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Enzymatic Resolution of *N*-Substituted-β-prolines

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Abstract:

A general and straightforward strategy for enzymatic resolution of *N*-substituted-β-proline has been successfully designed and developed in our research laboratories. A first affinity screen is followed by ratio enzyme/substrate optimization to source our chemistry groups *N*-substituted-β-proline in kilogram scale in an efficient and cost-effective way.

1. Introduction

Investigative pharmaceutical agents typically contain multiple functional groups and one or more stereocenters, and must be isolated in high chiral as well as chemical purity. Classical resolution or chiral chromatography can be useful in the preparation of these drug candidates in enantiomerically pure form. However, those approaches also show certain drawbacks. Hence, for instance, resolutions often suffer from low overall yield and normally require the presence of accessible acidic or basic functionalities. However, chiral chromatography can be sometimes solvent and time intensive. A selective synthesis that yields the desired functionalities in high chemo, enantio, and/or diastereo control is, therefore, greatly desirable. In this context, biocatalysis plays an increasing role due to the benefits associated with those types of processes, such as speed, selectivity, or ability to quantity.[1] Screen of commercially available enzymes can easily allow the rapid identification of hits. Further process development involving a more detailed study to optimize reaction parameters such as temperature, solvent composition, or rate of addition can then supply the desired synthetic intermediates in both high yield and high optical purity.

N-Substituted-β-prolines are of great interest because of their biological properties.[2] Hence, any valuable technology for the selective preparation of those type of substrates deserves special attention. For all these reasons, we decided to focus our efforts upon the development of an automated enzymatic screen and an efficient analytical methodology (that measures yield and the enantiomeric excess in a single experiment) capable to efficiently provide us those valuable key intermediates.

2. Results and Discussion

The synthesis of β-prolines (**3a**−**c**) was achieved by adjusting the procedure described in the literature (Scheme 1).[3−5] 1,3-Cycloaddition of *N*-methoxy-*N*-trimethylsilylbenzylamine (**1**) to methyl acrylate in acidic medium yielded pyrrolidine (**2**) in high yield.[3] Formation of corresponding hydrochloride salt **3a** allowed the easy isolation of highly pure material without any chromatographic step. Conventional debenzylation by hydrogenation yielded the desired pyrrolidine (**4**) in 98% yield (all attempts with the free amine always provided decomposition products).[4] β-Proline hydrochloride (**4**) was used as a key intermediate to prepare the two different pyrrolidines derivatives (*N*-boc **3b**[5] and *N*-Cbz **3c**[6]) envisioned to be included in our enzymatic screen because of their electronic behavior and synthetic availability.

A set of 13 commercial available hydrolytic enzymes was selected for a preliminary screen.[7] All tests were run in aqueous medium (phosphate buffer pH 7.5; C, 0.1 M, rt; Scheme 2).The conversion and optical purity were determined by achiral reversed-phase HPLC/MS and chiral GC, respectively.

The results for each substrate after 24 h of incubation are summarized in Table 1. For **3c** (Cbz), lipases PS, AY30, PPL, CCL, proteases P, PS, and acylase overreacted (conversion C ∼100%); lipases AK, AS, FAP15, and M (Table 1, entries 1−4) showed conversions close to 50% but only lipase M (*Mucor javanicus*) gave promising results: C, 60%; ee, 92%; and E,[8] 13 (Table 1, entry 2). When working with substrate **3a**, conversion could not be determined because of the difficulties found for the isolation of the corresponding carboxylic acid (zwitterionic behavior). Nevertheless, chiral GC allowed for the determination of the chiral purity of the remaining ester. Unfortunately, in all cases, ee was found to be <50% (Table 1, entries 5−17).

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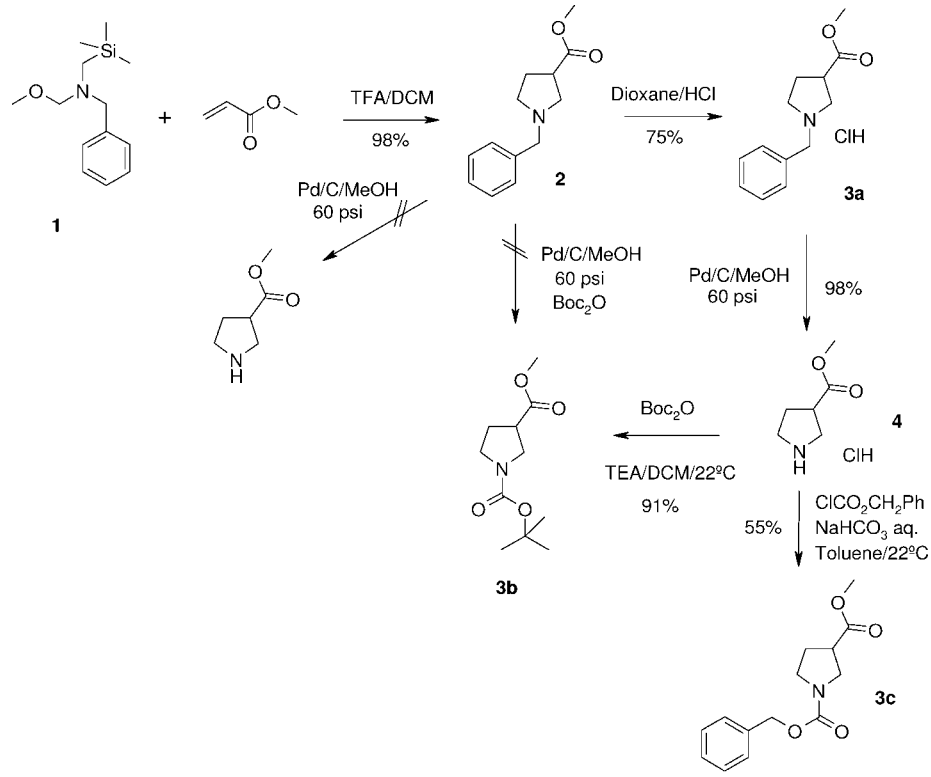
Scheme 1



Scheme 2

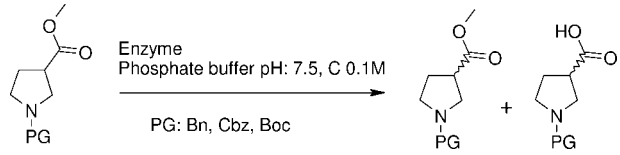


Table 1. **Screening for affinity study enzyme/substrate (24 h)**

entry	substrate	enzyme	conversion (%)	ee (%) (isomer favor)	E
1	**3c**	AK	>50	0	
2	**3c**	M	60	92 (E2)	13
3	**3c**	AS	16	15 (E2)	
4	**3c**	FAP15	60	5 (E2)	
5	**3a**	Acylase		12 (E1)	
6	**3a**	AK		5 (E2)	
7	**3a**	AY-30		44 (E2)	
8	**3a**	AS		2 (E1)	
9	**3a**	FAP15		1 (E2)	
10	**3a**	M		44 (E2)	
11	**3a**	N		21 (E2)	
12	**3a**	PPL		18 (E2)	
13	**3a**	Prot P		24 (E1)	
14	**3a**	PS		15 (E2)	
15	**3a**	R		1 (E1)	
16	**3a**	PGE		2 (E1)	
17	**3a**	CCL		46 (E2)	
18	**3b**	R	53	84 (E2)	19
19	**3b**	AS	53	96 (E2)	43
20	**3b**	PGE	25	12 (E2)	
21	**3b**	N	47	74 (E2)	28
22	**3b**	M	83	31 (E2)	
23	**3b**	FAP15	38	42 (E2)	

With substrate **3b** (Boc) (Table 1, entries 18−23), all selected enzymes showed positive reaction. Lipases AK, AY30, PPL, CCL, and acylase (Amano), and proteases PS and P overreacted. Lipases R, AS, FAP15, PGE, and M, and protease N showed conversions close to 50%. These six reactions were worked up, and samples were analyzed by HPLC and chiral GC. Excellent results were obtained with lipase AS: C, 53%; ee, 96%; and E, 43 (Table 1, entry 19). Compared to **3a** and **3c**, compound **3b** performed as the best substrate with the popular and inexpensive lipase AS (*Aspergillus niger*, Amano Co.) and was then selected to further refine our enzymatic approach.

Lipases offer several possibilities for synthetical purposes. Hence, screening in organic solvents allows enantioselective esterification of acids, this reaction being a different approach to also obtain the desired acid in enantiopure form. For this reason, we envisaged a parallel study of the enzymatic resolution of the racemic carboxylic acid (**5**) in an organic solvent[9] (MTBE). Required *N*-Boc-proline (**5**) was easily obtained by conventional hydrolysis of the available ester (**3b**) with NaOH/MeOH[10] (Scheme 3).

Enzymatic resolution screening was run using a set of 24 commercially available hydrolytic enzymes[11] in MTBE. The best results are summarized in Table 2. The three most promising assays, that showed conversion close to 50% by TLC/LCMS after 24−120 h in acidic conditions were worked up, and samples were analyzed by chiral HPLC. Unfortunately, low selectivity was observed in all cases (Table 2).

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(11) Hydrolytic enzyme screening kit (24 enzymes), ICR-2400 (Biocatalytics Co., U.S.A.).

Scheme 3

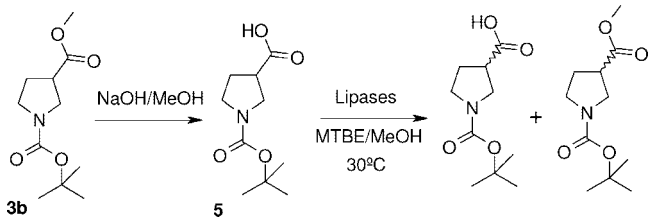

Table 2. **Results of enzymatic screening for ester formation (organic media)**

entry	enzyme	time (h)	conversion (%)	ester ee (%)	acid ee (%)	E
1	ICR-110	24	68	0	0	
2	ICR-113	120	39	33 (E1)	20 (A2)	
3	ICR-116	24	35	20 (E2)	15 (A1)	

Table 3. **Screening results with 24 hydrolytic enzymes**

entry	enzyme	conversion (%)	ester ee (%)	acid ee (%)	E
1	ICR-102	37	24 (E2)	44 (A1)	
2	ICR-103	35	36 (E2)	66 (A1)	
3	ICR-105	42	48 (E2)	74 (A1)	11
4	ICR-112	69	>98 (E2)	46 (A1)	11
5	ICR-118	45	44 (E1)	64 (A2)	
6	ICR-127	60	26 (E1)	20 (A2)	

The efforts involved in the simultaneous screen of multiple enzymes prompted us to take advantage of the TECAN platform available in our laboratories to enable the automation of both dispensation of stock solutions/suspensions and collection of aliquots for monitoring conversion ratio at different times. Besides this, intensive analytical studies resulted in the development of an efficient HPLC/MS method that achieved baseline resolution of the four isomers (product and reagent) in the same experiment.[12] Furthermore, MS detection assured easy and accurate identification of each of the components of the mixture. A, now automated, second screen with the selected 24 hydrolytic enzymes[11] was then carried out.

Conversion and optical purity were measured every 8 and 24 h hours, respectively. The best results are summarized in Table 3. Six assays afforded conversion close to 50% (determined by achiral reversed-phase LCMS under acidic conditions) and were worked up for chiral HPLC/MS analysis. ICR-112 (CAL-A) showed very promising results for the corresponding ester, C, 69%; ee >98%; and E, 11 (Table 3, entry 4).

Analysis of all data obtained clearly revealed lipases AS (C, 53%; ee, 96%) and ICR-112 (CAL-A) as the most suitable hydrolytic enzymes to perform resolution (C,: 69%; ee, >98%). Nevertheless, and for scale-up purposes, assay conditions (substrate/enzyme ratio, temperature, buffer concentration, or reaction time, among others) required further refinement. The goal was that the selected enzyme yielded conversion close to 50% and optical purity >97% ee in a reaction time of about 24 h. (Shorter reaction times are not convenient to avoid overreaction due to the impact of time required for analysis and workup steps.) With lipase AS, the optimization study was focused on reducing the substrate/enzyme ratio, in order to improve the workup and therefore decrease the final cost of the process. In the case of lipase ICR-112 (CAL-A), all efforts were focused on the adjustment of the observed 69% conversion.

Table 4. **Conversion and optical purity obtained with lipase AS at different S/E ratios and reaction times[a]**

entry	ratio S/E	time (h)	conversion (%)	ester ee (%)	acid ee (%)	E
1	2.5/1	24	65	>98 (E2)	57 (A1)	15
2	5/1	24	55	96 (E2)	91 (A1)	83
3	9/1	48	~40	73 (E2)	94 (A1)	71

[a] All assays were performed in 20 vol. of buffer at pH 7.5.

Table 5. **Conversion and optical purity obtained with lipase ICR-112 at different S/E ratios and reaction times[a]**

entry	ratio S/E	time (h)	conversion (%)	ester (ee) (%)	acid (ee) (%)	E
1	2.5/1	4.5	59	>98 (E2)	82 (A1)	46
2	2.5/1	8	61	>98 (E2)	74 (A1)	30
3	2.5/1	23	85	>98(E2)	50 (A1)	
4	5/1	4	47	76 (E2)	86 (A1)	30
5	5/1	7	51	>98 (E2)	80 (A1)	40
6	5/1	24	63	>98 (E2)	56 (A1)	15
7	9/1	4	29	34 (E2)	96 (A1)	
8	9/1	7	42	80 (E2)	88 (A1)	38
9	9/1	24	57	>98 (E2)	74 (A1)	30
10	18/1	4	18	14 (E2)	94 (A1)	
11	18/1	7	27	34 (E2)	98 (A1)	
12	18/1	24	52	94 (E2)	84 (A1)	40
13	18/1	48	55	>98 (E2)	72 (A1)	27

[a] All assays were performed in 20 vol. of buffer at pH 7.5.

For lipase AS, the ratios selected were 2.5/1, 5/1, and 9/1. Best results, after incubation at 30 °C and in Na_2HPO4/NaH_2PO4, pH 7.5, C, 0.2 M buffer, were obtained with 5/1 S/E ratio (C, 55%; ee, 96%; 24 h; Table 4, entry 2). For ICR-112 (CAL-A), the conversion obtained in the first screening was very high (69%); therefore, optimization was done by adjusting S/E ratios to 2.5/1, 5/1, 9/1, and 18/1. The best ratio to provide conversion close to 50% in 24 h was 9/1 (Table 5, entry 9). Other ratios also yielded good conversion and ee (Table 5, entries 1 and 5); however, reaction times were not convenient for scale-up (<7 h).

After full process optimization, we found that both selected enzymes provided the ester derivative **3b** in high optical purity. Because of its availability and affordability, lipase AS was selected for scale-up purposes. Assays were performed on 1 g, 10 g, 100 g, and 1 kg of substrate using 20% w/w (5/1 ratio) of lipase AS, at 30 °C, and Na_2HPO4/NaH_2PO4, pH 7.5, C, 0.2 M buffer. Reproducibility of results (Scheme 4) confirmed the robustness of the process. Configuration of the ester and acid so isolated was determined to be (*S*) and (*R*), respectively, by comparison of the elution profile of commercially available materials in the same chiral HPLC conditions selected for the monitoring of assays.

Final hydrolysis of the methyl ester was performed with different aqueous bases (NaOH, LiOH) in combination with organic solvents (MeOH, THF). Reaction with LiOH 2M/THF yielded the desired acid in 98% yield and >97% ee in a 10 g scale. It has to be noticed that reaction temperature in this step must be kept under control (below 0 °C during the addition of the basic reagent) to fully ensure the optical purity of the

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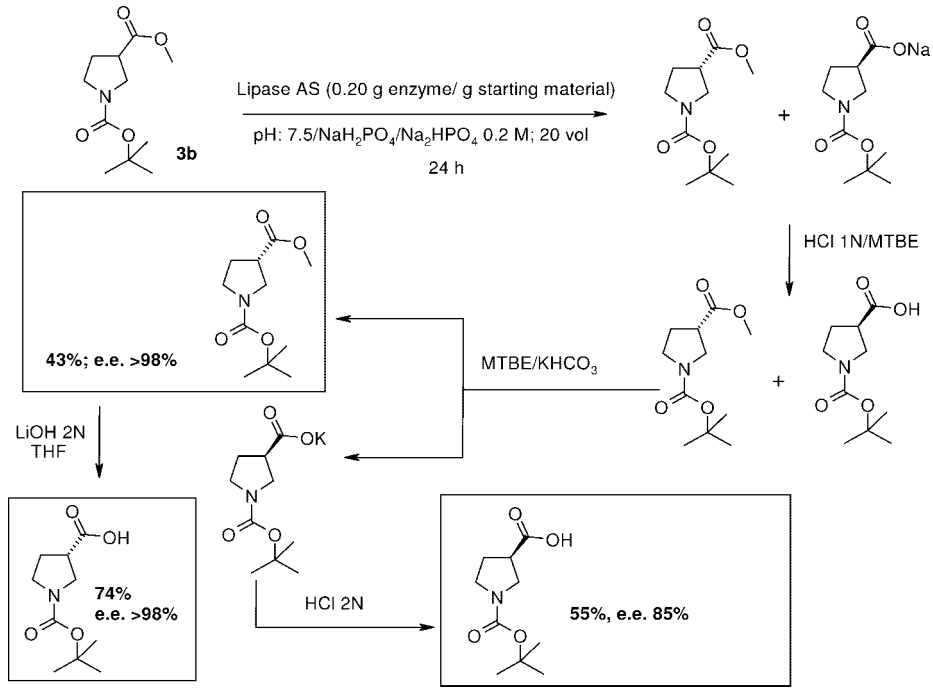
Scheme 4



material. This procedure was reproducible at an 845 g scale, providing the desired *N*-Boc-(*S*)-β-proline in 74% yield and >98% ee (Scheme 4).

3. Conclusions

We have designed and developed a simple and rapid high-throughput enzymatic approach for the efficient preparation of the expensive and low available *N*-substituted-β-proline **3b**. The process herein described has proven to be robust and reliable and has provided this key intermediate for research purposes in a multigram scale and at 98% ee.

4. Experimental Section

Enzymes and Reagents. All reagents were supplied by Biocatalytics Co. (U.S.A.) (Hydrolytic enzyme screening kit, ICR-2400), Amano Co. (Japan), and Sigma (U.S.A.).

Analytical Conditions. HPLC (DAD)/MS was used for the determination of reaction conversion and enantiomeric excess of both remaining substrate and resulting product.

All analytical studies were performed on a series 1100 liquid chromatography/mass selective detector LC/MSD (Agilent, Waldbronn, Germany) driven by ChemStation software (Rev. A10.02, Agilent Technologies).

Sample solution of reference materials and those products yielded by enzymatic screen was prepared by dissolving 2 mg in 1 mL of a 9/1 hexane/ethanol mixture.

Analyses were conducted using a CHIRALPAK AD unit from Daicel (Chiral Technologies Europe). Column dimension was 250 mm × 4.6 mm with the enantioselective phase coated onto a 10 μm silica-gel substrate. The mobile phase consisted on a 95/5 mixture of *n*-hexane (containing TFA at 0.05% v/v)/ethanol. Flow rate was set at 1 mL/min. All experiments were carried out at room temperature. The wavelength of UV detection was monitored from 190 to 500 nm, although chromatograms were recorded at 205,16 nm due to the poor UV response of target compounds. Mass spectra were recorded using API-APCI ionization (full scan in positive/negative modes, simultaneously). All of the four different components were resolved in the same experiment and easily identified by ion extraction (positive mode), which facilitated the interpretation of results. ^{1}H NMR spectra were acquired on a Brucker Avance DPX 300 MHz spectrometer.

General Procedure for Enzymatic Hydrolytic Screening. To 10 mg of each enzyme in a test tube was added 0.1 mmol of substrate dissolved/suspended in 1 mL of 0.1 M phosphate buffer at pH 7.5 (a stock solution/suspension can be used). The mixtures were incubated with agitation at 30 °C (sand bath for hood or Variomag with cooler for TECAN platform), and the progress of the reaction was monitored by any preferred method over a 24 h period (HPLC and/or TLC) to check the conversion of the ester to the carboxylic acid (recommended 8 h, 16 h, and 24 h). After 24 h, 1 N HCl (1 mL) and MTBE (2 mL) were added to each mixture. The organic phases were collected and filtered through a 0.45 nylon filter. The solvent was removed, and the conversion was monitored by LCMS (acidic methods).

Procedure for the Scale-Up. *Enzymatic Resolution of 1-tert-Butyl-3-methylpyrrolidine-1,3-dicarboxylate (**3b**).* To 50 L of reactor with mechanical stirring was added 1-*tert*-butyl-3-methylpyrrolidine-1,3-dicarboxylate (930 g, 4 mol) and 18 L of NaH_2PO4/Na_2HPO4 buffer (pH 7.5, C 0.2 M). The mixture was heated at 30 °C. Then, lipase AS (167 g) was added, and the reaction was stirred for 24 h at 30 °C. The reaction was checked by chiral chromatography after that time (ee of ester remaining: >98%). Then, 1 N HCl (5 L) was added. A white solid was observed. The mixture was extracted with MTBE twice (20 and 10 L). The organic layers were combined and filtered through celite. The filtrated was added to a separation funnel, and the aqueous phase was discarded. The organic layer was washed with 20% $KHCO_3$ twice (5 and 2 L), dried over

MgSO4, filtered, and concentrated to give (*S*)-1-*tert*-butyl-3-methylpyrrolidine-1,3-dicarboxylate (399 g, 1.74 mol, 43%, ee >98%) as an oil. ^{1}H NMR ($CDCl_3$): δ 1.45 (s, 9H), 2.10−2.15 (m, 2H), 3.00−3.10 (m, 1H), 3.28−3.40 (m, 1H), 3.44−3.68 (m, 3H), 3.71 (s, 3H).

The aqueous $KHCO_3$, which contained the acid, was acidified with 2 N HCl until pH 3. The solid obtained is filtered and washed with water, and dried to give (*R*)-1-(*tert*-butoxycarbonyl)pyrrolidine-3-carboxylic acid (481 g, 2.2 mol, 55%, ee 85%).

Hydrolysis of (S)-1-tert-Butyl-3-methylpyrrolidine-1,3-dicarboxylate. To a 22 L RBF with mechanical agitation, thermocouple, 2 L addition funnel, and cooling bath was added (*S*)-1-*tert*-butyl-3-methylpyrrolidine-1,3-dicarboxylate (845 g, 3.69 mol, ee >98%) and THF (8.5 L). The mixture was cooled to −10 °C with an acetone/ice bath. The 2 L addition funnel was charged with cold LiOH (1.47 L, 2.5 M in water, 3.69 mol), and this solution was added dropwise to the mixture, maintaining the internal temperature below 0 °C during the addition (2 h). When the addition was completed, the mixture was stirred for 2 h at −8 °C. Then cold MTBE (8.5 L) was added to the mixture, the layers were separated, and the organic layer was discarded. Then to the aqueous layer was added THF (4.25 L) and toluene (8.5 L) and cooled to −2 °C, followed by the addition of cold 1 N HCl until pH 3.5 (4 L added). The layers were separated, and the aqueous layer was discarded. The organic was washed with water (4.25 L) and concentrated under vacuum (adding toluene until dryness) to give (*S*)-1-(*tert*-butoxycarbonyl)pyrrolidine-3-carboxylic acid (585 g, 2.72 mol, 74%, ee > 98%) as a white solid. ^{1}H NMR (DMSO-d_6): δ 1.39 (s, 9H), 1.88−2.10 (m, 2H), 2.95−3.07 (m, 1H), 3.20−3.45 (m, 4H).

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Supporting Information Available

^{1}H NMR characterization for compounds **3a**−**c**, **4**, and **5**; standard chiral analysis for **3a** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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