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Resolution of (*RS*)-phenylglycinonitrile by penicillin acylase-catalyzed acylation in aqueous medium

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Abstract—A new strategy for the biocatalytic resolution of (*R*,*S*)-phenylglycinonitrile, a crucial intermediate in the antibiotic industry, has been developed. While former techniques exploit nitrilases or combinations of nitrile hydratases and amidases, manipulating with nitrile functionality, the current approach is based on a highly efficient and enantioselective acylation of the α-amino group with phenylacetic acid catalyzed by a well known enzyme, penicillin acylase from *E. coli*, in slightly acidic aqueous medium. It is shown that since the condensation product is poorly soluble, removal of (*S*)-phenylglycinonitrile from the reaction sphere is almost complete and irreversible, favoring kinetics of the process and making high conversion possible. The proposed approach is characterized by high space-time yield and extends the scope of enzymatic synthesis in aqueous medium.

1. Introduction

α-Aminonitriles are crucial intermediates in the production of the corresponding amides and acids. Within the framework of numerous applications of these compounds in fine chemistry, phenylglycinonitrile is of special interest since its derivative, (*R*)-phenylglycine amide, serves as an acyl chain donor in a biocatalytic ampicillin and cephalexin syntheses.[1] However, each particular application requires only one enantiomer and hence a problem of chiral resolution arises since α-aminonitriles are available via Strecker reaction only in a racemic form. The current strategies tackle this problem by using nitrilases or combining nitrile hydratases and amidases. The former, nitrilases, perform enantioselective conversion of a nitrile directly into the carboxylic acid while the latter imply (as a rule) nonspecific hydration of a nitrile to an amide with subsequent amidase-catalyzed hydrolysis of a single enantiomer.[2,3] Although a variety of strategies to cultivate strains, harboring activity towards a given nitrile, are available,[4,5] industrial applications of biocatalytic nitrile conversions are still handicapped due to the lack of a properly formulated biocatalyst: immobilized cells are prone to loose activity when recycling,[6] and purified enzymes are very capricious.[7] In addition only low concentrations of substrates could be converted by currently available techniques: the best examples show productivity (the so called space-time yield) of 0.1–0.3 kg of product per m^3 of reactor space per hour, which is quite low for industrial purposes.[4,6] With respect to phenylglycinonitrile there have been a number of studies on nitrilase and hydratase/amidase catalyzed resolutions.[6,8,9] It has been shown, that the latter approach works better with respect to yields and enantioselectivity. However, productivity of the process was still moderate even at high catalyst loading.[6]

Herein we report an alternative route of chiral phenylglycinonitrile resolution based on the enzymatic recognition of α-amino- rather than nitrile-functionality. Namely, we show how the amino group of the nitrile could be acylated enantioselectively and with nearly quantitative yield. For this purpose penicillin acylase from *E. coli*, an enzyme, well known for its affinity towards (*S*)-phenylglycine derivatives as external nucleophiles,[10–12] was used with phenylacetic acid as an acylating agent. The proposed approach allows the conversion of high substrate concentrations and enables easy separation of non-reactive (*R*)-phenylglycinonitrile from the acylated (*S*)-form by simple filtration due to very low solubility of the latter.

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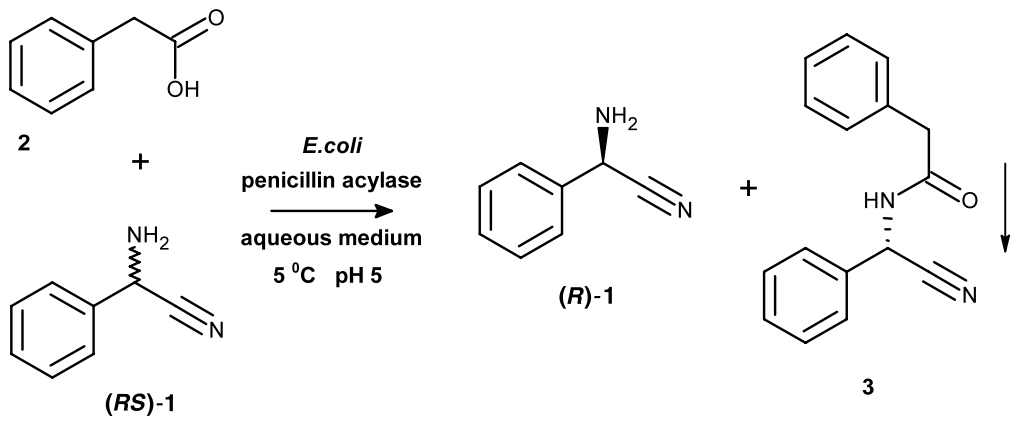

Scheme 1. The general scheme of α-phenylglycinonitrile resolution by penicillin acylase-catalyzed direct condensation with phenylacetic acid in aqueous medium.

2. Results

2.1. Effectiveness and enantioselectivity of acylation

We have found that direct condensation of racemic phenylglycinonitrile (Scheme 1) with phenylacetic acid catalyzed by penicillin acylase from *E. coli* in aqueous solution was highly enantioselective and nearly stoichiometric. Thus, condensation in the concentrated solution of phenylacetic acid 0.2 mol/kg (mol per kg of solution) and racemic **1** (0.4 mol/kg) at pH 5 gave (*R*)-phenylglycinonitrile with 99% e.e. at a conversion of the (*S*)-form higher than 49%. From the kinetic progress curve we have estimated *E* to be >500. Finally, the reaction mixture presented almost pure (*R*)-phenylglycinonitrile solution: it contained ~1 mM of phenylacetic acid (conversion >99.5%), less than 1 mM of the (*S*)-enantiomer and ~7 μM of phenylacetylated (*S*)-phenylglycinonitrile (equilibrium solubility of the compound) while the rest of **3** was in the precipitate phase. Condensation proceeded at a constant rate without inhibition effects (Fig. 1) up to the conversion degree dictated by thermodynamic equilibrium. No loss of enzyme activity was detected in the course of the reaction.

2.2. pH and temperature optimisation of the reaction

As the reaction proceeds in an aqueous medium where both components, the acyl donor and the aminocomponent, are able to ionize, there is a pH optimum of synthetic efficiency. In the case of phenylglycinonitrile condensation with phenylacetic acid the thermodynamic pH optimum is 4.45 (half sum of p*K* 4.3 of **2** and p*K* 4.6 of **1** at 5°C). In practice, condensation was nearly equally favorable over the pH range 4–5, while at pH >5 and at pH <4 ionization of acid and aminonitrile correspondingly reduces the synthetic efficiency. In our experiments we have chosen pH 5 in order to gain in enzyme activity.

Another factor, which has influenced the choice of appropriate reaction conditions was the stability of reagents. Aminoacid nitriles are prone to decompose via a retro-Strecker reaction at neutral and alkaline pH. As lowering the temperature contributes to the stability of α-aminonitriles, we have found that at 5°C and pH 5 decomposition of phenylglycinonitrile was negligible (monomolecular decomposition rate constant was 3.3×10^{-5} s^{-1}).

Another trap to be aware of is the spontaneous racemization of the remaining (*R*)-form. However in an independent experiment [with isolated (*R*)-form and (*R*)-phenylglycinonitrile tartarate salt] we have shown that racemization was even slower than spontaneous degradation: in 20 h at pH 5 and 5°C the value of e.e. dropped from 0.99 to 0.98 which is insufficient for acylation reaction having typical time 10 h.

3. Discussion

3.1. The principle of enzymatic resolution

Acylation of aminocompounds in aqueous medium could imply two options: acylation by a substituted

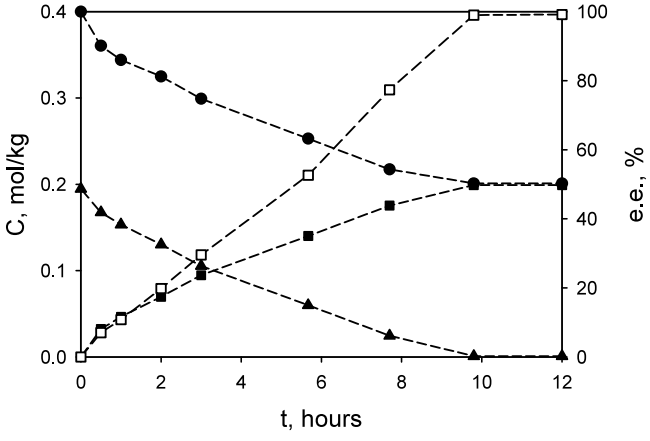

Figure 1. Kinetic progress curve of penicillin acylase-catalyzed direct condensation: (*RS*)-phenylglycinonitrile (●), phenylacetic acid (▲), acylation product **3** (■) (left axis); e.e. of (*R*)-phenylglycinonitrile (□) (right axis).

carboxyl derivative (an amide or an ester) known as acyl transfer, or direct condensation with a corresponding carboxylic acid. The principal difference between these processes is the following: while in the condensation reaction chemical equilibrium is attained gradually (e.g. the product is accumulated monotonously until the equilibrium is reached), in the former product accumulation may exceed the final equilibrium value if the acyl transfer itself is faster than the hydrolysis of the acyl donor and the acyl transfer product formed. The latter example describes the so-called kinetically-controlled synthesis, which is the common way to couple thermodynamically unfavorable (synthetic) reactions to the consumption of energetically-rich compounds (be it activated acyl donor or ATP and acetyl-CoA as in biological systems). As for the enzymatic reactions in vitro, there are examples of successful amine resolution by enantioselective acyl transfer in aqueous medium, catalyzed by penicillin acylase from *A. faecalis*.[13]

The choice of acylating agent for resolution purposes was predefined by the high affinity of penicillin acylase towards phenylacetic acid derivatives.[11–13] Since we considered reaction at pH 5 (enzyme activity permitted this) we might hope that **2** (pK 4.3), namely, its protonated form, could serve as an effective acylating agent. These findings demonstrate the feasibility of resolution of the aminoacid nitriles by enzymatic direct condensation in aqueous medium.

3.2. Thermodynamic considerations

Coincidence of thermodynamic pH optimum with p*K*s of **2** and **1** made condensation reaction not only kinetically, but also thermodynamically feasible. In contrast, peptide bond synthesis in an aqueous medium seems to work only via acyl transfer: due to the big difference in p*K* values of carboxylic ($\sim$2) and amino groups ($\sim$9–10) of amino acids direct condensation is thermodynamically unfavorable. However we should mention here that while favorable p*K*s of reagents make the reaction feasible, precipitation of the condensation product makes it practically irreversible, that is, stoichiometric. We have found that the solubility of **3** was 6.8 μM at 5°C, hence this example of enantiomer resolution could nicely represent an ideal of precipitation-driven catalysis.[15]

The last notion on the thermodynamics of aminonitrile resolution by direct condensation with carboxylic acid concerns the 'strength' of amide bond formed.[14,16] We have determined that apparent equilibrium constant characterizing synthesis of amide bond in phenylacetylated (*S*)-phenylglycinonitrile (expressed as the concentration ratio of the **3** with **1** and **2**) was 56 M^{-1}. We should mention that this value is characteristic for amide bonds formed by amines with low p*K*,[14,17,18,21] and in general can be considered as a quite moderate: for aliphatic amides and peptides the synthetic constant can be an order of magnitude larger.[16]

Experimental conversion degrees and the values of e.e. perfectly correlated with thermodynamic predictions. This meant that no kinetic obstacles (such as enzyme non-specificity, enzyme inactivation, strong inhibition effects, etc.) took place and true chemical equilibrium was attained, providing the best possible characteristics of enantiomer resolution. Note that if the product of condensation was soluble and hence the extra driving force of the reaction disappeared, conversion of the amino-compound would be sufficiently lower thus leading to lower e.e.

3.3. Kinetic features

Some kinetic features of the reaction should be mentioned. First of all we should point out that penicillin acylase from *E. coli* appeared to be stable and active under the described conditions. Surprisingly high activity of the enzyme in acidic medium has been exploited earlier for effective hydrolysis of benzylpenicillin and benzyldeacetoxycephalosporin hydrolysis in a two-phase 'aqueous solution–water immiscible organic solvent' medium.[14,20] This new example widens biocatalytic applications of penicillin acylase in acidic media.

As seen from the kinetic progress curve (Fig. 1), substrate consumption evolves at a constant rate until the equilibrium conversion is reached. This means that the reaction proceeds under enzyme saturation by substrate and is not complicated by product inhibition (since the product is poorly soluble). This feature favors scalability of the process towards higher concentrations of the substrates converted.

Note also that as far as the condensation reaction is seen at a pH close to the half-sum of reagents p*K*s, the pH shift during the reaction is insignificant and hence the reaction can be conducted per se in a simple batch mode without pH control and buffering.

Finally, we should comment on the substrate specificity. High enantioselectivity of penicillin acylase from *E. coli* towards (*S*)-phenylglycine and other (*S*)-amino acids as external nucleophiles in acyl transfer was known from our previous experience.[11,13] Herein we have shown that enzyme enantioselectivity towards (*S*)-phenylglycinonitrile (E >500) is comparable with selectivity to other amino acids and distinctly exceeds that for amino acid esters and aliphatic amines.

3.4. Prospects

The proposed approach of chiral resolution provides a high space-time yield $\sim$3 kg/m^3/h at a moderate biocatalyst charging used in our experiments. This productivity is at least an order of magnitude higher than using nitrile hydratases and amidases.[6,8] It should be emphasized that penicillin acylase is a very stable enzyme and a number of its immobilized forms (using different carriers, prepared by cross-linking enzyme crystals or enzyme aggregates) are easily available.

4. Conclusion

The proposed approach of α-aminonitrile resolution principally differs from the existing techniques: while the latter are targeted at selective hydrolysis of a nitrile group, converting one enantiomer into an amide or carboxylic acid, the former implies enzymatic acylation of α-amino group of the (*S*)-form. We have shown that penicillin acylase from *E. coli* was able to catalyze direct condensation of phenylglycinonitrile with phenylacetic acid in acidic medium (pH 5) and the reaction was exclusively enantioselective and effective, which allowed nearly stoichiometric acylation (with respect to both components). The quantitative conversion of reagents was due to the highly efficient precipitation of the acylation product, making condensation essentially irreversible. In addition, precipitation of the product has eliminated inhibition effects, so that the reaction proceeded at a constant rate until the complete conversion of the reagents. The proposed approach is scalable and allows for the conversion of high substrate concentrations. Further implications of precipitation-driven α-aminonitrile resolution by enzymatic direct condensation are under investigation.

5. Experimental

Solutions of penicillin acylase from *E. coli* were obtained from DSM Anti-Infectives, Delft, The Netherlands. The concentration of penicillin acylase active sites was determined as described earlier.[19] (*RS*)-Phenylglycinonitrile hydrochloride and (*R*)-phenylglycinonitrile tartarate salt were products of DSM. Phenylacetic acid was obtained from Aldrich. Eluent components were purchased at Reakhim (Moscow, Russia) and at Kriochrom (St. Petersburg, Russia). In all experiments MilliQ water was used.

5.1. HPLC analysis

Concentrations of the reactants were determined by HPLC using a Waters M6000 pump, Chrompack Nucleosil 100 C-18 column (150 mm×4.6 mm, 5 μm) and LKB 2138 uvicord S detector at 208 nm with an eluent containing 5 mM phosphate, acetonitrile 35% v/v, 0.25 g/L of sodium dodecylsulfate at pH 3.0. The flow rate was 0.8 mL/min. Retention times (in min): phenylacetic acid (4.7), phenylglycinonitrile (6.5), *N*-phenylacetyl-phenylglycinonitrile (18).

Chiral analysis was performed by HPLC using Waters M6000 pump, Crownpack® CR(+) column (150 mm×4 mm, 5 μm) and LKB 2138 uvicord S detector at 208 nm with an eluent containing 0.01 M aqueous solution of perchloric acid. The flow rate was 0.5 mL/min. Retention times, in min: (*S*)-phenylglycinonitrile (13), (*R*)-phenylglycinonitrile (17).

5.2. Enzymatic condensation of (*RS*)-aminonitrile with phenylacetic acid

α-Phenylglycinonitrile hydrochloride (2.65 mmol) and phenylacetic acid (1.325 mmol) were dissolved in water (6 mL) at 5°C and pH was adjusted to 5.0 with 10N NaOH. Reaction was carried out in the presence of 10 μM PA-*E. coli* under permanent stirring in a thermostatted cell of a pH-stat (Metrohm Titrino 719 S) charged with 2M aqueous HCl solution. Reaction was accompanied by precipitation of the condensation product.

Progress curves of the enzymatic acylation were followed by HPLC analysis on a reversed phase column (phenylacetic acid, aminonitrile and their condensation product were detected) and separately on a chiral column to check for e.e. of the remaining aminonitrile. For analysis on a reversed phase column 20 μL of heterogeneous reaction mixture were added to 0.98 mL of eluent in order to dissolve reactants and stop the reaction; 20 μL of obtained solution were added to 0.98 mL of eluent and the sample was injected into HPLC system. For chiral analysis 20 μL of heterogeneous reaction mixture were filtrated using microfiltration kit (membrane pore size 0.2 μm by Biochrom, Moscow, Russia) in combination with Eppendorf centrifuge 5415 D (5 s at 13.200 rpm). 10 μL of the filtrate were dissolved in 0.5 mL of eluent (for chiral analysis); 20 μL of obtained solution were diluted with 0.38 mL of eluent and subjected to chiral analysis.

Finally, the condensation product (*N*-phenylacetylated phenylglycinonitrile) was filtrated on the pore glass filter, washed for several times with water and dried in an exsiccator over concentrated H_2SO_4 overnight. Enantiomeric purity of *N*-phenylacetyl-(*S*)-phenylglycinonitrile has not been checked on a chiral column but we could suppose it was high enough (>99%) standing on mass balance data: conversion of acyl donor was ~99.5% and e.e. of residual (*R*)-phenylglycinonitrile was ~99% as if only the (*S*)-form was acylated.

The remaining (*R*)-phenylglycinonitrile was isolated as follows: in the filtrated mother liquor pH was brought to 7 and aminonitrile was extracted into 20 mL of ethyl acetate at 5°C. Then back extraction with 10 mL of 0.3 M HCl followed. Hydrochloride of (*R*)-phenylglycinonitrile was isolated from water phase by vacuum evaporation at 50°C and dried in a dessiccator over concentrated H_2SO_4 overnight.

5.2.1. (*R*)-Phenylglycinonitrile. (*R*)-Phenylglycinonitrile hydrochloride was isolated from the reaction mixture as described in 4.2. Yield 0.106 g (50%); 99% e.e.; mp 145–146°C (dec.); $[\alpha]_D^{25} = +51.0$ (*c* 1, 1N HCl); ^{1}H NMR (300 MHz, DMSO+CCl_4): δ 5.87 (s, 1H, CH), δ 7.45 (m, 3H, Ph), δ 7.75 (m, 2H, Ph), δ 9.85 (br. s, 3H, NH_3). MS *m*/*z*: 167 (5, M–H), 132 (85, M–HCl), 131 (100, M–HCl–H), 116 (26, PhCHCN), 106 (28, M–HCl–CN), 105 (15, M–HCl–HCN), 104 (40, M–HCl–H–HCN), 77 (47, Ph), 55 (20, M–HCl–Ph), 51 (30), 36 (25, HCl).

5.2.2. *N*-Phenylacetyl-(*S*)-phenylglycinonitrile. *N*-Phenylacetyl-(*S*)-phenylglycinonitrile was isolated from the reaction mixture as described in 4.2. Yield 0.3 g (90%); >99% e.e. (estimated from mass balance, see 4.2. for details); mp 118–119°C (dec.); $[\alpha]_D^{25} = +17.2$ (*c* 1, methanol); ^{1}H NMR (300 MHz, DMSO+CCl_4): δ 3.52s, 2H, CH_2), δ 6.05 (d, 1H, CH), δ 7.2–7.5 (m, 10H, Ph), δ 9.20 (d, 1H, NH). MS *m*/*z*: 250 (50, M), 116 (35, PhCHCN), 91 (100, $PhCH_2$), 77 (12, Ph), 65 (20).

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