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SINALEXIN, A PHYTOALEXIN FROM WHITE MUSTARD ELICITED BY DESTRUXIN B AND *ALTERNARIA BRASSICAE*

M. SOLEDADE C. PEDRAS* and KEVIN C. SMITH

Department of Chemistry, University of Saskatchewan, 110 Science Place, Saskatoon, SK S7N 5C9, Canada

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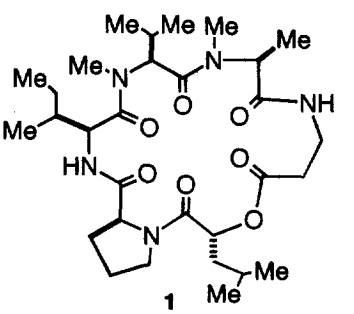
Key Word Index—*Sinapis alba*; cruciferae; white mustard; phytoalexin; sinalexin; *Alternaria brassicae*; alternaria blackspot; destruxin B; 4-hydroxybenzylisothiocyanate.

Abstract—Determination of the structure of sinalexin, a phytoalexin produced by white mustard (*Sinapis alba*) under biotic or abiotic elicitation, is described. Additionally, the isolation and synthesis of 4-hydroxybenzylisothiocyanate, the major antifungal component isolated from extracts of white mustard, and optimized conditions for the isolation of the phytotoxin destruxin B are described. © 1997 Elsevier Science Ltd

INTRODUCTION

It is well-documented that penetration and colonization of plant tissues by pathogenic fungi can involve fungal production and release of selective phytotoxine compounds [1]. We are evaluating the role of host-selective phytotoxins as part of a research program aimed at understanding mechanisms of plant disease resistance [2, 3]. Because white mustard (*Sinapis alba*) is resistant to alternaria blackspot, a significant fungal disease of the oilseed crops canola (*Brassica napus* and *B. rapa*) and rapeseed (*B. napus* and *B. rapa*) [4], we were interested in evaluating its response to the phytotoxins produced by the pathogenic organism. White mustard is a cruciferous plant grown as a source of condiment and industrial oil in several parts of the world [5]. The depsipeptide destruxin B (**1**) is the major host-selective phytotoxin produced both *in vitro* [6] and *in planta* [7] by the blackspot fungal pathogen *Alternaria brassicae* (Berk.) Sacc. Destruxin B (**1**) causes chlorotic and necrotic foliar lesions on diverse *Brassica* species and other cruciferous host-plants [8, 9]. Additional physiological effects, as for example selective reactions of pollen from various brassicas to destruxin B, have also been reported [10]. However, the molecular basis for the selective phytotoxicity of destruxin B and related toxins [6, 7] is not understood.

On the other hand, plant defense mechanisms involve the *de novo* biosynthesis of defense compounds having antimicrobial activity, some of which have low molecular weights and are known as phytoalexins [11, 12]. The phytoalexins from brassicas have an indole or indole related ring and at least one sulphur atom as common structural features [13, 14]. Most interestingly, crucifers appear to be the only plant family producing these sulphur metabolites. Several of these phytoalexins are produced by more than one *Brassica* spp., and can be elicited by diverse pathogens and/or abiotic factors. Brassinin (**2**), methoxybrassinin (**3**), and cyclobrassinin (**4**) were the first brassica phytoalexins to be reported [15]. Subsequently, several new phytoalexins have been isolated from cruciferous plants, including diverse *Brassica* spp.†; to the best of our knowledge no phytoalexins from white mustard have been reported to date. We analysed white mustard foliar tissue incubated with destruxin B. Here we wish to report the structure of a new phytoalexin we named sinalexin (**5**) produced by white mustard under elicitation by destruxin B, *A. brassicae*, or abiotic stress. Additionally, the isolation and synthesis of 4-hydroxybenzylisothiocyanate (**7**), the major antifungal component isolated from extracts of white mustard, and optimized conditions for the production and isolation of destruxin B are described.



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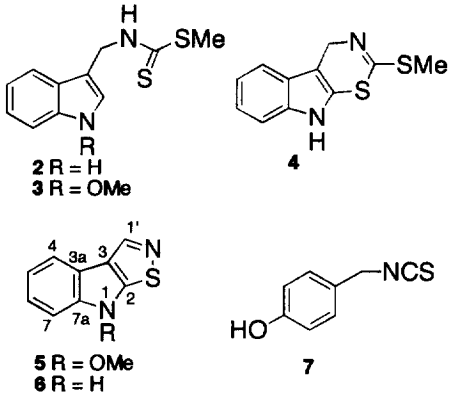
* Author to whom correspondence should be addressed.

† For recent reviews see references 13 and 14.



RESULTS AND DISCUSSION

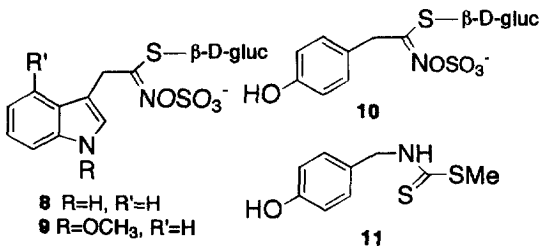
In preliminary experiments, leaves of white mustard were incubated with solutions of destruxin B isolated from cultures of *Alternaria brassicae*. Chlorotic tissues started developing in the areas where destruxin B (**1**) was placed two days after incubation, increasing slightly over a one-week period; areas treated with solvent showed no visible symptoms. The leaves were excised after one week and destruxin B treated chlorotic tissues were extracted with ethyl acetate; control leaf tissues were extracted similarly. The resulting extracts were compared by high performance liquid chromatography (HPLC) analysis under the conditions described in the experimental. Under those conditions, two peaks with R_t = 19.3 min and R_t = 20.2 min were present on the chromatogram of an extract of leaves incubated with destruxin B, which were not detected on the chromatogram of an extract of control tissues. The peak with R_t = 19.3 min could readily be attributed to destruxin B (**1**) by comparison (HPLC diode array detector) with an authentic sample. The peak with R_t = 20.2 min showed a UV spectrum rather different from that of destruxin B; comparison of that UV spectrum with UV spectra available in our HPLC-libraries [13] indicated it to be similar to that of the phytoalexin brassilexin (**6**) [16]. However, the R_t of brassilexin at 11.7 min was significantly different from that of the peak in question (20.2 min).

In order to obtain a reasonable amount of elicited tissue to identify the nature of the compound of R_t = 20.2 min unambiguously, white mustard leaves were sprayed with a copper chloride solution and incubated. Necrotic zones started developing on leaves one day after spraying, and increased steadily until the third day. The leaves were excised, necrotic tissues cut out and extracted with ethyl acetate; the HPLC chromatogram of this extract revealed a peak with R_t (20.2 min) and UV spectrum identical to that obtained previously from incubation with destruxin B (**1**). The extract obtained from copper chloride elicited leaves was fractionated and the fractions were analysed by HPLC and thin layer chromatography (TLC) (biodetection employing spores of *Cladosporium cucumericum*) [17]. Two of these fractions showed strong antifungal activity; one of these bioactive fractions showed a major peak with R_t = 20.2 min on the HPLC chromatogram, the other fraction showed a major peak with R_t = 15.3 min. While the antifungal compound with R_t = 15.3 min was also present in extracts of control (non-elicited) leaves, the antifungal compound with R_t = 20.21 min was detected only in extracts of elicited leaves and therefore represented a phytoalexin from white mustard. Each of these bioactive fractions was purified by preparative thin layer chromatography (prep. TLC) to give chromatographically homogeneous materials, whose chemical structures were elucidated as follows.

The NMR spectra of the phytoalexin displayed only aromatic signals, except for a resonance likely due to a methoxyl group (δ_H 4.15 and δ_C 64.8). The ^{1}H NMR spectrum displayed four signals clearly due to an indole moiety as well as an additional singlet at δ_H 8.66; the ^{13}C NMR spectrum corroborated the presence of an indole related system, likely a fused indole-isothiazole ring, as suggested by its UV spectrum. The final assignment of structure **5** to this plant metabolite was secured from HMBC, HMQC, FTIR, and HRMS data analysis. Furthermore, the location of the methoxyl group at N-1 (not at the isothiazole n) follows from nuclear Overhauser effect (NOE) experiments. That is, a positive NOE was observed for the proton at C-7 (δ_H 7.53) on irradiation of the methoxyl group (δ_H 4.15), and similarly, irradiation of the signal at δ_H 7.53 caused an enhancement of the signals due to the methoxyl group and to H-6 (δ_H 7.41). Additionally, irradiation of the proton at C-1′ (δ_H 8.66) caused a positive NOE on the proton at C-4 (δ_H 7.87). Structure **5** represents a new phytoalexin we named sinalexin, produced by *S. alba* under elicitation by *A. brassicae*, destruxin B, or copper chloride.

Structure **7** was assigned to the antifungal compound with R_t = 15.3 min; this was deduced by analysis of the spectroscopic data, and confirmed by synthesis. Namely, the ^{1}H and ^{13}C NMR spectra of **7** displayed signals characteristic of a 4-hydroxybenzyl group and the FTIR spectrum suggested the presence of an isothiocyanate group (2070 cm^{-1}) [18]. Isothiocyanate **7** was prepared from 4-hydroxybenzylamine, upon reaction with thiocarbonyl chloride, as previously described [19]. This compound is a well-known natural product previously isolated from white mustard seedlings and seeds [20], and reported to be responsible for the condiment value of white mustard seeds [21]. Moreover, isothiocyanate **7**, as well as other cruciferous isothiocyanates, are antifungal compounds known to result from enzymatic hydrolysis of the corresponding glucosinolates; that is 4-hydroxybenzylglucosinolate (**10**) is the biogenetic precursor isothiocyanate **7** [22].

In general, cruciferous plants including *Brassica* species produce complex glucosinolate mixtures; interestingly white mustard is known to produce only 4-hydroxybenzylglucosinolate (**10**) [22]. Since indole-



glucosinolates **8** and **9** are putative precursors of brassinins **2** and **3** [23], we anticipated that, in the case of white mustard, compound **10** could be a precursor of the corresponding 4-hydroxybenzyldithiocarbamate **11**. However, compound **11** (prepared from the corresponding amine, *cf.* experimental) was not detected in any of the leaf extracts obtained from white mustard.

Alternaria blackspot is the most destructive fungal disease of brassica oilseeds from a global perspective [4]. None of the commercially available rapeseed and canola cultivars show significant resistance to alternaria blackspot. However, if the blackspot resistance of white mustard is related to the presence of sinalexin (**5**) and/or 4-hydroxybenzylisothiocyanate (**7**), this resistance trait is potentially transferable to canola and other brassicas. In this context, it is worthy to note that production of the phytoalexin brassilexin (**6**) has been correlated with blackleg resistance [24] of the related brown mustard (*B. juncea*) [25].

EXPERIMENTAL

General. All chemicals were purchased from Aldrich Chemical Company, Inc., Madison, WI, or Sigma Chemical Company, St. Louis, MO. All solvents were HPLC grade and used as such. Prep. TLC: (Merck, Kieselgel 60 F_{254}), 20 × 20 cm × 0.25 mm; analytical TLC (Merck, Kieselgel 60 F_{254}, aluminum sheets) 5 × 2 cm × 0.2 mm; compounds were visualized by exposure to UV and by dipping the plates in a 5% aq. phosphomolybdic acid soln containing a trace of ceric sulphate and 4% H_2SO_4, followed by heating at 200°. Flash CC: silica gel Merck, grade 60, mesh size 230–400, 60 Å. HPLC analysis was carried out with a high performance Hewlett-Packard liquid chromatograph equipped with quaternary pump, automatic injector, and diode array detector (wavelength range 190–600 nm), degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 i.d. × 200 mm) equipped with an in-line filter. HPLC mobile phase: 75% H_2O–25% CH_3CN to 100% CH_3CN, for 35 min, linear gradient, and a flow-rate 1.0 ml min^{-1}. Samples for HPLC analysis were dissolved in acetonitrile (*ca* 0.5 mg ml^{-1}) and filtered through a cotton plug. NMR spectra were recorded on a Bruker AMX 500 or AM 300 spectrometer; for ^1H (300 or 500 MHz), δ values were referenced to $CHCl_3$ (7.27 ppm), $CHDCl_2$ (5.32 ppm), or CD_2HCN (1.94 ppm) and for ^{13}C (75.5 or 125.8 MHz) referenced to $CDCl_3$ (77.2 ppm), CD_2Cl_2 (54.0 ppm), or CD_3CN (118.7 ppm). Fourier transform infrared (FTIR) spectra were obtained on a Bio-Rad FTS-40 spectrometer using diffuse reflectance cell. Mass spectra (MS) were obtained on a VG 70 SE mass spectrometer [high resolution (HR), electron impact (EI) or chemical ionization (CI) with NH_3 as carrier gas], employing a solids probe.

Fungal culture and isolation of destruxin B. Alternaria brassicae isolate AB23 was obtained from G. Séguin-Swartz (Agriculture Canada Research Station, Saskatoon, Saskatchewan). The fungal isolate was grown on V8 agar [20% V8 juice, 0.75 g l^{-1} $CaCO_3$, 100 mg l^{-1} streptomycin sulphate, 4 mg l^{-1} Rose Bengal, 15 g l^{-1} agar] plates at 23 ± 2°, 12 hr photoperiod for 15 days. Liquid cultures were initiated by inoculating Fernbach flasks, containing sterile potato dextrose medium (1.68 g 70 ml^{-1}) and leaves (10.5 g cut in small pieces) of *Brassica napus* cv. Westar, with agar plugs (7 plugs with 7 mm diam per flask, 26 flasks). After incubating for 20 days in the dark, the contents of each flask were diluted with H_2O (100 ml/flask) and homogenized in a blender. The homogenate was centrifuged (3000 *g*, 20 min) and the supernatant was filtered through eight layers of cheese cloth. The ppt. was discarded and the filtrates were combined and extracted twice with equal vols of EtOAc; the combined organic layers were dried over Na_2SO_4 and concd under red. press. The residue (460 mg) was fractionated by FCC (hexane–Me_2CO, 70:30, 30 ml frs); frs 11 (8.2 mg), 12 (60.9 mg), 13 (32.2 mg), and 14 (27.7 mg) were combined and fractionated by FCC as previously. The frs containing destruxin B were combined and concd (121 mg); crystallization of this residue from hexane–Me_2CO yielded colourless needles of destruxin B (97.8 mg). The yields of destruxin B (54 mg l^{-1}) obtained by this method are significantly higher than those previously reported (*cf.* ref. 6 yield is *ca* 9 mg l^{-1}; ref. 7 yield is *ca* 27 mg l^{-1}).

Plant material and isolation of antifungal metabolites

Elicitation of sinalexin by Alternaria brassicae *and destruxin B* (**1**). Leaves from 24 day-old plants of *S. alba* cv. Ochre (in growth chamber, 16 hr day/8 hr night, 24 ± 2°) were punctured (3 leaves, 6 punctures/leaf, hypodermic needle, 19 gauge) and each puncture was inoculated with a 10 μl drop of either a spore suspension of *A. brassicae* or a soln of destruxin B (10^{-4} M in 22% aq. CH_3CN). After six days the leaves were excised and the lesions were cut out (#6 cork borer, 10 mm diam). Control tissue was similarly obtained after incubation with either distilled H_2O or 22% aq. CH_3CN for 6 days. The leaf tissue (0.20 g) was frozen in liquid N_2, crushed with a glass rod and extracted with EtOAc (50 ml, shaking for 16 hr). The EtOAc was removed by filtration, dried over Na_2SO_4 and concd under red. pres. The residue was dissolved in CH_3CN and analysed by HPLC.

Isolation of sinalexin (**5**). Sixty-three 27 day-old plants of *S. alba* cv. Ochre (in growth chamber, 16

hr day/8 hr night, 24 ± 2°) were sprayed with $CuCl_2$ solution (1.0×10^{-2} M) and after 3 days of growth, the leaf tissue (366 g) was frozen in liquid N_2 and extracted with EtOAc (3 l) as described above for 16 hr. The EtOAc was removed by filtration, dried over Na_2SO_4 and concd under red. pres. to give 3.66 g of oily residue. The residue was fractionated by FCC (CH_2Cl_2) and frs containing an HPLC peak at $R_f = 20.2$ were combined (25.8 mg) and bioassayed employing a spore culture of *C. cucumericum* isolate DAOM 146982 in double strength potato dextrose broth [17]. These combined frs were fractionated twice by prep. TLC (CH_2Cl_2, R_f 0.23, and hexane–Me_2CO, 7:3, R_f 0.45). The crude sinalexin (2.0 mg) was dissolved in CH_3CN (1 ml) and applied to a small C-18 reversed-phase silica gel (J. T. Baker, 40 μm) column (2 × 10 mm). The column was eluted with CH_3CN (3 × 1 ml), and the frs were combined and concd under red. press. to give sinalexin (**5**) (1.4 mg).

Isolation of 4-*hydroxybenzylisothiocyanate* (**7**). The leaf tissue (76.61 g) from eight 23 day-old *S. alba* plants was frozen in liquid N_2 and extracted with EtOAc (1 l) for 16 hr. The EtOAc was removed by filtration, dried over Na_2SO_4 and concd under red. press. to give an oily residue (426 mg). This residue was fractionated by FCC (CH_2Cl_2–MeOH, 49:1) and the frs were bioassayed employing a *C. cucumericum* spore suspension [17]. Prep. TLC (CH_2Cl_2–EtOAc, 49:1) of the most bioactive fr. (29.1 mg) yielded 4-hydroxybenzylisothiocyanate (23 mg).

Preparation of 4-*hydroxybenzylisothiocyanate* (**7**). Benzyl chloride (1.52 g, 12 mmol), K_2CO_3 (1.66 g, 12 mmol), and KI (33 mg, 0.2 mmol) were added to a soln of 4-hydroxybenzonitrile (1.19 g, 10 mmol) in DMF (10 ml) at 25°. After 2 hr, the reaction mixt. was diluted with H_2O (25 ml), extracted with Et_2O (2 × 30 ml), and the combined organic layers concd at red. pres. The crude product was crystallized from hexane–Et_2O to give 4-benzyloxybenzonitrile (2.03 g, 97%). The crystalline product (1.046 g, 5 mmol) was dissolved in Et_2O (10 ml) and added dropwise to a soln of $LiAlH_4/AlCl_3$ (228 mg, 6 mmol/800 mg, 6 mmol) in Et_2O (20 ml) and kept at 25° for 2 hr [26]. Water (25 ml) was added dropwise to the reaction mixt. and the aq. layer was extracted with Et_2O (30 ml). The combined organic layers were dried over Na_2SO_4 and concd under red. pres. to yield crude amine (1.04 g, 97%). A suspension of the crude amine (200 mg) and 10% Pd/C (50 mg) in EtOH–NH_4OH (10:3 ml) was stirred under H_2 (1 atm) for 2 hr at 0°. The reaction mixt. was filtered, concd under red. pres., and the crude product purified by FCC (CH_2Cl_2–MeOH–NH_4OH, 90:10:1) to give 4-hydroxybenzylamine (96 mg, 0.78 mmol, 83%). A soln of 4-hydroxybenzylamine (10 mg, 0.081 mmol) and Et_3N (24.7 mg, 0.24 mmol) in MeOH (1 ml) was added dropwise over 10 min to a soln of thiocarbonyl chloride (10.3 mg, 0.089 mmol) in $CHCl_3$ (1 ml) at 0°. After 60 min, the reaction mixt. was concd under red. pres., and the product purified by prep. TLC (CH_2Cl_2–EtOAc, 49:1) to yield isothiocyanate **7** (7.8 mg, 58%).

Preparation of methyl 4-*hydroxybenzyldithiocarbamate* (**11**). CS_2 (22.3 mg, 0.293 mmol) was added to a soln of 4-hydroxybenzylamine (30 mg, 0.244 mmol) in pyridine (0.2 ml) and Et_3N (54.4 mg, 0.538 mmol) at 0°. After 60 min at 0°, MeI (41.6 mg, 0.293 mmol) was added to the reaction mixt., and after a further 12 hr at 0° the reaction mixt. was poured onto 1.5 M H_2SO_4 (20 ml) and extracted with Et_2O (2 × 50 ml); the combined organic layers were dried over Na_2SO_4 and concd under red. pres. to give quantitatively a slightly yellow oil which crystallized on standing at −20°.

Sinalexin (**5**). R_f 0.35, hexane–acetone (4:1); ^1H NMR (CD_2Cl_2): δ 8.66 (*s*, H-1′), 7.87 (*m*, H-4), 7.53 (*m*, H-7), 7.41 (*m*, H-6), 7.28 (*m*, H-5), 4.15 (*s*, OCH_3); ^{13}C NMR (CD_2Cl_2): δ 64.8 (*q*, OCH_3), 109.8 (*d*, C-7), 117.8 (*s*, C-3a), 120.9 (*d*, C-4), 122.1 (*d*, C-5), 124.4 (*s*, C-3), 124.8 (*d*, C-6), 141.9 (*s*, C-7a), 148.2 (*d*, C-1′), 156.5 (*s*, C-2); HREIMS *m/z* (% relative abundance) measured: 204.0355 (204.0356 Calcd. for $C_{10}H_8N_2OS$); EIMS *m/z* (% relative abundance): 204 $[M]^+$ (87), 205 (10), 189 (27), 174 (24), 173 (100), 149 (43), 146 (38), 129 (24), 102 (13); FTIR ν_{max}: 2935, 1500, 1472, 1460, 1252, 1132, 752, 742 cm^{-1}.

4-*Hyroxybenzylisothiocyanate* (**7**). R_f 0.27, CH_2Cl_2–EtOAc, (49:1); ^1H NMR ($CDCl_3$): δ 7.20 (*m*, 2H), 6.85 (*m*, 2H), 4.63 (*s*, 2H); ^{13}C NMR ($CDCl_3$): 48.5 (*t*), 116.8 (*d*), 126.8 (*s*), 155.8 (*s*); HREIMS *m/z* (% relative abundance) measured: 165.0249 (165.0247 calcd. for C_8H_7NOS); EIMS *m/z* (% relative abundance): 165 $[M]^+$ (8), 107 (100), 106 (25), 78 (22), 77 (17), 59 (14), 52 (10); FTIR ν_{max}: 3419, 2172, 2091, 1613, 1599, 1515, 1439, 1345, 1172, 822 cm^{-1}.

Methyl 4-*hydroxybenzyldithiocarbamate* (**11**). R_f 0.35, CH_2Cl_2–MeOH, (19:1); ^1H NMR (CD_3CN): δ 7.15 (*m*, 2H), 6.91 (*s*, 1H, D_2O exchangeable), 6.75 (*m*, 2H), 4.75 (*d*, $J = 5.6$ Hz, CH_2), 2.53 (*s*, SCH_3); ^{13}C NMR (CD_3CN): δ 18.3 (*q*), 50.5 (*t*), 118.4 (*d*), 129.4 (*s*), 130.6 (*d*), 157.3 (*s*), 199.6 (*s*); HREIMS *m/z* (% relative abundance) measured: 213.0282 (213.0282 calcd. for $C_9H_{11}NOS_2$); EIMS *m/z* (% relative abundance): 213 $[M]^+$ (35), 107 (100); FTIR ν_{max}: 3330, 1515, 1380, 1328, 1214, 916 cm^{-1}.

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