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Moriyama, Takahiro
Graduate School of Pharmaceutical Sciences, Kyushu University

Yoritate, Makoto
Graduate School of Pharmaceutical Sciences, Kyushu University

Kato, Naoki
Faculty of Agriculture, Setsunan University

Saika, Azusa
Microbial Research Center for Health and Medicine, National Institutes of Biomedical Innovation, Health and Nutrition

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Linkage-Editing pseudo-Glycans: A Reductive α-Fluorovinyl-*C*-Glycosylation Strategy to Create Glycan Analogs with Altered Biological Activities

Takahiro Moriyama,[a§] Makoto Yoritate,[a§] Naoki Kato,[b,c*] Azusa Saika,[d,e] Wakana Kusuhara,[f,g] Shunsuke Ono,[a] Takahiro Nagatake,[d,h] Hiroyuki Koshino,[c] Noriaki Kiya,[a] Natsuho Moritsuka,[a] Riko Tanabe,[a] Yu Hidaka,[a] Kazuteru Usui,[a] Suzuka Chiba,[a] Noyuri Kudo,[a] Rintaro Nakahashi,[a] Kazunobu Igawa,[i] Hiroaki Matoba,[a] Katsuhiko Tomooka,[i] Eri Ishikawa,[f,g] Shunji Takahashi,[c] Jun Kunisawa,[d] Sho Yamasaki,[f,g] and Go Hirai[a,c*]

[a]Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

[b]Faculty of Agriculture, Setsunan University, 45-1 Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan

[c]RIKEN Center for Sustainable Resource Science, 2-1, Hirosawa, Wako, Saitama 351-0198, Japan

[d]Microbial Research Center for Health and Medicine, National Institutes of Biomedical Innovation, Health and Nutrition, 7-6-8 Asagi-Saito, Ibaraki, Osaka 567-0085, Japan

[e]Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, 11 Biopolis Way, Helios, Singapore 138667, Singapore

[f]Research Institute for Microbial Diseases, Osaka University, Yamadaoka, Suita, Osaka 565-0871, Japan

[g]Immunology Frontier Research Center, Osaka University, Yamadaoka, Suita, Osaka 565-0871, Japan

[h]Department of Life Sciences, School of Agriculture, Meiji University, 1-1-1 Higashi-Mita, Tama, Kawasaki, Kanagawa 214-8571, Japan

[i]Institute for Materials Chemistry and Engineering, IRCCS, Kyushu University, Kasuga, Fukuoka 816-8580, Japan

§ T.M. and M.Y. contributed equally to this work.

ABSTRACT: The acetal (*O*-glycoside) bonds of glycans and glycoconjugates are chemically and biologically vulnerable, and therefore *C*-glycosides are of interest as more stable analogs. We hypothesized that, if the *O*-glycoside linkage plays a vital role in glycan function, the biological activities of *C*-glycoside analogs would vary depending on their substituents. Based on this idea, we adopted a "linkage-editing strategy" for the creation of glycan analogs (pseudo-glycans). We designed three types of pseudo-glycans with CH_2- and CHF-linkages, which resemble the *O*-glycoside linkage in terms of bond lengths, angles, and bulkiness, and synthesized them efficiently by means of fluorovinyl *C*-glycosylation and selective hydrogenation reactions. Application of this strategy to isomaltose (IM), an inducer of amylase expression, and α-GalCer, which activates iNKT cells, resulted in the discovery of CH_2-IM, which shows increased amylase production ability, and CHF-α-GalCer, which shows opposite activity to native α-GalCer, serving as an antagonist of iNKT cells.

Introduction

C-Glycosides contain a stable C-C bond linkage at the anomeric position of carbohydrates instead of the usual *O*-glycoside bond. Among them, *C*-aryl glycosides (Csp^2-glycosides) have attracted considerable attention, and synthetic studies have led to the development of new sodium-glucose cotransporter-2 (SGLT2) inhibitors to treat type II diabetes,[1] as well as opening up new approaches to access glycoside natural products.[2–4] Csp^3-glycoside analogs of native glycans/glycoconjugates (pseudo-glycans) should also be of particular interest, because their increased stability against glycoside hydrolases makes them attractive as candidates for drug discovery and tools for functional analysis. Several but limited CH_2-linked glycan analogs have already been synthesized, and their interaction with binding proteins or their inhibitory activities towards glycoside hydrolases have been investigated to assess the extent to which they mimic the corresponding native glycans.[5–8] However, the potential of pseudo-glycan synthesis with C-glycoside linkage as a means for creating bioactive molecules that show effects at the cellular level or in mice remains a little explored.[9,10]

In contrast to other strategies for increasing resistance to glycoside hydrolases, such as building *S*-glycosides[11] or carbasugars,[12] *C*-glycosides have the additional advantage of enabling "linkage-editing" by the introduction of substituents on the C-atom, leading to perturbation of the glycan conformation. Here we consider three analogs containing sterically similar CH_2- and CHF-glycosidic linkages (Figure 1A).[13] Focusing on the dihedral angle φ, native *O*-glycosides mainly

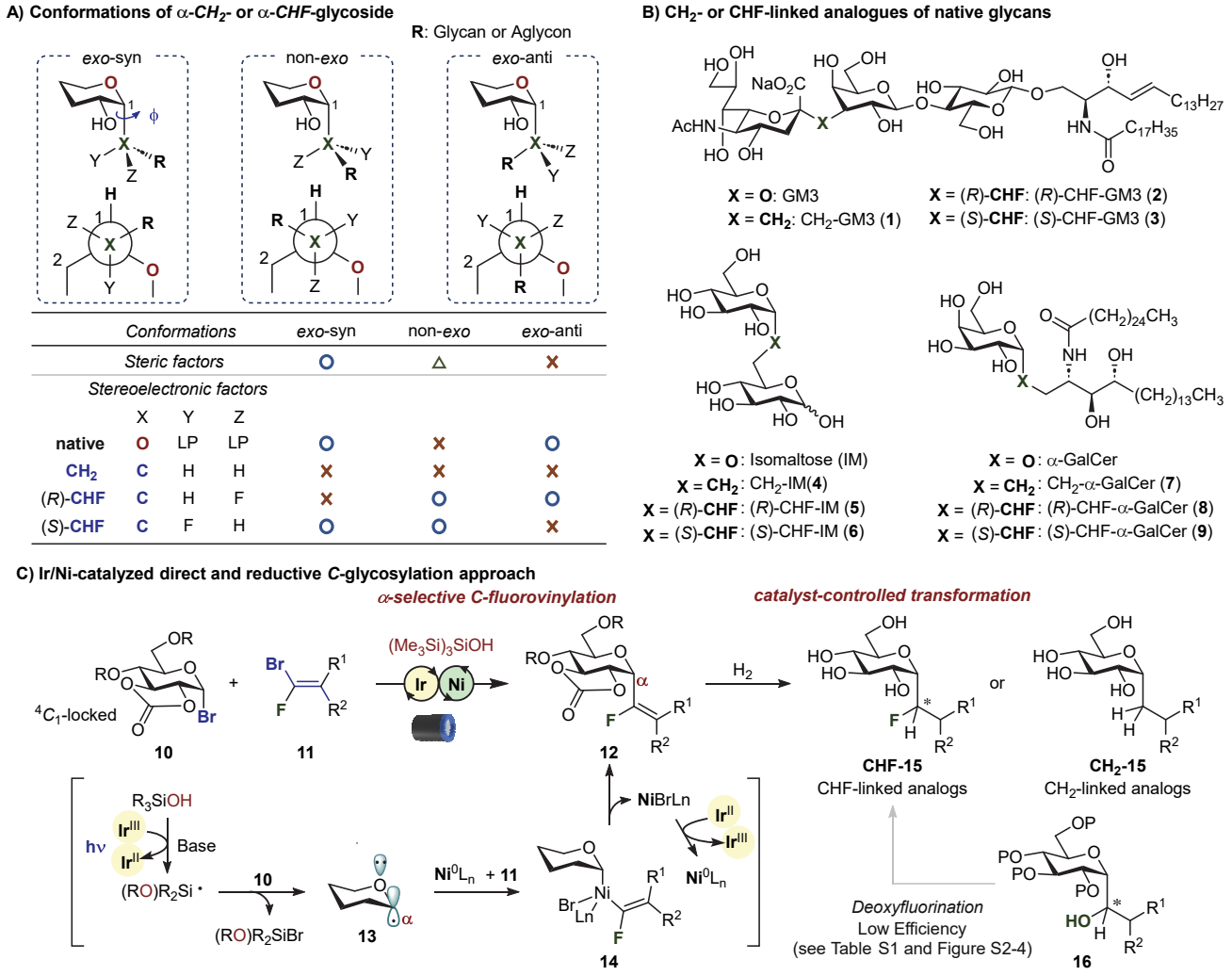


Figure 1. A) Steric and stereoelectronic factors in three staggered conformations of *O*-, *CH₂*- or *CHF*-glycosides. The conformations of these glycans are considered to be regulated by the balance between steric and stereoelectronic factors. LP: lone pair. B) Structures of GM3, isomaltose, and α-GalCer and their analogs with *CH₂*- or *CHF*-glycoside linkages. C) Synthetic strategy for α-*CH₂*- or α-*CHF*-glycoside analogs **15** based on reductive photoredox/Ni catalytic direct fluorovinyl *C*-glycosylation and hydrogenation.

adopt the *exo*-syn conformation due to both steric and *exo*-anomeric effects (Figure 1A), whereas the conformation of CH_2-glycoside analogs lacking the stereoelectronic effects would be more flexible.[5,6] On the other hand, the conformation of the CHF-glycoside analogs would be influenced by the gauche effect of the F atom, and the stereochemistry of the CHF-linkage.[14,15] Importantly, the native *O*-glycoside and the three *C*-glycoside analogs are each expected to have a different "conformational distribution" (Figure S1). We hypothesized that native glycans with *O*-glycosidic linkage are conformationally plastic and that not just a single specific stable conformation is biologically essential, but rather the conformational distribution is the key determinant of overall activity. If this is the case, we expected that the linkage-editing concept would allow us to create pseudo-glycans that might show different biological activities, mainly because of differences in their distributions of molecular conformations. It should be noted that this concept does not exclude the possibility that changes in affinity due to the interaction of the C-glycoside linkage with the target proteins might contribute to the resulting changes in biological phenomena.

We recently validated the above concept at the *O*-glycosidic linkage of Sia-α(2,3)-Gal structure in ganglioside GM3.[16] The synthesized compounds were the first analogs of a native glycoconjugate to have a different type of *C*-glycoside linkage as far as we know, though analogs of an artificial glycan have been developed.[13,15,17] Among them, (*S*)-CHF-linked pseudo-GM3 (**3**, Figure 1B) exhibited enhanced biological activity compared with native GM3 and was more potent than CH_2-linked **1** or (*R*)-CHF-linked **2**. NMR experiments confirmed that the conformational distributions of these CHF-isomers are different. Furthermore, CH_2-linked **1** was less active than native GM3, despite its sialidase-resistant structure. These results supported the idea that the biological activity of glycoconjugates could be modulated by conformational control according to our molecular design concept. However, because the glycosidic linkage in this system also influences the acidity of sialic acid, the activity differences between the CH_2 and CHF-linkages could not be interpreted wholly in terms of

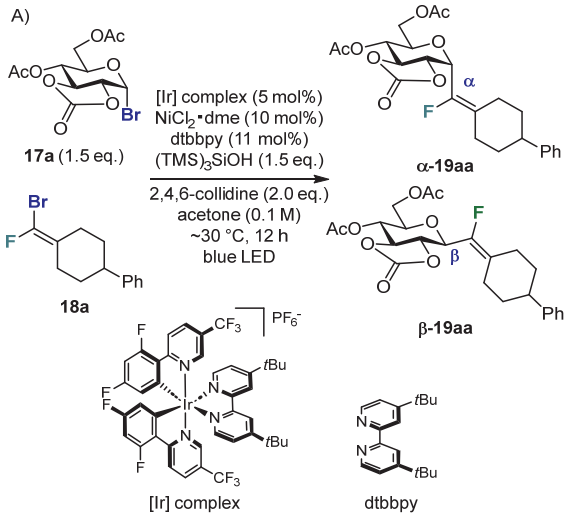
conformational factors. In addition, synthetic methods employed for the preparation of *C*-linked Sia-α(2,3)-Gal were not applicable to common glycan analogs. Herein, we describe an efficient methodology for the synthesis of a variety of pseudo-glycans/glycoconjugates. The activity changes of the newly synthesized C-linked pseudo-isomaltoses (**4**-**6**) and pseudo-α-galactosylceramides (**7**-**9**) were also examined.

Results and Discussion

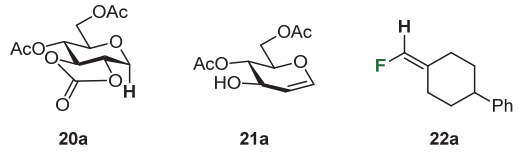
1. Development of α-selective fluorovinyl *C*-glycosylation

Although sophisticated synthetic methodologies, including Pd-catalyzed sp^2-sp^2 cross-coupling for preparation CH_2-linked glycans[18] and transition metal-catalyzed *C*-glycosylation of sp^2-acceptors with chemically stable C1-sp3 hybridized donors (direct C-glycosylation) for preparation of C(O)-linked glycans[19] have been reported, an efficient strategy applicable to both CH_2- and CHF-linked pseudo-glycan/glycoconjugates (**CHF-15** and **CH_2-15**, Figure 1C) is still required. In addition, we have found that the introduction of a fluorine atom at a position next to the anomeric position from the corresponding CH(OH)-glycosides **16** by deoxyfluorination is not always reliable (Table S1 and Figure S2-4),[20] although Walczak's group reported the synthesis of CF_2-linked gentiobiose by deoxy-fluorination of the corresponding C(O)-linked disaccharide.[19]

In the present study, we achieved efficient access to both **CHF-15** and **CH_2-15** by chemo- and stereoselective hydrogenation of the key common intermediate **12** (Figure 1C), which was obtained by direct metallo-photoredox-mediated fluorovinyl α-*C*-glycosylation of the bromofluoroolefin (BFO) acceptor **11** with glycosyl bromides **10**. The feasibility of the reaction with BFO **11** has been confirmed in α-selective coupling with 2-deoxyglycosyl trifluoroborates.[21,22] In contrast to 2-deoxy donors, however, α-selective cross-coupling via anomeric radicals of the standard 2-oxy-donor with the BFO acceptors remains unexplored. To achieve high α-selectivity, we aimed to employ **10**, which is conformationally restricted by the 2,3-carbonate.[23,24] Using **10** rather than the corresponding trifluoroborate is straightforward, but involves cross-electrophile coupling, and thus we decided to employ silanol as a reductant.[25] Catalytically generated silyl radicals formed from the silanol by irradiation in the presence of photo-catalyst and base triggers the formation of anomeric radical **13**[26–28] from **10**.[29] The 2,3-carbonate fixes the conformation of the donor to a chair-like $^4C_1$, so that the spin density of **13** takes α-orientation.[30] Oxidative addition to **11** and capture of **13** by the Ni-complex should afford the Ni(III) complex **14**, and then reductive elimination selectively delivers the α-C-glycoside **12**, along with Ni(I) complex, which can be reduced to Ni(0) by Ir(II). Despite recent reports on direct *C*-vinyl glycosylation,[31–36] our fluorovinyl *C*-glycosylation approach is distinct in that it provides access to three types of pseudo-glycans/glycoconjugates for biological evaluation.



entry	conditions	**19aa** (α:β)	**20a**	**21a**	**22a**
1	As left	84% (8.8/1)	2%	22%	5%
2	Na_2CO_3 i/o collidine	47% (8.4/1)	nd	nd	nd
3	pyridine i/o collidine	10% (9.0/1)	nd	nd	nd
4	2,6-lutidine i/o collidine	56% (8.4/1)	nd	nd	nd
5	$(TMS)_3SiH$ i/o $(TMS)_3SiOH$	50% (8.4/1)	54%	7%	22%
6	wo $NiCl_2$•dme, dtbbpy	0%	109%	0%	28%
7	wo $NiCl_2$•dme, dtbbpy, **17a**	—	—	—	91%



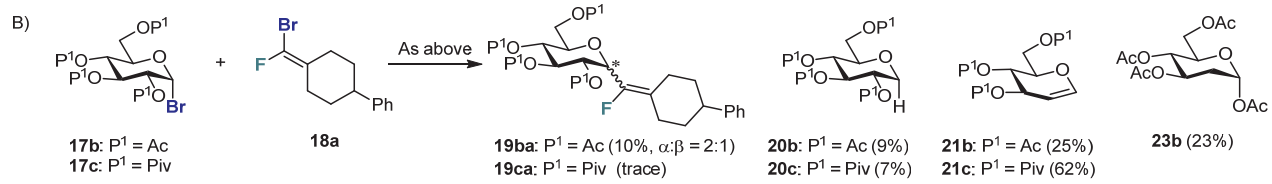


Figure 2. A) Optimization of reaction conditions for the fluorovinyl *C*-glycosylation of **17a** with **18a** (shown as NMR yields based on **18a**); B) Control experiments with acetyl or pivaloyl-protected glucosyl bromides **17b** and **17c**.

We prepared glucosyl bromide donor **17a** with a 2,3-carbonate moiety in 5 steps from commercially available thioglucoside (Figure S5). Tetra-substituted BFO **18a** was selected as a model acceptor and was prepared by Wittig-type olefination[37] (Figure S7). After intensive investigation of the reaction conditions (see Table S2-12), we employed the optimum conditions shown in Figure 2A. The use of 2,4,6-collidine as a base and acetone as a solvent was found to be

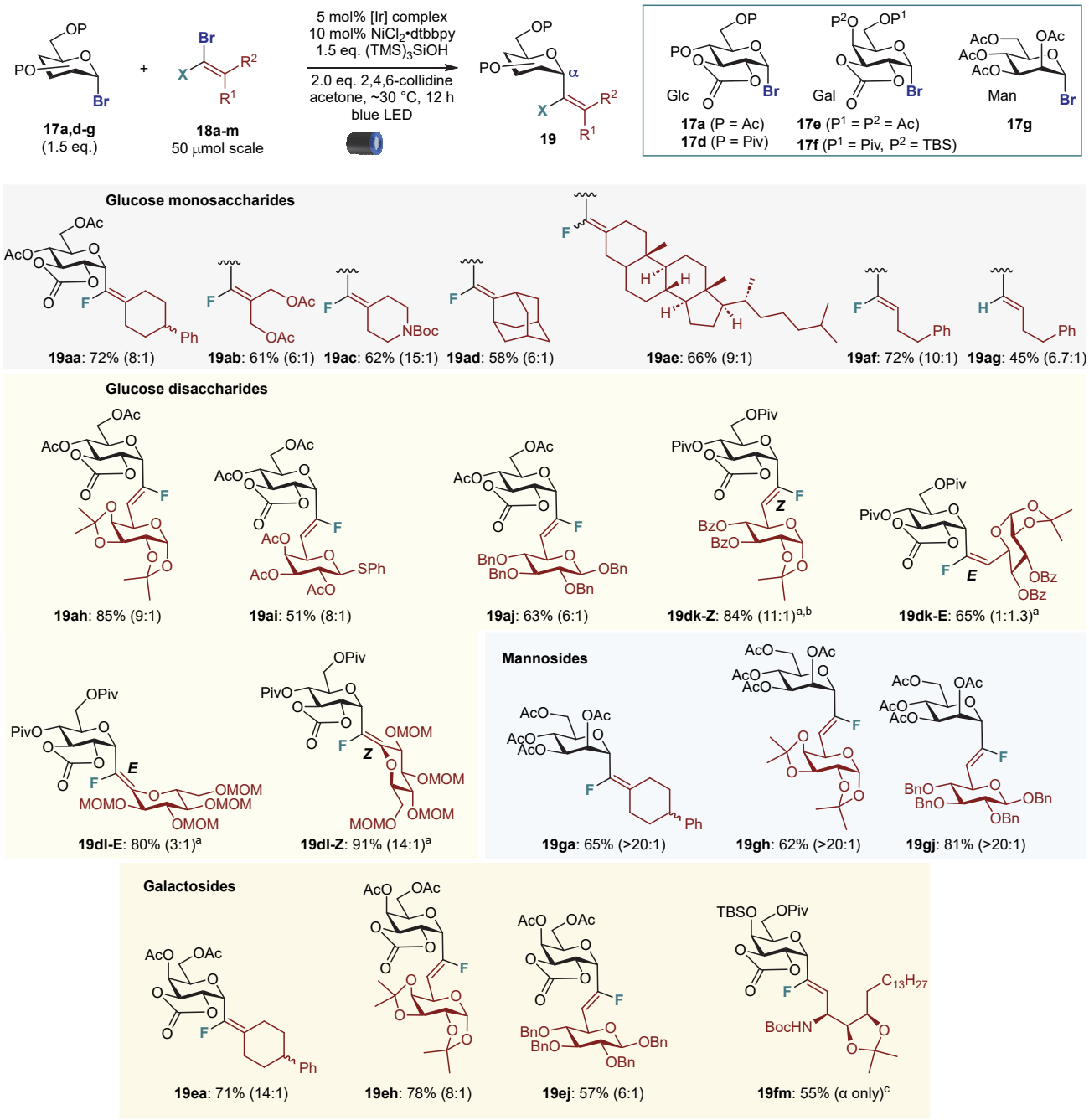


Figure 3. Fluorovinyl *C*-glycosylation using various donors **17a,d-g** and acceptors **18a-m** at 50 μmol scale (isolated yields). [a]Reactions performed with 2.0 equiv **17d** and supersilanol. [b]Reaction performed with Na_2CO_3 instead of 2,4,6-collidine. [c]Reaction perfomed with 1.8 equiv **17f** and supersilanol and with ethylene glycol dimethyl ether (DME) instead of acetone.

critical for the coupling with **18a**, affording **19aa** in a high yield with excellent α-selectivity (entry 1 vs entries 2-4, see Table S3-4). By-products included the reduced product **20a** (2%), glucal derivative **21a** (22%), and fluoroolefin **22a** (5%). $(TMS)_3SiOH$ was a better silyl radical source than $(TMS)_3SiH$,[38] which showed decreased coupling efficiency and generated a greater amount of reduced **20a** (54%, entry 5). Formation of **21a** was not observed in the absence of Ni complex, indicating that β-elimination of the intermediary anomeric Ni-complex takes place, as with **14** (entry 6). In contrast, the formation of fluoroolefin **22a** did not involve the Ni-complex, because irradiation of a mixture of **18a**, Ir complex, and $(TMS)_3SiOH$ afforded **22a** in 91% yield (entry 7). The 2,3-carbonate was also significant for both coupling efficiency and stereoselectivity. Namely, the reaction of acetyl-protected **17b** gave the desired product **19ba** (10%, α:β = 2:1) along with C-1 reduced **20b** (9%), glucal **21b** (25%), and acetyl-migrated[39,40] **23b** (23%). Only a trace amount of **19ca** was formed from pivaloyl-protected **17c** (Figure 2B). These experimental results also suggested that the steric factor of

O2 functionality would contribute to the reaction efficiency and high α-selectivity.

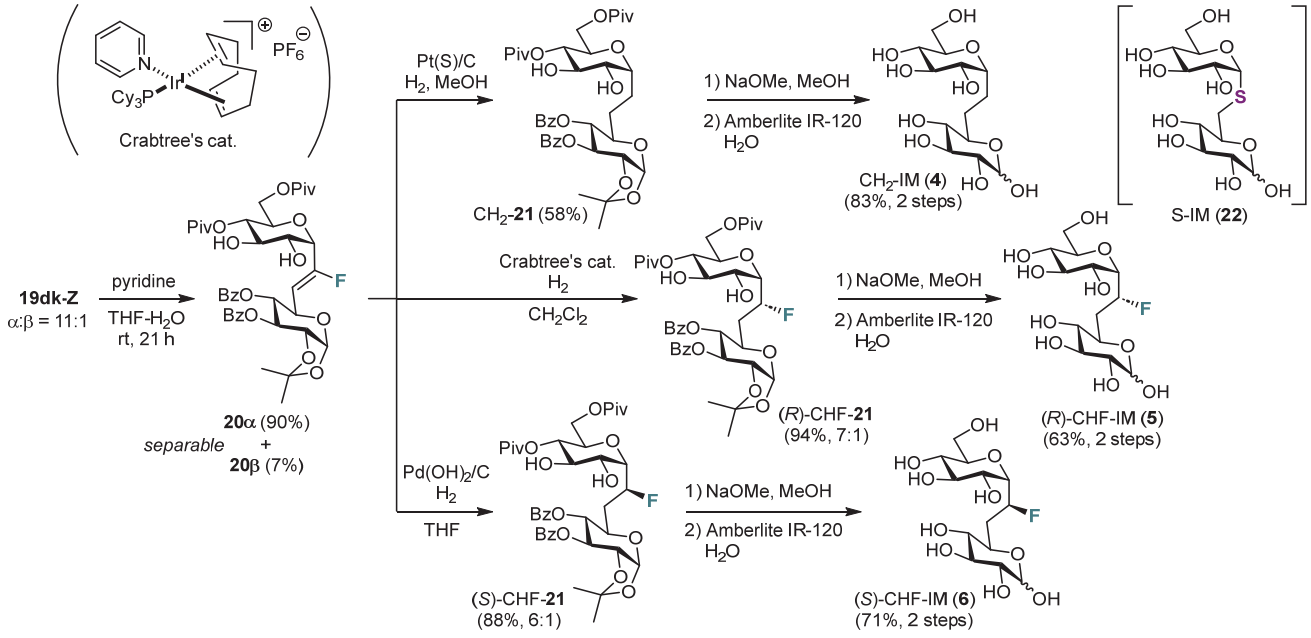
Various BFO acceptors **18** were applicable for the coupling reactions (Figure 3), although the optimum base was dependent on the acceptor (see Table S2 and S3). Coupling reaction of **17a** with **18a** gave **19aa** in 72% isolated yield (α:β = 8:1). Owing to the mild reaction conditions, base-sensitive cyclic carbonate (in **17**) or acetate (in **18b**) and acid-sensitive *N*-Boc (in **18c**) were tolerated, giving the corresponding fluorovinyl *C*-glycosides **19ab** and **19ac** in reasonable yields. Bulky adamantane-based BFO gave **19ad** with slightly lower α-selectivity, whereas glucosyl cholestane derivative **19ae** was obtained with high α-selectivity. Coupling with the trisubstituted BFO (*E*)-**18f** also proceeded smoothly to afford **19af** in a highly α-selective manner in 72% yield. The reactivity of the non-fluorinated (*E*)-bromoolefin **18g** was lower than that of the fluorinated (*E*)-**18f**, and **19ag** was obtained in 45% yield. It is noteworthy that the failure to obtain BFO **18c**, **18d**, and **18e** by Wittig-type reaction was overcome by use of the Julia-Kocienski reaction with the known sulfone[41] (Figure S8).

Next, coupling of **17a** with galactose-type BFO **18h** or **18i** afforded Glc-α(1,6)-Gal **19ah** or **19ai**, in which (*E*)-**18h** bearing dimethylene acetal showed better reactivity and the thio-glycoside in **18i** was tolerated. Glucose-type BFO (*E*)-**18j** with five benzyl groups resulted in the formation of Glc-α(1,6)-Glc **19aj** with slightly lower yield and stereoselectivity. We then prepared **17d** to block the β-face with the bulky pivaloyl group on O6 and obtained **19dk-Z** with higher yield and α-selectivity. The protecting group of (*E*)-**18k** was changed from that in (*E*)-**18j** for the latter process, which might also have contributed to the improvement of the reactivity. Despite the feasibility of the coupling with tetra-substituted BFO, the isomer of tri-subsituted BFO (*Z*)-**18k** was found to be an inappropriate acceptor, affording the corresponding **19dk-E** with only low stereoselectivity. It should be noted that only slight isomerization of the olefin geometry was observed, and thus the obtained α-isomer of **19dk-E** has the (*E*)-fluoroolefin. Not only analogs of 1,6-linked disaccaharide structures, but also other disaccharides could be constructed with this strategy. As a representative example, we here disclose the successful synthesis of *C*-linked Glc-α(1,1)-Glc structure. During the preparation of the BFO acceptor, we established the selective synthesis of (*E*)- or (*Z*)-BFO **18l** from the methoxylmethyl-protected gluconolactone derivative by Wittig-type or Julia-Kocienski-type reaction (Figure S7 and S8). Coupling reaction with **17d** afforded *C*-glycosides **19dl-E** and **19dl-Z** with moderate or high α-selectivity in good yields.

For galactose-type donor **17e** with 2,3-cyclic carbonate (Figure S6), coupling under optimum conditions gave monosaccharide **19ea** as well as disaccharides **19eh** and **19ej** in a highly α-selective manner. Conformational restriction by the cyclic carbonate was not necessary for the mannose-type donor, and the coupling of **17g** with the same acceptors provided the mono- or di-saccharides (**19ga**, **19gh**, and **19gj**) α-exclusively. These results indicated that the fluorovinyl α-*C*-glycosylation methodology can provide α-linked pseudo-glycans with various linkage modes.

2. Linkage-editing pseudo-Isomaltose: Enhanced and Disappeared biological activity

Next, pseudo-glycans with three different C-glycoside linkages were synthesized from the coupling products. The first target was an analog of one of the simplest glycans, isomaltose (IM). Basic hydrolysis of the carbonate group of the coupling product **19dk-E** with Glc-α(1,6)-Glc structure provided diol **20**, and a small amount of the β-isomer **20β**, which was easily separated at this stage (Scheme 1). Divergent transformation from **20α** to CH_2- or CHF-linked disaccharides **21** was achieved under a hydrogen atmosphere with appropriate catalysts. Namely, hydrogenation with $Pd(OH)_2/C$ selectively afforded (*S*)-CHF-**21**, while the reaction with Crabtree's catalyst produced (*R*)-CHF-**21** stereoselectively. The 2,3-hydroxy groups as a directing group in the reaction with Crabtree's



Scheme 1. Synthesis of C-linked isomaltose analogs **4**~**6** (pseudo-IM) and the structure of S-IM (**22**).

catalyst might be involved in switching the face-selectivity of hydrogenation. Determination of the stereochemistry was performed by means of X-ray crystallographic analysis, chemical transformations, and NMR analysis (Figure S10-13). Easy separation of the CHF-isomers suggests that the stereochemistry of the CHF group influences the chemical properties. Moreover, treatment with sulfur-poisoned Pt/C enabled hydrogenolysis of the fluoroalkene as well as hydrogenation of the olefin to give CH_2-**21** selectively. These results demonstrate the validity of fluorovinyl *C*-glycosylation methodology for pseudo-glycan synthesis. Stepwise removal of all protecting groups completed the synthesis of three pseudo-IMs (**4**-**6**). This is the first example of the synthesis of CHF-IMs **5** and **6**.

Native IM acts as an inducer of amylase production in *Aspergillus nidulans* (Figure 4A).[42] Glycoside hydrolases (GH) in *A. nidulans* synthesize IM by trans-glucosylation of maltose and are also involved in the degradation of IM. The mechanisms of gene expression of plant polysaccharide-degrading enzymes such as amylases or cellulases have been extensively studied in fungi,[43,44] but little is known about how signaling molecules such as IM activate pathway-specific transcription factors, owing to degradation of the signaling molecule itself. Thus, unhydrolyzable pseudo-IM, if it retains the physiological inducer function, would be a powerful tool for unveiling the regulatory mechanism of amylase production in microorganisms.

We found that amylase production was induced by exogenous addition of IM, but rapidly plateaued, probably because of the degradation of IM (Figure 4B). In contrast, CH_2-IM (**4**) induced prolonged amylase production, eventually showing a greater production ability than IM (Figure 4B), and thereby highlighting the potential value of glycoside hydrolase-resistant *C*-glycosides. Sulfur-linked isomaltose S-IM (**22**, Scheme 1) was completely inactive, probably owing to the difference of bond lengths and angles between *O*-glycoside and *S*-glycoside (Figure 4C and S18). This again emphasizes the utility of the *C*-glycoside linkage. Surprisingly, (*R*)- or (*S*)-CHF-IM (**5** and **6**) showed negligible amylase production ability, despite the single substitution of one H-atom to an F-atom. This might be due to the conformation-changing effect of the F-atom. Conformational flexibility of the glycosidic linkage in IM may be important for the biological actions of IM, including its membrane transportation and interaction with unidentified target protein(s).

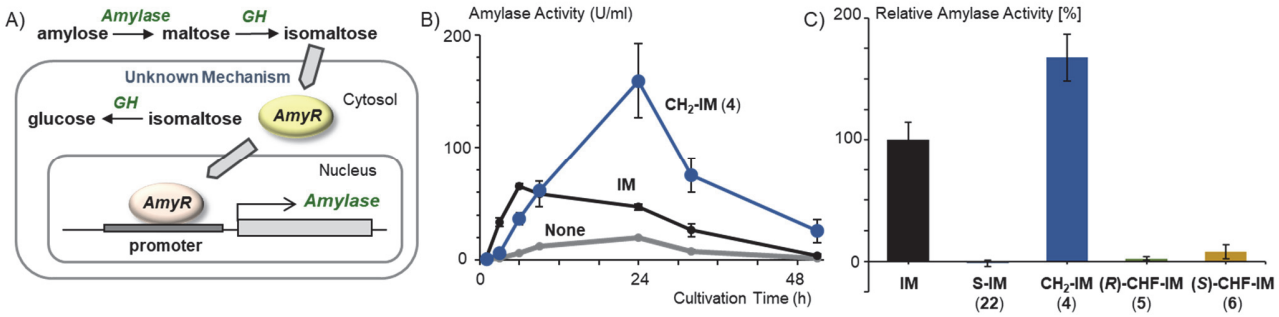


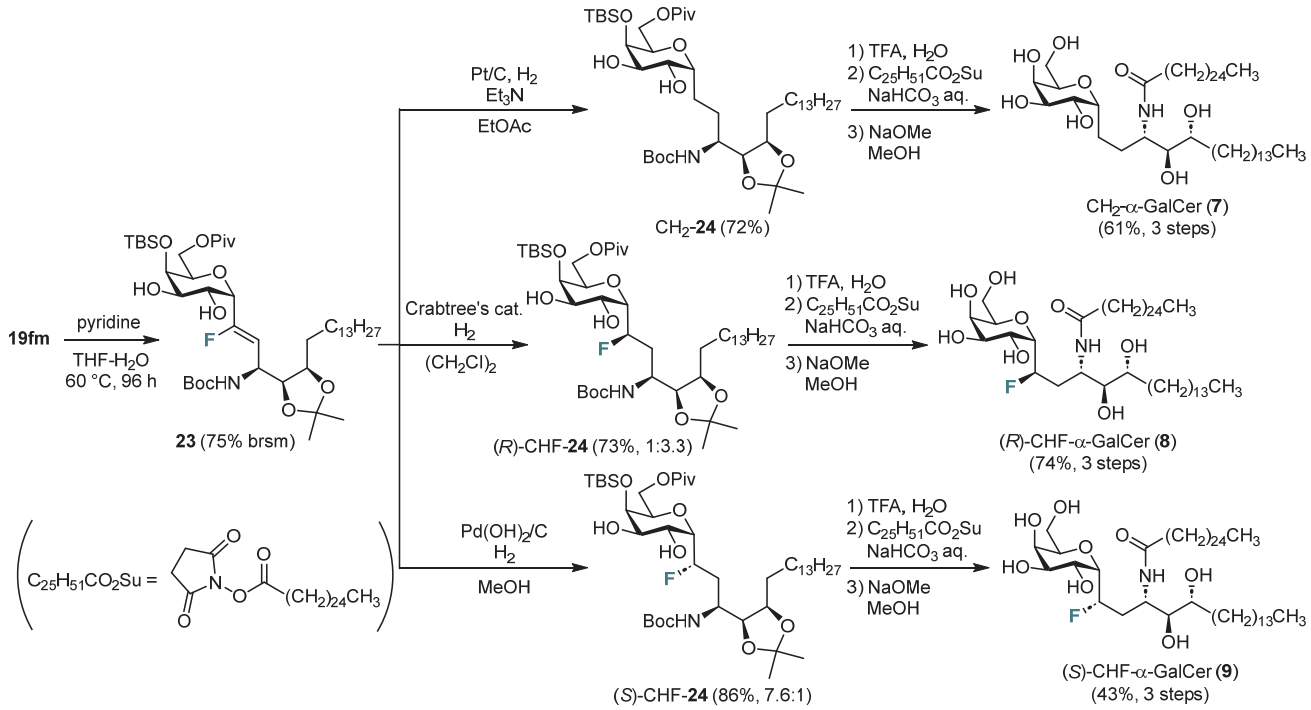
Figure 4. A) Schematic representation of the mechanism of induction of amylase mechanism by isomaltose (IM). GH: glycoside hydrolases (α-glucosidases). B) Time-dependent induction of amylase activity by isomaltose (IM) and CH_2-IM (**4**). C) Relative amylase activity at 24 hours after treatment with 10 μM IM, S-IM (**22**), CH_2-IM (**4**), (*R*)-CHF-IM (**5**), and (*S*)-CHF-IM (**6**).

3. Linkage-editing pseudo-α-galactosylceramide: Altered from an agonist to an antagonist

The glycolipid α-galactosylceramide[45] (α-GalCer, Figure 1B) is an immune stimulant that serves as an antigen presented on CD1d to activate invariant natural killer T (iNKT) cells through recoginition by T-cell receptors. The activated iNKT cells secrete Th1 (e.g. IFN-γ), Th2 (IL-4), and Th17 (IL-17) cytokines (Figure 5A),[46] leading to anti-tumor effects or immune-stimulating responses useful for vaccine adjuvants.[47] Franck and co-workers have previously synthesized the corresponding CH_2-α-GalCer (**7**) and found that it exhibited more potent antitumor and antimalarial activities than native α-GalCer *in vivo* and induced production of IFN-γ rather than Th-2 cytokines,[24,48–56] demonstrating the biological potential of *C*-glycoside analogs. Recently, (*R*)-CHF-α-GalCer (**8**) was reported to induce IL-17 in mouse serum,[57] though experimental details for the synthesis of **8** from the reported intermediate[55] were not disclosed. Here, we decided to synthesize the three C-linked α-GalCer analogs **7**∼**9** and compare their biological activities under the same conditions in order to further validate the linkage-editing strategy.

Our synthetic methodology enabled the synthesis of analogs **7**∼**9** from the same intermediate **23**, which was prepared from **19fm** (the coupling product of galactose donor **17f** and BFO **18m**, Figure 3, S6, S7, and S9, Table S9) by hydrolysis of the carbonate group (Scheme 2). Hydrogenation of **22** with Pt/C in the presence of Et_3N or $Pd(OH)_2$/C selectively afforded CH_2-**24** or (*S*)-CHF-**24**, respectively. Selective formation of (*R*)-CHF-**24** was difficult because of the substrate-controlled face-selectivity owing to the neighboring NHBoc group, but it was obtained as a minor product by hydrogenation with Crabtree's catalyst. The stereochemistry of (*R*)- and (*S*)-CHF-**24** was determined by $^3J_{H\text{-}F}$ and HOESY measurements (Figure S15-17). Selective removal of TBS, acetonide, and the Boc group, *N*-acylation of the resulting amine, and removal of the remaining pivaloyl group completed the synthesis of **7**∼**9** in reasonable yields.

The immunostimulating activity of C-linked α-GalCer analogs **7**∼**9** was evaluated in terms of adjuvant activity in mice. Mice



Scheme 2. Synthesis of C-linked α-GalCer analogs **7~9** (pseudo-α-GalCer).

were injected intraperitoneally with the antigen protein ovalbumin (OVA) and α-GalCer or C-linked α-GalCer as an adjuvant (2 μg, at day 0 and day 7), and then OVA-specific antibodies in serum were measured by ELISA at day 14. Injection of OVA with α-GalCer clearly induced OVA-specific IgG (IgG1) and IgM production compared to the levels without α-GalCer, demonstrating the adjuvant activity of α-GalCer (Figure 5B,and S19). Surprisingly, the use of C-linked **7**~**9** instead of α-GalCer diminished the generation of OVA-specific antibodies, and the adjuvant activity of CHF-linked **8** and **9** was lower than that of CH_2-linked **7** (Figure 5B). To examine the reason for this, splenocytes from C57BL6J mice were stimulated with these compounds in vitro for 48 hours and cytokine production was measured. As reported, production of IFN-γ, TNF, IL-4, IL-6 IL-10, and IL-17A was induced by native α-GalCer, while CH_2-linked **7** showed a weaker inducing activity (Figure 5C and S20). In contrast, production of these cytokines was negligible in the splenocytes treated with CHF-linked **8** or **9** (Figure 5C and S20). It should be noted that Ma and coworkers detected IL-17 in the serum of mice treated directly with (*R*)-CHF-linked **8**,[57] so the production of IL-17 might require mouse factors not present in splenocytes alone.

CH_2-Linked α-GalCer (**7**) is presented to CD1d and forms a complex with the TCR, despite having lower stability than the complex of native α-GalCer.[58–60] This suggests that CH_2-linked α-GalCer **7** is a weak activator of iNKT cells, which could explain the low cytokine production observed in the presence of **7**. On the other hand, the F-atom-containing compounds **8** and **9** did not induce cytokine production, suggesting that they induce little or no iNKT cell activation through the TCR complex, even when presented on CD1d. To test this hypothesis, we performed iNKT cell activation *in vitro* using the NFAT-GFP reporter gene assay.[61] While significant GFP expression by native α-GalCer was observed, indicating TCR-mediated iNKT cell activation (Figure 5D and S21), almost no GFP expression was observed in the presence of either C-linked α-GalCer in this system. Although CH_2-linked **7**[58,59] and (*R*)-CHF-linked **8**[57] have been shown to be presented to CD1d, these results suggest that the activation of iNKT cells via the TCR complex by C-linked α-GalCer is weak.

Thus, we next used NFAT-GFP reporter cells expressing CD1d and iNKT TCR to examine whether C-linked α-GalCer could suppress the activation of iNTK cells by native α-GalCer. Interestingly, while CH_2-linked **7** showed weak inhibitory activity, remarkable inhibition was observed by (*S*)-CHF-α-GalCer **9** and, even more significantly, by (*R*)-CHF-α-GalCer **8** (Figure 5E and S22). These results indicate that CHF-linked **8** and **9** act as antagonists of CD1d-TCR-mediated iNKT cell activation.[62] In other words, editing the *O*-glycoside bond of α-GalCer to CHF-glycoside alters the function of α-GalCer from an activator to an inactivator of iNKT cell activation. This is consistent with the idea that differences in the glycoside linkage and conformational distribution can induce dramatic changes in glycoconjugate functions.

Conclusion

We describe a robust divergent synthesis of C-linked glycans or glycolipids based on fluorovinyl *C*-glycosylation. Our evaluation of the synthesized compounds confirms the potential of the linkage-editing strategy for creating pseudo-glycans with enhanced or altered biological functions. We would particularly like to emphasize that pseudo-glycans generated by our strategy are only slightly linkage-modified versions of natural glycans, but nevertheless, their biological functions are drastically changed. These results support the potential value of glycoside hydrolase-resistant analogs and also suggest the importance of strict higher-order conformational

control even for flexible glycan structures. Our findings also raise the possibility that new bioactive molecules may be found by focusing on minor conformational structures of glycans. Further exploration of this strategy with other glycans and glycoconjugates is underway in this laboratory.

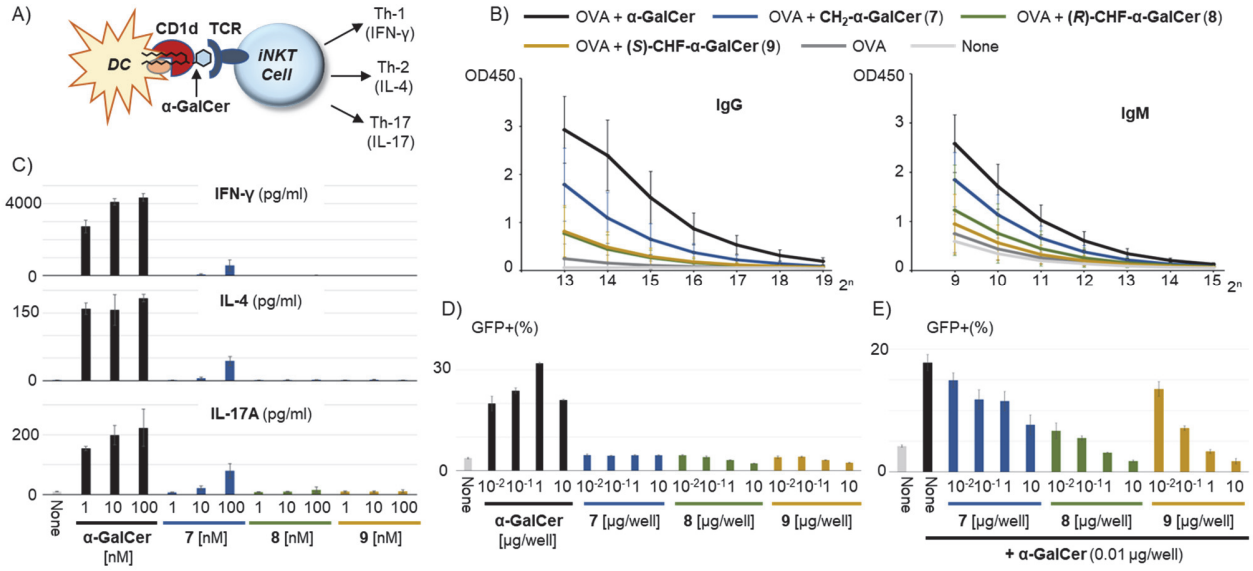


Figure 5. A) Schematic representation of iNKT cell activation by α-GalCer through CD1d. TCR: T cell receptors, DC: dendritic cells. B) Evaluation of adjuvant activity of α-GalCer, CH_2-α-GalCer (**7**), (*R*)-CHF-α-GalCer (**8**), and (*S*)-CHF-α-GalCer (**9**) by measuring OVA-specific IgG and IgM production in mice (n = 3x2/group). C) *In vitro* production of representative cytokines (IFN-γ, IL-4, and IL-17A) by splenocytes at 48 hours after treatment with 1-100 nM α-GalCer, CH_2-α-GalCer (**7**), (*R*)-CHF-α-GalCer (**8**), and (*S*)-CHF-α-GalCer (**9**). D) Activation of NFAT-GFP reporter cells expressing CD1d + mouse iNKT TCR induced by 0.01-10 μg/well of α-GalCer, CH_2-α-GalCer (**7**), (*R*)-CHF-α-GalCer (**8**), and (*S*)-CHF-α-GalCer (**9**), monitored in terms of GFP expression. E) Inhibition of the iNKT TCR activation induced by 0.01 μg/well α-GalCer in the presence of 0.01-10 μg/well CH_2-α-GalCer (**7**), (*R*)-CHF-α-GalCer (**8**), and (*S*)-CHF-α-GalCer (**9**), monitored in terms of GFP expression by NFAT-GFP reporter cells expressing CD1d + iNKT TCR.

Experimental Section

Evaluation of amylase induction by pseudo-glycans

Aspergillus nidulans ABTA16 (*pyrG89 biA1 wA3*, *pyroA4*, *argB*::*taaG2*)[42] was cultured at 37 °C for 18 hours in Aspergillus minimum medium with appropriate supplements and 2% (w/v) Bacto Peptone as a carbon source. The mycelia were collected by filtration, washed with the minimum medium lacking a carbon source, and suspended at about 1 g wet-weight of mycelia per 100 ml of fresh minimum medium containing 2% Bacto Peptone. For time-course experiments, 10-ml aliquots of the suspension were transferred to 50-ml conical tubes, and IM and pseudo-IMs were added to give the final concentration of 10 μM. The mycelial suspensions were further cultured at 37 °C on a rotary shaker at 200 rpm. The culture supernatants were collected at 1, 3, 6, 9, 24, 32, and 51 h after induction, dialyzed using a PD Spintrap G-25 (Cytiva) to remove the inducer, and assayed for α-amylase activity. For dose-response experiments, 1-ml aliquots of the suspensions were transferred to 96 deep-well plates, and the inducer was added at a concentration in the range of 0.01-250 μM. The mycelial suspensions were further cultured for 24 hours at 37 °C on a rotary shaker at 200 rpm. The culture supernatants were collected, dialyzed, and assayed. α-Amylase activity of the cultures was measured using an α-amylase assay kit (Kikkoman Biochemifa, Tokyo, Japan).

Mice

Female C57BL/6 mice (age, 8 weeks) were purchased from CLEA Japan (Tokyo, Japan) and kept for at least 1 week before the experiments in a specific pathogen-free environment at the National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN; Osaka, Japan). All animal experiments were conducted in accordance with the Animal Care and Use Committee guidelines of the NIBIOHN.

Immunization and ELISA

Mice were intraperitoneally injected with 5 μg of OVA (Sigma-Aldrich) in PBS with 2 μg of α-GalCer, CH_2-α-GalCer (**7**), (*R*)-CHF-α-GalCer (**8**), or (*S*)-CHF-α-GalCer (**9**) on days 0 and 7. 0.5% (v/v) ethanol in PBS was utilized as the vehicle control. On day 14, serum was collected for measurement of OVA-specific antibodies by ELISA as previously reported.[63] Briefly, 96-well immunoplates (Thermo Fisher Scientific Inc.) were coated with 1 mg/mL OVA in PBS for 16 hours at 4°C, then blocked with 1% (w/v) bovine serum albumin (BSA; Nacalai Tesque, Inc.) in PBS for 2 hours at room temperature, and washed three times with PBS containing 0.05% (v/v) Tween 20 (Nacalai Tesque, Inc). Serum samples were added to each well and incubated for 2 hours at room temperature. After another round of washing, horseradish peroxidase-conjugated anti-mouse IgG, IgG1, or IgM antibodies (SouthernBiotech) in PBS containing 1% (w/v) BSA and 0.05% (v/v) Tween 20 were added to the wells, and the plates were incubated for 1 hour at room temperature. The OVA-specific antibodies were detected by adding 3,3',5,5'-

tetramethylbenzidine peroxidase substrate (SouthernBiotech). The reaction was stopped by adding 0.5 M HCl to the wells, and the absorbance at 450 nm (OD_{450}) was measured using an iMark Microplate Absorbance Reader (Bio-Rad).

Cytokine secretion assay using mouse splenocytes

Cytokine secretion assay was conducted as described previously with some modification.[64] Briefly, splenocytes of C57BL/6J mice was prepared with cell strainers (size; 100 μm). The resulting suspensions were treated with 1 mL of red blood cell lysis buffer for 1 minute at room temperature. The splenocytes were diluted in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific), 1 mM sodium pyruvate solution (Nacalai Tesque), 1% penicillin–streptomycin mixed solution (Nacalai Tesque), and 0.1% 2-mercaptoethanol (Gibco, Thermo Fisher Scientific), and seeded into a 96-well plate (6 x 10^5 cells/well). Splenocytes were cultured with CH_2-α-GalCer (**7**), (*R*)-CHF-α-GalCer (**8**), and (*S*)-CHF-α-GalCer (**9**) at the indicated concentration for 48 hours at 37 °C in 5% CO_2. 0.5% (v/v) ethanol in the medium was utilized as the vehicle control. After incubation, the culture supernatant was collected for cytokine measurement. Cytokines were analyzed with a BD™ Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences) according to the manufacturer's protocol, followed by analysis with a MACSQuant Analyzer (Miltenyi Biotec).

Stimulation of NFAT-GFP reporter cells expressing T cell antigen receptor of iNKT cells with α-GalCer analogs

Mouse CD1d, TCRα and TCRβ chains of mouse iNKT T cell antigen receptor (TCR) (clone: DN32.D3) were cloned into retroviral vectors and transduced into mouse T cell hybridoma with an NFAT-GFP reporter gene.[65] α-GalCer, CH_2-α-GalCer (**7**), (*R*)-CHF-α-GalCer (**8**), and (*S*)-CHF-α-GalCer (**9**) were diluted in isopropanol and 20 μl aliquots of diluents containing 0.01-10 μg of glycolipids were added to each well of a 96-well flat-bottomed plate. After complete evaporation of the solvent, NFAT-GFP reporter cells were added and stimulated for 20 h. Propidium iodide was added at 2 μg/ml and the expression of GFP was analyzed using an Attune NxT flow cytometer (Thermo Fisher Scientific) and FlowJo software (BD Biosciences). For inhibition assay, NFAT-GFP reporter cells were stimulated with α-GalCer (0.01 μg per well) in the presence of 0.01-10 μg per well of CH_2-α-GalCer (**7**), (*R*)-CHF-α-GalCer (**8**), and (*S*)-CHF-α-GalCer (**9**) for 20 h. Induction of NFAT-GFP was analyzed as described above. All data are presented as mean ± SD of triplicate assays.

ASSOCIATED CONTENT

Supporting information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.xxxxx.

Figures S1-S22, Tables S1-S12, Experimental procedures and characterization data for all new compounds (PDF)

Accession Codes

CCDC2285079 contains the supplementary crystallographic data for this paper. This data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre,12 Union Road,Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

AUTHOR INFORMATION

Corresponding Author

Go Hirai – Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan; RIKEN Center for Sustainable Resource Science, 2-1, Hirosawa, Wako, Saitama 351-0198, Japan. orcid.org/0000-0003-3420-555X, e-mail: gohirai@phar.kyushu-u.ac.jp

Naoki Kato – Faculty of Agriculture, Setsunan University, 45-1 Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan; RIKEN Center for Sustainable Resource Science, 2-1, Hirosawa, Wako, Saitama 351-0198, Japan, orcid.org/0000-0003-4410-4090, e-mail: naoki.kato@setsunan.ac.jp

Authors

§ T.M. and M.Y. contributed equally to this work.

Takahiro Moriyama – Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Makoto Yoritate – Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Azusa Saika – Microbial Research Center for Health and Medicine, National Institutes of Biomedical Innovation, Health and Nutrition, Osaka 567-0085, Japan; Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, 11 Biopolis Way, Helios, Singapore 138667, Singapore

Wakana Kusuhara – Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan

Shunsuke Ono – Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Takahiro Nagatake – Microbial Research Center for Health and Medicine, National Institutes of Biomedical Innovation, Health and Nutrition, Osaka 567-0085, Japan; Department of Life Sciences, School of Agriculture, Meiji University, Kanagawa 214-8571, Japan

Hiroyuki Koshino – RIKEN Center for Sustainable Resource Science, Saitama 351-0198, Japan

Noriaki Kiya – Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Natsuho Moritsuka – Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Riko Tanabe – Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Yu Hidaka – Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Kazuteru Usui – Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Suzuka Chiba – Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Noyuri Kudo – Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Rintaro Nakahashi – Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Kazunobu Igawa – Institute for Materials Chemistry and Engineering, IRCCS, Kyushu University, Fukuoka 816-8580, Japan

Hiroaki Matoba – Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Katsuhiko Tomooka – Institute for Materials Chemistry and Engineering, IRCCS, Kyushu University, Fukuoka 816-8580, Japan

Eri Ishikawa – Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; ; Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan

Shunji Takahashi – RIKEN Center for Sustainable Resource Science, Saitama 351-0198, Japan

Jun Kunisawa – Microbial Research Center for Health and Medicine, National Institutes of Biomedical Innovation, Health and Nutrition, Osaka 567-0085, Japan

Sho Yamasaki – Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; ; Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan

Notes

Any additional relevant notes should be placed here.

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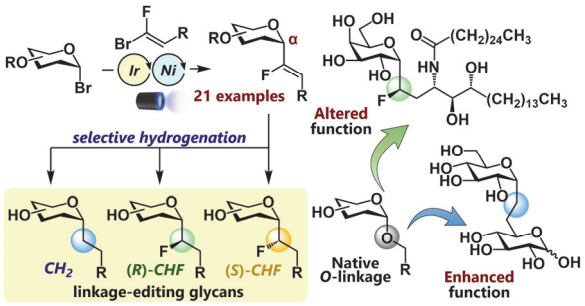
Ir
Ni
21 examples
selective hydrogenation
CH2
(R)-CHF
(S)-CHF
linkage-editing glycans
Altered function
Native O-linkage
Enhanced function