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Chemoenzymatic approaches to the dynamic kinetic asymmetric synthesis of aromatic amino acids

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Abstract—Enzymatic approaches for the production of amino acids by nitrilases are described. Dynamic kinetic asymmetric synthesis conditions were established for the aromatic aminonitriles, phenylglycinonitrile and 4-fluorophenylglycinonitrile, at high pH to produce the corresponding amino acid products in high enantiomeric excess. *N*-Acylation of aromatic aminonitriles led to spontaneous racemization at pH 8, allowing preferential enzymatic hydrolysis of the (*R*)-enantiomer to afford the product *N*-acyl-amino acids in up to 99% enantiomeric excess (ee).

1. Introduction

Amino acids have played a significant role in the fine chemical and pharmaceutical industries for several decades. They are used in a wide range of applications, including building blocks for the pharmaceutical industry, feed additives and human nutrition.[1] While fermentation-based routes have been more economical for the production of the naturally occurring proteinogenic L-amino acids, a niche exists for the use of chemoenzymatic methods in the production of unnatural D- and/or L-amino acids.[1] Chemoenzymatic routes include the use of aminoacylases,[2,3] hydantoinases,[4a] aminotransferases[4b] and amino acid dehydrogenases.[4c] Many of these methods are based upon kinetic resolution of racemic mixtures, requiring recovery and racemization of the undesired enantiomer. There is a distinct requirement for economic and efficient dynamic kinetic asymmetric synthesis methods for the production of these versatile compounds.

The Strecker synthesis of α-aminonitriles, followed by acid- or base-catalyzed nitrile hydrolysis, is one of the oldest and most well-known routes to racemic amino acids, having the advantages of cost effectiveness and readily available raw materials.[5] Asymmetric Strecker reactions are limited in many cases to the preparation of *N*-substituted aminonitriles and require expensive or difficult to obtain chiral catalysts[6,7] or chiral auxiliaries, while at the same time requiring harsh conditions of pH and temperature to hydrolyze the nitrile and remove the *N*-substituent. The stereoselective hydrolysis of aminonitriles by nitrilases represents an attractive approach for the production of amino acids. We have previously reported the use of nitrilases for the production of mandelic acid, phenyllactic acid and substituted carboxylic acids with very high ee (>97%).[8] These high enantiomeric purities are facilitated by reversible HCN loss and racemization of cyanohydrins at moderate pH and temperature. Racemization of aminonitriles turns out to be considerably more challenging.

There are few reports of amino acid production using nitrilases.[9–12] Our work focuses on determining conditions under which aromatic α-aminonitriles racemize and combining these conditions with a nitrilase-catalyzed hydrolysis of the aminonitrile, resulting in dynamic kinetic asymmetric synthesis.[13] Herein we report two approaches to nitrilase-catalyzed dynamic kinetic asymmetric synthesis: (i) hydrolysis of aromatic aminonitriles at high pH and (ii) hydrolysis of *N*-acyl

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aminonitriles at pH 8. Much of the research was carried out using phenylglycinonitrile **1a** as a model aromatic aminonitrile, with further development on the target compound, 4-fluorophenylglycinonitrile **1b**, an important chiral building block. These approaches demonstrate the feasibility of dynamic kinetic asymmetric synthesis for the production of amino acids using nitrilases.

2. Results and discussion

2.1. Chemical approaches to enhance racemization

Enantiomerically enriched aromatic aminonitrile salts are stable compounds. However, the corresponding free bases are configurationally labile and can racemize and decompose via a number of pathways, which include deprotonation, cyanide elimination and Schiff base formation (Scheme 1).[14] The challenge associated with developing a nitrilase-catalyzed dynamic asymmetric synthesis was to identify conditions under which racemization of the aminonitrile (k_{rac}) was faster than the nitrilase-catalyzed hydrolysis, and that both reactions occur under conditions, which minimize decomposition pathways.

The initial screen of our nitrilase collection involving **1a** at pH 8 identified a number of enzymes, which formed (*R*)-phenylglycine (*R*-**2a**) in high yield but with only moderate ee (55–65%). High conversions coupled with unreacted starting material of low ee indicated that partial racemization of the aminonitrile had occurred, but that the rate of racemization was significantly less than that of the enzymatic hydrolysis. The $t_{1/2}$ for racemization of (*R*)-**1a** was subsequently found to be 13 h at pH 8. Therefore, we set out to investigate conditions to enhance the rate of racemization. Enantiomerically enriched **1a** was used for surveying racemization conditions since both enantiomers can be easily prepared in high enantiomeric purity.[15†]

Racemization of amino acid derivatives using catalytic amounts of aldehydes or ketones has been coupled with a resolution process (enzymatic[16] or crystallization[17]) to effect high yielding dynamic kinetic asymmetric syntheses.[18] However, the addition of various aldehydes (benzaldehyde, salicylaldehyde, pyridoxal) to an aqueous solution of (*S*)-**1a** at pH 9.5 did not result in an acceleration of aminonitrile racemization. Treating (*S*)-**1a** under nonaqueous conditions with a series of eight different organic bases resulted in rapid racemization only in the case of DBU (pK_a 13.2);[19] the other bases (pK_a 8–11) showed no sign of racemization. DBU was not observed to racemize the aminonitrile under biphasic conditions, where the enzymatic reaction might operate. Initial results also indicated that the addition of cyanide and/or ammonia to solutions of **1a** resulted in rapid racemization. However, subsequent experiments showed that the rate enhancement was actually due to an increase in the pH of the reaction mixture. Further experimentation established that pH values >10 were required for the rapid racemization of aromatic aminonitriles (Fig. 1); $t_{1/2}$ for the racemization of **1a** at pH 10.5 was estimated to be 60 min compared to 4 h at pH 9.5. While the addition of cyanide did not enhance racemization, it did contribute to the stability of the aminonitrile at the high pH necessary for rapid racemization (Fig. 1), presumably by shifting the imine/aminonitrile equilibrium to the right and preventing decomposition by a retro-Strecker pathway. Similar results established the requirement for high pH in the racemization of **1b** (data not shown).

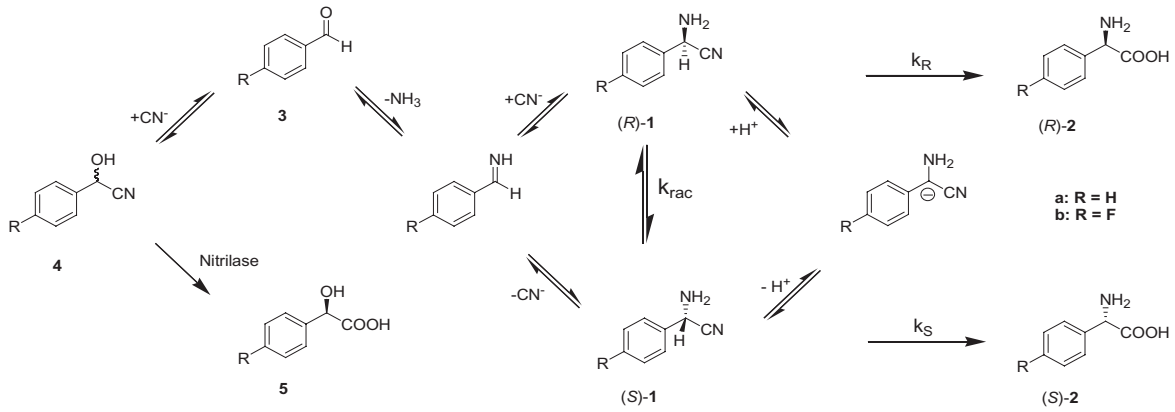
2.2. Nitrilase-catalyzed dynamic resolution at high pH

Our early results with Nitrilase 5086 had shown that the enzyme retained a considerable amount of activity at pH 9 (unpublished results). We investigated the effect of increasing pH on this enzyme and some of our other nitrilases. At pH 10.2, seven nitrilases were active, including Nitrilase 5086. Further exploration of this space showed that Nitrilase 5086 was still active at pH 10.8, which would be sufficient for racemization of **1a**. While activity up to pH 13 has been reported by a nitrile hydratase from *Rhodococcus* sp ATCC 39484,[20] we believe that this is the first report of an alkalophilic nitrilase. Further development of the hydrolysis of **1a** by this nitrilase led to reaction conditions, which afforded the desired product, (*R*)-**2a** in 91% ee and a yield of 60% at pH 10.6.[‡]

Unfortunately, the same enzyme was not successful with substrate **1b** at these pH levels. Our available collection of nitrilases was screened (total screened: 177) to determine the existence of other alkalophilic enzymes. By increasing the pH, we identified four enzymes that were active on the substrate at pH 11. Hydrolysis of **1b** by Nitrilase 5275 under controlled conditions at pH 10.8 was then investigated. Initially, lower yields (up to 67%) were encountered with two possible reasons postulated: (i) decomposition of the substrate by a retro-Strecker process, or (ii) high levels of 4-fluorobenzaldehyde **3b**, a possible enzyme inhibitor. Addition of 1 equiv of cyanide to the reaction enhanced the yield and lowered the level of aldehyde, through stabilization of the aminonitrile. The reaction temperature was also found to be significant, with lower temperatures (18 °C) providing higher yields. These conditions led to the achievement of 79.5% yield of (*R*)-**2b** with an ee of 96.3%. The addition of enzyme in two batches was found to be essential for: (i) maintaining the balance between the rates of hydrolysis and racemization that lead to high

† Enantiomerically enriched **1a** was isolated and stored as its tartrate salt and the free base generated before use. Compound **1b** gave inconsistent results when resolved under the same conditions. All new compounds were characterized by NMR and high resolution mass spectrometry. Ee was determined by chiral HPLC (i) Crownpak CR ($MeOH–HClO_4$) for compounds **1**and **2** and (ii) Chiralcel OD-RH ($MeCN–HClO_4$) for **8b**.

‡ Unless otherwise noted, typical reaction procedures are described in Ref. 8; for the high pH reactions, 0.1 M carbonate buffer was used.



Scheme 1.

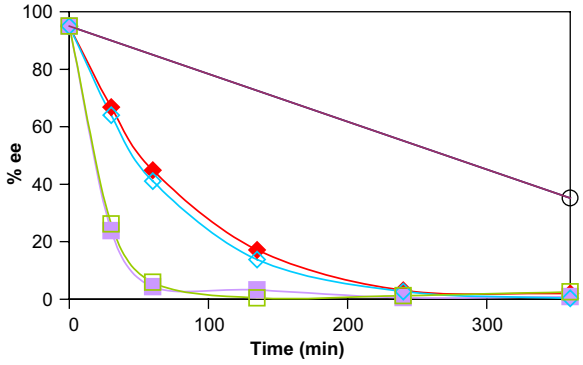


Figure 1. The pH dependence of (*S*)-**1a** racemization in the presence and absence of added cyanide. -◯- pH 9.6; -◆- pH 10.4; -■- pH 10.9; -◇- pH 10.4, 3 equiv CN; -□- pH 10.9, 3 equiv CN.

enantioselectivity, and (ii) to supplement enzyme lost through inactivation at high pH.

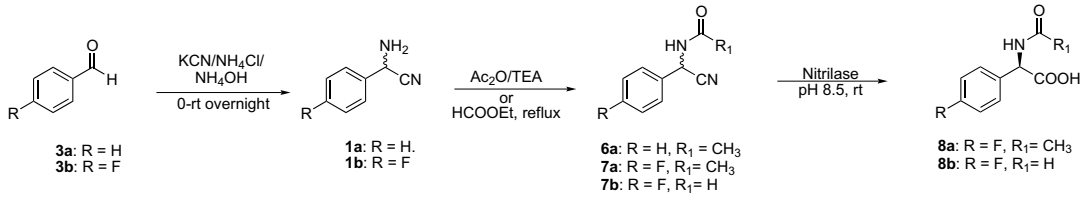
Following the relatively successful results reported above, the substrate concentration was scaled up to 100 mM. A continuous flow of enzyme into the reaction was maintained (0.23 mg/h decreasing to 0.15 mg/h at 6.5 h). Under these conditions, (*R*)-**2b** was isolated in 70% yield and 94.4% ee. While we believed that this approach showed significant promise, the lability of the aminonitrile substrate resulted in suboptimal yields while the purity of the product was compromised by low levels of the byproducts (**3**–**5**) shown in Scheme 1. Therefore, we decided to pursue the *N*-acyl aminonitrile approach described below.

2.3. Hydrolysis of aromatic *N*-acylaminonitriles

Unlike their parents, *N*-acylated aromatic aminonitriles are stable compounds, which racemize easily under mild conditions. In fact, (*S*)-*N*-acetyl phenylglycinonitrile **6a** racemizes with $t_{1/2} = 40$ min at pH 8. The nitrilase-catalyzed dynamic kinetic asymmetric hydrolysis of *N*-acyl aminonitriles (Scheme 2) is an attractive strategy, since *N*-acyl aminoacids are useful intermediates in peptide synthesis; the acyl group can be conveniently removed by a variety of methods, including an aminoacylase to yield the unprotected amino acids.[21]

Diversa's collection of nitrilases was screened for the hydrolysis of *N*-acetyl 4-fluorophenylglycinonitrile **7a**. The initial screen provided 19 active enzymes, of which Nitrilase 5086 was the most promising. Satisfactory results were obtained at 25 mM substrate concentration (95% yield of **8a**, 91% ee). However, in order to achieve high volumetric productivities, higher substrate solubility was required.

In general, our nitrilases had shown better activity on aminonitriles than on the corresponding *N*-acetyl aminonitrile. We, therefore, considered hydrolysis of a less sterically hindered derivative, *N*-formyl 4-fluorophenylglycinonitrile **7b**. This compound is more water soluble than the corresponding *N*-acetyl derivative while its stronger electron-withdrawing properties should enhance racemization. The 19 nitrilases that were active on **7a** were screened on **7b**. The screen identified three enzymes with high enantioselectivity. Further experimentation showed that one enzyme produced unacceptable levels of amide, while another showed low



Scheme 2.

conversions at higher substrate concentration (>25 mM). Nitrilase 5086 was the most promising, and high conversion was achieved at 100 mM substrate through the addition of two aliquots of enzyme. The presence of a conserved cysteine in the putative catalytic triad[22] suggested that the addition of a reducing agent might enhance enzyme stability. In the presence of 1 mM dithiothreitol (DTT), a single addition of enzyme at 1 mg/mL was possible; the level of DTT could be reduced to 0.1 mM when the reaction was performed under nitrogen. Further improvement of enzyme performance resulted from the addition of 15% MeOH and lowering the reaction temperature from 37 °C to ambient. Having established these conditions, the reaction was reproducibly scaled up to 100 mM (1 g of **7b**; 1 mg/mL lyophilized lysate of Nitrilase 5086 in 0.1 M Tris–HCl pH 8.5). Complete conversion of the substrate to (*R*)-*N*-formyl-4-fluorophenylglycine **8b** was typically observed in 24 h. After standard work-up, the product was isolated as a crude colorless solid, affording a yield of 87% and ee of 98–99%.

3. Conclusion

Two novel approaches to the chemoenzymatic production of aromatic amino acids have been described. The ability of nitrilases to function at high pH has been established, with phenylglycine and 4-fluorophenylglycine produced with high enantioselectivity. In order to increase the yields, *N*-acyl aminonitriles were explored and found to allow k_{rac} to exceed enzymatic hydrolysis, thereby establishing conditions for dynamic kinetic asymmetric synthesis. In one example, *N*-formyl-4-fluorophenylglycine was obtained in 87% yield and 99% ee. The versatility of our nitrilase collection has been further extended by demonstrating function at high pH and high specificity for derivatives of phenylglycinonitrile.

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