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Synthesis and evaluation of amino-threoses in D- and L-series: Are five membered ring amino-sugars more potent glycosidase inhibitors than the six membered ones?

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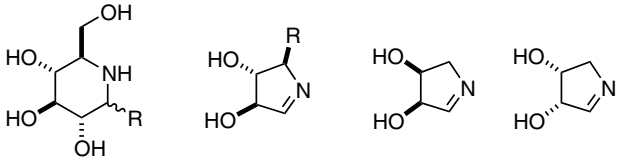
Abstract—Cyclic D- and L-4-aminothreose were synthesised from ethyl D- and L-tartrate, respectively. D-Aminothreose was a potent inhibitor of α-glucosidase and of α-mannosidase. From the glycosidase inhibition potencies of the four 4-amino-4-deoxy-tetroses, the contribution of binding of each functionality of the 5 and 6 membered ring amino-sugars towards the various glycosidases is discussed.

1. Introduction

Glycosidases are involved in the metabolism of saccharides and are essential biological tools for the trimming of glycosidic chains on the cell surface.[1,2] The first discovered piperidine amino-sugar was nojirimycin (**1a**), a potent α- and β-glucosidase inhibitor,[3–6] which was originally described as an antibiotic[7,8] (Scheme 1). This 5-amino-5-deoxyhexose is directly derived from glucose by replacement of the intracyclic oxygen atom by nitrogen. Other analogues have been studied in the mannose[5,9] and in the galactose series.[10,11] These amino-sugars are generally isolated as crystalline sulfite adducts (like **1b**[6,12]) which are more stable with retention of the inhibitory activity.[4]

Nectrisine (**2a**), a 5-membered ring analogue of **1a**, is likewise a potent inhibitor of glycosidases[14,15] and was first described as the immunomodulator FR 900483.[13] Surprisingly this amino-pentose **2a** is a more potent inhibitor of α-glucosidase[14] than nojirimycin (**1a**) itself. Concerning other amino-sugars in the simpler tetrose series, we have recently shown that the D- and L-erythrose analogues D-**3a** and L-**3a**[16] were very potent glycosidase inhibitors but not selective. We describe herein the synthesis and inhibitory properties of their isomers D- and L-amino-threose D-**4a** and L-**4a**, which are obtained from the corresponding protected nitrone D-**5** and L-**5** (Scheme 2).



1a R = OH
1b R = SO_3H
1c R = H

2a R = CH_2OH
D-**4a** R = H

L-**3a**

D-**3a**

Scheme 1.

By comparison of the inhibitory data of all four aminotetroses, the participation of the anomeric centre in the binding with various glycosidases is discussed, as well as the contribution of the diverse hydroxyl groups to the relative inhibitory potencies of the 5-membered ring amino-pentoses and the six-membered ring amino-hexoses.

2. Synthesis and inhibitory properties of amino-threoses

2.1. Nitrone D-5

Nitrone **5** has already been described as the L-[17–19] or the D-enantiomer[18] or as analogues displaying other

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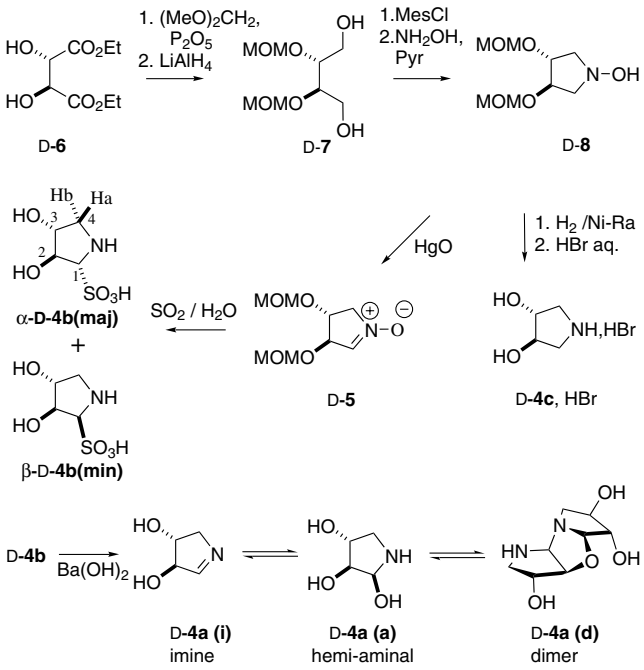


Scheme 2.

O-protective groups.[20] These compounds were particularly used in the synthesis of bicyclic imino-sugars by cycloaddition reactions.[18,21,22] We have obtained here both enantiomers of nitrone **5** by a new method which combined simple functional group transformations. The synthesis in the D-series is only presented in Scheme 2, but the synthesis in the L-series was carried out according to the same reactions.

Ethyl (*S*,*S*)-D-tartrate (D-**6**) was protected as the methoxymethyl (MOM) derivative[18,23] by the Fuji method[24] and reduced into diprotected threitol D-**7** as in[18,23,25] by $LiAlH_4$ in dry ether in an overall yield of 78% from D-**6**. D-**7** was then quantitatively bis-mesylated[18] and, without purification, directly cyclised into (*R*,*R*)-*N*-hydroxy-pyrrolidine D-**8** in 82% yield according to the method of Brandi[26,27] by nucleophilic disubstitution with hydroxylamine in boiling triethylamine.

Oxidation of D-**8** with HgO[20] gave quantitatively the pure nitrone D-**5** (64% overall yield from ethyl D-tartrate (D-**6**)). The enantiomer L-5 was obtained in a same manner starting from the ethyl (*R*,*R*)-L-tartrate (L-**6**).

2.2. Preparation of the amino-sugar D-4a

The reductive hydrolysis of nitrone D-**5** into the crystalline sulfite adduct D-**4b** was performed by simple action of SO_2 in water, or better in aqueous *N* sulfuric acid. Reaction conditions were critical and D-**4b** was obtained in acceptable yield when the reaction was performed 2 days at room temperature, higher temperature giving poor results. Sulfur dioxide plays a triple role in this reaction: it acts as reductant of the N–O bond, as acid catalyst for the O-deprotection and as reactant in the addition with the so-formed imine D-**4a** into the final sulfite adduct D-**4b**.

In contrast with the erythro-series,[16] D-**4b** was obtained as a 75/25 mixture of both α and β-anomers. Recrystallisation in water/ethanol did not change the anomer proportions. Consequently, these isomers must be in equilibrium in solution with the amino-sugar D-**4a** and SO_2. This phenomenon can explain the bad recovery of D-**4b** from solution and a still acceptable elemental analysis with a 10% loss of SO_2.

Reaction of the anomeric mixture of D-**4b** with barium hydroxide afforded the amino-sugar D-**4a** in aqueous solution with formation of insoluble barium sulfite. D-**4a** appeared as a mixture of dimer D-**4a**(**d**) and imine D-**4a**(**i**), with two minor species: amino-alcohol D-**4a**(**a**) and an unknown compound in 50/30/8/12 proportions. NMR data are compiled in Table 3 (see experimental part). Medium values (3–6 Hz) of the *J*(1,2) coupling constant in the amino-alcohol and in the dimer forms reveal a *cis*-relation between H–C(1) and H–C(2).[28,29] Accordingly, the amino-alcohol D-**4a**(**a**) must be α-configurated and the dimer D-**4a**(**d**) has the *anti*-tricyclic configuration (Scheme 2).

2.3. Stability of D-4a and D-4b in solutions

5-Deoxy-5-amino-hexoses have been assumed to be unstable in acidic solutions,[30,31] whereas *manno*-nojirimycin had been found to be enough stable under the conditions used for the enzymatic determinations.[5] For this reason, we have studied the stability of the synthesised compounds D-**4a**,**b** in the buffer solution used for the determination of the inhibition data.

2.3.1. Sulfite adduct D-4b, stability and configuration. No change was detectable by ^1H NMR for 24 h at room temperature as well as in aqueous solution (pH ca 4.5) or in the following buffer solutions: acetate (pH 4.5), phosphate (pH ca 7.0) or Hepes (pH ca 7.0). The proportions of the two α/β anomers were nevertheless depending on the buffer: 75/25 in D_2O, 70/30 in acetate buffer, 95/5 in phosphate buffer and 90/10 in Hepes buffer. Very similar ^1H NMR data were observed in D_2O or in acetate buffer (pH ca 4.5) on the one hand, and in phosphate or Hepes buffer (pH ca 7.0) on the other hand (Table 3).

^{13}C NMR data of both anomers in D_2O were very similar and, as well as for the major α-anomer in phosphate buffer, the C(1) signal appeared at ca. 78 ppm as for nojirimycin adduct (**1b**).[32,33]

The α-configuration (1,2-*trans*) for the major anomer was determined by NOE experiments in phosphate buffer (see Scheme 2, no effect was found in aqueous solution without buffer). Irradiation of H–C(1) at 4.01 ppm gave a weak signal enhancement on H–C(3) at 4.15 ppm, whereas irradiation of Ha-C(4) at 3.25 ppm induced a weak effect on H–C(1) and a clear effect (9%) on H–C(3) and showed that these protons are on

the same molecule side. Irradiation of Hb-C(4) at 2.95 ppm gave a 5% effect on H–C(2) at 4.33 ppm.

2.3.2. Amino-sugar D-4a. In D_2O solution (pH ca 8.0) or in Hepes buffer (pH ca 7.0), the major species were the dimer D-**4a**(**d**) and the imine D-**4a**(**i**), and no change was detected in the ^1H NMR spectrum even after 24 h at room temperature. In acetate buffer, numerous species were detected: the dimer D-**4a**(**d**), the good-defined hemi-aminal D-**4a**(**a**) and two other species with broad unresolved NMR signals. The only change, after 24 h at room temperature, was the disappearance of the dimer form. We have verified that no degradation occurred since a basification at pH 8 with barium hydroxide led to the same main species, imine and dimer, as in water at the same pH. In phosphate buffer, a similar mixture as in Hepes buffer was observed, nevertheless a slow disappearance of the different species, with $t_{1/2}$ around 16 h, seemed to correspond to a similar but therefore slower irreversible isomerisation/deuteration process as observed in the erythrose series.[16]

2.4. Inhibition of glycosidases

The inhibition data of the amino-sugars and sulfite adducts D-**4a**,**b** and L-**4a**,**b** as well as the 1-deoxy derivative D-**4c** are reported in Table 1. Partial data concerning the enantiomer pyrrolidinediol L-**4c** have already been published[34] and are also included. All these compounds are reversible competitive inhibitors. The kinetic constants (K_i values) were determined for the most potent inhibitors. In Table 1 were also reported the inhibition data of nojirimycin (**1a**)[5] and nectrisine (**2a**)[14] as well as of iminoarabinitol **2c**[14,36] (in Scheme 4).

As a first remark, the derivatives in the L-series are inactive against the studied glycosidases. On the contrary, the D-amino-sugars D-**4a**,**b** are potent α-glucosidase and α-mannosidase inhibitors, weak β-glucosidase inhibitors, but even as potent as nojirimycin (**1a**)[5] or *manno*-nojirimycin[5] against the corresponding glycosidases. Amine D-**4c** has only weak activities.

As noticed above, the sulfite adducts exist probably in an equilibrium mixture with the corresponding amino-sugar and SO_2, as it was already supposed for nojirimycin (**1a**)[4] and by us in the erythrose series.[16] At the concentrations of amino-sugars and sulfite adducts used for the inhibition studies (10^{-3} to 10^{-5} M), the same species, imine and amino-alcohol, can be then present in solution. This equilibrium might account for the very similar inhibitory potencies of the sulfite adduct and the corresponding amino-sugar that we observed here in the threose series. Analogous results were previously obtained in other pentose[16,35] and hexose[3,6,9] series. It has to be noticed, that the presence of a hydroxymethyl group in **2a** favours the recognition for the α- and β-glycosidases, but has no effect for the α-mannosidase, when compared to D-**4a**.

3. Inhibitory properties of pyrrolidine amino-sugars

Amino-sugars like nojirimycin (**1a**)[5,6] and nectrisine (**2a**)[14] are well-known potent glycosidase inhibitors. Nevertheless, they have been scarcely studied,[37] probably because of their relative instability in comparison to their stable 1-deoxy derivatives like deoxynojirimycin (**1c**)[38] or the iminoarabinitol **2c**.[14,36] The results disclosed in Table 1 deserve some comments and the following points are now discussed considering the inhibition data of all tetrose amino-sugars (see Table 2).

3.1. Participation of the anomeric hydroxyl group

In the pyrrolidine series, the equilibrium between both imine and hemi-aminal forms is known for a long time[30,31] and has a theoretical importance. The imine form, like D-**4a**(i) (Scheme 2), is indeed the perfect mimic of the oxonium cation which is formed during the glycoside hydrolysis (Fig. 1A, in the case of a retaining glycosidase[39]) and is thus a transition state analogue.[1,40] An important feature is that the reduction of the imine function, which corresponds for the hemi-aminal form D-**4a**(a) to the replacement of the anomeric hydroxyl by a proton, led to a dramatic decrease in the enzymatic activities as observed for D-**4c**, as well as in D-arabinose series for **2c**[14] (Table 1). The same effect has been already noticed in the glucose series for nojirimycin (**1a**) and its deoxy-derivative **1c**[37] as well as in the

Table 1. Inhibition data[a] (% at 1 mM or K_i in μM) of amino-threoses D,L-**4a**, their sulfite adducts D,L-**4b**, iminothreitols D-**4c**, L-**4c**[34] and, for comparison, nojirimycin (**1a**),[5] nectrisine (**2a**)[14] and imino-D-arabinitol **2c**[b14,36]

Enzymes	4-Amino-D-threose			4-Amino-L- threose			Others		
	D-**4b**	D-**4a**	D-**4c**	L-**4b**	L-**4a**	L-**4c**[34]	**1a**[5]	**2a**[14]	**2c**[14,36]
α-Glucosidase (yeast)	**25**	**2**	43%	**830**	nd	ni	**6.3**	0.04[c]	0.15[c]; 0,18[c]
β-Glucosidase (almond)	180[c]	200[c]	ni	2000[c]	nd	ni	**0.9**	2.2[c]	500[c]; 200[c]
α-Mannosidase (Jack bean)	**5**	**5**	ni	ni	nd	ni	**500**	3[c]	200[c]; 100[c]
β-Mannosidase (snail)	ni	50%	ni	28%	nd	ni	**900**[d]	42[d]	ni
α-Galactosidase (green coffee bean)	ni	40%	27%	20%	nd	ni			ni
β-Galactosidase (*Escherichia Coli*)	ni	nd	20%	ni	nd	ni			ni
α-Fucosidase (bovine kidney)	ni	nd	nd	23%	nd	ni[e]			ni

[a] ni, no inhibition or inhibition <20%, nd: no determined.
[b] Figure in Scheme 4.
[c] IC_{50}.
[d] β-Mannosidase of *Aspergillum ventii*.
[e] α-Fucosidase from bovine epididymis.

Table 2. Glycosidase inhibition data (K_i in μM) of amino-tetroses (amino-erythroses D,L-**3a** and amino-threoses D,L-**4a**)

	α-Glu'dase	β-Glu'dase	α-Man'dase	β-Man'dase	α-Gal'dase	β-Gal'dase	α- fuc'dase
D-**3a**	150[a]	ni	**20**	ni[b]	ni[b]	1000[a]	**3**
L-**3a**	ni	**0.3**	**2**	ni[b]	**0.2**	**0.2**	1000[ab]
D-**4a**	**2**	200[a]	**5**	1000[a]	ca 1000[a]	ni[b]	ni[b]
L-**4a**	**850**[b]	2000[ab]	ni[b]	ni[b]	ni[b]	ni[b]	ni[b]

Explanation of the dotted and continuous border, see the text.
[a]IC_{50}.
[b]Data for the corresponding adduct with SO_2.

D,L-erythrose series[16] and in the 5-deoxy-L-lyxose series.[35] This loss in activity can be quantified by a factor of nearly 5–100 and it appears consequently that this anomeric function is important for the binding in the active site of the enzymes.

It is assumed that two carboxylic functions are present in this active site of the enzymes and are involved in the catalytic process by interacting with the anomeric centre of the glycoside[1,2] (Fig. 1A). This enzyme-amino-sugar interaction can be depicted in several ways. As an amino-alcohol, the amino-sugar can interact through hydrogen bonds (Fig. 1B), or after H_2O elimination as an imine, it can interact through two ionic bonds (Fig. 1C) or even through both a covalent[39] and an ionic bond (Fig. 1D). Nevertheless, it appears that both the amine function and the anomeric centre are involved in the binding with the enzymes. The loss of the anomeric hydroxyl would lead to the lack of one binding functionality and therefore to the observed decrease in the inhibition potency.

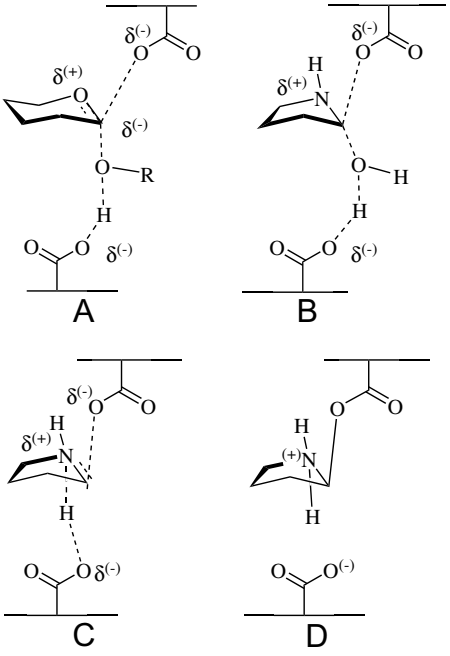
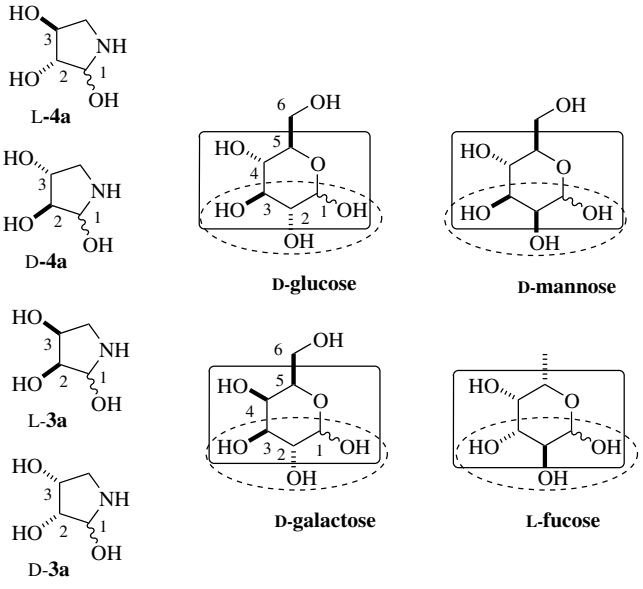


Figure 1. Expected interactions of the two carboxylic moieties of the active site of the enzyme with a glycoside (A in the case of a retaining glycosidase) or with an amino-sugar as amino-alcohol (B), or as imine with either an ionic (C) or a covalent bond (D).

3.2. Are five-membered ring amino-sugars more potent glycosidase inhibitors than six-membered ring amino-sugars? Interaction between inhibitor OH–C(2) and glycosidases

Surprisingly, the simple D-amino threose D-**4a** appears to be more active against α-glucosidase than nojirimycin (**1a**) itself, which theoretically displays the perfect shape to mimic the natural substrate (Table 1). Considering the four amino-tetroses, that is, the amino-threoses D- and L-**4a** and the amino-erythroses D- and L-**3a**,[16] their inhibitory data against several commercial glycosidases are compiled in Table 2. It is therefore interesting to compare the structure of each amino-tetrose with the structure of the hexoses corresponding to a given glycosidase (Scheme 3). For clarity, the amino-sugars are depicted in their hemi-aminal forms.

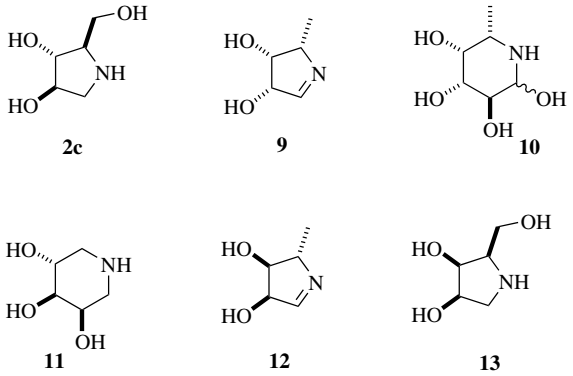


Scheme 3. Comparison of the OH orientation of the aminotetroses L-**3a**, D-**3a** and D-**4a** with those of the corresponding hexoses. The continuous lines correspond to the OH–C(1), OH–C(3), OH–C(4) moiety of these hexoses, the dotted lines to their OH–C(1), OH–C(2), OH–C(3) moiety.

3.2.1. General case: weak binding of OH–C(2) with the glycosidases. Considering the C(1), C(2), C(3) part, that is, the OH–C(1), OH–C(2), OH–C(3) moiety of the hexose sugars in the D-glucose, D-mannose, D-galactose or L-fucose series (in dotted line, Scheme 3), it is possible to correspond each moiety to the one of an aminotetrose having the same orientation of the OH-groups in the positions C(1),C(2),C(3). D-Glucose and D-galactose correspond to L-**4a**, D-mannose to L-**3a** and L-fucose to D-**4a**. According to the results disclosed in Table 2, it is clear that these aminotetroses are not potent inhibitors of the corresponding glycosidases (dotted border in Table 2); nevertheless as the sole exception, the α-mannosidase is potently inhibited by L-**3a**.

On the contrary, considering the OH–C(1), OH–C(3), OH–C(4) moiety of the hexose sugars (continuous line in Scheme 3), D-glucose and D-mannose correspond to D-**4a**, D-galactose to L-**3a** and L-fucose to D-**3a**. In all cases, glycosidases are potently inhibited by their respective aminotetroses (continuous border in Table 2), with the only exception of β-glucosidase and β-mannosidase. It thus seems that for the majority of these enzymes, the C(1), C(3), C(4) moiety is well recognised in the active site of the enzymes, but not the C(1), C(2), C(3) moiety. Consequently, the OH–C(2) of the hexose sugar substrate seems not to be involved in the binding, with the sole exception of the α-mannosidase.

As a consequence, the pyrrolidine 4-amino-pentoses (like nectrisine **2a**) should generally be as good inhibitors as the corresponding piperidine 5-amino-hexoses (like nojirimycin **1a**) for the corresponding enzymes, a positive or a negative interaction between OH–C(2) and the enzyme leading to slightly weaker or better inhibitors, respectively. Only partial data are available to compare these classes of amino-sugars, it is nevertheless possible to compare amino-sugars in the D-glucose and L-fucose series with the corresponding ones in the D-arabinose and L-lyxose series, respectively (Table 1): nectrisine (**2a**)[14] is an as good β-glucosidase inhibitor as nojirimycin (**1a**)[5] but a better α-glucosidase inhibitor. Likewise, in the case of the amino-sugars possessing the L-fucose structure (Scheme 4), the corresponding amino-L-lyxose **9** is a potent L-fucosidase inhibitor (K_i 10 nM[35]), while the L-*fuco*nojirimycin **10** is a more potent inhibitor (K_i 1 nM[41]). These two examples point out in the first case a neutral OH–C(2) interaction with the β-glucosidase, but a negative one for the α-glucosidase, and in the second case a clearly positive one.



Scheme 4.

This weak interaction between OH–C(2) and the active site of the enzymes, we observed here, has not been pointed out in literature yet. For example, energetic considerations had shown that the four hydroxyl groups of a glucoside, the α-isomaltoside, are equally binding with the baker yeast α-glucosidase.[42] On the other hand, glycosidases (or glycoside hydrolases, GH) have been classified in 85 families according to their aminoacid sequence similarity[43–45] and some crystal structures of enzyme-substrate complexes have already been published for glycosidases of the same family as the commercial enzymes used in this study. In most cases, all hydroxyl groups of the glycone moiety were linked by hydrogen bond with the enzymes. It is the case for some β-glucosidases of the family GH-1,[46,47] β-galactosidases of the family GH-27[48,49] and one α-fucosidase of the family GH-29,[50] but it is difficult to rationalise the relative strength of these hydrogen bindings. In the sole case of a *Zea mays* β-glucosidase complex with a thioglucoside, OH–C(2) was found to be not binding with the enzyme.[51] It had nevertheless been noticed that the removal of this hydroxyl group in D-glucose reduced only slightly the affinity of this sugar with almond β-glucosidase.[52]

An explanation for the weak interaction of OH–C(2) of most amino-tetroses with the diverse studied enzymes, except the α-mannosidase, could be advanced: this hydroxyl group would interact in the active site via a hydrogen bond with the carboxylic group which interacts itself strongly with the anomeric centre (Fig. 2 A′). Consequently, the loss of OH–C(2) would have no great influence on the total binding with the enzyme, this influence being either favourable or unfavourable. Some crystal structures of enzyme-substract complexes[46,47,49] displayed clearly such a hydrogen binding.

3.2.2. Case of the α-mannosidase. As shown above, the α-mannosidase, in which the OH–C(2) of the substrate sugar (i.e., the D-mannose) is in an axial position, is

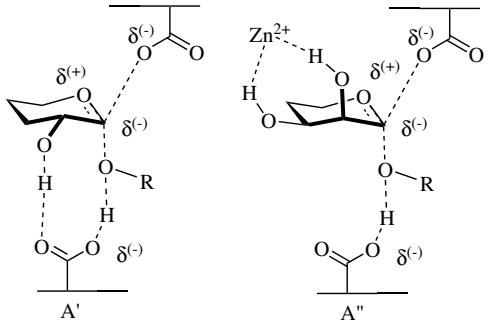


Figure 2. Expected interactions of the carboxylic groups of the active site of the enzyme in the general case of a glycoside with equatorial OH–C(2) (A′ in the case of a retaining α-glycosidase) and, in the case of a α-mannosidase, interactions of the OH–C(2) and OH–C(3) of an α-mannoside with the Zn^{2+} ion (scheme A″).

clearly a particular enzyme. α-Mannosidases are classified in two glycosidase families GH-38 and GH-47 which include retaining class II α-mannosidases and inverting class I α-1,2-mannosidases, respectively.[45,53] The Jack bean α-mannosidase is a Zn^{2+} metalloenzyme[54] and belongs to glycosidase family GH-38,[55] but no crystal structure has been published. However, crystal structure of *Drosophila* Golgi class II α-mannosidase, another family GH-38 α-mannosidase, has been obtained as a complex with the inhibitors deoxy*manno*-nojirimycin and swainsonine[56] and reveals that the Zn^{2+} ion is present in the active site of the enzyme and is directly bound to the OH–C(2) and OH–C(3) groups. The presence of a metal ion contributes to the specificity of the α-mannosidases since those of the second family GH-47 contain a Ca^{2+} ion.[57–59] It is necessary to admit that the coordination of these two *cis*-hydroxyl groups with the Zn^{2+} atom, as depicted in the Figure 2 A″, is a strong binding, and explains thus the strong inhibitions with the amino-erythroses L-**3a** and D-**3a** (Table 2).

3.3. Binding elements in five and six membered ring amino-sugar series

All the four amino-tetroses are strong glycosidase inhibitors with K_i in the micromolar range (Table 2). These compounds display a very simple structure and it must consequently be possible to outline the structure–activity relationships. For the strong inhibitors in the micromolar range, we assume that the amine group and all three other functionalities, that is, the two hydroxyl groups and the anomeric centre, participate in the binding with the enzymes. Thus, it must be possible to define experimentally which functionalities of the sugars bind strongly the diverse studied enzymes. As discussed above, some crystal structures of glycosidase-substrate complexes have already been published and might help to understand these interactions.

3.3.1. α-D-Glucosidase. Only the amino-D-threose D-**4a** inhibits strongly this enzyme. However, the addition of a supplementary hydroxymethyl group in the 4-position affords nectrisine (**2a**)[14] which is a more potent inhibitor (IC_{50} = 40 nM). This substituent is also a binding element. We can then define for this enzyme four binding elements which are all the functionalities present in nectrisine. They correspond for the glucose moiety to CH_2OH(6), OH–C(4), OH–C(3) and the anomeric centre. This conclusion is in agreement with energetic considerations deduced from diverse monodeoxy-glucosides.[42] Yeast α-glucosidases belong to the glycoside hydrolase family GH-13,[60] but no complexes with the enzyme have been still studied.

3.3.2. β-D-Glucosidase. Surprisingly, this enzyme is strongly inhibited by only the amino-L-erythrose L-**3a** in which the two hydroxyl groups in 2 and 3 positions are in *cis*-orientation and at the same side of the molecule as OH–C(3) and CH_2OH(6) groups of the D-glucose. Its *trans*-isomer D-**4a** is only a weak inhibitor and the homologue nectrisine (**2a**) is as potent as L-**3a**. It consequently seems, that the OH–C(3) group in L-**3a** plays a similar binding part to the CH_2OH group in nectrisine. In this latter compound as well as in the *trans* D-**4a**, the OH–C(3) (corresponding to OH–C(4) in glucose) seems not to bind to the enzyme. This feature differentiates β-glucosidase from α-glucosidase. Nectrisine displays therefore only three binding elements which correspond for the glucose moiety to CH_2OH(6), OH–C(3) and the anomeric centre. Previously, it had been noticed for the glucose itself that the methylation of OH–C(3) reduced strongly the affinity for the β-glucosidase, while modifications at OH–C(4) or OH–C(6) had only little effect.[52]

Almond β-glucosidase belongs to the glycosidase family GH-1[61] and crystal structures of enzyme-substrate complexes[51,46,47] in the same glycosidase family displayed only some flexibility for the binding of the diverse hydroxyl groups of the glucose moiety in the enzyme active site according to the substrate.

3.3.3. α- and β-D-mannosidase. Contrarily to the above glucosidases, several amino-tetroses, that is, both enantiomers of amino-erythrose D and L-**3a** and the amino-D-threose D-**4a**, strongly inhibit the α-mannosidase. In agreement with the presence of a Zn^{2+} ion in its active site,[54] this latter enzyme binds strongly the *cis*-2,3-dihydroxy group. By comparison of the inhibition data for D-**4a** and nectrisine (**2a**) (Table 1), a supplementary CH_2OH–C(4) group has a strong effect on the affinity towards β-mannosidase but no effect towards α-mannosidase. Analysing the inhibition data from Tables 1 and 2, it appears that OH–C(2) is a binding element for the α-mannosidase contrarily to the other glycosidases. Thus, four binding elements can be defined for the mannose moiety (OH–C(2), OH–C(3), OH–C(4) and the anomeric centre) but the CH_2OH(6) group can be excluded. However, in a study on α-mannosidase,[62] it had been proposed that hydroxyl groups which are topographically equivalent to cis OH–C(2) and OH–C(3) of mannose are necessary for the binding with the enzyme, the *trans* OH–C(4) and the CH_2OH(6) being not essential. Consequently, it seems that, for the six membered ring inhibitors, the interactions of OH–C(4) with the enzyme are weaker than those for the five membered ring ones and this is in accordance with the relative inhibition weakness of the deoxy*manno*-nojirimycin[5,56] and the inactivity of the imino-D-arabinitol **11**[63,64] which possesses the same configuration for the three OH-groups as mannose (see Scheme 4).

Jack bean α-mannosidase belongs to the retaining glycosidase family GH-38.[55] From the crystal structures of enzyme-substrate complexes,[56] an interesting feature is the presence of three aromatic residues (Trp95, Phe206, and Tyr727) in the binding pocket of the CH_2OH(6) moiety. This hydrophobic region can thus explain the important increase in inhibition of the 5-deoxy-amino-L-ribose **12** (K_i = 50 nM)[16] in comparison with L-**3a** and corresponding to the addition of the methyl(5) group. This methyl group interacts with the hydrophobic residues and becomes also a potent binding element. The optimal size of this substituent is still to be found.

In the case of the β-mannosidase, the poor inhibition potencies of all the amino-tetroses, but not of nectrisine

(**2a**), indicate probably that OH–C(3) is not a binding element but CH_2OH(6) is a potent one. β-Mannosidases belong to the glycosidase family GH-2, but no crystal structure has been reported.

3.3.4. α- and β-D-galactosidase. Among the four amino-tetroses, only the amino-D-erythrose L-**3a** inhibits strongly these enzymes. This compound presents the same OH-orientations in the 3 and 4-position as D-galactose. It is to notice that the related imino-D-lyxitol **13** (Scheme 4), which has all three substituents in a *cis*-position as in D-galactose, is a potent and selective inhibitor of α-galactosidase,[36,65,66] but a weak one of β-galactosidase.[36,65] Accordingly, it appears that the CH_2OH group is a binding element only for α-galactosidase. It can then be considered for this latter enzyme, that the four functionalities CH_2OH(6), OH–C(4), OH–C(3) and the anomeric centre of the D-galactose are binding elements.

For β-galactosidase, too few data are available; it seems nevertheless that only OH–C(4), OH–C(3), and the anomeric centre are binding elements.

Crystal structures of complexes with D-galactose were reported for α-galactosidases of *Oryza sativa* (rice)[49] and the fungus *Trichoderma reesei*[48] of the same glycosidase family GH-27 as the green coffee bean α-galactosidase.[67] In these complexes, all hydroxyl groups of the sugar are bound with the enzyme in its active site, and interestingly an aromatic Trp19 (resp. Trp16) is close to the hydrophobic site opposite to OH–C(3), OH–C(4), OH–C(5) of the galactose and hinders thus the binding with another sugar.

3.3.5. α-L-Fucosidase. Only the amino-D-erythrose D-**3a** inhibits strongly this enzyme. Addition of a methyl group in the 4-position led to the very potent inhibitor amino-L-lyxose **9**[35] (K_i 10 nM) (Scheme 4). It can be therefore considered that for this enzyme all four functionalities of the amino-lyxose **9** are binding elements, corresponding for the L-fucose moiety to CH_3(6), OH–C(4), OH–C(3) and the anomeric centre. Recently, synthesis and inhibition values of the *fuco*nojirimycin **10** have been reported (K_i 1 nM[41]). This compound is a more potent fucosidase inhibitor than **9** and the interaction of OH–C(2) with the enzyme is here positive.

All fucosidases belong to the retaining glycosidase family GH-29[50] and a crystal structure of a complex of marine bacterium *Thermotoga maritima* α-L-fucosidase with L-fucose has been recently reported.[50] All hydroxy groups of the sugar are bound in the active site of the enzyme and, as we have above noticed for the α-mannosidase, the methyl(6) group is enclosed in a hydrophobic domain with four aromatic residues (Phe32, Tyr171, Trp222, and Phe 290).

4. Conclusion

We have described the synthesis of 4-amino-4-deoxy-D- and L-threose in a ca. 30% overall yield from ethyl D- or L-tartrate, respectively. The D-enantiomer is a potent α-glucosidase and α-mannosidase inhibitor in the micromolar range, almost as potent as the known piperidine amino-sugar nojirimycin (**1a**) and *manno*-nojirimycin against the corresponding enzymes. It appears that the pyrrolidine amino-sugars, in the tetrose and pentose series, can display very potent glycosidase inhibition potencies.

It is therefore possible to define the notion of binding element as a functionality or a substituent which is strongly bound in the active site of the enzyme. A careful analysis of the data compiled in Tables 1 and 2 shows that such an element lowers the K_i values up to two orders of magnitude (factor 5–100). This value is in agreement with the literature evaluation[62,68] where it was been pointed out that one binding hydroxyl group corresponds to an average binding energy of ca 2.5 kcal/mole, that is, a factor of ca. 70 for the decrease of the K_i value. These binding elements can be hydroxyl, hydroxymethyl, methyl groups or the anomeric centre of the amino-sugars. More exactly for pyrrolidine amino-sugars or imino-alditols, the presence of three such binding elements on the molecule led to circa a micromolar range inhibition, four such elements giving a 10–50 nM value for the inhibition constant. It seems to be difficult to attempt a generalisation concerning the homologue amino-hexoses such as nojirimycin. Since, regarding the above discussion, OH–C(2) could be a weak to important binding element or an unfavourable one.

4.1. Experimental

4.1.1. General. Flash chromatography (FC): silica gel (Merck 60, 230–400 mesh). TLC: Al-roll silica gel (Merck 60, F_{254}). Mp: Kofler hot bench, corrected. IR spectra (ν in cm^{-1}): Perkin-Elmer 157 G, $[\alpha]_D$: Schmidt-Haensch Polartronic Universal polarimeter. ^1H and ^{13}C NMR (62.9 or 100 MHz) spectra: Bruker AC-F250 or 400; tetramethylsilane (TMS) in $CDCl_3$, or natrium (D_4)-trimethylsilylpropionate (D_4-TSP) in D_2O (^1H NMR) and $CDCl_3$, or (in D_2O) dioxane [$\delta(CDCl_3) = 77.0$, in D_2O δ(dioxane) = 67.4 with respect to TMS] (^{13}C NMR) as internal references; δ in ppm and J in Hz. High resolution FAB(HR)-MS were measured on a MAT-311 spectrometer at the University of Strasbourg, Pr. Vandorsselaer. Microanalyses were carried out by the Service Central de Microanalyses du CNRS, F-69390 Vernaison, or Service de microanalyse de l'ICSN-CNRS, F-91168 Gif sur Yvette.

4.1.2. Reagents and solvents. D- and L-Ethyl tartrate, Raney-nickel, aqueous suspension, SO_2 in bottle were obtained from Fluka, 45% (7N) aqueous HBr, methylal from Prolabo and $NH_2OH{\cdot}HCl$ from Merck. Usual solvents were freshly distilled, dry EtOH and MeOH distilled over Mg/MgI_2, dry Et_2O was distilled and stored over Na, dry *i*-PrOH distilled over CaO, CH_2Cl_2 was distilled over P_2O_5 and kept over Na_2CO_3. NEt_3 and methanesulfonyl chloride were distilled before use.

4.2. 2,3-Di-*O*-(methoxymethyl)-D-threitol (D-7)

Same procedure as for the L-enantiomer[18,23] with modifications:

1. *O*-Protection: to a stirred suspension of P_2O_5 (12 g, 85 mmol) in CH_2Cl_2 (20 ml) and methylal (22 ml, 0.25 mmol, 10 equiv) was added at rt a solution of ethyl tartrate D-**6** (5.0 g, 24 mmol) in CH_2Cl_2 (30 ml). After 1.5–2 h, 3 g P_2O_5 was added and the solution stirred until reaction completion (1–2 h). The solution was decanted, the residue washed with CH_2Cl_2 (2 × 50 ml) and the organic solutions stirred with Na_2CO_3 (5 g) for 1 h, filtered and evaporated to give as colourless resin (7.2 g, quant) used without purification for the next step.
2. Reduction of the ester: to a stirred suspension of $LiAlH_4$ (1.5 g, 39 mmol, 1.5 equiv) in Et_2O (100 ml) at 0 °C was added dropwise a solution of above protected ester (24 mmol) in Et_2O (75 ml). The solution was stirred at rt for 1–2 h until completion of the reaction (tlc, Et_2O/MeOH 9/1). $Na_2SO_4{\cdot}10H_2O$ (7 g, 22 mmol) was portionwise added, then 7 ml of 10% aq. NaOH, and the mixture stirred for 1 h, then filtered through Celite and the solids washed with Et_2O and then AcOEt (2 × 50 ml). Evaporation of the combined solutions gave crude diol (4.7 g, quant). Purification by dissolution in AcOEt, filtration, evaporation of the solvent, crystallisation in iPr_2O at 0 °C and washing with iPr_2O gave pure diol (3.98 g, 78%).

Diethyl 2,3-di-*O*-(methoxymethyl)-D-tartrate:

^{1}H NMR: ($CDCl_3$, 250 MHz, 300 K): 1.32 (t, 2 Me (Et)); 3.36 (s, 2 OMe); 4.26 (m, 2 CH_2 (Et)); 4.68 (d, 2 OCH*H*O); 4.71 (s, H–C(2), H–C(3)); 4.80 (d, 2 OC*H*-HO); $J(CH_2CH_3)$ = 7.2 Hz, J (OCH_2O) = 7.1 Hz. Similar data as in[18,23] for the L-enantiomer.

D-**7**: colourless needles, mp = 62–63 °C (iPr_2O) (lit.[18] 60–62 °C). $[\alpha]_D^{19} = +43$ (c = 1, $CHCl_3$) (lit.[18] $[\alpha]_D = +42.1$ (c = 1.8, $CHCl_3$)).

IR (KBr): 3440, 2930, 2880, 1478, 1448, 1368, 1210, 1155, 1092, 1020, 975, 910, 845, 810, 755 cm^{-1}.

^{1}H NMR ($CDCl_3$, 250 MHz, 300 K): 2.98 (s, OH–C(1), OH–C(4)); 3.44 (s, 2 OMe); ca 3.76 (m, H–C(2), H–C(3)); 3.76 (m, CH_2(1), CH_2(4)); 4.72, 4.77 (2 d, 2 OCH_2O). $J(OCH_2O)$ = 6.8 Hz. Similar data as for the L-enantiomer.[18,23,25]

Anal. calcd for $C_8H_{18}O_6$ (210.23) : calcd C 45.71, H 8.63; found C 45.7, H 8.6.

4.2.1. L-Enantiomer. Same procedure from L-**6** (10.3 g, 50 mmol) to give L-**7** (7.98 g, 76%). Mp = 62–63 °C (iPr_2O)(lit.[23] 64 °C, lit.[18] 60–62 °C, lit[25] 62–63 °C). $[\alpha]_D^{20} = -8$ (c = 2, MeOH), $[\alpha]_D^{20} = -42$ (c = 1, $CHCl_3$) [lit.[18] $[\alpha]_D$ −30.5 (c = 1, $CHCl_3$); lit.[25] $[\alpha]_D^{23} = -7.7$ (c = 3.4, MeOH); lit.[23] $[\alpha]_D^{21} = -7.9$ (c = 0.2, MeOH)].

4.3. 1,4-(Hydroxyl)imino 2,3-di-*O*-(methoxymethyl)-1,4-dideoxy-D-threitol (D-8)

1. Bis-mesylation according to the lit.[18] (for the L-enantiomer): to a stirred solution of D-**7** (3.73 g, 17.7 mmol) and NEt_3 (15 ml, 0.11 mol, 6 equiv) in dry CH_2Cl_2 (50 ml) at 0 °C was added dropwise a solution of $ClSO_2Me$ (4.2 ml, 54 mmol, 3 equiv) in dry CH_2Cl_2 (50 ml). After completion of the reaction (30 mn at 0 °C, tlc Et_2O/MeOH 9/1), the orange solution was poured in 200 g ice, the aqueous phase was extracted with Et_2O (3 × 20 ml), the organic phases were washed with water, dried ($MgSO_4$) and evaporated to give crude product (6.92 g, quant) which was used for the next step.
2. A stirred suspension of $NH_2OH.HCl$ (6.0 g, 86 mmol, 4 equiv) and of above dimesylate (17.7 mmol) in NEt_3 (60 ml) was refluxed for 1.5 h. Et_2O (60 ml) was added, the precipitate separated by decantation and washed with Et_2O (2 × 20 ml), and the combined organic phases were evaporated to give crude D-**8** (3.6 g, quant). Purification by dissolution in Et_2O, filtration and evaporation of the solvent gave pure D-**8** (3.0 g, 82%) after crystallisation and washing with iPr_2O at 0 °C.

1,4-Di-*O*-methanesulfonyl-2,3-di-*O*-(methoxymethyl)-D-threitol

Colourless resin. $[\alpha]_D^{20} = +10$ (c = 1, $CHCl_3$) (lit.[18] $[\alpha]_D^{20} = -9.0$ (c = 1, $CHCl_3$) for the L-enantiomer). Same ^{1}H NMR data as in[18] for the L-enantiomer.

D-**8**: colourless crystals, mp = 61–62 °C (iPr_2O). $[\alpha]_D^{19} = +16$ (c = 1, $CHCl_3$).

IR (KBr): 3150, 3110, 2935, 2880, 1465, 1440, 1430, 1385, 1250, 1225, 1210, 1200, 1150, 1115, 1063, 1032, 1008, 965, 938, 912 cm^{-1}.

^{1}H NMR ($CDCl_3$, 250 MHz, 300 K): 1.65 (br s, NOH); 3.07 (d large, Ha-C(1), Ha-C(4)); 3.38 (s, 2 OMe); 3.44 (m, Hb-C(1), Hb-C(4); 4.22 (t, H–C(2), H–C(3); 4.66, 4.72 (2 d, 2 OCH_2O). J(1a,1b) = 11.4, J(1a,2) = J(1b,2) = 4.8, $J(OCH_2O)$ = 6.8 Hz.

^{13}C NMR ($CDCl_3$, 62,9 MHz, 300 K): 55.5 (2 OMe); 63.0 C(1), C(4); 80.7 C(2), C(3); 95.8 (2 OCH_2O).

Anal. calcd for $C_8H_{17}NO_5$ (207.23): C 46.37, H 8.27, N 6.76; found: C 46.3, H 8.5, N 6.6.

4.3.1. L-Enantiomer. Same procedure from L-**7** (1.41 g, 6.7 mmol) to give L-**8** (1.4 g, quant). Mp = 60–61 °C (iPr_2O). $[\alpha]_D^{20} = -17$ (c = 1, $CHCl_3$). Anal. calcd for $C_8H_{17}NO_5$ (207.23): C 46.37, H 8.27, N 6.76; found: C 46.1, H 8.5, N 6.6.

4.4. (3*R*,4*R*)-Pyrrolidine-3,4-diol (1,4-imino-1,4-dideoxy-D-threitol, D-4c)

A solution of D-**8** (0.206 g, 1.0 mmoles) in water (2 ml) was hydrogenated over Raney-Nickel (0.3 g wet) for

16 h (tlc Et_2O/MeOH 9/1). The catalyst was discarded by centrifugation, washed with water (3 × 0.5 ml), 7 M HBr (1 ml) was added to the combined aqueous solutions and the solution warmed at 50 °C for 4–6 h. Evaporation of the solvent to dryness gave D-**4c.HBr** as crystals which were washed with dry *i*PrOH (0.105 g, 60%).

D-**4c.HBr**: brownish crystals, mp = 103–104 °C (*i*PrOH). $[\alpha]_D^{22} = 0$ ($c = 1$, H_2O); for the free base D-**4c** $[\alpha]_D^{22} = -22.3$ ($c = 0.53$, H_2O at pH 9) (lit.[34] $[\alpha]_D^{26} = +20.7$ ($c = 0.3$, MeOH) for the free base L-**4c**).

IR (KBr): 3390, 3009, 1625, 1444, 1421, 1303, 1250, 1109, 1029, 985 cm^{-1}.

^1H NMR (D_2O, 400 MHz, 295 K): 3.34 (d, Hb-C(2), Hb-C(5)); 3.58 (dd, Ha-C(2), Ha-C(5)); 4.39 (d, H–C(3), H–C(4)). J(2a,2b) = 13.0, J(2a,3) = 3.3 Hz. ^1H NMR at pH 9: same data as for the free base in lit.[34]

^{13}C NMR (D_2O, 100 MHz, 295 K): 51.4 (C(2), C(5)); 74.9 (C(3), C(4)).

Anal. calcd for $C_4H_{10}BrNO_2$ (184.03): C 26.11, H 5.48, N 7.61, Br 43.43; found: C 26.0, H 5.6, 7.5, Br 43.0.

4.5. 4-(Hydroxylamino)-2,3-di-*O*-(methoxymethyl)-4-deoxy-D-threose ((3*R*,4*R*)-3,4-Bis(methoxymethoxy)-1-pyrroline-*N*-oxid, D-5)

To a suspension of HgO (0.63 g, 2.9 mmol, 1.2 equiv) in CH_2Cl_2 (6 mL) was added D-**8** (0.50 g, 2.42 mmol) and stirred for 30 min at rt until completion (tlc: Et_2O/MeOH 9/1). The black precipitate of Hg was eliminated by centrifugation, washed with CH_2Cl_2 (2 × 1 ml) and the organic solutions evaporated to give nitrone D-**5** (0.47 g, 95%) as brownish resin. For analytical purpose, it was purified by FC (Et_2O/MeOH 9/1).

D-**5**: colourless resin. $[\alpha]_D^{20} = -40$ ($c = 1$, $CHCl_3$).

IR ($CHCl_3$): 3002, 2955, 2894, 1585, 1152, 1107, 1041, 919 cm^{-1}.

^1H NMR (D_2O, 250 MHz, 300 K): 3.44 (s, 2 OMe); 4.00 (ddt, Ha-C(4)); 4.50 (dm, Hb-C(4)); 4.57 (dt, H–C(3)); 4.80, 4.82 (2 d, J = 7.2 Hz, OCH_2O); 4.84, 4.86 (2d, J = 7.1 Hz, OCH_2O); 4.96 (m, H–C(2)); 7.39 (q, H–C(1)). J(1,2) = J(1,4a) = J(1,4b) = 1.8; J(2,3) = 2.4; J(2,4a) = 1.0; J(2,4b) = 1.6; J(3,4a) = 2.6; J(3,4b) = 6.8; J(4a,4b)= 14.2 Hz.

^1H NMR ($CDCl_3$, 250 MHz, 300 K): 3.39, 3.40 (2 s, 2 OMe); 3.87 (m, Ha-C(4)); 4.36 (m, Hb-C(4), H–C(3)); 4.74 (m, H–C(2)); 4.69, 4.75 (2 d, J = 7.0 Hz, OCH_2O); 4.70, 4.77 (2 d, J = 6.9 Hz, OCH_2O); 6.94 (t, J = 1.9 Hz, H–C(1)). Similar data as for the L-enantiomer.[17]

^{13}C NMR ($CDCl_3$, 62,5 MHz, 300 K): 55.6 (OMe); 55.8 (OMe); 67.0 (C(4)); 77.0 (C(3)); 82.7 (C(2)); 96.1, 96.2 (2 OCH_2O); 132.8 (C(1)). Partial data in lit.[19].

HRMS (FAB^+), $C_8H_{15}NO_5 + H^{(+)}$: *m*/*z* calcd 206.1028, found 206.1035.

4.5.1. L-Enantiomer. Same procedure with L-**8** (0.20 g, 1.15 mmol) to give L-**5** (0.193 g, 97%).

4.6. (2*S*,3*R*)-4-Amino-1,4-dideoxy-D-threose-1-sulfonic acid (D-4b)

A solution of D-**5** (0.20 g mmol) in aqueous N H_2SO_4 (1 ml) was stirred at 20-25 °C in SO_2 atmosphere (in glass vessel) for 2–2.5 days. The pink solution was evaporated to half by lyophilisation, EtOH (1 ml) was added, the solution saturated with SO_2 and let to crystallise at 0 °C. D-**4 b** was isolated by centrifugation and washed with EtOH (80 mg, 45%).

D-**4b**: Colourless crystals, mp above 200–210 °C (dec, H_2O/EtOH). $[\alpha]_D^{20} = +18$ ($c = 1$, H_2O).

IR (KBr): 3420, 3040, 2800, 1575, 1420, 1260, 1230, 1175, 1105, 1070, 1050, 1025, 990 cm^{-1}.

^1H NMR (D_2O, 400 MHz, 295 K): 75/25 mixture of α- and β-anomer: see Table 3.

^{13}C NMR (D_2O, 100 MHz, 295 K): α-anomer: 78.0, 76.7, 75.4 C(1), C(2), C(3); 51.2 C(4). β-anomer: 75.7, 74.6, 73.3 C(1), C(2), C(3); 53.0 C(4). In phosphate buffer (pH 7), α-anomer: 79.6, 79.2, 77.7 C(1), C(2), C(3); 50.8 C(4).

Anal. calcd for $C_4H_9O_5NS$ + 10% $C_4H_9O_3N$: C 27.18, H 5.13, N 7.65, S 16.3; found: C 27.0, H 5.3, N 7.6, S 15.8.

4.6.1. L-Enantiomer. Same procedure with L-**5** (0.21 g, 1.0 mmol) for 4 d at 15 °C to give L-**4b** (95 mg, 52%), mp = 200–210 °C.

4.7. Stability of sulfite adduct D-4b and amino-sugar, D-4a

The stability of D-**4b**, D-**4a** was assessed in D_2O at pH ca. 4.5 without buffer or in acetate buffer and at pH ca. 7 in phosphate buffer or in the non-nucleophilic Hepes buffer.

4.7.1. Sulfite adduct D-4b. To a ca. 8 10^{-3} M solution of D-**4b** (1.0 mg) in D_2O (0.7 ml) were added AcOK (5.0 mg) and AcOH (4.5μl) (acetate buffer, pH ca 4.5) or NaH_2PO_4 (5.0 mg) and $Na_2HPO_4{\cdot}3H_2O$ (8.0 mg) (phosphate buffer, pH ca 7) or Hepes (5.0 mg) and $NaHCO_3$ (5.0 mg) (Hepes buffer, pH ca 7). ^1H NMR data at natural pH (without buffer, pH ca. 5) or in buffered solutions are reported in Table 3. In acetate buffer two anomers (α/β 70/30) were observed in ^1H NMR (D_2O, 295 K); in phosphate and Hepes buffer, the α/β proportions were, respectively, 95/5 and 90/10. No transformations of the solutions were detected after 1 day at rt.

In order to compare the spectra in these various conditions, the spectrum of each solution of D-**4b** after incubation in phosphate or Hepes buffer was always performed as control at pH 4-5 by acidification with

Table 3. ^{1}H NMR data of D-**4b**, D-**4a** (major species: imine (i), amino-alcohol (a) and dimer (d)) in 8 mM solution (D_2O, 400 MHz, 295 K, δ in ppm, J in Hz) without buffer or in acetate, phosphate or Hepes buffer

	Buffer[a]	H–C(1)	H–C(2)	H–C(3)	Ha-C(4)	Hb-C(4)	J(1,2)	J(2,3)	J(3,4a)	J(3,4b)	J(4a,4b)
D-**4b**(α)	no	4.41	4.48	4.37	3.63	3.45	5.2	3.3	5.3	4.3	12.3
D-**4b**(β)	no	4.75	4.55	4.44	3.87	3.42	3.3	1.2	4.3	ca 1	12.8
D-**4b**(α)	P or H	4.01	4.33	4.15	3.25	2.94	4.8	5.5	6.4	7.6	11.2
D-**4b**(β)	P	4.42	4.41	e	3.53	3.02	4.0	e	5.0	2.5	12.0
D-**4b**(α)	A	4.37	4.46	4.34	3.58	3.39	5.3	3.0	5.2	4.6	12.1
D-**4b**(β)	A	4.75	4.54	4.43	3.86	3.41	ca4.0	ca1.0	4.1	ca 1.0	12.7
D-**4a**(i)[b]	no	7.72	4.68	4.28	4.14	3.70	1.0	3.0	6.0	3.4	16.6
D-**4a**(a)	no	[c]	3.95	[c]	3.32	[c]	5.8[d]	4.0[d]	6.8	[c]	11.0
D-**4a**(d)	no	ca 4.90	4.15	4.20	3.10	2.98	3.5	6.0	7.2	6.0	11.8
		ca 4.90	4.40	4.27	3.00	2.84	4.8	1.0	4.2	2.4	12.0
D-**4a**(i)	P or H	7.71	4.67	4.27	4.13	3.69	[c]	[c]	[c]	[c]	ca 17
D-**4a**(d)	H	5.23	4.10	4.19	[c]	[c]	5.0	ca 7	ca 7	ca 7	[c]
		5.21	4.44	4.27	3.41	3.54	5.0	ca 0	ca 3	ca 0	ca 12
D-**4a**(a)	A	5.03	3.43	4.11	3.16	3.19	6.5	2.2	4.2	8.6	13.4

[a] no, without buffer; A, acetate buffer, pH ca. 4,5; H, Hepes buffer, pH ca. 7.0; P, phosphate buffer, pH ca. 7.0.
[b] J(1,4a) = 2.2, J(1,4b) = 2.4, J(2,4a) = J(2,4b) = 1.0.
[c] not determinated.

AcOH. In both cases similar ^{1}H NMR spectra as in acetate buffer was observed.

4.7.2. Amino-sugar D-4a. Its solutions were prepared by addition of $Ba(OH)_2{\cdot}8H_2O$ (2.0 mg) to a 8 10^{-3} M solution of D-**4b** (1.0 mg) in D_2O (0.7 ml), stirring for 2 h, elimination of solids by centrifugation and then addition of the buffer components as above.

4.7.3. ^{1}H NMR studies. ^{1}H NMR spectral data of the main species in D_2O or in studied buffered solutions are compiled in Table 3. In D_2O solution at 295 K (natural, pH ca 8), D-**4a** is a mixture of 2 main species imine (i) and dimer (d), and two other minor species the amino-alcohol (a) and a unknown in proportions of 30/50/8/12 (in weight); by dilution at 0.8 10^{-3} M, these proportions become 63/22/15/0. In Hepes buffer, D-**4a** is a 1:2 mixture of 2 main species imine (i) and dimer (d). No notable evolution of these solutions was observed after 1 day at rt.

In acetate buffer, four main species were observed, dimer (d), a species assumed to be the amino-alcohol (a) and two other species with broad signals in proportions of 50/20/20/10. After 1 day at rt, the only evolution was the disappearance of the dimer. Ulterior basification to pH 8-9 with $Ba(OH)_2$ led to a similar ^{1}H NMR spectrum as without buffer, the main compounds being imine (i) and dimer (d).

In phosphate buffer (pH 7), similar mixture as in Hepes buffer was observed, but a irreversible evolution led to the disappearance of the signals of imine and dimer with a $t_{1/2}$ of ca 16 h at 22 °C.

4.8. Determination of the glycosidase activities

General conditions, see lit.[69]

Tested glycosidases: α-D-glucosidase (EC 3.2.1.20) from baker's yeast (K_m = 0.3 mM, pH 7.0), β-D-glucosidase (EC 3.2.1.21) from almonds (K_m = 1.3 mM, pH 5.0), α-D-mannosidase (EC 3.2.1.24) from Jack beans (K_m = 1.0 mM, pH = 4.5), β-D-mannosidase (EC 3.2.1.25) from snail, acetone powder (K_m = 1,3 mM, pH 4.0), α-L-fucosidase (EC 3.2.1.51) from bovine kidney (K_m = 0.3 mM, pH 5.5), α-D-galactosidase (EC 3.2.1.22) from green coffee beans (K_m = 0.25 mM, pH 6.5), and β-D-galactosidase (EC 3.2.1.23) from *Escherichia Coli* (K_m = 0.65 mM, pH 7.0).

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