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Sinalbins A and B, phytoalexins from *Sinapis alba*: elicitation, isolation, and synthesis

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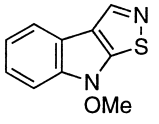
Abstract

The chemical structure and synthesis of sinalbin A is described. This cruciferous phytoalexin is produced by white mustard (*Sinapis alba*) after treatment with biotic and abiotic elicitors. In addition, a related metabolite, named sinalbin B, is present in extracts from elicited plants, but not in those from non-elicited controls. Sinalbin B, which was also synthesized, appears to be both a phytoalexin and a biosynthetic precursor of sinalbin A.

Keywords: *Sinapis alba*; Cruciferae; Crucifer; White mustard; Phytoalexin; Sinalexin; *Alternaria brassicae*; Alternaria blackspot; Destruxins; *Phoma lingam*; Blackleg

1. Introduction

Plant defense mechanisms may involve de novo biosynthesis of antimicrobial compounds, some of which have low molecular weights and are known as phytoalexins (Bailey and Mansfield, 1982; Brooks and Watson, 1985). The phytoalexins from crucifers have unique structures containing indole or indole related rings and at least one sulfur atom, thus far detected only in this plant family (Pedras et al., 2000).[1] Because white mustard (*Sinapis alba*) is resistant to economically important fungal diseases such as Alternaria blackspot and Phoma blackleg (Saharan, 1993; Hemingway, 1995), we have been studying its defense mechanisms, including phytoalexin production. We have reported the first phytoalexin produced by white mustard, sinalexin (**1**), under biotic and abiotic elicitation (Pedras and Smith, 1997). In continuation of these studies, we wish to report the elicitation, detection, and synthesis of two new metabolites, named sinalbins A (**5**) and B (**4**), produced by leaf and stem tissues of white mustard upon biotic elicitation with the blackleg [*Phoma lingam* (Tode ex Fr.) Desm., asexual stage of *Leptosphaeria maculans* (Desm.) Ces. et de Not.] and blackspot [*Alternaria brassicae* (Berk.) Sacc.] fungi, as well as through elicitation with destruxin B, homodestruxin B, hydroxydestruxin B, and $CuCl_2$.



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2. Results and discussion

HPLC chromatograms of extracts of elicited leaves of white mustard suggested that a compound with $t_R = 17.9$ min, not present in extracts of non-elicited leaves, was being produced over a period of several days. For example, upon elicitation with destruxin B, $CuCl_2$, *P. lingam*, or *A. brassicae* the maximum production of this compound occurred at 24 h, whereas elicitation with homodestruxin B or hydroxydestruxin B appeared to induce slower responses, at 48 and 96 h, respectively.

In order to obtain a reasonable amount of elicited tissue to identify the new compound unambiguously, white mustard plants were elicited with $CuCl_2$, and the leaves were extracted with EtOAc. The extract was fractionated, the fractions were analyzed by HPLC and further separated by preparative TLC, as described in the experimental. The ^1H and ^{13}C NMR spectra of the compound with $t_R = 17.9$ min displayed only aromatic

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[1] For a recent review see Pedras et al., 2000.

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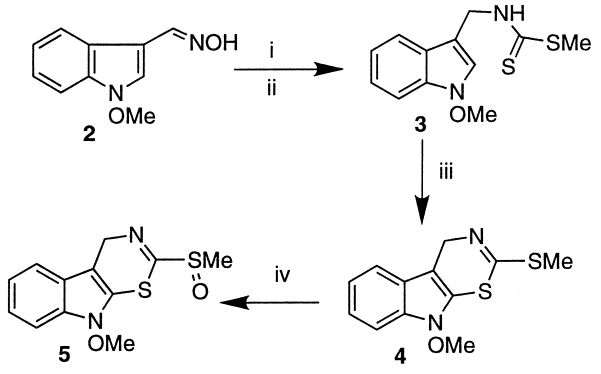
signals assignable to an indole moiety, except for three resonances due to CH_2 (δ_H 5.42 and δ_C 50.7), CH_3O (δ_H 4.06 and δ_C 66.5) and CH_3S (δ_H 2.85 and δ_C 42.0) groups. The assignment of structure **5** to this plant metabolite was secured from additional spectroscopic data (FTIR, UV, and HRMS) and corroboration by synthesis, as shown in Scheme 1.

The synthesis of **5** was accomplished (Scheme 1) in six steps from 1-methoxy indole, via oxime **2** and methoxybrassinin (**3**). The key step, which represents a significant improvement over a previously published synthesis of **3** (Somei et al., 1992; Yamada et al., 1993), is the reduction of oxime **2** to the corresponding amine and subsequent formation of methoxybrassinin (**3**) in 60% overall yield. Because the synthetic intermediate **4** appeared as a likely biogenetic precursor of **5**, we investigated whether this compound was also produced by elicited plant tissues. Interestingly, compound **4** ($t_R = 32.2$ min) was detected in extracts of white mustard stems elicited by *P. lingam,* but not in elicited leaf tissues; however, due to the relatively smaller amount present in these extracts, we were unable to isolate compound **4** from any of the plant tissues. It is likely that **4** is the biosynthetic precursor of **5**, thus absence from leaf tissues suggests its faster bioconversion in leaf tissue than in stem tissue. Upon standing at room temperature for 13 days (in CH_3CN), compound **5** yielded mostly **1** (90%), while **4** yielded undetermined products (80%). However, **1** appears to be a naturally occurring compound since its accumulation in elicited foliar tissue of *S. alba* depends on the elicitor, plant age, and plant cultivar (e.g. cv. Pennant accumulates sinalexin faster than cv. Ochre).

Finally, antifungal bioassays established that metabolites **4** and **5** were active against *P. lingam*. While **5** (5×10^{-4} M) caused complete inhibition of spore germination (ED_{50} 2×10^{-4} M at 48 h) for the duration of the assay (7 days), **4** showed moderate activity (ED_{50} 7×10^{-4} M at 48 h) at similar concentration (5×10^{-4} M), causing about 30% germination inhibition relative to controls, after 48 h. Further bioactivity studies are underway to better understand the role of these compounds in the resistance of *S. alba* to *P. lingam*, and *A. brassicae*, two of the most important pathogens of canola. To the best of our knowledge, compounds **4** and **5**, named sinalbin A (**5**) and sinalbin B (**4**), are new phytoalexins from white mustard, i.e. produced by elicited plant tissues, not detectable in non-elicited tissues, and having antifungal activity against *P. lingam*. Considering that the closely related phytoalexin cyclobrassinin (**6**) was shown to be a biosynthetic intermediate of the phytoalexin brassilexin (**7**) in *B. juncea* (Scheme 2) (Pedras et al., 1998), sinalbin B (**4**) may be biosynthetic precursor of sinalexin (**1**) in *S. alba*.



Scheme 1. (i) $TiCl_3/NaBH_3CN/NH_4Ac$; (ii) CS_2/Py,Et_3N; MeI, 60%; (iii) CH_2Cl_2/NBS, 41%; (iv) *m*-CPBA, 20%.

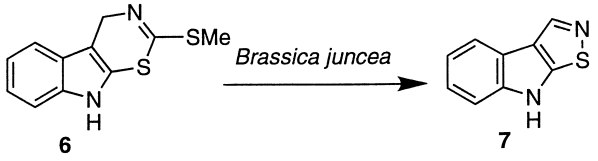
3. Experimental

3.1. General

All chromatographic conditions, including HPLC analyses, and spectroscopic techniques, were used as previously reported (Pedras and Okanga, 1999).

3.2. Elicitation of phytoalexins

Leaves from 21-day-old plants of *S. alba* cv. Ochre (in growth chamber, 16 h day/8 h night, 20°C constant) were punctured (5 leaves, 4 punctures/leaf, hypodermic needle, 19 gauge) and each puncture was inoculated with a 10 μl drop of spore suspension of *A. brassicae* (1.5×10^5 spores/ml). After 24 h, the leaf tissue (0.5 g) was frozen in liquid N_2, crushed with a glass rod and extracted with EtOAc (50 ml, shaking for 16 h). The EtOAc extract was separated by filtration, dried (Na_2SO_4) and concentrated under reduced pressure. The residue (6.4 mg) was dissolved in CH_3CN and analyzed by HPLC. Alternatively, leaves were cut, and the petioles partly immersed in solutions of destruxin B, homodestruxin B, or hydroxydestruxin B (2×10^{-5} M in 2% aq. CH_3CN) for solution uptake and incubated for different time periods (Pedras et al., 1999). Similar elicitation experiments were carried out with stems cut from 21-day-old plants and incubated with a spore suspension (5.5×10^7 spores/mL) of *P. lingam*, or the stems partly immersed in $CuCl_2$ (2×10^{-3} M) for solution uptake. After incubation for different times, the stems were extracted as described for leaves.



Scheme 2.

3.3. Antifungal activity assay

Bioassays to determine the effect of compounds **4** and **5** on the germination inhibition of spores of *P. lingam* were carried out after modification of a method previously reported (Pedras and Biesenthal, 1998). That is, Nunclon 4-well multidish plates containing 1×10^7 spores/mL in 500 μL solutions of compound to be tested (5×10^{-4}, 1×10^{-4}, 5×10^{-5} M in 3% Tween 80 and 2% DMSO in minimal medium) were incubated still under constant fluorescent light, at 24±1°C. The percentage of spore germination was determined by counting about 300 spores (in randomly selected fields) for each replicate after incubations of 24 h, 48 h and 7 days.

3.4. Isolation of sinalbin A (5)

Leaves of 21-day-old of *S. alba* cv. Ochre (120 plants) were sprayed with $CuCl_2$ solution (1×10^{-2} M); after 24 h the leaves were cut (750 g) and the tissue was frozen in liquid N_2. The leaf tissue was extracted with EtOAc (1.5 l) and processed as described above to yield an oily residue (3.32 g). The residue was fractionated by FCC (silica gel RP-18, CH_3CN–H_2O, 1:1) and fractions containing an HPLC peak at $t_R = 17.9$ min were combined (17.3 mg). Further fractionation (silica gel RP-18) of this residue gave 7.6 mg of material containing the peak of interest. This residue was then rinsed with CH_2Cl_2 (3 ml) and the CH_2Cl_2 soluble portion (2.7 mg) was separated by prep TLC (silica gel RP-8, CH_3CN–H_2O, 65:35, developed two times) to give (0.2 mg) of sinalbin A (**5**).

3.5. Synthesis of sinalbins A (5) and B (4)

1-Methoxyindole (Pedras and Sorensen, 1998) was formylated (Smith, 1954), and the corresponding oxime **2** prepared in quantitative yield. Sodium cyanoborohydride (738 mg, 12.5 mmol) and NH_4OAc (1.06 g, 13.7 mmol) were added to a cooled solution of **2** (237 mg, 1.25 mmol) in MeOH. To this mixture a neutralized solution (3.5 ml, 9.9 mmol) of $TiCl_3$ (30% by wt. in 2 M HCl) was added. After stirring for 15 min at 0°C, the reaction mixture was diluted with 10 ml water, neutralized with NaOH and extracted with CH_2Cl_2 (2×15 ml). The organic phase was dried (Na_2SO_4) and concentrated to dryness in vacuo to yield crude *N*-methoxyindole-3-methanamine (188 mg). To the cooled solution of crude amine in pyridine (0.5 ml), Et_3N (164 μl, 1.18 mmol) and CS_2 (64 μl, 1.1 mmol) were added. After 1 h of stirring at 0°C, MeI (67 μl, 1.1 mmol) was added and the reaction was then left at 5°C for 16 h. H_2SO_4 (2 ml) was added to the reaction mixture, the mixture was extracted with Et_2O (2×10 ml), the organic phase was separated, dried (Na_2SO_4) and concentrated to dryness in vacuo to yield residue (244 mg). The residue was separated by FCC (silica gel, CH_2Cl_2–hexane, 80:20) to yield 1-methoxybrassinin (**3**) 199 mg (60% yield from oxime). *N*-bromosuccinimide (7.8 mg, 0.044 mmol) was added to a stirred solution of 1-methoxybrassinin (**3**) (11.7 mg, 0.044 mmol) in anhydrous CH_2Cl_2 (0.5 ml) at room temperature. The reaction mixture was stirred for 10 min at room temperature, then Et_3N (13 μl, 0.088 mmol) was added and, after further stirring for 5 min, the solvent was removed under reduced pressure. The residue was separated by FCC (silica gel, CH_2Cl_2–hexane, 80:20) to yield sinalbin B (**4**) 5.5 mg (47% yield). *m*-CPBA (16.3 mg, 0.095 mmol) was added to a cooled stirred solution of **4** (16.7 mg, 0.06 mmol) in anhydrous CH_2Cl_2 (1 ml, 0°C). After 20 min at 0°C, Me_2S (250 μl) was added, the reaction mixture was allowed to warm up to room temperature (5 min) and the solvent was evaporated under reduced pressure. After standard work, the organic phase was dried (Na_2SO_4) and concentrated to dryness in vacuo, the residue (16.1 mg) was separated by prep TLC (silica gel RP-8, CH_3CN–H_2O, 70:30) to yield of sinalbin A (**5**) 3.5 mg (20% yield).

3.6. Sinalbin A (5)

HPLC $t_R = 17.9$ min; ^{1}H NMR (500 MHz, CD_2Cl_2) : δ 7.44 (*d*, $J = 8$ Hz, 1H), 7.43 (*d*, $J = 8$ Hz, 1H), 7.26 (*dd*, $J = 8$, 8 Hz, 1H), 7.16 (*dd*, $J = 8$, 8 Hz, 1H), 5.42 (s, 2H), 4.06 (s, 3H), 2.85 (s, 3H); ^{13}C NMR (125.8 MHz, CD_2Cl_2): δ 166.2 (*s*), 134.8 (*s*), 123.2 (*d*), 122.3 (*s*), 122.0 (*s*), 121.5 (*d*), 118.3 (*d*), 108.8 (*d*), 96.7 (*s*), 66.5 (*q*), 50.7 (*t*), 42.0 (*q*); HREIMS *m*/*z* (% relative abundance) measured: 280.0341 (280.0340 calcd. for $C_{12}H_{12}N_2O_2S_2$); EIMS *m*/*z* (% relative abundance): 280 $[M]^+$ (70), 248 (12), 217 (10), 191 (92), 160 (100), 117 (51), 101 (28); FTIR ν_{max}: 2933, 2842, 1728, 1632, 1443, 1325, 1231, 1126, 1066, 950, 921, 740 cm^{-1}; UV (CH_2Cl_2) λ_{max} (log ϵ) 231 (4.33), 280 (3.96) nm.

3.7. Sinalbin B (4)

HPLC $t_R = 32.2$ min; ^{1}H NMR (500 MHz, CD_2Cl_2) : δ 7.45 (*d*, $J = 8$ Hz, 1H), 7.42 (*d*, $J = 8$, Hz, 1H), 7.22 (*dd*, $J = 8$, 8 Hz, 1H), 7.14 (*dd*, $J = 8$, 8 Hz, 1H), 5.07 (s, 2H), 4.01 (*s*, 3H), 2.55 (*s*, 3H); ^{13}C NMR (125.8 MHz, CD_2Cl_2): δ 151.1 (*s*), 134.8 (*s*), 129.7 (*s*), 122.7 (*d*), 122.4 (*s*), 121.1 (*d*), 117.9 (*d*), 108.8 (*d*), 100.2 (*s*), 66.0 (*q*), 49.0 (*t*), 15.6 (*q*); HREIMS *m*/*z* (% relative abundance) measured: 264.0397 (264.0391 calcd. for $C_{12}H_{12}N_2OS_2$); EIMS *m*/*z* (% relative abundance): 264 $[M]^+$ (20), 232 (100), 191 (69), 159 (56), 117 (22), 89 (9); FTIR ν_{max}: 2925, 1593, 1448, 1409, 1322, 1215, 1058, 935, 740 cm^{-1}; UV (CH_2Cl_2) λ_{max} (log ϵ) 231 (4.13), 275 (3.80) nm.

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References

Bailey, J.A., Mansfield, J.W., (Eds.) 1982. Phytoalexins. Blackie & Son, Glasgow, UK, p. 334.

Brooks, C.J.W, Watson, D.G., 1985. Phytoalexins. Natural Product Reports, 427–459.

Hemingway, J.S., 1995. The mustard species: condiment and food ingredient use and potential as oilseed crops. In: Kimber, D., McGregor, D.I. (Eds.), Brassica Oilseeds. CAB International, Wallingford, UK, pp. 373–383.

Pedras, M.S.C., Biesenthal, C.J., 1998. Production of the host-selective toxin phomalide by isolates of *Leptosphaeria maculans* and its correlation with sirodesmin PL production. Canadian Journal of Microbiology 44, 547–553.

Pedras, M.S.C., Loukaci, A., Okanga, F.I., 1998. The cruciferous phytoalexins brassinin and cyclobrassinin are intermediates in the biosynthesis of brassilexin. Bioorganic & Medicinal Chemistry Letters 8, 3037–3038.

Pedras, M.S.C., Okanga, F.I., 1999. Strategies of cruciferous pathogenic fungi: detoxification of the phytoalexin cyclobrassinin by mimicry. Journal of Agricultural and Food Chemistry 47, 1196–1202.

Pedras, M.S.C., Okanga, F.I., Zaharia, I.L., Khan, A.Q., 2000. Phytoalexins from crucifers: synthesis, biosynthesis, and biotransformation. Phytochemistry 53, 161–176.

Pedras, M.S.C., Smith, K.C., 1997. Sinalexin, a phytoalexin from white mustard elicited by destruxin B and *Alternaria brassicae*. Phytochemistry 46, 833–837.

Pedras, M.S.C., Sorensen, J.L., 1998. Phytoalexin accumulation and production of antifungal compounds by the crucifer wasabi. Phytochemistry 49, 1959–1965.

Pedras, M.S.C., Zaharia, I.L., Gai, Y., Smith, K.C., Ward, D.E., 1999. Metabolism of the host-selective toxins destruxin B and homodestruxin B: probing a plant disease resistance trait. Organic Letters 1, 1655–1658.

Saharan, G.S., 1993. Disease resistance. In: Labana, K.S., Banga, S.S., Banga, S.K. (Eds.), Breeding Oilseed Brassicas. Springer-Verlag, Berlin, pp. 181–205.

Smith, G.F., 1954. Indoles. Part I. The formylation of indole and some reactions of 3-formylindole. Journal of the Chemical Society, 3842–3846.

Somei, M., Kobayashi, K., Shimizu, K., Kawasaki, T., 1992. A simple synthesis of a phytoalexin, methoxybrassinin. Heterocycles 33, 77–80.

Yamada, Y, Kobayashi, K., Shimizu, K., Aoki, N., Somei, M., 1993. A synthesis method of indole-3-methanamine and/or gramine from indole-3-carboxaldehyde, and its application for the synthesis of brassinin, its 4-substituted analogues and 1, 3, 4, 5-tetrahydro-pyrrolo[4,3,2-*de*]quinoline. Heterocycles 36, 2783–2804.