Remarkable incorporation of the first sulfur containing indole derivative: another piece in the biosynthetic puzzle of crucifer phytoalexins†,‡

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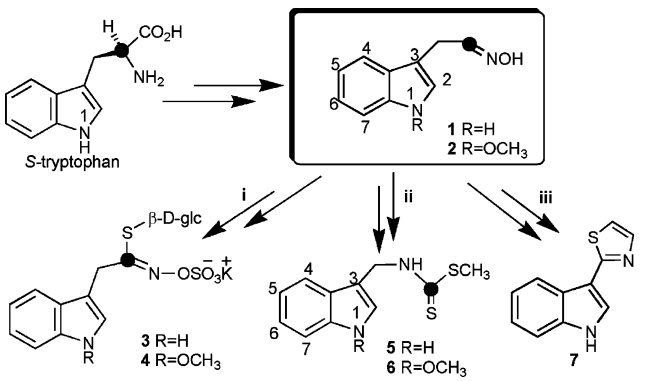
The first sulfur labelled compound, [$^2H_4$,^{34}S]indolyl-3-acetothiohydroxamic acid, is incorporated into the phytoalexins cyclobrassinin and spirobrassinin and the indole glucosinolate glucobrassicin, indicating that both biosynthetic pathways are closely related.

Plants have a complex range of defence responses that contribute to basal and specific disease resistance to many microbial pathogens. Phytoalexins are chemical defences produced *de novo* by plants to ward off pathogens and other stresses, including intense heat and UV irradiation.[1] Because crucifers are widely cultivated crops and include valuable oilseeds, vegetables, and condiments, their resistance to pests and diseases is an ongoing concern. Considering the negative impact of pesticides and fungicides on the environment, it is crucial to devise safer strategies to protect cropping systems. Evidently, safer and sustainable strategies must derive from a better molecular understanding of plant defences and their metabolic pathways. Toward this end, the biosynthetic pathways of crucifer phytoalexins and related metabolites are of great importance, albeit an ongoing challenge.[2] Specifically, the variety of phytoalexin structures[3,4] and some unique metabolic reactions[5] make mapping out these pathways very difficult. For example, the similarity between the chemical structures of brassinins **5** and **6** and indolyl glucosinolates **3** and **4** led to an earlier consensus that glucobrassicin (**3**) was a precursor of brassinin (**5**). Thus, it was rather surprising to find that glucobrassicin was not incorporated into brassinin.[6] This lack of incorporation suggested to us that if not glucobrassicin (**3**), one of its precursors would be the branch point between the brassinin and glucobrassicin pathways. Subsequently, indolyl-3-acetaldoxime (**1**) was shown to be a precursor of brassinin (**5**), methoxybrassinin (**6**) and related phytoalexins.[6]

The biosynthetic pathway of crucifer phytoalexins and that of indolyl glucosinolates starts with the conversion of tryptophan to indolyl-3-acetaldoxime (**1**) and appears to diverge into various branches, three of which are pertinent to consider here: (i) indolyl glucosinolates (*e.g.* **3**, **4**),[7] (ii) brassinins (*e.g.* **5**, **6**)[6,8] and (iii) camalexin (**7**, Scheme 1).[9] The biosynthesis of glucosinolates, due to their own importance, has been investigated for more than four decades.[10] Hence, most of the genes and intermediates of the pathway to glucobrassicin (**3**) are known. On the other hand, no genes of the brassinin branch have been reported.[2] However, camalexin (**7**) is produced in *Arabidopsis thaliana*, a wild species where a strong effort in functional genomics is concentrated. Thus, some intermediates and genes of the camalexin branch are known.[9]



Scheme 1 Biosynthetic precursors of (i) indolyl glucosinolates **3** and **4**, (ii) phytoalexins brassinin (**5**), methoxybrassinin (**6**), and (iii) camalexin (**7**).

Earlier work showed that the sulfur of glucobrassicin (**3**) was derived from cysteine. Furthermore, it was suggested that the *C–S* lyase that cleaves *S*-cysteinyl thiohydroximate is tightly coupled to the *S*-donating enzyme, and that the product of the *C–S* lyase is a thiohydroximic acid.[10] As well, Monde *et al.*[11] reported that the sulfur of the thiocarbonyl group of brassinin (**5**) was derived from cysteine and the SMe from methionine. Altogether the current information suggests to us that there should be a sulfur containing metabolite common to both brassinin (**5**) and glucobrassicin (**3**). Based on this reasoning, we synthesised ^2H and ^{34}S isotopically labelled indolyl-3-acetothiohydroxamic acid (**10a**) and administered it to UV-irradiated rutabaga tubers (*Brassica napus* L. ssp. *rapifera*). Remarkably, incorporation of this labelled compound into cyclobrassinin (**14**), spirobrassinin (**15**) and glucobrassicin (**3**) was established for the first time. Here we describe details of this biosynthetic investigation as well as the synthesis of the first doubly labelled indolyl thiohydroxamic acid. A detailed biosynthetic pathway for brassinin (**5**) is proposed that integrates all current knowledge.

Because indolyl-3-acetothiohydroxamic acid (**10**) had not been characterised,§ we synthesised first non-labelled material to determine its chemical stability in aqueous methanol solutions to use in plant feeding experiments. Thus, non-labelled **10** was

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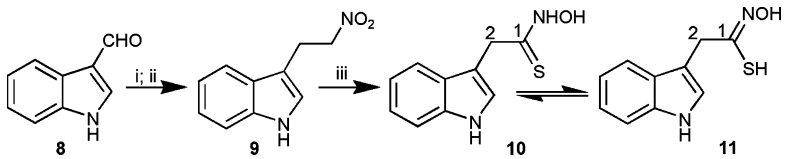
‡ Electronic supplementary information (ESI) available: Experimental section. See DOI: 10.1039/b714743k



Scheme 2 Synthesis of indolyl-3-acetothiohydroxamic acid (**10**). Reagents and conditions: (i) CH_3NO_2, NH_4OAc, 128–130 °C (98%); (ii) $NaBH_4$, i-PrOH, SiO_2, $CHCl_3$ (71%); (iii) KH, $(Me_3Si)_2S$, THF (74%).

prepared from indole-3-carboxaldehyde (**8**) in three steps. Indole-3-carboxaldehyde (**8**) was transformed to 3-(2′-nitrovinyl)-indole upon treatment with ammonium acetate in nitromethane.[12] Subsequent sodium borohydride reduction yielded 3-(2′-nitroethyl)-indole (**9**) which upon treatment with potassium hydride,[13] followed by hexamethyldisilathiane[14] yielded **10** in *ca.* 51% overall yield (Scheme 2). When sodium hydride was used instead of potassium hydride, only indolyl-3-acetonitrile (**18**) and indolyl-3-acetaldoxime (**1**) were obtained in a 1 : 1 ratio. The ^{13}C NMR data of indolyl-3-acetothiohydroxamic acid (**10**) indicated that structure **10** is the most likely tautomer present in solution, since the chemical shift of C-1 is 189.0 ppm. This conclusion is in agreement with current data.[15] In general, thiohydroximates (*e.g.* **3** or **4** or the sodium salt of **11**) have a lower δ_C for C-1 (*ca.* 162–172 ppm).¶[16]

With indolyl-3-acetothiohydroxamic acid (**10**) in hand, its chemical stability in aqueous solution was determined by HPLC analysis; **10** decomposed on standing (solution of water–methanol, 95 : 5, v/v) to indolyl-3-acetonitrile (**18**, *ca.* 50% in 7 h) with extrusion of sulfur. However, since the product of indolyl-3-acetothiohydroxamic acid (**10**) decomposition did not contain sulfur, any sulfur containing products obtained from incorporation of [4′,5′,6′,7′-$^2H_4$]indolyl-3-[^{34}S]acetothiohydroxamic acid (**10a**) would inevitably derive from **10a** itself. Nonetheless, to obtain a comprehensive map of these metabolic pathways, additional control experiments were carried out using tetradeuterated indolyl-3-acetonitrile (**18a**) as well.

The synthesis of [4′,5′,6′,7′-$^2H_4$]indolyl-3-[^{34}S]acetothiohydroxamic acid (**10a**) started with [4,5,6,7-$^2H_4$]indole-3-carboxaldehyde (**8a**)[17] and followed the steps shown in Scheme 2, but using $(Me_3Si)_2{}^{34}S$ (prepared from ^{34}S/Na and chlorotrimethylsilane),[18] as described in the ESI.‡ The synthesis of [4′,5′,6′,7′-$^2H_4$]indolyl-3-acetonitrile was carried out as previously described.[17]

Next, solutions of [4′,5′,6′,7′-$^2H_4$]indolyl-3-[^{34}S]acetothiohydroxamic acid (**10a**) and indolyl-3-acetothiohydroxamic acid (**10**) were separately added to UV-elicited rutabaga tubers, the tissues were incubated, extracted, and the extracts were fractionated to a non-polar fraction, containing phytoalexins, and a polar fraction, containing indolyl glucosinolates, as described in the ESI.‡ HPLC analysis of the fractions using photodiode array and mass detectors and comparison of the spectra of the components with those available in our metabolite library showed the presence of the phytoalexins cyclobrassinin (**14**), spirobrassinin (**15**), rutalexin (**16**) and brassicanate A (**17**) in the non-polar fraction, and the glucosinolates glucobrassicin (**3**), 1-methoxyglucobrassicin (**4**), and 4-methoxyglucobrassicin (**22**) in the polar fraction. LC-HRMS-ESI analysis of each fraction indicated the levels of deuterium and sulfur incorporation in each metabolite, as shown in Table 1. The $[M + 4]^{\pm}$ or $[M + 6]^{\pm}$ ion peaks were not detected in fractions of tubers incubated with non-labelled indolyl-3-acetothiohydroxamic acid (**10**).

Table 1 Metabolism of [4′,5′,6′,7′-$^2H_4$]indolyl-3-[^{34}S]acetothiohydroxamic acid (**10a**) in elicited tubers of rutabaga (*Brassica napus* ssp. *rapifera*)

Labelled product	Incorporation (% of ^2H and ^{34}S)[a]
^{34}S-[4′,5′,6′,7′-$^2H_4$]Cyclobrassinin (**14a**)[b]	7%
^{34}S-[4′,5′,6′,7′-$^2H_4$]Spirobrassinin (**15a**)[b]	7%
[4′,5′,6′,7′-$^2H_4$]Rutalexin (**16a**)[c]	10%
[4′,5′,6′,7′-$^2H_4$]Brassicanate A (**17a**)[c]	2%
^{34}S-[4′,5′,6′,7′-$^2H_4$]Glucobrassicin (**3a**)[c]	2%

[a] The % of ^2H and ^{34}S incorporation was established by HRMS-ESI according to the following equation: $\% = \{[M + n]^{\pm}/([M]^{\pm}+[M + n]^{\pm})\} \times 100$ ($n = 4$ or 6). [b] Positive ion mode. [c] Negative ion mode.

Additional experiments were carried out using [4′,5′,6′,7′-$^2H_4$]indolyl-3-acetonitrile (**18a**) and indolyl-3-acetonitrile (**18**), as reported above for acetothiohydroxamic acids **10a** and **10**. LC-HRMS-ESI analysis of each fraction showed the same components as above and indicated that none of the phytoalexins or indolyl glucosinolates contained deuterium. Interestingly, indolyl-3-acetonitrile (**18**) was completely metabolised within 24 h to indolyl-3-acetic acid (**19**), which in turn was metabolised to indole-3-carboxylic acid (**20**) and methyl indole-3-carboxylate (**21**).

Remarkably, as summarised in Table 1, LC-HRMS data indicated that cyclobrassinin (**14a**), spirobrassinin (**15a**) and glucobrassicin (**3a**) incorporated intact [4′,5′,6′,7′-$^2H_4$]indolyl-3-[^{34}S]acetothiohydroxamic acid (**10a**) since only the $[M + 6]^{\pm}$ peak was detected. By contrast, the phytoalexins rutalexin (**16a**) and brassicanate A (**17a**) showed incorporation of only four deuteria (only $[M + 4]^{\pm}$ peak) and no sulfur. Because rutalexin (**16**) is biosynthesised from cyclobrassinin (**14**),[19] these results indicate that the sulfur 34 of cyclobrassinin (**14**) was exchanged with unlabelled sulfur. Thus, it is likely that another sulfur donor (possibly also cysteine) was used in this conversion. A similar explanation could be used for brassicanate A (**17a**), since it appears to be derived from brassinin (**5**).[19] The fact that among the three indolyl glucosinolates only glucobrassicin (**3a**) showed incorporation of four deuteria and sulfur 34 is consistent with our previous findings[8] indicating that oxidation at position N-1 of indole occurs immediately after oxime formation, *i.e.* upstream of the step yielding thiohydroxamic acid **10**.

As expected, [4′,5′,6′,7′-$^2H_4$]indolyl-3-acetonitrile (**18a**) was not incorporated into any of the phytoalexins or glucosinolates present in elicited tissues (Table 2). This result is of interest since indolyl-3-acetonitrile (**18**) appears to be an intermediate in the biosynthesis of camalexin (**7**).[20] Nonetheless, since camalexin (**7**) is not produced in rutabaga we could not confirm that result.

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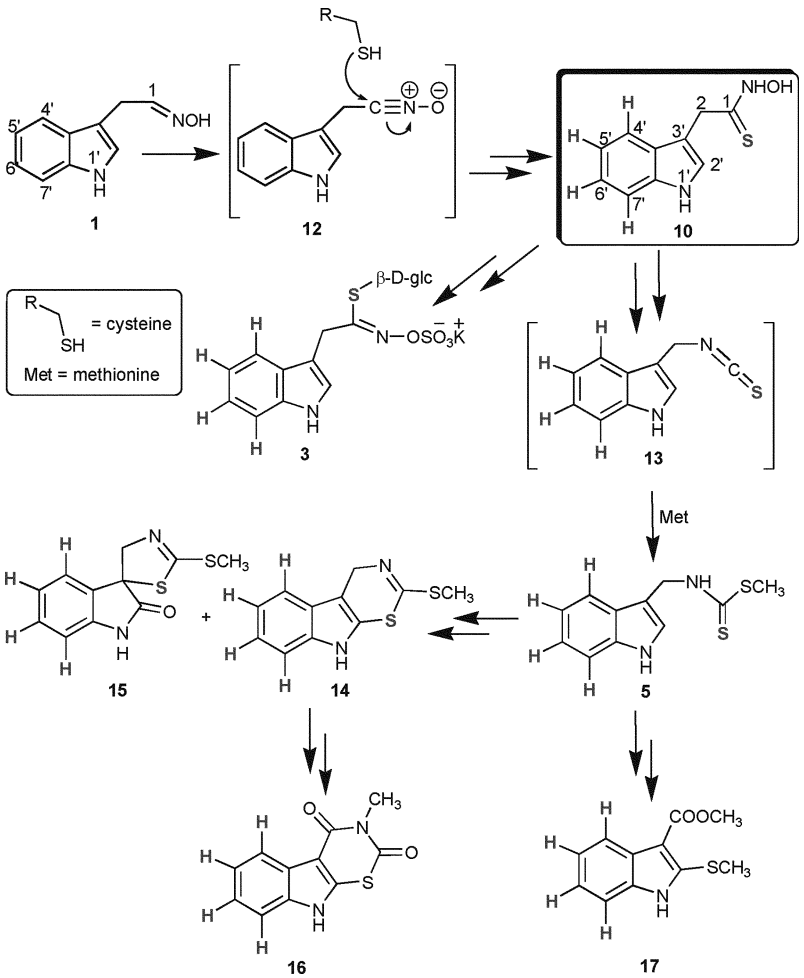
Table 2 Metabolism of [4′,5′,6′,7′-$^2H_4$]indolyl-3-acetonitrile (**18a**) in elicited tubers of rutabaga (*Brassica napus* ssp. *rapifera*)

Labelled product	Incorporation (% of ^2H)[a]
[4′,5′,6′,7′-$^2H_4$]Indole-3-carboxylic acid (**20a**)[b]	≥98%
Methyl [4′,5′,6′,7′-$^2H_4$]indole-3-carboxylate (**21a**)[b]	≥98%

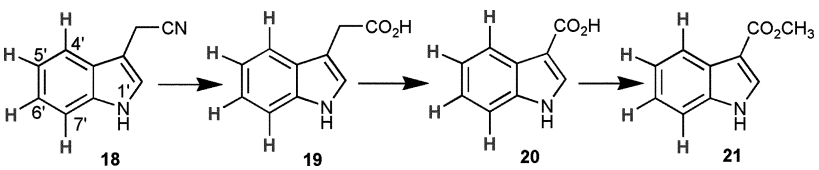
[a] The % of ^2H incorporation was established by HRMS-ESI according to the following equation: $\% = \{[M+4]^{\pm}/([M]^{+}+[M+4]^{\pm})\} \times 100$. [b] Negative ion mode.

Based on the results described above and their integration with reported data,[2,10] another intermediate (**10**) common to the pathways of brassinin (**5**) and glucobrassicin (**3**) can be proposed. Firstly, as depicted in Scheme 3, oxidation of indolyl-3-acetaldoxime (**1**) to the corresponding nitrile oxide **12** provides a putative intermediate suitable for nucleophilic attack by an alkylthiol (*e.g.* cysteine).[10] The resulting intermediate could be converted through a *C–S* lyase to the thiohydroxamic acid **10**, possibly the last intermediate common to both pathways. Indolyl-3-methylisothiocyanate (**13**) could then be thiomethylated to yield brassinin (**5**).

[4′,5′,6′,7′-$^2H_4$]Indolyl-3-acetonitrile (**18a**) appeared to be the main precursor of indolyl-3-acetic acid (**19a**), indole-3-carboxylic acid (**20a**) and methyl indole-3-carboxylate (**21a**) under the experimental conditions described in the ESI,‡ since these three metabolites were isolated fully tetradeuterated (about ≥98%, Table 2, Scheme 4). In agreement with these findings compounds **19–21** were not detected in control experiments.

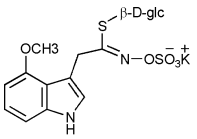


Scheme 3 Proposed biosynthetic pathway of glucobrassicin (**3**) and phytoalexins brassinin (**5**), cyclobrassinin (**14**), spirobrassinin (**15**), rutalexin (**16**) and brassicanate A (**17**); compounds **12** and **13** are proposed intermediates.



Scheme 4 Incorporation of [4′,5′,6′,7′-$^2H_4$]indolyl-3-acetonitrile (**18a**) into indolyl-3-acetic acid (**19a**), indole-3-carboxylic acid (**20a**) and methyl indole-3-carboxylate (**21a**).

As well, our findings have implications on the pathway of indole glucosinolates. The results described above reinforce our previous data,[6] which indicated that glucosinolates **4** and **22** derive from oxidation of indolyl-3-acetaldoxime (**1**) and not from glucosinolate **3**. This is likely because glucosinolates **4** and **22** did not incorporate the thiohydroxamic acid **10a**. Previously, it was proposed that these indole glucosinolates (**4** and **22**) resulted from enzymatic methoxylation of **3**.[21] In addition, our results suggest that brassinin (**5**) is perhaps only a couple of steps from the thiohydroxamic acid **10** (side-chain rearrangement and thiomethylation).



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Eventually, with a better understanding of the biosynthetic intermediates of crucifer phytoalexins and their corresponding enzymes and genes, it is expected that plants may be bred to produce a wider variety of chemical defences and display higher levels of resistance to stress.

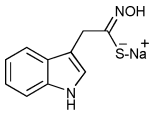
Selected spectroscopic data

Indolyl-3-acetothiohydroxamic acid (10). ^{1}H NMR (500 MHz $CDCl_3$): δ 8.26 (br s, 1H), 7.54 (d, J = 8 Hz, 1H), 7.44 (d, J = 8 Hz, 1H), 7.28 (dd, J = 7.5, 7.5 Hz, 1H), 7.17–7.24 (m, 2H), 4.26 (s, 2H). ^{13}C NMR (125.8 MHz $CDCl_3$): δ 189.0 (s), 136.7 (s), 126.9 (s), 124.5 (d), 123.5 (d), 121.0 (d), 119.0 (d), 111.9 (d), 107.2 (s), 36.9 (t). HRMS-ESI m/z measured 207.0579 (207.0586 calculated for $C_{10}H_{11}N_2OS$). HPLC-MS-ESI m/z (relative abundance) 207 $[M + H]^+$ (100), 189 (22), 174 (39), 130 (35). FTIR λ_{max}(KBr)/cm^{-1}: 3410, 3307, 3056, 2904, 1552, 1455, 1419, 1353, 1339, 1227, 1131, 1062, 971, 744.

[4′,5′,6′,7′-$^2H_4$]Indolyl-3-[^{34}S]acetothiohydroxamic acid (10a). ^{1}H NMR (500 MHz $CDCl_3$): δ 8.26 (br s, 1H), 7.20 (s, 1H), 4.27 (s, 2H). HRMS-ESI m/z measured 213.0797 (213.0795 calculated for $C_{10}H_7{}^2H_4N_2O^{34}S$). HPLC-MS-ESI m/z (relative abundance) 213 $[M + H]^+$ (100), 195 (15), 180 (13), 134 (18).

Notes and references

§ Sodium indolyl-3-thiohydroximate (**23**) was previously reported not to be stable in a buffer solution at pH 6.[16]



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¶ All compounds gave satisfactory spectroscopic data; in each case the percentage of deuterated synthetic compound was ≥98%. Labelled compounds are numbered with an additional letter **a** (*e.g.* **10a**).

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