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DIPHENYLPHOSPHINIC MIXED ANHYDRIDES IN SOLID-PHASE PEPTIDE SYNTHESIS

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Abstract - Diphenylphosphinic mixed anhydrides have been evaluated for use in solid-phase peptide synthesis using a phenolic polystyrene support. These mixed anhydrides were successfully used for the preparation of an amino terminal fragment of "Bombesin like" peptide and rapid, efficient acylation was observed during the assembly of the peptide on the support. No evidence of disproportionation or wrong way opening were observed.

The preparation of a phenolic solid-phase support for peptide synthesis was established several years ago.[1] Since that time little work has been published using this type of support, although a comparative study has been made in which the conventional chloromethyl polystyrene (Merrifield resin) was compared with the phenolic polystyrene.[2] It was found that in many respects the phenolic polymer was superior, particularly as the peptide chain may be removed from the support, either by peroxide catalysed cleavage,[3] or alternatively, the fragment may be removed by transesterification with dimethylamino ethanol.[4] The dimethylaminoethyl ester then being hydrolysed by treatment with aqueous sodium bicarbonate to give the free peptide acid.

Recently, new phenolic polymers based on a poly(acryloylmorpholine) matrix have been developed.[5-8] These resins have utilised the cleavage procedures mentioned above and also have used hydrazinolysis as a means of cleaving the peptide chain from the support with the concommitant formation of the peptide hydrazide which can then be used in azide fragment coupling.

In our current work we have used the original phenolic polystyrene resin,[1] which has been prepared by co-polymerisation of styrene and p-acetoxystyrene in the presence of divinylbenzene as the cross-linking agent. By this procedure we have prepared a phenolic resin substituted at a loading of approximately 1 mmol.g^{-1}.

In all the preceeding work using the phenolic polymer for peptide synthesis,[2,9] activation of the carboxyl group by DCCI has been the standard method of chain extension for assembly of the peptide on the resin. In our current work we have investigated mixed anhydride activation in place of the use of DCCI. We have examined the use of diphenylphosphinic mixed anhydrides,[10] which are formed by reaction of the N-methylmorpholine salt of an N-protected amino acid, with diphenylphosphinyl chloride (Dpp.Cl). It is clear that such mixed anhydrides have considerably greater stability than their corresponding carboxylic or carbonic mixed anhydride counterparts.[11,12] Thus, the phosphinic mixed anhydrides which are stable over relatively long periods of time at room temperature, are not prone to disproportionation. Also, because of a combination of steric and electronic effects they do not appear to undergo "wrong-way opening", which is a particular disadvantage of other types of mixed anhydrides. Such a problem would be particularly disadvantageous in a solid phase synthesis, as the 'wrong-way opening' would lead to chain termination, whereby the extending amino terminus would become blocked as the corresponding diphenylphosphinamide.

Recently it has become clear[13,14] that, like the diphenylphosphinyl mixed anhydrides, the dimethylphosphinothioyl (Mpt) mixed anhydrides can also be used as the means of activation in solid phase peptide synthesis, and on this occasion the conventional Merrifield resin was used as the support. Clearly, these mixed

anhydrides are of similar stability to the Dpp mixed anhydrides, and like the Dpp mixed anhydrides it is possible to isolate the mixed anhydride as a crystalline solid.

We therefore investigated the use of phenolic polymer as support, combined with activation by diphenylphosphinyl chloride as we proposed that this would lead to an efficient synthesis of fully protected peptide fragments.

In our synthesis we have used the highly acid labile p-biphenylyl-isopropyloxycarbonyl (Bpoc) protecting group for the α-amino functions with the side-chains being protected by tert-butyl based protecting groups. Thus, the first stage in synthesis was to attach the N-protected amino acid to the resin. We initially examined acylation of the phenolic hydroxyl group with Bpoc amino-acid N-hydroxy succinimide active esters, the acylation was carried out using a 2 molar excess of the active ester for up to forty-eight hours. Examples of the degree of incorporation, as indicated by amino-acid analysis, are given in Table 1.

	Incorporation ($mM.g^{-1}$)
Bpoc.Phe.ONSu	0.18
Bpoc.Gly.ONSu	0.25
Bpoc.Leu.ONSu	0.19

TABLE 1 Incorporation of N-protected amino-acid N-hydroxysuccinimide ester to the phenolic polymer (0.9 $mM.g^{-1}$).

A second treatment with Bpoc-Leu.ONSu was carried out in the third case in an attempt to increase the level of incorporation, however, quantitative amino acid analysis showed no improvement. As the resin loading was 0.9 $mM.g^{-1}$ it is clear that considerable wastage of resin capacity would occur if this method of incorporation were used.

In order to improve the situation, acylation was attempted using the 2,4,5-trichlorophenyl active ester with the addition of hydroxybenzotriazole.[15] The addition of HOBt is known to accelerate acylation when aromatic active esters are used, and as this has been found to be particularly effective in amide bond formation we considered that it might also be a means of improving the yield on ester formation. The active esters were prepared as a standard procedure using the protected (Bpoc) amino-acid, 2,4,5-trichlorophenol and DCCI. The coupling was carried out using a 3 molar excess of the active ester in the presence of 3 moles of hydroxybenzotriazole and in this case the level of incorporation is shown in Table 2.

	Incorporation ($mM.g^{-1}$)
Bpoc.Phe.OCp	0.38
Bpoc.Leu.OCp	0.30
Bpoc.Tyr(Bu^t).OCp	0.43
Bpoc.Glu(OBu^t).OCp	0.41

TABLE 2 Incorporation of N-protected amino-acid 2,4,5-trichlorophenyl ester to the phenolic polymer (0.9 $mM.g^{-1}$)

These loadings fall well within the limits suggested by Merrifield and co-workers in their study of polystyrene resins.[16] It is interesting that even with considerable steric hindrance a good degree of acylation was observed. As there were several remaining unacylated hydroxyl groups on the resin which would be available for further reaction, we treated the resin with acetic anhydride to block these groups permanently during synthesis.

The ease of removal of the Bpoc protecting group was then checked using 0.05 M HCl in dichloromethane as the cleavage agent, as this solution was fully compatible with the polystyrene resin. The cleavage was monitored by measurement of the UV absorbance of the deprotection washes, UV monitoring being at 275 nm. A detailed study of cleavage was carried out using Bpoc.Phe.O resin and it was found that four repeated cycles were required in order to bring about zero absorbance at 275 nm., using an alternate shrink/swell cycle between treatments. The very slow completion of the cleavage is somewhat surprising, as previous work[17] would suggest that Bpoc removal should be much more rapid. Ultimately, the Bpoc cleavage was carried out using two twenty minute treatments followed by two ten minute treatments with 0.05M HCl in dichloromethane. In the presence of sensitive amino acids such as methionine, tyrosine or tryptophan we have incorporated scavengers into the deprotection washes. When tyrosine alone was present anisole was used as a scavenger and for methionine and tryptophan, anisole plus ethanedithiol was used, the scavenger being in 50 molar excess over the resin bound peptide.

Although the phenyl ester linkage has a high degree of stability under acidic conditions, it does show slight susceptibility to cleavage under

basic conditions.[3] Thus, there is always a slight risk of peptide loss during the neutralization step, and this is particularly so at the di-peptide stage where the amino component can undergo an intramolecular reaction forming the stable diketopiperazine.

In order to overcome these problems an *in situ* neutralization, in combination with the coupling was carried out with the hindered base N-methylmorpholine. Thus, during the coupling reaction sufficient base was present to allow formation of the mixed anhydride and to permit neutralization of the peptidyl resin hydrochloride. Following a number of coupling steps it was clear that under these conditions residues were completely incorporated.

As discussed above, the DCCI method is the most generally found method of activation during solid phase synthesis, although recently the symmetrical anhydride method[18] has become increasingly favoured. The major disadvantage of the DCCI method lies in the fact that the resulting urea is frequently difficult to remove from the coupling vessel. The symmetrical anhydride also, although clean and rapid in it's acylation, is rather wasteful in that an equivalent of the protected amino acid is displaced as the leaving group during coupling, and is unable to take part in further acylation. Thus, when using three equivalents of the acylating species, six equivalents of the protected amino acids have to be used, with only one becoming incorporated into the growing peptide chain. It is for this reason that we considered the use of mixed anhydrides in solid phase synthesis using the diphenylphosphinic mixed anhydride where "wrong-way" opening and disproportionation are virtually eliminated. In fact, ^{31}P and ^{13}C NMR spectra showed that the Z.Gly.ODpp mixed anhydride was stable for at least forty-eight hours at room temperature, and peaks suggesting the formation of symmetrical anhydride were not found. This is in marked contrast to the related pivalic mixed anhydride of the same amino acid, which showed disproportionation after thirty minutes at 0°C. The mixed anhydride also retained full acylation potential after this extended period.

Two acylations of a resin bound amino component were studied in order to reveal how efficiently the diphenylphosphinic anhydride would react. Thus, pre-formed Bpoc.Val.ODpp and Bpoc.Gly.ODpp were reacted with phenylalanyl resin hydrochloride in dichloromethane, using *in situ* neutralization as indicated above with N-methylmorpholine in both cases a three molar excess was used. The mixed anhydrides were prepared using diphenylphosphinyl chloride and the N-protected amino acid in the presence of N-methylmorpholine for twenty minutes at 0°. The solution containing the mixed anhydride was then pumped into the solid phase reaction vessel. The incorporation of the protected amino acid was monitored by amino acid analysis, the ratio between the incorporated residue and phenylalanine being measured. In both cases a rapid acylation took place, the acylation with Bpoc.Val.ODpp was complete in twenty minutes using dichloromethane as the solvent, the progress of the reaction being as shown in Figure 1.

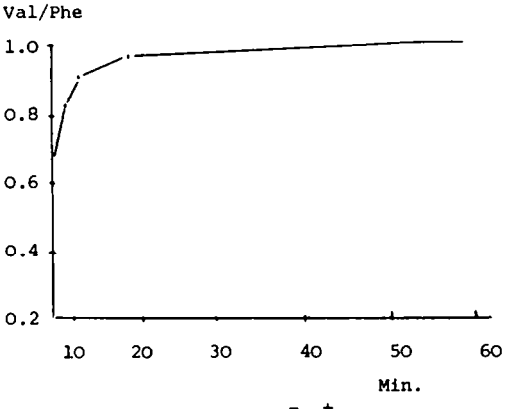

FIGURE 1 Acylation of $Cl^- H_2^+$.Phe.O Resin with Bpoc.Val.ODpp.

In the second example DMF had to be used as the solvent as Bpoc.Gly.OH is relatively insoluble in dichloromethane. The time course was again followed, but on this occasion only 60% acylation was achieved initially and a second acylation after a shrink/swell cycle using a further three equivalents of the pre-formed mixed anhydride was required in order to bring about complete reaction. This observation correlates well with the findings of Merrifield and co-workers using polystyrene resins,[16] in which it was found that the reduced solvation of the polystyrene matrix in DMF may limit incorporation.

Following these incorporation experiments, we devised the overall coupling and washing procedure which is indicated in Figure 2. This procedure combines the findings of our exploratory results for the phenolic polymer

1) CH_2Cl x 3	2) 0.05M HCl/ CH_2Cl_2 20 min	3) CH_2Cl_2 /^iPrOH x 3	4) CH_2Cl_2 x 2	5) 0.05M HCl/ CH_2Cl_2 20 min	6) CH_2Cl_2 /^iPrOH x 2	7) CH_2Cl_2 x 2
8) 0.05M HCl/ CH_2Cl_2 10 min	9) CH_2Cl_2 /^iPrOH x 2	10) CH_2Cl_2 x 2	11) 0.05M HCl/ CH_2Cl_2 10 min	12) CH_2Cl_2 /^iPrOH x 4	13) CH_2Cl_2 x 3	14) Bpoc.AA. ODpp 2 h
15) DMF x 3	16) CH_2Cl_2 /^iPrOH x 3	17) CH_2Cl_2 x 3	18) Bpoc.AA. ODpp 2 h	19) DMF x 3	20) CH_2Cl_2 /^iPrOH x 3	21) ^iPrOH x 2

FIGURE 2 Complete coupling cycle for the incorporation of one amino-acid residue

when using diphenylphosphinic mixed anhydrides for activation.

Monitoring of the deprotection steps could be carried out in the absence of scavengers, but as these have a high background absorbance at 275 nm difficulty was encountered in monitoring when scavengers were present. Also, we found that monitoring using ninhydrin[19], fluorescamine[20] and chloranil[21] was difficult when using Bpoc for amino protection. Thus, in order to monitor the progress of the reaction the amino acid analysis was checked at each stage using the 12M HCl/propionic acid (1:1) method,[22] although it is appreciated that amino-acid analysis is only accurate to ± 3%, and this is well outside the limits which would normally be tolerated during solid phase assembly.

As a test case we chose to examine the synthesis of the amino terminal region of Bombesin-like peptide.[23] Although a total synthesis of the Bombesin-like peptide has been published,[24] the amino terminal hepta-peptide was chosen for study as it was required for immunological work. In order to facilitate radiolabelling of this peptide we have added a tyrosine residue to the carboxyl terminus as this would not be expected to effect the raising of amino terminal antibodies. The total sequence proposed for synthesis is shown in Figure 3.

H.Ala.Pro.Val.Ser.Val.Gly.Tyr.OH

FIGURE 3 Modified Bombesin-like peptide (1 - 6) with C-terminal tyrosine extension.

The C-terminal tyrosine residue was attached to the resin using the active ester/HOBt catalysed procedure outlined above, the residues of the heptapeptide were then added sequentially using the protocol shown in Figure 2. The diphenylphosphinic mixed anhydrides required for chain assembly were prepared in a separate reaction vessel by reaction of the Bpoc amino-acid with N-methyl-morpholine and diphenylphosphinyl chloride at 0° for twenty minutes. The solution of the mixed anhydride being pumped directly into the solid phase synthesis vessel. Amino-acid analyses of the protected resin bound intermediates were carried out, the results being shown in Figure 4.

Serine was not recorded as under the conditions used for hydrolysis the serine was appreciably destroyed and extrapolation to zero time gave an unreliable figure. As a precaution we acetylated with acetic anhydride and pyridine after the addition of the serine residue. From the amino acid analyses it can be seen that the assembly of the peptide

	Ala	Pro	Ser	Val	Gly	Tyr
Gly.Tyr					1.00	1.00
Val.Gly.Tyr				1.00	1.00	1.00
Ser.Val.Gly.Tyr			-	1.01	1.02	0.97
Val.Ser.Val.Gly.Tyr			-	2.01	1.01	0.98
Pro.Val.Ser.Val.Gly.Tyr		1.02	-	1.95	1.03	1.03
Ala.Pro.Val.Ser.Val.Gly.Tyr	1.04	1.00	-	1.98	0.99	0.98

FIGURE 4 Amino-acid analyses of protected resin bound intermediates during synthesis.

had been achieved without complication.

After the peptide assembly had been completed removal of the peptide from the resin was investigated. Initially alkaline hydrolysis at pH 10.5 in the presence of hydrogen peroxide was attempted, using 90% aqueous DMF as the solvent. TLC indicated that over three and a half hours release of the peptide acid was very slow and thus we examined the alternative of transesterification using dimethylaminoethanol.[4] After stirring the peptidyl resin with 50% dimethylaminoethanol in DMF for four days at room temperature, the corresponding ester was recovered in 90% yield. Hydrolysis of this ester was then carried at pH 9.7 using DMF/water as the solvent. The reaction was found to be complete after eighteen hours and the solution was then acidified to pH 3.5 with 10% potassium hydrogen sulphate and the solvent evaporated. The white residue was extracted with DMF, filtered, washed and then applied directly to Sephadex LH20. A smooth elution profile was obtained indicating that the peptide was homogeneous, monitoring was carried out at 280 nm and by monitoring the optical rotation of the eluant. TLC, in several solvent systems, and amino acid analysis of the product were in agreement with the anticipated values.

The protected peptide was then treated with 90% trifluoroacetic acid in the presence of anisole and ethane-dithiol. After six hours the product was precipitated by the addition of ether, and then purified further on Sephadex G15 eluting with 10% aqueous acetic acid. Once again a symmetrical peak was observed on gel filtration, and the material corresponding to the Bombesin like peptide fragment was obtained by lyophilisation in 24% overall yield. The product was homogeneous by TLC in three solvent systems and by electrophoresis at pH 2.1 and pH 7. The amino-acid analysis after 6M acid hydrolysis and the 360 MHz proton NMR spectrum was consistent with the identity of the product.

An electron impact mass spectrum was also obtained after acetylation and permethylation, as we anticipated that any failure sequences would be evident from the resulting spectrum.

The spectrum shows the well defined loss of each of the sequence ions commencing from the carboxyl terminus, as well as the characteristic loss of CH_3OH which is frequently observed with serine containing peptides. The sequence ions are by far the strongest peaks in the spectrum, and there is no evidence to suggest that incorrect sequences are present. The spectrum also shows no evidence for the modification of tyrosine by either t-butyl or biphenylisopropyl carbonium ions.

The preparation of the homogeneous protected and deprotected heptapeptide sequence leaves no doubt as to the fact that activation by diphenylphosphinic mixed anhydrides is fully compatible with solid phase synthesis, using a phenolic polymer as the support. The synthesis confirms that a protected peptide acid can be assembled in the manner indicated and that transesterification using dimethylaminoethanol is the most satisfactory method of removing fragments from phenolic resins. The alternative simple phenyl ester cleavage using hydrolysis at pH 10.5 in the presence of hydrogen peroxide was found to be inefficient, this finding is consistent with the findings of other workers using phenolic polymer.[9]

The diphenyl phosphinic mixed anhydrides are clearly sufficiently active to efficiently acylate the extending amino terminus on a solid phase support, and as anticipated we did

not experience "wrong-way opening", or disproportionation. Thus, these mixed anhydrides provide an efficient economic method of solid phase peptide synthesis.

EXPERIEMENTAL

Abbreviations not in common use are as follows: DCCI, dicyclohexylcarbodiimide; DMAE, dimethylaminoethanol; DMF dimethylformamide; HOBt N-hydroxybenzotriazole; HOCp, 2,4,5-trichlorophenol; HONSu, N-hydroxysuccinimide; NMM, N-methylmorpholine; TEA, triethylamine; TFA, trifluoroacetic acid.

Amino-acid analysis was carried out on a Jeol JLC 6AH instrument with digital integrator; resin bound peptides were subjected to hydrolysis using 12M HCl/propionic acid (1:1) for 3½ h at 140° and free peptides were hydrolysed using 6M HCl for 18h at 110°.

Electrophoresis was carried out using an LKB 2117 Multiphor using cellulose acetate plates at a voltage of 1500v. Buffers used: pH 7 : 1 vol pH 6.7 phosphate buffer/7 vol 8M urea; pH 2.1 8% AcOH, 2% $H.CO_2H$.

NMR spectra (^1H) were recorded on a Perkin Elmer R34 (220MHz) and Bruker WH360 (360MHz), University of Edinburgh SERC service.

Mass spectra were recorded on a VG micromass 70 70-F mass spectrometer interfaced to a Finnigan Incos 2300 data system.

Solid phase synthesis : A reaction vessel fitted with sinters (porosity 3) at the inlet and outlet were shaken on a Chromatronix peptide shaker which was used in combination with a six way valve solvent delivery system operating by application of vacuum. In line UV monitoring being carried out using a Cecil CE212 variable wavelength instrument fitted with a flowcell.

INCORPORATION EXPERIMENTS

The phenolic resin (1.2g, 1.08mM) was placed in the reaction vessel and washed with CH_2Cl_2 (x 3). The resin was then treated with the Bpoc. amino-acid N-hydroxysuccinimide or 2,4,5-trichlorophenyl active ester (3.24mM), TEA 0.32g, 3.24mM and in the latter cases HOBt (0.43g, 3.24mM) was also added. The reaction was shaken for 24h using CH_2Cl_2 (10 ml) as solvent, then filtered and the resin washed with CH_2Cl_2 (x 3), $CH_2Cl_2/^iPrOH$ (x 3), ^iPrOH (x 2). After drying *in vacuo* quantitative amino-acid analysis was carried out using alanine as an internal standard.

DEPROTECTION STUDIES

The deprotection step was studied using 0.05M HCl in CH_2Cl_2 using an in line UV monitor (275 nm). Also the individual deprotection washes were collected and their optical density at 275 nm determined. Four treatments of 20 min., 20 min., 10 min and 10 min., respectively, with alternate $CH_2Cl_2/^iPrOH$ washes in between, were required as a minimum for complete deprotection. On a fifth treatment with 0.05M HCl no UV absorption at 275 nm was detected in the wash.

COUPLING STUDIES

(a) Formation of Bpoc Val.Phe.O resin.

Individual reactions of 5, 10, 20, 40, 60, 90 and 120 min. duration were run at room temperature, using a common acylating mixture, the volumes of which depended on the individual weights of $Cl^-H_2^+Phe.O$ resin used. The acylating reagent was formed in a separate vessel by adding diphenylphosphinyl chloride (0.25g, 1.05 mmol) to a solution of Bpoc Val.OH (0.37g, 1.05 mmol) and NMM (0.21g, 2.10 mmol) in CH_2Cl_2 (10 ml) and stirring the reaction for 20 min. at 0°C. Aliquots of this solution were added to the individual $Cl^-H_2^+Phe.O$ resin samples (approximately 0.05g) preswollen in an equal volume of DCM, to give a 3 x molar excess of the acylating species with respect to the resin bound amino component. The reactions were terminated by filtration, followed by washing the resin as follows:

1. CH_2Cl_2
2. DMF x 2
3. $CH_2Cl_2/^iPrOH$ x 3
4. ^iPrOH x 2

Each sample was dried under vacuum before amino acid analysis.

(b) Formation of Bpoc Gly.Phe.O resin.

1st Acylation.

In a similar manner to the previous experiment the following reactions were performed at room temperature over 5,10,15,20, 30,40,50,60, 90 and 120 min. The acylating reagent was formed by adding diphenylphosphinyl chloride (0.23g, 0.98 mmol) to a solution of Bpoc.Gly.OH (0.29g, 0.98 mmol) and NMM (0.21g, 2.10 mmol) in DMF (10 ml) and stirring the reaction for 20 min. at 0°C. Aliquots of this reagent were then added to individual preswollen $Cl^-H_2^+Phe.O$ resin samples. The

reactions were terminated by filtration and subjected to the following washing cycle:

1. DMF
2. DMF/^{i}PrOH x 3
3. ^{i}PrOH x 2

The samples were dried and then subjected to amino acid analysis.

2nd Acylation.

Four samples from the previous acylation reaction were chosen which all had a 60% incorporation of glycine. A second acylation was carried out employing the following reaction times: 15,30,60 and 90 min. The acylating reagent was again formed externally by the reaction of Bpoc Gly.OH (0.27g, 0.90 mmol), diphenylphosphinyl chloride (0.21g, 0.90 mmol) and NMM (0.18g, 1.80 mmol) in DMF (10 ml) for 20 min. at 0°C. The completed samples were filtered, washed and dried as described above and then subjected to amino acid analysis.

SYNTHESIS OF THE "BOMBESIN-LIKE" PEPTIDE: H.Ala.Pro.Val.Ser.Val.Gly.Tyr.OH.

Bpoc.Tyr(Bu^{t}).O Resin was prepared by the method outlined above using Bpoc.Tyr(Bu^{t})-OCp (2.1g, 3.24 mM) giving a substitution of 0.43 mM.g^{-1}.

The remaining free hydroxyl groups were acylated by treating the resin with TEA (1 ml, 7.1 mmol) in DMF (10 ml) for 1 h and then introducing acetic anhydride (0.7g, 6.9 mmol) in DMF (2 ml). After shaking for a further 2 h the resin was filtered and washed with CH_2Cl_2/ ^{i}PrOH (x 3).This was followed by a further treatment of the resin with TEA (1 ml, 7.1 mmol) and acetic anhydride (0.7g, 6.9 mmol) in DMF (10 ml) for 12 h. The resin was then washed as follows:

1. DMF x 3.
2. DMF/^{i}PrOH x 3.
3. CH_2Cl_2/^{i}PrOH x 3.
4. ^{i}PrOH x 2

and dried under vacuum.

Bpoc Ala.Pro.Val.Ser(Bu^{t}).Val.Gly.Tyr(Bu^{t}).O resin.

The sequence was built up according to the programe of coupling, deprotection and washes described above starting with Bpoc Tyr(Bu^{t}).O resin (1.17g, 0.5 mmol). Anisole (2.5 ml, 22.9 mmol) was added to the deprotection washes. All the coupling reactions were performed in CH_2Cl_2 with the exception of Bpoc Gly.OH which was performed in DMF. Acetylation was carried out after Bpoc Ser(Bu^{t}).OH coupling by treatment of the peptidyl resin with acetic anhydride (0.5g, 5 mmol) and pyridine (0.39 ml, 5 mmol) in DMF (10 ml) for 5h. Amino-acid analysis for the protected resin bound intermediates were recorded (see Figure 4).

Bpoc.Ala.Pro.Val.Ser(Bu^{t}).Val.Gly.Tyr(Bu^{t}).OH

The preceding resin bound heptapeptide (0.50g, 0.15 mmol) was stirred in 50% DMAE in DMF (30 ml) for four days at room temperature. After this time the resin was removed by filtration and washed with DMF. The combined filtrates were evaporated to yield an oil which was redissolved in 50% DMF/water (30 ml) and hydrolysed by the addition of 0.2M sodium hydroxide (1.7 ml) to maintain the pH at 9.7. TLC indicated that the hydrolysis was complete after 18h and the solution was cooled and acidified to pH 3.5 with 10% potassium hydrogen sulphate solution. The solvents were removed *in vacuo* leaving a white residue which was extracted with DMF, filtered and washed. The filtrate was evaporated and the oil obtained dissolved in a small volume of DMF and applied to a Sephadex LH20 column (V_t = 410 ml). The fractions corresponding to the product (V_e/V_t = 0.38) were pooled and evaporated to dryness.

Yield: 50 mg (32%)

TLC: nBuOH/AcOH/H_2O (3:1:1)
R_f = 0.89
CH_3CN/H_2O (4:1) R_f = 0.53

Amino acid analysis (theoretical values in brackets):

$Ala_{0.98(1)}Pro_{1.04(1)}Val_{2.00(2)}Ser^{+}_{0.63(1)}$-
$Gly_{1.01(1)}Tyr_{0.98(1)}$

+ no correction made for degradation.

H.Ala.Pro.Val.Ser.Val.Gly.Tyr.OH

The foregoing protected heptapeptide acid (50mg, 0.05 mmol) was dissolved in 90% aqueous TFA (2 ml) containing ethanedithiol (0.20 ml, 2.4 mmol) and anisole (0.26 ml, 2.4 mmol). The reaction was kept under nitrogen in a darkened centrifuge tube for 6h. The product was then precipitated by the

addition of dry Et_2O and isolated by centrifugation. The product was washed with dry Et_2O (x 4) and then dried *in vacuo*.

The product was dissolved in a small volume of 10% aqueous AcOH and applied to a Sephadex G15 column (V_t = 212 ml). Fractions corresponding to the product peak (V_e/V_t = 0.52) were pooled and lyophilized to give a white powder.

Yield: 24.5 mg (71%)

TLC: nBuOH/AcOH/H_2O (3:1:1) R_f=0.29
^{i}PrOH/H_2O (1:1) R_f=0.52
MeOH R_f=0.41

Amino acid analysis (theoretical values in brackets):

$Ala_{1.01(1)}Pro_{0.97(1)}Val_{2.02(2)}Ser^{+}_{0.71(1)}$-

$Gly_{1.01(1)}Tyr_{0.98(1)}$

+ not corrected for degradation.

α^{D}_{25} = -67.02° (c=0.56, water)
$E_{7.0}$ = -0.8
$E_{2.1}$ = 2.5 (both relative to EDNP Lys)
Mass Spectrum (E.I.) M/Z; 859 (0.02%), 828 (0.03%), 637 (0.24%), 605 (0.15%), 566 (0.48%), 534 (3.96%), 453 (2.78%), 421 (2.61%), 338 (25.57%), 225 (31.48%), 128 (100.00%).
U.V. $\lambda_{max.}$ (10% acetic acid) 274 and 278 (sh.) nm. NMR (δ, ppm) (360 MHz; d_4 acetic acid): 6.90 (AB , 4H) (Tyr aromatic), 4.40 - 4.86 (m, 6H), (Ala, Pro, Val x 2, Ser and TyrαCH), 3.55 - 4.17 (m, 6H) (Gly, Pro and Ser $-CH_2-$), 3.06 (m, 2H) (Tyr $-CH_2-$), 1.64 (m, 3H) (Ala $-CH_3$), 0.94 (m, 12H) (Val $-CH_3$). Pro CH_2CH_2-N and Val $-CH(CH_3)_2$ protons observed by solvent peak.

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