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PEPTIDES—XXXII[1]

THE USE OF PHENYL ESTERS IN PEPTIDE SYNTHESIS

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Abstract—Phenyl esters of N-terminal amino-protected peptides are valuable intermediates in synthesis of polypeptides. The phenyl ester function is stable during the customary manipulations of chain extension, and it can be removed selectively by treatment with one equivalent of hydrogen peroxide at pH 10.5 in a variety of solvents such as 80% acetone, dimethylformamide, hexamethylphosphoramide or trifluoroethanol. The method has always been satisfactory providing that dimethylsulphide is used as a scavenger.

Protection of amino-groups during peptide synthesis has received much attention since the modern age of peptide synthesis was ushered in by Max Bergmann and Leonidas Zervas.[2] An authoritative review[3] describes 145 distinct procedures often involving notable ingenuity, however many of them lack clear demonstration of practical utility. In contrast, the companion problem of carboxyl protection has been almost neglected, although 33 procedures are described.[4] Whilst tertiary-butyl esters are well established for protection of the side-chain functions of residues of aspartic and glutamic acids, methyl esters are generally considered to be adequate for "temporary" protection of C-terminal carboxyl functions during construction of the polypeptide chain. In our view the adequacy of methyl ester protection is doubtful.

Although the alkaline hydrolysis of C-terminal peptide methyl esters is facilitated by the relatively low pK_a(*ca.* 3.25) of the corresponding carboxy function, the hydrolytic conditions required may still cause undesired side-reactions. It has been clearly shown that residues of aspartic acid[5] and asparagine[6] are very susceptible to cyclisation to imides. Even earlier, similar observations in both the aspartic and glutamic series had been recorded[7] but the conditions of those reactions were more drastic. It appears that the risk of rearrangement is particularly high when benzyl esters are used for side-chain protection.[8] Moreover other intolerable side-reactions can obviously result from alkaline attack on residues of cysteine, serine, and threonine, be they "protected" or not. This point was made at the second European Peptide Symposium held in Munich in 1958, and subsequently the use of phenyl esters was proposed.[9] More recently we returned to the problem and were able to solve it, through a serendipitous observation based on traces of peroxide in a sample of dioxan.

Our preliminary communication of these results[10] gave sufficient information for those skilled in the art of the peptide synthesis, and we refrained from detailed publication until we had accumulated much further experience. Our two major projects, namely total synthesis of an analogue of lysozyme having a sequence of 129 residues and synthesis of compounds related to big gastrin (34 residues) are firmly based on the phenyl ester principle.

We believe that the method has stood us in good stead[11] and subsequent papers will clearly demonstrate the successful application of this form of carboxyl protection, though here we will only present the essence of the method.

Preparation of amino-acid phenyl esters is not difficult. Direct esterification is, of course, precluded, but N-protected amino-acids are easily converted into their phenyl esters. The comprehensive investigations by many workers into the use of aryl esters in carboxyl *activation*, in contrast to *protection*, during peptide synthesis[12] showed that diaryl sulphites and triaryl phosphites are both effective reagents for conversion of carboxylic acids into aryl esters.

Also recently the use of phosphonium salts, viz. the "BOP" reagent[13] has been introduced for the preparation of aryl esters.[14] In our work we have used these methods, but eventually we found that activation of the N-protected amino-acid by N,N'-dicyclohexylcarbodiimide sometimes with the addition of pyridine gave consistently high yields of the corresponding phenyl ester. The preparations of Z–Ala–OPh (1) and Z–Phe–OPh (2) are given in the experimental section as samples of phenyl ester preparation.

The protective benzyloxycarbonyl group can be removed either by hydrogen bromide in acetic acid [$Br^-H_2^+$–Phe–OPh (3)] or by hydrogenolysis in the presence of *p*-toluenesulphonic acid [$TosO^-H_2^+$–Ala–OPh (4)]. Further examples of phenyl esters which have been prepared in the course of out research are given in Table 1. Obviously amino-acid phenyl esters are more susceptible to dioxopiperazine formation, than are methyl esters, and therefore masking of the liberated amino-group by protonation is essential.

The above mentioned susceptibility of phenyl esters to nucleophilic attack necessitates some care in handling intermediates, although we have never encountered serious difficulties provided the following precautions are observed. Methanolic solvents should be avoided during recrystallisation and chromatography in order to eliminate the possibility of transesterification. Isopropyl alcohol is an acceptible alternative, but we have regularly

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Table 1. Phenyl ester derivatives. Method of preparation: (a) DCC/PhOH, (b) H_2, Pd/C 10% on Z-derivative, (c) DCCl/PhOH/Py, (d) HBr/HOAc2h on Z-derivative, (e) H_2Pd/C 5% on Z-derivative, (f) 5 M HCl/dioxane 40 min on Boc-derivative.

	Yield	mp	α_D	CHN	requires	found
Z.Ala.OPh[a]	86%	94-96°	$[\alpha]_D^{24}$=-48.2 (C=2), EtOH	$C_{17}H_{17}NO_4$	C, 68.21; H, 5.73; N, 4.68	C, 68.25; H, 5.63; N, 4.81
$TosO^-H_2^+$.Ala.OPh[b]	80%	158-160°	$[\alpha]_D^{21}$=+6.7° (C=2, MeOH)	$C_{16}H_{19}NO_5S$	C, 56.97; H, 5.68; N, 4.15	C, 56.62; H, 5.67; N, 4.33
Z.Arg(Adoc)$_2$.OPh[c]	80%	75-77°	$[\alpha]_D^{20}$=-6.5° (C=1, DMF)	$C_{42}H_{52}N_9O_8 \cdot 0.5\ H_2O$	C, 67.27; H, 7.12; N, 7.47	C, 67.54; H, 7.31; N, 7.79
Z.Gly.OPh[c]	88%	67-68°			mp. 67-68° [27]	
$Br^-H_2^+$.Gly.OPh[d]	95%	221-226°			mp. 230° [28]	
Z.Leu.OPh[c]	94%	oil				
$TosO^-H_2^+$.LeuOPh[b]	80%	172.5-175°	$[\alpha]_D^{24}$=+5.6° (C=2, DMF)	$C_{19}H_{25}NO_5S$	C, 60.14; H, 6.64; N, 3.69	C, 59.89; H, 6.63; N, 3.79
Boc.Met.OPh[a]	42%	73-75°	$[\alpha]_D^{21}$=-48.4° (C=1, MeOH)	$C_{16}H_{23}NO_4S$	C, 59.06; H, 7.13; N, 4.31	C, 59.06; H, 7.27; N, 4.35
Z.Phe.OPh[c]	77%	105-108°	$[\alpha]_D^{23}$=-18.9° (C=1, EtOH)	$C_{23}H_{21}NO_4$	C, 73.58; H, 5.64; N, 3.73	C, 73.55; H, 5.64; N, 4.00
$Br^-H_2^+$.Phe.OPh[d]	86%	232-233°	$[\alpha]_D^{27}$=+13.7° (C=1, H_2O)	$C_{15}H_{16}NO_2Br$	C, 55.90; H, 5.00; N, 4.34 Br, 24.80	C, 55.70; H, 5.20; N, 4.10 Br, 24.71
$TosO^-H_2^+$.Ser(Bu^t)OPh[e]	82%	158.5-159°	$[\alpha]_D^{24}$=-28.3° (C=2, $CHCl_3$)	$C_{20}H_{27}NO_6S$	C, 58.66; H, 6.65; N, 3.42	C, 58.51; H, 6.63; N, 3.26
Boc.Trp.OPh[c]	56%	154-155°	$[\alpha]_D^{24}$=-19.8° (C=1, DMF)	$C_{22}H_{24}NO_4$	C, 69.54; H, 6.36; N, 7.36	C, 69.34; H, 6.59, N, 7.26
$Cl^-H_2^+$.Trp.OPh[f]	99%	210° (d)	$[\alpha]_D^{24}$=+34.5° (C=1, DMF)	$C_{17}H_{17}ClN_2O_2$	C, 64.45; H, 5.41; N, 8.84	C, 63.80, H, 5.61; N, 8.60

used dimethylformamide, aqueous hexamethylphosphoramide[15] and N-methylpyrrolidine[16] in gel filteration.

The cardinal principle in protection of functional groups is that, while the protective group survives unscathed through all the preceding stages of synthesis, it can be removed efficiently without damage to the remainder of the molecule when it has served its purpose. In polypeptide synthesis, where a protective group may have to survive a hundred or more stages, these requirements are extraordinarily stringent. Our earlier studies[17] showed clearly that, in alkaline saponification of C-terminal peptide phenyl esters, while the rates had the expected superiority over those of alkyl esters, (half-life *ca.* 20 min, depending on structure, at pH 10.5 in 30% acetone/70% water), they were inadequate for practical use because severe racemisation of the C-terminal chiral centre ensued (e.g. 34% of racemate from Z–Gly–L–Phe–OPh). Initially we were forced to conclude that the method was limited to structures having C-terminal glycine unless the racemisation problem could be solved.

In a detailed re-investigation, the elegant method of Manning and Moore[18] was used for following racemisation by separation of diasterioisomers on ion-exchange columns using a standard amino-acid analyser. (Jeol 6AH, Column 0.6×60 cm, eluted at 57° with 0.2 M sodium citrate buffer pH 4.25 using a flow rate of 58 ml/hr.)

The results of an initial study of the racemisation observed during the hydrolysis of some benzyloxycarbonyl dipeptide methyl esters by one equivalent of sodium hydroxide are shown in Table 2, the solvent being 75% acetone, 25% water.

Having carefully checked the optical homogeneity of the starting compounds it was clear that a significant amount of racemisation was occurring during the hydrolysis, and that the chance of racemisation was highest for aromatic amino-acids. In the literature only isolated cases of such racemisation have been found[19] involving cysteine derivatives. Thus the question of the suitability of methyl ester protection for the carbonyl terminus during peptide synthesis must certainly be raised.

The solution to the problem of racemisation during hydrolysis of peptide phenyl esters came when, purely to overcome a solubility difficulty, a dioxan/water mixture was substituted for acetone/water resulting in an enhanced rate of hydrolysis and reduction of racemisation. The discrepancy was traced to the age of dioxan and attributed to the presence of peroxide impurities.

In Table 3 the extent of racemisation of Z–Ala–Phe–OPh (9) during alkaline hydrolysis at pH 10.5 in the presence of one equivalent of hydrogen peroxide is indicated.

From Table 3 it can be seen that a range of organic solvents could be used in the test case, also that the reaction was complete in a very short time, *ca.* 10 min. In addition it was clear that without the addition of hydrogen peroxide cleavage could be achieved but that racemisation was observed.

In kinetic studies it was found that the initial rate was rapid, the hydrolysis being about 95% complete in five minutes when 1.09 equivalents of hydrogen peroxide were used, and that the rate then levels off giving complete hydrolysis at about 20–25 min. With lower concentrations of hydrogen peroxide the initial rate was slower and total hydrolysis was not achieved. However, total hydrolysis was achieved when the hydrogen peroxide concentration rose above 0.82 equivalents indicating that the function of the peroxide must be to some extent catalytic.

The rapidity of attack by peroxide anion on aryl esters was already well documented in the elegant work of Jencks and Gilchrist,[20] and it is, of course, an example of the well-known "α-affect". Presumably the initially formed per-carboxylic acid (Scheme 1, summarising the process) is susceptible to relatively rapid hydrolysis, regenerating peroxide anion. There is rather little in-

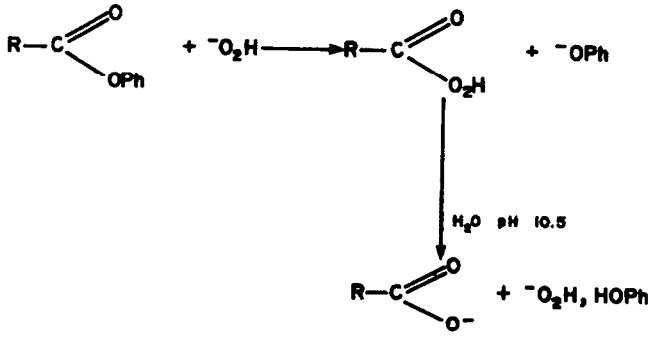
Table 2. Racemisation of methyl esters during hydrolysis

Compound	% Racemisation
Z-Leu-Ala-OMe (5)	0.8
Z-Leu-Leu-OMe (6)	1.0
Z-Ala-Phe-OMe (7)	2.8
Z-Ala-Tyr-OMe (8)	1.8

Table 3. Alkaline hydrolysis of Z-Ala-Phe-OPh(9)

solvent[a]	time(min)	% racemisation
dioxan	10	0
dioxan	13	2.2[b]
acetone	6	0
DMF	10	0

(a) Solvent 60% organic solvent 40% water
(b) Without addition of H_2O_2



Scheme 1.

formation about the stability of per-carboxylic acids under such conditions,[21] but there is some evidence that instability increases as the pK_a of the related carboxylic acid is reduced; the normal pK_a of a C-terminal peptide carboxy group lies around 3.2 compared with about 4.8 for normal aliphatic acids.

A very important question is the integrity of the α-amino-acids sequence when β- and γ-carboxyl functions of aspartic and glutamic acid are protected as alkyl esters.[5] The two protected dipeptides Z-Asp(OBu^t)-Gly-OPh (10) and Z-Glu(OBu^t)-Gly-OPh (11) were subjected to phenyl ester cleavage in 80% aqueous acetone at pH 10.5 in the presence of 1 equivalent of H_2O_2. After base uptake had ceased (about 10 min) the free acids were isolated and treated with 90% trifluoroacetic acid and finally hydrogenolysed over 10% Pd/C. The resulting free dipeptides were run on the amino-acid analyser and showed no trace of $\alpha \rightarrow \beta$ or $\alpha \rightarrow \gamma$ peptide rearrangement. In the absence of H_2O_2 the aspartyl dipeptide took 2 hr for complete cleavage at pH 10.5 and showed 1% $\alpha \rightarrow \beta$ rearrangement.

In addition to the question of side-reactions arising from rearrangement we examined the fate of methionine, S-acetamidomethyl cysteine and tryptophan as these residues are all sensitive to oxidative conditions. The results are summarised in Table 4. In all examples the cleavage was rapid, the rate being independent of the presence of dimethylsulphide which acted as a scavenger. Clearly the presence of such a scavanger is required for such amino-acids and in practice generally a fifty fold excess is used.

Whereas mixtures of acetone, acetonitrile, dioxan, etc. and water could be used in the preliminary work, more polar solvents are required for tackling current problems at the frontiers of peptide synthesis. We found that 10% was the minimum concentration of water for efficient cleavage in dimethylformamide or hexamethylphosphoramide; dimethyl-sulphoxide gave poor results. An alternative solvent, much favoured in difficult cases, is 2,2,2-trifluoroethanol, which automatically buffers at an apparent pH about 10.5 as it has a pK_a of 12.4.[22] When dimethylformamide or hexamethylphosphoramide is used, the pH is maintained by an auto-titrator which provides a convenient record of the course of reaction.

The rate of phenyl ester cleavage naturally depends on the structure of the substrate, normally between 5 and 20 min are sufficient for completion, even in the case of a relatively large peptide such as the 1–16 portion of our Lysozyme analogue which is cleaved in under 20 min when 90% trifluoroethanol is used as solvent. It should be emphasised that for larger peptides the rate is to some extent solvent dependent and follows the order DMF ≃ NMP < HMPA < TFE. The water content should not fall ourside the range 10–20%, as lesser proportions of water slow the hydrolysis down appreciably and higher proportions of water often cause precipitation of substrate.

That our conditions for the removal of the phenyl ester function do not disturb other protecting groups can be seen from the large number of examples provided in following papers in this series. It is clear that the phenyl ester group is fully compatible with t-butyl based side-chain protection and that providing a scavanger (dimethylsulphide) is used methionine, S-acetamidomethylcysteine and tryptophan are all unaffected by the cleavage conditions.

One fear about incorporation of C-terminal phenyl esters into peptide structures concerned us initially, but it proved to be illusory. If a sequence is constructed by stepwise addition from the C-terminus—the Bodansky tactic—there is a risk of dioxopiperazine formation during addition of the third residue. Although we initially used a 2+1 azide coupling to bypass this stage, many subsequent experiments have shown that the mixed anhydride method eliminates any problems that might exist. Also it is worth pointing out that when phenyl esters are used in the presence of hydroxy-benzotriazole no activation of the ester function is observed.[23]

The general reactivity of phenyl esters towards nucleophiles may, however, be turned to synthetic advantage. Thus phenyl esters may be readily hydrazinolysed to afford entry to the azide coupling of fragments; this has been employed in our big gastrin programme.[24] Also aminolysis of phenyl esters has been used to prepare peptide amides from the corresponding phenyl esters, for example C-terminal gastrin fragment Nps-Trp-Met-Asp(OBu^t)-Phe-NH_2 has been prepared by this method.[25]

To sum up, we regard the phenyl ester method as a versatile, powerful tool in polypeptide synthesis. It has enabled us to construct some of the largest polypeptide structures of varied sequence yet synthesised,[11] and it can be employed in the solid-phase technique by means of suitable phenolic resins.[26]

EXPERIMENTAL

The majority of abbreviations are those in common usage,[33] and all amino-acids are of the L-configuration. The abbreviations not in standard use are as follows: DCCI,N,N^1-dicyclohexylcarbodiimide; DCU,N,N^1-dicyclohexylurea; TEA, triethylamine; IBC, isobutylchloroformate; HOCp, 2,4,5-trichlorophenyl. All solutions were dried over $MgSO_4$ and evaporated *in vacuo* at the minimum temp. possible, an oil pump being used to remove DMF.

Z-Ala-OPh (1). Z-Ala-OH (22.3 g, 0.1 M) was dissolved in CH_2Cl_2 (200 ml) and the soln cooled to −20°. Phenol (9.4 g, 0.1 M) was added to the stirred soln followed by DCCI (22.7 g, 0.11 M),

Table 4. Effect of phenyl ester cleavage conditions on derivatives of sensitive amino-acids

Compound	Hydrolysis/time[a]	% recovery[b]	
Boc-Met-OPh (12)	20	12[c]	100[d]
Boc-Ala-Cys(Acm)-Gly-OPh (13)	15	5[c]	100[d]
Z-Trp-Gly-OPh (14)	5	69[c]	96[d]

(a) Time for complete hydrolysis at pH 10.5 in 80% aqueous dioxan (min)
(b) Estimated by running the deprotected peptide on the amino-acid analyser
(c) H_2O_2/NaOH
(d) H_2O_2/NaOH/30 equivalents $(CH_3)_2S$.

and the mixture allowed to warm to room temp. overnight. Three drops of glacial AcOH were added and after 0.5 hr the DCU was removed by filtration. The filtrate was evaporated and dissolved in EtOAc (200 ml), the soln was then washed with 5% $NaHCO_3$, 5% citric acid and water. After drying the product (1) was crystallised by the addition of petroleum ether to give (25.7 g, 86%, m.p. 94–96, $[\alpha]_D^{24}$–48.2° (c = 2, EtOH), (Found: C, 68.25; H, 5.63; N, 4.81. $C_{17}H_{17}NO_4$ requires: C, 68.21; H, 5.73; N, 4.68%).

Z–Phe–OPh (2). Z–Phe–OH (7.5 g, 25 mM), pyridine (1.94 ml, 25 mM) and phenol (2.7 g, 25 mM) were dissolved in EtOAc (75 ml) and cooled to −20°. DCCI (5.4 g, 26 mM) was added and the stirred soln allowed to attain room temp. overnight. A few drops of glacial AcOH were then added and after 0.5 hr the DCU was removed by filtration. The filtrate was washed with 5% $NaHCO_3$, 5% citric acid and water. The soln was dried and evaporated to give an oil which was crystallised from EtOAc petroleum ether yielding 2 (6.4 g, 68%) m.p. 105–108°, $[\alpha]_D^{22}$–18.9° (c = 1, EtOH), (Found: C, 73.55; H, 5.64: N, 4.00. $C_{23}H_{21}NO_4$ requires: C, 73.58; H, 5.64; N, 3.73%.

$Br^-H_2^+$–Phe–OPh (3). Z–Phe–OPh (28.2 g, 75 mM) was suspended in glacial AcOH (25 ml) and treated with a 50% soln of HBr in glacial AcOH (50 ml). After stirring under anhydrous conditions for 2 hr at room temp. 3 was precipated by the addition of Et_2O and drying was (20.8 g, 86%), m.p. 232–233°, $[\alpha]_D^{22}$ + 13.7°(c = 1, H_2O), (Found: C, 55.70; H, 5.20; N, 4.10; Br, 24.71. $C_{15}H_{16}NO_2Br$ requires: C, 55.90; H, 5.00; N, 4.34; Br,24.80%).

$TosO^-H_2^+$–Ala–OPh (4). Compound 1 (3.0 g, 10 mM) and *p*-toluenesulphonic acid monohydrate (1.9 g, 10 mM) were dissolved in a mixture of glacial AcOH (40 ml) and water (5 ml). This soln was hydrogenolysed overnight after the addition of Pd/C 10% catalyst (0.3 g). The soln was filtered through celite and evaporated, the residue being dissolved in MeOH and crystallised by the addition of Et_2O giving (4) (3.0 g, 80%), m.p. 158–160°, $[\alpha]_D^{21}$ + 6.7°(c = 2, MeOH), (Found: C, 56.62; H, 5.67; N, 4.33. $C_{16}H_{19}NO_5S$ requires: C, 56.97; H, 5.68; N, 4.15%).

The esters 5 to 8 were prepared by coupling Z–Leu–OH or Z–Ala–OH with the appropriate methyl ester hydrochloride using isobutyl-chloroformate, with TEA as base. Compound 5 yield 79%, m.p. 94–96°, $[\alpha]_D^{22}$ − 37.5°(c = 1, EtOH), lit.[29] m.p. 95–96°, $[\alpha]_D^{22}$ − 38.0°(c = 1, EtOH); Compound 6 yield 88%, m.p. 95–96°, $[\alpha]_D^{18}$ − 39.0°(c = 5.3, MeOH), lit.[30] m.p. 95–96°, $[\alpha]_D^{22}$ − 39.6° (c = 1, MeOH): Compound 7 yield 76%, m.p. 101–103°, $[\alpha]_D^{22}$ − 13.9° (c = 1, MeOH), lit.[31] m.p. 99–102°, $[\alpha]_D^{22}$ − 14.9° (c = 1, MeOH); Compound 8 yield 84%, m.p. 119–121°, $[\alpha]_D^{22}$ + 22.6°(c = 1, HOAc), lit.[32] m.p. 121–122°, $[\alpha]_D^{22}$ + 22.2° (c = 1.95, HOAc).

Z–Ala–Phe–OPh (9). Z–Ala–OH (2.2 g, 10 mM) and TEA (1.4 ml 10 mM) were dissolved in EtOAc (20 ml) and cooled to −20°. IBC(1.3 10 mM) was added and the soln stirred for 10 min. A soln of 3 (3.2 g, 10 mM) and TEA (1.4 ml, 10 mM) in DMF (20 ml) was added and the mixture allowed to attain room temp. overnight. The soln was evaporated and the residue dissolved in EtOAc. This soln was washed with 5% $NaHCO_3$ 5% citric acid and water then dried. Evaporation gave an oil which crystallised from EtOAc–petroleum ether giving 9 (1.1 g, 81%) m.p. 118–120°, $[\alpha]_D^{24}$ + 7.7°(c = 2, EtOH), (Found: C, 69.81; H, 5.90; N, 6.50. $C_{26}H_{26}N_2O_5$ requires: C, 69.94; H, 5.87; N, 6.27%).

Z–Asp(OBu^t)–Gly–OPh (10). Z–Asp(OBu^t)–OH (30 mM), prepared directly from the corresponding dicyclohexylammonium salt (15.1 g, 30 mM), was dissolved in EtOAc (100 ml) and cooled to −20°. TEA (4.2 ml), 30 mM) was added followed by IBC (3.8 ml, 30 mM). After 10 min activation a soln of $Br^-H_2^+$–Gly–OPh (9.7 g, 30 mM) and TEA (4.2 ml, 30 mM) in DMF (75 ml) was added and the mixture stirred overnight at room temp. Evaporation gave an oil which was dissolved in EtOAc. This soln was worked up in the usual way and 10 crystallised from Et_2O-petroleum ether giving (11.2 g, 68%), m.p. 93–94°, $[\alpha]_D^{22}$ − 32.2° (c = 1, DMF), (Found: C, 68.29; H, 6.30; N, 5.18. $C_{31}H_{34}N_2O_7$ requires: C, 68.11 H, 6.27; N, 5.13%).

Z–Glu(OBu^t)–Gly–OPh (11). Z–Glu(OBu^t)–OCp (5.2 g, 10 mM), $Br^-H_2^+$–Gly–OPh (2.3 g, 10 mM) and TEA (1.4 ml, 10 mM) were dissolved in DMF (25 ml) and stirred for 2 days at room temp. The solvent was evaporated and the residue dissolved in EtOAc, this soln was worked up by the same method as that used for 9. This gave the crystalline 11 (4.1 g, 86%), m.p. 75–77°, $[\alpha]_D^{22}$ − 13.2° (c = 1, DMF), (Found: C, 63.65; H, 6.51; N, 5.94. $C_{25}H_{30}N_2O_7$ requires: C, 63.81; H, 6.43; N, 5.95%).

Boc–Met–OPh (12). Boc–Met–OH dicyclohexylammonium salt (1.4 g, 3.2 mM) was suspended in EtOAc (50 ml) and washed with 50% citric acid (20 ml). The organic phase was washed with water (×2) and dried. Evaporation gave a pale yellow oil which was dissolved in CH_2Cl_2 (15 ml) and cooled to −20°. Phenol (0.3 g, 3.2 mM) and DCC1 (0.7 g, 3.6 mM) were added and the stirred mixture allowed to attain room temp. overnight. The urea was filtered and the filtrate washed with 5% $NaHCO_3$, 5% citric acid and water. After drying evaporation yielded an oil which was crystallised from EtOAc–petroleum ether yielding 12 (0.45 g, 42%), m.p. 73–75°, $[\alpha]_D^{21}$ − 48.4° (c = 1, MeOH), (Found: C, 59.06, H, 7.30; N, 4.40. $C_{16}H_{23}NO_4S$ requires: C, 56.06; H, 7.13; N, 4.31%).

Boc–Ala–Cys(Acm)–Gly–OPh (13). Boc–Cys(Acm)–OH (1.77 g, 6. mM) was dissolved in CH_2Cl_2 (25 ml) and cooled to −20°. TEA (0.84 ml, 6 mM) and IBC (0.78 ml, 6 mM) were consecutively added and 10 min allowed for activation. A soln of $Br^-H_2^+$–Gly–OPh (1.40 g, 6 mM) and TEA (0.84 ml, 6 mM) in DMF (60 ml) was added and the mixture stirred overnight. This soln was evaporated and the residue dissolved in EtOAc. This soln was washed with 5% $NaHCO_3$, 5% citric acid and water. After drying and evaporation an oil was obtained which could not be crystallised. This oil was dissolved in anhydrous trifluoroacetic acid and allowed to stand for 40 min at room temp. under N_2. Evaporation and trituration of the residue gave the corresponding trifluoroacetate (2.54 g, 5.7 mM) as an oil.

Boc–Ala–OH (1.08 g, 5.7 mM) was dissolved in DMF (25 ml) and cooled to −20°. TEA (0.80 ml, 5.7 mM) was added followed by IBC (0.74 ml, 5.7 mM) leaving 10 min for formation of the mixed anhydride. The above trifluoroacetate and TEA (0.8 ml, 5.7 mM) were dissolved in DMF (50 ml) and added to the mixed anhydride soln. This was stirred overnight and allowed to attain room temp. Evaporation gave oil which was worked up in the usual way by washing with 5% $NaHCO_3$ and 5% citric acid. The resulting oil was recrystallised twice from EtOAc–petroleum ether yielding the required protected tripeptide 13 (1.74 g, 68%), m.p. 114–117°, $[\alpha]_D^{22}$ − 53.0 (c = 2, MeOH), (Found: C, 52.92; H, 6.70; N, 11.00. $C_{22}H_{32}N_4O_7S$ requires: C, 53.22; H, 6.50; N, 11.28%), Amino-acid analysis: 6 M HCl/110°/24 hr $Gly_{1.00}$ $Ala_{1.00}$; APM, $Cys(Acm)_{0.99}$ $Gly_{1.01}$ $Ala_{1.00}$.

Z–Trp–OPh 14. Z–Trp–OH (3.4 g, 10 mM) was suspended in CH_2Cl_2 (60 ml) and TEA 1.4 ml, 10 mM) added. The soln was cooled to −20°, IBC (1.2 ml, 10 mM) added and 10 min allowed for activation. A soln of $Br^-H_2^+$–Gly–OPh (2.3 g, 10 mM) and TEA (1.4 ml, 10 mM) in DMF (25 ml) was added and the stirred mixture allowed to attain room temp. overnight. Evaporation gave an oil which was dissolved in EtOAc, this soln was processed in an identical manner to that used for 9 to give the required 14 (2.8 g, 59%), m.p. 124–127°, $[\alpha]_D^{21}$–17.6° (c = 2, MeOH) (Found: C, 68.90; H, 5.60; N, 8.89. $C_{27}H_{25}N_3O_5$ requires: C, 68.78; H, 5.34; N, 8.91%).

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