(s, 3), 2.6 (broad m, 1, disappears with D_2O), 1.0–2.1 (m, 11), 0.87 (m, 3); mass spectrum *m/e* 322 (M).

Anal. Calcd for $C_{18}H_{26}O_5$: C, 67.06; H, 8.13. Found: C, 66.94; H, 7.99.

Hexyl Alcohol 24. Oxidation of **14** (510 mg, 1.67 mmol) with osmic acid and potassium chlorate under reaction conditions analogous to those described for the oxidation of **12** yielded 1,5-diketone **21** (460 mg); ir ($CHCl_3$) 1710, 1685 (s), 1595, 1580, 1460, 1167, and 1155 cm^{-1}. Selective reduction of **21** as described for **19** gave keto alcohol **22**: ir ($CHCl_3$) 3600, 3400 (broad), 1690, 1597, 1580, 1460, 1170, and 1155 cm^{-1}. Tosylation of the crude keto alcohol **22** with 350 mg of *p*-toluenesulfonyl chloride in pyridine (4 ml), afforded the corresponding keto tosylate which was reduced with lithium aluminum hydride, as described for the tosylate of **20**, giving 214 mg of crude hexyl alcohol **24**. Chromatography on silica gel with chloroform gave 187 mg (35% from **10**) of hexyl alcohol **24**: ir ($CHCl_3$) 3350, 1620, 1600, 1480, and 1445 cm^{-1}; nmr ($CDCl_3$) δ 6.1 (s, 1), 3.8–4.1 (m, 4), 3.8 (s, 3), 3.75 (s, 3), 2.7 (m, 1, removed by D_2O), 1.0–2.2 (m, 11), 0.82 (m, 3); mass spectrum *m/e* 322 (M).

Kuhn–Roth Oxidation of 24 ([1-^{14}C]-Acetate Incorporation). Kuhn–Roth oxidation of **24** (average millimolar activity = 1.726×10^5 dpm) under mild conditions gave a mixture of caproic (60%), valeric (16%), butyric (3.3%), propionic (8.2%), and acetic acid (13%). Preparation of the *p*-bromophenacyl esters of the mixture of acids and chromatography on silica gel gave *p*-bromophenacylcaproate (7.711×10^4 dpm/mmol), valerate (5.615×10^4 dpm/mmol), butyrate (3.202×10^4 dpm/mmol), propionate (3.758×10^4 dpm/mmol), and acetate (1.914×10^4 dpm/mmol). The caproic and valeric acid were purified by preparative glpc and, on Schmidt degradation, gave carbon dioxide containing 1.463×10^4 dpm/mmol and 1.113×10^4 dpm/mmol, respectively.

Kuhn–Roth Oxidation of 23 ([2-^{14}C]-Acetate Incorporation). Kuhn–Roth oxidation of **23** (average millimolar activity = 4.994×10^5 dpm) gave a mixture of caproic and lower acids as reported for the oxidation of **24**. Preparation of the *p*-bromophenacyl esters of the acid mixture and chromatography on silica gel gave *p*-bromophenacylcaproate (1.426×10^5 dpm/mmol), valerate (1.432×10^5 dpm/mmol), butyrate (1.422×10^5 dpm/mmol), propionate (7.130×10^5 dpm/mmol), and acetate (7.046×10^5 dpm/mmol). The caproic and valeric acids were purified by preparative glpc and on Schmidt degradation afforded carbon dioxide containing 1.748×10^3 dpm/mmol and 1.767×10^3 dpm/mmol, respectively.

Acknowledgments. This work was supported by Contract No. PH 43-62-468 with the National Cancer Institute, National Institutes of Health. We wish to thank Professor R. I. Mateles and Messrs. J. A. Donkersloot and D. P. H. Hsieh, Massachusetts Institute of Technology, for the labeled aflatoxin and Dr. A. Brossi of Hoffmann La Roche, Inc. for his assistance.

Microanalysis by Successive Isotopic Dilution. A New Assay for Racemic Content[1]

D. S. Kemp, S. W. Wang, G. Busby, III, and G. Hugel

Contribution from the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received July 29, 1969*

Abstract: An isotopic dilution assay for racemate with sensitivity in the important 1–0.001% concentration range is outlined, and results are reported for its application to syntheses of the Anderson peptide, ethyl benzyloxycarbonylglycylphenylalanylglycinate, and the Young peptide, ethyl benzoylleucylglycinate, by isoxazolium salt couplings and by the acyl azide procedure. The Anderson test is used to explore the effect of solvent on racemate formation during *p*-nitrophenyl ester couplings, and the triethylamine-catalyzed racemization rate of the Anderson peptide in ethanol is reported. The Young test appears to have roughly ten times the sensitivity of the Anderson test. Under optimal conditions, acylazide and 3-acyloxy-2-hydroxy-N-ethylbenzamide couplings generate similar levels of racemate—0.015% by the Anderson and 0.25% by the Young assay.

Optical purity sets one of the more vexing limitations on synthetic approaches to large peptides. As the following expression indicates, trace levels of epimer at each of the *n* chiral sites of a polypeptide must detract nearly independently from its homogeneity, and as

$$(1 - X)^n = 1 - nx, \; x \text{ small}$$

has been stressed in recent reviews,[2] an average chiral purity of 99.5% per amino acid residue must be regarded as a marginally acceptable lower limit on the integrity required of starting materials and synthetic operations, if a reasonable yield of a large, chirally homogeneous peptide is to be attained.

The development in recent years of a variety[3] of peptide-coupling procedures which appear to be racemization free when judged by assays with limiting sensitivities of 0.5–2.0% racemate points up the need for a convenient, accurate assay for racemic or diastereomeric content in the range of 0.001 to 1.0%. Although much attention has been given the problem of assessing peptide optical purity,[4] only enzymatic assay[5] and tritium incorporation[6] appear to offer sensitivities in this range; unfortunately, neither is ideal. The enzymatic assay is

(1) This paper was reported in part at the Symposium on Racemization Mechanisms in Peptide Synthesis, 154th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1967, Abstract S33.

(2) (a) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley & Sons, Inc., New York, N. Y., 1961, p 945; (b) K. Hofmann and P. G. Katsoyannis in "The Proteins," H. Neurath, Ed., 2nd ed, Vol. I, Academic Press, New York, N. Y., 1963, p 104.

(3) (a) G. T. Young, *Proc. 8th Eur. Peptide Symp., Noordwijk, 1966*, 55 (1967); G. T. Young and J. H. Jones, *J. Chem. Soc.*, 436 (1968); (b) H. D. Jakubke and A. Voigt, *Chem. Ber.*, **99**, 2419 (1966); F. Weygand, A. Prox, and W. König, *ibid.*, **99**, 1451 (1966); (c) J. E. Zimmerman and G. W. Anderson, *J. Amer. Chem. Soc.*, **89**, 7151 (1967); (d) D. S. Kemp and S. W. Chien, *ibid.*, **89**, 2743 (1967); (e) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *ibid.*, **89**, 5012 (1967).

(4) For recent reviews, see: (a) M. Bodanszki and M. A. Ondetti, "Peptide Synthesis," Interscience Publishers, New York, N. Y., 1966, p 153; (b) E. Schröder and K. Lübke, "The Peptides," Academic Press, New York, N. Y., 1965, p 319; (c) T. Wieland and H. Determann, *Angew. Chem. Intern. Ed. Engl.*, **2**, 368 (1963); (d) B. Weinstein, "Proceedings of the First American Peptide Symposium, Yale, 1968," M. Dekker, Inc., New York, N. Y., in preparation.

(5) Reference 1a, p 1255.

(6) R. G. Denkewalter, *et al.*, *J. Amer. Chem. Soc.*, **88**, 3163 (1966).

demanding and is susceptible to contamination errors, while tritium incorporation is most satisfactorily carried out in protic solvents and under aprotic conditions may prove totally inappropriate if racemization rate can significantly exceed that of tritium incorporation.[7]

Racemization assays can be universally applicable, in that they can be designed to measure the chiral integrity of an arbitrary amino acid in a peptide chain. Methods of this type have involved hydrolysis, separation of the amino acid mixture, and assay of optical purity, either by an enzymatic method, or by separation of subsequently formed diastereomers. More common, and less powerful, are methods based on synthesis of model peptides for which separation of racemate or diastereomers is facile. Since our interests lay in evaluating the relative freedom from racemization of the "nonracemizing" coupling methods, and since we wished to compare our results with those previously reported for two of the commonly used model peptides, we sought means of increasing the sensitivity of these existing assays.

We wish to describe a generalizable multiple isotopic dilution assay for racemate, founded on the classic method of Berson and Ben-Efraim,[8] but related to it much as fractional crystallization is related to crystallization, or countercurrent distribution to simple extraction. Application of this assay to the well known Anderson[9] and Young[10] tests has extended the sensitivity of the latter to $\pm 0.05\%$ and the former to $\pm 0.001\%$. Further extension of sensitivity by at least two orders of magnitude should be possible without difficulty. The solubility conditions required by the method have been found to be rather widely met among peptide derivatives, and it appears likely that the method can be applied to a variety of other substances.

Outline of the Method

Besides an assay, a racemization test requires racemate-free starting materials. At first consideration the prospects for preparing a chiral substance with a known ratio of D to L enantiomer of less than 10^{-5} appear bleak; solid solution formation will likely render crystallization inefficient at this impurity level, and zone refining, the sole direct purification method of requisite power, is probably inapplicable to substances as heat labile as peptides.

Both for assay and for preparation of starting materials, the method to be described uses the fact that one can easily prepare an isotopically labeled chiral substance which contains negligible label in any contaminating enantiomer. As an example, consider benzoyl-L-leucine, the starting material for Young's racemization test. If this substance can be recovered in a state of greater than 99.5% purity from a solution containing 95% L and 5% D enantiomers, then the following operations can be envisaged. Benzoyl-L-leucine bearing an isotopic label is dissolved in and recovered from a solution containing *ca.* 10% of its weight of unlabeled racemate. The resulting L enantiomer will have 1/1.05 or 95% of the label that it possessed before the dilution; on the other hand, provided the samples initial chiral purity was greater than 99.5%, its D contaminant will now possess less than 0.005/0.055 or 9% of the fraction of isotope that it initially possessed, and the exchange with unlabeled racemate will have effected a selective extraction of isotopically labeled D enantiomer. Since the process may be repeated, one can clearly attain labeled L:D ratios of 10^6–10^7, provided the initial separability condition can be met.

The generalized racemization assay may be summarized.

(a). Preparation of Starting Materials. A radiolabeled substance, L–A, bearing a single chiral site, is repeatedly recovered from solutions containing 5–10% of its weight of unlabeled racemate, DL–A. Final purification yields a sample whose label is localized to any desired degree in the L enantiomer.

(b). Coupling and Recovery. The L–A prepared in a is converted to a substance B by a procedure whose stereospecificity is to be assessed. The product is labeled L–B, containing labeled D–B only to the extent that the procedure racemizes; n, the yield of product is determined, and product is dissolved in a solution containing an amount m of unlabeled DL–B, where m is less than $0.1n$. Racemate is recovered from this solution.

(c). Successive Dilution. The sample of racemate isolated in b is added to and recovered from a solution containing five to ten times its weight of unlabeled L–B. This dilution procedure is repeated until the specific activity of the recovered racemate reaches a constant value.[11]

The fraction, α, of the product B prepared in b which is racemate is given by expression 1, which for α small, reduces to the simpler expression, 2. The calculated

$$\alpha = \frac{2mx}{n(y - 2x) + mx} \quad (1)$$

$$\alpha = \frac{2mx}{ny}\ x \ll y \quad (2)$$

extent of racemization is thus dependent on four quantities all of which can be measured precisely: n is the total weight of L–B formed in b; m is the weight of racemate added in b; x is the limiting specific activity of racemate from c; and y is the specific activity of L–B formed in b. The method is limited only by recovery losses during the sequential dilutions of c and by the requirement that x exceeds the experimental background count or noise level.

Results

Control Experiments. From the foregoing it is clear that the realization of this multiple isotopic dilution assay stands or falls on the ease of separation of the corresponding racemates from excess enantiomeric starting material, A, and product B. Young's most

(7) For a review of isoracemization, see D. J. Cram in "Survey of Progress in Chemistry," Vol. 4, A. F. Scott, Ed., Academic Press, New York, N. Y., 1968, p 59.

(8) J. A. Berson and D. A. Ben-Efraim, *J. Amer. Chem. Soc.*, **81**, 4083 (1959); isotopic dilution was first applied to an amino acid problem by Graff, Rittenberg, and Foster: S. Graff, D. Rittenberg, and G. L. Foster, *J. Biol. Chem.*, **133**, 745 (1940); the work of Waterfield provides a recent application in the peptide field: W. R. Waterfield, *J. Chem. Soc.*, 2731 (1963), 541 (1964). For a general review of techniques of assessing chiral purity, see M. Raban and K. Mislow in "Topics in Stereochemistry," N. L. Allinger, and E. L. Eliel, Ed., Vol. 2, Interscience Publishers, New York, N. Y., 1967, p 199.

(9) G. W. Anderson and F. M. Callahan, *J. Amer. Chem. Soc.*, **80**, 2902 (1958).

(10) M. W. Williams and G. T. Young, *J. Chem. Soc.*, 881 (1963).

(11) For three successive dilutions of this kind, using a racemate excess enantiomer ratio of 0.1, and using 5% of racemate in step b, one will obtain a sample of racemate whose L activity is 3×10^{-6} times that of the initial sample of L–B. A fourth dilution will effect a further twentyfold diminution in L isotopic content (1.5×10^{-7} or 0.0002%).

widely used test for racemization involves coupling benzoyl-L-leucine with ethyl glycinate to form ethyl benzoylleucylglycinate, whose optical purity is assessed by optical rotation.[10] Anderson's test involves cou-

Young: Bz-L-LeuOH + GlyOEt ⟶ Bz-L-LeuGlyOEt

Anderson: ZGly-L-PheOH + GlyOEt ⟶ ZGly-L-PheGlyOEt

pling benzyloxycarbonylglycyl-L-phenylalanine with ethyl glycinate to form ethyl benzyloxycarbonylglycyl-L-phenylalanyl glycinate whose racemate can be separated from a large excess of enantiomer by fractional crystallization.[9] The Anderson test therefore guarantees the isolation of racemate for the critical case of the product, B. For the Young test, the feasibility of the corresponding separation of ethyl benzoylleucylglycinates remained to be demonstrated. Also needed were racemate separation procedures for the two starting acids, benzoylleucine and benzyloxycarbonylglycylphenylalanine, and a similar procedure or a racemization-free route from acid for benzoylleucylhydrazide and benzyloxycarbonylglycylphenylalanylhydrazide, and for the active ester, *p*-nitrophenyl benzyloxycarbonylglycylphenylalaninate.

In a solvent which minimizes solute–solute interactions, the solubility of a crystalline racemate should be governed by a mass-action expression and markedly diminished by the presence in solution of an excess of one enantiomer. That such a relationship holds in practice for the peptide derivatives, benzyloxycarbonylglycylphenylalanine in acetonitrile and ethyl benzyloxycarbonylglycylphenylalanylglycinate in ethanol is shown by the data of Figure 1.[12]

$$K = [L][D] \qquad (3)$$

Provided solid solutions or series of molecular compounds do not complicate the problem, three factors appear to facilitate the isolation of pure racemate from solutions containing a large excess of an enantiomer: (a) a frequently encountered higher stability of the racemate crystal lattice, reflected in its higher melting point and its smaller solubility; (b) the mass-action relationship 3; for the cases cited in Figure 1, this effect results in roughly a fourfold decrease in solvent power as the solvent is saturated with excess enantiomer; (c) the kinetic effect of selective seeding.

For the cases of benzoylleucine, benzyloxycarbonylglycylphenylalanine, ethyl benzoylleucylglycinate, and benzyloxycarbonylglycylphenylalanylhydrazide, the separability of racemate from enantiomer was demonstrated by using unlabeled L enantiomer, and racemate which was labeled with carbon-14 in its D enantiomer. The purity of recovered racemate was indicated by its specific activity, and the completeness of recovery by the isotopic balance. The specific activity of reisolated L enantiomer provided an assessment of the degree of contamination with unrecovered racemate. As an example, when a solution of benzyloxycarbonylglycyl-L-phenylalanine and 20% of its weight of D-labeled racemate was carefully seeded, solid corresponding to 90% of the weight of added racemate was recovered and found to contain 90% of the total activity. Solution of a further 8% of unlabeled racemate in the filtrate, followed by seeding, resulted in a total recovery in two steps of 99.6% of the initial D activity.[13]

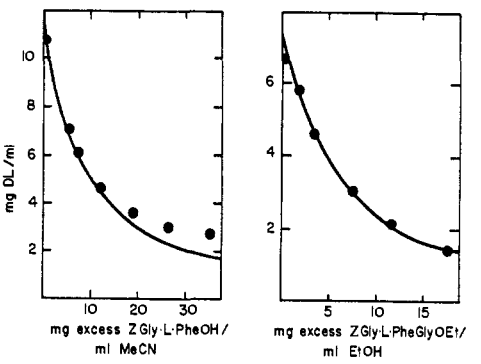


Figure 1. Solubility of ZGly-DL-PheOH at 30° in acetonitrile containing added ZGly-L-PheOH, and of ZGly-DL-PheGlyOEt at 30° in ethanol containing added ZGly-L-PheGlyOEt. Solid line is the calculated solubility for the mass action relationship, [L][D] = *K*.

Racemic *p*-nitrophenyl benzyloxycarbonylglycylphenylalaninate melts lower and shows a higher solubility than its enantiomer; it was therefore not surprising that separation experiments demonstrated the impracticality of racemate recovery. However, selective D labeling established the cleanness of the recovery of L ester from solutions containing less than 3% of racemate. For this substance one could effect the necessary dilution by a reverse recovery; the availability of two inclusive alternatives for the removal of D label from starting materials implies the applicability of this analysis to any chiral substance which is readily crystalline and which does not enter into intermediary crystal formation with its racemate.

Artifacts and contamination can arise with any analysis which does not actually isolate and characterize each impurity. In this study, isotopes have been introduced early in the reaction sequence leading to starting materials, and all intermediates have been carefully recrystallized to maximize the removal of extraneous labeled starting materials. In addition, the careful and repeated recrystallizations required during the successive dilution of racemic product with unlabeled enantiomer provide an added protection against errors of this kind. Nonetheless, strictly speaking, this analysis can be taken to yield only an upper bound on the actual racemic content of a sample. Two conditions argue against this conclusion. Errors of efficiency in removing D label from starting materials[14] are defined in the sense that the least amount of racemate detected in product from a given preparation of starting material establishes its purity—all racemate levels which exceed this minimum are real. Second, as will be seen, there appears to be a uniform relationship between results from the Anderson and Young assays; data from both tests which are mutually consistent are unlikely to be artifacts.

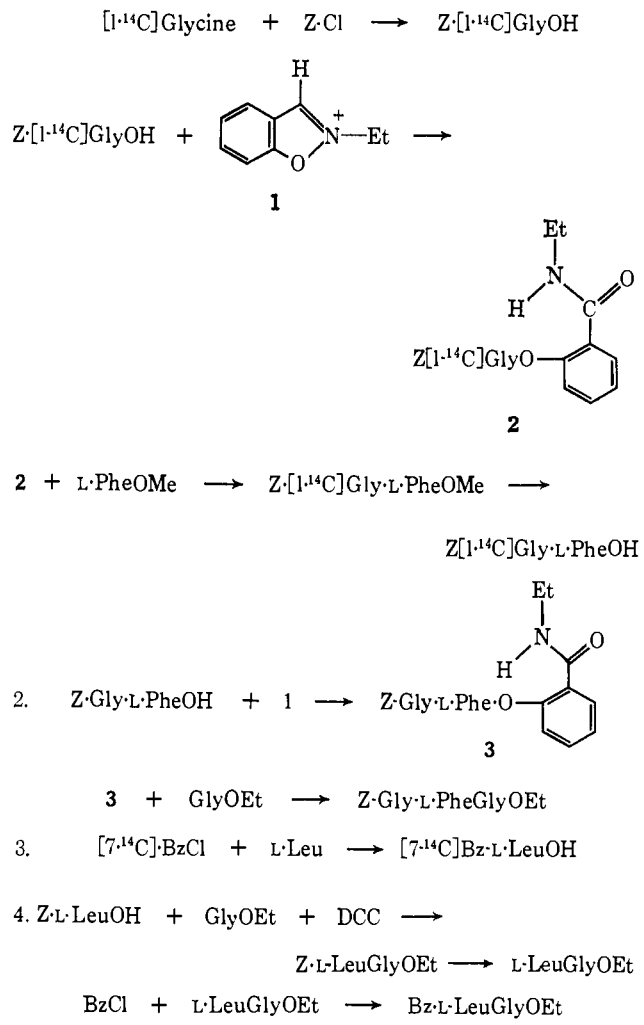
(12) The results of the figure were obtained with racemates selectively labeled with carbon-14 in their D enantiomers; the solubilities were thus obtained directly from the specific activities of the saturated solutions. At 0° the solubilities of DL and L Anderson tripeptide esters in ethanol were found to be 0.8 mg/ml and 5 mg/ml, respectively. From these numbers it can be estimated that a solution saturated in each at 0° would retain *ca.* 1.5% of its peptide as racemate; racemate levels below this cannot therefore be detected by direct isolation.

(13) Similar recovery experiments for the other three substances are outlined in the Experimental Section.

(14) Since direct evidence of the efficiency of racemate recovery during starting material purification is not available, the tests with D-labeled materials described above must be reproducible. For the cases studied, melting point and crystal form provide excellent independent indications of the success of the separations.

Starting materials and diluents were prepared as shown in Scheme I. The leucine dipeptide ester required for dilution was assumed to be optically pure; typical isotopic dilution Anderson test are given in Scheme II.

Scheme I. Preparation of Starting Materials



on the other hand, the phenylalanine tripeptide ester was prepared by a process which was shown subsequently to result in *ca.* 1% racemization. A bulk of this ester was carefully recrystallized to produce peptide used in all dilution experiments; its racemic content was estimated by noting the decrease in specific activity of a sample of D-labeled racemate which was recovered five times successively from six to ninefold excesses of the L peptide. The observed drop of 10% corresponds to the presence of 0.23% of racemate in the diluting L peptide. Although this source of error will rarely exceed 10% of the final value of α, it is easily corrected for by eq 4, and all data reported subsequently have been

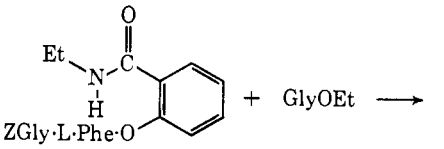
$$x = x_k \prod_{i=1}^{k} \left(\frac{m_i + \nu p_i}{m_i} \right) \quad (4)$$

$$y_k = \frac{2ny}{2n + m_i} \prod_{i=1}^{k} \left(\frac{m_i}{m_i + 2p_i} \right) \quad (5)$$

so corrected; x is the true specific activity of racemate, x_k is the observed specific activity after k dilutions, m_i is the weight of racemate at the ith dilution, p_i is the weight of L enantiomer at the ith dilution, ν is the fraction of racemate present in the diluent, and y_k is the residual L activity of racemate at the ith dilution. Results of a

Scheme II. Racemization during Coupling of Ethyl Glycinate with O-Benzyloxycarbonylglycyl-L-phenylalanyl-N-ethylsalicylamide

(1) Coupling



ZGlyPheGlyOEt
91%, 880 mg, 2.58 x 10^3 dpm/mg

(2) Isolation: 836 mg of the sample was combined with 59.4 mg of unlabeled racemate. 34.8 mg of racemate was recovered.

(3) Dilution: see Table I.

Table I

No.	DL wt, mg	L wt, mg	Recovered DL Mp, °C	Recovered DL Wt, mg	Recovered DL Activity, dpm/mg	Calculated L activity, dpm/mg
1	34.8	883.8	131–132	22.2		47.7
2	22.1	595.0	131–132	10.1	195	0.9
3	9.0	293.7	130–131	2.5	192	0.06

(4) Calculations

n = 836 mg, m = 59.4 mg,

$y = 2.58 \times 10^3$ dpm/mg, $x_3 = 1.93 \times 10^2$ dpm/mg

$x = 1.10 \times x_3 = 1.10 \times 1.93 \times 10^2 = 2.13 \times 10^2$

$\alpha = 1.4\%$

Although the initial control experiments necessary to establish its validity are somewhat involved, the assay itself is remarkably simple and expeditious, comparing favorably with the classical procedures in ease and speed of execution. In our opinion, it is probably the method of choice, even for conditions which result in the large amounts of racemate which are suitably handled by direct isolation.

Applications. The peptide activating agents which have been claimed to yield racemate-free products under synthetically useful conditions include the acyl azides, the isoxazolium salts, and a variety of active esters, of which the most important are probably the *p*-nitrophenyl esters, the N-hydroxysuccinimide esters, and the 8-hydroxyquinoline and catechol esters.[15] All of these methods deserve scrutiny, but only four types can be applied to an optically pure peptide acid: the azide procedure, the various isoxazolium salts,[16,17] the combination of N-hydroxysuccinimide and carbodiimides or mixed anhydrides,[18] and the mixed anhydride procedure when run under controlled conditions.[18] For other methods the optically pure activated ester

(15) For recent reviews, see: ref 4a, pp 146–155; ref 4b, p 325; M. Goodman and C. Glazer, "Proceedings of the First American Peptide Symposium, Yale, 1968," M. Dekker, New York, N. Y., in preparation.

(16) R. B. Woodward, R. A. Olofson, and H. Mayer, *J. Amer. Chem. Soc.*, **83**, 1010 (1961); R. B. Woodward, R. A. Olofson, and H. Mayer, *Tetrahedron, Suppl.*, **8**, 321 (1966).

(17) D. S. Kemp and R. B. Woodward, *Tetrahedron*, **21**, 3019 (1965).

(18) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **89**, 5012 (1967); J. E. Zimmerman and G. W. Anderson, *ibid.*, **89**, 7152 (1967).

must be prepared indirectly and subjected to the purification procedure outlined in the preceding section. In this study we have considered five coupling methods: the azide method, the 2-ethyl-5-(3′-sulfonatophenyl)isoxazolium (Woodward's reagent K),[16] the 2-ethylbenzisoxazolium cation,[17] the 7-hydroxy-2-ethylbenzisoxazolium cation,[3d] and the *p*-nitrophenyl ester method. For the latter study, the *p*-nitrophenyl ester of benzyloxycarbonyl[1-^{14}C]glycyl-L-phenylalanine was prepared by the method of Goodman and Steuben[19] and freed of D label by dilution with unlabeled racemate.

Although peptide *p*-nitrophenyl esters are recognized to be capable of retaining optical integrity under normal peptide coupling conditions, they have also been shown to be vulnerable toward tertiary amine-catalyzed racemization[19,20] and to be racemized in the presence of amine hydrochlorides.[20] Although their inaccessibility in optically pure form renders peptide *p*-nitrophenyl esters of small synthetic interest, they may be regarded as the archetype of the class of peptide active esters, and their racemization behavior under optimal coupling conditions provides a standard for judging other activated species.

Results of couplings conducted in a range of solvents between ethyl glycinate and the Anderson dipeptide *p*-nitrophenyl ester are shown in Table II.

Table II. Racemization in *p*-Nitrophenyl Ester Couplings

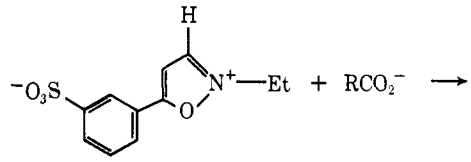
ZGly·L·Phe·O–C$_6$H$_4$–NO$_2$ + GlyOEt → ZGlyPheGlyOEt

Solvent[a]	Total yield (L + DL, %)	Racemate (% DL)
Toluene[b]	92	0.14
Pyridine	92	0.10
Acetonitrile	94, 99	0.23, 0.18
Dimethylformamide	93, 96	0.53, 0.53
DMF + 1,2,4-triazole (1.3 *N*)	82	0.45
Dimethyl sulfoxide	87	1.1, 2.0
Hexamethylphosphoramide	94	1.6
DMSO–water[b] 1:1, v/v	84, 94	4.9, 7.2

[a] Reactions run at 22–25° at 0.4–0.5 *M* active ester concentration, except for toluene and DMSO–H_2O;[b] 1.4 equiv of distilled ethyl glycinate; time, 24 hr. [b] Inhomogeneous reaction mixture.

The striking increase of racemate with solvent polarity is paralleled by Weygand's observations[21] for the more complex case of dicyclohexylcarbodiimide couplings, and by the work of Goodman and McGahren[22] with solvent effects on racemization and ring opening of oxazolones. Although the trend is also in accord with other generalizations,[23] it is nonetheless striking in that it represents a solvent effect on a rate *ratio*. The more than fiftyfold increase in the extent of racemate formation over the range of solvents studied must be considered in the perspective that coupling rates for ethyl glycinate with *p*-nitrophenyl benzyloxycarbonylglycinate in toluene, acetonitrile, and dimethyl sulfoxide show ratios of 1:1:24 at the initial amine concentrations of the racemization study.[24] If the coupling reactions of the Anderson *p*-nitrophenyl ester show a similar solvent sensitivity, then the racemization rate for this substance must increase several hundredfold when the solvent is varied from toluene to dimethyl sulfoxide.[25] Interesting further conclusions of this study are that the addition of 1,2,4-triazole[26] had no effect on the amount of racemate observed in the dimethylformamide coupling, and that only acetonitrile, pyridine, and to a marginal degree, dimethylformamide offer a tolerable combination of solvent power and freedom from racemization.

The isoxazolium salts prepared and studied by Woodward and coworkers, when used under controlled conditions,[27] provide a racemization-free route from peptide acids to enolic or phenolic esters. They, the combination of dicyclohexylcarbodiimide and N-hydroxysuccinimide,[18,28] and the acyl azide procedure at present constitute the generally applicable means of coupling block peptides without extensive racemization. The literature contains varied reports of the extent of racemization incurred with Woodward's reagent K, **4**. Neither Woodward and coworkers nor Williams and Young[29] observed significant racemization under optimal conditions, although Young reported detectible racemate from a benzoylleucine coupling run in nitromethane. On the other hand, Weygand, using his methyl trifluoroacetylvalylvalinate assay, observed substantially racemic product. Knowledge of the absolute levels of racemate formed during preparation of the Young and Anderson peptides by isoxazolium salt



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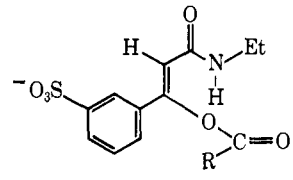

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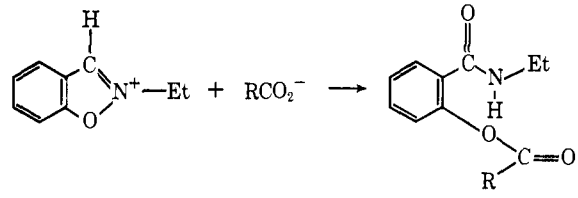


1 **7**

(19) M. Goodman and K. G. Steuben, *J. Org. Chem.*, **27**, 3409 (1962).

(20) M. W. Williams and G. T. Young, *J. Chem. Soc.*, 881 (1963); *ibid.*, 3701 (1964); A. L. Heard and G. T. Young, *ibid.*, 5807 (1963).

(21) F. Weygand, L. Schmidhammer, and W. Konig, *Angew. Chem.*, **75**, 282 (1963).

(22) M. Goodman and W. J. McGahren, *Tetrahedron*, **23**, 2031 (1967).

(23) Reference 4a, p 152.

(24) D. S. Kemp and R. Sitrin, unpublished observations.

(25) Evaluation of the exact magnitude of the solvent effect on racemization rate is complicated by measurement of racemate formation at the high reactant concentrations appropriate for practical synthesis. Since the more rapid of these reactions may be expected to result in a significant increase in temperature, part of the solvent effect on racemization may in fact be an effect of temperature on the rate ratio.

(26) H. C. Beyerman, *et al.*, *Rec. Trav. Chim. Pays-Bas*, **84**, 213 (1965).

(27) D. J. Woodman, Ph.D. Thesis, Harvard University, Cambridge, Mass., 1965.

(28) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **85**, 3039 (1963).

(29) M. W. Williams and G. T. Young, *J. Chem. Soc.*, 881 (1963).

Table III. Racemization during Isoxazolium Salt Couplings

Conditions	Racemate formed, % Anderson	Young
A. Woodward's reagent K, **4**.		
1. Et_3N salt of acid in MeCN is added to a suspension of WRK in MeCN at 0° with stirring. After 20–30 min,[a] distilled GlyOEt is added. Coupling run at 20° for 24 hr	2.1	8.5
2. Procedure 1, but GlyOEtHCl + 1 equiv of Et_3N replaced GlyOEt		9.5
3. WRK added slowly to a solution of Et_3N salt of acid in MeCN at 0°, then as with procedure 1.	4.2	
4. A portion of the activation mixture from 3 was concentrated at 0°, and the residue was washed repeatedly with ether to remove oxazolone, then as with 1	4.0	
B. O-Acyl-N-ethylsalicylamides, **7** (recrystallized)		
1. Ester **7** added to 1.1 equiv of distilled GlyOEt in MeCN, 22°, 24 hr		7.2
2. Procedure 1, but DMF replaced MeCN		13.8
3. Procedure 2, but 1.1 equiv of GlyOEtHCl and 1.1 equiv of Et_3N replaced GlyOEt	1.0, 1.4, 1.0, 1.0	9.0, 11.1
4. Procedure 3 with 1.0 equiv of Et_3N		6.6
5. Procedure 3 with 1.2 equiv of Et_3N		15.4
6. Procedure 3 with 2.2 equiv of Et_3N		33.2

[a] Woodward and Olofson report a 55-min reaction time.

routes is of critical importance for future users and for designers of related isoxazole-derived coupling reagents. Data are presented in Table III.

The striking result is the substantial racemization observed under optimal conditions with these reagents. In acetonitrile, for example, the enol ester **5** (R = ZGly-L-Phe) appears to be roughly ten times more racemization prone than the corresponding *p*-nitrophenyl ester. In dimethylformamide, the phenolic ester **7** (R = ZGly-L-Phe) is twice as vulnerable to racemization as the *p*-nitrophenyl ester. In an attempt to detect racemate derived from oxazolone which might have been formed in the activation step of a Woodward's reagent coupling, the crude intermediary ester **5** from reaction A4 was washed with ether, with which the oxazolone is miscible. That A3 and A4 gave the same result supports the view that the racemate formed in Woodward's reagent couplings derives from the enol ester **5**.

The sequence, peptide ester, acylhydrazide, acylazide, coupled peptide, is widely believed to occur without racemization, and indeed, when carried out under practical coupling conditions, none of the carefully applied conventional tests has revealed detectable racemate.[21,29] More recently, instances of tertiary amine-catalyzed racemization of acylazides[30] and of racemization of vulnerable peptides[31] have appeared. To our knowledge, the data of Table IV provide the first evidence of racemization during acylazide couplings under optimal conditions.[32]

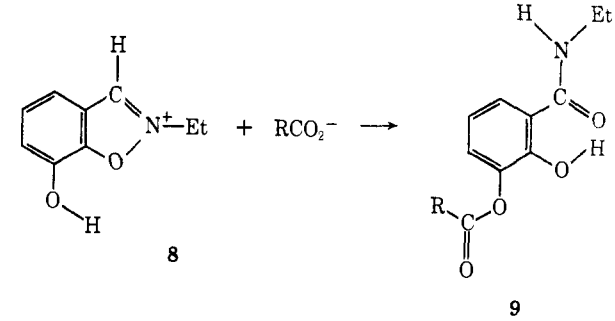
Table IV. Racemization during Acyl Azide Couplings

Conditions[a]	Amount of racemate,[b] % Anderson	Young
1. Acyl azide in Et_2O, 0–3° Distilled GlyOEt	0.011, 0.036	0.25, 0.35
2. Acyl azide in DMF, 0–3° Distilled GlyOEt	0.041	0.15, 0.50
3. Acyl azide in Et_2O, 0–3°, with 0.15 *M* Et_3N, 5 min, then GlyOEt	1.05	
4. Acyl azide in DMF, 0–3° GlyOEtHCl + 1 equiv Et_3N		0.66

[a] Acyl azides were prepared from the acyl hydrazides by reaction with $NaNO_2$–HCl in a water–ether mixture at 0–3°. For the Anderson tests the ether solution was washed at 0° with water and sodium bicarbonate, dried, and combined with 1.2–1.5 equiv of GlyOEt at 0° for 24 hr. For the Young tests, the procedure of Williams and Young[10] was followed: the ethereal solution from the diazotization was treated directly with 2 equiv of GlyOEt and the mixture was allowed to remain at room temperature for 24 hr. [b] Atypical fluctuations appear to result from varying acetic acid levels during the coupling step (D. S. Kemp and J. Rebek, unpublished observations).

It is clear from the data of Table IV that acyl azides do indeed yield small racemate, but not to a degree that they may be regarded as differing qualitatively from phenolic esters. For example, racemization for the Anderson azide coupling in ether at 0° is 0.02%, which is only fivefold less than the smallest result at 20° for the *p*-nitrophenyl ester couplings. Solvent effects appear to be small, and as expected, triethylamine results in a thirty to fiftyfold increase in racemate level.

Previously we have outlined[3d,33] a procedure which uses the 7-hydroxy-2-ethylbenzisoxazolium cation, **8**, to provide an oxazolone-free route from peptide acids to 3-acyloxy-2-hydroxy-N-ethylbenzamides, **9**, esters which undergo unusually facile aminolysis and unusually sluggish base-catalyzed racemization. Our preliminary report claimed no detectable racemate for Anderson peptide formation and only 1% racemization



8 9

(30) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **88**, 1338 (1966).

(31) H. Determann, J. Hener, P. Pfaender, and M. L. Reinartz, *Ann.*, **694**, 190 (1966).

(32) Since low levels of racemization were expected for azide couplings, the preparation of D-labeled free-starting materials was given unusually careful attention: [7-^{14}C]benzoyl-L-leucylhydrazide was prepared from [7-^{14}C]benzoyl-L-leucine, calculated to contain less than 0.005% of D-labeled enantiomer, and shown by limiting results with other couplings to contain less than 0.05% labeled enantiomer. Benzoyl-L-leucine was converted with diazomethane to its ester, which with hydrazine yielded the desired hydrazide. The leucine azide couplings were obtained with two independently prepared hydrazide samples. Benzyloxycarbonyl[1-^{14}C]glycyl-L-phenylalanylhydrazide was prepared by hydrazinolysis of the corresponding methyl or 2,3-dihydroxy-N-ethylbenzamide ester, and was subjected to a sequential dilution with unlabeled racemate. Material from the benzamide ester was estimated to contain considerably less than 0.001% D-labeled racemate. It should be noted that the methods of hydrazide synthesis require that racemate detected in the Anderson azide reaction must have been formed during the diazotization or coupling steps, while that observed in the Young azide coupling could have resulted from hydrazinolysis, diazotization, or coupling.

(33) D. S. Kemp, Abstracts, Joint Annual Meeting, The Chemical Society, ICI, RIC, Dublin, Ireland, April 1968, p 1.10; D. S. Kemp, Proceedings of the First American Peptide Symposium, Yale, 1968, M. Dekker, New York, N. Y., in preparation.

Table V. Racemization during Couplings with Esters, 9

Conditions[a]	Amount of racemate, %: Anderson	Young
1. Active ester was combined with 1.1 equiv of GlyOEtHCl and 1.1 equiv of Et_3N in DMF at 22° for 24 hr	0.13, 0.13, 0.14	0.77, 0.82
2. Active ester was combined with 1.1 equiv of distilled GlyOEt in DMF at 22° for 24 hr		0.67, 0.83
3. Active ester was combined in DMF with 0.4 *M* Et_3N for 12 hr at 22°. GlyOEtHCl was then added and reaction run for 12 hr at 22°	3.5	25.1
4. Procedure 2 was repeated at 0–3°	0.011, 0.013	0.22
5. Procedure 1 was repeated at 0–3°		0.23

[a] Active ester **9** was prepared by reaction of a purified sample of Z[1-^{14}C]Gly-L-PheOH or of [7-^{14}C]Bz-L-LeuOH with **8** in an aqueous pyridine buffer.

of **9**, R = ZGly-L-Phe, after 12 hr with 0.4 *M* triethylamine in dimethylformamide at 25°. Results for couplings with these esters are reported in Table V.

The important and extraordinary result is the minute extent of racemization observed when couplings with these esters are carried out in DMF at 0°. Comparison with data of Table IV indicates that the 3-acyloxy-2-hydroxy-N-ethylbenzamide couplings, for chiral integrity, are fully the equal of the most favorable acyl azide couplings. The effect of temperature is highly noteworthy—the amount of racemate increases five to tenfold as the reaction temperature is raised 20°. The chiral stability of these esters in the presence of triethylamine is confirmed, although the racemate level is three times our earlier estimate.

This stability of esters (**9**) under strongly basic conditions prompted our use[3d] of amino acid salts as amine components in coupling reactions.[34] We have reported[33] results using anhydrous tetramethylguanidine (TMG), which is capable of dissolving half of the common amino acids in dimethyl sulfoxide as their guanidinium salts. Couplings of glycine salts with esters (**9**) are conveniently run under any of three conditions: (1) by employing 2 equiv of tetraethylammonium glycinate in dimethyl sulfoxide solution, (2) by employing 1 equiv each of the glycine salt and TMG in dimethyl sulfoxide, or (3) by suspending glycine in dimethyl sulfoxide and stirring with the ester, **9**, and 2 equiv of TMG. Since glycine is sparingly soluble in the presence of excess TMG, the rate-limiting process is probably its solution, and procedure 3 provides a stringent racemization condition in which, throughout the coupling, TMG is present in excess.

The data of Table VI[35] indicate that when run at 0°, the coupling reactions of 3-acyloxy-2-hydroxy-N-ethylbenzamides with glycine salts equal or surpass those of acyl azides with ethyl glycinate in preservation of chiral integrity.

Table VI. Racemization during the Coupling of Esters (**9**) with Glycine Salts

Conditions[a]	Amount of racemate, %: Anderson	Young
1. DMSO 22°, 45 min		
(a) 2 equiv of $GlyO^-Me_4N^+$		1.1, 3.3
(b) 2 equiv of $GlyO^-Et_4N^+$		2.2
(c) 1 equiv of $GlyO^-Me_4N^+$ 1 equiv of TMG		3.7, 4.0
(d) 1 equiv of Gly 2 equiv of TMG	0.38, 0.40 1.04	9.9
2. DMSO–DMF 0°, 60 min, 1:1		
(a)[b] 2 equiv of $GlyO^-Me_4N^+$	0.009, 0.010	
(b) 2 equiv of $GlyO^-Et_4N^+$		0.14
(c) 1 equiv of $GlyO^-Me_4N^+$ 1 equiv of TMG	0.023	0.25
(d) 1 equiv of Gly 2 equiv of TMG	0.027	
(e) DMSO–H_2O 5:1 2 $GlyO^-Me_4N^+$		0.24

[a] Tetraalkylammonium salts were prepared by dissolving the amino acid in 1 equiv of aqueous tetraalkylammonium hydroxide and by lyophilizing the resulting solution, or azeotropically distilling its water with benzene. [b] Reaction run in DMF.

The foregoing assay experiments fully establish the high sensitivity, as well as the convenience and scope of this multiple dilution assay. As a still more stringent test of its sensitivity, we sought to measure the rate of racemization under mildly basic conditions of an amino acid residue imbedded in a peptide chain.

Starting material for this purpose was obtained from the low racemate experiments of Tables IV, V, and VI. The collected Anderson tripeptide ester was recrystallized and dissolved in ethanol containing triethylamine and salts, as indicated in Table VIII. Extrapolation of a plot of racemate *vs.* time to zero time revealed the presence of 0.0032% of labeled racemate in the ester sample.

Although the data must be regarded as preliminary, they clearly establish the practicality of the approach and indicate that under these conditions racemization proceeds with a half-time of roughly 300 years. Since the racemization process is only slightly slowed by triethylammonium ion, a large part of the catalytic effect must be attributed to triethylamine itself, rather than to ethoxide. Also striking is the failure to detect significant racemization after 27 days in dimethyl sulfoxide containing triethylamine; racemization in this solvent

(34) For a review of salt couplings see ref 4a, p 42 and 4b, p 69; also M. Bodanszky, *et al.*, *J. Amer. Chem. Soc.*, **85**, 991 (1963). The significant advance of using tetraalkylammonium salts, which are soluble in dipolar aprotic solvents, was reported by Wieland: T. Wieland and W. Kahle, *Ann.*, **691**, 212 (1966).

(35) The acids BzLeuGlyOH and ZGlyPheGlyOH obtained from the salt coupling reactions were converted without isolation to their ethyl esters by alkylation with triethyloxonium ion in an aqueous 2,6-lutidine buffer. To establish the accuracy of this conversion (yields for the alkylation lay in the range of 40–60%), a sample of ZGly-L-PheGlyOH containing 0.55% of Z[1-^{14}C]Gly-D-PheGlyOH was converted to ethyl ester, shown to contain 0.50% D enantiomer.

Table VII. Triethylamine-Catalyzed Racemation of ZGly-L-PheGlyOEt

Conditions[a]	Time, hr	Recovery, %	Amount of DL, %	Corrected[b] amount of DL, %	Calculated[c] DL, %
Ethanol					
1. 0.265 *M* Et_3N					
0.20 *M* $Et_4N^+Cl^-$					
a.	75.5	98	0.0050	0.0018	0.0017
b.	121.0	99	0.0059	0.0027	0.0027
c.	674.0	98	0.0185	0.0153	0.0153
Ethanol					
2. 0.266 *M* Et_3N	673.0	99	0.0130	0.0098	
0.028 *M* $Et_4N^+Cl^-$					
0.182 *M* $Et_3N^+HCl^-$					
3. DMSO	648	87	0.0042	0.001	
0.268 *M* Et_3N					

[a] Solutions were 2% in peptide; temp = 30.0°; μ = 0.2. [b] Corrected DL = observed DL − 0.0032. [c] Calculated for a pseudo-first-order rate constant of 2.3×10^{-7} hr^{-1} or 6.3×10^{-11} sec^{-1}.

appears to proceed at least ten times more slowly than in ethanol.

Discussion and Summary

The data of the preceding section in our opinion provide a clear demonstration of the sensitivity, convenience, and power of the "isotopic dilution microscope." Although the sensitivity of the method was not extended below ±0.002%, this limit was defined by the racemization levels encountered in the study; the Anderson test should be extendable without difficulty to 0.1 ppm if experimental situations requiring this sensitivity should arise.

As used here, the test employs separations of racemate from enantiomer, facilitated by a mass action effect. Extension to separations of diastereomers obviously will not possess this feature. In all other respects, the situations are comparable, and comparable sensitivity should be available for cases in which a reasonably clean separation of diastereomers is possible.

Although the test was devised with the peculiar requirements of the peptide synthetic problem in mind, it should have other interesting applications. The measurement of the rate of enolization of a peptide amide calls to mind earlier measurements of slow reaction rates,[36] and suggests measurement of the racemization rates of very weak chiral acids in water[37] for comparison with their more accessible exchange rates. The indispensable condition of racemate separability rests on the operation of the three factors of the first section, and it remains to be seen how generally these factors are met by substances whose crystallinity and solubility are such that the mass action effect can be encountered.

A striking general observation of this study is the correlation between results for the Anderson and Young tests. The average ratio for the nine cases which permit comparison is ten, and the most widely deviating ratios are four and twenty. The cases include some in which high levels of racemization occur by what is almost certainly an oxazolone mechanism, as well as others at a thirtyfold lower racemization level. Since it is difficult to rationalize a constant tenfold greater reactivity for the benzoylleucine system for processes as different as oxazolone formation and direct enolization, we regard this consistency as a cogent argument for a common oxazolone mechanism for all racemization phenomena of this study. The variation of extent of racemization with solvent for the *p*-nitrophenyl ester couplings and the remarkable variation with temperature for the 3-acyloxy-2-hydroxy-N-ethylbenzamide couplings require that the transition states for coupling and for racemization differ markedly, an observation which is not inconsistent with a racemization mechanism which proceeds through an equilibrated amide anion.[38]

Using either the Anderson or the Young dilution test, we were able to detect racemization incurred during azide couplings; the level lies roughly an order of magnitude below that observed with the best of the conventional active ester coupling reagents. When compared at 0° the 3-acyloxy-2-hydroxy-N-ethylbenzamides, **9**, are found to equal the acyl azides in minimizing racemization. The results obtained at low temperature for couplings between these esters and glycine salts in our opinion constitute the furthest refinement of the art of making peptide bonds with retention of configuration.

Experimental Section[39]

Starting Materials. Ethyl Benzyloxycarbonylglycyl-L-phenylalanylglycinate. a. O-Benzyloxycarbonylglycyl-N-ethylbenzamide. Recrystallized benzyloxycarbonylglycine, 60.0 g (0.29 mol), was dissolved in 300 ml of 1 *N* sodium hydroxide solution, the pH was brought to 4.5, and 100 ml of dichloromethane was added. The resulting mixture was chilled in ice and stirred vigorously as 70.0 g (0.30 mol) of finely powdered N-ethylbenzisoxazolium fluoroborate[40] was added slowly over the course of 2–3 min. After 5 min, the phases were separated, the aqueous phase was extracted with 3 × 100 ml of dichloromethane, and the organic phases were combined, extracted with 3 × 30 ml of aqueous sodium bicarbonate, dried, and evaporated *in vacuo* to a heavy syrup which was crystallized from ethyl acetate–cyclohexane to yield two crops, 90.0

(36) For a review, see: C. J. Collins, *Advan. Phys. Org. Chem.*, **2**, 40 (1964).

(37) A substance with a pK_a of 35 is expected to undergo exchange by reaction with hydroxide in water at a rate comparable to that observed for the triethylamine-catalyzed tripeptide ester racemization.

(38) D. S. Kemp and S. W. Chien, *J. Amer. Chem. Soc.*, **89**, 2745 (1967).

(39) Unless otherwise specified, solvents and reagents were spectro grade or reagent grade. Dimethyl sulfoxide was stored over Linde Molecular Sieves; dimethylformamide was distilled *in vacuo* at a pressure of 30 mm and stored over Molecular Sieves. Amino acids were Cal Bio Chem Co., A grade; [1-^{14}C] glycine and [7-^{14}C]benzoic acid were purchased from the New England Nuclear Co. Elemental analyses were performed by Scandinavian Microanalytical Laboratories of Copenhagen. A Packard 3375 Tri-Carbo liquid scintillation spectrometer was used for all counting experiments. Efficiencies were determined by internal or external standardization. Quenching effects for the peptide derivatives of the study were very small. For counting levels near background, averages of quadruplicate 200-min counts were taken. Counting liquid was prepared by dissolving 25 g of naphthalene, 1.75 g of 2,5-diphenyloxazole, and 75 mg of 1,4-bis-2(4-methyl-5-phenyloxazoyl)benzene in 250 ml of reagent grade dioxane.

(40) D. S. Kemp and R. B. Woodward, *Tetrahedron*, **21**, 3029 (1965).

g, mp 93.0–94.5°; 2.5 g, mp 92.5–94.0°; total yield, 95.5%. For analysis, the substance was recrystallized from dichloromethane–cyclohexane, mp 94.3–95.0°.

Anal. Calcd for $C_{19}H_{21}N_2O_5$: C, 64.03; H, 5.66; N, 7.86. Found: C, 63.75; H, 5.98; N, 8.01.

b. Benzyloxycarbonylglycyl-L-phenylalanine. To a solution of 60.0 g (0.28 mol) of methyl L-phenylalaninate hydrochloride,[41] mp 158.5–161.0°, in 120 ml of DMF was added 26 g (0.26 mol) of triethylamine, followed by 93 g (0.26 mol) of O-benzyloxycarbonylglycyl-N-ethylsalicylamide. After 36 hr the mixture was diluted with 300 ml of water and 150 ml of dichloromethane. The organic phase was separated, washed successively with 2 × 200 ml of water, 3 × 50 ml of 0.5 *N* hydrochloric acid, 3 × 200 ml of 0.5 *N* aqueous sodium hydroxide, and 100 ml of water. Concentration yielded *ca.* 100 g of oil which was taken up in 300 ml of 70% acetone–water; the resulting mixture was stirred during the continuous slow addition by syringe drive of 13 g of sodium hydroxide in 100 ml of water (4 hr). During the addition the pH as measured by short-range indicator paper did not exceed 10. The resulting solution was stripped of acetone on a rotary evaporator, extracted with 100 ml of ether, acidified to pH 1 with hydrochloric acid, and extracted with 3 × 30 ml of ethyl acetate. The pooled extracts were washed with 3 × 30 ml of 0.5 *N* hydrochloric acid and with 50 ml of water, then were diluted with 200 ml of ethyl acetate and evaporated *in vacuo* to a syrup which was crystallized from acetone–hexane to yield a first crop of 76.0 g and a second crop of 2.8 g (85%). The crude acid was recrystallized from acetonitrile (1.5 ml/g), from ethyl acetate (5 ml/g), and again from acetonitrile to yield 60 g, mp 129.5–131.0°, $[\alpha]^{25}D$ +39.2° (*c* 2.0, EtOH), lit. 126°, +41.9°.[42]

c. O-Benzyloxycarbonylglycyl-L-phenylalanyl-N-ethylsalicylamide. A solution of 40.5 g (0.114 mol) of finely powdered benzyloxycarbonylglycyl-L-phenylalanine in 110 ml of 1.0 *N* sodium hydroxide, 100 ml of water, and 10 ml of pyridine was brought to pH 5.5 with hydrochloric acid. The solution was chilled in ice, stirred vigorously, and 300 ml of dichloromethane was added, followed by 30 g (0.128 mol) of finely powdered N-ethylbenzisoxazolium fluoroborate, added as a fine, continuous stream over the course of 5 min. After a further 5 min of stirring the phases were separated, and the aqueous phase was extracted with 3 × 100 ml of ethyl acetate and discarded. The pooled organic phases were extracted with 3 × 50 ml of *3 N* hydrochloric acid, 2 × 20 ml of water, and 3 × 30 ml of saturated sodium bicarbonate, then were dried over magnesium sulfate and evaporated. Recrystallization of the resulting solid from ethyl acetate–hexane yielded two crops, 53.7 g, mp 138.0–140.0°, and 1.4 g, mp 136.0–137.5°; total yield, 55.1 g, 97.0%. For analysis the ester was recrystallized from ethyl acetate and acetonitrile, mp 141.4–142.0°, $[\alpha]^{22}D$ −22.5° (*c* 2.0, acetonitrile).

Anal. Calcd for $C_{28}H_{29}N_3O_6$: C, 66.78; H, 5.80; N, 8.35. Found: C, 66.78; H, 5.92; N, 8.44.

d. Ethyl Benzyloxycarbonylglycyl-L-phenylalanylglycinate. To a solution of 20 g (0.14 mol) of ethyl glycinate hydrochloride and 13 g (0.13 mol) of triethylamine in 100 ml of DMF (precipitated triethylammonium chloride was not removed) was added 53.7 g of O-benzyloxycarbonylglycyl-L-phenylalaninyl-N-ethylsalicylamide. After 24 hr the mixture was diluted with 300 ml of water and extracted with 4 × 40 ml of dichloromethane. The pooled extracts were extracted with 2 × 20 ml of 0.5 *N* hydrochloric acid, 3 × 80 ml of 1 *N* sodium hydroxide, and 3 × 100 ml of water, then were dried over magnesium sulfate and evaporated. The residue was recrystallized from 100 ml of ethanol to yield 40.7 g and a second crop of 2.5 g after concentration. The combined 43.2 g (91.7%) was recrystallized three times from 200 ml of ethanol to yield 38 g of solid, mp 118.0–119.5°, $[\alpha]^{25}D$ −12.5° (c 2.0, ethanol); lit. 118–119°, −12.7°.[43]

e. Benzyloxycarbonylglycyl-DL-phenylalanine and Ethyl Benzyloxyglycyl-DL-phenylalanylglycinate. Procedures analogous to those described above were followed. Reaction of O-benzyloxycarbonylglycyl-N-ethylsalicylamide with methyl DL-phenylalaninate hydrochloride and triethylamine, followed by slow saponification, yielded benzyloxycarbonylglycyl-DL-phenylananine, mp 161.5–162.0°, lit. 161°,[44] after two recrystallizations from acetonitrile. The volume of ethyl acetate used to extract this insoluble substance was increased to 15 ml/g. After acidic and water extractions, the phases were separated, and the organic phase was stripped *in vacuo;* additional ethyl acetate was added to remove water azeotropically. Use of chemical drying agents resulted in losses and contamination.

By procedure c, the DL acid was converted to its N-ethylsalicylamide ester, mp 126.8–127.8°.

Anal. Calcd for $C_{28}H_{29}N_3O_6$: C, 66.78; H, 5.80; N, 8.35. Found: C, 66.77; H, 5.92; N, 8.37. Reaction of this ester with ethyl glycinate by procedure d yielded ethyl benzyloxycarbonylglycyl-DL-phenylalanylglycinate, mp 131.7–132.2° after recrystallization from acetonitrile; lit. 132–134°.[43]

***p*-Nitrophenyl Benzyloxycarbonylglycyl-DL-phenylalaninate.** A hot solution of 1.1 g (3.1 mmol) of benzyloxycarbonylglycyl-DL-phenylalanine and 0.6 g (4.3 mmol) of *p*-nitrophenol in 8 ml of acetonitrile was quickly chilled in ice and treated with 0.7 g (3.4 mmol) of dicyclohexylcarbodiimide. After 3 hr, the solution was filtered and evaporated, and the residue was dissolved in 20 ml of dichloromethane. The resulting solution was extracted twice with 10 ml of sodium bicarbonate, once with 0.1 *N* hydrochloric acid, dried over magnesium sulfate, and evaporated. The residue was recrystallized from ethyl acetate–hexane to yield 1.1 g, mp 135.0–136.0° (75%), lit. 135.5–136.0°.[19]

***p*-Nitrophenyl Benzyloxycarbonylglycyl-L-phenylalaninate.** The procedure of Goodman and Steuben[19] was followed, with modifications. A solution of 5.0 g (12 mmol) of *p*-nitrophenyl benzyloxycarbonyl-L-phenylalaninate[45] in 25 ml of acetic acid containing 6 g of hydrogen bromide was allowed to stand for 20 min, then was treated with 100 ml of anhydrous ether. The resulting finely divided pale yellow salt was collected, washed with ether, and dried *in vacuo;* yield, 4.15 g (92%), mp 215–216° dec. The 4.15 g (11.4 mmol) was suspended in 25 ml of acetonitrile, chilled in ice, and swirled vigorously as 1.10 g (10.8 mmol) of triethylamine in 10 ml of acetonitrile was added in one portion. During *ca.* 20 sec the salt dissolved, and needles of triethylammonium bromide separated. A solution of 2.37 g (11.3 mmol) of benzyloxycarbonylglycine in 25 ml of acetonitrile, prepared by heating to effect solution, then chilling quickly in ice, was added before appreciable crystallization had occurred and was followed directly by a solution of 2.30 g of dicyclohexylcarbodiimide in 20 ml of acetonitrile. After a few minutes a curdy precipitate, presumably the benzyloxycarbonylglycine salt of the amino ester, separated and dissolved slowly over the course of 1 hr. After 6 hr the mixture was filtered, and the filtrate was evaporated. The residue was taken up in dichloromethane, and the solution was extracted with 2 × 10 ml of 0.1 *N* hydrochloric acid, 2 × 20 ml of saturated sodium bicarbonate, and a final 10 ml of acid, then was dried over magnesium sulfate and evaporated. The residue was crystallized by solution in the minimum volume of warm 1:1 dichloromethane–ethyl acetate followed by addition of hexane. Recrystallization from 40 ml of hot ethanol (kieselguhr) yielded 4.0 g of needles, mp 144.0–145.0°, 84%; lit. mp, 145–146°.[19] Material of this purity could be satisfactorily recrystallized from acetonitrile (*ca.* 2 ml/g), or from ethanol; best mp, 145.7–146.8°. Critical aspects of this preparation are the state of subdivision of the hydrobromide salt, which must dissolve immediately upon addition of triethylamine, the rapidity of addition of acid and diimide, and the care taken with the initial recrystallizations. Failure in any of these aspects can result in a low-melting, gel-prone ester which resists purification.

Preparation of D-Labeled DL-Phenylalanine Derivatives. 2a. Benzyloxycarbonyl[1-^{14}C]glycyl-D-phenylalanine + Benzyloxycarbonylglycyl-L-phenylalanine.[46] A sample of benzyloxycarbonyl-[1-^{14}C]glycine was prepared from a diluted sample of [1-^{14}C]-glycine[47] and recrystallized twice from chloroform, mp 119.5–120.5°, specific activity 1.08 μCi/mmol, 1.15 × 10^4 dpm/mg. This acid was converted by procedure 1a to its N-ethylsalicylamide ester, and thence by reaction with methyl D-phenylalaninate to methyl benzyloxycarbonyl[1-^{14}C]glycyl-D-phenylalaninate, which was saponified as described in 1b. The crude dipeptide acid thus obtained was dissolved in acetonitrile containing an equivalent weight of benzyloxycarbonylglycyl-L-phenylalanine. The precipitated ra-

(41) R. H. Boissonnas, S. Guttmann P. A. Jaquenand, and J. P. Walker, *Helv. Chim. Acta*, **39**, 1421 (1956).

(42) J. P. Greenstein and M. Winitz, "The Chemistry of the Amino Acids," John Wiley & Sons, Inc., New York, N. Y., 1961, p 1172.

(43) J. P. Greenstein and M. Winitz, "The Chemistry of the Amino Acids," John Wiley & Sons, Inc., New York, N. Y., 1961, p 1130.

(44) See ref 42.

(45) Mp 126.5–127.5°, prepared as described[19] from benzyloxycarbonyl-L-phenylalanine, mp 86.5–87.5°.

(46) Henceforth *DL will be used to symbolize a racemic mixture containing D enantiomer labeled with ^{14}C.

(47) H. E. Carter, R. L. Frank, and H. W. Johnston, *Org. Syn.*, **3**, 168 (1955).

cemate was recrystallized from acetonitrile, mp 160.5–162.5°, specific activity 0.53 μCi/mmol, 3.28×10^3 dpm/mg.

Recrystallization of 6.34 g of this substance from acetonitrile containing 3.19 g of unlabled L enantiomer resulted in the recovery, after an additional recrystallization, of 6.0 g racemate, specific activity 0.53 μCi/mmol. A sample of L recovered from the filtrate had specific activity of 2.9×10^{-3} μCi/mmol, hence the original sample of racemate contained 0.28% of its activity in its L enantiomer.

2b. Ethyl Benzyloxycarbonyl[1-C^{14}]glycyl-*DL-phenylalanylglycinate, Benzyloxycarbonyl[1-^{14}C]glycyl-*DL-phenylalanylhydrazide, and Benzyloxycarbonyl[1-^{14}C]glycyl-*DL-phenylalanylglycine. By procedure 1c, a sample of benzyloxycarbonyl[1-^{14}C]glycyl-*DL-phenylalanine was converted to its N-ethylsalicylamide ester, a portion of which was treated with 4 equiv of hydrazine in ethanol. After 20 min, the precipitated hydrazide was collected, recrystallized from ethanol, and dried, mp 170.0–171.5°, specific activity 0.51 μCi/mmol, 3.07×10^3 dpm/mg. The remaining ester was treated with ethyl glycinate as described in 1d to yield tripeptide ester, mp 131.7–132.2°, specific activity 0.52 μCi/mmol, 2.58×10^3 dpm/mg. From the extent of racemization determined subsequently for this coupling reaction, this sample was estimated to contain 0.6% of its activity in L enantiomer.

A sample of 0.263 g of the above tripeptide ester was combined with 0.212 g of inactive ester, dissolved in 30 ml of 70% acetone–water, and subjected to the slow addition by syringe drive of 1.05 equiv of 0.1 *N* sodium hydroxide. The acetone was removed *in vacuo*, and the resulting solution was extracted with ethyl acetate and acidified. The resulting solid was collected, dried, and recrystallized from ethyl acetate to yield 0.33 g of benzyloxycarbonyl-[1-^{14}C]glycyl-*DL-phenylalanylglycine, mp 146.5–148.0°, specific activity 0.28 μCi/mmol, 1.55×10^3 dpm/mg; calcd specific activity 1.53×10^3 dpm/mg.

2c. *p*-Nitrophenyl Benzyloxycarbonyl[1-^{14}C]glycyl-*DL-phenylalaninate. By the procedure given above for the L-*p*-nitrophenyl ester, carbobenzoxy[1-^{14}C]glycine was combined with *p*-nitrophenyl-D-phenylalaninate. The crude dipeptide ester was dissolved in ethyl acetate containing 1 equiv of pure unlabeled L-*p*-nitrophenyl ester. The resulting solid was recrystallized from ethyl acetate to yield asymmetrically labeled racemic ester, mp 135.5–136.5°, specific activity 1.9×10^3 dpm/mmol.

Control Experiments for the Anderson Test. Separability of Racemate from Benzyloxycarbonylglycyl-L-phenylalanine. A solution containing 1.036 g of benzyloxycarbonylglycyl-L-phenylalanine and 0.192 g of benzyloxycarbonyl[1-^{14}C]glycyl-*DL-phenylalanine, specific activity 4.98×10^2 dpm/mg in 40 ml of acetonitrile was stirred vigorously at 0° and treated with a microdrop of a freshly precipitated slurry of racemate crystals. After an hour, 0.170 g of solid was collected, mp 160–162°, specific activity 5.05×10^2 dpm/mg, 89.6% of the total D activity. In the filtrate was dissolved 0.073 g of unlabeled racemate, and cooling and seeding resulted in collection of a further crop of solid; 0.101 g, mp 159–161°, specific activity 0.95×10^2 dpm/mg; total recovered activity: 99.6%. Upon standing overnight the solution deposited 0.61 g of L enantiomer, mp 130–131°, specific activity 0.3 dpm/mg.

Separability of Racemate from Benzyloxycarbonylglycyl-L-phenylalanylhydrazide. A solution of 0.329 g of benzyloxycarbonylglycyl-L-phenylalanylhydrazide and 0.114 g of benzyloxycarbonyl[1-^{14}C]glycyl-*DL-phenylalanylhydrazide, specific activity 4.95×10^2 dpm/mg, in 50 ml of ethanol was stirred vigorously for 3 hr at 0° after seeding with a few drops of freshly precipitated racemate in ethanol; 0.098 g of solid was collected, mp 168.5–170.5°, specific activity 4.78×10^2 dpm/mg. A further 0.076 g of unlabeled racemate was dissolved in the filtrate, and seeding and chilling yielded after 6 hr, 0.081 g of solid, mp 169.0–170.5°, specific activity 0.92×10^2 dpm/mg; total recovered activity: 96.3%. The filtrate was taken to dryness, and the residue was crystallized from ethanol to yield 0.200 g of L enantiomer, specific activity 0.4 dpm/mg.

Separability of Racemate from *p*-Nitrophenyl Benzyloxycarbonylglycyl-L-phenylalaninate. A solution in 13 ml of ethanol of 0.376 g of *p*-nitrophenyl benzyloxycarbonylglycyl-L-phenylalaninate and 0.014 g of *p*-nitrophenyl benzyloxycarbonyl[1-^{14}C]glycyl-*DL-phenylalaninate, specific activity 1.9×10^3 dpm/mg, was cooled to 10° and seeded with L enantiomer. After 20 min, 0.211 g of solid, mp 145–147° was collected, and after 30 min at 0° an additional 0.072 g, mp 145.5–146.5 was obtained; recovery, 0.283 g, 75%, specific activity 0.6 dpm/mg. The isolated L ester contained less than 0.6% of the total activity.

Determination of the Racemate Concentration of Ethyl Benzyloxycarbonylglycyl-L-phenylalanine. A solution of 0.864 g of ethyl benzyloxycarbonylglycyl-L-phenylalanylglycinate and 0.0942 g of ethyl benzyloxycarbonyl[1-^{14}C]glycyl-*DL-phenylalanylglycinate, specific activity 2.59×10^3 dpm/mg, in 40 ml of ethanol was seeded with a microdrop of a freshly precipitated slurry of racemate in ethanol. After 6 hr at 15°, 0.0724 g of racemate was isolated, specific activity 2.50×10^3 dpm/mg. As indicated below (Table VIII) this procedure was repeated five times.

Table VIII. Purity of the Diluting Sample of Anderson Tripeptide

Recovery	Wt DL used, mg	Wt of L used, mg	Wt of DL isolated, mg	Sp act of DL, dpm/mg × 10^{-3}	Calcd[a] sp act, dpm/mg × 10^{-3}
0				2.59	2.59
1	94.2	864	72.4	2.50	2.54
2	69.8	477	68.3	2.49	2.50
3	61.3	405	40.2		2.47
4	40.2	339	31.3	2.28	2.42
5	25.3	115	15.9	2.39	2.39

[a] Calculated for an assumed 0.23% of racemate.

Solubility Determinations. Solutions of benzyloxycarbonylglycyl-L-phenylalanine in acetonitrile were prepared, and duplicate 3–5 ml aliquots of each solution were transferred to 10-ml ampoules containing *ca.* 100 mg of benzyloxycarbonyl[1-^{14}C]glycyl-*DL-phenylalanine. The tubes were sealed, and one ampoule of each pair was warmed until almost all solid had dissolved. The tubes were equilibrated for 20 hr with agitation in a bath at 30.0°. The tubes were then opened, and a 0.500-ml aliquot was withdrawn from each with a micropipet tipped with cotton and transferred to a counting vial. The data of Figure 1 are averages of values obtained for the duplicates. For runs at high concentrations of L enantiomer, aliquots were evaporated and residue weighed to determine the total solid in solution. For the case of the ethyl benzyloxycarbonylglycylphenylalanylglycinates, ethanol was used as the solvent and equilibration was continued for 50 hr. Even after this time, equilibration was not complete, and the warmed solutions contained an *ca.* 10% greater amount of racemate. Again, the average of values observed for the warmed and unwarmed duplicate values were used to approximate the equilibrium solubility.

The Anderson Test. 3. Preparation of Starting Materials, Free of Labeled D Enantiomer. a. Benzyloxycarbonyl[1-^{14}C]glycyl-L-phenylalanine. A solution of 4.0 g of benzyloxycarbonyl-[1-^{14}C]glycyl-L-phenylalanine, twice recrystallized from acetonitrile, and 0.80 g of unlabeled benzyloxycarbonylglycyl-DL-phenylalanine in 140 ml of acetonitrile was stirred vigorously at 0° and seeded with a finely ground, freshly precipitated slurry of racemate. After 2 hr, 0.90 g of solid was collected, and a further portion of 0.85 g of racemate was dissolved in the filtrate. The seeding and crystallization were carried out as before, and after 3 hr 0.80 g of solid was collected. A third 0.80-g portion was dissolved and 1.0 g of solid was collected. The filtrate was concentrated, and the residue was recrystallized from acetonitrile, then twice from ethyl acetate to yield 2.80 g of solid, mp 128.5–129.5°. Assuming the recovery of racemate to have been 90% for each dilution, and the initial and final racemic content to have been 0.5%, the sample is calculated to contain less than 0.0001% of its activity in D enantiomer. For processes involving moderate (0.1–5.0%) racemization, samples with specific activities in the range of $1–5 \times 10^3$ dpm/mg (0.2 μCi/mmol) were used; for the more sensitive assays, a specific activity of 2×10^4 dpm/mg (3 μCi/mmol) was employed.

b. Benzyloxycarbonyl[1-^{14}C]glycyl-L-phenylalanylhydrazide. The hydrazide was prepared by hydrazinolysis of methyl benzyloxycarbonyl[1-^{14}C]glycyl-L-phenylalaninate and was recrystallized from ethanol, mp 107° sh, 140.0–141.5°. A solution of 2.0 g of labeled L hydrazide in 50 ml of hot ethanol was diluted with 250 ml of cold ethanol and treated with a solution of 0.60 g of unlabeled benzyloxyglycyl-DL-phenylalanylhydrazide. The solution was cooled to room temperature, stirred, and seeded with a freshly precipitated fine slurry of racemate. After 12 hr, 0.42 g of DL hydrazide was collected, and the filtrate was combined with a second 0.60-g portion of unlabeled racemate, dissolved in 40 ml of ethanol. Seeding and stirring for 8 hr yielded 0.55 g of recovered racemate.

Addition of a third 0.60-g portion of racemate, followed by seeding and stirring for 12 hr yielded 0.83 g of recovered racemate. The filtrate was taken to dryness *in vacuo* and the resulting residue was recrystallized twice from 30 ml of ethanol to yield 1.47 g of L hydrazide, sh 107°, mp 142.5–143.5°. Provided the initial and final samples of hydrazide contained less than 0.2% racemate, and the recovered racemate was 80% pure, the final sample is calculated to contain less than 0.0001% of its activity in D enantiomer.

c. *p*-Nitrophenyl Benzyloxycarbonyl[1-^{14}C]glycyl-L-phenylalaninate. A solution of 4.0 g of L-*p*-nitrophenyl ester, prepared as described above, mp 144.0–145.0°, in 180 ml of ethanol containing 0.30 g of unlabled DL ester was cooled and carefully seeded with L ester. After 2 hr a first crop of 2.7 g of L ester, mp 145.0–146.0° was collected and recrystallized from 105 ml of ethanol containing 0.13 g of unlabeled DL ester. The resulting ester was recrystallized from acetonitrile to yield 1.70 g of L ester, mp 144.8–146.2°. Assuming the chiral purity of crystallized ester to be that of the separability experiment (>99.9%), the final sample of ester is calculated to contain less than 0.0002% of its activity in D enantiomer.

d. 3-Benzyloxycarbonyl[1-^{14}C]glycyl-L-phenylalanyloxy-2-hydroxy-N-ethylbenzamide. A solution of 2.73 g (7.67 mmol) of benzyloxycarbonyl[1-^{14}C]glycyl-L-phenylalanine, prepared as in 1a above, in 25 ml of 0.1 *N* sodium hydroxide and 1.5 ml of pyridine was brought to pH 4.5–5.0 by the addition of hydrochloric acid, overlayered with 30 ml of ethyl acetate, chilled, and stirred vigorously as 2.0 g (8.0 mmol) of 7-hydroxy-2-ethylbenzisoxazolium fluoroborate[32] was added slowly in 50–100-mg portions over 20 min. After the addition was complete, the solution was stirred for an additional 5 min, whereupon the layers were separated, and the aqueous layer was extracted twice with 10-ml portions of ethyl acetate. The pooled organic phases were extracted with two 20-ml portions of 1 *N* hydrochloric acid, 20 ml of 0.5 *N* sodium bicarbonate, and 10 ml of water. After drying over magnesium sulfate, the extracts were evaporated, and the residue was crystallized from ethyl acetate–hexane; yield, 3.6 g, 91%, mp 115.8–116.5°; $[\alpha]^{22}D$ −28.4° (*c* 2.0, acetonitrile).

Anal. Calcd for $C_{25}H_{27}N_3O_7$: C, 64.73; H, 5.63; N, 8.09. Found: C, 64.74; H, 5.83; N, 7.77.

4. Assay for Racemate. a. General Procedure. An appropriately activated derivative of isotopically pure benzyloxyglycyl[1-^{14}C]glycyl-L-phenylalanine of known specific activity *y* is combined with ethyl glycinate, and the neutral product is isolated by a suitable extraction procedure, with care taken to prevent fractionations arising from product crystallization. The product is transferred quantitatively to a 25-ml volumetric flask and dissolved in ethanol. A 1.00-ml aliquot of the solution is transferred to a supersaturated solution containing a known amount, *a*, of unlabeled ethyl benzyloxycarbonylglycyl-L-phenylalanylglycinate. A sample of ester from this solution is isolated, crystallized, and transferred to a counting vial. The weight of L-tripeptide ester formed is then

$$n = 25 \cdot \frac{az}{y - z}$$

where *z* is the observed specific activity.

The remaining 24 ml is transferred quantitatively to an erlenmeyer flask which contains an equal weight of unlabeled L-tripeptide ester and 5–10% of total L weight of an accurately weighed sample of unlabeled racemate (*m*).[48] Sufficient ethanol is added to make the solution 3–4% in total peptide, and the solution is heated to boiling to dissolve any seeds which may adhere to the walls and rim of the flask. When cool, the solution is carefully seeded with a trace of a freshly prepared slurry in ethanol of rapidly crystallized racemate.[49] The flask is sealed with aluminum foil and chilled at 15° for 4–6 hr with occasional gentle swirling. The resulting crop of racemate is collected, dried, and weighed, and its melting point checked. The racemate is added to an erlenmeyer containing an 8–10-fold weight excess of L-tripeptide, and sufficient ethanol is added to make a solution 2–4% in total peptide. The steps of dissolution, seeding, and collection are then repeated. After three (or in some cases, four) such dilutions, the isolated racemate is checked for melting point, recrystallized if necessary from a small volume of ethanol, and transferred to a vial for counting. The filtrate from the last dilution is concentrated and seeded with L peptide; the resulting solid is checked for melting point and counted. The activity of racemate is corrected, if necessary, for residual L activity, which is compared with that calculated from the dilution data.[50] The fraction racemized is then calculated from formula 1 (see text). The dilutions of this study employed L-tripeptide whose racemate level had been estimated as described to be 0.23% DL. Correction for dilution by the amount of D enantiomer in the L diluent can be made if desired by using formula 3. Use of this correction increased the value of α by around 10% for the cases studied.

For α small, the formula reduces to $\alpha = 2mx/ny$ where it is seen that errors in α are attributable in equal part to errors in *m*, *x*, and *y*. Of these numbers, *n* and *y*, the yield of L and the initial specific activity, are known with accuracy; *m*, the quantity of racemate added, is uncertain to the extent that the starting activated dipeptide derivative contained unlabeled racemate. Since it is unlikely from the manner of its purification and the checks on the separability of racemate that this amount could exceed 1%, this error could result at most in an uncertainty of 10–15% in the value of α. The most serious source of error for small values of α is the specific activity of *x* of the racemate obtained from the last dilution. Where background counts and residual L activity make major contributions to the observed racemate activity, the value of *x* is correspondingly uncertain. All disintegrations per minute reported for low activity samples are averages for 3–5 200-minute counting periods.

b. Racemization in the Coupling of 3-Benzyloxycarbonyl[1-^{14}C]-glycyl-L-phenylalanyloxy-2-hydroxy-N-ethylbenzamide with Ethyl Glycinate in DMF at 0–3°. Freshly distilled ethyl glycinate, 0.126 g (1.2 mmol), was weighed into a stoppered 10-ml round-bottomed flask, 3 ml of DMF was added, and the solution was cooled to *ca.* −20° and treated with a cooled solution of 0.530 g of the Anderson benzamide ester, (1.02 mmol), 1.24×10^4 dpm/mg, in 1 ml of DMF. The mixture was allowed to warm to 0–3° in the refrigerator. After 50 hr, the solution was transferred to a separatory funnel with the help of 5 ml of dichloromethane, 30 ml of water was added, the phases were separated, and the aqueous phase was extracted thrice with 3-ml portions of dichloromethane. The organic phases were pooled, washed with 30 ml of water, then with 10 ml of 0.1 *N* sodium hydroxide, then dried, and evaporated. The resulting residue was combined with 70.90 mg of unlabeled racemate and 455.7 mg of unlabeled L, and the whole dissolved in 40 ml of ethanol. Seeding and occasional swirling at 15° over an 8-hr period yielded 49.5 mg of racemate. From the filtrate was recovered L peptide, mp 118.5–119.5°, specific activity 6.73×10^3 dpm/mg, calcd yield, 93.4%. Dissolution of the racemate in 18 ml of ethanol containing 372.7 mg of L tripeptide yielded after seeding and cooling 41.43 mg of racemate, recrystallized from a solution containing 288.9 mg of L tripeptide in 12 ml of ethanol to yield 35.83 mg of racemate, recrystallized for the third time from 10 ml of ethanol containing 263.9 mg of L tripeptide. There was thus obtained 27.0 mg of racemate, mp 131.0–132.5°, specific activity 5.62 dpm/mg.[51] From the filtrate was isolated 138.9 mg of L tripeptide, mp 118.0–119.2°, specific activity 2.06 dpm/mg, calcd specific activity 1.8 dpm/mg; corrected DL activity: 5.62–1.03 = 4.6 dpm/mg; calcd racemization: $(2 \times 70.90 \times 4.6)/(421.0 \times 1.46 \times 104) \times 100 = 0.0107\%$; corrected racemization: 0.0113%.

c. Racemization during *p*-Nitrophenyl Ester or N-Ethylsalicylamide Ester Couplings. Samples of *p*-nitrophenyl benzyloxycarbonyl[1-^{14}C]glycyl-L-phenylalaninate, freed of isotopically labeled D enantiomer as described above, were used. Samples of O-benzyloxycarbonyl[1-^{14}C]glycyl-L-phenylalanyl-N-ethylsalicylamide were prepared from D label free dipeptide acid by procedure 1c. The

(48) As an alternative to the initial yield-determining dilution, a sample of L remaining in the filtrate from this solution may be reisolated, purified, and assayed.

(49) The seeding operation critically determines the ease and success of these fractional crystallizations. Best results are obtained by dissolving *ca.* 300 mg of racemate in a few milliliters of hot ethanol in a small test tube, then chilling the tube in an ice bath while vigorously triturating the mixture with a glass rod. The resulting fine cream is stored moist at 0° for no more than a day or two and diluted if necessary with a few drops of ethanol. Seeds are transferred to the supersaturate on the outside of a melting point capillary. Several weighings after allowing such residues to dry indicated that less than 0.05 mg of racemate is introduced, an amount which cannot affect the accuracy of the assay.

(50) Contamination of racemate by crystallizing L results in a drop in the efficiency of dilution which is manifested in a higher than calculated activity of L recovered from the final dilution. In the experiments here described these numbers always agreed to within ±15%. Occasionally massive spars or clusters of L crystals were observed among the flocculent microneedles of racemate. Rewarming, slight dilution, and careful reseeding resulted in separation of pure racemate.

(51) Racemate (26.96 mg) in 13 ml of dioxane-based counting solution gave 160.1 cpm with a background of 26.3 cpm and a counting efficiency of 88.4%.

active ester was added to a solution of 1.1 to 1.2 equiv of freshly distilled ethyl glycinate in the appropriate solvent. Initial reagent concentrations were 0.2–0.5 *M*. The temperature was ~22°, that of an air conditioned laboratory, with no attempt at precise control. After 24 hr the mixture was diluted with dichloromethane and extracted with 0.1 *N* hydrochloric acid, 0.5 *N* sodium carbonate, and water, then dried and evaporated.

d. Racemization during Couplings with Woodward's Reagent K. A solution of 573.9 mg (1.61 mmol) of isotopically purified benzyloxycarbonyl[1-^{14}C]glycyl-L-phenylalanine, and 160 mg of triethylamine (1.58 mmol) in 6 ml of acetonitrile was stirred and chilled in an ice bath, 410 mg (1.62 mmol) of N-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K, Aldrich) was added, and the slurry was stirred in the cold for 30 min. The solution was divided in two; half was concentrated at 0° and the resulting residue slurried and triturated three times in cold ether (20 ml) which was then discarded; the residue was then dissolved in 3 ml of acetonitrile. Both solutions were then treated with 0.13 g of distilled ethyl glycinate (0.13 mmol). After 24 hr at room temperature (22°) the solvent was removed, 10 ml of dichloromethane was added and then extracted successively with 0.1 *N* hydrochloric acid, 0.5 *N* sodium bicarbonate, and water, then dried and evaporated. The residue was then treated as described in the general procedure.

e. **Racemization during Acylazide Couplings.** To 0.637 g (1.72 mmol) of benzyloxycarbonyl[1-^{14}C]glycyl-L-phenylalaninylhydrazide (specific activity 1.43×10^4 dpm/mg) was added 8 ml of water and 1.5 ml each of 12 *N* hydrochloric acid and glacial acetic acid. The solution was cooled to −5–0° in an ice–salt bath, and the resulting paste was stirred vigorously and overlayered with 10 ml of diethyl ether, then treated dropwise with a solution of 0.25 g of sodium nitrite in 0.5 ml of water. After 5 min the ether layer was transferred to an ice-cooled separatory funnel and extracted with 8 ml of chilled water and 10 ml of chilled, saturated sodium bicarbonate. The ether phase was dried over magnesium sulfate and transferred to a chilled flask containing 210 mg of distilled ethyl glycinate in 5 ml of ether. After 24 hr at 0–3°, the ether was removed, and the residue was dissolved in 40 ml of ethanol along with 73.81 mg of DL tripeptide and 501.0 mg of L tripeptide. After three successive dilutions with inactive L tripeptide, there was obtained 17.02 mg of racemate, mp 131.3–132.3°, specific activity 16.2 dpm/mg; recovered L activity, 1.92 dpm/mg; corrected racemate activity, 15.2 dpm/mg; yield = 72.3%; $\alpha = (2 \times 73.8 \times 15.2)/(549 \times 1.20 \times 10^4) = 3.4 \times 10^{-4}$ or 0.034%.

f. **Racemization during Couplings with Glycine Salts.** A 50-ml erlenmeyer flask equipped with a large magnetic stirring bar was chilled in an ice–salt bath and filled with 0.237 g (2.05 mmol) of tetramethylguanidine in 3 ml of dimethyl sulfoxide containing 0.120 g (1.6 mmol) of finely divided glycine. To this vigorously stirred suspension was added 2 ml of DMF and 0.509 g (0.98 mmol, specific activity 1.24×10^4 dpm/mg) of 3-benzyloxycarbonyl[1-^{14}C]glycyl-L-phenylalanyloxy-2-hydroxy-N-ethylbenzamide. The mixture was stirred for 1 hr at −10–0°, then was poured into 20 ml of saturated sodium bicarbonate solution and extracted with five 20-ml portions of dichloromethane. After acidification to pH 1 the aqueous phase was extracted twice with 5 ml of dichloromethane, and the pooled organic phases were extracted with three 3-ml portions of saturated sodium bicarbonate. Acidification of these pooled bicarbonate extracts was followed by extraction with dichloromethane; the organic extracts were then evaporated, and the residue was dissolved in 15 ml of water, 0.8 ml of 1 *N* sodium hydroxide, and 0.5 ml of 2,6-lutidine. The solution was overlayered with 5 ml of diethyl ether and treated with 1.5 g of triethyloxonium fluoroborate. Sodium hydroxide solution was added to maintain the solution pH at 5.5–6.0. After 20 min the ether phase was separated, the aqueous phase was extracted with two 3-ml portions of dichloromethane, and the combined organic phases were extracted with aqueous bicarbonate, dried, and evaporated. The residue was taken up in 30 ml of ethanol containing 300.5 mg of L tripeptide and 59.08 mg of DL tripeptide. After three successive dilutions, 19.1 mg of racemate was isolated and counted: 5.71 dpm/mg; L tripeptide recovered from the final dilution had specific activity 0.89 dpm/mg; yield: 37.0%;[52] $\alpha = (2 \times 59.08 \times 5.26)/(160.5 \times 1.46 \times 10^4) = 2.66 \times 10^{-4}$ or 0.027%.

g. **Triethylamine-Catalyzed Racemization of Ethyl Benzyloxycarbonyl[1-^{14}C]glycyl-L-phenylalanylglycinate.** A solution of 0.901 g of triethylamine, 1.09 g of tetraethylammonium chloride, and 0.640 g of ethyl benzyloxy[1-^{14}C]glycyl-L-phenylalanylglycinate,[53] specific activity 6.28×10^3 dpm/mg, in 32.0 ml of absolute ethanol was maintained at 30.0° for 121.0 hr. To the solution was then added 1 ml of acetic acid and 87.15 mg of DL tripeptide. After three successive dilutions with unlabeled L tripeptide, racemate was isolated with activity 42.7, 43.5, and 45.1 cpm/13.3 mg; background count: 24.8 ± 2.0 cpm; efficiency: 87.8%; DL dpm/mg, 1.63 ± 0.16; activity of L tripeptide recovered from the third dilution: 0.80 dpm/mg (calcd, 0.70 dpm/mg); recovery of L tripeptide: 98%; $\alpha = (2 \times 87.15 \times (1.23 \pm 0.2))/(629 \times 6.28 \times 10^3) = (5.4 \pm 0.8) \times 10^{-5}$ or 0.0054%; corrected $\alpha = 0.0059\%$.

II. The Young Test. A. Preparation of Reagents. 1. Benzoyl-L-leucine and [7-^{14}C]benzoyl-L-leucine. The general procedure of Williams and Young[10] was followed. Initial preparations showed broad and low melting points, but seeding with a sample kindly supplied by Dr. G. T. Young resulted in material with mp 106–107°, mp cyclohexylammonium salt, 145–146°; lit. 106°, 145–146°.[10]

2. Ethyl Benzoyl-L-leucylglycinate. The procedure of Williams and Young was followed.[10] Ethyl benzyloxycarbonyl-L-leucylglycinate was prepared by the dicyclohexylcarbodiimide coupling of ethyl glycinate and benzyloxycarbonyl-L-leucine. The substance was recrystallized from ethyl acetate–petroleum ether, mp 102–103°, $[\alpha]^{24}$D −25.8° (*c* 5 ethanol) lit. 102–103°, −25.8°.[54] Catalytic hydrogenation in acetic acid, followed by benzoylation yielded solid, recrystallized from ethyl acetate–petroleum ether three times, mp 155.0–157.0°, $[\alpha]^{24}$D −33.3° (*c* 3.0, ethanol); lit. 156.5–157°,[10] −34.0°,[10] −32.5°.[55]

3. Benzoyl-DL-leucine, Ethyl Benzoyl-DL-leucylglycinate, [7-^{14}C]Benzoyl-*DL-leucine, and Ethyl [7-^{14}C]Benzoyl-*DL-leucylglycinate. Benzoyl-DL-leucine, mp 138–140°, was prepared by aqueous benzoylation of DL-leucine; the ethyl ester of benzoyl-DL-leucylglycine was prepared by reaction of crude 2-phenyl-4-isobutyloxazol-5-one, prepared from benzoyl-L-leucine and acetic anhydride, with ethyl glycinate; mp 145–146° after recrystallization from ethyl acetate–hexane. Asymmetrically labeled racemates were prepared from ^{14}C-labeled D-leucine derivatives by treatment with an equivalent of unlabeled L enantiomer. The sample of ethyl [7-^{14}C]benzoyl-*DL-leucylglycinate was estimated to contain 1% of its activity in its L enantiomer.

4. 3-Benzoyl-L-leucyloxy-2-hydroxy-N-ethylbenzamide. Benzoyl-L-leucine was combined with 7-hydroxy-2-ethylbenzisoxazolium fluoroborate in aqueous pyridine buffer by the procedure outlined in section 1c for preparation of the corresponding Anderson dipeptide ester. A yield of 85% was obtained, mp 148–149°, $[\alpha]^{25}$D −59.5° (*c* 2.0, chloroform), $[\alpha]^{25}$ 546 mμ −62.5°.

Anal. Calcd for $C_{22}H_{26}N_2O_5$: C, 66.32; H, 6.58; N, 7.03. Found: C, 66.09; H, 6.60; N, 6.82.

B. Racemate Recovery with Benzoylleucine Derivatives. 1. Benzoylleucine. A solution of 0.902 g of benzoyl-L-leucine and 0.186 g of [7-^{14}C]benzoyl-*DL-leucine in 3.5 ml of ethyl acetate was carefully seeded with racemate[49] and allowed to remain at 0–3° for 17 hr, at which time 0.226 g of solid was collected and found to contain 82.7% of the initially introduced ^{14}C. In the filtrate was dissolved 0.154 g of unlabeled racemate; seeding and cooling as before yielded 0.184 g of solid which contained 9.7% of the initial ^{14}C. A third addition of 0.085 g of racemate resulted in a recovery of 0.185 g of solid containing 4.1% of the initial ^{14}C; total recovery: 96.5%.

2. Ethyl Benzoylleucylglycinate. Ethyl benzoyl-L-leucylglycinate was found to be three times more soluble than its racemate. Of the solvents acetone, ethyl acetate, ethanol, and acetonitrile, ethyl acetate appears to be the most suited to recovery experiments; it dissolves *ca.* 25 mg/ml of the L enantiomer at 0–3°.

A solution of 0.151 g of ethyl benzoyl-L-leucylglycinate and 0.0335 g of ethyl [7-^{14}C]benzoyl-*DL-leucylglycinate, specific activity 751.5 dpm/mg, in 6 ml of ethyl acetate was cooled to 0–3° and carefully seeded with racemate.[49] After 3 hr, 28.7 mg of solid, mp 141–146°, was collected and recrystallized from ethyl acetate to yield 16.0 mg of crystals, mp 145–146°, specific activity 741.5 dpm/mg; estimated specific activity for purely D-labeled racemate: 744 dpm/mg.

In a second determination, symmetrically ^{14}C-labeled racemate, specific activity 1826 dpm/mg, 78.9 mg, was dissolved in 15 ml of

(52) The yield for the coupling step is >90%.

(53) Pooled L tripeptide, isolated from low racemization assays, was used in this racemization study, after recrystallization.

(54) J. R. Vaughn and R. L. Osato, *J. Amer. Chem. Soc.*, 74, 676 (1952).

(55) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *ibid.*, **89**, 5012 (1967).

ethyl acetate containing 369.8 mg of L enantiomer. Seeding and chilling to 0–4° for 4 hr yielded solid which was recrystallized from 3–4 ml of ethyl acetate to yield *ca.* 40 mg, mp 144.5–145.5°, specific activity 973 dpm/mg, calcd, 1000 dpm/mg, or 97.3% pure. A second dilution with 17.2 mg of recovered racemate, 115.3 mg of unlabeled racemate, and 577.6 mg of L enantiomer yielded racemate, mp 144–145.5°, specific activity 113.7 dpm/mg; calcd, 119.7 dpm/mg, 94.6%. A third dilution with 58.8 mg of racemate recovered from the second dilution and 248 mg of unlabeled L enantiomer yielded racemate, mp 144.5–146.0°, specific activity 114 dpm/mg; calcd, 118.6 dpm/mg; 96.4%.

C. The Young Test. Preparations and Control Experiments. Preparation of Starting Materials, Free of Labeled D Enantiomer. 1. [7-^{14}C]Benzoyl-L-leucine. A solution of 7.05 g of [7-^{14}C]-benzoyl-L-leucine and 1.02 g of benzoyl-DL-leucine in 28 ml of ethyl acetate was seeded with a drop of a slurry of freshly precipitated seeds of racemate[49] and chilled at 0–3° for 3 hr with occasional swirling. The precipitated solid, 1.07 g, was collected with the aid of 5–7 ml of ethyl acetate and the combined filtrate was concentrated to 30 ml and in it was dissolved a 0.38-g portion of unlabeled racemate. A second seeding and cooling yielded 0.55 g of solid. Again the filtrates were concentrated to 30 ml and used to dissolve 0.38 g of unlabeled racemate. Seeding and chilling yielded 0.50 g of solid. The filtrates were concentrated to *ca.* 20 ml, warmed, treated with petroleum ether to the cloud point, and seeded with benzoyl-L-leucine to yield 5.2 g of large prisms, mp 105–107°, $[\alpha]^{23}$°D −6.5° (*c*, 2.6; ethanol). A further 1.1 g of good quality product could be obtained by concentrating the mother liquors and crystallizing the residue from ether–petroleum ether.

2. [7-^{14}C]Benzoyl-L-leucylhydrazide. A specimen of [7-^{14}C]-benzoyl-L-leucine, freed of contaminating D-labeled enantiomer by the above procedure was converted with diazomethane to its methyl ester, and then by reaction with hydrazine[10] in ethanol to the hydrazide, mp 154–155°, lit. mp 155°.[10]

3. 3-[7-^{14}C]Benzoyl-L-leucyloxy-2-hydroxy-N-ethylbenzamide and O-[7-^{14}C]Benzoyl-L-leucyl-N-ethylsalicylamide. These esters were prepared from [7-^{14}C]benzoyl-L-leucine obtained from C1 and the corresponding benzisoxazolium salt by the procedures of Section 1 for the corresponding phenylalanine dipeptide derivative. See also Section A3 for characterization data.

General Procedure for the Isotopic Dilution Young's Test. The general procedure outlined in 4a was followed with a few major modifications. The product from a coupling between ethyl glycinate and isotopically pure [7-^{14}C]benzoyl-L-leucine was transferred, after removal of aliquot for dilution with ethyl benzoyl-L-leucylglycinate to determine yield, to an ethyl acetate solution containing a known amount of unlabeled racemate. The solution volume was adjusted to make the concentration 25 mg of L/ml and the amount of racemate used was 20% of the weight of L enantiomer. As with the Anderson test clean isolation of racemate was possible only with seeds prepared by ice chilling and vigorously triturating a hot saturated solution of racemate in ethyl acetate. A trace of the resulting moist slurry was immediately transferred to the supersaturated solution on the outside of a melting point capillary. After 3–4 hr at 0–3° with occasional swirling, the racemate was recovered, dried, and examined for melting point. Material melting below 144° was recrystallized from a small volume of ethyl acetate before being carried through further dilutions. From the amounts of materials used, the L activity can be calculated for each dilution and the measured activity of racemate can be corrected for activity due to labeled L enantiomer. The activities reported in this paper are mean values for the corrected activities from the second and third dilutions. In all cases these values agreed to within 20%. Although the greater susceptibility of benzoylleucine to racemization results in higher levels of racemate, the Anderson test was the easier to perform, and is probably preferable, despite the greater degree of dilution required to attain significant results.

6. Racemization during Acylazide Coupling in DMF. Young's procedure for the diazotization was followed.[10] A solution of optically pure [7-^{14}C]benzoyl-L-leucylhydrazide, 1.99×10^3 dpm/mg, 1.25 g, in 5 ml of water, 1 ml of 12 *N* hydrochloric acid, and 1 ml of acetic acid was cooled to 0° and treated with 0.7 g of sodium nitrite in a minimum volume of water. After a few minutes of stirring, the suspension was extracted with 4 × 7 ml of cold ether, and the pooled extracts were dried over magnesium sulfate and combined with 8 ml of cold DMF. The ether was removed at −10–0° in an ice–salt bath on the rotary evaporator, and the resulting solution was combined with 2 ml of DMF containing 1.01 g of triethylamine (1.0 mmol) and 1.42 g of ethylglycinate hydrochloride (1.0 mmol). After 12 hr at room temperature the mixture was poured into 100 ml of water and extracted with 4 × 10 ml of dichloromethane. The pooled extracts were washed with 2 × 20 ml of water, dried over magnesium sulfate, and evaporated. The residue was taken up in 25.0 ml of ethyl acetate and 1.00 ml of the solution was used to dissolve 93.7 mg of unlabeled ethyl benzoyl-L-leucylglycinate. From this was recovered material with specific activity 402 dpm/mg; yield = 820 mg, or 51%. To the 24.0 ml remaining was added 160.0 mg of unlabeled racemate and 6 ml of ethyl acetate; cooling and seeding yielded 155 mg of solid which was recrystallized twice from ethyl acetate, mp 143.5–145.5°, specific activity 724 dpm/mg; calcd for L, 1410 dpm/mg. In the first dilution, 63.5 mg of recovered racemate was dissolved in 12 ml of ethyl acetate containing 304.4 mg of L enantiomer. Seeding and cooling yielded 43 mg of racemate, specific activity 91.0 dpm/mg; calcd L activity, 133 dpm/mg. A second dilution with 25.4 mg of the above racemate, 32.8 mg of inactive racemate, and 247.3 mg of L enantiomer in 9 ml of ethyl acetate yielded material with specific activity 13.5 dpm/mg; calcd L activity, 6.1 dpm/mg. In a third dilution, 28.8 mg of recovered racemate was combined with 98.7 mg of L enantiomer in 4 ml of ethyl acetate to yield after seeding racemate which was recrystallized from ethyl acetate to yield 11.1 mg which had an activity of 128 cpm at a background count of 24.4 cpm and an efficiency of 86%: specific activity 10.8 dpm/mg; calcd L activity, 0.4 dpm/mg.

$$X_3 = 10.6\frac{25.4 + 32.8}{25.4} = 24.3 \text{ dpm/mg}$$

$$X_2 = (13.5 - 3.0)\frac{25.4 + 32.8}{25.4} = 24.0 \text{ dpm/mg}$$

$$X_1 = (91.0 - 66.5) = 24.5 \text{ dpm/mg}$$

$$\alpha = \frac{2 \times 160 \times 24.3}{(24/25)820 \times 1.55 \times 10^3} = 0.64\%$$

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