Microbiological Hydroxylation at Eight Individual Carbon Atoms of Marcfortine A

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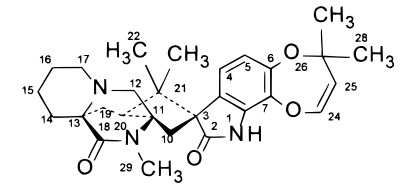
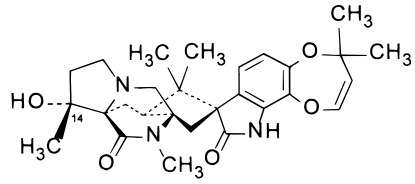
Hydroxylation of the indole alkaloid marcfortine A (**1**) at carbon atoms 5, 10, 12, 14, 15, 16, and 27 was realized by various soil-derived microorganisms. Fission at the C-12−N bond was also observed. The structural elucidation of the products (**2**−**12**) was accomplished by spectroscopic and semisynthetic means.

Helminths, especially parasitic nematodes, cause substantial health problems in humans and domestic animals. Currently, three distinct chemical classes are used for broad-spectrum control of gastrointestinal nematodes in veterinary medicine: benzimidazoles, imidazothiazoles, and macrocyclic lactones.[1] None of these drugs is ideally suited for all therapeutic situations, and each class has been challenged by the development of drug-resistant nematode strains.[2] Expansion of the anthelmintic arsenal is thus an urgent goal. Marcfortine A, paraherquamide A, and their analogues have potent antiparasitic activity.[3−10] Because the marcfortines and paraherquamides are unique both structurally and in their mode of action, they represent a promising new class of anthelmintics (Figure 1).

In 1991 at Upjohn, a new penicillium strain was discovered that produced marcfortine A (**1**),[11] originally isolated from *Penicillium roqueforti*.[12] Marcfortine A is closely related to paraherquamide A (**2**) first isolated by Yamazaki *et al.*[13] The difference between the two compounds resides only in ring G (Figure 1). While in **1** the G ring is six-membered with no substituents at C-14, in **2** the G ring is five-membered and has a hydroxyl and a methyl group at C-14. Introduction of a hydroxyl group in ring G of **1** would provide a convenient handle to correlate the two series of compounds and to further derivatize **1**.



Marcfortine A (**1**)



Paraherquamide A (**2**)

Figure 1.

Results and Discussion

Many reviews and hundreds of papers have been published on the use of mono-oxygenases for the introduction of oxygen atoms onto various substrates.[14] We were especially interested in nonactivated stereospecific carbon atom hydroxylation, and therefore, a large number of biotransformation experiments were carried out utilizing cultures reported in the literature and those randomly selected from the Upjohn culture collection and various soils. Screening was performed by adding a solution of **1** (10 mg) in DMF (0.4 mL) to vigorously growing 100 mL fermentations in 500 mL wide-mouth flasks. Incubation was conducted at 28 °C, and shaking was continued for 1−3 days depending on the culture. Isolations were achieved by thorough extraction with methylene chloride (CH_2Cl_2), centrifugation, and removal of solvents in high vacuum. The residues were analyzed by TLC, in three solvent systems, and also by HPLC. Promising samples were scaled up 10−100-fold and refermented in a Labraferm fermentation tank. Purifications were achieved by various chromatographic techniques, and structure elucidations were accomplished by NMR and MS. Whenever semisynthetic samples were available, a side by side comparison was also performed. The great majority of cultures screened either totally metabolized **1** or left it unchanged. However, a few cultures did provide hydroxylated products. Extensive efforts were undertaken to increase the yields of biotransformation products by changing the media, temperature, length of fermentation, etc. The highest yields were obtained for compounds **6**−**8**, which on scale-up gave a 10−15% yield in addition to recovered starting material (about 30%), which could be recycled.

Microbiological hydroxylation of **1** by three cultures (UC 7258, UC 4327, and UC 4998) (Table 1) all produced the same mixture consisting of **3** and **4** (Figure 2). The formyl derivative **3** is formed by the fission of the C-11,C-12 bond, and its structure is based on ^{1}H, ^{13}C, COSY, and HETCOR NMR experiments. In the ^{1}H spectrum a peak at δ 7.98 is indicative of the *N*-formyl functionality, further corroborated by the appearance of a new peak at δ 162 in the ^{13}C spectrum and the disappearance of the C-12 signal at δ 61. In the MS of **3** a gain of 14 mass units also agreed with the loss of

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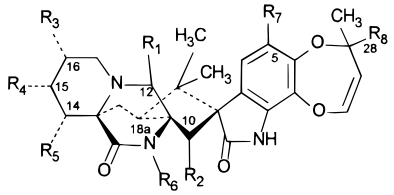
Table 1. Microbiological Hydroxylation of Marcfortine A

compd no.	culture
3 and **4**	UC 4327, UC 4998, UC 7258
5	UC 10750
6 and **7**	UC 11136
8	UC 11141, UC 5059, UC 11144
9 and **10**	UC 7330
11	UC 7602
12	UC 8622 and UC 11202



1 R_6= R_8=CH_3; R_1=R_2=R_3=R_4=R_5=R_7=H

4 R_1=OH; R_6= R_8=CH_3; R_2=R_3=R_4=R_5=R_7=H

5 R_2=OH; R_6= R_8=CH_3; R_1=R_3=R_4=R_5=R_7=H

6 R_3=OH; R_6= R_8=CH_3; R_1=R_2=R_4=R_5=R_7=H

7 R_4=OH; R_6= R_8=CH_3; R_1=R_2=R_3=R_5=R_7=H

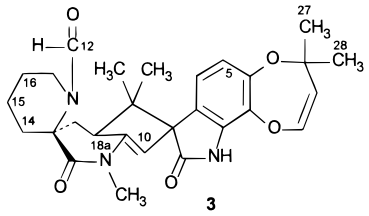
8 R_5=OH; R_6= R_8=CH_3; R_1=R_2=R_3=R_4=R_7=H

9 R_8=CH_3; R_1=R_2=R_3=R_4=R_5=R_6=R_7=H

10 R_1=OH; R_8=CH_3; R_2=R_3=R_4=R_5=R_6=R_7=H

11 R_7=OH; R_6=R_8=CH_3; R_1=R_2=R_3=R_4=R_5=H

12 R_8=CH_2OH; R_6=CH_3; R_1=R_2=R_3=R_4=R_5=R_7=H



3

Figure 2.

two hydrogen atoms and the addition of one oxygen. Compound **3** was identical with a semisynthetic sample prepared by the $FeCl_3/H_2O_2$ oxidation of **1**.[15] The precursor of **3** surely must be the 12-hydroxy compound **4**, whose structure was difficult to elucidate due to a misleading MS under FABMS. Thus, while the expected FABMS *m/z* is 494.2577 $(M + H)^+$ for **4**, the found value for FABMS *m/z* is 476.2579 $(M + H)^+$. This is a loss of 18 units (H_2O) and can be explained by a spontaneous dehydration in the mass spectrometer, an occurrence that is often seen in the MS of marcfortines possessing the NCOH moiety. The figure agrees well with the calculated figure $C_{28}H_{33}N_3O_4 + H_1 = 476.2549$. In the NMR of **4**, the C-12 is deshielded from δ 61 to 91, a figure in good agreement with the presence of the NCHOH moiety.

UC 10750 introduced a hydroxyl group at C-10 to provide **5**, a somewhat unexpected biotransformation due to the hindered nature of that carbon atom. Its structure proof rests on the downfield shift of the two hydrogens at C-10 from δ 1.8 and 2.3 (two doublets) to a broad singlet (1 hydrogen) at δ 4.7. COSY, HETCOR, and MS results are all in agreement with the postulated structure. Synthetically, this compound was not obtained by us.

UC 11136 was a most prolific metabolizer of **1**. At least five new spots were visible on TLC, in addition to unchanged starting material. Several scale-up experiments were carried out, and the two major constituents were isolated and identified as the 16-hydroxy (**6**) and the 15-hydroxy (**7**) derivatives of **1**. Despite the very hindered nature of the 14 position, cultures UC 5059, UC 11141, and UC 11144 were able to introduce the hydroxyl group at this position to give **8** (10−15% yield). The structure proofs for these three compounds (**6**−**8**) hydroxylated at positions 16, 15, and 14 rests again on the deshielding effect of the hydroxyl group in the NMR spectrum as described above. Compounds **6**−**8** were also identical with samples prepared from **1** by multistep synthetic procedures,[16] and the stereochemistries of these compounds were previously assigned.[16]

A somewhat different path was taken by UC7330. After scale-up, two major compounds were isolated. Compound **9** was characterized as marcfortine B, which lacks the very characteristic *N*-methyl group (δ 3.1) at position 18a. This material had been previously isolated from the naturally occurring mixture[17] of marcfortines. *N*-Demethylation is a well-known process that proceeds by hydroxylation to the NCH_2OH moiety followed by oxidation to a carbamic acid that spontaneously decomposes to the secondary amine. The second compound (**10**) was a 12-hydroxy derivative lacking a methyl group at position 18a and identified by comparison to the previously described **4**.

Of the two aromatic sites available for hydroxylation in **1**, UC 7602 substituted a hydroxyl group in the 5-position to provide **11**. Structural elucidation of **11** was based on the lack of a peak at δ 6.67, a characteristic of the aromatic hydrogen at C-5 in this series.

Two cultures, UC 8622 and UC 11202, hydroxylated one of the *gem*-dimethyl groups (C-27 or C-28) to provide **12**. Structure proof of **12** was deduced from the absence of a major peak at δ 1.43; the conformation of the methyl group carrying the hydroxyl functionality is unknown. In conclusion, microbiological hydroxylation of **1** was achieved on eight distinct carbon atoms, and three of those hydroxylations occurred in ring G. Except for **8**, all of these hydroxylated derivatives (**2**−**12**) are less active against *Hc* and *Tc* in our *jird* model than **1**.[18]

Experimental Section

General Experimental Procedures. Chemical reagents and solvents were obtained from commercial sources and used directly unless otherwise stated. ^1H and ^{13}C NMR were recorded on a Bruker ARX 400 NMR spectrometer at 400.13 and 100.12 MHz, respectively. FABMS were measured on a VG70-SE mass spectrometer. Thin-layer chromatography was performed on silica gel 60 F254 plates. Small-scale purifications were carried out by preparative TLC (Analtech silica gel GF plates, 20 × 20 cm, 1000 μm) using one of the solvent systems described above. For larger scale work flash chromatography over silica gel (EM Science, 230−400 mesh ASTM) was performed, and the fractions were analyzed by TLC and/or HPLC. ^1H NMR spectra were recorded at 300 or 400 MHz with TMS as internal standard; ^{13}C NMR spectra were recorded at 75 or 100 MHz. Fast atom bombardment mass spectrometry (FAB) was the only satisfactory method for obtaining high-resolution MS results.

Biotransformation Procedure. Agar plugs of fungi, actinomycetes, and other bacteria stored over liquid nitrogen were inoculated into 100 mL of sterile GS-7

medium contained in 500 mL wide-mouth fermentation flasks and incubated at 28 °C with shaking at 250 rpm for 2−3 days. The mature cultures were then used as inoculum for sterile GS-7 or a minimal medium (100 mL in 500 mL flasks) at a 1−5% rate and incubated as described above for 24 h, at which time 10 mg of **1** in 0.4 mL of DMF was added and further incubated for an additional 1−3 days. GS-7 contained 25 g of Parmamedia and 25 g of Cerelose per liter of tap H_2O with its presterilization pH adjusted to 7.2 with NH_4OH. The composition of the minimal salts medium was 20 g of sucrose, 100 mL of basal salts, 5 g of MOPS, and 50 mg of K_2HPO_4 per liter of tap H_2O. The presterilization pH was adjusted to 7.0 with KOH. The basal salts solution contained 46.7 g of NaCl, 10.7 g of NH_4Cl, 4.26 g of Na_2SO_4, 2.03 g of $MgCl_2 \cdot 6H_2O$, 290 mg of $CaCl_2 \cdot 2H_2O$, and 3 mg of $ZnCl_2$ per liter of deionized water. All media were sterilized by autoclaving for 30 min. The culture broth, which contained biotransformation product, was treated with an equal amount of CH_2Cl_2 and homogenized for 3−4 min at low speed. The resulting suspension was centrifuged for 10−50 min at 2500−4500 rpm, and the layers were separated. The residual mycelium was washed with CH_2Cl_2, and the combined CH_2Cl_2 layers were dried ($MgSO_4$), filtered, and evaporated to dryness under reduced pressure. The residual DMF was removed at 35 °C under high vacuum (0.03 mmHg), and the remaining extract was analyzed by TLC using the following three solvent systems: system A (30% Me_2CO:70% CH_2Cl_2), system B (5% MeOH: 95% CH_2Cl_2), and system C (5% MeOH:15% Me_2CO:80% CH_2Cl_2). Large-scale biotransformations (10 L and above) were carried out in a Labraferm fermentation tank kept at 28 °C, stirred at 250 rpm, and provided with air at a flow rate of 7−8 L per minute.

Organisms Used for Biotransformation Studies. Cultures of the following organisms provided identifiable products in yields of 2−15%: UC7602, *Cunninghamella echinulata* subsp. *elegans*; UC7330, *C. echinulata*; UC8622, *Nocardiopsis coeruleofusca*; UC7258, *Syncephalastrum racemosum*; UC4327, *S. racemosum*; UC4998, *Syncephalastrum sp.*; UC10750, fungus, unknown genus; UC5059, *Streptomyces cirratus*; UC11141, *Streptomyces* sp.; UC11144, *Streptomyces* sp.; UC11136, actinomycetes, unknown genus; and UC11202, actinomycetes, unknown genus.

Compounds 3 and 4. Culture broth (1800 mL) from the biotransformation brought about by UC4327 was extracted as described in the general procedure. The residue was flash chromatographed on silica gel (220 g) and eluted with CH_2Cl_2, followed by increasing amounts of CH_3OH in CH_2Cl_2 (5, 10, 20, and 30%). Fractions were pooled (based on TLC) after concentration. Further purification was achieved by repeated preparative TLC in solvent system A. Compound **3** was identical with a semisynthetic sample prepared by the $FeCl_3/H_2O_2$ oxidation of **1**.[8]

Compound 4: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.86 (s, 3H, H-23), 1.05 (s, 3H, H-22), 1.25 (brs, 2H), 1.43 (s, 3H, H-28), 1.45 (s, 3H, H-27), 1.75−2.11 (m, 3H), 2.45 (d, 1H, H-10B, $J_{A,B}$ = 16.2 Hz), 2.68 (d, 1H, H-10A), 2.75 (d, 1H, H-20), 3.12 (s, 3H, N−CH3), 3.23−3.31 (2d, 2H), 4.28 (d, 1H, H-12, $J_{12,OH}$ = 8.7 Hz), 4.9 (d, 1H, H-25, $J_{24,25}$ = 7.7 Hz), 6.34 (d, 1H, H-24), 6.68 (d, 1H, H-5, $J_{4,5}$ = 8.2 Hz), 6.84 (d, 1H, H-4), 8.13 (s, 1H, NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 19.60 (C-15), 20.47 (C-16), 22.18 (C-23), 23.77 (C-22), 25.56 (C-19), 29.03 (N−CH_3), 29.76 (C-28), 30.04 (C-27), 30.64 (C-14), 35.85 (C-10), 45.76 (C-17), 46.62 (C-21), 53.22 (C-20), 59.89 (C-3), 62.81 (C-11), 66.85 (C-13), 78.81 (C-26), 91.73 (C-12), 115.06 (C-5), 117.34 (C-25), 120.59 (C-4), 124.81 (C-9), 132.41 (C-7), 135.27 (C-8), 139.00 (C-24), 146.06 (C-6), 174.48 (C-18), 182.46 (C-2); HRMS (FAB) *m/z* 476.2579 ($C_{28}H_{33}N_3O_4$ + H requires 476.2549).

Compound 5. Culture broth (2000 mL) from the biotransformation brought about by UC10750 was extracted as described in the general procedure. The mixture was chromatographed on silica gel and eluted with 4% CH_3OH/CH_2Cl_2 to yield **5**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.89 (s, 3H), 1.14 (s, 3H, H-23), 1.20−1.95 (m, 8H), 1.39 (s, 3H, H-28), 1.45 (s, 3H, H-27), 2.04 (brd, 1H), 2.10−2.30 (m, 1H, H-17), 2.49 (brd, 1H, H-12B, $J_{A,B}$ = 12.1 Hz), 2.55−2.80 (m, 2H), 3.10 (s, 3H, N−CH_3), 3.80 (d, 1H, H-12A), 4.79 (s, 1H, H-10), 4.89 (d, 1H, H-25, $J_{24,25}$ = 7.7 Hz), 6.35 (d, 1H, H-24), 6.57 (d, 1H, H-5, $J_{4,5}$ = 8.2 Hz), 6.95 (d, 1H, H-4), 8.83 (s, NH); HRMS (FAB) *m/z* 494.2653 ($C_{28}H_{35}N_3O_5$ + H requires 494.2655).

Compounds 6 and 7. Culture broth (2000 mL) from the biotransformation brought about by UC 11136 was extracted as described in the general procedure. Several metabolites were present besides starting material, but only two could be positively identified. The crude extract was flash chromatographed (220 g silica gel) using CH_2Cl_2 to elute the column, followed by a CH_3OH/CH_2Cl_2 solvent gradient (1%, 2.5%, 5%, and 10%). Compounds **6** and **7** were identical with samples prepared from **1** by multisynthetic procedures.[9]

Compound 8. Culture broth (10 000 mL) from the biotransformation brought about by UC11141 was extracted as described in the general procedure. The residue (5.0 g) was flash chromatographed on silica gel (300 g) and eluted with CH_2Cl_2 to remove unchanged **1**, followed by increasing amounts of solvent system C in CH_2Cl_2 to provide a pure sample of **8** that was identical with an authentic sample prepared from **1** by multisynthetic procedures.[9]

Compounds 9 and 10. Culture broth (2000 mL) from the biotransformation brought about by UC 7330 was extracted by the general procedure. The residue was chromatographed on silica gel and eluted with increasing amounts of CH_3OH in CH_2Cl_2 (1−5%). Further purification of **9** was achieved by preparative TLC using solvent system B. A small sample was also purified by counter current chromatography. Compound **10** was also purified by preparative TLC using solvent system A. Compound **9** was identical with a sample prepared from **1** by multi synthetic procedures.[9]

Compound 10: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.85 (s, 3H, H-23), 1.04 (s, 3H, H-22), 1.15−1.87 (m, 6H), 1.42 (s, 3H, H-28), 1.46 (s, 3H, H-27), 1.90−2.20 (m, 4H), 2.58 (d, 1H, H-10A, $J_{10A,10B}$ = 15.8 Hz), 2.73 (m, 1H, H-17), 3.16 (brd, 1H, H-17), 3.30 (t, 1H, H-20), 4.19 (brd, 1H, H-12), 4.89 (d, 1H, H-25, $J_{24,25}$ = 7.7 Hz), 6.33 (d, 1H, H-24), 6.34 (s, NH), 6.67 (d, 1H, H-5, $J_{4,5}$ = 8.2 Hz), 6.84 (d, 1H, H-4), 7.73 (s, NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 19.53 (C-15), 20.74 (C-16), 21.97 (C-23), 23.86 (C-22), 25.43 (C-19), 29.68 (C-28), 30.09 (C-27), 38.42 (C-10), 45.85 (C-17), 46.63 (C-21), 54.44 (C-20), 60.74 (C-3), 62.84 (C-11), 63.88 (C-13), 79.78 (C-26), 90.46 (C-12),

115.11 (C-5), 117.37 (C-25), 120.60 (C-4), 124.89 (C-9), 132.45 (C-7), 135.27 (C-8), 139.00 (C-24), 146.06 (C-6), 177.33 (C-18), 182.66 (C-2); HRMS (FAB) *m/z* 462.2402 ($C_{27}H_{31}N_3O_4$ + H requires 462.2393).

Compound 11. Culture broth (1000 mL) from the biotransformation brought about by UC 7602 was extracted by the general procedure. The residue was chromatographed on silica gel, eluted with solvent system B, and further purified by preparative TLC using solvent system A: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.84 (s, 3H, H-23) 1.13 (s, 3H, H-22), 1.15−1.69 (m, 8H), 1.49 (s, 3H, H-28), 1.51 (s, 3H, H-27), 1.83 (d, 1H, H-10B, $J_{10A,10B}$ = 15.5 Hz), 2.05−2.19 (m, 1H), 2.21−2.38 (m, 1H), 2.41 (d, 1H), 2.68 (d, 1H, H-10A), 3.05 (t, 1H, H-20), 3.10 (s, 3H, NCH_3), 3.65 (d, 1H, H-12), 4.90 (d, 1H, H-25, $J_{24,25}$ = 5.8 Hz), 6.34 (d, 1H, H-24), 6.61 (s, 1H, H-4), 7.62 (s, NH); HRMS (FAB) *m/z* 494.2662 ($C_{28}H_{35}N_3O_5$ + H requires 494.2655.)

Compound 12. Culture broth (2000 mL) from the biotransformation brought about by UC 11202 was extracted by the general procedure. The residue was chromatographed on silica gel and eluted with increasing amounts of CH_3OH in CH_2Cl_2 (3−5%). Further purification was achieved using preparative TLC with ethyl acetate as an eluant: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.83 (s, 3H, H-23), 1.12 (s, 3H, H-22), 1.25 (s, 3H, H-28), 1.39 (s, 3H, H-27), 1.20−1.80 (m, 5H), 1.87 (d, 1H, H-10B, $J_{10A,10B}$ = 15.62 Hz), 1.95−2.1 (m, 1H), 2.12 (d, 1H, H-14), 2.20−2.38 (m, 1H), 2.40 (d, 1H, H-12B, $J_{12A,12B}$ = 10.08 Hz), 2.70 (d, 1H, H-10A), 3.04 (t, 1H, H-20), 3.11 (s, 3H, NCH_3), 3.55−3.75 (m, 2H), 4.87 (d, 1H, H-25, $J_{24,25}$ = 7.72 Hz), 6.51 (d, 1H, H-24), 6.69 (d, 1H, H-5, $J_{4,5}$ = 8.18 Hz), 6.83 (d, 1H, H-4), 8.15 (s, NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 20.78, 23.69, 23.90, 25.77, 26.40, 29.8 and 29.9 (C-27 & C-28), 31.57, 31.70, 37.11 (C-10), 46.62 (C-21), 52.85 (C-20), 54.56 (C-17), 60.49 (C-3), 61.51 C-12), 62.95 (C-11), 64.19 (C-13), 69.73 (CH_2OH), 82.55 (C-26), 110.82 (C-5), 116.89 (C-25), 120.72 (C-4), 126.0 (C-9), 133.0 (C-7), 135.1 (C-8), 141.63 (C-24), 145.20 (C-6), 173 (C-18), 182.33 (C-2); HRMS (FAB) *m/z* 494.2622 ($C_{28}H_{35}N_3O_5$ + H requires 494.2655).

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