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N-Bridged 1-deoxynojirimycin dimers as selective insect trehalase inhibitors

Laura Cipolla [a], Antonella Sgambato [a], Matilde Forcella [a], Paola Fusi [a], Paolo Parenti [b], Francesca Cardona [c], Davide Bini [a,*]

[a] Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milano, Italy
[b] Department of Earth and Environmental Sciences, University of Milano-Bicocca, Piazza della Scienza 1, 20126 Milano, Italy
[c] Department of Chemistry 'Ugo Schiff', University of Florence, Via della Lastruccia 3-13, I-50019 Sesto Fiorentino, Florence, Italy

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ABSTRACT

A small set of N-bridged 1-deoxynojirimycin dimers has been synthesized and evaluated as potential inhibitors of insect trehalase from midge larvae of *Chironomus riparius*, porcine trehalase as the mammalian counterpart and α-amylase from human saliva. All the tested compounds (**2**–**4**) proved to be active (micromolar range activity) against insect trehalase, showing selectivity toward the insect glycosidase. No activity was observed against α-amylase.

1. Introduction

Trehalase (α-glucoside-1-glucohydrolase, EC 3.2.1.28) is a specific glycosidase that catalyzes the hydrolysis of trehalose[1] (**1**, α-D-glucopyranosyl-α-D-glucopyranoside, Fig. 1) to the two constituent glucose units. This disaccharide is found in many organisms as diverse as bacteria, yeast, fungi, nematodes, plants, insects and some other invertebrates, but is absent in mammals. In lower organisms, trehalose may serve as a source of energy, a carbohydrate store, or an agent for protecting proteins and cellular membranes from inactivation or denaturation caused by a variety of environmental stress conditions. In insects, trehalose hydrolysis by trehalase is fundamental in various physiological processes including chitin synthesis during molting,[2] and thermotolerance in larvae.[3] Moreover, trehalase activity is the basis for flight metabolism,[1,4] trehalose being the principal hemolymph sugar in insects[5] that acts as an indispensable substrate for energy production and macromolecular biosynthesis.[6] Given these premises, insect trehalases are attractive targets for the search of inhibitors as potential novel and selective insecticides.[7] Some natural pseudodisaccharides, such as validoxylamine A,[8] trehazolin,[9] casuarine-6-*O*-α-D-glucoside[10,11] and its analogues[12] have been shown to be potent inhibitors of trehalase, together with synthetic trehalose analogues.[8,13–16]

In the search for new inhibitors that might be specific toward insect trehalase based on 1-deoxynojirimycin and its *N*-acyl derivatives[13] we herein propose the synthesis of N-bridged 1-deoxynojirimycin dimers (Fig. 1, **2**–**4**) and their biological evaluation toward both insect and porcine trehalase, compared to the human α-amylase enzyme (EC 3.2.1.1).

The synthesis of compounds **2**–**4** was performed straightforward from protected 1-deoxynojirimycin,[17] as outlined in Scheme 1. The reaction of compound **5** with oxalyl or succinyl chloride successfully afforded compounds **6** and **8** in 66% and 49% yields, respectively; in contrast, the reaction of **5** with malonyl chloride in the same reaction conditions gave compound **7** only in traces. The unexpected outcome of the reaction with malonyl chloride is probably due to by-products deriving from reaction of methylenic acidic protons of malonyl chloride with the basic pyridine used in the procedure. Dimer **7** was obtained in 70% yield by reaction of **5** with malonic acid in the presence of DCC. Direct hydrogenolysis of **6**–**8** quantitatively afforded the compounds **2**–**4**.

Compounds **2**–**4** were tested for their inhibitory activity against insect trehalase of midge larvae of *C. riparius*,[18] porcine trehalase (purchased from Sigma–Aldrich) as the mammalian counterpart and α-amylase from human saliva (purchased from Sigma–Aldrich), as a relevant glycolytic enzyme. Midge larvae are

* Corresponding author. Tel.: +39 02 64483427; fax: +39 02 64483565.
E-mail address: davide.bini@unimib.it (D. Bini).

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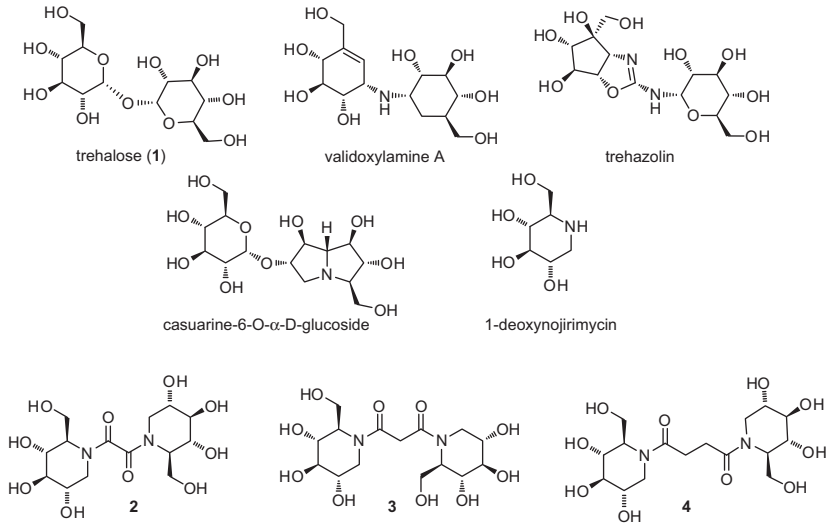
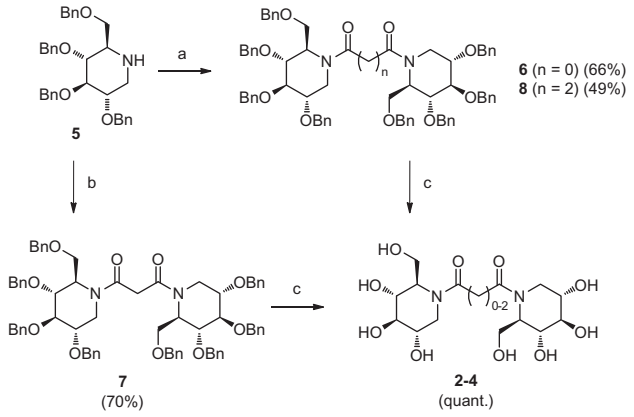


Figure 1. Structures of trehalose (**1**), validoxylamine A, trehazolin, casuarine-6-*O*-α-D-glucoside, 1-deoxynojirimycin, and the synthesized dimers **2**–**4**.



Scheme 1. Synthesis of nojirimycin dimers **2**–**4**. Reagents and conditions: (a) Oxalyl or succinyl chloride, pyridine, DCM, 0 °C→rt, 3 h; (b) Malonic acid, DCC, DMAP, *p*-TsOH, DCM, rt, 30 min; (c) $Pd(OH)_2/C$, H_2, EtOAc/EtOH = 1:1.

widespread in freshwater ecosystems, as sentinel organisms are widely used in ecotoxicological studies and environmental biomonitoring program and represent a good model for biochemical studies. To examine the potential of each 1-deoxynojirimycin dimer as trehalase inhibitor, preliminary screening assays at a fixed concentration (1 mM) of potential inhibitors were carried out, and dose–response curves were established for most active compounds in order to determine the IC_{50} values. Experiments were performed at a fixed substrate concentration, in the presence of increasing inhibitor concentrations. The inhibitory activity is shown in Figure 2 as IC_{50} value.

All the synthesized dimers were inactive against α-amylase, while they were similarly active against *C. riparius* trehalase, with the activity in the micromolar range. Compound **2** resulted to be the most active derivative of the series; all compounds **2**–**4** showed a slight selectivity toward the insect glycosidase, resulting more selective between mammalian and insect trehalase if compared to the parent compound 1-deoxynojirimycin.

We can conclude that despite the fact the both trehalase specifically hydrolyze trehalose, they might have significant differences in the catalytic pocket that can be exploited for the design and development of specific insect trehalase inhibitors, with potential follow up in the development of insecticides. However, further insights are needed into enzyme recognition features.

2. Experimental

2.1. General methods

All solvents were dried over molecular sieves, for at least 24 h prior to use, when required. When dry conditions were required, the reaction was performed under Ar or N_2 atmosphere. Thin-layer chromatography (TLC) was performed on silica gel $60F_{254}$ coated glass plates (Merck) with UV detection when possible, or spots were visualized by charring with a conc. H_2SO_4/EtOH/H_2O solution (10:45:45 v/v/v), or with a solution of $(NH_4)_6Mo_7O_{24}$ (21 g), $Ce(SO_4)_2$ (1 g), concd H_2SO_4 (31 mL) in water (500 mL) and then heating to 110 °C for 5 min. Flash column chromatography was performed on silica gel 230–400 mesh (Merck). Routine ^1H and ^{13}C NMR spectra were recorded on a Varian Mercury instrument at 400 MHz (^1H) and 100.57 MHz (^{13}C). Chemical shifts are reported in parts per million downfield from TMS as an internal standard; *J* values are given in Hz. Mass spectra were recorded on a System Applied Biosystems MDS SCIEX instrument (Q TRAP, LC/MS/MS, turbo ion spray) or on a System Applied Biosystem MDS SCIEX instrument (Q STAR elite nanospray). Elemental analyses (C, H, N) were performed with a Perkin–Elmer series II 2400 analyzer.

2.2. General procedure for the hydrogenolysis reaction

A 0.02 m solution of the appropriate dimer dissolved in EtOAc/EtOH (1:1) was treated with $Pd(OH)_2/C$ (100% in weight). The reaction was stirred for 5 d under a H_2 atmosphere. Palladium was then removed by filtration through a Celite pad followed by washing with EtOH and water. Evaporation of the solvents afforded the corresponding deprotected compounds in quantitative yields.

2.3. 1,2-Bis((2*R*,3*R*,4*R*,5*S*)-3,4,5-tris(benzyloxy)-2-((benzyloxy)methyl)piperidin-1-yl)ethane-1,2-dione (6)

To a solution of compound **5** (108 mg, 0.21 mmol) in dry DCM (1.1 mL), pyridine (33 μL, 0.41 mmol) and oxalyl chloride (9 μL, 0.10 mmol) were added at 0 °C. The temperature was slowly increased to rt (3 h); the mixture was then concentrated and the residue was purified directly on a silica gel column (petroleum ether/EtOAc, 65:35) affording **6** (74 mg, 66% yield). ^1H NMR ($CDCl_3$): δ = 7.37–7.01 (m, 40H, ArH), 4.77–4.19 (m, 18H, OCH_2Ph, H-5),

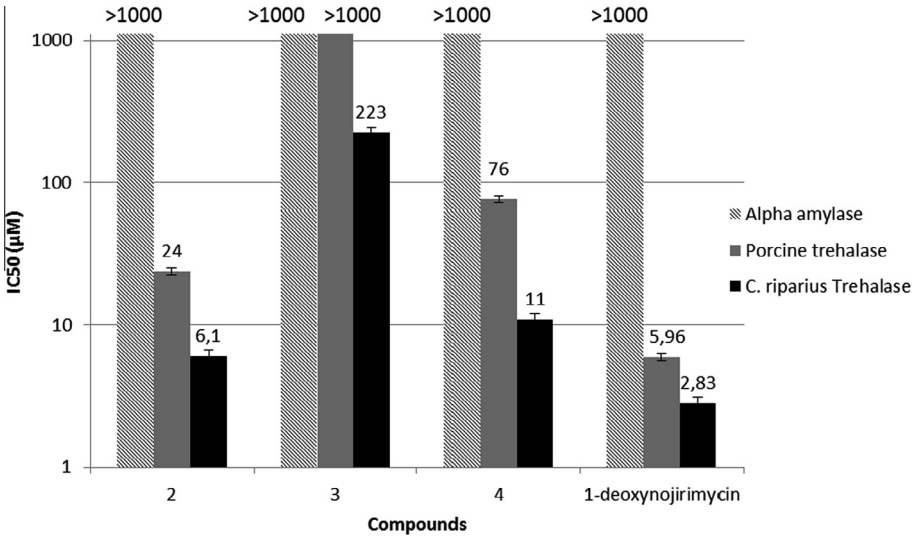


Figure 2. Histogram of the inhibitory activity of compounds **2**–**4**, compared to 1-deoxynojirimycin.

3.97–3.54 (m, 12H, H-1a, H-2, H-3, H-4, H-6), 3.14–3.02 (m, 2H, H-1b) ppm. ^{13}C NMR ($CDCl_3$): δ = 165.0, 164.7 (C═O), 138.5, 138.5, 138.3, 138.2, 138.2, 138.1, 138.1, 137.9 (C Ar), 128.7–127.6 (CH Ar), 82.4 (C-3), 79.1, 76.1 (C-2, C-4), 73.7–70.1 (OCH_2Ph), 68.9 (C-6), 58.6 (C-5), 43.8 (C-1) ppm. MS (TOF, *m*/*z*): $[M+H]^+$ calcd for $C_{70}H_{73}N_2O_{10}$, 1101.5; found 1101.5. $C_{70}H_{72}N_2O_{10}$ (1101.33): calcd C, 76.34, H 6.59, N 2.54; found C 76.49, H 6.58, N 2.55.

2.4. 1,3-Bis((2*R*,3*R*,4*R*,5*S*)-3,4,5-tris(benzyloxy)-2-((benzyloxy)methyl)piperidin-1-yl)propane-1,3-dione (7)

Compound **5** (67 mg, 0,12 mmol), malonic acid (6,4 mg, 0,06 mmol), DMAP (3 mg, 0.02 mmol) and *p*-toluenesulfonic acid (4.7 mg, 0.02 mmol) were dissolved in DCM (1.14 mL). DCC (32 mg, 0.15 mmol) was added and the solution was stirred for 30 min at room temperature. Then the DCC–urea was filtered off and washed with a small volume of DCM. The solvent was evaporated, and the residue was purified by flash column chromatography (petroleum ether/EtOAc, 62.5:37.5) giving pure **7** (48 mg, 70% yield). ^{1}H NMR ($CDCl_3$) δ = 7.34–7.15 (m, 40H, ArH), 4.77–4.59 (m, 6H, H-5, OCH_2Ph), 4.59–4.44 (m, 8H, OCH_2Ph), 4.41–4.25 (m, 4H, OCH_2Ph), 4.03–3.93 (m, 4H, H-2, H-1a), 3.80–3.56 (m, 10H, H-6, H-3, CH_2C=O, H-4), 3.56–3.46 (m, 2H, H-1b) ppm. ^{13}C NMR ($CDCl_3$) δ = 166.9 (C═O), 142.7–137.8 (C Ar), 128.4–127.5 (CH Ar), 80.9 (C-3), 78.1, 73.9 (C-2, C-4), 72.9–70.8 (OCH_2Ph), 68.0 (C-6), 54.2 (C-5), 44.4 (C-1), 41.9 (CH_2C═O) ppm. MS (TOF, *m*/*z*): $[M+H]^+$ calcd for $C_{71}H_{75}N_2O_{10}$, 1115.5; found 1115.5. $C_{71}H_{74}N_2O_{10}$ (1115.35): calcd C 76.46, H 6.69, N 2.51; found C 76.51, H 6.67, N 2.52.

2.5. 1,4-Bis((2*R*,3*R*,4*R*,5*S*)-3,4,5-tris(benzyloxy)-2-((benzyloxy)methyl)piperidin-1-yl)butane-1,4-dione (8)

To a solution of compound **5** (177 mg, 0.34 mmol) in dry DCM (1.9 mL), pyridine (55 μL, 0.67 mmol) and succinyl chloride (19 μL, 0.17 mmol) were added at 0 °C. The temperature was slowly increased to rt (3 h); the mixture was then concentrated and the residue was purified directly on a silica gel column (petroleum ether/EtOAc, 50:50) affording **8** (93 mg, 49% yield). ^{1}H NMR ($CDCl_3$): δ = 7.38–7.16 (m, 40H, ArH), 4.82–4.23 (m, 18H, OCH_2Ph, H-5), 4.05–3.41 (m, 12H, H-1a, H-2, H-3, H-4, H-6), 2.97–2.58 (m, 6H, H-1b, CH_2C═O) ppm. ^{13}C NMR ($CDCl_3$): δ = 171.8 (C═O), 138.4–137.8 (C Ar), 128.5–127.6 (CH Ar), 82.4 (C-3), 78.9, 74.3 (C-2, C-4), 73.3–71.1 (OCH2Ph), 68.3 (C-6), 54.5 (C-5), 44.0 (C-1), 32.0 (CH_2C═O) ppm. MS (TOF, *m*/*z*): $[M+H]^+$ calcd for $C_{72}H_{77}N_2O_{10}$, 1129.6; found 1129.6. $C_{72}H_{76}N_2O_{10}$ (1129.38): calcd C 76.57, H 6.78, N 2.48; found C 76.49, H 6.80, N 2.47.

2.6. 1,2-Bis((2*R*,3*R*,4*R*,5*S*)-3,4,5-trihydroxy-2-(hydroxymethyl)piperidin-1-yl)ethane-1,2-dione (2)

^{1}H NMR (D_2O): δ = 3.75–3.65 (m, 4H, H-6), 3.60–3.53 (m, 2H, H-2), 3.42–3.26 (m, 6H, H-1a, H-3, H-4), 3.02–2.98 (m, 2H, H-5), 2.82–2.72 (m, 2H, H-1b) ppm. ^{13}C NMR (D_2O): δ = 76.2 (C-3), 67.7, 66.9 (C-2, C-4), 60.0 (C-5), 57.6 (C-6), 45.8 (C-1) ppm. MS (TOF, *m*/*z*): $[M+H]^+$ calcd for $C_{14}H_{25}N_2O_{10}$, 381.1; found 381.4. $C_{14}H_{24}N_2O_{10}$ (380.35): calcd C 44.21, H 6.36, N 7.37; found C 44.27, H 6.34, N 7.38.

2.7. 1,3-Bis((2*R*,3*R*,4*R*,5*S*)-3,4,5-trihydroxy-2-(hydroxymethyl)piperidin-1-yl)propane-1,3-dione (3)

^{1}H NMR (D_2O) δ = 4.36 (d, 1H, *J* = 14.4 Hz, CH_2C═O), 3.96–3.52 (m, 16H, H-1, H-2, H-3, H-4, H-5, H-6), 3.20 (d, 1H, *J* = 14.8, CH_2C═O) ppm. ^{13}C NMR (D_2O) δ = 74.1 (C-3), 69.3, 68.4 (C-2, C-4), 63.0 (C-5), 59.8 (C-6), 47.7 (C-1), 40.6 (CH_2C═O) ppm. MS (TOF, *m*/*z*): $[M+H]^+$ calcd for $C_{15}H_{27}N_2O_{10}$, 395.2; found 395.3. $C_{15}H_{26}N_2O_{10}$ (394.37): calcd C 45.68, H 6.65, N 7.10; found C 45.74, H 6.63, N 7.11.

2.8. 1,4-Bis((2*R*,3*R*,4*R*,5*S*)-3,4,5-trihydroxy-2-(hydroxymethyl)piperidin-1-yl)butane-1,4-dione (4)

^{1}H NMR (D_2O): δ = 3.82–3.71 (m, 4H, H-6), 3.68–3.61 (m, 2H, H-2), 3.48–3.34 (m, 6H, H-1a, H-3, H-4), 3.09–3.05 (m, 2H, H-5), 2.86–2.80 (m, 2H, H-1b), 2.61–2.50 (m, 4H, CH_2C═O) ppm. ^{13}C NMR (D_2O): δ = 76.0 (C-3), 67.5, 66.7 (C-2, C-4), 59.7 (C-5), 57.4 (C-6), 45.6 (C-1), 28.6 (CH_2C═O) ppm. MS (TOF, *m*/*z*): $[M+H]^+$ calcd for $C_{16}H_{29}N_2O_{10}$, 409.2; found 409.4. $C_{16}H_{28}N_2O_{10}$ (408.40): calcd C 47.05, H 6.91, N 6.86; found C 47.14, H 6.89, N 6.87.

2.9. Biological assays

Trehalase activity was measured through a coupled assay with glucose-6-phosphate dehydrogenase and hexokinase according to Wegener at al.[19]. To examine the potential of each compound as a trehalase inhibitor, screening assays of potential inhibitors were

carried out at a fixed concentration of 1 mM and dose–response curves were established to determine the IC_{50} values. Experiments were performed at fixed substrate concentration close to the K_m value (0.5 mM for *C. riparius* trehalase and 2.5 mM for porcine trehalase), in the presence of increasing inhibitor concentrations. Initial rates as a function of inhibitor concentration were fitted to the following equation:

$$\frac{v_i}{v} = \frac{1}{1 + \left(\frac{[I]}{IC_{50}}\right)^n}$$

where v_i and v are the initial rate in the presence and in the absence of inhibitor, respectively, $[I]$ is the inhibitor concentration, IC_{50} is the inhibitor concentration producing half-maximal inhibition, and n is the Hill coefficient.

All enzyme assays were performed in triplicates at 30 °C by using sample volumes varying from 5 to 20 μL in 1 mL test and using a Cary3 UV/vis Spectrophotometer. Enzyme activities were analyzed by Cary Win UV application software for Windows XP. The α-amylase inhibition assay was performed at fixed starch concentration close to the K_m value (0.16% w/v), in the presence of increasing inhibitor concentration. Potential inhibitors were added to 0.5 mL enzyme solution (10 μg/mL). The reaction was initiated by adding 0.5 mL of starch dissolved in 0.02 mM sodium phosphate pH 6.9 and 6.7 mM sodium chloride and stopped after 3 min by adding 1 mL of 3,5-dinitrosalicylic acid reagent (43 mM 3,5-dinitrosalicylic acid, 1 M sodium potassium tartrate tetrahydrate, and 0.4 M sodium hydroxide in aqueous solution). The mixture was heated at 100 °C for 5 min. After cooling to room temperature, 10 mL of mQ water was added and absorbance was recorded at 540 nm using a Cary3 UV/vis Spectrophotometer.[20] Micromoles maltose released were determined from standard curve and the enzymatic activity was calculated using the following equation:

$$\frac{\text{Units}}{\text{mg}} = \frac{\text{micromoles maltose released}}{\text{mg enzyme in reaction mixture} \times 3 \text{ min}}$$

All enzyme assays were performed in triplicates at 30 °C.

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References

1. Elbein, A. D.; Pan, Y. T.; Pastuszak, I.; Carroll, D. *Glycobiology* **2003**, *13*, 17R–27R.
2. Tatun, N.; Singtripop, T.; Sakurai, S. *J. Insect Physiol.* **2008**, *54*, 351–357.
3. Tatun, N.; Singtripop, T.; Tungjitwitayakul, J.; Sakurai, S. *Insect Biochem. Molec.* **2008**, *38*, 788–795.
4. Legg, J. S.; Evans, D. R. *Science* **1961**, *134*, 54–55.
5. Thompson, S. N. *Adv. Insect Physiol.* **2003**, *31*, 203–285.
6. Friedman, S. *Annu. Rev. Entomol.* **1978**, *23*, 389–407.
7. Asano, N. *Glycobiology* **2003**, *13*, 93R–104R.
8. (a) Asano, N.; Takeuchi, M.; Kameda, Y.; Matsui, K.; Kono, Y. *J. Antibiot.* **1990**, *43*, 722–726; (b) Gibson, R. P.; Gloster, T. M.; Roberts, S.; Warren, R. A. J.; Storch de Gracia, I.; García, Á.; Chiara, J. L.; Davies, G. J. *Angew. Chem., Int. Ed.* **2007**, *46*, 4115–4119.
9. Kyosseva, S. V.; Kyossev, Z. N.; Elbein, A. D. *Arch. Biochem. Biophys.* **1995**, *316*, 821–826.
10. Kato, A.; Kano, E.; Adachi, I.; Molyneux, R. J.; Watson, A. A.; Nash, R. J.; Fleet, G. W. J.; Wormald, M. W.; Kizu, H.; Ikeda, K.; Asano, N. *Tetrahedron: Asymmetry* **2003**, *14*, 325–331.
11. Cardona, F.; Parmeggiani, C.; Faggi, E.; Bonaccini, C.; Gratteri, P.; Sim, L.; Gloster, T. M.; Roberts, S.; Davies, G. J.; Rose, D. R.; Goti, A. *Chem. Eur. J.* **2009**, *15*, 1627–1636.
12. Cardona, F.; Goti, A.; Parmeggiani, C.; Parenti, P.; Forcella, M.; Fusi, P.; Cipolla, L.; Roberts, S.; Davies, G. J.; Gloster, T. M. *Chem. Commun.* **2010**, 2629–2631.
13. Forcella, M.; Cardona, F.; Goti, A.; Parmeggiani, C.; Cipolla, L.; Gregori, M.; Schirone, R.; Fusi, P.; Parenti, P. *Glycobiology* **2010**, *20*, 1186–1195.
14. Bini, D.; Forcella, M.; Cipolla, L.; Fusi, P.; Matassini, C.; Cardona, F. *Eur. J. Org. Chem.* **2011**, 3995–4000.
15. Bini, D.; Cardona, F.; Forcella, M.; Parmeggiani, C.; Parenti, P.; Nicotra, F.; Cipolla, L. *Beilstein. J. Org. Chem.* **2012**, *8*, 514–521.
16. Bini, D.; Cardona, F.; Gabrielli, L.; Russo, L.; Cipolla, L. Trehalose Mimetics as Inhibitors of Trehalose Processing Enzymes In *Specialist of Periodical Reports, SPR Carbohydrate Chemistry*; Royal Society of Chemistry, 2011; Vol. 37, cap 10, pp 1–44. doi: 10.1039/9781849732765, ISBN-10: 1849731543, ISBN-13: 978-1849731546.
17. Wennekes, T.; Lang, B.; Leeman, M.; van der Marel, G. A.; Smits, E.; Weber, M.; van Wiltenburg, J.; Wolberg, M.; Aerts, J. M. F. G.; Overkleeft, H. S. *Org. Process Res. Dev.* **2008**, *12*, 414–423.
18. Forcella, M.; Mozzi, A.; Bigi, A.; Parenti, P.; Fusi, P. *Arch. Insect Biochem. Physiol.* **2012**, *81*, 77–89.
19. Wegener, G.; Tschiedel, V.; Schlöder, P.; Ando, O. *J. Exp. Biol.* **2003**, *206*, 1233–1240.
20. Bernfeld, P. *Methods Enzymol.* **1955**, *1*, 149–158.