70. A New Method for the Detection of Racemization during Peptide Synthesis: Stereoselective Hydrolysis of Diastereomeric Peptides by Leucine Aminopeptidase

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(27. XII. 72)

Summary. A suitably protected dipeptide of configuration L-D, *e.g.* Z-L-Ala-D-Ala is coupled with an all L alanine peptide, *e.g.* L-Ala-L-Ala-ONb[3]). The blocking groups are removed and the free peptide hydrolyzed by leucine amino peptidase (E.C. 3.4.1.1). This enzyme shows absolute L-specificity for the penultimate peptide bond from the amino end and therefore cleaves only the all L peptide formed through racemization. The amount of free alanine determined by amino acid analysis gives a multiple of the degree of racemization. The sensitivity of the test allows 0.1% of $(L\text{-Ala})_4$ to be detected in the synthesis of L-Ala-D-Ala-L-Ala-L-Ala. Coupling of Z-L-Ala-D-Ala and Z-L-Ala-D-Phe with di- and trialanine peptides has been studied using DCCI and DCCI + 1-hydroxybenzotriazole as coupling reagents. The degree of racemization was around 80% for the coupling by DCCI in DMF but was reduced to 0.2–0.4% in the presence of 2 equivalents of 1-hydroxybenzotriazole. Coupling using the succinimide esters Z-L-Ala-D-Ala-ONSu and Z-L-Ala-D-Phe-ONSu resulted in 0.8 to 10% racemization, depending on the solvent and base used.

Racemization is a dominating problem in the chemical synthesis of peptides. Unequivocal and sensitive methods for the detection of racemization are prerequisites for the synthesis of peptides which are to be used in biological studies. One approach to the problem is the physical separation of diastereomeric peptides formed during a racemization prone coupling reaction. Procedures which have been utilized include fractional recrystallization [1] and its combination with isotope dilution [2], gas-liquid chromatography [3], paper chromatography [4], thin layer chromatography [5], and ion-exchange chromatography [6]. Another possibility for differentiating asymmetric centers in peptides is the acidic hydrolsyis of the peptide and analysis of the optical purity of the free amino acids either by enzymic degradation (*e.g.* [7]), by gas-liquid chromatography on an asymmetric phase [8] or by ion-exchange chromatography after derivation of the free amino acids [9]. NMR.-analysis of diastereomeric mixtures [10] and proton-tritium exchange on the asymmetric C_α [11] have also been used successfully in recent years. Many of the physical separation methods rely on model amino acids and peptides which satisfy the special needs of the individual techniques. Analysis after acid hydrolysis [8] [9], isotope exchange [11] and enzymic degradation are more generally applicable. With few exceptions (*e.g.* [2] and [11])

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[3]) Abbreviations according to the IUPAC-IUB rules, 'Symbols for Amino-Acid Derivatives and Peptides, Recommendations (1971)', see *e.g.* J. biol. Chemistry *247*, 977 (1972). In particular the following abbreviations have been used: Z = benzyloxycarbonyl-, -ONb = *p*-nitrobenzyloxy-, -ONSu = succinimido-oxy-. Additional abbreviations are LAP = leucine aminopeptidase, DCCI = N,N'-dicyclohexylcarbodiimide, DMF = dimethylformamide.

the sensitivities of the cited methods are rather low; usually racemization below 1% goes undetected.

We report a highly sensitive enzymic method. It is based on the generally low side chain selectivity [12] and absolute L-specificity [13] which is found under certain conditions for LAP[3]). Peptides of the structure L-Ala-D-Ala-L-Ala-L-Ala ... in which the next to last amino acid residue from the amino end is of the D-configuration resist enzymic attack completely [13]. The principle of the procedure is depicted in the scheme.

Scheme

Z-L-Ala-D-Ala-OH + H-L-Ala-L-Ala-ONb

↓ coupling step, partially changes configuration at second alanine residue

Z-L-Ala-D-Ala-L-Ala-L-Ala-ONb, Z-(L-Ala)$_4$-ONb

↓ deprotection

L-Ala-D-Ala-L-Ala-L-Ala, (L-Ala)$_4$

↓ LAP hydrolysis, amino acid analysis

amount of free alanine corresponds to 4× (L-Ala)$_4$

A dipeptide of configuration L-D is coupled with an all L alanine peptide, the blocking groups are removed and the free peptide assayed with LAP. Any racemization during the coupling[4]) leads to a peptide with all L configuration which consequently is split into free alanine residues on digestion by LAP. The peptide with unchanged configuration resists LAP hydrolysis completely. The amount of alanine freed enzymically corresponds to a multiple of the degree of racemization occurred during the coupling step. The alanine is readily determined quantitatively by amino acid analysis. The sensitivity of the method depends on the amplification factor introduced by the amino fragment, the resolving power of the amino acid analysis and the optical purity of the D-amino acid used for the synthesis of the carboxyl fragment[5]). Under some of the reported conditions as little as 0.2% racemization could be detected.

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[4]) Only racemization *via* azlactone formation in the carboxyl fragment is considered. Racemization through proton abstraction at any other asymmetric C_α would result in different diastereometric products which consequently would obscure the result of the LAP hydrolysis. However, racemization of alanine residues *via* base catalyzed proton abstraction is extremely unlikely under the conditions employed here.

[5]) The stereochemical purity of the commercial D-amino acids was not checked. The amount of L-amino acid in the preparations used here must be below 0.1% since a configurational change of $^1/_{1000}$ could be detected. If greater accuracy is required or if commercial preparations are less pure, treatment by L-amino acid oxidase may provide adequate starting material.

Table 1. *Nanomoles of alanine freed enzymically from 2000 nanomoles each of* L-*Ala*-D-*Ala*-L-*Ala*-L-*Ala and* L-*Ala*-D-*Phe*-L-*Ala*-L-*Ala and from 50 nanomoles of* L-*Ala*-L-*Ala*-L-*Ala*-L-*Ala during a 24 h incubation with LAP*

The two L-D-L-L peptides contained 0.5% and 0.4%, respectively of the all L diastereomers.

Peptide	nanomoles of free alanine				
	0.1 h	0.5 h	1 h	3 h	24 h
L-Ala-D-Ala-L-Ala-L-Ala	18	36	34	40	44
L-Ala-D-Phe-L-Ala-L-Ala	12	22	26	24	28
L-Ala-L-Ala-L-Ala-L-Ala	90	220	195	215	

To test the method, the peptides Z-L-Ala-D-Ala-L-Ala-L-Ala-ONb, Z-L-Ala-D-Ala-L-Ala-L-Ala-L-Ala-ONb and Z-L-Ala-D-Phe-L-Ala-L-Ala-ONb were synthesized by fragment condensation as outlined in the scheme. Any unwanted separation of diastereomers by recrystallization was avoided. The crude peptides were hydrogenated and the free peptides collected by acetone precipitation. Hydrolysis by LAP was performed at pH 8.6 and 37° for at least 3 h. The substrate was 15 mMol and the enzyme concentration 0.025 mg per ml. The all L peptides were completely hydrolyzed within 30 to 60 min. under these conditions. Peptides containing the single D-residue in the second position remained intact over a 24 h incubation period. This was evident from the experiment summarized in Table 1. The small amount of alanine freed from the slightly 'racemized' L-D-L-L peptides appeared within the first hour of hydrolysis, this was the time necessary to degrade completely a L-L-L-L peptide. Furthermore, it has been shown earlier [13] that a great excess of L-D-L-L peptides does not inhibit the LAP hydrolysis of the all L peptides.

The products of the enzymic hydrolysis were qualitatively analyzed by high voltage paper electrophoresis at pH 1.4 where alanine peptides are separated according to their size [14]. The percentage of racemization was calculated from the amount of free alanine which was determined independently by amino acid analysis on the whole hydrolysis mixture. Samples from the hydrolysis of up to 2 μmol of original peptide were chromatographed on the long column of the amino acid analyzer [15]. Except for the peptide L-Ala-D-Ala-L-Ala-L-Ala-L-Ala, all hydrolysis resistant peptides were eluted after the alanine peak. The separation of the free amino acids from the intact peptides was in every case sufficient to allow heavy overloading of the column and detection of at least 1 part in 1000.

Table 2. *Racemization during the synthesis of Z-*L-*Ala*-D-*Ala*-L-*Ala*-L-*Ala-ONb and Z-*L-*Ala*-D-*Phe*-L-*Ala*-L-*Ala-ONb*

Succinimide esters were reacted in DMF (except where mentioned otherwise) overnight at ambient temperature; in all other cases reaction times were 1 h at −10° followed by 15 to 20 h at room temperature. Reaction mixtures were 0.2 M each in amino and carboxyl component; equivalents of other reagents are given in parenthesis. HOBt = 1-hydroxybenzotriazole, Et_3N = triethylamine, NMM = N-methylmorpholine.

	% racemization
Z-L-Ala-D-Ala + HBr · H-L-Ala-L-Ala-ONb DCCI (1.1), Et_3N (1)	80
Z-L-Ala-D-Phe + HBr · H-L-Ala-L-Ala-ONb DCCI (1.1), Et_3N (1)	90
Z-L-Ala-D-Ala + HBr · H-L-Ala-L-Ala-ONb DCCI (1.1), HOBt (2), NMM (2)	0.2
Z-L-Ala-D-Phe + HBr · H-L-Ala-L-Ala-ONb DCCI (1.1), HOBt (2), NMM (2)	0.4
Z-L-Ala-D-Ala-ONSu + HBr · H-L-Ala-L-Ala-ONb NMM (2)	1.0
same reaction but active ester not recrystallized	2.1
Z-L-Ala-D-Ala-ONSu + HBr · H-L-Ala-L-Ala-L-Ala-ONb NMM (2)	0.8
Z-L-Ala-D-Phe-ONSu + HBr · H-L-Ala-L-Ala-ONb NMM (2)	0.8
same reaction but Et_3N (1)	0.9
same reaction but Et_3N (2)	1.2
Z-L-Ala-D-Ala-ONSu + H-L-Ala-L-Ala dioxane/water 1:1, $NaHCO_3$ (2)	10.0

Results for a few coupling methods are given in Table 2. The high racemization during activation of a peptide with DCCI is well known. Addition of two equivalents of 1-hydroxybenzotriazole [16] avoided racemization almost completely. This is additional proof for the usefulness of this new 'additive' [17]. Remarkably little racemization was also observed when the carboxyl fragment was activated as a succinimide ester and coupled in DMF. The active esters Z-L-Ala-D-Ala-ONSu and Z-L-Ala-D-Phe-ONSu were synthesized by the DCCI method [18] in dioxane/ethyl acetate 1:1 using 2 equivalents of N-hydroxysuccinimide. The esters were once recrystallized from 2-propanol prior to their use in the coupling experiments. Coupling the crude active esters resulted in slightly higher quantities of the diastereomeric peptides.

Usually fragment condensation *via* the synthesis and isolation of an activated carboxyl component is very much racemization prone. Racemization through azlactone formation is possible during the activation step – usually an ester synthesis by means of DCCI – as well as during the coupling step when the active ester is exposed to bases and nucleophiles. Activation of peptides by succinimide ester formation and isolation of the activated species was applied in some rare cases (*e.g.* [19]) and was shown to be almost free of racemization [17]. Moreover, opening of the optically active azlactone 2-phenyl-L-4-benzyl-oxazolone by N-hydroxysuccinimide and other highly nucleophilic hydroxylamine derivatives occurred with high retention of configuration [20]. In our experiments racemic succinimide ester may have been formed during its synthesis[5]) and during the peptide bond formation. The percentage of racemization given in Table 2 is the sum of these two events. We have also considered the possibility (see [21]) that racemization during the coupling step is dependent on the ratio of the racemization rate of the succinimide ester *vs.* the rate of attack by the nucleophile (amino component). The high degree of racemization observed in dioxane/water with hydrogencarbonate as base can therefore not be unequivocally attributed to either a faster racemization of the ester or a slower coupling rate.

In summary our procedure of analyzing racemization during peptide synthesis has the following advantages: 1. Any coupling method as well as influences by solvents and bases may be checked. 2. The configuration change is amplified through the coupling with alanine peptides of varying length. This together with the high resolution power of the amino acid analysis makes the test one of the most sensitive known. 3. No manipulations which may lead to additional racemization (*e.g.* acid hydrolysis) are necessary before the enzymic hydrolysis. 4. Due to the broad specificity of LAP [12] the method may be extended to an array of different peptides.

Experimental Part

Materials. Leucine aminopeptidase (hog kidney), lot LAPC 2 FA, was from *Worthington*, Freehold, New Jersey. D-Alanine and 1-hydroxybenzotriazole were products of *Fluka*, Buchs, Switzerland; D-phenylalanine was from *British Drug House* and L-Ala-L-Ala from *Miles-Yeda*, Rehovot, Israel. Doubly distilled water was used for the enzymic assays.

Methods. Thin layer chromatography was performed on silicagel-coated alumina plates (*Riedel-DeHaen*, Hannover, Germany) in the systems chloroform/methanol 9:1, ethyl acetate/hexane 1:1 and diisopropyl ether/chloroform/acetone/2-propanol/water/formic acid 5:5:5:1:0.5:1. Spots were detected by charring.

Z-L-Ala-D-Ala. D-Ala (2.67 g, 30 mmol) and $NaHCO_3$ (5.05 g, 60 mmol) were dissolved in 75 ml of water, Z-L-Ala-ONSu [18] (8.9 g, 30 mmol) in 75 ml of dioxane was added and the clear solution allowed to stand at room temperature overnight. The mixture was concentrated to approximately 50 ml, acidified to pH 1 with 6N HCl and extracted four times with totally 500 ml of ethyl acetate. The organic extract was washed twice with 100 ml of water, with saturated NaCl-solution and was dried over $MgSO_4$. Evaporation left an oily residue which solidified in the presence of a little diisopropyl ether. Recrystallization from ethyl acetate/diisopropyl ether 1:5 yielded 5.9 g (67%) of colorless dipeptide, m.p. 115–116° (lit. 116.5° [22]), titration equivalent[6]: calc. 294.3, found 295.

The following compounds were synthesized accordingly.

Z-L-Ala-L-Ala, m.p. 148–150° (lit. 152–153° [23]), titration equivalent: calc. 294.3, found 285; *Z-L-Ala-L-Phe,* m.p. 123–124° (lit. 122° [24]), titration equivalent: calc. 370.4, found 379; *Z-L-Ala-D-Phe,* m.p. 45–47° (lit. 49–51° [25]), titration equivalent: calc. 370.4, found 372.

Z-L-Ala-D-Ala-ONSu. Z-L-Ala-D-Ala (1.47 g, 5 mmol) and N-hydroxysuccinimide (1.15 g, 10 mmol) were dissolved in 10 ml of dioxane and 5 ml of ethyl acetate and cooled in an ice-salt bath to −10°, DCCI (1.13 g, 5.5 mmol) was added as a solid into the well stirred solution. Stirring was continued overnight at 4°. The reaction mixture was filtered, and the filtrate was evaporated to dryness. The solid product was once recrystallized from 2-propanol. Yield 1.43 g (78%), m.p. 140–141°, $[\alpha]_D^{24} = 12.8°$ ($c = 4.53$, dioxane).

$C_{18}H_{21}N_3O_7$ (391.4) Calc. C 55.24 H 5.40 N 10.73% Found C 54.68 H 5.55 N 11.02%

The following active esters were prepared similarly: *Z-L-Ala-L-Ala-ONSu,* m.p. 145–146° (lit. 146° [26]), $[\alpha]_D^{24} = -46.4°$ ($c = 3.76$, dioxane).

$C_{18}H_{21}N_3O_7$ (391.4) Calc. C 55.24 H 5.40 N 10.73% Found C 55.01 H 5.76 N 11.18%

Z-L-Ala-L-Phe-ONSu, m.p. 136°, $[\alpha]_D^{24} = -24.6°$ ($c = 3.76$, dioxane).

$C_{24}H_{25}N_3O_7$ (467.5) Calc. C 61.66 H 5.39 N 8.99% Found C 61.27 H 5.60 N 9.30%

Z-L-Ala-D-Phe-ONSu, m.p. 146–147°, $[\alpha]_D^{24} = 7.4°$ ($c = 3.28$, dioxane).

$C_{24}H_{25}N_3O_7$ (467.5) Calc. C 61.66 H 5.39 N 8.99% Found C 61.67 H 5.50 N 9.20%

HBr · H-L-Ala-L-Ala-ONb and HBr · H-L-Ala-L-Ala-L-Ala-ONb. Stepwise synthesis of these peptides has been described earlier [14].

Fragment condensation by means of DCCI or DCCI + 1-hydroxybenzotriazole, Z-L-Ala-D-Ala-L-Ala-L-Ala-ONb. Z-L-Ala-D-Ala (295 mg, 1 mmol), HBr · H-L-Ala-L-Ala-ONb (395 mg, 1 mmol) and, depending on the coupling method chosen, 1-hydroxybenzotriazole (270 mg, 2 mmol) were dissolved in 6 ml of DMF. The hydrobromide was neutralized with 1 or 2 equivalents of a tertiary amine (triethylamine or N-methylmorpholine), and the whole mixture was cooled in an ice-salt bath to −10°, DCCI (206 mg, 1 mmol) was added as a solid into the well stirred solution and stirring was continued at 4° overnight. The reaction mixture was filtered, and the protected tetrapeptide was precipitated from the filtrate by adding 20 volumes of 0.05N HCl. The crude product was collected by filtration, washed with cold water and dried *in vacuo* over P_2O_5. *Z-L-Ala-D-Phe-L-Ala-L-Ala-ONb* was prepared accordingly.

Fragment condensation via *peptide succinimide esters. Z-L-Ala-D-Phe-L-Ala-L-Ala-ONb.* Z-L-Ala-D-Phe-ONSu (467 mg, 1 mmol) and HBr · H-L-Ala-L-Ala-ONb (395 mg, 1 mmol) were dissolved in 6 ml of DMF. The hydrobromide was neutralized with 1 or 2 equivalents of the desired tertiary amine and the whole mixture set aside at ambient temperature for 16 to 18 h. The reaction mixture was filtered, and the protected tetrapeptide was precipitated from the filtrate by adding 20 volumes of 0.05N HCl. The crude product was collected by filtration and dried *in vacuo* over P_2O_5.

Z-L-Ala-D-Ala-(L-Ala)$_{2,3}$-ONb and the various all L *isomers* were prepared in the same way. Occasionally the reaction mixture got jelly-like and was therefore stirred vigorously to achieve effective mixing.

Fragment condensation via peptide succinimide ester in dioxane/water. Z-L-Ala-D-Ala-L-Ala-L-Ala. L-Ala-L-Ala (160 mg, 1 mmol) and $NaHCO_3$ (168 mg, 2 mmol) were dissolved in 2 ml of water.

[6] Non-aqueous titration with sodium methoxide in DMF.

The clear solution was diluted with 2 ml of dioxane. Z-L-Ala-D-Ala-ONSu (391 mg, 1 mmol) was added into the well stirred solution. The active ester dissolved immediately, and the reaction was set aside for 16 h at ambient temperature. Finally the solution was concentrated on a rotary evaporator to about 1 ml, 20 ml of 0.1 N HCl were added. The product was collected by filtration after an additional hour at 4° and was dried *in vacuo* over P_2O_5.

Catalytic hydrogenation. The blocked peptides were dissolved in acetic acid (1 to 5 g per 100 ml according to solubility, slight heating was necessary in some cases to get clear solutions). Pd/C (10%, 200 mg per g peptide), suspended in a little water, was added and hydrogen bubbled through the stirred mixture for 3 to 4 h. The catalyst was removed by filtration, the filtrate evaporated, once more taken up in 20 to 50 ml of water and again evaporated to complete dryness. The residue was then suspended or partially dissolved in a small volume of water (*ca.* 5 ml per g peptide) and 10 to 20 volumes of acetone were added. After 2 h at 4° the free peptide was collected by filtration, washed with cold acetone and dried *in vacuo* over P_2O_5. Compounds obtained in this way were ready for LAP hydrolysis.

Enzymic digestion by LAP. Stock solutions of substrates were 30 mmol in water. The commercial LAP, delivered as a suspension, was diluted to 0.05 mg per ml with 50 mMol veronal-HCl of pH 8.6 containing $MnSO_4$ ($c = 10$ mM) and was preincubated at 37° for 1 h immediately before use. Equal volumes (usually 0.2 ml) of substrate and enzyme solutions were mixed in a small test tube covered by a piece of parafilm and incubated at 37° ± 1° for 3 h. The final concentrations in the assay mixture were: substrate 15 mM, LAP 0.025 mg/ml, veronal 25 mM and $MnSO_4$ 5 mM. One aliquote of 50 µl was pipetted into 0.2 ml of citrate buffer (0.2 M, pH 2.2) to give the sample ready for amino acid analysis. Another 50 µl aliquot was diluted with 50 µl of acetic acid and was used for high-voltage paper electrophoresis.

Amino acid analyses. These were performed on a *Beckman-Spinco* analyzer model 120 B equipped with the accelerated system (55 cm column and high sensitivity cuvettes). Buffers used for elution had pH 3.25 and 4.25, the total flow was 68 ml/h and the temperature 55°. Under these conditions elution volumes were: alanine 104 ml, phenylalanine 187 ml, $(Ala)_4$ (L-D-L-L) 130 ml and $(Ala)_5$ (L-D-L-L-L) 85 ml. Ala-Phe-Ala-Ala (L-D-L-L) was eluted only during recycling by alkali. The samples in citrate buffer (see digestion by LAP) were chosen so as to yield 10 to 100 nmol of free amino acids. For low degrees of racemization this corresponded to 1 to 2 µmol of original peptide. The percentage of racemization was calculated as $200 \times A/P \times$ amplification factor, where A is the amount of free amino acid determined in the analysis (accuracy ±5%) and P the original amount of peptide contained in the same sample. The latter value is based on the nitrogen analysis of the free peptide.

High voltage paper electrophoresis. Separation was carried out for 3 h on *Whatman* No. 1 paper at pH 1.4 and at a gradient of 30 V/cm. Other details have been described earlier [14]. Electrophoresis gave a readily available, qualitative picture of the hydrolysis products and was performed routinely beside the amino acid analysis.

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71. Konfigurationen und Konformationen von zwei 2,4,6,8-Tetrabrom-cyclooctan-1,5-dionen[1])

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Summary. The two isomeric tetrabromides, α-isomer mp. 198° and β-isomer mp. 226° described in [1], are identified as *cis,cis,trans*-2,4,6,8-tetrabromo-cyclooctane-1,5-dione (**2**) and *cis,trans,cis*-2,4,6,8-tetrabromo-cyclooctane-1,5-dione (**3**) by an analysis of their NMR.-spectra which also allows a derivation of their preferred conformations. Both exist in solution as boat-chair conformers, the geometries of which correlate well with the IR.- and UV.-spectra.

1. Einleitung. – Im Laufe der Synthese von Bishomochinonen aus Cyclooctan-1,5-dion (**1**) [1] isolierten wir zwei stereomere 2,4,6,8-Tetrabrom-cyclooctan-1,5-dione (das α-Isomer, Smp. 198°, und das β-Isomer, Smp. 226°), deren Eigenschaften und

[1]) Aus der Dissertation von *Jürg E. Heller*, Universität Zürich, 1973.
[2]) Stipendiat des Fonds zur Unterstützung von Doktoranden auf dem Gebiet der Chemie (1969/70).