Biosynthesis of Cruciferous Phytoalexins[†]

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Abstract: We report the results of biosynthetic studies on the sulfur-containing indole phytoalexins, antimicrobial compounds produced by plants after exposure to microorganisms. Feeding experiments with UV-irradiated sliced turnip root (Brassica campestris ssp. rapa) revealed that spirobrassinin (3) is formed via brassinin (1a) and that 1a is biosynthesized by the intramolecular rearrangement of L-tryptophan to form unstable indol-3-ylmethyl isothiocyanate (10a). Trapping experiments of 10a with NaSCH₃ and formation of PhCH₂NH-CS-SCH₃ (14) on administration of benzyl isothiocyanate supported the presence of the labile isothiocyanate intermediate. Feeding experiments of a mixture of L-[methyl-3H₃]methionine and L-[³⁵S]methionine revealed that the methylthio group of methionine is incorporated into 1a as an unusual intact unit. Incorporation of L-[35S]cysteine into 1a and 3 indicated the origin of another sulfur atom. These studies suggest that the biosynthetic pathway of cruciferous phytoalexins is at least partially linked to one of the indole glucosinolates which are typical components of crucifers. Structures of metabolites of 2-methylbrassinin (16) imply the presence of an oxidized intermediate between 1a and other cruciferous phytoalexins.

Introduction

Plants produce antimicrobial compounds called phytoalexins when they are exposed to microorganisms. Accumulation of phytoalexins around infection sites is considered as one of the important antimicrobial defense mechanisms of the plant. To date, more than 200 phytoalexins have been isolated from more than 20 plant families.¹ However, until our report² no phytoalexins had been obtained from the plant family Cruciferae even though members of this family include many economically important vegetables. The first three cruciferous phytoalexins isolated from Chinese cabbage (Brassica campestris L. ssp. pekinensis) inoculated with the bacterium Pseudomonas cichorii are named brassinin (1a), methoxybrassinin (1b), and cyclobrassinin (2) and have structurally unique features that include an indole ring and two sulfur atoms. Additional cruciferous phytoalexins have been isolated from Japanese radish (Raphanus sativus, spirobrassinin (3)),³ cabbage (B. oleracea L., 4-methoxybrassinin (1c), methoxybrassenin A (6a) and B (6b), brassicanal C (7b)),⁴ turnip (B. rapa or B. campestris),⁵ rape (B. napus L. var. oleifera L.),⁶ Indian mustard (B. juncea, brassilexin (5)),⁷ false flax (Camelina sativa, camalexin (8a), methoxycamalexin (8b)),⁸ arabidopsis (Arabidopsis thaliana L. Heynhold),9 and Chinese cabbage

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Figure 1. Structures of representative cruciferous phytoalexins: 1a, brassinin. 1b, methoxybrassinin; 1c, 4-methoxybrassinin; 1d, brassitin; 1e, methoxybrassitin; 2, cyclobrassinin; 3, spirobrassinin; 4, dioxibrassinin; 5, brassilexin; 6a, methoxybrassenin A; 6b, methoxybrassenin B; 7a, brassicanal A; 7b, brassicanal C; 8a, camalexin; 8b, methoxycamalexin.

(brassitin (1d), methoxybrassitin (1e), brassicanal A (7a)),¹⁰ as shown in Figure 1. All these phytoalexins are structurally related to indole or indole derivatives possessing one or two sulfur atoms.

Cruciferous plants contain thioglucosides called glucosinolates, which undergo enzymatic hydrolysis on crushing of the plant tissues to give isothiocyanates, nitriles, alcohols, and so on.¹¹ Mustard oil, which is a mixture of these decomposition products, has antimicrobial and anti-insect properties. The glucosinolates

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Scheme 1. Structures and Enzymatic Hydrolysis Products of Indole Glucosinolates



are therefore regarded as important defense compounds in crucifers against microorganisms and insects.¹²

Enzymatic hydrolysis converts the indole glucosinolates, neoglucobrassicin and glucobrassicin, to (1-methoxyindol-3-yl)methyl isothiocyanate (10b) and indol-3-ylmethyl isothiocyanate (10a), respectively (Scheme 1). Although the latter isothiocyanate, 10a, is too unstable to isolate,¹³ the addition of methanethiol, a known breakdown product in cruciferous plants, to isothiocyanates could formally yield 1b and 1a. However, these phytoalexins do not appear to be hydrolysis products of indole glucosinolates, because none of these compounds was detected in the extract of enzymatic hydrolysis products.^{2c} Therefore, a different biosynthetic pathway might be involved in the synthesis of these cruciferous phytoalexins. Structural similarities between the two types of compounds suggest that they may share some biosynthetic steps.

In a previous work based on feeding experiments of deuteriumand ¹³C-labeled compounds,¹⁴ we found that brassinin is biosynthesized from L-tryptophan by a Lossen-type molecular rearrangement and that it is a precursor of both cyclobrassinin and spirobrassinin. Moreover, time course studies^{5a} of phytoalexins and glucosinolates in UV-irradiated turnip root tissue (B. campestris L. ssp. rapa) indicated that the levels of both phytoalexins and indole glucosinolates increased in the UVirradiated tissue, whereas only the latter increased in nonirradiated control tissue. These facts suggest the possibility that a biosynthetic relationship may exist between the indolic phytoalexins and indole glucosinolates and that induction of additional enzymes would be required for phytoalexin formation.

In the present paper, we describe the results of detailed biosynthetic studies on cruciferous phytoalexins using UVirradiated turnip root tissue. Evidence for the isothiocyanate intermediate and the intact addition of the methylthio group of L-methionine are described. The results indicated a link between biosynthesis of indole phytoalexins and glucosinolates. Furthermore, a plausible diol or diol related intermediate linking la and



Figure 2. Feeding experiment methodology: (a) cutting turnips horizontally, (b) making hemispherical holes, (c) irradiation with ultraviolet for 10 min, (d) adding labeled compounds as a 0.1% Tween 80 aqueous solution, (e) HPLC analysis and harvesting.

other indolic phytoalexins is proposed from the results of a 2-methylbrassinin (16) feeding experiment. Structures of two minor phytoalexins isolated from Japanese radish also support the intermediate.

Results

Biosynthetic Correlation of Major Phytoalexins. In view of their structures, a plausible biogenesis of four representative phytoalexins is proposed. Brassinin (1a) is oxidatively cyclized to cyclobrassinin (2), and the latter is oxidized to dioxibrassinin (4), which has been isolated as a minor phytoalexin of P. cichoriiinoculated cabbage.^{4b} Finally, compound 4 cyclizes to give spirobrassinin (3). Chemical transformations of 1a to 2^{2b} and of 4 with thionyl chloride to 3^{15} have been accomplished.

Time course studies of the phytoalexins in UV-irradiated turnip tissue⁵^a have also suggested the same biosynthetic pathway. That is, the compounds **1a** and **1b** were first produced approximately 1 day after UV irradiation, and then 2 was produced with the decline of 1a after 2 days. Finally, 3 was produced with the decline of 2 and 1 week after the decline of 1a. These results are consistent with the proposed biogenesis.

To examine biogenesis, three deuterium-labeled phytoalexins (1a, 2, 4) were synthesized and several feeding experiments with the labeled compounds were carried out. Brassinin (1a) labeled at the methyl group of its side chain was readily synthesized from 3-(aminomethyl)indole¹⁶ by treatment with CS_2 and C^2H_3I . Cyclobrassinin (2) labeled at the same position was then synthesized from the labeled 1a. Deuterium-labeled 4 was synthesized from a corresponding amine¹⁷ by the same procedure. The ¹H-NMR spectra of synthetic [methyl-²H₃]brassinin, cyclobrassinin, and dioxibrassinin indicated the deuterium content at the methyl group to be at least 99%.

In a feeding experiment with the labeled brassinin, [methyl-²H₃]brassinin (0.88 mM, 800 mL) was administered to the UVelicited turnip tissue (Figure 2) and the progress of the biosynthesis was monitored using the HPLC method described in a previous paper.¹⁸ The aqueous phase was harvested at an earlier stage (27 h after the administration) to avoid dilution with increasing unlabeled natural 1a. The ethyl acetate extract from the water phase was separated by silica gel chromatography to give 2 mg of 2 and 11 mg of 3. The ¹H-NMR spectra of the two compounds indicated effective incorporation of the ²H label into cyclobrassinin (methyl-2H₃, 75%) and spirobrassinin (methyl-2H₃, 81%). Incorporation was estimated by the decline in signal area of the methyl group in each ¹H-NMR spectrum. Both 2 and 3 are therefore biosynthesized from 1a.

To confirm step 2 to 3, a feeding experiment with [methyl-²H₃]cyclobrassinin was conducted. Although the feeding experiments were done under several different conditions, [methyl-²H₃]cyclobrassinin was not incorporated into 3. Moreover,

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Figure 3. ²H-NMR spectrum (61.44 MHz, 10-mm tube) of spirobrassinin (3) derived from L-[4'-²H]tryptophan as a solution in CCl₄---cetone (9: 1).

 $[methyl-^{2}H_{3}]$ dioxibrassinin, a plausible precursor to 3, was not incorporated into spirobrassinin.

These results indicated that **1a** is an advanced precursor of **2** and **3** and that **3** is biosynthesized directly from **1a**, not by way of cyclobrassinin or dioxibrassinin. In the feeding experiment with [methyl-²H₃]brassinin, the incorporation ratio of spirobrassinin (81%) was higher than that of cyclobrassinin (75%), supporting the hypothesis that spirobrassinin is not biosynthesized via cyclobrassinin. In a later section, a different pathway from **1a** to **3** will be discussed.

Interestingly, the deuterium content of 4 recovered from the feeding experiment was 29%, in contrast to the initial content of 99%. This means that a facile exchange of the deuterium label in 4 occurred during the 6-h incubation period. On the other hand, high incorporation of the ²H label of brassinin (99%) into spirobrassinin (81%) and cyclobrassinin (75%) was realized, as described above. These results could be explained if we assume neighboring group participation by the hydroxyl group in 4 and also the presence of methanethiol or its equivalent in the water phase.

Origin of the Indole Ring of Brassinin. It is known that glucosinolates are biosynthesized through a common biosynthetic pathway from an amino acid precursor.¹⁹ In the case of indole glucosinolate, glucobrassicin (9a), it is biosynthesized from L-tryptophan.²⁰ Therefore, brassinin would be derived from L-tryptophan, though it is not clear whether the indole or indole related phytoalexins are biosynthesized from indole glucosinolates or directly from tryptophan. In order to confirm the origin of an indole moiety of 1a, incorporation of L-tryptophan was examined.

When L-[4'-2H] tryptophan $(4'-2H_3, 80\%)^{21}$ was fed to the UVelicited turnip tissue and incubated for 25 h, 1a and 3 were isolated as main metabolites. The ¹H-NMR spectrum of 3 showed a small decrease (18%) in the aromatic methine signal at C4, indicating the incorporation of the labeled L-tryptophan. To confirm the presence of ²H at C4 in spirobrassinin, the ²H-NMR was measured. Figure 3 shows the ²H-NMR spectrum of [4-²H]spirobrassinin. A clear signal in the aromatic region indicated unambiguously the incorporation of ²H into the benzene ring of 3. However, a clear ²H-NMR spectrum was not obtained in the case of 1a, because of a scanty amount of available 1a. Since the previous feeding experiments established that 3 is biosynthesized from 1a, the indole ring of 1a must originate from L-tryptophan.

Involvement of a Molecular Rearrangement in the Pathway from L-Tryptophan to Brassinin. The crucial point in the biosynthetic pathway leading to 1a is whether the thiocarbonyl carbon of 1a, therefore, the relevant imino carbon in 3, originates from the C2 carbon of L-tryptophan (Scheme 2). If this is the



Figure 4. Partial proton noise decoupled 67.5-MHz 13 C-NMR spectra of spirobrassinin (3): (a) after incorporation of [2- 13 C]tryptophan; (b) natural abundance.

Scheme 2. Biosynthetic Pathway of Cruciferous Phytoalexins



case, the biosynthetic pathway to 1a should involve a molecular rearrangement step since the thiocarbonyl carbon of 1a is separated from the methylene carbon by a nitrogen atom. To test this possibility, a feeding experiment with DL-[2-13C]tryptophan was carried out. Administration of the labeled tryptophan resulted in a 4-fold enhancement of the imino carbon NMR signal of 3 at δ 164 (Figure 4). This result indicates the involvement of a molecular rearrangement in the pathway from tryptophan to la and is suggestive of indol-3-ylmethyl isothiocyanate (10a) as a key intermediate to 1a. Although less labile (1-methoxyindol-3-yl)methyl isothiocyanate (10b) has been recently detected by mass spectrometry in the enzymatic hydrolysis products of neoglucobrassicin (9b),22 the unstable isothiocyanate (10a) has not been isolated to date. Only degradation products such as 3-indoleacetonitrile and 3-indolemethanol have been reported (Scheme 1).23

The Isothiocyanate Intermediate. In order to detect the labile indol-3-ylmethyl isothiocyanate (10a), a trapping experiment with

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Scheme 3. Direct and Stepwise Mechanisms of Methylthiolation to Brassinin (1a)



methanethiolate was attempted. Turnip roots were homogenized in a blender with 15% aqueous sodium methanethiolate in phosphate buffer (pH 7.0) for 5 min, and the homogenate was extracted immediately with ethyl acetate. Separation of the extract gave two adducts. Spectral data and direct comparison with the authentic samples revealed that they were la and PhCH₂-CH₂-NH-CS-SCH₃, which was named phenethylbrassinin (13).

Neither 1a nor phenethylbrassinin (13) was isolated in the absence of sodium methanethiolate. Isolation of 13 is in accord with the previous analytical studies of glucosinolates, 5ª where large amounts of a corresponding glucosinolate, gluconasturtiin, were detected in the turnip tissue. The present isolation of 1a suggests transient formation of 10a as a reaction intermediate.

To examine a possible role of isothiocyanates in the biosynthesis of cruciferous phytoalexins, moderately reactive benzyl isothiocyanate was chosen as a model substrate and administered to the turnip tissue without sodium methanethiolate. A new metabolite named benzylbrassinin (14) was isolated and identified as PhCH2-NH-CS-SCH₃ by direct comparison with a synthetic sample.

Formation of 14 in the turnip tissue indicates that isothiocyanate 10a is involved in the biosynthesis of 1a. It remains to be proved whether the isothiocyanate 10a can be formed via hydrolysis of 9a and/or directly from L-tryptophan, e.g. via thiohydroxamic acid

Intact Incorporation of the Methylthio Group of Methionine. To confirm the origin of the methyl group in brassinin (1a), an incorporation study of L-[methyl-2H3]methionine was carried out. The ¹H-NMR spectra of isolated 1a, 2, and 3 indicated unambiguously that each methyl group of 1a, 2, and 3 arose from L-methionine. Remarkable incorporation of the methyl group was evident from a decrease of respective methyl signal in each ¹H-NMR spectrum: 1a 35%, 2 34%, and 3 21%. These results indicated that methylation occurred in a late stage of the biosynthesis. This agrees with our proposal that 1a is biosynthesized via isothiocyanate 10a.

The methyl group of brassinin is derived from the methyl group of L-methionine, as described above. There are two possible biosynthetic routes from unstable isothiocyanate 10a to 1a (Scheme 3). Path A involves a direct attack by a methylthio group from L-methionine, whereas path B is a stepwise mechanism via a dithiocarbamic acid intermediate (15) or its equivalent.

In order to clarify whether the methylthio group in the phytoalexin is introduced directly or in a stepwise manner, a mixture of L-[methyl-3H3] methionine and L-[35S] methionine was fed to the UV-elicited turnip tissue. The mixture is considered doubly labeled L-[methyl-3H₃, 35S]methionine, provided a negligible isotope effect exists. The radioactive ratio of ${}^{35}S/{}^{3}H$ in the L-methionine mixture was 2.4. Forty eight hours after administration of the mixture, metabolites were extracted with ethyl acetate from the aqueous phase and separated to yield 0.2 mg of 1a and 2.4 mg of 3. The ratio of ${}^{35}S/{}^{3}H$ of isolated 1a and 3 was 2.5 and 2.2 at several concentrations, respectively. Therefore, the ratio is considered unchanged during biosynthesis of 1a and 3. This result indicates unambiguously that 1a is

Scheme 4. Preparation of 2-Methylbrassinin (16)^a

^a Reagents and conditions: (a) 1.6 equiv of NH₂OH·HCl, Na₂CO₃, H₂O-C₂H₅OH, reflux, 16 h, 75%; (b) Devarda's alloy, 1 M NaOH, H₂O-CH₃OH, 25 °C, 20 min; (c) 2.7 equiv of CS₂, triethylamine, dry pyridine, 0 °C, 1 h; (d) 2.5 equiv of CH₃I, 25 °C, 18 h, 72% from oxime.

biosynthesized by intact incorporation of the methylthio group from L-methionine to the isothiocyanate 10a. The same results were obtained when several experiments were done using L-methionine with different ratios of $^{35}S/^{3}H$.

This intact incorporation of the methylthio group of Lmethionine is unusual since many natural products with the methylthio group are derived by methylation of thiols,²⁴ which originate from the cysteine or glutathione conjugate of each acceptor molecule.

Since the two sulfur atoms of glucobrassicin (5) are derived from L-cysteine,²⁵ the sulfur atom of the isothiocyanate intermediate (10a) and the thiocarbonyl sulfur atom of 1a are most likely derived from L-cysteine.

To confirm the origin of the other sulfur atom, feeding experiments with L-[35S]cysteine were examined. The radioactivities of isolated 1a and 3 were clearly high. These results support the conclusion that the thiocarbonyl sulfur atom originates from L-cysteine.

An Intermediate Connecting Brassinin with Other Indole **Phytoalexins**. In a previous section, it was shown that 2 and 3 are biosynthesized from an advanced precursor 1a and that labeled 2 and 4 are not incorporated into 3. These results suggest that 2 and 3 are derived independently from 1a by way of a common intermediate. Production of a variety of indole or indole related phytoalexins can be explained by postulating a common intermediate which links 1a and other cruciferous phytoalexins.

To understand the intermediate, 2-methylbrassinin (16) was chosen as a probe. Since the compound 16 is blocked by a methyl group at the C2 position, the formation of a cyclobrassinin-type or a spirobrassinin-type compound is structurally prohibited. Therefore, possible isolation of a corresponding intermediate was expected. 2-Methylbrassinin was synthesized from 3-formyl-2methylindole by the same procedure used for 1a (Scheme 4). The model substrate 16 was administered to the UV-elicited turnip tissue. After a 27-h incubation, two new compounds, 17 (4 mg) and 18 (3 mg), were isolated (Scheme 5). Both compounds were not detected in the control tissue.

Compound 17, $[\alpha]^{25}_{D} = -21.8^{\circ}$, has a molecular formula of $C_{12}H_{12}N_2S_2$. The UV spectrum (λ_{max} (CH₃OH) 227 and 299(sh) nm)) suggests that it has an indolenine structure.²⁶ The ¹H-NMR spectrum shows the presence of four successive aromatic protons (δ 7.26 (1H, ddd, J = 7.3, 7.3, and 1.5 Hz), 7.37 (1H, ddd, J = 7.3, 7.3, and 1.1 Hz), 7.45 (1H, br d, J = 7.3 Hz), 7.53 (1H, br d, J = 7.3 Hz)), one methylthio group (δ 2.32 (3H, s)), one benzylic methyl group (δ 2.66 (3H, s)), and two methylene protons (δ 4.36 (1H, d, J = 15.8 Hz), 4.47 (1H, d, J = 15.8 Hz)). The coupling pattern (d, J = 15.8 Hz) of the methylene protons is suggestive of a methylene group attached to a spiro ring, as in

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Scheme 5. Mechanisms of Production of Compounds 17 and 18

3. The IR absorption $(\nu_{max} 1577 \text{ cm}^{-1})$ indicates the presence of a >C=N- group. These spectral data and eight degrees of unsaturation indicate that the product 17 is a tricyclic compound with a spiro ring system. An EI-MS fragment at m/z 175 (base peak, $(M - N=C-SCH_3)^+$) supports the partial structure $-N=C-SCH_3$. The full structure is therefore represented by formula 17. Results of difference NOE experiments are consistent with the proposed structure 17.

Compound 18, $[\alpha]^{25}D = -9.2^{\circ}$, has a molecular formula of C₁₂H₁₂N₂OS. Its ¹H- and ¹³C-NMR spectra also show the presence of four successive aromatic protons ($\delta_{\rm H}$ 6.84 (1H, dd, J = 7.8 and 7.3 Hz), 6.88 (1H, d, J = 8.3 Hz), 7.47 (1H, ddd, J = 8.3, 7.3, and 1.5 Hz, 7.58 (1H, d, J = 7.8 Hz), δ_{C} 112.9 (d), 119.3 (d), 124.8 (d), 137.7 (d)), one methyl group ($\delta_{\rm H}$ 1.33 (3H, s), $\delta_{\rm C}$ 20.8 (q)), one methylthio group ($\delta_{\rm H}$ 2.31 (3H, s), $\delta_{\rm C}$ 12.5 (q)), two methylene protons ($\delta_{\rm H}$ 3.56 (2H, d, J = 5.9 Hz) $\delta_{\rm C}$ 47.1 (t)), two D₂O exchangeable protons ($\delta_{\rm H}$ 5.02 (1H, br s), 5.68 (1H, br s, t-like, not completely exchangeable with D_2O)), and one sp³ quaternary carbon atom (δ_{C} 29.7 (s)). The UV spectrum $(\lambda_{max}$ (CH₃OH) 254(sh) and 391 nm) indicates that it must have an indoxyl nucleus.²⁷ These results suggest that the side chain of 2-methylbrassinin (16) rearranges to the C2 carbon atom of the indoxyl nucleus. The high-field shift of the methyl signal in the ¹H-NMR spectrum (from δ 2.64 in 16 to δ 1.33 in 18) and the presence of one sp³ quaternary carbon atom also support this indoxyl structure. Results of difference NOE experiments suggest that the methylene and one of the methyl groups are connected to the same C2 carbon atom. ¹H-¹H decoupling experiments indicate that the methylene is connected to a >NH group. The EI-MS spectrum of 18 does not show a molecular ion peak. However the FD-MS spectrum clearly indicates the presence of a molecular ion at m/z 250. Therefore, the residual side chain at C2 must include a thiocarbamate moiety. Hence, the structure of the second product is represented by formula 18.

These two metabolites, especially the compound 18, give important information pertaining to the intermediate in question. Formation of indoxyl (18) suggests that the immediate precursor to 18 is diol intermediate 19, which gives rise to 18 in a pinacoltype rearrangement (Scheme 5). The thiocarbamate side chain of 19 may be derived from the corresponding dithiocarbamate by oxidation. The supposed structure of diol intermediate 20 could account for the formation of metabolite 17. Dehydration of 20, followed by internal displacement could lead to spiro compound 17. Furthermore, the carbonyl group of thiocarbamate 19 is less nucleophilic as compared with the thiocarbonyl group of 20. Therefore, rearrangement would precede cyclization in diol 19.

Although epoxide 21 has the same oxidation state as diol 20, the latter is preferred as a more direct precursor because of the presence of an oxygen atom at the C2 position in spirobrassinin and in dioxibrassinin. Furthermore, the structures of 22 and 23, isolated from Japanese radish as minor phytoalexins,²⁸ support

the presence of a corresponding diol intermediate. That is, compound 22 could be biosynthesized by cyclization of the corresponding diol, which would be derived from methoxybrassinin (1b). Further methylation or methanolysis of 22 could yield compound 23. Isolation of the alcohol 22 could be explained by its resistance to further oxidation. Steric hindrance induced by the *N*-methoxyl group would be one reason for this resistance.

When the concept of a diol intermediate is applied to 1a, the corresponding diol intermediate 24 can be proposed (Figure 5). This diol precursor, 24, can explain the formation of a variety of indole or indole related phytoalexins. For example, internal displacement at the C2 carbon followed by dehydration can lead to cyclobrassinin (2), and oxidation of the hydroxyl group at C2 of diol 24 can give 4. Internal displacement of the hydroxyl at C3 followed by oxidation of the secondary hydroxyl group would yield 3. This biosynthetic pathway is consistent with the negative incorporation of 2 and 4 to 3.

Discussion

The biosynthetic pathway of cruciferous phytoalexins is summarized in Scheme 2. The fundamental phytoalexin, brassinin (1a), is biosynthesized and metabolized as follows. It originates from L-tryptophan with incorporation of one sulfur atom from L-cysteine, leading to the unstable intermediate, isothiocyanate 10a, which is directly methylthiolated to yield 1a. Brassinin (1a) is metabolized to 2, 3, and other indolic phytoalexins via an unstable oxidized intermediate.

The intact incorporation of the methylthio group from L-methionine to isothiocyanate 10a suggests that brassinin could be produced by nonenzymatic addition of methanethiol produced from L-methionine to the isothiocyanate. Although formation of methanethiol from broccoli under anaerobic conditions has been reported by Forney et al.,²⁹ detection by gas chromatography of methanethiol from UV-irradiated turnip tissue has not been successful thus far. Since methylthio groups in natural products are generally derived stepwise from methyl donors and sulfur donors, the direct methylthiolation is noteworthy. The direct methylthiolation also supports the presence of the labile intermediate, indole-3-ylmethyl isothiocyanate.

Methoxybrassinin (1b) is a representative of N-methoxylated cruciferous phytoalexins. This phytoalexin belongs to a rare group of N-methoxyindoles found in nature. It is not clear at the present stage whether 1b is derived from neoglucobrassicin (9b) or from 1a via N-hydroxylation followed by methylation. Although an incorporation experiment of labeled 1a to 1b has been undertaken, no incorporation has been observed so far.

Another type of cruciferous phytoalexins, brassilexin (5), has recently been isolated from Indian mustard (*Brassica nigra*),^{7a} but has not been isolated from our materials to date. Since brassilexin lacks a methylthio group, correlation between brassilexin and brassinin is not obvious. However, Barbier et al. have

⁽²⁷⁾ Sundberg, R. J.; Yamazaki, T. J. Org. Chem. 1967, 32, 290-294. (28) ¹H-NMR, IR, low- and high-resolution EI-MS, and UV are included as supplementary material. Monde, K.; Takasugi, M.; Shirata, A. Detailed results are in preparation for manuscript.

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demonstrated that brassilexin is derived chemically from cyclobrassinin by NaIO₄ oxidation.³⁰ According to their report, 5 is produced from cyclobrassinin disulfoxide,^{7b} which in turn is derived from 2. The co-occurrence of cyclobrassinin sulfoxide and 2 in the same crucifer^{7b} suggests that 5 is derived from 2 *via* cyclobrassinin sulfoxide and therefore from 1a, although no experimental evidence is available.

In the present paper, diol 24 is postulated as a key intermediate in the biosynthetic pathway to account for the formation of 2, 3, and other related phytoalexins. Although such a diol intermediate has been proposed by Scott in the biosynthetic studies of indole alkaloids,³¹ no experimental evidence for the diol intermediate has been presented. For example, it is possible that diol intermediate 24 is formed by a zwitterion followed by oxygen uptake and reduction of the ion, by enzyme-catalyzed dioxetane formation followed by reductive opening of the dioxetane ring,³² or by β -hydroxyindolenine formation followed by hydration.

The proposed biosynthetic pathway of the cruciferous phytoalexins indicates that a close relationship exists between the indole phytoalexins and indole glucosinolates. Time course studies on the sliced turnip tissue^{5a} show that the levels of both indole glucosinolates and phytoalexins increase significantly after UV irradiation, whereas only the former increases in nonirradiated control tissue. These results imply that the accumulated indole glucosinolates are converted to their respective isothiocyanate and then to 1a or 2 by methylthiolation through a UV-induced metabolic pathway. It is interesting to note that an increase in the levels of indole glucosinolates is accompanied by a decrease in the levels of aliphatic glucosinolates. Massive accumulation of the indole glucosinolates as well as a decrease in aliphatic glucosinolates has also been observed in wounded or insect-infected plant tissue.³³ These observations lead to the conclusion that wounded crucifers provide against possible microbial invasion an accumulation of indole glucosinolates, which serves as precursors of indole phytoalexins at the sacrifice of aliphatic glucosinolates. When the crucifers are exposed to pathogens, they produce indolic phytoalexins rapidly by using the accumulated indole glucosinolates.

Experimental Section

General Procedures. The ¹H-NMR (90 and 400 MHz) and ¹³C-NMR (100.6 MHz) spectra were obtained on JEOL JNM-EX90, JNM-EX400, and JNX-GX400 spectrometers. Chemical shifts are given in parts per million (ppm) relative to the TMS scale by reference to the solvent signal. IR spectra were recorded on a JASCO IR-700 spectrometer. UV spectra were obtained using a JASCO Ubest-30 spectrophotometer. Optical rotation measurements were done on a JASCO DIP-140 polarimeter. Low- and high-resolution EI-MS spectra were obtained using a JEOL JMS-DX300 spectrometer. FD-MS spectra were recorded using a JEOL JMS-01SG-2 spectrometer. Melting points were determined by a Yanagimoto MP micro melting point apparatus and were uncorrected. The HPLC analyses were performed on a JASCO HPLC system equipped with a Model 801-SC system controller, a Model 851-AS automatic sampler, a Model 880-50 line degasser, a Model 880-02 low-pressure gradient unit, a Model 865-CO column oven, a Model 880-PU pump, and a Model UVIDEC-100-V UV detector. A Shimadzu Chromatopac C-R6A integrator was used for measuring peak areas. Analytical conditions were used as previously described.¹⁸ Reagents and solvents were obtained from Nacalai tesque, Aldrich Chemical Co., and Wako Pure Chemical Industries. All chemicals of reagent grade were used without further purification unless otherwise noted. L-[4'-2H]Tryptophan (80%) was synthesized from L-tryptophan and D_2O (99%) by a reaction reported by Saito et al.²¹ [methyl-²H₃]Methyl iodide (99%), [methyl²H₃]methionine (99%), and DL-[2-¹³C]tryptophan (99%) were purchased from E. Merck AG., Aldrich Chemical Co., and MSD Isotope, respectively. L-[*methyl*-³H₃]Methionine, L-[³⁵S]methionine, and L-[³⁵S] cysteine were purchased from NEN Research Products.

Feeding Experiments with Labeled Precursors. Turnip roots (B. campestris L. ssp. rapa cv. Shirokamome) were cut horizontally, and hemispherical holes (2 cm in diameter) were made on each surface. After 12–24 h of incubation at 25 °C in moist plastic cases covered loosely with special polyethylene film (Aisaika film), the tissues were irradiated with a 15-W germicidal lamp for 10 min and incubated for an additional 6–12 h. Each hole was then filled with 0.1% Tween-80 aqueous solution (m⁻³. When the labeled compound could not be dissolved in Tween-80 aqueous solution, the compound was dissolved in small amounts of dimethyl sulfoxide (DMSO), and the DMSO solution was added carefully to the 0.1% Tween-80 aqueous solution with stirring (Figure 2).

Labeled compound was incorporated into the tissue to yield metabolites which diffused into the aqueous phase. The turnip tissue was incubated for the period indicated by HPLC analysis of sample aliquots. The aqueous phase was harvested at the optimum time and extracted with ethyl acetate. The extracts were separated by silica gel column chromatography and by preparative TLC on silica gel to give labeled metabolite(s).

Incorporation of [methyl-²H₃]Brassinin. Sample solutions were prepared as follows. Synthetic [methyl-²H₃]brassinin (99%) was dissolved in 1.6 mL of dimethyl sulfoxide (DMSO), and then the DMSO solution was carefully diluted with 850 mL of 0.1% Tween-80 aqueous solution with vigorous shaking. The resulting milky solution was used in the experiment. Each hole was filled with the 0.1% Tween-80 aqueous solution containing [methyl-²H₃]brassinin at a concentration of 1.0 mmol dm⁻³. Totally, 850 mL of sample solution was added. The tissues were then incubated for 37 h. The combined aqueous phase was extracted twice with 200 mL of ethyl acetate. The combined extract was dried over Na₂SO₄ and evaporated *in vacuo*. The residue (84 mg) was separated by silica gel column chromatography (methanol-dichloromethane, 1:99), followed by preparative TLC on silica gel to give 2 (2 mg) and 3 (11 mg).

2: ¹H-NMR (400 MHz, CDCl₃) δ 2.56 (0.75H, s), 5.09 (2H, d, J = 4 Hz), 7.14 (1H, ddd, J = 7, 7, and 2 Hz), 7.18 (1H, ddd, J = 7, 7, and 2 Hz), 7.33 (1H, dd, J = 7 and 2 Hz), 7.49 (1H, dd, J = 7 and 2 Hz), and 7.91 (1H, br s).

3: ¹H-NMR (CDCl₃) δ 2.62, (0.57H, s), 4.51 (1H, d, J = 15.1 Hz), 4.68 (1H, d, J = 15.1 Hz), 6.92 (1H, d, J = 7.8 Hz), 7.09, (1H, ddd, J = 6.8, 7.8, and 1.0 Hz), 7.26 (1H, ddd, J = 7.8, 7.8, and 1.5 Hz), 7.36 (1H, d, J = 6.8 Hz), and 8.61 (1H, br s, D₂O exchangeable).

Incorporation of [methyl-²H₃]Cyclobrassinin. Synthetic [methyl-²H₃]cyclobrassinin (99%, 60 mg) was dissolved in 0.5 mL of DMSO, and the DMSO solution was carefully diluted with 0.1% Tween-80 aqueous solution with vigorous shaking. The resulting milky solution was used in the experiment. Each hole was then filled with the 0.1% Tween-80 aqueous solution containing [methyl-²H₃]cyclobrassinin at a concentration of 0.84 mmol dm⁻³. Totally, 300 mL of sample solution was added. The tissues were then incubated for 32 h. The combined aqueous phase was extracted twice with 200 mL of ethyl acetate, and the combined extract was dried over Na₂SO₄ and evaporated *in vacuo*. The residue (53 mg) was separated by silica gel column chromatography (methanol-dichloromethane, 1:99), followed by preparative TLC on silica gel to give 3 (5 mg).

3: ¹H-NMR (CDCl₃) δ 2.62, (3H, s), 4.51 (1H, d, J = 15.1 Hz), 4.68 (1H, d, J = 15.1 Hz), 6.92 (1H, d, J = 7.8 Hz), 7.09, (1H, ddd, J = 6.8, 7.8, and 1.0 Hz), 7.26 (1H, ddd, J = 7.8, 7.8, and 1.5 Hz), 7.36 (1H, d, J = 6.8 Hz), and 8.61 (1H, br s, D₂O exchangeable).

Incorporation of DL-[methyl-²H₃]Dioxibrassinin. Synthetic DL-[methyl-²H₃]dioxibrassinin (99%, 105 mg) was dissolved with 1.0 mL of DMSO. Then the DMSO solution was carefully diluted with 500 mL of 0.1% Tween-80 aqueous solution with vigorous shaking. The resulting milky solution was poured into each hole at a concentration of 0.77 mmol dm⁻³. Totally, 500 mL of sample solution was added. The tissues were then incubated for 30 h, and the combined aqueous phase was extracted twice with 500 mL of ethyl acetate. The combined extract was dried over Na₂SO₄ and evaporated in vacuo. The residue (150 mg) was separated by silica gel column chromatography (methanol-dichloromethane 1:99), followed by preparative TLC on silica gel to give 3 (10 mg) and recovered 4 (43 mg). Both compounds were identified by ¹H-NMR, EI-MS, and HPLC retention time. The ¹H-NMR of isolated 3 from the aqueous solution revealed no incorporation of synthetic ²H-labeled 4 to 3.

3: ¹H-NMR (CDCl₃) δ 2.62, (3H, s), 4.51 (1H, d, J = 15.1 Hz), 4.68 (1H, d, J = 15.1 Hz), 6.92 (1H, d, J = 7.8 Hz), 7.09, (1H, ddd, J = 6.8,

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7.8, and 1.0 Hz), 7.26 (1H, ddd, J = 7.8, 7.8, and 1.5 Hz), 7.36 (1H, d, J = 6.8 Hz), and 8.61 (1H, br s, D₂O exchangeable).

4 (recovered after 6-h incubation): ¹H-NMR (acctone- d_6) δ 2.70 (0.87H, s), 4.00 (1H, dd, J = 14.2 and 3.9 Hz), 4.53 (1H, dd, J = 14.2 and 5.9 Hz), 5.58 (1H, br s), 7.06 (1H, d, J = 7.6 Hz), 7.15 (1H, dd, J = 7.6 and 7.6 Hz), 7.40 (1H, dd, J = 7.6 and 7.6 Hz), 7.55 (1H, d, J = 7.6 Hz), 8.97 (1H, br s), and 9.63 (1H, br s).

The tissues around the holes were also cut off and homogenized in a Waring blender with 1 L of ethyl acetate. The homogenized tissue was separated from the organic phase by centrifugation, and the resulting ethyl acetate layer was dried over Na₂SO₄ and evaporated *in vacuo*. The residue (653 mg) was separated by successive rounds of column chromatography, followed by preparative TLC on SiO₂ to give 3 (9 mg). The ¹H-NMR spectrum of 3 isolated from turnip tissue also revealed the same result as that from the aqueous solution.

Incorporation of L-[4'-2H]Tryptophan. Each hole was filled with 0.1% Tween-80 aqueous solution (450 mL) containing L-[4'-2H]tryptophan (80%) at a concentration of 1.2 mmol dm⁻³. After 14 h, the aqueous solution was harvested, and then additional aqueous solution (400 mL) of L-[4'-2H]tryptophan (0.8 mmol dm⁻³) was added. Totally, 176 mg of L-[4'-2H]tryptophan was added. The tissues were then incubated for 11 h. The combined aqueous solution was extracted twice with 600 mL of ethyl acetate, and the extract was dried over Na₂SO₄ and evaporated *in vacuo*. The residue (129 mg) was separated by silica gel column chromatography (methanol-dichloromethane, 1:99), followed by preparative TLC on silica gel to give 1a (2 mg) and 3 (11 mg).

1a: ¹H-NMR (CDCl₃) δ 2.65 (3H, s), 5.07 (2H, d, J = 4 Hz), 7.0 (1H, br s), 7.18 (1H, ddd, J = 8, 8, and 1 Hz), 7.25 (1H, ddd, J = 8, 8, and 1 Hz overlapped singlet 1H), 7.42 (1H, d, J = 8 Hz), 7.64 (0.95H, br d, J = 8 Hz), and 8.16 (1H, br s).

3: ¹H-NMR (CDCl₃) δ 2.62, (2.46H, s), 4.51 (1H, d, J = 15.1 Hz), 4.68 (1H, d, J = 15.1 Hz), 6.92 (1H, d, J = 7.8 Hz), 7.09, (1H, ddd, J = 6.8, 7.8, and 1.0 Hz), 7.26 (1H, ddd, J = 7.8, 7.8, and 1.5 Hz), 7.36 (0.82H, d, J = 6.8 Hz), and 8.61 (1H, br s, D₂O exchangeable); EI-MS m/z (%) 251 (M⁺ + 1, 11.1), 250 (M⁺, 36.1), 204 (M⁺ - SCH₃ + 1, 14.7), 203 (M⁺ - SCH₃, 56.6), 179 (M⁺ - C₂H₃NS + 2, 7.8), 178 (M⁺ -C₂H₃NS + 1, 25.7), and 177 (M⁺ - C₂H₃NS, 100).

Incorporation of DL-[2-¹³C]Tryptophan. Each hole was filled with 0.1% Tween-80 aqueous solution (400 mL) containing DL-[2-¹³C]tryptophan (99%, 98 mg) at a concentration of 1.2 mmol dm⁻³. The tissues were then incubated for 38 h. The aqueous solution was extracted twice with 400 mL of ethyl acetate. The combined extract was dried over Na₂SO₄ and evaporated *in vacuo*. The residue (67 mg) was separated by silica gel column chromatography (methanol-dichloromethane, 1:99), followed by preparative TLC on silica gel to give 1a (2 mg), 2 (0.4 mg), and 3 (3 mg). Each compound was identified by comparison of its ¹H-NMR spectrum and HPLC retention time with those of authentic samples.

3: 13 C NMR (100.6 MHz, CDCl₃, COM) δ 15.7, 64.6, 75.1, 110.1, 123.7, 124.6, 130.0, 131.3, 139.3, and 164.1.

Incorporation of L-[methyl-²H₃]Methionine. Each hole was filled with 0.1% Tween-80 aqueous solution (500 mL) containing L-[methyl-²H₃]methionine (99%) at a concentration of 2.0 mmol dm⁻³ and nonlabeled L-tryptophan at a concentration of 3.9 mmol dm⁻³. After 24 h, the aqueous solution was harvested, and additional aqueous solution (500 mL) of L-[methyl-²H₃]methionine (4.0 mmol dm⁻³) and non-labeled L-tryptophan (3.9 mmol dm⁻³) was added. Totally, 456 mg of L-[methyl-²H₃]methionine was added. Totally, 456 mg of L-[methyl-²H₃]methionine was extracted twice with 500 mL of ethyl acetate. The combined extract was dried over Na₂SO₄ and evaporated *in vacuo*. The residue (189 mg) was separated by silica gel column chromatography (methanol-dichloromethane, 1:99), followed by preparative TLC on silica gel to give 1a (2 mg), 2 (1 mg), and 3 (7 mg).

1a: ¹H-NMR (CDCl₃) δ 2.65 (1.95H, s), 5.07 (2H, d, J = 4 Hz), 7.0 (1H, br s), 7.18 (1H, ddd, J = 8, 8, and 1 Hz), 7.25 (1H, ddd, J = 8, 8, and 1 Hz overlapped singlet 1H), 7.42 (1H, d, J = 8 Hz), 7.64 (1H, br d, J = 8 Hz), and 8.16 (1H, br s).

2: ¹H-NMR (CDCl₃) δ 2.56 (1.98H, s), 5.09 (2H, d, J = 4 Hz), 7.14 (1H, ddd, J = 7, 7, and 2 Hz), 7.18 (1H, ddd, J = 7, 7, and 2 Hz), 7.33 (1H, dd, J = 7 and 2 Hz), 7.49 (1H, dd, J = 7 and 2 Hz), and 7.91 (1H, br s).

3: ¹H-NMR (CDCl₃) δ 2.62, (2.37H, s), 4.51 (1H, d, J = 15.1 Hz), 4.68 (1H, d, J = 15.1 Hz), 6.92 (1H, d, J = 7.8 Hz), 7.09, (1H, ddd, J = 6.8, 7.8, and 1.0 Hz), 7.26 (1H, ddd, J = 7.8, 7.8, and 1.5 Hz), 7.36 (1H, d, J = 6.8 Hz), and 8.61 (1H, br s, D₂O exchangeable).

Incorporation of Benzyl Isothiocyanate. Each hole was filled with 0.1% Tween-80 aqueous solution containing benzyl isothiocyanate (Nacalai tesque) at a concentration of 10 mmol dm⁻³. Totally, 450 mL

of sample solution was added. The tissues were then incubated for 24 h. The aqueous phase was extracted twice with 500 mL of ethyl acetate. The combined extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue (268 mg) was separated by silica gel column chromatography (benzene-hexane, 1:1) to give 3 mg of benzylbrassinin (14), which was identified by direct comparison with synthetic 14.

Incorporation of a Mixture of L-[methyl-³H₃]- and L-[³⁵S]Methionine. Each hole was filled with 1.0 mM cold L-methionine and 0.1% Tween-80 aqueous solution containing a mixture of L-[methyl-³H₃]methionine and L-[³⁵S]methionine (³H, 11.1 kBq/ μ mol; ³⁵S, 82.9 kBq/ μ mol). Totally, 300 mL of sample solution was added. The tissues were then incubated for 48 h at 25 °C. The combined aqueous phase was extracted twice with 300 mL of ethyl acetate, and the combined extract was dried over Na₂-SO₄ and evaporated *in vacuo*. The residue (30 mg) was separated twice by preparative TLC on silica gel to give labeled 1a (0.2 mg; R_f 0.53–0.55; methanol-dichloromethane, 2:98) and 3 (2 mg; R_f 0.13–0.16, methanoldichloromethane, 2:98; R_f 0.47–0.54; benzene-ethyl acetate, 1:1), after analyzing TLC with AMBIS 1000 radioanalytic imaging system (AMBIS). Radioactivity measurements were carried out on an Aloka LSC-3500 liquid scintillation counter with scintillation cocktail (Scintisol EX-H, Dojindo Laboratories).

Incorporation of L-[³⁵S]Cysteine. Each hole was filled with 1.0 mM cold L-cysteine in 0.1% Tween-80 aqueous solution containing L-[³⁵S]-cysteine (127 kBq/ μ mol). Totally, 700 mL of the sample solution was added. The tissues were then incubated for 48 h at 25 °C. The combined aqueous phase was extracted twice with 300 mL of ethyl acetate. The combined extract was dried over Na₂SO₄ and evaporated *in vacuo*. The residue (52 mg) was separated twice by preparative TLC on silica gel to give labeled 1a (1 mg; R_f 0.53–0.55; methanol-dichloromethane, 2:98; R_f 0.47–0.54; benzene-ethyl acetate, 1:1), after analyzing TLC with AMBIS 1000 radioanalytic imaging system. Radioactivity measurements were carried out on an Aloka LSC-3500 liquid scintillation counter with scintillation cocktail.

Incorporation of 2-Methylbrassinin (16). Each hole was filled with 0.1% Tween-80 aqueous solution of synthetic 2-methylbrassinin (16) with 400 μ L of DMSO (0.48 mmol dm⁻³, 700 mL). The tissues were then incubated for 27 h at 20 °C. The ethyl acetate extracts (2 × 500 mL) of the above aqueous solution were dried over Na₂SO₄. The solvent was evaporated *in vacuo*, and the residue (140 mg) was separated by silica gel column chromatography (methanol-dichloromethane, 1:99), followed by preparative TLC on silica gel to give 2-methyl-3-formylindole (2 mg), compound 17 (4 mg), and 18 (3 mg).

Compound 17: $C_{12}H_{12}N_2S_2$ (*m/z* 175.0466, M⁺), gum; $[\alpha]^{18}_D = -21.76^{\circ}$ (*c* 0.38, CHCl₃); IR (CHCl₃) 2926, 2852, 1577 (st), 1457, 1429, 1377, 1299, 1247, 992, and 944 cm⁻¹; UV (CH₃OH) 227 (ϵ 18900) and 299 nm (1590); ¹H-NMR (acetone-*d*₆, 400 MHz) δ 2.32 (3H, s), 2.66 (3H, s), 4.36 (1H, d, *J* = 15.8 Hz), 4.47 (1H, d, *J* = 15.8 Hz), 7.26 (1H, ddd, *J* = 7.3, 7.3, and 1.5 Hz), 7.37 (1H, ddd, *J* = 7.3, 7.3, and 1.1 Hz), 7.45 (1H, br d, *J* = 7.3 Hz), and 7.53 (1H, br d, *J* = 7.3 Hz); ¹H-NMR (CDCl₃, 400 MHz) δ 2.39 (3H, s), 2.64 (3H, s), 4.37 (2H, d, *J* = 1.5 Hz), 7.25 (1H, ddd, *J* = 7.3, 7.3, and 1.0 Hz), 7.36 (1H, ddd, *J* = 7.3, 7.8, and 1.5 Hz), 7.48 (1H, br d, *J* = 7.3 Hz); and 7.51 (1H, bdd, *J* = 7.8 Hz); EI-MS *m/z* (%) 248 (M⁺, 1.4), 233 (0.6), 201 (1.4), 176 (15), 175 ([C₁₀H₉NS]⁺, 100), 174 (13), 173 (11), 143 (10), 87 (15), 72 (19), and 44 (10).

Compound 18: $C_{12}H_{14}N_2O_2S (m/z 232.0697, M^+ - H_2O), gum; [<math>\alpha$]¹⁸_D = -9.21° (c 0.34, CHCl₃); IR (CHCl₃) 3342, 3320, 3008, 2926, 2852, 1681, 1617, 1488, 1468, 1323, and 965 cm⁻¹; UV (CH₃OH) 254 sh (ϵ 4850) and 391 nm (2330); ¹H-NMR (CDCl₃, 400 MHz) δ 1.33 (3H, s), 2.31 (3H, s), 3.56 (2H, d, J = 5.9 Hz), 5.02 (1H, br s, D₂O exchangeable), 5.68 (1H, br s, t-like, D₂O not completely exchangeable), 6.84 (1H, dd, J = 7.8 and 7.3 Hz), 6.88 (1H, d, J = 8.3 Hz), 7.47 (1H, ddd, J = 8.3, 7.3, and 1.5 Hz), and 7.58 (1H, d, J = 7.8 Hz); ¹³C-NMR (CDCl₃, DEPT, 100 MHz) δ 12.5 (q), 20.8 (q), 29.7 (s), 47.1 (t), 112.9 (d), 119.3 (d), 124.8 (d), 137.7 (d), and 160.8 (s); EI-MS m/z (%) 232 (M⁺ - H₂O, 3), 202 ([M-SCH₃-H]⁺, 14), 189 (13), 174 ([M-COSCH₃-H]⁺, 24), 130 (21), and 47 (100); FD-MS m/z (%) 250 (M⁺, 100).

Trapping Experiment of Indol-3-yimethyl Isothiocyanate (10a). Twenty pieces of turnip root were homogenized in a Waring blender with 2.5 L of phosphate buffer (pH 7.0, 25 mmol dm⁻³) containing 200 mL of 15% aqueous sodium methanethiolate for 5 min at room temperature. The homogenate was immediately poured into 1 L of ethyl acetate. The ethyl acetate layer was separated from the residue by centrifugation at 1000 rpm for 10 min. The organic phase was dried with sodium sulfate and evaporated *in vacuo*. The residue (1.075 g) was separated by silica gel column chromatography (benzene-ethyl acetate, 1:1), followed by

Biosynthesis of Cruciferous Phytoalexins

13: $C_{10}H_{13}NS_2$ (*m/z* 211.0486, M⁺), gum; IR (CHCl₃) 3376, 3062, 3026, 2960, 2922, 2854, 1493, 1453, 1383, 1336, 1307, 1260, 1217, 1095, 1030, 935, 908, 853, and 701 cm⁻¹; ¹H-NMR (90 MHz, CDCl₃) δ 2.62 (3H, s), 2.97 (2H, t, *J* = 6.8 Hz), 3.75 (1H, s, D₂O exchangeable), 4.00 (2H, m), and 7.29 (5H, m); EI-MS *m/z* (%) 211 (M⁺, 2), 163 ([M-SCH₃-H]⁺, 24), 105 ([PhCH₂CH₂]⁺, 23), 91 ([PhCH₂]⁺, 100), 77 (10), 72 (4), and 65 (12).

[methyl-²H₃]Brassinin. To a mixture of 3-(aminomethyl)indole¹⁶ (556 mg, 3.80 mmol, crude), dry triethylamine (580 µL, 4.16 mmol), and dry pyridine (2 mL) was added carbon disulfide (250 µl, 4.24 mmol) at 0 °C. The mixture was kept at 0 °C for 1 h, treated with [methyl-2H3]methyl iodide (99%, 260 µL, 4.01 mmol) at 0 °C, kept at room temperature for 18 h, and then poured into 1.5 M H₂SO₄ (50 mL). The mixture was extracted with ether $(2 \times 50 \text{ mL})$. The combined extract was washed with water (100 mL), saturated aqueous sodium hydrogen carbonate (100 mL), and water (100 mL), successively. The ethereal extract was dried over Na₂SO₄ and filtered, and the filtrate was concentrated in vacuo. The crystalline residue (436 mg) was recrystallized from dichloromethane to give [methyl-2H3]brassinin (311 mg, yield 34%). The ¹H-NMR spectrum of the synthetic [methyl-²H₃] brassinin revealed a 99% incorporation of ²H at the methyl group: ¹H-NMR (CDCl₃) δ 2.65 (0.01H, s), 5.07 (2H, d, J = 4 Hz), 7.00 (1H, br s), 7.18 (1H, ddd), 7.00 (1H, br s), 7.J = 8, 8, and 1 Hz), 7.25 (1H, ddd, J = 8, 8, and 1 Hz overlapped with singlet 1H), 7.42 (1H, d, J = 8 Hz), 7.64 (1H, br d, J = 8 Hz), and 8.16 (1H, br s).

[methyl-²H₃]Cyclobrassinin. To a solution of [methyl-²H₃]brassinin (99%, 283 mg, 1.18 mmol) in dry dichloromethane (24 mL) was added pyridinium bromide perbromide (380 mg, 1.18 mmol). The mixture was kept at 0 °C for 20 min, basified with 1,8-diazabicyclo[5.4.0]undec-7ene (DBU, 257 μ L, 1.72 mmol) at 0 °C, and then kept at room temperature for 4 h. The reaction mixture was evaporated, and the residue was separated by chromatography on silica gel (25 g) with dichloromethane to give crystalline material (117 mg), which on recrystallization from dichloromethane-hexane gave [methyl-²H₃]cyclobrassinin (69 mg) in 25% yield, indistinguishable from natural 2 except for the methyl signal. The ¹H-NMR spectrum of the synthetic [methyl-²H₃]cyclobrassinin revealed a 99% incorporation of ²H at the methyl group: ¹H-NMR (CDCl₃) δ 2.56 (0.01H, s), 5.09 (2H, d, J = 4 Hz), 7.14 (1H, ddd, J =7, 7, and 2 Hz), 7.18 (1H, ddd, J = 7, and 2 Hz), 7.33 (1H, dd J =7 and 2 Hz), 7.49 (1H, dd, J = 7 and 2 Hz), and 7.91 (1H, br s).

DL-[methyl-2H3]Dioxibrassinin. 3-(Aminomethyl)dioxindole hydrochloride¹⁷ (100 mg, 0.37 mmol) was dissolved in dry pyridine (500 μ L) and cooled down with an ice bath. Triethylamine (100 μ L, 0.74 mmol) and carbon disulfide (22 μ L, 0.37 mmol) were added to the solution, and the mixture was stirred for an additional 4 h at room temperature. The reaction mixture was treated with [methyl-2H3]methyl iodide (99%, 260 μ l, 4.01 mmol) at 0 °C, kept at room temperature for 18 h, then poured into 1 M HCl, and extracted with ethyl acetate three times. The combined extract was dried over MgSO4, and the solvent was evaporated in vacuo. The residue was purified by column chromatography on silica gel (15 g) with methanol-dichloromethane (5:95) as eluent to give [methyl-2H3]dioxibrassinin (94 mg, yield 78%). The ¹H-NMR spectrum of the synthetic [methyl-2H3]dioxibrassinin revealed a 99% incorporation of ²H at the methyl group: ¹H-NMR (acetone- d_6) δ 2.70 (0.01H, s), 4.00 (1H, dd, J = 14.2 and 3.9 Hz), 4.53 (1H, dd, J = 14.2 and 5.9 Hz), 5.58(1H, br s), 7.06 (1H, d, J = 7.6 Hz), 7.15 (1H, dd, J = 7.6 and 7.6 Hz), 7.40 (1H, dd, J = 7.6 and 7.6 Hz), 7.55 (1H, d, J = 7.6 Hz), 8.97 (1H, br s), and 9.63 (1H, br s).

Benzylbrassinin (14). Sodium methanethiolate (15% aqueous solution, 0.50 mL, 1.07 mmol) was added to a solution of benzyl isothiocyanate (113 mg, 0.76 mmol) in 5 mL of acetone and water (3:1), and the mixture was stirred for 10 min at room temperature. Acetone was removed *in vacuo*, and the mixture was poured into 20 mL of water. The mixture was extracted with ether (2×20 mL), and the combined ethereal extract was dried over Na₂SO₄. The solvent was evaporated *in vacuo* to leave almost pure 14 (136 mg, yield 91%): C₉H₁₁NS₂ (*m/z* 197.0311, M⁺), colorless oil; IR (CHCl₃) 3328, 3058, 3024, 3000, 2914, 1497, 1452, 1424, 1377, 1333, 1203, 1235, 1089, 1065, 1028, 935, 820, and 734 cm⁻¹; UV (CH₃OH) 254 (ϵ 11800) and 270 nm (11000); ¹H-NMR (CDCl₃) δ 2.66 (3H, s), 4.91 (2H, br d, J = 5.4 Hz), 7.11 (1H, br s, D₂O

exchangeable), and 7.34 (5H, s). The spectrum showed minor signals (1/3 the intensity of the major signals) due to a rotamer at δ 2.70 (s), 4.62 (d, J = 4.9 Hz), and 7.82 (br s, D₂O exchangeable); EI-MS m/z (%) 197 (M⁺, 2), 149 ([Ph-CH₂-NCS]⁺, 13), 91 (100), 65 (13), 48 (16), and 47 (18).

2-Methylindole-3-carboxaldehyde Oxime. Anhydrous sodium carbonate (1.10 g, 10.3 mmol) was added to a solution of hydroxylamine hydrochloride (700 mg, 10.1 mmol) in water (3 mL). 2-Methyl-3formylindole (1.024 g, 6.43 mmol) in 20 mL of ethanol was then added to the hydroxylamine solution. The mixture was refluxed for 16 h and was allowed to cool to room temperature. After removal of the solvent under reduced pressure, the residue was redissolved in ethyl acetate (300 mL), washed with brine $(2 \times 300 \text{ mL})$, dried over Na₂SO₄, and concentrated in vacuo. Residual crystals were recrystallized from ethyl acetate to afford first crystals (99 mg) and second crystals (633 mg, total yield 75%): C10H10N2O (m/z 174.0787, M⁺), mp 143-145 °C (AcOEt); IR (CHCl₃) 3370, 3272, 2970, 1612, 1574, 1552, 1455, 1413, 1309, 1246, 1154, 1140, 1102, 1028, 1010, 955, 906, 863, 830, 782, 746, and 664 cm⁻¹; UV (CH₃OH) 226 (\$\epsilon 23300), 267 (16600), and 287 nm (13500); ¹H-NMR (acetone- d_6 , 400 MHz) δ 2.64 (3H, s), 7.18 (2H, m), 7.43 (1H, d, J = 7.8 Hz), 8.12 (1H, d, J = 7.8 Hz), 8.47 (1H, s), 9.70 (1H, s), and 10.43 (1H, br s); EI-MS m/z (%) 174 (M⁺, 52), 158 (23), 157 (77), 156 (100), 155 (99), 142 (10), 130 (36), 129 (10), 128 (16), 102 (10), 101 (15), 78 (23), 77 (18), 51 (14), 43 (15), and 40 (34).

2-Methylbrassinin (16). An aqueous solution (8 mL) of 1 M sodium hydroxide was added to a solution of the oxime (102 mg, 0.590 mmol) in methanol (3 mL), and the mixture was stirred at room temperature. Devarda's alloy (630 mg) was added to the solution with vigorous stirring, and the mixture was allowed to stand for 20 min. The mixture was cooled to room temperature with a water bath and filtered through a Büchner funnel. The filtrate was extracted with ether $(3 \times 30 \text{ mL})$. The combined extract was dried over Na2SO4 and evaporated in vacuo. Almost pure amine was obtained and used in the next reaction without further purification. To a mixture of 3-aminomethyl-2-methylindole (102 mg, 0.638 mmol, crude), dry triethylamine (0.1 mL), and dry pyridine (1 mL) was added carbon disulfide (100 μ L, 1.70 mmol) at 0 °C, and the mixture was kept at this temperature for 1 h. The reaction mixture was then treated with methyl iodide (100 μ L, 1.61 mmol) at 0 °C, kept at room temperature for 18 h, poured into 0.25 M H₂SO₄ (50 mL), and extracted with ether $(3 \times 30 \text{ mL})$. The combined ether extract was washed with 0.25 M H₂SO₄ (100 mL), water (100 mL), saturated aqueous sodium hydrogen carbonate (2×100 mL), and water (100 mL), successively. The ethereal extract was dried over Na2SO4 and evaporated in vacuo. The resulting crystals (120 mg) were purified by column chromatography on silica gel (10 g) with benzene to give 16 (106 mg, 72% from oxime): C12H14N2S2 (m/z 143.0759, C10H9N), mp 119-121 °C (benzene); IR (CHCl₃) 3462, 3368, 3002, 2918, 1460, 1377, 1298, 1247, 1222, 1078, 1008, 956, and 929 cm⁻¹; UV (CH₃OH) 222 (¢ 36100) and 271 nm (15400); ¹H-NMR (CDCl₃, 400 MHz) & 2.44 (3H, s), 2.64 (3H, s), 4.98 (2H, d, J = 4.4 Hz), 6.91 (1H, br s), 7.12 (1H, dd, J = 7.8 and 6.8 Hz), 7.17 (1H, dd, J = 7.8 and 6.8 Hz), 7.30 (1H, d, J =7.8 Hz), 7.50 (1H, d, J = 7.8 Hz), and 7.97 (1H, br s). The spectrum showed minor signals (1/5 the intensity of the major signals) due to a rotamer at δ 2.17 (s), 2.83 (s), 4.70 (d, J = 4.5 Hz), and 7.81 (br s); EI-MS m/z (%) 144 (14), 143 (100), 142 (17), 128 (10), 116 (11), 115 (18), 107 (12), 102 (42), 76 (19), 75 (12), 60 (15), 59 (54), 51 (12), 50 (11), 48 (44), 47 (52), 45 (31), and 44 (11); FD-MS m/z (%) 250 (M+, 100).

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Supplementary Material Available: Text and diagrams describing the structures of compounds 22 and 23, text describing the experimental methods of characterizing 22 and 23, and a table of the radioactivity data of labeled compounds (9 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.