in 25 ml of benzene was shaken with a solution of 0.5 g of NaOH in 10 ml of water. The benzene layer was washed once with water and dried (Na_2SO_4), concentrated to half its volume, and chilled to 5°. To the well-stirred chilled solution a benzene solution of coumarin-6-carbonyl chloride (from 0.9 g of the acid) was added dropwise. A white solid began to separate within a few minutes and, after addition of the acid chloride was complete, the mixture was stirred in the cold bath for 30 min and at room temperature for 30 min, refluxed for 1.5 hr, and allowed to stand overnight at room temperature. After collection of 0.78 g of bis(2-chloroethyl)amine hydrochloride, the light yellow filtrate was treated with decolorizing carbon, concentrated, and left at room temperature for 2–3 hr. The filtered solution was heated just to boiling and carefully diluted with petroleum ether (30–60°). On cooling 1.25 g (83%) of the chloroamide separated as clusters of shiny white plates. Similar recrystallization raised the melting point to 111–112°. The infrared spectrum (KBr disk) showed amide absorption at 1647 cm^{-1} indicating that no rearrangement had occurred.

Anal. Calcd for $C_{14}H_{13}Cl_2NO_3$: C, 53.52; H, 4.17; Cl, 22.57; N, 4.46. Found: C, 53.60; H, 4.16; Cl, 22.50; N, 4.57.

6-Coumaryl Isocyanate (14).—Dry HCl was passed through a solution of 6 g of 6-aminocoumarin in 150 ml of dry toluene until precipitation of the hydrochloride was complete. After refluxing for 30 min dry $COCl_2$ was passed through the gently boiling suspension (hood and NaOH trap). Solution of the hydrochloride was essentially complete after 3 hr. On concentration to about 75 ml and filtering from a trace of solid, 6.3 g (90%) of the isocyanate separated as shiny white plates. Recrystallization from benzene raised the melting point to 166–167°, lit.[17] mp 163°.

Anal. Calcd for $C_{10}H_5NO_3$: C, 64.17; H, 2.69; N, 7.48. Found: C, 64.25; H, 2.62; N, 7.60.

N^1,N^1-Bis(2-chloroethyl)-N^3-(6-coumaryl)urea (15).—To a stirred suspension of 6-coumaryl isocyanate (1.9 g) in 100 ml of dry benzene a benzene solution of bis(2-chloroethyl)amine (from 2.0 g of the hydrochloride) was added. After refluxing for 6 hr the mixture was stirred for an additional 12 hr at room temperature. A granular brown solid (3.0 g) separated and was recrystallized from ethyl acetate to give the urea as clusters of fine white needles, mp 136–137°.

Anal. Calcd for $C_{14}H_{14}Cl_2N_2O_3$: C, 51.08; H, 4.29; Cl, 21.54; N, 8.51. Found: C, 50.92; H, 4.36; Cl, 21.60; N, 8.61.

6-Coumarylguanidine (16).—6-Aminocoumarin hydrochloride (12.9 g) was added to a solution of 16.5 g of 50% aqueous hydrogen cyanamide[29] in 150 ml of ethanol and the suspension was heated to boiling with stirring. Solution of the solid occurred within 10 min and immediately thereafter solid material separated. After refluxing with stirring for 4.5 hr and standing overnight at room temperature, 10.0 g of yellowish white granular material was collected and washed with ethanol. Water was added dropwise to a boiling suspension of the material in ethanol until it was all in solution. On treatment with decolorizing carbon and concentration of the solution, the hydrochloride of the guanidine, mp 296–297° dec, separated.

Anal. Calcd for $C_{10}H_{10}ClN_3O_2$: C, 50.11; H, 4.21; Cl, 14.80; N, 17.54. Found: C, 50.32; H, 4.34; Cl, 14.74; N, 17.61.

The free guanidine liberated from the hydrochloride melted at 208–209°. It was insoluble in common solvents and could not be recrystallized.

N^1-Nitro-N^3-(6-coumaryl)guanidine (18).—A suspension of 3.2 g of 6-aminocoumarin and 3.2 g of N^1-methyl-N^1-nitroso-N^3-nitroguanidine[20b] in 50 ml of 50% aqueous ethanol was refluxed with stirring for 1 hr. Solution was rapid and after 15 min solid separated from the red solution. After cooling 4.0 g of light brown material was collected with absolute ethanol and recrystallized from glacial acetic acid by chilling to give white granular crystals, mp 234°.

Anal. Calcd for $C_{10}H_8N_4O_4$: C, 48.39; H, 3.25; N, 22.58. Found: C, 48.62; H, 3.42; N, 22.38.

(29) Aero Cyanamide from Cyanamid of Canada Ltd., Montreal, Canada.

Synthesis of Potential Anticancer Agents. XIX. Nitrogen Mustards from 7-Hydroxycoumarin Derivatives[1,2]

ROBERT C. ELDERFIELD AND A. C. MEHTA

Department of Chemistry, The University of Michigan, Ann Arbor, Michigan 48104

Received April 7, 1967

7-Hydroxycoumarin derivatives when subjected to the Mannich reaction with iminodiethanol gave the expected eight Mannich bases which were in turn converted to nitrogen mustards. 7-Hydroxycoumarin-4-acethydrazide on reaction with representative 4-[N,N-bis(2-chloroethyl)amino]benzaldehydes gave the benzylidene mustards. The compounds prepared have been evaluated against cell cultures and experimental animal tumors.

In view of the suggestive experimental antitumor activity shown by certain coumarin nitrogen mustard derivatives,[2] it seemed advisable to explore possible cytotoxic properties of such coumarin derivatives somewhat further. Since there appeared to be no *a priori* reason to select one type of structure over others, accessibility of starting materials was the controlling factor in selection of compounds for synthesis. In this paper we present the synthesis of nitrogen mustards derived from 7-hydroxycoumarins and from 7-hydroxycoumarin-4-acetic acid together with the results of pharmacological evaluation of the cytotoxicity of the candidate compounds.

7-Hydroxycoumarin (**1a**) and its 4-methyl (**1b**) and 4-phenyl (**1c**) derivatives were subjected to the Mannich reaction with formaldehyde and iminodiethanol giving the 7-hydroxy-8-[N,N-bis(2-hydroxyethyl)aminomethyl]coumarins (**2a–c**) (see Table I). Optimum yields of **2a** and **2b** were obtained by the procedure of Cromwell[3] in which the hydroxycoumarin is heated with an activated stock solution of the reagent for 6 hr. Conventional Mannich reaction conditions gave better results in the preparation of **2c** but a 60-hr period of refluxing was required.

The Mannich reaction with **1b** and a variety of amines has been reported by Desai[4] who obtained either the expected product or a 7,8-(*m*-oxazino)coumarin depending on the nature of the secondary amine. It was assumed that the aminomethyl group entered the 8

(1) This work was supported by Research Grant CA-02961 from the National Cancer Institute, National Institutes of Health, to the University of Michigan.

(2) For the preceding paper in this series, see R. C. Elderfield and J. Roy, *J. Med. Chem.*, **10**, 918 (1967).

(3) N. H. Cromwell, *J. Am. Chem. Soc.*, **68**, 2634 (1946).

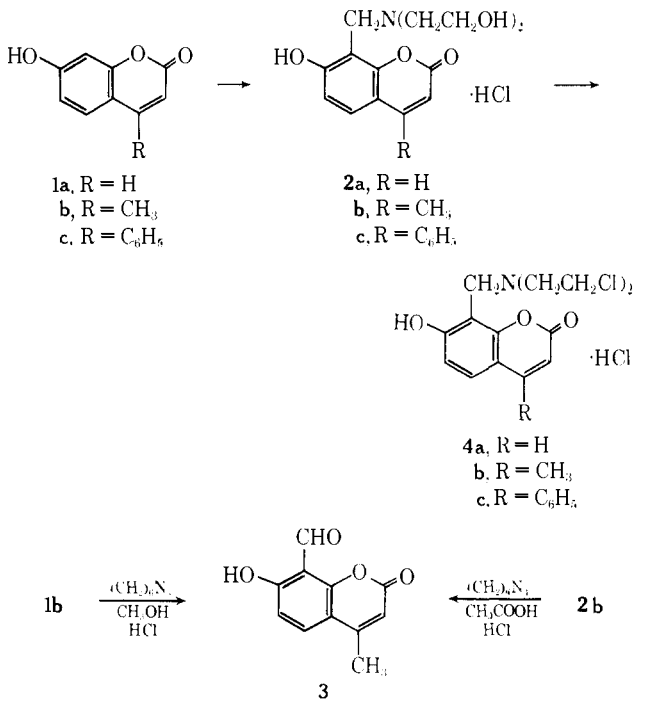
(4) R. B. Desai, *J. Org. Chem.*, **26**, 5251 (1961).

TABLE I
7-HYDROXYCOUMARIN DERIVATIVES

Compd	Formula	Calcd, % C	Calcd, % H	Calcd, % Cl	Calcd, % N	Found, % C	Found, % H	Found, % Cl	Found, % N
2a	$C_{14}H_{17}NO_5 \cdot HCl$	53.25	5.70	11.25	4.44	53.26	5.70	11.31	4.52
2b	$C_{15}H_{19}NO_5 \cdot HCl$	54.63	6.06	10.77	4.25	54.41	6.13	10.85	4.03
2c	$C_{20}H_{21}NO_5 \cdot HCl$	61.30	5.62	9.06	3.57	61.30	5.77	9.03	3.64
4a	$C_{14}H_{15}Cl_2NO_4 \cdot HCl$	47.67	4.54	30.22	3.97	47.87	4.65	30.03	3.90
4a (free base)	$C_{14}H_{15}Cl_2NO_5$	53.15	4.70	22.47	4.43	52.97	4.71	22.20	4.28
4b	$C_{15}H_{17}Cl_2NO_5 \cdot HCl$	49.11	4.91	29.06	3.82	49.08	4.86	29.17	3.90
4b (free base)	$C_{15}H_{17}Cl_2NO_5$	54.55	5.15	21.52	4.24	54.63	5.07	21.30	4.20
4c	$C_{20}H_{19}Cl_2NO_5 \cdot HCl$	56.01	4.67	24.85	3.20	56.17	4.56	24.70	3.34
4c (free base)	$C_{20}H_{19}Cl_2NO_5$	61.21	4.85	18.12	3.57	61.05	5.02	18.28	3.50

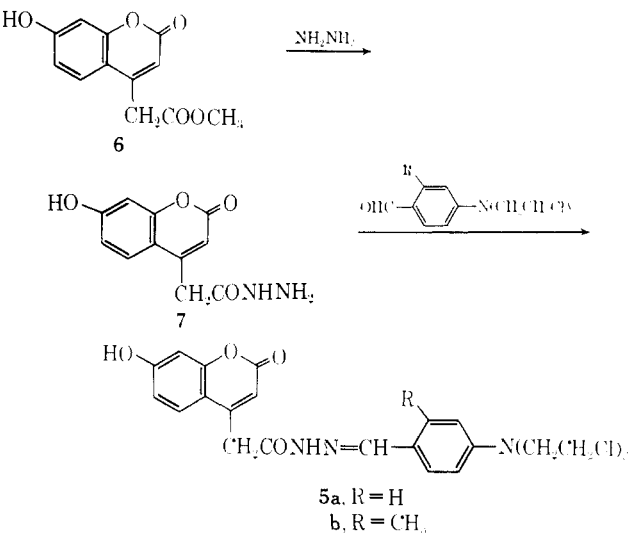
position of the coumarin by analogy with the orientation observed in formylation,[5] the Claisen rearrangement,[6] and the Fries rearrangement.[7] In order to confirm the structure of **2b**, the Mannich base was subjected to Duff's reaction[8] conditions with hexamethylenetetramine and acid whereby the aminomethyl group was displaced by a formyl group with the formation of **3** which was identical with authentic **3** prepared by formylation of **1b**[9] (see Scheme I). Conversion of **2a–c** to the mustards **4a–c** (see Table I) was readily accomplished by reaction with thionyl chloride in acetonitrile.

SCHEME I



Finally, we have prepared representative nitrogen mustards (**5a** and **b**) from the methyl ester of 7-hydroxycoumarin-4-acetic acid (**6**) which has not been reported previously (see Scheme II). Condensation of resorcinol with methyl acetonedicarboxylate readily gave the ester (**6**) from which the hydrazide (**7**) and mustards (**5a** and **5b**) were prepared by conventional methods. Inclusion of **5b** was on the basis of the observed greater cytotoxic activity of other mustards derived from 2-

SCHEME II



methyl-4-[*N*,*N*-bis(2-chloroethyl)amino]benzaldehyde.[9]

Biological Evaluation.—The above coumarin derivatives have been evaluated for cell culture cytotoxicity.[10,11] The results are shown in Table II.

TABLE II
CELL CULTURE TOXICITY

Compd	ED_{50},[a] μg/ml
4a·HCl	380
4b·HCl	250
4c·HCl	290
5a	1000
5b	361

[a] The concentration required to inhibit the growth of KB cells in culture to 50% of controls.[12]

All of the mustards were inactive against the Walker 256 carcinosarcoma: **5a** and **5b** at doses up to 200 mg/kg, **4a–c** at doses up to 17 mg/kg when excessive deaths ensued.

Experimental Section[14]

Stock solution was prepared according to Cromwell[8] by adding 6.6 g of paraformaldehyde to a solution of 21 g of iminodiethanol in

(5) (a) E. Späth and M. Pailer, *Ber.*, **68**, 940 (1935); (b) S. Rangaswami and T. R. Seshadri, *Proc. Indian Acad. Sci.*, **6A**, 112 (1937).

(6) W. Baker and O. M. Lothian, *J. Chem. Soc.*, 628 (1935).

(7) D. B. Limaye, *Ber.*, **65**, 375 (1932); **67**, 12 (1934).

(8) J. C. Duff and E. J. Bills, *J. Chem. Soc.*, 1987 (1932); 1305 (1934); J. C. Duff, *ibid.*, 547 (1941); 276 (1945).

(9) F. D. Popp, *J. Org. Chem.*, **26**, 1566 (1961).

(10) The evaluations were done through the facilities of the Cancer Chemotherapy National Service Center.

(11) These growth inhibition studies were carried out by the procedure of Eagle and Foley[12] as modified by the Cancer Chemotherapy National Service Center.[13]

(12) H. Eagle and G. F. Foley, *Cancer Res.*, **18**, 1017 (1958).

(13) *Cancer Chemotherapy Rept.*, **1**, 63 (1959).

(14) All melting points are uncorrected and were taken on a Thomas-Hoover melting point apparatus. Microanalyses are by Spang Microanalytical Laboratory, Ann Arbor, Mich.

absolute ethanol. The final volume was brought to 250 ml with absolute ethanol and the solution was stored in the refrigerator until needed.

7-Hydroxy-8-[N,N-bis(2-hydroxyethyl)aminomethyl]coumarin Hydrochloride (2a).—A mixture of 3.24 g of 7-hydroxycoumarin (umbelliferone),[15] 25 ml of stock solution, and 30 ml of absolute ethanol was heated on the steam bath without reflux for 6 hr. After removal of the solvent by evaporation, a solution of the residue in 20 ml of methanol was saturated with dry HCl. On refrigeration for 6 hr a pink crystalline solid separated on scratching. Recrystallization from methanol–ethyl acetate (charcoal) gave pink rectangular plates, mp 191–192°. The yield was 2.69 g.

7-Hydroxy-8-[N,N-bis(2-hydroxyethyl)aminomethyl]-4-methylcoumarin hydrochloride (2b) was prepared by the above procedure from 3.52 g of 7-hydroxy-4-methylcoumarin,[16] 25 ml of stock solution, and 20 ml of absolute ethanol. It crystallized from methanol (charcoal) as white needles which melted at 210–211°, then solidified at 215° and finally melted with decomposition at 265°. The yield was 3.42 g.

7-Hydroxy-8-[N,N-bis(2-hydroxyethyl)aminomethyl]-4-phenylcoumarin Hydrochloride (2c).—A mixture of 4.76 g of 7-hydroxy-4-phenylcoumarin,[17] 1.32 g of paraformaldehyde, 4 ml of iminodiethanol, and 100 ml of absolute ethanol was refluxed for 60 hr. After concentration to 20 ml the solution was saturated with dry HCl and 10 ml of ethyl acetate was added. Refrigeration gave a crystalline precipitate which was recrystallized from methanol–ethyl acetate (charcoal) to give 2.10 g of short white needles, mp 192–193°.

8-Formyl-7-hydroxy-4-methylcoumarin (3).—A mixture of 3 g of the Mannich base hydrochloride (**2b**), 6 g of hexamethylenetetramine, and 50 ml of glacial acetic acid was heated on the steam bath for 5 hr. After addition of 40 ml of 18% HCl the hot solution was allowed to stand for 10 min, cooled, and extracted ($CHCl_3$). The extract was washed (dilute $NaHCO_3$, H_2O) and dried (Na_2SO_4). Removal of $CHCl_3$ left a yellow solid which was recrystallized from ethyl acetate to give 100 mg of **3**; melting point and mixture melting point with an authentic sample of **3**[5b] was 176°. The infrared spectra of the two samples were identical.

7-Hydroxy-8-[N,N-bis(2-chloroethyl)aminomethyl]coumarin (4a).—To an ice-cold suspension of the coumarin hydrochloride (**2a**) in 50 ml of acetonitrile, 1.6 ml of $SOCl_2$ was added in one lot. After stirring for 2 hr at ice-bath temperature the mixture was refluxed for 2 hr and then stirred for 2 hr at room temperature. Addition of anhydrous ether precipitated solid material which on recrystallization from methanol–ethyl acetate (charcoal) gave 2.7 g of light pink granules, mp 169–170°.

The **free base** was obtained from the hydrochloride by macerating the latter with aqueous $NaHCO_3$. It crystallized from ethyl acetate–petroleum ether (bp 90–100°) as white glistening prisms, mp 141–142°.

7-Hydroxy-4-methyl-8-[N,N-bis(2-chloroethyl)aminomethyl]-coumarin Hydrochloride (4b).—From 1.0 g of the Mannich base hydrochloride (**2b**) 0.90 g of **4b** was obtained by the above procedure. The compound crystallized from methanol–ethyl acetate was white crystals, mp 193–194°.

The **free base,** obtained as in the preceding case formed white needles, mp 115–116°, from ethyl acetate–petroleum ether.

7-Hydroxy-4-phenyl-8-[N,N-bis(2-chloroethyl)aminomethyl]-coumarin Hydrochloride (4c).—From 1.00 g of the coumarin hydrochloride (**2c**) 0.85 of crystallized **4c** was obtained by the above procedure. The mustard crystallized as fine white needles, mp 201–202°, from methanol–ethyl acetate (charcoal).

The **free base,** obtained as above, melted at 155–156° from ethyl acetate–petroleum ether.

Methyl 7-Hydroxycoumarin-4-acetate (6).—Resorcinol (5.5 g) was dissolved in methyl acetonedicarboxylate (7.5 ml) by warming. The solution was then cooled in an ice–salt bath and added in small lots with stirring to 50 ml of concentrated H_2SO_4 at 0–5° over 30 min. After standing for 24 hr in the refrigerator the mixture was carefully poured over crushed ice with stirring. The light yellow solid which separated was collected, washed thoroughly with water, dried, and recrystallized from methanol (charcoal) to give 3.5 g of aggregates of small white needles, mp 221–223°, with preliminary sintering at 210°.

Anal. Calcd for $C_{12}H_{10}O_5$: C, 61.53; H, 4.30. Found: C, 61.31; H, 4.25.

7-Hydroxycoumarin-4-acethydrazide (7).—A solution of 2.34 g of the methyl ester (**6**) and 2 ml of 99–100% hydrazine in 80 ml of absolute ethanol was refluxed for 24 hr. The white solid which separated was collected and washed successively with boiling ethanol and hot acetone. The hydrazide crystallized from dilute acetic acid as white crystals, mp above 250°. The yield was 1.75 g.

Anal. Calcd for $C_{11}H_{10}N_2O_4$: C, 56.41; H, 4.27; N, 11.97. Found: C, 56.13; H, 4.29; N, 11.85.

7-Hydroxycoumarin-4-{4′[N,N-bis(2-chloroethyl)amino]benzylidene}acethydrazide (5a).—A solution of 0.234 g of the hydrazide (**7**) and 0.250 g of *p*-[N,N-bis(2-chloroethyl)amino]-benzaldehyde[18] in 10 ml of DMF was refluxed for 1 hr. After cooling anhydrous ether was added and the mixture was refrigerated for 1 hr. The light yellow precipitate was recrystallized twice from acetone (charcoal) giving 0.320 g of white crystals, mp 221–223° dec.

Anal. Calcd for $C_{22}H_{21}Cl_2N_3O_4$: C, 57.15; H, 4.54; Cl, 15.36; N, 9.09. Found: C, 57.29; H, 4.57; Cl, 15.41; N, 8.96.

7-Hydroxycoumarin-4-{2′-methyl-4′-[N,N-bis(2-chloroethyl)-amino]benzylidene}acethydrazide (5b).—From 0.234 g of **7** and 0.260 g of 2-methyl-4-[N,N-bis(2-chloroethyl)amino]benzaldehyde[9] 0.04 of **5b,** mp 210–211° dec with darkening at 198°, was obtained by the above procedure after recrystallization from methanol–acetone (charcoal). The compound turned yellow on standing.

Anal. Calcd for $C_{23}H_{23}Cl_2N_3O_4$: C, 57.98; H, 4.83; Cl, 14.92; N, 8.82. Found: C, 58.04; H, 4.90; Cl, 15.02; Cl, 15.02; N, 8.76.

(15) H. von Pechmann, *Ann.*, **264**, 284 (1891).

(16) A. Russell and J. R. Frye, "Organic Syntheses," Coll. Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1955, p 282.

(17) H. von Pechmann and C. Duisberg, *Ber.*, **16**, 2126 (1883).

(18) R. C. Elderfield, I. S. Covey, J. B. Geidushek, W. L. Meyer, A. B. Ross, and J. H. Ross, *J. Org. Chem.*, **23**, 1749 (1958).