Molecular Conformation of Ammonium 8-Anilino-1-naphthalenesulfonate Hemihydrate. A Fluorescent Probe for Thyroxine Binding to Thyroxine Binding Globulin

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The crystal and molecular structure of the ammonium hemihydrate salt of the fluorescent dye, 8-anilino-1-naphthalenesulfonic acid (ANS), has been determined. There are two conformationally distinct molecules in the triclinic $P\bar{1}$ lattice. The anilino nitrogen of one molecule has slightly distorted planar geometry, and the overall conformation of the molecule is similar to that observed for the potassium salt of the fluorescent dye 2-*p*-toluidinyl-6-naphthalenesulfonic acid (TNS). The anilino nitrogen of the other molecule has slightly distorted tetrahedral geometry and the overall conformation of the molecule is similar to that observed for the thyroid hormones T_3 and T_4. The observation of two distinct conformational modifications of ANS in this crystal structure determination has shed light on the conformational flexibility of the ANS molecule itself and on the mode by which it acts as a competitive inhibitor in thyroid hormone transport proteins and as a signal for hydrophobic areas in macromolecular systems.

Fluorescent probes have been defined as small molecules which undergo changes in one or more of their fluorescence properties as a result of noncovalent interaction with a protein or macromolecule.[1] Generally, the fluorescence of a probe varies with the polarity of the solvent, increasing as the solvent polarity decreases.[2-3] In aqueous solution, the same effects occur with the addition of protein. These dyes are then valuable because the fluorescence characteristics are sensitive to their local environment, and these effects indicate the hydrophobic nature of the probe–protein binding sites.

Fluorescent probes of the *N*-arylaminonaphthalenesulfonate type are the most widely utilized in contemporary biochemical research for the assessment of hydrophobicity of binding sites on proteins and as a means of monitoring conformational changes in biological macromolecules. As such, they have been used to explore the binding sites of many substrates, enzymes, lipids, fatty acids, membranes, and serum proteins.[4-10] Of this class of dyes, 8-anilino-1-naphthalenesulfonate (ANS) has been most extensively used. Since the fluorescence of the ANS dyes dissolved in nonpolar solvents is similar to that observed when they are absorbed to proteins and membranes, they are known as "hydrophobic" probes.

In addition to its important use as a fluorescent probe for hydrophobic sites, ANS has been shown to act as a competitive inhibitor with thyroxine for the binding sites on the thyroid hormone transport proteins, thyroxine binding globulin (TBG) and thyroxine binding prealbumin (TBPA).[11-13] This property of ANS has also made it useful in radioimmunoassay techniques for measuring thyroid hormones in unextracted serum by blocking triiodothyronine binding to thyroxine binding globulin.[14]

The crystal structure of ANS (Figure 1) was undertaken in order to compare its conformation with the thyroid hormones T_3 and T_4.

Experimental Section

Crystals of ammonium 8-anilino-1-naphthalenesulfonate (ANS), purchased from Eastman Kodak Chemicals, were grown at room temperature from an aqueous solution. A clear, well-formed crystal (0.2 × 0.1 × 0.1 mm) was selected for intensity data collection. All x-ray measurements were made on a Nonius CAD-4 automatic diffractometer using Cu Kα radiation. The unit cell dimensions were obtained from a least-squares calculation based on 2θ measurements of 71 hkl reflections having $2\theta > 60°$ using Cu Kα radiation. The crystal data are presented in Table I.

The intensities of 6333 (5892 observed) independent reflections with θ less than 75° were measured on the Nonius CAD-4. Reflections were considered unobserved if the net count was less than twice the standard deviation of the background. No significant changes were observed in the intensities of the standard reflections measured during data collection. Intensities were corrected for Lorentz and polarization factors.

Table I. Crystal Data for Ammonium 8-Anilino-1-naphthalenesulfonate Hemihydrate

Formula	$(NH_4)\cdot(C_{16}H_{12}NSO_3)\cdot 0.5H_2O$
Mol wt	650.77
Space group	$P\bar{1}$
a	11.0117 (9) Å
b	13.935 (2) Å
c	10.4519 (8) Å
α	99.811 (6)°
β	95.423 (6)°
γ	100.142 (8)°
V	1542.82 Å^3
d_{calcd}	1.373 g cm^{-3}
Z	4
Crystal size	0.2 × 0.1 × 1.0 mm
R	6.8% (5892 observed data)
R	7.0% all data
μ	19.94 cm^{-1}

The structure was solved by direct methods using MULTAN.[15] All nonhydrogen atoms for the two molecules of ANS were located in the resulting E maps from MULTAN. The ammonium ions and water molecule were located in the first Fourier map after three cycles of isotropic full-matrix least-squares refinement. Further cycles of anisotropic refinement of the nonhydrogen atoms by full-matrix least squares reduced the R index ($R = \Sigma||F_o| - |F_c||/\Sigma F_o$) to 0.08.

Acceptable hydrogen atom positions for all hydrogens were located from three-dimensional Fourier difference maps, calculated without the hydrogen atom contributions to the structure factors. Both the positional and isotropic thermal parameters for the hydrogen positions were refined. The complete structure was refined by full-matrix least squares in two passes. One block of parameters included one ANS molecule with hydrogens and the ammonium and water molecules, and the second block included the other ANS molecule along with the ammonium and water molecules. Refinement was terminated when there was no further improvement in the esd's.

All scattering factors were taken from the International Tables for X-Ray Crystallography (1962). The weighting scheme used in the final refinement was $w^{-1} = \sigma(F_o)$ where $\sigma(F_o)$ is defined by Stout and Jensen[16] (1968, eq H.14) and the instability correction was 0.06 rather than 0.01. The final R value was 0.068 (Table I).

The final fractional coordinates and anisotropic thermal parameters for ANS and the fractional coordinates and isotropic thermal parameters for all hydrogen atoms are listed in Tables II and III, respectively. (Observed and calculated structure factors can be obtained from the author upon request.)

Results

The estimated standard deviations for the nonhydrogen bond lengths of the ANS molecules range from 0.002 to

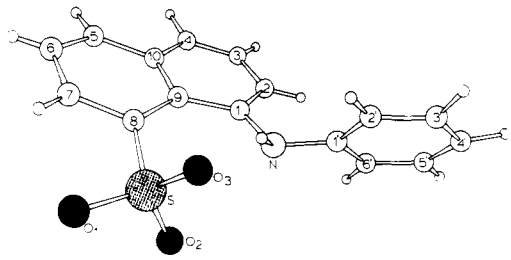

Figure 1. Ammonium 8-anilino-1-naphthalenesulfonate hemihydrate with numbering scheme.

0.008 Å with an average value of 0.004 Å, while the corresponding C–H values range from 0.03 to 0.04 Å with an average value of 0.035 Å. The esd's for the ammonium ions and water range from 0.03 to 0.07 Å and have an average value of 0.05 Å. The esd's for the corresponding bond angles range between 0.08 and 0.25° with an average value of 0.16° for the ANS molecules and between 0.81 and 1.1° with an average of 0.95° for the hydrogen atoms. The corresponding values for the ammonium and water range from 1.2 to 1.5° with an average value of 1.3°.

Most observed bonds and angles do not deviate significantly from their expected values and the individual bonds and angles in one molecule of ANS are within three standard deviations of their corresponding values in the other molecule.

One way in which the molecule can relieve steric strain is through the out-of-plane distortions involving the entire molecule. Deviations of the anilino nitrogen and sulfur atoms from the best least-squares plane through their respective rings are −0.24/−0.37 Å for N and 0.39/0.45 Å for S for molecules ANS(1) and ANS(2), respectively. The puckering of the naphthalene system is accomplished primarily by a twist about the central C(9)–C(10) bond of 5.6 and 6.4°, respectively, in the two ANS molecules. These values agree with those observed in other naphthalene derivatives.[17] The torsion angles made by the peri groups and the naphthalene ring are N–C(1)–C(9)–C(8) = −4.8, −10.5° and S–C(8)–C(9)–C(1) = −13.7,−15.1° for the two molecules, respectively.

The geometric details of the anilino linkage in ANS are listed in Table IV and are compared with the fluorescent dye potassium 2-*p*-toluidinyl-6-naphthalenesulfonate (TNS),[18] the thyroxine analogue 1-methyl-4′-methoxy-3,5-diiododiphenylamine (DN14),[19] and the thyroid hormone 3,5,3′-triiodo-L-thyronine (T_3).[20] As illustrated in Table IV, and in Figure 2, the C–N–C conformations in the individual molecules of ANS differ significantly from one another; however, the parameters of ANS(1) closely approximate those observed in DN14 and T3 while those of ANS(2) are similar to those observed in the structure of TNS.

The anilino nitrogen has slightly distorted tetrahedral geometry in ANS(1) as indicated by the C–N bond lengths, C–N–C angle, and the deviation of the hydrogen from the C–N–C plane. Similarly, the shorter C–N bond length, larger C–N–C angle, and smaller deviation of the hydrogen from the C–N–C plane indicate that the anilino nitrogen in ANS(2) has a slightly distorted trigonal geometry.

The shortening of the C–N distances (1.400 Å) from the expected single bond value of 1.471 Å suggests a significant degree of delocalization of the extended π-electron system between the phenyl and naphthalene rings. This extended π system has also been observed in other aromatic amines.[21]

The deviation of the C(1)–N–C(1′) plane from the naphthalene plane is significantly different (Table IV); and

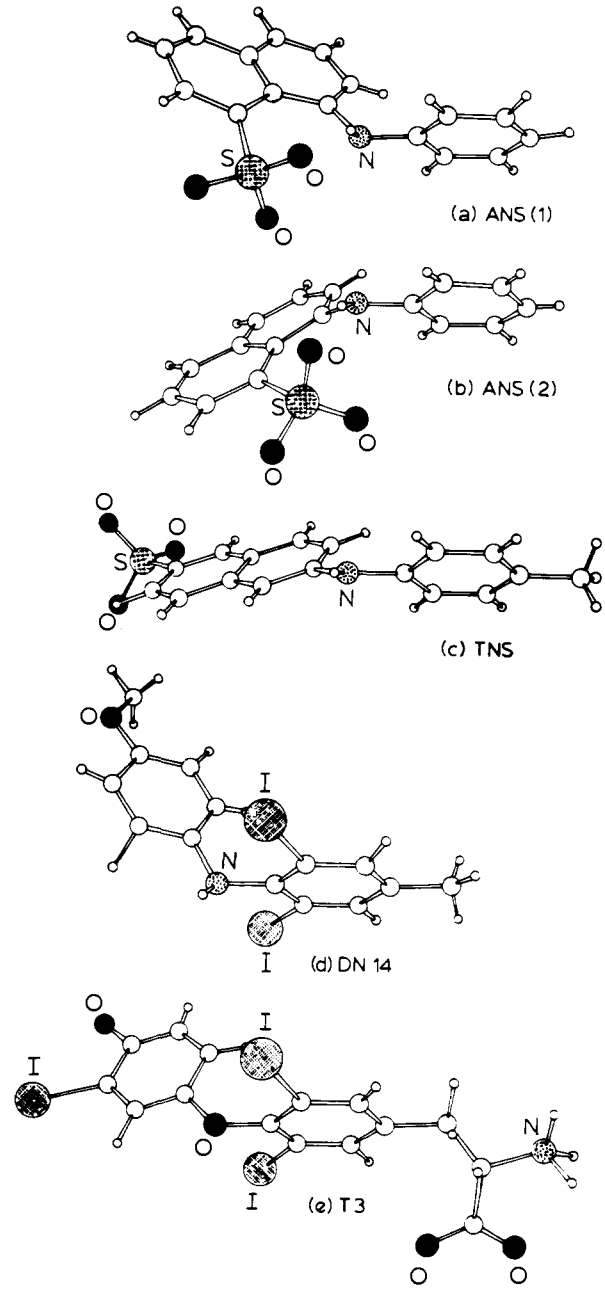

Figure 2. Conformational comparison of (a) ammonium 8-anilino-1-naphthalenesulfonate hemihydrate molecule 1, (b) molecule 2, (c) potassium 2-*p*-toluidinyl-6-naphthalenesulfonate, (d) 1-methyl-4′-methoxy-3,5-diiododiphenylamine, and (e) 3,5,3′-triiodo-L-thyronine.

the C(1)–N–C(1′) plane is nearly coplanar with the phenyl ring in molecule ANS(2) which as a nearly trigonal anilino nitrogen. In this form the C(1′)–N and C(1)–N distances are significantly shortened, indicating enhanced conjugation between the anilino group and the phenyl ring.

Since the C–N bonds in both ANS molecules (and to some degree those of ND14) show the same degree of double bond character, the angular deviation of the C(1)–N–C(1′) angle in ANS(2) from the trigonal 120° is not necessarily required to relieve any steric strain due to bond shortening.

The parameters of ANS(1) show the greatest similarity to the conformation observed in the structure of thyroid hormones.[20,22] As seen from Table IV, the C–N–C angle is near 120° and the conformation of the anilino linkage, as shown by the torsion angles, is near the ideal "skewed" geometry of 90/0° observed in the thyroid structures.

Table II. Positional and Thermal Parameters[a] and Their Estimated Standard Deviations for Ammonium 8-Anilino-1-naphthalenesulfonate Hemihydrate

Atom[b]	x/a	y/b	z/c	B_{11}	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
C(1)	0.4125 (2)	0.3275 (2)	0.2425 (2)	0.0067 (2)	0.0042 (1)	0.0081 (2)	0.0021 (2)	0.0024 (3)	0.0023 (2)
C(2)	0.4838 (3)	0.4122 (2)	0.3204 (3)	0.0082 (2)	0.0042 (1)	0.0092 (2)	0.0009 (3)	0.0021 (4)	0.0006 (3)
C(3)	0.4970 (3)	0.5056 (2)	0.2813 (3)	0.0088 (2)	0.0039 (1)	0.0115 (3)	0.0014 (3)	0.0024 (4)	0.0001 (3)
C(4)	0.4373 (3)	0.5130 (2)	0.1666 (3)	0.0099 (3)	0.0039 (1)	0.0137 (3)	0.0024 (3)	0.0051 (5)	0.0031 (3)
C(5)	0.2972 (3)	0.4429 (2)	−0.0345 (3)	0.0110 (2)	0.0057 (1)	0.0102 (2)	0.0059 (3)	0.0046 (4)	0.0066 (3)
C(6)	0.2208 (3)	0.3666 (2)	−0.1148 (3)	0.0109 (3)	0.0075 (2)	0.0081 (2)	0.0065 (3)	0.0019 (4)	0.0060 (3)
C(7)	0.2143 (3)	0.2699 (2)	−0.0914 (3)	0.0084 (2)	0.0063 (1)	0.0070 (2)	0.0027 (3)	0.0005 (4)	0.0031 (3)
C(8)	0.2804 (2)	0.2506 (2)	0.0172 (2)	0.0068 (2)	0.0041 (1)	0.0068 (2)	0.0013 (2)	0.0026 (3)	0.0023 (2)
C(9)	0.3533 (2)	0.3316 (2)	0.1149 (2)	0.0061 (2)	0.0040 (1)	0.0074 (2)	0.0027 (2)	0.0041 (3)	0.0020 (2)
C(10)	0.3655 (2)	0.4288 (2)	0.0825 (3)	0.0083 (2)	0.0042 (1)	0.0088 (2)	0.0028 (3)	0.0051 (4)	0.0034 (3)
N	0.3954 (3)	0.2378 (2)	0.2896 (2)	0.0133 (3)	0.0044 (1)	0.0070 (2)	−0.0036 (3)	−0.0029 (4)	0.0015 (2)
C(1′)	0.4104 (2)	0.2332 (2)	0.4243 (2)	0.0089 (2)	0.0035 (1)	0.0077 (2)	0.0025 (2)	0.0018 (4)	0.0013 (3)
C(2′)	0.5282 (2)	0.2536 (2)	0.4962 (3)	0.0073 (2)	0.0052 (1)	0.0087 (2)	0.0027 (3)	0.0018 (4)	0.0018 (3)
C(3′)	0.5404 (3)	0.2428 (2)	0.6260 (3)	0.0100 (2)	0.0059 (1)	0.0091 (3)	0.0064 (3)	−0.0000 (4)	0.0018 (3)
C(4′)	0.4368 (4)	0.2086 (2)	0.6826 (3)	0.0156 (4)	0.0065 (2)	0.0075 (2)	0.0049 (4)	0.0039 (5)	0.0036 (3)
C(5′)	0.3223 (3)	0.1878 (3)	0.6106 (3)	0.0112 (3)	0.0081 (2)	0.0093 (3)	−0.0001 (4)	0.0082 (4)	0.0016 (4)
C(6′)	0.3081 (3)	0.2003 (2)	0.4822 (3)	0.0081 (2)	0.0059 (2)	0.0081 (2)	0.0023 (3)	0.0025 (4)	−0.0003 (3)
O(1)	0.3961 (2)	0.1035 (1)	0.0276 (2)	0.0084 (2)	0.0050 (1)	0.0115 (2)	0.0035 (2)	0.0024 (3)	0.0020 (2)
O(2)	0.2047 (2)	0.0695 (2)	−0.1130 (3)	0.0136 (2)	0.0054 (1)	0.0084 (2)	−0.0005 (3)	−0.0030 (4)	−0.0007 (2)
O(3)	0.2028 (2)	0.0972 (2)	0.1216 (2)	0.0102 (2)	0.0055 (1)	0.0096 (2)	−0.0018 (2)	0.0058 (3)	0.0021 (2)
S	0.27028 (6)	0.12077 (4)	0.01456 (6)	0.00712 (5)	0.00395 (3)	0.00653 (5)	0.00045 (6)	0.00106 (8)	0.00093 (6)
C(1*)	0.8616 (2)	0.3903 (2)	0.4165 (2)	0.0063 (2)	0.0038 (1)	0.0083 (2)	0.0021 (2)	0.0012 (3)	0.0014 (3)
C(2*)	0.8306 (3)	0.4725 (2)	0.3751 (3)	0.0098 (2)	0.0047 (1)	0.0096 (3)	0.0034 (3)	0.0011 (4)	0.0020 (3)
C(3*)	0.8064 (3)	0.5526 (2)	0.4624 (4)	0.0095 (2)	0.0041 (1)	0.0150 (3)	0.0039 (3)	0.0014 (5)	0.0017 (4)
C(4*)	0.8186 (3)	0.5517 (2)	0.5930 (3)	0.0079 (2)	0.0044 (1)	0.0147 (3)	0.0027 (3)	0.0045 (5)	−0.0025 (4)
C(5*)	0.8729 (3)	0.4746 (3)	0.7788 (3)	0.0092 (2)	0.0078 (2)	0.0090 (3)	0.0019 (4)	0.0044 (4)	-0.0049 (4)
C(6*)	0.8990 (3)	0.3970 (3)	0.8286 (3)	0.0124 (3)	0.0100 (2)	0.0067 (2)	0.0056 (4)	0.0037 (4)	-0.0012 (4)
C(7*)	0.9021 (3)	0.3086 (3)	0.7438 (3)	0.0089 (2)	0.0081 (2)	0.0073 (2)	0.0039 (3)	0.0014 (4)	0.0010 (4)
C(8*)	0.8858 (2)	0.2997 (2)	0.6097 (2)	0.0058 (2)	0.0053 (1)	0.0067 (2)	0.0026 (2)	0.0011 (3)	0.0009 (3)
C(9*)	0.8655 (2)	0.3837 (2)	0.5526 (2)	0.0049 (2)	0.0044 (1)	0.0076 (2)	0.0020 (2)	0.0015 (3)	0.0001 (3)
C(10*)	0.8514 (2)	0.4700 (2)	0.6410 (3)	0.0061 (2)	0.0054 (1)	0.0095 (2)	0.0023 (3)	0.0032 (4)	−0.0017 (3)
N*	0.8983 (2)	0.3177 (2)	0.3278 (2)	0.0095 (2)	0.0043 (1)	0.0069 (2)	0.0038 (2)	0.0022 (3)	0.0008 (2)
C(1′*)	0.8417 (3)	0.2756 (2)	0.2016 (2)	0.0104 (2)	0.0040 (1)	0.0062 (2)	−0.0002 (3)	0.0044 (4)	0.0026 (2)
C(2′*)	0.7231 (3)	0.2881 (2)	0.1526 (3)	0.0093 (2)	0.0063 (2)	0.0083 (2)	−0.0010 (3)	0.0019 (4)	0.0026 (3)
C(3′*)	0.6706 (4)	0.2401 (3)	0.0268 (3)	0.0141 (4)	0.0085 (2)	0.0090 (3)	−0.0055 (5)	−0.0015 (5)	0.0048 (4)
C(4′*)	0.7305 (5)	0.1770 (3)	−0.0496 (3)	0.0279 (7)	0.0070 (2)	0.0075 (3)	−0.0056 (7)	0.0022 (8)	0.0004 (4)
C(5′*)	0.8458 (5)	0.1651 (3)	−0.0003 (3)	0.0289 (6)	0.0060 (2)	0.0082 (3)	0.0087 (5)	0.0096 (6)	0.0003 (4)
C(6′*)	0.9030 (4)	0.2152 (2)	0.1228 (3)	0.0171 (4)	0.0052 (1)	0.0088 (2)	0.0075 (4)	0.0081 (5)	0.0033 (3)
O(1*)	0.7652 (2)	0.1511 (1)	0.4273 (2)	0.0080 (2)	0.0050 (1)	0.0121 (2)	0.0014 (2)	−0.0006 (3)	−0.0001 (2)
O(2*)	0.8664 (2)	0.1125 (2)	0.6188 (2)	0.0175 (3)	0.0065 (1)	0.0112 (2)	0.0076 (3)	0.0047 (4)	0.0075 (2)
O(3*)	0.9897 (2)	0.1753 (2)	0.4602 (2)	0.0080 (1)	0.0063 (1)	0.0111 (2)	0.0063 (2)	0.0033 (3)	0.0002 (3)
S*	0.87582 (6)	0.17562 (4)	0.52134 (6)	0.00769 (5)	0.00424 (3)	0.00817 (5)	0.00360 (6)	0.00168 (8)	0.00231 (6)
N(1**)	0.0193 (2)	0.0193 (2)	0.2658 (2)	0.0078 (2)	0.0050 (1)	0.0073 (2)	0.0019 (2)	0.0029 (3)	0.0023 (2)
N(2**)	0.4225 (2)	0.9457 (2)	0.7884 (2)	0.0094 (2)	0.0060 (1)	0.0092 (2)	0.0035 (3)	0.0006 (4)	0.0020 (3)
O(3**)	0.4063 (3)	0.9865 (2)	0.3795 (3)	0.0163 (3)	0.0065 (2)	0.0221 (4)	0.0005 (4)	0.0144 (5)	0.0047 (4)

[a] Temperature factors are of the form $\exp[-(\beta_{11}h^2 + \beta_{22}k^2 + \beta_{33}l^2 + \beta_{12}hk + \beta_{13}hl + \beta_{23}kl)]$. [b] * indicates molecule two. ** indicates ammonium and water molecules.

Table III. Atomic Positional and Isotropic Thermal Parameters for the Hydrogen Atoms of Ammonium 8-Anilino-1-naphthalenesulfonate Hemihydrate

Atom[a]	x/a	y/b	z/c	B_{11}
H(2)	0.514 (3)	0.401 (2)	0.401 (3)	4.1 (6)
H(3)	0.559 (3)	0.568 (3)	0.339 (3)	5.9 (9)
H(4)	0.442 (3)	0.586 (2)	0.142 (3)	5.3 (8)
H(5)	0.310 (3)	0.512 (3)	−0.067 (3)	6.0 (8)
H(6)	0.187 (3)	0.377 (2)	−0.182 (3)	5.7 (8)
H(7)	0.166 (3)	0.218 (2)	−0.141 (3)	5.9 (7)
H	0.315 (3)	0.202 (3)	0.261 (3)	4.9 (8)
H(2′)	0.619 (3)	0.281 (3)	0.460 (3)	5.0 (9)
H(3′)	0.623 (3)	0.256 (2)	0.674 (3)	3.6 (6)
H(4′)	0.438 (3)	0.209 (3)	0.778 (3)	5.3 (8)
H(5′)	0.248 (4)	0.175 (3)	0.650 (4)	5.2 (10)
H(6′)	0.234 (3)	0.188 (2)	0.439 (3)	4.1 (7)
H(2*)	0.832 (3)	0.469 (3)	0.288 (3)	4.7 (8)
H(3*)	0.785 (3)	0.603 (2)	0.435 (3)	3.5 (6)
H(4*)	0.809 (3)	0.598 (3)	0.648 (3)	6.1 (8)
H(5*)	0.861 (4)	0.538 (3)	0.840 (4)	8.7 (9)
H(6*)	0.911 (3)	0.394 (3)	0.925 (3)	7.1 (8)
H*	0.953 (3)	0.277 (2)	0.361 (3)	4.5 (7)
H(2′*)	0.682 (3)	0.330 (3)	0.201 (3)	5.2 (8)
H(3′*)	0.595 (4)	0.234 (3)	−0.006 (4)	6.1 (9)
H(4′*)	0.700 (4)	0.154 (3)	−0.126 (4)	10.0 (10)
H(5′*)	0.888 (4)	0.127 (3)	−0.040 (4)	6.6 (10)
H(6′*)	0.969 (3)	0.203 (2)	0.846 (4)	6.0 (9)
H(1**A)	0.081 (3)	0.048 (3)	0.217 (3)	4.5 (7)
H(1**B)	0.049 (2)	−0.024 (2)	0.307 (2)	3.4 (5)
H(1**C)	0.017 (4)	0.065 (3)	0.322 (4)	6.6 (10)
H(1**D)	−0.034 (3)	0.004 (2)	0.214 (3)	3.9 (5)
H(2**A)	0.468 (3)	0.972 (3)	0.731 (4)	5.1 (8)
H(2**B)	0.466 (3)	0.913 (2)	0.820 (3)	6.1 (7)
H(2**C)	0.388 (4)	0.983 (3)	0.845 (4)	8.9 (10)
H(2**D)	0.341 (6)	0.899 (5)	0.739 (6)	16.7 (18)
H(3**A)	0.353 (3)	0.960 (3)	0.384 (4)	6.1 (8)
H(3**B)	0.402 (5)	1.033 (4)	0.424 (5)	9.3 (15)

[a] * indicates molecule two. ** indicates ammonium and water molecules.

Since ANS displaces the thyroid hormones from their carrier proteins, the adoption of a similar conformation allows ANS to compete more effectively at the binding sites.

The two ANS molecules, two ammonium ions, and one water molecule in the asymmetric portion of the lattice are held together by a network of eight intermolecular hydrogen bonds involving the eight ammonium hydrogens with the sulfonate and water oxygens. There are two intramolecular hydrogen bonds involving the anilino nitrogen with the sulfonate oxygens. In each case, the hydrogen bonds an analogous oxygen even though the conformations of the two sulfonate groups [C(9)–C(8)–S–O(3) = 68/77°, respectively] and the anilino geometries [C(9)–C(1)–N–H = −36/−22° respectively] differ slightly. Short H···H and H···O contact distances among the nonhydrogen bonded hydrogens are illustrated in Figure 3.

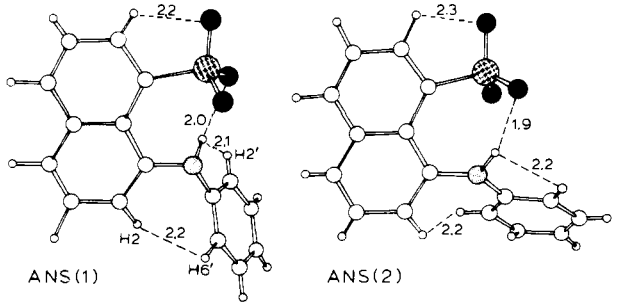

Figure 3. Intramolecular hydrogen bonded and H···H contact distances in the two molecules of ANS.

The N···O hydrogen bonded distances are well within the 2.52 Å predicted from the sum of van der Waals radii[23] suggesting a strong hydrogen bonded network. The same is true for the corresponding N···O distances when compared to the expected value of 3.07 Å. The only O···O contact is 3.08 Å, which is longer than the expected value of 3.04 Å, and is highly doubtful that this is a true hydrogen bond.

The ANS molecules are stacked with their sulfonate groups pointing into the hydrophylic channels formed by the water and ammonium ions.

Discussion

The structural similarities between the observed conformation of ANS(1) and those of the thyroid hormones (overlap of ring systems) may explain the effectiveness of ANS as a competitor for the thyroid hormone binding sites.[20,22,24] The average conformation of the diphenyl ether segment in the thyroid hormone structures observed crystallographically has a C–O–C angle of 119° and the torsion angles ϕ and ϕ' of 99°/16° for the transoid model and −90°/−24° for the cisoid model,[19] respectively. In addition, the conformation of ANS(1) most closely approximates the observed parameters for the distal conformer of the hormone 3,5,3′-triiodo-L-thyronine (T_3), thus providing the maximum overlap of ring surface area.

While the exact nature of the binding sites on the thyroid hormone transport proteins is not known, information on these binding sites can be based on the degree with which various thyroxine analogues and inhibitors bind to these proteins. The major considerations for binding to TBG[25,26] require that (1) the entire thyroxine molecule has surface contact with the protein, (2) a tetrasubstituted ring system is preferrable, (3) iodine (or halogen) is preferrable to alkyl substituents, and (4) the alanine side chain has the optimal length for maximum binding efficiency. In the case of TBPA, cystallographic protein data[27] show the binding site to be a channel or cavity into which the thyroxine is trapped.

In both instances, one feature which is of great importance is the requirement for halogen substitution and

Table IV. Geometry of the Anilino Linkage in Ammonium 8-Anilino-1-naphthalenesulfonate Hemihydrate

Property	ANS(1)	ANS(2)	TNS[a]	DN14[b]	T_3[c]
C(1′)-N, Å	1.415	1.386	1.402	1.433	1.37
C(1)-N, Å	1.409	1.393	1.391	1.415	1.43
C(1′)-N-C(1), deg	123.4	127.3	127.8	119.4	121.0
C(6′)-C(1′)-N-C(1), deg	112	171	151	−92	116
C(1′)-N-C(1)-C(2), deg	22	−46	−24	27	−21
H-N-C(1′)-C(2′), deg	−12	−27	−23	−48	
H-N-C(1)-C(9), deg	−26	−22	−27	−19	
Σ valency angles, deg	335.7	357.6	359.7	345.1	
H from C(1)-N-C(1′) plane, Å	−0.75	−0.28	0.08	−0.50	
N from C(1)-C(1′)-H plane, Å	0.35	0.11	−0.04	0.25	

[a] A. Camerman and L. H. Jensen, *J. Am. Chem. Soc.*, 92, 4200 (1970). [b] V. Cody and R. Mukherjee, *Acta Crystallogr., Sect. B*, 31, 2168 (1975). [c] V. Cody, *J. Am. Chem. Soc.*, 96, 6720 (1974) (oxygen replaces nitrogen).

the ability of these halogens to participate in charge-transfer complexes with the receptor protein. In this respect, the ANS dyes could compete favorably since another important property of this class of dyes is their ability to form intra- and intermolecular charge-transfer complexes. It has been shown that the increased ability to form charge-transfer complexes is followed by a decrease in the ionization potential of the amine or an increase in the electron affinity of the naphthalene ring system. As an anion, ANS dyes may interact with positively charged residues on the macromolecule and therefore could be important as a probe for charge as well as mobile dipoles. Studies with fluorescent dyes have suggested that a significant change in either charge distribution, hydrophobicity, or conformational state of molecular components may occur upon the initial coupled-electron transfer.

Although the mechanism of fluorescence enhancement in proteins is not clearly understood, it has been suggested that the relative orientation of the two ring systems in the probes may influence their fluorescence characteristics.[4,6,28-31] The results of an NMR study on ANS[30] suggest that the aromatic rings of ANS are more nearly coplanar in alcohol than in water and that the anilino hydrogen participates in a strong interaction with the sulfonate group in alcohol. The NMR study also shows that there is an anomalous behavior of the proton signals of the H(2), H(2′), and H(6′) protons in both solvents. Since the deshielding effects are more pronounced in alcohol, it is assumed that the rings of ANS are more nearly coplanar in this solvent. Finally, it is suggested from this study that solvents which favor intramolecular hydrogen bond formation will favor a conformation which has the rings nearly coplanar.

The presence of an intramolecular N—H···O hydrogen bond in each ANS molecule (Figure 3) indicates that both the coplanar and noncoplanar geometries of the anilino nitrogen are equally capable of hydrogen bond formation. The hydrogen bonds are nonlinear as predicted,[30] and the N—H···O angles are 146 and 153°, respectively.

As illustrated in Figure 3, there are short N···H intramolecular contacts involving H(2), H(2′), and H(6′) which are the hydrogens that gave rise to the anomalous proton signal behavior in both solvents studied. These close contacts, which could be relieved by a change in phenyl ring conformation, may be intrinsic to the molecule and explain why the anomalous NMR behavior is not solvent dependent. Since the hydrogen contacts to the sulfonate ion are essentially the same in both molecules despite the gross conformational change involving the anilino nitrogen, a change in the anilino geometry is not required to explain the anomalous deshielding effects seen in the NMR study.[30] The dihedral angles between the phenyl ring and naphthalene ring planes are 63 and 127°, respectively. Steric considerations of the peri groups based on space-filling models indicate that the smallest angle between the ring planes is 40°.[30]

It has been recently reported that in the protein crystal structure of horse liver alcohol dehydrogenase[32] there are two binding sites for ANS. The preliminary x-ray data show that the two ANS molecules bind in two different conformations (Eklund, personal communication).

If the relative orientation of the two rings has a significant influence upon the mechanism of fluorescence,[29-31] then the fluorescence properties of the two conformers observed here and in the protein receptor sites are probably different. The observation of two different noncoplanar conformers in these crystals as well as in the protein receptor sites suggests that a coplanar conformation of the two rings may not be essential to fluorescence.

In conclusion, the observation of two distinct conformational modifications of ANS in this crystal structure determination has shed light on the conformational flexibility of the ANS molecule itself and on the mode by which it acts as a competitive inhibitor in thyroid hormone transport proteins and as a signal for hydrophobic areas in macromolecular systems. The conformational flexibility observed in this structure points to the need to have a better understanding of the quantitative parameters which control fluorescence and the mechanisms by which this phenomenon occurs in these macromolecular systems.

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Supplementary Material Available: Table V, hydrogen bonds in ammonium 8-anilino-1-naphthalenesulfonate hemihydrate; Figure 4, bond distances and bond angles; Figures 5 and 6, hydrogen bonding and packing in the lattice (5 pages). Ordering information is given on any current masthead page.

References and Notes

(1) G. M. Edelman and W. O McClure, *Acc. Chem. Res.*, 1, 65 (1968).
(2) L. Stryer, *J. Mol. Biol.*, 13, 482 (1965).
(3) W. O. McClure and G. M. Edelman, *Biochemistry*, 5, 1908 (1966).
(4) L. Brand and J. R. Gohlke, *Annu. Rev. Biochem.*, 41, 843 (1972).
(5) G. Weber and E. Daniel, *Biochemistry*, 5, 1900 (1966).
(6) S. R. Anderson and G. Weber, *Biochemistry*, 8, 371 (1969).
(7) D. H. Haynes and H. Starek, *J. Membr. Biol.*, 17, 313 (1974).
(8) S. M. Aloj, K. C. Ingham, and H. Edelhoch, *Arch. Biochem. Biophys.*, 155, 478 (1973).
(9) D. A. Kolb and G. Weber, *Biochemistry*, 14, 4476 (1975).
(10) S. Tu and J. W. Hastings, *Biochemistry*, 14, 4310 (1975).
(11) R. F. Steiner, J. Roth, J. Robbins, *J. Biol. Chem.*, 241, 560 (1966).
(12) A. M. Green, J. S. Marshall, J. Pensky, and J. B. Stanbury, *Biochim. Biophys. Acta*, 278, 305 (1972).
(13) R. N. Ferguson, H. Edelhoch, H. A. Saroff, J. Robbins, and H. J. Cahnmann, *Biochemistry*, 14, 282 (1975).
(14) M. L. Brown and J. Metheany, *J. Pharm. Sci.*, 63, 1214 (1974).
(15) G. German, P. Main, and M. M. Woolfson, *Acta Crystallogr., Sect. A*, 27, 368 (1971).
(16) G. H. Stout and L. H. Jensen, "X-Ray Structure Determination", Macmillan, New York, N.Y., 1968, p 168.
(17) J. B. Robert, J. S. Sherfinski, R. E. Marshall, and J. O. Roberts, *J. Org. Chem.*, 39, 1152 (1974).
(18) A. Camerman and L. H. Jensen, *J. Am. Chem. Soc.*, 92, 4200 (1970).
(19) V. Cody and R. Mukherjee, *Acta Crystallogr., Sect. B*, 31, 2168 (1975).
(20) V. Cody, *J. Am. Chem. Soc.*, 96, 6720 (1974).
(21) O. Kennard, *Mol. Struct. Dimensions, Ser. A1*, 1 (1972).
(22) V. Cody, *J. Med. Chem.*, 18, 126 (1975).
(23) T. J. Koetzle in "Spectroscopy in Biology and Chemistry", S. H. Chen and S. Yip, Ed., Academic Press, New York, N.Y., 1974, p 177.
(24) V. Cody and J. Hazel, *Biochem. Biophys. Res. Commun.*, 68, 425 (1976).
(25) Y. Hao and M. Tabachnick, *Endocrinology*, 88, 81 (1971).
(26) V. Cody in "Thyroid Research", L. Braverman and J. Robbins, Ed., Excerpta Medica, Netherlands, 1976, p 290.

(27) C. C. F. Blake, M. J. Geisow, and I. D. A Swan, *J. Mol. Biol.*, 88, 1 (1974).
(28) T. Forster, *Naturwissenschaften*, **33**, 220 (1946).
(29) F. C. Green, *Biochemistry*, **14**, 747 (1975).
(30) G. R. Penzer, *Eur. J. Biochem.*, **25**, 218 (1972).
(31) R. P. Cory, R. R. Becker, R. Rosenbluth, and I. Isenberg, *J. Am. Chem. Soc.*, **90**, 1643 (1968).
(32) H. Eklund, B. Nordstrom, E. Zeppenzauer, G. Soderlund, J. Ohlsson, T. Boiwe, and C. I. Branden, *FEBS Lett.*, **44**, 200 (1974).

Cannabinoids. 1. 1-Amino- and 1-Mercapto-7,8,9,10-tetrahydro-6*H*-dibenzo[*b*,*d*]pyrans

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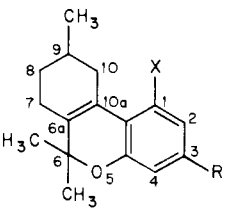
A series of 1-amino- and 1-mercapto-7,8,9,10-tetrahydro-6*H*-dibenzo[*b*,*d*]pyrans was synthesized and subsequently evaluated in three rodent test systems for CNS activity. The structure–activity data generated indicate that, in general, a change of the 1-hydroxy group to an amine results in a retention of pharmacological activity but that a change to sulfur results in loss of pharmacological activity. Derivatization of the 1-amino group with various functions decreased the activity of the parent compound. For optimum potency, in all series, the 3-position alkyl side chain should be either 1,1- or 1,2-dimethylheptyl. With either the 1-hydroxy- or 1-amino-7,8,9,10-tetrahydro-3-(1,1-dimethylheptyl)-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*]pyran (**4c** or **10c**), preparation of the optically active antipodes did not lead to any great degree of separation of activity. Both of the antipodes possess pharmacological activity as measured in these rodent test systems.

During the 1940's, two independent groups of workers began their studies on the chemistry of the natural products from marihuana: Adams and co-workers in the United States and Todd and co-workers in England. From their investigations into the structures of the natural products from *Cannabis sativa* L., extensive programs in syntheses of 7,8,9,10-tetrahydro-6*H*-dibenzo[*b*,*d*]pyrans (THDP) evolved. The resulting cannabinoid derivatives were tested in dog-ataxia and rabbit-corneal-areflexia assays for their pharmacological activity. An excellent summary of this work including tables of structure–activity data is provided by Mechoulam.[1]

A reexamination of the relative CNS potency of a series of 3-alkyl derivatives recently has been reported by Loev and co-workers.[2] Using rats as test animals, they observed structure–activity correlations that differed significantly from those reported by Adams and Todd for dogs and rabbits. Also, with specific relation to the 1 position of the synthetic THDP's, Loev and co-workers found that acetylation of the phenolic hydroxyl group diminished and methylation eliminated activity in their rat assays.

Additionally, in contrast to previously reported work,[3] Loev and co-workers reported that replacement of the 1-hydroxyl by hydrogen eliminated activity in the case where the 3-position side chain was *n*-pentyl but *not* where the 3-position side chain was 1,2-dimethylheptyl (1,2-DMH). In spite of this exception, it appears that a phenolic hydroxyl in the 1 position is a necessary requirement for pharmacological activity in most cannabinoids; removal of the hydroxyl or methylation leads to loss of activity.[2,4]

To our knowledge, no one has reported the effect on activity by replacement of the 1-position oxygen by other heteroatoms, namely, nitrogen and sulfur. We wish, therefore, to report on the synthesis and CNS activity of a series of 1-amino- and 1-mercapto-THDP's represented by the general formula



X = NR_1R_2 or SH
R = alkyl or cycloalkyl

Our choice of R was predicated on the basis of studies in our laboratories that indicated when the 1 position was substituted by a hydroxyl the greatest potency occurred when R was either 1,2- or 1,1-DMH. The same conclusion, about the relative CNS potency of 3-alkyl side chains, was reported by Loev and co-workers.[2] The cycloalkyl derivatives **4d** and **10d** were prepared in order to assess the effect of this modified side chain on CNS activity. Adams reported a significant increase in potency of the compound represented by the general formula above (X = OH and R = *n*-pentyl) when the optical center at carbon 9 was resolved.[5] From the more potent DMH side-chain structures, we reasoned that Adams' data would suggest even greater differences between isomers. Therefore, we prepared optically active antipodes of β-keto ester **1** (Scheme I) and incorporated these into 1-hydroxy- and 1-amino-3-(1,1-DMH)-THDP's.

Synthetic Chemistry. The synthetic routes to the 1-amino and 1-mercapto compounds are outlined in Scheme I. Unless otherwise indicated, all compounds are racemic when possible. Standard von Pechmann condensation of the β-keto ester **1** with the requisite resorcinols **2a–d** yielded the corresponding coumarins **3a–d** which subsequently were converted to the THDP's **4a–d** via a Grignard reaction described by Adams and co-workers.[6,7]

Conversion of the 1-hydroxy compounds **4a,b** to the 1-mercapto compounds was accomplished using Newman's method[8] for converting simple phenols to thiophenols.