# Aspartate Racemization in Synthetic Peptides. Part 2.[1] Tendency to Racemization of Aminosuccinyl Residue

**István Schön,*[a] Tamás Szirtes,[a] Attila Rill,[a] Gábor Balogh,[a] Zsolt Vadász,[b] János Seprödi,[b] István Teplán,[b] and in part† Naoyoshi Chino,[c] Kumiko Yoshizawa Kumogaye[c] and Shumpei Sakakibara[c]**

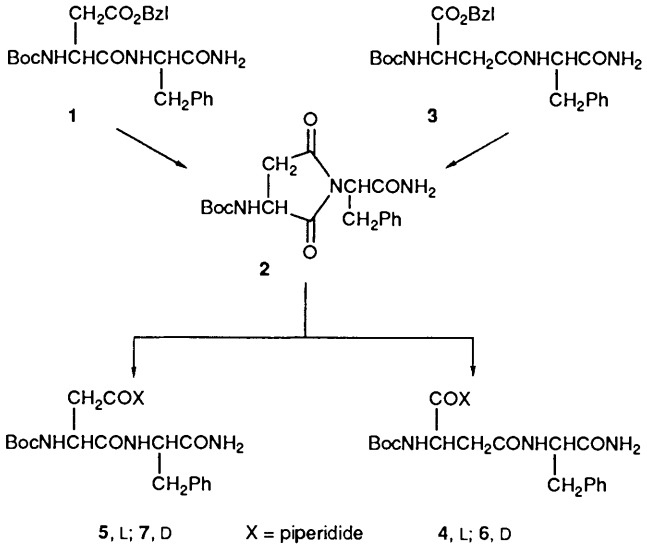
[a] *Chemical Works of Gedeon Richter Ltd., H-1475 Budapest, PO Box 27, Hungary*
[b] *1st Institute of Biochemistry, Semmelweis University Medical School, H-1444 Budapest, PO Box 260, Hungary*
[c] *Peptide Institute Inc., Protein Research Foundation, Minoh-shi, Osaka 562, Japan*

Aminosuccinyl (Asu) peptides, containing a strained ring system, are very vulnerable to epimerization and, during their formation, even as transients, in the presence of nucleophilic and non-nucleophilic bases, partial epimerization occurs. In the presence of nucleophilic bases, Asu-peptides transform to a partially epimerized mixture of α- and β-aspartyl peptides, with the preponderance of the β- over the α-peptides. In the absence of any internal basic functionality such as the protonated guanidino group, chirally pure Asu-peptides could be synthesized by heating of β-benzyl-aspartyl [Asp(OBzl)] peptides in dimethylformamide to elevated temperatures or, from aspartyl (Asp) peptides having a free carboxy group, by the usual treatment with pentafluorophenol or 1-hydroxybenzotriazole–dicyclohexylcarbodiimide. However, in the presence of the strongly basic acetate ion and/or a guanidinium group the probability of aspartate racemization is increased.

Our previous studies on base-catalysed transformations of Asp(OBzl)[2] and Asp(OBu$^t$)-peptides[3] caused by piperidine and ammonia, respectively, directed our further attention to an investigation of the reaction mechanism. Detailed TLC and HPLC analyses of ammonia-mediated reactions of some Asp(OBu$^t$)-peptides revealed a very complex series of parallel and consecutive reactions, including base-catalysed cyclization as well as transpeptidation *via* direct amidation and, indirectly, through transient esterification.[1] Additionally, epimerization made the entire process even more intricate. Data from amino acid analyses[4–6] and biochemistry/enzymology[7,8] support our earlier report[9] on the extraordinary ease of racemization of the aspartate residue. Simultaneously, we also presented some preliminary data on piperidinolysis under simulated deblocking conditions for the $N^{\alpha}$-fluorenylmethoxycarbonyl (Fmoc) group,[2,9] and these have relevance to the present studies.

For some time we have been interested in the synthesis of biologically active Asu-peptides,[10–12] and here we report many of the problems associated with their synthesis, purification, stability and optical purity, as well as the dependence of their chemical behaviour and biological activity on chirality. Throughout these studies, quantitative evaluation of results was mostly carried out by reversed-phase HPLC,[6] the superiority of which as an analytical method can be seen clearly in the following examples.

Synthesis and chemical characterization of model peptides Boc-Asp(OBzl)-Phe-$NH_2$ **1**, Boc-Asu-Phe-$NH_2$ **2** and Boc-Asp(Phe-$NH_2$)-OBzl **3** have been reported previously (Scheme 1).[2] These dipeptides were treated with 10 and 55% (v/v) mixtures of piperidine–dimethylformamide (DMF). In solution, the transformation of Boc-Asp(OBzl)-Phe-$NH_2$ **1** into Boc-Asu-Phe-$NH_2$ **2** and then into a mixture of Boc-L-Asp(Phe-$NH_2$)-X (**4**; X = piperidide) and Boc-Asp(X)–Phe-$NH_2$ **5** was followed by TLC. In these earlier experiments samples, taken at intervals, were evaporated at room temperature under reduced pressure and the residue, after being dried over $P_2O_5$, was dissolved in $CDCl_3$ ready for $^1H$ NMR and IR studies. A semiquantitative estimation of the extent of transformation, based on the relative intensities of the $CH_2$ resonances for benzyl (Bzl) alcohol and Bzl ester, respectively, showed a rapid disappearance of the starting material **1** with simultaneous formation of Asu-peptide **2** within the first hour. Piperidinolyses of Asu-peptide **2**, formed in this reaction mixture or used as a starting material, were complete within 24 h and yielded a mixture of piperidide **4** and **5**.[2]




**Scheme 1**

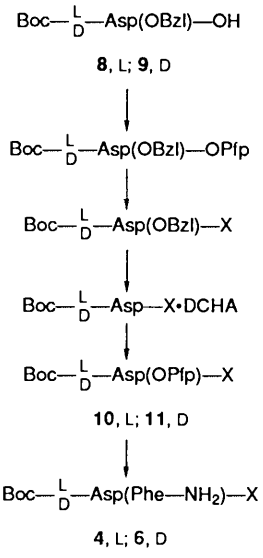
However, the more complex reaction mechanism found earlier in the ammonolysis of Asp(OBu$^t$)-peptides[1] prompted us to recheck the earlier results for piperidinolysis.

## Results and Discussion

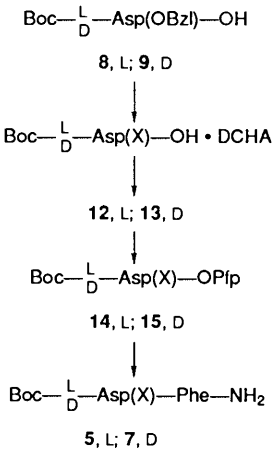
(a) *Piperidine-induced Rearrangement.*—Schemes 2, 3 and 5 show the synthetic routes leading to the various peptides

† Some reactions under standard HF conditions, and HPLC and qualitative stability studies of Asu-Phe-$NH_2$ **27** and **29**, were performed at the Peptide Institute Inc.

investigated here and Schemes 4, 6 and 7 indicate some of the chief transformation found. For detailed HPLC studies, the expected products of piperidinolysis were synthesized independently, *i.e.* piperidides **4** and **5**, and their epimeric counterparts, Boc-D-Asp(Phe-$NH_2$)-X **6** and Boc-D-Asp(X)-Phe-$NH_2$ **7** (Scheme 1; X = piperidide). Five-step synthesis of piperidides **4** and **6** started[13] from Boc-L- **8** and Boc-D-Asp(OBzl)-OH **9** and proceeded through the appropriate pentafluorophenyl (Pfp) esters, which were treated with less than 1 mol equiv. of piperidine (Scheme 2). Hydrogenolytic removal of the Bzl group gave the free β-carboxy compounds, which were purified as dicyclohexylammonium (DCHA) salts and were then converted into Pfp-esters **10** and **11**, respectively, by the usual dicyclohexylcarbodiimide (DCC) method.[14] For the synthesis of β-piperidides **5** and **7**, an alternative route was selected (Scheme 3). Prolonged piperidinolysis of Boc-L- **8** and Boc-D-Asp(OBzl)-OH **9** at 60 °C resulted in Boc-L- **12** and Boc-D-Asp(X)-OH **13**, which were purified as DCHA salts, then were transformed into Pfp-ester **14** and **15**, respectively. The active esters **10**, **11**, **14** and **15** were coupled with H-Phe-$NH_2$·HCl[1] to give the dipeptides **4**, **6**, **5** and **7**, respectively (Schemes 2 and 3). After piperidinolysis

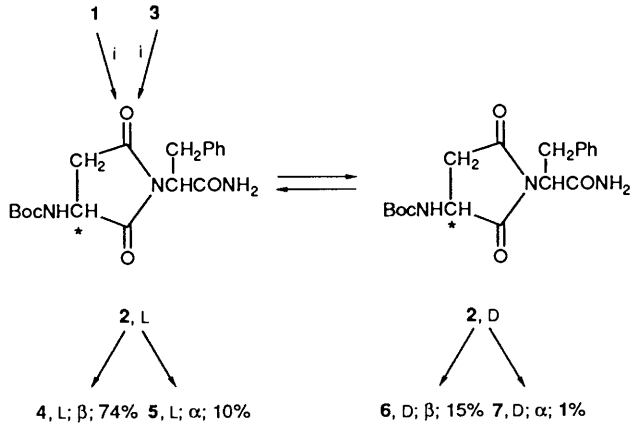



**Scheme 2** X = Piperidide




**Scheme 3** X = Piperidide

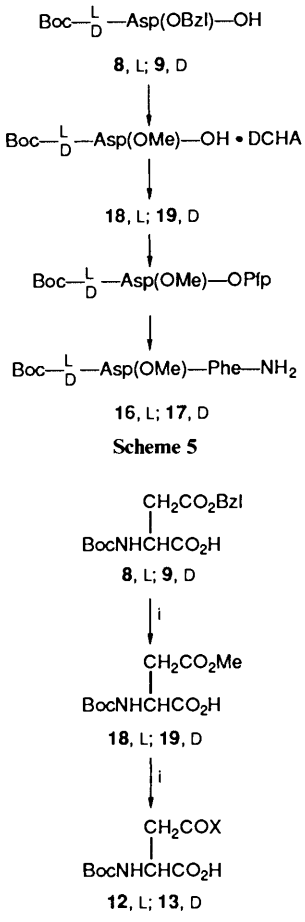
of Boc-Asp(OBzl)-Phe-$NH_2$ **1** and Boc-Asu-Phe-$NH_2$ **2**, HPLC revealed that a mixture of dipeptides (**4**, 74%; **5**, 10%; **6**, 15%; and **7**, 1%) had been formed. These results indicate that, in addition to our original observations,[2] piperidinolysis of both the Asp(OBzl)-containing compound **1** and Asu-peptide **2** led not only to transpeptidated compounds **4** and **5**, but also through base-catalysed epimerization to compound **6** and **7** as shown in Scheme 4. Since piperidinolysis of both compounds **1** and **2** led to the same ratios of products, and gave identical amounts of epimerized products **6** and **7**, we feel that our[1] and others' suggestions[7,8,15] are confirmed, *viz.* the very reactive and strained cyclic compound **2** (Scheme 4) is the key species leading to epimerization through a base-catalysed α-proton abstraction–enolization mechanism.[16] Simple racemization of starting materials and/or products, as well as of any ionized intermediates leading to and from cyclic compound **2**, can be experimentally and logically excluded.




**Scheme 4** *Reagent:* i, piperidine. The designations L, D refer to the centre marked with an asterisk; α, β refer to the type of peptide bond.

In accord with earlier observations[17] and assumptions,[2] formation of β-piperidides **5** and **7** is much less favoured. The very significant preponderance of α-piperidides **4** and **6** over the corresponding β-isomers may be due to additive effects from steric hindrance and electronic factors which promote attack of nucleophiles at the α-position. Relatively better accessibility by piperidine at the α-position in D-**2**, as compared with L-**2** to effect deprotonation yields a product ratio of 15:1 for the epimers **6** and **7**, whereas a ratio of 7.4:1 was observed for the unepimerized compounds **4** and **5**. In contrast, it is interesting that in ammonolyses of Asp(OBu$^t$)-peptides, normal (α) and isopeptides (β) are formed in nearly equal amounts and, in one case, there was a slight but definite preponderance in favour of the normal peptide.[1,3]

There is an added complication in the piperidinolytic experiments. Piperidinolysis of Boc-L- **8** and Boc-D-Asp(OBzl)-OH **9** was originally carried out in methanol (MeOH) with a nine-fold excess of piperidine with the aim of producing the dipeptides **5** and **7**, respectively. After 25 h, TLC showed that no starting material remained and the reaction mixture was worked up. The resulting compound was isolated, and subjected to a further series of reactions with final incorporation into various peptides. HPLC analysis of these model peptides showed an unexpected order of elution and unexplainable product ratios when compared with those formed with the corresponding amides as starting materials.[1] $^1$H NMR studies then revealed the problems in such an uncontrolled, mechanical approach and the products were actually shown to be Boc-L- **16** and Boc-D-Asp(OMe)-Phe-$NH_2$ **17** (Scheme 5). Repetition of the piperidinolysis of Boc-L-Asp(OBzl)-OH **8** in MeOH showed that it had been transformed into the corresponding β-methyl ester **18** and **19** (Scheme 6). Further slow conversion into piperidide **12** and **13** was observed only on prolonged heating to 60 °C. Attempted overnight piperidinolysis in EtOH yielded the corresponding ethyl ester in 10–20% yield but, in butan-1-ol, the butyl ester was formed only in trace amounts under similar




**Scheme 6** *Reagent:* i, piperidine. X = Piperidide.

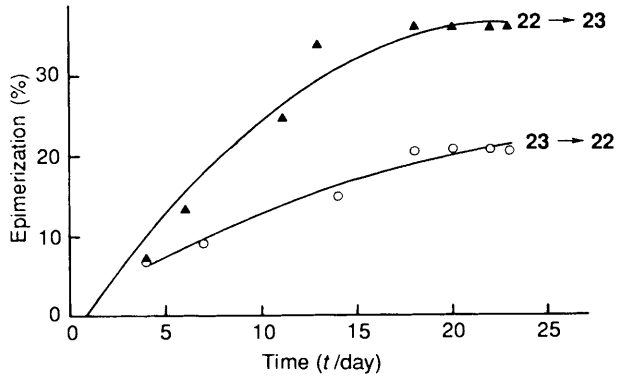
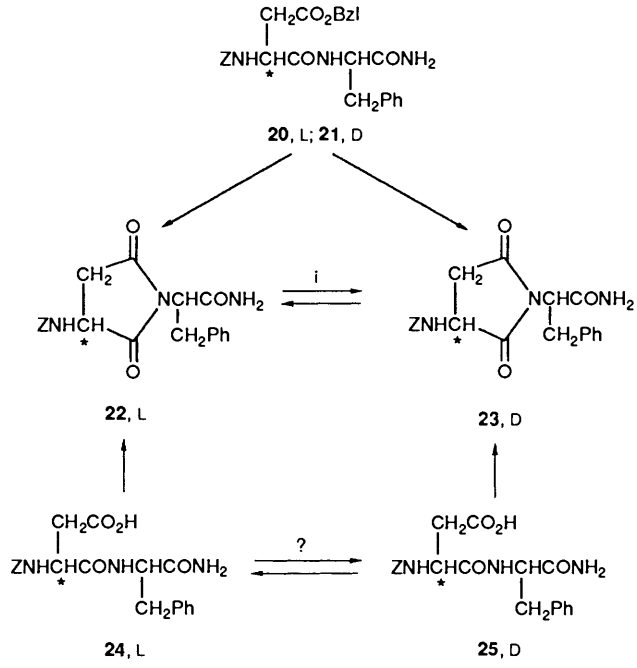



**Fig. 1** Epimerization of compounds **22** and **23** in 0.8% (w/v) solution in 0.1 mol $mol^{-1}$ $Et_3N$–DMF. Epimerization was followed by measuring the optical rotation.

conditions. Piperidinolysis in the dipolar-aprotic solvent DMF scarcely proceeded, even at 60 °C. Finally, piperidinolysis was accomplished in neat piperidine. Since overnight conversion at room temperature was slow, reaction mixtures were forced to completion at 60 °C in 40 h. These experiments can be explained simply by a piperidine-catalysed alcoholysis mechanism. Contribution of a push–pull mechanism cannot be excluded in the relatively fast reaction observed in MeOH. The observations are in good agreement with those reported for the ammonia-induced ring-opening reactions of Asu-peptides in similar solvents[1] as well as for the ammonia-catalysed methanolysis of ethyl prolinate and of peptides anchored to a polymer through Bzl-ester-type bonds.[18]

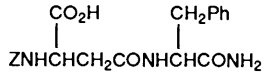
(b) *Racemization-free Preparation of Asu-peptides.*—To determine conditions for the racemization-free preparation of optically pure Asu-peptides and to examine their chiral stability, a multimodel approach was followed. At first, the thermal or triethylamine ($Et_3N$)-catalysed[19] cyclizations of Z-L-**20** and Z-D-Asp(OBzl)-Phe-$NH_2$ **21** to Z-Asu-Phe-$NH_2$ **22** and Z-D-Asu-Phe-$NH_2$ **23** were investigated and the ring closure of Z-L- **24** and Z-D-Asp-Phe-$NH_2$ **25** through various carboxyl-activation methods (Scheme 7). Dipeptides **20**, **21**, **24** and **25** were




**Scheme 7** *Reagent:* i, base. L, D Refer to the optically active centre marked with an asterisk.

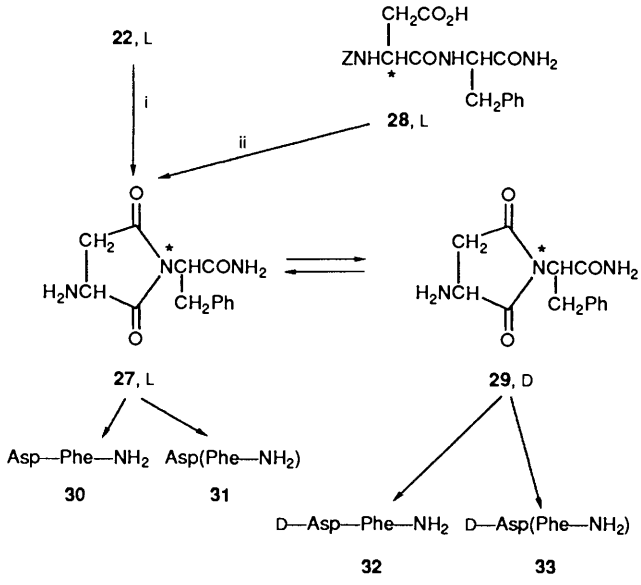
synthesized by conventional solution methods. Then a 10% (w/v) solution of the β-Bzl-ester **20** in DMF was heated at 60 °C for 10 days in the absence of $Et_3N$ to give the optically pure Asu-peptide **22**. No trace of any cyclized product was detected when solutions of the β-ester **20** in MeOH or EtOH were refluxed for 8 h. Similar treatment of the epimers **20** and **21** in the presence of a molar portion of $Et_3N$ remarkably yielded a mixture of the epimerized Asu-peptides **22** and **23**. Therefore (Scheme 7) base-catalysed cyclization of the Bzl-esters **20** and **21** gave a 35% yield of the D-isomer **23** and only a 20% yield of L-isomer **22**, respectively. The conventional mixed-anhydride activation of the Asp-peptide **24** with isobutyl chloroformate or with 6-chloro-2,4-dimethoxy-1,3,5-triazine[20] gave very complicated mixtures, which were not examined further. Reaction of compound **24** with *N*-hydroxysuccinimide–DCC over a period of 3 weeks yielded *ca.* a 10% yield of cyclized Asu-product. Best results were obtained with through-activation of compounds **24** and **25** with pentafluorophenol (HOPfp)–DCC (1:1 and 3:1)[21] and 1-hydroxybenzothiazole (HOBt)–DCC. Over a period of 2 weeks a less than 0.2% yield of epimerized products was formed. The chiral integrity of different batches was also checked by HPLC and by determination of the optical rotation. Fig. 1 shows the time dependence of epimerization of Asu-peptides **22** and **23** as 0.8% (w/v) solutions in 0.1 mol $dm^{-3}$ $Et_3N$–DMF (four-fold excess of $Et_3N$). This investigation was carried out for up to 23 days after which the solutions started to become coloured by (unidentified) decomposition; optical rotations were found to stabilize from the 18th day. It is interesting to note that the extent of epimerization (36%) of compound **22** is almost twice as much as that of the D-compound **23** (21%). These data are in good agreement with those (35% and 20%) obtained in HPLC studies.

(c) *Acidic Deprotection of Z-Asp-peptides.*—In 1979 we reported the formation of Asu-peptides by prolonged treatment of Asp(OBu$^t$)-peptides with strong acids.[22,23] Contrary to our earlier work[23] Sakakibara's standard HF deprotection procedure on Z-Asp-Phe-$NH_2$ **24** and Z-Asp(Phe-$NH_2$)-OH **26**



**26**

provided yields of only 0.4 and 0.3% for Asu-Phe-$NH_2$ **27**, respectively.



**Scheme 8** *Reagents:* i, $H_2$/Pd or HF; ii, HBr, AcOH

Samples of the Asu-peptide **27** were prepared in three different ways (Scheme 8). First, by catalytic hydrogenolysis of Z-Asu-Phe-$NH_2$ **22** in acetic acid (AcOH) for 2.5 h at room temperature. Second, by treatment of Z-Asu-Phe-$NH_2$ **22** with HF for 1 h at −2 °C and, third, by prolonged treatment of Z-Asp(OBu$^t$)-Phe-$NH_2$ **28** with HBr–AcOH at room temperature.[23] An isolated sample from the third procedure was kept in store for several months at room temperature. As no complete resolution of L-Asu-Phe-$NH_2$ **27** and D-Asu-Phe-$NH_2$ **29** by HPLC could be attained, uncertain estimation of the epimer content of these samples introduces similar uncertainty as to the exact source of racemization, which may arise from the procedure used for their preparation and/or from the work-up procedure or even from the HPLC technique. Nevertheless, to quantify the maximum epimer content, samples of the L-isomer **27** were hydrolysed with 1 mol dm$^{-3}$ aq. NaOH at room temperature for 30 s, and the epimer pairs of normal **30**/**32** and isopeptides **31**/**33** of Asp-Phe-$NH_2$ sequence (Scheme 8) were separated by HPLC. (These hydrolysis conditions may contribute to epimerization, as well.) The epimer content of these samples showed that D-Asp(Phe-$NH_2$) **33**, prepared through hydrolysis of samples obtained by hydrogenolysis or HF-acidolysis, amounted to 4.1 and 6.0%, respectively, whilst the ratio of Asp(Phe-$NH_2$) **31** to Asp-Phe-$NH_2$ **30** was about 7.5:2; the amount of D-Asp-Phe-$NH_2$ **32** was negligible. Furthermore it was apparent that AcOH also catalysed epimerization: a sample of Asu-Phe-$NH_2$ **27**, derived from hydrogenolysis of compound **22** in AcOH, was kept in AcOH at room temperature for 4 h, and at 37 °C for 1.2 h and was then hydrolysed with NaOH as above. This treatment led to an increase in D-epimer **33** from 4.1 to 9.3%. This catalytic effect of AcOH on epimerization is not unprecedented.[4]

Boc—Leu—Asu—Phe—$NH_2$ **34**

Boc—Leu—D—Asu—Phe—$NH_2$ **35**

Boc—Leu—Asp(OBzl)—Phe—$NH_2$ **36**

Boc—Leu—Asp(PheNH$_2$)—OBzl **37**

Boc—Glu(OBzl)—Asp(OBzl)—Ser—Gly—OBzl **38**

Boc—Glu(OBzl)—Asu—Ser—Gly—OBzl **39**

Boc—Glu(OBzl)—D—Asp(OBzl)—Ser—Gly—OBzl **40**

Boc—Glu(OBzl)—D—Asu—Ser—Gly—OBzl **41**

Further experiments showed that the above method for evaluating the extent of epimerization actually overestimates the true value. Syntheses of Boc-Leu-Asu-Phe-$NH_2$ **34** and its D-Asu-epimer **35** were accomplished in two ways, starting from the hydrobromide of Asu-Phe-$NH_2$ **27** (freshly prepared or stored for a long period) by the *N*-hydroxysuccinimide ester method, or by the *in situ* Pfp-ester method (see Experimental section), in the hydrogenolysis mixture of the protected Asu-dipeptide **22** and **23**, respectively. The values for the optical rotation of these samples of the Asu-tripeptide **34** varied over the range from −129.5° to −133.3° in DMF (within experimental error). HPLC analysis of the isolated products showed no evidence for the presence of the other epimer (the limit of detection was below 1%).

(d) *Synthesis of Other Peptides.*—Originally, Boc-peptide **34** was prepared from Boc-Leu-Asp(OBzl)-Phe-$NH_2$ **36** during a synthesis of a pentagastrin analogue.[10] Cyclization catalysed by $Et_3N$ was extremely slow (20% conversion in 7 months), whereas ring closure of Boc-Leu-Asp(Phe-$NH_2$)-OBzl **37** to give compound **34** reached 80% conversion in 2 days. Although these products **34** had the same optical rotation as those discussed in the previous paragraph, apparent yields as low as 42%, coupled with the much higher solubility of the D-epimer **35**, make it likely that the latter had been selectively removed during isolation. Therefore, because of such possible epimerization, the cyclization of the tripeptide **36** was examined at 50 mmol dm$^{-3}$ concentration in DMF in the absence and in the presence of 5 mol mol$^{-1}$ of $Et_3N$ at 60 °C. Surprisingly, both reactions were complete within 24 h. In the absence of $Et_3N$ no epimerization was detected but, in the presence of $Et_3N$, 25% of the D-epimer **35** was formed. These experiments clearly show a remarkable dependence of the rate of cyclization on temperature and illustrate the significant influence of $Et_3N$ on Asu-epimerization. Ring closure of Asp(OBzl)-peptides simply by recrystallization from hot EtOH has also been observed.[10,24]

During synthesis of Boc-Glu(OBzl)-Asp(OBzl)-Ser-Gly-OBzl **38** in DMF by the stepwise *N*-hydroxysuccinimide ester method, a significant quantity of a by-product was obtained, the amount of which increased even further during attempted purification by column chromatography. The well known problem of an Asp-Ser sequence[18] suggested that a ring-closure reaction had occurred. Pure tetrapeptide **38** was obtained from dichloromethane by the stepwise DCC method. In model experiments, the rate of cyclization of this tetrapeptide **38** to give Boc-Glu(OBzl)-Asu-Ser-Gly-OBzl **39** under different conditions was examined. At the same time, a search was made for the formation of the D-Asu-epimer **41**, an authentic sample of which was directly synthesized from Boc-Glu(OBzl)-D-Asp(OBzl)-Ser-Gly-OBzl **40**. Hence the tetrapeptide **38** as a 1% (w/v) solution in chloroform, ethyl acetate (AcOEt), or 1,4-dioxane does not rearrange to the Asu-peptide **39** either at room

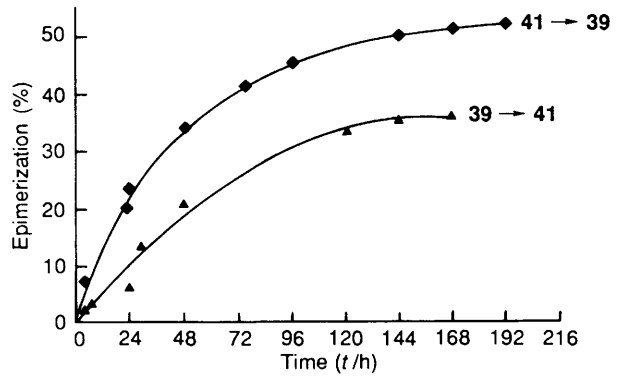



**Fig. 2** Epimerization of compounds **39** and **41** in 1% (w/v) $CHCl_3$ solution by the effect of 1 mol $mol^{-1}$ $Et_3N$. Epimerization was followed by HPLC.

temperature or at 60 °C in 24 h. However, in neat DMF at room temperature, 35 and 97.5% conversions of the tetrapeptide **38** into the Asu-peptide **39** were observed in 2 and 8 h, respectively. None of the D-epimer **41** was detected even after 24 h. In contrast to the experiments in $CHCl_3$ in the absence of base, a 41% conversion of the tetrapeptide **38** was observed in the presence of $Et_3N$ at room temperature in 24 h, and HPLC analysis showed that 39% of the Asu-peptide **39** and 2% of the D-epimer **41** had been formed. Whilst cyclization of tetrapeptide **38** in $CHCl_3$ was complete in 1 week in the presence of $Et_3N$, in DMF it required only 6 h. In DMF the equilibrium ratio **39**:**41** was 21:12. Fig. 2 shows the course of this epimerization. The rate of epimerization of the D-epimer **41** exceeded that of Asu-peptide **39**. Theoretically, at equilibrium, the same product ratio can be expected for both reaction mixtures but this result was not achieved because of decomposition. For this same reason, for the epimerization of the D-epimer **41**, the amount of Asu-peptide **39** exceeded 50% from 150 h onwards.

(e) *Syntheses of Gonadoliberin Analogues.*—Other examples of similar transformations were observed in the syntheses of gonadoliberin (GnRH) analogues **42**–**46**. Because of the higher biological potency of [6-isoAsp-OR]-GnRH(1–9)-ethylamides **42** and **43** over those of some α-amides, Seprödi assumed [25–28]

Glp—His—Trp—Ser—Tyr—Gly—Leu—Arg—Pro—Gly—$NH_2$

(GnRH)

Glp—His—Trp—Ser—Tyr—Asp(—Leu—Arg—Pro—NHEt)—OR

**42**, R = Me; **43**, R = Bzl

Glp—His—Trp—Ser—Tyr—X—Leu—Arg—Pro—NHEt

**44**, X = L-Asu; **46**, X = D-Asu

Glp—His—Trp—Ser—Tyr—Asp(OMe)—Leu—Arg—Pro—NHEt

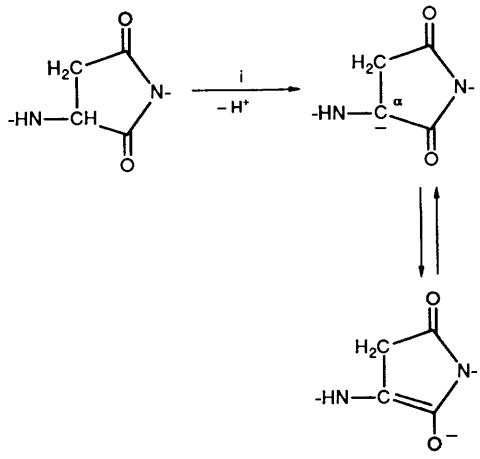
**45**

Boc—X—Leu—Arg—Pro—NHEt

X

**47** L-Asu
**48** L-Asp(OBzl)
**49** D-Asu
**50** D-Asp(OBzl)
**51** L-Asp

that this difference might come from the ease of cyclization of esters to yield [6-Asu]-GnRH(1–9)-ethylamide **44** as the species responsible for eliciting the biological effects. In a first synthesis of the nonapeptide **44**, the ring closure of [6-Asp(OMe)]-GnRH(1–9)-ethylamide **45** was carried out in the presence of a




**Scheme 9** *Reagent:* i, base

very large excess of $Et_3N$,[12] and HPLC revealed a large epimer content for species **46** in the resulting Asu-derivative.[29]

In an attempt to obtain the peptide **44** free from its epimer **46**, a 5 + 4 condensation was carried out with a *C*-terminal preformed-Asu-containing segment. Therefore, an epimerization-free synthesis was required for the 4-segment, Boc-Asu-Leu-Arg-Pro-NHEt **47**, from Boc-Asp(OBzl)-Leu-Arg-Pro-NHEt **48**.[30] Ring closure of the hydrochloride of compound **48** in DMF with $Et_3N$ at room temperature was followed by TLC. Conversions of 15, 70 and 90% were estimated after 3.5, 23 and 49 h. After complete cyclization, HPLC analyses after 69, 130 and 672 h showed the presence of 8, 10 and 18% of the epimer, Boc-D-Asu-Leu-Arg-Pro-NHEt **49**, respectively. Treatment of the hydrochloride of Boc-D-Asp(OBzl)-Leu-Arg-Pro-NHEt **50** with $Et_3N$ was accomplished under similar conditions in only 5 days, yielding the pure D-Asu-peptide **49**, due to its fortunate insolubility in DMF. Contrary to expectation, no cyclization of the hydrochloride of the L-peptide **48** occurred in DMF at room temperature after 24 h, whilst at 60 °C for 70 h, a 95% conversion into compound **47** was found together with the formation of 7.9% of D-Asu-peptide **49**. Surprisingly, a sample of the peptide **48**, purified by reversed-phase medium-pressure liquid chromatography (MPLC), using a mixture of propan-2-ol and aq. AcOH (10% v/v) for elution, was found to cyclize in 6 h at 60 °C, with 39% epimerization. Differences in the rates of ring closure and epimerization of the two samples of compound **38** were due probably to dissimilar counter-ions. In the second sample, acetate ion (being a stronger base) may contribute to proton abstractions from both peptidic NH and α-CH moieties.

For the preparation of epimer-free compound **47**, the tetrapeptide **48** was catalytically hydrogenolysed to give Boc-Asp-Leu-Arg-Pro-NHEt **51**. Table 1 presents the conditions and results of attempted cyclization to the Asu peptide **49**. Reactions were carried out in DMF with various carbodiimides and additives. Best results were obtained with the usual *in situ* 1-hydroxybenzotriazolyl and Pfp-ester procedures. Complete cyclizations without epimerization were detected after 26 h.

Several conclusions can be drawn from our results. In accordance with Clarke's suggestion,[7,8] we have unambiguously confirmed the propensity to racemization of the aspartimidyl residue under the usual conditions for chemical syntheses of peptides. This tendency to racemization of the five-membered succinimidyl ring may be similar to that of the well known oxazol-5(4*H*)-ones, 2,5-dioxopiperazines, and the pyroglutamyl group,[6,31] and may be due to charge delocalization on the α-carbon atom after deprotonation (Scheme 9). We have shown that experimental conditions, including solvent, temperature, and the presence of the non-nucleophilic base $Et_3N$, influence the rate of both cyclization and epimerization. Chirality and the

**Table 1** Cyclization of Boc-Asp-Leu-Arg(·AcOH)-Pro-NHEt, the acetate of compound **51**, in DMF[a,b] (method K: in water)

| Method | Reagent[c] | Additive (mol) | Reaction time (*t*/h) | Conversion (%) |
|---|---|---|---|---|
| A | DIC | HOBt (3) | 3.3 | 70 |
| | | | 26 | 100 |
| B | DIC | HOBt (3) | 0.66 | 80 |
| | | | 3.3 | 100[d] |
| C | DIC | HOBt (9) | 3.3 | 50 |
| | | | 26 | 98 |
| D | DIC | HOPfp (3) | 3.3 | 50 |
| | | | 26 | 100[d] |
| E | DIC | HOSu (3) | 3.3 | 2 |
| | | | 26 | 4 |
| F | DIC | none | 3.3 | 0 |
| | | | 26 | 0 |
| G | DCC | HOBt (3) | 3.3 | 60 |
| | | | 26 | 100[d] |
| H | DIC | HONp (3) | 3 | 1 |
| | | | 20 | 3 |
| I | EDC | HOBt (3) | 3 | 80 |
| | | | 20 | 99 |
| J | EDC | HOSu (3) | 3 | 10 |
| | | | 20 | 2 |
| K | EDC | HOBt (3) | 3 | 50 |
| | | | 20 | 10 |

[a] Conversion determined by TLC and epimerization tested by HPLC. [b] All experiments were carried out at room temperature, except for method B which was run at 45 °C. [c] 3 mol $mol^{-1}$ peptide, DIC = diisopropylcarbodiimide, EDC = *N*-ethyl-N'-[3-(dimethylamino)propyl]carbodiimide hydrochloride; [d] No epimerization detected.

neighbouring environment of aspartyl and/or aspartimidyl residues affect reactivities under different conditions. Differences in reactivity of epimeric compounds are probably related to steric factors. X-Ray analyses of peptides **22**[32] and **34**[33] have been reported recently. However, up to now, preparation of appropriate crystals of epimeric compounds **23** and **35** has not been achieved. Attention may be drawn to the increased sensitivity to racemization of an aspartimidyl ring in the presence of an arginine (guanidinium) group because the basicity of its counter-ion may substantially influence any reactions where reversible deprotonation/protonation occurs; this effect has been demonstrated for Asp(OBu$^t$)-OBu$^t$-residue.[34] Both base catalysis and the presence of arginine in a hypoglycaemic nonapeptide segment[35] of human growth hormone may contribute to a $^1$H NMR observation[36] that the α-NH proton of the Asu-residue had 'two chemical environments occupied in approximately equivalent amounts.'

*Conclusions.*—An ever increasing number of experimental proofs are combining to demonstrate the remarkable role of aspartate racemization in protein degradation,[37] thermoinactivation of enzymes,[38] ageing processes,[39] and even in Alzheimer's disease.[40] The present work supports these observations through appraisal of the more important parameters leading to racemization at aspartate residues.

## Experimental

M.p.s were taken on a Tottoli (Büchi) apparatus and are uncorrected. Optical rotations were determined at room temperature with a Perkin-Elmer 141 automatic photoelectric polarimeter. IR spectra were recorded as KBr disks on a Perkin-Elmer 257 IR spectrophotometer. NMR spectra were obtained on either a Varian EM 360 (60 MHz) or a Varian VXR300 (300 MHz) instrument, using $Me_4Si$ as internal reference. *J*-Values are given in Hz. TLC tests were run on precoated silica gel plates (Merck), using the appropriate solvent systems, of which some were made by mixing AcOEt and a stock solution of pyridine–AcOH–water (20:6:11) in the following ratios: (1) AcOEt–stock (99:1); (2) AcOEt–stock (19:1); (3) AcOEt –stock (9:1); (4) AcOEt–stock (4:1); (5) AcOEt–stock (3:2); (6) BuOH–stock (3:2). Other solvent systems were (7) $CHCl_3$–MeOH, (19:5); (8) $CHCl_3$–MeOH (9:1); (9) Pr$^i$OH–aq. 1 mol $dm^{-3}$ AcOH, (2:1). $R_f$-Values in these solvent systems are indicated as $R_f^n$ where *n* is the number corresponding to the solvent mixtures described here.

The chromatograms were developed by spraying of the plates with ninhydrin and then with *o*-toluidine–KI, after chlorination. For amino acid analysis, samples were hydrolysed in 6 mol $dm^{-3}$ HCl at 110 °C for 24 h. The ratio of amino acids was determined on a Biotronik LC 5001 instrument, using a 95 min automatic program. If not otherwise stated, analytical HPLC analyses were performed on a chromatograph consisting of Altex 110A pump, LABOR MIM Liquodet 308 (used at 210–225 nm) and Altex detector (used at 254 nm), Rheodyne 7125 loop injector (10 $mm^3$), and an MTA KUTESZ 175 multichannel recorder. Samples were analysed on a BST-SI-100-S $C_{18}$ column, 10 μm, 250 × 4.6 mm ID (BioSeparation Techn., Budapest). The mobile phases consisted of acetonitrile–trifluoroacetic acid (TFA)–0.001 mol $dm^{-3}$ ammonium acetate (pH 4.5) in the proportions 14.0–16.5:0.5:85.5–83.0. The chromatograph was operated isocratically at ambient temperature at a flow rate of 1.0 $cm^3$ $min^{-1}$. Reversed-phase MPLC was performed using a 400 × 25 mm ID column packed with Whatman LRP-1 ($C_{18}$; 13–24 μm), and 3 $cm^3$ $min^{-1}$ flow rate. Light petroleum refers to the fraction boiling in the range 40–60 °C.

Known amino acid derivatives were generally synthesized using literature procedures, with or without slight modification. Most of the reaction yields were not optimized and reported values are only indicative.

*Synthesis of Piperidides* **4** *and* **6**: *General Procedure.*—To a solution of Boc-Asp(OBzl)-OH **8** (3.23 g, 10 mmol) and HOPfp (1.84 g, 10 mmol) in AcOEt (25 $cm^3$) at 0 °C was added DCC (2.06 g, 10 mmol). The usual work-up[14] gave *Boc-Asp(OBzl)-OPfp* (4.35 g, 88.8%), m.p. 80–81 °C (from diethyl ether–light petroleum) (Found: C, 53.8; H, 4.15; N, 2.9. $C_{22}H_{20}F_5NO_6$ requires C, 54.0; H, 4.1; N, 2.9%); $R_f^8$ 0.85; $[\alpha]_D$ −19.5° (*c* 1, dioxane).

Piperidine (0.97 $cm^3$, 3.8 mmol) was added to a solution of this active ester (2.4 g, 4.9 mmol) in DMF (10 $cm^3$). After 3 h the solvent was removed. The residue was dissolved in $CHCl_3$ (50 $cm^3$) and the solution was washed successively with aq. HCl (1 mol $dm^{-3}$; 3 × 10 $cm^3$), aq. $NaHCO_3$ (5% w/v; 3 × 10 $cm^3$), and water (10 $cm^3$), and dried ($Na_2SO_4$). The solvent was removed, and the resulting oily Boc-Asp(Bzl)-piperidide ($R_f^8$ 0.85; $R_f^3$ 0.75) was catalytically hydrogenolysed in MeOH (50 $cm^3$) in the usual manner. The catalyst was filtered off and the solvent was evaporated off. To a solution of the residual oil in diethyl ether (20 $cm^3$) was added DCHA (1.0 $cm^3$, 5 mmol). The suspension was filtered to give a residue, which was twice recrystallized from AcOEt–light petroleum to yield Boc-Asp-piperidide·DCHA (1.6 g, 68%), m.p. 133–134 °C; $R_f^3$ 0.5; for DCHA: $R_f^3$ 0.05; $[\alpha]_D$ −9.8° (*c* 1, EtOH).

A suspension of this salt (1.4 g, 2.9 mmol) in diethyl ether (20 $cm^3$) was shaken with aq. $H_2SO_4$ (1 mol $dm^{-3}$; 10 $cm^3$) until it has dissolved. The organic layer was separated, washed with water (10 $cm^3$) and then evaporated to afford an oily residue, which was converted into its pentafluorophenyl ester **10** in the usual way but this was not isolated. To a solution of the ester **10** in DMF (10 $cm^3$) were added successively H-Phe-$NH_2$·HCl (0.44 g, 2.2 mmol) and $Et_3N$ (0.31 $cm^3$, 2.2 mmol). After 2 h, the solvent was removed and a solution of the residue in $CHCl_3$ (20 $cm^3$) was washed in the usual way. AcOEt was substituted for the $CHCl_3$ solvent, when some residual dicyclohexylurea could

be filtered off. Addition of diisopropyl ether precipitated Boc-Asp(Phe-$NH_2$)-piperidide **4** (0.52 g), which was recrystallized from $Pr^iOH$–diisopropyl ether (0.45 g) and then purified by flash column chromatography on silica gel (30 g) with a (2:23) mixture of AcOEt and the stock solution (see above) to give *Boc-Asp(Phe-$NH_2$)-piperidide* **4** (0.29 g, 22%), m.p. 131–132 °C (Found: C, 61.9; H, 7.8; N, 12.65. $C_{23}H_{34}N_4O_5$ requires C, 61.9, H, 7.7, N. 12.55%); $R_f$ [3] 0.5, $R_f$ [8] 0.4; $[\alpha]_D$ −42.5° (*c* 1, DMF); $\delta_H$[60 MHz; $CDCl_3$–$(CD_3)_2SO$] 1.43 (9 H, s, Bu$^t$), 1.54 (6 H, br, 3 × $CCH_2$), 2.5 (2 H, d, Asp-C$H_2$), 3.05 (2 H, m, Phe-C$H_2$), 3.42 (4 H, br, 2 × $NCH_2$), 4.2–5.0 (2 H, m, $CH_2$), 6.6 (2 H, d, $NH_2$, exchangeable with $D_2O$), 7.16 (5 H, s, ArH) and 8.96 (2 H, d, NH, exchangeable).

*Boc*-D-*Asp(Phe-$NH_2$)-piperidide* **6**.—This was prepared similarly to the piperidide **4**. Partial characterization of intermediates: D-Asp(OBzl), m.p. 199–201 °C; $R_f$ [5] 0.25; Boc-D-Asp(OBzl)-OH·DCHA **9**, m.p. 128–129 °C; $R_f$ [3] 0.5; Boc-D-Asp(OBzl)-OPfp (yield 87%), m.p. 76–78 °C; $R_f$ [7] 0.8; $[\alpha]_D$ +20.0° (*c* 1, dioxane); Boc-D-Asp-piperidide·DCHA (yield 66%), m.p. 133–134 °C; $R_f$ [3] 0.5; $[\alpha]_D$ +10.3° (*c* 1, EtOH). Boc-D-Asp(OPfp)-piperidide **11** was not characterized. *Boc*-D-*Asp(Phe-$NH_2$)-piperidide* **6** (yield 30%), m.p. 126–128 °C (from AcOEt–diisopropyl ether) (Found: C, 61.9; H, 7.65; N, 12.4. $C_{23}H_{34}N_4O_5$ requires C, 61.9; H, 7.7; N, 12.55%); $R_f$ [3] 0.5, $R_f$ [8] 0.45; $[\alpha]_D$ +6.8° (*c* 1, DMF); $\delta_H$[60 MHz; $CDCl_3$–$(CD_3)_2SO$] 1.42 (9 H, s, Bu$^t$), 1.54 (6 H, br, 3 × $CCH_2$), 2.4 (2 H, d, Asp-C$H_2$), 3.03 (2 H, m, Phe-C$H_2$), 3.4 (4 H, br, 2 × $NCH_2$), 4.3–4.9 (2 H, m, 2 × CH), 6.6 (2 H, d, $NH_2$, exchangeable), 7.2 (5 H, s, ArH) and 7.82 (2 H, d, 2 × NH, exchangeable).

*Synthesis of Piperidides* **5** *and* **7**: *General Procedure*.—Boc-Asp(OBzl)-OH **8** (3.23 g, 10 mmol) was treated with piperidine (25 $cm^3$) at 60 °C for 1 week, when the reaction mixture was evaporated, and a solution of the residue in AcOEt was washed in the usual way. The solvent was removed, and to a solution of the residual oil in diethyl ether (15 $cm^3$), was added DCHA (2 $cm^3$, 10 mmol) to give Boc-Asp(piperidide)-OH·DCHA **12** (3.5 g, 72.5%), m.p. 126–127 °C; $[\alpha]_D$ +16.3° (*c* 1, EtOH); $R_f$ [3] 0.3. This salt was liberated, then converted into Boc-Asp-(piperidide)-OPfp **14** (not characterized) in the usual way. The active ester **14** was coupled with H-Phe-$NH_2$·HCl in the usual way to yield the *dipeptide* **5**, m.p. 98–100 °C (from AcOEt) (Found: C, 61.85; H, 7.8; N, 12.65. $C_{23}H_{34}N_4O_5$ requires C, 61.9; H, 7.7; N, 12.55%); $R_f$ [3] 0.45, $R_f$ [8] 0.4; $[\alpha]_D$ −21.4° (*c* 1, DMF).

For Boc-D-Asp(piperidide)-Phe-$NH_2$ **7**, the partial characterization of intermediates was as follows: Boc-D-Asp(piperidide)-OH·DCHA **13** (41.5%), m.p. 124–125 °C (from AcOEt–diethyl ether); $R_f$ [3] 0.3; $[\alpha]_D$ −17.0° (*c* 1, EtOH). This salt was transformed into the active ester **15** in the usual way and the latter was coupled with H-Phe-$NH_2$·HCl to give amorphous *compound* **7** (Found: C, 61.75; H, 7.7; N, 12.45. $C_{23}H_{34}N_4O_5$ requires C, 61.9; H, 7.7; N, 12.55%); $R_f$ [3] 0.5, $R_f$ [8] 0.45; $[\alpha]_D$ −19.6° (*c* 1, DMF).

*Unwanted Synthesis of* β-*Methyl Esters* **16** *and* **17**.—A solution of Boc-Asp(OBzl)-OH **8** (3.23 g, 10 mmol) in MeOH (25 $cm^3$) was treated with piperidine (10 $cm^3$) at room temperature. After 3 days the reaction mixture was evaporated, and a solution of the residue in AcOEt was washed successively with aq. HCl (1 mol $dm^{-3}$) and water, and then dried and evaporated. To a solution of the resulting oil in diethyl ether (15 $cm^3$) was added DCHA (10 mmol). Boc-Asp(OMe)-OH·DCHA **18** was isolated in the usual way, m.p. 154–155 °C (from AcOEt); $R_f$ [3] 0.45; $[\alpha]_D$ +19.5° (*c* 1, EtOH).

Overnight reactions in EtOH and BuOH resulted in the appropriate ethyl (10–20%; $R_f$ [3] 0.50) and butyl ester (traces; $R_f$ [3] 0.55), respectively.

Partial characterization of other intermediates: Boc-D-Asp-(OMe)-OH·DCHA **19** (45.6%), m.p. 153–154 °C (from AcOEt); $R_f$ [3] 0.45; $[\alpha]_D$ −20.2° (*c* 1, EtOH).

*Boc-Asp(OMe)-Phe-$NH_2$* **16**.—This was prepared from Boc-Asp(OMe)-OPfp ($R_f$ [3] 0.9, not isolated) and H-Phe-$NH_2$·HCl in the usual way (69%), m.p. 169–171 °C (from EtOH) (Found: C, 58.2; H, 6.95; N, 10.6. $C_{19}H_{27}N_3O_6$ requires C, 58.0; H, 6.9; N, 10.7%); $R_f$ [3] 0.55, $R_f$ [8] 0.35; $[\alpha]_D$ −39.7° (*c* 1, DMF); $\delta_H$[60 MHz; $CDCl_3$–$(CD_3)_2SO$] 1.4 (9 H, s, Bu$^t$), 2.6 (2 H, m, Asp-C$H_2$), 3.0 (2 H, m, Phe-C$H_2$), 3.6 (3 H, s, Me), 4.1–4.7 (2 H, m, 2 × CH), 6.8 (2 H, d, $NH_2$, exchangeable), 7.2 (5 H, s, ArH) and 7.65 (2 H, br, 2 × NH, exchangeable).

*Boc*-D-*Asp(OMe)-Phe-$NH_2$* **17**.—Prepared similarly to compound **16** and purified by column chromatography with solvent system 2 (37%), m.p. 115–116 °C (from AcOEt–diisopropyl ether) (Found: C, 58.15; H, 7.05; N, 10.5. $C_{19}H_{27}N_3O_6$ requires C, 58.0; H, 6.9; N, 10.7%); $R_f$ [3] 0.50; $[\alpha]_D$ +8.1° (*c* 1, DMF); $\delta_H$[60 MHz; $CDCl_3$–$(CD_3)_2SO$] 1.43 (9 H, s, Bu$^t$), 2.6 (2 H, m, Asp-C$H_2$), 3.03 (2 H, m, Phe-$NH_2$), 3.58 (3 H, s, Me), 4.0–4.7 (2 H, m, 2 × CH), 6.9 (2 H, d, $NH_2$, exchangeable), 7.2 (5 H, s, ArH) and 7.8 (2 H, br, 2 × NH, exchangeable).

*Piperidinolyses of Compounds* **1** *and* **2**.—Either compound **1** (1 mmol) or compound **2** (1 mmol) was treated with a 55% (v/v) mixture of piperidine and DMF, as previously reported.[2] After 2 days, the reaction mixture was evaporated, and the residue was solidified by trituration with diisopropyl ether; composition of the residue was followed by HPLC analysis, using a 250 × 4 mm ID column (Bio Separation Technology, Budapest, Hungary), packed with 10 μm LiChrosorb RP-18 (Merck). Samples (20 $mm^3$) of 1 mg $cm^{-3}$ solutions in DMF were injected. A 7:18 mixture of acetonitrile–aq. $Na_2SO_4$ (5 mmol $dm^{-3}$) containing D-camphorsulphonic acid (0.5 mmol $dm^{-3}$) was applied with a flow rate of 1 $cm^3$ $min^{-1}$. Formation of piperidides **4** (74%), **5** (10%), **6** (15%) and **7** (1%) was calculated from peak-area normalization.

*Z-Asp(OBzl)-Phe-$NH_2$* **20**.—Z-Asp(OBzl)-OPfp was prepared in the usual way from Z-Asp(OBzl)-OH (92%), m.p. 95–96 °C (from AcOEt–light petroleum); $[\alpha]_D$ −3.5° (*c* 1, dioxane). This active ester and H-Phe-$NH_2$·HCl were allowed to react in DMF in the presence of $Et_3N$ in the usual way to give the *dipeptide* **20** (82%), m.p. 169–170 °C (from EtOH) (Found: C, 66.9; H, 5.9; N, 8.25. $C_{28}H_{29}N_3O_6$ requires C, 66.8; H, 5.8; N, 8.3%); $R_f$ [4] 0.80; $R_f$ [8] 0.60; $[\alpha]_D$ −23.5° (*c* 1, DMF), $[\alpha]_D$ −12.6° [*c* 1, dimethyl sulphoxide (DMSO)], $[\alpha]_D$ −8.8° (*c* 1, 1,1,1,3,3,3-hexafluoropropan-2-ol).

*Z*-D-*Asp(OBzl)-Phe-$NH_2$* **21**.—Z-D-Asp(OBzl)-OPfp was prepared in the usual way (89.6%), m.p. 92–93 °C (from AcOEt–light petroleum). This active ester was coupled with H-Phe-$NH_2$·HCl in the presence of $Et_3N$ in DMF in the usual way to give the *dipeptide* **21** (76.5%), m.p. 166–167 °C (from AcOEt) (Found: C, 66.65; H, 5.9; N, 8.45%); $R_f$ [8] 0.60; $[\alpha]_D$ 0° (*c* 1, DMF, DMSO, or hexafluoropropan-2-ol).

*Z-Asp-Phe-$NH_2$* **24**.—Z-Asp(OBu$^t$)-Phe-$NH_2$ **28**[41] (8.45 g, 18 mmol) was treated with HCl (4 mol $dm^{-3}$) in AcOH at room temperature for 15 min. The reaction mixture was evaporated, and the crude product was triturated with diethyl ether and then boiled with ethanol to give the *dipeptide* **24** (7.1 g, 95.4%), m.p. 218–219 °C (Found: C, 61.2; H, 5.6; N, 10.1. $C_{21}H_{23}N_3O_6$ requires C, 61.0; H, 5.6; N, 10.2%); $R_f$ [4] 0.20; $[\alpha]_D$ −38.6° (*c* 1, DMF).

*Z*-D-*Asp-Phe-$NH_2$* **25**.—Z-D-Asp(OBu$^t$)-Phe-$NH_2$ was syn-

thesized as described for compound **28** in ref. 41 (83.7%); m.p. 146–147 °C (from $Pr^iOH$); $R_f$[3] 0.65; $[\alpha]_D$ +9.85° (*c* 1, EtOH). The *dipeptide* **25** was prepared from Z-D-Asp(OBu$^t$)-Phe-$NH_2$ as described for compound **24** (86%); m.p. 176–178 °C (from MeOH) (Found: C, 61.15; H, 5.7; N, 10.35%); $R_f$[4] 0.20; $[\alpha]_D$ +7.3° (*c* 1, DMF).

*Z-Asp(Phe-$NH_2$)-OH* **26**.—Z-Asp(OSu)-OBu$^t$ was prepared from Z-Asp-OMe·DCHA[42] in the usual way (92%); m.p. 100–102 °C (from $Pr^iOH$–diisopropyl ether). The usual coupling of this active ester with H-Phe-$NH_2$·HCl resulted in Z-Asp(Phe-$NH_2$)-OBu$^t$ (74.3%), m.p. 162–165 °C; $R_f$[4] 0.80. This fully protected dipeptide was treated with HCl (4 mol $dm^{-3}$) in AcOH to afford *compound* **26** (75.5%), m.p. 192–193 °C [from EtOH–water (1:1)] (Found: C, 61.3; H, 5.75; N, 10.2. $C_{21}H_{23}N_3O_6$ requires C, 61.0; H, 5.6; N, 10.2%); $R_f$[4] 0.20; $[\alpha]_D$ −6.7° (*c* 1, DMF).

*Z-Asu-Phe-$NH_2$* **22**.—To an ice-cooled solution of compound **18** (0.47 g, 1 mmol) and HOPfp (0.19 g, 1 mmol) in DMF (5 $cm^3$) was added DCC (0.22 g, 1.05 mmol). The reaction mixture was kept at room temperature for 12 days and was then filtered and evaporated. A solution of the residue in chloroform was successively washed with aq. HCl (1 mol $dm^3$; 3×) and aq. $NaHCO_3$ (5% w/v; 5×) and was then dried and evaporated. Recrystallization of the residue from MeOH or EtOH gave the Asu-peptide **22** (0.25 g, 63.3%), m.p. 175–177 °C (Found: C, 63.8; H, 5.45; N, 10.5. $C_{21}H_{21}N_3O_5$ requires C, 63.8; H, 5.35; N, 10.6%); $R_f$[7] 0.40; $R_f$[8] 0.55; $[\alpha]_D$ −139.5° (*c* 1, DMF); $\delta_H$[300 MHz; $(CD_3)_2SO$] 2.47 (1 H, dd, $^3J_{\alpha\beta}$ 6.2, $^2J_{\beta\beta}$ 17.5, Asu-H$^\beta$), 2.95 (1 H, dd, $^3J_{\alpha\beta}$ 9.3, $^2J_{\beta\beta}$ 17.5, Asu-H$^{\beta'}$), 3.07 (1 H, dd, $^3J_{\alpha\beta}$ 10.8, $^2J_{\beta\beta}$ 13.9, Phe-H$^\beta$), 3.43 (1 H, dd, $^3J_{\alpha\beta}$ 4.1, $^2J_{\beta\beta}$ 13.9, Phe-H$^{\beta'}$), 4.36 (1 H, m, Asu-H$^\alpha$), 4.63 (1 H, dd, $^3J_{\alpha\beta}$ 4.1 and 10.8, Phe-H$^\alpha$), 5.07 (2 H, s, Z-C$H_2$), 7.13–7.42 (10 H, m, ArH) and 8.3 (1 H, d $^3J_{H^\alpha NH}$ 8.1, Asu-N*H*); $\delta_C$ 32.95 (Phe-C$^\beta$), 34.58 (Asu-C$^\beta$), 49.14 (Asu-C$^\alpha$), 54.55 (Phe-C$^\alpha$), 126.23 and 127.91 (Phe-C-4 and Z-C-4), 127.85, 128.18, 128.31 and 128.83 (aromatic C-2, -3, -5 and -6), 136.39 and 137.94 (aromatic C-1), 155.84 (Z-CO), 168.95 (Phe-CO) and 173.73 and 175.56 (2 × Asu-CO).

The same Asu-dipeptide **22**, with identical physical constants to these, was synthesized by the usual HOBt–DCC and HOPfp–DCC (3:1) (complex F)[21] methods. HPLC analysis of these three reaction mixtures showed no evidence for the epimer **23**.

*Z-D-Asu-Phe-$NH_2$* **23**.—This was prepared from the D-isomer **25** in the same way as above for compound **22**, from dipeptide **24** (50.5%), m.p. 135–136 °C (from AcOEt) (Found: C, 64.0; H, 5.5; N, 10.45%); $R_f$[3] 0.75; $R_f$[8] 0.50; $[\alpha]_D$ −86.7° (*c* 1, DMF); $\delta_H$[300 MHz; $(CD_3)_2SO$] 2.57 (1 H, dd, $^3J_{\alpha\beta}$ 5.6, $^2J_{\beta\beta}$ 17.8, Asu-H$^\beta$), 2.90 (1 H, dd, $^3J_{\alpha\beta}$ 9.4, $^2J_{\beta\beta}$ 17.8, Asu-H$^{\beta'}$), 3.10 (1 H, dd, $^3J_{\alpha\beta}$ 10.2, $^2J_{\beta\beta}$ 14.2, Phe-H$^\beta$), 3.46 (1 H, dd, $^3J_{\alpha\beta}$ 4.9, $^2J_{\beta\beta}$ 14.2, Phe-H$^{\beta'}$), 4.44 (1 H, m, Asu-H$^\alpha$), 4.65 (1 H, dd, $^3J_{\alpha\beta}$ 4.9 and 10.2, Phe-H$^\alpha$), 5.04 (2 H, s, Z-C$H_2$), 7.08–7.44 (10 H, m, ArH) and 7.94 (1 H, d, $^3J_{H^\alpha NH}$ 8.1, Asu-N*H*); $\delta_C$ 33.00 (Phe-C$^\beta$), 34.48 (Asu-C$^\beta$), 49.01 (Asu-C$^\alpha$), 54.58 (Phe-C$^\alpha$), 67.87 (Z-$CH_2$), 126.25 and 127.92 (aromatic C-4), 127.85, 128.25, 128.32 and 128.60 (aromatic C-2, -3, -5 and -6), 136.38 and 137.86 (aromatic C-1), 155.85 (Z-CO), 169.04 (Phe-CO) and 173.99 and 175.72 (2 × Asu-CO). None of the usual HOPfp–DCC, HOBt–DCC and complex F reactions were accompanied by detectable epimerization, *i.e.* no formation of epimer **22** was observed.

*Cyclization of Bzl-esters* **20** *and* **21**.—Solutions of esters **20** and **21** (0.50 g, 1 mmol) in DMF (5 $cm^3$) were kept at 60 °C for 10 days; >90% conversions were detected by both TLC and HPLC after 48 h. Solvent was removed and the residues were each recrystallized from the appropriate solvent to give Asu-peptides **22** and **23**, respectively. Samples **22** and **23** possessed the expected physical values and were shown to be enantiomerically pure by HPLC.

These reactions were repeated in the presence of $Et_3N$ (1 mol $dm^{-3}$). After 24 h at room temperature, 95% cyclization was observed and cyclization became complete after 30 h at 60 °C, as detected by TLC. Analysis (HPLC) showed the presence of different amounts of epimers in all samples, but these data will not be reported. The reaction mixtures were kept at room temperature for more than 4 weeks, by which time there was 35% of the D-Asu-peptide **23** in the cyclization mixture from the L-Bzl-ester **20**, and 20% of the L-Asu-peptide **22** in that from the D-Bzl-ester **21**.

Very similar product ratios were obtained when solutions of Asu-peptides **22** and **23** in DMF were kept in the presence of 1 mol $mol^{-1}$ $Et_3N$ at room temperature for 18 days. Epimerizations were calculated from optical rotations, as shown in Fig. 1.

No formation of the Asu-peptide **22** was observed when suspensions of the Bzl-ester **20** in MeOH and EtOH were boiled for 8 h. HPLC analyses were carried out with a 24:0.4:78.6 (v/v/v) mixture of acetonitrile–TFA–aq. 10 mmol $dm^{-3}$ sodium decane-1-sulphonate–$Na_2SO_4$ (10 mmol $dm^{-3}$), using the same conditions as described for piperidinolyses ($t_R$ 170 min for **23** and 188 min for **22**).

HF *Treatment of Dipeptide Acids* **24** *and* **26**.—This was carried out by Sakakibara's standard procedure[43] in the presence of anisole as scavenger at −2 °C for 1 h. HF was then evaporated off under reduced pressure and the residues were isolated by trituration with dry diethyl ether and were filtered off. The presence of 0.4 and 0.3% of cyclic product **27** was detected by HPLC, using a 250 × 4.6 mm ID column, packed with 5 μm Nucleosil $C_{18}$ (Chemcopak). Samples (2 $mm^3$) of 5 mg $cm^{-3}$ solutions in AcOH were injected. A 3:97 (v/v) mixture of acetonitrile–aq. potassium phosphate (0.1 mmol $dm^{-3}$) (pH 2), containing 50 mmol $dm^{-3}$ $Na_2SO_4$, was used with a 1 $cm^3$ $min^{-1}$ flow rate. Effluent was checked at 210 and 260 nm: $t_R$ 11 min for Asp-Phe-$NH_2$ **30**, 13 min for Asp(Phe-$NH_2$) **31**, 20.5 min for D-Asu-Phe-$NH_2$ **29**, and 21 min for Asu-Phe-$NH_2$ **27**.

The same HPLC system was used to detect the epimer impurity **29** of Asu-peptide samples **27**, prepared from Asp(OBu$^t$)-peptide **28** either by long-term acidolysis,[23] or from the protected Asu-peptide **22** by the standard HF procedure or by catalytic hydrogenolysis in AcOH. Owing to incomplete resolution of the Asu-peptides **27** and **29**, all of these samples were treated with aq. NaOH (1 mol $dm^{-3}$) at room temperature for 30 s, after which the hydrolysates were quenched with a large excess of AcOH and analysed by HPLC; results are reported in the text.

*Boc-Leu-Asp(OBzl)-Phe-$NH_2$* **36**.[10]—Boc-Asp(OBzl)-Phe-$NH_2$ **1**[2] (1.0 g, 2.1 mmol) was treated with TFA (10 $cm^3$) at room temperature for 30 min. The solvent was evaporated off and the residue was triturated with dry diethyl ether and was then filtered off to give H-Asp(OBzl)-Phe-$NH_2$·TFA (0.88 g); $R_f$[3] 0.30, $R_f$[5] 0.85. To a solution of this salt in DMF (10 $cm^3$) were added $Et_3N$ (0.28 $cm^3$, 2 mmol) and Boc-Leu-OSu[44] (0.65 g, 2 mmol). After 18 h the solvent was removed, a solution of the residue in $CHCl_3$ was washed in the usual way, the solution was concentrated, and the product was precipitated with diethyl ether. This suspension was filtered to give a crude product, which was recrystallized from EtOH (8 $cm^3$) to yield compound **36** (0.88 g, 83%), m.p. 155–158 °C (Found: C, 64.1; 7.15; 9.6. $C_{31}H_{42}N_4O_7$ requires C, 63.9; H, 7.3; N, 9.6%); $R_f$[3] 0.75; $[\alpha]_D$ −38.3° (*c* 1, DMF); amino acid analysis: Asp 0.97 (1), Leu 1.0 (1), Phe 1.02 (1).

*Boc-Leu-Asp(Phe-$NH_2$)-OBzl* **37**.—Boc-Asp(Phe-$NH_2$)-OBzl **3**[2] (1.0 g, 2.1 mmol) was treated with TFA (10 $cm^3$) in the

usual way. To a solution of the resulting TFA salt in DMF (10 $cm^3$) were added $Et_3N$ (0.30 $cm^3$, 2.1 mmol) and Boc-Leu-OSu (0.70 g, 2.1 mmol). The usual work-up procedure identical with that used for compound **36** gave the *tripeptide* **37** (0.55 g, 44%), m.p. 164–167 °C (with resolidification at 153 °C) (Found: C, 64.1; H, 7.15; N, 9.4. $C_{31}H_{42}N_4O_7$ requires C, 63.9; H, 7.3; N, 9.6%); $R_f$ [3] 0.75; $[\alpha]_D$ −14.3° (*c* 1, DMF); amino acid analysis: Asp 1.0 (1), Leu 0.98 (1), Phe 1.0 (1).

*Boc-Leu-Asu-Phe-NH*$_2$ **34**.[10]—*Method A.* To a solution of the hydrobromide of H-Asu-Phe-$NH_2$ [23] (**27**·HBr) (6.84 g, 20 mmol) in DMF (20 $cm^3$) were added $Et_3N$ (2.8 $cm^3$, 20 mmol) and Boc-Leu-OSu (6.56 g, 20 mmol). The usual work-up procedure gave a crude product, which was precipitated from a 3:3:4 mixture of EtOH–diethyl ether–hexane to give *compound* **34** (5.97 g, 62.8%), m.p. 200–201 °C (decomp.) (Found: C, 60.4; H, 7.05; N, 11.55. $C_{24}H_{34}N_4O_6$ requires C, 60.7; H, 7.2; N, 11.8%; $R_f$ [3] 0.70, $R_f$ [8] 0.60; $[\alpha]_D$ −131.8° (*c* 1, DMF); amino acid analysis: Asp 0.97 (1), Leu 1.0 (1), Phe 1.03 (1).

*Method B.* Hydrogen was bubbled through a stirred suspension of a mixture of the Z-Asu-peptide **22** (0.60 g, 1.5 mmol), Boc-Leu-OPfp [14] (0.86 g, 2 mmol) and Pd–charcoal catalyst (0.4 g) in DMF (10 $cm^3$), for 14 h. The catalyst was filtered off and the filtrate was evaporated to give a residue, which was dissolved in $CHCl_3$, and the solution was washed in the usual way and evaporated. Trituration of the residue with diethyl ether and recrystallization from EtOH (5 $cm^3$) gave the required tripeptide **34** (0.50 g, 70%), m.p. 199–200 °C; $[\alpha]_D$ −13.8° (*c* 1, DMF). HPLC conditions were identical with those used for analysis of the Asu-dipeptides **22** and **23**, with the exception that the eluent was a 22:0.8:77.2 (v/v/v %) mixture of acetonitrile–TFA–aq. 5 mol $dm^{-3}$ sodium decane-1-sulphonate–5 mol $dm^{-3}$ $Na_2SO_4$ ($t_R$ 260 min for **34** and 280 min for **35**). This sample was used for X-ray analysis;[33] $\delta_H$[300 MHz; $(CD_3)_2SO$] 0.85 (3 H, d, $^3J_{\gamma\delta}$ 6.9, Leu-H$^{\delta}$), 0.87 (3 H, d, $^3J_{\gamma\delta}$ 6.9, Leu-H$^{\delta'}$), 1.37 (9 H, s, Bu$^t$), 1.30–1.51 (2 H, m, Leu-H$^{\beta}$), 1.59 (1 H, m, Leu-H$^{\gamma}$), 2.39 (1 H, dd, $^3J_{\alpha\beta}$ 5.4, $^2J_{\beta\beta}$ 17.7, Asu-H$^{\beta}$), 2.96 (1 H, dd, $^3J_{\alpha\beta}$ 9.4, $^2J_{\beta\beta}$ 17.7, Asu-H$^{\beta'}$). 3.08 (1 H, dd, $^3J_{\alpha\beta}$ 10.7, $^2J_{\beta\beta}$ 14.1, Phe-H$^{\beta}$), 3.44 (1 H, dd, $^3J_{\alpha\beta}$ 4.4, $^2J_{\beta\beta}$ 14.1, Phe-H$^{\beta'}$), 3.97 (1 H, m, Leu-H$^{\alpha}$), 4.33 (1 H, m, Asu-H$^{\alpha}$), 4.62 (1 H, dd, $^3J_{\alpha\beta}$ 4.4 and 10.7, Phe-H$^{\alpha}$), 6.97 (1 H, d, $^3J_{H^{\alpha}NH}$ 8.2, Leu-NH), 7.16 (2 H, m, Phe 2- and 6-H), 7.20 (1 H, m, Phe 4-H), 7.24 (2 H, m, Phe 3- and 5-H), 7.22 and 7.35 (2 H, 2 × br, Phe-$NH_2$) and 8.56 (1 H, d, $J_{H^{\gamma}NH}$ 7.6, Asu-NH); $\delta_C$ 21.35 (Leu-C-δ), 22.77 (Leu-C-δ′), 24.07 (Leu-C-γ), 28.04 $CMe_3$), 32.77 (Phe-C-β), 34.55 (Asu-C-β), 40.45 (Leu-C-β), 47.88 (Asu-C-α), 52.34 (Leu-C-α), 54.44 (Phe-C-α), 78.07 ($CMe_3$), 126.22 (Phe-C-4), 128.19 (Phe-C-3 and -5), 128.73 (Phe-C-2 and -6), 138.08 (Phe-C-1), 155.26 (Z-CO), 169.15 (Phe-CO), 173.29 and 175.02 (2 × Asu-CO) and 174.12 (Leu-CO).

*Method C.* A solution of compound **36** (0.4 g, 0.7 mmol) and $Et_3N$ (0.1 ml, 0.7 mmol) in DMF (4 $cm^3$) was kept at room temperature for 2 weeks, after which the solvent was removed and the residue triturated with diethyl ether–hexane to yield compound **34** (0.17 g, 51%), m.p. 200–201 °C; $[\alpha]_D$ −133.3° (*c* 1, DMF).

*Method D.* The isoaspartyl tripeptide **37** was treated as described in Method C above to give compound **34** (42%), m.p. 195–198 °C; $[\alpha]_D$ −130.7° (*c* 1, DMF).

*Boc-Leu-*D*-Asu-Phe-NH*$_2$ **35**.—This was prepared from the D-Asu-dipeptide **23** as described for compound **34** using Method B. (64.6%), m.p. 116 °C (decomp., softening from 99 °C) (Found: C, 61.0; H, 7.1; N, 11.95. $C_{24}H_{34}N_4O_4$ requires C, 60.7; H, 7.2; N, 11.8%); $R_f$ [3] 0.70; $R_f$ [8] 0.70; $[\alpha]_D$ −106.6° (*c* 1, DMF); $\delta_H$[300 MHz; $CD_3)_2SO$] 0.84 (3 H, d, $^3J_{\gamma\delta}$ 6.6, Leu-H$^{\delta}$), 0.88 (3 H, d, $^3J_{\gamma\delta}$ 6.6, Leu-H$^{\delta'}$), 1.37 (9 H, s, Bu$^t$), 1.29–1.48 (2 H, m, Leu-H$^{\beta}$), 1.58 (1 H, m, Leu-H$_{\gamma}$), 2.44 (1 H, dd, $^3J_{\alpha\beta}$ 5.0, $^2J_{\beta\beta}$ 17.9, Asu-H$_{\beta}$), 2.86 (1 H, dd, $^3J_{\alpha\beta}$ 9.5, $^2J_{\beta\beta}$ 17.9, Asu-H$^{\beta'}$), 3.12 (1 H, dd, $^3J_{\alpha\beta}$ 10.2, $^2J_{\beta\beta}$ 14.1, Phe-H$^{\beta}$), 3.46 (1 H, dd, $^3J_{\alpha\beta}$ 5.0, $^2J_{\beta\beta}$ 14.1, Phe-H$^{\beta'}$), 3.94 (1 H, m, Leu-H$_{\alpha}$), 4.38 (1 H, m, Asu-H$^{\alpha}$), 4.67 (1 H, dd, $^3J_{\alpha\beta}$ 5.0 and 10.2, Phe-H$^{\alpha}$), 7.02 (1 H, d, $^3J_{H^{\gamma}NH}$ 7.8, Leu-NH), 7.14 (2 H, m, Phe 2- and 6-H), 7.20 (1 H, m, Phe 4-H), 7.24 (2 H, m, Phe 3- and 5-H), 7.17 and 7.35 (2 H, 2 × br, Phe-$NH_2$) and 8.72 (1 H, d, $^3J_{H^{\gamma}NH}$ 7.3, Asu-NH); $\delta_C$ 21.47 (Leu-C$^{\delta}$), 22.77 (Leu-C$^{\delta'}$), 23.98 (Leu-C$^{\gamma}$), 28.03 ($CMe_3$), 32.91 (Phe-C$_{\beta}$), 34.64 (Asu-C$^{\beta}$), 40.21 (Leu-C$^{\beta}$), 47.86 (Asu-C$^{\alpha}$), 52.24 (Leu-C$^{\alpha}$), 54.53 (Phe-C$^{\alpha}$), 78.06 ($CMe_3$), 126.29 (Phe-C-4), 128.25 (Phe-3 and -5), 128.57 (Phe-C-2 and -6), 137.95 (Phe-C-1), 155.31 (Z-CO), 169.22 (Phe-CO), 173.64 and 175.35 (2 × Asu-CO) and 174.17 (Leu-CO).

*Boc-Glu(OBzl)-Asp(OBzl)-Ser-Gly-OBzl* **38**.—This was prepared from H-Gly-OBzl by the usual stepwise strategy, using DCC activations (details of this synthesis will be published elsewhere). M.p. 108–111 °C; $R_f$ [1] 0.4; $[\alpha]_D$ −10.5° (*c* 1, $CHCl_3$).

*Boc-Glu(OBzl)-Asu-Ser-Gly-OBzl* **39**.—This was prepared from the non-cyclic precursor **38**, which was kept in DMF at room temperature for 24 h. The solvent was evaporated off and the residue was triturated with diethyl ether to give the required tetrapeptide **39**, m.p. 91–93 °C; $R_f$ [1] 0.27; $[\alpha]_D$ −41.8° (*c* 1, $CHCl_3$); $\delta_H$(300 MHz; $CDCl_3$) 1.42 (9 H, s, Bu$^t$), 1.90 and 2.06 (2 H, m, GluH$^{\beta}$), 2.45 (2 H, m, Glu-H$^{\gamma}$), 2.71 (1 H, dd, $^3J_{\alpha\beta}$ 4.7, $^2J_{\beta\beta}$ 18.3, Asu-H$^{\beta}$), 3.04 (1 H, dd, $^3J_{\alpha\beta}$ 9.2, $^2J_{\beta\beta}$ 18.3, Asu-H$^{\beta'}$), 3.92 (1 H, m, Gly-H$^{\alpha}$, overlapping), 4.03 (1 H, m, Ser-H$^{\beta}$, overlapping), 4.07 (1 H, m, Gly-H$^{\alpha}$, overlapping with the previous peaks), 4.24 (1 H, m, Glu-H$^{\alpha}$, overlapping with the next peak), 4.29 (1 H, m, Ser-H$^{\beta'}$, overlapping), 4.44 (1 H, m, Asu-H$^{\alpha}$), 4.86 (1 H, t [dd], $^3J^{\alpha\beta}$ 6.8, Ser-H$^{\alpha}$), 5.10 and 5.12 (4 H, 2 × s, Ph$CH_2$O), 5.49 (1 H, d, $^3J_{H^{\gamma}NH}$ 7.00, Glu-NH), 7.33 (10 H, m, *Ph*$CH_2$O), 7.48 (1 H, br t, Gly-NH) and 7.92 (1 H, d, $^3J_{H^{\gamma}NH}$ 6.8, Asu-NH); $\delta_C$ 27.11 (Glu-C-β), 28.26 ($CMe_3$), 30.20 (Glu-C-γ), 35.13 (Asu-C-β), 41.26 (Gly-C-α), 49.43 (Asu-C-α), 53.28 (Glu-C-α), 55.14 (Ser-C-α), 59.57 (Ser-C-β), 66.66 and 67.09 (Ph$CH_2$O), 80.63 ($CMe_3$), 128.17 and 128.21 (2 × OBzl-C-3 and -5), 128.34 and 128.43 (2 × OBzl-C-4), 128.58 (2 × OBzl-C-2 and -6), 135.21 and 135.55 (2 × OBzl-C-1), 155.99 (Boc-CO) and 167.97, 169.57, 173.04, 173.33, 174.39 and 175.71 (6 × CO).

*Boc-Glu(OBzl)-*D*-Asp(OBzl)-Ser-Gly-OBzl* **40**.—By the usual stepwise strategy, this was prepared from H-Gly-OBzl by the usual stepwise strategy, using DCC activations (details will be published elsewhere); m.p. 87–89 °C, $R_f$ [1] 0.40; $[\alpha]_D$ −7.2° (*c* 1, $CHCl_3$).

*Boc-Glu(OBzl)-*D*-Asu-Ser-Gly-OBzl* **41**.—This was prepared from the tetrapeptide **40** in DMF as described above for the L-epimer **39**, as an amorphous product; $R_f$ [1] 0.40; $[\alpha]_D$ −10.0° (*c* 1, $CHCl_3$); $\delta_H$(300 MHz; $CDCl_3$) 1.41 (9 H, s, $CMe_3$), 1.86 and 2.05 (2 H, m, Glu-H$^{\beta}$), 2.45 (2 H, m, Glu-H$^{\gamma}$), 2.77 (1 H, dd, $^3J_{\alpha\beta}$ 4.8, $^2J_{\beta\beta}$ 18.3, Asu-H$^{\beta}$), 3.10 (1 H, dd, $^3J_{\alpha\beta}$ 9.6, $^2J_{\beta\beta}$ 18.3, Asu-H$^{\beta'}$), 4.03 [3 H, m, Ser-H$^{\beta}$ (1 H) and Gly-H$^{\alpha'}$ overlapping], 4.24 (1 H, m, Glu-H$^{\alpha}$, overlapping), 4.25 [1 H, m, Ser-H$^{\beta'}$ (1 H), overlapping], 4.38 (1 H, m, Asu-H$^{\alpha}$), 4.89 (1 H, t [dd], $^3J_{\alpha\beta}$ 6.7, Ser-H$^{\alpha}$), 5.09 and 5.13 (4 H, 2 × s, Ph$CH_2$O), 5.34 (1 H, d, $^3J_{H^{\gamma}NH}$ 6.5, Glu-NH), 7.33 (10 H, m, *Ph*$CH_2$O), 7.62 (1 H, t, $^3J_{H^{\gamma}NH}$ 5.5, Gly-NH) and 7.91 (1 H, d, $^3J_{H^{\gamma}NH}$ 7.5, Asu-NH); $\delta_C$ 27.84 (Glu-C-β), 28.29 (C-$Me_3$), 30.07 (Glu-C-γ), 35.32 (Asu-C-β), 41.50 (Gly-C-α), 49.30 (Asu-C-α), 52.84 (Glu-C-α), 55.39 (Ser-C-α), 59.82 (Ser-C-β), 66.74 and 67.15 (Ph$CH_2$O), 80.51 ($CMe_3$), 128.24 and 128.31 (2 × OBzl-C-3 and -5), 128.39 and 128.46 (2 × OBzl-C-4), 128.62 and 128.63 (2 × OBzl-C-2 and -6), 135.26 and 135.57 (2 × OBzl-C-1),

155.66 (Boc-CO) and 167.92, 169.38, 173.22, 173.35, 174.35 and 175.64 (6 × CO).

*Epimerization of the Asu-peptide* **39**.—This peptide (100 mg, 0.15 mmol) in $CHCl_3$ (10 $cm^3$) was treated with $Et_3N$ (21 $mm^3$, 0.15 mmol) at room temperature. Time dependence for epimerization was checked by HPLC, using a 250 × 4.6 mm ID column, packed with 10 μm silica gel (Labor MIM, Budapest). A 97:2:1 (v/v/v) mixture of EtOAc–MeOH–AcOH was used with 1 $cm^3$ $min^{-1}$ flow rate; effluent was monitored at 254 nm: $t_R$ 8 min for **39** and $t_R$ 10 min for **41**.

*Boc-Asp(OBzl)-Leu-Arg(•HCl)-Pro-NHEt, Hydrochloride of Compound* **48**.—To an ice-cooled suspension of H-Leu-Arg-Pro-NHEt·2HCl (0.50 g, 1.0 mmol) and $Et_3N$ (0.14 $cm^3$, 1.0 mmol) in DMF (5 $cm^3$) was added Boc-Asp(OBzl)-OPfp (0.58 g, 1.2 mmol). After 3 h another portion of the active ester (0.2 g, 0.4 mmol) was added to the reaction mixture. After 18 h the solvent was removed and the residual oil was distributed between AcOEt (20 $cm^3$) and aq. $KHSO_4$ (1 mol $dm^{-3}$; 6 $cm^3$). The organic phase was washed successively with aq. $KHSO_4$ (2 × 6 $cm^3$) and saturated aq. NaCl (3 × 6 $cm^3$), and dried and evaporated. Trituration of the residue with diethyl ether gave the *hydrochloride of compound* **48** (0.53 g, 68.5%), m.p. 124–125 °C (Found: C, 55.4; H, 7.5; N, 14.6; Cl, 4.6. $C_{35}H_{57}ClN_8O_8$ requires C, 55.8; H, 7.6; N, 14.9; Cl, 4.7%); $R_f^5$ 0.60; $R_f^6$ 0.70; $R_f^9$ 0.90; $[\alpha]_D$ −69.5° (*c* 1, aq. 1 mol $dm^{-3}$ AcOH). The acetate of compound **48** was prepared from the corresponding hydrochloride by MPLC and a 9:11 mixture of $Pr^iOH$–aq. 10% AcOH. Elution gave the *acetate*, m.p. 114–117 °C (Found: C, 56.35; H, 7.8; N, 14.05. $C_{37}H_{60}N_8O_{10}$ requires C, 57.2; H, 7.8; N, 14.4%); $[\alpha]_D$ −69.8° (*c* 1, aq. 1 mol $dm^{-3}$ AcOH).

*Boc-D-Asp(OBzl)-Leu-Arg(•AcOH)-Pro-NHEt, Acetate of Compound* **50**.—This was prepared from H-Leu-Arg-Pro-NHEt·2HCl (1.45, 3.0 mmol) and Boc-D-Asp(OBzl)-OPfp (1.60 g, 3.3 mmol) in the same way as described for the hydrochloride of compound **48** (1.24 g, 55.0%). HPLC analysis showed the presence of an unidentified by-product (6.5%).

This hydrochloride salt (250 mg) was purified by MPLC as described previously for the acetate of compound **48** to give the pure *acetate of compound* **50** (54 mg), m.p. 113–116 °C (Found: C, 56.1; H, 7.55; N, 14.1. $C_{37}H_{60}N_8O_{10}$ requires C, 57.2; H, 7.8; N, 14.4%); $R_f^5$ 0.60; $R_f^5$ 0.75; $R_f^9$ 0.90; $[\alpha]_D$ −55.1° (*c* 0.25, aq. 1 mol $dm^{-3}$ AcOH).

*Cyclization of Compounds* **48** *and* **50** *to Compounds* **47** *and* **49** *Respectively, and Their Epimerization*.—For study of the $Et_3N$-catalysed ring closure, a solution of the hydrochloride of compound **48** (25 mg) in DMF (0.1 ml) was treated with $Et_3N$ (5 $mm^3$). Aliquots (6 $mm^3$) were taken at intervals and quenched with 10% aq. AcOH (0.9 $cm^3$) and were analysed by HPLC.

In other series of experiments, solutions of the hydrochloride or acetate of compound **48** (2 mg) in DMF (0.2 $cm^3$) were kept at room temperature or at 60 °C. A intervals, aliquots (20 $mm^3$) were taken, quenched with AcOH (80 $mm^3$), and kept at −20 °C until analysed.

*Boc-Asp-Leu-Arg(•AcOH)-Pro-NHEt, Acetate of Compound* **51**.— Hydrogen was bubbled through a solution of the hydrochloride of compound **48** (0.85 g, 1.13 mmol) in a mixture of EtOH (8 $cm^3$) and 50% (v/v) aq. AcOH in the presence of Pd–charcoal catalyst (0.2 g). After 90 min the catalyst was filtered off, the filtrate evaporated, and the residue was purified by silica gel column chromatography, using solvent system 5 for elution. Pure fractions were collected and evaporated, and product was isolated by trituration with diethyl ether to yield the *acetate of compound* **51** (0.47 g, 61%), m.p. 186–189 °C (Found: C, 51.9; H, 7.8; N, 16.0. $C_{30}H_{54}N_8O_{10}$ requires C, 52.5; H, 7.9; N, 16.3%); $R_f^5$ 0.36; $R^{f6}$ 0.64; $R^{f9}$ 0.83; $[\alpha]_D$ −84.3° (*c* 1, 1 mol $dm^{-3}$ AcOH); amino acid analysis: Asp 0.9 (1), Leu 1.0 (1), Pro 0.9 (1), Arg 0.7 (1).

*Cyclization of the Acetate of Compound* **51** *to Compound* **47**.—To solutions of the acetate of compound **51** (10 mg) in DMF (0.1 $cm^3$) were added solution(s) of the various reagents as presented in Table 1. Samples (8 $mm^3$) were quenched with 40% (v/v) AcOH before analysis by HPLC.

*Boc-Asu-Leu-Arg(•AcOH)-Pro-NHEt, Acetate of Compound* **47**.—To a solution of the acetate of compound **51** (0.3 g, 0.44 mmol) in DMF (2 $cm^3$) were added 1.5 mol $dm^{-3}$ HOPfp–DMF (0.9 $cm^3$, 1.35 mmol) and 1.3 mol $dm^{-3}$ diisopropylcarbodiimide (DIC)–DMF (1 $cm^3$, 1.3 mmol). The reaction mixture was stirred at room temperature for 44 h and then evaporated. Trituration of the residue with diethyl ether gave the crude acetate salt of compound **47** (230 mg), of which a large portion (174 mg) was purified by MPLC, using gradient elution with a mixture of $Pr^iOH$ and 10% (v/v) AcOH (36 mg), m.p. 153–156 °C (Found: C, 53.1; H, 7.6; N, 16.3. $C_{30}H_{52}N_8O_9$ requires C, 53.9; H, 7.8; N, 16.75%); $R_f^5$ 0.50; $R_f^6$ 0.64; $R_f^9$ 0.87; $[\alpha]_D$ −71.6° (*c* 0.22, 1 mol $dm^{-3}$ AcOH); $\nu_{max}$(KBr/$cm^{-1}$ 1777 (imide).

*Boc-D-Asu-Leu-Arg(•HCl)-Pro-NHEt, Acetate of Compound* **49**.—A solution of the hydrochloride of compound **48** (0.41 g, 0.54 mmol) in DMF (1.6 $cm^3$) was treated with $Et_3N$ (80 $mm^3$, 0.58 mmol) at room temperature for 5 days. The suspension was filtered and the precipitate was washed with cold DMF to yield the *hydrochloride of compound* **49** (0.28 g, 80%), m.p. 185–186 °C (Found: C, 52.1; H, 7.6; N, 17.2; Cl, 5.1. $C_{28}H_{49}ClN_8O_7$ requires C, 52.1; H, 7.7; N, 17.4; Cl, 5.5%); $R_f^5$ 0.50; $R_f^6$ 0.73; $R_f^9$ 0.93; $[\alpha]_D$ −62.4° (*c* 1, 1 mol $dm^{-3}$ AcOH); $\nu_{max}$(KBr)/$cm^{-1}$ 1777 (imide).

## Acknowledgements

We are indebted to Ms Zs. Muck, Ms I. Überhardt and Ms M. Darvas for their skilful technical assistance. We are very grateful to Professor R. A. W. Johnstone (University of Liverpool, Department of Chemistry) for revising and grammatically correcting the original manuscript.

## References

1 Part 1, I. Schön and A. Rill, *Collect. Czech. Chem. Commun.*, 1989, **54**, 3360.
2 I. Schön, R. Colombo and A. Csehi, *J. Chem. Soc., Chem. Commun.*, 1983, 505.
3 I. Schön and A. Rill, in *Peptides 1986*, ed. D. Theodoropoulos, Walter de Gruyter, Berlin, 1987, p. 83.
4 A. Neuberger, *Adv. Protein Chem.*, 1948, **4**, 298.
5 P. M. J. van der Oetelaar, J. R. C. M. van Beckhoven and H. J. Hoenders, *J. Chromatogr.*, 1987, **388**, 441.
6 G. Szókán, G. Mezö, F. Hudecz, Z. Majer, I. Schön, 0. Nyéki, T. Szirtes and R. Dölling, *J. Liq. Chromatogr.*, 1989, **12**, 2855.
7 P. N. McFadden and S. Clarke, *Proc. Natl. Acad. Sci. USA*, 1982, **79**, 2460.
8 E. D. Murray and S. Clarke, *J. Biol. Chem.*, 1984, **259**, 10722.
9 I. Schön and A. Rill, *Acta Chim. Hung.*, 1987, **124**, 191.
10 I. Schön, L. Kisfaludy, G. Holzinger and L. Varga, in *Peptides 1980*, ed. Brunfeldt, Scriptor, 1981, Copenhagen, p. 548.
11 L. Kisfaludy, O. Nyéki, I. Schön, L. Dénes, J. Ember, L. Szporny, Gy. Hajós and B. Szende, *Hoppe Seyler's Z. Physiol. Chem.*, 1983, **364**, 933.
12 J. Seprödi, I. Teplán, I. Schön, J. Érchegyi, Zs. Vadász, O. Nyéki, T. Szirtes, A. Selmeci and B. Kanyicska, *Hung. Pat.*, 194 280, 1985 (*Chem. Abstr.*, 1987, **107**, 218076d).

13 E. Bayer, G. Jung and H. Hagenmeier, *Tetrahedron*, 1968, **24**, 4853.
14 L. Kisfaludy, M. Löw, O. Nyéki, T. Szirtes and I. Schön, *Justus Liebigs Ann. Chem.*, 1973, 1421.
15 Y. C. Meinwald, E. R. Stimson and H. A. Scheraga, *Int. J. Pept. Protein Res.*, 1986, **28**, 79.
16 D. S. Kemp, in *The Peptides*, ed. E. Gross and J. Meienhofer, Academic, New York, 1979, vol. 1, p. 317.
17 M. Bodanszky and J. Martinez, in *The Peptides*, ed. E. Gross and J. Meienhofer, Academic, New York, 1983, vol. 5, p. 112.
18 T. Högberg, P. Ström, M. Ebner and S. Rämsby, *J. Org. Chem.*, 1987, **52**, 2033; H.-F. Federsel, E. Könberg, L. Lilljequist and B.-M. Swahn, *J. Org. Chem.*, 1990, **55**, 2254; M. Bodanszky and J. T. Sheehan, *Chem. Ind. (London)*, 1966, 1597; H. C. Beyerman, H. Hindriks and E. W. B. de Leer, *Chem. Commun.*, 1968, 1688.
19 M. Bodanszky and J. Z. Kwei, *Int. J. Pept. Protein Res.*, 1978, **12**, 67; J. Martinez and M. Bodanszky, *Int. J. Pept. Protein Res.*, 1978, **12**, 277.
20 Z. J. Kaminski, *Synthesis*, 1987, 917.
21 L. Kisfaludy, L. Ötvös, I. Schön and M. Löw, in *Peptides: Structure and Function*, ed. C. M. Deber, V. J. Hruby and K. D. Kopple, Pierce Chem. Co., Rockford, 1985, p. 221.
22 I. Schön and L. Kisfaludy, in *Peptides: Structure and Biological Function*, ed. E. Gross and J. Meienhofer, Pierce Chem. Co., Rockford, 1979, p. 277.
23 I. Schön and L. Kisfaludy, *Int. J. Pept. Protein Res.*, 1979, **14**, 485.
24 M. A. Ondetti, A. Deer, J. T. Sheehan, J. Pluscec and J. Kocy, *Biochemistry*, 1968, **7**, 4069.
25 J. Seprödi, I. Teplán, I. Mezö and J. Érchegyi, *Hung. Pat.*, 187 503, 1983 (*Chem. Abstr.*, 1985, **102**, 96087g).
26 J. Seprödi, J. Érchegyi, Zs. Vadász and I. Teplán, in *Peptides 1986*, ed. D. Theodoropoulos, Walton de Gruyter, Berlin, 1987, p. 509.
27 J. Seprödi, J. Érchegyi, Zs. Vadász, I. Teplán, I. Mezö, B. Kanyicska, M. Kovács and S. Vigh, *Biochem. Biophys. Res. Commun.*, 1987, **144**, 1214.
28 Zs. Vadász, J. Seprödi, J. Érchegyi, I. Teplán and I. Schön, *J. Chromatogr.*, 1989, **477**, 377.
29 Zs. Vadász, J. Seprödi, J. Érchegyi, I. Teplán and I. Schön, in *Peptides 1988*, ed. G. Jung and E. Bayer, Walter de Gruyter, Berlin, 1989, p. 40.
30 Zs. Vadász, Thesis for university doctor's degree, Loránd Eötvös University of Science, Budapest, 1990.
31 T. Szirtes, unpublished result.
32 L. Mazzarella, I. Schön, F. Sica and A. Zagari, *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.*, 1988, **44**, 880.
33 S. Capasso, L. Mazzarella, F. Sica and A. Zagari, *Biopolymers*, 1989, **28**, 139.
34 I. Schön, L. Kisfaludy, O. Nyéki, B. Herényi and S. Görög, in *Peptides 1988*, ed. G. Jung and E. Bayer, Walter de Gruyter, Berlin, 1989, p. 49.
35 M. T. W. Hearn, F. M.-W. Ng, V. M. J. Robson and M. F. O'Donoghue, *WO Pat.* 89/04323 (*Chem. Abstr.*, 1990, **112**, 151873u); V. M. J. Robson, I. D. Rae and F. Ng, *Hoppe Seyler's Z. Physiol. Chem.*, 1990, **371**, 423.
36 M. F. O'Donoghue, M. T. W. Hearn, F. Ng, I. D. Rae and B. A. Cross, in *Peptides 1988*, ed. G. Jung and F. Bayer, Walter de Gruyter, Berlin, 1989, p. 607.
37 J. L. Bada, *Methods Enzymol.*, 1984, **106**, 98; R. C. Stephenson and S. Clarke, *J. Biol. Chem.*, 1989, **264**, 6165; R. Shapira, K. O. Wilkinson and G. Shapira, *J. Neurochem.*, 1988. **50**, 649; B. L. Martin, D. Wu, L. Tabatabai and D. J. Graves, *Arch. Biochem. Biophys.*, 1990, **276**, 94.
38 M. Zhao, J. L. Bada and T. J. Ahern, *Bioorg. Chem.*, 1989, **17**, 36.
39 J. Loweson and S. Clarke, *Blood Cells*, 1988, **14**, 103.
40 K. K. Anderson, G. L. Perez, G. H. Fisher and E. H. Man, *Neurosci. Res. Commun.*, 1990, **6**, 45.
41 L. Kisfaludy, I. Schön, J. Náfrádi, L. Varga and V. Varró, *Hoppe Seyler's Z. Physiol. Chem.*, 1978, **359**, 887.
42 P. M. Hardy, J. C. Haylock and H. N. Rydon, *J. Chem. Soc., Perkin Trans 1*, 1972, 605.
43 S. Sakakibara, Y. Shimonishi, Y. Kishida and M. Okada, in *Peptides 1966*, eds. H. C. Beyerman, A. van de Linde and W. Maassen van den Brink, North-Holland Publ. Co., Amsterdam, 1967, p. 44.
44 G. W. Anderson, J. E. Zimmerman and F. M. Callahan, *J. Am. Chem. Soc.*, 1964, **86**, 1839.

*Paper* 1/00150G
*Received* 11*th January* 1991
*Accepted* 10*th July* 1991