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Chemoimmunotherapy of Cancer. 1†

A. H. Soloway,* I. Agranat, A. R. Chase, R. E. Hernandez, E. S. Kimball, T. Cascieri, Jr., and C. H. Cox

Department of Medicinal Chemistry, College of Pharmacy, Northeastern University, Boston, Massachusetts 02115.
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The use of a multiple-component system for the selective destruction of neoplastic cells is considered with emphasis on chemoimmunotherapy. The synthesis of potential tumor-tagging compounds is described against which antibodies can be prepared. The rationale is presented for the design and preparation of such compounds and the chemical features that are necessary.

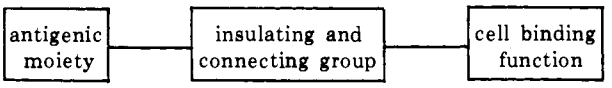
The rationale for using a multiple-component system for the selective destruction of cancer cells is founded on the premise that each agent of this system must be relatively innocuous but their interaction at the tumor cell level produces a cytotoxic reaction. This approach is the basis for the attempts to destroy neoplasms by neutron capture irradiation using ^{10}B and other neutron absorbers.[2-4] This same rationale is the foundation for the use of certain dyes, oxygen, and visible light in the photoradiation of malignancies.[5] Similarly related is the use of masked alkylating agents which are enzymatically activated within the tumor[6] and the use of radiation sensitizers.[7]

Chemoimmunotherapy is yet another possible multicomponent system for cancer therapy. The basic concept involves the preparation of tagging haptens which may be infused to label neoplastic cells. This is the first component of a two-component system. The second is a cytotoxic antibody which may be generated against these haptens. Alternative to such a soluble system is the stimulation of a cell-mediated response to these attached haptens. In either case, the combination of the antibody or sensitized lymphocyte with the antigen at the level of the tumor cell membrane may produce a cytotoxic reaction. This approach offers several advantages *vis-à-vis* conventional cancer chemotherapy. In the latter case, these drugs inhibit mitosis by modifying intracellular metabolism. The need for high intracellular concentrations has prevented the regression of certain solid tumors. Cellular destruction at the membrane level may obviate such restrictions. The background for this approach was that antimelphalan antibodies, when used with this mustard, produced cytotoxic effects in a murine ependymoblastoma. The combination was more effective than either agent alone.[8] Two distinct steps occurred: (1) the mustard became attached to the tumor by alkylation and (2) these labeled cells were destroyed by antihapten antibodies. This same approach has been used against Walker 256 carcinosarcoma.[9]

In order to refine this chemoimmunotherapeutic approach, the synthesis and evaluation of better haptens was undertaken. Ideally, one would want a tumor specific agent. Though we do not have such compounds yet, we can design haptens with several key attributes. In the first place, the compound must readily bind to tumor cells under *in vivo* conditions. For this purpose monofunctional alkylators were prepared since polyfunctional alkylating agents could crosslink proteins and produce mixtures of antigens and these compounds are frequently cytotoxic *per se*. This fact could becloud the question of whether

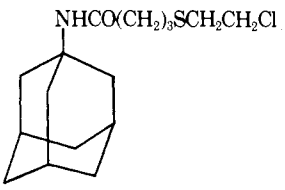
†Preliminary presentation of this work was made; see ref 1.

this procedure were a new form of cancer therapy. Secondly, it seemed imperative to have alkylating compounds of extremely short biological half-lives. The basis for this need is the fact that it would be undesirable to mark appreciably normal cells. Therefore the labeling compound would be infused into the neoplasm's arterial supply and, upon transit of the tumor, should be largely inactivated. Thirdly, it is essential that the compound contain a group capable of eliciting an antibody response. As a result of these considerations, the structures which were synthesized may be viewed as three-component compounds of the following generalized structure.

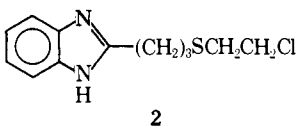


The cell binding function in all of the compounds described here is a sulfur mustard moiety. This group, in addition to having a single alkylating function, can have extremely short biological half-lives with values <1 min.[10] A variety of antigenic moieties are considered below and the insulating and/or connecting group joins both segments, preventing any activity change of the binding function.

Chemical Syntheses. In the compounds synthesized, the variable entity was largely the antigenic moiety. The following compounds (1–4) have been prepared. Com-



1



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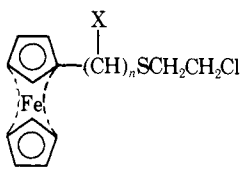
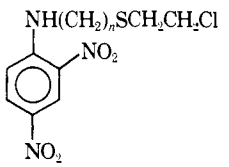


3a, X = H; $n = 2$
b, X = H; $n = 3$
c, X = CH_3; $n = 1$

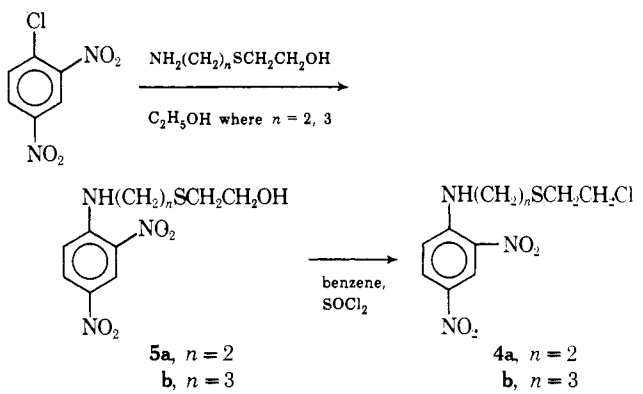


4a, $n = 2$
b, $n = 3$

pound 1 was initially isolated quantitatively as a hydrochloride, in the form of a 2:1 complex with benzene. Confirmation for this composition involving complexation with benzene was obtained by mass spectrometry. Transformation to **1** was achieved by refluxing the complex in a solution of toluene. This facile protonation of amides derived from 1-adamantanamine and the stability of their salts appear to be rather general. Amides such as 1-acetamidoadamantane and γ-chlorobutyroamidoadamantane also behaved in an entirely similar manner. However, chloroacetamidoadamantane failed to form such a stable salt under comparable conditions.

A second hapten, **2**, which was also synthesized is a sulfur mustard containing a benzimidazole moiety. In this compound, the alkylating portion is separated from the antigenic part, namely, the benzimidazole group, by three methylenic groups. Their function is both as a connecting group and as an insulator, thereby preventing the antigenic group from interfering with the rate of cyclization of the mustard. Standard synthetic schemes were utilized in the preparation of 1 and **2**. Both 1 and **2** suffer from a key limitation, namely, they are not chromophoric. A group absorbing in the visible region of the spectrum would offer the potentiality of a ready spectrophotometric measurement of the number of haptens in the hapten–protein conjugate. It is precisely for these reasons that **3** and **4** were synthesized. The first one has a ferrocene moiety within its structure and this nucleus has a characteristic absorption pattern in both the visible and ultraviolet spectra. Hopefully, this chromophoric constituent and its absorption maxima should be unaffected by protein conjugation. Additionally, however, the presence of iron in the ferrocene nucleus offers the opportunity of analyzing the number of haptens in the alkylated protein or tumor by atomic absorption spectroscopy or other analytical procedures. Ferrocene has been shown to elicit a strong antigenic response when conjugated to polypeptides[11] and therefore does have interesting potential as a part of a tumor-binding hapten. The various ferrocenes were prepared by standard methods starting with either vinylferrocene, β-ferrocenylpropanoic acid, or 1-hydroxyethylferrocene.

The 2,4-dinitrophenyl moiety has been studied immunochemically much more extensively than the ferrocenyl group.[12] This is certainly a powerful antigen and, as with ferrocenes, this chromophoric structure readily lends itself to direct spectrophotometric assay. The following synthetic procedure was used for the synthesis of **4**.



5a, $n = 2$
b, $n = 3$

4a, $n = 2$
b, $n = 3$

Protein-Binding and Mustard Hydrolytic Studies. For a viable therapeutic procedure, it is essential that suitable numbers of these sulfur mustard haptens be incorporated into the immunizing protein. Mustard–protein complexes have been described[13,14] and specific antisera produced against such conjugates. Immunogenic differences may be explained by the number of haptenic residues incorporated into the protein. Therefore, it has been necessary to bind such sulfur mustards to proteins and to measure their level of incorporation. In view of the extensive background with dinitrophenyl haptens and the ease of preparing **4a** and **4b**, protein-binding studies and rates of hydrolysis were carried out with these.

The proteins used for these binding studies were bovine serum albumin (BSA) and human gamma globulin (HGG). The method of conjugation involved dissolving the protein in a suitable buffer solution to which was added a dioxane aliquot of the mustard. From these studies, it is apparent that a major limitation in this binding work is the water solubility of these mustards. Their rate of incorporation was dependent upon dioxane concentrations. However, prolonged reaction times in the presence of high levels of dioxane raise the possibility of protein denaturation, and therefore concentrations in excess of 10% dioxane were not used. With these haptens a maximum of 3.5 mol/mol of protein was obtained; a higher hapten percentage was observed with BSA than HGG. Also, there were quantitative differences between the two.

Compound **4a** with three methylenes acting as insulating groups showed a higher incorporation number than **4a**. This result can be attributed to the greater alkylating action of 4b and is in agreement with studies on the half-lives of homologous series of sulfur mustards containing electron-withdrawing groups.[15] In those cases, the reactivity was increased by interposing additional methylene groups between the alkylating group and the electron-withdrawing function.

Even though the number of hapten groups incorporated into the protein was small, attempts were made to immunize rabbits against **4b**-HGG conjugate. The antisera obtained showed no evidence of *anti*-**4b** specific antibodies as determined by precipitin tests and tanned red cell hemolysis, though high titers were obtained for the presence of *anti*-HGG antibodies as observed by the hemolytic reactions. The results show that a major deficiency with the compound used is the inability to incorporate adequate numbers of haptens. In order to confirm this conclusion and determine whether antisera against any dinitrophenyl function would be cross reactive with **4b**–protein conjugates under *in vitro* conditions, 2,4-dinitrobenzenesulfonate was incorporated into HGG and BSA. The dinitrophenyl (DNP) conjugate of HGG was much more insoluble than the BSA counterpart and consequently the latter was characterized and used as the immunogen. Approximately 34 mol of DNP/mol of BSA was obtained. Immunization of guinea pigs with this immunogen resulted in the formation of *anti*-DNP-BSA antisera. The antisera were pooled and rendered specific for DNP by absorption with BSA. Passive hemolytic assays utilizing this antisera demonstrated that 4b–BSA and 4b–HGG had the same end point as DNP–HGG. These results certainly indicate that 4b–HGG, though behaving as a cross-reactive antigen, did not function effectively as a 4b immunogen. This result can be directly attributed to the small number of haptens which were incorporated into HGG and not the immunization protocol.

The apparent low reactivity of these sulfur mustards with proteins resulted in kinetic studies designed to determine their aqueous half-lives. The rates obtained were 15–30 times greater than predicted from the following alkylating agents: $CH_3CH_2CH_2SCH_2CH_2Cl$, $ClCH_2CH_2S(CH_2)_3CONH(CH_2)_3NHCO(CH_2)_3SCH_2CH_2Cl$.[15] The reason for the anomalous results may be attributed either to charge-transfer complexes between the sulfur atom and the aromatic nucleus or to the compound's water insolubility. It is apparent that the compounds which have been selected are inappropriate for evaluating this chemoimmunotherapeutic approach.

In conclusion, this concept of using a multicomponent system for the selective destruction of neoplastic cells remains attractive[9] but in need of more useful haptens. The attempted synthesis of tumor-seeking, water-soluble alkylating agents with ultrashort biological half-lives containing both chromophoric and immunogenic moieties is currently underway and will be reported subsequently.

Experimental Section

Chemical Syntheses. All melting points were determined using either a Fisher-Johns or Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were carried out by Schwarzkopf Microanalytical Laboratories, Woodside, N. Y., or Scandinavian Microanalytical Laboratory, Herlev, Denmark, and all reported compounds were within ±0.4% of theoretical values. Ir spectra were recorded on either a Perkin-Elmer 127 Infracord or a Beckman IR 110 spectrophotometer; uv measurements were carried out on a Beckman DB-G spectrophotometer; and nmr data were obtained by either a Varian A-60 or T-60.

1-[4-(2-Chloroethyl)thiobutyramido]adamantane (1) Hydrochloride. Reaction of 0.003 mol of 1-[4-(2-hydroxyethyl)-thiobutyramido]adamantane in 15 ml of anhydrous benzene with $SOCl_2$ at room temperature resulted in the precipitation of the hydrochloride of 1 in quantitative yield from the reaction mixture. The same salt was formed in 94% yield by direct reaction of the alcohol with dry HCl in benzene at room temperature. The product was a 2:1 mustard–benzene complex which could be recrystallized from benzene: mp 124° dec; ir_{max} 2200 (br), 1795, 1665 cm^{-1}. *Anal.* [$C_{38}H_{60}Cl_4N_2O_2S_2$[2($C_{16}H_{26}ClNOS$·HCl) C_6H_6]] C, H, Cl, N, S.

1-[4-(2-Chloroethyl)thiobutyramido]adamantane (1). Refluxing a solution of 622 mg of 1·HCl (0.0008 mol) in 70 ml of toluene followed by removal of the solvent *in vacuo* after 4 hr yielded a product which gave white needles: mp 55° (64% yield) from ether–pentane; ir_{max} 3310 (NH), 1637 cm^{-1} (CO); nmr ($CDCl_3$, TMS) 1.7 (s, br, 6 H, Ad, δ-H), 1.8–2.3 (m, 13 H, including a strong, broad singlet at 2.0, Ad, β-H γ-H, $COCH_2CH_2$), 2.6 (t, 2 H, $-CH_2CH_2SCH_2CH_2Cl$), 2.9 (t, 2 H, $-SCH_2CH_2Cl$), 3.7 (t, 2 H, $-SCH_2CH_2Cl$), 5.7 ppm (s, br, 1 H, NH). *Anal.* ($C_{16}H_{26}ClNOS$) C, H, Cl, N, S.

2-[3-(β-Chloroethyl)mercaptopropyl]benzimidazole (2) Hydrochloride. To a solution of 420 mg (0.0018 mol) of 2-[3-(β-hydroxyethyl)mercaptopropyl]benzimidazole in 10 ml of dry benzene was added 1.4 g of $SOCl_2$ in 2 ml of benzene. The mixture was refluxed for 1 hr and, upon cooling, **2** precipitated. The solution was filtered, washed with a small volume of cold benzene, and dried. The yield was quantitative. The product was recrystallized from chloroform–ethyl acetate: mp 149.5°; uv 281, 276, 270, and 242 nm. *Anal.* ($C_{12}H_{10}N_2SCl_2$) C, H, N, S, Cl.

General Method for the Preparation of Ferrocenyl Sulfur Mustards. To 1 g of the appropriate 2-hydroxyethyl sulfide (*ca.* 0.005 mol) in 10 ml of CH_2Cl_2 cooled to −30° (a Dry Ice–acetone bath) was added 1 ml each of 13% v/v $POCl_3$ in CH_2Cl_2 and 13% v/v PCl_3 in CH_2Cl_2 maintained at −30°. The solution was shaken to effect mixing and allowed to stand at −30° for 1 hr. The reaction mixture was allowed to rise to −15° and held at that temperature for 20 hr. The solution was poured into 40 ml of ice water and the aqueous layer immediately assumed a blue-green color. The CH_2Cl_2 was separated and the aqueous fraction was extracted with more CH_2Cl_2. The combined extracts were washed with H_2O and dried over K_2CO_3 at 0°. Solvent concentrated to approximately 1 ml was carried out at −35° and 0.5 mm. Purification was effected by preparatory tlc at 4°, eliminating in this manner polar components. The yield of pure mustard from this procedure was in the 40% range.

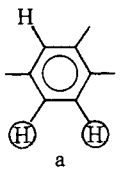
2-Chloroethyl-2-ferrocenylethyl sulfide (3a): yellow solid; mp 34°; nmr ($CHCl_3$, TMS) 2.7 (m, 4 H, $CH_2CH_2SCH_2CH_2Cl$), 2.9 (t, 2 H, $CH_2CH_2SCH_2CH_2Cl$), 3.75 (t, 2 H, $CH_2CH_2SCH_2CH_2Cl$), 4.2 ppm (s, 9 H, Fc protons). *Anal.* ($C_{14}H_{17}SClFe$) C, H, Cl.

2-Chloroethyl-3-ferrocenylpropyl sulfide (3b): orange oil; nmr ($CDCl_3$, TMS) 1.8 (q, 2 H, $-CH_2CH_2CH_2S$), 2.3–2.9 (m, 6, H, $-CH_2CH_2CH_2SCH_2CH_2Cl$), 3.6 (t, 2 H, $-SCH_2CH_2Cl$), 4.05 (s, 4 H, Fc protons), 4.1 ppm (s, 5 H, Fc protons). *Anal.* ($C_{15}H_{19}SClFe$) C, H, S, Cl.

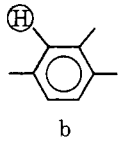
2-Chloroethyl-1-ferrocenylethyl sulfide (3c): orange oil; nmr ($CDCl_3$, TMS) 1.65 [d, 3 H, $-CH(CH_3)S-$], 2.75 [t, 2 H, $CH(CH_3)SCH_2CH_2Cl$], 3.3–3.9 [m, 3 H, $CH(CH_3)SCH_2CH_2Cl$], 4.05 (s, br, 4 H, Fc protons), 4.1 ppm (s, 5 H, Fc protons). *Anal.* ($C_{14}H_{17}SClFe$) C, H, S, Cl.

2-Hydroxyethyl-2-(2,4-dinitroanilinyl)ethyl Sulfide (5a). To a stirred solution of 17.5 g (0.086 mol) of 2,4-dinitrochlorobenzene in 300 ml of absolute ethanol was added 21.1 g (0.174 mol) of 2-hydroxyethyl-2-aminoethyl sulfide in 100 ml of absolute ethanol. The solution, which became darker yellow, remained at room temperature for 2 days. The solution was concentrated under reduced pressure and the residue was dissolved in ether. The ethereal solution was extracted with H_2O and the aqueous solution was reextracted with ether. The combined ether layers were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The resulting yellow oil crystallized, yielding 20.8 g (83%): mp 74.5–75°; ir ν_{max} 3400, 1615, and 1585 cm^{-1}. *Anal.* ($C_{10}H_{13}N_3O_5S$) C, H, N, S.

2-Hydroxyethyl-3-(2,4-dinitroanilinyl)propyl Sulfide (5b). The procedure was comparable for the synthesis of **5a**. The product obtained in a 65% yield was a yellow solid: mp 49–50°; ir ν_{max} 3400, 1615, and 1585 cm^{-1}; uv λ_{max} 346 nm; nmr ($CDCl_3$, TMS) 2.1 (m, 2 H, $-NHCH_2CH_2CH_2S-$), 2.5 (s, br, 1 H, $-SCH_2CH_2OH$), 2.8 (m, 4 H, $NHCH_2CH_2CH_2SCH_2CH_2OH$), 3.6 (t, 2 H, SCH_2CH_2OH), 3.8 (t, 2 H, $-NHCH_2Ch_2CH_2S-$), 7.1–8.3



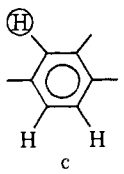
a



b

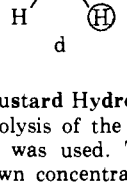
(m, 2 H, structure a), 8.7 (s, br, 1 H, -N*H*CH_2-), 9.1 ppm (d, 1 H, structure b). *Anal.* ($C_{11}H_{15}N_3O_5S$) C, H, N, S.

2-Chloroethyl-3-(2,4-dinitroanilinyl)propyl Sulfide (4b). A solution of 8.78 g (0.0336 mol) of **5b** in 4 ml of $SOCl_2$–50 ml of benzene was refluxed overnight. The mixture was filtered and concentrated under reduced pressure. The product was recrystallized from benzene yielding 6.5 g (60.8%) of a yellow solid: mp 78.5–79°; uv λ_{max} 346, 262, 243 nm; nmr ($CDCl_3$, TMS) 2.1 (m, 2 H, -NHCH$_2$C*H*$_2$CH$_2$S-), 2.8–2.9 (m, 4 H, -NHC*H*$_2$CH$_2$-CH$_2$SCH$_2$C*H*$_2$Cl), 7.0–8.3 (m, 2 H, structure a), 8.6 (s, br, 1 H, -N*H*CH$_2$CH$_2$CH$_2$S-), 9.1 ppm (d, 1 H, structure c). *Anal.* ($C_{11}H_{14}ClN_3O_4S$) C, H, Cl, N, S.

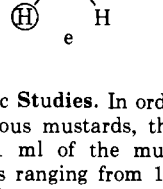


c

2-Chloroethyl-2-(2,4-dinitroanilinyl)ethyl Sulfide (4a). A similar procedure was used for the synthesis of this mutard as described for **4b**. A yellow solid (87% yield) was obtained after recrystallization from benzene: mp 91.5–92.5°: nmr ($CDCl_3$, TMS) 3.1 (m, 4 H, CH$_2$C*H*$_2$SC*H*$_2$CH$_2$Cl), 3.7 (m, 4 H, NHC*H*$_2$CH$_2$-SCH$_2$C*H*$_2$Cl), 7.0 (d, 1 H, structure d), 8.3 [d (split into d), 1 H, structure e], 8.8 (s, br, 1 H, -N*H*CH$_2$CH$_2$S-), 9.1 ppm (d, 1 H, structure f). *Anal.* ($C_{10}H_{12}ClN_3O_4S$) C, H, N, S, Cl.



d



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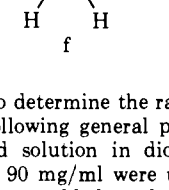

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Mustard Hydrolytic Studies. In order to determine the rate of hydrolysis of the various mustards, the following general procedure was used. To 1 ml of the mustard solution in dioxane (known concentrations ranging from 15 to 90 mg/ml were used) placed in a water bath maintained at 37° was added 1 ml of 5% 4-(4′-nitrobenzyl)pyridine[16] in dioxane and 0.5 ml of acetone. The solution was swirled and immediately attached to a condenser equipped with a drying tube and heated in a 70° water bath for 35 min. The solution was cooled in an ice bath and 0.5 ml of acetone was added. This solution was now stable for at least 20 min. The mixture was then transferred to a 5-ml volumetric flask, 1 ml of 50% triethylamine–acetone was added, and the solution was diluted to volume with acetone. An aliquot was transferred to a cuvette and the per cent transmittance at 560 nm determined.

Protein-Binding Studies. A variety of procedures were used for the conjugation of the mustards **4a** and **4b**. The following general experimental method was used. To a solution of 7.5 mg of HGG dissolved in 28.5 ml of 0.5 *M* carbonate buffer (pH 8.0) was added dropwise with stirring a solution of 14.8 mg of **4b** in 1.5 ml of dioxane. The solution became yellow and very turbid. The reaction vessel was covered with parafilm and tin foil and stirred overnight at room temperature. The major portion of the conjugate (25 ml) was dialyzed twice against 1 l. of 0.01 *M* phosphate (pH 7.5) at 4° for 8 hr each time in order to remove unconjugated mustard. Additional dialysis of the conjugate was unnecessary since lyophilization of subsequent dialysates contained no haptenic material as determined by optical density measurements at 362 nm. A slight suspension of yellow particulate material was present which analyzed for 0.012 μmol of HGG and 6.8 μmol of **4b**. Since this amount of HGG seems to precipitate nonspecifically from the controls, it would seem most likely that the suspension consists primarily of unreacted **4b**. This solid was removed by centrifugation at 5000 rpm for 15 min in a refrigerated centrifuge. The supernatant was stored at 4°.

Similar conjugation procedures were used for **4a** and dinitrobenzenesulfonic acid.

Immunization and Sera Isolation Procedures. Conjugates were mixed with an equal volume of Freund's complete adjuvant[17] by forcing the mixture in and out of a 1-ml syringe until a thick pastelike emulsion was formed. Initial injections containing 500 μg of conjugate/0.5 ml were made with this emulsion into the toe pads of all four feet of white male New Zealand rabbits (2–3 kg). An intravenous booster containing 500 μg of conjugate was given 3 weeks after the initial injection. The animals, which may have been fasted, were bled between the fifth and seventh days following the booster either by heart puncture or from the ear artery. The blood was allowed to stand at room temperature for 2 hr, the clot was rimmed, and the blood was then refrigerated for 21 hr. After that time interval, the defibrinated blood was spun between 3000 and 5000 rpm in a refrigerated centrifuge. The supernatant sera was removed by pipet and stored at refrigerator temperature wtih streptomycin added as a preservative. In view of the fact that the mustard–protein conjugates appeared to be nonimmunogenic, additional booster injections were administered at weekly intervals. No increase in the immunogenicity of this conjugate was observed.

Antibody Assays. Qualitative Precipitin Test. This test was performed according to an established procedure[17] in order to determine the optimal titer for use in Ouchterlony diffusion.

Ouchterlony Gel Diffusion. Reagents and plates were prepared according to the procedure of Campbell.[17] Purified agar (Difco Certified 0560-01) was used to prepare the diffusion plates. A 10% sodium azide stock solution[18] was prepared and added to the autoclaved agar solution to a final concentration of 0.1%. When the agar had hardened, wells were made in the plate by means of a template or a reasonable facsimile. Wells were approximately 8 mm in diameter and spaced 2 cm apart. Plates were stored at 4° until use. Sera or antigen, in a concentration determined by a qulitative precipitin test, was placed in the center well and 0.1 ml of the antigen or sera was placed in an outer well. Test plates were incubated usually at 37° from 12 to 24 hr. Tests requiring longer incubation were usually carried out at 4°.

Quantitative Precipitin Test. Antigen (1 ml, 0.01–0.50 mg/ml) was added to 1 ml of serum and mixed. Samples were incubated at 37° for 30 min and then refrigerated for 5 days. The precipitates were spun down at 2000 rpm in a refrigerated centrifuge. The precipitate was washed twice with 2.5-ml aliquots of isotonic saline and the sample was centrifuged. To each precipitate, 1.5 ml of biuret reagent was added and the solution was mixed well. Similarly, 1 ml of water was added, the tubes were incubated for 4 min at 37°, and spectral measurements were made at 550 nm.

Tanned Red Cell Hemolysis Procedure. Alsever's solution was prepared,[17] passed through a millipore filter, and stored in sterilized bottles at 4°. Sheep red blood cells which were obtained from the animal facility at the Massachusetts General Hospital were placed in an equal volume of Alsever's solution. Lyophilized guinea pig complement and restoring fluid, obtained from the Grand Island Biological Co., Grand Island, N. Y., were stored in the freezer.

Antisera and sheep red blood cells were prepared according to the procedure of Stavitsky[18] and the antisera was heated to inactivate residual complement. For the actual assay, the coated cells were washed and diluted to a 1% RBC suspension with a veronal buffer. To the serial dilutions of the adsorbed test sera in isotonic barbital buffer (0.5 ml per tube) was added 0.1 ml of the RBC suspension. The tubes were incubated at 37° for 15 min and 0.1 ml of cold guinea pig complement was added to each. The samples were partly mixed and incubated at 37° for 15–20 min, and then hemolysis was determined visually. Unsensitized cells were incubated with the antisera as controls and also a known positive serum was run with each assay in order to determine the validity of the tests with unknown sera. These controls were run in parallel studies.

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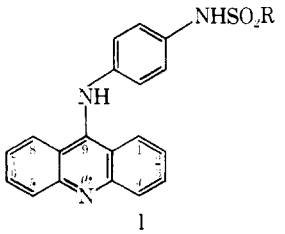
Potential Antitumor Agents. 14. Acridylmethanesulfonanilides

Bruce F. Cain,* Ralph N. Seelye, and Graham J. Atwell

Cancer Chemotherapy Laboratory, Cornwall Geriatric Hospital, Auckland, New Zealand. *Received January 29, 1974*

A series of 74 structural variants of 4′-(9-acridinylamino)methanesulfonanilide has been prepared and evaluated in the L1210 leukemia system. The intact acridine ring system appears essential for antileukemic activity. Lengthening the alkyl chain in either the 4′-(9-acridinylamino)-*n*-alkanesulfonanilides or 4′-(3-acetamidoacridin-9-ylamino)-*n*-alkanesulfonanilides caused a progressive decrease in antileukemic activity. Plots of ΔR_m, as a convenient measure of lipophilic–hydrophilic balance, against log biologic response for members of these homologous series provided a parabolic reference curve. Substituent effects on biologic activity were then gauged in relation to the activity predicted from the reference curve on the basis of the ΔR_m value of the variant. There appears to be bulk intolerance about positions 1(8) and 2(7) of the acridine ring, the activity of all 1- or 2-substituted variants being depressed below that expected from ΔR_m values or abolished. When allowance was made for the increase in lipophilic character due to hydrophobic 3-substituents, there was an increase in activity over that expected. It is predicted that more hydrophilic agents with hydrophobic 3-substituents may show greater activity. Analogies are drawn between the agents synthesized and the 9-phenylphenanthridinium salts; structural features as determined by X-ray crystallographic analyses are similar; both series have biologic activities in common and have the capacity to bind to and intercalate into DNA twin helices. Thirteen variants provided a proportion of indefinite survivors in early intraperitoneal (10^5) L1210 tests.

An earlier report from this laboratory demonstrated the excellent activity of **1** (R = CH_3) against the early intraperitoneal L1210 test system.[1] This communication is the first of a series in which this lead will be developed.



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Chemistry. The basic step involved in preparing all compounds listed in Table I was the coupling of the requisite γ-chloro heterocycle with a *p*-aminosulfonanilide component in acid medium (Scheme I).

The required 9-chloroacridines were synthesized by ring closure of *N*-arylanthranilic acids which were in turn produced by an Ullmann[2] synthesis (Scheme II). A modification of the conditions used for Ullmann synthesis has considerably simplified the application of this method without diminution of yields (see Experimental Section).

Use of meta-substituted anilines in this acridine synthesis (Scheme II) produces a mixture of 1- and 3-substituted 9-chloroacridines by ring closure in the two possible directions. Separation of isomers has been avoided when possible by using unequivocal syntheses from substituted 2-chlorobenzoic acid components. A different route can sometimes avoid isomer formation; for example, 3-methylacridone is conveniently prepared by condensing 3-methylcyclohexanone and anthranilic acid to 3-methyl-1,2,3,4-tetrahydroacridone[3,4] which on dehydrogenation (Pd/C; refluxing Dowtherm A) is quantitatively converted to 3-methylacridone. This dehydrogenation method is an improvement on an earlier one (Cu/air at 360°; 11% yield).[3]

Simple 9(10*H*)-acridones on treatment with $SOCl_2$ containing catalytic quantities of DMF are converted in excellent yield to the corresponding 9-chloro compounds; more vigorous conditions previously used (refluxing $POCl_3$) can then be avoided.

In the bulk of the examples listed (Table I) coupling of the γ-chloro heterocycle and the requisite aminosulfonanilide in refluxing EtOH–H_2O (method A) provided acceptable yields (61–96%). In certain cases low yields were found to result from competing hydrolysis of the chloroacridine to acridone. Coupling in anhydrous solvents (method B) then provided acceptable yields.