New scientific results

ENIKŐ RITA TŐKE

STUDY OF CHEMICAL AND ENZYMATIC INTERESTERIFICATION REACTIONS

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1. INTRODUCTION

Nowadays is an increasing demand for healthy products, which are produced from natural components, the manufacturing process is not poluant, or utilize the waste of other technologies.

Of course, for the industry are not indifferent the process costs or the quality and quantity of the produced wastes. In these cases the problems can be solved by chaging the parameters of the fundamental process, or changing the catalyst, but often the interpretation of the experimental observations or of the reaction mechanism gives the solution.

The other major duty of the synthetic organic chemistry is the economic synthesis of the increasing amount of biologically active compounds, and the preparation of the chiral molecules, stereoisomers in optically active form. Consequently the selective synthetic methods providing chiral molecules are deeply studied.

During my PhD work I was studied chemical and enzymatic interesterification reactions which are already used in the industry or what due to their efficacy can be considered by the industry. I deeply analyze the advantages or drawbacks of the chemical or enzymatic carry out of a given interesterification reaction, also interpreting the details which are not neglijible for the industry.

By utilizing the experimental observations and by molecular interpretation of the processes, all studied interesterification reaction could be optimized and could be adequate for the industrial application.

2. METHODS

2.1. Setup of the low temperature base catalysed interesterification reaction of triglicerols

The low temperature interesterification process was investigated experimentally by using a mixture of equal weights of trilaurin (L_3) and triolein (O_3) as starting triglycerides in test reactions (Fig. 1.). Due to the carbon number difference of the starting components (L_3 and O_3) and products (L_2O and LO_2), the progress of the reaction could easily be followed by gas chromatographic (GC) -analyses. After thorough drying (stirring at 90°C under 0.01 mmHg vacuum), an accelerator (2 %, if added) and sodium methanolate (0.2%) were added to the mixtures and the interesterification reactions were performed under argon. Analysis of the intermediate samples and the final mixtures showed that the reactions reached equilibrium.

2.2. Esterification reaction of plant β-sitosterol with enzyme preparations produced by solid state fermentation (SSF):

The preparations received from the solid state fermentation process after drying in air were used as such in the sterol esterification reaction, without any further downstream process. The SSF preaparations were added to the mixture of plant β-sitosterol and the apropriate acid in toluene.. At the end of the reaction, the enzyme was removed and the product was isolated by column chromatography. The esterification reaction can be followed by TLC and GC.

2.3. Enantiomer selective interesterification reaction of racemic alcohols with vinyl acetate:

The enzyme (lipase) was added to the mixture of the substrate in hexane:THF:vinyl acetate 2:1:1 solution. Usually, the interesterification reactions takes 1-48 hours, after removing the enzyme by filtration the products can be separated by extraction, crystallization or column chromatography technics. After regeneration by multiple wash and drying the enzyme can be reused.

2.4. Determination of the conversion of the enantiomer selective reactions and the enantiomer composition of the products by GC:

The conversion of the enantiomer selective reactions and the enantiomer composition (*ee %*) of the products were determined by GC using chiral columns. As standards we used the chemically prepared racemic materials.

2.5. Determination of the enantiomeric composition of optically pure compounds by ^{1}H-NMR using shift reagent:

In some cases the enantiomeric composition was determined by ^{1}H-NMR in the presence of praseodium-(D-3-heptaflorobutiryl-camphorate) shift reagent. The ^{1}H-NMR spectra of the optically pure or racemic compound (10 mg) was taken in $CDCl_3$-ban (600 µl) using increasing amounts of növekvő shift reagens (2-25 mg).

3. RESULTS

3.1. Study of the reaction mechanism of low temperature base catalysed interesterification reaction of triglycerols

Using isotope-marking techniques, we studied the mechanism of low temperature base catalysed interesterification reaction of triglycerols (Fig. 1). We conclude that from the three proposed mechanism theory present in the literature, -the $B_{Ac}2$ mechanism involving the diacylglicerolate anion, the enolate mechanism and the mechanism involving Claisen-condensation-, the later one can be excluded because the sodium salt of the ethyl acetoacetate (itself being a β-ketoester) at 90°C was not catalytically active.

The formation of the enolates in the interesterification mixture is supported by experimental data: the rate increasing effect of the acetone, the deuterium incorporation from the acetone-d_6 moecules into the α-position of triglycerols (Dijkstra et al, **2005**).

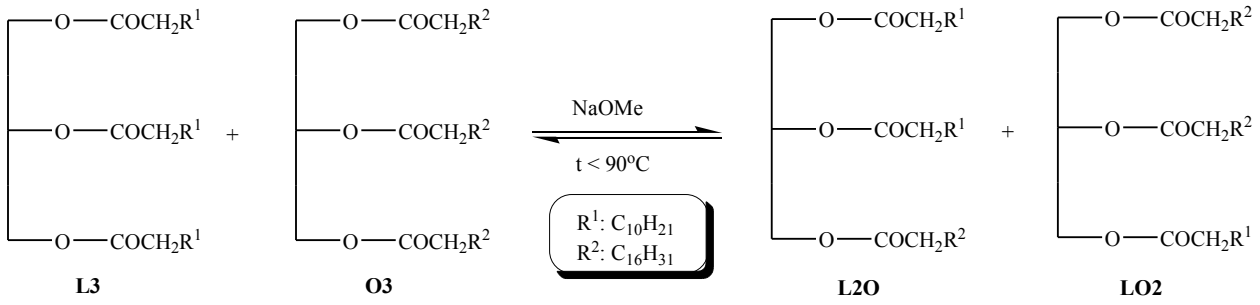


Fig. 1: *Low temperature base catalysed interesterification reaction of triglycerols*

The further mass spectroscopic and ^{2}H-NMR analyses for studying the degree of α-deuterium incorporation unambiguously demonstrated that the diacylglicerolate mechanism can be considered as the mechanism which drives this reaction, the formed enolates are product of

some equilibrique side-reactions which buffer a portion of the catalyst. (*data not yet published,under preparation)*

3.2. Production of novel sterol esterases by solid state fermentation (SSF) and synthetic application in esterification reaction of β-sitosterol

Using solid state fermentation we prepared novel sterol esterases from *Aspergillus* strains. These are effective and safe biocatalysts for use in food industry, their simple and cheap preparation can be considered an advantage.

Optimizing the fermentation conditions we were able to enhance the sterol esterase activity versus the lipase activity of the 24 enzyme preparation of two *Aspergillus* strains (*Aspegillus oryzae* NRRL 6270 and *Aspergillus sojae* NRRL 6271). Such – and also with genom analysis- we demonstrated that the sterol esterase and lipase activity belongs to distinct enzymes in these strains.The prepared biocatalysts in the esterification test reaction of β-sitosterol with lauric acid produced the β-sitosterol lauryl ester in 48 h with 45 % conversion compared to the initial 240 hours (Fig.2).

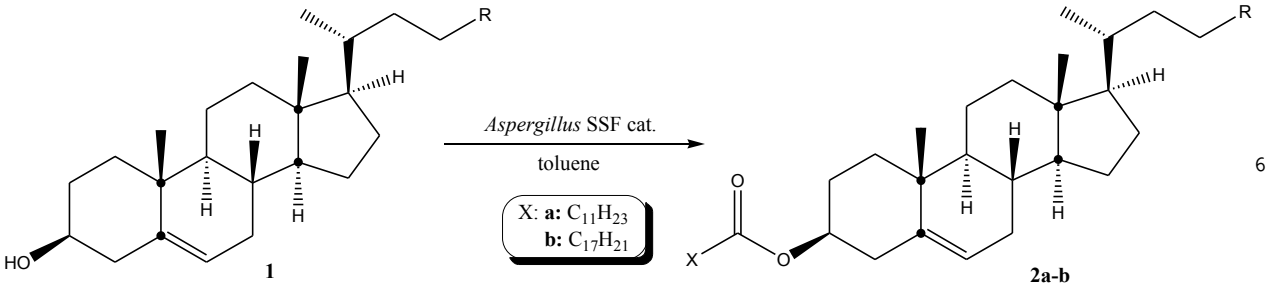


Fig. 2*: Esterification reaction of plant β-sitosterol in the presence of Aspergillus SSF preparations*

The synthetic usefulness of the prepared enzymes was proved by preparative scale preparation of lauryl and CLA (conjugated linoleic acid) of plant β-sitosterol. In this later case we showed the slight preference of *A. oryzae* NRRL 6270 toward the 10*E*,12Z isomer of CLA. (Tőke et al, **2007**).

3.3. Lipase catalysed kinetic resolution of 4-aryl- and 4-heteroarylbut-3-en-2-ols

The lipase-catalyzed enantiomer selective acetylation of the racemic (3*E*)-4-fenylbut-3-en-2-ol (*rac*-**3a**) and (3*E*)-4-(furan-2-il)but-3-en-2-ol (*rac*-**3b**) alcohols by vinyl acetate has been investigated with a crude lipase from submerged fermentation (SmF) of a thermophilic fungus, with several crude enzyme preparations from solid state fermentation (SSF) of mesophilic fungi and with several commercially available lipases. The commercial and SmF lipases and the majority of SSF preparations exhibited high but usual enantiomer selectivities and resulted in the formation of (*R*)-acetates [(*R*)-**4a,b**] according to the Kazlauskas' rule. From the own prepared biocatalysts particularly the SSF preparations of *Malbranchea pulchella* var. *sulfurea* and *Gliocladium catenulatum* NRRL 1093 were even more effective than the commercial lipases.. Several SSF preparations, however, behaved as selective anti-Kazlauskas catalysts (Fig.3).

Interestingly, replacement of the phenyl (in *rac*-**3a**) to a similarly bulky but more polar furan-2-yl moiety (in *rac*-**3b**) at position 4 of the racemic secondary allylic alcohol resulted in significant decrease of the enantiomer selectivity (*E*) in both the commercial and SSF lipases (Szigeti et al, **2008**).

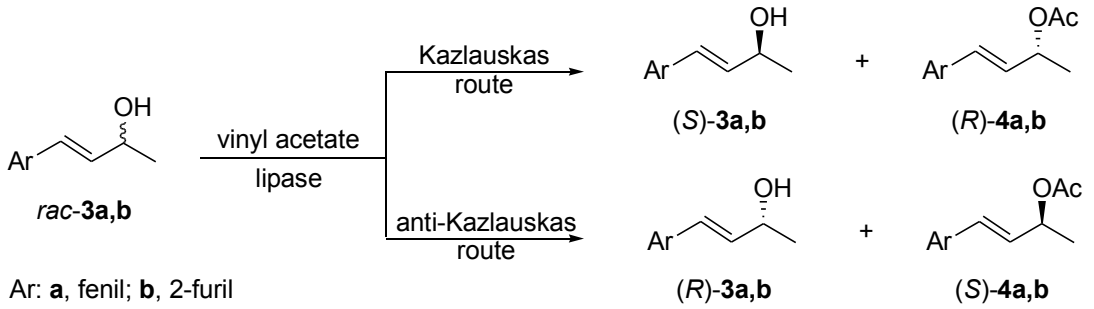
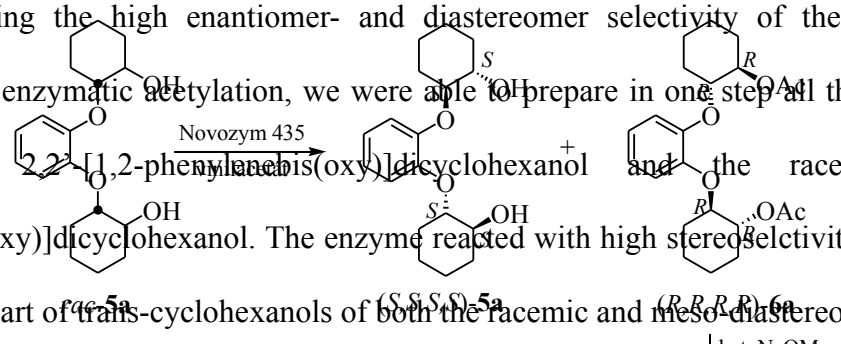


Fig. 3: *Lipase catalysed kinetic resolution of the racemic (3E)-4-phenylbut-3-en-2-ol (rac-**3a**) and (3E)-4-(furane-2-il)but-3-en-2-ol (rac-**3b**)*

3.4. *Lipase catalysed enantiomer and diastereomer separation of 2,2'-[1,2- and 1,3-phenylenebis(oxi)]dicyclohexanols*

Exploiting the high enantiomer- and diastereomer selectivity of the Novozym 435 enzyme, using enzymatic acetylation, we were able to prepare in one step all the stereomers of the racemic 2,2'-[1,2-phenylenebis(oxy)]dicyclohexanol and the racemic 2,2'-[1,3-phenylenebis(oxy)]dicyclohexanol. The enzyme reacted with high stereoselctivity with the (*R,R*) configuration part of trans-cyclohexanols of both the racemic and meso-diastereomers.



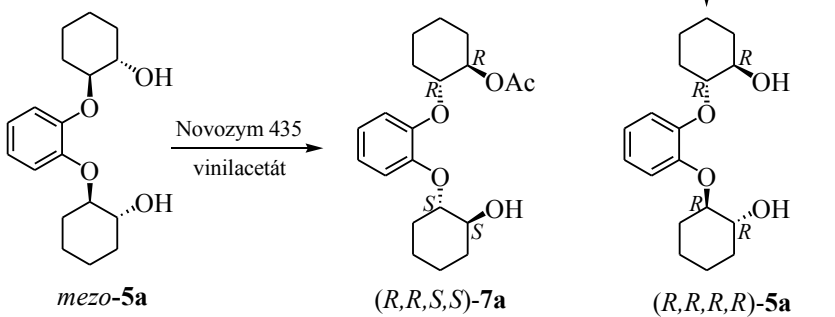


Fig. 4: Enantiomer selective acetylation of the r*acemic and meso 2,2'-[1,2-phenylenebis(oxi)] dicyclohexanol rac-**5a** and meso-**5a***

The Novozym 435 catalysed enantiomer selective acetylation of racemic 2,2'-[1,2-phenylenbis(oxi)] dicyclohexanol *rac*-**5a** resulted in enantiomeric pure (*S,S,S,S*)-**5a** diol (*ee* 96,5 %) and (*R,R,R,R*)-**6a** diacetate. The enzymatic hydrolysis of (*R,R,R,R*)-**6a gave** (*R,R,R,R*)-**5a** diol in optically pure form (*ee* >99 %). The enantiotop selective acetylation of *meso*-**5a** diastereomer resulted in pure (*R,R,S,S*)-**7a** monoacetate (*ee* >99 %) (Fig.4).

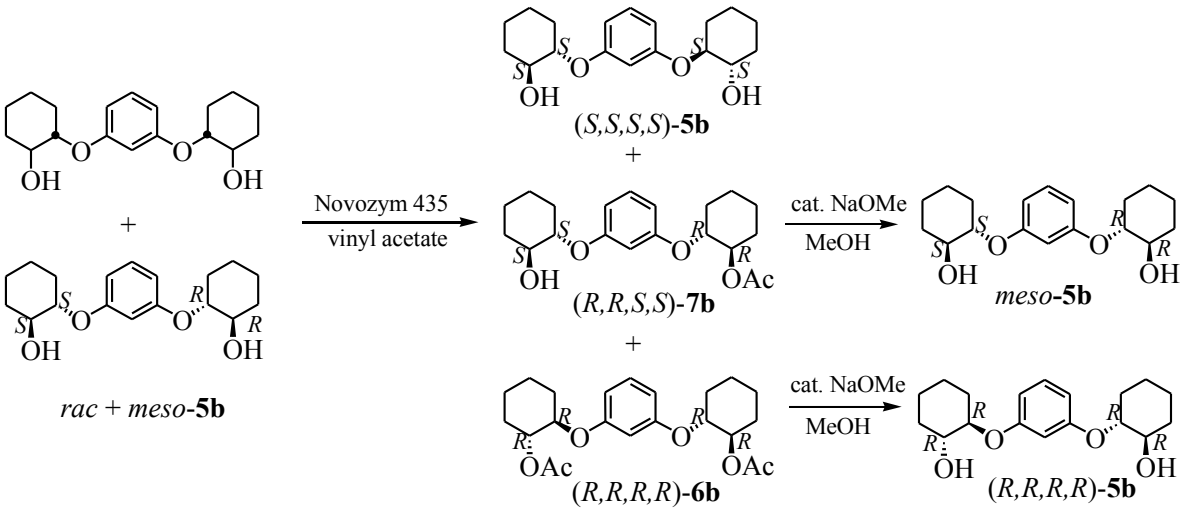


Fig. 5: Preparation of the stereomers of *2,2'-[1,3-phenylenebis(oxi)] dicyclohexanol rac-**5b** + meso-**5b***

The separation of the enantiomeric mixture of 2,2'-[1,3-phenylenebis(oxi)] dicyclohexanol *rac*-**5b** + *meso*-**5b**, was resolved in one step with the Novozym 435 catalysed enzymatic acetylation (Fig. 5). From the racemic *rac*-**5b** resulted enantiomerically pure diol (*S,S,S,S*)-**5b** (*ee* >98%) and diacetate (*R,R,R,R*)-**6b** and the meso-**5b** transformed in (*R,R,S,S*)-**7b** monoacetate (Fig. 5).

The molecular mechanic modeling of 2,2'-[1,2-phenylenebis(oxi)]dicyclohexanol *rac*-**5a** proved the (*R,R*) stereopreference of the enzyme and gave explication to the unusual long reaction time of these transformations: the dicyclohexanol molecule during the reaction from energetically favorable *trans*-diequatorial ring conformation goes to the more infavorable trans-diaxial conformation (Tőke et al, **2006**).

4. PUBLICATIONS

I. Articles and presentations related to the thesis

1. Dijkstra, A.J.; Tőke, E. R.; Kolonits, P.; Recseg, K.; Kővári, K.; Poppe, L.: The Base-Catalysed, Low-Temperature Interesterification Mechanism Revisited, *Eur. J. Lipid Sci. Technol.*, 107, **2005**, 912 – 921.

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2. Tőke, E. R.; Kolonits, P.; Novák, L., Poppe, L.: Lipase mediated enantiomer and diastereomer separation of 2,2'-[1,2-and 1,3-phenylenebis(oxy)]dicyclohexanols, *Tetrahedron: Asymmetry*, 17, **2006**, 2377 – 2385.

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3. Tőke, E. R., Nagy, V.; Recseg, K.; Szakács, Gy.; Poppe, L.: Production and application of novel sterol esterases from *Aspergillus* strains by solid state fermentation, *J. Am. Oil Chem. Soc.,* 84, **2007**, 907-915.

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4. Szigeti, M.; Tőke, E. R.; Turóczi, M.C.; Nagy, V.; Szakács, Gy.; Poppe, L.: Lipase-catalysed kinetic resolution of 4-aryl- and 4-heteroarylbut-3-en-2-ols, *Issue in Honor of Prof Csaba Szántay, Arkivoc,* (iii), **2008**, 54-65.

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5. Szatzker, G.; Pilbák, S.; Tőke, E.r.; Bódai, V.; Poppe, L.: Enantioselectivity in Candida antarctica lipase B reaction: transition states calculated by QM/MM methods, *30th FEBS Congress and 9th IUBMB Conference, FEBSJ*., 272, **2005**, 114.

6. Tőke, E. R.; Szatzker, G.; Huszthy, P.; Poppe, L.: Preparation of Highly Enantiopure Bis-Cyclohexanediol Derivatives Using Enzymatic Methods, *10th International Conference of Chemistry ,* (ISBN 973-7840-00-3), pp 234-236**, 2004** november 12-14., Cluj Napoca, Roumania.

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8. Tőke, E.R.; Szatzker, G.; Huszthy, P.; Poppe, L.: Magas enantiomertisztaságú bisz-ciklohexándiol származékok előállítása enzimatikus módszerekkel, *XXVII. Kémiai Előadói Napok*, **2004,** oktober 25., Szeged, Hungary.

9. TŐKE, E.R., PILBÁK, S., SZATZKER, G., BÓDAI, V., POPPE, L.: Stereoselectivity of *Candida antarctica* lipase B: calculations for enantiomeric pairs of alcohols, *Molecular Modeling in Chemistry and Biochemistry, Workshop*, **2005**, april 21-23, Cluj Napoca, Roumania.

10. PILBÁK, S., SZATZKER, G., TŐKE, E.R., BÓDAI, V., POPPE, L.: Stereoselectivity of *Candida antarctica* lipase B: QM/MM calculations for trans-cyclohexane-1,2-diol derivatives, *Biotrans Conference* **2005**, july 3-8, Delft, The Netherlands.

11. Tőke, E.R.; Dijkstra, A.J.; Kolonits, P.; Recseg, K.; Kővári, K.; Poppe, L.: A trigliceridek alacsony hőmérsékletű báziskatalizált átészterezése: új mechanizmus, *MTA Terpenoidkémiai és Elemorganikus Munkabizottság előadóülése*, **2005** september 9**,** Budapest, Hungary.

12. Tőke, E.R: A trigliceridek alacsony hőmérsékletű báziskatalizált átészterezése: új mechanizmus, *Doktoráns Konferencia, BME Vegyészmérnöki Kar,* **2006** february 7, Budapest, Hungary.

II. Articles and presentations not directly related to the thesis

1. Csajági, Cs.; Szatzker, G.; Tőke, E.R.; Ürge, L.; Darvas, F.; Poppe, L.: Enantiomer selective acylation of racemic alcohols by lipases in continuos-flow bioreactors, *Tetrahedron: Asimmetry*, 19, **2008**, 237-246.

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2. Nagy,V.; Tőke, E. R..; Keong, L.CH.; SZatzker, G.; Ibrahim, D.; Che Omar, I.; SZakács, GY.; Poppe, L.: Kinetic resolutions with novel, highly enantioselective fungal lipases produced by solid state fermentation, *Journal of Molecular Catalysis B: Enzymatic*, 39, **2006**,

141-148.

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3. KMECZ, I.; SIMÁNDI, B.; POPPE, L.; JUVANCZ, Z.; RENNER, K.; BÓDAI, V.; TŐKE, E. R.; CSAJÁGI, CS.; SAWINSKY, J.: Lipase-catalyzed Enantioselective Acylation of 3-Benzyloxypropane-1,2-diol in Supercritical Carbon Dioxide, *Biochem. Eng. J.,* 28, **2006**, 275-280.

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4. SZATZKER, G., TŐKE, E. R., NAGY, V.,SZAKÁCS, GY., POPPE, L.: Biocatalysis Using Novel Hidrolase Enzymes Produced by Solid State Fermentation, *11th International Conference of Chemistry,* (ISBN 973-7840-07-0), pp 312-315**,** **2005** november 11-13., Cluj Napoca, Roumania.

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6. NAGY, V., TŐKE, E.R., KEONG, L.CH., SZATZKER, G., IBRAHIM, D., CHE OMAR, I., SZAKÁCS, GY., POPPE, L.: Production of novel highly enantioselective lipases by solid state fermentation, *Biotrans Conference* **2005**, july 3-8, Delft, The Netherlands.

7. SZATZKER, G., TŐKE, E.R, KOLONITS, P., NOVÁK, L., HUSZTHY, P., POPPE, L.: Optikailag aktív mono- és bifunkciós piridin-alkoholok kemoenzimatikus előállítása, *MTA Terpenoidkémiai és Elemorganikus Munkabizottság előadóülése*, **2004,** april 2**.,** Budapest, Hungary.