Biotransformations of the Cardiovascular Drugs Mexrenone and Canrenone

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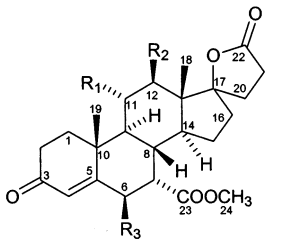
Microbial transformation studies of the cardiovascular drugs mexrenone (**1**) and canrenone (**2**) were conducted. Thirty-nine biotransformations of mexrenone and 84 biotransformations of canrenone were analyzed. Metabolism of the substrate was observed in the majority of these cases. Several monohydroxylated derivatives were detected by HPLC-MS-UV and subsequently identified. Two new mexrenone derivatives, 11α- (**3**) and 12*β*-hydroxymexrenone (**4**), and the known metabolite 6*β*-hydroxymexrenone (**5**) were isolated as major products produced by the *Beauveria bassiana* ATCC 13144 bioconversion (**3**) and the *Mortierella isabellina* bioconversion (**4** and **5**), respectively. Single-elimination products were also sought; however, only the production of the known metabolite $\Delta^{1,2}$-mexrenone (**6**) by several bacteria was confirmed. One new monohydroxylated derivative of canrenone, 9α-hydroxycanrenone (**7**), was isolated as a major product from the *Corynespora cassiicola* bioconversion. Structure elucidation of all metabolites was based on NMR and HRMS analyses.

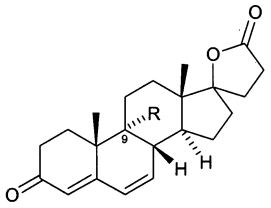
Mexrenone (**1**) and canrenone (**2**) belong to the steroid group containing a C-17 spirolactone side chain. They are diuretics and mineralocorticoid receptor antagonists which have been used for treatment of edematous states such as congestive heart failure and for treatment of essential hypertension.[1,2] Mexrenone (**1**), with a 7α-carbomethoxy function, is a more potent analogue than **2**.

The 9,11-epoxide of mexrenone was recently approved for treatment of high blood pressure (under the name Inspra). Inspra is synthesized via canrenone and mexrenone.[3] Dehydration of 9- or 11-hydroxylated canrenone or mexrenone would provide a useful route to the 9,11-epoxide function. Therefore, the objective of this work was to identify microorganisms capable of catalyzing the production of 9α- or 11α-hydroxymexrenone or hydroxycanrenone.

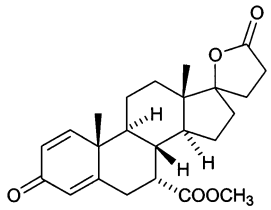
In the present study, we report biotransformation results for **1** and **2**, using commercially available microorganisms, and identification of three new (11α- (**3**) and 12*β*-hydroxymexrenone (**4**) and 9α-hydroxycanrenone (**7**)) and two previously reported derivatives (6*β*-hydroxy- (**5**) and $\Delta^{1,2}$-mexrenone (**6**)) of these substrates.[3,4] This is the first report of 6*β*- and 12*β*-hydroxylations of steroids by the fungus *Mortierella isabellina.*



1: $R_1 = R_2 = R_3 = H$
3: $R_1 = OH, R_2 = R_3 = H$
4: $R_1 = R_3 = H, R_2 = OH$
5: $R_1 = R_2 = H, R_3 = OH$



2: R = H
7: R = OH



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Results and Discussion

Eighty-four commercially available microbes (Table 1) were selected for the canrenone biotransformations, and 39 of these were tested using mexrenone as a substrate. Most of the microorganisms were selected on the basis of their documented ability to metabolize exogenously supplied steroid substrates, and many for their ability to vigorously hydroxylate steroids. About two-thirds of the screening group were filamentous fungi (for canrenone: 25 mitosporic fungi, 18 ascomycetes, 16 zygomycetes, and 2 basidiomycetes), and one-third were bacteria (actinomycetes or actinomycete-like).

Biotransformations were monitored by TLC during the initial stage of analysis, using a solvent system[5] that separated monohydroxylated products (lower R_f values) from single-elimination products and epoxides (higher R_f values, usually running just behind the substrate). On the basis of the disappearance of the substrate and/or the appearance of new spots on TLC, **1** and **2** were metabolized by 70% of the tested organisms (Table 1). All microbes that

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Table 1. Microbes Tested for the Ability to Metabolize Canrenone or Mexrenone and Selected Biotransformation Results

strain ID culture, source[a] (microbe type[b])	literature reference[c]	canrenone (**2**) TLC[d]	canrenone (**2**) monohydroxylation or elimination[e] (medium and harvest time[f])	mexrenone (**1**) TLC[d]	mexrenone (**1**) monohydroxylation or elimination[e] (medium and harvest time[f])
Absidia coerula 6647 (F)	11α[g]	Y	M 15.1, 15.5 (P4)	Y	M 11.8, 12.6, 13.5, 13.9 (P)
Absidia glauca 22752 (F)	11α[g]	Y	M 15.0; D 9.7, 11.3 (S48)	Nt	
Actinomucor elegans 6476 (F)	6β,9α[h]	Y		Nt	
Aspergillus flavipes 1030 (F)	6β,[i]11α[g]	N		N	
Aspergillus flavus 9170 (F)	6β,[i]11α[g]	Y		Y	
Aspergillus fumigatus 26934 (F)	6β,[i]11α[g]	Y		Nt	
Aspergillus nidulans 11267 (F)	6β,[i]11α[g]	Y	M 15.2, 16.1 (P48)	Nt	
Aspergillus niger 16888 (F)	6β,[i]11α[g]	Y	M 14.9, 15.1, 16.1; D 9.5, 11.5, 13.0 (S48)	Y	M 12.7, 13.9 (P)
Aspergillus niger 26693 (F)	6β,[i]11α[g]	Y	M15.2, 16.1; D10.0, 11.4 (P48)	Nt	
Aspergillus ochraceus 18500 (F)	6β,[j]9α[k] 11α[l,m]	Y	M15.3; D12.6, 13.3 (S48)	Nt	
Bacterium cyclo-oxydans 12673 (A)	9α,Δ[1,2 n]	Y	E22.1 (M5)	Y	E 19.4 (M)
Beauveria bassiana 7159 (F)	11α[o]	Y	M15.3 (S4)	Y	M 12.8, 14.2, 15.9 (P)
Beauveria bassiana 13144 (F)	6β[p]	Y	M 15.3, 18.1; D tr (P4)	Y	M 12.9, 14.9 (S)
Botryosphaeria obtusa 038560 (IMI) (F)	6β,9α,11α[q]	Y	M 17.0; D12.5, 13.0 (S5)	Y	M 12.5, 13.9 (P)
Calonectria decora 14767 (F)	6β,[o,r]12β[s,t]	Y	M 14.4, 15.1 (P4)	Nt	
Chaetomium cochliodes 10195 (F)		Y	M tr15.4 (P48)	Nt	
Cladosporium resinae 22712 (F)		Y	M tr13.9, 15.5 (P48)	Nt	
Colletotrichum lindemuthianum 12611 (F)		N		Nt	
Comomonas testosteroni 11996 (A)	9α,Δ[1,2 u]	Y	E22.5 (M48)	Y	E 19.4 (M)
Coniophora puteana 12675 (F)		N		Nt	
Cordyceps militaris 34164 (F)		N		Nt	
Corynespora cassiicola 16718 (F)	9α[v]	Y	M 14.7, 18.9 (P4)	N	
Cunninghamella blakesleeana 8688a (F)	6β,[o]9α[w]	Y	M 15.3, 17.1, 18.1; D 11.6, 12.7, 14.5 (P4)	Y	M 12.1, 13.9, 16.5 (P4)
Cunninghamella echinulata 3655 (F)	6β,[x]9α,[w]12β[x]	Y	M 15.2, 17.1, 18.1; D several (P4)	Y	M 12.1, 13.7, 16.4 (P4)
Cunninghamella elegans 9245 (F)	6β,9α,[w]11α[y]	Y	M 15.3, 17.1, 18.1;D some (P5)	Y	M 12.1, 13.8, 16.4 (P4)
Curvularia clavata 22921 (F)		Y	M 15.1, 15.6, 16.2, 16.9, 19.0, 20.2 (P48)	Nt	
Curvularia lunata 12017 (F)	6β,11α,[z]Δ[1,2aa]	Y		Y	
Cylindrocarpon radicicola 11011 (F)	9α	Y	E22.2 (P48)	N	
Epicoccum humicola 12722 (F)		Y	M 14.3, 15.3, 17.0, 19.0 (P48)	Y	
Epicoccum oryzae 12724 (F)		Y	M 18.1, 19.1, 20.3; D14.3 (P48)	Nt	
Fusarium oxysporum 7601 (F)	12β[bb]	Y	M 20.2 (P4)	Nt	
Fusarium oxysporum f.sp. cepae 11171 (F)	12β[bb]	Y	M 13.6, 14.5, 15.1, 16.3, 17.1, 20.4 (P48)	Nt	
Gibberella fujikuroi 14842 (F)		Y	M 13.4, 14.3, 15.0, 16.1, 17.0, 20.4 (P4)	Nt	
Gliocladium deliquescens 10097 (F)		Y		Nt	
Gongronella butleri 22822 (F)		Y	M 14.5 (S4)	Y	M 15.8 (P4)
Hypomyces chrysospermus 109891 (IMI) (F)	9α	Y	M 14.2, 15.2, 15.7, 17.0, 19.1 (P48)	Y	M 17.2 (S)
Lipomyces lipofer 10742 (F)		N		Nt	
Melanospora ornata 26180 (F)		Y	M 18.2 (S48)	Nt	
Mortierella isabellina 42613 (F)		Y	M 15.0, 15.3 (S4)	Y	M 12.4, 14.0, 16.0 (S)
Mucor griseo-cyanus 1207a (F)	6β,[h]9α[h]	Y		Nt	
Mucor mucedo 4605 (F)		Y	M 12.0 (M)	Y	
Mycobacterium fortuitum B8119 (NRRL) (A)	9α,Δ[1,2 cc]	N		Nt	
Myrothecium verrucaria 9095 (F)		Y	M 15.3, D14.5 (P48)	Nt	
Nocardia aurentia 12674 (A)	9α,Δ[1,2 dd]	Y		Y	E 19.8 (SF)
Nocardia canicruria 31548 (A)	9α,Δ[1,2 ee]	Y	M 14.7 (M48)	Y	E 20.6 (SF)
Paecilomyces carneus 46579 (F)		Y	E 22.0 (S48)	Y	
Penicillium chrysogenum 9480 (F)		Y		Nt	
Penicillium patulum 24550 (F)		Y	M 14.4 (S48)	Y	
Penicillium purpurogenum 46581 (F)		Y	M 15.3 (S48)	Y	
Pithomyces atro-olivaceus 6651 (IFO) (F)		Y	M 15.3, 17.0, 18.1; D 3 peaks (P48)	Y	M 13.7, 14.6, 16.6 (S)
Pithomyces cynodontis 26150 (F)		Y	M 19.5, 20.8 (P48)	N	
Phycomyces blakesleeanus 118496 (IMI) (F)		Y	M 14.6, 19.1 (P)	N	
Pycnosporium sp. 12231 (F)	12β[g,ff]	N		Nt	
Rhizopogon sp. 36060 (F)		N		Nt	
Rhizopus arrhizus 11145 (F)	6β,11α,12β[g,l,ff]	Y	M 15.3; D 12.0, 12.7 (P48)	Nt	
Rhizopus stolonifer 6227b (F)	11α[gg]	Y		Nt	
Rhodococcus equi 14887 (A)	9α[hh]	Y		Y	E 19.4 (M4)
Rhodococcus equi 21329 (A)	9α[hh]	Y		Y	E 20.3 (M)
Rhodococcus sp. 19070 (A)	9α[hh]	Y		Nt	
Rhodococcus rhodochrous 19150 (A)	9α,[hh]Δ[1,2 ii]	N		Nt	
Saccharopolyspora erythraea 11635 (A)		Y	M15.4, 17.1, 18.5 (SF)	Y	
Sepedonium ampullosporum 203033 (IMI) (F)	6β[ij]	Y	M 16.5 (P)	N	
Sepedonium chrysospermum 13378 (F)		N		N	

Table 1. Continued

strain ID culture, source[a] (microbe type[b])	literature reference[c]	canrenone (**2**) TLC[d]	canrenone (**2**) monohydroxylation or elimination[e] (medium and harvest time[f])	mexrenone (**1**) TLC[d]	mexrenone (**1**) monohydroxylation or elimination[e] (medium and harvest time[f])
Septomyxa affinis 6737 (F)	$\Delta^{1,2}$ [kk]	Y	M 14.7, 15.4, 21.8 (P4)	Nt	
Stachylidium bicolor 12672 (F)		Y	M 18.2 (P5)	Nt	
Streptomyces californicus 15436 (A)		N		Nt	
Streptomyces cinereocrocatus 3443 (NRRL) (A)		Y		Nt	
Streptomyces coelicolor 10147 (A)		N		Nt	
Streptomyces fradiae 10745 (A)	6β,11α[ll]	N		Nt	
Streptomyces griseus subsp. *griseus* 11984 (A)		Y		Nt	
Streptomyces griseus 13968 (A)	6β[mm]	Y		Y	
Streptomyces hydrogenans 19631 (A)		N		Nt	
Streptomyces hygroscopicus hygroscopicus 27438 (A)	6β[mm]	Y	M 15.2, 17.0, 18.0, 20.1 (M48)	Y	
Streptomyces lavendulae #105 (A)		N		Nt	
Streptomyces purpurascens 25489 (A)		Y		Y	
Streptomyces roseochromogenes 13400 (A)	6β[mm]	N		Nt	
Streptomyces spectabilis 27465 (A)		Y	M 20.3tr (M48)	Nt	
Stysanus microsporus 2833 (F)		N		Nt	
Syncephalastrum racemosum 18192 (F)	12β[s]	Y	M 15.2 (P4)	Nt	
Thamnidium elegans 18191 (F)		N		Y	
Thamnostylum piriforme 8992 (F)	6β,9α,[w]11α[h,nn]	Y	M 17.1; D 11.2, 11.6 (S48)	Y	
Thielavia terricola 13807 (F)		Y	M 14.3, 15.1, 19.0, 20.2 (P48)	Nt	
Trichoderma viride 26802 (F)		N		Nt	
Trichothecium roseum 12543 (F)	11α,[g]6β[o]	Y	M 14.5, 15.3; D 10.2, 11.7 (S48)	Y	
Verticillium theobromae 12474 (F)		Y		Nt	

[a] Commercial source is the American Type Culture Collection unless indicated otherwise. [b] A = actinomycete or actinomycete-like; F = fungus. [c] 9α = 9α-hydroxylation; 6β = 6β-hydroxylation; 12β =12β-hydroxylation; 11α = 11α-hydroxylation; $\Delta^{1,2}$ = $\Delta^{1,2}$-elimination. [d] Metabolism observed by TLC. Y = metabolism observed; N = no metabolism; Nt = not tested. [e] LCMS retention times (in minutes) of major peaks and indication whether mass-to-charge ratio indicates an elimination (E), monohydroxylation (M), or dihydroxylation (D); trace (tr). Mass-to-charge ratios (*m*/*z*): 417 and 357 (M + H for monohydroxylation of mexrenone and canrenone, respectively), *m*/*z* 399 and 339 (M + H for single elimination of mexrenone and canrenone, respectively), or *m*/*z* 373 (M + H for dihydroxylation of canrenone). The presence of the appropriate ammonium adduct was also checked. Retention times (minutes) for standards: canrenone, 24.0; 11α-OH-canrenone, 15.3; 9α-OH-canrenone, 15.1; mexrenone, 21.2; 9,11-dehydromexrenone, 19.3; 11α-OH-mexrenone, 13.9; 7,9-lactonized mexrenone, 14.7. (Retention times of standards varied due to changes in column pressure.) [f] Medium and bioconversion time for which LC-MS biotransformation results are reported: P = PYG; M = Mueller-Hinton; S = soybean; SF soybean + formate. [g] See main text footnote 3. [h] Hu, S.; Genain, G.; Azerad, R. *Steroids* **1995**, *60*, 337−52. [i] Iizuka, H.; Naito A.; Hattori, M. *Gen. Appl. Microbiol.* **1958**, *4*, 67−78. [j] Bell, A. M.; Browne, J. W.; Denny, W. A.; Jones, E. R. H.; Kasal, A.; Meakins, G. D. *J. Chem. Soc., Perkins Trans. 1* **1972**, *23*, 2930−2936. [k] Crabb, T. A.; Ratcliff, N. M. *J. Chem. Res. Synop.* **1986**, 48−49. [l] Holland, H.; Lakshmaiah, G. *J. Mol. Catal. B: Enzym.* **1999**, *6*, 83−88. [m] Shibahara, M.; Moody, J. A.; Smith, L. L. *Biochim. Biophys. 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accumulated putative metabolites of one or both of the substrates were refermented to confirm the results of the initial screen.

On the basis of TLC R_f values, several microbes produced possible monohydroxylation products of **1** and/or **2**; fewer microbes produced possible single-elimination products or epoxides (Table 1). The microbes accumulating a major monohydroxylated product in canrenone bioconversions were *Beauveria bassiana* ATCC 13144, *Penicillium purpurogenum*, *Rhizopus arrhizus*, *Septomyxa affinis*, and *Streptomyces fradiae*. For mexrenone bioconversions, this group included the three *Cunninghamella* species, *Penicillium patulum*, *P. purpurogenum*, *Phycomyces blakesleeanus*, *Rhodococcus equi* ATCC 21369 and 14887, *Sepedonium ampullosporum*, and *Streptomyces griseus*. Most of the producers of possible single-elimination products were actinomycetes or actinomycete-like bacteria; however, fungal producers of this product class in canrenone bioconversions were *Aspergillus fumigatus*, *Aspergillus niger*, *Cylindrocarpon radicicola*, *Paecilomyces carneus*, and *S. affinis* (S medium). Fungal producers of putative single-elimination products in mexrenone bioconversions were the

Table 2. ^{1}H NMR Spectral Data for **1**, **2**, **3**, **4**, and **7**[a]

proton	1	3	4	2	7
H-1a	2.23 m	2.76 dd, (14.5,3.5)	2.47 m	2.01 dd (11.8,4.7)	2.36 dd (13.8,5.3)
H-1b	1.82 m	1.98 dd (14.0,4.5)	2.02 m	1.68 m	1.77 m
H-2a	2.41 m	2.43 m	2.38 m	2.55 m	2.54 m
H-2b	2.41 m	2.30 m	2.34 m	2.42 m	2.48 m
H-4	5.69 s	5.65 s	5.69 s	5.65 s, br.	5.84 s, br.
H-6a	2.62 ddd (14.9,6.0,1.9)	2.60 ddd (14.6,5.8,1.7)	2.61 ddd (14.9,6.1, 1.7)	6.11 dd (9.9,2.3)	6.27 dd (9.8,2.9)
H-6b	2.47 m	2.48 m	2.41 m		
H-7	2.82 td (4.1,1.9)	2.78 m	2.81 m	6.05 d (10.2)	5.91 dd (9.9,1.9)
H-8	1.86 m	1.75 ddd (11.3,11.2,4.1)	1.78 m	2.28 m	2.63 dm (12.3)
H-9	1.78 m	1.89 m	1.87 m	1.18 ddd (10.0, 4.2,2.8)	
H-11a	1.66 m		1.78 m	1.66 m	1.72 m
H-11b	1.40 m	4.01 ddd (10.3,10.2,4.6)	1.41 m	1.46 dd (12.5,3.1)	1.65 m
H-12a	1.56 dt (11.7,3.1)	1.82 m	3.68 dd (10.7,4.6)	1.63 m	1.38 ddd (11.8,3.4,2.9)
H-12b	1.33 dd (12.2,3.7)	1.38 m		1.36 m	1.38 ddd (11.8,3.4,2.9)
H-14	1.43 m	1.62 m	1.39 m	1.35 m	1.97 m
H-15a	1.75 m	1.70 m	1.76 m	1.87 m	1.83 m
H-15b	1.41 m	1.37 m	1.48 m	1.59 m	1.55 dd (12.2,5.7)
H-16a	2.05 ddd (13.2,4.9,4.7)	2.22 ddd (14.6,12.2,3.5)	2.26 dd (13.7,3.2)	2.29 m	2.32 m
H-16b	1.84 m	1.83 m	1.79 m	1.86 m	1.88 m
H-18	0.95 s	0.97 s	0.97 s	1.01 s	1.02 s
H-19	1.20 s	1.33 s	1.20 s	1.11 s	1.21 s
H-20a	2.38 m	2.36 m	2.47 m	2.34 m	2.47 m
H-20b	1.89 m	1.92 m	1.92 m	1.90 m	1.95 m
H-21a	2.52 m	2.53 m	2.72 m	2.53 m	2.53 m
H-21b	2.52 m	2.46 m	2.50 m	2.47 m	2.49 m
$COOCH_3$	3.61 s	3.61 s	3.60 s		

[a] Data recorded at 500 MHz in $CDCl_3$ (δ in ppm, multiplicity, J in Hz).

two *B. bassiana* strains, the three *Cunninghamella* species, and *P. carneus*.

The extracts from all microbes reproducibly yielding an abundant metabolite observable by TLC were analyzed by reversed-phase LC-MS with electrospray ionization. In the LC-MS of these hydroxylated steroids, the molecular ion (M + H, base peak), and the ammonium adduct (M + 17) were observed as well as the successive loss of 18 mass units (H_2O). The abundance of the fragments relative to the molecular ion in the monohydroxylated steroid isomers varied with the position of the hydroxyl group; these quantitative differences (in combination with retention time) were consistent enough for diagnostic use in distinguishing regioisomers (data not shown). Bioconversion candidates selected on this basis were confirmed to be **3** and **7**. The retention times of monohydroxylated, dihydroxylated, and dehydration products are listed in Table 1 for selected bioconversions.

Following secondary analysis of the **1** and **2** bioconversions by LC-MS-UV, eight microorganisms were selected as candidates for production of biotransformation products of interest and were scaled up to produce material for structural analyses. Seven were bioconversions of **1**, which are listed here along with the biotransformation product or products that were subsequently identified: for monohydroxylated products, *B. bassiana* ATCC 13144 (**3**) and *M. isabellina* ATCC 42613 (**4** and **5**); for **6**, *B. cyclo-oxydans* ATCC 12673, *Comomonas testosteroni* ATCC 11996, *R. equi* ATCC 14887, *Nocardia aurentia* ATCC 12674, and *R. equi* ATCC 21329. The *Corynespora cassiicola* ATCC 16718 bioconversion of **2** was selected as a **7**-producing candidate.

Prior to determining the structures of the biotransformation products, the complete proton and carbon NMR assignments for **1** and **2** were made. NMR data sets for both compounds, including ^{1}H NMR, ^{13}C NMR, DEPT-135, COSY, HMQC, and HMBC experiments, were obtained. On the basis of analysis of the NMR data, the proton and carbon atoms for **1** were easily assigned. Carbon NMR data for **2** have been published.[6] Our data for **2** were in close agreement with the literature data except for the assignments of C9 and C14; we believe the published assignments should be interchanged. HMBC data revealed distinctive three-bond correlations from the methyl group (H19) at δ 1.11 to the carbon at δ 50.38 (C9) and from the methyl group (H18) at δ 1.01 to the carbon at δ 46.97 (C14). Our data also allowed the distinction between the C1/C2 methylene groups previously listed at δ 33.8. HMQC data showed correlations from protons at δ 2.55/2.42 and 2.01/1.68 to carbons at δ 33.85 and 33.89, respectively. In the HMBC, only the proton at δ 2.01 displayed a three-bond correlation to the carbon at δ 16.29 (C19 methyl group). This allowed assignment of δ 33.85 to C2 and δ 33.89 to C1. The complete NMR assignments for both compounds are listed in Tables 2 and 3. Using these data, comparisons could more easily be made to the metabolized substrates.

The bioconversion of **1** by *B. bassiana* ATCC 13144 yielded one major monohydroxylated product (**3**) in 67% overall yield. This material was subjected to the standard set of NMR experiments. Proton NMR analysis indicated that there was a new downfield-shifted signal (at δ 4.01) relative to the proton NMR spectrum of **1**. Carbon-13 NMR analysis confirmed the presence of a new methine at δ 69.1. Although no correlations to the proton at δ 4.01 were observed, there were several correlations to the carbon at δ 69.1 in the HMBC spectrum; H-9 and H-12a and b showed HMBC correlations to this carbon. These data, coupled with the COSY data showing correlations between δ 4.01 and H-9 and H-12a and b, suggested the assignment of this new methine as C-11. This is supported by the fact that *B. bassiana* strains have been reported to carry out 11α-hydroxylations of steroids.[7] All other data supported the assignment of this molecule as the new compound 11-hydroxymexrenone (**3**). Two large J values for H-11 suggested that this proton was in an axial position ($^3J_{HH}$ = 10.3, 10.2, 4.6 Hz). Therefore the hydroxyl group was assigned to the α position. This new compound is potentially useful as a synthetic intermediate for 9,11-epoxymexrenone.[3]

The *M. isabellina* bioconversion of **1** yielded two major monohydroxylated metabolites, **4** and **5**, in 50% and 33%

Table 3. ^{13}C NMR Spectral Data for **1**, **2**, **3**, **4**, and **7**[a]

carbon	1	3	4	2	7
C-1	35.47 t	37.26 t	35.43 t	33.89 t	27.21 t
C-2	33.87 t	34.07 t	33.78 t	33.85 t	33.60 t
C-3	198.77 s	199.52 s	198.67 s	199.35 s	199.01 s
C-4	125.57 d	126.10 d	125.78 d	123.94 d	127.72 d
C-5	167.50 s	167.70 s	166.74 s	163.00 s	159.17 s
C-6	35.29 t	36.31 t	35.25 t	128.31 d	128.94 d
C-7	42.73 d	42.32 d	42.14 d	139.38 d	135.76 d
C-8	37.64 d	36.95 d	36.74 d	37.75 d	41.34 d
C-9	46.11 d	52.51 d	44.78 d	50.38 d	75.06 s
C-10	38.20 s	39.34 s	38.06 s	35.99 s	42.63 s
C-11	20.60 t	69.13 d	30.61 t	20.06 t	24.87 t
C-12	31.21 t	43.41 t	72.20 d	31.58 t	26.33 t
C-13	45.83 s	46.13 s	49.57 s	46.40 s	46.27 s
C-14	45.50 d	45.21 d	44.39 d	46.97 d	40.17 d
C-15	22.69 t	22.51 t	22.16 t	22.42 t	22.15 t
C-16	35.29 t	35.40 t	36.00 t	35.43 d	35.60 d
C-17	95.53 s	94.95 s	95.33 s	95.32 s	95.32 s
C-18	14.12 q	15.39 q	8.54 q	14.38 q	13.50 q
C-19	17.80 q	18.38 q	17.72 q	16.29 q	19.24 q
C-20	31.21 t	31.18 t	31.16 t	31.13 t	31.56 t
C-21	29.19 t	29.09 t	29.47 t	29.20 t	29.29 t
C-22	176.63 s	176.48 s	177.12 s	176.53 s	176.67 s
C-23	173.16 s	172.73 s	172.94 s		
C-24	51.41 q	51.54 q	51.52 q		

[a] Data recorded at 125 MHz in $CDCl_3$.

overall yield, respectively. Analysis of the ^1H NMR spectrum of **4** immediately revealed many similarities with **1** (Table 2). The most obvious difference was a new downfield-shifted signal: a doublet of doublets at δ 3.68, suggesting the presence of an oxygenated methine carbon. Indeed, the ^{13}C NMR spectrum contained a methine signal at δ 72.2 (Table 3). From Tables 3 and 4, it was clear that an upfield-shifted methylene group had been replaced by this downfield-shifted methine. The downfield-shifted proton signal showed COSY correlations to H-11a and H-11b, suggesting that this proton was attached to C-12. The new signal correlated to one carbon in the HMBC spectrum: the C-18 methyl. Conversely, the C-18 methyl protons correlated with the C-12 in the HMBC spectrum. Interestingly, this proximal methyl group showed a carbon shift from 15.4 ppm (in **1**) to 8.5 ppm in **4**. These data indicated that the new hydroxyl group was at C-12. All additional connectivity data confirmed the assignment of this metabolite as **4**. The stereochemistry of the hydroxyl group was determined by examining coupling constants and NOESY data. The coupling constants for H-12 were 10.9 and 4.5 Hz, suggesting one diaxial coupling. H-12 also displayed NOESY correlations with H-9 and H-14, which are both 1,3-diaxial interactions. These data indicated that H-12 is in the axial position α to the ring, confirming the assignment of **4** as 12β-hydroxymexrenone.

The NMR data for **5** also suggested the presence of a new oxygenated methine carbon. Analysis of the 2D NMR data allowed assignment of this metabolite as the known synthetic compound 6β-hydroxymexrenone (**5**). Only a few of the proton NMR assignments for this compound were published;[2] therefore complete NMR assignments for this compound were made. 6β-Hydroxymexrenone (**5**) is the principal human and animal biotransformation product of **1**. It retains good anti-deoxycorticosterone acetate (DCA) potency when administered subcutaneously.[2] *M. isabellina* has not been reported previously to carry out either the 6β- or 12β-hydroxylation of steroids.

Five biotransformations of **1** to putative single-elimination products (Table 1) were scaled up to prepare material for structure determination: *B. cyclo-oxydans* ATCC 12673, *C. testosteroni* ATCC 11996, *R. equi* ATCC 14887, *N. aurentia* ATCC 12674, and *R. equi* ATCC 21329. The product (R_f 0.65) from the *B. cyclo-oxydans* biotransformation was purified in 15% overall yield. Once the single-elimination product of the *B. cyclo-oxydans* biotransformation of **1** was identified as the known synthetic compound $\Delta^{1,2}$-mexrenone (**6**), the remaining four extracts were analyzed to determine whether they contained **6** as well. An aliquot of each of the dried ethyl acetate extracts was analyzed by proton NMR without further purification; they all contained **6**. Only six proton NMR assignments for this compound were published previously;[1] therefore a complete NMR data set was obtained and all proton and carbon assignments were confirmed. Although **6** was previously prepared by a synthetic route,[1] this is the first report of its production by microbial biotransformation. It has slightly better oral potency in DCA-induced diuresis than **1**.[1] All of the producing genera have previously reported $\Delta^{1,2}$ dehydrogenase activity on steroids (Table 1).

The fermentation of *C. cassiicola* ATCC 16718 with canrenone yielded one major monohydroxylated product (**7**) in 30% overall yield. This material was subjected to the standard set of NMR experiments. Comparison of the ^{13}C NMR spectrum of **2** to the new spectrum revealed that the carbon signal at δ 50.38 was missing and instead a new quaternary carbon signal at δ 75.06 was observed. HMBC data showed correlations to this carbon from H7 (δ 5.91), H11a/b (δ 1.65 and 1.72), H12a/b (δ 1.38), and the methyl group H19 (δ 1.21). These correlations and all other NMR data confirmed the assignment of this metabolite as 9α-hydroxycanrenone (**7**). Complete proton and carbon assignments for **7** are listed in Tables 2 and 3. Hydroxylation of steroids in the 9α position by *C. cassiicola* has been reported previously.[8] Compound **7** was prepared concurrently using a totally synthetic route.[9] This is the first report of its structure determination and its preparation by biotransformation.

Experimental Section

General Experimental Procedures. ^1H and ^{13}C NMR spectra were recorded at 300 K on a Bruker Avance DRX 500 spectrometer operating at 500 and 125 MHz, respectively. The samples for NMR characterization were dissolved in $CDCl_3$ at a concentration of approximately 5−10 mg/mL. Standard pulse sequences were used for DEPT, COSY, HMQC, and HMBC (J = 8.3 Hz) experiments. NOESY experiments were run using a mixing time of 900 ms. Melting points were determined using an Electrothermal 9100 melting point apparatus and are uncorrected. IR data were acquired using a Perkin-Elmer 1600 Series FTIR spectrophotometer. A Perkin-Elmer 243B polarimeter was used to procure optical rotation data.

Silica gel 60 (250 μm) fluorescence-backed plates (254 nm) were used for TLC. EtOAc (500 μL) was added to each vial containing dried extract. Vials were vortexed, and 10 μL was spotted per lane under an air stream. Plates were developed in $CHCl_3/CH_3OH$ (95:5).[5] Mobilities of the standards relative to the solvent front (R_f) were as follows: 4-androsten-9α-hydroxy-3,17-dione, 0.36; 11α-hydroxycanrenone, 0.23; **2**, 0.64; **1**, 0.63; $\Delta^{9,11}$-canrenone, 0.65; $\Delta^{9,11}$-mexrenone, 0.62; 7,9-lactonized mexrenone, 0.53. Semipreparative reversed-phase HPLC was carried out on a Waters system equipped with a 600 controller and a 996 photodiode array detector. Separation was achieved on an EM Merck Lichrospher 100RP-18 column (250 × 10 mm, 10 μm, UV detection at 286 nm, flow rate of 10 mL/min). The following gradient was used to purify **6** and **7** from their respective whole cell bioconversions: 25% CH_3CN in H_2O to 60% CH_3CN over 14 min, then to 100% CH_3CN over 3 min. Fractions were collected every 30 s. High-resolution MS data were obtained using a Perceptive Biosystems Mariner ESI-TOF mass spectrometer. Low-resolution mass spectrom-

etry was carried out on a PE Sciex API3 mass spectrometer with the TurboIonSpray Source operated in positive mode for Q1 scans. The mass spectrometer was interfaced with a Gilson 233XL autosampler and Waters 600E HPLC and plumbed in parallel with a Waters 996 photodiode array detector. Separations were carried out on a Waters Nova-Pak C-18 (4 μm) RadialPak 4 mm cartridge using a linear gradient (25% CH_3CN in water to 75% CH_3CN over 25 min), with 2 mM ammonium acetate. The eluent was split 22:1 with the majority of the material directed into the PDA detector and the remainder directed into the mass spectrometer. The retention times of standards are reported in Table 1, footnote *e*.

Microorganisms and Media. All microorganisms were obtained from commercial culture collections. Strain identification information is presented in Table 1. Working stocks were prepared by freezing pure cultures at −80 °C in a spore storage solution: lactose 50 g, glycerol 100 g to 1 L. The following fermentation and bioconversion media were used: (a) soybean meal (S): dextrose 20 g, soybean meal 5 g, NaCl 5 g, yeast extract 5 g, K_2HPO_4 5 g, H_2O to 1 L, pH to 7.0; (b) peptone-yeast extract-glucose (P): bactopeptone 10 g, yeast extract 5 g, glucose 40 g, H_2O to 1 L; (c) Mueller-Hinton (M): beef infusion 300 g, casamino acids 17.5 g, starch 1.5 g, H_2O to 1 L. Fungi were grown in S and P media; actinomycetes and eubacteria were grown in SF (S medium, to which 0.9% sodium formate was added for the bioconversion period) and M media.

Small-Scale Fermentation Procedures and Bioconversions. Frozen spore stocks were used to inoculate starter cultures (20 mL of S medium in 250 mL Erlenmeyer flask). Starter cultures (24 or 48 h old) were used to inoculate bioconversion cultures (20 mL of media per 250 mL Erlenmeyer flask) with a 10−15% crossing volume, which were then fermented for 24−48 h before addition of substrate. Compound **2** was dissolved in MeOH at 10 mg/mL, filter sterilized, and added to the metabolism cultures to a final concentration of 0.1 mg/mL (2 mg; 100 μL per flask). Because **1** did not fully dissolve in MeOH, 200 μL of a 0.1 mg/mL unfiltered suspension (4 mg) was added per flask. No additions were made in the "no substrate" controls. All fermentations were shaken at 250 rpm (2 in. throw) at 26 °C and 60% relative humidity. For the screening work, biotransformations were harvested at 5 and 48 h or at 24 h after addition of substrate. Harvesting began with the addition of EtOAc (23 mL) to each flask. Flasks were shaken (250 rpm) for 20 min, and the contents of each flask were poured into a 50 mL conical tube. Tubes were centrifuged to separate the phases for 25 min at 4000 rpm in a room-temperature unit. The organic layer from each tube was transferred to a 20 mL borosilicate glass vial and evaporated under reduced pressure. Vials were capped and stored at −20 °C.

Scale-up Fermentations and Purification of Biotransformation Products. To obtain the material needed for structure determination, biotransformations were scaled-up by increasing the number of shake flasks and keeping media volume, substrate concentration, and extraction volumes constant. At the time of harvest (24 or 48 h after addition of substrate), EtOAc (23 mL) was added to each flask individually. The conditions for the different scale-up biotransformations were as follows (producing organism, scale-up size, medium, bioconversion time): *B. bassiana* ATCC 13144, 520 mL, S, 48 h (**3**); *M. isabellina*, 320 mL, S, 48 h (**4** and **5**); *B. cyclo-oxydans* ATCC 12673, 520 mL, SF, 24 h (**6**); and *C. cassiicola* ATCC 16718, 600 mL, P, 48 h (**7**). The respective fermentation broths were extracted with approximately equal volumes of EtOAc. The organic layers were dried under reduced pressure and lyophilized to generate the crude extracts. The yields of the crude extracts were 173 mg for **4** and **5**, 182 mg for **3**, 133.2 mg for **6**, and 101.9 mg for **7**. The other four mexrenone biotransformations producing **6** were all scaled-up at 600 mL and harvested after 24 h. The fermentation media and yields of the crude extracts are listed below: *C. testosteroni*, S, 39.1 mg; *R. equi* ATCC 14887, S, 193.5 mg; *N. aurentia*, S, 203.7 mg; and *R. equi* ATCC 21329, P, 145.8 mg.

To isolate **3**, EtOAc (8 mL) was added to the dried extract in aliquots (4 × 2 mL) and the suspension was filtered through glass wool. The EtOAc-insoluble material that dissolved in CH_2Cl_2 was collected and lyophilized to yield 70 mg of pure **3**. EtOAc (~3 mL) was added to the dried crude extract containing **4** and **5**. The solution was decanted, and the EtOAc-insoluble material was dried to yield 21 mg of pure **4**. An aliquot (54 mg) of the total EtOAc-soluble material (93 mg) was fractionated by silica gel preparative TLC ($CHCl_3$/MeOH, 95:5). Two bands were collected and dried. The faster migrating material (R_f 0.33) was identified as **5**, while the slower migrating material (R_f 0.27) yielded **4**. Compound **6** was isolated by adding ethyl acetate to the dried crude extract, resulting in a white insoluble material (66 mg) and a soluble fraction. The latter was fractionated, in aliquots, by semi-preparative HPLC. HPLC fractions were pooled (t_R 11.67 min) and dried to yield 14.9 mg of **6**. Aliquots of the crude extract containing **7** were dissolved in EtOAc (10 μL per mg). Fifteen semipreparative HPLC injections were made, and the fractions eluting at 7−8.5 min were pooled and dried. $CHCl_3$ was added to dissolve the dried material, leaving behind a brown, insoluble residue. The chloroform-soluble material was dried and lyophilized to yield 18.2 mg of **7**.

Mexrenone (17-hydroxy-3-oxo-17α-pregn-4-ene-7α,21-dicarboxylic acid 7-methyl ester γ-lactone, 1): ^1H and ^{13}C NMR, see Tables 2 and 3.

Canrenone (17β-hydroxy-3-oxo-4,6-androstadiene-17α-propionic acid γ-lactone, 2): ^1H and ^{13}C NMR, see Tables 2 and 3.

11α-Hydroxymexrenone (11α,17-dihydroxy-3-oxo-17α-pregn-4-ene-7α,21-dicarboxylic acid 7-methyl ester γ-lactone, 3): white solid; R_f 0.19; mp 229−230 °C; $[\alpha]^{20}_D$ +18.9° (*c* 0.0027, $CHCl_3$); IR (KBr) ν_{max} 3448, 2943, 1766, 1731, 1661, 1437, 1190, 1167, 1026, 914, 750 cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; HRESIMS *m*/*z* 417.2278 $[M + H]^+$ (calcd for $C_{24}H_{33}O_6$, 417.2277).

12β-Hydroxymexrenone (12β,17-dihydroxy-3-oxo-17α-pregn-4-ene-7α,21-dicarboxylic acid 7-methyl ester γ-lactone, 4): light brown powder; mp 244−248 °C; $[\alpha]^{20}_D$ +26.8° (*c* 0.0072, $CHCl_3$); IR (KBr) ν_{max} 3439, 2943, 1759, 1728, 1661, 1438, 1268, 1190, 1030, 751, 663 cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; HRESIMS *m*/*z* 439.2097 $[M + Na]^+$ (calcd for $C_{24}H_{32}O_6Na$, 439.2097).

6β-Hydroxymexrenone (6β,17-dihydroxy-3-oxo-17α-pregn-4-ene-7α,21-dicarboxylic acid 7-methyl ester γ-lactone, 5): ^1H NMR ($CDCl_3$) δ 2.05 (1H, m, H-1a), 1.81 (1H, m, H-1b), 2.51 (1H, m, H-2a), 2.44 (1H, m, H-2b), 5.79 (1H, s, H-4), 4.38 (1H, d, *J* = 2.1 Hz, H-6a), 2.93 (1H, dd, *J* = 4.6, 2.1 Hz, H-7), 2.34 (1H, m, H-8), 1.36 (1H, m, H-9), 1.66 (1H, m, H-11a), 1.50 (1H,.m, H-11b), 2.35 (1H, m, H-12a), 1.57 (1H, m, H-12b), 1.69 (1H, m, H-14), 1.84 (1H, m, H-15a), 1.28 (1H, m, H-15b), 2.26 (1H, m, H-16a), 1.84 (1H, m, H-16b), 0.99 (3H, s, H-18), 1.37 (3H, s, H-19), 2.35 (1H, m, H-20a), 1.88 (1H, m, H-20b), 2.56 (1H, m, H-21a), 2.49 (1H, m, H-21b), 3.62 (3H, s, H24); ^{13}C NMR ($CDCl_3$) δ 36.85 (t, C1), 34.20 (t, C2), 199.72 (s, C3), 127.82 (d, C4), 166.07 (s, C5), 73.71 (d, C6), 49.59 (d, C7), 32.09 (d, C8), 45.48 (d, C9), 37.60 (s, C10), 20.55 (t, C11), 31.27 (t, C12), 45.94 (s, C13), 45.64 (d, C14), 22.70 (t, C15), 35.32 (t, C16), 95.65 (s, C17), 14.28 (q, C18), 20.79 (q, C19), 31.14 (t, C20), 29.22 (t, C21), 176.68 (s, C22), 171.73 (s, C23), 51.58 (q, C24).

Δ^{1,2}-Mexrenone (17-hydroxy-3-oxo-17α-pregna-1,4-diene-7α,21-dicarboxylic acid methyl ester γ-lactone, 6): ^1H NMR ($CDCl_3$) δ 7.03 (1H, d, *J* = 10.2 Hz, H-1a), 6.22 (1H, dd, *J* = 10.1, 1.9 Hz, H-2a), 5.99 (1H, t, *J* = 1.5 Hz, H-4), 2.76 (1H, ddd, *J* = 13.7, 6.2, 1.5 Hz, H-6a), 2.58 (1H, m, H-6b), 2.90 (1H, td, *J* = 4.2, 2.1 Hz, H-7), 1.88 (1H, m, H-8), 1.98 (1H, td, *J* = 11.7, 4.1 Hz, H-9), 1.85 (1H, m, H-11a), 1.63 (1H, m, H-11b), 1.57 (1H, m, H-12a), 1.36 (1H, m, H-12b), 1.64 (1H, m, H-14), 1.62 (1H, m, H-15a), 1.40 (1H, m, H-15b), 2.23 (1H, m, H-16a), 1.79 (1H, m, H-16b), 0.98 (3H, s, H-18), 1.23 (3H, s, H-19), 2.33 (1H, td, *J* = 14.1, 3.4 Hz, H-20a), 1.87 (1H, m,

H-20b), 2.52 (1H, m, H-21a), 2.43 (1H, m, H-21b), 3.60 (3H, s, H24); ^{13}C NMR ($CDCl_3$) δ 154.86 (d, C1), 127.74 (d, C2), 185.71 (s, C3), 126.00 (d, C4), 164.09 (s, C5), 35.48 (t, C6), 43.96 (d, C7), 37.46 (d, C8), 44.75 (d, C9), 42.99 (s, C10), 22.04 (t, C11), 31.18 (t, C12), 45.99 (s, C13), 44.59 (d, C14), 22.85 (t, C15), 35.32 (t, C16), 95.45 (s, C17), 14.09 (q, C18), 18.98 (q, C19), 31.22 (t, C20), 29.19 (t, C21), 176.60 (s, C22), 172.37 (s, C23), 51.54 (q, C24).

9α-Hydroxycanrenone (9α,17*β*-dihydroxy-3-oxo-4,6-androstadiene-17α-propionic acid *γ*-lactone, 7): brownish flakes; R_f 0.38; mp 262−263 °C; $[\alpha]^{20}_D$ −41.2° (*c* 0.0034, $CHCl_3$); IR (KBr) ν_{max} 3482, 2961, 2351, 1765, 1649, 1610, 1266, 1178, 1122, 1017, 1011, 878, 754 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 2 and 3; HRESIMS *m*/*z* 357.2071 $[M + H]^+$ (calcd for $C_{22}H_{29}O_4$, 357.2066).

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