(silica gel, 20% ethyl acetate in hexane) to give the (*S*)-MTPA ester of **4** (224 mg, 98%); ^{1}H NMR ($CDCl_3$, 470 MHz) δ 1.15 (1 H, dd, *J* = 10.3, 8.6 Hz, H6a), 1.67 (1 H, d, *J* = 13.3 Hz, H4b), 1.75 (1 H, m, H6b), 1.83 (1 H, dd, *J* = 9.2, 8.8 Hz, H8a), 1.96 (1 H, m, H8b), 2.11 (1 H, m, H4a), 2.62 (1 H, m, H5), 2.91 (1 H, m, H1), 3.10 (1 H, dd, *J* = 8.7, 4.6 Hz, H2), 3.52 (3 H, MTPA-OCH_3), 3.61 (3 H, s, $COOCH_3$), 3.87 (4 H, m, OCH_2CH_2O), 5.78 (1 H, t, *J* = 4.6 Hz, H3), 7.40 (3 H, m), 7.55 (2 H, m).

(*S*)-α-Methoxy-α-(trifluoromethyl)phenylacetic Acid Ester of 7. To a solution of the (*S*)-MTPA ester of **4** (44 mg, 0.1 mmol) in tetrahydrofuran (1 mL) was added 2 N HCl (1 mL) and the mixture was stirred for 16 h. Ether (10 mL) was added and the organic phase was washed with water (10 mL) and saturated aqueous NaCl (10 mL), dried over $MgSO_4$, filtered, and evaporated to give the (*S*)-MTPA ester of **7** (39 mg, 95%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.56 (1 H, m), 1.74 (1 H, m), 2.2–2.6 (4 H, m), 2.85 (1 H, m), 3.06 (1 H, m), 3.24 (1 H, dd, *J* = 7.5, 4.5 Hz), 3.50 (3 H, MTPA-OCH_3), 3.64 (3 H, s, $COOCH_3$), 5.76 (1 H, dt, *J* = 4.5, 2.0 Hz), 7.4 (3 H, m), 7.47 (2 H, m).

(*R*)-α-Methoxy-α-(trifluoromethyl)phenylacetic acid esters of 4: prepared in the same manner of the (*S*)-MTPA ester; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.57 (1 H, dd, *J* = 10,9 Hz), 1.8–2.4 (5 H, m), 2.65 (1 H, m), 2.90 (1 H, m), 3.10 (1 H, dd, *J* = 9,5 Hz), 3.49 (3 H, s, $COOCH_3$), 3.52 (3 H, MTPA-OCH_3), 3.87 (4 H, m), 5.78 (1 H, t, *J* = 5 Hz), 7.40 (3 H, m), 7.55 (2 H, m).

(*R*)-α-Methoxy-α-(trifluoromethyl)phenylacetic acid ester of 7: prepared by aqueous 2 N HCl hydrolysis of (*R*)-MTPA ester of **4**; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.68 (1 H, m), 2.07 (1 H, m), 2.26 (1 H, m), 2.48 (2 H, m), 2.64 (1 H, m), 2.89 (1 H, m), 3.06 (1 H, m), 3.24 (1 H, dd, *J* = 7.5, 5 Hz), 3.45 (3 H, MTPA-OCH_3), 3.49 (3 H, s, $COOCH_3$), 5.73 (1 H, dt, *J* = 4.5, 3.0 Hz), 7.41 (3 H, m), 7.44 (2 H, m).

(*R*)-*O*-Methylmandelic Acid Ester of 4. (*R*)-*O*-Methylmandelic acid (40 mg, 0.24 mmol) was added to a mixture of oxalyl chloride (40 mg, 0.30 mmol) and dimethylformamide (30 mg, 0.40 mmol) in acetonitrile (1 mL) at 0 °C. The mixture was stirred for 10 min and then a solution of **4** (50 mg, 0.20 mmol) in pyridine (0.5 mL) was added dropwise. The mixture was stirred at 0 °C for 1 h after which ether (20 mL) and water (10 mL) was added. The organic phase was washed with saturated aqueous cupric sulfate (2 × 5 mL), dried over $MgSO_4$, filtered, and evaporated. The residue was purified by preparative TLC (silica gel, 2.0 mm, 20 cm × 20 cm plate, 20% ethyl acetate in hexane) to give the (*R*)-OMM ester of **4** (74 mg, 95%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.25–2.2 (6 H, m), 2.62 (1 H, m), 2.92 (1 H, m), 3.00 (1 H, m), 3.42 (3 H, s, OCH_3), 3.58 (3 H, s, $COOCH_3$), 3.92 (4 H, m, OCH_2CH_2O), 4.70 (1 H, s), 5.66 (1 H, t, *J* = 4 Hz), 7.3–7.45 (5 H, m).

(*R*)-*O*-Methylmandelic acid ester of 7: prepared by aqueous 2 N HCl hydrolysis of (*R*)-OMM ester of **4**; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.4 (1 H, m), 1.77 (1 H, m), 2.3–2.6 (4 H, m), 2.84 (1 H, m), 3.08 (1 H, m), 3.14 (1 H, m), 3.38 (3 H, s, OCH_3), 3.60 (3 H, s, $COOCH_3$), 4.65 (1 H, s), 5.62 (1 H, m).

(*S*)-*O*-Methylmandelic acid ester of 4: prepared in the same manner as the (*R*)-OMM ester of **4**; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.70–2.2 (6 H, m), 2.68 (1 H, m), 2.88 (1 H, m), 2.94 (1 H, m), 3.15 (3 H, s, OCH_3), 3.38 (3 H, s, $COOCH_3$), 3.95 (4 H, s, OCH_2CH_2O), 4.67 (1 H, s), 5.58 (1 H, t, *J* = 4 Hz), 7.3–7.45 (5 H, m).

(*S*)-*O*-Methylmandelic acid ester of 7: prepared by aqueous 2 N HCl hydrolysis of (*S*)-OMM ester of **4**; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.69 (1 H, m), 2.1–2.7 (6 H, m), 2.95 (1 H, m), 3.05 (1 H, m), 3.14 (3 H, s, OCH_3), 3.35 (3 H, s, $COOCH_3$), 4.60 (1 H, s), 5.56 (1 H, m), 7.37 (5 H, m).

Methyl (−)-(1*R*,2*S*,3*S*,5*S*)-3-Hydroxy-7,7-(ethylenedioxy)bicyclo[3.3.0]octane-2-carboxylate (8). To a solution of **4** (133 mg, 0.5 mmol) in dry methanol (5 mL) was added sodium (2 mg), and the mixture was stirred for 2 h. Solid $NaHCO_3$ (50 mg) was added followed by water (15 mL) and ether (30 mL). The organic phase was washed with saturated aqueous NaCl, dried over $MgSO_4$, filtered, and evaporated. The residue was purified by chromatography (silica gel, 30% ethyl acetate in hexane) to give (−)-**8** (125 mg, 95%): ^{1}H NMR, MS identical with that of (±)-**3**; $[\alpha]_D$ −27° (*c* 0.5, $CHCl_3$, 23 °C).

Methyl (+)-(1*R*,2*R*,3*S*,5*S*)-3-Hydroxy-7-oxobicyclo[3.3.0]octane-2-carboxylate (7): prepared by aqueous 2 N HCl hydrolysis of **4**; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.80 (1 H, dt, *J* = 14, 2 Hz), 2.10 (1 H, m), 2.3–2.6 (4 H, m), 2.94 (1 H, dd, *J* = 8.5, 3.5 Hz), 2.99 (1 H, m), 3.19 (1 H, m), 3.38 (1 H, m), 3.75 (3 H, s, $COOCH_3$), 4.54 (1 H, t, *J* = 4 Hz, CHOH); ^{13}C NMR ($CDCl_3$, 75 MHz) 37.47 (CH), 39.96 (CH), 41.46 (CH_2), 41.85 (CH_2), 46.10 (CH_2), 51.91 (CH_3), 53.39 (CH), 74.44 (CHOH), 174.03 ($COOCH_3$), 218.89 (CO); MS, M^+ = 198. Anal. Calcd for $C_{10}H_{14}O_4$: C, 60.59; H, 7.12. Found: C, 60.41; H, 6.99.

First-Order Rate Constants for the Racemization of Each Component in a Mixture of Isomeric Dipeptides and Their Diketopiperazines[1]

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L-Alanylglycine (L-Ala-Gly), glycyl-L-alanine (Gly-L-Ala), and c-L-Ala-Gly [diketopiperazine (DKP)] were racemized at 120 °C in aqueous phosphate-buffered solutions at pH 8.0, a pH value near maximum racemization. The kinetics were followed by regression analysis. The racemization of Ala-Gly and Gly-Ala closely followed reversible first-order kinetics. The initial rate of racemization of DKP was fast but soon slowed, likely because of hydrolysis to the dipeptides. The resulting rate was similar to that of the dipeptides. The observed racemization rate constants of the dipeptides and DKP were shown to be independent of the concentration of the peptides and the concentration of buffer. Component isolation studies using preparative TLC and chiral-phase GC analysis, coupled with computer analysis, showed an equilibrium existing between Ala-Gly, Gly-Ala, and DKP and the individual rates of racemization. At equilibrium, the mole fractions are as follows: Ala-Gly, 0.57; DKP, 0.22; Gly-Ala, 0.21. The rate constant for racemization of DKP was only 2 times that of Gly-Ala and 7 times the rate of Ala-Gly. Ala-Gly racemized 20 times and Gly-Ala 66 times faster than free alanine. The results support the influence of neighboring groups in the racemization of dipeptides. Factors that contribute to the rapid racemization (epimerization) are discussed.

Racemization of peptide-bound amino acids is a phenomenon of considerable importance to synthetic peptide chemists[2] and to biogeochemists.[3] Peptide chemists try to avoid conditions that might unfavorably alter the con-

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figuration of the amino acid constituents during either synthesis or chemical analysis. Biogeochemists study the factors affecting racemization to effectively apply racemization to geochronology or geothermometry.[3]

Dipeptides racemize faster than free or protein-bound amino acids.[4] Previously proposed explanations include inductive and/or field effects, intramolecular assistance, and diketopiperazine formation. The objectives of this research were (a) to determine the equilibrium mole fractions, (b) to determine equilibrium rate constants, (c) to determine racemization rate constants for Ala-Gly, Gly-Ala, and DKP, and (d) to give support to the hypothesis that intramolecular effects are partially responsible for the reactivity of dipeptides toward racemization.

Numerous studies have been reported on the racemization of free amino acids[5-8] and amino acid derivatives,[9,10] e.g. esters, amides, and *N*-acetyl derivatives. Racemizations during amino acid peptide syntheses have also been studied and are reported to occur by DKP formation.[2,10a-c,11,12] Many studies of diketopiperazines deal with either their synthesis[13,14] or their biological activity.[15] Others have proposed DKP formation in the racemization (epimerization) of dipeptides.[4a,10c,d]

Smith and de Sol[16] racemized 37 different dipeptides at pH 7.6 and 122 °C. Their results indicate that nonsterically hindered residues racemize faster at the COOH-terminal position, while sterically hindered residues racemize faster at the NH_2 position. Intramolecular effects were invoked to explain these results.

Racemizations of the dipeptides Ile-Gly and Gly-Ile at 132 °C were found to yield DKP and also the inverted dipeptides.[4a,10d] Steinberg and Bada[17] found that at most

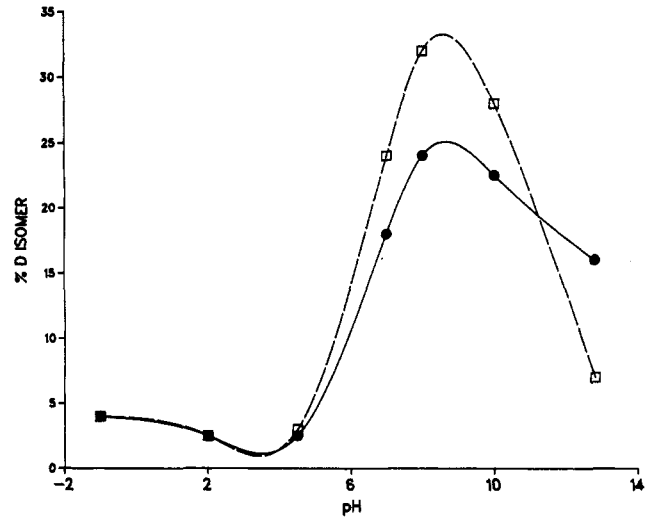
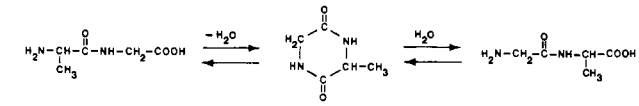


Figure 1. Effect of pH on the racemization of the dipeptides (122 °C, 8 h): ●, Ala-Gly; □, Gly-Ala.

pH values the DKP isolated was the most highly epimerized species. Their conclusion was as follows: "Because of the rapid reversible formation of the diketopiperazine, it is very difficult to evaluate the actual rate of epimerization of isoleucine at either COOH- or NH_2-terminal positions. Since the rate of both formation and epimerization of the diketopiperazine is sufficiently rapid, it is likely that, in these elevated temperature experiments, isoleucine epimerization occurs mainly in the diketopiperazine and not the actual dipeptides."[10d] We propose that individual racemization rate constants of dipeptides and of their DKP can be determined, and these data assist considerably in our understanding of why dipeptides racemize faster than amino acids and why some dipeptides react more readily than their isomers. Because rate constants for the individual species can be determined, it is no longer necessary to draw conclusions from the weighted average rate constants used previously in studying the racemization of dipeptides.

Results and Discussion

An equilibrium exists at elevated temperatures between Ala-Gly, Gly-Ala, and their DKP. The equilibrium constants for these interconversions are determined and reported in this paper.



ALA–GLY DKP GLY–ALA

Environmental Effects on Racemization. Figure 1 illustrates the amount of racemization of the L isomers of Ala-Gly and Gly-Ala as the pH is varied between −1.5 and +13.

The pK_2 values for the amino terminals of Ala-Gly and Gly-Ala are 8.24 and 8.23, respectively, at 25 °C.[17] (pK_1 is for the deprotonation of the carboxy terminal, and pK_2 is for deprotonation of the amino terminal.) These pK values are approximately the value of the pH at maximum racemization (Figure 1). At the pH of maximum racemization, there appears to be an optimum value or balance between the concentration of the most reactive dipeptide species, the zwitterion, and the concentration of the base.

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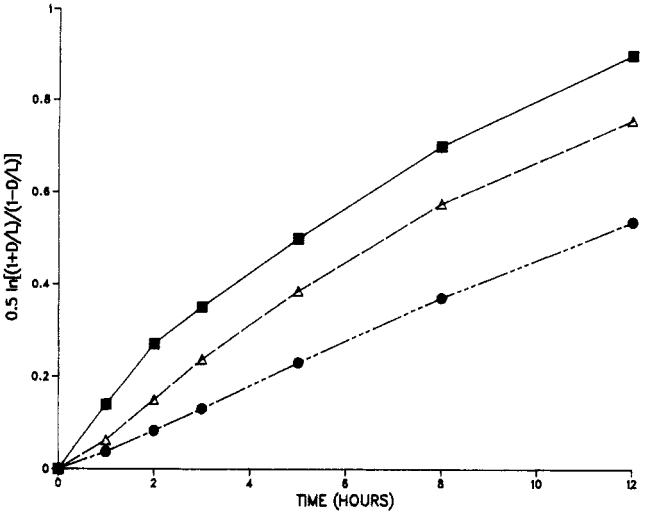

Figure 2. First-order kinetic plots of components racemized (pH 8.0, 120 °C): ●, Ala-Gly; ■, DKP; △, Gly-Ala.

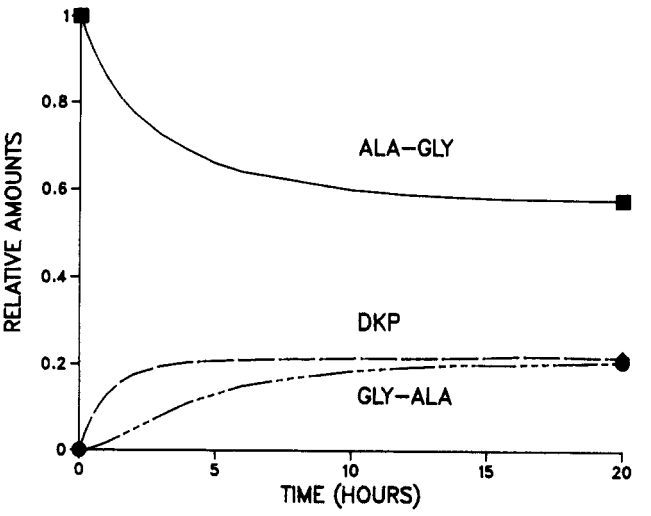

Figure 3. Relative concentrations of each peptide when racemizing Ala-Gly at pH 8.0 and 120 °C.

The zwitterion racemizes more quickly than the deprotonated species, but its concentration is reduced as the concentration of the hydroxide ion increases. At higher pH values, the concentration of the DKP may be reduced as a result of basic hydrolysis.

At approximately pH 8 these dipeptides showed the greatest rates of racemization. Therefore, pH 8.0 was used in further studies.

Racemization of Ala-Gly, DKP, and Gly-Ala is essentially independent of phosphate buffer[18] and peptide concentration.[18] The peptide concentration study, in conjunction with the buffer concentration and pH studies, all support the hypothesis that racemization of the dipeptides is a base-catalyzed reversible pseudo-first-order reaction and racemization of the DKP is a specific base-catalyzed first-order reversible reaction. Racemizations are shown to be pseudo-first-order as hydroxide ion concentration remains constant in the buffered solution.

Kinetics of Racemization. Analysis of Observed Rate Constants. A kinetic study of the racemization of Ala-Gly, Gly-Ala, and DKP was made to determine how well they followed first-order kinetics (Figure 2). It was not expected that these plots would be linear over all the time studied as the method used resulted in observed rate constant resulting from the weighted average of each species. Ala-Gly showed nearly linear reversible first-order kinetics. Deviation from linearity was noticeable for Gly-Ala and appreciable for DKP.

The initial rate of racemization of the DKP is faster than for the dipeptides. Hydrolysis of the DKP appears to take place, and the rate constant soon approaches a weighted average of the dipeptide constants.

Isolation Studies and Rate Constants. To determine the first-order rate constant for each species, it was necessary to isolate and identify the individual components in the racemization mixtures of the peptides. Preparative TLC was used to separate the dipeptides from the DKP. Thin-layer chromatography was used to determine the amount of each enantiomer with time, and capillary chiral-phase GC analysis was used to determine the extent of racemization of each species. The mole fractions and racemization rate constants for studies beginning with pure Ala-Gly and Gly-Ala after heating 8 h at pH 8 and 120 °C are given in Table I.

Table I. Mole Fractions, Percent D Present, and Observed Racemization Rate Constants for Ala-Gly and Gly-Ala at pH 8.0 and 120 °C for 8 h

	Ala-Gly		DKP		Gly-Ala		k,[b]
t, h	α[a]	% D	α	% D	α	% D	h^{-1}
0	1	0					
8	0.61	20.0	0.22	30.0	0.17	35.9	0.043
0					1	0	
8	0.46	27.8	0.23	34.1	0.31	33.8	0.061

[a] α represents calculated mole fractions. [b] k values are observed rate constants and were calculated from $k = {}^1/_2 \ln [(1 + D/L)/(1 - D/L)]$ h.

Table II. Mole Fractions, Percent D Present, and Observed Racemization Rate Constants for Equilibrium Mixtures of Ala-Gly, DKP, and Gly-Ala at pH 8.0 and 120 °C for 10 and 14 h

	Ala-Gly		DKP		Gly-Ala		k,[b]
t, h	α[a]	% D	α	% D	α	% D	h^{-1}
0	0.57	0	0.22	0	0.21	0	
10	0.57	28.0	0.22	38.2	0.21	37.8	0.052
14	0.57	33.1	0.22	44.0	0.21	44.1	0.050

[a] α represents calculated mole fractions. [b] k values are observed rate constants.

Scheme I

From these data (Table I) it was possible to calculate the equilibrium concentrations and the rate constants (k_1–k_4) for Ala-Gly, going reversibly to DKP and reversibly to Gly-Ala (Scheme I) at pH 8.0 and 120 °C. (See the Experimental Section.) To make these calculations, it was assumed that the ratios of forward and reverse rate constants were equal to the equilibrium constant (i.e., $k_f/k_r = K_{eq}$).

To verify these calculated rate constants and equilibrium concentrations, the peptides were heated beginning with these equilibrium concentrations (Scheme I) for 10 and 14 h (Table II). The concentrations (mole fraction) were found to remain constant after 10 and 14 h. Ala-Gly was found in the highest concentration (α = 0.57); Gly-Ala (α = 0.21) and DKP (α = 0.22) were found in approximately the same concentration. DKP is formed from the two

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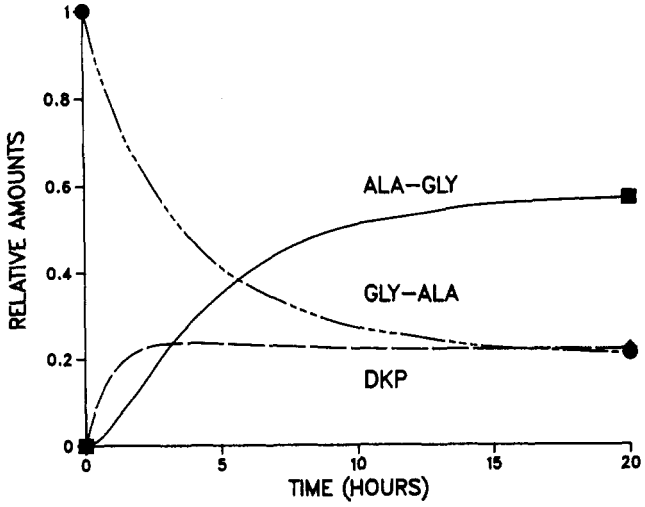
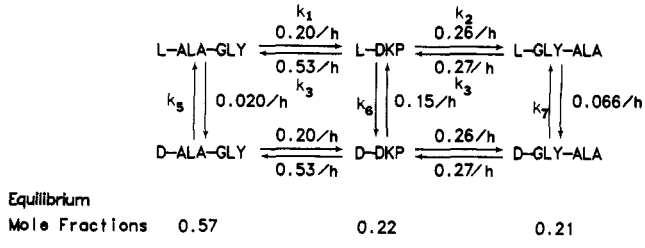


Figure 4. Relative concentrations of each peptide when racemizing Gly-Ala at pH 8.0 and 120 °C.

Scheme II



dipeptides at nearly the same rate, but once formed it is hydrolyzed twice as fast to Ala-Gly than to Gly-Ala (Scheme I).

Having determined the rate constants for equilibration, it was possible to calculate the relative concentrations of each compound present at any given time. This was done starting with Ala-Gly (Figure 3) and with Gly-Ala (Figure 4).

The relative amounts, or mole fractions, of each component were determined by summing the areas under the curves in increasingly smaller increments (see the Experimental Section for how these calculations were made and for a confirmatory method of calculation).

The rate constants for racemization of each dipeptide and DKP were calculated from the accumulated amounts of each peptide and the observed rate constants of racemization for the dipeptides at various time intervals (Scheme II). Scheme II shows the racemization rate constants of Ala-Gly, DKP, and Gly-Ala at pH 8.0 and 120 °C.

A comparison of experimental observations to calculated expectations is shown in Figure 5. The agreement between calculated and experimental results was good up to 8 h. Hydrolysis to free amino acids is a competing reaction and may explain the differences observed after 8 h.

Pure DKP was racemized to find the mole fractions and extent of racemization of each peptide after 4 and 6 h at pH 8.0 and 120 °C (Table III). The observed rate constant decreased with time (0.113–0.091). Although it never was as large as the initial rate constant of 0.15 calculated for DKP (Scheme II), this result supports the calculated initial rate constant of 0.15 as being correct for DKP. Figure 2 also supports the value of 0.15 as the rate constant of DKP racemization; the slope of the line equals the rate constant, and the initial slope for DKP approaches 0.15 at time zero.

There are many intramolecular factors that affect the observed rate of racemization of dipeptides. The two most

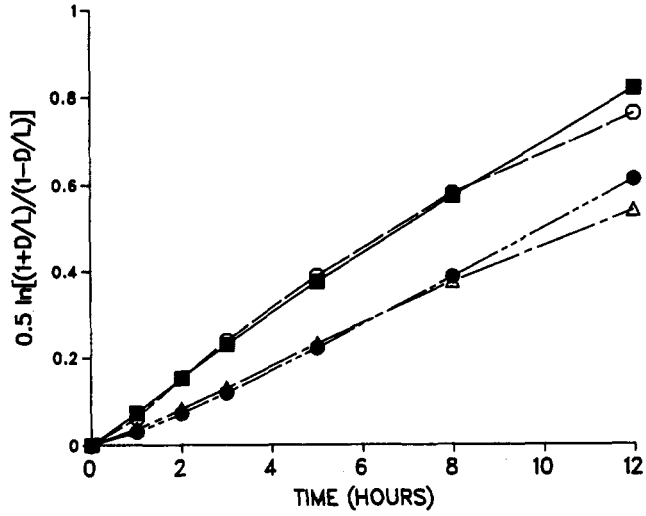


Figure 5. Experimental and calculated observed rate constants for racemization of Ala-Gly and Gly-Ala (pH 8.0 and 120 °C): ●, calculated Gly-Ala; ○, experimental Gly-Ala; ●, calculated Ala-Gly; △, experimental Ala-Gly.

Table III. Mole Fractions, Percent D Present, and Observed Racemization Rate Constants for DKP at pH 8.0 and 120 °C for 4 and 6 h

	Ala-Gly		DKP		Gly-Ala		k,[b]
t, h	α[a]	% D	α	% D	α	% D	h^{-1}
0			1	0			
4	0.40	24.9	0.33	34.0	0.27	32.1	0.113
6	0.50	27.5	0.22	37.6	0.28	40.0	0.091

[a] α represents calculated mole fractions. [b] k values are observed rate constants.

referred to of these intramolecular factors are the inductive effect by the positive charge on the amino group and the inductive effect of the negatively charged α-caroxylate group. Other influences are steric effects, formation of the DKP, inversion of the dipeptide to another dipeptide, intramolecular base effects, hydrolysis of the peptide linkage to the free amino acid, etc. The overall observed rate of racemization of dipeptides can be a combination of all these factors. Having determined the rate constant of racemization of the individual species (L-Ala-Gly, Gly-L-Ala, *c*-L-Ala-Gly), the problem of sorting out the various factors that influence the rate of racemization can be approached in a more logical and rational manner.

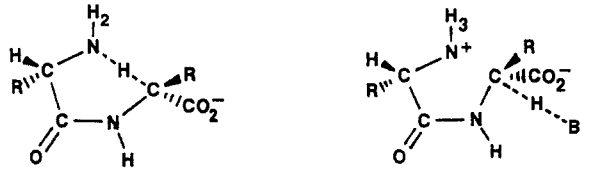
The inductive effect theory predicts that a positively charged nitrogen near the chiral center (α-methine carbon) would increase the rate of racemization and a carboxylate anion would decrease the rate. This theory has been verified in studies with amino acids. Esterification (or amide formation) of the carboxylate ion has been shown to *increase* the rate.[9] N-Acylation destroys the positive charge. This structural change has not been studied as thoroughly. On the basis of simplified inductive theory, N-terminal amino acids in dipeptides are expected to racemize faster than the C-terminal moiety. In studies of dipeptides of sterically hindered amino acids, N-terminal amino acids are found to racemize faster than their C-terminal isomers. For example, Ile-Gly and Ile-Gly epimerize faster than Gly-Ile or Gly-Val, respectively.[4,10d,16,18,19] However, this is not observed for all dipeptides. For dipeptides with less sterically hindered amino acids, the reverse is true. This paper demonstrates that the rate

(19) (a) Mitterer, R. M.; Kriausakul, N. *Science (Washington, D.C.)* **1978**, *201*, 1011. (b) Mitterer, R. M.; Kriausakul, N. *Geochim. Cosmochim. Acta* **1980**, *44*, 753.

constant for racemization of Gly-Ala is faster than that for Ala-Gly. A similar result was observed with other amino acids that are not sterically hindered.[16,18] The difference between Gly-Ala and Ala-Gly is of considerable interest. Some of the factors that appear to explain this will be discussed.

In Ala-Gly the positive charge on the nitrogen near the reactive site remains but the negatively charged carboxylate group is no longer near the incipient anion but is placed in a remote position from the intermediate α-C carbanion. In Gly-Ala the negative charge is close to the incipient carbanion while the positive charge on the nitrogen is remote. From the inductive theory involving only the σ-system, Ala-Gly would be expected to racemize faster than Gly-Ala. However, Gly-Ala reacts faster than Ala-Gly, which is opposite to what is predicted from the simplified inductive effect theory. This suggests that other factors are important.

It is proposed that the high racemization rate of Gly-Ala is a result of intramolecular assistance from the amine.[16]



1 2

amino-terminal intramolecular assistance

The deprotonated form can assist racemization by removing the α-hydrogen at the chiral center, **1**, by acting as an intramolecular base. The protonated form, **2**, can increase racemization by stabilizing the incipient carbanion generated during racemization through a charge–charge or charge–dipole interaction. Liardon and Ledermann support the intramolecular concept by employing it to explain their increased racemization reactivity of amino acids in dipeptides.[20] These researchers propose an intramolecular effect (intramolecular base) by the β-carboxylate group in forming the incipient carbanion to explain the rapid racemization of aspartic acid and invoked an intramolecular effect by aspartic acid causing the rapid racemization of proline in proteins.

The intramolecular effects shown in **1** and **2** are apparently very sensitive to steric factors. The large *sec*-butyl group prevents this influence from occurring with Ile-Gly and Gly-Ile allowing other factors, such as inductive effects, to play a more significant role. Removal of the negatively charged α-carboxylate group appears to have a greater accelerating effect than the removal of the positive charge on nitrogen has in decelerating the rate.

To add further evidence of the importance of removing the α-carboxyl negative charge, the rates of racemization of alanine and its β-naphthylamide were compared (Table IV). The amide was found to racemize 67 times faster. This ratio gives an indication of how effective removing the adjacent negative charge can be. With the dipeptide Ala-Gly the carboxylate group of alanine has been converted to its amide; however, the ratio of reactivity of this dipeptide to Ala is only 20:1 and not in the 60:1 as found with the naphthylamide. The remote carboxylate ion, apparently, has a negative effect on the racemization rate. Most likely this negative influence occurs through a direct field effect of the carboxylate ion on the incipient carbanion.

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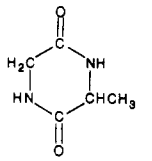
Table IV. Rate Constants for the Racemization of Alanine, Derivatives of Alanine, and Ala-Gly and Gly-Ala at pH 8.0 and 120 °C

compound	formula	k,[a] h^{-1}
alanine	$H_3N^+CHCOO^-$ CH_3	0.001 ± 0.0008
Ala-Gly	$H_3N^+CHCONHCH_2COO^-$ CH_3	0.020 ± 0.0004
Gly-Ala	$H_3N^+CH_2CONHCHCOO^-$ CH_3	0.066 ± 0.0004
acetylalanine	$CH_3CONHCHCOO^-$ CH_3	0.002 ± 0.0006
alanine β-naphthylamide	$H_3N^+CHCONHC_{10}H_7$ CH_3	0.067 ± 0.0004
Ala-Gly DKP		0.15 ± 0.0004

[a] Rate constants for Ala-Gly, Gly-Ala, and DKP are from computer analysis for the individual species (Scheme II).

All three of these compounds, alanine, Ala-Gly, and β-naphthylamide of alanine, have the positive charge still remaining on the nitrogen. At pH 8 the concentration of the zwitterion is only about 50%. The inductive theory predicts that this positive charge should increase the rate. Therefore, conversion of alanine to its *N*-acetyl derivative, where the positive charge has been removed, was expected to reduce the rate. Assuming hydrolysis did not occur and it is likely it did not, it had very little influence; in fact, it caused a slight increase (not decrease) in the rate (Table IV). Therefore, it is likely that the peptide bond in Gly-Ala, which also results in the removal of the positive charge, has only a slight effect in changing the rate of racemization of alanine in the dipeptide Gly-Ala. In summary, removing the negative charge on the carboxylate group has more of an effect in changing the rate of racemization than removing the positive charge on the nitrogen. The observed increased rate of racemization of Gly-Ala over Ala-Gly, however, requires a special explanation. This has already been discussed (see **1** and **2**).

DKP formation, its hydrolysis, and the factors contributing to the rate of its racemization (epimerization) will be discussed in some detail because of their considerable interest. Most DKP's racemize (epimerize) faster than amino acids and dipeptides.

The most important results to explain are the following: *c*-Gly-Ala racemizes only 2 times as fast as Gly-Ala but 7 times faster than Ala-Gly [0.15, 0.066, and 0.020 h^{-1}, respectively (Table IV)] and the equilibrium mole fractions reported in Scheme I.

Perhaps the most obvious structural change between DKP's and dipeptides is their difference in charge. Removal of the negative charge through DKP formation would increase reactivity noticeably, but removal of the positive charge may not affect the reactivity significantly. A combination of other factors likely has an even greater affect on reactivity. We will first consider the structure of cyclo dipeptides and their ease of hydrolysis.

DKP's possess atypical cis amide bonds. X-ray studies on crystalline DKP's and Dreiding models show them to be slightly flexible and can exist in either a flat or slightly puckered boat form. Substitution brings about a greater amount of the boat form. *c*-Gly-Gly is planar.[21] *cis*-*c*-L-

Ala-L-Ala adopts a slightly skewed boat conformation with methyl groups in the quasi-equatorial position.[22] Both c-L-Ala-Gly and c-D-Ala-Gly, discussed in this study, exist in the crystalline state in the planar conformation.[23] In contrast, DKP's involving proline adopt a nonplanar boat conformation both in the solid state[24] and in solution,[25,26] reflecting the constraints imposed on the DKP ring by fusion to the pyrrolidine ring. NMR has been used in solution studies. With large aromatic side chains, e.g. benzyl, a folded boat conformation is preferred.[27] The aromatic ring is held over the diketopiperazine ring by direct dipole-induced dipole effects amounting to approximately 12 kJ mol^{-1} of interaction energy.[28]

Recently, Ciarkowski calculated conformational energy using the semiempirical quantum mechanical CNDO/2 method.[29] The results confirm considerable flexibility of the DKP ring. As the degree of folding increases, twisted boat conformations with nonplanar peptide bonds tend to be more stable, while the more rigid regular boat conformations with planar peptide bonds tend to be less stable than a flat conformer. Kolaskar and Sarathy performed similar CNDO/2 calculations with comparative results.[30]

Sykes et al. have studied the acid- and base-catalyzed hydrolysis of diketopiperazines.[31] Second-order rate constants, k, for both acid and base catalysis were very fast. At 111.2 °C in 0.1 M HCl, $k = 5.49 \times 10^{-1}$ L mol^{-1} min^{-1}, and the rate was faster in 0.1 M NaOH, $k = 6.36 \times 10^{-1}$ L mol^{-1} min^{-1} at 25 °C. At 120 °C (temperature used in the present study) ring opening by hydrolysis is expected to be very fast. Sykes et al. reported the hydrolysis of the dipeptide was so slow it was of little importance. They attributed the high rate of hydrolysis of the DKP ring as evidence of strain in the ring. They suggested that the near-planar DKP structure and the higher energy of the cis configuration of the amide linkage contribute to its higher energy and that both of these factors lower the energy barrier to hydrolysis of DKP.

Purdie and Benoiton[32] have also reported the complete ring opening of the DKP ring at room temperature in 0.1–1.0 N alkali. They found negligible dipeptide fission to amino acids. The results reported in this study (Figure 2) also support that DKP's rapidly hydrolyze. Purdie and Benoiton[32] state, as the piperazinedione contains cis peptide bonds, the product containing the higher proportion of the cis isomer will be energetically favored. They state that in c-Ala-Gly inductive and steric effects would favor fission of the Gly-Ala bond over the Ala-Gly bond. Our study supports this prediction. Ala-Gly was formed in excess over Gly-Ala in a ratio of 2.7:1 (Scheme I). Steinberg and Bada's data support the rapid hydrolysis of DKP's.[10e] After heating Leu-Gly-Gly, they recovered 23% Leu-Gly-Gly, 26% c-Leu-Gly, 14% Gly-Leu-Gly, 22% Leu-Gly, and 13% Gly-Leu. A similar result was obtained when they started with Gly-Leu-Gly. They attributed cyclization to the DKP to aminolysis of the tripeptide and attributed dipeptide formation to hydrolysis.

The ease of base-catalyzed racemization (epimerization) of N-methylated peptides and DKP's was studied by Gund and Veber[33] using CNDO/2 calculations. The carbanions were studied with both sp^3 and sp^2 hybridization. In all cases, the sp^3-hybridized form was computed to be more stable by 3–24 kcal. They concluded the transition-state hybridization for racemization (epimerization) lay somewhere between sp^2 and sp^3 and proposed three carbanion stabilizing effects: (a) Structures that give transoid amido carbanions, e.g. cis peptides, react faster because transoid amido carbanions are more stable than cisoid. (b) N-Alkylation of a linear peptide increases the cis peptide present. (c) N-Alkylation stabilizes hyperconjugation. Solvent effects were not taken into account in the calculations but were not considered important. For DKP's, the incipient carbanion is held in the more favorable transoid conformation. N-Alkylation of diketopiperazines speed epimerization further by hyperconjugation. Diketopiperazine carbanions were calculated to be substantially more readily formed than those from peptide. Gund and Veber[33] attributed this to stabilization by the C α-substituent. p orbital overlap in cyclo dipeptides likely favors carbanion formation.

As discussed by Sykes et al.,[31] DKP's are readily hydrolyzed, which can be explained in part by strain in the substituted rings. Release of strain may also contribute to the fast racemization of DKP's as the carbanion is formed.

The intramolecular effects by the base (NH_2, 1) and/or the charge–dipole interaction by the NH_3^+ (2) cannot occur in DKP's. As this is a rate-enhancing effect, Gly-Ala reacts almost as rapidly (1:2) as c-Gly-Ala and 2 or 3 times faster then Ala-Gly. With Gly-Ile this type of intramolecular effect is likely precluded due to steric hindrance.

Smith and de Sol proposed that Gly-Ala racemized faster than Ala-Gly from analysis of observed rate constants only.[16] This study determines the racemization rate constants of Ala-Gly and Gly-Ala to be 0.020 and 0.066 h^{-1}, respectively. c-Gly-Ala racemized only twice as fast as Gly-Ala. The rate constants for the individual species confirm Smith and de Sol's study for Gly-Ala and Ala-Gly. For these nonsterically hindered amino acids, C-terminal amino acids racemize faster than N-terminal amino acids.

Summary

Computer analysis, coupled with preparative TLC and GC analyses, was employed to obtain equilibrium rate constants, equilibrium concentrations, and racemization rate constants for each component of the system Ala-Gly, c-Ala-Gly, and Gly-Ala.

The order of racemization reactivity is DKP > Gly-Ala > Ala-Gly > Ala (150:66:20:1). There are several logical reasons why the DKP reacts the fastest: transoid amido carbanion formation, removal of the α-carboxylate anion, p-orbital overlap, and, to a lesser extent, removal of ring strain by carbanion formation. The preferred position for the rapid hydrolysis of DKP's is at the less hindered amide position. This explains why Ala-Gly is formed preferentially in the hydrolysis of c-Ala-Gly.

Based on the simple inductive effect, Ala-Gly is expected to racemize faster than Gly-Ala. To explain why Gly-Ala

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reacts faster than Ala-Gly, an intramolecular effect (direct field effect) has been proposed. Ala-Gly reacts faster than alanine, in part because the negative charge on the carboxylate ion has been removed. DKP formation from either dipeptide and its racemization appears to play an important role in determining the extent of racemization, especially if only the overall racemization rate of a dipeptide is measured. With some dipeptides equilibrium rate constants in DKP formation may be faster than racemization rate constants. With others, the reverse may be true. When this occurs, peptides racemize appreciably during equilibration to their DKP's and isomeric dipeptides.

The computer programming developed in this study allows for quantitation of any three-component system in equilibrium. This research introduces a direct method of analyzing enantiomeric ratios of dipeptides having only one center of chirality, before hydrolyzing to the amino acids.

Experimental Section

Compounds Studied. **L-Alanylglycine and glycyl-L-alanine** were purchased from Vega Biochemicals, Tucson, AZ.

L-Alanine β-naphthylamide and alanine were purchased from U.S. Biochemical Corp. Cleveland, OH.

L-Alanylglycine Diketopiperazine (DKP). (*tert*-Butyloxycarbonyl)alanine (Boc-alanine) and glycine methyl ester were coupled by *N,N*-dicyclohexylcarbodiimide (DCC) in CH_2Cl_2.[34] Boc-alanylglycine-OMe was purified by column chromatography using 230–400-mesh silica gel and 7:3 hexanes/acetone eluent. The Boc protecting group was removed by adding a saturated HCl/CH_2Cl_2 solution at 0 °C and allowing it to react at room temperature overnight. The CH_2Cl_2 was removed by evaporation, and the methyl ester hydrochloride salt was precipitated and recrystallized from $MeOH/CH_2Cl_2$; mp 158–160 °C (lit.[35] mp 161–162.5 °C).

Ala-Gly-OMe·HCl was deprotonated with a saturated aqueous sodium bicarbonate solution. The water was evaporated under a stream of air at room temperature, and the ester was extracted with acetone. After evaporation of the acetone, the ester was refluxed in methanol under anhydrous conditions for 48 h.[14] (There was less than 1% racemization.) The resultant DKP was recrystallized from MeOH/EtOAc; mp 238–240 °C (lit.[36] mp 228–230 °C). Anal. Calcd for $C_5H_8N_2O_2$: C, 46.86; H, 6.31; N, 21.87. Found: C, 46.58; H, 6.18; N, 21.62.

Racemizations. Sample Preparation. The dipeptides, amino acids, amino acid derivatives, and DKP were prepared for racemization by making 0.02 M aqueous solutions in 0.05 M phosphate buffer. The pH was adjusted by adding the appropriate amount of phosphate buffers. Aliquots (approximately 3 mL) of the solutions were sealed in glass tubes and heated at 120 °C (plus or minus 0.5 °C) in a constant-temperature oil bath. The analyses were run in triplicate.

Temperature Control. The temperature of the samples during racemization was controlled to within 0.5 °C in a thermostatically controlled bath of heavy-duty synthetic aircraft oil. The bath was regulated via a proportional temperature controller (RFL Industries, Inc., Model 70A-115), and the iron–constantan thermocouples were calibrated against a National Bureau of Standards calibrated platinum resistance thermometer.

Separation of DKP from Dipeptides. Racemization mixtures were concentrated by a stream of air blowing overnight at room temperature. The concentrated aqueous mixtures were spotted on silica gel 60 F-254 preparative TLC plates and eluted with methanol. The plates were developed along one edge with ninhydrin/EtOH. It was found that DKP was located in the upper half and both dipeptides were located in the lower half of the plates. The plates were scraped and the compounds extracted with water. Most of the silica gel was removed by centrifugation. The water was removed by air evaporation at room temperature. The dry compounds were taken up in a small amount of water, transferred into vials for derivatization procedures, and blown dry at room temperature.

Derivatization for GC Analysis (*N*-Trifluoroacetyl Esters).[37] Amino Acids. To each dried amino acid residue (glycine and alanine from hydrolysis of dipeptides and DKP) was added 2-propanol/HCl (4 N, 1 mL). The tubes were resealed and heated for 2 h at 100 °C in an oil bath. The alcohol was evaporated under a stream of air to give the isopropyl ester. Derivatization was completed by adding 1–2 mL of trifluoroacetic anhydride in dichloromethane (30%) and allowing it to stand at room temperature overnight. There was no detectable racemization during the derivatizations.

Dipeptides. Hydrolyzing Dipeptides and DKP. To analyze for the total amount of racemization in a dipeptide–DKP mixture, small amounts of the original racemization mixtures were placed in glass tubes. An equivalent volume of 12 N HCl was added to give 6 N HCl solutions. The tubes were sealed and heated in an oil bath at 120 °C for 22 h. Blow drying yielded the free amino acids, which were derivatized as described above. Hydrolysis caused no detectable racemization.

Derivatizing Dipeptides and DKP. Dry samples of dipeptides or DKP were obtained in 4.5-cm length × 1.2-cm diameter screw cap vials from preparative TLC separation procedures or starting at this point for nonracemized samples. The samples were esterified by adding 3 mL of 3% w/w acetyl chloride/methanol and heating without caps at 90 °C in a heating block for 80 min.[10e,37] The remaining methanol was then blown off, 3–4 mL of trifluoroacetic anhydride in dichloromethane (30%) was added, and the resultant mixture was allowed to stand overnight at room temperature. This gave the *N*-TFA dipeptide methyl esters. (DKP was hydrolyzed to a 1:1 ratio of isomeric dipeptides during this derivatization.) No detectable racemization was found for any of the derivatizations.

GC Analysis of *N*-TFA Esters. All GC analyses were performed on a Hewlett-Packard 5880A with electronic integration and fid detector. The carrier gas was nitrogen. Isothermal conditions (see below) were always used. The amino acid derivatives were dissolved in CH_2Cl_2 and the derivatives of dipeptides in methanol for injection.

Nonchiral Phase Column. A 25-ft OV-101 fused silica capillary column was used to detect the ratios of isomeric derivatized dipeptides (e.g. *N*-TFA-Ala-Gly-OMe vs. *N*-TFA-Gly-Ala-OMe). Typical settings were 10 psi nitrogen and 115 °C.

Two-Phase Chiral Column. The enantiomeric ratios of alanine were detected on a stainless-steel capillary column (200 ft × 0.02 in.) coated with an optically active mixed phase consisting of 60% *N*-docosanoyl-L-valyl-*tert*-butylamide and 40% *N*-octadecanoyl-L-valyl-L-valylcyclohexyl ester.[37] Typical settings of 4 psi nitrogen and 120 °C were used.

Single-Phase Chiral Column. The enantiomers of Gly-Ala and Ala-Gly were separated on a stainless-steel capillary column (200 ft × 0.02 in.) loaded with only *N*-docosanoyl-L-valyl-*tert*-butylamide packing.[37] These compounds required approximately 20 psi nitrogen at 140 °C. Base-line separations were obtained with the following retention times: D-Ala-Gly, 63 min; L-Ala-Gly, 69 min; D-Gly-Ala, 78 min; L-Gly-Ala, 92 min.

Proton Nuclear Magnetic Resonance. All spectra were recorded on a JEOL FX90Q FT spectrometer.

Ala-Gly-OMe·HCl: (Me_2SO-d_6) δ 1.4 (d, 3), 3.7 (s, 3), 3.9 (m, 3), 8.4 (br s, 3), 9.1 (br t, 1).

Ala-Gly DKP: (Me_2SO-d_6) δ 1.3 (d, 3), 3.7 (m, 3), 7.9 (br s, 1), 8.1 (br s, 1); (D_2O) δ 1.3 (d, 3), 3.7 (m, 3).

Computer Analysis. All minimizations were performed with use of the IMSL routine ZXSSQ. This routine uses a modified Levenberg–Marquardt algorithm for nonlinear least-squares approximations.

Ala-Gly, Gly-Ala, and DKP. The concentration and amount of racemization of each dipeptide and DKP were determined by experimentation and calculation

$$R = AX + BY + CZ$$

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(35) Purdie, J. E.; Benoiton, N. L. *J. Chem. Soc., Perkin Trans. 2* **1973**, 184b.
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where R = total percent D (from hydrolysis of racemization mixture), A = mole fraction of Ala-Gly, X = percent D-Ala-Gly (from GC analysis of dipeptide), B = mole fraction of Gly-Ala, Y = percent Gly-D-Ala (from GC analysis of dipeptide), C = mole fraction of DKP, Z = percent D-DKP (from GC analysis of hydrolyzed DKP), and $M = A/B$ (from achiral GC analysis of dipeptides). From the following equations, derived by this study, it was possible to calculate all the mole fractions:

$$C = \frac{R(M+1) - MX - Y}{Z(M+1) - MX - Y}$$

$$A = (1 - C)/(1 + 1/M)$$

$$B = A/M$$

The first-order rate constants for this system were calculated from the concentrations of dipeptides and DKP after racemizing either Ala-Gly or Gly-Ala for 8 h. The analytical solution component for a three-component system with four steps for both forward and reverse reactions has been used.[38]

$$A_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} A_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} A_3$$

By setting the initial concentrations (mole fraction) $A_1 = 1$ and $A_2 = A_3 = 0$, we are able to calculate the relative concentrations (mole fraction at time t) and rate constants for equilibration using the following equations:

$$A_1 = \frac{k_2k_4}{\lambda_2\lambda_3} + \frac{k_1(\lambda_2 - k_3 - k_4)}{\lambda_2(\lambda_2 - \lambda_3)}e^{-\lambda_2 t} + \frac{k_1(k_3 + k_4 - \lambda_3)}{\lambda_3(\lambda_2 - \lambda_3)}e^{-\lambda_3 t}$$

$$A_2 = \frac{k_1k_4}{\lambda_2\lambda_3} + \frac{k_1(k_4 - \lambda_2)}{\lambda_2(\lambda_2 - \lambda_3)}e^{-\lambda_2 t} + \frac{k_1(\lambda_3 - k_4)}{\lambda_3(\lambda_2 - \lambda_3)}e^{-\lambda_3 t}$$

$$A_3 = \frac{k_1k_3}{\lambda_2\lambda_3} + \frac{k_1k_3}{\lambda_2(\lambda_2 - \lambda_3)}e^{-\lambda_2 t} - \frac{k_1k_3}{\lambda_3(\lambda_2 - \lambda_3)}e^{-\lambda_3 t}$$

where

$$\lambda_2 = (p + q)/2 \qquad \lambda_3 = (p - q)/2$$

and

$$p = k_1 + k_2 + k_3 + k_4 \qquad q = [p^2 - 4(k_1k_3 + k_2k_4 + k_1k_4)]^{1/2}$$

The experimentally determined relative concentrations of Ala-Gly, Gly-Ala, and DKP at 8 h were entered, and the computer varied the equilibrium constants until the best fit between computed and experimentally determined concentrations was found. The concentrations after starting with Ala-Gly were analyzed simultaneously with data from Gly-Ala. This resulted in the determinaton of the equilibrium rate constants (k_1–k_4), given in Scheme I.

From these equilibrium rate constants, the time course of the reaction could be reproduced to match the experimental data shown in Figures 3 and 4. To further substantiate these equilibrium rate constants, they were confirmed by an independent study using a different kinetic method (minimization routine) employed by Moore and Fife.[39]

The racemization rate constants were calculated by first determining the average mole fraction (α) of each component (dipeptide and DKP) present during several time spans (e.g. 1, 2, 3, 5, 8, 12 h; Figure 5) of racemization. This was done by taking 100 increments of time for each of the above, adding up the average mole fractions during each of the 100 time increments, and dividing by 100. This method is illustrated by Figure 6 and the sample calculation that follows. From these average mole fractions (α_1, α_2, α_3), the k_{obsd} at 1, 2, 3, 5, 8, and 12 h, and the

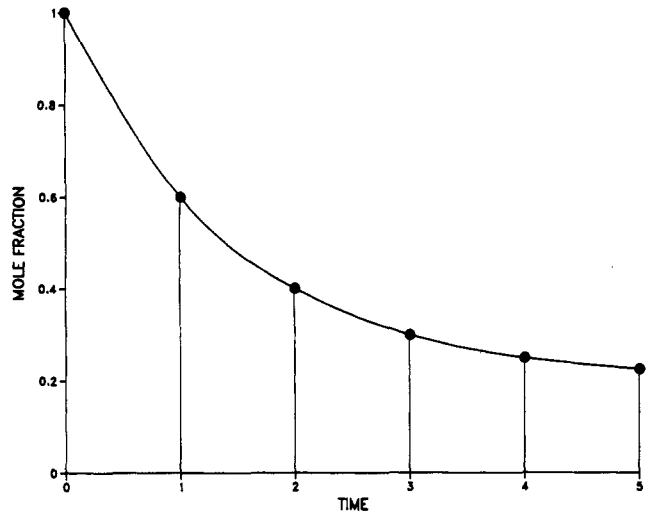

Figure 6. Hypothetical plot of mole fractions present during five increments of time.

IMSL minimization routine ZCSSQ, the racemization rate constants were calculated by

$$\sum k_{obsd}(\text{for 1, 2, 3, 5, 8, 12 h}) = \alpha_1k_5 + \alpha_2k_6 + \alpha_3k_7$$

where α_1 = average mole fraction for Ala-Gly, α_2 = average mole fraction for the DKP, and α_3 = average mole fraction for Gly-Ala. The values k_5, k_6, and k_7 are the racemization rate constants for Ala-Gly, DKP, and Gly-Ala. The computer varied the racemization rate constants until the best agreement between experimental and calculated values for total racemization was obtained.

The racemization rate constant values (k_5, k_6, k_7) and the method for their determination were supported by the following experiment. A mixture of the three components, Ala-Gly, Gly-Ala, and DKP, at their equilibrium concentrations, were heated at 120 °C for both 10 and 14 h, and the extent of racemization was determined. The k_{obsd} compared favorably to a calculated value, k_{calcd}, obtained by using the mole fraction values at equilibrium and these racemization rate constants (Table II). Further supportive evidence is given in Figure 5. The calculated values shown in Figure 5 were obtained from these racemization rate values.

The calculations used to obtain the average mole fraction using five time increments are as follows:

MF_1	(1.0 + 0.6)/2	=	0.8
MF_2	(0.6 + 0.4)/2	=	0.5
MF_3	(0.4 + 0.3)/2	=	0.35
MF_4	(0.3 + 0.25)/2	=	0.275
MF_5	(0.25 + 0.225)/2	=	0.2375
	total	=	2.1625

av mol fractn for hypothetical case = $\frac{\text{total MF}}{\text{no. time increments}} = \frac{2.1625}{5} = 0.4325$

The computer varied the racemization rate constants until there was the best agreement between experimental and calculated values for total racemization. The computer programming is available on supplementary material.

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Supplementary Material Available: Computer program used in the calculation of the mole fraction, the equilibrium rate constants, and the rate constants in the equilibration and racemization of the dipeptides (9 pages). Ordering information is given on any current masthead page.

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