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Discovery of Iminosugar Derivatives with Strong IFN-γ Inducing Activity

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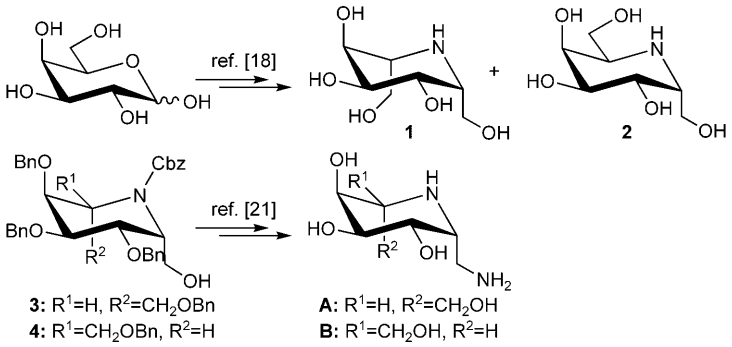
Through effective construction of a compound library and in situ cell-based screening, several iminosugar derivatives were discovered that show a strong ability to enhance IFN-γ secretion. In particular, compounds **A45** and **B8**, which exhibit remarkable activity in stimulating IFN-γ secretion, show good antibacterial activity in vivo and have the potential to be lead compounds for further studies in the search for drugs for the treatment of cancer and microbial infections.

Introduction

Interferon-γ (IFN-γ) is a cytokine secreted by lymphocytes that promote innate immunity, i.e. natural killer (NK) cells, and cells that are components of the adaptive immune system (specific subsets of T cells).[1,2] IFN-γ plays an important role in promoting innate and adaptive immune responses. For example, IFN-γ plays a critical and physiologically relevant role in promoting host resistance to microbial infection. The absence of IFN-γ production[3] or cellular responsiveness[4–8] in humans and experimental animals significantly predisposes the host to microbial infection. Furthermore, a role for IFN-γ in protecting against tumor development has recently been identified. Results have shown that endogenously produced IFN-γ is critical not only for rejection of transplantable tumors[9] but also preventing primary tumor development.[10–12]

Iminosugars, known as inhibitors of many carbohydrate processing enzymes,[13,14] are carbohydrate analogues in which the ring oxygen atom has been replaced by nitrogen. Iminosugars are found to be widespread in plants and microorganisms.[15] Iminosugars exhibit various biological activities;[16] however, so far their immunomodulating activities have been less explored and little is known about their effects on immune system responses.[17] Recently, some synthetic iminosugar derivatives that show potential immunosuppressive activity have been discovered by us[18,19] and others.[20] We found that iminosugars **1** and **2** (Scheme 1) remarkably decreased IFN-γ secretion in mice, whereas they were less toxic than cyclosporin A (CyA), a well–known immunosuppressive drug.

In our previous study, an iminosugar library based on the coupling of compounds **A** and **B** with various carboxylic acids by a parallel synthesis approach was effectively constructed on a micro–scale without any purification.[21] Herein we screened this library with a cytokine secretion assay. We were surprised to find that, in contrast to compounds **1** and **2**, the majority of these iminosugar derivatives show remarkable immunostimulating activities. Furthermore, some compounds with such excellent activities were evaluated by a mouse infection model to determine their potential as antibacterial therapeutics.



Scheme 1. Preparation of compounds **1**, **2**, **A**, and **B**.

Results and Discussion

As shown in Scheme 1, iminosugars **1** and **2** were synthesized from galactose according to our published procedure.[18] Simple transformations of **3** and **4** led to compounds **A** and **B**, respectively.

With compounds **A** and **B** in hand, a diversity-oriented library containing 100 members was assembled.[21] Compounds **A** and **B** reacted with 50 carboxylic acids. The reactions were

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performed in DMF on a microgram scale in the presence of (1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 1 equiv) and diisopropylethylamine (DIEA, 2 equiv), followed by medium dilution and screening without any purification. Each reaction was carried out in a 1.5-mL Eppendorf tube. Mass spectra and TLC analyses were employed to identify the products. The reactions proceeded at a concentration of 36 mM, and upon completion the reaction mixture was diluted 1000-fold with phosphate-buffered saline (PBS, final concentration: 36 μM) for screening.

To assay their effects on the secretion of cytokines (IFN-γ and IL-4), the diluted samples mentioned above were tested against splenocytes from BALB/c mice. The spleen cells were induced by concanavalin A with each sample at 37 °C, 5% CO_2 for 72 h. The secretion of IFN-γ was detected from the supernatant of spleen cells by the use of a mouse ELISA kit. Relative to the control, the levels of IFN-γ secretion were all increased remarkably. The assay of secretion of IL-4 from splenocytes was similar to the assay of IFN-γ. The supernatant of spleen cells were detected by a mouse IL-4 ELISA kit. Some samples showed inhibitory effects, whereas others showed enhancing effects on the secretion of IL-4. The effects on IL-4 are not very significant no matter whether they display inhibitory or enhancing effects (data not shown).

Based on the primary screening results described above, we selected several compounds which show good IFN-γ inducing activity and synthesized pure products for the second-round screening using splenocytes from BALB/c mice and C57B1/6 mice to confirm the activity. The compounds we selected were **A5**, **A6**, **A16**, **A17**, **A45**, **A47**, **B3**, **B5**, **B8**, **B21**, **B30**, **B31**, and **B33** (structures shown in Figure 1).

The effects of the 13 pure compounds on the secretion of cytokines from the splenocytes in mice were identified, and the results are shown in Figure 2. It is apparent that in all cases the level of IFN-γ secretion was enhanced by each compound at a concentration of 60 μM. Compounds **A45** and **B8** show the strongest IFN-γ inducing activity: IFN-γ secretion levels were enhanced 3.20- and 2.85-fold, respectively, in BALB/c mice, and 3.00- and 2.53-fold, respectively, in C57B1/6 mice. It was also found that all 13 compounds do not show a strong ability to change the IL-4 secretion level.

It was surprising to find that while compounds **1** and **2** show remarkable inhibitory effects, their derivatives show the complete opposite effects on IFN-γ secretion. It seems that IFN-γ secretion can be greatly enhanced by the introduction of an appropriate aromatic moiety (for example, compounds **A45** and **B8**). This discovery deserves further exploration. More compounds need to be synthesized to enable a better understanding of structure–activity relationships (SAR).

To further investigate the activities of compounds **A45** and **B8**, in vivo antibacterial effect tests were performed. Compounds **A45** (5 mg per mouse), **B8** (5 mg per mouse), and PBS were respectively injected to each group of mice two days before infection with virulent *Salmonella typhimurium C5*.[22] After infection, the survival rate of mice was observed daily. The results are shown in Figure 3; compound **B8** clearly increased the survival rate by nearly 50%. Compound **A45** also showed a strong ability to counteract the lethal effect of *S. ty-*

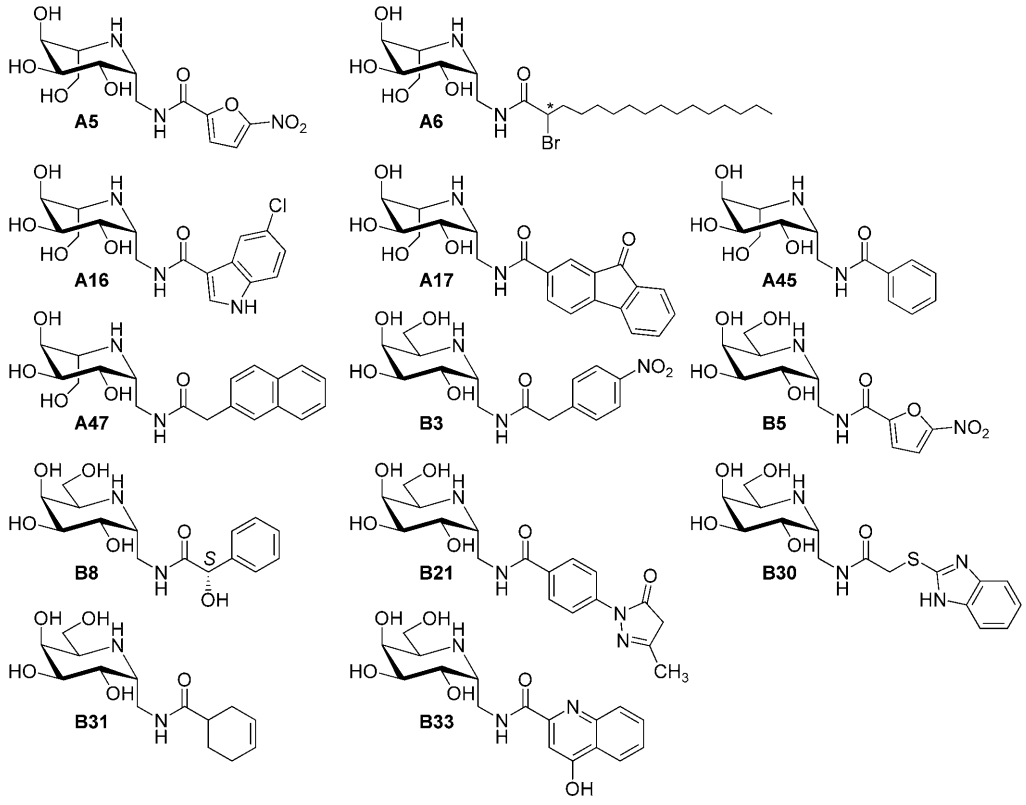

Figure 1. Structures of compounds **A5**–**B33**

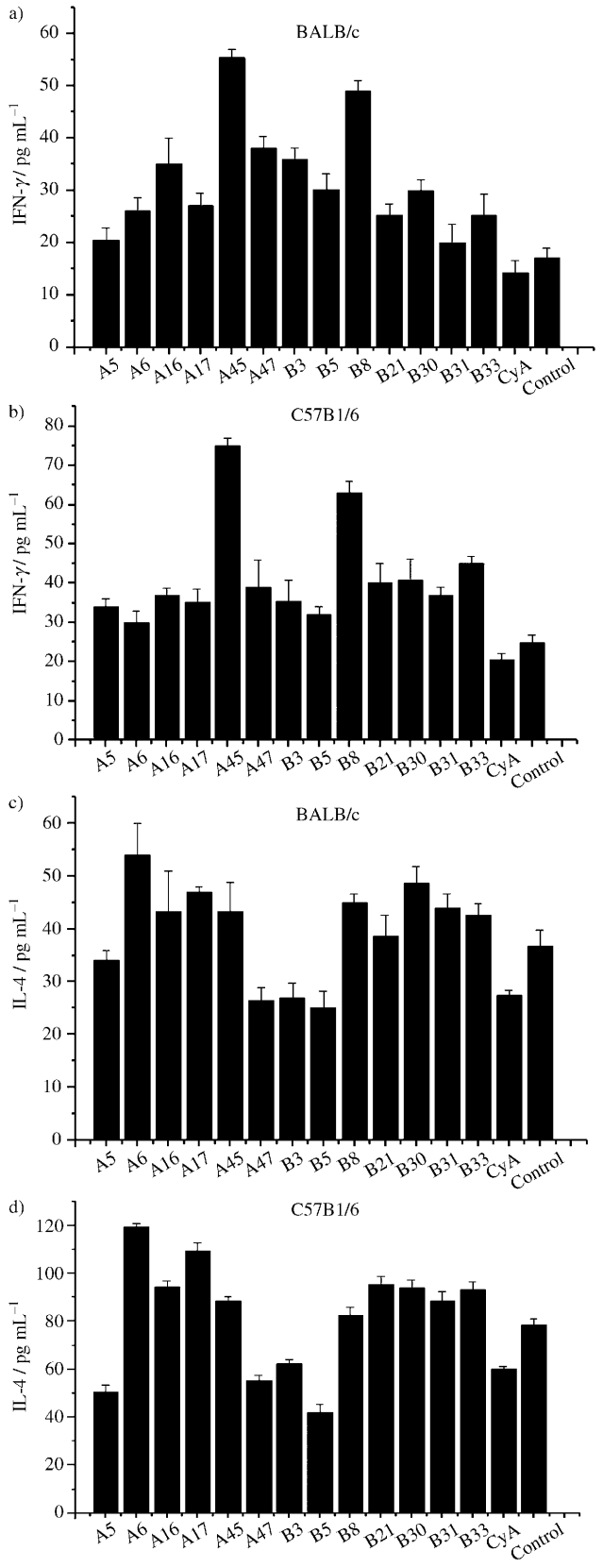

Figure 2. The effects on the secretion of IFN-γ and IL-4 in mice (indicated) by the 13 compounds **A5**–**B33** at 60 μM. Data represent the mean ±SEM of at least three independent experiments; $p < 0.05$.

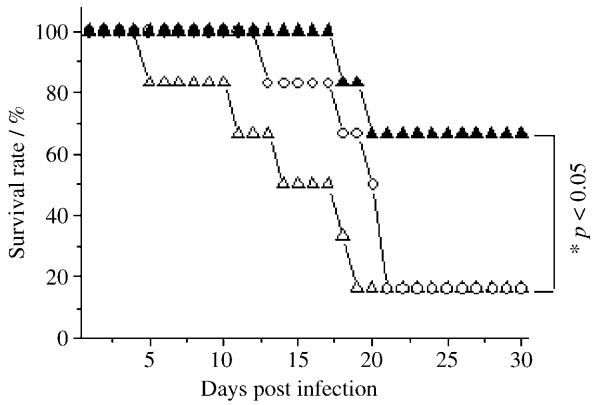

Figure 3. Survival rates of three groups of mice after *Salmonella* infection: △ PBS, ○ **A45**, ▲ **B8**.

phimurium C5, although the effect was not maintained after 20 days. This experiment demonstrated that **A45** and **B8**, especially iminosugar **B8**, show clear antibacterial effects in vivo, due to their strong IFN-γ inducing activity.

In fact, because intracellular bacteria, such as *Salmonella* spp., are quite demanding and variable targets of anti-infective chemotherapies, only a limited number of iminosugar derivatives aimed at inhibiting bacterial carbohydrate processing enzymes were found to be effective.[23,24] However, the compounds we discovered, such as compounds **A45** and **B8**, act in a totally different manner. Instead of killing the bacteria, our compounds promote the self-protection ability of the biological systems. Our strategy may become an efficient way to discover new chemical entities such as iminosugar derivatives with antibacterial activity. But the exquisite mechanism needs further exploration.

From our present and previous work, we disclosed that iminosugars have great potential as immunomodulating agents. Compounds **1** and **2** hold potential as immunosuppressive agents, whereas compounds **A45** and **B8** could be good drug lead compounds in treating cancer and microbial infection, due to their strong activities to induce the secretion of IFN-γ.

Conclusions

In the present study, by using a cell-based in situ screening strategy, we have detailed the discovery of several iminosugar derivatives which show excellent IFN-γ inducing activity in vitro and good antibacterial effect in vivo. The results demonstrate the effectiveness of our strategy to rapidly identify biologically active compounds. The SAR of these compounds deserves further exploration. In mice, IFN-γ has been shown to be an important mediator of natural resistance to *Salmonella* spp., and to inhibit intracellular bacterial growth.[25] Due to the important roles that IFN-γ plays in biological processes, these compounds which we have discovered might hold the potential for treating cancer and microbial infection.

Experimental Section

Chemistry

General: All reactions were monitored by analytical thin-layer chromatography (TLC) on silica gel 60 F_{254} pre-coated on aluminum plates (E. Merck). Column chromatography was performed on silica gel (200–300 mesh, Qingdao Chemicals). Reagents were of the highest commercial quality and were used without further purification, unless otherwise noted.

General procedures to prepare the compounds A5-B33: DIEA (30.0 μL, 0.171 mmol) was added to a solution of **A/B** (10.0 mg, 0.0518 mmol), HBTU (21.6 mg, 0.0570 mmol), and an acid (0.0570 mmol) in dry DMF (2 mL) at room temperature under N_2. The reaction mixture was stirred for 30 min and then evaporated in vacuo. The residue was purified by column chromatography with CH_2Cl_2/MeOH (10:1 *v*/*v*) to provide the product.

Compound A5: white solids; ^{1}H NMR (500 MHz, CD_3OD): δ = 7.53 (d, *J* = 3.5 Hz, 1H), 7.30 (d, *J* = 3.5 Hz, 1H), 3.91 (t, *J* = 3.5 Hz, 1H), 3.78–3.71 (m, 4H), 3.57 (dd, *J* = 13.5, 6.5 Hz, 1H), 3.41 (dd, *J* = 13.5, 7.5 Hz, 1H), 3.25 (td, *J* = 7.0, 1.5 Hz, 1H), 2.86 ppm (ddd, *J* = 10.5, 5.0, 3.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 159.3, 153.3, 149.3, 117.0, 113.3, 72.7, 71.4, 67.3, 62.7, 57.6, 54.1, 41.9 ppm; HRMS (*m*/*z*): calcd for $[C_{12}H_{17}N_3O_8+Na]^+$ 354.0908, found 354.0908.

Compound A16: white solids; ^{1}H NMR (300 MHz, CD_3OD): δ = 7.59 (d, *J* = 1.8 Hz, 1H), 7.40 (d, *J* = 8.7 Hz, 1H), 7.17 (dd, *J* = 8.7, 2.1 Hz, 1H), 7.02 (d, *J* = 0.9 Hz, 1H), 3.93 (t, *J* = 3.6 Hz, 1H), 3.82–3.60 (m, 5H), 3.39 (t, *J* = 6.9 Hz, 1H), 3.26 (t, *J* = 6.9 Hz, 1H), 2.93–2.87 ppm (m, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 164.4, 136.6, 133.5, 129.9, 126.8, 125.4, 121.8, 114.4, 103.9, 72.6, 71.2, 67.3, 62.7, 57.6, 54.5, 41.6 ppm; HRMS (*m*/*z*): calcd for $[C_{16}H_{20}ClN_3O_5+Na]^+$ 392.0984, found 392.0980.

Compound A17: yellow solids; ^{1}H NMR (300 MHz, CD_3OD): δ = 8.08–8.06 (m, 2H), 7.77–7.72 (m, 2H), 7.65–7.63 (m, 1H), 7.59 (td, *J* = 7.5, 1.2 Hz, 1H), 7.40 (td, *J* = 7.5, 0.9 Hz, 1H), 3.94 (t, *J* = 3.6 Hz, 1H), 3.85–3.72 (m, 4H), 3.65 (dd, *J* = 13.8, 6.9 Hz, 1H), 3.49 (dd, *J* = 13.8, 6.9 Hz, 1H), 3.37 (td, *J* = 6.6, 1.5 Hz, 1H), 3.04–2.97 ppm (m, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 194.2, 169.5, 148.7, 144.8, 136.5, 136.1, 135.7, 135.6, 135.3, 131.2, 125.2, 123.7, 122.6, 121.8, 72.2, 70.9, 66.8, 62.1, 57.8, 54.6, 41.7 ppm; HRMS (*m*/*z*): calcd for $[C_{21}H_{22}N_2O_6+H]^+$ 399.1551, found 399.1553.

Compound A45: white solids; ^{1}H NMR (300 MHz, CD_3OD): δ = 7.87 (d, *J* = 7.2 Hz, 2H), 7.56–7.45 (m, 3H), 3.95–3.91 (m, 4H), 3.79 (dd, *J* = 11.7, 6.3 Hz, 1H), 3.68–3.66 (m, 2H), 3.56 (t, *J* = 7.2 Hz, 1H), 3.25–3.18 ppm (m, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 171.3, 134.8, 133.1, 129.6, 128.5, 71.3, 69.8, 65.6, 60.7, 58.1, 55.3, 40.5 ppm; HRMS (*m*/*z*): calcd for $[C_{12}H_{17}N_3O_8+Na]^+$ 354.0908, found 354.0906.

Compound A47: white solids; ^{1}H NMR (500 MHz, CD_3OD): δ = 8.02 (dd, *J* = 8.5, 0.5 Hz, 1H), 7.87 (dt, *J* = 8.0, 0.5 Hz, 1H), 7.80 (t, *J* = 5.0 Hz, 1H), 7.53 (ddd, *J* = 8.5, 6.0, 1.5 Hz, 1H), 7.48 (ddd, *J* = 8.5, 6.0, 1.5 Hz, 1H), 7.44–7.43 (m, 2H), 4.02 (s, 2H), 3.84 (t, *J* = 3.5 Hz, 1H), 3.70 (dd, *J* = 11.0, 3.5 Hz, 1H), 3.68–3.63 (m, 2H), 3.60 (dd, *J* = 4.0, 1.5 Hz, 1H), 3.39 (dd, *J* = 13.5, 7.0 Hz, 1H), 3.13 (dd, *J* = 14.0, 7.0 Hz, 1H), 3.05 (td, *J* = 7.0, 1.5 Hz, 1H), 2.77 ppm (ddd, *J* = 10.5, 5.0, 3.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 174.7, 135.4, 133.6, 132.7, 129.7, 129.2, 129.0, 127.4, 126.8, 126.6, 124.8, 72.7, 71.4, 67.6, 63.0, 57.5, 54.0, 41.9, 41.4 ppm; HRMS (*m*/*z*): calcd for $[C_{19}H_{24}N_2O_5+H]^+$ 383.1577, found 383.1574.

Compound B3: white solids; ^{1}H NMR (500 MHz, CD_3OD): δ = 8.19–8.17 (m, 2H), 7.54 (dd, *J* = 8.5, 0.5 Hz, 2H), 3.90–3.87 (m, 2H), 3.68–3.65 (m, 4H), 3.58 (dd, *J* = 8.5, 3.0 Hz, 1H), 3.49 (dd, *J* = 14.0, 4.5 Hz, 1H), 3.38 (dd, *J* = 13.5, 10.0 Hz, 1H), 3.23–3.19 (m, 1H), 2.98 ppm (td, *J* = 5.5, 2.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 173.0, 148.4, 144.8, 131.4, 124.5, 72.8, 70.7, 70.3, 62.7, 55.8, 43.4, 37.6 ppm; HRMS (*m*/*z*): calcd for $[C_{15}H_{21}N_3O_7+Na]^+$ 378.1272, found 378.1275.

Compound B5: white solids; ^{1}H NMR (500 MHz, CD_3OD): δ = 7.52 (d, *J* = 4.0 Hz, 1H), 7.29 (d, *J* = 4.0 Hz, 1H), 3.97–3.92 (m, 2H), 3.72–3.66 (m, 3H), 3.64–3.58 (m, 2H), 3.37 (dd, *J* = 10.0, 5.0 Hz, 1H), 3.09–3.06 ppm (m, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 159.2, 149.4, 118.7, 116.8, 113.3, 72.7, 70.6, 70.2, 62.6, 55.8, 37.5 ppm; HRMS (*m*/*z*): calcd for $[C_{12}H_{17}N_3O_8+Na]^+$ 354.0908, found 354.0906.

Compound B8: white solids; ^{1}H NMR (500 MHz, CD_3OD): δ = 7.46–7.44 (m, 2H), 7.34–7.31 (m, 2H), 7.29–7.26 (m, 1H), 5.01 (s, 1H), 3.91–3.88 (m, 2H), 3.65 (d, *J* = 6.5 Hz, 2H), 3.58 (dd, *J* = 9.0, 3.0 Hz, 1H), 3.49 (dd, *J* = 14.0, 5.5 Hz, 1H), 3.42 (dd, *J* = 13.5, 9.5 Hz, 1H), 3.23–3.19 (m, 1H), 2.96 ppm (td, *J* = 6.5, 2.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 176.0, 141.7, 129.4, 129.1, 128.0, 75.6, 72.8, 70.8, 70.4, 62.9, 55.6, 37.1 ppm; HRMS (*m*/*z*): calcd for $[C_{15}H_{22}N_2O_6+Na]^+$ 349.1370, found 349.1369.

Compound B21: white solids; ^{1}H NMR (500 MHz, CD_3OD): δ = 7.89–7.86 (m, 4H), 4.01 (t, *J* = 2.5 Hz, 1H), 3.99 (dd, *J* = 8.0, 4.5 Hz, 1H), 3.84–3.69 (m, 4H), 3.63 (dd, *J* = 14.0, 9.0 Hz, 1H), 3.50–3.46 (m, 1H), 3.34 (s, 2H), 3.29–3.24 (m, 1H), 2.17 ppm (s, 3H); ^{13}C NMR (125 MHz, CD_3OD): δ = 170.4, 163.2, 152.3, 142.9, 131.0, 129.2, 121.0, 120.5, 118.9, 72.3, 70.1, 69.1, 61.4, 56.8, 55.4, 49.8, 38.1, 13.7 ppm; HRMS (*m*/*z*): calcd for $[C_{18}H_{24}N_4O_6+Na]^+$ 415.1588, found 415.1582.

Compound B30: white solids; ^{1}H NMR (500 MHz, CD_3OD): δ = 7.47 (dd, *J* = 6.0, 3.5 Hz, 2H), 7.20–7.16 (m, 2H), 3.95 (d, *J* = 2.5 Hz, 2H), 3.92–3.87 (m, 2H), 3.62–3.54 (m, 3H), 3.48 (dd, *J* = 14.0, 5.0 Hz, 1H), 3.42 (dd, *J* = 13.5, 10.0 Hz, 1H), 3.20–3.16 (m, 1H), 2.96–2.93 ppm (m, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 173.0, 171.2, 150.8, 123.5, 115.0, 72.8, 70.7, 70.4, 62.8, 55.6, 37.6, 36.5 ppm; HRMS (*m*/*z*): calcd for $[C_{16}H_{22}N_4O_5+Na]^+$ 405.1203, found 405.1199.

Compound B31: white solids; ^{1}H NMR (500 MHz, CD_3OD): δ = 5.68 (d, *J* = 2.0 Hz, 2H), 3.90–3.87 (m, 2H), 3.68–3.65 (m, 2H), 3.60 (dd, *J* = 8.5, 3.5 Hz, 1H), 3.47 (ddd, *J* = 13.5, 5.0, 3.5 Hz, 1H), 3.37–3.32 (m, 1H), 3.21–3.17 (m, 1H), 2.97 (br s, 1H), 2.47–2.41 (m, 1H), 2.24–2.18 (m, 1H), 2.15–2.09 (m, 3H), 1.89–1.86 (m, 1H), 1.70–1.62 ppm (m, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 179.5, 127.5, 126.5, 72.8, 70.8, 70.3, 62.8, 55.9, 42.4, 37.3, 29.2, 27.0, 25.8 ppm; HRMS (*m*/*z*): calcd for $[C_{14}H_{24}N_2O_5+Na]^+$ 323.1577, found 323.1580.

Compound B33: white solids; ^{1}H NMR (500 MHz, CD_3OD): δ = 8.23 (dd, *J* = 8.5, 1.5 Hz, 1H), 7.82 (d, *J* = 8.0 Hz, 1H), 7.74 (ddd, *J* = 8.5, 7.0, 1.5 Hz, 1H), 7.44 (ddd, *J* = 8.0, 7.0, 1.0 Hz, 1H), 6.84 (s, 1H), 3.98–3.94 (m, 2H), 3.75–3.61 (m, 5H), 3.40–3.36 (m, 1H), 3.09–3.06 ppm (m, 1H); ^{13}C NMR (75 MHz, CD_3OD): δ = 182.3, 165.5, 144.5, 141.8, 136.1, 128.0, 126.9, 126.5, 121.9, 108.9, 73.0, 71.5, 71.3, 64.0, 57.4, 55.8, 51.4, 38.5 ppm; HRMS (*m*/*z*): calcd for $[C_{17}H_{21}N_3O_6+H]^+$ 386.1323, found 386.1339.

Biology

Preparation and cultivation of splenocytes: The spleens from BALB/c mice and C57B1/6 mice were taken out in sterile conditions and soaked in non-serum-containing RPMI-1640 cell culture medium. The spleens were ground with a wire mesh. The cell suspension was filtered through a 200-mesh nylon net. The filtrate of the splenocytes was centrifuged at 2000 *g* for 10 min, and then

the supernatant was removed. The precipitate was dissolved in 5 mL Tris-NH_4Cl solution (pH 7.2) and incubated at 37 °C for 6–10 min in order to lyse the red cells. Then the cells were centrifuged at 2000 *g* for 7 min, and the cell pellets were dissolved in RPMI-1640 culture medium with 10% newborn bovine serum (NBS) and 10 g mL^{-1} concanavalin A. The cells were counted, and the concentration of cells was adjusted to 5×10^6 per mL; ~5×10^5 cells were added to each well of 96-well plates. Subsequently, various concentrations of each compound was added to the wells, and plates were incubated at 37 °C for 72 h under an atmosphere of 5% CO_2. The supernatant was collected and centrifuged at 2000 *g* for 5 min. The supernatant was collected and stored at −20 °C until assay.

Measurement of the secretion of IL-4 and IFN-γ from splenocytes: Mouse splenocytes were pretreated with concanavalin A (type IV) (Promega) at a final concentration of 10 μg mL^{-1} at 37 °C for 72 h in a medium containing 10% NBS and 5% CO_2. 96-well plates were coated with anti-mouse IFN-γ and IL-4 MAb in advance (commercial products). Various concentrations of compounds and IL-4 or IFN-γ standards (500, 250, 125, 62.5, 31.25, and 15.63 pg mL^{-1}) were added into each well. The wells were incubated at 20–25 °C for 120 min. The levels of IFN-γ and IL-4 secreted from immunized mice splenocytes were detected using the cytokine-specific ELISA kits. Standard curves were determined using known concentration of the IL-4 or IFN-γ. Using the standard curve, the concentration in the samples was then determined.

In vivo antibacterial effects of A45 and B8: The in vivo antibacterial activities of compounds **A45** and **B8** were determined in mice infected with *S. typhimurium C5*. Seven- to eight-week-old BALB/c male mice were obtained from the Animal Biosafety Level 3 Laboratory (ABSL-III) of Wuhan University School of Medicine. These mice were randomly divided into three groups (6 mice per group). Two days before infection, mice in each group were respectively inoculated intravenously in the tail vein with **A45** (5 mg per mouse), **B8** (5 mg per mouse) and PBS. Two days later, each mouse was infected with a lethal dose (i.e. 1×10^5 cfu) of virulent *S. typhimurium C5*. The survival rate was observed daily. The experimental protocols were performed in compliance with all guidelines and were approved by the Institutional Animal Care and Use Committee of Wuhan University.

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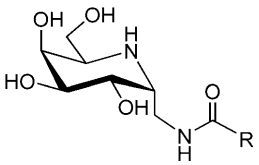
Discovery of Iminosugar Derivatives with Strong IFN-γ Inducing Activity



Through construction of an iminosugar library and in situ cell-based screening, several iminosugar compounds with the ability to stimulate IFN-γ secretion in vitro were discovered. Among these compounds, one was able to strongly induce IFN-γ secretion and showed remarkable antibacterial effects in vivo.

