BIOORGANIC CHEMISTRY **20,** 148–154 (1992)

Microbial Oxidation of the Antimalarial Drug Arteether

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Received December 5, 1991

Three oxidation products from the action of the enzymes in *Beauveria sulfurescens* on the antimalarial drug arteether were isolated and their structures established by mass spectrometry, and both 1D and 2D NMR. Two of the compounds, 14-hydroxyarteether and 9β-hydroxyarteether retained the peroxide bridge required for biological activity. The metabolites were employed as intermediates for the preparation of other products.

INTRODUCTION

More than one million deaths, primarily of children, occur worldwide each year from malaria and the problem grows worse in many underdeveloped countries (*1*) as more strains of *Plasmodium falciparum* develop resistance to drugs currently in use in Southeast Asia. To control the disease, the World Health Organization has promoted a search for new antimalarial drugs. One compound, artemisinin, **1**, identified by Chinese investigators as the active principle of a traditional herbal drug, *Artemisia annum* L., (*2*) has been shown to be active against strains of *P. falciparum* resistant to mefloquine and halofantrine, the most effective current antimalarials. Investigators in China and the United States (*3*) have prepared a variety of esters and ethers of dihydroartemisinin in their search for more promising compounds. Unfortunately, metabolic studies have shown that these groups were enzymatically hydrolyzed or oxidized to yield dihydroartemisinin (*4*). In order to prepare and investigate new types of derivatives it was necessary to introduce at various sites in the molecule another functional group. This process is complicated by the need to retain a reactive peroxide group that is essential for antimalarial activity. We report here the use of the fungus *Beauveria sulfurescens* to introduce hydroxyl groups at an unactivated methyl or methylene group of an artemisinin derivative, **2**.

Hufford *et al.* (*5*) investigated the microbial metabolism of arteether, **2**, with *Aspergillus niger* and *Nocardia corallina* and identified three metabolites, **3**, **4**, and **5**. Unfortunately, none had an intact peroxide bridge. They also examined the microbial metabolism of artemisinin (*6*) and here again found two metabolites in which the peroxide bridge had been destroyed. The metabolism of **2** by rat-liver microsomes was investigated by Baker *et al.* (*4*), who established dihydroartemisinin as the major metabolite. Small quantities of **3**, **4**, and **5** were formed, as were trace quantities of other products. The structures of the latter were not established.

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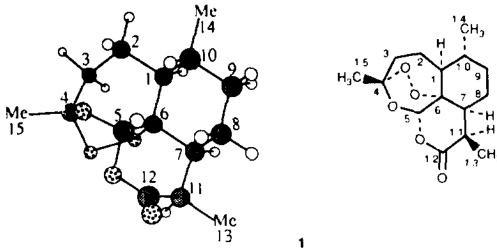

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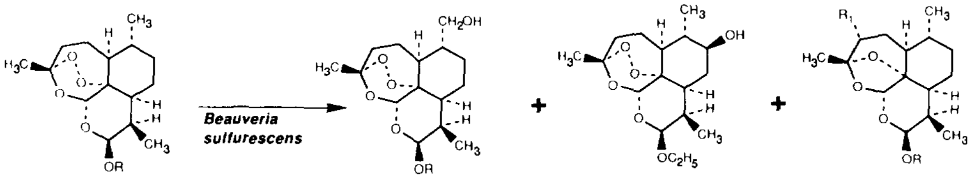


2 R=C_2H_5

6 R=CONHPh

7 R=CONHPh

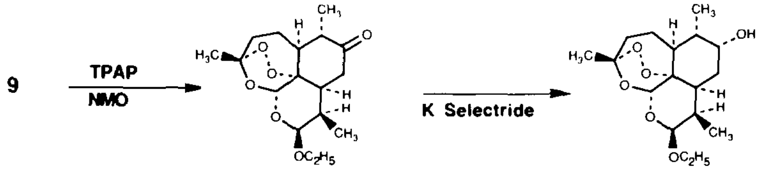
8 R=C_2H_5

9

3 R=C_2H_5; R_1=OH

4 R=R_1=H

5 R=H; R_1=OH



10

11

EXPERIMENTAL

Microbial Oxidation

A 1-liter Erlenmeyer flask containing 250 ml of medium (Sabouraud Liquid Broth Modified Antibiotic Medium 13, BBL, Cockeysville, MD) was innoculated with *B. sulfurescens* (ATCC 7159) grown on a slant made from the same medium containing 2% agar. The flask was shaken (200 rpm) at 27°C for 72 h. Aliquots of this culture were employed to innoculate each of twelve 1-liter Erlenmeyer flasks containing 250 ml of medium and approximately 100 μg of arteether as an inducer. The flasks were shaken (200 rpm) for 48 h at 27°C, and the mycelia were collected by filtration and resuspended in buffer (200 ml of 0.1 M KH_2PO_4, pH 7.5). Arteether (40 mg in 0.4 ml of ethanol) was added to each flask. The flasks were shaken for 3 days, the mycelia were removed by filtration, and the combined filtrates were extracted with ethyl acetate (3 × 600 ml). The extracts were dried (Na_2SO_4) and concentrated. The residue was purified by flash chromatography on silica gel by washing the column (2.5 × 19 cm) successively with (a) 500 ml of 15% ethyl acetate and 85% hexane, (b) 250 ml of 20% ethyl acetate and 80% hexane, and (c) 500 ml of 30% ethyl acetate and 70% hexane. The eluant was collected in 8-ml fractions and compounds were eluted in the following order. Unreacted **2**, 100 mg; 3α-

TABLE 1

Summary of Data on ^{1}H Chemical Shifts and Coupling Constants

H on carbon No.	J_{H-H}	3		8		9		11	
		δ	J(Hz)	δ	J(Hz)	δ	J(Hz)	δ	J(Hz)
1	1a,2a	a 1.40(m)	12.8	1.60(m)	11.8	1.37(m)	11.3	1.82(m)	11.7
	1a,10a		11.3		11.8		11.3		11.7
	1a,2e		5.2		4.9		6.1		6.7
2	2a,2e	e 1.85(m)	14.0	e 1.87(m)	14.3	e 1.88(m)	13.4	e 1.79(m)	13.4
	2e,3a				4.0		4.0		3.7
	2e,3e		1.8		3.2		3.1		2.6
	2a,3a	a 1.62(m)		a 1.55(m)	13.1	e 1.55(m)	13.4	a 1.49(m)	13.4
	2a,3e		4.0		4.6		4.9		4.8
3	3a,3e	e 3.56(q)		a 2.39(m)	14.7	2.37(m)	14.7	2.40(m)	14.6
				e 2.05(m)		2.06(m)		2.05(m)	
5		e 5.27(s)		5.42(s)		5.46(s)		5.42(s)	
7	7a,8e	a 1.88(m)	5.0	1.47(m)		1.61(m)	4.6	1.98(m)	3.9
	7a,8a		13.1				13.4		13.7
	7a,11e		5.0		4.6		4.9		4.5
8	8a,8e	e 1.82(m)	12.3	1.82–		e 2.00(m)	13.4	e 1.91(m)	13.9
	8e,9a		4.9	1.87(m)	4.8		4.6		
	8e,9e		3.4		3.4				3.9
	8a,9a	a 1.75(m)	13.4		12.5	a 1.94(m)	11.1	a 2.10(m)	
	8a,9e		3.4		3.4				2.4
9	9a,9e	a 0.98(m)	13.4	a 1.10(m)	12.5	a 3.11(m)		e 3.74(m)	
	9a,10a		13.4		12.5		9.6		
	9e,10a	e 1.72(m)	3.4	e 1.80(m)	3.4				2.7
10	10a,14	a 1.24(m)	6.4	1.47(m)	3.1	1.32(m)	5.8	1.53(m)	6.7
	10a,14′				5.8				
11	11e,7a	e 2.44(m)	5.0	2.62(m)	4.6	2.60(m)	4.9	2.65(m)	4.5
	11e,12a		4.3		3.1		3.4		3.1
12		a 4.76(d)		4.80(d)		4.80(d)		4.81(d)	
13	11e,13	0.92(d)	7.3	0.91(d)	7.3	0.91(d)	7.3	0.90(d)	7.3
14	14,14′	0.87(d)		3.73(q)	10.7	1.07(d)		1.04(d)	
				3.58(q)					
15		1.56(s)		1.44(s)		1.44(s)		1.44(s)	
16	16′,16	3.83(m)	9.8	3.86(m)	9.8	3.87(m)	9.8	3.87(m)	9.9
		3.44(m)		3.47(m)		3.48(m)		3.48(m)	
17	17,16	1.18(t)	7.0	1.18(t)	7.0	1.18(t)	7.0	1.18(t)	7.0
OH		2.05		1.44		1.58		1.52	

Note. ^{1}H, ^{13}C, ^{1}H COSY and HMQC spectra were measured with a Varian VXR-500S NMR spectrometer. The ^{13}C-DEPT spectra were obtained on a Varian VXR-300 spectrometer. Chemical shifts (δ, ppm) were measured relative to TMS (^{1}H, $\delta = 0.0$) or the solvent signal (^{13}C, $\delta = 77.0$).

hydroxydesoxyarteether, **3**, 27 mg; a mixture of 3α-hydroxydeoxyarteether and 9β-hydroxyarteether, **9**, pure 9β-hydroxyarteether $[\alpha]_D^{25°C}$ +177° (c 0.125 $CHCl_3$), 72 mg; and 14-hydroxyarteether, **8**, $[\alpha]_D^{25°C}$ +109.6 (c 0.12 $CHCl_3$), 110 mg.

Data on the ^{1}H and ^{13}C spectra of **2**, **3**, **8**, and **9** are summarized in Tables 1 and 2.

TABLE 2

Summary of Data on ^{13}C Assignments

Carbon	**2**[a]	**3**	**8**	**9**	**11**
1	52.8	42.5	47.0	44.2	44.6
2	24.7	30.4	24.2	24.5	24.5
3	36.6	69.7	36.3	36.3	36.5
4	104.0	107.8	104.0	104.1	104.1
5	87.9	93.6	87.8	87.6	87.2
6	81.2	84.2	81.2	80.4	80.8
7	44.7	40.8	44.4	42.0	37.0
8	24.6	25.0	24.2	33.5	31.7
9	34.8	34.8	28.7	74.1	69.9
10	37.6	34.8	44.4	50.1	40.6
11	31.0	30.4	30.8	30.5	30.2
12	101.7	99.8	101.6	101.6	101.9
13	13.1	12.3	12.9	12.9	12.9
14	20.4	18.8	64.8	15.2	16.5
15	26.3	21.0	26.1	26.1	26.2
16	63.8	64.0	63.8	64.0	63.8
17	15.1	15.2	15.2	15.2	15.2

[a] Assignment made by Hufford and ElSohly (*12*).

Experimental details for the oxidation of **8** to the corresponding aldehydes and the aldehyde subsequent reduction with sodium borodeuteride (or sodium borotritide) will be described elsewhere (*13*).

*Synthesis of 9-Oxoarteether, **10***

A mixture of **9** (5 mg), 4-methylmorpholine *N*-oxide (5 mg), and molecular sieves (5 mg) in dried methylene chloride (0.5 ml) was stirred for 15 min. A trace of tetra-*n*-propylammonium perruthenate was added and the disappearance of the starting material was followed by TLC. The reaction was complete after 45 min and the mixture was chromatographed on a short column of silica gel. The product was eluted with 30% ethyl acetate, 70% hexane. The eluate was concentrated to afford 9-oxoarteether (3 mg). Infrared ($CHCl_3$) 1710 cm^{-1}; CIMS *m/e* 344 ($M^+ + NH_4^+$).

*Preparation of 9α-Hydroxyarteether, **11***

A solution of 1 M potassium tri-*sec*-butylborohydride (K-Selectride) in THF (45 μl) was added to dry THF (0.6 ml) and the solution was cooled to −78°C. A solution of **10** (2 mg) in THF (0.6 ml) was added under N_2, and the cold solution was stirred for 2 h. The reaction mixture was then allowed to warm to room temperature and water (0.2 ml) and ethanol (0.3 ml) were added. After 10 min one drop of 2 N NaOH and three drops of 30% H_2O_2 were added and the solvent was removed *in vacuo*. The residue was treated with water and extracted into ethyl acetate. The ethyl acetate solution was concentrated and the product purified by preparative thin layer chromatography on silica gel using 20% ethyl acetate and 80% hexane. The band of R_f 0.34 was cut out and eluted with ethyl acetate to yield **11** (1.2 mg), $[\alpha]_D^{25°C}$ + 93.3° (c 0.06 $CHCl_3$), CIMS *m/z* 346 ($M^+ + NH_4^+$); ^1H and ^{13}C NMR data are summarized in Tables 1 and 2.

RESULTS

Although the earlier reports suggested that enzymes present in fungi catalyzed the rearrangement and destruction of peroxides, we nevertheless were fascinated by the idea of employing a fungus to introduce hydroxyl groups on an artemisinin derivative. Johnson (*7*) found that the fungus *Sporotrichum sulfurescens* hydroxylated methyl and methylene groups in a variety of amides. The same fungus renamed *B. sulfurescens* was later employed by Frustoss *et al.* (*8*) to oxidize a variety of bornyl, adamantyl, and norbornanyl amides. As the best substrates appeared to be amides we prepared the *N*-phenyl urethane of dihydroartemisinin, **6**, and investigated the action of the enzymes present in *B. sulfurescens* on this substrate (*9*). One metabolite was isolated in low yield; its molecular weight was shown by mass spectrometry (^{252}Cf plasma desorption) to be 442 D ($M^+ + Na^+$), corresponding to addition of 16 atomic mass units to the substrate. Examination of the ^1H NMR and ^{13}C DEPT spectra revealed that one of two secondary methyl groups on methine carbons had been converted into a hydroxymethyl group. The unoxidized methyl was identified by an ^1H COSY experiment which showed the proton on C-12 ($\delta = 5.78$) was coupled to the C-11 proton ($\delta = 2.6$) and this in turn was coupled to the C-13 methyl ($\delta = 0.94$). Therefore, C-14 must have been oxidized.

For the biotransformation to be of practical value, it was critical to increase the yield. In addition to investigating methods of optimizing the concentration of the enzyme involved, we also investigated the biotransformations of other dihydroartemisinin derivatives. When arteether, **2**, was employed as a substrate three products were isolated in 50% yield. They were separated and the ^1H NMR spectrum of the least polar metabolite showed it still contained all its methyl groups; however, a new resonance appeared at $\delta = 3.56$ consistent with formation of a new secondary alcohol. Its molecular weight was identical to that of the starting material. That information and the ^{13}C NMR spectrum were consistent with 3α-hydroxydeoxyarteether, **3**, a compound reported by Hufford *et al.* (*5*) in their studies of the microbial metabolism of **2**. We verified the previous stereochemical assignment of the hydroxyl group as α by determining all the ^1H coupling constants in the molecule (Table 1).

The ^1H NMR spectrum of the second metabolite, **8**, showed signals for only one methyl on a methine carbon and its ^{13}C DEPT spectrum indicated that one methyl had been converted into a hydroxymethyl group. The molecular weight of this metabolite was 16 Da greater than that of **2**. A ^1H COSY spectrum showed the proton on C-11 was coupled to both the protons on C-12 and C-13. Hydroxylation must have occurred on the C-14 methyl group.

The mass spectrum (CIMS) of the third metabolite, **9**, showed a molecular weight of 346 corresponding to the molecular weight of the starting material + NH_4^+ + 16 Da. Its ^1H NMR and ^{13}C spectra showed all methyl groups were still present and showed a new resonance at $\delta = 3.11$, indicating the presence of a new secondary alcohol. The identity of the methylene group was determined by ^1H COSY and HMQC spectra as C-9, and the stereochemistry of the hydroxyl group as β from an analysis of the proton chemical shifts and coupling constants (Table 1).

Our success in preparing two hydroxylated metabolites of **2** with intact peroxide groups in respectable yield prompted an investigation of their use in the preparation of other possibly useful compounds. One reaction sequence (*13*) was developed to introduce deuterium or tritium atoms on C-14 of **2**, a metabolically stable position, to help identify late metabolites of arteether (*4*). The hydroxymethyl group present in **8** was oxidized to the aldehyde, a potentially valuable intermediate in its own right, by treating it with catalytic quantities of tetra-*n*-propylammonium perruthenate and *N*-methylmorpholine *N*-oxide in methylene chloride. A single nonexchangeable deuterium (tritium) atom was introduced on C-14 by reducing the aldehyde with sodium borodeuteride (sodium borotritide) to form **8** containing either a deuterium or a tritium label. A sample of the unlabeled alcohol, **8**, was converted into its tosylate, which was reductively cleaved with sodium borodeuteride in DMSO to **2** labeled with a deuteron. If a sample of **2** of high specific activity were needed, use of both reactions would yield a sample of **2** containing either two deuterons or tritium atoms. Hutchins *et al.* (*11*) had shown that sodium borodeuteride in DMSO enhanced the rate of an S_N2 displacement by deuteride of tosylate, thus avoiding the use lithium aluminium hydride which could reduce the peroxide group.

9β-Hydroxyarteether was used to prepare 9-oxoarteether, **10**, and 9α-hydroxyarteether, **11**. Oxidation of 9-β-hydroxyarteether with tetra-*n*-propylammonium perruthenate and *N*-methylmorpholine *N*-oxide yielded **10**, a potentially useful intermediate. Stereoselective reduction of the carbonyl group in **10** with K-Selectride proceeded cleanly to yield **11**. The R_f values (on TLC) of the two alcohols differed as did their ^1H NMR spectra. An analysis of the ^1H chemical shifts and coupling constants of **11** established its stereochemistry and supported as well the one assigned to **9**. The ^1H and ^{13}C NMR data for these compounds in $CDCl_3$ are given in Tables 1 and 2. The antimalarial properties of these compounds are under investigation.

SUMMARY

The goal of employing microbially mediated oxidations to introduce functional (hydroxyl) groups on unactivated methyl and methylene groups has been realized for **2** and **6**. The metabolites were employed to generate potentially useful intermediates as well as to prepare an epimeric alcohol of one metabolite.

ACKNOWLEDGMENT

This research was supported in part by a grant from UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases.

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