Biosynthesis of Cruciferous Phytoalexins: the Involvement of a Molecular Rearrangement in the Biosynthesis of Brassinin[1]

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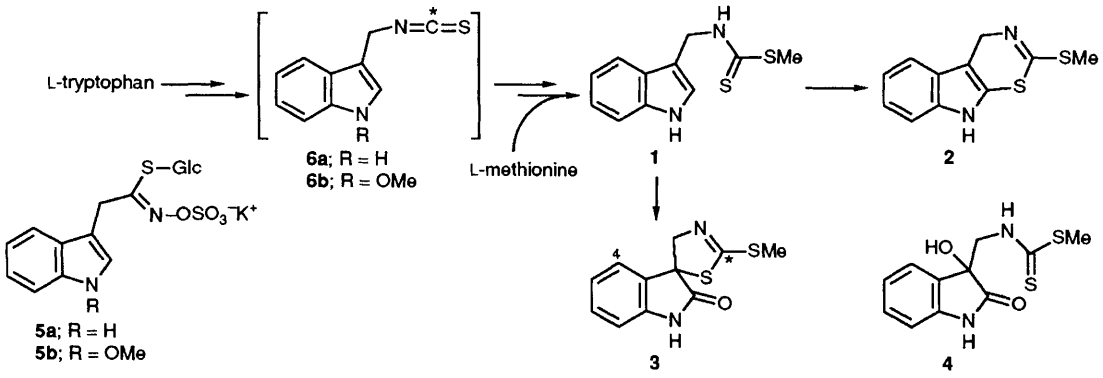
Incorporation of [4′-^{2}H], [2-^{13}C]tryptophan, and [*methyl*-$^2H_3$]brassinin **1** into spirobrassinin **3** by UV-irradiated turnip tissue indicates that spirobrassinin **3** is formed *via* brassinin **1**, which is biosynthesized from tryptophan by a pathway involving a molecular rearrangement and possible formation of indol-3-ylmethyl isothiocyanate **6a**.

Recently, we reported the first cruciferous phytoalexins,[2] antimicrobial compounds synthesized *de novo* by plants after their exposure to microorganisms,[3] from Chinese cabbage, radish and cabbage. These phytoalexins, represented by brassinin **1**, cyclobrassinin **2**, and spirobrassinin **3**, are characterized by the presence of an indolic nucleus with the appendage containing one[4] or two sulphur atoms. Time-course studies of phytoalexins and glucosinolates in UV-irradiated turnip root tissue (*Brassica campestris* L. ssp. *rapa*) indicated that both the levels of phytoalexins and indole glucosinolates increased in the UV-irradiated tissue whereas only the latter did in non-irradiated control tissue.[5] Glucosinolates, which are abundant among crucifers, give their respective isothiocyanates[6] on enzymic hydrolysis by the co-existing enzyme myrosinase, followed by Lossen-type rearrangement. Brassinin **1** shows a striking structural similarity to indol-3-ylmethyl isothiocyanate **6a**, the supposed enzymic hydrolysis product of an indole glucosinolate, glucobrassicin **5a**.[6] These facts suggest the possibility that some biosynthetic relationships may exist between the indolic phytoalexins and indole glucosinolates, and that induction of additional enzymes would be required for the phytoalexin formation. We describe here our biosynthetic studies of these cruciferous phytoalexins using UV-irradiated turnip root tissue.†

When L-[4′-^{2}H]tryptophan[7] was fed to the turnip tissue and incubated for 37 h, spirobrassinin **3** was isolated as the main metabolite. The ^{2}H NMR spectrum of the metabolite revealed the incorporation of the ^{2}H label into the oxindole nucleus of **3**. Decrease of 4-H signal intensity in the ^{1}H NMR spectrum of

† Turnip roots were cut horizontally and hemispherical holes (2 cm in diameter) were made in each surface. After 12 h of incubation at 25 °C, the tissues were irradiated with a 15 W germicidal lamp (Matsushita) for 10 min and incubated for additional 6–12 h. Each hole was then filled with 0.1% Tween 80 aqueous solution (or milky suspension) containing a sample at a concentration of 1–3 mmol dm^{-3}. The tissues were then incubated for the indicated periods, which were determined according to HPLC analysis[5] of the aliquots. The ethyl acetate extracts from the above aqueous phase were separated by silica gel column chromatography followed by preparative TLC on silica gel to give labelled metabolite(s).



Scheme 1 Biosynthetic pathway of cruciferous phytoalexins

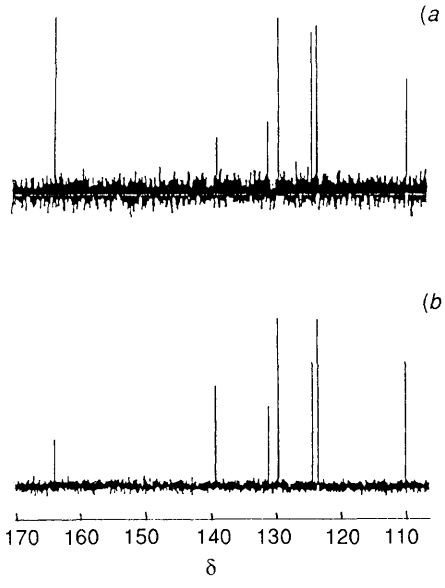


Fig. 1 Proton noise decoupled 67.5 MHz ^{13}C NMR spectra of spirobrassinin: (*a*) after incorporation of [2-^{13}C]tryptophan; (*b*) natural abundance

the metabolite showed a deuterium incorporation of 18% into **3**. A feeding experiment with L-[*methyl*-$^{2}H_3$]methionine indicated that all the methyl groups of brassinin **1**, cyclobrassinin **2** and spirobrassinin **3** arise from L-methionine.‡ Administration of [*methyl*-$^{2}H_3$]brassinin§ to the turnip tissue, followed by 27 h of incubation, led to effective incorporation of the ^{2}H label into cyclobrassinin **2** (*methyl*-$^{2}H_3$, 75%) and spirobrassinin **3** (*methyl*-$^{2}H_3$, 81%).¶ These results indicate that the biological origin of these phytoalexins is L-tryptophan and that brassinin **1** is an advanced precursor of cyclobrassinin **2** and spirobrassinin **3**. However, neither cyclobrassinin **2** nor dioxibrassinin **4**,[1] a plausible precursor to **3**, was incorporated into spirobrassinin **3**.¶

The crucial point in the biosynthetic pathway to brassinin **1** is whether the thiocarbonyl carbon of **1**, therefore, the relevant imino carbon in spirobrassinin, **3** originates from the C-2 carbon of tryptophan. If that is the case, then the biosynthetic pathway to brassinin **1** should involve a molecular rearrangement step since the thiocarbonyl carbon of **1** is separated from the methylene carbon by a nitrogen atom. Feeding the experiment with DL-[2-^{13}C]tryptophan resulted in a fourfold enhancement of the imino carbon NMR signal at δ 164 (Fig. 1) of spirobrassinin **3** and indicated the involvement of a molecular rearrangement in the pathway from tryptophan to brassinin **1**. This result is suggestive of the isothiocyanate **6a** as a key intermediate to **1**. Unstable **6a** has not been isolated although less labile isothiocyanate **6b** was detected recently,[8] using mass spectrometry, from enzymic hydrolysis products of neoglucobrassicin **5b**. To examine the possible role of isothiocyanates in the biosynthesis of cruciferous phytoalexins, benzyl isothiocyanate was chosen as a model substrate and administered to the turnip tissue. A new metabolite was isolated and identified as $PhCH_2NH$–CS–SMe **7** by direct comparison with a synthetic specimen. Formation of **7** in the turnip tissue suggested strongly that indol-3-ylmethyl isothiocyanate **6a** would be involved in the biosynthesis of brassinin **1**. It remains to be proved whether the isothiocyanate **6a** could be formed *via* hydrolysis of the indole glucosinolate **5a** and/or directly from tryptophan *e.g. via* thiohydroximic acid. Methylthiolation[9] of indolic isothiocyanates would lead to brassinin-type phytoalexins. The biosynthetic pathway is shown in Scheme 1.

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‡ Incubated for 30 h after the administration of the substrate. The incorporation of ^{2}H label in **1**, **2** and **3** was 35, 34 and 21%, respectively, based on signal intensity of each ^{1}H NMR spectrum.

§ The required ^{2}H-labelled compounds of **1**, **2** and **4** were prepared according to the procedures of refs. 2 and 1, respectively.

¶ Estimated by ^{1}H NMR signal intensities of the respective methyl groups.

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