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An Improvement of the Anderson Test by the Use of High Performance Liquid Chromatography

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Synopsis. The replacement of the *N*-benzyloxycarbonyl (Z) group in the Anderson peptide (Z-Gly-L/D-Phe-Gly-OEt) by Z-L-Ala and the subsequent separation of the diastereomers of the resulting tetrapeptide by reversed-phase HPLC offer a more convenient version with higher accuracy to the original Anderson test for studying racemization in peptide synthesis.

There have been known a variety of tests[1)] devised for the estimation of the racemization extent during peptide bond formation. Among them the model coupling of Z-Gly-L-Phe-OH[2)] with H-Gly-OEt proposed by Anderson et al.[3)] has widely been used for investigating coupling methods and conditions in peptide synthesis, through which extensive data have been accumulated.[4)] Since this test measures the weight of racemate isolated by fractional crystallization, the limit of detection has been shown to be ca. 1—2% racemate.[1a)] Moreover, in some cases the presence of by-products of the reaction is prone to make it difficult to separate the pure racemate. Kemp et al.[5)] applied to the original the multiple isotope dilution procedure, which extended the sensitivity of the test by several orders of magnitude.[1a)] However, this procedure still necessitates the isolation of a small amount of pure racemate by crystallization. Therefore, both the original and the improved procedures are not free from a troublesome manipulation of the separation. Thus, an improvement of the Anderson test is very desirable, which would omit the isolation of racemate and allow the analysis to be accomplished more conveniently and with higher accuracy.

Recently, we have found that the diastereomeric pairs of a series of protected tripeptides, Z-L/D-AA-Gly-L-Phe-OMe (where AA denotes various amino acid residues), can well be separated by reversed-phase high-performance liquid chromatography (RP-HPLC).[6)] Moreover, the kinetic resolution was found to be almost negligible in the coupling of Z-L/D-AA-OH with H-Gly-L-Phe-OMe, probably because achiral Gly occupies the coupling site in the amino component. Thus, it is reasonable to anticipate that the replacement of the Z group in the Anderson peptide (**1**) by an appropriate Z-L-AA will make it possible to separate the diastereomers of the resulting tetrapeptide (**2**) by RP-HPLC for the quantitation of the epimer in **1** (Scheme 1). As shown in Table 1, this was the case with the tetrapeptides **2** for AA=Ala, Val, Leu, and Pro. When the *N*-hydroxysuccinimide ester of Z-L-AA-OH (Z-L-AA-OSu) (for AA=Ala, Val, and Leu) was reacted with H-Gly-DL-Phe-Gly-OEt,[7)] the diastereomer ratio of the resulting tetrapeptide was close to 50 : 50.[8)]

Z-Gly-L/D-Phe-Gly-OEt → H-Gly-L/D-Phe-Gly-OEt
1

→ Z-L-AA-Gly-L/D-Phe-Gly-OEt
2

Scheme 1.

Based on the above results, we chose the simplest Z-L-Ala and decided on our version for the Anderson test as follows. The crude product obtained through the coupling of Z-Gly-L-Phe-OH with H-Gly-OEt by the method under consideration is hydrogenated over Pd-carbon in ethanol in the presence of 1 equiv. of concd hydrochloric acid. The debenzyloxycarbonylated product is then reacted with 2 equiv. of Z-L-Ala-OSu in DMF in the presence of 1 equiv. of triethylamine for 24 h. The resulting tetrapeptide, after suitable washings, is submitted to a separation of the diastereomers by RP-HPLC. The limit of detection

Table 1. HPLC Separation of Diastereomers of Z-AA-Gly-Phe-Gly-OEt (**2**)[a)]

AA	Mobile phase[b)]	k'[c)] L-L	k'[c)] L-D	α[d)]
Ala	A	9.49	11.17	1.18
Val	B	8.79	10.45	1.19
Leu	C	7.82	8.99	1.15
Pro	B	6.61	8.59	1.30
Phe	B	8.76	8.76	1.00

a) Conditions: column, Cosmosil $5C_{18}$ (4.6 mm I.D.×150 mm); flow rate, 1.0 ml min^{-1}; column temperature, 30 °C; detection, 254 nm. b) A, 55% MeOH aq; B, 60% MeOH aq; C, 65% MeOH aq. c) Capacity factor, $k'=(t_R-t_0)/t_0$, where t_0 denotes the void time (ca. 1.5 min). d) Separation factor, $\alpha=k'_{L\text{-}D}/k'_{L\text{-}L}$.

Table 2. Racemization Extent during the Coupling of Z-Gly-L-Phe-OH with H-Gly-OEt by the DCC Method plus Additive[a)]

Additive	D %[b)]
None	5.3
HOBt	<0.1[c)]
HOSu	<0.1[c)]
HONB	<0.1[c)]
Stearic acid	1.2
Oleic acid	2.8
Palmitic acid	2.8
Lauric acid	3.6
$CuCl_2$	<0.1[c)]

a) Reaction conditions: Z-Gly-L-Phe-OH/H-Gly-OEt/additive/DCC, 1 : 1 : 1 : 1 equiv.; solvent, THF (reactant concentration, 0.083 mol dm^{-3}); duration and temperature, 24 h at 5 °C. b) Obtained as L-D % of the tetrapeptide **2** for AA=Ala. c) Sensitivity range, 0.01 a.u.f.s.

was estimated as ca. 0.1% epimer.

Table 2 shows the results obtained by our improved procedure on the racemization suppressing effect of several additives in the dicyclohexylcarbodiimide (DCC) mediated couplings affording the Anderson peptide. By the use of copper(II) chloride[9] as well as the common *N*-hydroxy compounds such as 1-hydroxybenzotriazole (HOBt), *N*-hydroxysuccinimide (HOSu), and *N*-hydroxy-5-norbornene-*endo*-2,3-dicarboximide (HONB),[10] practically no racemization could be detected. On the other hand, the effect of the long-chain fatty acids, regarded as factors ordering the reaction medium, whose usefulness as a new type of additives had been demonstrated using the conventional Anderson test,[4g] proved to be considerable but not complete. Probably this discrepancy is partly due to the fact that the reaction conditions in both cases were not identical; primarily, though, it is due to the difference in the procedure for the quantitation of the epimer in the Anderson peptide.

A combination of the present improvement utilizing the HPLC separation of the diastereomers of the tetrapeptide derived from the Anderson peptide, and the multiple dilution procedure as applied by Kemp et al.[5] would increase the limit of detection in the Anderson test with greater precision.

Experimental

All melting points are uncorrected. Optical rotations were measured with a JASCO DIP-4 polarimeter. TLC and preparative TLC were performed on Merck Kieselgel 60 F_{254} and Kieselgel GF_{254} (Type 60), respectively. The liquid chromatograph employed was a Shimadzu LC-3A instrument, equipped with a Shimadzu SPD-2A variable wavelength UV monitor and a Shimadzu Chromatopac C-R1A data processor. A prepacked ODS column (Cosmosil $5C_{18}$) was consisted of a stainless steel tube, 4.6 mm I.D. and 150 mm long.

Z-Gly-DL-Phe-Gly-OEt. This was prepared by the reaction of Z-Gly-OSu with H-DL-Phe-Gly-OEt·HBr[11] in CH_2Cl_2; yield, 85%; mp 132—133 °C (acetone aq). [lit,[12] mp 132—133 °C].

Z-Gly-L-Phe-Gly-OEt. This compound has previously been prepared through the coupling of Z-Gly-L-Phe-OH with H-Gly-OEt by various methods (Anderson test), while its preparation by the stepwise elongation procedure has not been reported so far, to our knowledge. The reaction of Z-Gly-OSu with H-L-Phe-Gly-OEt·HCl[11] (prepared through the debenzyloxycarbonylation of Z-L-Phe-Gly-OEt[13] by hydrogenation in the presence of 5% Pd-C and concd HCl) in CH_2Cl_2 afforded white crystals; yield, 87%; mp 118—119 °C (EtOAc-hexane); $[\alpha]_D^{25}$ −12.5° (*c* 1.0, EtOH).

HPLC Separation of the Diastereomers of Z-AA-Gly-Phe-Gly-OEt. Z-L-AA-OSu (for AA=Ala, Val, Leu, Pro, and Phe) (0.125 mmol) and H-Gly-DL-Phe-Gly-OEt·HCl (prepared through the debenzyloxycarbonylation of Z-Gly-DL-Phe-Gly-OEt by hydrogenation in the presence of 5% Pd-C and concd HCl) (0.125 mmol) were stirred in DMF (1.5 ml) in the presence of TEA (0.125 mmol) at room temperature overnight. The neutral fraction was separated from the reaction mixture as usual. This fraction was submitted to separation of diastereomers by RP-HPLC under the conditions described in Table 1.

On the other hand, Z-L-AA-Gly-L-Phe-Gly-OEt (for AA=the same residues) was prepared from Z-L-AA-OSu and H-Gly-L-Phe-Gly-OEt·HCl[11] in a manner similar to that described above, and submitted to HPLC analysis.

Z-L-Ala-Gly-L-Phe-Gly-OEt. Z-L-Ala-OSu (298 mg) and H-Gly-L-Phe-Gly-OEt·HCl (prepared through the debenzyloxycarbonylation of Z-Gly-L-Phe-Gly-OEt by hydrogenation in the presence of 5% Pd-C and concd HCl) (310 mg) were stirred in the presence of TEA (91 mg) at room temperature for 3 d. To the reaction mixture was EtOAc added, and the resulting suspension was washed successively with 5% HCl, water, sat. $NaHCO_3$, and water, and dried over Na_2SO_4. Removal of the solvent afforded crystals, which were recrystallized from MeOH; yield, 304 mg (66%); mp 189—190 °C; $[\alpha]_D^{25}$ −9.6° (*c* 1.0, DMF). Found: C, 60.66; H, 6.08; N, 10.97. Calcd for $C_{26}H_{32}N_4O_7$: C, 60.93; H, 6.29; N, 10.93.

Z-D-Phe-Gly-OEt. This was prepared from Z-D-Phe-OH and H-Gly-OEt·HCl[11] by the DCC method in CH_2Cl_2; yield, 90%; mp 110—111 °C (EtOAc-petroleum ether); $[\alpha]_D^{25}$ +17.3° (*c* 2.0, EtOH). Cf. lit[13] for the L isomer, mp 110—110.5 °C, $[\alpha]_D^{25}$ −17.1° (*c* 2, EtOH).

Z-Gly-D-Phe-Gly-OEt. This was obtained by the coupling of Z-Gly-OSu with H-D-Phe-Gly-OEt·HCl[11] as described for the corresponding L isomer; yield, 90%; mp 118—119 °C (EtOAc-hexane); $[\alpha]_D^{25}$ +12.4° (*c* 1.0, EtOH). Found: C, 62.76; H, 6.18; N, 9.47. Calcd for $C_{22}H_{27}N_3O_6$: C, 62.57; H, 6.16; N, 9.52.

Z-L-Ala-Gly-D-Phe-Gly-OEt. This was prepared from Z-L-Ala-OSu and H-Gly-D-Phe-Gly-OEt·HCl[11] as described for the corresponding L-L isomer. The final purification was conducted by preparative TLC with $CHCl_3$-MeOH (9 : 1) to afford the desired compound as a syrup; yield, 77%; $[\alpha]_D^{25}$ +1.6° (*c* 1.0, DMF). Found: C, 60.71; H, 6.42; N, 10.88.

Z-Gly-L-Phe-OH. A solution of Z-Gly-OSu (12.25 g) in acetonitrile (70 ml) was added to a solution of H-L-Phe-OH (9.91 g) and $NaHCO_3$ (10.08 g) in water (180 ml) with stirring at room temperature. After 6 h of stirring, the reaction mixture was concentrated to ca. 120 ml and acidified with concd HCl to pH 2. The separated oil was extracted with EtOAc, washed with water, and dried over Na_2SO_4. Evaporation of the solvent in vacuo afforded crystals, which were recrystallized from EtOAc-petroleum ether; yield, 11.86 g (83%); mp, 128.5—129.5 °C; $[\alpha]_D^{25}$ +38.7° (*c* 1.0, EtOH). [lit,[14] mp 127—128 °C, $[\alpha]_D^{25}$ +36.6°±0.5° (*c* 5, EtOH)].

A part of the sample was esterified with diazomethane in ether, hydrogenated in the presence of 5% Pd-C and concd HCl, and then coupled[11] with Z-L-Ala-OH using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in the presence of HOBt in THF according to a previous report.[6] The HPLC analysis of the resulting product showed no presence of the D-L isomer, under the following conditions: mobile phase, 60% MeOH aq; flow rate, 1.0 ml min^{-1}; column temperature, 30 °C; detection, 254 nm; sensitivity range, 0.02 absorbance units full scale (a.u.f.s.). The L-L isomer of Z-Ala-Gly-Phe-OMe had t_R=11.10 min and the D-L isomer t_R=12.72 min.

Determination of Racemization in the Anderson Test. a) General Procedure: Z-Gly-L-Phe-OH (0.125 mmol) was coupled with H-Gly-OEt (0.125 mmol) by a certain coupling method. After suitable washes, the crude product obtained was dissolved in EtOH (5 ml), and hydrogenated in the presence of 5% Pd-C (15 mg) and concd HCl (0.011 ml). After filtration of the catalyst and a successive removal of the solvent, the residue was dried by the addition of MeOH followed by evaporation in vacuo and then dissolved in DMF (1 ml). To this solution Z-L-Ala-OSu (0.25 mmol) was added, followed by TEA (0.125 mmol). After the mixture had been stirred at 23 °C for 24 h, 1-(2-aminoethyl)piperazine (16 mg) was added and stirring was continued for 20 min. The mixture was distributed between EtOAc and 5% HCl, and the organic layer was further washed with 5% HCl, water, sat. $NaHCO_3$, and water, and dried over Na_2SO_4. After evaporation of the solvent, the residue was dissolved in MeOH (3 ml). This solution (5 μl) was injected through a microsyringe into the HPLC apparatus under the following conditions: mobile phase, 55% MeOH aq; flow rate, 1.0 ml min^{-1}; column temperature, 30 °C; detection, 254 nm; sensitivity range, 0.02 a.u.f.s. The L-L isomer of Z-Ala-Gly-Phe-Gly-OEt had t_R=16.37 min and the L-D isomer t_R=18.99 min, while the void time was t_0=1.56 min.

Thus, the percentage of D isomer in the original Anderson peptide was determined by the percentage of L-D isomer of the tetrapeptide. The limit of detection was estimated as ca. 0.1% epimer.

b) Racemization during the Coupling by the DCC Method plus Additive: Reactions were run by dissolving Z-Gly-L-Phe-OH, H-Gly-OEt, and additive where pertinent (1 : 1 : 1 equiv.) in THF (reactant concentration, 0.018 mol dm^{-3}) and adding 1 equiv. of DCC. After the mixture had been stirred at 5 °C for 24 h, it was diluted with EtOAc and treated as usual. The crude product was hydrogenated, and then reacted with Z-L-Ala-OSu as described above. The results are summarized in Table 2.

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2) Abbreviations given by the IUPAC-IUB Joint Commission (*Eur. J. Biochem.*, **138**, 9 (1984)) are used throughout. Additional abbreviations: Z, benzyloxycarbonyl; DMF, *N,N*-dimethylformamide; TEA, triethylamine; THF, tetrahydrofuran.

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