The Stereochemistry of Enzymic Hydration and of Chemical Cleavage of D-(+)-*cis*-12,13-Epoxyoleic Acid (Vernolic Acid)

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ABSTRACT

The absolute optical configurations of (+)-*threo*-12,13-dihydroxyoleic acid, derived by enzymic hydration of endogenous vernolic acid in crushed *Vernonia anthelmintica* seed, and of (−)-*threo*-12,13-dihydroxyoleic acid, derived by acetolysis-hydrolysis of vernolic acid, have been determined. The absolute configuration of the (+)-enantiomer is L-12,D-13-dihydroxyoleic acid and, as the parent vernolic acid is known to be D-12,D-13-epoxyoleic acid, the stereochemistry of the enzymic hydration is thus shown to involve attack by hydroxyl at the 12 position with inversion at that position. Chemical cleavage of vernolic acid, on the other hand, involves preferential nucleophilic attack, with inversion, at the 13 position.

INTRODUCTION

VERNOLIC ACID ((+)-*cis*-12,13-epoxyoleic acid) has been shown to occur in the seed oils of numerous species of the plant families Compositae, Euphorbiaceae, Onagraceae, Valerianaceae and Dipsacaceae (1). The enantiomer of vernolic acid, namely (−)-*cis*-12,13-epoxyoleic acid, also occurs naturally in the seed oils of a number of the Malvaceae (2). In *Vernonia anthelmintica* seed there is an epoxy acid hydrating enzyme which, on incubation of the crushed seed under moist conditions, cleaves the endogenous (+)-vernolic acid to give optically pure (+)-*threo*-12,13-dihydroxyoleic acid (3,4).

The absolute optical configuration of (+)-vernolic acid was recently established (5) as being D, i.e., 12-*S*,13-*R* in the Cahn-Ingold-Prelog system (6). It was then reasoned that (−)-*threo*-12,13-dihydroxyoleic acid, derived as the predominant enantiomer by acetolysis-hydrolysis of (+)-vernolic acid, was D-12,L-13-dihydroxyoleic acid, i.e., 12-*S*,13-*S*. Consequently, the (+)-*threo*-dihydroxy acid produced by enzymic cleavage was considered to be the L-12,D-13-enantiomer, i.e., 12-*R*,13-*R* and it was suggested that the enzymic attack must be at the 12 position, resulting in inversion at that position.

This paper describes the direct determination of the absolute optical configurations of the (+)- and (−)-enantiomers of *threo*-12,13-dihydroxyoleic acid. The reactions involved in this determination are summarized in Figure 1. They consist of the production of pairs of positionally isomeric hydroxy,tosyloxyoleates from vernolic acid, by epoxide cleavage with toluene-*p*-sulphonic acid, and from (+)- or (−)-*threo*-12,13-dihydroxyoleic acid, by partial tosylation with toluene-*p*-sulphonyl chloride. The stereochemistry of the starting materials and of these reactions is such that the pair of isomers produced from vernolic acid must be D-12-hydroxy,L-13-tosyloxy- and L-12-tosyloxy,D-13-hydroxyoleic acids, whereas the pair produced from the *threo*-dihydroxy acid must be either D-12-hydroxy,L-13-tosyloxy- and D-12-tosyloxy, L-13-hydroxy-oleic acids or the enantiomeric pair. Thus, each of the positional isomers obtained from vernolic acid must be optically identical with the corresponding positional isomer from one of the *threo*-dihydroxy acids and enantiomeric with the like isomer from the other *threo*-dihydroxy acid. The absolute configurations of the two positional isomers derived from vernolic acid are known so that, if it can be determined which of these isomers is which, then the configurational problem is solved. Reaction of tosyloxy groups with $LiAlH_4$ or $LiBH_4$ in boiling tetrahydrofuran results in the hydrogenolysis of the tosyloxy group. When this reaction is applied to the individual hydroxy, tosyloxyoleate isomers the corresponding hydroxyoleyl alcohols are produced. These, then, can be characterized by mass spectrometry (7) after conversion to the corresponding ketostearates.

The results obtained by this procedure are in accord with our previous deductions (5) as to the mechanism and stereochemistry of chemical cleavage of vernolic acid and of the hydration of vernolic acid by the enzyme present in *Vernonia* seed.

EXPERIMENTAL PROCEDURES

Methyl D-(+)-*cis*-12,13-epoxyoleate (methyl vernolate) was isolated from the mixed esters derived from *V. anthelmintica* seed oil by adsorption column chromatography, as described previously (8). (+)-*threo*-12,13-Dihydroxyoleic acid was obtained by incu-

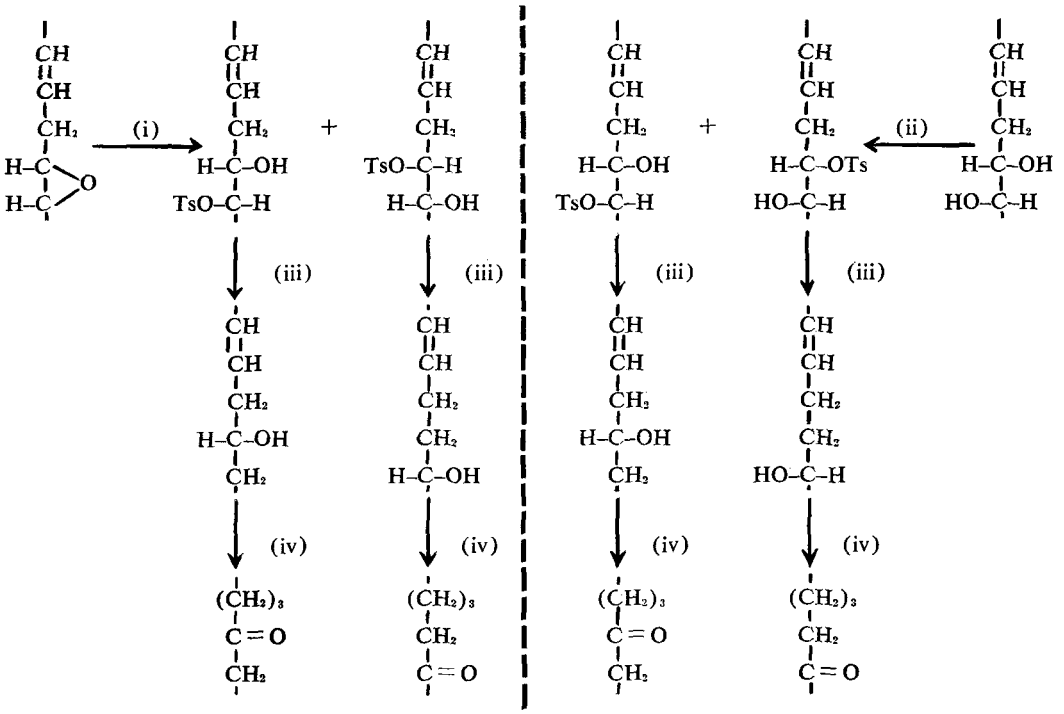

FIG. 1. Summary of reactions used in the determination of the absolute optical configuration of *threo*-12,13-dihydroxyoleate. Reactions are: (i) epoxide cleavage with toluene-*p*-sulphonic acid in diethyl ether; (ii) partial tosylation with toluene-*p*-sulphonyl chloride in pyridine; (iii) reduction with $LiAlH_4$ in tetrahydrofuran; (iv) catalytic reduction followed by oxidation with chromium trioxide in acetic acid.

bation of wetted, ground *V. anthelmintica* seed for three days at 25 C, in an atmosphere of nitrogen saturated with water (3). Total lipids were then extracted with chloroform–methanol (2:1), recovered, dissolved in ether, and free fatty acids were extracted into 10% aqueous KOH. The crude free acid fraction was recovered, esterified with diazomethane and fractionated by column chromatography on silica gel. The pure methyl *threo*–12,13–dihydroxyoleate, eluted with 30% ether in light petroleum, had $[\alpha]_{546.1\ m\mu}^{27°} = +20.3°$ (c = 1.0% in EtOH) and was evidently optically pure (2,3,9).

(–)–*threo*–12,13–Dihydroxyoleic acid was obtained from methyl vernolate by acetolysis with hot acetic acid followed by hydrolysis with 10% methanolic KOH, essentially as described by Gunstone (10). After two crystallizations from acetone, the chemically pure dihydroxyoleic acid was esterified with diazomethane and the methyl ester had $[\alpha]_{546.1\ m\mu}^{27°} = -7.6°$ (c = 2.5% in EtOH). Further recrystallization of the acid to obtain the pure optical enantiomer ($[\alpha]_D = -19°$), as described by Hopkins and Chisholm (2,9) was not attempted and this partially racemic ester was used for the subsequent work.

Preparation of Methyl Threo-12(13),13(12)-Hydroxy,tosyloxyoleates

Methyl vernolate (1.0/g) was dissolved in anhydrous diethyl ether (5 ml) and added to a solution of toluene–*p*–sulphonic acid (0.78/g) in anhydrous ether (15 ml). The reaction mixture was shaken for 30 min, allowed to stand at room temperature overnight, diluted with ether, washed twice with 10% aqueous KOH and then with water to neutrality. The solution was dried and evaporated to yield 940 mg (63%) of a pale yellow oil, which consisted almost entirely of hydroxy,tosyloxyoleates, as judged by TLC.

Methyl (+)– or (–)– *threo*–12,13–dihydroxyoleate (240 mg) was dissolved in pyridine (1ml) and added to a solution of toluene–*p*–sulphonyl chloride (143 mg) in pyridine (1 ml); the mixture was shaken for 30 min and allowed to stand at room temperature overnight. The mixture was diluted with water, extracted with diethyl ether and the extract

TABLE I

Characteristics of Products From Methyl Vernolate and (+)- and (−)-*Threo*-12,13-Dihydroxyoleates

Starting material	(+)-vernolate		(+)-*threo*-diOH-oleate		(−)-*threo*-diOH oleate	
[α] 546.1mμ	+5.4°		+20.3°		−7.6°	
Hydroxy, tosyloxyoleate	upper	lower	upper	lower	upper	lower
[α] 546.1mμ	−6.5°	−18.8°	−8.0°	+15.6°	+2.1°	−5.8°
Hydroxyoctadecenol						
[α] 546.1mμ	+1.6°	+2.4°			−0.3°	+0.8°
Ketostearate isomer	13	12			13	12

All optical rotations were measured in ethanol except that of methyl vernolate, which was in chloroform. The specific rotations of the partially racemic (−)-dihydroxyoleate and its products were all approximately one third of the specific rotations of the (+)-dihydroxyoleate and the products from it or from methyl vernolate, or both.

The terms "upper" and "lower" refer to the positionally isomeric hydroxy,tosyloxyoleates having, respectively, greater and lesser mobility on argentation TLC, as described in the text, and to the hydroxyoctadecenol and ketostearate products derived from them.

was washed twice each with water, dilute HCl, water, dilute aqueous KOH and finally with water to neutrality. The ether solution was dried and evaporated to yield ca. 300 mg of a pale yellow oil which, according to TLC, contained ca. 60% of hydroxy,tosyloxyoleate and also ditosyloxyoleate and unchanged dihydroxyoleate. The hydroxy,tosyloxyoleate fraction was isolated by preparative TLC.

Separation of Hydroxy, Tosyloxyoleate Positional Isomers

On normal unimpregnated silica gel layers, no appreciable separation of the two positionally isomeric *threo*-12(13),13(12)-hydroxy, tosyloxyoleates could be achieved, even on multiple development. However, on silica gel impregnated with silver nitrate there was clear separation of the mixture into two bands, after double development with diethyl ether–light petroleum (1:1). As in the case of cleavage with hydrogen halides (5), the two positional isomers derived from methyl vernolate were not formed in equal amounts and the less mobile isomer on argentation–TLC was the major component (60–70%). The two positional isomers derived from dihydroxyoleate were in approximately equal amounts.

The individual isomers from each product mixture were isolated by preparative argentation TLC, approximately 30 mg of mixture being separated on each 200 × 200 × 1 mm layer. The plates were developed twice with ether-light petroleum (1:1), the separated components were located under ultraviolet light after spraying with dichlorofluorescein and were eluted from the scraped-off adsorbent with pure diethyl ether. The separated positional isomers were all completely pure as judged by analytical argentation TLC.

Hydrogenolysis of Hydroxy, Tosyloxyoleate Isomers

Each of the pure hydroxy,tosyloxyoleate isomers (10–50 mg) was dissolved in anhydrous tetrahydrofuran (3 ml) and $LiAlH_4$ (50 mg) was added. The mixture was refluxed overnight, cooled and the complex then decomposed by the addition of excess dilute sulphuric acid. The product was extracted into ether, washed with water and the solvent was removed to yield a colourless oil. TLC indicated that each product was almost entirely a hydroxyoleyl alcohol, having suffered hydrogenolysis of the tosyloxy group and reduction of the ester group.

Preparation of Ketostearates

The hydroxyoleyl alcohols were each hydrogenated at atmospheric pressure in ethyl acetate solution over Adam's platinum oxide catalyst. The white solid hydroxystearyl alcohol products (5–10 mg) were each dissolved in a solution of 5% chromium trioxide in glacial acetic acid (1 ml) and shaken vigorously for 5 min. The solutions were then diluted with water, extracted with ether and the extracts washed thoroughly with water. The recovered products were each esterified with diazomethane and the ketostearates purified by preparative TLC.

Physical Methods

Optical rotations were measured in a 2.0 cm cell with an ETL/NPL Automatic Polarimeter (Type 143A), as solutions (concentrations 0.5–5.0%) in ethanol.

Mass spectra were obtained on an AEI MS12 instrument using the direct insertion sample probe technique and were interpreted on the basis of the published work of Ryhage and Stenhagen (7) and in comparison with the mass spectra of pure authentic samples of methyl 12– and 13–ketostearates.

RESULTS

A summary of the various products and of their specific rotations is provided in the Table I.

The two hydroxy,tosyloxyoleates derived from methyl vernolate both had the same sign of rotation, unlike the corresponding

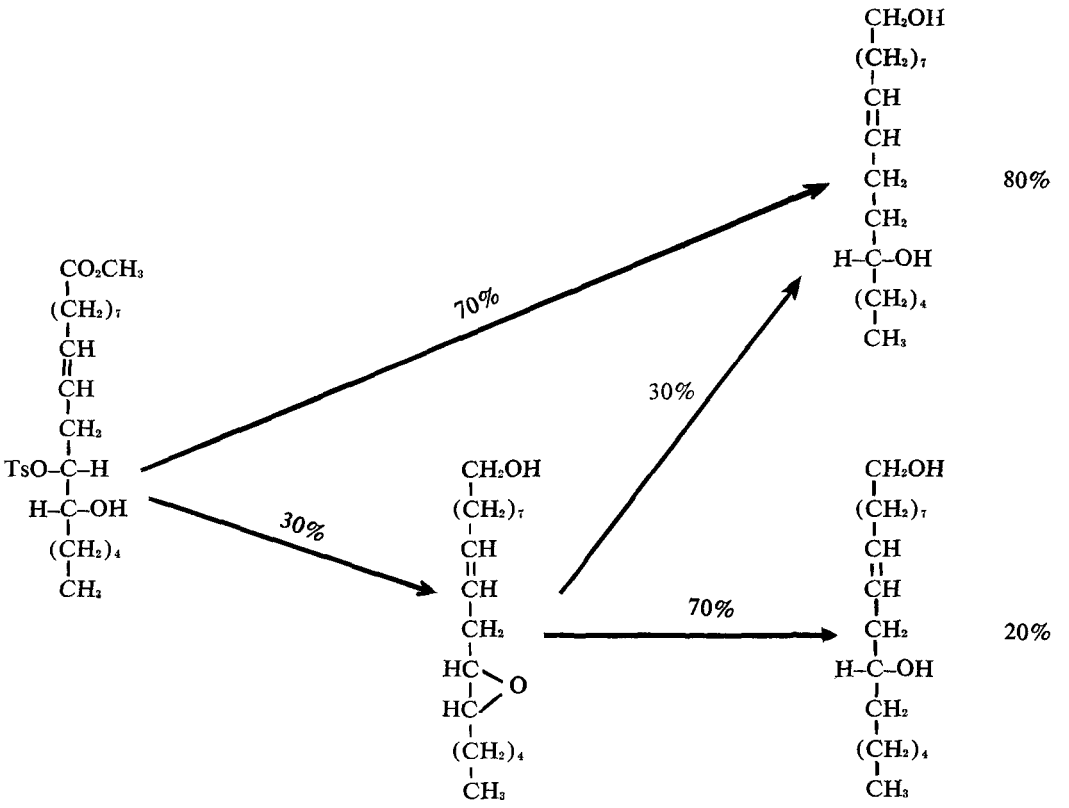

FIG. 2. Summary of postulated concurrent reactions and proportions of products in reduction of 13-hydroxy,12-tosyloxyoleate with $LiAlH_4$, in boiling tetrahydrofuran.

chlorohydroxyoleate derivatives which had opposite rotations (5). In each case, however, one isomer must be the D–12–hydroxy,L–13–tosyloxy– or –chloro–oleate and the other isomer must be the D–13–hydroxy,L–12–tosyloxy– or chloro–oleate, from the known stereochemistry of epoxide ring opening by acids (11). This is confirmed by the fact that the 12– or 13–hydroxyoleyl alcohols, produced on hydrogenolysis of the tosyloxy group from each isomer, were both dextrorotatory as is ricinoleyl alcohol ($[\alpha]_D$ = +3.0° in $CHCl_3$ (5)), which is known to be D–12–hydroxyoleyl alcohol.

The methyl ketostearate derived from the vernolate hydroxy,tosyloxy derivative of lower mobility on argentation TLC was proved by its mass spectrum to be almost entirely 12-ketostearate, with only a very small proportion of 13-ketostearate present. The other vernolate derived ketostearate, from the upper or more mobile hydroxy,tosyloxyoleate, was largely 13–ketostearate but it also contained a substantial proportion (ca. 20%) of 12–ketostearate. The position of the hydroxy group in the two derivatives from (–)–12,13–dihydroxyoleate was determined in the same way and with identical results from the mass spectra. The presence of these positionally isomeric ketostearate impurities in what should be pure, positionally homogenous products, is discussed below.

We have therefore now established that the less mobile (lower) hydroxy,tosyloxyoleate is the 12-hydroxy-isomer and if it is laevorotatory, it is the D–12–hydroxy,L–13–tosyloxy enantiomer. Similarly, the more mobile (upper) isomer is 13–hydroxy,12–tosyloxyoleate which if it is laevorotatory is the D–13–hydroxy,L–12–tosyloxy enantiomer.

Thus, of the products derived from (–) *threo*–12,13–dihydroxyoleate by partial tosylation, the less mobile isomer was laevorotatory and hence was D–12–hydroxy,L–13–tosyloxyoleate, identical with the corresponding vernolate product. The more mobile isomer, on the other hand, was dextrorotatory and therefore enantiomeric with the corresponding vernolate product, i.e., it was L–13–hydroxy, D–12-tosyloxyoleate. The configuration of the

substituents in both of these derivatives was D–12, L–13 and the parent (–)–dihydroxyoleate was thereby proved to be D–12,L–13–dihydroxyoleate, i.e., 12–*S*,13–*S*.

The (+)–*threo*–12,13–dihydroxyoleate produced by enzymic hydration of (+)–vernolic acid must be L–12,D–13–dihydroxyoleate, i.e., 12–*R*, 13–*R* and this was confirmed by the formation from it of D–13–hydroxy,L–12–tosyloxyoleate and L–12–hydroxy,D–13–tosyloxyoleate, these being respectively laevo– and dextrorotatory and hence identical and enantiomeric with the corresponding derivatives from vernolate.

DISCUSSION

Although the pair of positionally isomeric hydroxy,tosyloxyoleates showed no sign of separation on normal silica gel, they were quite readily separated from each other on silver nitrate impregnated silica gel. This separation implies a reduction in the silver ion-complexing ability of the double bond of the more mobile isomer, shown to be the 13–hydroxy,12–tosyloxyoleate. This is probably due to steric hindrance toward complexing exerted by the bulky tosylate group close to the double bond or to delocalization of the π– electrons of the double bond by this group or both.

The nucleophilic attack on the epoxy group of vernolic acid during reduction with $LiAlH_4$ has been shown (5) to occur predominantly at the 13 position to give the 12-hydroxy derivative. It was then argued that any nucleophilic reagent would likewise preferentially attack the 13 position. The present work has verified this in that the reaction of methyl vernolate with toluene–*p*–sulphonic acid gave unequal proportions of the hydroxy,tosyloxy isomers and the major product (60–70%) was again the 12–hydroxy isomer. The configurations previously assigned to the two chlorohydrin isomers from methyl vernolate (5) are thus confirmed.

One initially puzzling feature of the present work was the presence of the other isomer in each of the individual ketostearates, particularly of 12–ketostearate as a substantial impurity (ca. 20%) in the 13–ketostearate product. These findings were unexpected because each of the separated hydroxy,tosyloxyoleate isomers, from which these products were derived by seemingly straightforward reactions, was judged to be greater than 95% pure by argentation TLC. The reason for this is believed to be that the basic conditions of the hydrogenolysis reaction caused elimination of toluene–*p*–sulphonic acid from some proportion of the hydroxy, tosyloxyoleate thereby regenerating epoxyoleate which was then reductively cleaved in the normal way to give both 12– and 13–hydroxy isomers in approximately a 2:1 ratio. If some 30% of the 13–hydroxy,12–tosyloxyoleate went via this "epoxide pathway" on hydrogenolysis, as summarized in Figure 2, the product would consist of about 80% of the expected 13-hydroxyoleyl alcohol and 20% of the 12–hydroxy isomer. These proportions are close to the actual composition obtained, as determined by mass spectrometry of the derived ketostearate. On the same basis, the 12–hydroxy,13–tosyloxyoleate would give 90% of the desired 12–hydroxyoleyl alcohol and only about 10% of the 13–hydroxy isomer as impurity.

This work has demonstrated that the enzymic hydration of endogenous D–*cis*–12,13–epoxyoleic acid in crushed *Vernonia* seeds produces L–12,D–13–dihydroxyoleic acid. On the assumption that the stereochemistry of enzymic and chemical opening of the epoxide ring must be the same, namely inversion at the position of nucleophilic attack (and it is difficult to visualize any other stereochemistry), then the site of hydroxyl attack by the enzyme must be at the 12 position, as previously predicted (5). This is now being verified directly by incubation of crushed *V. anthelmintica* seeds in the presence of $H_2{}^{18}O$. Preliminary results indicate that the ^{18}O isotope is enriched exclusively in the 12–hydroxyl group of the (+)–*threo*–12,13–dihydroxyoleic acid product (13).

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