Synthesis of 1-Deoxy-1-hydroxymethyl- and 1-Deoxy-1-*epi*-hydroxymethyl Castanospermine as New Potential Immunomodulating Agents

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Two new C-1 epimeric hydroxymethyl castanospermine congeners **2a** and **2b**, synthesized by stereocontrolled intramolecular double reductive amination of D-glucose derived β-keto ester as a key step, showed impressive immuno-potentiating property. The bioactivity was mediated through up-regulation of T_{H1}/T_{H2} cytokine ratio. The finding suggested that immunmodulatory activity of polyhydroxylated indolizidine alkaloids can be tuned by minor structural/stereochemical alterations.

Introduction

Polyhydroxylated five-, six-, and seven-membered nitrogen heterocyclic compounds as well as bicyclic pyrrolizidine, indolizidine, and quinolizidine alkaloids, commonly called as iminosugars, are known to be promising glycosidase inhibitors.[1] Some iminosugars that are inhibitors of glycosidases and glycosyltransferases have shown promising chemotherapeutic applications against diabetes,[2] cancer,[3] Gaucher's disease,[4] and viral infections, including AIDS,[5] hepatitis C,[6] and HSV-1.[7] In addition, iminosugars also exhibit immonomodulatory property. In this respect, the immunosuppressive activity of indolizidine alkaloids such as castanospermine **1**, swainsonine **3**, and kifunensine **4** (Figure 1) has been reported.[8] More recently, synthetic analogues of nojirimycin (six-membered iminosugar) were also found to act as potential immunosuppressive agents.[9] Thus, applications of iminosugars as immunomodulators is an emerging area in drug development, however, it has received limited attention. Among iminosugars, castanospermine **1** has gained a lot of interest as it is a potent inhibitor of various glucosidases including lysomal α-glucosidase,[10] α- and β-glucosidase in fibroplast extracts,[10] the glycoprotein processing enzyme glucosidase I,[11] as well as a powerful inhibitor of β-xylosidase[10] and sucrase.[12] It was found that structural modifications in iminosugars induce significant changes in their biological activity, their potency, or their specificity as inhibitors of glycosidases[13] as well as immunomodulators.[9] As a part of our continuing interest in the synthesis and biological evaluation of iminosugars,[14] we report herewith the synthesis of hitherto unknown 1-deoxy-1-hydroxymethyl-castanospermine **2a** and 1-deoxy-1-epi-hydroxymethyl-castanospermine **2b**, using D-glucose-derived β-keto ester **5** as a substrate, and the study of their glycosidase inhibitory and immunomodulating activity. Our results provide a lead for further elucidation of the structure−activity relationship based on the hydroxymethyl substitution pattern, instead of hydroxyl substituent at C1, of castanosoermine on the biological activities.

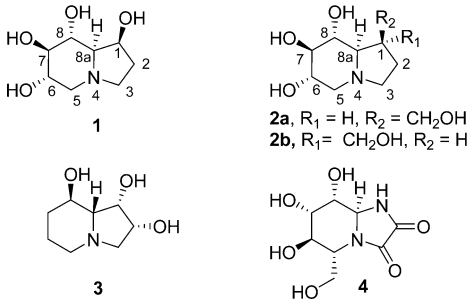

Figure 1. Iminosugar analogues.

Results and Discussion

Chemistry: As shown in Scheme 1, D-glucose was converted to the β-ketoester **5** as reported by us earlier.[15] Reaction of **5** with allyl bromide in the presence of sodium ethoxide at 60 °C afforded an inseparable C6-epimeric mixture of the allylated product **6** in 70% yield. Ozonolysis of **6** in dichloromethane at −78 °C afforded 6-carbethoxy-α-D-xylo-oct-5-ulo-1,8-dialdose as a C6-epimeric mixture (as evident from the ^{1}H NMR spectrum of the column purified product), which was immediately subjected to double reductive amino-cyclization with benzylamine and $NaBH_3CN$ to afford pyrrolidines **7a** and **7b** in the 1:2 ratio. In the ^{1}H NMR spectrum of **7a**, the H5 appeared at 3.67 δ as a triplet with $J_{5,6}$ = 9.4 Hz, while in **7b**, the H5 showed doublet of doublet at 3.66 δ with $J_{5,6}$ = 2.5 Hz, indicating *cis*- and *trans*-relative stereochemistry between the H5 and H6, respectively. However, this did not allow us to assign the absolute configuration at the newly generated stereocenters at C5 and C6 in **7a**,**b**. Fortunately, compound **7a** was obtained as a white solid, and the X-ray crystallographic data (Figure 2) showed the absolute configurations as (5*R*) and (6*S*).

The chemical shift values of the H5 in **7a** and **7b** were found to be nearly identical, therefore, we assumed the same D-gluco configuration in **7b**, and as the H5 and H6 were found to have *trans*-relative stereochemistry ($J_{5,6}$ = 2.5 Hz), the absolute configurations in **7b** were tentatively assigned as 5*R*,6*R*. The stereochemical assignment in **7b** was, however, confirmed by transforming it to the target molecule **2b** and its X-ray data (vide supra). The formation of the pyrrolidine ring skeleton in **7a** and **7b** with exclusive 5*R* configuration is interesting (the same configuration decides the stereochemical outcome at the

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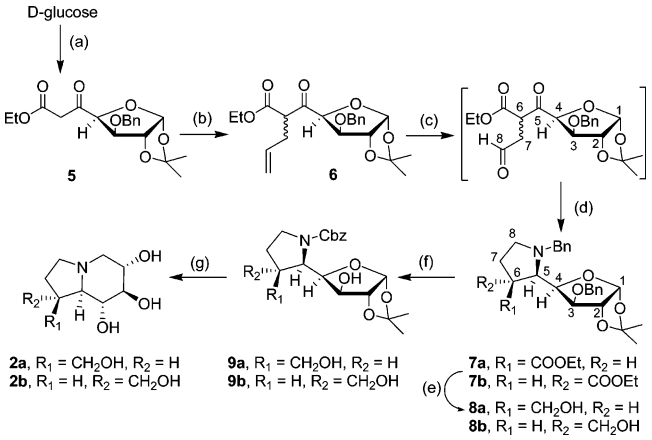
Scheme 1. Synthesis of **2a** and **2b**[a]



[a] Reagents and conditions: (a) see ref 15; (b) NaOEt, EtOH, allyl bromide, 60 °C, 2 h, 70%; (c) O_3, DCM, DMS, −78 °C, 1 h; (d) $BnNH_2$, AcOH, $NaCNBH_3$, MeOH, −78 °C to rt, 12 h; (e) LAH, THF, 0 °C, 2 h; (f) (i) 10% Pd/C, $HCOONH_4$, MeOH, 80 °C, 1 h; (ii) CbzCl, $NaHCO_3$, $MeOH/H_2O$, 0 °C to rt, 2 h; (g) (i) TFA/H_2O (3:2), rt, 2.5 h; (ii) H_2, 10% Pd/C, MeOH, 12 h.

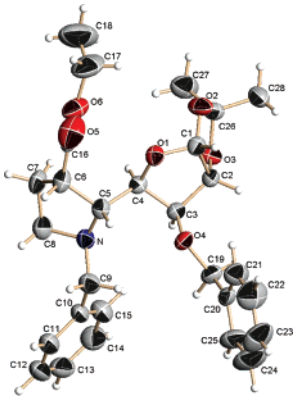


Figure 2. ORTEP drawing of compound **7a**.

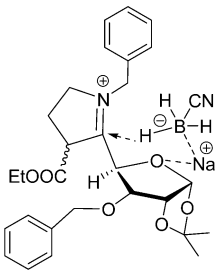


Figure 3. Explanation for observed stereoselectivity.

C-8a ring junction of **2**). We believe that the double reductive amino-cyclization of 5,8-ketoaldose involves prior formation of the five-membered cyclic-imminium ion in preferred (Figure 3) conformation wherein complexation of $NaCNBH_3$ with the lone pair of furan ring oxygen and the exclusive hydride delivery from preferred *Si* face, in a six-membered transition state as shown, led to the formation of pyrrolidine ring with 5*R* configuration. However, such complexation with C-3 OBn oxygen and attack of the hydride from the *Re* face is less likely due to the formation of the seven-membered crowded transition state having the *N*- and *O*-benzyl substituents.

In the next step, reduction of ester functionality of **7a** with $LiAlH_4$ gave alcohol **8a**, which on hydrogenolysis using ammonium formate, 10% Pd/C in methanol, followed by selective amine protection with benzyl chloroformate, and

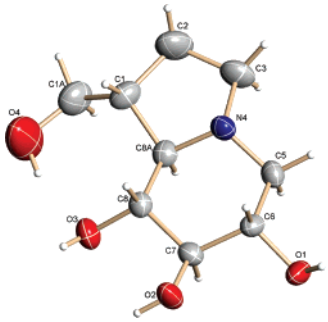


Figure 4. ORTEP drawing of compound **2b**.

sodium bicarbonate in MeOH afforded *N*-Cbz-protected diol **9a**. Finally, hydrolysis of the 1,2-acetonide functionality in **9a** with TFA−water followed by hydrogenation (H_2, 10% Pd/C in methanol) afforded 1(*S*)-hydroxymethyl castanospermine **2a**. Our attempts to deprotect the 1,2-acetonide functionality in **8a** with TFA−water and further hydrogenation did not afford the target product. As an alternative, *N*-benzyl group in **8a** was replaced with *N*-Cbz group to give **9a**, which on treatment with TFA−water and hydrogenation afforded a good yield of the target molecule. The spectral and analytical data of **2a** was in accordance with the assigned structure. The same sequence of reactions, as in the case of **7a** (LAH reduction and *N*-Cbz protection), was repeated with **7b** to give **8b** and **9b**. In the final step, acetonide cleavage and hydrogenation gave 1(*R*)-hydroxymethyl castanospermine **2b** as a white solid. The analytical and spectral data of **8b**, **9b**, and **2b** was in consonance with the assigned structures. The structure of **2b** was further confirmed by the single-crystal X-ray analysis (Figure 4), which established the absolute configurations at the newly generated stereocenters C-1 and C-8a as (*R*) and (*R*), respectively.

Enzyme Inhibition Study: The inhibitory activities of compounds **2a** and **2b** were studied against α-mannosidase, α-galactosidase, α-glucosidase, *β*-glucosidase, and *β*-galactosidase; however, both of the compounds showed no inhibition against any of the enzymes under study (data not shown). This indicated that the replacement of hydroxyl functionality at C-1 of castanospermine with hydroxymethylene substituent completely suppresses the inhibitory activity.[16]

Immunomodulatory Activity: The effect of the castanospermine analogues **2a** and **2b** on concavalin A (Con A[a])-induced proliferation of splenocytes of Swiss albino mice, as assessed by the MTT assay is shown in Figure 5. Our initial studies revealed that treatment of the mice splenocytes with Con A (10 μM) enhanced cell proliferation by ∼230% ($p < 0.01$) compared to the untreated cells. Hence, all subsequent experiments were carried out using the same concentration of Con A. The assays were conducted 72 h after the administration of **2a** and **2b** at five different concentrations (5, 10, 20, 75, 100 μM), using the Con A-treated splenocytes as the experimental control.

Up to a concentration of 20 μM, compound **2a** increased proliferation of the Con A-treated cells, with a maximum cell proliferation being observed at 5 μM. However, the increase in the cell proliferation was gradually reduced with increasing concentrations of **2a**. For example, compared to the Con A-treated control sample, treatment with Con A + **2a** (5, 10, and 20 μM) increased the cell proliferation by 161 ($p > 0.001$), 109 ($p > 0.01$), and 40% respectively. The inverse concentration

[a] Abbreviations: Con A, concavalin A; T_H, T helper; T_C, T cytotoxicity; IL, interleukin.

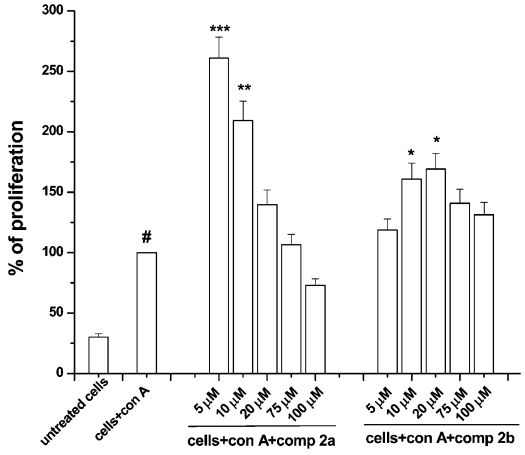


Figure 5. Proliferation of mouse splenocytes by Con A (10 μM) alone and in conjunction with different concentrations of compounds **2a** and **2b**. The cell proliferation was monitored by the MTT assay and the values (mean ± SEM, $n = 5$) are calculated considering that of the Con A-treated cells as 100. $^{\#}p < 0.01$ with respect to the untreated cells; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ with respect to the Con A-treated cells.

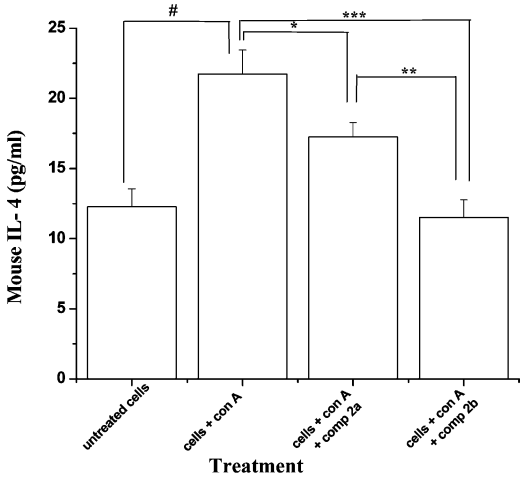


Figure 6. Effects of Con A (10 μM) alone and in conjunction with compounds **2a** (5 μM) and **2b** (10 μM) on the secretion of the IL-4 from normal mouse splenocytes (2×10^6 cells/well). The values are mean ± SEM ($n = 5$); *not significantly different, $^{**}p < 0.05$, $^{***}p < 0.01$, $^{\#}p < 0.001$.

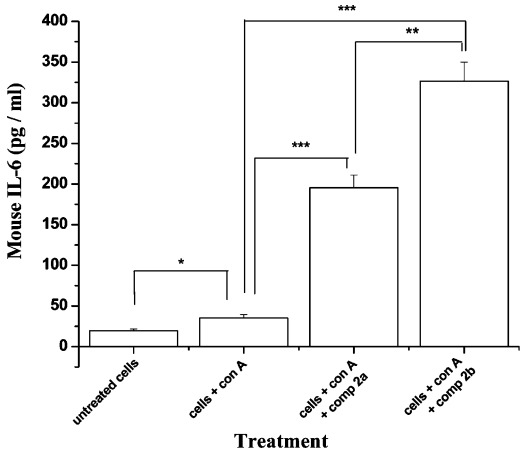


Figure 7. Effects of Con A (10 μM) alone, and in conjunction with compounds **2a** (5 μM) and **2b** (10 μM) on the secretion of the IL-6 from normal mouse splenocytes (2×10^6 cells/well). The values are mean ± SEM ($n = 5$); *not significantly different, $^{**}p < 0.05$, $^{***}p < 0.01$.

dependence was more pronounced at the highest test concentration (100 μM) of **2a**, when the cell proliferation was actually reduced by 27% compared to the control. In contrast, compound **2b** increased the cell proliferation concentration dependently up to 20 μM. Compared to the Con A-treated control sample, those treated with Con A + **2b** (5, 10, and 20 μM) showed increased cell proliferation by 19, 61 ($p < 0.05$), and 69% ($p < 0.05$), respectively. The increase in the cell proliferations at higher concentrations (75 and 100 μM) of **2b** were less (41 and 31%, respectively). However, it did not inhibit the cell proliferation. The cell proliferating capacity of **2a** was better than that of **2b**. Overall, compound **2a** showed maximum proliferation of the Con A-treated cells at a concentration of 5 μM, while compound **2b** was most active at 10−20 μM. Given that the extents of cell proliferation by **2b** at 10 μM and 20 μM were not significantly different, all subsequent studies were carried out with 10 μM of **2b**.

In separate experiments carried out without Con A-activation, the compounds, **2a** and **2b** did not show any significant cell proliferating capacities on their own. Thus, both of the compounds were nontoxic and showed synergistic effects to the Con A-induced activation, indicating their role as efficient protectors against activation-induced cell death. Next we assayed the effects of the compounds **2a** and **2b** on the secretion of various cytokines from the mouse splenocytes. For this, the spleen cells were induced by Con A (10 μM), and the secretion of the interleukins, IL-4, IL-6, IL-2, and interferon-γ (IFN-γ), was detected from the supernatant of spleen cells using mice ELISA kit. The assays were carried out using compounds **2a** and **2b** at their optimized effective concentrations (5 μM and 10 μM, respectively). Compared to the untreated cells, those treated with Con A (10 μM) showed significantly increased IL-4 secretion (77.1%, $p < 0.001$). However, treatment of the Con A-treated cells with **2a** and **2b** reduced the levels of IL-4 secretion by 20.6 and 47.0% ($p < 0.01$), respectively (Figure 6). The results with **2a** and **2b** were significantly different ($p < 0.05$).

Treatment of the mouse splenocytes with Con A (10 μM) did not alter the IL-6 secretion significantly. However, treatment of the Con A-treated cells with **2a** and **2b** increased ($p < 0.01$) IL-6 secretion >5.5-fold and >9-fold, respectively (Figure 7). The increase of IL-6 by **2b** was significantly more than that by **2a** ($p < 0.05$). Compared to the results with IL-4, modulation of IL-6 secretion by the compounds **2a** and **2b** showed an opposite trend.

With regard to IFN-γ, the Con A-activated cells showed significantly higher (4.25-fold) secretion of the cytokine compared to that in the unstimulated control cells ($p < 0.001$). Compound **2a** increased it further by 40.5% ($p < 0.01$), while the effect of compound **2b** was insignificant, increasing the cytokine production by 9.5% only. Thus, compound **2a** was a better inducer of IFN-γ than compound **2b** (Figure 8).

The Con A-activated cells showed significantly higher (~11-fold) secretion of IL-2 compared to that in the unstimulated cells ($p < 0.001$). Treatment of the Con A-activated cells with the compounds **2a** and **2b** increased the IL-2 level only marginally (6.6 and 3.3%, respectively). The results for the treatment with **2a** and **2b** were not significantly different from that of the treatment with Con A alone (Figure 9).

Con A is a specific activator of the T cells that play a central role in the cell-mediated immunity. The T cells contain two classes of T lymphocytes, T helper cells (T_H) and T cytotoxicity (T_C) cells. The T_H cells are further classified as T_{H1} and T_{H2}. The cytokine, IL-4, an anti-inflammatory cytokine secreted by

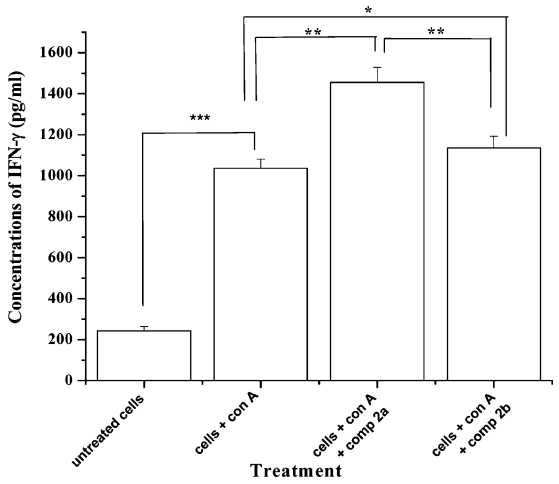


Figure 8. Effects of Con A (10 μM) alone, and in conjunction with compounds **2a** (5 μM) and **2b** (10 μM) on the secretion of the IFN-γ from normal mouse splenocytes (2×10^6 cells/well). The values are mean $\pm$ SEM ($n = 5$); *no significant difference, **$p < 0.01$, ***$p < 0.001$.

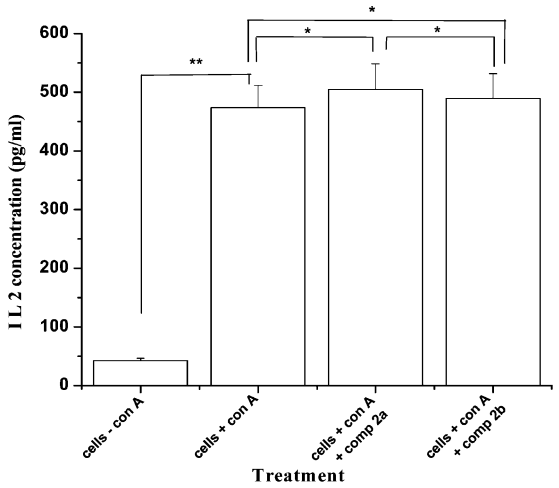


Figure 9. Effects of Con A (10 μM) alone, and in conjunction with compounds **2a** (5 μM) and **2b** (10 μM) on the secretion of the IL-2 from normal mouse splenocytes (2×10^6 cells/well). The values are mean $\pm$ SEM ($n = 5$); *not significantly different, **$p < 0.001$.

the T_{H2} cells, is a key regulator in humoral and adaptive immunity.[17] It is the hallmark cytokine produced by the T_{H2} cells and has many biological roles, including stimulation of the activated B-cell as well as proliferation and differentiation of T-cells. On the other hand, IL-6, a pro-inflammatory cytokine, is secreted by the T_{H1} cells and macrophages and stimulates immune response to trauma, especially burns or other tissue damage leading to inflammation. In terms of host response to a foreign pathogen, IL-6 has been shown to provide resistance to mice against the bacterium, *Streptococcus pneumoniae*.[18] Likewise, the immune hormone, IL-2 is instrumental in the body's natural response to microbial infection and is normally produced by the T_{H1} cells.[19] On the other hand, IFN-γ, the hallmark cytokine of the T_{H1} cells shows antiviral, immunoregulatory, and antitumor properties and is used to treat infectious diseases, although may precipitate autoimmunity. It increases expression of the chemokine receptors, which can mediate migration of immune cells to the site of inflammation.[20] The ratio of T_{H1} and T_{H2} cytokines is closely correlated with the outcome of many diseases. T_{H1} responses predominate in organ-specific autoimmune disorders, acute allograft rejection, and in some chronic inflammatory disorders. In contrast, T_{H2} responses predominate in Omann's syndrome, transplantation tolerance, chronic graft-versus-host disease, systemic sclerosis, and allergic diseases.

Based on the present results, it can be concluded that both **2a** and **2b** have a T_{H1} bias as they increase the ratio of the T_{H1}/T_{H2} cytokines (IL-6 and IL-4) in a similar way, albeit at different concentrations. The increase of the T_{H1}/T_{H2} ratio by the compounds is in tune with their excellent cell proliferating activity. Thus, the compounds appear to be good immunomodulators in controlling infection and might trigger an improved response to antibodies. IFN-γ mediates the T_{H1} type of cellular immune response, while IL-4 stimulates B cells to secrete antibodies. Compound **2a** had a marked ability to augment the secretion of both IL-6 and IFN-γ and only a minor inhibitory effect on the secretion of IL-4. Therefore, it might have stronger immuno-potentiating efficiency via both T_{H1}- and T_{H2}-mediated cellular as well as humoral immune reactions. On the other hand, compound **2b** had a stronger inhibitory effect on IL-4 secretion and a lesser potentiating effect of IFN-γ than that of compound **2a**. This may account for the better immuno-potentiating potency of compound **2a** compared to compound **2b**. With regard to the immunomodulatory property, castanospermine has been earlier reported to be an immunosuppressive agent.[8a−c] Our results revealed that insertion of a hydroxymethyl group at the C-1 position can convert it to an immuno-potentiating agent, which may have potential in controlling infection. Further, compound **2a** with a β-hydroxymethyl substituent was found to be a more potent immunomodulator being active at a lower concentration (5 μM) than **2b**, which showed lesser cell proliferation even at the higher concentrations of 10−20 μM. This established a key role of the C-1 stereochemistry of the test samples for the designated activity. However, a more detailed study is required to substantiate it.

In conclusion, we have demonstrated an efficient and stereocontrolled double reductive amino cyclization method for the synthesis of **1**(*S*)- and **1**(*R*)-hydroxymethyl castanospermines **2a** and **2b**. Interestingly, in contrast to castanospermine, its C-1-hydroxymethyl congeners **2a**,**b** showed significant immunopotentiating property, without any inhibition toward the glycosidase enzymes. Given that the compounds act synergistically with Con A, they may be suitable adjuvants with immuno-boosting drugs and vaccines. It is worth mentioning that the augmentation of IL-6 by **2a** and **2b** is very interesting given that the cytokine finds clinical application in cancer therapy.[21]

Experimental Section

(1*S*,6*S*,7*R*,8*R*,8a*R*)-1-(Hydroxymethyl)-6,7,8-trihydroxy-indolizidine (2a). A cooled (0 °C) solution of **9a** (0.12 g, 0.31 mmol) in TFA−H_2O (3 mL, 3:2) was stirred for 1 h, brought to 25 °C, and stirred for an additional 2 h. TFA was evaporated at high vaccum to yield the crude hemiacetal (0.11 g). A solution of the above product in dry methanol (4 mL) was hydrogenated in the presence of 10% Pd/C (0.03 g) at 80 psi for 12 h. The catalyst was filtered and washed with methanol, and the filtrate was concentrated to afford a thick liquid. Purification by column chromatography on silica gel (chloroform/methanol = 7/3) afforded **2a** (0.050 g, 82%) as a thick liquid. R_f 0.60 (chloroform/methanol = 3/2); $[\alpha]^{29.6}_D$ +46.1 (*c* 0.65, MeOH); IR (neat) 3600−3200 (broad, OH) cm^{-1}. ^1H NMR (300 MHz, D_2O): δ 1.23−1.40 (m, 1H, *H*-2a), 1.84−2.03 (m, 2H, *H*-5a and *H*-2b), 2.05−2.24 (m, 2H, *H*-8a and *H*-3a), 2.28−2.44 (m, 1H, *H*-1), 2.86 (br t, $J = 9$ Hz, 1H, *H*-3b), 2.96 (dd, $J = 11.1$ and 5.1 Hz, 1H, *H*-5b), 3.12 (t, $J = 9$ Hz, 1H, *H*-7), 3.28 (dd, $J = 10.8$ and 7.2 Hz, 1H, *H*-1a), 3.30 (t, $J = 9$ Hz, 1H, *H*-8), 3.42 (dt, $J = 9.0$ and 5.1 Hz, 1H, *H*-6), 3.62 (dd, $J = 10.8$ and 7.5 Hz, 1H, *H*-1a). ^{13}C NMR (75 MHz, D_2O): δ 25.9 (*C*-2),

39.0 (*C*-1), 52.6 (*C*-3), 55.2 (*C*-5), 62.3 (*C*-1a), 68.5 (*C*-8a), 69.6 (*C*-6), 69.8 (C-8), 78.4 (*C*-7).

(1*R*,6*S*,7*R*,8*R*,8a*R*)-1-(Hydroxymethyl)-6,7,8-trihydroxy-indolizidine (2b). Following a similar manner, compound **9b** (0.14 g, 0.35 mmol) was deacetalized with TFA−H_2O (3 mL, 3:2), and the resultant product was hydrogenated over 10% Pd/C (0.03 g) to furnish **2b** (0.06 g, 85%) as a colorless solid, after isolation and purification by column chromatography on silica gel (chloroform/methanol = 65/35). Mp: 199−200 °C. R_f 0.60 (chloroform/methanol = 3/2); $[\alpha]^{32.2}_D$ +16.0 (*c* 0.75, MeOH); IR (KBr) 3600−3200 (broad, OH) cm^{-1}. ^1H NMR (300 MHz, D_2O): δ 1.50−1.64 (m, 1H, *H*-2a), 1.96 (t, J = 9 Hz, 1H, *H*-8a), 1.97−2.04 (m, 1H, *H*-2b), 2.05−2.22 (m, 2H, *H*-5a and *H*-1), 2.36 (ddd Apq, J = 9.5 Hz, 1H, *H*-3a), 2.95 (dt, J = 9.5 and 1.8 Hz, 1H, *H*-3b), 3.13 (dd, J = 11.1 and 5.4 Hz, 1H, *H*-5b), 3.24 (t, J = 9 Hz, 1H, *H*-8), 3.29 (t, J = 9 Hz, 1H, *H*-7), 3.53 (dd, J = 10.8 and 6.3 Hz, 1H, *H*-1a), 3.53−3.62 (m, 1H, *H*-6), 3.65 (dd, J = 10.8 and 6.6 Hz, 1H, *H*-1b). ^{13}C NMR (75 MHz, D_2O): δ 25.8 (*C*-2), 44.1 (*C*-1), 51.5 (*C*-3), 54.4 (*C*-5), 63.6 (*C*-1a), 69.3 (*C*-8a), 69.7 (*C*-6), 73.6 (C-8), 78.4 (*C*-7).

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Supporting Information Available: General procedure for glycosidase inhibition assay, general experimental procedure, and experimental procedures for compounds **6**, **7a**, **7b**, **8a**, **8b**, **9a**, and **9b**. X-ray crystallographic data of compounds **7a** and **2b**, and copies of ^1H and ^{13}C NMR spectra of compounds **7a**, **7b**, **8a**, **8b**, **9a**, **9b**, **2a**, and **2b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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