Pergamon

Tetrahedron: *Asymmetry* 11 (2000) 1077–1083

TETRAHEDRON: *ASYMMETRY*

Penicillin acylase-catalyzed peptide synthesis: a chemo-enzymatic route to stereoisomers of 3,6-diphenylpiperazine-2,5-dione

Luuk M. van Langen,[a] Fred van Rantwijk,[a] Vytas K. Švedas[b] and Roger A. Sheldon[a,*]

[a]*Laboratory of Organic Chemistry and Catalysis, Delft University of Technology, Julianalaan 136, 2628 BL Delft, The Netherlands*

[b]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119899 Moscow, Russia*

Received 27 September 1999; revised 13 January 2000; accepted 14 January 2000

Abstract

Chiral dipeptides of phenylglycine were synthesized using immobilized *Escherichia coli* penicillin acylase. The high selectivity of penicillin acylase for L-amino acids as the nucleophile resulted in the efficient acylation of L-phenylglycine by D-phenylglycine amide at pH 9.7 to give D-phenylglycyl-L-phenylglycine in 69% yield. No isomers or tripeptides were formed. The low enantiospecificity of the enzyme for the acyl donor provided the possibility of preparing the corresponding L,L-dipeptides, starting from L-phenylglycine methyl ester as both donor and acceptor at pH 7.5, resulting in a 63% yield of L-phenylglycyl-L-phenylglycine methyl ester. The product precipitated under the reaction conditions; this effectively prevented the formation of oligomers as well as chemical transformation of the product.

The dipeptide esters of phenylglycine easily cyclized to diketopiperazines in aqueous methanol. L-Phenylglycyl-L-phenylglycine methyl ester formed L,L-3,6-diphenylpiperazine-2,5-dione (*cis*); the achiral *trans* isomer was obtained from D-phenylglycyl-L-phenylglycine methyl ester.

1. Introduction

Peptides and their derivatives constitute an interesting class of biologically active compounds, which find application as food additives and antibiotics;[1] one of them, the sweetener aspartame, has become an industrial commodity. The classical synthesis of peptides requires protection and activation of the donor and acceptor, whereas enzymatic routes allow the use of simple protecting and activating groups or even no protecting group at all. Activation of the acyl donor, however, is, for thermodynamic and kinetic reasons, inevitable.[2–4] Enzymatic peptide synthesis using

* Corresponding author. Tel: 31 15 2782675; fax: 31 15 2784700; e-mail: r.a.sheldon@stm.tudelft.nl

0957-4166/00/$ - see front matter
PII: S0957-4166(00)00027-6

proteases has been extensively studied in order to understand its mechanism as well as to improve the selectivity and reduce the hydrolytic side reactions in kinetically controlled processes.[5–7]

Proteases are restricted to L-amino acids, however, and do not convert unnatural amino acids, such as phenylglycine. We have already shown that penicillin acylase (EC 3.5.1.11) from *E. coli*, which is used industrially for the hydrolytic cleavage of penicillin into 6β-aminopenicillanic acid (6-APA) and phenylacetic acid,[8] is a promising catalyst for the kinetically controlled synthesis of phenylglycine-derived peptides.[9] D-Phenylglycine dipeptides have interesting properties and can be applied, for example, as tumor and tissue-dissolving compounds of low toxicity and as resolving agents for 1-arylethylamines.[10,11]

The unique substrate specificity of penicillin acylase requires careful consideration in this respect. The acyl donor binding subsite of penicillin acylase has a very high affinity for phenylacetic acid[12] and can accept derivatives of phenylglycine (α-aminophenylacetic acid), but shows moderate stereoselectivity.[13] The acyl acceptor subsite, however, shows an extremely high preference for the L-enantiomers of a very broad range of amino acids.[12,14,15]

In this paper we present a method for the synthesis of stereochemically pure dipeptides of phenylglycine without any need for protecting groups (Fig. 1). Dipeptide esters are known to readily undergo ring closure to the corresponding diketopiperazines.[16] The latter have interesting physiological properties and can be used as food additives,[17] as chitinase inhibitors[18] and as synthons for fungicidal, antiviral[19] and anti-allergic compounds.[20] We here report the first unambiguous characterization of two of the three possible stereoisomers of 3,6-diphenylpiperazine-2,5-dione.

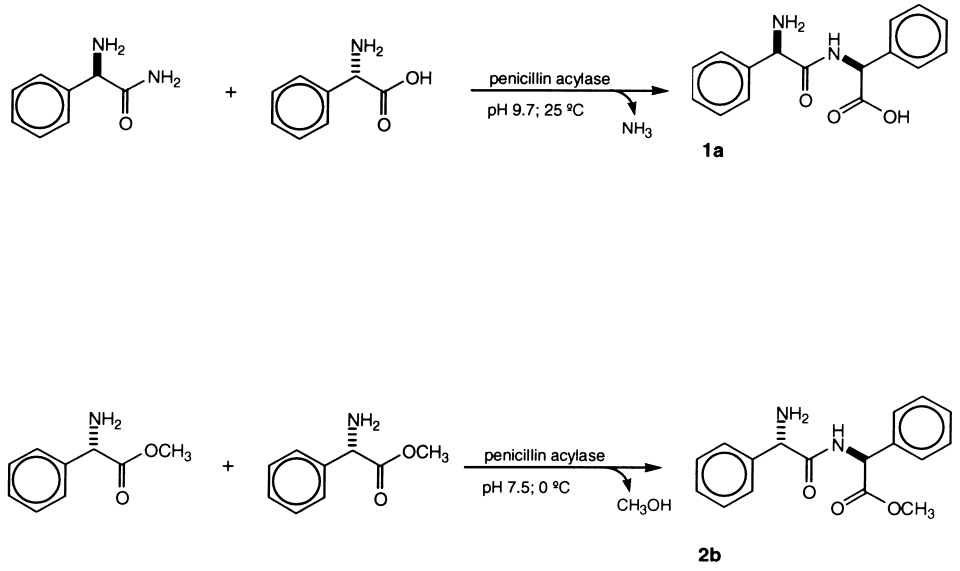

Figure 1. Synthesis of phenylglycine containing dipeptides using *E. coli* penicillin acylase

2. Results and discussion

As noted above, the nucleophile binding subsite of penicillin acylase is L-specific. This characteristic feature simplifies the synthesis of D-phenylglycyl-L-phenylglycine **1a** because any formation of products with D-phenylglycine at the C-terminus or tripeptides is not to be expected.

The possible formation of L-phenylglycyl-L-phenylglycine derivatives could be obviated by the use of free L-phenylglycine, which acts as acceptor at alkaline conditions, but is an extremely poor donor.[4] Accordingly, D-(−)-phenylglycine amide and L-(+)-phenylglycine smoothly reacted at pH 9.7 in the presence of immobilized penicillin acylase. The condensation product **1a** was obtained in 69% yield (Table 1) by acidification to pH 5.5. The ^{1}H and ^{13}C NMR spectra of the product were compared with its D,D-diastereomer, which had been prepared independently by chemical means (Fig. 2).[11]

Table 1
Penicillin acylase-catalyzed peptide synthesis

Donor	Acceptor	pH[a]	Product	Yield
D-Phg-NH_2	L-Phg-OH	9.7	D-Phg-L-Phg-OH	69%
L-Phg-OMe	L-Phg-OMe	7.5	L-Phg-L-Phg-OMe	63%

[a] For other reaction conditions see Experimental Section.

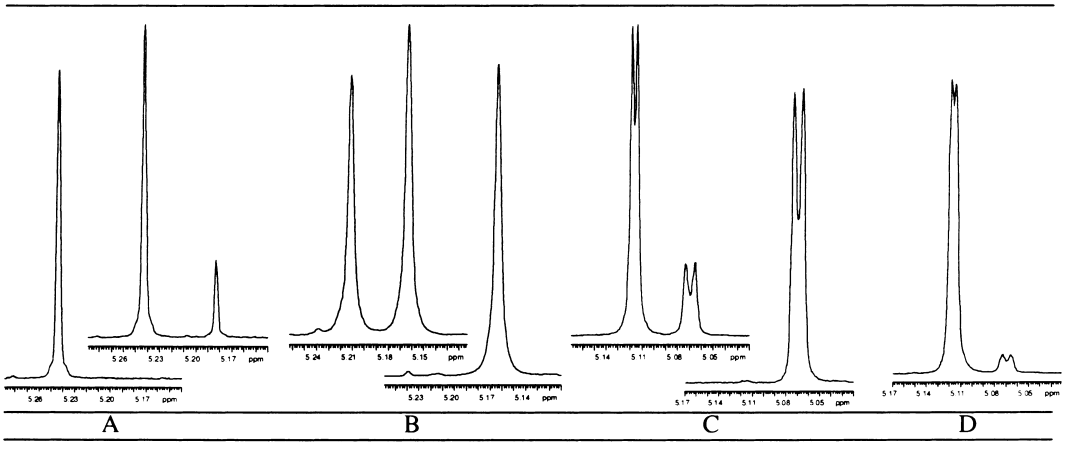

Figure 2. ^{1}H NMR spectra of obtained products, compared with samples mixed with their diastereomers: (**A**) C*H*(NH_3Cl) signal of enzymatically obtained D-phenylglycyl-L-phenylglycine **1a** and of a sample to which D-phenylglycyl-D-phenylglycine has been added; (**B**) C*H*(NH_3Cl) signal of enzymatically obtained L-phenylglycyl-L-phenylglycine methyl ester **2b** and of a sample to which D-phenylglycyl-L-phenylglycine methyl ester **2a** has been added; (**C**) C*H* signal of L,L-3,6-diphenylpiperazine-2,5-dione **3b**, obtained by ring closure of **2b** and D,L-3,6-diphenylpiperazine-2,5-dione **3a** with added **3b**; (**D**) C*H* signal of 3,6-diphenylpiperazine-2,5-dione obtained by heating D-phenylglycine methyl ester for 18 h at 190°C

Diastereomerically pure L-phenylglycyl-L-phenylglycine methyl ester **2b** was enzymatically synthesized in 63% yield from L-phenylglycine methyl ester, which acted both as activated acyl donor and nucleophile. The synthesis product precipitated during reaction at pH 7.5, which prevented possible tripeptide formation with **2b** acting as a nucleophile. Compound **2b** was isolated by extraction with ethyl acetate. Furthermore, precipitation prevented spontaneous degradation of the dipeptide ester during the course of its enzymatic synthesis.

Both **2b** and **2a**, obtained by esterification of **1a**, easily underwent ring closure to the corresponding diketopiperazines as solutions in aqueous alkaline methanol (Fig. 3). 3,6-Diphenylpiperazine-2,5-dione has been hitherto obtained by heating phenylglycine esters or their solutions under basic conditions,[21–23] by benzyloxylation and successive hydrogenolysis of 3,6-diphenyl-2,5-dichloropyrazines[23] or by decarboxylative coupling of Leuchs anhydrides catalyzed by aziridine.[24] The diketopiperazine of phenylglycine exists in three stereoisomeric forms, two of which are optically active. The diastereomers could be discriminated by thin layer chromatography, but their isolation and subsequent stereochemical characterization has never been reported.[23] Furthermore, it was observed that the diastereomers of 3,6-diphenylpiperazine-2,5-dione could not be discriminated by NMR (100 MHz),[23] in contrast to several other 3,6-dialkylpiperazine-2,5-diones.[23,25,26]

Due to the mild reaction conditions we were able to isolate and characterize stereochemically pure diketopiperazines obtained by ring closure of the corresponding dipeptide esters. The cyclization of D-phenylglycyl-L-phenylglycine methyl ester **2a** to the achiral diketopiperazine **3a** was 4.5 times as fast as the cyclization of the L,L-dipeptide ester **2b** to **3b** (Fig. 4). Furthermore, the formation of **3a** was not accompanied by any side product formation, whereas cyclization of **2b**

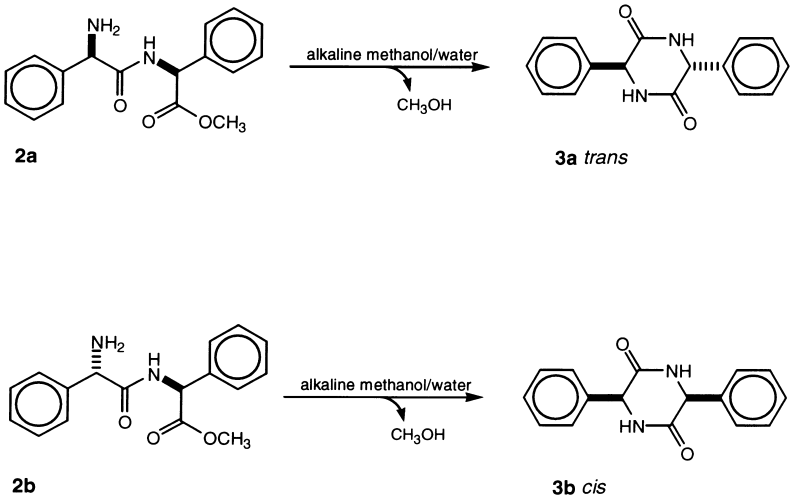

Figure 3. Diketopiperazine formation by ring closure of phenylglycine dipeptide esters

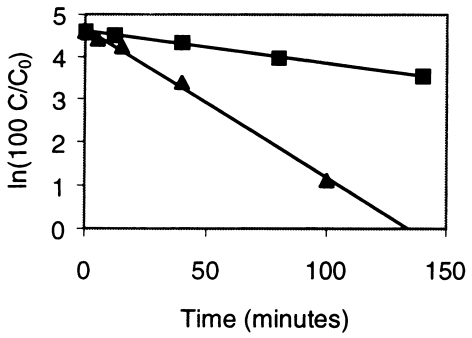

Figure 4. Rate of diketopiperazine formation by cyclization of D-phenylglycyl-L-phenylglycine methyl ester (▲) and L-phenylglycyl-L-phenylglycine methyl ester (■). Reaction conditions: a solution of 75 mM dipeptide ester in methanol:water (1:1) containing 0.75 M NH_3 was stirred at room temperature

gave, besides **3b**, a small amount of **3a**, which was removed by recrystallization. Presumably, **3a** is more stable and more easily formed due to the di-equatorial *trans* configuration of the phenyl groups. It has been claimed that D,D-3,6-diphenylpiperazine-2,5-dione was obtained by heating of D-phenylglycine methyl ester at 180–200°C.[19] In our hands, however, this procedure resulted in the predominant (>90%) formation of the D,L-diketopiperazine **3a** (Fig. 2).

3. Conclusion

Two of the possible stereoisomeric phenylglycine dipeptides have been synthesized in high yield in one step from readily available precursors. Ring closure of the phenylglycine dipeptide esters afforded the corresponding diketopiperazines as pure stereoisomers.

4. Experimental

Immobilized *E. coli* penicillin acylase, Assemblase® (315 U/g), was kindly donated by Gistbrocades (Delft, The Netherlands); one unit is defined as the amount of enzyme that hydrolyzes 1 μmol of penicillin-G per minute at pH 8.0 and 34°C. L-(+)-Phenylglycine and D-(−)-phenylglycine amide were received from DSM (Geleen, The Netherlands) as a gift. L-(+)-Phenylglycine methyl ester hydrochloride salt was obtained from Aldrich.

4.1. Synthesis of D-phenylglycyl-L-phenylglycine *1a*

D-(−)-Phenylglycine amide (8 g, 53.3 mmol) and L-(+)-phenylglycine (6 g, 39.7 mmol) were suspended in 120 ml water at 25°C. The pH was brought to 9.7 using 25% ammonia and 10 g immobilized *E. coli* penicillin acylase was added. The reaction was stirred with a turbine stirrer, while the pH was maintained at 9.7. After 30 min the enzyme was separated from the mixture by filtration. The dipeptide was obtained by lowering the pH of the solution to 5.5 using 3 M H_2SO_4 to yield 7.84 g (27.6 mmol, 69%) of **1a**. Dec. 270°C; as HCl salt: dec. 204°C; $[\alpha]_D$ +51.6 (*c* 1, methanol). ^{1}H NMR (400 MHz): δ 5.20 (s, 1H, C*H*(NH_3Cl)), 5.40 (d, 1H, C*H*NHCO), 7.20–7.55 (m, 10H, aromatic protons), 9.42 (d, 1H, N*H*CO); ^{13}C NMR (400 MHz): 171.05, 170.00, 136.37, 133.87, 128.91, 128.49, 128.33, 127.90, 127.69, 127.01, 56.18, 54.79. Anal. calcd for $C_{16}H_{17}N_2O_3Cl$: C, 59.91; H, 5.34; N, 8.73; Cl, 11.1. Found: C, 59.45; H, 5.21; N, 5.58; Cl, 10.8. IR (KBr) ν_{max}: 1685, 1671, 1516, 1491, 1369 cm^{-1}.

4.2. Synthesis of L-phenylglycyl-L-phenylglycine methyl ester *2b*

L-(+)-Phenylglycine methyl ester hydrochloride (5 g, 24.8 mmol) was dissolved in 60 ml water. The solution was cooled to 0°C and the pH was brought to 7.5 by addition of 25% ammonia; 5 g of immobilized *E. coli* penicillin acylase was added and the mixture was stirred with a turbine stirrer, while the pH was maintained at 7.5. The product precipitated in the course of reaction. After 2.5 h 40 ml water was added to the reaction mixture to maintain stirrability. After 4 h 100 ml ethyl acetate was added and the pH was raised to 8.5 using 25% ammonia and the immobilized enzyme was separated by filtration. The aqueous layer was extracted twice with 100 ml ethyl acetate and the combined organic layers washed with 100 ml water and dried over Na_2SO_4. After

evaporation of the ethyl acetate under reduced pressure the residue was recrystallized from hexane, yielding 2.32 g (7.8 mmol, 63%) of **2b**. M.p. 89–91°C; $[\alpha]_D$ +144.5 (*c* 1, methanol). ^{1}H NMR (300 MHz): δ 3.58 (s, 3H, OC*H*$_3$), 4.46 (s, 1H, C*H*(NH$_2$)), 5.22 (s, 1H, C*H*NHCO), 7.20–7.42 (m, 10H, aromatic protons), 8.92 (s, 1H, N*H*CO); as HCl salt: dec. 209–210°C; $[\alpha]_D$ +120.0 (*c* 1, methanol). ^{1}H NMR (400 MHz): δ 3.53 (s, 3H, OCH$_3$), 5.18 (s, 1H, C*H*(NH$_2$)), 5.24 (d, 1H, C*H*NHCO), 7.38–7.64 (m, 10H, aromatic protons), 9.68 (d, 1H, N*H*CO); ^{13}C NMR (300 MHz): 170.06, 167.12, 135.37, 133.73, 128.90, 128.66, 128.48, 127.91, 127.77, 56.37, 54.74, 52.21. Anal. calcd for $C_{17}H_{18}N_2O_3$: C, 68.44; H, 6.08; N, 9.35. Found: C, 68.69; H, 6.40; N, 9.32. IR (KBr) ν_{max}: 1736, 1638, 1519, 1324 cm^{-1}.

4.3. Synthesis of D-phenylglycyl-L-phenylglycine methyl ester hydrochloride **2a**

Compound **1a** (2 g, 7 mmol) was esterified by 4 h refluxing in 25 ml methanol/HCl. The methanol was evaporated and the product was crystallized from dioxane, yielding 1.0 g (43%) of **2a** hydrochloride. Dec. 145°C; $[\alpha]_D$ +40.4 (*c* 1, methanol). ^{1}H NMR: δ 3.66 (s, 3H, OCH$_3$), 5.22 (s, 1H, C*H*(NH$_2$)), 5.47 (d, 1H, C*H*NHCO), 7.27–7.53 (m, 10H, aromatic protons), 9.64 (d, 1H, N*H*CO); ^{13}C NMR (300 MHz): 170.31, 167.30, 135.39, 133.83, 128.94, 128.50, 128.24, 127.76, 127.31, 56.13, 54.79, 52.38. Anal. calcd for $C_{17}H_{19}N_2O_3Cl$: C, 60.99; H, 5.72; N, 8.37; Cl, 10.59. Found: C, 60.72; H, 6.02; N, 8.26; Cl, 10.33. IR (KBr) ν_{max}: 1736, 1685, 1553, 1453 cm^{-1}.

4.4. Synthesis of D,L-3,6-diphenylpiperazine-2,5-dione **3a**

Compound **1a** (2 g, 7 mmol) was esterified by 4 h refluxing in 25 ml methanol/HCl. To the reaction mixture 25 ml water was added and the pH was raised to 8.0 using KOH. After 3 h stirring at room temperature the product **3a** (1.68 g, 90%) was filtered off. Dec. 294–296°C; $[\alpha]_D$ +0.7 (*c* 1, DMSO). ^{1}H NMR (400 MHz): δ 5.11 (d, 2H, C*H*, *J*=2.0 Hz), 7.34–741 (m, 10H, aromatic protons), 8.62 (d, 2H, N*H*); ^{13}C NMR (400 MHz): 166.13, 138.95, 128.30, 127.83, 127.46, 58.51. HRMS calcd for $C_{16}H_{14}N_2O_2$: 266.1055. Found: 266.1061. IR (KBr) ν_{max}: 1670, 1450, 1303 cm^{-1}.

4.5. Synthesis of L,L-3,6-diphenylpiperazine-2,5-dione **3b**

Compound **2b** (1 g, 3.4 mmol) was dissolved in 40 ml methanol:water (1:1) and the reaction mixture was stirred for 18 h at room temperature. The solvents were evaporated and the crude product was recrystallized from dimethoxyethane, yielding 0.31 g (34%) of **3b**. Dec. 280–282°C; $[\alpha]_D$ +68.5 (*c* 1, DMSO). ^{1}H NMR (400 MHz): δ 5.07 (d, 2H, C*H*, *J*=3.2 Hz), 7.22–7.32 (m, 10H, aromatic protons), 8.67 (d, 2H, N*H*); ^{13}C NMR (400 MHz): 165.78, 138.32, 128.00, 127.60, 126.68, 58.13. HRMS calcd for $C_{16}H_{14}N_2O_2$: 266.1055. Found: 266.1057. IR (KBr) ν_{max}: 1690, 1678, 1448, 1327 cm^{-1}.

4.6. HPLC analysis

Samples were analyzed by HPLC using a Waters M6000 pump, a Nucleosil C-18 column and a Shimadzu SPD-6A UV detector at 215 nm. The eluent was prepared by adjusting the pH of a 1 g/l solution of sodium dodecylsulphate in methanol:water (70:30, v/v) to 3.5 with phosphoric

acid. The flow rate was 1.0 ml/min. Retention times (in minutes) were as follows: L,L-3,6-diphenylpiperazine-2,5-dione (3.2), phenylglycine (4.5), D,L,-3,6-diphenylpiperazine-2,5-dione (4.8), phenylglycine amide (5.2), D-phenylglycyl-D-phenylglycine (5.6), phenylglycine methyl ester (6.0), D-phenylglycyl-L-phenylglycine (6.8), L-phenylglycyl-L-phenylglycine methyl ester (7.6), D-phenylglycyl-L-phenylglycine methyl ester (8.2).

Acknowledgements

Financial support by DSM Life Science Products and the Netherlands Ministry of Economic Affairs is gratefully acknowledged. This work was financially supported by the Russian Foundation for Fundamental Research (Grant 97-04-48827). The authors wish to thank Professor Katsuyuki Ogura (Chiba University, Chiba, Japan) for a sample of chemically prepared D-phenylglycyl-D-phenylglycine. Thanks are due to Messrs. A. van Estrik and A. Sinnema for the NMR analyses.

References

1. Kleinkauf, H.; Von Döhren, H. In *Peptide Antibiotics*; Moo-Young, M., Ed. Comprehensive Biotechnology. Pergamon Press: Oxford, 1985; pp. 95–135.
2. Švedas, V. K.; Margolin, A. L.; Berezin, I. V. *Enzyme Microb. Technol.* **1980**, *2*, 138–144.
3. Diender, M. B.; Straathof, A. J. J.; Van der Wielen, L. A. M.; Ras, C.; Heijnen, J. J. *J. Mol. Catal. B: Enzym.* **1998,** *5*, 249–253.
4. Schroën, C. G. P. H.; Nierstrasz, V. A.; Kroon, P. J.; Bosma, R.; Janssen, A. E. M.; Beeftink, H. H.; Tramper, J. *Enzyme Microb. Technol.* **1999**, *24*, 498–506.
5. Frère, J. M.; Ghuysen, J. M.; Perkins, H. R.; Nieto, M. *Biochem. J.* **1973**, *135*, 483–492.
6. Kasche, V.; Haufer, U.; Riechmann, L. *Ann. NY Acad. Sci.* **1984**, *434*, 99–105.
7. Golobolov, B. Y.; Borisov, I. L.; Belikov, V. M.; Švedas, V. K. *Biotechnol. Bioeng.* **1987**, *32*, 866–872.
8. Verweij, J.; De Vroom, E. *Recl. Trav. Chim. Pays-Bas* **1993**, *112*, 66–81.
9. Shamolina, T. A.; Korennykh, A. V.; Van Langen, L. M.; Sheldon, R. A.; Švedas, V. K., submitted.
10. Biedermann, J.; Etschenberg, E.; Friehe, H.; Scheef, W.; Winkelmann, J. EP 0069894 A1, 1982.
11. Akazome, M.; Matsuno, H.; Ogura, K. *Tetrahedron: Asymmetry* **1997**, *8*, 2331–2336.
12. Švedas, V. K.; Savchenko, M. V.; Beltser, A. I.; Guranda, D. F. *Ann. NY Acad. Sci.* **1996**, *799*, 659–669.
13. Fuganti, C.; Rosell, C. M.; Servi, S.; Tagliani, A.; Terreni, M. *Tetrahedron: Asymmetry* **1992**, *3*, 383–386.
14. Kasche, V.; Michaelis, G.; Wiesemann, T. *Biomed. Biochim. Acta* **1991**, *50*, 38–43.
15. Didziapetris, R. J.; Švedas, V. K. *Biomed. Biochim. Acta* **1991**, *50*, 237–242.
16. Purdie, J. E.; Benoiton, N. L. *J. Chem. Soc., Perkin Trans. 2* **1973**, 1845–1852.
17. Shibuya, K. Japanese Patent, JP 58162260 A, 1983.
18. Izumida, H.; Nishijima, M.; Sano, H.; Yamagishi, M.; Oishi, K.; Ota, T.; Motosugi, M.; Kamimura, D.; Gotou, M. JP 08277203 A, 1996.
19. Svokos, R. G.; Angier, R. B. US Patent, US 3560483, 1971.
20. Shimazaki, N.; Henmi, K.; Hashimoto, S. Japanese Patent, JP 63290868 A, 1988.
21. Kossel, A. *Ber. Dtsch. Chem. Ges.* **1891**, *24*, 4145–4156.
22. Ovakimian, G.; Kuna, M.; Levene, P. A. *J. Biol. Chem.* **1940**, *135*, 91–98.
23. Ohta, A.; Akita, Y.; Nakane, Y. *Chem. Pharm. Bull.* **1979**, *27*, 2980–2987.
24. Rosenmund, P.; Kaiser, K. *Angew. Chem.* **1970**, *82*, 137–138.
25. Tsutsui, M.; Watanabe, T.; Ohta, A. *J. Heterocyclic Chem.* **1980**, *17*, 809–812.
26. Ohta, A.; Yamamoto, F.; Arimura, Y.; Watanabe, T. *J. Heterocyclic Chem.* **1982**, *19*, 781–784.