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Assembling the biosynthetic puzzle of crucifer metabolites: indole-3-acetaldoxime† is incorporated efficiently into phytoalexins but glucobrassicin is not

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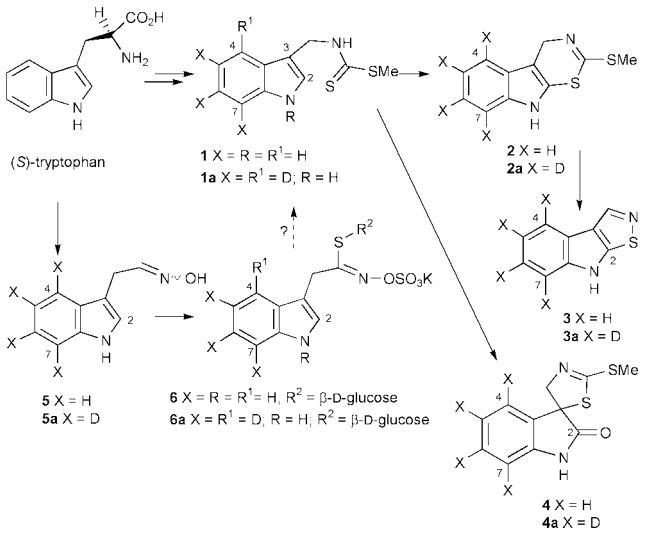
First biosynthetic studies utilizing tetradeuterated precursors indicate that the indole glucosinolate glucobrassicin is not a precursor of the phytoalexin brassinin, and that indole-3-acetaldoxime is an efficient precursor.

Plants have a complex arsenal of defense mechanisms to fight pathogen attack. A significant aspect of these defense mechanisms involves secondary metabolites, which may be either constitutive or biosynthesized *de novo* in response to diverse forms of stress, *i.e.* phytoalexins.[1] The plant family Cruciferae comprises a large number of economically important oilseed crops as well as vegetables. Besides their economic importance, crucifers are also interesting plant model-systems, containing the first and only example to date of a completely sequenced plant genome.[2] Chemical characterization of secondary metabolites from crucifers has unraveled a remarkable array of phytoalexins (*e.g.* **1**–**4**),[3] and a group of structurally related secondary metabolites known as glucosinolates (*e.g.* **6**).[4] This structural connection becomes more transparent considering that methoxy derivatives of brassinin **1** (*e.g.* R = OMe, R^1 = H) and glucobrassicin **6** (*e.g.* R = OMe, R^1 = H, R^2 = β–D–glucosyl) are naturally occurring within the same species and that their levels increase simultaneously in plants subjected to stress.[5] Furthermore, unambiguous biosynthetic studies have demonstrated that (*S*)-tryptophan is the precursor of both metabolites **1** and **6**.[3,6] In this connection a number of suggestions and attempts to establish a biogenetic relationship between indole glucosinolates such as glucobrassicin **6** and cruciferous phytoalexins have been reported.[3,7] Furthermore, a number of studies[8] have demonstrated that (*S*)-tryptophan is converted to **6** *via* indole-3-acetaldoxime **5** (Scheme 1).

Paradoxically, because glucosinolates are considered an undesirable group of metabolites in brassicas,[8] a large number of oilseed breeding programs are directed at obtaining plants containing low levels of glucosinolates. However, if indole glucosinolates such as glucobrassicin **6** are precursors of phytoalexins **1**–**4** (Scheme 1), then lowering glucosinolate contents in these oilseeds may pose a substantial ecological risk from a plant defense perspective. Nonetheless, despite a number of biosynthetic studies in crucifers, this biogenetic relationship has not been demonstrated.[3] Thus we became interested in establishing the possible biogenetic relationship between indole glucosinolate **6** and brassinin **1**, as well as the biosynthetic pathway to the phytoalexin brassilexin **3**.[9] Here we establish for the first time a biosynthetic relationship between indole-3-acetaldoxime **5** and phytoalexins **1**–**4** which, contrary to previous speculations,[3] do not appear to be derived from indole glucosinolate **6**.

To investigate the possible biogenetic relationship between phytoalexins and indole glucosinolates, [4,5,6,7-$^2H_4$]-compounds **1a**–**3a** were synthesized as previously reported;[9] [4,5,6,7-$^2H_4$]glucobrassicin **6a** was synthesized according to the previously reported route[10] but utilizing **7a**[9] as the starting material.[11] Initially, the cruciferous brown mustard (*Brassica juncea*) was selected to determine the proposed biosynthetic relationship because it produces phytoalexins **1**–**4** and indole glucosinolate **6**. Thus, [4,5,6,7-$^2H_4$]glucobrassicin **6a** was administered to leaves of brown mustard; after solution uptake the leaves were elicited with a spore suspension of the fungus *Phoma lingam* (perfect stage *Leptosphaeria maculans*), were incubated, and extracted.[9,12] HPLC analysis of the extracts indicated the presence of phytoalexins **2**–**4**; the extracts were fractionated by HPLC and the identity of each phytoalexin was confirmed by ^{1}H NMR and HRMS-EI. Deuterium incorporation in **2**–**4** (≤0.1% relative to a natural abundance control sample by HRMS-EI) was too small to establish **6a** as a precursor. Similar results were obtained when leaves were elicited with UV light instead of the fungal spores. This lack of deuterium incorporation was thought to be partly due to the substrate not reaching the appropriate cell site.

Subsequently, it was thought that a different plant tissue where precursors could be in contact with the tissue for a longer period (*i.e.* no uptake of the substrate solution *via* the plant vascular system) might lead to higher deuterium incorporation levels. Thus, [4,5,6,7-$^2H_4$]glucobrassicin **6a** was administered to UV-irradiated turnip root slices (*B. rapa*)[6] followed by incubation. Similar experiments were conducted with non-irradiated turnip roots. Extraction of the turnip tissue, fractionation, and HPLC analysis of extracts and fractions indicated the presence of phytoalexins **1**–**4**. Once again HRMS-EI analysis indicated that deuterium incorporation in **1**–**4** was too low to allow a reliable conclusion (≤0.1%). However, two additional compounds containing deuterium were separated and identified unambiguously as indole-3-carbaldehyde **7a** (34% ^{2}H incorporation) and methyl indole-3-carboxylate **8a** (26% ^{2}H



Scheme 1 Biosynthetic pathway from (*S*)-tryptophan to phytoalexins **1**–**4** and indole glucosinolate **6**.

† The IUPAC name for an acetaldoxime is acetaldehyde oxime.

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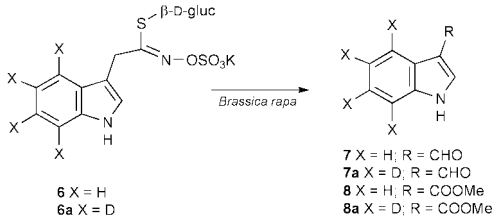
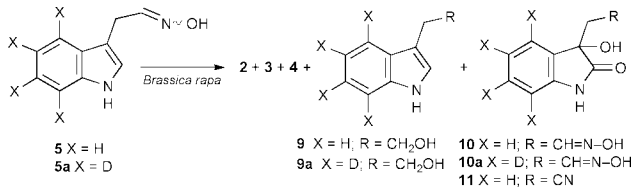
incorporation**)**. These results indicated that metabolism of **6a** to **7a** and **8a** occurred in turnip roots and that the metabolism was unrelated to phytoalexin biosynthesis (Scheme 2).[13] These results also revealed for the first time that **3** was produced in turnip roots.[14]



Scheme 2 Metabolism of [4,5,6,7-$^2H_4$]glucobrassicin (**6a**) in turnip roots (*B. rapa*).

Next, to confirm that turnip root was an appropriate tissue to study phytoalexin pathways, [4,5,6,7-$^2H_4$]brassinin **1a** was added to roots, the tissues were incubated, extracted, analyzed, and the extracts fractionated to yield phytoalexins **3** and **4**. HRMS-EI and ^{1}H NMR analysis indicated substantial deuterium incorporation into both spirobrassinin **4a** (15% ^{2}H incorporation) and **3a** (9% ^{2}H incorporation).[13] The incorporation level of deuterium into brassilexin was substantially higher than our early work utilizing *B. carinata* leaves,[9] thus demonstrating for the first time that in turnip, **1a** is also a precursor of **3a** and confirming that turnip root tissue is an adequate system for biosynthetic studies.[6]

Because of our unsuccessful attempt to demonstrate that **6a** is a precursor of **1a** or related phytoalexins **2a**–**4a**, we investigated the potential biogenetic relationship between **5a** and **1a**. Thus, tetradeuterated **5a** was administered to UV-irradiated turnip roots;[15] after incubation of the tissues, extraction, and fractionation of the extracts, **2**–**4** were obtained. HRMS-EI analysis indicated significant deuterium incorporation into **2a** (10% ^{2}H incorporation), **4a** (14% ^{2}H incorporation) and **3a** (2% ^{2}H incorporation). Furthermore, three additional compounds, subsequently established to be tryptophol **9a** and oxindoles **10a** and **11a**, were isolated and analyzed by HRMS-EI. Deuterium incorporation levels suggested that metabolites **9a**–**11a** were fully derived from metabolism of **5a** (Scheme 3, deuterium incorporation ⩽98%). This conclusion was consistent with the absence of compounds **9a**–**11a** in elicited control tissues. Additional studies indicated that oxime **10a** dehydrated upon standing to yield nitrile **11a**, suggesting that turnip tissue contained the enzyme system required to oxidize oxime **5a** to **10a**, and that **11a** might be an artifact of the isolation process.[16] Note that **11** was previously isolated from *B. oleracea*,[17] however its precursor oxindole oxime **10** has not been described to date.



Scheme 3 Metabolism of [4,5,6,7-$^2H_4$]indole-3-acetaldoxime (**5a**) in turnip roots (*B. rapa*).

In conclusion, our results established a most important biogenetic relation between phytoalexins **1**/**1a**–**4**/**4a** and aldoxime **5**/**5a**, whereas the postulated relationship between indole glucosinolate **6**/**6a** and these phytoalexins was not demonstrated. Thus, considering that **5** is also a precursor of **6**,[8] it is likely that the pathway to phytoalexins follows the tryptophan–aldoxime route and will branch out from the indole glucosinolate pathway a step(s) earlier than previously proposed.[3] Nonetheless, our results have strong implications on plant breeding to produce low glucosinolate content oilseed brassicas, *i.e.* it is essential not to delete the indole acetaldoxime formation steps, as aldoxime **5** is a close precursor of important cruciferous phytoalexins (*e.g.* **1**–**4**). Otherwise, ecologically unfit plants with higher disease susceptibility may be produced. Further work is necessary to find additional intermediates between aldoxime **5** and brassinin **1** as well as the branch point between the tryptophan pathway to indole glucosinolates and to phytoalexins.

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11 All compounds gave satisfactory spectroscopic data; in each case the percentage of tetradeuterated synthetic compound was ⩾99%.
12 Experiments were carried out as reported in ref. 9; each incorporation experiment was repeated at least twice. All compounds administered to plant tissues were tetradeuterated at concentrations of 6×10^{-4}–10^{-3} M.
13 Indole glucosinolate **6**/**6a** was stable on standing in solution. The % of deuterium incorporation was determined by HRMS-EI using the expression: $[M + 4]^+/([M]^+ + [M + 4]^+) \times 100$ (HRMS data indicated that $[M + 4]^+$ is not present in natural abundance samples).
14 In previous work we attributed lower incorporation of deuterated **2a** into **3a** to factors such as the low solubility and high phytotoxicity of **2a**,[9] however these factors are not likely to account for the absence of incorporation of **6a** into any of the phytoalexins **2a**–**4a**; **6**/**6a** was soluble in aqueous solution and did not show phytotoxicity to leaves of *B. juncea*.
15 Spectroscopic data of **5a**: δ_H (300 MHz, CD_3OD): 7.08 (s, 1H, H-2), 6.80 (t, *J* 5.3, 1H, CH_2-CH=N-OH), 3.80 (d, *J* 5.4, 2H, CH_2-CH=N-OH). δ_C (75.5 MHz, CD_3OD): 152.2, 138.3, 128.7, 123.8, 122.2 (t, $^1J_{C\text{-}D}$ 24), 119.4 (t, $^1J_{C\text{-}D}$ 24), 118.9 (t, $^1J_{C\text{-}D}$ 24), 112.4 (t, $^1J_{C\text{-}D}$ 24 Hz), 111.2, 22.5. HRMS-EI *m/z*: calcd. for $C_{10}H_6D_4N_2O$ 178.1044, found 178.1040. Synthesis to be published elsewhere.
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