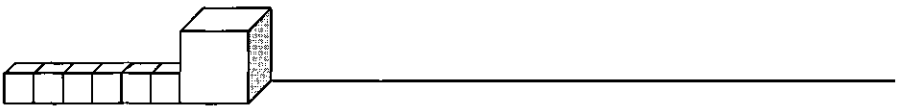
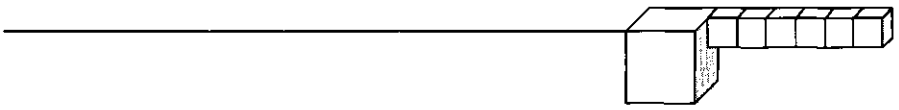


Lipase-catalyzed kinetic resolution of branched chain fatty acids and their esters

A study towards the production of enantiopure 4-methyloctanoic acid



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Lipase-catalyzed kinetic resolution of branched chain fatty acids and their esters

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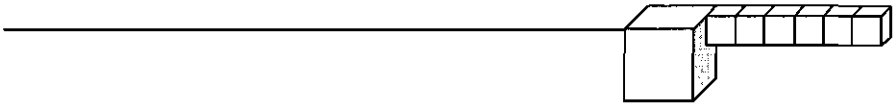

Lipase-gekatalyseerde kinetische resolutie van vertakte vetzuren en hun esters

Een studie naar de productie van enantiomeer zuiver 4-methyloctaanzuur

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus,
van Wageningen Universiteit,
dr. ir. L. Speelman,
in het openbaar te verdedigen
op woensdag 25 oktober 2000
des namiddags te half twee in de Aula

This work was carried out as part of the Innovation Oriented research Program on Catalysis (IOP-katalyse, no. IKA94001) sponsored by the Netherlands Ministry of Economic Affairs.

Heinsman, N.W.J.T. : Lipase-catalyzed kinetic resolution of branched chain fatty acids and their esters

A study towards the production of enantiopure 4-methyloctanoic acid

Thesis Wageningen University– with summary in Dutch

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NN08201, 2869

STELLINGEN

1. Het is opmerkelijk dat de enantiomeren van 4-methyloctaanzuur in schapenvlees in gelijke hoeveelheden worden aangetroffen.

   Dit proefschrift, hoofdstuk 7.

   Karl, V. *et al.* (1994). Stereoisomeric flavour compounds LXVIII. 2-, 3-, and 4-alkyl-branched acids, part 2: Chirospecific analysis and sensory evaluation. *Chirality*, **6**, 427-434.

2. Bij het onderzoek naar de sexferomonen van de neushoornkever lopen de schrijvers voorbij aan het feit dat er twee isomeren van 4-methyloctaanzuur bestaan.

   Hallet *et al.* (1995). Aggregation pheromone of coconut rhinoceros beetle, *Oryctes rhinoceros* (L.) (COLEOPTERA: SCARABAEIDAE). *J. Chem. Ecol.*, **21**, 1549-1570.

3. Het is verwarrend als in een veresteringsreactie de term "esterification rate" gebruikt wordt om de conversie aan te duiden.

   Tweddel, R.J. *et al.* (1998). Esterification and interesterification activities of lipases from *Rhizopus niveus* and *Mucor miehei* in three different types of organic media: a comparative study. *Enzyme Microb. Technol.*, **22**, 439-445.

4. Als bij een lipase-gekatalyseerde verestering van 2-methyloctaanzuur met butanol de verhouding van het teruggewonnen zuur gelijk is aan 0.806 (*S*-enantiomeer) : 0.194 (*R*-enantiomeer) dan is dit in tegenspraak met de bewering dat het *S*-enantiomeer sneller reageert dan het *R*-enantiomeer.

   Sonnet, P.E. and Welch Baillargeon, M. (1991). Methyl-branched octanoic acids as substrates for lipase-catalyzed reactions. *Lipids*, **26**, 295-300.

5. De chemische nomenclatuur die in de industrie wordt toegepast leidt in sommige gevallen voor organisch chemici tot nietszeggende termen.

   Arctander, S. (1969). Perfume and flavor chemicals. Volume I.

6. Er is onvoldoende bewijs voor de aanname van Laatsch *et al.* dat de bromering van phenylpyrrolen met behulp van het chloorperoxidase uit *Streptomyces aureofaciens* Tü 24 plaatsvindt in de actieve holte van het enzym.

Tweddel *et al.* (1994). Enzymatische Bromierung von Pseudilinen und verwandten Heteroarylphenolen mit der Chlorperoxidase aus *Streptomyces areofaciens* Tü 24. *Liebigs Ann. Chem.*, 65-71.

7. Meer adviezen of een groter aantal begeleiders tijdens een promotie leidt niet altijd tot een snellere promotie.

8. In de Amsterdam Arena is groen gras geen pleonasme.

9. Bij een vierdaagse werkweek verschuift het vrijdagmiddaggevoel naar de donderdagmiddag.

10. In de organisch chemische wereld is bekend dat enantiomeren een ander gedrag vertonen wanneer deze zich in een chirale omgeving bevinden. Echter, dat het energie kost en moeilijk is om een linkshandige op dezelfde wijze te laten schrijven als een rechtshandig persoon is helaas niet bij iedereen bekend.

11. Het uitbetalen van schadevergoedingen door sigarettenfabrikanten aan rokers die een ongeneeslijke ziekte ten gevolge van roken hebben opgelopen is verspilde moeite.

12. Vrouwen weten het beter

New Scientist, February 1, 1997; Applied Cognitive Psychology (in press); Intermediair, 20 februari 1997; The Sunday Times, February 2, 1997.

Stellingen behorende bij het proefschrift:
"Lipase-catalyzed kinetic resolution of branched chain fatty acids and their esters
A study towards the production of enantiopure 4-methyloctanoic acid"

Nicole W.J.T. Heinsman
Wageningen, 25 oktober 2000

met dank aan Marcel Verhoeven, Marcel van Aar & Maurice Franssen.

Sheep's Milk Ice Cream

Ingredients:

1 ½ cup sheep's milk or cow's milk with one drop of 4-methyloctanoic acid
½ cup light brown sugar
1 bag of vanilla sugar
pinch of salt
7 egg yolks
¼ cup granulated sugar
2 ¼ cups cream

Combine the milk, brown sugar, vanilla sugar and salt in a medium saucepan. Cook over moderately low heat, stirring occasionally, until the milk is steaming, about 8 minutes; do not let it boil. Remove from the heat and keep warm. Meanwhile, in a bowl, whisk the egg yolks with the granulated sugar until thick and pale. Whisk in half of the warm milk, then whisk in the remaining milk. Return the mixture to the saucepan and cook, stirring frequently, until the custard thickens slightly and coats the back of a spoon, 8 to 10 minutes. Stir in the cream. Strain the custard into a bowl and chill thoroughly. Transfer to an ice cream maker and freeze until use.

Ter nagedachtenis aan opa
Voor mijn ouders
Aan Marcel

Voorwoord

Zoals jullie weten bevat een proefschrift ofwel een voorwoord of een nawoord. Ik heb besloten om iedereen te bedanken in het voorwoord zodat niemand veel hoeft te bladeren om dat meest interessante stukje van het proefschrift te lezen.

Wie de voorkant van het proefschrift goed bekeken heeft zal zich afvragen hoe de schapen in verband te brengen zijn met de titel van het proefschrift. Collega's zullen het meteen begrijpen, de anderen zal ik het nog even uitleggen. De verbinding 4-methyloctaanzuur, waarmee ik heb gewerkt, is een geur- en smaakstof die onder andere voorkomt in schapenvlees en schapenkaas. Bij hoge concentraties heeft deze verbinding evenals zijn ester een doordringende geur die niet even van je handen is te verwijderen door ze te wassen. Ik kreeg daarom ook regelmatig opmerkingen naar mijn hoofd geslingerd dat het weer stonk in de gang en op het lab. Hoewel ik geen liefhebber ben van schapenvlees, en al helemaal niet van schapenkaas, ben ik toch van de geur gaan houden. Sorry mensen, voor de overlast.

Het voorwoord is natuurlijk dé gelegenheid om mensen te bedanken die een bijdrage hebben geleverd aan het tot stand komen van dit boekje, want de kreet "In Nijmegen studeer je niet alleen" kun je natuurlijk doortrekken naar "In Wageningen promoveer je niet alleen". Voor de dagelijkse begeleiding, de mogelijkheid om dit onderzoek uit te voeren, de waardevolle discussies en het vele correctiewerk bedank ik Albert en Maurice en mijn promotoren Klaas en Aede. Remko, jou wil ik ook nog speciaal bedanken voor de bijdrage aan hoofdstuk 4.

Aangezien mijn project een samenwerkingsverband was tussen organische chemie en proceskunde heb ik mijn vier jaren opgesplitst zodat ik de sfeer bij beide vakgroepen kon proeven. De eerste twee jaren heb ik doorgebracht in het souterrain van organisch. Allereerst zat ik met Dennis op de kamer, je was een echte Hagenees, maar wel één met een klein hartje. Al snel nadat jij weg was kreeg ik een nieuwe kamergenoot en jawel, nu een rasechte Rotterdammer. Matthew, hoewel we maar een korte tijd samen op één kamer hebben gezeten stelde ik je aanwezigheid erg op prijs.

Tijdens mijn aio-tijd ben ik in de gelukkige positie geweest om 6 studenten te begeleiden. Chris Marcelis, vanaf mijn allereerste dag was jij er. Jij hebt mij van alles geleerd over het werken met enzymen en ik heb je de kneepjes van de synthese

bijgebracht. Jouw werk staat beschreven in hoofdstuk 2. Ana Teixeira, you were my second student and a very good one. Part of your work is also presented in chapter 2 and 3. Na Ana kwamen Oscar Brugman en Paul van der Weide. Oscar, jouw werk aan malonesters is jammer genoeg niet in het proefschrift opgenomen. Paul, jij kwam bij organische chemie een afstudeervak doen toen mijn onderzoek in een flinke dip was beland. Maar ondanks alle tegenslagen heb je heel veel geprobeerd, inzet getoond en nieuwe ideeën aangevoerd. Ik was dan ook erg blij dat je tegelijk met mij naar proceskunde bent verhuisd om ook daar een afstudeervak bij mij te doen. Jouw bijdrage is verwerkt in hoofdstuk 6. Dankzij jouw enthousiasme kwam ook Henry Smienk een afstudeervak doen bij organische chemie. Henry, je liet al snel weten dat je graag zelfstandig werkt, dit is je goed afgegaan. Jij hebt laten zien dat topsport en een afstudeervak prima samen kunnen gaan. Ik heb er veel bewondering voor. Je hebt in ieder geval één keer de baas over mij mogen spelen tijdens één van onze volleybal trainingen. Een deel van jouw werk staat beschreven in hoofdstuk 5. In april 1998 Ana Valente visited Wageningen to work with me for 5 months. Ana you were fast in learning. All the unexpected results are now explained in chapter 4. Thank you for staying in your apartment during our visit to Portugal. We had a great time there.

Ik kan niet iedereen persoonlijk bedanken, dan zou ik een paar pagina's extra nodig hebben voor het voorwoord, vandaar dat ik een paar mensen in het bijzonder noem, maar ieder ander die hier niet bij naam genoemd is wordt bij deze ook bedankt voor de leuke tijd die ik op beide vakgroepen heb gehad. Bij organische chemie: Elbert, Pim, Harm, Bep, Hugo, Kees en Maarten, jullie wil ik bedanken voor de hulp bij GC/ HPLC, NMR, elementanalyse en 'massa'. Jurrie, Gert, Pleun en Ronald, bedankt voor al het (glas)blaaswerk, glaswerk en de chemicaliën die ik bij jullie gehaald heb. Het was altijd weer gezellig om 'even' met jullie te kletsen. Elly, Ineke, Ien, Wikje en Daniëlle van het secretariaat, bedankt voor de administratieve hulp. Bart, het was een grote schok te horen dat je er ineens niet meer was. Ik had je graag ook een boekje willen geven. Hannie en Gerrie, jullie zorgden ervoor dat wij altijd een schone werkplek hadden.

Tijdens het wekelijks uurtje zwemmen met Arie en Cindy N. kreeg ik alvast de mogelijkheid een selecte groep van de sportieve proceskunde vakgroep beter te leren kennen. Na twee jaar OC verruilde ik mijn kelderplek voor een kamer bij proceskunde op de 6^{e} verdieping van het biotechnion, alwaar ik toch iets verder weg kon kijken als ik

even inspiratie op wilde doen. Ik werd goed ontvangen op kamer 615, mede dankzij de brabantse gezelligheid van Suzanne en Marco, maar ook Ellen, Rolf (de computer expert) en Marieke (altijd in voor een praatje) hebben hier een belangrijke bijdrage aan geleverd. Later werd de groep nog uitgebreid met Marcel, Patricia en Nick. Pieter, het was erg prettig om van je Matlab specialiteiten gebruik te maken. Jos, jij hebt mij de weg gewezen in het lab op het 2e bordes, bedankt voor de experimenten die je voor mij hebt uitgevoerd. Joyce, Hedy en Lotte, ook jullie bedankt voor de administratieve zaken en de gezellige gesprekken. Hugo, de laatste afrondingen van mijn proefschrift heb ik uitgevoerd bij OC en wel bij jou op de kamer. Je was een gezellige kamergenoot.

De medewerkers van de bibliotheek, mediaservice, fotolokatie, werkplaats, kantine en automatisering wil ik hartelijk danken voor hun bijdrage.

Christian Orrenius, I specially want to thank you for your contribution to chapter 2. Yuri Belov (Supelco), many thanks for your willingness to test my compounds on your chiral columns. I used the chiral γ-dex a lot.

Zonder IOP-katalyse was deze aio-baan er niet geweest, ik bedank daarvoor ook met name Ward Mosmuller, Merlijn van Rijswijk en de heer J. Oelderik voor het initiatief dat IOP genomen heeft om de industrie en universiteit dichter bij elkaar te brengen. Ik heb ook met veel plezier aan jullie cursussen deelgenomen. Binnen het hydrolase cluster wil ik de begeleiders vanuit de industrie, de begeleiders van de universiteit en de collega AIO's bedanken voor de samenwerking. Cor Niedeveld, ik wil jou in het bijzonder bedanken voor de prettige samenwerking tijdens de afgelopen vier jaren. Het was een leuke ervaring om mijn stofje te proeven in een soep en smeerkaas.

Tijdens de laatste drie jaar van mijn aio-tijd was de 'reis' van Wijchen naar Wageningen een stuk gezelliger mede dankzij mijn carpoolmaten Frans Geurts, Willem van Berkel, Desiree Barten, Janneke Siebenga, en Dannie de Kleijn.

Mam, aan jou wil ik ook een woordje richten. Jij hebt ons altijd gestimuleerd om datgene te doen wat wij leuk vonden. Hoewel je vaak niet veel begreep van hetgeen waar ik mee bezig was heb je altijd veel interesse getoond.

Nu ik hopelijk iedereen gehad heb wil ik mijn laatste woord richten tot Marcel. Mars, je hebt mijn schrijfwerk regelmatig doorgelezen en van kritiek voorzien. Ik heb er veel van geleerd. Onze chemische binding is er niet zwakker door geworden.

Nicole

The role of IOP-Catalysis

The Dutch chemical industry is very interested in catalytic conversions because the use of catalysts reduces waste streams and production of undesired by-products. In addition, the use of catalysts saves energy and feedstocks. Nowadays more than 80% of the total volume of chemicals is produced using catalysts. However, in almost all cases, these catalysts are applied in the petroleum industry and the bulk chemistry. Therefore the Dutch ministry of economic affairs brought the program committee Innovation Oriented research Program on Catalysis (IOP-Catalysis) into being in 1989. The efforts of IOP-catalysis were directed to the introduction of novel catalytic routes in the fine chemical industry and to the precision in chemical conversions. With the intention to stimulate collaboration between universities, research institutes and the Dutch industries, IOP initiated research on catalysis by funding a number of projects, performed by graduate students and post-docs. Besides the transfer of knowledge and the synergistic contacts, these projects also provide a sense of industrial reality.
The program committee (PC) of IOP-catalysis is responsible for the planning and implementation of the IOP-catalysis research program. This PC consist of members from industry and university under the leadership of dr. ir. J.M. Oelderik.

Within the IOP-Catalysis three main research groups can be distinguished:

1 Catalyst preparation and reactor technology
2 Heterogeneous and homogeneous catalysis
3 Biocatalysis

The work described in this thesis belongs to the third category: biocatalysis. Twice a year this work was presented to a panel of industrial and academic experts (see below) and to other IOP-supported graduate students who study the application of hydrolytic enzymes (the 'hydrolase cluster'). The industrial relevance of the current project will be discussed in chapter six and seven of this thesis.

Chairman of the 'hydrolase cluster'

dr. G.J.M. van Scharrenburg (Solvay Pharmaceuticals, Weesp)

Industrial Experts

ir. C.J. Niedeveld (IFF Tilburg)

ing. H. Kierkels (DSM, Geleen)

dr. B. Kaptein (DSM, Geleen)

dr. E. de Vroom (Gist-brocades, Delft)

dr. H. Weenen (Quest, Naarden)

dr. ir. F.P. Cuperus (ATO-DLO, Wageningen)

Academic Experts

prof. dr. Ae. de Groot, prof. dr. ir. K. van 't Riet, prof. dr. ir. R.M. Boom, dr. M.C.R. Franssen, dr. ir. A. van der Padt (WAU)

prof. dr. H.M. Verheij (†), prof. dr. M. Egmond (UU), dr. A.J. Slotboom (UU)

Prof. dr. R.M. Kellogg, prof. dr. B.L. Feringa (UG)

dr. ir. J.A. Jongejan (TUD)

Graduate Students

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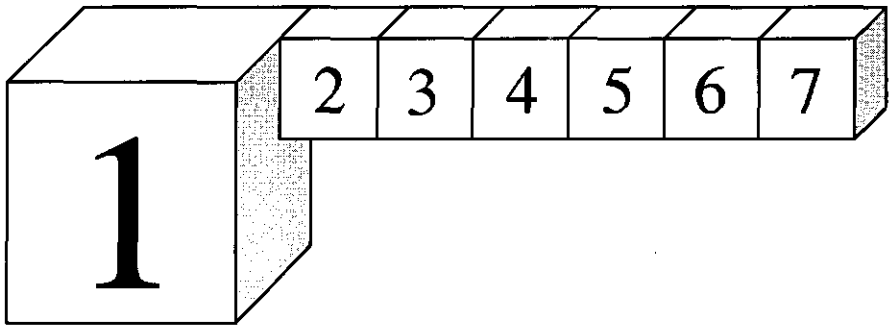
Dr. B.C. Koops (94005), UU

Dr. A. Cuiper (94042), RUG

Dr. ir. P.L.A. Overbeeke (94021), TUD

Contents



General introduction

SUMMARY

In this chapter a number of topics related to the research on the kinetic resolution of 4-methyloctanoic acid are discussed. In the first section, flavors & fragrances, *different types and examples of flavor and fragrance components are summarized. In the section* chirality in flavors & fragrances *the importance of chirality is emphasized. Connected to these subjects, the* branched chain fatty acids *are introduced. The occurrence and application of different acids is given. In the section* chiral synthesis and enantiomer separation *various techniques to obtain optically pure products are discussed. Because the research described in this thesis was performed using enzymes and in particular lipases, the next two paragraphs* (enzymes, lipases) *describe the pros and cons of enzymes, the structure and mechanism of lipases and lipase kinetics. In the next section* (sequential kinetic resolution) *it is shown that a two step reaction (esterification followed by hydrolysis) will lead to a higher enantiomeric excess compared to one reaction. In the section* Integration of reaction and separation *two possibilities are given for the production of enantiopure branched chain fatty acids. Finally, the* objective and justification *of the research and the* outline of the thesis *are presented.*

Flavors & Fragrances

The olfactory sense

Nature is full of flavors and fragrances, present in many living organisms like flowers, fruits, nuts and animals (Fenaroli (1994) and Claassen (1995)). The word flavor or fragrance refers to the effect perceived by the olfactory sense. This olfactory sense is one of the most important senses. To experience a flavor or fragrance by the olfactory sense, a certain threshold value has to be reached. This value varies from individual to individual (Ekborg-Ott and Armstrong, 1997). One of the important characteristics of the olfactory system of humans is selectivity; each person has its own odor and flavor sensation (salt, sweet, sour and bitter). These basic flavor perceptions may be altered, modified or completely masked by accessory responses (*e.g.* coolness, poignancy, astringency or texture). Since the sense of smell is closely related to the memory, a flavor or fragrance perception might also influence someone's behavior, emotion or elementary passion (food & sex). Finally, the olfactory system is also capable of warning us for dangerous situations (*e.g.* fire or rotten food products).

History of flavors and fragrances

The food ingredients *(i.e.* proteins, carbohydrates, fats and vitamins) that we need for a healthy life have a poor taste. The primitive people who used to eat plants and raw meat also experienced this. After the discovery of fire, they found out that heating of the food resulted in a better taste and better digestible products. These people also discovered preservation techniques such as drying, smoking, salting, bottling and marinating, necessary for survival during periods when fresh food was in shortage. Later, primitive people also added flavor ingredients (*e.g.* herbs, spices, honey, and salt) to the food during cooking to enhance its taste. After the introduction of distillation and extraction techniques, a more functional use of aromatic substances was realized. In the beginning of the nineteenth century the first small flavor and fragrance companies started with the processing of herbs, spices, fruits, ethereal oils, sticks of vanilla and other natural products. In 1870 the

first essence company was established in the Netherlands. Later the first fruit flavors were produced synthetically and many other flavors and fragrance ingredients have been introduced to the market since then.

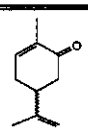
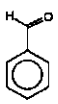
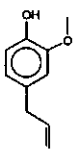
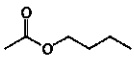
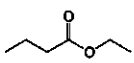
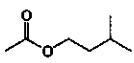
Types of flavors and fragrances

Most flavors are a fragrance at the same time. One flavor or fragrance usually consists of about hundred different chemical compounds. To obtain a single flavor ingredient, several techniques can be used. Traditionally the flavor and fragrance chemicals were obtained by chemical synthesis, isolation from natural sources (plants, meat) or fermentations. Nowadays, the consumers' demand for the production of natural flavors or fragrances has stimulated industries to use enzymes or micro-organisms for the production of these chemicals (Cheetham, 1997). The flavor ingredients obtained by these techniques can be classified into three categories:

1. *Natural flavors / fragrances*: chemical compounds having a natural origin.
2. *Nature identical flavors / fragrances*: synthetically produced compounds, which are structurally identical to the chemical compounds isolated from natural substances.
3. *Artificial or non-nature identical flavors / fragrances*: Products that originally are not present in food products. These compounds are only allowed when they are on a 'positive list', *e.g.* the flavors in Coca Cola.

A wide range of chemicals is known to be a flavor or fragrance compound. In table 1 examples are shown of different types of flavor or fragrance compounds, their presence in nature and their reported use.

Table 1: Flavors & fragrances present in nature and used in applications

component	name	natural occurrence and reported use
	carvone[1]	caraway seed (*S*-carvon), spearmint (*R*-carvon), perfumes, liqueurs, bread, candies, frozen dairy.
	benzaldehyde[1,2]	almonds[3], apricot, kernel[3], peach[3], hyacinth, cinnamon, ice-cream, chewing gum, fruit juice, baked goods.
	anethole[1]	fennel, pine oil, (star)anise, vinegar, alcoholic beverages, chewing gum, baked goods.
	eugenol[1]	cloves, laurel, cinnamon, chewing gum, confection, frosting, condiment, relish, meat products.
$C_3H_6O_3$	lactic acid	yogurt[4]
$C_2H_4O_2$ - $C_{10}H_{20}O_2$	acetic - capric acid	cheese[5]
$C_{18}H_{32}O_2$	linoleic acid	peanuts[6]
$C_{18}H_{34}O_2$	oleic acid	peanuts[6]
$C_{20}H_{40}O_2$	eicosanoic acid	peanuts[6]
	n-butyl acetate	fruity apple[5]
	ethyl butyrate	pineapple[5]
	isoamyl acetate	banana[5]

[1] (Fenaroli, 1994), [2] (Nakazawa *et al.*, 1981), [3](benzaldehyde present as amygdalin), [4](Laye *et al.*, 1993), [5](West, 1996), [6](Hashim *et al.*, 1993), [7](Ranadive, 1992).

Why are flavors and fragrances used?

The majority of the consumed flavor and fragrance compounds are already present in the food products. Only a small fraction (0.05%) is present as an additive.

To answer the question why we still add flavor and fragrance ingredients to food products while nature produces its own flavors and fragrances, three reasons can be mentioned. Firstly, the techniques used to conserve food products, like pasteurization, sterilization, canning and concentrating result in loss of aroma. Addition of a flavor or fragrance can compensate for this loss. Secondly, some products are tasteless, for example soy. Addition of a flavor to these products makes them more attractive. Finally, the demand for some flavor products, *e.g.* strawberries, exceeds the world market since not enough strawberries are available during the year. In such cases flavors are added to products, *e.g.* strawberry ice cream and pudding, to enhance the taste of these foodstuffs. In this manner flavors and fragrances are added to products like soda, liqueurs, ice cream, dairy products and bakery products.

Chirality in flavors & fragrances

In table 1 examples were given for different types of flavor & fragrance compounds. In addition to the diversity in chemical structures, there is another

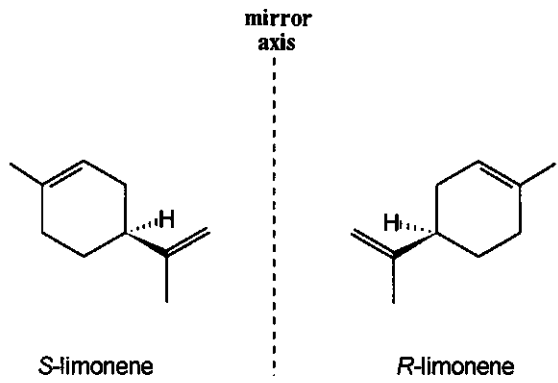


Figure 1: Mirror images of *R*- and *S*-enantiomer of limonene

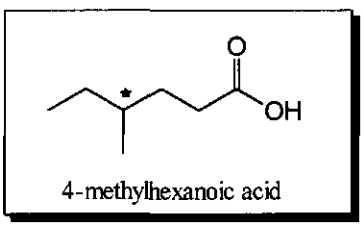
factor which is very important: chirality (Ahuja, 1997). The majority of the flavors and fragrances are chiral. Often the enantiomers have different sensory properties, for example limonene (figure 1, Boelens *et al.*, 1993). The *S*-enantiomer of limonene smells like lemon while the *R*-enantiomer has the odor of oranges. In the next section the occurrence and organoleptic characteristics of chiral branched chain fatty acids are given. Two of these compounds, *i.e.* 4-methylhexanoic acid and 4-methyloctanoic acid, were used for the research described in this thesis.

Branched Chain Fatty Acids

The chiral branched chain fatty acids (BCFAs) have been recognized as important flavor components. Table 2 summarizes the organoleptic characteristics, the natural occurrence and reported use of the alkyl-substituted C_3 to C_9 fatty acids and their derivatives. From the table it is clear that many BCFAs are involved in the cheese making process. In addition to this, Hallet and co-workers (1995) published that 4-methylalkanoic acids and esters are also pheromones for male coconut rhinoceros beetles, *Oryctes rhinoceros* (L.).

Two branched chain fatty acids (figure 2) were used for the research described in this thesis.

The stereoisomers of BCFAs have different sensory properties (Boelens *et al.*, 1993). Therefore it is worthwhile to separate these enantiomers. The different techniques to obtain chiral compounds in an optically pure form will be discussed in the next section.



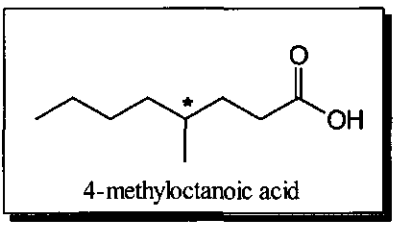


Figure 2: Chemical structures of 4-methylhexanoic and 4-methyloctanoic acid.

Table 2: Branched chain fatty acids

BCFA	organoleptic characteristics[1]	Natural occurrence and reported use
2-methylpropanoic acid		coffee[2,3], wine[4], bread[5]
3-methylpropanoic acid		bread[5]
2-methylbutanoic acid	pungent, acrid odor similar to Roquefort cheese: acrid taste; at low dilutions pleasant, fruity taste	angelica root oil[1], coffee[1,2,3], apples[6,7,8,9], brambles[10], papaya[7], cloudberries[10], strawberries[7,11,12], pineapple[7,8], fermented soy bean products[13,14], cheese (parmesan, French soft)[1,7,15,16], alcoholic beverages[7,9], apple products[7,9], puff pastries[17], black current juice[7], tobacco leaves[18].
2-methylbutanoates[a]		petunia flowers[19], lavender oil[1], Pimpinella anisum[20], apples[6,8,9], apple products[9], pineapples[8].
3-methylbutanoic acid		coffee[2,3], soy sauce[13], cheese (goat, parmesan)[15,21,22], wine[4], baked goods[5,17], tobacco leaves[18].
3-methylbutanoates[a]		Petunia flowers[19].
2-ethylbutanoic acid	faint, somewhat pleasant	parmesan cheese[15,16], baked goods[1], frozen dairy[1], pudding[1].
3-methylpentanoic acid	herbaceous, slightly green odor, sour	tobacco leaves[1,18], cheese (parmesan)[1,15], reconstituted vegetables[1], coffee[3], baked goods[1], candies[1].
4-methylpentanoic acid	unpleasant sour, penetrating	apple[1], barley[1], beef[1], cheese (Emmenthaler, camembert, parmesan)[1,15], cocoa beans[1], dry cured ham[1], hops[1], pecans[1], alcoholic beverages[1], soy beans[1], black tea[1], dairy products[1], condiment[1], pickles[1].
4-methylpentanoates[a]		dry cured ham[23].
2-methylhexanoic acid		baked goods[1], soft candies[1].
4-methylhexanoic acid		Italian cheese[15,16], mutton[22], tobacco leaves[18].
4-methylhexanoates[a]		Petunia flowers[18], dry cured ham[23] tobacco leaves[24].

Table 2 continued

5-methylhexanoic acid	fermented cheese odor	pork[1], strawberry[1], tea[1], baked goods[1], soups[1], snacks[1], imitation dairy[1].
5-methylhexanoates[a]		tobacco leaves[24,25], Petunia flowers[18]
2-ethylhexanoic acid		coffee[3], parmesan cheese[15], apples[8], pineapples[8].
2-methylheptanoic acid	fatty sour odor (rancid like) sour fruity, nut like flavor	baked goods[1], frozen dairy[1], gelatin[1], pudding[1], soft candies[1].
5-methylheptanoates[a]		tobacco leaves[24,25].
6-methylheptanoates[a]		Petunia flowers[18], tobacco leaves[24,25]
4-ethylheptanoic acid		parmesan cheese[15].
4-methyloctanoic acid	fatty, musty, plastic odor, sheepy notes, goaty	mutton[26-33], goat meat[27,29], sheep/goat cheese[21,22,34-36], sheep/goat milk[35,37], Italian cheese[15,16], frozen dairy[1], snack foods[1].
4-methyloctanoates[a]		Wine[38]
4-ethyloctanoic acid		goat meat, mutton[27,29,33], cheese (goat, parmesan)[16,21,22,34,35].
4-methylnonanoic acid		parmesan cheese[15,16], mutton[26,29-33], baked goods[1], snack food[1], imitation dairy[1].
8-methylnonanoic acid		parmesan cheese[15].

[a] Esters of the corresponding fatty acids.

[1](Fenaroli, 1994), [2](Schroder *et al.*, 1997), [3](Wohrman *et al.*, 1997), [4](Moret and Gambaro, 1996), [5](Hansen and Hansen, 1996), [6](Schumacher *et al.*, 1998), [7](Mosandl *et al.*, 1990), [8](Rettinger *et al.*, 1991), [9](Rettinger *et al.*, 1990), [10](Pyysalo *et al.*, 1977), [11](Wintoch *et al.*, 1991), [12](Barbeni *et al.*, 1992), [13](Ishihara *et al.*, 1996), [14](Tanaka *et al.*, 1998), [15](Ha and Lindsay, 1990), [16](Ha and Lindsay, 1991), [17](Gassenmeier and Schieberle, 1994), [18](Chuman and Noguchi, 1977) [19](Son *et al.*, 1994), [20](Karl *et al.*, 1992), [21](Le Quere *et al.*, 1998), [22](Le Quere *et al.*, 1996), [23](Barbieri *et al.*, 1992), [24](Ohya *et al.*, 1994), [25](Matsuzaki *et al.*, 1992), [26](Young *et al.*, 1997), [27](Ha and Lindsay, 1990), [28](Johnson *et al.*, 1977), [29](Wong *et al.*, 1975), [30](Sutherland and Ames, 1996), [31](Sutherland and Ames, 1995), [32](Brennand and Lindsay, 1992), [33](Brennand and Lindsay, 1993), [34](Pierre *et al.*, 1998), [35](Lamberet *et al.*, 1996), [36](Ha and Lindsay, 1991), [37](Ha and Lindsay, 1993), [38](Simpson and Miller, 1984).

Chiral Synthesis & enantiomer separation

Enantiopure compounds can be prepared according to several methods that are presented in figure 3 (Crosby, 1991, 1992; Koskinen, 1993; Sheldon, 1993; Ahuja, 1997).

A. The 'chiral pool' is a general term for the many optically pure compounds present in nature such as amino acids, sugars and terpenes. These compounds can be used as starting material in the synthesis of new optically active products.

B. Asymmetric synthesis is another way to produce enantiomerically pure compounds. In this way, a prochiral substrate is converted chemically or enzymatically to a chiral product with a high degree of efficiency (Crosby, 1991, 1992; Koskinen, 1993). The ways of introducing chirality can be subdivided into three groups:

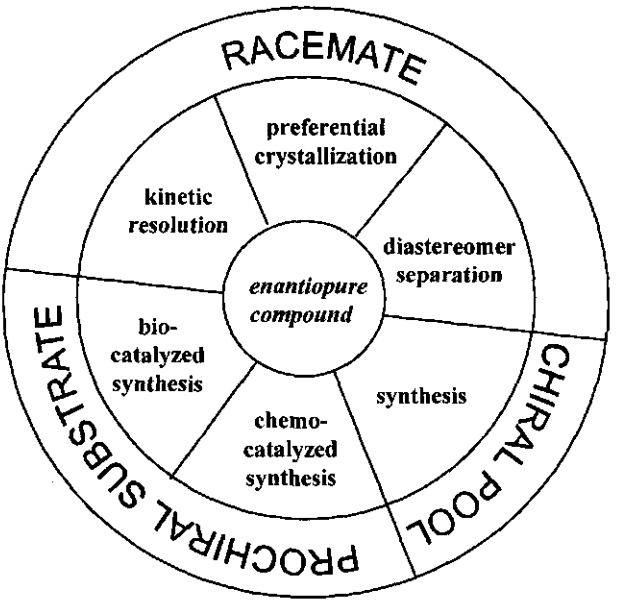


Figure 3: Synthesis of chiral compounds

1. *internal asymmetric induction:* a new chiral center is introduced into the molecule, induced by an already present chiral center.
2. *relayed asymmetric induction:* the chiral center is introduced into the molecule by chiral auxiliaries.
3. *external asymmetric induction:* the chiral center is introduced into the molecule by chiral catalysts. These can be either chemical catalysts or biocatalysts.

External asymmetric induction is economically the most desirable method since only a catalytic amount of a chiral catalyst is needed to introduce the chiral center into a molecule. This catalyst can be recovered after the reaction and is reusable for another batch reaction.

C. The third strategy involves the resolution of racemates. Racemates contain equal amounts of enantiomers and are therefore optically inactive. They also have identical physical properties in an achiral environment. To obtain optically pure compounds, it is necessary to separate these enantiomers. Methods of enantiomer separation can be divided into three categories.

1. One of the approaches is the conversion of enantiomers into diastereomers. Often chiral carboxylic acids are converted into diastereomers by formation of diastereomeric salts. However, it is also possible to use covalent adducts to reach this goal. As a result of different physical properties, these diastereomers can be separated, either by chromatography or by crystallization (Crosby, 1991, 1992; Sheldon, 1993). After separation the original enantiomerically pure compound has to be recovered from the diastereomer.
2. Preferential crystallization is a technique involving the seeding of a supersaturated solution of the conglomerate (*i.e.* equimolar mixture of two crystalline enantiomers) with one enantiomer which induces crystallization of that enantiomer (Jacques *et al.*, 1981; Sheldon, 1993; Ebbers, 1996).

3. Kinetic resolution is a separation technique based on the difference in reaction rates of the enantiomers with a chiral entity. From an economical point of view the entity should be a catalyst (Crosby, 1991, 1992; Sheldon, 1993; Schreier, 1997). This may be a chemical catalyst (chiral metal complex, chiral acid or base) or a biocatalyst (enzyme or microorganism). Kinetic resolution using biocatalysts meets the requirements of the flavor and fragrance industries, *i.e.* using food grade chemicals and catalysts, and therefore it is an attractive method to produce enantiomerically enriched compounds from racemates.

Enzymes

Throughout the ages fermentation processes have been used for brewing of beer, baking of bread and the production of alcohol and dairy products. Much later it was realized that microorganisms influenced these fermentation processes. Microorganisms produce a large variety of enzymes, most of which are produced only in small amounts and are involved in cellular processes. However, some enzymes are produced in large amounts by fungi and bacteria and are excreted into the medium. Enzymes became more and more important in this century. Nowadays enzymes like lipases, proteases, pectinases and amylases are used on large scale for applications in the textile industry, leather industry, pharmaceutical industry (penicillin production), dairy industry, paper and pulp industry and the

Table 3: Pros and cons of the use of enzymes[1]

Merits	Demerits
catalyze a broad spectrum of reactions	one enantiomeric form in nature
efficient	narrow operation parameters
act under mild conditions	highest activity in water
environmentally acceptable	accessible for inhibition effects
compatible with each other	may cause allergies
not bound to their natural role	

[1](Faber, 1997)

detergent industry (Brock and Madigan, 1988; Tramper, 1994; Godfrey and West, 1996). The use of enzymes does have a lot of advantages and some disadvantages, which are presented in table 3.
As already mentioned, lipases are produced on a large scale for different applications. In the next section the function of lipases, their structure and kinetics is described.

Lipases

Lipases (EC.3.1.1.3) are known as glycerol ester hydrolases. Their natural function is the hydrolysis of fats to produce fatty acids and glycerol (Bühler and Wandrey, 1987; Jensen *et al.*, 1990; Mukherjee, 1990). Besides, several other reactions are catalyzed by lipases (Cambou and Klibanov, 1984; Kloosterman *et al.*; 1988) such as hydrolysis of unnatural esters (Holmberg *et al.*, 1989; John and Abraham 1991), synthesis of esters (Seino *et al.*, 1984; Sonnet and Welch Baillargeon, 1987; van der Padt *et al.*, 1992; Valivety *et al.*, 1993), transesterifications (Zaks and Klibanov, 1985; Klibanov, 1990; Carrea *et al.*, 1992; Bovara *et al.*, 1993) and interesterifications (Balcâo, 1996; Faber 1997).
Lipases are very popular biocatalysts because of their availability, stability and applicability (Elferink *et al.*, 1991; Faber and Franssen, 1993). Two examples of the application of lipases in biotechnology are the involvement of lipase in the production of naproxen and ibuprofen, two anti-inflammatory drugs (Sheldon, 1993).

Structure of lipases

In the last decade the structures of different lipases and esterases have been solved (Brady *et al.*, 1990; Winkler *et al.*, 1990; Ollis *et al.*, 1992; Kazlauskas, 1994; Uppenberg *et al.* 1994). The proteins studied differ in size and the primary structures of these proteins have widely varying sequences. However, all lipases and esterases are folded in a similar way. Ollis and coworkers (1992) identified this fold as an α/β hydrolase fold consisting of a core of parallel β-sheets, flanked

on both sides by α-helices and a central catalytic domain. This domain is composed of a catalytic triad of Ser, His and either Glu or Asp and several oxyanion stabilizing residues. The catalytically active serine residue is situated in a hairpin loop between an α-helix and a β-sheet. The specific arrangement of the catalytic triad entails a lowering of the pK_a of the serine residue facilitating its nucleophilic attack on the carbonyl group of the substrate. In figure 4 the mechanism of lipase catalysis is given. The hydrolytic action of lipases can be described by a two-step reaction mechanism, referred to as the Bi Bi Ping Pong mechanism (Rakels *et al.* 1994; Martinelle and Hult, 1995). When substrate 1 ($R_2CO_2R_1$) enters the catalytic site of the free enzyme, a nucleophilic attack of the serine-OH residue takes place. The formed oxyanion is stabilized by several hydrogen bonds, facilitating the first transition state. After elimination of the R_1OH group, an acyl enzyme complex is formed, which can be attacked by a second nucleophile (R_3OH) giving another tetrahedral intermediate. After elimination of the product formed ($R_2CO_2R_3$), the free lipase is recovered, ready for a new cycle.

For most lipases the catalytic triad, which is close to the surface, but not directly accessible to the solvent, is covered with a helical segment, called the 'lid' (Brzozowski *et al.* 1991). This short α-helix is folded back when the lipase contacts an interphase of a biphasic polar/ apolar system (like water/ organic solvent). As a consequence, a conformational change of the lipase creates an active form of the enzyme. Thus a freely dissolved lipase resides in its inactive state in the absence of an aqueous/lipid interface (Sarda and Desnuelle, 1958; Patkar *et al.*, 1993; Egloff *et al.* 1995; Faber, 1997) and can be activated in the presence of this interface. Cutinase, Guinea pig pancreatic lipase (GPL) and *Candida antarctica* lipase B on the other hand do not show interfacial activation as a consequence of having a very small or no 'lid' (Martinelle *et al.*, 1995).

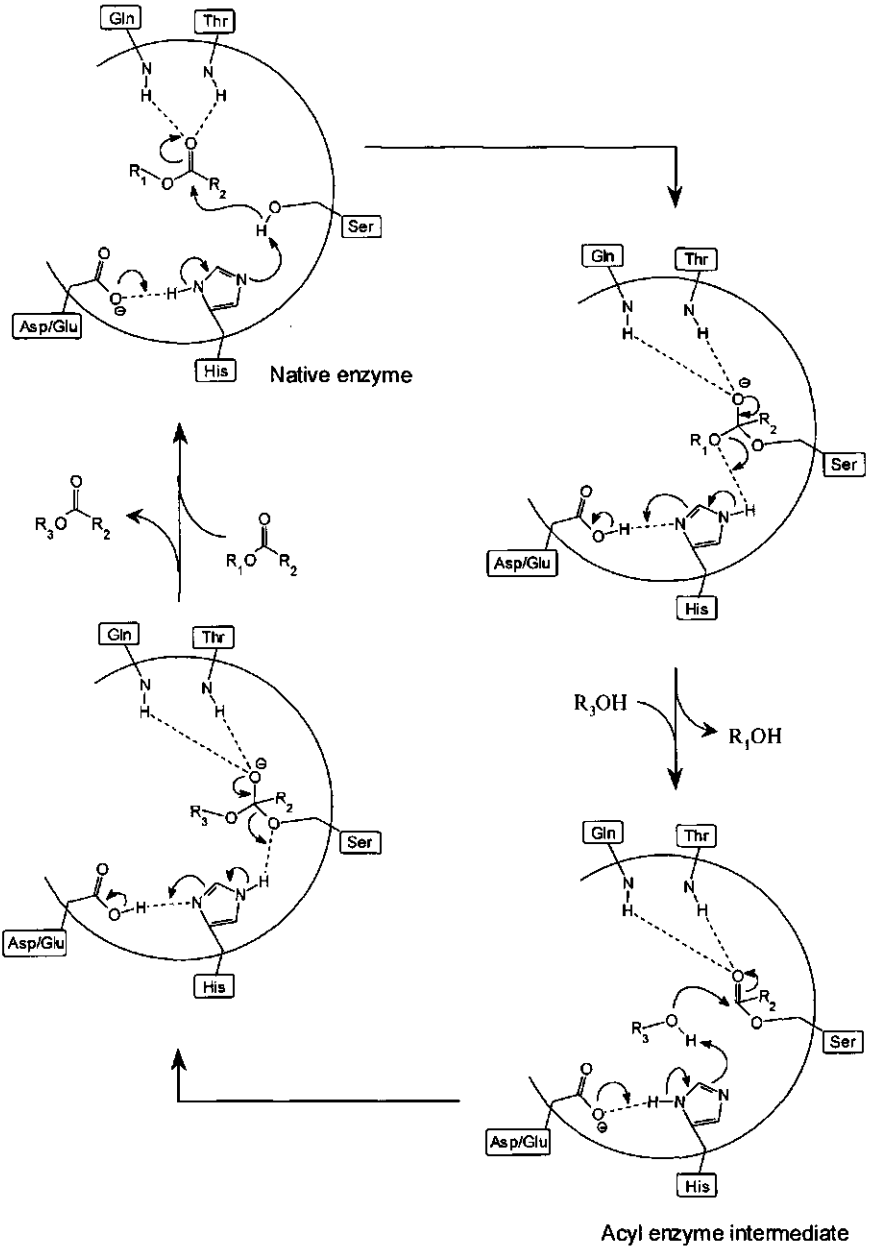


Figure 4: General mechanism of lipase catalysis. The catalytic triad is represented as Asp or Glu, His and Ser. The Gln and Thr function as oxyanion stabilizing residues.

Kinetics

Enzyme-catalyzed reactions can be quantitatively described (Cornish-Bowden, 1995). In the second half of the 19th century Michaelis and Menten introduced a simple model for the conversion of one substrate to one product:

$$\text{Enz} + \text{Sub} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{EnzSub} \xrightarrow{k_{cat}} \text{Enz} + \text{Prod}$$

In this model, the enzyme is represented by Enz, the substrate by Sub, the product by Prod and the enzyme-substrate complex by EnzSub. The kinetic parameters k_1 and k_{-1} are the rate constants representing the binding of substrate to enzyme and the release of substrate from the enzyme. The turnover number k_{cat} is the first-order rate constant of catalysis ($V_{max} = k_{cat} \cdot [Enz]_0$). The rate equation for the enzymatic reaction is $v = k_{cat}[EnzSub]$. Rewriting $[EnzSub]$ and replacing the terms $k_1/(k_{-1} + k_{cat})$, $[Enz]$ and $k_{cat} \cdot [Enz]_0$ by the reversed Michaelis constants $1/K_M$, $([Enz]_0 - [EnzSub])$ and V_{max} gives the well known Michaelis Menten equation:

$$v = \frac{V_{max}[Sub]}{K_M + [Sub]}$$

In this equation, K_M may be regarded as an apparent dissociation constant ($K_d = k_{-1}/k_1$) since the formation of the enzyme substrate complex is often rapid ($k_1 >> k_{cat}$). V_{max} is the reaction rate obtained under saturating substrate concentrations. The term V_{max}/K_M is called the specificity constant which equals the first order reaction constant at low substrate concentrations ($[Sub] << K_M$).

Often, the Michaelis Menten rate equation can also be used for chiral conversions. In this case the parameters V_{max} and K_M for the separate enantiomers have to be determined. To get information about the difference in rate for both enantiomers, the enantiomeric ratio (E) has been introduced:

$$E = \frac{V_{max}^{R} / K_{M}^{R}}{V_{max}^{S} / K_{M}^{S}}$$

The enantiomeric ratio E, a measure of the selectivity of the reaction, can thus be calculated by determining the rate constants for both enantiomers. However, when the enantiomers are not available in enantiopure form, other methods are available to calculate this E-value. For this purpose, Chen and coworkers (1982, 1987) mathematically linked the enantiomeric ratio E to conversion, enantiomeric excess and the equilibrium constant. For both irreversible (hydrolysis) and reversible ((trans)esterification) reactions the following equations are given in figure 5.

Irreversible reactions:

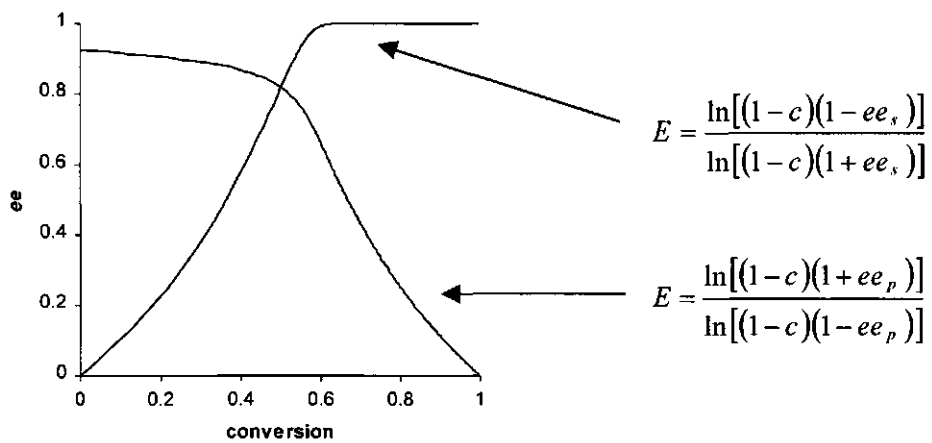
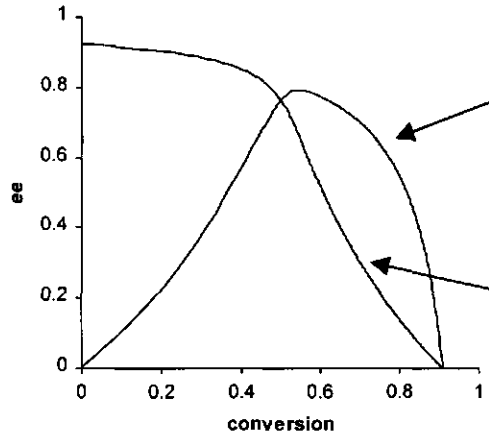


Figure 5a: Computer generated curves depicting the relationship between ee_s *versus* c and ee_p *versus* c for irreversible reactions (Chen *et al.*, 1982). Conversion; c = 1 – [Sub]/[Sub]$_0$, *ee* = enantiomeric excess of substrate (ee_s) or product (ee_p); *ee* =|R-S|/(R+S).

Reversible reactions:



$$E = \frac{\ln\left[1-(1+K)(c+ee_s\{1-c\})\right]}{\ln\left[1-(1+K)(c-ee_s\{1-c\})\right]}$$

$$E = \frac{\ln\left[1-(1+K)\cdot c(1+ee_p)\right]}{\ln\left[1-(1+K)\cdot c(1-ee_p)\right]}$$

Figure 5b: Computer generated curves depicting the relationship between ee_s *versus* c and ee_p *versus* c for reversible reactions (Chen *et al.*, 1982). Conversion; $c = 1 - [Sub]/[Sub]_0$, ee = enantiomeric excess of substrate (ee_s) or product (ee_p); $ee = |R\text{-}S|/(R+S)$, K = inverse of equilibrium constant K_{eq}; $K_{eq} = k_{-2}/k_2$ for Enz + Sub $\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}}$ Enz + Prod

It is assumed that the enantiomeric ratio remains constant in a reaction. For each catalyst under specific circumstances an E-value can be estimated.

Sequential kinetic resolution

The branched chain fatty acids used in this thesis have a chiral center positioned at the C_4 atom, which is remote from the reaction center. In case these substrates are esterified in a reaction catalyzed by enzymes, the enantioselectivity might be low (Wilson *et al.*, 1983). To enhance the enantiomeric excess, sequential kinetic resolution can be used (Chen *et al.*, 1982; Faber, 1997; Kroutil *et al.*, 1997; Vänttinen and Kanerva, 1997). In a two-step reaction (esterification followed by hydrolysis) the substrate is forced to enter the active site of the enzyme twice

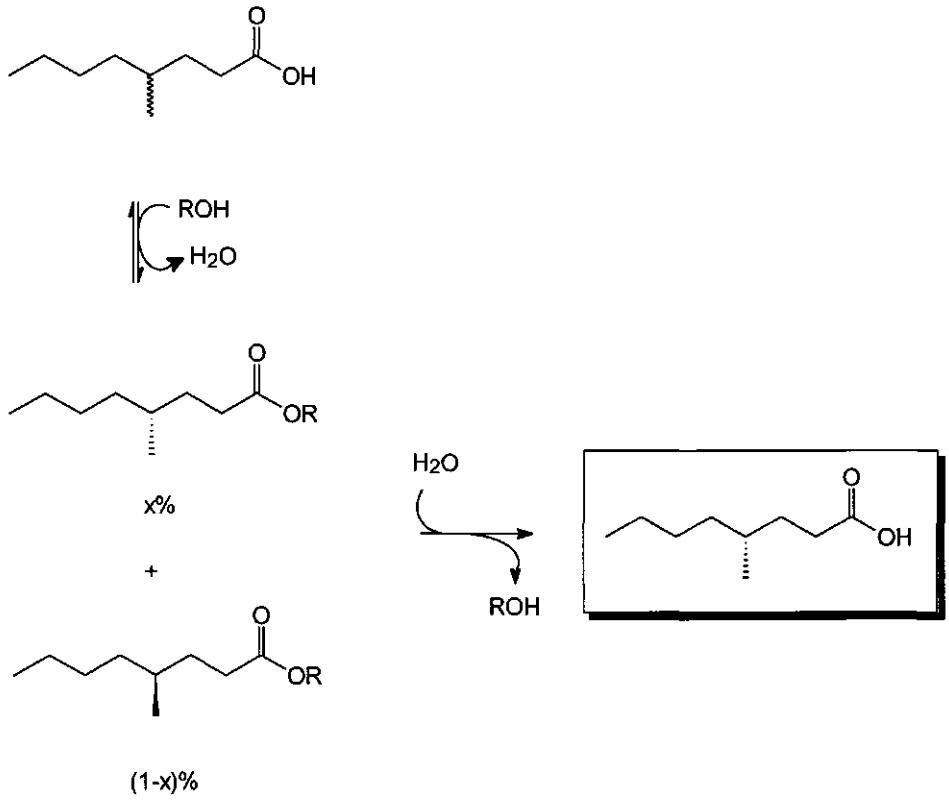


Figure 6: Sequential kinetic resolution for 4-methyloctanoic acid. After one reaction step (esterification) the product, consisting of x% *R*-enantiomer and (1-x)% of the *S*-enantiomer, can be isolated and used for hydrolysis. After two reaction steps the product may reach a high enantiomeric excess.

(figure 6). Suppose the enzyme used has a preference for the *R*-enantiomer. After one reaction step (esterification) the product consists of x% of the *R*-enantiomer and (1-x)% of the *S*-enantiomer, (2x-1)% then represents the enantiomeric excess of the product. If one reaction step is not enough to reach an acceptable enantiomeric excess of the product, the product can be isolated and used for a second reaction (hydrolysis). Starting with an enantiomerically enriched substrate (*R*: x%, *S*: (1-x)%) and using the same enzyme, thus having the same preference,

will lead to a product that is even more enriched with the *R*-enantiomer. For both the esterification and hydrolysis reaction, the enantiomeric ratio can be determined. The total enantiomeric ratio after two steps is approximately equal to half of the product of the values obtained in separate reaction steps (Faber, 1997):

$$E_{tot} \approx \frac{E_{esterification} \cdot E_{hydrolysis}}{2}$$

Objective & Justification

Branched chain fatty acids (BCFAs) like 4-methylhexanoic acid and 4-methyloctanoic acid are chiral compounds, which contribute to the characteristic taste and smell of mutton and sheep's cheese. The ***industrial*** and ***economic justification*** of this project is derived from the interest that the flavor and fragrance industry has in the production of enantiopure BCFAs. The ***objective*** was to find a scalable food-grade method for the resolution of γ-BCFA-enantiomers. Therefore, enzymatic kinetic resolution was investigated. The problem of low selectivity, which is to be expected in compounds with a chiral center that is remote from the reaction center, might be solved using sequential kinetic resolution (in our case: esterification followed by hydrolysis). For that purpose, *in situ* separation of the substrates and the products was investigated. For the design of a suitable reactor system it is necessary to gain information about enzyme kinetics and the behavior of substrates in the reaction mixture. Therefore, the following questions have to be answered. Firstly, which hydrolase can enantioselectively catalyze both the esterification of γ-BCFAs and the hydrolysis of their esters? Secondly, which food-grade alcohol is appropriate to create a stable two-phase system, which will facilitate downstream processing? In addition, what is the influence of solvent polarity on the kinetics of esterification? Finally, how can reaction and product separation be integrated?

Outline of the thesis

Enzyme screening for the transesterification of 4-methylhexanoic acid methyl ester and 4-methyloctanoic acid methyl ester to their butyl esters is described in chapter 2. Chapter 3 describes the esterification of 4-methyloctanoic acid using polyethylene glycols with different molecular weights. Partition experiments and enzymatic reactions were performed to find a stable two-phase system needed to run a continuous membrane reactor. In chapter 4 the esterification of 4-methyloctanoic acid with ethanol, catalyzed by Novozym 435™, is described. The effect of sorption of 4-methyloctanoic acid into the polymer matrix of Novozym 435™ on the observed *E*-value is studied in detail. The kinetic parameters V_{max}, K_M, and the *E*-ratio, determined in the esterification of 4-methyloctanoic acid and the hydrolysis of 4-methyloctanoic acid ethyl ester, are presented in chapter 5. It is shown that the amount of ethanol present in both reactions affects the enantioselectivity of the enzyme. Chapter 6 describes the esterification in a two-phase reactor. The transfer of the ester is studied using a trickle bed reactor and a distillation column. The thesis is concluded with a general discussion given in chapter 7, followed by a summary in English and Dutch.

REFERENCES

Ahuja, S., ed. (1997). Chiral separations and technology: an overview. In: *Chiral separations, applications and technology*, American Chemical Society, Washington DC, USA, 1-7.

Ahuja, S., ed. (1997). Chiral separation methods. In: *Chiral separations, applications and technology*, American Chemical Society, Washington DC, USA, 139-154.

Balcão, V.M., and Malcata, F.X. (1996). Reactors with immobilized lipase, mathematical modelling. In: *Engineering of/with lipases*, Malcata, F.X., ed., Kluwer Academic Publishers, The Netherlands, 435-454.

Barbeni, M., Allegrone, G., Cisero, M., and Guarda, P.A. (1992). GC and NMR enantiodiscrimination of 2-methyl substituted aliphatic acids via diastereomeric esterification with (*R*)-pantolactone. *Flavour Fragrance J.*, 7, 163-167.

Barbieri, G., Bolzoni, L., Parolari, G., Virgili, R., Buttini, R., Careri, M., and Mangia, A. (1992). Flavor compounds of dry-cured ham. *J. Agric. Food Chem.*, **40**, 2389-2394.

Boelens, M.H., Boelens, H., and Gemert, van, L.J. (1993). Sensory properties of optical isomers. *Perfumer and Flavorist*, **18**, 2-16.

Bovara, R., Carrea, G., Ottolina, G., and Riva, S. (1993). Effects of water activity on V_{max} and K_M of lipase catalyzed transesterification in organic media. *Biotechnol. Lett.*, **15**, 937-942.

Brady, L., Brzozowski, A.M., Derewenda, Z.S., Dodson, E., Dodson, G., Tolley, S., Turkenburg, J.P., Christiansen, L., Huge-Jensen, B., Norskov, L., Thim, L., and Menge, U. (1990). A serine protease triad forms the catalytic centre of a triacylglycerol lipase. *Nature*, **343**, 767-770.

Brennand, C.P., and Lindsay, R.C. (1992). Distribution of volatile branched-chain fatty acids in various lamb tissues. *Meat Sci.*, **31**, 411-421.

Brennand, C.P., and Lindsay, R.C. (1993). Influence of cooking on concentrations of species-related flavor compounds in mutton. *Food Sci. Technol.*, **25**, 357-364.

Brock, T.D., and Madigan, M.T., eds. (1988). Biology of microorganisms, 5th ed., Prentice Hall Inc., Englewood Cliffs, NJ, USA, 357-390.

Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G.G., Lawson, D.M., Turkenburg, J.P., Bjorkling, F., Huge-Jensen, B., Patkar, S.A., and Thim, L. (1991). A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. *Nature*, **351**, 491-494.

Bühler, M., und Wandrey, Ch. (1987). Enzymatische Fettspaltung. *Fett Wissensch. Technol.*, **89**, 156-164.

Cambou, B., and Klibanov, A.M. (1984). Comparison of different strategies for the lipase-catalyzed preparative resolution of racemic acids and alcohols: asymmetric hydrolysis, esterification and transesterification. *Biotechnol. Bioeng.*, **26**, 1449-1454.

Carrea, G., Ottolina, G., Riva, S., and Secundo, F. (1992). Effect of reaction conditions on the activity and enantioselectivity of lipases in organic solvents. In: *Biocatalysis in non-conventional media,* Tramper, J. *et al.*, eds., Elsevier, Amsterdam, The Netherlands, 111-119.

Cheetham, P.S.J. (1997). Combining the technical push and the business pull for natural flavors. In: *Biotechnology of aroma compounds*, Scheper, T. and Berger, R.G., eds., Springer-Verlag Berlin, Germany, 1-49.

Chen, C-S., Fujimoto, Y., Girdaukas, G., and Sih, C.J. (1982). Quantitative analyses of biochemical kinetic resolutions of enantiomers. *J. Am. Chem. Soc.*, **104**, 7294-7299.

Chen, C-S., Wu, S-H., Girdaukas, G., and Sih, C.J. (1987). Quantitative analyses of biochemical kinetic resolution of enantiomers. 2. Enzyme-catalyzed esterifications in water-organic solvent biphasic systems. *J. Am. Chem. Soc.*, **109**, 2812-2817.

Chuman, T., and Noguchi, M. (1977). The aroma constituents of Turkish tobacco. Part III. Acidic aroma components of turkish tobacco. *Agric. Biol. Chem.*, **41**, 1021-1030.

Claassen, J. (1995). Tussen neus en lippen, het hoe en waarom van geur en smaak. Tirion, Baarn, The Netherlands, 1-190.

Cornish-Bowden, A. (1995). Fundamentals of enzyme kinetics, Portland Press Ltd., London, U.K., 1-343.

Crosby, J. (1991) Synthesis of optically active compounds: a large scale perspective. *Tetrahedron*, **47**, 4789-4846.

Ebbers, E. (1997). Racemization and asymmetric transformation of α-substituted carboxylic acids, dissertation, Katholieke Universiteit Nijmegen.

Egloff, M-P., Ransac, S., Marguet, F., Rogalska, E., Tilbeurgh, van, H., Buono, G., Cambillau, C., et Verger, R. (1995). Enzymes lipolytiques et lipolyse. *OCL*, **2**, 52-67.

Ekborg-Ott, K.H., and Armstrong, D.W. (1997). Stereochemical analyses of food components. In: *Chiral separations, applications and technology*, Ahuja, S., ed., American Chemical Society, Washington DC, USA, 201-270.

Elferink, V.H.M., Breitgoff, D., Kloosterman, M., Kamphuis, J., Tweel, van den, W.J.J., and Meyer, E.M. (1991). Industrial developments in biocatalysis. *Recl. Trav. Chim. Pays-Bas,* **110**, 63-74.

Faber, K. (1997). Biocatalytic applications. In: *Biotransformations in organic chemistry, a textbook*, 3rd ed., Springer Verlag Berlin, Germany, 27-159.

Faber, K., and Franssen, M.C.R. (1993). Prospects for the increased application of biocatalysts in organic transformations. *TIBTECH,* **11**, 461-470.

Fenaroli, G. (1995). Fenaroli's handbook of flavor ingredients, 3rd ed., edited by Burdock, CRC Press, Florida, USA, **1, 2**.

Gassenmeier, K., and Schieberle, P. (1994). Comparison of important odorants in puff-pastries prepared with butter or margarine. *Lebensm. Wiss. Technol.*, **27**, 282-288.

Godfrey, T., and West, S.I. (1996). Introduction to industrial enzymology. In: *Industrial enzymology*, 2nd ed., Macmillan press Ltd., London UK, 1-8.

Ha, J.K. and Lindsay, R.C. (1990). Distribution of volatile branched-chain fatty acids in perinephric fats of various red meat species. *Lebensm. Wiss. Technol.*, **23**, 433-440.

Ha, J.K., and Lindsay, R.C. (1990). Method for the quantitative analysis of volatile free and total branched-chain fatty acids in cheese and milk fat. *J. Dairy Sci.*, **73**, 1988-1999.

Ha, J.K., and Lindsay, R.C. (1991). Contributions of cow, sheep, and goat milks to characterizing branched-chain fatty acid and phenolic flavors in varietal cheeses. *J. Dairy Sci.*, **74**, 3267-3274.

Ha, J.K., and Lindsay, R.C. (1991). Volatile branched-chain fatty acids and phenolic compounds in aged Italian cheese flavors. *J. Food Sci.*, **56**, 1241-1247, 1250.

Ha, J.K., and Lindsay, R.C. (1993). Release of volatile branched-chain and other fatty acids from ruminant milk fats by various lipases. *J. Dairy Sci.*, **76**, 677-690.

Hallet, R.H., Perez, A.L., Gries, G., Gries, R., Pierce Jr., H.D., Yue, J., Cameron Oehlschlager, A., Gonzales, L.M., and Borden, J.H. (1995). Aggregation pheromone of coconut rhinoceros beetle, *Oryctes rhinoceros* (L.) (Coleoptera: Scarabaeidae). *J. Chem. Ecol.*, **21**, 1549-1570.

Hansen, A., and Hansen, B. (1996). Flavour of sourdough wheat bread crumb. *Z. Lebensm. Unters. Forsch.*, **202**, 244-249.

Hashim, I.B., Koehler, P.E., and Kvien, C.K. (1993). Fatty acid composition, mineral content, and flavor quality of southern runner peanuts treated with herbicides and fungicides. *Peanut Sci.*, **20**, 106-111.

Holmberg, E., Szmulik, P., Norin, T., and Hult, K. (1989). Hydrolysis and esterification with lipase from *Candida cylindracea*. Influence of the reaction conditions and acid moiety on the enantiomeric excess. *Biocatalysis*, **2**, 217-223.

Ishihara, K., Honma, N., Matsumoto, I., Imai, S., Nakazawa, S., and Iwafuchi, H. (1996). Comparison of volatile components in soy sauce (Koikuchi shoyu) produced using *Aspergillus sojae* and *Aspergillus oryzae*. *J. Jpn. Soc. Food Sci. Technol.*, **43**, 1063-1074.

Jacques, J., Collet, A., and Wilen, S.H. (1981). Enantiomers, racemates, and resolutions, John Wiley and sons, New York, USA, 1-447.

Jensen, R.G., Galluzzo, D.R., and Bush, V.J. (1990). Selectivity is an important characteristic of lipases (acylglycerol hydrolases). *Biocatalysis*, **3**, 307-316.

John, V.T. and Abraham, G. (1991). Lipase catalysis and its applications. In: *Biocatalysts for industry*, Dordick, J.S., Plenum New York, USA, 193-217.

Johnson, C.B., Wong, E., Birch, E.J., and Purchas, R.W. (1977). Analysis of 4-methyloctanoic acid and other medium chain-length fatty acid constituents of ovine tissue lipids. *Lipids*, **12**, 340-347.

Karl, V., Schumacher, K., and Mosandl, A. (1992). Enantiomeric 2-methylbutanoates: sensory evaluation and chirospecific analysis. *Flavour Fragrance J.*, **7**, 283-288.

Kazlauskas, R.J. (1994). Elucidating structure-mechanism relationships in lipases: prospects for predicting and engineering catalytic properties. *TIBTECH*, **12**, 464-471.

Klibanov, A.M. (1990). Asymmetric transformations catalyzed by enzymes in organic solvents. *Acc. Chem. Res.*, **23**, 114-120.

Kloosterman, M., Elferink, V.H.M., Iersel, van, J., Roskam, J.-H., Meyer, E.M., Hulshof, L.A., and Sheldon, R.A. (1988). Lipases in the preparation of β- blockers. *TIBTECH*, **6**, 251-256.

Koskinen, A. (1993). Chirality, topology and asymmetric synthesis. In: *Asymmetric synthesis of natural products*. John Wiley and Sons Ltd., Chichester, U.K., 18-45.

Kroutil, W., Kleewein, A., and Faber, K. (1997). A computer program for analysis, simulation and optimization of asymmetric catalytic processes proceeding through two consecutive steps. Type 2: sequential kinetic resolutions. *Tetrahedron: Asymmetry*, **8**, 3263-3274.

Lamberet, G., Degas, C., Delacroix-Buchet, A., and Vassal, L. (1996). Effect of characteristics linked to A and F alphasl-casein alleles in goat milk on 'goat' flavour: cheesemaking with protein-fat exchange. *Lait*, **76**, 349-361.

Laye, I., Karleskind, D., and Morr, C.V. (1993). Chemical, microbiological and sensory properties of plain nonfat yogurt. *J. Food Sci.*, **58**, 991-995.

Martinelle, M., Holmquist, M., and Hult, K. (1995). On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginosa* lipase. *Biochim. Biophys. Acta*, **1258**, 272-276.

Martinelle, M., and Hult, K. (1995). Kinetics of acyl transfer reactions in organic media catalysed by *Candida antarctica* lipase B. *Biochim. Biophys. Acta*, **1251**, 191-197.

Matsuzaki, T., Shinozaki, Y., Hagimori, M., Tobita, T., Shigematsu, H., and Koiwai, A. (1992). Novel glycerolipids and glycolipids from the surface lipids of *Nicotiana benthamiana*. *Biosci. Biotechnol. Biochem.*, **56**, 1565-1569.

Moret, I., and Gambaro, A. (1996). Trend analysis in selection of aroma components as variables for chemometric studies of typical wines. *Ann.Chim.*, **86**, 309-318.

Mosandl, A., Rettinger, K., Weber, B., and Henn, D. (1990). Investigations on the enantiomer distribution of 2-methylbutanoic acid in fruits and other foodstuffs by multidimensional gas chromatography (MDGC). *Dtsche Lebensm. Rundsch.*, **86**, 375-379.

Mukherjee, K. D. (1990). Lipase-catalyzed reactions for modification of fats and other lipids. *Biocatalysis*, **3**, 277-293.

Nakazawa, Y., Wada, R., Izumitani, M., Nakumura, H., and Chikuma, K. (1981). Studies of vanilla flavours for food processing. IV. The use of vanilla components in commercial foods. *Jap. J. Dairy Food Sci.*, **30**, 161-167.

Ohya, I., Sinozaki, Y., Tobita, T., Takahashi, H., Matsuzaki, T., and Koiwai, A. (1994). Sucrose esters from the surface lipids of *Nicotiana cavicola*. *Phytochemistry*, **37**, 143-145.

Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J., Sussman, J.L., Verschueren, K.H.G., and Goldman, A. (1992). The α/β hydrolase fold. *Prot. Eng.*, **5**, 197-211.

Padt, van der, A., Sewalt, J.J.W., Ágoston. S.M.I., and Riet, van 't, K. (1992). *Candida rugosa* lipase stability during acylglycerol synthesis. *Enzyme Microb. Technol.*, **14**, 805-812.

Patkar, S.A., Bjørking, F., Zundel, M., Schulein, M., Svendsen, A., Heldt-Hansen, H.P., and Gormsen, E. (1993). Purification of two lipases from *Candida antarctica* and their inhibition by various inhibitors. *Indian J. Chem.*, **32**, 76-80.

Pierre, A., Quere, le, J.L., Famelart, M.H., Riaublanc, A., Rousseau, F., and Quere, le, J.L. (1998). Composition, yield, texture and aroma compounds of goat cheeses as related to the A and O variants of alphasl-casein in milk. *Lait*, **78**, 291-301.

Pyysalo, T., Suihko, M., and Honkanen, E. (1977). Odour thresholds of the major volatiles identified in cloudberry (*Rubus chamaemorus L.*) and arctic bramble (*Rubus arcticus L.*). *Lebensm. Wiss. Technol.*, **10**, 36-39.

Quere, le, J.L., Demaizieres, D., Negrello, C., Lesschaeve, I., Issanchou, S., Salles, C., and Quere, le, J.L. (1996). Goat cheese flavour: identification of the character impact compounds. In: *Proceedings of production and utilization of ewe and goat milk,* Crete, Greece 19-21 October 1995, 205-208.

Quere, le, J.L., Pierre, A., Riaublanc, A., Demaizieres, D., and Quere, le, J.L. (1998). Characterization of aroma compounds in the volatile fraction of soft goat cheese during ripening. *Lait*, **78**, 279-290.

Rakels, J.L.L., Caillat, P., Straathof, A.J.J., and Heijnen, J.J. (1994). Modification of the enzyme enantioselectivity by product inhibition. *Biotechnol. Prog.*, **10**, 403-409.

Ranadive, A.S. (1992) Vanillin and related flavor compounds in vanilla extracts made from beans of various global origins. *J. Agric. Food Chem.*, **40**, 1922-1924.

Rettinger, K., Karl, V., Schmarr, H.G., Dettmar, F., Hener, U., and Mosandl, A. (1991). Chirospecific analysis of 2-alkyl-branched alcohols, acids, and esters: chirality evaluation of 2-methylbutanoates from apples and pineapples. *Phytochem. Anal.*, **2**, 184-188.

Rettinger, K., Weber, B., and Mosandl, A. (1990). Stereoisomeric flavour compounds. XLII. Enantiomer distribution of 2-methylbutanoic acid in apples and apple-containing food. *Z. Lebensm. Unters. Forsch.*, **191**, 265-268.

Sarda, L., and Desnuelle, P. (1958). Action de la lipase pancréatic sur les esters en émulsion. *Biochim. Biophys. Acta,* **30**, 513-521.

Schreier, P. (1997). Enzymes and flavour biotechnology. In: *Biotechnology of aroma compounds*, by Scheper, T. and Berger, R.G., eds., Springer-Verlag, Berlin, Germany, 52-70.

Schroder, I., Stern, G., HojabrKalali, B., Schliekelmann, K., and Maier, H.G. (1997). Volatile minor acids in coffee. 2. Detection of steam treatment. *Dtsch. Lebensm. Rundsch.*, **93**, 216-218.

Schumacher, K., Asche, S., Heil, M., Mittelstadt, F., Dietrich, H., and Mosandl, A. (1998). Methyl-branched flavor compounds in fresh and processed apples. *J. Agric. Food Chem.*, **46**, 4496-4500.

Seino, H., Uchibori, T., Nishitani, T., and Inamasu, S. (1984). Enzymatic synthesis of carbohydrate esters of fatty acid (1) esterification of sucrose, glucose, fructose and sorbitol. *JAOCS*, **61**, 1761-1765.

Simpson, R.F., and Miller, G.C. (1984). Aroma composition of Chardonnay wine. *Vitis*, **23**, 143-158.

Son, K.C., Severson, R.F., Pair, S.D., and Kays, S.J. (1994). Comparison of the sucrose ester fatty acid components in flowers and flower buds of three Petunia X hybrida Hort. cultivars. *J. Korean Soc. Hort. Sci.*, **35**, 617-622.

Sonnet, P.E., and Welch-Baillargeon, M. (1987). Kinetic resolution of secondary alcohols with commercial lipases: application to rootworm sex pheromone synthesis. *J. Chem. Ecol.*, **13**, 1279-1292.

Sutherland, M.M., and Ames, J.M. (1995). The effect of castration on the headspace aroma components of cooked lamb. *J. Sci. Food Agric.*, **69**, 403-413.

Sutherland, M.M., and Ames, J.M. (1996). Free fatty acid composition of the adipose tissue of intact and castrated lambs slaughtered at 12 and 30 weeks of age. *J. Agric. Food Chem.*, **44**, 3113-3116.

Tanaka, T., Muramatsu, K., Kim, H., Watanabe, T., Takeyasu, M., Kanai, Y., and Kiuchi, K. (1998). Comparison of volatile compounds from Chungkuk-jang and Itohiki-natto. *Biosci. Biotechnol. Biochem.*, **62**, 1440-1444.

Tramper, J. (1994). 1. Applied biocatalysis: from product request to idea to product. In: *Applied biocatalysis*, Cabral, J.M.S. *et al.*, eds., Harwood academic publishers, Chur, Switzerland, 1-47.

Uppenberg, J., Öhrner, N., Norin, M., Hult, K., Kleywegt, G.J., Patkar, S., Waagen, V., Anthonson, T., and Jones, T.A. (1995). Crystallographic and molecular-modeling studies of lipase B from *Candida antarctica* reveal a stereospecificity pocket for secondary alcohols. *Biochemistry*, **34**, 16838-16851.

Valivety, R.H., Halling, P.J., and Macrae, A.R. (1993). Water as a competitive inhibitor of lipase-catalysed esterification in organic media. *Biotechnol. Lett,* **15**, 1133-1138.

Vänttinen, E., and Kanerva, L.T. (1997). Optimized double kinetic resolution for the preparation of (S)-solketal. *Tetrahedron: Asymmetry,* **8**, 923-933.

West, S. (1996). Flavour production with enzymes. In: *Industrial enzymology*, Godfrey, T. and West, S., eds., 2nd ed., Macmillan Press Ltd, London, UK, 209-225.

Wilson, W.K., Baca, S.B., Barber, Y.J., Scallen, T.J., and Morrow, C.J. (1983). Enantioselective hydrolysis of 3-hydroxy-3-methylalkanoic acid esters with pig liver esterase. *J. Org. Chem.*, **48**, 3960-3966.

Winkler, F.K., D'Arcy, A., and Hunziker, W. (1990). Structure of human pancreatic lipase. Nature, **343**, 771-774.

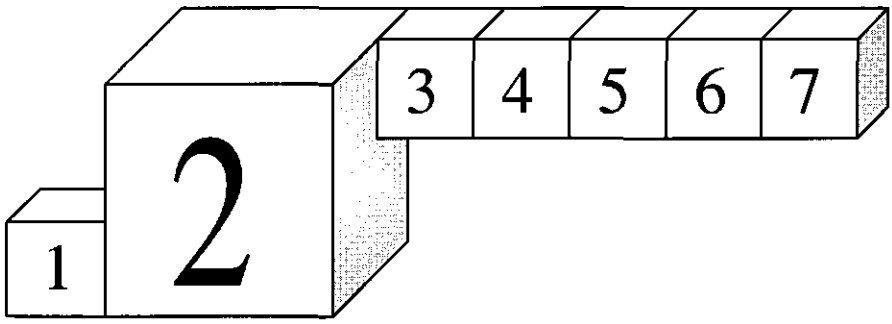
Wintoch, H., Krammer, G., and Schreier, P. (1991). Glycosidically bound aroma compounds from two strawberry fruit species, *Fragaria vesca f. semperflorens* and *Fragaria X ananassa, cv. Korona. Flavour Fragrance J.*, **6**, 209-215.

Wohrmann, R., HojabrKalali, B., and Maier, H.G. (1997). Volatile minor acids in coffee. 1. Contents of green and roasted coffee. *Dtsch. Lebensm. Rundsch.*, **93**, 191-194.

Wong, E., Johnson, C.B., and Nixon L.N. (1975). The contribution of 4-methyloctanoic (hircinoic) acid to mutton and goat meat flavour. *N.Z. J. Agric. Res.*, **18**, 261-266.

Young, O.A., Berdague, J.L., Viallon, C., Rousset-Akrim, S., and Theriez, M. (1997). Fat-borne volatiles and sheepmeat odour. *Meat Sci.*, **45**, 183-200.

Zaks, A., and Klibanov, A.M. (1985). Enzyme-catalyzed processes in organic solvents. *Proc. Natl. Acad. Sci. USA*, **82**, 3192-3196.



Lipase-mediated resolution of γ-branched chain fatty acid methyl esters

SUMMARY

*Kinetic resolution of the branched chain fatty acid (BCFA) esters 4-methylhexanoic acid methyl ester (**4**) and 4-methyloctanoic acid methyl ester (**5**) was investigated using a series of hydrolases as catalysts. In the transesterification of these methyl esters to their butyl esters, two enzymes showed good conversion and a moderate enantiomeric ratio (E). In the transesterification of (**4**), an E of 2 was obtained for the reaction catalyzed by Rhizomucor miehei lipase, whereas Candida antarctica lipase B (CALB) showed an E of 5. In the conversion of (**5**) to the butyl ester, Rhizomucor miehei lipase was not selective whereas CALB gave an E of 8. Apparently, changing from an ethyl group to a butyl group at the chiral center leads to an improved chiral recognition by CALB. The lipases displayed complementary enantiomeric preference. Rhizomucor miehei lipase favors the S-enantiomer of (**4**) while CALB preferentially transforms the R-enantiomer of both substrates. Molecular modeling studies supported the measured stereochemical preference of CALB. A decrease in reaction temperature from 45°C to 27°C led to a significant increase in enantiomeric ratio (E=23) in the transesterification of (**5**).*

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INTRODUCTION

The branched chain fatty acids 4-methylhexanoic acid and 4-methyloctanoic acid have been recognized as flavor components and the separate enantiomers of both compounds do have different sensory properties. Kinetic resolution is a technique that can be used to separate the enantiomers in order to study the characteristic smell and taste of the separate components.

In this chapter we focus on the enzymatic transesterification of racemic 4-methylhexanoic acid methyl ester and 4-methyloctanoic methyl ester to their butyl esters. The chiral center in 4-methylalkanoic acids is positioned at the γ-carbon with regard to the carboxyl group. This means that the enantiomeric ratio (*E*) might be low (Wilson *et al.*, 1983). In order to find good catalysts for the kinetic resolution of these compounds a total of 25 hydrolases were screened. The catalysts displaying the highest reaction rates were used to determine the enantiomeric ratio of the reactions. The results of the transesterification of 4-methyloctanoic acid methyl ester catalyzed by *Candida antarctica* lipase B (CALB) were compared with data obtained from molecular modeling studies of the tetrahedral intermediates of the two butyl 4-methyloctanoate enantiomers.

Here we present the first procedure for the lipase-mediated production of these enantiomerically enriched BCFA derivatives.

MATERIALS & METHODS

Enzymes

Rhizomucor miehei lipase and recombinant *Rhizomucor miehei* lipase were obtained from Fluka. *Rhizomucor miehei* lipase, *Chromobacterium viscosum* lipase, *Aspergillus niger* lipase, *Geotrichum candidum* lipase, *Pseudomonas fluorescens* lipase and pancreatic lipase were gifts from Biocatalysts Ltd., Pontypridd, UK. *Humicola lanuginosa* lipase (Novozym SP523™) and *Candida*

antarctica lipase B (Novozym 435™) were gifts from NOVO Nordisk (Bagsvaerd, DK). Lipase G, Lipase PS, Cholesterol esterase, Lipoprotein lipase, Lipase AP6, Lipase N, Lipase R10 and Protease N were gifts of Amano Pharmaceutical Co. Ltd. (Frankfurt, BRD). *Candida rugosa* lipase, porcine pancreatic lipase, Subtilisin Carlsberg, and *Bacillus thermoproteolyticus* protease were purchased from Sigma. Naproxen esterase was a gift of Gist brocades N.V. (Delft, NL). Chymotrypsine A4 and *Rhizomucor miehei* lipase (Chirazym L-9, c-f, lyo.) were obtained from Boehringer Mannheim GmbH (Penzberg, BRD).

Chemicals

4-Methyloctanoic acid (99%) was purchased from Oxford Chemicals. Methanesulfonyl chloride, 2-methyl-1-butanol and dichloromethane were purchased from Acros. Dimethyl malonate, methanol, n-octane and n-butanol (99%), sodium and lithium chloride were obtained from Merck. All solvents were distilled before use.

General Procedures

FT-IR-spectra were recorded on a Biorad FTS-7 infrared spectrometer. ^{1}H-NMR spectra were measured on a Varian EM-390 spectrometer (90 MHz) or a Bruker AC-E 200 (200 MHz) (Me_4Si as internal standard, $CDCl_3$ or C_6D_6 as solvent). Transesterification reactions were carried out in a New Brunswick Scientific Innova™ 4080 incubator shaker. Optical rotations were obtained from $CHCl_3$ solutions on a Perkin-Elmer 241 polarimeter.

Synthesis

***(R,S)-2-Methylbutyl-1-mesylate* (2)**

A solution of methanesulfonyl chloride (16 ml; 0.21 mol) in dichloromethane (16 ml) was added dropwise to a mixture of 2-methyl-1-butanol (22 ml; 0.20 mol), pyridine (17 ml; 0.21 mol) and dichloromethane (70 ml). After 2 hours a white precipitate was formed. The reaction mixture was stirred overnight at room

temperature. Water (150 ml) was added and the aqueous layer was extracted with dichloromethane (2x). The combined organic extracts were washed with 2M HCl (2x450 ml), water (1x450 ml), a saturated $NaHCO_3$ solution (2x450 ml) and water (1x450 ml). Drying ($MgSO_4$) was followed by concentration *in vacuo* to afford the crude mesylate in nearly quantitative yield. ^{1}H-NMR ($CDCl_3$, 90 MHz,): δ 0.90 (t, 3H, $C\underline{H}_3CH_2$, J = 6 Hz), 1.00 (d, 3H, $CH(C\underline{H}_3)$, J = 6 Hz), 1.5 (m, 3H, $CH_3C\underline{H}_2C\underline{H}$), 2.95 (s, 3H, $OSO_2C\underline{H}_3$), 4.05 (dd, 2H, $C\underline{H}_2OSO_2$, J = 3 Hz, J = 6 Hz) ppm. HRMS, calcd for $C_6H_{14}O_3S$ (M^+ -C_2H_5) *m/e* 137.0272, found *m/e* 137.0277.

***Dimethyl (R,S)-2-(2-methylbutyl)malonate* (3)**

(*R,S*)-2-Methylbutyl mesylate (31.4 g; 0.19 mol) was added dropwise to a solution of freshly prepared 2 M NaOMe (104 ml) and dimethyl malonate (21.6 ml; 0.19 mol). The mixture was heated under reflux for 16 hours. Excess MeOH was removed under reduced pressure and water (100 ml) was added to dissolve the mesylate salt. The organic layer was separated and the aqueous layer was extracted twice with ether (2x100 ml). The combined organic layers were washed with water, dried ($MgSO_4$) and concentrated *in vacuo* to give the crude product (yield 78%) which was sufficiently pure for further reactions. ^{1}H-NMR ($CDCl_3$, 90 MHz): δ 0.90 (t, 3H, $C\underline{H}_3CH_2$ J = 6 Hz), 1.00 (d, 3H, $CH(C\underline{H}_3)$, J = 6 Hz), 1.6 (m, 5H, $C\underline{H}_2C\underline{H}C\underline{H}_2$), 3.50 (dd, 1H, $C\underline{H}CO_2$ J = 3 Hz, J = 6 Hz), 3.70 (s, 6H, $CO_2C\underline{H}_3$) ppm. HRMS, calcd for $C_{10}H_{18}O_4$ (M^+ -OCH_3) *m/e* 171.1021, found *m/e* 171.1025.

***(R,S)-4-methyl-hexanoic methyl ester* (4)**

Water (2.7 ml; 0.15 mol) and LiCl (12.8 g; 0.30 mol) were added to a stirred solution of dimethyl (*R,S*)-2-(2-methylbutyl)malonate (30.5 gr; 0.15 mol) in DMSO (125 ml) at room temperature. After 15 hours of stirring at 180°C the reaction mixture was allowed to cool to room temperature. Ice water was added (200 ml) and the mixture was extracted with pentane (3x200 ml). The combined organic layers were washed with saturated NaCl (600ml), dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by distillation to give the methyl ester (yield 79%) as a colorless oil. Bp 52°C (10 mbar). ^{1}H-NMR ($CDCl_3$, 90

MHz): δ 0.90 (t, 3H, $C\underline{H}_3CH_2$, J = 6 Hz), 1.00 (d, 3H, $CHC\underline{H}_3$, J = 6 Hz), 1.5 (m, 5H, $C\underline{H}_2C\underline{H}C\underline{H}_2$), 2.30 (m, 2H, $C\underline{H}_2CO_2$), 3.70 (s, 3H, $CO_2C\underline{H}_3$) ppm. HRMS, calcd for $C_8H_{16}O_2$ (M^+) *m/e* 144.1146, found *m/e* 144.1153.

***(R,S)-4-methyl-octanoic acid methyl ester* (5)**

To a solution of racemic 4-methyloctanoic acid (7.9 g; 0.05 mol) in MeOH (25 ml), HCl (2 ml) was added. The reaction mixture was refluxed overnight. Excess of MeOH was evaporated under reduced pressure and the residue was dissolved in a mixture of water (50 ml) and ether (50ml). The organic layer was separated and the aqueous phase was extracted with ether (2x50 ml). The combined organic layers were washed with water (150 ml), saturated $NaHCO_3$ and brine. After drying ($MgSO_4$) and evaporation under reduced pressure, the ester was obtained as a colorless oil (8.3 g; 96%). ^{1}H-NMR (C_6D_6, 200 MHz): δ 0.81 (d, 3H, $CHC\underline{H}_3$ J = 6 Hz), 0.93 (t, 3H, $C\underline{H}_3CH_2$, J = 6 Hz), 1.4 (m, 9H, $(C\underline{H}_2)_3C\underline{H}(CH_3)C\underline{H}_2$), 2.21 (m, 2H, $C\underline{H}_2CO_2$), 3.43 (s, 3H, $CO_2C\underline{H}_3$) ppm. FT-IR ν 2956, 1743 cm^{-1}. HRMS, calcd for $C_{10}H_{20}O_2$ (M^+) *m/e* 172.1460, found *m/e* 172.1463.

Enzymatic Transesterification

A 4 ml screw-cap vial (Chrompack) was filled with 4-methylhexanoic acid methyl ester (**4**) (100 mM) or 4-methyloctanoic acid methyl ester (**5**) (100 mM), butanol (500 mM), cumene (40 mM) as internal standard and octane in a total volume of 1 ml. After thermostating the vial at 45°C for ten minutes in an incubator shaker, the reaction was started by adding 5, 20 or 100 mg of enzyme to the solution. The reactions were carried out at 45°C by shaking (350 rpm) for a specified time (see RESULTS & DISCUSSION).

To determine the enantiomeric excess in the transesterification of 4-methyloctanoic acid methyl ester, reactions were carried out on larger scale. 20 ml Vials (Wheaton) were filled with the same mixture as mentioned above. The total volume was 10 ml. Experiments were carried out for a specified time at 27°C or 45°C. The reactions were stopped by filtration of the enzyme. The methyl and butyl esters were separated by preparative GC and analyzed by NMR (see analytical methods).

Analytical Methods

Conversion

The conversion of the transesterification was measured either on a Varian 3300 GC or on a Fisons 8160 GC fitted with a flame ionisation detector. In case of **4**, a capillary DB1 megabore column (HP, length 30 m, ID 0.53 mm, d_f 0.88 μm) operated at 110°C was used with a nitrogen carrier gas flow rate of 15 ml/min. Peak areas were integrated electronically with a Hewlett Packard HP3395 integrator. The analysis of the transesterification of **5** was performed using a capillary DB-17 column from J&W (length 30 m, ID 0.25 mm, d_f 0.88 μm) at a temperature of 100°C and a hydrogen gas flow rate of 30 ml/min. A Fisons DP-700 integrator was used for integration.

Enantiomeric excess

To determine the enantiomeric excess in the transesterification of **4**, a chiral β-cyclodextrin column (Supelco β-dex 120, length 30 m, ID 0.25 mm, d_f 0.25 μm) was used. The analysis was carried out by using a temperature program (T_1 65°C, t_1 30 min., rate 1°C/ min, T_2 80°C, t_2 70 min, carrier gas H_2, gas flow 0.7 ml/min) (RT methyl ester: 24.45 min. and 24.90 min. [α = 1,03], RT butyl ester: 63.80 min and 64.33 min [α = 1,01]). In the transesterification of **5**, the enantiomeric excess was determined by separating the methyl and butyl esters using preparative GC (Becker Research GC type 3810, Alltech, superox 0.6 ON column, length 2m, ID 3/8", T 175°C, N_2 -gas flow 150 ml/min) and analyzing these esters by NMR using a chiral shift reagent ($Eu(hfc)_3$ in C_6D_6). To determine the enantiomeric ratio the enantiomeric excess versus c data were fitted using the Chen equation in the program SIMFIT (Jongejan *et al.*, 1991)

Optical rotation

To a solution of 4-methyloctanoic acid methyl ester (0.6445 g) in water (25 ml), immobilized *Candida antarctica* lipase B was added (100 mg). After 1½ hour, at

45°C and 350 rpm in a incubator shaker, the reaction was stopped by filtration of the enzyme. The reaction mixture was extracted with ethyl acetate and the organic layer was dried ($MgSO_4$) and concentrated *in vacuo*. Column chromatography (hexane/ethyl acetate 10:1) gave pure product (0.279 g) and remaining substrate (0.102 g). The optical rotation measured: 4-methyloctanoic acid methyl ester $[\alpha]_D$ = +0.83 ($CHCl_3$, c = 3.1), 4-methyloctanoic acid $[\alpha]_D$ = -0.21 ($CHCl_3$, c = 9.0) (*c.f.* pure compounds reported by Karl and co-workers (1994): (*R*)-4-methyloctanoic acid $[\alpha]_D$ = -1.53 ($CHCl_3$, c = 1.1), (*S*)-4-methyloctanoic acid $[\alpha]_D$ = +1.45 ($CHCl_3$, c = 1.1).

Molecular Dynamics Simulations

All modeling was performed on Silicon Graphics Indigo and 02 workstations using the Discover program version 2.97. Structures were viewed and analyzed in Biosym/MSI InsightII version 95.0. The molecular dynamics were performed using a distance dependent dielectric constant (ε = r) in the cvff forcefield with a non-bonded cut-off of 11 Å. Mainly, energy minimizations were performed with the conjugate gradient method. The procedure used here is similar to that of Norin and co-workers (1993). The calculation required approximately three days in cpu time for each enantiomer.

The enzyme starting structure was prepared from the 1TCA pdb structure found in the Brookhaven Protein Databank. The structure was downloaded including all crystal waters. Bond order was set and hydrogens added (pH 7) and subsequently partially minimized. The His 224 was replaced by the protonated form. The structure was subjected to pre-dynamics at 300 K for 5000 steps and water molecules at a distance longer than 3 Å from any protein atom were removed. Linked carbohydrates NAG1 and NAG2 were included in the simulations (potentials set manually). The structure was regularly checked with the Struct_check option in the InsightII Homology module.

An "oxyanion tetrahedral intermediate" transition state model with a serine (SerOγ attack on a butyl 4-methyloctanoate carbonyl) covalently bound was prepared. Geometry and structure was optimized and charge distribution calculated with the

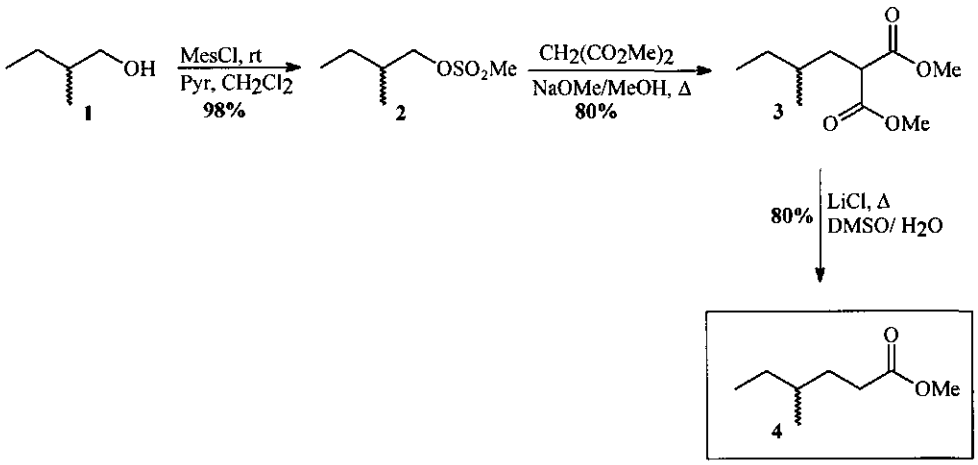
MOPAC 6.0 (Besler *et al.* 1990) program (MNDO type calculation, total charge -1). The included serine was replaced by Ser 105 and the Oγ charge corrected. The position of Ser 105, the charge relay system and the oxyanion hole restricted possible orientations of docking.

A combined energy minimization and-molecular dynamics protocol (a total of 105 ps at 300 K) was used to search the conformational space and to generate 10 structures, the first after 15 ps. No freezing or other restriction was imposed on any atom. No simulated annealing was employed. Note that the charge relay system was not perfected at the docking stage. However, generated low energy structures all displayed the hydrogen bond pattern required for catalysis (Ser 105 Oγ and butyl O to His 224 Nε2; complete oxyanion stabilization Thr 40 N and Oγ1 and Glu 106 N).

RESULTS & DISCUSSION

Synthesis of substrates

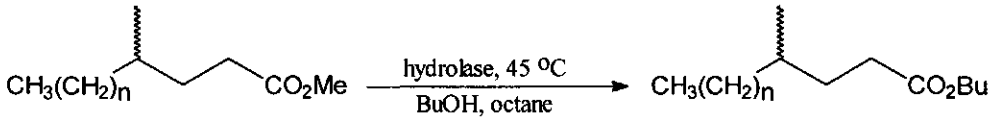
Racemic 4-methylhexanoic acid methyl ester (**4**) was synthesized in four steps (scheme 1) according to a modified literature procedure (Krapcho, 1982; Kelly & Buchecker, 1988). In the first step, racemic 2-methyl-1-butanol **1** was converted to mesylate (**2**), which was further reacted to dimethyl (2-methylbutyl) malonate (**3**). Methyl ester (**4**) was obtained by decarboxylation of the malonate (**3**) in a dipolar aprotic medium. 4-Methyloctanoic acid methyl ester (**5**) was prepared by esterification of commercially available 4-methyloctanoic acid (Tanner and Osman, 1987).



Scheme 1

Enzymatic resolution

Kinetic resolution of the two BCFA esters (**4**) and (**5**) was studied. We chose to perform the resolution by transesterification in an organic solvent rather than by hydrolysis in water, considering the ease of analysis and because resolutions in organic solvents are often more selective than in water (Klibanov, 1990).



4	n=1: (*R,S*)-4-methylhexanoic acid methyl ester	
	n=1: (*R,S*)-4-methylhexanoic acid butyl ester	**6**
5	n=3: (*R,S*)-4-methyloctanoic acid methyl ester	
	n=3: (R,S)-4-methyloctanoic acid butyl ester	**7**

Scheme 2

Twenty-five hydrolases were screened with respect to their ability to catalyze the transesterification between these two BCFA esters and butanol, with n-octane as a solvent (scheme 2).

Transesterification of 4-methylhexanoic acid methyl ester (4)

Racemic (**4**) was subjected to transesterification catalyzed by different enzymes. The conversion was monitored by GC. After 100 hours of incubation the reaction was stopped. The results of the screening are presented in table 1. Different amounts of enzyme were used, depending on the purity of the hydrolase. Of the 24 enzymes tested, only 6 enzymes were able to catalyze the transesterification (entries 2, 4, 10, 12, 20 and 21). Equilibrium after 100 hours was reached only for the reaction catalyzed by *Candida antarctica* lipase B (CALB) and *Rhizomucor miehei* lipase.

Enantiomeric ratio

The enantiomeric excess in the reactions catalyzed by one of the six lipases (entries 2, 4, 10, 12, 20, 21) was determined by GC using a chiral β-cyclodextrin column. Lipase G, lipase PS and cholesterol esterase were not enantioselective.

In table 2 the enantiomeric ratio is given for the three enzymes that catalyzed the transesterification with some selectivity. The enantiomeric ratio *E* was determined using the formulas of Chen and co-workers (1987).

Both *Rhizomucor miehei* lipase and recombinant *Rhizomucor miehei* lipase showed an *E* of 2 while CALB displayed a higher selectivity ($E = 5$). In order to determine the absolute configuration of the enzymatic product, the *S*-enantiomer of 4-methylhexanoic acid methyl ester was synthesized from *S*-2-methyl-1-butanol and injected on GC, together with racemic (**4**), in different ratios. From these chromatograms and the chromatograms of the transesterification reactions, we concluded that the *S*-enantiomer is the fast reacting enantiomer in the reaction catalyzed by *Rhizomucor miehei* lipase while *Candida antarctica* lipase B prefers the *R*-enantiomer.

Table 1: Transesterification of 4-methylhexanoic acid methyl ester (**4**) at 45°C

Nr	Enzyme	enzyme[1]	C(t=100h)[2]	t(c=0.5)[3]
		mg	*%*	*h*
1	*Aspergillus niger* lipase	20	<1	
2	*Candida antarctica* lipase B (CALB)	20	85	5
3	*Candida rugosa* lipase	100	19	
4	Cholesterol esterase	5	49	100
5	*Chromobacterium viscosum* lipase	5	1	
6	Chymotrypsin A4	5	0	
7	*Geotrichum candidum* lipase	20	0	
8	*Humicola lanuginosa* lipase	5	4	
9	Lipase AP6	100	2	
10	Lipase G (*Penicillium sp.*)	100	70	50
11	Lipase N	100	1	
12	Lipase PS	100	62	65
13	Lipase R 10	100	<1	
14	Lipoprotein lipase	5	0	
15	Naproxen esterase	5	0	
16	Pancreatic lipase (Biocatalysts)	20	0	
17	Porcine pancreatic lipase (Sigma)	100	9	
18	Protease N	100	0	
19	*Pseudomonas fluorescens* lipase	20	2	
20	Recombinant *Rhizomucor miehei* lipase	20	43	65
21	*Rhizomucor miehei* lipase (Biocatalysts)	20	85	15
22	*Rhizomucor miehei* lipase (Fluka)	5	4	
23	Subtilisin Carlsberg	5	<1	
24	Thermolysin	5	0	

[1] Amount enzyme means mg solid

[2] Conversion after 100 hours

[3] Time needed to reach 50% conversion

Table 2: *E*-ratio in transesterification of 4-methylhexanoic acid methyl ester (**4**) at 45°C

Enzyme	Supplier	*E*
Rhizomucor miehei lipase	Biocatalysts	2
Recombinant *Rhizomucor miehei* lipase	Fluka	2
Candida antarctica lipase B (CALB)	Novo	5

In summary, only a few hydrolases are able to catalyze the transesterification of 4-methylhexanoic acid methyl ester. Three enzymes were enantioselective. The enantiomeric ratio showed an *E* of *2* for both recombinant *Rhizomucor miehei* lipase and *Rhizomucor miehei* lipase and an *E* of 5 for *Candida antarctica* lipase B.

Transesterification of 4-methyloctanoic acid methyl ester

Racemic (**5**) was subjected to transesterification catalyzed by the enzymes mentioned in table 3.

Table 3: Transesterification of 4-methyloctanoic acid methyl ester (**5**) at 45°C

Enzyme	Supplier	c(t=200h)[1]	t(c=0.5)[2]
		%	*h*
Rhizomucor miehei lipase	Biocatalysts	85	15
Rhizomucor miehei lipase	Boehringer	[3]	10
Recombinant *Rhizomucor miehei* lipase	Fluka	85	50
Candida antarctica lipase B	Novo	85	5
Cholesterol esterase	Amano	85	7.5
Lipase PS	Amano	15	
Lipase G	Amano	10	

[1] Conversion after 200 hours

[2] Time needed to reach 50% conversion

[3] Reaction stopped after 65 hours

In this case only the enzymes were screened that also gave a moderate to good conversion in the transesterification of (**4**). The time course for the reactions is given in figure 1.
In comparison with the transesterification of (**4**), the reaction of **5** is much slower. Equilibrium is reached after approximately 150 hours. However, for both substrates, the reactions catalyzed by *Candida antarctica* lipase B reached 50% conversion after 5 hours.

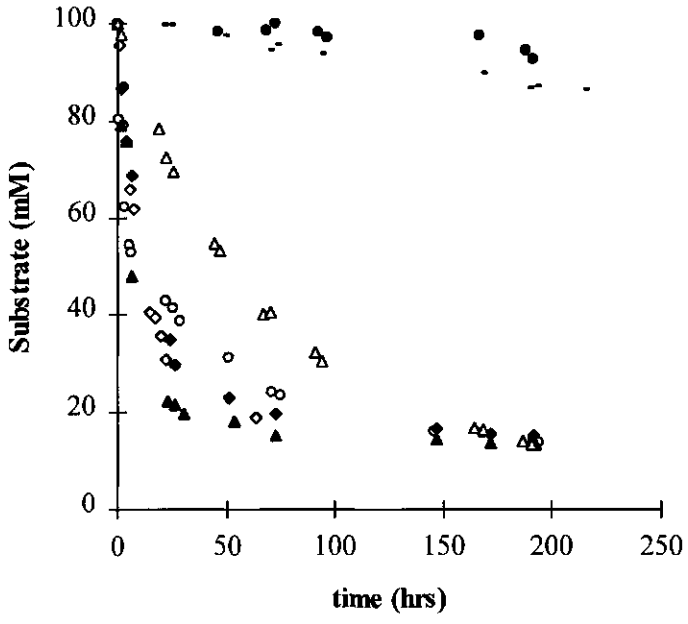


Figure 1: Transesterification of 4-methyloctanoic acid methyl ester (**5**). Substrate concentration of 4-methyloctanoic acid methyl ester versus time during the transesterification using different enzymes: ○ *Candida antarctica* lipase B, ● Lipase G, ◇ *Rhizomucor miehei* lipase (Boehringer), ◆ *Rhizomucor miehei* lipase (Biocatalysts), △ recombinant *Rhizomucor miehei* lipase, ▲ Cholesterol esterase, ━ Lipase PS.

Enantiomeric ratio

A chiral capillary GC column could determine the enantiomeric excess of neither the substrate nor the product. Preparative GC followed by NMR was used to determine the *ee* of the substrate as well as the *ee* of the product. Since in comparison with the transesterification of (**4**), lipase PS and lipase G showed low activity in the transesterification of 4-methyloctanoic acid methyl ester, only *Candida antarctica* lipase B and *Rhizomucor miehei* lipase (Boehringer) were used. An enantiomeric ratio of 8 was obtained in the transesterification catalyzed by CALB. *Rhizomucor miehei* lipase was not selective (E=1).

To determine which enantiomer was the fast reacting isomer, hydrolysis of the 4-methyloctanoic acid methyl ester was carried out since the optical rotation of the individual *R*- and *S*-enantiomer was known for the free acid (Karl *et al.*, 1994). After shaking for 1½ hour at 45°C, the enzyme was filtered off. After work up, the mixture was separated by liquid chromatography. The optical rotation revealed that the *R*-enantiomer is the fast reacting isomer.

Possible explanation of difference in enantiomeric ratio

When the two transesterification reactions discussed above are compared, it is clear that the enantiomeric ratio in the reaction catalyzed by CALB rises from 5 (4-methylhexanoic acid methyl ester) to 8 (4-methyloctanoic acid methyl ester). In the first mentioned case, the chiral center contains a methyl and an ethyl group. Apparently, it is difficult for the enzyme to differentiate between these two groups because of their comparable size. Therefore, both the *S*-enantiomer and the *R*-enantiomer will fit in the catalytic site, resulting in a low enantiomeric ratio. In the case of (**5**), the E-value is higher because the chiral center contains substituents which have bigger difference in volume (a methyl vs. a butyl group).

Temperature effect on enantioselectivity

To further optimize the transesterification of 4-methyloctanoic acid methyl ester, catalyzed by *Candida antarctica* lipase B, the temperature effect on enantioselectivity at 27°C was studied and compared with the reaction catalyzed

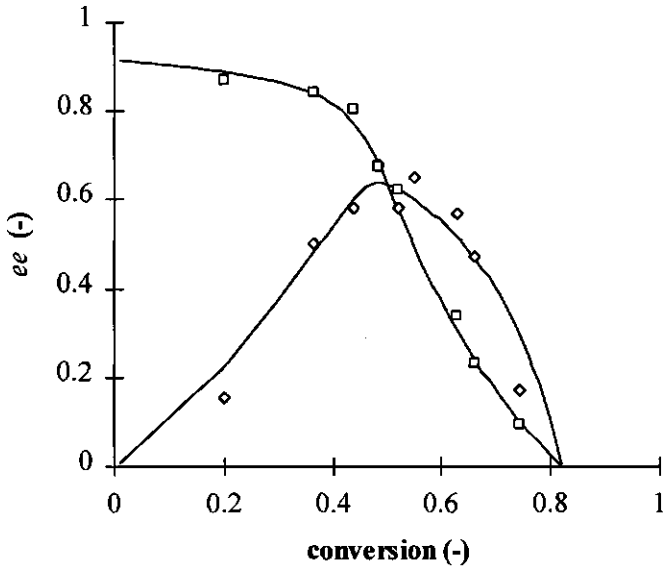


Figure 2: Transesterification of (**5**) by CALB at 27°C. Enantiomeric excess plotted versus conversion. Fitting the results revealed an *E* of 23, the curves are based on the formulas of Chen (1987) which were rectified by Engel (1992).

at 45°C. The enantiomeric excess was determined by NMR using a chiral shift reagent (see experimental section). An *E* of 23 was obtained for the reaction catalyzed at room temperature (figure 2).

In comparison with the transesterification at 45°C, the *E*-value increased with a factor 3. At 40% conversion an ee_p is obtained of approximately 82% (27°C) compared to 65% at 45°C.

Molecular modeling

The three-dimensional structure of *Candida antarctica* lipase B was solved by Uppenberg *et al.* (1994) and further investigated with a phosphonate inhibitor bound into the active site (Uppenberg *et al.*, 1995). The enzyme contains a

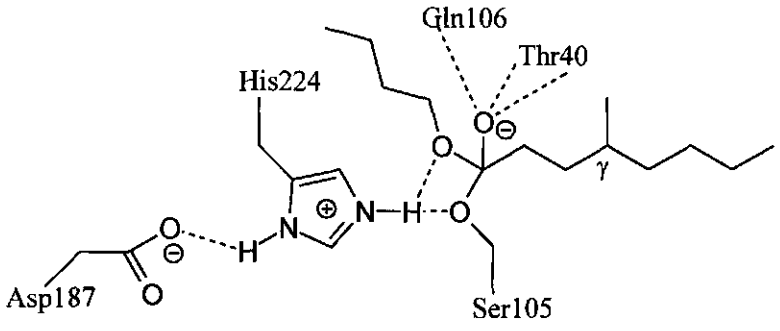


Figure 3: An oxyanion tetrahedral intermediate transition state model of 4-methyloctanoic acid butyl ester.

catalytic triad consisting of Ser 105, His 224 and Asp 187. Two other essential amino acids, Thr 40 and Gln 106, contribute to the stabilization of the tetrahedral intermediate oxyanion (figure 3).

The difference in reaction rate between the *R*- and the *S*-enantiomer of (**5**) was investigated by molecular modeling. Visualization of the binding of the *R*- and *S*-enantiomer of 4-methyloctanoic acid ester in the active site of *Candida antarctica* lipase B was realized by building a three dimensional computer model.

Ten structures obtained for each enantiomer were analyzed. Both lowest energy structures of the *R*- and *S*-complexes showed a valid charge-relay system. The *R*-structure was significantly lower in energy compared with the *S*-structure. This result is in agreement with the experimental data since the *R*-enantiomer is the fast reacting stereoisomer.

Experimentally, it was found that the enantiomeric ratio is dependent on the temperature and ranges from a value of 8 (45°C) to an *E* of 23 (at 27°C). The $\Delta\Delta G^{\ddagger}$ ($\Delta\Delta G^{\ddagger} = -RT\ln E$) can be separated into an enthalpic term, $\Delta\Delta H^{\ddagger}$, and an entropic term $T\Delta\Delta S^{\ddagger}$. In molecular modeling, *at best*, only the $\Delta\Delta H^{\ddagger}$ is taken into account ($\Delta\Delta G^{\ddagger} = \Delta\Delta H^{\ddagger}$). Thus the method is by definition qualitative and not quantitative.

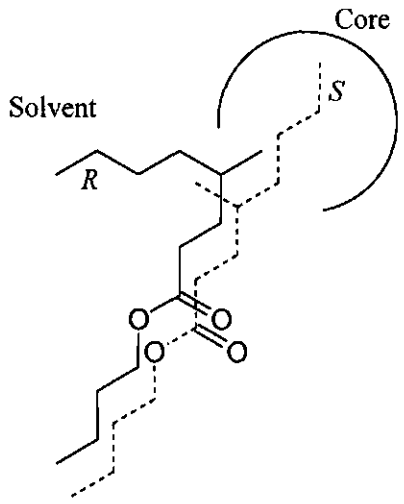


Figure 4: a) Schematic representation of both enantiomers located in the active site of CALB.

Visual inspection of the two enantiomeric structures and their minimum energy conformations provided information about the most plausible orientations. In figure 4 it can be seen that the long chain (butyl group) of the *R*- enantiomer turns toward the solvent, out of the active site, while the *S*-enantiomer places the methyl group in that direction.

Towards the core of the enzyme, the *R*-enantiomer contains only a methyl group. The *S*-enantiomer would orient its entire acyl chain inwards, resulting in severe steric crowding. Most of this is paid for by the substrate itself that is pushed outwards into a far from ideal conformation, 7 kcals/mol (29.4 kJ/mol) higher in energy compared to the *R*-substrate.

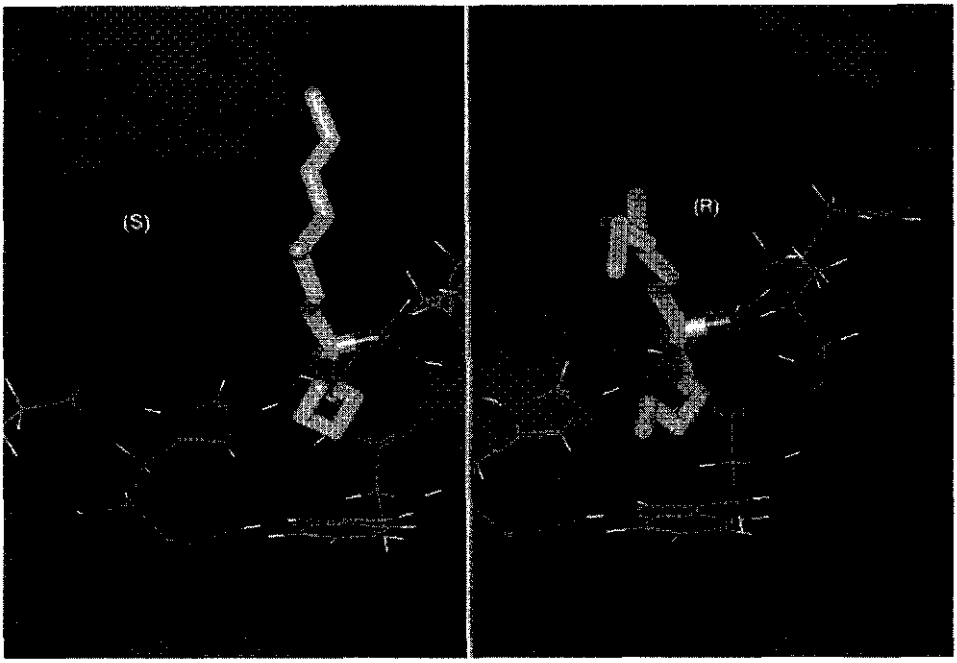


Figure 4: b) Molecular graphic of the resulting orientation of 4-methyloctanoic acid butyl ester in the catalytic site of CALB. The long chain (butyl group) of the *R*-enantiomer is pointing towards the solvent while, the S-enantiomer holds this acyl chain towards its core, which will result in a nonideal (*i.e.* high-energy) conformation of this acyl chain.

In conclusion, the enantiomeric ratio in the transesterification of 4-methyloctanoic acid methyl ester to its butyl ester can be risen to a value of 23 at a temperature of 27°C.

When the product obtained would be subjected to a second kinetic resolution, using the same enzyme, an enantiomeric excess of about 92% (at a conversion of 60%, T = 45°C) and 96% (at a conversion of 50%, T = 27°C) can be obtained according to the theory of Chen *et al.* (1982). This means that in a two-step kinetic resolution almost enantiomerically pure product could be obtained.

CONCLUSIONS

The kinetic resolution of 4-methyl branched chain fatty acid methyl esters was investigated. In the transesterification of 4-methylhexanoic acid methyl ester (**4**) and 4-methyloctanoic acid methyl ester (**5**), at 45 °C only two lipases (*Rhizomucor miehei* lipase, *Candida antarctica* lipase B) showed a good conversion and a moderate enantiomeric ratio ($2< E <8$). This moderate stereoselectivity might be explained by the fact that the chiral center is positioned at the C_4-atom, rather remote from the reaction center. In order to raise the enantiomeric excess, a two-step kinetic resolution would be the solution (Chen, 1982).

Rhizomucor miehei lipase and CALB showed a complementary enantiomeric preference. *Rhizomucor miehei* lipase favored the conversion of the *S*-enantiomer while CALB preferred the *R*-enantiomer of both (**4**) and (**5**). The enantioselectivity in the transesterification of (**5**), catalyzed by CALB was temperature dependent: the enantiomeric ratio increased from 8 to 23 upon decreasing the temperature from 45 °C to 27 °C.

The difference in enantiomeric ratio between (**4**) (E=5) and (**5**) (E=8) at 45 °C was explained by the fit of the substrate substituents in the catalytic site of the lipases. In the case of (**4**), the difference between an ethyl and a methyl group at the C_4-atom is small. Molecular modeling showed that both groups fit toward the core which resulted in a low enantiomeric ratio. In contrast, the butyl group of *S*-(**5**) occupied an energetically unfavorable position oriented towards the core of the enzyme, as opposed to an orientation into the solvent in case of *R*-(**5**).

REFERENCES

Besler, B., Mertz, K. and Kollman, P. (1990) Atomic charges derived from semi-empirical methods. *J. Comp. Chem.*, **11**, 431-439.

Chen, C-S., Fujimoto, Y., Girdaukas, G. and Sih, C. J. (1982) Quantitative analysis of biochemical kinetic resolutions of enantiomers. *J. Am. Chem. Soc.*, **104**, 7294-7299.

Chen, C-S., Wu, S-H., Girdaukas, G. and Sih, C. J. (1987) Quantitative analysis of biochemical kinetic resolution of enantiomers. 2. Enzyme-catalyzed esterifications in water-organic solvent biphasic systems. *J. Am. Chem. Soc.*, **109**, 2812-2817.

Engel, K-H. (1992) Lipase-catalyzed enantioselective acidolysis of chiral 2-methyl alkanoates. *JAOCS*, **69**, 146-150.

Jongejan, J.A., van Tol, J.B.A., Geerlof, A. and Duine, J.A. (1991) Enantioselective catalysis. 1. A novel method to determine the enantiomeric ratio. *Recl. Trav. Chim. Pays-Bas*, **110**, 247-254.

Karl, V., Kaunzinger, A., Gutser, J., Steuer, P., Angles-Angel, J. and Mosandl, A. (1994) Stereoisomeric flavour compounds LXVII. 2-, 3-, and 4-alkyl-branched acids, part 1: General approach to the synthesis of the enantiopure acids. *Chirality*, **6**, 420-426.

Kelly, S. M. and Buchecker, R. (1988) Ferroelectric liquid crystals. Chiral phenyl benzoates incorporating a trans-1,4-disubstituted cyclohexane ring. *Helv. Chim. Acta*, **71**, 451-460.

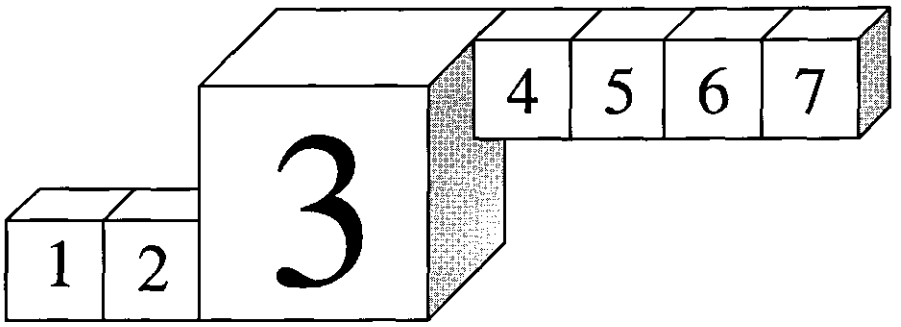
Krapcho, A.P. (1982) Synthetic applications of dealkoxycarbonylations of malonate esters, β-keto esters, α-cyano esters and related compounds in dipolar aprotic media-part 1. *Synthesis*, 805-822.

Norin, M., Mattson, A., Hult, K. and Norin, T. (1993) Molecular modelling of chymotrypsin-substrate interactions: Calculation of enantioselectivity. *Biocatalysis*, **7**, 131-147.

Uppenberg, J., Trier Hansen, M., Patkar, S. and Jones, A. (1994) The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida antarctica*. *Structure*, **2**, 293-308.

Uppenberg, J., Öhrner, N., Norin, M., Hult, M., Kleywegt, G.J., Patkar, S., *et al.* (1995) Crystallographic and molecular-modeling studies of lipase B from *Candida antarctica* reveal a stereospecificity pocket for secondary alcohols. *Biochemistry*, **34**, 16838-16851.

Wilson, W. K., Baca, S. B., Barber, Y. J., Scallen, T. J., and Morrow, C. J. (1983) Enantioselective hydrolysis of 3-hydroxy-3-methylalkanoic acid esters with pig liver esterase. *J. Org. Chem.*, **48**, 3960-3966.



Esterification of 4-methyloctanoic acid with polyethylene glycol at different a_w

SUMMARY

Novozym 435® (immobilized Candida antarctica lipase B) was used to esterify 4-methyloctanoic acid with polyethylene glycols (PEGs) of different molecular weights (PEG-600, PEG-3000 and PEG-20000) at different water activities and with different additives. A two-phase system of 4-methyloctanoic acid and PEG-600 was only obtained at a high water activity. Even at this water activity, PEG was still converted to its mono or diester, which proves the unique character of PEG. Unfortunately, the esters accumulated in the oil phase, hence, a straightforward downstream process could not be achieved.

Parts of this chapter have been submitted for publication, 1999.

INTRODUCTION

Biocatalysis in non-conventional media has received growing attention in the last decades (Laane *et al.*, 1987; Khmelnitsky *et al.*, 1988; Dordick, 1989; Halling, 1990; Halling and Valivety, 1992; Bell *et al.*, 1995; Koskinen and Klibanov, 1996 and Faber, 1997). Many reactions like (trans)esterification and interesterification have been carried out in apolar solvents, with a low thermodynamic water activity (a_w) and a log $P \geq 4$ (Laane *et al.*, 1987; Zaks & Klibanov, 1988; Bovara *et al.*, 1993; Janssen and Haas, 1994). In the literature, also a few examples are given for enzymatic reactions at high water activity performed in supercritical fluids (Sereti *et al.*, 1997; Almeida *et al.*, 1998; Tsitsimpikou *et al.*, 1998). Although these reactions have many advantages over organic solvents, from an economical point of view this technique might not be the first choice.

Working at low a_w makes it possible to shift thermodynamic equilibria, *e.g.* to favor esterification over hydrolysis. Although working in monophasic organic solvents seems to be the most logical medium for (trans)esterification reactions, several papers describe the preparation of esters in aqueous-organic two-phase systems (Klibanov *et al.*, 1977; Martinek *et al.*, 1981; Carrea, 1984; Janssen *et al.*, 1991; Janssen *et al.*, 1993; van der Padt *et al.*, 1993). The advantage of these systems is that the essential water layer around the enzyme is hardly affected, in contrast to the monophasic systems where inactivation is possible, especially when polar solvents (log P<2) are used. Furthermore, using a two-phase system facilitates downstream processing, *e.g.* by a membrane system.

This chapter describes the esterification of 4-methyloctanoic acid (4-MOA) with PEG in an aqueous-organic two-phase system using Novozym 435® (immobilized *Candida antarctica* lipase B) as the catalyst. PEG was used as the alcohol since it is a rather polar molecule and it was expected that the PEG-ester of 4-MOA would reside in the aqueous phase. In addition, PEG is known to stabilize enzymes just like glycerol and sorbitol (Khmelnitsky *et al.*, 1988; Boominathan *et al.*, 1995; Lambert *et al.*, 1997) and it is fully compatible with food industry. The partitioning of PEG in both phases and the equilibrium conversion was studied as

a function of the water activity and the presence of additives like apolar organic solvents or sugars.
We aimed at a two-phase system where the product should accumulate in the PEG-phase. Then membrane separation could facilitate continuous product removal.

MATERIALS & METHODS

Enzyme

Novozym 435® (immobilized *Candida antarctica* lipase B) was a generous gift from Novo Nordisk.

Chemicals

4-methyloctanoic acid was obtained from Oxford Chemicals, polyethylene glycol (average molecular weight: 600 g/mol; 3000 g/mol; 20,000 g/mol) was purchased from Merck. Acetonitrile was obtained from Labscan and Hydranal® from Riedel de Haën. Dextran T10 was obtained from Pharmacia, α-cyclodextrin and β-cyclodextrin were obtained from Avebe. Polyethylene was purchased from Aldrich and block copolymer Synperonic A3 was obtained from ICI. Chloroform, octane and hexane were used as p.a. quality.

General procedures

The water content in organic solvents was determined by Karl Fisher titration using Hydranal® for volumetric titration. Partition experiments and esterification reactions were carried out in a New Brunswick Scientific Innova™ 4080 incubator shaker at 350 rpm and 45°C, unless otherwise stated.
The conversion of 4-methyloctanoic acid was determined by HPLC using a RP-18 column (250 x 4.6 mm ID, S5-ODS2, flow 1 ml/min) with acetonitrile / water (80:20) as eluent (RT PEG-600: 2.50 min, 4-MOA: 4.02 min). The

chromatographic system consisted of a Separations model 300 high-pressure pump, a Separations model marathon (version 251) auto-sampler and a Separations RI 71 refractive index detector. The injection loop had a capacity of 20 µl. The computer program Gynkosoft (v5.3) from Separations monitored the column effluent.

Determination of the water content initially present in 4-MOA and PEG-600

The initial amount of water present in 4-methyloctanoic acid and PEG-600 was determined by Karl Fisher titration using 50 mg of 4-methyloctanoic acid or PEG-600 in a chloroform solution. PEG-600 had an average water content of 0.510 % (w/w). For all experiments, this initial amount of water was taken into account. The amount of water in 4-methyloctanoic acid was negligible.

Sorption isotherm

The amount of water in PEG-600 at different thermodynamic water activities (a_w), at room temperature was determined. For this purpose, four jars were filled with a layer of wetted salt with known a_w (LiBr: 0.064; LiCl: 0.113; $MgCl_2$: 0.328 and NaCl: 0.753). In each jar a vial containing PEG-600 was placed. Samples (100 mg) were taken from the vial at different times and analyzed until equilibrium was reached.

PEG-600: partition experiments and enzymatic esterifications

In 4 ml screw cap vials, PEG-600, water and 4-methyloctanoic acid were added in various amounts to a total weight of approximately 2 g (table 1). After mixing, the vials were either placed in an end over end incubator at room temperature or in an incubator shaker. After thermostatting the vials at 45°C for ten minutes, esterification was started by adding Novozym 435® to the solution. Samples from the organic and water layers for both the partition experiments and the enzymatic reactions were taken and analyzed by HPLC. Esterification was studied at three different water activities (0.02, 0.4 and 0.8). The water activities used represent the initial water activity in the reaction mixture at room temperature.

Table 1: Composition of the mixtures with varying water amounts and PEG-600

ratio 4MOA *vs* PEG/ water *w / w*	PEG-600 *g*	water *g*	4-MOA *g*	T *°C*	Novozym 435® *mg*
1 : 1	0.051 to 0.500	0.959 to 0.504	1.00	25	--
1 : 1	0.501 to 0.955	0.499 to 0.045	1.00	45	--
1 : 2	0.335 to 0.637	0.332 to 0.030	1.33	45	--
2 : 1	0.670 to 1.273	0.662 to 0.063	0.67	45	--
1 : 1	0.669 to 0.931	0.331 to 0.069	1.00	45	16.4
1 : 1	1.0005	0.0	1.00	45	14.3
1 : 1	0.9331	0.0670	1.07	45	15.1
1 : 1	0.6539	0.3485	1.06	45	15.4

PEG-3000: partition experiments and enzymatic esterifications

In 4 ml screw cap vials, PEG-3000, water and 4-methyloctanoic acid were mixed. The amounts used are presented in table 2.

In contrast to PEG-600, PEG-3000 is a solid substrate. To dissolve this substrate, the mixtures with a low water content were sonicated. To one series, Novozym 435™ was added in order to start the esterification. During the course of reaction 10 µl samples were taken, diluted with eluent to 1 ml and analyzed by HPLC.

Table 2: Composition of the mixtures with varying water amounts and PEG-3000

Ratio 4-MOA *vs* PEG/ water *w / w*	PEG-3000 *g*	water *g*	4-MOA *g*	T *°C*	Novozym 435® *mg*
1 : 1	0.654 to 0.903	0.349 to 0.100	1.00	45	--
1 : 1	0.648 to 0.900	0.348 to 0.100	1.00	45	20

Addition of apolar solvents

In order to regain a two-phase system at low water activity, different amounts of hexane and octane, ranging from 5% to 40% ($w/w_{4\text{-methyloctanoic acid}}$) were added to a mixture (1:1 w/w) of 4-methyloctanoic acid (organic phase) and PEG-600/water (water phase). In table 3 the composition of the mixtures is given.

Table 3: Composition at different a_w and varying amounts of apolar solvents

apolar solvent	PEG-600	water	4-MOA	a_w	Novozym 435®
	g	*g*	*g*	-	*g*
Hexane (5 to 40% w/w)[1]	0.98	0.02	1.00	0.2	
	0.93	0.07	1.00	0.4	
Octane (5 to 40% w/w)[1]	0.98	0.02	1.00	0.2	
	0.93	0.07	1.00	0.4	
40%	0.93	0.07	1.02	0.4	0.021
35%	0.84	0.16	1.01	0.6	0.033
40%	0.84	0.16	1.01	0.6	0.028

[1] The hexane and octane percentages were varied from 5 to 40% (w/w) with respect to 4-methyloctanoic acid.

Addition of sugars & block-copolymer

Sugars or a block-copolymer were added to a mixture of 4-methyloctanoic acid, water and PEG-600 to study their effect on the partitioning of PEG-600 at 45°C. After addition of Novozym 435® to the mixtures the esterification experiments were started. Samples (15 µl) were taken, diluted with 1.5 ml of eluent and injected on a HPLC column. In table 4 the conditions for the different experiments are given.

Table 4: Improvement of two-phase system using sugars or polymers

sugar/ polymer	# sugar / polymer	PEG-600	H_2O	4-MOA	Novozym 435®
	g	*g*	*g*	*g*	*g*
-	-	2.0941	0.1398	0.5275	
	-	1.9982	0.1392	0.5565	0.0263
β-cyclodextrin	0.2012	2.0794	0.1401	0.5386	
	0.2007	2.0445	0.1400	0.5391	0.0240
α-cyclodextrin	0.2099	2.0593	0.1386	0.5399	
	0.1986	2.0483	0.1409	0.5315	0.0230
dextran T10	0.1795	2.0905	0.1398	0.5422	
	0.1795	2.0440	0.1400	0.5439	0.0238
PE + BCP[1]	0.3625	2.0644	0.1372	0.5484	
	0.3718	2.0056	0.1415	0.5363	0.0248

[1] PE = polyethylene, BCP = block copolymer

RESULTS & DISCUSSION

Sorption isotherm of PEG-600

To relate the thermodynamic water activity (a_w) to the amount of water present in PEG-600 at room temperature, the water content at equilibrium was determined for four different water activities. In figure 1 the sorption isotherm for PEG-600 is shown. This plot was used to determine the initial water activity in the partition experiments and enzymatic reactions studied.

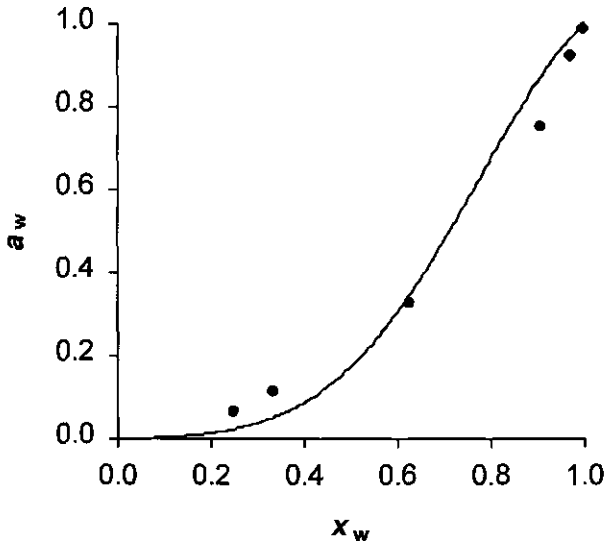


Figure 1: Sorption isotherm of PEG-600. The molar water fraction at equilibrium is plotted *versus* the thermodynamic water activity for the following salts: LiBr, LiCl, $MgCl_2$ and NaCl (●: experimentally determined, ◆ data determined by Voilley *et al.* (1989)). The line is calculated using the Norrish equation (Norrish, 1966).

Partition experiments and enzymatic esterifications using PEG

To perform a reaction in a membrane reactor it is of importance to have two separate phases. Therefore, both the partition of PEG and the enzymatic esterification were studied in a mixture of 4-methyloctanoic acid and water as a function of the water activity (a_w).

Partition experiments

Partition experiments were carried out to study the behavior of biphasic systems containing 4-methyloctanoic acid (organic layer) and PEG-600/water mixtures (water layer). The solutions were mixed thoroughly at room temperature for 1.5

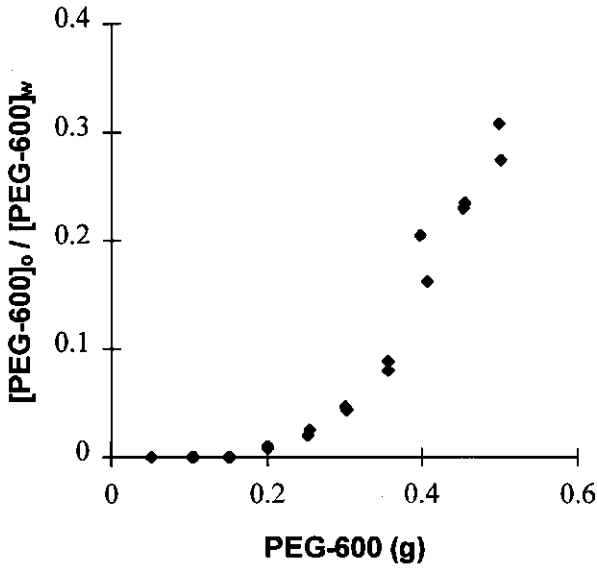


Figure 2: Partition coefficient of PEG-600 in water/4-methyloctanoic acid systems at room temperature plotted *versus* the initial amount of PEG-600 added to the mixture. The subscript *o* represents the organic phase and the subscript *w* represents the water phase.

hour in an end over end incubator. After separation of the two layers, the concentration of PEG-600 and 4-methyloctanoic acid in both layers was determined. As is shown in figure 2, upon raising the amounts of PEG-600 in the system the more PEG will diffuse to the organic phase. Almost no 4-methyloctanoic acid could be detected in the water layer. The same experiment was carried out in an incubator shaker using three different water activities and three different ratios of acid *versus* PEG/water. After approximately 20 hours a monophasic system was formed in the vials containing less than 30% (w/w) water ($a_w < 0.76$). The other systems remained biphasic. Increasing the ratios of acid phase to PEG-600/water phase did not have any influence on the miscibility of the system, but the volume of the water phase decreased in all vials during the experiments. The same behavior was observed when PEG-600 was replaced by PEG-3000. In a system containing PEG-molecules with a higher molecular weight of 20,000 (results not shown), highly viscous mixtures were obtained.

Furthermore, using PEG-20,000 as an alcohol is unpractical because in order to convert 1 g of 4-methyloctanoic acid, 63 g (in case of 100% di-ester formation) to 126 g (in case of 100% mono ester formation) of PEG is needed. For this reason PEG-20,000 was not studied any further.

Enzymatic esterification

Esterification of 4-methyloctanoic acid using PEG-600 or PEG-3000 was performed at water activities ranging from 0.02 to 0.8 using Novozym 435® as a catalyst. Remarkably, ester formation was observed in all systems, even at a_w = 0.8. Figure 3 shows that esterification of 4-methyloctanoic acid with PEG-600 at different water activities led to different equilibrium positions. The higher the water content, the lower the equilibrium conversions because the reverse reaction

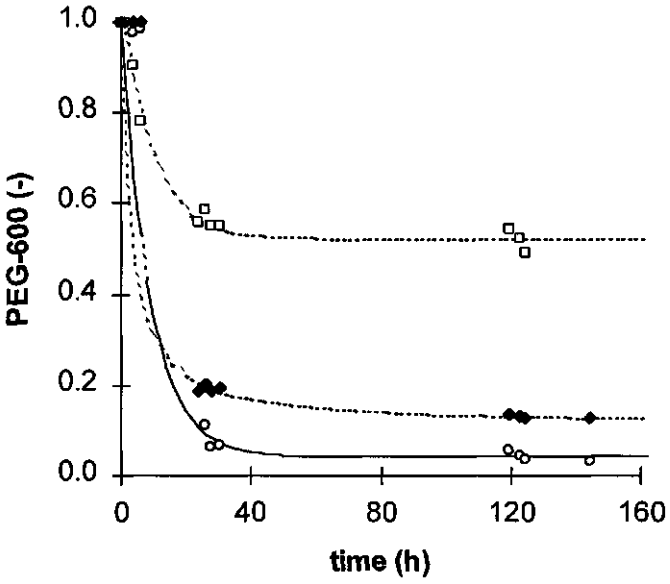


Figure 3: Esterification of 4-methyloctanoic acid with PEG-600. Decrease of PEG-600 in monophase (a_w = 0.02 and 0.4) or organic phase (a_w = 0.8) plotted *versus* time with ○ a_w = 0.02, ◆ a_w = 0.4 and □ a_w = 0.8. The y-axis represents the relative amount of PEG-600 remaining at time t.

(hydrolysis) is becoming more important. Only in the reaction systems having an a_w of 0.8, the system remained biphasic. Here the PEG-ester accumulated in the organic phase.

At varying water contents, the course of esterification of 4-methyloctanoic acid with PEG-600 and PEG-3000 was monitored during 500 hours. In the figure below, the equilibrium conversions of PEG-600 and PEG-3000 are plotted *versus* the water activity.

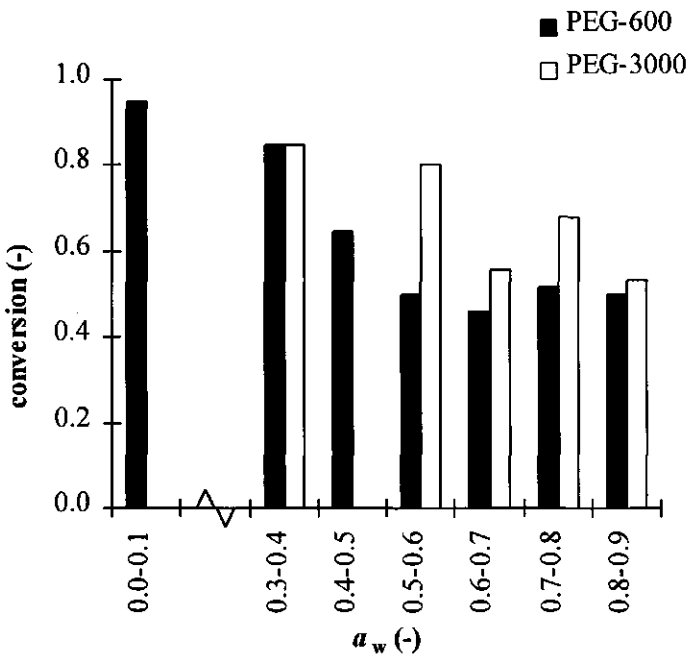


Figure 4: Conversions of PEG-600 and PEG-3000 at equilibrium plotted *versus* water activity.

The approximate water activities for PEG-3000 were calculated using the Norrish equation (Voilley *et al.*, 1989; Norrish, 1966) and the data of the PEG-600 reactions. As can be concluded from figure 4, the equilibrium conversion for PEG-600 is decreasing with increasing water activity until $a_w = 0.5$. For the higher water activities approximately 50% of PEG-600 was converted. Although fewer

experiments were performed for PEG-3000, it seems that even for this alcohol the same decrease is perceptible. At higher water activity more PEG-3000 is converted at equilibrium. The difference in equilibrium conversions might be explained by an entropy change during the formation and arrangement of the PEG-esters.

Addition of apolar solvents

In the previous section two PEG molecules of different molecular weight were compared in the partition experiments and the enzymatic esterification. It was shown that a two-phase system was only stable at high water activity. To decrease the miscibility of the system at low a_w, the addition of either hexane or octane was studied.

Partition experiments

At different water activities, the amounts of hexane or octane were varied from 5 to 40% (w/$w_{4\text{-methyloctanoic acid}}$) and added to a 1:1 mixture (w/w) of acid/(PEG-600 + water). After shaking for 4 days, the vials containing less than 30% of hexane or octane yielded one-phase systems. At higher octane or hexane percentages, two phases were present for longer periods. It was also observed that the higher the water activity and the hexane or octane concentration, the longer the systems remained biphasic. The vials containing 35 and 40% of octane and having an a_w of 0.4 or 0.6 still remained biphasic after one month. In conclusion, a more hydrophobic character of the organic layer contributes to the formation of a stable biphasic system.

Enzymatic esterification

The stable biphasic mixtures were also used to study the esterification of 4-methyloctanoic acid with PEG-600. 4-Methyloctanoic acid was indeed esterified, but the biphasic system disappeared, and after 13 hours one phase was present in all three cases (a_w 0.4, 40% (w/$w_{4\text{-methyloctanoic acid}}$) octane; a_w 0.6, 35%

($w/w_{4\text{-methyloctanoic acid}}$) octane and a_w 0.6, 40% ($w/w_{4\text{-methyloctanoic acid}}$)). A possible explanation for these results may be the formation of reversed micelles. Apparently, coupling of the apolar fatty acid chain to the polar PEG moiety converts the molecule into a surfactant.

Addition of sugars and a block copolymer

Since making the organic phase more apolar did not give the desired results, an attempt was made to modify the aqueous phase.

Partition experiments

Mixtures of 4-methyloctanoic acid, PEG-600, and sugars (α-cyclodextrin, β-cyclodextrin, dextran) or a block copolymer (polyethylene combined with synperonic A3) were incubated at room temperature. After 12 days, no interface was perceptible anymore. Apparently the addition of sugars or a polymer to the mixture of 4-methyloctanoic acid and PEG/water did not contribute to the stability of the biphasic system. Instead of increasing the difference in polarity between the organic phase and the water phase, addition of sugars or a block copolymer led to a better mixing of PEG-600 and 4-methyloctanoic acid.

Enzymatic esterification

In the presence of the sugars or a polymer, CALB-mediated esterification of 4-methyloctanoic acid with PEG-600 was studied. Unfortunately, in none of the cases an ester was formed. Obviously, the presence of these additives in the reaction medium inactivates the lipase.

CONCLUSIONS

In order to obtain a biphasic system, which is easy to process (*e.g.* by a membrane reactor), partition experiments and enzymatic esterifications were carried out with

4-methyloctanoic acid and polyethylene glycol (average molecular weight of 600 g/ mole and 3000 g/ mole). It appeared that only at high water activity the system remained biphasic. In this system the PEG-ester remained in the organic phase. Addition of octane improved the immiscibility of the system. In a system containing 35 or 40% of octane ($w/w_{4\text{-methyloctanoic acid}}$) at an a_w of 0.4 or 0.6, two phases were present for at least a month. However, when 4-methyloctanoic acid was esterified in the presence of CALB, the biphasic system slowly changed into one phase, presumably caused by the formation of reversed micelles. The attempt to add sugars to a mixture of PEG, water and 4-methyloctanoic acid resulted in inactivation of the enzyme.

In summary, for the use of PEG in a membrane reactor it is desirable to have the PEG-ester in the water phase and to have a stable biphasic system. Unfortunately this proved to be impossible. In the next chapters, a different approach for the esterification of 4-methyloctanoic acid and the hydrolysis of the ester formed are presented. However, PEG is a unique molecule and can be used to esterify 4-methyloctanoic acid at any water activity.

REFERENCES

Almeida, M.C., Ruivo, R., Maia, C., Freire, L., Corrêa de Sampaio, T. and Barreiros, S. (1998). Novozym 435 activity in compressed gases. Water activity and temperature effects. *Enzyme Microb. Technol.*, **22**, 494-499.

Bell, G., Halling, P.J., Moore, B.D., Partridge, J. and Rees, D.G. (1995). Biocatalyst behaviour in low-water systems. *TIBTECH*, **13**, 468-473.

Boominathan, R., Mishra, P. and Chand, S. (1995). Isolation of lipase from the culture filtrate of *Humicola lanuginosa*. *Bioseparation*, **5**, 235-239.

Bovara, R., Carrea, G., Ottolina, G. and Riva, S. (1993). Effects of water activity on V_{max} and K_M of lipase catalyzed transesterification in organic media. *Biotechnol. Lett.*, **15**, 937-942.

Carrea, G. (1984). Biocatalysis in water-organic solvent two-phase systems. *TIBTECH*, **2**, 102-106.

Dordick, J.S. (1989). Enzymatic catalysis in monophasic organic solvents. *Enzyme Microb. Technol.*, **11**, 194-211.

Faber, K. (1997). Enzymes in organic solvents, in: Biotransformations in organic chemistry, 2nd ed., Springer-Verlag, Berlin, Germany, 301-344.

Halling, P.J. (1990). Solvent selection for biocatalysis in mainly organic systems: predictions of effects on equilibrium position. *Biotechnol. Bioeng.* **35**, 691-701.

Halling, P.J. and Valivety, R.H. (1992). Physical-chemical nature of low water systems for biocatalysis: especially phase behaviour, water activity and pH, in: Biocatalysis in non-conventional media, eds. Tramper, Vermuë, Beeftink, and von Stockar, Elsevier, Amsterdam, The Netherlands, 13-21.

Janssen, G.G. and Haas, M.J. (1994). Lipase-catalyzed synthesis of oleic acid esters of polyethylene glycol 400. *Biotechnol. Lett.*, **16**, 163-168.

Janssen, A.E.M., Klabbers, C., Franssen, M.C.R. and Riet, van 't, K. (1991). Enzymatic synthesis of carbohydrate esters in 2-pyrrolidone. *Enzyme Microb. Technol.*, **13**, 565-572.

Janssen, A.E.M., Padt, van der, A. and Riet, van 't, K. (1993). Solvent effects on lipase-catalyzed esterification of glycerol and fatty acids. *Biotechnol. Bioeng.*, **42**, 953-962.

Janssen, A.E.M., Padt, van der, A., Sonsbeek, van, H.M. and Riet, van 't, K. (1993). The effect of organic solvents on the equilibrium position of enzymatic acylglycerol synthesis. *Biotechnol. Bioeng.*, **41**, 95-103.

Khmelnitsky, Y.L., Levashov, A.V., Klyachko, N.L. and Martinek, K. (1988). Engineering biocatalytic systems in organic media with low water content. *Enzyme Microb. Technol.*, **10**, 710-724.

Klibanov, A.M., Samokhin, G.P., Martinek, K. and Berezin, I.V. (1977). A new approach to preparative enzymatic synthesis. *Biotechnol. Bioeng.*, **19**, 1351-1361.

Koskinen, A.M.P. and Klibanov, A.M., (eds.) (1996). Enzymatic reactions in organic media, Blackie Academic & Professional, Glasgow, UK, 1-314.

Laane, C., Boeren, S., Vos, K. and Veeger, C. (1987). Rules for optimization of biocatalysis in organic solvents. *Biotechnol. Bioeng.*, **30**, 81-87.

Lambert, C. Pourplanche, C. and Larreta-Garde, V. (1997). Comparison of macromolecular and molecular effects of cosolvents on the catalytic behaviour of soybean lipoxygenase-1. *Biocatal. Biotransform.*, **15**, 39-55.

Martinek, K., Semenov, A.N. and Berezin, I.V. (1981). Enzymatic synthesis in biphasic aqueous-organic systems. 1. Chemical equilibrium shift. *Biochim. Biophys. Acta,* **658**, 76-89.

Norrish, R.S. (1966). An equation for the activity coefficients and equilibrium relative humidities of water in confectionery syrups. *J. Food Technol.*, **1**, 25-39.

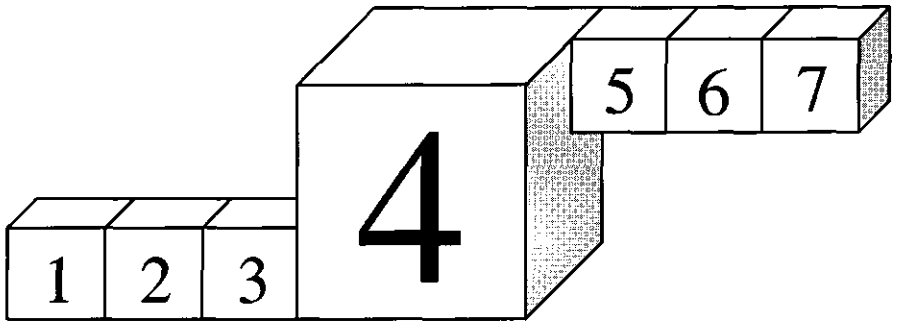
Padt, van der, A., Janssen, A.E.M., Sewalt, J.J.W. and Riet, van 't, K. (1993). Acylglycerol equilibrium in a two-phase system. *Biocatalysis*, **7**, 267-277.

Sereti, V., Stamatis, H. and Kolisis, F.N. (1997). Improved stability and reactivity of *Fusarium solani* cutinase in supercritical CO_2. *Biotechnol. Tech.*, **11**, 661-665.

Tsitsimpikou, C., Stamatis, H., Sereti, V., Daflos, H. and Kolisis, F.N. (1998). Acylation of glucose catalysed by lipases in supercritical carbon dioxide. *J. Chem. Technol. Biotechnol.*, **71**, 309-314.

Voilley, A., Blond, G. et Chevallier, G. (1989). Mesure et prediction de fortes activités de l'eau dans de systèmes eau-polymères. *Lebensm. -Wiss. u. Technol.*, **22**, 32-38.

Zaks, A. and Klibanov, A.M. (1988). The effect of water on enzyme action in organic media. *J. Biol. Chem.*, **263**, 8017-8021.



Substrate sorption into the polymer matrix of Novozym 435® and its effect on the enantiomeric ratio estimation

SUMMARY

Candida antarctica lipase B (Novozym 435®), was used to catalyze the enantioselective esterification of 4-methyloctanoic acid with ethanol. Solvents or substrates may cause the carrier material of Novozym 435® to swell. In this work, the addition of these substrates caused an increase of the volume of the enzyme beads. On the basis of microscopic observation, it was concluded that on average 0.0075 mol substrate (1.19 g) sorbed into 1 gram of enzyme beads. Hence, this part of the substrate is not available for the reaction resulting in an overestimated apparent conversion. As a result, the apparent enantiomeric ratios determined for the esterification reaction showed large confidence intervals. Correction of the conversion data for sorption and recalculation of the enantiomeric ratio showed correct values with an acceptable confidence interval. The optimum enantiomeric ratio (E=57) was observed for a 1 to 8 ratio of 4-methyloctanoic acid versus ethanol (mol:mol). In transesterification reactions, the enantiomeric ratio is equal for both the ee_s vs c and ee_p vs ee_s plots. Apparently, substrate sorption does not occur under conditions of a large excess of apolar organic solvent. In hydrolysis reactions, titration of the generated acid with KOH is the driving force for desorption of the acid from the enzyme beads, leading to conversion data, which are not influenced by swelling.

This chapter has been submitted for publication, 1999.

INTRODUCTION

Nowadays, lipases are well-accepted catalysts in organic chemistry, both in aqueous and non-aqueous media. Working with these enzymes in organic solvents has several potential advantages, *e.g.* the possibility to shift thermodynamic equilibria to favor esterification over hydrolysis (Arnold, 1990; Klibanov, 1990; Wescott & Klibanov, 1994; Koskinen & Klibanov, 1996; Faber, 1997). In this way, kinetic resolution of acids or alcohols can be performed by lipase-mediated esterification in organic solvents (Engel, 1991; Berglund *et al.* 1994, Anderson *et al.* 1998; Duan and Ching 1998, Nguyen and Hedenström, 1999).

To evaluate kinetic resolution in terms of enantioselectivity, the enantiomeric ratio (E) has been defined. This parameter corresponds to the ratio of the specificity coefficients, V_{max}/K_M, of the individual substrate enantiomers. Several methods exist for the determination of E (Chen *et al.*, 1982; 1987; Jongejan *et al.*, 1991; Rakels *et al.*, 1994; Straathof and Jongejan, 1997).

In this thesis we describe the esterification of 4-methyloctanoic acid, catalyzed by Novozym 435® (*Candida antarctica* lipase B, CALB). This enzyme showed the best results (E = 23) in the transesterification studies of 4-methyloctanoic acid methyl ester to its butyl ester (Heinsman *et al.*, 1998). In the present work, the sorption of 4-methyloctanoic acid into the hydrophobic beads proved to play an important role in determining the enantiomeric ratio (E) of the esterification reaction. The influence of this sorption behavior on hydrolysis and transesterification rates is also discussed.

MATERIALS & METHODS

Chemicals

4-Methyloctanoic acid (4-MOA) was purchased from Oxford Chemicals. Glacial acetic acid, toluene and octane were obtained from Acros. Tetradecane was

purchased from Aldrich, ethanol was obtained from Merck and dichloro dimethylsilane was supplied by Sigma.

Enzyme

Candida antarctica lipase B (Novozym 435®) is a product of NOVO Nordisk A/S Denmark. The support material consists of a DVB-crosslinked, hydrophobic macroporous polymer based on methyl- and butyl methacrylic esters. The lipase is adsorbed to the surface of the support material by hydrophobic interaction.

Apparatus

Microscope: Olympus automatic exposure photo micrographic system model PM-10AK, Olympus C35DA-2 photo camera (4x10), Magic Imaging MTI series 68.

Video: Olympus BH2 microscope, Sony model DXC-151AP camera.

Esterification of 4-methyloctanoic acid

Esterification was carried out in 4 ml screw-cap vials placed in a New Brunswick Scientific Innova™ incubator at 35°C and 350 rpm for up to 100 hours. The vials

Table 1: Reaction conditions for esterification of 4-methyloctanoic acid and ethanol

ratio A : E[1]	4-MOA		EtOH	
mol : mol	*g*	*mmol*	*g*	*mmol*
2:1	1.743	11.01	0.265	5.75
1:1	1.580	9.98	0.475	10.31
1:2	1.263	7.98	0.749	16.26
1:4	0.927	5.86	1.086	23.57
1:8	0.607	3.84	1.430	31.04
1:10	0.517	3.27	1.508	32.73
1:15	0.377	2.38	1.689	36.66
1:20	0.295	1.86	1.701	36.92

[1] A = 4-methyloctanoic acid, E = ethanol

contained the substrates 4-methyloctanoic acid and ethanol in different mol ratios as well as tetradecane (0.05 g of internal standard) and *Candida antarctica* lipase B (20 – 30 mg of CALB) in a total volume of approximately 2 ml. During the course of the reaction 20 µl samples were taken and diluted to 1 ml with octane, containing 0.5 % (v/v) acetic acid. The amounts used are given in table 1.

GC analysis

Aliquots of 0.1 µl were analyzed on a gas chromatograph using a GC-8000 series Fisons Instruments (8160) with a MFC 800 control unit and an A200S CE Instruments autosampler. The GC was equipped with a chiral γ-DEX™120 WCOT SUPELCO capillary column (30m x 0.25 mm ID, 0.25 mm) (Heinsman and Belov, 1997). Hydrogen was used as carrier gas (70 kPa). A temperature program was used to separate both the enantiomers of 4-methyloctanoic acid and 4-methyloctanoic acid ethyl ester (T_1=75°C, t_1=53 min, r_1=5°C/min, T_2=115°C, t_2=33 min). The peaks were detected by a FID (EL 980 Fisons Instrument) set at 200°C. The split temperature was 225°C. The GC was connected to the computer program Xchrom / Windows 4.0 version 2.11b for Windows NT (ThIS Labsystems) for online data acquisition (RT 4-methyloctanoic acid ethylester = 49.25 min, 49.79 min [α = 1.05], RT 4-methyloctanoic acid = 82.77 min, 84.10 min [α = 1.02]). The liner of the GC was replaced by a new silylated liner every week, since injection of fatty acids leads to a desilylation reaction which gives rise to adsorption of the fatty acids to the glass. Liners were silylated in a solution of dichlorodimethylsilane (5%) in toluene, rinsed with methanol and dried. A piece of silylated glasswool was placed into the liner to protect the capillary column from contamination.

Weight of CALB

The mean weight of 1 enzyme bead was determined by weighing 5 series of 10 beads and taking the average. One bead of Novozym 435® (*Candida antarctica* lipase B, CALB) did have an average weight of $5.4 \cdot 10^{-2}$ mg. One gram of Novozym 435® thus contains 18500 ± 2900 beads.

Diameter and volume

Approximately 300 'dry' CALB beads, 300 CALB beads soaked in 4-methyloctanoic acid and 300 CALB beads soaked in 4-methyloctanoic acid ethylester were scanned. For each bead the diameter was determined using the program RESULTS from Applied Imaging, version 5.1, 1991.

The Sauter mean diameter was calculated using the equation: $d_{32} = \frac{\sum n_i \cdot d_i^3}{\sum n_i \cdot d_i^2}$ and the mean volume was calculated using the equation: $V = \frac{1}{n}\sum_{k=1}^{n} \frac{4}{3}\pi \cdot r_k^3$, with n = 390 (dry CALB beads), 305 (CALB beads saturated with 4-methyloctanoic acid) and 381 (CALB beads saturated with 4-methyloctanoic acid ethyl ester).

Rate of swelling

The swelling of enzyme beads was recorded on a videotape. To one bead, viewed under the microscope, a drop of 4-methyloctanoic acid was added. The swelling was followed in time.

The volume was calculated as $V = \frac{1}{n}\sum_{k=1}^{n} \frac{4}{3}\pi \cdot r_k^3$. The relative volume was calculated as $\Delta V = \frac{V_{swollen}}{V_{original}}$

Determination of *E*-values

For the esterification of 4-methyloctanoic acid with ethanol, the enantiomeric ratio was determined only using enantiomeric excess data of the substrate and the extent of conversion, due to a poor separation of the 4-methyloctanoic acid ethyl ester enantiomers on GC. Plotting the ee_s *versus* the conversion in the computer program SIMFIT (Jongejan *et al.* 1991) gave the enantiomeric ratio.

To determine the peak areas of the 4-methyloctanoic acid enantiomers at t_0, a sample was taken from the mixture in the absence of lipase. However, in case of

substrate sorption, the initial amount of 4-methyloctanoic acid (mol/g) is different from the one determined by GC at t_0. Therefore the conversions calculated from the GC-data at time t and t_0 were corrected for the amount of 4-methyloctanoic acid (mol/g) sorbed into the enzyme beads. Plotting the ee_s *versus* the conversion in the SIMFIT program (Jongejan *et al.* 1991) gave new *E*-values.

RESULTS & DISCUSSION

Determination of the enantiomeric ratio in the esterification of 4-methyloctanoic acid

Esterification of 4-methyloctanoic acid was performed using a range of concentrations varying from 2:1 up to 1:20 mol fatty acid to mol ethanol. Remarkably, Novozym 435® catalyzed this reaction efficiently in the absence of solvent and in the presence of a large amount of polar substrate (ethanol, logP = -0.24). Under all circumstances, except for the 2:1 ratio, the reactions reached approximately 80% conversion of 4-methyloctanoic acid after 100 hours. The enantiomeric excess of the substrate was determined from the GC-data and plotted *versus* the measured conversion. The data were fitted using the computer program SIMFIT (Jongejan *et al.*, 1991), which uses the Chen equation for competitive Michaelis Menten kinetics for equilibrium reactions. This program calculates the enantiomeric ratio, the equilibrium constant and their confidence intervals. Figure 1 shows the calculated *E*-values and their confidence intervals for all fatty acid to ethanol ratios studied. In most of the cases there was poor agreement between model and data, resulting in large confidence intervals for the estimated *E*-values. For the ratio 1:8 a confidence interval is shown from 0-140 since the Chen equation could in this case not be used to fit the data. The poor fits could not be attributed to enzyme inactivation, since reuse of CALB showed the same enzyme activity (results not shown). The poor fits appeared to be caused by swelling of the enzyme beads. Due to swelling of polymers in the presence of organic solvents or

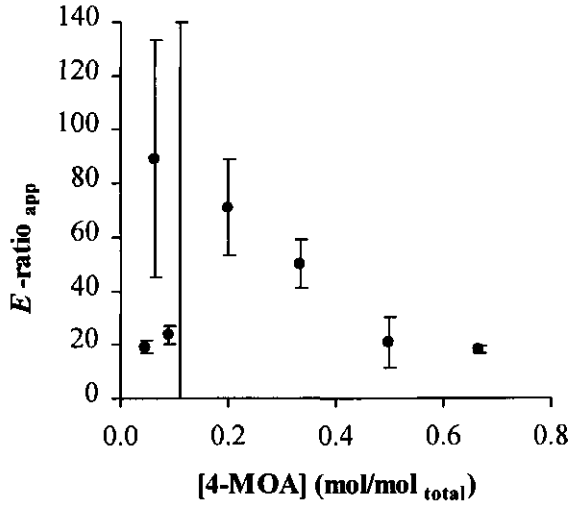


Figure 1: Esterification of 4-methyloctanoic acid with ethanol, catalyzed by Novozym 435® at different ratios varying from 2:1 up to 1:20 (mol fatty acid: mol ethanol). The estimated *E*-values and their confidence intervals were calculated using the equation of Chen and coworkers (1987) in the program SIMFIT (Jongejan *et al.*, 1991).

substrates, a part of the substrate is sorbed into the beads and is therefore not available for the reaction. This leads to an overestimation of the conversion.

Swelling of enzyme beads

Swelling of polymer materials in the presence of solvents is a common and widely known phenomenon. Andreopoulos, 1989; Sadler & Braddock, 1990; Charara *et al.*, 1992; Chen *et al.*, 1995; Johansson & Leufvén, 1997; Martin, *et al.*, 1998). Since in our case the swelling would give an explanation of the poor fits obtained, it was attempted to measure the swelling quantitatively. Different techniques were used to measure the volume increase that results from the diffusion of ester or acid into the macroporous beads of the polymer on which *Candida antarctica* lipase B is immobilized.

Characterization of the swelling

In order to monitor changes in the structure of the enzyme beads or increase in volume, Novozym 435® was equilibrated with pure 4-methyloctanoic acid or its ethyl ester. The hydrophobic material was studied under a microscope. As a result of sorption in the pores, the hydrophobic beads changed from opaque into a larger translucent structure (figure 2). At the same time, the mean diameter increased. A reliable mean value of the diameter was obtained by taking the average of approximately 300 beads of both enzyme beads equilibrated with 4-methyloctanoic acid or its ethyl ester, and 'dry' Novozym 435® beads. In figure 3 the distribution of the diameter was plotted for the three different cases. For the wetted enzyme beads the distribution shifted towards the right, which implies an increase in the diameter. A slight difference was observed between the enzymes wetted in 4-methyloctanoic acid and in 4-methyloctanoic acid ethyl ester. The Sauter mean diameter (d_{32}) was calculated using these diameter data (table 2). Assuming spherical beads the volume of both the swollen and 'dry' beads could also be calculated. From these calculations it can be concluded that the volume increased with 0.067 ± 0.017 µl. Assuming that this increase is caused by sorption of acid and no contraction takes place, the volume increase (175-250%) is caused by 0.0072 ± 0.0026 mol fatty acid per gram of enzyme beads. It has to be noticed that the reaction does not go to completion, *i.e.* at equilibrium there is still some acid left in the solution. Therefore, there is no driving force to release the acid from the immobilization material.

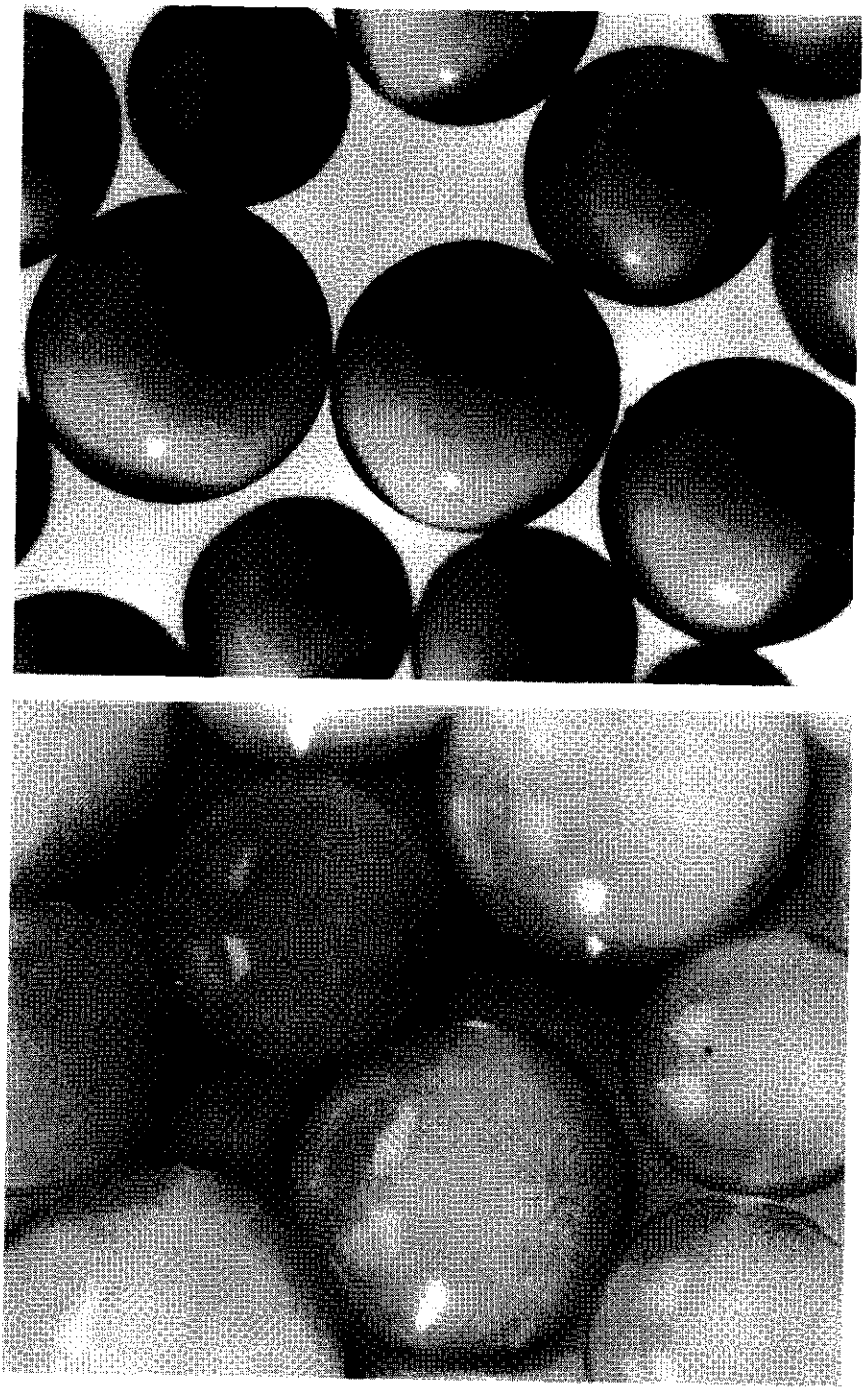


Figure 2: A) 'dry' Novozym 435®, B) swollen Novozym 435® equilibrated in 4-methyloctanoic acid.

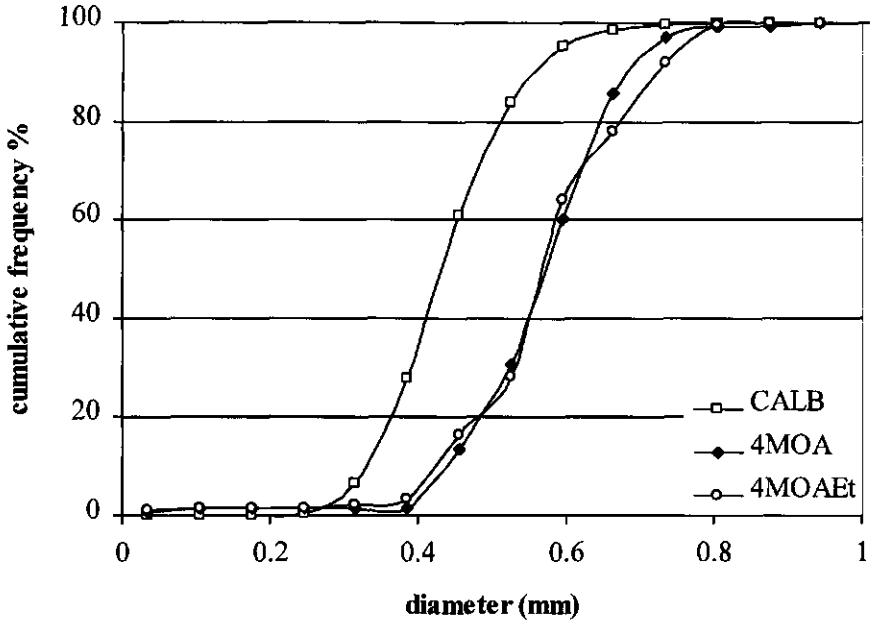


Figure 3: Frequency distribution of the calculated diameters from 'dry' CALB and CALB wetted either in 4-methyloctanoic acid or in 4-methyloctanoic acid ethyl ester.

Table 2: Sauter mean diameter (d_{32}) and volume of wetted and 'dry' Novozym 435®

	d_{32} μm	mean volume nl
dry beads	$5.07 \cdot 10^2 \pm 0.21 \cdot 10^2$	58.5 ± 4.0
beads wetted in acid	$6.50 \cdot 10^2 \pm 0.28 \cdot 10^2$	125.3 ± 12.4
beads wetted in ester	$6.15 \cdot 10^2 \pm 0.16 \cdot 10^2$	111.4 ± 5.9

Video recording of swelling with time

To determine the time needed for absorption/ swelling, the swelling of five different beads was taped on video. For each bead the relative volume with time is plotted in figure 4. Within two minutes the volume increase reached its maximum. Since our esterification reactions took place on a time scale of more than 100 hours it can be concluded that at the initial stage of the esterification reaction, the enzymes were saturated with 4-methyloctanoic acid. Although figure 4 shows a wide range of maximal relative volumes, the absolute volume increase, $\Delta V = 0.072 \pm 0.046$ µl, equaled the value calculated from table 2. Since only five beads were investigated, a large confidence interval was found.

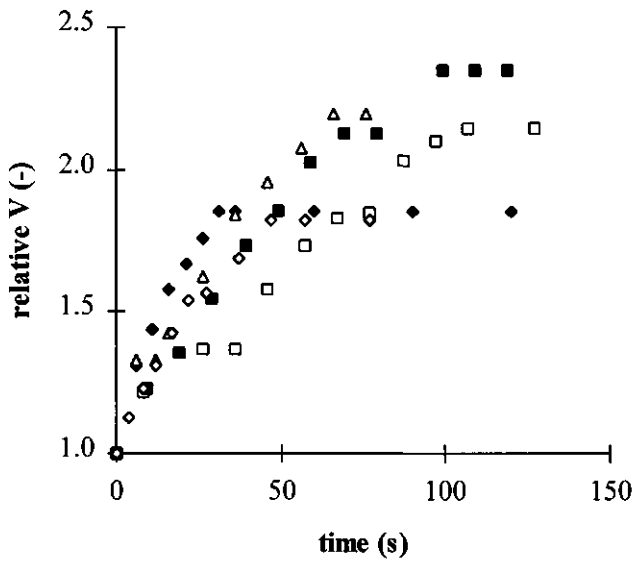


Figure 4: Swelling of Novozym 435™ due to sorption of 4-methyloctanoic acid. The swelling was recorded for 5 single beads (△, ■, □, ◆, ◇)

Correction of the enantiomeric ratio

As described above, during esterification of 4-methyloctanoic acid, a part of the substrate diffuses into the enzyme beads, causing swelling of these particles. Hence this part of the substrate does not take part in the reaction. Assuming that the amount of substrate sorbed by Novozym 435® beads must be subtracted from the initial substrate concentration, the true conversion can be calculated. The average value of 0.0072 mol/g lipase was used to correct the conversion for sorption and to estimate the real *E*-values (figure 5). The data could now be described adequately by the Chen equation. Figure 5 shows relatively small confidence intervals. Of course the *E*-ratio changes upon changing the ethanol concentration since the enzyme is sensitive to environmental changes (Tramper *et al.*, 1992; Berglund *et al.*, 1995; Edlund *et al,* 1996; Wehtje *et al.*, 1997).

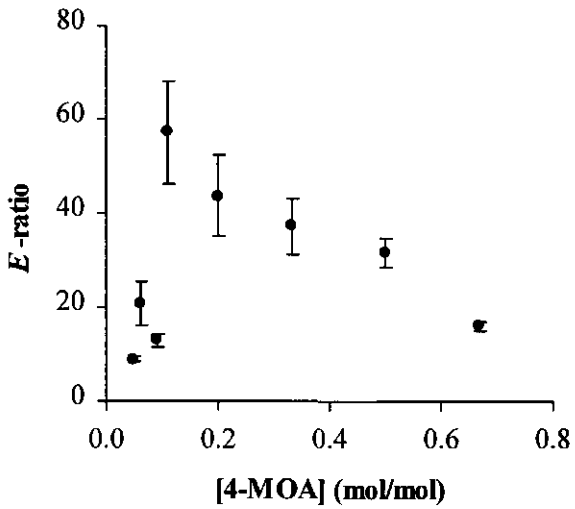


Figure 5: Esterification of 4-methyloctanoic acid with ethanol, catalyzed by Novozym 435® at different ratios varying from 2:1 up to 1:20 (mol fatty acid: mol ethanol). The estimated *E*-values and their confidence intervals were calculated using the equation of Chen and coworkers (1987) in the program SIMFIT (Jongejan *et al.*, 1991). Note the difference in scale in figure 1 and figure 5.

Figure 5 shows that upon increasing ethanol concentrations, hence decreasing 4-methyloctanoic acid concentrations, the enantiomeric ratio increases up to an optimum of E = 57 at 0.11 mol/mol. When the ethanol concentration is further increased (ratios: 1:10, 1:15 and 1:20 (mol:mol)) the enantiomeric ratio drops. These results suggest that the conformational stability of *Candida antarctica* lipase is dependent on the protein-solvent interaction and the enzyme structure. It might be possible that at low to moderate ethanol concentrations the enzyme will be in a more hydrophobic environment leading to a more rigid enzyme, thus enhancing the enantioselectivity. On the other hand, at very high ethanol concentrations the enzyme might become inactivated. This is confirmed by the time needed to reach the optimum enantiomeric excess. At molar ratios from 1:1 to 1:8 the optimum enantiomeric excess was reached within 30 hours while this maximum was reached between 40 and 85 hours for the higher molar ratios.

Correction for sorption in transesterification or hydrolysis ?

Previously, we presented an enantiomeric ratio of 23 in the transesterification of 4-methyloctanoic acid methyl ester to its butyl ester (Heinsman *et al.*, 1998), also catalyzed by Novozym 435®. In this reaction, the mixture for transesterification consisted of 500 mM n-butanol and 100 mM 4-methyloctanoic acid methyl ester in octane. A comparison of the estimated E for the plot of the measured ee_s *vs* c and the measured ee_s *vs* ee_p showed a value of 23 for both plots. Therefore, it can be concluded that substrate sorption did not play a role in this case. This could indicate that the beads were wetted with n-butanol and/or octane.

In hydrolysis reactions, the fatty acid production is commonly measured using in-line titration. Although 4-methyloctanoic acid ethyl ester sorbs into Novozym 435®, the acid produced is extracted to the water phase. In this bulk solution the liberated acid will be titrated with KOH, driving the reaction towards completion. This was confirmed by a separate experiment in which swollen beads (containing 4-methyloctanoic acid) were titrated. The total amount of acid sorbed into the beads was completely recovered by titration. In cases like this, the enantiomeric excess of the product should be used to determine the enantiomeric ratio.

CONCLUSIONS

Estimations of the enantiomeric ratio in the Novozym 435® catalyzed esterification of 4-methyloctanoic acid at different substrate concentrations, showed that the data were not correctly fitted to the Chen equation for equilibrium reactions. It was proven that during this esterification the substrate sorbed into the enzyme beads, resulting in swelling of the particles. Microscopic techniques showed that the amount of 4-methyloctanoic acid sorbed into Novozym 435® approximated a value of 0.0072 mol/g lipase. Since this amount does not take part in the esterification, a correction must be made to calculate the actual conversion. This resulted in reliable plots yielding correct *E*-values with an acceptable confidence interval. The best *E*-value (57) was found for an acid/ethanol ratio of 1:8 (mol:mol).

Correction for substrate sorption of fatty acids or their esters depends on the exact operation of the process and is therefore not always needed. For instance, transesterification of 4-methyloctanoic acid methyl ester in octane gave the same *E*-values for the plots of ee_s *versus* conversion and ee_s *versus* ee_p. During hydrolysis in a pH-stat, all the fatty acid produced is neutralized by KOH and can therefore be measured correctly in the water phase.

NOMENCLATURE

d_{32}	Sauter mean diameter	mm
n_i	number of beads	-
d_i	diameter of beads	mm
V	volume of beads	m^3
r_n	radius of beads	mm

REFERENCES

Anderson, E.M., Larsson, K.M., Kirk, O. (1998). One biocatalyst-many applications: the use of *Candida antarctica* B-lipase in organic synthesis. *Biocatal. Biotransform.*, **16**, 181-204.

Andreopoulos, A.G. (1989). Properties of poly(2-hydroxyethyl acrylate) networks. *Biomaterials*, **10**, 101-104.

Arnold, F.H. (1990). Engineering enzymes for non-aqueous solvents. *TIBTECH*, **8**, 244-249.

Berglund, P., Holmquist, M., Hult, K., and Högberg, H.-E. (1995). Alcohols as enantioselective inhibitors in a lipase catalysed esterification of a chiral acyl donor. *Biotechnol. Lett.*, **17**, 55-60.

Berglund, P., Vörde, C., Högberg, H-E. (1994). Esterification of 2-methylalkanoic acids catalysed by lipase from *Candida rugosa*: enantioselectivity as a function of water activity and alcohol chain length. *Biocatalysis*, **9**, 123-130.

Charara, Z.N., Williams, J.W., Schmidt, R.H., and Marshall, M.R. (1992). Orange flavor absorption into various polymeric packaging materials. *J. Food Sci.*, **57**, 963-972.

Chen, C.-S., Fujimoto, Y., Girdaukas, G., and Sih, C.J. (1982). Quantitative analyses of biochemical kinetic resolutions of enantiomers. *J. Am. Chem. Soc.*, **104**, 7294-7299.

Chen, C-S., Wu, S-H., Girdaukas, G., and Sih, C.J. (1987). Quantitative analyses of biochemical kinetic resolution of enantiomers. 2. Enzyme-catalyzed esterifications in water-organic solvent biphasic systems. *J. Am. Chem. Soc.*, **109**, 2812-2817.

Chen, X., Martin, B.D., Neubauer, T.K., Linhardt, R.J., Dordick, J.S., and Rethwisch, D.G. (1995). Enzymatic and chemoenzymatic approaches to synthesis of sugar-based polymer and hydrogels. *Carbohydr. Polym.*, **28**, 15-21.

Duan G, Ching, C.B. (1998). Preparative scale enantioseparation of flurbiprofen by lipase-catalysed reaction. *Biochem. Eng. J.*, **2**, 237-245.

Edlund, H., Berglund, P., Jensen, M., Hedenström, E., and H.-E. Högberg (1996). Resolution of 2-methylalkanoic acids. Enantioselective esterification with long chain alcohols catalysed by *Candida rugosa* lipase. *Acta Chem. Scan.*, **50**, 666-671.

Faber, K. (1997). Biocatalytic applications, in Biotransformations in organic chemistry, 3rd ed. Springer Verlag, Berlin Heidelberg, 27-160.

Heinsman, N., and Belov, Y. (1997). Enantiomeric analysis of 4-methyloctanoic acid by chiral gas chromatography, *The Reporter*, **16**, 9.

Heinsman, N.W.J.T., Orrenius, S.C., Marcelis, C.L.M., Sousa Teixeira, de, A., Franssen, M.C.R., Padt, van der, A., Jongejan, J.A., and Groot, de, Ae. (1998). Lipase mediated resolution of γ-branched chain fatty acid methyl esters, *Biocatal. Biotransform.*, **16**, 145-162.

Johansson, F., and Leufvén, A. (1997). Concentration and interactive effects on the sorption of aroma liquids and vapors into polypropylene. *J. Food Sci.*, **62**, 355-358.

Jongejan, J.A., van Tol, J.B.A., Geerlof, A., and Duine, J.A. (1991). Enantioselective enzymatic catalysis. 1. A novel method to determine the enantiomeric ratio. *Recl. Trav. Chim. Pays-Bas*, **110**, 247-254.

Klibanov, A.M. (1990). Asymmetric transformations catalyzed by enzymes in organic solvents. *Acc. Chem. Res.*, **23**, 114-120.

Koskinen, A.M.P., and Klibanov, A.M., eds. (1996). Enzymatic reactions in organic media, 1st ed., Blackie Academic & Professional, Glasgow, UK.

Martin, B.D., Linhardt, R.J., and Dordick, J.S. (1998). Highly swelling hydrogels from ordered galactose-based polyacrylates. *Biomaterials*, **19**, 69-76.

Nguyen, B-V., Hedenström. E. (1999). *Candida rugosa* lipase as an enantioselective catalyst in the esterification of methyl branched carboxylic acids: resolution of *rac*-3,7-dimethyl-6-octenoic acid (citronellic acid). *Tetrahedron: Asymmetry*, **10**, 1821-1826.

Rakels, J.L.L., Romein, B., Straathof, A.J.J., and Heijnen, J.J. (1994). Kinetic analysis of enzymatic chiral resolution by progress curve evaluation. *Biotechnol. Bioeng.*, **43**, 411-422.

Rakels, J.L.L., Straathof, A.J.J., and Heijnen, J.J. (1993). A simple method to determine the enantiomeric ratio in enantioselective biocatalysis. *Enzyme Microb. Technol.*, **15**, 1051-1056.

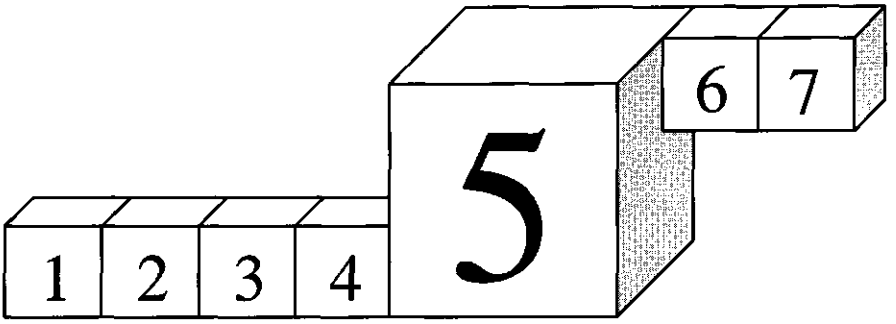
Sadler, G.D., and Braddock, R.J. (1990). Oxygen permeability of low density polyethylene as a function of limonene absorption: an approach to modeling flavor "scalping", *J. Food Sci.*, **55**, 587-588.

Straathof, A.J.J., and Jongejan, J.A. (1997). The enantiomeric ratio: origin, determination and prediction. *Enzyme Microb. Technol.*, **21**, 559-571.

Tramper, J., Vermuë, M.H., Beeftink, H.H., and Stockar, von, U. eds. (1992). Biocatalysis in non-conventional media, Elsevier Amsterdam, The Netherlands, 1-763.

Wehtje, E., Costes, D., and Adlercreutz, P. (1997). Enantioselectivity of lipases: effects of water activity. *J. Mol. Cat. B: Enzymatic*, **3**, 221-230.

Wescott, C.R., and Klibanov, A.M. (1994). The solvent dependence of enzyme specificity. *Biochim. Biophys. Acta*, **1206**, 1-9.



The effect of ethanol on the lipase-mediated kinetics of enantioselective esterification and hydrolysis

SUMMARY

The Novozym 435® catalyzed esterification and hydrolysis reactions of 4-methyloctanoic acid (ethyl ester) were investigated. In both the hydrolysis and esterification reactions, the increase of ethanol concentration led to an increase in enantiomeric ratio. For hydrolysis of the ethyl ester, the E increased from 5.5 (0% (v/v) EtOH) up to 12 (20% (v/v) EtOH). In case of esterification, the E was already 16 (14% (v/v) EtOH) and rose to 57 (73% (v/v) EtOH). An E-value of 57 is rather high, regarding the distance between the chiral center and the reaction center of the molecule (the carboxyl group). When combining these results of esterification and hydrolysis an enantiomeric ratio of 350 can be estimated for the sequential kinetic resolution of 4-methyloctanoic acid. In this way, enantiopure 4-methyloctanoic acid will be obtained after two consecutive reaction steps.

This chapter has been submitted for publication, 1999.

INTRODUCTION

A novel approach to produce optically active 4-methyloctanoic acid from a racemate is kinetic resolution using lipases. This branched chain fatty acid is in the interest of the flavor and fragrance industries since the two enantiomers have different sensory properties (Karl *et al.*, 1994a; 1994b). In enzyme-mediated kinetic resolutions, there is often a high enantioselectivity for one of the two stereoisomers when the chiral center is located close to the reaction center. However, the chiral center in 4-methyloctanoic acid is remote from the reaction center and therefore a low enantioselectivity may be expected (Wilson *et al.* 1983; Hughes *et al.* 1990). In order to enhance the selectivity, this compound may be subjected to sequential kinetic resolution (Fang and Li, 1996; Kroutil *et al.* 1997; Vänttinen and Kanerva, 1997). In this way, the enantiomerically pure product is obtained via two consecutive steps, *e.g.* esterification followed by hydrolysis. As a consequence, the substrate will pass the active site of the enzyme twice, resulting in a reinforcement of the chiral selection process.

In this chapter, we investigate the esterification of 4-methyloctanoic acid and the hydrolysis of its ethyl ester, catalyzed by Novozym 435® (immobilized *Candida antarctica* lipase B). Earlier results (chapter 2) revealed that this enzyme was the most enantioselective in the transesterification of 4-methyloctanoic acid methyl ester to its butyl ester. The kinetic parameters V_{max}^{app}, K_M^{app} and E are determined for both reactions. In order to use Michaelis Menten kinetics, the effect of droplet size and product inhibition on hydrolysis is investigated. From literature it is known that co-solvents in hydrolysis reactions may have an effect on the enantioselectivity of the enzyme (Björkling *et al.* 1986; Andrade *et al.*, 1991; Rakels *et al.*, 1994; Lundhaug *et al.*, 1998). For this purpose the effect of ethanol on the hydrolysis is investigated. The enantiomeric ratios obtained in both reactions are plotted *versus* the ethanol concentration in one graph.

MATERIALS & METHODS

Chemicals

4-Methyloctanoic acid (4-MOA) was purchased from Oxford Chemicals. Glacial acetic acid, toluene and octane were obtained from Acros. Tetradecane was purchased from Aldrich, ethanol was obtained from Merck and dichloro-dimethylsilane was supplied by Sigma.

Enzymes

Candida antarctica lipase B (Novozym 435®) is a product of NOVO-Nordisk A/S Denmark.

Phosphate buffer pH 7

30.5 ml of a 0.2 M Na_2HPO_4 solution was mixed with 19.5 ml of a 0.2 M NaH_2PO_4 solution and 50 ml water to get a 0.1 M phosphate buffer of pH 7.0.

Apparatus

pH-stat apparatus:	718 stat titrino (Metrohm), 718 stirrer (Metrohm), electrode pHC2412
Sonifier:	Sonics VC-300 sonifier
Equipment light scattering:	Lexel 150 mW multiline Ar-laser ALV-125 laserlight spectrometer ALV-5000 digital correlator ALV-800 transputerboard Microscan pentium pc

Enzymatic esterification of 4-methyloctanoic acid: initial rate experiments

Esterification was performed in either 4 ml or 20 ml screw-cap vials placed in a New Brunswick Scientific Innova™ incubator shaker at 35°C and 350 rpm. The incubations contained the substrates 4-methyloctanoic acid and ethanol in different mol ratios (table 1) as well as tetradecane (internal standard, 0.05 g) and Novozym 435 ® (5.5 mg/2 ml; 23.5 mg/5 ml) in a total volume of approximately 2 or 5 ml. Samples were taken during 6 hours. The 20 µl samples were diluted with octane (containing 0.5 % (v/v) glacial acetic acid) to 1 ml and analyzed on a gas chromatograph. The initial rate could be determined from the linear part of the [substrate] *versus* time plot. Data were not corrected for sorption (as was mentioned in chapter 4) since sorption does not influence the initial rate.

Table 1: Initial substrate concentrations in esterification of 4-methyloctanoic acid with ethanol

ratio A : E[1]	**4-MOA**		**EtOH**	
mol : mol	***g***	***mmol***	***g***	***mmol***
1:20	0.3007	1.90	1.7295	37.54
1:15	0.3794	2.40	1.6910	36.71
1:10	0.5142	3.25	1.5080	32.73
1:8	0.6067	3.83	1.4231	30.89
1:4	0.9352	5.91	1.0804	23.45
1:1	1.5828	10.00	0.4793	10.40
1:1	3.8794	24.52	1.1434	24.82
2:1	1.7418	11.01	0.2672	5.80
2:1	4.3636	27.58	0.6613	14.35
8:1	4.8225	30.48	0.1835	3.98
15:1	4.9076	31.01	0.1109	2.41
20:1	4.9324	31.17	0.0750	1.63

[1] A = 4-methyloctanoic acid, E = ethanol

Synthesis of 4-methyloctanoic acid ethyl ester

A 250 ml round bottom flask was filled with 4-methyloctanoic acid (23.52 g, 0.15 mol), 6 ml hydrochloric acid (0.07 mol) and ethanol (120 ml, 2.06 mol). The flask was placed in an oil bath at 90°C and the mixture was refluxed overnight. The excess of ethanol was removed under reduced pressure. Ether (150 ml) was added to the reaction mixture, the organic layer was extracted with a saturated sodium bicarbonate solution (150 ml) and washed with water (150 ml). After drying over Na_2SO_4, the mixture was filtered and ether was evaporated under reduced pressure using a rotavap. The crude ester (25.77 g, 92%) was obtained as yellow oil. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 0.9 (m, 6H, $C\underline{H}_3(CH_2)_3CHC\underline{H}_3(CH_2)_2CO_2CH_2CH_3$), 1.25 (m, 12 H, $CH_3(C\underline{H}_2)_3C\underline{H}CH_3C\underline{H}_2CH_2CO_2CH_2C\underline{H}_3$), 2.30 (t, 2H, $RCH_2C\underline{H}_2CO_2CH_2CH_3$), 4.15 (q, 2H, $CO_2C\underline{H}_2CH_3$). The ester was further purified by distillation. The ester was obtained as a colorless oil (0.05 mbar, 42°C).

Hydrolysis of 4-methyloctanoic acid ethyl ester

Initial rate experiments

During a time period of 30 minutes, hydrolysis of 4-methyloctanoic acid ethyl ester was studied in a pH-stat (pH 7). The pH-electrode was calibrated every day. The reaction vessel was filled with 25 ml of phosphate buffer (0.1 M). Different amounts of ester were added (0.006 - 0.167 M (45°C); 0.007 – 0.174 M (35°C)) to the solution and the mixture was sonicated (30 s, set at level 3, pulser set to 50%) using a microprobe. The emulsion was heated to either 35°C or 45°C. The hydrolysis of substrate was started by the addition of a proper amount of Novozym 435® (30 mg) and monitored by titration of the produced 4-methyloctanoic acid with a 0.1 M KOH solution. The reactions were performed in triplicate. The obtained data were sent to a connected computer using the Metrohm software (program 718). The initial rate could be determined from the linear part of the [substrate] *versus* time plot.

Droplet size determination

To a 0.1 M phosphate buffer different amounts of 4-methyloctanoic acid ethyl ester were added. The mixture was sonified during 45 seconds, resulting in a milky solution. Adding Novozym 435® to the solution started the reaction. At several points of time, in the linear area, 0.1 ml samples were taken from the reaction vessel and supplied with a Tween solution (7.5%, 10%, 15% w/w) to a total volume of 1 ml. The stabilized emulsions were characterized by dynamic light scattering. Samples were illuminated with a laser light beam. The scattered light intensity that will fluctuate due to particle movement was measured at a fixed angle of 90°. From these fluctuations, the diffusion coefficient and the particle radius could be determined.

Effect of racemic fatty acid on the hydrolysis of ester at 35°C

For this experiment the same procedure was used as described for the initial rate experiments. Different amounts of 4-methyl octanoic acid ethyl ester (0.013 - 0.119 M) and 10% (mol/mol) of 4-methyloctanoic acid were added to the buffer solution. The mixture was sonified and the pH-stat was started. After 30 seconds of a constant set pH, 30 mg of Novozym 435® was added in order to start the reaction. The reactions were followed for 5 to 10 minutes to determine the initial rates.

In order to compare the course of hydrolysis in case an initial amount of 4-methyloctanoic acid is present with the 'normal' hydrolysis, the reaction was followed for 5 hours. For this an initial amount of 4-methyloctanoic acid (0.1019 g; 0.644 mmol) was added to a solution of 0.0491 g of 4-methyloctanoic acid ethyl ester (0.263 mmol) in 25 ml phosphate buffer (0.1 M). After sonification, the reaction was started by the addition of 30.5 mg Novozym 435® to the mixture.

Ester hydrolysis in the presence of ethanol at 35°C

Hydrolysis reactions were performed with an initial amount of ethanol present (4%, 10% and 20% v/v). Reactions were carried out in a pH-stat (Metrohm) apparatus using the same procedure as described before. However, in the presence

of the ethanol concentrations used, the ethyl ester dissolved, therefore the solution was not sonicated. The initial volume in the vessel was kept at 25 ml. For different substrate concentrations, samples were withdrawn during the reactions and analyzed on GC after extraction.

Determination of enantioselectivity

The course of hydrolysis was followed in the pH-stat for three hours, using the same conditions as shown above. During the hydrolysis of 4-methyloctanoic acid ethyl ester (0.084 M), samples were taken from the mixture. Small-scale extraction was carried out by mixing 300 µl of reaction mixture with 75 µl hydrochloric acid (37%) and 150 µl octane (containing 0.75 µl glacial acetic acid and 0.12 µl tetradecane). The organic layer was used for GC analysis.

For low 4-MOAEt concentrations, lower KOH concentrations (<0.1 M) were used for titration. Using a straightforward mass balance, 100% conversion was never found for any of these titration reactions. This was caused by the change in ionic strength of the reaction mixture: the lower the KOH concentration, the lower the ionic strength. At low ionic strength the fatty acid formed will not dissociate completely, resulting in a lower conversion. Therefore, we omitted these measurements.

GC analysis

Aliquots of 0.1 µl were analyzed on a gas chromatograph using a GC-8000 series Fisons Instruments (8160) with a MFC 800 control unit and an A200S CE Instruments autosampler. The GC was equipped with a chiral γ-DEX™120 WCOT SUPELCO capillary column (ID: 30m x 0.25 mm, film: 0.25 µm) (Heinsman and Belov, 1997). Hydrogen was used as a carrier gas (70 kPa). A temperature program was used to separate both the enantiomers of 4-methyloctanoic acid and 4-methyloctanoic acid ethyl ester (T_1=75°C, t_1=53 min, r_1=5°C/min, T_2=115°C, t_2=33 min). The peaks were detected by an FID (EL 980 Fisons Instrument) set at 200°C. The split temperature was 225°C. The GC was connected to the computer program Xchrom / Windows 4.0 version 2.11b for Windows NT (ThIS Labsystems) for online data acquisition (RT 4-

methyloctanoic acid ethyl ester = 49.25 min, 49.79 min [α = 1.05], RT 4-methyloctanoic acid = 82.77 min, 84.10 min [α = 1.02]). The liner of the GC was replaced by a new silylated liner every week, since injection of fatty acids leads to a desilylation reaction which gives rise to adsorption of the fatty acids to the glass. Liners were silylated in a solution of dichlorodimethylsilane (5%) in toluene, rinsed with methanol and dried. A piece of silylated glasswool was placed into the liner to protect the capillary column from contamination.

Determination of *E*-values

The enantiomeric excess of the substrate and the conversion data were determined from GC data. Plotting the ee_s *versus* the conversion in the SIMFIT program (Jongejan *et al.*, 1991) gave the enantiomeric ratio.

RESULTS & DISCUSSION

Enzymatic esterification of 4-methyloctanoic acid at 35°C

To determine the kinetic parameters V_{max}^{app} and K_M^{app}, esterification of 4-methyloctanoic acid using different ratios of 4-methyloctanoic acid and ethanol was studied. Novozym 435® was used as the biocatalyst. From earlier experiments (chapter 2) it was known that this enzyme showed a preference for the *R*-enantiomer. From figure 1, V_{max}^{app} and K_M^{app} were calculated using the Michaelis Menten equation in the program Tablecurve 2D (table 2). As will be shown later in figure 6, the enantiomeric ratio varied within the range of 8.9 ± 0.6 to 57 ± 11, depending on the initial concentration of ethanol used.

Table 2: V_{max}^{app} and K_M^{app} values for esterification of 4-methyloctanoic acid at 35°C

V_{max}^{app}	2.17 ± 0.19[1]	mmol/min.g lipase
K_M^{app}	0.40 ± 0.09[1,2]	mol/mol

[1] 95% confidence interval, [2] 0.4 mol/mol is equal to 3.8 mol/l

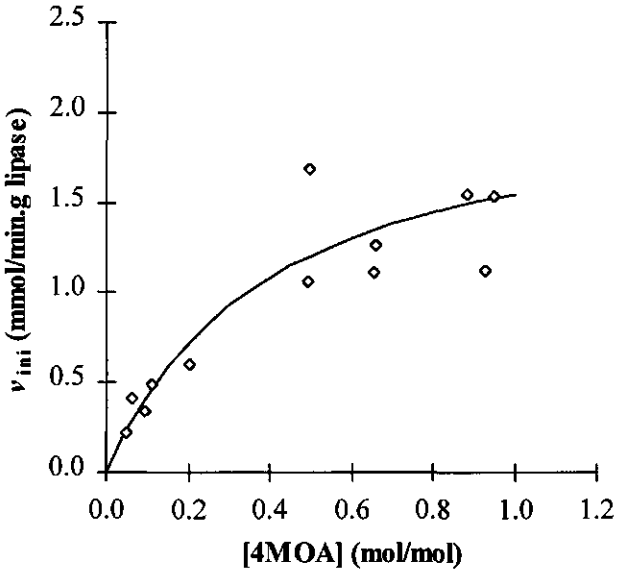


Figure 1: Initial rates in esterification of 4-methyloctanoic acid with ethanol, catalyzed by Novozym 435® at 35°C, plotted *versus* substrate concentration.

Enzymatic hydrolysis of 4-methyloctanoic acid ethylester

In order to obtain the kinetic parameters V_{max}^{app} and K_M^{app} for the hydrolysis of 4-methyloctanoic acid ethyl ester the following subjects were investigated. Firstly, the influence of 4-methyloctanoic acid ethyl ester droplet sizes on the kinetics was studied, since it is known from literature that the rate of lipase-mediated hydrolysis depends on droplet size in some cases (Armand *et al.* 1992; Borel *et al.*, 1994). Secondly, the effect of the presence of an initial amount of 4-methyloctanoic acid or ethanol in the reaction mixture on the reaction kinetics was determined. In case these effects are not perceptible, Michaelis Menten kinetics can be used to describe the hydrolysis of 4-methyloctanoic acid ethyl ester.

Influence of interfacial area on kinetics

Hydrolysis of 4-methyloctanoic acid ethyl ester was studied at pH 7. To find a relation between the interfacial area of 4-methyloctanoic acid ethyl ester and the amount of substrate, samples were taken at the initial phase of the reaction. After stabilizing these samples using a Tween solution (Kierkels *et al.*, 1990), the droplet size distribution could be determined using an optical particle sizer, based on light scattering (Irani *et al.*, 1963; Allen, 1975; Allen, 1997). From these data the total surface area was calculated. Figure 2 shows that in our case, there is a linear relation between the total interfacial area and the substrate concentration. Therefore, one cannot establish whether the increase of reaction rate is due to the increasing interfacial area or the increase of the amount of substrate itself. It is assumed that only the amount of ester determines the hydrolysis rate.

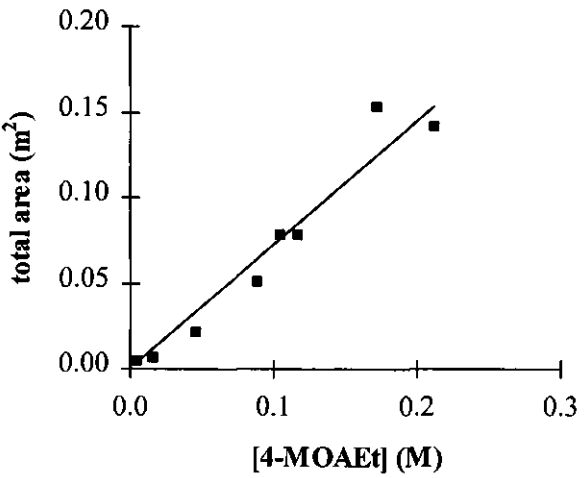


Figure 2: Correlation between concentration of 4-methyloctanoic acid ethyl ester and the total interfacial area (mean area of droplets · number of droplets).

Effect of 4-methyloctanoic acid on the activity of Novozym 435®

According to Rostrup-Nielsen and coworkers (1990) free fatty acids tend to inhibit lipase-catalyzed hydrolysis reactions. Therefore the hydrolysis of 4-methyloctanoic acid ethyl ester, catalyzed by Novozym 435®, was investigated in the presence of an initial amount (10 % mol/mol) of racemic 4-methyloctanoic acid (figure 3). Both curves (one with 4-methyloctanoic acid added to the starting mixture and one without) show the same course. From these results one can conclude that there was no inhibitory effect of 4-methyloctanoic acid. In addition, even in the presence of more than a double amount of 4-methyloctanoic acid (0.644 mmol 4-methyloctanoic acid *versus* 0.263 mmol 4-methyloctanoic acid ethyl ester) ester hydrolysis was still completed within three hours.

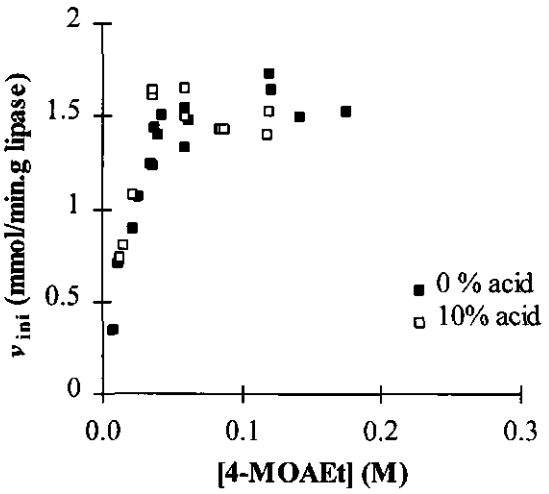


Figure 3: Initial hydrolysis rate of 4-methyloctanoic acid ethyl ester at 35°C *versus* initial substrate concentration. Comparison between 'normal' hydrolysis and hydrolysis in the presence of an initial amount of 10 mol % of racemic 4-methyloctanoic acid.

Influence of ethanol on the kinetic parameters V_{max}^{app} and K_M^{app}

Since product inhibition may occur in systems where the co-solvent is a product by itself (Rakels *et al.*, 1994), the influence of ethanol on the kinetic parameters V_{max}^{app} and K_M^{app} was investigated. Furthermore, a two-step process (esterification followed by hydrolysis) may be necessary for the production of enantiopure 4-methyloctanoic acid. In case the esterification is performed in a large excess of ethanol, it is also of importance to know what the effect of ethanol is on the kinetic parameters of the subsequent hydrolysis reaction. Moreover, several authors have demonstrated that the addition of co-solvents to aqueous media may change the enantioselectivity of the enzyme (Björkling *et al.*, 1986; Rakels *et al.* 1994; Lundhaug *et al.*, 1998).

We have investigated the hydrolysis of 4-methyloctanoic acid ethyl ester in water-ethanol mixtures up to 20% EtOH (v/v). The dependency of V_{max}^{app} and K_M^{app} on the substrate concentration was investigated by measuring the initial rates in the presence of ethanol concentrations of 4%, 10% and 20% (v/v). These results were compared with the results obtained for the hydrolysis of 4-methyloctanoic acid ethyl ester in the absence of ethanol. V_{max}^{app} hardly changes upon addition of ethanol (table 3, figure 4). However, the K_M^{app} increased with increasing ethanol concentration.

Table 3: Kinetic parameters for the hydrolysis of 4-methyloctanoic acid ethyl ester at 35°C in different solvent systems

# ethanol	V_{max}^{app}	K_M^{app}
% v/v	***mmol/min. g lipase***	***M***
0	1.85 ± 0.18[1]	0.018 ± 0.006[1]
4	2.09 ± 0.34[1]	0.032 ± 0.011[1]
10	1.79 ± 0.36[1]	0.042 ± 0.022[1]
20	1.63 ± 0.45[1]	0.077 ± 0.043[1]

[1] 95% confidence interval

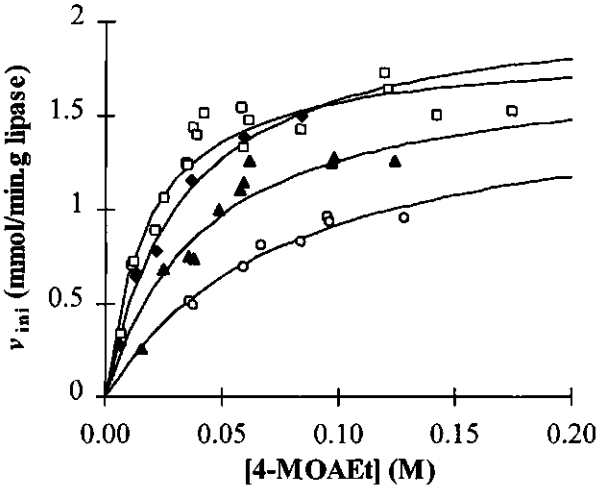


Figure 4: Initial hydrolysis rates of 4-methyloctanoic acid ethyl ester at 35°C, catalyzed by Novozym 453®, in the presence of different initial ethanol concentrations. □ 0% (v/v) ethanol, ◆, 4% ethanol, ▲ 10% ethanol, ○ 20% ethanol.

It is likely that an increase of the Michaelis Menten constant results from a weaker binding of the substrate to the enzyme, *i.e.* the partitioning of substrate from the aqueous phase into the enzyme active site is disfavored. This is caused by the increased solubility of the substrate in the aqueous phase: addition of ethanol to the reaction mixture led to a mono-phase.

Enzymatic hydrolysis of 4-methyloctanoic acid ethyl ester at 35°C and 45°C.

In the previous section it was shown that the presence of 4% ethanol (v/v) did not have a large influence on the hydrolysis of 4-methyloctanoic acid ethyl ester. Since the maximal substrate concentration used does not exceed 0.2 M, less than 1 % ethanol (v/v) will be formed during hydrolysis. Consequently no product inhibition will be observed. In addition, the presence of an initial amount of 4-methyloctanoic acid also did not affect the hydrolysis of 4-methyloctanoic acid

ethyl ester. From these results and the assumption that the substrate interfacial area does not have any effect on the hydrolysis reactions, it can be concluded that hydrolysis can be described by Michaelis Menten kinetics. Hydrolysis was performed at two temperatures, *viz* 45°C (optimum temperature for *Candida antarctica* lipase B) and 35°C (the same as used for the esterification). The results are shown in table 4 and figure 5. Although V_{max}^{app} of hydrolysis is almost equal to V_{max}^{app} of esterification, hydrolysis appears to be faster since K_M^{app} (in mol/l) differs a factor 100.

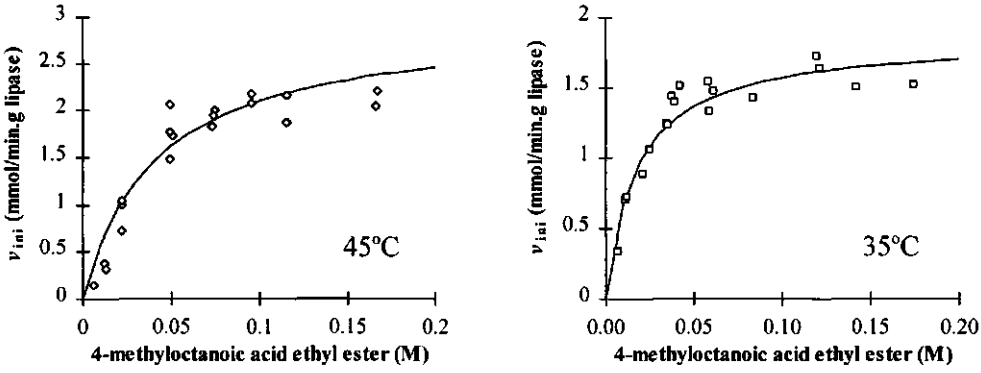


Figure 5: Initial rates of 4-methyloctanoic acid ethyl ester hydrolysis, catalyzed by Novozym 435® at 45°C and 35°C.

Table 4: V_{max}^{app} and K_M^{app} in hydrolysis of 4-methyloctanoic acid ethyl ester at 35°C and 45°C

T	V_{max}^{app}	K_M^{app}
°C	*mmol/min.g lipase*	*M*
35	1.85 ± 0.18[1]	0.018 ± 0.006[1]
45	2.93 ± 0.25[1]	0.042 ± 0.009[1]

[1] 95% confidence interval

Determination of enantiomeric ratio in hydrolysis

Hydrolysis was performed in a reaction vessel at 35°C or 45°C. The pH of the reaction mixture was kept constant during the reaction (pH ≅ 7). Hydrolysis was monitored until completion. Since only the enantiomeric excess of the product could be determined, the ee_p *versus* conversion data were fitted using the Chen equation. The ethyl ester of 4-methyloctanoic acid was hydrolyzed with a low enantioselectivity ($E = 5.5 \pm 2.5$) compared to esterification ($E = 8.9 \pm 0.6 - 57.2 \pm 11.0$). In this chapter, it was already shown that the kinetic parameters V_{max}^{app} and K_M^{app} in hydrolysis were affected due to the addition of ethanol to the reaction mixture. Wolff and coworkers (1997) showed that the kinetic parameters V_{max} and K_M of the separate enantiomers were influenced by addition of cosolvents to the chymotrypsin catalyzed hydrolysis of ethyl (*R*)- and (*S*)-3-phenyllactate. However, no overall effect of the cosolvent on the enantioselectivity could be observed. Contrary to Wolff and coworkers (1997), an increase of the enantiomeric ratio was found in the hydrolysis of 4-methyloctanoic acid ethyl ester upon raising the amount of ethanol. This change might be due to a conformational change of the enzyme.

Table 5: Enantiomeric ratio for Novozym 435® catalyzed hydrolysis at 35°C in different solvent systems

Ethanol (%v/v)	Enantiomeric ratio
0	5.5± 2.5[1]
4	8.6 ± 3.9[1]
10	8.1 ± 0.3[1]
20	12.2 ± 0.9[1]

[1] σ confidence interval

E-ratio in esterification and hydrolysis *versus* ethanol concentration

When the observed E-values for both the hydrolysis and esterification are plotted *versus* the ethanol concentration (figure 6) it is clear that the E-ratio increases with

increasing concentration of ethanol. In hydrolysis this effect might be explained by a conformational change of the active site. In the same way, upon increasing ethanol concentrations in esterification, the enantiomeric ratio increases up to an optimum of $E = 57 \pm 11$, which is rather high for a substrate with a remote chiral center. However, when the ethanol concentration is further increased (ratios: 1:10, 1:15 and 1:20 (mol:mol)) the enantiomeric ratio drops. The reason for this may be that at this point the enzyme is partially inactivated. Sequential kinetic resolution of 4-methyloctanoic acid will result in the production of enantiopure 4-methyloctanoic acid when using the optimal conditions in esterification and hydrolysis. When a 1:8 molar ratio of 4-methyloctanoic acid and ethanol is used, an E of 57 can be observed (at $c = 0.51$, $ee_s = 0.91$ and $ee_p = 0.87$). The optimal E for hydrolysis ($E = 12$) can be observed when 20% (v/v) ethanol is added to the reaction mixture. In this case the reaction has to be finished at a conversion of 52% ($ee_s = 0.74$, $ee_p = 0.69$). After two consecutive steps ($E \cong 350$) 4-methyloctanoic acid can be obtained with an enantiomeric purity of 99.9% and an overall yield of 27%.

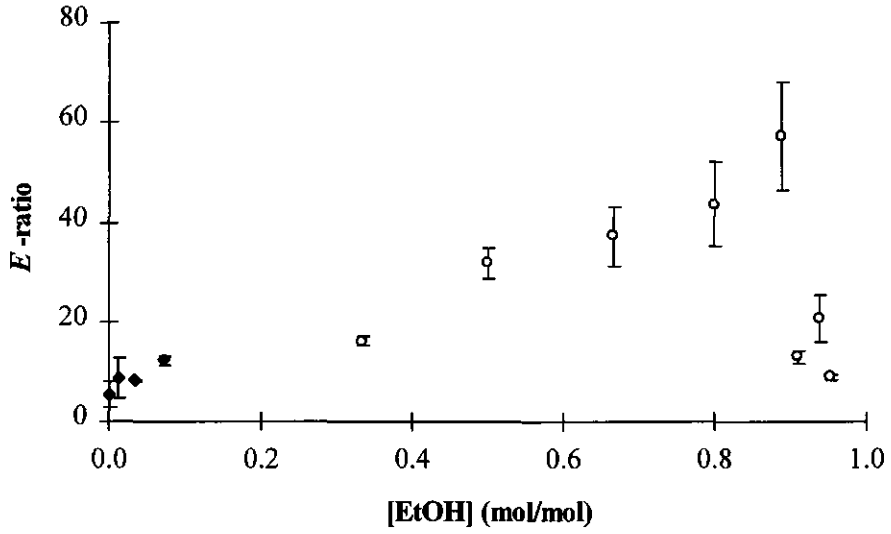


Figure 6: E-ratio in esterification (○) and hydrolysis (◆) plotted *versus* ethanol concentration.

CONCLUSIONS

We have determined the kinetic parameters V_{max}^{app}, K_M^{app} and E in the esterification and hydrolysis of 4-methyloctanoic acid (ethyl ester) catalyzed by Novozym 435®. The V_{max}^{app} for esterification (2.17 ± 0.19 mmol/min.g lipase) is comparable to the value obtained in hydrolysis (1.85 ± 0.18 mmol/min.g lipase). A difference in K_M^{app} explains why hydrolysis is much faster than esterification. The presence of 4-methyloctanoic acid at the initial state of the reaction does not influence the kinetics of the reaction. On the other hand, addition of ethanol in the hydrolysis of 4-methyloctanoic acid ethyl ester leads to a decrease in V_{max}^{app} and an increase in K_M^{app}. The addition of ethanol also leads to an increase in the enantiomeric ratio. This increase is also found when the ethanol concentration in esterification is raised. The optimum *E*-value (57 at an acid:EtOH ratio of 1:8) is rather high for a reaction involving a substrate with a remote chiral center. At a concentration of 77% EtOH (v/v) and higher the enzyme seems to be inactivated. The results of both reactions can be combined to find the optimal procedure for the production of enantiopure 4-methyloctanoic acid. Sequential kinetic resolution will result in an enantiomeric ratio of approximately 350 when esterification is performed in a molar ratio of 1:8 (mol 4-methyloctanoic acid: mol ethanol) and hydrolysis is carried out in the presence of 20% (v/v) ethanol.

REFERENCES

Allen, T. (1975). Radiation scattering methods of particle size determination, in: *Particle size measurement.* 2nd ed., Williams, J.C. (ed.), Chapman & Hall, London, UK, 314-327.

Allen, T. (1997). Measurement of the size distribution of drops in dispersions, in: *Particle size measurement.* 5th ed., Chapman & Hall, London, UK, 381-383.

Andrade, M.A.C., Andrade, F.A.C. and Phillips, R.S. (1991). Temperature and DMSO increase the enantioselectivity of hydrolysis of methyl alkyl dimethylmalonates catalyzed by pig liver esterase. *Bioorg. Med. Chem. Lett.*, **1**, 373-376.

Armand, M., Borel, P., Ythier, P., Dutot, G., Melin, C., Senft, M., Lafont, H. and Lairon, D. (1992). Effects of droplet size, triacylglycerol composition, and calcium on the hydrolysis of complex emulsions by pancreatic lipase: an in vitro study. *J. Nutrit. Biochem.*, **3**, 333-341.

Björkling, F., Boutelje, J., Gatenbeck, S., Hult, K., Norin, T. and Szmulik, P. (1986) The effect of dimethyl sulfoxide on the enantioselectivity in the pig liver esterase catalyzed hydrolysis of dialkylated propanedioic acid dimethyl esters. *Bioorg. Chem.*, **14**, 176-181.

Borel, P., Armand, M., Ythier, P., Dutot, G., Melin, C., Senft, M., Lafont, H. and Lairon, D. (1994). Hydrolysis of emulsions with different triglycerides and droplet sizes by gastric lipase in vitro. Effect on pancreatic lipase activity. *J. Nutrit. Biochem.*, **5**, 124-133.

Fang, J.W. and Li, Z.Y. (1996). Lipase-catalyzed double kinetic resolution of DL-2,4-dimethyl-1,5-pentanediol: a useful synthon in natural product synthesis. *Chin. Chem. Lett.*, 7, 129-130.

Heinsman, N., and Belov, Y. (1997). Enantiomeric analysis of 4-methyloctanoic acid by chiral gas chromatography. *The Reporter*, **16**, 9.

Hughes, D.L., Bergan, J.J., Amato, J.S., Bhupathy, M., Leazer, J.L., McNamara, J.M., Sidler, D.R., Reider, P.J. and Grabowski, E.J.J. (1990). Lipase-catalyzed asymmetric hydrolysis of esters having remote chiral/prochiral centers. *J. Org. Chem.*, **55**, 6252-6259.

Irani, R.R. and Callis, C.F. (1963). Light scattering as a measure of particle size, in: *Particle size: measurement, interpretation and application.* 1st ed., John Wiley & Sons, Inc. NY, USA, 138-139.

Jongejan, J.A., van Tol, J.B.A., Geerlof, A. and Duine, J.A. (1991) Enantioselective catalysis. 1. A novel method to determine the enantiomeric ratio. *Recl. Trav. Chim. Pays-Bas*, **110**, 247-254.

Karl, V., Kaunzinger, A., Gutser, J., Steuer, P., Angles-Angel, J. and Mosandl, A. (1994) Stereoisomeric flavour compounds LXVII. 2-, 3-, and 4-alkyl-branched acids, part 1: General approach to the synthesis of the enantiopure acids. *Chirality*, **6**, 420-426.

Karl, V., Kaunzinger, A., Gutser, J., Steuer, P., Angles-Angel, J. and Mosandl, A. (1994) Stereoisomeric flavour compounds LXVII. 2-, 3-, and 4-alkyl-branched acids, part 2: Chirospecific analysis and sensory evaluation. *Chirality*, **6**, 427-434.

Kierkels, J.G.T., Vleugels, L.F.W., Kern, J.H.A., Meijer, E.M. and Kloosterman, M. (1990). Lipase kinetics: on-line measurement of the interfacial area of emulsions. *Enzyme Microb. Technol.*, **12**, 760-763.

Kroutil, W., Kleewijn, A. and Faber, F. (1997) A computer program for analysis, simulation and optimization of asymmetric catalytic processes proceeding through two consecutive steps. Type 2: sequential kinetic resolutions. *Tetrahedron: Asymmetry*, **8**, 3263-3274.

Lundhaug, K., Overbeeke, P.L.A., Jongejan, J.A. and Anthonsen, T. (1998) Organic co-solvents restore the inherently high enantiomeric ratio of lipase B from *Candida antarctica* in hydrolytic resolution by relieving the enantiospecific inhibition of product alcohol. *Tetrahedron: Asymmetry*, **9**, 2851-2856.

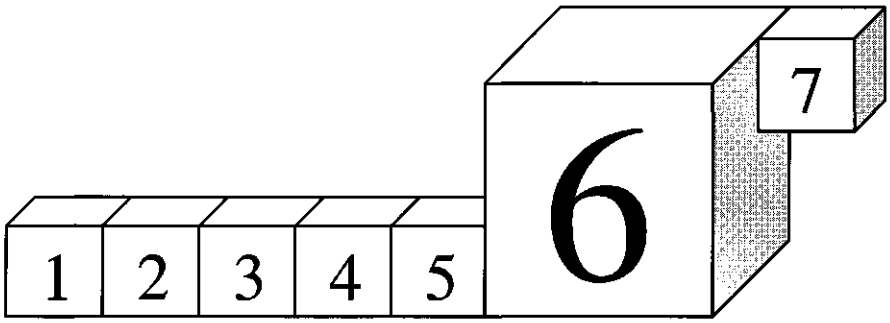
Rakels, J.L.L., Caillat, P., Straathof, A.J.J. and Heijnen, J.J. (1994) Modification of the enzyme enantioselectivity by product inhibition. *Biotechnol. Prog.*, **10**, 403-409.

Rostrup-Nielsen, T., Pedersen, L.S. and Villadsen, J. (1990) Thermodynamics and kinetics of lipase catalysed hydrolysis of oleyl oleate. *J. Chem. Tech. Biotechnol.*, Vol. 48, p. 467-482.

Väntinnen, E. and Kanerva, L.T. (1997) Optimized double kinetic resolution for the preparation of (*S*)-solketal. *Tetrahedron: Asymmetry*, **8**, 923-933.

Wilson, W.K., Baca, S.B., Barber, Y.J., Scallen, T.J. and Morrow, C.J. (1983). Enantioselective hydrolysis of 3-hydroxy-3-methylalkanoic acid esters with pig liver esterase. *J. Org. Chem.*, **48**, 3960-3966.

Wolff, A., Straathof, A.J.J., Jongejan, J.A. and Heijnen, J.J. (1997). Solvent induced change of enzyme enantioselectivity: rule or exception? *Biocatal. Biotransform.*, **15**, 175-184.



Reactor design for the Novozym 435®-catalyzed enantioselective esterification of 4-methyloctanoic acid

SUMMARY

The bench-scale Novozym 435®-catalyzed esterification of 4-methyloctanoic acid with ethanol was studied at 35°C. Esterification in a batch reactor (molar ratio of 1:8 (acid:EtOH)) resulted in the isolation of the enantiomerically enriched product (ee_p = 81%) and substrate (ee_s = 93%). In order to integrate reaction and separation, liquid-vapor equilibria calculations have been performed showing that an excess of ethanol results in an unfavorable distribution of ester over the liquid and vapor phase. Since this is undesirable for an integrated process of reaction and product removal, a repeated batch reaction was performed using a molar ratio of 10:1 (acid:EtOH). After 6 cycles the ee of 4-methyloctanoic acid ethyl ester turned out to be 80%. For different E-values the ee_p was calculated for batch and repeated batch reactions. It was shown that in all cases the ee_p was higher for the repeated batch reaction. However, the product is not enantiopure since the E-value of the reaction is rather low at low ethanol concentration. An alternative approach would be the continuous separation of the product during the reaction. A mathematic model was developed to describe esterification in a packed bed reactor integrated with product separation. This model shows that integration of reaction and product removal in advance is also not the best method to obtain an enantiomerically pure product. In conclusion, the preference is given to the batch reaction at high ethanol concentrations because in this case it is possible to perform the reaction at a high enantiomeric ratio.

To be submitted

INTRODUCTION

A convenient way to produce enantiomerically pure branched chain fatty acids is kinetic resolution catalyzed by lipases. Lipase-catalyzed kinetic resolution has almost exclusively been carried out in batch reactors (Indlekofer, *et al.*, 1996) although it might be commercially advantageous to design appropriate continuous production processes. In literature, the batch-mode resolution of 2-methylalkanoic acids is well described by Engel (1991), Holmberg *et al.* (1991) and Edlund *et al.* (1996). On a small scale (1.5 mmol), enantiomeric ratios (*E*) of 37 and 70 were obtained for the *Candida cylindracea* lipase-catalyzed esterifications of 2-methyldecanoic acid with 1-heptanol in cyclohexane and 2-methylpentanoic acid with octanol in heptane at room temperature. However, bench-scale esterification of 2-methyldecanoic acid (105 mmol) with heptanol resulted in a lower *E*-value of 15. Bench and large-scale resolution can result in lower *E*-values due to unforeseen causes like water accumulation.

It has also to be taken into account that the enantiomeric excess is dependent on the reactor configuration (*E*-ratio for batch process: Chen *et al.*,1982; *E*-ratio for CSTR: Rakels *et al.*, 1994). For example, the ee_p in a CSTR is lower compared to a batch reactor. This is due to the broad distribution of residence time in a CSTR (Rakels *et al.*, 1994). This type of reactor might only be profitable when a high enantiomeric ratio is to be expected. For low to moderate enantiomeric ratios, it is necessary to use a plug flow reactor for the continuous kinetic resolution of substrates. In literature different examples are given for continuous reactions in packed bed reactors. These reactors can be considered as plug flow reactors (Bornscheuer *et al.*, 1995; Indlekofer *et al.*, 1996; Shiraishi *et al.*, 1996; Chand *et al.*, 1997; Shimada e*t al.*, 1999). Besides the economical feasibility of continuous processes, these processes are even more interesting when reaction and separation of the products can be integrated in one process (Paiva and Malcata, 1994, 1997; Csányi *et al.*, 1998; Paiva *et al.*, 1998). In this manner, limiting factors like thermodynamic equilibria can be overcome.

Different categories of integration are possible including solid/liquid systems (*e.g.* membrane reactor), solid/vapor systems (reactants in vapor mode), solid/fluid systems (supercritical fluids) and solid/liquid/vapor systems (reactive distillation). This chapter aims at the design of a reaction system for the lipase-catalyzed esterification of 4-methyloctanoic acid with ethanol at the highest ee_p and yield. Bench scale esterifications of 4-methyloctanoic acid (0.5-1.0 mol) with ethanol, catalyzed by Novozym 435® (immobilized *Candida antarctica* lipase B) were performed in a batch and repeated batch mode. Calculations for batch and repeated batch reactions were performed in order to compare the enantiomeric excess of the product for both types of reactions. Finally, the esterification and *in situ* product removal were described using model calculations.

MATERIALS & METHODS

Chemicals

4-methyloctanoic acid was from Oxford Chemicals. Ethanol, octane and LiCl were from Merck. Tetradecane was from Aldrich and glacial acetic acid was from Boom b.v. Meppel. Dichlorodimethylsilane and toluene were from Sigma and Acros. Accurel® (400-1000 μm) was a gift from Akzo Nobel. All solvents were of p.a. quality.

Enzyme

Candida antarctica lipase B (Novozym 435®) was supplied by NOVO Nordisk.

Materials

The cryostate was from Haake, the thermostat from Lauda, the peristaltic and gear pumps from WM and Verder, the hollow fibre membrane was from Lobe and the membrane pump from Vacubrand.

Batch esterification

A 500 ml three-necked flask, equipped with a magnetic stirring bar and a reflux condensor was charged with 79.12 g (0.50 mol) 4-methyloctanoic acid, 186.39 g (4.05 mol) ethanol (35°C) and 3.21 g Novozym 435®. At regular intervals, samples (20 µl) were taken from the reaction mixture and diluted with octane (980 µl, containing 0.5% of glacial acetic acid and 0.06% tetradecane). After 19.5 hours the reaction was stopped by filtration of the enzyme. The excess of ethanol was evaporated under reduced pressure using a Rotavap. The crude reaction mixture was distilled *in vacuo* (0.11 mbar, 59-65°C: 4-methyloctanoic acid ethyl ester, 79-84°C: 4-methyloctanoic acid) to obtain pure ethyl ester (35.33 g; 0.19 mol) and pure 4-methyloctanoic acid (26.69 g; 0.17 mol). Since earlier experiments (chapter 4) have shown that part of the substrate sorbs into the polymer matrix of the Novozym 435® beads, the conversion was corrected for this amount (0.0072 mol 4-methyloctanoic acid/g Novozym 435®).

Repeated batch esterification

A 250 ml flask, equipped with a magnetic stirring bar and a reflux condensor, was charged with 158.24 g (1.0 mol) 4-methyloctanoic acid and 4.63 g (0.10 mol) ethanol. The mixture was heated to 35°C and the reaction was started by the addition of 1 g Novozym 435® to the mixture. The reaction was stopped after one hour by filtration of the enzyme. The produced ester was distilled *in vacuo* (0.09 mbar, 48°C). The residue was used for a new reaction of 4-methyloctanoic acid (0.91 mol/mol) and ethanol (0.09 mol/mol), catalyzed by fresh Novozym 435® (1 g). Reaction and distillation were repeated for 5 times until a conversion of 60% was reached.

In line product removal

Esterification in a packed bed reactor with stripping of the ester

In a 500 ml stirred vessel, 4-methyloctanoic acid (52.8 g; 0.3 mol) and ethanol (234,5 g; 5.1 mol) were mixed and fed to the packed bed reactor (35°C), filled

with a mixture of 75 mg Novozym 435® and 1.08 g Accurel®. Only a small amount of enzyme was used in order to adjust the reaction rate and product removal. Since this amount was not enough to completely fill the packed bed, the enzyme was mixed with Accurel®. After passing the packed bed reactor, the reaction mixture was fed to a strip column filled with stainless steel Raschig rings. Ethanol-saturated air was used as strip gas. The ethyl ester, stripped from the liquid phase, was collected in a cold water trap. Samples were taken from the reaction mixture during a period of ten days. The 20 µl samples were diluted with octane (containing 0.5 volume % of glacial acetic acid) to 1 ml. Both conversion and enantiomeric excess were determined from GC-data.

Esterification in a packed bed reactor with distillation of the ester

A packed bed reactor (9.57 g Novozym 435® and 2.25 g Accurel®) was placed in series with a pervaporation system (sweep gas: LiCl dried air) and a distillation flask (200°C). The pervaporation was necessary to prevent boiling point elevation during distillation. The reactor system, including the tubes, was filled with 4-methyloctanoic acid (4-MOA) up to 445.27 g (2.81 mol). The reaction was performed at 35°C and started after addition of a limiting amount of ethanol to the packed bed, in such a way that a molar ratio of 1:1 (4-MOA: ethanol) was achieved in the enzyme column. Samples (20 µl) were taken from the distillation flask, the cold trap and after water removal in the pervaporation membrane system. The samples were diluted with octane (containing 0.5 volume % of glacial acetic acid) up to 1 ml. Conversion and enantiomeric excess were determined from GC-data.

GC analysis

Aliquots of 0.1 µl were analyzed on a gas chromatograph using a GC-8000 series Fisons Instruments (8160) with a MFC 800 control unit and an A200S CE Instruments autosampler. The GC was equipped with a chiral γ-DEX™120 WCOT SUPELCO capillary column (ID: 30m x 0.25 mm, film: 0.25 µm) (Heinsman and Belov, 1997). Hydrogen was used as a carrier gas (70 kPa). A

temperature program was used to separate both the enantiomers of 4-methyloctanoic acid and 4-methyloctanoic acid ethyl ester (T_1=75°C, t_1=53 min, r_1=5°C/min, T_2=115°C, t_2=33 min). The peaks were detected by an FID (EL 980 Fisons Instrument) set at 200°C. The split temperature was 225°C. The GC was connected to the computer program Xchrom / Windows 4.0 version 2.11b for Windows NT (ThIS Labsystems) for online data acquisition (RT 4-methyloctanoic acid ethyl ester = 49.25 min, 49.79 min [α = 1.05], RT 4-methyloctanoic acid = 82.77 min, 84.10 min [α = 1.02]). The liner of the GC was replaced by a new silylated liner every week, since injection of fatty acids leads to a desilylation reaction which gives rise to adsorption of the fatty acids to the glass. Liners were silylated in a solution of dichlorodimethylsilane (5%) in toluene, rinsed with methanol and dried. A piece of silylated glasswool was placed into the liner to protect the capillary column from contamination.

Determination of *E*-values

To calculate the enantiomeric ratio, the enantiomeric excess of the substrate and the conversion, determined from GC data, were fitted in the SIMFIT program (Jongejan *et al.* 1991) using the Chen equation for equilibrium reactions.

Calculation of thermodynamic activities

The classical UNIFAC contribution method (Fredenslund *et al.*, 1997) was used to calculate thermodynamic activity coefficients of all the components in the mixture at phase equilibrium.

RESULTS & DISCUSSION

In the lab-scale esterification of 4-methyloctanoic acid (1.9–11.0 mmol) with ethanol, catalyzed by Novozym 435® (chapter 4) an enantiomeric ratio of 57 ± 11 could be estimated for the molar ratio of 1:8 (acid:EtOH). In this chapter, the

results are presented for the reaction on bench scale (0.5 mol), using the same conditions. To improve the resolution, a repeated batch esterification was designed and performed (molar ratio 10:1 (acid: ethanol)). Finally, a computer model was developed to describe the reaction and *in situ* product separation. These model calculations were verified using a packed bed reactor using both in line stripping and distillation.

Batch esterification

The Novozym 435® catalyzed esterification of 4-methyloctanoic acid (0.5 mol) with ethanol (4 mol) at 35°C was monitored up to 55% conversion. The reaction was stopped by filtration of the enzyme. The enantiomeric excess of the substrate was determined from the GC-data and plotted *versus* the corrected conversion (figure 1, for sorption correction see chapter 4). Since no data at conversions over 55% are present for the large-scale reaction, the *E*-value could not be estimated,

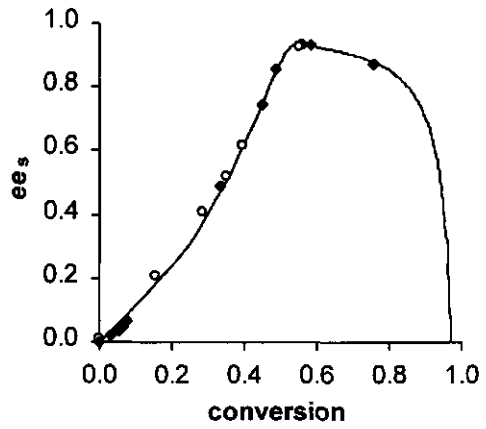


Figure 1: Large-scale esterification of 4-methyloctanoic acid (4-MOA) with ethanol (EtOH) catalyzed by Novozym 435® at 35°C. O represents the data obtained for the bench-scale reaction with an initial molar ratio of 1:8 (4-MOA:EtOH), ◆ represents the data obtained for the small-scale reaction with an initial molar ratio of 1:8 (4-MOA:EtOH). The line shows the estimated ee_S using the data from chapter 4.

because high conversion data are essential for correct calculation of the enantiomeric ratio. However, using the data of chapter 4, one line can describe the data of both the large and small-scale (E = 57) reaction. This implies that the large-scale esterification was also performed with a high enantiomeric ratio. After distillation *in vacuo* the enantiomerically enriched *R*-4-methyloctanoic acid ethyl ester (ee_p = 81%) and *S*-4-methyloctanoic acid (ee_s = 93%) could be obtained in a yield of 38% and 34%.

Repeated batch esterification

In order to design the integration of reaction and product separation, the composition of the vapor and liquid phases at equilibrium for a mixture of ethanol and ester were calculated using the UNIFAC contribution method. Calculations of the phase-equilibrium showed an unfavorable ratio of 4-methyloctanoic acid ethyl ester and ethanol in the vapor phase (figure 2). Only at mol fractions of ester in the liquid phase approaching to 1, ester transfer to the vapor phase will occur.

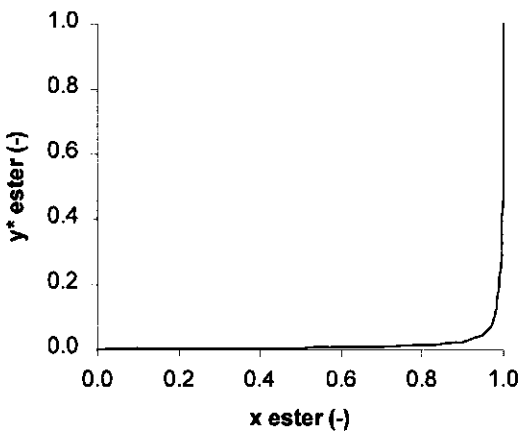


Figure 2: Vapor-liquid diagram for the ethanol/ester mixtures at 35°C and 10^5 Pa. The ester mol fraction is given in the liquid phase (x) and vapor phase (y*). The vapor pressure of ethanol used was $1.38 \cdot 10^4$ Pa at 35°C. Since the vapor pressure of 4-methyloctanoic acid ethyl ester was not known, the vapor pressure of the comparable octanoic acid methyl ester was used for these calculations ($9.47 \cdot 10^1$ Pa at 35°C).

The ratio of ester and ethanol remained unfavorable irrespective of temperature or pressure. From these calculations we concluded that the amount of ethanol should be reduced to selectively transfer the ester from the liquid to the vapor phase. Therefore, a molar ratio of 10:1 (acid:EtOH) was chosen. Since only 10% of ester can be produced using this molar ratio, a repeated batch esterification of 4-methyloctanoic acid with ethanol, catalyzed by Novozym 435®, was studied.

Repeated batch system

For different enantiomeric ratios (E) and a fixed equilibrium constant (K = 0.14, determined using the data from chapter 4), the enantiomeric excess of the substrate (ee_s) and of the product (ee_p) were calculated for both a batch and a repeated batch reaction (figure 3A,B). The ee_p in a batch reaction is high at the beginning of the reaction but decreases rapidly upon approaching 50% conversion. For this reason, ee_p is relatively low at the moment where ee_s is at its maximum (represented as horizontal lines in figure 3A). In the repeated batch reaction, however, ee_p remains high even at high conversion because the product is removed after every step, shifting the reaction equilibrium to the right. Therefore, the repeated batch esterification is preferred to a batch reaction. For the first few batches in the repeated batch system, the ee_s is low since the conversion is low. At 60% conversion (batch 6) the ee_s of the repeated batch system is in the same range as the batch system (see figure 3B).

To verify the repeated batch set up, 0.1 mol ethanol was added to 1 mol of 4-methyloctanoic acid. The reaction was stopped by filtration of the enzyme, the ester was distilled and the remaining fatty acid was used again for a new esterification reaction (4-MOA: 0.91 mol/(mol $_{total}$)); EtOH: 0.09 mol/mol $_{total}$). This process was repeated for 6 times. Distillation of only the small amount of ethyl ester produced proved to be successful. After each reaction step, 10% of acid (mol/mol $_{total}$) has been converted to the ethyl ester resulting in an increase of the

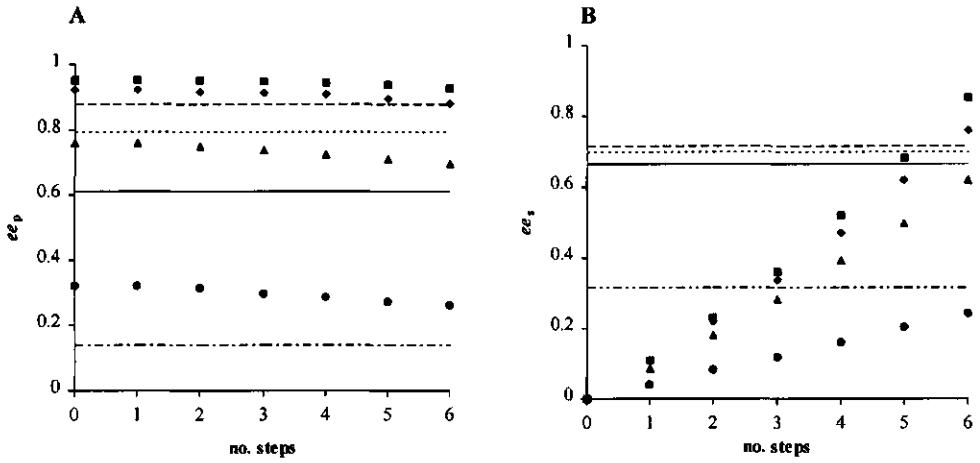


Figure 3: For different enantiomeric ratios and a fixed equilibrium constant (K = 0.14), the ee_p (figure 3A) and ee_s (figure 3B) of 4-methyloctanoic acid (ethyl ester) were calculated using the Chen equation (Chen *et al.* 1987; Jongejan 1991). The number of steps in both figures is proportional to the conversion (*e.g.* step 3 corresponds to a conversion of 30%). For each step in the repeated batch reaction, the ee_s and ee_p were calculated (■ (E=100); ◆ (E=40); ▲ (E=15); ● (E=2)). For the maximum ee_s in the batch esterification the accompanying ee_p is presented in figure A as a line (symbols: – – – (E=100); - - - (E=40); —— (E=15); – · – · – · (E=2)). In panel B, the lines signify the maximum attainable ee_s.

enantiomeric excess of the substrate up to 0.6 after 6 steps which is rather low compared to the batch system (figure 4). The enantiomeric excess of the ethyl ester decreased slightly after repeating reaction steps since the supply of *R*-4-methyloctanoic acid was running out. The experimental data from figure 4 must be compared to the estimated values using an enantiomeric ratio of 15, since this is the *E*-value at an 4-methyloctanoic acid/ethanol ratio of 10:1 (chapter 4). It can be concluded that the repeated batch esterification is well described by this model.

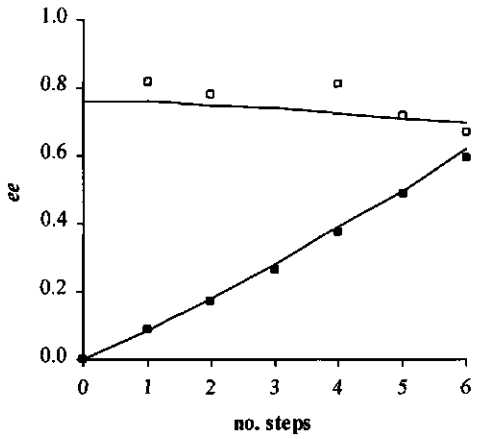


Figure 4: For the Novozym 435®-catalyzed repeated batch esterification of 4-methyloctanoic acid with ethanol, using a molar ratio of 10:1, the experimentally determined enantiomeric excess of 4-methyloctanoic acid (□) and 4-methyloctanoic acid ethyl ester (■) for each reaction step is plotted *versus* the number of reaction steps. The continuous lines in this figure represent the calculated values for both the ee_p and the ee_s belonging to an enantiomeric ratio of 15 (see figure 3).

Unfortunately, since the *E*-value in the repeated batch reaction is only 15 whereas the best batch reaction (ratio 1:8 mol acid: mol EtOH) has an *E*-value of 57, the latter system is preferable. Of course it can be questioned whether *in situ* or in line product removal is still preferable. Therefore, model calculations have been made in order to decide to perform the reaction in a batch reactor or to use an integrated system with product removal.

Simulation model for esterification

A computer model for the enantioselective esterification and *in situ* product removal was developed to predict the acid and ester concentrations in the reactor system (Figure 5). This model system consisted of three compartments: a storage

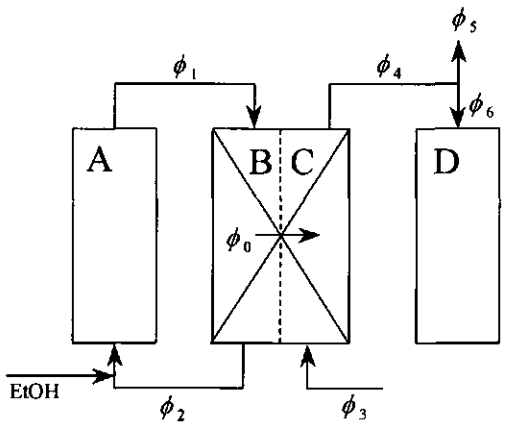


Figure 5: Simulation model for the integrated esterification of 4-methyloctanoic acid in a trickle bed reactor and separation of the produced ester from the reaction mixture.

vessel A, a trickle bed reactor B/C and a cold trap D. In the storage vessel A, a mixture of 4-methyloctanoic acid and ethanol is present. This mixture is transported (ϕ_1) from vessel A to trickle bed B/C. Esterification and separation are integrated in this compartment. The liquid phase and vapor phase are represented by the compartments B and C, respectively. Transfer from the liquid to the vapor phase is given by ϕ_0:

$$\phi_0 = k_n A \cdot (m_n \cdot [C]_B - [C]_C),$$

where k_n is the mass transfer coefficient, A is the area, m_n is the partition coefficient for n, and [C] is the concentration. The liquid outlet of the trickle bed (ϕ_2) is connected to compartment A. The gas phase inlet of the reactor is given as ϕ_3, and the outlet as ϕ_4. In compartment D the ester product accumulates.
Matlab® Simulink was used to implement the model described above. This model is based on mass balances of each compartment assuming that:

- The compartments A, B and C are ideally mixed
- $[EtOH]_A$ is constant
- Esterification in compartment B is based on Michaelis Menten kinetics
- Competitive inhibition takes place since both enantiomers compete for the same catalytic site of the enzyme
- Characteristic time for mass transfer is shorter than for kinetics

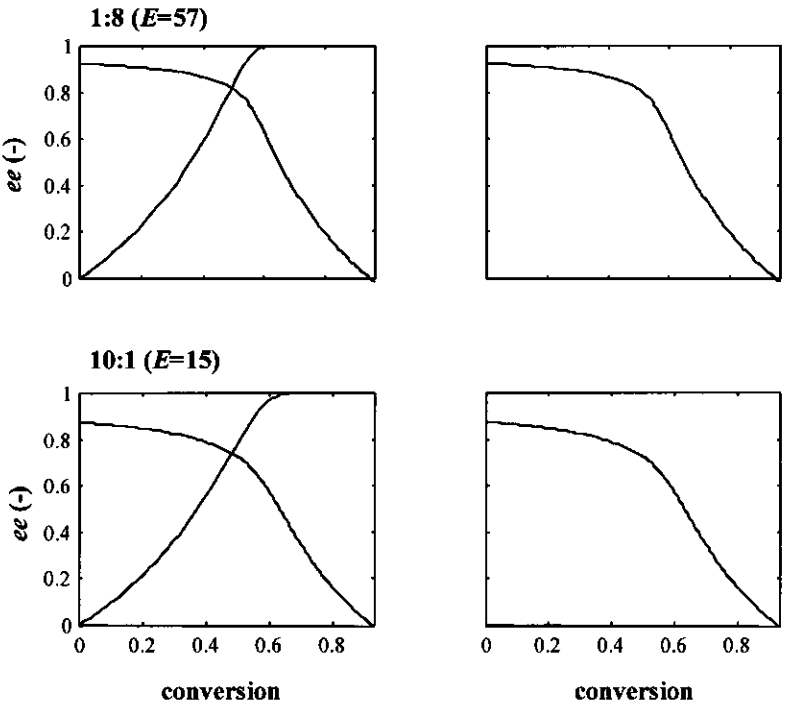


Figure 6: Enantiomeric excess of 4-methyloctanoic acid (ee_s) and its ethyl ester (ee_p) calculated according to the simulation model and plotted *versus* the conversion for a molar ratio of 1:8 (4-methyloctanoic acid:ethanol, (E=57)) and 10:1 (E=15). The left pictures are valid for the liquid phase present in the compartments A and B. The right pictures represent the enantiomeric excess of the product in the vapor phase. Initial mol fractions used: $x_{acid\,(R,S)}$: 0.1112, x_{EtOH}: 0.8888 (E=57); $x_{acid\,(R,S)}$: 0.9090, x_{EtOH}: 0.0910 (E=15).

The initial conditions and constants that are used are given in appendix I.
In figure 6 the enantiomeric excess of both the substrate and the product is plotted *versus* the conversion. The liquid phase (left pictures) contains both substrate and product. To overcome this problem, the mass transfer rate for the ester should be increased. In the vapor phase and cold trap only the product is observed (right pictures). Since product removal is accomplished, the equilibrium is shifted towards the right resulting in an irreversible reaction. For the 1 to 8 ratio both the ee_s and ee_p approach 0.8 at 0.5 conversion. In the cold trap D however, both ethanol and product accumulated in a ratio of 1:1. Since nearly all the ethanol reacted in the packed bed, no ethanol accumulation was observed for the 10:1 ratio. Unfortunately in this case the enantiomeric excess of both the product and the substrate was lower, due to a lower enantiomeric ratio of the enzymatic reaction. According to this model it is therefore concluded that the ratio of 1 to 8 is the best choice in order to obtain products with the highest enantiomeric excess.

Integration of reaction and separation

Esterification of racemic 4-methyloctanoic acid was performed in a packed bed reactor with a down flow mode, connected to a strip column or a distillation column. For the strip column system (figure 7), the starting mixture consisted of 4-methyloctanoic acid and ethanol (ratio 1:15 mol:mol). This mixture was esterified in the packed bed immobilized enzyme reactor. In the presence of a very small amount of Novozym 435® a conversion of 60% was reached after 10 days. The lipase showed a moderate enantioselectivity towards the substrate (E=11 ± 1). This value corresponds to the enantiomeric ratio determined for the lab-scale esterification (molar ratio 1:15 of acid:EtOH, chapter 4). Unfortunately, after ten days of reaction only 1 gram of ester was obtained in the cold trap, while an amount of approximately 30 grams of ester was present in the system. This was due to the unfavorable distribution of ethanol mixtures in the liquid and gas phase (figure 2). To enhance the separation of ester from the reaction mixture, the strip column was replaced by a distillation column (figure 8). since good results have been obtained for the repeated batch reaction (see figure 4). Limited amounts of

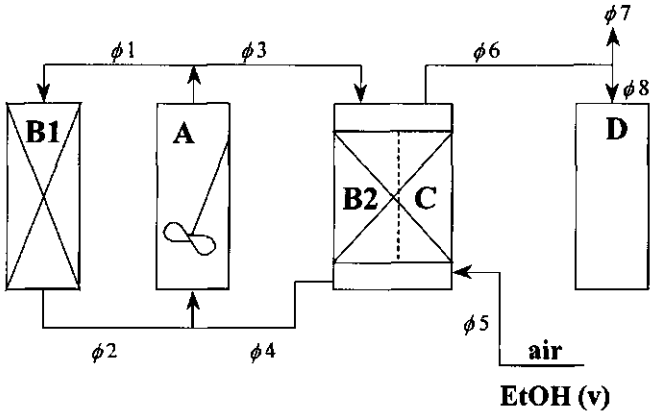


Figure 7: Esterification of 4-methyloctanoic acid with ethanol in a packed bed reactor (B_1) filled with immobilized enzyme (Novozym 435®) and stripping of the ethyl ester from the liquid phase to the gas phase (B_2/C). V_A: 250 ml; V_{B1}: 5.6 ml; $V_{B2/C}$: 450 ml; ϕ_1: $2.5 \cdot 10^{-7}$ m^3s^{-1}; ϕ_2: $2.5 \cdot 10^{-7}$ m^3s^{-1}; ϕ_3: $4.3 \cdot 10^{-6}$ m^3s^{-1}.

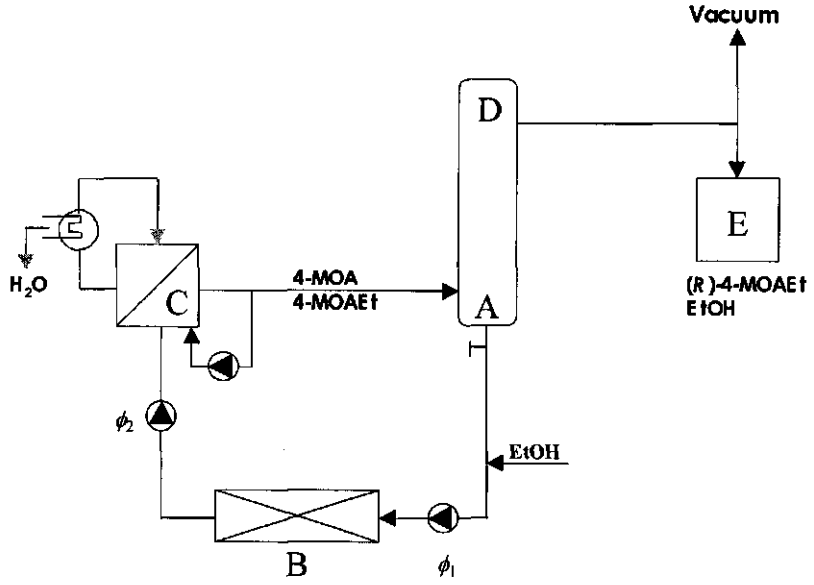


Figure 8: Esterification of 4-methyloctanoic acid with ethanol at 35°C in a packed bed reactor (B), containing Novozym 435® and distillation of the ethyl ester from the liquid phase (A) to the vapor phase (D). V_B: $3.4 \cdot 10^{-5}$ m^3; ϕ_1: $7.4 \cdot 10^{-10}$ m^3s^{-1}; ϕ_2: $5.5 \cdot 10^{-8}$ m^3s^{-1}.

ethanol were fed to a continuous flow of 4-methyloctanoic acid. The total amount of ethanol was esterified during a single pass through the packed bed immobilized enzyme reactor. The produced water was removed using pervaporation. Unfortunately, both ester and acid were distilled from the reaction mixture due to the absence of temperature control. Temperature control should enable product removal, however, due to the low ethanol concentration an ee_p of only 50% was measured.

CONCLUSIONS

The Novozym 435® catalyzed esterification of 4-methyloctanoic acid with ethanol was investigated using different reactor setups.

For the Novozym 435®-catalyzed batch esterification of 4-methyloctanoic acid with ethanol an enantiomeric ratio of 57 ± 11 was gained for both lab-scale (0.61 g, 3.84 mmol) and bench-scale reactions (79.12 g, 0.50 mol). In this case an initial molar ratio of 1:8 (4-methyloctanoic acid: ethanol) was used, resulting in a large excess of ethanol at the optimum ee_p (ee_p = 81% at 55% conversion). According to thermodynamic calculations it is unfavorable to have a large excess of ethanol present in the reactor when reaction and separation will be integrated. Therefore a ratio of 10:1 was chosen for a repeated batch experiment. Calculations showed that for different enantiomeric ratios the enantiomeric excess of the product for the repeated batch esterification was in all cases higher compared to the ee_p determined from the batch reactions at the same acid:ethanol ratio. This was confirmed by measuring the enantiomeric excess of six sequential batches (final conversion 60%). The average enantiomeric excess of the produced ethyl ester was 80%, while the substrate could be isolated with an enantiomeric excess of 60%. However, for a 10:1 molar ratio (acid:EtOH) an enantiomeric ratio of only 15 was obtained, which is rather low compared to the 1:8 molar ratio in the batch system. In the latter system, an *E*-value of 57 was obtained, leading to an ee_p of 81% and a maximum ee_s of 93% at c= 55%.

In addition, simulation and measurements of the lipase-mediated esterification and *in situ* or in line product removal did not improve the enantiomeric excess of the product. It is therefore recommended to perform the Novozym 435®-mediated esterification of 4-methyloctanoic acid with ethanol as a batch reaction instead of a repeated batch with product removal.

REFERENCES

Adachi, S., Nagae, K. and Matsuno, R. (1999). Lipase-catalyzed condensation of erythritol and medium-chain fatty acids in acetonitrile with low water content. *J. Mol. Catal. B: Enzymatic*, **6**, 21-27.

Almeida, M.C., Ruivo, R., Maia, C., Freire, L., Corrêa de Sampaio, T. and Barreiros, S. (1998). Novozym 435 activity in compressed gases. Water activity and temperature effects. *Enzyme Microb. Technol.*, **22**, 494-499.

Bornscheuer, U., Herar, A., Capewell, A., Wendel, V., Kreye, L., Scheper, T., Voß, E., Wünsche, K. and Meyer, H.H. (1995). Lipase-catalyzed kinetic resolution of 3-hydroxy esters: optimization, batch, and continuous reactions. *Ann. N.Y. Acad. Sci.*, **750**, 215-221.

Chand, S., Adlercreutz, P. and Mattiasson, B. (1997). Lipase-catalyzed esterification of ethylene glycol to mono- and diesters. The effect of process parameters on reaction rate and product distribution. *Enzyme Microb. Technol.*, **20**, 102-106.

Chen, C.-S., Fujimoto, Y., Girdaukas, G., and Sih, C.J. (1982). Quantitative analyses of biochemical kinetic resolutions of enantiomers. *J. Am. Chem. Soc.*, **104**, 7294-7299.

Chen, C-S., Wu, S-H., Girdaukas, G., and Sih, C.J. (1987). Quantitative analyses of biochemical kinetic resolution of enantiomers. 2. Enzyme-catalyzed esterifications in water-organic solvent biphasic systems. *J. Am. Chem. Soc.*, **109**, 2812-2817.

Coulombié, S., Tweddell, R.J., Condoret, J.-S. and Marty, A. (1998). Water activity control: a way to improve the efficiency of continuous lipase esterification. *Biotechnol. Bioeng.*, **60**, 362-368.

Csányi, E., Bélafi-Bakó, K., Harasek, M., Friedl, A., Sisak, Cs. and Gubicza, L. (1998). Ethanol removal from fermentation broth by enzymatic esterification. *Med. Fac. Landbouww. Univ. Gent*, **63**, 1549-1552.

Ducret, A., Trani, M. and Lortie, R. (1998). Lipase-catalyzed enantioselective esterification of ibuprofen in organic solvents under controlled water activity. *Enzyme Microb. Technol.*, **22**, 212-216.

Edlund, H., Berglund, P., Jensen, M., Hedenström, E. and Högberg, H.-E. (1996). Resolution of 2-methylalkanoic acids. Enantioselective esterification with long chain alcohols catalysed by *Candida rugosa* lipase. *Acta Chem. Scand.*, **50**, 666-671.

Engel, K.-H. (1991). Lipase-catalyzed enantioselective esterification of 2-methylalkanoic acids. *Tetrahedron: Asymmetry*, **2**, 165-168.

Fredenslund, A., Ghmeling, J., Rasmussen, P. (1977). Vapor-liquid equilibria using UNIFAC: a group-contribution method. Elsevier, Amsterdam, The Netherlands, 1-380.

Heinsman, N., and Belov, Y. (1997). Enantiomeric analysis of 4-methyloctanoic acid by chiral gas chromatography, *The Reporter.* **16**, 9.

Holmberg, E., Holmquist, M., Hedenström, E., Berglund, P., Norin, T., Högberg, H.-E. and Hult, K. (1991). Reaction conditions for the resolution of 2-methylalkanoic acids in esterification and hydrolysis with lipase from *Candida cylindracea. Appl. Microbiol. Biotechnol.*, **35**, 572-578.

Humeau, C., Girardin, M., Rovel, B. and Miclo, A. (1998). Effect of the thermodynamic water activity and the reaction medium hydrophobicity on the enzymatic synthesis of ascorbyl palmitate. *J. Biotechnol.*, **63**, 1-8.

Indlekofer, M., Brotz, F., Bauer, A., and Reuss, M. (1996). Stereoselective bioconversions in continuously operated fixed bed reactors: modeling and process optimization. *Biotechnol. Bioeng.*, **52**, 459-471.

Jongejan, J.A., van Tol, J.B.A., Geerlof, A., and Duine, J.A. (1991). Enantioselective enzymatic catalysis. 1. A novel method to determine the enantiomeric ratio. *Recl. Trav. Chim. Pays-Bas*, **110**, 247-254.

Kikkawa, S., Takahashi, K., Katada, T. and Inada, Y. (1989). Esterification of chiral secondary alcohols with fatty acid in organic solvents by polyethylene glycol-modified lipase. *Biochem. Int.*, **19**, 1125-1131.

Mensah, P., Gainer, J.L. and Carta, G. (1998a). Adsorptive control of water in esterification with immobilized enzymes: I. Batch reactor behavior. *Biotechnol. Bioeng.*, **60**, 434-444.

Mensah, P., Gainer, J.L. and Carta, G. (1998b). Adsorptive control of water in esterification with immobilized enzymes: II. Fixed-bed reactor behavior. *Biotechnol. Bioeng.*, **60**, 445-453.

Oguntimein, G.B., Anderson, W.A. and Moo-Young, M. (1995). Synthesis of geraniol esters in a solvent-free system catalysed by *Candida antarctica* lipase. *Biotechnol. Lett.*, **17**, 77-82.

Paiva, A.L. and Malcata, F.X. (1994). Process integration involving lipase-catalyzed ester synthesis reactions. *Biotechnol. Techn.*, **8**, 629-634.

Paiva, A.L. and Malcata, F.X. (1997). Integration of reaction and separation with lipases: An overview. *J. Mol. Catal. B: Enzymatic*, **3**, 99-109.

Pepin, P. and Lortie, R. (1999). Influence of water activity on the enantioselective esterification of (*R, S*)-Ibuprofen by *Candida antarctica* lipase B in solventless media. *Biotechnol. Bioeng.*, **63**, 502-505.

Rakels, J.L.L., Paffen, H.T., Straathof, A.J.J. and Heijnen, J.J. (1994). Comparison of enzymatic kinetic resolution in a batch reactor and a CSTR. *Enzyme Microb. Technol.*, **16**, 791-794.

Shimada, Y., Suenaga, M., Sugihara, A., Nakai, S., and Tominaga, Y. (1999). Continuous production of structured lipid containing γ-linoleic and caprylic acids by immobilized *Rhizopus delemar* lipase. *JAOCS*, **76**, 189-193.

Shiraishi, F., Hasegawa, T., Kasai, S., and Makishita, N. (1996). Characteristics of apparent kinetic parameters in a packed-bed immobilized enzyme reactor. *Chem. Engin. Sci.*, **11**, 2847-2852.

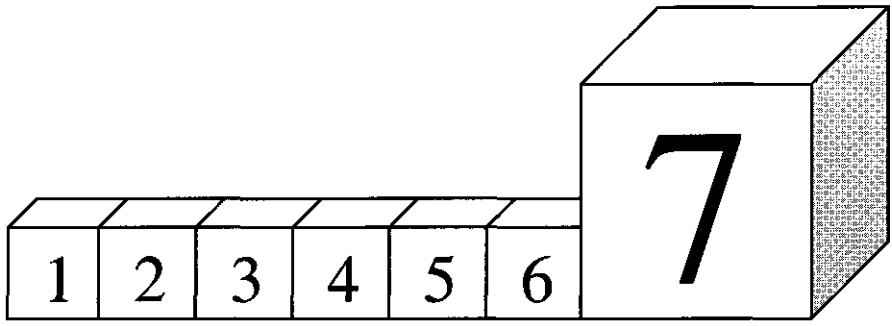
Valivety, R.H., Halling, P.J., Peilow, A.D. and Macrae, A.R. (1992). Lipases from different sources vary widely in dependence of catalytic activity on water activity. *Biochim. Biophys. Acta*, **1122**, 143-146.

APPENDIX I

Table I: Initial conditions and parameters for prediction of esterification in trickle bed reactor:

	Value		Unit	Motivation
	1:8	*10:1*		
[4-MOA (*R*)]	0.0556	0.4545	mol/mol	Based on lab-scale experiments
[4-MOA (S)]	0.0556	0.4545	mol/mol	Based on lab-scale experiments
[EtOH]	0.8888	0.0910	mol/mol	Based on lab-scale experiments
E-ratio	57	15	-	Based on lab-scale experiments
ε (gas)	0.4		-	Assumptions based on experiments
ε (liquid)	0.1		-	Assumptions based on experiments
ε (bed)	0.5		-	Assumptions based on experiments
m	$5 \cdot 10^{-3}$		$m^3_{(liq)}/m^3_{(gas)}$	Assumption
kA	$1 \cdot 10^{-7}$		s^{-1}	Assumption
V_A	$1.94 \cdot 10^{-4}$		m^3	Based on measurements of equipment
V_{BC}	$3.74 \cdot 10^{-5}$		m^3	Based on measurements of equipment
E	0.5		g	Assumption
ϕ_{V1}	$1 \cdot 10^{-6}$		m^3	Based on experimental data
ϕ_{V3}	$23 \cdot 10^{-6}$		m^3	Based on experimental data

E-ratio: enantiomeric ratio; ε: Volume fraction; m: partition coefficient; kA: 'lumped' mass transfer coefficient multiplied with specific area; V: volume of reactor; E: enzyme load; $\phi_{V1,3}$: volume flow into compartment 1 and 3



General discussion

In this thesis a study is presented towards the production of enantiomerically pure branched chain fatty acids using lipases. The kinetic resolution of branched chain fatty acids (BCFAs) using lipases in a two-phase reactor was considered to be an attractive method to produce pure enantiomers from racemates. In this chapter we return to the choices that we have made during the research to reach this goal:

DISCUSSION

- Branched chain fatty acids, why and which?
- Enantiomer separation
- Enantiomeric ratio estimation, choice of lipase (*chapter 2*)
- Sequential kinetic resolution
- Immobilization of enzyme
- Choice of reaction system
  - *Batch system (chapter 4,5)*
    - Gas-phase
    - Supercritical fluid system
    - Liquid & liquid-liquid system
  - *Repeated batch system (chapter 6)*
  - *Continuous system (chapter 3,6)*
    - Liquid-liquid system
    - Liquid-vapor system

RECOMMENDATIONS

- Optimization of large-scale esterification
- Lipase-catalyzed reactions with other BCFAs

DISCUSSION

Branched chain fatty acids, why and which?

Branched chain fatty acids make an important contribution to the smell and taste of a variety of food products (see *chapter 1*). Two of these branched chain fatty acids, *viz.* 4-methylhexanoic acid and 4-methyloctanoic acid, were used for the research described in this thesis. Both chiral fatty acids contribute to the characteristic taste and smell of mutton and sheep's cheese. Their methyl and ethyl esters play a role in the flavor of dry-cured ham and Chardonnay wine and their glucose and sucrose esters enhance the flavor of tobacco smoke.

The flavor and fragrance of the enantiomers of 4-methyloctanoic acid and its ethyl esters were independently evaluated by IFF (Tilburg, The Netherlands) and Quest (Naarden, The Netherlands). Both companies concluded that the enantiomers have different sensory properties. The *R*-enantiomers were more characteristic than the *S*-enantiomers (table 1). It is therefore worthwhile to separate these stereoisomers.

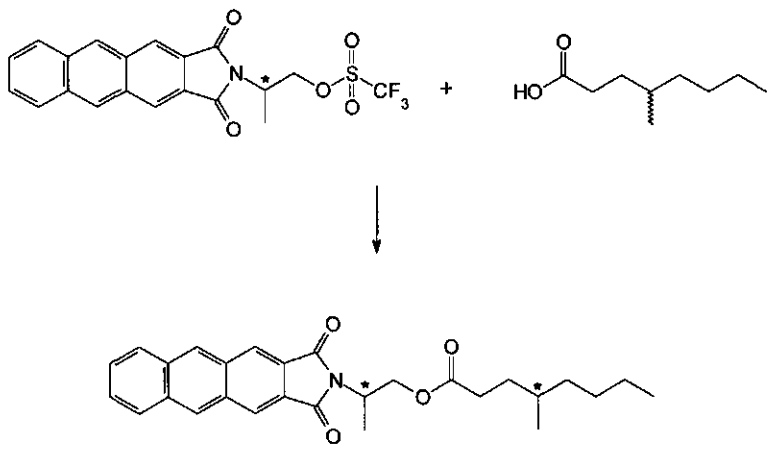
Table 1: Flavor and fragrance evaluation of *R*- and *S*-4-methyloctanoic acid and its ethyl esters

Compound[a]	Description
R-4-methyloctanoic acid	More goaty type character, typical mutton taste and smell, heavy, fuller, sweeter, 5 to 10 times stronger than *S*-enantiomer
S-4-methyloctanoic acid	Less warm goaty, fatty, whey, dairy, sheep's cheese, less interesting than *R*-enantiomer
R-4-methyloctanoic acid ethyl ester	Strong goaty, mutton note, tropical fruit, possibly interesting for sheep's cheese
S-4-methyloctanoic acid ethyl ester	Less interesting than *R*-enantiomer

[a] The compounds tested did either have a concentration of 10 ppm in H_2O or 100 ppb in H_2O

Enantiomer separation

Different techniques can be applied to obtain enantiomerically pure products (*chapter 1, page 10, 11*). However, in case the starting material is racemic only ***diastereomer separation*** (*i.e.* diastereomer crystallization (in case of a salt) or chromatographic methods (in case of covalent adducts)), ***preferential crystallization*** (*i.e.* seeding with the crystals of one enantiomer) or ***kinetic resolution*** (separation based on difference in reaction rates of the enantiomers with a chiral entity) can be applied. For the analytical separation of the enantiomers of branched chain fatty acids, different chiral columns were designed. Dietrich and coworkers (1992 a,b) developed chiral stationary phases for the enantioseparation of various branched chain fatty acids, amongst which 4-methyloctanoic acid. Akasaka and coworkers (1997) presented the enantiomeric separation of 4-methyloctanoic acid and many other branched chain fatty acids using a sensitive chiral fluorescent derivatization reagent (AP-OTf) (scheme 1). By coupling the fatty acid to this reagent, diastereomeric separation turned out to be possible on HPLC within 35 minutes (mobile phase; acetonitrile/ water (v/v) = 85/25, r.t. (min) = 33.1 (k' = 20.2), 35.7 (21.8), α = 1.08 and Rs = 1.2). Although good separation has been obtained using this method, one might expect problems for preparative applications (*e.g.* production costs, food safety).



Scheme 1: Coupling of AP-OTf with 4-methyloctanoic acid

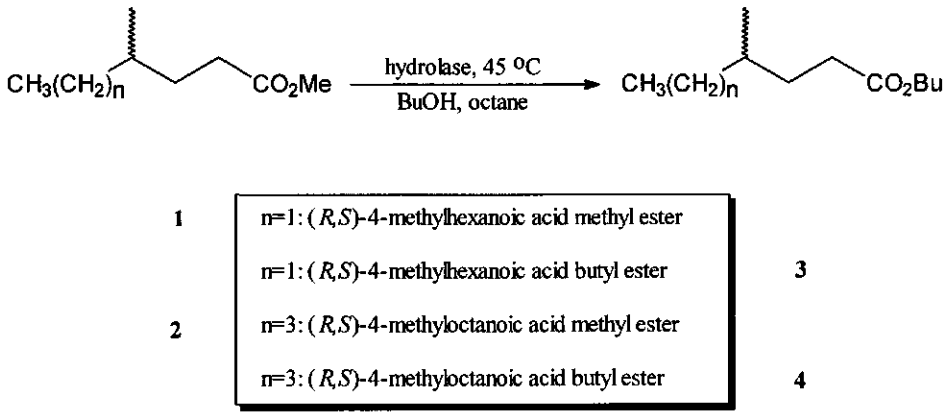
This thesis describes the enantiomer separation of 4-methylhexanoic acid and 4-methyloctanoic acid using kinetic resolution by enzymes. This technique meets the requirements of the flavor and fragrance industries (*i.e.* food grade catalysts).

Enantiomeric ratio estimation, choice of lipase

Gandhi (1997) reviewed the applications of lipases in industry, *e.g.* fat degradation, flavor production in dairy and related industries, medical applications and resolution of racemates. Lipase-catalyzed reactions are carried out at ambient pressure and temperature, resulting in purer products with a better color and reduced overall production costs. In case of flavors or fragrances also a better odor may be obtained. Nowadays, more and more reactions on an industrial scale are catalyzed by lipases.

The first step in kinetic resolution is the selection of an enzyme, and this can be done with the help of empirical rules, computer models or screening.

A set of empirical rules for secondary alcohols and α-substituted carboxylic acids was designed to predict the enantiopreference of the lipases as well as the maximum tolerated size of the substrate for many lipases (Kazlauskas, 1991; Ahmed *et al.*, 1994). The empirical rule for secondary alcohols seems to be valid for many lipases, but for complex substrates both rules are not reliable. The rule for carboxylic acids is also valid for reactions in organic solvents (Franssen *et al.*, 1998). A second approach is the use of computer models based on molecular modeling (Haeffner *et al.*, 1998; Orrenius *et al.*, 1998; Rottici *et al.*, 1998). Although these models indeed calculate the lowest ΔH for the fast reacting enantiomer, it is still difficult to calculate the enantiomeric ratio. This enantiomeric ratio is proportional to the difference in free energy between the enantiomers in the ground state and transition state ($\Delta\Delta G^{\ddagger} = -RT[\ln E] = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger}$). For molecular mechanics calculations, the entropic term (ΔS) is neglected whereas this term can contribute significantly to the enantiomeric ratio (Overbeeke *et al.*, 1998). The third possibility is screening, which is time consuming but is in many cases still the fastest method since the information for other kinds of substrates then secondary alcohols and α-substituted carboxylic acids is not available.



Scheme 2: Screening of lipases and esterases in the transesterification of 4-methylhexanoic acid methyl ester and 4-methyloctanoic acid methyl ester.

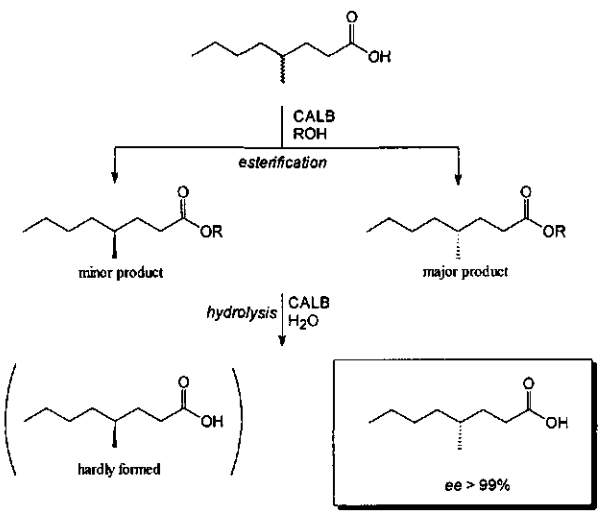
In order to find an efficient enzyme for the kinetic resolution of branched chain fatty acids, a variety of enzymes (lipases, esterases) were screened (*chapter 2*) for the transesterification of 4-methylhexanoic acid methyl ester and 4-methyloctanoic acid methyl ester to their butyl esters (scheme 2).

From the 25 enzymes screened, only *Rhizomucor miehei* lipase (Biocatalysts) and *Candida antarctica* lipase B (Novozym 435®, NOVO Nordisk) showed both activity and a moderate stereoselectivity ($2 < E < 8$). For the Novozym 435®-catalyzed transesterification of 4-methyloctanoic acid methyl ester with butanol, reduction of the temperature from 45°C to 27°C resulted in a much higher enantiomeric ratio ($E = 23$). Consequently, screening yielded Novozym 435® as the preferred biocatalyst. The differences in selectivity may be explained by the steric requirement of the substrate. Some lipases accept bulky substrates and exhibit therefore a low selectivity on small molecules, whereas other lipases are highly selective on small substrates having less steric requirements.

Sequential kinetic resolution

Since a low enantioselectivity was found in the (trans)esterification of 4-methylhexanoic acid (methyl ester) and 4-methyloctanoic acid (methyl ester), sequential kinetic resolution was used to enhance the enantiomeric excess of the product (scheme 3). The substrate passes the active site of the enzyme twice (once during esterification and the second time during hydrolysis) which enables the synthesis of enantiopure 4-methyloctanoic acid after a number of consecutive steps.

Figure 1 shows the predicted enantiomeric excess of the product for a number of reaction steps (Chen *et al.* 1982). It is clear that even reactions catalyzed by lipases having a low enantioselectivity can yield products with a high enantiomeric excess in case the reaction is repeated for a few times.



Scheme 3: Sequential kinetic resolution as a method to obtain optically pure branched chain fatty acids. After one reaction step (esterification) enantiomerically enriched ester is formed (in this example the *R*-enantiomer is the fast reacting one). This product is used for a second reaction step (hydrolysis) using the same lipase and resulting in enantiopure product.

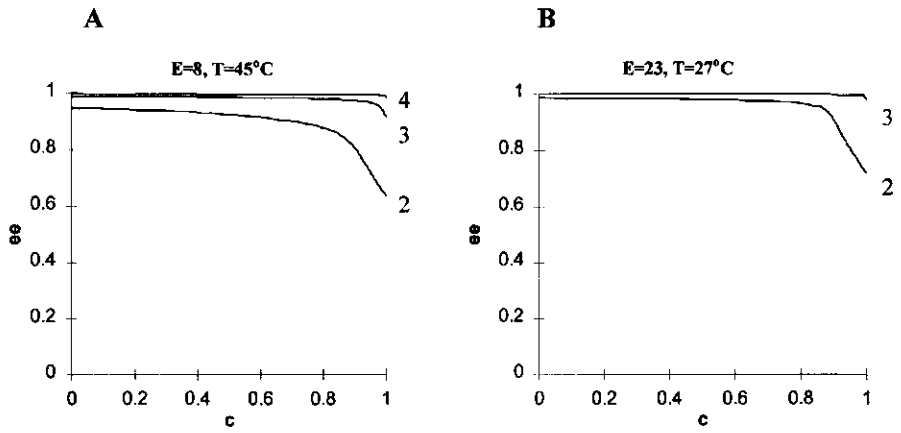


Figure 1: Predicted enantiomeric excess of product after 2, 3 or 4 reaction steps. These calculated lines are based on data from the transesterification reaction (A: T=45°C; c=0.6, ee_o=0.64 and B: T=27°C; c=0.5, ee_o=0.72; data from chapter 2), using Chen's expression (Chen *et al.* 1982)

However, when performing transesterification reactions, problems may arise during down-stream processing (separation of two esters). In chapter 5 it has been shown that sequential kinetic resolution (esterification followed by hydrolysis) may result in an overall enantiomeric ratio of approximately 350.

Immobilization of enzyme

To reduce the relatively high enzyme costs, the biocatalyst has to be reused. This can be achieved by immobilization of the enzyme onto a support. Besides the reusability, the advantages of immobilization includes the integration of a purification step in the process, a better process control, the use of a continuous process, and sometimes an increased stability of the enzyme (Brink *et al.*, 1988; Prazeres and Cabral, 1994). However, a disadvantage might be the possibility of substrate sorption into the immobilization material, resulting in incorrect interpretations of data (*chapter 4*). It was shown that part of the substrate did not take part into the reaction due to sorption into the polymer matrix of the Novozym 435® immobilization support. The swelling of the particles was recorded on photo

and video. Since sorption reached its maximum within two minutes, corrections had to be made to calculate the actual conversion of the substrate. After correction more reliable results were obtained. The sorption of substrate appeared to be no problem in hydrolysis and transesterification reactions.
Balcão and coworkers (1996) have reviewed the use of immobilized lipases in reactors. There are several possibilities to enclose immobilized enzymes in reactors, *e.g.* membrane reactors, batch stirred tank reactors and packed bed reactors. These three examples were options for the research presented in this thesis.

Choice of reaction system

Different reaction systems can be applied depending on the substrates used. One possibility is the use of a *batch system* in which reactions can occur in a supercritical fluid system, or in a liquid system. In addition, attention is paid to a *repeated batch system*. Finally, we will discuss *continuous systems*. Besides reaction in a gas phase, two other possibilities are given: a liquid-liquid system and a liquid-vapor system.

Batch system

Supercritical fluid system

Two decades ago another method was introduced, which make use of supercritical fluids, especially supercritical carbon dioxide (Rantakylä and Aaltonen, 1994; Michor *et al.*, 1996; Sereti *et al.*, 1997; Almeida *et al.*, 1998; Knez *et al.*, 1998; Tsitsimpikou *et al.*, 1998; Overmeyer *et al.*, 1999). Since the critical temperature is rather low (T_c = 31°C), carbon dioxide is an attractive medium for thermally unstable substrates or products and enzymes. Besides, supercritical CO_2 has many advantages over the conventional organic solvents: it has a low viscosity and a high diffusivity, it is easy to separate from the reaction mixture, it is available at low costs and it is non-toxic and non-flammable. A main drawback of reactions in

supercritical CO_2 is the poor solubility of polar substrates. Addition of co-solvents or the use of complexation agents (*e.g.* organoboric acids) can overcome this problem. In table 2, three examples of reactions in supercritical carbon dioxide are given. Although this new technology might be a promising technique for the resolution of racemates, its drawback will be the expensive equipment and thus high investments. Therefore, we chose another option for the resolution of branched chain fatty acids.

Table 2: Examples of reactions performed in supercritical carbon dioxide

	Example 1	**Example 2**	**Example 3**
Reaction type	Esterification	Esterification	Transesterification
Substrates	1-phenylethanol vinyl acetate	Glucose, lauric acid	menthol, isopropenyl acetate
Enzyme	CALB	CALB	esterase EP10
Conditions	90°C, 15 Mpa	60°C, 150 bar	100 bar, 50°C
Result	99% ee at 50% conv.	60% conv.	*E*-ratio = 180
Literature	Overmeyer *et al.*, 1999	Tsitsimpikou *et al.*, 1998	Michor *et al.*, 1996

Liquid & liquid-liquid system

For the kinetic resolution of 4-methyloctanoic acid, lab-scale reactions were performed in order to define the optimal conditions. At different substrate concentrations, the Novozym 435® catalyzed esterification of 4-methyloctanoic acid with ethanol was studied (*chapter 4*). The optimum enantiomeric ratio ($E = 57 \pm 11$) was estimated for the molar ratio 1:8 (4-methyloctanoic acid:ethanol).
A bench-scale batch esterification was also studied using the same conditions compared to the optimal lab-scale esterification (molar ratio 1:8 of acid: ethanol). Both reactions showed the same course. The substrate and product could be obtained with an ee_s of 93% and ee_p of 81% (*chapter 6*).

In hydrolysis reactions (*chapter 5*), the ethyl ester of 4-methyloctanoic acid was hydrolyzed with a low enantioselectivity ($E = 5.5 \pm 2.5$). However, the addition of ethanol affected the enantioselectivity of the enzyme. With increasing ethanol concentration, the enantiomeric ratio increased up to 12.2 ± 0.9 (for 20% v/v ethanol).

Repeated batch system

Considering the esterification of 4-methyloctanoic acid with ethanol integrated with product separation, the product can be removed making use of the low vapor pressures of ethanol and ester. Since calculations showed that for high ethanol concentrations only very small amounts of 4-methyloctanoic acid ethyl ester could be transferred to the vapor phase, a repeated batch reaction was studied using a molar ratio of 10:1 (fatty acid: ethanol). In *chapter 6* it was shown that the small amount of ester produced could be distilled in a controlled manner. After six cycles, an enantiomerically enriched product having an ee_p of 80% was obtained. For different E-values ($E = 2 \Rightarrow E = 100$) the ee_p was calculated both for the batch and repeated batch reaction. In chapter 6 it was shown that at 60% conversion the ee_p for the repeated batch was higher in all cases. Thus repeated batch integrated with distillation results in a product with a high enantiomeric excess.

Continuous system

Gas-phase

In the last decade, several articles have been published about the performance of enzymatic catalysis with reactants present in the gas phase (Ross and Schneider, 1991; Parvaresh *et al.*, 1992; Lamare and Legoy, 1993; 1995; Russell and Yang, 1996). Biocatalysis in solid-gas systems has certain advantages over catalysis in conventional media. Firstly, the biocatalyst is present in a solid state, and cannot diffuse into the continuous phase, making immobilization superfluous. Secondly,

no additional solvents are present which reduces the formation of by-products. Another advantage is the mass transfer, which is often more efficient in the gas phase than in the liquid phase. Of course this method also has its limitations. The major problem is to evaporate the substrates. For all reactions, the temperature, the pressure and the water activity play an important role. If these parameters are not optimized, thermal inactivation of the biocatalyst or a change in enzyme specificity may occur. Although biocatalysis in the gas phase seems to be very promising, this technology is not suitable for our branched chain fatty acids which have boiling points of around 300°C.

Liquid-liquid system

In order to esterify 4-methyloctanoic acid according to 'food-grade' conditions the alcohol has to be selected with care. In case both substrates have a high boiling point, the reaction occurs in the liquid phase. For lab-scale reactions it is possible to separate the product by extraction. At bench-scale, it is favorable to continuously separate the product from the mixture, which could be realized using a membrane reactor. To provide enantiopure products at least a set of two membrane reactors is necessary for the sequential kinetic resolution (esterification followed by hydrolysis). The first membrane reactor should consist of a two-phase system in which the lipase, immobilized on the membrane, would stereoselectively esterify the acid. After catalysis, the produced ester should diffuse from the organic layer to the water layer that circulates through the system. In a second membrane reactor the enriched product should be hydrolyzed with the same lipase.

For the production of 4-methyloctanoic acid ester polyethylene glycol (PEG) was used (*chapter 3*), since it was assumed that this ester would accumulate in the water phase. Unfortunately, an intensive study of the partition of PEG-600 in the PEG-water phase and the organic phase, together with a survey of the physicochemical properties of the PEG-ester, showed us that it is impossible to create a two-phase system in which the ester formed was present in the water layer. This is probably due to the amphiphilic character of PEG. Based on these

results we decided to use another alcohol for the kinetic resolution of 4-methyloctanoic acid.

Liquid-vapor system

Since polyethylene glycol was not suitable as alcohol, ethanol was chosen to esterify 4-methyloctanoic acid in the presence of Novozym 435®, in a solventless system. Although the log P-value of this alcohol amounts to –0.24 (Laane *et al.*, 1987), no inactivation or denaturation of Novozym 435® was observed (chapter 4). Esterification of racemic 4-methyloctanoic acid was performed in a packed bed reactor with a down-flow mode, connected to a strip column or distillation column (figure 2). For the integrated system of a packed bed reactor and a strip column a moderate enantiomeric ratio ($E = 11 \pm 1$) was measured for a 1:15 molar ratio of fatty acid *versus* ethanol (*chapter 6*). This value approached the one determined from the small-scale reactions (*chapter 4*). As was calculated, due to the excess of ethanol present, only a small amount of ester was observed in the vapor phase.

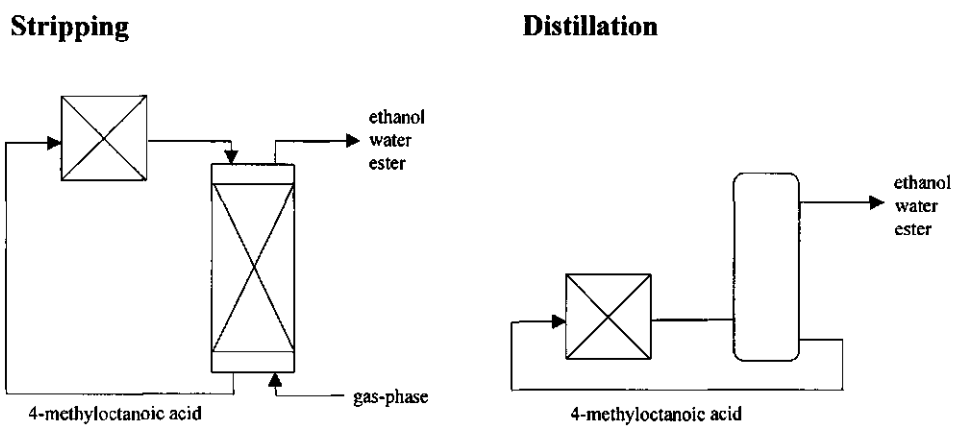


Figure 2: Scheme of the esterification of 4-methyloctanoic acid with ethanol in a packed bed reactor filled with Novozym 435®. The reaction mixture is fed into a strip column or distillation column to remove the ester from substrate phase.

Replacement of the strip column by a distillation column resulted in a better transfer of the ester from the liquid to the vapor phase (see repeated batch). However, it was shown that for an excess of 4-methyloctanoic acid, which was a necessary condition for a selective product transfer, the enantiomeric excess of the product is rather low. Since optimization of the system is still necessary some recommendations are given in the next section.

RECOMMENDATIONS

Optimization of large-scale esterification

Since a limiting amount of ethanol was present in the integrated system of esterification in the packed bed reactor and product removal using distillation, a low enantiomeric excess was estimated for the product obtained. A solution to this problem of a low ee_p might be the injection of an excess of ethanol into the packed bed reactor. At the end of the column this excess of ethanol has to be removed and regenerated for a new cycle. In this case, it might be possible to perform the

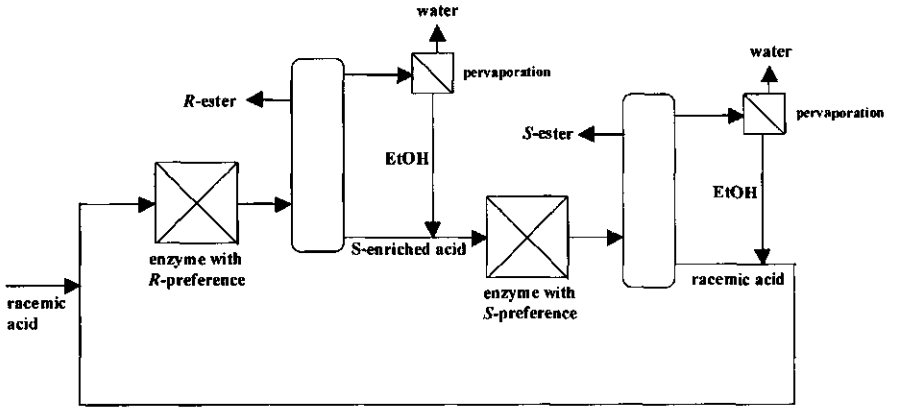


Figure 3: Proposed system for the production of 4-methyloctanoic acid ethyl ester with a high enantiomeric excess of the product. Two enzymes with the opposite enantiopreference are integrated into the system.

reaction with a 1:8 molar ratio resulting in a higher enantiomeric ratio (as was shown for the small-scale batch reactions).

For all reactions studied, the source of the *R*-enantiomer of 4-methyloctanoic acid runned out because this enantiomer was the fast reacting one. In the semi-continuous process this means that the *S*-enantiomer accumulates in the system. A possible solution to this problem is the introduction of another enzyme, with the preference for the *S*-enantiomer, into the system (figure 3). *Rhizomucor miehei* lipase (Biocatalysts) showed this preference, although with low selectivity (results not shown). In this case almost racemic acid will react in each cycle, resulting in a product having a higher ee_p compared to the previous mentioned methods.

Lipase-catalyzed reactions with other BCFAs

In this thesis, the branched chain fatty acid *4-methyloctanoic acid* was studied in detail. It was shown that chain length, the temperature and the substrate concentrations influenced the enantiomeric ratio. Branched chain fatty acids are of interest to flavor and fragrance industry, but they can also be used as chiral building blocks, *e.g.* for the synthesis of pheromones (Högberg *et al.* 1990; Holmberg *et al.* 1991; Nguyen and Hedenström, 1999). Therefore, it is interesting to investigate the influence of branching on the enantiomeric ratio, during lipase-catalyzed resolution. For that purpose, 2-, 3-, 5-, and 6-methyloctanoic acid can be prepared in order to perform (trans)esterification reactions. In literature, several synthesis routes have been published. Berglund and coworkers (1993) presented a synthesis route for racemic 2-methyloctanoic acid starting from hexylbromide and diethyl malonic ester. When 1-hexylbromide is replaced by 2-bromoheptane, 3-methyloctanoic acid or its ester can be prepared according to the same procedure (Berglund (1993)). A mixture of 3- and 5-methyloctanoic acid was synthesized in several steps from 3-methyl cyclohexanone (Descotes and Querou (1968)). Sonnet and coworkers (1990) presented different routes for the synthesis of 5- and 6-methyloctanoic acid. An overall yield of 14-15% could be reached for the synthesis of either racemic or enantiopure 5-methyloctanoic acid starting from 1,4-butanediol. Similarly to the synthesis of enantiopure 5-methyloctanoic acid, enantiopure 6-methyloctanoic acid could be synthesized from 1,5-pentanediol.

Although the routes for the production of enantiopure methyl alkanoic acids are useful, a simplified procedure for the synthesis of racemic 5-methyloctanoic acid can be used, starting from methylcyclopropyl ketone. In this way an overall yield of 35% can be reached (Sonnet *et al.*, 1990). Davidson and Schumacher (1993) described the synthesis of 6-methyloctanoic acid. This acid was synthesized in only three steps using a Wittig coupling of racemic 2-methylbutanal and an ylide. Until now, only a few groups have been working on the lipase catalyzed reactions of branched chain fatty acids (esterification (table 3), transesterification (table 4), hydrolysis (table 5) and acidolysis (table 6)). Most reactions studied were catalyzed by *Candida rugosa* lipase, although other lipases have also been used.

Table 3: Overview of lipase-catalyzed esterification of branched alkanoic acids.

Esterification[‡]				
Acid	***Alcohol***	***E***	***Scale mmol***	***Ref.***
2-methylbutanoic acid	Methanol	3	0.5	1
	Ethanol	3	0.5	1
	Cyclohexanol	3	0.5	1
	1-Heptanol[a]	4.6	0.75–15	2
	1-Octanol	4	0.5	1
	1-Octadecanol	5	0.5	1
	1-Hexadecanol	3.6	5.13	3
2-methylpentanoic acid	3-Methyl-2-buten-1-ol	20	0.5	1
	1-Octanol	70	0.5	1
	1-Octadecanol	35	0.5	1
2-methylhexanoic acid	Cyclohexylmethanol	14	0.5	1
	Ethanol	1.4	0.5	1
	1-Butanol	8	0.5	1
	Cyclohexanol	12	0.5	1
	1-Octanol	20	0.5	1
	(*R*)-(-)octan-2-ol	23	0.5	1
	(*S*)-(+)octan-2-ol	35	0.5	1
	1-Decanol	25	0.5	1
	1-Octadecanol	30	0.5	1
2-methylheptanoic acid	1-Hexadecanol[b]	23	5.13	3
2,6-dimethylheptanoic acid	1-Hexadecanol[b]	18	5.13	3

Table 3: continued

Acid	*Alcohol*	*E*	*Scale mmol*	*Ref.*
2-methyl-5-heptenoic acid	1-Hexadecanol[b]	51	5.13	3
2,6-dimethyl-5-heptenoic acid	1-Hexadecanol[b]	41	5.13	3
2-methyloctanoic acid	1-Butanol[c]	4.9	2.5	4
	1-Butanol[d]	>40	2.5	4
	1-Butanol	20 ± 2	3.75	5
	1-Heptanol	57 ± 7	3.75	5
	1-Decanol	79 ± 11	3.75	5
	1-Dodecanol	83 ± 11	3.75	5
	1-Dodecanol	23	127	6
	1-Tetradecanol	85 ± 11	3.75	5
	1-Hexadecanol	126 ± 19	3.75	5
	1-Hexadecanol[b,e]	92, 109	0.15 M	6,7
4-methyloctanoic acid	Ethanol [e,f]	8 – 57	2 – 11	---[f]
2,4-dimethyloctanoic acid	Hexadecanol[b,g]	1.6	0.15 M	3
3,7-dimethyl-6-octenoic acid	Hexadecanol[b,h]	24	0.15 M	3
3,7-dimethyloctanoic acid	Hexadecanol[b,h]	17	0.15 M	3
2-methyldecanoic acid	Ethanol	3 ± 0.4	1.5	5
	1-Propanol	6 ± 1	1.5	5
	1-Butanol	15 ± 3	1.5	5
	1-Pentanol	26 ± 3	1.5	5
	1-Hexanol	37 ± 5	1.5	5
	1-Heptanol	37 ± 5	1.5	5
	1-Heptanol	15	105	6
	1-Heptanol[e]	37, 81	0.15 M	6,8
	1-Heptanol[i]	40	0.75–15	2
	2-Heptanol	29 ± 4	1.5	5
	1-Octanol	34 ± 5	1.5	5
	1-Nonanol	23 ± 3	1.5	5
	1-Decanol	10 ± 1	1.5	5
	1-Decanol[e,j]	52 – 99	0.75	6,9
	1-Hexadecanol[b,k]	14, 28	0.15 M	6
	1-Octadecanol[b,k]	23, 135	0.15 M	6
	Eicosanol[b,k]	25, 36	0.15 M	6
4-methyldodecanoic acid	Eicosanol[b]	19	5.13	10

‡ Unless otherwise stated, all reactions were catalyzed by *Candida rugosa* lipase (CRL) with a preference for the *S*-enantiomer at room temperature. **a**. T = 6°C; **b**. a_w = 0.8; **c**. *Pseudomonas fluorescens* lipase; 30°C. **d**. *Rhizomucor miehei* lipase, 30°C; **e**. Amount of alcohol present influences the enantiomeric ratio; **f**. *Candida antarctica* lipase B (see chapter 4 and 5 of this thesis); **g**. *2R,4S*-enantiopreference; **h**. *R*-enantiopreference; **i**. T = 37°C; **j**. a_w = 0.16 or 0.8; **k**. Immobilization of CRL affects the enantiomeric ratio.

1.Engel (1991), **2**. Holmberg *et al.* (1991), **3**. Nguyen and Hedenström (1999), **4**. Sonnet and Welch Baillargeon (1991), **5**. Berglund *et al.* (1993), **6**. Edlund *et al.* (1996), **7**. Högberg *et al.* (1994), **8**. Berglund *et al.* (1994), **9**. Berglund *et al.* (1995), **10**. Lundh *et al.* (1996).

Table 4: Transesterification of 4-methylhexanoic and octanoic acid methyl ester

Transesterification‡,1				
Ester	***Alcohol***	***T***	***Scale***	***E***
4-methylhexanoic acid methyl ester	1-Butanol	45°C	0.1 mmol	5
4-methyloctanoic acid methyl ester	1-Butanol	45°C	0.1 mmol	8
	1-Butanol	27°C	0.1 mmol	23

‡ Transesterification reactions were catalyzed by *Candida antarctica* lipase B (CALB, Novozym 435®) with a preference for the *R*-enantiomer.

1. Heinsman *et al.* (1998).

Table 5: Overview of lipase- and esterase-catalyzed hydrolysis of branched alkanoic acid esters.

Hydrolysis				
Ester	***Enzyme***[a]	***E***	***Scale (mmol)***	***Pref.***
2-Methylbutanoic acid ethyl ester[1,b]	CRL	3.6	1.2 – 1.8	*S*
octyl ester[1,b,c]	CRL	3.6	1.2 – 1.8	*S*
1-phenylethyl ester[1,b]	CRL	3.6	0.6 – 0.9	*S*
3-Hydroxy-3-methylpentanoic acid methyl ester[2,d]	PLE	≥ 4.1	10	*R*
3-Hydroxy-3-methyl-5-hexenoic acid Me ester[2,d,e,f]	PLE	3.9 - 4.5	1.5, 10	*R*
3-Hydroxy-3-methyl-5-hexenoic acid Et ester[2,d,f]	PLE	3.1, 4.8	1.5, 10	*R*
2-methyloctanoic acid thiol ester[3,g]	CRL	1.9	10^{-5}–10^{-4} M	*S*
	RML	2.7	10^{-5}–10^{-4} M	*S*
	RDL	4.6	10^{-5}–10^{-4} M	*S*
	PFL	2.8	10^{-5}–10^{-4} M	*S*
	LPL	3.8	10^{-5}–10^{-4} M	*S*
2-methyloctanoic acid (*S*)-IPG ester[4,f]	CRL	30	1-2	*S*
2-methyloctanoic acid *p*-nitrophenyl ester[3,g]	CRL	2.2	10^{-5}–10^{-4} M	*S*
	RML	30.4	10^{-5}–10^{-4} M	*R*
3-methyloctanoic acid thiol ester[3,g]	RML	3.3	10^{-5}–10^{-4} M	*R*
	RDL	1.5	10^{-5}–10^{-4} M	*R*
	PFL	9.3	10^{-5}–10^{-4} M	*R*
	LPL	1.9	10^{-5}–10^{-4} M	*R*
4-methyloctanoic acid ethyl ester[5,h]	CALB	5.5 – 12	2.1	*R*
4-methyloctanoic acid thiol ester[3,g]	CRL	4.3	10^{-5}–10^{-4} M	*S*
	RML	2.0	10^{-5}–10^{-4} M	*R*
	RDL	1.5	10^{-5}–10^{-4} M	*R*
	PFL	1.4	10^{-5}–10^{-4} M	*R*
	PPL	2.1	10^{-5}–10^{-4} M	*R*
	LPL	1.2	10^{-5}–10^{-4} M	*R*
5-methyloctanoic acid thiol ester[3,g]	CRL	1.5	10^{-5}–10^{-4} M	*R*
	RML	1.5	10^{-5}–10^{-4} M	*S*
	PFL	4	10^{-5}–10^{-4} M	*R*
	PPL	3.1	10^{-5}–10^{-4} M	*R*
	LPL	6.3	10^{-5}–10^{-4} M	*R*

Table 5: continued

Ester		*Enzyme*[a]	*E*	*Scale (mmol)*	*Pref.*
6-methyloctanoic acid thiol ester[3,g]		CRL	6.2	10^{-5}–10^{-4} M	*R*
		RML	1.8	10^{-5}–10^{-4} M	*R*
		RDL	1.7	10^{-5}–10^{-4} M	*R*
		PFL	3.1	10^{-5}–10^{-4} M	*S*
		PPL	1.6	10^{-5}–10^{-4} M	*S*
		LPL	2.1	10^{-5}–10^{-4} M	*S*
3-hydroxy-3-methylnonanoic acid methyl ester[2,d]		PLE	3.5	10	*R*
2-methyldecanoic acid	ethyl ester[1,b,c]	CRL	<1	0.31-0.35	--
	hexyl ester[6,c]	CRL	33	0.1 M	*S*
	heptyl ester[6,c]	CRL	35	0.1 M	*S*
	octyl ester[1,6,c,i]	CRL	9	0.31 – 0.35	*S*
	octyl ester[1,6,i,j]	CRL	4.8	0.31 – 0.35	*S*

a. (CRL): *Candida rugosa* lipase, (PLE): Pig liver esterase, (RML): *Rhizomucor miehei* lipase, (RDL): *Rhizopus delemar* lipase, (PFL): *Pseudomonas fluorescens* lipase, (PPL): Porcine pancreatic lipase, (LPL): Lipoprotein lipase, (CALB): *Candida antarctica* lipase B. **b.** pH 7.5, room temperature; **c.** pH 8.0 + $CaCl_2$, room temperature, **d.** pH decreases from 9.0 to 7.2 during reaction, temperature not mentioned, **e.** pH 6.0, temperature not mentioned, **f.** pH 7.0, temperature not mentioned, **g.** pH 8.0, temperature not mentioned, **h.** pH 7, T = 35°C, an initial amount of ethanol results in an increase of *E*: 0% EtOH (v/v) ⇒ *E* = 5.5, 4% EtOH ⇒ *E* = 8.6, 10% EtOH ⇒ *E* = 8.1, 20% ⇒ *E* = 12.2 (see results in chapter 5 of this thesis), **i.** In the absence of $CaCl_2$ a low enantioselectivity (pH 8.0) and no enantioselectivity (pH 7.5) was observed, **j.** pH=8.0 + $CaCl_2$, room temperature.

1. Holmberg *et al.* (1991); 2. Wilson *et al.* (1983); 3. Sonnet and Welch Baillargeon (1991); 4. Sonnet (1993); 5. Heinsman *et al.* (1998); 6. Berglund *et al.* (1993).

Table 6: Overview of lipase-catalyzed acidolysis of branched alkanoic esters.

Acidolysis[f,i]			
Ester		***Acid***	***E***
2-Methylbutanoic acid	ethyl ester	Octanoic	6
	octyl ester	Octanoic	7
2-Methylhexanoic acid	ethyl ester	Octanoic	13
	ethyl ester	Cyclohexanoic	8
	ethyl ester	Phenylacetic	13
	ethyl ester	Dodecanoic	14
	ethyl ester	Octadecanoic	13
	ethyl ester	Oleic	12
	trifluoroethyl ester	Octanoic	15
	cyclohexyl ester	Octanoic	17
	octyl ester	Octanoic	52
	octyl ester	Oleic	49
	octadecyl ester	Octanoic	126
	octadecyl ester	Oleic	106

Table 6: Continued

Ester	*Acid*	*E*
Ethyl octanoate	2-Methylbutanoic	3
Ethyl octanoate	2-Methylhexanoic	6

‡ All reactions were catalyzed by *Candida rugosa* lipase (CRL) with a preference for the *S*-enantiomer, at room temperature using 0.5 mmol of ester and 0.5 mmol acid.

1. Engel *et al.* (1992)

It is clear from these tables that the reactions performed with 2-methylbutanoic acid show a low enantioselectivity in all cases. These reactions are independent of the alcohol or scale. In all reactions with other branched chain fatty acids or derivatives thereof the enantiomeric ratio is influenced by many parameters like substrate concentration, type of enzyme and alcohol. For the hydrolysis of methyloctanoic acid thiol esters for instance, one cannot draw any conclusion since upon increasing the distance between the ester group and the methyl group, the enantiomeric ratio slightly decreases, stays the same or shows an optimum dependent on the enzyme used. Thus, it is difficult to draw conclusions on forehand. It is clear though, that our best result for the esterification of 4-methyloctanoic acid ($E = 57$) is higher than expected. Maybe, for CALB and these kind of substrates, the enantioselectivity does not drop that dramatically upon increasing the distance between the chiral center and the carboxyl group.

For optimization of a reaction, aspects like the choice of a lipase, the use of free or immobilized lipase, the type of reaction, the concentrations of substrates, the chain length of the alcohol or alkyl chain, and the influence of water activity, pH and temperature can be studied in order to enhance the enantiomeric ratio. Since no computer program is available that predicts all these parameters, the only possibility is to review literature data in order to make assumptions for enzymatic reactions that still have to be done.

SUMMARY

The study towards the production of enantiopure 4-methyloctanoic acid, presented in this thesis, has resulted in several useful results:

1. A suitable lipase (Novozym 435®) for enantioselective (trans)esterification and hydrolysis of 4-methyloctanoic acid or derivatives has been found.
2. The enantiomeric ratio of the enzymatic reaction increases quite strongly with decreasing temperature (T = 45°C: E = 8, T = 27°C; E = 27).
3. A high enantiomeric ratio (E = 57 ± 11) was obtained for a 1:8 molar ratio of 4-methyloctanoic acid *versus* ethanol in small-scale batch reactions.
4. The enantiomeric ratio is the same for large-scale batch esterification.
5. The enantiomeric ratio in hydrolysis increased when ethanol was added. The optimal E (E=12) for hydrolysis was found in the presence of 20% (v/v) ethanol.
6. A good separation of the ester from the reaction mixture is possible using distillation, even when only small amounts of ester are present.
7. According to calculations, the ee_p in a repeated batch reaction is in all cases higher compared to the batch reaction.
8. In the experimental setup, the ee_p of both the batch and repeated batch reaction was approximately 80%. However, the enantiomeric ratio was much higher in the batch reaction (E = 57 compared to E = 15 in repeated batch).
9. The expectation that 4-methyloctanoic acid, having a remote chiral center, would only be resolved by lipases with low enantioselectivity, appeared to be not true.

These results can be used for optimization of the large-scale esterification of 4-methyloctanoic acid with ethanol, catalyzed by Novozym 435®.

REFERENCES

Ahmed, S.N., Kazlauskas, R.J., Morinville, A.H., Grochulski, P., Schrag, J.D. and Cygler, M. (1994). Enantioselectivity of *Candida rugosa* lipase toward carboxylic acids: a predictive rule from substrate mapping and x-ray crystallography. *Biocatalysis*, **9**, 209-225.

Akasaka, K., Meguro, H. and Ohrui, H. (1997). Enantiomeric separation of carboxylic acids having chiral centers remote from the carboxylic group by labelling with a chiral fluorescent derivatization reagent. *Tetrahedron Lett.*, **38**, 6853-6856.

Almeida, M.C., Ruivo, R., Maia, C., Freire, L., Sampaio, T.C. and Barreiros, S. (1998). Novozym 435 activity in compressed gases. Water activity and temperature effects. *Enzyme Microb. Technol.*, **22**, 494-499.

Balcão, V.M., Paiva, A.L. and Malcata, F.X. (1996). Bioreactors with immobilized lipases: State of the art. *Enzyme Microb. Technol.*, **18**, 329-416.

Berglund, P., Holmquist, M., Hedenström, E., Hult, K. and Högberg, H.-E. (1993). 2-Methylalkanoic acids resolved by esterification catalysed by lipase from *Candida rugosa*: Alcohol chain length and enantioselectivity. *Tetrahedron: Asymmetry*, **4**, 1869-1878.

Berglund, P., Holmquist, M., Hult, K. and Högberg, H.-E. (1995). Alcohols as enantioselective inhibitors in a lipase catalysed esterification of a chiral acyl donor. *Biotechnol. Lett.*, **17**, 55-60.

Berglund, P., Vörde, C. and Högberg, H.-E. (1994). Esterification of 2-methylalkanoic acids catalysed by lipase from *Candida rugosa:* enantioselectivity as a function of water activity and alcohol chain length. *Biocatalysis*, **9**, 123.

Brink, L.E.S., Tramper, J., Luyben, K.Ch.A.M. and van 't Riet, K. (1988). Biocatalysis in organic media. *Enzyme Microb. Technol.*, **10**, 736-743.

Chen, C-S., Fujimoto, Y., Girdaukas, G. and Sih, C. J. (1982) Quantitative analysis of biochemical kinetic resolutions of enantiomers. *J. Am. Chem. Soc.*, **104**, 7294-7299.

Davidson, B.S. and Schumacher, R.W. (1993). Isolation and synthesis of caprolactins A and B, new caprolactams from a marine bacterium. *Tetrahedron*, **49**, 6569-6574.

Descotes, G. and Querou, Y. (1968). Acylation de cyclohexanones substituées en 3. *Bull. Soc. Chim. France*, **8**,3395-3400.

Dietrich, A., Maas, B., Brand, G., Karl, V., Kaunzinger, A. and Mosandl, A. (1992a). Stereoisomeric flavor compounds, part LX: Diluted Modified Cyclodextrins as chiral stationary phases – The influence of the polysiloxane solvent. *J. High Res. Chrom.*, **15**, 769-772.

Dietrich, A., Maas, B., Messer, W., Bruche, G., Karl, V., Kaunzinger, A. and Mosandl, A. (1992b). Stereoisomeric flavor compounds, part LVIII: The use of heptakis (2,3-di-*O*-tert-butyldimethylsilyl)-β-cyclodextrin as a chiral stationary phase in flavor analysis. *J. High Res. Chrom.*, **15**, 590-593

Edlund, H., Berglund, P., Jensen, M., Hedenström, E. and Högberg, H.-E. (1996). Resolution of 2-methylalkanoic acids. Enantioselective esterification with long chain alcohols catalysed by *Candida rugosa* lipase. *Acta Chem. Scand.*, **50**, 666-671.

Engel, K.-H. (1991). Lipase-catalyzed enantioselective esterification of 2-methylalkanoic acids. *Tetrahedron: Asymmetry*, **2**, 165-168.

Engel, K.-H. (1992). Lipase-catalyzed enantioselective acidolysis of chiral 2-methylalkanoates. *JAOCS*, **69**, 146-150.

Franssen, M.C.R., Jongejan, H., Kooijman, H., Spek, A.L., Camacho Mondril, N.L.F.L., Boavida dos Santos, P.M.A.C. and Groot, de, Ae. (1996). Resolution of a tetrahydrofuran ester by *Candida rugosa* lipase (CRL) and an examination of CRL's stereochemical preference in organic media. *Tetrahedron: Asymmetry*, 7, 497-510.

Gandhi, N.N. (1997). Applications of lipase. *JAOCS*, **74**, 621-634.

Haeffner, F., Norin, T. and Hult, K. (1998). Molecular modeling of the enantioselectivity in lipase-catalyzed transesterification reactions. *Biophys. J.*, **74**, 1251-1262.

Heinsman, N.W.J.T, Orrenius, S.C., Marcelis, C.L.M., Sousa Teixeira, de, A., Franssen, M.C.R., Padt, van der, A., Jongejan, J.A. and Groot, de, Ae. (1998) Lipase mediated resolution of γ-branched chain fatty acid methyl esters. *Biocatal. Biotransform.* **16**, 145-162.

Högberg, H.-E., Berglund, P., Edlund, H., Fähgerhag, J., Hedenström, E., Lundh, M., Nordin, O., Servi, S. and Vörde, C. (1994). Biocatalysis as a useful tool in pheromone synthesis. Enantiomerically pure building blocks from baker's yeast reductions and enzyme catalysed resolutions. *Catalysis Today*, **22**, 591-606.

Högberg, H.-E., Hedenström, E., Wassgren, A.-B., Hjalmarsson, M., Bergström, G., Löfqvist, J., and Norin, T. (1990). Synthesis and gas chromatographic separation of the eight stereoisomers of diprionol and their acetates, components of the sex pheromone of pine sawflies. *Tetrahedron*, **8**, 3007-3018.

Holmberg, E., Holmquist, E., Berglund, P., Norin, T., Högberg, H.-E. and Hult, K. (1991). Reaction conditions for the resolution of 2-methylalkanoic acids in esterification and hydrolysis with lipase from *Candida cylindracea*. *Appl. Microbiol. Biotechnol.*, **35**, 572-578.

Kazlauskas, R.J., Weissfloch, A.N.E., Rappaport, A.T. and Cuccia, L.A. (1991). A rule to predict which enantiomer of a secondary alcohol reacts faster in reactions catalyzed by cholesterol esterase, lipase from *Pseudomonas cepacia*, and lipase from *Candida rugosa*. *J. Org. Chem.*, **56**, 2656-2665.

Knez, Z., Habulin, M. and Krmelj, V. (1998). Enzyme catalyzed reactions in dense gases. *J. Supercrit. Fluids*, **14**, 17-29.

Laane, C., Boeren, S., Vos, K. and Veeger, C. (1987). Rules for optimization of biocatalysis in organic solvents. *Biotechnol. Bioeng.*, **30**, 81-87.

Lamare, S. and Legoy, M-D. (1993). Biocatalysis in the gas phase. *TIBTECH*, **11**, 413-418.

Lamare, S. and Legoy, M-D. (1995). Working at controlled water activity in a continuous process: the gas/solid system as solution. *Biotechnol. Bioeng.*, **45**, 387-397.

Lundh, M., Smitt, O. and Hedenström (1996). Sex pheromone of pine sawflies: enantioselective lipase catalysed transesterification of *erythro*-3,7-dimethylpentadecan-2-ol, diprionol. *Tetrahedron: Asymmetry*, **7**, 3277-3284.

Michor, H., Marr, R., Gamse, T., Schilling, T., Klingsbichel, E. and Schwab, H. (1996). Enzymatic catalysis in supercritical carbon dioxide: comparison of different lipases and a novel esterase. *Biotechnol. Lett.*, **18**, 79-84.

Nguyen, B.-V. and Hedenström, E. (1999). *Candida rugosa* lipase as an enantioselective catalyst in the esterification of methyl branched carboxylic acids: resolution of *rac*-3,7-dimethyl-6-octenoic acid (citronellic acid). *Tetrahedron: Asymmetry*, **10**, 1821-1826.

Orrenius, C., Heusden, van, C., Ruiten, J., Overbeeke, P.L.A., Kierkels, H., Duine, J.A. and Jongejan, J.A. (1998). Simple conformation space search protocols for the evaluation of enantioselectivity of lipases. *Protein Eng.*, **11**, 1147-1153.

Overbeeke, P.L.A., Orrenius, S.C., Jongejan, J.A. and Duine, J.A. (1998). Enthalpic and entropic contributions to lipase enantioselectivity. *Chem. Phys. Lipids*, **93**, 81-93.

Overmeyer, A., Schrader-Lippelt, S., Kasche, V. and Brunner, G. (1999). Lipase-catalysed kinetic resolution of racemates at temperatures from 40°C to 160°C in supercritical CO_2. *Biotechnol. Lett.*, **21**, 65-69.

Parvaresh, F., Robert, H., Thomas, D. and Legoy, M-D. (1992). Gas phase transesterification reactions catalyzed by lipolytic enzymes. *Biotechnol. Bioeng.*, **39**, 467-473.

Prazeres, D.M.F. and Cabral, J.M.S. (1994). Enzymatic membrane bioreactors and their applications. *Enzyme Microb. Technol.*, **16**, 738-750.

Rantakylä, M. and Aaltonen, O. (1994). Enantioselective esterification of ibuprofen in supercritical carbon dioxide by immobilized lipase. *Biotechnol. Lett.*, **16**, 825-830.

Ross, N.W. and Schneider, H. (1991). Activities of *Candida rugosa* lipase and other esterolytic enzymes coated on glass beads and suspended in substrate and water vapor: enzymes in thin liquid films. *Enzyme Microb. Technol.*, **13**, 370-377.

Rottici, D., Haeffner, F., Orrenius, C., Norin, T., Hult, K. (1998). Molecular recognition of *sec*-alcohol enantiomers by *Candida antarctica* lipase B. *J. Mol. Cat. B: Enzymatic*, **5**, 267-272.

Russell, A.J. and Yang, F.X. (1996). Catalyze gas-phase reactions with enzymes. *CHEMTECH*, **26**, 24-27.

Sereti, V., Stamatis, H. and Kolisis, F.N. (1997). Improved stability and reactivity of *Fusarium solani* cutinase in supercritical CO_2. *Biotechnol. Tech.*, **11**, 661-665.

Sonnet, P.E. (1993). Lipase-catalyzed hydrolysis: effect of alcohol configuration on the stereobias for 2-methyloctanoic acid. *J. Agric. Food Chem., 41, 319-321.*

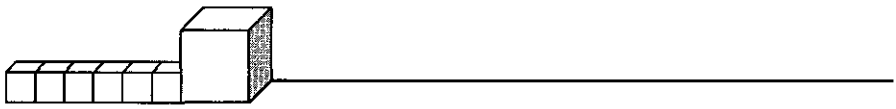
Sonnet, P.E., Gazzillo, J.A., Dudley, R.L. and Boswell, R.T. (1990). Synthesis and characterization of enantiomers of 5-methyloctanoic acid and 6-methyloctanoic acid. *Chem. Phys. Lipids*, **54**, 205-214.

Sonnet, P.E. and Welch Baillargeon, M. (1991). Methyl-branched octanoic acids as substrates for lipase-catalyzed reactions. *LIPIDS*, **26**, 295-300.

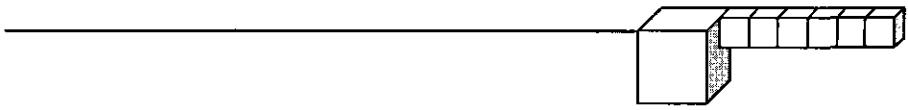
Tsitsimpikou, C., Stamatis, H., Sereti, V., Daflos, H. and Kolisis, F.N. (1998). Acylation of glucose catalysed by lipases in supercritical carbon dioxide. *J. Chem. Technol. Biotechnol.*, **71**, 309-314.

Weiss, J. (1995). *Ion chromatography*, 2nd ed., Weinheim New York, USA, 1-465.

Wilson, W.K., Baca, S.B., Barber, Y.J., Scallen, T.J. and Morrow, C.J. (1983). Enantioselective hydrolysis of 3-hydroxy-3methylalkanoic acid esters with pig liver esterase. *J. Org. Chem.*, **48**, 3960-3966.



SUMMARY

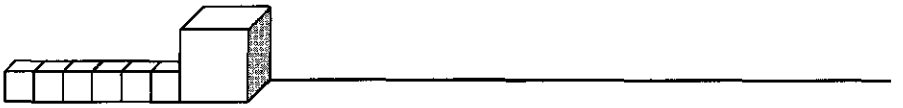


Branched chain fatty acids (BCFAs) and their derivatives are broadly distributed natural components. They occur in fruit, tobacco leaves and flowers, and make an important contribution to the taste and smell of mutton and sheep's cheese. Because of the branching, many of these compounds are chiral. Since the stereoisomers often have different sensory properties, flavor and fragrance industries have an interest in the food grade production of the separate enantiomers (BCFAs). The research described in this thesis was aimed at the design of a general scalable production process for enantiopure branched chain fatty acids. 4-Methyloctanoic acid was used as model compound and a lipase as a stereoselective catalyst for the separation of the enantiomers. In 4-methyloctanoic acid, the chiral center is relatively remote from the reaction center. This might be a reason that the chiral recognition by the enzyme leaves to be desired. A two-step process might therefore be a useful approach. In the first step of this so called sequential kinetic resolution, the substrate is esterified to give an enantiomerically enriched ester. In the second step, this enantiomerically enriched product is hydrolyzed. Since the enzymatic resolution proceeds through two consecutive steps, the substrate is forced to enter the active site of an enzyme twice, resulting in an increased enantiomeric excess of the product.

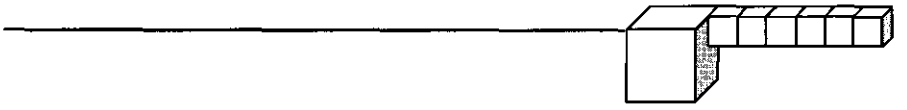
In *chapter one* a general introduction is given concerning flavors and fragrances, branched chain fatty acids, lipases and their use in the separation of racemates (kinetic resolution). *Chapter two* describes the screening of lipases in the kinetic resolution of the methyl esters of 4-methylhexanoic acid and 4-methyloctanoic acid by means of transesterification with n-butanol in octane. Only one enzyme, *viz. Candida antarctica* lipase B (Novozym 435®) showed both a good conversion and a good enantioselectivity. At 27°C an enantiomeric ratio of 23 could be obtained for 4-methyloctanoic acid methyl ester. Molecular modeling studies illustrated the preference of *Candida antarctica* lipase B for the *R*-enantiomer.
Candida antarctica lipase B was subsequently used to esterify 4-methyloctanoic acid with polyethylene glycols (PEGs) of different molecular weights, at different water activities and with different additives (*chapter three*). For the use of PEG in a membrane reactor, it was desirable to have the PEG-ester in the water phase and to have a stable biphasic system. In spite of the unique character of PEG (esterification was possible at any water activity), it appeared to be impossible to come up to these conditions. For that reason, another food grade alcohol, *viz.* ethanol, was used for the esterification reactions. Estimations of the enantiomeric ratio in the Novozym 435® catalyzed esterification of 4-methyloctanoic acid with ethanol, at different substrate concentrations, revealed data that could not be correctly fitted to the Chen equation for equilibrium reactions (*chapter four*). It was proven that substrate sorption into the polymer matrix of Novozym 435® affected the enantiomeric ratio estimation. After correction for substrate sorption, reliable fits could be obtained. An optimum enantiomeric ratio ($E = 57 \pm 11$) was observed for a 1 to 8 ratio of 4-methyloctanoic acid *versus* ethanol (mol:mol). In *chapter four* and *five* it is shown that ethanol has a great influence on the enantioselectivity of the enzyme. In both esterification and hydrolysis, the increase of ethanol led to an increase in enantiomeric ratio. The hydrolysis of 4-methyloctanoic acid ethyl ester in the absence of ethanol resulted in a low enantioselectivity ($E = 5.5 \pm 2.5$) while an initial amount of 20% ethanol (v/v) resulted in an increase to $E = 12.2 \pm 0.9$. When combining the results of esterification and hydrolysis a very good enantioselectivity can be obtained after two consecutive reaction steps ($E \cong 350$).

Integration of kinetic resolution and product separation was studied in *chapter six*. A comparison was made between the esterification of 4-methyloctanoic acid with ethanol in a batch reactor and in a repeated batch reactor. In the batch esterification (ratio 1:8 mol acid:mol ethanol) both the substrate and the product could be obtained with a high *ee* after distillation (c = 55%, ee_s = 93%, ee_p = 81 %, E = 57). Since an excess of ethanol appeared to be unfavorable for the integration of reaction and separation, the repeated batch reaction was performed using a ratio of 10:1 (mol acid:mol ethanol). As a result, after six steps, the enzymatic reaction turned out to be much less enantioselective (c = 60%, ee_s = 60%, ee_p = 80 %, E =15).

From all the obtained results, it can be concluded that the production of enantiopure 4-methyloctanoic acid leads to the best results in a batchwise esterification reaction with ethanol (molratio 1:8 (acid:EtOH)), using Novozym 435® as biocatalyst, at a temperature of 35°C.
The reaction should then be stopped at a conversion of 55% after which the product can be isolated using distillation. Provided that the *R*-acid is desired with a very high enantiomeric excess, a reaction can be performed in the presence of the same enzyme, at the same temperature and in the presence of 20% (v/v) ethanol.



SAMENVATTING



Vertakte vetzuren en hun derivaten zijn natuurstoffen met een wijde verspreiding. Zij komen voor in fruit, tabaksbladeren en bloemen en spelen een belangrijke rol bij de geur en smaak van schapenkaas en schapenvlees. Vanwege hun vertakking zijn het meestal chirale verbindingen. Omdat de stereisomeren vaak verschillende sensorische eigenschappen hebben, bestaat er interesse vanuit de geur- en smaakstoffenindustrie voor een 'food grade' bereidingswijze van de afzonderlijke enantiomeren. Het onderzoek beschreven in dit proefschrift was gericht op het ontwerpen van een algemeen, schaalbaar productieproces voor enantiomeer zuivere vertakte vetzuren. Hierbij is 4-methyloctaanzuur als modelstof genomen en is een lipase gebruikt als stereoselectieve katalysator voor de scheiding van de enantiomeren. In 4-methyloctaanzuur is het chiraal centrum relatief ver verwijderd van het reactiecentrum. Dit zou een reden kunnen zijn dat de chirale herkenning door het enzym te wensen overlaat. Een tweestaps proces zou dan een mogelijkheid kunnen zijn om de gewenste enantiomeerzuivere producten te verkrijgen. In de eerste stap van deze zogenoemde sequentiële kinetische resolutie, wordt het substraat veresterd zodat een enantiomeer verrijkte ester wordt gevormd. In de tweede stap wordt dit enantiomeer verrijkte product gehydrolyseerd. Aangezien de enzymatische resolutie in twee opeenvolgende

stappen verloopt, wordt het substraat gedwongen om twee keer de 'active site' van het enzym in te gaan, resulterende in een verhoogde enantiomere overmaat.

In *hoofdstuk één* wordt een algemene inleiding gegeven over geur- en smaakstoffen, vertakte vetzuren, lipasen en de scheiding van racematen hiermee (kinetische resolutie). *Hoofdstuk twee* beschrijft de screening van lipasen voor de kinetische resolutie van de methylesters van 4-methylhexaanzuur en 4-methyloctaanzuur d.m.v. omestering met n-butanol in octaan. Slechts één enzym, nl. *Candida antarctica* lipase B (Novozym 435®) vertoont zowel een goede conversie als een goede enantioselectiviteit. Voor 4-methyloctaanzuurmethylester is een enantiomere ratio gevonden van 23, bij 27°C. Het enzym heeft een voorkeur voor de *R*-isomeer, hetgeen bevestigd wordt m.b.v. molecular modelling studies.

Candida antarctica lipase B is vervolgens gebruikt voor de verestering van 4-methyloctaanzuur met polyethyleenglycolen (PEGs) van verschillend moleculair gewicht, bij verschillende wateractiviteiten en met verschillende toevoegingen (*hoofdstuk drie*). Voor het gebruik van PEG in een membraanreactor was het gewenst om een stabiel tweefasen systeem te creëren waarbij de PEG-ester zich in de waterfase zou moeten bevinden. Ondanks het unieke karakter van PEG (verestering was mogelijk bij iedere wateractiviteit) bleek het onmogelijk aan deze voorwaarde te voldoen.

Naar aanleiding van deze resultaten is een ander 'food-grade' alcohol (ethanol) gebruikt voor de veresteringsreactie. Voor de Novozym 435®-gekatalyseerde verestering van 4-methyloctaanzuur met ethanol bij verschillende substraat concentraties werd de enantiomere ratio bepaald. Deze *E*-waarden lieten zien dat de data niet goed gefit konden worden aan de Chen vergelijking voor evenwichtsreacties (*hoofdstuk vier*). Er is aangetoond dat sorptie van het substraat in de polymeer matrix van Novozym 435® invloed had op de bepaling van de enantiomere ratio. Na correctie voor substraat sorptie werden goede fits verkregen. Er werd een optimum voor de enantiomere ratio ($E = 57 \pm 11$) gevonden bij een ratio van 1:8 (mol 4-methyloctaanzuur:mol ethanol). In *hoofdstuk vier* en *vijf* is aangetoond dat ethanol een grote invloed heeft op de enantioselectiviteit van het enzym. In zowel de verestering van het zuur als de hydrolyse van de overeenkomstige ester leidde de toename van de ethanolconcentratie tot een

toename in de enantiomere ratio. In afwezigheid van ethanol resulteerde de hydrolyse van 4-methyloctaanzuur ethylester in een lage enantioselectiviteit. (E = 5.5 ± 2.5) terwijl een toename voor E te zien is tot een waarde van E = 12.2 ± 0.9 bij aanwezigheid van een initiële hoeveelheid van 20% ethanol (v/v). Als de resultaten van de verestering en hydrolyse gecombineerd worden kan een zeer hoge enantioselectiviteit worden verkregen na twee opeenvolgende stappen ($E \cong$ 350).

Integratie van kinetische resolutie en productscheiding is bestudeerd in *hoofdstuk zes*. Een vergelijking is gemaakt tussen de verestering van 4-methyloctaanzuur met ethanol in een batch reactor en in een 'repeated' batch reactor. Na destillatie konden zowel het substraat als het product in de batch verestering (ratio 1:8 mol zuur: mol EtOH) met een hoge *ee* verkregen worden (c = 55%, ee_s = 93%, ee_p = 81%, E = 57). Omdat een overmaat ethanol ongunstig bleek te zijn wanneer reactie en productverwijdering geïntegreerd zouden worden is de 'repeated batch' verestering uitgevoerd bij een ratio van 10:1 (mol zuur: mol EtOH). Een gevolg hiervan was dat na zes stappen de enzymatische reactie veel minder enantioselectief was (c = 60%, ee_s = 60%, ee_p = 80%, E = 15).

Uit alle verkregen resultaten kan geconcludeerd worden dat de productie van enantiomeerzuiver 4-methyloctaanzuur de beste resultaten oplevert wanneer een batchgewijze reactie wordt uitgevoerd met ethanol (molratio 1:8 (zuur:EtOH)), Novozym 435® als biokatalysator, bij een temperatuur van 35°C. De reactie dient dan bij een conversie van 55% te worden beëindigd waarna het product kan worden geïsoleerd met behulp van destillatie. Indien een hogere enantiomere overmaat gewenst is voor het *R*-zuur, kan een reactie worden uitgevoerd in aanwezigheid van hetzelfde enzym, bij dezelfde temperatuur en in aanwezigheid van 20% (v/v) ethanol.

LIST OF PUBLICATIONS

Thuring, J.W.J.F., Heinsman, N.W.J.T., Jacobs, R.W.A.W.M., Nefkens, G.H.L. and Zwanenburg, B. (1997) Asymmetric synthesis of all stereoisomers of demethylsorgolactone. Dependence of the stimulatory activity of *Striga hermonthica* and *Orobanche crenata* seed germination on the absolute configuration. *J. Agric. Food Chem.* Vol. 45, p. 507 - 513.

Heinsman, N. and Belov, Y. (1997) Enantiomeric analysis of 4-methyloctanoic acid by chiral gas chromatography, *The Reporter*, **16**, 9.

Heinsman, N.W.J.T, Orrenius, S.C., Marcelis, C.L.M., Sousa Teixeira, de, A., Franssen, M.C.R., Padt, van der, A., Jongejan, J.A. and Groot, de, Ae. (1998) Lipase mediated resolution of γ-branched chain fatty acid methyl esters, *Biocatal. Biotransform.*, **16**, 145-162.

Heinsman, N.W.J.T. Teixeira, A., Weide, van der, P.L.J., Franssen, M.C.R., Padt, van der, A., Groot, de, Ae. and Riet, van 't, K.. Esterification of 4-methyloctanoic acid with polyethylene glycol at different a_w. Submitted for publication in *Biocatalysis and Biotransformation.*

Heinsman N.W.J.T., Padt, van der, A., Franssen, M.C.R., Boom, R.M., Groot, de, Ae., Riet, van 't, K.. Substrate sorption into the polymer matrix of Novozym 435® and its effect on the enantiomeric ratio estimation. Submitted for publication in *Biotechnology and Bioengineering.*

Heinsman, N.W.J.T., Valente, A.M., Smienk, H.G.F., Padt, van der, A., Franssen, M.C.R., Groot, de, Ae. and Riet, van 't, K.. The effect of ethanol on the kinetics of lipase-mediated enantioselective esterification and hydrolysis. Submitted for publication in *Biotechnology and Bioengineering.*

Heinsman, N.W.J.T., Weide, van der, P.L.J., Padt, van der, A., Franssen, M.C.R., Groot, de, Ae. and Riet, van 't, K.. Reactor design for the Novozym 435®-catalyzed enantioselective esterification of 4-methyloctanoic acid. To be published.

CURRICULUM VITAE

Nicole Heinsman werd geboren op 12 juli 1969 in Goirle. In 1986 behaalde ze haar HAVO diploma aan de R.K. Scholengemeenschap Nijmegen-West waarna ze haar studie heeft voortgezet aan het Dominicus College te Nijmegen. In 1988 heeft ze haar middelbare schoolperiode afgerond met het VWO diploma. In datzelfde jaar werd begonnen met een studie scheikunde aan de Katholieke Universiteit Nijmegen. Tijdens haar studie is ze in 1991 begonnen met een uitgebreide hoofdvakstage bij de vakgroep biochemie (begeleiding: Jan van Groningen en Guido Swaart). Een tweede uitgebreide hoofdvakstage heeft zij gevolgd bij de vakgroep organische chemie onder begeleiding van Jan-Willem Thuring, Gérard Nefkens en professor Zwanenburg (1992-1994). In 1994 is zij tien weken naar Grinnell (Grinnell college, Grinnell Iowa, USA, prof. Mary M. Mader) geweest dankzij de KNCV prijs die zij in 1993 had ontvangen. Tijdens haar studie heeft ze diverse practica begeleid, de open dagen voor VWO-scholieren en de herfstcursussen georganiseerd. In april 1995 is zij begonnen als AIO aan de voormalige Landbouwuniversiteit Wageningen (nu Wageningen Universiteit). Vanaf april 1995 tot juni 1997 was zij werkzaam op de vakgroep organische chemie. In de periode juni 1997 tot april 1999 werkte ze op de afdeling proceskunde. Sinds 1 januari 2000 is zij werkzaam als training manager bij Quest International te Naarden.