Resolution of non-proteinogenic amino acids via microbial lipase-catalyzed enantioselective transesterification

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Abstract: A number of non-proteinogenic amino acids bearing aliphatic side chains were resolved with moderate to good enantioselectivities (E = 15–42) through the *Burkholderia cepacia* lipase-catalyzed enantioselective transesterification of the 2,2,2-trifluoroethyl esters of their *N*-benzyloxycarbonyl derivatives with methanol as a nucleophile in diisopropyl ether.

Key words: non-proteinogenic amino acids, microbial lipases, *Burkholderia cepacia* lipase, 2,2,2-trifluoroethyl ester, enantioselective transesterification, enantioselectivity.

Résumé : On a résolu un certain nombre d'acides aminés non-protéinogéniques portant des chaînes latérales aliphatiques, avec des énantiosélectivités allant de modérées à bonnes (E = 15–42), en faisant appel à une transestérification énantiosélective catalysée par la lipase *Burkholderia cepacia* des esters 2,2,2-trifluoroéthyles de leurs dérivés *N*-benzyloxycarbonyles et en utilisant le méthanol comme nucléophile dans l'éther diisopropylique comme solvant.

Mots-clés : acides aminés non-protéinogéniques, lipases microbiennes, lipase *Burkholderia cepacia*, ester 2,2,2-trifluoroéthylique, transestérification énantiosélective, énantiosélectivité.

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Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) have been employed as a very attractive group of enzymes for synthetic purposes (1) because they are stable and easy to handle and they have a rather broad substrate specificity. Moreover, as they are easily available from a variety of sources (especially bacteria and fungi), there must be a fair chance of finding a suitable enzyme for each transformation of interest in terms of catalytic activity and (or) selectivity. Since the first discovery of enzymatic activity in anhydrous organic media (2), 1ipase-catalyzed reactions, such as esterification and transesterification, have attracted an increasing attention in the field of organic synthesis. Thus, a large number of reports have dealt with the optical resolution of alcohols through lipase-catalyzed reactions in organic solvents. On the other hand, fewer examples have been accumulated for the resolution of carboxylic acids by means of the lipase-catalyzed esterification or transesterification procedure in organic milieu. We have previously reported on the resolution of 2-substituted carboxylic acids via the microbial lipase-catalyzed irreversible transesterification of their vinyl esters (3). We also have recently reported on the resolution of non-proteinogenic aliphatic amino acids through the *Carica papaya* lipase-catalyzed enantioselective transesterification of their *N*-benzyloxycarbonyl (*Z*) esters (4). In the present paper, we describe the details of our study on the resolution of non-proteinogenic amino acids via the similar enantioselective transesterification procedure mediated by microbial lipases (5). Homochiral non-proteinogenic amino acids are useful as building blocks for the synthesis of analogs of biologically active peptides (6) and as chiral auxiliaries for other synthetic purposes (7). We have already reported on the resolution of non-proteinogenic amino acids via the enantioselective hydrolysis of their *N*-*Z*-protected esters mediated by microbial and mammalian lipases (8) and via the enantioselective hydrolysis of their *N*-unprotected esters catalyzed by microbial lipases (9).

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Results and discussion

Initially, the transesterification between *N*-*Z*-DL-norvaline (2-aminopentanoic acid) 2,2,2-trifluoroethyl ester (DL-**1c**) and methanol in diisopropyl ether was chosen as a model reaction (Scheme 1; R = n-C_3H_7; R′ = CH_3), and a dozen lipases of microbial and mammalian origins were screened: *Burkholderia* (*Pseudomonas*) *cepacia* (Amano P), *Rhizopus javanicus* (Amano F), *Mucor javanicus* (Amano M), *Rhizopus japonicus* (Rhilipase A5), *Chromobacterium viscosum* (Asahikasei LP), *Aspergillus niger* (Amano A), *Candida rugosa* (Sigma Type VII, Meito MY, Amano AY), and porcine pancreas (Sigma Type II). Part of the results are summarized in Table 1. We already reported that in the *Carica papaya* lipase-catalyzed enantioselective transesterification the 2,2,2-trifluoroethyl ester was an ester of choice (4). Also in the present case, the transesterification reactions

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Scheme 1. Microbial lipase-catalyzed enantioselective transesterification between an *N*-*Z*-DL-amino acid 2,2,2-trifluoroethyl ester (DL-**1**) and an alcohol. See Table 4 for R.

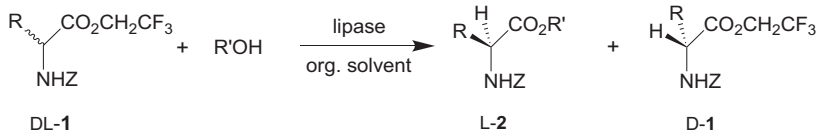

Table 1. Enantioselective transesterification between *N*-*Z*-DL-norvaline 2,2,2-trifluoroethyl ester (DL-**1c**) and methanol with different lipases in diisopropyl ether.

Lipase source	Conversion (%)	Time	ee (%)[a]	*E*
Burkholderia cepacia[b]	38	10 h	91	37
Rhizopus javanicus[c]	38	12 h	88	27
Mucor javanicus[d]	41	7 h	67	8.0
Rhizopus japonicus[e]	37	106 h	77	12
Chromobacterium viscosum[f]	43	38.5 h	68	8.4
Aspergillus niger[g]	21	21 d	61	4.9
Porcine pancreas[h]	31	88 d	51	3.8
Candida rugosa[i]	34	92 d	7.6	1.2

Note: Conditions: 0.4 mmol of DL-**1c**, 1.2 mmol of methanol, and 120 mg of a lipase preparation in 0.8 mL of diisopropyl ether at 25 °C.
[a]Enantiomeric excess of the newly formed methyl ester (**2c**).
[b]Amano P.
[c]Amano F.
[d]Amano M.
[e]Rhilipase A5 from Nagase ChemteX Corporation.
[f]Asahi Chemical Industry LP.
[g]Amano A.
[h]Sigma Type II.
[i]Sigma Type VII.

Table 2. *B. cepacia* lipase-catalyzed transesterification of *N*-*Z*-DL-norvaline 2,2,2-trifluoroethyl ester (DL-**1c**) with different alcohols as nucleophiles in diisopropyl ether.

Alcohol	Conversion (%)	Time (h)	ee (%)[a]	*E*
Methanol	38	10	91	37
Ethanol	40	11	90	35
Butanol	37	13	90	32
Heptanol	40	18	88	28
Tetradecanol	43	24	86	26

Note: Conditions: 0.4 mmol of DL-**1c**, 1.2 mmol of an alcohol, and 120 mg of *B. cepacia* lipase in 0.8 mL of diisopropyl ether at 25 °C.
[a]Enantiomeric excess of the newly formed ester.

of other esters, such as the 2-chloroethyl and 2,2,2-trichloroethyl esters, were extremely slow, and moreover, such an active ester as *N*-hydroxysuccinimide ester proceeded with almost no enantioselectivity. As can be seen form Table 1, the rate and enantioselectivity observed varied largely with the enzyme employed, while the stereochemical preference remained unchanged, the preferred absolute configuration of the newly formed methyl ester (**2c**) being L. Among the enzymes tested, 1ipases from *B. cepacia* and *R. javanicus* showed a rather high reaction rate together with a good enantioselectivity, as judged from the value of enantiomeric ratio, *E* (10). With *M. javanicus* lipase, the fastest reaction was accompanied by a poor enantioselectivity. With other lipases, the reactions were slow, and enantioselectivities were practically unacceptable. This includes *C. rugosa* lipase, which is known to show a high enantioselectivity toward carboxylic acids (11).

Next, the effect of alcohols as a nucleophile was examined on the *B. cepacia* lipase-catalyzed transesterification of DL-**1c** in diisopropyl ether. The results are shown in Table 2. The preferred configuration of the newly formed esters was L, irrespective of the alcohol employed. There was almost no difference between methanol and ethanol as the nucleophile in terms of the reaction rate and enantioselectivity. There was a tendency for the enantioselectivity, together with the reaction rate, to decrease with the chain length of the alcohol. This is similar to that observed in the *A. niger* lipase-catalyzed transesterification of vinyl 2-phenoxypropanoate (3), though the differences among the alcohols were rather small in the present case. A reverse tendency, i.e., an increase in enantioselectivity with increasing chain length of the alcohol, has been reported in the *C. rugosa* lipase-catalyzed esterification of 2-(4-chlorophenoxy)propanoic acid (12). The discrepancy may be attributable to the nature of the lipases used and (or) a difference in the type of reactions (transesterification vs. esterification).

Among the external factors influencing the catalytic activity and enantioselectivity of enzymatic reactions, much attention has recently been focused on the solvent effect (2). However, it is still difficult to predict the solvent effect, especially on the enantioselectivity of a certain reaction, and the best way is still to select an appropriate solvent after the screening experiment. Among many solvent parameters proposed so far is the hydrophobicity parameter, log *P* (13), and a correlation has sometimes been observed between the *E* values of an enzymatic resolution carried out in organic solvents and their log *P* values. We also have reported such a correlation, though rough, in the *A. niger* lipase-catalyzed transesterification of vinyl 2-phenoxypropanoate with methanol: the larger the log *P* value, the higher the *E* value (3). A number of solvents having different log *P* values were screened next in the *B. cepacia* lipase-catalyzed transesterification between DL-**1c** and methanol (Table 3). In the table, the solvents are arranged in decreasing order of log *P*. The stereochemical preference remained to be L with all the solvents examined. Good-to-excellent *E* values were obtained in most of the solvents except methanol and *N*,*N*-dimethylformamide. Thus, it is difficult to find a correlation between the *E* value and the log *P* value. The solvent effect on enantioselectivity observed here resembles that reported previously in the transesterification of 1-nitro-2-propanol with vinyl acetate cata1yzed by a lipase from *Pseudomonas* sp. (14) in that tetrahydrofuran, 1,4-dioxane, and benzene give excellent enantioselectivities. In the *A. niger* lipase-catalyzed transesterification of vinyl 2-phenoxypropanoate

Table 3. *B. cepacia* lipase-catalyzed transesterification between *N*-*Z*-DL-norvaline 2,2,2-trifluoroethyl ester (DL-**1c**) and methanol in different solvents.

Solvent	Log P^a	Conversion (%)	Time (h)	% ee^b	*E*
Cyclohexane	3.2	41	6.3	90	36
Toluene	2.5	39	75	90	34
Benzene	2.0	40	71	92	45
Chloroform	2.0	37	14 d	91	36
Diisopropyl ether	1.9	38	10	91	37
Diethyl ether	0.85	36	33	91	35
Pyridine	0.71	30	10 d	77	11
Tetrahydrofuran	0.49	40	129	92	45
Acetonitrile	–0.33	36	15.5	89	28
Methanol	–0.76	37	34	6	1.2
N,*N*-Dimethylformamide	–1.0	43	23	0	1.0
1,4-Dioxane	–1.1	40	95	91	39

Note: Conditions: 0.4 mmol of DL-**1c**, 1.2 mmol of methanol, and 120 mg of *B. cepacia* lipase in 0.8 mL of an anhydrous organic solvent at 25 °C.

[a]Hydrophobicity parameters are cited from ref. 13.

[b]Enantiomeric excess of the newly formed methyl ester (**2c**).

with methanol (3) and the *C. papaya* lipase-catalyzed transesterification of *N*-*Z*-amino acid esters (4), aliphatic hydrocarbons, especially cyclohexane, gave high enantioselectivities. Thus, the solvent effect on the enantioselectivity of lipase-catalyzed transesterifications of carboxylic acid esters is likely to be dependent largely on the lipase source. On the other hand, the solvent had a larger effect on the reaction rate of the present lipase-catalyzed transesterification. The reaction was fast in an aliphatic hydrocarbon, cyclohexane, while it was much slower in the aromatic hydrocarbons. It was extremely retarded in a halogenated methane, chloroform. In the acyclic ethers, the reaction proceeded rather smoothly. Although cyclohexane seemed to be the solvent of choice in terms of the reaction rate as well as enantioselectivity, it had a poor solubilizing power towards some of the substrates examined here. Thus, diisopropyl ether was chosen as the most practical solvent, taking into account not only the reaction rate and enantioselectivity but also the solubility of the substrates.

Based on these results, the transesterification between the 2,2,2-trifluoroethyl esters (DL-**1**) of the *N*-*Z*-derivatives of a number of non-proteinogenic amino acids and methanol in diisopropyl ether was examined using the two microbial lipases, *B. cepacia* lipase and *R. javanicus* lipase. The results are summarized in Tables 4 and 5, respectively, which include also those with proteinogenic amino acids for the purpose of comparison. The *B. cepacia* lipase-catalyzed transesterifications proceeded rather smoothly. The esters (DL-**1**) of amino acids carrying aliphatic side chains reacted generally with high enantioselectivities. One exception is the result with the alanine derivative (DL-**1a**). The enantioselectivity did not depend directly on the length of the side chain of amino acids. As the *E* values were larger than 15 in all these cases except **1a**, the recovered, unreacted substrate can be obtained with a practically high enantiomeric excess (ee) value when the reaction is allowed to proceed near or over ~60% conversion (10). In practice, this was confirmed experimentally in a gram-scale resolution of *N*-*Z*-norvaline mediated by *B. cepacia* lipase as described later. The enantiodiscrimination by *R. javanicus* lipase was poorer than that by *B. cepacia* lipase for each substrate. In comparison with the results with aliphatic amino acids, however, the enantioselectivities observed with aromatic amino acid derivatives were lower in the presence of both the microbial lipases. It is interesting to note that *R. javanicus* lipase showed better enantiodiscrimination than *B. cepacia* lipase for each aromatic substrate. In all the cases mentioned earlier, the preferential reaction of the L-enantiomers was confirmed by comparison with authentic samples prepared from the optically active amino acids, if available, on HPLC or suggested from the regularity of elution order of the enantiomers on HPLC (15). This stereochemical preference is the same as that observed in the lipase-catalyzed hydrolysis of the 2-chloroethyl or 2,2,2-trifluoroethyl esters of the *N*-*Z*-derivatives of amino acids (8) and that observed in the *C. papaya* lipase-catalyzed transesterification of the *N*-*Z*-amino acid 2,2,2-trifluoroethyl esters (4).

Furthermore, a gram-scale resolution of *N*-*Z*-DL-norvaline was carried out via the *B. cepacia* lipase-catalyzed transesterification of DL-**1c** with methanol in diisopropyl ether. After incubation of 9 h (33% conversion), the product methyl ester was isolated by column chromatography on silica gel, which after saponification yielded *N*-*Z*-L-norvaline with 92% ee. In another run, *N*-*Z*-D-norvaline with 97% ee was obtained from the remaining trifluoroethyl ester substrate separated after incubation of 35 h (55% conversion).

In the *C. papaya* lipase-catalyzed transesterification of *N*-*Z*-amino acid 2,2,2-trifluoroethyl esters, excellent enantioselectivities were obtained in cyclohexane. However, the enantioselectivity deteriorated by switching the solvent from cyclohexane to diisopropyl ether in which the starting esters were more soluble (4). On the other hand, although the enantioselectivities observed in the *B. cepacia* lipase-catalyzed transesterification of the same substrates were moderate to good, the fact that diisopropyl ether can be used as a solvent without lowering the enantioselectivity as compared with that in cyclohexane should make the present methodology applicable to more non-proteinogenic amino acids.

Table 4. *B. cepacia* lipase-catalyzed transesterification between *N*-*Z*-DL-amino acid 2,2,2-trifluoroethyl esters (DL-**1**) and methanol in diisopropyl ether.

Compound	R	Conversion (%)	Time (h)	ee (%)[a]	*E*
1a	CH_3	41	2.1	69	8.7
1b	C_2H_5	38	2.5	92	42
1c	n-C_3H_7	38	10	91	37
1d	n-C_4H_9	38	32	89	30
1e	n-C_5H_{11}	35	37	87	23
1f	n-C_6H_{13}	36	18	90	31
1g	CH_2=$CHCH_2$	42	2.5	79	15
1h	$C_6H_5CH_2$	42	14	73	11
1i	3-$FC_6H_4CH_2$	37	22	68	7.7
1j	4-$FC_6H_4CH_2$	43	18	55	5.1
1k	4-Thiazolylmethyl	36	11	73	9.5

Note: Conditions: 0.4 mmol of DL-**1**, 1.2 mmol of methanol, and 120 mg of *B. cepacia* lipase in 0.8 mL of diisopropyl ether at 25 °C.
[a]Enantiomeric excess of the newly formed methyl ester (**2**).

Table 5. *R. javanicus* lipase-catalyzed transesterification between *N*-*Z*-DL-amino acid 2,2,2-trifluoroethyl esters (DL-**1**) and methanol in diisopropyl ether.

Compound	Conversion (%)	Time (h)	ee (%)[a]	*E*
1a	37	31	56	4.8
1b	37	32	85	20
1c	38	8	88	27
1d	32	12	90	29
1e	44	18	83	21
1f	37	20	78	13
1g	40	17	79	14
1h	38	14	84	19
1i	38	21	73	9.9
1j	41	14	70	9.1
1k	36	11	73	9.5

Note: Conditions: 0.4 mmol of DL-**1**, 1.2 mmol of methanol, and 120 mg of *R. javanicus* lipase in 0.8 mL of diisopropyl ether at 25 °C.
[a]Enantiomeric excess of the newly formed methyl ester (**2**).

Conclusion

In this paper, we have demonstrated that a number of non-proteinogenic amino acids carrying aliphatic side chains can be resolved with moderate-to-good enantioselectivities through the microbial lipase-catalyzed enantioselective transesterification of the 2,2,2-trifluoroethyl esters of their *N*-*Z* derivatives. Although *C. rugosa* lipase is known to show high enantioselectivity toward carboxylic acids, *B. cepacia* lipase is a preferable enzyme in the present case, with methanol being the nucleophile in diisopropyl ether. This, together with the results reported earlier on the resolution of 2-substituted carboxylic acids via the lipase-catalyzed irreversible transesterification where lipases from *A. niger* and *B. cepacia* were utilized, demonstrates that the microbial lipase-catalyzed transesterification procedure in organic milieu must be useful for the resolution of various racemic carboxylic acids, taking into account that these lipase preparations are available easily and inexpensively.

Experimental

General

Thin-layer chromatography was run on precoated silica gel plates (Merck). Melting points were determined on a Yamato MP-21 apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 digital polarimeter. ^{1}H NMR spectra were obtained at 300 MHz on a Varian Unity 300 spectrometer using chloroform-*d* as a solvent with TMS as an internal standard. The liquid chromatograph employed was a Shimadzu LC-5A instrument, equipped with a Rheodyne 7125 sample injector and a Shimadzu SPD-2A variable wavelength UV monitor. A Shimadzu C-R1A data processor was used for data acquisition and processing.

Chemicals

The racemic amino acids were benzyloxycarbonylated using benzyloxycarbonyl chloride (*Z*-Cl) under the usual Schotten–Baumann conditions (16) to give *N*-*Z*-DL-amino acids, which were purified by recrystallization from appropriate solvents (e.g., ethyl acetate–petroleum ether) (8). They were converted to the 2,2,2-trifluoroethyl, 2-chloroethyl or 2,2,2-trichloroethyl esters by the EDC [l-ethy1-3-(3-dimethylaminopropyl)carbodiimide]-DMAP (4-dimethylaminopyridine) method (17) using the corresponding alcohols. All alcohols and organic solvents employed in this study were obtained commercially, and they were distilled following standard protocols and dried over molecular sieves prior to use.

Enzymes

Lipases from porcine pancreas (Type II) and *Candida rugosa* (Type VII) were purchased from Sigma-Aldrich Chemical Co. (USA). Lipases from *Candida rugosa* (MY), *Chromobacterium viscosum* (LP) and *Rhizopus japonicus* (Rhilipase A5) were supplied by Meito Sangyo Co. (Japan), Asahi Chemical Industry (Japan), and Nagase ChemteX Corporation (Japan), respectively. All other enzymes used were supplied by Amano Pharmaceutical Co. (Japan).

Table 6. TLC and HPLC separations of the 2,2,2-trifluoroethyl esters (**1**) and methyl esters (**2**, R′ = CH_3) of *N*-*Z*-amino acids.

	2,2,2-Trifluoroethyl ester		Methyl ester	
R[a]	TLC, R_f[b]	HPLC, t_R (min)[c]	TLC, R_f[b]	HPLC, t_R (min)[c]
CH_3	0.33	2.39	0.22	2.99
C_2H_5	0.38	2.69	0.29	3.50
n-C_3H_7	0.47	3.21	0.35	4.32
n-C_4H_9	0.50	4.42	0.38	5.78
n-C_5H_{11}	0.54	5.39	0.40	7.86
n-C_6H_{13}	0.52	5.59	0.41	11.37
CH_2=$CHCH_2$	0.45	2.92	0.31	3.86

[a]The side chain of an amino acid [–NHCH(R)CO–].
[b]Solvent: hexane–diethyl ether (2:1, *v*/*v*).
[c]HPLC conditions: column, Cosmosil 5C_{18} (4.6 mm i.d. × 150 mm); eluent, 75% aq. MeOH; flow rate, 1.0 mL min^{-1}; column temperature, 30 °C; detection, UV at 254 nm.

Reactions

General procedure for the lipase-catalyzed transesterification

In a typical experiment, a solution of the 2,2,2-trifluoroethyl ester of a racemic *N*-*Z*-DL-amino acid (DL-**1**; 0.4 mmol) and an alcohol (1.2 mmol) in an anhydrous organic solvent (0.8 mL) was stirred with a lipase preparation (120 mg) at 25 °C. The progress of the reaction was monitored by reversed-phase HPLC on a Cosmosil 5C_{18} column (4.6 mm i.d. × 150 mm; Nacalai Tesque, Japan) using aq. methanol as an eluent. When the desired degree of conversion (~40 % based on the racemic starting ester) had been achieved, the enzyme was filtered off and washed with diethyl ether. The error of conversion determined by the HPLC method was estimated as ~±1%. The filtrate and washing were combined and concentrated to a small volume, and the newly formed ester (**2**) was separated from the unchanged starting ester (**1**) by preparative TLC using hexane–diethyl ether (mainly 2:1, *v*/*v*) as a developing solvent. After the absence of the contaminant starting ester (**1**) had been confirmed by reversed-phase HPLC, the ester formed (**2**) was saponified by 0.1 mol/L NaOH in methanol as usual. The ee value of the resolved *N*-*Z*-amino acids (*Z*-AA) was determined by the following HPLC procedure (18): the obtained *Z*-AA was coupled with Gly-L-Phe-OMe to afford the diastereomeric tripeptide *Z*-AA-Gly-L-Phe-OMe, which was separated by reversed-phase HPLC. The peak area of each diastereomer separated was measured to calculate the ee value of the resolved amino acid. When the amino acid AA bears an aromatic ring, *Z*-AA was converted to *Z*-AA-Sar-L-Phe-OMe (Sar = sarcosine or *N*-methylglycine) for a better diastereomeric separation. The kinetic resolution during the couplings was found to be negligible, probably because achiral Gly or Sar occupies the coupling site in the amino component. When the product of the transesterification was the methyl ester, another procedure was also employed. The enantiomers of the methyl ester product were separated directly by normal-phase HPLC on a chiral column, Chiralcel OD (4.6 mm i.d. × 250 mm; Daicel Chemical Industry, Japan), using hexane–2-propanol as an eluent (15). The ee values obtained by these different procedures were consistent with each other within the experimental error (±2%). The ee value for each compound compiled in Table 4 is that obtained by the HPLC procedure utilizing the diastereomeric separation.

The TLC and HPLC separations of the 2,2,2-trifluoroethyl esters (1) and methyl esters (**2**, R′ = CH_3) of *N*-*Z*-amino acids carrying aliphatic side chains are shown in Table 6.

Gram-scale resolution of N-Z-*norvaline*

The 2,2,2-trifluoroethyl ester of racemic *N*-*Z*-DL-norvaline (DL-**1c**; 1.40 g, 4.2 mmol) was dissolved in diisopropyl ether (8 mL), followed by the addition of methanol (520 µL, 12.8 mmol) and then *B. cepacia* lipase (1.2 g). The reaction mixture was stirred at 25 °C. The reaction was stopped after 9 h (33% conversion) by removing the enzyme powder by filtration. The enzyme was washed with diethyl ether. Evaporation of the solvent in vacuo from the combined filtrate and the washing afforded a pale yellow oil, from which the product (**2c**) was isolated by column chromatography on silica gel (Wakogel C-300) using hexane–diethyl ether (10:1, *v*/*v*) as an eluent. Thus, the unreacted trifluoroethyl ester (**1c**) eluted first, and further elution afforded the methyl ester (**2c**) as a colorless oil. The latter was saponified by 0.1 mol/L NaOH, and the raw material was recrystallized from EtOAc–petroleum ether to give *N*-*Z*-L-norvaline as colorless crystals: 290 mg (27% yield); mp 87–88.5 °C; $[\alpha]_D^{25}$ –4.2° (*c* 1.0, acetone). *N*-*Z*-L-norvaline thus obtained was re-esterified with diazomethane, and the resulting methyl ester was analyzed by HPLC on a Chiralcel OD column: 92% ee. In another run, the reaction was stopped after 35 h (55% conversion), and the unreacted trifluoroethyl ester (**1c**) was separated from the newly formed methyl ester (**2c**) by column chromatography in the same manner as mentioned earlier. The trifluoroethyl ester (**1c**) thus obtained was saponified by 0.1 mol/L NaOH, and the raw material was recrystallized from EtOAc–petroleum ether to give *N*-*Z*-D-norvaline as colorless crystals: 410 mg (39% yield); mp 89.5–90 °C; $[\alpha]_D^{25}$ +4.6° (*c* 1.0, acetone); 97% ee by HPLC. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.94 (3H, t. *J* = 7.4 Hz, CH_3), 1.30–1.50 (2H, m, CH_2CH_3), 1.61–1.95 (2H, m, $CHCH_2$), 4.41 (1H, q-like, CH), 5.12 (2H, apparent s, $PhCH_2$), 5.27 (1H, d, *J* = 8.1 Hz, NH), 7.26–7.41 (5H, m, Ph), 10.48 (1H, br, OH). Anal. calcd. for $C_{13}H_{17}NO_4$: C, 62.14; H, 6.82; N, 5.57%. Found: C, 62.30; H, 6.89; N, 5.58%.

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