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Chemoimmunotherapy of Cancer. 2[1]

A. H. Soloway,* J. E. Wright, V. Subramanyam, and J. J. Gozzo

Department of Medicinal Chemistry and Pharmacology, Northeastern University, Boston, Massachusetts 02115.
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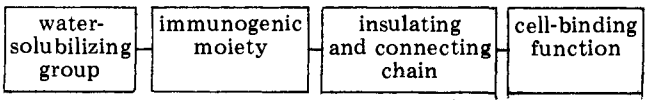
The preparation of a series of water-soluble mustard haptens for chemoimmunotherapy of cancer is described. Preliminary screening data are given, indicating some activity against P388 lymphocytic leukemia for those compounds containing the most potent immunogenic functional groups.

The current usage of chemotherapeutic anticancer agents at the maximum tolerated dose is based upon the theory that the fraction of target cells killed is linearly proportionate to dose,[2] i.e., follows first-order kinetics. A fundamental drawback to therapy based upon this model is the systemic toxicity that is encountered. In contrast, the cytotoxicity achieved with immunostimulants, such as MER[3] and levamisole,[4] is purported to follow zero-order kinetics, whereby the number of cells killed is proportionate to dose.[5] In such a case, the peak effective dose may be well below the maximum tolerated dose, and untoward systemic effects may be avoided. Thus, chemoimmunotherapy may offer another modality for the treatment of cancer.

Previously, we reported the preparation and characterization of several chemoimmunotherapeutic substances, designed for specific attachment to the cell membrane surface, where interaction with hapten-specific antibodies and complement might result in cellular destruction. Although these agents did prove to be immunopotent alkylators, possessing adequately short half-lives for our purposes, their poor water solubility made them most unsuitable markers for the cell membrane surface. In view of these results, the synthesis of water-soluble alkylators was undertaken. Furthermore, the previously reported compounds were designed solely for use in concert with antihapten antibodies, raised exogenously, against conjugates of the mustard with bovine serum albumin (BSA) and human immunoglobin G (HGG). While this approach remains attractive and under active study, we have sought to prepare as well other haptens possessing functional groups which may promote a tumor-specific, cell-mediated response. This report presents the synthesis and preliminary biological evaluation of two types of compounds which overcome the solubility limitations.

The compounds described herein may be viewed as having the following general structure.



* Address correspondence to this author at the College of Pharmacy, The Ohio State University, Columbus, Ohio 43210.

The cell-binding function retains the sulfur mustard structure which we have described previously.[1] The rationale for choosing such an entity is the excellent work of Seligman and his associates,[6] who have found that such mustards may have extremely short chemical half-lives. Therefore, these compounds may be useful for tagging tumor cells by perfusion into the vascular supply of the neoplasm. This technique serves to avoid concomitant attachment to normal tissues, which are remote vis-à-vis the site of administration. Also the short half-life may contribute significantly to the specific alkylation of the membrane surface and to exclusion from the intracellular medium. The connecting, insulating linkage has two purposes: (1) to prevent the immunogenic moiety from inhibiting the reactivity of the sulfur mustard and (2) to project the immunogenic moiety outward from the cell membrane surface, thus aiding recognition by the immune surveillance system. Two types of immunogenic determinants were prepared: (1) those which may elicit antihapten antibodies when conjugated with carrier proteins and (2) entities capable of stimulating the production of hapten-sensitized lymphocytes. Each type will be discussed separately below. The fourth component is a water-solubilizing function. The need for such a function, as established in our initial report,[1] lies in the enhanced hapten-to-carrier binding ratios of the immunizing conjugates and in the advantages of adminstering such solubilized conjugates to mammalian recipients. The compounds described below either have special solubilizing groups or the immunizing group is intrinsically soluble in aqueous systems.

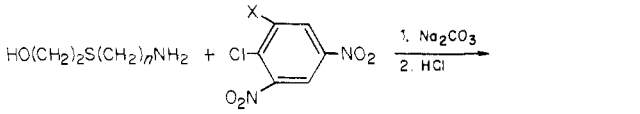
Chemical Syntheses. The dinitrobenzene mustards were prepared in several steps as shown in Chart I. Initially, the side chains were fashioned by the alkylation of 2-mercaptoethanol, using the ω-bromo derivatives of *n*-propyl- and *n*-butylammonium bromide in excess alkali. The resulting straight-chain alkylamines were subsequently arylated by the appropriate 2,4-dinitrochlorobenzenes, which contained the desired anionic solubilizing substituents in the 6 position. The resulting hydroxyethyl compounds were then chlorinated with $SOCl_2$ in benzene to give the target mustards.

Chart I. Mustard Preparation by Aromatic Nucleophilic Substitution

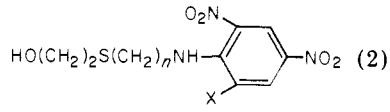
$$HO(CH_2)_2SH + Br(CH_2)_nNH_3Br \xrightarrow{NaOH} HO(CH_2)_2S(CH_2)_nNH_2 \quad (1)$$

1

n = 2 (1a) or 3 (1b)

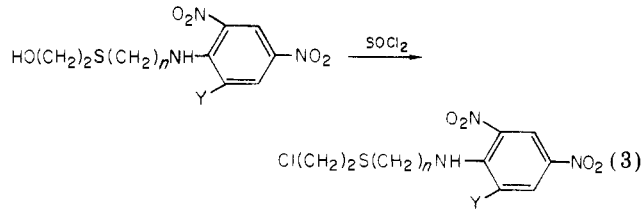


$$HO(CH_2)_2S(CH_2)_nNH_2 + \text{2,4-dinitrochlorobenzene derivative} \xrightarrow[2.\ HCl]{1.\ Na_2CO_3} \quad (2)$$



2, 3

X = SO_3Na; n = 2 (2a) or 3 (2b)
X = CO_2Na; n = 2 (3)



4, 5

Y = SO_3H; n = 2 (4a) or 3 (4b)
Y = CO_2H; n = 2 (5)

Chart II. Mustard Preparation by N-Acylation

$$HO(CH_2)_2SH + Br(CH_2)_nCO_2C_2H_5 \xrightarrow{NaOH} HO(CH_2)_2S(CH_2)_nCO_2C_2H_5 \quad (4)$$

6

n = 2 (6a) or 3 (6b)

$$HO(CH_2)_2S(CH_2)_nCO_2C_2H_5 \xrightarrow[2.\ HCl]{1.\ NaOH} HO(CH_2)_2S(CH_2)_nCO_2H \quad (5)$$

7

n = 2 (7a) or 3 (7b)

$$HO(CH_2)_2S(CH_2)_nCO_2H \xrightarrow[excess]{SOCl_2} Cl(CH_2)_2S(CH_2)_nCOCl \quad (6)$$

8

n = 2 (8a) or 3 (8b)

$$RNH_2 + Cl(CH_2)_2S(CH_2)_nCOCl \xrightarrow{DMF} RNHCO(CH_2)_nS(CH_2)_2Cl \quad (7)$$

9-13

R = $-CH(CH_3)_2$; n = 2 (9)
R = $-C_6H_4$-p-OH; n = 3 (10)
R = $-C_6H_4$-p-PO_3H_2; n = 2 (11a) or 3 (11b)
R = $-C_6H_4$-p-AsO_3H_2; n = 2 (12a) or 3 (12b)
R = $-C_6H_4$-p-$N(CH_3)_3^+I^-$; n = 2 (13a) or 3 (13b)

Preparation of the amide derivatives via the multistep synthesis outlined in Chart II also utilized the prior formation of side chain. Alkylation of 2-mercaptoethanol by ω-bromo-substituted ethyl propionate and ethyl butyrate was carried out with excess alkali. Subsequently, each of the free acids was prepared by saponification. Exhaustive chlorination with $SOCl_2$ led to the thioethers, possessing a chloroethyl group on one end and acid chloride at the other. These acid chlorides were used to acylate selected para-substituted anilines, and in the case of 2-propylamine, the target amide mustard was formed.

Table I. Evaluation of in Vivo Activity against P388 Lymphocytic Leukemia[a]

No.	Dose,[b] mg kg^{-1} inj^{-1}	TLC, %
4a	12.5	111
	25.0	124
	50.0[c]	124
	75.0	118
4b	6.25	111
	12.5	132
	25.0	136
	50.0[c]	128
11a	25.0	101
	50.0[c]	112
	100	98
11b	25.0	104
	50.0[c]	108
	100	105
12a	25.0	104
	50.0[c]	110
	100	107
12b	50.0	114
	100	116
	200	131
13a	12.5	109
	25.0	114
	50.0	52
13b	12.5	96
	25.0	113
	50.0	18
9	3.13	93
	6.25	105
	12.5	60
10	3.13	88
	6.25	94
	12.5	54
5	12.5	102
	25.0	111
	50.0[c]	106

[a] 3PS31 test system. [b] Given on days 1, 5, and 9. [c] Average of two experiments.

The success of this last step required a departure from conventional N-acylation procedures, which generally rely upon the use of bases for effecting the condensation. Such conditions could not be used in these instances since decomposition of the mustard group would occur. Using dry DMF as solvent, however, reaction of the acid chloride and amine gave adequate yields of the desired product in the absence of mustard-labile nucleophiles. The isolation of $(CH_3)_2NH_2Cl$ from the reaction mixture indicated that the HCl was neutralized in situ by a reaction involving decomposition of the solvent.

Biological Studies. Since these compounds are monofunctional alkylators, it was not anticipated that they would possess antineoplastic activity in the usual chemotherapeutic sense.[7] Nevertheless, they were submitted for evaluation by a conventional screening procedure,[8] in order to provide baseline data for comparison with later trials, using immunologically relevant protocols. The results are summarized in Table I. These results indicate, unexpectedly, some activity for compounds **4a** and **4b** but of a low order at the dosage levels used. One other compound, **12b**, has presumptive activity at the highest dosage tested, 200 mg kg^{-1}. Clearly, the findings are encouraging, though fragmentary, and have prompted us to undertake additional biological studies.

These compounds were bound covalently to proteins and to tissue specimens by procedures described in the Experimental Section. Analytical studies confirmed the covalent nature of the binding. Such results clearly indicate that these compounds are behaving as viable al-

Table II. Skin Graft Data for Transplants of B_6AF_1 Tissues to C3H/HeJ Mice. Percentage Viable on the Given Day in Each Group

Day	Group[a] (viability) A, %	B, %	C, %
7	100	100	100
8	60	90	100
9	30	60	60
10	0	40	40
11	0	10	30
12	0	0	10
13	0	0	0

[a] Ten mice per group. A = immunized, treated. B = unimmunized, treated. C = unimmunized, untreated.

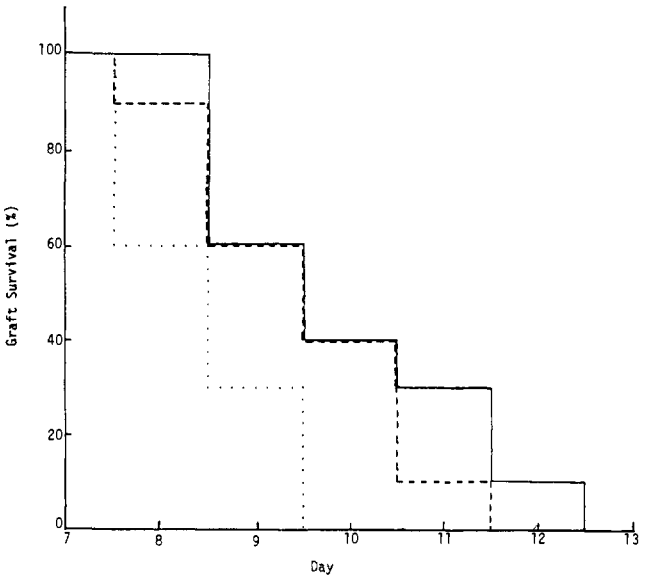


Figure 1. Ogive for allogratt survival in transplant of B_6AF_1 skin to C3H/HeJ mice. Percentage of grafts remaining on the given day following transplant: dotted line represents immunized, treated group (A); dashed line, unimmunized, treated (B); solid line, unimmunized, untreated (C). See Table II.

kylating agents and would therefore be appropriate for alkylating tumor cells in our planned future protocols.

Additionally, allogeneic skin grafts were used to demonstrate directly the cellular immune response "against hapten-bound tissue transplanted from male B_6AF_1 to male C3H/HeJ mice." Accelerated skin graft rejection was observed in the transplantation of hapten-bound allogeneic skin to mice previously sensitized against the hapten conjugated to HGG. In contrast, the same treated skin showed only slightly enhanced rejection by unsensitized mice in comparison with immunized controls receiving *un*treated skin grafts (see Figure 1 and Table II).

Experimental Section

Melting points were obtained on a Thomas-Hoover Unimelt apparatus using open capillary tubes and were corrected. Elemental analysis and molecular weight determinations were performed by Galbraith Laboratories, Inc. These are denoted by the appropriate symbols which indicate agreement with theory of ±0.4%. Infrared spectra were obtained by means of a Perkin-Elmer Model 137 spectrophotometer using the KBr wafer method for solids and salt plates for liquids. ^1H NMR spectra were obtained on a Varian T-60 instrument using the indicated solvents containing 1% tetramethylsilane as an internal standard. Thin-layer chromatography data were obtained using Analtech XLGF uniplates coated with 250 μ of fluorescent hard-layer silica gel. Eluent compositions are specified below for each of the relevant systems.

Starting materials for the side-chain amine and acid chloride intermediates were all obtained from the Aldrich Chemical Co. and were used without further purification. *p*-Arsanilic acid, which was obtained from the same source, did require recrystallization from water prior to use (lit. mp 232 °C, found 300 °C). The *p*-phosphanilic acid was prepared by the method of Grummitt et al.,[13] mp 242–244 °C. All of the remaining reagents were purchased from Eastman Organic Chemicals and purified as stated below. The thionyl chloride was distilled from (*R,S*)-limonene, 30 vol %. From a typical batch, starting with 250 mL of practical grade $SOCL_2$, the first 5-mL cut was discarded and the following 100-mL portion, boiling in the range of 75–76 °C, was collected for use. The *p*-hydroxyaniline was recrystallized under nitrogen from water containing a large portion of decolorizing charcoal. The (4-aminophenyl)trimethylammonium iodide hydrochloride was converted to the free base by stirring with a slight excess of Na_2CO_3 suspended in 70% aqueous EtOH until no more CO_2 evolved. The solution was evaporated to dryness and the solid recrystallized from absolute EtOH. The 2-chloro-3,5-dinitrobenzoic and benzenesulfonic acids were recrystallized from water.

5-Hydroxy-3-thiapentylamine[9] (1a). To a stirred solution containing 42 mL of 2-mercaptoethanol was added dropwise 61.5 g of 3-bromoethylamine hydrobromide (0.3 mol) dissolved in 150 mL of absolute EtOH. The mixture was heated in order to distill off 400 mL of the EtOH and then allowed to return to room temperature. The addition of 100 mL of Et_2O brought down a copious precipitate of NaBr. The mixture was filtered and the filtrate concentrated under reduced pressure until no solvent remained. The addition of 24.4 mL of concentrated HCl (0.3 mol) and 100 mL of Et_2O gave a precipitate of NaCl which was removed by filtration. The filtrate was again concentrated under reduced pressure to remove the ether and then distilled under high vacuum, collecting the 97–100 °C fraction at 0.43–0.45 Torr to yield 26.4 g (75%): IR 3350 and 3300 (NH and OH), 3250–3036 (OH and NH), 2995 and 2870 (CH_2), 1595 (NH_2), 1080–1030 cm^{-1}, (CCO); ^1H NMR ($CDCl_3$) δ 3.7 (t, CH_2OH), 3.3 (s, NH_2 and OH), 2.75 ppm (m, $HOCH_2CH_2SCH_2$).

Sodium 2-(5′-Hydroxy-3′-thiapentylamino)-3,5-dinitrobenzenesulfonate Monohydrate (2a). A mixture consisting of 30.4 g of sodium 2-chloro-3,5-dinitrobenzenesulfonate (0.1 mol), 8 g of Na_2CO_3 (0.08 mol), and 15.0 g of 1a (0.12 mol) in 300 mL of absolute EtOH was stirred for 24 h at room temperature. The solution was filtered and the residues were recrystallized from acetone–ether solution containing just enough water at the boiling point to effect solution. The yield of this initial crop of crystals was 16.8 g.

The filtrate was evaporated to dryness and the yellow residue recrystallized as before. The second precipitate weighed 7.6 g. The combined yield was 24.4 g of **2a** (60%): mp 156–157 °C; IR 3550–3200 (OH and NH), 3050 (CH_2), 2900, 2850 (C_6H_2), 1550 (SO_3^-), 1100, 1050 (C_6H_2), 940, 920 (CH_2), 860 (C_6H_2 and CH_2), 740 and 780 cm^{-1} (CSC); ^1H NMR [$CD_3OD(CD_3)_2CO$, 1:1] δ 8.7 (q, C_6H_2), 4.5 (H exchange), 3.7 (t, $HOCH_2$), 3.4–2.6 ppm ($CH_2SCH_2CH_2$). Anal. C, H, N.

2-(5′-Chloro-3′-thiapentylamino)-3,5-dinitrobenzenesulfonic Acid–Dimethylformamide Complex (4a). A 3.9-g portion of **2a** (0.01 mol) was suspended in a solvent mixture consisting of 100 mL of $CHCl_3$, 80 mL of $(CH_3)_2CO$, and 0.5 mL of $(CH_3)_2NCHO$. Then, 1.5 mL of $SOCl_2$ (0.02 mol) was added and the mixture stirred 1.5 h at room temperature. The solution was filtered to remove NaCl. The filtrate was concentrated to form a dark red gum, which was redissolved in warm acetone, boiled with decolorizing carbon, and filtered. To this 125-mL solution, 2 mL of DMF was added, followed by 150 mL of cyclohexane. The solution was refrigerated overnight. A bright yellow crystalline precipitate formed, the mixture was filtered, and the precipitate was dried in vacuo: yield, 2.75 g of **4a** (60%); mp 103–105 °C; ^1H NMR (polysol-D) δ 10.1 (s, SO_3H), 8.6 (s, C_6H_2), 8.0 (s, HCO), 3.7 (t, CH_2Cl), 3.0 [m, $CH_2S(CH_2)_2$], 2.9 ppm [d, $(CH_3)_2N$]. Anal. C, H, N, Cl.

2-(5′-Hydroxy-3′-thiapentylamino)-3,5-dinitrobenzoic Acid Sodium Salt (3). To a 200-mL aqueous suspension containing 12.4 g of 2-chloro-3,5-dinitrobenzoic acid (0.05 mol) was added 3.2 g of Na_2CO_3 (0.03 mol). The mixture was stirred as CO_2 evolved and the acid dissolved completely. The water was removed under reduced pressure and the residue recrystallized from absolute ethanol: yield, 12.1 g (90%).

To 6.0 g of this sodium salt (0.036 mol) was added 4.5 g of 1a (0.037 mol) in 150 mL of water. The mixture was stirred for 12 h; the solvents were removed under reduced pressure and the residue was recrystallized from acetone–ether (1:1): yield, 9.9 g (78%); mp 132–134 °C; IR 3600–3200 (OH and NH), 3090 (NH), 2920 (CH_2), 1620, 1580 (NO_2), 1540, 1520, 1330 (CO_2^-), 1060–1100 cm^{-1} (CH_2OH); ^1H NMR [CD_3OD–$(CD_3)_2CO$, 1:1] δ 8.8 (q, C_6H_2), 4.4 (H exchange), 3.7 (t, CH_2OH), 3.3–2.6 ppm [m, NH-$(CH_2)_2SCH_2$]. Anal. C, H, N.

2-(5′-Chloro-3′-thiapentylamino)-3,5-dinitrobenzoic Acid (5). To a well-stirred solution containing 3.53 g of **3** (0.01 mol) in 40 mL of dry acetone was added 3.0 g of $SOCl_2$ (0.025 mol). An immediate precipitate of NaCl resulted. The mixture was stirred at room temperature for 1.5 h and filtered, and the filtrate was evaporated to dryness under reduced pressure. The yellow solid was recrystallized from benzene, yielding 2.3 g (66%) of 5: mp 139–140 °C; IR 3220–3020 (NH), 2920–2880 (CH_2), 1670 (>NH_2^+), 1580 (CO_2^-, NO_2), 1520 (CO_2^-), 1500, 1400 (CCl), 1250 (NH), 1180 and 1110 cm^{-1} (C_6H_2); ^1H NMR ($CDCl_3$) δ 9.8 (s, CO_2H), 8.8 (q, C_6H_2), 3.7 (t, $ClCH_2$), 2.8–3.4 ppm (H exchange and $CH_2SCH_2CH_2$). Anal. C, H, N, Cl.

6-Hydroxy-4-thiahexylamine[7] **(1b).** To a mixture of 39.0 g of 2-mercaptoethanol (0.050 mol) and 54.7 g of 3-bromopropylammonium bromide (0.25 mol) was added 750 mL of 1 N methanolic KOH in a 2-L flask. The solution was refluxed with vigorous stirring for 3.5 h and then evaporated under reduced pressure in order to remove the volatile solvents. The resulting slurry was redissolved in 250 mL of 1 N aqueous KOH and extracted three times with 250-mL portions of $CHCl_3$. The extracts were dried over Na_2SO_4 (anhydrous), filtered, and concentrated at room temperature. The resultant thick syrup was vacuum distilled and the fraction distilling in the 86–88 °C range at 0.15 Torr was collected: yield, 30.1 g (89%); IR 3350 and 3300 (NH and OH), 2925 and 2870 (CH_2), 1595 (NH_2), 1080–1030 cm^{-1} (CCO); TLC, silica gel, elution with 2% methanolic HOAC gave a single spot by I_2 vapor detection at R_f 0.37; ^1H NMR ($CDCl_3$) δ 3.64 (t, CH_2OH), 3.16 (s, H exchange), 2.66 (q, $CH_2SCH_2CH_2CH_2$), 1.75 ppm (sextet, $CH_2CH_2CH_2$). Anal. C, H.

Sodium 2-(6′-Hydroxy-4′-thiahexylamino)-3,5-dinitrobenzenesulfonate Monohydrate (2b). The preparation of this compound did not vary significantly from that of compound **2a**, vide infra: yield, 76%; mp 106–109 °C; IR practically the same as for **2a**; ^1H NMR (acetone-d_6) δ 8.73 (C_6H_2–), 7.9 (t, –NH), 4.05 (s, H_2O and –OH), 3.7 (t, $-CH_2OH$), 3.15 (t, $-CH_2NH$), 2.5–2.9 (m, $-CH_2SCH_2$), 1.9 ppm (m, $-CH_2CH_2CH_2-$). Anal. C, H, N.

2-(6′-Chloro-4′-thiahexylamino)-3,5-dinitrobenzenesulfonic Acid (4b). Vide infra, compound 4a does not differ appreciably in its synthesis from **4b**: yield, 37%; mp 113–115 °C; IR practically identical with that for **4a**; ^1H NMR (CD_3OD) δ 8.6 (C_6H_2), 4.7 (s, H exchange), 3.7–2.4 (m, $CH_2CH_2CH_2SCH_2CH_2$), 1.9 ppm (m, $CH_2CH_2CH_2$).

Ethyl 5-Hydroxy-3-thiapentanoate (6a). This compound was reported previously,[14] but the method described herein seems superior due to its simplicity and higher yield (vide infra, compound **6b**). The properties of our sample confirm the data of the previous workers.

5-Chloro-3-thiapentanoyl chloride (8a): vide infra, compound **8b**, and reference.

4-Hydroxy-6′-chloro-4′-thiahexanilide (10). To a solution of 4.6 g of *p*-aminophenol (0.04 mol) in 75 mL of dry refluxing acetone was added 8.94 g of $Cl(CH_2)_2S(CH_2)_3COCl$ dissolved in 25 mL of acetone. The mixture refluxed for 10 min; then it was cooled to room temperature. The solvent was removed under reduced pressure until only a thick gum remained. This was refluxed in 100 mL of $CHCl_3$ and filtered. The filtrate was treated with 100 mL of mixed hexanes, giving a dark precipitate. The latter was reextracted with 100 mL of fresh $CHCl_3$, leaving a dark residue behind. The extracts were all combined and diluted to 2:1 with hexanes, slowly precipitating a mass of white crystals. The precipitate was filtered and dried in vacuo: yield, 4.6 g of 10 (40%); mp 86.0–87.0 °C; IR 3270 (OH), 3200 (NH), 2950, 2910 (CH_2), 1660 (CONH), 1600 (*p*-C_6H_4), 1640, 1620 (CCl), 1460, 1440, 1420, 1380 (CH_2), 1315, 1300, 1240, 1220 (C_6H_4OH), 1190, 1110, 1080 (*p*-C_6H_4), 840 [$(CH_2)_3$], 710 cm^{-1} (CSC); ^1H NMR (acetone-d_6) δ 8.7 (s, NH), 8.0 (s, OH), 6.7 (q, C_6H_4), 3.3 (t, $ClCH_2$), 2.6–1.9 (m, $CH_2SCH_2CH_2CH_2$), 1.6 ppm (q, $CH_2CH_2CH_2$). Anal. C, H, N.

4-(5′-Chloro-3′-thiapentamido)benzenephosphonic Acid (11a). A suspension of 2.6 g of *p*-phosphanilic acid (0.015 mol) in 15 mL of dry DMF was heated to 60 °C and 3.0 g of 8a (0.016 mol) in 3 mL of *p*-dioxane was added to the stirring mixture. The solids dissolved and heating was discontinued. The solution was allowed to return to room temperature and remain overnight. A solid was filtered off and saved for subsequent investigation. The filtrate was evaporated under high vacuum to give a thick syrup which was refluxed for 1.5 h in 125 mL of dry *p*-dioxane. The solution was filtered and the filtrate was refluxed briefly with 1 g of decolorizing carbon, refiltered, and concentrated to 50 mL. A 60-mL portion of cyclohexane was added and over the next week a powdery white solid formed. The precipitate was filtered and dried at 55 °C under 0.5 Torr for 24 h: yield, 1.1 g of 11a (22%); mp 143–144 °C; IR 3280 (NH), 2910 (CH_2), 1660 (C=O), 1600 (*p*-C_6H_4), 1530 (PO_3H_2), 1400 (CH_2), 1220–1150, 1000 (PO_3H_2), 940 (CH_2), 700 cm^{-1} (CSC); ^1H NMR (CD_3OD) δ 7.8, 7.7 (d, s, C_6H_4), 4.9 (s, H exchange), 3.6 (t, CH_2Cl), 3.1–2.6 (m, $CH_2SCH_2CH_2$). Anal. C, H, N.

4-(5′-Chloro-3′-thiapentamido)benzenearsonic Acid (12a). A solution of 2.2 g of *p*-arsanilic acid (0.01 mol) in 20 mL of dry DMF was warmed to 55 °C and 1.9 g (0.01 mol) of 8a was added to it and stirred for 3 h. The solvent was removed under vacuum. The residue was extracted with 100 mL of warm acetonitrile, filtered, and diluted with an equal volume of cyclohexane. The white cloudy solution was left in the refrigerator overnight. The white solid that separated was filtered and dried in vacuo: yield, 0.9 g (24%). On heating, the compound slowly decomposes without melting: IR 3280 (NH), 3100–2500 (OH, CH), 1680 (C=O), 1510 (AsO_3H_2), 1400 (CH_2), 1330–1220 (AsO_3H_2), 1180, 1110 (*p*-C_6H_4 and CH_2), 790–780 cm^{-1} (CSC); ^1H NMR (CD_3OD) δ 7.8 (d, C_6H_4), 4.9 (s, H exchange), 3.7 (t, $ClCH_2$), 2.6–3.05 ppm (m, $CH_2SCH_2CH_2$). Anal. C, H, N, Cl, mol wt.

4-(5′-Chloro-3′-thiapentamido)phenyltrimethylammonium Iodide (13a). To a solution of 2.5 g of 4-aminophenyltrimethylammonium iodide (0.009 mol) in 200 mL of acetonitrile was added 0.85 g of 8a (0.009 mol). The mixture was stirred for 2 h at room temperature and filtered. The solvent was removed under reduced pressure and the residue crystallized from dry acetone: yield, 1.1 g of 13a (28%); mp 132–136 °C; IR 3210–2990 (CH_2), 1680 (C=O), 1600 (NHCO), 1515–1500 (*p*-C_6H_4), 1410 (CH_2), 1360, 1310, 1260 [$N(CH_3)_3^+$], 1200, 1180, 1130 [*p*-C_6H_4, $(CH_2)_3$], 1120, 960, 940 (CH_2), 850, 840 (*p*-C_6H_4), 690 cm^{-1} (CSC); ^1H NMR (CD_3SOCD_3–CD_3COCD_3, 1:1) δ 7.9 (s, C_6H_4), 4.5 (s, NH), 3.7 [s, $(CH_3)_3N^+$], 3.1–2.7 ppm (m, $CH_2CH_2SCH_2CH_2$). Anal. C, H, N, Cl, I.

Ethyl 6-Hydroxy-4-thiahexanoate (6b). To a solution of 3.9 g of 2-mercaptoethanol (0.05 mol) refluxing in 55 mL of absolute EtOH containing 1 g of NaOH was added 7.53 g of ethyl 4-chlorobutyrate (0.04 mol) dropwise over a 1-h period. The mixture was allowed to cool to room temperature and placed in an ice bath for 0.5 h. The resulting NaCl precipitate was removed by filtration and the filtrate evaporated under reduced pressure. The addition of 50 mL of water dissolved the residual NaCl and the solution was extracted with three 50-mL portions of $CHCl_3$. The extracts were dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was distilled and the fraction at the 111–115 °C range at 0.3 Torr was collected: yield, 3.5 g (36%); IR 3250 (OH), 2910 (CH_2–), 2875 (CH_2–), 1720 (C=O), 1380 (CH_3–), 1218 cm^{-1} (butyrate); TLC, silica gel eluted with AcOH–$CHCl_3$ (1:25), a single spot at R_f 0.50; ^1H NMR (neat) 4.12 (s, OH), 4.04 (q, $CO_2CH_2CH_3$), 3.68 (t, CH_2OH), 2.50 (m, $CH_2SCH_2CH_2CH_2$), 1.87 (sextet, $CH_2CH_2CH_2$), 1.30 ppm (t, $CO_2CH_2CH_3$). Anal. C, H.

6-Hydroxy-4-thiahexanoic Acid (7b). A 33.1-g quantity of **6b** (0.17 mol) in 200 mL of ethanol was refluxed for 3.5 h with a solution of 13.8 g of NaOH in water. The mixture was cooled in an ice bath and acidified to pH 2 with concentrated HCl, and the ethanol was removed under reduced pressure. The resulting solution was clarified by addition of a minimum volume of water and then extracted three times with a 100-mL portion of Et_2O. The combined extracts were dried over Na_2SO_4, filtered, and evaporated at 50 °C under reduced pressure, first on a rotary evaporator and finally under high vacuum: yield, 10 g (40%); IR 3350 (OH), 3000 (CO_2H), 2940 (CH_2), 1710 (C=O), 1415

(CO_2H) 1240 cm^{-1} (CO_2H); TLC, silica gel, elution with AcOH–$CHCl_3$ (1:25), dark spot, R_f 0.77 (the acid), and pale spot, R_f 0.65 (a trace of impurity); ^1H NMR 6.51 (s, H exchange), 3.62 (t, CH_2OH), 2.47 (quintet, $CH_2SCH_2CH_2CH_2$), 1.80 ppm (m, $CH_2CH_2CH_2$). Anal. C, H.

6-Chloro-4-thiahexanoyl Chloride (8b). A suspension of 29.9 g of **7b** (0.18 mol) in 40 mL of benzene was cooled to 5 °C in an ice bath. To the stirring mixture was added 31 mL of $SOCl_2$ over a 30-min period. The mixture was then refluxed for 30 min and evaporated under reduced pressure to remove the benzene and SO_2. An additional 40 mL of benzene was introduced, followed by repeated evaporation, which was found to remove the remaining SO_2 and $SOCl_2$. Some preparations required three or four repetitions of this step for complete removal. The resulting syrup was vacuum distilled, collecting the 87–96 °C fraction at 0.15 Torr and a 101–107 °C fraction at 0.20 Torr. NMR and other data indicated that the three fractions were identical, so they were combined: yield, 16.8 g (47%); ^1H NMR ($CDCl_3$) 3.7 (t, $ClCH_2$), 2.9 (m, $CH_2SCH_2CH_2CH_2$), 2.0 ppm (m, $CH_2CH_2CH_2$). Anal. C, H.

4-(6′-Chloro-4′-thiahexamido)benzenephosphonic Acid (11b). To a suspension of 0.87 g of *p*-phosphanilic acid (0.005 mol) in 10 mL of dry refluxing DMF was added 1.67 g of **8b** (0.008 mol). Refluxing continued for 20 min; then the hot mixture was concentrated under high vacuum to give a thick gum. Stirring this gum with 20 mL of dry dioxane precipitated a white crystalline solid. The product was removed by filtration and recrystallized from 50 mL of CH_2Cl_2–petroleum ether (1:1). The resulting crystals were identified as dimethylammonium, based on their IR, NMR, and melting point. Following the addition of base, the odor was characteristic of the amine. The filtrate was diluted to 250 mL with benzene–dioxane (1:1) and filtered. A 150-mL portion of petroleum ether was added and a white powder precipitated slowly over the next 2 days. The solution was diluted to 600 mL with cyclohexane. A fluffy white product precipitated over the next 5 days. It was filtered and dried in vacuo: yield, 1.3 g of **11b** (67%); mp 122–124 °C; IR 3350 (NH), 2920 (CH_2), 1660–1640 (C=O), 1530 (PO_3H_2), 1590 (*p*-C_6H_4), 1400 (CH_2), 1340–1300 (PO_3H_2), 1050–900 (*p*-C_6H_4, CH_2), 840 (CH_2), 700 cm^{-1} (CSC); ^1H NMR (CD_3OD) δ 7.7 (m, C_6H_4), 5.1 (H exchange), 3.6 ($ClCH_2$), 3.0–2.3 (m, $CH_2SCH_2CH_2CH_2$), 2.0 ppm (m, $CH_2CH_2CH_2$). Anal. C, H, N, P, Cl, mol wt.

4-(6′-Chloro-4′-thiahexamido)benzenearsonic Acid (12b). A suspension of 2.18 g of *p*-arsanilic acid (0.010 mol) in 10 mL of anhydrous DMF was heated to 50 °C and stirred. When the solids had all dissolved, 3.25 g of **8b** (0.016 mol) was added and the temperature rose spontaneously to 75 °C. The solution was allowed to cool to 40 °C and was concentrated under high vacuum to give a thick gummy residue. This material was dissolved in 24 mL of anhydrous MeOH and diluted to 150 mL with *p*-dioxane, filtered, and concentrated to 100 mL under reduced pressure. Addition of 75 mL of cyclohexane gave a gummy precipitate over the next 5 h. Then, a fluffy solid began to precipitate. The supernatant was then decanted into a clean flask and the solid was allowed to precipitate over the next week. It was filtered and dried in vacuo: yield, 1.30 g of **12b** (26%); mp 150 °C dec; IR 3200 (NH), 3100–2500 (OH, CH_2), 1680 (C=O), 1590 (*p*-C_6H_4), 1510 (AsO_3H_2), 1400 (CH_2), 1330–1200 (AsO_3H_2), 1180, 1110 (*p*-C_6H_4 and CH_2), 880 (CH_2), 790–780 cm^{-1} (CSC); ^1H NMR (CD_3OD) δ 7.8 (d, C_6H_4), 4.9 (s, H exchange), 3.6 (t, $ClCH_2$), 3.0–2.3 (m, $CH_2SCH_2CH_2CH_2$), 2.0 ppm (q, CH_2CH_2CH). Anal. C, H, N, Cl, mol wt.

4-(6′-Chloro-4′-thiahexamido)phenyltrimethylammonium Iodide (13b). A 1.4-g portion of (4-aminophenyl)trimethylammonium iodide (0.005 mol) was stirred in 200 mL of CH_3CN until almost completely dissolved. The mixture was stirred while 1.8 g of **8b** (0.008 mol) was added slowly. A precipitate formed immediately. The mixture was stirred for 2 h at room temperature and filtered. The filtrate was evaporated to dryness under reduced pressure and then recrystallized from 100 mL of acetone containing a few milliliters of Et_2O. The pale yellow crystals of **13b** weighed 1.13 g (48%): mp 143–144 °C dec; IR 3220–2950 (CH_2), 1680 (C=O), 1600 (NHCO), 1540, 1520 (*p*-C_6H_4), 1415 (CH_2), 1375, 1340, 1260 [$N(CH_3)_3$], 1205, 1195, 1195, 1115 (*p*-C_6H_4), 940 [$(CH_2)_3$], 970 (CH_2), 850 (*p*-C_6H_4) 690 cm^{-1} (CSC); ^1H NMR (Me_2SO-d_6–acetone-d_6, 1:1) δ 11.6 (NH), 8.1 (s, C_6H_4), 3.8 [s, $(CH_3)_3N^+$], 3.7 (t, CH_2Cl), 3.3–2.4 (m, $CH_2SCH_2CH_2CH_2$), 2.1 ppm (q, $CH_2CH_2CH_2$). Anal. C, H, N, Cl.

Biological Testing. Anticancer Activity. In vivo activity against P388 leukemia was determined according to established procedures.[8,10] This testing was performed at Arthur D. Little, Inc., under the supervision of Mr. Isodore Wodinsky.

Cellular Immunity. The sensitization of mice against conjugates of compound **12b** with heterologous protein–hapten conjugate (HGG-M) was demonstrated by means of allogeneic skin grafting.

Preparation of HGG-M. Conjugates were prepared by addition of 0.057 g of **12b** to 0.025 g of human immunoglobin G (HGG) in 9 mL of concentrated phosphate buffer at pH 7.4. This buffer consisted of 291 mL of 0.6 M NaOH and 50 mL of 0.6 M KH_2PO_4, diluted to 100 mL. The mixture was allowed to remain 20 min at room temperature. It was then filtered over a column containing 8 g (dry weight) of Sephadex G75 expanded over 3 days in distilled water. After discarding the initial 30-mL void volume, the next 32 mL was collected. In this fashion, the conjugates were desalted and simultaneously freed of noncovalently bound mustard hydrolysates.[12] Arsenic and protein analyses of the covalent complex showed that a 6:1 mole ratio of hapten to HGG was achieved. Protein recovery was 36%. Detailed studies of the covalent attachment **12b** to HGG and bovine serum albumin (BSA) and rationale for the low protein recovery will be the subject of a separate paper.

Immunizations. One group (A) of ten each, male C3H/HeJ mice, 8–10 weeks old, were inoculated with 20 μL each of the conjugate, 378 μg mL^{-1}, with sufficient NaCl to give a 0.9% solution, and then emulsified (1:1) in complete Freund's adjuvant (CFA). The injections were distributed evenly over the four footpads of each mouse. Two control groups (B and C), each consisting of ten mice, received untreated HGG in normal saline at the same protein level, emulsified 1:1 in CFA.

Skin Grafting. To 20 fresh samples of skin, each 1 cm in diameter, obtained from several male B_6AF_2 mice 8–10 weeks old, in 50 mL of normal Tris buffer pH 7.4, was added 200 mg of **12b**. The suspension was stirred gently for 20 min at room temperature. The tissues were removed and washed in two 50-mL portions of Tris buffer. A graft was then adfixed to each of the mice in groups A and B. An additional ten grafts were prepared identically, except that the addition of compound **12b** to the first incubation in Tris was omitted. These skins were grafted to the C group recipients using the procedure of Billingham and Medawar,[11] as before. After 7 days, plaster casts were removed and viability of the grafts was monitored daily over the next 6 days by visual and tactile inspection, until necrosis was complete. These results were found to be reproducible in comparable animal experiments. Results are shown in Table II and Figure 1.

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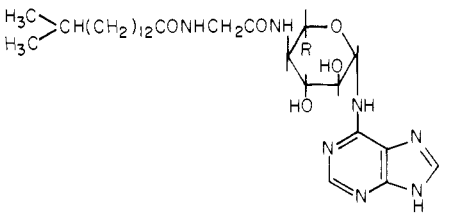
Antitumor Septacidin Analogues

Edward M. Acton,* Kenneth J. Ryan, and Arthur E. Luetzow

Life Sciences Division, Stanford Research Institute, Menlo Park, California 94025. *Received June 2, 1977*

In the first approach by total synthesis to the structure of the antitumor antibiotic septacidin, analogues have been obtained which show similar inhibition of RNA–DNA synthesis in cultured leukemia L1210 cells and similar activity against transplanted leukemia P388 in mice. In these analogues, the natural aminoheptose moiety is replaced by 4-amino-4-deoxy- and 4-amino-4,6-dideoxy-L-glucose, to retain the natural configuration of the pyranose ring. Also retained is the lipophilic fatty acid–amino acid side chain attached to the 4-amino group and glycosylation at the 6-NH_2 of adenine. If the fatty acid chain was shortened from C_{16} to C_6, if the fatty chain was shifted to the glycine unit, or if the glycine unit was omitted, activity was completely lost. However, activity was retained if the C_{16} chain was shortened only to C_{12} or if the glycine unit was extended to β-alanine. Both active and inactive analogues were nonbinding to DNA and nonmutagenic to *Salmonella* strains. The synthetic approach was to start with a suitably protected sugar (L-fucose and L-galactose), construct the adenine moiety at C-1, introduce a 4-amino group, and finally attach the preformed side chain.

The antitumor antibiotic septacidin[1-3] (1) is structurally unique, combining fatty acid, glycine, amino sugar, and adenine units. The amino sugar is a heptose, 4-amino-4-deoxy-L-glycero-L-glucoheptopyranose, not encountered elsewhere. It is linked to the adenine unit by glycosylation at the 6-amino group rather than at N-9 or other ring nitrogen as in the purine nucleosides. In published data,[1,3] septacidin was cytotoxic against Earle's L cells in culture; it was active against adenocarcinoma CA 755 in mice but was inactive against Walker carcinosarcoma 256 and L1210 lymphoid leukemia, two experimental mouse tumors used extensively by the National Cancer Institute for screening new agents.[4] These data could suggest either that the septacidin structure has no potential for cancer treatment or that it has an unusual spectrum of antitumor activity.



1, R = HOCH
CH₂OH

To explore the potential of 1 as a lead for cancer drug development, further studies required the flexibility of total synthesis for systematic structure variation. So far, the structure determination of septacidin rests entirely on elegant chemical degradation studies and spectral analyses.[1,5] Partial degradation permitted preparation of a number of septacidin analogues, through cleavage of the fatty acid–glycine side chain and reattachment of modified side chains and by chain shortening of the sugar unit to a hexopyranose or lengthening it to an octopyranose.[3] Activity vs. CA 755 was retained in a number of analogues, showing that certain structure changes are acceptable.

The synthesis and evaluation of any such structures have received surprisingly little attention. Only a handful of cases are reported[6-9] where a sugar is attached to the 6-amino group of adenine. Even the isomer of adenosine, 6-(β-D-ribofuranosylamino)purine, was only recently claimed.[10] Nor has 4-amino-4-deoxy-L-glucose previously been synthesized, a key degradation product in the structure proof. Comparison with the D enantiomer had to be relied upon.[5,11] This report describes the first totally synthetic approach to the septacidin structure, with the synthesis and biological evaluation of septacidin analogues **2–12** (Table I), of which all but **2** are completely new substances.

Chemistry. The general approach was to start with a sugar, construct on to it the adenine moiety, and attach the fatty acid–amino acid side chain last. In choosing the initial targets, the L-glycero-L-glucoheptose unit of 1 was replaced by hexoses (Table I, $R^1 = CH_2OH$ and CH_3) that were more accessible yet retained the L-gluco configuration of the pyranose ring. To introduce a 4-aminodeoxy function in the L-gluco configuration logically required 4-*O*-methanesulfonyl-L-galacto precursors (Scheme I, **22** → **23**). This would be as in the enantiomeric series in the synthesis of 4-aminodeoxy-D-glucose derivatives.[12,13] The most readily available L-galacto sugar as starting material was L-fucose (**14a**, 6-deoxy-L-galactose). Also, L-galactose (**14b**) was obtained by reduction of L-galactono-1,4-lactone (**13**).[14] The septacidin analogues **2–12** have been synthesized starting from these sugars.

Selective functionalization of the galactopyranose ring at the axial 4 position is readily attained.[12,15,16] In the fucose series (Scheme I, series **a**) the 2,3-di-*O*-benzoate 4-*O*-mesylate **16a** is formed in high yield.[16] Treatment of **16a** with hydrogen chloride in the presence of titanium chloride in benzene solution afforded a crystalline chloro