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Original article

Iminosugars as glycosyltransferase inhibitors: synthesis of polyhydroxypyrrolidines and their evaluation on chitin synthase activity

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Abstract

Chitin synthase is an enzyme involved in the biosynthesis of chitin, a major structural component of the cell wall of many fungi. Since chitin is absent in vertebrates, chitin synthase has been envisaged as a valuable target in the search for new antifungal agents. In this report, a series of C-2 substituted polyhydroxypyrrolidines were designed and synthesized with the aim of mimicking the glycosylcation involved at the transition state of the enzymatic reaction governed by chitin synthase. Some of these models displayed chitin synthase inhibition in the millimolar range. However, no significant antifungal activity was noted on a panel of fungal strains.

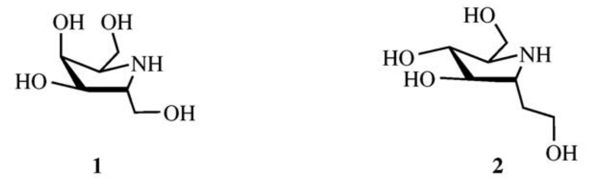
Keywords: Chitin synthase; Glycosyltransferase; Inhibition; Iminosugars; Difluoromethylphosphonoazasugars

1. Introduction

The rational design of potent glycosyltransferase inhibitors has attracted increasing interest during the last few years due to the pivotal role of these enzymes in the biosynthesis of polysaccharides and glycoconjugates [1]. Thus, inhibition of glycosyltransferases is a new approach for controlling invasive fungal infections [2], xenotransplantation rejection [3], inflammatory processes [4] or glycosphingolipid storage disorders like the Gaucher disease [5]. Since glycosyltransfer reactions are thought to proceed through transition states similar to those of glycosidases [6], iminosugars have been envisaged as potential inhibitors. These small molecules must be able to mimic, at the transition state, the charge and shape of the carbohydrate moiety of the sugar-nucleotide generally involved in these biological processes.

Among the iminosugars and related compounds which have been assayed against several glycosyltransferases [7], simple aza-pyranoses and furanoses displayed generally competitive inhibition towards the sugar-nucleotide donor with K_i's in the 10 mM range. Interestingly, Quiao et al. [8] demonstrated that the combination of an azasugar with the nucleotide-diphosphate product in the enzymatic assay resulted in potent synergistic effect, which reflects a better recognition of both the inhibitors taken together than each inhibitor itself [8]. In addition, some iminosugars, recently described, displayed affinities for glycosyltransferases in the micromolar range [7]. Among these, pyrrolidinol **1** (Fig. 1) had $K_i = 61$ μM against β-1,4-galactosyltransferase from bovine milk [9] and the natural 6-deoxy-homoDMDP **2** displayed strong inhibition of chitin synthase ($K_i = 38$ μM) [10]. Surprisingly, these compounds did not compete with the natural substrate but were shown to act as uncompetitive inhibitors. However, these pioneering results prompted us to design and develop new series of iminosugar-based glycosyltransferase inhibitors.

Accordingly, the rational design of new chitin synthase (EC 2.4.1.16) inhibitors [2] as antifungal agents led us to



1 **2**

β-1,4-GalTase, *K*i=61 μM   Chitin synthase, *K*i=38 μM

Fig. 1. Potent azasugar-type glycosyltransferase inhibitors.

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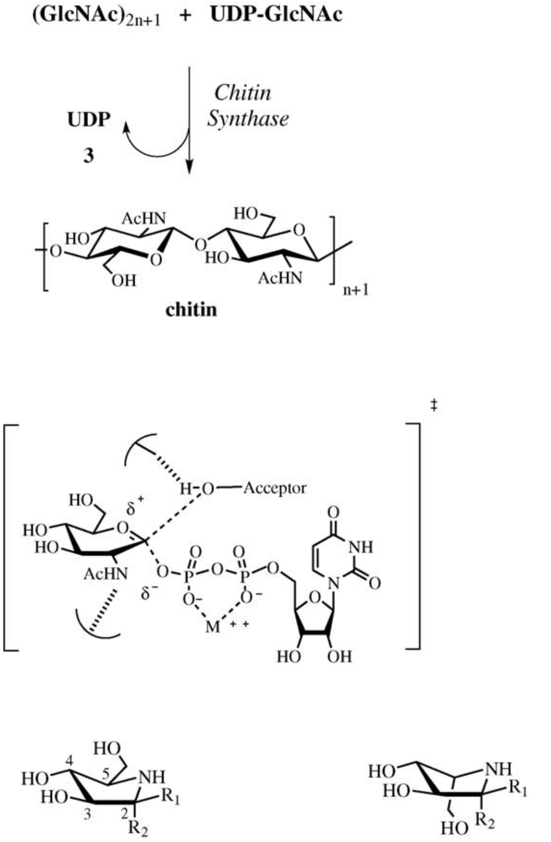

4: $R_1=CH_2OH$, $R_2=H$
5: $R_1=CF_2P(O)(OH)(OEt)$, $R_2=H$
6: $R_1=H$, $R_2=CF_2P(O)(OH)(OEt)$
7: $R_1=CH_2OH$, $R_2=H$
8: $R_1=CF_2P(O)(OH)(OEt)$, $R_2=H$
9: $R_1=H$, $R_2=CF_2P(O)(OH)(OEt)$

Fig. 2. Design of pyrrolidinols **4–9** as chitin synthase inhibitors.

envisage polyhydroxypyrrolidines **4–9** as possible mimics of the glycosylcation involved at the transition state of this *N*-acetylglucosamine-transfer reaction (Fig. 2). Several stereoisomers were targeted to investigate the influence of the stereochemistry at position C-2 or C-5. Thus, the D-*arabino* pyrrolidinols **4–6** might fit the configuration and the half-chair conformation of the D-*gluco*-configurated transition state. On the other hand, compounds **7–9** apparented to L-xylose, could induce an additional electrostatic interaction, the C-5 hydroxymethyl substituent replacing the NHAc group in a putative NH/enzyme hydrogen bond (Fig. 2). In addition, incorporation of a difluoromethylphosphonate group, mimicking the first portion of the pyrophosphate moiety of the donor, might exert binding forces increasing the inhibitory potencies. We report here the synthesis and kinetic evaluation of compounds **4–9** as inhibitors of chitin synthase as well as their bioevaluation against a variety of pathogenic fungi for antifungal activities.

2. Chemistry

2.1. Synthesis of iminosugars 4, 7

Pyrrolidinol **4** (2,5-dideoxy-2,5-imino-D-glucitol) had been synthesized at first in 1991 by a chemoenzymatic approach and was shown to exhibit potent inhibition of α-glucosidases [11]. We describe here a straightforward synthetic route to **4**, starting from *N*-benzyl-2,3,5-tri-*O*-benzyl-L-xylofuranosylamine **10** [12]. As reported in Fig. 3, addition of vinylmagnesium bromide to **10** gave the *syn* aminoalcool **11** in a very stereoselective manner (no trace of the *anti* isomer could be detected in the ^1H and ^{13}C NMR spectra) in an excellent yield (91%). The configuration at the newly formed stereocenter could be firmly assigned by NOE experiments on the corresponding vinylpyrrolidine **12** obtained by treatment of **11** with methanesulfonyl chloride. Ozonolysis was then performed on the sulfate salt of **12** to avoid oxidation of the free amine. Reduction of the crude aldehyde with $NaBH_4$ gave alcohol **13** in 56% yield (Fig. 3). Alternatively, the chemical transformation of **12–13** was also performed by bishydroxylation of **12** with osmium tetroxide in presence of *N*-methylmorpholine *N*-oxide, followed by $NaIO_4$ treatment in an $EtOH/H_2O$ solution and subsequent $NaBH_4$ reduction. Though the latter procedure was more time consuming, better yields (83%) could be obtained. The benzyl protecting groups were removed by extensive hydrogenolysis over Pd–C to afford **4** after purification by silica gel chromatography ($CHCl_3/MeOH/NH_4OH$, 60:30:5, v/v).

In addition to their potential as potent and specific glycosidase or glycosyltransferase inhibitors there is also considerable interest in using polyhydroxylated pyrrolidines, in particular those featuring C_2 symmetry, as chiral auxiliaries in asymmetric synthesis [13]. A former synthesis of 2,5-dideoxy-2,5-imino-L-iditol **7** was performed with this aim in view [14]. In our study, azasugar **7** was synthesized in good yield starting from the known vinylpyrrolidine **14** [15], prepared in two steps from commercial *N*-benzyl-2,3,5-tri-*O*-benzyl-D-arabinofuranosylamine (Fig. 3). The synthetic procedure described above for the preparation of **4** was applied to **14** and permitted to isolate pure **7**. The ^1H and ^{13}C NMR spectral data of compounds **4** and **7** as well as their optical rotations were similar to the previously reported ones [11,14].

2.2. Synthesis of difluoromethylphosphono-azasugars 5, 6, 8, 9 (Fig. 4)

In a previous paper we described the general synthetic approach to protected difluoromethylphosphono pyrrolidines [16]. Compounds **16–19** were prepared according to this procedure and we investigated here the experimental conditions leading to deprotected phosphonoazasugars **5**, **6**, **8**, **9** (Fig. 4). On the basis of previous work emphasizing the more efficient intracellular transport of mono-phosphonate esters when compared to their phosphonic acid counterparts [17], we intended to remove only one ethyl group in order to retain the lipophilic character of these antifungal candidates. Thus, the targeted monophosphonates might be obtained in two steps, i.e. hydrogenolysis of benzyl groups and mono-saponification of the so-obtained diethylphosphonates. However, the debenzylation step proved to be fairly difficult. Only the *N*-benzyl protecting group was cleaved by standard treat-

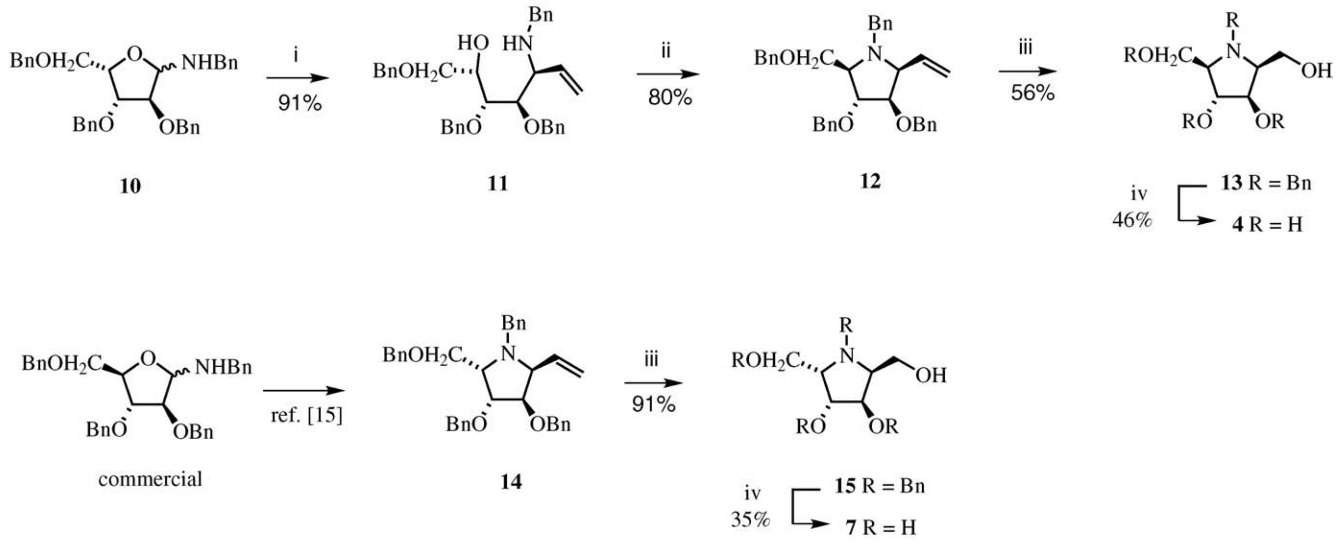

Fig. 3. Synthesis of azasugars **4**, **7**.
Reagents and conditions: (i) $H_2C=CH–MgBr$, THF; (ii) MsCl, pyr.; (iii) a) H_2SO_4, O_3, b) $NaBH_4$; (iv) H_2, Pd–C, HCl.

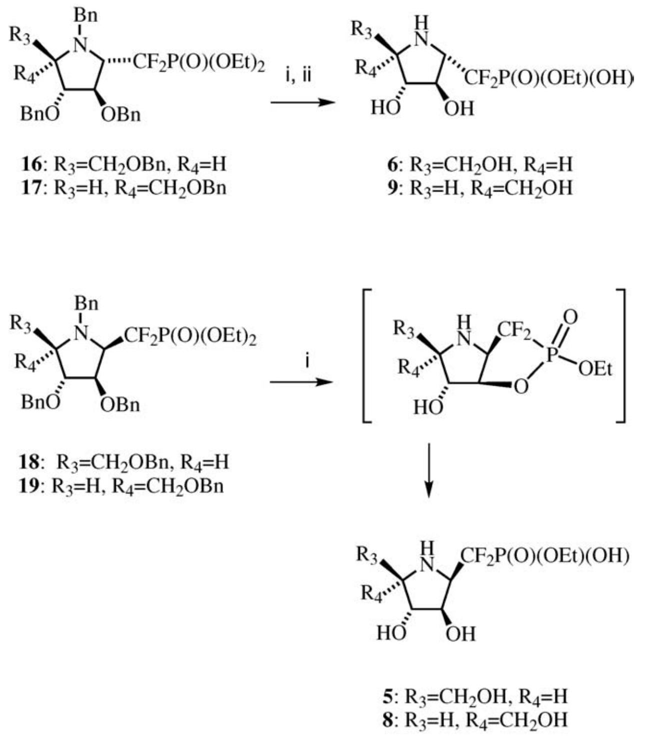

Fig. 4. Synthesis of difluoromethylphosphono-azasugars **5**, **6**, **8**, **9**.
Reagents and conditions: (i) HCO_2NH_4, 10% Pd/C, MeOH, 60 °C; (ii) 10% NH_4OH.

ment with Pd-C under hydrogen atmosphere. The use of black Pd as catalyst, AcOH as solvent or high pressure of hydrogen gave only partial deprotection. It is assumed that the secondary amine, formed during the process, might inhibit the catalytic properties of Pd [18]. Nevertheless, Pd(0)-catalyzed transfer hydrogenation in refluxing methanol, using ammonium formate as the hydrogen donor, allowed the cleavage of all the benzyl groups. The α-configurated mono-phosphonate esters **6** and **9** were obtained after subsequent monosaponification in aqueous ammonia. Surprisingly, this hydrolytic step was not required in the case of isomers **5** and **8** which were directly isolated after the debenzylation procedure. In this latter case, it is assumed that an in situ intramolecular transesterification occurred with assistance of the C-3 free hydroxyl group leading to a cyclic phosphonate ester which was readily hydrolized to the isolated monoesters.

3. Biological evaluation

3.1. Enzymology

Saccharomyces cerevisiae (X2180 strains) chitin synthase 1 activity was assayed on permeabilized cells by measuring the rate of formation of [^{14}C]-chitin from UDP-*N*-acetyl-[^{14}C]-glucosamine. The initial permeabilization of freshly grown cells was performed by osmotic shock, according to the procedure described by Crotti et al. [19].

Since chitin synthase 1 is present as its zymogen in the permeabilized cells, it must be activated prior to assay by partial proteolysis with trypsin and treatment with digitonin. Thus, an aliquot of permeabilized cell suspension (to a final concentration of 78 mg/ml) was incubated for 15 min in 30 mM Tris–HCl (pH 6.5) containing 55 mM GlcNAc, digitonin (5.2 mg/ml) and trypsin (1.0 mg/ml). Activation was stopped by adding soybean trypsin inhibitor (1.5 mg/ml). The resulting preparation was kept at 0 °C for assay.

The enzymatic assays were then carried out as reported in Section 6.

3.2. Antifungal evaluation

Antifungal activity of compounds **4–9** was tested on pathogenic fungi (*Cryptococcus neoformans*, *Trichophyton mentagrophytes*, *Microsporum canis*, *Aspergillus fumigatus*, *Candida albicans*, *S. cerevisiae*). MIC's were determined in broth macrodilution assays according to guidelines of the NCCLS M27-A protocol [20], with slight modifications [21]. Inocula for assays were prepared from stocks frozen at –80 °C by dilution in growth medium to give a final viable cell count of 2.5×10^3 CFU/ml. Each assay was performed with a duplicate series of drug dilutions. For each fungal strain, the assays were done in RPMI 1640 medium buffered to pH 7.0 with MOPS buffer, incubated at 35 °C for 48 h according to the described procedure [21]. *C. neoformans* and *A. fumigatus* were tested in the same assay, except that incubations were for 72 h. The dermatophytes *T. mentagrophytes* and *M. canis* were incubated at 30 °C for 7 days.

4. Results and discussion

The reaction rate of chitin synthase showed normal Michaelis–Menten kinetics and a K_m value of 0.62 mM was determined for UDP-GlcNAc (Fig. 5), which is in good agreement with previously published data [22]. In addition, the chitin synthase 1 activity was confirmed by positive control with nikkomycin Z.

The inhibitory activities of iminosugars **4–9** were examined at 1 mM of substrate UDP-GlcNAc and the results are reported in Table 1. For all the pyrrolidinols tested, an IC_{50} value (i.e. the concentration leading to half the original rate) was determined using Dixon plots, by assaying at least five drug dilutions of each compound.

From the data obtained in Table 1, the following conclusions can be drawn: azasugars **4**, **6**, **8**, **9** lowered significantly chitin synthase activity with IC_{50} ranging from 1.6 to 38 mM, whereas isomers **5** and **7** were completely inactive at the highest concentration tested. These data also point out important differences between parent iminosugars. Thus, compound **7** was totally inactive at the highest concentration tested (5 mM) whereas the epimer **4** inhibited enzyme activity by 50% at the same concentration. This effect was also observed for the phosphonoazasugars since compound **9** (IC_{50} = 1.6 mM) was significantly more active than its C-5 epimer **6** (IC_{50} = 38 mM) or its C-2 epimer **8** (IC_{50} = 4 mM).

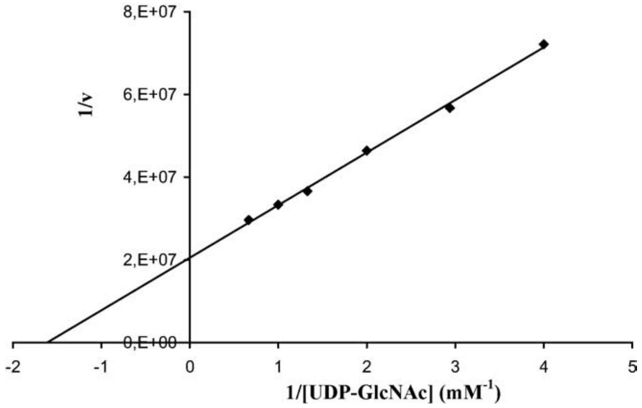

Fig. 5. K_m determination for UDP-GlcNAc.

Table 1
Inhibition of chitin synthase with iminosugars **4–9**

Compounds	CS1 inhibitionIC_{50} (mM)
4	5.7 ± 0.8
5	NI[a] at 8 mM
6	38 ± 4
7	NI[a] at 5 mM
8	4.0 ± 0.5
9	1.6 ± 0.2

[a] No inhibition.

Unfortunately, incorporation of a difluoromethylphosphonate moiety in the structure did not markedly increase the inhibitory potencies. Indeed, conflicting results might be noticed when comparing the affinities of pyrrolidinol **7** and its phosphonated analogue **8** (increase in inhibition) or compounds **4** and **5** (decrease in inhibition). Furthermore, the results obtained with phosphonoazasugar **6** were particularly disappointing since, in our minds, the structural feature of **6** was thought to reproduce at best the first portion of the pyrophosphate moiety as well as the configurations at C-1, C-3, C-4 and C-5 of UDP-GlcNAc. Clearly, among the compounds tested, best results were obtained with the L-*xylo* configurated difluoromethylphosphono azasugar **9**.

As noticed above, inhibition of glycosidases or glycosyltransferases by iminosugars is commonly attributed to the ability of mimicking both the conformational and the electronic features of the oxocarbenium ion involved at the transition state, in particular through protonation of the amine at physiological pH. However, the potent electronic effect of the difluoromethylene substituent might significantly influence the basicity of polyhydroxypyrrolidines **5**, **6**, **8**, **9** and thus prevent the requisite protonation. For this purpose we determined the dissociation constants for the fully deprotected difluoromethylphosphonic acid **20** prepared from **18** by treatment with bromotrimethylsilane and subsequent cleavage of the benzyl protecting groups (Fig. 6).

The pK_a values were evaluated with an automatic titrator using a 0.1 N sodium hydroxide solution. Among the three constants thus determined, two might be attributed to the phosphonic acid (1.73–3.71) and one to the ammonium salt (6.64). In consequence, the state of ionization of difluoromethylphosphono-azasugars **5**, **6**, **8**, **9** at pH 6.5 (standard pH of the enzymatic assay) differs significantly from that of "simple" iminosugars of type **4** or **7** (pK_a ca. 7.2). The lower basicity of azasugars **5**, **6**, **8**, **9** might thus explain, at least in part, the moderate affinities of these models for chitin synthase.

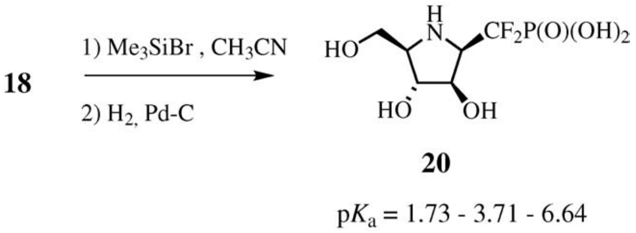

Fig. 6. Dissociation constants of difluoromethylphosphonic acid **20**.

In addition to the enzymatic evaluation, antifungal activity of compounds **4–9** was also tested on a panel of several pathogenic fungi (*C. neoformans*, *T. mentagrophytes*, *M. canis*, *A. fumigatus*, *C. albicans*, *S. cerevisiae*). Unfortunately, azasugars **4–9** showed no activity up to the maximum tested concentration of 128 µg/ml, whereas the standard compound terbinafine showed the expected activity [21]. Modest chitin synthase inhibition potencies as well as poor lipophilic character of the tested compounds might explain the absence of antifungal activity.

5. Conclusion

We designed and synthesized new difluoromethylphosphono-azasugars **5**, **6**, **8**, **9** as well as known pyrrolidinols **4**, **7** as chitin synthase inhibitors. Some of these models displayed encouraging enzyme inhibition with IC_{50} in the millimolar range. However, no antifungal activity was observed against a panel of pathogenic fungal strains. Furthermore, introduction of a difluoromethylphosphonate moiety in the structure, mimicking the first portion of the pyrophosphate moiety of the donor, did not improve the binding affinities. Due to the electronic effect of this substituent, only partial protonation of the pyrrolidine ring might occur in situ, which precludes efficient recognition of compounds **5**, **6**, **8**, **9** as transition state analogues. However, these pyrrolidinols with unprecedented substitution pattern, might be exploited as valuable tools for inhibition of parent glycosyltransferases utilizing a sugar-nucleotide donor.

6. Experimental protocols

6.1. Chemistry

Merck silica gel F254 (0.2 mm) was used for TLC plates, detection being carried out by spraying with an alcoholic solution of phosphomolybdic acid or an aqueous solution of $KMnO_4$ (2%)/Na_2CO_3 (4%), followed by heating. Flash column chromatography (FC) was performed on silica gel Merck 9385 (40–63 µM) Kieselgel 60. NMR spectra were recorded on a Brücker AC 250 spectrometer (250 MHz for ^{1}H, 62.5 MHz for ^{13}C). Chemical shifts are expressed in parts per million from TMS (^{1}H and ^{13}C) as internal standard. Coupling constants are in Hz and splitting pattern abbreviations are: b, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Optical rotations were determined with a Perkin–Elmer Model 241 polarimeter. Mass spectra were recorded on a Jeol D 30 spectrometer, at 70 eV.

6.1.1. *(3S,4R,5S,6S)-3-benzylamino-4,5,7-tribenzyloxy-6-hydroxyhept-1-ene **11***

To a stirred solution of glycosylamine **10** (3 g, 5.9 mmol) in THF (20 ml) at 0 °C, was added vinylmagnesium bromide (35 ml of a commercial 1 M solution, 35 mmol) and the resulting mixture was left to react at rt for 7 h. Saturated NH_4Cl was then added and the solution was extracted three times with Et_2O. The combined organic layers were dried ($MgSO_4$), evaporated and the crude aminoalcool was purified by FC (EtOAc/petroleum ether: 4:6; Rf = 0.5) to give pure **11** as a yellow oil (2.83 g, 91%). ^{1}H NMR ($CDCl_3$) 7.30–7.20 (m, 20H, Ar-H), 5.70 (ddd, *J* 18.8 2 × 9.4, C*H*=CH$_2$), 5.20 (dd, *J* 9.4 1.0, 1H, CH=C*H*$_2$), 5.10 (dd, *J* 18.8 1.0, 1H, CH=C*H*$_2$), 4.70–4.40 (m, 6H, 3xC*H*$_2$Ph), 4.10 (dd, *J* 6.2 5.0, 1H, H-6), 3.75 (d, *J* 6.2, 1H, H-5), 3.80–3.50 (m, 6H). – ^{13}C NMR ($CDCl_3$) 139.0–138.0 (Ar-C), 138.2 (*C*H=CH$_2$), 130.0–127.0 (Ar-C), 117.4 (CH=CH$_2$), 81.0 and 75.8 (C-4 and C-5), 78.9 , 73.6 and 73.1 (3 × *C*H$_2$Ph), 70.5 (C-7), 69.2 (C-6), 58.5 (C-3), 54.2 (N*C*H$_2$Ph).

6.1.2. *(2S,3R,4R,5R)-N-benzyl-3,4-dibenzyloxy-5-[(benzyloxy)methyl]-2-vinyl-pyrrolidine **12***

To a stirred solution of aminoalcool **11** (4.76 g, 8.86 mmol) in pyridine (13 ml) at 0 °C, was added methanesulfonyl chloride (1.3 ml, 1.1 eq) and the resulting mixture was left to react at rt for 16 h. The reaction was quenched with water and the solution was extracted twice with CH_2Cl_2. The combined organic layers were dried ($MgSO_4$), evaporated and the crude pyrrolidine was purified by FC (EtOAc/petroleum ether: 2:8; Rf = 0.7) to give pure **12** as a yellow oil (3.80 g, 80%). $[\alpha]_D$ = +30.1 (*c* 2.14, $CHCl_3$). – ^{1}H NMR ($CDCl_3$) 7.40–7.10 (m, 20H, Ar-H), 6.00 (ddd, *J* 18.8 2 × 9.4, C*H*=*C*H$_2$), 5.30 (dd, 1H, *J* 9.4 1.0, CH=C*H*$_2$), 5.25 (dd, *J* 18.8 1.0, CH=C*H*$_2$), 4.60–4.20 (m, 6H, 3 × C*H*$_2$Ph), 4.00 (d, 1H, J_{AB} 12.5, NC*H*$_2$Ph), 3.88 (d, 1H, *J* 2.2, H-4), 3.79 (d, 1H, *J* 4.6, H-3), 3.6 (d, 1H, J_{AB} 12.5, NC*H*$_2$Ph), 3.46 (m, 1H, H-2), 3.37 (dd, 1H, *J* 9.2 8.4, C*H*$_2$OBn), 3.12 (dd, 1H, *J* 8.4 4.5, C*H*$_2$OBn), 3.05 (m, 1H, *J* 8.4 4.5, H-5). – ^{13}C NMR ($CDCl_3$) 139.0–137.0 (Ar-C), 136.0 (*C*H=CH$_2$), 129.6–126.9 (Ar-C), 118.4 (CH=CH$_2$), 84.6 (C-3), 83.6 (C-4), 72.9 and 71.7 (2 × *C*H$_2$Ph), 71.6 (C-6), 70.8 (*C*H$_2$Ph), 69.9 (C-2), 67.8 (C-5), 56.7 (N*C*H$_2$Ph). – ESI-MS (NH_3) *m/z* 520 [(M + H)$^+$].

6.1.3. *(2S,3R,4R,5R)-N-benzyl-3,4-dibenzyloxy-5-[(benzyloxy)methyl]-2-hydroxymethyl pyrrolidine **13***

Concentrated H_2SO_4 was added drop by drop to a solution of alkene **12** in Et_2O at 0 °C to form the insoluble sulfate salt. The supernatant was then carefully removed and the residual orange oil was washed twice with 5 ml Et_2O, dissolved in 50 ml CH_2Cl_2 and subjected to ozonolysis at –50 °C. After 25 min, the reaction was quenched with Me_2S and the solution neutralized with 20 ml of sat. $NaHCO_3$. The organic layer was dried ($MgSO_4$), concentrated and dissolved in EtOH (10 ml). At 0 °C, $NaBH_4$ (225 mg) was added and to the resulting solution was stirred 1 h at rt. A saturated solution of NH_4Cl was then added and the organic layer was separated, dried and concentrated to give crude **13** which was purified by FC (EtOAc/petroleum ether: 2:8; Rf = 0.5) and isolated as a yellowish oil (450 mg, 56%, yellow oil). $[\alpha]_D$ = –21.8 (*c* 0.68, $CHCl_3$). – ^{1}H NMR ($CDCl_3$) 7.40–7.10 (m, 20H, Ar-H), 4.66 (d, 1H, J_{AB} 12.0, C*H*$_2$Ph), 4.58 (d, 1H, J_{AB} 12.0, C*H*$_2$Ph), 4.56 (d, 1H, J_{AB} 12.0, CH_2Ph), 4.50 (d, 1H, J_{AB} 12.0, CH_2Ph), 4.39 (s, 2H, CH_2Ph), 4.18 (t, 1H, *J* 2 × 5.0, H-4), 4.10 (dd, 1H, *J* 5.0 6.7, H-3), 3.86 (s, 2H, NCH_2Ph), 3.60 (dd, 1H, *J* 2.7 11.2, 1 × CH_2OH), 3.40 (m, 2H, 1 × CH_2OH and 1 × CH_2OBn), 3.32 (dd, 1H, *J* 4.0 10.0, 1 × CH_2OBn), 3.18 (ddd, 1H, *J* 2.7 4.7 6.7, H-2), 3.05 (ddd, 1H, *J* 4.0 4.5 5.0, H-5). – ^{13}C NMR ($CDCl_3$) 139.0–137.0 (Ar-C), 129.3–127.6 (Ar-C), 83.9 and 82.7 (C-3 and C-4), 73.5 (OCH_2Ph), 72.6 (OCH_2Ph), 72.5 (CH_2OBn), 70.4 (OCH_2Ph), 67.1 (C-5), 65.0 (C-2), 60.7 (CH_2OH), 59.2 (NCH_2Ph). – ESI-MS (NH_3) *m/z* 524 [(M + H)$^+$].

6.1.4. *2,5-Dideoxy-2,5-imino-D-glucitol **4***

A solution of benzylated pyrrolidine **13** (436 mg, 1.0 mmol), 10% Pd–C (800 mg) and aq. HCl (0.1 ml of a 1 M solution) in MeOH (4 ml) was stirred under H_2 at rt. After 48 h, the reaction mixture was filtered over a celite pad and the solvent was evaporated. The crude material was purified by silica gel FC ($CHCl_3$/MeOH/NH_4OH, 60:30:5) to give iminosugar **4** (75 mg, 46%) as a yellowish oil. $[\alpha]_D$ = + 22.7 (*c* 0.77, H_2O) [11]: $[\alpha]_D$ = +27.6 (*c* 1.3, MeOH),[23]: $[\alpha]_D$ = +25.1 (*c* 1.5, H_2O). – ^{1}H NMR (D_2O) 4.06 (dd, 1H, *J* 2.1 4.4), 3.82 (dd, 1H, *J* 2.1 3.6), 3.80–3.50 (m, 4H, H-2, H-6), 3.32 (q, 1H, *J* 5.0), 3.02 (dd, 1H). – ^{13}C NMR (D_2O) 81.2, 79.4, 67.6, 64.3, 63.6, 62.2. – ESI-MS (NH_3) *m/z* 186 [(M + Na)$^+$], 164 [(M + H)$^+$].

6.1.5. *(2S,3R,4R,5S)-N-benzyl-3,4-dibenzyloxy-5-[(benzyloxy)methyl]-2-vinyl pyrrolidine **14***

Pyrrolidine **14** was prepared as described [15] with slight modifications (replacement of Tf_2O by MsCl for cyclization). $[\alpha]_D$ = -39.4 (*c* 2.2, $CHCl_3$) (Ref. [15]: $[\alpha]_D$ = –28.6 (*c* 1; $CHCl_3$). ^{1}H NMR and ^{13}C NMR data were identical to those reported.

6.1.6. *(2S,3R,4R,5S)-N-benzyl-2-hydroxymethyl-3,4-dibenzyloxy-5-[(benzyloxy)methyl]-2-vinyl-pyrrolidine **15***

Ozonolysis of the sulfate salt of pyrrolidine **14** (1.097 g, 2.11 mmol) in CH_2Cl_2 (70 ml) was performed as described for the preparation of **13**. Subsequent reduction of the resulting aldehyde with $NaBH_4$ (280 mg) gave, after FC (EtOAc/petroleum ether: 2:8; Rf = 0.5), pure **15** (1.006 g, 91%) as a yellowish oil. $[\alpha]_D$ = –18.0 (*c* 5.28, $CHCl_3$). – ^{1}H NMR ($CDCl_3$) 7.40–7.20 (m, 20H, Ar-H), 4.80 (d, 1H, J_{AB} 12.0, CH_2Ph), 4.68 (d, 1H, J_{AB} 12.0, CH_2Ph), 4.51 (dd, 1H, *J* 3.7 7.0, H-3), 4.60 (m, 4H, 4 × CH_2Ph), 4.23 (t, 1H, *J* 7.0, H-4), 4.00 (s, 2H, NCH_2Ph), 3.65 (m, 3H, 3 × CH_2O–), 3.60 (dd, 1H, *J* 2.9 9.9, CH_2O), 3.40 (ddd, 1H, *J* 3.7 3.7 8.3, H-2), 3.35 (ddd, 1H, *J* 2.9 2.9 9.9, H-5). – ^{13}C NMR ($CDCl_3$) 139.3, 138.4, 138.2 (Ar-C), 128.3–126.9 (Ar-C), 84.4 (C-4), 83.2 (C-3), 73.4, 72.9, 72.6 (3 × OCH_2Ph), 66.2 (CH_2OBn), 63.3 (C-2), 60.3 (CH_2OH), 58.3 (C-5), 52.6 (NCH_2Bn). – ESI-MS (NH_3) *m/z* 524 [(M + H)$^+$].

6.1.7. *2,5-Dideoxy-2,5-imino-L-iditol **7***

Debenzylation of **15** (0.25 g, 0.48 mmol) in the conditions stated above for the synthesis of **4** gave pure **7** (25 mg, 35%) as a colorless precipitate. $[\alpha]_D$ = +16.8 (*c* 0.5, H_2O)[14] $[\alpha]_D$ = +14.3 (*c* 0.93, H_2O). – ^{1}H NMR (D_2O) 4.10 (d, 2H, *J* 3.7, H-3, H-4), 3.68 (dd, 2H, *J* 6.7 10.3, 2 × CH_2OH), 3.57 (dd, 2H, *J* 6.7 10.3, 2 × CH_2OH), 3.36 (dt, 2H, *J* 3.7 6.7 6.7, H-2, H-5). – ^{13}C NMR (D_2O) 77.1, 65.4, 60.0. – ESI-MS (NH_3) *m/z* 186 [(M + Na)$^+$], 164 [(M + H)$^+$].

6.1.7.1. General procedure for the debenzylation of phosphonoazasugars. A solution of each protected phosphonoazasugar **16–19** (0.44 g, 0.65 mmol) in MeOH (9 ml) was refluxed for 1 h in presence of 10% Pd–C (0.891 mg) and HCO_2NH_4 (2 × 0.286 g). The resulting mixture was filtered over a celite pad and the solvent was evaporated. Crude compounds **5** and **8** were obtained and purified at this stage by chromatographic separation on a hydrophobic HP20SS support (eluent, MeOH/H_2O: 3:7). Compounds **6**, **9** were obtained after subsequent treatment with 2 ml of 10% NH_4OH for 16 h, lyophilization of the solution and purification by chromatography on HP20SS (Prolabo).

*6.1.7.2. (2S,3R,4R,5S)-2-[(1-ethylphosphonyl)-1,1-difluoromethyl]-3,4-dihydroxy-5-hydroxymethyl-pyrrolidine **5**.* General procedure from **18** gave **5** as a white foam after purification (74%). $[\alpha]_D$ = +23.3 (*c* 0.9, H_2O). – ^{1}H NMR (D_2O) 4.35 (m, 1H, H-3), 4.10 (m, 2H, CH_2CH_3), 3.95 (m, 1H, H-4), 3.65–3.90 (m, 3H, H-2, H-6a, b), 3.20 (q, 1H, *J* 5.4, H-5), 1.30 (t, 3H, CH_3CH_2). – ^{13}C NMR (D_2O) 122,5 (m, CF_2), 80.4 (C-3), 79.1 (d, *J* 4.5, C-4), 67.8 (C-5), 66.3 (m, CH_2CH_3), 64.3 (qd, C-2), 64.1 (C-6), 18.7 (m, CH_3CH_2). – ^{19}F NMR (D_2O, 235 MHz) –107.6 (ddd, 1F, $^2J_{F,F}$ 300, $^2J_{F,P}$ 90, $^3J_{F,H}$ 11.5), –115.6 (ddd, 1F, $^2J_{F,F}$ 300, $^2J_{F,P}$ 90, $^3J_{F,H}$ 18.7). – ^{31}P NMR (D_2O, 101 MHz) 6.6 (bt). – ESI-MS (NH_3) *m/z* 313.9 [(M + Na)$^+$], 292 [(M + H)$^+$].

*6.1.7.3. (2R,3R,4R,5S)-2-[(1-ethylphosphonyl)-1,1-difluoromethyl]-3,4-dihydroxy-5-hydroxymethyl-pyrrolidine **6**.* General procedure from **16** gave **6** as a white foam after purification (45%). $[\alpha]_D$ = +21.5 (*c* 0.8, MeOH). – ^{1}H NMR (D_2O) 4.24 (t, 1H, *J* 7.0, H-3), 4.03 (m, 2H, CH_2CH_3), 3.86 (t, 1H, *J* 7.0, H-4), 3.8-3.4 (m, 3H, H-2, H-6a, b), 3.13 (m, 1H, H-5), 1.25 (t, 3H, CH_3CH_2). – ^{13}C NMR (D_2O) 122.6 (m, CF_2), 78.8 (C-3), 78.2 (C-4), 66.3 (m, CH_2CH_3), 65.4 (qd, C-2), 64.6 (C-5), 62.9 (C-6), 18.7 (m, CH_3CH_2). – ^{19}F NMR (D_2O, 235 MHz) –115.1 (ddd, 1F, $^2J_{F,F}$ 298, $^2J_{F,P}$ 90, $^3J_{F,H}$ 12.4), –120.2 (ddd, 1F, $^2J_{F,F}$ 298, $^2J_{F,P}$ 90, $^3J_{F,H}$ 3.3). – ^{31}P NMR (D_2O, 101 MHz) 6.5 (bt). – ESI-MS (NH_3) *m/z* 314 [(M + Na)$^+$], 292 [(M + H)$^+$].

*6.1.7.4. (2S,3R,4R,5R)-2-[(1-ethylphosphonyl)-1,1-difluoromethyl]-3,4-dihydroxy-5-hydroxymethyl-pyrrolidine **8**.* General procedure from **19** gave **8** as a white foam after purification (62%). $[\alpha]_D$ = +3.8 (*c* 1.36, H_2O). – ^{1}H NMR (D_2O) 4.50 (m, 1H, H-3), 4.31 (m, 1H, H-4), 4.10 (m, 1H, H-2), 3.97 (m, 2H, CH_2CH_3), 3.90–3.70 (m, 3H, H-5, H-6a, b), 1.25 (t, 3H, CH_3CH_2). – ^{13}C NMR (D_2O) 121.4 (m, CF_2), 77.8 (C-3), 77.2 (C-4), 66.6 (m, CH_2CH_3), 65.8 (C-5), 64.8 (qd, C-2), 60.5 (C-6), 18.8 (m, CH_3CH_2). – ^{19}F NMR (D_2O, 235 MHz) –103.8 (ddd, 1F, $^2J_{F,F}$ 302, $^2J_{F,P}$ 84, $^3J_{F,H}$ 8); –116.6 (ddd, 1F, $^2J_{F,F}$ 302, $^2J_{F,P}$ 86, $^3J_{F,H}$ 21). – ^{31}P NMR (D_2O, 101 MHz) 4.8 (bt). – FAB-MS (glycerol) *m/z* 314 [(M + Na)$^+$], 292 [(M + H)$^+$].

*6.1.7.5. (2R,3R,4R,5R)-2-[(1-ethylphosphonyl)-1,1-difluoromethyl]-3,4-dihydroxy-5-hydroxymethyl-pyrrolidine **9**.* General procedure from **17** gave **9** as a white foam after purification (61%). $[\alpha]_D$ = –26.7 (*c* 0.3, MeOH). – ^{1}H NMR (D_2O) 4.27 (dd, *J* 3.7 4.8, H-3), 4.20–4,00 (m, 3H, H-4, CH_2CH_3), 3.79 (dd, 1H, *J* 5.5 11.7, H-6a), 3.64 (dd, 1H, *J* 6.6 11.7, H6b), 3.53–3.34 (m, 2H, H-2, H-5), 1.28 (t, 3H, CH_3CH_2). – ^{13}C NMR (D_2O) 120.6 (m, CF_2), 79.1 (C-3, C-4), 68.4 (q, C-2), 66.1 (m, CH_2CH_3), 63.5 (C-5), 62.1 (C-6), 18.7 (m, CH_3CH_2). – ^{19}F NMR (D_2O, 235 MHz) –115.5 (ddd, 1F, $^2J_{F,F}$ 297, $^2J_{F,P}$ 92, $^3J_{F,H}$ 12.5), –118.7 (ddd, 1F, $^2J_{F,F}$ 297, $^2J_{F,P}$ 92, $^3J_{F,H}$ 17.2). – ^{31}P NMR (D_2O, 101 MHz) 6.6 (bt). – ESI-MS (NH_3) *m/z* 314 [(M + Na)$^+$], 292 [(M + H)$^+$].

6.1.8. (2S,3R,4R,5S)-2-[(1,1-difluoromethylphosphonic acid]-3,4-dihydroxy-5-hydroxymethyl-pyrrolidine 20

A solution of protected phosphonate **18** (0.64 g, 1.15 mmol) and Me_3SiBr (0.7 ml, 5.3 mmol) in CH_3CN was stirred at rt for 7 days. The solvents were evaporated and the residual oil was dissolved in EtOH (15 ml) and stirred for 3 days under H_2 in presence of 10% Pd–C (0.15 g). Filtration over a celite pad and evaporation of the solvents gave a brown oil from which **5** crystallized in the presence of EtOH (0.125 g, 53%). Analytically pure **5** was obtained by preparative RP-HPLC (eluent, H_2O). $[\alpha]_D$ = +47 (*c* 0.36, H_2O). – ^{1}H NMR (D_2O) 4.50 (dd, 1H, *J* 1.3 3.0, H-3), 4.20 (m, 1H, H-2), 4.10 (m, 1H, H-4), 3.95 (dd, 1H, *J* 5.5 12.2, H6a), 3.85 (dd, 1H, *J* 8.5 12.2, H6b), 3.60 (m, 1H, H-5). – ^{13}C NMR (D_2O) 120.2 (m, CF_2), 78.2 (C-4), 77.0 (d, *J* 4.5, C-3), 70.4 (C-5), 65.2 (m, C-2), 61.8 (C-6). – ^{19}F NMR (D_2O, 235 MHz) –105.2 (ddd, 1F, $^2J_{F,F}$ 304, $^2J_{F,P}$ 84, $^3J_{F,H}$ 8.5), –115.8 (ddd, 1F, $^2J_{F,F}$ 304, $^2J_{F,P}$ 84, $^3J_{F,H}$ 20). – ^{31}P NMR (D_2O, 101 MHz) 3.8 (t). – FAB-MS (glycerol) *m/z* 286 [(M + Na)$^+$], 264 [(M + H)$^+$].

6.2. Chitin synthase assay

Digitonin, trypsin, soybean trypsin inhibitor, UDP-GlcNAc were obtained from Sigma. UDP trisodium salt hydrate was from Aldrich and *N*-acetyl-D-glucosamine from Acros. [UDP^{14}C]-GlcNAc (289 mCi/mmol) came from Perkin–Elmer Life Sciences.

Assays were carried out at 30 °C in a volume of 60 µl which contained, in addition to the activated permeabilized cells preparation (40 µl), the following final concentrations of components: 30 mM Tris–HCl (pH 6.5), 35 mM GlcNAc, 3 mM $Mg(Ac)_2$, digitonin (3.5 mg/ml), cell suspension (52 mg/ml) and 1 mM UDP-[^{14}C]-GlcNAc (20,000 cpm).

Reaction was initiated by addition of the cell suspension. After 30 min incubation the reaction was stopped by adding 1 ml of 10% trichloroacetic acid and 950 µl of the resulting suspension were filtered through a glass-fiber filter (Whattman GF-C). The filter was washed 3 times with 1 ml 60% aqueous EtOH and dried. The ^{14}C-chitin formed was quantitated by liquid scintillation counting in 4 ml of ultima flow AP (Packard) scintillation fluid.

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