

sults. Since the urea hydrogen bonds to TC<sup>±</sup> in a manner which could be duplicated by guanine or its derivatives, hydrogen bonding to guanine residues may be important in the biological activity of tetracyclines. We are attempting at present to isolate other tetracycline adducts, particularly with guanine-type compounds, to test these hypotheses.

The optimum antimicrobial activity of tetracyclines is in the pH range<sup>25</sup> where the zwitterion is the predominant species.<sup>26</sup> The zwitterion is also required for adduct formation since protonation of O(am) would prevent adduct formation similar to that observed with urea. Consequently, zwitterion adduct formation could be a significant step in the therapeutic action of tetracyclines. However, whether adduct formation enhances lipid solubility and hence transport or is involved in the inhibition of protein synthesis can not yet be answered.

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**Supplementary Material Available:** Listing of observed and calculated structure amplitudes of tetracycline-urea tetrahydrate (14 pages). Ordering information is given on any current masthead page.

## References and Notes

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- (11) The abbreviations used in this paper are identical with the ones used in the preceding paper<sup>12</sup>. The abbreviations are TC<sup>±</sup>, tetracycline free base in the zwitterionic form; 5-HTC<sup>±</sup>, 5-hydroxytetracycline free base in the zwitterionic form; 6-DM-7-CLTC-HCl, 6-demethyl-7-chlorotetracycline hydrochloride; 7-CLTC-HCl, 7-chlorotetracycline hydrochloride; 5-HTC-HCl, 5-hydroxytetracycline hydrochloride; and 6-DH-5-HTC-HCl, 6-dehydroxy-5-hydroxytetracycline hydrochloride.
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## Preparation, Crystalline Structure, and Spectral Properties of the Fluorescent Probe 4,4'-Bis-1-phenylamino-8-naphthalenesulfonate<sup>1a</sup>

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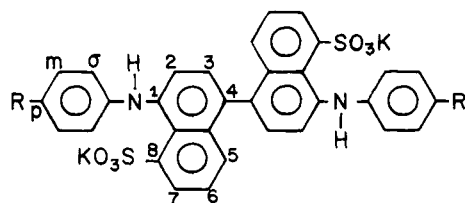
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**Abstract:** A method for the preparation and purification of the covalent dimers of 1-phenylamino- and 1-toluidylaminonaphthalenesulfonic acids is described. X-ray analysis of the crystals of the potassium salt of the former (bis-ANS) showed this to be 4,4'-bis-1-phenylaminonaphthalene-8-sulfonate. By similarity of the NMR spectra and optical properties the second dimer is recognized to be 4,4'-bis-1-toluidylaminonaphthalene-8-sulfonic acid. NMR spectra, molar absorption coefficients, fluorescent lifetimes, and the detailed structural parameters derived from the x-ray data are presented. Some of the uses of the compounds as fluorescent probes in solutions and crystals of proteins are briefly discussed.

## Introduction<sup>1d</sup>

Rosen and Weber<sup>2</sup> isolated a covalent dimer of 1-phenylaminonaphthalenesulfonic acid (bis-ANS) by treatment of 1-aminonaphthalene-8-sulfonic acid (ANS) with nitrous acid. The covalent character of the dimer was confirmed by elemental analysis and measurements of molecular weight by osmometry and mass spectrometry, and by the rotational

diffusion coefficient deduced from measurements of polarized fluorescence and fluorescence lifetime. However, attempts to determine the coupling positions in the monomers by NMR did not yield an unequivocal answer. As bis-ANS is finding application as a fluorescence probe of protein structure, we have now prepared bis-ANS, and also bistoluidylaminonaphthalenesulfonate (bis-TNS), by an improved method and



R = H : bisANS

R = CH<sub>3</sub> : bisTNS

Figure 1.

determined the three-dimensional structure of the former compound. Crystals of the potassium salt of bis-ANS of appropriate size were obtained from butanol solutions and the x-ray crystallographic analysis showed these to be 4,4'-bis(1-phenylamino)naphthalene-8-K<sup>+</sup>-sulfonate (Figure 1).

### Experimental Section

**Preparation of Bis-ANS.** The magnesium salt of 1-phenylamino-8-naphthalenesulfonic acid (ANS) (2 g) is dissolved in glacial acetic acid (20 mL), and concentrated HCl (20 mL) is added with stirring in a large test tube, followed by sodium nitrite (2.5 mL, 0.2 M) with vigorous agitation in a Vortex mixer. A purple color develops. After standing for 1 h, barium acetate (160 mL, 1 M) is added, and the solution is left at 4 °C overnight. The precipitated barium bis-ANS salt is centrifuged off, then dissolved in boiling water (40 mL). The barium salt of the dimer (bis-ANS) is insoluble in the cold, but soluble in boiling water (pH 6.0), but the barium salt of ANS is much more soluble in cold water. To obtain bis-ANS almost free from ANS, three to five precipitations as the barium salt are required, a procedure best followed by thin layer chromatography. The barium salt is finally decomposed by potassium sulfate (1 M, 8 mL/g original ANS) and the barium sulfate accumulated in the cold (4 °C) is separated. The supernatant is shaken with 1-butanol (1/2 volume). The butanol layer contains the potassium salt of bis-ANS and strongly colored impurities. The butanol volume is reduced to 1/2 to 1/3 of the original and enough water is added to cause separation of a strongly colored water phase, which is discarded. Extraction with water is continued until this is only lightly colored. The butanol is then removed, and the residue is dissolved in hot water (5 mL) and allowed to crystallize. A second crystallization from water yields the dimer as about 10% of the original ANS.

**Chromatographic Purification of Bis-ANS.** Bis-ANS moves slowly when eluted with sodium phosphate buffer (0.2 M at pH 6) from a Sephadex G-75 column previously equilibrated with the buffer, but is rapidly eluted with distilled water. After introduction of the bis-ANS solution (~10 mg in 3 mL of buffer) development with buffer is continued until a blue fluorescent band (ANS) and sometimes a second, very small, band have passed. On elution with water bis-ANS is washed out leaving a small, dark band on top of the column.

**Thin Layer Chromatography.** Satisfactory separation of ANS and bis-ANS was achieved by ascending chromatography in silica gel papers (Eastman, 6061), using a mixture of 1-butanol, glacial acetic acid, and water (60:20:20). The top layer which separates after some days is used to develop the chromatogram. ANS (blue fluorescence) runs close to the solvent front. Bis-ANS (*R* ~ 0.4) shows as a spot of yellow-orange color and fluorescence. Dark impurities removed by water extraction of the butanol solutions of bis-ANS do not move in this solvent.

Bis-TNS was prepared by a method entirely similar to that of bis-ANS, in a comparable or slightly better yield.

**X-Ray Analysis of the 2:1 1-Butanol Solvate of the Dipotassium Salt of Bis-1-phenylamino-naphthalene-8-sulfonic Acid (Bis-ANS).** The crystals of the solvated potassium salt of bis-ANS are dark yellow and transparent with a plate-like shape. The diffraction pattern did not extend to a very high  $2\theta$  angles and thus gave evidence either of high thermal motion in the crystal or of crystal disorder. An untwinned crystal with dimensions 0.4 × 0.2 × 0.1 mm was used for data collection. Crystal data follow: C<sub>32</sub>H<sub>22</sub>O<sub>6</sub>N<sub>2</sub>S<sub>2</sub>·2K<sup>+</sup>·2C<sub>4</sub>H<sub>10</sub>O, mol wt 821.1, triclinic, *a* = 9.526 (3), *b* = 14.536 (4), *c* = 17.523 (5) Å,  $\alpha$  = 121.47 (2),  $\beta$  = 89.05 (3),  $\gamma$  = 103.02 (2)°, *V* = 2001.4 Å<sup>3</sup>, *Z* =

2,  $\rho_c$  = 1.36 g/cm<sup>3</sup>,  $\mu$ (Cu K $\alpha$ ) = 34.5 cm<sup>-1</sup>, *F*(000) = 860, space group *P* $\bar{1}$  or *P*1,  $\lambda$ (Cu K $\alpha$ ) = 1.541 78 Å.

Intensity data were collected on a computer-controlled four-angle Syntex P2<sub>1</sub> diffractometer using a  $2\theta$  scan mode with variable scan speeds. A total of 5941 independent reflections was scanned in the  $2\theta$  sphere from 0 to 120° with graphite-monochromatized Cu K $\alpha$  radiation ( $\lambda$  = 1.541 78 Å). Out of these reflections, 3390 were considered to be observed at the  $2\sigma$  level, as determined by counting statistics. No correction was made for absorption. There was no evidence for crystal deterioration during the period of data collection.

The structure was solved by direct methods,<sup>3</sup> assuming the space group *P* $\bar{1}$ . Two 1-butanol solvent molecules were found in the crystal asymmetric unit. Full-matrix least-squares refinement of the positional and anisotropic thermal parameters for the nonhydrogen atoms and positional and isotropic thermal parameters for the atoms of the solvent molecules converged to an *R* factor of 0.201 and *R*<sub>w</sub> of 0.156. As we had some difficulty in obtaining a satisfactory model for the solvent molecules, we introduced occupancy factors for the atoms of the butanol molecules by taking the average value of the refined occupancy factors for each atom in a single butanol molecule while keeping the positional and thermal parameters constant in the first few cycles of refinement. These factors were 0.85 for one molecule and 0.60 for the other, and were not refined thereafter. The positions of the hydrogens, except those in the solvent molecules, were located from a difference map. Inclusion of the hydrogen atoms with fixed positional and isotropic thermal parameters in the least-squares refinement gave values for *R* and *R*<sub>w</sub> of 0.103 and 0.130. A difference map calculated at this stage showed a large peak 2.7 Å from the potassium ion and quite close to the site of the oxygen atom of the butanol molecule with lower occupancy (0.60); the K<sup>+</sup> - -O (butanol) distance is 3.12 (1) Å. We interpreted this peak as either a water molecule or the oxygen of a butanol molecule in a somewhat different orientation from the 0.60 occupancy butanol. As we were unable to find well-defined positions for the remainder of a butanol molecule that would include this oxygen atom, we included only this atom as an oxygen with an occupancy of 0.40. Full-matrix refinement of the positional and anisotropic thermal parameters for the nonhydrogen atoms in the cations and anions and the positional and isotropic thermal parameters for the nonhydrogen atoms of the solvent molecules (with fixed occupancies as described above) and for the hydrogen atoms in the anion gave final values for *R* and *R*<sub>w</sub> of 0.086 and 0.089, respectively, on all observed reflections. The value of  $[\sum w\Delta^2/(m-n)]^{1/2}$  was 2.2. The scattering curves were taken from the analytical expression used in the International Tables for X-Ray Crystallography.<sup>4</sup> The final values for the coordinates are given in Table I.

### Results and Discussion of the Crystal Structure

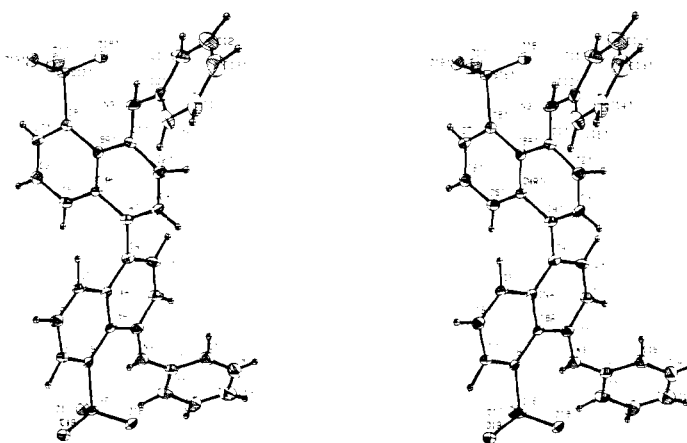
While incomplete occupancy and some possible loss of solvent from the crystals and a relative shortage of intensity data place some limitations on the conclusions to be drawn from the x-ray analysis, a number of important questions are resolved. The results of the x-ray analysis clearly demonstrate that bis-ANS is formed through the 4 position of the naphthalene group. A stereoscopic view of a single bis-ANS anion is shown in Figure 2. The full list of bond lengths and angles has been deposited. The C-C lengths in the aromatic rings range from 1.33 (1) to 1.49 (1) Å with a mean value of 1.395 Å and a root mean square deviation of 0.036 Å. The C(sp<sup>2</sup>)-N, C-S, and S-O lengths range from 1.33 (1) to 1.42 (1), 1.77 (1) to 1.78 (1), and from 1.44 (1) to 1.47 (1) Å, respectively. The ten carbon atoms of each of the two naphthalene rings are reasonably coplanar within the accuracy of the analysis; the two naphthalene rings are in a slightly cis orientation (Figure 3a) and inclined at an angle of 78.5°. In the optically inactive, B form of binaphthyl,<sup>5</sup> the naphthalene rings adopt a cis configuration with a dihedral angle of 68°, while in the (+)-2,2'-dihydroxyl-1,1'-binaphthyl-3,3'-dicarboxylic acid dimethyl ester,<sup>6</sup> there is a similar configuration with a dihedral angle of 77°. By contrast, in 8,8'-biquinolyl the rings are in a trans configuration (Figure 3b) with the dihedral angle being 96.8°.<sup>7</sup>

In a recent report<sup>8</sup> on the crystal structure of the ammonium salt of monomeric 8-anilino-1-naphthalenesulfonic acid

**Table I.** Final Atomic Parameters for the 2:1 1-Butanol Solvate of the Dipotassium Salt of Bis-1-phenylaminonaphthalene-8-sulfonic Acid

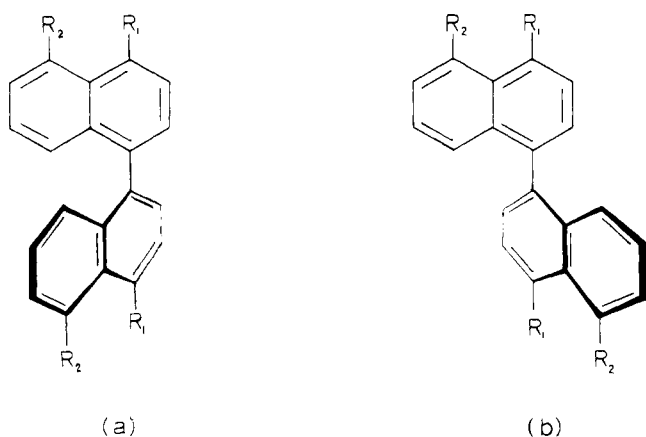
| Atom | x            | y            | z           | Atom               | x            | y           | z           |
|------|--------------|--------------|-------------|--------------------|--------------|-------------|-------------|
| K1   | 0.8168 (2)   | 0.5178 (2)   | 0.4975 (1)  | C15'               | -0.2797 (11) | -0.0010 (9) | -0.2837 (7) |
| K2   | 0.4972 (3)   | 0.7106 (2)   | 0.4858 (2)  | S16'               | 0.1413 (3)   | -0.2186 (2) | -0.4111 (1) |
| C1   | 0.5063 (9)   | 0.4024 (7)   | 0.1730 (6)  | O17'               | -0.0130 (7)  | -0.2734 (5) | -0.4193 (5) |
| C2   | 0.5196 (11)  | 0.4025 (8)   | 0.0955 (6)  | O18'               | 0.1583 (7)   | -0.1501 (5) | -0.4498 (4) |
| C3   | 0.4328 (11)  | 0.3251 (8)   | 0.0151 (6)  | O19'               | 0.2327 (8)   | -0.2953 (5) | -0.4459 (4) |
| C4   | 0.3241 (10)  | 0.2331 (7)   | 0.0064 (5)  | BO1 <sup>a,b</sup> | -0.019 (1)   | 0.515 (1)   | 0.632 (1)   |
| C4A  | 0.3097 (9)   | 0.2259 (7)   | 0.0841 (5)  | BC2                | -0.023 (1)   | 0.607 (1)   | 0.720 (1)   |
| C5   | 0.2057 (10)  | 0.1326 (7)   | 0.0738 (5)  | BC3                | 0.096 (1)    | 0.638 (1)   | 0.787 (1)   |
| C6   | 0.1823 (10)  | 0.1230 (7)   | 0.1461 (6)  | BC4                | 0.093 (1)    | 0.738 (1)   | 0.882 (1)   |
| C7   | 0.2570 (10)  | 0.2034 (7)   | 0.2300 (5)  | BC5                | 0.218 (2)    | 0.778 (1)   | 0.955 (1)   |
| C8   | 0.3628 (9)   | 0.2962 (6)   | 0.2450 (5)  | BO6                | 0.228 (1)    | 0.690 (1)   | 0.373 (1)   |
| C8A  | 0.3950 (9)   | 0.3091 (6)   | 0.1687 (5)  | BC7                | 0.237 (2)    | 0.715 (2)   | 0.301 (1)   |
| N9   | 0.5946 (8)   | 0.4799 (6)   | 0.2483 (5)  | BC8                | 0.194 (2)    | 0.627 (2)   | 0.229 (2)   |
| C10  | 0.6645 (10)  | 0.5894 (7)   | 0.2758 (6)  | BC9                | 0.184 (3)    | 0.640 (2)   | 0.147 (2)   |
| C11  | 0.7887 (10)  | 0.6414 (8)   | 0.3380 (6)  | BC10               | 0.203 (3)    | 0.558 (3)   | 0.079 (2)   |
| C12  | 0.8614 (13)  | 0.7520 (9)   | 0.3704 (7)  | BO6' <sup>c</sup>  | 0.324 (2)    | 0.757 (2)   | 0.398 (1)   |
| C13  | 0.8101 (13)  | 0.8105 (9)   | 0.3401 (8)  | H2 <sup>d</sup>    | 0.598 (8)    | 0.469 (6)   | 0.109 (5)   |
| C14  | 0.6891 (14)  | 0.7594 (9)   | 0.2797 (8)  | H3                 | 0.439 (7)    | 0.326 (5)   | -0.045 (4)  |
| C15  | 0.6106 (12)  | 0.6475 (8)   | 0.2467 (7)  | H5                 | 0.166 (6)    | 0.081 (5)   | 0.008 (4)   |
| S16  | 0.4365 (2)   | 0.3914 (2)   | 0.3593 (1)  | H6                 | 0.134 (7)    | 0.194 (5)   | 0.143 (4)   |
| O17  | 0.5937 (6)   | 0.4053 (5)   | 0.3646 (4)  | H7                 | 0.217 (7)    | 0.199 (5)   | 0.289 (4)   |
| O18  | 0.3918 (6)   | 0.4926 (4)   | 0.3864 (4)  | H9                 | 0.616 (7)    | 0.468 (6)   | 0.269 (5)   |
| O19  | 0.3718 (7)   | 0.3409 (5)   | 0.4098 (4)  | H11                | 0.852 (9)    | 0.616 (7)   | 0.372 (6)   |
| C1'  | 0.0538 (9)   | 0.0176 (7)   | -0.2489 (5) | H12                | 0.954 (9)    | 0.780 (7)   | 0.404 (5)   |
| C2'  | 0.0376 (10)  | 0.1228 (8)   | -0.1831 (6) | H13                | 0.856 (9)    | 0.880 (7)   | 0.357 (6)   |
| C3   | 0.1241 (10)  | 0.1886 (7)   | -0.1025 (6) | H14                | 0.659 (9)    | 0.790 (7)   | 0.258 (5)   |
| C4'  | 0.2305 (9)   | 0.1551 (7)   | -0.0811 (5) | H15                | 0.537 (6)    | 0.600 (5)   | 0.192 (4)   |
| C4A' | 0.2538 (9)   | 0.0478 (6)   | -0.1454 (5) | H2'                | -0.041 (8)   | 0.161 (6)   | -0.188 (5)  |
| C5'  | 0.3619 (10)  | 0.0128 (7)   | -0.1218 (6) | H3'                | 0.108 (7)    | 0.256 (6)   | -0.065 (5)  |
| C6'  | 0.3883 (10)  | -0.0873 (8)  | -0.1801 (6) | H5'                | 0.409 (7)    | 0.050 (5)   | -0.067 (4)  |
| C7'  | 0.3144 (10)  | -0.1537 (7)  | -0.2649 (5) | H6'                | 0.463 (7)    | -0.107 (5)  | -0.162 (4)  |
| C8'  | 0.2082 (9)   | -0.1250 (7)  | -0.2945 (5) | H7'                | 0.331 (9)    | -0.210 (7)  | -0.301 (5)  |
| C8A' | 0.1693 (9)   | -0.0208 (6)  | -0.2316 (5) | H9'                | -0.057 (13)  | -0.106 (10) | -0.376 (8)  |
| N9'  | -0.0429 (8)  | -0.0461 (6)  | -0.3277 (4) | H11'               | -0.139 (10)  | -0.048 (8)  | -0.474 (6)  |
| C10' | -0.1760 (10) | -0.0259 (7)  | -0.3427 (6) | H12'               | -0.380 (10)  | -0.028 (8)  | -0.495 (6)  |
| C11' | -0.2064 (12) | -0.0379 (10) | -0.4249 (7) | H13'               | -0.543 (9)   | 0.002 (7)   | -0.414 (6)  |
| C12' | -0.3409 (14) | -0.0264 (11) | -0.4463 (8) | H14'               | -0.478 (10)  | 0.012 (7)   | -0.268 (6)  |
| C13' | -0.4408 (13) | -0.0021 (10) | -0.3871 (9) | H15'               | -0.252 (8)   | 0.025 (6)   | -0.226 (5)  |
| C14' | -0.4060 (11) | 0.0113 (9)   | -0.3080 (8) |                    |              |             |             |

<sup>a</sup> The atom labels preceded by B refer to atoms in the butanol molecules. <sup>b</sup> The atoms BO1–BC5 were included in the calculations at 0.85 occupancy; the atoms BO6–BC10 were included with 0.60 occupancy. <sup>c</sup> The atom BO6' corresponds to the atom at the alternate site for BO6 and was included at 0.40 occupancy. <sup>d</sup> The hydrogen atoms were given the numbers of the atoms to which they are attached.

**Figure 2.** Stereoscopic view of a single bis-ANS anion.

(ANS), the geometry of the two crystallographically independent ANS anions was described. Many of the differences in the two conformations centered around the geometry at the nitrogen atoms, one of the molecules exhibiting a tetrahedral geometry while the other was approximately planar (deviation of N from the plane of its bonded neighbors was 0.11 Å). In the present structure, the location of the (N)H protons is rather

imprecise, but is indicative of a reasonably planar arrangement at nitrogen in both halves of the molecule; N(9) lies 0.06 Å and N(9') lies 0.04 Å from the planes defined by their respective bonded neighbors. An approximately planar arrangement was found in the molecule of 2-*p*-toluidinyl-6-naphthalenesulfonic acid (TNS) (deviation of N, 0.04 Å),<sup>9</sup> and also in the ANS anion in the hexaaquamagnesium salt (deviation of N, 0.08



**Figure 3.** (a) cis form of a binaphthyl derivative; (b) trans form of a binaphthyl derivative.

Å).<sup>10</sup> In the unprimed portion of the molecule, the normals to the best planes through the naphthalene and phenyl rings make angles of 26.9 and 24.1°, respectively, with that of the plane defined by N(9) and its two bonded carbon atoms; in the primed molecule, the corresponding angles are 14.7 and 44.3°. These angles have values such that significant overlap could take place between the p orbital of a planar, or near planar, nitrogen atom and the  $\pi$  orbitals of the aromatic rings.

There may be intramolecular N-H...O hydrogen bonding between the nitrogen and the sulfonate group in both halves of the molecule. In the unprimed half, the N(9)...O(17) and H(9)...O(17) distances are 2.76 (1) and 2.28 (9) Å, respectively, and the N-H...O angle is 153 (10)°; in the primed half, the N(9')...O(18') and H(9')...O(18') distances are 2.89 (1) and 2.43 (11) Å, respectively, and the N-H...O angle is 116 (11)°. While these dimensions, particularly the latter set, are not particularly convincing, they may be considerably affected by uncertainties in the hydrogen positions.

A stereoscopic view of the packing in the crystal looking in the *x* direction is shown in Figure 4. The structure can be described as consisting of layers of the large, mainly nonpolar anions that run in the *ab* planes. The boundaries of these layers in the *c* direction are characterized by the sulfonate groups and the N-H groups. The butanol molecules have their nonpolar regions buried in layers with the hydroxyl groups also located at the boundaries. Between successive layers there is a layer of potassium ions that are complexing to the oxygen atoms of the sulfonate groups and the hydroxyl groups of the butanol molecules. One can readily appreciate that a molecule such as 1-butanol must have been necessary to form crystals both to fill spaces left by the awkwardly shaped anions and to provide additional complexing sites for the potassium ions.

A view of the coordination of the potassium ions is shown in Figure 5. One of the potassium ions, K(1)<sup>+</sup>, is apparently coordinated to seven oxygen atoms with K<sup>+</sup>...O distances ranging from 2.69 (1) to 2.88 (1) Å (Table II). The other cation, K(2)<sup>+</sup>, appears to coordinate to only four oxygen atoms with distances from 2.64 (1) to 2.78 (1) Å. The other side of the K(2)<sup>+</sup> ion is protected by two aniline rings, C(10)-C(15) in the reference molecule and C(10')-C(15') in the molecule at 1 + *x*, 1 + *y*, 1 + *z*. The K<sup>+</sup>...ring distances are 3.616 and 3.275 Å, respectively; the shortest K<sup>+</sup>...C distance is 3.326 Å involving (C12') in the molecule at 1 + *x*, 1 + *y*, 1 + *z*. K(2)<sup>+</sup> is complexed to BO(6') at a distance of 2.69 (2) Å, while the K(2)<sup>+</sup>...BO(6) distance of 3.12 (1) Å appears rather long for a strong coordination. As BO(6) is not involved in coordination to K(1)<sup>+</sup>, its replacement in some of the cells by a water molecule or another orientation of the butanol molecule would seem reasonable.

Apart from the possible intramolecular N-H...O(S) hydrogen bonds, discussed earlier, there appear to be two hydrogen bonds involving the butanol molecules. One of these involves BO(6) which forms a hydrogen bond to O(18') in the molecule at *x*, 1 + *y*, 1 + *z*; the BO(6)...O(18') distance is 2.92 (2) Å and the BC(7)-BO(6)...O(18') angle is 119.7 (1.1)°. It appears that this hydrogen bond can be replaced by one between BO(6') and the same O(18'); the BO(6') and O(18') distance is also 2.92 (3) Å. The other hydrogen bond appears to involve the two butanol molecules. The distance between BO(1) and BO(6) in the molecule at -*x*, 1 - *y*, 1 - *z* is 3.14 (2) Å and the corresponding BC(2)-BO(1)...BO(6) angle is 103.8 (7)°. This hydrogen bond does not represent a particularly strong interaction, but it is unlikely that BO(6') can substitute for BO(6) in it. It therefore appears that hydrogen bonding between butanol molecules favors the BO(6) position, while metal coordination favors the BO(6') location for an oxygen. Whether BO(6') is provided by an alternative orientation for the butanol or by a water molecule is uncertain.

### NMR Spectra of Bis-ANS and Bis-TNS

Although it was evident that two ring protons became absent as a result of the coupling of the ANS or TNS molecules to form bis-ANS and bis-TNS, respectively, they could not be assigned with any degree of certainty to the aromatic carbons. However, the recognition of the coupling positions in bis-ANS was rendered obvious by the x-ray crystallographic study. The proton assignments worked out in this fashion, by comparison of the spectra of ANS, TNS, bis-ANS, and bis-TNS, are shown in Figure 6. The same naphthyl proton resonances, ca. 1728 ppm, disappear in both bis-ANS and bis-TNS and on this basis and on the chemical composition and general similarity of the optical spectroscopic properties (see Tables III and IV) bis-TNS is assigned the structure shown in Figure 1, namely, that of 4,4'-bis-1-toluidylaminonaphthalene-8-sulfonate.

### Optical Spectroscopic Properties

The absorption and fluorescence spectra of bis-ANS and bis-TNS in the ultraviolet and visible regions resemble those of the monomers, but with a shift to longer wavelengths (see Table III). The relatively minor spectral differences indicate a small perturbation of each anilinonaphthyl chromophore by the presence of the other and are well explained by the x-ray crystallographic observation of the two linked naphthalene rings occupying planes almost normal to each other. The fluorescence polarization spectrum,<sup>2</sup> which shows a value close to 0.45 for excitation on the longest wavelength absorption band, is consistent with a transition moment for the S<sub>0</sub> → S<sub>1</sub> transition in the naphthalene ring plane and lying either along the dimer axis (*a* direction in Figure 1) or a normal to it (*b* direction). In the first case energy transfer between the two halves of the molecule would leave the transition moment unchanged and in the second case the probability of transfer would be reduced to 1/25 by the unfavorable orientation. The fluorescence lifetimes of ANS, bis-ANS, and bis-TNS in several solvents are shown in Table IV. The fluorescence lifetimes of the dimers in solvents in which these molecules have high fluorescence efficiencies (alcohols, dimethylformamide) are about one-half of that of the monomers in agreement with an increased oscillator strength of similar magnitude for the last absorption transition.

### Areas of Application of the Dimer Compounds

The two dimers exhibit somewhat larger fluorescence enhancement than the monomers when transferred from water

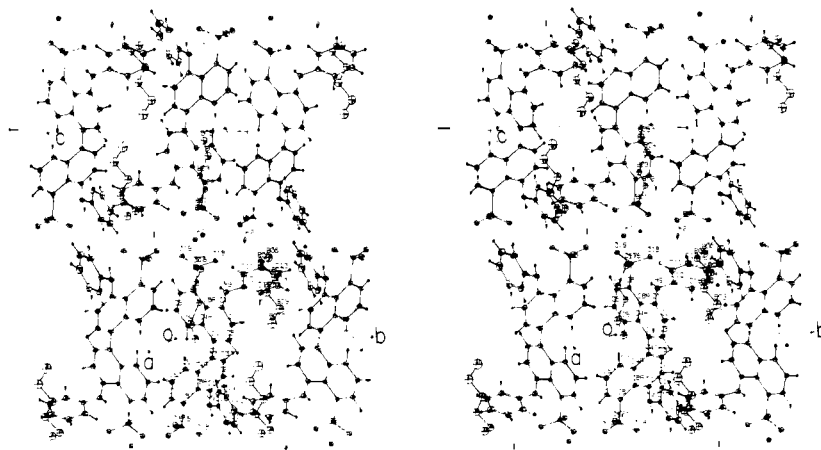


Figure 4. Stereoscopic view of the packing in the crystal.

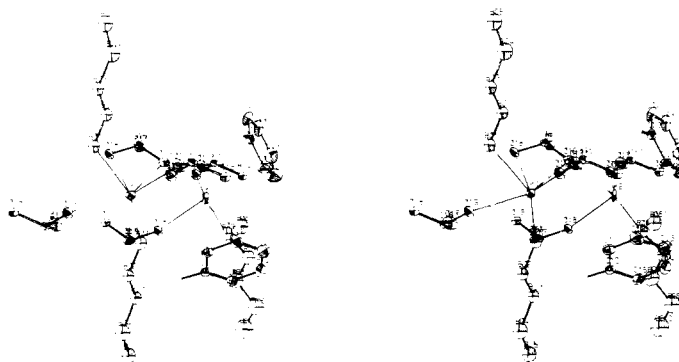


Figure 5. A stereoscopic view of the environment of the two  $K^+$  ions.

Table II. Short  $K^+ \cdots O$  Distances ( $\text{\AA}$ )<sup>a</sup>

|                              |      |                            |      |
|------------------------------|------|----------------------------|------|
| $K(1)^+ \cdots O(17)$        | 2.69 | $K(2)^+ \cdots O(18)$      | 2.64 |
| $K(1)^+ \cdots BO(1)^I$      | 2.88 | $K(2)^+ \cdots BO(6)$      | 3.12 |
| $K(1)^+ \cdots O(17')^{II}$  | 2.69 | $K(2)^+ \cdots BO(6')$     | 2.69 |
| $K(1)^+ \cdots O(19')^{III}$ | 2.79 | $K(2)^+ \cdots O(19')^V$   | 2.78 |
| $K(1)^+ \cdots O(18)^{IV}$   | 2.86 | $K(2)^+ \cdots O(19)^{IV}$ | 2.72 |
| $K(1)^+ \cdots O(19)^{IV}$   | 2.86 |                            |      |
| $K(1)^+ \cdots BO(1)^{IV}$   | 2.87 |                            |      |

<sup>a</sup> I represents the molecule at  $1 + x, y, z$ ; II represents the molecule at  $1 + x, 1 + y, 1 + z$ ; III represents the molecule at  $1 - x, -y, -z$ ; IV represents the molecule at  $1 - x, 1 - y, 1 - z$ ; V represents the molecule at  $x, 1 + y, 1 + z$ .

Table III. Molar Absorption Coefficients of the Potassium Salts of Bis-ANS and Bis-TNS in Water and Ethanol Solutions<sup>a</sup>

| Bis-ANS   |            | Bis-TNS   |            |
|-----------|------------|-----------|------------|
| $\lambda$ | $\epsilon$ | $\lambda$ | $\epsilon$ |
| Water     |            |           |            |
| 385 M     | 16 790     | 390 M     | 16 540     |
| 351 S     | 12 240     | 356 S     | 12 840     |
| 316 m     | 5380       | 317 m     | 5910       |
| 270 M     | 34 230     | 272 M     | 33 640     |
| 244 m     | 19 910     | 243 m     | 18 330     |
| 221 M     | 73 940     | 221 M     | 65 590     |
| Ethanol   |            |           |            |
| 397 M     | 21 120     | 400 M     | 20 270     |
| 360 S     | 13 450     | 362 S     | 13 290     |
| 320 m     | 4760       | 321 m     | 5470       |
| 276 M     | 32 340     | 277 M     | 32 120     |
| 246 m     | 16 580     | 246 m     | 15 580     |
| 222 M     | 66 190     | 222 M     | 66 020     |

<sup>a</sup>  $\lambda$ , wavelength in nanometers;  $\epsilon$ , molar absorption in  $\text{cm}^2/\text{mmol}$ ; M, maximum; m, minimum; S, shoulder.

Table IV. Fluorescence Lifetimes<sup>d</sup>

| Solvent                       | ANS                     | Bis-ANS           | Bis-TNS           |
|-------------------------------|-------------------------|-------------------|-------------------|
| Formamide                     | 2.14                    | 2.0               | 0.83              |
| Water                         | 0.22 <sup>a</sup>       | 0.14 <sup>a</sup> | $\sim 0.09^{a,b}$ |
| <i>N,N</i> -Dimethylformamide | 8.3, 8.7 <sup>c</sup>   | 3.8               | 4.3               |
| Propanol                      | 10.0, 10.2 <sup>c</sup> | 4.5               | 4.8               |

<sup>a</sup> Measurements with light modulated at 30 MHz. <sup>b</sup> Estimated from fluorescence polarization data to be between 30 and 80 ps. <sup>c</sup> Modulation measurements. <sup>d</sup> Unless indicated measurements are by phase fluorometry<sup>18</sup> on excitation by 385-nm light modulated at 14.2 MHz, at room temperature ( $\sim 23^\circ\text{C}$ ). Figures are in nanoseconds rounded to standard error. This is 0.1 ns for values greater than 5 ns, down to 30 ps for values of 2 ns or less.

to favorable solvents like ethanol. Fluorescence spectra of bis-ANS and bis-TNS in water and ethanol solutions were obtained using a scanning photon-counting spectrofluorometer.<sup>11</sup> For equal number of exciting photons the integrated emissions in ethanol were 220 times and 1090 times greater than in water for bis-ANS and bis-TNS, respectively. The extremely small fluorescence efficiency of bis-TNS in water becomes particularly evident when its fluorescence is visually compared with that of bis-ANS in water, on excitation through a Wood's filter. Bis-ANS and bis-TNS appear to have a remarkable affinity for the nucleotide binding sites of proteins, typically 5–100 times as large as the monomers. These effects include observations on glutamic dehydrogenase,<sup>12</sup> ATCase,<sup>13</sup> and myosin-ATPase,<sup>14</sup> and unpublished observations of binding to DNA polymerase I<sup>15</sup> and Erythrocyte Na-K ATPase.<sup>16</sup> The higher affinity of bis-ANS for the NAD-linked dehydrogenases permits observations of depolarization by energy transfer among the bound fluorophores which can be

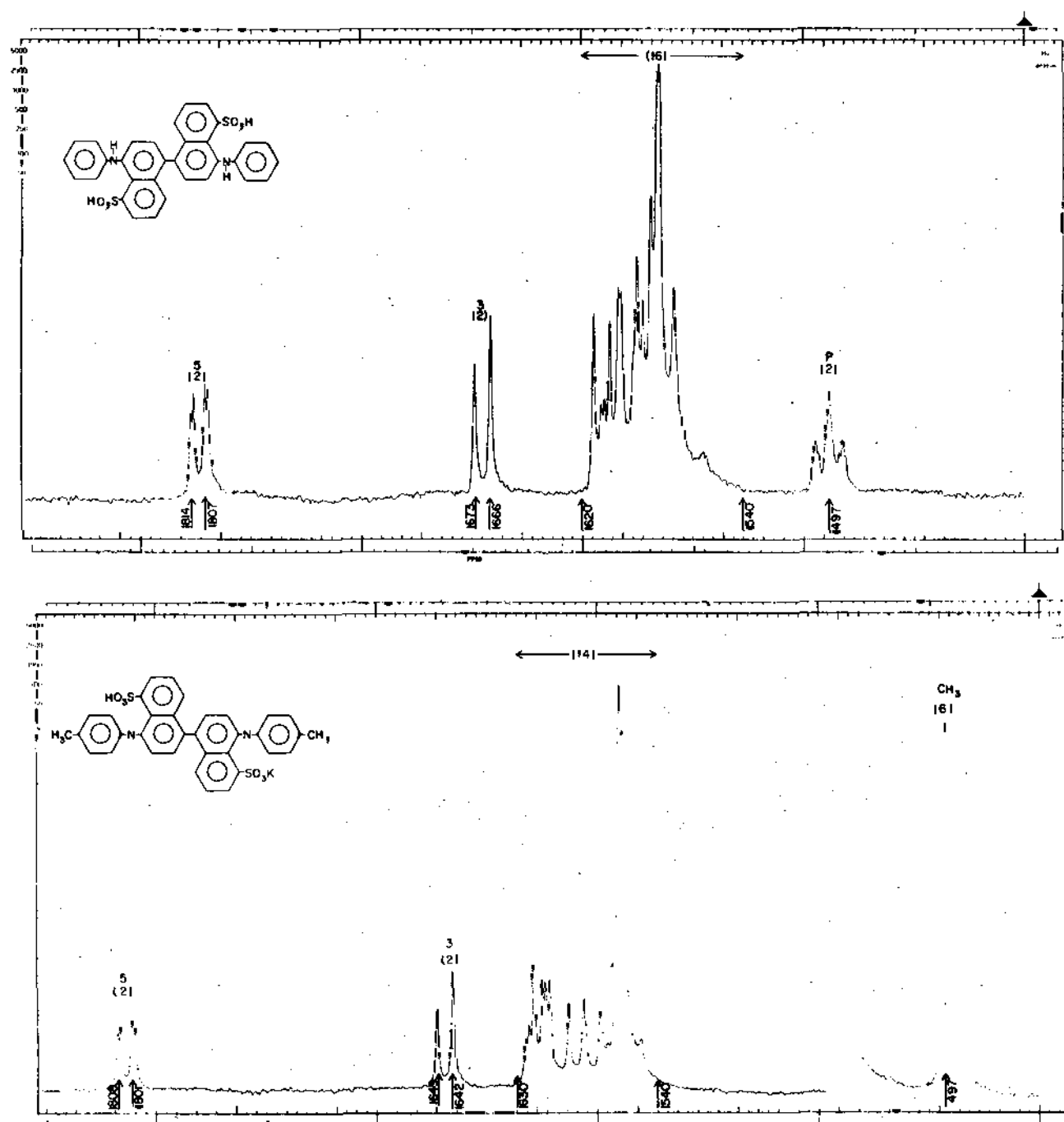


Figure 6. 220-MHz NMR spectra of bis-ANS (a) and bis-TNS (b). Number or letter above resonance indicates the position of proton shown in Figure 1. Numbers in parentheses are integrated numbers of protons under envelope.

used to estimate the distribution of the ligands among the protein molecules.<sup>17</sup> The complete crystallographic determination of the structure of bis-ANS will also facilitate the study of the complexes that may be formed with proteins in the crystal. The possibility exists that these very large ligands with their multiple rigid aromatic rings may change the conformation of the peptide chain in the process of maximizing the interaction with the protein. Observation of this process in crystals could provide useful information as to changes in structure and internal binding energies of proteins upon ligand binding.

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**Supplementary Material Available:** Lists of the bond lengths and angles, thermal parameters, and structure factors (24 pages). Ordering information is given on any current masthead page.

## References and Notes

- (1) (a) This compound can be equivalently named 5-5'-bis-8-phenylamino-1-sulfonic acid. Because the optical spectroscopic properties are critically dependent upon the substitution position of the amino group in the naphthalene ring and only marginally upon the substitution position of the sulfonic group, we have chosen the name that stresses the derivation of these compounds from 1-naphthylamine. (b) Department of Chemistry. (c) Department of Chemistry. (d) Abbreviations used: ANS, 1-phenylamino-naphthalene-8-sulfonic acid; TNS, 1-toluidylaminonaphthalene-8-sulfonic acid.
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