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Specific inhibition of an α-galactosyltransferase from *Trypanosoma brucei* by synthetic substrate analogues

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Since the α-D-galactose-(1→3)-D-galactose epitope has been identified to be the major target in the process of hyperacute rejection of xenografts transplanted from nonprimate donors to humans, specific inhibitors of α-galactosyltransferases are of broad interest. Using *Trypanosoma brucei,* a protozoan parasite causing sleeping sickness and Nagana, we have a very useful model system for the investigation of α-galactosyltransferase inhibitors, since the variant surface glycoprotein (VSG) accounts for about 10% of the total cell protein an this parasite expresses many different galactosyltransferases including the one catalysing the formation of the Galα1→3Gal epitope. In order to study inhibition of galactosylation on the VSG from *Trypanosoma brucei,* we designed, synthesized and tested substrate analogues of trypanosomal α-galactosyltransferases. Effective inhibitors were a pair of diastereoisomeric UDP-galactose analogs, in which the galactose residue is linked to UDP via a methylene bridge rather than an ester linkage. Hence, galactose cannot be transferred to the respective acceptor substrate VSG or the synthetic acceptor substrate Manα1→6Manα1S-$(CH_2)_7$-CH_3, which was previously proven to replace VSG effectively [Smith et al. (1996) *J Biol Chem* 271:6476–82]. Inhibitors have been prepared starting from 1-formyl galactal. The final condensation was performed using UMP morpholidate leading to a pair of diastereomeric compounds in 39% or 30% yield, respectively. These compounds were tested using α-galactosyltransferases prepared from *T. brucei* membranes and lactose synthetase from bovine milk. While the K_M-value for UDP-galactose was determined as 59 μM on bovine lactose synthetase, the K_I-values for both inhibitors were 0.3 mM and 1.1 mM respectively, showing that these inhibitors are unable to inhibit enzyme activity significantly. However, using the N-glycan specific α-galactosyltransferase from trypanosomes, the K_M-value was determined as 20 μM, while the K_I-values were 34 μM and 21 μM respectively. Interestingly, other trypanosomal α-galactosyltransferases, which modify the GPI membrane anchor, are 2 orders of magnitude less effected by the inhibitor.

Keywords: **Trypanosoma brucei, α-galactosyltransferases, sugar donor analogs, competitive inhibitors**

Abbreviations: **UDP, uridine-5′-diphosphate; UDP-Gal, uridine-5′-diphosphate galactose; VSG, variant surface glycoprotein; αGalT, α-galactosyltransferase; Galα1,3GalT, UDP-Gal:β-D-Gal(1 → 4)-D-GlcNAc-α(1,3)-galactosyltransferase (EC 2.4.1.51); Galα1,3ManT, UDP-Gal:GPI-anchor-α(1,3)-galactosyltransferase; Man, D-mannose; Gal, D-galactose; Man_2-S-C_8, Manα(1-6)Manα1S-$(CH_2)_7$-CH_3; GlcNAc, D-N-acetylglucosamine; LacNAc, D-N-acetyllactosamine; Galα1,3Gal, α-D-Gal(1→3)-D-Gal epitope; GPI, glycosyl-phosphatidylinositol; DTT, dithiothreitol; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid).**

Introduction

Several approaches have been made to solve the problem of hyperacute rejection of xenografts [1–6]. Here the epitope "Galα1→3Galβ1→4GlcNAc-R", which is present on the surface of many cells except those from old world monkeys and primates including man, has been identified to be mainly responsible for this process [7–9]. Since the gene coding for the respective enzyme, i.e., UDP-Gal:β-D-Gal1→4-D-GlcNAc-α1→3 galactosyltransferase; EC 2.4.1.51 (GalαGalT), appears to be a pseudogene, humans are able to express a specific anti-Gal antibody, which is constitutively present in the serum and accounts for about 1% of the total IgG content [10]. Mutation of this gene, which occurred some 20 million years ago after the spatial separation of new world and old world monkeys, was a major advantage in evolutionary terms, because most parasites expressing this epitope could be recognized and eradicated *a prima vista.* These days, however, the advantage became a major obstacle against xenotransplantation and causes hyperacute reac-

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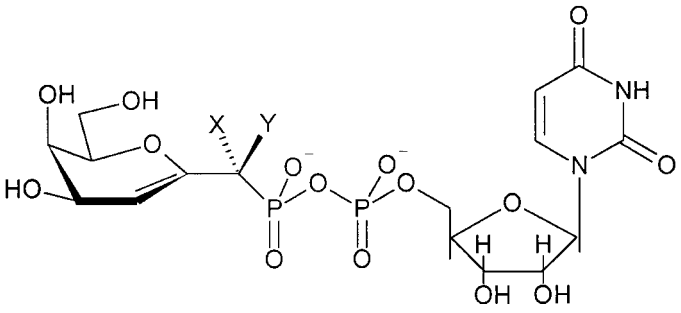
tion, leading to ill functioning transplants and graft rejection within hours [11,12]. Thus there is a need for highly efficient GalαGalT inhibitors, which are not available so far.

African trypanosomes, the causative agents of sleeping sickness and economically important livestock endemics (Nagana), also express α-galactosyltransferases (αGalT) leading to Galα1→3Gal or Galα1→3Man epitopes [13–15]. These parasites do not reside within host cells but move freely within the bloodstream and the lymphatic system of the infected mammal. Protection against the immune system is acquired by a glycoprotein, the so called variant surface glycoprotein (VSG), which forms a protective barrier around the parasite cell. This coat consists of about 10^7 copies of a single protein variant encoded by a distinct gene. After 8 to 10 days, following the formation of a specific antibody, all cells expressing this variant are recognized and eventually destroyed, while only cells survive which express a different VSG gene. Since up to 1000 immunologically different VSG genes exist within the trypanosome genome and can be sequentially expressed, antigenic variation leads to a sustained infection and finally, if untreated, to the death of the host. VSG molecules contain a glycosyl-phosphatidylinositol (GPI) membrane anchor and one or several N-linked glycans [16]. Interestingly, both glycan structures (the GPI anchor and internal N-glycans) may contain Galα1→3Gal epitopes, introduced by different αGalTs. We have recently isolated and characterized an αGalT acting on N-glycans, which recognizes the usual N-acetyllactosamine (LacNAc) residue (Galβ1→4GlcNAc-R) and adds a galactose residue via UDP-Gal [17]. Additionally, an αGalT acting on the anchor by specifically recognizing the mannose containing epitope was characterized [15,18,19] and is currently investigated [Kolb & Duszenko, unpublished results]. Both carbohydrate structures are usually not exposed to antibodies in live trypanosomes but hidden within the surface coat. Although a conclusive physiological function for the glycans was not determined so far, they are thought to be involved in the dense arrangement of VSG molecules and thus in the formation of the protective coat.

Here we report on two diastereoisomeric UDP-Gal analogues, labeled (R)-**1** and (S)-**1** (Scheme 1), which specifically inhibit the isolated N-glycan α-galatosyltransferase, an anchor specific αGalT and the bovine lactose synthetase [EC 2.4.1.22]. Bovine lactose synthetase forms the Galβ1→4Glc epitope and is commercially available. Whereas the UDP-Gal analogues show K_I-values between 21 and 34 μM for the N-glycan specific αGalT, the K_I-values for bovine lactose synthetase are 0.3 mM and 1.1 mM respectively.



Scheme 1

Materials and methods

Chemicals

Gal, Man, LacNAc, Triton X-100, Dowex1X8 100–200 mesh, UDP-hexanolamine-Sepharose, PIPES, ATP, DTT, protease inhibitors, UDP, UDP-Gal and lactose synthetase were purchased from Sigma (Deisenhofen); UDP-[^{14}C]-Gal was from Amersham (Braunschweig); Aluminum backed HPTLC-60 and Kieselgel 60 F_{254} plates were from Merck (Darmstadt); ISOLUTE C18 EC columns were from ICT (Bad Homburg); Manα1→6Manα1S-$(CH_2)_7$-CH_3 (Man_2SC_8) was a generous gift from T. Ziegler, University of Cologne, Germany. Solvents were dried before use.

Synthesis of inhibitors

Dibenzyl-(4,5,7-tri-O-acetyl-2,6-anhydro-3-deoxy-D-galacto-hept-2-enitol-1-yl)phosphonate, (S)-**3** and *Dibenzyl-(4,5,7-tri-O-acetyl-2,6-anhydro-3-deoxy-D-talo-hept-2-enitol-1-yl) phosphonate* (R)-**3**

Aldehyde **2** [20,21] (347 mg, 1.16 mmol) was dissolved in toluene (10 mL). Dibenzyl phosphite (285 μL, 1.27 mmol) and triethylamine (20 μL) were added. Crystals appeared after 10 min. The mixture was concentrated after 2 hours of stirring and recrystallized from ethanol. The mother liquor was concentrated again and taken up in little ethyl acetate. Petrol ether was added until crystals were formed which were removed, recrystallized from ethanol and added to the first batch to give 348 mg (53%) of (S)-**3** (M.P. 166°C). The mother liquors were collected, concentrated and purified over silica gel (toluene:ethyl acetate = 5:2 to 0:1) to give 190 mg (29%) of (R)-**3** as an oil. (S)-**3:** R_f = 0.18 (silica gel, ethyl acetate:petrol ether = 1:3), $[\alpha]_D = -15°$ (c = 1.0 in $CHCl_3$). ^{1}H-NMR (250.13 MHz, $CDCl_3$): δ = 2.00 (s, 6 H), 2.03 (s, 3 H), 3.34 (dd, J = 3.0, 3.0 Hz, 1 H), 4.11-4.33 (m, 3 H), 4.42 (dd, J = 13.2, 3.0 Hz, 1 H), 4.99 (bs, 1 H), 5.08-5.12 (m, 4 H), 5.37 (bd, J = 4.7 Hz, 1 H), 5.52–5.57 (m, 1 H), 7.34–7.35 (m, 10 H). $C_{27}H_{31}O_{11}P$ (562.52): calcd. C 57.65, H 5.55; found C 57.51, H 5.61. (R)-**3:** R_f = 0.18 (silica gel, ethyl acetate:petrol ether = 1:3), $[\alpha]_D = -32°$ (c = 1.0 in $CHCl_3$). ^{1}H-NMR (250.13 MHz, $CDCl_3$): δ = 1.97 (s, 3 H), 1.99 (s, 3 H), 2.00 (s, 3 H), 4.11–4.34 (m, 4 H), 4.44 (dd, J = 11.9, 6.2 Hz, 1 H), 5.03–5.06 (m, 1 H), 5.09–5.13 (m, 4 H), 5.36–5.38 (m, 1 H), 5.51–5.53 (m, 1 H), 7.34–7.36 (m, 10 H), $C_{27}H_{31}O_{11}P$ (562.52): calcd. C 57.65, H 5.55; found C 57.71, H 5.71.

Disodium (2,6-anhydro-3-deoxy-D-galacto-hept-2-enitol-1-yl)phosphonate, (S)-**4.** Compound (S)-**3** (200 mg, 356

μmol) was dissolved in a mixture of ethyl acetate (5 mL) and methanol (20 ml). Palladium on charcoal (10 mg, 10% Pd) was added and the mixture was stirred intensely under a hydrogen atmosphere at normal pressure. The catalyst was filtered off and washed with methanol when the consumption of hydrogen had ceased (about 40 min). Filtrate and washings were combined and 1 M sodium methoxide in methanol (1 mL) was added. The mixture was centrifuged after 10 min, the precipitate washed with acetone and dried under reduced pressure to give 106 mg (quant.) of an amorphous powder, (S)-**4:** R_f = 0.55 (cellulose, acetone:0.05 M ammonium bicarbonate = 1:1), ^{1}H-NMR (250.13 MHz, D_2O): δ = 3.63 (dd, J = 3.3, 11.8 Hz, 1 H), 3.77 (bd, J = 4.5 Hz, 1 H), 3.84 (dd, J = 8.8, 11.8 Hz, 1 H), 3.88 (d, J = 13.7 Hz, 1 H), 3.97 (bdd, J = 8.8, 3.3 Hz, 1 H), 4.36 (bs, 1 H), 4.67 (bs, 1 H), ^{31}P-NMR (161.70 MHz, D_2O): δ = 16.21 (s), MS (FAB, negative mode), matrix: glycerol:acetic acid:DMSO = 1:1:1, m/z: 255 $(M+H^+)^-$.

Disodium (2,6-anhydro-3-deoxy-D-talo-hept-2-enitol-1-yl)phosphonate (R)-**4.** Diastereomer (R)-**3** 33 mg (59 micromol) was treated as described above to yield 16 mg (90%) of an amorphous powder. (R)-**4:** R_f = 0.55 (cellulose, acetone:0.05 M ammonium bicarbonate = 1:1), ^{1}H-NMR (250.13 MHz, D_2O): δ = 3.54 (dd, J = 3.6, 12 Hz, 1 H), 3.73 (ddd, J = 1.5, 1.5, 4.6 Hz, 1 H), 3.79 (dd, J = 5.0 Hz, 1 H), 3.81 (d, J = 12.2 Hz, 1 H), 3.96 (dddd, J = 5.0, 1.5, 3.6 Hz, 1 H), 4.26 (ddd, J = 1.0, 1.0, 4.6 Hz, 1 H), 4.68 (bs, 1 H). ^{31}P-NMR (161.70 MHz, D_2O): δ = 16.75 (s), MS (FAB, negative mode), matrix: glycerol:acetic acid:DMSO:H_2O = 1:1:1:1, m/z: 277 $(M+Na^+)^-$.

*(−)-R-α-Hydroxyphosphono acetic acid (**5**).* Sodium salt (R)-**4** (37 mg, 123 μmol) was dissolved in water (2 mL). Tetrabutylammonium hydroxide solution in methanol (230 μl, 0.8 M) was added and the mixture stirred with IRC-176 (1 g, H^+-form) until the mixture was neutralised. The solvents were removed after filtration. The residue was taken up in methanol (25 mL) and cooled to −60°C. A stream of oxygem containing about 2.5 g/h of ozone was passed through the solution for 2 min. The solution was warmed to room temperature after dimethyl sulfide (150 μL) had been added. Sodium hydroxide solution (1 ml, 1 M) was added. The mixture was centrifuged, the residue washed with methanol, taken up in water, stirred with IR-120 (H^+-form), filtered and lyophilized to give 6 mg of an oil. **5:** $[\alpha]_D$ = −21° (c = 0.5 in H_2O), ^{1}H-NMR (250.13 MHz, D_2O): δ = 4.36 (d, J = 18.0 Hz). MS (FAB negative mode, matrix: glycerol:acetic acid:DMSO:H_2O = 1:1:1:1) m/z = 155 $(M-H^+)^-$.

*Disodium O^P[(2,6-anhydro-3-deoxy-D-galacto-hept-2-enitol-1-yl)phosphonyl]-uridine-5′-phosphate, (S)-**1.*** Compound (S)-**4** (33 mg, 109 μmol) was dissolved in water (0.5 mL) and passed through a small column filled with IR-120 (triethylammonium-form). The eluates were combined and lyophilized. The remainder was dried in vacuo together with N,N′-dicyclohexyl-4-morpholincarboxamidinium UMP-morpholidate (112 mg, 164 μmol, Sigma). Molecular sieves (100 mg) and dry pyridine (2 mL) were added and the mixture stirred for ten days. The mixture was concentrated and purified by HPLC. The fractions containing product were collected, lyophilized, dissolved in water, brought into the Na^+-form by IR-120 and lyophilized again to yield 26 mg (39%) of an amorphous powder.

(S)-**1:** t_R = 8.8 min (HPLC: prep. RP-18 column, flow: 8 ml/min, 0.1 M triethylammonium bicarbonate buffer containing 2% acetonitrile). ^{1}H-NMR (250.13 MHz, D_2O): δ = 3.57 (dd, J = 4.1, 11.9 Hz, 1 H), 3.72–3.75 (m, 1 H, 5″-H), 3.75 (dd, J = 8.2, 11.9 Hz, 1 H), 3.92 (dd, J = 4.1, 8.2 Hz, 1 H), 4.04–4.16 (m, 3 H), 4.13 (d, J = 14.3 Hz, 1 H), 4.16–4.20 (m, 2 H), 4.32 (bs, 1 H), 4.72 (bs, 1 H), 5.77 (d, J = 7.5 Hz, 1 H), 5.79 (d, J = 4.5 Hz, 1 H), 7.77 (d, J = 7.5 Hz, 1 H). ^{31}P-NMR (161.70 MHz, D_2O): d = −9.82 (d, J = 28.1 Hz), 8.01 (d, J = 28.1 Hz). MS (FAB, negative mode, matrix: glycerol:acetic acid:DMSO = 1:1:1): m/z = 583 $(M+Na^+)^-$.

Disodium O^P-[(2,6-anhydro-3-deoxy-D-talo-hept-2-enitol-1-yl)phosphonyl]-uridine-5′-phosphate, (R)-**1**

Compound (R)-**4** (58 mg, 192 μmol) was treated as described for (S)-**4** to yield 29 mg (30%) of an amorphous powder. (R)-**1:** t_R = 8.4 min (HPLC: prep. RP-18 column, flow: 8 ml/min, 0.1 M triethylammonium bicarbonate buffer containing 2% acetonitrile). ^{1}H-NMR (250.13 MHz, D_2O): δ = 3.59 (dd, J = 3.7, 12.1 Hz, 1 H), 3.76–3.79 (m, 1 H), 3.80 (dd, J = 6.8, 12.1 Hz, 1 H), 3.99 (dd, J = 3.7, 6.8 Hz, 1 H), 4.01–4.14 (m, 3 H), 4.17 (d, J = 14.9 Hz, 1 H), 4.14–4.21 (m, 2 H), 4.32 (bs, 1 H), 4.77 (bs, 1 H), 5.80 (d, J = 8.1 Hz, 1 H), 5.82 (d, J = 4.6 Hz, 1 H), 7.79 (d, J = 8.1 Hz, 1 H). ^{31}P-NMR (161.70 MHz, D_2O): δ = −7.98 (d, J = 27.5 Hz), 10.03 (d, J = 27.5 Hz). MS (FAB, negative mode, matrix: glycerol:acetic acid:DMSO = 1:1:1) m/z = 583 $(M+Na^+)^-$.

Stock solutions

1. Acceptor substrate: 200 mM LacNAc and 100 mM of a synthetic substrate analog Man_2SC_8 in distilled water. Aliquots of these stock solutions were frozen and kept at −30°C until use.
2. Donor substrates: a) donor substrates for the N-glycan GalT-assay: UDP-Gal (100 mM in distilled water) was frozen and kept at −30°C until use and UDP-[^{14}C]-Gal (spec. activity 303 mCi $mmol^{-1}$). The enzyme reaction was started by adding a mixture of radiolabelled (final activity: 25 or 50 nCi) and unlabelled UDP-Gal (in a concentration range between 7 to 500 μM) to the assay. b) donor substrate for GPI-anchor GAlT-assay: UDP-[^{14}C]-Gal (spec. activity 303 mCi $mmol^{-1}$; final concentration 3.3 μM, final activity 50 nCi).
3. Inhibitors: a) Inhibitors for N-glycan αGalT: (S)-**1** was tested in the range of 3 to 100 μM, (R)-1 was tested in the range of 10 to 300 μM. Lyophilized inhibitors were dissolved in distilled water as 10fold concentrated stock solutions and used as described. b) Inhibitors for GPI

anchor αGalT: 5 mM stock solutions of (S)-**1** and (R)-**1** in distilled water.

Preparation of trypanosome membrane fractions

Bloodstream forms of *Trypanosoma brucei* strain 427 variant MITat 1.4 were isolated from infected rats, lysed for 5 min in 10 mM PIPES buffer (pH 6.5) containing 1 mM dithiothreitol and protease inhibitors (chymostatin, leupeptin, and pepstatin; 1 μM each). The lysate was centrifuged (14,000 × g; 10 min, 4°C) and the pellet was resuspended in 50 mM PIPES buffer (pH 6.5), containing 15 mM $MnCl_2$, 1 mM dithiothreitol, 1 mM ATP, and protease inhibitors as before.

Enzyme assay for the N-glycan specific αGalT

The αGalT was isolated from *T. brucei* as described [17]. Active fractions, collected after affinity chromatography using a UDP-hexanolamine-Sepharose™column (Pharmacia, Freiburg) were incubated in PIPES buffer (70 mM, pH 6.5) containing $MnCl_2$ (20.5 mM), ATP (1.37 mM), DTT (1 mM), Triton X-100 (0.1% v/v), and the protease inhibitors pepstatin, leupeptin and chymostatin in final concentrations of 1 μM each. We used a mixture of UDP-Gal and UDP-[^{14}C]-Gal as the donor substrate and LacNAc as an acceptor substrate. Inhibitors were added from stock solutions immediately before the assay was started.

For each assay, PIPES buffer (36.5 μL) was mixed on ice with 5 μL acceptor substrate and either 5 μL of distilled water or inhibitor stock solution. The enzymatic assay was started by adding 3.5 μL of the donor substrate and the mixture was incubated at 37°C for 15 min. The reaction was terminated by adding 400 μL ice cold distilled water to the assay immediately followed by a passage through a Bio-Rad-poly-prep chromatography column containing 1 mL Dowex 1-X8 (Cl-form, 100 to 200 mesh). The column was washed three times with 500 μL distilled water; the flow through and the washing water was collected, thoroughly mixed with 3 mL of scintillation cocktail (Packard Ultimagold XR) and counted using a liquid scintillation counter.

Enzyme assay for the the GPI anchor specific αGalTs

αGalT activities, specific for GPI anchor modification, were assayed in a total volume of 50 μL PIPES buffer (50 mM, see above) containing up to 38 μL membrane fraction, 0.025 % Triton X 100, 50 nCi UDP-[^{14}C]-Gal (3.3 μM), 20 mM Man_2SC_8, and 500 μM of either (S)-**1** or (R)-**1.** Controls were made either without inhibitors, or without Man_2SC_8 or without both. Mixtures were incubated for 60 min at 35°C. The reaction was stopped by adding 50 μL of icecold acetone and the denatured protein was removed by centrifugation (5,000 × g, 4°C, 10 min). The supernatant was diluted with 900 μL acetic acid (100 mM) and applied onto an ISOLUTE C-18 EC solid phase extraction column (ICT, Bad Homburg). The column was washed 5 times with 1 mL acetic acid (100 mM) each before the reaction products were eluted 3 times with 250 μL methanol. 100 μL of the combined eluates assayed for radioactivity using 1 mL of Ultimagold liquid scintillation cocktail (Packard, Frankfurt). The remaining eluates (650 μL) were dried in a rotation evaporator and used for glycosidase digestions or directly applied to aluminium backed 20 cm × 20 cm silica gel HPTLC plates (E. Merck, Darmstadt).

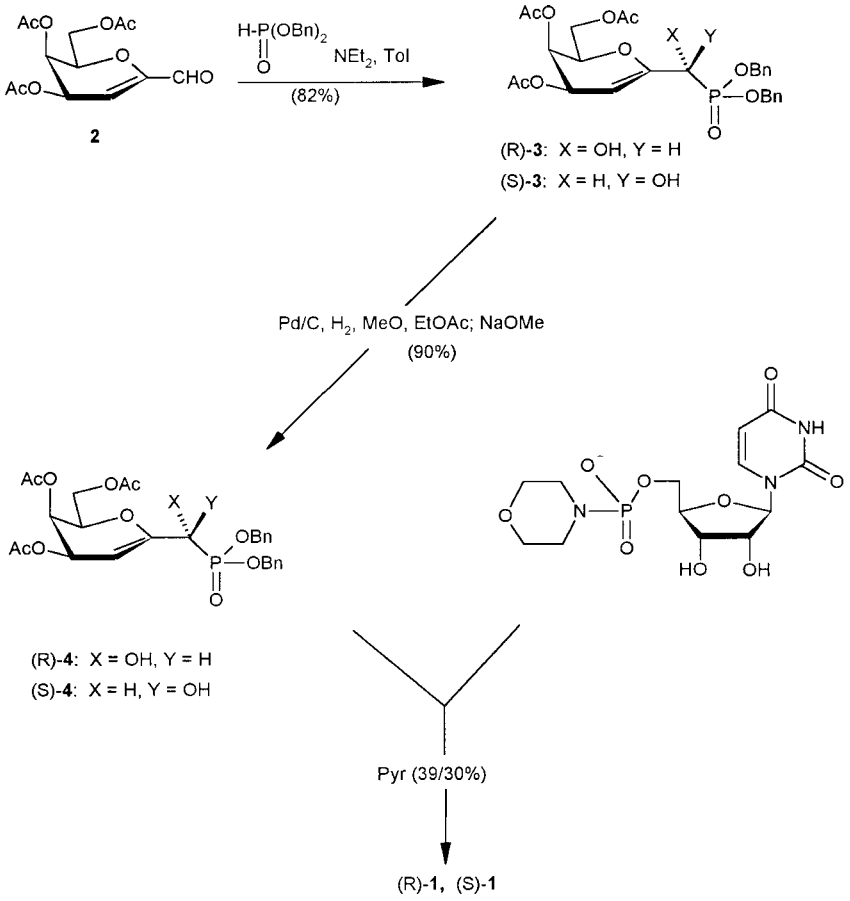
Autoradiography

Autoradiography of radiolabeled samples was performed on thin layer plates using En[^{3}H]ance® spray (NEN Life Science, Boston, USA) and Biomax® film sheets (Kodak, Stuttgart, Germany); exposure time was between 40 and 60 days.

Results and discussion

We used two synthetic substrate analogues of UDP-Gal to inhibit the αGalTs from *Trypanosoma brucei.* These analogues, (R)-**1** and (S)-**1,** which are a pair of diastereoisomers designed to fit into the active center of the enzyme without being converted, i.e., inhibiting transfer of the sugar unit to the disaccharide LacNAc. The inhibitors have been synthesized starting from known aldehyde **2** [20, 21]. Pudovic-reaction [22] with dibenzyl phosphite led to a mixture of the diastereomeric α-hydroxy-phosphonates (R)-**3** and (S)-**3** which could be separated by crystallization. Hydrogenolytic debenzylation, ensuing deacetylation (leading to (R)-**4** and (S)-**4**) and condensation with UMP-morpholidate in the presence of pyridine afforded compounds (R)-**1** and (S)-**1** in considerable yields (Scheme 2). Determination of the configuration of the new stereocenter was based on Mosher's method [23,24]. Additionally, (R)-**4** was transformed into the soluble tetrabutylammonium hydroxide salt which was treated with ozone in methanol; the resulting ester was saponified with sodium hydroxide without isolation to yield (−)-α-hydroxy-phosphonoacetic acid **5** (Scheme 3) after acid treatment [24,25].

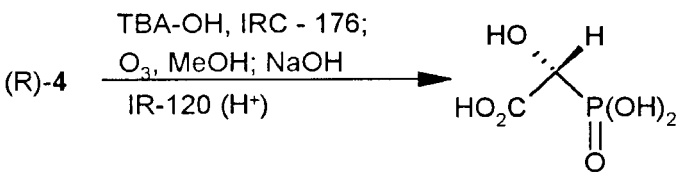
Using trypanosomal membrane preparations containing active αGalTs, Gal is transferred from the sugar donor UDP-Gal to a suitable acceptor (e.g. LacNAc). In the case of hydrophilic sugar acceptors, the enzyme product is easily separated by solid phase anion exchange chromatography. Here the uncharged product (as well as the uncharged acceptor) does not bind the resin, whereas the charged UDP-Gal remains bound to the Dowex material. With amphiphilic acceptors, such as Man_2SC_8, the reaction product is adsorbed on a hydrophobic matrix, whereas the UDP-Gal appears in the flow through. Both methods have been used to investigate the inhibitory effects of the two syn-



Scheme 2

thetic UDP-Gal analogues. The products have been specifically identified and quantified by using UDP-[^{14}C]-Gal and thin layer chromatography.

Using LacNAc and a mixture of radiolabelled and non-labelled UDP-Gal we have been able to show that the synthetic substances (S)-**1** and (R)-**1** have significant inhibitory effects on the purified GalαGalT prepared from *Trypanosoma brucei* MITat 1.4 membranes. Analyses of the corresponding Lineweaver-Burk plots (prepared by using inhibitor concentrations in a range from 3 to 300 μM) revealed K_I-values of 34 μM for (S)-**1** and 21 μM for (R)-**1** for



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Scheme 3

this enzyme, whereas a K_M of 20 μM was observed for UDP-Gal. Additionally, since $1/V_{max}$ remains constant, both compounds work as competitive inhibitors (Fig. 1a and b). Commercially available bovine lactose synthetase showed a similar K_M value for UDP-Gal of 59 μM, while inhibition of the enzyme was at least one order of magnitude less effective (K_I values of 0.3 mM for (R)-**1** and 1.1 mM for (S)-**1** respectively; data not shown). The results show that both inhibitors compete effectively with the sugar donor, but have a significantly higher specificity for GalαGalT as compared with bovine lactose synthetase.

We further investigated the effect of (S)-**1** and (R)-**1** on UDP-Gal:GPI-anchor α(1,3)-galactosyltransferase (GalαManT). For this purpose, trypanosomal membrane fractions were incubated together with UDP-[^{14}C]-Gal and the acceptor Man_2SC_8, which is galactosylated by action of αGalTs but not βGalTs [18]. In control experiments, the incubation of purified GalαGalT, Man_2SC_8 and UDP-[^{14}C]-Gal did not result in the formation of radiolabelled products. Radioactivity was measured after separation of the reaction products from UDP-[^{14}C]-Gal by solid phase extraction on ISOLUTE™ C18 reversed phase columns. Addition of 500 μM of either (S)-**1** or (R)-**1** to the assay reduced the incorpo-

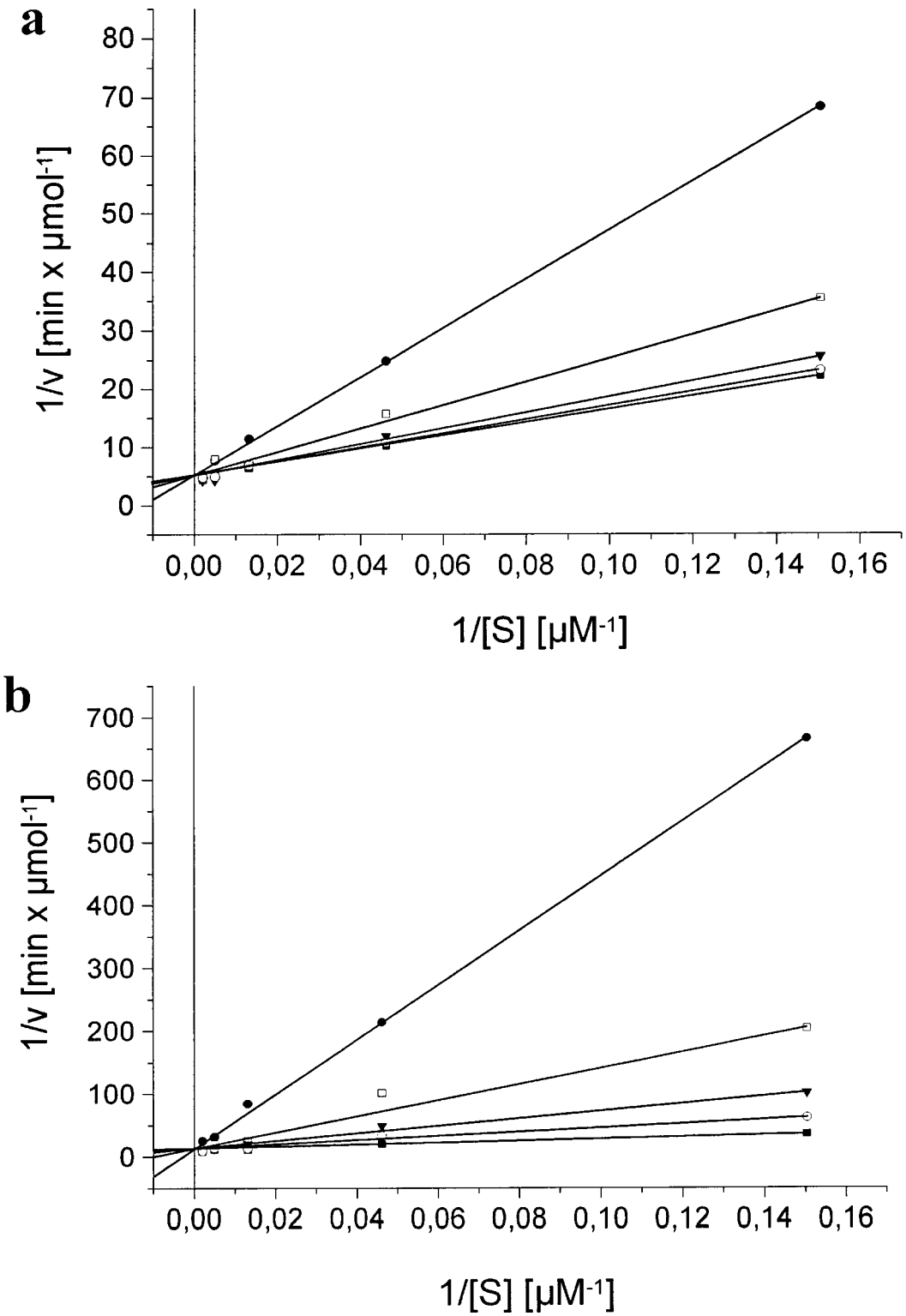

Figure 1. Lineweaver-Burk Plots for (S)-**1** and (R)-**1:** The purified trypanosomal GalαGalT was incubated for 15 min at 37°C in the presence of 20 mM N-LacNAc and 7 to 500 µM UDP-Gal. **a)** with various concentrations of (S)-**1:** 3 µM (○), 10 µM (▼), 30 µM (□), 100 µM (●) or without (S)-**1** (■). **b)** with various concentrations of (R)-**1:** 10 µM (○), 30 µM (▼), 100 µM (□) and 300 µM (●) or without (R)-**1** (■).

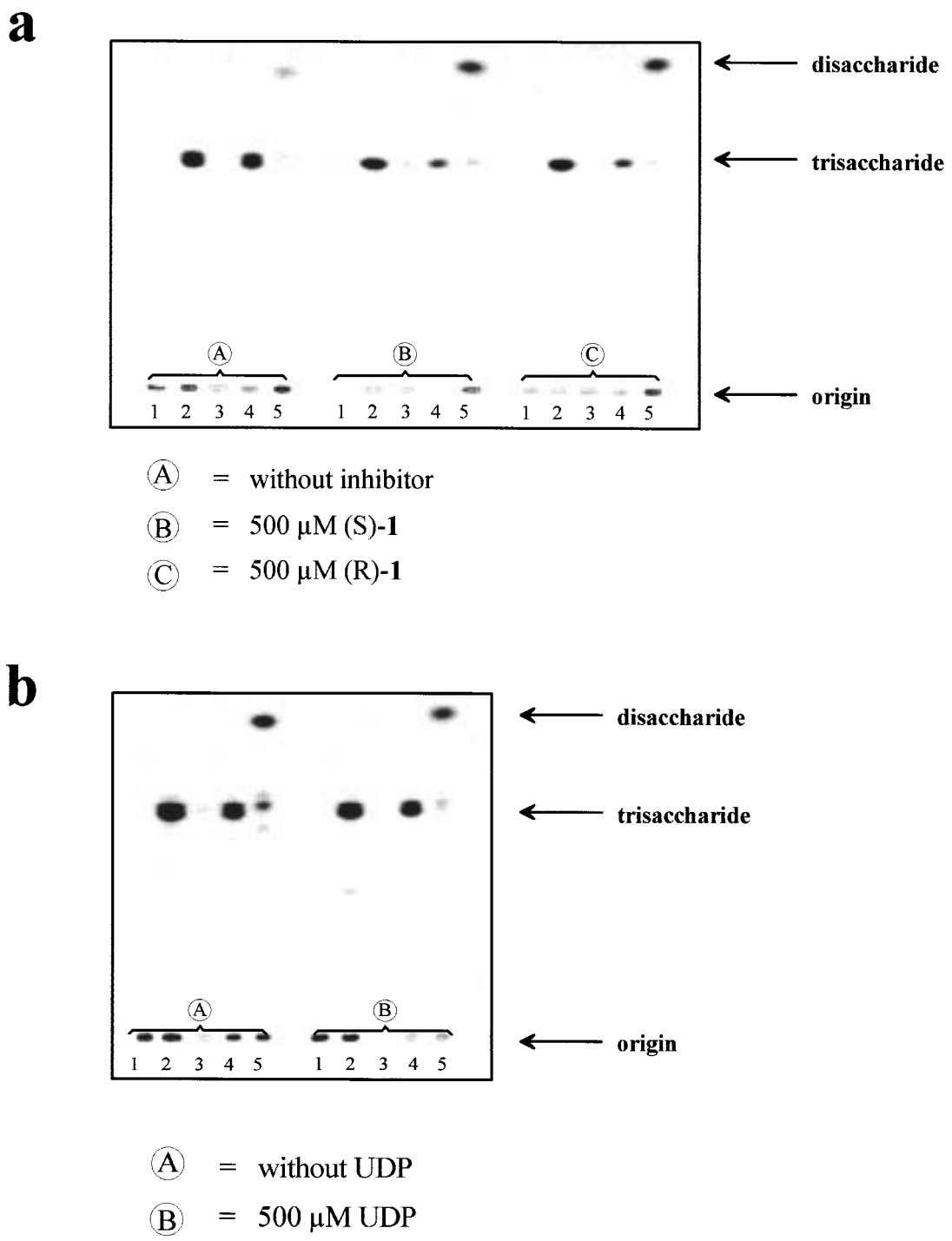

Figure 2. Product analysis of the galactosylation of Man_2SC_8 by trypanosomal membrane fractions **a)** without inhibitor (A), 500 µM (S)-**1** (B), and (R)-**1** (C); **b)** without inhibitor (A), and 500 µM UDP (B). Lane 1: control assays without acceptor (Man_2SC_8); lane 2: control assays with acceptor (Man_2SC_8); lane 3: with acceptor (Man_2SC_8) and α-galactosidase; lane 4: with acceptor (Man_2SC_8) and β-galactosidase; lane 5: with acceptor (Man_2SC_8) and α-mannosidase.

ration of radioactivity into the product fraction by 48% for (S)-**1** and 43% for (R)-**1.** To determine the type of linkage, the reaction products were treated with α- or β-galactosidase or α-mannosidase respectively and analysed by thin layer chromatography as described earlier [19]. Autoradiographs of the corresponding thin layer plates clearly revealed that galactose is indeed added from UDP-gal to Man_2SC_8 in an α-glycosidic linkage and additionally showed

that after incubation with (S)-**1** or (R)-**1** approximately 45% of radioactivity disappeared (Fig. 2a). To test whether this effect is caused solely by the UDP moiety in (S)-**1** and (R)-**1,** we repeated the experiments with 500 μM UDP. However, although a minor reduction of the total incorporation of radioactivity was observed (less than 15%), UDP alone cannot cause the observed inhibition of the enzyme (Fig. 2b). From these results we conclude that 500 μM (S)-**1** or (R)-**1** inhibit at least one other GalT in *Trypanosoma brucei* in a specific way and according to the substrate used (Man_2SC_8) that this enzyme is likely to be the GPI anchor specific Galα-ManT. Since in this assay the enzymatic substrate UDP-Gal is only present at a final concentration of 3.3 μM which is about 150 times lower than the final concentration of (S)-**1** and (R)-**1,** the inhibitory effect of (S)-**1** and (R)-**1** on the trypanosomal UDP-Gal:GPI-Anchor α(1,3)-galactosyltransferase is much less effective than on the N-glycan specific counterpart GalαGalT.

Obviously, compounds (S)-**1** and (R)-**1** exhibit quite selective inhibition effects on the various galactosyltransferases investigated in this study. Therefore, structural modifications of **1** seems to be an attractive aim for further work.

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