Vol. 130, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1985 Pages 350-357

INTRODUCTION OF THE DANSYL GROUP INTO HISTIDINE AND TYROSINE RESIDUES IN PEPTIDES AND PROTEINS

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Received May 13, 1985

Two dansyl derivatives: 1-(5-dimethylaminonaphthalene) sulfonyl (4-amino)-benzyl amine and 1-(5-dimethylaminonaphthalene) sulfonyl β(4-aminophenyl) ethylamine, have been recently synthesized. Reaction of these compounds with nitrous acid lead to the corresponding dansyl-bearing diazonium salts. The latter derivatives can couple, under mild basic conditions, to the imidazole moiety of histidine, the phenolic ring of tyrosine and to the ε-amino function of lysine. The applicability of the two reagents was tested in the modification of several peptides, including [D-Phe^6]LHRH, [D-Gln^6]LHRH, Leu-enkephalin and Tyr-tuftsin, and proteins such as calmodulin, bovine serum albumin and nerve growth factor.

The application of fluorescent probes in analytical, structural and functional studies of proteins and bioactive peptides is now well established. One such compound is the 1-dimethylaminonaphthalene-5-sulfonyl (dansyl) group (1). Due to its beneficial spectroscopic features in aqueous media (1) it is often used in protein and peptide research. The dansyl moiety is commonly introduced into these molecules using dansyl chloride as a reagent (2). Chemical modification occurs primarily at free amino functions, ε-groups of Lys and α-amino groups. Other side chains of amino acids carrying hydroxyl, sulfhydryl or imidazole groups may, however, also be modified, thus leading to complex products, and sometimes to spectroscopic misinterpretation. To avoid chemical, and hence physical ambiguity, reagents capable of selective dansylation should be employed. Towards this aim, several compounds have been developed and most successfully utilized to specifically dansylate sulfhydryl functions of proteins (3). The selective danyslation of a reactive Met residue in protein was also described (4,5).

0006-291X/85 $1.50

The imidazole side chain of His and the phenolic moiety of Tyr play an important role in a variety of functions of many proteins and peptides, e.g. in the mechanism of action of enzymes (6). Specific chemical modification of these residues may help in revealing the mode of their involvement in protein and peptide action. We wish to report in this paper, the synthesis of two new reagents aimed at the specific dansylation of His and Tyr residues, and its application to the modification of several biologically active peptides and proteins.

MATERIALS AND METHODS

Dansylchloride was obtained from Sigma (St. Louis, Mo., USA). p-nitrobenzyl amine hydrochloride and (4-aminophenyl)ethylamine were purchased from Aldrich (Milwaukee, Wis., USA). UV-visible spectra were recorded on a Gilford 250 spectrophotometer. Fluorescence spectra were recorded on a Perkin-Elmer L-30 spectrofluorimeter, and Aminco Bowman spectrofluorimeter. All spectra presented are uncorrected. Mass spectra were recorded on a Varian MAT 731 high resolution mass spectrometer. Thin layer chromatography (TLC) was performed on silica gel plates, with and without fluorescent indicator (Riedel-De Haen, Hanover), using the solvent systems: I, acetonitrile-water (9:1; v/v); II, n-butanol-acetic acid-water-ethylacetate (4:1:1:1; v/v); III, n-butanol-acetic acid-water (4:1:1; v/v). Detection was achieved under the UV-lamp, by ninhydrin, Pauly's reagent, iodine vapors, or by charring over a flame. Melting points were determined using a Gallenkamp capillary apparatus and are uncorrected. Amino acid analysis was carried out using a Dionex amino acid analyzer. Samples were hydrolyzed with 6N HCl containing phenol (3% w/v), for 24 h at 110°C, in evacuated sealed tubes.

Synthesis

1-(5-dimethylaminonaphthalene) sulfonyl (4-amino)benzyl amine [DANS-ABA]

Dansylchloride (1.02g; 3.8 mmol) was dissolved in dioxane (20 ml) containing triethylamine (1.05 ml; 7.6 mmol) and p-nitrobenzylamine hydrochloride (0.71g; 3.8 mmol). The mixture was stirred for 4 h at room temperature. The dioxane was evaporated in-vacuo and the residue left dissolved in ethylacetate (70 ml). The organic solution was extracted, consecutively, with 5% aqueous $NaHCO_3$ (40 ml; x2) and with saturated NaCl solution (40 ml; x2), dried over anhydrous Na_2SO_4 and evaporated in-vacuo to yield an oily residue. Crystallization from ethylacetate-petroleum ether (40-60°C) (1:1, v/v) yielded a bright yellow material (1.06g; 73%); mp 185-187°C. Mass spectrum: M^+ (parent ion), 385; calculated 385; m/e 171$(-SO_2NHCH_2-C_6H_4-NO_2)^+$. Anal. calcd. for $C_{19}H_{19}N_3O_4S$: C, 59.21; H, 4.97 N, 10.90; S, 8.32%. Found: C, 59.30; H, 4.96 N, 11.05; S, 8.56%. TLC, Rf_I 0.91; Rf_{II} 0.94. The product (0.53g; 1.4 mmol) was dissolved in methanol (25 ml) containing 1N aqueous HCl (4 ml), and Pd/C (10%; 50 mg; Fluka) was added. Hydrogen gas was bubbled through the stirred suspension. Reduction of the nitro group was completed within 4 h at room temperature, as revealed by TLC in solvent system II. The catalyst was filtered off, the solvent evaporated in-vacuo, and the residue crystallized, as the hydrochloride salt, from 2N HCl in dioxane. Yield 0.5g (94%); mp 205-206°C. Mass spectrum: M^+355 (β-HCl is released under mass spectrometry conditions). Anal. calcd. for $C_{19}H_{23}N_3O_2S$ HCl 2.5 H_2O: C, 52.23; H, 6.23; S, 7.34; Cl, 8.11%. Found: C, 52.38; H, 5.31; S, 7.63; Cl, 7.87%. TLC, Rf_{II} 0.74; Rf_{III} 0.76.

1-(5-dimethylaminonaphthalene) sulfonyl β(4-aminophenyl) ethylamine [DANS-APEA]

A solution of dansyl chloride (1.25g; 4.6 mmol), triethylamine (0.64 ml; 4.6 mmol) and (4-aminophenyl)ethylamine (0.64g; 4.7 mmol) in peroxide-free dioxane (25 ml) was allowed to stand for 4 h at room temperature. The reaction mixture was worked out as described for DANS-ABA, and the oily product obtained crystallized as the hydrochloride salt from 2N HCl in dioxane. Yield 1.83g (90%); mp 186-188°C. Mass spectrum: M^+369. Anal. calcd. for $C_{20}H_{23}N_3O_2S$ HCl $3H_2O$: C, 52.22; H, 6.57; N, 9.14; S, 6.97%. Found: C, 52.27; H, 6.62; N, 9.49; S, 7.30%. TLC, Rf_I 0.92; Rf_{III} 0.92.

N^{α}-benzyloxycarbonyl 2-monoazo dansyl-β-phenyl ethylamine L-tyrosine

To a cold solution of DANS-APEA (178 mg; 0.5 mmol) in 1N HCl (2.5 ml) a solution of $NaNO_2$ (34.5 mg; 0.5 mmol) in distilled water (1.5 ml). The homogeneous mixture was kept at 4°C for 10 min and then combined with a cold solution of N^{α}-benzyloxycarbonyl-L-tyrosine (158 mg; 0.5 mmol) in 1.5 ml of 1N $KHCO_3$, and the pH was adjusted to 9 with 1N NaOH. Diazo-coupling proceeded for 6 h at 4°C and then left overnight at room temperature. The reaction mixture was cooled to 4°C, acidified to pH 2 with 1N HCl, and the precipitate formed, collected redissolved in 1N $KHCO_3$ and reprecipated with 1N HCl. This procedure was repeated twice. The product was finally dissolved in ethanol and precipitated with petroleum ether (40-60°C). Yield 117 mg; mp 132-135°C (dec). $[\alpha]_D^{23}$ -13.0 (c, 0.1, ethanol). Anal. calcd. for $C_{32}H_{32}N_5O_7S$ $3H_2O$: C, 59.27; H, 5.78; N, 9.34; S, 4.28%. Found: C, 59.15; H, 5.60; N, 9.26; S, 4.27%.

N^{α}-benzyloxycarbonyl monoazo dansyl β-phenyl ethylamine L-histidine

DANS-APEA was diazotized and coupled to N^{α}-benzyloxycarbonyl L-histidine (145 mg; 0.5 mmol) using the procedure described above. The product was crystallized from ethanol by petroleum ether. Yield 72 mg. mp 145-147°C (dec); $[\alpha]_D^{23}$ -138.0 (c, 0.1, ethanol). Anal. calcd. for $C_{34}H_{36}N_7O_6S$ $6H_2O$: C, 52.50; H, 6.09; N, 12.67; S, 4.14%. Found: C, 52.73; H, 5.68; N, 12.24; S, 4.48%.

Diazo couplings of peptides and proteins with DANS-ABA and DANS-APEA

Coupling of the two fluorescent reagents with a variety of synthetic amino acid derivatives and peptides (including: N^{α}-Ac-L-His; N-Z-Tyr-Ala; N-Z-Ala-Tyr-OMe; H-Gly-Gly-Tyr-OH; tuftsin; Tyr-tuftsin; [His^4]tuftsin; LHRH; [$D\text{-}Phe^6$]LHRH; [$D\text{-}Gln^6$]LHRH and Leu-enkephalin), and proteins (BSA, calmodulin and nerve growth factor) was performed essentially as described for Z-Tyr. Modified peptides were isolated by passage through Sephadex, and checked for purity by TLC and HVPE. Modified proteins were passed through Sephadex G-100 and extensively dialysed upon completion of the reaction.

RESULTS AND DISCUSSION

The synthesis of the reagents, 1-(5-dimethylaminonaphthalene)sulfonyl-(4-amino)benzyl amine [DANS-ABA;(R1)] and 1-(5-dimethylaminonaphthalene)-sulfonyl-β(4-aminophenyl) ethylamine [DANS-APEA;(R2)], is illustrated in Figs. 1a and 1b, respectively. The spectral properties of the two reagents are very similar (Table 1), and close to those reported in the literature for other dansyl containing reagents (4). The absorption and fluorescent spectra of DANS-APEA are given in Fig. 2.

DANS-ABA and DANS-APEA were diazotized with nitrous acid and coupled in equal molar ratios, under mild basic conditions (pH 9.0), with Z-Tyr and with Z-N^{α}-Z

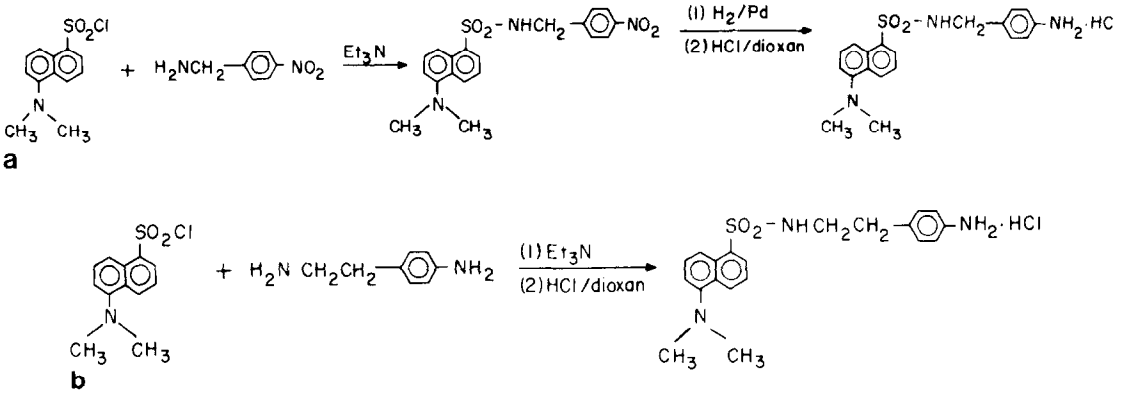

Figure 1: (a) Synthesis of 1-(5-dimethylaminonaphthalene) sulfonyl-(4-amino) benzyl amine, DANS-ABA.
(b) Synthesis of 1-(5-dimethylaminonaphthalene) sulfonyl-β-(4-aminophenyl) ethylamine, DANS-APEA.

His. The corresponding monoazo derivatives obtained were isolated, purified and characterized. Absorption and fluorescent spectra of Z-Tyr (monoazo-R_1), and of Z-His (monoazo-R_2) are given in Figs. 3 and 4, respectively. Reaction of Z-Tyr and Z-His with large molar excesses (up to 20-fold) of diazotized reagents did not yield diazo-derivatives, probably due to the bulkiness of the reagents as judged by spectral analysis, thin layer chromatography, and isolation of products.

A series of peptides containing Tyr and His residues, including several biologically active peptides, were coupled with the diazotized dansyl reagents and the products isolated, purified and analyzed. Amino acid analyses (data not shown) relate to Tyr and His, the reaction targets, Lys, Arg and Ser, residues potentially sensitive to diazotized compounds, and Gly, as a marker. Three consequences are noted: Tyr and His residues are indeed modified; Lys and Ser

Table 1: Spectral properties of dansyl reagents

	Absorption		Fluorescence	
	λ_{max} (nm) (EtOH)	($M^{-1}cm^{-1}$) (ϵ)	λ_{ex} (nm) (EtOH)	λ_{em} (nm)
DANS-ABA (R1)	338	4000	338	520
DANS-APEA (R2)	334	4400	334	520

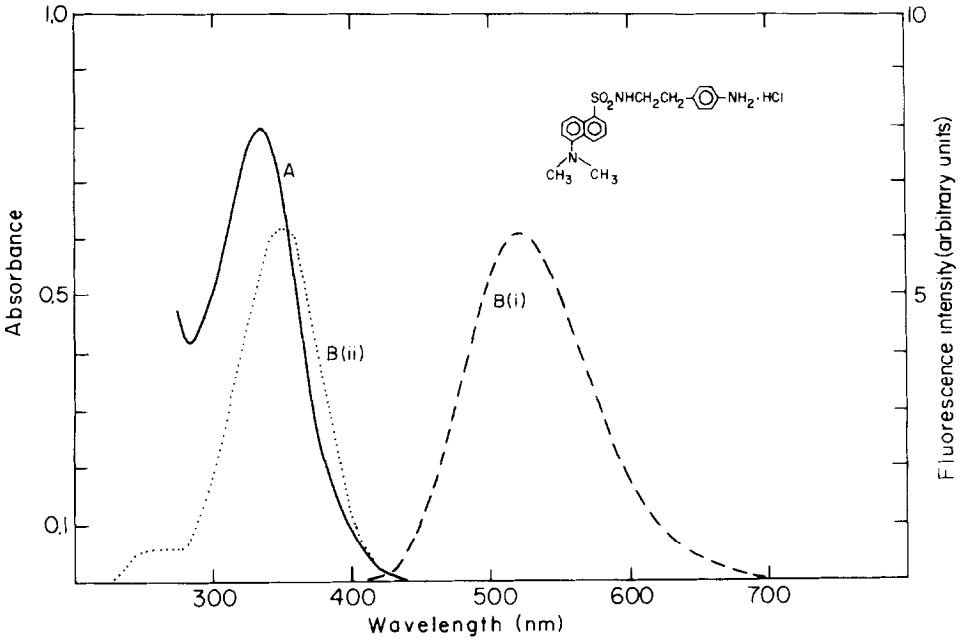

Figure 2: Absorption and fluorescence spectra of DANS-APEA in EtOH. A, Absorption spectrum; B(i), Fluorescence emission spectrum, λ_{ex}= 334 nm; B(ii), Fluorescence excitation spectrum, λ_{em}=518 nm.

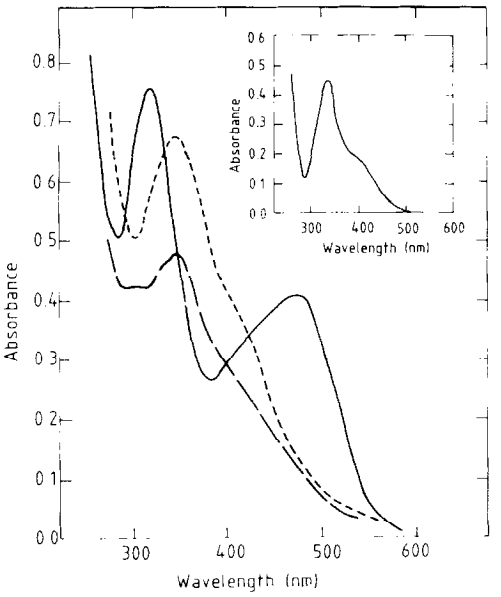

Figure 3: Absorption spectrum of Z-L-Tyr(monoazo-R1) 0.1 mM at ——— pH 13.0, ------ pH 7.0, —— —— pH 2.0. Inset: Absorption spectrum of Z-L-Tyr(monoazo-R1) in EtOH.

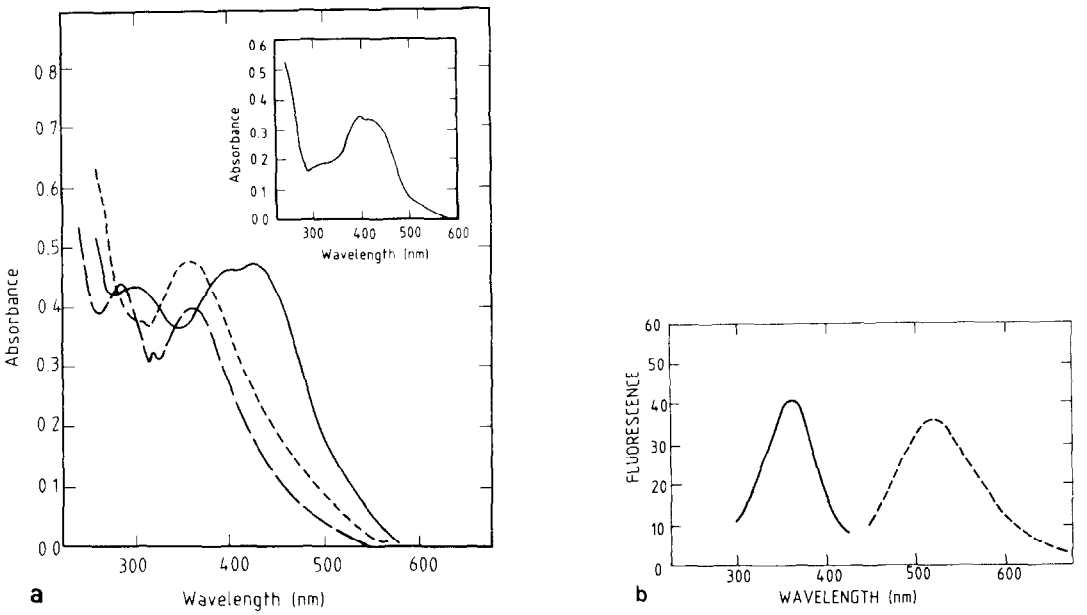

Figure 4: (a) Absorption spectrum of Z-L-His(monoazo-R2) 0.1 mM at ——— pH 13.0, ------ pH 7.0, —— —— pH 2.0. Inset: Absorption spectrum of Z-L-His(monoazo-R2) in EtOH.
(b) Fluorescence emission and excitation spectrum of Z-L-His(monoazo-R2) in EtOH. A similar spectrum is seen for Z-L-Tyr(monoazo-R1).

are not affected; free α-amino functions (e.g.,H-Gly-Gly-Tyr-OH) are, practically not reactive. Moreover, amino acid analysis reveal that all other residues contained in the peptides studied were not modified.

Three proteins: calmodulin (from rat testis), bovine serum albumin (BSA) and mouse nerve growth factor (NGF) were treated with various molar excesses of diazotized DANS-ABA and DANS-APEA. Amino acid analyses of the modified proteins are given in Table 2. In addition to modification of Tyr and His residues, extensive reaction occurred at the ε-side chain of Lys. All other amino acids (not shown) are practically unaffected. Dansylation of calmodulin, in the presence or absence of specific additives, (Table 2), is of particular interest. In the absence of Ca^{2+} (<1 ppm), there is only slight modification of His. With small excesses of reagent, and Ca^{2+} (>0.2 mM) increased dansylation of His was observed.

A marked difference was observed between the pattern of diazo-coupling to peptides and to proteins. In the former case, modification occurred almost exclusively at Tyr and His residues with no apparent difference in the

Table 2: Proteins modified by diazotized dansyl reagents

Protein	Reagent	[Reagent]	Specific Reaction [Protein] Conditions	Amino Acid Analysis					
				Tyr	His	Lys	Arg	Ser	Gly
Calmodulin	-	-	-	1.40(2.0) [b]	1.02(1.0)	6.88(7.0)	5.22(6.0)	4.48(4.0)	11.0(11.0)
Calmodulin	1	170[a]	no $CaCl_2$ (<1 ppm)	0.88(1.40)	0.76(1.02)	3.12(6.88)	2.58(5.22)	4.88(4.48)	11.0(11.0)
Calmodulin	2	5[a]	0.2mM $CaCl_2$	0.87(1.40)	0.55(1.02)	2.08(6.88)	5.04(5.22)	4.44(4.48)	11.0(11.0)
Calmodulin	2	10[a]	40mM $CaCl_2$	1.23(1.40)	0.0(1.02)	4.42(6.88)	5.71(5.22)	4.69(4.48)	11.0(11.0)
Calmodulin	2	5[a]	no $CaCl_2$;2mM EGTA	0.70(1.40)	0.51(1.02)	2.08(6.88)	4.92(5.22)	4.20(4.48)	11.0(11.0)
BSA [c]	-	-	-	17.12(19.0)	17.18(17.0)	59.15(59)	23.94(23)	29.05(29)	15.0(15.0)
BSA	2	1[a]	-	13.5(17.0)	11.1(15.1)	38.0(52.2)	20.3(23)	26.00(29)	15.0(15.0)
NGF	-	-	-	2.0(2.0)	3.7(4.0)	10.3(8)			5.0(5.0)
NGF	2	16[a]	-	0.58(2.0)	1.85(2.0)	3.12(10.3)			5.0(5.0)

[a] Values represent molar ratios of reagent to protein with respect to Tyr + His content.

[b] Values in parentheses correspond to unmodified proteins used.

[c] Product purification by dialysis and Sephadex G-75.

reactivity of the two moieties, at pH 9. In proteins however, extensive reaction took place, as well, at ε-amino side chains of Lys, as judged by the loss of Lys revealed by amino acid analysis. It seems from our experiments with peptides that the reactivity of the phenolic and imidazole functions greatly exceeds that of ε-amino group. The feasibility of using the Tyr and His directed reagents for probing macromolecules is being further studied for different ratios of reagent/ protein and the effect of salt concentrations and pH. It should be noted that modification of any protein will require its own reaction conditions.

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