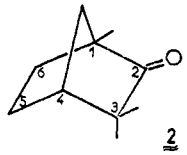
The peak heights of the A, B, F, and G lines are larger in the presence of (−)-fenchone than in the presence of (+)-fenchone (reproducibility in ten experiments is better than 5%). It can be concluded that (+)-(1*S*,4*R*)-fenchone (**2**) is preferentially included in β-cyclodextrin in



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50% water–50% Me_2SO at 15 °C. We have checked this result by a precipitation method; when racemic fenchone is complexed with β-cyclodextrin, the filtrate is enriched in (−)-fenchone. If the concentration of the inclusion complex in solution is neglected, the optical rotatory power of the filtrate gives the optical purity P of the uncomplexed fenchone: $P = 0.054$.

The (+)-fenchone thus is selectively included in the precipitate, in agreement with ESR results. The precipitation experiment gives $K_d/K_1 = 1.3 \pm 0.2$ for fenchone–cyclodextrin association constants at 20 °C in solution containing 80% water–20% Me_2SO.

Conclusion

This is the first direct spectroscopic evidence of a difference in the association of a cyclodextrin with the two enantiomers of a chiral molecule: the specific association of a chiral diamagnetic molecule can thus be observed by studying the displacement of the association equilibrium of a nitroxide biradical with cyclodextrin. This suggest that ESR can similarly be used to study chiral recognition by other receptors.[15,16]

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Avidin–Biotin Interaction. Synthesis, Oxidation, and Spectroscopic Properties of Linked Models

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Abstract: In order to help uncover the basis of the strong biotin–avidin binding and to identify any contributory biotin–tryptophan interaction, we have synthesized model compounds, Biot-C_3-Ind (**4a**) and Biot-C_4-Ind (**4b**), having trimethylene and tetramethylene chains between the biotin ring system and indole, by starting with (+)-biotin and (+)-homobiotin. The UV spectroscopic effect of the proximate biotin moiety in Biot-C_3-Ind compared with 3-propylindole, both in cyclohexane (with 1% ethanol), is similar to the effect of biotin bound to avidin. Interaction of the biotin ring system and indole was also evidenced by a red shift in λ_{em} in comparing the fluorescence emission of 3-propylindole with that of Biot-C_3-Ind or Biot-C_4-Ind in cyclohexane (1 or 0.1% ethanol), but the proximate biotin ring system caused no quenching of fluorescence. We have found no evidence of strong interaction between the biotin ring system and the indole of tryptophan such as would help account for the magnitude of the avidin–biotin association constant. The diastereomeric sulfoxides of Biot-C_3-Ind were prepared, and the stereochemistry of each was established. ^1H NMR spectral comparisons with the methyl esters of (+)-biotin and its two sulfoxides confirmed the stereochemical assignments, as did X-ray single-crystal analysis (the sequel). With the ^1H NMR chemical shift assignments and the X-ray-confirmed stereochemistry, we have developed correlations with ^{13}C NMR chemical shifts that should prove useful in assigning stereochemistry to other asymmetric sulfoxides. A quantitative study of the *N*-bromosuccinimide oxidation of model compounds and of avidin, biotin, and the avidin–biotin complex established the relative rates of oxidation and the stereochemistry of oxidation of the biotin moicty in various environments. The protection of the tryptophans in the avidin–biotin complex against NBS oxidation is due only partially to consumption of the oxidizing agent by the bound biotin. Biotin in the avidin–biotin complex is oxidized by aqueous NBS, yet it is more protected than free biotin in aqueous solution. Oxidation of biotin in the avidin–biotin complex yields predominantly the α-sulfoxide, indicating steric limitation of the approach of oxidant to bound biotin.

The avidin–biotin complex,[1] which is one of the tightest biological complexes known, is characterized by a dissociation constant, K_D, of approximately 10^{-15} M.[2] The high affinity of avidin for biotin explains the toxic effect of ingested uncooked egg white in animals,[3] since it renders biotin (vitamin H), an essential growth factor,[4,5] unavailable to the test animals. Avidin binds not only biotin and its derivatives and analogues[1] but also biotin conjugated to protein, which makes avidin a very useful tool for the study of biotin-dependent enzymes.[6-8] Biotin- or biocytin-bound solid supports serve as effective affinity chromatographic columns for the purification of avidin,[9,10] and avidin–Sepharose columns can be used for the purification of biotinyl enzymes[11-13] or for the separation of subunits of biotinyl enzymes.[14] A general application of avidin–biotin complexation has been described by Hofmann in which biotin is used as an anchor to bind biological ligands

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to an avidin–Sepharose solid matrix, and the solid matrix can then serve as an affinity column for separation of the receptor of the ligand.[15,16] Cells labeled with biotin can be retrieved selectively from complex media.[17] Biological membrane proteins labeled with biotin can be visualized with the electron microscope by using avidin–ferritin conjugate as a staining reagent.[18,19] Biotin–avidin–ferritin has been used similarly in the localization of receptors for lectin and antibody on erythrocytes[20] and of the position of 4s RNA genes in HeLa cell mitochondrial DNA.[21] Several methods of electron microscopic gene mapping and gene enrichment involving avidin–biotin–cytochrome and avidin–biotin–ferritin have been reported from Davidson's laboratory.[22-25] Fluorescence microscopy can be used to localize biotin-labeled biomolecules when fluorescence-modified avidin is used.[26]

Despite these many valuable applications of the strong avidin–biotin complexation, the nature of the binding of avidin and biotin has not been established, although the effects of various chemical modifications of the biotin molecule on avidin binding have been known for some time.[1,27,28] Each of the four subunits of the glycoprotein avidin binds one biotin molecule, which enhances the association of the subunits. According to Green, the results of ultraviolet absorption studies "combined with those of chemical modification studies suggest that three or four tryptophan residues in each subunit interact directly with biotin".[1] This conclusion was based upon the ultraviolet difference spectra observed for avidin vs. the complexes of avidin with biotin, its derivatives, and analogues.[29-31] That the spectroscopic changes are not the result of conformational changes induced by biotin attachment distant from the tryptophans in avidin is suggested, in the solid, by the isomorphous nature of the crystals of avidin and of the avidin–biotin complex[32] and, in solution, by their similar sedimentation and diffusion coefficients and by the fact that a variety of chemical modification reactions of avidin, including amino-group acylation and carboxyl-group esterification, does not affect its binding of biotin.[33,34] The conclusion concerning the proximity of biotin to tryptophans was also based on the oxidation of tryptophans in avidin by oxidizing agents such as *N*-bromosuccinimide, sodium metaperiodate, and ozone, and their protection from oxidation by the same reagents in the avidin–biotin complex.[29,30,35-37]

In order to help uncover the basis of the strong biotin–avidin binding and to identify any contributory biotin–tryptophan interaction, we synthesized model compounds that were designed to answer the questions: (1) What perturbations are observed, both spectroscopically and chemically, when the biotin ring system and the tryptophan ring system, namely, indole, are brought into proximity? (2) What are the indications of strong interaction between the biotin ring system and indole? In the sequel[38] is described the single-crystal X-ray examination of the same questions.

In the past, we have used polymethylene bridges, and in particular the trimethylene bridge, $-(CH_2)_3-$, as synthetic spacers to determine the intramolecular interactions between nucleic acid bases, between the component heterocyclic rings of certain coenzymes, and between indole and nucleic acid bases.[39] For this study, we have synthesized compounds in which the biotin or homobiotin side chain is utilized as the precursor for a tri- or tetramethylene chain that joins the biotin ring system to indole at the 3 position. These models, in which the stereochemistry of *d*- or (+)-biotin has been preserved, were examined, together with the corresponding half molecules, by ultraviolet absorption, fluorescence emission, and NMR spectroscopy. The diastereomeric sulfoxides of the model compounds were prepared, and the stereochemistry of each was established. Proton magnetic resonance spectral comparisons with the methyl esters of (+)-biotin and its two sulfoxides confirmed the stereochemical assignments, as did X-ray single-crystal analysis.[38] The methyl esters of (+)-biotin and its two sulfoxides were examined by ^{13}C magnetic resonance spectroscopy, and correlations were developed that should prove useful in assigning stereochemistry to other asymmetric sulfoxides. A quantitative study of the *N*-bromosuccinimide oxidation of model compounds and of avidin, biotin, and the avidin–biotin complex established the relative rates of oxidation and the stereochemistry of oxidation of the biotin moiety in various environments.

Experimental Section

General. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Microanalyses were performed by Mr. J. Nemeth and his staff, who also weighed samples for quantitative electronic absorption studies. Infrared spectra were recorded on a Perkin-Elmer Model 337 spectrophotometer. Low-resolution mass spectra were run on a Varian-MAT CH-5 spectrometer. Field desorption and high-resolution mass spectra were obtained on a Varian MAT 731 spectrometer, coupled with a 620i computer and STATOS recorder. ^1H NMR spectra were recorded on a Varian HR-220 spectrophotometer, and ^{13}C NMR spectra were obtained on a Varian XL-100 spectrophotometer, in both cases with tetramethylsilane (Me_4Si) as an internal standard. For the detection of biotin compounds on thin layer chromatographic plates, 4-dimethylaminocinnamaldehyde in 1% H_2SO_4 in ethanol[40,41] was used as a spray reagent.

Electronic Absorption. Electronic absorption spectra were recorded on a Beckman Acta MVI spectrophotometer. For quantitative measurements, a specified amount of material weighed to the nearest 0.001 mg was dissolved in 10% ethanol–90% cyclohexane. Aliquots of these solutions were then diluted with cyclohexane to give 1% ethanol–99% cyclohexane or 0.1% ethanol–99.9% cyclohexane solutions. The final solutions had a concentration of about 7×10^{-5} or 7×10^{-6} M. Spectrograde cyclohexane was employed. Difference spectra were acquired on the same spectrophotometer operating in the difference mode.

Fluorescence Emission. Fluorescence emission spectra were acquired for 7×10^{-6} M solutions on a Spex Fluorolog spectrofluorometer and were corrected for monochromator efficiency and photomultiplier response. For determinations of quantum yields, the solutions were saturated with argon and the fluorometer was purged with nitrogen.

Materials. (+)-Biotin (**1a**, $n = 4$) and avidin were purchased from Sigma Chemical Co. (+)-Homobiotin (**1b**, $n = 5$) was a gift from Hoffmann-La Roche, Inc., Nutley, N.J. (+)-[*carbonyl*-^{14}C]Biotin (57 mCi/mmol) was from Amersham/Searle Corp. 3-Propylindole (Ind^3-C_3 or, simply, $Ind-C_3$),[42] (+)-biotin methyl ester (**2a**, $n = 4$),[43] and (+)-biotinol (**5a**, $n = 4$)[44] were prepared as described previously.

[3a*S*-(3aα,4β,6aα)]4-[3-(Indol-3-yl)propyl]hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole (Biot-C_3-Ind) (4a). To a stirred solution of 500 mg (1.94 mmol) of (+)-biotin methyl ester (**2a**) in 50 mL of dry methylene chloride (freshly distilled over P_2O_5) at −70 °C was added dropwise, with a syringe, 5 mL of 1 M diisobutylaluminum hydride in hexane (Aldrich) under dry nitrogen over a period of 30 min. The reagent was decomposed with methanol (5 mL) and the solution was evaporated to a gel under reduced pressure at 20 °C. The gel was dissolved in 50 mL of glacial acetic acid, phenylhydrazine hydrochloride (285 mg, 1.95 mmol) and boron trifluoride etherate (0.23 mL, 1.94 mmol) were added, and the mixture was heated at 90 °C under argon for 1.5 h. The reaction mixture was evaporated to dryness under reduced pressure, the residue was taken up in water, and the mixture was extracted with diethyl ether. The combined extracts were washed with 5% aqueous $NaHCO_3$, followed by water, and were dried over sodium sulfate. Evaporation of the solvent gave a brown gum which was chromatographed on silica gel (30 g, slurry packed in chloroform). The column was first eluted with chloroform (500 mL) and then with 2% methanol–98% chloroform (500 mL). Elution with 5% chloroform–95% methanol afforded the desired compound (252 mg). Two recrystallizations from aqueous ethanol yielded 175 mg (30% yield overall) of microcrystalline, colorless solid: mp 165.5–166.5 °C; MS (70 eV) *m/e* (rel intensity) 301 (M^+) (34.65), 130 ($IndCH_2^+$) (100); indole portion NMR (25% CD_3OD–75%$CDCl_3$) δ 1.78 (m, 2, $IndCH_2CH_2$), 2.82 (t, 2, $IndCH_2$), 7.00 (s, 1, 2′-H), 7.10 (2 m, 2, 5′- and 6′-H), 7.37 (d, 1, $J = 7.4$ Hz,

4′-H), 7.58 (d, 1, J = 7.7 Hz, 7′-H). Anal. Calcd for $C_{16}H_{19}N_3OS$: C, 63.79; H, 6.31; N, 13.95; S, 10.63. Found: C, 64.00; H, 6.34; N, 13.91; S, 10.70.

Methyl Ester of (+)-Homobiotin (2b). A solution of 200 mg (0.74 mmol) of (+)-homobiotin (**1b**),[44] the homologue of natural (+)-biotin, in 20 mL of anhydrous methanol containing 1 g of HCl was heated at reflux for 2.5 h. The solvent was evaporated. The residue was taken up in water and neutralized with sodium bicarbonate, and the mixture was extracted with ethyl acetate. The combined extracts were dried over sodium sulfate. The solvent was evaporated, leaving 184 mg (88%) of colorless solid which was used for the preparation of compound **4b** without further purification: mp 144–146 °C; MS (70 eV) m/e 272 (M^+).

[3a*S*-(3aα,4β,6aα)]-4-[4-(Indol-3-yl)butyl]hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole (Biot-C$_4$-Ind) (4b). Compound **4b** was prepared from **2b** in 30% yield using the same procedure as for **4a**: mp 141–143 °C; MS (70 eV) m/e (rel intensity) 315 (M^+) (30.19), 130 ($IndCH_2^+$) (100); NMR (20% CD_3OD–80% $CDCl_3$) δ 1.53, 1.74 (multiplets, 6, Biot$CH_2CH_2CH_2$), 2.71 (d, 1, J = 13.0 Hz, 6β-H) 2.79 (t, 2, $IndCH_2$), 2.90 (dd, 1, J = 13.0, 4.8 Hz, 6α-H), 3.16 (m, 1, 4-H), 4.25 (dd, 1, 3a-H), 4.46 (m, 1, 6a-H), 7.00 (s, 1, 2′-H), 7.11 (2 m, 2, 5′- and 6′-H), 7.36 (d, 1, J = 6.9 Hz, 4′-H), 7.58 (d, 1, J = 7.3 Hz, 7′-H). Anal. Calcd for $C_{17}H_{21}N_3OS$: C, 64.76; H, 6.67; N, 13.33; S, 10.16. Found: C, 64.47; H, 6.69; N, 13.39; S, 10.25.

[3a*S*-(3aα,4β,6aα)]-4-Pentylhexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole (Biot-C$_5$) (6a). To a suspension of 100 mg (0.43 mmol) of (+)-biotinol (**5a**)[44,45] in 3 mL of pyridine cooled in an ice–water bath was added *p*-toluenesulfonyl chloride (92 mg, 0.48 mmol) in portions. The solution was stirred at 25 °C for 4 h and kept at 0 °C overnight. The reaction solution was poured into 10 mL of ice–water with vigorous stirring. The white precipitate was collected, washed with water, and dried in vacuo (0.1 Torr) at 25 °C for 24 h to give 109 mg of solid biotinol tosylate, mp 128–132 °C. This compound (50 mg) in 5 mL of dry tetrahydrofuran was added to a solution of 25 mg of lithium aluminum hydride in 5 mL of dry ethyl ether. The solution was refluxed for 2 h. The reagent was decomposed with ethyl acetate, 10 mL of 1 N HCl was added, and the solution was extracted with ethyl ether. The combined extracts were washed with 5% aqueous $NaHCO_3$, then water, and dried over sodium sulfate. Evaporation of the solvent gave 33 mg of colorless solid (71% from biotinol). Two recrystallizations from ethanol afforded an analytically pure sample (15 mg, 35%): mp 184.5–186 °C; MS (70 eV) m/e 214 (M^+); NMR (20% CD_3OD–80% $CDCl_3$) δ 0.88 (t, 3, CH_3), 1.32 (multiplets, 6 $CH_2CH_2CH_2CH_3$), 1.61 (m, 2, BiotCH_2), 2.71 (dd, 1, J = 12.8 and <1 Hz, 6β-H), 2.93 (dd, 1, J = 12.8, 4.4 Hz, 6α-H), 3.18 (m, 1, 4-H), 4.28 (dd, 1, J = 8.9, 4.0 Hz, 3a-H), 4.50 (ddd, 1, J = 8.9, 4.4, 1.0 Hz, 6a-H). Anal. Calcd for $C_{10}H_{18}N_2OS$: C, 56.07; H, 8.41; N, 13.08; S, 14.95. Found: C, 56.00; H, 8.58; N, 12.85; S, 15.04.

Biotin (+)-sulfoxide (7) and its methyl ester (9) were prepared from (+)-biotin (**1a**) and its methyl ester (**2a**), respectively, essentially as described.[46]

Biotin (−)-Sulfoxide (8). To a solution of 100 mg (0.41 mmol) of (+)-biotin in 10 mL of 0.1 M phosphate buffer, pH 6.8, at 0 °C was added 80 mg (0.45 mmol) of *N*-bromosuccinimide in portions, and the solution was stirred at 0 °C for 1 h and then evaporated to dryness. Two recrystallizations from water yielded 16 mg of polymorphic plates, mp 239–242 °C (lit.[46] 238–241 °C).

Methyl Ester (10) of Biotin (−)-Sulfoxide. To a solution of 426 mg (1.65 mmol) of the methyl ester (**2a**) of (+)-biotin in 50 mL of anhydrous methanol at 0 °C was added 324 mg (1.82 mmol) of *N*-bromosuccinimide in portions. The solution was kept at 0 °C for 1 h and was allowed to stand at room temperature overnight. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel (40 g, packed in chloroform). Elution with 20% methanol–80% chloroform afforded 376 mg (83%) of colorless solid. Recrystallization from ethanol yielded analytically pure, colorless, long needles, mp 197–199 °C (lit.[46] 196–198 °C).

[3a*S*-(3aα,4β,6aα)]-4-[3-(Indol-3-yl)propyl]hexahydro-2,5α-dioxo-1*H*-thieno[3,4-*d*]imidazole (11). Compound **4a** (50 mg, 0.166 mmol) was treated with 0.17 mmol of hydrogen peroxide in 4 mL of glacial acetic acid at room temperature for 4 h. Thin layer chromatography indicated the formation of two products. The solvent was evaporated and the residue was chromatographed on silica gel (50 g, packed in chloroform). Elution with 10% methanol–90% chloroform afforded the first product (40 mg). Two recrystallizations from methanol yielded colorless crystals: mp 240–240.5 °C; UV (EtOH) λ_{max} 291 nm (ϵ 4670), 282 (5460); 275 sh; λ_{min} 245 (1240); MS (10 eV) m/e (rel intensity) 317 (M^+) (4.88), 300 (M^+ − OH) (23.04), 183 (100), 130 ($IndCH_2^+$) (34.27); NMR (25% CD_3OD–75% $CDCl_3$) δ 1.90 (multiplets, 4, Biot(SO)CH_2CH_2), 2.85 (t, 2, $IndCH_2$), 7.01 (s, 1, 2′-H), 7.07 and 7.16 (2 m, 2, 5′- and 6′-H), 7.37 (d, 1, J = 7.7 Hz, 4′-H), 7.57 (d, 1, J = 7.7 Hz, 7′-H). See text for assignments in the biotin sulfoxide moiety. Anal. Calcd for $C_{16}H_{19}N_3O_2S$: C, 60.57; H, 5.99; N, 13.25; S, 10.09. Found: C, 60.67; H, 5.86; N, 13.07; S, 10.22.

[3a*S*-(3aα,4β,6aα)]-4-[3-(Indol-3-yl)propyl]hexahydro-2,5β-dioxo-1*H*-thieno[3,4-*d*]imidazole (12). Further elution of the column described above with 20% methanol–80% chloroform afforded the second oxidation product of compound **4a**, Biot-C$_3$-Ind. The product recovered from these fractions was dissolved in methanol and precipitated with ethyl acetate to give a light yellow solid: mp 230 °C dec; field desorption mass spectrum m/e 317 (M^+); high resolution 317.1201 (found), 317.1194 (calcd for $C_{16}H_{19}N_3O_2S$); NMR (25% CD_3OD–75% $CDCl_3$) δ 1.96 (multiplets, 3, Biot(SO)$CHCH_2$), 2.90 (t, 2, $IndCH_2$), 7.07 (m, 2, 2′-H and 5′- or 6′-H), 7.15 (m, 1, 6′- or 5′-H), 7.37 (d, 1, J = 7.8 Hz, 4′-H), 7.57 (d, 1, J = 7.8 Hz, 7′-H). See text for other assignments.

Quantitative Comparison of the Relative Oxidation Rates of Biotin and Indole Compounds in the Sodium Periodate Oxidation. 1. Biot-C$_3$-Ind (**4a**), Biot-C$_5$ (**6a**), biotin methyl ester (**2a**), or 3-propylindole (Ind^3-C$_3$) (80 μmol), weighed to the nearest 0.001 mg, was dissolved in 15 mL of CH_3OH–H_2O (v/v, 1:1). To the stirred solution was added 340 μL of 0.24 M aqueous sodium metaperiodate. The reaction solution was kept at 25 °C, and the course of the reaction was followed by standard titration methods.[47]

2. Two solutions (CH_3OH–H_2O, v/v, 1:1) of similar concentration, one containing Biot-C$_3$-Ind and the other containing an equimolar mixture of Biot-C$_5$ and Ind-C$_3$, were treated with exactly 1 molar equiv of sodium metaperiodate in aqueous solution. The extent of the reaction of the indole portion in each solution was determined by UV spectroscopy.

***N*-Bromosuccinimide (NBS) Oxidation of the Avidin–Biotin Complex.** The homogeneity of the commercial avidin was checked by SDS gel electrophoresis, which exhibited a single, separate band plus a very weak band (<5%). The latter is either an impurity in the sample or avidin–biotin complex that survived the denaturation conditions for the gel electrophoresis. A solution of avidin was extensively dialyzed to remove any potential low molecular weight contaminant that could react with NBS. The binding capacity of this avidin sample was determined spectrophotometrically by the method of Green based on the use of the dye 4-hydroxyazobenzene-2′-carboxylic acid.[48,49] This gave a value of 13.0 units/mg protein. The protein solution consumed 2.4 molar equiv of NBS per mol of tryptophan residue.

A 2-mL solution of avidin–biotin complex (2.6 μM) in 0.1 M acetate buffer, pH 4.5, containing [*carbonyl*-^{14}C]biotin (0.85 μCi) was prepared. The amount of biotin used corresponds to a 6% excess of that required for total saturation of the binding site determined as above. To the solution was added dropwise with gentle stirring 85 μL of freshly prepared NBS in water (2 mM, NBS recrystallized from water prior to use). After 15 min, the remaining NBS was quenched with 800 μL of the avidin solution (2.6 μM). To the solution was then added 2 mg of a mixture of biotin, (+)-sulfoxide, and (−)-sulfoxide (2:1:1) and the solution was autoclaved at 120 °C for 30 min in order to denature[1] the complex. The solution was concentrated to ca. 500 μL, applied to a P2 column (2.5 × 40 cm), and eluted with water. Fractions (2 mL) were collected, and the fractions were checked for radioactivity, UV absorption, and conductivity. It could be concluded on the basis of recovery of radioactivity that all of the complex had been denatured by the autoclaving procedure. That is, the radioactivity was nearly quantitatively recovered from the P2 column in fractions containing biotin and its sulfoxides. There was neither radioactivity nor UV absorption (A_{280} < 0.01) at the elution position calibrated for avidin. The P2 column eliminated about 70% of the salt from the biotin compounds. Fractions containing radioactivity were pooled, evaporated, applied to a TEAE-cellulose column (2.5 × 29 cm), and eluted with a linear gradient of 0.01 (1 L) to 0.3 M (1 L) triethylammonium bicarbonate, pH 8.0. Fractions (2 mL) were checked for radioactivity. The elution profile is shown in Figure 1. The order of elution was (−)-sulfoxide, (+)-sulfoxide, biotin with increasing volume of eluant. The identity of each compound was established by TLC. On the TEAE-cellulose column, the sulfoxides were well separated from biotin whereas the two sulfoxides were not completely

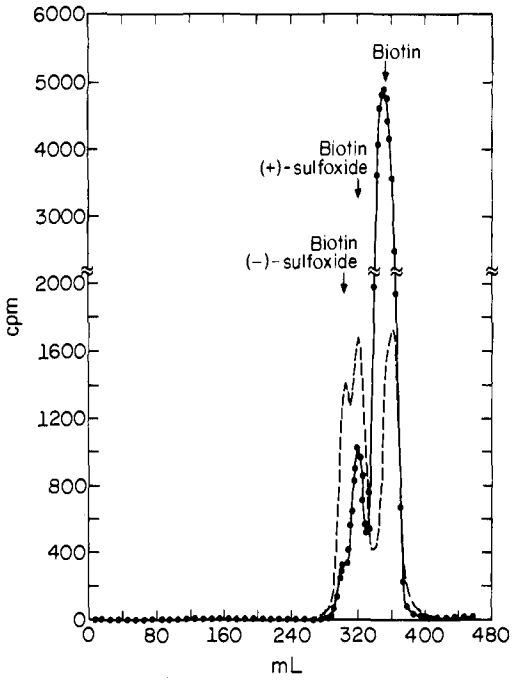
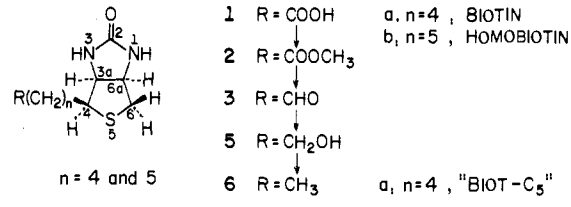


Figure 1. Anion-exchange chromatographic separation of (+)-biotin and its sulfoxides (a) from the reaction of the avidin–biotin complex with NBS, after denaturation by autoclaving (–·–·–); (b) from the reaction of biotin with 0.5 molar equiv of NBS in water, pH 6.8 (– – –).

resolved. For quantitation of the two sulfoxides, the fractions containing these compounds were pooled, evaporated to dryness, coevaporated with ethanol to remove the TEAB salt, and spotted on a TLC plate (Eastman, silica gel). Development with *n*-BuOH–AcOH–H_2O (5:2:3) separated the two sulfoxides, the ratios of which were determined by scintillation counting.

Results and Discussion

Synthesis. Our method of synthesis of the requisite model compounds having trimethylene and tetramethylene chains between the biotin ring system and indole was based on (+)-biotin (**1a**) and (+)-homobiotin (**1b**) in order to preserve correct stereochemistry in the biotin moiety. We considered that the Fischer indole synthesis would serve most effectively for the construction of the attached indole and that two carbons of the biotin and homobiotin side chains could be used in constructing the pyrrole ring of the indole. Reduction of the methyl ester (**2a**) of biotin to the aldehyde (**3a**) was effected by diisobutylaluminum hydride in methylene chloride at −70 °C. Since the aldehyde was not a stable compound and extensive decomposition resulted on attempts at isolation, the Fischer indole synthesis was applied directly on the crude reduction mixture and the synthesis of compound **4a** from **2a** became



essentially a "one-pot" preparation. The preferred conditions involved heating an acetic acid solution of the crude aldehyde, to which phenylhydrazine hydrochloride and boron trifluoride etherate had been added, under argon (**3a** → **4a**). We chose an abbreviation for compound **4a**, Biot-C_3-Ind, consistent with past usage[39] and as a space saver for the complete name that describes not only structure but also stereochemistry, namely, [3a*S*-(3aα,4β,6aα)]-4-[3-(indol-3-yl)propyl]hexahydro-2-

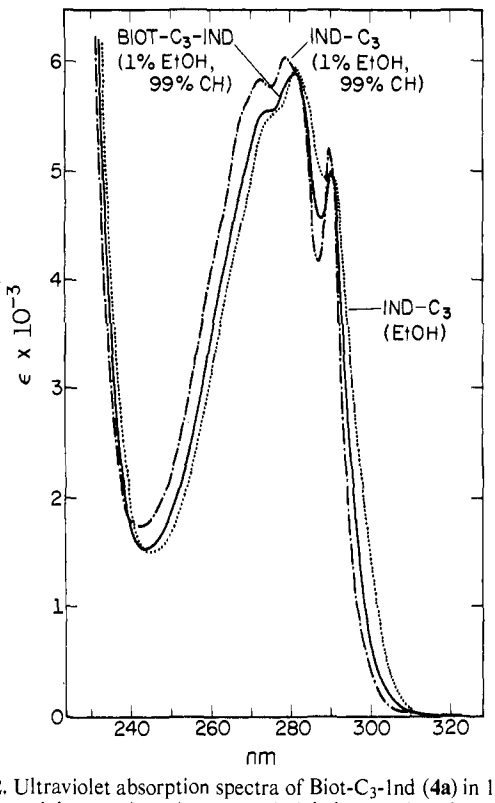
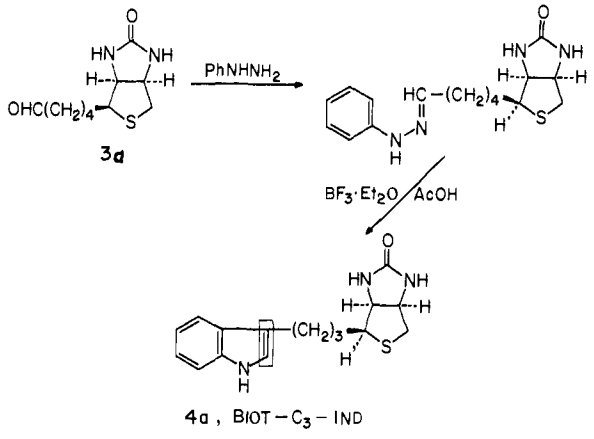


Figure 2. Ultraviolet absorption spectra of Biot-C_3-Ind (**4a**) in 1% ethanol–99% cyclohexane (——), 3-propylindole in 1% ethanol–99% cyclohexane (— · — · —), and 3-propylindole in ethanol (· · · · · · · ·).

oxo-1*H*-thieno[3,4-*d*]imidazole. The same sequence of reactions was used to synthesize Biot-C_4-Ind (**4b**) from **1b** through **2b** and **3b**.



The structures of the products were confirmed by their UV (Table I, Figure 2), NMR, and mass spectra. The indole compounds included in this study always exhibited an intense peak, usually the base peak, at *m/e* 130 corresponding to the "indolylmethyl" ion, $C_9H_8N^+$, a characteristic of 3-alkylindoles. For comparison studies, it was necessary to synthesize the corresponding half-molecules, 3-Propylindole, Ind[3]-C_3, has been used before in this laboratory,[39n] and Biot-C_5 (**6a**) was prepared by a complete reduction of the side chain involving conversion of the intermediate (+)-biotinol (**5a**) to its *p*-toluenesulfonate followed by reduction with lithium aluminum hydride. For spectroscopic studies, the compounds were purified extensively by chromatography and recrystallization and their purity was checked by microanalysis and thin layer chromatography using several solvent systems.

Electronic Absorption and Fluorescence Emission. For the purpose of detecting possible interaction between the bicyclic

Table I. Quantitative Electronic Absorption Data of Indole Derivatives[a]

compd	solvent	λ_{max} (ϵ)	λ_{min} (ϵ)	λ_{max} (ϵ)	λ_{min} (ϵ)	λ_{max} (ϵ)	λ_{max} (ϵ)	λ_{min} (ϵ)	λ_{max} (ϵ)
Ind^3-C_3	1% EtOH–99% CH	221 (31 500)	242 (1770)	272.5 (5790)	275 (5750)	279 (6100)	282 sh (5910)	287 (4090)	290 (5190)
Biot-C_3-Ind (**4a**)	1% EtOH–99% CH	221 (31 600)	242 (1490)	274 sh (5510)		281 (5840)		288 (4540)	290 (4960)
Biot-C_4-Ind (**4b**)	1% EtOH–99% CH	221 (31 500)	243 (1550)	273 sh (5540)		281 (5820)		288 (4490)	290 (4840)
Ind^3-C_3	EtOH	222 (31 900)	245 (1530)	275 sh (5470)		282 (5870)			290 (4950)

[a] Abbreviations: λ, wavelength (nm); ϵ, molar extinction coefficient; sh, shoulder; CH, cyclohexane.

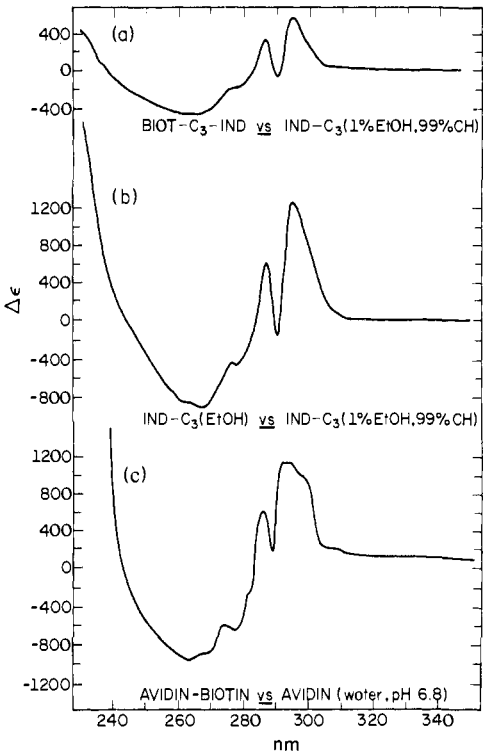


Figure 3. Difference UV spectra for (a) Biot-C_3-Ind vs. 3-propylindole in 1% ethanol–99% cyclohexane; (b) 3-propylindole in ethanol vs. 3-propylindole in 1% ethanol–99% cyclohexane; (c) avidin–biotin complex vs. avidin in water, pH 6.8.

biotin ring system and indole, the model compounds Biot-C_3-Ind (**4a**) and Biot-C_4-Ind (**4b**) were studied spectroscopically at concentrations low enough to preclude intermolecular interactions so that perturbations associated with any internal 1:1 interaction of the two parts of the molecules could be characterized. The UV absorption and fluorescence emission spectra of solutions of Biot-C_n-Ind (**4**) were compared with those of solutions containing the two half molecules Biot-C_5 and Ind-C_3. Biot-C_5 (**6a**) has no UV absorption above 225 nm (ϵ (220) < 100); accordingly, electronic transitions of the indole moiety are responsible for the UV absorption of Biot-C_n-Ind in the region of interest. There was no detectable evidence of interaction in the UV absorption and fluorescence emission of Biot-C_n-Ind in water, the solvent in which we have observed maximal stacking interactions between nucleic acid bases and between indole and nucleic acid bases in our previous examination of synthetic spectroscopic models.[39] Nor was any interaction detectable spectroscopically in ethanol, acetonitrile, or ether as solvents. However, there was obvious intramolecular interaction detectable in hydrocarbon solvents such as cyclohexane. Since it was not possible to guarantee complete dissolution of all the compounds in cyclohexane, for quantitative comparisons we dissolved the compounds in ethanol or ethanol–cyclohexane and diluted with cyclohexane to either 99 or 99.9% cyclohexane.

The UV absorption spectral data for Biot-C_n-Ind and Ind-C_3 in 1% ethanol–99% cyclohexane and for Ind-C_3 in ethanol are given in Figure 2 and Table I. The UV absorption spectrum of Biot-C_3-Ind in 1% ethanol–99% cyclohexane is less resolved than that of Ind-C_3 in the same solvent, the 280-nm band is narrower, and there is a red shift in the main transition band. The perturbation of indole absorption caused by the proximate biotin unit in the model compound **4a** in this solvent system follows the trend of solvent influence on indole absorption in going from cyclohexane or other hydrocarbon solvent to ethanol.[50-53] This can be seen in Figure 2 in a comparison of the two curves for the 1% ethanol–99% cyclohexane solutions vs. the curve for Ind-C_3 in ethanol. The UV spectroscopic effect of the proximate biotin moiety in Biot-C_3-Ind compared with Ind-C_3 is similar to the effect of biotin bound to avidin, which produces a red shift and a narrowing of the 280-nm band of avidin.[1] The same relationships are shown in the difference spectra (Figure 3) for Biot-C_3-Ind (**4a**) vs. Ind-C_3 (with or without an added 1 molar equiv of Biot-C_5) in 1% ethanol–99% cyclohexane, Ind-C_3 in ethanol vs. Ind-C_3 in 1% ethanol–99% cyclohexane, and avidin–biotin vs. avidin in water (see also ref 1). The UV spectroscopic results obtained with Biot-C_4-Ind (**4b**) are practically identical with those of Biot-C_3-Ind (**4a**). The near-ultraviolet spectrum of indole compounds consists of two overlapping electronic transitions, the ^1L_a and ^1L_b bands, which have been qualitatively distinguished by solvent perturbation[51,54] and have been resolved by magnetic circular dichroism[55] and excitation polarization.[56-58] It is the ^1L_a band, which is more sensitive to solvent polarity, that has been implicated by Green[1] in the UV red shift caused by the binding of biotin to avidin. Additional perturbation of the ^1L_a band in the spectrum of the avidin–biotin complex has been ascribed to the negatively charged carboxyl group of biotin and in part to the sulfur atom;[1] the removal of either of these features (using biotin methyl ester or dethiobiotin) gives a much closer approach to the derivative spectrum.[1,29,30]

The interaction of the biotin ring system and indole is also evident in the fluorescence emission spectrum of the model compounds **4a** and **4b** in hydrocarbon solvent (Table II). In 1% ethanol–99% cyclohexane, the emission maximum corresponding to excitation of Ind-C_3 at 290 nm is 325 nm while λ_{em} for both Biot-C_3-Ind and Biot-C_4-Ind is 335 nm. Biot-C_4-Ind (**4b**) is more soluble in cyclohexane than its trimethylene counterpart **4a**, and a solution of sufficient concentration for accurate spectrofluorometry could thus be obtained in 0.1% ethanol–99.9% cyclohexane. In this solvent, the λ_{em} values obtained for Ind-C_3 and Biot-C_4-Ind were 310 and 330 nm, respectively. Hence, the proximate biotin moiety in Biot-C_4-Ind causes a red shift of 20 nm in fluorescence emission of indole. This may be explained on the basis of the interaction of biotin with the excited-state indole, similar to the effect of polar solvent molecules on indole itself,[59-69] as can be seen from

the three λ_{em} values for Ind-C_3 in Table II. It is of interest to note (Table II) that an increment of ethanol concentration from 0.1 to 1% (in cyclohexane) causes a red shift of λ_{em} of Ind-C_3 with a magnitude close to that induced by a connected nearby polar biotin moiety (in Biot-C_4-Ind, 0.1% EtOH–99.9% CH). This indicated that polar solvent molecules, 1% in concentration, compete favorably with biotin for interaction with excited-state indole. It is conceivable, then, that concentration of polar component in the solvent shell of indole is greater than the bulk solvent as was suggested by Eisinger and Navon.[64] While a shift in λ_{em} was observed, the proximate biotin ring caused no quenching of fluorescence, as seen from the comparable values of the quantum yields for Biot-C_n-Ind and Ind-C_3 in the same solvent (Table II). The fluorescence results thus ruled out an intramolecular, dark complex, and the detailed emission spectra also ruled out the existence of an exciplex. Moreover, examination of scale molecular models indicated that hydrogen-bonding interaction within the Biot-C_n-Ind (n = 3, 4) molecules could not be an important factor.

We examined the series **2a, 6a,** and **4a** in extremely dilute solutions of $CDCl_3$ by Fourier transform ^1H nuclear magnetic resonance spectroscopy at 220 MHz in order to determine whether any of the biotinyl protons were appreciably shielded or deshielded by the anisotropic indole ring separated from the biotin portion by the trimethylene bridge. The β attachment of the $CH_2CH_2CH_2$Ind side chain to the biotin rings of **4a** (the same is true for the $CH_2CH_2CH_2CH_2$Ind in **4b**), while it has the advantage of retaining the stereochemistry of biotin, precludes the existence of folded conformations with the indole lying over, or exo to, the fused five-membered rings. It does permit the indole to lie under, or endo to, the fused rings. According to molecular models, the biotinyl hydrogens that can most readily experience deshielding by the indole in feasible folded conformations are those at 3a(α) and N1 and N3. The chemical shift for the 3a proton in both biotin methyl ester and Biot-C_5 (**6a**) is 4.32 and that for Biot-C_3-Ind (**4a**) is 4.27. This small shift might be considered to indicate shielding, but the value observed for **4a** in the more polar 20% CD_3OD–80% $CDCl_3$ solution was 4.25 ± 0.01. No appreciable changes in chemical shift in $CDCl_3$ were observed for the other α protons of compounds **2a** and **6a** vs. **4a,** nor was there any change for the 6β proton. However, there was a change in the chemical shifts of the N–H's of Biot-C_5 (**6a**), δ 4.92 and 4.85, brought about by attachment of the indole as in **4a**: N–H's δ 4.61 and 4.57. All of the the signals were singlets for equivalently very dilute solutions in $CDCl_3$. The NMR values are suggestive of the proximity of the indole to the N–H's of the biotin as described above and therefore of the possible existence of folded conformations in dilute solution in $CDCl_3$, the least polar solvent we were able to use. However, it must be recognized that these N–H signals are very sensitive to concentration, and thus to dimer $\rightleftarrows$ monomer equilibria, and to adventitious moisture.

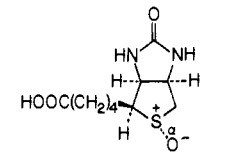
Thus, in the spectroscopic work we have shown that perturbations due to biotin–indole proximity can be observed in hydrophobic media. The response of the ultraviolet absorption in particular is similar to that observed in the avidin–biotin complex. We have found no evidence of strong interaction between the biotin ring system and the indole of tryptophan such as would help account for the very low dissociation constant of the avidin–biotin complex.

Sulfoxide of Biot-C_3-Ind. During the purification of Biot-C_n-Ind (n = 3, 4), it was found that the model was converted to a more polar compound in aerated solution. The major conversion product was found to be sulfoxide by IR, UV, NMR, and mass spectra. Since two sulfoxides are possible, it was of interest to determine whether their proportions would be influenced by the presence of the indole moiety in the same molecule compared, for example, with the presence of the normal ester side chain of **2.** For any interpretation to be attached to potentially differing proportions of sulfoxides formed by various oxidants of **4,** it was necessary to determine the configurations of the sulfoxides. For comparison, the two sulfoxides of (+)-biotin (**1a**), named for convenience according to their specific rotations (biotin (+)-sulfoxide (**7**) and biotin (−)-sulfoxide (**8**)),[46] were prepared as were the corresponding

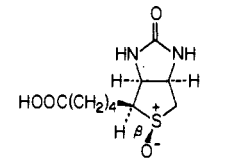
Table II. Quantitative Fluorescence Emission Data[a]

compd	solvent	λ_{em}, nm	rel quantum yield
Ind^3-C_3	EtOH	345	1
Biot-C_3-Ind (**4a**)	1% EtOH–99% CH	335	0.84
Ind^3-C_3	1% EtOH–99% CH	325	0.85
Biot-C_4-Ind (**4b**)	0.1% EtOH–99.9% CH	330	0.72
Ind^3-C_3	0.1% EtOH–99.9% CH	310	0.76

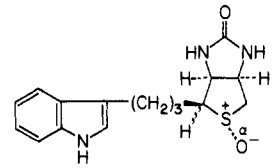
[a] Excitation 290 nm; deaerated by purging with argon; 25 °C. Abbreviation: CH, cyclohexane.



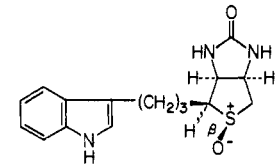
7
BIOTIN (+)-SULFOXIDE
9, METHYL ESTER



8
BIOTIN (−)-SULFOXIDE
10, METHYL ESTER



11



12

two sulfoxides (**9** and **10**)[46] of the methyl ester of (+)-biotin, by hydrogen peroxide and NBS oxidation, respectively. Both sulfoxides of the methyl ester of (+)-biotin were obtainable by oxidation with hydrogen peroxide in glacial acetic acid followed by separation, with the predominance of **9,** the α isomer, over **10,** the β isomer. When Biot-C_3-Ind (**4a**) was treated with hydrogen peroxide in acetic acid, two sulfoxides were formed in a ratio of 4:1, and the major isomer was identical with the product of air oxidation.

The proton magnetic resonance data for the two sulfoxides of Biot-C_3-Ind and those for biotin methyl ester are listed in Table III together with those for the parent compounds **4a** and **2a**. In general, the protons on the carbons of the bicyclic ring of biotin and its sulfoxides are well resolved. The 6β-H appears as either a doublet or a doublet of doublets with very small coupling constants (J = 0–2.8 Hz) to 6a-H, which is trans to the 6β-H at a dihedral angle close to 90° in the model. The 6α-H appears as a doublet of doublets with larger coupling constants (J = 4.9–6.6 Hz) to 6a-H. The 4-H is a multiplet coupled to both 3a-H and the first methylene protons of the side chain, and 3a-H and 6a-H are doublets of doublets or multiplets. The assignments were confirmed by decoupling experiments. It is clear from the values in Table III that compounds **11** and **12** have the same sulfoxide configurations as compounds **9** and **10,** respectively, because of the close correspondence in chemical shifts and coupling constants for the respective protons in the same solvent used throughout, $CDCl_3/CD_3OD$ = 3/1. The similarities also appeared when CD_3OD was used as the solvent and all solutes were in the same concentration. The coupling constants $J_{H_{3a}H_4}$, $J_{H_{6a}H_{6\alpha}}$, and $J_{H_{6a}H_{6\beta}}$ indicated that the conformation of the tetrahydrothiophane ring of the sulfoxides in solution is similar to that of

Table III. Proton Magnetic Resonance Data[a]

compd	H_{6a}	H_{3a}	H_4	$H_{6\alpha}$	$H_{6\beta}$	H_7[b]
(+)-biotin methyl ester (**2a**)	4.51 (dd) J = 4.9, 7.0	4.31 (dd) J = 5.0, 7.0	3.17 (m)	2.94 (dd) J = 4.9, 13.0	2.75 (d) J = 13.0	1.69 (m)
biotin (+)-sulfoxide methyl ester (**9**)	4.71 (m)	4.64 (dd) J = 6.0, 8.5	3.10 (m)	3.09 (dd) J = 6.0 13.5	3.44 (dd) J = 2.6, 13.5	1.86 (m)
biotin (−)-sulfoxide methyl ester (**10**)	4.87 (dd) J = 5.7, 7.5	4.72 (dd) J = 6.5, 7.5	2.67 (m)	2.86 (dd) J = 5.7, 15.1	3.47 (d) J = 15.1	2.16 (m) 1.95 (m)
Biot-C_3-Ind (**4a**)	4.46 (dd) J = 4.9, 7.9	4.25 (dd) J = 4.4, 7.9	3.19 (m)	2.90 (dd) J = 4.9, 12.9	2.71 (d) J = 12.9	1.78 (m)
Biot(SO)-C_3-Ind (**11**)	4.61 (m)	4.50 (dd) J = 5.4, 8.9	3.10 (m)	3.02 (dd) J = 6.4, 13.5	3.31 (dd) J = 2.8, 13.5	1.90 (m)
Biot(SO)-C_3-Ind (**12**)	4.78 (dd) J = 6.6, 8.2	4.62 (dd) J = 6.7, 8.2	2.65 (m)	2.77 (dd) J = 6.6, 15.3	3.46 (d) J = 15.3	2.16 (m) 1.95 (m)

[a] 220 MHz; $CDCl_3$–CD_3OD (v/v 3:1). Internal reference Me_4Si, 0.08 M solutions. [b] H_7 = Biot-$CH_2(CH_2)_n$–.

Table IV. ^{13}C Nuclear Magnetic Resonance[a]

compd	C_2	C_{11}	C_{3a}/C_{6a}		C_4	C_6	$C_7/C_8/C_9$			C_{10}	C_{12}
biotin methyl ester (**2a**)	165.85	174.54	63.28	61.53	56.87	40.98	29.65	29.42	25.88	34.51	51.94
biotin (+)-sulfoxide methyl ester (**9**)	163.93	175.42	58.27	55.25	71.80	59.29	26.43	28.04	25.72	34.32	51.96
biotin (−)-sulfoxide methyl ester (**10**)	163.22	175.41	63.79	61.43	67.92	57.90	24.06	28.54	25.82	34.38	51.94

[a] CD_3OD; internal reference, Me_4Si. The numbering 7–12 starts with the methylene carbon adjacent to the ring.

biotin and is probably an envelope conformation with the sulfur atom at the tip (endo). This is the conformation of biotin in the solid state as determined by X-ray structure analysis.[70] In this conformation, the side chain is in the more favorable pseudoequatorial orientation. The S–O is therefore in the pseudoequatorial position in the α-sulfoxides and the pseudoaxial position in the β-sulfoxides.

In a thorough NMR study of the conformation and configuration of bicyclic sulfides and sulfoxides, including biotin compounds, Lett and Marquet[71,72] found that, for all of the sulfoxides studied, the J_{gem} for methylene protons adjacent to the S–O group is smaller for the equatorial sulfoxides compared with the respective axial sulfoxides. Based on this J_{gem} relation, they assigned the α configuration to the (+)-sulfoxide and the β configuration to the (−)-sulfoxide in the biotin series, as pictured in **7** and **9** vs. **8** and **10**. Additional support for the assignments, and also those for **11** and **12**, lies in the fact that the 7-H's of **10** and **12** are more deshielded than those of their counterparts **9** and **11** and are split. The proton on a second carbon removed from the S–O group and in a diaxial relationship is generally strongly deshielded.[72] When the side chain is on the same side of the ring as the S–O (**10** and **12**) it may experience a greater barrier to rotation with one of the 7-H's in an effective diaxial orientation to the S–O group.

The ^{13}C NMR chemical shifts for the methyl esters of (+)-biotin and its two sulfoxides in CD_3OD are summarized in Table IV. The carbon assignments were established by proton-coupled spectra (off-resonance decoupling) and selective proton-decoupled spectra. The chemical shift assignments for C_{3a} and C_{6a} have not been made unequivocally for each compound, but it is probably safe to assume that the chemical shift for C_{3a} is the larger value since there is additional substitution[73] on the carbon adjacent to C_{3a}. The assignments for C_7, C_8, and C_9 (reading away from the biotin ring) were made tentatively on the assumption that C_8 and C_9 should be relatively insensitive to the change from sulfide to sulfoxides. The chemical shift for the carbonyl carbon, C_2, does not change appreciably from sulfide to sulfoxides and the shift of C_2 is not sensitive to the configuration of the sulfoxides. This is in agreement with the earlier finding that there is little if any transannular S–C_{CO} or S–O–C_{CO} interaction.[70,74] The C_{3a}, C_{6a}, and C_4 signals appeared as doublets; the C_6 and the methylene carbons of the side chain, as triplets; and the methoxy carbon, as a quartet in off-resonance decoupled spectra. The C_4 and C_6 chemical shifts are widely separated ($\Delta\delta$ = 10–16 ppm) owing to the difference in substitution on these carbons adjacent to sulfur.[75]

The (−)-sulfoxide isomer, which has C_4 and C_6 signals at higher field than the corresponding carbons in the (+)-sulfoxide of biotin methyl ester, was assigned the β-sulfoxide configuration (**10**). The ^{13}C NMR data are in agreement with the general observation that the carbons adjacent to an axial sulfoxide resonate at higher field than those in a corresponding equatorial sulfoxide.[76,77] This so-called "β effect" has been rationalized on the basis of the electric field effect.[77] The C_{3a} and C_{6a} carbons are more shielded in the (+)-sulfoxide of biotin methyl ester, which is assigned the α-sulfoxide configuration (**9**) in accordance with the observed anti-periplanar effect of sulfoxide oxygen on carbons γ to the oxygen.[76] An attendant X-ray crystal study of the Biot-C_3-Ind sulfoxide **11** confirms and establishes the α configuration and the pseudoequatorial conformation of the S–O group.[38]

Quantitative Study of the Oxidation of Biot-C_3-Ind (4a). The oxidations of Biot-C_3-Ind, Biot-C_5, and Ind-C_3 with sodium metaperiodate were compared in order to determine the effect, if any, of the linked indole of Biot-C_3-Ind on the rate and stereochemistry of the oxidation of biotin and of the linked biotin on the oxidation of indole. Sodium metaperiodate is known to oxidize sulfides in general[78] and biotin in particular[72] to their sulfoxides. The reagent also oxidizes indole derivatives[79] and tryptophan residues in avidin but not those in the avidin-biotin complex.[30] When Biot-C_3-Ind (**4a**) was treated with sodium metaperiodate in aqueous methanol, in which we had found no spectroscopic evidence of biotin-indole interaction, the α-sulfoxide (**11**) and β-sulfoxide (**12**) were formed in a ratio of approximately 9:1. Only a trace of another product was formed, probably with the indole ring modified[79] since it gave a negative Erlich's test. When biotin methyl ester (**2a**) was treated with sodium metaperiodate under the same conditions, the α-sulfoxide (**9**) and β-sulfoxide (**10**) were formed in a ratio of approximately 9:1.[80] 3-Propylindole alone was oxidized to a single product, assigned the structure 1-(2-formamidophenyl)-1-butanone by analogy,[79] under the same conditions but at a much slower rate. For a comparison of the relative rates of oxidation, the rate of sodium metaperiodate consumption in each reaction mixture was determined by standard titration methods,[47] and the rate of oxidation of the indole was determined by monitoring the decrease in UV absorption at 280 nm. The similar rates of oxidation for Biot-C_3-Ind (**4a**), biotin methyl ester (**2a**), and Biot-C_6 (**6a**) showed the absence of any appreciable effect of the linked indole on the biotin oxidation in methanol-water. For example, when a 5.6×10^{-3} M solution of any of these compounds in methanol-water (v/v, 1:1) at 25 °C was treated with 1 molar equiv of sodium metaperiodate, the time required for consumption of one-half the oxidant was the same within experimental error, 65 ± 3 min. When either of two solutions of the same concentration, one containing Biot-C_3-Ind and the other containing an equimolar mixture of Biot-C_5 and Ind-C_3, was treated with 1 molar equiv of sodium metaperiodate, the decrease in A_{280} was 8 ± 3% relative to the total decrease possible for oxidation of 3-propylindole with 1 molar equiv of periodate. Thus, we confirmed that the linking of the biotin and indole rings as in **4a** did not influence the periodate oxidation of *either* ring component in aqueous methanol.

N-Bromosuccinimide is known to oxidize tryptophan and indole derivatives[81] and to oxidize sulfides to sulfoxides.[82,83] We found that biotin methyl ester (**2a**) could be oxidized stereoselectively to the β-sulfoxide (**10**), with only a trace of the α isomer, by addition of solid NBS to a solution of **2a** in methanol. By addition of solid NBS to a solution of biotin (**1a**) in water, it was also possible to obtain biotin β-sulfoxide (**8**) as the major product (>80%). The high stereoselectivity of the reaction when conducted in this manner can be rationalized as the result of electrophilic attack by NBS on sulfur to form an intermediate bromosulfonium ion,[84-87] which then undergoes reaction with solvent with inversion to form the β-sulfoxide (Scheme I, top). Electrophilic reactions on thianes generally proceed by equatorial attack,[84,88] a selectivity that is enhanced in the case of biotin and its methyl ester since axial attack is much more sterically hindered. When an aqueous solution of NBS was used, the oxidation reaction became unselective. Thus, the oxidation of an aqueous solution (1.5×10^{-2} M) of biotin or biotin methyl ester with an aqueous solution of NBS at 25 °C was complete in 5 min and resulted in a 1:1 mixture of the α- and β-sulfoxides. The apparent lack of stereoselectivity implies β-attack or the existence of a second, equally rapid, route to sulfoxide, namely, the α-equatorial attack on the sulfide by the hypobromous acid, which is formed in an aqueous solution of NBS as with moist bromine,[88] and conversion to the α-sulfoxide without inversion of the intermediate (Scheme I, bottom). Application of the NBS oxidation in aqueous solution to Biot-C_3-Ind (**4a**) indicated, from the complexity of the mixture of products which included the two sulfoxides **11** and **12**, that the biotin and indole units were both being oxidized.

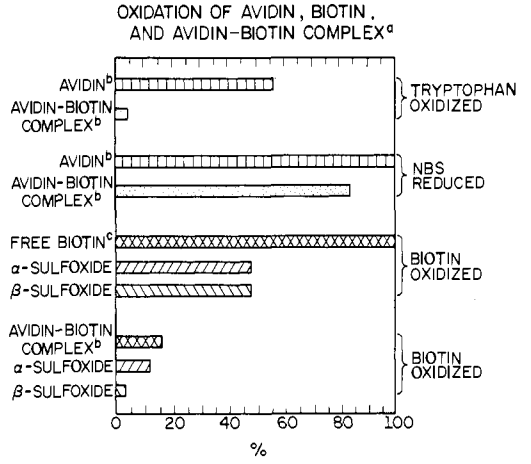
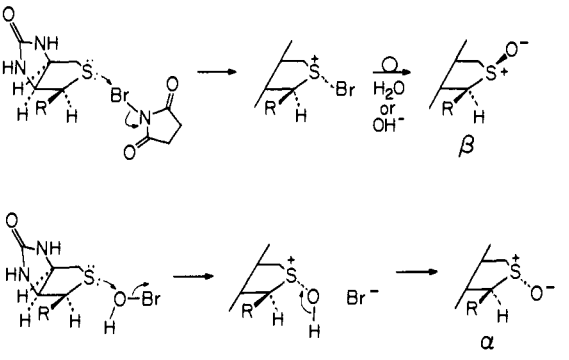


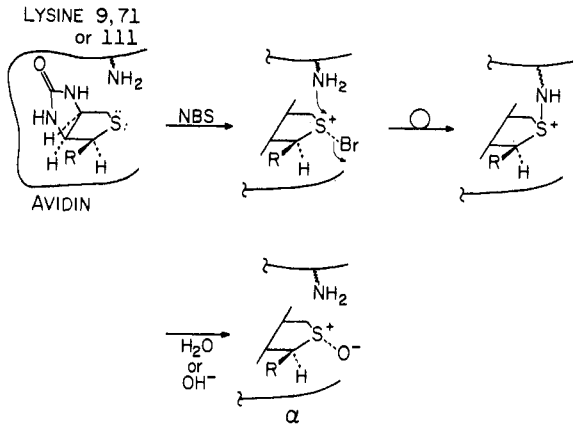
Figure 4. Oxidation of avidin, biotin, and avidin-biotin complex with *N*-bromosuccinimide. (a) Reaction time: 15 min at 25 °C, pH 4.5, 0.1 M acetate buffer, except for biotin which was at pH 6.8 in phosphate buffer. (b) 2 mol *N*-bromosuccinimide/mol tryptophan. (c) 1 mol NBS/mol biotin.

Scheme I



Oxidation of Biotin in the Avidin-Biotin Complex by *N*-Bromosuccinimide. As a consequence of our finding that both biotin and indole moieties were oxidized by aqueous NBS in our linked model (**4a**), we decided to investigate the fate of biotin in the avidin-biotin complex under the same conditions. It appears that no one has examined what happens to the biotin, although it is well recognized that its presence in the complex inhibits the oxidation of the tryptophans of avidin.[1] We designed our experiments to answer the following questions with regard to the oxidant NBS: (1) Is the protection of the tryptophan residues in the avidin-biotin complex due partially to the sparing effect of biotin, i.e., by consumption of the oxidizing agent by biotin? (2) Is the biotin in the complex also protected from or less reactive toward NBS oxidation? (3) If biotin in the avidin-biotin complex is oxidized, which diastereomeric sulfoxide is formed? When a solution of avidin-[*carbonyl*-^{14}C]biotin complex in 0.1 M acetate buffer at pH 4.5 (see Experimental Section) was treated with an aqueous solution of NBS (2 molar equiv per tryptophan residue) at 25 °C for 15 min, 15 ± 1% of the biotin was oxidized to sulfoxides. The ratio of α-sulfoxide (**7**) to β-sulfoxide (**8**) was 4:1 (Figure 4; see also Figure 1). Free biotin was 100% oxidized in 15 min. During the same reaction time, there was only a 4% decrease in A_{280}, corresponding to the oxidation of tryptophan. This figure was in contrast to the 56% decrease in A_{280} observed

Scheme II



almost instantaneously when avidin alone was treated under the same conditions. If the reaction was allowed to continue, an additional decrease of 12% in A_{280} was observed after 24 h and no further decrease thereafter. Thus, a true protection of tryptophan residues was evident in the avidin–biotin complex as previously noted.[1,35] Biotin was also less reactive in the very stable complex than when free in aqueous solution, suggesting a location in the interior of the protein and not as accessible to oxidizing agents. During the oxidation of 15% of the biotin and 4% of the tryptophan in the avidin–biotin complex, 83% of the NBS was reduced (Figure 4), as determined by a quenching "titration" with pure avidin. While tryptophan residues in the complex are protected, other amino acid residues of avidin, e.g., methionine and tyrosine, are oxidized.

Biotin and biotin sulfoxides are chemically stable and both sulfoxides are configurationally stable under the autoclaving conditions (120 °C, 30 min)[89] used to free the biotin and biotin sulfoxides from the complex for chromatographic analysis. The radioactive tracer technique provided accuracy for the reported percentages of biotin oxidized and for the ratios of the two sulfoxides **7** and **8** isolated (Figures 1 and 4). The predominance of the biotin α-sulfoxide (**7**) over the β-sulfoxide (**8**) is real whether we consider both compounds to result from NBS oxidation of biotin in the avidin–biotin complex or of complexed biotin plus free biotin. We used a slight excess (6%) of biotin relative to avidin. If we assume that all of the excess, therefore free, biotin was oxidized, it would have given the two sulfoxides in a 1:1 ratio (Figure 4). Accordingly, the 4:1 ratio of α- to β-sulfoxide (12%:3%) from NBS oxidation of the avidin–biotin complex (plus 6% excess biotin) represents a *minimum* for the actual stereoselectivity, which is therefore inferred to be much greater. The high degree of stereoselectivity indicates that one of the two approaches, equatorial or axial, to sulfide sulfur is hindered in the avidin–biotin complex, and this, in turn, suggests a relatively fixed orientation of biotin within the avidin–biotin complex. The predominance of the α-sulfoxide (**7**) as the NBS oxidation product indicates that the α-pseudoequatorial approach site is more exposed. Involvement of hypobromite in the oxidation (Scheme I, bottom) could account entirely for the product. The yield of β-sulfoxide, which is lower than expected for equivalent involvement of NBS in α-equatorial attack, as in Scheme I, top, could be explained if there is a nucleophile available in the binding site, such as the ε-amino group of a lysine residue, so positioned that it can react with the bromosulfonium ion intermediate to yield a second unstable intermediate, which is then hydrolyzed with net retention of configuration (α) (Scheme II). A lysine residue has been implicated in the binding site.[1] This may be one of the three lysine residues which are adjacent to tryptophans in the avidin sequence.

Now, we may answer the questions posed earlier in this section. The protection of the tryptophans in the avidin–biotin complex against NBS oxidation is due only partially to consumption of the oxidizing agent by the bound biotin. Biotin in the avidin–biotin complex is oxidized, a fact which had apparently not been recognized earlier, yet it is more protected from NBS oxidation than free biotin in aqueous solution. Whereas biotin in solution is oxidized to equal portions of α- and β-sulfoxides, the biotin in the avidin–biotin complex that is oxidized by NBS yields the α-sulfoxide predominantly, indicating a steric limitation of the approach of oxidant to bound biotin. The suggestion can be made that the biotin molecule almost fills a hydrophobic pocket of avidin with a jigsaw fit, with one side of the biotin more exposed than the other.

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Avidin–Biotin Interaction. Crystal and Molecular Structures of Two Linked Models[1]

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Abstract: The nature of the strong interaction between biotin (**1**) and the glycoprotein avidin has been examined by X-ray analysis of two model compounds containing the biotin ring system separated by a trimethylene bridge from an indole ring that represents the tryptophan molecules present in the binding site of avidin. Crystals of Biot-C_3-Ind (**2**) hemihydrate are orthorhombic ($P2_12_12_1$) with $a = 46.229$ (13), $b = 7.533$ (2), and $c = 9.012$ (2) Å, and the structure containing two molecules (**2** and **2′**) in the asymmetric unit was refined to an R factor of 0.067 on 2285 nonzero reflections. The crystals of Biot(SO)-C_3-Ind (**6**) are also orthorhombic ($P2_12_12_1$) with $a = 10.634$ (3), $b = 13.641$ (2), and $c = 10.847$ (3) Å, and the structure has been refined to an R factor of 0.045 on 1595 nonzero reflections. One of the molecules (**2**) has a gauche arrangement around the central bond of the trimethylene bridge, while **2′** and **6** have a fully extended arrangement. There is no evidence for a significant intramolecular interaction between the biotin and the indole rings in any of the three molecules, nor is there any evidence for a strong intermolecular hydrogen bonding interaction between the rings. In the crystal of Biot-C_3-Ind, one of the molecules, **2′**, fits snugly into a hydrophobic V-shaped crevice formed by two indole rings. It is postulated that such an interaction may be significant in the avidin–biotin complex.

Introduction

The nature of the binding of biotin (**1**) and avidin, which together form one of the strongest biological complexes known, has not been established. It has been concluded, however, that spectroscopic changes and the protection of tryptophans from oxidation in the glycoprotein avidin which are brought about by complex formation with biotin are due to interaction between biotin and the tryptophans of each subunit of avidin.[2]

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