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# Synthesis and Properties of a New Cleavable Nucleic Acid–Protein Crosslinking Reagent

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A new compound, dithiobis[9-(2-ethylenecarbamoylethylamino)-2,3-dimethoxy-6-azidoacridine], was synthesized and used in some preliminary experiments to form cleavable complexes between nucleic acids and proteins. In a first step the azidoacridine moiety of the reagent intercalates between the bases of nucleic acids and is then bound by reaction of the azido group. The disulfide group of the reagent is simultaneously converted under reducing conditions into a thiol which, in a second step, can be bound by oxidation to –SH groups of a vicinal protein (additional –SH groups can be inserted in the protein using 2-iminothiolane). The nucleic acid–protein complexes thus formed can be redissociated by reduction. The potential applications of this new cleavable crosslinking reagent could be extended to topographical investigations of any biological structure composed of nucleic acids and proteins.

## INTRODUCTION

Chemical methods are often used to investigate the structure of complex systems of biological macromolecules. Thus for instance in ribosomes the spatial arrangement of many proteins could be established using bifunctional crosslinking reagents (*1*). Similar studies of neighborhood relationship between proteins and nucleic acids are more difficult for different reasons, among others the lower chemical reactivity of nucleic acids compared to proteins. It seemed therefore useful to synthesize a new reagent especially designed for a heterologous crosslinking of these two types of macromolecules.

To this end it seemed advisable to design a compound composed of two fragments, each of them able to interact specifically with one of these macromolecules. These fragments should not necessarily be simple chemical functions. In particular in the one designed to react with the less reactive nucleic acids, the alkylating group should be associated to a ligand having some affinity for nucleic acids to ensure a specific binding to the latter before covalent substitution. The other part of the reagent should be able to react with proteins but not with nucleic acids. Such reagents having not merely two chemical functions but rather a



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nucleic acid-specific and a protein-specific moiety could be termed *bispecific* rather than bifunctional reagents.[1]

In the reagent described in the present paper, specificity for nucleic acids is based upon the property of some fused-ring heterocyclic compounds to interact with nucleic acids. Thus one end of the reagent contains an acridine derivative chosen as the intercalating moiety. To allow conversion of the preliminary binding by intercalation into covalent binding to nucleic acids, the acridine nucleus bears an azido group. The latter was chosen for the following reasons: (i) the chemically rather inert azido group does not have the disadvantage of many common alkylating or acylating reagents, which can be hydrolyzed or undergo undesired side reactions before reaching the target (*2–4*); (ii) photoactivation converts azides to highly reactive nitrenes able to substitute even unreactive receptor sites (*5–8*); the lack of specificity of the reaction of azides has no importance in this case since after intercalation of the azidoacridine any kind of linking to nucleic acid was acceptable. Such a biphasic binding process based on physicochemical affinity can in a sense be compared to affinity labeling based on biological affinity.

The acridine further bears a side chain of variable length with a terminal thiol group which can be bound to protein –SH groups by oxidation (if necessary, additional –SH groups can be introduced into the protein using 2-iminothiolane (*1*)). In view of this methodology thiols can also be considered as precursor functional groups which are unreactive under the usual experimental conditions; and thus reaction with protein –SH groups has to be triggered. An additional advantage is the possibility of splitting the disulfide bond under mild, controlled conditions to dissociate the crosslinked species in order to allow individual identification (*9*).

It was found more convenient to isolate the reagent denoted AASH[2] (azidoacridine-SH) in the more stable disulfide ($(AAS)_2$) form (compound **VIII**) which can generate *in situ* the thiol form for use as a protein–nucleic acid crosslinker. The disulfide also offers the additional opportunity of using the reagent as a cleavable alkylating DNA bis-intercalator.

The present paper describes the synthesis and properties of this new reagent together with some preliminary experiments demonstrating its ability to crosslink nucleic acids with proteins, performed on ribosomal macromolecules.

## EXPERIMENTAL

The purity of all the synthesized compounds was tested by thin-layer chromatography on Merck silica gel 60 $F_{254}$ plastic sheets using the following solvent

[1] According to this nomenclature a protein–nucleic acid crosslinker would be a heterobispecific reagent while homo- as well as heterobifunctional protein reagents or DNA bis-intercalators would be homobispecific.

[2] Abbreviations used: AASH and $(AAS)_2$, respectively, monomeric (thiol) and dimeric (disulfide) forms of the reagent (structure shown in Fig. 1); $C_4SH$, iminothiolane (*1*); TP50, ribosomal 50 S proteins; PF, proflavine; DMSO, dimethylsulfoxide; MS, mass spectrum.

systems: (A) benzene–methanol–acetic acid, 24: 4: 2; (B) *n*-butanol–acetic acid–water, 15: 9: 6. Spectral determinations were performed on the following instruments: infrared (KBr disks), Perkin Elmer 720; ultraviolet: Cary 15 or Zeiss PMQ II; proton magnetic resonance, Cameca 250 MHz; mass, AEI MS-30; fluorescence, Aminco Bowman spectrofluorimeter. Melting points were determined on a Reichert hot-stage apparatus.

The buffers were: Buffer A—30 m*M* Tris–HCl, pH 7.6; 10 m*M* magnesium acetate; 100 m*M* KCl; 6 m*M* mercaptoethanol; Buffer B—30 m*M* phosphate buffer, pH 7.5; 10 m*M* magnesium acetate; 350 m*M* KCl; 6 m*M* mercaptoethanol. Buffer C—30 m*M* phosphate buffer, pH 7.5; 30 m*M* EDTA; 2.5 *M* NaCl.

(*1*) *Synthesis of the Reagent* (*see Fig. 1*)

*2-(3,4-Dimethoxyphenylamino)-4-nitrobenzoic acid, I.* A solution of 12.25 g (0.08 mol) of 3,4-dimethoxyaniline in 50 ml of warm (60°C) ethanol was added to a homogeneous mixture of 16.13 g (0.08 mol) of 2-chloro-4-nitrobenzoic acid and 11.06 g (0.08 mol) of potassium carbonate in 18 ml of water at 60°C. Copper powder, 0.8 g, was then introduced, the solvent was evaporated from the stirred mixture until the distillation temperature reached 92°C, and stirring under reflux was continued for 5 hr. Water, 250 ml, and 0.8 g of charcoal were added, the suspension was stirred and heated to boiling, and filtered while hot. The solid was again extracted with 100 ml of boiling water, filtered, and washed with 25 ml of hot water. The combined warm orange filtrates were acidified to pH 4 with 2 *N* hydrochloric acid and the green precipitate was filtered, washed thoroughly with boiling water, and dried shortly. It was then dissolved in 110 ml of tetrahydrofuran, the solution poured on a 1.4 × 50-cm column of Florisil 60/100, and chromatographed with 200 ml of tetrahydrofuran. The eluate was concentrated to 100 ml and rechromatographied as above on a similar column. The eluate was diluted with an equal volume of water, concentrated, the orange precipitate was filtered, washed with water, and dried *in vacuo* over KOH then over $P_2O_5$ at 80°C, yielding 11.6 g (45.4%) of product, mp 226.5–228°C (decomp.), sublimation point 192°C (lit.: mp 222–224°C (*10*); mp 221–223.5°C (*11*)), $R_f$ in solvent A, 0.41.

*Anal.* Calcd for $C_{15}H_{14}N_2O_6$: C, 56.61; H, 4.43; N, 8.80. Found: C, 56.56; H, 4.49; N, 8.58.

*2,3-Dimethoxy-6-nitro-9-chloroacridine, II.* A solution of 13.8 g (0.043 mol) of 2-(3,4-dimethoxyphenylamino)-4-nitrobenzoic acid in 138 ml (231 g, 1.5 mol) of freshly distilled phosphorus oxychloride was refluxed in a dry atmosphere for 2.5 hr. Phosphorous oxychloride was then evaporated at 60°C under 15 mm Hg, ice was added to the residue, and the brown solid was filtered and washed several times with water, then suspended in 400 ml of 0.1 *M* sodium bicarbonate and stirred until effervescence ceased. The product was filtered, washed several times with water, dried shortly *in vacuo* at 80°C over KOH and $P_2O_5$, dissolved in 250 ml of boiling pyridine, and chilled. The yellow solid was filtered, washed three times with a total volume of 60 ml of pyridine, and dried *in vacuo* over KOH and $P_2O_5$ at 100°C. Yield was 10.45 g (75.7%), $R_f$ in solvent A, 0.54, in solvent B, 0.78, mp 267–268°C (decomp.) (lit.: mp 252–253°C (decomp.) (*10*); mp 246–248°C (*11*)).

*Anal.* Calcd for $C_{15}H_{11}N_2O_4Cl$: C, 56.53; H, 3.48; N, 8.79; Cl, 11.12. Found: C, 56.6; H, 3.5; N, 8.4; Cl, 11.4.

The percentage of nitrogen was somewhat low, in agreement with a previous statement (*11*) that products of this series often do not give concordant values for nitrogen. However, the compound thus obtained can be used in the next step of the synthesis. A correct value for nitrogen was obtained when the product was sublimated at 208–209°C. Found: C, 56.5; H, 3.6; N, 8.9; Cl, 11.0. ir: 2860, 1645, 1355, 855, 830, and 750 $cm^{-1}$. uv (ethanol), $\lambda_{max}$ nm ($\epsilon_M$): 255 (54,000), 315 (28,300), 385 (16,800). MS (70 *e*V), *m/e* (rel. int.): 318 (88), 319 (15), 320 (30), 272 (29), 86 (100), 58 (42), 42 (75), 36 (52), 30 (41), 29 (35).

*2,3-Dimethoxy-6-nitro-9-(2-carboxyethylamino)acridine, III.* 2,3-Dimethoxy-6-nitro-9-chloroacridine (11.15 g, 0.035 mol) in 33 g of phenol was stirred at 85°C until the product dissolved (30–45 min). β-Alanine (7.4 g, 0.083 mol) was added and stirring at 85°C was continued for 1 hr 45 min. The solution was cooled to 40–45°C, 450 ml of acetone was added, and after 1 hr at 4°C the precipitate was filtered, washed five times with a total volume of 100 ml of cold acetone, and dried. It was further washed four times with a total amount of 160 ml of water at 80°C, then stirred in 200 ml of boiling pyridine. The suspension was chilled, the solid filtered, washed with pyridine and with acetone, and dried *in vacuo* over KOH, then over calcium chloride at 100°C. Yield 10.9 g (83.7%) of fine orange needles, mp 242–243°C (decomp.), $R_f$ in solvent B, 0.63. ir: 3100–2950, 2870, 1645, 1600, 1360, 840, and 835 $cm^{-1}$. uv ($10^{-2}$ *M* NaOH): 218 (13,000), 259 (44,000), 329 (20,000), 385 (7600), 440 (3600). MS (70 *e*V): 371 (31), 284 (25), 269 (40), 79 (30), 72 (29), 55 (32), 44 (100), 29 (19).

*Anal.* Calcd for $C_{18}H_{17}N_3O_6$: C, 58.22; H, 4.61; N, 11.32. Found: C, 58.1; H, 4.6; N, 11.2.

*2,3-Dimethoxy-6-amino-9-(2-carboxyethylamino)acridine, IV.* 2,3-Dimethoxy-6-nitro-9-(2-carboxyethylamino)acridine (10.9 g, 0.029 mol) in 750 ml of ethanol containing 5 ml of 10 *N* sodium hydroxide was hydrogenated at room temperature and atmospheric pressure in the presence of 3 g of 5% palladium on charcoal. After absorption of 3 mol of hydrogen (2.5 hr) the catalyst was filtered off, washed with ethanol, and the solution was acidified to pH 1.5 with 2 *N* hydrochloric acid. The yellow precipitate was filtered, washed twice with diluted (pH 1.5) hydrochloric acid and with acetone, and dried *in vacuo* over KOH then over $P_2O_5$ at 100°C for 6 hr. Hydrochloride (9.2 g) of product IV was obtained (83% yield). $R_f$ in solvent B: 0.57, mp 249–250°C (decomp.). ir: 3485, 3400, 2870, 1740, 1720, 1650 $cm^{-1}$. uv (water): 225 (20,500), 271 (42,900), 364 (14,200), 415 (7700).

*Anal.* Calcd for $C_{18}H_{19}N_3O_4 \cdot HCl$: C, 57.22; H, 5.34; N, 11.12; Cl, 9.38. Found: C, 56.91; H, 5.43; N, 11.14; Cl, 9.24.

*2,3-Dimethoxy-6-azido-9-(2-carboxyethylamino)acridine, V.* Sodium nitrite (2 g, 0.029 mol) in 25 ml of water was added dropwise in 20 min to a stirred ice-cold solution of 9.2 g (0.024 mol) of 2,3-dimethoxy-6-amino-9-(2-carboxyethylamino)acridine hydrochloride in 180 ml of water and 6.2 ml of concentrated hydrochloric acid. After 30 min the red solution was treated with 0.5 g of urea and stirring was continued for 10 min. A solution of 3.2 g (0.048 mol) of sodium azide in 25 ml of water was then introduced dropwise in 40 min. A yellow solid

precipitated at once and nitrogen was evolved. After stirring for 1 hr the product was filtered, washed with water, dissolved in 160 ml of 2 *N* sodium hydroxide, and the stirred solution was slowly acidified to pH 1 with 2 *N* hydrochloric acid. The precipitate was filtered, washed thoroughly with water, dried *in vacuo* over KOH and $P_2O_5$ at room temperature, and finally for 1 hr at 60°C. Yield 9.2 g (94%) of hydrochloride of product **V**, $R_f$ in solvent B, 0.54. The product decomposes at 121°C with evolution of gas. ir: 2870, 2140, 1710, 1650 $cm^{-1}$. uv ($10^{-2}$ *M* NaOH): 237 (24,000), 286, (64,500), 356 (7500), 372 (11,500), 404 (7000).

*Anal.* Calcd for $C_{18}H_{17}N_5O_4 \cdot HCl$: C, 53.54; H, 4.49; N, 17.34; Cl, 8.78. Found: C, 53.32; H, 4.48; N, 17.44; Cl, 8.94.

After 48 hr exposure to daylight, the solution of the product in $10^{-2}$ *N* NaOH shows the following uv spectrum: 226 (22,700), 245 (24,500), 285 (18,000), 330 (11,500).

Under the same conditions, using an equivalent amount of sulfuric acid instead of hydrochloric acid to dissolve compound **IV**, product **V** was obtained in the same yield in the form of neutral sulphate.

*Anal.* Calcd for $(C_{18}H_{17}N_5O_4)_2 \cdot H_2SO_4$: C, 51,92; H, 4.36; N, 16.82; O, 23.05; S, 3.85. Found: C, 51.8; H, 4.6; N, 16.6; O, 23.1; S, 3.8.

*2,3-Dimethoxy-6-azido-9-(2-chloroformylethylamino)acridine, VI.* One milliliter of *N,N*-dimethylacetamide was added dropwise with stirring to an ice-cold suspension of 2 g (0.005 mol) of 2,3-dimethoxy-6-azido-9-(2-carboxyethylamino)acridine hydrochloride in 40 ml of thionyl chloride under a dry atmosphere and the mixture was allowed to warm to room temperature. The solid dissolved at 17°C. The solution was stirred at 20°C for 5 hr. The resulting suspension was treated with 40 ml of benzene, kept for 0.5 hr at 4°C, the solid was collected, washed repeatedly with benzene, then with acetone, and dried over KOH and $P_2O_5$, yielding 1.91 g (91%) of fine yellow needles of hydrochloride of compound **VI**. The product decomposes with effervescence at 138°C. ir: 2150, 1803, 1650 $cm^{-1}$.

*Anal.* Calcd for $C_{18}H_{16}N_5O_3Cl \cdot HCl$: C, 51.20; H, 4.06; N, 16.58. Found: C, 51.3; H, 4.2; N, 16.4.

*2,3-Dimethoxy-6-azido-9-[2-(2-triphenylmethylthioethylcarbamoyl)-ethylamino]acridine, VII.* 2,3-Dimethoxy-6-azido-9-(2-chloroformylethylamino)-acridine hydrochloride (1.67 g, 0.0039 mol) was added in small portions in 1.75 hr to a stirred solution of 2.5 g (0.0078 mol) of *S*-tritylcysteamine and 0.8 g (0.0079 mol) of triethylamine in 33 ml of dry (4-Å molecular sieve) *N,N*-dimethylacetamide at 40°C. Each addition was made only after the preceding portion dissolved. Heating and stirring were continued for 0.5 hr and the solution was allowed to cool to room temperature. Seventy milliliters of acetone was added, followed by 375 ml of ether, and after 2 hr at 4°C the precipitate was filtered and washed with ether. The solid was dissolved in 250 ml of chloroform and the solution was shaken vigorously with 85 ml of 0.2 *N* hydrochloric acid. The aqueous phase was extracted with three 10-ml portions of chloroform and the combined chloroform solutions were washed twice with 10 ml of water, dried over sodium sulfate, and evaporated to 50 ml. Acetone (200 ml) was added with stirring and, after 2 hr at 4°C, the bright yellow precipitate of hydrochloride of product **VII**

was filtered and washed with acetone. Yield 1.55 g (56%). The product decomposes at 167–170°C with evolution of gas. ir: 2145, 1650, 705 $cm^{-1}$.

*Anal.* Calcd for $C_{39}H_{36}N_6O_3S \cdot HCl$: C, 66.42; H, 5.29; N, 11.91. Found: C, 66.24; H, 5.20; N, 12.08.

*Dithiobis[9-(2-ethylenecarbamoylethylamino)-2,3-dimethoxy-6-azidoacridine hydrochloride], VIII (compound $(AAS)_2$).* A solution of 0.81 g (0.0025 mol) of mercuric acetate in 90 ml of ethanol was added to a solution of 0.9 g (0.00127 mol) of hydrochloride of product VII in 90 ml of chloroform and the resulting suspension was stirred at room temperature for 1.5 hr, evaporated, and the residue was triturated with 40 ml of ether. The yellow solid was filtered, washed with ether, and dried. It was then suspended in 6 ml of dimethylformamide, oxygen was bubbled for 0.5 hr, 15 ml of a ca. 6 *N* solution of dry hydrochloric acid in dimethylformamide was added, and oxygenation was continued until the Toennies and Kolb test (*12*) showed complete conversion of thiol to disulfide. The product was centrifuged, washed several times with dimethylformamide, once with acetone, and dried over KOH and $P_2O_5$. Yield 0.432 g (74%) (dihydrochloride). $R_f$ in solvent B, 0.64, mp (rapid heating) 240°C (decomp.). ir: 2140, 1650, 1600, 1570, 1520, 1290 $cm^{-1}$. uv ($1.6 \times 10^{-5}$ *M* in 1% DMSO–water): 234 (44,500), 289 (94,700), 360 (32,500), 403 (17,500), 425 (15,000). Irradiation decreases all the absorptions, e.g., when irradiated at 254 nm, the absorption at 289 nm decreases to half of its value in 45 min and in 30 min under 366 nm irradiation. nmr (DMSO-$d_6$): δ 2.55 (m, $C_{12}H$ and $C_{16}H$), 2.95 (t, $C_{11}H$, or $C_{15}H$), 4.12 (s, 3, $OCH_3$), 4.06 (s, 3, $OCH_3$), 7.36 (s, 1, $C_1H$, or $C_4H$), 7.46 (quadr. 1, $J$ = 9.5 and 2.5 Hz, $C_7H$), 7.60 (d, 1, $J$ = 2.5 Hz, $C_5H$), 7.98 (s, 1, $C_1H$ or $C_4H$), 8.54 (m, 1, $N_{14}H$), 8.70 (d, 1, $J$ = 9.5 Hz, $C_8H$), 9.44 ppm (m, 1, $N_{10}H$). Fluorescence spectrum: see below.

*Anal.* Calcd for $C_{40}H_{44}N_{12}O_6S_2Cl_2$: C, 52.00; H, 4.80; N, 18.19; S, 6.94; Cl, 7.67. Found: C, 51.6; H, 4.9; N, 17.9; S, 6.7; Cl. 7.9.

### (*2*) *Biological Preparations*

*Escherichia coli* 70 S and 50 S ribosomes, labeled with tritiated amino acids or unlabeled, were prepared by classical methods (*13, 14*). 50 S ribosomal proteins (TP50) were extracted from $^3H$-labeled 50 S ribosomes according to reference (*15*). Total ribosomal RNA was prepared by phenol extraction of 70 S ribosome (*13*). *E. coli* tRNAs were purchased from Boehringer-Mannheim.

### (*3*) *Spectrofluorimetry*

All fluorescence measurements were made under the following conditions: scanning speed 100 nm/min; excitation slit 0.5 mm (spectral band width 2.7 nm); emission slit 0.5 or 1 mm. The samples (1 or 2 ml) were thermostatized at 22°C. Unless otherwise stated proflavine was excited at 445 nm and the reagent AASH at 370 nm. For both compounds the emission was recorded at 504 nm.

### (*4*) *Irradiation*

Photoreactions were performed at room temperature in a vessel (1 cm outer

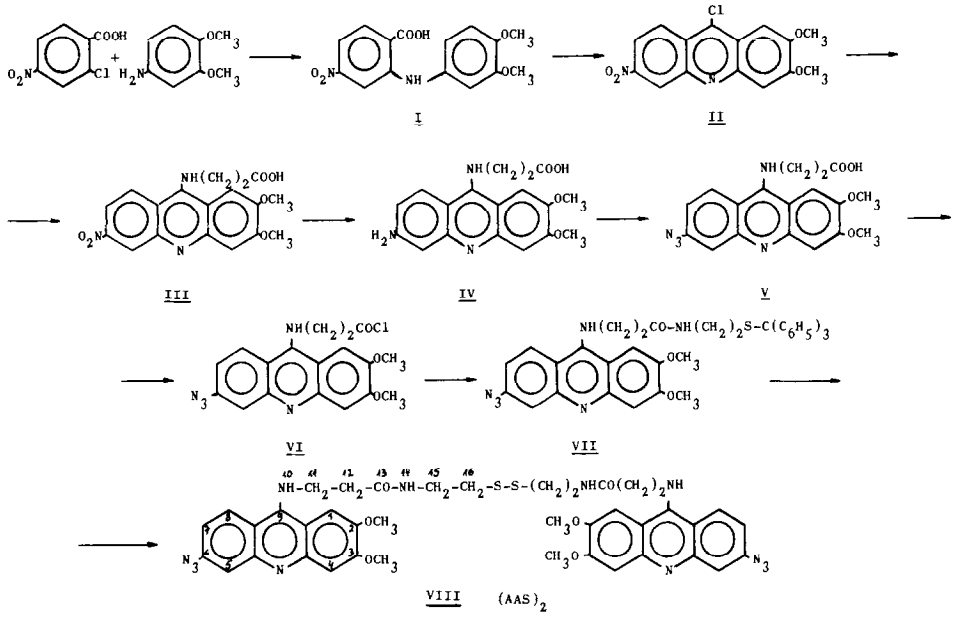

FIG. 1. Synthesis of dithiobis [9-(2-ethylenecarbamoylethylamino)-2,3-dimethoxy-6-azidoacridine] ($(AAS)_2$).

diameter Pyrex test tube) filtering wavelengths below 350 nm. The samples were irradiated for 5 min without shaking at a distance of 1 cm by a uv lamp (Sylvania, F8T5/BLB). The samples (final volume 1 ml) contained the required quantity of RNA in buffer A and the reagent (PF or AASH) dissolved in buffer A containing 1% of DMSO. Final DMSO concentrations <0.1%. Before irradiation the mixtures were thermically equilibrated for 10 min.

### (5) *Reaction of Ribosomal Proteins ($^3H$)TP50 with $C_4SH$*

A reaction mixture containing 7.5 μg of ($^3H$)TP50 (240,000 counts/min) and 150 μg of unlabeled TP70 in 150 μl of buffer B was incubated for 20 min at 0°C in the presence of $C_4SH$ (10 μl of a molar stock solution in buffer B). Excess iminothiolane was eliminated by dialysis against buffer B.

### (6) *Formation and Dissociation of Complexes between RNA and Ribosomal Proteins*

In a final volume of 1 ml (buffer B), 1.6 mg, 40 $A_{260}$ units of total ribosomal RNA were incubated for 1 hr at 43°C in the presence of 7.5 μg of ($^3H$)TP50 (240,000 counts/min) and 150 μg of unlabeled TP70. Under these conditions 17% (40,800 counts/min) of the labeled proteins bind to RNA to form the complex. Under Results it will be specified if RNA and the proteins were previously treated with AASH or $C_4SH$, respectively. The complexes were dissociated after dialysis against buffer C and incubation in the same buffer for 1 hr at 45°C (*16*).

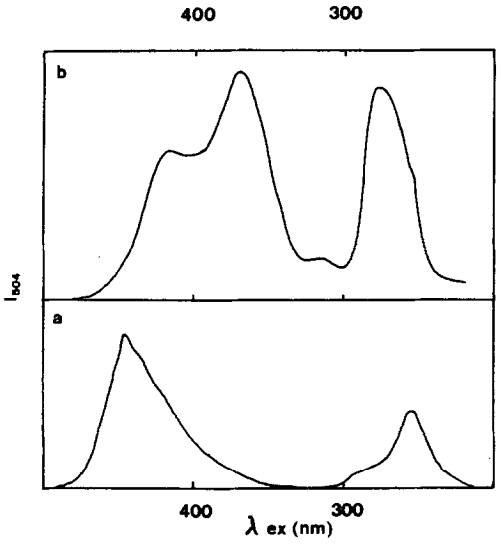


FIG. 2. Excitation spectrofluorimetry of proflavine and of compound AASH. (a) Proflavine 3.55 $\mu M$. (b) AASH 2.16 $\mu M$. The emission of each compound was recorded at 504 nm.

### (7) *Isolation of the Complexes*

The complexes in a 0.5-ml volume were poured on a 14 × 1-cm Bio-Gel A 1.5 m (Bio-Rad Lab.) column equilibrated with buffer B or C and eluted at 25 ml/hr by the same buffer. Fractions of 0.5 ml were collected. RNA, either free or complexed with labeled proteins, was eluted in fraction 9 (maximum absorbance and maximum radioactivity). Free proteins ($^3$H-TP50) were eluted in fraction 22 (maximum radioactivity).

## RESULTS AND DISCUSSION

### (1) *Synthesis*

Although the synthesis of the reagent is a multistep procedure (see Fig. 1), most of the reactions can be performed quickly and easily in an overall yield of 8%. Only the first two steps leading to compound II are comparatively time consuming. Since the latter product is commercially available it was initially hoped that the whole synthesis could be simplified. Unfortunately we have found that commercial 2,3-dimethoxy-6-nitro-9-chloroacridine is impure. Attempts to purify it or to use it without purification failed, and this compound also had to be synthesized.

The preparation of product I had been described by different authors (*10, 11, 17*). Although all these syntheses are based on the same principle outlined in Fig. 1, the experimental procedures were different; and the products obtained varied in purity. Therefore we had to reinvestigate the synthetic procedure; and

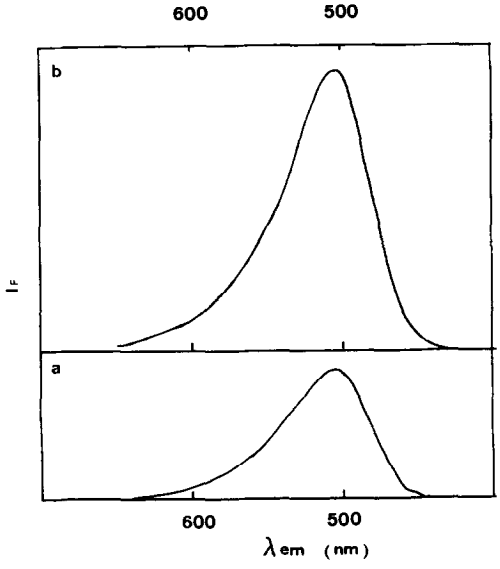


FIG. 3. Emission spectrofluorimetry of proflavine and of compound AASH. (a) Proflavine 3.55 $\mu M$; $\lambda_{ex}$ = 445 nm. (b) AASH 2.16 $\mu M$; $\lambda_{ex}$ = 370 nm.

this led to the working method described under Experimental, which gives pure product I in a 45% yield.

The first part of the synthesis of the reagent, i.e., the construction of the acridine moiety, could not be achieved with a chlorine atom at position 9, and compound II had to be linked to β-alanine[3] before conversion of the 6-nitro substituent to an azido group. The first step of this conversion, i.e., reduction of the nitro group, can also be performed with stannous chloride, leading to a complex of two molecules of product IV with one of stannic chloride. Although this complex is suitable for use in the next steps of the synthesis, only the simpler preparation of compound IV by catalytic hydrogenation of the nitro derivative is described above.

For the conversion of the carboxylic group in product V to the corresponding acyl chloride VI with thionyl chloride, the addition of a small amount of *N*,*N*-dimethylacetamide was necessary to obtain a pure product in a high yield.

### (2) *Fluorescence Spectra of the Reagent AASH*

The fluorescence properties of the reagent in the reduced AASH form were compared with those of proflavine. Excitation and emission spectra were recorded on 3.55 × $10^{-6}$ *M* solutions of proflavine and 2.16 × $10^{-6}$ *M* solutions of AASH in 1% aqueous DMSO containing mercaptoethanol (6 m*M*) in order to maintain AASH in the reduced form. Figures 2 and 3 show that the excitation

[3] An ω-amino acid was chosen because of the ready availability of a homologous series of these compounds allowing an easy variation of the length of the reagent within the general synthetic framework described here.

spectrum of AASH differs from that of proflavine in its general shape (shoulder at 418 nm) as well as in the maximum excitation wavelength (370 nm instead of 445 nm for proflavine). Both compounds have the same emission maximum (504 nm). The molar quantum yield ($\Phi$) of AASH calculated according to Parker and Rees (*18*) is 0.24 times that of proflavine. However, this value largely depends on the solvent (e.g., in buffer A, $\Phi$ AASH = 0.7 × $\Phi$ proflavine). The decomposition of AASH in solution stored in the dark at 4°C is accompanied, after a week or more, by some precipitation and an important change of its excitation spectrum, consisting in a redshift of the 370- and 418-nm absorption bands and an inversion of the 370/418-nm intensity ratio. Use of freshly prepared solutions is thus recommended.

### (*3*) *Interaction of AASH with Nucleic Acids and with Ribosomes*

(*a*) *Interaction with transfer ribonucleic acids.* The interaction of AASH with nucleic acids was evaluated by comparison with proflavine. Interaction of the latter with nucleic acids results in important modifications of the visible and ultraviolet spectrum of the dye (*19, 20*) and can be easily followed by fluorescence measurements. Actually the intensity of fluorescence at 504 nm, corresponding to maximum emission of proflavine, decreases as a function of the concentration of nucleic acids. We made use of this method to study the RNA-binding properties of the reagent AASH.

The experiment consists of measuring the relative intensity of fluorescence ($I_{504}$) of mixtures of proflavine or AASH and tRNA at different concentrations. Figure 4 shows that for both compounds $I_{504}$ decreases with increasing concentrations of nucleic acids. Thus AASH interacts with tRNAs in a way similar to proflavine.

(*b*) *Interaction with ribosomes.* The same method was used to evaluate the binding of AASH to *E. coli* ribosomes. In these experiments the amounts of proflavine or of AASH were varied with respect to a constant amount of ribosomes, and $I_{504}$ was followed as a measure of the interaction of these compounds with ribosomal nucleic acids. As can be seen in Fig. 5, AASH interacts with

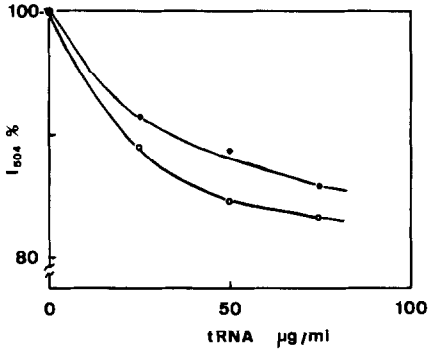


FIG. 4. Interaction of proflavine and of AASH with tRNAs. (○) Proflavine (3.55 $\mu M$); (●) AASH (2.16 $\mu M$). $I_{504}$: relative intensity of fluorescence at 504 nm.

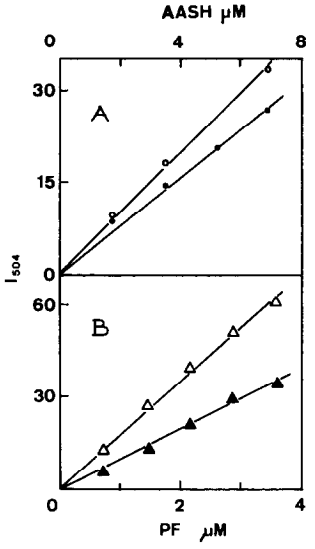


FIG. 5. Interaction of proflavine and of AASH with ribosomes. (A) AASH (○) without ribosomes; (●) in presence of 70 S ribosomes. (B) Proflavine (△) without ribosomes; (▲) in presence of 70 S ribosomes. $I_{504}$: relative intensity of fluorescence at 504 nm. 70 S ribosomes: 1.1 $\mu M$.

ribosomes, although the variations of fluorescence are smaller than those observed with proflavine. Nevertheless it can be concluded that the characteristics of intercalation of the reagent AASH in nucleic acids are qualitatively similar to those of proflavine.

### (4) *Irreversible Binding of AASH to Ribosomal RNA*

To check if the reagent is able to bind irreversibly to nucleic acids, a mixture of ribosomal RNA and AASH was irradiated at $\lambda \geq 350$ nm. The unbound reagent

TABLE 1

REACTION OF AASH WITH RIBOSOMAL RNA

| Sample | $h\nu$ | Percentage $I_{504}$ after dialysis | Reagent/RNA molar ratio |
|---|---|---|---|
| Proflavine | + | 0.05 | — |
| Proflavine + RNA (1) | − | 1.2 | 0.7 |
| Proflavine + RNA (1) | + | 1.2 | 0.7 |
| AASH | + | 2.0 | — |
| Irradiated AASH + RNA (2) | | 10.0 | 2.3 |
| AASH + RNA (2) | + | 59.0 | 13.6 |
| AASH + RNA (2) | − | 45.0 | 10.4 |
| AASH + denaturated RNA (2) | + | 20.0 | 4.6 |

*Note.* Initial concentrations: proflavine, 3.5 $\mu M$; AASH, 3.68 $\mu M$; RNA (1), 0.062 $\mu M$; RNA (2), 0.16 $\mu M$. $I_{504}$: relative intensity of fluorescence at 504 nm. $I_{504}$ of proflavine or of AASH before dialysis = 100%. Irradiation: $h\nu \geq 350$ nm, 5 min. Sample and dialysis buffer: buffer A.

was then eliminated by dialysis (20 hr against 3 × 1000 vols), and the amount of reagent undissociable from the substrate was determined by fluorescence measurements. Except for the irradiation step the whole experiment was performed in the dark to minimize uncontrolled photoactivation of the azido group.

The results in Table 1 show that with or without irradiation the control reagent proflavine is eliminated to the extent of about 99%. Also the reagent AASH after irradiation in the absence of RNA is almost entirely (98%) eliminated by dialysis, thus showing that irradiation does not result in formation of high-molecular-weight derivatives of the reagent. If AASH is irradiated before mixing with RNA, a small amount of the reagent resists dialysis under the above conditions, perhaps due to a strong acridine–RNA interaction.

Irradiation of a AASH–RNA mixture leads to a much higher proportion of undialyzable complexes (59% of the reagent remains associated to RNA). When irradiation is omitted, 45% of the reagent remains bound to its substrate. Thus it is

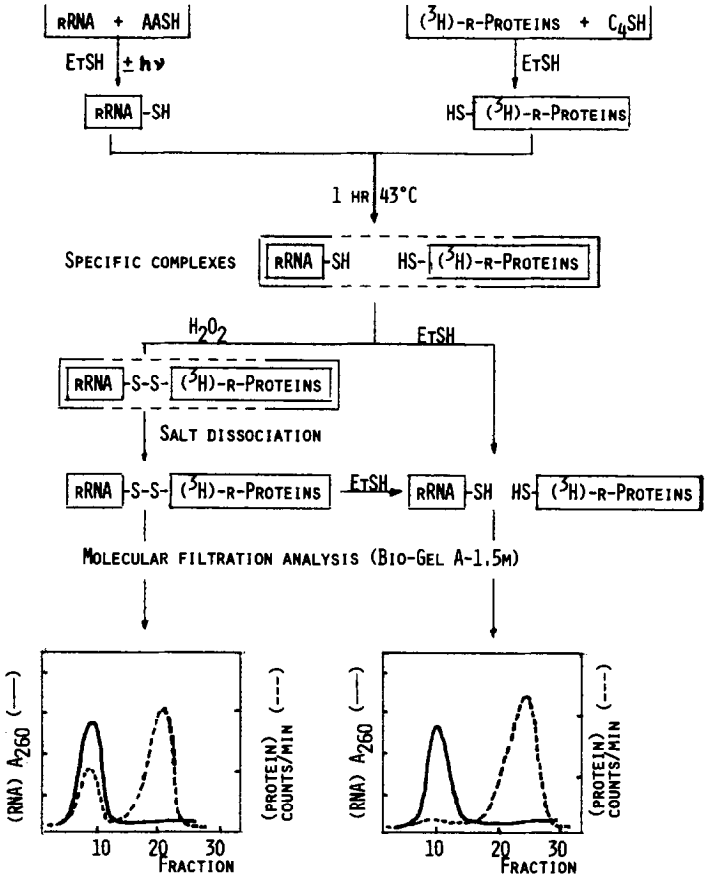


FIG. 6. Scheme of the experiment leading to the formation of covalent complexes between nucleic acids and proteins. EtSH: mercaptoethanol; oxidation: $H_2O_2$, 0.3% final concentration, 20 min at 4°C; reduction: 6 $\mu M$ mercaptoethanol.

TABLE 2

FORMATION OF CLEAVABLE RNA–PROTEIN CROSSLINKS

| Sample $^3$H-TP50 + RNA | Successive treatments | | | | | | Percentage undissociable complexes (yield %) |
|---|---|---|---|---|---|---|---|
| | AASH | $h\nu$ | $C_4SH$ | EtSH | $H_2O_2$ | EtSH | |
| 1 | − | − | − | + | − | − | 2–4 |
| 2 | − | − | − | − | + | − | 2–4 |
| 3 | + | + | + | + | − | − | 2–4 |
| 4 | + | + | + | − | + | − | 24–29 |
| 5 | + | + | + | − | + | + | 2–4 |

*Note.* EtSH: mercaptoethanol 30 m*M*, 30 min at room temperature; $H_2O_2$: 0.3% final concentration, 20 min at 4°C; $h\nu \geq 350$ nm. The yield is given as percentage of the amount of complex initially formed, as described under Experimental.

possible that the azido group of the azidoacridine moiety tightly bound to RNA reacted also in a purely chemical way. Earlier findings in the literature show that the azido group can react intramolecularly with nucleic acid bases in the dark (*21, 22*) and that this reaction may occur under mild conditions (*23*). The possibility that such a nonphotochemical reaction took place in our case is now being investigated in more detail on model compounds and will be reported later. In any case, in whatever form the reagent binds, it shows the ability to produce complexes with nucleic acids sufficiently stable for crosslinking experiments.

On the other hand when ribosomal RNA was denatured by 4 *M* urea and 0.1 *M* EDTA before mixing with AASH, subsequent irradiation led to a reduced amount of undissociable complexes (only 20% of the reagent was bound to the denatured substrate). These results suggest that AASH can react spontaneously with RNA after intercalation in double-stranded regions or in regions in which the bases are stacked.

### (*5*) *Formation of Cleavable RNA–Protein Crosslinks*

In the AASH form the side chain of the reagent has a terminal thiol group which should be able to form disulfide bridges with any product containing –SH groups. This property was tested using the specific complexes formed between ribosomal 23 S RNA and some ribosomal proteins (*24*). Since these proteins contain few free –SH groups, additional thiols were introduced by treating the proteins with 2-iminothiolane (see Experimental). These modified proteins are still able to form specific complexes with 23 S RNA, which is consistent with earlier statements that even extensive chemical modification (methylation) of lysine residues in ribosomal proteins does not prevent the latter from forming specific complexes with RNA (*25*); in addition, reaction of C4SH with ribosomal proteins under the usual conditions modifies only a small number of lysine residues (*26*).

The experiments were performed in several steps (Fig. 6): (i) intercalation and reaction of AASH with 23 S RNA; (ii) under reducing conditions (mercapto-

ethanol), formation of specific noncovalent complexes between 23 S RNA charged with the –SH groups of AASH and ribosomal proteins ($^3$H-TP50) enriched with –SH groups; (iii) oxidation ($H_2O_2$) of the RNA–proteins complexes to form disulfide bridges; (iv) treatment of the complexes under drastic dissociation conditions (*16*) in the absence of reducing agents; (v) molecular filtration (Bio-Gel A-1.5 m) to determine the proportion of complexes resisting salt dissociation both under oxidative and reducing conditions.

The results of these experiments are shown in Table 2. It appears clear that the specific RNA–protein associations can be completely cleaved under the indicated dissociation conditions (*16*). Also the complexes formed between RNA and proteins charged, respectively, with AASH and C4SH can be totally dissociated under reducing conditions, i.e., in the presence of mercaptoethanol. In contrast, after oxidative treatment ($H_2O_2$), these complexes resist dissociation (24–29%) but can be totally dissociated after reduction. Thus, these results confirm the existence of covalent links of the disulfide bridge type between RNA and proteins.

Inter-protein or inter-RNA crosslinks were not observed in this work. However, in view of the fact that in our system the true crosslinking species is –SH, their formation cannot be excluded and would limit the bispecific character of AASH. The design of a similar but hopefully more strictly bispecific reagent is now in progress. Nevertheless, in the light of the above preliminary evaluation of the properties of AASH it seems that this reagent is well suited for topological studies of biological systems composed of nucleic acids and proteins (ribosomes, chromatine, viruses, etc.) while the dimeric $(AAS)_2$ form could serve as a cleavable nucleic acid bis-intercalator and inter-nucleic acids crosslinker.

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