108824-13-1; 1·2Cl⁻/sodium 2-naphthylenesulfonate, 114274-59-8; 1·2Cl⁻/trimethyl(1-naphthyl)ammonium fluorosulfonate, 114274-60-1; 2·2I⁻, 114274-52-1; 2·2Cl⁻, 114274-35-0; 2·2Cl⁻/25, 114274-57-6; 4·2Cl⁻, 114274-36-1; 4·2I⁻, 114274-55-4; 5, 99407-79-1; 6, 108787-21-9; 7a, 108787-22-0; 7b, 114274-48-5; 8a, 108787-23-1; 8b, 114274-49-6; 9a, 108787-24-2; 9b, 114274-50-9; 10a, 108787-25-3; 10b, 114274-51-0; 11, 114274-37-2; 12, 114274-38-3; 13, 114274-39-4; 14, 114299-60-4; 15, 108787-26-4; 16, 114274-40-7; 17, 114274-41-8; 19a, 108787-27-5; 19b, 114274-53-2; 20, 105400-47-3; 21, 114274-43-0; 22, 114274-44-1; 23, 114274-54-3; 23·2HBr, 114299-61-5; 24, 114274-45-2; 25, 605-62-9; 26, 6549-14-0; 27, 1523-11-1; 28, 26177-06-0; 29, 114274-46-3; 30, 114274-47-4; α-chymotrypsin, 9004-07-3; 1,4-dichlorobutane, 110-56-5; 1,6-dichlorohexane, 2163-00-0; 5-methoxyisophthalic acid, 46331-50-4; N-hydroxysuccinimide, 6066-82-6; dimethyl 5-(benzyloxy)isophthalate, 53478-04-9; pentafluorophenol, 771-61-9; 5-acetoxyisophthalic acid, 90466-78-7; N-(benzoyoxycarbonyl)-N-(carboxymethyl)glycine, 17335-88-5; bromoacetyl bromide, 598-21-0; 3,5-bis(chloroformyl)anisole, 35227-77-1; 2-naphthol, 135-19-3; p-nitrotoluene, 99-99-0; p-tolunitrile, 104-85-8; 2-methoxy-6-naphthonitrile, 67886-70-8; sodium 2-naphthylenesulfonate, 532-02-5; trimethyl(1-naphthyl)ammonium fluorosulfonate, 93254-42-3.

Aspartame Decomposition and Epimerization in the Diketopiperazine and Dipeptide Products as a Function of pH and Temperature

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Cyclization and hydrolysis of aspartame were studied over a range of pH and temperatures by using an HPLC method which allows simultaneous analysis of the diastereomeric dipeptide and diketopiperazine products. The pH dependence of aspartic acid and phenylalanine racemization rates in the dipeptide–diketopiperazine system resulting from aspartame decomposition was determined. On the basis of these studies a general scheme of relative epimerization rates of amino acids in diketopiperazines and in the various positions and ionic states of peptides is presented. This scheme is discussed in terms of the carbanion mechanism of amino acid racemization and found to be consistent with it. Racemization rates in the diketopiperazine were greater than those of all ionic forms of the free amino acids and dipeptides except for fully protonated free amino acids and protonated terminal amino acids of peptides. In the neutral pH range the relative racemization rates in the DKP and dipeptides were DKP > amino terminal > carboxy terminal. Apparently contradictory results reported in the literature from dipeptide heating experiments were reanalyzed in terms of dipeptide–diketopiperazine-inverted dipeptide conversions. Viewed in this light, the literature is self-consistent and supports the generality of our scheme of relative racemization rates and mechanistic conclusions.

Intramolecular aminolysis of dipeptides and their derivatives to form cyclic dipeptides (diketopiperazines or DKPs) occurs readily in aqueous solution.[1,2] The ubiquitous nature of this reaction has become apparent to workers in the fields of peptide chemistry and biogeochemistry. Rapid rates of internal aminolysis via DKP formation at the amino terminal of peptides has lead to the suggestion that this process may play a major role in the abiotic decomposition of proteins in fossils.[3,4] Kinetic and mechanistic studies of peptide hydrolysis[5] and amino acid racemization in proteins[2,6] have been complicated by DKP formation and peptide sequence inversion. In order to properly interpret results from studies wherein DKPs may form, a clear understanding of the dipeptide–DKP system is necessary.

The purpose of this study was to develop a general qualitative scheme of relative epimerization rates in the various positions and ionic forms of peptides. Such a scheme will assist in the interpretation of results from investigations of the geochemical decomposition of proteins, as well as in the design of experiments to model peptide decomposition and epimerization. The literature contains a number of contradictory conclusions about relative epimerization rates in DKPs and dipeptides, with widely differing mechanistic interpretations.[2,6,7] We have drawn together results from the literature and shown that they are consistant with our general scheme and with the accepted mechanistic explanation of amino acid epimerization.

In this study the decomposition and subsequent epimerization of aspartame (L-aspartyl-L-phenylalanine methyl ester) have been investigated. We have determined the rates of decomposition of this dipeptide derivative, the relative stability of the DKP and dipeptide products, and overall epimerization rates over the pH range 3–10 at temperatures ranging from 6 °C to 100 °C. Besides its low cost and ready availability, aspartame (AP-OMe) was chosen as the model dipeptide for a number of reasons. The presence of two chiral centers permits chromatographic separation of diastereomers without derivatization or hydrolysis. The Phe moiety provides a chromophore for UV detection at a convenient wavelength so that peptide hydrolysis can be followed during heating experiments. These properties enabled us to develop an analytical method for the direct, simultaneous analysis of the methyl ester and its various diastereomeric decomposition products.[8] This affords an insight into the relative rates of epimerization of the DKP and dipeptide products not accessible from analysis of D/L ratios for the amino acids in hydrolyzed samples.

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Experimental Section

General Procedures. Buffers were prepared from oxalate, succinate, phosphate, or borate salts. The ionic strength of each buffer was adjusted to 0.5 with sodium chloride. The actual pH of each sample solution was measured after the addition of aspartame. The pH was measured again after each heating period; no significant change was observed. The pH values of each solution at the experimental temperatures were determined as described elsewhere.[9]

Temperatures in all heating experiments were maintained to ±0.5 °C in laboratory heating blocks containing silicon oil. The 6 °C samples were maintained to within ±0.5 °C in an insulated water bath placed in a coldroom.

The high performance liquid chromatograph (HPLC) used was an Altex Model 310 gradient liquid chromatograph equipped with an Altex Model 155 variable-wavelength UV detector or a Gilson Spectraglo fluorescence detector. Analyses were carried out on a 5-μm C18 Alltech Econosphere column (4.5 mm × 250 mm).

GC analyses were made on an HP Model 5710A gas–liquid chromatograph equipped with a splitter and a fused silica capillary column (30 m × 0.25 mm) coated with OV 101.

Chromatographic data were collected on a strip chart recorder for peak height measurements and a DEC PDP-1103 computer for peak area measurements.

Aspartame was obtained from Sigma, L-phenylalanyl-L-aspartic acid from Bachem, and L-aspartyl-L-phenylalanine from Vega Biotechnologies.

Decomposition Experiments. Solutions of AP-OMe (0.01 M) were prepared in pH 3.4, 6.6, 9.1, and 10.0 buffers. Immediately after mixing, 2-mL samples were saled in Pyrex glass ampules and placed in temperature baths at 6 °C, 35 °C, and 100 °C. Samples for the decomposition time series were removed from the temperature blocks at various intervals. The pH was adjusted immediately to pH 3–4 with phosphoric acid and the samples were filtered and injected directly into the HPLC. The time between removal from the heating block and injection was 4–10 min; during this period the samples were kept on ice until just prior to injection.

Sample chromatograms showing the HPLC separation of the diastereomeric decomposition products are presented in Figure 1. Chromatographic conditions and identification of the products are described in detail elsewhere.[8] The HPLC eluent was monitored for UV absorbance at 215 nm and quantification was by peak-height and peak-area measurements. The calibration curves relating these measurements to concentrations are described in ref 8.

Samples of L-phenylalanyl-L-aspartic acid were prepared in the pH 3.4 buffer and analyzed in the manner described above for AP-OMe.

Enantiomeric Ratios in Hydrolyzed Samples. AP-OMe (0.01 M) samples (2 mL) were prepared in buffers at several pHs from 2 thru 10, sealed in Pyrex glass ampules, and heated at 100 °C for various lengths of time. Upon removal from the heating block, samples were dried under vacuum and hydrolyzed in doubly distilled 6 M hydrochloric acid for 6 h at 100 °C. After removing the acid under vacuum, the samples were redissolved in water and desalted on Dowex 50W-X8 cation exchange resin. The amino acids were removed from the resin with 1–2 M ammonium hydroxide and dried under vacuum. Enantiomeric ratios of aspartic acid (Asp) and phenylalanine (Phe) were determined by either GC analysis of the *N*-(trifluoroacetyl)-L-prolyl chloride (TPC) derivatives or by HPLC analysis of the *o*-phthaldialdehyde *N*-acetyl-L-cysteine (OPA-NAC) derivatives.

The TPC reagent was obtained from Aldrich and GC derivatives were synthesized by the method of Hoopes et al.[10] Chromatographic conditions used for separation of the TPC diastereomers are described by Boehm and Bada.[11] Enantiomeric ratios were calculated from peak-height measurements.

Synthesis of the HPLC derivative and chromatographic conditions for analysis of Asp followed the method of Aswad.[12] The OPA-NAC Phe diasteromers do not separate under the conditions used for Asp. They were separated by isocratic elution with 50% methanol, 50% pH 5.9 acetate buffer (0.005 M) containing a 1:2 Cu(II)–L-proline chelate[13] (0.005 M). D/L ratios were calculated from peak-height and peak-area measurements.

Unheated AP-OMe standards were hydrolyzed and analyzed by both the GC and HPLC methods; the enantiomeric ratios obtained were used to correct for the racemization which occurs during sample processing and derivatization.

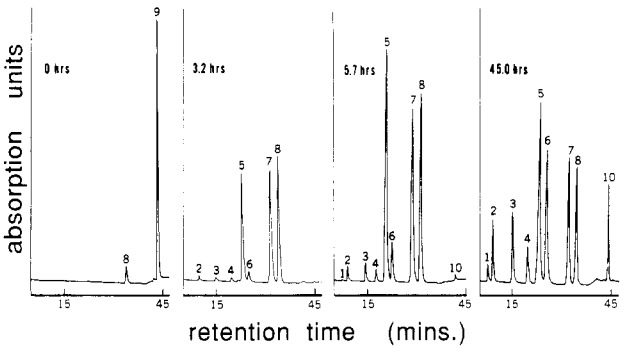

Figure 1. HPLC chromatograms of AP-OMe and its diastereomeric decomposition products: time series at pH 8.8, 100 °C. Chromatographic conditions are described elsewhere.[8] Varying concentrations of the sample solutions were injected, so that only relative peak heights of the components should be compared between chromatograms. Peak identifications:[8] 1 = Phe, 2 = L-Phe-L-Asp + D-Phe-D-Asp; 3 = D-Phe-L-Asp + L-Phe-D-Asp; 4= Asp-β-Phe (tentative identification); 5 = L-Asp-L-Phe + D-Asp-D-Phe; 6 = D-Asp-L-Phe + L-Asp-D-Phe; 7 = D-Asp-L-Phe-DKP + L-Asp-D-Phe-DKP; 8 = L-Asp-L-Phe-DKP + D-Asp-D-Phe-DKP; 9 = AP-OMe; 10 = unidentified final degradation product (this product appears to form irreversibly from the DKP at elevated temperatures).

Results and Discussion

Decomposition: Products and pH Dependence. Scheme I shows the major pathways of AP-OMe decomposition and the equilibria between the various diastereomeric products. Sample chromatograms from the time series at pH 8.8 (100 °C) are presented in Figure 1. Figure 2 shows the build-up of decomposition products over time at 100 °C and several pH values. At all pHs the major pathway for AP-OMe decomposition is DKP formation.

At low pH (3.4) the dominant reactions are cyclization of the ester to the DKP, subsequent opening of the ring to the dipeptides and rapid hydrolysis to the free amino acids. At this pH peptide hydrolysis is much faster than ring opening and the dipeptide concentrations remain low. At pH 6.6 the DKP is produced rapidly from cyclization of the ester; the proportion of DKP then decreases slightly as the equilibrium between the DKP and the dipeptides is attained. The rate of peptide hydrolysis is slightly slower than the opening of the DKP, so that the dipeptides tend toward their equilibrium proportions. The concentration of Phe increases linearly over time. At pH 8.8 rapid cyclization to the DKP is again apparent, but at this pH the direct hydrolysis of AP-OMe to aspartylphenylalanine (AP) is rapid enough to compete with the cyclization reaction. The DKP is still, however, the dominant decomposition product. After its initial formation from AP-OMe the DKP concentration decreases with a corresponding increase in AP and the inverted dipeptide, phenylalanylaspartic acid (PA).[14] Again, peptide hydrolysis is

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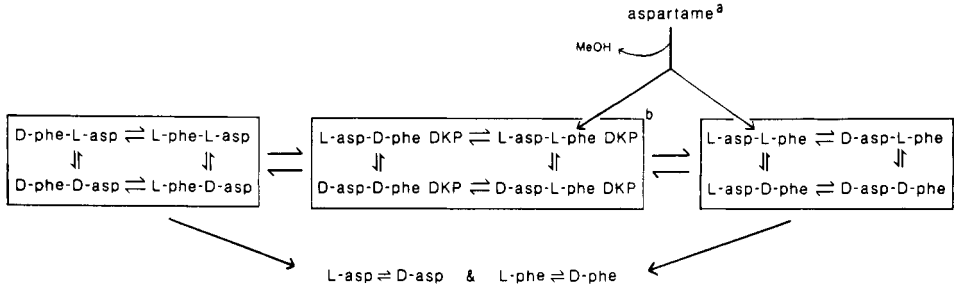
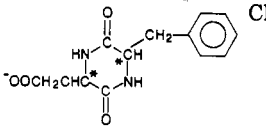
(14) The continual decrease in DKP concentration is due to its irreversible degradation to an unidentified product (peak 10 in Figure 1, 45 h).

Scheme I. Major Decomposition Pathways of AP-OMe and Epimerization in the Products



[a] Theoretically, epimerization may occur in aspartame as well as in the dipeptides; internal aminolysis and hydrolysis are so much faster, however, that epimerization in aspartame cannot be detected. [b] Asp-Phe-DKP =  Chiral centers are indicated by an asterisk.

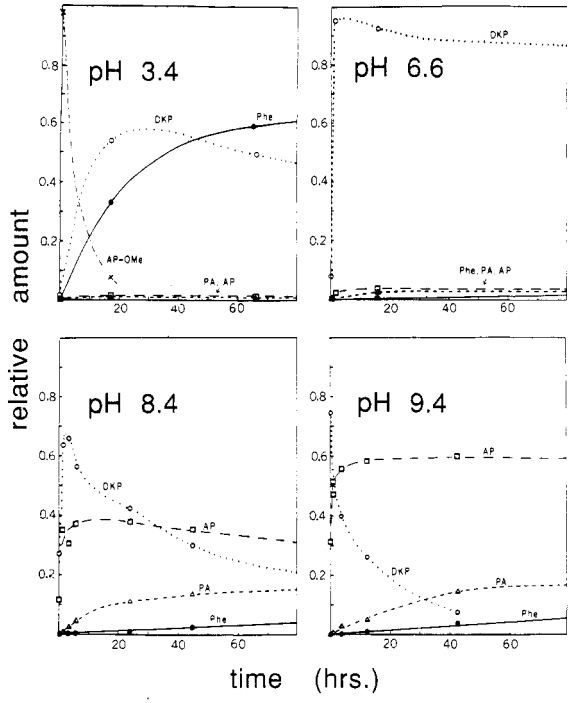


Figure 2. Build-up of the decomposition products of AP-OMe with time at 100 °C as a function of pH: DKP (··○··), AP (–□–), PA (– –△– –), Phe (–●–). Curves are extended offscale to data points taken up to 700–900 h. The concentration of Asp was not measured, but should be equal to that of Phe.

much slower than ring opening. At pH 9.8 ester decomposition is so fast that within the 9 min at room temperature required for sample preparation (i.e. the t = 0 h sample) the DKP has already reached its maximum and is decreasing while AP and PA increase. The rate of ester hydrolysis has increased relative to DKP formation but is still significantly slower. Peptide hydrolysis is again much slower than ring opening at this pH.

Approximations of first-order, or pseudo-first-order, rate constants (k_{DKP}) for DKP formation from AP-OMe at several temperatures and pHs are given in Table I. Purdie et al.[1] observed a degree of second-order dependence for DKP formation from several dipeptide esters which they attributed to self catalysis by the unprotonated amino group of the dipeptide ester. For cyclization of AP-OMe at pH 3.4, 35 °C, our data show good correlation (r = 0.993) for the first-order rate plot of five measurements. At the higher pHs where one might expect self catalysis to be more important the rates were too fast to confirm first-order kinetics in our system. The significant increase in the rate of DKP formation with increasing pH is evident, whether it is due to base catalysis, the increasing concentration of the reactive species, or both.

Table I. First-Order Rate Constants (k_{DKP}) for DKP Formation from Aspartame at Various pHs

pH (22 °C)	T (°C)	k_{DKP} (h^{-1})[a]
3.4	6	7.6×10^{-6}
	35	4.0×10^{-4}
	100	0.15
6.6	6	3.0×10^{-3}
	25	7.8×10^{-2}
	35	0.13
	100	≥3.4
8.8	6	8.1×10^{-2}
	25	0.82
	35	≥1.2
	100	≥2.2
9.8	25	≥22

[a] Rate constants determined from two to five measurements of AP-OMe concentrations. See paragraph at end of paper about supplementary material. The uncertainty in these values from measurement uncertainties is ±10%; ≥ indicates that when the earliest measurement was made AP-OMe was entirely decomposed. [b] At pH 3.4 and 6.4 the rate of DKP formation is approximately equal to the rate of AP-OMe decomposition. At pH 8.8 and 9.8 ester hydrolysis begins to compete with DKP formation; minimum values of k_{DKP} at these pHs were calculated from

$$k_{DKP} = \frac{k_{dec}}{(1 + AP/DKP)}$$

using the initial measurements of AP/DKP, where k_{dec} is the first-order rate constant for AP-OMe decomposition. This relationship is derived from the rate expressions for two parallel first-order reactions and disregards the AP–DKP equilibrium.

DKP–Dipeptide Equilibrium. Figure 2 illustrates a dramatic increase in stability of the DKP relative to the dipeptides with decreasing pH within the range 3.4–9.4. This can be understood in terms of the overall dipeptide–DKP equilibrium at these pHs:[15]

$$DKP + OH^- \rightleftharpoons dipeptide^{0(-)-} \quad (1)$$

$$DKP + OH^- \rightleftharpoons dipeptide^{+(-)-} + OH^- \quad (2)$$

At pHs much greater than the pK_a of the dipeptide amino group the equilibrium composition will tend toward $K_{equil.1}$ where

$$K_{equil.1} = \frac{[DKP][OH^-]}{[dipeptide^{0(-)-}]} \quad (3)$$

Thus at basic pH the equilibrium will be increasingly shifted in the direction of the dipeptide with increasing base concentration. At pHs lower than the pK_a of the dipeptide amino group the equilibrium composition will tend toward $K_{equil.2}$ where

$$K_{equil.2} = \frac{[DKP]}{[dipeptde^{+(-)-}]} \quad (4)$$

Thus at acidic pH the equilibrium ratio DKP/dipeptide will tend toward $K_{equil.2}$ which is independent of base concentration.

Also apparent in Figure 2 is the greater stability of AP compared to PA. Molecular models illustrate that for the dipeptide PA at neutral pH in the trans conformation there is a degree of electrostatic repulsion between the negatively charged β-carboxy group of Asp and the terminal carboxy group. This repulsion is relieved in the DKP where the peptide bond is frozen in the cis conformation. No similar destabilizing factor exists in AP where the β-carboxy group is farther from the terminal carboxy group. Thus the stability of the DKP relative to PA is greater than its stability relative to AP.

Epimerization. The mechanism of amino acid racemization, as proposed by Neuberger,[16] involves removal of the α-hydrogen by a base to form a carbanion intermediate. This mechanism is supported by isotope exchange studies.[17,18] The stability of the carbanion intermediate will depend on the electron-withdrawing and resonance-stabilizing effects of the α-carbon substituents. Racemization rates have been shown to depend strongly on the ionic state of the amino acid[19,20] and on the inductive influence of the α-carbon substituents[21] in accordance with the carbanion mechanism. A negatively charged, unprotonated carboxy group has an inhibitory effect, whereas a positively charged protonated amino group enhances racemization rates.

Average racemization rates for the Phe and Asp in the AP-OMe system were determined from the total D/L ratios in hydrolyzed samples. For racemization of a free amino acid with one asymmetric center and an initial D/L ratio of zero, the first-order rate constant for interconversion of the D and L enantiomers, k_i, is given by[17]

$$2k_i t = \ln\left[\frac{1 + D/L}{1 - D/L}\right] \quad (5)$$

First-order rate plots of this form for the racemization of Asp and Phe in the AP-OMe system would be expected to show curvature, i.e. changing values of the slope ($2k_i$) with time. This curvature reflects the changing species composition of the racemizing mixture described in the first section. Furthermore, average values of the racemization rate constants at each pH should reflect the variations in both species distribution and the predominant ionic states with pH.

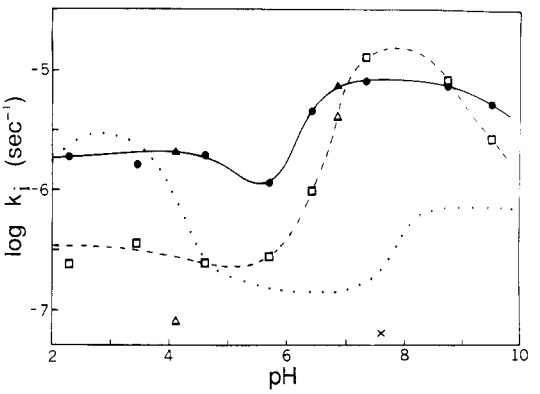


Figure 3. pH dependence of the Asp and Phe interconversion rates (k_i) in the AP-OMe solutions at 100 °C: Asp in the AP-OMe system (–●–), Phe in the AP-OMe system (–□–) (▲ and △ points determined by Boehm and Bada[11]); free Asp (···) (from ref 19); free Phe (×) (from ref 22).

Table II. Epimerization Rate Constants (100 °C) of the DKP at Various pHs

pH (100 °C)	k_{epim} (h^{-1})[a]	pH (100 °C)	k_{epim} (h^{-1})[a]
3.4	0.0081	8.4	0.54
6.6	0.020	9.4	≥1.05

[a] k_{epim} values were determined from the first-order rate equation for epimerization of diastereomeric dipeptides[21]

$$\ln \frac{(1 + D,L)/(LL + DD)}{1 - 1/K(D,L/LL + DD)} = (1 + 1/K)k_{epim}t$$

where D,L represents D-Asp-L-Phe-DKP + L-Asp-D-Phe-DKP. The ratios D,L/(LL + DD) were calculated from the peak-height measurements of peaks 7 and 8 in Figure 1. Rates were determined from three time points at pHs 3.4 and 6.6 and two points at pHs 8.4 and 9.4. K is the equilibrium constant,

$$K = \left[\frac{D,L}{LL + DD}\right]_{equil}$$

for epimerization. K = 1.2 was estimated from the heating experiments and is used in these calculations. The values given at pH 8.4 and 9.4 are not absolute epimerization rates, due to exchange with the dipeptides. Since the epimerization rates of the dipeptides are slower than those of the DKP, these are minimum rates.

Figure 3 shows average values of the interconversion rates for Phe and Asp at 100 °C plotted against pH. Also shown is the pH dependence of the rate constant for free Asp.[19] At low pH the rate of Asp racemization in the AP-OMe system is similar to that for free Asp. From the decomposition experiments at pH 3.4 we know that the DKP and free amino acids are present in comparable amounts during the heating period (Figure 2). Thus, according to Figure 3, the racemization rate of Asp in the DKP must then be similar to that of free Asp at this pH. This conclusion is supported by the data in Table II. The DKP epimerization rate constant at pH 3.4 should be equal to the sum of the interconversion rate constants of Asp and Phe in the DKP. This is the case; the DKP value (0.0081 h^{-1}) is nearly equal to the sum of the individual interconversion rates for Asp and Phe (0.0076 h^{-1}; see Figure 3) measured in the heated aspartame solutions at pH 3.4. Bada has calculated absolute rate constants for racemization of the various ionic species of Asp.[19] The curve for free Asp in Figure 3 reflects the opposing influences of increasing rates due to the base catalysis and decreasing rates due to increasing proportions of the more slowly racemizing unprotonated forms of Asp. Comparison of the absolute rate constants for racemization of the +(0)0 and +(0)– ionic forms of Asp[19] with our average rate constant

(15) In the notation used for ionic states of amino acids, dipeptides, and dipeptide methyl esters the charges are ordered as follows: amino terminal (β-carboxy group of Asp) carboxy terminal. Thus at neutral pH the notation for the dominant ionic form of aspartic acid would be +(–)–.

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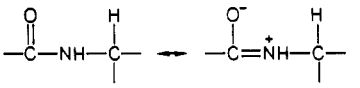
at pH 2.1 shows that Asp racemization in the DKP is slower than in the +(0)0 form and faster than in the +(0)– form of the free amino acid. This agrees with suggestions that high rates of racemization in DKPs must be due in part to lack of the rate-inhibiting negative charge on the terminal carboxy group.[16]

A pH 3.4 solution of the dipeptide PA was also heated (100 °C) and analyzed for DKP formation and epimerization. This study showed clearly that at pH 3.4 the epimerization rate of PA is greater than that of the DKP. If only inductive effects are considered one would expect relative enhancement of the Phe racemization rate in PA by the positively charged amino group, and depression of the Asp racemization rate by the negatively charged α-carboxy group. The relative importance of these two effects in PA depends on the relative magnitude of the respective inductive effects, as well as on how completely the carboxy terminal is deprotonated. The pK_a of the carboxy terminal in the dipeptide should be greater than in free Asp.[23]

Figure 3 shows a slight drop in Asp racemization rates in the AP-OMe system between pH 4 and 6; this drop parallels the behavior of free Asp and is probably due to increasing ionization of the β-carboxy group. As noted in the previous section, at pH 6 the AP-OMe system is dominated by the DKP. An increase in rate constants compared to the free amino acids (Figure 3) around pH 6 illustrates the enhanced racemization rates in the DKP compared to the +(–)– and 0(–)– forms of the free amino acids. The increase in the rate of Phe racemization above that of Asp at neutral pH is to be expected from inductive effects on the carbanion intermediate. The aromatic group of Phe is less electron withdrawing than the prtonated β-carboxy group of Asp but more electron withdrawing than the unprotonated β-carboxy group.[21]

Base catalysis of DKP epimerization is indicated by the increase in epimerization rates with increasing pH (Table II). If the DKP were the only species present, then the racemization rates of Asp and Phe in Figure 3 would be expected to increase continuously with increasing base concentration. The leveling out of the curves around pH 7.5 and decrease in rates around pH 8 reflects the increasing proportions of dipeptides present in the racemizing mixture at basic pHs (Figure 2). This illustrates clearly that the racemization rates of both carboxy-terminal (C-terminal) and amino-terminal (N-terminal) amino acids in the dipeptides are slower than the rates in the DKP at neutral to basic pH. The average racemization rate of Phe decreases more than that of Asp above pH 8. Because the concentration of AP is more than twice that of PA at basic pH (Figure 2) Phe is predominantly in the C-terminal position; the negatively charged carboxy group of Phe then retards its racemization rate relative to Asp. The chromatograms in Figure 1 show explicitly that the DKP epimerizes faster than AP at pH 8.8; the chromatograms at pHs 6.6 and 9.8 show this as well. PA formed from hydrolysis of the DKP does not begin to appear until the DKP is almost fully epimerized; accordingly, the ratio of PA diastereomers is thus inherited from the DKP from which it has formed.

The exact pK_a values of AP and PA at 100 °C are not known, but at neutral pH these dipeptides must exist as some mixture of the +– and 0– forms.[23] That the amino acids in the DKP racemize more slowly than protonated N-terminal Phe in PA was noted earlier. We now see that amino acids in the DKP racemize more rapidly than unprotonated N-terminal amino acids in the dipeptides. Similarly, Steinberg[3] found that leucine in Gly-Leu-DKP racemizes more quickly than N-terminal leucine in the tripeptide Leu-Gly-Gly at pHs as low as 6.8. It is apparent that, in addition to inductive stabilization and destabilization of the carbanion intermediate, other factors must play a role in enhancing racemization rates in DKPs. Neuberger proposed that the dipolar resonance hybrid of the amide linkage



makes a significant contribution to enhancing racemization rates in DKPs and at internal positions in peptides.[16] He also proposed that the longer the peptide chain, the greater the stability of the polar resonance form, and thus the greater the expected rate of racemization for amino acids near the center of the chain. This theory predicts that racemization rates at internal positions of large peptides should be greater than, or at least comparable to, those in DKPs.

Racemization rates for Asp in poly-Asp at various pHs were determined by Steinberg et al.[24] Although these rates, as well as those presented here, are only rough estimates, and the true analogue to poly-Asp would be Asp-Asp-DKP, it is interesting to make the comparison. At 100 °C and pH 6.65 and 7.65 interconversion rates in poly-Asp are 3.98×10^3 h^{-1} and 3.58×10^2 h^{-1}, respectively. Our values for interconversion of Asp in Asp-Phe-DKP are significantly higher: 6.77×10^3 h^{-1} at pH 6.4, 1.07×10^4 h^{-1} at pH 6.8[11] and 1.45×10^3 h^{-1} at pH 7.3.[25] Data presented by Steinberg[3] show clearly that racemization rates in Gly-Leu-DKP are significantly higher than that for internal leucine in the tripeptide Gly-Leu-Gly. Gund and Veber[26] report CNDO/2 calculations on model peptide systems which may afford a reasonable explanation for enhanced racemization rates in DKPs relative to internal and unprotonated N-terminal amino acid moieties. They suggest that the transoid conformationa of the amido carbanion grouping which results during epimerization of a cis peptide is 6–7 kcal more stable than the cisoid conformation which results during epimerization of a trans peptide. Thus epimerization in a DKP where the peptide bond is frozen in the cis conformation should be more rapid than that in an open chain peptide which is predominantly in the trans conformation.

To summarize the above discussion, we propose that the relative rates of epimerization in free and peptide-bound amino acids in various ionic forms are as follows:

$$\text{COOH(terminal)}, \text{NH}_3^+\text{(terminal)}, +0 \text{ free} > \text{DKP} > \text{internal}, +- \text{ free} > \text{NH}_2\text{(terminal)} > \text{COO}^-\text{(terminal)}, 0- \text{ free}$$

It is clear that the observed rate of amino acid racemization

(23) The carboxy-terminal pK_as of dipeptides are consistantly greater than those of the corresponding free amino acids by about 0.7–1.0 pK unit. The amino terminal pK_as of dipeptides tend to be significantly lower than those of the corresponding free amino acids. They range from about 7.5 to 8.5 at 25 °C and decrease with increasing temperature. Martell, A. E.; Smith, R. M. *Critical Stability Constants, Volume 1: Amino Acids*; Plenum Press: New York, 1974.

(24) Steinberg, S. M.; Masters, P. M.; Bada. J. L. *Bioorg. Chem.* **1984**, *12*, 349–355.

(25) The rate constants compared are the observed interconversion rates, k_i, divided by the base concentration, eliminating differences due to base catalysis. The pH 6.4 and 6.8 rates were estimated from data in Figure 3 assuming the DKP is the only racemizing species (Figure 2). The pH 7.3 value is the initial rate of racemization corresponding to maximum concentration of DKP. These estimates may therefore be too low since the rates of racemization of the other species which might be present are lower than the rate in the DKP.

(26) Gund, P.; Veber, D. F. *J. Am. Chem. Soc.* **1979**, *101*, 1885–1887.

in any system will depend on the relative amounts of each species and their rates of interconversion. The difficulties of generalizing racemization rates determined in model systems with small peptides to complex geochemical systems is apparent.

Reassessment of the Literature. There has been some confusion in the recent literature on the relative amino acid racemization rates in dipeptides and diketopiperazines. Smith and Sol[7] came to the conclusion that amino acids in the C-terminal position of some dipeptides racemize faster (pH 7.6) than in the N-terminal position. They invoked neighboring group stabilization of the carbanion intermediate to explain this trend. Mitterer and Kriausakul,[6] on the other hand, have concluded that N-terminal isoleucine (Ile) epimerizes faster than C-terminal Ile at pH 6. Our results and mechanistic interpretation predict this latter observation. Mitterer and Kriausakul also state, however, that the rate of epimerization of Ile in a DKP is less than that for N-terminal Ile and approximately equal to that of C-terminal Ile at pH 8; this is in contradiction to expectations from our results which indicate that at this pH the epimerization rate in the DKP is faster than at both positions of the dipeptide. Steinberg and Bada[2] suggested that the observed rates of epimerization in dipeptides may be largely dependent on the degree of DKP formation rather than their original positions in the dipeptides. A review of these apparently contradictory results in light of the present study will show that they are actually consistant with the mechanistic analysis given above and lend support to the generality of that analysis.

Mitterer and Kriausakul[6] have examined epimerization in the Ile-Gly system, measuring D/L ratios in Ile-Gly, Gly-Ile, and their DKP. At pH 8 and 131 °C their data shows that the relative extent of Ile racemization in each component is

$$\text{DKP} > \text{N-terminal} > \text{C-terminal}$$

as would be predicted by our results. Furthermore, in heating experiments with the tripeptides Ile-Gly-Gly and Gly-Gly-Ile they found the extents of epimerization of Ile in the DKP formed[27] to be greater than that of Ile in the Ile-Gly-Gly, which was in turn greater than that of Ile in Gly-Gly-Ile. Again this supports our expectations about the relative rates of epimerization at this pH. However Mitterer and Kriausakul interpret these results as implying that the relative rates of epimerization are

$$\text{N-terminal Ile} > \text{DKP} \sim \text{C-terminal Ile}$$

and claim that the high degree of epimerization observed in the DKP is inherited from N-terminal Ile. It is true that the degree of epimerization in the DKP reflects the rate of epimerization in the open peptides as well as in the DKP. However, it is impossible for Ile in the DKP to be consistantly more highly epimerized than in either dipeptide or tripeptide unless it has the highest rate of epimerization. The relative rates of epimerization must be

$$\text{DKP} > \text{N-terminal} > \text{C-terminal}$$

We have seen that in heating experiments with dipeptides, the dipeptide–DKP-inverted dipeptide conversions may be much more rapid than epimerization in the original dipeptide. In order to study epimerization in small peptides it is imperative to have some understanding of the rates of DKP formation and hydrolysis, and the relative stability of the dipeptide and its sequence-inverted analogue. The observation that racemization rates of amino acids appear to be faster when they are in the C-terminal position of certain dipeptides than when they are in the N-terminal position[7] can be explained in these terms. If a particular dipeptide has a faster rate of DKP formation than its inverted analogue, then even its initial rate of epimerization can be enhanced, by fast epimerization in the DKP, over the rate observed when the inverted analogue is heated. Thus if the D/L ratio for Leu in Gly-Leu after 8 h of heating is greater than that found after heating Leu-Gly for 8 h, it does not mean that Leu racemizes faster in the C-terminal position. Rather it shows that Gly-Leu is being converted more quickly, or more extensively, to the DKP than Leu-Gly. In other words, either the rate constant for conversion of Gly-Leu to DKP is greater than that for conversion of Leu-Gly to DKP or the equilibrium

$$\text{Gly-Leu} \rightleftarrows \text{DKP} \rightleftarrows \text{Leu-Gly}$$

lies more toward Leu-Gly than Gly-Leu. For the later case, in order for the system to reach equilibrium from 100% Gly-Glue it must pass more extensively through the DKP than if one begins with 100% Leu-Gly.

Racemization rates and enantiomeric ratios of amino acids in heating experiments with various dipeptide systems are collected from the literature and presented in Table III. Values determined in heating experiments beginning with a dipeptide X-Y are paired with those from heating experiments beginning with Y-X. This table shows that, for dipeptides with two chiral amino acids wherein an amino acid appears to racemize more rapidly in the C-terminal than in the N-terminal position, what is actually being observed is an apparent difference in the epimerization rate between the two dipeptides. This is consistant with the example given above for Leu-Gly. Both amino acids have significantly higher racemization rates in the DKP than in either of the dipeptides; the dipeptide that yields the DKP most rapidly or most extensively during the heating period will exhibit higher overall racemization rates for both amino acids. On the other hand, for dipeptides with very slow rates of DKP formation the initial racemization rates will reflect the relative rates in the starting dipeptide so that for a given amino acid the rate will be faster in the N-terminal than in the C-terminal position of the dipeptide. The data in Table III should reflect the relative stabilities of the dipeptides and their inverted analogues, as well as their rates of DKP formation.

Little data exist on the relative stability of dipeptides and their inverted analogues or their rates of conversion to the DKPs. These equilibria will be largely controlled by the degree of steric hindrance to hydrolysis of the two peptide bonds in the DKP. This in turn will depend on the bulkiness of the side chains of the two amino acids and on their conformation in the DKP. Other factors which may affect these equilibria and the rates of DKP formation are electrostatic repulsion such as that described above for Phe-Asp, the ease with which a dipeptide assumes the cis conformation, and stabilizing factors in the DKP such as that alloted by interaction between the aromatic side chains of Phe or Tyr and the dipoles of the DKP.[29]

Table IV presents information from the literature concerning the relative stabilities and rate of DKP formation for several dipeptides. From molecular models it can be seen that for dipeptides containing one bulky amino acid,

(27) Steinberg and Bada[4] have shown that DKPs are formed directly from internal aminolysis during heating of peptides and that this reaction may be faster than direct hydrolysis of the peptide bond, although this will depend on the amino acid residues in the peptide.

(28) Kriausakul, N.; Mitterer, R. M. *Science (Washington, D.C.)* **1978**, *201*, 1011–1014.

(29) Kopple, K. D.; Marr, D. H. *J. Am. Chem. Soc.* **1967**, *89*, 6193–6200.

Table III. Racemization of Amino Acid Residues in Dipeptide–DKP Systems: Observations from the Literature[a]

	N-terminal position	C-terminal position
	Group I Dipeptides	
Ala-Gly	27.5 (Ala)	
Gly-Ala		34.5 (Ala)
Leu-Gly	20.3 (Leu)	
Gly-Leu		25.6 (Leu)
Phe-Gly	22.7 (Phe)	
Gly-Phe		34.1 (Phe)
Met-Gly	29.4 (Met)	
Gly-Met		34.8 (Met)
Phe-Val	11.8 (Phe)	2.2 (Val)
Val-Phe	16.7 (Val)	19.5 (Phe)
Leu-Val	11.1 (Leu)	3.5 (Val)
Val-Leu	18.1 (Val)	21.0 (Leu)
Ala-Val	20.2 (Ala)	5.6 (Val)
Val-Ala	26.0 (Val)	32.5 (Ala)
Asp-Val	28.1 (Asp)	3.1 (Val)
Val-Asp	29.5 (Val)	40.9 (Asp)
Asp-Gly	39.4 (Asp)	
Gly-Asp		42.3 (Asp)
	Group 2 Dipeptides	
Ile-Gly	20.6 (Ile)	
Gly-Ile		5.6 (Ile)
Ile-Ala	[5.30] (Ile)	nd[b]
Ala-Ile	nd	[2.77] (Ile)
Ile-Tyr	[3.40] (Ile)	nd
Tyr-Ile	nd	[0.38] (Ile)
Ile-Phe	[3.57] (Ile)	nd
Phe-Ile	nd	[0.36] (Ile)
	Group 3 Dipeptides	
Ala-Pro	43.2 (Ala)	nd
Pro-Ala	nd	26.5 (Ala)
Leu-Pro	44.1 (Leu)	nd
Pro-Leu	nd	16.8 (Leu)
Glu-Pro	33.0 (Glu)	nd
Pro-Glu	nd	15.8 (Glu)
Ile-Pro	52.8 (Ile)	nd
Pro-Ile	nd	0.9 (Ile)
Val-Pro	34.6 (Val)	nd
Pro-Val	nd	1.1 (Val)
Phe-Pro	48.7 (Phe)	nd
Pro-Phe	nd	12.2 (Phe)

[a] Unbracketed numbers are % D amino acid after heating dipeptides for 8 h at 122.5 °C, pH 7.6; taken from Smith and Sol.[7] Bracketed numbers are the initial epimerization rate constants ($h^{-1} \times 10^{-2}$) at 152 °C, pH 8; from Kriausakul and Mitterer.[28] [b] Not determined.

X, and another less bulky amino acid, Y, steric hindrance to hydrolysis in the DKP will cause the dipeptide X-Y to be formed more readily than Y-X. This is reflected in Table IV where Ile-Gly and Leu-Gly are more stable than Gly-Ile and Gly-Leu. Similarily Ala-Gly is more stable than Gly-Ala.[30] We would thus expect Phe-Gly to be more stable than Gly-Phe, Met-Gly to be more stable than Gly-Met, etc.

The data in Table III reflect the relative stabilities listed in Table IV; for all cases in group 1 the enhanced epimerization rate of one dipeptide in a pair can be explained by the position of the dipeptide-inverted dipeptide equilibrium, as described for Gly-Leu above. Val-X dipeptides in Table III consistantly epimerize more rapidly than X-Val dipeptides. By the above analysis this would imply that Val-X dipeptides are less stable, or form the DKP more rapidly, than X-Val dipeptides. Table IV indicates that Gly-Val is more stable, or at least as stable, as Val-Gly.[31] The electrostatic repulsion discussed previously for X-Asp dipeptides predicts that X-Asp dipeptides will have faster apparent epimerization rates than Asp-X dipeptides due to their faster formation of the DKP. This expectation is confirmed by the Asp containing dipeptides in group 1.

Table IV. Relative Stabilities of Some Dipeptides and Their Sequence Inverted Analogues

dipeptide	stability	inverted dipeptide
Ile-Gly	>	Gly-Ile[a]
Gly-Val	≥	Val-Gly[b]
Phe-Gly	>	Gly-Phe[c]
Asp-Phe	>	Phe-Asp[d]
Leu-Gly	>	Gly-Leu[c]
Pro-Gly	>	Gly-Pro[c]
Ala-Gly	>	Gly-Ala[e]

[a] From Mitterer and Kriausakul.[6] [b] Val-Gly was formed during heating of the hexapeptide Phe-gly-Leu-gly-Val-Gly; the ratio Val-Gly/Gly-Val decreased to ~1, but had not yet reached equilibrium when the experiment ended.[3] [c] From Steinberg.[3] This experiment was not carried out to full equilibrium between the two dipeptides; the observation was that Pro-Gly forms the DKP more slowly than Gly-Pro. [d] This study. [e] From Smith and Baum.[30]

The X-Ile dipeptides in group 2 do not show the enhanced epimerization rates over Ile-X which might be expected based on their relative stabilities. The rates of DKP formation for Ile containing dipeptides are slow enought3 that initial racemization rates reflect the position of the amino acid in the starting dipeptide. Thus in group 2 all amino acids racemize faster when the dipeptide containing them at the N-terminal is heated than when the experiment begins with them in the C-terminal position.

It is to be expected that X-Pro dipeptides would form the DKP more rapidly than Pro-X dipeptides, as the secondary amino group of Pro-X is a poorer nucleophile for internal aminolysis than the primary amino group of X-Pro. Indeed, Gly-Pro forms the DKP more rapidly than Pro-Gly (Table IV). The data in group 3 of Table III are consistant with these relative rates: the apparent racemization rate for X in X-Pro dipeptides is greatly enhanced over its racemization rate in Pro-X dipeptides due to more rapid DKP formation for the X-Pro dipeptides.

Conclusion

This study has illustrated the rapid rate of DKP formation from the dipeptide derivative aspartame at neutral and basic pHs. The products from the decomposition of aspartame are dominated by the DKP at neutral pH, with the dipeptides becoming more important with increasing pH. Our results indicate that DKP formation from PA is accelerated by electrostatic repulsion between the two

(30) Recently, Smith and Baum (Smith, G. G.; Baum, R. *J. Org. Chem.* **1987**, *52*, 2248–2255) studied the Ala-Gly, Gly-Ala, DKP system and found that Ala-Gly was more stable than Gly-Ala. The also found that the rate of DKP formation and the subsequent hydrolysis into the original dipeptide and its sequence-inverted analogue were much faster than the racemization of Ala at either the N-terminal or C-terminal positions. In addition, they reported that Ala racemized fastest within the DKP, which supports the conclusions which we have discussed here. Smith and Baum attempted to calculate the Ala racemization rates in Ala-Gly, Gly-Ala, and the DKP, but as we have discussed here because of the complex nature of the exchanges which take place within a dipeptide–DKP-inverted dipeptide system these values should be viewed with caution.

(31) NMR conformational analyses[29] of DKPs indicate that for most nonaromatic amino acids the side chains exist in a fully extended position where they may inhibit access to the adjacent carbonyl by hydroxide ion or water. The Val side chain, however, appears to be positioned with one of its methyl groups folded over the top of the DKP, minimizing the degree of steric inhibition to attack at the adjacent carbonyl.[32] In this case steric inhibition of DKP formation may be more important than steric inhibition of DKP hydrolysis. There is more steric interferance to internal aminolysis at the carbonyl of Gly-Val than at that of Val-Gly, so that DKP formation from Val-Gly should be more rapid than from Gly-Val.

carboxy groups which forces the dipeptide into the cis conformation. A general scheme indicating the relative rates of racemization of amino acids in DKPs and in the various ionic forms and positions of peptides has been presented. At the neutral pHs of natural systems our results indicate that amino acids in DKPs will be more rapidly racemized than those at the N-terminal, internal, or C-terminal positions of peptides. Amino acids at the C-terminal position racemize much more slowly than N-terminal or internally bound amino acids at neutral pH. A review of the literature has shown that these relative rates are not reflected by analysis of enantiomeric ratios in the various components of a peptide system due to the rapid rates of interconversion between DKPs and dipeptides. However, an interpretation of these ratios in terms of the factors expected to affect these interconversions has revealed trends fully consistant with our scheme.

High rates of DKP formation in aqueous solution at neutral pH have important implications for the biogeochemical cycles of amino acids and proteins. Our results, as well as those of Steinberg,[3] Steinberg and Bada,[4] and Kriausakul and Mitterer[33] imply that DKP formation from peptides occurs rapidly enough to constitute a major pathway for degradation of proteins in natural waters, sediments, and fossils. It is possible that DKPs may constitute a temporary sink for degrading peptides in biogeochemical systems.

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Supplementary Material Available: Tables of the data used in calculating the rate constants in Table I (2 pages). Ordering information is given on any current masthead page.

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Rates of Hydrogen Atom Abstraction from Benzyltrimethylsilanes by Bromine and *tert*-Butoxy Radical. The Question of the Stability of the α-(Trimethylsilyl)benzyl Radical

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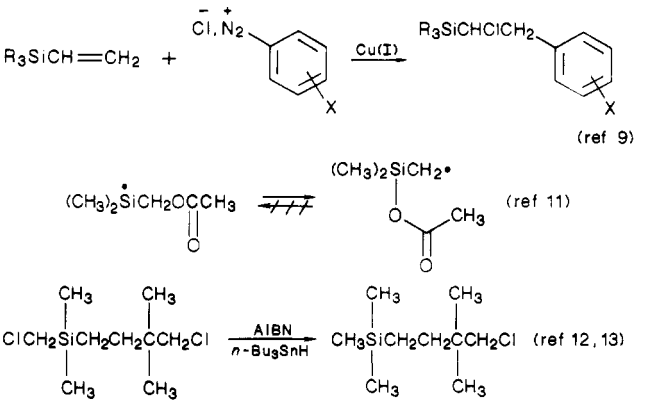
The benzylic position of benzocyclobutene derivatives shows enhanced reactivity in free-radical bromination reactions when a benzylic hydrogen is replaced by a trimethylsilyl group. However, the rates of free-radical brominations (*N*-bromosuccinimide/AIBN) of the respective benzyltrimethylsilanes are accelerated by a factor of only $\simeq 7$ relative to the respective toluene. Furthermore, the ρ value (−1.33) is not significantly different from that reported ($\rho = -1.38, -1.42$) for the reaction of *N*-bromosuccinimide with substituted toluenes. The absolute rate constants for benzylic hydrogen abstraction from benzyltrimethylsilanes by *tert*-butoxy radical are $\simeq 5 \times 10^6$ L/mol·s as measured by laser flash spectroscopy. On a per hydrogen basis, these values are a factor of about 10 times faster than the rates measured for *tert*-butoxy radical abstraction from the α-position of tetraethylsilane, $\simeq 3 \times 10^5$ L/mol·s, and a factor of 5–15 times faster than those of the reactions with the analogous toluenes. Although substitution of a methylene hydrogen with a trimethylsilyl group is often synthetically beneficial for effecting free-radical reactions at this center, there is only a small increase in the ease of formation of radicals generated α to a trimethylsilyl group as compared to an alkyl group.

Introduction

Trisubstituted silyl groups are often synthetically advantageous because they facilitate certain transformations and are then readily replaced, most commonly by a hydrogen[1] atom or hydroxyl[2] group. Extensive use has been made of silicon-derived reagents in cationic and anionic processes since a β-trialkylsilyl group is known to stabilize a positively charged center and an α-trisubstituted silyl group stabilizes anionic centers.[3] Recently, the effect of α-trimethylsilyl groups on radical centers has been of interest, and several reports suggest that radical stabilization in such systems is small or negligible.[4-6c,7] Thus, the C–H bond strength of tetramethylsilane is only 0.5 kcal/mol weaker than that of neopentane,[4] and ESR measurements

Scheme I. Selected Reactions Involving α-Silyl Radical Intermediates



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