imide would be preferable since both steps would occur under mild conditions with a selective reagent. Unfortunately, as shown here, this reaction will not produce a quantitative amount of amine. Nevertheless the yields are relatively high and repeated treatment of some proteins has given encouraging results.[20]

From previous studies[13] it is clear that the particular soluble carbodiimide used here is not essential for the reaction, and other compounds, *e.g.*, the commercially available 1-ethyl-3-dimethylaminopropylcarbodiimide, would be equally efficient in these reactions.

Side Reactions. It is clear that the above procedure will produce a quantitative yield of the amine provided side reactions do not occur. These are very easy to avoid in the case of a simple free carboxylic acid in solution but are not so easy to avoid in the case of a complex polyfunctional protein. The possibility of inter- and intramolecular reactions of amino acid residues with reactive intermediates is, of course, not predictable unless the protein structure is known. The three-dimensional shape of the protein may place reactive groups in immediate proximity or it may prevent any such favorable orientation. From the rate constants obtained with methyl isocyanate and ammonium ion or alcohol it is seen that the main intermolecular reactions between two protein molecules would be slow at concentrations $\leq 10^{-3}$ *M* and by further dilution could be eliminated. However, the rate constants are sufficiently great so that the juxtaposition of a lysine side chain or an unusually reactive serine could produce intramolecular cross-linking reactions. Since the data indicate that a favorable conformation would be required for a trapping of the intermediate isocyanate, the finding of such cross-linking reactions would be an interesting clue to the geometry of the molecule. If such intramolecular reactions did occur, chromatographic separation of the cross-linked material from the remaining material might be necessary but the study might be rewarding in regard to its information on the structure of the molecule.

Implications of the Rearrangement. The rearrangement outlined here would allow one to convert the carboxylic acid group in proteins to a group of opposite charge and approximately the same steric size. This should be unusually interesting in studies on the conformational properties of proteins, in problems of the association of protein subunits with each other, in problems involved with the folding of protein, *i.e.*, the determination of three-dimensional structure from primary structure, and in problems related to carboxylic acid groups at active sites. Recently it has been shown that the specificity of a protein can be altered by the modification of protein side chains,[21] in that case a conversion of the hydrophobic methionine side chain to the polar methionine sulfoxide side chain. Perhaps similar results can be obtained by the conversion of the negatively charged carboxyl side chains of glutamic and aspartic acids to the positively charged 2,4-diaminobutyric or 2,3-diaminopropionic acid residues.

The conversion of the C-terminal carboxylic acid groups to an amine would lead to an aldehyde on acid hydrolysis and this might allow the development of a quantitative C-terminal procedure.

Acknowledgment. The authors are grateful for support of this research by the National Institutes of Health and the National Science Foundation.

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(21) H. Weiner, C. W. Batt, and D. E. Koshland, Jr., *J. Biol. Chem.*, **241**, 2687 (1966).

Synthesis and Fluorescent Properties of Some N-Methyl-2-anilino-6-naphthalenesulfonyl Derivatives[1]

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Contribution from the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331. *Received July 31, 1967*

Abstract: N-Methyl-2-anilino-6-naphthalenesulfonyl chloride has been synthesized and the fluorescent properties of its derivatives have been studied. The quantum yield of the sulfonamide is extremely sensitive to the polarity of its environment. A bovine serum albumin derivative has a high quantum yield in the native state which is significantly lowered when the protein is denatured with 5 *M* guanidine hydrochloride. When the protein is denatured with acid, the emission increases. The binding of the chromophore to the protein is covalent.

Weber and Laurence[3] found that the quantum yield of anilinonaphthalenesulfonates was extremely sensitive to the environment. The noncovalent binding of these salts to proteins was accompanied by a large increase in quantum yield.[4-6]

Of the methods of attaching such compounds covalently, that using the sulfonyl chloride suggests itself

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(2) National Institutes of Health Postdoctoral Fellow, 1966-1967.

(3) G. Weber and D. J. R. Laurence, *Biochem. J.*, **56**, xxxi (1954).

(4) J. E. Fildes, D. J. R. Laurence, and V. H. Rees, *ibid.*, **56**, xxxi (1954).

(5) J. A. Gally and G. M. Edelman, *Biochim. Biophys. Acta*, **94**, 175 (1965).

(6) L. Stryer, *J. Mol. Biol.*, **13**, 482 (1965).

since it is a common way to bind them to amino groups. However, presumably due to the presence of the secondary amine, the sulfonyl chloride could not be made. We have, therefore, prepared two N-methylanilinonaphthalenesulfonates and found that one of these can be activated to the sulfonyl chloride. This chloride has been used to prepare the amide and a bovine serum albumin (BSA) derivative. The amide showed large changes in quantum yield with changes in the polarity of the solvent. The BSA derivative showed changes in the presence of denaturants.

The synthesis of 2-*p*-toluidinylnapthalene-6-sulfonyl chloride has recently been reported[7] and the quantum yield of the conjugates are sensitive to environmental polarity.

In the nomenclature currently used, anilinonaphthalenesulfonic acid salts are known as ANS. Since we wish to make derivatives and to refer to various salts, it seems desirable to introduce a slightly different form. In this paper, the sodium salt of 1-anilino-8-naphthalenesulfonic acid will be called sodium 1,8-ansate. Likewise, N-methyl-2-anilino-6-naphthalenesulfonic acid (sodium salt) will be called sodium 2,6-mansate; the sulfonyl chloride, 2,6-mansyl chloride; and the sulfonamide, 2,6-mansylamide.

Experimental Section

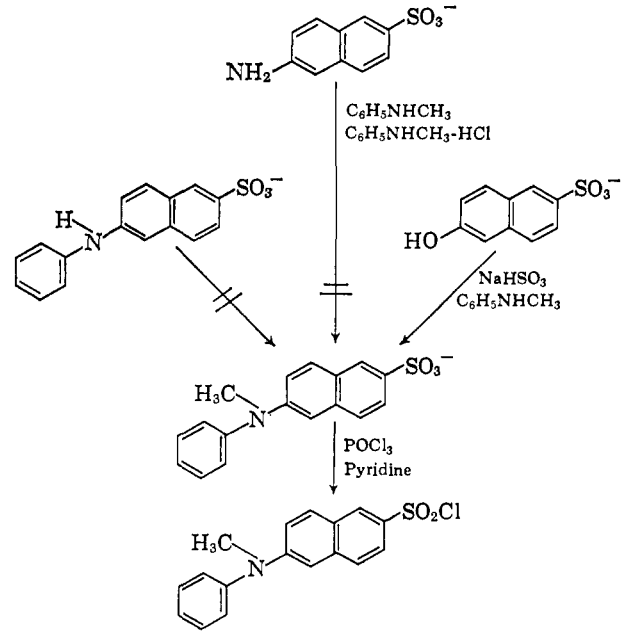
The naphthalenesulfonic acid salts used in this work were obtained from commercial sources and recrystallized from water. Where indicated, they were converted from the sodium or ammonium salts to the magnesium salt, or were specially synthesized as indicated below. Nmr spectra were obtained by Dr. F. T. Bond, Oregon State University, using solutions in deuterium oxide, employing a Varian Model A-60 nmr spectrometer. Ultraviolet spectra were obtained using a Cary Model 14 spectrophotometer. Fluorescence spectra were obtained using an Aminco-Bowman spectrophotofluorometer with an IP121 photomultiplier and Hruska photometer. Emission spectra are uncorrected. Melting points are uncorrected. Elemental analyses were performed by the Schwarzkopf Microanalytical Laboratory.

Magnesium 1,8-Mansate. Magnesium 1,8-ansate (12.93 g, 0.042 mol) was suspended in 125 ml of acetone, and 5.5 ml of dimethyl sulfate was added. The mixture was refluxed for 4 hr. Then 10 g of anhydrous sodium carbonate and 10 ml of dimethyl sulfate were added, and the mixture was refluxed for 20 hr. The mixture was dried *in vacuo* to remove the acetone and excess dimethyl sulfate. The product was crystallized from a hot 1% solution of magnesium sulfate and recrystalized from hot water. The yield was 2.53 g (19%). The nmr spectrum was like that of the starting material with an added three-proton band at 160 cps.

Magnesium 2,6-Ansate.[8] A mixture of 50 g (0.214 mol) of 6-amino-2-naphthalenesulfonic acid (magnesium salt), 100 g of aniline hydrochloride, and 300 ml of freshly distilled aniline were refluxed for 48 hr. The mixture was cooled and extracted with carbon tetrachloride to remove excess aniline, and the aqueous portion was filtered. The precipitate was crystallized from 3 l. of hot 1% magnesium sulfate solution and recrystallized from hot water. The yield was 18 g (27%).

Sodium 2,6-Mansate. A mixture of 70 g (0.282 mol) of 6-naphthol-2-sulfonic acid (sodium salt), 250 g of sodium bisulfite, 300 ml of N-methylaniline, and 1 l. of water was refluxed for 48 hr. The aqueous portion was filtered off through glass wool while keeping the mixture near the boiling point. The organic layer with solids was washed with chloroform after cooling and then filtered. The precipitate was washed thoroughly with chloroform and crystallized from a hot 1% sodium hydroxide solution. Recrystallization was from hot water. The aqueous phase of the reaction mixture was refluxed with fresh N-methylaniline for 6 days, and the work-up was repeated. The yield was 31 g (34%) for the first batch and 11.2 g (12%) for the second batch (Scheme I). The nmr spectrum showed a 3-proton singlet at 161 cps and 11 aromatic protons. The ultraviolet spectra of the 2,6-ansate and 2,6-mansate are shown in Figure 1.

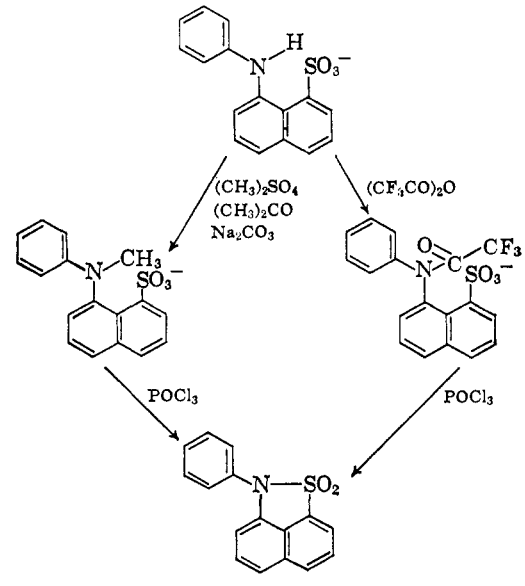
Scheme I



2,6-Mansyl Chloride. Sodium 2,6-mansate (19 g, 0.031 mol) was suspended in 100 ml of pyridine, and 10 ml of phosphorus oxychloride was added. The mixture was refluxed for 1.5 hr, cooled, and evaporated to dryness, without heating, *in vacuo*. The acetone-soluble fraction was evaporated, redissolved in acetone, reevaporated, then crystallized and recrystallized from acetone–water. The crystals were washed with boiling water, then dried *in vacuo*. The yield was 4.5 g (44%), mp 128–133°. A single spot was found on thin layer chromatography using silica gel F_{254} (Brinkmann Instruments) upon elution with carbon tetrachloride–chloroform, 3:1; R_f 0.85. The mansyl chloride was nonfluorescent but strong emission appeared after treatment with ammonia. Treatment with sodium hydroxide produced an ultraviolet spectrum identical with that of the starting material. *Anal.* Calcd for $C_{17}H_{14}ClNO_2S$: C, 61.5; H, 4.2; Cl 10.7; N, 4.2. Found: C, 62.2; H, 4.2; Cl, 10.1; N, 4.2.

2,6-Mansylamide. A solution of 1.0 g (3 mmol) of 2,6-mansyl chloride in 25 ml of acetone was treated with 15 ml of concentrated ammonium hydroxide solution, and the mixture was allowed to stand in the dark for 1 hr. It was then evaporated to a water suspension which was extracted with ethyl acetate. The ethyl

Scheme II



(7) W. O. McClure and G. M. Edelman, Seventh International Congress of Biochemistry, Tokyo, Japan, 1967, Abstracts, p 799.

(8) F. K. Beilstein, "Handbuch der organischen Chemie," Vol. XIV, Springer-Verlag, Berlin, 1931, p 762.

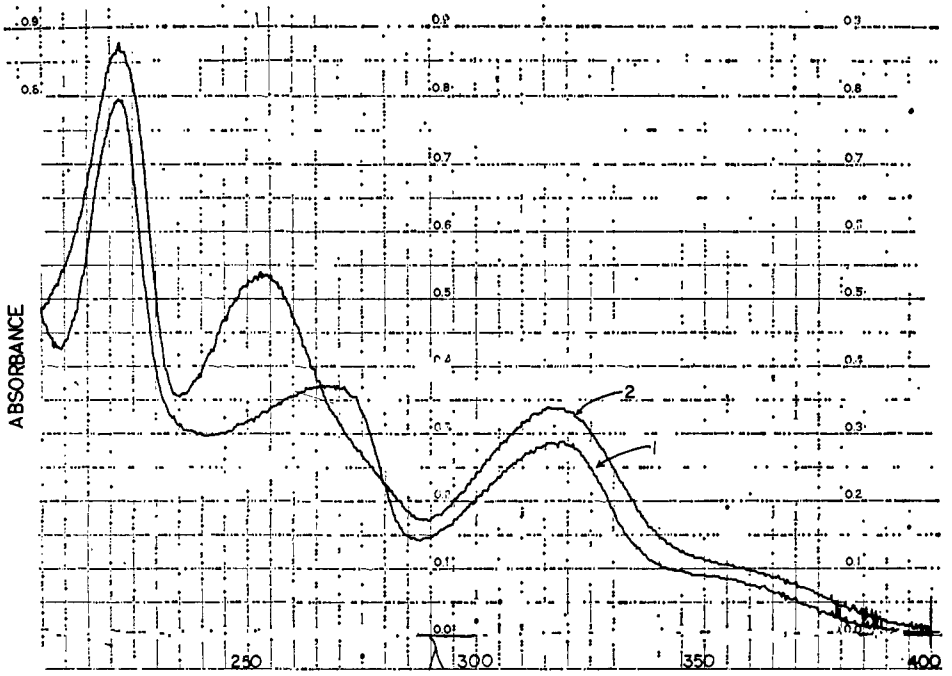


Figure 1. Ultraviolet spectra: 1, magnesium 2,6-ansate, 1.9×10^{-5} *M* in water; 2, sodium 2,6-mansate, 1.9×10^{-5} *M* in water. Absorbance *vs.* wavelength (λ) in nanometers.

acetate fraction was dried with sodium sulfate. Petroleum ether was added until the solution became cloudy and the sample was cooled. The yield was 0.50 g (53%) of light yellow crystals, mp 157–159.5°. *Anal.* Calcd for $C_{17}H_{16}N_2O_2S$: C, 65.5; H, 5.15; N, 8.95. Found: C, 65.5; H, 4.95; N, 8.84.

Other Attempts at Synthesizing Mansyl Compounds. Magnesium 1,8-mansate (2.18 g, 6.7 mmol) was refluxed for 24 hr with 25 ml of phosphorus oxychloride. The excess phosphorus oxychloride was removed *in vacuo*, and ice water was added. The suspension was extracted with ethyl acetate. The ethyl acetate fraction was dried with sodium sulfate and evaporated. The product was crystallized and recrystallized from carbon tetrachloride–petroleum ether. The product was identified as the N-phenyl-1,8-naphthalenesultam (mp 160–164°, lit.[9] mp 158°). *Anal.* Calcd for $C_{16}H_{11}NO_2S$: C, 68.3; H, 3.9; N, 5.0. Found: C, 68.4; H, 3.9; N, 5.2.

An attempt to make the 1,8-mansyl chloride through the N-trifluoroacetyl-1,8-ansic acid also gave the sultam (Scheme II).

Many attempts were made to make 2,6-mansic acid salts by direct methylation similar to that used for making magnesium 1,8-mansate above. Reactions were run using acetone, dimethylformamide, and pyridine as solvents; dimethyl sulfate and methyl iodide as methylating agents; and sodium carbonate, sodium bicarbonate, sodium hydroxide solution, barium acetate, and magnesium carbonate as bases. In all cases, only starting material was recovered. Also, an attempt was made to substitute N-methylaniline and N-methylaniline hydrochloride for aniline and aniline hydrochloride in the magnesium 2,6-ansate procedure, but only starting material was recovered.

Mansylation of Bovine Serum Albumin. Monomeric BSA (15 mg), prepared from Pentex Lot No. 12 by gel filtration on Sephadex G-200, was placed in each of two flasks and dissolved in 15 ml of 0.1 *M* sodium bicarbonate solution (pH 8.4). To one flask was added 2.2×10^{-4} g (3 mol/mol of BSA) of mansyl chloride in 0.1 ml of acetone, and to the other the same amount of sodium 2,6-mansate in 0.1 ml of water. Both were stirred for 24 hr at 4°. Then both were dialyzed for 48 hr against 1 l., with one buffer change, 0.1 *M* acetic acid–sodium acetate buffer at pH 5.5. Then for 48 hr, they were dialyzed against 800 ml of 0.12 *M* acetic acid, with one change, and then against the acetate buffer, pH 5.5, for 24 hr. A BSA control was made by dissolving 15 mg of monomeric BSA in 15 ml of the acetate buffer.

Results

We have made N-methyl-2-anilino-6-naphthalenesulfonyl chloride, a new compound which can be covalently attached to amino groups. The amide derivative shows changes in quantum yield with changes in its environment qualitatively similar to 1,8-ansate.[6] The mansyl moiety has been covalently attached to BSA.

The synthesis of the magnesium 1,8-mansate led to no useful derivatives, due to sultam formation whenever sulfonyl chloride preparation was attempted. However, the compound did show that methyl substitution of the anilino nitrogen did not destroy the sensitivity of quantum yield to changes in environment. We found that the 2,6-mansate also had this attribute and proceeded to make its sulfonyl chloride. The sulfonamide was made also and its fluorescent properties studied. A series of spectra using 2-propanol–water mixtures and chloroform[10] are shown in Figure 2.

The covalent nature of the binding of the 2,6-mansyl chloride was shown by the retention of 2.2 mansyl residues per BSA after dialysis against 0.12 *M* acetic acid as compared with 0.35 molecule of sodium 2,6-mansate per BSA. The 2,6-mansylated BSA showed a relatively high quantum yield, a not unexpected result considering the work with 1,8-ansate.[3] The quantum yield of the mansyl moiety in BSA was approximately equal to that of the 2,6-mansylamide in 100% 2-propanol. However, the mansyl BSA derivative is blue shifted by 15 nm compared to the emission spectrum of the amide in 2-propanol. The fluorescent emission of mansylated BSA at pH 2 was significantly higher than for the same material at pH 5.5, whereas the 2,6-mansylamide quantum yield in 2-propanol was lowered by the addition of 10^{-2} *M* hydrochloric acid, even when corrected for that change in quantum yield caused by dilution.

Mansyl BSA, denatured by 5 *M* guanidine hydrochloride, had about 25% of the quantum yield of the native

(9) W. Koenig and E. Wagner, *Ber.*, **57B**, 1056 (1924).

(10) We have observed that emission spectra of mansylamide in so-called gas chromatographically pure chloroform without ethanol preservative frequently showed very small quantum yields. High quantum yields were obtained with chloroform with preservative and with methylene chloride. We therefore ascribe the low quantum yields to interaction with an impurity, possibly phosgene.

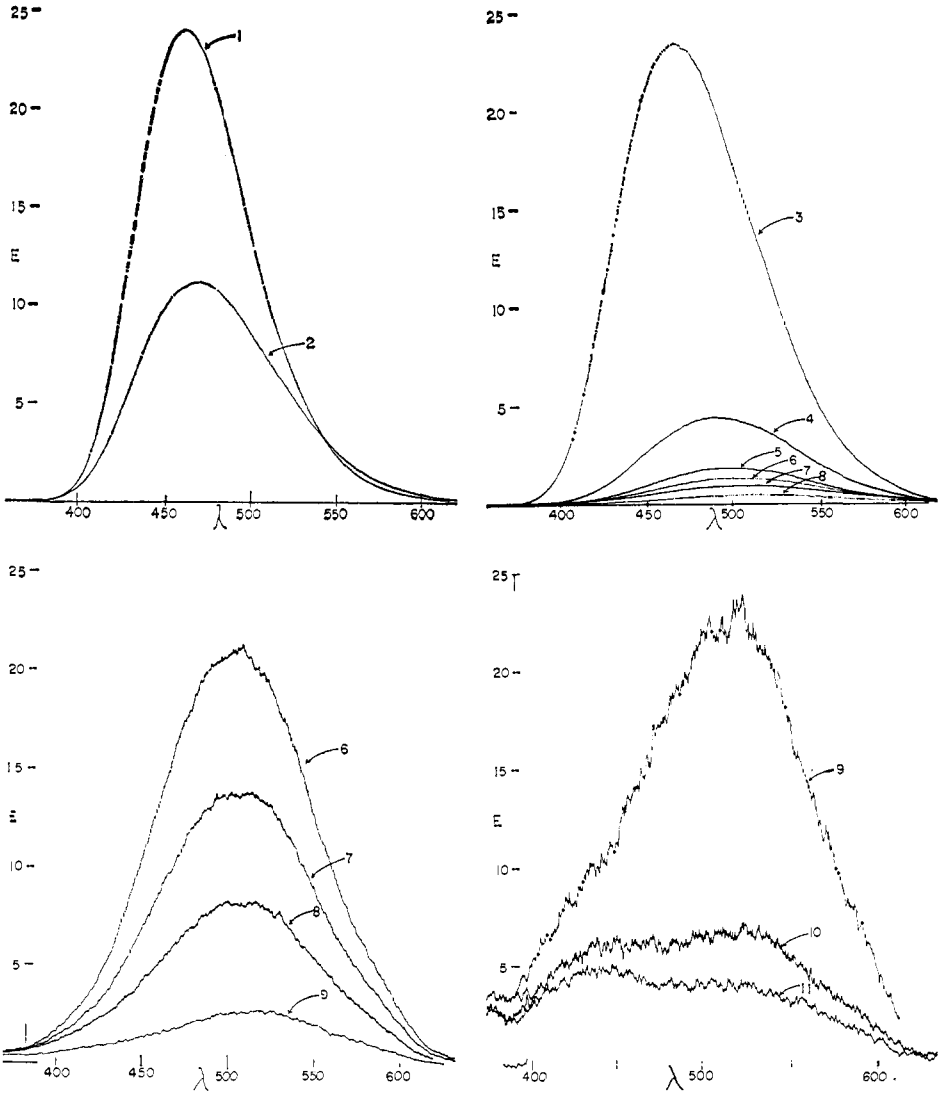


Figure 2. Fluorescence emission spectra of 2×10^{-5} *M* 2,6-mansylamide in various solvents. Emission (*E*) in relative units *vs.* wavelength (λ) in nanometers. Excited at 324 nm; solvents: 1, chloroform; 2 and 3, 100% 2-propanol; 4, 80% 2-propanol in water; 5, 60% 2-propanol; 6, 50% 2-propanol; 7, 40% 2-propanol; 8, 30% 2-propanol; 9, 20% 2-propanol; 10, 10% 2-propanol; 11, 4% 2-propanol.

derivative (Figure 3). It is difficult to estimate how much of this decrease is due to the direct action of guanidine hydrochloride on the mansyl chromophore. The most significant control would be to compare the effect of guanidine hydrochloride on the emission of mansylamide in 100% 2-propanol, *i.e.*, under conditions in which the mansylamide quantum yield is about that of the mansyl BSA derivative. However, guanidine hydrochloride is not sufficiently soluble in 2-propanol to do this. It is, however, possible to make a 5 *M* guanidine hydrochloride solution in 33% 2-propanol. In this solvent, the emission of 2,6-mansylamide was lowered by about 20%. This observation is not decisive and a more direct control would be desirable. Nevertheless, these data suggest that in the denaturation of the mansyl BSA by guanidine hydrochloride direct action of the salt on the mansyl moiety lowers the quantum yield by a small amount, but the major decrease probably results from an unfolding of the protein.

Fluorescent activation spectra, taken on native 2,6-mansyl BSA, showed a peak at the tryptophan absorption band indicating energy transfer from the protein to the mansyl chromophore.

The ultraviolet spectral data on many of these compounds are summarized in Table I.

Table I. Ultraviolet Spectral Data of Mansyl Derivatives

Compound	Solvent	λ, nm	$\epsilon \times 10^{-4}$
2,6-Mansyl-amide	2-Propanol	225	4.2
		255	3.0
		321	2.3
	10% 2-propanol	224	4.2
		255	2.6
		326	2.0
	Chloroform	258	2.6
		328	2.3
Sodium 2,6-mansate	Water	222	4.5
		254	2.8
		317	1.8
2,6-Mansyl chloride	Chloroform	283	1.5
		378	2.0
2,6-Mansyl BSA	0.1 *M* acetate, pH 5.5	330	...

Discussion

We find that the fluorescent quantum yield of N-methylanilinonaphthalenesulfonic acid derivatives is very

sensitive to the environment of the chromophore. It is qualitatively similar to the emission properties of the anilinonaphthalenesulfonates studied by others.[3-6] These compounds have been useful in studying protein structure, but according to Weber and Laurence[3] they can be used only with proteins which bind the chromophore rather tightly. We feel that the mansyl moiety, covalently attached, may provide some advantages in the use of protein fluorescent probes. In the preliminary studies reported here it has been shown that denaturing the protein leads to marked changes in the quantum yield of the chromophore. As would be expected in the presence of 5 *M* guanidine hydrochloride, the emission is decreased when the protein acquires a more random structure. This may be due to the decreased interaction of the mansyl residue with nonpolar regions of the protein.

However, the results obtained by comparing the emission of the mansyl BSA at pH 5.5 and pH 2.0 were not expected. Foster[11] reported that BSA is unfolded at pH 2.0. Wishnia and Pinder[12] found that butane and pentane were bound to BSA only 20% as much at pH 2 as at pH 5.5. Their data suggest that there are fewer effective hydrophobic binding sites at pH 2 than at pH 5.5. Our data suggest that upon denaturation by acid the covalently bound mansyl moeity is in a less polar environment. There is, thus, no conflict between these results; each technique probably measures a different property of BSA.

(11) J. F. Foster in "The Plasma Proteins," F. W. Putnam, Ed., Academic Press Inc., New York, N. Y., 1960, pp 179–239.
(12) A. Wishnia and T. Pinder, *Biochemistry*, 3, 1377 (1964).

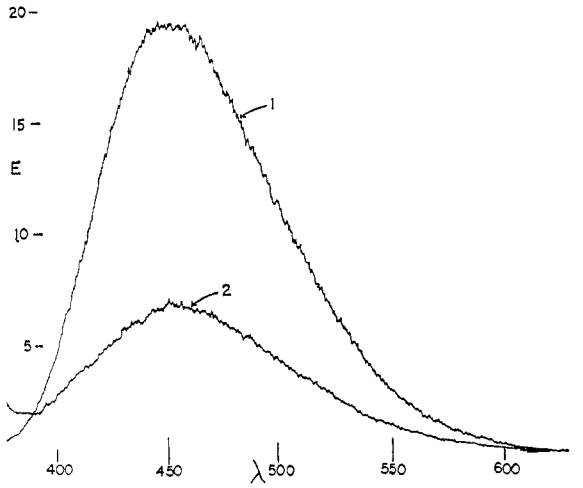


Figure 3. Effect of 5 *M* guanidine hydrochloride on the fluorescence emission of 2,6-mansyl BSA. Emission (*E*) in relative units *vs.* wavelength (λ) in nanometers. Excited at 360 nm; curve 1, mansyl BSA in buffer at pH 5.5; curve 2, mansyl BSA in 5 *M* guanidine hydrochloride at pH 5.5. Both samples are 6×10^{-6} *M* in mansyl.

McClure and Edelman[13] reported a rise in the fluorescence of 2-*p*-toluidinylnapthalene-6-sulfonate as the pH was lowered. However, their data may not be comparable to ours since, in our case, the chromophore is covalently linked to the protein.

Acknowledgment. The authors thank Dr. F. T. Bond for nmr spectra.

(13) W. O. McClure and G. M. Edelman, *ibid.*, 6, 559 (1967).

Communications to the Editor

The Stereospecific Total Synthesis of *dl*-Lycopodine

Sir:

Lycopodine (I) is one of many structurally related alkaloids found in numerous *Lycopodium* species.[1] We now report the total synthesis of *dl*-lycopodine.

The initial goal of our approach was the substituted quinolone II. Reaction of *m*-methoxybenzaldehyde with ethyl acrylate and triphenylphosphine[2] gave ethyl 4-(*m*-methoxyphenyl)-3-butenoate, bp 125–128° (0.1 mm), which on subsequent heating with ethyl acetoacetate (sodium ethoxide–ethanol overnight) followed by hydrolysis and decarboxylation (15% aqueous potassium carbonate, 30-hr reflux) led in 36% yield to 5-(*m*-methoxybenzyl)-1,3-cyclohexanedione (III), mp 110–110.5°. The dione was converted *via* the usual lithium aluminum hydride reduction of the ethyl enol ether into 5-(*m*-methoxybenzyl)cyclohexenone (IV), bp 139–146° (0.1 mm), λ^{film} 5.85 μ, and the latter then led (methylmagnesium iodide, catalytic amount of cupric chloride in ether) in 90% yield to *trans*-3-(*m*-methoxybenzyl)-5-methylcyclohexanone[3] (V), bp 140–142° (0.1 mm), λ^{film} 5.85 μ, δ 0.96 (d, $J \sim 7$ cps).

Transformation of V into II was accomplished by reaction of the pyrrolidinenamine of V with acrylamide (overnight reflux in dioxane followed by addition of water and heating 2 hr). The two isomeric quinolones anticipated from this reaction[4] could be readily separated by crystallization which gave in about 20–25% yield the desired II, mp 132–133°, λ^{CHCl_3} 6.0 μ, δ ($CDCl_3$) 0.95 (3 H d, $J = 5$ cps), accompanied by the incompletely purified lower melting isomer II′ (= II with methyl and methoxybenzyl interchanged), δ 1.0 (d, $J \sim 7$ cps). The structural assignment follows from the mass spectra which show, in addition to the molecular ion at *m/e* 285, a much larger peak at M − 121 (loss of methoxy benzyl) than at M − 15 (loss of methyl) for II, while the reverse is true of II′.

(1) *Cf.* K. Wiesner, *Fortschr. Chem. Org. Naturstoffe*, 20, 271 (1962).
(2) *Cf.* R. Oda, T. Kawabata, and S. Tanimoto, *Tetrahedron Letters*, 1653 (1964).
(3) *Cf.* N. L. Allinger and C. K. Riew, *ibid.*, 1269 (1966).
(4) This method of synthesis of 3,4,5,6,7,8-hexahydro-2-quinolones was first investigated by Dr. G. P. Moss of these laboratories, using the pyrrolidine enamine of cyclohexanone.