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The glucosinolate–myrosinase system. New insights into enzyme–substrate interactions by use of simplified inhibitors

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Myrosinase, a thioglucoside glucohydrolase, is the only enzyme able to hydrolyse glucosinolates, a unique family of molecules bearing an anomeric *O*-sulfated thiohydroximate function. Non-hydrolysable myrosinase inhibitors have been devised and studied for their biological interaction. Diverse modifications of the *O*-sulfate moiety did not result in a significant inhibitory effect, whereas replacing the D-glucopyrano residue by its carba-analogue allowed inhibition to take place. X-Ray experiments carried out after soaking allowed for the first time inclusion of a non-hydrolysable inhibitor inside the enzymatic pocket. Structural tuning of the aglycon part in its pocket is being used as a guide for the development of simplified and more potent inhibitors.

Introduction

Glucosinolates **1** are naturally occurring thiosugars mainly found in the botanical order *Brassicales*. The structural framework of glucosinolates invariably results from a combination of three parts: a β-D-glucopyranosyl unit, an unique *O*-sulfated anomeric thiohydroximate function and a broad library of aglycons, whose structure varies in the vegetal kingdom depending on the species (Fig. 1).[1] Myrosinase (thioglucoside glucohydrolase EC 3.2.3.1) is the only enzyme able to hydrolyze those unusual thiosaccharidic compounds and one of the few enzymes able to hydrolyse a thioglycosidic bond. Myrosinase and glucosinolates are stored in different parts of the plant, but especially in seeds. Mixing of the enzyme and the substrate induces glucosinolate hydrolysis with retention of the anomeric configuration. Myrosinase belongs to family 1 of glycoside hydrolases, albeit an atypical member of this family: whereas classical glycosidases activate the glycosidic oxygen by a catalytic acid residue in the glycosylation step, no such activation appears necessary nor indeed possible for plant myrosinase. The glycosyl intermediate formed in the process is hydrolysed by a water molecule perfectly positioned by a *glutamine* residue (and not a *glutamate* residue as in classical glycosidases).[2] The interaction between enzyme and substrate has been determined using various analogues of glucosinolates. These have been prepared with structural modifications on the glycosidic moiety,[2] on the aglycon chain[3] or on the anionic site.[4] Most of those modifications proved useful in clearly demonstrating the specificity of myrosinase towards the D-glucopyranosyl moiety and the flexibility with regard to the aglycon moiety. So far, 2-fluoro-2-deoxy-glucotropaeolin **2a** was the only good inhibitor acting through the formation of a covalent glucosyl–enzyme intermediate. It allowed studies of the activity of myrosinase, notwithstanding a tendency to slow-rate hydrolysis.[2f] This slow hydrolysis allowed the trapping of the glucosyl–enzyme intermediate during structural analysis by X-ray crystallography. This allowed an understanding of the interaction of the D-glucosyl residue with the active site. However, the fast hydrolysis of the aglycon part of 2-fluoro-2-deoxy-glucotropaeolin is the reason why an X-ray analysis[5] of the myrosinase–substrate interaction was precluded.[2b]

In order to improve our knowledge of substrate conformation and binding inside the active site, a non-hydrolyzable substrate would be an invaluable tool. But mainly, an improved inhibitor would be an important tool in the physiological study of glucosinolate–myrosinase location and interaction in plants. We chose glucotropaeolin (GTL) **2c** as the starting point because of its current use as an European Union official standard for the analysis of glucosinolates.[3b] The first attempt with a *C*-glucopyranosyl analogue **2b** of GTL **2c**[3b] was unsucessful inasmuch as it did not interact with myrosinase. In this article we present two novel approaches: one consists of the variation of the anionic part of the aglycon, whereas the second, while keeping the aglycon structure of GTL **2c**, consists of the modification of the carbohydrate moiety into a non-hydrolysable structure.

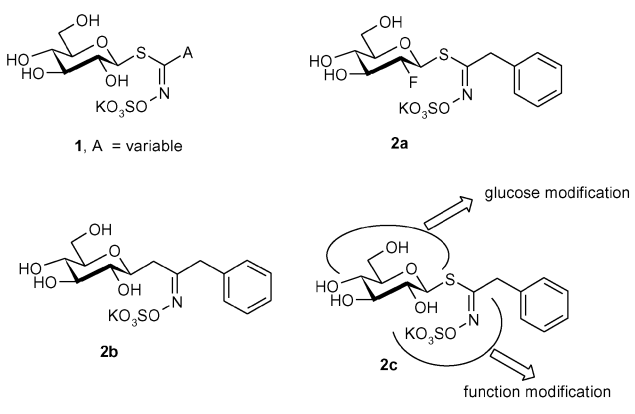

Fig. 1 Glucosinolates **1**, glucotropaeolin **2c** and modified analogues.

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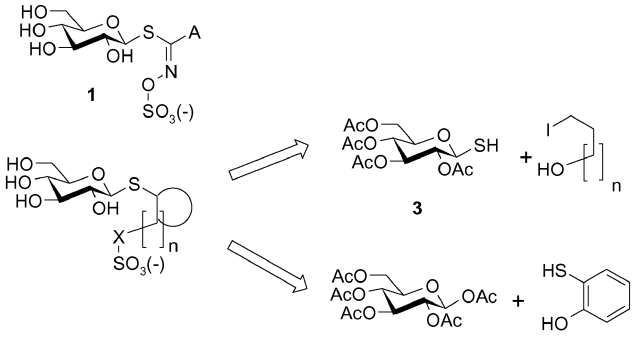
Results and discussion

Synthesis of inhibitors. Modification of the thiohydroximate function

We have explored the modification of the thiohydroximate function and its importance in the interaction with the enzyme: for example, the imino double-bond system was replaced either by a flexible 2- or 3-carbon acyclic alkyl chain or by a more constrained aryl moiety. Furthermore, we replaced the sulfate group with a sulfonate group (Scheme 1). Two synthetic pathways were needed to introduce such aglycon modifications (see retrosynthetic plans in Scheme 1).



Scheme 1 Retrosynthetic scheme to sulfate analogues.

For most compounds, a three-step synthetic sequence was implicated (Scheme 2). *S*-Alkylation of commercially available protected 1-thio-β-D-glucopyranose **3** using alkyl iodides proceeded with moderate to excellent yields under standard conditions[6] and subsequent *O*-sulfatation and glucose deprotection yielded acyclic derivatives **7** and **8** in reasonable overall yields. The sulfonate analogue **9** was obtained through a very efficient two-step procedure starting from **3**. The constrained chain was introduced using *o*-hydroxythiophenol in a standard Lewis acid-catalysed thioglycosylation on per-*O*-acetylated β-D-glucopyranose, yielding intermediate **10**, which was first *O*-sulfated, then de-*O*-acylated to give compound **11** in high yield.

Synthesis of inhibitors. Modification of the carbohydrate subunit

We have previously published the synthesis of a non-hydrolyzable *C*-glucoside analogue **2b** (*C*-GTL) (Fig. 2), which did not inhibit myrosinase activity.[3b] This result showed the importance of the anomeric sulfur atom in the thiohydroximate function and prompted us to design and synthesize a range of non-hydrolyzable substrate analogues of myrosinase, which preserve the anomeric sulfur atom. We first prepared the carba-glucosinolate **12**, which would maintain both the D-gluco conformation and the thiohydroximate function intact but preclude hydrolysis by myrosinase. Further exploration oriented us towards a family of simplified structures, in which the carbohydrate moiety itself would be replaced by acyclic alkyl chains (from ethyl to ω-hydroxybutyl).

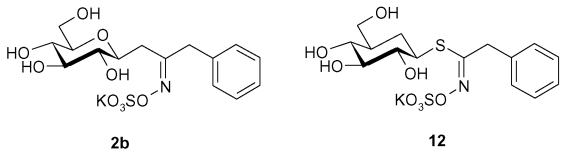


Fig. 2

The general approach to build the *O*-sulfated thiohydroximate structures followed a two-step sequence (Scheme 3 and Table 1): a) condensation on selected thiol derivatives of phenylacethydroximoyl chloride **13** prepared according to a previously developed methodology;[7] and b) sulfation with sulfur trioxide–pyridine complex followed, without intermediate purification, by potassium cation exchange using $KHCO_3$. The functionalized derivatives **14** required further steps: peracetylation to yield intermediates **15** then selective deprotection of the thiohydroximate function to form monoacetates **16**. Final *O*-sulfation, followed by *in situ* cation exchange with $KHCO_3$ and MeOK-catalysed transesterification led to sulfates **17**.

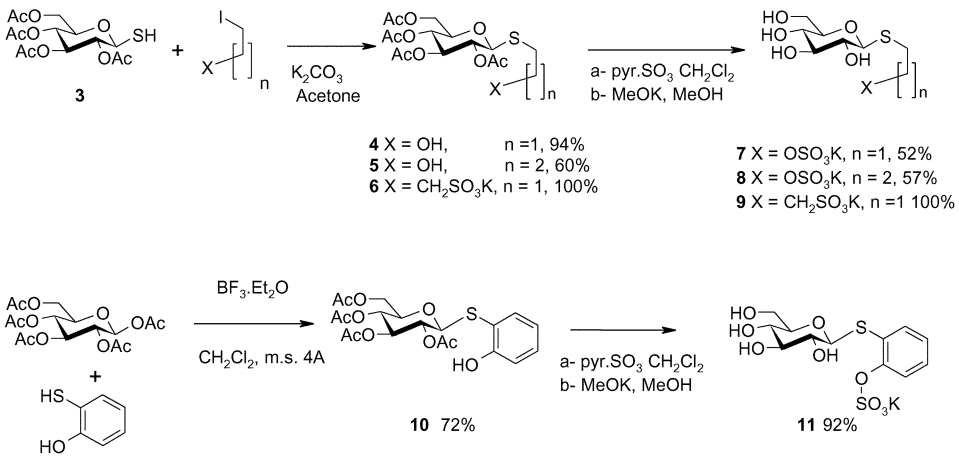
Most of the starting thiols were commercially available, with the exception of protected 1-thio-5*a*-carba-β-D,L-glucopyranose **21**. Following Ogawa's procedure, 2,3,4,6-tetra-*O*-benzoyl-5*a*-carba-α-D,L-glucopyranose **18** was first prepared.[8] Sulfur atom introduction through a pseudo α to β epimerisation at the pseudo-anomeric center was realised *via* a two-step sequence involving formation of triflate **19**, then nucleophilic displacement with thiourea to provide the isothiouronium salt **20** with a 60% overall yield. Further hydrolysis yielded thiol **21**, which was condensed with the hydroximoyl chloride **13** as described above to yield thiohydroximate **14f**. After sulfation–cation exchange, the benzoyl groups were removed through transesterification to yield the required carba-glucosinolate **12** (Scheme 4).[9]

Inhibition studies

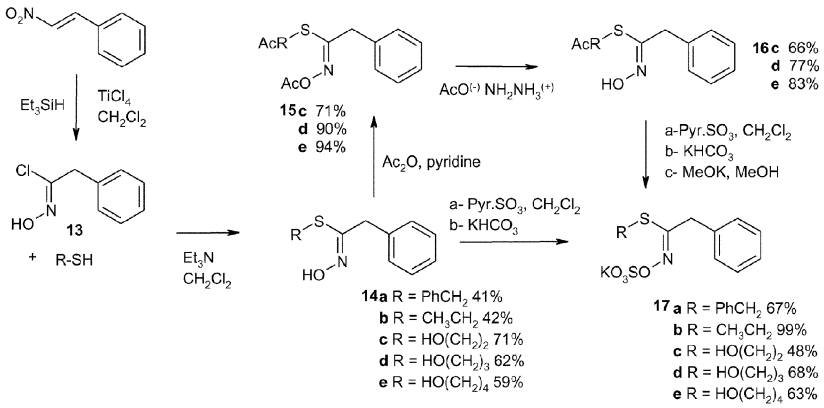
The activity of *Sinapis alba* myrosinase towards the different glucosinolate analogues was determined by titration of released glucose from sinigrin. The simplest method for assaying

Table 1 Modified anionic subunit, inhibition determination

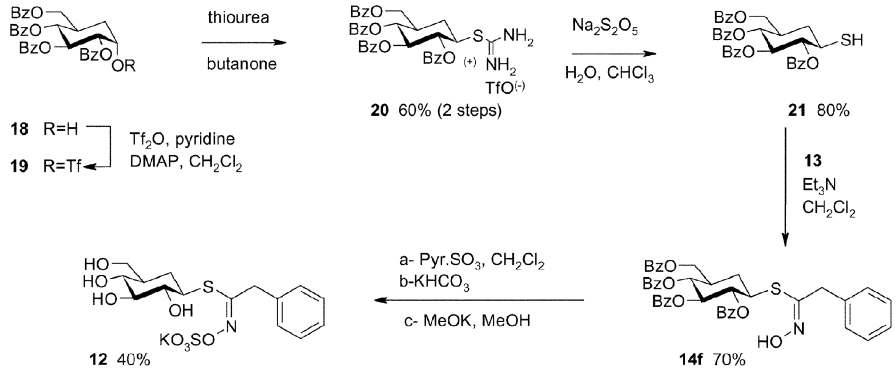
Substrate	**7**	**8**	**9**	**11**
Inhibition	30% at 10 mM	IC_{50} = 5 mM	15% at 10 mM	No inhibition at 10 mM



Scheme 2



Scheme 3



Scheme 4

myrosinase is to record the decrease of the UV absorption of sinigrin at 227.5 nm (DSA assay),[10] but this was inappropriate in our case since all the inhibitors absorbed strongly at the above wavelength. We selected the glucose oxidase–peroxidase coupled enzyme assay[11] as an alternate method, which was found to be more sensitive than the pH-stat assay[11] and less costly than the glucose deshydrogenase–hexokinase coupled enzyme assay.[12] The K_m (Michaelis constant) of myrosinase with sinigrin as substrate was found to be 0.3 mM, in agreement with the reported value of 0.156 mM when using the DSA assay.[10] The glucose oxidase–peroxidase system is known to undergo inhibition by the myrosinase activator ascorbic acid,[11,12] but no inhibition was observed with the compounds tested. The drawback of this method is that some compounds (**7**, **8**, **9** and **11**) may act as poor substrates and their hydrolysis will lead to the production of glucose which will be detected in the assay.

The IC_{50} (molar concentration for 50% inhibition of myrosinase under the conditions described in the experimental section) and percentage of inhibition values for the compounds modified at the thiohydroximate are reported in Table 1. It is of importance to note that the four compounds showing low levels of inhibition did not act as substrates. Only the sulfate derivative **8** shows an interesting IC_{50} value. Comparison of sulfate **7** and sulfonate **9**, which have the same chain length, showed no significant differences. The rigidified structure of **11** showed no inhibition, the hydroxy-phenyl moiety seeming to hamper the interaction with the enzyme probably due to the displacement of the water molecule which interacts with Arg259 and the hydroximate nitrogen (Fig. 3a).

We were pleased to observe that the non hydrolysable carba-glucosinolate **12** (Table 2) shows an inhibition similar to earlier values determined for classical glycosidase inhibitors.[3c]

The IC_{50} values for the ethyl derivative **17b** and its hydroxylated homologues **17c–17e** are reported in Table 2. Surprisingly, the ethyl derivative **17b** acts as a better inhibitor than the carba-glucosinolate **12**. The 2-hydroxyethyl derivative **17c** is a weaker inhibitor than **17b**, indicating a detrimental effect due to the presence of the hydroxyl group; however, the higher homologues 3-hydroxypropyl and 4-hydroxybutyl derivatives **17d** and **17e** respectively, appeared to be stronger inhibitors. We presume that the presence of a hydroxyl group at an appropriate distance from the sulfur atom may be involved in stabilizing hydrogen bonds with the protein and that a longer aliphatic chain may show less conformational flexibility due to an interaction with the benzyl ring.

Crystallographic studies

The electron density (Fig. 3a) showed that the carba-glucosinolate **12** bound in the active site of *Sinapis alba* myrosinase. Electron density for the benzyl ring, the sulfate group and the glucopyranose ring are clearly visible. We observed the biologically active D-enantiomer, which was selectively bound from the D/L-mixture used in the experiment. Despite this, in the crystal only about half of the active sites are occupied with the inhibitor and the glucose ring is even more disordered. The low occupancy of the inhibitor despite a concentration of 20 mM used in the soak may be due to competition with the high sulfate ion concentration present in the crystal and binding of glycerol from the cryoprotectant.

Table 2 Modified glucose subunit, inhibition determination

Starting thiols RSH	Inhibition at 1 mM (%)	IC_{50}/mM
17a $PhCH_2SH$	-[a]	—
17b CH_3CH_2SH	67	0.58 ± 0.09
17c $HO(CH_2)_2SH$	23 (at 10 mM)	nd
17d $HO(CH_2)_3SH$	70	0.44 ± 0.02
17e $HO(CH_2)_4SH$	88	0.25 ± 0.01
12	50	0.99 ± 0.01

[a] Not determined due to solubility problems.

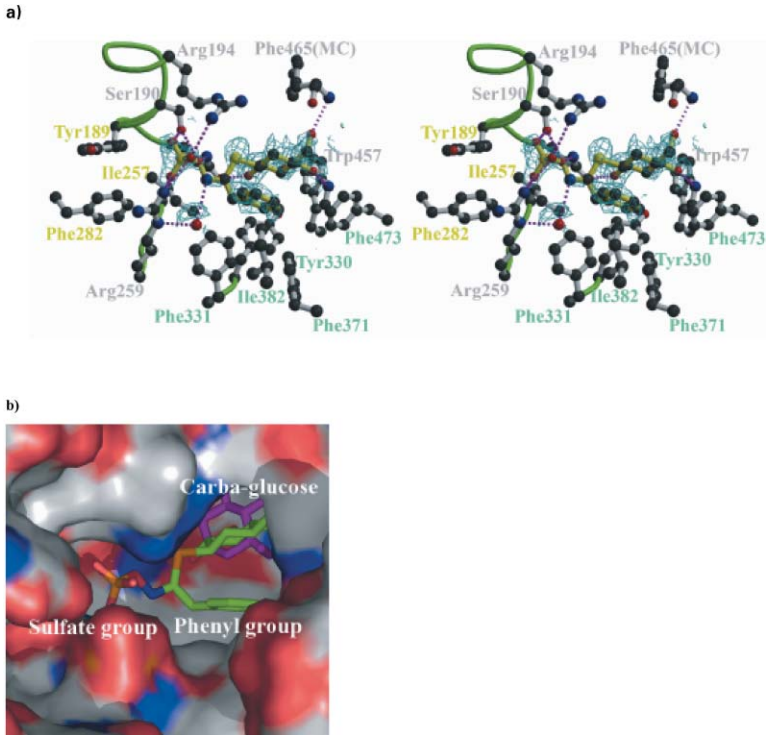


Fig. 3 a) Stereoview of the myrosinase active site. The refined structure of the carba-glucosinolate **12** (yellow) and the residues of the active site interacting with the inhibitor (white) are shown. Residues involved in hydrogen bonds are labelled in grey, residues forming the hydrophobic pocket binding the phenyl group are labelled in dark green and residues contributing to the hydrophobic surface next to the sulfate group are shown in light green. For clarity, Gln187 at the back is not labelled. The averaged electron density (as described in the methods section) of a F_{inh}–F_{nat} Fourier synthesis contoured at 2.5 σ around the inhibitor is shown in cyan. Hydrogen bonds are shown as dotted lines. b) The active site is represented by its accessible surface coloured according to atom types, the carba-glucosinolate inhibitor **12** is shown in a stick representation (carbon atoms in green). The position of the glucopyranosyl group in the 2-F glucosyl enzyme[5] is shown in magenta.

The benzyl group is bound in a hydrophobic pocket formed by residues Phe331, Phe371, Phe473, Ile382 and Tyr330 as predicted.[5] The sulfate group establishes hydrogen bonds to Arg259(Nη2), and Ser190(Oγ) as well as Gln187(Nε2), the residue replacing the catalytic glutamic acid residues classically present in glucosidases. The positive charge of Arg194 interacts more weakly with the sulfate group. Apart from these polar interactions, the group is located on top of a hydrophobic surface formed by Tyr189, Phe282 and Ile 257. A water molecule bridges the nitrogen atom of the hydroximate function to Arg259(Nε) through hydrogen bonds.

There are only three interactions of the glucose group with myrosinase, involving i) the hydroxyl group in position 2 and Gln187 Oε1, ii) the 4-hydroxyl group and the indole nitrogen of Trp457 and iii) the 6-hydroxyl and the main chain amide of Phe465 (Fig. 3a). The weak interaction combined with a lack of order of this group suggested that the glucose moiety did not significantly contribute to the affinity of the inhibitor. Furthermore, its position differs from the one of the 2-deoxy-2-fluoroglucopyranosyl group in the covalent glucosyl enzyme complex (Fig. 3b).[5,13] These observations led us to design and synthesize simplified inhibitors of the ethyl derivative series, which, as expected, proved to be better inhibitors than the carba-glucosinolate **12**.

A crystal structure of the complex of myrosinase with the ethyl derivative **17b** showed similar electron density for the sulfate and the benzyl ring as observed for **12**, but only low electron density for the ethyl group (data not shown). The interactions of sulfate and benzyl groups of **17b** with the enzyme correspond to those observed for **12** (Fig. 3a).

The substrate analogue **12** binds to myrosinase in a conformation corresponding probably to a state of prebinding as the position of the glucosyl group differs from that observed for the glucosyl–enzyme (Fig. 3a). A transient movement of the glucosyl group to this position combined with a distortion towards a boat conformation will then lead with a high commitment to catalysis.

In this combined study of inhibition and crystal structure using a carba-glucosinolate, we were, for the first time, able to observe directly an enzyme–substrate analogue complex and the interactions of the aglycon with the myrosinase active site. The previous studies[5,13] only exhibited structures of the glucosyl–enzyme intermediate as well as myrosinase complexed with several transition state analogues. Attempts to bind substrate analogues had failed so far.[13,14]

Conclusions

Our inhibition studies suggest that the thiohydroximate function is important for substrate recognition by myrosinase. The anionic charge by itself was not sufficient to induce inhibition: even a slight modification of the function dramatically reduces binding. This is not surprising as all polar atoms of the function interact directly or indirectly through hydrogen bonds with the active site (Fig. 3a). A similar loss of binding was observed using *C*-GTL **2b**, in which the sulfur atom of the thioglucosidic linkage is replaced by a methylene group.[3b] In contrast, when the endo-cyclic oxygen of the carbohydrate moiety is replaced by a methylene group, the ability to bind to myrosinase (IC_{50} = 1 mM) is preserved, pointing the way to drastic simplification of the structure to an ethyl group, which even improved the inhibitory effect (IC_{50} = 0.6 mM). This shows the unimportance of the glucosyl group during the initial interaction with the active site. Increasing the chain length (propyl and butyl) and adding a ω-hydroxyl function further improved inhibition up to an IC_{50} of 0.2 mM. These inhibitors may be more conformationally constrained due to a hydrophobic contact between the alkyl chain and the benzyl ring and in addition be able to engage one or more hydrogen bonds with the enzyme. The detailed knowledge on the interaction

of the sulfate group, the hydroximate function and the benzyl aglycon with the myrosinase active site will facilitate further design and improvement of myrosinase inhibitors.

Experimental

Chemical synthesis

Melting points were determined on a Köfler hot-stage apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX250 at 250 MHz and 62.89 MHz respectively. The chemical shifts (δ) are reported in ppm downfield from TMS as internal standard or from the residual solvent peak. Coupling constants (J) are reported in Hz. Specific rotations were measured at 20 °C using a Perkin-Elmer polarimeter 141. Low resolution mass spectra (MS) were recorded by the ICOA Analytical Service on a Perkin-Elmer SCIEX API 300 (ion spray: IS) and HRMS spectra were recorded on a VG analytical 70 SV. Elemental analysis was performed by the analytical service of the CNRS, Vernaison. Evaporation was conducted *in vacuo* with a Büchi rotary evaporator. Analytical TLC was carried out on precoated silica gel 60F-254 plates (E. Merck) and spots were detected by UV light (254 nm) and by heat treatment with a 10 : 85 : 5 mixture of sulfuric acid, ethanol and water. Flash column chromatography was performed on Kieselgel 60 (230–400 mesh) silica gel (E. Merck).

General protocol for *S*-alkylation of protected 1-thio-β-D-glucopyranose 3 to compounds 4, 5 and 6

The thiol **3** (1 eq.) was dissolved in acetone (DMF in the case of **6**) and alkylated according to Cerny *et al.*[6] using 1.1 eq. of the iododerivative and 1 eq. of K_2CO_3. After completion, the reaction medium was extracted with CH_2Cl_2, washed with water, then brine and evaporated to a crude mixture which was purified on silica gel.

(2-Hydroxy)ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside **4** was obtained with 94% yield according to the literature.[6]

(3-Hydroxy)propyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside 5. Obtained with 60% yield as a white solid; mp: 75–77 °C, $[\alpha]_D^{25} = -20$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$): 1.81 (m, 2H, H-2′), 1.98 (s, 3H, CH_3CO), 2.00 (s, 3H, CH_3CO), 2.03 (s, 3H, CH_3CO), 2.06 (s, 3H, CH_3CO), 2.78 (m, 2H, H-1′), 3.70 (m, 3H, H-5, H-3′), 4.12 (dd, 1H, $J_{6b-5} = 2.6$ Hz, $J_{6b-6a} = 12.4$ Hz, H-6b), 4.21 (dd, 1H, $J_{6a-5} = 4.7$ Hz, H-6a), 4.47 (d, 1H, $J_{1-2} = 10.0$ Hz, H-1), 5.02 (t, 1H, $J_{2-3} = 9.2$ Hz, H-2), 5.04 (t, 1H, $J_{4-5} = 10.0$ Hz, H-4), 5.20 (t, 1H, $J_{3-4} = 9.4$ Hz, H-3); ^{13}C NMR ($CDCl_3$): 21.0, 21.1 (OAc), 26.6 (C-2′), 32.5 (C-1′), 60.9 (C-3′), 62.4 (C-6), 68.6 (C-4), 70.1 (C-2), 74.1 (C-3), 76.3 (C-5), 83.9 (C-1), 169.8, 169.9, 170.5, 171.1 (CO); MS (IS+): m/z: 423,0 [$M^+ + H$]; elemental analysis calcd for $C_{17}H_{26}O_{10}S$: C, 48.33; H, 6.20. Found: C, 48.13; H, 6.18%.

(3-Sulfonato)propyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside, potassium salt 6. Obtained quantitatively; mp = 236–238 °C, $[\alpha]_D^{25} = +15$ ($c = 1.0$ in MeOH), ^{1}H NMR ($DMSO_{d6}$): 1.81 (m, 2H, H-2′), 1.92, 1.96, 1.99, 2.01 (4s, 12H, 4 CH_3CO), 2.52 (m, 2H, H-3′), 2.70 (m, 2H, H-1′), 3.96–4.01 (m, 2H, H-5 and H-6b), 4.12 (dd, 1H, $J_{6a-5} = 5.8$ Hz, $J_{6a-6b} = 12.6$ Hz, H-6a), 4.77 (t, 1H, $J_{2-3} = 10.0$ Hz, H-2), 4.88 (t, 1H, $J_{4-5} = 10.0$ Hz, H-4), 4.90 (d, 1H, $J_{1-2} = 10.0$ Hz, H-1), 5.23 (t, 1H, $J_{3-4} = 9.4$ Hz, H-3); ^{13}C NMR ($DMSO_{d6}$): 21.1, 21.2, 21.3, 21.4 (OAc), 26.7 (C-2′), 30.0 (C-1′), 50.9 (C-3′), 62.8 (C-6), 69.0 (C-4), 70.7 (C-2), 73.9 (C-3), 75.1 (C-5), 83.1(C-1), 170.0, 170.1, 170.4, 171.0 (CO), MS (IS-): m/z: 485 [$M^+ - H$]; elemental analysis calcd for $C_{17}H_{25}O_{12}S_2K$: C, 38.93; H, 4.81. Found: C, 38.67; H, 4.79%.

General protocol for sequential sulfation/de-*O*-acylation to 7, 8, 9

To a solution of **4** or **5** (1 eq.) in dichloromethane, pyridine–SO_3 complex (10 eq.) was added under dry conditions. The solution was stirred at room temperature for 20 h, then an aqueous solution of $KHCO_3$ (15 eq.) was added and stirring was maintained for 1 h. After removal of the solvents, the crude residue was purified over silica gel. The resulting compounds were de-*O*-acylated using catalytic potassium hydroxide in methanol at room temperature. After overnight reaction, Dowex H^+ resin was added and after filtration and evaporation, the crude material was purified over silica gel.

(2-Sulfato)ethyl 1-thio-β-D-glucopyranoside 7. Obtained as a gum in 52% yield from **4**; $[\alpha]_D^{25} = -9$ ($c = 1.0$ in H_2O); ^{1}H NMR (D_2O): 2.96 (m, 2H, H-1′), 3.22 (t, 1H, $J_{2-3} = 8.7$ Hz, H-2), 3.32–3.44 (m, 3H, H-3, H-4, H-5), 3.62 (dd, 1H, $J_{6b-5} = 4.9$ Hz, H-6b), 3.81 (dd, 1H, $J_{6a-6b} = 12.4$ Hz, $J_{6a-5} = 1.5$ Hz, H-6a), 4.15 (m, 2H, H-2′), 4.51 (d, 1H, $J_{1-2} = 9.8$ Hz, H-1); ^{13}C NMR (D_2O): 29.3 (C-1′), 61.2 (C-6), 68.7 (C-2′), 69.8 (C-4), 72.6 (C-2), 77.5 (C-3), 80.2 (C-5), 85.8 (C-1); MS (IS-): m/z: 319.0 [$M^- - K$]; HRMS calcd for $C_8H_{15}KO_9S_2$: 357.9794. Found: 357.9798.

(3-Sulfato)propyl 1-thio-β-D-glucopyranoside 8. Obtained as a gum in 57% yield from **5**; $[\alpha]_D^{25} = -9$ ($c = 1.0$ in H_2O); ^{1}H NMR (D_2O): 2.06 (m, 2H, H-2′), 2.88 (m, 2H, H-1), 3.34 (t, 1H, $J_{2-3} = 8.7$ Hz, H-2), 3.43–3.56 (m, 3H, H-3, H-4, H-5), 3.73 (dd, 1H, $J_{6b-5} = 5.3$ Hz, H-6b), 3.92 (dd, 1H, $J_{6a-6b} = 12.4$ Hz, $J_{6a-5} = 1.7$ Hz, H-6a), 4.19 (m, 2H, H-2′), 4.57 (d, 1H, $J_{1-2} = 9.8$ Hz, H-1); ^{13}C NMR (D_2O): 26.6 (C-2′), 29.6 (C-1′), 61.2 (C-6), 67.9 (C-2′), 69.8 (C-4), 72.6 (C-2), 77.5 (C-3), 80.1 (C-5), 85.8 (C-1); MS (IS-): m/z: 333.0 [$M^- - K$]; HRMS calcd for $C_9H_{17}KO_9S_2$: 371.9951. Found: 371.9957.

(3-Sulfonato)propyl 1-thio-β-D-glucopyranoside 9. Obtained as a gum in quantitative yield from **6**; $[\alpha]_D^{25} = -7$ ($c = 1.0$ in H_2O); ^{1}H NMR (D_2O): 2.00 (m, 2H, H-2′), 2.78 (m, 2H, H-1′), 2.94 (m, 2H, H-3′), 3.21 (dd, 1H, $J_{2-3} = 8.8$ Hz, H-2), 3.33–3.47 (m, 3H, H-3, H-4, H-5), 3.60 (dd, 1H, $J_{6b-5} = 4.8$ Hz, H-6b), 3.79 (d, 1H, $J_{6a-6b} = 12.5$ Hz, H-6a), 4.45 (d, 1H, $J_{1-2} = 9.8$ Hz, H-1); ^{13}C NMR (D_2O): 25.3 (C-2′), 29.2 (C-1′), 49.9 (C-3′), 61.2(C-6), 69.8 (C-4), 72.6 (C-2), 77.5 (C-3), 80.2 (C-5), 85.8 (C-1); MS (IS-): m/z: 317.0 [$M^- - K$]; HRMS calcd for $C_9H_{17}KO_8S_2$: 356.0002. Found: 355.9997

(2-Hydroxy)phenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside 10. 1,2,3,4,6-Penta-*O*-acetyl-β-D-glucopyranose (0.5 g, 1.28 mmol) in acetone (10 mL) was reacted with 2-hydroxythiophenol (0.139 g, 1.34 mmol) in the presence of $BF_3{\cdot}Et_2O$ (227 µL, 1.8 mmol) first at 0 °C, then overnight at room temperature. After hydrolysis with ice and dichloromethane extraction, the organic phases were collected, dried over $MgSO_4$ and evaporated. The crude material was purified over silica gel giving a 72% yield; $[\alpha]_D^{25} = -6$ ($c = 1.0$ in $CHCl_3$); mp: 124–126 °C; ^{1}H NMR ($CDCl_3$): 1.95 (s, 3H, CH_3CO), 1.98 (s, 3H, CH_3CO), 2.07 (s, 3H, CH_3CO), 2.10 (s, 3H, CH_3CO), 3.70 (m, 1H, H-5), 4.14 (d, 2H, $J_{6-5} = 3.9$ Hz, H-6), 4.59 (d, 1H, $J_{1-2} = 10.0$ Hz, H-1), 4.90 (t, 1H, $J_{2-3} = 9.2$ Hz, H-2), 4.99 (t, 1H, $J_{4-5} = 9.8$ Hz, H-4), 5.20 (t, 1H, $J_{3-4} = 10.0$ Hz, H-3), 6.84 (td, 1H, $J = 1.3$ Hz, $J = 7.5$ Hz, Har), 6.96 (dd, 1H, $J = 1.3$ Hz, $J = 8.3$ Hz, Har), 7.02 (s, 1H, OH), 7.26–7.38 (m, 2H, Har); ^{13}C NMR ($CDCl_3$): 20.6, 20.7, 20.8 (OAc), 61.7 (C-6), 67.9 (C-4), 69.7 (C-2), 73.7 (C-3), 76.1 (C-5), 86.4(C-1), 114.3 (Car), 116.3, 120.7, 132.6, 137.3 (CHar), 160.6 (Car), 169.1, 169.3, 170.1, 170.6 (CO); MS (IS+): m/z: 474 [$M^+ + NH_4$], 479 [$M^+ + Na$]; elemental analysis calcd for $C_{20}H_{24}O_{10}S$:C, 52.63; H, 5.30. Found: C, 52.32; H, 5.27%.

2′-Sulfatophenyl-1-thio-β-D-glucopyranoside 11. Pyridine·SO_3 complex (0.991 g, 6.2 mmol) was added to a dichloromethane solution of the phenolic compound **10** (284 mg, 0.62 mmol). The resulting mixture was heated for 1 h, then, after cooling to room temperature, quenched with

aqueous $KHCO_3$ (1.3 g, 13 mmol) for 1 h. The resulting slurry was suspended in MeOH, filtrated on celite, then evaporated to dryness. The crude residue was dissolved again in MeOH (5 mL) and MeOK (1M solution in MeOH) was added until pH 8 was reached. After 2 h, Dowex 50H was added, then eliminated through filtration. The crude material obtained after evaporation was purified over silica gel, yielding **11** (0.233 g, 0.57 mmol) 92%; $[a]_D^{25} = -27$ ($c = 1.0$ in H_2O); ^{1}H NMR (D_2O): 3.38–3.57 (m, 4H, H-2, H-3, H-4, H-5), 3.69 (dd, 1H, J_{6b-5} = 5.1 Hz, H-6b), 3.88 (dd, 1H, J_{6a-6b} = 12.4 Hz, J_{6a-5} = 1.9 Hz, H-6a), 4.97 (d, 1H, J_{1-2} = 9.6 Hz, H-1), 7.27 (m, 3H, Har), 7.64 (dd, 1H, J = 1.7 Hz, J = 7.5 Hz, Har); ^{13}C NMR (D_2O): 61.0 (C-6), 69.6, 72.3, 77.4, 80.1 (C-2, C-3, C-4, C-5), 86.4 (C-1), 122.2, 127.3, 129.1, 131.7 (CHar), 127.1 (Car), 158.9 (Car); MS (IS-): m/z: 367 [M^- − K]; HMRS calcd for $C_{12}H_{15}KO_9S_2$: 405.9795. Found: 405.9793.

General protocol for synthesizing thiohydroximates 14

A dichloromethane solution of β-nitrostyrene and Et_3SiH (2.1 eq.) was reacted with $TiCl_4$ (2.2 eq.) under Ar for 16 h according to Kulkarni.[7] The mixture was hydrolyzed with ice, extracted with dichloromethane, dried over $MgSO_4$ and evaporated to dryness. The crude residue was dissolved in dichloromethane and Et_3N (3 eq.) before the thiol (1.2 eq.) was added. At the optimum time (TLC monitoring), the resulting mixture was directly purified over a silica gel column.

(*Z*) *S*-Benzyl phenylacetothiohydroximate 14a. (41%); mp: 134–135 °C; ^{1}H NMR ($CDCl_3$): 3.78 (s, 2H, CH_2C=N), 3.92 (s, 2H, CH_2S), 7.23–7.31 (m, 10H, Har), ^{13}C NMR ($CDCl_3$): 33.9 (S*C*H$_2$), 39.2 (Ph*C*H$_2$C=N), 127.1, 127.5, 128.3, 128.7, 128.8, 135.9, 136.3 (CHar and Car), 154.7 (C=N); MS (IS+): m/z: 258 [M^+ + H]; elemental analysis calcd for $C_{15}H_{15}NOS$ C, 70.01; H, 5.87; N, 5.44. Found: C, 69.76; H, 5.85; N, 5.42%.

(*Z*) *S*-Ethyl phenylacetothiohydroximate 14b. (42%); oil;^{1}H NMR ($CDCl_3$): 1.18 (t, 3H, J = 7.5 Hz, CH_3), 2.73 (q, 2H, CH_2S), 3.82 (s, 2H, CH_2Ph), 7.21–7.35 (m, 5H, Har), 8.9 (ls, 1H, OH); ^{13}C NMR ($CDCl_3$): 14.7 (CH_3), 23.8 (CH_2S), 39.1 (CH_2Ph), 127.1, 128.4, 128.8, 136.2 (CHar and Car), 155.3 (C=N); MS (IS+): m/z: 196 [M^+ + H]; elemental analysis calcd for $C_{10}H_{13}NOS$ C, 61.50; H, 6.71; N, 7.17. Found: C, 61.22; H, 6.68; N, 7.14%.

(*Z*) *S*-(2-Hydroxyethyl) phenylacetothiohydroximate 14c. (71%); oil; ^{1}H NMR (CD_3OD): 2.91 (t, 2H, J = 6.4 Hz, CH_2S), 3.60 (t, 2H, CH_2O), 3.93 (s, 2H, CH_2Ph), 7.27–7.36 (m, 5H, Har); ^{13}C NMR (CD_3OD): 32.2 (CH_2S), 39.1 (CH_2Ph), 62.0 (CH_2O), 127.6, 128.9, 129.4, 137.6 (CHar and Car), 154.8 (C=N); MS (IS+): m/z: 212 [M^+ + H]; elemental analysis calcd for $C_{10}H_{13}NO_2S$ C 56.85; H, 6.20; N, 6.63. Found: C 56.70; H, 6.19; N, 6.61%.

(*Z*) *S*-(3-Hydroxypropyl) phenylacetothiohydroximate 14d. (62%); oil; ^{1}H NMR ($CDCl_3$): 1.70 (qt, 2H, J = 6.6 Hz, CH_2), 2.32 (ls, 1H, OH), 2.81 (t, 2H, J = 7.2 Hz, CH_2S), 3.59 (t, 2H, J = 6.6 Hz, CH_2O), 3.82 (s, 2H, CH_2Ph), 7.22–7.34 (m, 5H, Har), 9.67 (ls, 1H, OH); ^{13}C NMR ($CDCl_3$): 25.9 (CH_2), 32.3 (CH_2S), 39.1 CH_2Ph), 60.7 (CH_2O), 127.1, 128.4, 128.9, 136.2 (CHar and Car), 155.1 (C=N); MS (IS+): m/z: 226 [M^+ + H]; elemental analysis calcd for $C_{11}H_{15}NO_2S$ C, 58.64; H, 6.71; N, 6.22. Found: C, 58.45; H, 6.82; N, 5.93%.

(*Z*) *S*-(4-Hydroxybutyl) phenylacetothiohydroximate 14e. (59%); oil; ^{1}H NMR ($CDCl_3$): 1.48–1.52 (m, 4H, CH_2), 2.67 (t, 2H, J = 6.6 Hz, CH_2S), 2.99 (ls, 1H, OH), 3.48 (t, 2H, J = 5.6 Hz, CH_2O), 3.79 (s, 2H, CH_2Ph), 7.18–7.31 (m, 5H, Har), 10.27 (ls, 1H, OH); ^{13}C NMR ($CDCl_3$): 26.0, 29.0 (CH_2), 31.2 (CH_2S), 38.9 (CH_2Ph), 61.8 (CH_2O), 127.0, 128.2, 128.7, 136.2 (CHar and Car), 155.1 (C=N); MS (IS+): m/z: 240 [M^+ + H]; elemental analysis calcd for $C_{12}H_{17}NO_2S$ C, 60.22; H, 7.16; N, 5.85. Found: C, 59.95; H, 7.13; N, 5.83%.

(*Z*) 1-*S*-[2,3,4,6-Tetra-*O*-benzoyl-5*a*-carba-β-D-glucopyranosyl] phenylacetothiohydroximate 14f. (70%);^{1}H NMR ($CDCl_3$): 1.69 (dd, 1H, $J_{5'b-1}$ = 11.0 Hz, $J_{5'b-5'a}$ = 13.5 Hz, H-5′b), 1.89 (ddd, 1H, $J_{5'a-1}$ = $J_{5'a-5}$ = 3.8 Hz, H-5′a), 2.13–2.4 (m, 1H, H-5), 3.74 (d, 1H, J = 15.7 Hz, CH_2Ph), 3.82 (d, 1H, CH_2Ph), 3.82–3.93 (m, 1H, H-1), 4.15 (dd, 1H, J_{6b-5} = 4.7 Hz, J_{6a-6b} = 11.6 Hz, H-6b), 4.28 (dd, 1H, J_{6a-5} = 4.1 Hz, H-6b), 5.46 (dd, 1H, J_{3-2} = 9.4 Hz, J_{3-4} = 10.6 Hz, H-3), 5.52 (dd, 1H, J_{2-1} = 11.4 Hz, H-2), 5.64 (dd, 1H, J_{4-5} = 9.5 Hz,H-4), 7.17–7.56 (m, 17H, Har), 7.70–7.98 (m, 8H, Har); ^{13}C NMR ($CDCl_3$): 33.0 (C-5′), 40.0 (C-5), 40.7 (CH_2Ph), 43.7 (C-1), 63.7 (C-6), 72.0 (C-2), 74.0 (C-3), 75.2 (C-4), 128.3, 128.4, 128.5, 8.6, 128.7, 128.8, 128.9, 129.1, 129.2, 129.7, 129.8, 129.9, 130.0, 133.1, 133.2, 135.8 (CHar and Car), 151.8 (C=N), 165.5, 165.6, 166.0, 166.3 (CO); MS (IS+): m/z: 744.5 [M^+ + H], 766.5 [M^+ + Na]; elemental analysis calcd for $C_{43}H_{37}NO_9S$, C, 69.43; H, 5.01. Found: C, 69.81; H, 5.02%.

Preparation of di-*O*-acetates 15

The thiohydroximates **14** were acetylated in dry pyridine with Ac_2O added dropwise. After 12 h at room temperature, the mixtures were co-evaporated with toluene and purified by silica gel column chromatography to obtain di-*O*-acetates **15**

(*Z*) *O*-Acetyl-*S*-(2-acetoxyethyl) phenylacetothiohydroximate 15c. (71%); oil; ^{1}H NMR ($CDCl_3$): 1.76 (s, 3H, N–OAc), 1.93 (s, 3H, CH_2OAc), 2.75 (t, 2H, J = 6.2 Hz, CH_2S), 3.80–3.82 (m, 4H, CH_2O, CH_2Ph), 7.01–7.10 (m, 5H, Har); ^{13}C NMR ($CDCl_3$): 18.6, 20.0 ($COCH_3$), 27.8 (CH_2S), 38.0 ($PhCH_2$), 62.0 (CH_2O), 126.8, 127.6, 128.3, 134.3 (CHar and Car), 162.7 (C=N), 167.0, 169.7 (CO); MS (IS+): m/z: 296 [M^+ + H]; elemental analysis calcd for $C_{14}H_{17}NO_4S$ C, 56.96; H, 5.80; N, 4.74. Found: C, 56.76; H, 5.78; N, 4.73%.

(*Z*) *O*-Acetyl-*S*-(3-acetoxypropyl) phenylacetothio hydroximate 15d. (90%): oil; ^{1}H NMR ($CDCl_3$): 1.75 (qt, 2H, J = 6.6 Hz, CH_2); 1.98, 2.19 (2s, 2 × 3H, OAc); 2.78 (t, 2H, J = 7.4 Hz, CH_2S); 3.98–4.03 (m, 4H, OCH_2, CH_2Ph); 7.23–7.32 (m, 5H, Har); ^{13}C NMR ($CDCl_3$): 19.0, 20.4 ($COCH_3$); 26.1 (CH_2); 28.2 (CH_2S); 38.6 ($PhCH_2$); 61.9 (CH_2O); 127.0, 127.8, 128.6, 134.7 (CHar and Car); 163.6 (C=N); 167.3, 170.3 (CO); MS (IS+): m/z: 310 [M^+ + H]; elemental analysis calcd for $C_{15}H_{19}NO_4S$ C 58.23; H, 6.19; N, 4.53. Found: C 58.11; H, 6.30; N, 4.37%.

(*Z*) *O*-Acetyl-*S*-(4-acetoxybutyl) phenylacetothiohydroximate 15e. (94%); oil; ^{1}H NMR ($CDCl_3$): 1.47–1.63 (m, 4H, CH_2CH_2), 2.00, 2.21 (2s, 2 × 3H, OAc), 2.74 (t, 2H, J = 7.0 Hz, CH_2S), 3.93–3.98 (m, 4H, OCH_2, CH_2Ph), 7.25–7.33 (m, 5H, Har); ^{13}C NMR ($CDCl_3$): 19.2, 20.8 ($COCH_3$), 25.7, 27.3 (CH_2CH_2), 29.3 (CH_2S), 39.0 ($PhCH_2$), 63.3 (CH_2O), 127.2, 128.0, 128.8, 134.9 (CHar and Car), 164.0 (C=N), 167.7, 170.8 (CO); MS (IS+): m/z: 324 [M^+ + H]; elemental analysis calcd for $C_{16}H_{21}NO_4S$ C, 59.42; H, 6.54; N, 4.33. Found: C, 59.11; H, 6.71; N, 4.05%.

Protocol for selective de-*O*-acetylation to monoacetates 16. Under dry conditions, di-*O*-acetates **15** were dissolved in DMF and stirred at 60 °C for 4 h in the presence of hydrazinium monoacetate. After completion, the reaction medium was extracted with ethyl acetate, washed with water to remove DMF and salts, then washed with brine, dried over $MgSO_4$ and evaporated to a crude residue which could be used without purification for the next step. Structure checking was effected by MS and ^{1}H NMR.

(*Z*) *S*-(2-Acetoxyethyl) phenylacetothiohydroximate 16c. (66%);^{1}H NMR (CD_3OD): 1.97 (s, 3H, OAc), 2.94 (t, 2H, J = 6.6 Hz, CH_2S), 3.87 (s, 2H, CH_2Ph), 4.00 (t, 2H, J = 6.6 Hz, CH_2O), 7.20–7.30 (m, 5H, Har); MS (IS+): m/z: 254 [M^+ + H].

(*Z*) *S*-(3-Acetoxypropyl) phenylacetothiohydroximate 16d. (77%);^{1}H NMR ($CDCl_3$): 1.75 (qt, 2H, J = 6.8 Hz, CH_2), 1.99

(s, 3H, OAc), 2.76 (t, 2H, $J = 7.4$ Hz, CH_2S), 3.82 (s, 2H, CH_2Ph), 4.01 (t, 1H, $J = 6.2$ Hz, CH_2O), 7.22–7.33 (m, 5H, Har), 9.98 (bs, 1H, OH); MS (IS+): *m/z*: 268 [$M^+ + H$].

(*Z*) *S*-(4-Acetoxybutyl) phenylacetothiohydroximate 16e. (83%); oil; ^1H NMR ($CDCl_3$): 1.50–1.59 (m, 4H, CH_2CH_2), 2.00 (s, 3H, OAc), 2.71 (t, 2H, $J = 7.0$ Hz, CH_2S), 3.82 (s, 2H, CH_2Ph), 3.95 (t, 2H, CH_2O), 7.22–7.29 (m, 5H, Har); MS (IS+): *m/z*: 282 [$M^+ + H$].

Sulfation protocol

Thiohydroximates **16** were dissolved in CH_3CN under dry Ar then reacted with pyridine–SO_3 complex in excess (5 eq.). After 1 h heating at 50 °C, the mixture was cooled and an aquous solution of $KHCO_3$ (10 eq.) was added. The crude mixture was evaporated to dryness and purified by chromatography to yield the final compound **17**. In the case of acetylated intermediates **16c-e**, de-*O*-acylation was performed through MeOK-catalysed methanolysis. Compounds **17** were purified by silica gel chromatography. The same protocol was applied to prepare the carbaglucosinolate **12**.[9]

(*Z*) *S*-Benzyl phenylacetothiohydroximate-*O*-sulfate 17a. (67%);^1H NMR (CD_3OD): 3.78 (s, 4H, 2 × CH_2Ph), 6.94–7.22 (m, 10H, Har); ^{13}C NMR (CD_3OD): 35.0 (CH_2S), 39.6 (CH_2Ph), 128.2, 128.6, 129.2, 129.3, 129.7, 129.8, 130.0, 137.2, 137.3 (CHar and Car), 162.0 (C=N); MS (IS-): *m/z*: 336 [$M^- - K$]; HRMS calcd for $C_{15}H_{14}KNO_4S_2$: 375.001. Found 374.9997.

(*Z*) *S*-Ethyl phenylacetothiohydroximate-*O*-sulfate 17b. (99%);^1H NMR (CD_3OD): 1.1 (t, 3H, $J = 7.6$ Hz, CH_3), 2.74 (q, 2H, CH_2S), 3.95 (s, 2H, CH_2Ph), 7.13–7.35 (m, 5H, Har); ^{13}C NMR (CD_3OD): 14.5 (CH_3), 24.8 (CH_2S), 39.4 (CH_2Ph), 128.0, 129.2, 129.7, 137.1 (CHar and Car), 162.9 (C=N); MS (IS-): *m/z*: 274 [$M^- - K$]; HRMS calcd for $C_{10}H_{12}KNO_4S_2$ 312.9845. Found 313.9852.

(*Z*) *S*-(2-Hydroxyethyl) phenylacetothiohydroximate-*O*-sulfate 17c. (48%);^1H NMR (CD_3OD): 2.87 (t, 2H, $J = 6.6$ Hz, CH_2S), 3.53 (t, 2H, $J = 6.6$ Hz, CH_2O), 3.99 (s, 2H, CH_2Ph), 7.24–7.40 (m, 5H, Har); ^{13}C NMR (CD_3OD): 33.2 (CH_2S), 39.5 (CH_2Ph), 62.0 (CH_2O), 128.1, 129.3, 129.8, 137.2 (CHar and Car), 162.4 (C=N); MS (IS-): *m/z*: 290 [$M^- - K$]; HRMS calcd for $C_{10}H_{12}KNO_5S_2$ 328.9794. Found 328.9788.

(*Z*) *S*-(3-Hydroxypropyl) phenylacetothiohydroximate-*O*-sulfate 17d. (68%);^1H NMR (CD_3OD): 1.64 (qt, 2H, $J = 6.8$ Hz, CH_2), 2.82 (t, 2H, $J = 7.3$ Hz, CH_2S), 3.50 (t, 2H, $J = 6.2$ Hz, CH_2O), 3.97(s, 2H, CH_2Ph), 7.21–7.40 (m, 5H, Har); ^{13}C NMR (CD_3OD): 27.1 (CH_2), 33.6 (CH_2S), 39.4 (CH_2Ph), 60.9 (CH_2O), 128.1, 129.3, 129.7, 137.2 (CHar and Car), 162.6 (C=N); MS (IS-): *m/z*: 304 [$M^- - K$]; HRMS calcd for $C_{11}H_{14}KNO_5S_2$ 342.9951. Found 342.9947.

(*Z*) *S*-(4-Hydroxybutyl) phenylacetothiohydroximate-*O*-sulfate 17e. (63%);^1H NMR (CD_3OD): 1.46–1.51 (m, 4H, CH_2), 2.75 (t, 2H, $J = 7.1$ Hz, CH_2S), 3.44 (t, 2H, $J = 6.0$ Hz, CH_2O), 3.96 (s, 2H, CH_2Ph), 7.25–7.40 (m, 5H, Har); ^{13}C NMR (CD_3OD): 27.0, 30.3 (CH_2), 32.3 (CH_2S), 39.5 (CH_2Ph), 62.0 (CH_2O), 128.1, 129.3, 129.8, 137.3 (CHar and Car), 162.6 (C=N); MS (IS-): *m/z*: 318 [$M^- - K$]; HRMS calcd for $C_{12}H_{16}KNO_5S_2$: 357.0107. Found 357.0112.

Preparation of the 1-thio-carbaglucose derivative 21

2,3,4,6-Tetra-*O*-benzoyl-1-*O*-trifluoromethanesulfonyl-5*a*-carba-α-DL-glucopyranose **19**: under dry conditions, 2,3,4,6-tetra-*O*-benzoyl-5*a*-carba-α-DL-glucopyranose **18** (0.15 g, 0.25 mmol) was dissolved in dichloromethane containing pyridine (0.124 mL, 1.51 mmol) and DMAP (6 mg, 0.05 mmol). After cooling to −78 °C, triflic anhydride (0.124 mL, 0.76 mmol) was added and the mixture was stirred for 30 min at −78 °C and then allowed to return to room temperature and stirred overnight. Work-up using ice and 10% K_2SO_4 followed by dichloromethane extraction gave an organic phase which was washed with sat. $NaHCO_3$, water, dried over $MgSO_4$ and evaporated. Crude **19** was pure enough for the next step. ^1H NMR ($CDCl_3$): 2.18 (ddd, 1H, $J_{5'b,5} = 13.6$ Hz, $J_{5'b,1} = 2.0$ Hz, H-5′b), 2.54 (ddd, 1H, $J_{5'a,5'b} = 15.8$ Hz, $J_{5'a,1} = 3.6$ Hz, $J_{5'a,5'} = 4.0$ Hz, H-5′a), 2.78–2.89 (m, 1H, H-5), 4.38 (dd, 1H, $J_{6b,5} = 4.5$ Hz, H-6b), 4.51 (dd, 1H, $J_{6a,6b} = 11.7$ Hz, $J_{6a,5} = 3.5$ Hz, H-6a), 5.49 (dd, $J_{2,1} = 2.7$ Hz, H-2), 5.60 (sl, 1H, H-1), 5.72 (dd, $J_{3,2} = 10.4$ Hz, H-3), 6.11 (dd, 1H, $J_{3,4} = J_{4,5} = 10.1$ Hz, H-4), 7.21–7.61 (m, 12H, Har), 7.78–8.03 (m, 8H, Har); MS (IS+): *m/z*: 599 [$M^+ - TfOH + Na$], 594 [$M^+ - TfOH + NH_4$], 577 [$M^+ - TfOH + H$].

1-*S*-(2,3,4,6-Tetra-*O*-benzoyl-5*a*-carba-β-DL-glucopyranosyl) isothiouronium triflate 20. Under dry conditions, the crude triflate **19** and thiourea (46 mg, 0.6 mmol) were dissolved in butanone. The solution was heated at 80 °C for 5 h, then cooled and evaporated. The crude mixture was directly purified over silica gel, to produce **20** (0.17 g, 0.02 mmol, 67% yield). ^1H NMR (CD_3OD): 2.08–2.23 (m, 1H, H-5′b), 2.55 (ddd, 1H, $J_{5'a,5'b} = 13.5$ Hz, $J_{5'a,1} = J_{5'a,5} = 4.0$ Hz, H-5′a), 2.65–2.81 (m, 1H, H-5), 4.26–4.35 (m, 1H, H-1), 4.41 (dd, 1H, $J_{6b,5} = 4.7$ Hz, H-6b), 4.48 (dd, 1H, $J_{6a,6b} = 11.5$ Hz, $J_{6a,5} = 4.4$ Hz, H-6a), 5.71–5.88 (m, 3H, H-2, H-3, H-4), 7.21–7.59 (m, 12H, Har), 7.69–7.70 (m, 2H, Har), 7.83–803 (m, 6H, Har); ^{13}C NMR (CD_3OD): 31.4 (C-5′), 40.8 (C-5), 47.4 (C-1), 65.4 (C-6), 73.4, 75.7, 76.2 (C-2, C-3, C-4), 129.3, 129.7, 129.8, 129.9, 130.2, 130.4, 130.6, 130.7, 134.3, 134.5, 134.6, 134.9 (CHar and Car), 166.7, 167.0, 167.1, 167.7 (CO), 185.4 (C=N); MS (IS+): *m/z*: 653 [$M^+ - TfO$]; HRMS calcd for $C_{37}H_{33}F_3N_2O_{11}S_2$ 802.1447. Found 802.1453

2,3,4,6-Tetra-*O*-benzoyl-1-thio-carba-β-DL-glucopyranose 21. The isothiouronium salt **20** (0.69 g, 0.86 mmol) was suspended in water (8 mL) containing $Na_2S_2O_5$ (0.33 g, 1.72 mmol) and heated to 85 °C before $CHCl_3$ (7 mL) was added. The resulting solution was heated for another 3 h in order to complete hydrolysis. After usual work-up, extraction with $CHCl_3$ followed by washes with water and brine, silica gel column purification gave a 80% yield of thiol **21** (0.5 g, 0.82 mmol). MS (IS+): *m/z*: 633 [$M^+ + Na$]; ^1H NMR ($CDCl_3$) 1.74–1.87 (m, 2H, H-5′b, SH), 2.38–2.47 (m, 2H, H-5′a, H-5), 3.14–3.19 (m, 1H, H-1), 4.23 (dd, 1H, $J_{6b,5} = 5.9$ Hz, H-6b), 4.39 (dd, 1H, $J_{6a,6b} = 11.4$ Hz, $J_{6a,5} = 3.7$ Hz, H-6a), 5.40 (dd, $J_{2,1} = 9.7$ Hz, $J_{2,3} = 10.6$ Hz, H-2), 5.54–5.67 (m, 2H, H-3, H-4), 7.10–7.67 (m, 12H, Har), 7.38–7.95 (m, 8H, Har); ^{13}C NMR ($CDCl_3$): 35.2 (C-5′), 39.7 (C-1), 40.5 (C-5), 64.0 (C-6), 72.3, 74.95 (C-3, C-4), 76.9 (C-2), 128.3, 128.4, 128.5, 128.6, 129.0, 129.1, 129.3, 129.7, 129.8, 129.9, 133.1, 133.2, 133.3, 133.4 (CHar and Car), 165.7, 165.8, 166.0, 166.4 (CO); MS (IS+): *m/z*: 633 [$M^+ + Na$]; elemental analysis calcd for $C_{35}H_{30}O_8S$ C, 68.83; H, 4.96. Found: C, 67.83; H, 4.88%.

Enzyme assay

All reagents were purchased from Sigma. The buffer was 0.1 M sodium phosphate at pH 7. Myrosinase from *Sinapis alba*[10,15] was a gift from Dr R. Iori (CRA, ISCI Bologna, Italy). The enzyme kinetics were carried out using a method adapted from the literature.[11] Myrosinase (10 μL, 36 U mL^{-1}) was incubated at 37 °C in the absence or presence of inhibitor (38 μL at final concentrations of 10^{-3} to 10 mM in H_2O) with sinigrin (302 μL at final concentration of 2.3 mM in buffer), glucose oxidase (10 μL at concentration of 55U mL^{-1} in buffer), peroxidase (10 μL at concentration of 131U mL^{-1} in buffer), and *o*-dianisidine (2.5 mg mL^{-1} in H_2O). The release of glucose was measured by following the variation of the absorbance at 436 nm. Classical Michaelis–Menten kinetics were observed under the conditions used, and the IC_{50} determined using the GRAFIT® software.

Crystallographic studies

Sinapis alba myrosinase crystals were grown as described.[5] Crystals were soaked for some hours in their mother liquor (66% sat. ammonium sulfate, 100 mM HEPES pH 7.5) with 20 mM of inhibitor **12** or 10 mM of **17b**. Data (resolution between 1.5 and 1.8 Å, R_{cryst} typically 0.07, completeness 99%) were collected at cryogenic temperature at the ESRF synchrotron (Grenoble, France) on beamlines ID14-1 and ID14-2 and reduced using standard crystallography software as described.[13] The experiments were interpreted with initial F_{inh}–F_{nat} difference Fourier maps where F_{inh} are the structure factor amplitudes of a dataset collected on a crystal soaked with inhibitor, whereas F_{nat} and the phases were obtained from a native myrosinase structure (pdb entry 1e4m[13]). The signal in these maps is reduced due to the presence of a well ordered glycerol molecule and several water molecules in the active site. In the case of the glucosinolate inhibitor, difference maps of three independent experiments were averaged in order to improve the signal to noise ratio of the electron density for the inhibitor (software MAPMAN,[16]). The inhibitor structures (compound **13** and **17b**) have been built into the electron density and subjected to refinement using CNS[17] at a resolution of 1.7 and 1.6 Å. The crystallographic *R*-factors are 0.175 and 0.179 (R_{free} 0.191 and 0.181). Due to an occupancy of only about 0.5 of the carba-glucosinolate inhibitor, alternate structures in presence and in absence of the inhibitor have been modeled into the active site. Coordinates and structure factors have been submitted to the PDB (entries 1w9b and 1w9d) where further details about data collection and refinement can be found. Illustrations were made using BOBSCRIPT[18] and PyMol (Delano Scientific).

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