Preparation and Some Biochemical Properties of Neoglycoproteins Produced by Reductive Amination of Thioglycosides Containing an ω-Aldehydoaglycon†

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ABSTRACT: A new method of preparing neoglycoproteins containing thioglycosides is described. We have previously prepared a series of thioglycosides having an ω-aldehydo group in the aglycon: *N*-[*S*-(β-D-galactopyranosyl)thioacetyl]-aminoacetaldehyde (Gal-AD) and *N*-[*S*-(β-D-galactopyranosyl)thiohexanoyl]aminoacetaldehyde (Gal-HD) [Lee, R. T., & Lee, Y. C. (1979) *Carbohydr. Res.* (in press)]. These thioglycosides were attached to proteins by reductive amination using $NaCNBH_3$ as the reducing agent. Optimal coupling of Gal-AD to bovine serum albumin (BSA) was obtained at pH 6–7, using a 5–20-fold excess of $NaCNBH_3$ over the aldehyde compound. The concentration of $NaCNBH_3$ was kept below 0.1 M to avoid undesirable side reactions. Unlike the coupling of sugars to proteins by reductive amination in which reducing sugars were used as the source of the aldehyde group, [Gray, G. (1974) *Arch. Biochem. Biophys. 163*, 426–428], the coupling of sugars by this method proceeded rapidly, presumably because an unmasked aldehyde was used. The bulk of the reaction was finished within 2 h at 37 °C, and 24 mol of the thioglycoside was incorporated into BSA in 2 h when a 300-fold molar excess of Gal-AD was used over the protein. As in the case of coupling sugar imidate to proteins [Lee, Y. C., Stowell, C. P., & Krantz, M. J. (1976) *Biochemistry 15*, 3956–3962; Krantz, M. J., Holtzman, N. A., Stowell, C. P., & Lee, Y. C. (1976) *Biochemistry 15*, 3963–3968], only limited numbers of lysyl residues in the proteins were available to these aldehydo reagents. Incorporation of Gal-AD into enzymes did not cause inactivation even when a large excess of the reagent was used, so long as active sites were properly protected. As expected, neoglycoproteins containing D-galactosyl residues were found to be good inhibitors of asialoorosomucoid (ASOR) binding to the rabbit liver plasma membrane. The ability of a neoglycoprotein to inhibit the ASOR binding was directly related to the galactoside density on the protein. Asialotransferrin, which is a poor inhibitor, became an excellent inhibitor after coupling thiogalactoside to it. At the same low sugar density, bovine serum albumin modified with the 9-atom aglycon was found to be a better inhibitor than the 5-atom aglycon. Therefore, the distance of the sugar residue from the protein surface might be of some importance at lower levels of sugar density.

Neoglycoproteins (Lee et al., 1976) have been of great value in studying the role of carbohydrates in various glycoconjugates (Stowell & Lee, 1979). Ideal methods for coupling sugars to proteins should be such that the coupling proceeds efficiently in the moderate pH range, that the products formed retain the original overall charge distribution with minimal introduction of hydrophobicity, and that the newly formed linkage be of reasonable chemical stability. For these reasons the imidate coupling method previously reported from this laboratory (Lee et al., 1976) is an ideal procedure. Neoglycoproteins prepared by this method have been used (Krantz et al., 1976; Stowell & Lee, 1978) to study the D-galactose-binding protein of the rabbit liver membrane (Ashwell & Morell, 1974). This type of neoglycoprotein contains a thioglycosidic linkage; its advantage over the *O*-glycosidic linkage has been discussed elsewhere (Chipowsky et al., 1973; Stowell & Lee, 1979). One disadvantage of the imidate method is the difficulty in preparing the imidate of carbohydrates with low solubility in dry methanol. Such carbohydrates include those which are larger than disaccharide and those which contain charged groups and some oligosaccharides which contain acetamide groups.

Of various methods used for modification of proteins, reductive amination is known to preserve protein structure close to its native state (Means & Feeney, 1968). As shown in Scheme I, reductive amination modifies lysyl residues (and N-terminal residues) to produce first a secondary amine (eq 1a) and then a tertiary amine (eq 1b), so that the original positive charge of the lysyl residues is retained. Gray (1974) was first to use reductive amination for coupling sugars to proteins, using an existing aldehyde group of a disaccharide. We have prepared several thioglycosides containing an ω-aldehydo group in the aglycon (Lee & Lee, 1979) (Chart I). Unlike the imidate thioglycosides, the final stages of preparation of these ω-aldehydo thioglycosides are conducted in aqueous media, so that the method is applicable to a wider range of carbohydrates.

In this paper we describe the procedure for coupling such thioglycosides to proteins and discuss the advantage of this method over the imidate method and over direct coupling of sugars by reductive amination. We describe also the effect of sugar attachment on enzymatic properties and use of these neoglycoproteins to study the rabbit liver D-galactose-binding protein.

Materials and Methods

The following chemicals were obtained commercially and used without purification: BSA[1] (Sigma Chemical Co. and

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[1] Abbreviations used: BSA, bovine serum albumin; LDH, lactate dehydrogenase; TLC, thin-layer chromatography; TNBS, 2,4,6-trinitrobenzenesulfonic acid; Gal-AA, *N*-[*S*-(β-D-galactopyranosyl)thioacetyl]-aminoacetaldehyde dimethyl acetal; Gal-AD, *N*-[*S*-(β-D-galactopyranosyl)thioacetyl]aminoacetaldehyde; Gal-HA, *N*-[*S*-(β-D-galactopyranosyl)-6-thiohexanoyl]aminoacetaldehyde dimethyl acetal; Gal-HD, *N*-[*S*-(β-D-galactopyranosyl)-6-thiohexanoyl]aminoacetaldehyde; ASOR, asialoorosomucoid; AST_f, asialotransferrin; RIP, relative inhibitory power; Gal-AD– or Gal-HD–protein, the protein to which thiogalactosides are incorporated by reductive amination using Gal-AD or Gal-HD.

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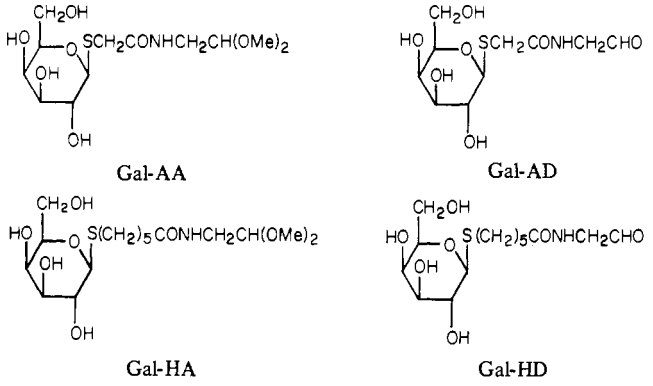
Scheme I

$$RCHO + NH_2R' \xrightarrow{NaCNBH_3} RCH_2NHR' \quad (1a)$$

$$RCHO + RCH_2NHR' \xrightarrow{NaCNBH_3} (RCH_2)_2NR' \quad (1b)$$

Chart I



Pentex, fatty acid free); $NaCNBH_3$ (Aldrich Chemical Co. and Alfa-Ventron); rabbit muscle L-LDH, porcine pancreatic α-amylase, ovalbumin, NADH, N^{α}-acetyl-L-lysine, and pyruvic acid (all from Sigma); NAD^+ (Li^+ salt, Boehringer); soluble starch (Merck); sodium [^{125}I]iodide, carrier free in 0.1 M NaOH (New England Nuclear).

Maltopentaose was kindly provided by Anheuser-Busch, Inc.; human serum transferrin and orosomucoid were provided by the American Red Cross, and neuraminidase from *Arthrobacter ureafaciens* was provided by Dr. Y. Uchida. α-Amylase from *Asperigillus oryzae* was prepared according to the previously published method (McKelvy & Lee, 1969). Thioglycosides, Gal-AA and Gal-HA (Chart I), were prepared according to the method of Lee & Lee (1979). Briefly, they are prepared by first acylating aminoacetaldehyde dimethyl acetal with ω-haloacid to form an N-(ω-haloalkanoyl)aminoacetaldehyde dimethyl acetal, which was in turn reacted with per-O-acetylated 1-thioglycose. Deblocking of protecting groups produced desired products.

Orosomucoid was desialylated as described earlier (Krantz et al., 1976). Transferrin (30 mg) was desialylated by using 0.5 unit[2] of the neuraminidase in 2 mL of 0.1 M sodium acetate buffer, pH 5.0, at 37 °C for 48 h. The desialylated transferrin was dialyzed, saturated with ferric ion, and purified on a column of DEAE-cellulose as described by Wong & Regoeczi (1977). The sialic acid content of untreated and neuraminidase-treated orosomucoid and transferrin was determined by using the method of Aminoff (1961) as modified by Uchida et al. (1977). Sialic acid contents were as follows: orosomucoid, 15.6; ASOR, 1.2; transferrin, 5 mol/mol of protein. AST_f was completely devoid of sialic acid.

Protein concentrations were determined by the microbiuret method (Zamenof, 1957) using fatty acid free BSA (from Pentex) which had been dried in a heated vacuum desiccator (40 °C) as the standard. Protein concentrations were also determined from UV absorbance with $E_{1cm}^{1\%}$ of 6.6 and 16.8 at 280 nm for BSA and *A. oryzae* α-amylase (McKelvy & Lee, 1969), respectively, and $E_{1cm}^{1\%}$ of 13.0 at 278 nm for iron transferrin (Lane, 1971). Values obtained from UV absorption and from the microbiuret method agreed well.

Neutral sugar was quantitated by a modified phenol–sulfuric acid method (McKelvy & Lee, 1969) and amino group was quantified by modification of the TNBS method (Lee, 1978). Aldehydo group was determined by the neocuproine method (Dygert et al., 1965). TLC was done on silica gel F254 or Kieselguhr precoated on aluminum sheets (Merck). High-voltage electrophoresis (Savant Instruments, Inc.) was carried out on Whatman 3 MM paper and stained with 3% ninhydrin in acetone. Protein samples were hydrolyzed with 5.7 M HCl in vacuo at 105 °C for 24 h and were analyzed for amino acid contents by a Durrum D-500 automatic amino acid analyzer.

Deacetalization of 1-Thioglycoside ω-Aldehydo Dimethyl Acetal. Due to instability of the aldehydo group during long-term storage, the glycosides were stored mostly in the form of dimethyl acetal derivatives, which were converted to the corresponding aldehydes immediately before the coupling to proteins. The dimethyl acetal derivative was dissolved in a minimum volume of 0.05 M HCl or trifluoroacetic acid in a screw-capped tube (13 × 100 mm) with a Teflon-lined cap and heated in a heating block at 100 °C for 20 min. Hydrolysis was essentially over in 10 min with 0.05 M HCl and in 15 min with 0.05 M trifluoroacetic acid, as determined by the neocuproine method, and the aldehyde content stayed at a constant value up to 1 h. After the reaction, the acid was neutralized with the equivalent amount of NaOH. In the case of trifluoroacetic acid, the mixture can be evaporated completely, if so desired. Neutralized solution was used directly in the coupling reaction.

Determination of Thiogalactoside Incorporation into Proteins. For determination of the efficiency of sugar coupling to BSA under different conditions, a small column (2 × 34 cm) of Sephadex G-25 was used in the cold for separation of derivatized BSA from small molecules in the reaction mixtures. The eluant was 0.1 M NaCl and fractions of 4.5 mL were collected. The flow rate was between 0.8 and 1.2 mL/min and the elution was usually completed within 1.5–2 h. Effluent fractions were analyzed for carbohydrate by the phenol–sulfuric acid method and for protein by absorbance at 280 nm. The amount of sugar coupled (moles/mole) was estimated by three methods. *Method A*: knowing the quantities of the protein and the thiogalactoside used, we calculated the amount of sugar coupled from the ratio of protein-associated thiogalactoside to that of thiogalactosides of lower molecular size (as determined with the phenol–sulfuric acid method). *Method B*: the quantities of protein (absorbance at 280 nm) and the thiogalactoside associated with the protein peak (phenol–sulfuric acid method) were individually measured. *Method C*: the protein peak was dialyzed against water and lyophilized. Protein was analyzed by the microbiuret method and galactose was analyzed by automated sugar analysis (Lee, 1972) after hydrolysis of the thioglycosidic linkage with mercuric acetate (Krantz & Lee, 1976).

Enzyme Assays. Activity of the α-amylases was determined by the neocuproine method based on the method of Strumeyer (1967) routinely using reduced starch as the substrate. Reduced maltopentaose was also used as the substrate. Maltopentaose (20 mg in 0.4 mL of water) was reduced according to the method of Strumeyer (1967) using 10 mg of $NaBH_4$ at room temperature overnight.

LDH activity was determined by measuring the decrease in absorbance at 340 nm with a Perkin-Elmer 576 spectrophotometer based on the method of Kornberg (1955).

Assay of Neoglycoprotein Binding by Liver Plasma Membrane. The efficiency of binding of neoglycoproteins to liver plasma membrane was determined by measuring their ability to inhibit the binding of ASOR to membranes. A modified version (Krantz et al., 1976) of the method described by Van

[2] One unit of neuraminidase liberated 1 μmol of N-acetylneuraminic acid per min from (N-acetylneuraminyl)lactose at pH 5.0 and 37 °C.

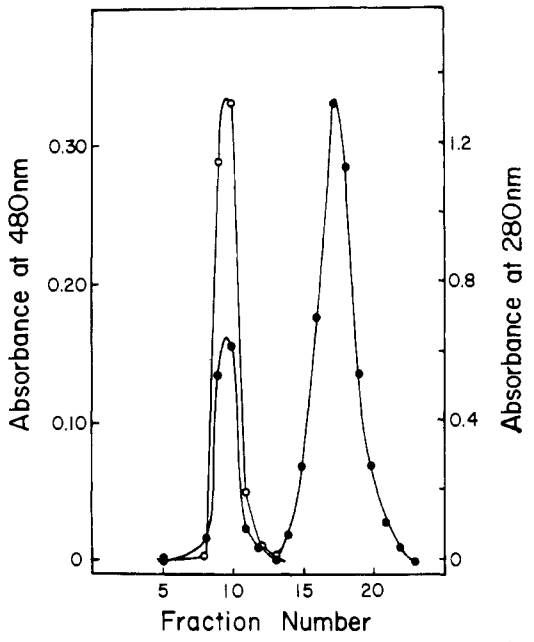


FIGURE 1: Typical elution profile of a reductive amination mixture on the column (2 × 34 cm) of Sephadex G-25. (●) Absorbance at 480 nm; sugar determination by phenol–sulfuric acid method. (○) Absorbance at 280 nm.

Lenten & Ashwell (1972) was used. The relative inhibitory power (RIP) of a neoglycoprotein was defined as the ratio of the amount of ASOR producing 50% inhibition to the amount of neoglycoprotein producing 50% inhibition (Krantz et al., 1976).

Results

Factors Affecting Coupling of Gal-AD to BSA. Several factors which affect the coupling of ω-aldehydo thioglycoside to proteins were investigated by using Gal-AD and BSA. A neutralized acid hydrolysate of Gal-AA (4 mg, 12 μmol) (see previous section) was dissolved in a solution (2 mL) of BSA (25 mg, 0.36 μmol) in 0.2 M sodium borate buffer, pH 8.0. After the addition of about 5 mg (~80 μmol) of $NaCNBH_3$, the mixture was incubated for 19–22 h at 37 °C. The extent of coupling was determined by passing the reaction mixture through the Sephadex G-25 column, as described under Materials and Methods. A typical elution profile is shown in Figure 1. Under these conditions, about 20% of the total carbohydrate in the effluent was found in the protein peak (the first peak in Figure 1), indicating that 7 mol of sugar was incorporated per mol of BSA (method A). Analysis of the same protein peak by method B gave an incorporation value of 8 mol/mol of BSA. The effects of pH, the molar ratio of $NaCNBH_3$ to Gal-AD, time, and temperature on the coupling reaction (see below) were studied under essentially the same conditions described above except for the parameter that is varied.

(*a*) *Effect of pH.* The pH values of the reaction medium were varied from 6 to 9 by using 0.2 M sodium phosphate buffers (pH 6–8) and 0.2 M sodium borate buffers (pH 8–9). The pH of the reaction medium was unchanged after overnight incubation. Figure 2 summarizes the results of these experiments. On the basis of these results, 0.2 M sodium phosphate buffer, pH 7.0, was used in subsequent experiments.

(*b*) *Molar Ratio of $NaCNBH_3$ to Gal-AD.* Figure 3 shows the results of varying the ratio of $NaCNBH_3$ to Gal-AD in the coupling reaction. Under the conditions described in the figure legend, the level of thioglycoside coupled was maximal at a ratio of $NaCNBH_3$/aldehyde of (10–15):1 and a $NaCNBH_3$ concentration of 0.1 M. Further increases in the ratio (or the concentration of $NaCNBH_3$) resulted in lower levels of coupling.

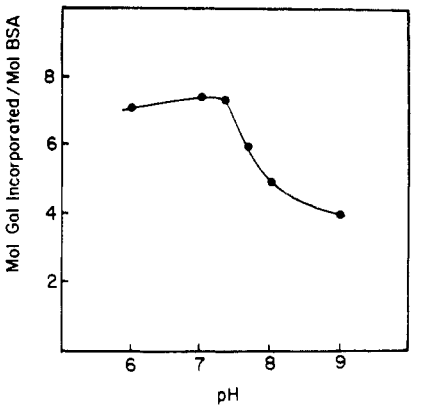


FIGURE 2: Thiogalactoside incorporation into BSA as a function of the pH of the reductive amination mixture. Each reaction mixture contained 20 mg (0.29 μmol) of BSA, 4.4 mg (13 μmol) of hydrolyzed Gal-AA, and 25 mg (400 μmol) of $NaCNBH_3$ in 1.2 mL of 0.2 M sodium phosphate or sodium borate buffer.

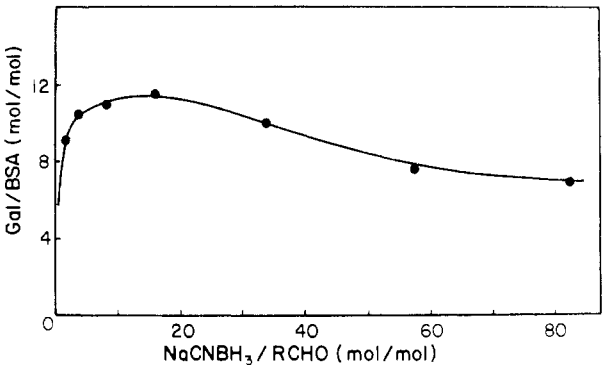


FIGURE 3: Effect of $NaCNBH_3$ on the incorporation of thiogalactoside into BSA. Reaction mixtures contained 4.4 mg (13 μmol) of acid-hydrolyzed Gal-AA, 25 mg (0.36 μmol) of BSA, and various quantities of $NaCNBH_3$ as shown in the figure in 2 mL of 0.2 M sodium phosphate buffer, pH 7.0. The mixtures were incubated at 37 °C for 24 h.

In another experiment, $NaCNBH_3$ was added either all at once or in three equal portions during the 24-h incubation period. There was no appreciable increase in the coupling efficiency by such a multiple addition of $NaCNBH_3$ over the single addition reaction.

(*c*) *Time Course of Reductive Amination.* The time course of the coupling reaction was investigated under two different sets of conditions. The molar ratio of the aldehyde to BSA was 40:1 in the first set (low coupling) and 300:1 in the other (high coupling). The ratio of $NaCNBH_3$/aldehyde was 11:1 for the low-coupling and 5:1 for the high-coupling reaction. As shown in Figure 4, in both experiments a large portion of total incorporation occurred within the first few hours, followed by a slower increase extending over 2 days.

(*d*) *Effect of Temperature.* The coupling reaction was carried out at three different temperatures, using a molar ratio of BSA/aldehyde/$NaCNBH_3$ = 1:66:760 and an incubation time of 24 h. Incorporation of thiogalactoside was 12, 9, and 6 mol/mol of BSA at 37, 23, and 4 °C, respectively. Obviously, the reaction proceeded faster at higher temperatures.

(*e*) *Sequential Addition of the Aldehyde and $NaCNBH_3$.* In an attempt to increase the concentration of the purported aldimine (Schiff's base) formed between the aldehyde and the ε-amino group of lysyl residues in the protein, BSA was preincubated with the aldehyde at 37 °C for various lengths

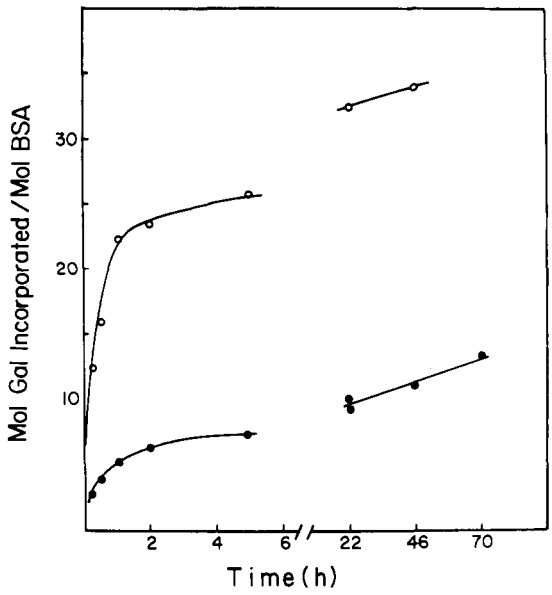


FIGURE 4: Time course of thiogalactoside incorporation into BSA. (●) A 40-fold molar excess of Gal-AD over BSA was used (molar ratio of Gal-AD to amino group was 0.7). (○) A 300-fold molar excess of Gal-AD over BSA was used (molar ratio of Gal-AD to amino group was 5.0).

Table I: Coupling of Gal-AD to BSA by Reductive Amination

experiments	ratio of Gal-AD to BSA[a]	ratio of $NaCNBH_3$ to Gal-AD	vol of reaction mixture (mL)	thiogalactoside incorpd (mol/mol of BSA)
I	32	20	6	12.6
II	96	17	6	22.1
III	176	11	6	31.4
IV	288	9	6	35.7
V	496	6	7	41.8
VI	800	5	7	52.0

[a] BSA used in each experiment was 69 mg (1 μmol).

of time before the reducing agent was added. Incubation periods of up to 1 h did not change the coupling efficiency.

Preparation of Neoglycoproteins with Different Levels of Sugars. On the basis of the results presented above, a series of neoglycoproteins were prepared from Gal-AD and BSA. Reactions were carried out by using ~1 mol (60 mg) of BSA at 37 °C in 0.2 M sodium phosphate buffer, pH 7.0, for 24 h. The modified BSA samples were dialyzed exhaustively against water in the cold, freeze-dried, and analyzed by method C (without column fractionation) for galactose content. Table I summarizes the levels of sugar incorporated under different reaction conditions.

Neoglycoproteins containing D-glucose, D-mannose, and 2-acetamido-2-deoxy-D-glucose were also prepared by using conditions similar to those described above. In each case, the amount of incorporated sugar per mole of BSA was within 10% of the values presented in Figure 5. BSA was also modified with Gal-HD. The level of sugar incorporation was similar to that obtained with Gal-AD. α-Amylases, LDH, and AST_f were modified with Gal-AD under similar conditions, except that coupling was done at room temperature and the $NaCNBH_3$ concentrations were kept below 0.1 M. After the reductive amination, *A. oryzae* α-amylase and LDH were dialyzed in the cold against water. The pancreatic α-amylase was dialyzed in the cold against 5 mM sodium phosphate buffer, pH 7, containing 5 mM NaCl and AST_f against 10 mM Tris-HCl buffer, pH 8. All proteins were freeze-dried after

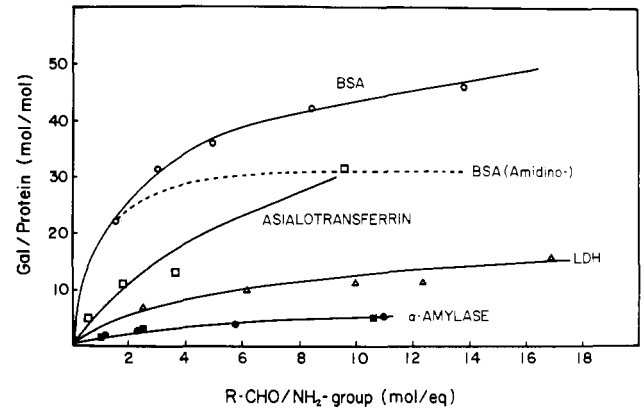


FIGURE 5: Incorporation of thiogalactoside into various proteins as a function of the molar ratio of Gal-AA to ε-amino group of proteins. (○) BSA; (●) *A. oryzae* α-amylase; (■) porcine pancreatic α-amylase; (△) LDH; (□) AST_f. The dashed line indicates the incorporation of thiogalactoside into BSA by the imidate method.

dialysis. The thioglycoside content in these neoglycoproteins was determined by method C without prior fractionation on the column of Sephadex G-25 (see Materials and Methods). Figure 5 summarizes the results of the sugar incorporation as a function of the ratio of aldehyde to amino groups of various proteins.

Characterization of Neoglycoproteins Formed by Reductive Amination. A model compound, N^{α}-acetyl-L-lysine (1 mmol) was treated with 2 mmol of Gal-AD and 20 mmol of $NaCNBH_3$ in 10 mL of 0.2 M sodium phosphate buffer, pH 7.0, overnight at 37 °C. The reaction mixture was acidified with 1 mL of 60% acetic acid and fractionated on a column (5 × 215 cm) of Sephadex G-25 using 0.1 M acetic acid as the eluant. Three well-separated peaks, containing 10, 40, and 50% of the total carbohydrate, were obtained.

The last peak was a double peak and was shown to contain Gal-AD and another compound of much higher R_f (presumably the reduction product of Gal-AD) by TLC on silica gel by using 3:2:1 (v/v) ethyl acetate–acetic acid–water as the solvent. The first peak contained one component (R_f 0.12) and the middle peak contained one major component (R_f 0.38) and a small amount (~10%) of a contaminant (R_f 0.18) by TLC on Kieselguhr by using the same solvent system. The order of elution from the Sephadex column and the mobility in TLC suggested that peaks 1 and 2 contained the di-N^{ϵ}-substituted and mono-N^{ϵ}-substituted derivatives of N^{α}-acetyl-L-lysine, respectively.

The following experiment was carried out to obtain further support for this conclusion. The fractions of peaks 1 and 2 were evaporated separately to dryness. The dried residues from peaks 1 and 2 were hydrolyzed in 6 M HCl at 100 °C for 2 h. The hydrolysates were analyzed by the TNBS method for primary amino groups (using 6-aminohexanoic acid as the standard), and these values were compared to the thiogalactoside contents measured with the phenol-H_2SO_4 method prior to the acid hydrolysis (using Gal-AA and Gal-AD as the standards). The ratio of amino group to thiogalactoside was 1.47 for the hydrolysate of the material in peak 1 and 1.9 for the hydrolysate of the material in peak 2. These results strongly suggest that peaks 1 and 2 are di- and mono-N^{ϵ}-substituted N^{α}-acetyl-L-lysine, respectively, and strong acid hydrolysis yielded N^{ϵ},N^{ϵ}-di(2-aminoethyl)-L-lysine (**1**) and N^{ϵ}-(2-aminoethyl)-L-lysine (**2**), respectively (Chart II).

Identification of Mono- and Disubstituted Lysine Residues in the Neoglycoproteins. Compounds **1** and **2** could be separated from each other and from all the usual amino acids

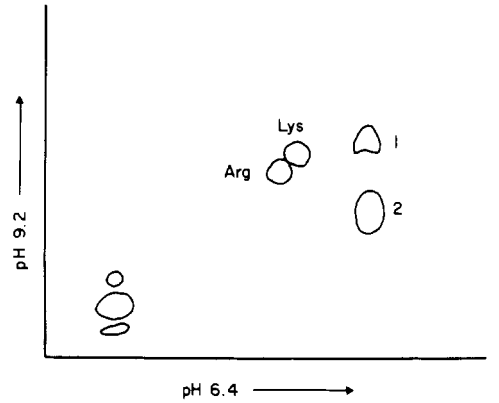
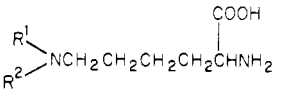


FIGURE 6: Two-dimensional electrophoresis of the acid hydrolysate of Gal-AD–BSA VI (Table I). The first direction: pyridine–acetic acid buffer, pH 6.4 (pyridine, 100 mL; acetic acid, 4 mL; water, 900 mL); 1500 V; 30 min. The second direction: 1% sodium borate buffer, pH 9.2; 1500 V; 25 min.

Chart II



1, R^1, $R^2 = NH_2CH_2CH_2-$
2, $R^1 = NH_2CH_2CH_2-$; $R^2 = H$

(including lysine and arginine) by two-dimensional paper electrophoresis. Figure 6 shows the ninhydrin-stained electrophoretogram of such a separation. The acidic and neutral amino acids migrated near the origin.

The preparations of BSA modified with Gal-AD (Gal-AD–BSA) (Table I) were subjected to the standard acid hydrolysis for amino acid analysis and then to the two-dimensional electrophoresis. Preparation VI contained both **1** and **2** while II contained **2** only. A very small amount of **1** was evident in the Gal-AD–BSA IV hydrolysate.

The acid hydrolysates were also analyzed with the amino acid analyzer. Under the conditions used for the separation of normal amino acids, **1** was eluted exactly at the same position as arginine, while **2** was eluted ahead of arginine with a partial overlap. Amino acid compositions (Cys, Tyr, and Arg not included) of Gal-AD–BSA preparations presented in Table I are shown in Table II.

The level of unmodified lysine decreased as the level of thiogalactoside incorporation increased, and the decrease in the number of lysyl residues agreed well with the number of thiogalactosides incorporated up to ~40 mol/mol of BSA. Arginine content of Gal-AD–BSA preparations could not be measured accurately because of the overlaping of arginine with **1** and **2**. The level of **2** increased as thiogalactoside incorporation increased. However, hydrolysates of Gal-AD–BSA's V and VI had very similar contents of **2**.

Effect of Sodium Cyanoborohydride Concentration on Enzymes. Both α-amylases and LDH were treated overnight with various concentrations of $NaCNBH_3$ at pH 7.0 and room temperature. Both amylases were fully active when treated with 0.2 M $NaCNBH_3$, and the LDH activity was not affected by 0.1 M $NaCNBH_3$ (higher concentrations were not tested). Pancreatic amylase showed a slight decrease in activity after 48 h treatment with 0.3 M $NaCNBH_3$.

Effect of Thiogalactoside Incorporation on Enzymatic Activity. Because of possible side effects of $NaCNBH_3$ on the enzymatic activities, reductive amination was routinely carried out by using $NaCNBH_3$ concentrations of less than 0.1 M. Effects of thiogalactoside incorporation on the activities of enzymes modified with the reductive amination are shown

Table II: Amino Acid Composition of Gal-AD-BSA Preparations Described in Table I

amino acid	BSA[a]	Gal-BSA[b] I	II	IV	V	VI
Asp	54	49.7	54.8	53.0	54.2	53.7
Thr	34	39.3	41.2	36.9	36.5	39.7
Ser	28	27.6	27.6	26.4	26.4	27.9
Glu	79	70.7	80.1	77.7	78.1	78.4
Pro	28	27.6	37.5	37.5	38.3	33.6
Gly	15	16.3	16.8	15.8	16.3	16.3
Ala	46	45.9	47.1	46.0	44.5	45.5
Val	36	32.2	35.0	32.7	32.7	32.0
Met	4	4.4	4.5	4.3	4.6	4.2
Ile	14	11.4	12.2	11.6	11.6	11.6
Leu	61	61.0	61.0	61.0	61.0	61.0
Tyr	19	19.6	20.1	19.5	19.3	19.3
Phe	27	25.6	26.5	25.5	24.7	26.0
His	17	20.0	21.5	22.0	21.5	20.9
Lys	59	44.7	37.2	19.8	14.9	11.5
ΔLys[c]		14	22	39	44	47.5
Gal[d]		12.6	22.1	35.7	41.8	52

[a] Amino acid composition of BSA given by Peters (1975). [b] Amino acid composition calculated by setting the content of leucine as 61.0. [c] Decrease in the lysine content of the Gal-AD-BSA as compared to that of the unmodified BSA. [d] Thiogalactoside content as shown in Table I.

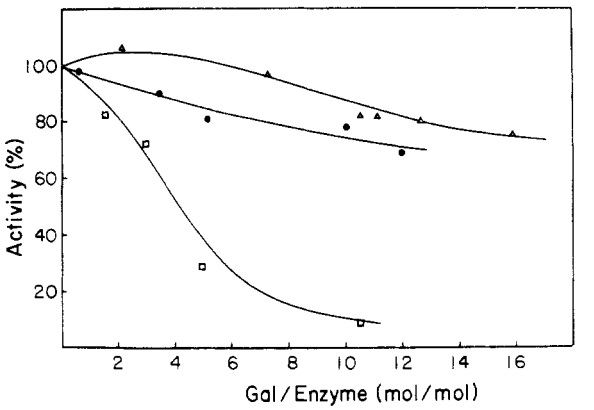


FIGURE 7: Effect of thiogalactoside incorporation by reductive amination on the enzymatic activities. (●) *A. oryzae* α-amylase; (□) porcine pancreatic α-amylase; (△) LDH (expressed as thiogalactoside incorporation per subunit). Activities were determined at the end of the 24-h reductive amination period before dialysis.

in Figure 7. Both LDH and *A. oryzae* α-amylase showed a gradual decrease in activity as the level of thiogalactoside incorporation increased. However, the activities did not decrease below 68% even under conditions where a large excess (>350 molar excess over the proteins) of the thiogalactoside was used in the coupling reaction. On the contrary, porcine pancreatic α-amylase lost about 80% of its activity under high coupling conditions.

Inclusion of maltose (0.1 M) in the reaction mixture during reductive amination did not afford any protection for the *A. oryzae* α-amylase, when assayed with reduced starch. On the other hand, activity of the pancreatic α-amylase was protected considerably by the presence of maltose during reductive amination. As shown in Table III, the higher the concentration of maltose in the reaction mixture, the more activity was preserved. However, there was no difference in the number of thiogalactosides incorporated in the presence and absence of maltose, both samples having 11.4 mol of thiogalactoside per mol of the enzyme.

The remaining α-amylase activity was found to be dependent on the substrates used. When the α-amylases were assayed with a smaller substrate, reduced maltopentaose, *A. oryzae*

Table III: Effect of Maltose on the Activity of Procine Pancreatic α-Amylase

maltose concn (M)	% act. remaining	
	assayed with starch	assayed with maltopentaose
0	12	12
0.009	18	NA[a]
0.047	49	42
0.094	70	83
0.46	82	120

[a] NA, not assayed.

enzyme retained full activity at all levels of sugar incorporation with or without maltose being present. On the other hand, as shown in Table III, the pancreatic enzyme exhibited the same low activity toward reduced starch and maltopentaose, when reacted under the high-coupling condition in the *absence* of maltose. Increasingly higher concentrations of maltose during the reductive amination afforded more protection for the enzyme against the loss of activity. At the highest concentration of maltose (0.46 M), the pancreatic α-amylase appeared to retain full activity toward this small substrate.

As shown in Figure 7, the activity of LDH also decreased slightly at higher levels of thiogalactoside incorporation. This small loss of activity was completely protected by the presence of 5 mM each of NAD^+ and pyruvate in the reaction mixture. When a highly modified LDH (>11 mol/mol of subunit) was dialyzed against water and stored at 4 °C for several days, it lost activity completely and the inactive LDH was eluted as a single peak from a Sephadex G-100 column (1.3 × 40 cm) at an elution volume expected for the dissociated subunits, near the elution volume of ovalbumin.

Gal-AD- and Gal-HD-Neoglycoproteins as Inhibitors of ASOR Binding to Rabbit Liver Plasma Membrane. Results of the liver membrane binding assay are summarized in Figure 8. For comparison of the effectiveness of various thiogalactoside-containing proteins as inhibitors of ASOR binding, the RIP values were plotted (in logarithmic scale) against thiogalactoside content expressed as the moles of thiogalactoside per 10000-dalton peptide on the abscissa. A linear relationship was observed within each group of inhibitors, indicating that the RIP value increased exponentially with an increase in D-galactose density. The longer armed Gal-HD–BSA's were better competitors of the [^{125}I]ASOR binding to the liver membrane than the shorter armed Gal-AD–BSA's in the lower range of sugar density. Interestingly, RIP values of AST_f and Gal-AD–AST_f fell on a line close to that of Gal-HD–BSA. Similar plots for ASOR and Gal-AD–α-amylase showed that both were close to the line generated by Gal-AD–BSA's.

Discussion

Reductive amination is one of the most successful techniques for modifying proteins with little or no alteration of their innate structure (Means & Feeney, 1968). As an alternative to our already established method of using the imidate thioglycosides for preparation of neoglycoproteins (Lee et al., 1976), we have now developed another useful method of preparing neoglycoproteins through reductive amination, using thioglycosides bearing an ω-aldehydoaglycon.

Gray and co-workers (Gray, 1974; Schwartz & Gray, 1977) were the first to use reductive amination in the preparation of neoglycoproteins, utilizing reducing disaccharides as the source of the aldehyde. The slow rate of sugar incorporation in these reactions was suspected to be due to very low concentrations of acyclic sugars (free aldehyde) in aqueous solution. Unmasked aldehydes are expected to react much faster than cyclic sugars. Our synthetic glycosides having unmasked aldehydo groups (e.g., Gal-AD and Gal-HD) indeed reacted very much faster than the disaccharides. Thus, under a comparable molar ratio of the aldehyde to BSA (~300 to 1), 24 mol of Gal-AD was incorporated into BSA within 2 h, whereas only 4.5 mol of maltose was incorporated into BSA during 24 h (Schwartz & Gray, 1977).

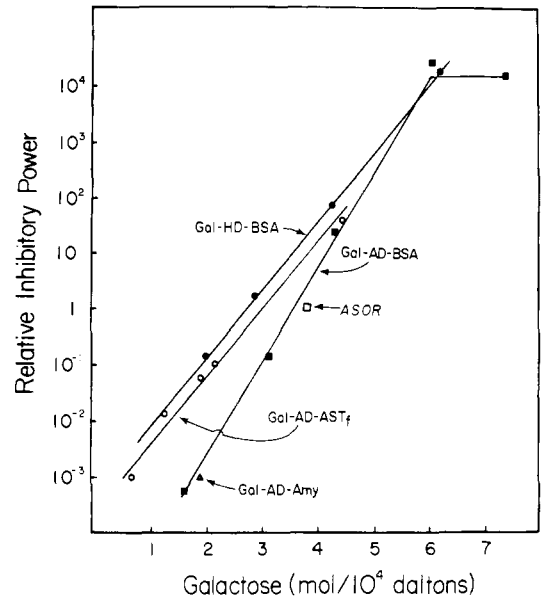


FIGURE 8: Relative inhibitory power of various thiogalactoside-containing neoglycoproteins as a function of galactose density. (■) Gal-AD–BSA; (●) Gal-HD–BSA; (○) Gal-AD–AST_f; (△) Gal-AD–α-amylase (*A. oryzae*); (□) ASOR.

Earlier workers have shown that the reaction of aldehydes with a primary amino group in the presence of excess reagents can lead to both mono- (secondary amine) and di-N-substitution (tertiary amine) (Borch et al., 1971; Schwartz & Gray, 1977). However, with the exception of formaldehyde, di-N^ϵ-substitution has not been observed with lysyl residues of proteins (Means & Feeney, 1968). In our reductive amination, incorporation of Gal-AD into BSA (and other proteins) followed a biphasic curve, as seen in Figure 5. Hydrolysis of the Gal-AD–BSA's shown in Figure 5 (and Table I), followed by electrophoretic separation, showed that the tertiary amine was indeed formed but not until the level of thiogalactoside coupling reached 35–40 mol/mol of BSA, where the second phase of the curve started. The amount of tertiary amine steadily increased beyond this level of thiogalactoside coupling. Modification of BSA with the imidate-containing thioglycosides (Lee et al., 1976) reached a plateau at 30–35 mol of thiogalactoside per mol of BSA, beyond which no further sugar incorporation took place even when a large excess of the reagent was used (Figure 5).

From these observations, the course of reaction of Gal-AD with BSA can be most reasonably interpreted as follows. The readily available lysyl side chains react with Gal-AD to form secondary amines until a saturation point (~35 mol/mol) is reached. In this phase the coupling efficiency is comparable to that of the imidate thioglycoside coupling (Lee et al., 1976). At this point unavailability of the remaining lysyl residues makes the secondary amines quite competitive toward the remaining reagents, so that a significant quantity of tertiary amine begins to form.

The number of lysyl residues readily available to Gal-AD is smaller than that available to smaller aldehydes, such as

formaldehyde, which can easily modify almost all the lysyl residues in most of the proteins studied (Means & Feeney, 1968). It is interesting that the imidate thioglycosides and the ω-aldehydo glycosides described here both can modify only 60–70% of the lysyl residues in BSA, which may be a reflection of the three-dimensional structure of this protein. The number of available lysyl residues in other proteins seems to be even more limited than BSA. Thus, only ~43 and ~33% of the lysyl residues are readily available to Gal-AD in LDH and the α-amylases, respectively. It is known from the X-ray crystallographic studies that ~65% (19 out of 29 residues per subunit) of lysyl residues in dogfish LDH-M_4 lie near the surface of the enzyme (Holbrook et al., 1975). Using [(2,4-dinitrophenyl)amino]hexanimidate as a modifying reagent with a reporter group, Kapmeyer et al. (1977) observed that of the modifiable lysyl residues (~67% of total) of porcine LDH-H_4, 50–80% (33–54% of total), are more readily available to the reagent. The latter values compare fairly well with our value.

Because of the di-N^{ϵ}-substitution in reductive amination, a BSA derivative containing as many as 52 mol of thiogalactoside per mol of BSA has been obtained. As a consequence, the current method can provide an ~50% higher level of sugar attachment than the imidate method, thus increasing the efficiency of the resultant neoglycoproteins as ligands in carbohydrate-binding systems (see below). Our reagents have added advantages in that the newly formed linkage is very stable and the operative pH range is near neutral. Compared to $NaBH_4$, $NaCNBH_3$ is a superior, selective reducing agent for imines, especially at neutral pH values where the rate of imidinium ion formation is maximal (Borch et al., 1971). At pH below 6, the reduction of aldehydes and ketones becomes much faster. For this reason the reductive amination involving Gal-AD and BSA was not investigated below pH 6. In the reductive amination described here, the optimal pH was indeed near neutral, in contrast to that observed by Schwartz & Gray (1977). In their reductive amination using a disaccharide, coupling increased with an increase in pH from 6 to 9. Perhaps the reduction of aldimine was not the rate-limiting step in their case.

The use of high concentrations of $NaCNBH_3$ does cause undesirable side reactions. First, it decreases the amount of thiogalactoside incorporated (Figure 3), probably due to the reduction of the aldehyde to an alcohol by the excess $NaCNBH_3$, and thus lowers the concentration of the aldehyde available for coupling. Schwartz & Gray (1977) reported the formation of lactitol from lactose in their reductive amination mixture. Second, the high concentrations of $NaCNBH_3$ can affect the native protein structure. In one experiment in which 0.36 M $NaCNBH_3$ (~60-fold excess over Gal-AD) was used in the reductive amination of *A. oryzae* α-amylase, 70% of enzymatic activity was lost, though only 3 mol of thiogalactoside was incorporated per mol of protein (see below for the effect of reductive amination on the enzymatic activity of the α-amylase). Moreover, much of the protein precipitated out upon dialysis against water, suggesting an extensive modification in the structure.

The enzymatic activities of the three enzymes used in this study were unaffected by 0.1 M $NaCNBH_3$. Since reductive amination proceeds effectively with 0.1 M $NaCNBH_3$, this concentration was used as a general rule. BSA, having very stable disulfide bonds (Peters, 1975), appears to tolerate $NaCNBH_3$ concentrations of 0.5 M or higher. As shown in Table I, Gal-AD–BSA's containing different levels of thiogalactoside were prepared by using the following general guidelines. For lower levels of modification, the $NaCNBH_3$/aldehyde ratio of (10–20):1 was used, while keeping the concentration of $NaCNBH_3$ below 0.1 M, and for higher levels of modification concentrations of $NaCNBH_3$ as high as 0.6 M were used, while keeping the ratio of $NaCNBH_3$/aldehyde to 5:1.

Although it is known that there is a lysyl residue situated in or near the active site of LDH (Chen & Engel, 1975), the most highly modified LDH was fully active if the reductive amination was carried out in the presence of NAD^+ and pyruvate. Similarly, the pancreatic α-amylase retained 100% activity against a small substrate, maltopentaose, when it was modified in the presence of maltose. On the other hand, *A. oryzae* α-amylase was fully active against maltopentaose even without such protection, suggesting the lack of a critical lysyl residue at its active site. Both α-amylases exhibited a decrease in activity toward starch (~70%) when highly modified with Gal-AD. This portion of lowered activity could not be protected by maltose. The decreased activity toward starch was probably due to interference by the bulky substituents on the lysyl side chains with the binding of starch and the dissociation of the hydrolytic products, thus making the enzymatic process less efficient.

The neoglycoproteins prepared by the present method competed effectively with [^{125}I]ASOR for the binding to the rabbit liver membranes. As found in the case of the neoglycoproteins prepared by the imidate method (Krantz et al., 1976), increasingly higher levels of thiogalactoside on proteins led to increasingly higher RIP values. The exponential increase in the RIP values shown in Figure 8 suggests that the density of thiogalactoside on proteins is the most important factor in determining their effectiveness as competitors. The RIP values of neoglycoproteins prepared from BSA by the imidate method (Gal-AI–BSA) are similar to those of Gal-AD-modified BSA's (Gal-AD–BSA). Spacings between the ε-nitrogen of lysyl residue and the 1-sulfur of the thiogalactose moieties in Gal-AI–BSA, Gal-AD–BSA, and Gal-HD–BSA, are 2, 5, and 9 atoms, respectively. Since the Gal-HD–BSA's are significantly better competitors at lower levels of galactose density (Figure 8), the distance of the thiogalactoside from the protein surface appears to be an important factor when the sugar density on the protein surface is low. If the binding of neoglycoproteins required a certain degree of proximity between sugars, the longer and thus more flexible arms may allow a better chance of such clustering. Of several desialylated serum glycoproteins studied by Ashwell & Morell (1974), ASOR was bound very strongly and AST_f was bound very poorly to liver plasma membranes. It is interesting to note that ASOR and AST_f, representing two extremes in binding affinity, appear similar when compared on the basis of the D-galactose density.

To date the highest reported RIP values for glycoproteins and neoglycoproteins are in the range of 10^2 (Krantz et al., 1976). The highest RIP value obtained in this work reached the range of 10^4. Whether any higher RIP values can be attained by any neoglycoproteins of higher sugar density awaits further investigation.

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Synthesis of Dicytidylyl-(3′–5′)-1,2-di(adenosin-N^6-yl)ethane and Dicytidylyl-(3′–5′)-1,4-di(adenosin-N^6-yl)butane: Covalently Joined Terminals of Two Transfer Ribonucleic Acids and Their Behavior toward Snake Venom Phosphodiesterase†

Jiří Žemlička

ABSTRACT: The chemical synthesis of the title bridged trinucleoside diphosphates **3e** and **3f** along with the corresponding dinucleoside phosphates **3c** and **3d** is described. Bridged nucleosides **3a** and **3b** gave on treatment with triethyl orthoformate in the presence of *p*-toluenesulfonic acid in dimethylformamide the cyclic orthoesters **2a** and **2b**. Condensation of **2a** and **2b** with *N*,2′,5′-*O*-triacetylcytidine 3′-phosphate (**1**) using dicyclohexylcarbodiimide in pyridine afforded after deblocking and chromatographic separation products **3c–f**. The latter were readily degraded with pancreatic RNase, but **3c** and **3e** were completely resistant toward snake venom phosphodiesterase whereas **3d** and **3f** were digested to the extent of 65 and 43%, respectively. The major product of degradation of **3f** with phosphodiesterase was compound **3d** resulting from the combined action of phosphodiesterase and contaminating phosphomonoesterase. The results are explained in terms of stacking of terminal bridged nucleoside units in **3c–f**. The implications of these findings for the function of snake venom phosphodiesterase are discussed.

Previous studies have established that suitably functionalized bridged adenosines are valuable spacer probes for ribosomal peptidyltransferase from *Escherichia coli* (Li et al., 1978). The results of those investigations led us to propose a possible "transition state" for the reaction of peptidyl- and aminoacyl-tRNA[1] catalyzed by peptidyltransferase which includes an intercalation of both 3′ C-A terminals (Li et al., 1978). In order to examine this possibility, it was necessary to prepare the appropriate oligonucleotides derived from the 3′ terminal of tRNA and linked covalently between their purine moieties. Such models are of additional interest as substrates for nucleolytic enzymes involved also in the metabolism of tRNA. The present report describes chemical synthesis of the requisite models which incidentally constitute the first oligonucleotides

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[1] Abbreviations used: TLC, thin-layer chromatography; DEAE, diethylaminoethyl; NMR, nuclear magnetic resonance; RNase, ribonuclease; OD, optical density; UV, ultraviolet; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; *N*-AcPhe-tRNA, *N*-acetyl-L-phenylalanyl-tRNA; CD, circular dichroism; CPK models, Corey–Pauling–Koltun models; TosOH, *p*-toluenesulfonic acid; Et, ethyl; Ac, acetyl; other abbreviations conform with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (1971).

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