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A simple O-sulfated thiohydroximate molecule to be the first micromolar range myrosinase inhibitor

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ABSTRACT

New non-hydrolyzable analogues of glucosinolates have been prepared. Myrosinase inhibition was observed with modified aglycon moieties, even bulky phenothiazine analogue **6** gave reasonable inhibition. The simplest structure **8** derived from dimethylaminoethanethiol has shown to be the most potent inhibitor with an IC_{50} of 3.32 μM.

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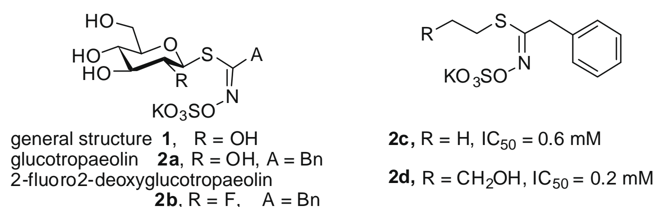
Glucosinolates **1** are naturally occurring thiosugars mainly found in the botanical order *Brassicales*. The structural framework of glucosinolates invariably results from a combination of three segments: a D-glucopyrano unit, an O-sulfated anomeric thiohydroximate function and a broad library of aglycons, whose structure diversifies in the vegetal kingdom according to species.[1] Glucosinolates are substrates of a specific enzyme, myrosinase (thioglucoside glucohydrolase EC 3.2.1.147), the only enzyme able to hydrolyze those thiosaccharidic compounds. Via concomitant expulsion of sulfate ion and D-glucose release, glucosinolate hydrolysis delivers a wide range of aglycon moieties modified into isothiocyanates, nitriles, 1,3-oxazolidine-2-thiones, etc. which usually take part in the defense mechanism of plants and may have some impact on human nutrition.[2] The influence of glucosinolates and their breakdown products on health and more specifically the role they can play in cancer chemoprevention are still not perfectly well understood.[3] With a view to developing new chemical tools for biological and pharmaceutical applications, we have explored the enzyme-substrate recognition mechanism and developed various classes of glucosinolate analogues. Previously, some substrate mimics have been prepared with structural modifications on the glycosidic moiety,[4] on the aglycon chain[5] or on the anionic site.[6] Most of these modifications proved useful in clearly demonstrating the specificity of myrosinase towards the D-glucopyrano moiety and the flexibility with regard to the aglycon moiety. More recently, glucosinolates bearing modified aglycons have been synthesized and studied as new pro-drugs able to deliver cytotoxic isothiocyanates.[7] So far, only 2-fluoro-2-deoxy-glucotropaeolin **2b** was found as a good inhibitor acting through the formation of a covalent glucosyl–enzyme intermediate. Glucotropaeolin (GTL) **2a** was taken as a model because of its current use as a EU official standard for glucosinolate analysis (Scheme 1).[8]

GTL **2a** appeared to be a good template to develop non-hydrolyzable substrates by keeping the benzyl thiohydroximate function and modifying the osidic moiety. We have previously investigated synthetic modifications involving carbasugars or more simple alkyl derivatives, among which the most potent inhibitor was detected.[9] The non-hydrolyzable GTL mimic **2c** was shown to be the best inhibitor inducing 88% myrosinase inhibition at 1 mM. We have decided to follow two different approaches aimed at



Scheme 1. Structure of O-sulfated thiohydroximates.

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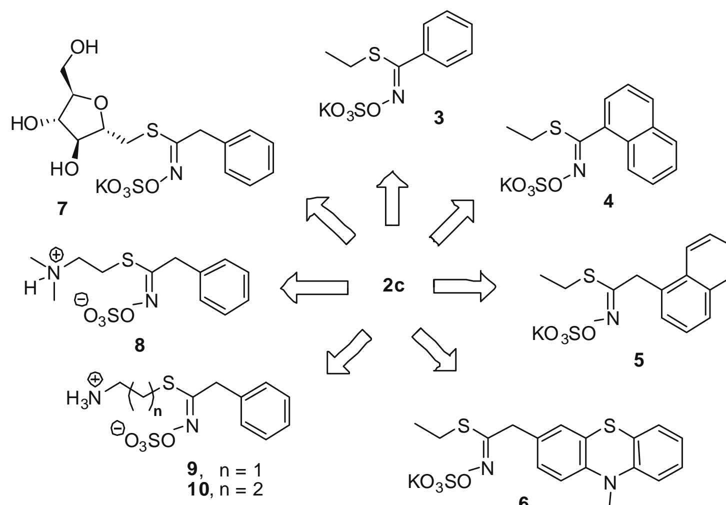


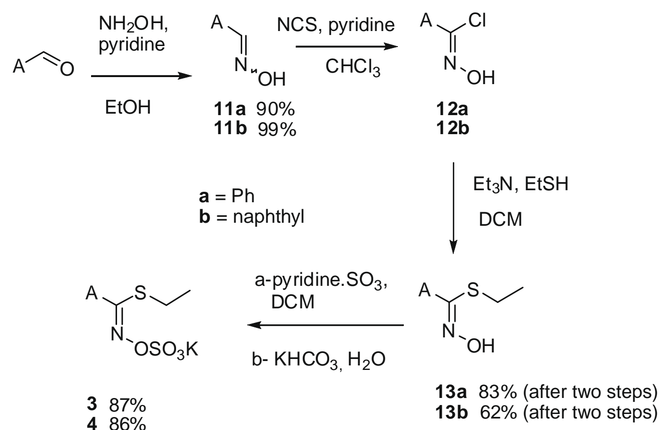
Scheme 2. Panel of myrosinase inhibitors.

improving myrosinase inhibition, either through modification of the aglycon moiety (**3–6**) or by developing transition state analogues (Scheme 2).

We have synthesized a panel of glucosinolate-like molecules in which aryl-type aglycons replace the benzyl group: **3** and **4** analogues lack the benzylic methylene 'knuckle' while **5** and **6** introduce bulky and fluorescent aromatic entities. On the other hand, modifications of the carbohydrate in **2a** afford a panel of geometric mimics of the transition state formed during myrosinase catalysis. These include the 2,5-anhydro-D-mannitol derivative **7** and the oxonium mimics **8–10** which bear ammonium ions distal from the benzylic moiety.

The expected O-sulfated thiohydroximates were obtained by standard sulfation of a precursor synthesized via a key reactive species—a hydroximoyl chloride intermediate. This electrophilic partner could be prepared either from nitroalkene derivatives following Kulkarni's method[10] (Scheme 4) or by substitutive chlorination of aldoximes[11] (Scheme 3).

Hydroximoyl chlorides devoid of a methylene 'knuckle' were prepared from aldoximes **11a–b** (Scheme 3) by reaction with *N*-chlorosuccinimide:[11] the unstable intermediates **12a–b** were engaged in the next step without further purification and reacted with ethyl mercaptan. Thiohydroximates **13a** and **13b** obtained, respectively, in 83% and 62% yields over two steps were sulfated



Scheme 3. Synthesis of thiohydroximates via oxime chlorination.

using standard conditions. After careful ionic exchange using $KHCO_3$ in excess, the O-sulfated thiohydroximates **3** and **4** were obtained in similarly good yields.

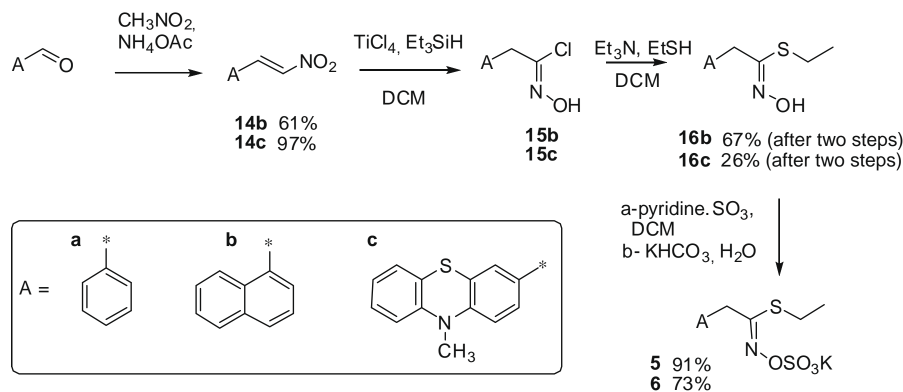
Hydroximoyl chlorides bearing a methylene 'knuckle' were prepared following a different reaction sequence. A Henry-type procedure[12] was first applied to produce the nitroalkene derivatives **14b–c**, which in turn were converted into chloro-oximes **15b–c** by using the protocol described by Kulkarni.[10] *S*-Ethyl naphthylacetothiohydroximate **16b** was isolated in 67% yield, but the *N*-methyl phenothiazinyl counterpart **16c** was isolated in only 26% yield.[13] Finally, the derived O-sulfates **5** and **6** were obtained in good yields.

All built on a common phenylacetothiohydroximate frame of the GTL-type, other glucosinolate mimics **7–10** were obtained through condensation of diverse mercaptans on a unique hydroximoyl chloride **15a** (Scheme 5) previously used to make **2a–d**. The anhydromannitol-derived thiol **20** was synthesized from D-glucosamine **17** according to a previously described sequence.[14] Under nitrosation conditions, **17** underwent ring contraction leading to 2,5-anhydro-D-mannose, which was reduced with $NaBH_4$ to yield 2,5-anhydro-D-mannitol **18** (Scheme 5). Selective monotosylation and further peracetylation gave a 35% yield of compound **19**, which was further converted into the corresponding thiol **20** in three steps (64% overall yield): (1) substitutive iodination (2) formation of the thiouronium salt (3) hydrolysis under reductive conditions.

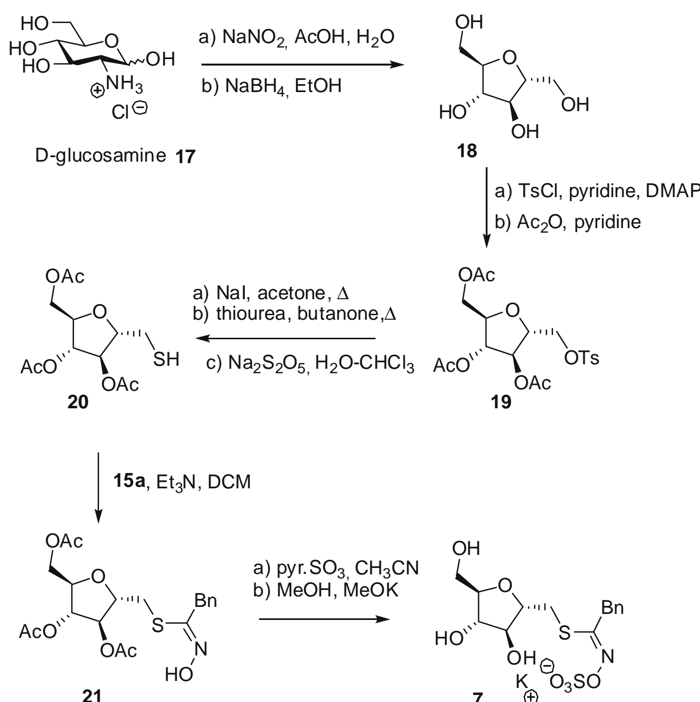
1-Thio-3,4,6-tri-*O*-acetyl-2,5-anhydro-D-mannitol **20** was condensed with **15a** to give thiohydroximate **21** in 50% yield. A final two-step sulfation/de-O-acetylation sequence gave the glucosinolate analogue **7** in 73% yield.

The unprecedented ammonium analogues **8–10** were prepared following a similar sequence involving stereospecific addition of the corresponding aminothiols to the transient nitrile oxide generated from **15a**,[15] followed by standard sulfation.[9] A slight modification was needed for the synthesis of **9**, where the starting aminothiol was used in the *N*-*t*Boc protected form.[16] Sulfate **23** obtained was deprotected in the last step to generate zwitterionic **9** (Scheme 6).

This amino acid **9** was easily isolated, thanks to its precipitation in the aqueous reaction medium but unfortunately on re-solubilization in DMSO,[17] a spontaneous decomposition occurred: intramolecular nucleophilic addition of the amine onto the O-sulfated thiohydroximate resulted in the formation of thiazoline **24** through sulfamic acid elimination.[18] Preparation of the amino acid **10** was



Scheme 4. Synthesis of thiohydroximates from a nitroalkene precursor.

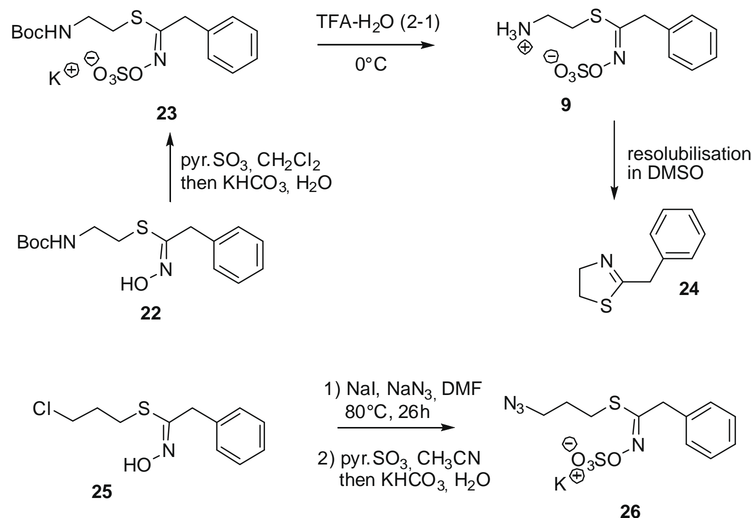


Scheme 5. Synthesis of the anhydromannitol-derived thiohydroximate **7**.

attempted from the corresponding azidopropyl derivative **26**, obtained in two steps and 61% yield from thiohydroximate **25**. Unfortunately on catalytic hydrogenation of **26** and similarly to **9**, rapid degradation occurred. On the contrary, the *N*,*N*-dimethylammonium derivative **8**, which could be smoothly prepared in only two steps and 47% yield, proved fairly stable in solution and could therefore be tested.

The activity of *Sinapis alba* myrosinase towards the different glucosinolate analogues was determined by titration of released glucose from sinigrin (Table 1).[8] From this small panel of molecules, a major information related to the inhibition of myrosinase could be put into light. Comparison with both standard compounds **2c** and **2d** demonstrated the importance of the methylene knuckle: only a small 20.8% inhibition was observed at 1 mM with the phenyl group (compound **3**, entry 3) while no activity was detected with a more bulky naphthyl group (compound **4**, entry 4). Introducing back a methylene knuckle restored good inhibitory properties in compound **5** (entry 5), while even a bulkier phenothiazinyl moiety (compound **6**, entry 6) did not significantly hamper the inhibition capacity. Neither the transition state mimic **7**, bearing the 2,5-anhydro-D-mannitol moiety (Table 1, entry 7), nor the azide **26** (Table 1, entry 9) displayed a significant ability to inhibit myrosinase. Surprisingly, the most potent myrosinase inhibitor is the most simple constructed one. The *N*,*N*-dimethylammonium-based transition state mimic **8** displayed an IC_{50} of 3.32 μM and at 1 mM it completely ablated myrosinase activity. The potency shown by **8** is approximately 100-fold greater than shown by the previous millimolar inhibitors developed in our laboratories.

Major features to create new potent inhibitors could thus be extracted from the above results: the presence of a methylene knuckle, the possibility of bulky fluorescent moieties and, more importantly, the introduction of a dialkylamino group to mimic the transition state and mostly to introduce stability. Further exploration on designing and studying new non-hydrolyzable inhibitors of myrosinase is ongoing and will be published in due course.



Scheme 6. Synthesis of the ammonium-type thiohydroximates.

Table 1
Myrosinase inhibition results

Entry	Molecules	% Inhibition at 1 mM	IC_{50} (mM)
1	**2d**	88	0.2
2	**2c**	67	0.6
3	**3**	20.8	n.d.[b]
4	**4**	0	n.d.[b]
5	**5**	77.3	n.d.[b]
6	**6**	44	n.d.[b]
7	**7**	20.9	n.d.[b]
8	**8**	100	3.32×10^{-3}[a]
9	**26**	26.1	n.d.[b]

[a] $IC_{50} = 3.32 \pm 0.13$ μM.
[b] n.d.: not determined.

Acknowledgements

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15. In a representative synthetic sequence, the crude phenylacetohydroximoyl chloride **15a** (0.67 mmol)[9] under argon was dissolved in dichloromethane (10 mL) and NEt_3 (280 μL, 2 mmol) just before commercial *N-t*Boc-amino-ethanethiol (135 μL, 0.8 mmol) was added. After stirring for 5 h, the mixture was evaporated in vacuo. Flash chromatography (petroleum ether/AcOEt, 7:3) purification of the residue gave compound **22** (0.14 g, 0.45 mmol, 67%) as a white solid, mp: 143–145 °C. IR (KBr): ν 3343, 2980, 1672, 1524, 1286, 1163 cm^{-1}. ^1H NMR (DMSO-d_6): δ 11.15 (s, 1H, OH), 7.35–7.20 (m, 5H, H_{arom}), 7.05 (t, 1H, J = 5.6, NH), 3.83 (s, 2H, CH_2Ph), 3.06–2.98 (m, 2H, CH_2 N), 2.70 (t, 2H, J = 7.2, CH_2S), 1.39 (s, 9H, CH_3). ^{13}C NMR (DMSO-d_6): δ 155.5 (C=O), 150.8 (C=N), 137.1 (C_{arom}), 128.4, 128.1, 126.5 (CH_{arom}), 77.9 (Me_3C–), 40.7 (CH_2 N), 37.2 (CH_2Ph), 28.1 (CH_3), 27.9 (CH_2S). ESI-HRMS calcd for $C_{15}H_{22}N_2O_3S$ $[M+H]^+$: 311.1429, found: 311.1454.
16. Compound **22** was O-sulfated according to a previously described protocol.[9] Flash chromatography (AcOEt/MeOH, 9:1) purification of the residue gave compound **23** (91%) as a white solid, mp: 151–155 °C (dec). IR (KBr): ν 3357, 2977, 1700, 1574, 1409, 1294, 1070 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.33–7.23 (m, 5H, H_{arom}), 7.07 (t, 1H, J = 5.6, NH), 3.90 (s, 2H, CH_2Ph), 3.05–2.97 (m, 2H, CH_2 N), 2.68 (t, 2H, J = 7.0, CH_2S), 1.39 (s, 9H, CH_3). ^{13}C NMR (DMSO-d_6): δ 155.5 (C=O or C=N), 155.2 (C=N or C=O), 136.4 (C_{arom}), 128.5, 127.9, 126.8 (CH_{arom}), 78.0 (Me_3C–), 40.5 (CH_2 N), 37.1 (CH_2Ph), 28.3 (CH_2S), 28.1 (CH_3). ESI-HRMS calcd for $C_{15}H_{21}N_2O_6S_2$ $[M]^+$: 389.0841, found: 389.0826.
17. To the protected compound **23** (0.3 g, 0.7 mmol) dissolved in water (2 mL) at 0 °C was added TFA (4 mL). After stirring at room temperature for 4 h, the formed white precipitate of zwitterionic **9** was filtered and rinsed with water and acetone (0.61 mmol, 88%). ^1H NMR (DMSO-d_6): δ 7.79 (br s, 3H, NH_3^+), 7.40–7.24 (m, 5H, H_{arom}), 3.88 (s, 2H, CH_2Ph), 2.96–2.87 (m, 4H, CH_2 N and CH_2S). ^{13}C NMR (DMSO-d_6): δ 153.8 (C=N), 136.0 (C_{arom}), 128.6, 128.1, 126.9 (CH_{arom}), 39.0 (CH_2 N), 37.3 (CH_2Ph), 26.2 (CH_2S).
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