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Five-Membered Ring Azasugars as Potent Inhibitors of α-L-Rhamnosidase (Naringinase) from *Penicillium decumbens*

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Abstract—Five-membered ring azasugars with the L-rhamnose configuration were synthesized as inhibitors of α-L-rhamnosidase from *Penicillium decumbens*. All compounds tested were in the μM or sub-μM range. Substitution at the nitrogen shifted the inhibition mechanism from mixed to competitive.

Introduction

Glycosidases are involved in several metabolic pathways and the development of selective inhibitors has become an important strategy towards the treatment of numerous diseases.[1,2] The great potential of polyhydroxylated pyrrolidines and piperidines as inhibitors of glycosidases is well recognized and many azasugars have been used to investigate the hydrolytic mechanism of glycosidases.[3–6]

α-L-Rhamnosidase (EC 3.2.1.40) and β-D-glucosidase (EC 3.2.1.21) are the two active enzymes in naringinase from *Penicillium decumbens*.[7,8] The former cleaves rhamnose from naringin, the main bitter component of several citrus juices, to liberate prunin. Naringinase is thus used commercially as debittering agent.[9] Rhamnose is also a constituent of many other natural flavonoid glycosides (e.g. poncirin, hesperin, rutin),[10] plant tissues and bacterial cell walls. The specificity of this rhamnosidase[7] as well as that from other sources[11,12] is known and the enzyme was successfully used in the structure determination of a complex polysaccharide.[13–15] As part of our efforts towards the development of new glycosidase inhibitors in general, this paper reports the synthesis of a series of new azasugars, compounds **1–4**, for use to study the inhibition of α-L-rhamnosidase.

Results and Discussion

The five-membered ring azasugars **1–4** are expected to have the advantages of both six-membered (nojirimycin-type) and five-membered (polyhydroxylated pyrrolidine) systems. The nojirimycin-type azasugars (e.g. **5**) can mimic the transitory charge generated during hydrolysis of glycosides while the pyrrolidine-type azasugars also mimic the shape of the postulated flattened half-chair transition state (Figure 1).

Imine **1** was synthesized from 2,3-*O*-isopropylidene-D-xylose diethyl dithioacetal[16] (**6**) obtained in three steps from D-xylose (Scheme I). The amino group was introduced via an azide displacement of the primary tosylate, followed by inversion at C-4 through aziridine formation. Subsequent deprotection provided imine **1** which upon dissolution in water exists in equilibrium between hydrated and dimeric forms[17–21] (Scheme II).

The equilibrium favors the imine form under basic conditions as determined by the ^{13}C NMR spectrum (Figure 2). Similar equilibrium behavior was also observed for the immunomodulator FR 900483,[22,23] its epimer[24] and a fuco-imine analog.[25]

Reduction of imine **1** with sodium cyanoborohydride in methanol gave azasugar **2** which was appropriately substituted in order to get azasugars **3** and **4**[26] (Scheme III).

L-1-Deoxyrhamnojirimycin was synthesized both chemically and enzymatically in order to investigate different results reported by other groups.[27,28] The chemical synthesis was accomplished starting from D-gulonolactone according to the procedure of Fleet *et al.*,[27,29] and enzymatically according to the procedure reported from this laboratory[4] using a recombinant rhamnulose-1-phosphate aldolase from *E. coli* K12 (Scheme IV).

All kinetic studies on α-L-rhamnosidase[30] were done in 40 mM sodium acetate buffer, pH 5.0, which provides an efficient buffering capacity while no significant effect was observed by varying the ionic strength (5–300 mM Coulomb[2]) of the buffer. The results of the inhibitory studies are presented in Table 1.

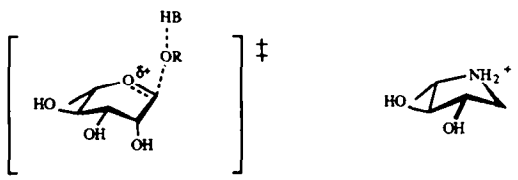
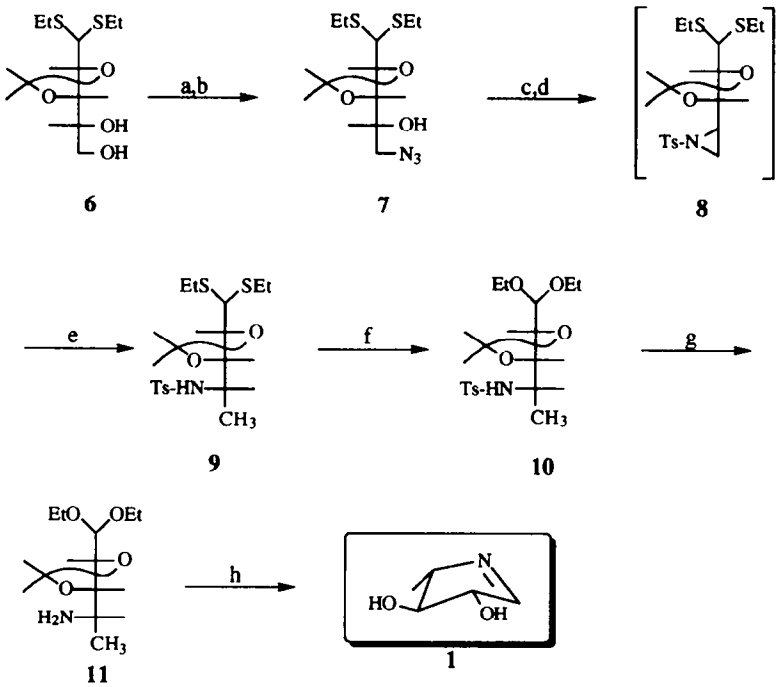
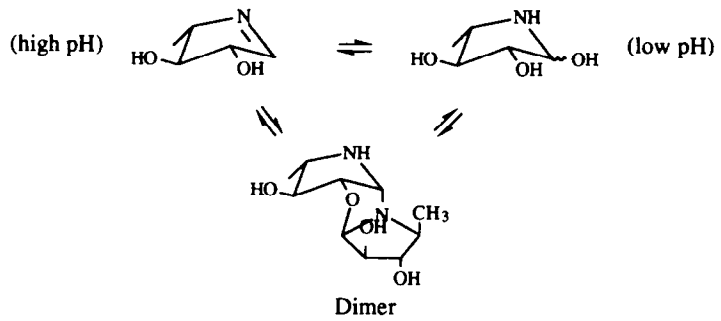


Figure 1. Comparison of a pyrrolidine-type azasugar with the proposed transition state of the rhamnosidase reaction.

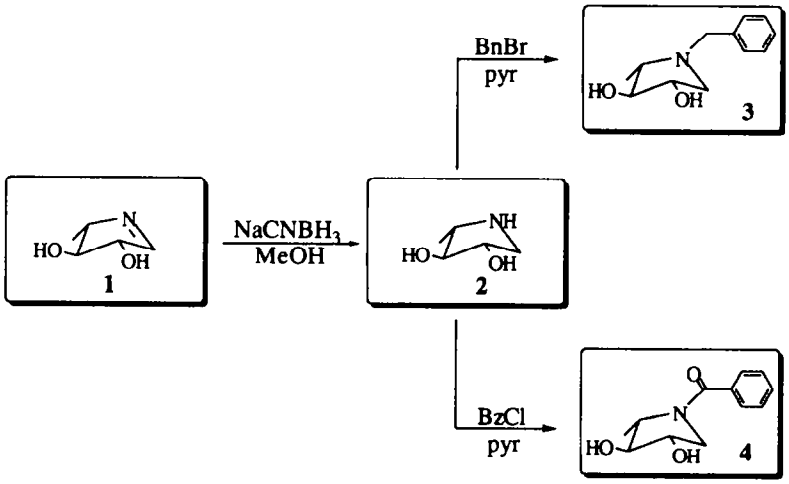


[a](a) *p*-TsCl (1.2 eq.), pyr. (b) NaN_3 (6 eq.), DMF, 80 °C. (c) PPh_3 (1.0 eq.) toluene, reflux. (d) TsCl (1.3 eq.) TEA (1.3 eq.) CH_2Cl_2. (e) Red-Al (1.2 eq.). (f) $HgCl_2$ (2.0 eq.) HgO (2.5 eq.) EtOH. (g) Na/NH_3. (h) 1 N HCl/MeOH.

Scheme I[a].



Scheme II.



Scheme III.

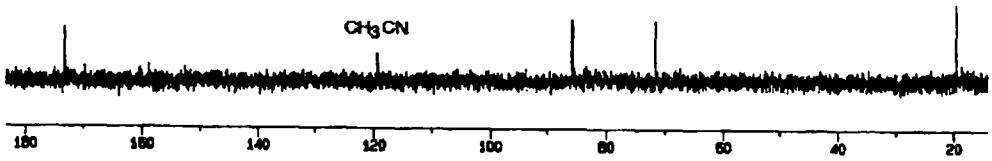
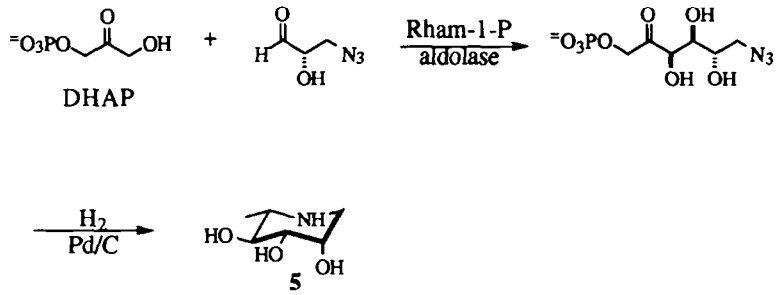


Figure 2. ^{13}C NMR spectrum of **1**.



Scheme IV.

Table 1. Inhibition Study of Glycosidases by Azasugars **1**–**5**

INHIBITOR	α-L-Rhamnosidase K_i[a] μM	α-L-Fucosidase K_i[b] mM
1	K_{is} = 0.14 K_{ii} = 1.2	N.D.
2	K_{is} = 5.5 K_{ii} = 8.5	K_{is} = 7.6 K_{ii} = 17.6
3	11.5	N.D.
4[c]	46	N.D.
5	62[d] 490[e]	N.D.

[a]40 mM NaOAC buffer, pH 5.0; [b]50 mM NaOAc buffer, pH 5.5; [c]3:1 *N*-rotamers; [d]Enzymatic synthesis; [e]Chemical synthesis, average of two assays.

Imine **1** is the best inhibitor reported thus far for α-L-rhamnosidase with K_i in the submicromolar range (Table 1, Figure 3). Reduction of the imine decreases the inhibitory potential by a factor of 40 for azasugar **2**. Further study of the inhibition of **1** revealed a time-dependent inactivation of rhamnosidase (Figure 4). Perhaps a nucleophilic group in the enzyme active site reacts with **1** to form a covalent adduct. Since the inactivation was not first order, the inactivation rate constant was not determined. Both the imine and its reduced form have mixed inhibition patterns while the *N*-aryl-substituted derivatives **3** and **4** are competitive inhibitors. It is possible that the former two compounds can bind to both glycoside and aglycon pockets in the active site. *N*-Benzylation of **1** did not significantly affect the inhibition as expected[31] and clearly shows that either nitrogen substitution or presence of a methylenehydroxy group alpha to the nitrogen is required for competitive inhibition. Consistent with this result, compounds **12**–**15** (Scheme V) are all competitive inhibitors of α-L-fucosidase from bovine kidney.[32]

One interesting result is the inhibitory activity of azasugar **4**. It is usually observed that *N*-carbonyl azasugars such as *N*-acetylated compound lose most of their activity as inhibitors of glycosidases due to the inability of the nitrogen to be protonated. However, *N*-benzoylation of azasugar **1** provided sufficient hydrophobic character to help bind the inhibitor to the active site of the enzyme, thus mimicking the natural substrate naringin which possesses a large hydrophobic group. When protonation at the nitrogen is restored as in azasugar **3**, better inhibition is observed. These results also show that stereochemistry of the remaining two hydroxyl groups on the five-membered ring azasugars is very important since azasugar **2** with the L-rhamnose configuration is a much better inhibitor with α-L-rhamnosidase (μM range) than α-L-fucosidase (mM range).

The L-1-deoxyrhamnojirimycin chemically prepared is a weaker inhibitor (K_i 0.49 mM) than the one prepared from enzymatic synthesis (K_i 62 μM) and confirms both results reported by Fleet[27] and Honek[28] (Figure 5). Both inhibition studies were done in 40 mM NaOAc buffer at pH 5.0. This discrepancy is hard to explain since the spectroscopic data are identical, although the optical rotations are slightly different. This discrepency may be the result of a small amount (< 5 %) of C-5 epimer resulting from the reductive amination step. This possibility is under current investigation.

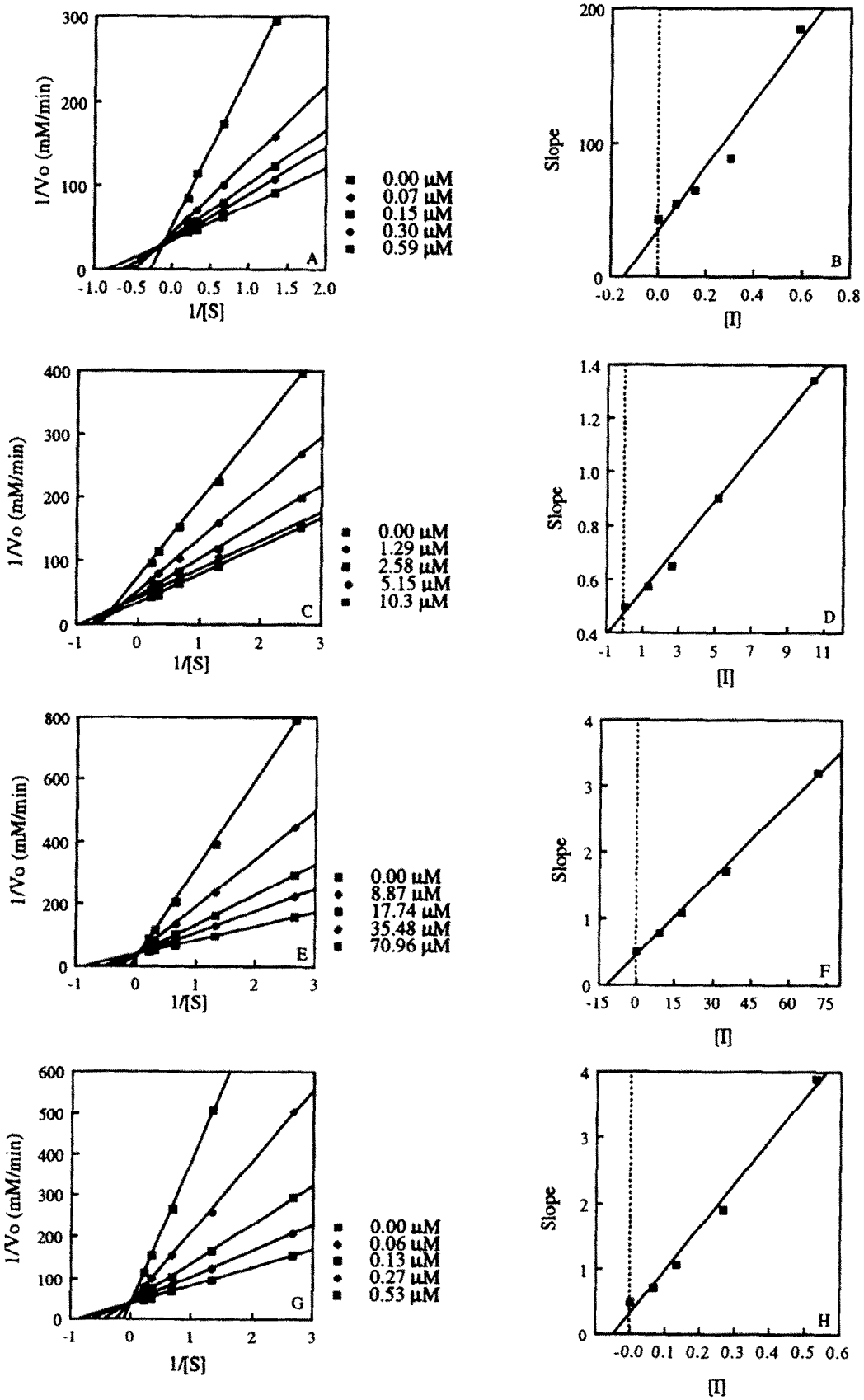


Figure 3. Inhibition analysis of rhamnosidase with azasugars 1–4. Graphs A and B: azasugar **1**; C and D: azasugar **2**; E and F: azasugar **3**; G and H: azasugar **4**.

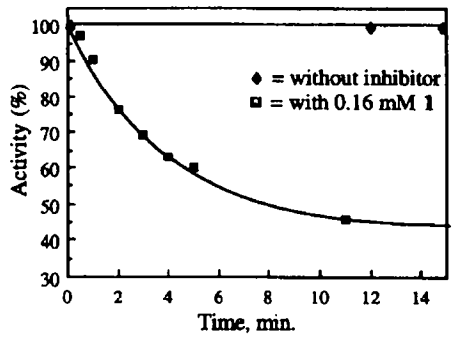
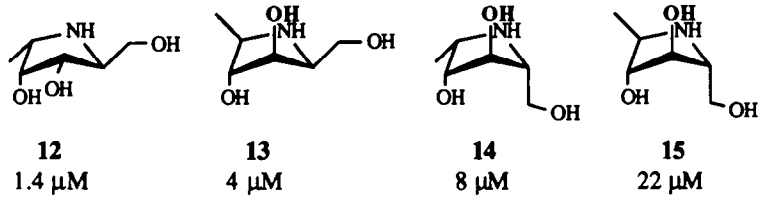


Figure 4. Time-dependent inactivation of rhamnosidase by **1** (see experimental for details).

In summary, this paper presents the first five-membered ring azasugars which are better inhibitors of L-rhamnosidase than their six-membered counterpart with K_i values in the submicromolar range.

Experimental

All reactions were run under an argon atmosphere, unless otherwise stated. NMR Spectra were obtained with a Bruker 250 MHz or 400 MHz instrument. Chemical shifts are reported as parts per million downfield from internal tetramethylsilane. For D_2O, HOD = 4.65 ppm ^1H, and internal CH_3CN = 1.3 ppm ^{13}C were used. IR Spectra



Scheme V.

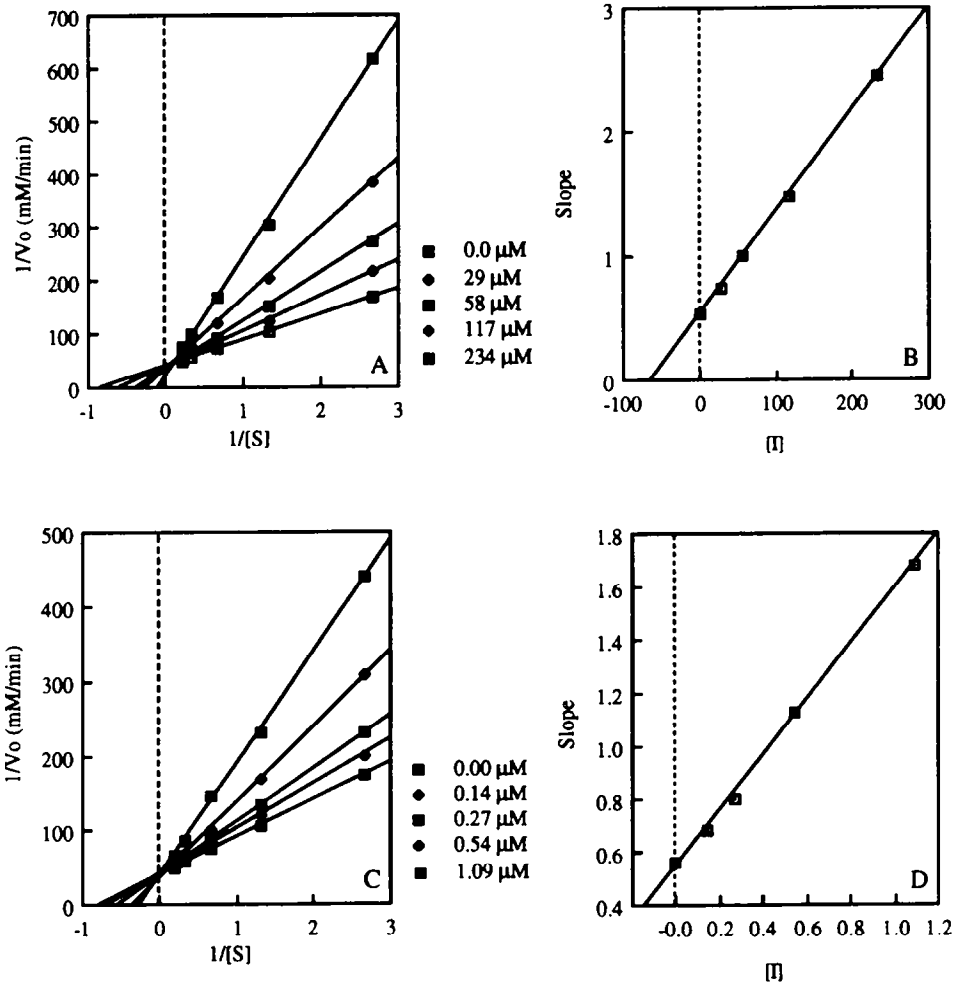


Figure 5. Inhibition analysis of α-rhamnosidase with azasugar **5**. Graphs A and B: enzymatically synthesized azasugar **5**; C and D: chemically synthesized azasugar **5**.

were obtained with a Perkin Elmer FTIR 1600 series spectrophotometer. Solids were run as KBr disks and liquids as neat films. Melting points were recorded on a Thomas Hoover capillary melting point apparatus, and are uncorrected. Boiling points are from Kugelrohr distillations. Optical rotations were obtained with a Perkin-Elmer 141 polarimeter or an Autopol III automatic polarimeter.

2,3-O-Isopropylidene-D-xylose diethyl dithioacetal (6)[33]

To a stirred solution of D-xylose (50.31g, 335 mmol) in 12 N aqueous HCl (50 mL) at 0 °C was added EtSH (55 mL) and the solution stirred at 0 °C for 1 h. H_2O (500 mL) was added and the solution was basified with 14.8 N aqueous NH_4OH (~42 mL). The aqueous solution was extracted with hexanes (2 × 50 mL) and the combined organic layers concentrated to give D-xylose diethyldithioacetal as an oil which slowly crystallized (65.17 g, 76 %). R_f 0.23 (EtOAc). Mp 58 °C (Lit.[16] 59–61 °C). IR ν 3363 (br s, OH), 2961, 2928, 2870, 1653, 1457, 1421 (br), 1375, 1266, 1201, 1121 (s), 1040 (s), 979 (s), 840, 793, 747, 625 cm^{-1}. ^1H NMR (250 MHz, D_2O) δ 1.10 (6H, t, J = 7.4 Hz), 2.50–2.68 (4H, m), 3.47 (1H, dd, J = 11.6 Hz, J' = 6.8 Hz), 3.57 (1H, dd, J = 11.6 Hz, J' = 4.4 Hz), 3.68 (1H, dt, J = 6.7 Hz, J' = 4.1 Hz), 3.75 (1H, t, J = 5.2 Hz), 3.86 (1H, t, J = 4.4 Hz), 3.95 (1H, d, J = 5.8 Hz) ppm. ^{13}C NMR (62.5 MHz) δ 14.4, 14.5, 24.0, 25.5, 55.0, 64.1, 70.5, 73.4, 73.9 ppm. HRMS calcd for $C_9H_{20}O_4S_2Na$: 279.0701. Found $[M+Na]^+$ 279.0700.

Reaction of D-xylose diethyl dithioacetal (25.258 g, 98.52 mmol), 2,2-dimethoxypropane (65 mL), acetone (130 mL) and *p*-toluene sulfonic acid monohydrate (6.184 g, 32.51 mmol) gave 2,3:4,5-di-*O*-isopropylidene-D-xylose diethyl dithioacetal[34] (30.89 g, 92 %). R_f 0.44 (2:8 EtOAc: Hexanes). Bp 138 °C (0.7 mm Hg) (Lit.[33]128 °C/0.05 mm Hg). $[\alpha]_D^{26}$ –49.0° (*c* 1.53, $CHCl_3$) [Lit.[33] –65° (*c* 1.91, acetone)]. IR (neat) ν 2985, 2931, 1455, 1379, 1370, 1249 (br), 1219 (br), 1158, 1070, 992, 969, 885, 850, 751 cm^{-1}. ^1H NMR δ 1.27 (3H, t, J = 7.4 Hz), 1.28 (3H, t, J = 7.4 Hz), 1,38 (3H, s), 1.43 (6H, s), 1.47 (3H, s), 2.60–2.90 (m, unresolved), 3.91 (1H, d, J = 5.3 Hz), 3.95 (1H, d, J = 7.7 Hz), 4.06 (1H, dd, J = 8.1 Hz, J' = 6.7 Hz), 4.15 (1H, dd, J = 7.4 Hz, J' = 3.2 Hz), 4.30–4.38 (2H, m) ppm. ^{13}C NMR (100 MHz) δ 14.2, 14.3, 24.9, 25.3, 25.5, 26.1, 27.1, 27.3, 53.0, 65.9, 75.2, 78.6, 80.0, 109.5, 110.0 ppm. HRMS calcd for $C_{15}H_{28}O_4S_2Na$: 359.1327. Found: $[M+Na]^+$ 359.1320.

To a stirred solution of the above di-*O*-isopropylidene derivative (8.71 g, 25.9 mmol) in methanol (60 mL) and H_2O (12 mL) was added *p*-toluene sulfonic acid monohydrate (2.46 g, 12.9 mmol) and the solution was stirred for 2.5 h. The solution was made basic by addition of triethylamine (1.85 mL) and then concentrated to give a crude oil (11.66 g). The residue was taken up in ethyl acetate (200 mL) and washed with H_2O (2 ×), saturated aqueous $NaHCO_3$ (1 ×), H_2O (1 ×), brine (1 ×), dried ($MgSO_4$) then concentrated[35] to give dithioacetal (**6**) (6.94 g, 90 %). R_f 0.29 (1:1 EtOAc:Hexanes). $[\alpha]_D^{26}$ –62.9° (*c* 1.50, $CHCl_3$) [Lit.[16] $[\alpha]_D^{21}$ –60° (*c* 3.4, $CHCl_3$)]. Bp 150 °C (0.9 mm Hg). IR (neat) ν 3417 (br, OH), 2983, 2929, 2872, 1636 (w), 1455, 1372, 1245 (br s), 1217 (br s), 1165, 1072, 977, 887, 831, 751 cm^{-1}. ^1H NMR δ 1.27 (3H, t, J = 7.4 Hz), 1.28 (3H, t, J = 7.4 Hz), 1.44 (3H, s), 1.47 (3H, s), 2.20 (1H, dd, J = 8.4 Hz, J' = 4.1 Hz), 2.50 (1H, d, J = 9.1 Hz), 2.64–2.86 (4H, m), 3.69–3.85 (2H, m, unresolved), 3.86–3.96 (2H, m), 4.14 (1H, dd, J = 7.6 Hz, J' = 2.1 Hz), 4.39 (1H, dd, J = 7.6 Hz, J' = 6.0 Hz) ppm. ^{13}C NMR (62.5 MHz) δ 14.2, 14.3, 24.9, 25.3, 27.0(7), 27.1(2), 52.8, 65.1, 70.1, 79.4, 80.9, 110.0 ppm. HRMS calcd for $C_{12}H_{24}O_4S_2Cs$: 429.0170. Found: $[M+Cs]^+$ 429.0183.

2,3-O-Isopropylidene-5-O-p-tolylsulfonyl-D-xylose diethyl dithioacetal

To a stirred solution of dithioacetal (**6**) (6.17 g, 20.81 mmol) in dry pyridine (90 mL) at 0 °C was added *p*-TsCl (4.76g, 24.97 mmol). The solution was then stirred at room temperature for 4.5 h. Ethyl ether (175 mL) was added to the solution which was then washed with 1 N aqueous $CuSO_4$ (4 ×), H_2O (1 ×), 0.2 N aqueous NaOH (2 ×), H_2O (1 ×), brine (1 ×), dried ($MgSO_4$), then concentrated to give the corresponding tosylate as a crude oil (18.23 g, 88 %). This tosylate is unstable at room temperature and forms the epoxide over 1–2 days. A small quantity was purified for analytical purposes and the bulk of the product was used without further purification. R_f 0.26 (3:7 EtOAc:Hexanes). IR (neat) ν 3519 (br, OH), 3036 (br w), 2983, 2929, 2872, 1598, 1455, 1371 (br s), 1244, 1215, 1190, 1177, 1097, 1068, 974, 896, 815, 666 cm^{-1}. ^1H NMR δ 1.26 (3H, t, J = 7.3 Hz), 1.27 (3H, t, J = 7.3 Hz), 1.39 (3H, s), 1.41 (3H, s), 2.32 (1H, br t, J = 3.7 Hz), 2.45 (3H, s), 2.60–2.85 (4H, m), 3.87 (1H, d, J = 6.3 Hz), 4.00–4.15 (4H, m), 4.30 (1H, t, J = 7.0 Hz), 7.36 (2H, d, J = 8.1 Hz), 7.82 (2H, d, J = 8.1 Hz) ppm. ^{13}C NMR (62.5 MHz) δ 14.2, 14.4, 21.6, 25.0, 25.4, 27.0, 27.1, 52.9, 68.1, 71.3, 78.6, 78.9, 110.2, 128.0, 129.9, 132.7 ppm.

5-Azido-5-deoxy-2,3-O-isopropylidene-D-xylose diethyl dithioacetal (7)

To a stirred solution of tosylate (**7**) (8.18 g, 18.15 mmol) in dry DMF (50 mL) at room temperature was added NaN_3 (3.54 g, 3.54 mmol). The solution was stirred at 80 °C for 4 h. The solvent was removed under reduced pressure and H_2O (50 mL) was added to the residue. The aqueous phase was extracted with CH_2Cl_2 (4 ×) and the organic phase was dried (cotton plug) and concentrated to give a crude oil (7.17 g). Flash chromatography (SiO_2 250 g, 2:8 EtOAc:Hexanes) yielded azido dithioacetal (**7**) as a clear oil (4.20 g, 72 %). R_f 0.30 (2:8 EtOAc:Hexanes). $[\alpha]_D^{27}$ –65.6° (*c* 1.37, $CHCl_3$). IR (neat) ν 3475 (br OH), 2983, 2929, 2871, 2101 (s, N_3), 1455, 1372, 1258 (br s), 1217, 1165, 1120, 1065, 1004, 976, 883, 829, 782, 751 cm^{-1}. ^1H NMR δ 1.27 (3H, t, J = 7.4 Hz), 1.28 (3H, t, J = 7.4 Hz), 1.45 (3H, s), 1.47 (3H, s), 2.29 (1H, d, J = 9.3 Hz, OH), 2.63–2.87 (4H, m, unresolved), 3.34 (2H, dd, J = 12.6 Hz, J' = 5.2 Hz), 3.48 (2H, dd, J = 12.6 Hz, J' = 7.4 Hz), 3.91 (1H, d, J = 6.3 Hz), 3.95–4.07 (1H, m, unresolved), 4.10 (1H, dd, J = 9.4 Hz, J' = 1.8 Hz), 4.30 (1H, dd, J = 7.5 Hz,

J' = 6.4 Hz) ppm. ^{13}C NMR (62.5 MHz) δ 14.2, 14.3, 24.9, 25.3, 27.0(8), 27.1(2), 53.0, 54.5, 69.6, 79.2, 79.6, 110.1 ppm. HRMS calcd for $C_{12}H_{23}N_3O_3S_2Na$: 344.1079. Found: $[M+Na]^+$ 344.1079.

4,5-Dideoxy-4,5-imino-2,3-O-isopropylidene-L-arabinose diethyl dithioacetal[36] *and 4,5-dideoxy-2,3-O-isopropylidene-4,5-(tosyl)imino-L-arabinose diethyl dithioacetal (8)*

To a stirred solution of Ph_3P (781 mg, 2.98 mmol) in dry toluene (10 mL) was added a solution of azido dithioacetal (**7**) (1.016 g, 2.98 mmol) in dry toluene (5 mL) and the reaction mixture refluxed for 4 h. The toluene was removed *in vacuo* and the triphenylphosphine oxide was precipitated using ethyl ether (13 mL). Filtration followed by concentration gave imino dithioacetal as an oil (1.115 g). R_f 0.23 (1:1 EtOAc:Hexanes). ^1H NMR (250 MHz, $CDCl_3$) δ 1.27 (3H, t, J = 7.4 Hz), 1.29 (3H, t, J = 7.4 Hz), 1.43 (3H, s), 1.45 (3H, s), 1.67 (1H, d, J = 3.3 Hz, H_5, *trans*), 1.83 (1H, d, J = 5.6 Hz, H_5, *cis*), 2.19–2.24 (1H, m), 2.68–2.83 (4H, m), 3.79 (1H, t, J = 6.5 Hz), 3.96 (1H, d, J = 4.9 Hz), 4.24 (1H, dd, J = 7.2 Hz, J' = 4.9 Hz) ppm. ^{13}C NMR (62.5 MHz) δ 14.3, 14.4, 22.8, 25.0, 25.5, 27.0, 27.2, 52.9, 80.8, 83.9, 109.9 ppm. HRMS calcd for $C_{12}H_{23}NO_2S_2Na$: 300.1068. Found: $[M+Na]^+$ 300.1073.

The title compound **8** was obtained by adding *p*-TsCl (738 mg, 3.87 mmol) to a solution of aziridine (1.115 g) and TEA (535 μL, 394 mg, 3.87 mmol) in CH_2Cl_2 (20 mL) at 0 °C. The solution was stirred at 25 °C for 12 h. Et_2O (40 mL) was added and the precipitate (Ph_3PO) was filtered. The solvent was evaporated and the crude oil (2.019 g) was flash chromatographed [SiO_2 80 g, 1:9 EtOAc:Hexanes, 1 % (v/v) TEA] to give sulfonamide **8** (1.135 g, 88 % over 2 steps) as a clear oil. R_f 0.28 (2:8 EtOAc:Hexanes). $[\alpha]_D^{23}$ –62.6° (*c* 1.18, $CHCl_3$). IR (ν) 2984, 2929, 2871, 2102 (w), 1597, 1455, 1373, 1328 (vs), 1236 (br vs), 1163 (vs), 1091 (vs), 982, 926, 903, 882, 816, 752, 721 (vs) cm^{-1}. ^1H NMR (250 MHz, $CDCl_3$) δ 1.24 (3H, t, J = 7.4 Hz), 1.28 (3H, t, J = 7.4 Hz), 1.40 (3H, s), 1.42 (3H, s), 2.46 (1H, d, J = 4.3 Hz), 2.60–2.80 (5H, m), 2.98 (1H, dt, J = 6.9 Hz, J' = 4.3 Hz), 3.75 (1H, d, J = 3.5 Hz), 3.84 (1H, t, J = 7.0 Hz), 4.22 (1H, dd, J = 7.3 Hz, J' = 3.5 Hz), 7.36 (2H, d, J = 8.1 Hz), 7.81 (2H, d, J = 8.3 Hz) ppm. ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 14.3, 21.6, 25.0, 25.3, 26.8, 26.9, 31.7, 39.4, 52.0, 78.2, 83.8, 110.5, 128.0, 129.8, 134.2, 145.0 ppm. HRMS calcd for $C_{19}H_{29}NO_4S_3Na$: 554.1156. Found: $[M+Na]^+$ 554.1161.

4,5-Dideoxy-2,3-O-isopropylidene-4-N-tosylamino-L-arabinose diethyl dithioacetal (9)[37]

To a stirred solution of **8** (960 mg, 2.22 mmol) in dry THF (30 mL) at –78 °C was added Red-Al (3.4 M in toluene, 0.8 mL, 2.67 mmol). The solution was stirred at –78 °C for 45 min, at 0 °C for 2 h, then at 25 °C for 21 h. The reaction was carefully quenched with 1 N KNa-tartrate (Rochelle's salt, 30 mL) then extracted with EtOAc (5 × 20 mL). The organic phase was washed with brine (1 ×), dried ($MgSO_4$), then concentrated to give sulfonamide (**9**) (1.017 g, quant.) as an oil which slowly crystallized. R_f 0.23 (2:8 EtOAc:Hexanes). $[\alpha]_D^{23}$ –55.9° (*c* 1.34, $CHCl_3$). IR (ν) 3271 (s, NH), 2984, 2930, 2872, 1598, 1494, 1455, 1380, 1334 (vs), 1241, 1216, 1163 (vs), 1089 (br vs), 1020, 974, 911, 883, 815, 757(vs) cm^{-1}. ^1H NMR (250 MHz, $CDCl_3$) δ 1.06 (3H, d, J = 6.6 Hz), 1.26 (3H, t, J = 7.4 Hz), 1.27 (3H, t, J = 7.4 Hz), 1.37 (3H, s), 1.38 (3H, s), 2.43 (3H, s), 2.60–2.85 (4H, m), 3.58–3.71 (1H, m), 3.85 (1H, d, J = 5.7 Hz), 3.91 (1H, dd, J = 6.8 Hz, J' = 5.6 Hz), 4.06 (1H, dd, J = 6.8 Hz, J' = 5.7 Hz), 4.57 (1H, d, J = 9.3 Hz), 7.31 (2H, d, J = 8.1 Hz), 7.77 (2H, d, J = 8.3 Hz) ppm. ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 14.3, 14.4, 16.8, 21.5, 25.2, 25.3, 27.0, 27.2, 51.7, 53.3, 81.4, 81.6, 109.8, 127.0, 129.7, 138.0, 143.4 ppm. HRMS calcd for $C_{19}H_{31}NO_4S_3Na$: 456.1313. Found: $[M+Na]^+$ 456.1320.

4,5-Dideoxy-2,3-O-isopropylidene-4-N-tosylamino-L-arabinose diethyl acetal[19] *(10)*

To a stirred solution of sulfonamide (**9**) (240 mg, 0.55 mmol) in dry EtOH (6 mL) at 24 °C was added $HgCl_2$ (300 mg, 1.38 mmol) and HgO (300 mg, 1.38 mmol). This mixture was stirred at 50 °C for 25 h, filtered through Celite, and concentrated. The residue was taken up in CH_2Cl_2, washed with 1 N aqueous KI (3 ×), H_2O (1 ×), dried (cotton plug), and concentrated to give a crude oil. Flash chromatography (SiO_2 10 g, 3:7 EtOAc:Hexanes) yielded **10** (205 mg, 92 %) as a clear oil. R_f 0.32 (3:7 EtOAc:Hexanes). $[\alpha]_D^{26}$ +3.4° (*c* 1.15, $CDCl_3$). IR ν 3213 (br, NH), 2980, 2936, 1598 (w), 1454, 1370, 1335, 1257, 1214, 1163 (br, s), 1093 (br s), 1060, 984, 870, 816, 667 cm^{-1}. ^1H NMR (400 MHz) δ 1.21 (3H, d, J = 6.4 Hz), 1.26 (6H, t, J = 7.2 Hz), 1.27 (3H, s), 1.33 (3H, s), 2.42 (3H, s), 3.30 (1H, sextet, J = 6.5 Hz), 3.49 (1H, dd, J = 7.1 Hz, 5.7 Hz), 3.55–3.71 (3H, m, CH_2 + H_3), 3.75–3.83 (m, 2H), 4.41 (1H, d, J = 5.7 Hz), 5.65 (1H, d, J = 5.6 Hz), 7.29 (2H, d, J = 7.9 Hz), 7.76 (2H, d, J = 8.3 Hz) ppm. ^{13}C NMR (62.5 MHz) δ 15.0, 15.1, 18.0, 21.4, 26.6, 52.2, 62.5, 65.1, 78.8, 79.7, 102.4, 109.8, 127.0, 129.5, 137.6, 143.1 ppm. HRMS calcd for $C_{19}H_{31}NO_6SCs$: 534.0926. Found: $[M+Cs]^+$ 534.0926.

4- Amino-4,5- dideoxy-2,3-O- Isopropylidene -L- arabinose diethyl acetal (11)

To a stirred solution of **10** (1.504 g, 3.75 mmol) in dry THF (5 mL) at –78 °C was added NH_3 (50 mL) followed by the addition of freshly cut solid sodium. A deep blue color persisted for 30 min at which time solid NH_4Cl was added and the ammonia evaporated. The residue was taken up in THF (50 mL) and TEA (2 mL) was added. The mixture was filtered through Celite and concentrated to give **11** (1.068 g, quant.). Further purification was performed by flash chromatography (SiO_2, 40 g, $CHCl_3$, 2 % MeOH, 1 % TEA). R_f 0.13. $[\alpha]_D^{25}$ +9.4° (*c* 1.80, $CDCl_3$). IR ν 3365 (w, NH), 2976 (s), 2930, 2888, 1599, 1451, 1378, 1250, 1211, 1153 (br), 1066 (br s), 872 cm^{-1}. ^1H NMR (250 MHz, $CDCl_3$) δ 1.14 (3H, d, J = 6.6 Hz), 1.22 (3H, t, J = 7.1 Hz), 1.24 (3H, t, J = 7.1 Hz), 1.41 (6H, s), 1.63 (2H, br s, NH_2), 2.99–3.09 (1H, m), 3.52–3.99 (4H, m, 2 × CH_2), 3.86 (2H, dd, J = 3.5 Hz, J' = 1.2 Hz), 4.51 (1H, dd, J = 3.6 Hz, J' = 1.4 Hz) ppm. ^{13}C NMR

(62.5 MHz) δ 15.2, 15.3, 19.0, 27.0, 27.3, 49.0, 62.8, 64.7, 79.2, 82.8, 103.2, 109.2 ppm. HRMS calcd for $C_{12}H_{25}NO_4Cs$: 380.0838. Found: $[M+Cs]^+$ 380.0846.

4-Amino-4,5-dideoxy-L-arabinose, hydrochloride salt (1)

Amine **13** (205 mg, 0.83 mmol) was stirred in 1:1 MeOH:1 N HCl (10 mL) at 24 °C for 18 h. The solution was concentrated ($T < 30$ °C) then dried under vacuum (2 days) to give **1** (142 mg, quant.) as a white solid. Mp 95–98 °C (decompose). R_f 0.41 (7:3:1 CH_2Cl_2:MeOH:28 % NH_4OH (aq)). IR ν 3600–2500 (br s, OH), 2985, 2933, 1718 (w), 1636 (w), 1617 (w), 1507 (m), 1388 (w), 1210, 1139 (m), 1052 (br s) cm^{-1}. Upon dissolution in water, this compound exists as an equilibrium of 3 compounds. Under basic conditions, the imine form is predominant (peak at 7.35 ppm). ^1H NMR (400 MHz, D_2O + 1 drop 30 % NaOD in D_2O) [mixture imine (major), azasugar (minor) and dimer (minor)] δ 7.35 (1H, s, H1), 4.32 (1H, s), 3.43 (2.5 H, br s), 1.95 (2.9H, br s), 0.8–1.1 (4H, m) ppm. ^{13}C NMR (62.5 MHz, D_2O + 1 drop 30 % NaOD in D_2O) δ 19.60, 71.80, 85.71, 85.98, 173.28; HRMS calcd for $C_5H_{11}NO_3$ 116.0712. Found: $[M+H]^+$ 116.0716 (imine); HRMS calcd for $C_5H_{11}NO_3$ 134.0817. Found: $[M+H]^+$ 134.0823 (azasugar); HRMS calcd for $C_{10}H_{18}N_2O_4$ 231.1345. Found: $[M+H]^+$ 231.1350 (dimer).

(3S, 4S)-Dihydroxy-2-(S)-methylpyrrolidine (2)

The hydrochloride salt of amine **1** (666 mg, 4.07 mmol) was dissolved in dry MeOH (25 mL) and the pH adjusted to 6.0 with methanolic NaOH. After addition of $NaCNBH_3$ (256 mg, 4.07 mmol), the solution was stirred at 25 °C for 16 h. The pH was readjusted to 6.0 with methanolic HCl, molecular sieves (3 Å) were added and stirring continued for 34 h. The solution was carefully quenched with 12 N HCl, filtered through Celite, and concentrated. The residue was stirred with Dowex 1X2-400 (OH^- form) in H_2O (30 mL) for 10 h at 25 °C. The mixture was filtered and concentrated to give a crude oil (963 mg). Purification by flash chromatography (SiO_2 50 g, 7:3:1 CH_2Cl_2:MeOH:28 % aq. NH_4OH) gave **2** (246 mg, 52 %) as a thick oil. R_f 0.25. $[\alpha]_D^{25}$ +4.4° (*c* 1.13, MeOH). ^1H NMR (400 MHz, D_2O) δ 1.24 (3H, d, J = 6.9 Hz), 3.00 (1H, dd, J = 12.6 Hz, J' = 3.2 Hz), 3.19 (1H, t, J = 6.2 Hz), 3.25 (1H, dd, J = 12.6 Hz, J' = 5.6 Hz), 3.71 (1H, br t, J = 4.4 Hz), 4.10 (1H, dt, J = 5.6 Hz, J' = 3.4 Hz) ppm. ^{13}C NMR (62.5 MHz, CD_3OD) δ 18.0, 52.7, 62.8, 78.3, 83.9 ppm. HRMS calcd for $C_5H_{12}NO_2$ 118.0868. Found: $[M+H]^+$ 118.0868.

N-Benzyl-(3S,4S)-dihydroxy-2-(S)-methylpyrrolidine (3)

To a stirred solution of **2** (31 mg, 0.26 mmol) in dry pyridine (1 mL) at 0 °C was added benzyl bromide (31 μL, 45 mg, 0.26 mmol). The ice-bath was allowed to warm up to room temperature and the solution was stirred for 3 days. The solvent was evaporated and the crude residue (107 mg) was purified by preparative TLC (Merck SiO_2, 0.5 mm) using 8:2 EtOAc:Hexanes as eluent. This provided pyrrolidine **3** (10 mg, 18 %). R_f 0.62 (8:2 EtOAc:Hexanes). $[\alpha]_D^{25}$ +106° (*c* 1.0, CD_3OD). ^1H NMR (400 MHz, D_2O) δ 1.13 (3H, d, J = 6.3 Hz), 2.30 (1H, quintet, J = 6.6 Hz), 2.53 (1H, dd, J = 11.5 Hz, J' = 2.3 Hz), 2.62 (1H, dd, J = 11.6 Hz, J' = 7.3 Hz), 3.20 (1H, d, J = 12.8 Hz), 3.49 (1H, dd, J = 7.7 Hz, J' = 4.0 Hz), 3.85 (1H, d, J = 12.8 Hz), 3.88 (1H, dd, J = 3.9 Hz, J' = 2.8 Hz), 7.18–7.28 (5H, m) ppm. ^{13}C NMR (62.5 MHz, CD_3OD) δ 16.6, 58.9, 60.9, 67.3, 76.9, 86.1, 128.4, 129.3, 130.5, 138.8 ppm. HRMS calcd for $C_{12}H_{18}NO_2$: 208.1338. Found: $[M+H]^+$ 208.1345.

N-Benzoyl-(3S,4S)-dihydroxy-2-(S)-methylpyrrolidine (4)

To a stirred solution of **2** (107 mg, 0.91 mmol) in dry pyridine (2 mL) at 0 °C was added dropwise benzoyl chloride (1061 μL, 128 mg, 0.91 mmol). The solution was stirred at 0 °C for 2 h then at room temperature for 12 h. Small pieces of ice were added to the solution which was then concentrated. The residue was purified by preparative TLC (Merck SiO_2, 0.5 mm) using 9:1 CH_2Cl_2/MeOH (1 % of 28 % aq. NH_4OH) as eluent. This provided pyrrolidine **4** (11.7 mg, 6 %) (rotamers mixture 2.5:1). R_f 0.25. $[\alpha]_D^{25}$ +59.8° (*c* 1.17, MeOH). ^{13}C NMR (62.5 MHz, CD_3OD) δ (major rotamer) 17.6, 56.0, 61.9, 76.1, 82.1, 127.6, 129, 131.3, 137.9, 172.6; (minor rotamer) 18.8, 53.8, 64.0, 75.5, 82.8, 127.9, 129.6, 131.0, 138.3, 179.9 ppm. HRMS calcd for $C_{12}H_{16}NO_3$: 222.1130. Found: $[M+H]^+$ 222.1130.

L-1-Deoxyrhamnojirimycin (5)

Chemical synthesis[27]. Free amine isolated by flash chromatography. R_f 0.18 (7:3:1 CH_2Cl_2:MeOH:28 % aq. NH_4OH). $[\alpha]_D^{25}$ +41.6° (*c* 1.01, H_2O). ^1H NMR (250 MHz, D_2O) δ 1.24 (3H, d, J = 6.5 Hz), 2.91 (1H, dq, J = 10.0 Hz, J' = 6.5 Hz), 3.01 (1H, dd, J = 13.7 Hz, J' = 1.4 Hz), 3.17 (1H, dd, J = 13.6 Hz, J' = 3.0 Hz), 3.45–3.54 (2H, m), 4.06 (br m, J = 1.0 Hz) ppm. ^{13}C NMR (62.5 MHz, D_2O) δ 15.1, 48.0, 55.8, 66.7, 70.8, 72.8 ppm. HRMS calcd for $C_6H_{14}NO_3$: 148.0974. Found: $[M+H]^+$ 148.0970. Hydrochloride salt. $[\alpha]_D^{25}$ +39.3° (*c* 1.09, H_2O).

Enzymatic synthesis using recombinant rhamnulose-1-phosphate aldolase. To a stirred solution of *S*-2-*O*-acetoxy-3-azidopropanal diethyl acetal (938 mg, 4.06 mmol, > 99 % *e.e.*) in MeOH (2 mL) was added a solution of K_2CO_3 (1.12 g, 8.11 mmol) in H_2O (2 mL). After stirring at 25 °C for 1 h, (hydrolysis monitored by TLC, SiO_2, using 1:9 EtOAc:Hexanes, R_f 0.13; SM R_f 0.28), the solution of *S*-3-azido-2-hydroxypropanal diethyl acetal was concentrated ($T < 45$ °C) and 1 N HCl (8.0 mL) was added to the residue and the solution was made acidic by further addition of 12 N HCl. This solution was stirred at 30–35 °C for 17 h (TLC-monitored hydrolysis, 1:10 MeOH:CH_2Cl_2; R_f 0.39). The pH of the solution of *S*-3-azido-2-hydroxypropanal was adjusted to 7.0 using 0.5 N NaOH, and then sterilized[28] by filtration through a 0.2 μm filter. To this solution was added a filtered-sterilized solution of DHAP (135 mM in H_2O, 15.0 mL, 2.03 mmol, pH 7.0 adjusted with 0.5 N NaOH), followed by a filtered-sterilized solution of rhamnulose-1-phosphate aldolase (5 Units, recombinant enzyme aldolase from *E. coli* K12) and

60 mL of 1 mM aq. $ZnCl_2$ solution. The solution was gently stirred for 5 days under argon (94 % completion determined by DHAP assay). The pH of the solution was adjusted to 7.0 and an equal amount of MeOH (125 mL) was added. Evaporation of the solvent ($T < 40$ °C) gave a residue which was dissolved in H_2O (20 mL), pH 7.0, and a solution of $BaCl_2$ (1 g) in H_2O (5 mL) was added. The precipitate was centrifuged (2500 rpm, 10 min) and the supernatant (25 mL) was added to an equal amount of acetone (25 mL). The suspension was kept in the freezer (−5 °C) for 1 h, and the precipitate was isolated by centrifugation (2500 rpm, 10 min). After traces of acetone were removed under vacuum for 2 h, the solid was dissolved in H_2O (20 mL) and treated with Dowex 50-X8-100 (H^+ form) until pH 1.0. The mixture was further stirred at 25 °C for 40 min. The mixture was filtered, the solution was adjusted to pH 6.0 with aqueous NaOH. Pd/C (10 %) (100 mg) was added to the solution which was then agitated under H_2 (50 psi) for 40 h. The mixture was filtered through Celite and methanol (50 mL) was added to the filtrate which was then concentrated. The residue was triturated with methanol (10 mL) and the precipitate was removed by centrifugation (2500 rpm, 10 min). The supernatant was concentrated to give crude product (176 mg). Purification by flash chromatography (SiO_2 8 g, 7:3:1 CH_2Cl_2:MeOH:28 % aq. NH_4OH) gave pure L-1-deoxyrhamnojirimycin (79.1 mg, 27 %) as a hygroscopic solid. R_f 0.18 (reveal with 2 % $KMnO_4$ in 0.2 M NaOH then heat). $[\alpha]_D^{25}$ +46.0° (*c* 0.615, H_2O). ^1H NMR (400 MHz, D_2O) δ 1.26 (3H, d, J = 6.5 Hz), 2.91 (1H, quintuplet, J = 7.8 Hz), 3.03 (1H, d, J = 13.6 Hz), 3.19 (1H, dd, J = 13.6 Hz, J' = 3.0 Hz), 3.47–3.55 (2H, m), 4.07 (1H, br m, J = 1.0 Hz) ppm. ^{13}C NMR (62.5 MHz, D_2O) δ 15.2, 48.1, 55.9, 66.8, 70.9, 72.8 ppm. HRMS calcd for $C_6H_{14}NO_3$: 148.0974. Found: $[M+H]^+$ 148.0970.

Kinetic and inhibitory studies

The α-L-fucosidase (EC 3.2.1.51) from bovine kidney, α-L-rhamnosidase (naringinase) (EC 3.2.1.40) from *Penicillium decumbens*, *p*-nitrophenyl-α-L-rhamnopyranoside and *p*-nitrophenyl-α-L-fucopyranoside were purchased from Sigma Co. The α-L-fucosidase was assayed as described by Dumas *et al.*[38] Inhibitory studies were performed at various concentrations of substrate (0.1, 0.2, 0.4 and 0.8 mM, K_m = 0.53 mM) at 25 °C in 50 mM acetate buffer (pH 5.5) in a total assay volume of 0.4 mL. An end-point technique was used and each enzymatic reaction was quenched with 0.8 mL of glycine buffer (pH 10.5) after an incubation time of 5 min. The concentration of liberated *p*-nitrophenoxide was determined by measuring the optical absorbance at 400 nm ($\varepsilon_{400,\,pH\,10.5}$ = 18.4 mM^{-1} cm^{-1}). The α-L-rhamnosidase was assayed at various concentrations of substrate (0.5, 1.0, 2.0, 4.0 and 6.75 mM) in 40 mM acetate buffer (pH 5.0) at 25 °C for 5 min (total volume of assay: 0.4 mL) then quenched with 0.6 mL of glycine buffer (pH 10.5). The optical absorbance at 400 nm was measured to determine the amount of liberated *p*-nitrophenoxide.

Time-dependent inactivation of rhamnosidase

A standard enzyme solution containing 28.5 mg naringinase dissolved in 1 mL buffer (40 mM NaOAc, pH 5.0) and a standard substrate solution containing 22.7 mg *p*-nitrophenyl α-L-rhamnopyranoside dissolved in 10 mL buffer (40 mM NaOAc, pH 5.0) were prepared. This produced a standard substrate solution of 8.0 mM which is more than 5 times K_m. (A high dilution with a high concentration of substrate is preferable. Under these conditions the free enzyme is protected against further inactivation.) An inhibitor solution was prepared by dissolving 0.7 mg of **1** in 1 mL of buffer solution (40 mM NaOAc, pH 5.0). At t = 0 min, 20 μL of the inactivator solution was added to 500 μL of the enzyme solution and the solution was vortexed. At t = 0.5 min a 20 μL aliquot of the enzyme/inhibitor solution was removed and added to 1000 μL of the substrate solution, contained in a disposable cuvette, and the solution was vortexed. After 5 min at room temperature (t = 5.5 min) the reaction was quenched with 480 μL of glycine buffer (2 M, pH 10.5) and the absorbance was measured at 400 nm. This procedure was repeated with aliquots being removed at t = 1, 2, 3, 4, 5 and 11 min. At t = 12 min a 20 μL aliquot was removed from the original enzyme solution and added to 1000 μL of substrate solution. After 5 min at room temperature the reaction was quenched with glycine buffer (480 μL) and the absorbance measured. This value was identical to the t = 0 value.

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