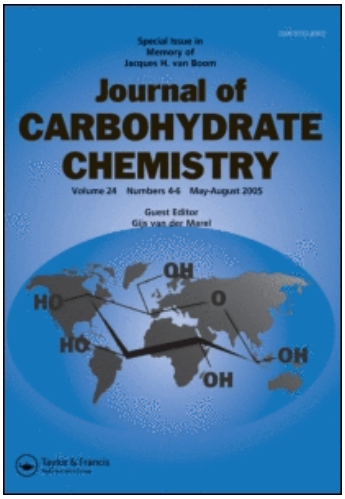

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AN IMPROVED METHOD FOR THE SYNTHESIS OF GDP-HEXANOLAMINE DERIVATIVES, KEY REAGENTS FOR THE PURIFICATION AND CHARACTERISATION OF CARBOHYDRATE PROCESSING ENZYMES

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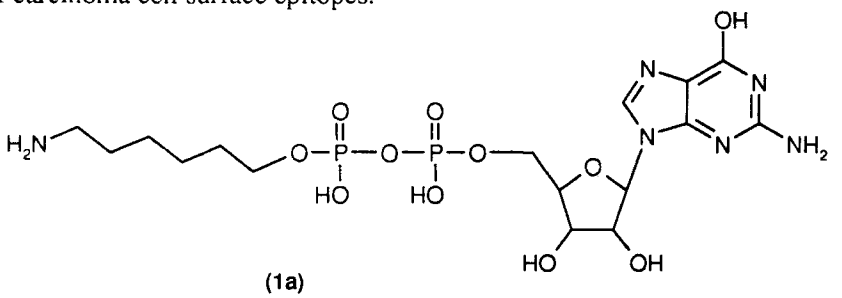
ABSTRACT

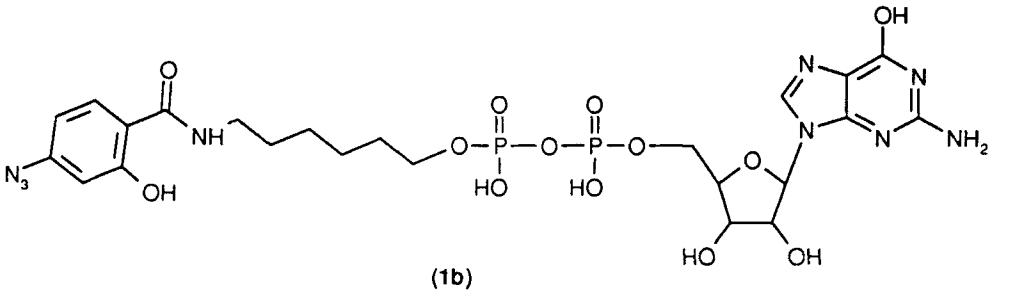
As part of our ongoing studies on the isolation and characterisation of α-(1,3)-fucosyltransferase (FucT) enzymes, an improved method for the synthesis and purification of the key biological tool GDP-hexanolamine (**1a**), together with detailed experimental conditions and product characterisation are reported. We demonstrate the viability of the product obtained, by its competitive inhibition of an α-(1,3)-fucosyltransferase. Subsequently, we are able to use it to derivatise a sepharose gel column which we show is highly efficient in the purification of a recombinant α-(1,3)-fucosyltransferase from a crude preparation. The GDP-hexanolamine is converted to the photoaffinity probe GDP-hexanolaminyl-4-azidosalicylate (**1b**), useful in the characterisation of fucosyltransferase enzymes.

INTRODUCTION

Fucose is observed extensively in mammalian glycoconjugates. It is found at non-reducing terminal positions on the periphery of many oligosaccharide chains, and as such is implicated in a number of recognition events involving carbohydrates.[1] Of particular interest to us is the presence of the fucose-containing tetrasaccharide unit sialyl Lewis X (sLex) at the terminus of N-linked glycoproteins on the surface of neutrophils.[2] This moiety is implicated in the initial stage of recruitment of these and other immune cells to sites of inflammation and therefore has profound clinical implications.[3] The final stage in the biosynthesis of the sLex epitope is believed to be the α-fucosylation of the 3-OH of the distal *N*-acetylglucosamine unit of the 3'-sialyl-lactosamine (SLN) oligosaccharide. This reaction is mediated by an α-(1,3)-fucosyl-transferase (FucT).[4] Such enzymes utilise the fucose donor GDP-fucose and have a high affinity for the GDP nucleotide (Ki = 23 μM).[5] There are five known human α-(1,3)-FucT isotypes, each capable of forming different fucosylated carbohydrate products. In order to help isolate and characterise the α-(1,3)-FucT responsible for the synthesis of the sLex present on the surface of neutrophils, we sought to synthesise the GDP-fucose-mimicking affinity ligand GDP-hexanolamine[5] (**1a**). This was required for use in affinity chromatographic purification of enzyme and subsequent derivatisation to the photoprobe GDP-hexanolaminyl-4-azidosalicylate (**1b**) (GDP-hexanolaminyl-ASA), as used by Holmes *et al.*[5] for the α-(1,3)-FucT involved in the biosynthesis of carcinoma cell surface epitopes.



(1a)



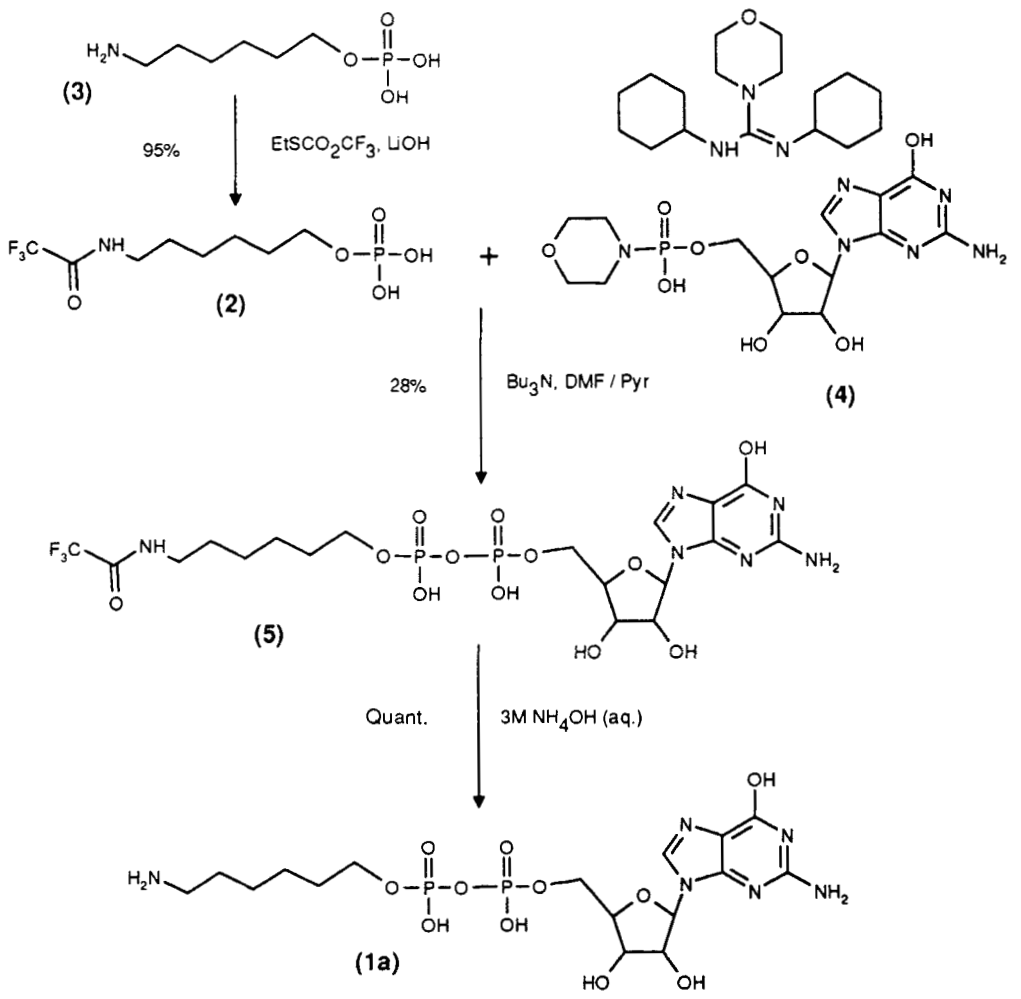
(1b)

Our initial investigations into the synthesis of (**1a**) demonstrated to us several features; 1) the relatively few reports on the synthesis of such compounds; 2) the lack of detailed experimental conditions[6,7] and characterisation of products; 3) the difficulty associated with the formation of the key diphosphate bond, and; 4) the complex nature of the purification methods for the products. Here we report an improved method for the synthesis and purification of the key biological tool GDP-hexanolamine (**1a**), together with detailed experimental conditions and product characterisation. We demonstrate the viability of the product obtained, by its inhibition of an α-(1,3)-FucT. Subsequently, we are able to use it to derivatise a sepharose gel column which we show is highly efficient in the purification of a recombinant α-(1,3)-FucT. The GDP-hexanolamine produced by the improved methodology is converted into the photoaffinity probe (**1b**), a compound useful in the identification of fucosyltransferases.

RESULTS AND DISCUSSION

The synthesis of GDP-hexanolamine has been described by Beyer *et al.*[8] using a method similar to that employed by Barker *et al.*[9] Neither authors quote analytical details of the product or intermediates, and utilise lengthy purification techniques. Our initial attempts to reproduce the work of Barker met with very low yields at the stage of the diphosphate bond formation, so wasting valuable material and making the purification of product difficult. This procedure is dependent on the activation of the phosphate group of *N*-trifluoroacetyl-*O*-phosphoryl-6-amino-1-hexanol (**2**) (Scheme) as its imidazolide prior to reaction with GMP under strictly anhydrous conditions. An alternative method is to activate the phosphate of GMP prior to reaction with the phosphate group of the protected hexanolamine; this approach has been applied with varying degrees of success in related systems including by activation of the phosphate as its phosphinothioic anhydride, 2-hydroxypyridyl ester, mixed phosphate anhydride and its morpholidate.[6,7] GMP-morpholidate is a stable compound which is commercially available[10] so greatly simplifying the procedure for diphosphate bond formation. Hindsgaul *et al.*[6] give a detailed preparative procedure for its use in the synthesis of GDP-sugars, but for these compounds purification procedures are extensive.

Our synthesis (Scheme) of GDP-hexanolamine (**1a**) utilises a modification of the approach of Barker *et al.* to incorporate the use of GMP-morpholidate in a highly efficient overall route. Compound (**2**) was synthesised from the commercially



Scheme

available[10] 6-amino-1-hexanol-phosphate (**3**) according to the method of Barker, by reaction with *S*-ethyl trifluorothioacetate (1.5 eq.) at pH 9.5. Following acidification with TFA, excess lithium trifluoroacetate was removed by stirring with Dowex (H^+) resin to give, after filtration and concentration, the desired product in 95% yield. This was reacted as its tributylammonium salt, with GMP-morpholidate 4-morpholine-*N,N'*-dicyclohexylcarboxamidine salt (**4**). Initial attempts using anhydrous pyridine as solvent gave low yields, possibly due to poor solubility of substrate. Solubility was achieved using a mixed pyridine-DMF solvent, and resulted in much improved yields of

Table 1. FucT VI inhibitory activities of GDP-hexanolamine **1a** and its derivative **5.**

Compound	Concentration (μM)	Inhibition (%)
5	0	0
	250	20
	500	30
1a	0	0
	200	60
	400	75

(**5**). Improvements in such reactions when using DMF as solvent have previously been observed by Sadler *et al.*[11] It was possible to monitor the reaction by TLC on silica using an isopropyl alcohol / ammonium hydroxide / water solvent system, observing product by UV. Of equal importance, the product (**5**) was conveniently purified by preparative HPLC using TFA as solvent modifier, obviating the need for extensive ion exchange chromatography and the associated desalting procedures. A moderate isolated yield of 28% was consistently achieved using this convenient method.

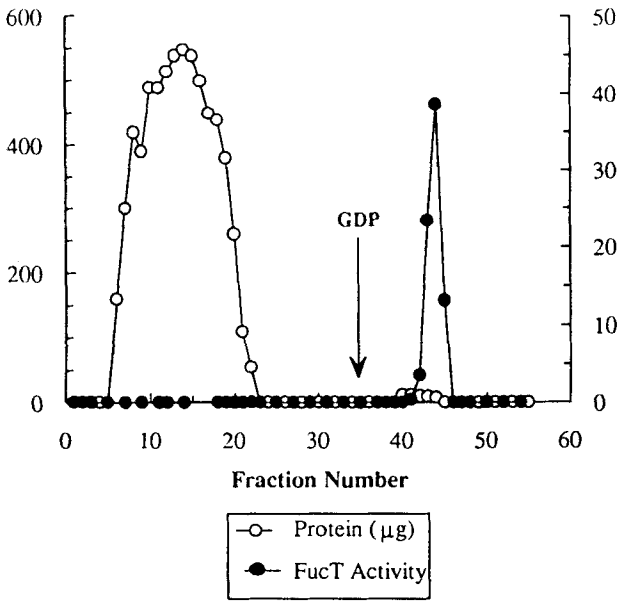
The latent primary amino function was liberated to give the desired GDP-hexanolamine (**1a**) under basic conditions. The use of sodium hydroxide according to the method of Barker *et al.* resulted in the desired product, but heavily contaminated with an unknown species. We found that the use of 3M aqueous ammonia[12] gave only the desired compound in quantitative yield. In addition, the reagent was readily removed by repeated co-evaporation with water; due to the high level of purity of the starting material (**5**), no further purification of product was required prior to subsequent use.

The viability of our GDP-hexanolamine (**1a**) as a reagent for affinity column formation for purification of α-(1,3)-FucT, and as an intermediate for conversion to an affinity probe for this enzyme, was investigated by looking at its competitive inhibition of FucT VI (Table 1). The competitive inhibitory activity of the trifluoroacetamide derivative (**5**) was also investigated.

Both compounds, and in particular compound **1a**, are inhibitory to and therefore presumably show affinity for FucT VI. This gave us additional confidence that our material would be effective in affinity column (Figure) and photoaffinity probe applications. Thus, GDP-hexanolamine (**1a**) was reacted with cyanogen bromide-

activated sepharose; unreacted sites were capped by subsequent reaction with ethanolamine. The GDP-hexanolamine-sepharose was packed into a column, washed and equilibrated with buffer. This was used in the purification of a crude preparation of recombinant FucT VI extracted from baculovirus-infected Sf9 cells; all low affinity protein was washed off using buffer, prior to elution with a GDP-containing buffer system. Eluted protein was measured using the Biorad DC method, with concomitant evaluation of α-(1,3)-FucT activity. Results are shown in the figure below. Eluted α-(1,3)-FucT activity corresponds excellently with a small protein band retained by the column and displaced by GDP. The purification (74-fold) is depicted in Table 2.

Our GDP-hexanolamine (**1a**) was also used in the preparation of the photoaffinity probe GDP-hexanolaminyl-ASA (**1b**). Compound (**1a**) was reacted[13] with *N*-hydroxysuccinimido-ASA in aqueous DMF-triethylammonium bicarbonate buffer in the dark to give the desired product (**1b**)[14] in 77 % yield following sequential silica and Biogel-P2 column chromatographic purification. The use of this material in the characterisation of the active site of α-(1,3)-FucT will be the subject of a future publication.



Scale: 0 - 600 μg protein / fraction; 0 - 50 nmol/min.mg FucT activity.

Figure: Purification of recombinant FucT VI on GDP-hexanolamine sepharose.

Table 2. Purification of recombinant FucT VI using a GDP-hexanolamine-sepharose affinity column.

	Protein (mg)	Total Activity (nmol/min)	Specific Activity (nmol/min.mg)	Fold Purification	Recovery (%)
Cell lysate	8.2	4.31	0.526	1	100
Peak fraction	0.009		38.68	73.5	
Pooled fractions	0.049	0.73			16.9

CONCLUSION

This report constitutes an improved and convenient synthetic and purification procedure for obtaining GDP-hexanolamine and a photo-labelled derivative. It includes extensive physico-chemical and biological characterisation. In particular we have demonstrated the viability of the GDP-hexanolamine so produced in the formation of an affinity column and its highly efficient use in the purification of a fucosyltransferase isotype. The ability to readily obtain quantities of the photo-affinity probe will allow more extensive exploration of the enzyme active site.

EXPERIMENTAL

General Methods. Analytical TLC was performed on Silica Gel 60-F_{254} glass-backed plates (E. Merck, Darmstadt) with detection by UV, or ninhydrin; eluent 7 : 1 : 2 isopropyl alcohol : 0.880 NH_4OH : water. Silica column chromatography was performed using Silica Gel (9384, Merck Kieselgel) with flash elution. All reagents were used as supplied. ^1H NMR spectra were recorded at 250 MHz (Bruker AM250) or 400 MHz (Bruker AM400) in D_2O solvent with DOH (δ4.80) internal standard. Mass spectra were recorded using VG Autospec (FAB +ve), Hewlett Packard HP Engine (TSP), VG Platform (ES) or VG Autospec QM (HRMS) spectrometers. IR spectra were obtained using a Nicolet 5SXC FTIR spectrometer, and a Hewlett Packard 8452A diode array spectrophotometer was used to obtain UV spectra.

Analytical HPLC was performed on an Inertsil ODS2 column with mobile phase consisting of 'A' = 0.1 % H_3PO_4 (aq.), 'B' = 95 : 5 acetonitrile : 0.1 % H_2PO_4 (aq.); Gradient 0 % 'B' for 5 min to 100 % 'B' in 40 min and then 100 % 'B' for 10 min. Preparative HPLC was performed on a Kromasil C8 column (1" diameter x 25 cm) with a mobile phase consisting of 'A' = 0.1 % TFA in CH_3CN and 'B' = 0.1 % TFA (aq.). Gradient 15 % 'B' for 40 min. A flow rate of 5mL/min was used with detection at 253 nm.

***N*-Trifluoroacetyl-6-amino-1-hexanol phosphate (2).** A solution of 6-amino-1-hexanol phosphate (1.00 g, 5.07 mmol) in water (32 mL) was cooled to 9 °C. *S*-Ethyl trifluorothioacetate (1.0 mL, 1.2 g, 7.8 mmol) in acetone (5 mL) was added and the reaction was cooled to 5 °C. The solution was kept at pH 9.5 by addition of sat. aq. LiOH and stirred at the reduced temperature under nitrogen until testing with ninhydrin proved negative (2.5 h). The solution was adjusted to pH 4 by addition of trifluoroacetic acid and the excess *S*-ethyl trifluorothioacetate was removed at reduced pressure. The water was removed by freeze drying to give a cream solid (>100%). The residue was dissolved in water (150 mL) and stirred with Dowex 50W-X8 (H^+) resin (*ca.* 10 g) at 21 °C for 30 min. The resin was removed by filtration and the filtrate freeze dried to give (**2**) (1.42 g, 95 %) as a white solid: ^{1}H NMR (250 MHz) δ 1.40 (m, 4H, $O(CH_2)_2C\underline{H}_2C\underline{H}_2(CH_2)_2N$), 1.60 (m, 4 H, $OCH_2C\underline{H}_2$ and $C\underline{H}_2CH_2N$), 3.32 (t, 2 H, J = 7 Hz, $C\underline{H}_2N$), 3.85 (q, 2 H, J = 6 Hz x 3, $POC\underline{H}_2$). TSP +ve MS: 311 $(M + NH_4)^+$; HRMS: $(M + H)^+$ obs. = 294.0718 (calcd $C_8H_{16}NO_5F_3P$ = 294.0718); IR (Nujol) 1699 cm^{-1}; HPLC (RT = 15.2 min).

***N*-Trifluoroacetyl-GDP-hexanolamine (5).** Tributylamine (162 μL, 0.68 mmol) was added to a solution of (**2**) (200 mg, 0.68 mmol) in anhydrous pyridine (6 mL). The solution was stirred under nitrogen at 21 °C for 30 min. The mixture was treated with guanosine 5'-monophosphomorpholidate 4-morpholine-*N,N'*-dicyclohexylcarboxamidine salt (**4**) (594 mg, 0.81 mmol) in anhydrous pyridine (14 mL) and anhydrous DMF (14 mL) and the reaction stirred under nitrogen at 21 °C for 5 d. The solvent was removed *in vacuo* by co-evaporation with toluene, and the residue purified by preparative HPLC to give (**5**) (120 mg, 28%) as a white solid: ^{1}H NMR (250 MHz) δ 1.3 (m, 4H, $O(CH_2)_2C\underline{H}_2C\underline{H}_2(CH_2)_2N$), 1.6 (m, 4 H, $OCH_2C\underline{H}_2$ and $C\underline{H}_2CH_2N$), 3.3 (t , 2 H , J = 5 Hz, $C\underline{H}_2N$), 3.95 (q, 2 H, J = 6 Hz x 3, $POC\underline{H}_2$), 4.2-4.45 (m, 3 H, H-5' and H-4'), 4.50 (dd, 1H, J = 4 Hz, J = 4 Hz, H-3') 6.1 (d, 1 H, J = 6 Hz, H-1'), 9.15 (br s, 1 H, H-8 guanosine). FAB -ve MS: 637 $(M - H)^-$; ES -ve MS: 637 $(M-H)^-$; ES +ve MS: 639 $(M+H)^+$; UV $\lambda_{max.}$ = 253nm (water); HPLC (RT = 13.8 min).

GDP-Hexanolamine (1a). Compound (**5**) (15.0 mg, 0.02 mmol) was dissolved in 3M NH_4OH (15 mL) and stirred under nitrogen at 21 °C for 3 h. The solvent was removed *in vacuo* and the residue repeatedly co-evaporated with water and then freeze dried to give (**1a**) (11.0 mg, quant) as a white solid: ^1H NMR (250 MHz) δ 1.3 (m, 4H, $O(CH_2)_2C\underline{H}_2C\underline{H}_2(CH_2)_2N$), 1.6 (m, 4 H, $OCH_2C\underline{H}_2$ and $C\underline{H}_2CH_2N$), 2.95 (t, 2 H, J = 7 Hz, $C\underline{H}_2N$), 3.9 (q, 2 H, J = 6 Hz x 3, $POC\underline{H}_2$), 4.2 (dd, 2 H, J = 3 Hz, J= 4 Hz, H-5'), 4.35 (m, 1 H, H-4'), 4.55 (dd, 1 H, J = 3 Hz, J = 5 Hz, H-3'), 5.95 (d, 1 H, J = 6 Hz, H-1'), 8.10 (s , 1 H, H-8 guanosine); FAB +ve MS: 543 $(M + H)^+$; HRMS: $(M + H)^+$ obs.= 543.1363 (calcd $C_{16}H_{29}N_6O_{11}P_2 + H^+$ = 543.1370; HPLC (RT = 5.92 min).

GDP-Hexanolaminyl-4-azidosalicylic acid (1b). To a solution of (**1a**) (5 mg, 9.2 μmol) in triethylammonium bicarbonate buffer (0.5 mL, 1.0 M, Sigma) was added a solution of *N*-hydroxysuccinimido-4-azidosalicylate (6.0 mg, 21.73 μmol) in DMF (0.25 mL). The mixture was stirred in the dark at 21 °C for 24 h. The solvent was removed *in vacuo* to leave a yellow solid. Flash column chromatography (7:2:1 2-propanol-conc. ammonia-water) gave a white solid (6.0 mg). The product was further purified by using a Biogel-P2 column and the appropriate fractions pooled, and freeze dried to give (**1b**) (5.0 mg, 77%): ^1H NMR (400 MHz) δ 1.3 (m, 4 H, $O(CH_2)_2C\underline{H}_2$ and $C\underline{H}_2(CH_2)_2N$), 1.6 (m, 4 H, $OCH_2C\underline{H}_2$ and $C\underline{H}_2CH_2N$), 3.28 (t, 2 H, J = 6 Hz, $C\underline{H}_2N$), 3.90 (q, 2 H, 6 Hz x 3, $POC\underline{H}_2$), 4.20 (dd, 2 H, J = 3 Hz, J= 4 Hz, H-5'), 4.35 (m, 1 H, H-4'), 4.48 (dd, 1 H, J = 4 Hz, J = 4 Hz, H-3'), 4.68 (dd, 1 H, J = 6 Hz , J = 4 Hz, H-2'), 5.85 (d, 1 H, J = 6 Hz, H-1'), 6.45 (d, 1 H, J < 2 Hz, ASA), 6.50 (d, 1 H, J = 8 Hz, *ortho* H of ASA), 7.66 (dd, 1 H, J = 8 Hz, J < 2 Hz), 8.02 (s, 1 H, H-8 guanosine); ES -ve MS: 702 $(M + H)^-$; UV $\lambda_{max.}$ = 263 nm (water); HPLC (RT = 21.7 min).

Preparation of GDP-hexanolamine-sepharose. CNBr-activated sepharose (1.0 g) was resuspended in HCl (200 mL of 1 mM). GDP-hexanolamine (**1a**) (25 mg) was dissolved in buffer (0.5 M NaCl in 100 mM $NaHCO_3$ pH 8.3) and added to the washed sepharose gel. The reactants were mixed end-over-end for 2 h at 23 °C before treatment with ethanolamine (1 M, aq.) for 2 h at 23 °C. GDP-hexanolamine-sepharose was packed into a glass column (3 x 1 cm) and washed sequentially with 100 mM sodium acetate pH 4 and 50 mM Tris-HCl, pH 8.0 containing 0.3 M NaCl prior to equilibration in column buffer.

Source of α–1,3-fucosyltransferase. Recombinant α–(1,3)-FucT-VI was extracted from baculovirus-infected Sf9 cells by solubilisation in detergent-containing

medium (2% (v/v) Triton X-100, 25% (v/v) glycerol, 150 mM NaCl, 5 mM $MnCl_2$ buffered to pH 7.0 with 50 mM Tris-HCl) coupled with probe sonication (4 x 15 sec).

Purification of recombinant FucT VI. GDP-hexanolamine-sepharose 4B (3 x 1 cm) was washed in 0.3 M NaCl in 50 mM Tris-HCl pH 7.8 and equilibrated in column buffer (0.1% (v/v) Triton X-100, 10% (v/v) glycerol, 100 mM NaCl, 2 mM DTT buffered to pH 7.4 with 20 mM Tris-HCl). Solubilised FucT VI was diluted 1:1 (v/v) with column buffer and applied to the column at a flow rate of 0.3 $mL.min^{-1}$. Once loaded, the column was washed extensively in column buffer until no further protein eluted. FucT VI was eluted by washing the column in elution buffer (0.2 mM GDP, 1 mM $MgCl_2$ buffered to pH 7.4 with 20 mM Tris-HCl).

Measurement of α–(1,3)-fucosyltransferase activity. α–(1,3)-Fucosyltransferase activity was measured in medium containing GDP-β-fucose (either 20 μM for purified protein activity determination or 100 μM for determination of inhibitory activities of compounds **1a** and **5**), 0.1 μCi GDP-[^{3}H]-fucose, 1 mM ATP, 5 mM $MnCl_2$, buffered to pH 7.2 with 50 mM Hepes-NaOH. The acceptor saccharide (Galβ(1-4)GlcNAc) was added to a final concentration of 5 mM. The reaction was started by the addition of enzyme and transfer to a 37 °C water bath. After 1 hour the reaction was terminated by the addition of 1 mL of a Dowex slurry (Dowex 1-X8 (Cl^- form) 1:4 distilled water (w/v)), vortexed and centrifuged, and the radioactivity in the supernatant (600 μL) measured by liquid scintillation counting. Non-specific breakdown of GDP-fucose was assessed by duplicating the enzyme assay in the absence of acceptor saccharide.

Protein Measurement. Protein was measured using the BioRad DC method with human IgG as a standard.

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