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Report

Microbial Biotransformation of Retinoic Acid by *Cunninghamella echinulata* and *Cunninghamella blakesleeana*

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Vitamin A (retinol) is needed by higher animals for the maintenance of normal epithelium and growth, and retinoic acid (**1**) has been proposed to be the active metabolite. Microbial models are useful for the study of mammalian metabolism of xenobiotics. Two species of the fungal genus *Cunninghamella* afforded products of greater polarity than **1** when fed **1** in a two-stage fermentation procedure. The products obtained were principally the result of oxidation of the trimethylcyclohexenyl ring. Although most of the isolated metabolites of **1** have been previously seen in mammalian studies, two novel compounds, 2-hydroxyretinoic acid (**2**) and 2,3-dehydro-4-oxoretinoic acid (**4**), were isolated.

KEY WORDS: retinoic acid; metabolism; microbial models; retinoids; chemoprevention.

INTRODUCTION

Higher animals require vitamin A (retinol) for normal growth and proper epithelial cell differentiation (1). An oxidized metabolite of retinol, retinoic acid (**1**), is capable of supporting the biological functions of the parent compound. This metabolite has shown greater activity than retinol in the maintenance of epithelial tissue differentiation (2). Although **1** has been studied extensively in the areas of cancer chemoprevention and chemotherapeutics, its mechanism of action is still not fully understood. Further, **1** and its analogues, such as isotretinoin and etretinate, have recently found utility as dermatological agents (3).

Whether **1** needs to be further metabolized in order to exert its actions remains unknown. However, only the metabolites retinoyl-β-glucuronide and 13-*cis*-retinoic acid have been shown to be equipotent to **1** in assays for vitamin A-like activity (2,4). Other metabolites have decreased activity compared to **1** (5); however, one or more of these metabolites may be responsible for the teratogenic or toxic effects of **1** (6). In addition, the stereospecificity of certain of the metabolic transformations of **1** is unknown.

Recently, several additional metabolites of retinoic acid, some with unknown structure and activity, have been isolated from rats receiving physiological doses of retinoic acid (7). Therefore, the ultimate functional form of vitamin A necessary for epithelial differentiation remains uncertain.

Microbial models are useful in the study of mammalian metabolism of various xenobiotics (8,9). Microorganisms possess enzymes similar in nature to those of mammals and, therefore, can produce similar metabolites in many instances. We have recently reported our studies using the structurally similar β-ionone as a model substrate for microbial transformation of **1** (10).

In this report, we extend our results to the study of the microbial metabolism of **1**. Information about the stereochemistry of known metabolites of **1** may shed light on their mechanism of action and may assist in the design of analogues of **1** as therapeutic agents.

MATERIALS AND METHODS

General

Nuclear magnetic resonance spectra were obtained as $CDCl_3$ solutions on an IBM AF250 spectrometer with $CHCl_3$ as the internal standard. Nuclear Overhauser effect difference spectra (11) were done on an IBM AF270 spectrometer. Fast atom bombardment (FAB) mass spectra were determined on a Kratos MS-30 mass spectrometer. The FAB mass spectra were obtained using dithiothreitol:dithioerythritol (3:1 or 5:1), with up to 20% CH_3OH in $CHCl_3$, as the solvent (12). Optical rotations were measured employing a Perkin–Elmer 241 polarimeter. The ultraviolet spectra were obtained using a Beckman DU-40 spectrophotometer. Infrared spectra were recorded with a Beckman 4320 infrared spectrophotometer as liquid films. TLC was performed on 0.25-mm silica gel 60 F_{254}-precoated glass plates from EM reagents. Column chromatography was done by employing silica gel as the stationary phase (70–230 mesh, EM Science). Preparative TLC was also done on precoated glass-backed plates utilizing silica gel 60 F_{254} (1 mm, 20 × 20 cm) as the stationary phase (EM Science). HPLC was carried out on a Beckman 332 gradient liquid chromatograph equipped with a Beckman 164 UV detector using a DuPont Zorbax-Sil (4.6 mm × 25-cm) column at a flow rate of 1.5 ml/min. Preparative HPLC was done utilizing the above instrumentation with a DuPont Zorbax-Sil (9.4 mm × 25-cm) column at a

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flow rate of 3 ml/min. All reagent chemicals were purchased from Aldrich Chemical Co. All manipulations of retinoids were conducted under yellow light to minimize possible photoisomerization.

Culture and Screening Methods

Thirteen fungal cultures obtained from the American Type Culture Collection were initially screened. The fungal cultures were maintained at 4°C on agar slants of Mycophil (Difco) and transferred every 2–3 months to fresh agar to ensure continued viability. A two-stage fermentation procedure identical to our earlier method was employed for the screening and biotransformation (10). Surface growth from 7-day-old agar slants was suspended in normal saline and used to inoculate Stage I shake cultures. Aliquots (10 ml) of 2-day-old Stage I cultures were used as inocula for Stage II shake cultures; 10 mg of **1** was added in 1 ml of EtOH to each of the cultures 1 day after the initiation of Stage II. All shake cultures were grown in a broth medium consisting of the following (per liter of H_2O): Pharmamedia (Traders Oil Mill Co., Memphis, TN), 10 g; yeast extract, 5 g; D-glucose, 20 g; NaCl, 5 g; and K_2HPO_4, 5 g. Incubations were done on a rotary shaker at 25°C and 250 rpm.

After incubation with **1** for 7 days, the Stage II cultures were filtered and the filtrate was exhaustively extracted with EtOAc. The organic extracts were combined and washed with brine, dried (Na_2SO_4) and concentrated. Those organisms which produced promising metabolites when analyzed by TLC (10% CH_3OH/CH_2Cl_2 on original extracts or 50% EtOAc/hexane after esterification with diazomethane) were selected for further study. Confirmation that no methylated metabolites were overlooked by treatment with diazomethane was obtained by comparison of the native metabolites with their methylated counterparts in various solvent systems (3% acetone/CH_2Cl_2, 50% EtOAc/hexane, and 50% Et_2O/hexane).

Fungal organisms (with ATCC numbers) which showed production of apparent transformation products of **1** included the following: *Aspergillus ochraceus* (18500), *Beauvaria sulfurescens* (7159), *Cunninghamella blakesleeana* (8688a), *C. echinulata* (9244), *C. elegans* (9245), *Mucor rouxii* (24905), and *Syncephalastrum racemosum* (18192). The following organisms showed no obvious transformation: *Aspergillus flavipes* (11013), *A. niger* (9142), *A. niger* (11394), *A. niger* (16888), *Beauvaria bassiana* (13144), and *Penicillium citrinum* (16040).

Microbial Biotransformation of Retinoic Acid

After initiation of the Stage II cultures as described above, 10 mg of **1** was added to each of 30 flasks of *Cunninghamella blakesleeana* ATCC 8688a as a 1-ml solution in EtOH. The products were isolated as described above (280 mg) and methylated with diazomethane. The resulting orange oil was crudely fractionated via silica gel column chromatography. Elution was performed in a stepwise fashion, with 20% EtOAc/hexane being the initial eluent (100 ml); followed by 40% (50 ml), 60% (50 ml), 80% (50 ml), and finally, 100% EtOAc (150 ml).

In a similar manner, the biotransformation of **1** by *Cunninghamella echinulata* ATCC 9244 was also accomplished. In this case 270 mg of an orange oil was recovered. A crude fractionation was also done utilizing conditions identical to that described for *C. blakesleeana*. The metabolite product mixture appeared to be unchanged qualitatively but increased in total concentration for both organisms throughout the fermentation, as indicated by periodic sampling of the fermentation flasks during the incubation.

Summary of Physical and Chemical Data for Compounds Isolated

In each case the compounds isolated were mainly the all-*trans* isomer, but significant amounts of the 13-*cis* isomer were present. An additional minor unidentified double-bond isomer was also detected in many cases. All compounds were analyzed as their methyl esters. Some unmetabolized starting material was recovered in each case: *C. blakesleeana* (51.6 mg) and *C. echinulata* (85.0 mg). The yields of the compounds isolated and their optical rotations, if applicable, are found in Table I.

Methyl 2-Hydroxyretinoate (2) (C. blakesleeana). Further purification via column chromatography (35–50% EtOAc/hexane) and preparative TLC (75% CH_2Cl_2/EtOAc) yielded **2**. UV ($CHCl_3$) λ_{max} = 360 nm (ϵ = 29139); IR (neat) cm^{-1} 3420, 1710, 1700; ^1H NMR ($CDCl_3$) δ 1.04 (s, 3, 1-CH_3), 1.08 (s, 3, 1-CH_3), 1.71 (s, 3, 5-CH_3), 1.74 (brt, 2, 4-H), 1.97 (s, 3, 9-CH_3), 2.07, 2.36 (2s, 3, 13-*cis* and all-*trans* 13-CH_3), 2.14 (brdd, 2, 3-H), 3.54 (brd, 1, 2-H), 3.72 (s, 3, OCH_3), 5.64, 5.77 (2brs, 1, 13-*cis* and all-*trans* 14-H), 6.04–6.32 (m, vinyls), 6.98 (2dd, 1, 13-*cis* and all-*trans* 11-H), 7.76 (d, 1, 13-*cis* 12-H); MS *m/z* (%): 329 (M^{-2+1}, 7.0), 135 (69.3), 85 (100).

Methyl 2-Hydroxy-4-oxoretinoate (3) (C. blakesleeana). This metabolite was also purified by column chromatography (50% EtOAc/hexane) and preparative TLC (75% EtOAc/CH_2Cl_2). This compound exhibited the same spectral properties as that which was isolated from *C. echinulata*.

Methyl 2,3-Dehydro-4-oxoretinoate (4) (C. blakesleeana). Purification was achieved in the same manner as described for **3** above. UV ($CHCl_3$) λ_{max} = 364 nm (ϵ = 30406); IR (neat) cm^{-1} 2940, 1740, 1720; ^1H NMR ($CDCl_3$) δ 1.26 (s, 6, 1,1-CH_3), 1.99 (s, 3, 5-CH_3), 2.04 (s, 3, 9-CH_3)

Table I. Microbial Biotransformation Products of **1**

Organism	Compound	Yield (%)[a]	Optical rotation[b]
C. blakesleeana	**2**	5.5 (2.2)	+96.0°
	3	0.9 (0.3)	—[c]
	4	3.0 (1.2)	—
	5	3.2 (1.3)	+10.8°
C. echinulata	**6**	8.0 (4.3)	—
	2	5.0 (2.7)	+4.2°
	3	17.6 (9.5)	−2.8°
	4	11.0 (5.9)	—

[a] All yields are reported in milligrams. The products were purified as isomer mixtures, in which the ratio of all-*trans* to 13-*cis* was approximately 2:1 in all cases.

[b] The optical rotations were measured at 22°C in CH_2Cl_2 except for the **3** from *C. echinulata*, which was measured in (CH_3OH).

[c] Insufficient material available to make a determination.

2.08, 2.36 (2s, 3, 13-*cis* and all-*trans* 13-CH_3), 3.79 (s, 3, OCH_3), 5.68, 5.82 (2brs, 1, 13-*cis* and all-*trans* 14-H), 6.22 (d, 1, 3-H), 6.25–6.52 (m, vinyls), 6.76 (d, 1, 2-H), 6.98 (2dd, 1, 13-*cis* and all-*trans,* 11-H), 7.84 (d, 1, 13-*cis,* 12-H); MS *m/z* (%) 327 (M^{-2+1}, 27.6), 135 (40.6), 119 (100), 85 (87.2).

Methyl 16-Hydroxy-4-oxoretinoate (5) (C. blakesleeana). Preparative TLC (75% (EtOAc/CH_2Cl_2 followed by preparative HPLC (50% EtOAc/hexane) afforded **5**. UV ($CHCl_3$) λ_{max} = 364 nm (ϵ = 9406); IR (neat) cm^{-1} 3470, 1740, 1680; ^1H NMR ($CDCl_3$) δ 1.16 (s, 3, 1-CH_3), 1.65 (m, 2, 2-H), 1.86 (s, 3, 5-CH_3), 2.01 (s, 3, 9-CH_3), 2.35 (s, 3, 13-CH_3), 2.56 (m, 2, 3-H), 3.44, 3.74 (m, 2, CH_2OH), 3.70 (s, 3, OCH_3), 5.68 and 5.80 (2s, 1, 13-*cis* and all-*trans* 14-H), 6.14–6.40 (m, vinyls), 6.33 (s, 1, 7-H), 6.96 (dd, 1, all-*trans* 11-H), 7.82 (d, 1, 13-*cis* 12-H); MS *m/z* (%) 345 (M^{+1}, 27.8), 165 (29.5), 85 (100).

Methyl 4-Oxoretinoate (6) (C. echinulata). Column chromatography (20% EtOAc/hexane) and preparative TLC (80% CH_2Cl_2/EtOAc) afforded **6**. This isolate had spectroscopic properties similar to those of synthetic **6** (13,14).

Methyl 2-Hydroxyretinoate (2) (C. echinulata). Purification via column chromatography (25% EtOAc/hexane) and preparative TLC (50% CH_2Cl_2/EtOAc) gave **2**. This compound possessed physical properties similar to those obtained for **2** from *C. blakesleeana.*

Methyl 2-Hydroxy-4-oxoretinoate (3) (C. echinulata). Column chromatography (30% EtOAc/hexane) afforded pure **3**. UV (CH_3OH) λ_{max} = 360 (ϵ = 35100), 285 (ϵ = 11800), 231 (ϵ = 8150); IR (neat) cm^{-1} 3420, 1740, 1715; ^1H NMR ($CDCl_3$, δ 1.18 (s, 3, 1-CH_3), 1.21 (s, 3, 1-CH_3), 1.84 (s, 3, 5-CH_3), 2.01 (s, 3, 9-CH_3), 2.06, 2.33 (2s, 3, 13-*cis* and all-*trans,* 13-CH_3) 2.59, 2.78 (2dd, 2, 3-H), 3.70 (s, 3, OCH_3), 3.87 (m, 1, 2-H), 5.68, 5.80 (2brs, 1, 13-*cis* and all-*trans,* 14-H), 6.22–6.37 (m, vinyls), 6.95 (2dd, 1, 13-*cis* and all-*trans,* 11-H), 7.82 (d, 1, 12-H); MS *m/z* (%) 345 (M^{+1}, 8.9), 185 (29.6), 93 (100), 58 (63.0).

Methyl 2,3-Dehydro-4-oxoretinoate (4) (C. echinulata). Purification was done in a manner similar to that described for **2** above except 75% CH_2Cl_2/EtOAc was used as the preparative TLC solvent system. This isolate had properties similar to that seen for **4** from *C. blakesleeana.*

RESULTS AND DISCUSSION

The functional form of vitamin A, in epithelial tissue differentiation, is still a subject of debate. Retinoic acid is known to be more active than retinol in assays of epithelial tissue differentiation (2). Whether **1** is further metabolically activated remains unanswered.

Two readily obtainable known metabolites of **1**, 4-hydroxyretinoic acid (**7**) (13) and 4-oxoretinoic acid (**6**) (14,15), were synthesized from **1**, as previously described, to serve as TLC standards for the metabolic transformation screens. Thirteen fungi were screened for their ability to transform **1** to products of polarity resembling that of **7** and **6**. A two-stage culture method was employed for the biotransformation studies (9,10). Over the course of the 7-day incubations, the distribution of products was monitored by TLC after extraction using ethyl acetate. From these 13 species, **2** were selected for further study, *Cunninghamella blakesleeana* ATCC 8688a and *Cunninghamella echinulata* ATCC 9244.

C. blakesleeana and *Aspergillus niger* ATCC 16888 were previously employed for our studies on the microbial metabolism of the structurally related β-ionone (10). However, *A. niger,* which produced the 4-hydroxy and 4-oxo compounds from β-ionone, did not metabolize **1** to any great extent.

Utilizing the two *Cunninghamella* species, these fermentations were repeated on a larger scale to facilitate the identification of the transformation products. After 7 days, the cultures were extracted with ethyl acetate following our earlier procedures (10). In order to enhance solubility in organic solvents, allowing more efficient adsorption chromatography and spectroscopic analysis, the entire product mixture was treated with diazomethane to give the methyl esters (16). No methylated metabolite possessed an R_f equivalent to the native metabolites. The resulting methyl retinoates were purified by silica gel column chromatography and, in some cases, further purified by utilizing preparative TLC and HPLC. The products and their yields, are shown in Fig. 1 and Table I). Many of the metabolites in which stereocenters were introduced exhibited an optical rotation, suggesting stereoselective metabolism of **1** (Table I). All metabolites were identified by comparison with available data for known compounds and by employing appropriate spectroscopic techniques.

The metabolites, 2-hydroxy-4-oxoretinoic acid (**3**), as its 13-*cis* isomer (**17**), and 16-hydroxy-4-oxoretinoic acid (**5**) (18), have been previously isolated and identified from studies examining the mammalian metabolism of **1**. The spectra recorded for both isolated compounds matched those reported in the above studies. Independently, we confirmed the assigned structures by utilizing NOE difference spectroscopy. 4-Oxoretinoic acid (**6**) has also been detected previously in mammalian metabolic studies (19).

The two remaining products isolated, **2** and **4**, have not been reported as mammalian metabolites of **1**. However, Mikami *et al.* have reported structurally similar products from microbial biotransformation studies of β-ionone by *Aspergillus niger* JTS 191 (20). The apparent similarity in the spectroscopic data for the β-ionone transformation products and the 2-hydroxyretinoic acid (**2**) and 2,3-dehydro-4-oxoretinoic acid (**4**) isolated here, facilitated structural assignment. Again, NOE difference spectra were employed to confirm the structural assignments.

In our recent NMR (21) and conformational studies

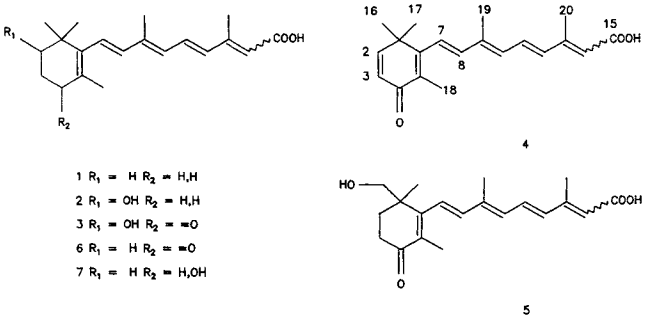

Fig. 1. Metabolites isolated from the biotransformation of **1** which were analyzed as their methyl esters.

(R. W. Curley, Jr., and J. W. Fowble, unpublished results) of 1, we have found that a significant NOE is observed at the C2 methylene protons with a considerably smaller enhancement at C3 when the methyl protons at C1 are irradiated. A measurable NOE was also found between the C5 methyl protons and the C4 methylene protons, as well as a smaller NOE to C3. These data proved useful in the assignment of the position of the hydroxyl group in 2-hydroxylated metabolites and the position of the double bond in 4.

For example, irradiation at the resonance frequency corresponding to the geminal methyl groups of 2 resulted in an enhancement of the signal at 3.54 ppm. This downfield methine proton resonance is not enhanced upon irradiation of the 5-CH_3 group and thus corresponds to the methylene protons at C2 confirming the assignment of the structure. Irradiation of the 5-CH_3 group of 4 resulted in an enhancement of only the signal for the vinyl proton tentatively designated as being at C3 (6.22 ppm). In addition, irradiation at the resonance corresponding to the methyl groups at C1 caused a sizable NOE to the doublet at 6.76 ppm. Thus this doublet has been assigned as the C2 vinyl proton. These results exclude the isomeric structure 2-oxo-3,4-dehydroretinoic acid for metabolite 4.

In all cases where identical metabolites were isolated from both organisms, yields of metabolites were significantly higher from *C. echinulata* than *C. blakesleeana*. However, polarimetry results show that *C. blakesleeana* has produced 2 with greater stereoselectivity than *C. echinulata* (Table I). Attempts to clarify the stereochemistry of 2-hydroxylation on the basis of ^1H NMR studies of the alcohol acylated with optically pure α-methoxy-α-(trifluoromethyl)phenylacetate (Mosher's reagent) (10,22) did not succeed in the present studies. Apparently, steric hindrance by the adjacent geminal methyl groups prevented 2-hydroxy acylation under conditions that did not decompose the metabolite. While 5 was also produced stereoselectively as evidenced by its significant optical rotation, insufficient material was present to allow the assignment of stereochemistry.

Three of the five metabolites isolated from microbial biotransformations of 1 have been reported by other workers studying the metabolism of 1 (23), but only 6 was assayed for vitamin A-like activity. This 4-oxo metabolite represents a prominent mammalian metabolite of 1 which is thought to retain little favorable activity (5) but may contribute to the teratogenicity of 1 (6). All of the other known metabolites isolated here have also been found in samples of human blood and urine after treatment with 1 or its 13-*cis* analogue (18,24), but little information exists concerning their biological activity. Thus, microbial metabolism of 1 represents a valuable approach for the production of known and new metabolites of 1 in sufficient quantities to permit evaluation of their relative contribution to the activity/toxicity of 1.

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