Trapping Experiment with Aniline for a Biosynthetic Intermediate of Sulfur-Containing Cruciferous Phytoalexins†

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Introduction

Plants produce antimicrobial compounds called phytoalexins after exposure to microorganisms.[1−4] The accumulation of phytoalexins is considered to be one of the most important defense mechanisms of plants. Cruciferous plants are cultivated worldwide and include important vegetables such as cabbage, broccoli, cauliflower, rape seed, mustard, radish, Japanese radish, Chinese cabbage, and Arabidopsis, an experimental plant. The phytoalexins produced by crucifers[5,6] have been shown to be indole or indole-related compounds that contain one or two sulfur atoms (Figure 1).[7−11] Recent studies of biosynthetic pathways[12] have revealed that the cruciferous phytoalexin brassinin (**3**) is synthesized from L-tryptophan with the incorporation of one sulfur atom from L-cysteine and another as a methylthio group from L-methionine. The related phytoalexins cyclobrassinin (**5**) and spirobrassinin (**6**) are derived from **3**. However, it is not clear how phytoalexins that contain only one sulfur atom, such as brassicanal A (**7**),[9] B (**8**),[9] and C(**9**),[11] are biosynthesized. This group of phytoalexins is thought to be biosynthesized from indole phytoalexins that contain two sulfur atoms, although no experimental evidence has been reported. In this paper, we describe the unique trapping of a potential biosynthetic intermediate to brassicanal A (**7**) in which aniline or acetanilide is added to UV-irradiated turnip tissue. The structure of the trapping product **1** strongly suggests that compound **2** is a biosynthetic intermediate from **3** to **7**.

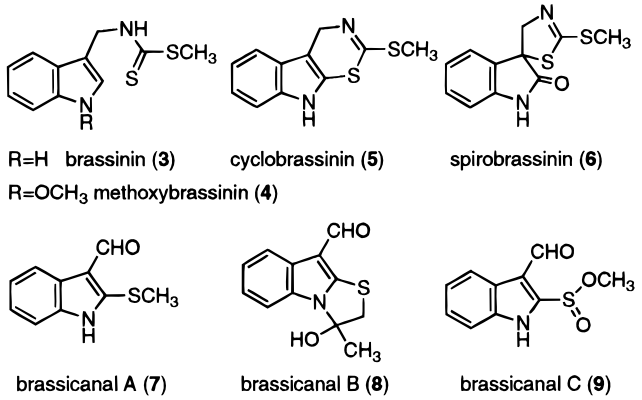

Figure 1.

Results and Discussion

Since brassicanal A (**7**) has a formyl group, aniline[13] was chosen as a trapping agent and was expected to produce a Schiff base. Aniline was administered to turnip root tissue (*Brassica campestris* ssp. *rapa* cv. Tamasato) that had been activated by slicing or by slicing followed by UV irradiation. A time-course analysis by HPLC of ethyl acetate extracts of UV-irradiated slices indicated a series of new peaks that were not detected with intact turnip root tissue. Except for two peaks, these new peaks were also observed in the extracts from UV-nonirradiated and aniline-fed turnip slices.[14] One of the two peaks was attributed to spirobrassinin (**6**), which is a major phytoalexin of turnip.[15] This means that the biosynthesis of turnip phytoalexins proceeds in UV-irradiated and aniline-fed turnip slices.

A yellow compound **1** isolated from the UV-irradiated slices corresponded to the other of the two peaks. Compound **1**, mp 211−213 °C, has a molecular formula of $C_{15}H_{12}N_2S$. The ^{1}H NMR spectrum showed a D_2O-exchangeable proton signal at δ 13.58 (d, J = 13.5 Hz, H-9) coupled to a proton (d, J = 13.5 Hz, H-8) at δ 8.44, which became a singlet upon D_2O exchange. The spectrum further showed the presence of one additional D_2O exchangeable proton signal at δ 8.95 (H-1) and of one monosubstituted and one *o*-disubstituted benzene rings. The two separated benzene rings were correlated by NOE and HMBC experiments. Irradiation of the proton at δ 8.44 (H-8) caused signal enhancements at a proton at δ 7.51 (H-4) in one ring and also at protons at δ 7.33 (H-2′ and H-6′) on the other ring. The HMBC experiment revealed the complete structure of **1.** This structure shows that compound **1** is formed by the condensation of 3-formyl-2-mercaptoindole (**2**) or its thione tautomer with aniline (Scheme 1). Compound **1** was also obtained when the UV-irradiated tissue was treated with acetanilide instead of aniline. Since the homogenate from either UV-irradiated or nonirradiated turnip slices promoted the hydrolysis of acetanilide to aniline, acetanilide should be deacetylated in the plant tissue and then condensed with compound **2** or its equivalent to yield the trapping product **1**.

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(14) These peaks were shown to be due to phenylthioureas derived from aniline and respective isothiocyanates from corresponding glucosinolates (Tanaka, A. Unpublished results).
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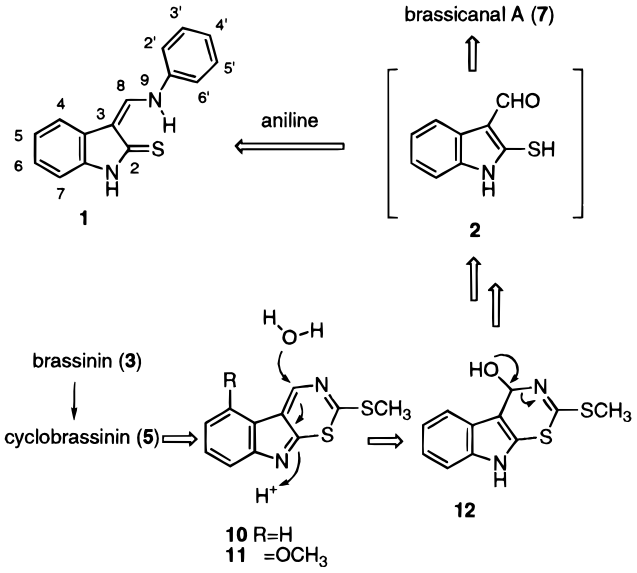
Scheme 1



Previous studies on phytoalexins from crucifers showed that these phytoalexins can be classified into three types: brassinin (**3**), methoxybrassinin (**4**), and brassicanal series. In contrast to the former two series, brassicanals **7**−**9** contain only one sulfur atom. Although the biosynthetic pathway from brassinin (**3**) to cyclobrassinin (**5**) and spirobrassinin (**6**) has recently been reported,[12] that of brassicanals remains to be elucidated. The isolation of **1** from UV-irradiated and aniline-fed turnip slices indicated that aniline trapped a potential biosynthetic precursor **2** to brassicanal A (**7**), which upon *in vivo* methylation should yield **7**. The expected precursor **2** or its thione tautomer may be derived by hydrolysis of dehydrocyclobrassinin (**10**), which in turn would be formed from **5** via enzymatic dehydrogenation (Scheme 1). Although **10** has not yet been isolated, a methoxy derivative **11** was recently isolated from *Pseudomonas cichorii*-inoculated turnip root.[16] Co-occurrence of **7** with **5** in *P. cichorii*-inoculated Chinese cabbage supports this pathway. These results also suggest the possibility that other nucleophilic or electrophilic reagents may trap potential biosynthetic precursors in plant tissue systems. Elucidation of such trapping products will provide valuable information on plant biosynthetic pathways that is otherwise difficult to obtain.

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Experimental Section

General Methods. ^{1}H (400 MHz) and ^{13}C (100.6 MHz) NMR spectra were obtained on a JNM-EX400 spectrometer. Chemical shifts are given in ppm relative to TMS by referencing to the solvent signal. IR spectra were recorded on a JASCO IR-700 spectrometer. UV spectra were obtained using a JASCO Ubest-30 spectrophotometer. Low- and high-resolution EI-MS spectra were obtained using a JEOL JMS-DX300 spectrometer. Melting points were determined using a Yanagimoto MP micro melting point apparatus and were uncorrected. HPLC analyses[15] 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.

Feeding Experiments. Turnip roots (*B. campestris* ssp. *rapa* cv. Tamasato, 2.4 kg) were cut transversely into slices (3 mm thick) and incubated at 25 °C for 24 h under humid conditions. The aged slices were irradiated on both sides with a 15 W germicidal lamp (0.5 mW/cm^2) for 10 min and then dipped into a fine 0.1 M suspension of aniline or acetanilide[13] in 0.1% Tween 80 aqueous solution containing small amounts of DMSO. The slices were removed from the solution, incubated for an additional 24 h under the same conditions, homogenized in ethyl acetate, and then centrifuged. Sequential column chromatography of the ethyl acetate extract (1.1 g) on silica gel ($CHCl_3$), Sephadex LH-20 (CH_3OH), and μ-Porasil (CH_2Cl_2) gave **1** (10 mg): ^{1}H NMR ($CDCl_3$) δ 13.58 (1H, d, J = 13.5 Hz, D_2O exchangeable, H-9), 8.95 (1H, s, D_2O exchangeable, H-1), 8.44 (1H, d, J = 13.5 Hz, H-8) 7.51 (1H, d, J = 7.5 Hz, H-4), 7.46 (2H, dd, J = 7.5, 8.5 Hz, H-3′ and H-5′), 7.33 (2H, dd, J = 1.0, 8.5 Hz, H-2′ and H-6′), 7.22 (1H, dd, J = 1.0, 1.0 Hz, H-4′), 7.18 (1H, dd, J = 7.0, 7.3 Hz, H-6), 7.14 (1H, dd, J = 7.3, 7.5 Hz, H-5), and 7.10 (1H, dd, J = 7.0, 7.0 Hz, H-7); ^{13}C NMR ($CDCl_3$) δ 178.3 (C2), 141.4 (C8). 139.2 (C1′), 137.8 (C7a), 130.0 (C3′ and C5′), 127.5 (C3a), 125.5 (C4′), 124.3 (C6), 122.1 (C5), 117.6 (C2′ and C6′), 115.4 (C4), 109.6 (C7), and 108.9 (C3); UV−vis (CH_3OH) λ_{max} 227 nm (ϵ 14 000), 301 (19 500), 358 (7300), and 430 (9300); IR ($CHCl_3$) 3432, 3014, 1646, 1597, and 1300 cm^{-1}; HREI-MS m/z (M^+) calcd for $C_{15}H_{12}N_2S$ 252.0721, found 252.0704; EI-MS m/z 252 (M^+, 100), 160 (35), and 77 (27).

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