N-Methylamino Acids in Peptide Synthesis. IV. Racemization and Yields in Peptide-bond Formation[1]

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The racemization of an *N*-methylamino-acid residue during peptide-bond formation and mixed-anhydride activation has been investigated using Ala-MeLeu-Gly and Ala-MeLeu as model peptides. The results were compared with those for Ala-Leu-Gly, Ala-Leu, and Ala-Pro. The extents of racemization were determined by analysis of the diastereomeric products of the reactions after deprotection, using an amino-acid analyzer. Extensive racemization was detected after the hydrolysis of the mixed anhydrides of Bz-MeLeu, Z-Ala-MeLeu, and Z-Ala-Leu, but not of Boc-Ala-Pro and Z-MeIle. Significant racemization (2.8–39%) was observed when Z-Ala-MeLeu was coupled with Gly-OBzl by various methods in the presence of salts such as triethylamine hydrochloride or *p*-toluenesulfonate. Only coupling through the *N*-hydroxysuccinimide (HONSu) ester gave stereochemically pure product. In the absence of salt, less racemization was observed, but only couplings using *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline and *N,N'*-dicyclohexylcarbodiimide–HONSu gave essentially pure products. Polar solvents promoted racemization, but excess base did not. Chemical evidence that the racemization intermediate is an oxazolium-5-oxide has been obtained by trapping the intermediate as an addition product (a pyrrole) in 85% yield.

The yields obtained by various coupling methods have been determined for several model peptides. Couplings at the carboxyl group of an *N*-methylamino acid gave high yields only in the absence of salt, except for coupling by the HONSu ester method. Couplings to an *N*-methylamino group gave high yields, except for coupling by the *p*-nitrophenyl ester method. Couplings to Ala-MeLeu-OBu^t gave higher yields than couplings to Ala-MeLeu-OBzl, presumably due to piperazine-dione formation by the latter.

La racémisation d'un reste acide aminé *N*-méthyle lors de la formation de la liaison peptidique et de l'activation par anhydride mixte, a été étudiée en utilisant les peptides modèles Ala-MeLeu-Gly et Ala-MeLeu. Les résultats ont été comparés à ceux obtenus pour Ala-Leu-Gly, Ala-Leu et Ala-Pro. L'étendue de racémisation a été déterminée par analyse des diastéréoisomères après déprotection et emploi d'un analyseur d'acide aminé. Une racémisation importante a été détectée après hydrolyse des anhydrides mixtes de Bz-MeLeu, Z-Ala-MeLeu et Z-Ala-Leu mais pas pour Boc-Ala-Pro et Z-MeIle. Une racémisation notables (2.8–39%) a été observée lors du couplage du Z-Ala-MeLeu sur le Gly-OBzl par les diverses méthodes en présence de sels tels que le chlorohydrate ou para toluène sulfonate de triéthylamine. Seul le couplage par l'ester de la *N*-hydroxy succinimide (HONSu) conduit à un produit de pureté stéréochimique. En l'absence de sel, la racémisation est plus faible mais seuls les couplages par le *N*-éthoxycarbonyl éthoxy-2 dihydro-1,2 quinoléine et le complexe *N,N'*-dicyclohexylcarbodiimide–HONSu donnent des produits essentiellement purs. Les solvants polaires favorisent la racémisation mais pas un excès de base. L'intermédiaire de racémisation oxyde-5 d'oxazolium a été mis en évidence par piegeage sous forme de produits d'addition (un pyrrole) avec un rendement de 85%.

Les rendements obtenus dans les divers couplages ont été déterminés sur différents peptides modèles. Les couplages sur le carboxyle d'un acide aminé *N*-méthylé se font avec des rendements élevés en l'absence de sel sauf pour la méthode de l'ester HONSu. Les couplages sur le groupe *N*-méthylamino se font avec des rendements élevés sauf pour la méthode de l'ester *p*-nitrophényle. Les couplages de l'Ala-MeLeu-OBu^t sont meilleurs que ceux de l'Ala-MeLeu-OBzl probablement à cause de la formation par ce dernier d'une pipérazine dione. [Traduit par le journal]

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Peptides containing *N*-methylamino acids form an important group of antibiotics (2) and are also of interest as analogs of peptide hormones (3). Many peptides of *N*-methylamino acids have been synthesized, but systematic studies in this area of peptide synthesis have not been carried out. The synthesis of these peptides is attended with the problems of the lack of crystallinity of intermediates, the tendency of intermediates to cyclize and the lesser reactivity of the methylamino group or steric hindrance (4), though the latter does not always manifest itself. Racemization has generally been assumed to be less of a problem than for ordinary peptides, because of the inability of *N*-acyl *N*-methylamino-acid de-

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rivatives to form oxazolones (5), and because of their structural similarity to derivatives of proline whose resistance to racemization is well known. However, several observations are completely inconsistent with this assumption. Both *N*-methylphenylalanine and proline can be racemized by acetic anhydride in acetic acid (6), but only *N*-methyltryptophane and not proline was racemized by ketene at acid p*H* (7). Z-Gly-MePhe[2] was partially racemized as was Z-Gly-Phe during the formation of the *p*-nitrophenyl ester using tris-(*p*-nitrophenyl) phosphite but Z-Gly-Pro was not (9). And finally, a product with a lower specific rotation than that of the same product prepared by a different method was obtained from the DCCI-mediated coupling of Z-Cys(Bzl)-MeTyr with IleOMe (10). In the previous paper we have demonstrated the tendency of *N*-methylamino-acid derivatives to racemize during saponification and acidolysis (1). In this paper, we present a study on the racemization of *N*-methylamino-acid residues during peptide-bond formation, and a comparison of the yields obtainable by different methods for several model peptides.[3]

Our interest in racemization of *N*-methylamino acids during peptide synthesis arose from the observation that a mixed-anhydride coupling of Bz-MeLeu with *p*-nitroaniline gave a product devoid of optical activity (12). In preliminary experiments, it was then found that no optical activity remained after the mixed anhydride formed from Bz-MeLeu and ethyl chloroformate at 0° had been hydrolyzed. The extents of racemization for some other derivatives subjected to the same admittedly rather drastic activation conditions followed by hydrolysis are given in Table 1. Z-MeLeu racemized very little, but the extents of racemization of Z-Ala-MeLeu and Bz-MeLeu approached those of Z-Ala-Leu and Bz-Leu. Boc-Ala-Pro resisted racemization, as expected, but Bz-Pro racemized sufficiently to indicate that one should not disregard completely the possibility that proline residues might

TABLE 1. Extent of racemization during the hydrolysis of mixed anhydrides*

Compound	Diastereomer or D isomer formed (%)
Bz-Leu	50†
Bz-MeLeu	45†
Bz-Pro	6.5†
Z-MeIle	<0.5‡
Z-Ala-MeLeu	26.5‡
Z-Ala-Leu	38‡
Boc-Ala-Pro	0.1‡

*Mixed anhydride formed by reaction with ROCOCl–Et_3N in THF at 0° for 5 min. Water was then added.
†Determined by measurement of optical rotation. EtOCOCl used.
‡Determined by analysis after deprotection. Bu^tOCOCl used.

racemize during couplings.[4] The consistently low values observed for the two proline derivatives show that proline is atypical in not behaving like other imino acids, as has already been observed in the previous paper (1).

Further studies were then carried out on actual peptide synthesis using several standard coupling methods. The extents of racemization and the yields have been obtained throughout this work by determining the deprotected diastereomeric products of the reaction using an amino-acid analyzer. This method was chosen because the sensitivity and speed of this type of analysis have been demonstrated (14, 15) and because of our own experience with the analyzer (16). The elution conditions used and the elution times and ninhydrin color yields for the various diastereomeric pairs are given in Table 2.[5] The elution times and relative color yields for each pair were determined by analysis of the peptides prepared from the racemic amino acid, and the identity of the peaks and the absolute color yields for each isomer were established by analysis of the L,L-peptide. The standard instrument column and buffers,[6] plus the Aminex A-5 column which has been a standard part of our instrument for several years, sufficed for separating all the

[2]The abbreviations for the amino-acid and peptide derivatives are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (8). When not indicated, the amino-acid symbols represent the L isomer, except for glycine. Other abbreviations used: DCCI, *N*,*N*′-dicyclohexylcarbodiimide; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; HONSu, *N*-hydroxysuccinimide; NMM, *N*-methylmorpholine; NEPIS, *N*-ethyl-5-phenylisoxazolium-3′-sulfonate; THF, tetrahydrofuran.

[3]A preliminary account of this work has appeared (11).

[4]Partial racemization of a proline residue during coupling has in fact been reported (for the DCCI-mediated coupling of Bz(NO_2)-Pro with *p*-nitrophenol) (13).

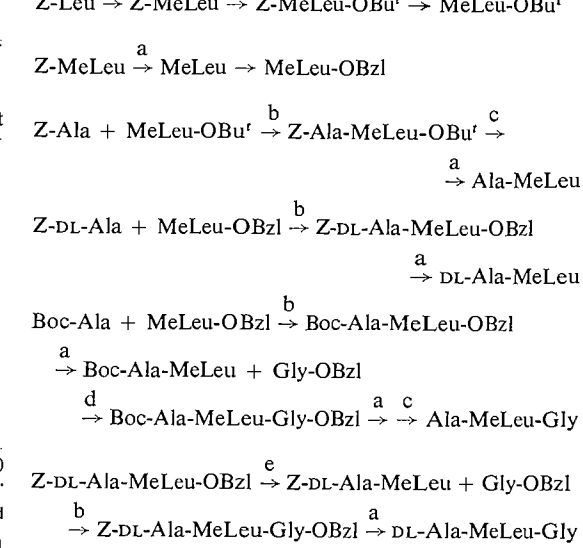
[5]It is interesting to note the decrease in ninhydrin color constants in going from Ala-Leu-Gly (24.3), to Gly-Ala-MeLeu (13.6), Ala-MeLeu-Gly (5.7), Ala-MeLeu (1.1), and MeAla-Leu (<0.05).

[6]The concentrations given for the p*H* 4.25 buffer in refs. 11, 16, and 17 are in error. The correct concentration is 0.20 *N*.

TABLE 2. Chromatographic data for analysis of diastereomers*

Compound	Elution time (min)	Color constant
Ala-MeLeu	43.5	1.1
Ala-D-MeLeu	34.5	1.0
Ala-Leu	40	17.2
Ala-D-Leu	31	14.6
Ala-MeLeu-Gly	35	5.7
Ala-D-MeLeu-Gly	41	5.2
Ala-Leu-Gly	33, 96†	24.3
Ala-D-Leu-Gly	38, 120†	21.9
Ala-Pro‡	158	7
Ala-D-Pro‡	142	9
MeIle§	63	5.2
D-Me*a*Ile§	57	4.5

*Beckman model 120B amino-acid analyzer, Aminex A-5 (15 cm) resin, eluted with 0.20 *N* sodium citrate, p*H* 4.25, at 68 ml/h; 57 °C.
†AA-15 (50 cm) resin.
‡AA-15 (50 cm) resin, eluted with p*H* 3.28 buffer (85 min), followed by p*H* 4.25 buffer.
§Elution at 34 ml/h (16). Other constants at 34 ml/h: Ile, 39.1 (92 min); *a*lle, 39.3 (83 min); MeAla-Leu, 0.3 (82 min).



SCHEME 1. a, hydrogenation; b, mixed anhydride; c, trifluoroacetic acid; d, DCCI–HONSu; e, saponification.

diastereomeric pairs. Though no experiments designed to test sensitivity were carried out, the peptide peaks were well enough separated to allow detection of about 1 part in 1000 of the other isomer, as has been reported by other workers (14, 15). The *N*-methylisoleucine–*N*-methyl-*allo*-isoleucine pair were less well separated, allowing detection of about 1 part in 200.

The methods used for the synthesis of the *N*-methylleucine peptides are shown in Scheme 1. Points to note are that the syntheses of the L,L-isomers had to be carried out without using saponification or acidolysis with hydrogen bromide, both of which cause racemization (1), and that the tripeptide was obtained by using DCCI–HONSu as the coupling agent for the reason which is apparent from Table 4.

The coupling of Z-Ala-MeLeu to Gly-OBzl was used to determine the extent of racemization during peptide-bond formation by various procedures. The crude product of the reaction was deprotected by hydrogenation, and the resulting tripeptide analyzed. The initial series of couplings were done in the presence of triethylamine *p*-toluenesulfonate or hydrochloride, derived from the salt of the appropriate Gly-OBzl. The results are shown in Table 3, together with those obtained for coupling Z-Ala-Leu to Gly-OBzl for comparison. Racemization occurred in varying degrees for all the coupling methods studied, except the HONSu ester method. Moreover, in all cases but one, the *N*-methylamino acid suffered more racemization than did the corresponding amino acid in the analogous peptide. The low extents of racemization obtained for Z-Ala-Leu-Gly-OBzl using DCCI–HONSu and EEDQ are in accord with the literature (15, 18, 19) except for the report of Kemp *et al.* (20). In the case of the DCCI–HONSu coupling, racemization clearly occurred during the formation of the active ester, since no diastereomer was formed when the HONSu ester was coupled. When *N*-methylmorpholine was used as base for the mixed-anhydride couplings, instead of depressing the degree of racemization as it normally does (21), it had the opposite effect.

In Table 4 are shown the results for couplings carried out using Gly-OBzl in the absence of salt of tertiary base. In this later work, crystalline Boc-Ala-MeLeu was used in preference to the oily benzyloxycarbonyl derivative. In a test case (DCCI), the yield and extent of racemization were, as expected, very similar for the two compounds. DCCI–HONSu and EEDQ caused much less racemization than in experiments with salt present, the former yielding a stereochemically pure product. The enhancement of racemization by salts (22, 23) has been attributed to the basicity of the anion (the chloride ion in particular) (13), the increased ionic strength of the medium (24),

TABLE 3. Yields and extents of racemization during couplings to Gly-OBzl in the presence of salt*

Coupling method	Z-Ala-Leu	Z-Ala-MeLeu	
	$TosOH.Et_3N$	$TosOH.Et_3N$	$HCl.Et_3N$
DCCI–HONSu	0.4 (93)	2.8 (77)	11 (71)
HONSu ester			<0.1 (91)
EEDQ	0.5 (78)	15 (68)	7.7 (74)
Bu^tOCOCl–Et_3N	2.0 (84)	7.0 (66)†	8.2 (63)
DCCI	16 (89)	15 (64)	27 (58)
NEPIS‡		39 (45)	

*Percent of L,D-peptide formed, determined by analysis after deprotection. The yields (figures in parentheses) were determined by weighing the neutral product of the reaction. Couplings were carried out in THF in the presence of the designated salt.
†13 (74) in AcOEt; 13 (70) using NMM as base; 29 (74) in AcOEt using NMM as base.
‡In CH_3CN.

TABLE 4. Yields and extent of racemization during the coupling of Boc-Ala-MeLeu with Gly-OBzl*

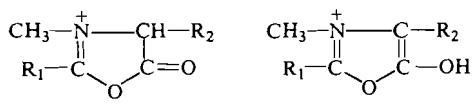
Coupling method	Solvent	L,D-Peptide formed (%)	Yield (%)
DCCI–HONSu	THF	<0.1	93
EEDQ	THF	0.5	86
DCCI	THF	15	78
DCCI	AcOEt	26	82
DCCI + Et_3N†	THF	14	55
Bu^tOCOCl–Et_3N‡	THF	6.4	60
Bu^tOCOCl–Et_3N‡	AcOEt	14	65
Bu^tOCOCl–Et_3N§	THF	3.9	36
Bu^tOCOCl–NMM‡	THF	15	68
NEPIS	CH_3CN	20	52

*Free base. Results determined by analysis after deprotection.
†One molar equivalent.
‡90-s activation, −10°.
§30-s activation, −15°.

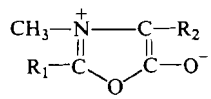
and more recently to both of these (25). We have shown that the addition of an equivalent amount of triethylamine to a DCCI coupling did not increase the percentage of L,D-isomer. Thus the racemization is not base-catalyzed, and only the increased ionic strength can be appropriate as an explanation here. The seemingly anomalous results obtained with *N*-methylmorpholine may be due to the higher solubility in THF of *N*-methylmorpholine hydrochloride compared to triethylamine hydrochloride, leading to an increased ionic strength of the solution. The mixed-anhydride coupling at −15° using a 30-s activation time (the conditions for minimum racemization) (21) gave less diastereomer but at the expense of a considerably reduced yield.

Racemization was considerably enhanced for both the mixed-anhydride and DCCI couplings when ethyl acetate was used instead of the less polar THF. This dependence on solvent polarity could explain why high extents of racemization were obtained for the NEPIS coupling where the solvent was acetonitrile. It is interesting to note that with this reagent under similar conditions (absence of chloride), Smart *et al.* were able to couple the racemization-prone Bz-Leu without diastereomer formation (22).

The absence of base catalysis indicates that the mechanism involved is not a simple α-hydrogen abstraction. *N*-Acyl *N*-methylamino acids cannot form oxazolones, and this has led to the oxazolonium salt **1** being proposed as the racemization intermediate (9, 13, 26). The oxazolonium (or more correctly the 5-oxo-Δ^2-oxazolinium) cation can racemize by loss of proton to give the mesoionic oxazolium-5-oxide **3** or by tautomerism to the 5-hydroxy-oxazolium cation **2**. The resonance stabilization of these two derivatives (the oxazolium ring is in fact pseudoaromatic (27)) could contribute to the ease with which *N*-methylamino-acid derivatives racemize.



1 **2**



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Oxazolonium derivatives were thought to be extremely unstable, and evidence for their existence was derived from cryoscopic data obtained with Bz-MeGly in sulfuric acid (28) and from their implication in the mechanism of the Dakin–

West reaction (29). Recently, however, several stable oxazolonium perchlorates have been prepared by treating *N*-substituted *N*-acylamino acids in acetic anhydride with perchloric acid (30).

The oxazolonium cation is a powerful acylating agent, giving a transient yellow color, ascribed to structure **3** when it is added to an amine (31, 32). We observed an immediate yellow color when we prepared the mixed anhydride of Bz-MeLeu or added DCCI to Bz-MeLeu. The oxazolium-5-oxides, prepared *in situ*, also undergo a 1,3-dipolar cycloaddition reaction with activated double and triple bonds (31), and we have made use of this reaction to show that oxazolonium-type intermediates can be formed under peptide coupling conditions. Z-Ala-MeLeu and DCCI were left in THF at room temperature for 10 min. Upon addition of methyl propiolate **4**, carbon dioxide was immediately evolved, and *N*,*N*′-dicyclohexylurea and the crystalline pyrrole **5** were obtained in about 85% yield. The plausible "classical" products of the

$$HC{\equiv}C{-}CO_2CH_3$$

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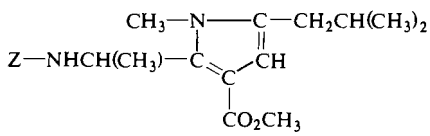

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reaction would have been the *N*-acylurea derivative, which could not have been formed in large amounts, and the symmetrical anhydride of the protected peptide (33). But neither of these has the structural features required for a 1,3-dipolar cycloaddition reaction, therefore the pyrrole must have come from the oxazolium-5-oxide (**3**: R_1 = Z-NHCH(CH_3)—; R_2 = —$CH_2CH(CH_3)_2$), presumably formed in the reaction by cyclization to the oxazolonium cation followed by loss of a proton. This mechanism is consistent with the observations that the extent of racemization is (i) markedly dependent on solvent polarity and, seemingly, ionic strength which would be expected for a charged intermediate, and (ii) not affected by base, base catalysis being unnecessary for cyclization to an oxazolonium cation, the presumed rate-determining step.

The deprotonated mesoionic ring structure **3** is extremely unstable, and decomposes by dimerization (acylation at C-4) when unsubstituted at C-4(R_2 = H) (32). Treatment of Bz-MeGly with DCCI yielded the dimer resulting from α-*C*-acylation (34). It follows that if the oxazolonium cation is an intermediate in the coupling of *N*-methylamino-acid residues, the possibility that α-*C*-acylation might take place when sarcosyl is the coupling residue should be kept in mind. A practical implication of ignoring this danger might be that in the design of a synthesis, the decision to condense peptide fragments at sarcosyl residues in order to avoid racemization could prove to be more of a nuisance than an expedience.

It thus transpires that *N*-methylamino-acid residues seem to be just as prone to racemize during couplings as amino-acid residues, and even more so in the presence of salts. This is contrary to the generally accepted notions, which stem from their apparent structural similarity to prolyl residues, and could be attributed to the formation of the oxazolonium intermediate by the *N*-methylamino-acid derivative. The striking difference in behavior between the *N*-methylamino acid and proline could be due to the failure of the latter to form the oxazolonium intermediate. This in fact was proposed by Goodman and Stueben to account for the difference in behavior observed in the formation of the *p*-nitrophenyl esters (9). They suggested that the rigidity of the proline ring prevents the formation of a second contiguous ring. This appears to us a plausible explanation, but it must be borne in mind that under appropriate conditions, an oxazolonium intermediate can be formed even from a proline derivative. This had been suggested by Williams and Young (13) and has now been proved by Boyd and Wright who prepared the oxazolonium perchlorate from Bz(NO_2)-Pro (30).

The yields of tripeptide were determined in conjunction with the extents of racemization from the amino-acid analysis chromatogram (except as indicated in Table 3) for all the foregoing coupling reactions, and the results appear in Tables 3 and 4. They varied from 93% (DCCI–HONSu) to 52% (NEPIS) in the absence of triethylammonium salt and from 77% (DCCI–HONSu) to 45% (NEPIS) in the presence of salt. The yield was lowered by the presence of salt in all but the conventional mixed-anhydride case, where salt is always present. It is noticeable that

TABLE 5. Yields for couplings to an *N*-methylamino-acid ester component*

Coupling method	MeLeu-OBzl†	Ala-MeLeu-OBzl‡	Ala-MeLeu-OBu^t‡
DCCI	96	46	96
DCCI–HONSu	96	61	98
Bu^{t}OCOCl–Et_3N	92	68	92
EEDQ	91	93	95
p-Nitrophenyl ester	29	67	94

*Yields (%) determined by analysis after deprotection.
†Z-Ala coupled to the ester *p*-toluenesulfonate.
‡Z-Gly coupled to the ester hydrochloride. Ninhydrin color constant for Gly-Ala-MeLeu, 13.6.

the two racemization-free coupling methods (DCCI–HONSu and EEDQ) gave the best yields. It is known that β-alanyl derivatives can be formed as side-products during DCCI–HONSu couplings when the formation of the intermediate HONSu ester is slow (35). A good correspondence was found between the yield of the tripeptide calculated from the amino acid analysis and the weight of the total neutral product, indicating that the amount of any such impurities in our case could not have been appreciable.

The yields for couplings between three other pairs of reactants were determined and the results are given in Table 5. Excellent yields of the dipeptide were obtained in all but the *p*-nitrophenyl-ester coupling. Allusions to the lesser reactivity of an *N*-methylamino group in couplings have been made (4, 36). From our findings it appears that couplings to an *N*-methylamino group proceed smoothly, with the exception of a classical activated-ester coupling, but that only moderate yields result when a *C*-terminal *N*-methylamino acid is activated for synthesis. More experiments with different peptides are needed to confirm this generalization.

The two tripeptides in Table 5 were used to investigate the importance of cyclization to the piperazine-2,5-dione, which is known to be a problem in synthesis with both proline and *N*-methylamino-acid derivatives (4). The dipeptide *t*-butyl ester gave very high yields indicating that the *t*-butyl ester provided adequate protection against this side reaction, whereas the dipeptide benzyl ester gave considerably lower yields, presumably because of the competing cyclization reaction (the piperazine-dione was not isolated). A high yield was nevertheless obtained with the benzyl ester when EEDQ was the coupling reagent. The explanation for this is not apparent to us.

Experimental

Materials and Methods

Amino acids were obtained from General Biochemicals, Chagrin Falls, Ohio; Boc-Ala and HONSu from Pierce Chemical Company, Rockford, Illinois; DCCI from Schwarz-Mann, Orangeburg, New York; EEDQ and NEPIS from Aldrich Chemical Company, Milwaukee, Wisconsin; methyl propiolate from K & K Laboratories Inc., Plainview, New York; Aminex A-5 resin from Bio-Rad Laboratories, Richmond, California. Benzyloxycarbonylamino acids were prepared by the original procedure as described by Greenstein and Winitz (ref. 37, p. 891). Solvents were purified and dried by standard methods before use. Light petroleum refers to the fraction boiling between 30–60°. Organic solvent extracts were dried with $MgSO_4$ and evaporated under reduced pressure using a rotary evaporator. When not specified, hydrogenations were carried out at atmospheric pressure in 80% aqueous acetic acid in the presence of 10% palladium on charcoal catalyst for 4 h. The catalyst was removed by filtration, the solution was evaporated, the evaporation was repeated several times after the addition of water, and the residue was dried *in vacuo* over KOH pellets. Optical rotations were determined with a Perkin Elmer model 141 polarimeter using a 1 dm tube. Proton nuclear magnetic resonance (n.m.r.) (Varian T-60) and t.l.c. on silica gel GF_{254} in chloroform–methanol (9:1) were used to confirm the identity and purity of oils. Diastereomeric amino acids and peptides were analyzed with a Beckman model 120B amino-acid analyzer according to Spackman *et al.* (38) using the conditions described in Table 2. Both the extent of racemization and the yield of a reaction were calculated from the same chromatogram of an aliquot of deprotected peptide using the data in Table 2.

The following compounds were prepared as indicated: Bz-Leu and Bz-Pro (ref. 37, p. 1266), Bz-MeLeu (40), Z-MeLeu (41), Boc-Ala-Pro (1), Z-Ala-Leu and Z-DL-Ala-Leu (42), Gly-OBzl.TosOH, Gly-OBzl.HCl, and Gly-OBzl (ref. 37, p. 928), Z-Ala-MeLeu (oil, containing 0.4% of the L,D-isomer) from the *t*-butyl ester (1).

MeLeu-OBzl.TosOH

A mixture of *N*-methyl-L-leucine (3.3 g, from the hydrogenation of Z-MeLeu in aqueous acetic acid), *p*-toluenesulfonic acid monohydrate (4.87 g), benzyl alcohol (20 ml), and benzene (60 ml) was heated under reflux for 36 h, the water formed being trapped in a Dean–Stark tube (44). The benzene was evaporated off,

ether (100 ml) and light petroleum (200 ml) were added, and the solution was left overnight at 0°. The crystals were collected, and recrystallized from ethyl acetate – methanol – light petroleum. Yield 7.0 g (72%), m.p. 116–117°, $[\alpha]_D^{25}$ −2.2° (*c*, 1 in ethanol).

Anal. Calcd. for $C_{21}H_{23}NO_5S$: 61.9; H, 7.2; N, 3.4 Found: C, 61.5; H, 7.1; N, 3.4.

Boc-Ala-MeLeu

Boc-Ala (1.89 g) and MeLeu-OBzl.TosOH (4.04 g) were coupled by the mixed-anhydride method using Bu^{t}OCOCl (1.3 ml) and triethylamine (1.38 ml) in ethyl acetate (20 ml) at −10° as described below. The neutral product was an oil (3.6 g; 92%), giving a single spot on t.l.c., and whose n.m.r spectrum was consistent with the structure (Boc-Ala-MeLeu-OBzl). The oil was hydrogenated in ethyl acetate for 8 h. Removal of the solvent left an oil which crystallized upon trituration with light petroleum, and which was recrystallized from ether – light petroleum. Yield 2.0 g (75%), m.p. 107–109°, $[\alpha]_D^{28}$ −55.1° (*c*, 1 in ethanol).

Anal. Calcd. for $C_{12}H_{28}N_2O_5$: C, 56.9; H, 9.0; N, 8.9. Found: C, 56.4; H, 9.1; N, 8.4.

A sample (20 mg) was left in trifluoroacetic acid (0.5 ml) for 30 min and then analyzed. The product contained none (< 0.1%) of the diastereomer.

Reference Compounds

The elution times and relative ninhydrin color yields for each diastereomeric pair of peptides were obtained by chromatography of the peptides synthesized from a racemic starting material. For convenience, in the case of the two *N*-methylamino acid peptides, one isomer of each diastereomeric pair was actually the enantiomorph of the L,D-isomer which was analyzed for. Moreover, some racemization undoubtedly occurred during the synthesis of these peptides at the mixed-anhydride coupling and saponification stages; however, the products would still be a mixture of diastereomers in equal amounts *i.e.*, L,L + D,D = D,L + L,D. The assignments of the two peaks obtained during chromatography and the absolute color yields of the isomers were then established by chromatography of a known amount of the stereochemically pure L,L-isomer.

N-methyl-L-isoleucine and *N*-methyl-D-*allo*-isoleucine were obtained by hydrogenation of Z-MeIle and Z-D-MeaIle prepared by the room-temperature methylation of Z-Ile and Z-D-*a*Ile (41). Ala-DL-Leu and Ala-DL-Pro were prepared from L-alanine *N*-carboxyanhydride and the free amino acid by the method of Manning and Moore (14), and used for analysis without purification. Ala-Leu was obtained by leaving Boc-Ala-Pro in trifluoroacetic acid for 30 min, evaporating the solution, and drying the residue *in vacuo* over KOH.

DL-Ala-Leu-Gly was obtained by hydrogenation (8 h) of Z-DL-Ala-Leu-Gly-OBzl, prepared by the mixed-anhydride (Bu^{t}OCOCl–Et_3N) coupling of Z-DL-Ala-Leu with Gly-OBzl.TosOH in THF. Ala-Leu-Gly was obtained by hydrogenation (8 h) of Z-Ala-Leu-Gly-OBzl prepared by the DCCI–HONSu-mediated coupling of Z-Ala-Leu with Gly-OBzl.TosOH as described below.

DL-Ala-MeLeu, as the hydrochloride, was obtained by a mixed-anhydride (Bu^{t}OCOCl–Et_3N) coupling of Z-DL-Ala (0.545 g) and MeLeu-OBzl.TosOH (1.06 g) in ethyl acetate (15 ml). The neutral product was isolated as an oil (1.07 g, 97%) which was hydrogenated in 80% aqueous acetic acid containing 2*N* HCl (1 mol. equiv.).[7] Ala-MeLeu was obtained by hydrogenation of Z-Ala-MeLeu.

DL-Ala-MeLeu-Gly was obtained by hydrogenation (8 h) of Z-DL-Ala-MeLeu-Gly-OBzl. The latter was the neutral product (oil, 0.55 g, 80%) obtained by the mixed-anhydride (Bu^{t}OCOCl–Et_3N) coupling of Z-DL-Ala-MeLeu (0.465 g) and Gly OBzl.TosOH (0.453 g) in THF as described below. The Z-DL-Ala-MeLeu (oil) was the acidic product (0.74 g, 77%) obtained by the saponification (2 h) of Z-DL-Ala-MeLeu-OBzl (0.97 g) in a mixture of methanol (8 ml) and 4 *N* NaOH (2.5 ml). Ala-MeLeu-Gly was the product obtained from the DCCI–HONSu-mediated coupling of Boc-Ala-MeLeu and Gly-OBzl described below. This was the first coupling to have given pure L,L-peptide.

Racemization during Hydrolysis of Mixed Anhydrides

The test compound (1–2 mmol) was dissolved in THF (5 ml) containing triethylamine (1 mol. equiv.) at 0°. Isobutyl chloroformate (1 mol. equiv.) was added with stirring, followed by water (2 ml) 5 min later. After 1 h at 0°, the THF was evaporated, and the residue was partitioned between ether (5 ml) and 5% aqueous $NaHCO_3$ (5 ml). The aqueous layer was acidified to p*H* 1 (p*H* 3 for Boc-Ala-Pro) with 3 *N* HCl, extracted with ethyl acetate, and the extract was dried and evaporated. Benzyloxycarbonyl derivatives were deprotected by hydrogenation, Boc-Ala-Pro by treatment with trifluoroacetic acid for 30 min, and the products were analyzed. The optical rotations of the benzoyl derivatives were measured, with the following results: Bz-Leu, $[\alpha]_D^{24}$ 0.0° (*c*, 1.8 in dimethylformamide); Bz-MeLeu, $[\alpha]_D^{24}$ −6.5° (*c*, 1 in dimethylformamide); Bz-Pro, $[\alpha]_D^{25}$ −83.3° (*c*, 4 in ethanol). Original values were, respectively: +7.0°, −54.0°, and −95.4°.

Coupling Methods for Racemization Experiments

The following methods were employed for coupling Z-Ala-Leu, Z-Ala-MeLeu, and Boc-Ala-MeLeu (0.5 mmol) with Gly-OBzl (0.5 mmol) (Tables 3 and 4). When salts of Gly-OBzl were used, triethylamine (0.5 mmol) was added to the ester salt. After the reaction, the solvent was removed by evaporation, the residual oil was taken up in ethyl acetate (10 ml), and the solution was washed with *N* HCl (2 × 5 ml), 5% aqueous $NaHCO_3$ (2 × 5 ml), and water (2 × 5 ml). The extract was dried and concentrated to an oil which was analyzed after deprotection by hydrogenation for 8 h. For couplings with Boc-Ala-MeLeu, aqueous citric acid was used instead of HCl for washing, and the hydrogenation was followed by treatment with trifluoroacetic acid (1 ml) for 30 min.

(i) DCCI (45)

DCCI (110 mg) was added to the reactants in the appropriate solvent (2 ml) and the mixture was stirred at room temperature for 18 h. The mixture was cooled, filtered, and the filtrate was evaporated.

[7]In the absence of HCl, only a small amount of ninhydrin-positive material was formed, presumably due to cyclization of the ester to the piperazine-dione after loss of the benzyloxycarbonyl group.

(ii) DCCI–HONSu (18)

As for the DCCI coupling except that HONSu (57 mg) was added to the reaction mixture before the DCCI.

(iii) HONSu Ester (46)

As for the DCCI–HONSu coupling except that the Gly-OBzl.HCl (0.101 g) in THF (1 ml) containing triethylamine (0.069 ml) was added 1 h after the DCCI and HONSu.

(iv) Mixed Anhydride (47)

The carboxy-containing component, base (0.069 ml of triethylamine or 0.056 ml of *N*-methylmorpholine), and isobutyl chloroformate (0.065 ml) were stirred in the solvent (2 ml) at −10° C for 90 s. The ester in THF (2 ml) was added, and the mixture was stirred at −10° for 1 h, and at room temperature for 1 h. One coupling was done under different conditions (Table 4).

(v) EEDQ (19)

A solution of the two reactants and EEDQ (129 mg; 5% excess) in THF (2 ml) was stirred at room temperature for 18 h. The neutral product was isolated as described above except that four washings with *N* HCl were carried out to remove the quinoline.

(vi) NEPIS (48)

The carboxy-containing component was stirred with NEPIS (127 mg) and triethylamine (0.069 ml) in acetonitrile (2.5 ml) at 0 °C for 1 h. The ester in cold acetonitrile (1 ml) was added, the mixture was stirred at 0° for 2 h, and then overnight at room temperature.

Couplings of Z-Ala + MeLeu-OBzl

Z-Ala (112 mg; 0.5 mmol) and MeLeu-OBzl.TosOH (202 mg) were coupled in THF (5 ml) using the methods described above. The neutral product was isolated and deprotected by hydrogenation in 80% aqueous acetic acid containing *N* HCl (0.5 ml) for 8 h. A solution of Z-Ala-ONp (39), MeLeu-OBzl.TosOH (202 mg), and triethylamine (0.069 ml) in dimethylformamide (2 ml) was kept at 37 °C for 36 h (43). The total neutral product was deprotected for analysis.

Couplings of Z-Gly + Ala-MeLeu-OR

Ala-MeLeu-OBzl.HCl (0.63 g; 91%; m.p. 157–160°; hygroscopic) was obtained by leaving Boc-Ala-MeLeu-OBzl (0.81 g) in dioxane containing hydrogen chloride (3.9 *N*, 10 ml) at room temperature for 1 h and evaporating the solution.

Ala-MeLeu-OBu^t.HCl (0.56 g; 70%; m.p. 176–178°) was prepared by hydrogenation of Z-Ala-MeLeu-OBu^t (1.1 g) (1) in *t*-butanol containing HCl (1 mol. equiv.) for 4 h. The product was obtained as a white solid by evaporating the solvent and drying the residual gel *in vacuo* over KOH.

Z-Gly (0.063 g) and Ala-MeLeu-OBzl.HCl (0.104 g) or Ala-MeLeu-OBu^t.HCl (0.092 g) were coupled in THF (2 ml) using the general methods described above, with triethylamine to neutralize the HCl. The nitrophenyl-ester coupling was carried out in dimethylformamide at 37° for 24 h. The neutral products were isolated to give Z-Gly-Ala-MeLeu-OBzl and Z-Gly-Ala-MeLeu-OBu^t as oils which were hydrogenated for 8 h. The *t*-butyl ester was treated with trifluoroacetic acid (1 ml) for 1 h before hydrogenation.

The color constant for Gly-Ala-MeLeu was determined from the deprotected product of the mixed-anhydride coupling of the benzyl ester. The product was washed with ether and dried *in vacuo* over KOH.

Trapping of Racemization Intermediate

Z-Ala-MeLeu (0.85 g) and DCCI (0.502 g) were stirred in THF (20 ml) at room temperature for 10 min. The mixture was cooled in ice, and methyl propiolate (0.50 ml) was added. There was an immediate evolution of carbon dioxide. The mixture was stirred at 0 °C for 2 h and then evaporated. The residue was suspended in acetone (20 ml), the mixture was kept at −10° for 3 h, and the *N,N'*-dicyclohexylurea (0.45 g; 83%) was filtered off. The filtrate was evaporated, the residue was taken up in ethyl acetate, and the solution was washed twice with aqueous $NaHCO_3$ and water, dried, and evaporated. The residual oil was crystallized (0.80 g) from ether – light petroleum and recrystallized from methanol–water. Yield 0.71 g (79%), m.p. 106–108°, n.m.r. δ($CDCl_3$–TMS): 7.31 (s, 5, $-C_6H_5$); 6.49 (s, 1, pyrrole); 5.11 (s, 2, $-CH_2O-$); 4.9 (b, 2, $-NHCH\langle$); 3.73 (s, 3, $-OCH_3$); 3.42 (s, 3, $\rangle NCH_3$); 2.84 (d, 2, $-CH_2-$, J = 7.0 Hz); 1.8 (m, 1, $\rangle CH-$); 1.52 (d, 3, CH_3-, J = 6.0 Hz); 0.92 (d, 6, $(CH_3)_2=$, J = 6.2 Hz).

Anal. Calcd. for $C_{21}H_{28}N_2O_4$: C, 67.7; H, 7.6; N, 7.5. Found: C, 67.4; H, 7.8; N, 7.3.

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1. J. R. McDERMOTT and N. L. BENOITON. Can. J. Chem. This issue.
2. E. SCHRÖDER and K. LÜBKE. The peptides. Vol. II. Academic Press, New York, N.Y. 1966. p. 396.
3. J. RUDINGER. *In* Drug design. *Edited by* E. J. Ariens. Vol. II. Academic Press, New York, N.Y. 1971. p. 319.
4. J. RUDINGER. *In* Peptides: Proc. of the 5th European Peptide Symposium, Oxford. 1962. *Edited by* G. T. Young. Oxford University Press, Oxford. 1963. p. 133.
5. M. BODANSZKY and M. A. ONDETTI. Peptide synthesis. Interscience Publishers, New York, N.Y. 1966. p. 137.
6. H. E. CARTER and C. M. STEVENS. J. Biol. Chem. **133**, 117 (1940).
7. R. W. JACKSON and W. M. CAHILL. J. Biol. Chem. **126**, 37 (1938).
8. IUPAC–IUB commission on biochemical nomenclature. J. Biol. Chem. **247**, 977 (1972).
9. M. GOODMAN and K. C. STUEBEN. J. Org. Chem. **27**, 3409 (1962).
10. R. L. HUGUENIN and R. A. BOISSONNAS. Helv. Chim. Acta, **44**, 213 (1961).
11. J. R. McDERMOTT and N. L. BENOITON. *In* Chemistry and biology of peptides: Proc. of the 3rd American Peptide Symposium, Boston, 1972. *Edited by* J. Meienhofer. Ann Arbor Publishers, Ann Arbor, Mich. 1972. p. 369.
12. N. L. BENOITON. Unpublished results.
13. M. W. WILLIAMS and G. T. YOUNG. J. Chem. Soc. 3701 (1964).

14. J. M. MANNING and S. MOORE. J. Biol. Chem. **243**, 5591 (1968).
15. N. IZUMIYA and M. MURAOKA. J. Am. Chem. Soc. **91**, 2391 (1969); N. IZUMIYA, M. MURAOKA, and H. AOYAGI. Bull. Chem. Soc. Jap. **44**, 3391 (1971).
16. J. R. COGGINS and N. L. BENOITON. J. Chromatogr. **52**, 251 (1970).
17. N. L. BENOITON and J. R. COGGINS. *In* Progress in peptide research, Vol. 2; Proc. of the 2nd American Peptide Symposium, Cleveland, 1970. *Edited by* S. Lande. Gordon and Breach, New York, N.Y. 1972. p. 145.
18. E. WÜNSCH and F. DREES. Chem. Ber. **99**, 110 (1966); F. WEYGAND, D. HOFFMANN, and E. WÜNSCH. Z. Naturforsch. **21b**, 426 (1966).
19. B. BELLEAU and G. MALEK. J. Am. Chem. Soc. **90**, 1651 (1968).
20. D. S. KEMP, Z. BERNSTEIN, and J. REBEK. J. Am. Chem. Soc. **92**, 4756 (1970).
21. G. W. ANDERSON. J. E. Zimmerman, and F. M. CALLAHAN. J. Am. Chem. Soc. **89**, 5012 (1967).
22. N. A. SMART, G. T. YOUNG, and M. W. WILLIAMS. J. Chem. Soc. 3902 (1960).
23. G. W. ANDERSON, J. BLODINGER, and A. D. WELCHER. J. Am. Chem. Soc. **74**, 5309 (1952); F. WEYGAND, A. PROX, L. SCHMIDHAMMER, and W. KÖNIG. Angew. Chem. **75**, 282 (1963).
24. M. GOODMAN and W. J. MCGAHREN. Tetrahedron, **23**, 2031 (1967).
25. A. W. WILLIAMS and G. T. YOUNG. J. Chem. Soc. Perkin I, 1194 (1972).
26. A. NEUBERGER. Adv. Protein Chem. **4**, 297 (1948).
27. R. HUISGEN. *In* Aromaticity. Chem. Soc. Special Publ. No. 21, London. 1967. p. 51.
28. J. L. O'BRIEN and C. NIEMANN. J. Am. Chem. Soc. **79**, 80 (1957).
29. J. W. CORNFORTH and D. F. ELLIOT. Science, **112**, 534 (1950).
30. G. V. BOYD and P. H. WRIGHT. J. Chem. Soc. Perkin I, 909 (1972).
31. R. HUISGEN, H. GOTTHARDT, H. O. BAYER, and F. C. SCHAEFER. Angew. Chem. (Int. ed.), **3**, 136 (1964).
32. G. V. BOYD and P. H. WRIGHT. J. Chem. Soc. Perkin I, 914 (1972).
33. E. SCHRÖDER and K. LÜBKE. The peptides. Vol. I. Academic Press, New York, N.Y. 1965. p. 109.
34. C. V. GRECO, R. P. GRAY, and V. G. GROSSO. J. Org. Chem. **32**, 4101 (1967).
35. F. WEYGAND, W. STEGLICH, and N. CHYTIL. Z. Naturforsch. **23b**, 1391 (1968); H. GROSS and L. BILK. Tetrahedron, **24**, 6935 (1968).
36. P. A. JAQUENOUD. Chimia, **14**, 373 (1960).
37. J. P. GREENSTEIN and M. WINITZ. Chemistry of the amino acids. John Wiley and Sons, New York, N.Y. 1962.
38. D. H. SPACKMAN, W. H. STEIN, and S. MOORE. Anal. Chem. **30**, 1190 (1958).
39. F. MARCHIORI, R. ROCCHI, and E. SCOFFONE. Gazz. Chim. Ital. **93**, 834 (1963).
40. J. R. COGGINS and N. L. BENOITON. Can. J. Chem. **49**, 1968 (1971).
41. J. R. MCDERMOTT and N. L. BENOITON. Can. J. Chem. **51**, 1915 (1973).
42. E. KLIEGER, E. SCHRÖDER, and H. GIBIAN. Ann. **640**, 157 (1961).
43. M. BODANSZKY. Acta Chim. Acad. Sci. Hung. **10**, 335 (1957).
44. J. D. CIPERA and R. V. V. NICHOLLS. Chem. Ind. 16 (1955).
45. J. C. SHEEHAN and G. P. HESS. J. Am. Chem. Soc. **77**, 1067 (1955).
46. G. W. ANDERSON, J. E. ZIMMERMAN, and F. M. CALLAHAN. J. Am. Chem. Soc. **85**, 3039 (1963).
47. R. A. BOISSONNAS. Helv. Chim. Acta, **34**, 874 (1951); J. R. VAUGHAN. J. Am. Chem. Soc. **73**, 3547 (1951); T. WIELAND and H. BERNHARD. Ann. **572**, 190 (1951).
48. R. B. WOODWARD, R. A. OLOFSON, and H. MAYER. J. Am. Chem. Soc. **83**, 1010 (1961).