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Factors Affecting the Rate of Racemization of Amino Acids and Their Significance to Geochronology[1a]

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Geological samples have been dated on the basis of the degree of racemization of the constituent amino acids by extrapolating rate data from simulated diagenetic studies. The validity of this extrapolation has been questioned, since it is known that, as well as the problem of estimating accurate diagnetic temperatures, factors such as pH and metal ions significantly affect rates of racemization. Results from this study show that further variables affecting the rate of racemization of free amino acids in aqueous solution are ionic strength, the buffer, and buffer concentration. Because of the possible variability in phosphate content in fossil bones, it is significant that racemization increased with increasing concentration of phosphate buffer. Furthermore, since geological matrices vary from sample to sample, and ionic strength may simulate to some extent such matrix changes, it is interesting that, although increasing ionic strength has no effect on racemization rate for a pH of 7.2, at pH 10.0 racemization increases with increasing ionic strength. These data for free amino acids in solution emphasize the necessity to use only the bound amino acid fraction for the dating of fossil specimens. Furthermore, the results suggest that a cautious approach be taken in applying amino acid racemization kinetics of even bound amino acids to geochronology and geothermometry. Concerning the basic mechanism of racemization, our results indicate that steric and proximity effects are equally as important as the inductive strength of the α substituent in determining the relative order of racemization of the amino acids.

Amino acids are ubiquitous and the L-enantiomers of the amino acids have become associated with the presence of life. In recent years there has been a renewed interest in the diagenesis of proteins and, in particular, in the diagenetic racemization of protein amino acids as applied to geochronology and geothermometry. A current investigation of these applications originated in the work of Hare and Abelson[1b] who found increasing proportions of D-amino acids, resulting from the racemization of the constituent L-amino acids, in a series of fossil shells of increasing age. Hare and Mitterer[2] then demonstrated that extrapolation of racemization rate data for L-isoleucine in heated Mercenaria shells agreed well with the data for a series of radiocarbon dated shells at ambient temperatures.

Application of the amino acid dating technique to other geological samples and environments has revealed inherent complications. For the case of deep-sea sediments, it has been shown that the racemization of L-isoleucine does not follow first-order reaction kinetics beyond a few 100 000 years.[3,4] In a study of fossil corals,[5] it was observed that the "D/L ratios in many of the samples investigated did not conform to the concept of increasing racemization being associated with increasing fossil age". Modern contamination was suggested as a problem factor.

The most successful application of amino acid racemization data has been in the dating of fossil bones. In particular, Bada has taken up this application demonstrating the linearity of the kinetics,[6] overcoming the uncertainty of a temperature fluctuation using a "calibration procedure"[7] and presenting evidence for the concordance of the aspartic acid dates so obtained, with known collagen-based radiocarbon dates.[8] Even this data has not been free of criticism, however. Hare has demonstrated the sensitivity of amino acid racemization in bone to water content and has pointed out discrepancies in Bada's results.[9] It was Hare's conclusion that "probably few, if any, of the published amino acid dates are reliable. Some are possibly off by an order of magnitude".[9] Despite such criticism, Bada and Helfman concluded that "because of the close correlation between temperatures calculated from in situ racemization rates (under diverse environmental conditions) and actual mean annual temperatures at various sites throughout the world . . . that factors other than temperature have very small effects on the reaction rate".[10]

In view of the potential problems associated with the technique, we have undertaken to examine more closely parameters which might affect the rate of racemization of amino acids during diagenesis. This involves a two-part study: firstly, with free amino acids in aqueous solution as the simplest system, and, secondly, a study of "protein-bound" amino acids. We here report on the first of these two objectives. We

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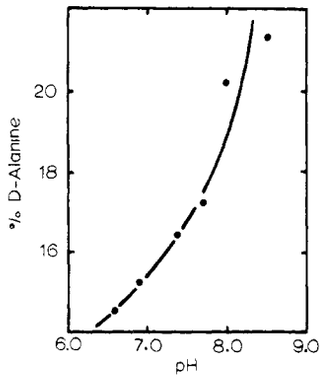

Figure 1. The effect of pH on the racemization of L-alanine at 125 °C, ionic strength 0.20, phosphate buffer 0.03 M.

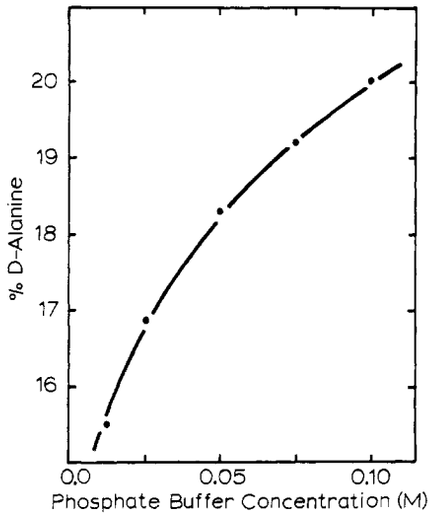

Figure 2. The effect of increasing phosphate buffer concentration on the racemization of L-alanine at 126 °C for 5 days, pH 7.6, ionic strength 0.50.

have attempted to relate our results to geochronology and to a better understanding of the mechanism of amino acid racemization.

Results and Discussion

Kinetics of Amino Acid Racemization. The general racemization reaction for amino acids may be expressed by

$$\text{L-amino acid} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{D-amino acid} \quad (1)$$

where k_1 and k_2 are the forward and reverse rate constants, respectively. The rate expression for this reaction is

$$\frac{-\mathrm{d[L]}}{\mathrm{d}t} = k_1[\mathrm{L}] - k_2[\mathrm{D}] \quad (2)$$

where [L] and [D] are the respective concentrations of the L and D enantiomers. Equation 2 may be integrated to give eq 3

$$\ln\left(\frac{1 + [\mathrm{D}]/[\mathrm{L}]}{1 - K'([\mathrm{D}]/[\mathrm{L}])}\right) = (1 + K')k_1 t + C \quad (3)$$

where $K' = 1/K = k_2/k_1$, and C is the integration constant.

In general, for free amino acids and aqueous solution, k_1 and k_2 and eq 3 reduces to

$$\ln\left(\frac{1 + [\mathrm{D}]/[\mathrm{L}]}{1 - [\mathrm{D}]/[\mathrm{L}]}\right) = 2kt + C \quad (4)$$

However, for L-isoleucine, which racemizes (or more correctly epimerizes) to its diastereomer D-alloisoleucine, $k_1 > k_2$ and thus $K > 1.00$. The importance of the value assigned the isoleucine equilibrium constant will be discussed later in this article.

Table I. Percent D-Alanine Obtained from Racemizing Alkaline Solutions of L-Alanine at Different Ionic Strengths[a]

Sample	% D-Ala			
	μ = 0.2	μ = 0.7	μ = 1.2	μ = 2.2
1	20.8	22.4	25.3	28.6
	20.7	22.3	25.3	28.5
2	21.0	23.2	25.2	28.6
	21.1	23.3	25.3	28.6
3	20.8	23.5	25.1	28.5
	20.8	23.3	25.1	28.5

[a] Conditions: After adjusting the ionic strength of a 0.024 M aqueous solution of L-alanine with sodium chloride, the pH was carefully brought to a pH of 10.0 using 0.05 M phosphate buffer solution. The samples were each heated at 148 °C for 18 h.

Buffer and pH. It has been claimed that in the pH range of 5 to 8 racemization is independent of pH.[11] However, we have found that in the range of 6.5 to 8.5 that there is an increase in racemization with increasing pH for phosphate-buffered alanine (Figure 1). This pH range is that which would most often be encountered during natural diagenetic racemization. Consequently, it has been suggested that by choosing fossil material with either a calcareous or an hydroxylapatite matrix the effects of pH might be minimized, since the matrix would act to buffer the fossil against pH fluctuations.[12] In view of the data illustration in Figure 1, this appears to be a sensible precaution.

Since the racemization reaction is believed to be general-base catalyzed,[26] a study was made on the effect of increasing buffer concentration on the rate of racemization of alanine at constant pH. As the concentration of the phosphate buffer was increased (at constant ionic strength), there was a corresponding increase in racemization of L-alanine (Figure 2). Thus, this data supports the general-base catalyzed mechanism. Furthermore, it lends credence to the possibility discussed by Schroeder and Bada[26] that "various anionic species of carbonate and phosphate may serve as nucleophiles in skeletal material".

At first we also thought that the carbonate–bicarbonate buffer system resulted in a substantial increase in racemization over other buffers. However, it was later realized that the pH was not constant in this study, which further illustrates the sensitivity of amino acid racemization to pH change. Bicarbonate solutions are very unstable and lose carbon dioxide to give carbonate with a resulting increase in pH. Because of the difficulties encountered in maintaining a constant pH, we discontinued further work with the carbonate buffer.

Unbuffered amino acid solutions also do not maintain a constant pH when heated presumably because of some decarboxylation of the amino acid at elevated temperatures. Solutions of leucine were titrated to a pH of 8.0 and 3.7 and then heated to ca. 118 °C for 6 days. The pH was measured again and found to have increased to 8.8 and 8.7, respectively. Solutions of alanine which were treated in the same way also increased in pH to ca. 8.7. An interesting observation, however, was that samples of nonbuffered alanine which were heated under identical conditions as the phosphate-buffered samples of Figure 1 underwent much less racemization. Figure 1 would indicate that at pH 8.7 alanine should racemize to give more than 22% D-alanine. Unbuffered samples were found to form only 13.7% of the D enantiomer. This result is in agreement with the trend evident in Figure 2 for phosphate buffer, and may be of interest in the application of amino acid racemiza-

Table II. Values Reported for the Isoleucine Epimerization Equilibrium Constant, K_{Ile}

K_{Ile}	Temp (°C)	Environment	Reference
1.30	~2	Early Miocene foraminifera	King and Hare[16]
1.40	~2	Miocene sediments	Wehmiller and Hare[3]
1.29	130	6 N HCl	Nakaparksin et al.[17]
1.30	140	Mercenaria shells	Hare[18]
1.25	*a*	Aqueous solution	Hare and Mitterer[2]
1.38	148	Modern bones	Bada[19]
1.28	150	Fossil bone	Dungworth et al.[14]
1.40	*a*	Alkaline solution	Hare and Mitterer[20]
1.40	126	Aqueous solution, pH 7.6, ionic strength 0.50	Smith et al., this paper

[a] Temperature not specified.

Table III. The Effect of K_{Ile} on the Half-Life (τ) Determinations for Isoleucine Racemization[a]

K_{Ile}	$k_1 \times 10^7$ (s^{-1})	τ^b
1.00	143.4	6.7
1.25	50.4	21.2
1.30	48.2	22.6
1.35	46.4	23.8
1.40	44.9	25.0

[a] T = 161.9 °C, pH 7.6, ionic strength 0.50. [b] A general expression for the half-life of amino acid racemization is $\tau = (\ln 2)/[(1 + K')k_1]$.

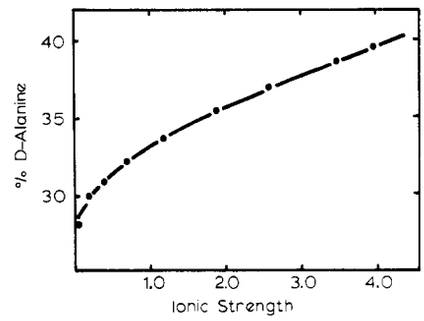

Figure 3. The effect of changes in ionic strength on the racemization of L-alanine at pH 10.7 (unbuffered), at ~130 °C for 3 days.

tion to geochronology. A modern dry bone is ca. 50% calcium phosphate.[13] Since the phosphate content of fossil bones may be changing with time, it might be predicted that the amino acids would initially racemize rapidly in the presence of the high phosphate concentration and then as the percent phosphate decreased so too would the rate of racemization. This expectation may be supported by a report which implies that the racemization rate of alanine in fossil bone is decreasing with time, the decrease being ca. 30% over the time period datable by radiocarbon.[14] (However, we have only established the effect of phosphate on the racemization of "free" alanine. Collagen-bound alanine may not be susceptible to the effects of phosphate. This study has yet to be carried out. For a discussion of the significance of "bound" and "free" amino acids, reference may be made to a recent review by Williams and Smith.)[15]

Ionic Strength. The effect of changes in ionic strength on racemization rates of alanine was studied to partially simulate matrix changes found in the geological environment. The effects were measured at pH 7.2, 10.0, and 10.7. At pH 7 (phosphate buffer) it was thought that the rate decreased with an increase in ionic strength. However, this proved to be a pH effect not an ionic-strength effect. The addition of sodium chloride also changed the pH. When the pH was carefully adjusted to pH 7.2 *after* the sodium chloride was added, no change in the rate was observed with a change in ionic strength. However, at pH 10 and 10.7, the ionic strength altered the racemization rate. At pH 10 (phosphate buffered) an increase in the ionic strength appreciably increased the racemization rate (Table I). Also, at pH 10.7 the same relative result was observed with a greater amount of racemization occurring at the higher pH (Figure 3). These effects are predicted from theory which states that reactions between like-charged ions [in this case $RCH(NH_2)COO^-$ and B^-] proceed more rapidly as the ionic strength increases.

The Effect of K on Racemization Rate Data for Isoleucine. It has already been mentioned that, in general, $k_1 = k_2$ and, consequently, $K = 1.0$, but that for the racemization of L-isoleucine to D-alloisoleucine k_1 is greater than k_2 and, hence, $K > 1.0$. Table II lists values for K_{Ile} which have appeared in the literature and the conditions under which they were determined. In other reports using the kinetics of isoleucine racemization, the value of K_{Ile} has been arbitrarily assigned.[5] We wondered if a significant error has been introduced into reported rate data by assuming an incorrect figure for K_{Ile}. The effect of K_{Ile} on the half-life of the isoleucine epimerization at an elevated temperature (161.9 °C) is demonstrated in Table III. Extrapolation of our Arrhenius data to 25 °C gives a half-life of 9000 or 8200 years, depending on whether K_{Ile} is assigned a value of 1.30 or 1.40, respectively. With the scatter in values reported for K_{Ile} (Table II), it is easily evident that isoleucine-derived age estimates have a minimum uncertainty of ca. 10% even if all other parameters are accurately known. The racemization of isoleucine has been used extensively for dating shells and sediments because it is relatively stable against diagenetic degradation,[27,28] has a suitable half-life, and because L-isoleucine may be resolved from D-alloisoleucine by conventional ion-exchange chromatographic procedures. We wish to emphasize the need for an accurate evaluation of K_{Ile} for each system under study to avoid unnecessary errors in age estimations. There is also a need to determine whether K_{Ile} is temperature dependent. We are currently investigating this possibility.

Arrhenius Parameters: Mechanism of Amino Acid Racemization. Amino acid racemization proceeds via base abstraction of the α-proton leading to formation of a planar carbanion, which may be resonance stabilized.[21] This mechanism is supported by the results of deuterium-exchange studies. It has been shown that the rates of racemization and of deuterium-exchange at the α-carbon parallel each other.[22,23]

The observed order of amino acid racemization at elevated temperatures is Asp > Glu > Phe > Ala > Leu > Ile > Val. The electron-withdrawing capacity of the R substituent has been invoked as the principle factor in determining this order by several authors.[6,17] Sato et al.[24] concluded from their base-catalysis studies that the relative susceptibility of a series

Table IV. Reported Arrhenius Parameters for the Racemization of Free Amino Acids in Aqueous Solution

Amino acid	Registry no.	Dungworth et al.[a] (pH 7.0) E_a(kcal/mol)	Dungworth et al.[a] (pH 7.0) A (s^{-1}) × 10^{-9}	Bada[b] (pH 7.6) E_a (kcal/mol)	Bada[b] (pH 7.6) A (s^{-1}) × 10^{-10}	Smith et al.[c] (pH 7.6) E_a (kcal/mol)	Smith et al.[c] (pH 7.6) A (s^{-1}) × 10^{-8}	Nakaparksin et al.[d] (6 N HCl)[b] E_a (kcal/mol)
Asp	6899-03-2			31.0	17.03			
Ala	6898-94-8	29.4	3.15	30.9	4.09	28.5	20.72	25
Val	7004-03-7	29.1	0.69			28.6	8.36	31
Leu	7005-03-0	29.3	2.03			27.7	5.85	25
Ile	7004-09-3	28.9	1.19	31.4	3.03	27.9[c]	4.57	
Phe	3617-44-5			28.6	0.356	24.0	0.173	
α-Ab[d,g]	1492-24-6							25

[a] Reference 16. [b] Reference 11. [c] This paper. [d] Reference 17. [e] A values not reported. [f] For K = 1.40 at all temperatures. [g] α-amino-n-butyric acid.

Table V. The Eyring Parameters for the Racemization of Free Amino Acids in Aqueous Solution

Amino acid	$\Delta H^\dagger$ (kcal/mol)	$\Delta S^\dagger$ (esu)
Phe	23.0 ± 0.3	−28.4 ± 0.8
Ala	28.6 ± 0.4	−16.3 ± 1.0
Leu	27.5 ± 0.3	−19.8 ± 0.8
Ile[a]	27.1 ± 0.6	−21.6 ± 1.4
Val	28.0 ± 0.4	−19.8 ± 1.0

[a] For K_{Ile} = 1.40 at all temperatures.

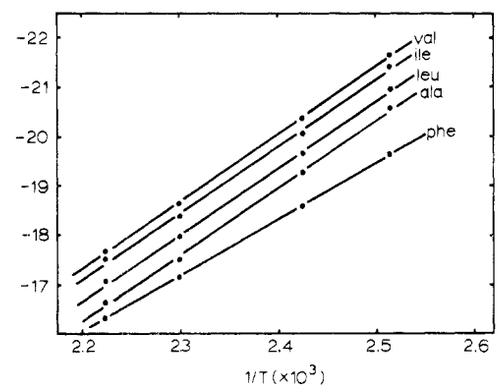

Figure 4. Arrhenius plots for the racemization of valine, isoleucine, leucine, alanine, and phenylalanine, at pH 7.6, ionic strength 0.50 in phosphate-buffered solution (0.05 M).

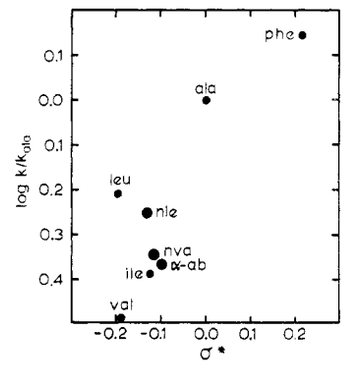

Figure 5. The relationship between the inductive strength (σ^*) of the amino acid side chain and the relative rate constants for the racemization of phenylalanine, leucine, isoleucine, alanine, valine, norvaline, norleucine, and α-amino-n-butyric acid at 161.9 °C, pH 7.6, ionic strength 0.50, and phosphate buffer 0.05 M. The data for norvaline, norleucine, and α-ab are less accurate as indicated.

of N-benzoyl amino acid anilides to racemization "is proportional to the electronegativity of the α-substituent unless the α-substituent exerts a steric effect". A linear result was obtained when log (k/k_{Ala}) was plotted against σ^* for the derivatives of phenylglycine.

The differences in rates of racemization for each amino acid may be attributed to differences in $\Delta H^\dagger$ and/or $\Delta S^\dagger$. If inductive or resonance effects determine the rate, they will be reflected in a change in $\Delta H^\dagger$; if steric considerations are important, they will alter either $\Delta H^\dagger$ or $\Delta S^\dagger$; field effects will change $\Delta H^\dagger$. Finally, any differences in the ordering of the solvent during racemization will effect the relative rates via an entropy contribution.

In order to determine if the electron-withdrawing capacity of the α-substituent accounts for the observed order of amino acid racemization, accurate activation parameters are required. (It should also be emphasized that accurate Arrhenius parameters are essential for amino acid dating, since a 1% error in the activation energy will give a corresponding 20% error in the calculated age.) Since previously reported Arrhenius parameters did not agree well[11,12] (Table IV), we determined activation parameters under carefully controlled conditions of temperature, ionic strength, pH, and buffer concentration. Furthermore, baseline resolution was obtained between the D- and L-enantiomeric peaks for all amino acids studied. Consequently, we believe this data to be reliable. Our results (Figure 4, Tables IV and V) indicate that the order of amino acid racemization cannot be accounted for simply by inductive effects.

For phenylalanine $\Delta H^\dagger$ is the determining factor in the racemization rate overcoming the large negative entropy. In this case, the electron-withdrawing strength of the benzyl group apparently accounts for the high comparative rate constant. However, for the other amino acids this is not the case. For leucine, isoleucine, and valine (whose σ^* values are very similar), both the $\Delta H^\dagger$ and $\Delta S^\dagger$ values are the same within experimental error, and it is by no means clear what determines the rate. For alanine, which appears to have a slightly higher $\Delta H^\dagger$, the rate must be determined by its comparatively lower $\Delta S^\dagger$, since alanine actually racemizes more rapidly than leucine, isoleucine, and valine. Although phenylalanine shows the fastest racemization rate of the amino acids studied, its rate is not as fast as it would be expected from the value of its $\Delta H^\dagger$ alone. The bulk of the $PhCH_2^-$ group apparently alters the $\Delta S^\dagger$ value. It was found to be the most negative of all the amino acids studied (Table V).

Although the reason for the observed order of racemization is not completely clear, it is apparent that to account for this order on the sole basis of the inductive strength of R is unrealistic. A plot of log (k/k_{Ala}) against σ^* for six amino acids (161.9 °C; Figure 5) shows no correlation, further emphasizing this point. (Recently published σ^* values have again been questioned. Therefore, plots of σ^* may not necessarily be significant.[29]) Racemization rates of the amino acids are de-

termined by a complex combination of inductive, resonance, steric, proximity, and solvent effects.

Conclusions

It is clear from this study of factors affecting the rate of racemization of free amino acids in aqueous solution that diagenetic racemization involves a complex interaction of many variables.

We have identified ionic strength, pH, buffer, and buffer concentration as factors which influence racemization rates. These data help explain why, for example, linear first-order kinetics are only observed over short ranges for total sediment hydrolysates. The results for free amino acids also show why it is essential to examine only the racemization of the bound amino acid fraction of fossil specimens. Furthermore, although bound amino acids should be less susceptible to variation of some of these parameters, the results suggest that a cautious approach be taken in applying amino acid racemization kinetics of even bound amino acids to geochronology and geothermometry.

This study supports the contention that the order of racemization of amino acids is not only determined by the inductive strength of the α-substituent, but also by steric, proximity, and solvent effects.

Of importance to the use of isoleucine for amino acid dating is the value assigned to K_{Ile}. This should be accurately defined for each system studied to avoid unnecessary errors in fossil age estimations. There is a need for an investigation of whether or not there is any temperature dependence of K_{Ile} for protein-bound isoleucine.

Experimental Section

Temperature Control. Temperatures of the samples were controlled to ±0.2 °C by immersion in thermostatically controlled baths of heavy-duty motor oil. The baths were regulated via proportional temperature controllers (RFL Industries, Inc., Model 70-115).

Sample Preparation. The amino acid solutions were prepared such that the final buffer concentration was 0.05 M and the ionic strength was brought to 0.50 using sodium chloride solution (5.00 M). The pH was adjusted by the addition of an appropriate volume of sodium hydroxide or hydrochloric acid solution. Aliquots (1.00 mL) of the amino acid solutions were sealed in glass tubing and heated in the oil bath for the required times. The tubes were cooled and opened, and the water was evaporated under a nitrogen stream at 80–90 °C. The last traces of moisture were removed by azeotroping with dichloromethane and by then placing the tubes in a vacuum oven (30 min, 80 °C) or by keeping them over anhydrous calcium chloride in a vacuum dessicator overnight.

Sample Derivatization. *N*-Trifluoroacetylamino Acid Isopropyl Esters. To each dried amino acid residue was added 2-propanol/HCl (4 N; 600 μL). The tubes were sealed, ultrasonicated (70 °C, 20 min) and heated in an oil bath (120 min, 100 °C). The 2-propanol was removed under a stream of nitrogen, dichloromethane was added, and the solvent was evaporated again. Derivatization was completed by the addition of trifluoroacetic anhydride (200 μL) and dichloromethane (200 μL). After 2 h at room temperature, the reagent was removed as previously, and the residue was taken up in dichloromethane (0.5 mL) and transferred to the GLC vials.

Gas Chromatography. GLC analyses were run under appropriate isothermal conditions using stainless-steel capillary columns (150 ft × 0.02 in.), coated with the optically active liquid-phase *N*-lauroyl-L-valyl-*tert*-butylamide.[25] In all cases, baseline resolution was obtained for the D- and L-enantiomeric amino acid peaks. The gas chromatograph was an HP 5830A with an HP automatic injector and electronic integration.

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