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(54) Title:
PRIMARY AMIDE SYNTHESIS FROM CARBOXYLIC ACIDS WITH A LIPASE

(57) Abstract

The invention relates to a method of preparing a reaction product such as a primary amide, by means of two reagents A and B, which reagents form a salt that impedes fast conversion. The concentrations of the reagent A and the reagent B are chosen such that they comply with formula (I) wherein [A] represents the concentration of the first reagent A in relation to the total volume taken up by the reagents A, B, and the solvent; [B] represents the concentration of the second reagent A in relation to the total volume taken up by the reagents A, B, and the solvent; k is the solubility product for the salt AB formed between the first reagent A and the second reagent B, and F is a factor between 1/10 and 10. It has been shown that by controlling the concentrations this accurately conversion suffers the least possible impediment.
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The present invention relates to a method of preparing a reaction product by means of an enzymatically catalyzed reaction of a first reagent A and a second reagent B in a solvent, the first reagent A and the second reagent B each being soluble in the solvent, and the first reagent A and the second reagent B are capable of forming a salt AB that precipitates in the solvent.

Such a method is impeded by the fact that the precipitated substrates A and B are not available to the enzyme that catalyzes the reaction between A and B. The commonly used practice in the organic chemistry of raising the temperature of the solvent in order to aid the solubility of the reagents A, B is, in connection with the thermic instability of the enzyme, often only possible to a limited extent.

The object of the present invention is to provide a method of the kind mentioned in the preamble, which accelerates the conversion of A and B.

To this end the method according to the invention is characterized in that the concentrations of the first reagent A and the second reagent B are chosen such that they comply with the formula (I)

\[ [A]_T = [B]_T + (F-1) \cdot \sqrt{\frac{K}{F}} \]

wherein

- \([A]_T\) represents the concentration of the first reagent A in relation to the total volume taken up by the reagents A, B, and the solvent;
- \([B]_T\) represents the concentration of the second reagent A in relation to the total volume taken up by the reagents A, B, and the solvent.
K is the solubility product for the salt AB formed between the first reagent A and the second reagent B,

and F is a factor between 1/10 and 10.

In chemistry, reagents are basically brought together in stoichiometric amounts. There may be reasons for departing from this principle, for instance to suppress side reactions. Also, if the yield of reactions does not approximate 100%, considerations regarding costs may result in an excess of the cheapest reagents being added. In practice however, when stoichiometric amounts are indeed involved, there is always a deviation from the optimal stoichiometric proportions. Applicant has surprisingly found that minor deviations in the ratio between the reagents A and B have considerable consequences for the rate at which the reagents A and B are converted by the enzyme. Selecting the concentrations for A and B such that they are within the range defined in accordance with the invention by the factor F, proved to produce good yields more quickly.

The term concentration in the present application refers to the molar concentration. Furthermore, reagents are only included in the formula if they are available for precipitation to salt AB or if they are present as salt AB. The invention is in particular applicable to those reactions in which the salt AB has a solubility product of, for example, less than $10^{-3} \text{ M}^2$, such as less than $3 \times 10^{-4} \text{ M}^2$ and in particular less than $10^{-4} \text{ M}^2$ or $3 \times 10^{-5} \text{ M}^2$.

To avoid the problem of reagents precipitating as salt, the prior art preparation of primary amides using an enzymatically catalyzed reaction involves esterification of the carboxylic acid prior to contacting the ester with the ammonium compound.

De Zoete et al. (Ref. 1) posit that direct ammoniolyis of octanoic acid does not to produce any octanoic amide because of the formation of salts. For this reason they carry out a two-step reaction via the ethylester of octanoic acid.
Öhrner et al. (Ref. 2) state that octanoic acid might lead to the undesirable formation of salts, which is the reason why they, too, use ethyl octanoate.

The formation of primary amides is an important application of the method.

According to a favourable embodiment, $F$ lies between $1/5$ and $5$, preferably between $1/3$ and $3$, and more preferably between $1/2$ and $2$.

Choosing such restricted ranges results in even faster conversion rates. However, it should be noted that with respect to the concentrations of the two reagents, within the ranges mentioned it may be the reaction-kinetic aspects that cause the deviation from the optimal value of 1 for $F$, as will be obvious to the person skilled in the art.

According to a very favourable embodiment the first reagent A and the second reagent B are added as their salt. This is a simple and effective way of achieving a value of 1 for $F$.

An important application of the method according to the invention relates to the preparation of a reaction product wherein the first reagent A is an acidic reagent and the second reagent B is a basic reagent. These will readily precipitate in an organic solvent. If the reaction is a condensation reaction, the solvent will preferably be substantially free of the compound that is released during the condensation reaction as simple molecule, such as water, and which might function as solvent (or as medium aiding dissolution).

According to a preferred embodiment, the first acidic reagent A is a carboxylic acid and the second basic reagent B is a compound selected from the group comprising i) ammonia; and ii) a primary amine.

According to an interesting embodiment, the ammonia is added in the form of ammonium bicarbonate, ammonium carbamate, urea, or ammonia-loaded ion exchange resin.
In this way the ammonia becomes available for reaction more gradually. An interesting aspect of the use of urea as source for ammonia, for example by enzymatic hydrolysis, is that water released during the condensation reaction is removed, which promotes the formation of the amide. For every eliminated water molecule two ammonia molecules are released, which means that there is a certain degree of control regarding the release of ammonia. If desired, this or another embodiment described in the present application, may be provided with an ammonia sensor or may employ a chromatographic technique for measuring the concentration of free ammonia.

As enzyme, an enzyme is used selected from lipases, esterases, and peptidases, such as preferably the lipase Candida antarctica lipase B.

This is an efficient manner of forming a primary amide.

The invention will now be elucidated with reference to the following exemplary embodiment.

EXAMPLE 1
A solution of 73 mg of butyric acid dried over 3 Ångstrom molecular sieves in 10 ml of methylisobutyl ketone dried over 3 Ångstrom molecular sieves, is stirred at 35°C with 80 mg ammonium carbamate (as ammonia source) and 15 mg Candida antarctica lipase B (Novozym 435, activity 11000 PLU/g, Novo Nordisk, Bagsvaerd, Denmark). The solution also comprises 10 μl dodecanee per ml solution as internal standard for analysis. Samples were taken at various intervals and immediately after centrifugation (14,300 rpm for 1 min.) analyzed with the aid of gas chromatography. After three days, the initial butyric acid concentration of 83 mmol/l is reduced to 7 mM, and 75 mM of butyramide is formed.

EXAMPLE 2
Four ml of a solution of 395 mg of butyric acid and 120 μl of dodecanee (as internal standard) in methylisobutyl ketone dried over 3 Ångstrom molecular sieves is mixed with dried methylisobutyl ketone saturated
with ammonia (230 mM) in an amount indicated in the table below and made up to 30 ml with dried methylisobutyl ketone. Thus this solution contains 150 mM of butyric acid, and a concentration of ammonia as indicated in the table. The suspension is stirred at 25°C with 45 mg of *Candida antarctica* lipase B (Novozym 435, activity 11000 PLU/g, Novo Nordisk, Bagsvaerd, Denmark). Under these conditions $K = 1.3 \cdot 10^{-4}$ M$^2$.

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<th>NH$_3$ -satur. MIBK$'$ (ml)</th>
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<th>[NH$_3$] (mM)</th>
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<td>3</td>
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<td>2</td>
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<tr>
<td>4</td>
<td>26</td>
<td>0</td>
<td>199</td>
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') dried methylisobutyl ketone

The experiments 3 and 4 are comparative experiments. In the figure the concentration product (butyramide) is plotted against time (in hours). It can be clearly seen that a deviation from the stoichiometric ratio strongly affects the rate at which the product is formed. In experiment 3, the concentration butyric acid is 150 mM and the concentration NH$_3$ is 184 mM. Although the concentration ammonia is only 21% higher, the conversion rate is more than a factor 2 lower than in experiment 1 (F is 0.09 and 0.8, respectively).

**EXAMPLE 3**

**Preparation of acetamide, the reagents being added as a salt:**

In a 33 ml closed glass vessel 1.25 mmol ammonium acetate (98% pure from J.T. Baker) was stirred in 25 ml dry methylisobutyl ketone containing 10 µl/ml dodecane as
an internal standard (F_{value} = 1). The reaction was started by the addition of 25 mg immobilized Candida antarctica Lipase B (Novozym 435) with a catalytic activity of 11 PLU/mg preparation (which was a kind gift of NOVO Nordisk. Presently this enzyme is marketed by Roche Diagnostics GmbH, Mannheim, Germany). The reaction mixture was stirred at 35°C for 3 days. After that a sample was taken through a septum, centrifuged, and analyzed by GC. The yield was 98%, as determined from the measured acetamide concentration using dodecane as internal standard. GC procedure: acetamide (standard from J.T. Baker) was 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 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.
CLAIMS

1. A method of preparing a reaction product by means of an enzymatically catalyzed reaction of a first reagent A and a second reagent B in a solvent, the first reagent A and the second reagent B each being soluble in the solvent, and the first reagent A and the second reagent B capable of forming a salt AB that precipitates in the solvent, characterized in that the concentrations of the first reagent A and the second reagent B are chosen such that they comply with the formula (I)

\[
[A]_T = [B]_T + (F-1) \cdot \sqrt{\frac{K}{F}}
\]

wherein

[A]_T represents the concentration of the first reagent A in relation to the total volume taken up by the reagents A, B, and the solvent;

[B]_T represents the concentration of the second reagent A in relation to the total volume taken up by the reagents A, B, and the solvent;

K is the solubility product for the salt AB formed between the first reagent A and the second reagent B,

and F is a factor between 1/10 and 10.

2. A method according to claim 1, characterized in that F lies between 1/5 and 5, preferably between 1/3 and 3, and more preferably between 1/2 and 2.

3. A method according to claim 2, characterized in that the first reagent A and the second reagent B are added as salt AB.

4. A method according to one of the preceding claims, characterized in that the first reagent A is an acidic reagent and the second reagent B is a basic reagent and the solvent an organic solvent.
5. A method according to claim 4, characterized in that the first acidic reagent A is a carboxylic acid and the second basic reagent B is a compound selected from the group comprising i) ammonia; and ii) a primary amine.

6. A method according to claim 4 or 5, characterized in that the ammonia is added in the form of ammonium bicarbonate, ammonium carbamate, urea, or ammonia-charged ion exchange resin.

7. A method according to one of the claims 4 to 6, characterized in that as enzyme for the enzymatically catalyzed reaction an enzyme is used selected from lipases, esterases, and peptidases.

8. A method according to claim 7, characterized in that the lipase is Candida antarctica lipase B.
Fig. 1
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12P13/02 C12P1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched: (classification system followed by classification symbols)

IPC 7 C12P C07C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>CEROVSKY, V. ET AL.: &quot;C-terminal peptide amidation catalyzed by orange flavedo peptide amidase.&quot; ANGEWANDTE CHEMIE. INTERNATIONAL EDITION., vol. 37, no. 13/14, 1998, pages 1885-7, XP002144585 cited in the application the whole document</td>
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Patent family members are listed in annex.

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Date of the actual completion of the international search

9 August 2000

Date of mailing of the international search report

28/08/2000

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