Enantioselective Cleavage of *meso*-Nitrodiol Diacetates by an Esterase Concentrate from Fresh Pig Liver: Preparation of Useful Nitroaliphatic Building Blocks for EPC Syntheses**

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Abstract: Diacetates of *meso*-2-nitro-1,3-diols, readily available from nitroalkanes and aldehydes, are saponified to crystalline monoacetates of > 97% *ee* by pig liver esterase (PLE). A general discussion of this type of EPC synthesis (EPC: enantiomerically pure compounds) is given, and a specific example is described in detail: a procedure for the preparation of a very inexpensive PLE concentrate and of a PLE solution from fresh liver, and a procedure for preparing 20 g amounts of enantiomerically pure (1*S*, 2*S*, 3*R*)-3-hydroxy-2-nitro-cyclohexyl acetate.

1. Ester Cleaving Enzymes

The numerous applications of biological chemical methods in enantioselective organic synthesis can be ordered in three groups[1]: (i) The use of enzymes which do not require cofactors. (ii) The use of whole cells (from microorganisms, plants, animals) in which part or the entire cell metabolism is supplied. (iii) The use of complex systems in which several enzymatic steps or enzymatic and non-enzymatic steps are coupled to a «cycle» achieving the desired transformation. It does not require prophetic abilities to predict that for everyday laboratory practice (with varying substrates and target molecules) the first approach will become the most important one. Of the enzymes not requiring coenzymes the most generally applicable ones seem to be those cleaving or making ester bonds. They can be used for the preparation of simple chiral starting materials by kinetic resolution of racemic mixtures or – better – by enantioselective conversions of *meso*-precursors to monoesters of dicarboxylic acids or of diols.

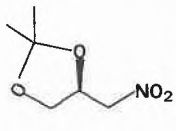
The kinetic resolution of (±)-mandelic ester by pig liver esterase (PLE) was described by *Dakin*[2] as early as 1903. Pioneering work in this area was published in 1959 by *Cohen* et al. who used chymotrypsin[3]. *Sih* was the first to realize the potential of this approach[4] in the synthesis of enantiomerically pure compounds (EPC)[5]. More recently, the groups of *Gais*[6], *Jones*[7], *Klibanov*[8], *Ohno*[9], *Schneider*[10], *Tamm*[11], and *Whitesides*[12] (in alphabetic order!) have employed it for the preparation of various useful starting materials or auxiliaries for EPC syntheses – see also the accompanying articles[13, 14]. The enzymes employed in these investigations are esterases [for instance PLE], lipases [for instance from pig pancreas (PPL) or from yeasts (*candida* species)], and peptidases [for instance chymotrypsin]. The techniques and conditions vary from low concentrations in water, through mixtures containing 50% substrate and 50% water, to the use of organic solvents containing < 2% of water, all the way to immobilized enzymes.

The most attractive applications on a preparative scale are those using a substrate of *meso*-configuration (ideally no separations and no recycling necessary), with an inexpensive *or* highly effective form of the enzyme which should be accessible to *every* laboratory. Batchwise use of purified commercial PLE (a mixture of isoenzymes) can be quite costly: for 100 mmole of a substrate 10 mg of enzyme (1000 units, ca. $ 10.00) are usually required. There are two solutions to this problem: the immobilization of purified PLE or the use of crude PLE. Disadvantages of immobilization are the high costs of most carriers, loss of activity during the immobilization, and by thermal, mechanical, and chemical «stress» during the reaction. Possible complications with the crude PLE are low selectivity due to the action of several esterases, formation of byproducts by other enzymes, difficulties in the isolation procedure, and extraction of cellular material. In the application described in the following sections these last mentioned problems with crude PLE are not encountered or can be readily overcome, and we strongly recommend fresh pig liver from the butcher to become a standard reagent for the synthetic laboratory – just like baker's yeast[15].

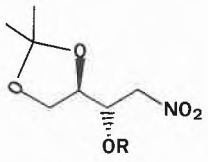
2. Nitroaliphatic Compounds in EPC Syntheses

While diastereoselective reactions of nitroaliphatic compounds have recently been developed (for reviews see ref.[5b, 16, 17]), there are only a few enantioselective conversions involving this class of synthetically useful derivatives, see **1**–**3** and Scheme 1, and the references given therein.

1 (ref.[18])



2 (ref.[19])



3 (ref.[20–22])

In our own work, we have been especially interested in the *different variations* of Michael additions to nitroolefins for EPC syntheses (see Scheme 1). The most successful approach to date was the addition of enamines from cyclohexanones or β-tetralones and 2-methoxymethyl-pyrrolidine («prolinol methyl ether») to nitroolefins[5b, 26–29], see for example the formation[29] of the bicyclo[3.3.1]nonanone **4** from such an enamine and an NPP[31] derivative.

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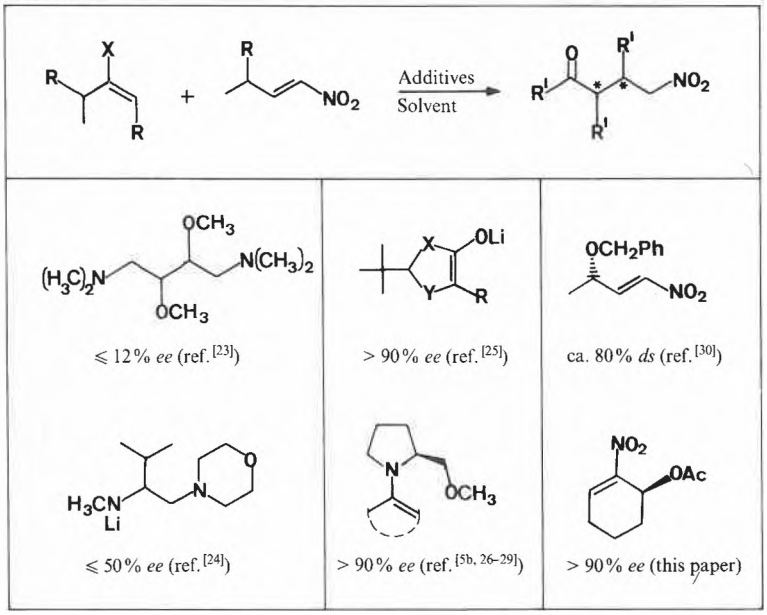
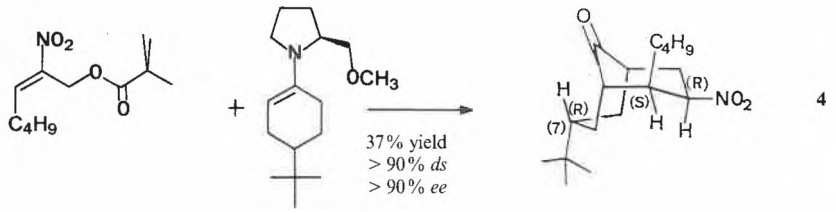
Scheme 1

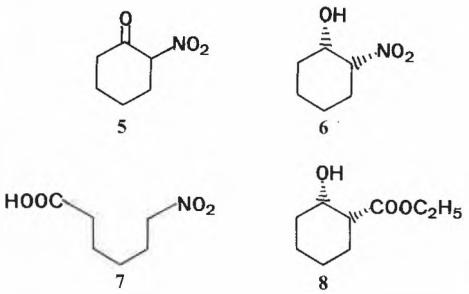




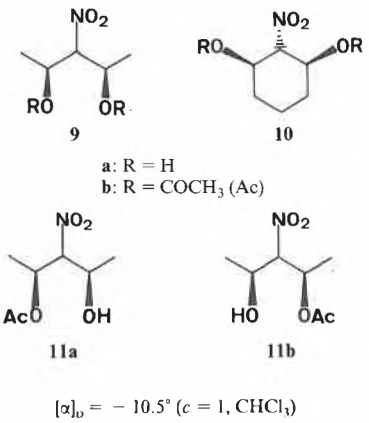
3. *Microbial and Enzymatic Conversions of Nitroaliphatic Compounds*

Results and Discussion

Initially, we tried to reduce α-nitroketones such as the nitrocyclohexanone **5** with yeast. Instead of the desired nitroalcohol **6,** which we isolated in yields ranging from 5 to 30% with > 95% enantiomeric excess (*ee*), the main product was the nitroacid **7** resulting from retro-acylation. The configuration of **6** (assignment see formula) is analogous to that of the yeast reduction product **8** of cyclohexanone carboxylic ester[32] (cf. the similarity of $RCO_2^{\ominus}$ and RNO_2 in biological systems[33]).



We then employed the diacetates of *meso*-nitrodiols from double Henry reactions of nitromethane with aldehydes or dialdehydes[34]. *meso*-Diols **9a** (of hitherto unknown configuration on C-3) and **10a** from acetaldehyde and from glutaraldehyde are readily prepared on large scale in 20% and 60% yield, respectively, after crystallization[35, 36]. The corresponding diacetates **9b** and **10b** are also crystalline compounds suitable for purification and separation from stereoisomers.



$[\alpha]_D = -10.5°$ ($c = 1$, $CHCl_3$)

Stereoselective saponification of the diacetates **9b** and **10b** is readily achieved with PLE. The monoacetates **11** and **12** crystallize from the crude product mixtures, they are isolated in yields of 50–80% of recrystallized material, *and they are enantiomerically pure* (> 98% *ee* by ^{1}H-NMR spectroscopy in the presence of chiral shift reagent [Eu(dcm)$_3$]). The nitrocyclohexanediol derivative **10b** was also hydrolized with several lipases, acylases, and with α-chymotrypsin, but the reaction times were much longer than with PLE, even if we used 100 times as much enzyme.

We have not yet determined the configuration of the open chain product **11.** The single stereoisomer we have isolated must be one of four, (3*R*)- or (3*S*)-**11a** or the enantiomers (3*S*)- or (3*R*)-**11b.** The structure of the cyclic monoacetate **12** was determined by single crystal X-ray diffraction[37] of the camphanic acid[38, 39] ester **17** (the all-*trans* configuration of the starting *meso*-diol **10a** had been assigned previously[36]). Thus, PLE hydrolizes the (*Re*)-acetate group of the diacetate **10b** preferentially. From the monoacetate **12** esters of either (*S*)- or (*R*)-2-nitrocyclohex-2-en-1-ol are available, see **13** and **14,** respectively, in Scheme 2. Hydrolysis of the nitroolefinic acetate **13** to the parent allylic alcohol (**15**) and hydrogenation gives the same nitrocyclohexanol **6** which was obtained by yeast reduction of the nitroketone **5** (see above), which in turn was correlated with the known[42] *cis*-2-aminocyclohexanol (**16**), thus establishing the structure of the yeast product as well.

The nitroallylic esters **13** and **14** are multiple coupling reagents, as outlined for achiral and racemic analogues in previous papers[31]. We have found that the enantiomerically pure derivatives **13** and **14** react with certain nucleophiles to give optically active substitution products (Scheme 3).

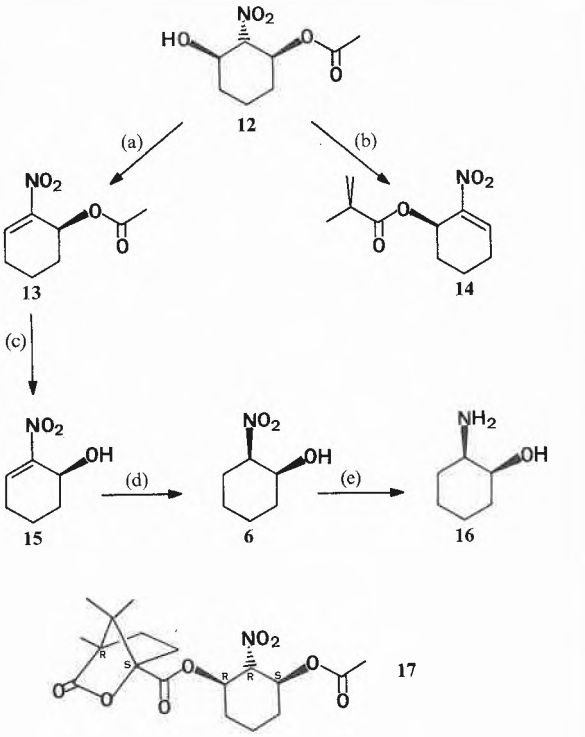
Applications of the now readily available chiral nitroalcohols and nitroolefins and the steric course of the reactions are being studied.

Crude Pig Liver Enzyme Concentrate

At the beginning of our investigation we used to work with immobilized PLE[43]. This technique became quite expensive, since several times denaturation of the protein occurred (possibly caused by nitroolefins), with loss of the expensive support (Eupergit C, 67 mg/mg PLE)[44]. We therefore looked for a less elaborate form of the enzyme, such as porcine liver acetone powder (sometimes abbreviated PLAP[14], ca. $15.00/25 g) or another crude concentrate. We decided to start from the «real thing», i.e. pig liver itself which should be available to every chemist, and which is very inexpensive in our country (ca. $ 1.5/kg). There are many published procedures for the isolation and purification of PLE from pig liver. We found the one by *Horgan* et al.[45] to be most convenient for the preparation of a crude concentrate. It involves draining of *masticated* fresh liver with acetone and removal of lipids with methylene chloride, see detailed procedure below.

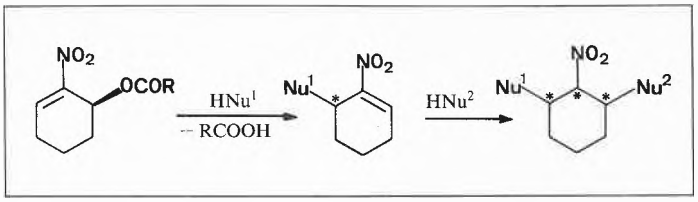
The dried product (ca. 300 g from 1 kg of liver) thus obtained can be kept in a freezer for months without significant loss of activity. An amount of 20 g of this material, if used directly, has the same activity in our reaction (**10b** → **12**) as 40 mg (4000 units) of commercially available purified PLE (ca. $ 40.00). Since the reaction mixture is heterogeneous, either filtration or centrifu-

Scheme 2



(a) DCC/CuCl/EtOEt/room temperature/48 h[40].
(b) $(Piv)_2O/H_2SO_4$; CH_3OH/H_2SO_4; then analogous to (a).
(c) CH_3OH/H_2SO_4.
(d) Hantzsch ester/C_6H_6/SiO_2[41].
(e) Raney Ni/H_2/50 °C/26 atm[18].

Scheme 3



gation is required before continuous extraction of the product (**12**); we have recently carried out the centrifugation before the reaction, with loss of ca. 50% of the activity. Thus, 50 g of the diacetate **10b** (204 mmol) are enantioselectively saponified with 100 g of the crude concentrate in 1.4 L water within 8 h[46].

4. Experimental

(A) Preparation of pig liver acetone methylene chloride powder (PLAMP): Six 100 g pieces of a fresh (preferably still warm) pig liver are mixed in turn at 4°C with 150 mL precooled acetone in a 1L-waring blendor for not more than 2 min. The brown fine mash is transferred into six centrifuge vessels and centrifuged at high speed (ca. 20 000 *G* or 10 000 rpm with 29 cm diameter) for 20 min. The supernatant is discarded and the residue is returned to the waring blendor. Each portion is mixed with 150 mL of precooled methylene chloride for 2 min and centrifuged again. After decanting and discarding the supernatant the residual solid is dried at 0.01 Torr at room temperature for about 2 h, the solid turning light brown. To obtain a powder the mass is ground with a pestle and mortar and dried for another 2 h at high vacuum. The powder prepared in this way shows an activity of about 200 units/g and can be stored in a freezer for months without any significant loss of activity.

(B) Preparation of a clear buffered PLAMP solution: In a 2L Erlenmeyer flask 27.2 g (0.2 mol) potassium dihydrogenphosphate is dissolved in 1L of distilled water and the pH is adjusted to 7.0 by adding about 9 g of potassium hydroxide. About 60 g of the PLAMP (see *(A)*) are added to the vigorously stirred buffer solution. After about 1 h the hetereogeneous mixture is centrifuged at high speed for 20 min. The clear yellow supernatant is returned to the Erlenmeyer flask. This solution should *not* be stored, but rather used immediately, since the PLE is not stable under these conditions.

(C) Enantioselective saponification of trans-trans-3-acetoxy-2-nitro-cyclohexyl acetate (**10b**) *to (1S,2S,3R)-3-hydroxy-2-nitro-cycloheyl acetate* (**12**): The diacetate **10b** (*m.p.* 88–89°C) was prepared by the procedures of *Lichtenthaler* et al.[36] in an overall yield of about 50% from glutaraldehyde. To 1 L of a well-stirred PLAMP solution (see *(B)*) 30 g (0.12 mol) of diacetate **10b** are added. The pH, measured with a pH-meter, decreases during the reaction from 7.0 to 5.7. As soon as the pH is almost constant (about 8 h are required at ca. +25°C) the reaction mixture is transferred to a continous extractor. Since the organic solvent will denaturate most of the proteins it is advisable to use a 2L extractor to overcome problems with emulsions. After 20 h of extraction with ether the organic phase is separated, dried, and concentrated evaporatively to a pale brown oil that solidifies on standing. An ethereal solution of the product is finally filtered through a bed of silica gel (ca. 10 × 3 cm), concentrated, and the residue dried at high vacuum to yield about 22 g (89%) of colorless monoacetate **12**. The $[\alpha]_D$ is +9.0° (*c* = 1.0, $CHCl_3$), but can be as high as +9.5°. Recrystallization from boiling ether/pentane yields a product of $[\alpha]_D$ = +9.8° (*c* = 1.0, $CHCl_3$), *m.p.* 89–91°C. – IR (Nujol): 3420 br m, 1720s, 1545s, 1370s, 1250s, 1085w, 1025m, 955w. – ^{1}H-NMR (300 MHz): δ = 5.2–5.1 (6 line system, 1 H, CHOAc), 4.4 (3 line system 1:2:1, 1 H, $CHNO_2$), 4.1 (9 line system, 1 H, CHOH), 2.7 (d, *J* = 5.5 Hz, 1 H, OH), 2.2–2.1 (m, 2 H, ring), 2.0 (s, 3 H, OAc), 1.9–1.3 (m, 4 H, ring). – MS: *m/z* 203 (M^+ < 1%), 149 (1), 113 (2), 112 (2), 98 (4), 97 (46), 96 (4), 79 (20), 70 (4), 69 (7), 67 (12), 61 (12), 43 (100), 44 (5).

(D) Determination of the enantiomeric excess of **12:** The *ee* of the monoacetate **12** was determined by ^{1}H-NMR spectroscopy of the nitroolefin **13** in the presence of chiral shift reagent on a 300 MHz spectrometer: 1 mg of (±)-**13** is dissolved in 0.5 mL [$^2H_6$]benzene. On addition of 4.5 mg $Eu(dcm)_3$, the signal for the OAc methyl group is split into two baseline separated singletts at $\delta \approx$ 1.8. The same experiment done with (−)-**13** shows a single signal corresponding to an *ee* of at least 97%.

(E) Esterification of (1S,2S,3R)-3-hydroxy-2-nitro-cyclohexyl acetate (**12**) *with camphanic acid:* In 50 mL methylene chloride 1.0 g (4.9 mmol) alcohol **12** and 1.0 g (5.0 mmol) (−)-camphanic acid are dissolved and cooled to 0°C. A mixture of 1.2 g (5.8 mmol) dicyclohexylcarbodiimid (DCC) and 0.05 g (0.4 mmol) 4-dimethylamino-pyridine in 10 mL methylene chloride is added by syringe. After 30 min at 5–10°C the mixture is treated with 60 mL tetrachloromethane and filtered. The filtrate is acidified with 10 mL 1 M HCl, washed with 10 mL brine, dried and evaporated. The crystalline mixture is chromatographed on silica gel with ether/pentane (1:1) to yield 1.7 g (90%) of camphanic ester **17.** For the X-ray analysis the product was recrystallized twice from a mixture of benzene, ether, and pentane. $[\alpha]_D$ = −25.5° (*c* = 1.0, $CHCl_3$); *m.p.* 168°C (with decomposition); space group: monocline, $P2_1$, *a* = 6.369 Å, *b* = 15.277 Å, *c* = 10.793 Å, β = 104.44°.

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