Microbial Hydroxylation of a Dihydroartemisinin Derivative

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The fungus *Beauveria sulfurescens* has been employed to convert the 14-methyl group of the phenylcarbamoyl of dihydroartemisinin to the corresponding 14-hydroxymethyl derivative; the structure of the product was established by mass and 2D NMR spectroscopy.

The formidable problems encountered in developing a malaria vaccine[1] and the ability of *Plasmodium falciparum* to become resistant to new drugs[2] have stimulated interest in using combinations of drugs for treating this disease, which claims more than a million lives a year.[3] One drug of current interest for which *P. falciparum* has not developed resistance is artemisinin **1** (qing hao su).[4] First isolated by Chinese investigators[5] from a traditional medicinal herb, *Artemisia annua L.*, it was also isolated from Artemisia species in the United States.[6] In order to prepare new and novel derivatives of **1**, the fungus *Beauveria sulfurescens* was employed in an effort to introduce hydroxy groups onto unactivated methyl or methylene groups.[8]

Earlier studies with *B. sulfurescens* found that very poor substrates could be converted into acceptable ones by introducing an amide group which appears to enhance bonding between substrate and the hydroxylating enzyme complex. The substrate chosen, **3** (a derivative of dihydroartemisinin prepared by the procedure of Brossi *et al.*[9]), when incubated with *B. sulfurescens*, yielded a hydroxylated derivative **4**, which retained the peroxide grouping required for biological activity. Its structure was established by mass spectroscopic, and ^1H and ^{13}C NMR studies.

Earlier studies by Hufford *et al.*[10] on the action of a number of fungi on **1** and **5** identified several transformation products in which the peroxide bridge had been destroyed. Our selection of *B. sulfurescens* was based on reports that the fungus accepted a variety of substrates[11] and on our own studies with *p*-alkyl-*N*-acetylaniline. The fungus was grown in three litre flasks each containing 250 ml of media, prepared from 20 g of dextrose and 10 of neopeptone per litre, for two days. The mycelia were then separated and the contents of each flask resuspended in 200 ml of phosphate buffer (0.1 mol l^{-1}), pH 7.5, and **3** was added (3 × 100 mg in 0.8 ml of dimethylformamide). The mixture was shaken for 3 days, the mycelia and buffer were extracted with ethyl acetate and a UV-absorbing transformation product was isolated by flash chromatography on silica gel, with ethyl acetate–hexane (40:60) as eluent. Further purification by HPLC using the same solvent system yielded **4** $\{[\alpha]_D^{25} -22.8°$ (*c* 0.1, MeOH)$\}$ in 3.4% yield. Its mass spectrum (^{252}Cf plasma desorption) showed a peak at *m/z* 442, $(M + Na)^+$, corresponding to

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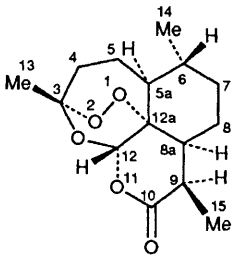

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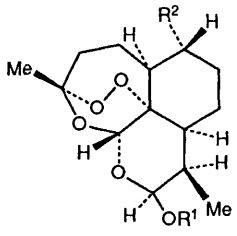
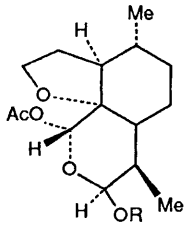


2 R^1 = H, R^2 = Me
3 R^1 = CONHPh, R^2 = Me,
4 R^1 = CONHPh, R^2 = CH_2OH
5 R^1 = Et, R^2 = Me



6a R = CONHPh
6b R = Et

Table 1 Summary of ^{13}C chemical shift assignments

Carbon	**1** δ	**3** δ	**4** δ	**6a** δ	**6b** δ
3	105.22	104.55	104.53	168.46	169.3
4	35.77	36.33	36.22	68.76	68.6
5	24.79	24.66	24.19	27.6	27.8
5a	49.90	51.63	45.96[a]	54.99	55.7
6	37.42	37.35	44.29[a]	33.95	30.6
7	33.45	34.19	28.36	35.46	35.9
8	23.32	22.02	21.73	22.59	24.7
8a	44.80	45.41	45.22	47.40	47.1
9	32.78	31.85	31.89	30.42	33.3
10	171.92	93.08	93.10	94.51	101.7
12	93.62	91.55	91.40	90.98	88.4
12a	79.38	80.20	80.27	80.07	80.6
13	25.10	25.83	25.90	21.39	21.6
14	19.74	20.24	64.64	20.45	20.5
15	12.47	12.15	12.20	12.01	12.5
1′		137.70	137.51	137.44	
2′,6′		118.88	118.84	118.94	
3′,5′		128.91	129.03	129.02	
4′		123.47	123.71	123.75	
C–O		152.05	151.89	151.60	

[a] May be interchanged.

addition of 16 mass units, consistent with the insertion of an oxygen atom. The ^1H spectrum showed the presence of only two methyl groups with a new multiplet (2H) at δ 3.48. One of the methyl groups, the singlet at δ 1.4, corresponds to the C-13 methyl. The doublet at δ 0.94 was assigned to the C-15 methyl by a multiple quantum filtered COSY spectrum, which showed that the multiplet at δ 2.6 (H-9) is coupled both to the doublet at δ 0.94 and to that at δ 5.78 (H-10). The methyl at C-6 had therefore been converted into a hydroxymethyl group. A DEPT experiment and heteronuclear correlation spectrum confirmed that the methylene carbon at δ 64.6 bears the protons at δ 3.48. The presence of the peroxide group, implied by the molecular weight, was deduced from a comparison of the ^{13}C chemical shifts for the quaternary carbons (δ 104.5 and 80.2) at the terminus of the peroxide bridge, in **3** and **4**. The relevant chemical shifts (Table 1) were essentially identical, establishing the continued presence of the group.

Since a significant amount of starting material was recovered using the above reaction conditions, a number of small-scale experiments were conducted in an effort to increase the yield in the biotransformation. It was found that if the mycelia obtained from 250 ml of media were resuspended in 100 ml of buffer and reacted with 20 mg of the substrate, the yield of the biotransformation increased to 15%. The role the media plays in stimulating the production of high levels of the hydroxylating enzyme complex was also examined. *B. sulfurescens* was grown on corn steep liquors as described by Johnson.[11] The mycelia was isolated, resuspended in phosphate buffer at pH 7.5, and **3** was added. After three days the reaction mixture was worked up as described above to yield a different metabolite **6a**, with a molecular weight which indicated that it was isomeric with the starting material. Its structure was assigned by ^1H and ^{13}C NMR spectroscopy. Hufford *et al.*[10b] reported the formation of a similar metabolite, **6b**, from **5**. The ^{13}C chemical shifts of **1, 3, 4, 6a** and **6b** and their assignments are given in Table 1.

The ability of the fungus *B. sulfurescens* to hydroxylate the C-14 methyl of artemisinin derivatives has enabled us to employ the reaction product obtained from arteether, the ethyl ether of dihydroartemisinin, in conjunction with conventional chemistry to incorporate one or two deuterium (tritium) atoms on the C-14 methyl group of arteether.[11] Since very little of a label at this position is removed during the drug's metabolism, labelled arteether may be useful in a variety of biological studies. That work will be published elsewhere. Derivatives of these hydroxylated dihydroartemisinin derivatives will be prepared for testing as antimalarial agents. Preliminary results of the antimalarial activity of **4** support the conclusion of earlier structure–activity studies that the intact peroxide bridge is important for antimalarial activity.

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