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Solution Synthesis of a Dimeric Pentapeptide: Diketopiperazine Cyclisation of Glu-Asp Dipeptide Esters and Asp-Racemisation During Segment Condensation

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Abstract: A haemoregulatory peptide analogue derived from the cystine-dimerised pentapeptide Glp-Glu-Asp-Cys-Lys-OH, in which the cystine residue has been replaced by an isosteric *L,L*-2,7-diaminosuberic acid moiety, was prepared by segment condensation in solution. The preparation of protected Glp-Glu-Asp-OH tripeptides was found to be hampered by the surprising ease with which *t*-butyl side-chain protected H-Glu-Asp-OR dipeptide esters underwent diketopiperazine cyclisation. A number of Asp esters were compared in this respect. Reaction conditions for the segment condensation were investigated in terms of racemisation as well as chemical yield and product purity.

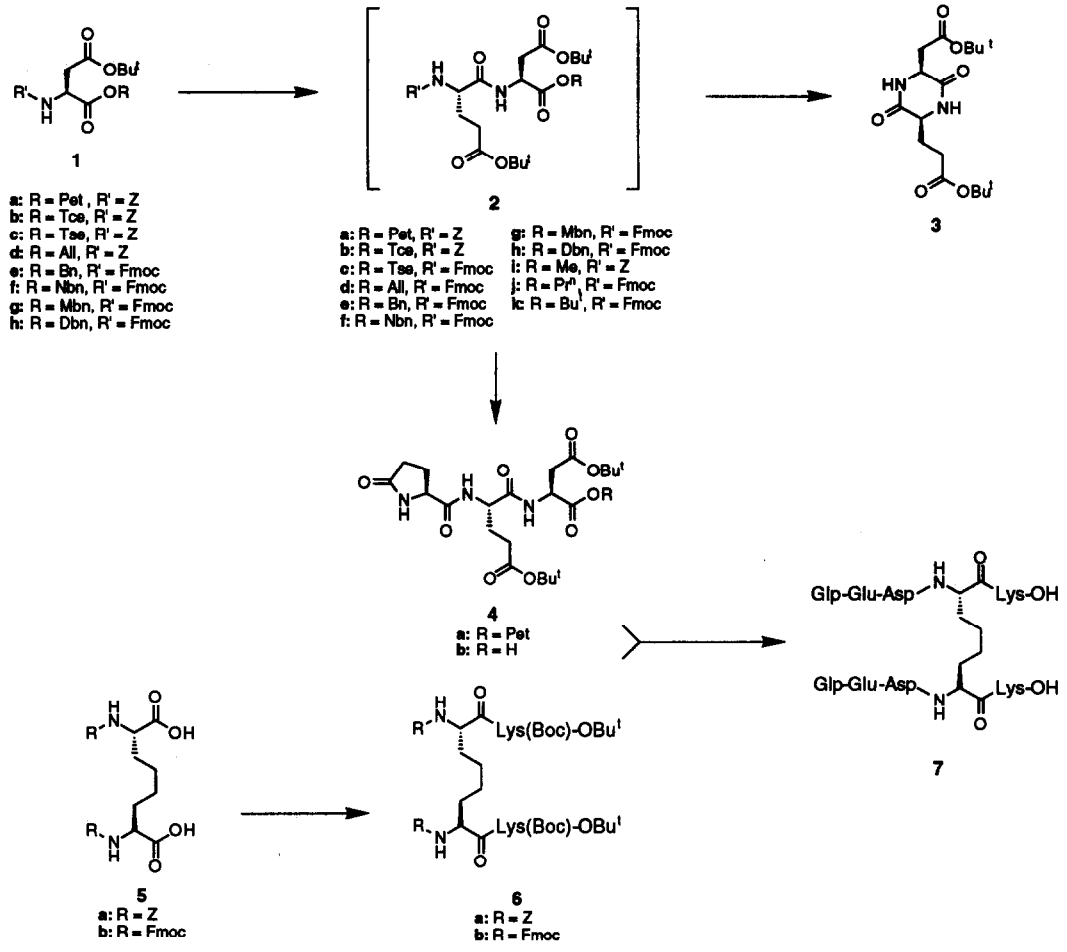
INTRODUCTION

The haemoregulatory pentapeptide Glp-Glu-Asp-Cys-Lys-OH has been reported to inhibit selectively proliferation of myelopoietic cells. The disulphide-linked peptide dimer derived from this peptide by oxidation, on the other hand, has been found to have potentially useful therapeutic properties in that it can stimulate haemopoietic bone marrow cells and cause proliferation of epithelial and epidermal cells.[1]

We were interested in a solution synthesis strategy of the peptide containing an isosteric α,α'-diaminosuberic acid (Dsa) substitution for the cystine residue.[2] In this strategy protected segments corresponding to the Glp-Glu-Asp-OH tripeptide and the dimeric H_2-Dsa-(Lys-OH)$_2$ dipeptide were to be condensed (refer Scheme 1). For ease of final deprotection of the fully assembled peptide we chose side-chain and carboxy terminus protecting groups based on *t*-butyl alcohol. Use of the ß-*t*-butyl ester of Asp was also desirable due to its lesser propensity toward aspartimide formation than *e.g.* the more conventional benzyl ester.[3] For temporary N^{α}-protection we envisaged benzyl carbamates since they can be cleaved cleanly under neutral conditions to yield the free amino acid esters/peptides. Here we describe the synthesis of suitably protected fragments, including the somewhat surprising propensity of certain intermediate Glu-Asp dipeptide esters towards diketopiperazine formation.

Although completely racemisation-free coupling of peptide segments using chemical methods is probably impossible and might only be achieved using enzymatic methods,[4] according to a recent study[5] it could be expected that a side-chain protected *C*-terminal Asp residue would be comparatively insensitive to racemisation during segment condensation. In the present study we have assessed a number of different segment condensation methods with respect to the extent of racemisation. This was possible with the aid of authentic peptides, prepared using solid-phase synthesis methods, containing *L*- and *D*-Asp.

Scheme 1



Non-standard abbreviations: All, allyl; Dbn, *o,p*-dimethoxybenzyl; Mbn, *p*-methoxybenzyl; Nbn, *p*-nitrobenzyl; Pet, 2-(2-pyridyl)-ethyl; Tce, 2-(2,2,2-trichloro)-ethyl; Tse, 2-(toluene-*p*-sulphonyl)-ethyl.

RESULTS AND DISCUSSION

Diketopiperazine formation. Because of the traditional success with azide couplings in convergent syntheses, and despite the fact that the stability of the ß-*t*-butyl ester function of Asp residues to strong nucleophiles is somewhat suspect,[6] it was decided to investigate if elaboration of the tripeptide α-methyl ester *via* the hydrazide to the azide and segment condensation *via* this reactive species was feasible. Surprisingly, it was found that the dipeptide methyl ester **2i**, while stable during the hydrogenolysis reaction, did not lead to the target tripeptide **4** upon the following attempted acylation with Glp-OH/*N,N'*-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt)/Pr^i_2NEt. The only product emerging from the work-up of this

reaction showed, amongst other things, loss of the methoxy signal in the ^{1}H-NMR spectrum and a shortfall corresponding to the mass of methanol of the expected molecular ion upon FAB-MS. Clearly diketopiperazine cyclisation had occurred under the reaction conditions of chain elongation.

The driving force for the quite facile formation of the diketopiperazine **3** is not clear, considering that this structure contains two *cis*-peptide bonds as well as two rather bulky *cis*-ring substituents. While carrying out the experiments described in this paper we became aware of a recent report[7] of a very facile diazomethane-induced cyclisation of spaglumic acid (Ac-Asp-Glu-OH) upon attempted methylation, resulting in a diketopiperazine product similar to compound **3** described here.

Table 1. ***Influence of alcohol portion R of H-Glu(OBu^t)-Asp(OBu^t)-OR dipeptide esters on the ease of diketopiperazine formation.***

Compound	R	R'	Diketopiperazine formation index[a]	
			During N$^\alpha$-deprotection[b]	During acylation[c]
2a	Pet	Z	-	-
2b	Tce	Z	-[d]	++++
2c	Tse	Fmoc	+	++
2d	All	Fmoc	++	++
2e	Bn	Fmoc	++++[e]	N/A
2f	Nbn	Fmoc	++++[e]	N/A
2g	Mbn	Fmoc	+++	++
2h	Dbn	Fmoc	+	-
2i	Me	Z	-	++++
2j	Pr^n	Fmoc	++	++
2k	Bu^t	Fmoc	+	+

[a] Measured by thin-layer chromatography (TLC) analysis: observation of relative intensities of spots due to diketopiperazine (chlorine/dicarboxidine stain). Reaction conditions were kept constant from experiment to experiment and a known quantity of authentic diketopiperazine was always co-chromatographed. No cyclisation is indicated by (-), the extent of cyclisation varied from very little (+) to practically complete reaction (++++).

[b] Fmoc: 50 % Diethylamine in dichloromethane, overnight reaction at room temperature. Z: with 0.4 g/mmol 10 % palladium on charcoal in methanol under an hydrogen atmosphere.

[c] With one equivalent each of Glp-OH, DCC and HOBt in dichloromethane overnight.

[d] Decomposition both during hydrogenolysis and acylation was observed.

[e] Reaction was complete after two hours.

These results prompted us to investigate some other esters commonly used in peptide synthesis. The results obtained with such dipeptide esters are summarised in Table 1. Both the benzyl esters **2e** and **2f**, as well as the alkyl ester **2j**, furnished the diketopiperazine **3** in almost quantitative yield after work-up of the Fmoc-deprotection reaction products. The *n*-propyl ester **2j** was obtained as one of the products of hydrogenolysis of Z-Glu(OBu^t)-Asp(OBu^t)-OAll in an as yet unpublished study whose purpose was to investigate if selective hydrogenolysis under controlled conditions of Z groups in the presence of allyl esters is possible, a question of importance in relation to the utility of allyl esters in glycopeptide synthesis.[8] More surprising was the fact

that even the *t*-butyl ester **2k**, while almost stable to cyclisation during Fmoc-deprotection, afforded appreciable quantities of the cyclic product **3**, apart from the desired tripeptide **4**, upon carbodiimide-mediated elongation with pyroglutamic acid. *t*-Butyl esters are considered quite stable to nucleophilic attack and are generally regarded as unreactive in intramolecular cyclisations.

Like phenacyl esters, which are deprotected similarly with Zn in acidic solution and are known to undergo cyclisation through intramolecular attack by the amino nucleophile of dipeptide esters, the trichloroethyl ester can be regarded as an activated methyl ester. The finding that the dipeptide ester **2b** underwent diketopiperazine formation upon attempted acylation was thus not surprising. Similarly the tosylethyl ester **2c** was incompletely resistant to intramolecular nucleophilic attack. Of the alkoxysubstituted benzyl esters, which are relevant to solid-phase peptide synthesis, because there they are used as acid-labile linkers,[9] the mono-substituted ester **2g** gave rise to considerable quantities of **3**, whereas the di-substituted equivalent **2h** was almost completely stable to cyclisation. Indeed it was possible to synthesise the tripeptide **4b** without any problems starting from Fmoc-Asp(OBu^t)-O(SASRIN)-resin.[10] The only other ester which turned out to be useful in terms of its resistance to cyclisation was the pyridylethyl ester **2a**.

Segment condensation. As is always the case when attempting acylations with activated peptide segments rather than activated carbamate-protected amino acids, it was clear from the outset that the main issue to be addressed here would be racemisation of the Asp residue during segment condensation. For this reason it was desirable to have authentic samples of the possible peptide **7** Asp-diastereomers. These were accessible readily by three separate step-wise solid-phase peptide synthesis (SPPS) experiments using either *L*-, *D*-, or *L/D*-Asp derivatives. Fortunately the peptide diastereomers so produced were separable by RP-HPLC (Fig. 1) and it was thus possible to quantitate accurately the extent of racemisation caused by various segment condensation conditions.

The results obtained using various different such conditions for the condensation between **4b** and N^{α}-deprotected **6** are summarised in Table 2. HOBt was included throughout in the reaction mixtures except in the mixed anhydride experiments since its racemisation-suppressing effect has been established both for carbodiimide-,[11] as well as BOP-[12] mediated couplings. Castro's original reagent BOP and the various related coupling reagents suggested later were of interest because of their known capacity to permit very efficient acylation reactions in SPPS. BOP itself is known to be problematic in segment condensations from the point of view of racemisation but BOP's successor PyBOP was reported to perform significantly better in this respect.[13] In our case PyBOP-mediated reactions, when HOBt and a tertiary base (1 equiv. each) were included, were relatively efficient in terms of chemical yield and purity. However, racemisation was very high at around 16 %. The addition of certain metal salts as racemisation suppressants has been reported to be highly efficient in carbodiimide-mediated couplings[14] as well as segment condensation with mixed anhydrides.[15] We found that addition of $CuCl_2$ to the standard PyBOP coupling mixture here also significantly lowered the extent of racemisation; unfortunately this gain was linked to a loss in product yield and purity. It is clear that the presence of a tertiary base in BOP- and PyBOP-mediated couplings has an influence both on coupling efficiency[16] as well as extent of racemisation.[17] We found no significant difference between NMM and Pr^i_2NEt with respect to racemisation. It is generally held that deprotonation of the amino component by an organic base is necessary in such reactions: our results show that PyBOP-coupling without auxiliary base is in fact possible albeit resulting in less 'clean' reaction. The fact that

racemisation is also halved under these conditions once again emphasises the implication of added base in the maintenance of stereochemical integrity during peptide bond formation reactions. As expected,[18] DCC/HOBt gave a similar extent of racemisation. Of all the reagents tested *i*-butyl chloroformate[19] and EEDQ,[20] *i.e.* those reagents giving rise to mixed carbonic anhydrides, gave the least amount of racemisation. The results obtained with EEDQ were particularly good since the reaction yield and product purity approached those achievable with the more modern coupling reagents. Another benzotriazine-derived coupling reagent, HBTU,[21] afforded the chemically purest but optically most impure product. Similarly the oxopyridine-derived reagent TPTU, which was specifically suggested as a possible reagent for segment condensations,[22] also exhibited high racemisation.

Table 2. ***Influence of reagent choice on chemical and optical purity, as well as yield of product from fragment condensation between Glp-Glu(OBu^t)-Asp(OBu^t)-OH and H$_2$-Dsa-(Lys(Boc)-OBu^t)$_2$.***

Expt. No.	Conditions[a]	Crude Yield[b] (mg)	Purity[c] (%)	Chemical Yield (%)	Racemisation[c] (%)
1	PyBOP/HOBt/NMM	22	60.4	45	15.9
2	PyBOP/HOBt/Pr$^i_2$NEt	26	61.1	54	16.9
3	PyBOP/HOBt/NMM/$CuCl_2$	13	33.2	15	2.3
4	PyBOP/HOBt	17	42.8	25	8.8
5	DCC/HOBt	11	58.9	22	7.3
6	Bu^{i}OCOCl/NMM	9	51.4	16	0.5
7	EEDQ	22	53.5	40	0.8
8	HBTU/HOBt/Pr$^i_2$NEt	22	65.5	49	24.2
9	TPTU/HOBt/Pr$^i_2$NEt	19	63.6	41	22.7

Abbreviations: EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; NMM, *N*-methylmorpholine; PyBOP, benzotriazol-1-yl-oxy-*tri*(pyrrolidino)phosphonium hexafluoro phosphate; TPTU, *O*-(1,2-dihydro-2-oxo-1-pyridyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate.

[a] One equivalent (25 μmol) of the dimeric dipeptide segment and two equivalents of the tripeptide segment and the reagents listed were reacted in DMF.

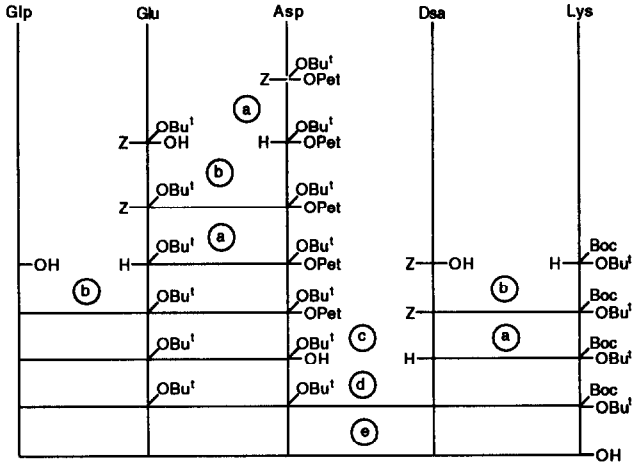
[b] Weight of peptide material from Et_2O-precipitation after reaction work-up and acidolytic deprotection.

[c] Determined from integration of appropriate peak areas in HPLC chromatograms (λ = 215 nm).

Optimised synthesis of 7. The details of the sequence of reactions finally chosen for the synthesis of our target peptide are summarised in Scheme 2. The pyridylethyl ester **1a** was the starting point. In our case hydrogenolysis of the Z group in the presence of the pyridylethyl moiety, which can apparently be difficult, proceeded smoothly. The deprotected Asp residue was then coupled with Z-Glu(OBu^t)-OH using the mixed anhydride method with *i*-butyl chloroformate. The resulting dipeptide ester **2a** was again deprotected by hydrogenolysis and then acylated with the Glp-OH mixed anhydride to furnish the tripeptide **4a**, whose solubility in organic solvents was very high thus permitting purification by normal phase chromatography. Deprotection of the pyridylethyl ester proceeded uneventfully. Alkylation of the pyridine nitrogen took four

days to go to completion; during this time the *N*-methyl-pyridinium intermediate could be observed to precipitate from solution. Removal of this group was then achieved cleanly with morpholine/methanol. The tripeptide acid **4b**, unlike all of the oily precursors, could be obtained in solid form by precipitation from diethyl ether. The yield, based on the Asp derivative **1a**, *i.e.* over 5 reaction steps and one chromatographic step was 68 %.

Scheme 2



Reagents: (**a**) Hydrogenolysis over Pd/C in MeOH or DMF, (**b**) acylation with preformed mixed anhydride from Bu^{i}OCOCl/NMM in THF or DMF at -10 °C, (**c**) MeI, MeCN then morpholine/MeOH, (**d**), segment condensation with EEDQ in DMF, (**e**) acidolysis with 2 % H_2O/CF_3COOH.

Carbamate (Z and Fmoc) protection of the amino groups of Dsa[23] using conventional methods failed but could be achieved after silylation with Me_3SiCl/1,1,1,3,3,3-hexamethyldisilazane(HMDS) and reaction with the appropriate chloroformate using a procedure developed in our laboratory for the amino-protection of diaminodicarboxylic acids. Condensation of the protected Dsa **5a** with H-Lys(Boc)-OBu^t.HCl, again using the mixed anhydride method, gave the protected dipeptide **6a** in 69 % yield (based on free Dsa), which could be purified by recrystallisation.

EXPERIMENTAL SECTION

General. TLC was carried out using glass plates precoated with silica G60-F254. Compounds were visualised variously using UV light, ninhydrin spray reagent as well as chlorination/dicarboxidine[24] stain. Flash chromatography was carried out as described.[25] HPLC was carried out using Beckman System Gold apparatus and Vydac 218TP54 analytical columns. Gradient elution was carried out with MeCN in 0.1 % CF_3COOH/H_2O at 1 mL/min. NMR experiments were carried out using a Varian 300 MHz spectrometer. Samples were dissolved in $CDCl_3$. Optical rotations were measured on a Perkin-Elmer model 241 polarimeter. Melting points are not corrected.

Table 3. ***Analytical Details of Amino Acid Derivatives and Peptides.***

Compound	State[a]	m.p. (°C)	$[\alpha]_D^{20}$ (*c*=1)	Isolation[b]	Formula	M_r	FAB-MS[c]	TLC R_f
1a	o		-18.5 (M)	FC (5:95 M/D)	$C_{23}H_{28}N_2O_6$	428.5	429.2 s	0.56 (1:9 M/C)
1b	c	< 70	-18.8 (M)	RC (E/H)	$C_{18}H_{22}NO_6Cl_3$	454.7	456 m	0.49 (1:2 E/H)
1c	o		-3.0 (M)	FC (1:1 E/H)	$C_{25}H_{31}NO_8S$	505.6	1013.1 s[d]	0.40 (1:1 E/H)
1d	o		-15.4 (5:95 A/M)	FC (1:2 E/H)	$C_{19}H_{25}NO_6$	363.4	364.7 m	0.45 (1:2 E/H)
1e	c	107-108	-19.4 (DMF)	FC (1:2 E/H)	$C_{30}H_{31}NO_6$	501.6	502. 3 s	0.32 (1:2 E/H)
1f	c	141	-15.7 (DMF)	RC (E/H)	$C_{30}H_{31}N_2O_8$	529.4	530.5 m	0.27 (1:2 E/H)
1g	c	117-118	-16.2 (DMF)	RC (Et_2O/M)	$C_{31}H_{33}NO_7$	531.6	532.2 w	0.34 (1:2 E/H)
1h	c	105-106	-10.1 (DMF)	FC (1:2 E/H)	$C_{32}H_{35}NO_8$	561.6	562.3 m	0.29 (1:2 E/H)
2a	o		-11.8 (DMF)	not isolated	$C_{32}H_{43}N_3O_9$	613.7	614.5 s	0.56 (1:9 M/C)
2b	o		-18.0 (M)	FC (1:2 E/H)	$C_{27}H_{37}N_2O_9Cl_3$	640.0	[e]	0.33 (1:2 E/H)
2c	c	87-92	-8.0 (DMF)	FC (1:1 E/H)	$C_{39}H_{46}N_2O_{11}S$	750.9	752.3 w	0.30 (1:1 E/H)
2d	o		-13.7 (M)	FC (1:1 E/H)	$C_{35}H_{44}N_2O_9$	636.7	637.5 m	0.40 (1:1 E/H)
2e	c	99	-14.5 (DMF)	FC (3:5 E/H)	$C_{39}H_{46}N_2O_9$	686.8	687.3 s	0.35 (3:5 E/H)
2f	c	97-98	-11.1 (DMF)	FC (1:1 E/H)	$C_{39}H_{45}N_3O_{11}$	731.8	732.3 w	0.32 (1:1 E/H)
2g	c	84	-24.1 (DMF)	FC (1:2 E/H)	$C_{40}H_{48}N_2O_{10}$	716.8	717.5 w	0.24 (1:2 E/H)
2h	o		-8.8 (DMF)	FC (1:1 E/H)	$C_{41}H_{50}N_2O_{11}$	746.9	747.5 w	0.38 (1:1 E/H)
2i	o		-9.4 (DMF)	FC (4:96 M/D)	$C_{26}H_{38}N_2O_9$	522.6	523.4 s	0.71 (85:10:5 C/M/A)
2j	f		-12.5 (DMF)	FC (4:5 E/H)	$C_{35}H_{46}N_2O_9$	638.8	639.4 s	0.46 (4:5 E/H)
2k	f		-17.9 (DMF)	PP (E/H)	$C_{36}H_{48}N_2O_9$	652.8	653.4 s	0.27 (1:2 E/H)
3	c	196-198	-24.5 (M)	FC (5:95 M/D)	$C_{17}H_{28}N_2O_6$	356.4	357.2 s	0.43 (1:9 M/C)
3 (free diacid)				RP-HPLC	$C_9H_{12}N_2O_6$	244.2	245.1	
4a	o		-11.5 (DMF)	FC (15:185 M/D)	$C_{29}H_{42}N_4O_9$	590.7	591.3 s	0.45 (1:9 M/C)
4b	c	166-170	-8.2 (DMF)	FC (85:10:5 C/M/A)	$C_{22}H_{35}N_3O_9$	485.5	508.4 w[f]	0.18 (85:10:5 C/M/A)
5a	c	169-170	-10.8 (DMF)	RC (Et_2O)	$C_{24}H_{28}N_2O_8$	472.5	473.2 s	0.37 (85:10:5 C/M/A)
5b	c	200-208	-10.8 (DMF)	RC (Et_2O)	$C_{38}H_{36}N_2O_8$	648.7	649.6 s	0.45 (85:10:5 C/M/A)
6a	c	120-121	-10.0 (DMF)	RC (EtOH/H_2O)	$C_{54}H_{84}N_6O_{14}$	1041.3	941.5 [g]	0.65 (85:10:5 C/M/A)
6b	c	130-134	-7.9 (DMF)	RC (EtOH/H_2O)	$C_{68}H_{92}N_6O_{14}$	1217.5	1240.2 w[f]	0.80 (85:10:5 C/M/A)
7				RP-HPLC	$C_{48}H_{74}N_{12}O_{22}$	1171.2	1171.7 s	

[a]) Codes: c, crystalline; f, foam; o, oil. [b]) Isolation methods: FC, flash chromatography; RC, recrystallisation; PP, precipitation. Solvents: A, acetic acid; C, chloroform; D, dichloromethane; DMF, *N,N*-dimethylformamide; E, ethyl acetate; Et_2O, diethyl ether, EtOH, ethanol; H, hexane; M, methanol.. [c]) The molecular ion $[M + H]^+$ is given, except [d]) doubly charged ion only, [e]) no satisfactory spectrum was obtained, [f]) only $[N + Na]^+$ observed, [g]) only $[M - Boc]^+$ observed. Signal strengths, w, weak; m, medium; s, strong.

Peptide synthesis grade solvents were obtained from Rathburn (Scotland). Amino acid derivatives and peptide synthesis resins were obtained from Bachem AG and Novabiochem AG (Switzerland) or were prepared according to literature procedures. Analytical details of all intermediates are collected in Table 3.

Esters of *N*-Z or *N*-Fmoc-protected ß-*t*-butyl aspartic acid were obtained as follows: the pyridylethyl ester **1a** with 2-(2-pyridyl)ethanol and DCC/4-(dimethylamino)pyridine (DMAP) in $CHCl_3$,[26] the trichloroethyl ester **1b** with trichloroethanol and DCC/pyridine,[27] the toluenesulphonylethyl ester **1c** similarly from 2-(toluene-*p*-sulphonyl)ethanol,[28] the allyl ester **1d** with allyl bromide and dicyclohexylamine in boiling $CHCl_3$,[29] the benzyl esters **1e** and **1f** from the corresponding benzyl bromides and Pr^i_2NEt in EtOAc,[30] and **1g** and **1h** from the appropriate benzyl alcohols with the aid of DCC/DMAP in CH_2Cl_2.[31] The dipeptide esters **2** were obtained using standard procedures of Z- and Fmoc-deprotection (hydrogenolysis over 10 % Pd(C) in MeOH, EtOH and/or DMF and 50 % Et_2NH in CH_2Cl_2 or DMF/CH_2Cl_2, respectively), followed by coupling with Z- or Fmoc-Glu(OBu^t)-OH in THF and/or DMF using the Bu^{i}OCOCl/NMM mixed carbonic anhydride method.

Diketopiperazine of H-Glu(OBu^t)-Asp(OBu^t)-OH (**3**). Analytical samples of this compound were obtained by flash chromatography (5:95 MeOH/CH_2Cl_2) and recrystallisation from Et_2O/hexane after Et_2NH-deprotection of various Fmoc dipeptide esters as well as attempted acylations of free amino-dipeptide esters. ^{1}H-NMR: 1.45 (s, 18 H, Bu^t), 2.2 (md, 2 H, ß-CH_2 Glu), 2.45 (m, 2 H, γ-CH_2 Glu), 2.8 (dm, 2 H, ß-CH_2 Asp), 4.1 (m, 1 H, α-CH, Glu), 4.35 (m, 1H, α-CH Asp), 6.85 (br s, 1H, CONH Glu), 7.25 (br s, 1 H, CONH Asp).

Solution synthesis of tripeptide esters (**4**). *Glp-Glu(OBu^t)-Asp(OBu^t)-OPet* (**4a**). Z-Asp(OBu^t)-OPet (2.93 mmol) was submitted to hydrogenolysis in MeOH with 200 mg 10 % Pd(C) catalyst for 1 h. The catalyst was removed and the filtrate was evaporated. Z-Glu(OBu^t)-OH (988 mg, 3.93 mmol) and NMM (0.32 mL, 2.93 mmol) were dissolved in THF (25 mL) and the solution cooled to -10°. Bu^{i}OCOCl (0.4 mL, 3.08 mmol) was then added and 5 min later the precooled solution of the above H-Asp(OBu^t)-OPet in THF (25 mL). The cooling bath was removed and the mixture stirred for 30 min. NMM.HCl was then filtered off and the filtrate was evaporated. The residual Z-Glu(OBu^t)-Asp(OBu^t)-OPet was Z-deprotected as above. The mixed carbonic anhydride from Glp-OH (379 mg, 2.93 mmol) was formed as before (in 25 mL THF and 3 mL DMF) and was treated with the above H-Glu(OBu^t)-Asp(OBu^t)-OPet in THF (25 mL) for 30 min. The mixture was filtered and the filtrate was evaporated to dryness. The residue was partitioned between 2 M aq NaCl (50 mL) and CH_2Cl_2 (2 x 50 mL). The combined organic phases were dried and evaporated. The product was purified by flash chromatography (4.5 cm diam. column, 7.5 % MeOH in CH_2Cl_2). The title compound was obtained in analytically pure form (1.42 g, 81.9 %). ^{1}H-NMR: 1.45 (2 s, 18 H, Bu^t), 1.99 (dm, 2 H, β-CH_2 Glu), 2.34 (m, 6 H, γ-CH_2 Glu & β,γ-CH_2's Glp), 2.85 (qd, 2 H, β-CH_2 Asp), 3.15 (t, 2 H, α-CH_2 Py), 4.2 (m, 2 H, α-H's Glp & Glu), 4.55 (m, 3 H, α-H Asp & β-CH_2 Py), 4.78 (m, 1 H, CONH Asp), 7.15 (m, 1 H, CONH Glu), 7.44 (m, 3 H, Ar-H β,γ-Py), 8.05 (s, 1 H, CONH Glp), 8.55 (d, 1 H, Ar-H α-Py).

Glp-Glu(OBu^t)-Asp(OBu^t)-OH (**4b**). Glp-Glu(OBu^t)-Asp(OBu^t)-OPet (0.5 mmol) was dissolved in MeCN (10 mL) and MeI (0.47 mL, 7.5 mmol) was added. The clear mixture was stirred at room temperature; after 32 h precipitated material could be observed. After a total reaction time of 4 d, conversion to the *N*-methyl pyridinium derivative was complete as indicated by TLC . The reaction mixture was then evaporated

and taken to dryness under high vacuum. The residue was redissolved in 2:8 morpholine/MeOH (25 mL) and the solution was stirred overnight. It was then rotary evaporated and redissolved in CH_2Cl_2 (50 mL). This solution was extracted twice with 10 % aq citric acid (25 mL each), after each extraction a few mL of MeOH were added to the organic phase to counteract gel formation of the product. The CH_2Cl_2 phase was dried on $MgSO_4$, filtered and evaporated to dryness under high vacuum. The resulting glassy solid was triturated with Et_2O (25 mL), the suspension cooled to -20° and centrifuged (3 min, 2,500 r.p.m.). The ethereal supernatant was decanted and the pellet was washed once more with Et_2O in the same manner. The product was dried and the title compound was obtained as a white amorphous powder (202 mg, 83.3 %).

Solid-phase synthesis of tripeptide Glp-Glu(OBu^t)-Asp(OBu^t)-OH (**4b**). Fmoc-Asp(OBu^t)-O-SASRIN resin (0.67 mmol/g loading; 2 g, 1.34 mmol) was washed with CH_2Cl_2 and DMF (2 x 50 mL each), Fmoc-deprotected with 1:1 piperidine/DMF (50 mL, 15 min) and washed again (DMF, CH_2Cl_2 and DMF; 2 x 50 mL each). Fmoc-Glu(OBu^t)-OH (2.4 g, 5.36 mmol), PyBOP (4 eq), HOBt (4 eq) and NMM (6 eq) were then added in DMF (10 mL) and the acylation allowed to proceed for 1 h. The peptidyl resin was washed as before. After further Fmoc-deprotection and washing, Glp-OH (0.69 g, 5.36 mmol) was coupled with PyBOP, HOBt and NMM as usual. After 1 h reaction time the resin was washed and recoupling performed with Glp pentachlorophenyl ester (2 g, 5.36 mmol) and HOBt (4 eq). After 3 h reaction time acylation was apparently complete. The peptidyl resin was again washed with DMF, CH_2Cl_2, and finally with Et_2O. An aliquot of this material (1 g) was treated with 1 % CF_3COOH in CH_2Cl_2 (20 mL) for 15 min. The resin was then filtered off and the filtrate neutralised immediately by the addition of Et_3N (1 mL). This process was repeated 3 times (the resin appeared pink after the second, and dark purple after the fourth cleavage reaction). The combined neutralised filtrates were evaporated to dryness. The residue was purified by flash chromatography (3.5 cm column, 85:10:5 $CHCl_3$/MeOH/AcOH). The title compound was obtained as a glassy solid (TLC R_f 0.23 in flash chromatography eluant). ^{1}H-NMR (d_6-DMSO): 1.45 (2 s, 18 H, Bu^t), 1.85 (dm, 2 H, β-CH_2 Glu), 2.12 (m, 2 H, γ-CH_2 Glu), 2.27 (m, 4 H, CH_2's Glp), 2.65 (qd, 2 H, β-CH_2 Asp), 4.15 (m, 1 H, α-H Glp), 4.35 (m, 1 H, α-H Glu), 4.45 (m, 1 H, α-H Asp), 7.85 (s, 1 H, CONH Glp), 8.15 (d, 1 H, CONH Glu), 8.25 (d, 1 H, CONH Asp).

Solution synthesis of dimeric dipeptide fragments (**6**). *Z$_2$-Dsa-(OH)$_2$* (**5a**). H_2-Dsa-(OH)$_2$.2 HCl (380 mg, 1.37 mmol) was suspended in HMDS (1.5 mL). The mixture was flushed with N_2 and Me_3SiCl (0.2 mL) was added. The reaction mixture was heated at 120° (under N_2) for 2.5 h (dissolution was complete after 2 h). It was cooled and evaporated at 50° under oil-pump vacuum. The residual colourless oil was redissolved in dry CH_2Cl_2 (20 mL) and cooled in an ice bath. Z-Cl (0.49 mL of fresh reagent) in CH_2Cl_2 (10 mL) was then added and the mixture stirred and allowed to reach room temperature. After a further 4 h stirring it was rotary evaporated and redissolved in 2:8 water/THF (25 mL) and stirred again for 2 h. THF was then removed by rotary evaporation and the residue taken to complete dryness under high vacuum. The semi-solid residue was triturated with Et_2O (25 mL) and hexane (20 mL) was added. After cooling to -20° the product was filtered and dried *in vacuo*. TLC analysis of this material revealed the presence of some ninhydrin-positive polar component. This was removed as follows: The solid material was redissolved in CH_2Cl_2 (50 mL) by the addition of MeOH (5 mL). This solution was extracted with 10 % aq citric acid (2 x 25 mL), addition of MeOH (2 mL) to the organic phase after each extraction was necessary to counteract product precipitation.

After drying over $MgSO_4$, filtration and rotary evaporation, the residue was kept under high vacuum until solidification started. It was then triturated with Et_2O (30 mL), cooled to -20° and filtered. The title compound was obtained as a white powder (478 mg, 73.8 %). ^{1}H-NMR (d$_6$-DMSO): 1.25 (m, 4 H, γ-CH_2), 1.60 (m, 4 H, β-CH_2), 3.92 (m, 2 H, α-CH), 5.00 (s, 4 H, Ar-CH_2), 7.30 (m, 10 H, Ar-H), 7.58 (d, 2 H, -CONH-), 12.50 (br s, 2 H, -COOH).

Z_2-Dsa-[Lys(Boc)-OBu^t]$_2$ (**6a**). Z_2-Dsa-$(OH)_2$ (473 mg, 1 mmol) was dissolved in THF (25 mL) and NMM (0.22 mL, 2 mmol). The solution was cooled to -15° when Bu^{i}OCOCl (0.27 mL, 2.1 mmol) was added. After 5 min a precooled solution of H-Lys(Boc)-OBu^t.HCl (678 mg, 2 mmol) and NMM (0.22 mL, 2 mmol) in THF (25 mL) was added. The entire mixture was stirred for a total of 3 h during which time it was allowed to reach room temperature. NMM.HCl was then filtered off and the filtrate was evaporated. The residue was redissolved in CH_2Cl_2 (100 mL), extracted successively with 5 % aq $NaHCO_3$, 10 % aq citric acid and 2 M aq NaCl (25 mL each), dried over $MgSO_4$, filtered and evaporated to dryness. The crude product was obtained as a glassy solid and was recrystallised from EtOH/H_2O as a white powder. The yield was 970 mg (93.2 %).

Fmoc$_2$-Das-(OH)$_2$ (**5b**). This was prepared from H_2-Das-$(OH)_2$.2 HCl (500 mg, 1.8 mmol) using the same procedures as for **5a** except that Fmoc-Cl (930 mg, 3.6 mmol) instead of Z-Cl was used. The title compound was obtained by trituration with Et_2O as an off-white amorphous powder (0.98 g, 83.9 %).

Fmoc$_2$-Das-[Lys(Boc)-OBu^t]$_2$ (**6b**). This was prepared from Fmoc$_2$-Das-$(OH)_2$ (**5b**; 500 mg, 0.77 mmol) and H-Lys(Boc)-OBu^t.HCl (522 mg, 1.54 mmol) with Bu^{i}OCOCl/NMM in THF in the same way as the Z-derivative **6a**. The title compound was obtained as a very white powder in 626 mg (65 %) yield after crystallisation from EtOH/H_2O.

Segment condensations. An aliquot (25 μmol) of Z_2-Dsa-[Lys(Boc)-OBu^t]$_2$ **6a** was dissolved in DMF (5 mL) and hydrogenolysed for 1 h over 10 % Pd on charcoal. The catalyst was then removed by filtration through Celite filter aid. The filtrate was cooled to 4 °C and added to a solution of Glp-Glu(OBu^t)-Asp(OBu^t)-OH **4b** (50 μmol), coupling reagent (50 μmol) and, if used, additives (50 μmol) in DMF (1 mL), which had been preactivated at 0 ° (-15 ° for *i*-butyl carbonate mixed anhydride method) for 5 min. The mixture was then blanketed with N_2 and stirred overnight at room temperature. DMF was evaporated under high vacuum at 40 ° and the residue partitioned between 5 % aq $NaHCO_3$ (10 mL) and CH_2Cl_2 (10 mL). The aqueous layer was extracted once with CH_2Cl_2 (10 mL). The combined organic fractions were extracted with 10 % aq citric acid (10 mL), dried over $MgSO_4$, filtered and evaporated to dryness. The residue was dissolved in 2 % H_2O/CF_3COOH and stirred under N_2 for 90 min. The mixture was then evaporated and the residue triturated with Et_2O (20 mL). Precipitated peptide was collected by centrifugation (2,500 r.p.m, 3 min) and decantation of the supernatant. The crude peptide **7** so obtained was washed once more with Et_2O in a similar fashion before being dried under high vacuum.

An aliquot (5 mg) of the crude product from the best experiment (No. 7 in Table 3) was purified by semi-preparative RP-HPLC (Vydac 218TP1022; 22 x 250 mm) using a gradient of 0 to 10 % MeCN in 0.1 % CF_3COOH/H_2O over 60 min at a flow rate of 9 mL/min (λ = 230 nm). Analytically pure (99.5 % by anal. RP-HPLC) title peptide (2.4 mg, 48 %) was obtained after lyophilisation of the appropriate peak fractions.

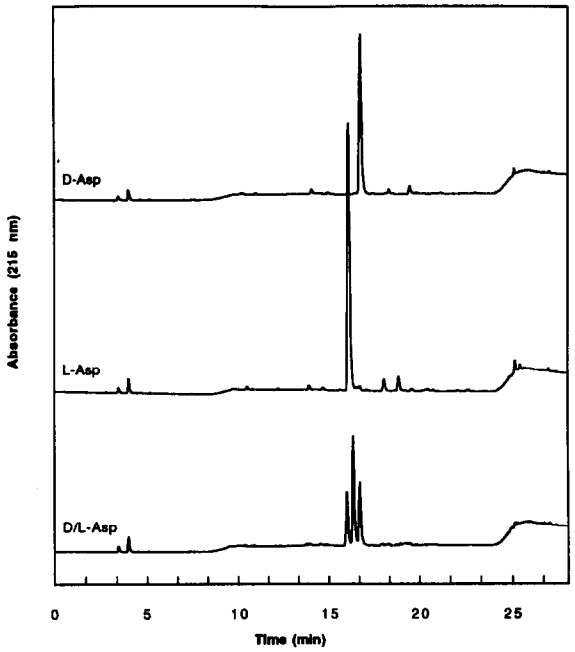


Figure. 1. ***Solid-Phase Peptide Synthesis of Dimeric Peptide 7 Optical Isomers.*** Standard Fmoc/*t*-butyl SPPS was carried out starting with resin-bound Fmoc-Lys(Boc)-OH. In three separate syntheses either Fmoc-*D*-Asp(OBu^t)-OH, Fmoc-*L*-Asp(OBu^t)-OH or an equimolar mixture of the two was used in the acylation at position 3. The crude reaction products, after acidolytic deprotection/resin detachment and precipitation from diethyl ether, were chromatographed using anal. RP-HPLC (gradient 0 to 18 % MeCN over 20 min; λ=215 nm). As expected, two syntheses gave rise to single non-identical main products whereas the latter synthesis yielded approximately equal quantities of these two diastereomeric products in addition to the mixed *D/L*-Asp peptide in roughly the amount expected.

Solid-phase synthesis of diastereomeric peptides (7). *Fmoc$_2$-Dsa-[Lys(Boc)]$_2$-O-SASRIN resin*:. Fmoc-Lys(Boc)-O-SASRIN resin (6 g, 0.6 mmol/g) was Fmoc-deprotected, washed and acylated as follows: Fmoc$_2$-Dsa-(OH)$_2$ (0.97 g, 1.5 mmol) and HOBt (1.01 g, 7.5 mmol) in DMF (50 mL) were added to the drained resin. The mixture was agitated and DCC (1.55 g, 7.5 mmol) was added. The reaction was allowed to proceed overnight when a very slightly positive Kaiser test was recorded. The resin was washed again and further reacted for 1 h with 7.5 mmol each of HOBt and DCC. The resin was again washed with DMF and MeOH/CH_2Cl_2 (8:2) before being acetylated for 30 min with Ac_2O/DMF (1:9). The product was washed extensively with DMF, CH_2Cl_2 and Et_2O before being dried.

(Glp-Glu-Asp)$_2$-Dsa-(Lys-OH)$_2$ (7). Fmoc-Sub-Lys(Boc)-OSASRIN resin aliquots (1 g, *ca.* 0.3 mmol each) were deprotected for 15 min with 20 % piperidine/DMF (50 mL) and washed (4 x 2 min, 25 mL DMF each). The resin samples were then acylated for 1 h with Fmoc-*L*-Asp(OBu^t)-OH, Fmoc-*D*-Asp(OBu^t)-OH or an equimolar mixture of the two (total 987 mg, 2.4 mmol in each case), preactivated with PyBOP (1249 mg, 2.4 mmol), HOBt (324 mg, 2.4 mmol) and NMM (0.4 mL, 3.6 mmol) in DMF (5 mL). The resin samples were then washed and deprotected as above. Each peptidyl resin sample was further elongated successively

with Fmoc-Glu(OBu^t)-OH and Glp-OH. The final peptidyl resins were washed with CH_2Cl_2, MeOH and Et_2O, before being dried in vacuo. The resin samples were then stirred for 90 min with 1 % H_2O in CF_3COOH (40 mL). The resins were filtered off, washed with a little neat CF_3COOH and the combined filtrates and washings were rotary evaporated to a small volume. The peptides were precipitated by addition of Et_2O (50 mL each) and collected by centrifugation (2 min, 2,500 r.p.m.). The ethereal supernatants were decanted and the pellets washed once more with Et_2O. The crude peptides were dried in a stream of N_2 and then under high vacuum. They were obtained as white powders: 275 mg (L-Asp isomer), 324 mg (D-Asp isomer) and 350 mg (L/D-Asp isomer).

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