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SYNTHESIS OF THE T [β-D-Gal-(1→3)-α-D-GalNAc]-ANTIGENIC DETERMINANT IN A FORM USEFUL FOR THE PREPARATION OF AN EFFECTIVE ARTIFICIAL ANTIGEN AND THE CORRESPONDING IMMUNOADSORBENT

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ABSTRACT

The T antigenic determinant was synthesized in the form 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-α-D-galactopyranoside (**6**) (β-D-Gal-(1→3)-α-D-GalNAcO$(CH_2)_8CO_2Me$). This T-hapten was used to prepare a T-BSA artificial antigen (**7**) and an immunoadsorbent (**8**), which were shown to possess the expected immunological properties. Nuclear Overhauser enhancements of the signals for *anti*-periplanar H-2′ and the *syn*-axial H-3′ of the β-D-galactopyranosyl group were observed on saturation of H-1′. The signal for H-3 of the 2-acetamido-2-deoxy-α-D-galactopyranoside residue was also enhanced.

INTRODUCTION

Over fifty years ago, Thomsen[1] observed that human red cells, *in vitro*, could become transformed such that the cells became agglutinated by normal ABO-compatible sera. After extensive investigation, Friedenreich[2] concluded that Thomsen's agent was a bacterial enzyme that degraded a natural antigenic determinant to liberate the so-called T-receptor—a structure bound by an agglutinin of general occurrence in human sera. It is now established that the enzyme responsible for the transformation is a neuraminidase, which exposes the T-determinant by removing *N*-acetyl-α-neuraminic acid (α-sialoside) residues from certain sialoglycoproteins[3].

In 1966, the structure of the determinant was shown by Kim and Uhlenbruck[4] to be the disaccharide β-D-Gal-(1→3)-α-D-GalNAc which, in the glycoprotein, is glycosidically linked to a threonine or serine residue. The T-determinant is now known to occur in a wide variety of glycoproteins[5,6]. The agglutinins occurring in human sera that bind T-antigens are antibodies which occur probably as the result of stimulation by antigens of the individual's intestinal flora[7]. As the anti-T antibodies

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occur in the sera of normal individuals, the T-determinant structural units found in human glycoproteins must have only a transient existence as intracellular intermediates toward the completed glycoprotein that emerges from the Golgi apparatus. The T-determinant is particularly accessible from the MN specific glycoproteins[8] of the human red-cell antigens, where it is liberated by neuraminidase to produce the so-called T-receptor[9]. Recent findings that this structure occurs in tumor-associated antigens[10-13] has caused a resurgence of interest in its investigation. In this regard, we report here the synthesis of the T-disaccharide in a form suitable for the preparation of an artificial antigen and the corresponding immunoadsorbent[14,15]; that is, the synthesis of 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-α-D-galactopyranoside (**6**).

The preparation of 2-amino-2-deoxy-α-D-galactosides from *O*-protected 2-azido-2-deoxy-β-D-galactopyranosyl chlorides was demonstrated by Paulsen and co-workers[16]. Following this approach, we developed the azidonitration of D-galactal triacetate to provide ready access to 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-β-D-galactopyranosyl chloride[17] (**1**). Condensation **1** with 8-methoxycarbonyloctanol to produce **2**, followed by catalytic reduction of the azido group, subsequent *N*-acetylation and, finally, *O*-deacetylation provided 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**3**). The doublet at 4.95 p.p.m. with a spacing of 3.0 Hz in the ^{1}H-n.m.r. spectrum for **3** required the presence of an α linkage, as also indicated by the high, positive optical rotation.

Benzylidenation of **3** with α,α-dimethoxytoluene afforded a crystalline product (**4**) which, when condensed with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide in the presence of mercuric cyanide, produced the blocked disaccharide **5** as a foam. Hydrolytic removal of the benzylidene group and *O*-deacetylation gave the desired, crystalline hapten (**6**). The doublet signal for H-1′ (J = 7.63 Hz) at 4.77 p.p.m. in the ^{1}H-n.m.r. (200 MHz) spectrum of **6** required the β configuration for the inter-sugar linkage.

The structure of **6** was confirmed by nuclear Overhauser enhancement studies[18,19]. Saturation of the signal (δ = 5.18, J = 3.7 Hz) for H-1 of the α-D-GalNAc residue caused enhancement (20%) of the signal for H-2 at δ 4.61 p.p.m. (spacings 3.8 and 10.9 Hz) and thereby confirmed the α configuration, which places these two hydrogen atoms in *syn*-clinal orientation. A weak enhancement of the signal for one hydrogen atom centered at 3.78 p.p.m. was shown to arise from one of the hydrogen atoms of the aglyconic methylene group by finding that it was coupled to the aliphatic signal centered at 1.90 p.p.m. Saturation of H-1′ caused the expected enhancement (17%) of the *syn*-axial H-3′ at 3.93 p.p.m., with spacings of 3.4 and 10.1 Hz, and thereby confirmed the β configuration. The signal for H-5′ was not clearly evident in the difference spectrum, but may have been present as a weak, broad band near 4.3 p.p.m. However, the signal for H-3 (aglyconic) centered at δ 4.32 p.p.m., with spacings of 3.2 and 10.9 Hz, was strongly enhanced (11%). Moreover, the signal for the *anti*-periplanar H-2′ was enhanced (9%). This result was at first unexpected because of the distance between the proton nuclei of H-1′ and H-2′. However,

nuclear Overhauser enhancements are dependent not only on this distance, but also on the distances between the proton whose signal is enhanced from the other protons in the molecule. In general, the more isolated the proton is from other protons, the greater is the enhancement. As it happens, H-2′ in **6** is well separated from the other hydrogen atoms in the molecule[20].

Recently, Kaifu and Osawa[21] reported the synthesis of 3-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-*N*-tosyl-L-serine by way of tri-*O*-acetyl-2-deoxy-2-(2,4-dinitroanilino)-α-D-galactopyranosyl bromide. Since then, Ferrari and Pavia[22] have reported the synthesis of the 3-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl) derivatives of L-serine and L-threonine using the azido chloride (**1**). Furthermore, Kaifu and Osawa[23] have now reported the synthesis of the T-determinant attached to the 3-*O*-position of *N*-tosyl-L-serine and studied the binding of the compound with a variety of plant lectins. Springer and co-workers[13] have reported the preparation of T-antigen from human blood-group O, MN red blood-cells. We now report briefly the use of the T-hapten (**6**) to raise in rabbits anti-T antibodies that cross-react well with this natural T-antigen, and the preparation from **6** of an immunoadsorbent that proved highly effective both for the isolation of anti-T antibodies from sera and for the isolation of the anti-T lectin present in peanuts[24].

The disaccharide hapten (**6**) was converted into the hydrazide derivative, which was coupled, *via* the acyl azide method[14], to bovine serum albumin (BSA) and to silylaminated[25], calcined diatomaceous earth, to yield an artificial antigen (**7**) and immunoadsorbent (**8**), respectively. The biological activities of these substances were demonstrated as follows.

The agglutination of neuraminidase-treated O erythrocytes[26] by human anti-T (titer 1/32) was totally inhibited by compound **6** at a concentration of 1 mg/mL, whereas methyl β-D-galactopyranoside and 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**3**) showed no inhibition at this concentration.

A single passage of 55 mL of human serum (original titer 1/32) through a 6-g column of the T-adsorbent (**8**) removed all detectable anti-T activity. The bound antibodies eluted from this column by using 1% ammonium hydroxide were found, after neutralization, dialysis, and concentration, to agglutinate neuraminidase-treated cells strongly, but did not agglutinate untreated cells.

High-titer animal anti-T sera were obtained on immunization of rabbits with the artificial T-antigen (**7**), using previously reported procedures[14]. The anti-T antibodies were isolated by affinity chromatography in the usual way and, as expected, agglutinated only neuraminidase-treated human red cells.

It was possible to demonstrate the binding of the rabbit anti-T antibodies with a natural T-antigen* by using a solid-phase radioimmunoassay[27]. Thus, polystyrene tubes coated with the purified rabbit anti-T-BSA antibodies bound[28] the ^{125}I-labelled,

*The natural T-antigen was isolated from human erythrocytes and generously donated by Professor G. F. Springer.

natural T-antigen strongly and the binding was specifically inhibited by the artificial T-BSA antigen.

Natural T-antigens have been located by using the peanut agglutinin (*Arachus hypogea*)[24]. As expected, the T-immunoadsorbent had a strong affinity for this lectin. The isolated lectin strongly agglutinated neuraminidase-treated erythrocytes.

In view of the foregoing results, it was not surprising that the rabbit anti-T antibodies were found by immunofluorescence staining[29] to bind selectively to certain human stomach carcinomas.

EXPERIMENTAL

The general procedures and analytical methods used were the same as previously described[14].

8-Methoxycarbonyloctyl 2-acetamido-2-deoxy-α-D-galactopyranoside (3). — A solution of 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-β-D-galactopyranosyl chloride[17] (14.0 g) in benzene (20 mL) was added to a mixture of 8-methoxycarbonyloctanol (8.46 g), mercuric cyanide (11.77 g), Drierite (42 g), and 1 : 1 (v/v) dry benzene–nitromethane (225 mL). This mixture was stirred for 72 h at 45–50°, at which time the solids were removed by filtration through a Celite pad. The filtrate was evaporated to a syrup that was dissolved in dichloromethane (200 mL). The resulting solution was washed with water (2 × 100 mL), dried, and evaporated to a syrup (17.8 g). Without further purification, this material was dissolved in acetic acid (50 mL) and hydrogenated at 100 lb.in^{-2} and room temperature in the presence of 5% palladium-on-charcoal for 4 h. Acetic anhydride (2 mL) was added and the catalyst was removed by filtration. The filtrate was diluted with toluene and evaporated to a foam (17 g). Removal of the *O*-acetyl groups by transesterification with a catalytic amount of sodium methoxide in methanol (30 mL), followed by removal of the sodium ions with Amberlite IR-120 (H^+) resin and evaporation, gave a foam (10.1 g). Crystallization from hot water provided pure 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-α-D-galactopyranoside (3, 5.1 g) in 32% overall yield, m.p. 138–140°, $[\alpha]_D^{25}$ +130.4° (*c* 1.25, methanol); ^{1}H-n.m.r. (D_2O): δ 4.95 (d, 1 H, $J_{1,2}$ 3.0 Hz, H-1), and 2.12 (s, 3 H, NAc); ^{13}C-n.m.r. (CH_3OD): δ 98.6 (C-1), 62.7 (C-6), and 51.6 (C-2).

Anal. Calc. for $C_{18}H_{33}NO_8 \cdot 0.5\ H_2O$: C, 53.98; H, 8.56; N, 3.50. Found: C, 53.98; H, 8.31; N, 3.46.

8-Methoxycarbonyloctyl 2-acetamido-2-deoxy-β-D-galactopyranoside. — The mother liquor from the aforementioned crystallization appeared to contain additional quantities of 3, together with ~20% overall yield of the β-D anomer. Consequently, the material was acetylated for chromatography on a column of silica gel developed with 6:4:1 hexane–ethyl acetate–ethanol. The fraction that appeared to contain the β anomer of *O*-acetylated 3 was conventionally *O*-deacetylated with sodium methoxide in methanol. The product crystallized readily from methanol–diethyl ether, m.p. 178–179.5°, $[\alpha]_D^{25}$ −3.2° (*c* 0.9, methanol); ^{1}H-n.m.r. (CD_3OD): δ 4.40 (d, 1 H,

$J_{1,2}$ 7.6 Hz, H-1) and 2.01 (s, 3 H, NAc); ^{13}C-n.m.r. (CD_3OD): δ 102.9 (C-1), 62.3 (C-6), and 54.2 (C-2).

Anal. Calc. for $C_{18}H_{33}NO_8$: C, 55.22; H, 8.50; N, 3.58. Found: C, 55.44; H, 8.73; N, 3.62.

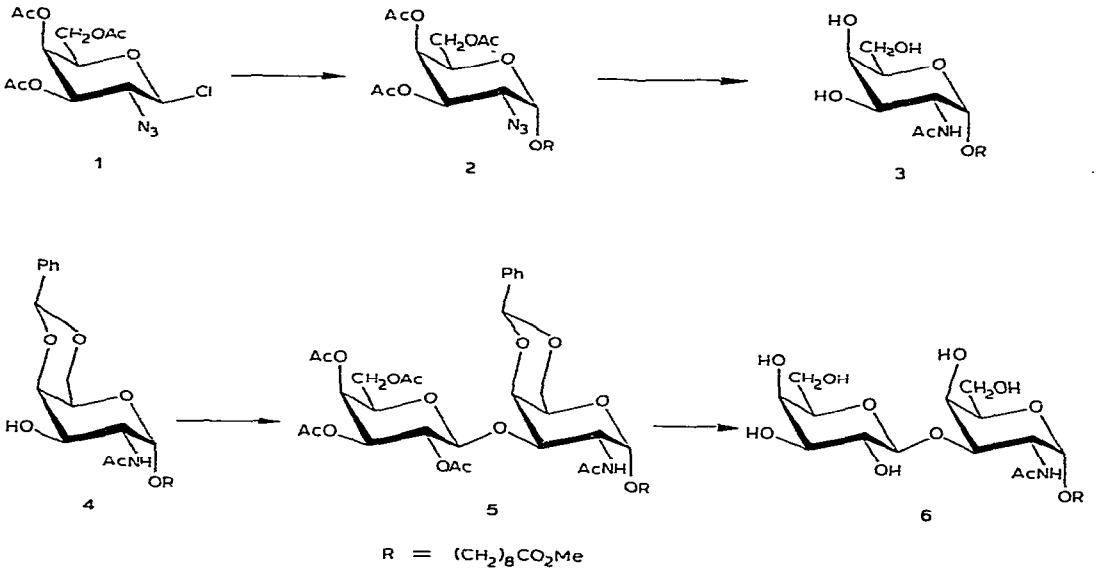
This compound is more readily prepared *via* the use of 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl bromide[17].

8-Methoxycarbonyloctyl 2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (**4**). — Compound **3** (5.0 g) was dissolved in *N*,*N*-dimethylformamide (20 mL) containing α,α-dimethoxytoluene (8 mL) and *p*-toluenesulfonic acid (0.10 g). This mixture was heated for 5 h at 50°, at which time triethylamine (0.5 mL) was added and the solution was taken to dryness under vacuum to give an amorphous glass (5.2 g), which was treated with pentane (2 × 50 mL). Crystallization of this solid from ethyl acetate–pentane gave the title compound (4.5 g), m.p. 145–146°, $[\alpha]_D^{25}$ +101° (*c* 1, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 5.74 (d, 1 H, $J_{NH,2}$ 10.0 Hz, NH), 5.56 (s, 1 H, *CH*Ph), 4.92 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), and 2.02 (s, 3 H, CH_3): ^{13}C-n.m.r. ($CDCl_3$); δ 101.2 (*CH*Ph), 98.6 (C-1), 62.9 (C-6), and 50.5 (C-2).

Anal. Calc. for $C_{25}H_{37}NO_8$: C, 62.61; H, 7.78; N, 2.92. Found: C, 62.65; H, 7.75; N, 2.79.

8-Methoxycarbonyloctyl 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-α-D-galactopyranoside (**6**). — A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (0.315 g) in benzene (2 mL) was added to a mixture of compound **4** (0.30 g), mercuric cyanide (0.18 g), Drierite (0.97 g), and 1:1 (v/v) dry benzene–nitromethane (50 mL). This mixture was stirred for 3 h at 50°, at which time another portion of the bromide (0.05 g) was added and the reaction was continued for an additional hour. The solids were removed and the filtrate was diluted with dichloromethane (100 mL), washed with water (2 × 50 mL), and dried. Solvent removal left a foamy product (**5**, 0.50 g) that resisted crystallization. The material was dissolved in dichloromethane (5 mL) and 90% aqueous trifluoroacetic acid (1 mL) was added. After 2 min at room temperature, toluene (5 mL) was added and then the solvent was removed under vacuum at 30°. The residue was applied to a column (20 × 1.5 cm) of silica gel (40 g), which was eluted with 3:3:1 benzene–ethyl acetate–ethanol. The main fraction provided a syrup (0.30 g) [^{13}C-n.m.r. ($CDCl_3$): 97.7 (C-1) and 101.7 (C-1′)] that was *O*-deacetylated with a catalytic amount of sodium methoxide in methanol, followed by the removal of the sodium ions with Amberlite IR-120 (H^+) resin. The residue (0.175 g, 51% yield) on solvent removal appeared homogeneous (t.l.c.) and crystallized from butanol–ethanol, m.p. 208–210°, $[\alpha]_D^{25}$ +93° (*c* 1, water): ^{1}H-n.m.r. (D_2O, 400 MHz): δ 5.18 (d, 3.7 Hz, H-1), 4.77 (d, 7.6 Hz, H-1′), 4.61 (dd, 3.7, 10.9 Hz, H-2), 4.54 (dd, <1, 2.9 Hz, H-4), 4.32 (dd, 2.9, 10.9 Hz, H-3), 4.29 (m, H-5), 4.21 (dd, <1, 3.2 Hz, H-4′), 3.93 (dd, 3.2, 9.9 Hz, H-3′), 3.81 (dd, 7.6, 9.9 Hz, H-2′), and the signals expected for the 8-methoxycarbonyloctyl and acetyl groups; ^{13}C-n.m.r. (CD_3OD): δ 98.1 (C-1) and 105.7 (C-1′).

Anal. Calc. for $C_{24}H_{43}NO_{13} \cdot H_2O$: C, 50.42; H, 7.93; N, 2.45. Found: C, 50.62; H, 8.00; N, 2.35.



Preparation of the T-artificial antigen. — The procedure used was identical to that previously described[14] *via* the acyl hydrazide obtained from compound **6** through treatment with hydrazine hydrate. The hydrazide was coupled to BSA to give the artificial T-antigen, which had 22 equivalents of hapten per mole of BSA, as determined by the phenol–sulfuric assay[28].

Preparation of the T immunoadsorbent. — Cristobalite, 100–120 mesh (Johns–Manville, Denver, Colorado) was silylaminated with 3-aminopropyltriethoxysilane (Aldrich Chemical Company, Milwaukee, WI) according to a previously described procedure[25]. The acyl hydrazide of compound **6** was coupled to this silylaminated Cristobalite by the azide coupling method[14]. Incorporation of the hapten was 0.52 μmol/g, as determined by the phenol–sulfuric method[25].

Antisera and antibodies. — Human serum was obtained by recalcification of plasma separated from outdated whole-blood supplied by the Canadian Red Cross. Rabbits were immunized as previously described[14]. All affinity columns were operated at 4° and antibodies were eluted from affinity columns with 1 % ammonium hydroxide. Receptor-destroying enzyme (neuraminidase) was obtained from GIBCO, Grand Island, NY. Agglutination titers were determined by the saline quick-spin method[30].

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REFERENCES

1 O. THOMSEN, *Z. Immun. Forsch.*, 52 (1927) 85–107.
2 V. FRIEDENREICH, *The Thomsen Hemagglutination Phenomenon*, Levin & Munksgaard, Copenhagen, 1930.
3 C. M. CHU, *Nature (London)*, 161 (1948) 606–607.
4 Z. KIM AND G. UHLENBRUCK, *Z. Immun. Forsch.*, 130 (1966) 88–99.
5 R. R. RACE AND R. SANGER, *Blood Groups in Men*, 6th edn., Blackwell, Oxford, 1978, pp. 486–487.
6 P. VAITH AND G. UHLENBRUCK, *Z. Immun. Forsch.*, 154 (1978) 1–14.
7 K. B. FRASER, *J. Pathol. Bacteriol.*, 70 (1955) 13–33.
8 G. F. SPRINGER, P. R. DESAI, AND I. BANATWALA, *J. Natl. Cancer Inst.*, 54 (1975) 335–339.
9 G. F. SPRINGER AND P. R. DESAI, *Biochem. Biophys. Res. Commun.*, 61 (1974) 470–475.
10 G. F. SPRINGER AND P. R. DESAI, *Transplant. Proc.*, 9 (1977) 1105–1111.
11 J. H. ANGLIN, JR., M. P. LERNER, AND R. E. NORDQUIST, *Nature (London)*, 269 (1977) 254–255.
12 R. E. NORDQUIST, J. H. ANGLIN AND M. P. LERNER, *Science*, 197 (1977) 366–367.
13 G. F. SPRINGER, P. R. DESAI, M. S. MURTHY, AND E. F. SCANLON, *J. Surg. Oncol.*, 11 (1979) 95–106.
14 R. U. LEMIEUX, D. R. BUNDLE, AND D. A. BAKER, *J. Am. Chem. Soc.*, 97 (1975) 4076–4083.
15 R. U. LEMIEUX, D. A. BAKER, AND D. R. BUNDLE, *Can. J. Biochem.*, 55 (1977) 507–512.
16 H. PAULSEN, C. KOLAR, AND W. STENZEL, *Angew. Chem. Int. Ed.*, 15 (1976) 440–441.
17 R. U. LEMIEUX AND R. M. RATCLIFFE, *Can. J. Chem.*, 57 (1979) 1244–1251.
18 R. RICHARZ AND K. WÜTHRICH, *J. Magn. Resonance*, 30 (1978) 147–150.
19 R. U. LEMIEUX AND K. BOCK, *Jpn. J. Antibiot.*, 32 (1979) s-163.
20 J. H. NOGGLE AND R. E. SCHIRMER, *The Nuclear Overhauser Effect*, Academic Press, New York, 1971, pp. 44–75..
21 R. KAIFU AND T. OSAWA, *Carbohydr. Res.*, 58 (1977) 235–239.
22 B. FERRARI AND A. A. PAVIA, *Carbohydr. Res.*, 79 (1980) c1–c7.
23 R. KAIFU AND T. OSAWA, *Carbohydr. Res.*, 69 (1979) 79–88.
24 S. LEVIN, E. C. RUSSELL, D. BLANCHARD, N. B. MCWILLIAMS, H. M. MAURER, AND T. MOHANAKUMAR, *Blood*, 55 (1980) 37–39.
25 H. H. WEETALL, *Methods Enzymol.*, 44 (1976) 140.
26 H. H. GUNSON, F. STRATTON, AND G. W. MULLARD, *Br. J. Haematol.*, 18 (1970) 309–316.
27 K. COTT AND G. W. TREGEOR, *Science*, 158 (1967) 1570–1577.
28 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
29 W. M. WEINSTEIN AND C. M. SWITZER, personal communication.
30 *Technical Manual of the American Association of Blood Banks*, 7th edn., W. V. MILLER (Ed.-in-chief), J. B. Lippincott, Philadelphia, 1977, p. 167.