Pergamon

SCIENCE DIRECT®

Tetrahedron: *Asymmetry* 14 (2003) 3105–3109

TETRAHEDRON: *ASYMMETRY*

Diastereoselective microbial reduction of (*S*)-[3-chloro-2-oxo-1-(phenylmethyl)propyl]carbamic acid, 1,1-dimethylethyl ester

Ramesh N. Patel,* Linda Chu and Richard Mueller

Process Research and Development, Bristol-Myers Squibb Pharmaceutical, Research Institute, PO Box 191, New Brunswick, NJ 08903, USA

Received 2 May 2003; accepted 25 July 2003

Abstract—The chiral intermediate (1*S*,2*R*)-[3-chloro-2-hydroxy-1-(phenylmethyl)propyl]carbamic acid, 1,1-dimethylethyl ester **2a** was prepared for the total synthesis of the HIV protease inhibitor Atazanavir. The diastereoselective reduction of (1*S*)-[3-chloro-2-oxo-1-(phenylmethyl)propyl] carbamic acid, 1,1-dimethyl-ethyl ester **1** was carried out using microbial cultures among, which *Rhodococcus*, *Brevibacterium*, and *Hansenula* strains reduced **1** to **2a**. Three strains of *Rhodococcus* gave >90% yield. A diastereomeric purity of >98% and enantiomeric excess of >99.3% were obtained for alcohol **2a**.

1. Introduction

An essential step in the life cycle of human immunodeficiency virus (HIV-1) is the proteolytic processing of its precursor proteins. This processing is accomplished by HIV-1 protease, a virally encoded enzyme. Inhibition of HIV-1 protease arrests the replication of HIV in vitro. Thus, HIV-1 protease is an attractive target for chemotherapeutic intervention.[1–6] Atazanavir is an acyclic aza-peptidomimetic that is a potent HIV protease inhibitor.[6–11] The original process was utilized for the small scale preparation of Atazanavir to support initial development[12–14] and subsequently an improved process for the large scale synthesis of Atazanavir was developed.[15]

Herein we describe the diastereoselective microbial reduction of (1*S*)-[3-chloro-2-oxo-1-(phenylmethyl)-propyl]carbamic acid, 1,1-dimethylethyl ester **1** to homochiral chlorohydrin **2a**, a key intermediate in the total synthesis of Atazanavir as illustrated in Scheme 1.

2. Results and discussion

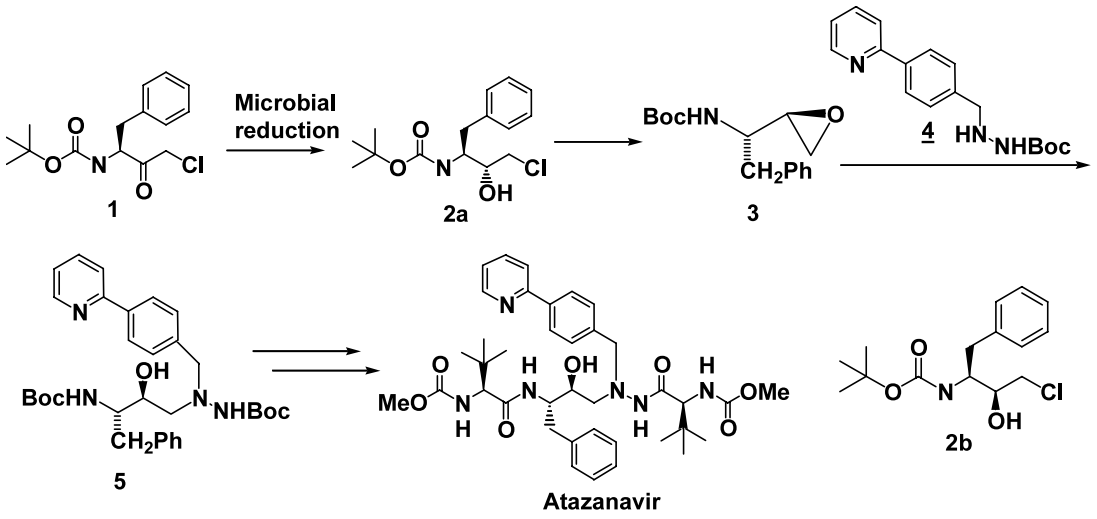
Chemical reduction of chloroketone **1** using $NaBH_4$ produces primarily the undesired chlorohydrin **2b**.[16,17] As a result, the use of microbial reduction to produce the desired isomer was investigated. A number of alcohol oxidoreductases from many organisms, including those from yeast,[18–23] horse liver,[24] *Thermoanerobic brockii*,[25,26] *Lactobacillus kefir*,[27] *Pseudomonas* sp.,[28] *Geotrichum candidum*,[29,30] *Hansenula polymorpha*,[31,32] *Mortierella rammaniana*,[33] and *Sulfolobus* solfataricus[34] have proven useful for the enantioselective reduction of ketones to alcohols.

About 120 microorganisms were screened for the diastereoselective reduction of **1** (typically with e.e. >99%) to **2a**. The reaction yield and diastereomeric purity of **2a** obtained with the eight best cultures are shown in Table 1; lower diastereomeric purities (<90%) and reaction yields (<12%) were obtained with other cultures. *Brevibacterium* sp. SC 16101, *Hansenula anomala* SC 13833, *Hansenula saturnus* SC 13829, *Rhodococcus erythropolis* SC 13845, *Rhodococcus erythropolis* SC 116236, *Rhodococcus* sp. SC 13810, and *Rhodococcus* sp. SC 16002 gave >30% reaction yields and >94% diastereomeric purities of product **2a**; chlorohydrin **2b** was formed as the minor diastereomer.

Rhodococcus erythropolis SC 13845 and *Rhodococcus* sp. 16002 were investigated further. Cells of *Rhodococcus erythropolis* SC 13845 and *Rhodococcus* sp. 16002 were grown in a 25 L fermentor for 48 h. The cells were collected and suspended in 70 mM potassium phos-

* Corresponding author. E-mail: patelr@bms.com

0957-4166/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetasy.2003.07.016



Scheme 1.

phate buffer (pH 7.0), and the resulting cell-suspensions were used to carry out the two-stage process for biotransformation of **1** as described in Section 3. After 49 h, reaction yields of >92%, diastereomeric purities of >98% and enantiomeric excesses of 99.3–99.4% for alcohol **2a** were obtained (Table 2).

A more efficient single-stage fermentation-biotransformation process was developed for the reduction of ketone **1** with cells of *Rhodococcus erythropolis* SC 13845 as described in Section 3. A reaction yield of 95%, diastereomeric purity of 98.2% and enantiomeric excess of 99.4% for alcohol **2a** were obtained (Table 3). From a 12 L reaction mixture, 9.1 g of alcohol **2a** was isolated in 76% overall yield.

Cell extracts of *Rhodococcus erythropolis* SC 13945 were then evaluated for the reduction of ketone **1**. Glucose dehydrogenase in the presence of glucose and NADP or formate dehydrogenase in the presence of formate and NAD were used to regenerate the cofactor (NADPH or NADH respectively) required for the reduction reaction. After 6 hr of reaction, higher reaction yields and diastereomeric purities were obtained for chiral alcohol **2a** (49% yield, 98.6% diastereomeric purity) when NADH was used as a cofactor compared to NADPH (Table 4).

The oxidoreductases from yeast,[18–23] horse liver,[24] and *T. brockii*[26] transfer the pro-*R* hydride to the *re*-face of the carbonyl to give (*S*)-alcohols, a process described by Prelog's rule.[35] In contrast, oxidoreductases from *Lactobacillus kefir*[27] and two *Pseudomonas* sp.[28] exhibit anti-Prelog specificity, transferring the pro-*R* hydride to form (*R*)-alcohols. We have described above the reduction of ketone **1** to the corresponding (*S*)-alcohol by *Rhodococcus erythropolis*. NADH was a better cofactor compared to NADPH, affording higher reaction yields and diastereomeric purities when cell extracts were used for this reduction process.

3. Experimental

Starting substrate **1** and reference compounds **2a** and **2b** were synthesized by colleagues in the Chemical Process Research Department, Bristol-Myers Squibb Pharmaceutical Research Institute. The physico-chemical properties including spectral characteristics (^{1}H NMR, ^{13}C NMR, mass spectra) were in full accord for all these compounds.[36] The proton magnetic resonance (^{1}H NMR) and carbon magnetic resonance (^{13}C NMR) were recorded on a Brucker AM-300 spectrometer.

3.1. Microorganisms

Microorganisms (Table 1) were obtained from the culture collection of the Bristol-Myers Squibb Pharmaceutical Research Institute. Microbial cultures were stored at −90°C in vials.

Table 1. Diastereoselective microbial reduction of ketone **1**

Microorganisms	Yield of **2a** (%)	Diastereomeric purity of **2a** (%)
Brevibacterium sp. SC 16101	99	94
Hansenula anomala SC 13833	33	98
Hansenula saturnus SC 13829	35	98.5
Rhodococcus erythropolis SC 13845	98	98.9
Rhodococcus erythropolis SC 16236	74	98
Rhodococcus sp. SC 13810	91	98
Rhodococcus sp. SC 16002	97	98
Trichoderma viridae SC 13826	12	98

Table 2. Diastereoselective microbial reduction of ketone **1**: two-stage process

Microorganisms	Reaction time (h)	Yield of **2a** (%)	Diastereomeric purity of **2a** (%)	Enantiomeric excess of **2a** (%)
Rhodococcus erythropolis SC 13845	20	62		
	49	97	98.5	99.4
Rhodococcus erythropolis SC 16236	20	60		
	49	92	98.2	99.3

Table 3. Diastereoselective microbial reduction of ketone **1**: single-stage process

Microorganisms	Reaction time (h)	Yield of **2a** (%)	Diastereomeric purity of **2a** (%)	Enantiomeric excess of **2a** (%)
Rhodococcus erythropolis SC 13845	21	38		
	48	52		
	72	72		
	93	95	98.2	99.4

Table 4. Diastereoselective microbial reduction of ketone **1**: cell-free system

Regeneration system	Reaction time (h)	Yield of **2a** (%)	Diastereomeric purity of **2a** (%)
NAD, formate, formate dehydrogenase	6	49	98.6
NADP, glucose, glucose dehydrogenase	6	22	60

3.2. Growth of microorganisms

For screening purposes, one vial of each culture was used to inoculate 100 mL of medium A containing 1% malt extract, 1% yeast extract, 2% glucose and 0.3% peptone. The medium was adjusted to pH 6.8 before sterilization. Cultures were grown at 28°C and 280 rpm for 48 h.

Cultures were harvested by centrifugation at 18,000×*g* for 15 min, washed with 70 mM potassium phosphate buffer pH 7.0, and used for reduction studies.

3.3. Reduction of 1 by cell-suspensions

Cells of various microorganisms were suspended separately in 70 mM potassium phosphate buffer (pH 7.0) at 20% (w/v, wet cells) cell concentration and supplemented with 1 mg/mL of **1** and 30 mg/mL of glucose. Reduction was conducted at 28°C and 150 rpm. Periodically, samples of 1 mL were taken and extracted with 5 mL of *tert*-butylmethylether: toluene (60:40). After centrifugation, the separated organic phase was collected and dried with a nitrogen stream. The oily residue obtained was dissolved in 1 mL of ethanol, filtered through a 0.2 μm LID/X filter and analyzed by HPLC.

3.4. Two-stage process for reduction of 1

Rhodococcus erythropolis SC 13845 and *Rhodococcus erythropolis* SC 16236 culture were grown in a 25 L fermentor containing 15 L of medium A containing 0.025% UCON antifoam. Growth consisted of several inoculum development stages and fermentation. Inoculum development consisted of F1 and F2 stages. In the F1 stage, a frozen vial of each culture was inoculated into 100 mL of medium A contained in a 500-mL flask. Growth was carried out at 28°C and 280 rpm for 48 h on a rotary shaker. In the F2 stage, 10 mL of F1 stage culture was inoculated into 1 L of medium A and incubated at 28°C and 280 rpm for 24 h.

Fermentors containing 15 L of medium A were inoculated with 1 L of inoculum of each culture from an F2 stage. Fermentation was conducted at 25°C and 500 rpm with 15 lpm (liter per min) aeration for 36 h. After 48 h fermentation, cells were collected and stored at −90°C until further use. About 1 kg of wet cell pastes was collected from each fermentation.

Frozen cells from the above batches were used to conduct the reduction of **1** in a 5 L reactor. Cell suspensions (10% w/v, wet cells) in 3 L of 70 mM potassium phosphate buffer (pH 7.0) were used. Compound **1** (30 g) and glucose (75 g) were added to the reactor and the reduction was carried out at 28°C and 160 rpm with 1 lpm aeration for 49 h. The pH was maintained between 6.8–7.0. Periodically, samples were prepared as described above and analyzed by HPLC to determine the % conversion of **1** to **2a**. The diastereomeric purity of **2a** was determined by HPLC.

3.5. Isolation of 2a

At the end of two-stage bioreduction, 3 L of the reaction mixture was extracted with 6 L of *tert*-butylmethylether: toluene (60:40). The separated organic phase was washed with 2 L of 0.1 M sodium chloride, dried over anhydrous sodium sulfate and evaporated under reduced pressure to obtain 24 gram of crude product which was recrystallized from ethyl acetate to afford 22.5 g (75% overall yield) of **2a**. The diastereomeric purity and the enantiomeric purity of the isolated chiral alcohol **2a** were >98% and 99.5%, respectively. ^{1}H NMR ($CDCl_3$) δ 1.4 (S, 9H), 2.9 (d, 2H, $J=13$ Hz), 3.2 (S, 1H); 3.6 (m, 2H), 3.85 (m, 1H), 4.5 (S, 1H), 7.2–7.4 (m, 5H). MS m/z 302 $(M+H)^+$ (calcd for $C_{15}H_{22}ClNO_2$, 301).

3.6. Single-stage process for reduction of 1

Rhodococcus erythropolis SC 13845 was grown in a 25 L fermentor containing 15 L of medium A as described above. After 30 h, 15 g of ketone **1** was added to the fermentor and the biotransformation process was continued for 93 h. The pH was maintained at 7.0. Periodically, samples were prepared as described above and analyzed by HPLC to determine the % conversion of **1**. The diastereomeric purity of **2a** was determined by HPLC.

3.7. Analytical methods

Analysis of **1**, **2a**, and **2b** were carried out using a Hewlett Packard HPLC. A Vydak column (100×4.6 mm, ID 5 m) was used under the following conditions. Mobile phase was 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in 70% acetonitrile, 30% water (solvent B) used in a gradient:

Time (min)	Solvent A (%)	Solvent B (%)
0	50	50
0.1-20 (gradient)	50	100
20-25	0	100
25.1-26	50	50
26-30	50	50

The flow rate was 1 mL/min and the detection wavelengths were 224, 250 and 280 nm. The retention times for substrate **1**, compounds **2a**, and **2b** were 12.9, 10.9 and 9.7 min, respectively.

The enantiomeric excess of chiral alcohol **2a** was also determined by chiral HPLC. A Bakerbond chiralpak AD column (100×4.5 mm, ID 5 m) was used at ambient temperature; Injection volume was 10 μL; the mobile phase was 97.5% hexane: 1% cyclohexanol: 1.5% ethyl acetate mixture; flow rate was 0.8 mL/min; and the detection wavelength was 210 nm. The retention times for the compounds **2a**, **2b** were 14 min, 15.5 min, respectively.

3.8. Preparation of cell extracts of *Rhodococcus erythropolis* SC 13845

Preparation of cell extracts were carried out at 4–7°C. Cells were washed with 50 mM potassium phosphate buffer (pH 7.0) and washed cells (30 g) were suspended in 200 mL of buffer containing 10% glycerol and 1 mM ethylenediamine tetraacetic acid (EDTA). Cell suspensions (15% w/v, wet cells) were disintegrated with a Microfluidizer (Microfluidics, Inc) at 12,000 psi (two passages), and the disintegrated cells were centrifuged at 25,000×g for 30 min to obtain the cell extract. Protein in cell extracts was estimated using Bio-Rad protein reagent with bovine serum albumin as a standard. The assay mixture contained 1–10 μL of enzyme fraction, 0.8 mL water and 0.2 mL Bio-Rad reagent. After mixing, the absorbance of the solution was measured at 595 nm. Cell extracts were evaluated for biotransformation of ketone **1**. The reaction mixture contained 1 mg/mL substrate, 2 mM nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP), 8 units glucose dehydrogenase, and 2 mg/mL glucose or 1 mM formate and 3 units formate dehydrogenase to regenerate reduced cofactor (NADH or NADPH) required for the reduction. Samples were incubated at 28°C,105 rpm for 6 h. Concentrations of **1**, **2a**, and **2b** were estimated by HPLC.

Acknowledgements

We would like to acknowledge Dr. John Wasylyk for development of the HPLC assay system and Dr. Thomas LaPorte for fermentation of microbial cultures. We would like to thank Dr. John Scott for reviewing this manuscript and providing valuable suggestions.

References

1. Yoshimura, K.; Kato, R.; Kavlick, M. F.; Nguyen, A.; Maroun, V.; Maeda, K.; Hussain, K. A.; Ghosh, A. K.; Gulnik, S. V.; Erickson, J. W.; Mitsuya, H. *J. Virol.* **2002**, *76*, 1349–1358.
2. Hoetelmans, R. M. W.; Meenhorst, P. L.; Mulder, J. W.; Burger, D. M.; Koks, C. H. W.; Beijnen, J. H. *Pharm. World Sci.* **1997**, *19*, 159–175.
3. Prasad, J. V. N. V.; Lunney, E. A.; Para, K. S.; Tummino, P. J.; Ferguson, D.; Hupe, D; Domagala, J. M.; Erickson, J. W. *Drug Des. Discov.* **1996**, *13*, 15–28.
4. Tomasselli, A. G.; Thaisrivongs, S.; Heinrikson, R. L. *Adv. Antiviral Drug Des.* **1996**, *2*, 173–228.
5. Kageyama, S.; Hoekzema, D. T.; Murakawa, Y.; Kojima, E.; Shirasaka, T.; Kempf, D. J.; Norbeck, D. W.; Erickson, J. W.; Mitsuya, H. *AIDS Res. Hum. Retroviruses* **1994**, *10*, 735–743.
6. Fassler, A.; Bold, G.; Capraro, H. G.; Cozens, R.; Mestan, J.; Poncioni, B.; Rosel, J.; Tintelnot-Blomley, M.; Lang, M. *J. Med. Chem.* **1996**, *39*, 3203–3216.
7. Bold, G.; Fassler, A.; Capraro, H. G.; Cozens, R.; Klimkait, T.; Lazdins, J.; Mestan, J.; Poncioni, B.; Rosel,

J.; Stover, D.; Tintelnot-Blomley, M.; Acemoglu, F.; Beck, W.; Boss, E.; Eschbach, M.; Hurlimann, T.; Masso, E.; Roussel, S.; Ucci-Stoll, K.; Wyss, D.; Lang, M. *J. Med. Chem.* **1998**, *41*, 3387–3401.
8. Robinson, B. S.; Riccardi, K. A.; Gong, Y.-F.; Guo, Q.; Stock, D. A.; Blair, W. S.; Terry, B. J.; Deminie, C. A.; Djang, F.; Colonno, R. J.; Lin, P.-F. *Antimicrobial Agents Chemotherapy* **2000**, *44*, 2093–2099.
9. Gong, Y.-F.; Robinson, B. S.; Rose, R. E.; Deminie, C.; Spicer, T. P.; Stock, D.; Colonno, R. J.; Lin, P.-F. *Antimicrobial Agents Chemotherapy* **2000**, *44*, 2319–2326.
10. Drusano, G. L.; Bilello, J. A.; Preston, S. L.; O'Mara, E.; Kaul, S.; Schnittman, S.; Echols, R. *J. Infect. Diseases* **2001**, *183*, 1126–1129.
11. Rabasseda, X.; Silvestre, J.; Castaner, J. *Drugs of the Future* **1999**, *24*, 375–380.
12. Giordano, C.; Pozzoli, C.; Benedetti, F. 2001, WO Patent 0127083, CAN 134:311112.
13. Fassler, A. Bold, G. Capraro, H-G; Steiner, H. **1997**, W. O. Patent 9746514, CAN 128:48504
14. Fassler, A.; Bold, G.; Steiner, H. *Tetrahedron Lett.* **1998**, *39*, 4925–4928.
15. Xu, Z.; Singh, J.; Schwinden, M. D.; Zheng, B.; Kissick, T. P.; Patel, B.; Humora, M. J.; Quiroz, F.; Dong, L.; Hsieh, D.-M.; Heikes, J. E.; Pudipeddi, M.; Lindrud, M. D.; Srivastava, S. K.; Kronenthal, D. R.; Mueller, R. H. *Org. Proc. Res. Development* **2002**, *6*, 323–328.
16. Chen, P.; Cheng, P. T. W.; Alam, M.; Beyer, B. D.; Bisacchi, G. S.; Dejneka, T.; Evans, A. J.; Greytok, J. A.; Hermsmeier, M. A.; Humphreys, W. G.; Jacobs, J. A.; Kocy, A.; Lin, P.-F.; Lis, K. A.; Marella, M. A.; Ryono, D. E.; Sheaffer, A. K.; Spergel, S. H.; Sun, C.-Q.; Tino, J. A.; Vire, G.; Colono, R. J.; Zahler, R.; Barrish, J. C. *J. Med. Chem.* **1996**, *39*, 1991–2007.
17. Gordon, E. M.; Barrish, J. C.; Bisacchi, G. S.; Sun, C. Q.; Tino, J. A.; Vite, G. D.; Zahler, R. Preparation of diphenyl-substituted amino alcohols as protease inhibitors. Eur. Pat. Appl. (1994), 393 pp. CAN 122:290438 AN 1995:511385.
18. Ward, O. P.; Young, C. S. *Enzyme Microb. Technol.* **1990**, *12*, 482–493.
19. Csuk, R.; Glanzer, B. I. *Chem. Rev.* **1991**, *91*, 49–97.
20. Sih, C. J.; Chen, C. S. *Angew. Chem.* **1984**, *96*, 556–566.
21. Sih, C. J.; Zhou, B.; Gopalan, A. S.; Shieh, W. R.; VanMeddlesworth, F. In *Selectivity, a Goal for Synthetic Efficiency*, Proceedings, 14th workshop conference, Hoechst, Batman, W.; Trost, B. M., Eds.; Verlag Chemie: Weinheim, 1984; pp. 250–261.
22. Hummel, W.; Kula, M. R. *Eur. J. Biochem.* **1989**, *184*, 1–13.
23. Jones J. B.; Back, J. F. In *Applications of Biochemical Systems in Organic Synthesis*; Jones, J. B.; Sih, C. J.; Perlman, D., Eds. John Wiley & Sons: New York 1976, pp. 248–376.
24. Jones, J. B. *Mechanisms of Enzymatic Reaction, Stereochemistry*; Frey, P. A., Eds.; Elsevier Science: Amsterdam, 1986, pp. 3–14.
25. Keinan, E.; Hafeli, E. K.; Seth, K. K.; Lamed, R. *J. Am. Chem. Soc.* **1986**, *108*, 162–168.
26. Kula, M.-R.; Kragl, U. In *Stereoselective Biocatalysis*; Patel, R. N. Marcel Dekker: New York, 2000; pp. 839–866.
27. Bradshaw, C. W.; Hummel, W.; Wong, C.-H. *J. Org. Chem.* **1992**, *57*, 1532–1536.
28. Bradshaw, C. W.; Fu, H.; Shen, G.-J.; Wong, C.-H. *J. Org. Chem.* **1992**, *57*, 1526–1532.
29. Nakamura, K.; Kawai, Y.; Oka, S.; Ohno, A. *Bull. Chem. Soc. Jpn.* **1989**, *62*, 631–3632.
30. Nakamura, K.; Kawai, Y.; Miyai, T.; Ohno, A. *Tetrahedron Lett.* **1990**, *31*, 3631–3632.
31. Patel, R. N.; Banerjee, A.; McNamee, C. G.; Szarka, L. *J. Appl. Microbiol. Biotechnol.* **1993**, *40*, 241–245.
32. Patel, R. N.; Banerjee, A.; Howell, J. M.; McNamee, C. G.; Brzozowski, D.; Mirfakhrae, D.; Nanduri, V.; Thottathil, J. K.; Szarka, L. J. *Tetrahedron: Asymmetry*. **1993**, *4*, 2069–2084.
33. Patel, R. N.; Banerjee, A.; Liu, M.; Hanson, R. L.; Ko, R.; Howell, J. M.; Szarka, L. J. *Biotechnol. Appl. Biochem.* **1993**, *17*, 139–153.
34. Trincone, A.; Lama, L.; Lanzotti, V.; Nicolaus, B.; DeRosa, M.; Rossi, M.; Gambacorta, A. *Biotechnol. Bioeng.* **1990**, *35*, 559–564.
35. Prelog, V. *Pure Appl. Chem.* **1964**, *9*, 119–123.
36. Kronenthal, D.; Schwinden, Mark. Process for the preparation of α-chloroketones from amino acid phenyl esters and sulfur ylides. PCT Int. Appl. 2002, 18 pp. WO 0214256, A1 20020221, CAN 136:183620, AN 2002:142651.