Toward the Synthesis of a Heme Octapeptide Occurring in Cytochrome c[1,2]

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The protected octapeptides carbobenzoxy-S-benzyl-L-cysteinyl-L-alanyl-L-glutaminyl-S-benzyl-L-cysteinyl-*im*-benzyl-L-histidyl-L-threonyl-L-valyl-L-glutamic dibenzyl ester and its corresponding N-tosyl derivative have been synthesized. Two synthetic approaches have been tried in order to avoid possible racemization, the DCC method for the N-terminal "stepwise" elongation of the peptide chain and the azide one for "fragment condensation." Concerning the synthesis of related porphyrin peptides, modified procedures for the formation of the porphyrin thioether bond have been investigated. 2,4-a,a'-Bis(cysteine methyl ester)mesoporphyrin di-*p*-nitrobenzyl ester and porphyrin c have been synthesized.

The amino acid sequence around the heme-containing prosthetic group of cytochrome c has been known for some time. After the tryptic digest of cytochrome c Tuppy and Bodo[3] isolated a heme octapeptide with the structure CySH-Ala-Glu(NH_2)-CySH-His-Thr-Val-Glu. The two cysteine residues occurring in the chain are linked to the side chains 2 and 4 of the ferriporphyrin prosthetic group *via* two thio ether bridges. The isolated heme octapeptide constitutes part of the active center of the cytochrome c enzyme, and consequently interest has been focused on the synthesis of such related peptides in order to correlate structure and activity.

Porphyrin c and nonbridging simple porphyrin dipeptides have been prepared from porphyrin dibromide and cysteine or cysteine dipeptides under conditions involving fusion of the reactants[4] or heating to 120° over a prolonged period of time.[5] Nevertheless, these conditions are too drastic to be employed in joining the porphyrin ring to the above octapeptide. Therefore, the purpose of this paper is to report the synthesis of the octapeptide fragment and to describe some modified procedures, which would most likely permit the total synthesis of the heme octapeptide.

For the synthesis of the octapeptide fragment, we have selected synthetic steps which would avoid possible racemization on one hand, and on the other hand block groups removable from the final octapeptide derivative in one operation.

Esterification of L-valyl-L-glutamic acid[6] with benzyl alcohol in the presence of *p*-toluenesulfonic acid afforded the corresponding ester, L-valyl-L-glutamic dibenzyl ester *p*-toluenesulfonate (I) in 71.9% yield. Coupling of I with carbobenzoxy-L-threonine[7] by the DCC method,[8] produced carbobenzoxy-L-threonyl-L-valyl-L-glutamic dibenzyl ester (II) in high yield. Removal of protecting groups of II by catalytic hydrogenation,[9] gave L-threonyl-L-valyl-L-glutamic acid (III), isolated as the semihydrate. The latter, on esterification as above, afforded L-threonyl-L-valyl-L-glutamic dibenzyl ester *p*-toluenesulfonate (IV) in 60% yield. Its optical homogeneity was tested according to Crofts, *et al.*:[10] thus a sample of IV was completely hydrolyzed by acid and the rotation of this solution was found to be identical with that of an equimolar solution of the respective amino acids.

Coupling of IV with N-trityl-*im*-benzyl-L-histidine[6] by the DCC method, produced the corresponding N-trityl tetrapeptide ester, which was detritylated by mild acid solvolysis to give *im*-benzyl-L-histidyl-L-threonyl-L-valyl-L-glutamic dibenzyl ester di-*p*-toluenesulfonate (V).

An attempt to prepare V by condensation of formyl-*im*-benzyl-L-histidine and IV by the DCC method is being studied. It should be noted that the water-soluble formyl-*im*-benzyl-L-histidine can serve for the formation of a histidyl peptide bond. This was demonstrated by the synthesis of formyl-*im*-benzyl-L-histidylglycine benzyl ester in 50% yield.

The particular property of formyl-*im*-benzyl-L-histidine in conjunction with the use of water-soluble carbodiimides[11] as the condensing agents, could be employed for the modification of protein molecules.

As the next step, formyl-L-glutamine was coupled with S-benzyl-L-cysteine benzyl ester[12] by means of Woodward's reagent[13] to give formyl-L-glutaminyl-S-benzyl-L-cysteine benzyl ester as a crystalline compound. Attempts to hydrolyze the formyl group selectively, including the use of hydrogen chloride[14] or *p*-toluenesulfonic acid in the presence of benzyl alcohol were unpromising. The dipeptide ester underwent cyclization to give the corresponding pyrolidone derivative. Also the hydrogen bromide–acetic acid[15] removal of the carbobenzoxy group of carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteine ethyl or benzyl ester, led to a complex product mixture which probably contained some of sulfoxide derivative.

An attempt was made to synthesize the amino acid sequence Ala-Glu(NH_2), which occupies positions 2 and 3 of the octapeptide fragment. Thus, carbobenzoxy-L-glutamine[16] was converted into its *p*-nitrobenzyl ester, according to our new procedure[7] in high yield.

In a similar manner the *p*-nitrobenzyl esters of carbobenzoxy-L-asparagine, formyl-L-glutamine, and formyl-

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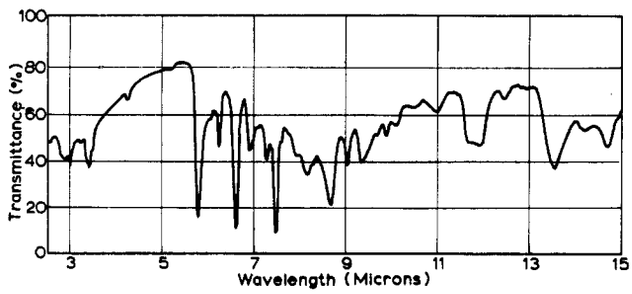

Figure 1.—Infrared spectra of haematoporpyrin di-*p*-nitrobenzyl ester.

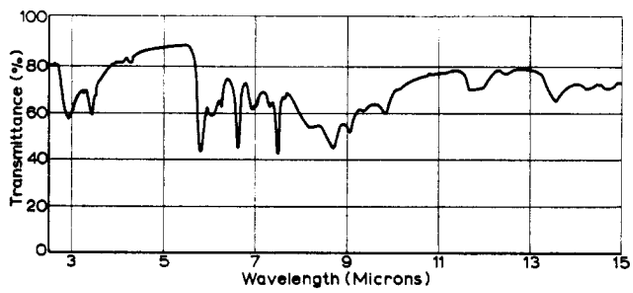

Figure 2.—Infrared spectra of 2,4,a,a′-bis(cysteine methyl ester)-mesoporphyrin di-*p*-nitrobenzyl ester.

L-asparagine were prepared. Treatment of the carbobenzoxy esters with hydrogen bromide–acetic acid afforded the desired *p*-nitrobenzyl ester hydrobromides of L-glutamine and L-asparagine. These compounds can serve as useful intermediates for the synthesis of glutamine and asparagine peptides. Using Woodward's reagent as the coupling agent, the *p*-nitrobenzyl esters of carbobenzoxyglycyl-L-glutamine, carbobenzoxy-L-alanyl-L-glutamine, carbobenzoxy-L-asparaginyl-L-glutamine, carbobenzoxy-L-asparaginyl-L-asparagine, and carbobenzoxy-L-glutaminyl-L-asparagine were prepared.

It should be noted that these *p*-nitrobenzyl esters are extremely labile even in the presence of organic bases. When carbobenzoxy-L-alanyl-L-glutamine *p*-nitrobenzyl ester was recrystallized from not previously purified dimethylformamide, the ester was converted into an oily product consisting mainly of the cyclic imide, as confirmed by paper electrophoresis of a hydrogenated sample.

The facile formation of the imide structure is probably due to the electron-withdrawing *p*-nitro group on the esterified carboxylate, which facilitates nucleophilic attack by the amide nitrogen.

Coupling of the tetrapeptide ester V with carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteine azide produced the hexapeptide, carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-*im*-benzyl-L-histidyl-L-threonyl-L-valyl-L-glutamic dibenzyl ester (VI).

In connection with the synthesis of the above-mentioned azide, it should be noted that its intermediate hydrazide was prepared from the methyl ester of carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteine under milder conditions than those reported.[17] In our experience, the extensive boiling of the ester in 1-butanol in the presence of hydrazine results in some desulfurization of the cysteine residue.

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For the final steps, the azides of carbobenzoxy-S-benzyl-L-cysteinyl-L-alanine and tosyl-S-benzyl-L-cysteinyl-L-alanine were coupled, respectively, with decarbobenzoxylated VI to produce carbobenzoxy-S-benzyl-L-cysteinyl-L-alanyl-L-glutaminyl-S-benzyl-L-cysteinyl-*im*-benzyl-L-histidyl-L-threonyl-L-valyl-L-glutamic dibenzyl ester and its corresponding N-tosyl octapeptide derivative.

Complete hydrolysis of the carbobenzoxy octapeptide followed by paper chromatography revealed the presence of alanine, glutamic acid, threonine, valine, histidine (very weak), *im*-benzylhistidine, and S-benzyl cysteine. In addition, a distinct spot in the position of cysteic acid was observed, the appearance of which was also detected on acid hydrolysis of a sample of S-benzyl-L-cysteine. This should be kept in mind, when analyzing quantitatively a complete hydrolysate of S-benzylcysteine peptides.

As part of our work toward the synthesis of the heme octapeptide, we have been investigating modified procedures for the preparation of porphyrin peptides, since the drastic conditions of methods used with simple dipeptides or cysteine would eventually damage the sensitive octapeptide fragment.

In this connection, we have decided to replace porphyrin dibromide in the coupling reaction, with a more or equally reactive porphyrin component, which in contrast to the former, is very soluble in organic solvents and thus permits the reaction to proceed at low temperature.

For the first step, haematoporphyrin was converted into its di-*p*-nitrobenzyl ester (VII), according to our new procedure.[7] Its identity was confirmed by elementary analysis, and absorption spectrum, which exhibited ϵ_{mol} 21,910 at 270 mμ in methanol, compared with ϵ_{mol} 3920 for the haematoporphyrin. The obtained ester VII was very soluble in organic solvents. Its protecting *p*-nitrobenzyl groups could be selectively removed by reduction with sodium in liquid ammonia, which process does not affect the conjugated double bonds of the porphyrin nucleus. Indeed, haematoporphyrin was isolated, after the reductive cleavage of VII, almost in quantitative yield. (See Figure 1.)

For the conversion of the ester VII into its dichloride, 2,4-a,a′-dichloromesoporphyrin di-*p*-nitrobenzyl ester (VIII), we have found the thionyl chloride method to be most effective. It is presumed, according to the literature,[18] that the dichloride VIII has the opposite configuration to that of the alcohol, but bimolecular displacements (SN2) on the dichloride VIII might be expected to produce derivatives retaining the configuration of the original alcohol. (See Figure 2.)

Reaction of dichloride VIII with the sulfhydryl group of cysteine methyl ester proceeded smoothly in chloroform solution at 0° in the presence of an equivalent amount of triethylamine which was used to bind the liberated hydrogen chloride.

The crude 2,4-a,a′-bis(cysteine methyl ester)mesoporphyrin di-*p*-nitrobenzyl ester obtained in this manner was fractionated on a chromatographic column of a weak cation-exchange resin Amberlite IRC-50 (H). Its analytical data and absorption and infrared spectra confirmed its identity.

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In conclusion another experiment, which involved the reaction of dichloride VIII with cystine previously reduced with the equivalent amount of sodium in liquid ammonia, should be mentioned. The product 2,4-a,a′-bis(cysteine)mesoporphyrin di-*p*-nitrobenzyl ester (IX), was not isolated but was immediately reduced with sodium in liquid ammonia to afford porphyrin c in 18% yield. Even though the yield of the desired porphyrin c was rather low, this process might have a wider application in the synthesis of porphyrin peptides and is now under study.

Experimental Section

Melting points were taken in capillary tubes and are uncorrected. All peptides tested were hydrolyzed with 6 *N* hydrochloric acid at 105° for 24 hr followed by paper chromatography. The solvent system used was 1-butanol–acetic acid–pyridine–water (15:3:10:12). Chromatograms were run at 20°. Whatman No. 1 paper was used.

L-Valyl-L-glutamic Dibenzyl Ester *p*-Toluenesulfonate (I).—A mixture of 2.46 g (0.01 mole) of L-valyl-L-glutamic acid and 2.1 g (10% excess) of *p*-toluenesulfonic acid monohydrate was dissolved in 12 ml of benzyl alcohol by heating under reflux in a steam bath. Benzene (60 ml) was added and the flask was fitted with a Dean–Stark tube to remove entrained water. Benzene and benzyl alcohol were removed under high vacuum. The residue, upon addition of 20 ml of ether and 100 ml of petroleum ether (bp 30–60°) and cooling overnight, solidified: yield 5.8 g (96.9%), mp 80–90°. The solid was recrystallized from equal volumes of ethyl acetate–petroleum ether (20:20): yield 4.3 g (71.9%), mp 111–114°, R_f 0.97 (single spot), $[\alpha]^{23}D$ +6.0° (*c* 1.9, $CHCl_3$).

Anal. Calcd for $C_{31}H_{38}N_2O_8S$: C, 62.18; H, 6.39; N, 4.67. Found: C, 62.34; H, 6.58; N, 4.70.

Carbobenzoxy-L-threonyl-L-valyl-L-glutamic Dibenzyl Ester (II).—Into 50 ml of methylene chloride were added consecutively L-valyl-L-glutamic dibenzyl ester *p*-toluenesulfonate (5.98 g 0.01 mole), dry triethylamine (1.01 g), carbobenzoxy-L-threonine (2.5 g 0.01 mole), and DCC (2 g). The mixture remained at room temperature overnight and then dicyclohexylurea was removed by filtration. The filtrate was diluted with 100 ml of methylene chloride and this solution was washed successively with 5% bicarbonate solution and water and dried (Na_2SO_4). The solvent was removed *in vacuo* and the remaining residue was dissolved in 10 ml of acetone. Traces of dicyclohexylurea were removed by filtration and the filtrate was evaporated to dryness. The residue upon addition of petroleum ether solidified, yield 5.6 g (86%), mp 125–128° (softens at 95°). Recrystallization from ethyl acetate–petroleum ether (30:45) gave 4.8 g (72.7%) of product: mp 132–136°, $[\alpha]^{28}D$ −26 ± 1° (*c* 2, $CHCl_3$), $[\alpha]^{28}D$ −40.1 ± 0.5° (2 *c*, MeOH).

Anal. Calcd for $C_{36}H_{43}N_3O_9$: C, 65.33; H, 6.53; N, 6.35. Found: C, 65.68; H, 6.46; N, 6.52.

L-Threonyl-L-valyl-L-glutamic Acid (III).—The above ester II (3.3 g, 0.005 mole) was dissolved in a mixture of ethyl alcohol–dimethylformamide (40:10) by gentle heating and was hydrogenated in the presence of 0.35 g of palladium black. The hydrogenation was continued for 0.5 hr after CO_2 evolution had ceased. The mixture was gently heated to dissolve the partly precipitated free peptide and the catalyst was removed by filtration. The catalyst was washed several times with hot water and the combined filtrate was evaporated to dryness under high vacuum at 40°. The peptide was collected by addition of dry acetone: yield 1.4 g (81%); mp 237–238° dec (softens at 210°); R_f 0.45 (single spot), R_{fval} 0.5, R_{fthr} 0.38, and R_{fglu} 0.24. A sample (100 mg) was dissolved in 4 ml of water, filtered, and concentrated to one-fourth of its volume *in vacuo*. Upon addition of 30 ml of acetone and cooling, the product precipitated. The recrystallized material (90 mg, 90% yield) had mp 248–250° dec, $[\alpha]^{23}D$ −33 ± 1° (*c* 1, water). Its analytical data were best in accord with a semihydrate.

Anal. Calcd for $C_{14}H_{25}N_3O_7 \cdot 0.5H_2O$: C, 47.18; H, 7.41; N, 11.79. Found: C, 47.18; H, 7.35; N, 11.73.

L-Threonyl-L-valyl-L-glutamic Dibenzyl Ester *p*-Toluenesulfonate (IV).—This compound was prepared from L-threonyl-L-valyl-L-glutamic acid semihydrate (2 g, 0.01 mole) in a similar manner to that described for compound I, yield 6.3 g (90%), mp 146–150°. The product was recrystallized by dissolving it in a 50-ml mixture of hot ethyl acetate–ethyl alcohol (4:1) and adding 60 ml of petroleum ether with cooling. The product (2.9 g 61% yield) had mp 169–172°, $[\alpha]^{20}D$ −27.2° (*c* 1.9, MeOH), R_f 0.96 (single spot). The mother liquor upon standing in the refrigerator gave an additional amount of 0.5 g of product, mp 160–170° (chromatographically pure).

Anal. Calcd for $C_{35}H_{45}N_3O_{10}S$: C, 60.58; H, 6.63; N, 6.07. Found: C, 60.80; H, 6.46; N, 6.07.

The optical purity of the peptide ester was established as follows. The ester (0.0418 g) was heated, with 6 *N* HCl (2 ml) in a sealed tube at 110° for 24 hr. After filtration through Whatman No. 1 the solution had $[\alpha]^{30}D$ +8.13 ± 0.2°; hydrolysis of the peptide was shown by paper chromatography to be complete.

A mixture equimolar to the above solution, made of L-threonine (0.0075 g), L-valine (0.0065 g), L-glutamic acid (0.0088 g), *p*-toluenesulfonic acid monohydrate (0.0114 g), benzyl alcohol (0.0064 ml), and treated in a similar manner, had $[\alpha]^{30}D$ +8.08°. The latter without being heated gave $[\alpha]^{30}D$ +8.10°.

Carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteine Ethyl Ester.—Into a suspension of 2.8 g (0.01 mole) of carbobenzoxy-L-glutamine in 25 ml of absolute acetonitrile were added 1.01 g (0.01 mole) of triethylamine and 2.5 g (0.01 mole) of Woodward's reagent with stirring. After 10 min the almost-clear solution was mixed with 2.75 g (0.01 mole) of S-benzyl-L-cysteine ethyl ester hydrochloride and 1.01 g (0.01 mole) of triethylamine in 15 ml of the above solvent. Even though the peptide derivative began to precipitate in the first 15 min, stirring was continued for about 4 hr. After that time the mixture was poured into 500 ml of water, cooled, filtered, and washed with 5% bicarbonate solution and water, yield 2.8 g (56%), mp 194° dec (with previous softening at 186°). The product was recrystallized from 50 ml of isopropyl alcohol–water (3:1), yield 2.5 g (54%), mp 195–196° dec (lit.[17] mp 196–198°), $[\alpha]^{18}D$ −40.7° (*c* 1.09, glacial acetic acid).

Carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteine Hydrazide.—Into a mixture of 1 5 ml of dimethylformamide and 5 ml of absolute ethanol were added 1.25 g (0.005 mole) of carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteine ethyl ester and 0.5 ml of hydrazine hydrate. The mixture was heated in a steam bath until all of the peptide derivative was dissolved and then was allowed to remain at room temperature for 24 hr. The product was precipitated by addition of 100 ml of water, cooled, filtered, and washed several times with water, yield 0.9 g (73%), mp 210° (softens at 204°). For purification the product was suspended into a 50-ml mixture of isopropyl alcohol–water (3:1) and heated under reflux for 0.5 hr. It was then cooled overnight, filtered, and washed with a cold isopropyl alcohol–water mixture, yield 0.8 g (68%), mp 220° dec (lit.[17] mp 233–234°).

***im*-Benzyl-L-histidyl-L-threonyl-L-valyl-L-glutamic Dibenzyl Ester Di-*p*-toluenesulfonate.**—To 25 ml of methylene chloride were added 2.8 g (0.005 mole) of trityl-*im*-benzyl-L-histidine diethylammonium salt, 3.5 g (0.005 mole) of tripeptide ester IV, and 1 g of DCC. After 24 hr dicyclohexylurea was removed by filtration and the filtrate was washed with bicarbonate solution (ten 10-ml portions) and water. It was then dried over Na_2SO_4 and the solvent was removed *in vacuo*. The residue was detritylated with 2.1 g (0.011 mole) of *p*-toluenesulfonic acid monohydrate in 5 ml of isopropyl alcohol. After remaining for 1 hr at room temperature, ether was added, the precipitating product was well triturated, and the ether layer was decanted. This was repeated three times and then it was dried *in vacuo* over P_2O_5. For analysis a sample was dissolved in isopropyl alcohol, reprecipitated with ether, and dried under high vacuum for 3 days over P_2O_5; it had R_f 0.9, $[\alpha]^{30}D$ −18.8° (*c* 2, MeOH).

Anal. Calcd for $C_{55}H_{66}N_6O_{11}S_2$: N, 7.98; S, 6.09. Found: N, 7.50; S, 5.82.

Formyl-*im*-benzyl-L-histidine.—An ice-cold solution of 2.45 g (0.01 mole) of *im*-benzyl-L-histidine in 20 ml of formic acid (98–100%) was treated with 8 ml of acetic anhydride added gradually with constant stirring over a 20-min period. Stirring was continued for 1 hr at room temperature, then portions of 10 ml of water were added at intervals and removed *in vacuo* until the residue gave no more traces of acetic or formic acid. The residue solidified upon addition of a mixture of ethyl alcohol–ether (1:2). After cooling, it was filtered and washed with ether, yield 1.85 g (68%), mp 190–192°. It was recrystallized from dimethylformamide–ether (10:50 ml), yield 1.65 g (60%), $[\alpha]^{30}D$ +47.9 ± 1° (*c* 2.4, H_2O).

TABLE I

p-NITROBENZYL ESTERS OF N-CARBOBENZOXY PEPTIDES

Substituent	Yield, %	Mp, °C	Formula	Carbon, % Calcd	Carbon, % Found	Hydrogen, % Calcd	Hydrogen, % Found	Nitrogen, % Calcd	Nitrogen, % Found
L-Glutaminyl-L-asparagine[a,b,c]	65	188–191	$C_{24}H_{27}N_5O_9$	54.43	54.47	5.13	5.27	13.22	13.37
L-Asparaginyl-L-glutamine[a,d]	53	228–230	$C_{24}H_{27}N_5O_9$	54.43	54.17	5.13	5.06	13.22	13.16
L-Asparaginyl-L-asparagine[a,e]	53	188–190	$C_{23}H_{25}N_5O_9$	56.05	56.42	5.11	5.33	14.21	13.91
L-Glutaminylglycine[c,f,g]	68	195–196	$C_{22}H_{24}N_4O_8$	55.91	55.96	5.01	5.40	11.86	11.75

[a] For purification it was triturated with chloroform. [b] $[\alpha]^{28}D$ −23.1° (*c* 1, DMF). [c] For the free peptides, see K. H. Miller and H. Waelsch, *Arch. Biochem. Biophys.*, **35**, 176 (1952); J. Rudinger, *Collection Czech. Chem. Commun.*, **19**, 375 (1954); M. J. Swan and V. du Vigneaud, *J. Am. Chem. Soc.*, **76**, 3110 (1954); J. Rudinger, J. Honzl, and M. Zaoral, *Collection Czech. Chem. Commun.*, **21**, 202 (1956). [d] $[\alpha]^{28}D$ −10.8° (*c* 1, DMF). [e] $[\alpha]^{28}D$ −27.5° (*c* 1, DMF). [f] It was recrystallized from 50% methanol. [g] $[\alpha]^{30}D$ −18.9° (*c* 1, DMF).

The product was found to be soluble in water and insoluble in absolute ethanol or ether. Mixture melting point with *im*-benzyl-L-histidine (mp 250°) showed a softening at 184° with complete melting at 205°.

Anal. Calcd for $C_{14}H_5N_3O_3$: C, 61.52; H, 5.53; N, 15.38. Found: C, 61.62; H, 5.45; N, 15.33.

Formyl-*im*-benzyl-L-histidylglycine Benzyl Ester.—Formyl-*im*-benzyl-L-histidine (1.36 g, 0.005 mole) was dissolved in 15 ml of dimethylformamide containing 0.5 g (0.005 mole) of triethylamine. To this solution were added 1.74 g (0.005 mole) of glycine benzyl ester *p*-toluenesulfonate and 1 g of DCC, and the mixture was allowed to remain at room temperature overnight. Dicyclohexylurea was removed by filtration and the desired product was precipitated by addition of water. The solid was washed with 5% bicarbonate solution and water, yield 1.4 g (66%), mp 169–173° (softens at 163°). It was recrystallized from 50 ml of dimethylformamide–ether (1:4) and had mp 176–180°, yield 1.05 g (50%).

Anal. Calcd for $C_{23}H_{24}N_4O_4$: C, 65.79; H, 5.75; N, 13.32. Found: C, 65.98; H, 5.94; N, 13.53.

Formyl-L-glutaminyl-S-benzyl-L-cysteine Benzyl Ester.—Formyl-L-glutamine (1.7 g 0.01 mole) and S-benzyl-L-cysteine benzyl ester *p*-toluenesulfonate (4.7 g, 0.01 mole) were coupled by means of Woodward's reagent in acetonitrile in the usual manner, yield 2.95 g (65%), mp 117–122°. Recrystallization from slightly warm dimethylformamide–ether yielded 2.2 g (50%), mp 185–187°.

Anal. Calcd for $C_{23}H_{28}N_3O_5S$: C, 60.24; H, 6.15; N, 9.16. Found: C, 60.05; H, 5.99; N, 9.13.

Carbobenzoxy-L-alanyl-L-glutamine *p*-Nitrobenzyl Ester.—A portion of 1.1 g (0.005 mole) of carbobenzoxy-L-alanine was activated with the equivalent amount of Woodward's reagent in the usual manner. This solution was mixed with an ice-cold mixture of 1.89 g (5% excess) of L-glutamine *p*-nitrobenzyl ester hydrobromide, neutralized by dropwise addition of 0.5 g (0.005 mole) of triethylamine. After 10 min the product began to precipitate while stirring was continued for 4 hr at room temperature. Water was added and the product was collected by filtration, washed with cold 5% solution of bicarbonate and water, and dried. Trituration with 50% methanol gave 1.6 g (66%), mp 192–194°.

Anal. Calcd for $C_{23}H_{26}N_4O_8$: C, 56.7; H, 5.3; N, 11.5. Found: C, 56.5; H, 5.6; N, 11.7.

In a similar manner the compounds listed on Table I were prepared.

Carbobenzoxy-L-glutamine *p*-Nitrobenzyl Ester.—Into 25 ml of dry acetone was dissolved 2.8 g (0.01 mole) of carbobenzoxy-L-glutamine by addition of 1.01 g (0.01 mole) of triethylamine. To this solution was added 3.1 g (0.01 mole) of *p*-nitrobenzyl tosylate and the mixture was heated under reflux. After 10 min triethylammonium *p*-toluenesulfonate began to precipitate. At the end of 45 min the product was precipitated by addition of 500 ml of water, cooled, filtered, washed with 5% bicarbonate solution, and water. The crude ester (4.0 g, 96.6%) had mp 125–127°; upon recrystallization from 80 ml of ethyl alcohol–ethyl ether (1:3) the product had mp 138–142°, yield 2.9 g (70%), $[\alpha]^{22}D$ −7.3 ± 0.5° (*c* 2, DMF).

Anal. Calcd for $C_{20}H_{21}N_3O_7$: C, 57.82; H, 5.09; N, 10.11. Found: C, 58.12; H, 5.24; N, 10.22.

Carbobenzoxy-L-asparagine *p*-Nitrobenzyl Ester.—This compound was prepared from carbobenzoxy-L-asparagine (2.7 g, 0.01 mole) as described previously. The crude product (3.7 g, 92%, mp 159°) was recrystallized from 40 ml of ethyl alcohol–ether (1:3) yielding 2.3 g (80%), mp 167–169, $[\alpha]^{20}D$ −12.5 ± 1° (*c* 2, DMF).

Anal. Calcd for $C_{19}H_{19}N_3O_7$: C, 56.86; H, 4.73; N, 10.47. Found: C, 56.48; H, 4.75; N, 10.60.

Formyl-L-glutamine *p*-Nitrobenzyl Ester.—This was prepared as described previously, using dimethylformamide–acetone (1:6) as the solvent, yield 1.7 g (55%), mp 130–132°. Recrystallization from 120 ml of ethyl alcohol–ether (1:3) yielded 1.1 g (36%), mp 152–153°, $[\alpha]^{20}D$ −19.4 ± 0.5° (*c* 2, DMF).

Anal. Calcd for $C_{13}H_{15}N_3O_6$: C, 50.64; H, 4.54; N, 13.31. Found: C, 50.84; H, 4.71; N, 13.27.

Formyl-L-asparagine *p*-Nitrobenzyl Ester.—Similarly prepared as above this compound had mp 160–162°, $[\alpha]^{20}D$ −20.5 ± 1° (*c* 2, DMF), yield 0.6 g (40%).

Anal. Calcd for $C_{12}H_{13}N_3O_6$: C, 48.97; H, 4.08; N, 14.28. Found: C, 49.22; H, 4.16; N, 14.19.

L-Glutamine *p*-Nitrobenzyl Ester Hydrobromide.—Carbobenzoxy-L-glutamine *p*-nitrobenzyl ester (4.15 g, 0.01 mole) was added to a freshly prepared solution of 2.5 *N* hydrogen bromide in glacial acetic acid (10 ml). The mixture remained at room temperature for 30 min during which time decarbobenzoxylation ensued followed by evolution of CO_2, while the reaction product was partly precipitated. The mixture was poured into 150 ml of dry ether, and the precipitate was filtered and washed several times with ether, yield 2.95 g (82%), mp 160°. Recrystallization was effected by dissolving the solid in 20 ml of slightly warm isopropyl alcohol and precipitating it by addition of 120 ml of petroleum ether, yield 2.55 g (70%), mp 163–165°, $[\alpha]^{30}D$ +5.6 ± 0.5° (*c* 2, DMF), R_f 0.68.

Anal. Calcd for $C_{12}H_{16}BrN_3O_5$: C, 39.79; H, 4.45; N, 11.60. Found: C, 39.51; H, 4.41; N, 11.25.

L-Asparagine *p*-Nitrobenzyl Ester Hydrobromide.—Following the directions given above, carbobenzoxy-L-asparagine *p*-nitrobenzyl ester (4.09, 0.01 mole) was converted into its *p*-nitrobenzyl ester hydrobromide, yield 3.0 g (87%), mp 165°. After recrystallization from isopropyl alcohol–ether, the product (2.9 g, 83%) had mp 172–175°, $[\alpha]^{22}D$ +7.7 ± 0.5° (*c* 2, DMF), R_f 0.66.

Anal. Calcd for $C_{11}H_{14}BrN_3O_5$: C, 38.10; H, 4.97; N, 12.03. Found: C, 38.32; H, 4.48; N, 11.90.

Carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-*im*-benzyl-L-histidyl-L-threonyl-L-valyl-L-glutamic Dibenzyl Ester.—A solution of 5.5 g (0.005 mole) of the tetrapeptide ester di-*p*-toluenesulfonate in 20 ml of dimethylformamide was neutralized with 1.01 g (0.01 mole) of triethylamine and mixed with carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteine azide (0.35 g). The latter was previously dissolved in the same solvent. The mixture was allowed to remain at room temperature overnight, filtered to remove traces of insoluble material, and diluted with ice-cold water. The precipitated product was filtered and washed several times with water, yield 5.3 g, mp 189–190° (softens at 170°). For recrystallization it was heated under reflux in 100 ml of 95% methanol and filtered, and the filtrate was concentrated to a 20-ml volume. Addition of ether and cooling afforded 2.5 g of product, mp 195–196°, $[\alpha]^{17}D$ −9.6 ± 1° (*c* 1.9, DMF).

Anal. Calcd for $C_{64}H_{75}N_9O_{13}S$: C, 63.5; H, 6.2; N, 10.4. Found: C, 63.9; H, 6.1; N, 10.4.

A sample was hydrolyzed with 6 *N* HCl for 24 hr at 110°. Paper chromatography revealed the presence of glutamic acid, threonine, valine, *im*-benzylhistidine, histidine, S-benzylcysteine, and cysteic acid (intense spot).

Carbobenzoxy-S-benzyl-L-cysteinyl-L-glutaminyl-S-benzyl-L-cysteinyl-*im*-benzyl-L-histidyl-L-threonyl-L-valyl-L-glutamic Di-

benzyl Ester.—The carbobenzoxy hexapeptide ester (6 g) was decarbobenzoxylated with 1.5 *N* HBr in 20 ml of acetic acid for 15 min at room temperature. It was then triturated with ether and immediately dried under high vacuum over P_2O_5, R_f 0.92.

The above ester dihydrobromide (3.1 g) was treated with 0.5 g (0.005 mole) of triethylamine in 20 ml of dimethylformamide and was mixed with 1.1 g of carbobenzoxy-S-benzyl-L-cysteinyl-L-alanine azide.[19] The next day the mixture was diluted with water and the precipitated product was washed with 1 *N* HCl (two 10-ml portions), bicarbonate solution (twice), and water, yield 1.8 g. The product was recrystallized from dimethylformamide–ether (20:100), yield 1.5 g (40%), mp 180° (softens at 165°), $[\alpha]^{28}D$ −28.8° (*c* 2, acetic acid). Complete hydrolysis with 6 *N* HCl, followed by paper chromatography, revealed the expected amino acids, plus a distinct spot at the position of cysteic acid.

Anal. Calcd for $C_{77}H_{91}N_{11}O_{15}S_2$: C, 62.71; H, 6.22; N, 10.44. Found: C, 63.10; H, 6.22; N, 10.21.

Tosyl-S-benzyl-L-cysteinyl-L-alanyl-L-glutaminyl-S-benzyl-L-cysteinyl-*im*-benzyl-L-histidyl-L-threonyl-L-valyl-L-glutamic Di-benzyl Ester.—This was prepared from tosyl-S-benzyl-L-cysteinyl-L-alanine azide, prepared from the corresponding hydrazide[2] in the usual manner, and the hexapeptide ester, as described above, yield 40%, mp 180° (lit.[2] mp 180°), $[\alpha]^{30}D$ −35.7° (*c* 2, acetic acid).

Haematoporphyrin Di-*p*-nitrobenzyl Ester. A.—A portion of 3 g (0.005 mole) of haematoporphyrin was dissolved into a mixture of 40 ml of acetone–dimethylformamide (2:4) and to this solution was added 0.4 g of sodium hydroxide, dissolved in 5 ml of water. The mixture was heated to reflux and into the clear solution was added 3.01 g (0.01 mole) of *p*-nitrobenzyl tosylate. Heating was continued for 2 hr and the reaction mixture was poured into 1 l. of water. The precipitated product, after having being cooled, was filtered and washed with 5% bicarbonate solution and water, yield 3.85 g (88%), mp 133–140° dec. The product was recrystallized from chloroform–ether (2:5) and gave 2.1 g (50%), mp 144–148° dec.

B.—This ester was also prepared from haematoporphyrin (3 g), triethylamine (1.4 ml), and di-*p*-nitrobenzyl tosylate (3.01 g) from a mixture of acetone–dimethylformamide (3:2) as above, yield 41%.

Anal. Calcd for $C_{48}H_{48}N_6O_{10}$; C, 66.3; H, 5.5; N, 9.7. Found: C, 66.7; H, 5.7; N, 9.9.

2,4-a,a′-Dichloromesoporphyrin Di-*p*-nitrobenzyl Ester.—Into an ice-cold solution of 0.87 g (1 mmole) of haematoporphyrin di-*p*-nitrobenzyl ester in 10 ml of dry chloroform were added consecutively 0.28 ml of triethylamine and 0.14 ml of thionyl chloride. The mixture after having being cooled for 10 min at 0°, was kept for 0.5 hr at room temperature. The solution was then washed with cold water quickly and the chloroform layer was dried over Na_2SO_4. Evaporation of the solvent *in vacuo* and addition of petroleum ether afforded 0.85 g (94%) of product which gave analytical data close to those expected and was used further without purification.

Anal. Calcd for $C_{48}H_{46}Cl_2N_6O_8$: N, 9.27; Cl, 7.82. Found: N, 9.6; Cl, 7.1.

2,4-a,a′-Bis[cysteine methyl ester]mesoporphyrin Di-*p*-nitrobenzyl Ester.—Into 20 ml of dry chloroform, cooled to 0°, were added 0.68 g (100% excess) of cysteine methyl ester hydrochloride, 0.40 g of triethylamine, and 0.90 g of dichloride VIII. The mixture was allowed to remain in an ice bath for 1 hr and then overnight at room temperature. The following day the chloroform layer was washed with water and dried (Na_2SO_4). The solvent was evaporated *in vacuo* at 35° and the remaining residue (0.95 g) was fractionated on a chromatographic column of a weak cation-excange resin Amberlite IRC-50 (H). A 200-mg sample was dissolved in 70 ml of DMF–water (2:5) and applied to the column (2.5 × 40 cm), which was washed with a mixture of 140 ml of 2 volumes of DMF and 5 volumes of water (eluate 1), 300 ml of 2 volumes DMF and 5 volumes of 0.5 *N* acetic acid (eluate 2), and 300 ml of equal volumes of DMF and 10 *N* acetic acid (eluate 3); the eluates were collected separately. Eluate 3, on removal of the solvent under high vacuum and at 30–35°, gave 100 mg of the desired product, which was further diluted in acetic acid, centrifuged, and lyophilized. Its absorption spectra was in good agreement with that reported for the porphyrin c tetramethyl ester. The Soret maximum was at 405 mμ and gave an extinction coefficient of 2.82×10^5.

Anal. Calcd for $C_{56}H_{62}N_8O_{12}S_2$: N, 10.15; S, 5.81. Found: N, 10.23; S, 6.05.

Reduction of Haematoporphyrin Di-*p*-nitrobenzyl Ester with Sodium in Liquid Ammonia.—A suspension of 0.86 g (1 mmole) of haematoporphyrin di-*p*-nitrobenzyl ester in 100 ml of liquid ammonia was treated with 0.1 g (about 100% excess) of sodium, added in small pieces. This was followed by the appearance of several changing colors, varying between green and blue. Finally the reaction mixture was set aside and the ammonia was allowed to evaporate slowly at room temperature. The remaining residue was dissolved in water and by adjusting the pH to 3.7 with acetic acid the desired haematoporphyrin was precipitated. The product was filtered, washed with water, dried, and triturated with methanol–ether (1:2), yield 0.53 g (86%), mp 267–275° dec. Its visible and ultraviolet absorption spectra were identical with those of a sample of haematoporphyrin.

Porphyrin c.—A suspension of 0.96 g (4 mmoles) of cystine in 100 ml of liquid ammonia was treated with 0.18 g (16 g-atoms) of sodium, added in pieces. When the blue color had disappeared 0.90 g (1 mmole) of dichloride VIII, diluted in 10 ml of dry chloroform, was added dropwise with stirring. The reaction mixture was set aside and the ammonia was allowed to evaporate at room temperature. The remaining residue was washed with water to remove the unreacting sodium salt of cysteine and then was acidified with dilute acetic acid. Sulfur and nitrogen analysis indicated it to be a mixture consisting mainly of 2,4-a,a′-bis-(cysteine)mesoporphyrin di-*p*-nitrobenzyl ester (IX).

Anal. Calcd for $C_{54}H_{58}N_8O_{12}S_2$: N, 10.29; S, 5.87. Found: N, 10.12; S, 4.21.

The above mixture (0.92 g) was reduced with 0.17 g (about 100% excess) of sodium in liquid ammonia. The reduced product, after the ammonia had been evaporated, was diluted in water and acidified to pH 4 with dilute acetic acid. The precipitated product was filtered, washed with water, and fractionated on a column of purified Celite according to Neilands and Tuppy;[4] over-all yield based on the dichloride VIII used was 0.14 g (18%). It melted at 300–310° in accord with the literature.[4] The Soret maximum of the product in 1 *N* HCl was at 406 mμ and gave an extinction coefficient of 3.05×10^5, which is in good agreement with that reported.[4]

Anal. Calcd for $C_{40}H_{48}N_6O_8S_2$: N, 10.44; S, 7.95. Found: N, 10.75; S, 7.81.

(19) The hydrazide was converted into the azide in the usual manner, and was precipitated with ice-cold water. It was filtered and dried under high vacuum over P_2O_5 for 5 hr before it was used.