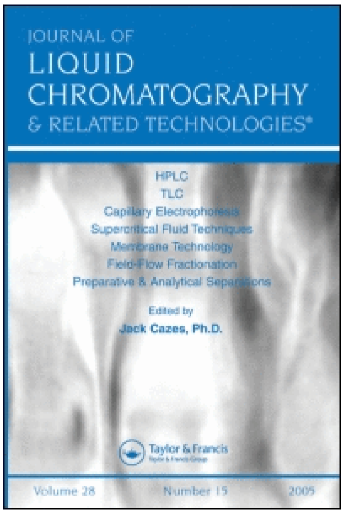

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation and HPLC Analysis of Diastereomers and Rotational Isomers of L-*N*-(Butyloxycarbonyl)-3-(3-Hydroxyethyl-4-(Benzyloxy)-Phenyl) Alanine Benzyl Ester

Donna Chamely-Wiik[a]; Charles E. Carraher Jr[a]; George Kamel[a]; Jerome E. Haky[a]
[a] Department of Chemistry and Biochemistry, Florida Atlantic University, Boca Raton, Florida, USA

To cite this Article Chamely-Wiik, Donna , Carraher Jr, Charles E. , Kamel, George and Haky, Jerome E.(2006) 'Separation and HPLC Analysis of Diastereomers and Rotational Isomers of L-*N*-(Butyloxycarbonyl)-3-(3-Hydroxyethyl-4-(Benzyloxy)-Phenyl) Alanine Benzyl Ester', Journal of Liquid Chromatography & Related Technologies, 29: 13, 1877 — 1890

To link to this Article: DOI: 10.1080/10826070600757680
URL: http://dx.doi.org/10.1080/10826070600757680

Journal of Liquid Chromatography & Related Technologies®, 29: 1877–1890, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070600757680

Separation and HPLC Analysis of Diastereomers and Rotational Isomers of L-*N*-(Butyloxycarbonyl)-3-(3-Hydroxyethyl-4-(Benzyloxy)-Phenyl) Alanine Benzyl Ester

Donna Chamely-Wiik, Charles E. Carraher, Jr., George Kamel, and Jerome E. Haky
Department of Chemistry and Biochemistry, Florida Atlantic University,
Boca Raton, Florida, USA

Abstract: We have synthesized intermediates towards the preparation of a dimeric derivative of L-dopa for the potential treatment of Parkinson's disease. We synthesized L-*N*-(butyloxycarbonyl)-3-(3-hydroxy-ethyl-4-(benzyloxy)-phenyl)alanine benzylester, a compound containing a secondary alcohol moiety that has a unique set of characteristics. Upon reduction of the precursor, which contained a ketone moiety, we synthesized a material that when formed, contained a pair of diastereomers of the secondary alcohol, each diastereomer also exhibiting two individually stable conformational isomers. We believe that the conformational isomers were generated by rotation of the C-N bond of the BOC carbamate, and were so stable that they could be separated by normal phase HPLC techniques.

Energy optimization studies and molecular modeling techniques were performed using HyperChem, and rotational barrier energy values were calculated for the different conformational isomers for each of the diastereomers. HPLC and NMR techniques were also used to obtain information about these materials. Using the calculated data from these studies, and analyzing the HPLC chromatograms and NMR spectra we were able to fully determine the assignments for the diastereomers and the individual conformational isomers. We discovered that the SS form was synthesized preferentially over the SR form and, that in both cases. The E conformation was energetically more stable than the Z form.

Keywords: Diastereomers, Rotational isomers, HPLC, Separation, Dimer, Levodopa

Address correspondence to Donna Chamely-Wiik, Department of Chemistry and Biochemistry, Florida Atlantic University, Boca Raton, FL 33432, USA. E-mail: dchamely@fau.edu

INTRODUCTION

Parkinson's disease (PD) is characterized by the progressive degeneration of pigmented dopaminergic neurons. These neurons are responsible for the synthesis and storage of dopamine, and when the neurons begin to decline, it leads to a decrease in the amounts of available dopamine. The decline in striatal dopamine synthesis and release is largely responsible for motor signs and symptoms of PD.[1] Many treatments associated with this disease include dopamine replacement therapy. However, direct treatment with dopamine, either orally or intravenously is not effective, since dopamine does not cross the blood-brain barrier (BBB).[2]

Levodopa (L-dopa), the metabolic precursor of dopamine does cross the barrier in small amounts (1%); as such, large doses are standard in treatment of this disease.[3] These high doses of L-dopa administered to patients, lead to a number of unwanted side effects and motor fluctuations. The main factors responsible for the problems associated with L-dopa as a drug include low water solubility, low lipid solubility, and high susceptibility of the drug to chemical and enzymatic degradation. Limitations associated with L-dopa including poor bioavailability, variations in plasma levels, and various side effects associated with treatment, have led to much research in the area of improving drug treatments.[4]

The purpose of our project was to address these issues by synthesizing a dimeric derivative of L-dopa, with improved pharmacological properties over L-dopa itself. We developed a synthetic strategy of a compound, which we believe, should be more soluble in water and lipids and allow the maintenance of L-dopa in a sustained release manner over a longer period, before being converted into dopamine. In the process of our synthetic strategy, we introduced a second chiral center into one of our intermediate compounds, and came across some interesting chemistry associated with this intermediate synthesized. We discovered a material that contained two diastereomers and rotational isomers of each diastereomer, compounds that were so stable that they were separable by HPLC and NMR techniques. In this paper, we report the methodology by which we were able to separate the diastereomers and analyze the rotational isomers by normal phase HPLC. We describe our attempts at completely assigning the chromatograms associated with these compounds, to their respective diastereomers and rotational isomers. These attempts were based on theoretical studies performed using HyperChem and calculation of rotational barrier energies.

EXPERIMENTAL

Reagents

All reagents were used without further purification. L-tyrosine (99%), anhydrous aluminum chloride, palladium on carbon (10%) (50% wet with water), sodium perborate tetrahydrate, and boron trifluoride etherate were purchased from Acros Chemical Co. Nitrobenzene, tetraethylammonium iodate (98%), benzyl bromide (98%), acetyl chloride, ammonia 2 M solution in methyl alcohol, di-*tert*-butyl dicarbonate (97%), and acetyl chloride were purchased from Aldrich Chemical Co. Silica gel 60, (particle size 0.015–0.040 mm) was purchased from EM Science. P-Toluenesulfonic acid and sodium borohydride, sodium thiosulfate, sodium bicarbonate, sodium chloride, sodium sulfite, potassium chloride, anhydrous potassium carbonate, anhydrous magnesium sulfate, anhydrous sodium sulfate, and solvents (HPLC grade) were purchased from Fisher Chemical Co.

Instrumentation

HPLC was performed using a 4.6 × 250 mm Altech Econosphere silica (5 μ) column using spherical packing on a Perkin Elmer LC 235 with a Perkin Elmer Series 410 LC BIO Pump, using Peak Simple software. The flow rate utilized was 1 mL min^{-1} and a column temperature of 25°C.

Medium performance liquid chromatography, MPLC was performed on a packed glass column using Silica gel 60 (particle size 0.015–0.040 mm) with a Milton Roy Constametric IIG flow meter and a LDC/Milton Roy Spectro-Monitor D variable wavelength detector, and charted with a Houston, Omni-Scribe chart recorder.

Mass spectrometry was performed using a ThermoFinnigan LCQ-quadrupole ion trap in electrospray ionization (ESI) mode. High resolution mass spectrometry analysis was carried out on a ThermoFinnigan MAT 95Q hybrid sector mass spectrometer. Chemical ionization (CI) was carried out at 150 eV using a direct insertion probe in the presence of methane.

^{1}H NMR and ^{31}P NMR spectra of the samples were obtained on a Varian Inova 500 MHz or 400 MHz spectrometer using 3 mm and 5 mm o.d. tubes, respectively. Sample concentrations were about 2% (w/v) in $CDCl_3$. Variable temperature studies were performed on the Varian Inova 500 MHz spectrometer using 3 mm tubes and a variable temperature probe.

Melting points were obtained using MelTemp II Laboratory device and Corning Pyrex Melting point tubes.

Infrared spectra were obtained using a Mattson Instruments, Galaxy Series 4020 FTIR spectrometer using films, neat, and potassium bromide KBr pellets. All spectra were recorded at an instrumental resolution of 4 cm^{-1} using 32 scans.

Synthesis

Synthesis was performed using L-tyrosine [1], as the starting material.

Synthesis of L-3-(3-Acetyl-4-Hydroxyphenyl)Alanine Hydrochloride [2]

The reaction was set up following the procedure indicated by Boger et al.[5] NMR and mass spectral results were consistent with the published data.[5]

Synthesis of N-(*tert*-Butyloxycarbonyl)-3-(3-Acetyl-4-Hydroxyphenyl)-L-Alanine [3]

The material was prepared according to the protocol by Chen et al.[6] The percent yield of the product, melting point, +ESI-MS and NMR were consistent with published data.[6]

Synthesis of N-(*tert*-Butyloxycarbonyl)-3-(3-Acetyl-4-Benzyloxyphenyl)-L-Alanine Benzyl Ester [4]

The material was prepared according to the protocol by Chen et al.[6] The +ESI-MS and NMR were consistent with published data.[6]

Synthesis of L-*N*-(Butyloxycarbonyl)-3-(3-Hydroxy-Ethyl-4-(Benzyloxy)-phenyl) Alanine Benzyl Ester [5]

The material was prepared according to the protocol by Boger et al.[5] To a solution of the ketone [4] (3.05 g, 5.87 mmol) in dry methanol (27 mL), sodium borohydride (0.3402 g, 5.87 mmol) was added at 10°C. The reaction was effervescent. The reaction mixture was allowed to stir at 25°C for 2 hours and monitored by TLC. The reaction mixture was then poured onto 5% HCl, and extracted with methylene dichloride (3 × 50 mL). The organic extracts were collected, washed with saturated aqueous sodium chloride, dried with magnesium sulfate, and concentrated in vacuo to afford a colorless oil [5]. FT-IR, mass spectral data and NMR data was consistent with the expected analyses of the structure (manuscript in preparation).

Synthesis of n-(*tert*-Butyloxycarbonyl)-3-(3-Hydroxy-4-Benzyloxyphenyl)-L-Alanine Benzyl Ester [6]

The following material was prepared according to the protocol of Kabalka et al.[7] +ESI-MS and NMR data were consistent with the structure.[7]

Synthesis of 3-(4-Benzyloxy-3-{[2-Benzyloxy-5-(2-Benzyloxycarbonyl-2-*tert*-Butoxy Carbonylamino-Ethyl)-Phenoxy]-Phenyl-Phosphinoyloxy}-Phenyl)-2-*tert*-Butyloxy Carbonyl Amino-Propionic Acid Benzyl Ester[8]

This material was synthesized with a modification of the Spivack's[8] procedure and Li et al.[9] DMAP (0.257 g, 0.002 mol) was dissolved in 10 mL of toluene. (1.01 g, 0.002 mol) of [6] was dissolved in 10 mL of toluene and the mixture was added to the DMAP solution. Over a 30 minute period, phenylphosphonic dichloride (0.112 mL 0.001 mol) in 25 mL of toluene was added to the mixture and the reaction was allowed to stir for 48 hours, while being monitored by TLC. After 48 hours of the reaction, water was added to the reaction mixture, and allowed to stir overnight. To the reaction mixture, 20 mL of water was added and the reaction was allowed to stir for 3 hours at room temperature. The resultant phases were separated and the organic phase was dried with anhydrous sodium sulfate. The material was concentrated in-vacuo and the residue was purified on silica to give a colorless oil [8]. The molecular weight, FT-IR and ^{31}P analyses were consistent with the structure of the material.

Purification

Products were purified using a glass chromatography column (24/40 outer joint, 500 mL reservoir, 1.5 inch (W) × 18 inch (L). The column was packed with Silica gel 60 (particle size 0.015–0.040 mm), filled with hexane, and the solvent was allowed to run through the solid support. The silica was packed under the force of gravity and assisted with the use of nitrogen pumped into the column. The sample was placed on the top of the column of silica gel support. A gradient mobile phase beginning with 10% ethyl acetate/hexane solution, moving up to 50% ethyl acetate/hexane solution, was used to purify and separate the products through the column. Nitrogen was pumped into the column to increase the rate at which the solvent infiltrated through the column, dramatically decreasing the time needed to purify the sample. The pure materials were collected separately as they emerged from the bottom of the column.

Molecular Modeling

Molecular modeling and calculation of energy values and dipole moments of optimized structures were performed using HyperChem Professional 7.5: Tools for Molecular Modeling, 2002 from Hypercube Inc. The force field chosen was the MM+ force field, a general purpose force field.

Optimization studies were run under two sets of conditions for the secondary alcohol, utilizing both the N-C=O structure as well as the corresponding resonance structures involving the N=C-OH moiety in the compound, to account for the partial double bond character that causes these conformational isomers. Dipole moments were calculated assuming free rotation around single bonds, and reflect an average value for the rotational isomers.

RESULTS AND DISCUSSION

The synthetic strategy for the production of the new compound is shown in Figure 1. Structure [5] is where we focus our attention on for this paper. Step

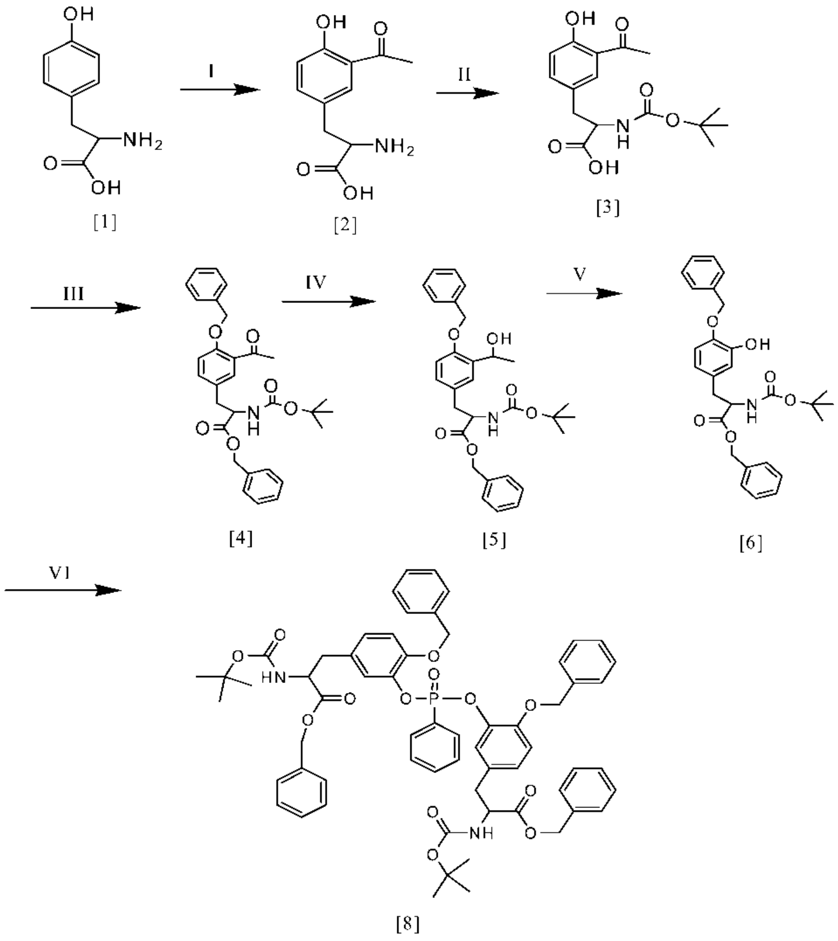

Figure 1. Synthetic strategy for production of a dimer of L-dopa.

IV involved the reduction of a ketone [4] to a secondary alcohol [5], a new compound using sodium borohydride according to the protocol by Boger et al.[5]

Once the experiment was completed, the impure material [5] was run through an HPLC system for analysis. The HPLC chromatographic analysis of the mixture showed four peaks, suggesting the existence of four species. Figure 2 shows the HPLC chromatogram of the secondary alcohol mixture [5].

The two different sets of mixtures were separated and purified and the products were collected and reanalyzed on HPLC for purity. The existence of the four products were attributed to the presence of two diastereomers of the secondary alcohol [5], as well as two conformational isomers that appeared to be stable enough to be separable by HPLC. Only two

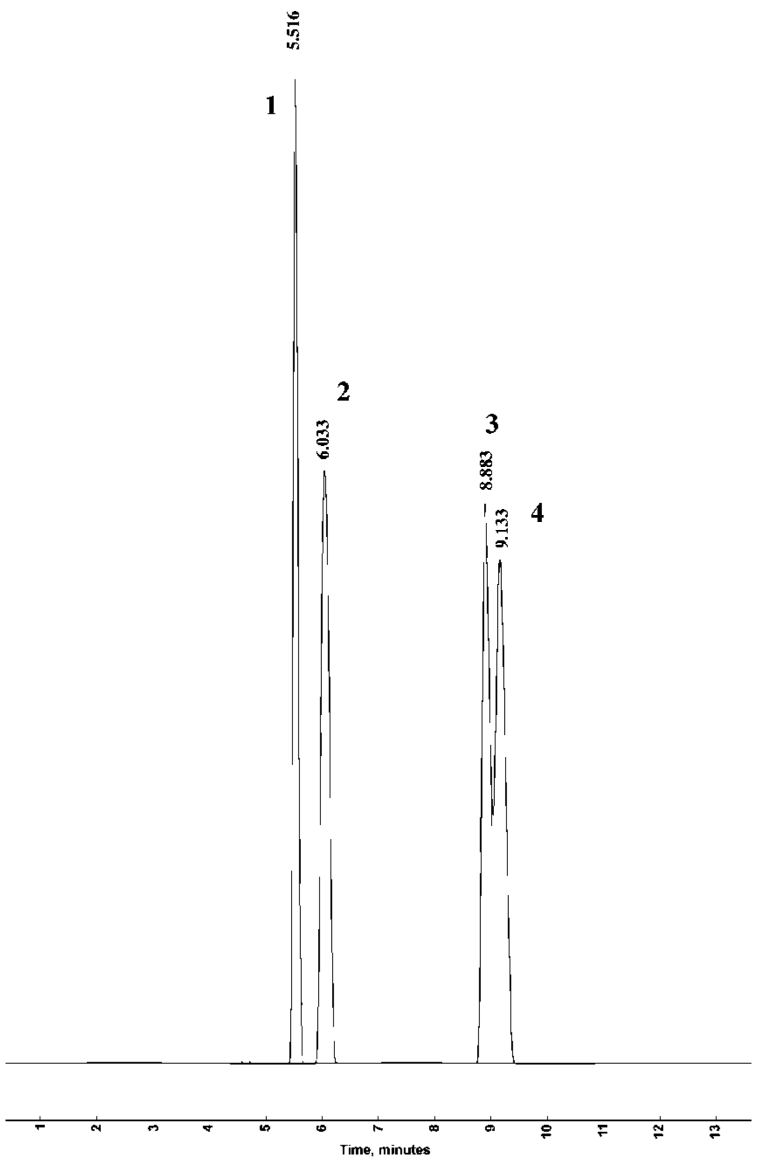

Figure 2. HPLC of L-*N*-(butyloxycarbonyl)-3-(3-hydroxy-ethyl-4-(benzyloxy)-phenyl) alanine benzyl ester [5].

diastereomers were formed once the reduction had been completed since L-tyrosine with a fixed stereocenter was used as the starting material.

The presence of two conformers could have been generated by rotation of the C-N bond of the BOC carbamate and appeared as separate compounds on the HPLC chromatograph. This is an unusual finding, as in many cases, when two conformations of the same structure are present in the material they usually appear as a broad peak on the chromatograph.[10,11] Previous published work has suggested that hindered rotation around the amide bond could lead to the formation of two conformational isomers, which are partially separable by HPLC at room temperature,[11] and can appear as diastereomers on the chromatographic time scale.[10] These findings indicate that even though the stereoisomers do interconvert by rotation about the C-N bond, the rate of interconversion is slow enough, allowing the separation of the isomers on the chromatographic time scale. Figure 3 shows the mechanism of formation of the two conformational isomers.

Utilizing the mechanism, we were able to derive four structures for our compounds, which can be seen in Figure 4.

Assignment of Individual Peaks of Diastereomers and Conformational Isomers

We were able to make complete assignments by 1. analysis of the stabilities of the products formed using HyperChem,[12] 2. determination of polarity of the products formed, and 3. analysis of the energies of the conformational isomers.

Analysis of the Stabilities of the Products Formed Using HyperChem

Geometric optimization studies were performed on the products of the reduction to the secondary alcohol [5]. From the optimized structures, energy calculations on both the SR and SS diastereomers were performed

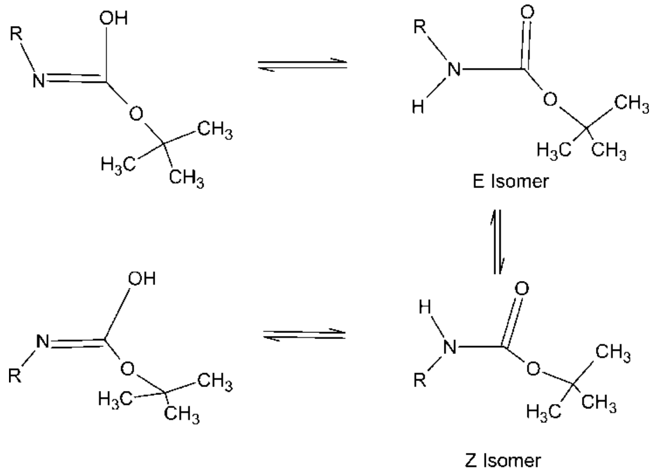

Figure 3. Illustration for the interconversion of the Z and E isomers.

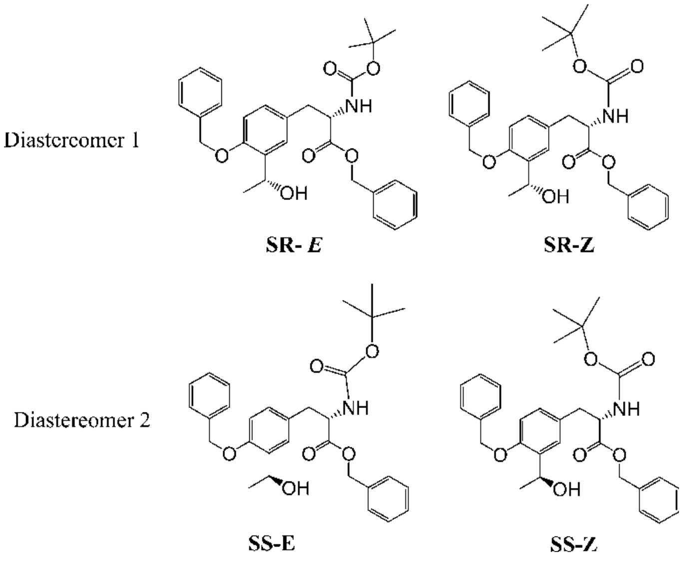

Figure 4. E and Z isomers formed by rotation about the carbamate bond.

utilizing HyperChem for the products of the secondary alcohol [5]. The energy values obtained are shown in Table 1.

The energy values that were obtained for the diastereomers where we utilized the N-C=O structure are lower than the values for each individual isomer where we utilized the full double bond N=C-OH structure. Since these conformational isomers are a result of a partial double bond, we believe the true energies probably lie between these two values. In either case, the lower energy values for the SS diastereomers indicate their greater stability over the SR diastereomers. We predict that the SS diastereomers would be the preferred conformation over the SR diastereomers.

Table 1. Energy values for optimized structures of isomers

Structure	Free energy, kcal mol^{-1} ($\pm 5 \times 10^{-4}$ kcal mol^{-1})
Ketone [4]	2.4348
SR diastereomers N-C=O	−7.8186
S diastereomers N-C=O	−9.0555
SR-Z	−4.8738
SR-E	−4.9184
SS-Z	−4.6308
SS-E	−4.9588

From the HPLC chromatograph, we determined the integration values of the two mixtures. Mixture I had a combined integration of 1.7 times the corresponding values for Mixture II, forming in excess of mixture II. When NMR analysis was performed the ratios of the NMR peaks also seemed to be fairly consistent with the ratios observed in the HPLC chromatogram. We can, therefore, assign the SS diastereomer to Mixture I corresponding to the first two peaks in the HPLC chromatogram and the SR diastereomers is then assigned to Mixture II.

The energy values also indicate that the E conformation of both diastereomers exhibit a lower free energy value than the corresponding Z isomer counterpart. We can infer that the E conformational isomer is the preferred conformation of the two and would be formed preferentially over the Z conformer. This is consistent with Deetz et al. assertion that carbamates and amides strongly prefer the E conformation.[13] The energy values for the SS diastereomers showed a greater energy difference between the two conformational isomer energy values, a difference of 0.328 kcal mol^{-1}, with the SS-E conformer being the preferred conformation. From this comparison, we can also infer the assignments of the SS-E compound to the first peak of the SS diastereomers and the SS-Z compound to the second, less predominant peak of the SS diastereomers. Comparing the energy values of the SR conformational isomers, we find that the SR-E value is slightly more energetically favored than the SR-Z isomer, with an energy difference of 0.045 kcal mol^{-1}. We can, therefore, assign the SR-E compound to the first peak of the SR diastereomers and the SR-Z compound to the second peak, noting that there is little thermodynamic favoring between these two isomers contributing to the almost even distribution of the conformational isomers.

Determination of Polarities of the Products Formed

In both the analytical and preparative normal phase HPLC chromatographs, which were used to separate the two diastereomers, one of the two diastereomers eluted from the column before the other diastereomer. Since normal phase HPLC works on partition/adsorption mode, the elution order indicates that the less polar substances should elute from the column before the more polar substances. Using HyperChem we were able to calculate the dipole moments of the two diastereomers SS and SR., determined by the molecular charge distribution. The dipole moment associated with the diastereomer can be seen in Table 2.

Table 2. Values of the dipole moments for the two diastereomers

Structure	Dipole moment
SR diastereomers	5.2628
SS diastereomers	3.2499

Table 3. Calculated values of X and defined parameters from variable temperature ^{1}H NMR study

Compound	ΔP	X	T_c(°K)	δv (Hertz)
1 (SS)	0.543	2.350	303.0	3.498
2 (SR)	0.045	1.587	303.0	4.998

These values are consistent with the assignments of the SS diastereomer to Mixture I, which eluted from the column before Mixture II assigned to the SR diastereomer, the more polar of the two diastereomers.

Analysis of the Energies of the Conformational Isomers

^{1}H NMR methods including a variable temperature study was performed on [5] and the coalescence temperature was used to calculate the barriers of rotation in the diastereomers. Since there is an unequal isomer population in the compounds we were studying, this was an indication that the barriers of rotation for the conversion of one isomer to the other would be different from the reverse processes in the compounds. We have calculated these barriers of rotation from the coalescence temperatures using equations developed by Shanan-Atidi et al.[14] T_c represents the coalescence temperature of the ^{1}H NMR signals arising from the two rotamers and δv represents the frequency difference in Hertz between the two NMR signals. ΔP represents the population difference between the two rotamers and X is a value determined by successive approximations.[14] Table 3 shows the results from determining the value of X for the two diastereomers labeled as compound 1 and 2 and defining all other parameters to solve for $\Delta G^{\neq}_{E/Z}$ and $\Delta G^{\neq}_{Z/E.}$. Once X was determined, we used the value to solve for $\Delta G^{\neq}_{E/Z}$ and $\Delta G^{\neq}_{Z/E}$ for both diastereomers.

The results for the rotational barrier energies to hindered rotation of the two diastereomers are given in Table 4.

These calculated rotational barriers (ΔG) are consistent with the calculated ΔG values of other carbamates (~16 kcal mol^{-1}).[14] The difference between the rotational barrier energies of the SS conformational isomers were significant, an indication that it would be harder for the SS-E

Table 4. Free energies of activation to hindered rotation of diastereomers

Compound	$\Delta G^{\neq}_{E/Z}$ kcal mol^{-1} ± 0.3 kcal mol^{-1}	$\Delta G^{\neq}_{Z/E}$ kcal mol^{-1} ± 0.3 kcal mol^{-1}	$\Delta G^{\neq}_{E/Z} - \Delta G^{\neq}_{Z/E}$ kcal mol^{-1} ± 0.3 kcal mol^{-1}
1 (SS)	17.27	16.54	073
2 (SR)	16.37	16.32	0.05

conformation to be converted to the SS-Z. This is consistent with the greater percentage of the E conformer formed in the reaction.

The calculated energy values determined from HyperChem, along with the calculation of dipole moments and the integration values from the HPLC chromatogram, enable us to fully assign the diastereomers and conformational isomers to each peak present on the HPLC chromatogram, and this complete assignment can be seen in Figure 5.

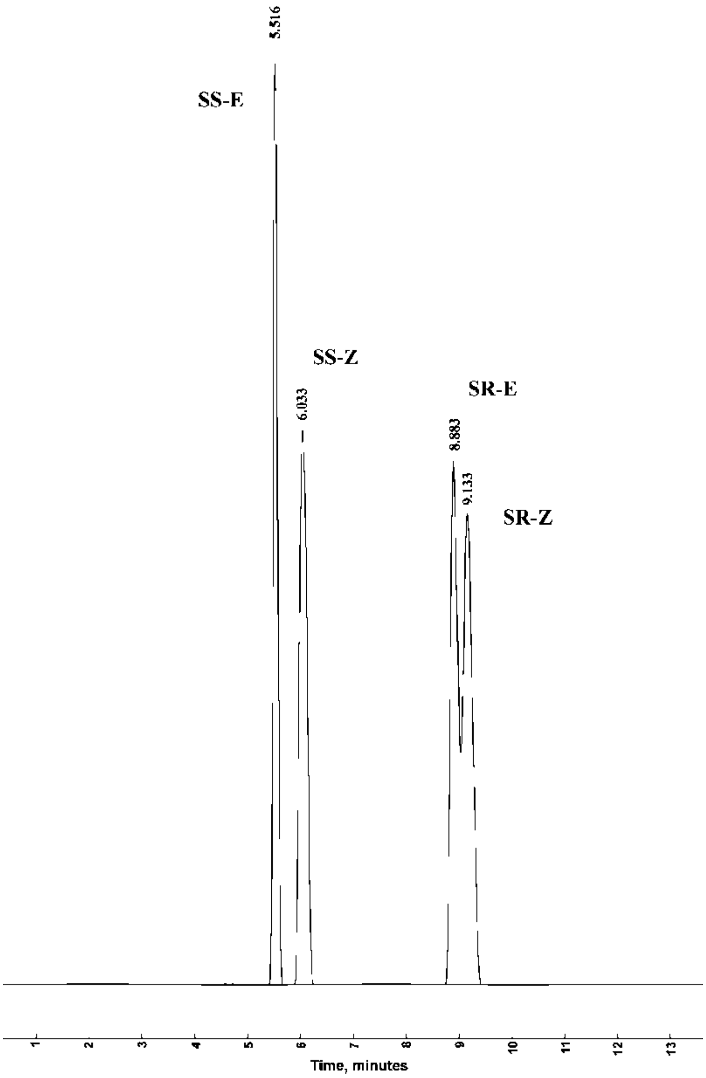

Figure 5. HPLC of [5] the secondary alcohol assigning the diastereomers and conformational isomers.

CONCLUSIONS

In the process of synthesizing a dimeric derivative of L-dopa, we came across an intermediate which appeared to have a unique set of characteristics. Upon analysis of the HPLC and NMR data, we discovered the presence of two diastereomers of the secondary alcohol, as well as two conformational isomers that appeared surprisingly stable. We believe that the conformational isomers were generated by rotation of the C-N bond of the BOC carbamate, and were so stable that they could be separated by HPLC and distinguished by NMR techniques.

We used HyperChem software to perform energy optimization studies, dipole moment calculations, and molecular modeling techniques. We calculated rotational barrier energy values for the different conformational isomers for each of the diastereomers. Using the calculated data from these studies, we were able to fully assign the spectra to determine the assignments for the diastereomers and the individual conformational isomers. We discovered the SS form was synthesized preferentially over the SR form and, in both cases, the E conformation was preferred over the Z form. This intermediate alcohol also proved to be the key step in the completion of the synthesis to form our target compound, a dimerized version of L-dopa containing a phosphonate moiety.

REFERENCES

1. Djaldetti, R.; Melamed, E. New therapies for Parkinson's disease. J. Neurol. **2001**, *248*, 357–362.
2. Juncos, J.L. Levodopa: Pharmacology, pharmacokinetics and pharmacodynamics. Neurol. Clin. **1992**, *2*, 487–509.
3. Jankovic, J. Levodopa strengths and weaknesses. Neurology **2002**, *58* (4), 1–24.
4. Djaldetti, R.; Melamed, E. New drugs in the future treatment of Parkinson's disease. J. Neurol. **2002**, *249*, 1130–1135.
5. Boger, D.L.; Yohannes, D. Selectively protected L-dopa derivatives: Application of the benzylic hydroperoxide rearrangement. J. Org. Chem. **1987**, *52*, 5283–5286.
6. Chen, C.; Zhu, Y.F.; Wilcoxen, K. An improved synthesis of selectively protected L-dopa derivatives from L-tyrosine. J. Org. Chem. **2000**, *65*, 2574–2576.
7. Kabalka, G.W.; Reddy, N.K.; Narayana, C. Sodium perborate: A convenient reagent for benzylic hydroperoxide rearrangement. Tett. Lett. **1993**, *34* (48), 7667–7668.
8. Pastor, S.D.; Spivack, J.D. Medium-sized heterocycles: Synthesis of a 2,10-bis(alkoxycarbonyl)-substituted 6-oxo-2Hdibenzo[d,g][1,3,2]dioxaphosphocin. J. Hetero. Chem. **1991**, *28*, 1561–1564.
9. Li, X.; Li, J.; Wang, Y.; Matsuura, T.; Meng, J. Synthesis and photochromic behaviors of novel bis-spiro-naphthooxazines connected through a phosphoryl group. Mol. Crys. Liq. Crys. **2000**, *344*, 295–300.
10. Cardoza, L.A.; Cutak, B.J.; Ketter, J.; Larive, C.K. High Performance liquid chromatographic-nuclear magnetic resonance investigation of the isomerization of alachor-ethanesulfonic acid. J. Liq. Chromatogr. A. **2004**, *1022*, 131–137.

11. Heberle, S.A.; Aga, D.S.; Hany, R.; Muller, S.R. Simultaneous quantification of acetanilide herbicides and their oxanilic and sulfonic acid metabolites in natural waters. Anal. Chem. **2000**, *72*, 840–845.
12. Teppen, B. HyperChem, release 2: Molecular modeling for the personal computer. J. Chem. Inf. Com. Sci. **1993**, *32* (6), 757–760.
13. Deetz, M.J.; Forbes, C.C.; Jonas, M.; Mlaerich, J.P.; Smith, B.D. Unusually low barrier to the carbamate C-N rotation. J. Org. Chem. **2002**, *67*, 3949–3952.
14. Shanan-Atidi, H.; Bar-Eli, K.H. A convenient method for obtaining free energies of activation by the coalescence temperature of an unequal doublet. J. Phys. Chem. **1970**, *74*, 961–963.

Received February 14, 2006
Accepted March 8, 2006
Manuscript 6831