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Regio- and enantioselectivity of the enzyme-catalyzed hydrolysis of citric acid derivatives

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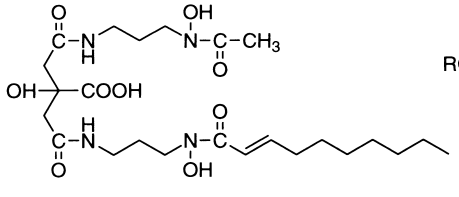
Abstract

The hydrolysis of triethyl citrate in the presence of three serine proteases (chymotrypsin, subtilisin BPN′, subtilisin carlsberg) is highly regioselective and gives the symmetric diester. Several lipases and proteases have the complementary regioselectivity and give the chiral diester. Pig liver esterase, *Aspergillus niger* lipase and *Candida antarctica* lipase give the chiral (*R*)-diester with good regio- and enantioselectivity. The stereoselective hydrolysis of the meso citric derivatives **7a**,**b** in the presence of *Candida antarctica* lipase gives the corresponding (*R*)-monoester.

The citric sub-unit is found in various natural products[1–8] or synthetic bioactive compounds.[9,10] For instance, rhizobactin 1021[1] (**1**) and rhizoferrin,[2,3] siderophores isolated from fungi, are chiral citrate derivatives. Viridiofungins[4] (**2**) and squalestatins[5–7] (also called zaragozic acids) are members of two families of squalene synthase inhibitors and antifungal natural products incorporating the citrate moiety. Recently, Bergeron et al.[11] reported the resolution of 1,2-dimethyl citrate by fractional crystallization of the corresponding (−)-brucine salts, and determined the absolute configuration by single crystal X-ray diffraction. Also, the citric acid derivative **8a** was resolved via fractional crystallization of the (*R*)- or (*S*)-α-methylbenzylamine salts.[12] We report here a study on the regio- and enantioselectivity of enzyme-catalyzed hydrolysis of citric acid derivatives.



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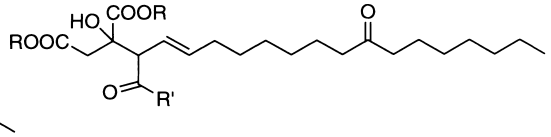

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First, we found that the hydrolysis of triethyl citrate **3** in the presence of three serine proteases (α-chymotrypsin, subtilisin BPN′ and subtilisin carlsberg) was highly regioselective, and that the symmetric

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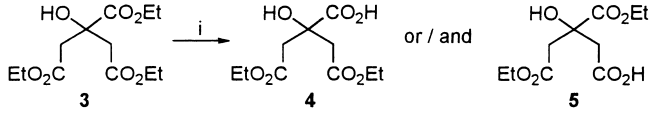
diester **4** was obtained as the sole product (Scheme 1). Anthonsen et al.[13] reported the regioselective hydrolysis of citrates by a subtilisin of unspecified origin.



Scheme 1. *Reagents*: (i) enzyme, r.t., (see Table 1)

Next, we did some screening to find enzymes which were able to distinguish the enantiotopic groups of triethyl citrate. Many lipases and proteases showed the complementary regioselectivity and gave chiral diester **5** as the sole or the major product, but the enantioselectivities were low (0–50%).[14]

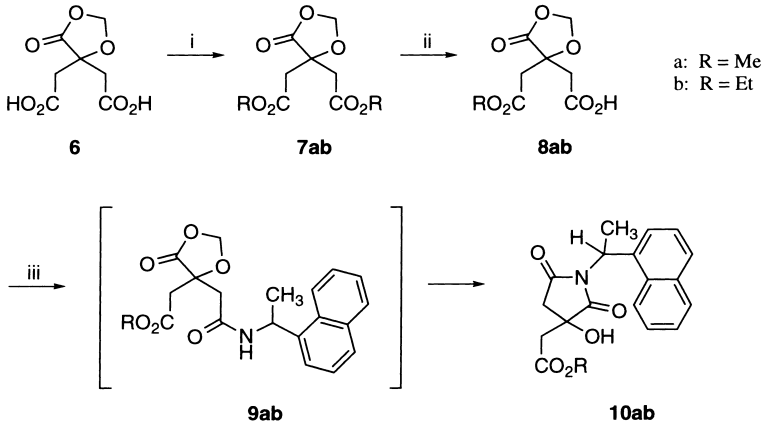
Hydrolysis of **3** in the presence of pig liver esterase (PLE) rapidly gave (2 h) the chiral diester **5** with fair regioselectivity (4/1) and good enantioselectivity (ee 90%). Lipase from *Aspergillus niger* gave **5** with high regio- (19/1) and enantioselectivity (ee 90%), but the reaction was very slow. *Candida antarctica* lipase produced **5** as the sole product with good enantioselectivity (ee 90%) in a short reaction time (3 h) (Table 1). The isomer ratio 5:4 was measured by ^{1}H NMR analysis and the diesters **4** and **5** were easily separated by standard flash chromatography. The enantiomeric composition of **5** was determined by reaction with (*S*)-(−)-(1-naphthyl)ethylamine in the presence of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC), followed by NMR analysis of the resulting diastereomeric amides.

Following from this, we investigated the desymmetrization of the citric acid derivative **7**. The diacid **6**[10] was esterified with methanol or ethanol in the presence of the corresponding 2,2-dialkoxypropane and an acidic resin to give esters **7a**,**b** (Scheme 2). Hydrolysis of **7a**,**b** in the presence of pig liver esterase in phosphate buffer provided monoesters **8a**,**b** in good yield (80–85%) and high enantioselectivity (ee 90–92%). The enantiomeric composition of **8a**,**b** was determined by reaction with (−)-(naphthyl)ethylamine in the presence of EDC, followed by NMR analysis of the resulting rearranged amides. The initially formed amides **9a**,**b** were rearranged to cyclic imides **10a**,**b** by intramolecular ring-opening of the 1,3-dioxolan-**4**-one.[15] A similar reaction was observed by Gilbert et al.[10]

The absolute configurations of **5** and **8a**,**b** were determined by comparison or correlation with (*R*)-(+)-**11** and (*R*)-(+)-**8a** of known absolute configurations[11,12] (Scheme 3). Ring-opening of the 1,3-dioxolan-4-one ring by refluxing **8a**,**b** with the appropriate alcohol and triethylamine gave (*R*)-**11** and

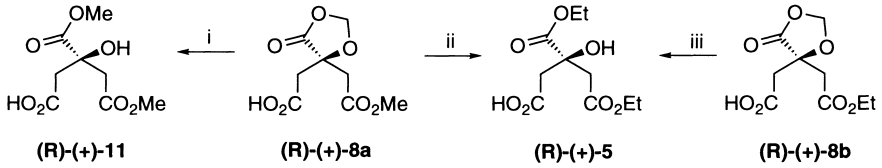
Table 1
Enzymatic hydrolysis of triethyl citrate

Enzyme	Time (days)	Yield (%)	Regioselectivity ratio 5/4	ee (%)	abs. conf.
Proteases					
Chymotrypsin	4	85	4 only	-	-
Subtilisin carlsberg	0.5	80	"	-	-
Subtilisin BPN′	4	80	"	-	-
Lipases or esterases					
Candida antarctica	3	82	5 only	90	R
Aspergillus niger	38	85	19/1	90	R
Pig liver esterase	(2 hours)	85	4/1	90	R



Scheme 2. *Reagents*: (i) ROH, H^+, $(CH_3)_2C(OR)_2$, reflux, 85%; (ii) pig liver esterase, phosphate buffer, r.t., 80%; (iii) NEA, EDC, DMAP, r.t.

(*R*)-**5**, respectively. Treatment of **8a** with sodium ethoxide in ethanol yielded **5** (ring opening and transesterification). These correlations proved that compounds **5**, **8a**,**b** have the *R* configuration.



Scheme 3. *Reagents*: (i) MeOH, Et_3N, reflux, 60%; (ii) EtONa, EtOH, r.t., 50%; (iii) EtOH, Et_3N, reflux, 60%.

1. Experimental

NMR spectra were recorded using a Bruker AC-300 instrument. Optical rotations were measured on a JASCO DIP-360 digital polarimeter (c as g of compound per 100 ml). All proteases were obtained from Sigma. Lipases were purchased from Amano International Enzyme Co. except for porcine pancreas lipase (Sigma) and *Pseudomonas fluorescens* lipase (Fluka). Pig liver esterase was from Sigma or Amano. *Candida Antarctica* lipase (novozym 435) was a gift from Novo Nordisk.

1.1. Enzymatic hydrolysis of triethyl citrate — general procedure

In a typical experiment, a suspension of triethyl citrate (150 mg, 1.84 mmol) was hydrolyzed with PLE (50 mg) in a phosphate buffer (pH 7) at room temperature. The enzymatic reaction was indicated by the decrease of pH, which was maintained at its initial value by the addition of a 0.1 M NaOH solution. The reaction was monitored by the consumption of base, and terminated when one ester equivalent was hydrolyzed. The aqueous solution was extracted with ether to remove any remaining starting material. The aqueous solution was acidified with 5 N HCl to pH 2, and the hydrolysis products were extracted with ethyl acetate. The isomeric diesters **4** and **5** were separated by flash chromatography on silica gel using hexane:ethyl acetate 4.5:5.5 as eluant. **Compound 4**: IR (neat) 3600–3040, 2980–2860, 1750, 1370, 1190, 1020, 850, 780 cm^{-1}. ^1H NMR ($CDCl_3$) 1.25 (t, J=7.1 Hz, 6H), 2.91 (m, 4H), 4.15 (q, J=7.1 Hz, 4H). ^{13}C NMR ($CDCl_3$) 177.13, 170.09, 73.16, 61.36, 42.76, 14.01. **Compound 5**: $[\alpha]_D^{25}$ +3.8 (c 1.84, MeOH); IR (neat) 3600–3040, 2980–2860, 1750, 1370, 1120, 1020, 830 cm^{-1}. ^1H NMR ($CDCl_3$)

1.25 (t, J=9.1 Hz, 6H), 2.86 (m, 4H), 4.12 (q, J=9.1 Hz, 2H), 4.28 (q, J=9.1 Hz, 2H). ^{13}C NMR ($CDCl_3$) 174.76, 174.03, 169.64, 73.12, 62.39, 60.86, 42.88, 13.89.

*1.2. Preparation of **7a**,**b***

Diacid **6**[10] (1.0 g, 4.90 mmol) was dissolved in the selected alcohol (methanol or ethanol, 20 ml), then the corresponding acetal (dimethoxypropane or diethoxypropane, 4 ml) and an acidic ion-exchange resin (Dowex 50WX4, 0.5 g) were added. The mixture was heated to reflux for 24 h. The reaction mixture was filtered to remove the resin and evaporated. The crude product was dissolved in ethyl acetate (100 ml) and washed with aq. Na_2CO_3 (2×20 ml) and brine (2×20 ml). The organic phase was dried ($MgSO_4$) and evaporated. The crude product was purified by flash chromatography (AcOEt:hexane 3:10) to give corresponding diester. **Diester 7a**: yield 86%, m.p. 60–63°C; IR (KBr) 3000, 2960, 1800, 1740, 1440, 1370, 1240, 1180, 1060, 990 cm^{-1}; ^{1}H NMR (acetone-d_6) 2.97 (s, 4H), 3.66 (s, 6H), 5.50 (s, 2H); ^{13}C NMR (acetone-d_6) 174.9, 171.0, 96.3, 77.1, 52.3, 41.8. **Diester 7b**: yield 85%, oil; IR (KBr) 2980, 2930, 1800, 1740, 1375, 1180, 1060, 1020 cm^{-1}; ^{1}H NMR (acetone-d_6) 1.21 (t, J=7.0 Hz, 6H), 2.94 (s, 4H), 4.11 (q, J=7.0 Hz, 4H), 5.51 (s, 2H); ^{13}C NMR (acetone-d_6) 173.3, 169.4, 95.4, 76.3, 61.4, 41.6, 14.2.

*1.3. Enzymatic hydrolysis of **7a**,**b***

Preparation followed the general procedure described above: **7a**,**b** (0.43 mmol), pig liver esterase (50 mg), reaction time: 2 h. **Compound 8a**: $[\alpha]_D^{25}$ +8.4 (c 2.46, MeOH); IR (KBr) 3500–2500, 1790, 1720, 1180, 1145, 1000 cm^{-1}; ^{1}H NMR ($CDCl_3$) 2.90 and 2.95 (2s, 4H), 3.71 (s, 3H), 5.55 (s, 2H), 7.97 (s, 1H); ^{13}C NMR ($CDCl_3$) 173.4, 172.8, 168.9, 95,0, 75.3, 52.2, 40.8. **Compound 8b**: $[\alpha]_D^{25}$ +10.2 (c 1.84, MeOH); IR (KBr) 3500–2500, 1790, 1720, 1180, 1145, 1000 cm^{-1}; ^{1}H NMR ($CDCl_3$) 1.20 (t, J=7.0 Hz, 3H), 2.83 and 2.89 (2s, 4H), 4.10 (q, J=7,0 Hz, 2H), 5.49 (s, 2H), 8.92 (s, 1H); ^{13}C NMR ($CDCl_3$) 173.6, 172.8, 168.4, 75.3, 61.3, 41.1, 40.8, 13.9.

*1.4. Ring opening of compound **8a**,**b***

Compound **8a** or **8b** (0.85 mmol) was dissolved in dry methanol or ethanol (20 ml). Freshly distilled triethylamine (236 μl, 1.70 mmol) was added and the solution was heated at reflux for 24 h. After allowing to cool to room temperature, the solution was evaporated to dryness. The excess of amine was neutralized with 1 N HCl. The aqueous solution was extracted with ethyl acetate (4×15 ml). The organic solution was washed with brine (2×20 ml), dried ($MgSO_4$) and evaporated to yield **5** or **11**. **Compound 5**: $[\alpha]_D^{25}$ +3.8 (c 1.84, MeOH), data described earlier. **Compound 11**: $[\alpha]_D^{25}$ +3.9 (c 1, MeOH); lit.[11,12] $[\alpha]_D^{25}$ +4.0 (c 1, MeOH). Spectral data are in agreement with those reported in the literature.[10,11]

*1.5. Ring opening and transesterification of compound **8a***

Sodium (47 mg, 2.06 mmol) was added to dry ethanol cooled to 0°C. A solution of **8a** (180 mg, 0.825 mmol) in dry ethanol was added and the mixture was allowed to warm to room temperature and stirred overnight. The solution was evaporated to dryness and the residue was dissolved in ethyl acetate. The organic layer was neutralized with 1 N HCl and washed with brine. The organic phase was dried ($MgSO_4$) and evaporated to give **5** ($[\alpha]_D^{25}$ +3.8 (c 1.84, MeOH)).

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15. Data for compound **10a** (one diastereoisomer): IR (KBr) 3420, 1780, 1700 cm^{-1}; ^{1}H NMR (acetone-d_6) 1.89 (d, J=7.0 Hz, 3H), 2.57 (d, J=18 Hz, 1H), 3.10 (m, 3H), 3.49 (s, 3H), 6.10 (q, J=7.0 Hz, 1H), 7.48 (m, 3H), 7.88 (m, 3H), 8.10 (m, 1H). ^{13}C NMR (acetone-d_6) 178.7, 175.0, 170.9, 135.5, 134.6, 132.1, 129.5, 129.0, 127.1, 126.8, 126.2, 125.8, 123.8, 72.5, 51.9, 47.1, 42.4, 41.2, 17.2.