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Metabolism and Metabolites of Dithiocarbamates in the Plant Pathogenic Fungus *Leptosphaeria maculans*

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Supporting Information

ABSTRACT: Synthetic compounds containing a dithiocarbamate group are known to have a variety of biological effects and applications including antifungal, herbicidal, and insecticidal application. *Leptosphaeria maculans* is a fungal pathogen of crucifers able to detoxify efficiently the only plant natural product containing a dithiocarbamate group, the phytoalexin brassinin. To evaluate the effects of dithiocarbamates on *L. maculans*, a number of structurally diverse *S*-methyl dithiocarbamates containing indolyl, biphenyl, and benzimidazolyl moieties were synthesized, and their antifungal activities and metabolism by *L. maculans* were investigated. All dithiocarbamates were transformed by *L. maculans* through hydrolysis to the corresponding amines, which were less antifungal than the parent compounds. Two dithiocarbonates were shown to be much less antifungal than the corresponding dithiocarbamates. Results of this investigation indicate that *S*-methyl dithiocarbamates are not useful inhibitors of *L. maculans* and that their rates of transformation by *L. maculans* did not correlate with the antifungal activity of the particular compound.

KEYWORDS: *antifungal, Brassicaceae, biotransformation, brassinin, crucifer, detoxification, dithiocarbamate, Leptosphaeria maculans, Phoma lingam, phytoalexin*

INTRODUCTION

Crop protectants against microbial diseases have been used in agriculture for more than a century. The early fungicides included salts of copper and mercury and sulfur dust.[1] The first patent awarded to Tisdale and Williams in 1934 represents a landmark related to the development of dithiocarbamates as fungicides, which were until then used as vulcanization accelerators in the rubber industry.[1] Besides fungicidal activity, dithiocarbamates are known to have a variety of biological effects and applications that include herbicidal and insecticidal activity, regulation of apoptosis, pro- and antioxidant effects, and therapy of alcohol aversion.[2] The discovery of the first plant natural product containing a dithiocarbamate group, brassinin (**1**), established a curious link to the use of dithiocarbamates in crop protection. Brassinin (**1**) is a phytoalexin produced by cruciferous species, mainly oilseeds such as canola (*Brassica napus* L., *Brassica rapa* L., *Brasssica juncea* L.) and rapeseed (*B. napus*, *B. rapa*, *B. juncea*) and horticultural crops, most of which belong to *Brassica* species.[3] Brassica crops are of enormous importance worldwide as major sources of vegetable oils, food, feed, and fuel. In addition to brassinin (**1**), brassicas produce phytoalexins such as cyclobrassinin (**2**) and brassilexin (**3**), which are biosynthetically derived from brassinin (**1**) and camalexin (**4**, Figure 1). Somewhat surprisingly, to date no other plant species have been reported to produce metabolites containing dithiocarbamates.

Although an important part of the defensive mechanisms of plants involves phytoalexins, these metabolites can be counteracted by detoxifying enzymes produced by phytopathogenic fungi.[4] In this connection, brassinin (**1**) was investigated and found to be detoxified by diverse fungal species, including host-specific and nonspecific species. The blackleg fungus

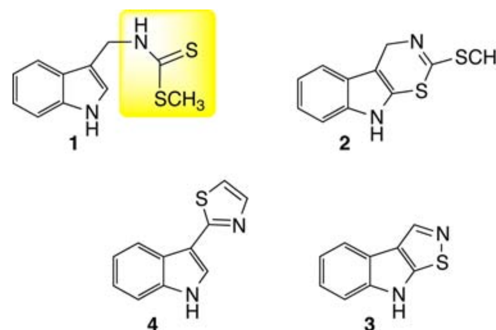

Figure 1. Chemical structures of phytoalexins from crucifers: brassinin (**1**), cyclobrassinin (**2**), brassilexin (**3**), and camalexin (**4**).

[*Leptosphaeria maculans* (Desm.) Ces. et de Not. [asexual stage *Phoma lingam* (Tode ex Fr.) Desm.], one of the major pathogens of canola (*B. napus* L., *B. rapa* L.), is able to detoxify brassinin (**1**) by converting it to indole-3-carboxaldehyde (**6**, Figure 2).[5] All other fungal species investigated so far (*Alternaria brassicicola*, *Leptosphaeria biglobosa*, *L. maculans* (isolates virulent on brown mustard, *B. juncea* L.) and *Botrytis cinerea*) converted brassinin (**1**) to the corresponding amine **9**, followed by acetylation (Figure 2).[3] The enzyme involved in the transformation of brassinin (**1**) to aldehyde **6**, brassinin oxidase (BOLm), was purified to homogeneity and shown to be inducible and substrate specific.[5] Screening of more than 60 compounds for inhibition of BOLm showed that the

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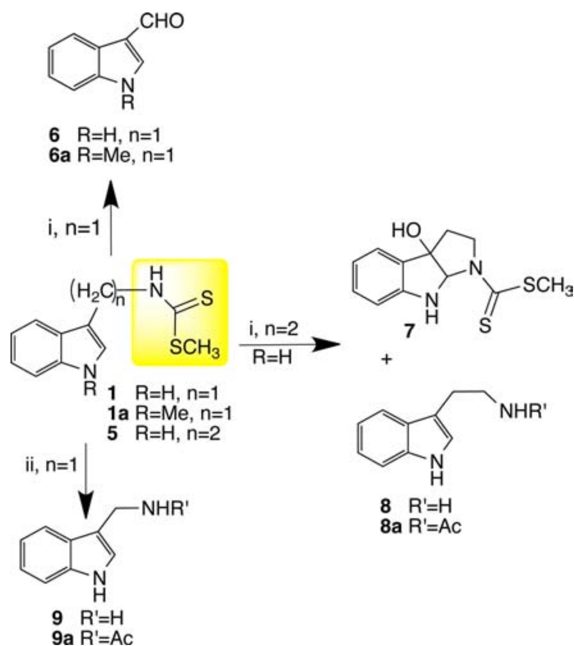

Figure 2. Biotransformation of brassinin (**1**) by plant pathogenic fungi: (i) *Leptosphaeria maculans* (isolates virulent on canola); (ii) *Alternaria brassicicola*, *L. biglobosa*, and *L. maculans* (isolates virulent on brown mustard) and *Botrytis cinerea*.

phytoalexins cyclobrassinin (**2**), brassilexin (**3**), and camalexin (**4**) were competitive inhibitors.[5] The best synthetic inhibitors of BOLm developed to date possess a scaffold based on camalexin[6] or brassilexin (**3**).[7] The enzymes involved in the hydrolysis of brassinin (**1**), brassinin hydrolases (BHs), were isolated from *L. maculans* (BHLm, from isolates virulent on brown mustard) and from *A. brassicicola* (BHAb);[8] both hydrolases were substrate specific and were inhibited competitively by the phytoalexin cyclobrassinin (**2**) but not by camalexin.

1-Methylbrassinin (**1a**) was transformed both in cultures of *L. maculans* and by BOLm to the corresponding aldehyde **6a** at a rate similar to that of brassinin (**1**). *S*-Methyl tryptamine dithiocarbamate (**5**), a homologue of brassinin (**1**), was not a substrate of BOLm, but in cultures of *L. maculans* dithiocarbamate **5** was transformed via different reactions: (i) oxidative cyclization to dithiocarbamate **7** (not transformed further) and (ii) hydrolysis to tryptamine (**8**) (Figure 2).[9] These results suggest that *L. maculans* produces in culture, in addition to BOLm, other enzymes that catalyze the hydrolysis of dithiocarbamates and the oxidation of the indole nucleus. Previously, three benzyl dithiocarbamates were also hydrolyzed by *L. maculans* to the corresponding amines, which were then acetylated in culture.[10] That is, except for brassinin (**1**), the transformation of dithiocarbamates by *L. maculans* appears to occur via hydrolysis to amines; in all examples, these transformations were detoxification reactions.

Considering the broad use of dithiocarbamates in agriculture, and the need to design inhibitors of brassinin degradation by *L. maculans*, it was of interest to investigate the potential metabolism of structurally diverse dithiocarbamates. Here we report that indolyl, biphenyl, and benzimidazolyl dithiocarbamate transformations in *L. maculans* are mediated by hydrolases, the products of which are amines. All amines are less toxic to the fungus than the corresponding dithiocarbamates; that is, all hydrolytic transformations occurring in cultures of *L. maculans* are detoxifications.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON, Canada; solvents were of HPLC grade and used as such. Flash column chromatography (FCC) was carried out using silica gel grade 60, mesh size 230−400 Å or WP C_{18} prepscale bulk packing 275 Å (J. T. Baker, Phillipsburg, NJ, USA).

Instruments. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker 500 MHz Avance spectrometers, for ^1H at 500.3 MHz and for ^{13}C at 125.8 MHz; chemical shifts (δ) are reported in parts per million (ppm) relative to TMS; spectra were calibrated using the solvent peaks; spin coupling constants (J) are reported to the nearest 0.5 Hz. Fourier transform infrared (FT-IR) data were recorded on a Bio-Rad FTS-40 spectrometer, and spectra were measured by the diffuse reflectance method on samples dispersed in KBr. MS [high-resolution (HR), electron impact (EI)] were obtained on a VG 70 SE mass spectrometer employing a solids probe.

HPLC analysis was carried out with Agilent high-performance liquid chromatographs equipped with quaternary pump, automatic injector, diode array detector (DAD, wavelength range 190−600 nm), and degasser. For method A (for neutral extracts), the column used was a 150 mm × 4.6 i.d., 5 μm, Eclipse XDB-C18 (Agilent), having an in-line filter, and mobile phase 50% H_2O/50−100% MeOH, for 25.0 min, linear gradient, at a flow rate of 0.75 mL/min. For method B (for amines and basic extracts), the column used was a 100 mm × 3.0 i.d., 3.5 μm, Zorbax ODS (with both solvents containing 0.01% *n*-propanamine) 40% H_2O/60−100% MeOH, for 10.0 min, linear gradient, at a flow rate of 0.50 mL/min.

Synthesis and Characterization of New Compounds. All synthetic compounds were purified using flash column chromatography (FCC) on silica gel; satisfactory spectroscopic data identical to those previously reported were obtained for all reported compounds. Organic extracts were dried over Na_2SO_4. Syntheses of brassinin[11] isobrassinin (**1**),[12] 1-methoxyisobrassinin (**13a**),[12] compounds **5**,[13] **11**,[14] and **20**,[11] were carried out as reported in the respective publications, and compounds **16**, **18**, and **21** were prepared as reported below and summarized in Figure 3.

*S-Methyl 4-Biphenyl-4-methyl Dithiocarbamate (**16**).* A solution of $NH_2OH \cdot HCl$ (757 mg, 11.0 mmol) and Na_2CO_3 (873 mg, 8.23 mmol) in water (4 mL) was added to a solution of 4-biphenylcaboxaldehyde (**15**, 1000 mg, 5.48 mmol) in EtOH (10 mL) and heated to reflux for 30 min. The reaction mixture was concentrated to one-third, diluted with water, and extracted with EtOAc. The combined organic extract was concentrated to yield a mixture of (*E*,*Z*)-4-biphenylcarboxaldoximes (1049 mg, 5.32 mmol) in 97% yield. $NaBH_4$ (234 mg, 6.18 mmol) and $NiCl_2 \cdot 6H_2O$ (245 mg, 1.03 mmol) were added to a cooled solution of the oximes (202 mg, 1.03 mmol) in MeOH (8 mL), and the reaction mixture was stirred at room temperature. After 15 min, the reaction mixture was diluted with 1% NH_4OH (30 mL) and filtered. The filtrate was extracted with EtOAc (3 × 40 mL), and the organic extract was combined, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was separated by FCC (SiO_2; MeOH/CH_2Cl_2/NH_4OH, 20:80:1, v/v/v) to afford 4-biphenylmethanamime (**24**, 170 mg, 0.93 mmol) in 91% yield. CS_2 (68 μL, 1.13 mmol) and MeI (88 μL, 1.40 mmol) were added to a solution of amine **24** (170 mg, 0.94 mmol) and triethylamine (256 μL, 1.88 mmol) in pyridine (2 mL) and stirred at room temperature. After 30 min, the reaction mixture was diluted with toluene and concentrated under reduced pressure. The residue was separated by FCC (SiO_2, EtOAc/hexane, 20:80, v/v) to yield **16** (185 mg, 72%, 0.68 mmol): mp 84 °C; HPLC t_R = 23.9 min (method A); ^1H NMR (500 MHz, $CDCl_3$) δ 7.60 (d, J = 8 Hz, 4H), 7.48 (dd, J = 8, 8 Hz, 2H), 7.42−7.38 (m, 3H), 7.27 (br s, NH), 4.98 (d, J = 5 Hz, 2H), 2.69 (s, 3H); signals for a minor rotamer were found at δ 4.66 and 2.72; ^{13}C NMR (125 MHz, $CDCl_3$) δ 199.5, 141.3, 140.7, 135.4, 129.1, 128.9, 127.8, 127.7, 127.3, 51.1, 18.5; FT-IR (KBr) υ_{max} (cm^{-1}) 3339, 3224, 2919, 1486, 1328, 1088, 926, 761, 697; UV (HPLC,

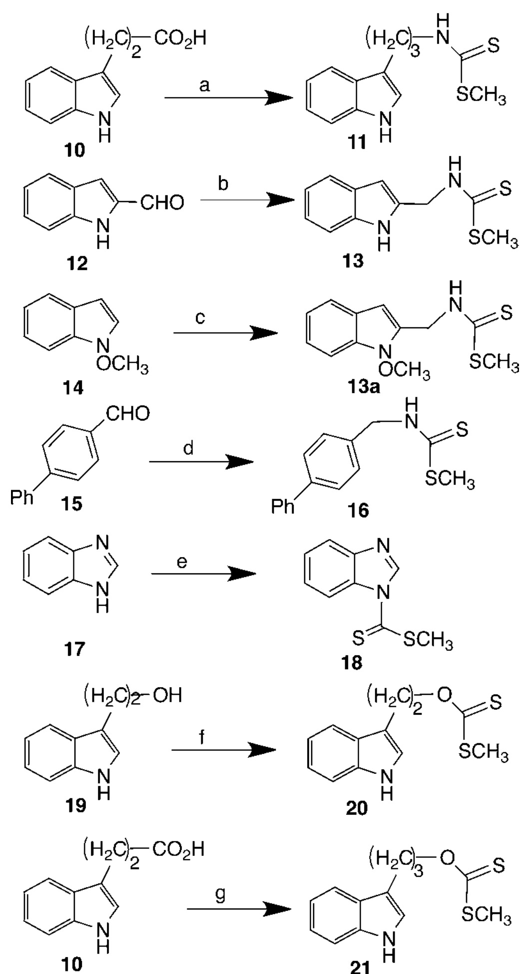

Figure 3. Syntheses of dithiocarbamates and dithiocarbonates. Reagents and reaction conditions: (a) (i) EtOH, H_2SO_4, reflux, 96%; (ii) DiBAl-H, toluene, −78 °C, 90%; (iii) $NH_2OH·HCl$, Na_2CO_3, EtOH/H_2O, 97%; (iv) $NaBH_4$, $NiCl_2·6H_2O$, MeOH; (v) Pyr, Et_3N, CS_2, CH_3I, 43%; (b) (i) EtOH, H_2SO_4, reflux, 87%; (ii) LAH, THF, 0 °C; (iii) MnO_2, DCM, 80%; (iv) $NH_2OH·HCl$, Na_2CO_3, EtOH/H_2O, 94%; (v) $NaBH_4$, $NiCl_2·6H_2O$, MeOH; (vi) Pyr, Et_3N, CS_2, CH_3I, 44%; (c) (i) *t*-BuLi, THF, −78 °C; (ii) DMF, 86%; (iii) $NH_2OH·HCl$, Na_2CO_3, EtOH/H_2O, 93%; (iv) $NaBH_3(CN)$, $TiCl_3$, NH_4OAc, MeOH; (v) Pyr, Et_3N, CS_2, CH_3I, 51%; (d) (i) $NH_2OH·HCl$, Na_2CO_3, EtOH/H_2O, 97%; (ii) $NaBH_4$, $NiCl_2·6H_2O$, MeOH, 91%; (iii) Pyr, Et_3N, CS_2, CH_3I, 72%; (e) (i) NaH, THF, 0 °C; (ii) CS_2, CH_3I, 72%; (f) (i) NaH, THF, CS_2; CH_3I, 90%; (g) (i) EtOH, H_2SO_4, reflux, 96%; (ii) LAH, THF, 0 °C, 95%; (iii) NaH, THF, 0 °C; (iv) CS_2, CH_3I, 73%.

MeOH/H_2O) λ_{max} (nm) 204, 260; HRMS-EI calcd 273.0646 for $C_{15}H_{15}NS_2$ ([M]$^+$), found 273.0646; MS (EI) m/z (% relative intensity) 273 [M$^+$] (16), 225 (20), 167 (100).

*S-Methyl Benzimidazolyl Dithiocarbamate (**18**).* A solution of benzimidazole (**17**, 100 mg, 0.85 mmol) in THF (1 mL) was added to a stirred suspension of NaH (26 mg, 1.1 mmol) in THF (1 mL). After 5 min, CS_2 (61 μL, 1.02 mmol) and MeI (80 μL, 1.27 mmol) were added to the reaction mixture at room temperature, which was further stirred for 20 min; the reaction mixture was diluted with ice-cold water and extracted with EtOAc. The combined organic extract was concentrated under reduced pressure to yield a residue that was separated by FCC (SiO_2; EtOAc/hexane, 20:80, v/v) to yield *S*-methyl benzimidazolyl dithiocarbamate (**18**, 126 mg, 0.61 mmol) as a yellow solid in 72% yield: mp 63 °C (lit. 58 °C,[15]); HPLC t_R = 11.2 min (method A); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.88 (s, 1H), 8.54 (d, *J* = 7.5 Hz, 1H), 7.81 (d, *J* = 8.5 Hz, 1H), 7.44−7.38 (m, 2H), 2.85 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 199.1, 145.5, 141.8, 133.0, 125.9, 125.3, 121.2, 115.8, 20.1; FT-IR (KBr) ν_{max} (cm^{-1}) 3072, 1511, 1446, 1350, 1277, 1221, 1182, 1048, 1018, 825, 742; UV (HPLC, MeOH/H_2O) λ_{max} (nm) 210, 262, 315; HRMS-EI calcd 208.0129 for $C_9H_8N_2S_2$ ([M]$^+$), found 208.0127; MS (EI) m/z (% relative intensity) 208 [M$^+$] (100), 161 (64), 134 (27), 91 (100), 63 (8).

*S-Methyl 3-(3-Indolyl)propyl Dithiocarbonate (**21**).* A solution of acid **10** (500 mg, 2.67 mmol) in EtOH and H_2SO_4 was heated to reflux for 20 h, and the solvent was removed under vacuum. The reaction mixture was diluted with water and extracted with EtOAc, the combined organic extract was concentrated, and the crude reaction mixture was separated by FCC (SiO_2; EtOAc/hexane, 10:90, v/v) to yield ethyl indolyl-3-propanoate (545 mg, 2.51 mmol) in 96%. LAH (116 mg, 3.06 mmol) was added in portions to a cooled solution of the ethyl ester (550 mg, 2.55 mmol) in dry THF (5 mL), and the reaction mixture was stirred at room temperature for 40 min. The reaction mixture was quenched with NaOH solution (1 M, 5 mL), and the precipitate was vacuum filtered. The filtrate was extracted with EtOAc, and the combined organic extract was concentrated using a rotary evaporator under reduced pressure. The residue was separated by FCC (SiO_2; 30% EtOAc/hexane) to afford indolyl-3-propanol (420 mg, 2.40 mmol) as a pale yellow oil in 95% yield. A solution of indolyl-3-propanol (30 mg, 0.17 mmol) in THF (0.5 mL) was added to a suspension of NaH (6 mg, 0.21 mmol) in THF (2 mL) and stirred at room temperature for 5 min. Next, CS_2 (19 μL, 0.31 mmol) followed by MeI (20 μL, 0.31 mmol) was added, and the reaction mixture was stirred at room temperature. After 20 min, the reaction mixture was diluted with ice-cold water and extracted with EtOAc; the combined organic extract was concentrated under reduced pressure using a rotary evaporator to yield a residue that was separated by FCC (SiO_2; EtOAc/hexane, 20:80, v/v) to afford *S*-methyl 3-(3-indolyl)propyl dithiocarbonate (**21**, 33 mg, 0.12 mmol) as a yellow viscous material in 72% yield: HPLC t_R = 21.4 min (method A); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.94 (br s, NH), 7.64 (d, *J* = 8 Hz, 1H), 7.38 (d, *J* = 8 Hz, 1H), 7.25 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.17 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.01 (s, 1H), 4.69 (t, *J* = 6.5 Hz, 2H), 2.94 (t, *J* = 7.5 Hz, 2H), 2.60 (s, 3H), 2.25 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 216.1, 136.5, 127.4, 122.2, 121.7, 119.4, 118.9, 115.1, 111.3, 73.8, 28.7, 21.6, 19.1; FT-IR (KBr) ν_{max} (cm^{-1}) 3414, 2921, 1456, 1223, 1056, 742; UV (HPLC, MeOH/H_2O) λ_{max} (nm) 220, 278; HRMS-EI calcd 265.0595 for $C_{13}H_{15}NOS_2$ ([M]$^+$), found 265.0587; MS (EI) m/z (% relative intensity) 265 [M$^+$] (33), 232 (52), 218 (32), 157 (52), 130 (100).

General Procedure for Acetylation of Amines 22−25. Acetic anhydride (1.1 equiv) was added to a solution of each amine (1 equiv) and pyridine (1.5 equiv) in CH_2Cl_2 (2 mL) at 0 °C and stirred at room temperature, as described below. After complete conversion of the starting material, the reaction mixture was diluted with toluene (5 mL) and concentrated using a rotary evaporator to afford crude substituted acetamides that were separated by FCC (SiO_2; EtOAc).

*N_b-Acetyl-2-indolylmethanamine (**22a**).* Acetylation of indolyl-2-methanamine (**22**, 30 mg, 0.20 mmol) using the above procedure afforded compound **22a** (29 mg, 0.15 mmol) in 75% yield: mp 109 °C; HPLC t_R = 6.2 min (method A); ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.05 (br s, NH), 7.57 (d, *J* = 8 Hz, 1H), 7.33 (d, *J* = 8 Hz, 1H), 7.17 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.09 (dd, *J* = 7.5, 7.5 Hz, 1H), 6.32 (s, 1H), 6.18 (br s, NH), 4.45 (d, *J* = 6 Hz, 2H), 2.00 (s, 3H);^{13}C NMR (125 MHz, $CDCl_3$) δ 171.9, 136.6, 127.8, 122.2, 120.5, 119.9, 111.3, 100.7, 37.6, 23.2; FT-IR (KBr) ν_{max} (cm^{-1}) 3392, 3269, 3060, 1651, 1531, 1456, 1423, 1288, 749; UV (HPLC, MeOH/H_2O) λ_{max} (nm) 219, 272; HRMS-EI m/z measured 188.0947 ([M] $^+$, calcd 188.0950 for $C_{11}H_{12}N_2O$); MS (EI) m/z (% relative intensity) 188 [M$^+$] (100), 145 (46), 130 (40), 118 (51).

*N_b-Acetyl-1-methoxy-2-indolylmethanamine (**23a**).* Acetylation of 1-methoxy-2-indolylmethanamine (**23**, 209 mg, 1.19 mmol) using the general procedure afforded compound **23a** (223 mg, 1.02 mmol) in 84% yield as a pale yellow semisolid: HPLC t_R = 8.9 min (method A); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.52 (d, *J* = 8 Hz, 1H), 7.39 (d, *J* = 8 Hz, 1H), 7.24 (dd, *J* = 8, 8 Hz, 1H), 7.11 (dd, *J* = 8, 8 Hz, 1H), 6.32 (br s, NH), 6.24 (s, 1H), 4.58 (d, *J* = 6 Hz, 2 H), 4.04 (s, 3H), 1.98 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.3, 133.3, 132.7, 123.7,

122.5, 121.0, 120.5, 108.2, 97.4, 65.5, 34.7, 23.2; FT-IR (KBr) ν_{max} (cm^{-1}) 3275, 3060, 2940, 1655, 1541, 1452, 1288, 971, 744; UV (HPLC, MeOH/H_2O) λ_{max} (nm) 220, 270; HRMS-EI m/z measured 218.1050 ([M] $^+$, calcd 218.1055 for $C_{12}H_{14}N_2O_2$); MS (EI) m/z (% relative intensity) 218 [M^+] (28), 187 (44), 169 (27), 145 (100), 129 (12), 118 (70), 89 (14).

*N_b-Acetyl-3-(3-indolyl)propanamine (**24a**).* Acetylation of 3-(3-indolyl)propanamine (**24**, 150 mg, 0.867 mmol) using the above procedure afforded compound **24a** (138 mg, 0.64 mmol) in 74% yield as yellowish crystals: mp 93−95 °C (lit.[16] 97−99 °C); HPLC t_R = 21.4 min (method A); ^1H NMR (500 MHz, $CDCl_3$) δ 8.54 (br s, NH), 7.58 (d, J = 8 Hz, 1H), 7.34 (d, J = 8 Hz, 1H), 7.20 (dd, J = 8, 7 Hz, 1H), 7.12 (dd, J = 8, 7 Hz, 1H), 6.90 (s, 1H), 5.86 (br s, NH), 3.28 (m, 2H), 2.77 (t, J = 7 Hz, 2H), 1.91 (s, 3H), 1.89 (quint, J = 7 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.6, 136.6, 127.4, 121.9, 121.8, 119.1, 118.8, 115.2, 111.5, 39.7, 29.8, 23.3, 22.7; FT-IR (KBr) ν_{max} (cm^{-1}) 3403, 3283, 2930, 1652, 1550, 1456, 743; UV (HPLC, MeOH/H_2O) λ_{max} (nm) 221, 282; HRMS-EI m/z measured 216.1266 ([M] $^+$, calcd 216.1263 for $C_{13}H_{16}N_2O$); MS (EI) m/z (% relative intensity) 216 [M^+] (39), 157 (35), 144 (37), 130 (100).

*N-Acetyl-4-biphenylmethanamine (**25a**).* Acetylation of 4-biphenylmethanamine (225 mg, 1.24 mmol) using the above procedure afforded compound **25a** (**25**, 251 mg, 1.11 mmol) in 90% yield: darkening 158 °C; mp 184 °C (lit.[17] 180−182 °C); HPLC t_R = 18.3 min (method A); ^1H NMR (500 MHz, $CDCl_3$) δ 7.64 (d, J = 8 Hz, 2H), 7.61 (d, J = 8 Hz, 2H), 7.46 (dd, J = 7, 8 Hz, 2H), 7.36 (m, 3H), 6.85 (br s, NH), 4.36 (d, J = 6 Hz, 2H), 1.92 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.0, 142.0, 140.9, 140.3, 130.3, 129.3, 128.7, 128.3, 128.2, 43.6, 23.4; FT-IR (KBr) ν_{max} (cm^{-1}) 3292, 3091, 1638, 1554, 1443, 1373, 1294, 1094, 756; UV (HPLC, MeOH/H_2O) λ_{max} (nm) 206, 252; HRMS-EI m/z measured 225.1148 ([M] $^+$, calcd 225.1154 for $C_{15}H_{15}NO$); MS (EI) m/z (% relative intensity) 225 [M^+] (100), 182 (66), 167 (34), 106 (20).

Antifungal Bioassays and Metabolism of Dithiocarbamates and Dithiocarbonates by *Leptosphaeria maculans*. The antifungal activity of compounds was determined using a mycelial radial growth bioassay.[11] In brief, isolates of *L. maculans* (isolate BJ-125 or UAMH-9410) were grown on V8 agar plates. Potato dextrose agar (PDA) sterile tissue culture 6-well plates (33 mm diameter) containing 0.50, 0.20, and 0.10 mM of each test compound (dissolved in CH_3CN) were used; control plates contained only 1% CH_3CN in PDA. Wells were inoculated with 4 mm mycelial plugs, cut from 7-day-old V8 agar plates of *L. maculans*, placed upside down on the center of each plate and incubated under constant light for 5 days. Mycelial growth (diameter) in each treatment was measured, and the percent inhibition was calculated according to the following equation: % inhibition = 100 − [(growth diameter on amended medium/growth diameter in control medium) × 100]. All bioassay experiments were carried out in triplicate, at least twice.

For investigation of metabolic transformations, Erlenmeyer flasks (150 mL) containing minimal medium[10] (50 mL) were inoculated with spores of *L. maculans* (isolate BJ-125, 10^6/mL) and incubated at 23 °C on a shaker at 120 rpm under constant light. After 2 days, compounds dissolved in CH_3CN were added to the cultures (final concn 0.10 mM) as well as to noninoculated medium (stability control). Samples (2 or 5 mL) were withdrawn immediately and at intervals up to 72 h and were either frozen or immediately extracted with EtOAc, the organic extracts were concentrated, and the residue was dissolved in CH_3CN and analyzed by HPLC using method A. The extracted broth was basified by adding NH_4OH (pH ≥9) and extracted with $CHCl_3$/MeOH (95:5). The concentrated extract was analyzed by HPLC using method B. Calibration curves of each compound were built for quantitative analysis using HPLC-DAD.

RESULTS AND DISCUSSION

Chemical Synthesis. Syntheses of brassinin (**1**) and dithiocarbamates **5**, **11**, **13**, **13a**, and **16** were accomplished by treatment of the corresponding amines with CS_2, followed by methylation with MeI as summarized in Figure 3. Syntheses of dithiocarbonates **20** and **21** were carried out by treatment of the corresponding alcohols with carbon disulfide, followed by methylation with methyl iodide, as summarized in Figure 3.

Antifungal Activity. The antifungal activity of each compound against *L. maculans* was determined, and results are summarized in Table 1. *S*-Methyl benzimidazolyl dithiocarbamate (**18**) and *S*-methyl tryptamine dithiocarbamate (**5**) were the most potent mycelial growth inhibitors among all compounds tested, displaying significantly higher inhibitory activity than brassinin (**1**). The inhibitory activity of dithiocarbonates **20** and **21** was similar to that of 1-methoxyisobrassinin (**13a**) and the lowest among all

Table 1. Antifungal Activities of Brassinin, Dithiocarbamates, and Dithiocarbonates against *Leptosphaeria maculans*

compound	inhibition ± SD (%)[a]		
	0.50 mM	0.20 mM	0.10 mM
brassinin (**1**)	55 ± 3 d	21 ± 3 fg	10 ± 4 def
S-methyl tryptamine dithiocarbamate (**5**)	ci b	71 ± 3 c	43 ± 2 b
S-methyl 3-(3-indolyl)propyl dithiocarbamate (**11**)	67 ± 3 c	46 ± 5 d	21 ± 5 c
isobrassinin (**13**)	43 ± 4 e	22 ± 4 f	13 ± 3 de
1-methoxyisobrassinin (**13a**)	34 ± 2 f	15 ± 3 g	7 ± 3 e,f
S-methyl 4-biphenyl-4-methyl dithiocarbamate (**16**)	46 ± 4 e	32 ± 4 e	14 ± 4 d
S-methyl benzimidazolyl dithiocarbamate (**18**)	ci b	85 ± 3 b	43 ± 5 b
S-methyl 3-(3-indolyl)propyl dithiocarbonate (**20**)	32 ± 4 f	15 ± 2 g	7 ± 3 ef
S-methyl tryptophol dithiocarbonate (**21**)	31 ± 2 f	18 ± 2 fg	5 ± 3 f

[a]The percentage of inhibition was calculated using the following formula: % inhibition = 100 − [(diameter of mycelia on amended/diameter of mycelia on control) × 100]; values are averages of three independent experiments conducted in triplicate; ci = complete inhibition. For statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; n = 6; entries with the same letters (b−f) within each column are not significantly different (P < 0.05).

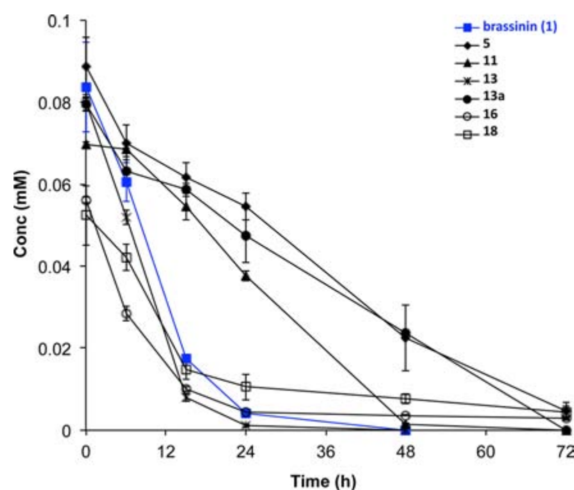

Figure 4. Progress curves for transformation of brassinin (**1**) and dithiocarbamates **5**, **11**, **13**, **13a**, **16**, and **18** by *Leptosphaeria maculans*. Concentrations were determined by HPLC using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

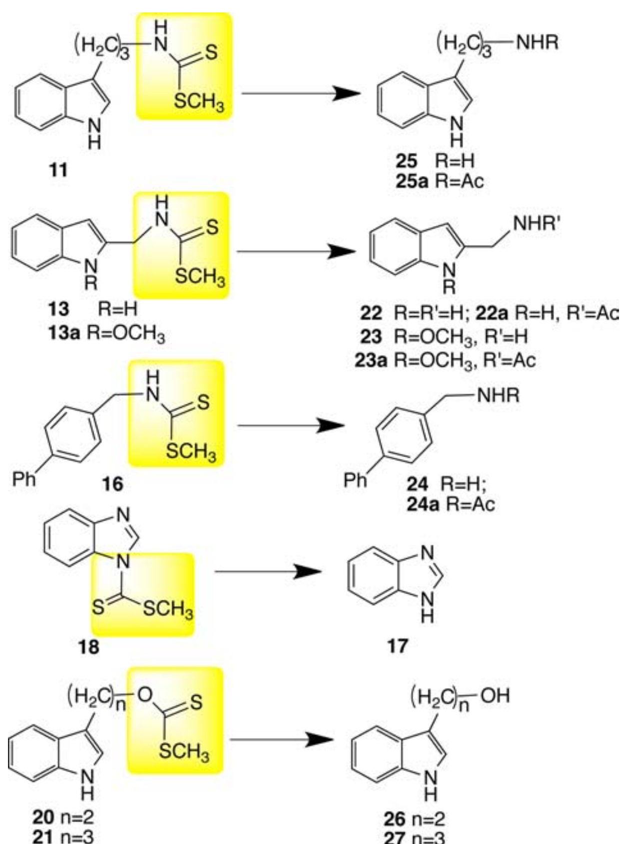

Figure 5. Biotransformation reactions of synthetic dithiocarbamates **11**, **13**, **13a**, **16**, and **18** and dithiocarbonates **20** and **21** by *Leptosphaeria maculans* (isolates virulent on canola).

compounds; however, dithiocarbamate **11** was also more active than **1**.

Metabolism of Dithiocarbamates 5, 11, 13, 13a, 16, and 18 and Dithiocarbonates 20 and 21. To determine if synthetic dithiocarbamates **5**, **11**, **13**, **13a**, **16**, and **18** and dithiocarbonates **20** and **21** were metabolized by *L. maculans*, each compound dissolved in acetonitrile was added to cultures and to noninoculated media (stability control) to obtain a final concentration of 0.10 mM. HPLC analysis of neutral and basic extracts after different incubation times revealed that all compounds were hydrolyzed by the fungus in the medium, albeit at different rates (all compounds were stable in medium alone, giving comparable recoveries). Progress curves for the transformation of each dithiocarbamate (Figure 4) indicated that tryptamine dithiocarbamate (**5**), 3-(3-indolyl)propyl dithiocarbamate (**11**), and 1-methoxyisobrassinin (**13a**) were metabolized substantially more slowly than brassinin (**1**), whereas isobrassinin (**13**), 4-biphenyl-4-methyl dithiocarbamate (**16**), and benzimidazolyl dithiocarbamate (**18**) were metabolized at rates similar to that of brassinin (**1**) (Figures 4 and 5). Dithiocarbonate **20** was metabolized to the corresponding alcohol **26** at a somewhat slower rate than **21** (24 vs 48 h, respectively), which was metabolized to **27**. Except for brassinin (**1**), which yielded aldehyde **6**, all dithiocarbamates were hydrolyzed to the corresponding amines; amines **22**−**25** were further transformed to the corresponding acetyl derivatives **22a**−**25a**, whereas benzimidazole (**17**) was not transformed further and remained in culture for the duration of the experiment (72 h) (Figures 4 and 5). In addition, as previously shown,[9] dithiocarbamate **5** was mainly transformed through two pathways that yielded dithiocarbamate **7** and tryptamine (**8**). Dithiocarbamate **7** remained in culture for the duration of the experiment, thus appearing to be the only dithiocarbamate that *L. maculans* is unable to transform. These studies, that is, products of each transformation, amounts, and reaction times, are summarized in Table 2.

To establish if the transformations of dithiocarbamates **5**, **11**, **13**, **13a**, **16**, and **18** and dithiocarbonates **20** and **21** by *L. maculans* were detoxifications, the products of each transformation, that is, amines **22**, **23**, and **25**, acetyl amines **22a**, **23a**, and **25a**, benzimidazole (**17**), and alcohols **26** and **27** were assayed for mycelial growth inhibition of *L. maculans* (Table 3). All of these products were less inhibitory than the parent compounds; 4-biphenyl-4-methanamine (**24**) was not soluble in the agar medium and thus could not be evaluated. Considering the substantially higher inhibitory activity of dithiocarbamates **5**, **11**, **13**, **13a**, **16**, and **18** than those of their biotransformation products, it is concluded that *L. maculans* is able to detoxify these dithiocarbamates to less toxic products. On the other hand, the inhibitory activities of the products of transformation of dithiocarbonates **20** and **21** were similar to those of the parent compounds.

Table 2. Products of Metabolism of Dithiocarbamates and Dithiocarbonates in Cultures of *Leptosphaeria maculans*

compound	recovery after 24 h (%)	major products of metabolism, molar %[a] (time)
brassinin (**1**)	<5	indole-3-carboxaldehyde (**6**), 40% (15 h)
S-methyl tryptamine dithiocarbamate (**5**)	55	methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-*b*]indol-1(2*H*)-yl carbodithioate (**7**), 38%; tryptamine (**8**), <5%; N_b-acetyltryptamine (**8a**), 20% (72 h)
S-methyl 3-(3-indolyl)propyl dithiocarbamate (**11**)	37	3-(3-indolyl)propanamine (**25**), <5%; N_b-acetyl-3-(3-indolyl)propanamine (**25a**), 6% (24 h)
isobrassinin (**13**)	<5	2-indolylmethanamine (**22**), <5%; N_b-acetyl-2-indolylmethanamine (**22a**), 30% (48 h)
1-methoxyisobrassinin (**13a**)	47	1-methoxy-2-indolylmethanamine (**23**), <5%; N_b-acetyl-1-methoxy-2-indolylmethanamine (**23a**), 37% (48 h)
S-methyl 4-biphenyl dithiocarbamate (**16**)	<5	4-biphenylmethanamine (**24**), <5%; *N*-acetyl-4-biphenylmethanamine (**24a**), 21% (72 h)
S-methyl benzimidazolyl dithiocarbamate (**18**)	10	benzimidazole (**17**), 62% (24 h)
S-tryptophol dithiocarbonate (**20**)	15	tryptophol (**26**), 32% (24 h)
S-methyl 3-(3-indolyl)propyl dithiocarbonate (**21**)	nd	3-(3-indolyl)-1-propanol (**27**), 60% (24 h)

[a]The percentage of each product was determined using calibration curves and is the average of at least three experiments conducted in triplicate.

Table 3. Antifungal Activity of Amines, Acetylamines, Benzimidazole, and Alcohols against *Leptosphaeria maculans*

compound	inhibition ± SD (%)[a]		
	0.50 mM	0.20 mM	0.10 mM
tryptamine (**8**)	28 ± 3 bc	15 ± 5 bcd	12 ± 4 bc
N_b-acetyltryptamine (**8a**)	15 ± 3 e	7 ± 3 e	ni d
benzimidazole (**17**)	35 ± 3 b	20 ± 2 b	11 ± 3 b
2-indolylmethanamine (**22**)	33 ± 2 b	19 ± 4 b	ni d
N_b-acetyl-2-indolylmethanamine (**22a**)	33 ± 4 b	18 ± 3 bc	7 ± 2 bcd
1-methoxy-2-indolylmethanamine (**23**)	19 ± 3 de	8 ± 4 de	ni d
N_b-acetyl-1-methoxy-2-indolylmethanamine (**23a**)	15 ± 2 e	11 ± 3 cde	4 ± 3 cd
N-acetyl-4-biphenylmethanamine (**24a**)	30 ± 5 bc	17 ± 4 bc	9 ± 4 bc
3-indolylpropanamine (**25**)	27 ± 5 bc	14 ± 4bcd	ni d
N_b-acetyl-3-(3-indolyl)propanamine (**25a**)	24 ± 3 cd	13 ± 4 bcde	10 ± 3 bc
tryptophol (**26**)	29 ± 5 bc	16 ± 4 bcd	ni d
3-(3-indolyl)-1-propanol (**27**)	18 ± 3 de	14 ± 3 bcde	7 ± 2 cd

[a]The percentage of inhibition was calculated using the following formula: % inhibition = 100 − [(growth on amended/growth in control) × 100]; ni = no inhibition. For statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; n = 6; entries with the same letters (b−f) within each column are not significantly different ($P < 0.05$).

In summary, the potential metabolism of dithiocarbamates containing indolyl, biphenyl, and benzimidazolyl moieties by *L. maculans* and their antifungal activities were investigated. All dithiocarbamates were transformed via hydrolysis to the corresponding amines, unlike brassinin (**1**), which was transformed to indole-3-carboxaldehyde (**6**). That is, whereas the transformation of brassinin (**1**) was catalyzed by an oxidase,[5,6] the transformations of dithiocarbamates **5**, **11**, **13**, **13a**, **16**, and **18**, evaluated for the first time in this work, were likely catalyzed by hydrolases. It is likely that these hydrolases are "house-keeping" enzymes used in the metabolism of xenobiotics. The products of these hydrolyses (amines **8**, **22**, **23**, and **25**) were less toxic to the fungus than the corresponding dithiocarbamates; that is, these hydrolase-mediated transformations occurring in *L. maculans* are detoxifications. It is worth noting that the rates of transformation of dithiocarbamates **5**, **11**, **13**, **13a**, **16**, and **18** did not correlate with their inhibitory activities. For example, *S*-methyl tryptamine dithiocarbamate (**5**), one of the most inhibitory dithiocarbamates tested, was transformed at a rate similar to that measured for 1-methoxyisobrassinin (**13a**), one of the least inhibitory compounds.

On the basis of the results reported above, it is concluded that, regardless of the antifungal activity of dithiocarbamates against *L. maculans*, this important plant pathogen is able to detoxify these chemicals, which indicates that dithiocarbamates are not useful to treat fungal infections caused by this plant pathogen.

■ ASSOCIATED CONTENT

Supporting Information

Progress curves for transformation of dithiocarbonates **20** and **21** by *Leptosphaeria maculans* and ^{1}H and ^{13}C NMR spectra of new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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