, R_F 0.37). The yield of **8** was 62% (based on the pseudothiourea derivative). Aliquots of a solution of **8** were analyzed by the neocuproine assay after mild hydrolysis with acid; the ratio of D-mannose to aldehyde group was found to be 1.13 : 1.0.

*[(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyl)thioglycolyl]aminoacetaldehyde dimethyl acetal* (**9**) *and [(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-thioglycolyl]aminoacetaldehyde dimethyl acetal* (**10**). — These compounds were prepared in the same way as **2** and **3**. The reactions proceeded very similarly to those of the D-galactose and D-glucose counterparts. Overnight storage of the *O*-deacetylation mixture in the cold produced crystalline **10** (1.07 g, 2.8 mmol; from 8 mmol of the pseudothiourea derivative). The filtrate was made neutral with 60% acetic acid, evaporated, and then fractionated on a column of Sephadex G-25, as for **3**; the overall yield of **10** from the pseudothiourea derivative was 72%. The material,

recrystallized from 95% ethanol, was pure by t.l.c. in solvent *B* (R_F 0.27) and had m.p. 198–199°. Neocuproine assay after acid hydrolysis of **10** yielded a molar absorbance value of 2.25×10^4; p.m.r. data: δ 1.80 (s, 3, CCH_3), 3.23 (s, 6, OCH_3), 3.49 (s, 4, CCH_2), and 4.28–4.4 (m, ring protons). It appears that the anomeric proton signal and the methine proton signal overlap at ~4.45 to 4.65 p.p.m.

[*(2-Acetamido-2-deoxy-β-*D*-glucopyranosyl)thioglycolyl*]*aminoacetaldehyde*(**11**). — Compound **11** was prepared as described for **4**. A solution of the dimethyl acetal **10** (0.76 g, 2 mmol) in 0.05M HCl (4 mL) was heated in a boiling-water bath for 20 min, cooled, made neutral with 2M NaOH (0.1 mL), and kept overnight in the cold, to yield crystalline **11** in 54% yield; m.p. 218–222°.

[*6-(2,3,4,6-Tetra-*O*-acetyl-β-*D*-galactopyranosylthio)hexanoyl*]*aminoacetaldehyde dimethyl acetal* (**12**). — A solution of 6-bromohexanoic acid (3.9 g, 20 mmol), aminoacetaldehyde dimethyl acetal (2.6 mL, 24 mmol), and EDAC (4.6 g, 24 mmol) in dry dichloromethane (40 mL) was kept overnight at room temperature, washed successively with cold, 0.6M H_2SO_4 (twice) and cold, saturated $NaHCO_3$, dried (Na_2SO_4), and evaporated. The resulting, thin syrup was dissolved in 1:1 (v/v) acetone–water (80 mL), and treated with per-*O*-acetyl-1-thio-D-galactose as described for **2**, using 12 mmol of the pseudothiourea and corresponding amounts of K_2CO_3 and $NaHSO_3$. After being kept overnight at room temperature, the mixture was partitioned between chloroform and water, and the organic phase was washed as described for **2**. The chloroform solution was evaporated, and the residue was dissolved in 95% ethanol and fractionated on a column of Sephadex LH-20. Fractions containing **12** (t.l.c. in solvent *A*) were combined, evaporated, and dried in a vacuum desiccator, to yield syrupy, but homogeneous, **12** (4.4 g, 8.64 mmol) in 72% yield (from the pseudothiourea derivative); p.m.r. data ($CDCl_3$): δ 1.5–1.7 (m, 6, C-CH_2-C), 1.96–2.13 (m, 14, 4 CH_3CO and CH_2CO), 2.6–2.73 (m, 2, CH_2-S), 3.33 (m, 8, 2 OCH_3 and $NHCH_2$) and 4.27–4.37 [t, 1, *J* 10 Hz, $CH(OMe)_2$].

[*6-(β-*D*-Galactopyranosylthio)hexanoyl*]*aminoacetaldehyde dimethyl acetal* (**13**). — Deacetylation of **12** was conducted as described for **3**. Gel filtration of the deacetylation product on a column of Sephadex G-25 produced homogeneous **13** (t.l.c. in solvent *B*) in 81% yield; this was crystallized from ethanol, to yield slightly hygroscopic crystals having m.p. 74–76°. After mild hydrolysis with acid, crystalline **13** yielded 94% of the theoretical quantity of D-galactose, and a molar absorbance of the neocuproine color of 1.9×10^4 M^{-1}.

Attachment of glycosides to BSA. — Attachment of these ω-aldehydo 1-thioglycosides to proteins by reductive amination was investigated, using **4** and BSA. To a solution (2 mL) of BSA (20 mg, ~0.3 μmol) and **4** (1.8 mg; 5.9 μmol) in 0.2M sodium phosphate buffer (pH 7.0) was added $NaCNBH_3$ (80 μmol in 50 μL of the buffer), and the mixture was incubated for 22 h in a water bath at 37°. The mixture was fractionated in the cold on a column (2 × 34 cm) of Sephadex G-25, which removed uncoupled reagents. From the absorbances at 280 and 480 nm, the D-galactose incorporation was calculated to be ~14 mol per mol of BSA. Incorporation of D-galactose was also confirmed by analysis of dialyzed, mercury-hydrolyzed[11],

neoglyco-BSA with the neutral-sugar analyzer. All of the ω-aldehydo 1-thioglycosides described here could be coupled to proteins under similar conditions.

DISCUSSION

Of the methods available for modification of amino groups (in proteins) that cause minimal changes in the innate structure of a protein, formation of amidino compounds with imidates[13] and formation of secondary and tertiary amines *via* reductive amination[14] have thus far proved the most useful. Both of these methods have been applied to attachment of carbohydrates to proteins[3,15]. Although, when the aldehydes were reducing sugars, reductive amination using sodium cyanoborohydride was more effective than with sodium borohydride, the reaction may nevertheless require several days or longer[16]. The slow reaction may be attributed to the minuscule concentration of the aldehydo form of the sugar in solution. It follows, that the reaction should be accelerated by using preformed glycosides possessing a free aldehyde group in the aglycon. After trial of several possible designs for such glycosides, we arrived at the compromise detailed here.

Even for the structural design finally adopted, several synthetic routes can be employed. For example, preparation of **1** has also been accomplished by chloroacetylation of aminoacetaldehyde dimethyl acetal with chloroacetyl chloride. Similarly, the bromo analog of **1** could be prepared by acylation of aminoacetaldehyde dimethyl acetal with bromoacetyl bromide; this analog behaved similarly to **1** in subsequent reactions with 1-thio sugars. However, the method described in detail here gave the best results in our hands, because of its ease of operation, as well as its high yield.

Among the 1-thioglycosides prepared, only the D-mannose derivative has the α-D anomeric configuration, whereas the other glycosides are of the β-D configuration. In earlier work[17], we found that the addition of 1-thio-α-D-mannose to an activated double bond, using pyridine as the catalyst, was considerably slower, and the yield of the adduct was much lower, than that of 1-thio-β-D-glycoses. In contrast, in the present *S*-alkylation reaction, where K_2CO_3 was used instead of pyridine, only a small difference between 1-thio-β-D-glucose and 1-thio-α-D-mannose was observed as regards the yield of the final products.

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