A MODIFICATION OF THE IZUMIYA–MURAOKA PEPTIDE RACEMIZATION TEST

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A new highly sensitive test for racemization in peptide synthesis was introduced recently by Izumiya and Muraoka.[1] These authors condensed Z-Gly-L-Ala-OH† with L-Leu-OBzl by the particular coupling procedure under investigation, and the crude product Z-Gly-Ala-Leu-OBzl was then catalytically hydrogenated to form the free tripeptide which was applied to a Hitachi KLA-3B amino acid analyser. The D-L diastereoisomer resulting from any racemization is readily separated from the predominant L-L peptide, and the degree of racemization determined. In this system, neither of the tripeptide peaks overlapped those of glycylalanine or leucine, which might be present as a result of incomplete coupling. One part of D-L isomer in 1000 could be detected by the method.

Employment of catalytic hydrogenation in the second stage could be a disadvantage in some circumstances, partly on grounds of convenience, and, more particularly, if coupling procedures involving sulphur compounds were being studied. An alternative approach would be the use of a more acid-labile ester of L-leucine than the benzyl derivative, with subsequent removal of the protecting groups in the tripeptide intermediate by the action of hydrogen bromide in acetic acid under mild conditions.

The t-butyl ester would probably be selected most frequently for this purpose, but the proposed modification of the Izumiya–Muraoka test conditions is illustrated here using the 2,4,6-trimethylbenzyl carboxyl-protecting group.[2,3] This acid-labile ester group was also employed for the synthesis of various protected intermediates and reference peptides required in the work.

The optically pure tripeptide H-Gly-L-Ala-L-Leu-OH was obtained by the action of 2N hydrogen bromide in acetic acid on Z-Gly-L-Ala-L-Leu-OTMB, which was synthesized in stepwise manner from L-Leu-OTMB,HCl[4] through the *o*-nitrophenylsulphenyl derivative NPS-L-Ala-L-Leu-OTMB.[2,3] The tripeptide gave a single peak in the Beckman 120B analyser with an elution time of 108 min in pH 4·25 citrate buffer.

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† Abbreviations used are : Z, benzyloxycarbonyl; NPS, *o*-nitrophenylsulphenyl; Bzl, benzyl; TMB, 2,4,6-trimethylbenzyl; NP, *p*-nitrophenyl; DCHA, dicyclohexylamine; DMF, dimethylformamide.

[1] Izumiya, N., and Muraoka, M., *J. Am. chem. Soc.*, 1969, **91**, 2391.

[2] Stewart, F. H. C., *Aust. J. Chem.*, 1966, **19**, 1067.

[3] Stewart, F. H. C., *Aust. J. Chem.*, 1968, **21**, 2831.

[4] Ledger, R., and Stewart, F. H. C., *Aust. J. Chem.*, 1968, **21**, 1101.

A mixture of the L-L and D-L diastereoisomers of the protected tripeptide was prepared by the mixed carbonic anhydride method from Z-Gly-DL-Ala-OH,[5] and a sample treated with the hydrogen bromide reagent. The D-L tripeptide was separated from the L-L isomer on the analyser, and had an elution time of 131 min.

The method was checked with a peptide coupling procedure in which the degree of racemization is known to be very low. Z-Gly-L-Ala-OH* was coupled with L-Leu-OTMB using Woodward's reagent K essentially as described in the literature.[1,7] The presence of D-L diastereoisomer corresponding to 0·7% racemization was readily detected. Izumiya and Muraoka[1] reported 1·8% racemization for this reaction with the benzyl ester.

The free dipeptides H-Gly-L-Ala-OH and H-L-Ala-L-Leu-OH were prepared for reference purposes from the corresponding protected 2,4,6-trimethylbenzyl derivatives. Their elution times on the analyser were 62 and 122 min, respectively.

Experimental

The microanalyses were carried out by the Australian Microanalytical Service, Melbourne. Melting points are uncorrected. Thin-layer chromatography was performed on Silicagel G plates with n-butanol–acetic acid–water (3 : 1 : 1) as the solvent system.

(*a*) o-*Nitrophenylsulphenyl*-L-*alanyl*-L-*leucine 2,4,6-Trimethylbenzyl Ester*

The compound was obtained in 95% yield from NPS-Ala-OH,DCHA [8] and Leu-OTMB,HCl [4] according to the standard dicyclohexylcarbodiimide coupling procedure described by Zervas, Borovas, and Gazis,[8] and recrystallized from ethyl acetate–light petroleum, m.p. 113·5–114·5°; $[\alpha]_D^{20\cdot5}$ −19·8° (*c*, 1·0; DMF) (Found; C, 61·3; H, 6·5; N, 8·5. Calc. for $C_{25}H_{33}N_3O_5S$: C, 61·6; H, 6·8; N, 8·6%).

Treatment of the product with 2N hydrogen bromide in acetic acid for 15 min at room temperature, and dilution with ether, gave L-alanyl-L-leucine hydrobromide, which was converted into the free dipeptide on a Dowex 3 column in the usual way.[2] The yield was 65%, and the compound was recrystallized from methanol–ethyl acetate, m.p. 256–257·5° (dec.); $[\alpha]_D^{20}$ −15·6° (*c*, 0·5; H_2O); R_F 0·59. Lit.[9] $[\alpha]_D^{21}$ −17·0°.

(*b*) L-*Alanyl*-L-*leucine 2,4,6-Trimethylbenzyl Ester Hydrochloride*

The foregoing *o*-nitrophenylsulphenyl derivative was treated with 1N hydrogen chloride in dioxan (2·5 equiv.).[2,3] The product was obtained in 99% yield, and recrystallized from methanol–ether, m.p. 191·5–192°; $[\alpha]_D^{20\cdot5}$ −25·0° (*c*, 0·5; MeOH); R_F 0·68 with a very faint trace of impurity at 0·50 (Found: Cl, 9·4; N, 7·4. Calc. for $C_{19}H_{31}ClN_2O_3$: Cl, 9·6; N, 7·6%).

(*c*) *Benzyloxycarbonylglycyl*-L-*alanyl*-L-*leucine 2,4,6-Trimethylbenzyl Ester*

The tripeptide derivative was prepared in 92% yield from Z-Gly-ONP and Ala-Leu-OTMB,HCl by the active ester procedure,[2,3] and recrystallized from ethyl acetate–cyclohexane, m.p. 161·5–163°; $[\alpha]_D^{20\cdot5}$ −16·4° (*c*, 1·0; DMF) (Found: C, 66·2; H, 7·8; N, 7·9. Calc. for $C_{29}H_{39}N_3O_6$: C, 66·3; H, 7·4; N, 8·0%).

* Conveniently prepared here by selective cleavage of the 2,4,6-trimethylbenzyl group in Z-Gly-L-Ala-OTMB with trifluoroacetic acid at room temperature.[6]

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[7] Woodward, R. B., Olofson, R. A., and Mayer, H., *J. Am. chem. Soc.*, 1961, **83**, 1010.

[8] Zervas, L., Borovas, D., and Gazis, E., *J. Am. chem. Soc.*, 1963, **85**, 3660.

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(*d*) *Glycyl-L-alanyl-L-leucine*

The benzyloxycarbonyl tripeptide 2,4,6-trimethylbenzyl ester was converted into the free tripeptide as in (*a*) with a reaction time of 1 hr. The yield was 85%, and the product was recrystallized from aqueous ethanol, m.p. 226–228° (dec.); $[\alpha]_D^{20}$ −86·5° (*c*, 1·0; 0·1N HCl); R_F 0·45. Lit.[10] $[\alpha]_D^{22}$ −85·0°.

(*e*) *Benzyloxycarbonylglycyl-L-alanine 2,4,6-Trimethylbenzyl Ester*

The compound was prepared from Z-Gly-ONP and Ala-OTMB,HCl[2] in 96% yield by the active ester method as in (*c*), and recrystallized from ethyl acetate–cyclohexane, m.p. 94·5–95·5°; $[\alpha]_D^{20.5}$ −12·8° (*c*, 1·0; DMF) (Found: C, 66·8; H, 6·7; N, 6·5. Calc. for $C_{23}H_{28}N_2O_5$: C, 67·0; H, 6·8; N, 6·8%).

Glycyl-L-alanine was obtained in 66% yield by treatment of this derivative with the hydrogen bromide reagent as before. The free dipeptide was recrystallized from aqueous ethanol, m.p. 234–236·5° (dec.); $[\alpha]_D^{20.5}$ −61·0° (*c*, 1·0; 0·5N HCl). Lit.[11] $[\alpha]_D^{25}$ −59·3°.

(*f*) *Benzyloxycarbonylglycyl-L-alanine*

The 2,4,6-trimethylbenzyl derivative (900 mg) was treated with trifluoroacetic acid (5·0 ml) and anisole (1·0 ml) for 30 min at room temperature, and the mixture worked up as described previously.[6] The product was obtained in 94% yield, and recrystallized from ethyl acetate–cyclohexane, m.p. 136·5–137·5°; $[\alpha]_D^{19.5}$ −15·4° (*c*, 1·0; EtOH). Lit.[5] m.p. 133°; $[\alpha]_D^{19}$ −9·5°.

(*g*) *Racemization Test Procedure*

(i) Z-Gly-DL-Ala-OH[5] was coupled with Leu-OTMB in dimethylformamide solution by the mixed carbonic anhydride method. The mixture of diastereoisomeric protected tripeptides produced was isolated by extraction with ethyl acetate followed by standard acidic and basic washing procedures. The crude amorphous product was washed with light petroleum (yield, 78%).

A sample (50 mg, *c.* 100 μmole) was treated with 2N hydrogen bromide in acetic acid (0·5 ml) for 1 hr at room temperature. The solution was diluted with water and lyophilized prior to application to the amino acid analyser in pH 4·25 citrate buffer.

(ii) Z-Gly-L-Ala-OH was coupled with Leu-OTMB using Woodward's reagent K in acetonitrile solution.[1,7] The yield of crystalline product was 87%, and this material was treated as in (i).

Acknowledgments

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