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MICROBIAL HYDROXYLATION OF QUADRONE TO 8a-HYDROXYQUADRONE[1]

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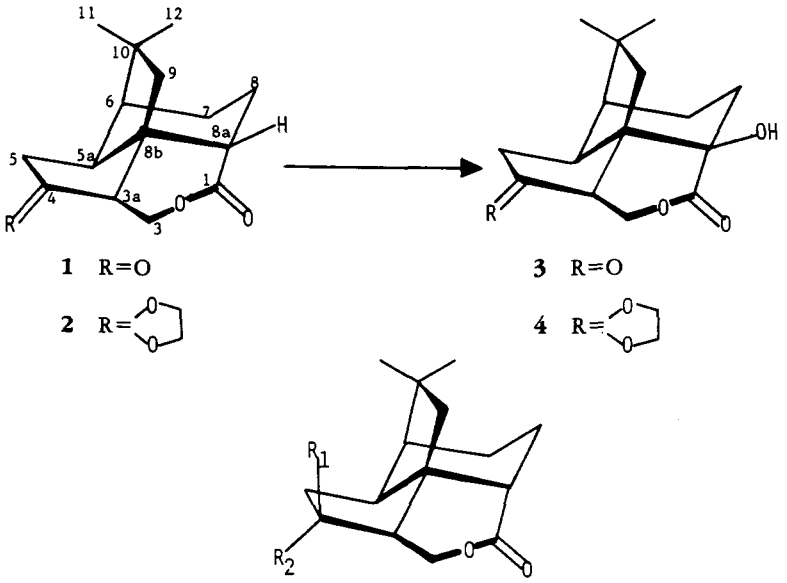
ABSTRACT.—Microbial transformation of quadrone [**1**] and its 4-ethylene ketal [**2**] with *Cunninghamella echinulata* afforded the 8a-hydroxyquadrone [**3**] and its 4-ethylene ketal [**4**], respectively.

The sesquiterpene lactone quadrone [**1**] produced by cultures of *Aspergillus terreus* Thom (1,2) possesses antitumor and antibacterial activity. The unusual tetracyclic lactone skeleton of quadrone has been the target of several synthetic chemical efforts (3-13), and derivatives of quadrone are being examined to establish structure-activity relationships and to afford new quadronoids with useful biological activity. Structural analogs of quadrone are difficult to obtain by total or partial chemical synthetic means, and the microbial transformation approach (14) was explored in an effort to prepare new analogs of the sesquiterpene lactone. A previous report (15) from this laboratory described the reduction of the ketone functional group of quadrone [**1**] to the isomeric quadronols [**5a** and **5b**] as the most common metabolic transformation reaction by many microorganisms. This report describes the hydroxylation of quadrone [**1**] and its 4-ethylene ketal [**2**] to their respective 8a-hydroxy derivatives **3** and **4** by cultures of *Cunninghamella echinulata*.



1 R=O
2 R=
3 R=O
4 R=
5a R_1=OH, R_2=H
5b R_1=H, R_2=OH

FIGURE 1. Structures of quadrone [**1**] and various derivatives

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EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined in open capillary tubes with a Thomas Hoover melting point apparatus and are uncorrected. Ir spectra were recorded in KBr using a Beckman IR 4240 spectrophotometer. ^{1}H- (360 MHz) and ^{13}C-nmr (90 MHz) spectra were recorded in $CDCl_3$ solutions with a Bruker WH-360 FT spectrometer using TMS (δ 0) and $CDCl_3$ (δ 77.0) as internal reference standards. Low resolution ei (70 ev) and ci (NH_3 as the reagent gas) mass spectra were obtained using a Nermag-S-a spectrometer. High resolution mass spectra were obtained on a Kratos AEI MS-50 instrument through the mass spectral services of the Midwest Center for Mass Spectrometry, Department of Chemistry, University of Nebraska, Lincoln, Nebraska. Optical rotations were determined in 95% EtOH solution with a Perkin-Elmer 141 polarimeter.

Quadrone was obtained as a generous gift from Dr. Gary Calton of Purification Engineering, Inc., Columbia, Maryland, and the purity and the identity of the compound were confirmed by chromatographic and spectral comparison with reported values (1,2).

PREPARATION OF QUADRONE-4-ETHYLENE KETAL (3).—A total of 1 g of Dowex-50 (H^+ form), sulfonic acid cation exchange resin beads was added to a solution of 1 g of quadrone [**1**] in 50 ml dry C_6H_6 containing 2 ml of ethylene glycol. The reaction vessel was equipped with a Dean-Stark separator, and the mixture was refluxed for 5 h. The reaction was cooled, the resin beads removed by filtration, and the filtrate was washed five times each with 100 ml of H_2O. Evaporation of the C_6H_6 solution afforded 1.2 g of pure quadrone-4-ethylene ketal [**2**] as colorless needles: mp 167-168°; $[\alpha]_D^{25}$ −78° (c=1); eims *m/z* (% rel. int.) 292.1677 (67.13, M+, Calc for $C_{17}H_{24}O_4$ 292.16745), 277.1443 (38.52), 235.0971 (10.82), 179.1071 (13.78), 153.0916 (25.79), 138.0681 (43.24), 119.0861 (11.43), 99.0446 (100), 86.0374 (59.79), 55.0205 (19.91); ir (KBr, cm^{-1}) 3430 (C=O overtone), 3000, 2990, 2950, 2940, 2590, 2860 (C-H), 1730 (δ-lactone), 1190, 1150, 1105 (C-O-C ketal); ^{13}C nmr, Table 1; ^{1}H nmr Table 2.

TABLE 1. ^{13}C-nmr Chemical Shifts of Quadrone and Its Derivatives

Carbon	Chemical Shift Assignments (ppm)					
	1	**2**	**4**	8a-Hydroxyquadrone [**3**]		
1	173.95	173.87	173.06	172.41[a]	171.75[b]	172.49[c]
3	65.00	64.69	65.62	66.84	66.89	66.98
3a	51.90	51.28	50.12	51.95	52.09	52.13
4	216.50	118.30	118.54	218.07	217.68	218.10
5	42.90	39.79	40.04	42.88	42.91	43.00
5a	52.20	53.89	54.29	50.67	50.74	50.81
6	48.43	48.13	47.28	47.21	47.30	47.39
7	27.84	27.64	29.93	29.73	29.71	29.89
8	19.05	18.57	29.05	28.61	28.76	28.72
8a	45.63	45.40	76.92	76.31	(−)[d]	78.36
8b	49.61	51.13	54.29	54.33	54.33	54.49
9	52.11	53.24	47.20	46.51	46.73	46.71
10	40.25	39.44	38.90	40.20	40.34	40.34
11	26.62	26.71	27.15	26.69	26.80	26.82
12	34.54	35.65	35.15	34.70	34.82	34.84
Ketal-CH_2		64.09	64.65			
Ketal-CH_2		64.17	65.22			

[a]8a-Hydroxyquadrone [**3**] obtained from the metabolism of quadrone [**1**].

[b]8a-Hydroxyquadrone [**3**] obtained from the metabolism of quadrone-4-ethylene ketal [**2**] and subsequent hydrolysis of 8a-hydroxyquadrone-4-ethylene ketal [**4**].

[c]8a-Hydroxyquadrone [**3**] from Hayano *et al.* (18).

[d]Under the $CHCl_3$ peak.

CHROMATOGRAPHY.—Tlc was performed on 0.25 mm thick silica gel GF_{254} (E. Merck) plates prepared with a Quickfit Industries spreader. Plates were air dried and oven activated at 120° for 30 min prior to use. The plates were developed with the following solvent systems: (A) $CHCl_3$-Me_2CO-C_6H_6-HCOOH (95:5:5:1) and (B) $CHCl_3$-EtOAc (3:1). After development, the spots were visualized by spraying with

TABLE 2. Partial ^{1}H-nmr Spectral Data For Quadrone [**1**] and Its Derivatives

Proton	Chemical Shift Assignment (ppm)			
	1	**2**	**3**	**4**
11	1.22, s	1.15, s	1.19, s	1.14, s
12	1.28, s	1.24, s	1.30, s	1.28, s
6	2.00, t J=3 Hz	—	1.96, t J=3 Hz	—
5α	2.66, dd J=17, 14 Hz	—	2.75, dd J=17, 13 Hz	—
3α	4.65, dd J=12, 1 Hz	4.42, dd J=12, 1 Hz	4.52, dd J=11, 1 Hz	4.35, dd J=11, 1 Hz
3β	4.23, dd J=12, 6 Hz	4.20, dd J=12, 5 Hz	4.87, dd J=11, 6 Hz	4.86, dd J=11, 5 Hz

p-anisaldehyde-HOAc-H_2SO_4 (1:120:1) followed by warming sprayed plates with a heat gun. Color intensities were enhanced by again spraying the warm plates with 50% H_2SO_4. The Rf values of various compounds in solvent systems A and B and their colors after spraying were as follows: Quadrone (0.48, 0.60, yellow), quadrone-4-ethylene ketal (0.40, 0.50, yellow), 8a-hydroxyquadrone [**3**] (system B, 0.45, gray), 8a-hydroxyquadrone-4-ethylene ketal [**4**] (system B, 0.40, gray).

Column chromatography was performed using silica gel (40-140 mesh, Baker analyzed 3404) activated at 120° for 30 min prior to use.

MICROORGANISMS.—All cultures were grown and maintained on Sabouraud maltose agar slants and stored in a refrigerator at 4° in sealed, screw-cap tubes until needed. Cultures and the culture collections from which they were obtained were: *Beauveria sulfurescens* ATCC 7159, *Cunninghamella echinulata* NRRL 3655, *Rhizopus arrhizus* QM-1032, *Streptomyces griseus* ATCC 10137, *S. griseus* ATCC 13273, and *Streptomyces punipalus* NRRL 3529.

FERMENTATION.—Fermentations were carried out according to a standard two-stage protocol (16) in a soybean-meal/glucose medium of the following composition: glucose 20 g, yeast extract 5 g, soybean meal 5 g, NaCl 5 g, potassium phosphate dibasic 5 g, distilled water 1000 ml, finally adjusted to pH 7.0 with 6N HCl. Screening experiments were performed in 125-ml DeLong culture flasks containing 25 ml of medium. Cultures were incubated at 27° with shaking at 250 rpm in a New Brunswick Scientific Co., gyrotary shaker. Quadrone substrates (10 mg dissolved in 0.1 ml DMF) were added to each flask containing 24-h-old stage II cultures (16). After 24, 48, and 72 h, 4 ml of the complete incubation mixtures was withdrawn, extracted with EtOAc (1 ml), and 30 μl of the extracted layer spotted on tlc plates for analysis.

MICROBIAL HYDROXYLATION OF QUADRONE [**1**] TO 8A-HYDROXYQUADRONE [**3**].—A total of 500 mg of quadrone [**1**] was dissolved in 10 ml of DMF and distributed equally among ten, 1-liter DeLong flasks each holding 100 ml of 24-h-old, stage II *C. echinulata* culture. Substrate containing cultures were incubated at 250 rpm for 72 h when tlc (solvent B) showed that quadrone was almost entirely consumed. The entire culture was combined and filtered through cheesecloth, and the cells and fermentation solids were washed with H_2O. The combined aqueous filtrates were acidified to pH 4, 1 g of NaCl was added, and the mixture was extracted four times each with 1 liter of $CHCl_3$. The organic layer was dried over anhydrous Na_2SO_4, filtered, and the solvent evaporated under reduced pressure to afford 560 mg of a brown oil.

The brown oil was purified on a column of silica gel (25 g, 2×28 cm) packed in $CHCl_3$ and eluted by a mixture of $CHCl_3$-EtOAc (5:1) while 3-ml fractions were collected. The eluate between 60-123 ml gave unreacted quadrone (**1**, 73 mg), while elution volumes 123-195 ml gave 8a-hydroxyquadrone (**3**, 62 mg, 12% yield). Further elution between volumes 255-480 ml afforded a mixture of quadrone-4-ols (**5a** and **5b**, 28 mg, 15% yield) and unidentified products.

The isolated 8a-hydroxyquadrone [**3**] gave the following physical properties: high resolution eims (*m/z*, % intensity) 264.1429 (M+, calcd for $C_{15}H_{20}O_4$ 264.13616), 247.1346 (2.53, M-OH), 236.1414 (20.37, M-CO), 220.1466 (44.61, M-CO_2), 218.1312 (27.43, M-CO-H_2O), 208.1463 (17.54, M-2CO), 205.1233 (28.85, M-CO_2-CH_3), 192.1524 (18.37, M-CO-CO_2), 192.1167 (30.05, M-CO-CHO-CH_3), 190.1357 (24.78, M-2CO-H_2O), 177.1268 (30.47, M-CO-CO_2-CH_3), 176.1202 (26.54, M-CH_3-CHO-CO_2), 174.1039 (100, M-CO-CHO-H_2O-CH_3); ^{13}C nmr Table 1; ^{1}H nmr Table 2.

MICROBIAL HYDROXYLATION OF QUADRONE-4-ETHYLENE KETAL [**2**] TO 8A-HYDROXYQUADRONE-4-ETHYLENE KETAL [**4**].—A total of 137.5 mg of quadrone-4-ethylene ketal [**2**] was dissolved in

5.5 ml of 95% EtOH and distributed equally among eleven 1-liter DeLong flasks each holding 200 ml of 24-h-old, stage II cultures of *C. echinulata*. Substrate containing cultures were incubated for 72 h at which time the entire culture was extracted three times each with 2 liters of EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and filtered. Evaporation of the solvent under reduced pressure afforded 136 mg of a gummy residue that was further purified by column chromatography over silica gel (20 g, 3×25 cm) which was slurry packed and eluted with $CHCl_3$. Elution volumes 36-108 ml gave 8a-hydroxyquadrone-4-ethylene ketal (**4**, 36 mg, 25% yield).

This chromatographically pure metabolite gave the following physical data: high resolution eims M+ 308.1630 (calc. for $C_{17}H_{24}O_5$ 308.16238); eims *m/z* (% intensity) 308 (15.3, M+), 293 (22.0, M-CH_3), 291 (7.7, M-OH), 280 (5.7, M-CO), 264 (3.6, M-CO_2), 252 (3.6, M-2CO), 249 (3.3, M-CO_2-CH_3), 236 (3.1, M-CO-CO_2), 235 (3.8, M-CHO-CO_2), 221 (5.0, M-CO-CO_2-CH_3), 219 (5.1, M-2CO-H_2O-CH_3), 208 (4.3), 207 (7.0), 205 (7.0), 180 (13.6), 179 (13.4), 167 (11.3), 153 (22.6), 149 (18.3), 139 (22.0), 138 (17.5), 135 (10.1), 134 (13.9), 129 (20.2), 126 (18.3), 125 (17.5), 121 (15.4), 119 (14.1), 113 (11.3), 112 (11.7), 107 (18.0), 105 (16.3), 100 (10.1), 99 (87.8), 86.0 (100), 55 (59.1); ^{1}H nmr, 1.14 (3H, s, H-11), 1.28 (3H, s, H-12), 1.4-1.6 (2H, m), 1.6-1.71 (2H, m), 1.78 (1H, bs), 1.98 (1H, dd, J=12, 6 Hz), 2.12 (1H, dd, J=12, 12 Hz), 2.30-2.34 (2H, m), 2.41 (1H, d, J= 5 Hz, H-3a), 2.54-2.58 (1H), 2.8 (1H, bs, OH), 3.85-4.10 (4H, m, -O-CH_2-CH_2-O-), 4.35 (1H, dd, J=11, 1 Hz, H-3α), 4.86 (1H, dd, J=11, 5 Hz, H-3β); ^{13}C nmr (Table 1).

HYDROLYSIS OF 8A-HYDROXYQUADRONE-4-ETHYLENE KETAL [**4**] TO 8A-HYDROXYQUADRONE [**3**].—A solution of 30 mg of 8a-hydroxyquadrone-4-ethylene ketal [**4**] in 40 ml of Me_2CO containing 100 mg of *p*-toluene sulfonic acid was stirred for 16 h at room temperature. The reaction mixture was concentrated to 1.5 ml and purified on a silica gel column (10 g 1×10 cm) which was eluted with $CHCl_3$-EtOAc (3:2). Elution volumes 3-15 ml afforded 7 mg of 8a-hydroxyquadrone [**3**] completely identical (tlc, ^{13}C and ^{1}H nmr, ms) with **3** obtained by the direct hydroxylation of quadrone. Further elution of the column gave 15 mg of unreacted **4** between volumes 15-60 ml.

RESULTS AND DISCUSSION

Previous microbial transformation of quadrone [**1**] with 100 microorganisms resulted in the reduction of the ketone carbonyl functional group to 4*S* and/or 4*R* alcohols [**5a** and **5b**] as the major and most common biotransformation reaction (15). In an effort to obtain different types of quadrone metabolites, the ketone functional group which appears to be the primary site of metabolic activity was protected with an ethylene ketal blocking group. By removal of the ketone moiety, it was hoped that other useful types of reactions such as hydroxylation would be observed. Nine microorganisms known to hydroxylate a variety of steroid and alkaloid substrates were screened for their abilities to biotransform quadrone-4-ethylene ketal [**2**]. Of the cultures examined, the following gave similar patterns of polar derivatives of **2** as evidenced by tlc (solvent system B): *R. arrhizus* QM-1032, *B. sulfurescens* ATCC-7159, *C. echinulata* NRRL-3655, *S. griseus* ATCC-10137, *S. griseus* ATCC-13273, and *S. punipalus* NRRL-3529.

C. echinulata appeared to give the best and most consistent yield of metabolites, and it was selected for preparative scale transformation with **2**. The major metabolite was isolated and purified by column chromatography and identified by spectral analysis (see below) as 8a-hydroxyquadrone-4-ethylene ketal [**4**]. Hydrolysis of **4** with *p*-toluenesulfonic acid afforded 8a-hydroxyquadrone [**3**], which was characterized as described below.

Encouraged by the results with **4**, quadrone [**1**] was subjected to microbial transformation with the four microorganisms (QM-1032, NRRL-3529, NRRL-3655, and ATCC-10137) that appeared to give best yields of hydroxylated products with **2**. Two cultures, *C. echinulata* NRRL 3655 and *S. griseus* ATCC 10137, consistently produced one more polar product as the major metabolite along with a mixture of quadronols [**5a** and **5b**]. A preparative scale incubation was performed with *C. echinulata*, the major metabolite was isolated and purified by chromatography, and the structure was established as 8a-hydroxyquadrone [**3**] by spectral analysis.

The mass spectra of the metabolites 8a-hydroxyquadrone [**3**] and 8a-hydroxyquadrone-4-ethylene ketal [**4**] clearly indicated that a single oxygen atom had been introduced into each of the substrate structures. ^{13}C nmr (Table 1) indicated that one of the methine carbon atoms (CH) of quadrone [**1**] and its ketal [**2**] were converted to tertiary alcohols (C-OH) in their respective metabolites. Quadrone and its ketal contain four methine carbon atoms, and these occur at positions 3a, 5a, 6, and 8a. Comparison of the ^{13}C-nmr spectral data of **1** and the ketal [**2**] and their respective hydroxylated metabolites **3** and **4** supported 8a as the most likely position of hydroxylation. Carbons 8 and 8b experienced significant downfield shifts (β-effect) of about 10 and 4 ppm, respectively. Carbon 9, on the other hand, experienced an upfield shift of about 6 ppm typical for an expected γ-*gauche* effect. Further support for the structures of the hydroxylated metabolites is obtained by comparing features of their ^{1}H-nmr spectra (Table 2). In 8a-hydroxyquadrone [**3**] the 5α proton is clearly observed at 2.75 ppm where it exhibits splitting by the adjacent 5a and 5β protons. Thus, the 5a position must be unsubstituted in the metabolite. The 6-hydrogen atom which resonates at 1.96 ppm exhibits the same splitting pattern in **1** and the metabolite **3**, thus excluding this as the hydroxylation site. The 3α and 3β protons have exactly the same splitting patterns in the substrates and metabolites, thus making it possible to exclude 3a as the hydroxylation site. However, the relative chemical shifts of the 3α and 3β protons of the metabolites are significantly changed when compared to their respective quadrone precursors (Table 2). The 3β protons of the metabolites **3** and **4** resonate at 4.87 and 4.86 ppm, respectively, deshielded by 0.64 and 0.66 ppm from their respective substrates **1** and **2**. On the other hand, the 3α proton resonating at 4.52 and 4.35 ppm in **3** and **4**, respectively, are only slightly more shielded (upfield shifts of about 0.13 and 0.07 ppm) relative to their respective substrates. Examination of Dreiding models of **3** and **4** indicates that the 3β proton would experience a through space paramagnetic interaction with 8a-hydroxy groups in the metabolites. Thus, the newly introduced hydroxyl group in the metabolites **3** and **4** would have the same stereochemical orientation as the 8a hydrogen in quadrone [**1**] and its ketal.

The nmr spectral observations indicate that no inversion of stereochemistry occurs at position 8a during the microbial hydroxylation reaction. This appears to be a common feature of microbial hydroxylation reactions where the introduced hydroxyl group assumes the same stereochemical configuration as the replaced hydrogen atom (17). In the course of this work, 8a-hydroxyquadrone [**3**] was isolated as a natural product from cultures of *A. terreus* (18). This would indicate that *A. terreus* is also able to catalyze the hydroxylation of quadrone and suggests that the hydroxylation reaction occurs as a late step following complete assembly of the quadrone skeleton. 8a-Hydroxyquadrone was found devoid of antineoplastic and cytotoxic activity when tested against P-388 lymphocytic leukemia (18).

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