under anaerobic conditions for the reduction of the flavopapain by these substrates.

In summary, using *N*-alkyl-1,4-dihydronicotinamides as reductants and a variety of appropriate electron acceptors we have shown that a flavoenzyme exhibiting high catalytic efficiency both in terms of k_{cat}/K_m and of the value of the turnover number can be constructed by utilizing the active and binding sites of the hydrolytic enzyme papain.

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Trichothecene Metabolism Studies: Isolation and Structure Determination of 15-Acetyl-3α-(1′β-D-glucopyranosiduronyl)-scirpen-3,4β,15-triol

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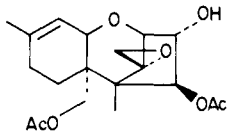
The epoxytrichothecene mycotoxins are a group of fungal metabolites that exhibit a range of significant biological properties including cyto- and phytotoxicity.[3] These mycotoxins are potent inhibitors of protein synthesis in eukaryotes and have been implicated in a number of diseases of plants, animals, and humans.[3a] Certain members of this group, including anguidine (**1**) and T-2 toxin (**2**), have also received considerable notoriety as a consequence of the "yellow rain" controversy.[4]



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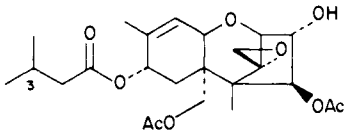
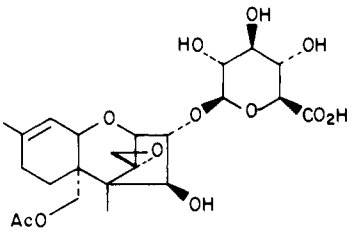


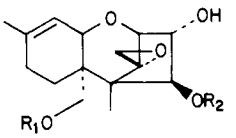
2

In spite of the toxicological significance of the epoxytrichothecenes, relatively little is known about their metabolic fate in mammalian systems.[3,5] Anguidine, for example, has been examined in dogs and monkeys mainly for toxic manifestations.[6,7]

The only metabolic transformations documented prior to the initiation of our studies had been deacylation reactions of the trichothecene esters mediated by microsomal esterases,[5c,8] the cytochrome P_{450} catalyzed oxidations of T-2 and HT-2 toxin leading to 3′-hydroxyl derivatives,[5a,b] and the deoxygenation of the 12,13-epoxide of deoxynivalenol.[5d] We report herein the isolation and structure determination of glucuronide **3**, the major



3



4, R_1=Ac, R_2=H
5, R_1=H, R_2=Ac

metabolite of anguidine in in vitro studies, and also present evidence that glucuronidation occurs in vivo.[9] This is the first rigorous demonstration that glucuronidation is a significant pathway for trichothecene metabolism.

[^{3}H]Anguidine (12 μM, 0.16 μCi)[10] was incubated with uridine 5′-diphosphoglucuronic acid (UDPGA, 12 mM), β-naphthoflavone-induced hepatic microsomes from male Long-Evans rats (0.6 mg of protein/mL),[11] $MgCl_2$ (2.5 mM), and K_2HPO_4 (10 mM, pH 7.7) at 37 °C. HPLC analysis of the mixture after 1.5 h indicated that three new products were present: glucuronide **3** (38–50%; R_t = 12.5 min), 15-acetoxyscirpendiol (**4**) (43–32%; R_t = 22 min), and 4-acetoxyscirpendiol (**5**) (18–19%; R_t = 18 min).[12-14] After a 3.3-h incubation, the mixture consisted of **3** (68–73%), **4** (1–4%), and **5** (22–23%).[15] Scaleup of this procedure[16] (12 mg of anguidine) afforded 9 mg (56%) of **3**[17] following HPLC purification.

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(9) A complete account of our investigation of the metabolism of anguidine will be reported separately: Marletta, M. A.; Roush, W. R.; Recchia, J.; Russo-Rodriguez, S., manuscript in preparation. We have looked for, but failed to find any evidence of, metabolic products stemming from cytochrome P_{450} oxidation, epoxide hydrolysis, or glutathione conjugation.

(10) [^{3}H]Anguidine (13.4 mCi/mmol) was prepared by Dr. T. J. Caggiano by NaB^3H_4 reduction of 4β,15-diacetoxyscirpen-3-one. We thank Dr. Doyle of Bristol Laboratories for providing a copy of this procedure prior to publication.

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(12) The microsomes were removed by centrifugation at the end of the incubation. The metabolites were concentrated on a C18 SEP-PAK cartridge (Waters Assoc.) that had been preequilibrated in water, eluted with MeOH, and then separated by HPLC (see ref 13).

(13) A μBondapak C18 column (3.9 mm × 30 cm; Waters Assoc.) was used for all analyses and isolations (100% H_2O for 2 min, 0–45% MeOH linear ramp for 13 min, and a 45–60% MeOH linear ramp for 15 min; 1.5 mL/min).

(14) Metabolites **4** and **5** were identified by GC/MS analysis with comparison to authentic samples. We thank Dr. J. S. Wishnok for assistance with these analyses.

(15) These data suggest that anguidine is rapidly hydrolyzed to a mixture of monoacetates **4** and **5** and that glucuronidation then occurs with **4** in a subsequent step. In support of this hypothesis we have found that incubation of [^{3}H]-**4** with liver microsomes and UDPGA, or **4** with [^{14}C]-UDPGA, according to the procedure described in the text also afforded glucuronide **3**.

(16) UDPGA (12 mM), anguidine (645 μM), and 1.16 mg/mL (29 mg total) of microsomal protein were incubated for 3.3 h.

(17) Data for **3**: $[\alpha]^{22}_D$ – 44.7° (*c* 0.44, 99.8% MeOH); ^{1}H NMR (MeOH-d_4, 250 MHz) δ 5.44 (br d, 1 H, *J* = 5.2 Hz, H-10), 4.67 (d, 1 H, *J* = 7.8 Hz, H-1′), 4.43 (m, 2 H, H-3 and H-4), 4.20 and 3.88 (AB, 2 H, *J* = 12.3 Hz, H-15), 4.00 (d, 1 H, *J* = 5.4 Hz, H-11), 3.67 (d, 1 H, *J* = 4.5 Hz, H-2), 3.61 (m, 1 H, sugar), 3.45 (m, 2 H, sugar), 2.95 and 2.77 (AB, 2 H, *J* = 4.1 Hz, H-13), 2.04 (s, 3 H, -OAc), 1.70 (s, 3 H, H-16), 0.80 (s, 3 H, H-14); FT-IR (solid film on CaF_2 window) 3500–3200 (br, OH), 2966, 2910, 1739, 1653, 1616, 1613, 1419, 1415, 1387, 1374, 1366, 1242, 1159 cm^{-1}; mass spectrum (FAB, glycerol dispersion), *m/e* 501 (MH^+), 307, 265.

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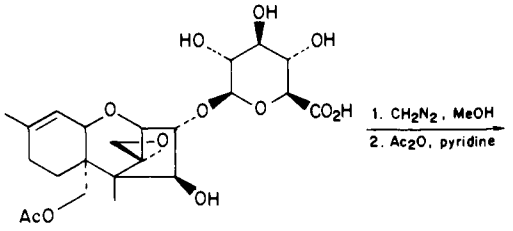
The FAB mass spectrum[18] of **3** is consistent with a 1:1 adduct of a monoacetoxyscirpendiol and glucuronic acid [*m/e* 501 (MH^+), 307 ($M^+ - C_6H_9O_7$), 265 ($M^+ - C_6H_9O_7 - C_2H_2O$)], a conclusion supported by ^1H NMR data which showed a single acetyl resonance.[17] Comparison of the ^1H signals for H-4 (δ 4.43), H-3 (4.43), and H-15 (4.20 and 3.88) of **3** measured in MeOH-d_4 with those of **4** (δ 4.21, H-4; 4.06, H-3; 4.15 and 3.91, H-15) and **5** (δ 5.54, H-4; 4.19, H-3; 3.93 and 3.45, H-15) suggested that **3** is a glucuronide derivative of **4**. This assignment was verified by incubation of [^3H]-**3** (3.7 μM) with limpet β-glucuronidase (40 units, 200 units/mL) in 0.2 M NaOAc (pH 4.5) for 18 h (37 °C) which afforded **4** in 73% yield (HPLC analysis).[19] The linkage between the trichothecene nucleus and glucuronic acid was determined following conversion to the peracetate methyl ester derivative **6**.[20] This compound proved to be identical with an



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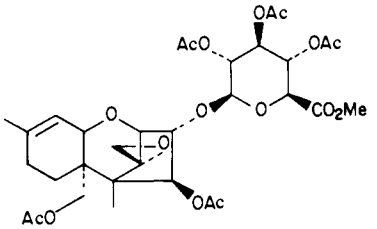

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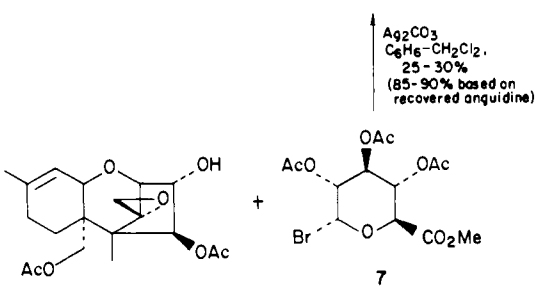

1 **7**

authentic sample synthesized from anguidine (**1**) and bromo sugar **7** by a Koenigs–Knorr reaction (25–30% yield; 85–90% based on recovered anguidine).[21] These experiments rigorously establish that the glucuronic acid residue is attached to C3–OH of **4** and that the glycosidic linkage is β.

A combination of in vitro and in vivo studies are necessary to fully understand the pathways of foreign compound metabolism. In vitro studies are particularly useful in defining the types of transformations expected in vivo, thereby facilitating structural characterization of in vivo metabolites. For example, evidence from a number of laboratories has pointed toward the production and excretion of very polar trichothecene metabolites.[5e,g,h] On the basis of our work it is now reasonable to speculate that one or more of these as yet unidentified compounds may be glucuronides. Indeed, we have found that mice treated with [^3H]-anguidine excrete most of the dose in urine and that one of the major urinary metabolites is a glucuronide (substrate for limpet β-glucuronidase).[22] This, together with our finding that **3** is a poor inhibitor of protein synthesis relative to anguidine,[9] highlights the need to fully understand the nature and scope of metabolic transformations responsible for trichothecene modifications since these processes undoubtedly influence the biological half-life and the observed toxicity. Such studies are in progress and will be reported in due course.

Acknowledgment. This research was supported by the U.S. Army Medical Research and Development Command (Contract DAMD 17-82-C-2235).

(18) The FAB mass spectral measurements were performed by Dr. C. Costello and S. Maleknia using the facility supported by NIH Research Grant RR00317 from the Biotechnology Resources Branch, Division of Research Resources (Principal Investigator, Prof. K. Biemann).

(19) Glucuronide **3** is not a substrate for bovine liver or *E. coli* β-glucuronidases.

(20) Data for **6**: mp 94.5–97 °C (CH_2Cl_2); $[\alpha]^{23}_D$ – 11° (*c* 1.61, $CHCl_3$); ^1H NMR ($CDCl_3$, 250 MHz) δ 5.71 (d, 1 H, *J* = 2.8 Hz, H-4), 5.47 (br d, 1 H, *J* = 5.3 Hz, H-10), 5.25 (m, 2 H, H-3′ and H-4′), 5.09 (dd, 1 H, *J* = 7.7, 9.2 Hz, H-2′), 4.76 (d, 1 H, *J* = 7.5 Hz, H-1′), 4.35 (dd, 1 H, *J* = 2.9, 4.9 Hz, H-3), 4.14 and 4.02 (AB, 2 H, *J* = 12.3 Hz, H-15), 3.96 (m, 2 H, H-5′ and H-11), 3.74 (d, 1 H, partially obscured by CO_2Me, H-2), 3.71 (s, 3 H, CO_2Me), 3.05 and 2.75 (AB, 2 H, *J* = 3.9 Hz, H-13), 2.11–2.00 (five s, 15 H, OAc), 1.68 (s, 3 H, H-16), 0.72 (s, 3 H, H-14); IR ($CHCl_3$) 2980, 2950, 2930, 2910, 1750 (br), 1440, 1370, 1250–1170 (br), 1070–1020 (br) cm^{-1}; mass spectrum (FAB, glycerol dispersion), *m/e* 682 (M^+), 622 (M^+ – HOAc); EI mass spectrum, *m/e* 622 (M^+ – HOAc). Anal. Calcd for $C_{32}H_{42}O_{16}$: C, 56.28; H, 6.20. Found: C, 56.10; H, 6.37.

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(22) The FAB mass spectrum (glycerol dispersion) of this metabolite shows a molecular ion at *m/e* 523 (MNa^+) and the product of β-glucuronidase treatment is monoacetate **4** (HPLC analysis). However, HPLC coelution studies have shown that this metabolite is not **3**. A rigorous structure proof which will establish the position of glucuronidation (apparently at C4) is in progress.

Picosecond Time-Resolved Raman Studies of Photodissociated Carboxymyoglobin

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X-ray crystallographic studies of myoglobin reveal that in liganded Mb the protein environment about the heme is different from that of the equilibrium deoxy species.[1a,b] These structural changes are qualitatively similar to the more exaggerated changes observed in X-ray crystallographic comparisons between deoxy and carboxy hemoglobin.[2] The structural changes in the heme environment induced by ligand binding can be studied using time-resolved resonance Raman spectroscopy.[3] Transient absorption studies[4–6] have shown that within a fraction of a picosecond subsequent to ligand photolysis from hemoglobin and myoglobin the heme absorption spectrum qualitatively resembles that of equilibrium deoxyheme. Protein reorganization in response to changes at the heme is expected to occur more slowly. Na-

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