

**Synthesis and comparative conformational energetics of  
D-phenylalanylsarcosine and its cyclic dehydration product,  
(R)-1-methyl-3-(phenylmethyl)-2,5-piperazinedione**

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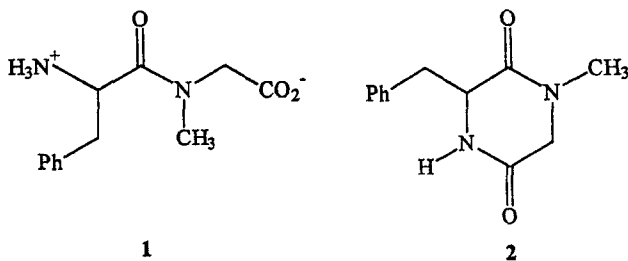
**Summary.** Synthetic protocols are presented both for D-PheSar and the corresponding cyclised diketopiperazine, prepared from N-t-butoxycarbonyl-protected D-PheSar. Deprotection conditions could be manipulated to yield either D-Phenylalanylsarcosine or (R)-1-methyl-3-(phenylmethyl)-2,5-piperazinedione. Molecular modelling revealed several low energy conformers which contained a Z-peptide bond and which were readily amenable to cyclisation. Cyclisation was found by HPLC to be fastest in strongly acidic conditions.

**Keywords:** Amino acids – Diketopiperazine – Conformational analysis – Sarcosine – Acid cyclisation

**Abbreviation:** HBTU, o-Benzotriazolyl-tetramethyluronium hexafluorophosphate

**Introduction**

The dipeptide phenylalanylsarcosine has been reported to have biological activity, specifically against enkephalinase from mouse striatum for which L-PheSar showed 50% inhibition at 100  $\mu$ M (Llorens et al., 1980). We recently prepared samples of D-PheSar (**1**) by standard peptide synthetic procedures, and found that the product isolated was sometimes the cyclic dehydrated form, the 2,5-piperazinedione (**2**).



In view of the report of the biological activity of PheSar, we felt it appropriate to describe well-authenticated routes to both **1** and **2**. We also describe HPLC analysis of the ready cyclisation of **1** to **2** under acidic conditions, along with a conformational analysis of **1** to explore the cyclisation process.

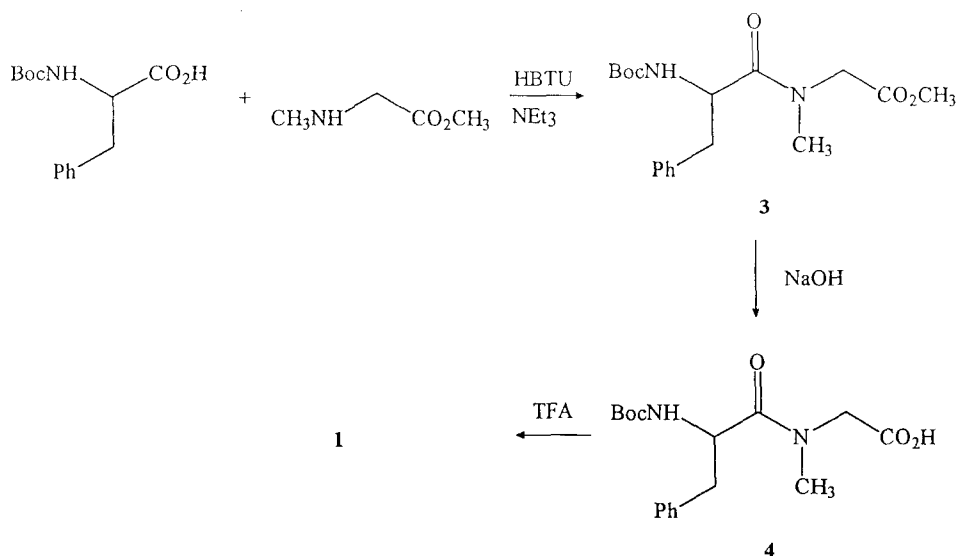
## Materials and methods

### Synthesis

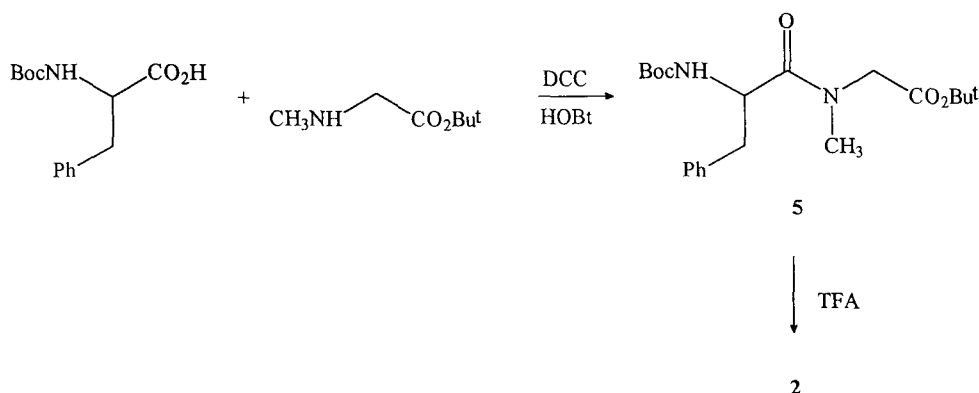
For coupling reactions, anhydrous reactants and dry solvents were used. All amino acid derivatives were purchased from Nova Biochem (UK). All remaining materials were supplied by Aldrich and were of reagent grade.  $^1\text{H}$  NMR spectra were recorded on a Jeol JNM EX270 spectrometer operating at 270 MHz. Chemical shifts are reported in parts per million (ppm) relative to TMS. Splitting patterns are abbreviated as: s, singlet; d, doublet; t, triplet; q, quartet; m, unresolved multiplet and br, broad. Fast atom bombardment mass spectra (FAB-MS) were obtained by means of a Kratos-Concept Instrument (Xe-beam bombardment).

HPLC analysis was performed on a system consisting of a Kontron 32X pump and Kontron 332 detector operating at 210 nm linked to a Philips PM 8251 chart recorder. Preparative HPLC was effected using a Whatman Magnum 20 column (22 mm  $\times$  50 cm) with ODS3 Partisil 10  $\mu\text{m}$  packing at a flow rate of 10 ml/min using a gradient elution system consisting of 0.1% trifluoroacetic acid in water (solvent A) and 60% acetonitrile and 0.1% trifluoroacetic acid in water (solvent B). The elution was increased from 0–30% solvent B in 150 minutes. Analytical HPLC used a Hichrom Kromasil 100-5C18 column (4.6 mm  $\times$  25 cm) with 5  $\mu\text{m}$  ODS3 packing. The flow rate was 1 ml/min and the gradient elution system used was 0–80% solvent B in 32 minutes.

D-PheSar (**1**) and (R)-1-methyl-3-(phenylmethyl)-2,5-piperazinedione (**2**) were synthesised according to Schemes 1 and 2 respectively.



**Scheme 1.** Synthesis of D-PheSar



**Scheme 2.** Synthesis of (R)-1-methyl-3-(phenylmethyl)-2,5-piperazinedione

(R)-1-methyl-3-(phenylmethyl)-2,5-piperazinedione (**2**)

To a solution of Boc-D-Phe (1.0 g, 3.77 mmol) in dichloromethane (20 ml) were added H-Sar-OBu<sup>t</sup> (685 mg, 3.77 mmol) and hydroxybenzotriazole (577 mg, 3.77 mmol) to form a suspension, which was dispelled by adding the minimum volume of dimethylformamide. This solution was cooled in an ice-water bath and stirred whilst dicyclohexylcarbodiimide (855 mg, 4.15 mmol) was added. The reaction mixture was allowed to warm to room temperature and stirred overnight. The precipitated dicyclohexylurea was removed by filtration and the solvent removed *in vacuo* to give an oil, which was taken up in water (100 ml) and this solution extracted with ethyl acetate (3 × 50 ml). The combined organic phases were washed with saturated NaHCO<sub>3</sub> (2 × 50 ml), water (50 ml), 10% citric acid (2 × 50 ml), water (50 ml), and finally with brine (2 × 50 ml). The organic phase was dried with MgSO<sub>4</sub>, filtered and evaporated to dryness to give crude product **5** (1.32 g, 89%).

The protected dipeptide was taken up in neat trifluoroacetic acid and stirred for one hour at room temperature. The excess trifluoroacetic acid was removed *in vacuo* to give an oil, which was triturated with diethyl ether. The oily solid obtained was dissolved up in water and freeze-dried, to give crude product **2** as an off-white powder (750 mg, 85%). This was then purified by preparative HPLC.

<sup>1</sup>H NMR (CDCl<sub>3</sub>): 8.12 (br s, 1H, NH), 7.15–6.95 (m, 5H, Ph-Phe), 4.0 (m, 1H, CH- $\alpha$ -Phe), 3.32 (d, 1H, CH<sub>2</sub>- $\alpha$ -Sar), *J* = 16.9 Hz), 3.26 (s, 3H, N-CH<sub>3</sub>-Sar), 2.45–2.95 (m, 2H, CH<sub>2</sub>- $\beta$ -Phe), 2.45 (d, 1H, CH<sub>2</sub>- $\alpha$ -Sar, *J* = 17.2 Hz).

HR-MS: calculated for C<sub>12</sub>H<sub>14</sub>N<sub>2</sub>O<sub>2</sub> 218.1055, found 218.1054. HPLC retention time: 18 min. Optical rotation: –117° (*c* = 1.0, water).

N-Boc-D-Phenylalanylsarcosine methyl ester (**3**)

To a solution of SarOMe.HCl (0.26 g, 1.9 mmol) and triethylamine (0.22 g, 2.2 mmol) in acetonitrile (10 ml) was added Boc-D-Phe (0.50 g, 1.9 mmol). The solution was cooled to 0°C, and HBTU (0.78 g, 2.0 mmol) added to form a milky solution. The reaction was then allowed to stir at room temperature for 16 hours. The solvent was evaporated *in vacuo*, the residual solid taken up in water (25 ml) and this solution extracted with ethyl acetate (3 × 25 ml). The combined organic phases were washed with saturated NaHCO<sub>3</sub> (2 × 25 ml), water (25 ml), 10% citric acid (2 × 25 ml), water (25 ml), and finally brine (2 × 25 ml). The organic phase was dried with MgSO<sub>4</sub>, filtered and evaporated to dryness to give protected **3** (0.4 g, 60%). This product was used in the subsequent steps without further purification.

<sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.35–7.10 (m, 5H, Ph-Phe), 5.40 (d, *J* = 13 Hz, 1H, NH-Phe), 4.80 (m, 1H, CH-Phe), 4.0 (q, *J* = 23 Hz, 2H, CH<sub>2</sub>- $\alpha$ -Sar), 3.65 (s, 3H, N-CH<sub>3</sub>-Sar), 2.90 (m, 2H, CH<sub>2</sub>- $\beta$ -Phe), 1.37 (s, 9H, CH<sub>3</sub>(Boc)-Phe).

### N-Boc-D-Phenylalanylsarcosine (**4**)

To a solution of N-Boc-D-phenylalanylsarcosine methyl ester (**3**) (150 mg, 0.429 mmol) in tetrahydrofuran (1.3 ml) was added sodium hydroxide (0.43 ml of 1 M, 1 eq.) and the two-phase mixture stirred for 3.5 hours at ambient temperature. Water (1.3 ml) was added to the reaction mixture and organic solvent removed *in vacuo*. The aqueous portion was acidified to pH 3 with 1 M hydrochloric acid, extracted with ethyl acetate and the organic solution dried over anhydrous magnesium sulphate, filtered and evaporated to give **4** as a gum (110 mg, 76%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.26–7.16 (m, 5H, Ph-Phe), 6.47 (br s, 1H, OH), 5.64 (m, 1H, NH-Phe), 4.60–4.91 (m, 1H, CH- $\alpha$ -Phe), 4.05 (q, J = 20 Hz, 2H, CH<sub>2</sub>-Sar), 3.02–2.91 (m, 5H, CH<sub>2</sub>- $\beta$ -Phe and NCH<sub>3</sub>-Sar), 1.38–1.35 (m, 9H, CH<sub>3</sub>(Boc)-Phe).

### D-Phenylalanylsarcosine (**1**)

N-t-Butoxycarbonyl-D-phenylalanylsarcosine (46 mg, 0.137 mmol) was dissolved in a solution of trifluoroacetic acid: dichloromethane (1.5 ml of a 1:1 mixture) and stirred at room temperature. Deprotection of the amino group was quantitative within 15 minutes (as monitored by NMR). Evaporation *in vacuo* afforded D-PheSar **1** as the hygroscopic trifluoroacetic acid salt (58 mg, quant).

<sup>1</sup>H NMR (D<sub>2</sub>O): 7.24–7.07 (m, 5H, Ph), 4.59 (t, J = 7 Hz, 1H, CH- $\alpha$ -Phe), 3.93 (q, J = 18 Hz, CH<sub>2</sub>-Sar), 3.01 (m, 2H, CH<sub>2</sub>- $\beta$ -Phe), 2.74, (s, 3H, N-CH<sub>3</sub>-Sar).

HR-MS (FAB): calculated for C<sub>12</sub>H<sub>17</sub>N<sub>2</sub>O<sub>3</sub> (M + 1) 237.1239, found 237.1240. HPLC retention time: 16 min. Optical rotation: –21° (c = 0.9, water).

### Computational methods

Conformational searches were carried out on a Silicon Graphics Indigo machine using SYBYL (SYBYL 6.2, 1995), using the standard Tripos force-field. The systematic grid search method described by Schiller was employed (Wilkes and Schiller, 1987; Schiller et al., 1993). The procedure was briefly as follows. A starting structure of the zwitterionic peptide **1** was fully minimised. Each rotatable bond of **1** was then scanned in 30° increments using the Systematic Search facility of SYBYL, which eliminates conformations solely on the basis of unfavourable Van der Waals' contacts. From the set of conformations produced, those within 5 kcal/mol of the lowest energy conformer were selected for further processing. This resulting ensemble of conformations was then grouped into families containing structures whose torsion angles differed by  $\pm 30^\circ$ . The lowest energy structure within each family was then fully minimised to yield the final set of conformers. A distance-dependent dielectric constant of 78 was used in the energy minimisations to simulate an aqueous environment.

### HPLC analysis of cyclisation

Stock solutions of **1** (0.5 mM) were made up in buffer solutions as follows: pH 1.16 (0.1 M trifluoroacetic acid), pH 3.96 (0.1 M acetic acid solution, adjusted with sodium hydroxide), pH 6.88 (1:1 v/v mixture of 0.05 M sodium hydrogen phosphate and potassium dihydrogen phosphate solutions), pH 9.37 (0.1 M sodium borate solution) and pH 12.94 (0.1 M sodium hydroxide solution). An aliquot (50  $\mu$ l) of each solution was mixed with an aliquot (50  $\mu$ l) of a standard solution of benzoic acid (0.82 mM in water) and a portion (20  $\mu$ l) loaded onto the HPLC column (HPLC conditions as described above). This process was repeated at intervals of 0, 1, 6, 15 and 22 days. The height of the standard peak varied by 25% over the course of the experiment. A calibration curve of concentration vs height was also determined for stock solutions of **2**. Data was converted from peak height to percentage conversion of **1** to **2** assuming a 1:1 stoichiometry. Where appropriate, data were fit to a monoexponential decay function using FigP (BIOSOFT, Cambridge, UK) to obtain the pseudo-first order rate constant for the conversion.

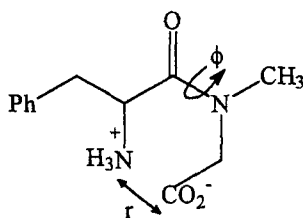
## Results and discussion

Initial attempts to synthesise D-PheSar yielded solely the diketopiperazine **2**, as shown in Scheme 2. The reactions of Scheme 1 allowed us to monitor the individual stages of the N- and C-deprotection of **3**. Removal of the C-terminal methyl ester, using aqueous sodium hydroxide, yielded the anticipated t-butoxycarbonyl-protected derivative, **4**. Deprotection of **4** in neat trifluoroacetic acid also yielded **2** as the sole isolated product after work-up. However, deprotection using a solution of trifluoroacetic acid in dichloromethane (1:1, v/v), yielded D-PheSar (**1**) quantitatively. Thus it is possible to direct product formation between **1** or **2** by selection of the deprotection conditions. It is worth noting that initially-pure aqueous solutions of **1** showed signs (by HPLC) of **2** (*vide infra*).

A previous synthesis of L-PheSar has been communicated, but with no analytical or characterisation details were given [1517]. Facile cyclisation of sarcosyldehydrophenylalanine to the corresponding diketopiperazine 1-methyl-3-trans-benzylidenepiperazine-2,5-dione in water has been reported [1522]. Synthesis of **2** has been reported via the conventional route of diketopiperazine formation from the methyl ester of racemic PheSar [1520]. A diketopiperazine has been reported as a weak endothelin antagonist with an  $IC_{50}$  value of  $22.5\mu M$ , selective for the  $ET_A$  receptor [1524], but it is not yet apparent whether the seat of biological activity is the diketopiperazine function or if this serves as a peptidomimetic fragment. In view of the potential of diketopiperazines to provide endothelin receptor binding, it is also possible that the anti-enkephalinase activity reported in [1517] may arise from a contribution by the diketopiperazine of PheSar and/or PheSar itself.

The ease of cyclisation of **4** to **1** is surprising. Normally, diketopiperazine formation is spontaneous only if the N-protection is removed first, to leave the C-terminal ester in place [1523]. This promotes intramolecular nucleophilic attack of the free amino group on the ester. In view of this we analysed the conformational basis of the cyclisation of **1** to **2** in order to determine the relative orientation of the amino group and carboxyl groups.

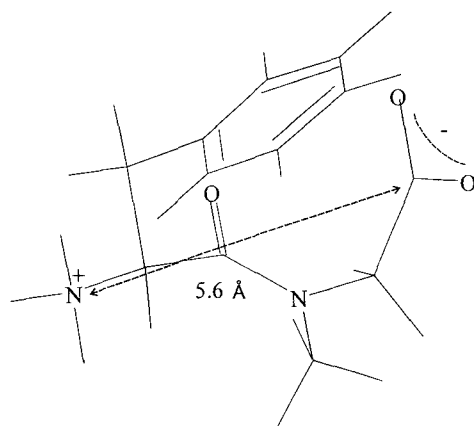
Table 1 shows the torsional angle ( $\phi$ ) of the peptide bond, and distance between the amino nitrogen and carboxyl carbon atoms ( $r$ ) for the ten lowest energy conformers of **1** as defined in **6**.



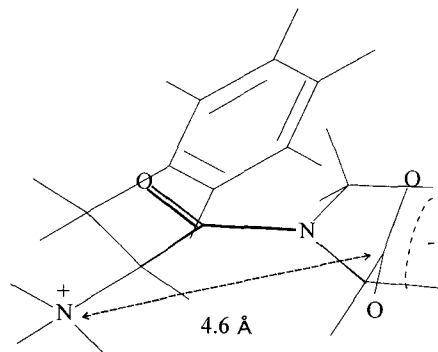
The minimum energy conformation of **1** (conformer number 1) possesses a E-peptide bond (see Fig. 1). The next lowest energy conformer has a Z peptide bond (see Fig. 2). A Z-peptide bond would be expected to be geometrically more amenable to cyclisation than an E peptide bond. The closest

**Table 1.** Peptide bond torsional angle  $\phi$ , and distance between the protonated nitrogen and carboxyl carbon atom ( $r$ ), for the ten lowest energy conformers of **1** as defined by **6**

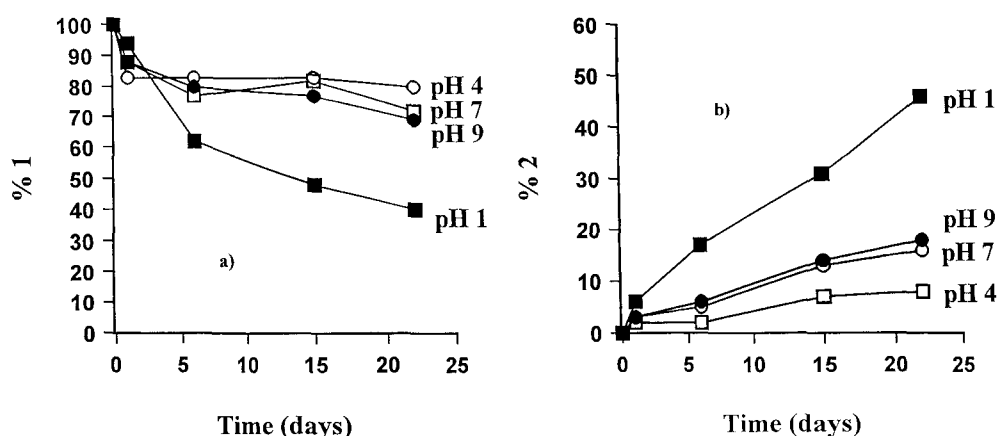
Conformer number	$\phi(^{\circ})$	$r(\text{\AA})$	Energy (kcal/mol)	E or Z Peptide bond
1	179	5.6	3.63	E
2	4	4.6	3.83	Z
3	179	5.4	4.11	E
4	0	4.1	4.22	Z
5	176	4.9	4.40	E
6	3	4.4	4.57	Z
7	180	5.0	4.64	E
8	180	5.2	4.87	E
9	1	4.3	5.05	Z
10	177	5.1	5.19	E



**Fig. 1.** Lowest energy conformation for **1**, showing the distance from the carboxylate carbon atom to the protonated nitrogen atom



**Fig. 2.** Conformer 2 (Z-peptide bond) of **1** showing the distance from the carboxylate carbon atom to the protonated nitrogen atom



**Fig. 3.** Graphs showing **a)** percentage loss of **1** and **b)** percentage formation of **2** with time at varying pH, as monitored by HPLC

approach possible for the E-peptide (Fig. 1) of the carboxyl carbon and the ammonium nitrogen is 5.6 Å. However, for the Z-peptide bond (Fig. 2) this distance is smaller (4.6 Å) and energetically inexpensive rotation of the  $\text{N}(\text{CH}_3)\text{-CH}_2(\text{CO}_2^-)$  bond allows close approach of the N and C heavy atoms which must bond for cyclisation. Table 1 also shows that there are several low energy conformers of **1** containing a Z-peptide bond, all of which have lower values of  $r$  than the E-peptides, and thus there would be expected to be a significant population of Z-peptides in solution. This type of conformational analysis has been applied previously to an unusual process for diketopiperazine formation from tripeptides, with intermediate formation of a 9-membered ring with subsequent rearrangement (Carpenter et al., 1994).

In order to explore further the differential product formation under various deprotection conditions, we investigated the pH-dependence of the cyclisation by HPLC over the range pH1–13. Figure 3a shows a graph of the percentage loss of **1** with time as a function of the pH of the solution. Assuming that pseudo-first order conditions applied, the observed rate constant of the acid-catalysed cyclisation at pH1 was  $0.05 \text{ day}^{-1}$ , giving a half life for the conversion of 14 days. Within the accuracy of the experiment, there was little significant difference in the rate of loss of **1** at pH4, 7 or 9. There was no observable formation of **2** at pH13 over the period that the reaction was monitored. The formation of **2** broadly mirrored the loss of **1**, as shown in Figure 3b. Thus it can be seen that the intramolecular cyclisation is accelerated in acidic conditions.

### Conclusion

We report the synthesis and characterisation of authentic samples of **1** and **2** and have shown that the ready, acid-promoted, cyclisation of **1** to **2** occurs by way of a conformationally accessible low energy Z-peptide bond conformer of **1**.

### Acknowledgements

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