

The biosynthesis of crucifer phytoalexins: unprecedented incorporation of a 1-methoxyindolyl precursor

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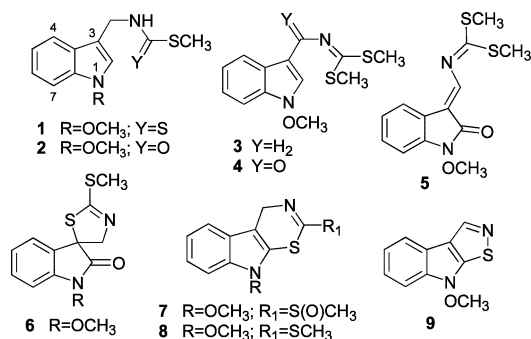
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The first biosynthetic studies revealing that 1-methoxy-3-indolylacetaldehyde oxime is an early precursor of 1-methoxyindole containing phytoalexins.

1-Methoxyindole containing alkaloids are an intriguing group of natural products produced by both plants and microorganisms. The large array of chemical structures incorporating the 1-methoxyindole motif suggests a broad range of biological activities. Indeed, pharmacologically active natural products such as the apicidins,¹ neoxaline,² and HUN-7293,³ as well as synthetic agonists of melatonin⁴ containing a 1-methoxyindolyl moiety have been identified. We have been interested in a group of indole alkaloids involved in the interaction of crucifer plants with their pathogenic fungi.⁵ Crucifers comprise a very large number of economically important oilseed and condiment crops such as rapeseed (*Brassica napus* and *B. rapa*) and mustards (*B. juncea*, *B. carinata*, *Sinapis alba*), and many vegetable species including turnip (*B. rapa*), broccoli (*B. oleracea* var. *botrytis*), cauliflower (*B. oleracea* var. *italica*), kale (*B. oleracea* var. *acephala*), radish (*Raphanus sativus*), and cabbages (*B. oleracea*). Besides their economic importance, crucifers are also interesting plant model-systems, containing the first example of a completely sequenced plant genome.⁶ Interestingly, a number of epidemiological studies suggest that cruciferous vegetables protect against cancer by modulating carcinogen metabolism.⁷ Related studies attributed this modulation to indole-containing metabolites⁸ such as 1-methoxyindole-3-carbinol, which appears to show higher efficiency in the induction of cytochrome P450 hepatic enzymes.⁹ Chemical characterization of secondary metabolites biosynthesized in crucifers has unraveled a remarkable array of 1-methoxyindole/oxindole containing phytoalexins, **1–9**,⁵ and a group of structurally related secondary metabolites known as glucosinolates, including 1-methoxyindoles **13**¹⁰ and **14**.¹¹ Phytoalexins are induced plant defenses particularly active against phytopathogenic fungi, whereas glucosinolates are constitutive metabolites playing a role in plant defense namely as attractants and deterrents against herbivorous insects.¹²

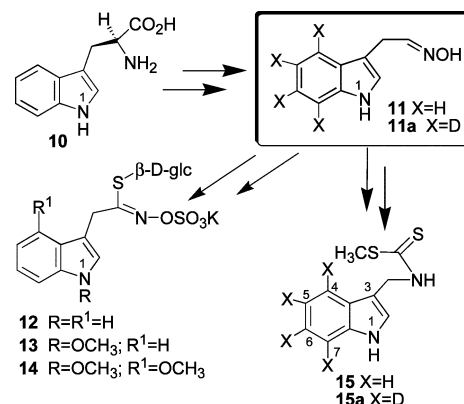


A number of biosynthetic studies has demonstrated that *S*-tryptophan is the precursor of both phytoalexins¹³ and indole glucosinolates¹² via the common intermediate indolyl-3-acetaldehyde oxime¹⁴ (**11**, Scheme 1). Oxime **11** appears to be the metabolic branch point between the tryptophan pathways to phytoalexins and to indole glucosinolates. However, in the case of 1-methoxyindole/oxindole containing phytoalexins **1–9**, tryptophan is the only known precursor.⁵ Despite the importance and

number of naturally occurring compounds containing this moiety, the first 1-methoxyindolyl intermediate(s) of such biosynthetic pathways remains unidentified to date. This knowledge is essential for an effective genetic manipulation of the biosynthetic pathways of phytoalexins and related metabolites. We are investigating the biosynthetic pathway of cruciferous phytoalexins and wish to report here results of the unprecedented incorporation of 1-[D₃]-methoxyindolyl-3-acetaldoxime (**18**), the first 1-methoxy intermediate identified, into the phytoalexin 1-[D₃]-methoxybrassinin (**1a**).

We have established previously¹⁴ that biosynthetic incorporation of intermediates/precursors into phytoalexins is more effective in turnip root tubers than in leaves or stems, likely due to faster intake of the solutions (*i.e.* no uptake of the substrate solution via the plant vascular system is necessary). Hence, to identify precursors of 1-methoxyindolyl phytoalexins **1–9**, first we searched for cruciferous tubers that produce these phytoalexins.^{5a} Root tubers of rutabaga, turnip, kohlrabi, and radish† were treated, elicited and extracted as previously described.¹⁵ HPLC analysis of the extracts of each plant using photodiode array detection and comparison of the UV spectra of the components with those in our phytoalexin library indicated that rutabaga tubers produced a larger amount of 1-methoxybrassinin (**1**) than any of the other plants. In our hands radish, kohlrabi and turnip tubers produced variable to undetectable amounts of 1-methoxybrassinin (**1**) and spirobrassinin (**6**, R = H), although kohlrabi was reported to produce also 1-methoxyspirobrassinin (**6**).¹⁶ Thus, we used rutabaga and kohlrabi to identify potential biosynthetic precursors of 1-methoxybrassinin (**1**), the likely precursor of most of the currently known 1-methoxy phytoalexins **1–9**.⁵

Next, [4,5,6,7-*D*₄]-brassinin (**15a**) and [4,5,6,7-*D*₄]-indolyl-3-acetaldoxime (**11a**) were synthesized as previously reported.¹⁵ Because 1-methoxyindolyl-3-acetaldoxime (**18a**, X = H) was unknown, a short synthesis from tryptamine (**16**) was developed (Scheme 2). First, oxidation of *N*-1 to the corresponding 1-hydroxytryptamine followed by methylation¹⁷ with (CD₃)₂SO₄ afforded 1-[D₃]-methoxytryptamine (**17**). The resulting amine **17** was further oxidized¹⁸ to yield the corresponding oxime **18**.‡ Solutions of each compound§ were separately added to UV-elicited tubers of kohlrabi and rutabaga, the tissues were incubated, extracted,

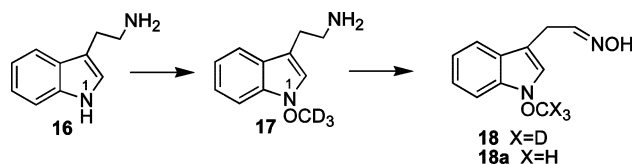


Scheme 1 Biosynthetic relationship of tryptophan (**10**), indolyl-3-acetaldoxime (**11**), glucobrassicin (**12**), and brassinin (**15**).

Table 1 Metabolism of [4,5,6,7-D₄]-brassinin (**15a**), [4,5,6,7-D₄]-indolyl-3-acetaldoxime (**11a**) and 1-[D₃]-methoxyindolyl-3-acetaldoxime (**18**), in rutabaga (*Brassica napus*) and kohlrabi (*B. oleracea*) tubers

Precursor	Plant, incubation time	Labeled products (total amount of D incorporation ^a)
[4,5,6,7-D ₄]-brassinin (15a)	rutabaga, 3 days	spirobrassinin (6 , R = H) (76%)
[4,5,6,7-D ₄]-brassinin (15a)	kohlrabi, 4 days	spirobrassinin (6 , R = H) (89%) 1-methoxybrassinin (1) (3%)
[4,5,6,7-D ₄]-indolyl-3-acetaldoxime (11a)	rutabaga, 3 days	spirobrassinin (6 , R = H) (7%)
[4,5,6,7-D ₄]-indolyl-3-acetaldoxime (11a)	kohlrabi, 4 days	spirobrassinin (6 , R = H) (10%)
1-[D ₃]-methoxyindolyl-3-acetaldoxime (18)	rutabaga, 3 days	1-methoxybrassinin (1) (5%)
1-[D ₃]-methoxyindolyl-3-acetaldoxime (18)	kohlrabi, 4 days	1-methoxybrassinin (1) (4%)

^a The % of D (deuterium) incorporation was established by HRMS-EI according to the following equation: % of D_n = [M + n]/([M]⁺ + [M + n]⁺) × 100 (n = 3 or 4).



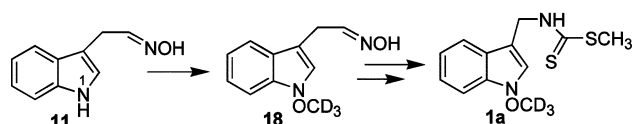
Scheme 2 Synthesis of 1-[D₃]-methoxyindolyl-3-acetaldoxime (**18**).^{17,18}

analyzed, and the extracts were fractionated, as described in a previous report,¹⁵ to yield phytoalexins. HRMS-EI analysis indicated the levels of deuterium incorporation shown in the table.

Most importantly, we established for the first time that both indolyl-3-acetaldoxime (**11**) and 1-methoxyindolyl-3-acetaldoxime (**18a**) are precursors of 1-methoxybrassinin (**1**) (Table 1). It is likely that aldoxime **11** is converted to the 1-methoxy aldoxime **18a** which is further transformed into 1-methoxybrassinin (**1**) (Scheme 3). Considering that 1-methoxybrassinin (**1**) is the likely biosynthetic precursor of phytoalexins **2–9**, by analogy to brassinin (**15**),⁵ and that crucifers produce both 1-methoxyindole and 1*H*-indole phytoalexins, it is likely that the enzyme(s) involved in the conversion of **11** to **18a** are widespread among crucifers. Furthermore, the incorporation level of [4,5,6,7-D₄]-brassinin (**15a**) into spirobrassinin (**6**, R = H) in both rutabaga and kohlrabi confirmed previous results obtained in turnip,¹³ that is brassinin is a precursor of spirobrassinin. As well, the lack of incorporation of [4,5,6,7-D₄]-brassinin (**15a**) into 1-methoxybrassinin (**1**) corroborates that this biosynthetic relationship does not exist, *i.e.* **15** is not a precursor of **1**.⁵

In addition, our findings have implications for the pathway to indole glucosinolates **12–14**. We surmise that 1-methoxy aldoxime **18a** may also be an intermediate in the biosynthesis of 1-methoxyindole glucosinolate **13**. Since glucosinolate **12** is biosynthesized from **11**,¹² **13** is likely derived from **18a**, by analogy with brassinin (**15**) and 1-methoxybrassinin (**1**) (Schemes 1 and 3). Nonetheless, this hypothesis remains to be demonstrated, as we did not isolate or detect metabolite **13**. However, given that the production of 1-methoxyindole glucosinolate **13** in *Arabidopsis thaliana* has been reported recently to increase substantially in the presence of jasmonates,¹⁹ corroboration of our hypothesis in *A. thaliana* should be possible. Although it was proposed that indole glucosinolates **13** and **14** result from enzymatic methoxylation of **12**,¹⁹ it now appears likely that these enzymes could use as substrate the indole acetaldoxime **11** and not the glucosinolate **12**.

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Scheme 3 Biosynthetic relationship of indolyl-3-acetaldoxime (**11**), 1-[D₃]-methoxyindolyl-3-acetaldoxime (**18**), and 1-[D₃]-methoxybrassinin (**1a**).

spectroscopic determinations, and A. Loukaci and Y. Xu in the synthesis of **15a** and **11a**, respectively.

Notes and references

† Purchased from local markets.

‡ Selected data for 1-[D₃]-methoxyindolyl-3-acetaldoxime (**18**): *R*_f 0.3 (CH₂Cl₂-CH₃OH, 95 : 5 v/v). UV (CH₂Cl₂) λ_{max} (log ε) 229 (4.4) and 291 (3.7). FTIR ν_{max} 3255, 2921, 1450, 1319 and 1091 cm⁻¹. HRMS-EI *m/z* (% relative abundance): measured: 207.1089 [M⁺] (100) (207.1087 calcd. for C₁₁H₉D₃N₂O₂), 163.0955 (36), 155.0608 (24), 129.0574 (48). ¹H NMR (500 MHz, CD₂Cl₂) δ 3.65 (d, *J* = 6 Hz, 0.5 H), 3.84 (d, *J* = 6 Hz, 1.5 H), 6.94 (t, *J* = 6 Hz, 1 H), 7.14 (t, *J* = 7.5 Hz, 1 H), 7.21 (s, 1 H), 7.28 (t, *J* = 7.5 Hz, 1 H), 7.46 (d, *J* = 7.5 Hz, 1 H), 7.60 (d, *J* = 7.5 Hz, 1 H), 8.03 (br s, D₂O exchangeable). ¹³C NMR (125.8 MHz, CD₂Cl₂) δ 21.7, 26.2, 107.3, 108.8, 119.5, 119.6, 120.3, 122.0, 123.2, 124.3, 133.2, 150.9, 151.5.

§ All compounds gave satisfactory spectroscopic data; in each case the percentage of deuterated synthetic compound was ≥ 99%.

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