En route to erucalexin: a unique rearrangement in the crucifer phytoalexin biosynthetic pathway

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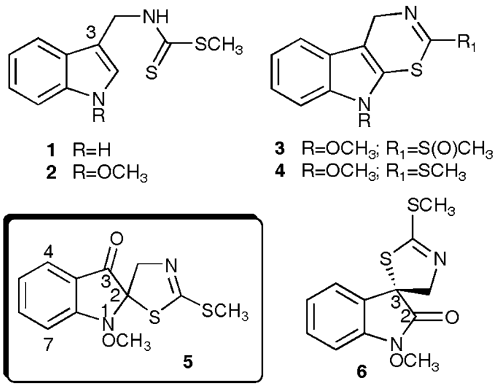
The first biosynthetic studies revealing that both 1-methoxybrassinin and sinalbin B are close precursors of the phytoalexins erucalexin and 1-methoxyspirobrassinin.

In contrast with more than 30 crucifer phytoalexins known to date (*e.g.* **1–4**),[1] erucalexin (**5**) contains a unique indoxyl system substituted at C-2 rather than at C-3.[2] Sequential oxidation and 1,2-carbon–carbon bond migration from C-3 to C-2 of the indolyl moiety were proposed to rationalize the biosynthesis of this new skeleton.[2] Both (+)-erucalexin (**5**) and its regioisomer (+)-1-methoxyspirobrassinin (**6**) are produced by the wild crucifer dog mustard (*Erucastrum gallicum*), a source of genetic resistance to stem rot disease.[2] The occurrence of both metabolites in the same plant poses an exceptional opportunity to establish yet another step in the biosynthetic puzzle created by crucifer phytoalexins.



We are interested in phytoalexins as crucial components of the interaction of crucifer plants with their pathogenic fungi.[1,2] Crucifers comprise a vast group of economically important crops, which include rapeseed (*Brassica napus* and *B. rapa*), mustards (*B. juncea, B. carinata, Sinapis alba*), and vegetables (*e.g.* turnip, *B. rapa*, broccoli, *B. oleracea*, cauliflower, *B. oleracea*, radish, *Raphanus sativus*). Recent epidemiological studies suggest that crucifer vegetables can protect against cancer by modulating carcinogen metabolism, and that some of the crucifer phytoalexins appear to contribute to this immuno-protective role.[3] Phytoalexins are plant chemical defenses produced *de novo* in response to stress, which are particularly active against phytopathogenic microbial systems.[4] In order to generate crucifer plants with improved disease resistance traits, an understanding of the biosynthetic pathways of phytoalexins is essential.

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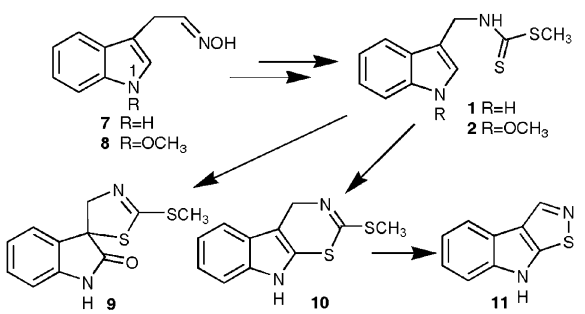
It was established previously that indolyl-3-acetaldehyde oxime[5] (**7**) and 1-methoxyindolyl-3-acetaldehyde oxime (**8**)[6] are precursors of crucifer phytoalexins. Brassinin (**1**) and 1-methoxybrassinin (**2**) are the first members of the biosynthetic sequence that generates a great variety of structures. For example, brassinin (**1**) is a precursor of both cyclobrassinin (**10**) and spirobrassinin (**9**),[7] and cyclobrassinin (**10**) is a precursor of brassilexin (**11**)[8] (Scheme 1) but not of spirobrassinin (**9**).[7] Nonetheless, the precursor role of 1-methoxybrassinin (**2**) in the biosynthetic sequence of 1-methoxy-substituted phytoalexins remains to be demonstrated.[6] Toward this end, we have investigated the biosynthesis of erucalexin (**5**) and 1-methoxyspirobrassinin (**6**) in dog mustard using perdeuterated precursors. Here we wish to report that, albeit unexpectedly, both **5** and **6** are biosynthetically derived from the phytoalexins 1-methoxybrassinin (**2**) and sinalbin B (**12**).

Although the metabolic incorporation of labeled precursors into phytoalexins is more effective in tubers than in leaves or stems,[6] dog mustard does not form tubers. In addition, tuberous plants (rutabaga, radish, kohlrabi, turnip) did not produce detectable amounts of **5** or **6**. Thus, to obtain reasonable precursor incorporation, various experimental conditions using dog mustard were investigated. Eventually, it was determined that double-elicitation of leaves was crucial to maximize leaf production of phytoalexins (Table 1). HPLC analyses of extracts of doubly-elicited leaves indicated that production of erucalexin (**5**) for at least five consecutive days was substantially higher than production of 1-methoxyspirobrassinin (**6**), and that maximum production was detected after 48 h incubation (Table 1).

Perdeuterated 1-methoxybrassinins **2a** and **2b** were synthesized as reported previously.[9]† Perdeuterated sinalbins B **12a** and **12b** were prepared from the respective perdeuterated 1-methoxybrassinins,[9] using *N*-bromosuccinimide, as reported for sinalbin B.[10]†



Scheme 1 Biosynthetic relationship of indolyl-3-acetaldoximes **7** and **8**, brassinin (**1**), 1-methoxybrassinin (**2**), spirobrassinin (**9**), cyclobrassinin (**10**) and brassilexin (**11**).

Table 1 Production of erucalexin (**5**) and 1-methoxyspirobrassinin (**6**) in doubly-elicited leaves[a] of dog mustard (*Erucastrum gallicum*)

Phytoalexin	Incubation time	Total amount of phytoalexin[b] (μmol/100 g of fresh tissue)
Erucalexin (**5**)	24 h	5.0–6.7
	48 h[c]	**13.2–16.6**[c]
	72 h	3.9–7.7
	96 h	5.3–5.5
	120 h	1.8–3.5
1-Methoxyspirobrassinin (**6**)	24 h	3.7–3.8
	48 h[c]	**3.1–3.8**[c]
	72 h	0.7–1.5
	96 h	1.4–2.7
	120 h	0.67–0.68

[a] 1) 4-Week-old plants sprayed with $CuCl_2$ and incubated for 24 h in a growth cabinet, 2) leaves excised at the base of petiole, placed in vials containing distilled water, sprayed again with $CuCl_2$ and incubated under constant light. [b] Amounts present in extracts were determined by HPLC using calibration curves prepared from pure compounds **5** and **6**. [c] Time chosen for incorporation experiments with deuterated compounds.

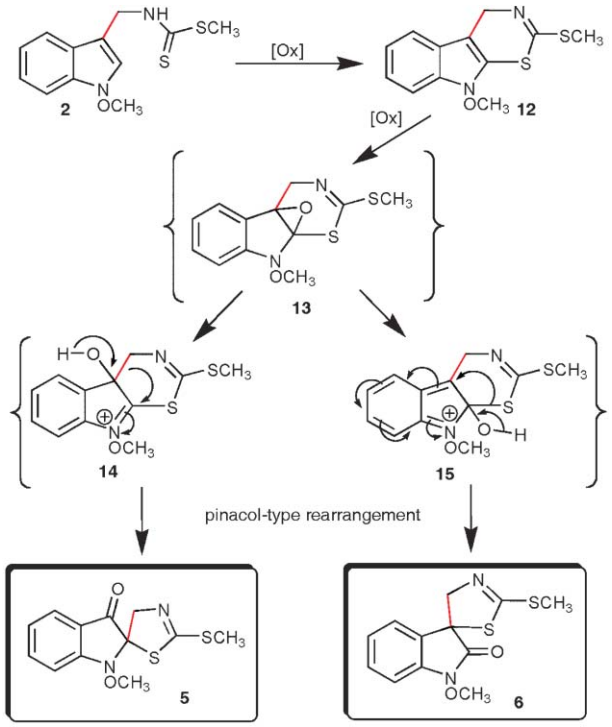
Solutions of each compound (5×10^{-4} M) dissolved in H_2O–MeOH–Tween 80 (95/5/0.05, v/v) were taken up by doubly-elicited leaves of dog mustard (conditions summarized in Table 1), the leaves were incubated under constant light for 48 h and were extracted. Control experiments were carried out using: (1) doubly-elicited leaves incubated with 1-methoxybrassinin (**2**) and sinalbin B (**12**),‡ (2) doubly-elicited leaves incubated with carrier solution only,‡ and (3) non-elicited leaves incubated with carrier solution only.‡ After the leaves were extracted, the extracts were subjected to a preliminary clean up and the fractions containing phytoalexins were analyzed by HPLC-DAD and HPLC–MS-ESI. HRMS-ESI analyses were used to determine the levels of deuterium incorporation into erucalexin (**5**) and 1-methoxyspirobrassinin (**6**), as shown in Table 2.

The substantially higher percentages of incorporation (Table 2) of perdeuterated compounds **2a**, **2b**, **12a** and **12b** into 1-methoxyspirobrassinin (**6**) than into erucalexin (**5**), reflect the lower amount of natural 1-methoxyspirobrassinin (**6**) produced (Table 1) and the rates of formation of **5** and **6**. The lower percentage of incorporation of sinalbins B (**12a** and **12b**) relative to 1-methoxybrassinins (**2a** and **2b**) is consistent with the lower solubility and stability of **12**.[10] Furthermore, the incorporation of perdeuterated

Table 2 Metabolism of 1-methoxybrassinin (**2**) and sinalbin B (**12**) in elicited leaves of dog mustard (*Erucastrum gallicum*)

Deuterated precursor	Labeled products (% amount of D incorporation[a])
[4,5,6,7,SCD_3-D_7]-1-Methoxybrassinin (**2a**)	Erucalexin (**5**) (4%) 1-Methoxyspirobrassinin (**6**) (65%)
[SCD_3]-1-Methoxybrassinin (**2b**)	Erucalexin (**5**) (6%) 1-Methoxyspirobrassinin (**6**) (55%)
[4,5,6,7-D_4]-Sinalbin B (**12a**)	Erucalexin (**5**) (5%) 1-Methoxyspirobrassinin (**6**) (19%)
[SCD_3]-Sinalbin B (**12b**)	Erucalexin (**5**) (2%) 1-Methoxyspirobrassinin (**6**) (10%)

[a] The % of D (deuterium) incorporation was established by HRMS-ESI according to the following equation: % of $D_n = [M+n]^+/([M]^+ + [M+n]^+) \times 100$ (n = 3, 4, 7).



Scheme 2 Proposed biosynthesis of erucalexin (**5**) and 1-methoxyspirobrassinin (**6**).

1-methoxybrassinins **2a** and **2b** into 1-methoxyspirobrassinin (**6**) confirmed, as expected, that **2** is a precursor of **6**, similar to the pathway of NH-containing phytoalexins (Scheme 1). On the other hand, it was rather surprising to find incorporation of perdeuterated sinalbins B **12a** and **12b** into 1-methoxyspirobrassinin (**6**). Due to the likely similarity between the biosynthetic pathways of 1-methoxy-substituted and NH-containing phytoalexins, both erucalexin (**5**) and 1-methoxyspirobrassinin (**6**) were proposed to derive from 1-methoxybrassinin (**2**).[2] Therefore, it appears that either these two parallel pathways, *i.e.* NH and 1-methoxy phytoalexins, differ in this step, or cyclobrassinin (**10**) is a precursor of spirobrassinin (**9**) as well, a conclusion inconsistent with previous findings.[7] However, before definite conclusions can be drawn, additional experiments using other plant tissues that make both NH and 1-methoxy phytoalexins need to be carried out.

In conclusion, based on these results (Table 2), the proposed sequence of biosynthetic steps *en route* to erucalexin (**5**) and 1-methoxyspirobrassinin (**6**) includes two oxidative steps followed by a pinacol-type rearrangement (Scheme 2). First, oxidation of 1-methoxybrassinin (**2**) to sinalbin B (**12**), followed by oxidation of **12** to epoxide **13** and subsequent rearrangement of **14**, a C-3 to C-2 bond migration to yield **5**, or **15**, a C-2 to C-3 bond migration to yield **6**, integrates and rationalizes our findings (Scheme 2). Although the C-3 to C-2 pinacol-type rearrangement is unprecedented in the crucifer phytoalexins, other indole alkaloids, such as the brevianamides are known to result from similar rearrangement.[11]

Finally, considering that erucalexin (**5**) is strongly active against economically significant plant pathogens,[2] and that *Sinapis alba* produces sinalbin B (**12**),[10] our results suggest

that engineering the erucalexin pathway into *S. alba* or related species is a reasonable proposition to increase the plant's disease resistance traits.

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Notes and references

† All compounds gave satisfactory spectroscopic data; in each case the percentage of deuterated synthetic compound was $\geqslant 99\%$.
‡ HRMS-ESI showed that the molecular ions $[M+3]^+$, $[M+4]^+$ or $[M+7]^+$ were not present in any of the controls.

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