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Chemo-enzymatic preparation of chiral 3-aminopyrrolidine derivatives

Hans Iding,[a,*] Beat Wirz[a] and Mark Rogers-Evans[b]

[a]*Non-clinical Development—Biotechnology, F. Hoffmann-La Roche Ltd, Basel, Switzerland*
[b]*Pharmaceutical Research Basel—Discovery Chemistry, F. Hoffmann-La Roche Ltd, Basel, Switzerland*

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Abstract—A new simple method for the enantioselective enzymatic hydrolysis of N-protected D-asparagine esters suitable for the use on the preparative scale is presented. Due to major obstacles observed under conventional reaction conditions—racemization of the retained ester and a strong enzyme inactivation—a comparatively low pH together with an organic co-solvent had to be employed. Under these conditions, nearly all tested proteases demonstrated good activity and excellent enantioselectivity giving access to the corresponding D-esters and L-asparagines in high optical purities (>95% ee) and good chemical yields (>40%). From the unnatural D-asparagine derivative, sequential cyclization, selective deprotection and reduction yielded efficiently benzyl protected (*R*)-3-aminopyrrolidine, a homo-chiral building block utilized in numerous drug candidates.

1. Introduction

Natural amino acids as well as their antipodes provide the source of chirality in the production of many enantiopure compounds and their efficient preparation is a continuing source of interest. The preparation of D-asparagine, for example, is conducted principally by the resolution of its racemate (or close derivatives) via chromatography,[1] diastereomeric salt separation[2] or industrially via whole cell biotransformation[3] (there are no asymmetric syntheses described).

Besides these classical resolution methods there exist further known enzymatic routes to enantiomerically pure D-asparagine derivatives: the common enzymatic hydrolysis of D,L-5-substitued hydantoins using a D-hydantoin hydrolase and a *N*-carbamoyl-D-amino acid hydrolase.[4] In addition, the newly discovered D-aminoacylase from the genera *Sebekia* and *Amycolatopsis* might be applied as catalysts for the enzymatic preparation of D-asparagine[5] though the purified enzymes have a specific activity towards *N*-acetyl-D-asparagine of only 1.4% and 19%, respectively, as compared to *N*-acetyl-D-methionine (100%).

However, surprisingly, the most conventional method, the enzymatic hydrolysis of racemic asparagine ester derivatives had remained unexplored to the best of our knowledge. Herein we describe the identification of active and selective hydrolases for the resolution of N-protected D,L-asparagine esters and the development of a scalable biotransformation under technically relevant conditions (Scheme 1).

2. Results and discussion

2.1. Screening of enzymatic hydrolysis activity and selectivity

The substrate esters were prepared from the racemic N-protected D,L-asparagines **3** and **4** by esterification with thionylchloride of the respective alcohol. All proteases tested displayed a reasonable activity with racemic ester **1a** in a well plate assay based on the color change of a pH-indicator. Re-evaluation of the four cheapest enzymes in a pH-stat experiment revealed only moderate selectivites (Table 1) when stopped after approximately 50% conversion. A repetition of these experiments at a higher conversion degree of approximately 55% did not result in an increased enantiomeric purity for the remaining ester **1a** (Table 1) as one would expect according to Sih and Girdaukas.[6]

2.2. pH-dependent racemization

As such modest enantioselectivities of proteases towards an amino acid ester substrate would be unusual, and based on the fact the ee-values for **1a** tended to be lower with prolonged reaction time (Fig. 1) we suspected racemization

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* Corresponding author. Tel.: +41-61-688-0486; fax: +41-61-688-1673; e-mail address: hans.iding@roche.com

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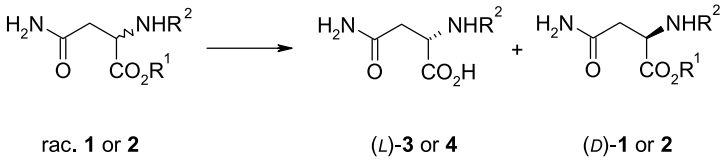


compound	R^1	R^2
1 a	Me	Cbz
1 b	Et	Cbz
1 c	Bu	Cbz
2 b	Et	Bz
2 d	Bn	Bz
3	-	Cbz
4	-	Bz

Scheme 1. Resolution of different N-protected D,L-asparagine esters.

Table 1. Screening for the resolution of *N*-benzyloxycarbonyl-D,L-asparagine methyl ester rac. **1a** with different proteases

Protease[a]	Conversion (%)	Reaction time (h)	ee of **1a** (%)	~*E*
ALC	50	0.6	82	25
NUE	50	1.0	79	20
PZ	50	1.2	77	18
SP539	50	20	57	6
ALC	55	5	75	9.5
NUE	54	10	63	6
PZ	55	9	74	9

[a] The abbreviations are given in Section 4.2. Reaction conditions were similar to General procedure (Section 4.4), except for (i) a lower substrate concentration of 30 mg rac. **1a**, (ii) a protease aliquot of only 3 mg lyophilisate or 30 μl solution and (iii) a pH of 7.0 was used.

of the remaining optical enriched ester to be a possible explanation.

Reducing the pH of the reaction resulted indeed in higher enantiomeric excess of **1a** as was demonstrated by the protease Alcalase, but, as expected, at the cost of enzymatic activity (data not shown). A pH of 6.5 was chosen as a suitable compromise between the stability of the remaining ester **1a** with respect to racemization and sufficient enzymatic activity. In addition, one has to avoid also acidic conditions during the work up of the enantiomerically pure ester **1a**, because we recognized a slow racemization of analytical samples at pH~2. The cheap bulk protease Alcalase and several other microbial proteases turned out to be highly enantioselective catalysts for the synthesis of enantiopure remaining methyl ester **1a** (Table 2).

After verifying the former working thesis that the proteases are highly enantioselective catalysts under reaction conditions preventing the racemization of the optically pure

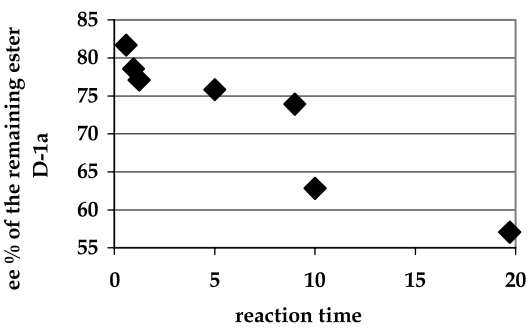


Figure 1. The enantiomeric excess of the retained methyl ester **1a** from different biotransformations (Table 1) in dependence of the reaction time.

ester, the transformation should be transferred to a preparative scale.

2.3. Enzyme inhibition at higher substrate concentrations

On a more preparative scale at higher substrate concentrations the enzymatic hydrolysis unexpectedly stopped after a few percent conversion with several proteases and ester substrates (rac. **1a**; **1b**, **1c**, **2b**; **2d**). The enzyme inhibition seemed to be a robust feature in this biocatalytic conversion of asparagine derivatives and could be observed already at a substrate concentration of 2% (Table 3).

When we tried to overcome the poor substrate solubility by adding an organic co-solvent we found—again unexpectedly—that this inactivation was overcome in the presence of the co-solvent. The addition of an organic co-solvent like THF in a concentration of ~16% before addition of the enzyme eliminated inhibition previously encountered with the different substrates and proteases. The effect was observed with water- miscible and immiscible solvents. Interestingly, the inhibition seems to be irreversible. In control experiments with addition of an organic co-solvent after the inhibition had set in the enzyme activity was not recovered. Therefore, the prevention of the inhibition can not be explained with substrate solubilization as mode of action of the solvent (Table 3).

The different organic co-solvents affected the activity of the

Table 2. Preparation of enantiopure remaining methyl ester **1a** with different proteases at approximately 50% conversion

Protease[a,b]	Microbial origin	Reaction time (h)	ee of **1a** (%)
ALC	*Bacillus licheniformis*	0.4[b]	>99
SAV	*Bacillus*	0.8[b]	>99
EA	not defined	2.0[b]	>99
FGF	*Bacillus subtilis*	1.4[b]	>99
BPN	*Bacillus subtilis*	0.5[a]	>99
NUE	not defined	2.3[a]	>99
K	*Tritirachium album*	2.1[a]	94
PZ	*Aspergillus melleus*	1.5[a]	98

[a] The abbreviations are given in Section 4.2. Reaction conditions were similar to General procedure (Section 4.4), except for (i) a lower substrate concentration of 30 mg rac. **1a**, (ii) a protease aliquot of only 3 mg lyophilisate or 30 μl solution and (iii) a pH of 7.0 was used.

[b] The abbreviations are given in Section 4.2. Reaction conditions were similar to General Procedure (Section 4.4), except for (i) a lower substrate concentration of 15 mg rac. **1a**, (ii) a protease aliquot of only 3 mg lyophilisate or 30 μl solution and (iii) a pH of 7.0 was used.

Table 3. Enzyme inhibition and the influence of the addition of an organic co-solvent

#	Protease	Organic co-solvent	Compound	Amount enzyme	Substrate concentration (%)	Conversion[a] (%)	Reaction time (h)	ee ester (%)
a	ALC	—	**1a**	100 μL	5	13	Reaction stops, no work up	
b	ALC	Ethanol	**1a**	100 μL	5	48	22	98
c	ALC	Ethyl acetate	**1a**	100 μL	5	54	3.5	>99
d	ALC	TBME	**1a**	100 μL	5	48	5.5	>99
e	ALC	Acetone	**1a**	100 μL	5	49	5.0	>99
f	SAV	—	**1a**	100 μL	5	<1	Reaction stops, no work up	
g	SAV	THF	**1a**	100 μL	5	50	18	>99
h	K	—	**1b**	4.0 mg	2	7	Reaction stops, no work up	
I	K	THF	**1b**	4.2 mg	2	48	3.2	97
j	PSG	—	**1b**	4.0 mg	2	5	Reaction stops, no work up	
k	PSG	THF	**1b**	4.0 mg	2	48	12	92
l	ALC	—	**2b**	50 μL	2	10	Reaction stops, no work up	
m	ALC	THF	**2b**	50 μL	2	53	17	97
n	SAV	—	**2b**	50 μL	2	11	Reaction stops, no work up	
o	SAV	THF	**2b**	50 μL	2	45	46	93
p	ALC	—	**1c**	50 μL	2	3	Reaction stops, no work up	
q	ALC	Dioxane	**1c**	50 μL	2	51	48	98
r	ALC	—	**2d**	50 μL	2	14	Reaction stops, no work up	
s	ALC	Acetone	**2d**	50 μL	2	47	10	>99

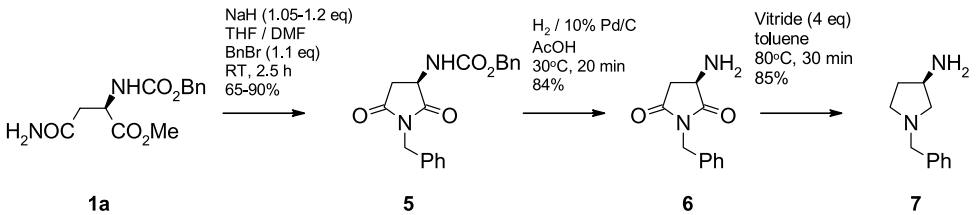
[a] The conversion was calculated from the base consumption in the pH-static experiments. Reaction conditions, see General procedure (Section 4.4). The abbreviations are explained in Section 4.2.

proteases with the methyl ester **1a**, but the selectivity remained nearly absolute. Ethanol as co-solvent reduced the activity roughly four times stronger as compared to the best water-miscible solvent acetone. The water-immiscible solvent TBME provided nearly the same activity as acetone. Homogeneity of the transformation mixture did not seem to play a role: the bi-phasic co-solvent ethyl acetate afforded the highest activity (Table 3). As a further result of the experiments shown in Table 3, the methyl ester **1a** turned out to be the best substrate with respect to enzymatic activity.

Nevertheless, different esters (**1a**; **1b**; **1c**; **2b** and **2d**) were synthesized in gram-amounts with high enantiomeric purities (>95% ee) and in a good chemical yields (>40%). Besides, also the formed acids **3** and **4** were isolated in high optical purities (>99% ee) and good chemical yields (>40%). The separation and isolation of the enantiomerically pure reaction products, D-esters and L-acids, was achieved by conventional extraction.

On the preparative scale substrate concentrations above 7% (overall) became problematic due to the viscosity of the suspension formed. Therefore, an even larger scale the stirring conditions are expected to be a critical issue.

With these esters in hand, it was possible to elaborate them into key precursors, for example ester **1a** was submitted to sequential cyclization, selective deprotection and reduction yielding efficiently the benzyl protected (*R*)-3-amino-pyrrolidine,[7] a homo-chiral building block utilized in the preparation of numerous drug candidates (Scheme 2).[8] In particular, we have exploited the use of this pyrrolidine in the assembly of a broad spectrum cephalosporin.[7a] Although each step had some literature precedent, some modifications were necessary; thus although a one-pot cyclization and benzylation of ester **1a** to benzylmide **5** has been described for its enantiomer (aq. NaOH/BnBr/cat. NBu_4I),[9] it proved not possible to perform this procedure without incurring significant racemization (47% yield, 55.4% ee). However replacing the base with NaH gave only minor racemization which could be rectified by one re-crystallization (65% yield, >99.9% ee). Additionally deprotection of the Cbz-protected imide **5** had been described for its enantiomer using $Pd(OH)_2$[9] or Pd/C[10] in MeOH. We found no conversion using these systems; however employing AcOH as the solvent, allowed the hydrogenation to proceed smoothly in just 20 min at room temperature, facilitating the isolation of the unstable amine **6** as the corresponding acetate salt in 84% yield, which could be stored without problems. Carefully controlled liberation of the free amine at pH 8 (quantitative) and immediate reduction with Vitride in toluene (borane led to decomposition and $LiAlH_4$ gave isolation problems) resulted in the recovery of the desired amine **7** in 87% yield with no racemization.



Scheme 2. Synthesis of *N*-benzyl (*R*)-3-aminopyrrolidine from enantiopure ester **1a** via cyclyzation, selective deprotection and reduction.

3. Conclusion

An attractive chemo-enzymatic route for the preparation of N-protected (*R*)-3-aminopyrrolidines has been established based on an efficient racemic resolution of N-protected D,L-asparagine esters with proteases as the key step. Beside the retained D-esters also the respective L-acids were obtained in high enantiomeric purity and chemical yield by this enzymatic step. The enzyme inhibition encountered at higher substrate concentrations could be overcome by application of an organic co-solvent. The reaction has been successfully carried out on the multi-gram scale.

4. Experimental

4.1. General

NMR-spectra: Bruker DPX 400 MHz. IR-spectra: Nicolet, FT-IR 20 SXB. EI-MS-spectra: SSQ7000 (Finnigan MAT). EI-, ISN- and ISP-MS-spectra. Optical rotations: Perkin–Elmer Polarimeter 241.

4.2. Materials

The proteases used for the preparative experiments were Savinase 6.0T (*Bacillus*, *SAV*); *SP539* (*Bacillus*); Alcalase 2.4L (*Bacillus sp.*, *ALC*); *NUE* 6.0 S, Typ 1; Esperase 6.0 T (*EA*) from Novo Nordisk, subitilsin *BP N*(Nagarse) from Sigma, Protase *K* (*Tritichium album*); Pronase E (*Streptomyces griseus*, *PSG*) from Fluka, Prozyme 6 (*Aspergillus melleus*, *PZ*); Proleather *FG-F* from Amano. All other reagents were purchased from Fluka or Merck and used directly.

4.2.1. *N*-Benzyloxycarbonyl-D,L-asparagine methyl ester 1a. A suspension of 40.0 g (150.2 mmol) *N*-benzyloxycarbonyl-D,L-asparagine **3** [Bachem C-1310] in 500 mL methanol was cooled to 0 °C. Then 34.0 mL (462.6 mol, 3.08eq.) of thionyl chloride was added dropwise so that the temperature remained below 10 °C. The mixture was stirred at 0 °C for another 15 min. The reaction mixture was evaporated together with 1 L toluene (after 500 mL of volume was reached, 200 mL methanol was added and evaporation continued; 40 °C bath temperature). The resulting white, crystalline residue was triturated/digested overnight in 600 mL TBME. The residue was filtered off and dried at HV to give 38.45 g of the rac. methyl ester **1a** as a white crystalline powder (yield: 91%). Analytics: SFC: 99.5% area. EI-MS: 280.1, 221.2 ($M-CO_2Me$), 173.1 ($M-COPh$). IR (Nujol) (NH) 3318, (CO_2Me) 1742, (NCO_2) 1682, (NCO) 1665 and 1558, 1219, 757, 698 cm^{-1}. ^1H NMR ($CDCl_3$): 2.76 (dd, J=4, 17 Hz, 1H, CH), 2.98 (dd, J=4, 17 Hz, 1H, CH), 3.76 (s, 3H, OCH_3), 4.60 (m, 1H, CHCOO), 5.13 (s, 2H, $-CH_2O$), 5.45 and 5.55 (2×bs, 2H, $CONH_2$), 6.00 (bd, $J\sim8$ Hz, <1H, OCONH), 7.26–7.37 (m, 5H, Ph).

4.2.2. *N*-Benzyloxycarbonyl-D,L-asparagine ethyl ester 1b. Similar to the above (Section 4.2.1) the racemic crystalline ethyl ester **1b** was prepared with a yield of 79%. Analytics: HPLC: >99% area. ISP-MS: 317.2 ($M+Na^+$), 295.3 ($M+H^+$). ^1H NMR (DMSO): 1.15 (t, J=6 Hz, 3H, CH_3), 2.42–2.58 (m, ~2H, CH_2), 4.07 (q, J=6 Hz, 2H, CH_2O), 4.39 (m, 1H, CH), 5.03 (s, 2H, CH_2O), 6.93 (bs, 1H, $CONH_2$), 7.31–7.37 (m, 6H, Ph and $CONH_2$), 7.60 (d, $J\sim8$ Hz, 1H, CONH).

4.2.3. *N*-Benzyloxycarbonyl-D,L-asparagine butyl ester 1c. Similar to the above (Section 4.2.1) the racemic solid butyl ester **1c** was prepared with a yield of 67%. Analytics: HPLC: >99% area. ISP-MS: 345.3 ($M+Na^+$), 323.3 ($M+H^+$). ^1H NMR (DMSO): 0.87 (t, J=6 Hz, 3H, CH_3), 1.31 (m, 2H, CH_2), 1.51 (m, 2H, CH_2), 2.42–2.59 (m, ~2H, CH_2), 4.03 (m, 2H, CH_2O), 4.40 (m, 1H, CH), 5.03 (m, 2H, CH_2O), 6.93 (bs, 1H, $CONH_2$), 7.29—7.39 (m, 6H, Ph and $CONH_2$), 7.60 (d, $J\sim4$ Hz, 1H, CONH).

4.2.4. *N*-Benzoyl-D,L-asparagine 4. A solution of 20.0 g (0.15 mol) D,L-asparagine, 120 mL deionized H_2O, 20 g sodium carbonate and 20 mL THF was heated to 55 °C. A solution of 20 mL THF and 21 mL of benzoyl chloride (0.18 mol) was added dropwise during 2 h at 55 °C. The reaction mixture was stirred for another 1 h and then cooled to 15 °C. The pH was adjusted to 2.5 and the reaction mixture was stirred for 0.5 h at 15 °C. The crystalline asparagine **4** was filtered, washed thrice with 75 mL H_2O and dried at HV. The yield was 32.4 g (87%). Analytics: HPLC: 95.9% area. ISN-MS: 235.2 ($M-H^-$), 217.2 ($M-H_2O$). ^1H NMR (DMSO): 2.50–2.72 (m, 2H, CH_2), 4.72 (m, 1H, CH), 6.94 (bs, 1H, $CONH_2$), 7.39 (bs, 1H, $CONH_2$), 7.46–7.85 (m, 5H, Ph), 8.64 (d, J=8 Hz, 1H, CONH), 12.6 (s, 1H, COOH).

4.2.5. *N*-Benzoyl-D,L-asparagine ethyl ester 2b. Similar to the above (Section 4.2.1) the racemic crystalline ethyl ester **2b** was prepared with a yield of 65%. Analytics: HPLC: >99% area. EI-MS: 264.2 (M), 191.2 ($M-COOEt$). ^1H NMR (DMSO): 1.16 (t, J=8 Hz, 3H, CH_3), 2.57–2.72 (m, 2H, CH_2), 4.09 (q, J=8 Hz, 2H, CH_2O), 4.75 (m, 1H, CH), 6.95 (bs, 1H, $CONH_2$), 7.40 (bs, 1H, $CONH_2$), 7.46–7.85 (m, 5H, Ph), 8.75 (d, J=8 Hz, 1H, CONH).

4.2.6. *N*-Benzoyl-D,L-asparagine benzyl ester 2d. Similar to the above (Section 4.2.1) the racemic crystalline benzyl ester **2d** was prepared with a yield of 33%. Analytics: HPLC: >99% area. ISP-MS: 349.4 ($M+Na^+$), 327.3 ($M+H^+$). ^1H NMR (DMSO): 2.61–2.78 (m, 2H, CH_2), 4.84 (m, 1H, CH), 5.14 (s, 2H, OCH_2), 6.98 (bs, 1H, $CONH_2$), 7.30–7.85 (m, 11H, 2xPh and $CONH_2$), 8.84 (d, J=8 Hz, 1H, CONH).

4.3. Activity assay

Into 96 well plates pre-loaded with 0.5 mg enzyme/well a buffered pH-indicator solution (190 μl, 7.5 mM Tris/HCl, pH 8.0, 0.02% NaN_3, 50 mg/l cresol red) and the substrate solution (1 mg **1a** in a mixture of 2.5 μl DMF and 7.5 μl EtOH) were added with a liquid handler (Lissy; Zinsser Analytics). The color change of the indicator from red to yellow was monitored for up to 1 day at 410 nm with a well plate reader (Tecan Sunrise) and a well plate autosampler (Twister).

4.4. General procedure for the preparation of different N-protected D-asparagine esters (cf. Table 3)

A suspension of 0.5 g (1.76–1.53 mmol) [2 g (7.0 mmol)] *N*-protected asparagine ester in 23 mL [28 mL] 0.1 M sodium chloride solution, 2 mL [4 mL] 0.1 M sodium phosphate buffer pH 7.0 and in the presence or absence (Tables 1 and 2) of 4 mL [8 mL] organic co-solvent was vigorously stirred. The pH was adjusted to 6.5 with 1.0 N hydrochloric acid and the reaction started by addition of the protease (Table 3). The pH was maintained at 6.5 under vigorous stirring by the controlled addition (pH-stat) of 1.0 N sodium hydroxide solution. The experiments without organic co-solvent showing incomplete conversion (Table 3) were not worked up, and the experiments with organic co-solvent were treated as follows: After approximately 50% conversion the reaction mixture was extracted with 2×25 mL [3×30 mL] dichloromethane. The combined organic phases were dried on anhydrous sodium sulfate and evaporated at 35 °C. The residue containing the retained ester was subjected to ee-determination (methods see the corresponding experiments on a larger scale).

4.4.1. *N*-Benzyloxycarbonyl-D-asparagine methyl ester 1a. A suspension of 18.5 g (65.7 mmol) methyl ester rac. **1a** (99.5%) in 190 mL 0.1 M sodium chloride solution, 40 mL 0.1 M sodium phosphate buffer pH 6.5 and 25 mL THF was vigorously stirred. 1.0 mL Alcalase 2.4 L was added and the pH maintained at 6.5 under vigorous stirring by the controlled addition (pH-stat) of 1.0 N sodium hydroxide solution. The hydrolysis was terminated in 8 h with a consumption of 32.0 mL (49% conversion) of titrating agent. After 16 h the reaction mixture was extracted trice with 350 mL dichloromethane. The combined organic phases were washed with 500 mL 20 mM sodium phosphate buffer pH 7.0, dried on anhydrous sodium sulfate and evaporated (at 25 °C bath temperature). The residue was triturated overnight in 200 mL TBME. The suspension was filtered and the filter cake dried at HV to give 8.2 g the methyl ester **1a** as a white crystalline powder (yield: 46%). Enantiomeric excess: >99.5% (Chiracel ODH, 15 cm×300 μm, 85% *n*-hexane+15% isopropanol, 6 μl/min, 30 °C, 210 nm). $[\alpha]_D$=+0.97 (*c*=1.6; AcOH). SFC: 98.8% area. EI-MS: 280.2 (M), 221.1 (M−CO_2Me). IR (Nujol): (NH) 3349, (CO_2Me) 1741, (NCO_2) 1665, (NCO) 1550, 733, 698 cm^{-1}. ^{1}H NMR (CDCl3): 2.76 (dd, *J*=4, 16 Hz, 1H, CH), 2.98 (dd, *J*=4, 16 Hz, 1H, −CH), 3.76 (s, 3H, OCH_3), 4.60 (m, 1H, $CHCO_2$), 5.13 (s, 2H, CH_2O), 5.42 and 5.53 (2×bs, 2H, $CONH_2$), 6.00 (bd, *J*~8 Hz, <1H, OCONH), 7.29–7.37 (m, 5H, Ph).

4.4.2. *N*-Benzyloxycarbonyl-L-asparagine 3. In analogy to Section 4.4.1 10.0 g (35.1 mmol) of the methyl ester rac. **1a** (98%) was suspended in 140 mL 0.1 M sodium chloride solution, 20 mL 0.1 M sodium phosphate buffer pH 6.5 and 40 mL THF under vigorous stirring. 0.5 mL Alcalase 2.4 L was added and the pH maintained at 6.5 under vigorous stirring by the controlled addition (pH-stat) of 1.0 N sodium hydroxide solution. After a consumption of 16.42 mL of 1.0 N sodium hydroxide solution after 2.1 h (corresponds to 47% conversion) the reaction mixture was extracted with 3×200 mL dichloromethane to remove the uncleaved methyl ester. The aqueous phase was concentrated to approximately 50 mL volume using 200 mL of toluene. The pH was adjusted to 3.5 with 25% hydrochloric acid. The formed precipitate was filtered off and triturated overnight in 300 mL of deionized water. The suspension was filtered and the filter cake dried on HV to give 4.07 g (44%) of **3** as white crystals. Analytics: enantiomeric excess: >99% (Chiracel ODH, 25 cm×4.6 mm, 85% *n*-heptane+15% isopropanol, 0.8 mL/min, room temperature, 220 nm). $[\alpha]_D$=+5.4 (*c*=2.0; AcOH)[†] ($[\alpha]_D$=−6.7 (*c*=1.1; DMSO) determined in another experiment). HPLC: >99% area. ISN-MS: 265.3 ($M-H^-$). IR (Nujol): (NH) 3337, (CO_2H) 1697, (NCO_2) 1643, 1536, 1268, 737, 695 cm^{-1}. ^{1}H NMR (DMSO): 2.41–2.58 (m, 2H, CH_2), 4.34 (m, 1H, CH), 5.03 (s, 2H, CH_2O), 6.92 (bs, 1H, $CONH_2$), 7.26–7.40 (m, 6H, Ph and $CONH_2$), 7.44 (bd, *J*~8 Hz, 1H, OCONH), 12.67 (bs, 1H, −COOH).

4.4.3. *N*-Benzyloxycarbonyl-D-asparagine ethyl ester 1b. A suspension of 10.0 g (34.0 mmol) ethyl ester rac. **1b** in 460 mL 0.1 M sodium chloride solution, 40 mL 0.1 M sodium phosphate buffer pH 7.0 and 80 mL THF was vigorously stirred. The pH was adjusted to 6.5 with 1.0 N hydrochloric acid and the reaction started by addition of 1.0 mL Savinase 16 L. The pH was maintained at 6.5 under vigorous stirring by the controlled addition (pH-stat) of 1.0 N sodium hydroxide solution. After a consumption of 16.45 mL 1.0 N sodium hydroxide solution (48.2% conversion; after 17 h) the reaction mixture was extracted with 2×500 mL dichloromethane. The combined organic phases were dried on anhydrous sodium sulfate, evaporated at 35 °C and the residue dried on HV to give 5.02 g of the ethyl ester **1b** as white crystals (yield: 50%). Enantiomeric excess: 98.9% (Chiracel ODH, 15 cm×2.1 mm, 87% *n*-heptane+13% isopropanol+0.1% TFA, 100 μl/min, room temperature, 220 nm). $[\alpha]_D$=+12.2 (*c*=1.0; EtOH). HPLC: >99% area. ISP-MS: 317.2 ($M+Na^+$), 295.3 ($M+H^+$). ^{1}H NMR (DMSO): 1.16 (t, *J*=8 Hz, 3H, CH_3), 2.42–2.58 (m, ~2H, CH_2), 4.07 (q, *J*=8 Hz, 2H, CH_2O), 4.39 (m, 1H, CH), 5.03 (m, 2H, CH_2O), 6.93 (bs, 1H, $CONH_2$), 7.31–7.37 (m, 6H, Ph and $CONH_2$), 7.60 (d, *J*~8 Hz, 1H, CONH).

4.4.4. *N*-Benzyloxycarbonyl-D-asparagine *n*-butyl ester 1c. A suspension of 4.0 g (12.41 mmol) *n*-butyl ester rac. **1c** in 180 mL 0.1 M sodium chloride solution, 16 mL 0.1 M sodium phosphate buffer pH 7.0 and 30 mL dioxan was vigorously stirring. The pH was adjusted to 6.5 with 1.0 N hydrochloric acid and the reaction started by addition of 400 μl Alcalase 2.4 L. The pH was maintained at 6.5 under vigorous stirring by the controlled addition (pH-stat) of 1.0 N sodium hydroxide solution. After 66 h and 87 h an additional 200 μl of Alcalase-solution was added to the reaction. After a total consumption of 5.73 mL 1.0 N sodium hydroxide solution (46% conversion; after totally 91 h) the reaction mixture was extracted with 2×200 mL dichloromethane. The combined organic phases were dried on anhydrous sodium sulfate, evaporated at 35 °C and the residue triturated overnight in 50 mL TBME. The solid was filtered off, the filter cake washed with TBME and dried on HV to give 1.61 g of the butyl ester **1c** as white solid (yield: 40%). Enantiomeric excess: 95% (Chiracel ODH, 15 cm×0.3 mm, 90% *n*-heptane+10% isopropanol, 5 μl/min, 30 °C, 210 nm). $[\alpha]_D$=+19.9 (*c*=1.0; DMSO). HPLC:

98.9% area. ISP-MS: 345.3 ($M+Na^+$), 323.3 ($M+H^+$). ^1H NMR (DMSO): 0.87 (t, J=8 Hz, 3H, CH_3), 1.31 (m, 2H, CH_2), 1.52 (m, 2H, CH_2), 2.43–2.59 (m, ~2H, CH_2), 4.03 (m, 2H, CH_2O), 4.40 (m, 1H, CH), 5.03 (m, 2H, CH_2O), 6.93 (bs, 1H, $CONH_2$), 7.29–7.39 (m, 6H, Ph and $CONH_2$), 7.60 (d, J~12 Hz, 1H, CONH).

4.4.5. *N*-Benzoyl-D-asparagine ethyl ester 2b. A suspension of 3.5 g (13.2 mmol) ethyl ester rac. **2b** in 55 mL 0.1 M sodium chloride solution, 5 mL 0.1 M sodium phosphate buffer pH 7.0 and 8 mL THF was vigorously stirred. The pH was adjusted to 6.5 with 1.0 N hydrochloric acid and the reaction started by addition of 350 μl Alcalase 2.4 L The pH was maintained at 6.5 under vigorous stirring by the controlled addition (pH-stat) of 1.0 N sodium hydroxide solution. After a consumption of 6.44 mL 1.0 N sodium hydroxide solution (49% conversion; after 23 h) the reaction mixture was extracted with 3×50 mL dichloromethane and 3×50 mL ethyl acetate. The combined organic phases were dried on anhydrous sodium sulfate, evaporated at 35 °C and the residue dried on HV to give 1.63 g of the ethyl ester **2b** as a white solid (yield: 47%). Enantiomeric excess: 98.2% (Chiralpak-AD, 25 cm×4.6 mm, 75% *n*-heptane+25% EtOH+0.2% TFA, 1 mL/min, room temperature, 220 nm). $[\alpha]_D$=+12.0 (c=1.1; DMSO). HPLC: >99% area. ISP-MS: 287.1 ($M+Na^+$), 265.3 ($M+H^+$). ^1H NMR (DMSO): 1.17 (t, J=8 Hz, 3H, CH_3), 2.57–2.72 (m, 2H, CH_2), 4.19 (q, J=8 Hz, 2H, CH_2O), 4.75 (m, 1H, CH), 6.95 (bs, 1H, $CONH_2$), 7.40 (bs, 1H, $CONH_2$), 7.46–7.85 (m, 5H, Ph), 8.75 (d, J=8 Hz, 1H, CONH).

4.4.6. *N*-benzoyl-D-asparagine benzyl ester 2d and *N*-benzoyl-L-asparagine 4. A suspension of 2.50 g (7.66 mmol) benzyl ester rac. **2d** in 115 mL 0.1 M sodium chloride solution, 10 mL 0.1 M sodium phosphate buffer pH 7.0 and 20 mL acetone was vigorously stirred. The pH was adjusted to 6.5 with 1.0 N hydrochloric acid and the reaction started by addition of 250 μl Alcalase 2.4 L. The pH was maintained at 6.5 under vigorous stirring by the controlled addition (pH-static) of 1.0 N sodium hydroxide solution. After a consumption of 3.479 mL 1.0 N sodium hydroxide solution (46% conversion; after 17.9 h) the reaction mixture was extracted with 2×125 mL dichloromethane. The combined organic phases were dried on anhydrous sodium sulfate, evaporated (at 35 °C bath temperature) and the residue triturated in 20 mL TBME overnight. The solid was filtered off and dried on HV to give 1.10 g of the benzyl ester **2d** as a white solid (yield: 44%). Enantiomeric excess >99% (Chiracel-ODH, 15 cm×2.1 mm, 87% *n*-heptane+13% iPrOH+0.1% TFA, 0.1 mL/min, room temperature, 220 nm). $[\alpha]_D$=+13.2 (c=1.2; DMSO). HPLC: >99% area. ISP-MS: 349.5 ($M+Na^+$), 327.3 ($M+H^+$). ^1H NMR (DMSO): 2.61–2.78 (m, 2H, CH_2), 4.84 (m, 1H, CH), 5.14 (s, 2H, OCH_2), 6.97 (bs, 1H, $CONH_2$), 7.32–7.84 (m, 11H, 2×Ph and $CONH_2$), 8.83 (d, J=8 Hz, 1H, CONH).

The aqueous phase was acidified to pH 2 with 25% hydrochloric acid. The formed precipitate was stirred at 1 °C overnight and filtered off. The filter cake was washed with 10 mL 10 mM hydrochloric acid and dried on HV to give 0.77 g of **4** as a white powder (yield: 43%). Enantiomeric excess: >99% (Chiralpak-AD, 25 cm×4.6 mm, 85% *n*-heptane+15% EtOH+0.12% TFA, 0.7 mL/min, room temperature, 220 nm). $[\alpha]_D$=−16.5 (c=1.0; DMSO). HPLC: 99% area. ISN-MS: 235.2 ($M-H^-$). ^1H NMR (DMSO): 2.57–2.72 (m, 2H, $-CH_2-$), 4.72 (m, 1H, −CH−), 6.94 (bs, 1H, $CONH_2$), 7.39 (bs, 1H, $CONH_2$), 7.46–7.85 (m, 5H, Ph), 8.64 (d, J=8 Hz, 1H, −CONH−), 12.6 (s, 1H, COOH).

4.4.7. (*R*)-(1-Benzyl-2,5-dioxo-pyrrolidin-3-yl-carbamic acid benzyl ester 5. A suspension of 1.12 g of 60% NaH in 75 mL of THF is treated with 7.50 g of methyl ester **1a** (99.9% (*R*)-isomer) over 5 min at room temperature. After 20 min, 3.57 mL of benzyl bromide was added followed by 120 mL of DMF. After 3 h, HPLC indicated conversion was complete. The reaction was quenched with 150 mL H_2O and extracted three times with 120 mL of toluene. The organic layer was washed with H_2O, dried over $MgSO_4$, filtered and the filtrate evaporated to dryness. The residue was triturated in 100 mL of TBME, the resultant suspension filtered and dried (35 °C/10 mbar) to give 8.13 g (90%) of the pure benzyl imide **5** as white crystals. Analytics: enantiomeric excess: 93% (Chiracel ODH, 15 cm×300 μm, 85% *n*-hexane+15% isopropanol, 6 μl/min, 30 °C, 210 nm). SFC: 100% area. Mp 143.3–144.5 °C (lit. 140.5 °C).[11] SFC: 100% area. ISP-MS: 339.2 ($M+H^+$). IR (Nujol): (NH) 3321, (NCO) 1773, 1706, (CO_2Bu) 1685 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): 2.77 (dd, J=18, 4 Hz, 1H, $COCH_2$), 3.05 (dd, J=18, 9 Hz, 1H, $COCH_2$), 4.27 (m, 1H, NCH), 4.70 and 4.65 (2× d, J=12 Hz, 2H, NCH_2), 5.08 (bs, 2H, OCH_2), 5.23 (bd, 1H, NH), 7.37–7.25 (m, 10H, Ph). Optically pure material could be obtained from crystallization from CH_2Cl_2/*n*-hexane, 72% recovery. Analytics: enantiomeric excess 99.9%. Mp 145.9–146.7 °C. Anal. Calcd for $C_{19}H_{18}N_2O_4$: C, 67.45; H, 5.36; N, 8.28. Found: C, 67.25; H, 5.27; N, 8.29.

4.4.8. (*R*)-3-Amino-1-benzyl-pyrrolidine-2,5-dione diacetic acid salt 6. A solution of 7.80 g of the benzyl imide **5** (93% (*R*)-isomer) in 160 mL of acetic acid was treated with 0.78 g of 10% Pd/C (Degussa 1835) and hydrogenated at 30 °C for 20 min whereupon TLC and HPLC indicated the reaction was complete. The reaction mixture was filtered, evaporated and the residue crystallized from EtOAc and *n*-hexane to give 5.80 g (78%) of the pure amine acetate salt **6** as white crystals. Analytics: enantiomeric excess: 91% as trifluoroacetamide (GC (BGB-177): 15 m×0.25 mm, carrier gas: He; program: 150 °C-200 °C at 1 °C /min; injector temp. 210 °C; FID: 220 °C). HPLC 100% area. GC 99.8% area (as free amine). ISP-M.S: 205.2 ($M+H^+$). ^1H NMR ($CDCl_3$, 1.6 equiv. AcOH) 2.08 (s, 2×CH_3CO_2, 6H), 2.50 (dd, J=18, 5.4 Hz, $COCH_2$, 1H), 3.05 (dd, J=7.8, 18 Hz, $COCH_2$, 1H), 3.92 (dd, J=5.4, 7.8 Hz, 1H, NCH), 5.64 (bs, 4H, NH), 4.65 (s, 2H, $PhCH_2$, 7.32 (m, 5H, Ph). MA (1.6 equiv. AcOH): Calcd for $C_{11}H_{12}N_2O_2$. 1.6 equiv. $C_2H_4O_2$: C, 56.79; H, 6.18; N, 9.33. Found: C, 56.66; H, 6.33; N, 9.23.

4.4.9. (*R*)-1-Benzyl-pyrrolidin-3-ylamine 7. A solution of 10.87 g of the amine salt **6** in 100 mL of H_2O was treated with 100 mL of CH_2Cl_2 followed by 67.60 mL of 1 N NaOH at room temperature to pH 8.0. After saturation with NaCl, the mixture was extracted seven times with 100 mL of CH_2Cl_2, dried over $MgSO_4$ and evaporated at 35 °C/10 mbar to give 6.32 g (97%) of the free base as a pale

yellow solid. NMR ($CDCl_3$, 250 MHz): 2.43 (dd, J=5, 17.5 Hz, 1H, $COCH_2$), 3.04 (dd, J=7.5, 17.5 Hz, 1H, $COCH_2$), 3.88 (dd, J=5, 7.5 Hz, 1H, NCH), 4.64 (s, 2H, $PhCH_2$), 7.30 (m, 5H, Ph).

5.90 g of this yellow oil was treated at 0 °C over 20 min with 33 mL of a 3.5 M solution of Vitride in toluene and the resultant yellow–orange solution was warmed to 80 °C for 30 min (MS indicated the reaction was complete), cooled to 0 °C and treated with 80 mL of 1 N NaOH solution. The phases were separated and the aqueous phase extracted with two further portions of 15 mL toluene. The combined organic phases were washed with 76 mL 1 N NaOH, 70 mL brine, dried and evaporated to give 4.42 g (87%) of the NMR pure amine **7**, as a light brown oil. Analytics: enantiomeric excess 93% as trifluoroacetamide (GC (BGB-177): 15 m×0.25 mm, carrier gas: He; program: 150–200 °C at 1 °C/min; injector temp. 210 °C; FID: 220 °C). GC 97% area. ISP-MS: 177.1 ($M+H^+$). IR (Film): (NH) 3357, (NCH) 2789. ^1H NMR ($CDCl_3$, 250 MHz): 1.45 (bm, 3H, NH_2 and CH_2), 2.15 (m, 1H, CH_2), 2.24 (dd, J=4.5, 9.5 Hz, 1H, NCH_2), 2.44 (m, 1H, NCH_2), 2.69 (m, 2H, 2×NCH_2), 3.48 (m, 1H, $CHNH_2$), 3.60 (dd (AB), 2H, $PhCH_2$), 7.28 (m, 5H, Ph).

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