separate experiment, measuring the pH of the hemin suspension as a function of the volume of phosphoric acid added. The identical pH changes could thus be effected anaerobically within the cuvette previously described.[10d] The second-order carbon monoxide rate constants were then obtained at each pH by the usual flash photolysis technique. At the conclusion of the pH-rate profile, the pH of the solution was measured to verify that the final pH was correct. In these experiments heme concentrations were 2-7 μM and carbon monoxide concentrations were 15-30 μM. The methods of determining the kinetics of reversible reaction of dioxygen with the chelated hemes have been described in detail.[10c,d]

Titrations of Hemes with Carbon Monoxide. Carbon monoxide binding to hemes was followed by observing optical density changes at the heme Soret absorption. Titrations were performed with 10 mL of the heme solution (8 μM) in 2% $CetMe_3NBr$-0.1 M phosphate buffer, pH 7.3, in a tonometer similar to that previously described,[10d] which consisted of a cuvette attached to a bulb and a stopcock, such that the volume of the entire apparatus was 550 cm^3. A calibrated space between stopcocks allows 0.45 mL of gas at a measured pressure to be admitted. The CO could then be swept and expanded into the evacuated tonometer by a pulse of argon, thus obtaining a known pressure of CO over the solution. Each addition introduced an amount of CO which was at least five times the amount of heme present. After stirring and shaking for at least 10 min, the spectrum was recorded. This operation was repeated for successive additions of CO, thus obtaining $P_{1/2} = 0.25$ Torr for **3**. This method affords only a lower limit for K_{CO} values which are greater than 10^7 M^{-1}.

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Avidin-Biotin Affinity Columns. General Methods for Attaching Biotin to Peptides and Proteins

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Abstract: The A-B catch principle is a novel technique for the preparation of affinity columns that is based on the high affinity of the **B**-complex vitamin biotin for the egg-white protein avidin. One advantage of the technique involves the attachment of biotin to biologically active molecules in a targeted manner that does not impair biological activity. This communication relates synthetic routes by biocytinamide and to N^{α}-*p*-aminobenzoylbiocytinamide, two compounds that are useful for the biotinylation of biologically active molecules. Also described is a synthetic route to [25-biocytin]-$ACTH_{1-25}$-amide, a biotinylated derivative of $ACTH_{1-24}$. This compound exhibits the same adrenocortical potency with isolated bovine adrenocortical cells as the parent compound, and binds to avidin attached to Sepharose 4B.

In two previous communications[1,2] we have presented a novel approach to affinity columns for receptor studies which appears to be broadly applicable. This technique is based on the remarkably strong noncovalent interaction between the B-complex vitamin biotin and the egg-white protein avidin ($K_D \simeq 10^{-15}$ M). The basic steps of the procedure are outlined in

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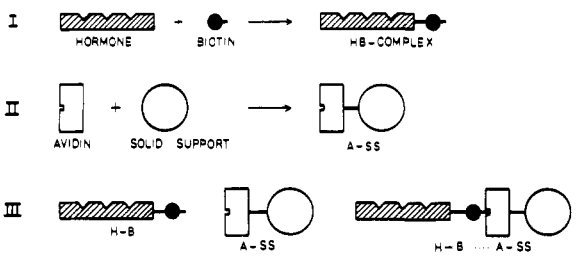
Table I. Elemental Analyses

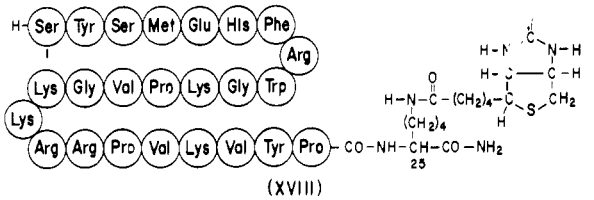
Boc-*p*-aminobenzoic acid	
Anal. Calcd for $C_{12}H_{15}NO_4$:	C, 60.8; H, 6.4; N, 5.9
Found:	C, 61.0; H, 6.3; N, 5.7
N-Hydroxysuccinimido Boc-*p*-aminobenzoate (XI)	
Anal. Calcd for $C_{16}H_{18}N_2O_6$:	C, 57.5; H, 5.4; N, 8.4
Found:	C, 57.6; H, 5.5; N, 8.5
N^{α}-Boc-N^{ϵ}-Z-Lys-amide (IV)	
Anal. Calcd for $C_{19}H_{29}N_3O_5$:	C, 60.14; H, 7.7; N, 11.1
Found:	C, 60.29; H, 7.8; N, 11.1
N^{α}-Boc-biocytinamide (VI)	
Anal. Calcd for $C_{21}H_{37}N_5O_5S$:	C, 53.5; H, 7.9; N, 14.8; S, 6.8
Found:	C, 53.4; H, 8.1; N, 14.6; S, 6.7
N^{α}-Z-N^{ϵ}-Boc-Lys-amide (VIII)	
Anal. Calcd for $C_{19}H_{29}N_3O_5$:	C, 60.14; H, 7.7; N, 11.1
Found:	C, 59.6; H, 7.9; N, 11.3
N^{α}-Z-Biocytinamide (X)	
Anal. Calcd for $C_{24}H_{35}N_5O_5S$:	C, 57.0; H, 7.0; N, 13.8; S, 6.3
Found:	C, 56.8; H, 7.2; N, 13.5; S, 6.6
Biocytinamide acetate (I)	
Anal. Calcd for $C_{16}H_{29}N_5O_3S{\cdot}CH_3COOH$:	C, 50.1; H, 7.7; N, 16.2; S, 7.4
Found:	C, 50.3; H, 7.6; N, 16.5; S, 7.7
Boc-*p*-aminobenzoylbiocytinamide hydrate (XII)	
Anal. Calcd for $C_{28}H_{41}N_6O_6S{\cdot}H_2O$:	C, 55.3; H, 7.1; N, 13.8; S, 5.3
Found:	C, 55.7; H, 7.0; N, 13.5; S, 5.5
p-Aminobenzoylbiocytinamide acetate (II)	
Anal. Calcd for $C_{25}H_{38}N_6O_6S$:	C, 54.5; H, 7.0; N, 15.3; S, 5.8
Found:	C, 54.0; H, 6.7; N, 16.0; S, 6.2

Scheme I



Scheme I. First, biotin is covalently attached to a biologically active molecule, for example, a hormone at a site that does not interfere with biological activity to form a hormone-biotin complex (H-B). Second, avidin is covalently attached to a solid support, for example, Sepharose 4B, to form an avidin-solid support complex (A-SS). Third, the biotinylated hormone (H-B) is mixed with the immobilized avidin (A-SS) to form Sepharose 4B particles that are coated with the hormone.

This paper describes syntheses of biocytinamide (I), N^{α}-*p*-aminobenzoylbiocytin (II) and of [25-biocytin]-$ACTH_{1-25}$-amide (XVIII). Reagents at hand for the



(XVIII)

introduction of biotin into polyfunctional molecules by way of carboxyl groups (biocytinamide), amino groups (*N*-hydroxysuccinimido biotinate), and finally via azo coupling to exposed tyrosin and histidine side chains of proteins (N^{α}-*p*-aminobenzoylbiocytinamide.)

Experimental Section[3]

Materials and General Methods. *d*-Biotin was obtained from Dr. W. E. Scott of Hoffmann-la Roche, Inc., Nutley, N.J. N^{α}-Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH and *H*-Lys-(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys-(Boc)-Val-Tyr-Pro-OH were gifts from Dr. W. Rittel, Ciba-Geigy Corporation, Basel, Switzerland. N^{α}-Boc-N^{ϵ}-Z-L-Lys and N^{α}-Z-N^{ϵ}-Boc-L-Lys were purchased from Fluka Corporation, Buchs, Switzerland. CMC (carboxymethylcellulose) was from Bio-Rad Corporation, Richmond, Calif., and Amberlite IRA-400 was from Mallinckrodt Chemical Works, St. Louis, Mo. Thin-layer chromatography (TLC) was performed on glass plates coated with silica gel G (type 60) (E. Merck and Co., Darmstadt, German Federal Republic) in the following solvent systems: R_f^{I} = 1-butanol-glacial acetic acid-water, 60:20:20; R_f^{II} = chloroform-methanol-water, 8:3:1 (lower phase); R_f^{III} = 1-butanol-pyridine-glacial acetic acid-water 30:20:6:24. Compounds were visualized on thin-layer plates by fluorescamine[4] and the chlorine reagent.[5] Compounds containing *p*-aminobenzoic acid exhibit a blue fluorescence when thin-layer plates are exposed to a UV lamp. *p*-Aminobenzoylbiocytinamide was also visualized by spraying plates developed in solvent system I with 1% sodium nitrite followed by a 1% solution of β-naphtol in 0.1 N NaOH. The ensuing spots are bright orange. Biotin was determined according to Green.[6] Melting points are uncorrected. Optical rotations were performed with a Zeiss precision polarimeter. Measurements were performed with a mercury lamp at 546 and 576 nm and extrapolated to the 589 sodium line. Elemental analyses were by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y. (Table I). Acid hydrolyses were performed in constant boiling HCl at 110 °C for 24 h in evacuated tubes. The amino acid composition of acid hydrolysates was determined with a Beckman-Spinco amino acid analyzer.

Syntheses. Boc-*p*-aminobenzoic Acid. To a solution of *p*-aminobenzoic acid (4.1 g, 30 mmol) in DMF (25 mL) was added 1 N NaOH (30 mL) followed by di-*tert*-butyl dicarbonate[7] (7.6 g, 35 mmol) and the mixture was stirred at room temperature for 42 h when the solvent was evaporated. The solid residue was distributed between water and ethyl acetate and the suspension was acidified with 20% citric acid. The organic layer was washed with five portions of 10% citric acid, two portions of saturated NaCl, and two portions of water and concentrated to a small volume. The product was precipitated by addition of petroleum ether, washed with petroleum ether, and dried. This material was dissolved in EtOH, 0.1% aqueous citric acid was added to the cloud point, and the mixture was kept at room temperature for 12 h. The crystals were collected, washed with 30% EtOH, and dried: yield 3.9 g (55%); mp 197–198 °C dec; R_f^{II} 0.7. Anal. ($C_{12}H_{15}NO_4$): C, H, N.

***N*-Hydroxysuccinimido Boc-*p*-aminobenzoate (XI).** To a solution of Boc-*p*-aminobenzoic acid (2.37 g, 10 mmol) and *N*-hydroxysuccinimide (1.15 g, 10 mmol) in THF (50 mL) was added a solution of DCC (2.06 g, 10 mmol) in THF (10 mL) and the mixture was stirred at room temperature for 20 h. The DCU was removed by filtration,

the clear filtrate was evaporated to dryness, and the residue was recrystallized from ethyl acetate/petroleum ether: yield 2.68 g (80%); mp 172-173 °C. Anal. ($C_{16}H_{18}N_2O_6$): C, H, N.

N^{α}-Boc-N^{ϵ}-Z-Lys-amide (IV). N^{α}-Boc-N^{ϵ}-Z-Lys DCHA salt (III) (5.62 g, 10 mmol) was suspended in ice-cold ethyl acetate (250 mL) and ice-cold 0.5 N citric acid (180 mL) was added. The mixture was stirred for 30 min; the organic layer was separated and washed with two portions of 0.5 N citric acid, ten portions of saturated sodium chloride, and two portions of water and dried over sodium sulfate. The solvent was evaporated, the residue was redissolved in ethyl acetate (70 mL), and the solution was cooled at −10 to −15 °C. To this solution was added TEA (1.39 mL, 10 mmol) followed by isobutyl chloroformate (1.31 mL, 10 mmol) and the mixture was stirred for 10 min at −10 to −15 °C. To this mixed anhydride solution was added 28% aqueous ammonia (5 mL) and the mixture was stirred for 2 h at 0 to −10 °C. The solid was collected and dried; 3.02 g. The filtrate (organic layer) was evaporated to give additional material: 0.82 g. Recrystallization from ethyl acetate gave clusters of needles: yield 3.5 g (92%); mp 140-141 °C; no measurable rotation in DMF; R_f^{I} 0.8; R_f^{II} 0.7; R_f^{III} 0.7. Anal. ($C_{19}H_{29}N_3O_5$): C, H, N.

N^{α}-Boc-biocytinamide (VI). (a) From N^{α}-Boc-Lys-amide Acetate (V) and *N*-Hydroxysuccinimido Biotinate. N^{α}-Boc-N^{ϵ}-Z-Lys-amide (IV) (556 mg, 1.47 mmol) was hydrogenated over palladium in MeOH (7 mL) and 10% acetic acid (1 mL). After 5 h the catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was evaporated several times with MeOH and was dried over P_2O_5 and KOH. The residue was then dissolved in DMF (7 mL), *N*-hydroxysuccinimido biotinate[8] (500 mg, 1.47 mmol) was added, and the mixture was stirred at room temperature until a clear solution was obtained. TEA (0.20 mL, 1.44 mmol) was then added and the mixture was stirred at room temperature for 44 h. The DMF was evaporated, the residue was distributed between 0.5 N citric acid and 1-butanol, and the butanol layers were washed with 0.5 N citric acid, saturated sodium chloride, 5% sodium bicarbonate, and water and the butanol was evaporated. The residue (677 mg) was recrystallized from aqueous EtOH: yield 426 mg (62%); mp 166-168 °C; $[\alpha]^{22}D$ + 45.7° (*c* 1.035, MeOH); R_f^{I} 0.5; R_f^{II} 0.4; R_f^{III} 0.7. Anal. ($C_{21}H_{37}N_5O_5S$): C, H, N, S.

(b) From N^{α}-Boc-Lys-amide Acetate (V) and Biotin with *N,N'*-Carbonylbisimidazole. A stirred suspension of biotin (976 mg, 4 mmol) in dry DMF (16 mL) was heated at 80 °C to give a clear solution. To this solution was added *N,N'*-carbonylbisimidazole (648 mg, 4 mmol) and the mixture was kept at 80 °C until evolution of CO_2 had ceased. The solution was cooled at room temperature and soon formed a thick gel. A solution of N^{α}-Boc-Lys-amide acetate (1.31 g, 4.3 mmol) in DMF (3.5 mL) was added followed by TEA (0.6 mL, 4.3 mmol) and the mixture was stirred at room temperature for 20 h. The DMF was evaporated, the residue was distributed between 1-butanol and 0.5 N citric acid, and the product was isolated as described under a above. The material was recrystallized from aqueous EtOH: yield 1.21 g (60%); mp 165-166 °C; $[\alpha]^{22}D$ +47.4° (*c* 1.0, MeOH); R_f^{I} 0.5; R_f^{II} 0.4; R_f^{III} 0.7.

$N\alpha$-Z-N^{ϵ}-Boc-Lys-amide (VIII). The title compound was prepared from N^{α}-Z-N^{ϵ}-Boc-Lys-DCHA salt (VII) (5.62 g, 10 mmol) in the manner described for the preparation of IV. The product was recrystallized from a mixture of MeOH and petroleum ether: yield 2.98 g (79%); mp 143-143.5 °C; $[\alpha]^{25}D$ +3.6° (*c* 1.10, DMF); R_f^{I} 0.8; R_f^{II} 0.8. Anal. ($C_{19}H_{28}N_3O_5$): C, H, N.

N^{α}-Z-Biocytinamide (X). N^{α}-Z-N^{ϵ}-Boc-Lys-amide (VIII) (1.52 g, 4 mmol) was dissolved in ice-cold 90% trifluoroacetic acid (7 mL) and the solution was kept at 0 °C for 45 min. The trifluoroacetate (IX) was precipitated by addition of ether, was washed with ether, and dried over P_2O_5 and KOH: yield 1.40 g (89%); R_f^{I} 0.6.

To an ice-cold stirred solution of biotin (855 mg, 3.5 mmol), N^{α}-Z-Lys-amide trifluoroacetate (1.38 g, 3.5 mmol), and 1-hydroxybenzotriazole (1.07 g, 7.0 mmol) in DMF (65 mL) was added *N*-ethyldiisopropylamine (0.60 mL, 3.5 mmol) followed by 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (1.34 g, 7.0 mmol). The mixture was stirred for 1 h at 0 °C and for 20 h at 20 °C. The ensuing precipitate was collected, washed with water, and dried. The material was recrystallized from hot DMF/MeOH: yield 1.54 g (87%); mp 223-224 °C; R_f^{I} 0.7; R_f^{II} 0.1 ; $[\alpha]^{28}D$ +44.6° (*c* 0.42, Me_2SO). Anal. ($C_{24}H_{35}N_5O_5S$): C, H, N, S.

Biocytinamide Acetate (I). (a) By Hydrogenation of N^{α}-Z-Biocytinamide (X). N^{α}-Z-Biocytinamide (506 mg, 1 mmol) was hydrogenated over palladium in DMF (140 mL) containing water (5 mL) and *N*-ethyldiisopropylamine (0.68 mL). After 24 h the catalyst was removed by filtration and the solvents were evaporated. The solid residue was dissolved in 10% acetic acid, insoluble material was removed, and the filtrate was lyophilized. The residue was dissolved in MeOH; the product was precipitated with ethyl acetate, washed with ethyl acetate, and dried: yield 269 mg (62%); $[\alpha]^{25}D$ +55.9° (*c* 0.37, MeOH); R_f^{I} 0.3; R_f^{III} 0.6. Anal. ($C_{16}H_{29}N_5O_3S{\cdot}CH_3COOH$): C, H, N, S.

(b) From N^{α}-Boc-biocytinamide (VI) with TFA. N^{α}-Boc-biocytin (1.0 g) was dissolved in 90% TFA (10 mL) and the solution was kept at room temperature for 30 min. The bulk of the TFA was evaporated and the product was precipitated with ether, washed with ether, and dried. This material was dissolved in 10% acetic acid (5 mL). TFA ions were exchanged for acetate ions on Amberlite IRA-400 in the usual manner and fractions containing the desired material were lyophilized: yield 1.03 g. The residue was dissolved in MeOH and the product was precipitated with ethyl acetate: yield 711 mg (77%); $[\alpha]^{27}D$ +56.2° (*c* 0.373, MeOH); R_f^{I} 0.3; R_f^{III} 0.6; identical with the material prepared according to a above.

Biocytinamide Tosylate. Biocytinamide acetate (I) (110 mg, 0.255 mmol) was dissolved in MeOH (8 mL) and the solution was cooled at 0 °C. An ice-cold solution of *p*-toluenesulfonic acid hydrate (48.5 mg, 0.255 mmol) in pyridine (0.70 mL) was added and the mixture was evaporated at a bath temperature of 10 °C. The residue was dissolved in MeOH and the solution was again evaporated. This procedure was repeated several times until the product solidified. The solid residue was triturated with ethyl acetate and dried in vacuo at 50 °C over P_2O_5: yield 140 mg (101%).

Boc-*p*-aminobenzoylbiocytinamide Hydrate (XII). *N*-Hydroxysuccinimido Boc-*p*-aminobenzoate (XI) (734 mg, 2.0 mmol) was added at room temperature to a solution of biocytinamide acetate (862 mg, 2.0 mmol) and TEA (0.278 mL, 2.0 mmol) in DMF (20 mL) and the solution was stirred at room temperature for 24 h. At this point TLC showed the presence of unreacted biocytinamide, and 70 mg of *N*-hydroxysuccinimido Boc-*p*-aminobenzoate was added and the mixture was stirred for an additional 66 h. Insoluble material was removed by filtration, the bulk of DMF was evaporated, and the product was precipitated with ethyl acetate and dried. The solid residue (1.15 g) was dissolved in glacial acetic acid (5 mL) and water was added to the cloudpoint. After standing in a refrigerator for several hours the solid was collected, washed with 10% acetic acid, and dried: yield 1.07 g (88%); $[\alpha]^{25}D$ +40.4° (*c* 1.034, glacial acetic acid); R_f^{I} 0.6; R_f^{III} 0.7. Anal. ($C_{28}H_{41}N_6O_6S{\cdot}H_2O$): C, H, N, S.

***p*-Aminobenzoylbiocytinamide Acetate (II).** The protected compound (800 mg) was dissolved in 90% TFA (10 mL) and the solution was kept at room temperature for 30 min. The bulk of the TFA was evaporated and the product precipitated with ether, washed with ether, and dried. TFA ions were exchanged for acetate ions in Amberlite IRA-400 using 50% acetic acid as the solvent. Evaporation of the acetic acid left a crystalline solid which was dried and recrystallized from aqueous ethanol: yield 347 mg (48%); mp 236-238 °C; $[\alpha]^{24}D$ +40.7° (*c* 1.055, 50% acetic acid); R_f^{I} 0.4; R_f^{III} 0.6. Anal. ($C_{25}H_{38}N_6O_6S$): C, H, N, S.

N^{α}-TFA-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OH (XIII). Ethylthiol trifluoroacetate (0.098 mL) was added to a solution of *H*-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OBu^{t} triacetate (870 mg, 0.38 mmol) and *N*-ethyldiisopropylamine (0.13 mL, 0.76 mmol) in DMF (10 mL) and the mixture was stirred for 3 h at room temperature. The product was precipitated with ether (500 mL) and was collected, washed with ether, and dried: yield 836 mg (95%); $[\alpha]^{26}D$ −71.6° (*c* 0.44, MeOH); R_f^{I} 0.6; R_f^{III} 0.8. This material (820 mg) was dissolved in 90% TFA (10 mL) and the mixture was kept at room temperature for 1 h. The bulk of the solvents was removed and the product was precipitated with ether, washed with ether, and dried: yield 825 mg (96%); R_f^{III} 0.4; $[\alpha]^{27}D$ −32.4° (*c* 0.48, DMF). This material (820 mg, 0.337 mmol) was dissolved in DMF (14 mL) and *N*-ethyldiisopropylamine (0.288 mL, 1.685 mmol) and di-*tert*-butyl dicarbonate (588 mg, 2.696 mmol) were added. The mixture was stirred at room temperature for 18 h, the solvent was evaporated, and the product was precipitated with ether, washed with ether, and dried.

The material was then dissolved in 50% acetic acid and TFA ions were exchanged for acetate ions on Amberlite IRA-400 in the usual manner. Chlorine positive eluates were pooled and lyophilized and the residue was washed with ethyl acetate and redried: yield 731 mg (96%); $[\alpha]^{25}D$ −60.8° (*c* 0.50, MeOH); R_f^{I} 0.5; R_f^{III} 0.8. The di-

acetate (390 mg) was converted to the ditosylate according to the procedure of Brundish and Wade:[9] yield 430 mg.

N^{α}-TFA-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-Bct-amide Diacetate (XIV). N^{α}-TFA-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Tyr-Pro-OH ditosylate (430 mg, 0.172 mmol), biocytinamide tosylate (187 mg, 0.344 mmol), and 1-hydroxybenzotriazole monohydrate (158 mg, 1.032 mmol) were dissolved in a mixture of DMF (10 mL) and pyridine (5 mL), and DCC (71 mg) was added with stirring. Additional 71-mg portions of DCC were added after 1 and 2 h (total addition 213 mg, 1.032 mmol). The mixture was stirred for 40 h at room temperature. The solvent was removed, the residue was precipitated by addition of ethyl acetate, and the precipitate was washed with ethyl acetate and dried. The material was dissolved in 50% acetic acid and TFA ions were exchanged for acetate ions on Amberlite IRA-400 in the usual manner. Chlorine positive eluates were pooled; the solution was concentrated to a small volume and lyophilized: yield 645 mg. This material was dissolved in 50% aqueous MeOH (250 mL) and the solution was applied to a column of CMC (3 × 10 cm) previously equilibrated with 50% MeOH. The column was eluted with 50% MeOH (250 mL), MeOH/0.06% acetic acid, 1:1 (300 mL), and MeOH/0.1% acetic acid, 1:1 (500 mL). Individual fractions (10 mL each) were collected and monitored spectroscopically at 280 nm and by TLC. Fractions 90–136 were pooled and evaporated to dryness. The residue was lyophilized from dilute acetic acid: yield 384 mg (85%); $[\alpha]^{27}D$ −30.6° (*c* 0.50, MeOH); R_f^{I} 0.3; R_f^{III} 0.7; amino acid ratios in acid hydrolysate, $Lys_{4.9}Arg_{1.9}Pro_{3.0}Gly_{1.1}Val_{3.2}Tyr_{0.9}$ (91%).

***H*-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-Bct-amide Tritosylate (XV).** The protected peptide amide diacetate (XIV) (310 mg, 0.118 mmol) was dissolved in 1 M methanolic piperidine (8 mL), 1 M aqueous piperidine (40 mL) was added, and the solution was stirred at room temperature for 8 h. 1-Butanol (20 mL) was added to the reaction mixture and the solvents were evaporated at a bath temperature of 20 °C. The residue was triturated with ethyl acetate and the solid was washed with ethyl acetate and dried. This material was dissolved in 50% acetic acid and TFA ions were exchanged for acetate ions on Amberlite IRA-400 in the usual manner. Chlorine positive fractions were pooled, the solvent was removed, and the residue was washed with ethyl acetate and dried: yield 297 mg (97%); $[\alpha]^{27}D$ −20.9° (*c* 0.43, DMF); R_f^{I} 0.2; R_f^{III} 0.7. The compound was converted to the tritosylate according to the method of Brundish and Wade.[9]

Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-Bct-amide Triacetate (XVII). N^{α}-Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH tosylate (XVI) (275 mg, 0.169 mmol), the tritosylate of XV (450 mg, 0.154 mmol), and 1-hydroxybenzotriazole (117 mg, 0.76 mmol) were dissolved in a mixture of DMF (16 mL) and pyridine (8 mL), and DCC (64 mg) was added with stirring. Additional 64-mg portions of DCC were added after 1 and 2 h (total addition 192 mg, 0.924 mmol). The mixture was stirred at room temperature for 24 h, the solvents were evaporated, and the residue was triturated with ethyl acetate and dried. The material was dissolved in 50% acetic acid and TFA ions were exchanged by acetate ions on Amberlite IRA-400 in the usual manner. Chlorine-positive fractions were pooled, evaporated to a small volume, and lyophilized: yield 680 mg. This material was dissolved in 50% MeOH (300 mL), a small amount of insoluble material was removed by filtration, and the clear solution was applied to a CMC column (3 × 8 cm) which was previously equilibrated with 50% MeOH. The column was eluted with 50% MeOH (150 mL), MeOH/0.2% acetic acid, 1:1 (500 mL), MeOH/0.4% acetic acid, 1:1 (750 mL), MeOH/2% acetic acid, 1:1 (500 mL), and MeOH/20% acetic acid, 1:1 (500 mL). Fractions (10 mL each) were monitored by absorbancy at 280 nm and TLC. Fractions 97–176 were pooled and evaporated and the residue was dissolved in dilute acetic acid and the solution was lyophilized: yield 416 mg (67%); $[\alpha]^{27}D$ −21.0° (*c* 0.4, DMF); R_f^{I} 0.3; R_f^{III} 0.7; amino acid ratios in acid hydrolysate, $Ser_{2.03}Tyr_{1.94}Met_{1.01}Glu_{1.05}His_{0.97}Phe_{1.04}Arg_{2.91}Gly_{2.02}Lys_{4.96}Pro_{3.07}Val_{3.02}NH_{3,0.97}$ (85%).

[25-Biocytin]-ACTH$_{1-25}$-amide 7-CH$_3$COOH (XVIII). The protected peptide (XVII) (306 mg) was dissolved in ice-cold 90% TFA (5 mL) containing ethanethiol (0.1 mL) and the solution was stirred at 0 °C for 10 min and at room temperature for 50 min. The bulk of the solvent was removed, the residue was triturated with ether, and the solid product was collected, washed with ether, and dried. This material was dissolved in 10% acetic acid (20 mL) and the solution was passed through a column (2 × 13 cm) of Amberlite IRA-400 (acetate form) which was washed with 10% AcOH until the eluates were fluorescamine-negative. Pooled fluorescamine-positive eluates were lyophilized and the residue was dried. This material was dissolved in 1% aqueous thioglycolic acid (15 mL) and the solution was incubated for 24 h at 35 °C. The solution was then applied to an acetate cycle Amberlite IRA-400 column (2 × 17 cm) previously equilibrated with 10% acetic acid and eluted with water until the elutes were fluorescamine-negative. Fluorescamine-positive fractions were combined and lyophilized and the residue was dried: yield 281 mg; R_f^{III} 0.4; $[\alpha]^{26}D$ −65.2° (*c* 9.46, 1% AcOH). Amino acid ratios in 24-h acid hydrolysate: $Lys_{5.00}His_{0.98}Arg_{2.90}Ser_{2.10}Glu_{1.01}Pro_{3.03}Gly_{2.02}Val_{3.01}Met_{0.98}Tyr_{1.94}Phe_{1.01}NH_{3,1.32}$.

Results and Discussion

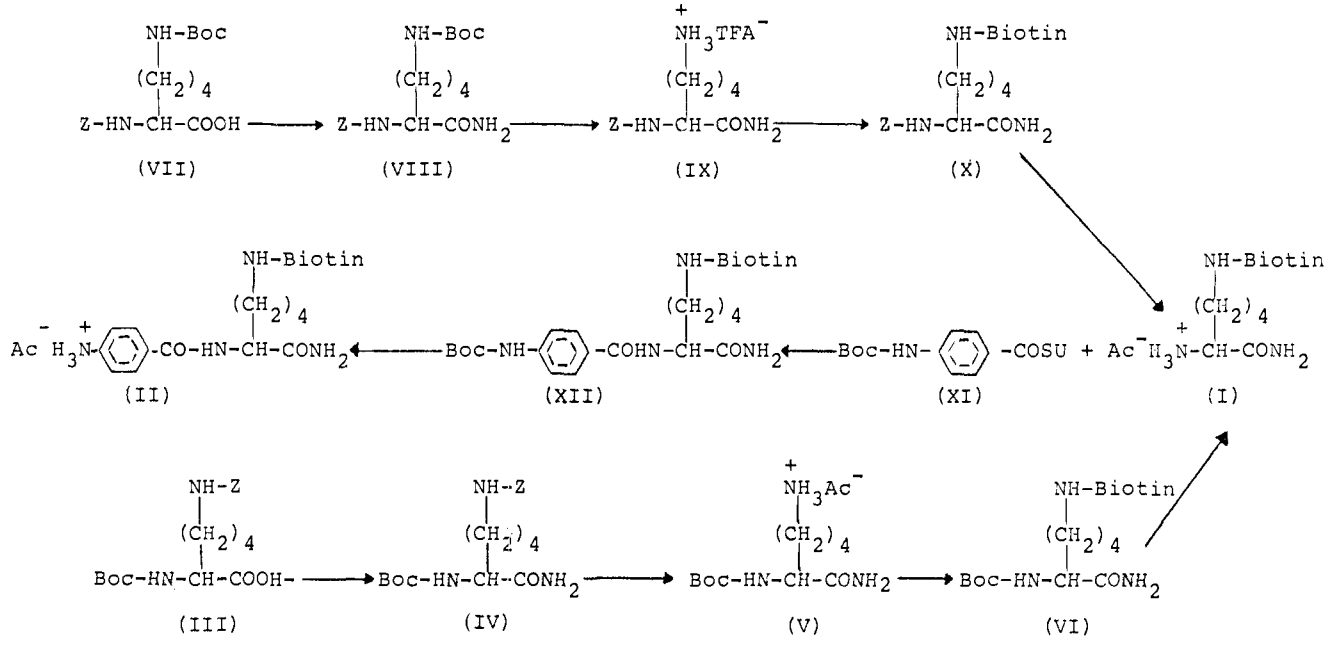
The observation that biotin-containing carboxylating enzymes bind very firmly to immobilized avidin[10] aroused our interest in exploring the biotinylation of biologically active molecules in order to exploit the high affinity of avidin for biotin for the development of affinity columns for hormone–receptor studies. Since columns containing immobilized avidin "catch" biotinylated molecules we suggest the term A–B catch principle to delineate this route to affinity columns. Thus far we have biotinylated $ACTH_{1-24}$[1] and insulin,[2] but the methodology is obviously applicable to a wide variety of molecules and may be useful for the identification of drug receptors.

Many of the presently available procedures for attaching peptide and protein hormones to solid supports[11] are not targeted and result in the formation of complex mixtures in which the positions of the covalent bonds between peptide and support are not defined. Although such preparations have been used successfully in isolation work it is desirable to have available more selective, targeted methods for attaching peptides or protein hormones to solid supports. Such procedures should satisfy the following criteria: (1) The hormone should be attached to the support via a single, defined site that is not involved in its biological function. (2) The synthetic route employed for anchoring the hormone to the support should be unequivocal. (3) Chemical manipulations should be performed with the free hormone, and thus their effect on binding and biological activity can be readily assessed by bioassay. (4) The ultimate step involved in attaching the hormone to the support should proceed in high yield. The attachment of $ACTH_{1-24}$ to immobilized avidin which was the subject of a previous communication[1] satisfies these criteria. In order to exhibit maximal receptor affinity it appears crucial that the linkage to the support be through the carboxyl end of $ACTH_{1-24}$ since this portion of the molecule is not essential for biological function.[12] In the biotinyl enzymes the biotin is covalently attached to the ϵ-amino group of lysine residues and ϵ-biotinyl-L-lysine has become known as biocytin (Bct). Biocytin was originally synthesized by Wolf et al.[13] Recently Bodanszky and Fagan[14] prepared the *p*-nitrophenyl ester of biotin by exposure of the vitamin to *p*-nitrophenyl trifluoroacetate. This reaction results in the formation of an *N*-trifluoroacetyl derivative of the *p*-nitrophenyl ester that is converted to the *p*-nitrophenyl ester by exposure to ethanol. N-Acylation is not observed when biotin is converted to the *N*-hydroxysuccinimide ester according to the procedure of Jasiewicz et al.,[8] a procedure that was employed in the present investigation.

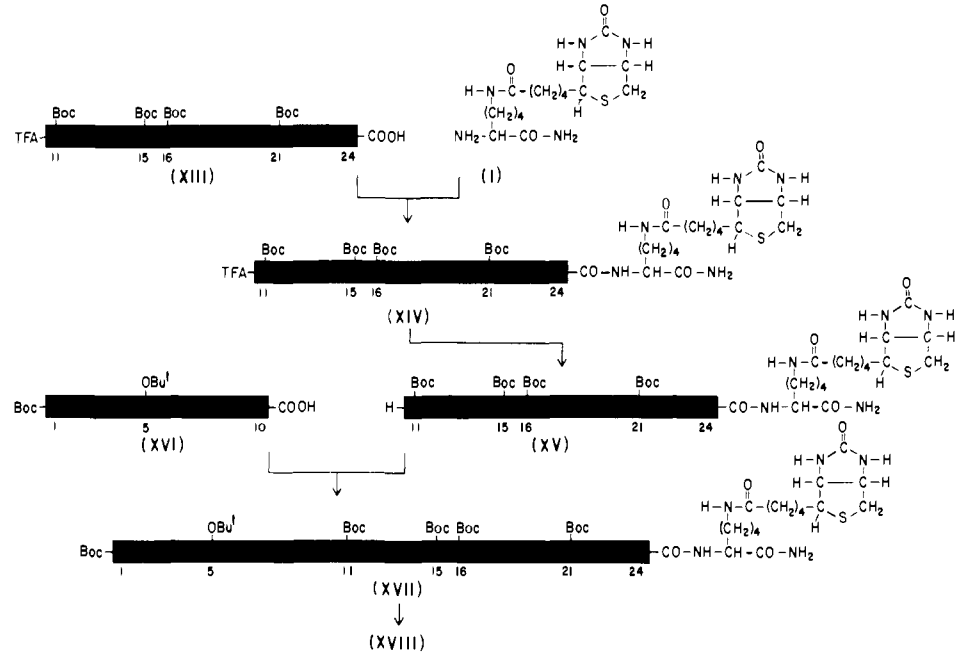
We selected biocytinamide (I) as a suitable intermediate for the biotinylation of $ACTH_{1-24}$ since the side chain of lysine provides a sizable spacer removing the biotin from the biologically active region of the ACTH molecule. Two routes (Scheme II) were employed to prepare biocytinamide (I), a key intermediate for attaching biotin to $ACTH_{1-24}$ to form [25-biocytin]-$ACTH_{1-25}$-amide (XVIII).

The preferred route to biocytinamide utilizes N^{α}-Boc-N^{ϵ}-

Scheme II



Scheme III



Z-Lys (III) as the starting material. This compound is converted to the amide IV by the method of mixed anhydrides. Hydrogenolysis of IV in the presence of acetic acid affords V, which is acylated either with the *N*-hydroxysuccinimido ester of biotin or with *N*-biotinylimidazole obtained from biotin and *N,N'*-carbonylbisimidazole.[8] Removal of the Boc group from VI with 90% TFA followed by exchange of TFA ions by acetate ions on acetic acid cycle Amberlite IRA-400 affords biocytinamide (I) in the form of its acetate salt. The synthesis of I was also performed using N^{α}-Z-N^{ϵ}-Boc-Lys (VII) via the intermediates VIII, IX, and X. Hydrogenolysis of X over palladium in MeOH containing *N*-ethyldiisopropylamine[15] afforded the free base of I but difficulties were encountered in this step and this route was abandoned. For the synthesis of *p*-aminobenzoylbiocytinamide (II) biocytinamide is acylated with the *N*-hydroxysuccinimido ester of Boc-*p*-aminobenzoic acid (XI) to form XII. Attempts to prepare Boc-*p*-aminobenzoic acid by the use of Boc-azide[16] were unsuccessful but this compound is readily obtained when *p*-aminobenzoic acid is reacted with di-*tert*-butyl dicarbonate[7] in the presence of base. The *N*-hydroxysuccinimido ester of Boc-*p*-aminobenzoic acid was readily prepared in the usual manner.[17] Removal of the Boc group from XII with 90% TFA gives the TFA salt of II which is converted to the acetate salt with acetate cycle Amberlite IRA-400. The diazonium chloride of II forms a brilliant red dye when coupled with alkaline β-naphthol and this reaction served to locate the compound on thin-layer plates.

The synthetic route to [25-biocytin]-ACTH$_{1-25}$-amide (XVIII) is illustrated in Scheme III. Starting material is *H*-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OBu^{t} which is trifluoroacetylated with ethylthiol trifluoroacetate.[18] The *tert*-butyl ester and the Boc groups are then removed with 90% TFA and the ensuing product is exposed to di-*tert*-butyl dicarbonate in the presence of *N*-ethyldiisopropylamine to afford XIII. Biocytinamide (I) is acylated with XIII using the DCC/HOBt procedure of König and Geiger[19] to give XIV which is partially deprotected

by exposure to piperidine in aqueous methanol.[20] The ensuing compound, XV, in the form of the tritosylate salt is then coupled with the tosylate of N^{α}-Boc-Ser-Tyr-Ser-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH (XVI) by the DCC/HOBt procedure[19] to afford protected [25-biocytin]-$ACTH_{1-25}$-amide (XVII) which is purified by chromatography on CMC. Finally, XVII is deprotected with 90% TFA, TFA ions are exchanged for acetate ions on acetate cycle IRA-400, and the product is exposed to aqueous thioglycolic acid[21] to remove trace quantities of the *S*-sulfoxides of Met and biotin. The biotin content of [25-biocytin]-$ACTH_{1-25}$-amide (XVIII) as determined by the dye assay[6] was 94% of theory based on the average amino acid recovery in acid hydrolysates of the peptide. [25-Biocytin]-$ACTH_{1-25}$-amide is as active as $ACTH_{1-24}$ in stimulating steroidogenesis in isolated bovine adrenal cortical cells and binds to avidin attached to Sepharose 4B.[1]

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Protein Rotational Correlation Times Determined in Aqueous Solution by Carbon-13 Rotating Frame Spin–Lattice Relaxation in the Presence of an Off-Resonance Radiofrequency Field

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Abstract: An NMR off-resonance rotating frame relaxation technique is presented and applied to the study of rotational tumbling of protein molecules in solution. The experimental observable consists of the ^{13}C resonance peaks in the carbonyl region of the NMR spectrum for the protein. The ratio of the peak intensities in the presence and absence of the off-resonance rf field is related to both the Zeeman spin-lattice relaxation rate ($1/T_1$) and to a relaxation rate (designated $1/T_{1\rho}^{off}$) which is produced by the off-resonance irradiation. It is shown that the assumption of random isotropic rotation of the protein molecules allows this ratio of peak intensities to be interpreted in terms of a rotational correlation time. To illustrate the $T_{1\rho}^{off}$ technique, rotational correlation times for lysozyme, chymotrypsinogen A, concanavalin A, human methemoglobin A, bovine serum albumin, and immunoglobulin A were determined. The correlation time values obtained via the off-resonance $T_{1\rho}$ technique compare favorably in all cases with values determined by other experimental methods.

Several techniques have been applied to study rotational motions of proteins in aqueous solution including dielectric relaxation,[1] polarized light scattering,[2] fluorescence depolarization,[3a] electric birefringence,[3b] water proton NMR[4] spin–lattice relaxation dispersion,[5] and ^{13}C NMR relaxation.[6,7] We have recently developed an NMR technique which entails rotating frame spin–lattice relaxation in the presence of an off-resonance radiofrequency field;[8] this technique is useful for investigating rotational motions of macromolecules and appears to have potential for studying internal motions in macromolecules.

The work presented here utilizes our off-resonance $T_{1\rho}$ technique to study the rotational reorientation of a series of proteins in aqueous solution. The ^{13}C resonances of the envelope composed of carbonyls predominately on the protein backbone are observed. These carbons are expected to be relaxed via the dipole–dipole mechanism with nearby protons in a 23.5 kG magnetic field.[9] The backbone carbonyls were

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