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Exploration of Lipase-catalyzed Direct Amidation of Free Carboxylic Acids with Ammonia in Organic Solvents

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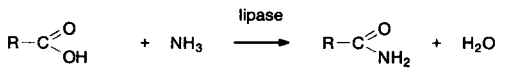
Abstract: Lipase-catalyzed direct amidation of free carboxylic acids is possible with ammonia in organic solvents. For butyric acid as a model compound the reaction proceeds well despite precipitation of ammonium butyrate, provided that the added molar amounts of butyric acid and ammonia are in the same range. The addition of ammonium salts is a convenient way to ensure suitable ammonia concentrations. Using *Candida antarctica* lipase B as the biocatalyst, the amidation proceeds well for various carboxylic acids and is very enantioselective in the amidation of 4-methyloctanoic acid.

INTRODUCTION

Amides are important derivatives of several types of carboxylic acids. Fatty acid amides are used as lubricants in the plastics industry and they are produced in thousands of metric tons per year.[1] Oleamide has recently been described as a sleep inducing drug.[2] Enantioselective amidation of chiral acids may be used as an alternative to esterification in kinetic resolution processes. In order to generate sensitive amides such as oleamide without degradation, and to perform enantioselective amidation, enzymatic methods are being developed. Secondary amides have been obtained using lipases in the aminolysis of esters[3] or the amidation of free carboxylic acids with amines.[4] Enzymatic ammoniolysis of esters has been reported by De Zoete et al.[5] For direct amidation of free carboxylic acids with ammonia, however, only harsh chemical conditions (200 °C, 7 bar anhydrous ammonia)[1] have been described, because in an organic solvent at low temperature the carboxylic acids were expected to precipitate as ammonium salts, making them unavailable to an enzyme.[5,6] In a recent report[7] we have challenged this commonly held belief by demonstrating that butyramide and oleamide can be synthesized by direct lipase-catalyzed reaction of the carboxylic acids with ammonia in an organic solvent (Scheme 1). The present paper explores the conditions for successful direct enzymatic amidation of carboxylic acids. Suitable conditions are used for the amidation of various carboxylic acids. A convenient procedure for the lipase-catalyzed synthesis of oleamide from oleic acid and ammonia is presented.



Scheme 1.

THEORETICAL

A favorable reaction combines a short reaction time and a high yield. In order to obtain a suitable reaction rate in the amidation of a carboxylic acid with ammonia, it is important that both substrates are present in reasonable concentrations. For an equilibrium-controlled amidation a high yield requires a large excess of ammonia, for example by saturating the organic solvent with ammonia. However, under such conditions it is likely that most of the acid will precipitate as its ammonium salt, which means that the dissolved acid concentration will be very low. As a result, the reaction rate will be low. The solubility of ammonium butyrate

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in methyl isobutyl ketone (MIBK) at 25 °C can be described by its solubility product:[8]

$$K_{sol} = c_{ammonia} \cdot c_{acid} \quad (1)$$

where K_{sol} = 1.35·10^{-4} mol^2·l^{-2}, which corresponds to a solubility of 11.6 mmol·l^{-1}. Figure 1 gives a graphical representation of Equation (1), which makes it clear that high concentrations of dissolved ammonia lead to low concentrations of dissolved butyric acid. For example, if MIBK is saturated with ammonia ($c_{ammonia}$ = 230 mmol·l^{-1})[8] then the dissolved butyric acid concentration is far less than 1 mmol·l^{-1}. This will cause the enzymatic reaction to be very sluggish.

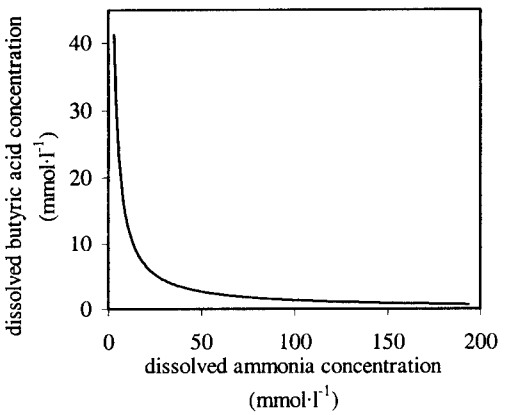

Fig. 1. Calculated dissolved butyric acid concentration as a function of the dissolved ammonia concentration in dry MIKB at 25 °C. The curve was calculated using the solubility product K_{sol} = 1.35·10^{-4} mol^2·l^{-2}.

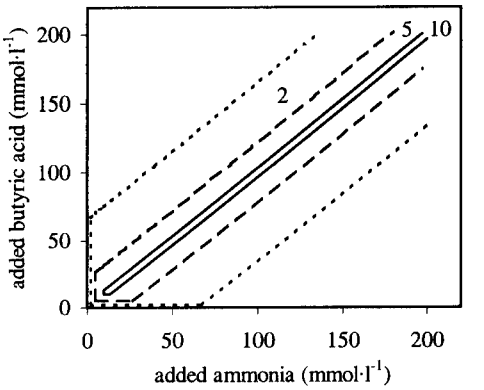

Fig. 2. Window of operation for direct enzymatic amidation of butyric acid with ammonia in dry MIBK at 25 °C. The areas within the drawn lines indicate conditions where the dissolved concentration of the least abundant substrate is larger than 2 mmol·l^{-1} (···), 5 mmol·l^{-1} (---), and 10 mmol·l^{-1} (—), respectively.

From Equation (1) in combination with mass balances over ammonia and butyric acid it can be calculated that relatively small differences between the added amounts of these substrates may cause the ratio of the dissolved substrate concentrations to deviate strongly from unity. This means that the dissolved concentration of the least abundant substrate is very low. For example, if the concentration of the least abundant substrate in solution is to be kept above 10 mmol·l^{-1}, then the range of substrate amounts to choose from is very limited (Figure 2). If a minimum substrate concentration of 5 mmol·l^{-1} is sufficient, then the window of operation is larger. For a minimum concentration as low as 2 mmol·l^{-1} the window of operation is large but certainly not unlimited, as indicated. For other reaction systems, with a different value of K_{sol}, the windows of operation are larger (for a higher value of K_{sol}) or smaller (for a lower value of K_{sol}).

The reaction system where 50 mmol·l^{-1} ammonia and 50 mmol·l^{-1} butyric acid are added to dry MIBK at 25 °C is situated within the narrowest window of operation depicted in Figure 2. The dissolved concentration of both substrates is 11.6 mmol·l^{-1}, the rest is precipitated as ammonium butyrate. If the reaction is started by adding enzyme, the disappearance of both substrates from the solution is replenished by the dissolution of ammonium butyrate. Since the molar ratio of the substrates in the ammonium salt is equal to the stoichiometry of the reaction, the ratio of the dissolved substrate concentrations remains at the initial value.

The predictions regarding the influence of differences in the available molar amounts of ammonia and

carboxylic acid on the dissolved concentrations, and the reaction rate, are verified experimentally for the enzymatic amidation of butyric acid.

RESULTS AND DISCUSSION

Influence of the supply of ammonia on the course of the enzymatic amidation

Figure 3 shows the influence of the concentration of ammonia on the initial rate of butyramide formation. The reactions were performed in dry MIBK at 25 °C, and precipitation of ammonium butyrate was observed before addition of immobilized *Candida antarctica* lipase (CALB) as the biocatalyst. It is striking that increased concentrations of ammonia decrease the reaction rate. This observation can be explained by an increased precipitation of ammonium butyrate leading to a decreased concentration of dissolved butyric acid, assuming that the enzymatic reaction rate is not determined by the dissolved ammonia concentration but by the dissolved butyric acid concentration. This explanation is confirmed by the linear relation of the initial reaction rate (as determined from the slope of the concentration of butyramide versus time) with the measured concentrations of dissolved butyric acid (legend of Figure 3). We are aware that other explanations for the decrease in reaction rate at increased concentrations of ammonia are possible. For example, high concentrations of ammonia may slow down the enzymatic reaction due to substrate inhibition or inactivation of the enzyme. However, the activity of CALB in the ammoniolysis of esters is not decreased by the presence of even 2.5 mol·l^{-1} ammonia in *tert*-butanol.[9] The ionisation state of the substrates, too, may have an influence on the reaction rate. Maugard et al.[10] report that the reaction rate in the CALB-catalyzed amidation of oleic acid with *N*-methylglucamine in organic solvents decreases at acid/amine ratios higher than 1, because the medium is acidic and the amino group is mainly in its unreactive protonated form. In these experiments

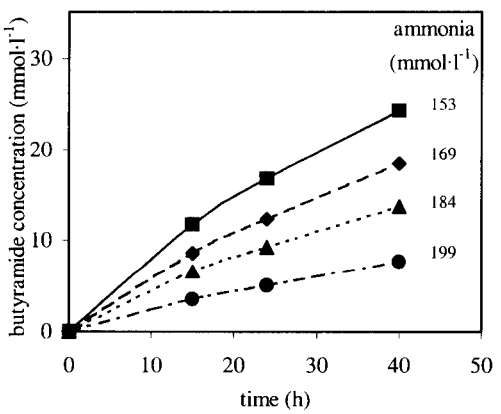

Fig. 3. Dissolved concentrations of butyramide in the early stage of the formation of butyramide from 150 mmol·l^{-1} butyric acid and various initial concentrations of ammonia. The measured concentrations of dissolved butyric acid (averages of three samples at 15, 24, and 40 hours reaction time) for the initial ammonia concentrations of 153, 169, 184, and 199 mmol·l^{-1} are 11.6, 10.3, 9.1, and 8.0 mmol·l^{-1}, respectively. The reactions were performed in 30 ml dry MIBK at 25 °C with 45 mg immobilized CALB.

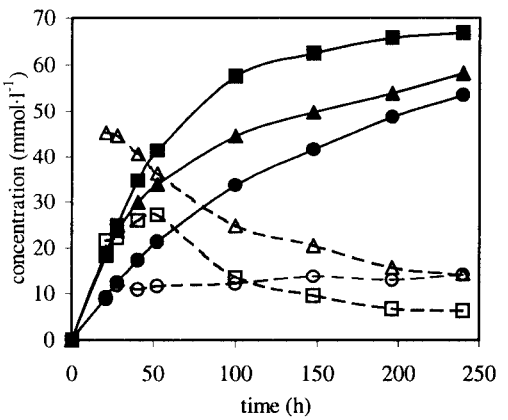

Fig. 4. Measured concentrations of butyramide (filled markers) and dissolved butyric acid (open markers) for the CALB-catalyzed amidation of 70 mmol·l^{-1} butyric acid with various sources of ammonia: 57.5 mmol·l^{-1} ammonium carbamate (■,□), 115 mmol·l^{-1} ammonium bicarbonate (▲,△), and 115 mmol·l^{-1} dissolved ammonia gas (●,○). The reactions have been performed in 20 ml dry MIBK at 25 °C with 24 mg immobilized CALB.

precipitation of the reactants was avoided by using a relatively polar solvent (*tert*-amylalcohol) at 90 °C. Similarly, the ionisation state of butyric acid and ammonia may also play a role in our reaction system, but we expect that their influence is of minor importance. From our results the importance of the dissolved butyric acid concentration is, however, clear.

The measured butyric acid concentrations (Figure 3) are higher than expected from calculations using the solubility product of ammonium butyrate (Equation (1)) and mass balances over butyric acid and ammonia. This may be explained by an increased solubility of ammonium butyrate in the presence of reactants such as butyramide and water. In addition, the equilibration between solid and liquid may be slow.

It is expected that if the ammonia is made available gradually during the reaction instead of all added instantaneously at the start, then the dissolved ammonia concentration will be lower and less butyric acid will precipitate. This should lead to increased reaction rates. To verify this, a reaction of 70 $mmol \cdot l^{-1}$ butyric acid with ammonia in MIBK was performed with three different sources of ammonia, each with the same total amount (i.e. 115 $mmol \cdot l^{-1}$) of ammonia present in the reaction system. In two experiments ammonia was added as a solid ammonium salt (ammonium bicarbonate and ammonium carbamate, respectively) which dissolved slowly during the reaction. In the third experiment ammonia was added instantaneously as a dissolved gas. Figure 4 shows that the rate of formation of butyramide is indeed significantly higher in the experiments where ammonia is dissolved gradually than in the experiment where all ammonia was dissolved initially. The gradual release of ammonia apparently creates a situation where the combination of ammonia and butyric acid concentrations is more favorable. The experiment with 115 $mmol \cdot l^{-1}$ ammonia in the form of ammonium carbamate was repeated with an extra addition of 115 $mmol \cdot l^{-1}$ dissolved ammonia gas. The reaction rate decreased dramatically, confirming that high concentrations of ammonia slow down the reaction instead of accelerating it (result not shown). The yield of butyramide on butyric acid is higher with ammonium carbamate than with ammonium bicarbonate as the source of ammonia. This can be explained by the release of water upon dissolution of ammonium bicarbonate, which limits the equilibrium conversion of butyric acid to butyramide.[7,8] With ammonium carbamate the yield of butyramide from butyric acid is 96 %.

Similar to the data collected in Figure 3, the measured concentrations of butyric acid are somewhat higher than expected. A mass balance between butyric acid and butyramide indicates that with ammonium bicarbonate as the source of ammonia there is hardly any precipitated ammonium butyrate during the course of the reaction. The ammonia that is released by the dissolution of ammonium bicarbonate and the butyric acid clearly react rather than precipitate. With ammonium carbamate as the source of ammonia, a mass balance between butyric acid and butyramide indicates that during the first 50 hours of the reaction some precipitated ammonium butyrate may be present. The dissolved butyric acid concentration increases slightly within this time interval (possibly because of an increased polarity of the reaction medium), and decreases after all the ammonium butyrate has dissolved.

Enzymatic amidation of other carboxylic acids

As the enzymatic amidation of butyric acid with ammonia proceeds with good yields, the scope of this reaction was investigated in a broader sense. Table 1 presents an overview of the results that have been obtained for various reaction systems. Ammonium carbamate was used as the source of ammonia, except for the preparation of acetamide where ammonium acetate provided both ammonia and acetic acid. A variety of other carboxylic acids are efficiently converted into their amides. The amidations of 2-chloropropionic acid and phenylacetic acid proceeded sluggishly at 35 °C, which may be due to their low pK_a values (which are 2.8 and 4.3, respectively; the pK_a of the other acids is approximately 4.8). This may mean that an increased

fraction of the acid is present in the carboxylate form, leading to increased precipitation of the ammonium salt. Also, these two carboxylic acids are not very good substrates for CALB. At 60 °C the reaction proceeded better, probably due to a better dissolution and a higher enzyme activity. Immobilized CALB is highly stable even at 60 – 80 °C.[11] *Candida rugosa* lipase (CRL) and porcine pancreas lipase (PPL) were also used for the amidation of oleic acid but gave poor results at 35 °C. These enzymes were not tested any further.

Table 1. Enzymatic Amidation of various Carboxylic Acids

substrate	enzyme [a)]	temperature (°C)	time (days)	yield (%)
acetic acid [b)]	CALB	35	3	98
butyric acid	CALB	35	3	91
oleic acid	CALB	35	3	94
oleic acid	CRL [c)]	35	7	2
oleic acid	PPL [c)]	35	7	4
oleic acid	none	60	7	0
2-chloropropionic acid	CALB	35	3	5
2-chloropropionic acid	CALB	60	3	24
phenylacetic acid	CALB	35	4	10
phenylacetic acid	CALB	60	5	87
(*rac*)-4-methyloctanoic acid	CALB	35	3	52[d)]

Standard reaction conditions: 50 mmol·l^{-1} carboxylic acid in 25 ml dry MIBK, 1.3 mmol solid ammonium carbamate, 25 mg CALB.

[a)] CALB = *Candida antarctica* lipase B, CRL = *Candida rugosa* lipase, PPL = pig pancreas lipase

[b)] substrate is ammonium acetate, no ammonium carbamate added

[c)] 100 mg enzyme

[d)] enantiomeric excess[12] of remaining (*S*)-acid = 95 %

The CALB-catalyzed amidation of racemic 4-methyloctanoic acid in MIBK at 35 °C is very enantioselective towards the (*R*)-enantiomer. The enantiomers of the product were not sufficiently separated on GC. For this reason the enantiomeric ratio[12] E was calculated from the enantiomeric excess of the remaining substrate versus the degree of conversion for 5 data points at less than 45 % conversion (because of the reversibility of the reaction) using the program SimFit.[13] The calculated value is $E = 76$. This means that the CALB-catalyzed amidation of 4-methyloctanoic acid is much more enantioselective than the transesterification of its methyl ester to the butyl ester by the same enzyme (E = 23 in octane at 27 °C).[14] Figure 5 shows the course of the enantiomeric excess of the (*S*)-acid during this reaction. Under the applied experimental conditions the (*S*)-acid could not be obtained in an enantiomeric excess of more than 95 % as a result of the reaction equilibrium. Upon addition of 3 Å molecular sieves to remove the water that was formed and thereby improve the reaction equilibrium, a non-selective chemical amidation reaction took place. No further options for improving the reaction equilibrium were tested. Still, the enzymatic amidation with ammonia may be an interesting method for the synthesis of enantiomerically pure branched chain fatty acids and their amides.

Amino acids and peptides are also interesting targets for amidation, but without protection of the amino group the solubility of these compounds in organic solvents will be much less than that of unsubstituted carboxylic acids. For this reason we do not think that our system will be suitable for direct amidation of amino

acids and peptides. Low to moderate yields have been achieved with protected dipeptides in acetonitrile containing 5 % water.[15]

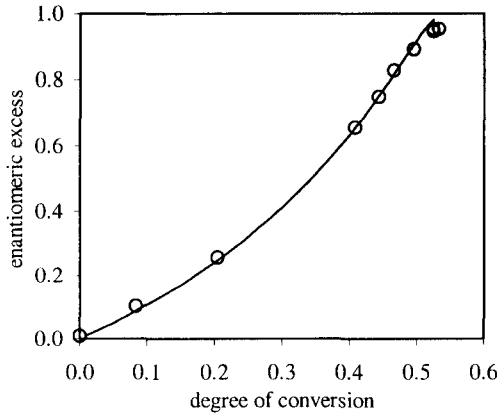

Fig. 5. Enantiomeric excess of the remaining (*S*)-acid versus the degree of conversion in the CALB-catalyzed amidation of 50 mmol·l^{-1} racemic 4-methyloctanoic acid. The reaction was performed in dry MIBK at 35 °C with ammonium carbamate as the source of ammonia. Markers are measured data, line is model calculation[12] for $E = 76$.

Synthesis of oleamide in diisopropylether

The examples shown in Table 1 indicate that the enzymatic amidation of carboxylic acids with ammonia offers interesting opportunities, but the employed substrate concentrations and the observed reaction rates are too low for practical applications. A rapid preparative synthesis of oleamide from oleic acid and ammonia was designed to demonstrate the practical use of this reaction. A different organic solvent had to be chosen because a combination of high temperatures and acid catalysis may cause ammonia to react with MIBK, which may result in the formation of condensates. The solvent should be polar enough to dissolve small quantities of ammonium salts and its boiling point should exceed 60 °C (to support a reaction at this temperature). *tert*-Butanol, *tert*-amylalcohol, and diisopropylether were compared using a standardized amidation protocol at 60 °C (data not shown). The latter solvent appeared to be the most suitable. A concentrated solution of oleic acid (4.8 g) in diisopropylether (25 ml) was converted using 20 % excess of ammonia (from ammonium carbamate) and 3 Å molecular sieve to remove the reaction water. At 60 °C only 6 hours reaction time sufficed to reach 93 % conversion using a moderate amount of CALB. Isolation of crystalline oleamide from the reaction mixture was straightforward and gave 79 % overall yield.

CONCLUSIONS

Lipase-catalyzed direct amidation of carboxylic acids with ammonia in organic solvents is an attractive procedure for the synthesis of primary amides under mild conditions. The reaction may be useful for the kinetic resolution of chiral carboxylic acids. The supply of carboxylic acid and ammonia should be performed in such a way that neither substrate concentration becomes extremely low because of the precipitation of the ammonium salt. The addition of ammonia in the form of a dissolving solid salt is a convenient way to establish suitable ammonia concentrations.

EXPERIMENTAL PROCEDURES

Materials

Immobilized *Candida antarctica* Lipase B (Novozyme 435) with a catalytic activity of 11 PLU/mg preparation was a kind gift of NOVO Nordisk. Presently this enzyme is marketed by Roche Diagnostics GmbH, Mannheim, Germany. *Candida rugosa* lipase (Type VII) with a catalytic activity of 835 units/mg

solid and crude porcine pancreas lipase (Type II) with a catalytic activity of 53 units/mg solid were obtained from Sigma (units based on hydrolysis of olive oil). Methyl isobutyl ketone (99 %), *n*-hexane (95 %) and *n*-dodecane (99 %) were from Merck. Diisopropylether (99 %), ammonium bicarbonate (99 %), and 2-chloropropionic acid (98 %) were from Aldrich. Butyramide (99 %) was from Acros. Ammonium carbamate (99.5 %) and phenylacetic acid (99 %) were from Fluka. Oleic acid (99 %) and oleamide (99 %) were from Sigma. Butyric acid (99 %), ammonium acetate (98 %), and acetamide were from J.T. Baker. 2-chloropropionic acid (98 %) was from Janssen. 4-Methyloctanoic acid (99 %) was from Oxford Chemicals. All solvents were dried over 3 Å molecular sieves before use.

Analytical methods

Non-chiral acids and amides were analyzed on a Shimadzu GC-17A gas chromatograph with a flame ionization detector and a Shimadzu AOC-17 Auto Injector (0.5 µl injection volume). Helium was used as the carrier gas at a split ratio of 1:100. The column used was a Hewlett Packard FFAP Cross-linked Polyethylene Glycol-TPA column (25 m x 0.32 mm) with a retention gap. For analysis of butyric acid and butyramide the column temperature was 70 °C for 4 min, raised by 15 °C·min^{-1} to 130 °C, kept for 2 min, and then raised by 15 °C·min^{-1} to 160 °C. The column pressure was 0.6 bar for 5 min and then raised by 0.2 bar·min^{-1} to 1.0 bar. For oleic acid and oleamide the column temperature was 110 °C for 1 min and then raised by 15 °C·min^{-1} to 230 °C. The column pressure was 1.2 bar. For acetic acid and acetamide the column temperature was 80 °C for 6 min and then raised by 15 °C·min^{-1} to 110 °C. The column pressure was 1.2 bar. For 2-chloropropionic acid and its amide the column temperature was 110 °C for 1 min and then raised by 15 °C·min^{-1} to 170 °C. The column pressure was 1.0 bar. The same column conditions were used for phenylacetic acid and phenylacetamide, except that the column pressure was 1.2 bar.

Chiral analysis of 4-methyloctanoic acid was performed on a Hewlett Packard 5890A gas chromatograph with a flame ionization detector and a HP 7673 GC-injector (1 µl injection volume). Helium was used as the carrier gas at a split ratio of 1:30. The column used was a Supelco gamma-DEX-120 column (30 m x 0.25 mm). The column temperature was 75 °C for 50 min and then raised by 5 °C·min^{-1} to 115 °C (kept for 20 min) and then raised by 5 °C·min^{-1} to 175 °C. The column pressure was 1.0 bar.

^{1}H NMR spectra were recorded in $CDCl_3$ on a Varian Unity Inova 300 MHz spectrometer with tetramethylsilane (0 ppm) as an internal standard.

Enzymatic amidation of butyric acid

A saturated solution of ammonia in MIBK was prepared by bubbling 99.9 % pure ammonia gas through MIBK at 25 °C for two hours.[8] The resulting ammonia concentration was 230 mmol·l^{-1}. Butyric acid, MIBK, and dodecane (internal standard) were mixed with solid ammonium bicarbonate, solid ammonium carbamate, or the ammonia solution in 33 ml closed glass vessels with a septum in the lid. The reactions were started by adding CALB and the solutions were stirred at 25 °C. Samples were taken using a syringe through the septum to prevent the escape of gas, and they were centrifuged to remove solids. Concentrations of butyric acid and butyramide were measured by GC.

Amidation of various carboxylic acids with ammonium carbamate

In a 33 ml closed glass vessel 1.25 mmol carboxylic acid was dissolved in 25 ml dry MIBK containing 10 µl/ml dodecane as an internal standard. The reaction was started by the addition of 1.3 mmol solid ammonium carbamate and the indicated enzyme preparation. The reaction mixture was stirred at the indicated

temperature for the indicated time. Samples were taken through a septum, centrifuged, and analyzed by GC. The yields were determined from the measured amide concentration using dodecane as internal standard. In the amidation of acetic acid the substrates were supplied as ammonium acetate, and no ammonium carbamate was added. In the enantioselective amidation of 4-methyloctanoic acid the extent of conversion and the enantiomeric excess of the acid was determined by GC. This amidation experiment was repeated with 100 mg 3 Å molecular sieves as an extra addition, and as a blank with 100 mg 3 Å molecular sieves instead of the enzyme preparation.

Synthesis of oleamide

Oleic acid (4.80 g; 17.0 mmol), ammonium carbamate (0.80 g; 10.2 mmol), CALB (600 mg), 3 Å molecular sieves (3 g), and diisopropylether (25 ml) were stirred at 60 °C in a closed glass vessel with a septum in the lid. After 6 hours the pressure that was built up due to carbon dioxide formation was released using a 100 ml syringe. The reaction was stopped at 93 % conversion by carefully opening the vessel and filtration of the solids. The filter cake was washed with solvent to dissolve solidifying product. The solvent was evaporated and recrystallization from hexane gave oleamide as a white powder. The purity was 99 % according to GC, the main impurity being oleic acid. The formation of oleamide was confirmed by ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.86 – 0.90 (t, 3 H, CH_3), 1.27 – 1.31 (m, 20 H, 10 × CH_2), 1.62 (m, 2 H, $CH_2CH_2CONH_2$), 2.00 – 2.02 (m, 4 H, 2 × CH_2CH=CH), 2.19 – 2.24 (t, 2 H, CH_2CONH_2), 5.34 (m, 2 H, HC=CH, 2 H, NH_2, broad).[9a]

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