Pergamon

Tetrahedron: Asymmetry, Vol. 8, No. 4, pp. 527–536, 1997

Printed in Great Britain
PII: S0957-4166(97)00016-5
0957-4166/97 $17.00 + 0.00

Asymmetric synthesis of L-thienylalanines

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Abstract: L-Thienylalanines were prepared *via* the hydantoin and azlactone routes with the key step consisting of the microbial transamination of 2-hydroxy-3-thienylacrylic with L-aspartic acid as amino donor. The transamination reaction was performed by a genetically engineered *E. coli* strain on scales up to 100 g of L-3-(2-thienyl)alanine **1a** and is also applicable to the preparation of the isomeric amino acid **1b** and some ring-substituted derivatives. © 1997 Elsevier Science Ltd. All rights reserved.

Introduction

Optically active, non-proteinogenic amino acids are highly important as pharmaceuticals and as crop protection agents. Examples are L-dopa against Parkinson's Disease, α-methyldopa against hypertension or L-phosphinothricin as biologically active component of a herbicidal active substance.[1] Moreover, optically active amino acids are synthesis precursors, in particular for pharmaceuticals, such as, for example, D-phenyl-glycine or D-*para*-hydroxyphenylglycine in the preparation of semisynthetic pencillins. They are used, more-over, as chiral synthesis units for other chiral fine chemicals and for incorporation into modified biologically active peptides.[2] Since the non-proteinogenic optically active amino acids cannot be obtained by fermentation or from natural sources, they have hitherto been prepared by conventional synthesis followed by racemate resolution, by asymmetric synthesis using chiral auxiliaries, or by biotransformation of chiral or prochiral precursors.[1] Examples of processes used for the commercial synthesis of non-proteinogenic amino acids[3] are the amidase process,[4] in which the racemic amino acid amides are cleaved by hydrolysis using an L-specific aminopeptidase, and the acylase-catalyzed enantioselective hydrolysis of N-acetyl-D,L-amino acids.[5] The shortcoming of both processes, which are employed industrially on ton-scales, is that the yield of the optically active reaction product is not more than 50%. In a more advanced biotechnological process, namely the microbial hydrolysis of D,L-monosubstituted hydantoins,[6] the chiral precursor of the amino acid can be converted into the L-amino acid with a yield of more than 50% by the presence of a racemase. Disadvantages of this process are, on the one hand, that synthesis of the hydrogenated hydantoin precursors is particularly difficult when sulfur containing moieties are present in amino acids, such as in the thienylalanines **1** (Figure 1), which result in inactivation of noble metal hydrogenation catalysts and require less convenient electrochemical reduction processes for providing the precursors.[7] On the other hand, in this process three enzymes are involved which have different activities at certain pH values and certain temperatures, which means that the whole process requires laborious optimization.[8] A further possibility for carrying out the enantiospecific synthesis of amino acids **1** is the transamination of prochiral α-keto acid precursors. Such processes were described mainly for the synthesis of natural amino acids such as L-leucine[9] and L-phenylalanine[10], and also for non-proteinogenic amino acids on very small scales. Examples are the preparation of L-*tert.*-leucine and L-phosphinothricine using a genetically modified *E. coli* strain,[11] and the transamination of a series of α-keto acids using an isolated aspartate aminotransferase from *E. coli*.[12]

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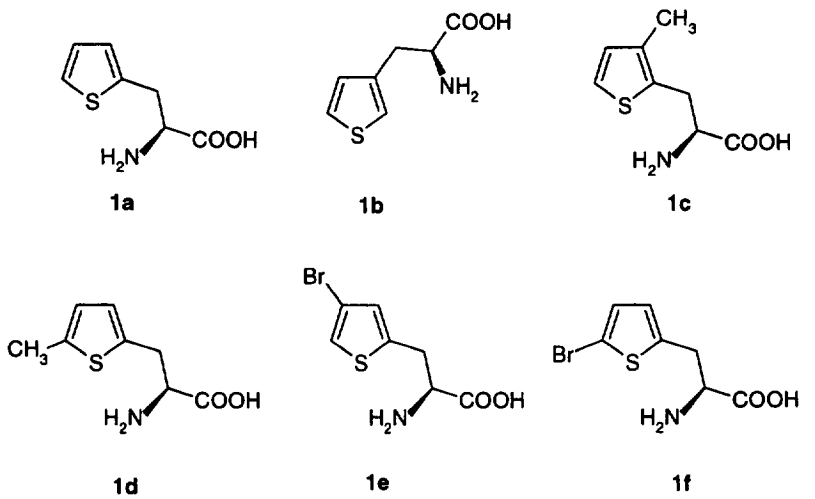

Figure 1. L-Thienylalanines **1**.

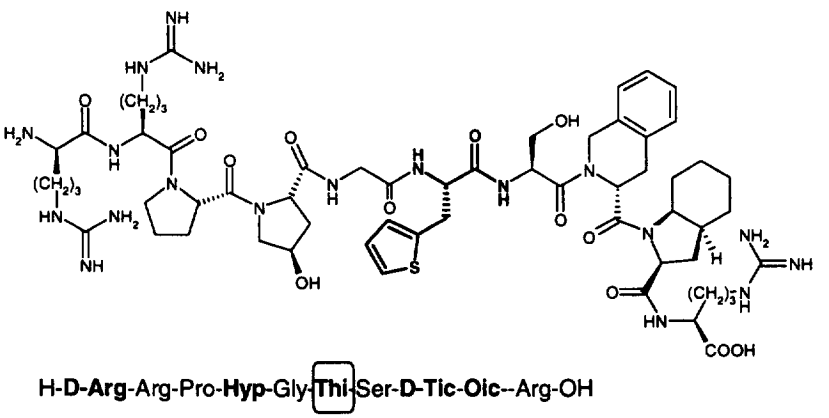

Figure 2. Structure of the bradykinin antagonist HOE 140. The non-proteinogenic amino acids are depicted in bold.

The non-proteinogenic amino acid L-3(2-thienyl)alanine **1a** is a key building block of the bradykinin antagonist HOE 140[13] which is under development as an anti-inflammatory and anti-allergic agent (Figure 2). Recent syntheses of the optically active, non-proteinogenic L-3(2-thienyl)alanine **1a** were performed through traditional racemate resolution by crystallization of diastereomeric salts,[14] enantioselective hydrolysis of lower alkyl esters by means of α-chymotrypsin[15] or resolution of the corresponding amide with an amino-peptidase.[16] These resolutions and the hydantoinase process[6,7] have the abovementioned shortcomings in terms of yield and accessibility of the 5-thienylmethylhydantoin precursor, respectively. One further approach reported recently consists of the addition of ammonia onto *trans*-thienylacrylic acids catalyzed by *Rhodotorula glutinis* yeast, providing **1a** and **1b** in 32% and 25% yield, respectively, as determined by means of a TLC-scanner.[17] Clearly, this procedure is not suitable for syntheses on an industrial scale. Here we describe a technically more accessible route to the homochiral compounds **1** based on the transamination of 2-hydroxy-3-thienylacrylic acids **2** in the presence of L-aspartic acid as amino donor which is suitable for laboratory scale as well as for larger scale preparations.

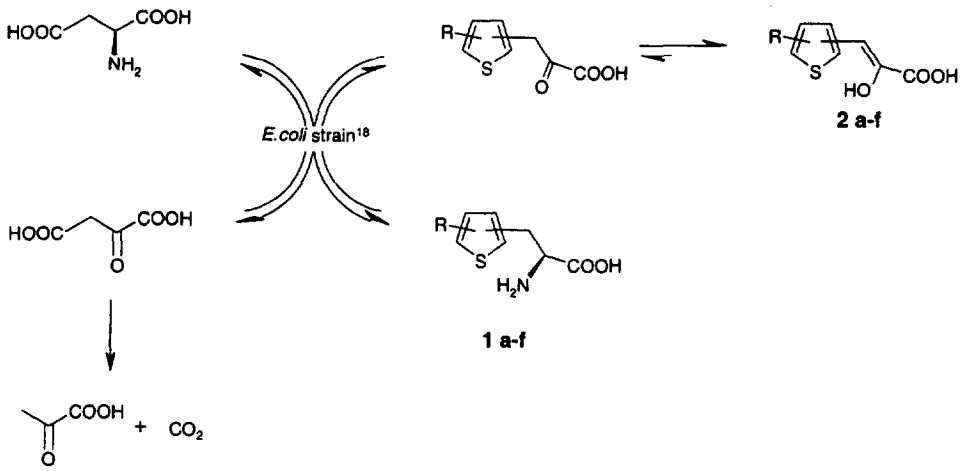

Figure 3. Biotransformation of 2-hydroxy-3-thienylacrylic acids **2** to amino acids **1**. The decarboxylation of oxaloacetate shifts the equilibrium of transamination towards the desired products.

Table 1. Preparative results for the asymmetric synthesis of amino acids **1** from precursors **2**

educt **2** (scale)	amino acid **1**	yield (%) (HPLC)	isolated yield (%)	purity (%) (HPLC)	1-D (%)	$[\alpha]^{D}_{20}$
2a (30 g)	**1a**	82	57	>98	<0.2	-30.1 (c=1, H_2O)
2a (800 g)	**1a**	55	31	>99.5	<0.2	-30.3 (c=1, H_2O)
2b (20g)	**1b**	61	40	>90	<0.2	-38.9 (c=1, H_2O)
2c (0.3 g)	**1c**	70	61	>95	<0.2	-8.8 (c=1, 1*N* NaOH)
2d (0.3 g)	**1d**	71	52	>95	<0.2	-19.6 (c=1, H_2O)
2e (0.75 g)	**1e**	n.d.	10	>95	<0.2	-29.3 (c=1, H_2O)
2f (1 g)	**1f**	n.d.	3	>95	<0.2	n.d.

Preparative results

The preparation of the pure L-amino acids **1** was achieved by means of the bacterial strain *E. coli* ATCC 11303 which was additionally transformed by cloning the *tyrB* gene encoding an aromatic transaminase onto a multi-copy plasmid as described.[18] L-aspartate was used as amino group donor, the irreversible decarboxylation of oxaloacetate to pyruvic acid under the conditions of incubation driving the equilibrium transamination reaction towards completion (Figure 3).[19] Preferably, the biotransformation was carried out using a suspension of the whole intact cells, after removal of the nutrient broth, at pH 8 at 40°C. It was not found necessary to permeabilize the microorganism before or during the biotransformation. High reaction rates and yields were achieved in particular when the batch was incubated with the microorganism under the exclusion of atmospheric oxygen. Under these conditions, for example, the yield of **1a** was 82% and no starting material **2a** could be detected, as determined by HPLC analysis. Customary work-up procedures employing anion exchange chromatography provided the pure L-amino acid **1a** in 57% isolated yield (Table 1). Further scale-up of the reaction almost to the kg-scale was feasible, but the (unoptimized) isolated yield of pure product dropped considerably to 31%.

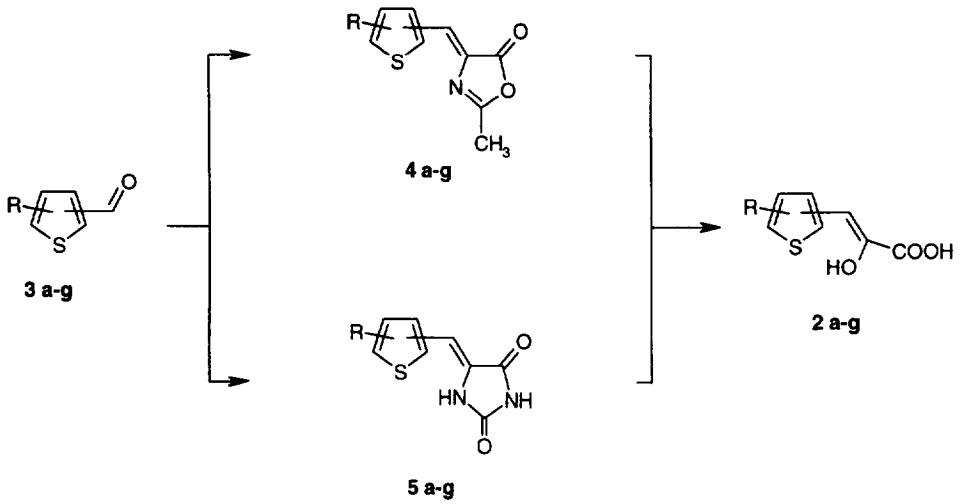

Figure 4. Preparation of 2-hydroxy-3-thienylacrylic acids **2**.

The biotransformation was then successfully applied to the synthesis of the L-3-(3-thienyl)alanine **1b** and some ring-substituted derivatives **1c–f**, the brominated products being isolated in quite poor yields only. In principle, the biotransformation could also be carried out using cell extracts or the purified transaminases, however, the batch-wise procedure described here was preferred due to its simplicity.

Another important feature of this process is the short and flexible synthetic route to provide the 2-hydroxy-3-thienylacrylic acid precursors **2** which was established in close analogy to procedures known for the synthesis of α-ketocarboxylic acids.[20–24] As shown in Figure 4, compounds **2** were then obtained by hydrolytic ring opening of the corresponding azlactones **4** or hydantoins **5** which were prepared in high yields by condensation of the thienylaldehydes **3** with acetylglycine/acetic anhydride/sodium acetate[26] or with unsubstituted hydantoin,[25,26] respectively. The synthesis of the enolcarboxylic acids **2** from the hydantoin derivatives **5** was hitherto not described and, surprisingly, proceded in high yields by simply heating the hydantoins in aqueous bases up to the boiling point of the aqueous solution. The hydrolysis of azlactones **4** can be carried out by heating the substances in aqueous acids or bases,[20] but the hydrolysis of the derivatives **4** was favourably carried out in two steps:[21] Reaction with gaseous HCl in dioxane in the presence of one equivalent of water precipitated the 2-acetylamidoacrylates which were then hydrolyzed by aqueous hydrochloric acid. Both, the hydantoin and the azlactone route, could be performed on kg-scales if the hydrolysis conditions were carefully controlled. Moreover, the crude precipitated acids **2** could then be introduced directly into the biotransformation reaction.

Interestingly, the biotransformation substrates **2** were uniformly present in their tautomeric enol forms, the 2-hydroxy-3-thienylacrylic acids, as drawn in Figure 3. This was demonstrated by ^{1}H NMR spectroscopy, within the detection limits of this analytical technique, for wide pH ranges and in particular under the incubation conditions of the transamination at pH 8 (Table 2). In general, substantial amounts of aryl-substituted β-pyruvic acids are present in the tautomeric enol form only in a very basic medium, and under physiological pH conditions, the enol content is very low.[22] For example, the sodium salt of phenylpyruvic acid is present almost exclusively in the keto acid form.[27] In contrast, compound **2a** for example has been described by the corresponding keto acid structural formula.[24]

The enzymatic transamination can be divided into two half-reactions, first the binding of the L-aspartate to the coenzyme pyridoxal-5′-phosphate (PLP) with subsequent release of oxaloacetate

Table 2. Analytical data of the transamination precursors **2**

2-hydroxy-3-(...)-acrylic acids **2**	mp [°C]	^{1}H NMR (100 MHz, DMSO-d_6, 20°C)
2a (2-thienyl)	166-168	7.52, 7.25, 7.02 (3dd, 3H), 6.75 (s, 1H, H-3)
2b (3-thienyl)	178-180	7.75, 7.45 (2m, 3H), 6.50 (s, 1H, H-3)
2c (3-methyl-2-thienyl)	184-185	7.45, 6.90, (2d, 2H), 6.70 (s, 1H, H-3), 2.23 (s, 3H, CH_3)
2d (5-methyl-2-thienyl)	184-186	7.05 (d, 1H), 6.70 (dd, 1H), 6.68 (s, 1H, H-3), 2.43 (s, 3H, CH_3)
2e (4-bromo-2-thienyl)	194-195	7.63, 7.25 (2m, 2H), 6.75 (s, 1H, H-3)
2f (5-bromo-2-thienyl)	156-158	7.05 (d, 1H), 6.75 (d, [br], 1H), 6.70 (s, 1H, H-3)
2g (5-nitro-2-thienyl)	192-193	8.06, 7.34, (2d, 2H), 6.87 (s, 1H, H-3)

and pyridoxamine, and second the reaction of the latter with the accepting α-keto acid.[19a] For the transamination substrates **2e,f** containing electron-withdrawing bromine atoms in the thienyl ring, the keto–enol equilibrium may be shifted too far in favour of their enol tautomers, which can explain the low yields obtained in these cases (Table 1). Indeed, with the even more electron-withdrawing nitro group present in the substrate **2g** (Table 2), no transamination reaction could be achieved at all, which is in accord with this interpretation. In summary, a highly efficient asymmetric synthesis of the non-proteinogenic L-thienylalanines **1**, useful as starting materials for chiral fine chemicals and for incorporation in biologically active peptides has been developed.

Experimental part

General

^{1}H NMR spectra were recorded at 100 MHz in DMSO-d_6, except for the amino acids **1**, for which the ^{1}H NMR spectra were recorded at 300 MHz and ^{13}C NMR at 75 MHz, both in D_2O. Chemical shifts are given in δ [ppm]. Melting points are uncorrected.

Fermentation of the E. coli *strain*[18]

Feed-batch fermentations of *E. coli* BpIMS 4006[18] (controlled feeding of glucose) were performed in a 30 l stirred tank at 37°C under air atmosphere (0.67 vvm) and 400 rpm in order to achieve high cell densities. The transaminase activity reached 60,000 U/l after 15 h at *ca.* 50 g/l weight of biomass.

Enzyme assay

0.5 ml of the assay solution [200 mM Tris·HCl (pH 7.0), 200 mM L-asp, 400 μM pyridoxal phosphate, 0.4 U malate dehydrogenase (MDH)] were diluted with 0.4 ml bidistilled water and mixed with 0.1 ml of the sample at 30°C. The consumption of NADH was measured photometrically at 340 nm and therefrom the enzyme activity was calculated. 1 U corresponds to 1 μmol NADH produced per min.

Isolation and immobilization of the aromatic amino acid amino transferase

20 g of the cells were suspended in 60 ml of buffer (20 mM K_2HPO_4/KH_2PO_4, 10 μM pyridoxal phosphate, 5 mM 2-mercaptoethanol, 10 mM EDTA and 12 mg of lysozyme). The suspension was stirred at a low speed for 10 min at 30°C. The resulting suspension was centrifuged for 15 min at 4°C, and the clear supernatant was used for the subsequent work-up. First, a precipitation was carried out for 1 h at 4°C using 30% $(NH_4)_2SO_4$, and the mixture was subsequently centrifuged for 15 minutes at 10,000 g/4°C. The precipitate was discarded, the supernatant was subjected to a precipitation with

70% $(NH_4)_2SO_4$ (1h / 4°C), and the suspension was centrifuged as above. The precipitate contained most of the transaminase and was taken up in the coupling buffer (see below).

The silica gel support was activated as follows: 10 g of support (silica gel XWP 50 MP from Grace) were suspended in 100 ml of distilled water, and 2.30 g of 3-aminopropyltriethoxysilane were added. The mixture was brought to pH 2.5 using HCl and heated for 2 h at 65°C. The mixture was subsequently filtered and washed with water until neutral, and the silica gel was dried for 24 h at 90°C. The dry support was taken up in 160 ml of 0.25 M potassium phosphate buffer (pH 8) containing 2% of glutardialdehyde, and the mixture was incubated for 2 h under a water pump vacuum with gentle stirring. The mixture was subsequently refiltered and washed thoroughly with water. The activated support was taken up in 200 ml of 1 M potassium phosphate buffer with 10 µM pyridoxal phosphate and 130 mg/10,000 U enzyme and incubated overnight at 4°C. The coupling yield was approximately 80%.

Preparation of 2-hydroxy-3-(2-thienyl)-acrylic acid ***2a***

a) *From the azlactone* ***4a***: 420 g (4.90 mol) of anhydrous sodium acetate and 500 g (4.27 mol) of acetylglycine were suspended in 1300 g of acetic anhydride, and 719 g (6.42 mol) of thiophene-2-aldehyde were added. The batch was heated to the boil under inert gas, then refluxed for 1.5 h and subsequently cooled in an ice-bath, and the product which had precipitated was filtered off with suction. This was washed four times using 300 ml of ice–water, and the residue was dried in a vacuum shelf dryer at 50°C. Precipitated product from the combined filtrates was treated in the same manner. Total azlactone **4a** yield: 730 g (88%); mp. 137–140°C; ^{1}H NMR: 8.2–6.8 (several m, 4H, arom.-H, =CH), 4.60 (s, 3H, CH_3). 193 g (1.00 mol) of **4a** were introduced into 1.5 l of boiling hydrochloric acid. After 0.5 h under reflux, most of the educt was dissolved. The mixture was filtered while hot and washed with a little water. The reddish-brown residue was composed of the 2-(N-acetylamino)-acrylate (55 g, 26%). The filtrate was cooled to 0°C while stirring, and **2a** and some more 2-(N-acetylamino)-acrylate precipitated. The mixture was refiltered with suction and washed with a little cold water. The residue remaining on the frit was triturated several times with ether and filtered off with suction. The solid was 2-(N-acetylamino)-acrylate (50 g, 25%). The desired **2a** was isolated from the ether phase after evaporation and drying in 46 g (27%) yield; analytical data see Table 2. Alternatively, **5a** was hydrolyzed in the two-step procedure as described in the following: 96.5 g (0.50 mol) of **4a** were dissolved in 0.5 l of dioxane and 1 l of water. HCl gas was subsequently passed in. The 2-(N-acetylamino)-acrylate started to crystallize out and was in the form of a thick suspension when the reaction had ended 40 min later. Then 1 l of diethyl ether was added with stirring, and the hydrogen chloride was purged by passing in nitrogen. The product was filtered off with suction, washed thoroughly with ether and freed *in vacuo* from remains of solvent to yield 99.5 g (94%) of the 2-(N-acetylamino)-acrylate; mp.: 232–235°C; ^{1}H NMR: 9.25 (s, 1 H, NH), 7.75 (d, 1 H, arom.-H), 7.70 (s, 1 H, =CH), 7.50 (d, 1 H, arom.-H), 7.10 (dd, 1 H). The hydrolysis with HCl was effected as described above to yield 59.5 g (70%) of **2a**. b) *From the 5-(2-thienyliden)-hydantoin* ***5a***: 1.2 l of 5 N NaOH were heated to reflux while stirring and passing in inert gas. 77.7 g (0.40 mol) of **5a** were introduced, and the mixture was left to reflux for 1 h, then cooled in an ice-bath and slowly treated with 0.5 l of concentrated hydrochloric acid. Some of the product **2a** precipitated directly, the remainder was extracted from the aqueous filtrate using ether. Total yield of dried product **1a**: 45.5 g (67%).

Preparation of 2-hydroxy-3-(3-thienyl)-acrylic acid ***2b***

The synthesis was carried out analogously as described for **2a** (data see Table 2). The analytical data of the intermediates are given in the following: 5-(3-Thienyliden)-2-methyl-3-oxazolin-4-one **4b**: mp. 112–116°C; ^{1}H NMR: 8.34, 7.90, 7.68 (3 dd, 3 H, arom.-H), 7.28 (s, 1 H, =CH), 2.37 (s, 1 H, CH_3). 2-(N-Acetylamino)-3-(3-thienyl)-acrylic acid: mp. 205°C; ^{1}H NMR: 7.93, 7.60, 7.41 (3 dd, 3 H, arom.-H), 7.33 (s, 1 H, =CH), 2.00 (s, 1 H, NAc). 5-(3-Thienyliden)-hydantoin (**5b**): mp. 270°C; ^{1}H NMR: 7.95 (m, 1 H, arom.-H), 7.68–7.42 (4 dd, 2 H, arom.-H), 6.50 (s, 1 H, =CH).

Preparation of 2-hydroxy-3-(3-methyl-2-thienyl)-acrylic acid 2c

The synthesis was carried out analogously as described for **2a** (data in Table 2). Analytical data of intermediates: 5-(3-Methyl-2-thienyliden)-2-methyl-3-oxazolin-4-one **4c**: mp. 145–146°C; ^{1}H NMR: 7.90 (d, 1 H, arom.-H), 7.35 (s, 1 H, =CH), 7.03 (d, 1 H, arom.-H), 2.40, 2.25 (2 s, 6 H, CH_3). 2-N-Acetylamino-3-(3-methyl-2-thienyl)-acrylic acid: mp. 231–232°C; ^{1}H NMR: 9.25 (s, [br], 1 H, NH), 7.65 (d, 1 H, arom.-H), 7.60 (s, 1 H, =CH), 7.00 (d, 1 H, arom.-H). 5-(3-Methyl-2-thienyliden)-hydantoin (**5c**): mp. 212–214°C; ^{1}H NMR: 11.23, 10.02 (2 s, 2 H, NH), 7.65, 7.00 (2 d, 2 H, arom.-H), 6.60 (s, 1 H, =CH), 2.29 (s, 3 H, CH_3).

Preparation of 2-hydroxy-3-(5-methyl-2-thienyl)-acrylic acid 2d

The synthesis was carried out analogously as described for **2a** (data in Table 2). Analytical data of intermediates: 5-(5-Methyl-2-thienyliden)-2-methyl-3-oxazolin-4-one **4d**: mp. 280°C (decomp.); ^{1}H NMR: 7.57 (d, 1 H, arom.-H), 7.48 (s, 1 H, =CH), 6.93 (d, split, 1 H, arom.-H), 2.50; 2.32 (2 s, 6 H, CH_3). 2-N-Acetylamino-3-(5-methyl-2-thienyl)-acrylic acid: mp. 257°C; ^{1}H NMR: 9.20 (s, [br], 1 H, NH), 7.60 (s, 1 H, =CH), 7.30 (d, 1 H, arom.-H), 6.80 (d, split, 1 H, arom.-H), 2.00 (s, 3 H, NAc). 5-(5-Methyl-2-thienyliden)-hydantoin (**5d**): mp. 263–264°C; ^{1}H NMR: 11.20, 10.22 (2 s, 2 H, NH), 7.40, 6.85 (2 d, 2 H, arom.-H), 6.45 (s, 1 H, =CH), 2.45 (s, 3 H, CH_3).

Preparation of 2-hydroxy-3-(4-bromo-2-thienyl)-acrylic acid 2e

The synthesis was carried out analogously as described for **2a** (data in Table 2). Analytical data of intermediates: 5-(4-Bromo-2-thienyliden)-2-methyl-3-oxazolin-4-one **4e**: mp. 142–145°C; ^{1}H NMR: 8.05, 7.75, 7.54 (3 m, 3 H, arom.-H, =CH), 2.37 (s, 3 H, CH_3). 2-N-Acetylamino-3-(4-bromo-2-thienyl)-acrylic acid: mp. 213–215°C; ^{1}H NMR: 7.30 (s, [br], 1 H, NH), 7.80, 7.65, 7.50 (3 m, 3 H, arom.-H, =CH), 2.00 (s, 3 H, NAc). 5-(4-Bromo-2-thienyl)-hydantoin **5e**: mp. 220–221°C; ^{1}H NMR: 11.35, 10.52 (2 s, 2 H, NH), 7.77, 7.65 (2 m, 2 H, arom.-H), 6.48 (s, 1 H, =CH).

Preparation of 2-hydroxy-3-(5-bromo-2-thienyl)-acrylic acid 2f

The synthesis was carried out analogously as described for **2a** (data in Table 2). Analytical data of intermediates: 5-(5-Bromo-2-thienyliden)-2-methyl-3-oxazolin-4-one **4f**: mp. 192°C; ^{1}H NMR: 7.55 (d, 1 H, arom.-H), 7.50 (s, 1 H, =CH), 7.35 (d, 1 H, arom.-H), 2.35 (s, 3 H, CH_3). 2-N-Acetylamino-3-(5-bromo-2-thienyl)-acrylic acid: mp. 221–222°C; ^{1}H NMR: 9.30 (s, 1 H, NH), 7.75 (s [br], 1 H, =CH), 7.35, 7.25 (2 d, 2 H, arom.-H), 2.03 (s, 3 H, NAc). 5-(5-Bromo-2-thienyliden)-hydantoin **5f**: mp. 235–236°C; ^{1}H NMR: 11.3, 10.4 (2s, 2H, NH), 7.42, 7.30 (2 d, 2H, arom.-H), 6.52 (s, 1H, =CH).

Preparation of 2-hydroxy-3-(5-nitro-2-thienyl)-acrylic acid 2g

The synthesis was carried out analogously as described for **2a** (data in Table 2). Analytical data of intermediates: 5-(5-Nitro-2-thienyliden)-2-methyl-3-oxazolin-4-one **4g**: mp. >300°C (decomp.); ^{1}H NMR: 8.05 (d, 1 H, arom.-H), 7.68 (s, 1 H, =CH), 7.48 (d, 1 H, arom.-H), 2.16, 2.05 (2 S, 3 H, CH_3). 2-N-Acetylamino-3-(5-nitro-2-thienyl)-acrylic acid: mp. 220°C; ^{1}H NMR: 8.15 (d, 1 H, arom.-H), 7.75 (s, 1 H, =CH), 7.60 (d, 1 H, arom.-H), 2.06 (s, 1 H, NAc).

HPLC analysis of transamination reactions and determination of the amino acid purity

HPLC-column Grom Amino-OPA (150×4.6 mm) with identical precolumn (20×4.6 mm) were used. Flow rate: 1.5 ml/min. Detection: 340 nm. Gradient: 10% (A) to 50% (B) in 13 min. (A): 12.5 mM potassium phosphate pH 7.2, (B): acetonitrile. The *ortho*-phthaldialdehyde (OPA) derivatization was carried out as follows: Solution (1): 50 mg/ml OPA (analytical grade) in ethanol. Solution (2): 100 mg/ml Boc-L-cysteine in analytical grade ethanol. Solution (3): 1 M potassium borate buffer pH 10.4. Immediately prior to use, 10 µl of each (1) and (2) were added to 980 µl of (3). The solution was stable for approximately 48 h at 4°C. Derivatization: 10 µl of the suitable dilute sample were mixed with 90 µl of reagent. After 2 min, the sample was injected. The detection limit for the D-enantiomeric amino acids was approximately 0.2%, i.e. the content of **ent-1** in all cases was found to

be below 0.2% (Table 1). The purities given for the products **1** in Table 1 refer to the content of the respective amino acid, the remainder are inorganic salts.

General procedure for batch-wise transaminations and preparation of L-*3-(2-thienyl)alanine* ***1a***

30.0 g (0.176 mol) of 2-hydroxy-3-(2-thienyl)-acrylic acid (**2a**), 29 g (0.218 mol) of L-aspartate and 40 mg of pyridoxal phosphate were dissolved in 700 ml of distilled water, and the pH was brought to 8 using NaOH (solution 1, temp. 4°C). In a 1 l reactor, 100 ml of a suspension of the *E. coli* cells[18] (corresponds to approxim. 6 g of dry matter or 3000 U transaminase activity, as determined above) were introduced together with 200 ml of distilled water (solution 2). The pH of this suspension was brought to 8, and the mixture brought to 40°C. A stream of N_2 was passed in to keep the mixture under an inert atmosphere. Solution 1 was metered into this suspension with stirring in the course of 16 h. The pH was kept constant by means of $NaOH/H_2SO_4$. The reaction course was monitored by HPLC. After 24 h, the concentration of **1a** was 24.6 g/l (82% yield); no educt **2a** was detectable any more. The reaction mixture was centrifuged (15 minutes, 8000 g), the supernatant was brought to pH 1.5, then 5 g of active charcoal were added, and the suspension was stirred for 0.5 h at 70°C. It was subsequently cooled to 4°C, and the precipitate was filtered off. The filtrate was brought to pH 12 using NaOH and applied to a column packed with 1 l of Dowex 1 x 2 (Cl^-). It was rinsed with 2 l of water (brought to pH 9 using ammonia). Subsequently the amino acids L-aspartate and **1a** were eluted using 4 l of 0.105% acetic acid. The eluates containing **1a** were concentrated *in vacuo* to 300 ml, and the resulting concentrate was lyophilized to yield 17.8 g (57%) of **1a**. Analytical data see Table 1. ^{13}C NMR: 174 (C=O), 136, 128, 127, 126 (arom.-C), 56 (Cα), 30 (Cβ); ^1H NMR: 7.26 (d, 1 H, arom.-H), 6.88 (m, 2 H, arom.-H), 3.87 (*pseudo*-t, 1 H, Hα), 3.35 (m, 2 H, Hβ). Up-scaling to 800 g (4.70 mol) **2a**: See results shown in Table 1.

Continuous preparation of ***1a*** *using the immobilized transaminase*

10.0 g (58.7 mmol) of **2a**, 9.30 g (69.8 mmol) of L-aspartic acid, 20 mg of pyridoxal phosphate and 1 ml of mercaptoethanol were dissolved in 1 l of dist. water, and the mixture was brought to pH 8 using NaOH. The solution was cooled to 4°C and used for the reaction. The immobilized biocatalyst had a specific activity of *ca.* 500–800 U per gram of dry support. 10 ml of the immobilizate were introduced into a column (40×18 mm internal diameter) which was heated at 40°C using a heating jacket. The reaction solution was pumped through the column at a flow-rate of 1.5 ml/h and recooled to 4°C. The yield obtained was 0.80 mg/l/h at a conversion rate of 50–60%. These values were achieved continuously over a period of 800 h. The product **1a** was isolated analogously to the batch experiment described above.

Preparation of L-*3-(3-thienyl)alanine* ***1b*** *(batch process)*

The procedure was largely as described above, however, solution 1 contained only 20 g (0.117 mol) of **2b** and 19 g (0.142 mol) of L-aspartate. The product concentration reached 11.5 g/l (61%) after the reaction, and after ion exchange chromatography 7.9 g (40%) of **1b** were obtained. Analytical data see Table 1. ^1H NMR: 7.38, 7.18, 6.97 (3 s,[br], 3 H, arom.-H), 3.88 (*pseudo*-t, 1 H, Hα), 3.16 (m, 2 H, Hβ).

Preparation of L-*3-(3-methyl-2-thienyl)alanine* ***1c*** *(batch process)*

The reaction was carried out analogously to the general procedure. Solution 1: 0.30 g (1.63 mmol) of **2c**, 0.29 g (2.18 mmol) of L-aspartate, 4 mg of pyridoxal phosphate, and H_2O to 35 ml. Solution 2: 5 ml of cell suspension and 10 ml dist. water. After a reaction time of 6 h, 0.21 g (70%) of product were detected. Prep. HPLC (analog. to **1e**) gave 0.184 g (61%) of **1c**. Analytical data see Table 1. ^1H NMR: 7.18, 6.82 (2 d, 2 H, arom.-H), 3.78 (dd, 1 H, Hα), 3.23 (2 dd, 2 H, Hβ), 2.10 (s, 2 H CH_3); ^{13}C NMR: 175 (C=O), 137, 131, 130, 124 (arom.-C), 56 (Cα), 29 (Cβ), 13 (CH_3).

Preparation of L-*3-(5-methyl-2-thienyl)alanine* ***1d*** *(batch process)*

The conversion of **2d** was carried out analogously to the procedure described above for **2c**. After 6 h, 0.21 g (71%) of product were detected by HPLC. Prep. HPLC gave 0.15 g (52%) of **1d**. ^{1}H NMR: 7.30, 6.92 (2 d, 2 H, arom.-H), 3.94 (m, 1 H, Hα), 3.40 (m, 2 H, Hβ), 2.20 (s, 2 H CH_3); ^{13}C NMR: 140, 135, 134, 128 (arom.-C), 59 (Cα), 32 (Cβ), 17 (CH_3).

Preparation of L-*3-(4-bromo-2-thienyl)alanine* ***1e*** *(batch process)*

The reaction was carried out as out-lined in the general procedure, however, this was done on a smaller scale, and the isolation step was modified. Solution 1: 0.75 g (3.00 mmol) of (**3e**), 0.72 g (5.40 mmol) of L-aspartate, 4 mg of pyridoxal phosphate, and distilled water to 35 ml. Solution 2: 5 ml of cell suspension and 10 ml of distilled water. After a reaction time of 6 h, the cells were separated off and the mixture decolorized using active charcoal, the solution was rendered neutral, and the volume was reduced *in vacuo* to 10 ml. The mixture was purified by means of prep. HPLC on a Nucleosil C-18 column (10 cm×40 mm×250 mm); flow rate 20 ml/min; eluent A: H_2O; eluent B: acetonitrile; detection at 254 nm; gradient 0% of B to 20% of B in 80 min, subsequently to 50% of B in 60 min. The product fractions were concentrated *in vacuo* to give 10 ml of aqueous residue, which was lyophilized to yield 70 mg (10%) of **1e**. Analytical data see Table 1. ^{1}H NMR: 7.40, 7.00 (2 s, 2 H, arom.-H), 4.00 (*pseudo*-t, 1 H, Hα), 3.42 (m, 2 H, Hβ). ^{13}C NMR: 173 (C=O), 138, 130, 124, 109 (arom.-C), 56 (Cα), 30 (Cβ).

Preparation of L-*3-(5-bromo-2-thienyl)alanine* ***1f*** *(batch process)*

The reaction was carried out according to the general procedure. Solution 1: 1.00 g (4.00 mmol) of **2f**, 0.97 g (7.27 mmol) of L-aspartate, 4 mg of pyridoxal phosphate, and H_2O to 35 ml. Solution 2: 5 ml of cell suspension and 10 ml of dist. water. After 6 h, 43 mg (4.3%) of product were detected by HPLC. Prep. HPLC (performed as for **1e**) gave 27 mg (2.7%) of **1f**. Analytical data: Table 1. ^{1}H NMR: 7.50–6.95 (m, 2 H, arom.-H), 3.85 (m, 1 H, Hα), 3.38 (m, 2 H, Hβ).

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(Received in UK 20 November 1996; accepted 7 January 1997)