Phosphorus-based Reagents in Peptide Synthesis: Synthesis of Methionine-Enkephalin and the Solution Conformation of its *N*-Diphenylphosphinoyl Derivative

D. David Smith,[a] Kenneth G. Boyd,[b] David Hopton,[b] Robert L. Baxter*[,b] and Robert Ramage[b]

[a] *Division of Biochemistry, Department of Biological Sciences, Creighton University, 2500 California St., Omaha, Nebraska 68178, USA*

[b] *Edinburgh Centre for Molecular Recognition, Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, UK*

Met[5]enkephalin **1** was synthesized by solution phase synthesis using the diphenylphosphinoyl (Dpp) group for α-amino group protection and diphenylphosphinoyl–carboxylic acid mixed anhydrides for carboxylate activation. These reagents proved to be compatible with both tyrosine and methionine side chain functionalities. A ^{1}H NMR study of the solution conformation of the N-terminal-protected pentapeptide DppMet[5]enkephalin **2** in [$^2H_6$]dimethyl sulfoxide suggests that the peptide backbone of this derivative adopts a major conformation possessing a type I′ β-bend between residues Gly[2]–Gly[3] with the side chains of the Tyr[1] and Phe[4] lying on the same side of the plane of the bend. The predominant conformations of the Phe[4] and Met[5] side chains appear to be tg^+.

Historically the most widely employed protecting groups for sequential protection of the α-amino function in solution-phase peptide synthesis have been the benzyloxycarbonyl[1] (Z) and the *tert*-butoxycarbonyl[2] (Boc) groups. The Z group can generally be removed by hydrogenolysis or by hydrolysis with a strong acid. Removal of Boc protection can be achieved under less forceful conditions using trifluoroacetic (TFA) or hydrochloric acid in an organic solvent. However, a serious drawback associated with use of these acid-labile protecting groups is the generation, under acidolysis conditions, of benzyl and *tert*-butyl carbocations which can react with the side chain functional groups of tyrosine, tryptophan, cysteine and methionine.[3] More recently the base-labile[4] fluorenylmethoxycarbonyl (Fmoc) protecting group has proved to be a useful alternative in both solution[5] and solid-phase[6] peptide synthesis. The base lability of the Fmoc group allows acid-labile groups to be used for protection of side chain functional groups introducing orthogonality into the strategy. However, dibenzofulvene, produced by cleavage of the Fmoc group, can polymerise to afford insoluble products which can be difficult to remove. An alternative approach involves the use of the acid-labile diphenylphosphinoyl (Dpp) group[7] for sequential α-amino protection. Preliminary studies in which this methodology was used in the synthesis of the C-terminal tetrapeptide of gastrin showed that use of the diphenylphosphinoyl group is compatible with phosphinic–carboxylic acid mixed anhydride coupling methods[8] and with the presence of methionine and tryptophan side chain functional groups.[7] To be generally useful in solution-phase peptide synthesis, however, the diphenylphosphinoyl protecting group has to be applicable to syntheses of peptides containing other combinations of potentially reactive side chains. Here we describe the application of this methodology to the synthesis of Met[5]enkephalin **1**, which contains both potentially reactive methionine and tyrosine residues, and conformational studies of its synthetic precursor, the N-terminal-blocked diphenylphosphinoyl derivative DppMet[5]enkephalin, **2**.

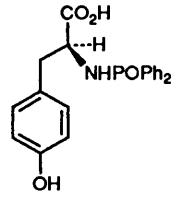
H–Tyr–Gly–Gly–Phe–Met–OH **1**

H–Tyr–Gly–Gly–Phe–Leu–OH **1a**

Dpp–Tyr–Gly–Gly–Phe–Met–OH **2**

Results and Discussion

Synthesis of DppMet[5]enkephalin **2** *and Met[5]enkephalin* **1**.—The synthesis of amino acid diphenylphosphinoyl derivatives was carried out essentially as previously described.[7] Optimisation of the work-up and purification procedures in the preparation of DppTyr **3** from tyrosine methyl ester, which had proved troublesome in earlier studies,[7] have enabled this conversion to be carried out in 65% overall yield. A perceived advantage of diphenylphosphinic–carboxylic mixed anhydride methodology was that the potentially reactive phenolic group of tyrosine might be protected *in situ* during the activation step. Thus treatment of DppTyr **3** with two molar equivalents of diphenylphosphinoyl chloride should afford the *N*,*O*-bis(diphenylphosphinoyl)tyrosine mixed anhydride **4** which would offer effective protection of the phenolic hydroxy group.



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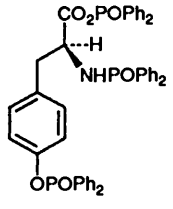

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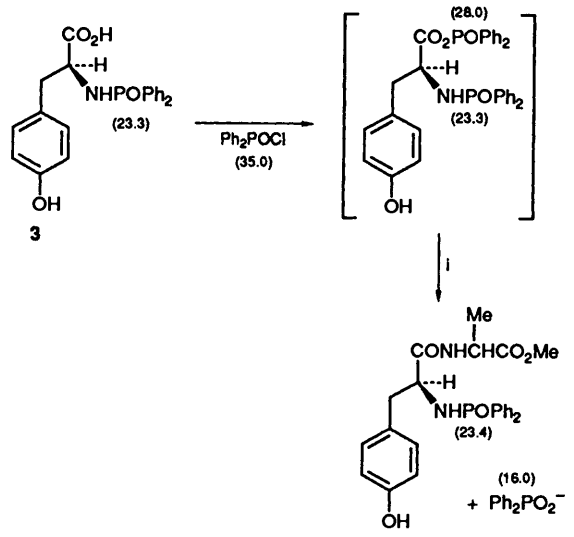
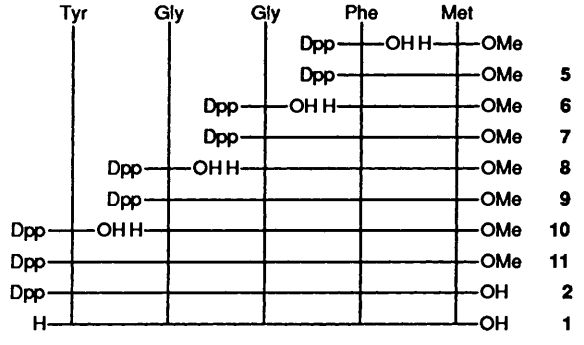
To see if this was indeed the case a series of trial coupling reactions with DppTyr **3** and L-alanine methyl ester was carried out and the formation of reaction products was monitored by ^{31}P NMR spectroscopy (Scheme 1). The ^{31}P NMR spectrum of the reaction mixture of DppTyr **3** with two molar equivalents of diphenylphosphinoyl chloride showed two signals, at δ_P 28.0 and 23.3, corresponding to the Ph_2PO_2CO– (mixed anhydride) and Ph_2PONH– groups of DppTyr–diphenylphosphinic carboxylic acid mixed anhydride, respectively, and a signal at δ_P 35.0 corresponding to unchanged diphenylphosphinoyl chloride. Even after 2 h no significant signal corresponding to formation of the Ph_2PO_2Ar group of the expected triderivatised tyrosine could be detected [*cf.* the ^{31}P NMR spectrum of *N*,*O*-bis(diphenylphosphinoyl)tyrosine methyl ester in which the resonance of the phosphinate is observed at δ_P 30.6].[7] On addition of L-alanine methyl ester the signals due to the mixed



Scheme 1 Diphenylphosphinic anhydride coupling of DppTyr **3** with alanine methyl ester. The signals observed in the 24 MHz ^{31}P NMR spectrum of the reaction mixture are shown in parentheses. *Reagent:* i, $MeCH(NH_2)CO_2Me$.

anhydride and diphenylphosphinoyl chloride rapidly diminshed with concomitant appearance of a signal at δ_P 16.0 due to formation of diphenylphosphinate anion. Although it appears that no protection of the tyrosine hydroxy group is conferred under these conditions it is significant that the phenolic oxygen is not sufficiently nucleophilic to react with the mixed anhydride.

The overall strategy employed for the synthesis of DppMet[5] **2** and hence Met[5] **1** is outlined in Scheme 2. The first three



Scheme 2 Solution-phase synthesis of Met[5]enkephalin **1**

coupling reactions were carried out in a straightforward manner to afford DppGlyGlyPheMetOMe **9** from MetOMe hydrochloride[9] in an overall yield of 33%. Despite the fact that the synthesis of compound **9** was carried out without chromatographic purification of intermediates, HPLC analysis of the product indicated a purity of > 98% at this stage.

In contrast to the trial reactions between DppTyr and L-alanine methyl ester described above, coupling of DppTyr with GlyGlyPheMetOMe **10**, using two molar equivalents of diphenylphosphinoyl chloride, gave a mixture of the desired product, DppTyrGlyGlyPheMetOMe **11**, and a less polar derivative. Spectral data on the mixture suggested the presence of an additional diphenylphosphinoyl group in the impurity, which we conjecture to be the *O*-diphenylphosphinoyl derivative of compound **11**. Acid hydrolysis of the mixture afforded only TyrGlyGlyPheMetOMe, which lends support to this interpretation. When the reaction was repeated using a single molar equivalent of diphenylphosphinoyl chloride only the desired product **11** was obtained. Why partial derivatisation of the phenolic hydroxy group of the tyrosine side chain should occur in this reaction but not in the model studies is unclear but it should be noted that similar results have been observed in reactions with carboxylic acid-based mixed anhydride reagents.[10]

Mild alkali hydrolysis of the ester **11** afforded DppMet[5] **2** which was our target compound for conformational studies. Completion of the synthesis of Met[5]enkephalin was achieved by acid hydrolysis of derivative **2**. To prevent esterification of the free peptide this reaction was carried out using 2 mol dm^{-3} HCl in dioxane–water rather than the methanolic HCl procedure used during construction of the chain. Under these conditions removal of the diphenylphosphinoyl group is significantly slower, requiring 6 h for completion rather than the 2 h typically required for methanolic HCl hydrolysis. Purification of the free pentapeptide was achieved by sequential gel filtration and partition chromatography to give compound **1** in 7% overall yield from MetOMe. The results of the synthetic work indicate that the diphenylphosphinoyl protecting group and diphenylphosphinic acid mixed anhydride methodology are suitable for the construction of peptides containing phenolic side chains.

Conformational Studies of DppMet[5] **2**.—The opiate activity of Met[5] and Leu[5] enkephalins (**1** and **1a**, respectively) was originally ascribed to their binding to the morphine μ receptor from which it was suggested that their conformations at the site of binding might resemble that of the rigid opium alkaloid.[11] More recently it has been shown that there are at least three subtypes of opiate receptors (μ, δ and κ)[12] each of which has been postulated to couple with a specific G_i protein.[13] The fact that enkephalins are known to bind to both μ and δ receptors implies that the unmodified peptides may adopt two or more different, and distinct, conformations in their binding to different receptors.[12,14] The 'two-stage capture model' proposed for peptide hormone–receptor interaction[15] requires two distinct conformational changes in the effector peptide; the first is effected by trapping of the molecule by the interfacial environment of the cell membrane and extracellular fluid and the second by binding to the receptor site itself. It would be congruent with this theory if the peptides were shown to adopt distinct conformations under different conditions mimicking the environments of the membrane surface and receptor binding sites. Indeed both minimum-energy calculations[16] and the structural evidence obtained to date do indicate that these peptides can adopt a range of different conformations under very similar environmental conditions. For example two distinct structural types appear to be stabilised in the solid state. Crystallographic studies of both natural enkephalins and the bromo derivatives, TyrGlyGly(4'-Bromo)PheLeu and BocTyrGlyGly(4'-Bromo)PheMet, have shown that these molecules can adopt a dimeric antiparallel β sheet structure.[17] A second crystalline conformer, exhibited by both Leu[5]enkephalin and TyrGlyGly(4'-Bromo)PheMet,[18] is characterised by a type I' β bend between the adjacent glycine residues which is stabilised by TyrNH···PheCO and TyrCO···PheNH hydrogen-bonding interactions. Evidence for several conformational forms has also been found in solution studies. ^1H NMR studies have suggested that in dimethyl sulfoxide (DMSO) solution, a medium suggested to mimic the interfacial environment,[19,20b] both Met[5] and Leu[5] adopt either a structure similar to the Gly^2–Gly^3 type I' β bend found in the solid state[20] or one characterised by a type I' (or type I) β bend between the Gly^3 and Phe^4 residues stabilised by hydrogen bonding between the Gly^2 carbonyl and the Met [or Leu] amide NH.[21] Recently, based on evidence from TRNOE studies, it has been suggested that in a phospholipid environment both Leu[5] and the

Table 1 ^{1}H NMR assignment of DppMet^5 in [$^2H_6$]DMSO[a]

Residue	H^α	H^β	NH	Other
Tyr^1	3.41 (8.2)	2.90, 2.88	5.87 (8.2)	7.06, 6.78 (ABA'B')
Gly^2	3.71, 3.80 (17.2)		8.56 (5.8, 5.8)	
Gly^3	3.65, 3.75 (17.3)		8.73 (5.8, 5.8)	
Phe^4	4.54 (4.7, 7.7, 9.0)	3.03, 2.81 (4.7, 9.0)	7.87 (7.7)	7.2
Met^5	4.21 (3.9, 8.5, 9.5)	1.98 (3.9, 9.5)	8.26 (8.5)	2.45, 2.03

[a] Coupling constants are shown in parenthesis.

Table 2 Summary of ROE and NOE interactions for DppMet^5 **2** in [$^2H_6$]DMSO

	Tyr^1	Gly^2	Gly^3	Phe^4	Met^5
$\delta_{N,N}(i, i+2)$		+			
$\delta_{N,N}(i, i+1)$	+	+	+	+	
$\delta_{\alpha,N}(i, i+1)$	+	+	+	+ +	
$\delta_{\alpha,N}(i, i)$	+	+	+	+	+
$\delta_{\beta,N}(i, i)$	+			+	+
$\delta_{N,ArH}(i, i)$	Dpp ArH				

enkephalin agonist Tyr(D-Ala)GlyPheLeu adopt a fourth type of conformation, involving a type II' β bend between the Gly^3 and Phe^4 residues and a γ turn at the Gly^2 (or D-Ala^2) residue.[22]

In view of the conformational flexibility inherent in these peptide structures the crystal structures determined for these molecules, dominated as they are by crystal-packing forces or intermolecular interactions, may be of limited relevance to the membrane interface or receptor environments. While perhaps more relevant to physiological conditions, earlier ^{1}H NMR studies of enkephalin derivatives have been restricted in that they have, with few exceptions,[20b,22] been based on single-dimensional spectra obtained in solutions of relatively high concentration, a condition which can give rise to association.[21d]

We chose to examine the Dpp-blocked derivative of Met^5-enkephalin rather than the free peptide for two reasons: first, while functionalisation of the N-terminal amine inhibits formation of dimers by denying amine–carboxylate ionic interactions, derivatisation of the terminal amine position does not necessarily lead to loss of activity.[23] Secondly, the presence of a bulky hydrophobic N-terminal blocking group should help to stabilise a single conformation.

Conformational Analysis of DppMet^{5}enkephalin.—The assignments of resonances and coupling constants in the ^{1}H NMR spectrum of DppMet^{5}enkephalin in [$^2H_6$]DMSO are shown in Table 1. While the resonances of the Tyr^1, Phe^4 and Met^5 residues could be assigned in a straightforward manner on the basis of single-dimension spin-decoupling experiments and 2D-COSY interactions, discrimination of the Gly^2 and Gly^3 spin systems required consideration of inter-residue through-space interactions (Table 2). In the two-dimensional ^{1}H NMR rotating-frame NOE spectrum (2D-ROESY) the lower-frequency glycine NH triplet, at δ 8.56, showed connectivity with the H^α of Tyr^1, allowing assignment of this signal to the Gly^2 residue. This was substantiated by the observation of a ROESY interaction between the H^α of Gly^3 and the NH of Phe^4. The presence of inter-residue ROEs between the NH protons of Gly^2 and Gly^3 and of Gly^3 and Phe^4 are also indicative of adoption of a locally folded conformation. These

Table 3 Temperature coefficients for DppMet^5 **2** amide protons in [$^2H_6$]DMSO solution

Amide proton	Tyr^1	Gly^2	Gly^3	Phe^4	Met^5
Temperature coefficient (ppm/° × 10^3)	+8.4	+4.1	+6.6	+4.6	+5.0

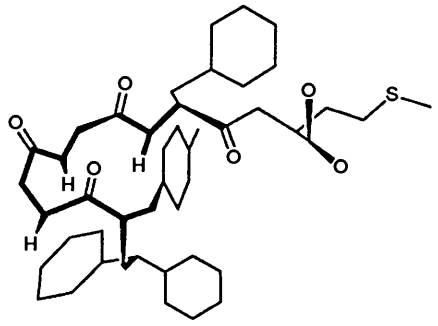


Fig. 1 Backbone conformation of DppMet^5 **2** from NMR data. For clarity only the peptide carbonyl oxygens and the amide hydrogens of Gly^2, Gly^3 and Phe^4 are shown.

ROE interactions, supported by single-dimension NOE difference measurements, are consistent with adoption of a Gly^2–Gly^3 type I' (or type I) β turn which would bring these protons within approximately 2.5 Å of each other. In contrast, the presence of a stable type II' β turn, as proposed for the conformation of Leu^{5}enkephalin in a phospholipid environment,[22] would preclude the observed interaction of the Gly^2 and Gly^3 NH protons. The presence of a type I' β turn conformation is also supported by the 17.3 Hz geminal coupling constant of the Gly^3 methylene protons, which indicates a ψ torsion angle of close to 0° (or 180°). While this torsion angle for Gly^3 is in agreement with the value predicted for a type I' (0°) β turn [or possibly a type I (± 30°) β turn] it can hardly be accommodated in a type II' β turn which requires a ψ angle of ± 120°. Adoption of a favoured peptide backbone major conformation is also suggested by other through-space ^{1}H–^{1}H interactions and by coupling-constant data. The Phe^4 H^α proton has a strong NOE interaction with the NH of Met^5, suggesting in-plane geometry, and this relative rigidity is supported by the Phe^4 H^αNH coupling and observation of an ROE interaction between the Phe^4 H^α and one of the Phe H^β protons which can be best accommodated where the Phe^4 θ dihedral angle (HNCH$^\alpha$) is between 150 and 180°. The 8.2 Hz coupling between the H^α and NH protons of the Tyr^1 residue suggests a θ angle for this residue of close to 0° (a θ angle of around 180° being precluded by interactions of the diphenylphosphinoyl group with the peptide backbone) and this geometry is also supported by the presence of an ROE interaction between the Tyr^1 NH and the NH of Gly^2. Modelling of the structure using the NMR data gives a type I' β turn backbone conformation similar to that suggested for Leu^{5}enkephalin from the X-ray diffraction study of Smith and Griffin[18a] (Fig. 1). While a type I β-turn cannot be precluded by the NMR data alone this conformation is highly disfavoured by the severe steric interactions it confers to the N-terminal region of the molecule. A logical inference from this model would be that the type I' β turn structure could be stabilised by a weak (out of plane) TyrCO–PheNH hydrogen bond. This is supported to some extent by the observation of a lowered temperature coefficient for the Phe^4 amide proton (Table 3). While the similar value for the temperature coefficient of the Gly^2 amide proton cannot be explained by H-bonding this is also consistent with the type I' β turn model which suggests that

the mobility of this proton may be restricted by the close proximity of one of the rings of the diphenylphosphine group.

It is noteworthy that this backbone conformation has previously been advanced for Met^{5}enkephalin in ^2H_2O solution on the basis of the observation of an inter-residue NOE between the aromatic protons of the Tyr^1 and Phe^4 residues[20a] which can only occur where the side chains of both these residues have *gg* conformations. However, in the case of DppMet^5 no NOE or ROE interaction could be detected between these aromatic protons, suggesting that the predominant conformations of the side chains are dissimilar from the model proposed for the free peptide. Consideration of the $H^{\alpha}H^{\beta}$ coupling constants permits evaluation of the averaged conformations of the Phe^4 and Met^5 side chains of the peptide. Application of the treatment described by Pachler[24] to the observed Phe^4 and Met^5 $H^{\alpha}H^{\beta}$ coupling constants gives an approximate distribution of 75% *tg* and 25% *gg* for both side chains. Using these values to limit the solutions predicted by the Feeney equations[25] allows prediction of rotamer populations of 63% tg^+, 12% tg^- and 25% *gg* for the Phe^4 side chain and 69% tg^+, 2% tg^- and 29% *gg* for the Met^5 side chain. Complications imposed by long-range P–H coupling and lack of significant shift dispersion between the H^{β}s of Tyr^1 even at 600 MHz precluded evaluation of rotamer populations for the tyrosine side chain. The presence of the bulky diphenylphosphinoyl group dominates the conformational options available for the N-terminal residue side chain, and, indeed, energy minimisation using a model in which the peptide backbone was fixed within the angular constraints derived from the NMR data suggests adoption of a predominant tg^+ side chain conformation for the Tyr^1 residue.

Although the data are quite consistent with a structure for DppMet^5 similar to that suggested for Leu^{5}enkephalin by Smith and Griffin[18a] these should not be taken to imply that Dpp Met^5 exists in a single unique backbone conformation in solution. Our interpretation is that the Gly^2–Gly^3 type I′ β bend structure represents the major and most highly structured conformational state available to the molecule in dilute solution.

Experimental

M.p.s were determined using a Buchi 510 melting point apparatus and are uncorrected. Optical rotations were measured on a AA1000 polarimeter, and $[\alpha]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}. IR spectra were recorded on a PE781 spectrophotometer, on mulls prepared with $CHBr_3$; UV spectra were obtained on solutions in MeOH using a Pye Unicam SP8-400 spectrophotometer. NMR spectra were recorded using Varian VXR600, Bruker WH360 and JEOL FX-60-Q instruments. Unless otherwise stated spectra were recorded in $CDCl_3$. Spectral data on DppMet^5 **2** were obtained on a 5.2 mmol dm^{-3} solution in [$^2H_6$]DMSO. In the COSY experiments the data sets of consisted of 256 t_1 increments of 32 transients and those in the ROESY experiments of 256 t_1 increments of 16 transients with a mixing time of 200 ms. In both cases spectra were resolution-enhanced by zero filling and sine bell multiplication. The programs ALCHEMY and SYBYL were used for molecular modelling of DppMet^5 **2**. FAB Mass spectra were obtained by using a Kratos MS50TC spectrometer and a glycerol matrix. Amino acid analyses were carried out on hydrolysates (6 mol dm^{-3} HCl; 110 °C; 18 h) using an LKB Alpha analyser and no particular precautions were taken to minimise oxidation of methionine during hydrolysis. Analytical HPLC separations were carried out on a Waters HPLC system using 5μ Hypersil C-18 columns and, unless otherwise stated, a linear gradient of 0.05% TFA–water to 0.05% TFA–MeCN over a period of 25 min starting immediately after sample injection. TLC was carried out on 0.25 mm silica 60 GF-254 layers using the following eluents: (A) MeOH–$CHCl_3$ (1:9), (B) HOAc–MeOH–$CHCl_3$ (1:40:60), (C), BMOH–HOAc–water (3:1:1) and (D) BuOH–HOAc–water (4:1:5). All solvents were distilled from appropriate drying agents before use. Organic extracts were typically dried over anhydrous Na_2SO_4, filtered, and evaporated at reduced pressure on a Buchi rotary evaporator. Light petroleum refers to the fraction boiling in the range 60–80 °C.

N,O-*Bis(diphenylphosphinoyl)tyrosine Methyl Ester*.—A solution of diphenylphosphinoyl chloride (40.8 g, 173 mmol) in CH_2Cl_2 (90 cm^3) and *N*-methylmorpholine (28.5 cm^3) was added to an ice-cold suspension of tyrosine hydrochloride methyl ester (20 g, 86 mmol) in CH_2Cl_2. The stirred solution was left at 5 °C for 1 h and was then allowed to warm to room temperature over 1 h. The solvent was evaporated off, the residue was partitioned between $CHCl_3$ and water, and the organic extract was washed sequentially with saturated aq. $NaHCO_3$ (75 cm^3 × 3), water (75 cm^3), 5% aq. citric acid (75 cm^3 × 2), water (75 cm^3), saturated aq. $NaHCO_3$ (75 cm^3 × 2), water (75 cm^3), cold aq. NaOH (0.25 mol dm^{-3}; 75 cm^3 × 2), water (75 cm^3) and brine (75 cm^3) and was then dried. Evaporation of the solution and crystallisation of the residue from $CHCl_3$–light petroleum afforded the desired product (43.3 g, 85%), m.p. 184–186 °C (lit.,[7] 189–190 °C); $[\alpha]_D$ 20.3 (1.0, MeOH); ν_{max}/cm^{-1} 3380, 1740, 750 and 695; λ_{max}/nm 207 (49 810), 223 (45 480), 260 (3900), 267 (4550) and 274 (3460); δ_H 2.95 (2 H, d), 3.55 (4 H, m), 3.95 (1 H, m), 7.05 (4 H, ABA′B′) and 7.2–7.9 (20 H, m); δ_C 40.36, 40.38, 51.9, 54.6, 120–133 (m), 149.9, 150.1, 172.8 and 173.0; δ_P 23.3 and 30.6; R_f 0.6 (A); HPLC t_R 23 min.

N-(*Diphenylphosphinoyl)tyrosine* **3**.—*N,O*-Bis(diphenylphosphinoyl)tyrosine methyl ester (5.96 g, 10 mmol) was dissolved in peroxide-free 1,4 dioxane (70 cm^3) and treated with aq. NaOH (1 mol dm^{-3}; 25 cm^3). The solution was stirred at room temperature for 5 h, evaporated to 50 cm^3 and adjusted to pH 3 with sat. aq. citric acid to precipitate a colourless gum. The latter was extracted with EtOAc (30 cm^3 × 3) and the extract washed with water (20 cm^3 × 2) and brine (20 cm^3 × 2), dried and evaporated. The resultant residue was dissolved in EtOAc (10 cm^3) and treated with dicyclohexamine (2.5 cm^3) to afford the dicyclohexamine salt of **3** (4.22 g, 75%), m.p. 218–220 °C (lit.,[7] 218–220 °C); $[\alpha]_D$ 14.2 (1.0, MeOH) (Found: C, 70.4; H, 7.7; N, 5.0·$C_{33}H_{43}N_2O_4P$ requires C, 70.4; H, 7.7; N, 5.0%). A suspension of *N,O*-bis(diphenylphosphinoyl)tyrosine methyl ester (2.63 g, 4.67 mmol) in EtOAc (20 cm^3) was shaken with saturated aq. $NaHCO_3$ (20 cm^3) until the material dissolved. The EtOAc layer was separated, washed successively with 5% aq. citric acid (20 cm^3 × 2) and water (20 cm^3 × 2), dried, and concentrated to 10 cm^3. Addition of hexane (with cooling) afforded crystals of the dihydrate of *compound* **3** (1.75 g, 90%), m.p. 85–86 °C; $[\alpha]_D$ −151 (1.0, MeOH); ν_{max}/cm^{-1} 3600–2200br, 3260, 1745, 750 and 700; λ_{max}/nm 204 (23 000), 225 (24 100), 265 (2600) and 273 (2580); δ_H([$^2H_6$]DMSO) 2.80 (2 H, m), 3.75 (1 H, m), 3.90 (1 H, m), 6.7 (4 H, ABA′B′) and 7.05–7.70 (10 H, m); δ_C 38.9, 39.0, 55.4, 115.1–133.7 (m), 156.2 and 174.0; *m/z* 381.1130 (M^+) (Found: C, 60.1; H, 5.8; N, 3.4%; $C_{21}H_{20}NO_4P$·$2H_2O$ requires C, 60.4; H, 5.8; N, 3.4%; $C_{21}H_{20}NO_4P$: M, 381.1130); R_f 0.2 (C); t_R 17.6 min.

N-(*Diphenylphosphinoyl)phenylalanylmethionine Methyl Ester* **5**.—A solution of diphenylphosphinoyl chloride (1.42 g, 6 mmol) in CH_2Cl_2 (20 cm^3) and *N*-methylmorpholine (0.66 cm^3, 6 mmol) were added to a stirred solution of *N*-(diphenylphosphinoyl)phenylalanine (2.19 g, 6 mmol) in CH_2Cl_2 (35 cm^3) at 0 °C. After 15 min a solution of methionine methyl

ester hydrochloride (1.0 g, 5 mmol) in dimethylformamide (DMF) (10 cm^3) was added immediately followed by addition of *N*-methylmorpholine (0.55 cm^3, 5 mmol). The mixture was stirred for 1 h at 0 °C and was then allowed to come to room temperature over a period of 1 h. The solvent was evaporated off and the oily residue was partitioned between EtOAc (50 cm^3) and water (50 cm^3). The organic layer was sequentially washed with saturated aq. $NaHCO_3$ (50 cm^3 × 3), water (50 cm^3), 5% aq. citiric acid (50 cm^3 × 2), water (50 cm^3) and brine (50 cm^3 × 2), dried, and concentrated under reduced pressure. Addition of light petroleum to the solution afforded *compound* **5** (2.1 g, 82%) as crystals, m.p. 155–157 °C; $[\alpha]_D$ −69.7 (1.0, MeOH); ν_{max}/cm^{-1} 3360, 3260, 1740, 1665, 750 and 699; λ_{max}/nm 207 (25 940), 220 (16 130), 264 (1430) and 272 (920); δ_H 2.00 (5 H, m), 2.50 (2 H, t), 3.20 (2 H, m), 3.25 (1 H, m), 3.75 (4 H, m), 4.65 (1 H, m), 7.1–7.9 (15 H, m) and 8.19 (1 H, d); δ_C 15.1, 29.9, 31.0, 39.3, 39.5, 51.9, 52.1, 56.2, 126.7–137.0 (m), 171.8 and 172.0 (Found: C, 63.1; H, 6.0; N, 5.4. $C_{27}H_{31}N_2O_4PS$ requires C, 63.5; H, 6.1; N, 5.5%); amino acid analysis—Phe 1.0, Met 0.4; R_f 0.6 (A); t_R 19.1 min (under a 20–100% gradient).

N-(*Diphenylphosphinoyl*)*glycylphenylalanylmethionine Methyl Ester* **7**.—Methanolic HCl (34.5 cm^3; 4 mol dm^{-3} HCl) was added to a stirred solution of compound **5** (11.2 g, 23 mmol) in MeOH. The reaction was monitored by ^{31}P NMR spectroscopy. After 2 h, when the reaction had gone to completion, the mixture was evaporated to dryness and the residue was washed with diethyl ether (50 cm^3 × 3) to remove diphenylphosphinic acid. The solid residue, hydrochloride **6**, was stored over P_2O_5 overnight. A solution of diphenylphosphinoyl chloride (5.4 g, 23 mmol) in CH_2Cl_2 (10 cm^3) and *N*-methylmorpholine (2.5 cm^3, 6 mmol) were added to a stirred solution of *N*-(diphenylphosphinoyl)glycine (6.5 g, 23 mmol) in CH_2Cl_2 (200 cm^3) at −5 °C. After 15 min a solution of compound **6** (7.6 g, 22 mmol) in DMF (10 cm^3), containing 2,6-lutidine (2,6-dimethylpyridine) (2.67 cm^3, 23 mmol) and *N*-methylmorpholine (2.5 cm^3, 23 mmol) was added slowly to the mixture, which was stirred for 1 h below 0 °C and then was allowed to come to room temperature and was stirred for a further 1 h. The solvent was evaporated off and the oily residue was partitioned between EtOAc (50 cm^3) and water (50 cm^3). The organic layer was sequentially washed with saturated aq. $NaHCO_3$ (50 cm^3 × 3), water (50 cm^3), 5% aq. citric acid (50 cm^3 × 2), water (50 cm^3) and brine (50 cm^3 × 2), dried, and evaporated to give an oil, which solidified on trituration with hexane. Crystallisation from $CHCl_3$–hexane afforded *compound* **7** (9.0 g, 70%) as a microcrystalline solid, m.p. 143–144 °C; $[\alpha]_D$ −26.1 (1.0, MeOH); ν_{max}/cm^{-1} 3240, 3200, 1750, 1660, 750 and 695; λ_{max}/nm 203 (51 560), 257 (2110), 265 (2390) and 272 (1780); δ_H 2.00 (5 H, m), 2.40 (2 H, t), 3.10 (2 H, d), 3.55 (3 H, m), 3.60 (4 H, m), 4.60 (1 H, m), 7.1–7.9 (16 H, m) and 8.70 (1 H, d); δ_C 15.1, 29.9, 31.1, 37.6, 44.6, 51.6, 52.0, 54.4, 126.6–136.6 (m), 170.1, 170.8 and 171.8; δ_P 25.1; *m/z* 567.1950 (M^+) ($C_{29}H_{34}N_3O_5PS$ requires M, 567.1957) (Found: C, 61.3; H, 6.0; N, 7.4. $C_{29}H_{34}N_3O_5PS$ requires C, 61.4; H, 6.0; N, 7.4%); amino acid analysis—Gly 1.0, Phe 1.0, Met 0.4; R_f 0.55 (A); t_R 21.4 min.

Glycylphenylalanylmethionine Methyl Ester Hydrochloride **8**.—A solution of compound **7** (11 g, 19.4 mmol) in methanolic HCl (29 cm^3, 4 mol dm^{-3} HCl) was stirred at 35 °C for 30 min, at which time TLC of the mixture showed that complete hydrolysis of the diphenylphosphinamide had occurred. The solvent was evaporated off, and the residue was washed with diethyl ether and dissolved in MeOH. Addition of diethyl ether resulted in precipitation of *compound* **8** as a microcrystalline solid (6.74 g, 86%), m.p. 176–177 °C; $[\alpha]_D$ −8.4 (1.0, MeOH); ν_{max}/cm^{-1} 3500–2500, 3300, 3200, 1750, 1660, 745 and 695; λ_{max}/nm 203 (17 280); $\delta_H([^2H_6]DMSO)$ 1.95 (2 H, m), 2.05 (3 H, s), 2.50 (2 H, m), 3.09 (2 H, m), 3.45 (2 H, AB), 3.65 (3 H, s), 4.44 (1 H, m), 4.65 (1 H, m), 7.25 (5 H, m), 8.00 (3 H, m), 8.65 (1 H, d) and 8.70 (1 H, d); $\delta_C([^2H_6]DMSO)$ 14.5, 29.5, 30.3, 37.6, 39.9, 51.1, 51.8, 54.0, 126–137 (m), 165.5, 170.8 and 171.8 (Found: C, 49.7; H, 6.5; N, 10.3. $C_{17}H_{26}ClN_3O_4 \cdot 0.5H_2O$ requires C, 49.4; H, 6.3; N, 10.2%); amino acid analysis—Gly 1.0, Phe 1.0, Met 0.5; R_f 0.18 (D).

N-(*Diphenylphosphinoyl*)*glycylglycylphenylalanylmethionine Methyl Ester* **9**.—A solution of diphenylphosphinoyl chloride (4.55 g, 19.25 mmol) in CH_2Cl_2 (14.2 cm^3) was added slowly to a stirred solution of *N*-diphenylphosphinoylglycine (5.2 g, 19.25 mmol) and *N*-methylmorpholine (2.12 cm^3, 19.25 mmol) in DMF (20 cm^3) at −5 °C. After 15 min a chilled solution of compound **8** (6.95 g, 17.2 mmol), 2,6-lutidine (2.24 cm^3, 19.25 mmol) and *N*-methylmorpholine (1.9 cm^3, 17.2 mmol) in DMF (50 cm^3) was added slowly to the mixture. The mixture was stirred for 1 h below 0 °C, then was allowed to come to room temperature and was stirred for a further 1 h. The reaction mixture was concentrated by evaporation and worked up by using the procedure described for compound **7** above to afford *compound* **9** as a solid (m.p. 168–169 °C) which could be crystallised (6.5 g, 60%); $[\alpha]_D$ −23.5 (1.0, MeOH); ν_{max}/cm^{-1} 3300–3200, 1740, 1660, 750 and 695; λ_{max}/nm 208 (42 400), 259 (1500), 265 (1870) and 272 (1370); δ_H 1.95 (5 H, m), 2.30 (2 H, t), 3.05 (2 H, m), 3.60 (5 H, m), 3.90 (2 H, s), 4.55 (2 H, m), 4.80 (1 H, m), 7.0–7.9 (16 H, m), 8.05 (1 H, t) and 8.1 (1 H, d); δ_C 15.1, 29.9, 31.0, 37.9, 43.1, 43.9, 51.4, 54.4, 126.5–136.7 (m), 168.7, 170.9, 171.1 and 172.0; δ_P 27.6; *m/z* 625.2250 $[M + 1]^+$. ($C_{29}H_{38}N_3O_5PS$ requires MH, 625.2250); amino acid analysis—Gly 2.0, Phe 1.0, Met 0.7; R_f 0.50 (A); t_R 20.6 min.

N-(*Diphenylphosphinoyl*)*tyrosylglycylglycylphenylalanylmethionine Methyl Ester* **11**.—Compound **9** (6.4 g, 10.25 mmol) was deprotected using methanolic HCl as described for compound **7** above, and afforded the hydrochloride **10** which was precipitated from MeOH with diethyl ether to give a solid, which was washed with diethyl ether and dried under reduced pressure overnight; amino acid analysis—Gly 2.1, Phe 1.0, Met 0.9.

A solution of *N*-(diphenylphosphinoyl)tyrosine dihydrate **3** (4.7 g, 11.28 mmol) in DMF (30 cm^3) was dried over 4 Å molecular sieves for 3 h and was then decanted and cooled to −5 °C. To the stirred solution a solution of diphenylphosphinoyl chloride (2.67 g, 11.28 mmol) and *N*-methylmorpholine (1.24 cm^3, 11.28 mmol) in CH_2Cl_2 (5 cm^3) was slowly added and the mixture was stirred for 15 min, after which a chilled solution of compound **10** (prepared above) in DMF (30 cm^3) was added, followed by addition of 2,6-lutidine (1.31 cm^3, 10.28 mmol) and *N*-methylmorpholine (1.13 cm^3, 10.25 mmol). After being stirred for 1 h at 0 °C and for 1 h at ambient temperature the reaction mixture was concentrated under reduced pressure and worked-up as described for compound **7** above. The *product* **11** (4.9 g, 61%) separated as a microcrystalline solid on cooling of the concentrated EtOAc extract to −20 °C; m.p. 116–118 °C; $[\alpha]_D$ −60 5 (1.0, MeOH); ν_{max}/cm^{-1} 3300, 1740, 1655, 750 and 695; λ_{max}/nm 213 (23 500), 225 (21 000), 266 (2100) and 273 (2150); δ_H 1.90 (2 H, m), 2.0 (3 H, s), 2.30 (2 H, m), 2.95 (2 H, m), 3.10 (2 H, m), 3.6–3.9 (8 H, m), 4.35 (1 H, m), 4.65 (1 H, m), 4.90 (1 H, m), 6.8 (4 H, ABA'B'), 7.0–7.8 (18 H, m) and 8.45 (1 H, s); δ_C 15.1, 29.7, 31.0, 37.8, 39.0, 43.1, 43.7, 51.6, 52.1, 54.6, 57.0, 115.5–136.8 (m), 155.9, 169.5, 170.1, 171.3, 171.8 and 174.4; δ_P 27.6; *m/z* 788.2883 $[M + 1]^+$ ($C_{40}H_{47}N_5O_8PS$ requires MH, 788.2883) (Found: C, 60.0; H, 6.1; N, 8.6. $C_{40}H_{46}N_5O_8PS \cdot H_2O$ requires C, 59.6; H, 6.0; N, 8.7%); amino acid analysis—Gly 2.1, Tyr 1.0, Phe 1.0, Met 0.5; R_f 0.50 (A); t_R 17.2 min (20–100% gradient).

N-(*Diphenylphosphinoyl*)*tyrosylglycylglycylphenylalanylmethionine* **2**.—The methyl ester **11** (2 g, 2.54 mmol) was dissolved in DMF (10 cm^3) and aq. NaOH (5.3 cm^3; 1 mol dm^{-3}) was added to the stirred solution. After 6 h the solvent was removed under reduced pressure, the residue was dissolved in water (5 cm^3) and the solution was adjusted to pH 3 with saturated aq. citric acid. The resultant gel was extracted with EtOAc (15 cm^3 × 3) and the extract was washed successively with water (10 cm^3 × 2) and brine (10 cm^3 × 2) and dried. Evaporation of the organic extract and trituration of the residue with light petroleum afforded *compound* **2** as a solid (1.78 g, 91%); $[\alpha]_D$ −54.0 (1.0, MeOH); ν_{max}/cm^{-1} 3650–2700, 3300, 1725, 1660, 750 and 700; λ_{max}/nm 211 (22 700), 225 (17 800), 267 (1800) and 274 (2050); *m/z* 774.2726 $[M + 1]^+$ ($C_{39}H_{45}N_5O_8PS$ requires MH, 774.2726), 478, 421, 336 and 201; amino acid analaysis—Tyr 0.9, Gly 2.0, Phe 1.0, Met 1.0; R_f 0.5 (B); t_R 19.0 min. For NMR data see text.

Met^5*enkephalin* **1**.—Compound **2** (0.774 mg, 1 mmol) was dissolved in a solution of aq. HCl (4 cm^3; 3 mol dm^{-3}) and peroxide-free 1,4-dioxane (2 cm^3) and the course of the reaction was monitored by NMR spectroscopy. After 6 h, when the reaction was complete, the solution was freeze dried and the residue was dissolved in the minimum volume (~2 cm^3) of 5% HOAc–water and applied to a Sephadex G-15 column (5 × 100 cm^3). Elution with 5% HOAc–water and lyophilisation of the appropriate fractions afforded compound **1** (361 mg) which was shown by HPLC to be contaminated with a minor impurity. Purification was achieved by partition chromatography on Sephadex G-25 with BuOH–HOAc–water (4:1:5) as eluent. Evaporation of the appropriate fractions afforded *compound* **1** as a powder (220 mg, 40%); $[\alpha]_D$ −20.8 (0.5, water); ν_{max}/cm^{-1} 3600–2500, 3300 and 1750–1630; $\delta_H([^2H_6]DMSO)$ 1.90 (2 H, m), 2.05 (3 H, s), 2.50 (2 H, m), 2.85 (2 H, m), 2.95 (2 H, m), 3.6–3.9 (4 H, m), 4.00 (1 H, m), 4.35 (1 H, m), 4.60 (1 H, m), 6.9 (4 H, ABA'B', *J* 8.4), 7.25 (5 H, m), 7.99 (1 H, dd, *J* 4.5, 6.0), 8.01 (1 H, d, *J* 8.0), 8.16 (1 H, d, *J* 8.5) and 8.50 (1 H, dd, *J* 4.0, 6.0); *m/z* 574.2335 $[M + 1]^+$ ($C_{27}H_{36}N_5O_7S$ requires MH, 574.2335); amino acid analysis—Gly 2.01, Tyr 0.95, Phe 1.05, Met 1.01; t_R 21.4 min (0–60% gradient).

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References

1 M. Bergmann and L. Zervas, *Ber. Dtsch. Chem. Ges.*, 1932, **65B**, 1192.
2 F. C. McKay and N. F. Albertson, *J. Am. Chem. Soc.*, 1957, **79**, 4686; G. W. Anderson and A. C. McGregor, *J. Am. Chem. Soc.*, 1957, **79**, 6180.
3 M. Low, L. Kisfaludy, E. Jaeger, P. Thamm, S. Knof and E. Wunsch, *Hoppe-Seyler's Z. Physiol. Chem.*, 1978, **359**, 1637; B. F. Lundt, N. L. Johansen and J. Markussen, *Int. J. Pept. Protein Res.*, 1979, **14**, 344; R. J. Vourek, L.-H. Hsi, E. J. York, M. E. Hall and J. M. Stewart, *Peptides*, 1981, **2**, 303; J. A. Stekol, *J. Biol. Chem.*, 1941, **140**, 827.
4 C. A. Dekker, S. P. Taylor and J. S. Fruton, *J. Biol. Chem.*, 1949, **180**, 155; C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T.-B. Lo and J. Ramachandran, *J. Am. Chem. Soc.*, 1961, **83**, 4449.
5 L. A. Carpino and G. Y. Han, *J. Am. Chem. Soc.*, 1970, **92**, 5748; *J. Org. Chem.*, 1972, **37**, 3404.
6 J. Meienhofer, M. Waki, E. P. Heimer, T. J. Lambros, R. C. Makofse and Ch. Chang, *Int. J. Pept. Protein Res.*, 1979, **13**, 35; E. Atherton, C. J. Logan and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1981, 538.
7 R. Ramage, D. Hopton, M. J. Parrott, G. W. Kenner and G. A. Moore, *J. Chem. Soc., Perkin Trans. 1*, 1984, 1357.
8 R. Ramage, D. Hopton, M. J. Parrott, R. S. Richardson, G. W. Kenner and G. A. Moore, *J. Chem. Soc., Perkin Trans. 1*, 1985, 461.
9 E. Pietrzick, *Justus Liebigs Ann. Chem.*, 1977, 609.
10 L. Kisfaludy in *The Peptides*, ed. E. Gross and J. Meienhofer, Academic Press, New York, 1980, vol. 2, pp. 418–438; P. Stelzel in *Methoden der Organischen Chemie*, ed. E. Muller, G. Thieme Verlag, Stuttgart, 1974, vol. 15/2, p. 169–259.
11 A. S. Horn and J. R. Rodgers, *Nature*, 1976, **260**, 795; A. F. Bradbury, D. G. Smyth and C. R. Snell, *Nature*, 1976, **260**, 165.
12 S. L. Patterson L. E. Robson and H. W. Kosterlitz, *Br. Med. Bull.*, 1983, **39**, 31.
13 W. A. Klee and M. Nirenberg, *Nature*, 1976, **263**, 609.
14 H. W. Kosterliitz and S. L. Patterson, *Philos. Trans. R. Soc. London, Ser. B*, 1985, **308**, 291.
15 H.-U. Gremlich, U.-P. Fringeli and R. Schwyer, *Biochemistry*, 1983, **22**, 4257; R. M. Epand, *Trends Biochem. Sci.*, 1983, **8**, 205; K. Wakamatsu, A. Okada, T. Miyazawa, Y. Masui, S. Sakakibara and T. Higashijima, *Eur. J. Biochem.*, 1987, **163**, 331.
16 M. Vasquez and H. A. Scheraga, *Biopolymers*, 1985, **24**, 1437; J. L. DeCoen, C. Humblet and M. H. J. Koch, *FEBS Lett.*, 1977, **73**, 38.
17 I. L. Karle, J. Karle, D. Mastropaolo, A. Camerman and N. Camerman, *Acta Crystallogr., Sect. B*, 1983, **39**, 625; A. Camerman, D. Mastropaolo, I. L. Karle, J. Karle and N. Camerman, *Nature*, 1983, **306**, 447; M. Doi, T. Ishida, M. Inoue, T. Fujiwara, K. Tomita, T. Kimura and S. Sakakibara, *FEBS Lett.*, 1984, **170**, 229; J. F. Griffin, D. A. Langs, G. D. Smith, T. L. Blundell, I. J. Tickle and S. Bedarkar, *Proc. Natl. Acad. Sci. USA*, 1986, **83**, 3272.
18 (*a*) G. D. Smith and J. F. Griffin, *Science*, 1978, **199**, 1214; (*b*) T. Ishida, M. Kenmotsu, Y. Mino, M. Inoue, T. Fujiwara, K. Tomita, T. Kimura and S. Sakakibara, *Biochem. J.*, 1984, **218**, 677.
19 A. Motta, M. A. C. Morreli, N. Goud and P. A. Temussi, *Biochemistry*, 1989, **28**, 7996.
20 (*a*) G. Gupta, M. H. Sarma, R. H. Sarma and M. M. Dhingra, *FEBS Lett.*, 1986, **198**, 245; (*b*) A. Motta, D. Picone, T. Tancredi and P. Temussi, *Tetrahedron*, 1988, **44**, 975.
21 (*a*) B. P. Roques, C. Garbay-Jaureguiberry, R. Oberlin, M. Anteunis and A. K. Lala, *Nature*, 1976, **262**, 778; (*b*) C. R. Jones, W. A. Gibbons and V. Garsky, *Nature*, 1976, **262**, 779; (*c*) E. R. Stimson, Y. C. Meinwald and H. A. Scheraga, *Biochemistry*, 1979, **18**, 1661; (*d*) M. A. Khaled, M. M. Long, W. D. Tompson, R. J. Bradley, G. B. Brown and D. W. Urry, *Biochem. Biophys. Res. Commun.*, 1977, **76**, 224.
22 A. Milon, T. Miyazawa and T. Higashjima, *Biochemistry*, 1990, **29**, 65.
23 G. Gacel, M. C. Fournie-Zaluski, E. Fellion, B. P. Roques, B. Senault, J. M. Lecomte, B. Malfroy, J.-P. Swerts and J. C. Schwartz, *Life Sci.*, 1979, **24**, 725.
24 K. G. R. Pachler, *Spectrochim. Acta*, 1964, **20**, 581.
25 J. Feeney, *J. Magn. Reson.*, 1976, **21**, 473.

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