Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 901–909
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Preparative separation and identification of derivatized β-methylphenylalanine enantiomers by chiral SFC, HPLC and NMR for development of new peptide ligand mimetics in drug discovery

Lisa M. Nogle [a,*], Charles W. Mann [b], William L. Watts Jr. [a], Yingru Zhang [c]

[a] *Discovery Analytical Chemistry, Chemical and Screening Sciences, Wyeth Research, 500 Arcola Road, Collegeville, PA 19426, USA*
[b] *Discovery Medicinal Chemistry, Chemical and Screening Sciences, Wyeth Research, 500 Arcola Road, Collegeville, PA 19426, USA*
[c] *Discovery Analytical Sciences, Pharmaceutical Research Institute, Bristol-Myers Squibb Company, P.O. Box 4000, Princeton, NJ 08648, USA*

Received 17 October 2004; received in revised form 23 August 2005; accepted 23 August 2005
Available online 18 October 2005

Abstract

A direct preparative purification of all four isomers of the unnatural amino acid β-methylphenylalanine was achieved using supercritical fluid chromatography (SFC) with stacked-injection. Final purification of the Cbz-methyl ester derived isomers was performed on a Daicel Chiralpak AD-H column (20 mm × 250 mm), using 50:50 methanol/ethanol as the organic modifier and resulted in purification of over 3.4 g of material in 6.25 h with >90% total recovery. The absolute stereochemical assignment of the purified amino acids was determined through a combination of chiral HPLC, NMR and optical rotation studies. To our knowledge, this is the first reported preparative approach that has yielded all four compounds in a single chromatographic run.

Keywords: Supercritical fluid chromatography; Chiral chromatography; β-Amino acids; NMR spectroscopy; Enantiomer identification; Diastereomers

1. Introduction

In the last decade much progress has been made toward the development of selective peptide receptor ligands for various therapeutic targets. However, a major challenge for scientists in medicinal chemistry is the ability to design structures that possess both optimal pharmacological (e.g. receptor binding) and optimal pharmaceutical properties (e.g. membrane permeability and metabolic stability). Amino acids, in particular, unnatural amino acids, have been recognized as major tools for the preparation of peptide ligand mimetics with both enhanced biological activity and proteolytic resistance. These molecules represent a nearly infinite array of diverse building blocks for the development of new pharmacological leads [1–6]. However, amino acids are also chiral molecules. Consequently, with the increasing demands for production of enantiomerically pure compounds in the fields of pharmaceuticals, peptide research, etc., these chiral separations have become an important analytical task [7].

Successful resolution of amino acids has been achieved using either direct or indirect methodologies [8–10]. In the indirect approach, optically pure chiral derivatizing reagents are applied to form diastereomers that are subsequently separated on achiral columns. In the direct approach, chiral stationary phases (CSPs) are most often employed. This latter method is greatly preferred due to complications associated with removal of the derivatizing reagents.

While various CSPs, including cyclodextrin-bonded, quinine-derived, and most recently, glycopeptide antibiotic-bonded varieties, have been used to effect separation of amino acids by high performance liquid chromatography (HPLC), these separations often require special conditions such as the addition of mobile phase modifiers, pH adjustments, and stringent temperature controls. In addition, these methods have been utilized almost exclusively for analytical scale separations and have shown only modest success in the separation of

* Corresponding author. Tel.: +1 484 865 2674; fax: +1 484 865 9398.
E-mail address: noglel@wyeth.com (L.M. Nogle).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.08.034

all isomers of amino acids containing two chiral centers [11–14]. Moreover, only one report has thus far demonstrated a semi-preparative HPLC resolution of β-methylphenylalanine racemic pairs (threo or erythro), albeit with poor recovery [15].

Herein, we report the first direct multi-gram purification of all four isomers of the unnatural amino acid β-methylphenylalanine using supercritical fluid chromatography (SFC) with stacked-injection. SFC has several advantages over other conventional chromatographic techniques like HPLC [16–22]. For example, the increased diffusivity observed with SFC leads to sharper peak shapes and thus increased resolution. Moreover, the low viscosity of SFC eluents allows for faster separations, a significant benefit in preparative-scale isolations. As demonstrated through this work, SFC has proven to be the most effective means of β-methylphenylalanine enantiopurification to date.

2. Experimental

2.1. Chemicals

Carbon dioxide (industrial grade) was obtained from Airgas Inc. (Radnor, PA, USA). HPLC grade methanol, acetonitrile and isopropyl alcohol were purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA), and absolute ethyl alcohol was purchased from Pharmco Products Inc. (Brookfield, CT, USA). Water was purified using a Millipore Milli-Q-plus purification system. β-Methylphenylalanine hydrochloride, reported as a 99% mixture of ∼67% threo/33% erythro isomers (product no. 217034) and carbobenzoxy chloride (product no. 119938) were obtained from Aldrich (St. Louis, MO, USA) and copper(II) sulfate pentahydrate (product no. VW3312-2) was obtained from VWR Scientific (West Chester, PA, USA). Deuterated DMSO-d_6 (99.9%) was purchased from Cambridge Isotopes (Andover, MA, USA).

2.2. Instrumentation

Supercritical fluid chromatography was performed using both analytical and preparative Berger instruments (Mettler Toledo, Newark, DE, USA). The Berger Analytical SFC Instrument consisted of a model FCM-1200 fluid control module with a six-position modifier switching valve, a TCM-2000 column oven with a six-port column switching valve, a model 719 autosampler, an Agilent G1315A Diode Array Detector (Palo Alto, CA, USA) equipped with a high-pressure flow cell (400 bar), and SFC ProNTo software. The analytical SFC was operated at 2 mL/min and 35 °C, with an outlet pressure of 100 bar. The Berger multigram SFC system consisted of two Varian SD-1 pumps (Walnut Creek, CA, USA), one modified to pump CO_2, a Julabo FT401 chiller (Labortechnik GmbH. Seelback, Germany), a model SCM 2500 phase separator (Berger Instruments), a model ECM 2500 electronic control module (Berger Instruments), and a Knauer 2501 variable wavelength detector with high-pressure flow cell. Solute was introduced via the modifier line prior to CO_2 mixing. The multigram SFC was run at 50 mL/min, 35 °C and 100 bar outlet pressure. Analytes were detected at a wavelength of 220 nm. Chiral HPLC analyses were performed on a Hewlett Packard 1090 liquid chromatograph system (Wilmington, DE, USA).

Nuclear magnetic resonance (NMR) Spectroscopy was performed on a Bruker Avance 300 MHz spectrometer (Billerica, MA, USA) equipped with a 5 mm auto-switchable quad nucleus (QNP) probe at 25 °C. NMR data were collected in DMSO-d_6 and standard Bruker zg30 and noesyst NMR pulse programs were used for the ^{1}H and NOESY experiments, respectively. Chemical shifts are referenced to tetramethylsilane (TMS).

Optical rotations were measured with a JASCO P-1020 polarimeter (Easton, MD, USA).

2.3. Protection/deprotection of β-methylphenylalanine

The carboxy termini of the β-methylphenylalanine mixture were protected by conversion to methyl esters using standard methods (MeOH, cat HCl). Following esterification, the amino termini were also protected by conversion to *N*-benzylcarbamate (Cbz) derivatives (CbzCl, $NaHCO_3$, aq THF). Following purification of the individual isomers by preparative SFC, mild hydrolysis (1 M LiOH, aq THF) was used to deprotect the carboxy moieties while hydrogenolysis (HCO_2NH_4, 10% Pd/C, iPrOH, 75 °C, 1 h) was used to deprotect the amino termini, yielding the enantiomerically pure free amino acids.

2.4. Chiral stationary phases (CSPs)

The Daicel Chiralcel OD-H, Chiralcel OJ-H, Chiralpak AD-H and Chiralpak AS-H analytical columns (4.6 mm × 250 mm, 5 μm) and the preparative scale Chiralpak AD-H column (20 mm × 250 mm, 5 μm) were purchased from Chiral Technologies Inc. (Exton, PA, USA). A Phenomenex Chirex D-penicillamine phase 3126 (4.6 mm × 250 mm) ligand exchange column (Torrence, CA, USA) was used for the free amino acid chiral analyses.

2.5. Chromatographic mobile phase and sample preparation

Analytical and preparative SFC samples were dissolved in 100% MeOH and 50:50 MeOH/EtOH, respectively. For SFC, the mobile phase was comprised of carbon dioxide (80%), and various organic modifiers (20% MeOH, EtOH, CH_3CN, IPA, or mixed modifiers) were examined to adjust the mobile phase elution strength. The amino acids standards (Aldrich) were prepared as 1 mg/mL solutions in 50:50 H_2O/CH_3CN, and their subsequent chiral HPLC analyses (20 μL injection volume) were conducted on the Chirex 3126 column using 2 mM $CuSO_4$ in 85% H_2O/15% CH_3CN as the isocratic mobile phase.

3. Results and discussion

Naturally occurring peptides are highly flexible molecules in their native state but interact with their receptors in very precise conformations. As the number of characterized ligand–receptor complexes is very limited or nonexistent in some cases, the synthesis of different conformationally restricted peptide analogs

is an indirect strategy used to study the features of these complex bioactive forms [1–3]. Unnatural amino acids are often employed to affect these constraints. In particular, stereospecific β-methyl substitution is a widely used technique for constraining aromatic side-chains [4–6]. Additionally, it is most advantageous to examine only stereochemically pure substances, thereby precluding ambiguous physicochemical and biological data that may arise from the testing of enantiomeric/diastereomeric mixtures. Thus, to support our synthetic drug discovery efforts in peptide research, we set out to find a straightforward and efficient scheme for the large-scale purification of all four isomers of β-methylphenylalanine.

Chiral chromatography is an area where SFC has frequently demonstrated advantages over HPLC [16–18]. In an industry fueled by the need for chiral purity to validate biological and metabolic data, the higher efficiency, improved resolution and shorter separation times often observed in chiral SFC separations prompted us to explore SFC as a means for affecting this preparative purification.

3.1. Chiral SFC method development

Polysaccharide stationary phases based on cellulose or amylose have proven to be the most successful and widely used groups of CSPs for chiral HPLC and SFC separations, largely due to their separation efficacy and high loading capacity. Thus, an initial chiral screening of Cbz/methylester-derivatized β-methylphenylalanine was automated on a Berger analytical SFC using four different polysaccharide CSPs (amylose-derived ChiralPak AD and AS and cellulose-derived ChiralCel OD and OJ) coupled with four different organic modifiers. Derivatization of the mixture was performed using standard chemical methods to aid in both the elution and detection of the four stereoisomers.

As shown in Fig. 1, selectivity for this series of compounds varied considerably among the tested CSPs, with the greatest potential for preparative purification readily demonstrated on the ChiralPak AD-H column. Therefore, further separation studies were reserved for this CSP alone. In addition to the influence of

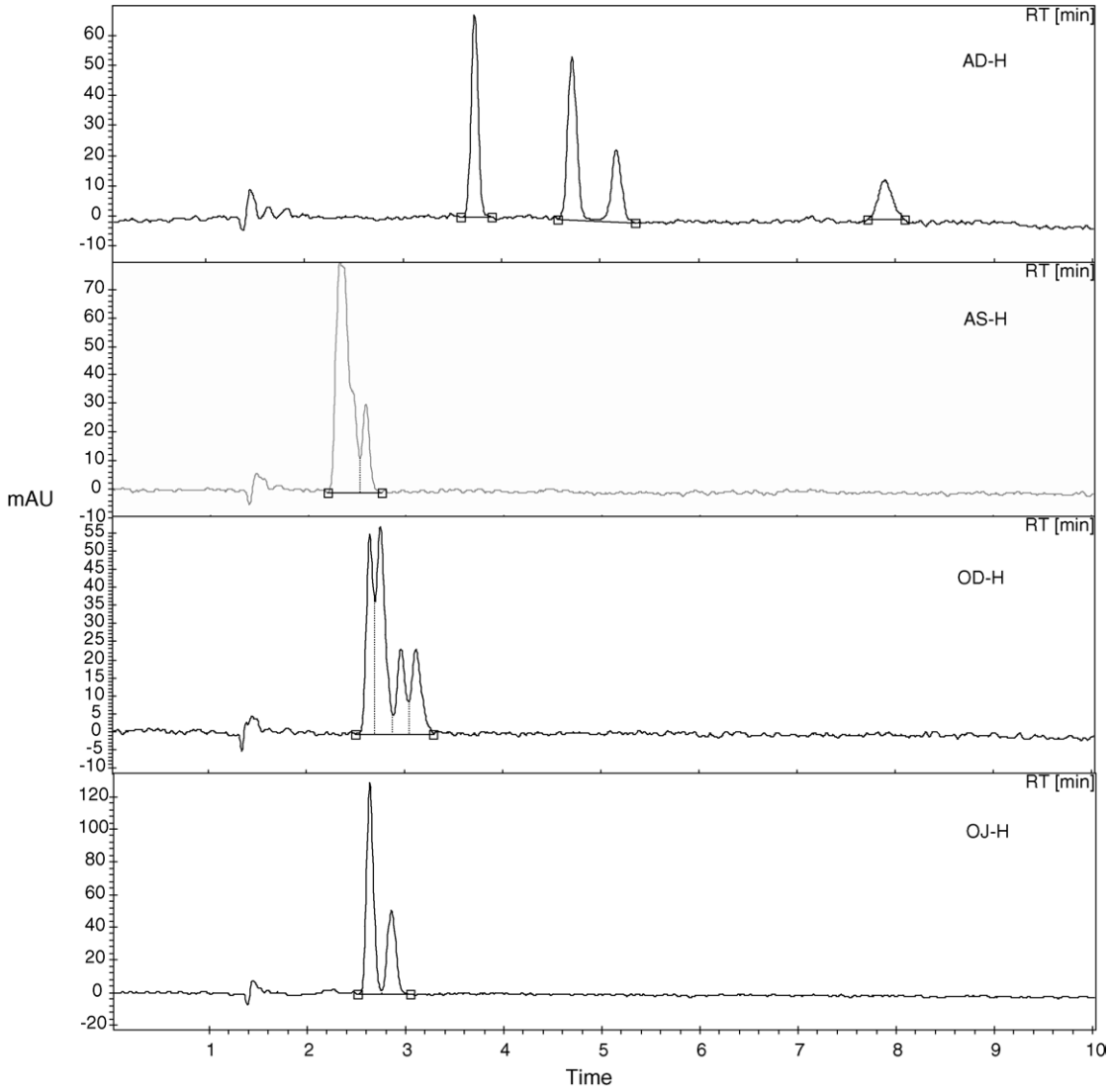

Fig. 1. SFC comparison of the β-methylphenylalanine *N*-benzylcarbamate methyl ester isomers on four enantioselective columns. Chromatographic conditions are as follows: 20% MeOH/80% CO_2 at 2 mL/min, 35 °C, 220 nm detection, 100 bar outlet pressure.

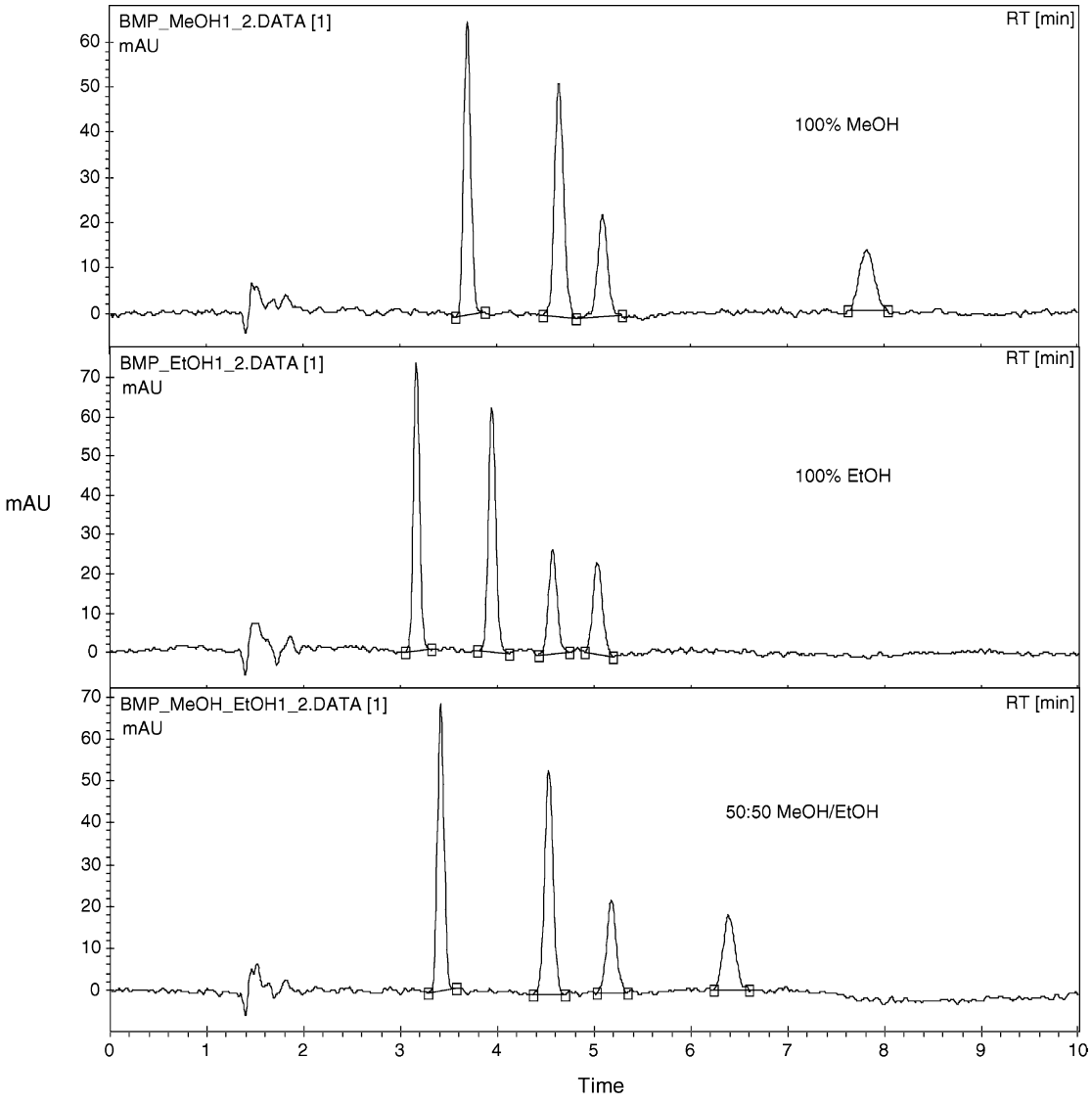

Fig. 2. SFC of the β-methylphenylalanine *N*-benzylcarbamate methyl ester isomers on Chiralpak AD-H using various organic modifiers. Conditions: 20% modifier/80% CO_2 at 2 mL/min, 35 °C, 220 nm detection, 100 bar outlet pressure.

the CSP, selectivity could be further adjusted by the choice of solvent modifier (Fig. 2). Specifically, alcohol modifiers have been shown to impart profound selectivity differences under HPLC conditions on the Chiralpak AD CSP [23]. This phenomenon also appears applicable to SFC. By employing 100% MeOH as the CO_2 modifier, good separation was observed between peaks 1 and 2 (α 1.41) while the separation of peaks 2 and 3 was insufficient for preparative scale purposes (α 1.14). Moreover, peak 4 was excessively retained on this CSP with 100% MeOH. Switching from MeOH to 100% EtOH produced two notable chromatographic changes, both an increased resolution between peaks 2 and 3 (α 1.24) and a dramatically decreased retention of peak 4 ($\alpha_{4,3}$ 1.15). Therefore, it seemed logical to exploit the positive attributes of the two solvents, and a 50:50 MeOH/EtOH solvent modifier was examined in turn. By combining modifiers, the desired separation of all four peaks ($\alpha_{2,1}$ 1.56, $\alpha_{3,2}$ 1.21, $\alpha_{4,3}$ 1.23) in a shortened run time (compared with MeOH alone) was achieved.

Having affected significant improvements in the elution and resolution of the four isomers through use of a mixed modifier, the influence of percent composition of this mixture was then examined. Interestingly, the MeOH/EtOH ratio had relatively little impact on separation of the chromatographic peaks, mainly affording slight increases in the separation between peaks 1 and 2, and slight decreases in the separation between peaks 3 and 4 with increasing percent EtOH. It was initially concluded from this study that a modifier composition of 40:60 MeOH/EtOH would provide the most efficient separation.

3.2. Preparative isolation of the four stereoisomers

While the analytical SFC results indicated that a mixed solvent modifier of 40:60 MeOH/EtOH would produce adequate peak resolution in minimal run times, peak broadening was observed when the method was extended to preparative scale SFC (Fig. 3) and slight modifications were

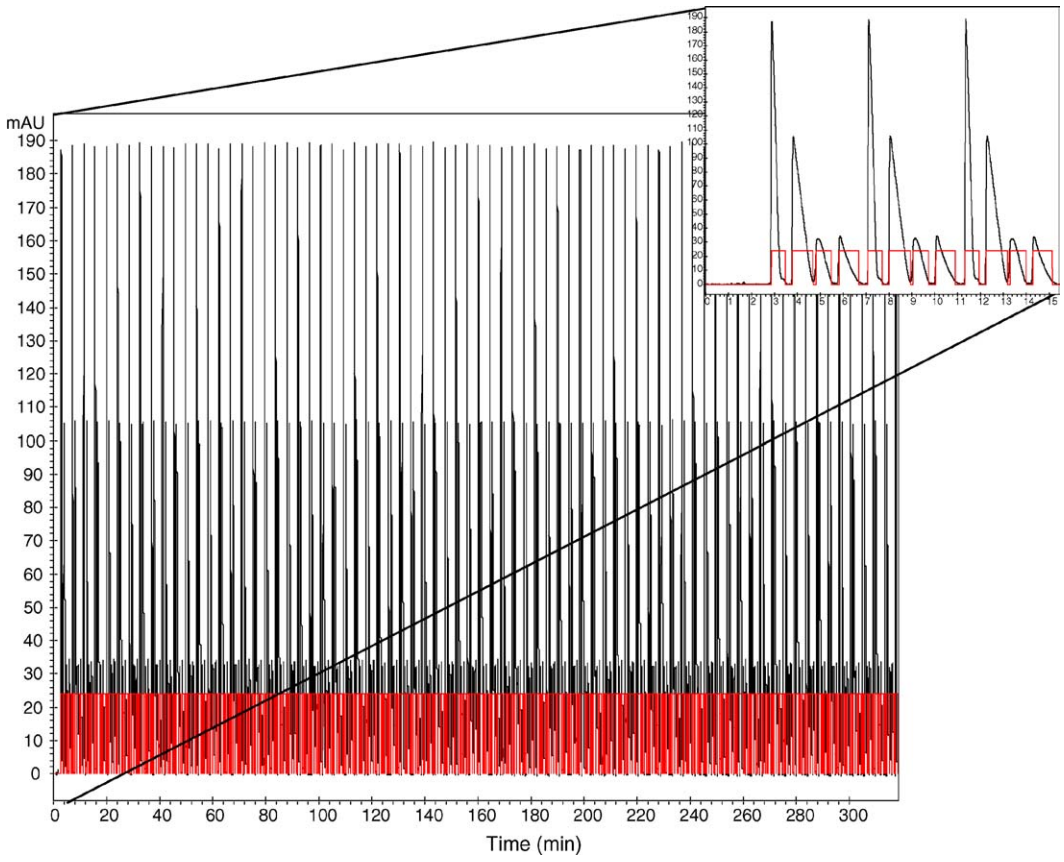

Fig. 3. Preparative chiral SFC separation of the β-methylphenylalanine derivatives using the ChiralPak AD-H (20 mm × 250 mm). Conditions: 15% modifier (50:50 MeOH/EtOH)/85% CO_2 at 50 mL/min, 35 °C, 220 nm detection, 100 bar outlet pressure. Approximately 39 mg of material (dissolved in 300 μL 1:1 MeOH/EtOH) was injected every 4.25 min, processing this 3.43 g batch of material in approximate 6.25 h.

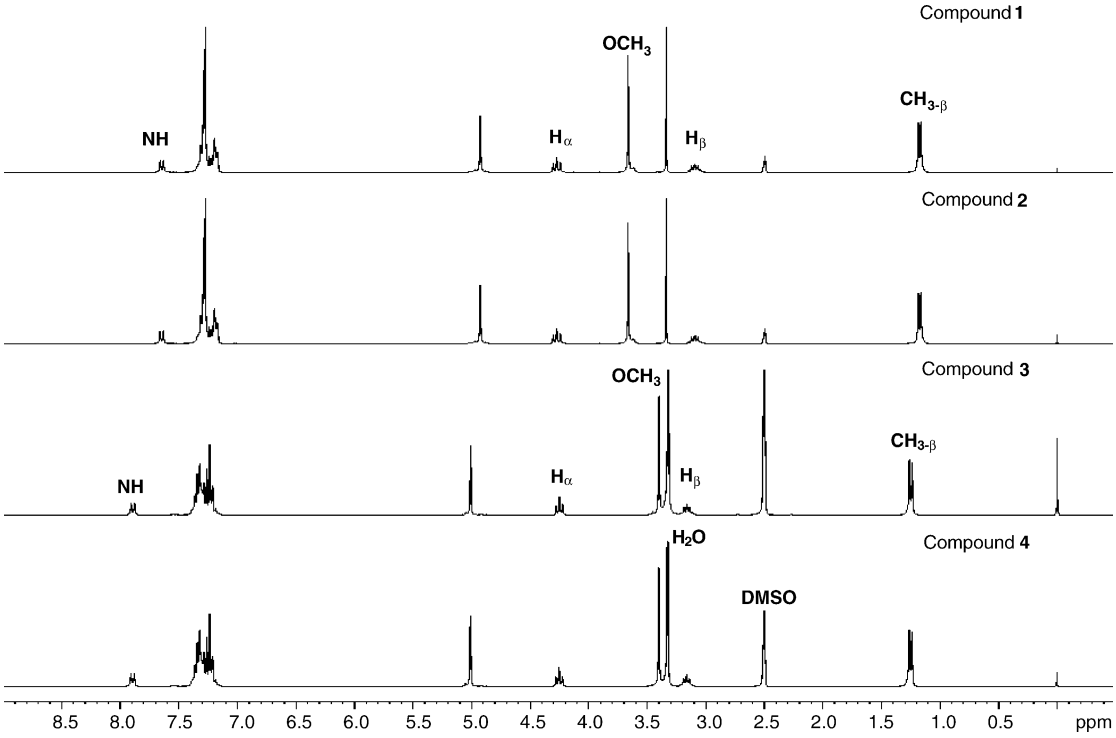

Fig. 4. 1D proton NMR spectral comparison of the four SFC purified compounds, demonstrating compounds **1** and **2** are enantiomeric pairs, as are compounds **3** and **4**.

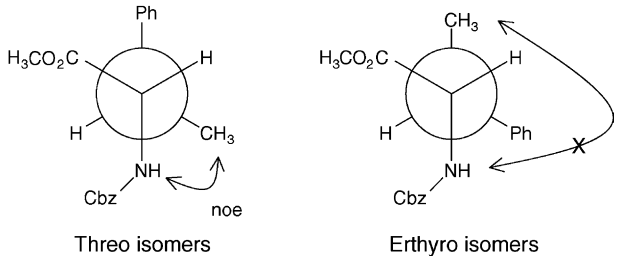

Fig. 5. Expected NOE correlations based on the predicted conformations of threo and erythro diastereomers. Proton coupling constant analyses supported an anti relation between alpha and beta protons for both compounds **1** and **3**, indicating the predicted conformations were correct ($^3J_{\alpha,\beta}$ (compound) **1** = 9.3 and $^3J_{\alpha,\beta}$ (compound **3**) = 8.6).

required for the preparative purification (15% modifier (50:50 MeOH/EtOH)/85% CO_2 at a total flow of 50 mL/min). Under these conditions, 39 mg of material could be injected at 255 s intervals using injection stacking. The four individual isomers were collected as methanol dissolved solutes into separate pressurized collection vessels using the four-peak detection/collection setting in the SFC software. This afforded purification of one particular 3.4 g batch of material in approximately 6.25 h with >90% total recovery. Subsequently, this method has been used in the chiral purification of over 10 g of derivatized β-methylphenylalanine.

Aliquots from the preparative collection vessels were removed and the individually isolated peaks were examined for chiral purity using the original analytical method (20% 50:50 MeOH/EtOH, 80% CO_2); each was found to be greater than 99.9% pure by diode array detection.

3.3. NMR analysis of the individual amino acids

Given the reported diastereomeric ratio of 67% threo/33% erythro and the observed relative areas of SFC peaks 1 and 2 compared to peaks 3 and 4 (3:1, respectively), it appeared that compounds **1** and **2** were likely threo (*R*,*S* or *S*,*R*) while **3** and **4** were erythro (*R*,*R* or *S*,*S*). To readily confirm the enantiomeric pairs, ^{1}H NMR spectra were collected on all four compounds. As shown in Fig. 4, 3 compounds **1** and **2** displayed identical proton spectra and were therefore enantiomers; likewise, compounds **3** and **4** were identical. Significant conformational distinctions between the diastereomeric pairs were also indicated by chemical shifts differences observed for several proton signals (NH and OCH_3 protons, for example).

Based on predicted conformations for the underivatized amino acids [24] (Fig. 5) and the spectral differences observed by 1D ^{1}H NMR, it appeared that 2D NOESY NMR experiments could be used to easily confirm if compounds **1** and **2** were the threo isomers and compounds **3** and **4**, the erythro pair. In the threo conformation, an NOE enhancement is expected to exist between the NH proton and the β-methyl protons. This correlation would not be observed for the erythro isomers. Hence, NOESY experiments were run on compounds **1** and **3**. While only one compound did show the predicted NOE cross-peak, the results were quite unexpected; the NOE correlation was observed in compound **3**, not compound **1** as anticipated (Fig. 6).

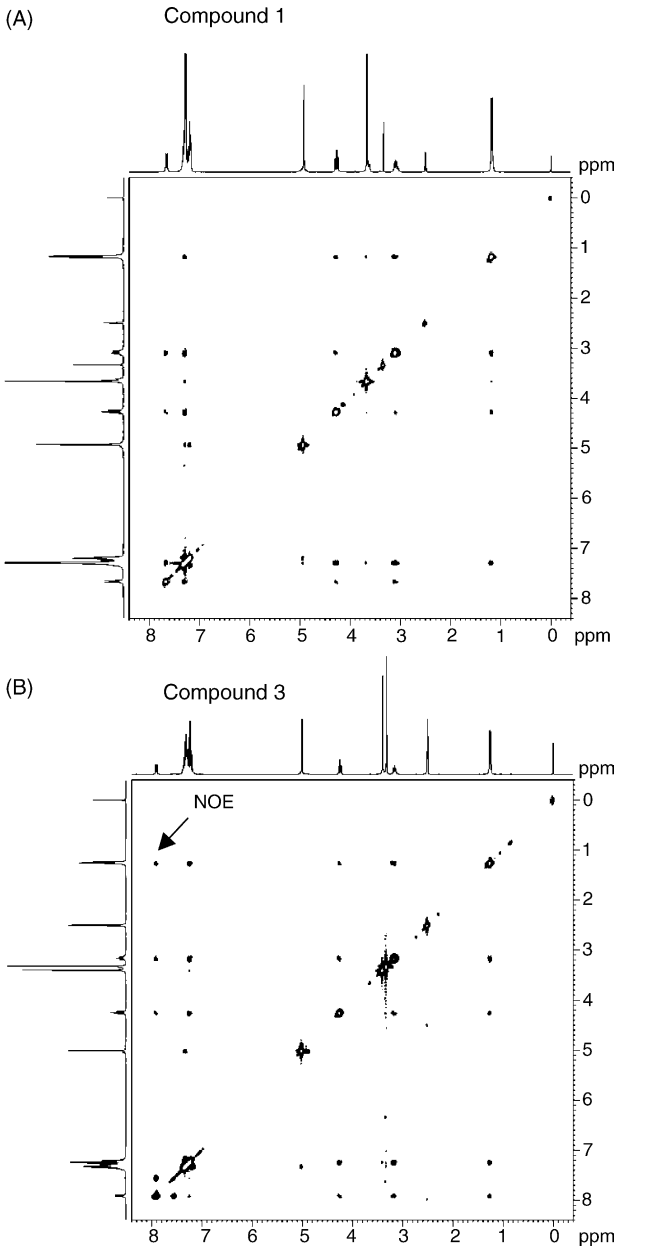

Fig. 6. NOESY spectral comparison of (A) compound **1** and (B) compound **3**. An NOE correlation is observed between the NH and β-methyl protons only in compound **3**.

3.4. Investigation of contradictory results: amino acid deprotection, chiral HPLC and optical rotation analyses

In order to absolutely confirm stereochemical configurations and to clarify conflicting results: (1) a portion of each purified compound was separately deprotected to yield its corresponding free amino acid, (2) a chiral analytical HPLC method was developed to separate the four isomers using the standard mixture (starting material) obtained from Aldrich, and (3) this

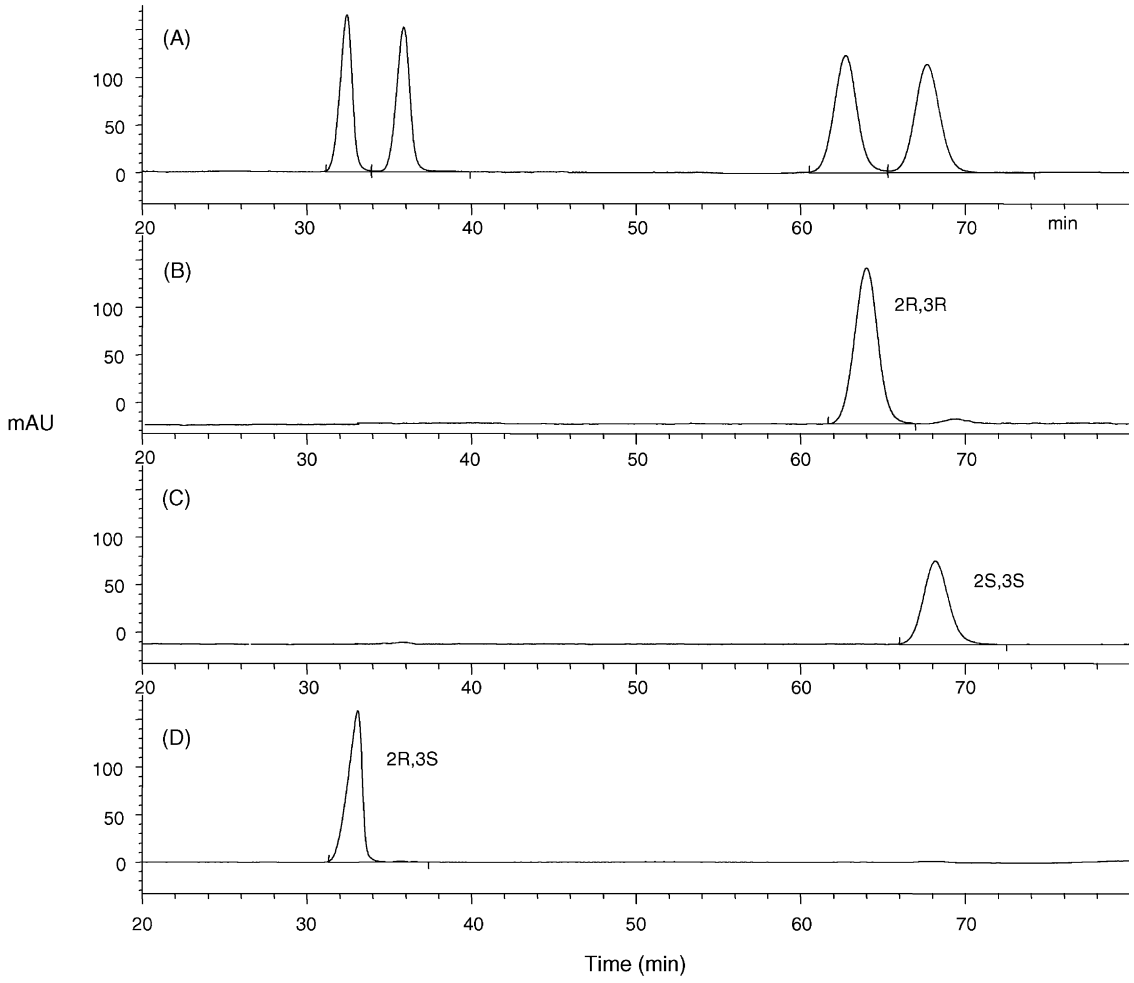

Fig. 7. Chiral HPLC analyses of (A) Aldrich amino acids standard, (B) deprotected compound **1**, (C) deprotected compound **2**, (D) deprotected compound **4** were performed on an Agilent 1090 using a Chirex phase 3126 (4.6 mm × 250 mm) column using 2 mM $CuSO_4$ in 85% H_2O/15% CH_3CN, 1.0 mL/min, λ = 210 nm, column oven 35 °C; 20 μL of the standard and 5 μL of each free amino acid were injected for analysis (solutions were prepared as a 1 mg/mL in 50:50 H_2O/CH_3CN).

method was used to determine the purity of the deprotected amino acids prior to obtaining optical rotation measurements.

A Chirex phase 3126 analytical HPLC column was employed to separate the four free amino acid isomers in the standard mixture (Fig. 7A). Deprotected compounds **1**, **2** and **4** were then analyzed using this same method to determine chiral purity (Fig. 7B, C and D, respectively) and each was found to be >98% pure. Optical rotation values were then measured and compared with those obtained from the literature [25].

The optical rotation of compound **1**, +28.3°, was found to be in excellent agreement with the literature value determined for 2*R*,3*R* erythro-D-β-methylphenylalanine ($[\alpha]_D^{23} = +28.4°$). Similarly, compound **2** ($[\alpha]_D^{22} = -24.6°$) was in accord with 2*S*,3*S* erythro-L-β-methylphenylalanine ($[\alpha]_D^{23} = -29.0°$), while compound **4** ($[\alpha]_D^{21} = +7.1°$) coincided with 2*R*,3*S* threo-D-β-methylphenylalanine ($[\alpha]_D^{23} = +7.3°$). These results confirmed that the NMR data were indeed correct; the first two peaks eluting from the preparative SFC were erythro isomers, in contradiction to our original expectations based on the 2:1 threo/erythro ratio reported for the starting material. A closer examination of the Aldrich material revealed the source of this inconsistency.

Three different lot numbers of β-methylphenylalanine were examined for threo/erythro content (each run in duplicate). Not only did the ratio between the different lots vary, but also more importantly, the ratio never approached the reported 67% threo/33% erythro (Fig. 8). Moreover, examination of five separate bottles from the same lot also displayed variations in their threo:erythro content. These discrepancies likely result from deficient manufacturing quality control.

3.5. Future directions

Further research into the facile SFC preparation of simplified β-methylphenylalanine derivatives is currently underway with promising preliminary results. For instance, the four isomers could not be resolved under our original separation conditions when only the N-termini were protected (Cbz-derivatized, no esterification). However, by simply exploring an alternative alcohol modifier such as butanol and methylpropanol, excellent resolution was once again readily achieved (Fig. 9).

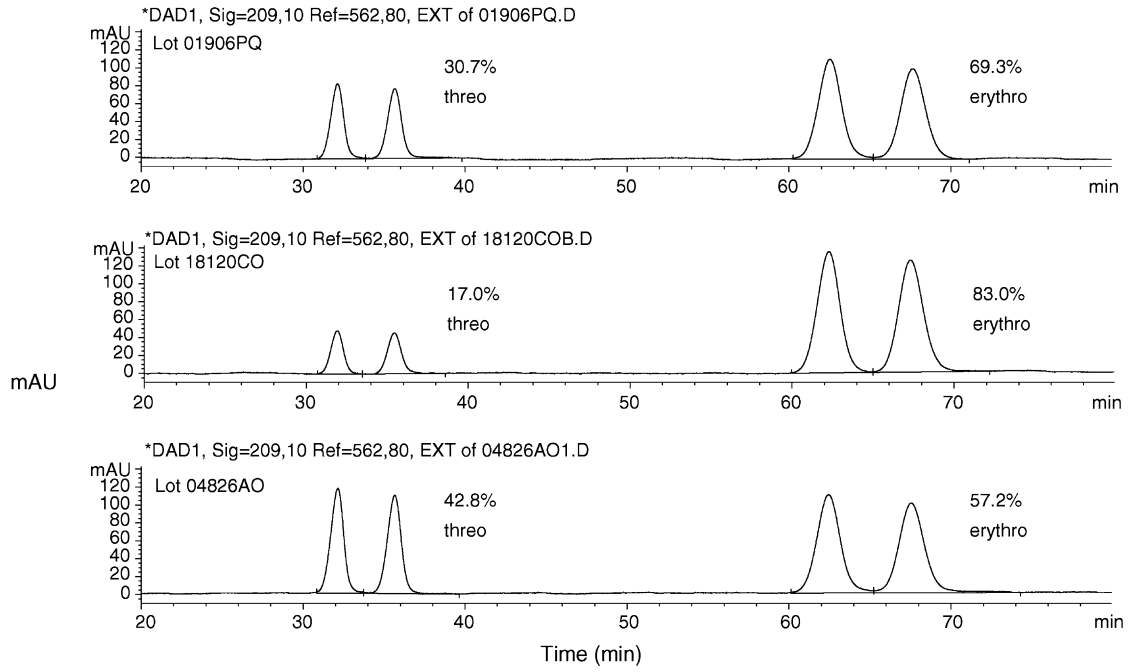

Fig. 8. Chiral HPLC analysis of three separate lots of commercial β-methylphenylalanine hydrochloride (see Fig. 7 for HPLC conditions).

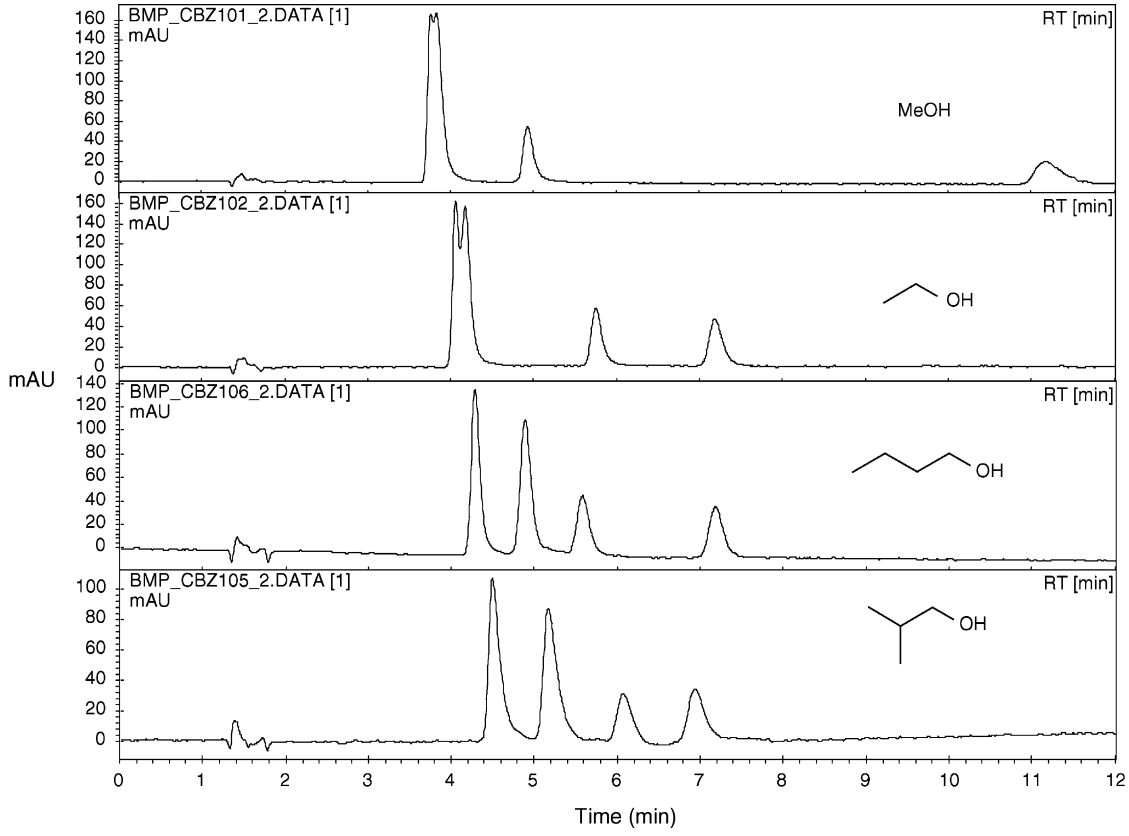

Fig. 9. Chiral SFC analysis of *N*-benzylcarbamate β-methylphenylalanine isomers using various alcohol modifiers on the ChiralPak AD-H (4.6 mm × 250 mm). Conditions: 20% modifier/80% CO_2 at 2 mL/min, 35 °C, 220 nm detection, 100 bar outlet pressure.

4. Conclusion

In conclusion, a preparative scale SFC separation was developed for the purification of the four stereoisomers of β-methylphenylalanine. The absolute stereochemical identity of the individual amino acids was determined through a combination of techniques including 1- and 2D NMR, chiral HPLC and optical rotation comparisons. Through use of this newly

developed method, over 10 g of material have been purified with greater than 90% total recovery. This derivatized material is now available in pure enantiomeric form (>99.9%) for use in the construction of new chemical entities for various drug discovery endeavors.

References

[1] J. Rizo, L.M. Gierasch, Annu. Rev. Biochem. 61 (1992) 387–418.
[2] P. Balaram, Curr. Opin. Struct. Biol. 2 (1992) 845–851.
[3] S. Hanessian, G. McNaughton-Smith, H.G. Lombart, W.D. Lubell, Tetrahedron 53 (1997) 12789–12854.
[4] V.J. Hruby, Acc. Chem. Res. 34 (2001) 389–397.
[5] R. Guerrini, G. Calo, R. Bigoni, D. Rizzi, A. Rizzi, M. Zucchini, K. Varani, E. Hashiba, D.G. Lambert, G. Toth, P.A. Borea, S. Salvadori, D. Regoli, J. Med. Chem. 44 (2001) 3956–3964.
[6] G.G. Bonner, P. Davis, D. Stropova, S. Edsall, H.I. Yamamura, F. Porreca, V.J. Hruby, J. Med. Chem. 43 (2000) 569–580.
[7] W.H. De Camp, J. Pharm. Biomed. Anal. 11 (1993) 1167–1172.
[8] H. Bruckner, M. Wachsmann, J. Chromatogr. A 998 (2003) 73–82.
[9] A. Peter, G. Torok, P. Cosmos, G. Bernath, F. Fulop, J. Chromatogr. A 761 (1997) 103–113.
[10] A. Peter, A. Arki, E. Vekes, D. Tourwe, L. Lazar, F. Fulop, D.W. Armstrong, J. Chromatogr. A 1031 (2004) 171–178.
[11] M.H. Hyun, Y.J. Cho, J.S. Jin, J. Sep. Sci. 25 (2002) 648–652.
[12] A. Peter, G. Torok, D.W. Armstrong, J. Chromatogr. A 793 (1998) 283–296.
[13] A. Peter, G. Torok, J. High Resol. Chromatogr. 23 (2000) 628–636.
[14] G. Torok, A. Peter, D.W. Armstrong, D. Tourwe, G. Toth, J. Sapi, Chirality 13 (2001) 648–656.
[15] M. Alias, M.P. Lopez, C. Cativiela, Tetrahedron 60 (2004) 885–891.
[16] K.L. Williams, L.C. Sander, S.A. Wise, J. Pharm. Biomed. Anal. 15 (1997) 1789–1799.
[17] J.L. Bernal, L. Toribio, M.J. del Nozal, E.M. Nieto, M.I. Montequi, J. Biochem. Biophys. Meth. 54 (2002) 245–254.
[18] M. Maftouh, C. Granier-Loyaux, E. Chavana, J. Marini, A. Pradines, Y.V. Heyden, C. Picard, J. Chromatogr. A 1088 (2005) 67–81.
[19] K. Anton, C. Berger (Eds.), Supercritical Fluid Chromatography with Packed Columns: Techniques and Applications, Marcel Dekker Publishing, New York, 1998, pp. 19–58, 223–249.
[20] K.W. Phinney, Anal. Chem. 72 (2000) 204A–211A.
[21] G. Terfloth, J. Chromatogr. A 906 (2001) 301–307.
[22] Y. Zhang, D. Wu, D.B. Wang-Iverson, A.A. Tymiak, Drug Discovery Today 10 (2005) 571–577.
[23] T. Wang, R.M. Wenslow Jr., J. Chromatogr. A 1015 (2003) 99–110.
[24] R. Dharanipragada, K. VanHulle, A. Bannister, S. Bear, L. Kennedy, V.J. Hruby, Tetrahedron 48 (1992) 4733–4748.
[25] Y. Kataoka, Y. Seto, M. Yamamoto, T. Yamada, S. Kuwata, H. Watanabe, Bull. Chem. Soc. Jpn. 49 (1976) 1081–1084.