solvent program, at a flow rate of 1 mL/minute, was 5 min isocratic, 15 min linear increase in water from 0.5 to 1.4, and 20 min isocratic at the final solvent composition of 6:8:1.4. We achieved excellent separation (Figures 1 and 2) in 10 min less time beginning with a solvent composition of 6:8:0.5 and running a linear increase in water to a final composition of 6:8:1.5, flow rate 1.5 mL/min.

The methods for phospholipids of red blood cell ghosts and squid axions did not separate sufficient quantities for further analysis. The data in Table I show that our method separates sufficient quantities of the phospholipids for quantitation by colorimetric phosphorus determination. The data also confirm the predominance of PC and PE in wheat root membranes observed by Willemot (1975). Willemot's results are based on incorporation of radioactive phosphorus into phospholipids separated by TLC.

The fatty acid compositions of PE and PC were nearly identical (Table II). Phospholipids are synthesized via a common intermediate, PA. PC and PE can be synthesized by condensation of digylceride, derived from PA, with either CDP-ethanolamine or CDP-choline (Kates and Marshall, 1975). Some evidence suggests that both these condensation reactions are catalyzed by the same enzyme. PC can also arise from stepwise methylation of PE. Our data suggest that in wheat roots, biosynthetic reactions for PC and PE subsequent to PA are not selective in regard to acyl chain composition.

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Tritium Labeling of Avermectins B_1a and B_2a via Stereoselective Reduction of 5-Ketoavermectins

The potent parasiticides avermectins B_1a and B_2a have been labeled in the 5 position by stereoselective reduction of the corresponding 5-ketoavermectins using sodium borotritide. The 5-ketoavermectins were prepared in 40–55% yields by manganese dioxide oxidation of the natural products.

Recent reports from these laboratories have described the discovery (Burg et al., 1979), isolation (Miller et al., 1979), and structure determination (Albers-Schönberg, et al., 1981; Springer et al., 1981) of the avermectins, a new family of complex macrocyclic lactones elaborated by the soil actinomycete *Streptomyces avermitilis*. The avermectins (Figure 1) exhibit potent activity against a wide variety of parasitic helminths (Egerton et al., 1979; Blair and Campbell, 1979a,b; Campbell and Blair, 1979), arachnids (Centurier and Barth, 1980), and insects (Ostlind et al., 1979; James et al., 1980), apparently by selective blockade of γ-aminobutyric acid mediated neurotransmission (Fritz et al., 1979; Pong et al., 1980; Pong and Wang, 1980; Kass et al., 1980). Such activity is unprecedented among macrocyclic lactones (Burg et al., 1979; Masamune et al., 1977; Zahner, 1977). The further study of the mode of action of the avermectins, as well as metabolic, pharmacokinetic, and tissue residue analyses, required radiolabeled materials, and we wish to report the preparation of 5-[^{3}H]avermectins B_1a (1) and B_2a (2) via redox cycling through the 5-ketoavermectins **3** and **4**, respectively, a convenient method which should be applicable to a variety of avermectin derivatives.

EXPERIMENTAL SECTION

Materials. Avermectins B_1a (**5**) and B_2a (**6**) were supplied by the Natural Products Isolation Department of the Merck Sharp & Dohme Research Laboratories and were >99% pure. Activated manganese dioxide (Winthrop Laboratories) and sodium [^{3}H]borohydride (New England Nuclear Corp.) were used as received. All solvents were of high-performance liquid chromatography (HPLC) or

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spectroscopic grade and were used as received. Thin- and thick-layer chromatography (TLC) was performed on silica gel (SG) GF chromatography plates (Analtech), and column chromatography was performed by using SG (E. Merck SG 60, 70–230 mesh). Sephadex LH-20 (Pharmacia Fine Chemicals AB) was swollen under methanol for 18 h, poured into a column, and washed with 2 column volumes each of methanol and 6–8:1:1 (v/v/v) hexane–toluene–methanol before use. Resin volumes were measured after the final wash.

Spectra. The 300-MHz ^1H NMR spectra were recorded by using a Varian SC-300 NMR spectrometer in the FT mode in $CDCl_3$ with $(CH_3)_4Si$ as internal standard. The mass spectra were recorded by using an LKB Model 9000 mass spectrometer at 70-eV ionizing voltage. The IR spectra were recorded on neat films by using a Perkin-Elmer 267 grating spectrophotometer calibrated against polystyrene film. The UV spectra were recorded in methanol solutions by using a Cary 118 spectrophotometer, and optical rotations were determined by using a Perkin-Elmer 241 digital polarimeter in a 1-mL, 1-dm cell.

High-Performance Liquid Chromatography. Analytical HPLC was performed on (1) a Chromegabond C_{18} reverse-phase column (30 cm × 4.6 mm i.d.) monitored at 245 nm, eluted with 78:21:1 (v/v/v) acetonitrile–water–acetic acid for 1 and 5 and 62:18:20 (v/v/v) acetonitrile–methanol–water for 2 and 6, and (2) a μBondapak C_{18} reverse-phase column (30 cm × 7.8 mm i.d.) monitored at 254 nm, eluted with 85:18 (v/v) methanol–water for 1–6. Preparative HPLC of 2 was performed by using a Chromegabond C_{18} column (30 cm × 9.6 mm i.d.) eluted with 62:18:20 (v/v/v/) acetonitrile–methanol–water. Purities were determined as percent of total UV areas.

5-Ketoavermectin B_1a (3). A solution of 5 (3.00 g, 3.44 mmol) in ether (250 mL) was stirred 18 h at 25 °C with activated manganese dioxide (15 g, 35 mmol), filtered, and washed with ethyl acetate (4 × 100 mL). The combined filtrates were evaporated in vacuo and chromatographed on a column of silica gel (250 g) eluted with 1:1 (v/v) ethyl acetate–benzene. Fractions of 28 mL each were collected, and 3 was isolated by pooling fractions 21–36, evaporating, and drying (25 °C; 1.0 torr; 18 h) to afford 1.68 g (56%) of 3. An analytical sample was prepared by chromatography on Sephadex LH-20 (1 L of resin/g of 3) eluted with 7:1:1 (v/v/v) hexane–toluene–methanol: IR 3600–3200 (OH), 1730 (lactone C=O), 1685 (ketone C=O) cm^{-1}; UV λ_{max} 242 nm (ε 29 100), 252 sh (20 500); $[\alpha]_D$ +28.2° (*c* 0.655, $CHCl_3$), -22.2° (*c* 0.670, CH_3OH); MS (Scheme I) *m/e* 870 (M^+), 725, 582, 564, 305, 289, 259, 221, 145, 127, 113. Anal. Calcd for $C_{48}H_{70}O_{14}$: C, 66.19; H, 8.10. Found: C, 65.82; H, 8.26.

5-Ketoavermectin B_2a (4). A solution of 6 (250 mg, 281 μmol) in ether (20 mL) was stirred 18 h at 25 °C with activated manganese dioxide (1.1 g, 13 mmol), filtered, and washed with ethyl acetate (3 × 6 mL). The combined filtrates were evaporated in vacuo and purified by preparative TLC [three 1000-μm SG GF plates developed with 4:1 (v/v) chloroform–tetrahydrofuran] and then rechromatographed [three 1000-μm Sg GF plates developed 3 times with 9:1 (v/v) chloroform–tetrahydrofuran] to provide 198 mg (43%) of 4. An analytical sample was prepared by chromatography on Sephadex LH-20 (1 L of resin/g of substrate) eluted with 7:1:1 (v/v/v) hexane–toluene–methanol: IR 3600–3200 (OH), 1735 (lactone C=O), 1685 (ketone C=O); UV λ_{max} 241 nm (ε 26 300), 251 sh (20 700); $[\alpha]_D$ +9.4° (*c* 0.585, $CHCl_3$), -35.1° (*c* 0.655, CH_3OH); MS (Scheme I) *m/e* 888 (M^+), 743, 600, 582, 564, 323, 305, 289, 259, 239, 221, 145, 127, 113. Anal. Calcd for $C_{48}H_{72}O_{15}$: C, 64.84; H, 8.16. Found: C, 64.45; H, 8.32.

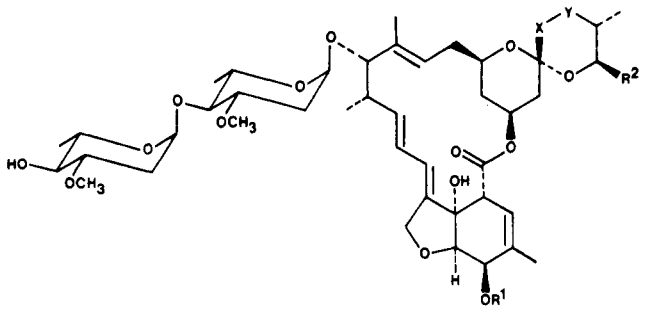
5-[^3H]Avermectin B_1a (1). A solution of 3 (310 mg, 356 μmol) in methanol (3.0 mL) at -15 °C was treated with NaB^3H_4 (13.4 mg, 356 μmol, 4.0 Ci), and stirred 20 min at 25 °C. The mixture was cooled to 5–10 °C, treated dropwise with H_2O (10 mL), warmed to 25 °C, and stirred 1 h, with precautions to remove HT gas by catalytic reduction. The product was filtered off, washed with 3:1 (v/v) water–methanol, and air-dried to provide 249 mg (714 mCi) of 1, ~80% pure. Crude 1 (111 mg) was purified by preparative TLC [twelve 250-μm SG GF plates developed twice with 4:1 (v/v) chloroform–tetrahydrofuran], followed by Sephadex LH-20 chromatography [1.3 L; 8:1:1 (v/v/v) hexane–toluene–methanol], to provide 81 mg (210 mCi, 65%) of 1, 99% pure.

5-[^3H]Avermectin B_2a (2). A solution of 4 (90 mg, 100 μmol) in ethanol (2 mL) was cooled to -15 °C and treated with NaB^3H_4 (4.0 mg, 106 μmol, 860 mCi). After 15 min the reaction mixture was quenched by addition of 0.1 N acetic acid (10 mL), stirred 30 min with precautions to remove HT gas by catalytic reduction, filtered, washed with water, and dried in vacuo at 25 °C. The product was purified by preparative TLC [eight 250-μm SG GF plates developed with 87:13 (v/v) chloroform–tetrahydrofuran], followed by preparative HPLC, to provide 23.5 mg (55 mCi, 26%) of 2, 99% pure.

RESULTS AND DISCUSSION

Oxidation of 5 or 6 with manganese dioxide provides 5-ketoavermectins 3 and 4 as 80–85% pure materials in 70–85% yields. The ketones streak on silica gel, however, and subsequent purification on this support resulted in about a 30% loss of material. Ketones 3 and 4 are stable solids which exhibit no tendency to isomerize to α,β-unsaturated lactones or to aromatize. Treatment of 3 or 4 with triethylamine or potassium carbonate for 18 h at 25 °C in alcoholic solution returned the 5-ketones unchanged. The structures of the ketones were determined by comparison of their spectral properties with those of the natural products (Albers-Schönberg et al., 1981). Their 300-MHz ^1H NMR spectra were particularly informative and are illustrated in Figure 2. Both 3 and 4 showed loss of the 5 proton of 5 and 6 and identical downfield shifts in H-2, H-3, and H-6 expected upon conversion of an allylic alcohol to an α,β-unsaturated ketone. All other protons in 3 and 4 were identical in shift and multiplicity with those of 5 and 6, respectively. The presence of an α,β-unsaturated ketone was confirmed by the presence of a new absorption at 1685 cm^{-1} in their IR spectra, in addition to the lactone carbonyl absorption at 1735 cm^{-1}. The mass spectral fragmentation patterns of 3 and 4 (Scheme I) also agree with a loss of two hydrogen atoms from C-5 and the 5-OH.

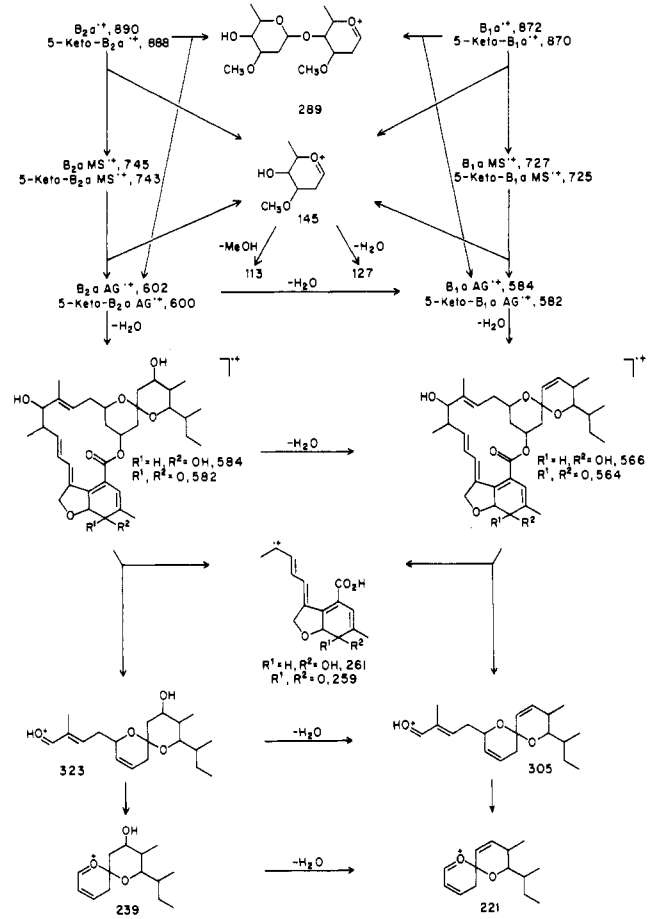
Models of 3 and 4 indicated that the α face was much less hindered, suggesting that hydride reduction would provide largely 5 and 6 and not their 5 epimers. In fact, reduction of 3 and 4 with sodium borohydride provided mixtures containing ~70% of the desired isomers, from which 90% pure materials were isolated by silica gel chromatography. Samples of >95% purity were prepared by subsequent chromatography on Sephadex LH-20. The reduction products were identical with the natural products in their spectral, chromatographic, and biological (Ostlind, 1977) properties. Use of NaB^3H_4 for reduction introduced the desired radiolabel, which was found to be metabolically stable in vivo (Green and Jacobs, 1979).



Avermectin	R^1	X-Y	R^2
A_1a	CH_3	CH=CH	(S)-secC_4H_9
A_1b	CH_3	CH=CH	isoC_3H_7
A_2a	CH_3	(S)-$CH_2CH(OH)$	(S)-secC_4H_9
A_2b	CH_3	(S)-$CH_2CH(OH)$	isoC_3H_7
B_1a (5)	H	CH=CH	(S)-secC_4H_9
5-^{3}H: 1	H	CH=CH	(S)-secC_4H_9
B_1b	H	CH=CH	isoC_3H_7
B_2a (6)	H	(S)-$CH_2CH(OH)$	(S)-secC_4H_9
5-^{3}H: 2	H	(S)-$CH_2CH(OH)$	(S)-secC_4H_9
B_2b	H	(S)-$CH_2CH(OH)$	isoC_3H_7

Figure 1. Structures of the avermectins and 5-[^{3}H]avermectins.

Scheme I. Mass Spectral Fragmentation Patterns of Avermectins B_1a (5) and B_2a (6) and 5-Ketoavermectins B_1a (3) and B_2a (4)



The low overall yields of 1 and 2 prepared by this redox sequence reflect the losses of material entailed in purifying the final products to 99% purity required for biological evaluation. Nevertheless, this sequence is rapid, mild, and selective and should be applicable to a wide variety of 5-hydroxylated avermectin derivatives.

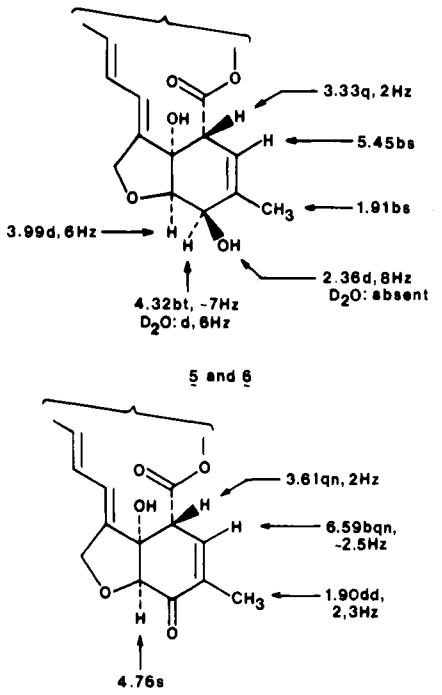


Figure 2. 300-MHz ^{1}H NMR spectral assignments for avermectins B_1a (5) and B_2a (6) and 5-ketoavermectins B_1a (3) and B_2a (4). s = singlet, d = doublet, t = triplet, q = quartet, qn = quintet, dd = doublet of doublets, and b = broad. Values in hertz are apparent coupling constants.

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Inhibitors of Trypsin and Chymotrypsin in Winged Bean (*Psophocarpus tetragonolobus*) Tubers

Trypsin and α-chymotrypsin inhibitors were present in winged bean (*Psophocarpus tetragonolobus* var. Chimbu) tubers. Compared with those of mature seeds, the levels of inhibitors were approximately the same. Tuber inhibitors could be easily inactivated by wet heat with more than 90% loss of activity after a 2-min heating of a water suspension of freeze-dried tubers in boiling water. The two types of inhibitors exhibited similar inactivation patterns. Affinity chromatography demonstrated that the two proteinase inhibitors are distinct and different and do not have overlapping activities. The presence of these inhibitors point to the necessity of processing winged bean tubers before consumption to eliminate these toxic factors.

A survey of underexploited tropical plants by a panel of the National Academy of Sciences (1975) brought attention to the winged bean (*Psophocarpus tetragonolobus*) as a promising source of protein and oil for both animals and humans in the humid tropics. It is interesting to note that the potential of this crop as a possible substitute for soybean was pointed out as early as 1929 by Agcaoili (1929), who published probably the first chemical composition of the mature seed.

The plant is grown and eaten in many parts of Southeast Asia and Melanesia (Burkill, 1906; Purseglove, 1968) and other parts of the tropics. The tender, green pods are the most popular part of the plant and used as a green vegetable, either raw or cooked, in most of these places. All parts of the plant are claimed to be edible and nutritious and efforts are being made to provide information on their chemical composition (Onuma Okezie and Martin, 1980). The leaves and the flowers are popular in many areas where they are eaten either raw or cooked and added to salads and soups (Claydon, 1978).

The tubers of winged bean are consumed as human food but this appears to be limited to Burma and New Guinea highlands (Claydon, 1978). It is possible that the limited use of the tuber in certain areas is due to the fact that people are not aware of the tubers because of the low yield of certain varieties and the adverse effects of tubers when eaten raw (Claydon, 1978). The tubers are reported to be eaten raw or cooked (Burkill, 1906). Chemical composition data reported so far show that the tubers have relatively much higher protein than other edible plant roots. To our knowledge, there have been no studies on antinutritional factors in the tubers. The presence of such compounds in the tubers can be very important due to its consumption in the raw state as pointed out. We are reporting here the presence of trypsin and chymotrypsin inhibitors in winged bean and their heat inactivation patterns.

MATERIALS AND METHODS

Seeds and Tubers. Winged bean seeds (var. Chimbu), were supplied to us by Louis Lazaroff of the International Council on Development of Underutilized Crops, Orinda, CA. The seeds were planted in April 1979 in the University greenhouse and flowered in November, and the dried pods and tubers were harvested in the first week of Jan 1980. Periodic partial pruning by removing young shoots, flowers, and pods is claimed to increase tuber yield (Khan et al., 1977), but since the main objective was to multiply seeds, this was not done in these plants. The average yield of tuber was ~60 g/plant. The seeds used in the experiments for comparison were obtained from the same plants.

Extraction and Determination of Trypsin and Chymotrypsin Inhibitors. The extraction and determination of trypsin inhibitor from the seed were done as reported previously (de Lumen and Salamat, 1980). Typically, 1 g of ground sample was extracted with 20 mL of ice-cold distilled water by homogenizing for 2 min with a Potter-Elvehjem homogenizer run with an electric drill. The extract was centrifuged at 4 °C for 30 min at 13600*g* and the supernatant was used for the assay after appropriate dilution.

The whole tubers, after washing and drying, were homogenized in a blender for ~2 min without the addition of additional water and freeze-dried. This material was extracted the same way as the seeds.

Trypsin activity was measured by its hydrolysis of *p*-toluenesulfonylarginine methyl ester (TAME) at 25 °C, pH 8.1, and measuring the absorbance of *p*-toluenesulfonylarginine at 247 nm with a Cary 14 recording spectrophotometer. One unit of trypsin activity is defined as 1 μmol of product released per min. One unit of trypsin activity inhibited is defined as 1 inhibitor unit (TIU).

Chymotrypsin activity was determined by hydrolysis of benzoyl-L-tyrosine ethyl ester (BTEE) as described in the Worthington Biochemical Corp. (1977) manual. The increase in absorbance at 256 nm was recorded as described above. One unit of chymotrypsin activity is equivalent to 1 μmol of substrate hydrolyzed per min at pH 7.8 and 25 °C. One unit of enzyme activity inhibited is defined as 1 chymotrypsin inhibitor unit (CIU).

Trypsin (2× crystallized) was obtained from Sigma Chemical Co. (St. Louis, MO) while chymotrypsin was obtained from Worthington Biochemical Corp. (Freehold, NJ).

Crude fat and crude protein were carried out according to AOAC (1975) procedures. Acid detergent fiber was done according to Van Soest (1963).

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