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Chemo-enzymatic approach to D-allo-isoleucine

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Abstract—A mixture of D-alloisoleucine and L-isoleucine derivatives are selectively hydrolysed by enzymatic catalysis (alcalase) allowing the recovery of the D-allo stereoisomer in excellent d.e. and yields. Thus, *N*-formyl-D-allo-isoleucine benzylester is obtained after enzymatic hydrolysis of the diastereoisomeric mixture or by a crystallisation procedure.

1. Introduction

D-Allo-isoleucine (D-allo) is a non-proteinogenic amino acid, found in nature which is probably formed by time dependent epimerisation of L-isoleucine (L-ileu).[1] It has recently been found as a component unit in a number of biologically active depsipeptides[2–13] and it has been employed in the synthesis of oxytocin analogues[14] and as a chiral synthon for the preparation of isostatins and of natural cytotoxic depsipeptides such tamandarins.[15] In view of the growing interest in the synthesis of peptide mimetics involving non-natural amino acids, several synthetic schemes leading to D-allo have been proposed. A total synthesis from (*S*)-2-butanol, requires among other steps, a TEMPO catalysed oxidation and condensation with (*R*)-(+)-menthyl *p*-toluenesulphinate (Andersen's reagent).[16] Since a roughly 1:1 mixture of D-allo and L-ileu (mix-ile **1**) is easily formed by epimerisation of the natural amino acid, separation of D-allo from that mixture is a logical approach to compound **4**. Thus, several reports along this line have been published, exploiting specific properties of compounds obtained reacting the mixture of diastereoisomers with enantiomerically pure compounds (α-phenylethylamine,[17] L-phenylalanine,[18] tartaric acid derivatives[19]) and separating them by fractional crystallisation or chromatography. Moreover the enzymatic hydrolysis of appropriate derivatives of the diastereoisomeric mixture has been applied using papaine,[20] hog kidney acylase[21,22] or hydantoinase.[23] Indeed L-specific hydrolytic enzymes hydrolyse L-isoleucine derivatives, leaving behind the D-allo stereoisomer. Epimerization of the recovered L-ileu allows the repetition of the separation process.

We report here a practical preparation of D-allo as such, and in synthetically useful protected forms, starting from **1** with an approach that is both chemical and enzymatic.

2. Results and discussion

Biocatalysis has reached a stage of mature methodology for application on the industrial scale.[28,29] It is widely recognised that the bottleneck in applications depends on the enzymes availability.[28,29] Solution for the obtention of affordable biocatalysts is the in house development of suitable enzyme sources[30] or simply the application of commercially available enzyme preparations of low cost. Alcalase is a commercial preparation of a partly purified protease from *B. licheniformis* produced by NOVOzyme for detergent applications, it is commercially available at low cost, and it has an extremely broad substrate specificity.[24–27] As most enzymatic systems acting on amino acid derivatives[31–34] it shows L-selectivity. Most of those enzymes have been applied in the kinetic resolution of amino acid derivatives often coupled with racemisation techniques to improve the efficiency.[35] We prepared a series of derivatives of the epimeric forms of isoleucine and submitted them to alcalase hydrolysis with the aim of obtaining as

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a residue from the enzymatic hydrolysis D-alloisoleucine or a synthetically useful protected form thereof (Scheme 1).

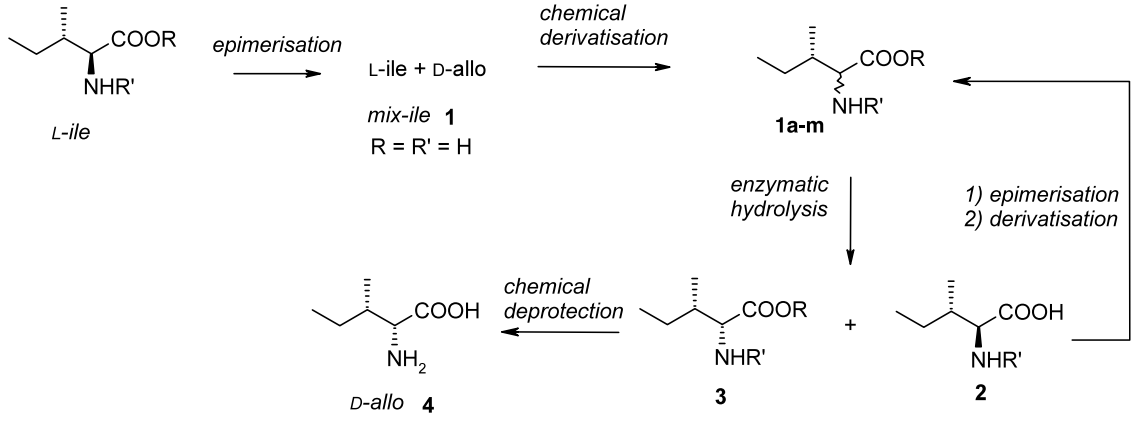
Starting with L-ile, an approximately equimolar mixture (*mix-ile*) of the two epimers was generated as described in the experimental. The corresponding esters **1a–d** were prepared as substrates for the enzyme. Preliminary assays at various pH and temperatures ranging from 20 to 40°C in the absence of the enzyme, showed those substrates which were prone to spontaneous hydrolysis above pH 7.5 and 25°C. Accordingly, alcalase catalysed hydrolysis of a 0.2 M solution of substrates **1a–d** allowed the recovery of the corresponding derivatives **3** with unsatisfactory d.e.s. Thus under the conditions described in the experimental part, compound **1c** was hydrolysed at a rate of 4 mmol/h living at approximately 50% conversion **3c** of 70% d.e., as determined by HPLC. Compound **1d**, which was transformed at a rate approximately 25 times slower, gave the corresponding compound **3d** with about the same d.e. as before. This result confirms that the lack of selectivity is due to concomitant chemical hydrolysis.

We turned our attention to the *N*-acyl derivative of mixtures of **1** and substrates **1e–m** were prepared through conventional methods. Although the substrates are poorly soluble in water, the enzymatic hydrolysis requires no added cosolvent and the reactions are run in a suspension with a formal concentration of 0.2 M. Higher concentrations were occasionally equally efficient. Preliminary experiments in the absence of the enzyme showed that no detectable chemical hydrolysis occurred on those substrates at pHs up to 9 and temperatures up to 40°C. Among esters **1e–h**, chloroethyl esters were hydrolysed about 25 times faster than benzyl esters, methyl and ethyl lying in between. The specificity for L-ile was quite high allowing compounds of type **3** to be obtained as residues in >98% d.e. Table 1 summarises the results observed with various L-isoleucine derivatives. It appears from these data that compounds **1e–m** allow the D-allo derivatives to be obtained with excellent d.e.

Table 1. Results of the alcalase catalysed hydrolysis of L-isoleucine derivatives

Substrate	Product	Yield (%)	D.e. (%)	Time (h)
1c	**3c**	50	70	2
1d	**3d**	40	60	40
1e	**3e**	30	98	20
1f	**3f**	30	98	21
1g	**3g**	40	96	2.5
1h	**3h**	41	98	22
1j	**3j**	50	99	3.3
1m	**3m**	20	98	28

In Figure 1, the enzymatic hydrolyses of the *N*-formyl derivatives **1f–h** are compared. The graph suggests that recovery of D-allo of high d.e. is possible for high conversion in the **f** and **g** series, while the hydrolysis profile of the benzyl ester indicates a sharp selectivity. Indeed at 50% conversion (as determined from NaOH uptake at pH 7.8), compound **3h** was obtained in almost quantitative yield and a diastereomeric excess higher than 98%. Comparison of benzyl esters of differently *N*-protected compounds are reported in Figure 2. Mixture **1j** proved to be the best substrate for the hydrolysis. Reaction was fast and extremely selective allowing the obtainment of **3j** with a d.e. higher than 98%. Moreover the remaining **2i** is easily recycled in the preparation of the epimeric mixture **1j**. However, the results with **1h** should also be considered for the easier



compound	R'	R	compound	R'	R
a	H	Me	**g**	CHO	Cl-Et
b	H	Et	**h**	CHO	CH_2Ph
c	H	Cl-Et	**i**	$COCH_3$	H
d	H	CH_2Ph	**j**	$COCH_3$	CH_2Ph
e	CHO	Me	**l**	Z	H
f	CHO	Et	**m**	Z	CH_2Ph

Scheme 1.

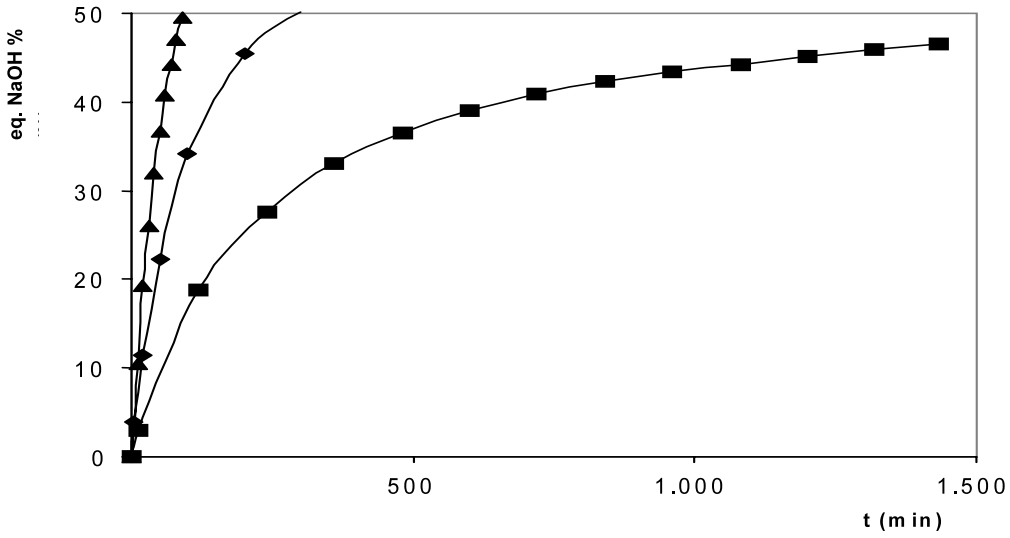


Figure 1. Enzymatic hydrolysis (alcalase) of *N*-formyl-D-allo- and *N*-formyl-L-isoleucine esters (◆ *O*-methyl **1f**, ▲ *O*-chloroethyl **1g**, ■ *O*-benzyl **1h**).

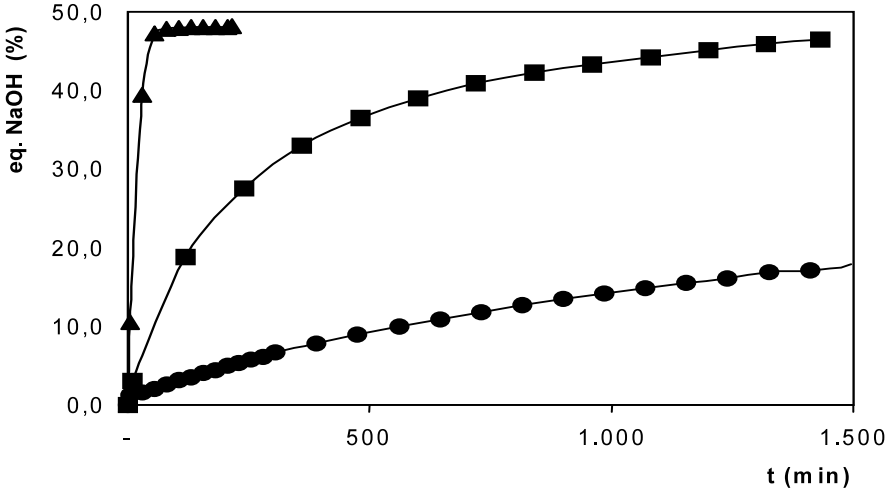


Figure 2. Enzymatic hydrolysis (alcalase) of D-allo- and L-isoleucine benzylester derivatives (▲ *N*-acetyl **1j**, ■ *N*-formyl **1h**, ● *N*-Cbz **1m**).

removal of the formyl group compared with the *N*-acetyl derivative. The hydrolysis of mixture **1m** is exceedingly slow. Thus the procedure from **1j**/**1h** to D-allo has been applied on a multigram scale. The effect of substrate and enzyme concentration was evaluated. Best selectivity was obtained with low enzyme/substrate ratio (see Section 4). L-ile obtained from the hydrolysis could be easily epimerised under several conditions both at the acid and at the ester level. Thus *N*-formyl-*O*-benzylester was epimerised to give the thermodynamic mixture in the presence of catalytic K*t*BuO in MTBE as solvent. Thus in successive steps L-ile can in principle be converted to the diastereoisomer D-allo in quantitative yield.

Complementary to this method, we found that the two diastereomers often exhibit quite different physical properties. Compound **3d·HCl** for instance, is a nice crystalline material, while the L-liso epimer is a glassy solid difficult to crystallise. An epimeric mixture of **1d** dissolved in ethyl ether at 0°C, was saturated with gaseous HCl. **3d·HCl** separated in white needles, while the epimer remained in solution. The solid was thus recovered in two successive experiments, accounting for >40% of the expected compound. The d.e. of the solid compound was >98% from HPLC. Thus, this simple crystallisation method complemented well the above separations based on selective enzymatic hydrolysis.

3. Conclusions

N-Acyl-esters of mixtures of the epimeric isoleucines are easily enzymatically hydrolysed in water to the *N*-acyl-L-forms allowing the recovery of the D-allo compounds in high diastereoisomeric excess. Although similar methods have been applied before,[20–23] the present approach seems convenient for large scale

application due to the nature of the biocatalyst (alcalase, an industrial grade proteolytic enzyme), the possibility of running the reaction in water at concentrations of 30–50 g/l, and for the useful D-allo derivatives obtained.[17,41,42] In fact both *N*- or *C*-protected forms of D-allo can be easily prepared in one step from the material surviving to enzymatic hydrolysis. A simple crystallisation technique allows to obtain **3d** directly from the epimeric mixture.

4. Experimental

Alcalase 2.5L DX from NOVOZYMES was used in all experiments. ^{1}H NMR analyses were carried out on a 250 MHz *Varian EMX* instrument. EI-MS were recorded on a *TSQ mass spectrometer*. Optical rotation data were measured with a *Propol automatic digital polarimeter* at 20°C. Gradient HPLC was performed on a Agilent 1100 Series instrument equipped with a SEDERE Sedex 75 evaporative light scattering detector (T = 40°C, Gain = 4, P = 3 bar) using a reversed phase column (Agilent ZORBAX XDB-C_8) at 25°C and with a 1 ml/min flow. Method 1a: eluent TFA 0.1%/acetonitrile; from 0 to 35% of acetonitrile in 7 min, followed by acetonitrile 35% for 2 min. Method 1b: eluent TFA 0.1%/acetonitrile 75/25. HPLC evaluation of the diastereoisomeric excess of the *N*-protected esters was carried out on a Merck-Hitachi L6000 Intelligent Pump equipped with a L600 UV-detector using a chiral column (Daicel Chiralcel OD) with a 0.6 ml/min flow. Method 2a: $\lambda = 220$ nm, eluent hexane/isopropanol 95/5. Method 2b: $\lambda = 240$ nm, eluent hexane/isopropanol 90/10. Method 2c: $\lambda = 254$ nm, eluent hexane/isopropanol 90/10. In enzymatic reactions automatic pH control was performed by a Metrohm 718 STAT Titrino Isoleucine derivatives were prepared according to general procedures found in the literature.[36–40]

4.1. D-allo- and L-isoleucine *mix-ile* 1

50 g of L-isoleucine were dissolved in 250 g of glacial acetic acid in the presence of 2 g of benzaldehyde, and the mixture heated at 100°C in a nitrogen atmosphere for 2 h. The mixture was cooled to 50°C and filtered on a Celite pad. The solution was concentrated under vacuum to a volume of 50 ml. 160 ml of 2-propanol were then added and the mixture stirred for 1 h. The mixture was filtered and washed with 2×40 ml of 2-propanol. The solid was dried under vacuum to give 45 g (90%) of the epimeric mixture. The diastereoisomeric composition was evaluated by polarimetry by comparison with a pure sample of D-alloisoleucine {$[\alpha]_D = -37.4$ (*c* 2 in 5 M HCl)} and L-isoleucine {$[\alpha]_D = +39.0$ (*c* 2 in 5 M HCl}. The $[\alpha]_D$ value of 0.8 (*c* 2 in 5 M HCl) of the mixture showed a slight predominance (51%) of the D-allo isomer, which is consistent with the composition found for the *N*-acyl esters (generally D-allo/L-iso 54:46).

4.2. D-Allo- and L-isoleucine methyl ester hydrochloride 1a·HCl

To a stirred suspension of the mixture of L-isoleucine and D-alloisoleucine (15 g,114 mmol) in methanol (100 ml) 5 ml of thionyl chloride were dropped at 0°C within 15 min. The solution was refluxed for 7 h. After removal of the solvent, the residue was dissolved in water (100 ml) and adjusted to pH 10 with 50% aqueous potassium carbonate. After extraction with diethyl ether (4×200 ml), the organic layers were dried over sodium sulphate and evaporated. The liquid was taken up in diethyl ether (150 ml) and converted to the hydrochloride with gaseous hydrochloric acid at 0°C. The precipitate was filtered and washed with diethyl ether, giving 16.6 g (91.8 mmol, 84%) of a white solid. NMR (D_2O) δ: 0.9–1.1 ppm, 6H, m; 1.2–1.6 ppm, 2H, m; 2.0–2.2 ppm, 1H, m; 3.9 ppm, 3H, s; 4.2 ppm, 1H, m HPLC (Method 1a) t_R: 4.79 min **2a** and 4.96 min **3a**.

4.3. D-Allo- + L-isoleucine ethyl ester hydrochloride 1b·HCl

A suspension of the epimeric mixture of L-isoleucine and D-alloisoleucine (15 g,114 mmol) in ethanol (250 ml) was treated with gaseous hydrochloric acid and refluxed for 8 h. After evaporation of the solvent, the oily residue was taken up in water (150 ml), adjusted to pH 9.5 with NH_3 and extracted with ethyl ether (3×100 ml). The organic layers were washed with brine (2×100 ml), dried over sodium sulphate, filtered and concentrated (200 ml). The solution was treated with gaseous hydrochloric acid at 0°C and evaporated to give 18.52 g (95 mmol, 83%) of a white solid. NMR (D_2O) δ: 0.85–1.05 ppm, 6H, m; 1.2–1.3 ppm, 3H, t; 1.2–1.6 ppm, 2H, m; 2.0–2.2 ppm, 1H, m; 4.10 ppm, 1H, m; 4.30 ppm, 2H, m. HPLC (Method 1a) t_R: 6.26 min **2b** and 6.46 min **3b**.

4.4. D-Allo- and L-isoleucine chloroethyl ester hydrochloride 1c·HCl[36]

To a chilled suspension of the epimeric mixture of L-isoleucine and D-alloisoleucine (15 g,114 mmol) in 2-chloroethanol (80 ml) thionyl chloride (96 g, 1.19 mol) was added dropwise within 30 min. The suspension was kept at rt for 3 days and then heated at 50°C for 7 h. After evaporation of the solvent, the residue was treated according to the procedure described for the ethyl ester **2** to give 18.6 g (81 mmol, 71%) of a white solid. NMR (D_2O) δ: 0.80–1.15 ppm, 6H, m; 1.15–1.6 ppm, 2H, m; 2.0–2.3 ppm, 1H, m; 3.8–4.0 ppm, 2H, t; 4.20 ppm, 1H, m; 4.55 ppm, 2H, m. HPLC (Method 1a) t_R: 7.36 min **2c** and 7.54 min **3c**.

4.5. D-Allo- and L-isoleucine benzyl ester *p*-toluenesulphonate 1d·*p*-TsOH[37]

To 100 ml of toluene an epimeric mixture of L-isoleucine and D-alloisoleucine (10 g, 76 mmol), *p*-toluenesulphonic acid monohydrate (15 g, 79 mmol)

and benzyl alcohol (40 ml) were added and the suspension was refluxed for 18 h collecting water with a Dean–Stark apparatus. After cooling, toluene (150 ml) and ethyl ether (300 ml) were added to the solution. After 2 h at 4°C, the precipitate was filtered and washed with diethyl ether, giving 18.6 g (47 mmol, 62%) of a white solid. A second crop (5.6 g, 14 mmol, 19%) was obtained after concentration of the filtered solution and addition of ethyl acetate and hexane (24.2 g, total yield 81%).

4.6. D-Alloisoleucine benzyl ester hydrochloride 3d·HCl

The mixture D-allo- and L-isoleucine benzyl ester *p*-toluenesulphonate (24.2 g, 62 mmol) was suspended in water (100 ml) and ethyl acetate (100 ml). The pH was adjusted at 8.5 with K_2CO_3, the organic layer was separated and the ester mixture was extracted from the aqueous phase with other ethyl acetate (2×100 ml). After drying and evaporation of the solvent the oily residue was dissolved in ethyl ether and converted to the hydrochloride with anhydrous HCl at 0°C. After filtration and washing with ethyl ether, the solid was used to seed the organic phase concentrated to half the volume and the mixture was left overnight at 5°C. The two crops were pooled and the solid crystallised from a mixture of ethyl ether and ethanol giving D-alloisoleucine benzyl ester hydrochloride **3d·HCl** (8 g, 30 mmol, 41%, mp 122.6–123.8°C). NMR (D_2O) δ: 0.85 ppm, 3H, d; 0.85 ppm, 3H, t; 1.25 ppm, 1H, m; 1.45 ppm, 1H, m; 2.10 ppm, 1H, m; 4.20 ppm, 1H, d; 5.3 ppm, 2H, q; 7.45 ppm, 5H, m. HPLC (Method 1b) t_R: 8.20 min **3d** and 10.7 min **2d**.

4.7. Enzymatic hydrolysis of compounds 1a–d

A solution of the substrate in distilled water (0.18 M) was heated at 39°C and adjusted to pH 7.8 by addition of 0.35 M sodium hydroxide. After Alcalase 2.5L was added (0.23 g/ml) the reaction mixture was kept at pH 7.8 by the automatic pH-stat addition of 0.35 M sodium hydroxide while the temperature was maintained at 39°C. The reaction course was followed by HPLC with an evaporative light scattering detector (t_R: D-allo, 3.23 min and L-ile 3.36 min, method 1a).

4.8. *N*-Formyl-D-allo- and L-isoleucine methyl ester 1f

To a chilled solution of **1** (2.98 g, 23 mmol) in formic acid (120 ml), acetic anhydride (21 ml) was added within 15 min, keeping the temperature at 20°C. The solution was stirred for 1 h without any further cooling (the temperature raised to 65°C). The solvent was evaporated and the residue dissolved in methanol (100 ml) and treated with a cold solution of diazomethane in diethyl ether, at 0°C. The solvent was removed, and the residue dissolved in ethyl acetate (100 ml). The solution was washed with dilute hydrochloric acid (2×15 ml), dried over sodium sulphate and evaporated, yielding 3.07 g (18 mmol, 78%) of an oil, which slowly solidified at 4°C. NMR ($CDCl_3$) δ: 0.9–1.0 ppm, 6H, m; 1.05–1.15 ppm, 1H, m; 1.15–1.30 ppm, 1H, m; 2.0 ppm, 1H, m; 3.9 ppm, 3H, s; 4.85 ppm, 0.5H, dd; 4.95 ppm, 0.5H, dd; 6.20 ppm, 1H (broad); 8.10 ppm, 0.5H, s; 8.20 ppm, 0.5H, s. MS (EI) m/z: 174 (M+1), 142 (M-OMe), 117 (Mc-Lafferty). HPLC (Method 2a) t_R: 24.5 min **3f** (53%) and 26.9 min **2f** (47%).

4.9. *N*-Formyl-D-allo- and L-isoleucine chloroethyl ester 1g

8.0 g of the mixture of L-isoleucine and D-alloisoleucine chloroethyl ester hydrochloride **3** (35 mmol) was dissolved in water (100 ml). After the addition of ethyl acetate (200 ml), the biphasic system was cooled with an ice bath and adjusted to pH 9 by the addition of sodium carbonate. After separation of the phases, the product was extracted from the aqueous solution with ethyl acetate. The combined organic layers were dried over sodium sulphate and evaporated, yielding 6.73 g (35 mmol, 100%) of a mixture of L-isoleucine and D-alloisoleucine chloroethyl ester as a pale yellow oil. The mixture was dissolved in formic acid cooling with an ice-bath between 5 and 10°C. Acetic anhydride (24 ml) was added dropwise at 5°C and the solution was left at rt for 23 h. After evaporation of the solvent, the residue was taken up in chilled water (100 ml) and the product extracted with ethyl acetate (3×100 ml). The organic layers were washed successively with brine (50 ml), aqueous 5% sodium hydrogen carbonate (50 ml) and again with aqueous sodium chloride (50 ml). Drying over sodium sulphate and evaporation of the solvent gave 7.3 g (33 mmol, 94%) of a white solid. NMR ($CDCl_3$) δ: 0.85–1.0 ppm, 6H, m; 1.15–1.35 ppm, 1H, m; 1.35–1.55 ppm, 1H, m; 2.0 ppm, 1H, m; 3.7 ppm, 2H, m; 4.4 ppm, 2H, m; 4.85 ppm, 0.5H, dd; 4.95 ppm, 0.5H, dd; 6.35 ppm, 1H (broad); 8.20 ppm, 0.5H, s; 8.30 ppm, 0.5H, s. HPLC (Method 2b) t_R: 20.0 min **3g** and 22.5 min **2g**.

4.10. *N*-Formyl-D-allo- and L-isoleucine benzyl ester 1h

5.0 g (23 mmol) of the mixture of L-isoleucine and D-alloisoleucine benzyl ester-prepared via the *p*-toluensulphonate salt as described for product **4** was dissolved in formic acid (100 ml) cooling with an ice-bath at 15°C. Acetic anhydride (50 ml) was added dropwise at 10°C and the solution was left at rt for 3 h. After evaporation of the solvent, the residue is taken up in chilled aqueous sodium chloride (100 ml) and the product was extracted with ethyl acetate (3×100 ml). The combined organic layers were washed successively with aqueous sodium chloride (50 ml) solution, aqueous 5% sodium hydrogen carbonate (50 ml) and again with aqueous sodium chloride (50 ml). Drying over sodium sulphate and evaporation of the solvent gave 5.6 g (22 mmol, 96%) of a pale yellow oil. NMR ($CDCl_3$) δ: 0.8–1.0 ppm, 6H, m; 1.1–1.3 ppm, 1H, m; 1.3–1.5 ppm, 1H, m; 1.95 ppm, 1H, m; 4.75 ppm, 0.5H, dd; 4.85 ppm, 0.5H, dd; 5.20, 2H, m; 6.15 ppm, 1H (broad); 7.35 ppm, 5H, m; 8.20 ppm, 0.5H, s; 8.30 ppm, 0.5H, s. MS (EI) m/z: 250 (M+1), 114 (M-$PhCH_2COO$), 91 ($PhCH_2$). HPLC (Method 2c) t_R: 21.0 min **2h** (46%) and 22.5 min **3h** (54%).

4.11. *N*-Acetyl-D-allo- and L-isoleucine benzyl ester 1j

To 5.0 g (23 mmol) of **1d**, dissolved in acetic anhydride (25 ml) in an ice-bath, dimethylaminopyridine (1 mg) and triethylamine (3.5 ml) were added and the solution left at rt for 2.5 h. After evaporation of the solvent, the residue was taken up in cold brine (50 ml) and the product extracted with ethyl acetate (3×50 ml). The combined organic layers were washed successively with 0.2 M aqueous hydrochloric acid (50 ml), aqueous 5% sodium hydrogen carbonate (50 ml) and with sodium chloride (50 ml). Drying over sodium sulphate and evaporation of the solvent gave 5.74 g (22 mmol, 96%) of a white solid. HPLC (Method 2c) t_R: 13.7 min **3j** (54%) and 15.1 min **2j** (46%).

4.12. *N*-Carbobenzyloxy-D-allo- and L-isoleucine benzyl ester 1m

To 100 ml of a 1 M $NaHCO_3$, 5.0 g (23 mmol) of **1d**, benzyl chloroformate (4.2 ml, 30 mmol) were added dropwise and the mixture stirred at 5–10°C. After 45 min, the reaction was quenched with 2 M aqueous hydrochloric acid (21 ml) and, after separation of the phases, the aqueous one was extracted with ethyl acetate (2×100 ml). The combined organic layers were washed with 0.2 M aqueous hydrochloric acid (50 ml) and with brine (2×50 ml). Drying over sodium sulphate and evaporation of the solvent gave a residue, which yielded 7.65 g (22 mmol, 96%) of a pale yellow oil after chromatography. NMR ($CDCl_3$) δ: 0.75–1.0 ppm, 6H, m; 1.0–1.25 ppm, 1H, m; 1.25–1.5 ppm, 1H, m; 1.95 ppm, 1H, m; 4.40 ppm, 0.5H, dd; 4.95 ppm, 0.5H, dd; 5.0–5.4 ppm, 5H, m; 7.35 ppm, 10H, m. MS (EI) m/z 356 (M+1), 220 (M–$PhCH_2OCO$), 91 ($PhCH_2$). HPLC (Method 2c) t_R: 9.4 min **2m** (47%) and 24.0 min **3m** (53%).

4.13. Enzymatic hydrolysis of compounds 1f–m

A suspension of the substrate in distilled water (0.18 or 0.36 M) was heated at 39°C and adjusted to pH 7.8 by the addition of NaOH 0.35 M. Alcalase 2.5L was then added (0.21 or 0.43 g/ml) and the reaction mixture was kept at pH 7.8 by automatic addition of NaOH 0.35 M. When the consumption of NaOH reached about 50% (or more) the reaction was stopped and the unreacted substrate was recovered by extraction with *tert*-butylmethylether. The acidic product could be recovered by extraction or precipitation after acidification of the aqueous phase with citric acid or dilute hydrochloric acid.

4.14. *N*-Formyl-D-alloisoleucine methyl ester 3f

[Substrate]: 0.18 M; [Enzyme]: 0.043 (g/ml) The reaction was stopped after 23 h (64% of sodium hydroxide consumption). Yield: 30% (pale yellow oil) d.e.(HPLC, Method 2a): 98% $[\alpha]_D=-32.5$ (c 1 in $CHCl_3$) NMR ($CDCl_3$) δ: 0.85 ppm [d, $J=7.0$ Hz, 3H, $CH_3CH_2CH(\mathbf{CH_3})CH(NHCOH)COOCH_3$]; 0.95 ppm, [t, $J=7.0$ Hz , 3H, $\mathbf{CH_3}CH_2CH(CH_3)CH(NHCOH)COOCH_3$]; 1.1 ppm, [m, 1H,$CH_3\mathbf{CH_2}CH(CH_3)$ CH (NHCOH)$COOCH_3$]; 1.25 ppm [m, 1H, $CH_3\mathbf{CH_2}CH(CH_3)CH(NHCOH)COOCH_3$]; 2.0 ppm, [m, 1H, $CH_3CH_2\mathbf{CH}(CH_3)CH(NHCOH)COOCH_3$]; 3.9 ppm, [s, 3H, $RCOO\mathbf{CH_3}$]; 4.95 ppm, [dd, $J_1=9.4$ Hz, $J_2=3.5$ Hz, 1H, $CH_3CH_2CH(CH_3)\mathbf{CH}(NHCOH)COOCH_3$]; 6.2 ppm, [d broad, $J=9.4$ ppm, 1H, $CH_3CH_2CH(CH_3)CH(\mathbf{NH}COH)COOCH_3$]; 8.15 ppm, [s,1H, $CH_3CH_2CH(CH_3)CH$ (NH**COH**) $COOCH_3$]. Anal. calcd for $C_8H_{15}NO_3$: C, 55.47%; H, 8.73%; N, 8.09%. Found: C, 55.40%; H, 8.71%; N, 8.11%

4.15. *N*-Formyl-D-alloisoleucine chloroethyl ester 3g

[Substrate]: 0.18 M [Enzyme]: 0.043 (g/ml) The reaction was stopped after 2.5 h (59% of sodium hydroxide consumption). After recovery by extraction the unreacted substrate was purified by chromatography (hexane/ethyl acetate 1:1). Yield: 40% (pale yellow oil) d.e.(HPLC, method 2b): 96% $[\alpha]_D=-13.3$ (c 1 in $CHCl_3$); NMR ($CDCl_3$) δ: 0.9 ppm, [d, $J=7.1$ Hz, 3H, $CH_3CH_2CH(\mathbf{CH_3})CH(NHCOH)COOR$]; 0.95 ppm, [t, $J=7.5$ Hz, 3H, $\mathbf{CH_3}CH_2CH(CH_3)CH(NHCOH)COOR$]; 1.1–1.3 ppm, [dq, $J_1=7.1$ Hz, $J_2=$not determined, $CH_3\mathbf{CH_2}CH(CH_3)CH(NHCOH)COOR$]; 1.35–1.55 ppm, [dq, $J_1=7.1$ Hz, $J_2=$not determined, $CH_3\mathbf{CH_2}CH(CH_3)CH(NHCOH)COOR$]; 2.0 ppm, [m, 1H, $CH_3CH_2\mathbf{CH}(CH_3)CH$ (NHCOH)COOR]; 3.70 ppm, [t, $J=5.6$ Hz, $RCOOCH_2\mathbf{CH_2}Cl$]; 4.40 ppm, [m, 2H, $RCOO\mathbf{CH_2}$ CH_2Cl]; 4.85 ppm, [dd, $J_1=9.4$ Hz, $J_2=4.1$ Hz, 1H, $CH_3CH_2CH(CH_3)\mathbf{CH}(NHCOH)COOR$]; 6.35 ppm, [d (broad), $J=9.0$ Hz, 1H, $CH_3CH_2CH(CH_3)CH(\mathbf{NH}COH)COOBn$]; 8.25 ppm, [s, 1H, CH_3 $CH_2CH(CH_3)CH(NH\mathbf{COH})COOCH_3$]. Anal. calcd for $C_9H_{16}ClNO_3$: C, 48.76%; H, 7.27%, Cl 15.99%; N, 6.32%. Found: C, 48.65%; H, 7.22%, Cl 15.76%; N, 6.33%.

4.16. *N*-Formyl-D-alloisoleucine benzyl ester 3h

[Substrate]: 0.18 M [Enzyme]: 0.021 (g/ml) The reaction was stopped after 22.5 h (47% of sodium hydroxide consumption). After recovery by extraction the unreacted substrate was purified by chromatography (hexane/ethyl acetate 6:4). Yield: 41% (pale yellow oil) d.e. (HPLC, method 2c): 98% $[\alpha]_D=-7.7$ (c 1 in $CHCl_3$); NMR ($CDCl_3$) δ: 0.8 ppm, [d, $J=7.2$ Hz, 3H, $CH_3CH_2CH(\mathbf{CH_3})CH(NHCOH)COOBn$]; 0.9 ppm, [t, $J=7.2$ Hz, 3H, $\mathbf{CH_3}CH_2CH(CH_3)CH(NHCOH)COOBn$]; 1.1–1.3 ppm, [dq, $J_1=7.2$ Hz, $J_2=6.4$ Hz, $CH_3\mathbf{CH_2}CH(CH_3)CH(NHCOH)COOBn$]; 1.3–1.5 ppm, [dq, $J_1=7.2$ Hz, $J_2=6,4$ Hz, $CH_3\mathbf{CH_2}CH$ $(CH_3)CH(NHCOH)COOBn$]; 1.95 ppm, [m, 1H, $CH_3CH_2\mathbf{CH}(CH_3)CH(NHCOH)$ $COOBn$]; 4.85 ppm, [dd, $J_1=8.8$ Hz, $J_2=4.0$ Hz, 1H, $CH_3CH_2CH(CH_3)\mathbf{CH}(NHCOH)$ $COOBn$]; 5.15 ppm, [AB system, $J_{AB}=16.0$ Hz, 2H, $RCO\mathbf{CH_2}Ph$]; 6.30 ppm, [d (broad), $J=8,0$ Hz, 1H, CH_3CH_2CH $(CH_3)CH(\mathbf{NH}COH)COOBn$]; 7.35 ppm, [m, 5H, $RCOCH_2\mathbf{Ph}$]; 8.20 ppm, [s, 1H, $CH_3CH_2CH(CH_3)$ $CH(NH\mathbf{COH})COOCH_3$]. Anal. calcd for $C_{14}H_{19}NO_3$: C, 67.45%; H, 7.68%; N, 5.62%. Found: C, 67.35%; H, 7.72%; N, 5.60%.

4.17. *N*-Acetyl-D-alloisoleucine benzyl ester 3j

[Substrate]: 0.18 M [Enzyme]: 0.021 (g/ml) The reaction was stopped after 3.3 h (48% of sodium hydroxide consumption). After recovery by extraction the unreacted substrate was purified by chromatography (hexane/ethyl acetate 6:4). Yield: 50% (white solid) d.e. (HPLC, Method 2c): L-isomer was not detectable $[\alpha]_D = -13.8$ (*c* 1 in $CHCl_3$); NMR ($CDCl_3$) δ: 0.8 ppm, [d, $J = 7.0$ Hz, 3H, $CH_3CH_2CH(\mathbf{CH_3})CH(NHCOCH_3)$-$COOBn$]; 0.9 ppm, [t, $J = 7.4$ Hz, 3H, $\mathbf{CH_3}CH_2CH$-$(CH_3)CH(NHCOCH_3)COOBn$]; 1.1–1.3 ppm, [dq, $J_1 = 7.4$ Hz, $J_2 = 6.5$ Hz, $CH_3\mathbf{CH_2}CH(CH_3)CH(NHCO$ $CH_3)COOBn$]; 1.3–1.5 ppm, [dq, $J_1 = 7.4$ Hz, $J_2 = 6.5$ Hz, $CH_3\mathbf{CH_2}CH(CH_3)CH(NHCOH)COOBn$]; 1.95 ppm, [m, 1H, $CH_3CH_2\mathbf{CH}(CH_3)CH(NHCOCH_3)$ $COOBn$]; 2.05 ppm, [s, 3H, $CH_3CH_2CH(CH_3)CH$-$(NHCO\mathbf{CH_3})COOCH_3$]; 4.75 ppm, [dd, $J_1 = 9.2$ Hz, $J_2 = 4.4$ Hz, 1H, $CH_3CH_2CH(CH_3)\mathbf{CH}(NHCOCH_3)$-$COOBn$]; 5.15 ppm, [AB system, $J_{AB} = 16.1$ Hz, 2H, $RCO\mathbf{CH_2}Ph$]; 6.05 ppm, [d (broad), $J = 9.2$ Hz, 1H, $CH_3CH_2CH(CH_3)CH(\mathbf{NH}COCH_3)$ $COOBn$]; 7.35 ppm, [m, 5H, $RCOCH_2\mathbf{Ph}$]. Anal. calcd for $C_{15}H_{21}NO_3$: C, 68.42%; H, 8.04%; N, 5.32%. Found: C, 68.46%; H, 8.06%; N, 5.24%.

4.18. *N*-Acetyl-D-alloisoleucine 3i

600 mg (2.3 mmol) of *N*-acetyl-D-alloisoleucine benzyl ester **3j** were dissolved in ethanol (100 ml), and hydrogenated on 10% Pd–C (400 mg) at 60 psi for 2 h. The catalyst was removed by filtration and washed with ethanol. Evaporation the solvent gave *N*-acetyl-D-alloisoleucine **3i**, (290 mg, 1.7 mmol, 74%) as a white solid. $[\alpha]_D = -19.0$ (*c* 2 in EtOH) (Ref.: −20.5);[6] NMR (CD_3OD) δ: 0.8 ppm, [d, $J = 6.8$ Hz, 3H, CH_3CH_2-$CH(\mathbf{CH_3})CH(NHCOCH_3)COOH$]; 0.9 ppm [t, $J = 7.7$, 3H, $\mathbf{CH_3}CH_2CH(CH_3)CH(NHCOCH_3)COOH$]; 1.1 ppm, [dq, $J_1 = 7.7$, $J_2 = 7.4$, 1H, $CH_3\mathbf{CH_2}CH$-$(CH_3)CH(NHCOCH_3)COOH$]; 1.25 ppm, [dq, $J_1 = 7.7$, $J_2 = 6.8$, 1H, $CH_3\mathbf{CH_2}CH$ $(CH_3)CH$ $(NHCOCH_3)$-$COOH$]; 1.8 ppm, [m, 1H, $CH_3CH_2\mathbf{CH}(CH_3)CH$-$(NHCOCH_3)$ $COOH$]; 2.1 ppm, [s, 1H, CH_3CH_2CH-$(CH_3)CH(NHCO\mathbf{CH_3})COOH$]; 4.3 ppm, [dd, $J_1 = 8.6$ Hz, $J_2 = 4.7$ Hz, 1H, $CH_3CH_2CH(CH_3)\mathbf{CH}(NH$-$COCH_3)COOH$]; 7.9 ppm, [d broad, $J = 8.6$ Hz, 1H, $CH_3CH_2CH(CH_3)$ $CH(\mathbf{NH}COCH_3)COOH$]. Anal. calcd for $C_8H_{15}NO_3$: C, 55.47%; H, 8.73%; N, 8.09%. Found: C, 55.37%; H, 8.76%; N, 8.28%.

4.19. D-Alloisoleucine 4

4.19.1. From *N*-acetyl-D-alloisoleucine 3i obtained from 3i. *N*-Acetyl-D-alloisoleucine **3i** (1.0 g, 3.8 mmol) was dissolved in dioxane (10 ml) and 5% aqueous hydrochloric acid (10 ml) and refluxed 8 h. After cooling, the solution was washed with diethyl ether (2×30 ml) and adjusted to pH 6.5 with sodium carbonate. After filtration D-alloisoleucine was recovered as a white solid. $[\alpha]_D = -31.2$ (*c* 2 in HCl 5 M).

4.19.2. From *N*-formyl-D-alloisoleucine benzyl ester 3h. *N*-Formyl-D-alloisoleucine benzyl ester **3h** (1.0 g, 3.8 mmol) was dissolved in dioxane (10 ml) and 5% aqueous hydrochloric acid (10 ml) and refluxed 11 h. After cooling, the solution was washed with ethyl acetate (2×30 ml) and adjusted to pH 7–8 with sodium carbonate. Extraction of the product with ethyl ether (3×30 ml) gave, after drying and evaporation of the solvent, D-alloisoleucine benzyl ester **3d** (0.726 g, 1.2 mmol, 32%) as a solid. 660 mg (6.0 mmol) of D-alloisoleucine benzyl ester **3d** was dissolved in water (50 ml) and ethanol (100 ml), and hydrogenated on 10% Pd–C (400 mg) at 70 psi for 1.5 h. The catalyst was removed by filtration and washed with ethanol/water 1:1. Evaporation of the solvent gave D-alloisoleucine (360 mg, 2.7 mmol, 90%). $[\alpha]_D = -36.6$ (*c* 2 in HCl 5M) (−37.4 on a commercial sample; lit.: −37.8).

4.19.3. From *N*-formyl-D-alloisoleucine benzyl ester 3h. A suspension of *N*-formyl-D-alloisoleucine benzyl ester **3h** (1.0 g, 4.0 mmol) in 1 M hydrochloric acid (80 ml) was heated at 90°C for 12 h. After cooling, the solution was washed with ethyl acetate (2×50 ml) and adjusted to pH 9 with sodium carbonate. Extraction of the product with ethyl acetate (3×50 ml) gave, after drying and evaporation of the solvent, D-alloisoleucine benzyl ester **3d** (0.76 g, 3.4 mmol, 85%) as an oil. 660 mg (6.0 mmol) of D-alloisoleucine benzyl ester **3d** was dissolved in water (50 ml) and ethanol (100 ml), and hydrogenated on 10% Pd–C (400 mg) at 70 psi for 1.5 h. The catalyst was removed by filtration and washed with ethanol/water 1:1. Evaporation of the solvent gave D-alloisoleucine (360 mg, 2.7 mmol, 90%). $[\alpha]_D = -31.2$ (*c* 2 in HCl 5 M).

4.19.4. From *N*-formyl-D-alloisoleucine chloroethyl ester 3g. A suspension of *N*-formyl-D-alloisoleucine chloroethyl ester **3g** (1.71 g, 7.7 mmol) in HCl 1 M (70 ml) was heated at 90°C for 5 h. After cooling, the solution was washed with ethyl acetate (2×50 ml) and adjusted to pH 9 with sodium carbonate. Extraction of the product with ethyl acetate (3×50 ml) gave, after drying and evaporation of the solvent, D-alloisoleucine chloroethyl ester **3c** (1.3 g, 6.7 mmol, 87%) as an oil. 1.17 g (6.0 mmol) of D-alloisoleucine chloroethyl ester **3c** was dissolved in dioxane (10 ml). After addition of sodium hydroxide 1 M (10 ml) the solution was kept at rt for 15 h, then refluxed for 1.5 h. After cooling the solution was washed with *tert*-butylmethylether (2×20 ml) and then evaporated. The residue was taken up in water (6 ml) and the pH adjusted to 6.2 with 6 M hydrochloric acid. After filtration D-alloisoleucine was recovered as a white solid. $[\alpha]_D = -31.2$ (*c* 2 in HCl 5 M).

References

1. Bada, J. L.; Zhao, M.; Steinberg, S.; Ruth, E. *Nature* **1986**, *319*, 314–316.
2. Bodanzky, M.; Perlman, D. *Science* **1969**, *163*, 352–354.
3. Tsuda, M.; Shimbo, K.; Kubota, T.; Mikami, Y.; Kobayashi, J. *Tetrahedron* **1999**, *55*, 10305–10314.

4. Noda, H.; Sakai, K.; Murakami, N. *Jpn. Pat. Appl.* 1999 (CA 131:157989).
5. Roy, M. C.; Ohtani, I. I.; Ichiba, T.; Tanaka, J.; Satari, R.; Higa, T. *Tetrahedron* **2000**, *56*, 9079–9092.
6. Murakami, Y.; Takei, M.; Shindo, K.; Kitazume, C.; Tanaka, J.; Higa, T.; Fukamachi, H. *J. Nat. Prod.* **2002**, *65*, 259–261.
7. Kawabata, T.; Fuji, K. *Jpn. Pat. Appl.* 2002 (CA 137:47441).
8. Kitagawa, I.; Kobayashi, M.; Lee, N. K.; Shibuya, H.; Kawata, Y.; Sakiyama, F. *Chem. Pharm. Bull.* **1986**, *34*, 2664–2667.
9. Kitagawa, I.; Lee, N. K.; Kobayashi, M.; Shibuya, H. *Tetrahedron* **1991**, *47*, 2169–2180.
10. Kobayashi, M.; Lee, N. K.; Shibuya, H.; Momose, T.; Kitagawa, I. *Chem. Pharm. Bull.* **1991**, *39*, 1177–1184.
11. Kobayashi, M.; Kanzaki, K.; Katayama, S.; Ohashi, K.; Okada, H.; Ikegami, S.; Kitagawa, I. *Chem. Pharm. Bull.* **1994**, *42*, 1410–1415.
12. Nakamura, H.; Kobayashi, J.; Nakamura, Y.; Ohizumi, Y.; Kondo, T.; Hirata, Y. *Tetrahedron Lett.* **1986**, *27*, 4319–4322.
13. Li, S.; Dumdei, E. J.; Blunt, J. W.; Munro, M. H. G.; Robinson, W. T.; Pannell, L. K. *J. Nat. Prod.* **1998**, *61*, 724–728.
14. Flouret, G.; du Vigneaud, V. *J. Am. Chem. Soc.* **1965**, *87*, 3775–3777.
15. Liang, B.; Portonovo, P. S.; Vera, M. D.; Xiao, D.; Joullié, M. M. *Org. Lett.* **1999**, *1*, 1319–1322.
16. Portonovo, P; Liang, B.; Joullié, M. M. *Tetrahedron: Asymmetry* **1999**, *10*, 1451–1455.
17. Flouret, G.; Nakagawa, S. H. *J. Org. Chem.* **1975**, *40*, 2635–2637.
18. Shiraiwa, T.; Ikawa, A.; Sakaguchi, K.; Kurokawa, H. *Bull. Chem. Soc. Jpn.* **1984**, *57*, 2234–2237.
19. Noda, H.; Ohsaka, K.; Sakai, K.; Murakami, H.131:170636 CA Eur. Pat. Appl.
20. Yokouchi, Y.; Ohno, Y.; Nakagomi, K.; Tanimura, T.; Kabasawa, Y. *Chromatography* **1998**, *19*, 374–375.
21. Doherty, D. G.; Popenoe, E. A. *J. Biol. Chem.* **1951**, *189*, 447.
22. Lloyd-Williams, P.; Monerris, P.; Gonzalez, I.; Jou, G.; Giralt, E. *J. Chem. Soc., Perkin Trans. 1* **1994**, 1969–1974.
23. Keil, O.; Schneider, M. P.; Rasor, J. P. *Tetrahedron: Asymmetry* **1999**, *6*, 1257–1260.
24. Chen, S.-T.; Wang, K.-T.; Wong, C.-H. *J. Chem. Soc., Chem. Commun.* **1986**, *20*, 1514–1516.
25. Chen, S.-T.; Chen, S.-Y; Hsiao, S. C.; Wang, K. T. *Biotechnol. Lett.* **1991**, *13*, 773.
26. Chen, S.-T.; Wang, K.-T. *J. Chin. Chem. Soc.* **1999**, *46*, 301–311.
27. Chen, S.-T.; Huang, W.-T.; Wang, K.-T. *Chirality* **1994**, *6*, 572–576.
28. Koeller, K.; Wong, C.-H. *Nature* **2001**, *409*, 232–240.
29. Schmid, A; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B. *Nature* **2001**, *409*, 258–268.
30. Shaw, N. M.; Robins, K. T.; Kiener, A. *Adv. Synth. Catal.* **2003**, *245*, 425–435.
31. Schulze, B.; de Vroom, E. In *Enzyme Catalysis in Organic Synthesis*; Drauz, K.; Waldmann, H., Eds. Wiley-VCH, 2002; pp. 716–741.
32. Bommarius, A. S. In *Enzyme Catalysis in Organic Synthesis*; Drauz, K.; Waldmann, H., Eds., Wiley-VCH, 2002; pp. 741–761.
33. Pietsch, M.; Syldatk, C. In *Enzyme Catalysis in Organic Synthesis*; Drauz, K.; Waldmann, H., Eds. Wiley-VCH, 2002; pp. 761–796.
34. Rozzell, J. D.; Bommarius, A. S. In *Enzyme Catalysis in Organic Synthesis*; Drauz, K.; Waldmann, H., Eds.; Wiley-VCH, 2002; pp. 873–892.
35. Schnell, B.; Faber, K.; Kroutil, W. *Adv. Synth. Catal.* **2003**, *245*, 653–666.
36. Brenner, M.; Huber, W. *Helv. Chim. Acta* **1953**, *36*, 1109–1115.
37. Tschaen, D. M.; Turos, E.; Weinreb, S. M. *J. Org. Chem.* **1984**, *49*, 5058–5064.
38. Kunz, H.; Bucholz, M. *Chem. Ber.* **1979**, *112*, 2145–2157.
39. Zervas, L.; Winitz, M.; Greenstein, J. P. *J. Org. Chem.* **1957**, *22*, 1515–1520.
40. Schmidlin, T.; Burckhardt, P. E.; Waespe-Sarcevic, N.; Tamm, C. *Helv. Chim. Acta* **1983**, *66*, 450–465.
41. Huffman, W. A.; Ingersoll, A. W. *J. Am. Chem. Soc.* **1951**, *13*, 3366–3369.
42. Kessler, H.; Schudok, M. *Synthesis* **1990**, 457–458.