Synthesis and Structural Elucidation of Analogs of Ochratoxin A

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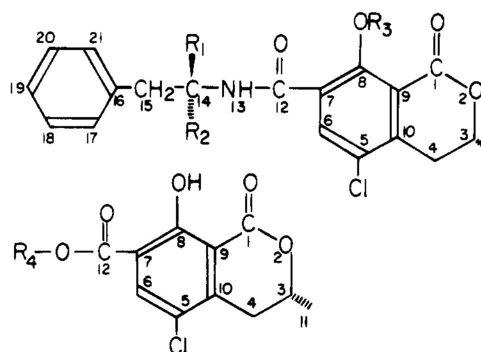
Five analogs of ochratoxin A (OA) including the ethylamide of OA (OE-OA), the D-phenylalanine form of OA (*d*-OA), the decarboxylated OA (DC-OA), the *O*-methyl ether of OA (OM-OA), and the methyl ester of ochratoxin α (M-Oα) were synthesized using OA or ochratoxin α (Oα) as the starting material. The reactions involved activation of OA to the *N*-hydroxysuccinimide ester (OA-NHS) and of Oα to acyl chloride (Oα-Cl) followed by nucleophilic substitution with primary amines, amino acids, and alcohols to form corresponding amides and esters. All analogs were obtained in pure forms, and all but OM-OA were crystallized. A simplified procedure for the isolation and crystallization of Oα was also developed. The chemical structures of all analogs were elucidated and/or confirmed using EI-MS and ^{1}H NMR. Other physicochemical parameters such as melting point, UV–vis absorption, fluorescence, and HPLC elution pattern for each analog are presented. The procedures that have been developed for the synthesis of the analogs of OA from OA or Oα are simple and efficient. The reactions generally result in high yields of the desired compounds. The overall yields of final products range approximately from 85 to 90% of the starting materials. The analogs synthesized together with the natural analogs of OA can be used to establish the structure–activity relationship of OA and for metabolic and immunological studies.

Keywords: *Ochratoxin A; synthetic analogs; synthesis; structure*

INTRODUCTION

Ochratoxin A (OA), the 7-(L-β-phenylalaninylcarbonyl)-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3*R*-methylisocoumarin (Figure 1), is a secondary metabolite of some toxigenic species of *Aspergillus* and *Penicillium*. This mycotoxin is of concern as it is hepatotoxic, nephrotoxic, teratogenic, and carcinogenic to most animal species (Kuiper-Goodman and Scott, 1989) and occurs worldwide in many agricultural commodities (Dwivedi and Burns, 1986). On the basis of information from the literature, the toxicity of OA may be the result of three major effects: (1) inhibition of ATP synthesis; (2) enhanced lipid peroxidation; and (3) inhibition of protein synthesis (Marquardt and Frohlich, 1992; Röschenthaler *et al.*, 1984). Several hypotheses for the mechanism of action of OA have been proposed. Creppy *et al.* (1983, 1984) reported that OA competitively inhibited phenylalanine tRNA synthetase, resulting in inhibition of protein synthesis. Enhanced lipid peroxidation in animals treated with OA has been observed, suggesting that free radicals or active oxygen species may be involved in ochratoxicosis (Rahimtula *et al.*, 1988; Omar *et al.*, 1990). Ochratoxin A was reported to be a competitive inhibitor of succinate dehydrogenase and cytochrome *c* oxidase in cellular respiration, resulting in mitochondrial dysfunction (Wei *et al.*, 1985). Ochratoxin A is metabolized to hydroxy-OAs (OA-OH) by a microsomal cytochrome P_{450} dependent enzyme system (Stormer *et al.*, 1983,). Conclusive evidence, however, has not been provided on the mode of action of OA and if its toxicity is the result of a direct action of OA or its bioactivation in the animal. A structure–activity relationship study was therefore initiated to determine the roles of each functional groups in OA toxicity. The design and synthesis of the OA analogs were directed to remove or to block the functional groups and to modify the hydrophobic side chain of OA. This study describes simple and efficient procedures for preparation of modified forms of OA useful for further toxicological studies.



	ANALOG	R1	R2	R3	R4
I	OA	COOH	H	H	—
II	OE-OA	$CONHCH_2CH_3$	H	H	—
III	DC-OA	H	H	H	—
IV	OM-OA	COOH	H	CH_3	—
V	d-OA	H	COOH	H	—
VI	M-Oα	—	—	—	CH_3
VII	Oα	—	—	—	H

Figure 1. Structure of OA and its analogs. OA, ochratoxin A; OE-OA, ethylamide of OA; DC-OA, decarboxylated OA; OM-OA, *O*-methyl ether of OA; *d*-OA, D-phenylalanine form of OA; Oα, ochratoxin Oα; M-Oα, methyl ester of Oα.

EXPERIMENTAL PROCEDURES

Chemicals. Reagent grade OA, methyl ester of D-phenylalanine (D-Phe) containing 5% of L-phenylalanine (L-Phe), *N*,*N*-carbonyldiimidazole (CDI), N-hydroxysuccinimide (NHS), di-

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cyclohexycarbodiimide (DCC), phenylethylamine, and ethylamine were purchased from Sigma (St. Louis, MO.). Thionyl chloride ($SOCl_2$) was purchased from Aldrich (Milwaukee, WI.). Dry, powdered sodium metal (dry Na) was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Diazomethane was prepared according to the method described by De Boer and Backer (1963).

Instrumentation. Reversed-phase (KC18 5 × 20 cm) and silica gel (LK5D 5 × 20 cm) TLC plates were from Whatman, Inc. (Clifton, NJ). The reversed-phase column (C_{18}) for the HPLC system was from Beckman Canada Ltd. (Mississauga, ON), while the photodiode array UV–vis detector and the fluorescent detector (RF-535) were from Shimadzu (Kyoto, Japan). Unless specified in the text, all of the analytes were eluted from the HPLC system under such conditions: 65% solvent A (methanol/2-isopropanol, 9:1) and 35% solvent B (double-distilled H_2O, pH 2.1) at a temperature of 39 °C with a flow rate of 1.5 mL/min. The synthetic products were dried using a vacuum dryer (AS160-Savant, Farmingdale, NY). The melting point (mp) for each compound was determined using an uncorrected capillary melting point apparatus (Unimelt, Philadelphia, PA). UV–vis spectra were recorded using a DU-8 spectrophotometer (Beckman, Irvine, CA). Microscopic observations of crystalline OA and its analogs were carried out according to the procedure described by Marquardt *et al.* (1989). ^1H NMR spectra were recorded at 300 MHz (Bruker AM300, Germany) with tetramethylsilane (TMS) as a reference standard, and EI-MS were obtained using a 7070EHF organic mass spectrometer (VG Analytical, Manchester, England) at the Department of Chemistry, University of Manitoba.

Preparation of Ochratoxins A (OA, Figure 1, I), B (OB), and α (Oα, Figure 1, VII). OA and OB were produced using solid-phase fermentation of wheat inoculated with *Aspergillus ochraceus* NRRL 3174. In general, 2 kg of wheat was separated into 50 g samples and added individually to 40 flasks (500 mL). The wheat was adjusted to 45% moisture (w/w), and the flasks were autoclaved for 30 min. The wheat was cooled, inoculated with *A. ochraceus*, sealed with a sponge plug, and incubated at 39 °C for 4 weeks. The fermented wheat was autoclaved, dried, and prepared for the isolation of OA and OB using a modification of the procedure described by van der Merwe *et al.* (1965). The fermented wheat (350 g) was soaked with 350 mL of 0.1 N HCl and extracted with 2.5 L of $CHCl_3$ in a 4 L separation funnel with constant shaking for 30 min. The $CHCl_3$ fraction was collected, evaporated to 500 mL, and mixed with 1 L of 0.1 M Na_2CO_3. The basic aqueous fraction which contained OA was separated from the $CHCl_3$ fraction, acidified with 6 N HCl to a pH of 1, and re-extracted with 500 mL of $CHCl_3$. The $CHCl_3$ fraction containing the ochratoxins was dried using a rotatory evaporator (Buchi, Brinkman, Rotavapor R110), and the residual toxins were dissolved in 10–20 mL of benzene. The benzene fraction was applied onto a silica gel column (25 × 5 cm, 40 μm), and the toxins were eluted (10 mL/min) with acidified benzene (benzene/acetic acid, 95:5, v/v). The flow rate of the column was controlled with pressurized air. The fraction containing OA was dried and then dissolved in 5 mL of hot benzene. Crystalline OA (Figure 2) formed within 30 min. Recrystallization (three times) in hot benzene (500 mg/mL) yielded pure OA, which was dried using a vacuum dryer (AS160-Savant) at 50 °C for 24 h to remove the associated benzene. The final yield of OA crystals from 350 g of fermented wheat was 1650 mg (approximately 5 g of OA/kg of dry matter). Some of the properties of OA were as follows: HPLC elution time, 6.9 min; UV λ_{max} (in ethanol), 215 nm (e = 28 000), 333 nm [e = 5500, (lit. 6 400; van der Merwe *et al.*, 1965)]; fluorescence, 333 nm (excitation), 452 nm (emission); mp (from benzene), 118–122 °C (lit. 121 °C; van der Merwe *et al.*, 1965); EI-MS, *m/z* 403 (M^+, 100%), 405 ($M^+ + 2$, 35%), 358 (45%), 255 (50%), 241 (25%), 239 (70%); ^1H NMR ($CDCl_3$) δ 1.59 (3H, d, $J_{11,3}$ = 6.3 Hz, 3-CH_3), 2.85 (1H, dd, $J_{4,4}$ = 17.5 Hz, $J_{4\text{-}pro\text{-}R,3}$ = 11.5 Hz, 4-*pro-R*-H), 3.22 (1H, dd, $J_{15,15}$ = 14.1 Hz, $J_{15\text{-}pro\text{-}S,14}$ = 7.4 Hz, 15-*pro-S*-H), 3.28 (1H, dd, $J_{4,4}$ = 17.5 Hz, $J_{4\text{-}pro\text{-}S,3}$ = 3.3 Hz, 4-*pro-S*-H), 3.36 (1H, dd, $J_{15,15}$ = 14.1 Hz, $J_{15\text{-}pro\text{-}R,14}$ = 5.3 Hz, 15-*pro-R*-H), 4.75 (1H, m, 3-H), 5.04 (1H, m, 14-H), 7.2–7.4 (5H, m, 17–21-H), 8.42 (1H, s, 6-H), 8.48 (1H, d, $J_{13,14}$ = 6.7 Hz, 13-H), 12.76 (1H, s, 8-OH).

A blue fluorescent fraction containing OB was eluted (10 mL/min) from the column after elution of OA. OB crystallized in methanol (50 mg/mL) over 24 h at 0 °C. Pure white crystalline OB (Figure 2) was obtained following recrystallization in methanol. OB was also prepared by catalytic dechlorination of OA following the method described by Bredenkamp *et al.* (1989). The total yield of OB from 350 g of fermented wheat was 500 mg (approximately 1500 mg/kg of dry matter). Some of the properties of OB were as follows: HPLC elution time, 4.5 min; UV λ_{max} (in ethanol) 218 nm (e = 32 000), 320 nm (e = 6900); fluorescence, 320 nm (excitation), 458 nm (emission); mp (from methanol), 217–220 °C; EI-MS, *m/z* 369 (M^+, 80%), 324 (25%), 221 (30%), 205 (100%); ^1H NMR ($CDCl_3$), δ 1.55 (3H, d, $J_{11,3}$ = 6.3 Hz, 3-CH_3), 2.98 (2H, m, 4-H), 3.23 (1H, dd, $J_{15,15}$ = 14.2 Hz, $J_{15\text{-}pro\text{-}S,14}$ = 7.7 Hz, 15-*pro-S*-H), 3.34 (1H, dd, $J_{15,15}$ = 14.2 Hz, $J_{15\text{-}pro\text{-}R,14}$ = 5.3 Hz, 15-*pro-R*-H), 4.75 (1H, m, 3-H), 4.97 (1H, m, 14-H), 6.84 (1H, d, $J_{5,6}$ = 8.0 Hz, 5-H), 7.22–7.35 (5H, m, 17–21-H), 8.35 (1H, d, $J_{6,5}$ = 8.0 Hz, 6-H), 8.54 (1H, d, $J_{13,14}$ = 6.5 Hz, 13-H), 12.72 (1H, s, 8-OH).

Ochratoxin α was prepared using a modification of the procedure described by van der Merwe *et al.* (1965). Ochratoxin A (250 mg) was heated with 100 mL of 6 N HCl and refluxed for 72 h. The hydrolyzed Oα crystallized from 6 N HCl at 25 °C over 12 h without purification. The crystals of Oα were harvested by the filtration and were washed repeatedly with distilled water to remove L-Phe. Pure Oα crystals were obtained following recrystallization of Oα (100 mg/mL) in the mixture of methanol and water (1:1). Residual Oα from the acid–water fraction were separated from phe by partitioning into 250 mL of $CHCl_3$. The $CHCl_3$ fraction containing Oα was evaporated to dryness, and Oα (50 mg) was reconstituted with 0.5 mL of methanol/ethyl ether (75:25 v/v ether) at 60 °C. Crystals of Oα (Figure 2) formed at 4 °C over 48–72 h. Residual OA was completly removed following recrystallization of Oα in methanol. The total yield of Oα from OA was approximately 60%. Some of the properties of Oα were as follows: HPLC elution time, 3.1 min; UV λ_{max} (in ethanol), 218 nm (e = 23 000), 335 nm (e = 6200); fluorescence, 335 nm (excitation), 433 nm (emission); mp (from methanol), 245–246 °C; EI-MS, *m/z* 256 (M^+, 45%), 258 ($M^+ + 2$, 15%), 214 (35%), 212 (100%), 194 (70%); ^1H NMR ($CDCl_3$) δ 1.63 (3H, d, $J_{11,3}$ = 6.3 Hz, 3-CH_3), 2.91 (1H, dd, $J_{4,4}$ = 17.6 Hz, $J_{4\text{-}pro\text{-}R,3}$ = 11.6 Hz, 4-*pro-R*-H), 3.30 (1H, $J_{4,4}$ = 17.6 Hz, $J_{4\text{-}pro\text{-}S}$ = 3.5 Hz, 4-*pro-S*-H), 4.82 (1H, m, 3-H), 8.41 (1H, s, 6-H), 10.45 (1H, broaden, 7-COOH), 13.30 (1H, s, 8–OH).

Preparation of the *N*-Hydroxysuccinimide Ester of OA (OA-NHS). OA-NHS was used as an activated intermediate for the synthesis of the analogs from OA. NHS (500 mg) and OA (500 mg) were dissolved with 2 mL of anhydrous tetrahydrofuran (THF) in a 10 mL reaction vial and 1000 mg of DCC was then added to the solution at 25 °C and left to react for 1 h. The mixture was evaporated under nitrogen gas and reconstituted with 4 mL of anhydrous $CHCl_3$ to dissolve the OA-NHS. The precipitate was discarded, and the $CHCl_3$ fraction was stored in a sealed vial at −20 °C until used for subsequent reactions. OA-NHS when subjected to TLC (silica) using neutral and anhydrous $CHCl_3$ as the mobile phase had an R_F value of 0.88 and exhibited a blue fluorescence under UV light (300–400 nm). Unreacted OA was retained at the origin.

Preparation of Acyl Chloride of Oα (Oα-Cl). Oα-Cl was used as an activated intermediate for the synthesis of several analogs of OA from Oα and was prepared by reacting Oα with $SOCl_2$ under reduced pressure. Oα (500 mg) was added to a 10 mL reaction vial, dissolved in 4 mL of anhydrous $CHCl_3$ containing 10% $SOCl_2$, and allowed to react at 25 °C for 2 h. The reaction mixture was dried with the aid of a vacuum dryer (AS160-Savant) at 25 °C for 12 h to remove residual SO_2 and HCl. This product was used for subsequent reactions. The presence of Oα-Cl was established by adding a drop of absolute ethanol to 1 μL of the reaction mixture. The ethyl ester of Oα, which was the product of the reaction, migrates with the

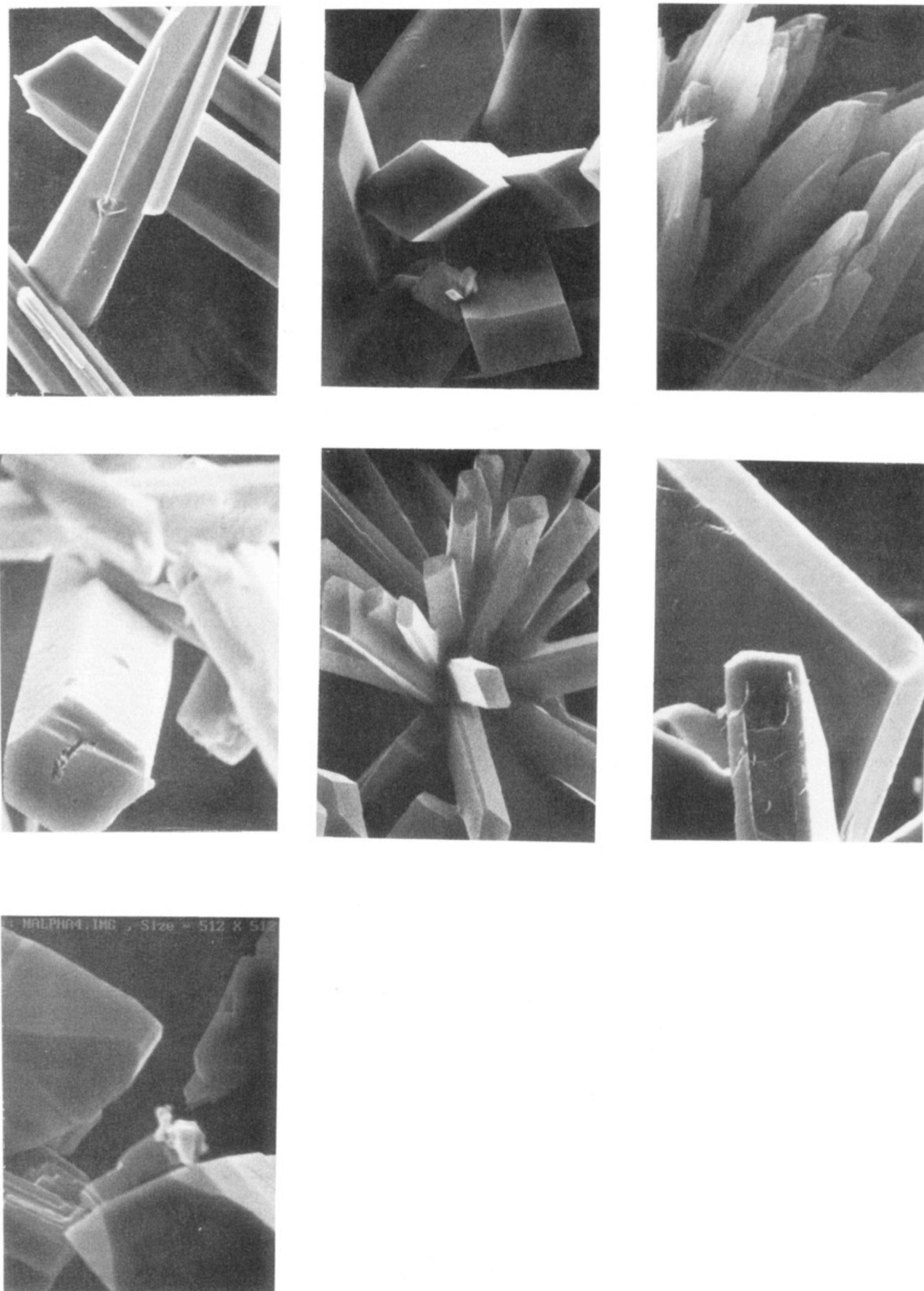

Figure 2. Electron micrograph images of crystalline structures of OA and analogs. Top: OA (left), OB (center), Oα (right). Middle: OE-OA (left), *d*-OA (center), DC-OA (right). Bottom: M-Oα.

solvent (neutral $CHCl_3$) during TLC (silica), while unreacted Oα stays at the origin.

Synthesis of Ethylamide of OA (OE-OA, Figure 1, II). Ethylamine and OA-NHS were used for synthesis of OE-OA. Ethylamine (500 μL) was diluted with 2 mL of anhydrous $CHCl_3$, and the mixture was added to a 10 mL reaction vial containing 500 mg of OA-NHS and allowed to react at 25 °C for 4 h. The reaction mixture was diluted to 100 mL with $CHCl_3$, and OE-OA was isolated from the mixture by partitioning with 100 mL of acidified water (pH 0.5−1) to remove the excessive ethylamine and with 100 mL of neutral water. The chloroform fraction containing OE-OA was evaporated to approximately 5 mL and applied to a silica gel column (25 × 5 cm, 40 μm) for purification. OE-OA was eluted from the

column with neutral chloroform (10 mL/min). The purified OE-OA was dried and crystallized in benzene (100 mg/mL) at 25 °C over 2 h (Figure 2). The final yield of OE-OA crystals was approximately 460 mg, corresponding to a conversion rate of approximately 90% of OA. Some of the properties of OE-OA were as follows: HPLC elution time (neutral mobile phase), 9.2 min (acidic mobile phase, pH 2.1) 6.9 min; UV λ_{max} (in ethanol), 215 nm (e = 36 000), 381 nm (e = 8500); fluorescence, 381 nm (excitation), 442 nm (emission); mp, 156–158 °C; EI-MS, m/z 430 (M^+, 25%), 432 (M^+ + 2, 10%), 360 (20%), 358 (60%), 255 (20%), 241 (35%), 239 (100%), 176 (30%); ^{1}H NMR ($CDCl_3$) δ 1.02 (3H, t, $J_{25,24}$ = 7.2 Hz, CH_3), 1.59 (3H, d, $J_{11,3}$ = 6.3 Hz, 3-CH_3), 2.87 (1H, dd, $J_{4,4}$ = 17.4 Hz, $J_{4\text{-}pro\text{-}R,3}$ = 11.5 Hz, 4-*pro-R*-H), 3.07–3.32 (5H, m, 4-*pro-S*-H, 15-H, CH_2), 4.77 (2H, m, 14-H, 3-H), 5.68 (1H, broaden, 14-CONH), 7.20–7.35 (5H, m, 17–21-H), 8.41 (1H, s, 6-H), 8.60 (1H, d, $J_{13,14}$ = 7.4 Hz, 13-H), 12.8 (1H, s, 8-OH).

Synthesis of *d*-Ochratoxin A (d-OA, Figure 1, V). This compound was prepared by reacting the methyl ester of D-Phe with Oα-Cl. The hydrochloride salt of the methyl ester of D-Phe (500 mg) was deprotonated using fine dry Na metal powder in 4 mL of anhydrous $CHCl_3$. The mixture was added to 250 mg of Oα-Cl in a 10 mL reaction vial and allowed to react at 25 °C for 2 h. The methyl ester of *d*-OA was then separated from the mixture by partitioning into 100 mL of $CHCl_3$ in the presence of 100 mL of acidic water (pH 0.5) to remove excessive D-Phe and then with 100 mL of neutral water to remove unreacted Oα. The methyl ester of *d*-OA in the $CHCl_3$ fraction was dried and hydrolyzed to *d*-OA with 100 mL of 0.5 N NaOH at 25 °C for 12 h. The mixture was then acidified with 6 N HCl to pH <1.0 and allowed to stand for 12 h at 20 °C followed by extraction of *d*-OA with 100 mL of $CHCl_3$ in a 500 mL separation funnel. The $CHCl_3$ fraction containing *d*-OA was dried, and *d*-OA was crystallized in benzene (100 mg/mL) at 25 °C in 12 h. The crystals, however, contained approximately 5% OA (the L form of OA) as indicated by the HPLC profile. The L form of OA was not removed by recrystallization. *d*-OA, however, was separated from OA by HPLC (C_{18} reversed phase preparatory column) following elution with 50% solvent A and 50% solvent B (pH 2.1) at a flow rate of 10 mL/min. Pure *d*-OA crystals (Figure 2) were then obtained by recrystallized from benzene (100 mg/mL) at 25 °C. The total yield of *d*-OA from Oα following HPLC was approximately 85%. The properties of *d*-OA were as follows: HPLC elution time, 8.62 min; UV λ_{max} (in ethanol) 228 nm (e = 18 000), 333 nm (e = 6700); fluorescence, 333 nm (excitation), 456 nm (emission); mp, 184–186 °C; EI-MS, m/z 403 (M^+, 5%), 257 (35%), 255 (100%), 241 (30%), 239 (90%), 120 (15%); ^{1}H NMR ($CDCl_3$) δ 1.59 (3H, d, $J_{1,3}$ = 6.3 Hz, 3-CH_3), 2.87 (1H, dd, $J_{4,4}$ = 17.4 Hz, $J_{4\text{-}pro\text{-}R,3}$ = 11.6 Hz, 4-*pro-R*-H), 3.24 (1H, $J_{15,15}$ = 14.4 Hz, $J_{15\text{-}pro\text{-}R,14}$ = 7.3 Hz, 15-*pro-R*-H), 3.28 (1H, dd, $J_{4,4}$ = 17.4 Hz, $J_{4\text{-}pro\text{-}S,3}$ = 3.5 Hz, 4-*pro-S*-H), 3.32 (1H, dd, $J_{15,15}$ = 14.1 Hz, $J_{15\text{-}pro\text{-}S,14}$ = 5.3 Hz, 15-*pro-S*-H), 4.75 (1H, m, 3-H), 5.02 (1H, q, $J_{14,15}J_{14,13}$ = 7.0 Hz, 14-H), 7.20–7.38 (5H, m, 17–21-H), 8.42 (1H, s, 6-H), 8.47 (1H, d, $J_{13,14}$ = 6.7 Hz, 13-H), 12.75 (1H, s, 8-OH).

Synthesis of the *O*-Methyl Ether of OA (OM-OA, Figure 1, IV). This compound was obtained by direct hydrolysis of the ester of the methyl ester of OM-OA in 0.5 N NaOH. The methyl ester of OM-OA was synthesized according to the method described by van der Merwe *et al.* (1965). OA (600 mg) was mixed with 1000 mg of diazomethane in 10 mL of methanol and allowed to react at 25 °C for 12 h to form the methyl ester of OM-OA. After evaporation of the solvent and the excess of diazomethane, the oily ester was dissolved in 10 mL of methanol and was then hydrolyzed to OM-OA in 100 mL of 0.5 N NaOH at 25 °C for 12 h. The hydrophobic impurities were removed from the alkaline solution by partitioning into 100 mL of $CHCl_3$. The aqueous phase was then acidified with 6 N HCl to pH 0.5–1 for 12 h at 20 °C and extracted with 100 mL of $CHCl_3$ to obtain pure OM-OA. The total yield of OM-OA from the methyl ester of OM-OA was approximately 95% by weight. Some of the properties of OM-OA were as follows: HPLC elution time, 6.3 min; UV λ_{max}, 216 nm (e = 30 100), 310 nm (e = 3200); EI-MS, m/z 417 (M^+, 15%), 269 (45%), 255 (35%), 253 (100%); ^{1}H NMR ($CDCl_3$) δ 1.54 (3H, d, $J_{11,3}$ = 6.3 Hz, 3-CH_3), 2.85 (1H, dd, $J_{4,4}$ = 17.3 Hz, $J_{4\text{-}pro\text{-}R,3}$ = 11.5 Hz, 4-*pro-R*-H), 3.20 (1H, dd, $J_{15,15}$ = 14.4 Hz, $J_{15\text{-}pro\text{-}S,14}$ = 7.6 Hz, 15-*pro-S*-H), 3.20 (1H, dd, $J_{4,4}$ = 17.3 Hz, $J_{4\text{-}pro\text{-}S,3}$ = 2.9 Hz, 4-*pro-S*-H), 3.40 (1H, dd, $J_{15,15}$ = 14.4 Hz, $J_{15\text{-}pro\text{-}R,14}$ = 5.3 Hz, 15-*pro-R*-H), 3.60 (3H, s, 8-OCH_3), 4.56 (1H, m, 3-H), 5.06 (1H, q, $J_{14,13}$ $J_{14,15}$ = 7.3 Hz, 14-H), 7.20–7.35 (5H, m, 17–21-H), 8.35 (1H, d, $J_{13,14}$ = 7.7 Hz, 13-H), 8.36 (1H, s, 6-H).

Synthesis of Decarboxylated OA (DC-OA, Figure 1, III). DC-OA was prepared by reacting phenylethylamine with Oα-Cl. Phenylethylamine (500 μL) was diluted with 2 mL of anhydrous $CHCl_3$ and reacted with 200 mg of Oα-Cl at 0 °C for 2 h. DC-OA was separated from the mixture by partitioning into 100 mL of $CHCl_3$ in the presence of 100 mL of acidified water (pH 0.5–1) to remove the excess phenylethylamine and then with 100 mL of water (pH 7) to remove the unreacted Oα. The $CHCl_3$ fraction containing DC-OA was evaporated, and DC-OA was dissolved in 1 mL of benzene. Crystals of DC-OA (Figure 2) were obtained at 4 °C over 30–60 min. Pure DC-OA was obtained by recrystallization (50 mg/mL) in methanol/water (9:1 v/v). The total yield of DC-OA from Oα was approximately 95% as determined by HPLC. Some of the properties of DC-OA were as follows: HPLC elution time, 12.2 min; UV λ_{max} (in ethanol), 218 nm (e = 30 000), 333 nm (e = 5700); fluorescence, 333 nm (excitation), 462 nm (emission); mp, 154–155 °C; EI-MS, m/z 359 (M^+, 90%), 361 (M^+ + 2, 30%), 257 (15%), 255 (50%), 241 (35%), 239 (100%), 104 (55%); ^{1}H NMR ($CDCl_3$) δ 1.59 (3H, d, $J_{11,3}$ = 6.3 Hz, 3-CH_3), 2.84 (1H, dd, $J_{4,4}$ = 17.4 Hz, $J_{4\text{-}pro\text{-}R,3}$ = 11.6 Hz, 4-*pro-R*-H), 2.93 (2H, t, $J_{15,14}$ = 7.0 Hz, 15-H), 3.28 (1H, dd, $J_{4,4}$ = 17.4 Hz, $J_{4\text{-}pro\text{-}S,3}$ = 3.4 Hz, 4-*pro-S*-H), 3.74 (2H, q, $J_{14,15}J_{14,13}$ = 7.0 Hz, 14-H), 4.74 (1H, m, 3-H), 7.18–7.36 (5H, m, 17–21-H), 8.06 (1H, broaden, 13-H), 8.45 (1H, s, 6-H), 12.66 (1H, s, 8-OH).

Synthesis of Methyl Ester of Oα (M-Oα, Figure 1, VI). Oα-Cl (250 mg) was allowed to directly react with 1 mL of absolute methanol at 4 °C for 2 h to yield M-Oα. The residual HCl and unreacted Oα were removed by partitioning into 100 mL of $CHCl_3$ in the presence of 100 mL of neutral distilled water. M-Oα in the $CHCl_3$ fraction was dried and crystallized (Figure 2) in a mixture of ethyl acetate/methanol (2:8 v/v) at 25 °C. The total yield of M-Oα from Oα was approximately 99%. Some of the properties were as follows: HPLC elution time, 4.57 min; UV λ_{max} (in methanol), 218 nm (e = 24 000), 335 nm (e = 5800); fluorescence, 335 nm (excitation), 466 nm (emission); mp, 141–142 °C; EI-MS, m/z 270 (M^+, 100%), 272 (M^+ + 2, 35%), 239 (45%), 194 (80%); ^{1}H NMR ($CDCl_3$) δ 1.58 (3H, d, $J_{11,3}$ = 6.3 HZ, 3-CH_3), 2.84 (1H, dd, $J_{4,4}$ = 17.4 Hz, $J_{4\text{-}pro\text{-}R,3}$ = 11.6 Hz, 4-*pro-R*-H), 3.27 (1H, dd, $J_{4,4}$ = 17.4 Hz, $J_{4\text{-}pro\text{-}S,3}$ = 3.3 Hz, 4-*pro-S*-H), 3.94 (3H, s, 12-OCH_3), 4.72 (1H, m, 3-H), 8.11 (1H, s, 6-H), 12.19 (1H, s, 8-OH).

Biological Assay. The biological activity of OA and its analogs was tested using a bacterial disk diffusion assay system as described by Madhyastha *et al.* (1994).

RESULTS AND DISCUSSION

Isolation of OA, OB, and Oα. The isolation of ochratoxins from a solid-phase fermentation medium is usually more difficult than from a liquid-phase medium due to the complexity of the culture medium. Nevertheless, OA and OB were produced using solid-phase fermentation as the procedure is simple and results in high yields of toxins (OA, 5000 mg/kg; OB, 1500 mg/kg). Several different organic solvents such as methanol, acetonitrile, and $CHCl_3$ can be used for extraction of the ochratoxins. Regardless of its toxicity, $CHCl_3$ was selected as it not only efficiently extracts the toxins at a low pH but is also immiscible with water, which facilitates partitioning of compounds between the aqueous and organic phases. The pigments and lipids in the crude $CHCl_3$ extract can be efficiently separated by partitioning the toxins into carbonate buffer. The ochratoxins in the carbonate fraction are readily recovered by extraction with $CHCl_3$ at low pH. High-resolution flash chromatography (10 mL/min) using a

column packed with fine silica (40 μm) gel and a benzene/acetic acid (95:5 v/v) mobile phase resulted in a rapid and efficient separation of OA and OB (loading >2 g of OA and OB/400 mL gel). Pure OA and OB following column chromatography are readily crystallized in benzene and methanol, respectively. The associated solvents in the crystals can be removed by heating at 45 °C under reduced pressure. These modified procedures are suitable for the large-scale production and isolation of OA and OB. Acid hydrolysis of OA in concentrated HCl yields Phe and Oα (van der Merwe *et al.*, 1965). The recovery of Oα from the hydrolysate in the current study was relatively high (>90%) with only trace amounts (<1%) being oxidized when the sample was refluxed in concentrated HCl. The procedure for isolation of Oα is relatively simple as Oα crystallizes in 6 N HCl while the other product, Phe, remains in the solution. Crystalline Oα is easily separated from Phe by filtration and by repeated washing with distilled water. Oα can also be crystallized in methanol/ether (75:25 v/v), and the traces of unhydrolyzed OA can be removed by recrystallization in the same solvent. The HPLC (50% solvent A, 50% solvent B, pH 2.1) elution profile as detected by a photodiode array UV–vis detector (50 μg/injection) demonstrated that Oα crystals from the acidified water were highly pure as only a single peak was observed having a UV spectrum (λ_{max}, 335 nm) identical to that of Oα. The structure and physicochemical properties of OA, OB, and Oα, as outlined under Experimental Procedures, agree with those reported in the literature (van der Merwe *et al.*, 1965) and further confirmed their identity and purity.

Activation of OA and Oα. In the current study, synthesis of an activated carboxyl intermediate of OA using $SOCl_2$ and CDI resulted in the generation of isomers as indicated by the HPLC profiles of their products. Both reactions resulted in racemization of the L-Phe moiety of OA and, therefore, could not be used for the synthesis of stereospecific products from OA. The reactions, however, are highly efficient and relatively rapid and provide high yields of products which are useful for preparation of immunoconjugates of OA and of other carboxylated haptens (unpublished data). OA-NHS was selected as the activated intermediate for the synthesis of analogs from OA as this reaction yields stereospecific products (Rousseau *et al.*, 1984). Thionyl chloride, however, can be used for the activation of the carboxyl group of Oα. It forms an intermediate, the acyl chloride of Oα (Oα-Cl), which is highly reactive to primary amines and primary alcohols to yield the corresponding amides and esters. The reaction does not involve an asymmetric center and therefore did not produce isomers. This intermediate could be readily produced in high purity as the reagent ($SOCl_2$) and its degraded products (HCl and SO_2) are volatile and, therefore, readily removed under reduced pressure. Oα-Cl is a white powder which should be prepared fresh as it decomposes in the presence of trace amounts of water. Nondissociable and anhydrous organic solvents should be used in these synthetic reactions. $CHCl_3$ is an ideal solvent for the reactions as essentially only the desired product is formed with very small amounts of oxidation products of Oα being formed. The use of $SOCl_2$ for synthesis of OA from Oα has been reported by Breitholtz-Emanuelsson *et al.* (1992). Their procedure was not used in the current study as it is relatively complicated and involves several unnecessary steps. Other procedures for the activation of Oα were not used in the current study as they all have limitations relative to that outlined above. Activation of Oα with CDI also resulted in a slow rate of reaction and a low yield of product. Activation of Oα using NHS in contrast to CDI resulted in high yield of products, but they were considerably more difficult to isolate. The NHS procedure has been used for the synthesis of ^{14}C-labeled OA from Oα and [^{14}C]Phe (Rousseau *et al.*, 1984).

Synthesis of OE-OA. Ethylamine is an excellent nucleophile and readily reacts with OA-NHS to form the ethylamide of OA (OE-OA). A 5-fold dilution of ethylamine with anhydrous $CHCl_3$ reduced the oxidation of OA and optimized the reaction. The purification procedure requires considerable effort as the reagents in the reaction medium cannot be separated from OE-OA through partition chromatography. The HPLC profile of the reaction mixture showed peaks for unreacted OA at 5.6 min (5% of total area) and another peak at 9.2 min for OE-OA (92%) when eluted with a neutral mobile phase. The two peaks for OA and OE-OA merged at 6.9 min when they were eluted at a low pH (pH 2.1). This indicated that OE-OA was more strongly adsorbed to the stationary phase (C_{18}) under neutral as compared to acidic condition, while the opposite pattern was observed for OA. MS analysis of OE-OA indicates that OE-OA has a mass of 430. The fragment ions of OE-OA that correspond to the isocoumarin moiety are identical to those of OA, indicating ethylamine is covalently linked to the carboxyl group of OA. The ^{1}H NMR spectrum of OE-OA, as compared to that of OA, has three additional protons at 1.0 ppm (triplet, the CH_3 protons of the ethyl group), two at 3.2 ppm (multiplets, the CH_2 protons of the ethyl group), and one at 5.7 ppm (broaden, the NH proton of ethyl amide), indicating that ethylamine is linked to the carboxyl group of OA through an amide bond. OE-OA is relatively stable under acidic or basic condition (pH 2–12).

Synthesis of *d*-OA. Coupling the methyl ester of D-Phe to Oα-Cl generates HCl, which in turn protonates the amino group of D-Phe and retards the reaction. The efficiency of this reaction, however, was greatly increased by the use of dry Na metal powder as a deprotonating agent. The methyl ester of *d*-OA was readily isolated from the mixture by partition chromatography. Complete hydrolysis of *d*-OA methyl ester to *d*-OA with 0.5 N NaOH requires approximately 12 h which may also open the lactone ring of *d*-OA. The medium should therefore be acidified to pH 1.0 and left for 12 h to restore the lactone structure before extraction with $CHCl_3$. This conclusion is based on the observation that *d*-OA exhibits an UV λ_{max} at 345 nm (the open-ring form) after hydrolysis in alkaline solution but shifts back to 333 nm in a time-dependent manner when the solution is acidified, indicating that an intramolecular esterification reaction may occur. The phenolic group of *d*-OA dissociated in 0.5 N NaOH solution, exhibiting an instantaneous red shift in UV λ_{max} from 333 to 380 nm, followed by a time-dependent blue shift from 380 to 345 nm. A similar pattern of time-dependent changes in UV λ_{max} of *d*-OA was not observed in 0.5 M Na_2CO_3 ($11 > pH > 9$, λ_{max} 380 nm). The red shift was not observed with OM-OA, a form in which the phenolic group is blocked. These observations suggest that the red shift in UV absorption in 0.5 NaOH is attributed to the dissociation of the phenolic group (Chu *et al.*, 1972) and the blue shift (λ_{max} 380 to 345 nm) possibly to the

base hydrolysis of the lactone of *d*-OA. The HPLC profile of the reaction mixture indicated the presence of the L form of OA (<5%). Both OA and *d*-OA can be crystallized in benzene, with the appearance of crystalline OA (fine needle) being different from that of *d*-OA (rectangular). The presence of small amounts of OA in this reaction cannot be attributed to racemization caused by the use of Oα-Cl but to the contamination of D-Phe with L-Phe, since the same proportion of OA and *d*-OA was also observed in the reaction using the Oα-NHS ester, which was known to be stereospecific (Rousseau *et al.*, 1984). Steyn *et al.* (1967) reported that Oα-Cl caused extensive racemization in the preparation of OA, but the procedure was different from that in the current study. The stereospecificity of this reaction was also confirmed using the pure methyl ester of L-Phe and Oα-Cl for resynthesis of OA. Under such conditions only OA but no *d*-OA was formed as indicated by the HPLC profile of the reaction mixture. Pure *d*-OA (>99%) can be obtained following the removal of residual Oα and OA by HPLC using a preparatory reversed-phase column (C_{18}). The EI-MS of *d*-OA showed the same molecular and fragment ions as those for OA but with different intensity. The NMR spectrum of *d*-OA showed identical chemical shifts and coupling constants to that of OA. Although the two compounds cannot be readily distinguished on the basis of NMR or MS, they exhibit differences in their HPLC elution profile (6.9 and 8.6 min, respectively) and in the appearance of their crystal structure. *d*-Ochratoxin A is stable in most organic solvents and in water over long periods of time.

Synthesis of DC-OA. Phenylethylamine is the decarboxylated form of Phe and will readily react with Oα-Cl to form the phenylethylamide of Oα, the decarboxylated form of OA (DC-OA). The HPLC profile of the reaction mixture shows an Oα peak at 3.1 min (<5%) and a peak for DC-OA at 12.2 min (>95%), indicating that the yield of DC-OA in this reaction was high. DC-OA was readily isolated from the mixture by partitioning into $CHCl_3$ with acidified water (pH <2) to remove excessive phenylethylamine and then with neutral water to remove unreacted Oα. DC-OA in the $CHCl_3$ fraction was relatively pure and could be directly crystallized from benzene or methanol/water (8:2 v/v). EI-MS of DC-OA yielded a molecular ion having a *m/z* of 359. The linkage between phenylethylamine and Oα was confirmed to be an amide bond as indicated by the ^1H NMR spectra of DC-OA. The spectrum of DC-OA as compared to that of OA demonstrates that DC-OA does not have an asymmetric carbon center but has one extra proton at the 14-C position. The higher field chemical shift of protons at the 14-C and 13-N positions of DC-OA as compared to those of OA also indicates the absence of the carboxyl group.

Synthesis of OM-OA. OM-OA was readily obtained by hydrolysis of the ester bond of the methyl ester *O*-methyl ether of OA (methyl ester of OM-OA) with 0.5 N NaOH. The procedure for the synthesis of the ester has been described by van der Merwe *et al.* (1965). Caution should be taken with this procedure as diazomethane used in the reaction is a volatile, explosive, and toxic gas. Again, as discussed above, restoration of the ring structure from the alkaline condition must be considered before the extraction of OM-OA from 0.5 N NaOH as a time-dependent shift of UV λ_{max} from 295 (open-ring form) to 310 nm (closed-ring form) was also evident when the NaOH solution was acidified. The UV λ_{max} (310 nm) of OM-OA is the same in 0.1 N HCl (pH <2) and in 0.5 N Na_2CO_3 (pH >9). The ^1H NMR spectrum of OM-OA showed one extra singlet with three protons at 4 ppm (8-OCH_3) and the absence of the singlet for the phenolic hydroxyl proton as compared to the spectrum of OA, indicating that the phenolic hydroxy group but not the carboxyl group was methylated. The EI-MS of OM-OA showed a molecular ion with a mass of 417, corresponding to the additional mass of the methyl group. The fragment ions attributed to the isocoumarin moiety of OM-OA as compared to those for OA also had an additional mass equal to that of the methyl group, further confirming that it was not the carboxyl but the phenolic group that was methylated. OM-OA did not emit visible light when excited at 200–400 nm. OM-OA is relatively stable in water and other common organic solvents.

Synthesis of M-Oα. The HPLC profile of the reaction mixture showed only a single peak for M-Oα at 4.6 min, indicating that the conversion rate of this reaction was almost 100%. After the solvent was dried under reduced pressure, M-Oα could be crystallized in methanol without further purification. The molecular ion of M-Oα as determined by EI-MS was observed at 270 *m/z* (100%) with the other fragment ions of M-Oα being identical to those observed for Oα. The ^1H NMR spectrum of M-Oα as compared to that of Oα revealed an additional singlet (three protons) at 4 ppm, corresponding to the additional methyl group and the absence of the exchangeable carboxyl proton of Oα at 10 ppm. This suggested that a methyl group was linked to the carboxyl of Oα. M-Oα is not stable in water at a pH above 8 due to hydrolysis of the ester bond. Displacement of the methyl group by other primary alcohols was also observed. Storage of M-Oα in a dry crystalline state is highly recommended.

Biological Activity of the Synthetic Analogs. The antimicrobial activity of OA and its synthetic analogs was determined using *Bacillus brevis* as a model in a disk difussion assay system. The assay determined the minimum dose (in micrograms per disk) of each analog that causes a visible and measurable inhibition zone of growth (in millimeters). The results indicated that the parent toxin (OA) had the same toxicity as *d*-OA (12 mm, 2 μg/disk), 4-folds less toxicity than OE-OA (12 mm, 0.5 μg/disk), 25-fold more toxicity than M-Oα (11 mm, 50 μg/disk), and 50-fold more toxicity than OM-OA (11 mm, 100 μg/disk). The decarboxylated form of OA, DC-OA, was not toxic to the bacteria at doses of 100 μg/disk. A systemic study on the structure–activity relationship in cytotoxicity, nephrotoxicity, genotoxicity, and immunotoxicity of OA and its analogs (including some natural analogs) is in progress.

Summary. Simple and efficient methods for the synthesis, isolation, and purification of five analogs of OA were developed. The structures and the major chemical properties of these analogs were characterized using HPLC, UV–vis, EI-MS, and ^1H NMR. These compounds together with the natural analogs of OA will be used for further study to establish the structure–activity relationships and the molecular mechanism of action of OA.

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