Koninginin B: A Biologically Active Congener of Koninginin A from *Trichoderma koningii*

Horace G. Cutler,*,† David S. Himmelsbach,† Boris Yagen,‡ Richard F. Arrendale,§ John M. Jacyno,† Patsy D. Cole,† and Richard H. Cox∥

Richard B. Russell Research Center, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 5677, Athens, Georgia 30613, School of Pharmacy, Faculty of Medicine, The Adolph Weinberger Building, The Hebrew University of Jerusalem, P.O. Box 12065, Jerusalem 91120, Israel, Glaxo Incorporated, 5 Moore Drive, Research Triangle Park, North Carolina 27709, and Philip Morris U.S.A. Research Center, P.O. Box 26583, Richmond, Virginia 23261

Koninginin B, a biologically active natural product related to koninginin A, was isolated from *Trichoderma koningii*, an organism isolated from the roots and soil line of a *Diffenbachia* sp. The compound, which inhibited the growth of etiolated wheat coleoptiles 100% at 10^{-3} M, was a white, finely crystalline compound with molecular formula $C_{16}H_{26}O_4$ and a melting point of 72–74 °C. The structure was established as 8-hydroxy-2-(1-hydroxyheptyl)-3,4,5,6,7,8-hexahydro-2*H*-1-benzopyran-5-one by comparison with koninginin A, UV, IR, ^1H NMR, ^{13}C NMR, and MS.

INTRODUCTION

In the course of searching for new, biologically active, fungal metabolites with agrochemical potential, we examined *Trichoderma koningii*, which was found in the soil associated with a wilting *Diffenbachia*. A strain of this microorganism has also recently been the subject of a study by Simon and Dunlop (Simon et al., 1988; Dunlop et al., 1989). Our bioassay-directed fractionation resulted in the isolation of three metabolites (differing from those reported by Simon and Dunlop, vide supra) which were active in the etiolated wheat coleoptile assay: koninginin A (Cutler et al., 1989), cyclonerodiol (Cutler et al., 1991), and koninginin B. We new report the details concerning the identification and biological activity of koninginin B.

EXPERIMENTAL PROCEDURES

Materials and Equipment. IR spectra were obtained with a Beckman IR 4210 spectrometer from thin films on KBr. UV spectra were obtained from 95% ethanolic solution by using a Beckman 35 instrument. Standard ^1H and ^{13}C 1D and 2D spectra for samples dissolved in $CDCl_3$ were gathered on Varian XL300 and Bruker MSL300 spectrometers; an inverse (^1H) detected heteronuclear multiple bond (HMBC) 2D spectrum and 1D projections were obtained on a Bruker AM500 as previously described (Himmelsbach, 1990). Direct probe, EI mass spectra were determined by using a Hewlett-Packard 5985B mass spectrometer. GC–MS experiments were performed on the same instrument equipped with a laboratory-constructed, cold on-column inlet system, attached to an SE-54, fused-silica, capillary column (30 m × 0.3 mm).

Melting points were determined on a Kofler block apparatus and are uncorrected. TLC was performed on Merck silica gel 60, F-254 plates; spots were visualized with UV light (254 or 366 nm) or by spraying with anisaldehyde/H_2SO_4 and heating. C_{18} reverse-phase silica gel used in column chromatography was from a Waters PrepPak 500.

Production, Isolation, and Purification of Metabolites. *T. koningii* (ATCC No. 46314) was cultured on potato–dextrose agar slants for 10 days, then transferred to 2.8-L Fernbach flasks, each containing 100 g of shredded wheat, 200 mL of Difco mycological broth, 2% yeast extract, and 20% sucrose (Kirksey and Cole, 1974), and incubated for 14 days at 21–22 °C. About 300 mL of acetone was then added to each flask, the mixture pulped with a Super Dispax homogenizer (Tekmar Co., Cincinnati, OH), and the slurry filtered through Whatman No. 1 filter paper under suction. The filtrate was concentrated and extracted with ethyl acetate. After evaporation of the solvent, the residue was chromatographed on silica gel, eluting successively with benzene, diethyl ether, ethyl acetate, acetone, and acetonitrile. The ether and ethyl acetate fractions, which exhibited activity in the coleoptile assay, were combined and rechromatographed on silica gel, initially eluting with benzene, followed by an increasing gradient of acetone. The biologically active fractions were combined and rechromatographed on hydrated silica gel (20 mL of H_2O/100 g) by using benzene–ethyl acetate (5:4). Active fractions were pooled and chromatographed on C_{18} reverse-phase silica gel with acetonitrile–water (1:1). The fractions with biological activity were again combined and chromatographed on silica gel containing 2% $AgNO_3$, eluting with CH_2Cl_2–acetone (9:1) to finally yield koninginin B.

The isolations of koninginin A and cyclonerodiol, which were also obtained from *T. koningii*, have been described elsewhere (Cutler et al., 1989, 1991).

Derivative Synthesis and Analysis. Derivatives of koninginin B were prepared as follows: The peracetate was made by dissolving koninginin B (5 mg) in dry pyridine (1 mL), adding acetic anhydride (1 mL), and heating for ~4 h at 60 °C, under nitrogen. After evaporation under high vacuum, the crude product was purified by Chromatotron. The trimethylsilyl (TMS) derivative was synthesized by dissolving koninginin B in an excess of bis(trimethylsilyl)trifluoroacetamide–dimethylformamide mixture (1:1) and heating for 30 min at 76 °C. *p*-Bromobenzoates were prepared by treating koninginin B with excess *p*-bromobenzyl bromide and pyridine in dichloromethane solution (18 h at room temperature, followed by 5 h at reflux). The mixture was diluted with EtOAc and then washed sequentially with brine, 1 N HCl, brine, and saturated $NaHCO_3$. The organic solution was dried (Na_2SO_4) and evaporated in vacuo to give crude product. This was purified by Chromatotron to give the mono- and dibromobenzoates. Koninginin B was converted to its dihydro derivative by bubbling H_2 into a solution of koninginin B in ethanol containing 2% H_3PO_4, in the presence of 10% Pd–C, until TLC showed the disappearance of starting material. The mixture was filtered and the filtrate poured into saturated $NaHCO_3$. This mixture was extracted with EtOAc, and then the extracts were dried ($MgSO_4$) and evaporated to give crude product.

The bromobenzoates and hydrogenation product were characterized by IR, NMR, and MS. The peracetate and TMS derivatives were analyzed by GC–MS under the following

† Richard B. Russell Research Center.
‡ The Hebrew University of Jerusalem.
§ Glaxo Incorporated.
∥ Philip Morris U.S.A. Research Center.

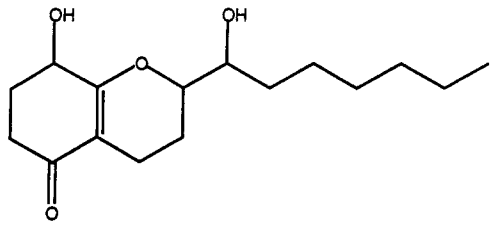

Figure 1. Structure of koninginin B.

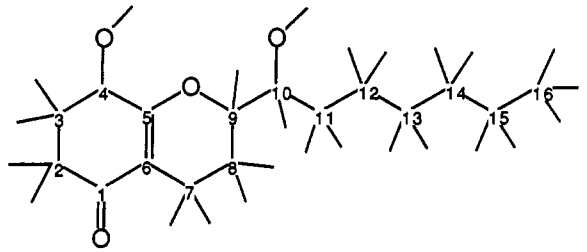

Figure 2. Structural skeleton of koninginin B showing atom labels used in NMR assignments [using nonsystematic numbering system of Dunlop et al. (1989)].

conditions: peracetate, 90 °C for 1 min and then 90–280 °C at 6 °C/min, flow rate 40 cm/s; TMS derivative, 100 °C for 1 min and then 100–300 °C at 4 °C/min, flow rate 40 cm/s.

Bioassays. The primary bioassay was the etiolated wheat coleoptile (Hancock et al., 1964) which was used throughout the isolation and purification process to determine biological activity. This assay was performed by germinating wheat (*Triticum aestivum* L., cv. Wakeland) on moist sand at 22 ± 1 °C for 4 days in the dark. Seedlings were removed and prepared under a green safelight: shoots were fed into a Van der Weij guillotine, the apical 2 mm was cut and discarded, and the next 4 mm of the coleoptile was removed for bioassay. Ten 4-mm sections were placed in each test tube with phosphate–citrate buffer (pH 5.6) containing 2% sucrose and the fraction to be tested (generally a 25-μL aliquot from a chromatography cut that had been evaporated under nitrogen). The test tubes were placed in a roller tube apparatus for 20 h at 22 °C. Then the coleoptiles were taken out and blotted dry and their magnified (×3) images measured. All data were analyzed statistically. Additional details concerning this assay are given in a previous publication (Cutler et al., 1982).

Microbial bioassays utilized Gram-positive and Gram-negative bacteria: *Bacillus subtilis* (+), *B. cereus* (+), *Mycobacterium thermosphactum* (+), *Escherichia coli* (−), *E. cloacae* (−), and *Citrobacter freundii* (−). Each organism was heavily seeded onto diagnostic sensitivity test agar (DST) in glass Petri dishes, to ensure a dense lawn, and to these were added 4-mm disks that had been impregnated with various concentrations of koninginin B at 500, 250, and 50 μg. The metabolite was dissolved in acetone and added to the disks, which were dried and then placed on the seeded agar surface. Plates were incubated at 37 °C for 18 h.

RESULTS AND DISCUSSION

Biological. Etiolated wheat coleoptiles were inhibited 100% ($P < 0.01$) by koninginin B at 10^{-3} M, but there was no inhibition at lower concentrations (Figure 3). This degree of inhibition is comparable to that exhibited by a number of "commercial" herbicides in this bioassay (Jacyno and Cutler, unpublished results); insufficient sample precluded more extensive tests for herbicidal activity. Gram-positive bacteria, *B. subtilis*, *B. cereus*, and *M. thermosphactum*, were inhibited at 500 μg/disk (Table II). While koninginin B is only marginally active against Gram-positive bacteria and inactive against Gram-negative strains, it does show activity relative to its congener, koninginin A, which was completely inactive against these organisms.

Chemical. Fifty-six flasks of culture media yielded 154 mg of koninginin B as a colorless, crystalline solid, mp

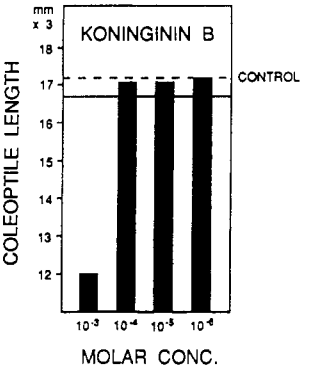

Figure 3. Effects of koninginin B on etiolated coleoptiles (*Triticum aestivum* L., cv. Wakeland). The initial coleoptile length (×3) was 12 mm. (Dotted line) Control; (below solid line) significant inhibition ($P < 0.01$).

Table I. ^{1}H and ^{13}C NMR Data for Koninginin B[a]

position	δ ^{13}C[b]	position	δ ^{1}H[c]	$J_{H,H}$,[c] Hz
1	198.10			
2	27.14	2a	2.50	$J_{2a,2b} = -17.0$
		2b	2.56	$J_{2a,3a} = 14.4$; $J_{2a,3b} = 2.3$; $J_{2b,3a} = 5.1$; $J_{2b,3b} = 5.2$
3	20.02	3a	1.79	$J_{3a,3b} = 12.9$
		3b	2.34	$J_{3a,4} = -12.1$; $J_{3b,4} = 4.8$
4	71.02	4	4.05	
5	171.58	5		
6	109.09	6		
7	17.70	7a	2.09	$J_{7a,7b} = -15.9$
		7b	2.57	$J_{7a,8a} = 11.6$; $J_{7a,8b} = 6.1$; $J_{7b,8a} = 13.2$; $J_{7b,8b} = 2.1$
8	22.59	8a	1.68	$J_{8a,8b} = -13.0$
		8b	1.96	$J_{8a,9} = 5.2$; $J_{8b,9} = 1.9$
9	80.80	9	3.80	$J_{9,10} = 11.2$
10	72.89	10	3.68	$J_{10,11} = 5.9$
11	32.76	11	1.55	
12	25.44	12	1.30	
			1.55	
13	29.25	13	1.30	
14	31.75	14	1.30	
15	22.59	15	1.30	$J_{15,16} = 6.8$
16	14.06	16	0.89	

[a] ^{1}H–^{13}C long-range coupling ($J = 5$ Hz) gave the following results: C-4 (H-3a,3b); C-10 (H-11,12); C-9 (H-11,8a,8b); C-6 (H-2a,2b,7a,7b,8a,8b). Assignments based on ^{13}C–^{13}C, ^{1}H–^{13}C, ^{1}H–^{1}H correlations and ^{1}H decoupling experiments. [b] Obtained at 125 MHz in $CDCl_3$. Multiplicities were determined from DEPT experiments. [c] Obtained at 500 MHz in $CDCl_3$. Values were calculated from simulated spectra by using the PANIC program.

Table II. Effects of Koninginin B against Gram-Positive (+) and Gram-Negative (−) Bacteria[a]

	concn, μg/disk[b]		
organism	50	250	500
B. subtilis (+)	−	−	++
B. cereus (+)	−	−	++
M. thermosphactum (+)	−	−	++
E. coli (−)	−	−	−
E. cloacae (−)	−	−	−
C. freundii (−)	−	−	−

[a] 4-mm disks impregnated with selected concentrations of koninginin B. [b] ++, inhibition ≥12 mm in diameter; −, no inhibition.

72–74 °C. The R_f of this compound in toluene–ethyl acetate–formic acid (5:4:1) was 0.47–0.50; spots on TLC plates quenched fluorescence when irradiated at 254 nm but were invisible to anisaldehyde/H^+/heat treatment. Spectroscopic data were as follows: UV λ_{max} 262 (log ϵ = 4.24); IR ν_{max}, 3450, 2960, 2930, 2860, 1650, 1612, 1453, 1402, 1381, 1287, 1242, 1196, 1152, 1078, 1048, 981, 860, 728, 692 cm^{-1} EI-MS m/z 282 (M^+), 264 ($M^+ - 18$), 246

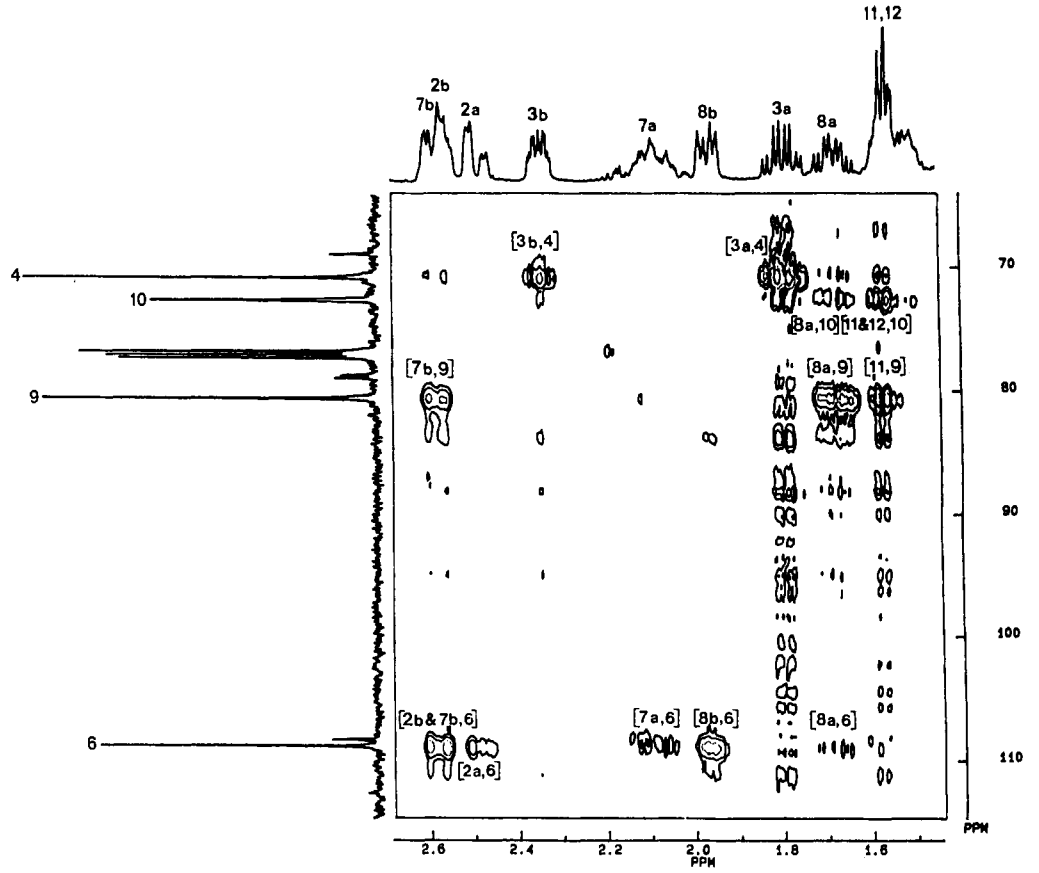

Figure 4. Inverse (^{1}H) detected heteronuclear multiple bond (HMBC) 2D spectrum of koninginin B showing the two- and three-bond ^{13}C–^{1}H connectivities between carbons 4–6 and protons 14–7.

($M^+ - 18 - 18$). ^{1}H and ^{13}C NMR data are shown in Table I.

The ^{1}H spectrum showed 24 nonexchangeable and 2 exchangeable protons. The ^{13}C data showed 16 unique carbons: 1 methyl, 9 methylenes, 3 methines, and 3 quaternaries. Two of the quaternaries (at 171.58 and 109.09 ppm) are olefinic, and the third (at 198.10 ppm) corresponds to a carbonyl. These data, together with the apparent M^+ at m/z 282 in the MS, suggested the molecular formula $C_{16}H_{26}O_4$, implying a structure with four double bonds/rings.

The UV (λ_{max} 262 nm) and IR data (absorptions at 1650 and 1612 cm^{-1}) were consistent with an α,β-unsaturated cyclohexenone fragment, which is also supported by the ^{13}C data showing olefinic and carbonyl resonances. The integration of the ^{1}H spectrum (not shown) of a sample of koninginin B after hydrogenation was consistent with the presence of two additional protons. Evidence of the presence of two hydroxyl groups in the molecule came from the IR (absorption at 3450 cm^{-1}), ^{1}H NMR (two signals displaying variable chemical shifts and disappearing upon addition of D_2O), and MS [ions at m/z 264 ($M^+ - 18$) and m/z 246 ($M^+ - 18 - 18$)]. Further information concerning the hydroxyl groups was adduced by examining the peracetylated and TMS derivatives of koninginin B. The GC–MS data for the peracetate indicated a molecular ion peak at m/z 366 (koninginin B, diacetate), and fragments at 324 ($M^+ - CH_2CO$), 306 ($M^+ - CH_3CO_2H$), and 246 [$M^+ - 2(CH_3CO_2H)$]. Data obtained from the TMS derivative indicated an M^+ at m/z 426 (koninginin B, diTMS), with fragments at m/z 411 ($M^+ - CH_3$), a very prominent ion peak at m/z 339 ($M^+ - TMS - CH_3$), and m/z 321 ($M^+ - HOTMS - CH_3$).

The ^{1}H spectrum of the mono-*p*-bromobenzoate derivative showed a 1.50 ppm downfield shift for H-4 (see Figure 2). The spectrum of the di-*p*-bromobenzoate exhibited an additional downfield shift for H-10. These results indicated that the hydroxylic protons were located on the same carbon atoms as H-4 and H-10 and that the H-4 was in the most accessible portion of the structure.

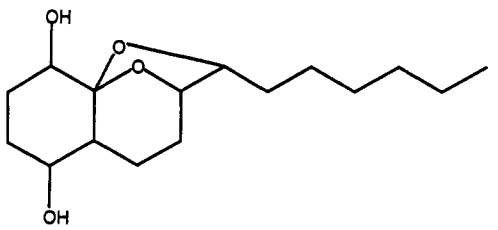

Figure 5. Structure of koninginin A.

The results of the decoupling experiments, together with those of a 2D {^{1}H, ^{1}H} homonuclear correlation experiment, suggested the following proton interactions: 16–15–11–10–9, 7–8–9–10, and 2–3–4. The critical experiment was the HMBC (Figure 4) that fixed the positions of protons 8a, 8b, 7a, 7b, 2a, and 2b relative to C-6 at 109.09 ppm. This then placed C-5 at the other end of the double bond, giving rise to a signal at 171.58 ppm and leaving C-1 as the carbonyl at 198.10 ppm.

These observations led to the placing of the carbons and protons as shown in Figure 2, which becomes the final structure (Figure 1) when the hydroxyl protons are added to the oxygens attached to C-4 and C-10 and C-1 is made a carbonyl.

In structure, koninginin B is thus closely related to koninginin A (Figure 5), as well as to 4,8-dihydroxy-2-(1-hydroxyheptyl)-3,4,5,6,7,8-hexahydro-2*H*-1-benzopyran-5-one, a metabolite recently reported by Dunlop et al. (1989) from another isolate of *T. koningii*. Relative to koningi-

nin A, koninginin B has a double bond at the bridgehead and no dioxolane ring. Additionally, one of the hydroxyls in the cyclohexane ring is oxidized to a carbonyl, making the ring a cyclohexanone. Thus, the structure of koninginin B more closely resembles that of the metabolite reported by Dunlop, differing from the latter in the absence of a hydroxyl at C-4 (Figure 2). Computer-generated, energy-minimized models (generated by using Alchemy II, Tripos Associates) of koninginin B and the Dunlop metabolite indicate "half-chair" conformations for both molecules where one set of geminal protons, H-2a and H-3a, is transdiaxial. This is consistent with the proton coupling constant $J_{2a,3a}$ = 14.1 Hz, observed in koninginin B. Here H-4 makes angles of 177.6° and 56.9° with H-3a and H-3b, respectively, in agreement with the observed coupling, $J_{3a,4}$ = 12.1 Hz and $J_{3b,4}$ = 4.8 Hz. Thus, although there is a conformational difference between these structures that gives rise to considerable spectral differences, the relative configuration at C-4 is unchanged. The NMR data indicate that there is little conformational difference in the dihydropyran rings of these two structures. The hydroxyheptyl side chain in koninginin B is, however, twisted somewhat from that observed in the C-4 hydroxylated analogue as indicated by an increase in $J_{9,10}$ to 11.2 Hz. All the data suggest that koninginin B possesses the same relative stereochemistry for its three asymmetric centers.

It will be interesting to see if other koninginin congeners will be found in *T. koningii* and other fungi and what their specific biological activities will be.

LITERATURE CITED

Cutler, H. G.; Crumley, F. G.; Cox, R. H.; Davis, E. E.; Harper, J. L.; Cole, R. J.; Sumner, D. R. Prehelminthosporol and Prehelminthosporol Acetate: Plant Growth Regulating Properties. *J. Agric. Food Chem.* **1982**, *30*, 658–662.

Cutler, H. G.; Himmelsbach, D. S.; Arrendale, R. F.; Cole, P. D.; Cox, R. H.; Koninginin A: A Novel Plant Growth Regulator from *Trichoderma koningii*. *Agric. Biol. Chem.* **1989**, *53*, 2605–2611.

Cutler, H. G.; Jacyno, J. M.; Phillips, R. S.; VonTersch, R. L.; Cole, P. D.; Montemurro, N. Cyclonerodiol from a Novel Source, *Trichoderma koningii*: Plant Growth Regulatory Activity. *Agric. Biol. Chem.* **1991**, *55*, 243–244.

Dunlop, R. W.; Simon, A.; Sivasithamparam, K.; Ghisalberti, E. L. An Antibiotic from *Trichoderma koningii* Active Against Soilborne Plant Pathogens. *J. Nat. Prod.* **1989**, *52*, 67–74.

Hancock, C. R.; Barlow, H. W.; Lacey, H. J. The East Malling Coleoptile Straight Growth Test Method. *J. Exp. Bot.* **1964**, *15*, 116–176.

Himmelsbach, D. S.; VanHalbeek, H.; Arrendale, R. F.; Severson, R. F. Assignment of Proton and Carbon-13 Spectra of a Sucrose Ester from Tobacco by Inverse Detected Two-Dimensional Heteronuclear Correlated NMR Spectroscopy. *Magn. Reson. Chem.* **1990**, *28*, 682–687.

Kirksey, H. W.; Cole, R. J. Screening for Toxin-producing Fungi. *Mycopathol. Mycol. Appl.* **1974**, *54*, 291–296.

Simon, A.; Dunlop, R. W.; Ghisalberti, E. L.; Sivasithamparan, D. *Trichoderma koningii* Produces a Pyrone Compound with Antibiotic Properties. *Soil Biol. Biochem.* **1988**, *20*, 263–264.

Received for review August 6, 1990. Revised manuscript received November 13, 1990. Accepted November 30, 1990.

Registry No. Koninginin B, 133471-72-4; koninginin B, diacetate, 133471-73-5; koninginin B, diTMS, 133471-74-6; koninginin, B, mono-*p*-bromobenzoate, 133471-75-7; koninginin B, di-*p*-bromobenzoate, 133471-76-8.