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Chemoenzymatic Synthesis of GDP-L-Fucose Derivatives as Potent and Selective α-1,3-Fucosyltransferase Inhibitors

Yu-Nong Lin,[a,b] Daniel Stein,[a] Sheng-Wei Lin,[a,d] Sue-Ming Chang,[a,b] Ting-Chien Lin,[a,c] Yu-Ruei Chuang,[a,c] Jacquelyn Gervay-Hague,[e] Hisashi Narimatsu,[f] and Chun-Hung Lin[a,b,c,*]

[a] Institute of Biological Chemistry, Academia Sinica, No. 128 Academia Road Section 2, Nan-Kang, Taipei 11529, Taiwan
Fax: (+886)-2-2651-4705; phone: (+886)-2-2789-0110; e-mail: chunhung@gate.sinica.edu.tw
[b] Institute of Biochemical Sciences, National Taiwan University, Taipei, 10617, Taiwan
[c] Department of Chemistry, National Taiwan University, Taipei, 10617, Taiwan
[d] Genomics Research Center, Academia Sinica, Taipei, 11529, Taiwan
[e] Department of Chemistry, University of California, Davis, CA 95618, USA
[f] Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan

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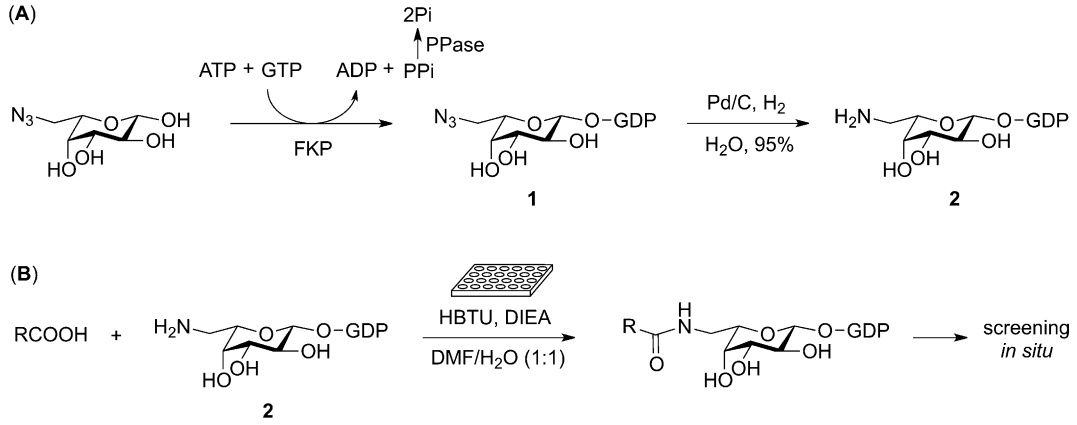
Abstract: Fucosyltransferases (FucTs) usually catalyze the final step of glycosylation and are critical to many biological processes. High levels of specific FucT activities are often associated with various cancers. Here we report the development of a chemoenzymatic method for synthesizing a library of guanosine diphosphate β-L-fucose (GDP-Fuc) derivatives, followed by *in situ* screening for inhibitory activity against bacterial and human α-1,3-FucTs. Several compounds incorporating appropriate hydrophobic moieties were identified from the initial screening. These were individually synthesized, purified and characterized in detail for their inhibition kinetics. Compound **5** had a K_i of 29 nM for human FucT-VI, and is 269 and 11 times more selective than for *Helicobacter pylori* FucT ($K_i = 7.8$ μM) and for human FucT-V ($K_i = 0.31$ μM).

Keywords: enzyme catalysis; enzymes; guanosine diphosphate β-L-fucose (GDP-Fuc) derivatives; inhibitors; transferases

Introduction

Cell-surface glycoconjugates play an important role in numerous physiological and pathological processes, including cell-cell adhesion, cell differentiation, immune response, fertilization, viral and bacterial infection, and tumor progression.[1–4] Fucosylated glycans are critical to a variety of cell events, and fucosylation of cell-surface glycans is often the final step in the biosynthesis of an oligosaccharide. For instance, the tetrasaccharide sialyl Lewis x (sLe^x) is well known for its physiological importance in the inflammatory response and the recruitment of leukocytes, and in tumor metastasis.[5] α-2,3-Sialyltransferase and α-1,3-fucosyltransferase (α-1,3-FucTs) sequentially catalyze the last two steps in the corresponding biosynthesis. Aberrant expression of sLe^x and enhanced FucT activities are found in many carcinomas.[6–9] Developing FucT inhibitors as potential anti-inflammatory and anti-tumor therapies is therefore a promising strategy.[10]

In humans, there are 12 FucTs that catalyze the formation of various fucosyl linkages (α-1,2-, α-1,3-, α-1,4- and α-1,6-glycosidic bonds);[11] thus, selective inhibition of a specific FucT(s) offers a prospective strategy for developing a therapeutic agent. FucTs catalyze the transfer of L-fucose (Fuc) from guanosine diphosphate β-L-fucose (GDP-Fuc) to various sugar-acceptor substrates. Several approaches have been developed for the preparation of FucT inhibitors, such as the design and synthesis of substrate- and transition state-based analogues.[12–17] Rational design is mainly impeded by the limited structural information available, however. We previously reported the first X-ray structures of *Helicobacter pylori* α-1,3-FucT (HP-FucT); this remains the only study on the enzyme-substrate and enzyme-product complex structures to date.[18] Recently, potent FucT inhibitors have been



Scheme 1. Three-step preparation of the 94 GDP-Fuc derivatives investigated for FucT inhibition. (**A**) Chemoenzymatic synthesis of GDP-6-amino-Fuc (**2**) by FKP. PPase = inorganic pyrophosphatase. (**B**) Synthesis of GDP-Fuc derivatives by amide-forming reactions in microtiter plates, followed by the *in situ* FucT activity assay.

developed separately by Wong and by Nishimura, by applying click chemistry to make GDP and GDP-Fuc derivatives,[19–21] respectively. These were subsequently assayed for inhibition activity. In their studies, GDP-alkyne or GDP-6-alkynylfucose was prepared to couple with different azide derivatives *via* 1,3-dipolar cycloaddition. Potent inhibition was thus achieved by incorporation of a suitably hydrophobic group to either the phosphate of GDP or the fucose residue of GDP-Fuc.

Previous studies indicated that sugar nucleotides and analogues often offer satisfying inhibition potency against glycosyltransferases because they provide the necessary hydrogen bonding and electrostatic interactions.[22–25] However, their synthesis is not trivial. Particularly it usually requires numerous trials for optimizing the yields when introducing the mono- or diphosphate moiety, and purifying the intermediates or/and products. The development of an efficient synthetic procedure will bring a beneficial impact to the fields of glycosyltransferases and related inhibition studies.

In this report, we have established a three-step chemoenzymatic method for the rapid assembly of various GDP-Fuc derivatives from 6-azido-L-fucose. To examine the inhibition and selectivity of these derivatives, three α-1,3-FucTs were selected, including HP-FucT, human FucT-VI (FucT-VI), and human FucT-V (FucT-V). Of these products, compound **5** was found to be a potent inhibitor of FucT-VI ($K_i = 29$ nM), with highly selective inhibition compared to that of FucT-V and HP-FucT.

Results and Discussion

Chemoenzymatic Synthesis of GDP-6-amino-L-fucose (2)

In previous studies, GDP or GDP-Fuc analogues have mainly been synthesized using click chemistry strategies. Instead, we used amide-forming reactions, and GDP-6-amino-L-fucose (**2**) was designed as the core template to couple to various carboxylic acids. This study allowed us to determine if the amide coupling products are able to produce potent inhibition. The bifunctional enzyme L-fucokinase/GDP-Fuc pyrophosphorylase (FKP), from *Bacteroides fragilis*,[26–28] was used for the synthesis of compound **2**, and it offers the following advantages: (i) FKP is capable of synthesizing GDP-Fuc analogues in one step, directly from the corresponding Fuc analogues; (ii) the operation is simple compared to the chemical method;[29,30] and (iii) FKP is easy to prepare with high yield and purity. Thus, N-terminally His-tagged FKP was cloned, overexpressed in *Escherichia coli* and purified in one step, giving a yield of 40 mg of FKP with >95% homogeneity from 1 L of culture medium.

Despite the flexible substrate tolerance of FKP, 6-amino-L-fucose turned out to be a very poor substrate. An alternative two-step reaction was therefore employed in which GDP-6-azido-L-fucose (**1**) was synthesized by the FKP-catalyzed reaction from 6-azido-L-fucose, followed by reduction of the azido group to give **2** (Scheme 1). The synthesis can be carried out at the 100 mg scale and gives 68% overall yield.

Preparation of Various GDP-Fuc Derivatives and *in situ* Screening for Inhibition Activity

Compound **2** was subjected to diversity-oriented synthesis, i.e., the amide-forming reactions of compound **2** with 94 carboxylic acids (see the Supporting Information, Figure S1 for the carboxylic acid structures) were carried out in the presence of *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) and *N,N*-diisopropylethylamine (DIEA) in wells of 96-well microtiter plates. The coupling reactions were monitored by thin-layer chromatography (TLC). Assuming the reaction yields to be quantitative (as judged by TLC), the reaction mixtures were diluted and directly assayed for the inhibition activity of HP-FucT and FucT-VI without product purification. We used radioisotope-labeled substrate and TLC analysis for the activity assay because of the difficulty in preparing human FucT, and because of the low enzyme activity and high cost of GDP-Fuc. Furthermore, fluorescence-coupled enzymatic assays[31] are not suitable for large-scale screening owing to the limited volume. Thus, enzyme activity was quantified by the transfer of L-[^{14}C]Fuc to the acceptor substrate *N*-acetyllactosamine (LacNAc). None of the reagents used for the amide-forming reactions were found to generate any substantial inhibition (see the Supporting Information, Figure S3). The concentration of each coupling product was kept at 1 μM for preliminary screening. Most of the products derived from the 48 carboxylic acids in groups A through D (see the Supporting Information, Figure S1) showed no inhibitory effect for either FucT-VI or HP-FucT (see the Supporting Information, Figure S2), revealing that simple, aliphatic extensions from C-6 of Fuc may not improve FucT inhibition. In contrast, the reaction products of several of the 12 compounds in group G (see the Supporting Information, Figure S1) showed good inhibition potency against FucT-VI, and their inhibitory activities (at the concentration of 1 μM) are shown in Figure 1. In particular, the amide product of **G6** (designated **G6z** in Figure 1 and compound **5** in the following text) and **G7** resulted in the most potent inhibition against FucT-VI (98% and 32% inhibition, respectively), and those of **E11** and **E12** (designated **E11z** and **E12z** in Figure 1 and compounds **3** and **4** in the following text, respectively) showed moderate inhibition (approximately 20%), as shown as the closed bars in Figure 1. Interestingly, these products exhibited a different inhibition pattern for HP-FucT (shown as open bars in Figure 1). Compounds **3** and **4** were the most potent inhibitors of HP-FucT, with 32% and 60% inhibition at 1 μM, respectively. Conversely, the coupling products derived from the carboxylic acids in group G (see the Supporting Information, Figure S1) exhibited minimal inhibition (<5%) under the conditions tested.

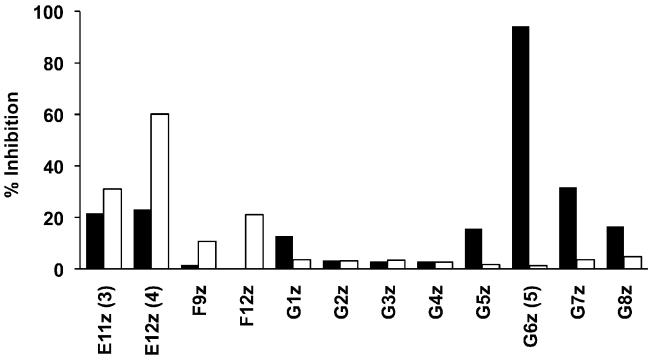


Figure 1. Inhibition of FucT-VI (closed bars) and HP-FucT (open bars) by 12 selected compounds. The synthesized GDP-Fuc derivatives were screened *in situ* for inhibitory activity against the two FucTs. Results for compounds discussed below or for those showing higher inhibition activities are shown. Amide products are labeled by adding "z" to the end of the corresponding acid names. For example, **G1z** is the product of coupling the carboxylic acid **G1** and compound **2**. For the complete screening results, see Figure S2 in the Supporting Information.

Evaluation of Inhibitory Activities of Compounds 3 through 5

We individually synthesized and purified compounds **3**, **4** and **5** (Figure 2) for further characterization. The carboxylic acids of **G6**, **E11** and **E12**, were converted to the corresponding succinimide esters using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), followed by the coupling reaction with **2**. The desired products (**3**–**5**) were purified by anion exchange chromatography and characterized by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry. These amide products were stable in the assay condition (50 mM Tris-HCl buffer at pH 7.5) for at least three weeks, with no sign of degradation according to the observation by ^{1}H NMR.

Lineweaver–Burk plot analysis indicated that compounds **3**–**5** all exhibited competitive inhibition with respect to GDP-Fuc (Figure 3 and Supporting Information, Figure S4–Figure S10). The IC_{50} and K_i values for the three FucTs are listed in Table 1. Compound **5** was found to be an excellent inhibitor for FucT-VI with a K_i of 29 nM. It had K_i values of 7.8 and 0.31 μM for HP-FucT and FucT-V, respectively, indicating that it is 269 and 11 times more selective for FucT-VI than for HP-FucT and FucT-V, respectively. In contrast, although compound **4** was a potent inhibitor of HP-FucT ($K_i = 0.22$ μM), it had no substantial selectivity for the three FucTs examined. Similar results were obtained for **3**. Regarding the inhibi-

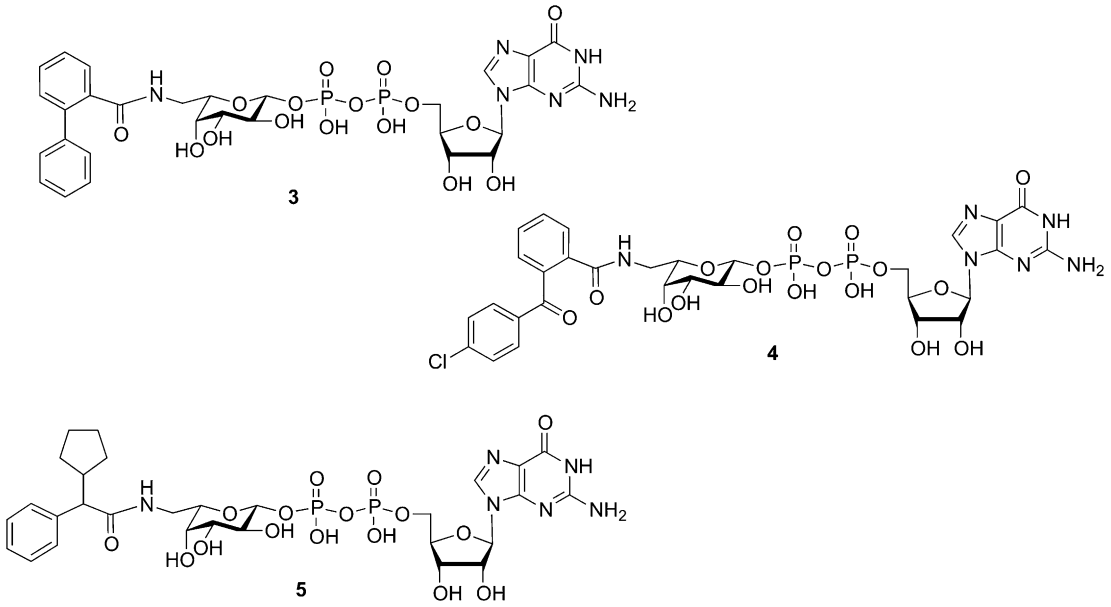


Figure 2. The structures of compounds **3**–**5**.

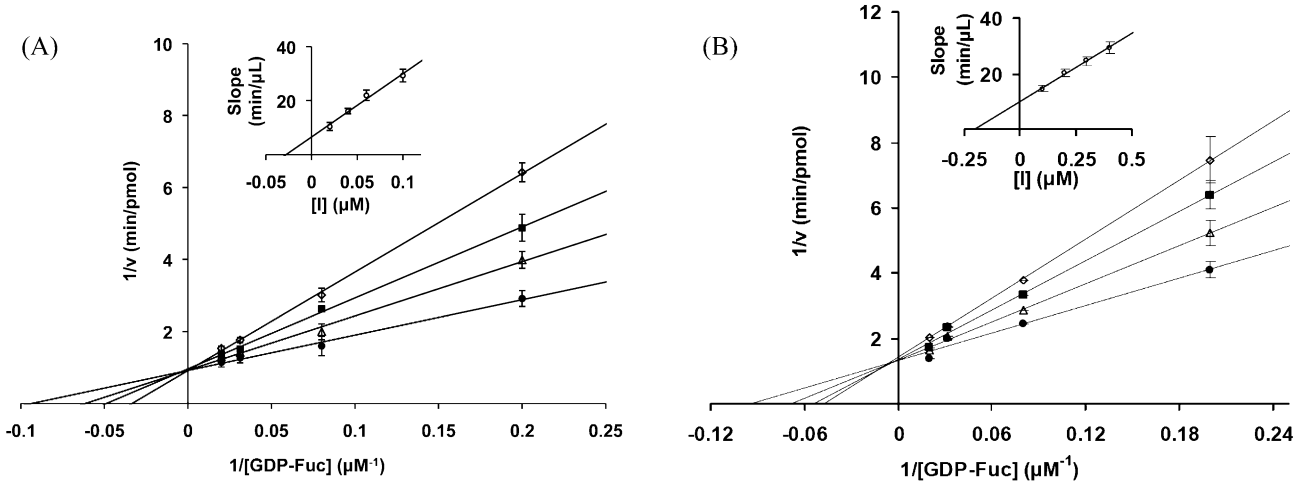


Figure 3. Lineweaver–Burk analysis of the steady-state kinetics of FucT-VI and HP-FucT in the presence of compounds **5** and **3**, respectively. In both experiments, the concentration of GDP-Fuc was varied while that of LacNAc was fixed. The resulting slopes were plotted against inhibitor concentration (shown as inserts). (**A**) Kinetics for FucT-VI in the presence of compound **5** at 0.01 (●), 0.02 (△), 0.06 (■) and 0.1 μM (◇). The insert indicates that **5** is a competitive inhibitor of FucT-VI with a K_i of 29 nM. (**B**) Kinetics for HP-FucT in the presence of compound **4** at 0.1 (●), 0.2 (△), 0.3 (■) and 0.4 μM (◇). The insert indicates that **4** is a competitive inhibitor of HP-FucT with a K_i of 0.22 μM.

Table 1. IC_{50} and K_i values (μM) of compounds **3**–**5** against various FucTs.

	3		**4**		**5**		GDP-Fuc
Enzyme	IC_{50}	K_i	IC_{50}	K_i	IC_{50}	K_i	K_m
FucT-V	2.7	0.81 ± 0.15	1.2	0.52 ± 0.11	0.69	0.31 ± 0.09	13.8 ± 2.3
FucT-VI	5.4	1.9 ± 0.7	2.1	1.1 ± 0.29	0.094	0.029 ± 0.009	13.2 ± 2.5
HP-FucT	3.9	0.9 ± 0.2	0.78	0.22 ± 0.06	26	7.8 ± 0.7	11.3 ± 2.2

tion of HP-FucT, the available X-ray complex structure provides useful insight to account for the different potencies of compounds **3**–**5**. Computational modeling of HP-FucT (PDB code 2NZY) complexed to **4** (Figure 4) suggests that a deep pocket formed by hydrophobic residues, including Trp33, Trp34, Phe42,

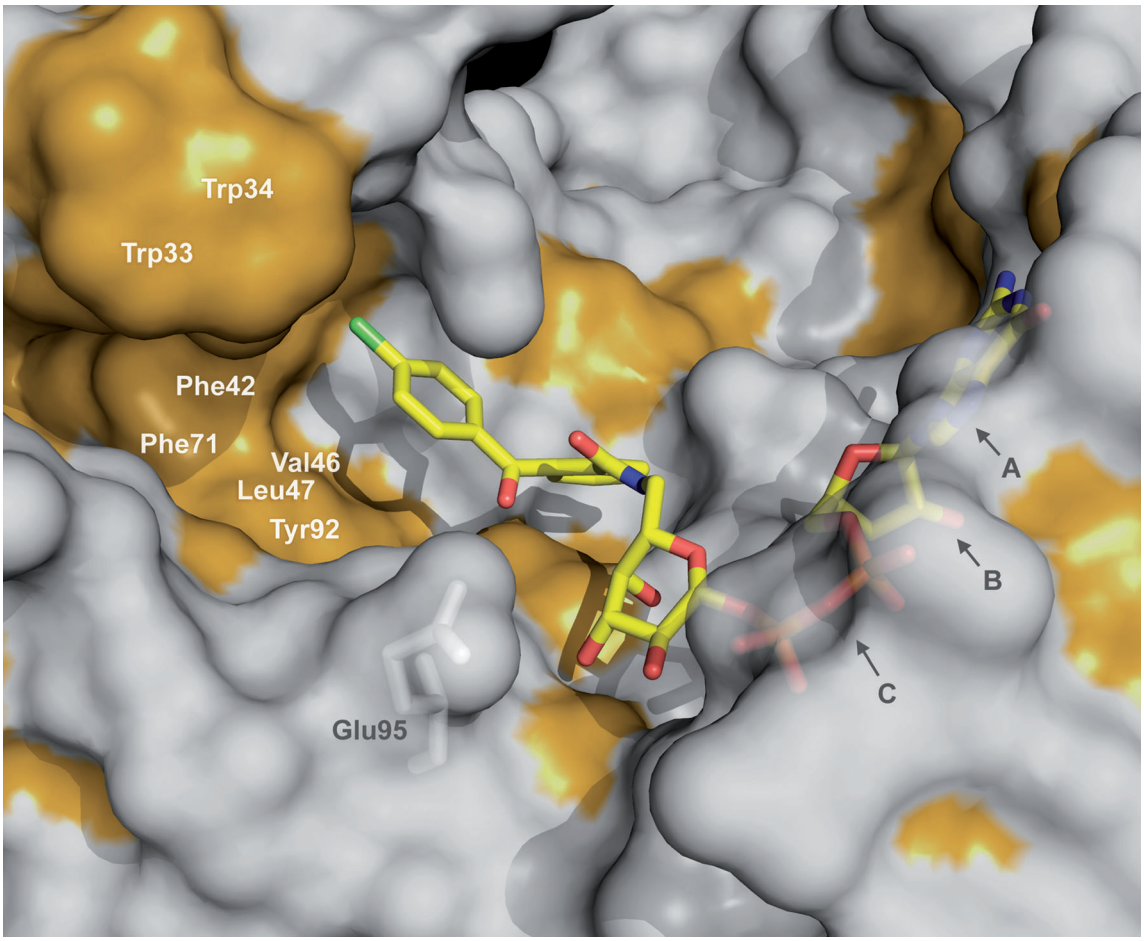


Figure 4. Molecular model of compound **4** in the active site of HP-FucT. The molecular structure of **4** is depicted in yellow (carbon), red (oxygen), blue (nitrogen), green (chlorine) and orange (phosphorus). Hydrophobic residues of HP-FucT (PDB code 2NZY) are shown in orange and other residues in white. A deep hydrophobic pocket consisting of residues Trp33, Trp34, Val46, Leu47, Phe42, Phe71, and Tyr92 is proposed to interact with the hydrophobic group of compound **4**. Arrows A–C indicate the guanine, ribose, and diphosphate moieties of compound **4**, respectively. Glu95 acts as a general acid/base in the catalysis. The molecular model was created and displayed using Discovery Studio v2.5 and PyMOL v1.4.

Val46, Leu47, Phe71, and Tyr92, is near the active site. The benzophenone moiety of **4** ($K_i = 0.22\ \mu M$) may fit well into the hydrophobic pocket and thereby enhance the binding. Compound **3** ($K_i = 0.9\ \mu M$), however, may be a weaker fit for the pocket owing to its bulky biphenyl group. The branched cyclopentyl group of **5** ($K_i = 7.8\ \mu M$) was found to be a poor fit for the hydrophobic pocket, and this would explain its low affinity.

We previously had success in developing potent and selective inhibitors for α-fucosidase[32–34] and β-hexosaminidase.[35,36] In these studies, even though the same type of enzymes share high sequence similarity and use the same catalytic mechanism, their protein surfaces are very different. Thus, achieving selective inhibition is possible by choosing a suitable group(s) for optimizing the binding interactions. A similar strategy was applied for this study. We expected the GDP-Fuc moiety to occupy the conserved active site because compounds **3**–**5** are all proved to be competitive inhibitors (Figure 3 and Supporting Information, Figure S4–Figure S10). A variety of carboxylic acids were incorporated as amides and examined to match the different surfaces of the three α-1,3-FucTs. As a result, compounds **4** and **5** have highly improved affinities for HP-FucT and FucT-VI, respectively, compared to GDP-Fuc ($K_m = 11.3\ \mu M$ for HP-FucT, and $K_m = 13.2\ \mu M$ for FucT-VI; Table 1). The additional structural motifs of GDP-Fuc and **5** apart from GDP (i.e., the groups of fucose and hydrophobically C-6-substituted fucose) contribute a 2.5- and 1100-fold enhancement of inhibition against FucT-VI, as compared to GDP ($K_i = 33\ \mu M$). This large difference in binding affinity underscores the validity of our approach of efficiently enhancing inhibition potency.

Previous studies have suggested that the binding interaction between GDP-Fuc and FucT could be enhanced by the addition of a suitably hydrophobic

group to the C-6 of Fuc, or directly to GDP without the sugar residue.[19,21] This suggests that both the incorporated hydrophobic group and the triazole may contribute to the improved inhibition. Maeda et al. prepared two GDP-Fuc-derived compounds by applying either click chemistry or the amide-forming reaction to couple GDP-Fuc with a naphthylmethyl group.[37] Despite their similar structures, the triazole-containing compound was found to be an inhibitor of FucT-VI, whereas the amide-containing compound turned out to be a good substrate. Instead of being substrates, compounds **3–5** are potent FucT inhibitors. Conversely, compound **2** is neither a substrate nor an inhibitor for the α-1,3-FucTs tested. We observed a similar phenomenon in the case of FKP, which accepted a wide range of Fuc analogues but failed to utilize 6-amino-L-fucose as a substrate. The same molecule is neither an inhibitor of FKP. It is intriguing that compound **2** showed no activity for the FucTs, yet it was converted to a potent inhibitor after incorporation of the optimal hydrophobic moiety. In addition, high selectivity was also achieved, which enabled distinction between different FucTs.

Conclusions

In summary, this chemoenzymatic method was developed to discover potent and selective FucT inhibitors, and assess them *in situ* using an activity assay. Despite improved inhibition potency, several critical issues remain to be addressed. For instance, the pyrophosphate group needs to be replaced to allow delivery across the cell membrane. To increase stability, substitution of the glycosidic linkage of compounds **3–5** is currently being investigated, and our progress will be reported in due course.

Experimental Section

Materials

Chemical reagents were used as purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise indicated. Reactions and assays were monitored by TLC on silica gel 60 F254 (Merck, Darmstadt, Germany) and visualized under UV, cerium ammonium molybdate stain, or by isotope signal. GDP-L-[U-^{14}C]fucose (GDP-[^{14}C]Fuc) was purchased from Perkin–Elmer (Boston, MA). NMR spectra were recorded routinely on a Bruker AV-400 (400 MHz) or AVII-500 (500 MHz) spectrometer with D_2O ($\delta_H = 4.80$) as the internal standard.

B. fragilis NCTC9343 genomic DNA was obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). Restriction enzymes and alkaline phosphatase were purchased from New England Biolabs (Ipswich, MA). Inorganic pyrophosphatase was purchased from Sigma–Aldrich. Anion exchange chromatography was performed on a HiTrap Q Sepharose column (GE Healthcare Life Sciences, Uppsala, Sweden).

Plasmid Construction, Protein Expression and Purification

DNA manipulations were carried out according to standard procedures. For the construction of pET28-FKP, the 3-kb coding sequence of FKP was amplified from *B. fragilis* NCTC9343 genomic DNA by PCR using the forward primer, 5′-AACTCTGACCATATGCAAAAACTACTATCTTTACCGTCC-3′, and reverse primer, 5′-CCAATACTCGAGTTATGATCGTGATACTTGGAATCCCTT-3′, to create the underlined *NdeI* and *XhoI* restriction enzyme sites, respectively. After digestion with *Nde*I and *Xho*I, the PCR fragment was first ligated into the pGEM-T EASY vector (Promega, Fitchburg, WI) and then subcloned into the pET28a vector to yield pET28-FKP. The desired constructs were verified by DNA sequencing. *E. coli* BL21 (DE3) was transformed with pET28-FKP to overexpress the N-terminal hexahistidine (His_6)-tagged FKP. The clone harboring pET28-FKP was cultured in lysogeny broth (containing 50 μg mL^{-1} kanamycin) overnight at 37 °C with vigorous shaking. The overnight culture was then diluted 1:1000 (v/v) with the same medium and grown at 37 °C until cells reached an A_{600} of 0.7. Protein expression was induced by addition of isopropyl-1-thio-β-D-*galacto*-pyranoside to a final concentration of 0.2 mM, and the culture was grown for an additional 24 h at 16 °C. Cells were harvested by centrifugation at 10000 × *g* for 10 min at 4 °C, resuspended in phosphate-buffered saline, and lysed using a French Press. Recombinant FKP was purified by a single step of Ni^{2+} affinity chromatography. The desired protein fractions were collected, concentrated and desalted (against 50 mM pH 8.0 Tris-HCl) using a 50 kDa molecular weight cut-off Amicon Ultra-15 centrifugal filter (Millipore, Taiwan). For the preparation of human FucT-V and -VI, the DNA fragment encoding the desired FucT was amplified by PCR, and the sequence corresponding to N-terminal transmembrane domain was removed. The amplified fragment was digested with *Nde*I and *Xho*I and ligated into the pFLAG-CMV™-3 vector (Sigma–Aldrich). HEK293T cells were transfected with the FucT plasmid using calcium phosphate. The stable 293T cell lines expressing FLAG-tagged FucT were selected and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg mL^{-1} streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

For purification of FucT, cells were harvested by treatment with trypsin/EDTA. After washing with phosphate-buffered saline twice, the cell pellets were suspended in lysis buffer containing 0.5% Nonidet P-40, 25 mM hydroxyethyl piperazineethanesulfonic acid (Hepes, pH 7.9), 200 mM KCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 10 mM NaF, 0.1 mM Na_3VO_4, 5% glycerol and a protease inhibitor cocktail (Sigma–Aldrich). After 20 min on ice, cell suspensions were homogenized by sonication then centrifuged at 13,000 × *g* for 10 min at 4 °C to remove the nuclear and cellular debris. The resulting supernatant was incubated with an antibody against FLAG that had been coupled to agarose beads (Sigma–Aldrich) at

4 °C for 2 h. After extensive washing with lysis buffer, FLAG-tagged FucT was eluted with 100 ng/mL 3× FLAG peptide (Sigma–Aldrich). HP-FucT was prepared as described.[18]

Synthesis of Guanosine 5′-Diphospho-6-amino-β-L-fucopyranoside (2)

Guanosine 5′-diphospho-6-azido-β-L-fucopyranoside, **1**, was enzymatically synthesized by FKP from 6-azido-β-L-fucose (Scheme 1). Reduction of the azide to an amine was performed over Pd/C to give **2**. A 25 mL mixture of 50 mM Tris-HCl (pH 8.2), 10 mM 6-azido-L-fucose (36 mg, 200 μmol), 11 mM ATP (113 mg, 220 μmol), 5.5 mM GTP (108 mg, 220 μmol), 10 mM $MnCl_2$, inorganic pyrophosphatase (20 units), and FKP (5 units) was incubated at 37 °C for 12 h. After the reaction was complete, as judged by TLC using a 7:1:3 (v/v/v) solution of *i*-PrOH:AcOH:H_2O, the sample was treated with 5 units of alkaline phosphatase for 5 h at 37 °C to hydrolyze the remaining nucleoside phosphates. After removing the precipitated magnesium phosphate by centrifugation, the resulting sample was applied to the HiTrap Q Sepharose column. The column was initially washed with 10 mL water, then the retained materials were eluted with a 40 mL linear gradient of 50–200 mM NH_4HCO_3. Eluents were monitored by UV absorption at 260 nm. The desired fractions were pooled, concentrated under reduced pressure, and lyophilized to yield 101 mg of **1** as a white solid in 72% yield. For the synthesis of **2**, a solution of **1** (30 mg, 300 μmol) and 10% Pd/C (8 mg) in water (10 mL) was stirred under a hydrogen atmosphere for 15 min (the product decomposed when the reaction was carried out in methanol). The mixture was filtered, and the filtrate was lyophilized to afford **2** as a white foam; yield: 28 mg (95%). ^{1}H NMR (400 MHz, D_2O): δ = 3.23–3.35 (m, 2H, H-6α″, H-6β″), 3.63 (dd, 1H, J = 9.5, 7.8 Hz, H-2″), 3.71 (dd, 1H, $J_{2'',3''}$ = 10.0 Hz, $J_{3'',4''}$ = 3.2 Hz, H-3″), 3.92–3.98 (m, 2H, H-4″, H-5″), 4.22 (m, 2H, H-5α′, H-5β′), 4.36 (m, 1H, H-4′), 4.51-4.53 (m, 1H, H-3′), 4.97 (dd, 1H, $J_{1'',2''} = J_{1'',P}$ = 7.6 Hz, H-1″), 5.94 (d, 1H, $J_{1',2'}$ = 6.0 Hz, H-1′), 8.15 (s, 1H, H-8); ^{13}C NMR (125 MHz, D_2O): δ = 39.7, 50.2, 65.3, 68.4, 70.3, 71.0, 72.0, 73.6, 83.6, 86.7, 98.4, 116.1, 137.4, 151.7, 153.8, 158.8; HR-MS (ESI): *m/z* = 603.0930, calcd. for $C_{16}H_{25}N_6O_{15}P_2$ $[M-H]^-$: 603.0848.

Amide-Forming Reactions of GDP-Fuc Derivatives and *in-situ* FucT Activity Assay

Dimethylformamide (DMF, 12 μL) was aliquoted into each well of a 96-well microtiter plate. The following reagents were then added into each well: 1.1 μL of each carboxylic acid (from a 50 mM stock solution in DMF), 1.1 μL of 50 mM *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) dissolved in DMF (1.1 equivalents), and 2 μL of 50 mM DIEA dissolved in DMF (2 equivalents). The reaction was initiated by adding 10 μL of a 5 mM aqueous solution of **2** to each well, and progress was monitored by TLC using a 7:1:3 (v/v/v) solution of *i*-PrOH:AcOH:H_2O. After 3 h, a 1 μL aliquot was withdrawn from the reaction and mixed with 199 μL of water, i.e., a final concentration of 10 mM of the compound in each well of the microtiter plate. To each well of another microtiter plate, 8 μL of reaction buffer containing 50 mM pH 7.5 Tris-HCl buffer, LacNAc (due to the different K_m values of each enzyme, the concentration of LacNAc was 20, 5 and 2 mM for FucT-V, FucT-VI and HP-FucT, respectively), 10 mM $MnCl_2$, 50 μunit of FucT were mixed with an aliquot (1 μL) of the mixture from previous microtiter plate to give ~1 μM inhibitor concentration. Assays were initiated by adding 1 μL of GDP-Fuc (from a 100 mM stock solution containing 25% GDP-[^{14}C]Fuc) to each well at 37 °C. Enzyme activity was detected by measuring the incorporation of radioactive label from GDP-[^{14}C]Fuc into the reaction product. Samples were taken at defined time points (30, 90, 120 min) and analyzed by TLC using a 7:1:3 (v/v/v) solution of *i*-PrOH:AcOH:H_2O. Radioactivity was detected by phosphoimaging using a BAS-MS 2040 imaging plate and BAS-1500 PhosphorImager (Fujifilm, Tokyo, Japan). Signals were evaluated and quantified using the software Image Gauge, v4.0 (Fujifilm).

Synthesis of Compounds 3–5

Compounds displaying strong inhibition activity were selected, synthesized, and purified for further investigation. To avoid formation of side products that are troublesome to remove, DIEA was not used, and the NHS-ester was added in 5-fold excess for the amide-forming reactions. To synthesize **5**, a mixture of **G6** (20 mg, 100 μmol), EDC (172 mg, 150 μmol), and NHS (21 mg, 110 μmol) in 10 mL CH_2Cl_2 was stirred at room temperature for 1 h. After TLC analysis with hexane:ethyl acetate (v/v = 1:1) to indicate the reaction was complete, the mixture was washed with a saturated solution of $NaHCO_3$, then dried over $MgSO_4$, concentrated under vacuum, and precipitated by the slow addition of diethyl ether at 4 °C. The precipitate was washed with diethyl ether to give the NHS-activated **G6** (**G6**-OSu) as a white powder; yield: 29 mg (96%).

G6-OSu (25 mg, 83 μmol, dissolved in 1 mL of DMF) was mixed with **2** (10 mg, 16 μmol, dissolved in 0.5 mL of 50 mM sodium phosphate buffer, pH 8.1) and stirred at 50 °C. The reaction was monitored by TLC using a 7:1:3 (v/v/v) solution of *i*-PrOH:AcOH:H_2O. After the reaction was complete, the mixture was washed with CH_2Cl_2 to remove DMF and the excess **G6**-OSu. The solution was lyophilized, reconstituted in water, and the product purified using HiTrap Q Sepharose anion exchange chromatography as described above to give **5** as a white powder; yield: 11.8 mg (90%). Compounds **3** and **4** were synthesized in the same manner; yield: 92 and 83%, respectively.

Compound 3: ^{1}H NMR (400 MHz, D_2O): δ = 3.42–3.50 (m, 2H, H-6α″, H-6β″), 3.60–3.68 (m, 4H, H-2″, H-3″, H-4″, H-5″), 4.25–4.28 (m, 2H, H-5α′, H-5β′), 4.39 (m, 1H, H-4′), 4.57 (m, 1H, H-3′), 5.00 (dd, 1H, $J_{1'',2''} = J_{1'',P}$ = 7.7 Hz, H-1″), 5.95 (d, 1H, $J_{1,2'}$ = 6.0 Hz, H-1′), 7.46–7.65 (m, 9H, aromatic), 8.13 (s, 1H, H-8); ^{13}C NMR (125 MHz, D_2O): δ = 41.5, 65.2, 68.5, 70.2, 71.2, 72.1, 72.3, 73.7, 83.6, 86.7, 98.3, 161.2, 127.2, 127.3, 128.1, 128.3, 129.1, 130.5, 130.8, 132.4, 137.4, 140.9, 151.7, 154.2, 159.2, 169.7; HR-MS (ESI): *m/z* = 783.1505, calcd. for $C_{29}H_{33}N_6O_{16}P_2$ $[M-H]^-$: 783.1423.

Compound 4: ^{1}H NMR (400 MHz, D_2O): δ = 3.39-3.46 (m, 2H, H-6α″, H-6β″), 3.61–3.72 (m, 2H, H-2″, H-3″), 3.82–3.89 (m, 2H, H-4″, H-5″), 4.20–4.21 (m, 2H, H-5α′, H-5β′), 4.34 (m, 1H, H-4′), 4.55–4.59 (m, H-3′), 5.94 (d, 1H, $J_{1',2'}$ =

6.0 Hz, H-1′), 7.40–7.89 (m, 8H, aromatic), 8.13 (s, 1H, H-8); ^{13}C NMR (125 MHz, D_2O): δ = 42.5, 65.1, 68.2, 70.1, 70.8, 70.9, 71.8, 73.4, 83.4, 83.6, 86.7, 98.2, 116.5, 127.4, 128.8, 129.8, 130, 130.5, 132.7, 135.8, 137.5, 151.7, 153.9, 159.1, 165.8; HR-MS (ESI): *m/z* = 845.0972, calcd. for $C_{30}H_{32}ClN_6O_{17}P_2$ $[M-H]^-$: 845.0982.

Compound 5: ^{1}H NMR (500 MHz, D_2O): δ = 0.93–0.95, 1.19–1.27, 1.46–1.55, 1.76, 2.54–2.56 (m, 9H, cyclopentane), 3.26–3.34 (m, 2H, H-6α″, H-6β″), 3.52–3.66 (m, 5H, H-2″, H-3″, H-4″, H-5″, CH), 4.20–4.24 (m, 2H, H-5α′, H-5β′), 4.32–4.34 (m, 1H, H-4′), 4.50 (dd, 1H, $J_{2'',3''}$ = 4.5, $J_{3'',4''}$ = 3.8 Hz, H-3′), 4.93 (dd, 1H, $J_{1'',2''} = J_{1'',P}$ = 7.9 Hz, H-1″), 5.90 (d, 1H, $J_{1',2'}$ = 6.0 Hz, H-1′), 7.29–7.42 (m, 5H, aromatic), 8.14 (s, 1H, H-8); ^{13}C NMR (125 MHz, D_2O): δ = 24.4, 24.7, 30.0, 31.2, 41.2, 42.8, 57.1, 65.2, 68.4, 70.3, 70.9, 71.0, 72.0, 73.6, 83.6, 83.7, 86.7, 98.3, 116.2, 126.9, 128.1, 128.3, 137.5, 139.5, 151.7, 154.0, 159.1, 170.3; HR-MS (ESI): *m/z* = 789.1817, calcd. for $C_{29}H_{39}N_6O_{16}P_2$ $[M-H]^-$: 789.1892.

Measurement of IC_{50} and K_i Values

To measure IC_{50} values, the activity assays were carried out in 10 μL solutions containing 50 mM Tris-HCl (pH 7.5), 10 mM $MnCl_2$, 20 μM GDP-Fuc (containing 25% [GDP-[^{14}C]Fuc), LacNAc (20 mM, 5 mM, and 2 mM for FucT-V, FucT-VI, and HP-FucT, respectively), 50 μunits of FucT, and a variable inhibitor concentration (0.01–50 μM). Reactions were conducted at 37 °C, and enzyme activity was detected by monitoring the incorporation of radioactive label from GDP-[^{14}C]Fuc by TLC as described above.

The experiments to determine K_i values were performed with various concentrations of GDP-Fuc when one inhibitor concentration was fixed. Assays were carried out in 10 μL solutions containing 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MnCl_2$, LacNAc (20, 5 and 2 mM for FucT-V, FucT-VI and HP-FucT, respectively), 50 μunits of FucT, GDP-Fuc (within the range of 5–50 μM, each contained 2.5 μM GDP-[^{14}C]Fuc), and inhibitor (0.01–20 μM). Reactions were conducted at 37 °C, and enzyme activity was detected by radio-TLC as described previously. Lineweaver–Burk plot analysis was then carried out to determine the K_i values.

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