Specific Covalent Fixation of Chelating Agents on Peptides

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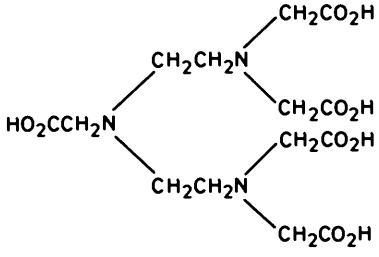
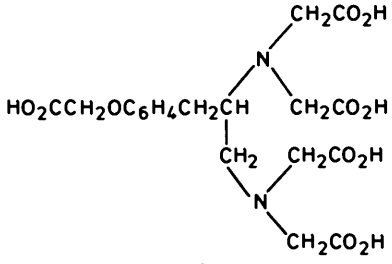
The synthesis of two selectively protected bifunctional analogues of ethylenediaminetetra-acetic acid (EDTA) is described. In these analogues the four carboxy groups involved in chelation are protected as benzyl or t-butyl esters, while the fifth one, which is free, allows their coupling in a predetermined position to a nucleophilic group. An example of their use in solid phase peptide synthesis is given.

Covalent fixation of metal chelates to macromolecules has recently received considerable attention.[1-7] These chelates generally bind metals such as technecium 99m or indium 111 whose availability and favourable decay allow their use as radiopharmaceuticals. Homobifunctional chelates such as (**1a**)[8,9] or (**1b**) take advantage of the properties of monoclonal antibodies to achieve highly specific imaging.



(**1a**)



(**1b**)

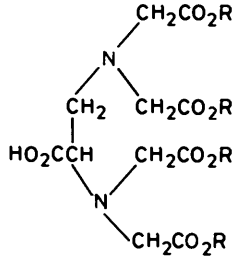
However, these chelates suffer from two main disadvantages: (i) being homobifunctional agents, the carboxylic groups which are involved in chelation can be implicated during the coupling reaction, leading to the formation of chelating structures having decreased affinities and (ii) the target reacting groups on the proteins (mainly ε-NH_2 groups of lysine side chains) which are modified during the coupling reaction, can sometimes be necessary to maintain the biological activity or the affinity of the macromolecule.

Although the first problem could be solved by use of various heterobifunctional chelates, the second one remains largely unsolved. Our goal was to achieve the labelling of small peptides, such as hormones or neuropeptides with metal chelates. In such peptides, structure-activity relations generally show that, while several identical residues often co-exist in the same molecule, their importance for biological activity can be completely different. Non-specific modification of one class of residues could thus lead to a heterogeneous population of peptide-chelates whose activity and affinity for receptors could be greatly different. While in the case of proteins, such an approach seems at the moment the sole possibility, the incorporation of a chelating structure in a predetermined position could be of great interest in the case of molecules such as peptides which, owing to their moderate size, can now be easily obtained by chemical synthesis.

We describe in this paper the preparation of the selectively protected homobifunctional chelates (**2**) and (**3**) which can be used in solid phase peptide synthesis and allow an unequivocal anchoring of the chelating group. In these compounds, the four carboxy groups of the chelating moiety are protected by esterification while the fifth one, which remains free can be activated and coupled to a nucleophilic function.

(**2**) R = CH_2Ph

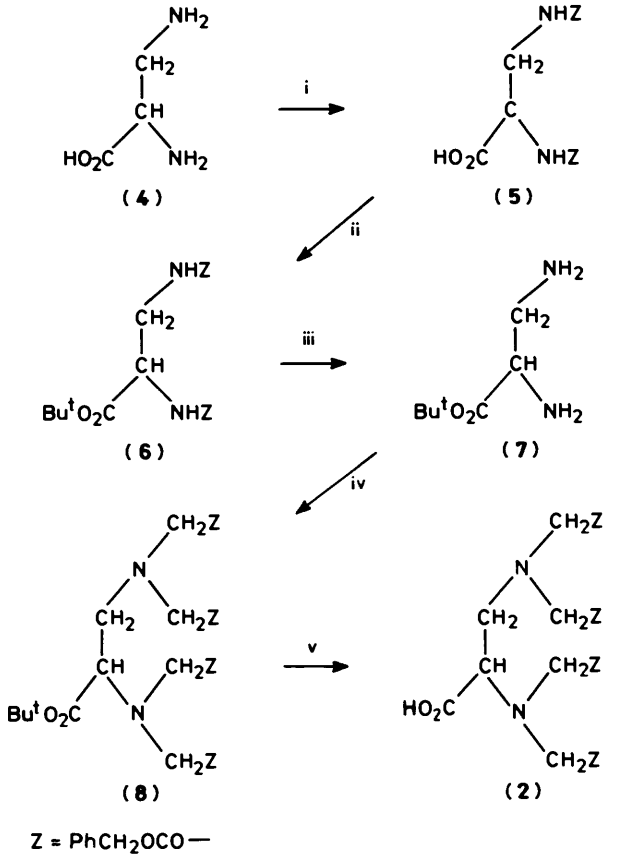
(**3**) R = Bu^t



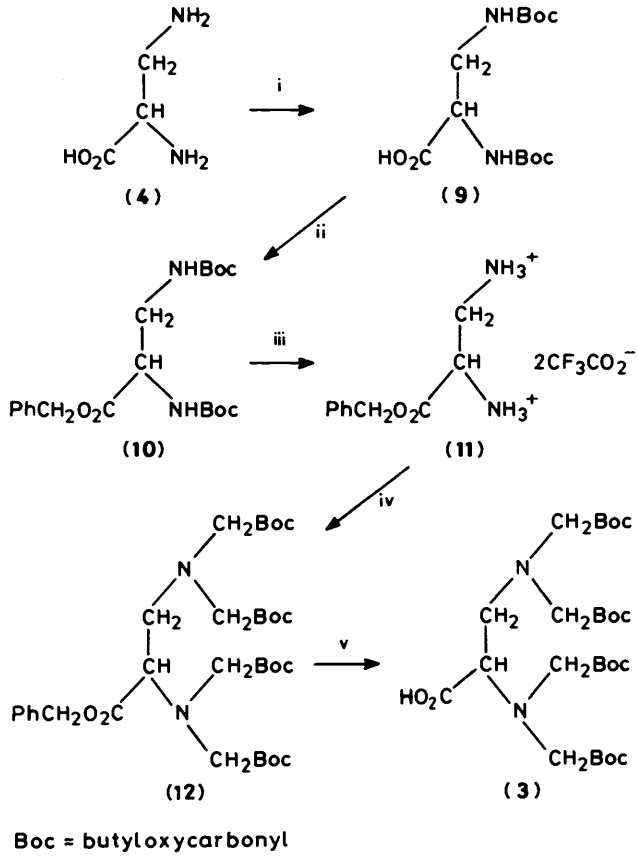
Compounds (**2**) and (**3**) can thus be used in the two most popular methods of solid phase peptide synthesis introduced by Merrifield:[10] (i) In the first method, *N*-α-Boc temporary protection and repetitive acid-catalysed deprotections generally using trifluoroacetic acid (TFA) are associated with benzyl type groups for permanent side chain protection and anchoring to the resin. Final cleavage and deprotection are generally performed using anhydrous hydrogen fluoride. Under these conditions, compound (**2**) can be used to acylate the ε-NH_2 group of a lysine residue located inside the growing peptide chain (such a free ε-NH_2 group can be obtained by using *N*-α-Boc,*N*-ε-Fmoc† lysine which allows selective base deprotection of the side chain), while compounds (**2**) and (**3**) can both be used to acylate the NH_2 terminal group, and (ii) The second method makes use of *N*-α-Fmoc temporary protection and repetitive basic deprotection while t-butyl type groups are used for permanent side chain protection.[11] A protolytic treatment (TFA) at the end of the synthesis allows complete deprotection and cleavage from the resin. In these conditions, compound (**3**) can be used to acylate the α-NH_2 terminal group and will be deprotected during the final TFA treatment.

Synthesis of 2,3-*Bis*[*di*(*benzyloxycarbonylmethyl*)*amino*]*propionic Acid* (**2**).—The preparation of compound (**2**) was per-

† Fmoc = fluoren-9-ylmethoxycarbonyl.



Scheme 1. *Reagents:* i, $PhCH_2OCOCl$; ii, Bu^tOH–$POCl_3$; iii, H_2–C–Pd; iv, $BrCH_2CO_2CH_2Ph$, pH 6 ± 0.5; v, TFA.



Scheme 2. *Reagents:* i, $(Boc)_2O$; ii, CsOH–$PhCH_2Cl$; iii, TFA; iv, $BrCH_2CO_2Bu^t$, pH 6 ± 0.5; v, H_2–C–Pd.

formed according to a five step protocol (Scheme 1). Owing to the poor solubility of 2,3-diaminopropionic acid (**4**), direct esterification to give compound (**7**) was not possible. The acid (**4**) was instead first converted into the benzyl ester (**5**) by reaction with benzyloxycarbonyl chloride. Compound (**5**) was then converted into the t-butyl ester (**6**) by treatment with $POCl_3$ and 2-methylpropan-2-ol in pyridine. Compound (**6**) was submitted to hydrogenolysis in the presence of palladium on activated carbon to give the diamine (**7**) which was then treated with benzyl 2-bromoacetate at 75 °C, the pH of the reaction medium being maintained between 5.5 and 6.5 by the addition of triethylamine, thus allowing deprotonation of the amino groups while avoiding hydrolysis of benzyl esters. Treatment of the fully protected compound (**8**) with TFA, led to the tetrabenzyl ester (**2**) which is suitably protected for unequivocal coupling in peptide synthesis.

Synthesis of 2,3-*Bis*[*di*(*t-butyloxycarbonylmethyl*)*amino*]*propionic Acid* (**3**).—The preparation of the acid (**3**) was performed according to Scheme 2. In this case, compound (**4**) was first converted to the di-Boc derivative by reaction with di-t-butyl dicarbonate. Reaction of the caesium salt of the diester (**9**) with benzyl chloride led to the triester (**10**) under mild conditions. Compound (**10**) was then deprotected by reaction with TFA to give the salt (**11**) which was alkylated with t-butyl 2-bromoacetate at 75 °C, the pH of the reaction medium being maintained between 5.5 and 6.5 by the addition of triethylamine. Final treatment of resulting pentaester (**12**) with hydrogen in the presence of palladium on activated carbon allowed for the selective cleavage of the benzyl ester group to give the suitably protected compound (**3**).

Use in Solid Phase Peptide Synthesis.—In order to demonstrate the usefulness of these derivatives, compound (**3**) was incorporated on the terminal NH_2 group of Substance P: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2. Modification in this position, even with bulky structures is known to have little effect on the biological activity of this peptide.[12] The solid phase synthesis was performed on a benzhydrylamine polystyrene resin.[10] *N*-α-Boc protection was used for all amino-acids, TFA–CH_2Cl_2 (4:6) for deprotection, and ethanedithiol (5%) was added as a scavenger. The side chain of trifunctional amino-acids were protected as follows: Lys,Z and Arg,Ts. At the end of the synthesis, compound (**3**) was incorporated by coupling with dicyclohexylcarbodi-imide (DCC), in the presence of hydroxybenzotriazole (HOBT). After treatment with HF, the peptide was purified by gel filtration (Trisacryl GF05, 5% aqueous acetic acid) and reverse phase h.p.l.c. (μ Bondapack C18). The purified product was controlled by analytical h.p.l.c. and amino-acid analysis of the acidic hydrolysate. The presence of the intact chelating structure on the peptide was confirmed by FAB mass spectroscopy which showed the expected protonated ion at *m/z* 1 665.

Experimental

M.p.s were determined on a BUCHI melting point apparatus and are uncorrected. ^1H N.m.r. spectra were obtained by using a JEOL 60 Mz spectrometer; mass spectra were obtained from the University of Sciences, Lille (France), micro-analysis and FAB mass spectrometry from CNRS (France).

2,3-*Di*(*benzyloxycarbonylamino*)*propionic Acid* (**5**).—To a

solution of 2,3-diaminopropionic acid monohydrochloride (**4**)* (2.67 g, 19 mmol) in aqueous NaOH (4M; 10 ml), at O °C, were alternately added dropwise to aqueous NaOH (4M; 3 × 5 ml) and benzyloxycarbonyl chloride (3 × 2.9 ml, 0.06 mol). After 30 min at 0 °C, water (400 ml) was added and the reaction mixture washed twice with diethyl ether. The aqueous layer was acidified to pH 3 with 1M-HCl and extracted with ethyl acetate. The extract was dried (Na_2SO_4) and evaporated under reduced pressure to yield a crude compound (6.7 g, 95%) which recrystallized from ethyl acetate–hexane to give the pure *compound* (**5**), (6.1 g, 87%), m.p. 126 °C; n.m.r. ($CDCl_3$) 7.3 (10 H, s, ArH), 5.5 and 5.3 (2 × 1 H, m, 2 × NH), 5.2 (4 H, m, CH_2Ph), 4.2 (1 H, m, CH), and 3.2 (2 H, m, CH_2) (Found: C, 61.3; H, 5.35; N, 7.5. $C_{19}H_{20}N_2O_6$ requires C, 61.28; H, 5.41; N, 7.52%).

2,3-*Di*(*benzyloxycarbonylamino*)*propionic Acid t-Butyl Ester* (**6**).—To a solution of the acid (**5**) (4 g, 10.7 mmol) in pyridine (9.5 ml) and 2-methylpropan-2-ol (22 ml) at −5 °C, was added $POCl_3$ (1.16 ml) with vigorous stirring. The mixture was then stirred at room temperature for 4 h after which time the solution was partitioned between water and ethyl acetate. The organic layer was separated, washed with 1M-HCl (100 ml), shaken with 2% aqueous $NaHCO_3$ (200 ml), dried ($MgSO_4$), and evaporated to dryness to give a yellow oil (2.3 g, 50%) which slowly crystallized (from light petroleum b.p. 40—65 °C) m.p. 75—76 °C; δ($CDCl_3$) 7.3 (10 H, s, ArH), 5.5 and 5.3 (1 H, m, NH), 5.2 (4 H, m, CH_2Ph), 4.2 (1 H, m, CH), 3.2 (2 H, m, CH_2), and 1.4 (9 H, s, CMe_3) (Found: C, 64.35; H, 6.6; N, 6.55. $C_{23}H_{28}N_2O_6$ requires C, 64.47; H, 6.58; N, 6.53%).

2,3-*Diaminopropionic Acid t-Butyl Ester* (**7**).—Palladium-on-activated charcoal catalyst (5%; 0.3 g) was added to a solution of the ester (**6**) (3 g, 7 mmol) in methanol (30 ml). The mixture was stirred in an atmosphere of hydrogen until no more gas was absorbed (about 3 h). The end of the transformation was determined by t.l.c. [SiO_2; toluene–ethyl acetate (8:2)], the catalyst was then removed by filtration and the filtrate concentrated under reduced pressure to yield a yellow oil (0.7 g, 65%) which was used without further purification.

2,3-*Bis*[*di*(*benzyloxycarbonylmethyl*)*amino*]*propionic Acid t-Butyl Ester* (**8**).—Benzyl 2-bromoacetate (10.1 g, 0.44 mol) was added dropwise to a refluxing solution of compound (**7**) (1.5 g, 9.3 mmol) in absolute ethanol (80 ml). The pH of the mixture was maintained between 5.5 and 6.5 for *ca.* 1 h by repeated addition of triethylamine. The solvent was then removed and the oily residue diluted with ethyl acetate and water. The organic layer was separated, dried, and evaporated to dryness. The residue was chromatographed on a column of silica gel; elution with light petroleum (b.p. 40—65 °C) diethyl ether (7:3) gave the *title compound* (**8**) as an oil, (1.5 g, 20%), δ($CDCl_3$) 7.3 (20 H, s, ArH), 5.2 (8 H, s, CH_2Ph), 4.2 (1 H, m, CH), 3.7 (8 H, s, CH_2N), 3.2 (2 H, m, CH_2), and 1.4 (9 H, s, CMe_3); *m/z* 752 (M^+), 661 ($M^+ - CH_2Ph$), 651 ($M^+ - CO_2CMe_3$), 617, and 561 (Found: C, 68.7; H, 6.3; N, 3.7. $C_{43}H_{48}N_2O_{10}$ requires C, 68.60; H, 6.42; N, 3.72%).

2,3-*Bis*[*di*(*benzyloxycarbonylmethyl*)*amino*]*propionic Acid* (**2**).—The ester (**8**) (1.5 g, 2 mmol) was treated with a solution of TFA in CH_2Cl_2 (2:3) for 30 min. The TFA and solvent were removed under reduced pressure, the residue dissolved in ethanol, and the pH of the mixture brought to 5 by the addition of triethylamine. The oily residue was purified by preparative t.l.c. [SiO_2; light petroleum (b.p. 40—65 °C–diethyl ether (6:4)] and showed δ($CDCl_3$) 7.3 (20 H, s, ArH), 5.2 (8 H, s, CH_2Ph), 4.2 (1 H, m, CH), 3.7 (8 H, s, NCH_2) 3.2 (2 H, m, CH_2).

2,3-*Di*(*butyloxycarbonylamino*)*propionic Acid* (**9**).—According to the method of Pless *et al.*,[1] di-t-butyl dicarbonate (15.5 g, 0.07 mol) was added with stirring to a solution of 2,3-diaminopropionic acid monohydrochloride (**4**) (2.5 g, 18 mol) in aqueous $KHCO_3$ (1M; 30 ml) and 2-methylpropan-2-ol (35 ml). The pH of the reaction mixture was maintained between 8 and 9 by the addition of aqueous Na_2CO_3 (1M). The transformation was monitored by t.l.c. [SiO_2; methanol–ethyl acetate (1:2)]. After 12 h, the 2-methylpropan-2-ol was removed, and the turbid solution was diluted with water (50 ml) and extracted with pentane (3 × 25 ml). The aqueous phase was acidified (pH 2—3) by the addition of aqueous $KHSO_4$ and extracted with ethyl acetate (2 × 100 ml). The combined organic layers were dried and evaporated and the oily residue was crystallized from light petroleum (b.p. 40—65 °C) to give *compound* (**9**) (4.8 g, 90%), m.p. 160 °C; δ($CDCl_3$) 9.1 (1 H, s, CO_2H), 5.2 (2 H, m, 2 × NH), 4.2 (1 H, m, CH), 3.2 (2 H, m, CH_2), and 1.4 (18 H, s, 2 × CMe_3) (Found: C, 51.25; H, 8.0; N, 9.15. $C_{13}H_{24}N_2O_6$ requires C, 51.30; H, 7.94; N, 9.20%).

2,3-*Di*(*butyloxycarbonylamino*)*propionic Acid Benzyl Ester* (**10**).—According to the method of Wang *et al.*,[2] the pH of a solution of the acid (**9**) (9.8 g, 32 mmol) in ethanol (100 ml) and water (50 ml) was adjusted to 7 by the addition of 10% aqueous CsOH. The solution was evaporated to dryness, the residue dissolved in DMF (20 ml), and benzyl 2-bromoacetate (4.6 ml, 0.038 mol) added with stirring. After 12 h at room temperature, DMF was removed under reduced pressure, and water and ethyl acetate were added. The organic layer was separated, dried, and evaporated and the residue was recrystallized from ethyl acetate–hexane to yield *compound* (**10**) (10.3 g, 81%) m.p. 126 °C, δ($CDCl_3$) 7.3 (5 H, s, ArH), 5.5 and 5.3 (each 1 H, m, NH), (2 H, s, CH_2Ph), 4.2 (1 H, m, CH), 3.5 (2 H, m, CH_2), and 1.4 (18 H, s, 2 × CMe_3) (Found: C, 60.75; H, 7.7; N, 7.15. $C_{20}H_{30}N_2O_6$ requires C, 60.89; H, 7.66; N, 7.10%).

2,3-*Diaminopropionic Acid Benzyl Ester Trifluoroacetate* (**11**).—Compound (**10**) (10.2 g, 26 mmol) was dissolved in a solution (70 ml) of TFA in CH_2Cl_2 (2:3) and the mixture stirred for 30 min. The TFA and solvent were then removed under reduced pressure. The residue was washed several times with anhydrous diethyl ether to yield the trifluoroacetate (**11**) (10.5 g, 98%) which was used without further purification.

2,3-*Bis*[*di*(*t-butoxycarbonylmethyl*)*amino*]*propionic Acid Benzyl Ester* (**12**).—The pH of a solution of the acetate (**11**) (5.7 g, 13 mmol) in absolute ethanol (150 ml) was adjusted to 7 by the addition of triethylamine. t-Butyl 2-bromoacetate (10.5 ml, 0.064 mol) was added dropwise to the refluxing solution and the pH of the mixture was maintained between 5.5 and 6.5 for *ca* 1 h by further addition of triethylamine. The solvent was then removed and the oil residue treated with ethyl acetate–water; the organic layer was separated, dried, and evaporated to dryness. An oily product (**12**) was isolated by column chromatography [SiO_2; light petroleum (b.p. 40—65 °C–diethyl ether (7:3)] (2.1 g, 23%), δ($CDCl_3$) 7.3 (5 H, s, ArH), 5.2 (2 H, s, CH_2Ph), 4.2 (1 H, m, CH), 3.7 (8 H, s, CH_2N), 3.2 (2 H, s, CH_2), and 1.4 (36 H, s, CMe_3); 650 (M^+), 606 ($M^+ - CO_2$), 549 ($M^+ - CO_2 - CMe_3$), 515 ($M^+ - C_6H_5CH_2CO_2$) (Found: C, 62.5; H, 8.45; N, 4.35. $C_{34}H_{54}N_2O_{10}$ requires C, 62.75; H, 8.36; N, 4.30.

2,3-*Bis*[*di*(*t-butoxycarbonylmethyl*)*aminopropionic Acid* (**3**).—Palladium on activated carbon (0.5 g) was added to a solution of compound (**12**) (0.8 g, 1.2 mmol) in methanol (10

* Racemic DL-2,3-diaminopropionic acid was used and no attempt was made to resolve racemates.

ml). The mixture was stirred in an atmostphere of hydrogen until no more gas was absorbed (*ca.* 3 h). The end of the transformation was determined by t.l.c. [SiO_2; toluene–ethyl acetate (4:1)], the catalyst was removed by filtration, and the filtrate concentrated under reduced pressure. Preparative t.l.c. eluting with light petroleum (b.p. 40—65%)–diethyl ether (7:3) gave the acid (**3**) as an oil, (0.40 g, 50%), n.m.r. spectroscopy confirmed the removal of the benzyl group; *m/z* 560 (M^+), 516 ($M^+ - CO_2$), 459 ($M^+ - CO_2O - CMe_3$).

Peptide Synthesis.—The peptidyl-resin was prepared on a benzhydrylamine–functionalized polymer using a previously described protocol.[10] After deprotection and neutralization of the α-amino group of arginine, compound (**3**) was coupled in two-fold excess using DCC and HOBT for 24 h. The peptidyl-resin was then treated with anhydrous HF in the presence of dimethyl sulphide (5%) and *p*-cresol (10%) for 1 h at 0 °C. The resin was washed with diethyl ether and extracted with aqueous acetic acid (5%). After careful evaporation of the liquid hydrogen fluoride the crude peptide was purified by gel filtration on trisacryl GF 0.5 followed by preparative reversed chromatography (μ Bondapack C18) using 0.1% aqueous TFA–MeCN (gradient from 95:5 to 40:60). The purity was checked by t.l.c. (eluting with butanol–pyridine–acetic acid–water (75:15:3:12), analytical h.p.l.c. [μ Bondapack C18, 215 nm (PO_4^{3-}, water, MeCN; 3—50%)], amino-acid analysis of acidic hydrolysate (6M-HCl; 24 h) and FAB mass spectrometry.

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