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AN AZIDOARYL THIOGLYCOSIDE OF SIALIC ACID. A POTENTIAL PHOTOAFFINITY PROBE OF SIALIDASES AND SIALIC ACID-BINDING PROTEINS*

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ABSTRACT

An azidoaryl thioglycoside of sialic acid was prepared, as a potential photoaffinity probe reagent for the analysis of sialidases and sialic acid-binding proteins, by treatment of the glycosyl chloride of *N*-acetylneuraminic acid methyl ester with potassium thioacetate to give, in 70% yield, methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-2,3,5-trideoxy-2-thio-α-D-*glycero*-D-*galacto*-2-nonulopyranosonate. Selective hydrolysis of the thioacetate ester, followed by condensation with 4-fluoro-3-nitrophenyl azide, *O*-deacetylation, and hydrolysis gave (4-azido-2-nitrophenyl)- 5-acetamido-2,3,5-trideoxy-2-thio-α-D-*glycero*-D-*galacto*-2-nonulopyranosidonic acid.

INTRODUCTION

Biochemical and metabolic processes involving sialic acid, its O- and N-acetyl derivatives, and its conjugated forms are currently of great interest (for a review, see ref. 1). In order to investigate various aspects of sialic acid biochemistry at a molecular level, we have prepared a novel azidoaryl glycoside of thiosialic acid to be employed as a photoaffinity probe reagent. The thioglycosyl linkage was utilized as it is presumably resistant to enzyme degradation[2]. Thus, the photoreactive functionality and the sialic acid residue would remain intact, even in crude tissue or cellular homogenates that may contain sialidase activities. Also, the thioglycosyl linkage permits the incorporation of ^{35}S into the molecule to provide a convenient radioactively labeled compound with high specific radioactivity. Upon exposure to visible light, the arylazide residue is converted into the chemically reactive arylnitrene residue which can, by a variety of mechanisms, form covalent adducts with molecules in close proximity[3]. The photosensitive reagent described herein may facilitate the purification and characterization of a number of proteins that specifically recognize sialic acid or its derivatives and cannot otherwise be purified by

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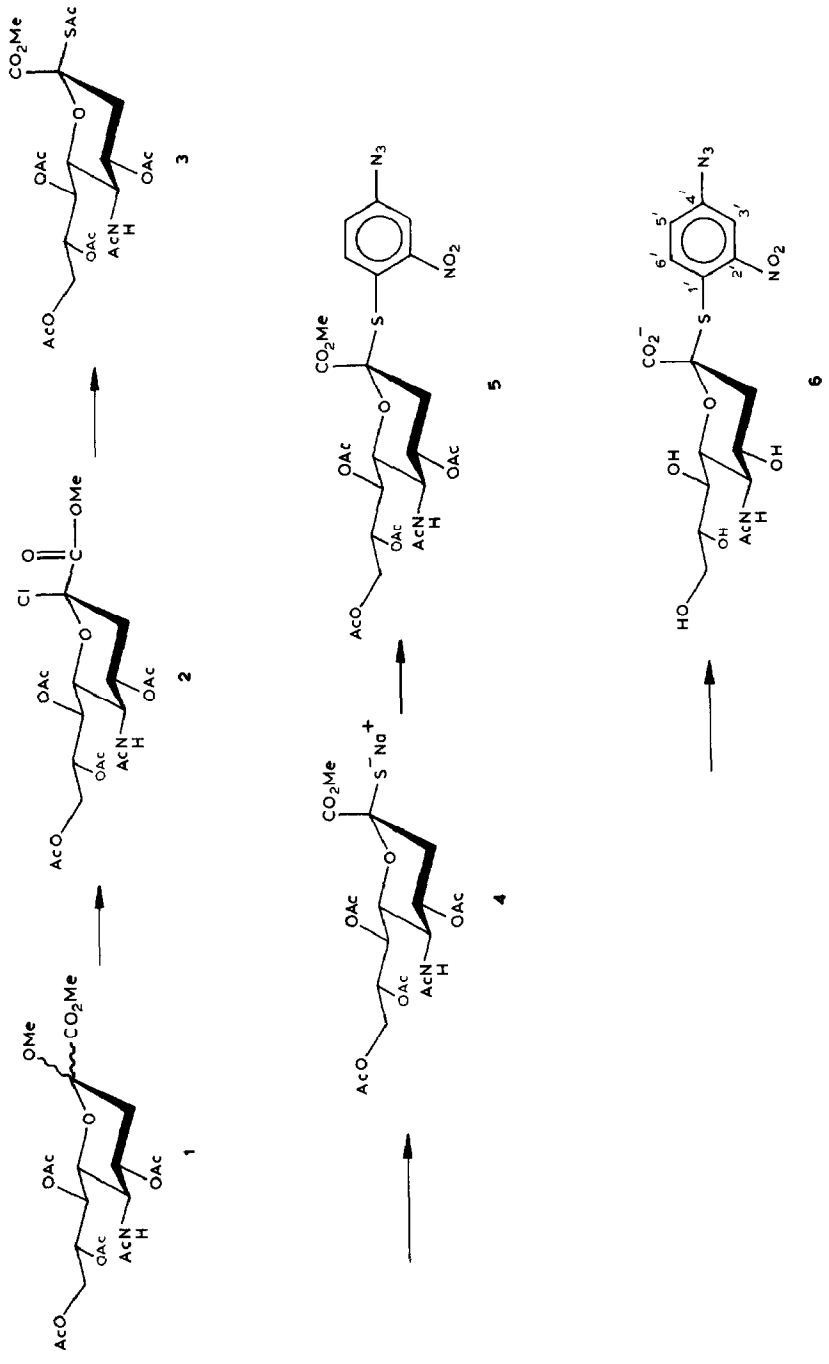
CO2Me
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CO2
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1
OMe

conventional methods. Photolabeling provides a means of incorporating a radioactive tracer into the polypeptide chain which can be used as a marker for following the progress of purification. Some proteins that are attractive candidates for investigation with this aproach include mammalian lysosomal sialidases which, due to extreme thermal sensitivity, have not been obtained in purified form[4,5]; sialic acid-binding proteins such as the hemagglutinin protein of influenza C that specifically recognizes 9-*O*-acetylsialic acid, which is difficult to assay because it requires the involved synthesis of a radioactive substrate[6]; and sialic acid trans-membrane transport proteins or sialic acid-specific lectins, which do not exhibit catalytic activity and consequently the purification of which cannot be followed by enzyme assay[7,8].

The procedure described herein utilizes the potassium salt of thioacetic acid for introducing the sulfhydryl group into the sialic acid molecule at C-2. Subsequent selective base hydrolysis of the thioacetate ester and condensation of the resulting sodium thiolate salt with 4-fluoro-2-nitrophenyl azide in *N,N*-dimethylformamide, followed by deacetylation and ester hydrolysis gave the desired product[9] **6**.

RESULTS AND DISCUSSION

To date, only a single report describing the preparation of aryl thioglycosides of sialic acid has appeared[2]. In this procedure, the pseudothiourea of peracetylated neuraminic acid methyl ester was synthesized in high yield (89%) by treating the peracetylated glycosyl chloride of neuraminic acid methyl ester with thiourea, followed by base hydrolysis to give the sodium thiolate salt. We initially explored this reaction under a variety of conditions and observed that the preparation of the pseudothiourea adduct was not straightforward and low yields (25%) were obtained. Further, upon reaction with 4-fluoro-3-nitrophenyl azide, no coupled product was detected. On the other hand, we found that reaction of the glycosyl chloride with potassium thioacetate provides a convenient method for introduction of the sulfhydryl residue into sialic acid in good yield. In contrast to other thiosugars such as 1-thiogalactose which react rapidly under mild conditions with 4-fluoro-3-nitrophenyl azide[10], the reaction of the thiolate salt of peracetylated sialic acid methyl ester (**4**) proceeded slowly, unless *N,N*-dimethylformamide was employed as solvent. This is not surprising as the thio group is linked to a tertiary carbon atom, and steric factors probably account for its reduced nucleophilicity. Also, polar, aprotic solvents such as *N,N*-dimethylformamide are well known for their ability to accelerate nucleophilic displacement reactions.

The aryl thioglycosides were less susceptible to acid hydrolysis than aryl glycosides which are extremely acid labile. This facilitated the purification and manipulation of these compounds. For example, chromatography on the strongly acid ion-exchange resin, Dowex 50, was possible and the thioglycosyl linkage remained intact. In contrast, glycosides of *N*-acetylneuraminic acid undergo hydrolytic cleavage under moderately acidic conditions[11] (pH 4.0).

The ^{1}H-n.m.r. spectra of the novel compounds described herein were com-

pared with those of sialic acid derivatives of closely related structure[12]. The spectrum of **3** was similar to those of other peracetylated sialic acids, except that the *S*-acetyl methyl signal at δ 2.3 was at a notably lower field than the signals of *O*-acetyl residues, which are usually at δ 2.1–2.2 (ref. 12). The assignment of the *S*-acetyl methyl resonance was also confirmed by the absence of the signal in the spectrum of the reaction product **5**, and the presence of similar signals in this region given by thioacetic acid and other thioacetates[13].

The ^{1}H-n.m.r. spectrum of **6** was consistent with its expected structure and it was similar to those of glycosides[11,14]. The assignments for H-3*a* and H-3*e* were confirmed by irradiation of the doublet of doublets at δ 1.911 resulting in the collapse of the signal at δ 2.855 to a doublet (J 4.5 Hz), and by irradiation at δ 2.855 to form a doublet at δ 1.911 (J 11 Hz). The α configuration of the thioglycoside may be inferred from the chemical shift of H-3*e* at δ 2.855; in contrast, β-D-glycosides, generally give[12] signals at a higher field, in the range δ 2.1–2.5. It is possible to make a similar correlation with *S*-linked derivatives. For example, Ponpipom *et al.*[15] reported that H-3*e* of a β-linked alkyl thioglycoside of sialic acid gave a signal up field relative to that of the corresponding α anomer. Also, the reaction of thioacetate salts with glycosyl halides of a number of sugars is known to proceed with inversion of configuration at the anomeric carbon atom[16], which is subsequently unaffected by hydrolysis of the thioacetate ester. Thus, it seems plausible that reaction of the β-chloride of peracetylated sialic acid methyl ester occurred in a similar manner, ultimately resulting in α-linked glycosides. Assignments for the signals in the aromatic region were made by decoupling experiments. The signal at δ 7.396 was assigned to H-5′, which is coupled to both lower-field signals, thus giving rise to a doublet of doublets (J 2.5 and 8.5 Hz) coupling to H-3′ and H-6′, respectively.

In summary, the ability of the aryl azides to act as effective photo-affinity labeling reagents will depend upon the specific protein or enzyme system tested. The relative affinity of the protein for the substrate, the protein purity, and the nature of the amino acids in closest proximity to the photo-generated aryl nitrene will determine the efficiency and specificity of labeling[17,*].

EXPERIMENTAL

General methods. — Optical rotations were measured with a Rudolph Autopol 111 polarimeter for solutions in a 1-dm cell at 25°. I.r. spectra were recorded on 1% KBr pellets with a Perkin–Elmer model 1320 spectrometer, calibrated with a polystyrene sheet. ^{1}H-N.m.r. spectra were recorded with a Bruker model AM 400 MHz spectrometer operating in the F.t. mode, at a sweep width of

*Hasegawa *et al.*[18] reported the synthesis of a series of alkyl thioglycosides of sialic acid using a method similar to that developed herein.

+/−2500 Hz. In some cases, the spectra were recorded with a Varian EM-360 at 60 MHz. The samples were prepared in (^{2}H)chloroform or ($^2H_4$)methanol and tetramethylsilane as reference. The aqueous samples were subject to four freeze-thaw cycles in the presence of deuterium oxide (99.9% ^{2}H, Merck Isotopes, Inc., St. Louis, MO.) and the spectra recorded for solutions in deuterium oxide with acetone as internal standard, relative to external sodium 4,4-dimethyl-4-sila-(2,3-$^2H_4$)pentanoate, at ambient temperature. T.l.c. was performed on precoated silica gel-coated 20 × 20 cm glass plates (Whatman). The plates were developed in one of the following solvent systems: (*A*) 7:3 chloroform–acetone 2, (*B*) 2:1:1 butanol–acetic acid–water, (*C*) 1:1 chloroform–methanol, and (*D*) 3:2 chloroform–methanol (all v/v). After development, the plates were dried under a stream of warm air, and the carbohydrates and their derivatives detected with the resorcinol spray-reagent[20]. Other detection systems included exposure of the plate to I_2 vapor (general detection); orcinol spray (1% orcinol in 50% H_2SO_4), followed by heating at 150° for 8 min (general detection); and immersion of the plate in 2% $AgNO_3$ in 9:1 (v/v) acetone–water, followed by air drying (thio esters give a yellow color after several hours). Liquid chromatography under pressure (l.c.) was carried out with a Varian liquid chromatograph model 5000 (Varian Associate, Palo Alto, CA,) using the flow-through u.v. detector supplied with the chromatograph. All solvents were reagent grade or better and were obtained from Mallinkrodt Inc. (Paris, KY.) *N,N*-Dimethylformamide, silylation grade, and 4-fluoro-2-nitrophenyl azide were from Pierce Chemical Co. (Rockford, IL). Thioacetic acid, acetyl chloride, and acetic anhydride were from Aldrich Chemical Co. (Milwaukee, WI). *N*-Acetylneuraminic acid, grade VI (Neu5NAc) isolated from *E. coli*, was from Sigma Chemical Co. (St. Louis, MO) or, in some cases, the material was isolated from edible bird nest soup according to the procedure of Martin *et al.*[19]. Potassium thioacetate was prepared by mixing equimolar amounts of thioacetic acid and KOH in dry methanol at 4°, followed by removal of the excess solvent under vacuum. The salt was thoroughly dried prior to use. Elemental analysis was performed by Galbraith Laboratories (Knoxville, TN).

*Methyl 5-acetamido-2,4,7,8,9-penta-*O*-acetyl-2,3,5-trideoxy-*D-glycero-D-galacto-*2-nonulopyranosonate* (**1**). — *N*-Acetylneuraminic acid (1.0 g, 3.2 mmol) was esterified in dry methanol (100 mL) in the presence of the cation-exchange resin AG 50W-X8 (H^+) for 3 h at room temperature according to the method of Kuhn *et al.*[21]. The resin was removed by filtration and washed with additional methanol, and the filtrate evaporated under vacuum to give a white solid (~0.93 g, 91% yield) showing a single major spot on t.l.c. (*B*).

The methyl ester was dried overnight and, in the presence of P_2O_5 under vacuum, acetylated according to the following modification of the method of Sharma and Eby[22]. The dried methyl ester was suspended in acetic anhydride (10 mL) and dry pyridine (10 mL) at 4°. The mixture was kept for 4 h in the cold and for 24 h at room temperature. Cold methanol (20 mL) was added and the mixture maintained for 1 h at 4°, and then for 0.5 h at room temperature. Excess acetic

anhydride, pyridine, and methyl acetate were removed under vacuum. The residue was dissolved in chloroform (150 mL), and the solution washed with saturated $NaHCO_3$, water (3 times), dried $(Na_2SO_4)_2$, and evaporated. The residue was purified on a silicic acid column (2.5 × 35 cm, Sil A 200, Sigma Chemical Co.), eluted with chloroform, and then a gradient of chloroform with increasing amounts of 1:1 (v/v) chloroform–acetone. The elution was monitored by t.l.c. of the collected 1.5-mL fractions. The appropriate fractions were pooled, the solvent was removed, and the residue dried (P_2O_5) to give a glass (70% yield), homogeneous by t.l.c. (R_F 0.22, *A*); ^{1}H-n.m.r. ($CDCl_3$): δ 1.9 (s, 3 H, $NCOCH_3$), 2.02–2.05 (s, 9 H, $3OCOCH_3$), 2.1 (s, 6 H, $2OCOCH_3$) and 3.8 (s, 3 H, OCH_3).

*Methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,3,5-trideoxy-β-*D*-glycero-*D*-galacto-nonulopyranosyl chloride)onate* (**2**). — Dried **1** was dissolved in acetyl chloride (25 mL), the solution cooled to −5° and dry HCl bubbled through for 10 min with constant cooling and mechanical stirring. The reaction vessel was sealed and maintained for 48 h at 4°. Excess HCl was removed under a stream of N_2 and the residual acetyl chloride under vacuum. The homogeneous chloride (R_F 0.34, *A*) was used without further purification, ^{1}H-n.m.r. ($CDCl_3$): δ 1.8 (s, 3 H, $NCOCH_3$), 2.0 (s, 9 H, $3OCOCH_3$), 2.1 (s, 3 H, $OCOCH_3$), and 3.9 (s, 3 H, OCH_3).

*Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-2,3,5-trideoxy-2-thio-α-*D*-glycero-*D*-galacto-nonulopyranosonate* (**3**). — A solution of **2** (0.67 g, 131 μmol) in dry acetone (5 mL) at 4° was added to a suspension of potassium thioacetate (0.225 g, 197 μmol) in acetone (5 mL) at 4°. The mixture was brought to room temperature and kept for 1.0 h with vigorous mechanical mixing. KCl and excess thioacetate were removed by filtration and the salts washed with chloroform. The combined filtrate was evaporated under vacuum, the residue dissolved in chloroform (150 mL), the solution washed with 0.1M HCl, and then water (3 times), dried (Na_2SO_4), and evaporated. Purification of the residue on a silicic acid column, as described earlier, gave **3** (70% yield) showing a single spot on t.l.c. (R_F 0.30, *A*), $[\alpha]_D^{25}$ +54° (*c* 0.82, methanol); ^{1}H-n.m.r. ($CDCl_3$) 1.9 (s, 3 H, $NCOCH_3$), 2.0 (s, 6 H, $2OCOCH_3$), 2.1 (s, 6 H, $2OCOCH_3$), 2.3 (s, 3 H, $SCOCH_3$) and 3.8 (s, 3 H, OCH_3).

Anal. Calc. for $C_{22}H_{30}NO_{13}S$: C, 48.17; H, 5.51; N, 2.55; O, 37.92; S, 5.84. Found: C, 48.10; H, 5.81; N, 2.45; O, 37.70; S, 5.95.

*Methyl (4-azido-2-nitrophenyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,3,5-trideoxy-2-thio-α-*D*-glycero-*D*-galacto-2-nonulopyranosid)onate* (**5**). — Compound **3** (80 mg, 146 μmol), sodium methoxide (7.1 mg, 146 μmol), and 4-fluoro-3-nitrophenyl azide (52 mg, 291 μmol) were dried overnight (P_2O_5) under vaccum in separate reaction vials fitted with septum caps. Each of the reactants was suspended in dry methanol (0.5 mL) and anhydrous conditions were maintained by momentarily purging the solution with dry N_2. All manipulations with the aryl azide were carried out with the exclusion of light and the reactions were conducted in amber reaction vials. The methanolic solutions of sodium methoxide and **3** were combined. After 5 min at room temperature, the resulting sodium thiolate salt, **4**

was condensed directly with the phenyl azide without isolation, and the mixture maintained for 10 min at room temperature. The solvent was removed under N_2, the residue placed under vacuum in the presence of P_2O_5 for 15 min, and then suspended in *N,N*-dimethylformamide (0.5 mL). After 1 h at room temperature the solvent was removed under vacuum, the mixture dissolved in chloroform, and the product purified on a silicic acid column (1.5 × 15 cm) by elution first with chloroform (50 mL), and then a gradient of chloroform–acetone (2.0-mL fractions). Further purification was carried out by l.c. on a C-18 reverse phase column equilibrated in 1:1 (v/v) acetonitrile–water (Altec Ultrasphere, 4.5 × 250 mm, Beckman, Inc. Carlsbad, CA). The partially purified product was suspended in 1:1 (v/v) acetonitrile–water, and the column eluted at a flow rate of 3.0 mL/min with a linear gradient of increasing amounts of acetonitrile (3.3% increase/min for 15 min, up to 100% acetonitrile). The eluate was monitored at 210 nm, and the appropriate fractions were pooled to give **5** (~50 mg, 49% yield), $[\alpha]_D^{25}$ +76° (*c* 1.12, methanol); R_F 0.34 (*A*); ν_{max}^{KBr} 2125, and 2110 (sh) cm^{-1} (N_3); ^{1}H-n.m.r. ($CDCl_3$): δ 1.9 (s, 3 H, $NCOCH_3$), 2.0 (s, 9 H, $3OCOCH_3$), 2.1 (s, 3 H, $OCOCH_3$), 3.6 (s, 3 H, OCH_3), 7.3 (d, 1 H, arom.), 7.4 (s, 1 H, arom.), and 7.8 (d, 1 H, arom.).

Anal. Calc. for $C_{26}H_{30}NO_{14}S$: C, 46.71; H, 4.52; N, 10.47; O, 33.50; S, 4.80. Found: C, 46.64; H, 4.75; N, 10.15; O, 33.31; S, 5.16.

*(4-Azido-2-nitrophenyl 5-acetamido-2,3,5-trideoxy-2-thio-α-*D-glycero-D-ga-lacto-*2-nonulopyranosid)onic acid* (**6**). — Deacetylation and ester hydrolysis was accomplished by suspension of **4** in 1:1 (v/v) methanol–0.5M NaOH (1.5 mL). After 30 min at 37°, the mixture was applied to a column (1.0 × 2.5 cm) containing AG 50 W-X8 (H^+), the product eluted with water (10 mL) and the solution lyophilized. The homogeneous product (R_F 0.49, *B*) was obtained in 73% yield (33 mg) or 24% yield based on **3**, $[\alpha]_D^{25}$ +171° (*c* 0.51, water); ν_{max}^{KBr} 2125 and 2110 (sh) cm^{-1} (N_3); ^{1}H-n.m.r., see Table I.

Anal. Calc. for $C_{17}H_{20}N_5O_{10}S \cdot 2\ H_2O$: C, 39.08; H, 4.63; N, 13.41; O, 36.75; S, 6.14. Found: C, 39.24; H, 4.61; N, 13.28; O, 36.64; S, 6.23.

TABLE I

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

Atoms	δ	*Signal intensity (Theor./Exper.)*	*Signal type*[b]
H-3*a*	1.911	1.0/1.1	dd
$NCOCH_3$	2.025	3.0/2.9	s
H-3*e*	2.855	1.0/1.0	dd
H-4–H-9[b]	3.507–3.906	7.0/7.3	
H-5′	7.396	1.0/1.0	dd
H-3′	7.624	1.0/0.8	d
H-6′	7.902	1.0/1.0	d

[a]Spectra were acquired at 400 MHz at ambient temperature. Coupling constants were taken directly from the observed line spacing from Gaussian-resolution enhanced spectra. The sample was prepared in deuterium oxide with acetone as internal standard relative to external sodium 4,4-dimethyl-4-sila-(1,3-$^2H_4$)pentanoate (δ 2.234). Signal assignments were based on double irradiation experiments of each signal, respectively. Pulses of ~2μsec (~−40° flip angle) were used with a delay between pulses of 16s, with 16 acquisitions. [b]Ring proton signals were not assigned.

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