Schnarr, G., & Maurizot, J.-C. (1982) *Eur. J. Biochem. 128*, 515–520.
Schulz, G., & Schirmer, R. H. (1979) *Principles of Protein Structure*, Springer-Verlag, New York.
Staehelin, T., Durrer, B., Schmidt, J., Takacs, B., Stocker, J., Miggiano, V., Stähli, C., Rubinstein, M., Levy, W. P., Hershberg, R., & Pestka, S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1848–1852.
Sternberg, M. J. E., & Cohen, F. E. (1982) *Int. J. Biol. Macromol. 4*, 137–144.
Tanaka, S., & Scheraga, H. A. (1976a) *Macromolecules 9*, 159–167.
Tanaka, S., & Scheraga, H. A. (1976b) *Macromolecules 9*, 812–833.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.
Wetlaufer, D. B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 697–701.
Wetlaufer, D. B. (1981) *Adv. Protein Chem. 34*, 61–92.
Wetzel, R., Johnston, P. D., & Czarniecki, C. W. (1983) in *The Biology of the Interferon System* (De Maeyer, E., & Schellekens, H., Eds.) pp 101–112, Elsevier, Rotterdam.
Zav'yalov, R. P., & Denesyuk, A. I. (1982) *Immunol. Lett. 4*, 4–14.

# Syntheses of Biotinylated and Dethiobiotinylated Insulins†

Klaus Hofmann,* Wei Jun Zhang,‡ Hana Romovacek, Frances M. Finn, Aksel A. Bothner-By, and P. K. Mishra

ABSTRACT: The 600-MHz proton spectrum of dethiobiotin (prepared from *d*-biotin with Raney nickel) was measured in order to gain information pertaining to its stereochemical homogeneity. The spectrum demonstrated clearly that the material is a 6:1 mixture of two stereoisomers. The cis compound, corresponding to the stereochemistry of *d*-biotin, is the major isomer. Two biotinyl- and two dethiobiotinylinsulins were prepared in which the distance between the biotins and insulin was varied by interposition of spacer arms. The synthesis of these compounds involved repeated *N*-hydroxysuccinimido ester condensations. Biotin *N*-hydroxysuccinimido ester, dethiobiotin *N*-hydroxysuccinimido ester, 6-aminohexanoic acid, and *N*-[3-[(3-aminopropyl)carboxyamino]propyl]succinamic acid *N-tert*-butyl ester served as the building blocks for the spacers. The latter compound was prepared from *N*-[3-[(3-aminopropyl)amino]propyl]succinamic acid sulfate by the use of a selective amino-protecting method based on the differential stability toward acid of citraconyl and *tert*-butoxycarbonyl amino-protecting groups. The structure of *N*-[3-[(3-aminopropyl)amino]propyl]succinamic acid sulfate was established unequivocally by X-ray diffraction. The attachment of the biotinylated spacers to the insulin was exclusively at the $N^{\alpha,B1}$ position. Homogeneity of the final products as well as of the intermediates used in their synthesis was established by thin-layer chromatography, by high-pressure liquid chromatography, and in most instances by elemental analysis. The ratio of 6-aminohexanoic acid to lysine in hydrolysates of the insulin derivatives was in agreement with theory. The insulin derivatives were required for a study on the effect of avidin on their ability to interact with insulin receptors on rat epididymal adipocytes, which is described in the accompanying paper.

In a recent paper (Hofmann et al., 1982), we reported that the attachment of insulin to the carboxyl group of biotin exerts a rather dramatic effect on its ability to bind to succinoylavidin. The half-time for dissociation of biotin from avidin is approximately 200 days (Green, 1975); we have determined the half-time for dissociation of biotin from succinoylavidin as approximately 127 days (Finn et al., 1980). Most surprising was the observation that the avidin and succinoylavidin complexes of $N^{\alpha,B1}$-biotinylinsulin dissociate rather rapidly with a half-time for dissociation of approximately 3 h. The attachment of the bulky insulin molecule to biotin appears to exert a steric impediment to avidin binding. As a consequence, it became of interest to have available a series of biotinylated insulins in which the distance between biotin and insulin is systematically increased by insertion of spacers.

Avidin interferes with the ability of biotinylinsulin to stimulate lipogenesis in rat epididymal adipocytes but has no effect on insulin-stimulated lipogenesis (Hofmann et al., 1977). May et al. (1978) described similar observations with $N^{\epsilon,B29}$-biotinylinsulin. This finding suggests that avidin weakens the ability of biotinylinsulin to interact with insulin receptors on the adipocyte, and it seems logical to assume that this interference should be inversely proportional to the distance between the biotin and insulin in the analogue. In the present paper, we describe the synthesis of four insulin derivatives (Figure 1) in which spacers of increasing chain length separate the biotin or dethiobiotin from the insulin. An accompanying paper explores the dissociation behavior of succinoylavidin and streptavidin complexes of these compounds and relates their ability to stimulate glucose oxidation in rat epididymal adipocytes in the presence or absence of avidin, succinoylavidin, and streptavidin.

## Experimental Procedures

### *Materials and Methods*

Biotin was a gift from Dr. W. E. Scott of Hoffmann-La Roche Inc., Nutley, NJ, and $Boc_2$-insulin[1] (bovine) was a gift

† From the Protein Research Laboratory and the Department of Medicine, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15261 (K.H., W.J.Z., H.R., and F.M.F.), and the Department of Chemistry, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213 (A.A.B. and P.K.M.). *Received September 14, 1983.* Supported by National Institutes of Health Grants AM 21292, AM 16532, and RR 00292 and the Juvenile Diabetes Foundation International. A preliminary account of this work was presented at the 21st Symposium on Peptide Chemistry in Tsukuba, Japan, Oct 26, 1983.
‡ Present address: Institute of Organic Chemistry, Academia Sinica, Shanghai, The People's Republic of China.

0006-2960/84/0423-2547$01.50/0 © 1984 American Chemical Society

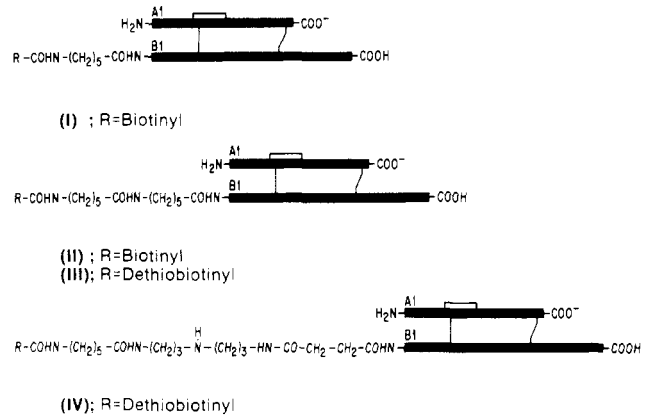


FIGURE 1: Simplified structures of $N^{\alpha,B^1}$-[6-(biotinylamido)hexanoyl]insulin (I), $N^{\alpha,B^1}$-[6-[6-(biotinylamido)hexanamido]hexanoyl]insulin (II), $N^{\alpha,B^1}$-[6-[6-[6-(5-methyl-2-oxo-4-imidazolidinyl)hexanamido]-hexanamido]hexanoyl]insulin (III), and $N^{\alpha,B^1}$-[*N*-[3-[[3-[6-[6-(5-methyl-2-oxo-4-imidazolidinyl)hexanamido]hexanamido]propyl]-amino]propyl]succinamoyl]insulin (IV).

from Professor R. Geiger of Höchst A. G., Frankfurt am Main, West Germany. Diethylaminoethylcellulose DE-52 was from Whatman Ltd., Springfield Hill, Maidstone, Kent, England. The solvent systems for ascending TLC on silica gel (E. Merck and Co., Darmstadt, West Germany) were (I) 1-butanol–glacial acetic acid–water (60:20:20), (II) chloroform–methanol–water (8:3:1), (III) 1-butanol–pyridine–glacial acetic acid–water (30:20:6:24), and (IV) 1-propanol–29% aqueous ammonia (3:2). Compounds were visualized on thin-layer plates by fluorescamine (Udenfriend et al., 1972), the chlorine reagent (exposure to hypochlorite followed by spraying with a 1:1 mixture of 0.4% KI and 1% starch solution in water), *p*-(dimethylamino)cinnamaldehyde (McCormick and Roth, 1970), and the nitroprusside–acetaldehyde reagent (Feigl et al., 1966). Elemental analyses were by Schwarzkopf Microanalytical Laboratory, Woodside, NY. High-pressure liquid chromatography (HPLC) was performed with a Waters system with a Model 660 solvent programmer. For amino acid analysis, samples were hydrolyzed with constant-boiling HCl containing 0.1% phenol at 110 °C for 24 h. Proton NMR spectra were obtained on the 600-MHz spectrometer at the NMR Facility for Biomedical Studies, Pittsburgh, PA, operating in the rapid-scan correlation mode. The samples were at 1–2 mM concentration in deuterated dimethyl sulfoxide, with 2% $Me_4Si$ added as an internal reference and lock.

*Synthetic Aspects*

*6-(Biotinylamido)hexanoic Acid.* TEA (0.153 mL, 1.1 mmol) was added with stirring to a suspension of *N*-hydroxysuccinimido biotinate (Jasiewicz et al., 1976) (341 mg, 1 mmol) and methyl 6-aminohexanoate hydrochloride (200 mg, 1.1 mmol) in DMF (5 mL), and the solution was stirred at room temperature for 20 h. The DMF was evaporated in vacuo, 1 N sodium hydroxide (3 mL) and enough methanol were added to give a clear solution, and the mixture was stirred at room temperature for 2 h. The solution was concentrated to a small volume, water (10 mL) was added, and the solution was acidified to Congo red with concentrated HCl and placed in a refrigerator. The crystals were collected, washed with a small volume of ice–water, and dried. The compound was recrystallized from boiling water: yield 327 mg (92%); mp 213–215 °C dec; $R_f^{II}$ 0.3 (minor contamination of $R_f^{II}$ 0.7). Anal. Calcd for $C_{16}H_{27}N_3O_4S$: C, 53.76; H, 7.61; N, 11.75; S, 8.97. Found: C, 53,41; H, 7.41; N, 11.07; S, 9.48.

*N-Hydroxysuccinimido 6-(Biotinylamido)hexanoate.* *N*-Hydroxysuccinimido trifluoroacetate (reagent) (Sakakibara & Inukai, 1965) (354 mg, 1.68 mmol) was added to a solution of 6-(biotinylamido)hexanoic acid (300 mg, 0.84 mmol) in pyridine (9 mL), and the solution was stirred at room temperature for 1 h when an additional amount (354 mg, 1.68 mmol) of reagent was added. After the mixture was stirred for 4 h, TLC showed the absence of starting material. The pyridine was evaporated, and the residue was triturated with three portions of ether and crystallized from 2-propanol: yield 300 mg (79%); mp 160–162 °C dec; $R_f^{II}$ 0.56. Anal. Calcd for $C_{20}H_{32}N_4O_6$: C, 52.85; H, 7.10; N, 12.33; S, 7.04. Found: C, 52.78; H, 6.95; N, 11.99; S, 7.17.

*6-[6-(Biotinylamido)hexanamido]hexanoic Acid.* TEA (10% in DMF) (0.77 mL, 0.55 mmol) was added to a stirred solution of *N*-hydroxysuccinimido 6-(biotinylamido)hexanoate (227 mg, 0.5 mmol) and methyl 6-aminohexanoate hydrochloride (100 mg, 0.55 mmol) in DMF (2.5 mL), and the solution was stirred at room temperature for 20 h. The DMF was evaporated, the residue was suspended in 1 N sodium hydroxide (3 mL), and enough methanol was added to give a clear solution, which was stirred for 2 h at room temperature. The bulk of the methanol was removed in vacuo, and the solution was acidified to Congo red with concentrated HCl. The ensuing suspension was placed in a refrigerator to complete crystallization. The crystals were collected, washed with a small volume of ice–water, and dried: yield 234 mg (99%); mp 185–191 °C dec. A sample for analysis was recrystallized from water: $R_f^{I}$ 0.45, $R_f^{II}$ 0.28. Anal. Calcd for $C_{22}H_{38}N_4O_5S \cdot {}^1/_2H_2O$: C, 55.09; H, 8.13; N, 11.90; S, 6.81. Found: C, 55.26; H, 7.93; N, 11.63; S, 6.66.

*N-Hydroxysuccinimido 6-[6-(Biotinylamido)hexanamido]hexanoate.* *N*-Hydroxysuccinimido trifluoroacetate (reagent) (89 mg, 0.42 mmol) was added at room temperature to a suspension of 6-[6-(biotinylamido)hexanamido]hexanoic acid (100 mg, 0.21 mmol) in dry pyridine (5 mL), and the solution was stirred at room temperature for 1 h when TLC showed the presence of starting material. Reagent (89 mg, 0.42 mmol) was added, and stirring was continued for 6 h, when a third portion (89 mg) of reagent was added. After this was stirred for 12 h, the insoluble material (unreacted acid) was removed by filtration (73 mg), and the pyridine was evaporated. The residue was triturated with three portions of ether and recrystallized from 2-propanol: yield 45 mg (38%); mp 135–138 °C dec; $R_f^{II}$ 0.45. Anal. Calcd for $C_{26}H_{41}N_5O_7S$: C, 55.01; H, 7.28; N, 12.34; S, 5.65. Found: C, 54.84; H, 7.53; N, 11.68; S. 5.84.

*6-[6-[6-(5-Methyl-2-oxo-4-imidazolidinyl)hexanamido]-hexanamido]hexanoic Acid.* TEA (0.153 mL, 1.1 mmol) was added at room temperature to a stirred solution of *N*-hydroxysuccinimido 6-[6-(5-methyl-2-oxo-4-imidazolidinyl)-hexanamido]hexanoate (IX) (Hofmann et al., 1982) (242.5 mg, 1 mmol) and methyl 6-aminohexanoate hydrochloride (200 mg, 1.1 mmol) in DMF (5 mL), and the solution was stirred at room temperature for 23 h. The DMF was removed in vacuo, the residue was suspended in 1 N sodium hydroxide (3 mL), and enough methanol was added to give a clear solution, which was stirred at room temperature for 1 h. The bulk of the methanol was evaporated, and the solution was diluted with water and was acidified to Congo red with concentrated HCl. The ensuing suspension was placed in a re-

[1] Abbreviations: Boc, *tert*-butoxycarbonyl; $Boc_2$-insulin, $N^{\alpha,A^1}$,$N^{\epsilon,B^{29}}$-bis(*tert*-butoxycarbonyl)insulin; DCC, *N*,*N*'-dicyclohexylcarbodiimide; $Me_2SO$, dimethyl sulfoxide; HPLC, high-pressure liquid chromatography; OSu, *N*-hydroxysuccinimido ester; TEA, triethylamine; TLC, thin-layer chromatography; DMF, dimethylformamide; THF, tetrahydrofuran; TFA, trifluoroacetic acid; $Me_4Si$, tetramethylsilane; dethiobiotin, 6-(5-methyl-2-oxo-4-imidazolidinyl)hexanoic acid.

frigerator to complete crystallization, and the crystals were collected, washed with a small volume of ice–water, and dried: yield 433 mg (98%); mp 147–150 °C; $R_f^I$ 0.5, $R_f^{II}$ 0.5, $R_f^{III}$ 0.6. A sample for analysis was recrystallized from water. Anal. Calcd for $C_{22}H_{40}N_4O_5$: C, 59.97; H, 9.15; N, 12.72. Found: C, 59.97; H, 9.20; N, 12.44.

*N-Hydroxysuccinimido 6-[6-[6-(5-Methyl-2-oxo-4-imidazolidinyl)hexanamido]hexanamido]hexanoate.* N-Hydroxysuccinimido trifluoroacetate (reagent) (287 mg, 1.36 mmol) was added to a suspension of 6-[6-[6-(5-methyl-2-oxo-4-imidazolidinyl)hexanamido]hexanamido]hexanoic acid (300 mg, 0.68 mmol) in dry pyridine (9 mL). The suspension was stirred for 1 h at room temperature when TLC showed the presence of starting material. Additional reagent (287 mg, 1.36 mmol) was added, and the suspension was stirred for 4 h at room temperature. Insoluble material was removed by filtration, and the pyridine was evaporated. The residue was triturated with three portions of ether and was dissolved in boiling 2-propanol. Insoluble material was removed by filtration, and the clear filtrate was concentrated to a volume of approximately 5 mL for crystallization. The crystals were collected, washed with ice-cold 2-propanol and ether, and dried: yield 176 mg (48%); mp 130–132 °C; $R_f^{II}$ 0.67. Anal. Calcd for $C_{26}H_{43}N_5O_7$: C, 58.08; H, 8.08; N, 13.03. Found: C, 57.71; H, 8.10; N, 12.76.

*N-[3-[(3-Aminopropyl)amino]propyl]succinamic Acid Sulfate (1:1) (VII).* 3,3′-Iminobis(propylamine) (V) (7.0 mL, 50 mmol) was dissolved in water (60 mL), and the pH of the solution was adjusted to 6.0 with 5 N sulfuric acid. The solution was cooled in an ice bath, and succinic anhydride (VI) (8.0 g, 80 mmol) in dioxane (120 mL) was added slowly with vigorous stirring, the pH being maintained at 6.0 by addition of 5 N NaOH. The mixture was stirred for 1 h at 0 °C and for 16 h at 4 °C, the inorganic salts that had precipitated were removed by filtration, and the filtrate was concentrated in vacuo to a small volume. The solution was acidified to pH 3.0 with 5 N sulfuric acid, and the volume was adjusted to approximately 75 mL with water. Methanol (~175 mL) was added, the heavy precipitate of inorganic salts was removed by filtration, and the clear filtrate was placed in a refrigerator where crystallization occurred. The crystals were collected, washed with water/methanol (15:35), and dried. For recrystallization, the crystals were dissolved in water (5 mL/g), methanol (13 mL/g) was added, and the solution was placed in a refrigerator to complete the crystallization: average yield 4.9 g (30%); mp 219–220 °C; $R_f^I$ 0.16, $R_f^{III}$ 0.31, $R_f^{IV}$ 0.20 (fluorescamine-, nitroprusside–acetaldehyde-, and chlorine-positive spot). The relative fluoresence with fluorescamine was 67% that of methyl 6-aminohexanoate hydrochloride. Anal. Calcd for $C_{10}H_{23}N_3O_7S \cdot {}^1/_2H_2O$: C, 35.49; H, 7.15; N, 12.42; S, 9.48. Found: C, 35.57; H, 7.20; N, 12.34; S, 9.80.

*Bis(dinitrophenyl) Derivative of VII.* The sulfate salt of VII (116 mg, 0.5 mmol) and sodium bicarbonate (225 mg, 3 mmol) were dissolved in water (1.5 mL). To this was added a solution of dinitrofluorobenzene (186 mg, 1 mmol) in ethanol (3 mL), and the mixture was stirred at room temperature in the dark for 16 h, the pH being kept at 9.0 by the addition of 1 N sodium hydroxide. The suspension was extracted with ether, the water phase was acidified with 2 N HCl to pH 2–3, and the solid was collected, washed with ice–water, and dried. The material was recrystallized from 2-propanol: mp 118–124 °C (unsharp); $R_f^I$ 0.67. Anal. Calcd for $C_{22}H_{25}N_7O_{11}$: C, 46.89; H, 4.47; N, 17.40. Found: C, 46.51; H, 4.59; N, 17.08.

*N-[3-[(3-Aminopropyl)carboxyamino]propyl]succinamic Acid N-tert-Butyl Ester (VIII).* The sulfate of VII (5.08 g, 15 mmol) was dissolved in water (10 mL), 1 N $NaHCO_3$ (50 mL) was added, and the pH was adjusted to 8.3 with 5 N NaOH. The solution was cooled in an ice bath, and citraconic anhydride (1.9 mL, 21.0 mmol) in dioxane (8 mL) was added dropwise with stirring, the pH being maintained at 8.2–8.4 by addition of 5 N NaOH. The mixture was stirred for 2.5 h at ice-bath temperature, then dioxane (40 mL) and di-*tert*-butyl dicarbonate (5.25 g, 24.0 mmol) in dioxane (15 mL) was added at pH 8.5, and the mixture was stirred at room temperature for 20 h. The mixture was cooled in an ice bath, and the precipitated inorganic salts were removed by filtration. The salts were washed with three portions of 50% aqueous dioxane, and filtrate and washings were pooled and evaporated to a small volume (~80 mL) in vacuo. The solution was cooled at 0 °C, acidified to pH 3.0 with saturated potassium hydrogen sulfate, and stirred for 2 h at room temperature. The solution was then extracted with four 50-mL portions of ethyl acetate, and the extracts were discarded. The aqueous phase was then extracted with four 100-mL portions of 1-butanol (previously equilibrated with water). The butanol extracts were washed with five 25-mL portions of saturated NaCl and evaporated to a small volume. Inorganic salt was removed by filtration, water was added, and the butanol was evaporated. The residue was diluted with 10% acetic acid (50 mL), and the solution was passed through a column of acetate-cycle IRA-400 (2.5 × 30 cm), which was eluted with 10% acetic acid. Chlorine-positive eluates were pooled, evaporated to dryness, and dried in vacuo over $P_2O_5$ and KOH pellets. The residue was crystallized from methanol–ethyl acetate: yield 1.93 g, (39%); mp 168–169 °C; $R_f^I$ 0.3, $R_f^{II}$ 0.1, $R_f^{III}$ 0.7, $R_f^{IV}$ 0.7 (fluorescamine positive, nitroprusside–acetaldehyde negative). The compound is soluble in water, methanol, ethanol, 1-butanol, and 2-propanol. It is sparingly soluble in ethyl acetate and acetonitrile. The relative fluorescence with fluorescamine was 278% that of methyl 6-aminohexanoate hydrochloride. A sample for analysis was dried in vacuo at 100 °C for 12 h. Anal. Calcd for $C_{15}H_{29}N_3O_5$: C, 54.36; H, 8.82; N, 12.68. Found: C, 54.50; H, 8.90; N, 12.67.

*N-[3-[Carboxy[3-[6-[6-(5-methyl-2-oxo-4-imidazolidinyl)hexanamido]hexanamido]propyl]amino]propyl]succinamic Acid N-tert-Butyl Ester (X). (a) In Water.* N-Hydroxysuccinimido 6-[6-(5-methyl-2-oxo-4-imidazolidinyl)hexanamido]hexanoate (IX) (Hofmann et al., 1982) (272 mg, 0.64 mmol) was added to a solution of VIII (232 mg, 0.70 mmol) and $NaHCO_3$ (130 mg, 1.54 mmol) in 2-propanol–water (1:1) (8 mL), and the clear solution was stirred 12 h at room temperature. Water (10 mL) was added, and the solution was applied to a column of the ion exchanger AG 1X2 (acetate cycle) (0.9 × 28 cm). The column was eluted with water until the eluates were fluorescamine negative and then with 5% acetic acid. Eluates containing the product (biotin test on TLC) were pooled and evaporated. For removal of acetic acid, the oily residue was evaporated with five 5-mL portions of water and two 5-mL portions of methanol and dried in vacuo: yield 417 mg (92%); $R_f^I$ 0.5 (single biotin-positive, fluorescamine- and nitroprusside–acetaldehyde-negative spot). A sample for analysis was dried in vacuo at 100 °C for 12 h. Anal. Calcd for $C_{31}H_{56}N_6O_8 \cdot {}^1/_2H_2O$: C, 57.29; H, 8.84; N, 12.92 (C/N ratio 5.16). Found: C, 57.28; H, 8.40; N, 13.19 (C.N ratio 5.07).

*(b) In DMF.* N-Hydroxysuccinamido 6-[6-(5-methyl-2-oxo-4-imidazolidinyl)hexanamido]hexanoate (IX) (136 mg, 0.32 mmol) was added to a solution of VIII (116 mg, 0.35 mmol) and TEA (0.1 mL, 0.70 mmol) in DMF (4 mL), and the solution was stirred at room temperature for 22 h. The

solvent was removed in vacuo, the residue was dissolved in water (10 mL), and the solution was applied to a column of AG 1×2 (acetate cycle) (0.9 × 12 cm), which was eluted with water until the effluents were fluorescamine negative. Elution with 5% acetic acid removed the desired product. Eluates containing the product (biotin test on TLC) were pooled, the solvent was removed, and the residue was evaporated with water (3 times) and with methanol (3 times) and was dried in vacuo: yield 222 mg (98%); $R_f^1$ 0.5.

*tert-Butyl [3-[6-[6-(5-Methyl-2-oxo-4-imidazolidinyl)-hexanamido]hexanamido]propyl][3-[3-[(succinimidooxy)-carbonyl]propionamido]propyl]carbamate (XI).* DCC (52 mg, 0.25 mmol) was added at room temperature to a solution of X (155 mg, 0.25 mmol) and *N*-hydroxysuccinimide (29 mg, 0.25 mmol) in DMF (2 mL), and the mixture was stirred for 20 h at room temperature. The solvent was removed in vacuo, and 2-propanol (2 mL) was added to the residue. The suspension was kept in a freezer for 48 h and was then centrifuged. The clear supernatant was collected, evaporated to dryness, and dried in vacuo: yield 170 mg of oily material; $R_f^{II}$ 0.64 [contaminated by a higher running material (acylurea) of $R_f^{II}$ 0.73 and unchanged acid of $R_f^{II}$ 0.31].

*N-[3-[[3-[6-[6-(5-Methyl-2-oxo-4-imidazolidinyl)hexanamido]hexanamido]propyl]amino]propyl]succinamic Acid.* A sample of X (217 mg) was dissolved in TFA (2 mL), and the solution was kept at room temperature for 30 min. The TFA was removed in vacuo, and TFA ions were exchanged for acetate ions on a column (1.5 × 20 cm) of the ion exchanger Amberlite IRA-400. Biotin-positive eluates were pooled and evaporated to dryness in vacuo: yield 166 mg (89%) of an oil; $R_f^{II}$ 0.25 (biotin-positive, fluorescamine-negative spot; forms a dark blue color with the sodium nitroprussive–acetaldehyde reagent). A sample for analysis was dried in vacuo at room temperature. Anal. Calcd for $C_{26}H_{48}N_6O_6 \cdot H_2O$: C, 55.89; H, 9.02; N, 15.04. Found: C, 55.83; H, 9.15; N, 15.73.

*Preparation of Insulin Derivatives.* The insulin derivatives I–IV were prepared as follows. 4-Methylmorpholine (40 μL, 400 μmol) was added at room temperature to a solution of $Boc_2$-insulin (ox) (62 mg, 10 μmol) and the appropriate active ester (80 μmol) in $Me_2SO$ (4 mL), and the solution was stirred for 20 h at room temperature. The products were isolated as described previously (Hofmann et al., 1982). The average yield ranged from 50 to 60 mg. The HPLC patterns are shown on Figure 6. Compounds whose HPLC pattern indicated the presence of major impurities were purified on diethylaminoethylcellulose DE-52 (Hofmann et al., 1977). The ratios of 6-aminohexanoic acid to lysine in acid hydrolysates were (compound I) 0.88, (compound II) 1.87, (compound III) 2.06, and (compound IV) 0.95.

## Results and Discussion

The dethiobiotin used in this and a previous study (Hofmann et al., 1982) was prepared from *d*-biotin with Raney nickel (Melville et al., 1943), and its stereochemical homogeneity has, to this date, not been ascertained. The configuration of synthetic *dl*-dethiobiotin has been determined by X-ray crystallography (Chen et al., 1976) and is shown in Figure 2. The substituents on the imidazolidone ring are cis oriented as they are in *d*-biotin (Traub, 1956; DeTitta, 1976). In order to gain information pertaining to the stereochemical homogeneity of the dethiobiotin used in this investigation, we have measured its 600-MHz proton spectrum, which is shown in Figure 3. The spectrum demonstrates clearly that this material is a 6:1 mixture of two stereoisomers. The assignments of the peaks are given in Table I. These assignments were confirmed for

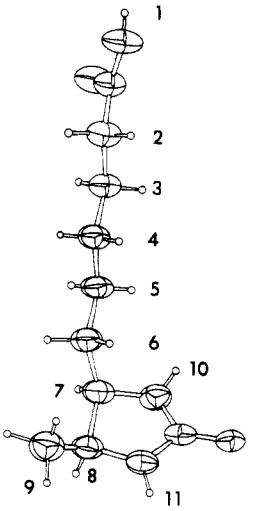


FIGURE 2: Crystal structure of *d*-dethiobiotin (Chen et al., 1976).

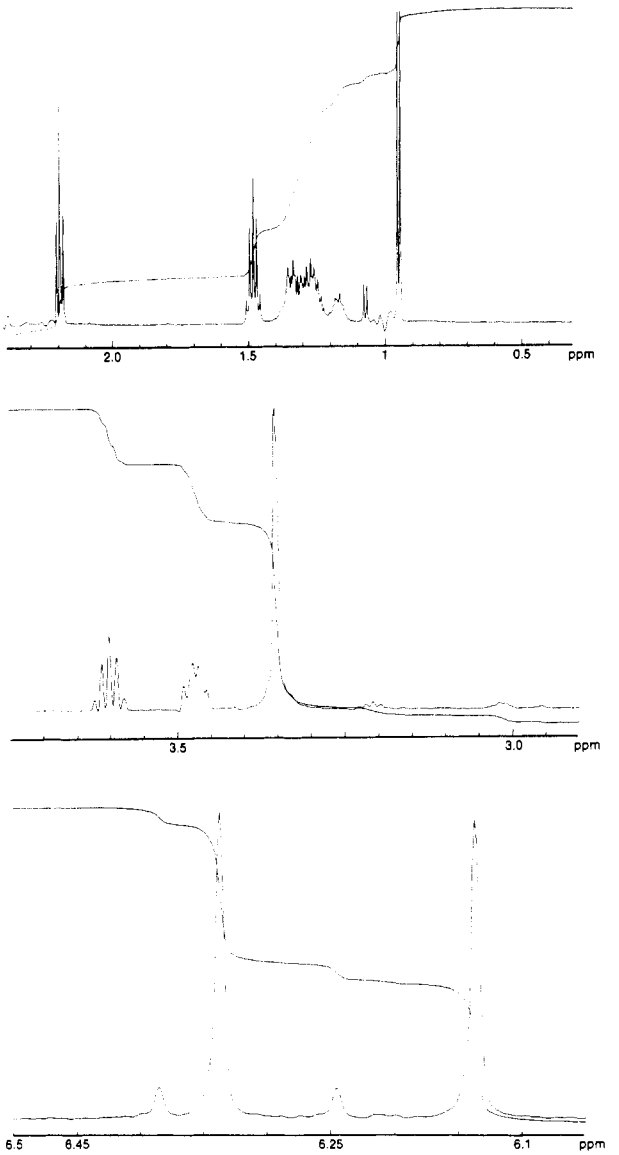


FIGURE 3: NMR spectrum of dethiobiotin from biotin by Raney nickel desulfuration.

both isomers by decoupling and by the observation of nuclear Overhauser effects.

The most likely explanation of the presence of the two isomers is that during the Raney nickel desulfurization process partial inversion of the configuration at either C(7) or C(8)

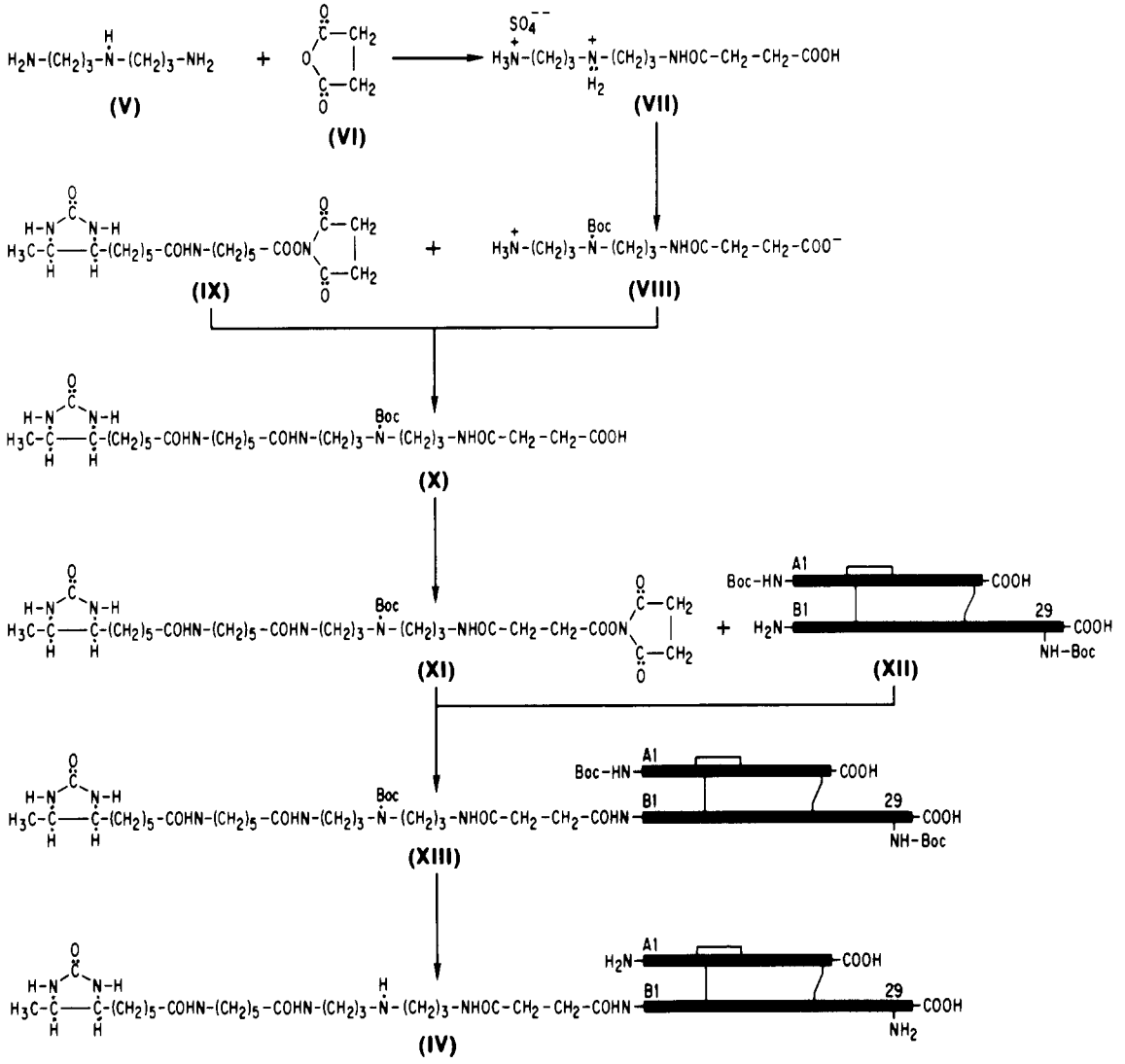


FIGURE 4: Synthetic route to compound IV.

Table I: NMR Analysis of Dethiobiotin Derived from *d*-Biotin[a]

| protons | δ (major) | multiplicity | δ (minor) | multiplicity |
|---|---|---|---|---|
| 1 | 12.01 | s | 12.01 | s |
| 2 | 1.70 | t | 1.70 | t |
| 3 | 1.48 | quintet | 1.48 | quintet |
| 4, 5, 6 | 1.1–1.4 | not resolved | 1.1–1.4 | not resolved |
| 7 | 3.48 | dt ($J_{78}$ ~ 7 Hz) | 3.01 | dt ($J_{78}$ ~ 6 Hz) |
| 8 | 3.60 | dq | 3.21 | dq |
| 9 | 0.95 | d | 1.07 | d |
| 10 | 6.34 | s | 6.39 | s |
| 11 | 6.14 | s | 6.25 | s |

[a] Decouplings for 2{3}, 6{7}, 8{7}, and 8{9} of both isomers. Overhauser effects for 7{10} and 11{8} of both isomers. δ, chemical shift in parts per million; s, singlet; d, doublet; t, triplet; dq, double quartet; dt, double triplet.

takes place. The cis configuration of C(6) and C(9) relative to the five-membered ureido ring occurring in biotin would thereby be changed to the trans configuration. NMR does not distinguish whether inversion occurs at C(7) or C(8), and it is not excluded that partial inversion occurs at both sites. The assignment of the major and minor isomers may be made by comparison with the shifts in the *dl* and meso cyclic carbonates of 2,3,-dihydroxybutane (Anet, 1962). In the *dl* isomer, the C(2) and C(3) protons yield a signal at higher field and the methyl protons a signal at lower field than do the corresponding protons in the meso isomer. Thus, the major isomer of dethiobiotin is the cis compound corresponding to the stereochemistry in biotin, and the minor isomer is the trans isomer. Consequently, the dethiobiotin derivatives described in this and a previous paper (Hofmann et al., 1982) are contaminated with the trans isomer. The presence of *trans-dl*-dethiobiotin in dethiobiotin obtained from *d*-biotin with Raney nickel is of considerable interest in connection with our avidin binding studies. As far as we could ascertain, pure *trans-dl*-dethiobiotin has not been described in the literature, and hence, its affinity for avidins is unknown. The synthesis of I requires little comment since it follows the scheme described previously for the preparation of similar compounds (Hofmann et al., 1982). The OSu ester of biotin served to acylate methyl 6-aminohexanoate, and the ensuing methyl 6-(biotinylamido)hexanoate was saponified. The resulting acid was converted to the OSu ester, which was used to acylate $Boc_2$-insulin. Removal of the protecting groups from the reaction product with TFA afforded I. An identical approach with successive active ester couplings was employed to prepare compounds II and III.

The synthetic route to compound IV is illustrated in Figure 4. 3,3′-Iminobis(propylamine) (V) was succinoylated with 1.6 equiv of succinic anhydride (VI) in dioxane–water at pH 6. TLC of the reaction mixture showed the presence of a small proportion of starting material (V), the desired product (VII), and higher running materials (di- and trisuccinoylated products?); these were not investigated further. *N*-[3-[(3-

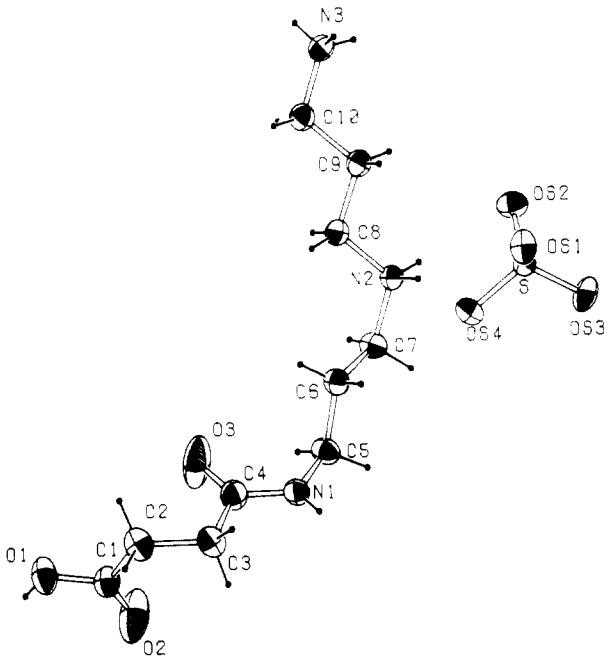


FIGURE 5: Crystal structure of *N*-[3-[(3-aminopropyl)amino]-propyl]succinamic acid sulfate (1:1) (Suh et al., 1984).

Aminopropyl)amino]propyl]succinamic acid (VII) is readily separated from the other products by crystallization of the sulfate salts from methanol–water. This separation is facilitated when only small proportions of triamine are present and it is for this reason that 1.6 equiv of succinic anhydride is employed. The crystal structure of compound VII as determined by X-ray diffraction (Suh et al., 1984) is shown in Figure 5. This structure analysis proves unequivocally the correctness of the assigned structure.

The approach to the synthesis of VIII is based on the differential stability toward acid of citraconyl and *tert*-butoxycarbonyl amino-protecting groups. Citraconic anhydride has been used for the transient protection of amino groups in proteins (Dixon & Perham, 1968); it reacts with amino groups to form citraconyl derivatives that are stable under alkaline conditions (pH 8–10). When subjected to weakly acidic conditions (pH 2–3), such derivatives are readily cleaved with regeneration of free amino groups. *tert*-Butoxycarbonyl protecting groups are stable to weak acids.

With TLC as a guide, the reaction of VII with citraconic anhydride and di-*tert*-butyl dicarbonate was explored on a microscale. When VII ($R_f^{IV}$ 0.20) was reacted with 1.2–1.3 equiv of citraconic anhydride, a material ($R_f^{IV}$ 0.38) was generated that produced a strong blue color with the nitroprusside–acetaldehyde reagent and developed weak fluorescence with fluorescamine. It was concluded that this represented material that was citraconylated on the primary amino group contaminated with unacylated product. Reaction with di-*tert*-butyl dicarbonate at pH 9.5 converted this material into a compound ($R_f^{IV}$ 0.59) that developed neither fluorescence nor a blue color with the appropriate reagent and thus appeared to be citraconylated mainly on the primary and *tert*-butoxycarbonylated on the secondary amino group. At pH 3.0, this material is converted to VIII ($R_f^{IV}$ 0.70). On exposure to 5 equiv of citraconic anhydride, compound VII was transformed into a material ($R_f^{IV}$ 0.30) in which both the primary as well as the secondary amino groups appear to be citraconylated. This compound failed to react with di-*tert*-butyl dicarbonate and on exposure to acid regenerated VII. These experiments showed, as was to be expected, that both the primary as well as the secondary amino groups of VII are susceptible to citraconylation. Since the p*K*'s of aliphatic primary and secondary amines are very similar, the small difference in reactivity of the primary and the secondary amino group of VII appears to be the result of a steric effect.

On a preparative scale, VII was reacted with 1.4 equiv of citraconic anhydride at pH 8.3 to protect the primary amino group, and partially protected material was acylated in situ at pH 8.5 with di-*tert*-butyl dicarbonate. The reaction mixture was acidified with potassium hydrogen sulfate to pH 3.0, and compound VIII was isolated from the reaction mixture in crystalline form.

The final steps in the synthesis of IV involved chain elongation and coupling to $Boc_2$-insulin. Accordingly, VIII was acylated with *N*-hydroxysuccinimido 6-[6-(5-methyl-2-oxo-4-imidazolidinyl)hexanamido]hexanoate (IX) to form X, which was converted to the OSu ester. This ester served to acylate $Boc_2$-insulin.

We resorted to the ester exchange method of Sakakibara & Inukai (1965) for the preparation of OSu esters. The results were satisfactory, however; the conversion of 6-[6-(biotinylamido)hexanamido]hexanoic acid to its OSu ester afforded a low yield presumably because of the poor solubility of this acid in pyridine. In our hands, the ester exchange method was not applicable to molecules containing *tert*-butoxycarbonyl groups. Thus, the conversion of the acid (X) to its OSu ester (XI) had to be performed by the DCC procedure, affording a product that was contaminated (TLC) with a second material, most likely the *N*-acylurea. Compounds X and XI as well as the four insulin derivatives (Figure 1) were not obtained in crystalline form, and some comments are in order pertaining to their purification and structure proof. For the isolation of compound X, we resorted to ion-exchange chromatography on the resin AG 1X2, which we had used extensively in the purification of analogues of ribonuclease A S-peptide derivatives (Hofmann et al., 1970). The reaction mixture resulting from the acylation of VIII by XI, which contained an excess of the amino component (VIII), was diluted with water and was applied to a column of AG 1X2 in the acetate cycle. Elution of the column with water removed the amino component. Elution with 5% acetic acid removed the desired product as a chromatographically (TLC) homogeneous oil. The elemental analysis of the product was in good agreement with theory. A sample of X subjected to TFA for removal of the *tert*-butoxycarbonyl group eluted with water from the AG 1X2 column and was cleanly separated from the starting material (experiment not shown). The elemental analysis of this oily product was in agreement with theory.

As concerns the acylation of $Boc_2$-insulin by the various active esters, we have observed the following: under the conditions used, i.e., $Me_2SO$ as the solvent and 4-methylmorpholine as the base),[2] the acylations work well with the *N*-hydroxysuccinimido esters of biotin, 6-(biotinylamido)-hexanoic acid, 6-[6-(5-methyl-2-oxo-4-imidazolidinyl)hexanamido]hexanoic acid, 6-[[6-(biotinylamido)hexanoyl]-amido]hexanoic acid, and 6-[6-[6-(5-methyl-2-oxo-4-imidazolidinyl]hexanamido]hexanamido]hexanoate. The products contained only minor impurities on HPLC (Figure 6). As concerns acylation of $Boc_2$-insulin with *N*-[3-[carboxy[3-[6-[6-(5-methyl-2-oxo-4-imidazolidinyl)hexanamido]-hexanamido]propyl]amino]propyl]succinamic acid *N*-*tert*-butyl ester (XI), the yield of the product was low and extensive purification was necessary to obtain a homogeneous product.

[2] In more recent experiments, we have used imidazole as the base with excellent results.

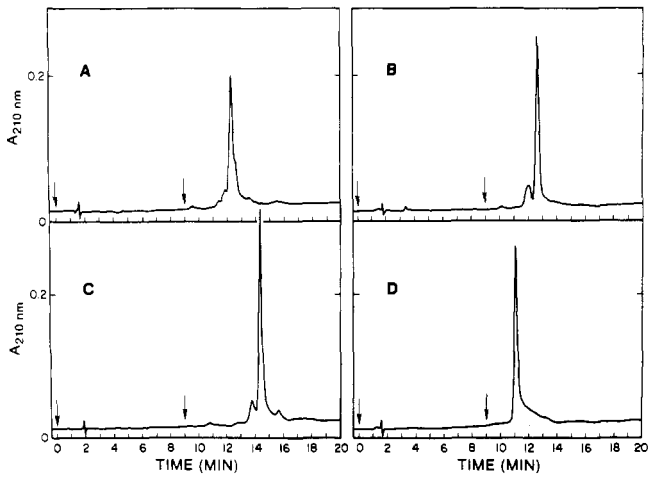


FIGURE 6: HPLC of bovine insulin derivatives: (panel A) $N^{\alpha,B^1}$-[6-(biotinylamido)hexanoyl]insulin; (panel B) $N^{\alpha,B^1}$-[6-[6-(biotinylamido)hexanamido]hexanoyl]insulin; (panel C) $N^{\alpha,B^1}$-[6-[6-[6-(5-methyl-2-oxo-4-imidazolidinyl)hexanamido]hexanamido]hexanoyl]-insulin; (panel D) $N^{\alpha,B^1}$-[*N*-[3-[[3-[6-[6-(5-methyl-2-oxo-4-imidazolidinyl)hexanamido]hexanamido]propyl]amino]propyl]-succinamoyl]insulin. Left arrow shows site of injection; right arrow shows position of bovine insulin. A Bondapak $C_{18}$ column with a solvent system of (pump A) 0.1% $H_3PO_4$ and (pump B) 50% acetonitrile in 0.1% $H_3PO_4$ was employed. The gradient was from 50–90% pump B over 20 min with a pumping speed of 2 mL/min. Samples of 10–20 μg were applied.

In view of the fact that the insulin derivatives contained 6-aminohexanoic acid, we have employed the ratio of this acid to lysine in acid hydrolysates as an additional indicator of homogeneity (Hofmann et al., 1982).

## Acknowledgments

We thank Dr. Kurt L. Loening, Nomenclature Director, Chemical Abstracts Service, for his help concerning nomenclature.

## References

Anet, F. A. L. (1962) *J. Am. Chem. Soc. 84*, 747–752.

Chen, C.-S., Parthasarathy, R., & DeTitta, G. (1976) *J. Am. Chem. Soc. 98*, 4983–4990.

DeTitta, G., Edmonds, J. W., Stallings, W., & Donohue, J. (1976) *J. Am. Chem. Soc. 98*, 1920–1926.

Dixon, H. B. F., & Perham, R. N. (1968) *Biochem. J. 109*, 312–314.

Feigl, F., Anger, V., & Oesper, R. E., (1966) in *Spot Tests in Organic Analysis*, p 251, Elsevier, Amsterdam, London, and New York.

Finn, F. M., Titus, G., Montibeller, J. A., & Hofmann, K. (1980) *J. Biol. Chem. 255*, 5742–5746.

Green, N. M. (1975) *Adv. Protein Chem. 29*, 85–133.

Hofmann, K., Visser, J. P., & Finn, F. M. (1970) *J. Am. Chem. Soc. 92*, 2900–2909.

Hofmann, K., Finn, F. M., Friesen, H.-J., Diaconescu, C., & Zahn, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2697–2700.

Hofmann, K. Titus, G., Montibeller, J. A., & Finn, F. M. (1982) *Biochemistry 21*, 978–984.

Jasiewicz, M. L., Schoenberg, D. R., & Mueller, G. C. (1976) *Exp. Cell. Res. 100*, 213–217.

May, J. M., Williams, R. H., & de Haën, C. (1978) *J. Biol. Chem. 253*, 686–690.

McCormick, D. B., & Roth, J. A. (1970) *Anal. Biochem. 34*, 226–236.

Sakakibara, S., & Inukai, N. (1965) *Bull. Chem. Soc. Jpn. 38*, 1979–1984.

Suh, J. S., Xu, Z. B., Hofmann, K., Yoo, C. S., & Sax, M. (1984) *Acta Crystallogr., Sect. C: Cryst. Struct. Commun. C40*, 131–134.

Traub, W. (1956) *Nature (London) 178*, 649–650.

Udenfriend, S., Stein, S., Böhlen, P., Dairman, W., Leimgruber, W., & Weigele, M. (1972) *Science (Washington, D.C.) 178*, 871–872.