AMMONOLYSIS MEDIATED SIDE REACTIONS OF β-TERT-BUTYL ASPARTYL PEPTIDES*

István SCHÖN and Attila RILL

Chemical Works of Gedeon Richter, Ltd., Lajos Kisfaludy Research Laboratory, H-1475 Budapest, P.O. Box 27, Hungary

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Dedicated to the memory of Dr Karel Bláha.

Ammonolysis of Z-Asp(OBu^t)-Phe-NH_2 and Boc-Leu-Asp(OBu^t)-Phe-NH_2, as well as their diastereomers, resulted not only in transpeptidated, but also in epimerized peptides through a complex mechanism. Key compounds of these transformations are presumably very reactive cyclic aminosuccinyl derivatives. In some cases, the amount of α-peptide formed approached that of the β-peptide, in one case it exceeded this amount.

Conservation of the chemical and optical integrity of aspartyl and asparaginyl residues in peptides remains a great and not always recognized challenge for peptide and protein chemists. The exceptional cyclization tendency of these derivatives represents a real danger during the synthesis and isolation, as well as purification of peptides[1]. Recently, we reported in a preliminary communication[2] formation of a heterogeneous reaction mixture completely lacking the expected product during the synthesis of a pentagastrin analog, when the β-tert-butyl-aspartyl residue containing peptide chain had been ammonolyzed for the carboxy terminal amidating cleavage under the usual conditions of solid phase synthesis[3]. Now we wish to report here complete experimental details of ammonolysis of some β-tert-butyl-aspartyl peptides, resulting not only in transpeptidated, but also in epimerized products. In addition, we present the most probable reaction sequence and mechanism of these transformations in accordance with previous findings.

In order to study the influence of usual conditions of ammonolysis on transformations, including the aspartyl residue, benzyloxycarbonyl-β-tert-butyl-aspartyl--phenylalaninamide of L-L (*I*), L-D (*II*) and D-L (*III*) diastereomeric sequences and tert-butyloxycarbonyl-leucyl-β-tert-butyl-aspartyl-phenylalaninamide of L-L-L (*IV*) and L-D-L (*V*) epimeric sequences have been selected as model dipeptides and tripeptides, respectively. All these compounds were synthesized in solution by means

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of successive acylations using either the N-hydroxysuccinimide or pentafluorophenyl ester method. In addition to β-tert-butyl-aspartyl peptides *I* to *V*, aminosuccinyl peptides (Z-Asu-Phe-NH_2 (*VI*)[4], Z-D-Asu-Phe-NH_2 (*VII*)[4] and Boc-Leu-Asu-Phe--NH_2 (*VIII*)[5]), as possible intermediates in the transpeptidation process, were also ammonolyzed. Physico-chemical characterizations and synthesis of *VI* to *VIII* will be published elsewhere.

Z-Asp(OBu^t)-Phe-NH_2

I, L-L
II, L-D
III, D-L

Boc-Leu-Asp(OBu^t)-Phe-NH_2

IV, L-L-L
V, L-D-L

Z-Asu-Phe-NH_2

VI, L-L
VII, D-L

Boc-Leu-Asu-Phe-NH_2

VIII, L-L-L

Ammonia gas was bubbled at 0°C through solutions of compounds *I* to *VIII* in dry methanol, then the reaction mixtures were kept in closed vessels at room temperature until the completion of conversions, at least for 16 h. Reactions were followed by TLC. The resulted suspensions were evaporated to dryness, the residual crystals were triturated with ether and then filtered off. To study the effect of solvents on transformations, *I* was treated with ammonia in ethanol, 1-butanol and dimethylformamide, too.

The procedure of the tedious and gradual identification of the ammonolytic products was briefly mentioned previously[2]. Ammonolyses of dipeptides *I*, *II*, *III*, *VI*, and *VII* resulted in various mixtures of benzyloxycarbonylasparaginyl-phenylalaninamides of L-L (*IX*, α-L-L), α-D-L (*X*), α-L-D (*XI*) and α-D-D diastereomers and benzyloxycarbonyl-isoasparaginyl-phenylalaninamides of β-L-L (*XII*), β-D-L (*XIII*), β-L-D (*XIV*) and β-D-D. In the tripeptide series, mixtures of normal peptides tert--butyloxycarbonyl-leucyl-asparaginyl-phenylalaninamide of α-L-L-L (*XV*) and α-L-D-L (*XVI*) and isopeptides tert-butyloxycarbonyl-leucyl-isoasparaginyl-phenylalaninamide of β-L-L-L (*XVII*) and β-L-D-L (*XVIII*) formed by ammonolyses of *IV*, *V* and *VIII*. All these peptides were synthesized employing usual methods and characterized by melting points, optical rotation, ^1H and ^{13}C NMR spectra.

Z-Asn-Phe-NH_2

IX, L-L
X, D-L
XI, L-D

Z-iAsn-Phe-NH_2

XII, L-L
XIII, D-L
XIV, L-D

Boc-Leu-Asn-Phe-NH_2

XV, L-L-L
XVI, L-D-L

Boc-Leu-iAsn-Phe-NH_2

XVII, L-L-L
XVIII, L-D-L

Product ratios of the heterogeneous mixtures obtained after ammonolyses of *I*, *III*, *VI*, and *VII*, including experiments on the investigation of solvent dependence, were determined by HPLC, using area normalization. Results are summarized in Table I. Similarly, Table II presents the percentage of tripeptide (*IV*, *V* and *VIII*) ammonolyses product ratio. In the latter cases, no HPLC systems were found for resolution of the protected tripeptide end products, while successful separations were accomplished when samples *XV* to *XVIII* and their mixtures had been subjected to acidolysis with trifluoroacetic acid for 1 h and, after removal of the solvent, the deprotected derivatives were analysed. Details of HPLC studies will form the subject of another publication[6].

Several conclusions can be drawn from results presented in Tables I and II. Besides the solvent effect, we examined the effect of the in-chain position of β-tert-butyl--aspartyl residue and chirality of the molecules on the ammonolytic products ratio, i.e. on the ratio of α/β peptides, and the extent of epimerization.

The rate of the overall ammonolysis of *I* is significantly influenced by the solvent (Table I). The contribution of the push-pull mechanism may explain the observed differences in the reaction rates. In its absence in dipolar-aprotic dimethylformamide, the slowest rate of transformation was experienced. It is interesting that in protic alcohols the order of extent of epimerization corresponds to that of the reaction rate, however, epimerization in dimethylformamide is faster than in butanol. Dimethylformamide afforded the highest selectivity in favour of β-peptide formation in the ring opening reaction. According to our opinion, the conclusions on the solvent effects mentioned above, may be generalized.

On the other hand, the following conclusions related to chemical structures are not intended for generalization, they may concern only the examples presented. Results of experiments carried out in methanol are compared. In the dipeptide series (Table I), ammonolysis of enantiomeric *II* (L-D) and *III* (D-L) resulted in identical overall amounts of α-peptides and epimerized products within the range of experimental error. Ammonolysis of *I* (L-L) yields more epimerized products (35%) than its epimers *II* (L-D) and *III* (D-L) (>30%) and significantly less α-peptides (26% versus 36%). Among the products *IX* to *XIV*, α- and β-D-D, we have noticed an interesting difference. While the ratios α-L-L/β-L-L and α-D-D/β-D-D are lower than 1/4, the same ratios of the enantiomeric pairs α-D-L/β-D-L and α-L-D/β-L-D are about 3/4. These data point to the expected significant selectivity in the former and its lack in the latter cases.

In the tripeptide series (Table II), ammonolysis of diastereomeric *IV* (L-L-L) and *V* (L-D-L) reflected also significant differences. The α-products *XV* and *XVI* from *IV* amount only 30%, referring to the usual selectivity in favour of the formation of β-products *XVII* and *XVIII*, while ammonolysis of *V* (L-D-L) yields α- and β-peptides (*XV*, *XVI* and *XVII*, *XVIII*, respectively) in nearly identical quantities (47% and

TABLE I

Product ratios of ammonolyses of dipeptides *I* (L-L), *II* (L-D), *III* (D-L), *VI*, and *VII*

Starting dipeptide	Solvent	Reaction time, days	Product, %						
			α-peptides[a]			β-peptides[a]			Epimerized products
			α-L-L, *IX* (α-D-D)	α-D-L, *X* (α-L-D, *XI*)	Σ	β-L-L, *XII* (β-D-D)	β-D-L, *XIII* (β-L-D, *XIV*)	Σ	
I	MeOH	1	10	16	26	55	19	74	35
	EtOH	2	10	7	17	70	13	83	20
	BuOH	7	11	5	16	76	8	84	13
	DMF	14	5	4	9	76	15	91	19
II	MeOH	1	(5)	(31)	36	(22)	(42)	64	27
III	MeOH	1	6	30	36	24	40	64	30
VI	MeOH	1	11	14	25	60	15	75	29
VII	MeOH	1	(3)	(36)	39	(17)	(44)	61	20

[a] Data parenthesized correspond to compounds parenthesized.

53%, respectively) almost without selectivity. Moreover, while among the products (*XV* to *XVIII*) the ratio of α-L-L-L/β-L-L-L is usual, i.e. less than 1/3, the ratio α-L-D-L/β-L-D-L is approximately 1·3. As far as we know, this is the first case reported, where the quantity of an α-peptide surpasses that of its β-twin, pointing to a slightly reversed selectivity.

Ammonolysis of aminosuccinyl peptides *VI*, *VII* and *VIII* resulted in product ratios very similar to those of *I*, *III*, and *IV*, respectively, (Tables I and II), thus the intermediacy of the former in ammonolytic transformation of the latter seems to be firmly supported. However, the observed differences in overall amounts of the epimerized products may originate from the simpler reaction mechanism operating in ammonolysis of the aminosuccinyl peptides (*VI*, *VII*, and *VIII*), as compared to the appropriate tert-butyl esters (*I*, *III*, and *VI*).

A deductive and conclusive analysis always requires consequent retrospection. The starting point of this investigation, i.e. ammonolysis for deaminating cleavage of a β-tert-butyl-aspartyl peptide anchored to a solid support, refers to our ignorance or incomplete knowledge of the previous literature. This statement involves two facts: (i) there is no perfect method for survey of literature on potential side reactions[7], and (ii) during designing the original synthesis we did not take into consideration the sensitivity of β-tert-butyl-aspartyl peptides to basic hydrolysis. Side chain saponification in an Asp(OBu^t)-containing peptide had been reported to be faster than that of the carboxy-terminal methyl ester[8]. This side reaction was rediscovered again 20 years later[9]. A damage of side chain tert-butyl esters by the supernucleophile effect of hydrazinolysis was reported[10], but as far as we know, the harmful effect of ammonolysis has not been mentioned in the literature.

On the other hand, when in 1984 we assumed (and in the next year confirmed) simultaneous epimerization at the aspartyl residue during transpeptidation of the

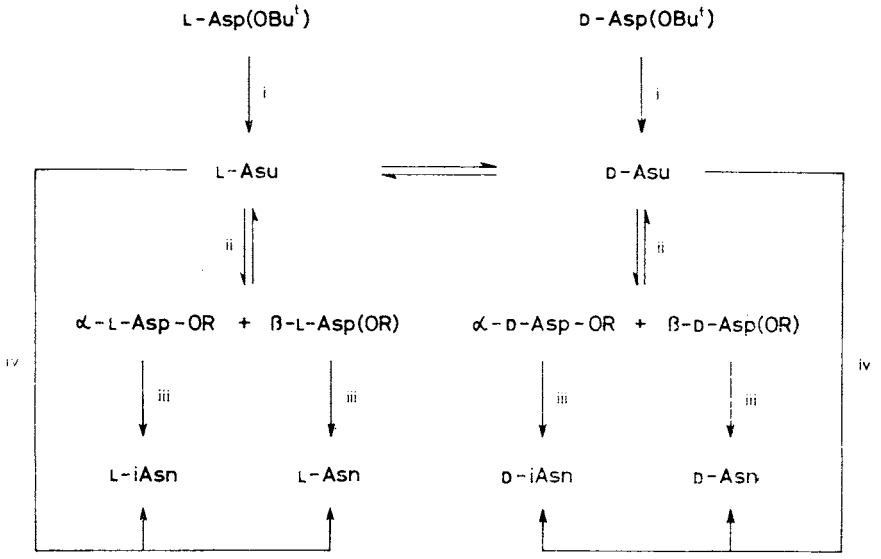
TABLE II

Product ratios of ammonolyses of tripeptides *IV* (L-L-L), *VIII* and *V* (L-D-L)

Starting tripeptide	Product, %						
	α-peptides			β-peptides			Epimerized peptides
	α-L-L-L (*XV*)	α-L-D-L (*XVI*)	Σ	β-L-L-L (*XVII*)	β-L-D-L (*XVIII*)	Σ	
IV, L-L-L	12	18	30	55	15	70	33
VIII	14	22	36	47	17	64	39
V, L-D-L	6	41	47	21	32	53	27

original model peptide[11], we were really surprised, even though the significant proneness to racemization of aspartic acid had been reported long ago[12] and the assumption[13,14] of Clarke and coworkers on the increased sensitivity of the cyclic aminosuccinyl residue to racemization from the aspect of a biochemical approach missed our attention.

We can add a new finding to the earlier proved and/or assumed reaction sequence and mechanism of the transpeptidation of aspartyl peptides and their derivatives[15] (Scheme 1). A qualitative TLC study revealed an unexpected, but easily explainable reaction sequence in the transpeptidation process in methanol. Compound *I* completely disappeared from the reaction mixture within 1 h, cyclizing to the expected



SCHEME 1

General mechanism of ammonia mediated transformations of β-tert-butyl-aspartyl peptides (R = CH_3, C_2H_5 or C_4H_9). Residues are considered to be incorporated into the peptide chain, if either Asn or iAsn derivatives are at CO_2H-terminus: i irreversible cyclization; ii reversible esterification/cyclization; iii ester ammonolysis; iv direct ammonolysis

aminosuccinyl derivative *VI*. The direct participation of tert-butoxy group in a substitution by one of the external nucleophiles can be excluded. This fast ring closure observed is in good agreement with our previous experience on the piperidinolysis of β-benzyl-aspartyl peptides[16]. In both cases the base-catalyzed ring closure initiates the complex reaction sequence.

In accordance with Clarke's suggestion[13,14] and with reference to significant racemization during deamidation of the Asn-Gly sequence[17] we assume that epimeri-

zation takes place in this very reactive and stressed cyclic intermediate through α-proton abstraction-enolization mechanism[18], as the tendency of both aducts and products to the epimerization, as well as that of the ionized transitional state species resulting in or from the aminosuccinyl derivative, can be logically excluded. Recently, we afforded first experimental proofs on the high vulnerability to epimerization of isolated aminosuccinyl peptides *VI* and *VII* in dimethylformamide by the effect of nonnucleophilic triethylamine[4]. Ammonolysis of the aminosuccinyl ring is not the next step of transpeptidation, but a faster base catalyzed ring opening reaction by the effect of methanol, yielding the appropriate α- and β-methyl-aspartyl peptides takes place. The chirality of the latter was not identified. Since end products of ammonolysis of *I* started to appear slowly, direct ammonolysis of the succinimidyl ring cannot be excluded. The exact mechanism, of course, depends on the relative rates of these reaction steps.

Furthermore, we suppose that the solvent dependence of the overall reaction rate comes from the slower rate of formation and/or amidation of ethyl and 1-butyl esters. In dimethylformamide only the direct, very slow route of amidation operates. The fastest overall transformation observed in methanol may involve the contribution of the push-pull mechanism.

It is interesting to note, that these results are in good accordance with 20 year old analogous observations obtained in solid phase synthesis. Suspensions of Z-Pro-Leu--Gly-polymer in methanol and ethanol were treated with ammonia. Amide formation via the appropriate soluble esters, solvent dependence on the reaction rate and base catalysis in transesterification were reported[19,20].

The final result of this base-catalyzed complex process is the formation of partially epimerized asparaginyl and isoasparaginyl peptides. The preponderance of the latter has always been emphasized[1,7] as electronic effects favour the attack of α-carbonyl carbon atom by nucleophilic agents. Literature survey revealed one exception – α- and β-Asp-Gly were detected in equal quantities, however, previous recrystallization step with a 40% yield puts uncertainity to this observation[21].

In several cases the significantly decreased or slightly reversed selectivity in ring opening reactions of one of the diastereomeric intermediates contradicts the accepted knowledge and experience. Recent theoretical approach to the explanation of the dominance of isopeptide formation generally concentrates on electronic differences in the polarities of α- and β-carbonyl groups. Now, as an additional possibility, we propose the significance of sterical and conformational factors in influencing the ring opening of succinimidyl ring by the effect of nucleophiles.

Physico-chemical structural analysis of some aminosuccinyl peptides was reported by Mazzarella's group[22–24]. In our cooperation X-ray analysis of *VI* was recently published[25]. Similar structure analyses for *VII*, *VIII* and the latter's L-D-L epimer, the comparison of the structures of the epimer pairs may explain the differences observed in the product ratios of ammonolytic reactions.

EXPERIMENTAL

Melting points were determined on a Tottoli (Büchi) apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer 241 automatic polarimeter at 22 to 25°C. Ascending TLC was performed on precoated silica gel plates (Merck). Solvent systems were prepared by mixing ethyl acetate (AcOEt) and a stock solution of pyridine–acetic acid–water, 20 : 6 : 11 in the following ratios: AcOEt/stock, 9 : 1 (S1); AcOEt/stock, 4 : 1 (S2); AcOEt/stock, 7 : 3 (S3); AcOEt/stock, 3 : 2 (S4); AcOEt/stock, 1 : 1 (S5). The plates were vizualized by spraying with ninhydrin, then tolidine/KI after chlorination. HPLC studies will be reported elsewhere[6]. IR spectra were recorded on a Perkin–Elmer 257 IR spectrophotometer. NMR spectra ($(CD_3)_4SO$, ppm) were obtained on a Bruker WM 250 instrument, using TMS as internal standard. The known substances were prepared according to procedures described in literature: Z-Asp(OBu^t)-Phe-NH_2 (*I*) (ref.[26]), Boc-Phe-NH_2 (ref.[27]), Z-Asp(OBu^t)-OSu (refs[28,29]), Boc-Leu-Asp(OBu^t)-Phe-NH_2 (*IV*) (ref.[30]), Boc-Leu-OSU (ref.[31]), Z-Asu-Phe-NH_2 (*VI*) (ref.[4]), Z-D-Asu-Phe-NH_2 (*VII*) (ref.[4]), H-Asu-Phe-NH_2.HBr (ref.[30]), Z-Asn-OPfp (ref.[32]), Boc-Asn-OPfp (ref.[32]).

Z-Asp(OBu^t)-Phe-NH_2 (*I*) (ref.[26])

^{1}H NMR: 1·36 s, 9 H (Bu^t); 2·40 and 2·64 dd and dd, 2 H (Asp-CH_2); 2·85 and 3·05 dd and dd, 2 H (Phe-CH_2); 4·40 m, (Asp- + Phe-CH, overlapping with solvent peak); 5·05 ABq, 2 H (Z-CH_2); 7·21 s, 5 H (Phe-aromatic); 7·35 s, 5 H (Z-aromatic); 7·14 and 7·41 d, 2 H (NH_2, exchangeable with D_2O); 7·58 broad, 1 H (Asp-NH, exchangeable with D_2O); 7·83 broad, 1 H (Phe-NH, exchangeable with D_2O).

Z-Asp(OBu^t)-D-Phe-NH_2 (*II*)

Boc-D-Phe-NH_2 4·3 g, (16·3 mmol) (prepared as the L-antipod with a yield of 81·3%, m.p. 132–135°C) was treated with 40 ml of HCl/dioxane for 30 min. Then the solvent was removed and the residual crystals were twice recrystallized from methanol–ether, yielding 2·65 g (81·0%) of H-D-Phe-NH_2.HCl, m.p. 227–230°C, $[\alpha]_D$ −29·4° (*c* 1, dimethylformamide), R_F 0·4 (S5). 2·41 g (12·0 mmol) of H-D-Phe-NH_2.HCs and 5·20 g (12·4 mmol) Z-Asp(OBu^t)-OSu reacted in 35 ml of tetrahydrofuran for 3 h in the presence of 1·70 ml (12·0 mmol) of triethylamine. The solvent was removed and a solution of the residue in ethyl acetate was successively washed with 1 M-HCl, 5% $NaHCO_3$ solution and water, then dried over Na_2SO_4 and evaporated to dryness. Recrystallization of the residue from 2-propanol resulted in 4·1 g (72·8%) of *II*, m.p. 146–147°C; $[\alpha]_D$ −10·0° (*c* 1, ethanol); R_F 0·65 (S1). ^{1}H NMR: 1·34 s, 9 H (Bu^t); 2·29 and 2·47 dd and dd, 2 H (Asp-CH_2); 2·82 and 3·06 dd and dd, 2 H (Phe-CH_2); 4·37 m, 1 H (Asp-CH); 4·45 m, 1 H (Phe-CH); 5·04 s, 2 H (Z-CH_2); 7·20 s, 5 H (Phe-aromatic); 7·34 s, 5 H (Z-aromatic); 7·0 to 7·5, 2 H (NH_2, exchangeable with D_2O); 7·52 d, 1 H (Asp-NH, exchangeable with D_2O); 8·03 d, 1 H (Phe-NH, exchangeable with D_2O). ^{13}C-NMR: 29·4 (Bu^t-CH_3), β-carbon atoms overlap with solvent signal, 53·5 (Asp-α), 55·4 (Phe-α), 67·3 (Z-CH_2), 81·4 (Bu^t-qC), 127·9, 129·2, 129·4, 129·6, 130·0, 130·9, 138·6, 139·3 (aromatic), 157·3 (Z-CO), 171·0, 171·8, 174·2 (3 CO). For $C_{25}H_{31}N_3O_6$ (469·52) calculated: 63·94% C, 6·66% H, 8·95% N; found: 64·07% C, 6·61% H, 8·89% N.

Z-D-Asp(OBu^t)-Phe-NH_2 (*III*)

To a suspension of 2·0 g (10 mmol) of H-Phe-NH_2.HCl (prepared in the same way as the D-antipod, m.p. 228–230°C, $[\alpha]_D$ +28·0° (*c* 1, dimethylformamide)) 4·2 g (10 mmol) of Z-D-

-Asp(OBu^t)-OSu (prepared in the same way as the L-antipod, m.p. 148–150°C) in 30 ml of tetrahydrofuran was added. Reaction mixture was processed as described for *II*, yielding 1·97 g (42·0%) of *III*, m.p. 145–147°C; $[\alpha]_D$ +10·7° (*c* 1, ethanol); R_F 0·65 (S1). For $C_{25}H_{31}N_3O_6$ (469·52) calculated: 63·94% C, 6·66% H, 8·95% N; found: 63·98% C, 6·50% H, 9·06% N.

Boc-Leu-D-Asp(OBu^t)-Phe-NH_2 (*V*)

To a solution of 1·7 g (3·62 mmol) of *III* in 50 ml of methanol 0·2 g Pd on charcoal was added and hydrogen was bubbled through the suspension. The progress of the reaction was followed on TLC (R_F 0·07 (S1) for H-D-Asp(OBu^t)-Phe-NH_2 while R_F 0·15 for L-L diastereomer). The catalyst was removed, the solvent was evaporated and to a solution of the residual oil in 20 ml of ethyl acetate 1·19 g (3·62 mmol) of Boc-Leu-OSu was added. After completion, the reaction mixture was washed with 1M-HCl, 5% $NaHCO_3$ solution and water, then dried, and the solvent was evaporated. A suspension of the residue in ether was filtered. Recrystallization of the crude product from ethanol–ether resulted in 1·32 g (66·7%) of *V*, m.p. 163–163°C, $[\alpha]_D$ 0° (*c* 1, dimethylformamide), R_F 0·70 (S1). For L-L-L diastereomer *IV*: m.p. 169–170°C; $[\alpha]_D$ −38·7° (*c* 1, ethanol); $[\alpha]_D$ −38·3° (*c* 1, dimethylformamide); R_F 0·70 (S1). ^{13}C NMR: 23·4 and 24·5 (Leu-δC), 26·0 (Leu-γC), 29·4 and 29·9 (Bu^t-CH_3), 39·2 (Leu + Phe βC overlapping with the solvent peak), 42·7 (Asp-βC), 51·4 (Phe-αC), 55·0 (Leu-αC), 55·8 (Asp-αC), 80·0 and 81·9(Boc-qC), 127·9, 129·7, 130·8, 139·6 (aromatic), 157·1 (Boc-CO). For $C_{28}H_{44}N_6O_7$ (548·66) calculated: 61·29% C, 8·08% H, 10·21% N; found: 61·42% C, 8·00% H, 9·97% N.

Boc-Leu-Asu-Phe-NH_2 (*VIII*)

To a solution of 6·84 g (20 mmol) of H-Asu-Phe-NH_2.HBr and 6·56 g (20 mmol) of Boc-Leu-OSu 2·80 ml (20 mmol) triethylamine was added. On the next day the reaction mixture was evaporated and a solution of the residue in 100 ml of chloroform was washed with 1M-HCl, 5% $NaHCO_3$ solution and water, then dried, and evaporated. The residue was isolated with ether. Recrystallization of the crude product (7·55 g) from a mixture of ethanol, ether and hexane gave 5·97 g (62·8%) of *VIII*, m.p. 205–209°C; $[\alpha]_D$ −131·8° (*c* 1, dimethylformamide); R_F 0·65 (S1); IR (KBr): 1 780 cm^{-1} (imide); ^{13}C NMR: 23·2 and 24·4 (Leu-δC), 25·9 (Leu-γC), 29·8 (Boc-CH_3), 34·7 (Leu-βC), 36·4 (Phe-βC), 42·2 (Asu-βC), 49·8 (Phe-αC), 54·6 (Leu-αC), 56·3 (Asu-αC), 80·0 (Boc-qC), 127·9, 129·3, 130·4, 139·3 (aromatic), 156·8 (Boc-CO), 170·8 ($CONH_2$), 175·1, 175·8, 176·7 (3 CO). For $C_{24}H_{34}N_4O_6$ (474·54) calculated: 60·74% C, 7·22% H, 11·81% N; found: 60·55% C, 7·32% H, 11·75% N.

Z-Asn-Phe-NH_2 (*IX*, α-L-L)

To a suspension of 1·0 g (5·0 mmol) of H-Phe-NH_2.HCl and 2·16 g (5·0 mmol) of Z-Asn-OPfp (ref.[32]) ($[\alpha]_D$ −6·5° (*c* 1, dioxane)) in 15 ml of dimethylformamide, 0·70 ml (5·0 mmol) of triethylamine was added. After 2 h the reaction mixture was evaporated, and the crystalline residue was triturated with ether, yielding 1·42 g (68·9%) of *IX*, m.p. 251–256°C; $[\alpha]_D$ −24·5° (*c* 0·5, dimethylformamide); R_F 0·20 (S1). Reported[33] m.p. 235–236°C, $[\alpha]_D$ −21·9° (*c* 1, dimethylformamide). ^{1}H NMR: 2·34 and 2·50 dd, 2 H (Asn-CH_2); 2·83 and 3·08 dd and dd, 2 H (Phe-CH_2); 4·35 m, 2 H (Asn + Phe-CH, overlapping with the solvent peak); 5·08 s, 2 H (Z-CH_2); 7·10 d, 2 H (NH_2, exchangeable with D_2O); 7·20 s, 5 H (Phe-aromatic); 7·37 st 5 H (Z-aromatic); 7·45 d, 1 H (Asp-NH), exchangeable with D_2O) 8·0 d, 1 H (Phe-NH, exchangeable with D_2O). For $C_{21}H_{24}N_4O_5$ (412·43) calculated: 61·15% C, 5·87% H, 13·59% N; found: 61·18% C, 5·75% H, 13·30% N.

Z-D-Asn-Phe-NH_2 (*X*, α-D-L)

To a suspension of 1·20 g (6·0 mmol) of H-Phe-NH_2.HCl and 3·0 g (6·95 mmol) of Z-D-Asn--OPfp (prepared in the same way as the L-antipod[32], m.p. 144—146°C) in 20 ml of dimethylformamide 0·84 ml (6·0 mmol) of triethylamine was added. On the next day the suspension was diluted with chloroform, the mixture was kept in the refrigerator, and then filtered. The precipitate was triturated with hot ethanol, then filtered off to yield 2·0 g (80·5%) of *X*, m.p. 267—269°C; $[\alpha]_D$ −13·6° (*c* 1, dimethylformamide), R_F 0·23 (S1). For $C_{21}H_{24}N_4O_5$ (412·43) calculated: 61·15% C, 5·87% H, 13·59% N; found 61·08% C, 5·97% H, 13·41% N.

Z-Asn-D-Phe-NH_2 (*XI*, α-L-D)

Compound *XI* was prepared as described for *X*, m.p. 269—272°C, $[\alpha]_D$ +13·1° (*c* 1, dimethylformamide). ^{1}H NMR: 2·29 d, 2 H (Asn-CH_2); 2·80 and 3·05 dd and dd, 2 H (Phe-CH_2); 4·30 q, 1 H (Asn-CH); 4·40 m, 1 H (Phe-CH, overlapping with the solvent peak); 5·02 s, 2 H (Z-CH_2); 7·1—7·5 3 H (NH + NH_2, broad, overlapping, exchangeable with D_2O); 7·29 m, 5 H (Phe--aromatic); 7·35 s, 5 H (Z-aromatic); 8·05 d, 1 H (Phe-NH). ^{13}C NMR: Asn + Phe βC overlap with the solvent peak, 53·6 (Asn-αC), 55·5 (Phe-αC), 67·3 (Z-CH_2), 127·9, 129·3, 129·4, 130·0, 130·8, 138·6, 139·5 (aromatic), 157·4 (Z-CO), 172·6, 173·1, 174·4 (3 CO). For $C_{21}H_{24}N_4O_5$ (412·43) calculated: 61·15% C, 5·87% H, 13·59% N; found: 61·02% C, 5·74% H, 13·79% N.

Z-iAsn-Phe-NH_2 (*XII*, β-L-L)

To a suspension of 0·65 g (3·25 mmol) of H-Phe-NH_2.HCl in 15 ml of dimethylformamide, 0·46 ml (3·26 mmol) of triethylamine and 1·40 g (3·23 mmol) of Z-iAsn-OPfp (prepared in the same way from Z-iAsn-OH (ref.[34]), as described for Z-Asn-OPfp (ref.[32]), yield 73·9%, m.p. 139—140°C; $[\alpha]_D$ −28·6° (*c* 1, dioxane)) were successively added. After 1 h the solvent was removed, and trituration of the residue with hot ethanol resulted in 0·88 g (66·1%) of *XII*, m.p. 250—254°C; $[\alpha]_D$ −5·5° (*c* 1, dimethylformamide); R_F 0·20 (S1). ^{1}H NMR: 2·35 and 2·60 dd and dd, 2 H (iAsn-CH_2); 2·80 and 3·05 dd and dd, 2 H (Phe-CH_2); 4·30 q, 1 H (iAsn-CH); 4·40 q, 1 H (Phe-CH); 5·02 s, 2 H (Z-CH_2); 7·1—7·5 broad, 10 H (overlapping with NH, aromatic); 7·2 d, 2 H (NH_2, exchangeable with D_2O); 7·3, 1 H (iAsn-NH, broad, exchangeable with D_2O); 8·12 d, 1 H (Phe-NH, exchangeable with D_2O). ^{13}C NMR: 39·6 (iAsn-βC), 43·5 (Phe-βC), 53·5 (iAsn-αC), 55·6 (Phe-αC), 67·3 (Z-CH_2), 127·8, 129·2, 129·7, 129·9, 130·7, 138·6, 139·8 (aromatic), 157·3 (Z-CO), 171·0, 174·6, 174·9 (3 CO). For $C_{21}H_{24}N_4O_5$ (412·43) calculated: 61·15% C, 5·87% H, 13·58% N; found: 60·98% C, 5·89% H, 13·50% N.

Z-D-iAsn-Phe-NH_2 (*XIII*, β-D-L)

To a suspension of 1·0 g (5·0 mmol) of H-Phe-NH_2.HCl in 20 ml of dimethylformamide, 0·70 ml (5·0 mmol) of triethylamine and 2·35 g (5·56 mmol) of Z-D-iAsn-OPfp (m.p. 135—137°C) were successively added. On the next day the reaction mixture was diluted with chloroform, the precipitate was filtered off, and its trituration with 10 ml of hot ethanol gave 1·72 g (83·5%) of *XIII*, m.p. 259—261°C; $[\alpha]_D$ −22·0° (*c* 0·99, dimethylformamide), R_F 0·20 (S1). ^{1}H NMR: 2·45 d, 2 H (iAsn-CH_2); 2·75 and 3·05 dd and dd, 2 H (Phe-CH_2); 4·25 q, 1 H (iAsn-CH); 4·45 m, 1 H (Phe-CH); 5·01 s, 2 H (Z-CH_2); 7·22 s, 5 H (Phe-aromatic); 7·35 s, 5 H (aromatic signals overlap with NH, (Z-aromatic)); 7·1 d, 2 H (NH_2; exchangeable with D_2O); 7·3—7·3, 1 H (iAsn-NH, broad, exchangeable with D_2O, overlapping); 8·06 d, 1 H (Phe-NH, exchangeable with D_2O, overlapping). For $C_{21}H_{24}N_4O_5$ (412·43) calculated: 61·15% C, 5·87% H, 13·59% N; found: 61·07% C, 5·70% H, 13·39% N.

Z-iAsn-D-Phe-NH_2 (*XIV*, β-L-D)

To a suspension of 0·34 g (1·7 mmol) of H-D-Phe-NH_2.HCl in 6 ml of dimethylformamide, 0·24 ml (1·7 mmol) triethylamine and then 0·80 g (1·85 mmol) of Z-iAsn-OPfp were added. After 2 h the solvent was removed, the residue was triturated with chloroform, then with hot ethanol to yield 0·54 g (77·1%) of *XIV*, m.p. 258–260°C; $[\alpha]_D$ +22·0° (*c* 1, dimethylformamide); R_F 0·20 (S1). For $C_{21}H_{24}N_4O_5$ (412·43) calculated: 61·15% C, 5·87% H, 13·59% N; found: 61·37% C, 5·90% H, 13·58% N.

Boc-Leu-Asn-Phe-NH_2 (*XV*, α-L-L-L)

To a suspension of 1·0 g (5·0 mmol) of H-Phe-NH_2.HCl in 10 ml of dimethylformamide, 0·70 ml (5·0 mmol) of triethylamine and then 2·2 g (5·5 mmol) of Boc-Asn-OPfp (ref.[32]) were added. After 2 h the solvent was removed, the residue was triturated with ether, then filtered off. The crude product was recrystallized from ethanol yielding 1·46 g (77·0%) of Boc-Asn-Phe-NH_2 (m.p. 192–194°C, R_F 0·35 (S2)). A small sample recrystallized from hot ethanol melted at 202–203°C.

Boc-Asn-Phe-NH_2 (1·30 g, 3·43 mmol) was treated with trifluoroacetic acid for 30 min, the reaction mixture was evaporated and the residual oil was triturated with ether. The suspension was filtered to yield 1·30 g of a very hygroscopic, amorphous free dipeptides, which was dissolved in 10 ml of dimethylformamide. To this solution 0·56 ml (4·0 mmol) of triethylamine and then 1·31 g (4·0 mmol) of Boc-Leu-OSu were added. Next day the reaction mixture was evaporated, the residue was triturated with chloroform and ethanol. Yield 1·37 g (81·9%), m.p. 198–201°C; $[\alpha]_D$ −31·7° (*c* 1, dimethylformamide), R_F 0·70 (S3). For $C_{24}H_{37}N_5O_6$ (491·58) calculated: 58·64% C, 7·59% H, 14·25% N; found: 58·45% C, 7·48% H, 14·31% N. ^{1}H NMR: 0·825 and 0·85 dd, 6 H (Leu-CH_3); 1·21–1·42 m, 2 H (Leu-CH_2); 1·37 s, 9 H (Bu^t); 1·57 m, 1 H (Leu-γCH); 2·39 and 2·41, 2·54 and 2·59 dd and dd, 2 H (Asn-CH_2); 2·78 and 2·80, 3·12 and 3·13 dd and dd, 2 H (Phe-CH_2); 3·94 m, 1 H (Leu-αCH); 4·31 m, 1 H (Phe-CH); 4·64 q, 1 H (Asn-CH); 6·93 d, 1 H (Leu-NH, exchangeable with D_2O); 7·03 and 7·11 d, 2 H (Phe-NH_2, exchangeable); 7·10 to 7·27 m, 5 H (Phe-aromatic); 7·48 s, 2 H (Asn-NH_2, exchangeable); 7·99 s, 1 H (Asn-NH, exchangeable); 8·11 d, 1 H (Phe-NH, exchangeable). ^{13}C NMR: 21·21 and 23·01 (Leu-δC), 24·01 (Leu-γC), 28·08 (Boc-CH_3), 36·58 (Phe-βC), 36·82 (Asn-βC), 40·63 (Leu-βC), 49·32 (Asn-αC), 52·51 (Leu-αC), 53·86 (Phe-αC), 77·92 (Boc-qC), 125·97 (4′-aromatic), 127·96 (3′ and 5′-aromatic), 128·8 (2′ and 6′-aromatic), 138·1 (1′-aromatic), 155·23 (Boc-CO), 170·37 (Asn-CH_2-CO), 171·87 (Asn-CO), 172·28 (Leu-CO), 172·73 (Phe-CO).

Boc-Leu-D-Asn-Phe-NH_2 (*XVI*, α-L-D-L)

To a solution of 1·70 g (4·12 mmol) of *X* in 50 ml of dimethylformamide, 0·25 g of Pd on charcoal was added. Hydrogen was bubbled through the stirred suspension until completion of the deprotection, then the catalyst was removed, the solvent evaporated, and a solution of the residue in hot etanol was treated with charcoal, and filtered. The filtrate was concentrated to a volume of 20 ml, and diluted with ether, resulting in 1·0 g (87·0%) of H-D-Asn-Phe-NH_2 (m.p. 177–178°C, R_F 0·15 (S4)). To a solution of 0·80 g (2·86 mmol) of H-D-Asn-Phe-NH_2 in 15 ml dimethylformamide, 0·98 g (3·0 mmol) of Boc-Leu-OSu was added. On the next day, the reaction mixture was evaporated, the residue was triturated with water, then filtered off. Trituration of the crude product with hot ethanol, then with a 1 : 4 mixture of dimethylformamide and ethanol resulted in 1·0 g (70·9%) of *XVI*, m.p. 218–219°C; $[\alpha]_D$ +24·8° (*c* 1·02, dimethylformamide); R_F 0·70 (S3). For $C_{24}H_{37}N_5O_6$ (491·58) calculated: 58·64% C, 7·59% H, 14·25% N; found: 58·80% C,

7·72% H, 14·11% N. ^{1}H NMR: 0·85 and 0·87 dd, 6 H (Leu-CH_3); 1·20—1·50 m, 2 H (Leu-CH_2); 1·37 s, 9 H (Bu^t); 1·59 m, 1 H (Leu-γCH); 2·31 and 2·36, 2·40 and 2·42 dd and dd, 2 H (Asn-CH_2); 2·76 and 2·79, 3·11 and 3·12 dd and dd, 2 H (Phe-CH_2); 3·93 m, 1 H (Leu-αCH); 4·31 m, 1 H (Phe-CH); 4·44 q, 1 H (Asn-CH); 6·93 d, 1 H (Leu-NH, exchangeable with D_2O); 6·99 and 7·22 br and br, 2 H (Phe-NH_2, exchangeable); 7·15—7·25 m, 5 H (Phe-aromatic); 7·36 d, 2 H (Asn-NH_2, exchangeable); 7·84 d, 1 H (Asn-NH_2. exchangeable); 8·04 d, 1 H (Phe-NH, exchangeable). ^{13}C NMR: 21·51 and 22·87 (Leu-δC), 24·09 (Leu-γC), 28·09 (Boc-CH_3), 36·92 (Phe-βC), 36·98 (Asn-βC), 40·45 (Leu-βC), 49·37 (Asn-αC), 52·51 (Leu-αC), 54·18 (Phe-αC), 78·20 (Boc-qC), 126·09 (4′-aromatic), 127·95 (3′ and 5′-aromatic), 129·01 (2′ and 6′-aromatic), 137·96 (1′-aromatic), 155·34 (Boc-CO), 170·56 (Asn-CH_2-CO), 171·62 (Asn-CO), 172·31 (Leu-CO), 172·63 (Phe-CO).

Boc-Leu-iAsn-Phe-NH_2 (*XVII*, β-L-L-L)

To a solution of 1·0 g (2·43 mmol) of *XII* in 30 ml of dimethylformamide, 0·2 g of Pd on charcoal was added. Through the stirred suspension hydrogen was bubbled until completion of the reaction, then the catalyst was removed, the solvent evaporated. A solution of the residue in ethanol was charcoaled, then filtered, concentrated to a volume of 20 ml and kept in the refrigerator. Yield 0·60 g (89·6%) of H-iAsn-Phe-NH_2, m.p. 187—188°C; R_F 0·25 (S4).

To a solution of 0·50 g (1·8 mmol) of H-iAsn-Phe-NH_2 in 10 ml of dimethylformamide, 0·62 g (1·9 mmol) of Boc-Leu-OSu was added. On the day next the reaction mixture was evaporated, the residue was twice recrystallized from ethanol, resulting in 0·58 g (65·5%) of *XVII*, m.p. 214—217°C; $[\alpha]_D$ −31·8° (*c* 1, dimethylformamide); R_F 0·70 (S3). For $C_{24}H_{37}N_5O_6$ (491·58) calculated: 58·64% C, 7·59% H, 14·25% N; found: 58·82% C, 7·48% H, 14·19% N. ^{1}H NMR: 0·83 and 0·86 dd, 6 H (Leu-CH_3); 1·30—1·47 m, 2 H (Leu-CH_2); 1·38 s, 9 H (But); 1·60 m, 1 H (Leu-γCH), 2·31 and 2·32, 2·61 and 2·63 dd and dd, 2 H (Asn-CH_2); 2·74 and 2·76, 3·50 and 3·65 dd and dd, 2 H (Phe-CH_2); 3·85 q, 1 H (Leu-αCH); 4·36 m, 1 H (Phe-CH, overlapping); 4·41 m, 1 H (iAsn-CH, overlapping); 7·06 and 7·24 br and br, 2 H (iAsn-NH_2, exchangeable with D_2O); 7·08 d, 1 H (Leu-NH, exchangeable); 7·11 and 7·23 br and br, 2 H (Phe-NH_2, overlapping, exchangeable); 7·15—7·28 m, 5 H (Phe-aromatic); 8·13 d, 1 H (iAsn-NH, exchangeable); 8·19 d, 1 H (Phe-NH, exchangeable). ^{13}C NMR: 21·43 and 22·86 (Leu-δC), 24·09 (Leu-γC), 28·07 (Boc-CH_3), 36·97 (Phe-βC), 37·12 (iAsn-βC), 40·10 (Leu-βC), 49·51 (iAsn-αC), 53·18 (Leu-αC), 53·86 (Phe-αC), 78·24 (Boc-qC), 126·03 (4′-aromatic), 127·93 (3′ and 5′-aromatic), 128·92 (2′ and 6′-aromatic), 138·12 (1′-aromatic), 155·66 (Boc-CO), 169·60 (iAsn-CH_2-CO), 172·87 (iAsn-CO), 172·27 (Leu-CO), 172·93 (Phe-CO).

Boc-Leu-D-iAsn-Phe-NH_2 (*XVIII*, β-L-D-L)

Through a stirred solution of 1·50 g (3·63 mmol) of *XIII* in 50 ml of dimethylformamide hydrogen was bubbled in the presence of 0·2 g Pd on charcoal. After deprotection the catalyst was removed, the solvent evaporated and a solution of the residue in 50 ml of ethanol was charcoaled and filtered. The filtrate was evaporated to a volume of 20 ml, then diluted with 20 ml of ether. The resulting suspension was filtered to yield 0·78 g (77·0%) of H-D-iAsn-Phe-NH_2, m.p. 187—188°C; $[\alpha]_D$ −14·9° (*c* 1, dimethylformamide); R_F 0·15 (S4).

A solution of 0·60 g (2·15 mmol) of the free dipeptide in 10 ml of dimethylformamide, 0·76 g (2·30 mmol) of Boc-Leu-OSu was added. On the next day the reaction mixture was evaporated, the residue was triturated with hot ethanol, then with a 4 : 1 mixture of hot ethanol and dimethylformamide, resulting in 0·86 g (81·1%) of *XVIII*, m.p. 231—232°C; $[\alpha]_D$ −14·6° (*c* 1, dimethylformamide); R_F 0·70 (S3). For $C_{24}H_{37}N_5O_6$ (491·58) calculated: 58·64% C, 7·59% H, 14·25% N;

found: 58·82% C, 7·73% H, 14·25% N. ^{1}H NMR: 0·82 and 0·83 dd, 6 H (Leu-CH_3); 1·33—1·41 m, 2 H (Leu-CH_2, overlapping); 1·36 s, 9 H (But); 1·53 s, 1 H (Leu-γCH); 2·45 m, 2 H (iAsn-CH_2); 2·75 and 2·77, 3·05 and 3·06 dd and dd, 2 H (Phe-CH_2); 3·84 q, 1 H (Leu-αCH); 4·42 m, 2 H (iAsn- and Phe-CH overlapping); 7·1 br, 3 H (Leu-NH and Phe-NH_2 overlapping, exchangeable with D_2O); 7·15—7·27 m, 5 H (Phe-aromatic, overlapping); 7·20 and 7·40 d and d, 2 H (iAsn-NH_2 overlapping, exchangeable); 8·06 d, 1 H (iAsn-NH, exchangeable); 8·24 d, 1 H (Phe-NH, exchangeable). ^{13}C NMR: 21·80 and 22·66 (Leu-δC), 23·97 (Leu-γC), 28·07 (Boc-CH_3), 36·82 (Phe-βC), 37·31 (iAsn-βC), 39·81 (Leu-βC), 49·50 (iAsn-αC), 52·98 (Leu-αC), 53·56 (Phe-αC), 78·16 (Boc-qC), 126·06 (4′-aromatic), 127·91 (3′ and 5′-aromatic), 128·93 (2′ and 6′-aromatic), 137·99 (1′-aromatic), 155·73 (Boc-CO), 169·14 (iAsn-CH_2-CO), 172·31 (Leu-CO), 173·14 (iAsn--CO), 172·75 (Phe-CO).

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