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Metabolism of the Host-Selective Toxins Destruxin B and Homodestruxin B: Probing a Plant Disease Resistance Trait

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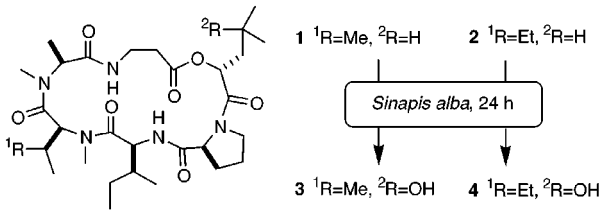
ABSTRACT



Metabolism of the host-selective toxins destruxin B (1) and homodestruxin B (2) by plants resistant and susceptible to Alternaria blackspot (caused by the fungal pathogen *Alternaria brassicae* (Berk.) Sacc.) was established using synthetic radiolabeled compounds. The toxins are transformed into the less phytotoxic hydroxydestruxins 3 and 4. The rate of metabolic transformation was correlated with the plant's disease resistance, i.e., significantly faster rates were observed for plants resistant to the pathogen. Efficient syntheses of 1, 2, 3, and 4 are described.

Despite the continuing achievements of plant biotechnology, engineering plants with disease resistance traits remains a significant challenge. Recent strategies to control fungal diseases using biotechnology have resulted in a race to obtain plant resistance genes and transfer them to susceptible, but agronomically valuable, crops.[1] Different approaches have been used for cloning such genes, a particularly difficult task in the absence of a gene product. Nonetheless, host-selective toxins (HSTs) might be one of the most attractive targets to determine specific disease resistance traits in plants and to guide the cloning of the corresponding disease resistance gene(s).[2,3] Subsequent plant genetic engineering with such gene(s) can be carried out to obtain plants with the particular resistance trait, similar to examples of herbicide, fungicide, and insect resistance.[4] HSTs are secondary metabolites produced by plant pathogens which facilitate colonization of host plants by the producing microorganism but do not significantly affect non host plants. Host plants may be resistant to a particular pathogen if they produce one or more specific enzymes that metabolize and detoxify the pathogen's HST(s). Consequently, HST-detoxifying enzymes can confer disease resistance traits to plants and are desirable gene products useful for cloning disease resistance genes. Perhaps not surprisingly, this potential application of HSTs has been demonstrated in very few instances.[3a]

As part of a research program aimed at understanding disease resistance mechanisms, we have been evaluating the role of HSTs produced by fungal pathogens of important crops.[5] Because destruxin B and homodestruxin B are HSTs

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(1) For recent reviews, see, inter alia: (a) Staskawicz, B. J.; et al. *Science* **1995**, *268*, 661. (b) Cornelissen, B. J. C.; Melchers, L. S. *Plant Physiol.* **1993**, *101*, 709. (c) Lamb, C. J.; et al. In *Biotechnology in Agriculture*; You, C. B., Chen, Z., Ding, Y., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands; 1993; p 45.

(2) Johal, G. S.; Briggs, S. P. *Science* **1992**, *258*, 985.

(3) For recent reviews, see: (a) Walton, J. D. *Plant Cell* **1996**, *8*, 1723. (b) Walton, J. D.; Panaccione, D. G. *Annu. Rev. Phytopathol.* **1993**, *31*, 275 and references therein.

(4) Yoneyama, K.; Anzai, H. In *Biotechnology in Plant Disease Control*; Wiley-Lyss, 1993; p 115.

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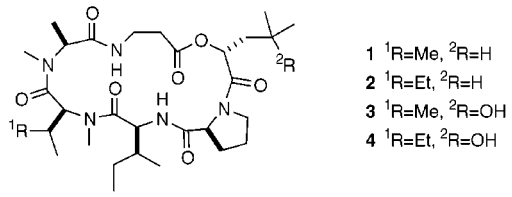
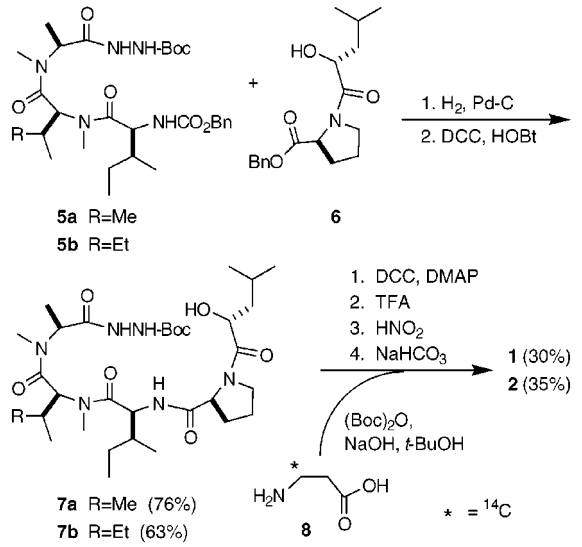
produced by one of the most damaging plant pathogenic fungi (*Alternaria brassicae* (Berk.) Sacc.) of economically important oilseed crops (e.g., rapeseed and canola, *Brassica napus*, *B. rapa*),[6] we became interested in investigating the metabolism of destruxins by plants. Here we wish to report the metabolism and detoxification of destruxin B (**1**) and homodestruxin B (**2**) by plants resistant and susceptible to the fungal pathogen, as well as the structure elucidation and chemical synthesis of the metabolic products and their phytotoxic activity. In addition, a new methodology for synthesis of ^{14}C-radiolabeled destruxins is summarized.



The metabolism of destruxin B (**1**) and homodestruxin B (**2**) by plant tissues was investigated using radiolabeled compounds[7] prepared as shown in Scheme 1.[8] The previously

Scheme 1



reported[9] route for synthesis of destruxin B was modified to allow introduction of a radiolabeled residue in the penultimate step. Thus, deprotection and coupling of **5a**[9] and **6**[9] gave the pentadepsipeptide **7a** in good yield. Without purification of the intermediates, [^{14}C-2]-β-alanine[7] (**8**) was converted into the Boc derivative and coupled with **7a**, and the resulting hexadepsipeptide was subjected to cyclization[9] to give **1**[7] in 30% overall yield. Radiolabeled **2** was similarly prepared from **5b**.[10]

The metabolism of **1** and **2** by leaves of plants resistant (*Sinapis alba* cultivar Ochre) and susceptible (*B. napus* cultivar Westar) to *A. brassicae* was investigated. Thus, solutions of radiolabeled toxins were administered to petiolated leaves; after incubation in a growth chamber for various periods, the leaves were individually extracted and the extracts analyzed by LSC[11] (ca. 70−100% recovery of radioactive material) and by HPLC (equipped with a radiodetector) (Table 1).[12] HPLC analysis[13] indicated that both toxins were almost completely metabolized by *S. alba* within 24 h; destruxin B (t_R = 19.3 min) was biotransformed to a metabolite with t_R = 10.4 min, here on called metabolite A, and homodestruxin B (t_R = 21.5 min) was biotransformed to a metabolite with t_R = 12.8 min, here on called metabolite B. *B. napus* did not appear to metabolize any of the destruxins to a detectable level within the same time period; about 10% transformation occurred in 96 h.

A close inspection of chromatograms shown in Figure 1 indicated that the transformation of **2** occurred within 24 h for cultivar Ochre. Similar chromatograms were obtained for **1** incubated with Ochre. The data shown in Table 1 indicated that the peaks observed in chromatograms corresponding to metabolites A (**3**) and B (**4**) represented ca. 90% of the toxins. That is, both destruxin B (**1**) and homodestruxin B (**2**) were biotransformed by leaves of *S. alba* to metabolites significantly more polar than each of the toxins within 24 h.

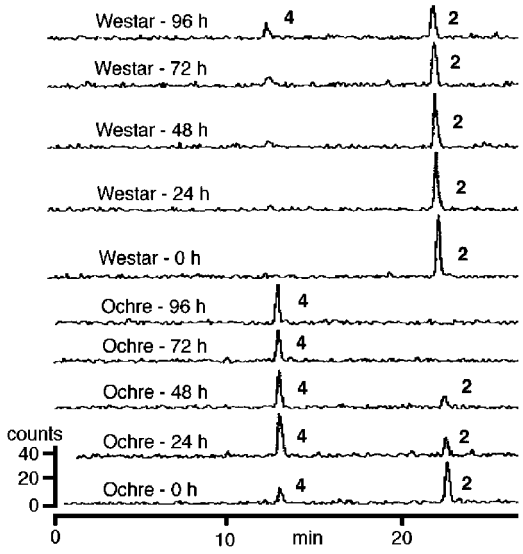


Figure 1. HPLC chromatograms (radiodetection) of extracts of leaves of *Sinapis alba*, cultivar Ochre (resistant to Alternaria blackspot), and *Brassica napus*, cultivar Westar (susceptible to Alternaria blackspot), incubated with ^{14}*C*-homodestruxin B (**2**) up to 96 h. For HPLC conditions, see ref 13.

(5) (a) Pedras, M. S. C. *Recent Res. Devel. Agric. Food Chem.*, **1998**, *2*, 513. (b) Pedras, M. S. C.; Biesenthal, C. J. *Can. J. Micro.* **1998**, *44*, 547. (c) Ward, D. E.; Vázquez, A.; Pedras, M. S. C. *J. Org. Chem.* **1999**, *64*, 1657.

(6) Saharan, G. S. In *Breeding Oilseed Brassicas*; Labana, K. S., Banga, S. S., Banga, S. K., Eds.; Springer-Verlag: Berlin, 1993; p 181. Canola refers to varieties of rapeseed containing very low amounts of erucic acid and glucosinolates.

(7) [^{14}C-2]-β-Alanine (250 μCi, 55.5 × 10^7 dpm) was diluted with cold material: 6 mg was used for **1**; 10 mg was used for **2**. Specific activities: destruxin B, 1.28 × 10^7 dpm/mg; homodestruxin B, 7.94 × 10^6 dpm/mg.

(8) All new compounds gave satisfactory spectroscopic data.

(9) Ward, D. E.; Lazny, R.; Pedras, M. S. C. *Tetrahedron Lett.* **1997**, *38*, 339.

Table 1. Metabolism of Host-Selective Toxins (HST) Destruxin B (**1**) and Homodestruxin B (**2**) by Leaves of *Sinapis alba*, Cultivar Ochre (resistant to *A. brassicae*), and *Brassica napus*, Cultivar Westar (susceptible to *A. brassicae*)

HST (radioactivity[a] added per leaf[b])	incubation period	HST conversion (%) per leaf[b] determined by HPLC[c]		% of total radioactivity recovered per leaf[b] (LSC)	
		Ochre	Westar	Ochre	Westar
destruxin B (**1**) (1.44×10^5)	0 h	22 ± 8	no conversion	97 ± 2	97 ± 4
	24 h	87 ± 25	no conversion	96 ± 4	81 ± 21
	48 h	complete conversion	3 ± 4	82 ± 7	96 ± 3
	72 h	complete conversion	6 ± 3	93 ± 3	95 ± 2
homodestruxin B (**2**) (1.22×10^5)	0 h	43 ± 2	no conversion	92 ± 1	93 ± 3
	24 h	97 ± 2	no conversion	76 ± 11	92 ± 1
	48 h	90 ± 20	4 ± 3	77 ± 21	91 ± 1
	72 h	complete conversion	9 ± 6	70 ± 8	93 ± 5

[a] Units are in disintegration per minute (dpm). [b] Results are averages of experiments conducted in triplicate. [c] HPLC analysis as described in footnote 13; areas of peaks determined in counts and converted to dpm utilizing a calibration curve. The total HPLC peak areas were within 10−20% of the LSC total counting.

To isolate the metabolites, scale-up experiments were carried out with nonlabeled destruxins utilizing ca. 300 leaves per toxin (1 mL per leaf, 2×10^{-5} M in 2% aqueous CH_3CN, v/v). Following an incubation period of 48 h, the leaves were extracted with EtOAc and the extracts fractionated, while monitoring the presence of metabolites A and B by HPLC. Multiple chromatographic fractionations yielded ca. 2 mg of each metabolite.[14] HR-EIMS analysis of metabolite A ($C_{30}H_{51}N_5O_8$) indicated an additional oxygen compared to destruxin B ($C_{30}H_{51}N_5O_7$). ^{1}H and ^{13}C NMR spectroscopic data[15] of metabolite A (**3**) were very similar to those of destruxin B except for the presence of two distinct methyl singlets at δ_H 1.33, δ_C 31.4 and δ_H 1.30, δ_C 29.5 and an additional quaternary carbon at δ_C 70.0. Thus, the spectroscopic data suggested that metabolite A contained a hydroxyl group located either at the valyl or at the 2-hydroxy-3-methylpentanoyl residue. Further proton-decoupling experiments allowed the unambiguous assignment of structure **3** to metabolite A. Similarly, the spectroscopic data of metabolite B obtained from homodestruxin B indicated it to have structure **4**. Although structures of at least 30 destruxins are known,[16] neither compound **3** or **4** appears to have been previously reported. As expected, parallel experiments carried out with leaves of *B. napus* and *S. alba* incubated under similar conditions but without destruxins did not allow detection of metabolites **3** or **4**.

(10) Prepared in 65% overall yield from Cbz-MeAla-NHNH−Boc, Cbz-MeIle-OH, and Cbz-Ile-OH using PyBrop/DIEA for coupling.[9]

(11) Liquid scintillation counting was performed on a Beckman LS-6500 connected to a Wyse WY-370 data system, and results are quench corrected and reported in dpm.

(12) Leaves were cut at the base of their petiole and immediately placed in Eppendorf tubes containing the ^{14}C-toxin (1 mL of 2.0×10^{-5} M dissolved in 2% aqueous CH_3CN). After the solution was taken up (this time = t_0), an additional 0.5 mL of H_2O was added to ensure the complete uptake of compound. Leaves were incubated in a growth chamber under fluorescent lighting (16-h light (22 °C)/8-h dark (18 °C) cycle) for 12, 24, 48, 72, or 96 h, keeping the petiole immersed in dd H_2O.

(13) HPLC analysis was carried out with a high performance Hewlett-Packard liquid chromatograph equipped with quaternary pump, automatic injector, and photodiode array detector (wavelength range 190−600 nm) connected with a Canberra Packard Radiomatic 150TR flow scintillation analyzer (fitted with a 210 μL high performance flow cell Solarscint), degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 i.d. × 200 mm), equipped with an in-line filter. 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. The ^{14}C counting window was 15−100 keV with an update time of 6 s.

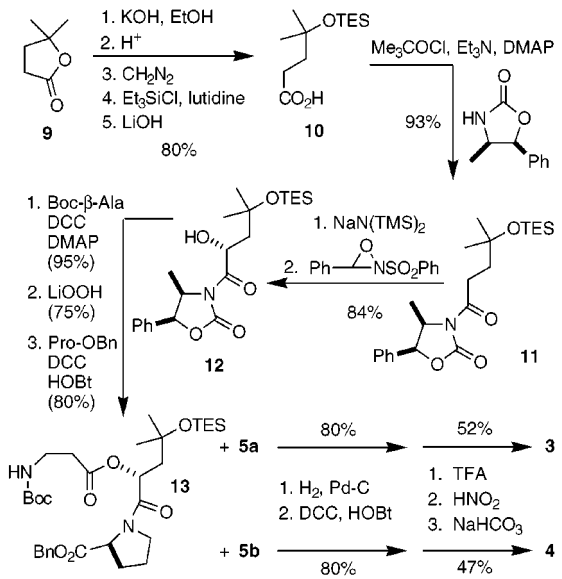
(14) The EtOAc residue was fractionated by multiple column chromatography (C-18 reversed phase silica gel, J. T. Baker, 40 μm) eluting with acetonitrile−water, gradient elution 30:70 to 100:0. The fractions were combined after HPLC analysis and further fractionated by PTLC on silica gel, 250 μm, developed in acetone−hexane (65:35) to yield chromatographically homogeneous material (TLC and HPLC).

(15) Spectroscopic data of metabolite A (**3**): HPLC t_R = 10.2 min; $[\alpha]_D^{25.5} = -255$ (*c* 0.10, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.20 (d, J = 8.5 Hz, 1H), 7.18 (d, J = 9 Hz, 1H), 5.19 (br q, J = 7 Hz, 1H), 5.17 (dd, J = 8.5, 3 Hz, 1H), 4.95 (d, J = 11 Hz, 1H), 4.88 (dd, J = 9, 6.5 Hz, 1H), 4.66 (d, J = 7 Hz, 1H), 4.05 (m, 1H), 3.90 (br t, J = 9 Hz, 1H), 3.61 (br q, J = 9 Hz, 1H), 3.23 (s, 3H), 3.08 (dddd, J = 13.5, 11.5, 2, 2 Hz, 1H), 2.72 (s, 3H), 2.68 (ddd, J = 18.5, 11, 2 Hz, 1H), 2.56 (ddd, J = 18.5, 5, 2 Hz, 1H), 2.47 (m, 1H), 2.32 (dsept, J = 11, 6.5 Hz, 1H), 2.11 (dd, 15, 8.5 Hz, 1H), 2.07−1.91 (m, 4H), 1.87 (dd, 15, 3 Hz, 1H), 1.43 (ddd, J = 13.5, 7.5, 3.5 Hz, 1H), 1.33 (s, 3H), 1.31 (d, J = 5 Hz, 3H), 1.30 (s, 3H), 1.26 (m, 1H), 0.93 (d, J = 6.5 Hz, 3H), 0.89 (d, J = 6.5 Hz, 3H), 0.87 (d, J = 7 Hz, 3H), 0.86 (t, J = 7 Hz, 3H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 173.8 (s), 173.6 (s), 171.3 (s), 171.2 (s), 170.0 (s), 169.9 (s), 71.1 (d), 70.0 (s), 61.0 (d), 58.3 (d), 55.7 (d), 53.9 (d), 46.7 (t), 43.2 (t), 37.7 (d), 34.8 (t), 33.4 (t), 31.4 (q), 31.1 (q), 29.5 (q), 29.2 (t), 28.3 (q), 27.5 (d), 24.6 (t), 24.3 (t), 20.2 (q), 19.9 (q), 15.6 (q), 15.4 (q), 11.6 (q); EIMS *m*/*z* (%) 609 (M^+, 10), 552 (28), 86 (60), 70 (100); HREIMS *m*/*z* calcd for $C_{30}H_{51}N_5O_8$ (609.3738), found 609.3735; FTIR (cm^{-1}) 3382, 3300, 2966, 1729, 1668, 1631, 1516, 1442, 1182. Spectroscopic data of metabolite B (**4**): HPLC t_R = 12.2 min; $[\alpha]^{24}_D = -196$ (*c* 0.14, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.23 (d, J = 8.5 Hz, 1H), 7.17 (d, J = 9 Hz, 1H), 5.18 (br q, J = 6.5 Hz, 1H), 5.17 (dd, J = 8.5, 3 Hz, 1H), 5.02 (d, J = 11 Hz, 1H), 4.87 (dd, J = 9, 6.5 Hz, 1H), 4.66 (d, J = 7 Hz, 1H), 4.05 (m, 1H), 3.90 (br t, J = 9 Hz, 1H), 3.61 (br q, J = 9 Hz, 1H), 3.20 (s, 3H), 3.08 (dddd, J = 13.5, 11.5, 2, 2 Hz, 1H), 2.72 (s, 3H), 2.68 (ddd, J = 18.5, 11, 2 Hz, 1H), 2.58 (ddd, J = 18.5, 5, 1.5 Hz, 1H), 2.46 (m, 1H), 2.11 (dd, 15, 8.5 Hz, 1H), 2.07−1.91 (m, 5H), 1.87 (dd, 15, 3 Hz, 1H), 1.50−1.37 (m, 2H), 1.32 (s, 3H), 1.30 (d, J = 5.5 Hz, 3H), 1.29 (s, 3H), 1.28 (m, 1H), 0.93 (m, 1H), 0.91 (m, 3H), 0.86 (t, J = 7.5 Hz, 3H), 0.85 (d, J = 6.5 Hz, 6H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 173.8 (s), 173.6 (s), 171.3 (s, 2×), 169.9 (s, 2×), 71.1 (d), 70.1 (s), 61.0 (d), 57.0 (d), 55.7 (d), 53.9 (d), 46.7 (t), 43.2 (t), 37.7 (d), 34.8 (t), 33.8 (d), 33.4 (t), 31.4 (q), 31.2 (q), 29.5 (q), 29.2 (t), 28.3 (q), 26.0 (t), 24.7 (t), 24.3 (t), 16.5 (q), 15.6 (q), 15.4 (q), 11.6 (q), 11.3 (q); EIMS *m*/*z* (%) 623 (M^+, 12), 566 (28), 524 (18), 467 (10), 196 (14), 100 (100); HREIMS *m*/*z* calcd for $C_{31}H_{53}N_5O_8$ (623.3894), found 623.3891; FTIR (cm^{-1}) 3385, 3297, 2961, 1730, 1672, 1631, 1514, 1441, 1230, 1182.

(16) All the destruxins reported so far were isolated from diverse fungal species; for recent reports see, for example: (a) Krasnoff, S. B.; Gibson, D. M. *J. Nat. Prod.* **1996**, *59*, 485. (b) Cai, P.; Smith, D.; Katz, B.; Pearce, C.; Venables, D.; Houck, D. *J. Nat. Prod.* **1998**, *61*, 290.

Next, to establish if the hydroxylation of destruxins represented in fact a metabolic detoxification, it was crucial to determine the phytotoxicity of hydroxydestruxins **3** and **4** to plants resistant and susceptible to *A. brassicae*. Total synthesis of **3** and **4** was necessary because of the lengthy and difficult separations required to obtain these hydroxydestruxins from plant tissue. The required (2*R*)-2,4-dihydroxy-4-methylpentanoic acid residue has been obtained in very low yield by resolution but was found to be highly prone to lactonization and racemization.[17] To prevent these processes, the silyl ether **10** was prepared from the readily available lactone **9**[18] (Scheme 2).[8] Enantioselective hydroxylation of **10** using the method of Evans et al.[19] provided **12** in good yield. We were unable to hydrolyze **12** without extensive concomitant γ-lactonization. By contrast, acylation of **12** with Boc-β-Ala followed by hydrolysis and coupling with Pro-OBn gave the tridepsipeptide **13** in good overall yield. In analogy with the previously described synthesis of **1**,[5a] coupling of fragments **5a** and **13** followed by cyclization provided **3** in 41% overall yield; similar processing of **5b** and **13** gave **4** in 37% yield. As expected, the chemical synthesis of both hydroxydestruxins **3** and **4** provided products identical in all respects with metabolites A (**3**) and B (**4**), thus corroborating the chemical structure assignments.

Scheme 2



With both compounds **3** and **4** in hand, leaf puncture and leaf uptake bioassays were carried out; results of both bioassays indicated that the hydroxylated compounds were less phytotoxic than each of the destruxins. In addition, a bioassay with cell suspension cultures of both *B. napus* and *S. alba* was employed to compare **1** and **3**; the toxicity of both **1** and **3** was evaluated by determining the percentage of viable cells after exposure to each compound for different periods. The results of these bioassays were similar to those observed with the leaf assays and indicated that the percentage of viability of cells exposed to destruxin B (**1**) was about 50% lower than the percentage of viability of cells exposed to hydroxydestruxin B (**3**). Moreover, **3** was no more phytotoxic to the susceptible cultivar Westar than to the resistant cultivar Ochre. Altogether these bioassay results indicate that the metabolism of destruxins **1** and **2** to the respective hydroxylated products are detoxifications occurring at a faster rate in Ochre plants, i.e., plants resistant to *A. brassicae*. Most importantly, investigation with two additional plant cultivars, a disease resistant and a susceptible, showed similar behavior, indicating that destruxin and homodestruxin B detoxification correlated with Alternaria blackspot resistance. Consequently, it is probable that this biochemical trait is involved in the blackspot resistance shown by Ochre; if this is true Westar might be expected to become blackspot resistant if transformed with the Ochre destruxin B hydroxylating gene.

Acknowledgment. Financial support from the Natural Sciences and Engineering Research Council of Canada (Strategic Project Grant to M.S.C.P. and D.E.W.) and the University of Saskatchewan is gratefully acknowledged.

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(17) Mori, K.; Takigawa, T.; Matsuo, T. *Tetrahedron* **1979**, *35*, 933.

(18) Stevens, C. M.; Tarbell, D. S. *J. Org. Chem.* **1954**, *19*, 1996.

(19) Evans, D. A.; Morrissey, M. M.; Dorow, R. L. *J. Am. Chem. Soc.* **1985**, *107*, 4346.