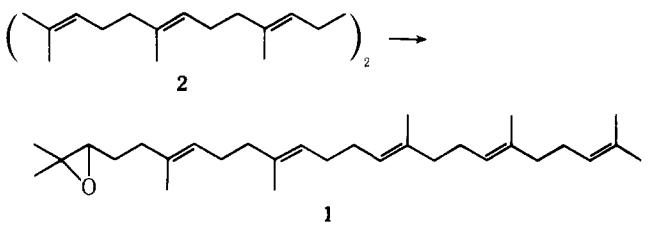
Enzymic Epoxidation of Squalene Variants

Sir:

Although the action of squalene 2,3-oxide cyclase on structural modifications of the normal substrate (**1**) has been broadly surveyed,[1] very little is known about the effect of squalene epoxidase on squalene (**2**) variants.[2] The present study reveals that (a), as in the cyclase series,[3] certain types of structural deviations, e.g., the absence of several π-bonds, do not preclude enzyme action, (b) highly selective terminal attack always occurs in successful cases, and (c) an unnatural substance must approximate the size and shape of squalene. However, in contrast to epoxide cyclizations, where little modification in the heterocyclic area is tolerated,[4] considerable variation at the epoxidation site in squalene-like systems is permitted, with little effect on product yields.



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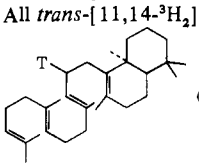
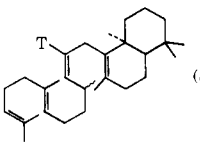
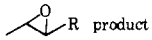
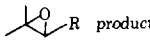
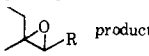
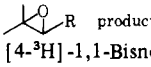
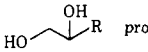
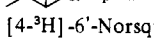
Enzyme was prepared by homogenizing Sprague-Dawley rat liver tissue in twice its weight of 0.08 *M* phosphate buffer (pH 7.43) then centrifuging for 10 min at 9000 × gravity. Such amounts of cofactors were added to the supernatant as to provide a solution 0.00167 *M* in glucose 6-phosphate, 0.00067 *M* in NADP, 0.03 *M* in nicotinamide, and 0.004 *M* in $MgCl_2$. All manipulations were carried out at ≤4°. Incubations were carried out by mixing 5 ml of enzyme solution, 2 IU of glucose 6-phosphate dehydrogenase, and a Tween 80 suspension of 25 μg of cyclase inhibitor squalene-2,3-imine[5] and incubating at 37° for 5 min in a Dubnoff shaker. Radiolabeled substrate, solubilized in H_2O with six times its weight of Tween 80, was then added, and the incubation continued for 90 min. After quenching by addition of 5 ml of 1 *N* KOH-MeOH, each incubation mixture was worked up by extracting exhaustively with ether. Small aliquots of the extracts were removed for measuring recovery of radioactivity, and the remainder was thin-layer chromatographed. Bands corresponding to products (bisepoxides, monoepoxides, and corresponding glycols)[6] and starting materials were usually scraped directly into scintillation vials and counted. Products were identified by TLC (silica gel) comparison, using at least two solvent systems, with authentic samples and usually by periodate cleavage of epoxide-derived glycols to aldehydes, each of which in turn was compared with a specimen of known structure. As indicated by the squalene and other cases, chromatographic methods (e.g., silica gel-ethyl acetate-petroleum ether on derived glycol) are sensitive enough to differentiate between terminal and internal oxidation products. Total epoxide yields were calculated according to the formula

Table I. Enzymic Terminal Epoxidation Aptitudes of Olefins

No.	Substrate	No. of trials	Relative epoxide yield	% Average recovery of radioactivity[b]
1	[4-^{3}H] Squalene		1.00	76.1
2[2b]	[2,4-$^3H_2$] 2,3-Dihydrosqualene	6	0.25	71.2
3[a,8]	All *trans*-[9,10',11,14,15',16-$^3H_4$] 6,7,18,19-tetrahydrosqualene	9	0.32–0.57	72.0
4[a,9]	All *trans*-[11,14-$^3H_2$] 10,11,14,15-tetrahydrosqualene	6	0.10–0.22	77.7
5[10]	 (*dl*)	3	0.02	77.0
6[10]	 (*dl*)	3	<0.05	76.7
7[11]	[4-^{3}H] Bigeranyl (cis–trans mixture)	6	<0.03	9.0 (35.7)[c]
8[4]	[4-^{3}H]-1-Norsqualene (cis:trans ~ 1:3)	6		62.7
	 product + glycol		0.52	
	 product		0.46	
9[12]	[4-^{3}H]-1-Methylsqualene (cis:trans ~ 1:3)	3		82.6
	 product		0.11	
	 product		0.56	
10[4]	[4-^{3}H]-1,1-Bisnorsqualene	6		69.5
	 product		0.50	
	 product		0.40	
11[1]	[4-^{3}H]-6'-Norsqualene (product isomers not separated)	3	0.72	68.6
12	[4-^{3}H]-2-Methyltetradec-2-ene	6	<0.03	7.2 (34.8)[c]
13	[9,10'-$^3H_2$]-2,6,10-Trimethyldodeca-2,6-diene (*dl*)	9	<0.03	5.7 (30.0)[c]

[a] Mixture of racemates. [b] Except for cases 7, 12, and 13, radioactivity recovery reflects only starting hydrocarbon and identified oxidation product(s). [c] Radioactivity extractable only after acidification of enzyme mixture.

$$\% \text{ yield} = \frac{\text{dpm glycol} + 2(\text{dpm bisepoxide}) + \text{dpm monoepoxide}}{\text{total dpm}} \times 100$$

Table I summarizes the yield data, compared to squalene (1.00). All substrates had an initial concentration of 8.2×10^{-5} *M*, and concurrent squalene controls and boiled controls were always run. The usual yield of squalene oxide was 70–85%. Time vs. yield studies showed that all epoxidase activity was lost by the end of the normal 90-min incubations. That product does indeed arise by squalene oxidase action, and not by that of some other liver enzyme, is indicated by the efficient inhibition[7] by squalene in two exemplary cases: in the presence of two-fold excess of squalene, oxidation of either polyene **3** or **4** is >80% inhibited. In general, while hydrocarbons in the C_{28}–C_{31} range underwent exclusive terminal epoxidation, terpenoids in the C_{15-20} range suffered extensive oxidative degradation, resulting in much lower recovery of radioactivity. In one set of structures (**2–7**), the isopropylidine unit was maintained as the epoxidation site while the remainder of the molecule was varied with respect to general size, number of double bonds, and/or presence of rings. Compared to squalene, the absence of one or two double bonds (**2, 3,** and **4**) does not affect markedly the oxidation rate. However, incorporation of bicyclic moieties as unreactive termina (**5** and **6**), or removal of two isoprenoid units (**7**), does result in distinctly lower or unobservable reactivity. In a second series of substrates (**8–10**) the alkyl substitution pattern on one terminal olefin unit was varied, thus allowing internally competitive rate determinations. Again, the product yields imply that the enzymic oxidation rates are not strongly influenced by either (1) the purely chemical, nucleophilic properties of the reactive double bonds or (2) steric factors due to methyl groups in the vicinity of the oxidation site.

With farnesyl methyl ether (FME), methyl *cis,trans*- and *trans,trans*-farnesate, oxidation by the rat liver enzyme system led to a low recovery of a mixture of products, of which 10,11-dihydroxyfarnesic acid (and/or methyl ester) was the only identifiable $\Delta^{10,11}$ oxidation product (2–4% yields). Similar preliminary results were obtained with 2,3- and 6,7-dihydro FME as well as 10,11-oxido- and 10,11-dihydroxy FME. Moreover, the absence of inhibition of the FME oxidation phenomena by even large excesses of squalene revealed that squalene epoxidase was probably not responsible for $\Delta^{10,11}$ oxidation, and therefore these sesquiterpenoid results must be regarded as negative in that sense. Also, no epoxide or glycol was produced from 2-methyltetradec-2-ene (**12**) or 2,6,10-trimethyldodeca-2,6-diene (**13**).

Previously, the abiological, highly selective terminal oxidation of squalene, farnesol, farnesic ester, and related terpenoids,[13] was taken to mean that positional control might not be an important factor in epoxidase action.[14] The results herein indicate that incorrectness of this surmise and suggest instead that, in spite of the apparent relative nonspecificity of the epoxidising moiety per se (Table I, **8–10**), comparatively strict overall substrate structural requirements must be met for successful epoxidase action (**5–7, 12, 13**) indicating rather specific binding of the entire substrate molecule.

Acknowledgment. The authors gratefully acknowledge financial support from the National Institutes of Health (Grant GM 10421).

References and Notes

(1) For the most recent contribution from this laboratory on the subject, see E. E. van Tamelen, J. A. Smaal, and R. B. Clayton, *J. Am. Chem. Soc.*, **93**, 5279 (1971).

(2) (a) E. J. Corey and W. E. Russey, *J. Am. Chem. Soc.*, **88**, 4751 (1966), have reported the enzymic conversion of all-*trans*-10,11-dihydrosqualene to a mixture of 2,3-oxide, 22,23-oxide, and 2,3:22,23-dioxide. (b) The biological conversion under aerobic conditions of 2,3-dihydrosqualene and 1,1′,2-trisnorsqualene to steroids with modified side chains, first observed by E. E. van Tamelen, K. B. Sharpless, J. D. Willett, R. B. Clayton, and A. L. Burlingame, *ibid.*, **89**, 3920 (1967), reveals successful oxidase action on these squalene variants.

(3) E. E. van Tamelen, XXIIIth International Congress of Pure and Applied Chemistry, **5**, 85 (1971).

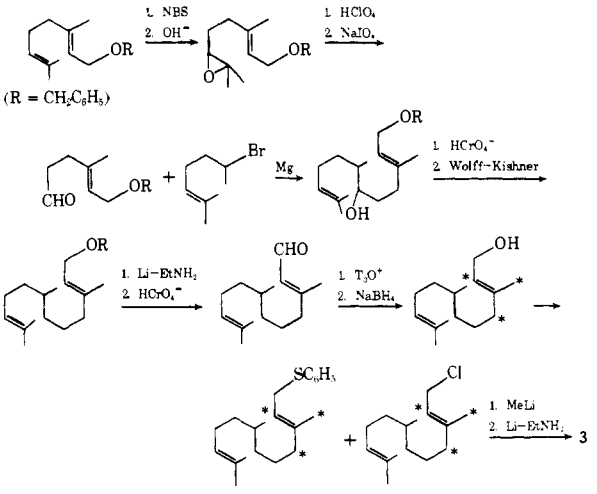
(4) R. B. Clayton, E. E. van Tamelen, and R. G. Nadeau, *J. Am. Chem. Soc.*, **90**, 820 (1968).

(5) (a) E. J. Corey, P. R. Ortiz de Montellano, K. Lin, and P. D. G. Dean, *J. Am. Chem. Soc.*, **89**, 2797 (1967); (b) L. Avruch and A. C. Oehlschlager, *Synthesis*, 622 (1973).

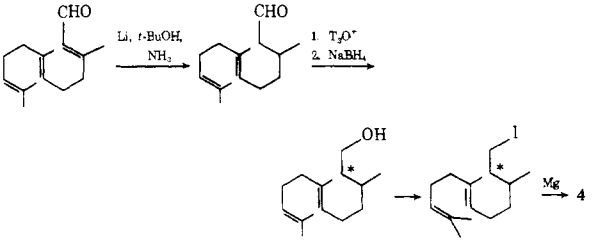
(6) Liver enzyme mediated hydration of 1,2-epoxides is well precedented. See for example, ref 4 and E. J. Corey, K. Lin, and M. Jautelat, *J. Am. Chem. Soc.*, **90**, 2724 (1968). For extensive studies see F. Oesch, D. M. Jerina, and J. W. Daly, *Arch. Biochem. Biophys.*, **144**, 253 (1971); F. Oesch, N. Kaubisch, D. M. Jerina, and J. W. Daly, *Biochemistry*, **10**, 4858 (1971).

(7) For studies of liver microsomal epoxidations, which undoubtedly are not due to squalene oxidase action, see (a) D. T. Wong and L. C. Terriere, *Biochem. Pharmacol.*, **14**, 375 (1965); (b) K. C. Leibman and E. Ortiz, *Mol. Pharmacol.*, **4**, 201 (1968); (c) T. Watabe and E. W. Maynett, *Pharmacologist*, **10**, 203 (1968); (d) K. C. Liebman and E. Ortiz, *J. Pharmacol. Exp. Ther.*, **173**, 242 (1970).

(8) Synthesis

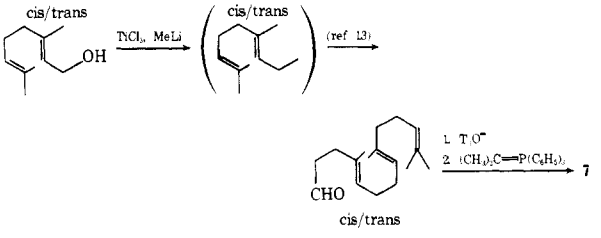


(9) Synthesis



(10) E. E. van Tamelen, R. A. Holton, R. E. Hopla, and W.-E. Konz, *J. Am. Chem. Soc.*, **94**, 8228 (1972). Samples kindly supplied by R. E. Hopla.

(11) Synthesis



(12) E. E. van Tamelen, R. B. Clayton, and O. Crosby, *Chem. Commun.*, 532 (1969).

(13) E. E. van Tamelen and T. J. Curphey, *Tetrahedron Lett.*, 121 (1962).

(14) E. E. van Tamelen and K. B. Sharpless, *Tetrahedron Lett.*, 2655 (1967).

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