Tetrahedron Letters,Vol.25,No.1,pp 91 - 94,1984 0040-4039/84 $3.00 + .00
Printed in Great Britain

ELECTROOXIDATIVE SIMULATION OF STEREOSELECTIVITY IN MICROSOMAL ALLYLIC HYDROXYLATION[1]

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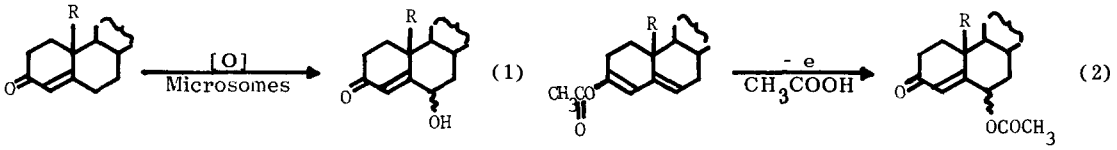
A comparison of the stereochemistry of liver microsomal γ-hydroxylation of some cyclic α,β-unsaturated ketones with that of electrochemical γ-acetoxylation of the corresponding dienol esters and with that of peracid oxidation of the dienol esters has been carried out.

One of the important processes in metabolism is oxidation catalyzed by the cytochrome P-450 monooxygenase system. Secondary and tertiary amines and amides are oxidized to give N-dealkylated amines and amides,[2] while the oxidation at saturated aliphatic carbon atoms leads to the formation of hydroxylated metabolites.[3]

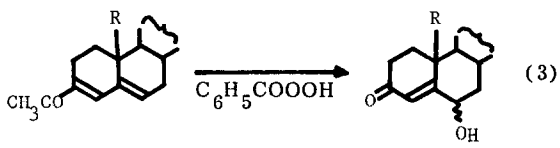
We have suggested previously that an electron transfer process is involved in the liver microsomal N-dealkylation of amines and amides[4,5] and electrooxidative method is one of the best tools to simulate the microsomal N-dealkylation.[4,6]

On the other hand, microsomal hydroxylation at aliphatic carbons has been suggested to proceed with homolytic hydrogen abstraction by an iron-oxygen species.[7] In a number of studies on the stereochemistry of enzymatic hydroxylation at primary,[8] secondary,[9] and tertiary carbon atoms,[10] the configuration of hydroxylated products has been discussed, whereas the mechanism controlling stereoselectivity of hydroxylation still remains obscure.[11]

Previously, we have shown that anodic allylic acetoxylation proceeds with a remarkable stereoselectivity.[12] Since both the electrode reactions and the enzymatic reactions have similar heterogeneous characters, we compared in the present study the stereoselectivity of liver microsomal γ-hydroxylation of cyclic α,β-unsaturated ketones (eq.1) with that of electrochemical γ-acetoxylation of the corresponding dienol esters (eq.2) and with that of peracid oxidation (γ-hydroxylation) of the dienol esters (eq.3) to find an efficient method to simulate the metabolism of steroidal α,β-unsaturated ketones catalyzed by the cytochrome P-450 monooxygenase system, and also to get a stereochemical insight into the cytochrome P-450 catalyzed hydroxylation.



The substrates used in the present study were Δ^4-3-ketosteroids (**4**-**7**)[13] and their analogs (**1**-**3**)[14] shown in Table 1. The microsomal hydroxylation of the cyclic α,β-unsaturated ketones (**1**-**7**) was carried out with incubation of the substrates with rat liver microsomes[15] prepared under conventional centrifugal conditions.[16] The incubation mixture contained 4 mM glucose 6-phosphate, 0.5 mM NADPH, 5 mM $MgCl_2$, 0.5 unit/ml of glucose 6-phosphate dehydrogenase, 0.1 M Tris hydrochloride buffer (pH 7.4), microsomal suspension containing 3.0 mg of protein/ml, and substrate in the concentration of 50 μM (**1**, **2**, and **3**) or 1 μM (^{3}H-labeled **4**, **5**, **6**, and ^{14}C-labeled **7**). After the mixtures were incubated for 10 min at 37°C, the reaction was terminated by the addition of methanol. The anodic oxidation of dienol acetates[17] was carried out at room temperature under conditions of controlled potential at 2.0 V *vs.* SCE with a platinum electrode. After 2 *F*/mol of electricity was passed through the solution of 0.5 mM dienol acetate and 10 mM potassium acetate as a supporting electrolyte in acetic acid, the reaction mixture was poured into sodium bicarbonate solution and the products were extracted with ether. The enol acetates were also oxidized chemically with perbenzoic acid in refluxing ether for 16 h to compare the stereochemistry with microsomal and anodic oxidations.[18] The ethereal solution was washed with sodium bicarbonate, dried and evaporated. γ-Hydroxylated metabolites produced by microsomal oxidation, γ-acetoxylated products obtained by anodic oxidation and γ-hydroxylated products obtained by chemical oxidation were detected by comparative TLC[19] and HPLC[20] with the reference compounds[21] and the isomer ratios of γ-substituted products were determined by HPLC.



(3)

The following results are shown in Table 1.

(1) With respect to all the compounds examined, microsomal γ-hydroxylation and anodic γ-acetoxylation gave β-isomers almost exclusively.

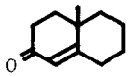
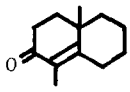
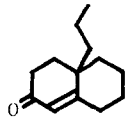
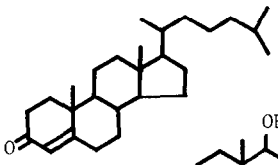
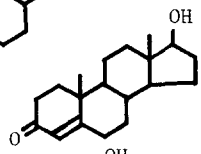
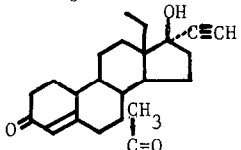
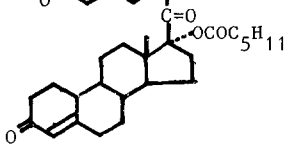
(2) Good accordance of the stereoselectivity between microsomal γ-hydroxylation and anodic γ-acetoxylation was observed.

(3) Comparing with peracid oxidation, a much greater stereoselectivity was observed in the electrochemical oxidation.

These results strongly suggest that the present electrooxidative method is the most effective to simulate the metabolism of some steroidal α,β-unsaturated ketones catalyzed by liver cytochrome P-450 monooxygenase system.

In a number of studies, the microsomal hydroxylation at C-6 position of steroids has been reported to proceed with β-orientation[22] as same as the present study. The stereoselectivity of hydroxylation may be determined either at the step of formation of cytochrome P-450-substrate complex or at some step after the complex is formed. On the other hand, we have proposed previously that the initial active species in the anodic allylic substitution reaction is a cation radical formed from one-electron oxidation of the substrate.[12] Compared with chemical allylic benzoyloxylation known as the Kharasch-Sosnovsky reaction (*cis*/*trans*=0.2-0.3), a remarkable *cis*-selectivity observed in the anodic allylic acetoxylation of methylcyclohexenes (*cis*/*trans*=2.8-3.4) has been explained by a selective adsorption of methylcyclohexenes to the anode from the less sterically

Table 1. Stereoselectivity in Microsomal Hydroxylation, Electrooxidative Acetoxylation and Chemical Oxidation

Substrate		Product Ratio (β-isomer/α-isomer)[a]		
		Microsomal Method	Electrooxidative Method	Chemical Method
	**1**	14.1 ± 1.2	13.9 ± 0.9	3.0 ± 1.6
	**2**	7.3 ± 1.6	7.2 ± 0.4	3.2 ± 0.8
	**3**	13.5 ± 2.1	13.0 ± 0.8	2.9 ± 0.6
	**4**	11.8 ± 2.1	12.3 ± 0.9	2.8 ± 1.1
	**5**	12.1 ± 2.6	13.3 ± 1.7	3.9 ± 0.7
	**6**	8.4 ± 2.7	10.3 ± 1.1	2.9 ± 1.0
	**7**	10.8 ± 1.9	9.0 ± 1.8	3.5 ± 0.5

[a]Anodic oxidation, peracid oxidation and the incubation of substrates with liver microsomes of a rat were carried out three times for each substrates. The isomer ratios (β-isomer/α-isomer) of γ-substituted products were given as means ± SD.

hindered side, namely the reverse side with respect to the methyl group, followed by the introduction of an acetoxyl group to the allylic position from the side of solution, that is, the side of the methyl group to give predominantly the *cis*-isomer of allylically acetoxylated methylcyclohexenes. Since the anodic γ-acetoxylation of dienol acetates has been proposed to proceed through the similar mechanism,[23] the β-oriented stereoselectivity observed in the present study suggests the similar steric interaction of electrode-substrate-nucleophile. Thus, the good accordance of the stereoselectivity between microsomal γ-hydroxylation and anodic γ-acetoxylation shown in Table 1 may

suggest that the similar stereoselective approach of the substrates to the surface of cytochrome P-450 enzyme is involved in the stereoselectivity determining step of the microsomal γ-hydroxylation of steroidal α,β-unsaturated ketones, while such a control of stereochemistry is not expected in the chemical oxidation in a homogeneous solution using peracid.

Acknowledgment. One of the authors (T.S.) wishes to thank the Ministry of Education, Science, and Culture, Japan, for the Grant-in-Aid for Special Project Research (1) (No. 521313, 56109011, and 57102010).

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5. As for the electron transfer mechanism of the cytochrome P-450 catalyzed oxidation of amines, see also: (a) R.B. Silverman, S.J. Hoffman, and W.B. Catus, III, *J. Am. Chem. Soc.*, **102**, 7126 (1980); (b) P. Shannon and T.C. Bruice, *ibid.*, **103**, 4580 (1981).
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10. S. Burstein and M. Gut, *Adv. Lipid Res.*, **9**, 291 (1971).
11. C.E. Castro, "Cytochrome P-450," ed by R. Sato, T. Omura, Academic Press, New York (1978), p. 547.
12. T. Shono and A. Ikeda, *J. Am. Chem. Soc.*, **94**, 7892 (1972).
13. ^{3}H- And ^{14}C-labeled and unlabeled substances were supplied from Schering AG (Berlin, GFR).
14. The analogs of Δ^{4}-3-ketosteroids were prepared from the reaction of 2-substituted cyclohexanone with corresponding vinyl ketone.
15. Liver microsomal fractions were prepared from the liver homogenates of male Sprague-Dawley rats. Microsomal protein was determined by the method of Lowry.
16. P. Mazel, "Fundamentals of Drug Metabolism and Drug Disposition," ed by B.N. La Du, H.G. Mandel, E.L. Way, Williams & Wilkins, Baltimore (1971), p. 527.
17. Dienol acetates were prepared from the corresponding α,β-unsaturated ketones.
18. The peracid oxidation of dienol ether prepared from a cyclic α,β-unsaturated ketone has been described to give γ-hydroxylated products: H.L. Holland and B.L. Auret, *Can. J. Chem.*, **53**, 2041 (1975).
19. TLC was carried out on silica gel plates with a solvent system of ethyl acetate-hexane and visualized under UV (254 nm). Rs values varied with the substrates are as follows: dienol acetates; α,β-unsaturated ketones; 1.0; β-isomer of γ-acetoxy derivative; 1.1-0.9, α-isomer of γ-acetoxy derivative; 0.9-0.8, β-isomer of γ-hydroxy derivative; 0.5-0.4, α-isomer of γ-hydroxy derivative; 0.5-0.3.
20. HPLC was monitored with UV absorbance at 235 nm or with measurements of radioactivity of effluents collected at 0.5 min intervals, with a mobile phase containing 0.5-1% methanol in dichloromethane by using a Zorbax SIL column. The retention volumes of β-isomers were always smaller than those of α-isomers.
21. The reference compounds for microsomal γ-hydroxylation were prepared in large scale by using isolated perfused rat livers: T. Toda and N. Oshino, *Drug Metab. Dispos.*, **9**, 108 (1981). The allylic hydroxylated metabolites in the perfusate were separated by TLC. NMR ($CDCl_3$) for γ-hydroxylated derivatives of **1-7** (δ-values varied somewhat with the substrates); β-isomer, δ 5.80-5.87 (s, 1, α-CH), 4.32-4.39 (t, 1, γ-CH) and α-isomer, δ 6.18-6.20 (d, 1, α-CH), 4.10-4.40 (m, 1, γ-CH). The reference compounds for anodic γ-acetoxylation were obtained by preparative electrolysis of dienol acetates. NMR ($CDCl_3$) for γ-acetoxylated derivatives of **1-7**; δ 5.90-5.97 (s, 1, α-CH), 5.40-5.45 (t, 1, γ-CH), 2.04-2.06 (s, 3, acetate).
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23. The initiation process of this anodic oxidation has been proposed to be the electron transfer from dienol esters to anode yielding cationic species: T. Shono, I. Nishiguchi, S. Kashimura, and M. Okawa, *Bull. Chem. Soc. Jpn.*, **51**, 2181 (1978).

(Received in Japan 29 September 1983)