Organic &
Biomolecular Chemistry

ROYAL SOCIETY OF CHEMISTRY

PAPER

Cite this: *Org. Biomol. Chem.*, 2015, **13**, 886

New synthesis and biological evaluation of uniflorine A derivatives: towards specific insect trehalase inhibitors†

Giampiero D'Adamio,‡[a] Antonella Sgambato,‡[b] Matilde Forcella,‡[b] Silvia Caccia,‡[c] Camilla Parmeggiani,[a,d] Morena Casartelli,[c] Paolo Parenti,[e] Davide Bini,[b] Laura Cipolla,[b] Paola Fusi*[b] and Francesca Cardona*[a]

Received 22nd September 2014,
Accepted 29th October 2014

DOI: 10.1039/c4ob02016b

www.rsc.org/obc

7-Deoxy-uniflorine A (**6**), synthesized *ex novo* with a straightforward and simple strategy, and the analogues **4**, **5** and **7**, were evaluated as potential inhibitors of insect trehalase from *Chironomus riparius* and *Spodoptera littoralis*. All the compounds were tested against porcine trehalase as the mammalian counterpart and α-amylase from human saliva as a relevant glucolytic enzyme. The aim of this work is the identification of the simplest pyrrolizidine structure necessary to impart selective insect trehalase inhibition, in order to identify new specific inhibitors that can be easily synthesized compared to our previous reports with the potential to act as non-toxic insecticides and/or fungicides. All the derivatives **4–7** proved to be active (from low micromolar to high nanomolar range activity) towards insect trehalases, while no activity was observed against α-amylase. In particular, the natural compound uniflorine A and its 7-deoxy analogue were found to selectively inhibit insect trehalases, as they are inactive towards the mammalian enzyme. The effect of compound **6** was also analyzed in preliminary *in vivo* experiments. These new findings allow the identification of natural uniflorine A and its 7-deoxy analogue as the most promising inhibitors among a series of pyrrolizidine derivatives for future development in the agrochemical field, and the investigation also outlined the importance of the stereochemistry at C-6 of pyrrolizidine nucleus to confer such enzyme specificity.

Introduction

Casuarine **1** (Fig. 1) is a naturally occurring highly oxygenated pyrrolizidine alkaloid obtained from *Casuarina equisetifolia*. Together with its 6-α-glucopyranosyl conjugate (**2**),[1] which is found in *C. equisetifolia* and *Eugenia jambolana*,[2] it is a potent and specific α-glucosidase inhibitor.[3] In particular, both casuarine and its glucosyl derivative **2** were shown to be effective trehalase inhibitors.[4] Trehalases [EC 3.2.1.28] are retaining α-glucosidases in charge of the hydrolytic cleavage of trehalose (**3**, α-D-glucopyranosyl α-D-glucopyranoside), which is a disaccharide with several functions in different organisms such as fungi, mycobacteria, and insects. Trehalases are present in mammals and are responsible for the hydrolysis of ingested trehalose (intolerance to mushrooms is ascribed to the absence or deficit of trehalases) because this disaccharide is absent in their metabolism. The absence of trehalose in the metabolism of mammals, together with the physiological relevance of trehalose hydrolysis in insects, makes insect trehalase inhibition a relevant target for the development of selective insecticides that are potentially non-toxic to mammals.[5] Moreover, the design of trehalase inhibitors may help in the elucidation of the structure of the protein active site of the enzyme from different organisms, which are featured by the same substrate specificity but possess some degree of variability because they can be selectively inhibited, as shown by the previous data.[4,6]

To date, only the X-ray structure of *Escherichia coli* trehalase is known (PDB entry 2WYN);[7] therefore, structural data justifying the reasons for selective inhibition are still scarce.

[a]Dipartimento di Chimica "Ugo Schiff", University of Florence, Via della Lastruccia 3-13, 50019 Sesto Fiorentino, FI, Italy. E-mail: francesca.cardona@unifi.it; Tel: +390554573504
[b]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milano, Italy. E-mail: paola.fusi@unimib.it
[c]Department of Biosciences, University of Milano, Via Celoria 26, 20133 Milano, Italy
[d]CNR-INO, U.O.S. Sesto Fiorentino and LENS, via Nello Carrara 1, 50019 Sesto Fiorentino, FI, Italy
[e]Dipartimento di Scienze dell'Ambiente e del Territorio e di Scienze della Terra, University of Milano-Bicocca, Piazza della Scienza 1, 20126 Milano, Italy

† Electronic supplementary information (ESI) available: ^{1}H and ^{13}C NMR spectra of compounds **6**, **9**, **10** and **11**, inhibition kinetics of compounds **4–7** and bioassay data. See DOI: 10.1039/c4ob02016b
‡ These authors contributed equally to this work.

 This journal is © The Royal Society of Chemistry 2015

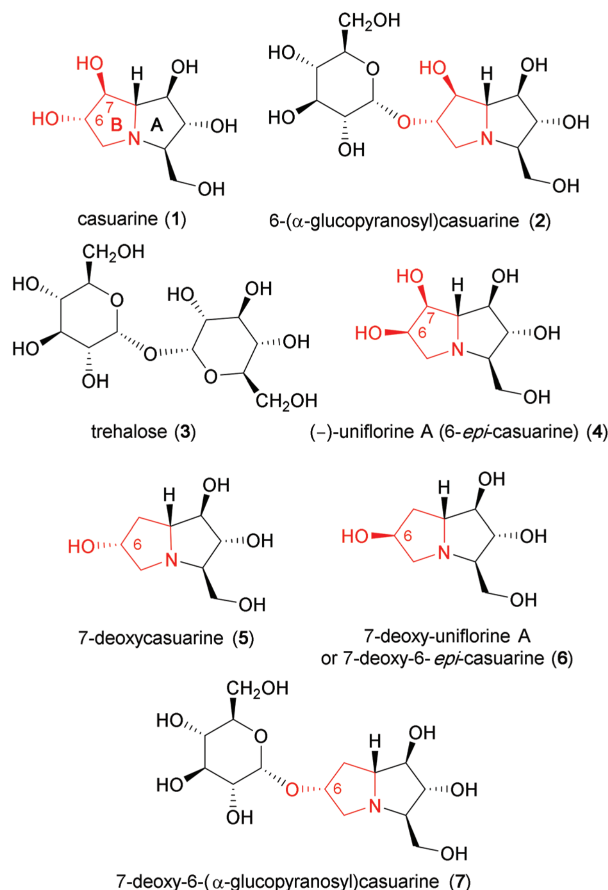


Fig. 1 Chemical structure of casuarine (**1**), its glucopyranosyl derivative (**2**), compounds **4–7** used in the present study, and trehalose (**3**), which is the natural substrate of trehalases.

If specificity is an important issue to be addressed with any inhibitor, it is of particular significance with respect to trehalase inhibitors. Indeed, the design of a viable insecticide, which targets trehalase, needs to be safe for plants, mammals, and for insects, which are beneficial in nature; thus, the inhibitor should target only specific insect trehalases.

Our previous findings showed that with *E. coli* trehalase (for which some X-ray structures of the complex enzyme inhibitors were obtained), casuarine based inhibitors bind the primary catalytic site with the **A** ring of the pyrrolizidine nucleus, which mimics the natural glucose configuration. Moreover, simple modifications at the C-7 of the **B** ring (OH, H, CH_2OH) were able to influence both the potency and specificity of inhibition.[4a,c] It was therefore expected that modifications at the C-6 of the B ring could also disclose interesting results in terms of the potency/specificity of trehalase inhibition, and this is the aim of the present work. Moreover, the preparation of new inhibitors by simple and low cost synthetic routes is of great importance to investigate them by *in vivo* tests, and if effective, in agri-food industry.

To briefly investigate modifications at the position C-6 of the B ring pyrrolizidine nucleus, we propose here a novel synthetic approach for the preparation of 7-deoxy-uniflorine A, and the biological evaluation of analogues **4–6** and of 7-deoxy-6-(α-glucopyranosyl)casuarine **7** as selective trehalase inhibitors.

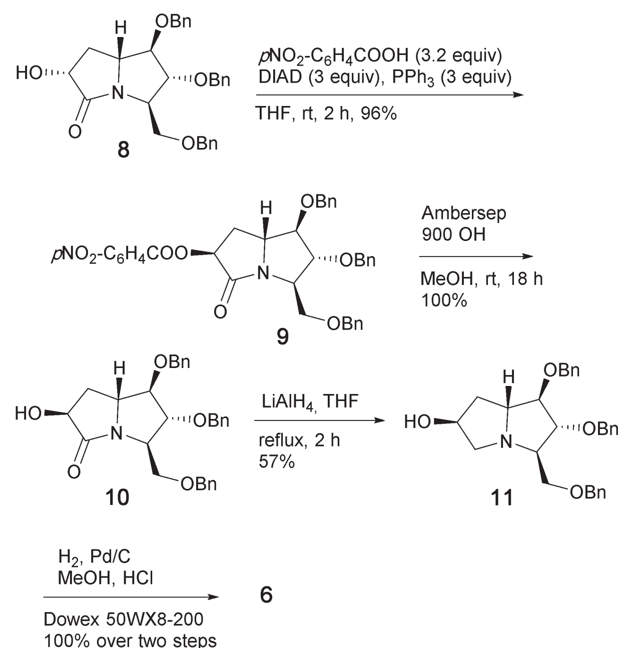
Results and discussion

Chemistry

Derivatives **4**, **5** and **7** were synthesized as already described. In particular, natural (−)-uniflorine A (**4**) was isolated in 2000 from the leaves of *E. uniflora* L. (Myrtaceae), an evergreen tree widely found in Paraguay, Uruguay, Argentina and Brazil. From the leaves, a natural Paraguayan medicine named Nangapiry is produced and used in folk medicine as an antidiarrheic, diuretic, antirheumatic, antifebrile and antidiabetic agent. The α-glucosidase, maltase and sucrose inhibitor activities of **4** have also been reported.[8]

The total synthesis of **4** was accomplished in nine steps by us with 11% overall yield[9] from a carbohydrate-based nitrone by exploiting the nitrone cycloaddition chemistry.[10] Cycloaddition with a different dipolarophile on the same nitrone allowed the synthesis of 7-deoxycasuarine (**5**).[11] A selective α-glucosylation on intermediate **8**[11] (Scheme 1) after proper synthetic elaborations provided 7-deoxy-6-(α-glucopyranosyl)-casuarine (**7**).[4c]

Derivative **6** was synthesized *ex novo*, as reported in Scheme 1, to test a non-natural derivative bearing the same configuration at C-6 of native (−)-uniflorine A, but lacking the hydroxyl group at C-7. Our aim was to verify if (i) stereochemistry at C-6 may have an effect on inhibition in terms of potency and selectivity, (ii) the presence of another hydroxyl group at C-7 is fundamental for activity, (iii) an additional glucosyl moiety at C-6 is essential, considering that both the



Scheme 1 Synthesis of 7-deoxy-uniflorine A (**6**).

introduction of an OH group at C-7 and a glucosyl moiety at C-6 considerably elongate the total synthesis, which requires more synthetic steps and lowers the overall yield of the final compound.

To accomplish the desired inversion of configuration at C-6, a Mitsunobu reaction on intermediate **8** appeared to be the best and simple straightforward strategy. *p*-Nitrobenzoic acid (3.2 equiv.) was used as the nucleophile for the Mitsunobu reaction, in THF as solvent and in the presence of triphenylphosphine (3 equiv.) and diisopropylazodicarboxylate (3.2 equiv., Scheme 1). After 2 hours at room temperature, lactam **8** was converted into ester **9** in 96% yield. Treatment with the strongly basic resin Ambersep 900 OH provided lactam **10** in quantitative yield with an opposite configuration at C-6 with respect to **8**. The reduction of the C=O bond with $LiAlH_4$ in refluxing THF produced pyrrolizidine **11** in 57% yield.[12] The final catalytic hydrogenation followed by treatment with ion exchange resin quantitatively afforded 7-deoxy-6-*epi*-casuarine (**6**) (Scheme 1). The synthesis of **6** had been previously reported by Behr and co-workers with a different synthetic strategy, which also yielded 7-deoxycasuarine (**5**) in approximately 1 : 1 ratio.[13,14]

The synthetic strategy reported herein afforded **6** in 54% yield over 4 steps from lactam **8**, thus allowing the production of this compound on a considerably larger scale, as required for *in vivo* tests.

In vitro enzymatic assays

To evaluate the specific inhibition of the synthesized compounds against insect trehalases and to briefly explore the importance of the configuration at C-6 in the selectivity, a set of different α-glucosidases were selected for comparison. Derivatives **4–7** were then assayed for their activity against α-amylase from human saliva, trehalase from porcine kidney, purified *C. riparius* trehalase[15] and trehalases present in different tissue homogenates from *S. littoralis* larvae. The biological activity is listed in Table 1.[16] *S. littoralis* is a serious lepidopteran pest responsible for significant crop losses worldwide. Results obtained on trehalases from *S. littoralis* were in accordance with the effects on *C. riparius* trehalase, suggesting that *C. riparius* is a good model for biochemical studies. For a given compound, IC_{50} values of the same order of magnitude are obtained in the two insect species, although a marked but small difference was detected between trehalase from the midgut and Malpighian tubules of *S. littoralis* probably due to enzyme isoforms.

None of the tested compounds inhibited α-amylase from human saliva, suggesting the possibility of identifying non-toxic insecticides/fungicides among these compounds. Casuarine derivatives **5** and **7**, possessing the stereochemistry at carbon 6 corresponding to native casuarine, did not show the inhibition of α-amylase even at the highest concentration (1 mM), while they were active on both porcine and insect trehalases. In particular, the glucosylated derivative **7** showed IC_{50} values in the nanomolar range, whereas 7-deoxy-casuarine (**5**) was able to inhibit both the trehalases only in the low micromolar range, indicating the important role exerted by the glucosyl moiety in terms of the potency of inhibition. However, for both compounds, a considerable activity towards the mammalian enzyme was maintained and only a slight selectivity toward insect trehalases could be observed.

Much more interestingly, uniflorine derivatives (or 6-*epi*-casuarines **4** and **6**) showed total selectivity against insect trehalases, as they were completely inactive toward α-amylase and porcine trehalase at 1 mM concentration. In both the cases, IC_{50} was in the high nanomolar range, independent of the substituent (7-hydroxyl or 7-deoxy) at position 7 and an additional glucosyl moiety at C-6. The observed trend in the inhibitory activity of the compounds against the mammalian and insect trehalase suggests that the stereochemistry at position 6 is a key issue in discriminating the two enzymes and that the presence of an additional glucosyl moiety at C-6, which considerably elongates the total synthesis of the compounds, is not essential.

Inhibition kinetics

Inhibition kinetics revealed that all the compounds tested interact with the active site of the insect trehalase. To evaluate the inhibitory pattern produced by the compounds studied, kinetics experiments as a function of trehalose concentration at different inhibitor concentrations were performed for each of the compounds. Results showed different inhibitory patterns by the compounds, compounds **4** and **5** being hyperbolic mixed-type, compound **6** pure competitive and compound **7** linear mixed-type (see ESI†). To simplify the diagnosis of the inhibition type, data were plotted according to the Lineweaver-Burk plot and replots were built to calculate inhibition constants and additional kinetic parameters (Table 2).

Kinetic analysis revealed that compounds **4** and **5** produce hyperbolic mixed-type inhibition. Hyperbolic mixed-type inhibition is considerably rare; however, it can be overlooked if a sufficient inhibition concentration range is not explored.[17] Detailed kinetic analysis performed in structure–activity

Table 1 IC_{50} of compounds **4–7**

Compd.	α-Amylase	Porcine trehalase	*C. riparius* trehalase	*S. littoralis* midgut trehalase	*S. littoralis* Malpighian tubule trehalase
4	>1 mM	>1 mM	177 ± 18 nM	261 ± 24 nM	43 ± 10 nM
5	>1 mM	20.6 ± 2.2 μM	1.22 ± 0.08 μM	9.84 ± 0.49 μM	1.34 ± 0.27 μM
6	>1 mM	>1 mM	175 ± 12 nM	560 ± 40 nM	330 ± 90 nM
7	>1 mM	479 ± 45 nM	44 ± 1.0 nM	96 ± 15 nM	49.7 ± 6.8 nM

 This journal is © The Royal Society of Chemistry 2015

Table 2 Kinetic parameters of the inhibition of *C. riparius* trehalase. The factors α is the factor by which K_S changes when the inhibitor occupies the enzyme, and the factor β is the factor by which the rate constant for the breakdown of enzyme–substrate complex changes when the inhibitor is bound. The factor β is determined from 1/Δ intercept replot. The replot has an intercept on the 1/Δ intercept-axis of $\beta V_{max}/(1-\beta)$. The factor α is determined from 1/Δ slope replot: this replot has an intercept on the 1/Δ slope-axis of $\beta V_{max}/K_m(\alpha-\beta)$. When 1/Δ intercept = 0, the intercept on the 1/[I]-axis gives $-\beta/\alpha K_i$. With α and β known, K_i can be calculated (see ESI)

Inhibitor	K_i (nM)	α Factor	β Factor
Compound **4**	48 ± 4	4.8	0.4
Compound **5**	810 ± 16	2.5	0.5
Compound **6**	118 ± 12	—	—
Compound **7**	34 ± 2	5.8	—

relationships studies may disclose this behavior.[17] This type of inhibition occurs when both E and EI complex bind with S but with different affinities; moreover, both ES and ESI complexes are productive but with different rates. The primary plot of the hyperbolic mixed-type inhibition is not different from that of classical mixed-type, *i.e.*, the family curves pivot above the 1/[S] axis; however, secondary plots are hyperbolic instead of linear type. The system states that at any inhibitor concentration, an infinitely high [S] will drive all the enzyme to a mixture of EI and ESI. Because ESI is less productive by a factor β, V_{max} decreases. The value of β ranges from 0 to less than 1. At any infinitely high [I], all the enzyme is again driven to EI and ESI. Because EI has a lower affinity to [S], K_S is increased by a factor $\alpha > 1$. Because ESI is productive, the velocity cannot be driven to zero by increasing [I]. To calculate residual activity in the presence of hyperbolic mixed-type inhibitor, appropriate rate equations have been developed.[18] Applying these equation to compounds **4** and **5**, at a concentration 20-fold higher than the K_i value, the enzyme still retains 12% and 19% of residual activity, respectively.

Considering the data obtained on *C. riparius* trehalase, the stereochemistry of the pyrrolizidine ring around carbon 6 appears to be crucial for inhibition, suggesting a more precise fitting of the inhibitor when the stereochemistry of compound **6** is adopted, as confirmed by the K_i being 6.8-fold higher for compound **5** compared to compound **6**. This also holds true for *S. littoralis* enzyme; however, if a hydroxyl group is present on carbon 7, as in compound **4**, the inhibition becomes mixed-type, although a slight decrease of the K_i value is observed. The addition of a glucosyl moiety produces a stronger inhibitor in terms of the potency of inhibition due to a much closer structural similarity with the natural substrate. However, compound **7** was able to inhibit porcine trehalase, while the compounds **4** and **6** were not, thus revealing that in terms of selectivity towards the insect trehalases, the presence of an additional glucosyl moiety is not essential.

Bioassays on *S. littoralis* larvae

Due to the promising activity and selectivity detected by *in vitro* assays on insect trehalases, we decided to test the *in vivo* activity of casuarine derivative **6**. Different doses of the inhibitor were intra-hemocoelically injected in sixth instar *S. littoralis* larvae. Inhibitor concentrations were chosen according to similar experiments reported in the literature on the insecticidal activity of trehalase inhibitors.[19,20] Effects on larval and pupal weight, larval, pupal and adult morphology and adult emergence, longevity and fertility were monitored. Contrary to the expectation, in these preliminary experiments, no effects were detected on the parameters analyzed (see ESI†). The treatment of fifth instar larvae with the higher doses applied for sixth instar bioassay also failed to impair *S. littoralis* development (data not shown). The preliminary and promising *in vitro* responses of putative bioinsecticides may go against *in vivo* effectiveness, both in qualitative and quantitative terms, due to the complexity of the *in vivo* context. Having made that preliminary remark, the lack of effects at this point could be due to both the low inhibition of *S. littoralis* trehalases in the *in vivo* conditions (*e.g.* high hemolymphatic trehalose concentration) and/or rapid degradation of the derivative in the hemolymph. It is thus pivotal to take into account these hypotheses for the improvement in the design of inhibitors, including effective delivery vectors that preserve their activity in the *in vitro* context.[21]

Conclusions

A series of casuarine analogues (*i.e.* (−)-uniflorine A (**4**), 7-deoxycasuarine (**5**), its glucosyl derivative **7** and the newly synthesized 7-deoxy uniflorine A (or 7-deoxy-6-*epi*-casuarine, (**6**)) were evaluated as the potential specific inhibitors of insect trehalase from *C. riparius* and *S. littoralis*. None of the compounds inhibited α-amylase from human saliva, showing their potential as non-toxic insecticides/fungicides. Compounds **5** and **7** proved to be active against both insect and porcine trehalases, with a slight selectivity towards the insect enzyme, while compounds **4** and **6** behaved as exclusive inhibitors of insect trehalases (they did not inhibit the mammalian porcine trehalase). Although the glucosylated derivative **7** represents the most active inhibitor in terms of potency of inhibition, the results outlined in this work demonstrate that the presence of an additional glucosyl moiety is not essential for targeting the selective inhibitors of the insect trehalase. Indeed, in terms of selectivity, compounds **4** and **6** were identified as the most interesting compounds. The specificity of compounds **4** and **6** suggests that stereochemistry at position 6 is a key issue in discriminating porcine and insect trehalases. However, additional factors, such as the absence of substitution at position 7, could be critical in producing high affinity true competitive inhibitors. Due to their selectivity towards insect trehalases, compounds **4** and **6** are the most promising for future development as insecticides. In particular, the straightforward and practical synthesis of **6** disclosed in this work will allow further investigation of its activity for other *in vivo* studies. If the inefficacy of **6** in the bioassays on *S. littoralis* trehalase is due to the high *in vivo* trehalose concentrations, it would be of

great importance to develop a pure competitive inhibitor based on the structure of **6** with a lower K_i, and work is underway in our laboratories in this direction.

Experimental section

Chemistry

Commercial reagents were used as received. All reactions were carried out with magnetic stirring and monitored by TLC on 0.25 mm silica gel plates (Merck F_{254}). Column chromatographies were carried out on Silica Gel 60 (32–63 μm) or on silica gel (230–400 mesh, Merck). Yields refer to spectroscopically and analytically pure compounds unless otherwise stated. ^{1}H NMR spectra were recorded on a Varian Mercury-400 or on a Varian INOVA 400 instrument at 25 °C. ^{13}C NMR spectra were recorded on a Varian Gemini-200. Chemical shifts are reported relative to TMS (^{1}H: δ = 0.00 ppm) and $CDCl_3$ (^{13}C: δ = 77.0 ppm). Integrals are in accordance with assignments, and coupling constants are given in Hz. For detailed peak assignments, 2D spectra were measured (COSY, HSQC, NOESY, and NOE if necessary). IR spectra were recorded with a BX FT-IR Perkin-Elmer System spectrophotometer. ESI-MS spectra were recorded with a Thermo Scientific™ LCQ fleet ion trap mass spectrometer. Elemental analyses were performed with a Perkin-Elmer 2400 analyzer. Optical rotation measurements were performed on a JASCO DIP-370 polarimeter.

(1*R*,2*R*,3*R*,6*S*,7a*R*)-1,2-Bis(benzyloxy)-3-[benzyloxy)methyl]-6-*p*-nitrobenzoyl-5*H*-pyrrolizin-5-one (9). To a solution of **8** (108 mg, 0.23 mmol) in dry THF (5 ml), triphenylphosphine (181 mg, 0.69 mmol) was added under nitrogen atmosphere. The reaction mixture was cooled to 0 °C and DIAD (140 mg, 0.69 mmol) was added dropwise. After the addition of *p*-nitrobenzoic acid (43 g, 0.26 mmol), the temperature of the mixture was raised to room temperature and stirred under nitrogen atmosphere for 2 h until TLC analysis (AcOEt–EP 3 : 1) showed the disappearance of the starting material (R_f = 0.35) and the formation of a new product (R_f = 0.66). The solvent was removed under reduced pressure and the crude was purified by flash column chromatography (EP–AcOEt 4 : 1) to afford pure **9** (R_f = 0.14, 138 mg, 0.22 mmol, 96%) as a colorless oil. $[\alpha]_D^{24}$ = −34.4 (c = 1.17, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.20–8.17 (m, 2H, Ar), 8.13–8.10 (m, 2H, Ar), 7.31–7.19 (m, 15H, Ar), 5.42–5.39 (m, 1H, H-6), 4.56–4.46 (m, 6H, Bn), 4.32 (dd, J = 4.9, 3.9 Hz, 1H, H-2), 4.06 (q, J = 3.9 Hz, 1H, H-3), 4.02 (q, J = 6.6 Hz, 1H, H-7a), 3.69–3.64 (m, 2H, H-1 and Ha-8), 3.55 (dd, J = 9.7, 3.9 Hz, 1H, Hb-8), 2.29–2.26 (m, 2H, H-7) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 169.4 (s, C=O), 164.0 (s, C=O), 150.8 (s, C–Ar), 137.9, 137.6, 137.4 (s, 3C, C–Ar), 134.7 (s, C–Ar), 131.1 (d, 2C, C–Ar), 128.5–127.5 (d, 15C, C–Ar), 123.5 (d, 2C, C–Ar), 88.3 (d, C-1), 87.0 (d, C-2), 75.7 (d, C-6), 73.4 (t, C–Bn) 72.5 (t, C–Bn), 72.4 (t, C–Bn), 69.0 (t, C-8), 62.1 (d, C-7a), 59.5 (d, C-3), 32.8 (t, C-7) ppm. IR ($CDCl_3$) $\tilde{\nu}$ = 3086, 3060, 3032, 2922, 2866, 1730, 1706, 1530, 1454, 1348, 1269, 1102 cm^{-1}. MS (ESI): m/z (%) = 645.37 [M + Na]$^+$. $C_{36}H_{34}N_2O_8$ (622.66): calcd C 69.44, H 5.50, N 4.50; found C 69.15, H 5.89, N 4.02.

(1*R*,2*R*,3*R*,6*S*,7a*R*)-1,2-Bis(benzyloxy)-3-[(benzyloxy)methyl]-6-hydroxyhexahydro-5*H*-pyrrolizin-5-one (10). A solution of **9** (73 mg, 0.12 mmol) in 12 ml of methanol was stirred with the ionic exchange resin Ambersep 900 OH (600 mg) at rt for 15 h until TLC analysis (AcOEt–EP 3 : 1) showed the disappearance of starting material (R_f = 0.73) and the formation of a new product (R_f = 0.43). After filtration through Celite®, the solvent was removed under reduced pressure, and the crude was purified by flash column chromatography (AcOEt–EP 2 : 1) to afford pure **10** (R_f = 0.26, 55 mg, 0.12 mmol, 100%) as a colorless oil. $[\alpha]_D^{25}$ = −45.2 (c = 1.47, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.37–7.25 (m, 15H, Ar), 4.60–4.52 (AB system, J = 11.7 Hz, 2H, Bn), 4.59–4.52 (AB system, J = 12.2 Hz, 2H, Bn), 4.56–4.48 (AB system, J = 11.7 Hz, 2H, Bn), 4.34–4.32 (m, 1H, H-6), 4.31 (dd, J = 4.9, 3.9 Hz, 1H, H-2), 4.05 (q, J = 7.0 Hz, 1H, H-7a), 4.03–4.00 (m, 1H, H-3), 3.64 (dd, J = 7.8, 4.9 Hz, 1H, H-1), 3.61 (dd, J = 9.8, 5.9 Hz, 1H, Ha-8), 3.57 (dd, J = 9.8, 4.4 Hz, 1H, Hb-8), 3.40 (d, J = 2.5 Hz, 1H, OH), 2.21 (ddd, J = 14.1, 6.9, 2.5 Hz, 1H, Ha-7), 2.05 (ddd, J = 14.1, 6.3, 7.3 Hz, 1H, Hb-7) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 174.5 (s, C=O), 137.9, 137.8, 137.7 (s, 3C, C–Ar), 128.5–127.7 (d, 15C, C–Ar), 88.7 (d, C-1), 87.5 (d, C-2), 73.4 (t, C–Bn), 73.3 (d, C-6), 72.4 (t, C–Bn), 72.3 (t, C–Bn), 69.0 (t, C-8), 62.1 (d, C-7a), 58.8 (d, C-3), 34.5 (t, C-7) ppm. IR ($CDCl_3$): $\tilde{\nu}$ = 3365, 3088, 3066, 3032, 2926, 2866, 1688, 1496, 1454, 1308, 1277, 1111, 1103 cm^{-1}. MS (ESI): m/z (%) = 496.36 [M + Na]$^+$. $C_{29}H_{31}NO_5$ (473.56): calcd C 73.55, H 6.60, N 2.96; found C 73.38, H 6.17, N 2.63.

(1*R*,2*R*,3*R*,6*S*,7a*R*)-1,2-Bis(benzyloxy)-3-[(benzyloxy)methyl]-6-hydroxyhexahydro-1*H*-pyrrolizine (11). A solution of **10** (107 mg, 0.23 mmol) in 2 ml of dry THF was stirred under nitrogen atmosphere at 0 °C and $LiAlH_4$ (1 M solution in THF, 0.92 ml, 0.92 mmol) was added dropwise. The temperature of the mixture was raised to rt and refluxed for 2 h until TLC analysis (AcOEt) showed the disappearance of the starting material (R_f = 0.53) and the formation of a new product (R_f = 0.20). The reaction was then quenched with 2 ml of a saturated aqueous solution of Na_2SO_4 at 0 °C, and after extraction with AcOEt (3 × 25 ml), the organic layers were dried over Na_2SO_4 and concentrated at reduced pressure. The crude was purified by flash column chromatography (AcOEt) to afford pure **11** (R_f = 0.20, 60 mg, 0.13 mmol, 57% yield) as a white solid. M.p. 95–97 °C. $[\alpha]_D^{24}$ = +9.5 (c = 1.02, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.35–7.23 (m, 15H, Ar), 4.68–4.45 (AB system, J = 11.7 Hz, 2H, Bn), 4.56 (d, J = 11.7 Hz, 2H, Bn), 4.51 (s, 2H, Bn), 4.48–4.45 (m, 1H, H-6), 4.06 (dd, J = 7.0, 5.5 Hz, 1H, H-2), 3.84 (t, J = 5.5 Hz, 1H, H-1), 3.72–3.66 (m, 1H, H-7a), 3.57 (dd, J = 9.3, 4.7 Hz, 1H, Ha-8), 3.50 (dd, J = 9.4, 6.2 Hz, 1H, Hb-8), 3.14–3.11 (m, 1H, Ha-5), 3.00 (td, J = 6.6, 5.0 Hz, 1H, H-3), 2.93 (dd, J = 11.7, 4.3 Hz, 1H, Hb-5), 2.05 (ddt, J = 13.2, 7.0, 1.6 Hz, 1H, Ha-7), 1.95 (bs, 1H, OH), 1.80 (ddd, J = 13.7, 9.2, 5.1 Hz, 1H, Hb-7) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 138.1, 137.9, 137.7 (s, 3C, C–Ar), 128.1–127.1 (d, 15C, C–Ar), 88.3 (d, C-1), 85.8 (d, C-2), 73.7 (d, C-6) 73.0 (t, C–Bn), 72.2 (t, C–Bn), 71.9 (t, C-8), 71.6 (t, C–Bn), 68.7 (d, C-3), 65.9 (d, C-7a), 63.4 (t, C-5), 40.4 (t, C-7) ppm. IR ($CDCl_3$): $\tilde{\nu}$ = 3390, 3088, 3065, 3031, 2926, 2857, 1496, 1454, 1366, 1260, 1206, 1100, 1076 cm^{-1}. MS (ESI): m/z (%) =

 This journal is © The Royal Society of Chemistry 2015

460.36 $[M + 1]^+$. $C_{29}H_{33}NO_4$ (459.58): calcd C 75.79, H 7.24, N 3.05; found C 75.49, H 7.14, N 3.04.

(1*R*,2*R*,3*R*,6*S*,7a*R*)-1,2,6-Trihydroxy-3-(hydroxymethyl)-hexahydro-1*H*-pyrrolizine (6). To a solution of **11** (43 mg, 0.094 mmol) in 10 ml of methanol, 22 mg of Pd on activated carbon (10% Pd) and two drops of 37% HCl were added with stirring under nitrogen atmosphere, and then the mixture was left under hydrogen atmosphere at rt for 3 days. After TLC analysis (CH_2Cl_2–MeOH 10 : 1) showed the disappearance of starting material (R_f = 0.43) and the formation of a new product (R_f = 0.00), the mixture was filtered through Celite®, and the solvent was removed under reduced pressure to afford a crude yellow oil (40 mg). Free amine was obtained by eluting the hydrochloride salt through a Dowex 50WX8 ion-exchange resin. Elution with 6% ammonia afforded the free base **6** (18 mg, 0.094 mmol, 100% yield over two steps) as a white solid. M.p. 159–162 °C. $[\alpha]_D^{21}$ = +25.4 (c = 0.31, H_2O). ^1H NMR (400 MHz, D_2O): δ = 4.45 (pseudo q, J = 4.2 Hz, 1H, H-6), 3.74–3.68 (m, 2H, H-2 and H-1), 3.65 (dd, J = 11.7, 3.9 Hz, 1H, Ha-8), 3.50 (dd, J = 11.7, 6.9 Hz, 1H, Hb-8), 3.26 (q, J = 7.4 Hz, 1H, H-7a), 2.87 (dd, J = 11.7, 3.4 Hz, 1H, Ha-5), 2.81 (dd, J = 11.7, 4.4 Hz, 1H, Hb-5), 2.66–2.62 (m, 1H, H-3), 1.94 (ddd, J = 13.1, 7.3, 3.9 Hz, 1H, Ha-7), 1.86 (ddd, J = 13.2, 7.3, 5.2 Hz, 1H, Hb-7) ppm. ^{13}C NMR (50 MHz, D_2O): δ = 80.6 (d, C-2), 77.8 (d, C-1), 72.2 (d, C-6) 70.1 (d, C-3), 65.3 (d, C-7a), 62.6 (t, C-8), 61.6 (t, C-5), 37.8 (t, C-7) ppm. MS (ESI): m/z (%) = 190.1 $[M + 1]^+$. $C_8H_{15}NO_4$ (189.21): calcd C 50.78, H 7.99, N 7.40; found C 50.97, H 7.89, N 7.59.

Biology

***In vitro* assays with different α-glucosidases.** Compounds **4–7** were tested for their inhibitory activity against the insect trehalase of midge larvae of *C. riparius*[15] and of the midgut and Malpighian tubules of *S. littoralis* with porcine trehalase (purchased from Sigma-Aldrich) as the mammalian counterpart and α-amylase from human saliva (purchased from Sigma-Aldrich) as a relevant glucolytic enzyme.

S. littoralis larvae (first day of sixth instar) were anesthetized with CO_2 prior to dissection. The midgut and Malpighian tubules of *S. littoralis* were isolated in 20 mM Tris-HCl of pH 6.8 and homogenized using Teflon Potter-Elvehjem homogenizer with 5 volumes of the same buffer in a glass, two time 9-strokes at 2000 rpm, with separation for 1 min in ice. The crude homogenate was centrifuged at 13 000g at 4 °C for 30 min. The resulting supernatant was used to measure enzyme activities. Proteins were measured according to Bradford using bovine serum albumin as a standard.[22]

Trehalase activity was measured through a coupled assay with glucose-6-phosphate dehydrogenase and hexokinase, according to Wegener *et al.*[23] 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 IC_{50} values. Experiments were performed at fixed substrate concentration close to the K_m value (0.5 mM for *C. riparius* and *S. littoralis* trehalases 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 rates in the presence and 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.

Kinetic experiments were performed using *C. riparius* trehalase, measuring enzymatic activity at different trehalose concentrations from 0.25 to 10 mM in the presence of fixed inhibitor concentrations. Kinetic parameters were calculated using a multiparameter, iterative, non-linear regression program based on the Levenberg-Marquardt algorithm (Sigma Plot, Jandel, CA). Data are given as ±S.D. of three independent experiments.

All enzyme assays were performed in triplicates at 30 °C using sample volumes varying from 5 to 20 μL in 1 mL cuvette 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 (0.6% w/v) in the presence of increasing inhibitor concentration. Potential inhibitors were added to 0.5 mL enzyme solution (10 μg mL^{-1}). The reaction was initiated by adding 0.5 mL of starch dissolved in 20 mM sodium phosphate of pH 6.9 and 6.7 mM sodium chloride, and the reaction was stopped after 3 minutes at room temperature 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 minutes. After cooling to room temperature, 10 mL of MilliQ water was added and absorbance was recorded at 540 nm using a Cary3 UV/Vis Spectrophotometer.[24] All enzyme assays were performed in triplicates. Micromoles of maltose released were determined from standard curve, and the enzymatic activity was calculated using the following equation:

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

Bioassays with α-glucosidases inhibitor. *S. littoralis* larvae (first day of fifth or sixth instars) were intra-hemocoellically injected by a Hamilton syringe (Hamilton, Nevada, USA). Briefly, larvae were anesthetized with CO_2 and injected with a single dose of casuarine derivative **6** (50, 150 and 300 μg per larva and 300 μg per larva in sixth instar and fifth instar, respectively). Casuarine derivative **6** was dissolved in 1 : 1 H_2O : DMSO. Control larvae were injected with equal amounts of solvent. Larval weight was recorded daily, and pupal weight and morphology, adult emergence, fertility and longevity were also monitored.

Acknowledgements

We gratefully acknowledge University of Milano-Bicocca, FAR 2009 and PRIN 2010–2011, 2010L9SH3 K 006.

Notes and references

1 A. Kato, E. Kano, I. Adachi, R. J. Molyneux, A. A. Watson, R. J. Nash, G. W. J. Fleet, M. W. Wormald, H. Kizu, K. Ikeda and N. Asano, *Tetrahedron: Asymmetry*, 2003, **14**, 325.

2 M. R. Wormald, R. J. Nash, A. A. Watson, B. K. Bhadoria, R. Langford, M. Sims and G. W. J. Fleet, *Carbohydr. Lett.*, 1996, **2**, 169.

3 R. J. Nash, P. L. Thomas, R. D. Waigh, G. W. J. Fleet, M. R. Wormald, P. M. Q. Lilley and D. J. Watkin, *Tetrahedron Lett.*, 1994, **35**, 7849.

4 (*a*) F. Cardona, C. Parmeggiani, E. Faggi, C. Bonaccini, P. Gratteri, L. Sim, T. M. Gloster, S. Roberts, G. J. Davies, D. R. Rose and A. Goti, *Chem. – Eur. J.*, 2009, **15**, 1627; (*b*) M. Forcella, F. Cardona, A. Goti, C. Parmeggiani, L. Cipolla, M. Gregori, R. Schirone, P. Fusi and P. Parenti, *Glycobiology*, 2010, **20**, 1186; (*c*) F. Cardona, A. Goti, C. Parmeggiani, P. Parenti, M. Forcella, P. Fusi, L. Cipolla, S. Roberts, G. J. Davies and T. M. Gloster, *Chem. Commun.*, 2010, **46**, 2629.

5 (*a*) D. Bini, F. Cardona, L. Gabrielli, L. Russo and L. Cipolla, *Carbohydr. Chem.*, 2012, **37**, 259; (*b*) D. Bini, F. Cardona, L. Gabrielli, L. Russo and L. Cipolla, in *Specialist of Periodical Reports, SPR Carbohydrate Chemistry*, 2011, vol. 37, p. 259.

6 (*a*) D. Bini, M. Forcella, L. Cipolla, P. Fusi, C. Matassini and F. Cardona, *Eur. J. Org. Chem.*, 2011, 3995; (*b*) D. Bini, F. Cardona, M. Forcella, C. Parmeggiani, P. Parenti, F. Nicotra and L. Cipolla, *Beilstein J. Org. Chem.*, 2012, **8**, 514; (*c*) L. Cipolla, A. Sgambato, M. Forcella, P. Fusi, P. Parenti, F. Cardona and D. Bini, *Carbohydr. Res.*, 2014, **389**, 46.

7 R. P. Gibson, T. M. Gloster, S. Roberts, R. A. J. Warren, I. S. de Gracia, A. García, J. L. Chiara and G. J. Davies, *Angew. Chem., Int. Ed.*, 2007, **46**, 4115.

8 T. Matsumura, M. Kasai, T. Hayashi, M. Arisawa, Y. Momose, I. Arai, S. Amagaya and Y. Komatsu, *Pharm. Biol.*, 2000, **38**, 302.

9 C. Parmeggiani, D. Martella, F. Cardona and A. Goti, *J. Nat. Prod.*, 2009, **72**, 2058.

10 A. Brandi, F. Cardona, S. Cicchi, F. M. Cordero and A. Goti, *Chem. – Eur. J.*, 2009, **15**, 7808.

11 C. Bonaccini, M. Chioccioli, C. Parmeggiani, F. Cardona, D. Lo Re, G. Soldaini, P. Vogel, C. Bello, A. Goti and P. Gratteri, *Eur. J. Org. Chem.*, 2010, 5574.

12 The enantiomer of **11** was previously reported: G. Podolan, L. Kleščíková, L. Fišera, J. Kožíšek and M. Fronc, *Synlett*, 2011, 1668.

13 J.-B. Behr, A. Erard and G. Guillerm, *Eur. J. Org. Chem.*, 2002, 1256.

14 The inhibition of chitin synthase by **6** was reported: J.-B. Behr, A. Gainvors-Claisse and A. Belarbi, *Nat. Prod. Res.*, 2007, **21**, 76.

15 M. Forcella, A. Mozzi, A. Bigi, P. Parenti and P. Fusi, *Arch. Insect Biochem. Physiol.*, 2012, **81**, 77.

16 For the activity of compounds **5–7** against a panel of commercially available glycosidases see: G. D'Adamio, C. Parmeggiani, A. Goti, A. J. Moreno-Vargas, E. Moreno-Clavijo, I. Robina and F. Cardona, *Org. Biomol. Chem.*, 2014, **12**, 6250 and ref. 11.

17 C. M. González Tanarro and M. Gütschow, *J. Enzyme. Inhib. Med. Chem.*, 2011, **26**, 350.

18 Calculated using the Baici equation: A. Baici, *Eur. J. Biochem.*, 1981, **119**, 9.

19 N. Asano, M. Takeuchi, Y. Kameda, K. Matsui and Y. Kono, *J. Antibiot.*, 1990, **43**, 722.

20 O. Ando, M. Kifune and M. Nakajima, *Biosci., Biotechnol. Biochem.*, 1995, **59**, 711.

21 (*a*) B. C. Bonnin and N. P. Chougule, *Trends Biotechnol.*, 2014, **32**, 91; (*b*) F. Pennacchio, B. Giordana and R. Rao, in *Parasitoid viruses: symbionts and pathogens*, ed. N. E. Beckage and J.-M. Drezen, Elsevier, 2012, p. 269.

22 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248.

23 G. Wegener, V. Tschiedel, P. Schlöder and O. J. Ando, *Exp. Biol.*, 2003, **206**, 1233.

24 P. Bernfeld, *Methods Enzymol.*, 1955, **1**, 149.

 This journal is © The Royal Society of Chemistry 2015