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A One-Pot Enantioselective Chemo-Enzymatic Synthesis of Amino Acids in Water

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Abstract: The combination of immobilised Rh-MonoPhos (**1**-AlTUD-1) and acylase I afforded a chemoenzymatic, one-pot process for the enantioselective synthesis of amino acids in water, without the need for isolation of intermediates. In addition, the enzymatic hydrolysis increases the enantiopurity of the product from 95% ee to >98% ee. Compatibility studies revealed that for optimum results compartmentalisation of the catalysts is required.

Keywords: aminoacylase; asymmetric catalysis; cascade reaction; hydrogenation; immobilisation

Introduction

During the last decades an increasing urgency has arisen to develop greener and economically competitive processes for the industrial synthesis of chemicals.[1] Especially in the production of pharmaceuticals or agrochemicals, where the waste generation can surpass 100 kg/kg product, this is a pressing necessity.[2] A very important tool in the chemist's arsenal to achieve more environmentally benign processes is catalysis. Not only does the remarkable progress in (enantioselective) chemocatalysis continue, but the potential of biocatalysts is also increasingly being recognized by the fine chemical industry.[3] However, either of these catalysts only solve part of the problem, since they are usually part of a complex multistep synthesis where the majority of reaction steps still consist of classic stoichiometric chemistry. Additionally, these processes usually require wasteful and expensive isolation as well as purification of intermediates. For genuinely sustainable processes, the majority of the steps should be catalytic and, ideally, intermediate purification and isolation steps should be circumvented. Cascade reactions offer a unique opportunity to address these issues, in particular when carefully orchestrated, involving enzymes and/or chemocatalysts.[4–6] These two types of catalysts complement each other: transition metals are very versatile for oxidations and reductions (tasks often difficult to perform with enzymes, due to problems with cofactor regeneration) and enzymes readily perform hydrolytic reactions and their reverse (whereas a chemo-catalytic approach often requires drastic conditions and generates large amounts of salts as waste).

To overcome the common incompatibility of reagents and conditions, smart solutions need to be found: immobilisation of the catalyst, as a form of compartmentalisation or in combination with other compartmentalisation approaches, is often an efficient strategy. A noteworthy example was recently published by Gelman et al.[7] By immobilising a lipase and a rhodium complex in two separate sol-gel matrices they were able to perform a one-pot esterification and hydrogenation reaction leading to saturated esters in good yields. In contrast, when only the enzyme was immobilised yields decreased almost 7-fold. Thus, the support of the catalyst in this example has a similar function to a membrane in a cell – it inhibits the interaction of the incompatible reagents.

Recently, we reported in a communication the successful immobilisation of the asymmetric hydrogenation catalyst, Rh-MonoPhos, on AlTUD-1 ([**1**-AlTUD-1] Figure 1).[8] This catalyst is based on the synthetically readily accessible MonoPhos ligand,[9] which was immobilised *via* straightforward ionic interactions with the surface of the three-dimensional mesoporous material, AlTUD-1.[10] In this manner, the need for modification

of the ligand prior to immobilisation is circumvented. In addition to the obvious advantages of easy separation and improved recyclability of the catalyst, this methodology also opened up the possibility to use water as a reaction medium, thereby creating the unique opportunity for a chemo-enzymatic cascade, since water is the ideal solvent for enzyme-catalysed hydrolyses. We now report on the successful combination of the chemocatalytic asymmetric hydrogenation with enzymatic hydrolysis of the product to afford an one-pot green synthesis of enantiopure amino acids in water.

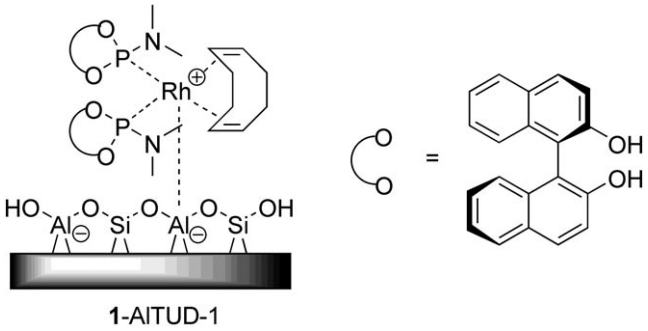
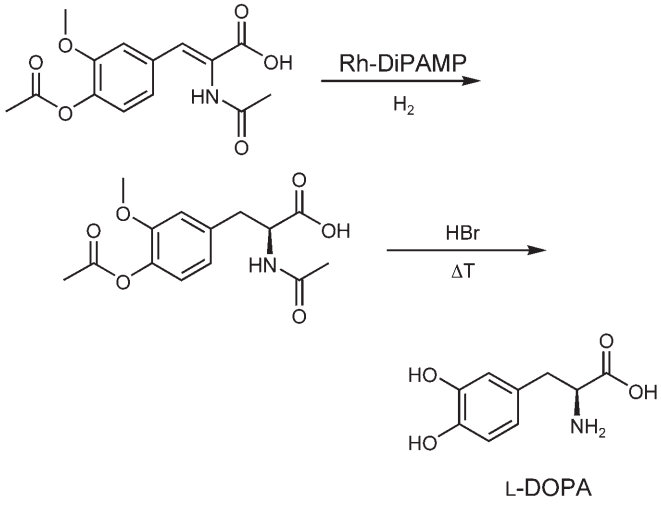


Figure 1. Rh-MonoPhos immobilised on AlTUD-1.

The benchmark Monsanto L-DOPA process (Scheme 1) perfectly illustrates the problem: an elegant enantioselective chemical reduction is followed by a waste-generating chemical hydrolysis.[11] A chemo-enzymatic cascade would greatly improve the sustainability of this type of amino acid synthesis: the ideal process would be entirely catalytic, require only one work-up and eliminate the use of organic solvents and the stoichiometric formation of salts.



Scheme 1. The Monsanto L-DOPA process.

Results and Discussion

To test our concept we chose the synthesis of L-alanine, starting from methyl 2-acetamidoacrylate (see Figure 2). The ideal candidates for the enzymatic part of the cascade are the well-known aminoacylases,[12] since they were evolved to catalyse this type of hydrolysis. We selected both *Aspergillus melleus* (AM) and porcine kidney (PK) as sources for this type of enzyme, since these acylases are highly active,[13] commercially available and, in the case of *Aspergillus melleus*, inexpensive.

In the first form of cascade, which we investigated, the hydrogenation and hydrolysis reactions were separated by a straightforward filtration. A schematic representation of this set-up is given in Figure 2. The results of the hydrogenations as well as the hydrolyses can be found in Table 1.

In the first step, i.e., the asymmetric hydrogenation, the intermediate **3a** is produced with an enantiomeric excess (ee) of 95% as reported earlier.[8] The second step is initiated by simply passing the reaction mixture through the filter and adding the enzyme with a concentrated phosphate buffer (10 vol %, pH = 7.5, 1.1 M). Given that the substrate of the hydrolysis reaction (**3a**) has 2 functional groups that can be hydrolysed, namely the amide group and the ester group, several products (Figure 3) can be formed. From these possible products, **3c** was never detected, corresponding to the earlier findings that acylase I requires a terminal carboxylate group for its hydrolytic action on the *N*-acyl group.[13] Advantageously, both acylases demonstrated ester hydrolysis activity for the *N*-acylamino acid esters, giving rise to **3b**. This monohydrolysed product is sequentially and efficiently converted by both enzymes into the desired free amino acid, **4**. A similar sequence was observed by Liljeblad et al. in the hydrolyses of the methyl esters of racemic *N*-acylvaline and *N*-acylmethionine.[14] AM is clearly more active in this hydrolysis sequence than PK, even though more units for PK were used. AM reaches a conversion of **3a** of 98% within 4 h, whereas PK only achieves 14% in the same time-frame. With both enzymes there is still a significant quantity of **3b** present after 4 h. The conversion of **3a** with AM remains at 98% after 24 h, but now **4** is the only product. As is described in the literature, acylase I demonstrates high enantioselectivity for the ester as well as the amide hydrolysis,[14,15] resulting in a maximum conversion of 98%, since the remaining 2% of the substrate is the wrong enantiomer. The enantioselectivity of the hydrolysis is confirmed by the enantiopurity of **4** (ee of $>98\%$, as determined by chiral HPLC). The same analysis for PK showed that this enzyme also exhibits a very high selectivity ($>98\%$ ee). Thus, by using this chemo-enzymatic cascade, we not only reduced the total number of steps and made the synthesis entirely catalytic, but we also enhanced the enantiopurity of the product.

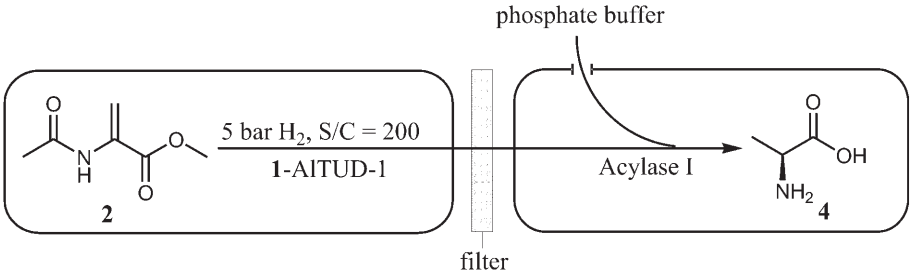


Figure 2. Schematic representation of the filtered cascade.

Table 1. Results of the various chemo-enzymatic cascades in the asymmetric synthesis of **4**.

Enzyme[a]	Conversion to **3a** [%]	ee of **3a** [%]	Conversion[b] of **2** to **3a** [%]	Ratio **4**/**3b**[b]	ee of **4**[b] [%]
AM filtered	100	95	98 (98)	100:0 (89:11)	>98 (>98)
PK filtered	100	95	82 (16)	87:13 (63:37)	>98
AM unfiltered	100	95	98 (98)	100:0 (85:15)	>98 (>98)
PK unfiltered	100	95	40 (14)	98:2 (85:15)	>98

[a] AM = acylase I from *Aspergillus melleus* (175 U); PK = acylase I from porcine kidney (700 U); filtered and unfiltered refer to the respective protocol. [1 U corresponds to the amount of enzyme which hydrolyses 1 μmol *N*-acetyl-L-methionine per minute at pH 7.5 and 22 °C.]
[b] After 24 h of hydrolysis; results in brackets are after 4 h of hydrolysis.

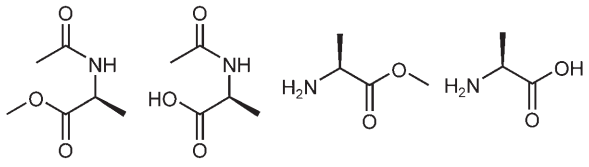


Figure 3. Substrate and possible products of the hydrolysis step.

In our systematic exploration towards a genuine cascade process for the asymmetric synthesis of the amino acid, we also investigated the possibility to perform the reaction without intermediate removal of the rhodium catalyst. The hydrolysis is simply initiated by addition of enzyme and buffer. Consequently, the hydrogenation catalyst is still present in the hydrolytic phase. The activity and selectivity of AM was unaffected by **1**-AlTUD-1 (Table 1). In contrast, PK exhibited significantly lower conversions, especially after 24 h. The selectivity of both enzymes on the other hand remained excellent. Thus, the unfiltered system provides the same advantages as the filtered protocol, e.g., enhanced enantiopurity, compared to the stepwise synthesis. Additionally, the process is further simplified by eliminating the filtration step.

We also investigated if it would be possible to perform a genuine cascade process with all the ingredients, rhodium catalyst as well as buffer and enzyme, already present from the beginning. Under these conditions **2** was subjected to hydrogenation at 5 bar H_2 and 22 °C. However, after 24 h only a negligible amount of hydrogen was consumed. Visual inspection of the one-pot reaction revealed that the enzyme had been adsorbed onto the support of the hydrogenation catalyst, thereby greatly diminishing accessibility of the substrate. To determine whether the buffer also influenced the hydrogenation activity, the hydrogenation of **2** utilizing **1**-AlTUD-1 was conducted separately in the phosphate buffer. This revealed that the phosphate buffer is indeed part of the problem. In this medium 24% conversion with an ee of 74% was reached, compared to a conversion of 100% with an enantioselectivity of 95% in water.

In order to circumvent this problem, we modified the substrate. By switching from the ester to the sodium salt of **3a**, the hydrolysis reaction becomes pH neutral, thus eliminating the need for a buffer. AM requires the presence of buffer for the hydrolysis of **3a**. The drop in pH caused by the ester hydrolysis completely deactivates AM in non-buffered media. Furthermore, immobilization of the enzyme will overcome its adsorption on the TUD-1 surface. After screening several immobilized acylases, the crosslinked enzyme aggregate (CLEA) of AM was selected[16] due to its superior activity. This modified one-pot reaction is depicted schematically in Figure 4.

Unfortunately, the hydrogen consumption was very slow under these conditions. After 24 h merely 7% conversion of the substrate was reached. However, the product consisted entirely of **4**, demonstrating that the enzyme does not lack activity in this system, even though accurate data on the enzyme activity cannot be derived from these results. Surprisingly, the enzyme did not hydrolyse the remainder of the substrate, the unhydrogenated **2**, as no side products were detected in the reaction

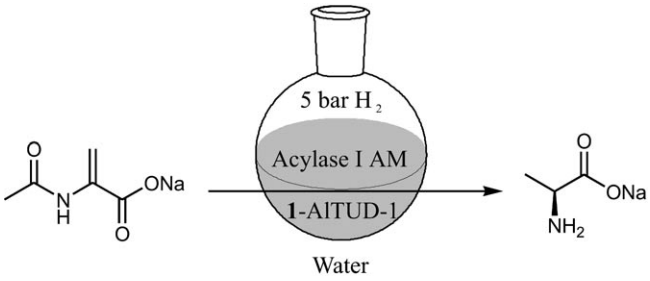


Figure 4. Schematic representation of the modified one-pot procedure.

mixture. The spatial requirements of the unsaturated amino acid most likely hinder the productive docking of the substrate into the active site. Apparently the activity of **1**-AlTUD-1 in this system is still inhibited, despite the absence of phosphate buffer or free enzyme. An explanation for these results is suggested by the report of Malmström et al.,[17] who demonstrated that the rates of the rhodium-catalysed hydrogenation of 2-acetamidoacrylic acid are strongly dependent on the pH of the mixture. They showed that the steepest change occurred between pH of 4.5 and 3.2, which coincides with the pK_a value of 2-acetamidoacrylic acid. From their NMR data it could be deduced that by going from protonated to completely unprotonated substrate, the coordination mode of the olefin changes. At low pH the chelate complex involves the double bond and the amide carbonyl, while at higher pH it consists of the double bond and carboxylate anion. This carboxylate complex undergoes oxidative addition of hydrogen much slower, which explains the poor hydrogenation activity in our modified one-pot setup.

Conclusion

We have successfully combined immobilised Rh-MonoPhos (**1**-AlTUD-1) and acylase I, leading to a chemo-enzymatic cascade for the enantioselective synthesis of amino acids. This entirely catalytic sequence offers several major advantages compared to the classic amino acid synthesis *via* asymmetric hydrogenation followed by chemical hydrolysis. By employing this cascade, the formation of stoichiometric amounts of salts and the use of organic solvents are eliminated and the number of reaction steps is reduced to one. In addition to creating a genuinely sustainable process, this methodology also produces a superior product, since the enantiopurity is greatly enhanced. This truly demonstrates the power of smart reaction design. The key to the successful combination is the compartmentalisation of both catalysts. We have demonstrated the feasibility of a one-pot procedure, superior results being obtained in a sequential protocol. Even when comparing the two investigated sequential protocols, with and without filtration, the one with the higher degree of compartmentalisation (filtered) is preferred, since it offers the possibility to recycle the hydrogenation catalyst. Having demonstrated the effectiveness of compartmentalisation by catalyst immobilisation in the amino acid synthesis to achieve sustainable processes, we believe it to be widely applicable in chemical synthesis. We also note that the process is amenable to operation in a membrane reactor in which the organometallic catalyst and the enzyme are compartmentalised on different sides of an ultrafiltration membrane.

Experimental Section

General Remarks

Reactions and manipulations involving air-sensitive compounds were performed under an atmosphere of dry nitrogen using standard Schlenk-type techniques. Dry solvents for the synthesis of the catalysts were purchased from Aldrich and deoxygenated before use. Solvents used in dehydrogenation were also deoxygenated before use. Methyl 2-acetamidoacrylate,[10] bis(1,5-cyclooctadiene)rhodium tetrafluoroborate,[18] (*R*)-MonoPhos,[19] $[Rh^I(cod)((R)\text{-MonoPhos})_2]BF_4$[9] and acylase CLEA[16] were prepared according to literature procedures. Chloro(1,5-cyclooctadiene)rhodium dimer was purchased from Strem. Acylase I from *Aspergillus melleus* was obtained from Fluka and acylase I from porcine kidney grade II from Sigma. All other reagents were purchased from Aldrich, Acros or Fluka and used without further purification. Hydrogenations were performed in a 100-mL Parr hastelloy C autoclave (A1128HC). NMR spectra were recorded on a Varian Inova 300 MHz or a Varian VXR 400 s spectrometer, relative to TMS. Enantiomeric excesses of **3a** were determined by chiral GC using a Shimadzu GC-17A, equipped with a Chiralsil DEX CB column (25 m × 0.32 mm, df = 0.25 μm), He as carrier gas, split injector (36/100) at 220 °C and FID at 220 °C. Retention times (min) at 95 °C isotherm: 2-acetamidoacrylate (**2**) (5.4), (*S*)-methyl 2-acetamidopropanoate (*S*-**3a**) (7.5) and (*R*)-methyl 2-acetamidopropanoate (*S*-**3a**) (8.1). Enantiomeric excesses of **4** were determined by chiral HPLC using a Crownpak CR (+) column (150 × 4 mm) with $HClO_4$ (pH = 1) as eluent, a flow of 0.5 mL/min at 0 °C and UV detection at 215 nm. Retention times (min): D-alanine (4.6) and L-alanine (10.8). The rhodium content of the immobilised catalysts were measured using instrumental neutron activation analysis (INAA), which was performed at the Interfaculty Reactor Institute (IRI), Delft. The "Hoger Onderwijs Reactor", with a neutron flux of 10^{17} neutrons s^{-1} cm^{-2}, was used as a source of neutrons and the gamma spectrometer was equipped with a germanium semiconductor as detector.

Immobilization Procedure for $[Rh^I(cod)((R)\text{-MonoPhos})_2]BF_4$

AlTUD-**1** (1.0 g) was dried at 200 °C under vacuum for 2 h. To the dried support 2-propanol (45 mL) was added. After 30 min stirring, Rh-MonoPhos (0.15 g, 0.15 mmol) in 2-propanol

(40 mL) was added and the resulting suspension stirred for 3 h. The solid was collected by filtration and washed thoroughly with portions of 30 mL 2-propanol until the washings were colourless (approx. 5 times). Finally, the catalyst was dried at 55 °C under vacuum for 2 h. Rh loading was determined by INAA: 11 mg Rh/g support, which corresponds to an $Al_{tetrahedral}$/Rh ratio of approximately 10.

Hydrogenation Reaction of Cascade

All hydrogenation experiments were performed with 0.1 g of immobilized catalyst (~1 wt % Rh). **1**-AlTUD-1 (0.1 g) was transferred to the autoclave under a nitrogen atmosphere, followed by substrate solution (50 mL water, 0.05 M **2**). The sealed autoclave was purged with hydrogen by pressurising to 7 bars while stirring at 300 rpm, followed by release of pressure. This cycle was repeated 5 times and finally the desired pressure was applied and the stirring speed was increased to 1000 rpm. After 1 h the remaining hydrogen pressure was released and the autoclave was purged three times with nitrogen.

Enzymatic Hydrolysis Reaction of Cascade

After an optional filtration of the hydrogenation mixture (50 mL), phosphate buffer (5 mL, pH 7.5, 1.1 M) was added followed by AM (205 mg, 175 U) or PK (90 mg, 700 U). This mixture was shaken for 24 h during which samples were obtained. The samples were adjusted to pH 5 with 1 M HCl, heated to 60 °C with Norit, filtered over Celite and lyophilised. Conversions were determined by ^{1}H-NMR. Before determining the enantioselectivity, the samples were passed over a DOWEX-50 (H^+) column, which was rinsed with water until neutral, followed by ammonia (1 M). The ammonia layer was again lyophilised.

One-Pot Procedure

1-AlTUD-1 (0.1 g) and acylase I from *Aspergillus melleus* (82 mg) were transferred to the autoclave under a nitrogen atmosphere, followed by substrate solution [50 mL, 0.05 M in phosphate buffer (pH 7.5, 0.1 M) for the one-pot or in water for the modified one-pot protocol, respectively]. The sealed autoclave was purged with hydrogen by pressurising to 7 bars while stirring at 300 rpm, followed by release of pressure. This cycle was repeated 5 times and finally the desired pressure was applied and the stirring speed was increased to 1000 rpm. After 24 h the remaining hydrogen pressure was released and the autoclave was purged three times with nitrogen.

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