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# NO INVOLVEMENT OF METHOXYBRASSININ IN THE BIOSYNTHESIS OF CYCLOBRASSININ*

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**Key Word Index**—*Raphanus sativus*; Cruciferae; Japanese radish; phytoalexin; biosynthesis; brassinin; methoxybrassinin; cyclobrassinin.

**Abstract**—Feeding experiments with *Pseudomonas cichorii*-inoculated Japanese radish roots indicated that methoxybrassinin is not derived from brassinin, and that cyclobrassinin is not biosynthesized from methoxybrassinin.

## INTRODUCTION

Accumulation of phytoalexins at infection sites is considered as one of the important defence mechanisms of plants against microorganisms [2]. Members of the family Cruciferae include many economically important crops. The first three cruciferous phytoalexins, brassinin (**1**), methoxybrassinin (**2**), and cyclobrassinin (**3**) were isolated from Chinese cabbage (*Brassica campestris* subsp. *pekinensis*) inoculated with the bacterium *Pseudomonas cichorii* [3]. These compounds have structurally unique features that include an indole ring with two sulphur atoms. Additional phytoalexins [4] of the Cruciferae have been isolated from the Chinese cabbage, Japanese radish (*Raphanus sativus* var. *hortensis*), cabbage *B. oleracea* var. *capitata*), turnip (*B. campestris* subsp. *rapa*), Indian mustard (*B. juncea*), black mustard (*B. nigra*), false flax (*Camelina sativa*), and *Arabidopsis thaliana*.

A biosynthetic relationship among brassinin (**1**), cyclobrassinin (**3**), and spirobrassinin (**4**) has recently been elucidated by using UV irradiated sliced turnip roots [4, 5]. Namely, **3** and **4** are derived independently from **1**, which, in turn, is biosynthesized from tryptophan, methionine, and cysteine. The biosynthesis of methoxybrassinin (**2**) has not been reported to date although it is an important phytoalexin of Chinese cabbage [3], Japanese radish [6], and oilseed rape (*B. napus*) [7]. The co-occurrence of **2** with **1** and **3** in *P. cichorii*-inoculated Chinese cabbage [3] and Japanese radish [6] suggests the possibility that *N*-hydroxylation of **1** followed by biological methylation gives **2**, which leads to **3** through eliminative cyclization. Somei emphasizes the importance of the *N*-hydroxyl or *N*-methoxyl group as a leaving group in the biogenesis of substituted natural indoles [8]. A recent time course study on UV-irradiated sliced turnip root has not clarified the relationship among **1**–**3** [9]. Dahiya and Rimmer have also examined the time course on phytoalexin formation in *B. juncea* callus tissue [10] and reported that the formation of **2** preceded that of **3** by 4 days. However, they did not detect the formation of **1**. In this paper, we represent evidence for the non-participation of methoxybrassinin (**2**) in the biosynthesis of cyclobrassinin (**3**) from brassinin (**1**).

## RESULTS AND DISCUSSION

Methoxybrassinin (**2**) was shown to be a minor phytoalexin of turnip when it was elicited by UV irradiation [9] or by inoculation with *Pseudomonas cichorii*. Therefore, Japanese radish roots were used in this study. UV irradiation was not adequate as an elicitor since it induced the formation of cyclobrassinin (**3**) but not **2**. Compounds **2** and **3** were induced effectively when the tissue was inoculated with *P. cichorii*. Because **2** was also induced by an autoclaved culture of the bacterium, oxidation at the indolic nitrogen atom is caused by the plant tissue.

Feeding experiments were carried out by using *S*-methyl deuterated brassinin (**1′**) [4] and methoxybrassinin (**2′**). Japanese radish roots were cut transversely and hemispherical holes were made on each cut surface. After being aged for 20 hr, each hole was inoculated with *P. cichorii* and incubated for an additional 7 hr. To the holes was added **1′** ( > 99% $d_3$ from FD-MS) as a milky aqueous suspension. Since prolonged incubation may result in the decrease of **2′** by further metabolism, the aqueous phase and the tissue around the holes were harvested after 7 hr of feeding, although most of **1′** remained unchanged. Cyclobrassinin and methoxybrassinin were

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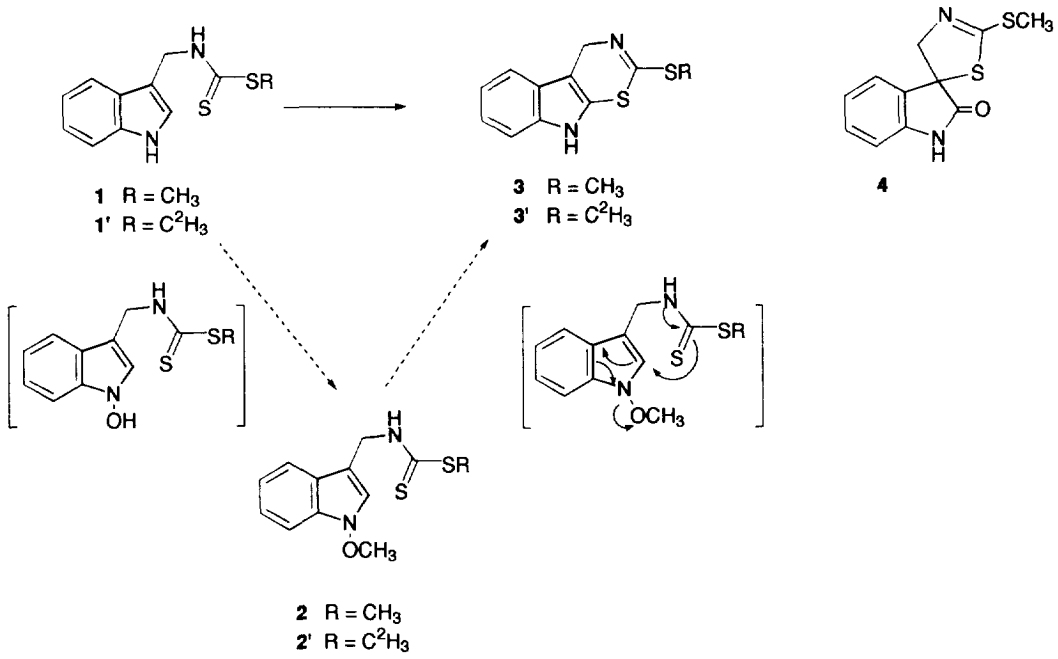


isolated from the aqueous phase and the tissue. FD-mass spectral measurement of the cyclobrassinin showed two molecular ion peaks at *m/z* 234 (rel. int, 59%) and 237 (100%, trideuterated). This result indicates that **3** is biosynthesized from **1** as reported in the case of turnip roots [4, 5]. If **3** is biosynthesized through **2**, the latter should contain the deuterium label. However, the isolated methoxybrassinin showed a single molecular ion peak at *m/z* 266 on FD-mass spectrometry, indicating the lack of incorporation of **1** into **3** through **2**. A feeding experiment with **2'** supported this conclusion. The $^1$H NMR spectrum of the isolated cyclobrassinin showed the relative signal intensity of the methylthio and the methylene groups in the precise ratio of 3 to 2. Furthermore, the FD-mass spectrum of the isolated cyclobrassinin showed the absence of a molecular ion peak due to **3'**. All these results indicate that cyclobrassinin (**3**) is not biosynthesized from methoxybrassinin(**2**), and that **2** is not derived from brassinin (**1**).

## EXPERIMENTAL

*Synthesis of* [S-*Me*-$^2H_3$] *methoxybrassinin* (**2'**). To a mixture of 3-(aminomethyl)-1-methoxyindole (272 mg) [11], pyridine (3 ml), and triethylamine (215 $\mu$l) was added $CS_2$ (93 $\mu$l) at 0°. The mixture was kept at 0° for 2 hr and at room temp for 2 hr, treated with [Me-$^2H_3$]MeI (96 $\mu$l, Isotec, 99.5% $^2H_3$), kept at room temp for 16 hr, and then poured into 1.5 M $H_2SO_4$. Ether extracts from the aq. soln gave **2'** (309 mg) after CC on silica gel ($CH_2Cl_2$–hexane, 1: 1). FD-MS *m/z* (rel. int): 269 (100%, $[M]^+$) and no $[M]^+$ at 266.

*Feeding experiment with* [*Me*-$^2H_3$] *brassinin.* Nine Japanese radish roots *Raphanus sativus* var. *hortensis* cv Aokubitaibyo, total fr. wt 6 kg, were surface sterilized with 1% NaOCl aq. soln, cut transversely (3 cm thick), and 7 hemispherical holes (1.5 cm in diameter) were made on each surface. After 20 hr of aging at 20°, the holes were inoculated with a suspension of *Pseudomonas cichorii.* After 7 hr of inoculation, each hole was filled with a milky suspension of 3 mM [Me-$^2H_3$] brassinin in 0.1% Tween 80 aq. soln (total of 700 ml), and the tissue was incubated at the temp for 7 hr. The EtOAc extracts (646 mg) from the aq. phase gave cyclobrassinin (6.5 mg) after CC separation on silica gel (1, 5% hexane in $CH_2Cl_2$; 2, $CH_2Cl_2$) and on $\mu$-Bondapak $C_{18}$($H_2O$–MeOH–MeCN). The EtOAc extracts (524 mg) from the tissue around the holes gave methoxybrassinin (0.2 mg) and cyclobrassinin (0.2 mg) after similar treatments. Identification of each phytoalexin was carried out by direct HPLC-UV comparison with authentic samples. FD-MS *m/z* (rel. int): cyclobrassinin, 237 (100%,$[M]^+$) and 234 (59%,$[M]^+$); methoxybrassini, 266 (100%,$[M]^+$).

*Feeding experiment with* [S-*Me*-$^2H_3$] *methoxybrassinin* (**2'**). Three Japanese radish roots were processed as described above. After being aged at 20° for 12 hr, the holes were inoculated with *P. cichorii* and incubated for an additional 9 hr. To each hole was added **2'** as an aq. 3 mM suspension in 0.1% Tween 80 (total of 263 ml). After 4 days, the aq. phase and the tissue around the holes were harvested, and extracted with EtOAc. Repeated CC of the EtOAc extracts (680 mg) on silica gel (1, hexane–$Et_2O$; 2, $C_6H_6$–hexane) gave cyclobrassinin (2.9 mg). FD-MS *m/z* (rel.int): 234 (100%, $[M]^+$).

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