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Rapid discovery of potent α-fucosidase inhibitors by in situ screening of a library of (pyrrolidin-2-yl)triazoles

Pilar Elías-Rodríguez, Elena Moreno-Clavijo, Ana T. Carmona, Antonio J. Moreno-Vargas* and Inmaculada Robina

The synthesis of a small library of (pyrrolidin-2-yl)triazoles via copper catalysed cycloaddition of an alkynyl iminocyclopentitol and a set of commercial and synthetic azides has been achieved. The in situ screening for the activity towards α-fucosidase of the resulting triazoles has allowed the identification of one of the most potent and selective pyrrolidine derived inhibitors of this enzyme ($K_i = 4$ nM).

Introduction

α-Fucosidases are glycosidases involved in the biosynthesis of cell surface O-fucosylated oligosaccharides which play an important role in cell recognition, bacterial adhesion and viral invasion. Thus, α-fucosidases are associated to certain disorders as inflammation, metastasis of certain cancer cells and cystic fibrosis. Recently, it has been reported that the activity of human α-fucosidase 2 is critical for the pathogenesis of Helicobacter pylori including gastric cancer among other diseases. Moreover, α-fucosidases have been found in human seminal plasma and in the membrane of human sperm cells and facilitate sperm transport and sperm-egg interactions. For these reasons, α-fucosidases are clinically important targets and the design of efficient routes for the synthesis of potent and selective inhibitors remains an attractive goal.

Over recent years, we have been actively working on the development of new iminocyclitols with inhibitory activity towards α-fucosidases. We have shown that the presence of an additional aromatic or heteroaromatic binding component close to the five membered iminocyclitol increases notably their inhibitory activity: ($1^{10}$ vs $2^8$, Figure 1). This fact has been also shown by Behr in the case of pyrrolidine $3^{11}$ and by Wong in the case of six-membered iminocyclitols ($4^9$ vs $5$). Nevertheless, attempts to explore chemical diversity on five-membered iminocyclitols by the systematic variation of the aromatic group remains a cumbersome task.

The Cu(I)-catalyzed alkyne-azole cycloaddition ($\text{CuAAC}$) has become a widely used strategy for chemical space exploration in drug design due to its quickness at room temperature and compatibility with water-rich solvent systems, which makes it very suitable for in situ screening. In the search for potent glycosidase inhibitors, the strategy of in situ screening of a library of compounds generated by a combinatorial procedure has been very scarcely explored to date. Thus, the search for α-mannosidase inhibitors has been performed through imine condensation of the appropriate configured pyrrolidine carbaldehyde and amines. In the case of α-fucosidase inhibitors, L-fucopiperidine derivatives were sought through amide condensations from 1-aminofoconojirimycin as starting material. For the synthesis of pyrrolidine derivatives as hexosaminidase inhibitors, amide condensation and non-catalyzed Huisgen cycloaddition were used starting from convenient 1-amino- and 1-azido-iminocyclitols.
respectively. Particularly, CuAAC has been only used in the in situ screening of aminocyclitols as glucocerebrosidase (a type of β-glucosidases) inhibitors, in spite that CuAAC has been broadly used in the last years as click reaction in the preparation of multivalent glycosidase inhibitors.

We report herein the application of the CuAAC followed by in situ screening towards α-fucosidase for the preparation of analogues of compound 2 with improved inhibitory properties. As far as we are aware, no other optimization of the glycosidase inhibitory properties of iminocyclitols using this strategy has been reported. To achieve this goal and choose the appropriate lead compound, we planned two possible strategies (Scheme 1): 1) CuAAC between an unprotected alkenyl iminocyclitol with synthetic or commercial azides, and 2) CuAAC between an aziimidethyl iminocyclitol and terminal alkynes. In both cases the biological analysis of the combinatorial library should be carried out by in situ screening, allowing determining whether the resulting substituted pyrrolidine-triazole derivatives are α-fucosidase inhibitors and if they could improve the activity of the parent pyrrolidines.

Results and discussion

Synthesis of the lead compound for the generation of the library.

Protected precursors of the above iminocyclitols were prepared as described in Scheme 2. The synthesis of pyrroline 6 was carried out from D-lyxose following the procedure reported by Behr but using the tert-butylidiphenylsilyl ether as protecting group. Treatment of 6 with TBAB followed by stereoselective hydrogenation of 7 over Pd/C in MeOH at 200 psi gave 8. N-Boc protection and oxidation furnished carbaldehyde 10 which was transformed into alkyn derivative 11 after reaction with the Bestmann-Ohira reagent. On its side, N-Chz protection of 8 and introduction of the azido moiety through tosylation and displacement, provided azido derivative 13.

Scheme 2. Synthesis of protected alkynyl/azidomethyl pyrrolidines.

In order to choose the best strategy (strategy 1 or 2, Scheme 1) for the combinatorial preparation of a library of potential inhibitors, alkyn 11 and azide 13 were transformed into isomeric triazoles 14a and 15, respectively. Thus, CuAAC of 11 with benzyl azide using Cu/DIPEA followed by acide deprotection afforded triazole 14a (Scheme 3). Azide 13 was transformed into triazole 15 by CuAAC with phenyl acetylene followed by deprotection. The inhibitory activity of both triazoles 14a and 15 was studied towards eleven commercial glycosidases (Table 1). Triazole 14a was twenty times better fucosidase inhibitor than the isomeric triazole 15. The presence of a methylene group between the pyrrolidine skeleton and the triazole moiety proved to be detrimental for the inhibition of fucosidase. Thus, strategy 1 was chosen for the generation of the combinatorial library. For this reason, unprotected alkyne 14 was also synthesized from 11 and biologically analyzed.

Scheme 3.

Table 1. Inhibitory activities of compounds 14, 14a and 15 towards glycosidases. Percentage of inhibition at 1 mM of inhibitor, IC50 and Kᵢ. Optimal pH for each enzyme, 37 ºC. a, b

<table>
<thead>
<tr>
<th>Compartes/Enzyme</th>
<th>14</th>
<th>14a</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-L-fucosidase (from bovine kidney)</td>
<td>IC50 = 0.5 µM</td>
<td>IC50 = 1 µM</td>
<td>IC50 = 2.1 µM</td>
</tr>
<tr>
<td>IC50 = 0.3 µM</td>
<td>Kᵢ = 44 nM</td>
<td>Kᵢ = 24 nM</td>
<td>Kᵢ = 500 nM</td>
</tr>
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</table>

a No inhibition was detected towards β-galactosidase from Aspergillus oryzae and from Escherichia coli, α-glucosidase from rice, α-mannosidase from jack beans, β-N-acetylglucosaminidase from jack beans, α-galactosidase from coffee beans, amyloglucosidase from Aspergillus niger, α-glucosidase from Saccharomyces cerevisiae, β-glucosidase from almonds and β-mannosidase from snail.

b Competitive inhibition was observed in all the cases, according to the Lineweaver-Burk plots (See Supporting Information).

Generation of a library of (pyrrolidin-2-yl)triazoles and in situ screening as α-fucosidase inhibitors.

In order to obtain a library of triazoles from alkyn 14, alkyl and aryl azide reactants (Scheme 4) were selected from commercial sources or prepared from commercially available materials following standard protocols (see ESI). As we had previously reported that appropriate configured furyl iminocyclitols showed good α-fucosidase inhibition, a batch of synthetic azides containing the furan moiety (compounds 0-u) was also used.

Parallel CuAAC between (2-ethynyl)iminocyclitol 14 and azides a-u were carried out under the same reaction conditions using H2O2-BuOH as solvent system (Scheme 4). The in situ screening of the resulting crude (pyrrolidin-2-yl)triazoles towards α-fucosidase was carried out in a 96-well microtiter plate. Each inhibition assay was performed in a well containing 0.5 µM of the potential inhibitor (see Experimental for details). Blank experiments with the CuAAC reagents (CuSO4 and sodium ascorbate) and with each of the azides a-u were also carried out: no inhibition was observed. Interestingly, several
potent inhibitors were found among the 21 library members studied, their % inhibition values at 0.5 µM are shown in Figure 2. All the triazole derivatives resulted to be better inhibitors than the alkaline precursor. Compounds containing the triazole motif linked to other aromatic moieties showed better inhibition than when linked to non-aromatic ones (% inhibition of 14b,d,e,o-t >85% vs % inhibition of 14h-1 < 68%). It is worth noting the high inhibition presented by the derivatives bearing the furan moiety (14o-14u), being 14p the best inhibitor of the library (93% inhibition at 0.5 µM). These results were used as criteria for preliminary screening and compound selection.

In order to perform a more accurate inhibition study, selected triazole 14p was synthesized in higher scale and purified by column chromatography. Its inhibition properties were studied towards eleven commercial glycosidases (footnote in Table 1). At 1 mM concentration, 14p only showed inhibition towards α-fucosidase from bovine kidney, being the IC50 = 17 nM and Ki = 4 nM (competitive inhibition), which confirms the high potency of this inhibitor.

In conclusion, we have demonstrated that the fucosidase inhibitory activity of a library of (pyrrolidin-2-yl)triazoles generated by CuAAC can be in situ analysed after the click reaction, avoiding the tedious isolation/purification steps. By this strategy, one of the best α-fucosidase inhibitor reported so far belonging to the pyrrolidine-iminosugar family has been identified. This is the first combinatorial method for the rapid discovery of α-fucosidase inhibitors that employs the combination of a CuAAC click reaction and in situ screening.

Experimental

General methods.

Optical rotations were measured in a 1.0 cm or 1.0 dm tube with a Jasco P-2000 spectropolarimeter. Infrared spectra were recorded with a Jasco FTIR-410 spectrophotometer. 1H and 13C NMR spectra were recorded with a Bruker AMX300, AV300, AV500 and AVIII500 for solutions in CDCl3, CD2OD and DMSO-d6, at room temperature except when indicated. δ are given in ppm and J in Hz. All the assignments were confirmed by COSY and HSQC experiments. Mass spectra (Cl and LSI) were recorded on Micromass AutoSpecQ and QTRAP spectrometers. The LSI was performed using thioglycolate as the matrix. NMR and Mass spectra were registered in CITIUS (University of Seville). TLC was performed on silica gel HF254 (Merck), with detection by UV light charring with H2SO4, p-anisaldehyde, vanillin, ninhydrin or with Pancaldi reagent [(NH4)3MoO4, Ce(SO4)2, H2SO4, H2O]. Silica gel 60 (Merck, 63-200 µm) was used for preparative chromatography.

(3R,4S,5S)-5-Hydroxymethyl-3,4-O-isopropylidene-2-methyl-1-pyrroline-3,4-diol (7).

To a solution of 6 (3.6 g, 8.5 mmol) in THF (20 mL), TBAF (1 M in THF, 8.5 mL, 8.5 mmol) was added. After stirring at r.t. for 3 h, the solvent was evaporated and the residue purified by chromatography on silica gel (CH2Cl2/MeOH, 15:1) to give 7 (1.5 g, 8.1 mmol, 95%) as a colourless oil. NMR and IR data are in accordance with those of its enantiomer.22 [α]D27 = 78.2 (c 0.96, CH2Cl2). IR (ν cm−1) 3181 (OH), 2932, 2826, 1648, 1207, 1069, 868. 1H-NMR (300 MHz, CDCl3, δ ppm, J Hz) δ 4.88 (d, 1H, J1a = 5.6, H-3), 4.58 (d, 1H, H-4), 4.16 (br.s, 1H, H-5), 3.87 (dd, 1H, J1,2 = 11.6, J2,3 = 3.3, H-1'a), 3.76 (dd, 1H, J1,2 = 3.5, H-1'b), 2.76 (br.s, 1H, OH), 2.09 (d, 3H, J3,4 = 1.1, Me), 1.35 (s, 3H, -(C2H5)2), 1.34 (s, 3H, -(C2H5)2). 13C-NMR (75.4 MHz, CDCl3, δ ppm) δ 176.1 (C-2), 111.9 (-C(CH3)2), 87.4 (C-3), 80.8 (C-4), 78.1 (C-5), 62.6 (C-1'), 27.0 (-C(CH3)2), 25.8 (-C(CH3)2), 17.1 (Me). LSIMS m/z 186 [33%, (M+H)+] HRLSIMS m/z found 186.1134, calc. for C8H16NO5: 186.1130.

(2S,3S,4R,5S)-2-Hydroxymethyl-3,4-O-isopropylidene-5-methylpyrroline-3,4-diol (8).

A solution of pyrroline 7 (2.0 g, 10.8 mmol) in MeOH (40 mL) was stirred under H2 (200 psi) in the presence of 10% Pd/C. After 24 h, the catalyst was filtered through celite and washed with MeOH. The solvent was evaporated and the residue purified by chromatography on silica gel (CH2Cl2/MeOH, 15:1→10:1, 1% Et2N) to give 8 (1.6 g, 8.6 mmol, 80%) as a white solid. NMR and IR data are in accordance with those of its enantiomer.22 [α]D27 = −13.3 (c 0.89, CH2Cl2). IR (ν cm−1) 3239 (OH, NH), 2977, 2874, 1369, 841. 1H NMR (300 MHz, CDCl3, δ ppm, J Hz) δ 4.48 (dd, 1H, J3,4 = 5.4, J1a,b = 3.9, H-4), 4.40 (d, 1H, H-3), 3.50 (dd, 1H, J1,2,3 = 10.2, J1a,b = 5.3, H-1'a), 3.32-3.24 (m, 2H, H-1'b, H-2), 3.09 (qd, 1H, J3,4 = 6.6, H-5), 2.93 (s, 2H, NH, OH), 1.47, 1.29 (2s,

Figure 2. Inhibitory activities towards bovine kidney α-fucosidase (pH 6, 37 ºC) measured for triazole derivatives 14a-u at 0.5 µM on the well.
(25,35,4R,5S)-N-tert-Butoxy carbonyl-2-hydroxymethyl-3,4-O-isopropylidene-5-methyl pyrrolidine-3,4-diol (9).

To a solution of 8 (198 mg, 1.06 mmol) in MeOH (8 mL), (Boc)O (302 mg, 1.46 mmol) was added. The mixture was left for r.t. for 6 h. The solvent was evaporated and the obtained residue was purified by column chromatography on silica gel (CH2Cl2/MeOH, 40:1→5:1) to give 9 (281.2 mg, 0.98 mmol, 92%) as a colourless oil. [α]D25 +145.6 (c 0.41, CH2Cl2); IR (υ cm⁻¹) 2986, 2933, 1706 (C=O), 1366, 1163, 1024, 859. 1H NMR (300 MHz, CDCl3, δ ppm, J Hz) δ 6.61 (d, 1H, J = 11.7 Hz), 5.18 (d, 1H, J = 10.0 Hz), 4.93 (d, 1H, J = 4.6 Hz), 3.89 (s, 3H, Me); 3.71 (d, 1H, J = 10.0 Hz), 2.79 (t, 2H, J = 6.8 Hz), 2.26 (s, 3H, -CH3); 1.27 (s, 3H, -CH3). 13C NMR (75.4 MHz, CDCl3, δ ppm) δ 167.1 (C-1), 136.8 (C-2), 126.4 (C-3, C-4), 116.3 (C-5), 60.3 (C-6), 36.0 (C-7), 26.3, 24.1 (-CH2CH3), 13.4 (Me). MS: m/z 311 [M+H]+. HRMS m/z found 311.2499, [M+H]+. HCRIMS m/z found 311.2479, [M+H]+. HCRIMS m/z observed 311.2474.

(25,35,4R,5S)-N-Benzoyloxy carbonyl-2-hydroxymethyl-3,4-O-isopropylidene-5-methyl pyrrolidine-3,4-diol (12).

To a solution of compound 8 (265.2 mg, 1.42 mmol) in EtOH:H2O (1:1, 20 mL), NaHCO3 (120 mg, 1.43 mmol) and CbCl2 (224 μL, 1.56 mmol) were added. After stirring 2 h at r.t., sat. aq. sol. of NaHCO3 was added and the mixture was extracted with ethyl acetate. The organic phase was dried over Na2SO4, filtered and concentrated. The resulting residue was purified by chromatography on silica gel (EtOAc/cyclohexane, 1:2) to give 12 (385.7 mg, 1.20 mmol, 85%) as a colourless oil. [α]D25 +93.1 (c 0.53, CH2Cl2). IR (υ cm⁻¹) 3447 (OH), 2986, 2938, 1661 (C=O), 1410, 1210, 1026, 697. 1H NMR (300 MHz, DMSO-d6, 363 K, δ ppm, J Hz) δ 7.37-7.31 (m, 5H, H-aromat.), 5.10 (d, 1H, J = 12.5 Hz, CH2 of Cbz), 5.05 (d, 1H, CH2 of Cbz), 4.68-4.60 (m, 3H, H-3, H-4, OH), 3.92-3.84 (m, 2H, H-2, H-5), 3.56-3.53 (m, 2H, H-1′a, H-1′b), 1.39, 1.29 (2s, 3H each, -CH2CH3), 1.27 (d, 3H, J = 6.6 Hz, Me). 13C NMR (75.4 MHz, DMSO-d6, 363 K, δ ppm) δ 154.1 (C=O of Cbz), 136.6, 127.9, 127.4, 127.2 (C-aromat.), 109.9 (-CH2CH3), 80.5, 79.8 (C-3, C-4), 65.5 (CH2 of Cbz), 64.3, 56.9 (C-2, C-5), 60.0 (C-1′), 25.5, 24.6 (-CH2CH3), 14.9 (Me). MS: m/z 322 [2%, (M+H)+]. 290 [22%, (M-CH2OH)]. HCRIMS m/z observed 322.1647, calc. for C22H24O3N: 322.1654.

(25,35,4R,5S)-N-Benzyloxy carbonyl-2-azidomethyl-3,4-O-isopropylidene-5-methyl pyrrolidine-3,4-diol (13).

To a 0ºC solution of the alcohol 12 (602 mg, 1.87 mmol) in dry pyridine (16 mL), TsCl (717 mg, 3.76 mmol) was slowly added. After stirring at r.t. overnight, the mixture was cooled to 0 ºC, water was slowly added, and the mixture was allowed to warm to r.t. Solvent was then removed and the residue was diluted with EtOAc, washed with HCl (1N), sat. aq. sol. of NaHCO3 and brine, dried, filtered, and concentrated. To a solution of this compound in DMF (16 mL), NaN3 (305 mg, 4.69 mmol) was added. After heating at 70 ºC for 2 h, the solvent was evaporated and the residue diluted with CH2Cl2 and washed with water and brine. The organic phase was dried, filtered, and concentrated. Purification by chromatography column (EtOAc:cyclohexane 1:6) afforded 13 (464 mg, 1.34 mmol, 72%) as a colourless oil. [α]D25 +60.4 (c 0.55, CH2Cl2), IR (υ cm⁻¹) 2986, 2938, 2103 (N=), 1693 (C=O), 1403, 1210, 1026, 697. 1H NMR (300 MHz, DMSO-d6, 363 K, δ ppm) δ 7.38-7.30 (m, 5H, H-aromat.), 5.14 (d, 1H, J = 12.5 Hz, CH2 of Cbz), 5.08 (d, 1H, CH2 of Cbz), 4.68 (t, 1H, J = 6.2 Hz, H-4), 4.56 (dd, 1H, J = 1.0, H-3), 4.01-3.98 (1m, 1H, H-2), 3.90 (q, 1H, J = 1.0 Hz), 2.65 (d, 1H, J = 6.5 Hz), 2.36 (dd, 1H, J = 6.0 Hz, J = 6.0 Hz, 1H), 1.53 (dd, 1H, J = 3.5 Hz, J′ = 1.4 Hz), 1.40 (3s, 3H each, (-C(=O)CH3)2), 1.28 (d, 3H, Me). 13C NMR (75.4 MHz, DMSO-d6, 363 K, δ ppm) δ 153.6 (C=O of Cbz), 136.2, 127.8, 127.4, 127.2 (C-aromat.), 110.4 (-CH2CH3), 80.5 (C-3), 79.4
Evaporated. The resulting crude was purified by silica gel (Toluene/acetone, 10:1), affording the corresponding layer was dried over Na$_2$SO$_4$ and washed with sat. aq. sol. of NaHCO$_3$. Compound 11 (117.5 mmol) and CuI (7 mg, 0.037 mmol) were added. The mixture was stirred at r.t. for 2.5 h. Evaporation of the solvent and chromatographic purification on Dowex 50WX8 eluting with MeOH (50 mL), H$_2$O (50 mL) and NH$_4$OH 10% afforded 14 (19.2 mg, 0.14 mmol, 87% as a yellow solid. [α]$_D^{28}$ = -10.6 (c 0.83, MeOH); IR (ν cm$^{-1}$) 3306 (OH), 1662, 1454, 1190, 1344, 844, 799, 724. H NMR (300 MHz, MeOD, δ ppm, J Hz) δ 4.17 (dd, 1H, J$_{1,4}$ = 6.8, J$_{3,4} = 4.4$, H-3), 3.91 (t, 1H, J$_{2,3} = 2.3$, H-2), 3.37-3.28 (1H, H-5), 2.73 (d, 1H, H-2'), 1.16 (d, 3H, J$_{Me} = 6.7$, Me). $^{13}$C NMR (75.4 MHz, MeOD, δ ppm) δ 85.3 (C-1'), 81.0 (C-3), 74.5 (C-4), 73.2 (C-2'), 56.6 (C-5), 54.3 (C-2), 14.6 (Me). CIMS m/z found 142 [M+H]+. HRCIMS m/z found 142.0866, calc. for C$_7$H$_{14}$O$_2$N$_2$: 142.0868.

1,2,3-triazole-4-yl)-5-methylpyrrole-3,4-diol (14a).

To a solution of alkyne 14 (13.9 mg, 0.098 mmol) and azide p (32.1 mg, 0.118 mmol) in a 2:1 mixture of BuOH/H$_2$O (3.3 mL) treated with a catalytic amount of CuSO$_4$ (0.54 mg, 3.37·10$^{-3}$ mmol) followed by sodium ascorbate (2 mg, 0.011 mmol). After 24 h at r.t., the mixture was concentrated under reduced pressure. The resulting residue was purified by chromatography column on silica gel (CH$_2$Cl$_2$/MeOH, 8:1) to give 14p (22 mg, 0.053 mmol, 33%) as a yellow solid. [α]$_D^{22}$ = -33.2 (c 0.52, MeOH); IR (ν cm$^{-1}$) 3346 (OH, NH), 2912, 1711, 1218, 1076, 770, 623. $^{1}$H NMR (500 MHz, DMSO-d$_6$, 363K, δ ppm, J Hz) 6.790 (s, 1H, H-5'), 7.41-6.76 (5H, H-rotamat.), 6.76 (s, 1H, H-3''), 5.56 (s, 2H, H-1'''), 5.28 (s, 2H, -CH$_2$Ph), 4.13-4.12 (2H, H-2, H-3), 3.83 (t, 1H, J$_{2,3} = 4.0$, H-4), 3.33-3.31 (1H, H-5), 2.52 (s, 3H, Me), 1.07 (d, 3H, J$_{Me} = 6.5$, Me). $^{13}$C NMR (125.7 MHz, DMSO-d$_6$, 363K, δ ppm) 162.1 (C=O), 158.8, 149.3, 146.8, 135.8, 127.9, 127.5, 112.7, 113.4 (C-rotamat.), 121.3 (C-5'), 109.8 (C-3''), 78.1, 57.7 (C-2', C-3), 72.8 (C-4), 65.0 (-CH$_2$Ph), 54.7 (C-5), 45.0 (C-1''), 14.7 (Me), 12.9 (Me). HRCIMS m/z found 413.1822, calc. for C$_{27}$H$_{31}$O$_2$N$_4$: 413.1825.

In situ screening towards α-fucosidase. Glycosidase inhibition assays.

To a solution of alkyne 14 (0.3 mL, 30 mM in BuOH-H$_2$O (2:1)) in an eppendorf, a solution of the corresponding azide (a-u) was added (0.1 mL, 108 mM in BuOH) followed by 25 μL of an aqueous solution of sodium ascorbate (40 mM) and 25 μL of an aqueous solution of CuSO$_4$ (12 mM). The final concentration of the alkyne in each eppendorf was 20 mM. The resulting mixtures were left at room temperature for 24 h (the reactions were monitored for completion by TLC). Then, the reactions were diluted to the desired concentration in order to use it in the enzymatic assays. In the preliminary screening of the resulting crude (pyrrolidine-2-yl)triazoles, % of inhibition towards α-fucosidase from bovine kidney (EC 3.2.1.51) was determined in the presence of 0.5 μM of the inhibitor on the well (concentration corresponding to the IC$_{50}$ of the alkyne 14) with p-nitrophenyl α-L-fucopyranoside (Sigma-Aldrich) as
substrate. Each enzymatic assay (final volume 0.12 mL) contains 0.01 to 0.5 units/mL of the enzyme and 10 mM aqueous solution of the appropriate p-nitrophenyl glycoside substrate buffered to the optimal pH of the enzyme. Enzyme and inhibitor were preincubated for 5 min at rt, and the reaction started by addition of the substrate. After 20 min of incubation at 37 °C, the reaction was stopped by addition of 0.1 mL of sodium borate buffer (pH 9.8). The p-nitrophenol formed was measured by visible absorption spectroscopy at 405 nm.

Under these conditions, the p-nitrophenolate released led to optical densities linear with both reaction time and concentration of the enzyme. Blank experiments with sodium ascorbate/CuSO₄ and with each of the azides a-u were also carried out. An aqueous solution of sodium ascorbate (0.66 μM), CuSO₄ (1 μM), a solution of CuSO₄-sodium ascorbate (0.5 μM and 0.33 μM, respectively) and a solution of each azide (1.8 μM) were prepared in different wells. Each solution was assayed towards α-fucosidase as described above and no inhibition was observed for all cases. For the highest inhibition rate (triazole 14p) and for compounds 14, 14a and 15, the IC₅₀ value (concentration of inhibitor required for 50% inhibition of enzyme activity) and Kᵢ towards α-fucosidase were calculated. IC₅₀ values were calculated from slopes of percentage of inhibition versus inhibitor concentration and the Kᵢ values were determined from the Lineweaver-Burk plots (See ESI for details). All the experiments were performed by duplicate. In the case of the other ten enzymes, % of inhibition was determined at 1 mM of inhibitor on the well with the appropriate p-nitrophenyl glycosides as substrates. Commercially available glycosidases from Sigma-Aldrich: β-galactosidase (EC 3.2.1.23) from Aspergillus orizae and from Escherichia coli, α-mannosidase (EC 3.2.1.24) from Jack beans, β-N-acetylglucosaminidase (EC 3.2.1.30) from Jack beans, α-galactosidase (EC 3.2.1.22) from coffee beans, amyloglucosidase (EC 3.2.1.3) from Aspergillus niger, α-glucosidase (EC 3.2.1.20) from rice, α-glucosidase (EC 3.2.1.20) from Saccharomyces cerevisiae, β-glucosidase (EC 3.2.1.21) from almonds and β-mannosidase (EC 3.2.1.25) from snail.

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Notes and references
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Electronic Supplementary Information (ESI) available: experimental details for the preparation of 6 and azides a-u, enzymatic test details, 1H- and 13C-NMR spectra. See DOI: 10.1039/b000000x/

1 M. Sperrandio, FEBJS, 2006, 273, 4377.


18 See ESI for details of the synthetic route.


21 TLC showed complete conversion of all the reactions except for azides c and m, probably due to steric and stereoelectronic reasons.