Studies on Conformationally Restricted, Disiloxane Bridged Analogues of the Enkephalins

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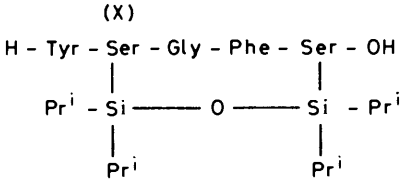
Solution-phase synthesis of protected [Ser^2, Ser^5]- and [D-Ser^2, Ser^5]-enkephalins, followed by treatment with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane [$Pr^i_2Si(Cl)$–O–$Si(Cl)Pr^i_2$] in base have yielded cyclic bridged disiloxane analogues showing biological activity in an *in vitro* assay. Highfield ^1H n.m.r. studies augmented by n.O.e. difference spectroscopy, temperature dependence studies of the amide protons, and the use of computer graphics support the presence of a 2→5 H-bonded β-bend conformation for the most biologically potent of the cyclic analogues.

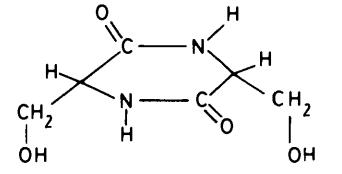
Of the many examples reported[1] for the modification of the natural enkephalin opiate peptides, restricting the natural mobility of the peptide backbone through the formation of cyclic or bridged analogues has given rise to a number of examples with increased biological activity and receptor binding characteristics. Attempts at restricting the conformational freedom of the enkephalin pentapeptides have been quite varied and include: head-to-tail cyclisation between the terminal functional groups;[2,3] cyclisation between an amino group in the side-chain of the amino-acid in position 2 and the terminal carboxy group;[4] cyclisation between a D-cysteine or D-penicillamine residue in position 2 and equivalent residues in position 5 to give the most selective δ-agonist so far reported;[5] bridging of 2,5-dipenicillamine analogue with various spacer units;[6] an azo link between the *N*-terminal tyrosyl ring and the phenylalanyl ring at position-4.[7] Direct comparisons of biological potencies in the same test situation are rather difficult to make, but some features governing structure–activity relationships have emerged. For example, preserving the conformational freedom of the *N*-terminal tyrosyl residue and keeping acyclic features at the *C*-terminus appear to be desirable.

As part of a longer term study on improving the transport of peptides by derivatisation with silyl reagents, we have investigated the feasibility of restricting the conformation freedom of the enkephalin peptide backbone by bridging two seryl residues in an enkephalin analogue using a disiloxane bridge. The study provided molecules with restricted mobility for investigation by n.m.r. techniques, so that a comparison could be made between biologically active conformations. The recently reported bifunctional disiloxane reagent 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane $Pr^i_2Si(Cl)$–O–$Si(Cl)Pr^i_2$ ($TIPDSiCl_2$) had been shown in ribonucleoside work[8] to be specific for primary alcohols but capable of bridging to a secondary alcohol in the correct spatial environment. The target molecules in the present study therefore became the cyclic disiloxane analogues (**1**) and (**2**).

The feasibility of bridging two seryl side chains in close proximity by a disiloxane bridge was readily proven by

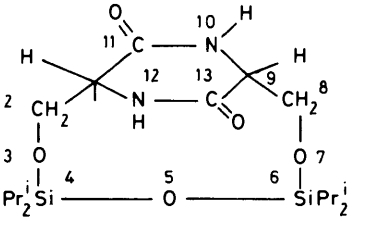


(**1**) X = L-form (**2**) X = D-form



(**3**)

pyridine
$TIPDSiCl_2$



(**4**)

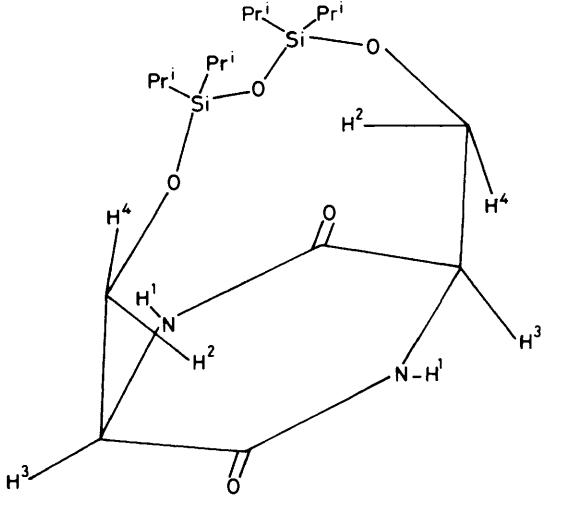


Figure 1. $J_{gem}(H^2\text{-}H^4)$ = 10.2 Hz; $J_{vic}(H^2\text{-}H^3$ or $H^3\text{-}H^4)$ = 5.6 Hz or 5.0 Hz

subjecting 3,6-bis(hydroxymethyl)piperazine-2,5-dione[9] (**3**) to treatment with the $TIPDSiCl_2$ reagent in pyridine giving the bicyclic compound (**4**) in a non-optimised 25% yield.

Only 7% of the non-bridged analogue corresponding to introduction of two disiloxane residues was formed as by-product.

The use of highfield ^1H n.m.r. (360 MHz) and decoupling experiments on compound (**4**) allowed assignments for all the protons in this compound to be made and the conformation of this molecule deduced from these experiments is shown in Figure 1.

The strategy chosen for the synthesis of (**1**) and (**2**) required the introduction of the disiloxane bridge at the pentapeptide stage. The absence of detailed information about the ultimate stability of the disiloxane bridge led to the avoidance of aqueous acid- and base-sensitive protecting groups after the bridge had been incorporated. The synthetic approach taken involved the use of *N*-protection by the *N*-fluorenylmethyloxycarbonyl (Fmoc) group,[10] *C*-terminal and tyrosyl side-chain protection being afforded by the benzyl group. Seryl residues were protected as t-butyl ether derivatives. Figure 2 summarises the synthetic scheme initially attempted.

This approach while successfully giving a pure sample of (**1**; X = L) suffered from a very low yielding step at stage 3. The basic conditions used for removal of the Fmoc group, known to give good results in the solid-phase approach[11] gave rise to significant amounts of piperazinedione from the *C*-terminal dipeptide fragment, thus lowering the yield. Evidence for piperazinedione formation was obtained by analysing the deprotection of Fmoc-Phe-Ser(Bu^t)-OBzl with piperidine in an n.m.r. tube using $(CD_3)_2SO$ as solvent. The development of a signal in the n.m.r. spectrum for the methylene group of benzyl alcohol at the expense of the methylene peak due to the benzyl ester confirmed that cyclisation to a piperazinedione was up to 50% even after only 20 min exposure to base.

A better yield of peptide (**5**; X = L) and its D-Ser^1 analogue

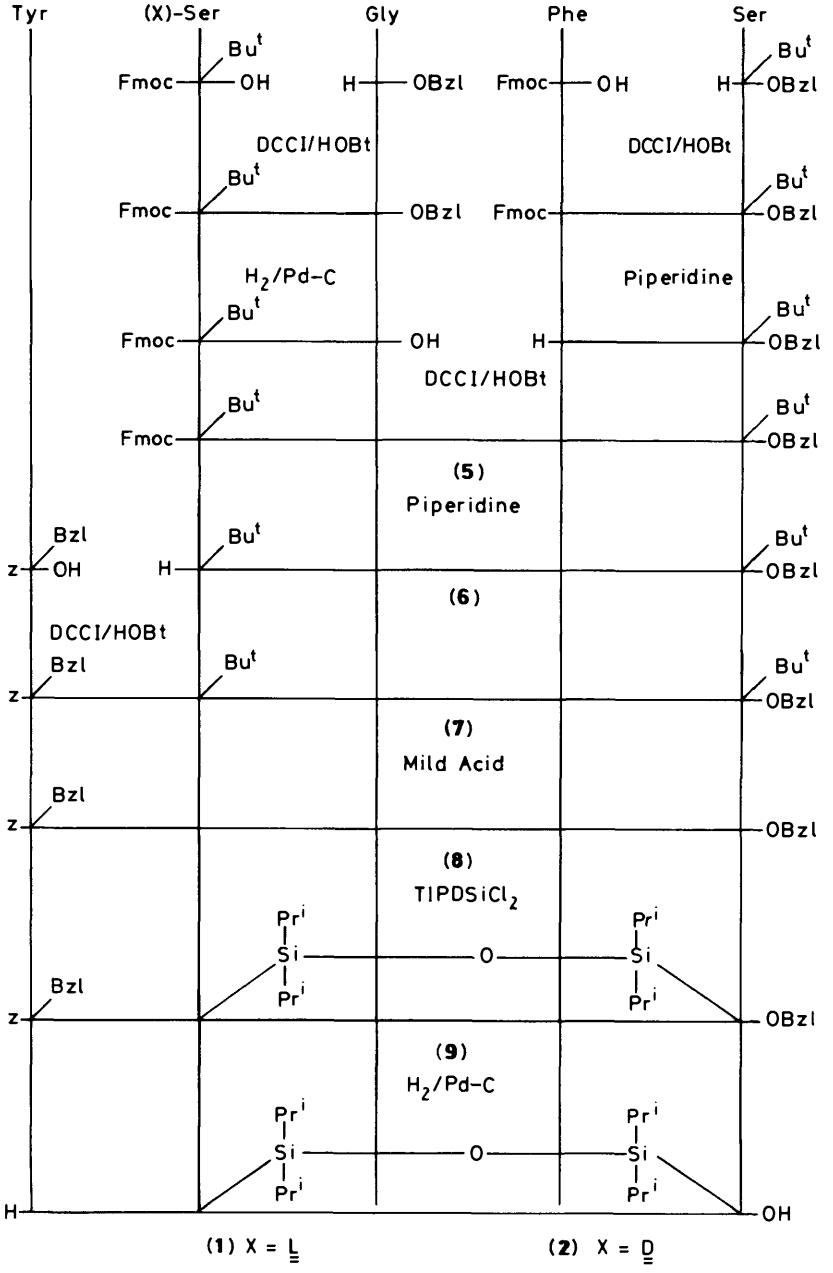


Figure 2.

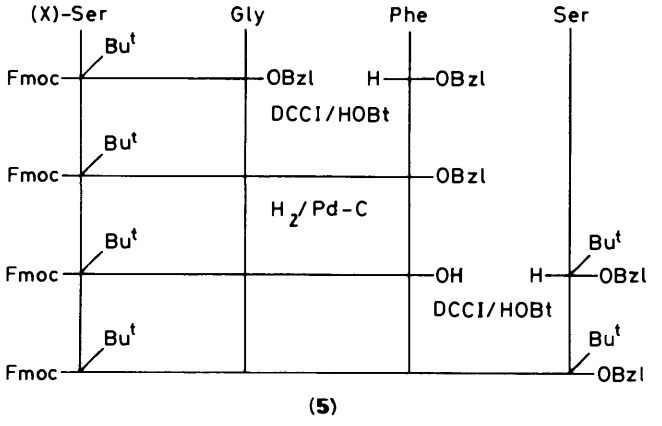


Figure 3.

was eventually obtained by following a 3 + 1 fragment coupling approach as summarised in Figure 3.

Bridging of the two free seryl hydroxy groups in derivative (**8**) was effected by the slow addition of $TIPDSiCl_2$ (0.5 eq) every 24 h until a two-fold excess of the reagent had been added. Two major u.v.-absorbing components were separated by column chromatography, corresponding to two components which had given peaks on normal phase h.p.l.c. The slower running component (with a purity of >95%) was identified by f.a.b.–m.s. to be the cyclic compound (**9**; X = L) showing a characteristic peak at *m/z* 1 116, free from any contamination from open chain analogues. The isolated yield of (**9**; X = L) was 30%.

When the sequence of reactions summarised in Figures 2 and 3 were carried out for the D-Ser^2 analogue to yield (**2**; X = D) a totally different solubility profile for the cyclic species was obtained. While the cyclic compound (**9**; X = L) was found to be soluble in non-polar solvents such as methylene dichloride, its D-Ser^2 analogue (**9**; X = D) was very insoluble in such solvents. Only polar solvents such as dimethyl sulphoxide (DMSO) or dimethylformamide would dissolve the material which led initially to be the belief that polymerisation might have occurred. However, subsequent analysis and reactions showed that the monomeric cyclic compound was formed.

For the last stage, deprotection of the benzyl and benzyloxycarbonyl groups hydrogenation over palladium–charcoal was carried out in two different solvents for the L-Ser^2 and D-Ser^2 analogues. Methylene dichloride–chloroform (1:1) was a suitable solvent for the former which gave the cyclic siloxane derivative (**1**) in 65% yield for the hydrogenation step. For the formation of (**2**) hydrogenation was carried out in DMSO followed by purification by h.p.l.c. This gave a lower yield of (**2**) mainly due to the solubility difficulties encountered earlier in the synthetic sequence leaving extra purification requirements to the last stages. H.p.l.c. and f.a.b.–m.s. results confirmed the purity of (**1**) and (**2**).

In order to compare their biological activities to those of the cyclic analogues, the acyclic pentapeptides H-Tyr-Ser-Gly-Phe-Ser-OH and H-Tyr-D-Ser-Gly-Phe-Ser-OH were also prepared from the protected pentapeptide (**8**; X = L), and its D-Ser^2 analogue (**8**; X = D) by hydrogenation in glacial acetic acid over Pd–C catalyst.

In Vitro *Biological Assays*.—The potency of the four enkephalin analogues synthesised in this study were assessed using mouse vas deferens (mvd) isolated tissue assay[12] (carried out at I.C.I. Pharmaceuticals Division, Macclesfield). The results were originally reported relative to [D-Thr^2, Leu^5, Thr^6]-enkephalin (DTLET) a potent δ-selective agonist. In Table 1 the potencies have also been compared with [Met]-enkephalin by taking the IC_{50} value[13] (the concentration of opiate required to inhibit the electrically induced response by 50%) for DTLET to be 0.58 and that for [Met]-enkephalin[14] to be 13.0. Two trends can be seen in the results. As expected, we see the lack of activity in the all-L linear enkephalin analogue, but replacement of an L-Ser for a D-Ser at position-2 gives the enhanced activity usually associated with such a modification. Increased resistance to enzymic attack and more efficient binding to the opioid receptors have been put forward as an explanation for this well known phenomenon. However, the second trend to be noted is that bridging the two seryl units *via* the siloxane link in the all L-compound increases the activity by the same amount as changing from the L to the D-analogue. Although relatively low in activity, the siloxane analogue (**1**) did show a true opiate effect since its agonist activity was reversed by the μ-selective antagonist naloxone and the δ-selective antagonist *N*,*N*-diallyl-Tyr-Aib-Phe-Leu (Aib = α-aminoisobutyric acid). The cyclic D-analogue (**2**) showed even better enhancement of activity, showing 60 times the activity of its linear analogue and appears to be more potent than [Met]-enkephalin. In addition the results indicate a K_e value of 38.5 nM against naloxone for the linear D-Ser^2 analogue which is typical of a δ-selective agonist, and a value of 13.7 nM for cyclic analogue (**2**) which would make it either δ- or K-selective.

Table 1.

Compound	Potency relative to DTLET	Potency relative to [Met]-enkephalin
H-Tyr-Ser-Gly-Phe-Ser-OH	Not active	Not active
Siloxane derivative (**1**)	0.001	0.02
H-Tyr-D-Ser-Gly-Phe-Ser-OH	0.001	0.02
Siloxane derivative (**2**)	0.06	1.34

There is no doubt therefore that disiloxane-bridging across the 2—5 seryl residues in these enkephalin analogues give enhanced opiate activity which suggests that cyclisation fixes the conformation of the linear molecules into a series of conformations which mimic more closely the pharmacologically active conformations of the enkephalins. Unfortunately *in vivo* studies on these compounds could not be carried out owing to lack of facilities, but the *in vitro* experience in handling the cyclic siloxane analogues and their properties in chromatography systems confirm that the analogues seem to have much improved lipophilic quantities which might be important in transport through membranes.

Conformational Analysis using N.M.R. Techniques and Computer Graphics.—Conformational work on these analogues have been concentrated in the main on the cyclic siloxane D-Ser analogue (**2**). General correlations and comparisons between the four analogues proved to be difficult to carry out from the limited machine time available to us. Nevertheless, the data in Table 2 summarise the broad similarities and differences in the signals in the four analogues. Our initial aim to correlate biological activity with subtle changes in conformation was, however, thwarted by the overlap of α-protons even at highfield so that evaluation of side-chain orientations *via* couplings between α-protons and amide protons was not possible.

Concentrating on the n.m.r. data at highfield for the cyclic analogue (**2**), the 360 MHz spectrum in $(CD_3)_2SO$ is reproduced in Figure 4. Two-dimensional n.m.r. techniques, and nuclear Overhauser effect (n.O.e.) difference spectroscopy[15] studies on the amide protons greatly assisted the interpretation of the spectra and signal correlation. The application of n.O.e. difference techniques to confirm the existence of a β-bend is based on the phenomenon illustrated in Figure 5.

Table 2.

Residue		L H-Tyr-Ser-Gly-Phe-Ser-OH	D H-Tyr-Ser-Gly-Phe-Ser-OH	L H-Tyr-Ser-Gly-Phe-Ser-OH Pr^i_2Si—O—$SiPr^i_2$	D H-Tyr-Ser-Gly-Phe-Ser-OH Pr^i_2Si—O—$SiPr^i_2$	L Z-Tyr-Ser-Gly-Phe-Ser-OBzl OBzl, $SiPr^i_2$—O—$SiPr^i_2$
Tyrosine	α-H	3.60d[a]	3.30—3.90[a]	3.80—4.10[a]	3.80—4.00[a]	4.27—4.43[a]
	β-H	2.62dd	2.53dd	2.64dd	2.65	2.68dd
		2.92dd	2.83dd	2.94dd	2.94	3.00dd
	NH/NH_3^+	Exchanged	Exchanged	Exchanged	Exchanged	Either 8.44, 8.16, 7.76, or 7.54
	OH	Exchanged	Exchanged	9.25s	9.24s	
	Aromatic	6.69d	6.64d	6.67d	6.64d	6.89d[b]
		7.00d	6.98d	7.06d	7.00d	7.18d[b]
Serine	α-H	4.04[c]	3.30—3.90[c,a]	4.42[d]	4.34[e]	Either at 4.33—4.27 or 4.10—3.90 or dd at 3.82
	β-H	3.50—3.70[a]	3.30—3.90[a]	3.80—4.10[a]	3.81—4.00[a]	Not assigned
	NH	8.00d[b]	Not assigned	7.35	8.34[e]	Either 8.44, 8.16, 7.76, or 7.54
Glycine	α-H	3.60[a,b]	3.30—3.90[a]	3.80—4.10[a]	3.80—4.00[a]	3.53 2 × dd
	NH	8.04t	8.07t	8.38t	8.46t	8.12[e]
Phenylalanine	α-H	4.52[f]	4.40[f]	4.34[f]	4.43[f]	4.33—4.27[a]
	β-H	2.74dd	2.76dd	2.83dd	3.80dd	2.81dd
		2.92dd	3.01dd	3.11dd	3.12dd	3.17dd
	NH	8.20d	8.18d	8.26d[g]	8.10[e]	8.12
Serine	α-H	4.29[c]	4.21[c]	4.10—3.80[a]	4.14[e]	Either 4.33—4.27 or 4.10—3.90 or dd at 3.82
	β-H	3.50—3.80[a]	3.30—3.90[a]	3.80—4.10[a]	3.80—4.00[a]	Not assigned
	NH	8.39	7.51d	8.84	7.47[e]	Either 8.44, 8.16, 7.76, or 7.54
	CO_2H	Exchanged	Exchanged	Exchanged	Exchanged	

[a] Overlapping with other protons. [b] Based on COSY spectra. [c] Assignment based on difference in spectra of the two linear pentapeptides, the biggest change in δ occurs for the α-H of the residue whose optical activity has been inverted. [d] Based on having similar coupling to pentapeptides. [e] Based on n.O.e and decoupling experiments, overlaps with another NH. [f] Six line symmetrical multiplet. [g] Overlaps with Gly^3 NH.

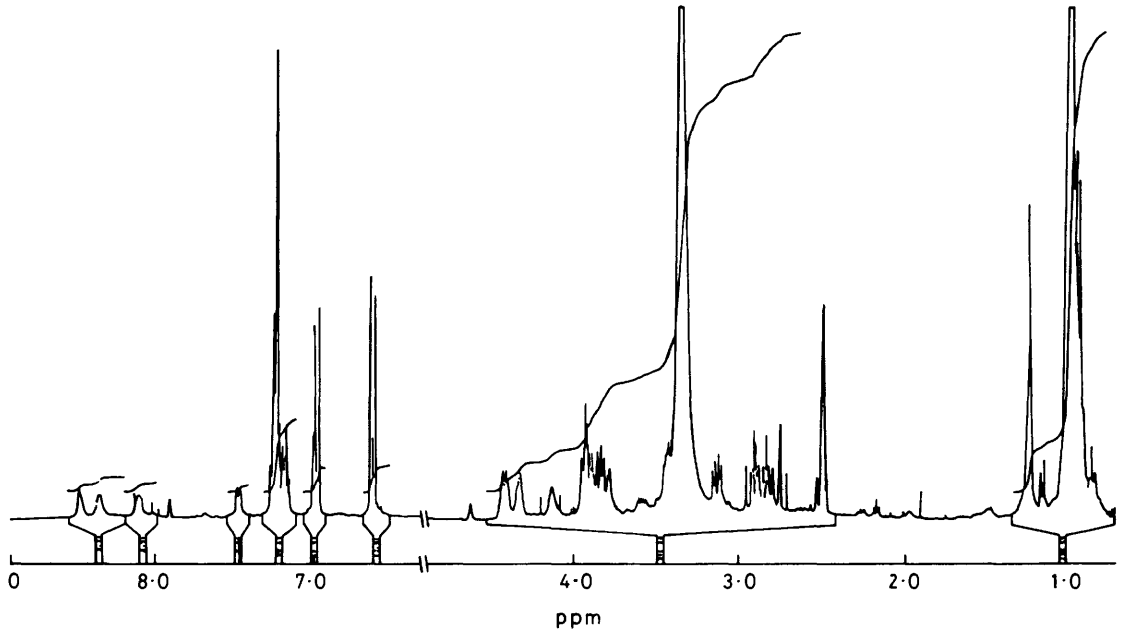


Figure 4. 360 MHz, ^{1}H N.m.r. of the D-Ser^2 cyclic analogue

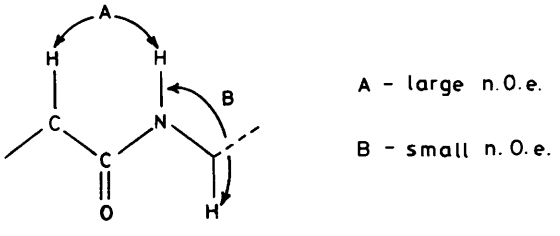


Figure 5.

A β-bend brings the α-H of the preceding residue into close proximity with the neighbouring amide and so a large n.O.e. is observed. Conversely, a small n.O.e. is observed between the amide and the α-H of the same residue where a β-bend would hold these functionalities apart. For compound (**2**) the n.O.e. studies can be summarised as follows. (*a*) Irradiation of the Gly^{3}N-H (a triplet) at δ 8.46 showed a significant enhancement of the α-H of D-Ser^2 and a much smaller enhancement of the α-H's of Gly^3. (*b*) Irradiation of the N–H signal at δ 8.34 produced a small enhancement of the α-H of D-Ser^2. Thus the δ 8.34 signal must be due to the amide N–H or D-Ser^2. (*c*) Irradiation of the N–H signal at δ 7.47 gives a significant enhancement at the α-H of Phe^4 and a smaller enhancement at the α-H of L-Ser^5, thus identifying the signal as being due to the L-Ser^5 amide proton. (*d*) The amide N–H at δ 8.10 must, therefore, be that of Phe^4 and is confirmed by a small enhancement of the α-H of Phe^4 together with a stronger and broader enhancement in the Gly^3 α-H's region.

The n.O.e. results seem, therefore, to justify the speculation that the compound might exhibit β-bend conformational forms in solution. Further proof was obtained from a study of the temperature dependence of the chemical shifts of the individual amide N–H signals. The results have been plotted in Figure 6.

Apart from the NH's of Gly^3 and Ser^5 the temperature dependences of the other amides are almost linear over the temperature range. In the case of Gly^3 and Ser^5 a dramatic shift occurs in the range 30—45 °C in the case of Ser^5 amide and in the range 30—60 °C in the case of the Gly^3 amide. A linear dependence of these protons is then maintained to the maximum temperature studied. It seems likely that a conformational change occurs in the molecule in the 30—50 °C range, but on assessing the linear portions of the plots, the temperature dependence results (p.p.m./°C) indicate it is only the L-Ser^5 amide proton with a temperature dependence below 3×10^{-3} p.p.m./°C (published data[16] suggest <0.003—solvent shielded or H-bonded protons, >0.0045—free or solvent exposed protons), that could be involved in reasonably strong hydrogen bonding. This further leads to the conclusion that a 5→2 hydrogen bond exists which stabilises a β-bend conformation.

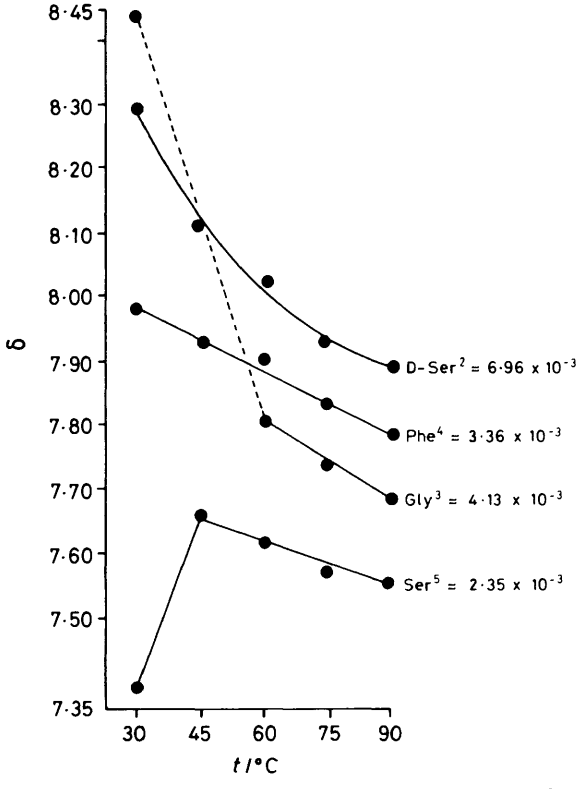


Figure 6. Amide proton temperature dependence of the D-Ser^2 cyclic analogue

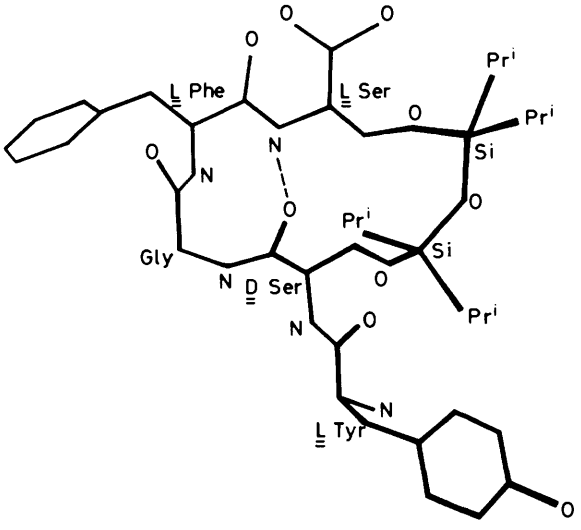


Figure 7. Computer-generated conformation of the disiloxane bridged analogue (**2**) (X = D)

The n.m.r. results were further strengthened by a computer graphics low-energy optimisation (carried out at Roche Products Ltd., Welwyn Garden City) which predicted the structure deduced from the n.m.r. experiments. This computer generated structure is shown in Figure 7.

In summary, the bridging of the Ser^2, Ser^5-enkephalin analogues using disiloxane bridge to give a 17-membered cyclic ring analogue seems to lead to a reasonably stabilised β-bend conformational form with enhanced biological potency. The relative ease of cyclisation of the acyclic precursors using the $TIPDSiCl_2$ reagent would suggest that the enkephalins can readily flex their backbone to bring the seryl hydroxy side-chains sufficiently close together for a facile bridging of the two ends of the molecule to occur. The extra stabilisation of a β-bend by bridging may be considered to be further support to the theory that the natural enkephalins might need to adopt such a conformation at the receptor site.[17]

Experimental

M.p.s were determimined on a Gallenkamp capillary m.p. apparatus and are uncorrected. I.r. spectra were determined with a Pye-Unicam SP 1050 spectrophotometer using KBr discs or sodium chloride plates. Optical rotations were obtained with a Perkin-Elmer 141 automatic polarimeter using the sodium D line at 589 nm. ^1H N.m.r. spectra were determined at 100 MHz using a Varian HA 100 and XL 100 instruments and at 360 MHz with a Bruker WH-360. Tetramethylsilane was used as internal standard, except in the case of certain silylated derivatives for which CH_2Cl_2 was employed. Mass spectra were obtained with an A.E.I. MS902 with a f.a.b. source. Computer graphics were obtained using a VAX 11/750 computer attached to a Megatek 7000 display employing the Cambridge Crystal Data File. H.p.l.c. was carried out using an LDC/Milton Roy constametric III pumps, a spectromonitor D detector, a CI-10B integrator and Sekonic printer plotter. The reversed phase column used was a Spherisorb-C_8 (25 cm × 0.43 cm) and the normal phase column was Hypersil 5μ (25 cm × 0.43 cm). T.l.c. was carried out on Merck Kieselgel 60 F_{254} pre-coated plates while column chromatography used Merck Kieselgel 60.

4,4,6,6-*Tetraisopropyl*-3,5,7-*trioxa*-10,12-*diaza*-4,6-*disilabicyclo*[7.2.2.]*tridecane*-11,13-*dione*.—A sample of 3,6-bis(hydroxymethyl)piperazine-2,5-dione[18] (**3**) (50 mg, 0.287 mmol) that had been thoroughly dried (100 °C, 2 mmHg, 48 h) was dissolved in dry pyridine (1 ml) under an N_2 atmosphere. The solution was stirred at room temperature and 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (99 μl, 1 equiv.) was added. The solvent was removed under reduced pressure (0.5 mmHg, 25 °C) after 24 h, the residue dissolved in CH_2Cl_2 and filtered and the crude product was purified by column chromatography (10% MeOH–CH_2Cl_2) to yield the *title product* (**4**) (33 mg, 25%) as white crystals, m.p. 224—226 °C (Found: C, 52.0; H, 8.9; N, 6.4; M^+ = 417.1. $C_{18}H_{36}N_2O_5Si_2$ requires C, 51.9; H, 8.7; N, 6.7%; *M*, 416.66), t.l.c. R_F = 0.21 (10% MeOH–CH_2Cl_2); δ ($CDCl_3$; 360 MHz) 5.95 (2 H, br s, NH), 4.37 (2 H, dd, β-H's deshielded by carbonyl), 4.09 (2 H, m, α-H), 3.95 (2 H, dd, β-H's), and 1.10 (28 H, s, $SiPr^i_4$). A by-product, the corresponding non-bridged analogue with two siloxane residues (15 mg, 7%) was obtained as a gum, t.l.c. R_F = 0.32 (10% MeOH–CH_2Cl_2); δ($CDCl_3$) 7.05 (2 H, br s, NH), 4.30—4.10 (6 H, m, α-H's and β-H's), and 1.10 (56 H, s, 2 × Pr^i_4 and 2 × Si–OH).

Standard Conditions for Coupling Reactions.—The *N*-fluorenylmethoxycarbonyl-protected amino acid/peptide (2 mmol) in dry THF (10 ml) was cooled to 0 °C. *N,N'*-Dicyclohexylcarbodi-imide (DCCI) (2 mmol) and 1-hydroxybenzotriazole[19] (HOBt) (2 mmol) were both added as cooled solutions (0 °C) in dry THF (2 ml). The *C*-terminal component (usually as a salt) (2 mmol) was suspended in dry THF (10 ml) with stirring and upon addition of *N,N*-di-isopropylethylamine (2 mmol), dissolution was achieved. The resultant solution was cooled to 0 °C and added over a period of 10 min to the solution containing the *N*-terminal component at 0 °C with stirring. Once addition was complete the reaction was stirred for 2 h during which the solution was allowed to warm to room temperature. The solution was filtered to remove precipitated dicyclohexylurea and the solvent removed under reduced pressure. The residue was then taken up in CH_2Cl_2, washed with 0.1M HCl (25 ml), saturated $NaHCO_3$ (25 ml), 0.1M HCl (25 ml), and water (25 ml) and dried ($MgSO_4$). The crude peptides were purified by column chromatography.

Synthesis of N-*Fluorenylmethoxycarbonyl*-O-*t-butyl*-L-*seryl*-*glycyl*-L-*phenylalanyl*-O-*t-butyl*-L-*serine Benzyl Ester* (**5**; X = L) *by* 2 + 2 *Fragment Condensation*.—N-*Fluorenylmethoxycarbonyl*-O-*t-butyl*-L-*seryl glycine benzyl ester*. *N*-Fluorenylmethoxycarbonyl-*O*-t-butyl-L-serine[20] and glycine benzyl ester toluene *p*-sulphonate[21] were coupled together using the standard method to yield the *title compound* (6.69 g, 76%) as a white crystalline solid, used without further purification, m.p. 100—102 °C (Found: C, 70.0; H, 6.5; N, 5.3. $C_{31}H_{34}N_2O_6$ requires C, 70.2; H, 6.5; N, 5.3%), t.l.c. R_F = 0.33 (10% ether–CH_2Cl_2); δ($CDCl_3$) 7.90—7.10 (14 H, m, fluorenyl ArH, $CH_2C_6H_5$ and N*H* glycine or seryl), 5.80 (1 H, br, N*H* glycine or seryl), 5.20 (2 H, s, CH_2Ph), 4.50—4.10 (4 H, m, $CHCH_2$, NHC*H* seryl), 4.05 (2 H, d, $NHCH_2CO$), 3.60 (2 H, 2 × dd, CH_2OBu^t), and 1.20 (9 H, s, Bu^t); $[\alpha]_D^{25}$ +159.5° (*c* 0.019 in CH_2Cl_2); h.p.l.c. R_F 3.08 [normal phase column, EtOAc–CH_2Cl_2 (3:2) 1 ml/min, 264 nm].

N-*Fluorenylmethoxycarbonyl*-L-*phenylalanyl*-O-*t-butyl*-L-*serine benzyl ester*. The *C*-terminal component was obtained from *N*-fluorenylmethoxycarbonyl-*O*-t-butyl-L-serine benzyl ester[20] (0.471 g, 0.996 mmol) by dissolution of the latter in piperidine–DMF (1:1; 2 ml) and stirring at room temperature for 10 min. Solvents were removed by azeotroping with DMF on a rotary evaporator (4 times at 25 °C, 0.5 mmHg). The product, *O*-t-butyl serine benzyl ester was used in the next stage without further characterisation. This benzyl ester (1.6 g, 6.35 mmol) was coupled with *N*-fluorenylmethoxycarbonyl-L-phenylalanine (2.45 g, 6.35 mmol) using DCCI (1.31 g, 6.35 mmol) and HOBt (0.85 g, 635 mmol) according to the standard

procedure followed by column chromatography (10% ether–CH_2Cl_2) to give the *title compound* (1.90 g, 50%) as a white crystalline solid, m.p. 118—120 °C (Found: C, 74.1; H, 6.8; N, 4.6. $C_{38}H_{40}N_2O_6$ requires C, 73.5; H, 6.5; N, 4.5%); t.l.c. R_F = 0.55 (product), traces at 0.84, 0.73, and 0.28 (fluorenyl cleavage by-products) (10% ether–CH_2Cl_2); δ($CDCl_3$) 7.80—7.10 (18 H, m, fluorenyl ArH, C_6H_5 benzyl ester, C_6H_5 phenylalanyl), 6.60 (1 H, br d, NH not assigned), 5.50 (1 H, br d, NH not assigned), 4.80—4.00 (5 H, NHC*H* phenylalanyl, NHC*H* serine and C*H*CH_2), 3.60 (2 H, 2 × dd, OCH_2Ph), 3.00 (2 H, d, CH_2, phenylalanyl), and 1.00 (9 H, s, Bu^t).

N-*Fluorenylmethoxycarbonyl*-O-*t*-*butyl*-L-*serylglycine*. *N*-Fluorenylmethoxycarbonyl-*O*-t-butyl-L-serylglycine benzyl ester (6.69 g, 12.6 mmol) was dissolved in EtOH–EtOAc to achieve a concentration of 0.2M (63 ml required), and hydrogenated with a gentle stream of H_2, using 10% Pd–C catalyst (0.364 g, 29 mg/mmol). The reaction was followed by t.l.c. and was complete after 1 h, during which no cleavage of the fluorenylmethoxycarbonyl group was observed. The catalyst was removed under suction using a filter aid and the solvent evaporated to give the *title compound* (5.30 g, 95%) as a clear glass which was used without further purification. This compound was characterised as the dicyclohexylamine salt and the data listed is for this salt, m.p. 190 °C (Found: C, 69.3; H, 8.6; N, 6.9. $C_{36}H_{51}N_3O_6$ requires C, 69.5; H, 8.3; N, 6.75%); δ($CDCl_3$) 7.90—7.20 (9 H, m, fluorenyl ArH and N*H* either glycine or seryl), 5.90 (1 H, br d, N*H* glycine or seryl), 4.50—4.20 (4 H, m, C*H*CH_2 and NHC*H* seryl), 3.90 (4 H, d, overlapping with 2 × dd, $NHCH_2$ and CH_2OCMe_3), 3.00 (2 H, m, cyclohexylamine), 2.20—1.25 (22 H, m, cyclohexylamine), 1.10 (9 H, s, Bu^t); $[\alpha]_D^{25}$ +13.6° (*c* 0.022 in MeOH).

N-*Fluorenylmethoxycarbonyl*-O-*t*-*butyl*-L-*seryl*-*glycyl*-L-*phenylalanyl*-O-*t*-*butyl*-L-*serine Benzyl Ester* (**5**; X = L).—Using the piperidine-catalysed deprotection of the Fmoc group as previously described, L-phenylalanine-*O*-t-butyl-L-serine benzyl ester (2.64 mmol) was generated from *N*-fluorenylmethoxycarbonyl-L-phenylalanyl-*O*-t-butyl-L-serine benzyl ester (1.7 g). Coupling of this residue with *N*-fluorenylmethoxycarbonyl-*O*-t-butyl-L-serylglycine (1.20 g, 2.64 mmol) was effected with DCCI (0.82 g, 2.64 mmol) and HOBt (0.365 g, 2.64 mmol) using the standard coupling procedure. Purification by column chromatography (40% EtOAc–CH_2Cl_2) yielded the *title compound* (**5**; X = L) (0.86 g, 38%) as a white crystalline solid which was used without further purification. The yield quoted was the maximum obtained, and yields were found to vary in the range 10—38% even using standardised conditions; m.p. 152—153 °C (Found: C, 68.7; H, 7.0; N, 6.8. $C_{47}H_{56}N_4O_9$ requires C, 68.7; H, 6.9; N, 6.8%), t.l.c. R_F 0.43 (40% EtOAc–CH_2Cl_2); δ($CDCl_3$; 360 MHz) 7.75—7.16 (19 H, m, fluorenyl ArH, C_6H_5 phenylalanyl, C_6H_5 benzyl ester, and N*H*), 6.68 (1 H, br d, N*H*), 6.49 (1 H, br d, N*H*), 5.72 (1 H br d, N*H*), 5.16 (2 H, dd, CH_2 benzyl ester), 4.68 (2 H, m, α-H), 4.42 (2 H, d, CH_2CH fluorenyl), 4.23 (2 H, t, >CH_2CH fluorenyl, and α-*H*), 3.92 (2 H, dd, β-H or glycyl CH_2), 3.78 (2 H, dd, β-H or glycyl CH_2), 3.47 (2 H, dd, β-*H* or glycyl CH_2), 3.38 (2 H, dd, β-H), 3.07 (2 H, t, β-H), 1.18 (9 H s, Bu^t), 1.03 (9 H, s, Bu^t); $[\alpha]_D^{25}$ +65.4° (*c* 0.0179 in CH_2Cl_2); h.p.l.c. R_T = 6.59 [normal phase column, EtOAc–CH_2Cl_2 (60:40) 1 ml/min, 264 nm]. The D-Ser[1] analogue of (**5**; X = D) was synthesised using the same procedures as outlined for (**5**; X = L). At most of the intermediate states the D-analogues had similar physical data to the corresponding L-analogues except for the following details.

N-*Fluorenylmethoxycarbonyl*-O-*t*-*butyl*-D-*serylglycine* (90% yield) was obtained as a 'glass,' m.p. 185 °C.

N-*Fluorenylmethoxycarbonyl*-O-*t*-*butyl*-D-*seryl*-*glycyl*-L-*phenylalanine benzyl ester* (68%) was obtained as a white solid, m.p. 106 °C, t.l.c. R_F 0.46 (40% EtOAc–CH_2Cl_2), $[\alpha]_D^{25}$ = −5 (*c* 0.02 in CH_2Cl_2), h.p.l.c. R_T 3.99 [normal phase column EtOAc–CH_2Cl_2 (3:2) 1 ml/min].

N-*Fluorenylmethoxycarbonyl*-O-*t*-*butyl*-D-*seryl*-*glycyl*-*phenylalanine* (93%) was obtained as a clear glass and used without further purification to give N-*fluorenylmethoxycarbonyl*-O-*t*-*butyl*-D-*seryl*-*glycyl*-L-*phenylalanyl*-O-*t*-*butyl*-L-*serine benzyl ester* (60%), m.p. 110 °C, t.l.c. R_F 0.48 (40% EtOAc–CH_2Cl_2), h.p.l.c. R_T = 5.05 [normal phase, EtOAc–CH_2Cl_2 (3:2) 1 ml/min].

Synthesis of N-*Benzyloxycarbonyl*-O-*benzyl*-L-*tyrosyl*-O-*t*-*butyl*-L-*serylglycyl*-L-*phenylalanyl*-O-*t*-*butyl*-L-*serine benzyl ester* (**6**; X = L).—Using the procedure described previously, *O*-t-butyl-L-serylglycyl-L-phenylalanyl-O-t-butyl-L-serine benzyl ester (3.44 mmol) was generated from *N*-fluorenylmethoxycarbonyl-*O*-t-butyl-L-serylglycyl-L-phenylalanyl-*O*-t-butyl-L-serine benzyl ester (2.82 g). *N*-Benzyloxycarbonyl-*O*-benzyl-L-tyrosine (1.39 g, 3.44 mmol) was coupled to this using DCCI (0.71 g, 3.44 mol) and HOBt (0.49 g, 3.44 mmol) using the standard coupling procedure and purified by column chromatography to give the *title compound* (2.47 g, 70%) as a white crystalline solid and used without further purification, m.p. 165—167 °C (Found: C, 68.2; H, 7.2; N, 7.0. $C_{56}H_{67}N_5O_{11}$ requires C, 68.2; H, 6.8; N, 7.1%), t.l.c. R_F 0.25 (40% EtOAc–CH_2Cl_2); δ($CDCl_3$; 360 MHz) 7.40—7.16 (15 H, m, C_6H_5 urethane, C_6H_5 phenylalanyl and C_6H_5 benzyl ester), 7.08 (2 H, d, tyrosyl ArH), 7.00 (1 H, t, N*H* glycyl), 6.87 (2 H, d, tyrosyl ArH), 6.79 (1 H, d, NH), 6.76 (1 H, d, NH), 6.54 (1 H, d, NH), 5.27 (1 H, d, NH), 5.16 (2 H, dd, CH_2 benzyl ether, benzyl ester or urethane), 5.09 (2 H, dd, CH_2 benzyl ether, benzyl ester or urethane), 5.00 (2 H, s, CH_2 benzyl ether, benzyl ester or urethane), 4.71 (1 H, q, α-H), 4.66 (1 H, m, α-H), 4.40 (1 H, q, α-H), 4.35 (1 H, m, α-H), 3.84 (2 H, 2 × dd, β-H), 3.74 (2 H, dd, β-H), 3.47 (1 H, dd, β-H), 3.37 (1 H, dd, β-H), 3.09 (2 H, 2 × dd, β-H), 3.01 (2 H, dd, β-H), 1.13 (9 H, s, Me_3), and 1.01 (9 H, s, Me_3); $[\alpha]_D^{25}$ −20.0° (*c* 0.045 in CH_2Cl_2); h.p.l.c. R_T 3.99 (reverse phase 0.1M NH_4HCO_3–MeCN 1 ml/min).

N-*Benzyloxycarbonyl*-O-*benzyl*-L-*tyrosyl*-O-*t*-*butyl*-D-*seryl*-*glycyl*-L-*phenylalanyl*-O-*t*-*butyl*-L-*serine Benzyl Ester*.—Using identical quantities, conditions and purification procedures as for the L-seryl[2] diastereoisomer, the *title compound* (2.45 g, 68%) was obtained as a white crystalline solid and used without further purification; it had m.p. 96—98 °C, t.l.c. R_F = 0.22 (40% EtOAc–CH_2Cl_2); δ ($CDCl_3$ 360 MHz) 7.42—6.82 (17 H, m, C_6H_5 urethane, C_6H_5 phenylalanyl, C_6H_5 benzyl ester, C_6H_4 tyrosyl and 3 × N*H*), 6.62 (1 H, d, NH), 6.15 (1 H, d, NH), 5.12 (2 H, dd, CH_2 or either benzyl ether, benzyl ester or urethane), 5.04 (2 H, dd, CH_2 benzyl ether, benzyl ester or urethane), 4.99 (2 H, s, CH_2 benzyl ether, benzyl ester or urethane), 4.71 (1 H, q, α-H), 4.63 (1 H, m, α-H), 4.29 (2 H, m, α-H), 3.81 (2 H, 2 × dd, β-H), 3.68 (2 H, dd, β-H), 3.42 (1 H, dd, β-H), 3.27 (1 H, dd, β-H), 3.13—2.92 (4 H, m, β-H), 1.14 (9 H, s, Bu^t), and 1.02 (9 H, s, Bu^t); $[\alpha]_D^{25}$ −176.0° (*c* 0.048 in CH_2Cl_2).

N-*Benzyloxycarbonyl*-O-*benzyl*-L-*tyrosyl*-L-*serylglycyl*-L-*phenylalanyl*-L-*seryl*-*Benzyl Ester* (**8**; X = L).—The protected ester (**5**) (2.95 g, 2.99 mmol) was dissolved in TFA–CH_2Cl_2 (1:1, 40 ml) and stirred at room temperature. The reaction was followed by t.l.c. (EtOAc), and was complete after 1.5 h. The solvents were removed under reduced pressure (2 mmHg, 25 °C) overnight to give an amorphous solid which was recrystallised from methanol–ether to give the *title compound* (**8**; X = L) (2.00 g, 76%) as a white crystalline solid which was used without further purification; it had m.p. 206—207 °C (Found: C, 65.5; H, 6.1; N, 7.9. $C_{48}H_{51}N_5O_{11}$ requires C, 66.0; H, 5.9; N, 8.0%); δ([$^2H_6$]DMSO) no signal corresponding to the Bu^t groups was

observed, broad signals being obtained in the spectra which did not allow assignments to be made.

N-*Benzyloxycarbonyl*-O-*benzyl*-L-*tyrosyl*-D-*serylglycyl*-L-*phenylalanyl*-L-*seryl-Benzyl Ester* (**8**; X = D).—Using identical quantities, conditions, and purification procedures as for the L-seryl[2] diastereoisomer, the *title compound* (1.84 g, 70%) was obtained as a white crystalline solid which was used without further purification; it had m.p. 204—206 °C. All the other data were identical with those of the L-Ser[2] analogue.

N-*Benzyloxycarbonyl*-O-*benzyl*-L-*tyrosyl*-[L-Ser[2],L-Ser[5]-O-(1,1,3,3-*tetraisopropyldisiloxane*-1,3-*diyl*)-L-*serylglycyl*-L-*phenylalanyl*-L-*serine*] *Benzyl Ester* (**9**; X = L).—A dried sample (100 °C, 2 mmHg, 48 h) of *N*-benzyloxycarbonyl-*O*-benzyl-L-serylglycyl-L-phenylalanyl-L-serine benzyl ester (**8**; X = L) (0.949 g, 1.09 mmol) was dissolved in dry pyridine (60 ml) under an N_2 atmosphere. 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (187 μl, 0.5 equiv.) was added at intervals of 24 h until a two-fold excess of this reagent had been introduced. Upon completion of the final addition, the resultant mixture was stirred for a further 6 days, after which the solvent was removed under reduced pressure (0.5 mmHg, 25 °C) and the crude product purified by column chromatography (40% EtOAc–CH_2Cl_2) to yield the *title compound* (**9**; X = L) (0.377 g, 31%) as a white crystalline solid, m.p. 85—88 °C [Found: C, 63.8; H, 7.3; N, 5.7%. $(MH)^+$, 1 116.5. $C_{60}H_{77}N_5O_{12}Si_2$ requires: C, 64.5; H, 6.95; N, 6.3%; *M*H, 1 116.51]; t.l.c. R_F = 0.32 (40% EtOAc–CH_2Cl_2); δ($CDCl_3$) listed in Table 2; h.p.l.c. R_T 4.53 (normal phase, 30% EtOAc–CH_2Cl_2 + 2% MeOH, 1 ml/min).

N-*Benzyloxycarbonyl*-O-*benzyl*-L-*tyrosyl*-[L-Ser[2]-Ser[5]-O-(1,1,3,3-*tetraisopropyldisiloxane*-1,3-*diyl*)-D-*serylglycyl*-L-*phenylalanyl*-L-*serine*] *Benzyl Ester* (**9**; X = D).—Using the same experimental technique described for the synthesis of the L-Ser[2] analogue, *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-D-serylglycyl-L-phenylalanyl-L-serine benzyl ester (**8**; X = D) (1.00 g, 1.15 mmol) was treated with 1,3-dichloro-1,1,3,3-tetraisopyldisiloxane[8] (4 × 198 μl) in dry pyridine (60 ml). After removal of the solvent under reduced pressure (0.5 mmHg, 25 °C), the resultant amorphous residue was found to be insoluble in most solvents, the exception being DMSO. Thus, chromatographic purification of the crude product could not be effected and the *title compound* (**9**; X = D) (1.10 g, 86%), m.p. 70—90 °C was characterised by ^1H n.m.r. and used without further purification.

L-*Tyrosyl*-[L-Ser[2],L-Ser[5]-O-(1,1,3,3-*tetraisopropyldisiloxane*-1,3-*diyl*)-L-*serylglycyl*-L-*phenylalanyl*-L-*serine*] (**1**).—*N*-Benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-[L-Ser[2],L-Ser[5]-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-L-serylglycyl-L-phenylalanyl-L-serine] benzyl ester (**9**; X = L) (0.191 g, 0.171 mmol) was dissolved in CH_2Cl_2–MeOH (50:50, 3 ml) and hydrogenated overnight using 10% Pd–C (0.019 g). Analysis by t.l.c. (20% MeOH–CH_2Cl_2) indicated the absence of starting material. The catalyst was filtered off and the filtrate evaporated under reduced pressure. The crude product obtained by hydrogenation was dissolved in MeOH (300 μl) and applied in aliquots (6 μl) to an analytical reverse-phase h.p.l.c. column. Two fractions were collected, both having a purity of >98% by h.p.l.c. The solvent was removed from both fractions using MeCN to azeotrope residual water. Both fractions were then placed in a drying pistol containing P_2O_5 (2 mmHg, 25 °C) for several days before being submitted for further analysis. The first fraction was identified by f.a.b.–m.s. to be the *title compound* (**1**) (0.089 g, 65%); t.l.c. R_F = 0.46 (20% MeOH–CH_2Cl_2); f.a.b.–m.s. [Found: $(M + 2H)^+$, 803.0. $C_{38}H_{61}N_5O_{10}Si_2$ requires $(M + 2H)$, 803.43]; δ values ([$^2H_6$]DMSO) listed in Table 2; h.p.l.c. R_T 3.23 [reverse phase, 0.1M NH_4HCO_3–CH_3CN (50:50), 1 ml/min]; $[\alpha]_D^{25}$ +61.9° (*c* 0.06 in methanol). Remained at origin in electrophoresis (pH = 3.5, 2 000 V, 20 mA, 90 min). The second component isolated (0.006 g) gave t.l.c. R_F = 0.46 (20% MeOH–CH_2Cl_2); 816 $(MH)^+$. δ Values ([$^2H_6$]DMSO) were essentially the same as the other component fraction; h.p.l.c. R_T 3.88 (eluant as for other fraction).

L-*Tyrosyl*-[D-Ser[2],L-Ser[5]-O-(1,1,3,3-*tetraisopropyldisiloxane*-1,3-*diyl*)-D-*serylglycyl*-L-*phenylalanyl*-L-*serine*] (**2**).—The crude benzyl ester (**9**; X = D) (0.100 g, 0.89 mmol) dissolved in DMF (6 ml) was hydrogenated for 4 h using 10% Pd–C (0.01 g). Analysis by t.l.c. (20% MeOH–CH_2Cl_2) indicated the absence of starting material. The catalyst was filtered off and the filtrate evaporated under reduced pressure (25 °C, 0.4 mmHg). The resulting residue was found to be only partially soluble in MeOH (150 μl) and this solution was applied in aliquots (10 μl) to an analytical reverse-phase h.p.l.c. column [0.1M NH_4HCO_3–MeCN (1:1)]. The major component was collected and processed in a similar manner to (**1**), and was subsequently identified by f.a.b.–m.s. as the *title compound* (**2**) (0.022 g, 31%); t.l.c. R_F 0.40 (20% MeOH–CH_2Cl_2) [Found: $(MH)^+$, 802. $C_{38}H_{60}N_5O_{10}Si_2$ requires (MH), 802.42]; δ values ([$^2H_6$]DMSO) are listed in Table 2; h.p.l.c. R_T 2.31 [reverse phase, 0.1M NH_4HCO_3–MeCN (1:1), 1 ml/min]. The compound remained at the origin in electrophoresis (pH 3.5, 2 000 V, 20 mA, 90 min, visualised with ninhydrin).

L-*Tyrosyl*-L-*serylglycyl*-L-*phenylalanyl*-L-*serine*.—*N*-Benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-L-serylglycyl-L-phenylalanyl-L-serine benzyl ester (**8**; X = L) (0.10 g, 0.224 mmol) dissolved in glacial acetic acid–methanol (1:1, 2 ml) with warming was hydrogenated for 1 h at 60 °C using 10% Pd–C (10 mg). The catalyst was filtered off and the filtrate evaporated under reduced pressure (0.5 mmHg, 30 °C). Crystallisation of the residue was effected with methanol to yield the *title compound* (0.051 g, 80%) as a solid; δ values ([$^2H_6$]DMSO) are listed in Table 2; [Found: $(MH)^+$, 559.9. Calc. for $C_{26}H_{33}N_5O_9$: (MH), 560.234]. The molecular ion region of the spectra contained no other significant peaks. Electrophoresis: 3.5 cm (pH = 3.5, 2 000 V, 20 mA, 90 min, visualised with ninhydrin); h.p.l.c. R_T 2.14 [reverse phase, 0.01M NH_4HCO_3–MeCN (50:50), 1 ml/min].

L-*Tyrosyl*-D-*serylglycyl*-L-*phenylalanyl*-L-*serine*.—The benzyl ester (**8**; X = D) (0.100 g, 0.224 mmol) was hydrogenated and processed under conditions identical with those employed for the synthesis of the L-Ser[2] analogue. The crude product was dissolved in MeOH (150 μl) and aliquots (10 μl) applied to an analytical reverse-phase h.p.l.c. column (solvents as described below). The *title compound* (0.038 g, 60%) was obtained as an amorphous solid; δ values ([$^2H_6$]DMSO) are listed in Table 2; [Found: $(MH)^+$, 560.0. $C_{26}H_{33}N_5O_9$ requires (MH), 560.234]. The only other significant peak in the molecular ion region of the spectra was found at 581.8 and corresponds to $(M + Na)^+$. Electrophoresis: 3.5 cm (pH 3.5, 2 000 V, 20 mA, 90 min, visualised with ninhydrin); h.p.l.c. R_T 2.19 [reverse phase 0.1M NH_4HCO_3–MeCN (1:1), 1 ml/min].

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