Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 1339–1342

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Powerful Probes for Glycosidases: Novel, Fluorescently Tagged Glycosidase Inhibitors[1]

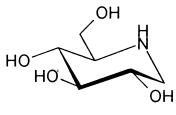
Albin Hermetter,[a] Hubert Scholze,[a] Arnold E. Stütz,[b,*] Stephen G. Withers[c] and Tanja M. Wrodnigg[b]

[a]*Institut für Biochemie der Technischen Universität Graz, Petersgasse 12, A-8010 Graz, Austria*
[b]*Glycogroup, Institut für Organische Chemie der Technischen Universität Graz, Stremayrgasse 16, A-8010 Graz, Austria*
[c]*Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, BC, Canada V6T 1Z1*

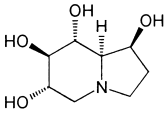
Received 7 February 2001; accepted 26 March 2001

Abstract—1-Amino-1,2,5-trideoxy-2,5-imino-D-mannitol was fluorescently tagged by reaction with dansyl chloride at N-1 or by attachment of a dansyl amide bearing spacer to this centre. Compounds obtained are highly potent inhibitors of β-glucosidase exhibiting K_i values in the single figure nanomolar range. The 1-*N*-dansyl substituted inhibitor was successfully exploited for binding studies with β-glucosidase from *Agrobacterium* sp. employing fluorescence spectrometric methods. © 2001 Elsevier Science Ltd. All rights reserved.

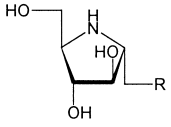
Iminosugars are potent reversible inhibitors of glycoside hydrolases and consequently important tools in glycobiology and glycotechnology.[2] 1-Deoxynojirimycin (1,5-dideoxy-1,5-imino-D-glucitol, **1**),[2] the paradigmatic D-glucosidase inhibitor in this class of inhibitors, exhibits similar properties as castanospermine (**2**),[2] another important glucosidase inhibiting sugar mimetic.[2] These and related compounds cause notable biological effects such as anti-diabetes, anti-retroviral as well as anti-tumour properties.[2]



1



2



3 R = OH
4 R = NH_2

The iminoalditol 2,5-dideoxy-2,5-imino-D-mannitol (**3**),[3] yet another typical representative of sugar analogues with basic nitrogen instead of oxygen in the ring, is a powerful reversible inhibitor of D-glucosidases and invertase.[2]

In order to extend our understanding of the structure–activity relationships that are operative in this range of compounds, some examples of analogues of **3** bearing lipophilic side chains attached to C-1 were prepared and screened.[4,5] In the course of this programme we also became interested in derivatives of **3** featuring extended aromatic systems, in particular fluorescent tags. Such compounds should bear great potential for the direct investigation and mapping of glycosidase active sites as well as for specific labelling of particular enzymes and, consequently, for diagnostic purposes.

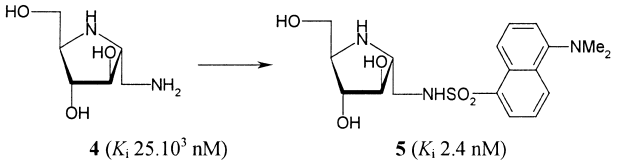
Exploiting the Amadori rearrangement reaction of readily available 5-azidodeoxy-D-glucofuranose with dibenzyl amine followed by catalytic hydrogenation with concomitant intramolecular reductive amination of the resulting 5-azido-1-(dibenzyl)amino-1,5-dideoxy-D-fructopyranose and nitrogen deprotection provided 1-amino-1,2,5-trideoxy-2,5-imino-D-mannitol (**4**), the 1-aminodeoxy derivative of **3** in 75% yield.[4] Based on this simple synthetic approach, a range of C-1 modified derivatives of compound **3** became available for evaluation of their glucosidase inhibitory activities.[5] In particular, analogues bearing lipophilic substituents showed interesting biological properties.[6]

These encouraging observations have now led to the design and synthesis of novel, fluorescently labelled analogues such as **5**, which were easily obtained[7] by

*Corresponding author. Tel.: +43-316-873-8241; fax: +43-316-873-8740; e-mail: stuetz@orgc.tu-graz.ac.at

0960-894X/01/$ - see front matter © 2001 Elsevier Science Ltd. All rights reserved.
PII: S0960-894X(01)00209-8

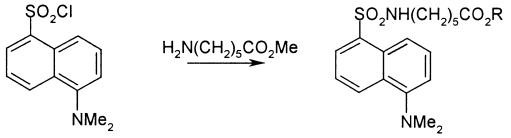
coupling dansyl chloride directly to N-1 of the inhibitor **4** (Scheme 1). Alternatively, in the case of spacer-armed compound **6**, a convergent synthesis was performed by coupling iminoalditol intermediate **4** with the fluorescently labelled spacer arm **8** employing HBTU [(*O*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate][8] as the coupling reagent.



4 (K_i 25.10^3 nM) **5** (K_i 2.4 nM)

Scheme 1.

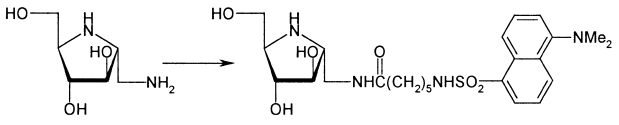
Compound **8** was prepared from dansyl chloride and methyl 6-aminohexanoate to give methyl ester **7** which was subsequently saponified (Schemes 2 and 3).



7 R = Me
8 R = H

Scheme 2.

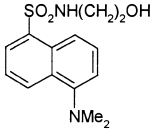
In both cases, the primary amine in compound **4** reacted highly selectively in the presence of the unprotected ring nitrogen furnishing the desired 1-*N*-acylated products **5** and **6**.



4 **6** (K_i 2.1 nM)

Scheme 3.

In order to probe any undesired inhibitory effect of the dansylamino moiety itself, *N*-dansyl ethanolamine (**9**) was prepared.



9 (K_i 5.10^5 nM)

Strong inhibition was observed with both new derivatives of compound **4** employing β-glucosidase from *Agrobacterium* sp. (Abg) at pH 6. Their inhibitory power was found to be two orders of magnitude better than parent compound **3** (**3**: K_i 200 nM; **5**: K_i 2.4 nM; **6**: K_i 2.1 nM) and thus amongst the most potent of this type of reversible β-glucosidase inhibitors.[3,9]

N-Dansyl ethanolamine (**9**) exhibited a K_i value of 500 μM documenting the importance of the sugar mimicking iminoalditol moiety for the inhibitory activity found with compounds **5** and **6**.

Characterisation of fluorescence properties of compound **5** as well as the corresponding complex with *Agrobacterium* sp. β-glucosidase was conducted as follows.[10] Different amounts of enzyme were incubated with compound **5** and electrophoresis was performed on native polyacrylamide gel without detergent (Fig. 1A). Other proteins such as β-galactosidase from *Aspergillus oryzae*, glucose isomerase, as well as albumin served as probes for the selectivity of **5** for β-glucosidase activity.

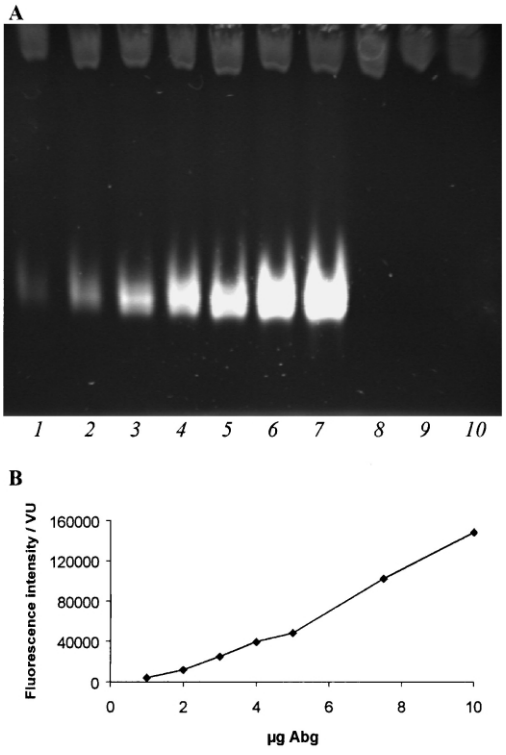


Figure 1. Visualization of the fluorescent complex of **5** with *Agrobacterium* sp. β-glucosidase (Abg) on native PAGE. (A) Different amounts of the enzyme and other related enzymes were preincubated with 2 nmol of **5** at room temperature for 15 min. Lanes: 1, 1 μg Abg; 2, 2 μg Abg; 3, 3 μg Abg; 4, 4 μg Abg; 5, 5 μg Abg; 6, 7.5 μg Abg; 7, 10 μg Abg; 8, 40 μg galactosidase; 9, 40 μg glucose isomerase; 10, 40 μg albumin. (B) Plot of fluorescence intensity versus enzyme mass.

Only the desired glucosidase–inhibitor complex could be detected as a single strongly fluorescing band nicely demonstrating the purity of the enzyme with respect to glucosidase activity as well as the selectivity of the inhibitor.

A plot of fluorescence intensity versus concentration of enzyme showed a linear concentration-dependent effect (Fig. 1B).

The quenching of the intrinsic tryptophan fluorescence of a defined amount of *Agrobacterium* sp. β-glucosidase by titration with compound **5** was also examined. The dansyl fluorophor is an energy acceptor for the first

excited singlet state of tryptophan. Consequently, binding of dansyl labelled inhibitor **5** to the enzyme suppresses the protein fluorescence highly efficiently.

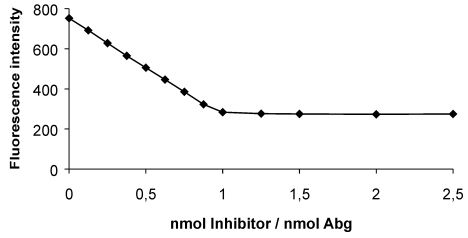


Figure 2. Quenching of the intrinsic tryptophan fluorescence of *Agrobacterium* sp. β-glucosidase (Abg) by titration with compound **5**.

Gratifyingly, the plot of the tryptophan fluorescence intensity versus the inhibitor/enzyme ratio exhibited maximal quenching at a 1:1 ratio of enzyme to inhibitor (Fig. 2). Larger amounts of inhibitor **5** did not further reduce the protein fluorescence.

Thus, it may be concluded that one inhibitor molecule binds to the active site of a single polypeptide without any additional adsorbtion of label at the surface of the protein.

Analogue **6** exhibited analogous behaviour.[11]

In conclusion, compounds **5** and **6** represent a novel type of reversible iminoalditol-based glycosidase inhibitor featuring not only powerful inhibitory activities but also excellent chemo-optical properties which, by their unprecedented combination, are clearly eminently suitable for exploitation in a wide range of new applications in glycobiology.

Acknowledgements

Synthetic studies were kindly supported by the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (FWF), Project P 13593 CHE. Enzymatic studies were supported by funds from the Protein Engineering Network of Centres of Excellence of Canada. T.M.W. thanks the FWF for a Hertha-Firnberg-Fellowship (T-18 CHE).

References and Notes

1. Dedicated to Herbert Stütz (1944–2000), a dedicated chemist and good friend.
2. *Iminosugars as Glycosidase Inhibitors*; Stütz, A. E., Ed.; VCH-Wiley: Weinheim, 1999, and references cited therein.
3. Welter, A.; Jadot, J.; Dardenne, G.; Marlier, M.; Casimir, J. *Phytochemistry* **1976**, *15*, 747.
4. Wrodnigg, T. M.; Stütz, A. E.; Withers, S. G. *Tetrahedron Lett*. **1997**, *38*, 5463.
5. Wrodnigg, T. M.; Gaderbauer, W.; Greimel, P.; Häusler, H.; Sprenger, F. K.; Stütz, A. E.; Virgona, C.; Withers, S. G. *J. Carbohydr. Chem*. **2000**, *19*, 975.
6. Wrodnigg, T; Withers, S. G.; Stütz, A. E. *Bioorg. Med. Chem. Lett*. **2001**, *11*, 1063.
7. Experimental procedures: Compound **5**. To a 3% solution of compound **4** in CH_3CN/MeOH 1:4 (v/v), 1.0 equiv of dansyl chloride (Aldrich) was added and the mixture was kept at ambient temperature until all starting material was consumed (TLC: MeOH/$CHCl_3$/NH_4OH concd 25:75:2). Concentration of the solution under reduced pressure and chromatographic purification of the residue (silica gel, Merck 60; MeOH/$CHCl_3$/NH_4OH concd 10:80:1) furnished sulfonamide **7** in 40% yield. $[\alpha]_D^{20}$ 22.1 (*c* 1.0 in MeOH). ^{13}C NMR (50.9 MHz, CD_3OD, 25 °C): δ 79.5, 78.3, 63.3, 62.2, 61.5, 45.1, 44.6 (2C). ^1H NMR (200 MHz, CD_3OD): δ 3.80–3.58 (m, 2H, H-3, H-4), 3.65–3.42 (m, 2H, $J_{6,6'} = 11.0$ Hz, $J_{5,6} = 4.0$ Hz, $J_{5,6'} = 6.1$ Hz, H-6, H-6′), 3.37–3.27 (m, 1H, NH), 3.08–2.79 (m, 10H, H-1, H-1′, H-2, H-5, NMe_2). Compound **6**. To a 3% solution of dansyl chloride in CH_2Cl_2, triethylamine (2.1 equiv) and methyl 6-aminohexanoate hydrochloride (1.0 equiv, Aldrich) were added and the mixture was kept at ambient temperature for 2 h. The mixture was partitioned between CH_2Cl_2 and 6% aqueous HCl and the organic layer was washed with aqueous sodium bicarbonate until neutral, dried (Na_2SO_4) and concentrated under reduced pressure. Conventional chromatography of the residue gave **7** (quantitative). To a 10% solution of this compound in dioxane/water 1:1 (v/v), aqueous NaOH (10%) was added dropwise until pH 10 was reached and the mixture was kept at ambient temperature for 4 h. The solution was neutralised with ion exchange resin Amberlite IR 120 [H^+], filtered and concentrated under reduced pressure to give the free acid **8** (85%). To a 10% methanolic solution of this compound, compound **4**, triethylamine, and HBTU (1 equiv each) were added and the mixture was kept at ambient temperature for 30 min (TLC in $CHCl_3$/MeOH/NH_4OH 75:25:2) and concentrated under reduced pressure. Chromatographic purification ($CHCl_3$/MeOH/NH_4OH 70:10:1) gave compound **6** (70% from **4**). $[\alpha]_D^{20}$ 12.2 (*c* 1.6 in MeOH). ^{13}C NMR (50.9 MHz, CD_3OD, 25 °C): δ 175.4, 79.9, 78.1, 63.6, 62.0, 61.7, 44.6 (2C), 42.5, 41.9, 35.7, 29.1, 26.0, 25.1. Compound **9**: Reaction of dansyl chloride with 2-aminoethanol (1.5 equiv) in CH_2Cl_2 in the presence of triethylamine (2 equiv) gave compound **9**, which, after concentration of the reaction mixture under reduced pressure, was isolated in 98% yield by chromatography ($CHCl_3$/MeOH 60:1). ^{13}C NMR (50.9 MHz, CD_3OD, 25 °C): δ 60.8, 44.9, 44.6 (2C). Synthetic procedures are not optimised. For all compounds reported, the signals of the aromatic system were found in the expected region.
8. Dourtoglou, V.; Ziegler, J.-C.; Gross, B. *Tetrahedron Lett*. **1978**, *15*, 1269; Li, P.; Xu, J.-C. *Tetrahedron* **2000**, *56*, 4437.
9. For other, related types of highly potent β-glucosidase inhibitors see: Jespersen, T. M.; Dong, W.; Sierks, M. R.; Skrydstrup, T.; Lundt, I.; Bols, M. *Angew. Chem*. **1994**, *106*, 1858; *Angew. Chem., Int. Ed. Engl*. **1994**, *33*, 1778; Legler, G.; Finken, M.-T.; Felsch, S. *Carbohydr. Res*. **1996**, *292*, 91; Panday, N.; Canac, Y.; Vasella, A. *Helv. Chim. Acta* **2000**, *83*, 58.
10. Characterisation of binding of compound **7** to *Agrobacterium* sp. β-glucosidase (Abg) and separation on native polyacrylamide gel: gels: 3.6% stacking gel, 6% resolving gel. For the labelling experiment, the enzyme (4 mg/mL) was diluted with 0.1 M potassium phosphate buffer pH 7.0 to a final concentration of 0.5 mg/mL. Aliquots from 2 to 20 μL were used and brought to a final volume of 20 μL with 0.1 M potassium phosphate buffer pH 7.0. Compound **5** was also dissolved in 0.1 M potassium phosphate buffer pH 7.0 to a concentration of 1 mM. Two millilitres of the stock solution were pipetted to the enzyme samples and incubated for 15 min at room temperature (final concentration 0.09 nmol/μL). Seven millilitres dissociation buffer (10 mM Tris/HCl pH 7.4, 50% glycerol, 1% Triton X-100, 0.1% bromophenol blue) were added and samples were applied onto the gel after 10 min. Images were taken using a Herolab EASY gel documentation system with UV excitation at 254 nm. Fluores-

cence was detected for 0.32 s corresponding to eight iteration steps. Quenching of the intrinsic tryptophan fluorescence of *Agrobacterium* sp. β-glucosidase (Abg) by titration with compound **5**. The enzyme was diluted with 0.1 M potassium phosphate buffer pH 7.0 to a final concentration of 1 nmol/mL (50 μg/mL). Two millilitres were used for the experiment. Instrument (Shimadzu RF-5301 PC, spectrofluorophotometer) settings were excitation 280 nm, emission 335 nm, slits 3 nm/3 nm. The inhibitor was dissolved in 0.1 M potassium phosphate buffer pH 7.0 (31 μM, detected by the absorption spectrum at 335 nm, $\varepsilon = 4600$ in methanol) and added stepwise to the cuvette under stirring. The fluorescence intensity was detected 2 min after the addition of the inhibitor.

11. Influences of the nature as well as of the length of the spacer-arm on enzyme–inhibitor interactions are currently being investigated.