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Amino Acids and Peptides. III. Synthesis of Model Peptides Related to Cytochrome P-450[1)]

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Various tetra- and pentapeptides with Cys and Ser (Thr, Tyr, His) at the N- and C-terminals, respectively, were synthesized as model peptides of the apoprotein of cytochrome P-450. The optical spectra of the synthetic peptide–hemin complexes were measured and the results are discussed.

Keywords——cytochrome P-450; synthetic Cys-containing peptide; peptide-hemin complex; optical spectrum of peptide–hemin complex

Among various cytochromes, P-450 has characteristic optical and electron spin resonance (ESR) spectra ascribable to its heme complex. Unlike other cytochromes, P-450 has a thiolate as the 5th ligand of heme iron.[2)] The 6th ligand has not been identified, but it has been assumed to a hydroxy group or an imidazole of the apoprotein.[3)] To study the iron-apoprotein complex of cytochrome P-450, we synthesized several tetra- and pentapeptides with Cys and His, Cys and Ser, Cys and Thr, and Cys and Tyr at N- and C-terminals, respectively, as model peptides of apoP-450. We expected that the N-terminal Cys and C-terminal amino acid would form an intramolecular coordination structure with hemin.

The peptides were synthesized as shown in Figs. 1—3. The key intermediate, Boc–Cys(MBzl)–Ala–Gly–$NHNH_2$, was synthesized by stepwise elongation from the carboxyl terminal employing the mixed anhydride method,[4)] as shown in Fig. 1. The tripeptide hydrazide was coupled with an amino acid (Ser, Thr, His, Tyr) to form a tetrapeptide as shown in Fig. 2. The tripeptide hydrazide was also coupled with a dipeptide (Ala–Ser, Ala–

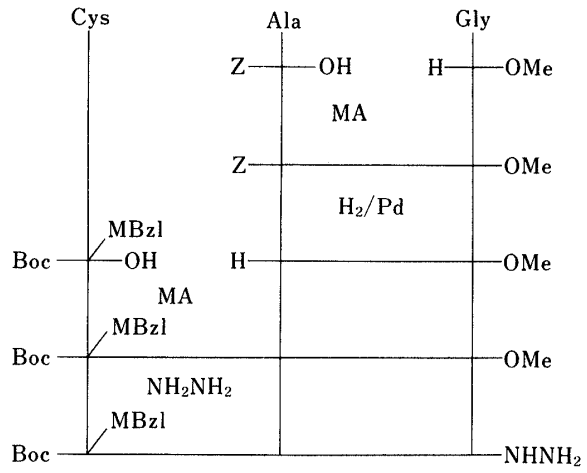

Fig. 1. Synthetic Scheme for the Tripeptide Hydrazide

MA: the mixed anhydride method.

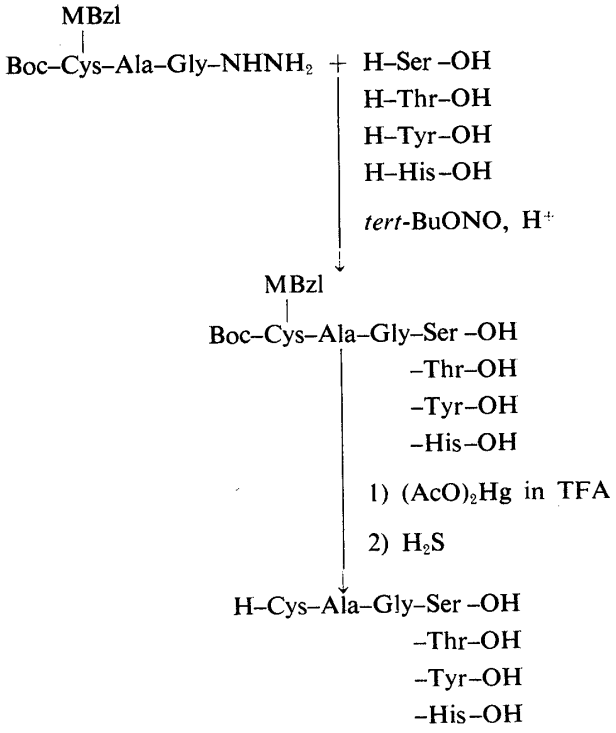

Fig. 2. Synthetic Scheme for the Tetrapeptides

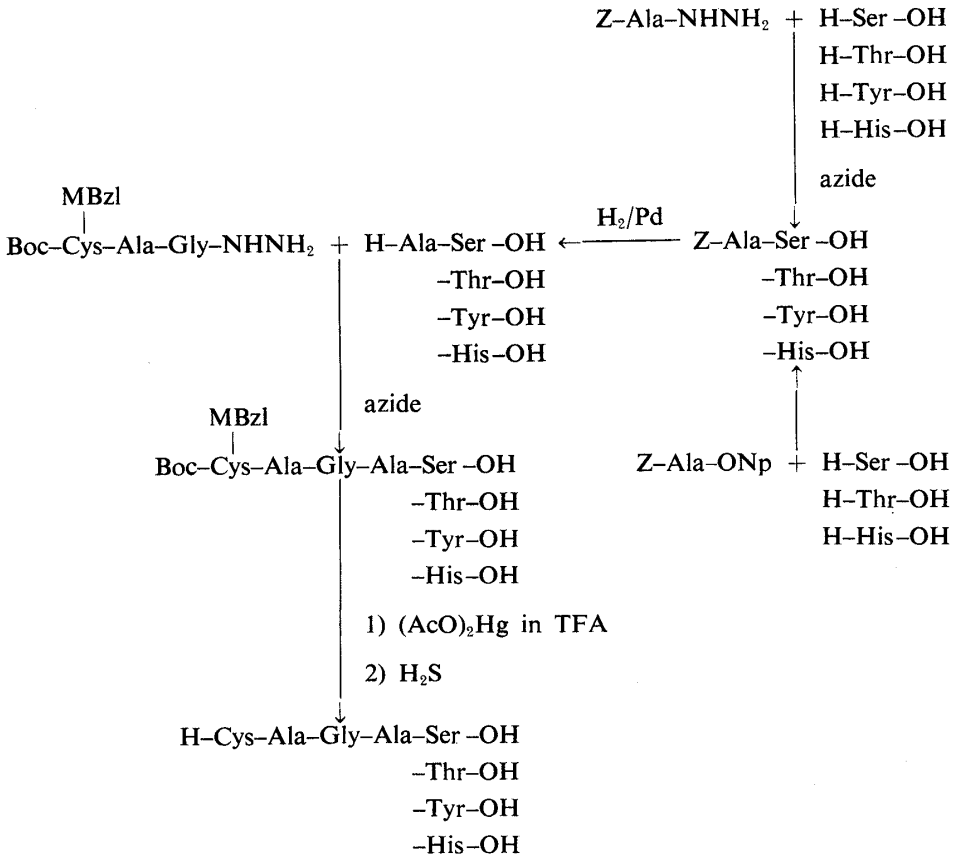

Fig. 3. Synthetic Scheme for the Pentapeptides

Thr, Ala–His, or Ala–Tyr) in the same manner to form a pentapeptide as shown in Fig. 3. The dipeptides were formed by the *p*-nitrophenyl ester method[5] and the azide method.[6] Better coupling yields were obtained by the latter method. Z–Ala–Tyr–OH was prepared only by the azide method in order to avoid O-acylation in the *p*-nitrophenyl ester method.[7] The protected tetra- and penta peptides (except His-containing peptides) were easily purified by extraction with AcOEt. The protected His-containing tetra- and pentapeptides were not extractable with AcOEt, but were purified by partition chromatography between AcOH-containing *n*-BuOH and H_2O on a Sephadex G-25 column. The protecting groups on synthetic tetra- and pentapeptides were removed by treatment with mercuric acetate in TFA[8] followed by H_2S treatment.

The complexes of the deblocked peptides with hemin in the oxidized form [Fe(III)] were prepared as reported elsewhere[9] and the optical spectra of these peptide–hemin complexes were measured. The results are summarized in Table I and an optical spectrum of peptide–hemin complex is shown in Fig. 4. As judged from the optical spectra in aqueous solutions at pH 7.5 and 9.6, ferric heme complexes in high- and low-spin states were formed. The absorption bands of low-spin species were similar to those of cytochrome P-450 enzymes. The appearance of a Soret band of a complex depended on the type of peptide, the Soret bands of the complexes containing Cys and Tyr being very similar to those of cytochrome P-450 enzymes. The spectra of the hemin complexes containing Cys–Ala–Gly–Ser, Cys–Ala–Gly–Thr, Cys–Ala–Gly–Ala–Ser, Cys–Ala–Gly–Ala–Thr, and Cys–Ala–Gly–Ala–Tyr at pH 9.6 showed an additional absorption band near 442 nm, suggesting the formation of a small amount of dithiolate-hemin complex consisting of 2 mol of peptide and hemin.[10]

ESR spectra of hemin complexes were also measured. The results suggested that some complexes consisted of an intramolecular peptide–hemin 1 : 1 complex including a thiolate and a imidazole nitrogen coordination bond; intramolecular coordination of the peptides to

TABLE I. Spectral Properties of Peptide–Hemin Complexes at Room Temperature

Peptide	pH	λ_{max} (nm) Peptide + hemin							
Cys–Ala–Gly–Ser	7.5	364	412			531	566 s	637 s	
	9.6	365	414	441 s		543	570 s	629 s	
–Thr	7.5	366	410		510			639 s	
	9.6	365	410 s	442 s		537	571	636 s	
–His	7.5	365	414			534	566 s	636 s	
	9.6	364	414			538	569 s	633 s	
–Tyr	7.5	365	419			538	568 s	639 s	
	9.6	364	419			540	568 s	642 s	
–Ala–Ser	7.5	363	412			531	566 s	639 s	
	9.6	364	413	442 s		539	569 s	636 s	
–Ala–Thr	7.5	362	412			531	566 s	636 s	
	9.6	363	411	444 s		540	568 s	639 s	
–Ala–His	7.5	361	414			534	566 s	635 s	
	9.6	362	413			539	566 s	633 s	
–Ala–Tyr	7.5	367	417			534		647 s	
	9.6	367	417	443 s		543		640 s	
P-450 (Camphor)[18]	7.4	391			520	540		645	(High-spin)
	7.4		417			535	571		(Low-spin)
P-450 (Liver microsomes)[18]	7.4	394			517	540 s			(High-spin)
	7.4		417			534	568		(Low-spin)

s: shoulder.

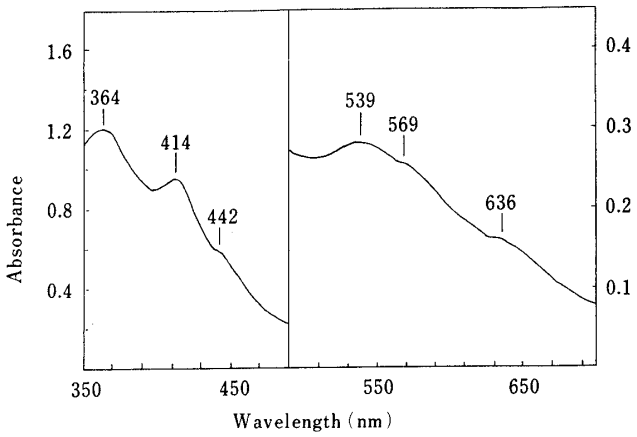

Fig. 4. Optical Spectrum of (Cys–Ala–Gly–Ala–Ser)–Hemin Complex at pH 9.6
Peptide 7.36 mM + hemin 25 μM.

hemin through the thiolate and an oxygen of Ser, Thr, or Tyr seems unlikely to occur under the conditions investigated. The details of these studies will be published elsewhere.[11]

Experimental

Melting points are uncorrected. Solvent systems for ascending thin-layer chromatography on Silica gel G (type 60, E. Merck) are indicated as follows: Rf^1 = *n*-BuOH–AcOH–H_2O (4 : 1 : 5, upper phase), Rf^2 = *n*-BuOH–pyridine–AcOH–H_2O (4 : 1 : 1 : 2), Rf^3 = $CHCl_3$–MeOH–H_2O (8 : 3 : 1, lower phase), Rf^4 = AcOEt–benzene (1 : 1). Acid hydrolyses were performed in constant-boiling HCl at 110 °C for 24 h in evacuated tubes. The amino acid compositions of acid hydrolysates were determined with a JEOL JLC-6AH amino acid analyzer. Optical rotations were measured with an automatic polarimeter, model DIP-180 (Japan Spectroscopic Co., Ltd.). Optical spectra were measured with a Union SM-302 spectrometer at room temperature.

Z–Ala–Gly–OMe——Prepared by the mixed anhydride method[4] in the usual manner. The product was precipitated from AcOEt/petro. ether; yield 71%, mp 96—98 °C, $[\alpha]_D^{27}$ −23.2° (c = 1.0, MeOH), Rf^3 = 0.92, Rf^4 = 0.56. *Anal.* Calcd for $C_{14}H_{18}N_2O_5$: C, 57.1; H, 6.2; N, 9.5. Found: C, 56.9; H, 6.3; N, 9.3. [lit.[12] Prepared by the DCC method.[13] mp 98—99 °C, $[\alpha]_D^{15}$ −25.1° (c = 5.0, MeOH)].

Boc–Cys(MBzl)–Ala–Gly–OMe——Z–Ala–Gly–OMe (1.18 g) was hydrogenated over Pd in a mixture of 1 N HCl (4 ml) and MeOH (15 ml) in the usual manner. The hydrogenated material was lyophilized and dissolved in a mixture of DMF (8 ml) and Et_3N (0.55 ml). Next, Et_3N (0.55 ml) and iso-BuOCOCl (0.53 ml) were added to a solution of Boc–Cys(MBzl)–OH[14] (1.37 g) in THF (14 ml) at −10 °C and the mixture was stirred for 10 min. This mixture was combined with the solution of the hydrogenated dipeptide ester described above and the whole was stirred overnight in a cold room. The solvent was evaporated off and the residue was extracted with AcOEt. The AcOEt layer was washed successively with 10% citric acid, H_2O, 10% Na_2CO_3 and H_2O, then dried over Na_2SO_4, and evaporated down. The residue was precipitated from AcOEt/petro. ether; yield 1.18 g (61%), mp 109—113 °C, $[\alpha]_D^{22}$ −22.8° (c = 1.0, MeOH), Rf^1 = 0.78, Rf^3 = 0.73. *Anal.* Calcd for $C_{22}H_{33}N_3O_7S$: C, 54.6; H, 6.9; N, 8.7. Found: C, 54.5; H, 6.9; N, 8.5.

Boc–Cys(MBzl)–Ala–Gly–NHNH$_2$——$NH_2NH_2 \cdot H_2O$ (0.3 ml) was added to a solution of Boc–Cys(MBzl)–Ala–Gly–OMe (1 g) in MeOH (6 ml) and the mixture was stirred overnight. The resulting precipitate was collected by filtration and recrystallized from EtOH; yield 0.77 g (77%), mp 98—99 °C, $[\alpha]_D^{22}$ −2.4° (c = 1.0, MeOH), Rf^1 = 0.62, Rf^2 = 0.72, Rf^3 = 0.63. *Anal.* Calcd for $C_{21}H_{33}N_5O_6S$: C, 52.2; H, 6.9; N, 14.5. Found: C, 52.1; H, 7.0; N, 14.5.

Z–Ala–Ser–OH——A) The Azide Procedure: 6 N HCl/dioxane (12.5 ml) and *tert*-butylnitrite (2.95 ml) were added to a solution of Z–Ala–$NHNH_2$ (5.93 g)[15] in DMF (50 ml) at −10 °C and the mixture was stirred for 10 min. The mixture was neutralized with Et_3N (10.4 ml) and combined with a solution of Ser (2.63 g) in a mixture of H_2O (15 ml) and Et_3N (3.45 ml). The whole was stirred overnight in a cold room and evaporated down. The residue was extracted with H_2O and the H_2O layer was washed with AcOEt 3 times. The H_2O layer was acidified with conc. HCl and the resulting precipitate was collected by filtration. Recrystallized from EtOH; yield 5.12 g (66%), mp 205—207 °C (lit.[16] mp 204—205 °C, prepared by the *N*-hydroxysuccimide ester method), $[\alpha]_D^{23}$ −2.0° (c = 1.0, MeOH), Rf^1 = 0.69, Rf^2 = 0.67, Rf^3 = 0.46. *Anal.* Calcd for $C_{14}H_{18}N_2O_6$: C, 54.2; H, 5.9; N, 9.0. Found: C, 54.0; H, 6.1; N, 8.8. Amino acid ratio in an acid hydrolysate: $Ala_{1.00}Ser_{0.83}$ (average recovery 78%).

B) *p*-Nitrophenyl Ester Method: Z–Ala–ONp (7.1 g)[17] dissolved in dioxane (15 ml) was added to a solution of

Ser (2.7 g) in a mixture of H_2O (12 ml) and Et_3N (3.5 ml) and the whole was stirred overnight. The solvent was evaporated off and the residue was purified by the same procedure as described in the azide method; yield 3.78 g (59%), mp 206 °C, $[\alpha]_D^{22}$ −2.0° (c=1.0, MeOH), Rf^1=0.69, Rf^2=0.67, Rf^3=0.46. Amino acid ratio in an acid hydrolysate: $Ala_{1.00}Ser_{0.80}$ (average recovery 75%). This product was identical with the sample prepared by the azide procedure (infrared (IR) spectrum and mixed melting point determination).

Z–Ala–Thr–OH——Prepared by the same procedures as described for Z–Ala–Ser–OH. The product was recrystallized from AcOEt/petro. ether; yield 68% (the azide procedure) or 54% (the *p*-nitrophenyl ester procedure), mp 130—132 °C, $[\alpha]_D^{23}$ −8.4° (c=1.1, MeOH), Rf^1=0.72, Rf^3=0.51. *Anal.* Calcd for $C_{15}H_{20}N_2O_6$: C, 55.6; H, 6.2; N, 8.6. Found: C, 55.4; H, 6.3; N, 8.7. Amino acid ratio in an acid hydrolysate: $Ala_{1.00}Thr_{1.03}$ (average recovery 89%).

Z–Ala–His–OH——A) The Azide Method: Z–Ala–$NHNH_2$ (5.93 g) and His·HCl (4.8 g) were coupled by the azide method in the usual manner. After the reaction, the solvent was evaporated off and the residue was dissolved in H_2O. The H_2O layer was washed with AcOEt and evaporated down. The residue was dissolved in 0.05 M pyridine–acetate buffer (pH 3.5) and the resulting precipitate was collected by filtration (1.58 g). The filtrate was applied to a Dowex 50 column (H^+, 5.5×25 cm) and the column was developed with pyridine–acetate buffer by the pH gradient method (0.05 M, pH 3.5→0.1 M, pH 5.8). Ninhydrin-negative and Pauly test-positive fractions were pooled, concentrated and lyophilized (3.21 g). The collected precipitate (1.58 g) and lyophilized material (3.21 g) were combined and recrystallized from H_2O; yield 4.31 g (48%), mp 123—128 °C, $[\alpha]_D^{30}$ +26.4° (c=1.1, MeOH), Rf^1=0.22, Rf^2=0.63. *Anal.* Calcd for $C_{17}H_{20}N_4O_5 \cdot 1.2H_2O$: C, 53.5; H, 5.9; N, 14.7. Found: C, 53.4; H, 6.2; N, 14.8. Amino acid ratio in an acid hydrolysate: $Ala_{1.00}His_{0.93}$ (average recovery 91%).

B) The *p*-Nitrophenyl Ester Method: Z–Ala–ONp and His·HCl were coupled in the usual manner. The product was purified in the same manner as described in A). Yield 28%, mp 122—125 °C, $[\alpha]_D^{30}$ +26.1° (c=1.0, MeOH), Rf^1=0.22, Rf^2=0.63.

Boc–Cys(MBzl)–Ala–Gly–Ser–OH——6 N HCl/dioxane (1.9 ml) and *tert*-butylnitrite (0.44 ml) were added to a solution of Boc–Cys(MBzl)–Ala–Gly–$NHNH_2$ (1.65 g) in DMF (20 ml) at −25 °C and the mixture was stirred for 10 min. The mixture was neutralized with Et_3N (1.6 ml) and combined with a solution of Ser (0.4 g) and Et_3N (0.5 ml) in H_2O (2 ml). The whole was stirred overnight in a cold room and the solvent was evaporated off. The residue was extracted with H_2O, and the H_2O layer was washed with AcOEt, acidified with citric acid, and extracted with AcOEt. The AcOEt layer was washed with H_2O, dried over Na_2SO_4 and evaporated down. The residue was triturated with AcOEt/petro. ether; yield 1.54 g (80%), mp 179—181 °C, $[\alpha]_D^{30}$ −4.1° (c=1.3, MeOH), Rf^1=0.61, Rf^2=0.71, Rf^3=0.15. *Anal.* Calcd for $C_{24}H_{36}N_4O_9S$: C, 51.8; H, 6.5; N, 10.1. Found: C, 52.0; H, 6.6; N, 9.8. Amino acid ratios in an acid hydrolysate: $Ala_{1.00}Gly_{1.03}Ser_{0.85}$ (average recovery except Cys, 81%).

Boc–Cys(MBzl)–Ala–Gly–Thr–OH——Prepared in the same manner as described above; yield 77%, mp 80 °C, $[\alpha]_D^{30}$ −6.8° (c=1.0, MeOH), Rf^1=0.83, Rf^2=0.85, Rf^3=0.47. *Anal.* Calcd for $C_{25}H_{38}N_4O_9S$: C, 52.6; H, 6.7; N, 9.8. Found: C, 52.4; H, 6.9; N, 9.6. Amino acid ratios in an acid hydrolysate: $Ala_{1.02}Gly_{1.00}Thr_{0.78}$ (average recovery except Cys, 87%).

Boc–Cys(MBzl)–Ala–Gly–Tyr–OH——Prepared by the same procedure as described above; yield 63%, mp 161—166 °C, $[\alpha]_D^{31}$ +4.5° (c=1.0, MeOH), Rf^1=0.88, Rf^2=0.83, Rf^3=0.40. *Anal.* Calcd for $C_{30}H_{40}N_4O_9S$: C, 57.0; H, 6.4; N, 8.9. Found: C, 57.0; H, 6.3; N, 8.8. Amino acid ratios in an acid hydrolysate: $Ala_{1.10}Gly_{1.00}Tyr_{0.73}$ (average recovery except Cys, 76%).

Boc–Cys(MBzl)–Ala–Gly–His–OH——Boc–Cys(MBzl)–Ala–Gly–$NHNH_2$ (3.2 g) was coupled with His·HCl (0.7 g) by the azide procedure as described above. After the reaction, the solvent was evaporated off and the residue was extracted with H_2O. The H_2O layer was washed with AcOEt, concentrated and lyophilized. The residue was dissolved in the upper phase of *n*-BuOH: AcOH : H_2O (4 : 1 : 5) and the solution was applied to a Sephadex G-25 column (3×95 cm) equilibrated with the lower phase of the above solvent. The column was developed with the above *n*-BuOH layer and the fractions containing material with Rf^1=0.34 (Pauly test) were pooled and evaporated down. The residue was lyophilized from H_2O; yield 1.73 g (68%), hygroscopic powder, $[\alpha]_D^{24}$ −2.0° (c=1.0, H_2O), Rf^1=0.34, Rf^2=0.74, Rf^3=0.17. *Anal.* Calcd for $C_{27}H_{38}N_6O_8 \cdot 4.5H_2O$: C, 51.7; H, 6.6; N, 11.2. Found: C, 51.6; H, 6.7; N, 10.9. Amino acid ratios in an acid hydrolysate: $Ala_{1.11}Gly_{1.00}His_{1.08}$ (average recovery except Cys, 82%).

Boc–Cys(MBzl)–Ala–Gly–Ala–Ser–OH——Boc–Cys(MBzl)–Ala–Gly–$NHNH_2$ (5.98 g) was converted to the azide in DMF (100 ml) in the usual manner. The azide solution was combined with a solution of Ala–Ser (2 g, prepared from Z–Ala–Ser–OH by hydrogenation) in a mixture of H_2O (50 ml) and Et_3N (1.6 ml). The whole was stirred overnight in a cold room and the solvent was evaporated off. The residue was extracted with H_2O, and the H_2O layer was washed with AcOEt followed by acidification with citric acid. The resulting precipitate was extracted with AcOEt and the AcOEt layer was washed with H_2O, dried over Na_2SO_4 and evaporated down. The residue was triturated with AcOEt/petro. ether; yield 4.05 g (57%),mp 135 °C, $[\alpha]_D^{30}$ −18.2° (c=1.1, MeOH), Rf^1=0.66, Rf^3=0.27. *Anal.* Calcd for $C_{27}H_{41}N_5O_{10}S$: C, 51.7; H, 6.6; N, 11.2. Found: C, 51.6; H, 6.7; N, 10.9. Amino acid ratios in an acid hydrolysate: $Ala_{2.02}Gly_{1.00}Ser_{0.85}$ (average recovery except Cys, 80%).

Boc–Cys(MBzl)–Ala–Gly–Ala–Thr–OH——Prepared by the same procedure as described above; yield 76%, mp 183 °C, $[\alpha]_D^{30}$ −16.8° (c=1.0, MeOH), Rf^1=0.72, Rf^3=0.71. *Anal.* Calcd for $C_{28}H_{43}N_5O_{10}S \cdot 1/2H_2O$: C, 51.7; H, 6.8; N, 10.8. Found: C, 51.7; H, 6.8; N, 10.8. Amino acid ratios in an acid hydrolysate: $Ala_{2.22}Gly_{1.00}Thr_{1.08}$ (average

TABLE II. Yields and Properties of the Deprotected Peptides

Peptide	Yield of deprotection procedure (%)	$[\alpha]_D$ in H_2O	Temp. (°C)	Rf^2	Amino acid ratios in an acid hydrolysate						
					Cys+(Cys)$_2$[a]	Ala	Gly	Ser	Thr	Tyr	His
Cys–Ala–Gly–Ser–TFA	54	−17.8 (c=1.0)	35	0.28	0.92	1.04	1.00	0.79			
Cys–Ala–Gly–Thr–TFA	68	−34.9 (c=1.0)	35	0.38	0.97	1.04	1.00		0.77		
Cys–Ala–Gly–Tyr–TFA	85	−4.1 (c=1.0)	35	0.38	0.80	1.00	1.00			0.74	
Cys–Ala–Gly–His–2TFA	88	−8.1 (c=0.9)	30	0.23	0.96	1.04	1.00				1.01
Cys–Ala–Gly–Ala–Ser–TFA	74	−40.9 (c=1.0)	30	0.26	0.79	1.92	1.00	0.87			
Cys–Ala–Gly–Ala–Thr–TFA	74	−39.6 (c=0.9)	30	0.36	0.85	1.98	1.00		0.88		
Cys–Ala–Gly–Ala–Tyr–TFA	92	−21.0 (c=1.0)	34	0.49	0.74	1.90	1.00			0.85	
Cys–Ala–Gly–Ala–His–2TFA	76	−24.9 (c=1.0)	30	0.25	0.85	1.95	1.00				0.96

a) Acid hydrolysis of cysteine converted cysteine partially into cystine. Cystine was added as 2 cysteine.

recovery except Cys, 76%).

Boc–Cys(MBzl)–Ala–Gly–Ala–Tyr–OH——Prepared by the same procedure as described above; yield 84%, mp 183—187 °C, $[\alpha]_D^{31}$ −6.0° (c=1.0, MeOH), Rf^1=0.90, Rf^3=0.17. *Anal.* Calcd for $C_{33}H_{45}N_5O_{10}S \cdot H_2O$: C, 54.9; H, 6.6; N, 9.7. Found: C, 54.7; H, 6.8; N, 10.1. Amino acid ratios in an acid hydrolysate: $Ala_{2.10}Gly_{1.00}Tyr_{0.75}$ (average recovery except Cys, 79%).

Boc–Cys(MBzl)–Ala–Gly–Ala–His–OH——Boc–Cys(MBzl)–Ala–Gly–$NHNH_2$ (4 g) and Ala–His (2.03 g, prepared from Z–Ala–His–OH by hydrogenation) were coupled by the same procedure as described above. After the reaction, the solvent was evaporated off and the residue was extracted with H_2O. The H_2O layer was washed with AcOEt and evaporated down. The residue was purified by partition chromatography as described for the purification of Boc–Cys(MBzl)–Ala–Gly–His–OH; yield 3.1 g (50%), mp 126—128 °C, $[\alpha]_D^{30}$ +9.5° (c=0.9, MeOH), Rf^1=0.35, Rf^3=0.15. *Anal.* Calcd for $C_{30}H_{43}N_7O_9S \cdot 1.5H_2O$: C, 51.1; H, 6.6; N, 14.0. Found: C, 51.1; H, 6.9; N, 13.7. Amino acid ratios in an acid hydrolysate: $Ala_{1.94}Gly_{1.00}His_{0.94}$ (average recovery except Cys, 87%).

Deprotection of the Synthetic Peptides——The protecting groups on the synthetic peptides were removed by treatment with mercuric acetate in TFA according to the procedure reported by Nishimura *et al.*[8] The Hg salt of the peptide was treated with H_2S in aqueous AcOH and the mixture was filtered. The filtrate was lyophilized repeatedly. All the deblocked peptides were hygroscopic. Yields in the deprotection procedure, $[\alpha]_D$ and Rf^2 values and amino acid ratios are shown in Table II. Preparation of synthetic peptide–hemin complexes and optical studies were carried out as reported previously.[9]

References and Notes

1) Amino acids and peptides and their derivatives mentioned in this paper are of L-configuration. Abbreviations used in this paper are: Z=benzyloxycarbonyl, Boc=*tert*-butoxycarbonyl, MBzl=*p*-methoxybenzyl, TFA=trifluoroacetic acid, ONp=*p*-nitrophenyl ester, DMF=dimethylformamide.
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