were taken at 4 hr and at 24 hr. Levels of cyclophosphamide were respectively 157 and 34 μg/ml and of 4-ketocyclophosphamide, 5.4 and 4.7 μg/ml.

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Synthesis and Antitumor Activity of 6-Trifluoromethylcyclophosphamide and Related Compounds

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In an attempt to increase the combined toxicity of the metabolic end-products [acrolein (**4**) and phosphoramide mustard (**3**)] from cyclophosphamide (**1**), the analog 2-[bis(2-chloroethyl)amino]tetrahydro-6-trifluoromethyl-2*H*-1,3,2-oxazaphosphorine 2-oxide (**2**, 6-trifluoromethylcyclophosphamide) was synthesized and its metabolism and antitumor activity studied. Following metabolism of **2** by rat liver microsomes the predicted formation of 4,4,4-trifluorocrotonaldehyde (**5**) was confirmed by isolation and identification, by mass spectrometry, of its dinitrophenylhydrazone. The therapeutic indices (LD_{50}/ID_{90}) for **2** against the ADJ/PC6 mouse tumor and the Walker 256 tumor in the rat were 28.6 and 7.7, respectively, and were lower than the corresponding values for **1** (91.8 and 33.2, respectively) although the toxicities toward Walker cells in a bioassay system of **1** and **2** following microsomal metabolism were similar. In order to study the toxicities of **4** and **5** released under drug metabolizing conditions independently of the production of a toxic mustard the analogs **18** [2-(diethylamino)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide] and **6** [2-(diethylamino)tetrahydro-6-trifluoromethyl-2*H*-1,3,2-oxazaphosphorine 2-oxide] were also synthesized. The release of **5** from **6** following metabolism was confirmed and shown by use of the bioassay system to be an event of similar toxicity to release of **4** from **18**; in vivo, however, **6** (LD_{50} 330 mg/kg) was more toxic to mice than **18** (LD_{50} >500 mg/kg).

Since cyclophosphamide [2-[bis(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (**1**)] was first synthesized[1] in 1958 and found to be an effective antitumor agent, many analogs and derivatives have been prepared,[2] but none has activity superior to that of the parent drug. However, recent advances[3-5] in the understanding of the metabolism and possible mode of action of the drug may permit a more rational design of agents and investigation of structure–activity relationships than has been possible hitherto. We now report on the synthesis and biological activity of 6-trifluoromethylcyclophosphamide [2-[bis(2-chloroethyl)amino]tetrahydro-6-trifluoromethyl-2*H*-1,3,2-oxazaphosphorine 2-oxide (**2**)].

The metabolism of cyclophosphamide is believed[3-5] to proceed via initial formation of 4-hydroxycyclophosphamide, the open-chain tautomer (aldophosphamide) of which yields the cytotoxic products phosphoramide mustard (**3**) and acrolein (**4**). Other metabolites of cyclophosphamide, in particular the major urinary excretion products, carboxyphosphamide and 4-ketocyclophosphamide, are not significantly more toxic than the parent drug.[5] The relatively selective toxicity of cyclophosphamide toward tumor cells in vivo has been tentatively attributed[5] to the preferential release of the alkylating agent **3** therein, and the concomitant production of an equimolar amount of acrolein may also be of importance with regard to the antitumor activity.[6,7] On a molar basis acrolein is 40-fold less toxic than phosphoramide mustard (see Results) and it is thus probable that it plays only a minor role in the antitumor effect of cyclophosphamide. Indeed the analog of cyclophosphamide where the bis(2-chloroethyl)amino group is replaced by a diethylamine residue also liberates acrolein in a microsomal system[7] but is inactive as an antitumor agent,[2] showing acrolein to be ineffective when formed in isolation. However, if an α,β-unsaturated carbonyl compound of far greater toxicity than acrolein were released in association with

the mustard 3 inside neoplastic cells, it is possible that toxicity due to alkylation by 3 might be enhanced by that due to the acrolein analog. Ideally such a compound should be at least of the same order of toxicity as 3.

The toxicity of acrolein is presumably due to reaction with important cellular nucleophiles (such as SH groups) and the compound would become more reactive toward such groups if the electrophilic character of the conjugated system were increased. This possibility may be investigated by attaching an electron-withdrawing group, resistant to metabolism, to cyclophosphamide in a position (5 or 6) that should not interfere significantly with the essential initial metabolic step, namely, 4-hydroxylation. The trifluoromethyl group fulfils the electronic conditions and should be resistant to metabolism. 6-Trifluoromethylcyclophosphamide (2) was first investigated and was expected to yield, after 4-hydroxylation, phosphoramide mustard (3) and 4,4,4-trifluorocrotonaldehyde (5). Compound 5 is not readily available by synthesis, having been reported[8] only once and having been characterized only as its dinitrophenylhydrazone, and, therefore, in order to investigate the cytotoxicity of 4,4,4-trifluorocrotonaldehyde released under drug metabolizing conditions, the analog (6) of 6-trifluoromethylcyclophosphamide in which the bis(2-chloroethyl)amine group is replaced by a diethylamine residue, and which therefore cannot generate the cytotoxic mustard 3, was also investigated.

Synthesis. The route of synthesis used for 6-trifluoromethylcyclophosphamide (Scheme I) paralleled that generally employed for cyclophosphamide derivatives and involved the condensation of the dichlorophosphoramidate (7) with 4-amino-2-hydroxy-1,1,1-trifluorobutane (8). Compound 8 was obtained by the following route. 1,1,1-Trifluoropropan-2-one (9) was converted[9] into 3-bromo-1,1,1-trifluoropropan-2-one (10), which was reduced with $NaBH_4$ to give 3-bromo-2-hydroxy-1,1,1-trifluoropropane (11). Temperature control was necessary in the bromination of 9; when the reaction was carried out as described by Haas,[9] the dibromo compound was the major product which yielded 3,3-dibromo-2-hydroxy-1,1,1-trifluoropropane after reduction. Treatment of 3-bromo-2-hydroxy-1,1,1-trifluoropropane (11) with boiling aqueous ethanolic sodium cyanide gave 4,4,4-trifluoro-3-hydroxybutyronitrile (12), which was reduced with $LiAlH_4$ to afford 8.

The condensation of 8 with bis(2-chloroethyl)phosphoramidic dichloride (7) proceeded smoothly in CH_2Cl_2, giving 6-trifluoromethylcyclophosphamide (2). The ^{19}F NMR spectrum of 2 showed two fluorine resonances (doublets, J = 6 Hz, ratio 1:2.25) attributable to the two possible diastereoisomers. An additional product was the triamidate (13). As an analogous product was not formed during the preparation of cyclophosphamide from 7 and 3-aminopropan-1-ol (19), it appears that the inductive effect of the trifluoromethyl group lowers the tendency of the vicinal hydroxyl group to react with the P–Cl function thus allowing the competing intermolecular reaction leading to 13.

The analog (6) of 6-trifluoromethylcyclophosphamide was prepared from 4-amino-2-hydroxy-1,1,1-trifluorobutane (8) and *N,N*-diethylphosphoramidic dichloride (14). The resulting racemic diastereoisomers of 6, isolated as crystalline solids following column chromatography on silicic acid, each showed a single fluorine resonance (doublet, J = 7 Hz). Again, a triamidate (15) was isolated as a side product.

Metabolism and Biological Results. Metabolism of 6-trifluoromethylcyclophosphamide (2) by rat liver washed microsomes gave (TLC) a compound having mass spectral properties consistent with the structure 4-ethoxy-6-trifluoromethylcyclophosphamide (16). This structure was assigned since under comparable conditions 4-ethoxycyclophosphamide is formed from cyclophosphamide. The 4-ethoxy derivative[5] is thought to be formed by the reaction of ethanol (used as a protein precipitant) with the 4-hydroxy derivative (possibly as its acyclic tautomer) which is the first product of metabolism. No further oxidation of the 4-hydroxy derivative occurred under our experimental conditions, all soluble enzymes having been removed from the microsomal preparation. The mixtures of metabolites separately formed from 2 and from 6 reacted with acidic 2,4-dinitrophenylhydrazine to give a compound (R_f 0.43, TLC, C_6H_6) having the mass spectral characteristics expected for the 2,4-dinitrophenylhydrazone of 4,4,4-trifluorocrotonaldehyde (17). Thus, the predicted metabolic pathway (see the introduction) is operative for 2 and 6 and their biological activity was therefore investigated.

Table I shows the results of a bioassay[5] in which Walker 256 ascites tumor cells were incubated with each compound prior to their injection (ip) into Wistar rats. Toxicities are expressed as the concentration required to give a 20% in-

Scheme I

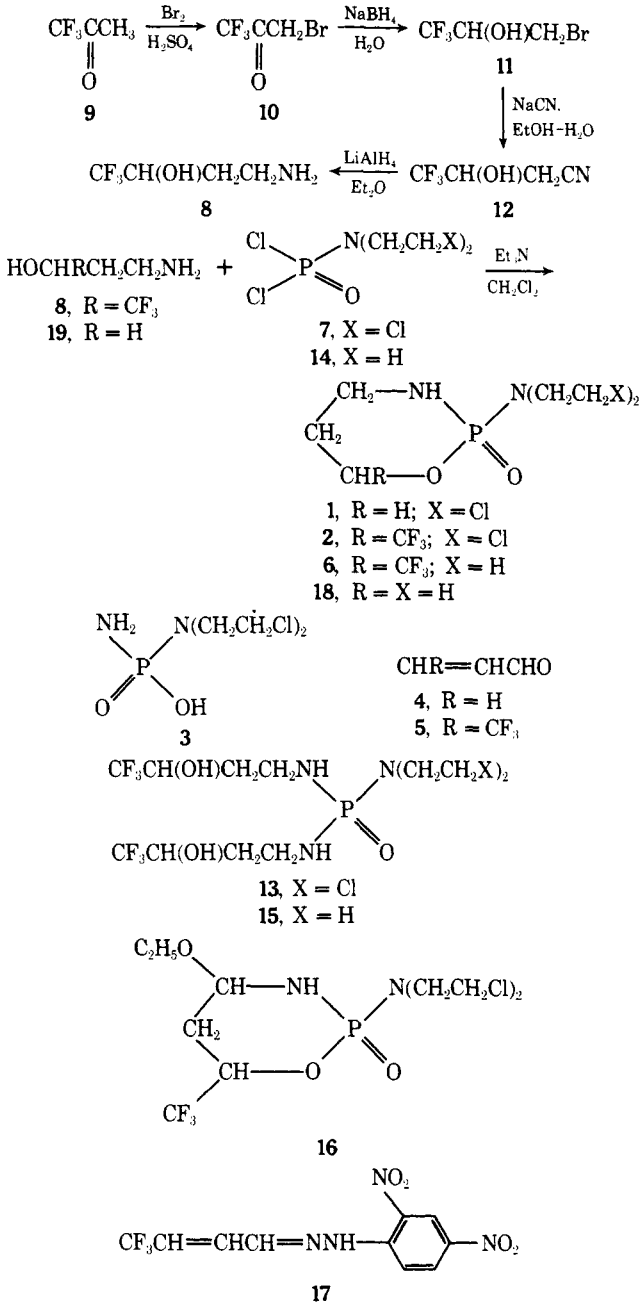

Table I. Bioassay[a] Results

Compound	Substrate concn for 20% increase in survival time, μg/ml	μM
Cyclophosphamide (1)	400	1538
Phosphoramide mustard (3)[b]	0.5	1.6
Acrolein (4)	3.5	63
1 + microsomes + cofactors[c]	1.5	5.8
6-Trifluoromethylcyclophosphamide (2)	450	1372
2 + microsomes + cofactors	1.35	4.1
6	>800	>3077
6 + microsomes + cofactors	70	269
18	>800	>4167
18 + microsomes + cofactors	60	313

[a]The bioassay technique involved treatment of Walker ascites cells in vitro, injection (ip) of these cells into rats, and measurement of the survival in days (control 7 days). [b]Phosphoramide mustard cyclohexylammonium salt was kindly supplied by Dr. H. Wood of Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Washington, D.C. [c]Microsomes were prepared from sodium phenobarbital pretreated rats. Cofactors supplied an NADPH generating system utilizing glucose 6-phosphate (see ref 5).

crease in survival time of the animals, compared to controls, and correspond to a 75% cell kill. Cyclophosphamide (1), 6-trifluoromethylcyclophosphamide (2), and the analogs 6 and 18 were each essentially nontoxic, in the absence of metabolic activation. In the presence of microsomes and cofactors, however, a 75% cell kill was achieved with 1 and 2 at 1.5 and 1.35 μg/ml, respectively. The diethylamino analogs 6 and 18 showed a considerably smaller metabolic activation in the presence of microsomes and cofactors, the respective values being 70 and 60 μg/ml. This reflects the fact that these analogs cannot generate a reactive mustard and also suggests that the release of 4,4,4-trifluorocrotonaldehyde (from 6) is not a significantly more toxic event in the bioassay than the release of acrolein (from 18). The triamidates 13 and 15 were essentially nontoxic both in the presence and absence of microsomes and cofactors.

The results of in vivo testing against the advanced ADJ/PC6 plasma cell tumor in mice and the Walker 256 tumor in rats are shown in Table II. The therapeutic indices (28.6 and 7.7, respectively) of 6-trifluoromethylcyclophosphamide for these tumors were lower than those (91.8 and 33.2, respectively) of cyclophosphamide, although the diethylamino analog (6) of 6-trifluoromethylcyclophosphamide (major diastereoisomer) had a very slight effect against the PC6 tumor which was not seen with the corresponding cyclophosphamide analog 18; toxicity to the host was also higher for 6 than for 18, the respective LD_{50} values being 330 and >500 mg/kg.

6-Trifluoromethylcyclophosphamide (NSC 235769) was also tested against the L1210 leukemia according to the standard protocol[10] by the National Cancer Institute; optimal activity was at 125 mg/kg (single dose), which gave a 65% increase in survival time over controls. Further antitumor testing is in progress.

In conclusion, even though the predicted metabolic pathways are operative for the compounds synthesized, this initial attempt to improve upon the antitumor effect of cyclophosphamide by producing a situation where the cytotoxicity of the metabolic end product phosphoramide mustard is significantly enhanced by the simultaneous production of a toxic acrolein analog has not produced a compound with improved therapeutic index in the in vivo test systems studied so far. An extension and evaluation of the present approach involve the search for a synthetically available acrolein analog which is of much greater toxicity than acrolein and which could also be liberated by metabolism of an appropriate cyclophosphamide analog.

Table II. Antitumor Tests

Compound	LD_{50}, mg/kg	ID_{90}, mg/kg	Therapeutic index (LD_{50}/ID_{90})
PC6 Tumor in Mice			
Cyclophosphamide (1)	450	4.9	91.8
6-Trifluoromethylcyclophosphamide (2)	400	14	28.6
6	330	455	0.7
18	>500		
Walker 256 Tumor in Rats			
Cyclophosphamide (1)	283	8.5	33.2
6-Trifluoromethylcyclophosphamide (2)	224	29	7.7

Experimental Section

Melting points were determined with a Kofler hot-stage apparatus and are corrected. Infrared spectra were recorded with a Perkin-Elmer 257 grating spectrophotometer and NMR spectra with a Perkin-Elmer R-10 spectrometer operating at 60 MHz (for ^1H resonance) (internal Me_4Si) and 56.458 MHz (for ^{19}F resonance). Deuterium exchange was accomplished by equilibration of the sample (0.5 ml) with D_2O (2 drops) at 33.5° for 30 min. Mass spectra were determined using an AEI MS-12 instrument by the direct insertion method with an ionizing voltage of 70 eV and ion-source temperature of 100–120°. Merck Kieselgel 60 was used for column chromatography and for thin-layer chromatography (TLC) on coated glass plates (20 × 5 cm).

3-Bromo-1,1,1-trifluoropropan-2-one (10). 1,1,1-Trifluoropropan-2-one (9, 25.3 g, 0.226 mol) was brominated by the method of Haas,[9] except that the reaction mixture was cooled in ice–water during the initial stages of reaction, prior to warming to 55° under reflux for 1.5 hr. The product 10 (32.4 g, 75%), isolated by fractional distillation, had bp 82–86° (lit.[11] bp 85.0–86.8°). When the bromination was carried out under conditions of reflux throughout[9] only 3,3-dibromo-1,1,1-trifluoropropan-2-one, bp 110–112° (lit.[11] bp 111–113°), was isolated (45%).

3-Bromo-2-hydroxy-1,1,1-trifluoropropane (11). 3-Bromo-1,1,1-trifluoropropan-2-one (10, 31.4 g, 0.165 mol) was reduced with $NaBH_4$ as described by McBee[12] giving 11 (20.3 g, 64%), bp 124–126° (lit.[12] bp 124–124.5°). When 3,3-dibromo-1,1,1-trifluoropropan-2-one was reduced in the same way 3,3-dibromo-2-hydroxy-1,1,1-trifluoropropane (68%), bp 155–158°, was obtained. Anal. ($C_3H_3Br_2F_3O$) C, H, Br, F.

3-Hydroxy-4,4,4-trifluorobutyronitrile (12). A solution of 3-bromo-2-hydroxy-1,1,1-trifluoropropane (11, 75 g, 0.39 mol) in EtOH (70 ml) was added during 40 min to a solution of NaCN (20 g, 0.41 mol) in water (28 ml) at 95°. The mixture, which rapidly became dark brown, was heated under reflux for 14 hr. Ethanol was then evaporated, the residue was diluted with water, and the mixture was extracted continuously with $CHCl_3$ (700 ml) for 5 hr. After removal of $CHCl_3$, the product was distilled to give 12 (20.5 g, 38%): bp 112–120° (13–17 mm); ν_{max}^{film} 2270 cm^{-1} (C≡N); ^1H NMR data (Me_2CO-d_6) δ 6.1 (m, 1 H, exchanges in D_2O, OH), 4.5 (m, 1 H, H-3), 2.9 (m, 2 H, 2H-2). The compound became discolored on storage, even after redistillation, and a satisfactory ele-

mental analysis could not be obtained. The recovery of 22% of starting material suggested that a longer reaction time may be advisable for optimum yield of product.

4-Amino-2-hydroxy-1,1,1-trifluorobutane (8). A solution of 3-hydroxy-4,4,4-trifluorobutyronitrile (**12**, 19.5 g, 0.14 mol) in dry Et_2O (340 ml) was added slowly to a stirred suspension of $LiAlH_4$ (6.45 g, 0.17 mol) in dry Et_2O (34 ml) at 0–5°. The mixture was stirred at this temperature for a further 30 min, heated under reflux for 45 min, and stirred again at room temperature for 1.75 hr. Saturated aqueous sodium sulfate (25.5 ml) was then added slowly with rapid stirring and cooling, and, after further stirring (1 hr), the white precipitate was collected and washed well with Et_2O. The combined filtrate and washings were concentrated to an oil (13 g) which solidified. Distillation gave **8** (9.4 g, 47%), bp 102° (18 mm), which solidified immediately, mp 50–55°. Anal. ($C_4H_8F_3NO$) C, H, F, N.

2-[Bis(2-chloroethyl)amino]tetrahydro-6-trifluoromethyl-2*H*-1,3,2-oxazaphosphorine 2-Oxide [6-Trifluoromethylcyclophosphamide (2), NSC 235769]. A solution of 4-amino-2-hydroxy-1,1,1-trifluorobutane (**8**, 4.5 g, 31.4 mmol) and dry Et_3N (6.30 g, 62.4 mmol) in dry CH_2Cl_2 (120 ml) was added during 1.5 hr to a stirred solution of *N,N*-bis(2-chloroethyl)phosphoramidic dichloride[13] (**7**, 7,71 g, 30 mmol) in dry CH_2Cl_2 (120 ml) at 0–5°. The mixture was stirred at room temperature for 22 hr and then concentrated to an oil, a solution of which in water (120 ml) was acidified to pH 3 with dilute HCl and extracted with $CHCl_3$ (3 × 100 ml). The extract was dried (Na_2SO_4) and concentrated, and the residue was applied to a column (4 × 42 cm) of silicic acid (300 g) equilibrated in EtOH–$CHCl_3$ (2:98) and eluted with the same solvent mixture (10-ml fractions). Fractions 120–190 contained the major product (R_f 0.46, TLC, EtOH–$CHCl_3$, 1:9), which was crystallized from Et_2O–petroleum ether (bp 30–40°) giving **2** (4.66 g, 48%), mp 111–115°. A slight variation of melting point for different batches possibly reflected different diastereoisomeric composition: ^1H NMR data ($CDCl_3$) δ 4.8 (m, 1 H, H-6), 3.8–2.9 (m, 11 H, H-4,4′, $2CH_2CH_2Cl$, NH), 2.0 (m, 2 H, H-5,5′); ^{19}F NMR data ($CHCl_3$ + C_6F_6) 82.1 and 82.4 ppm downfield from C_6F_6 (2 d, J = 6 Hz, ratio 2.25:1); mass spectrum *m/e* 328 (1.8%, $M^{\cdot+}$), 309 (1.3, $[M - F]^+$), 293 (1.4, $[M - Cl]^+$), 279 (100, $[M - CH_2Cl]^+$), 188 (3, $[M - N(CH_2CH_2Cl)_2]^+$), 92 (65, $[CH_2{=}NHCH_2CH_2Cl]^+$). Anal. ($C_8H_{14}Cl_2F_3N_2O_2P$)† C, H, N; Cl: calcd, 21.55; found, 22.18.

Further elution of the column with EtOH–$CHCl_3$ (1:9, 800 ml) gave a second product (R_f 0.37, TLC, EtOH–$CHCl_3$, 1:9) which was crystallized from Et_2O–petroleum ether (bp 30–40°) giving *N,N*-bis(2-chloroethyl)-*N′,N″*-di(3-hydroxy-4,4,4-trifluorobut-1-yl)phosphorotriamidate (**13**), mp 109–112°. Anal. ($C_{12}H_{22}Cl_2F_6N_3O_3P$) C, H, Cl, N.

2-(Diethylamino)tetrahydro-6-trifluoromethyl-2*H*-1,3,2-oxazaphosphorine 2-Oxide (6). A solution of 4-amino-2-hydroxy-1,1,1-trifluorobutane (**8**, 2.86 g, 20 mmol) and dry Et_3N (4.04 g, 40 mmol) in dry CH_2Cl_2 (80 ml) was added during 1 hr to a stirred solution of *N,N*-diethylphosphoramidic dichloride[14] (**14**, 3.78 g, 20 mmol) in dry CH_2Cl_2 (80 ml) at 0–5°. The mixture was stirred at room temperature for 46 hr and then worked up as described above for 6-trifluoromethylcyclophosphamide. Products were isolated by chromatography on a column (4 × 40 cm) of silicic acid (270 g). After elution with EtOH–$CHCl_3$ (1:99, 2200 ml), the three major products were eluted in turn with EtOH–$CHCl_3$ (1:19) and crystallized from Et_2O–petroleum ether (bp 30–40°).

Diastereoisomer A (0.34 g, 7%, R_f 0.43, TLC, EtOH–$CHCl_3$, 1:9) of **6**, eluted by the first 400 ml of solvent, had mp 122–125°: ^1H NMR data ($CDCl_3$) δ 4.3 (m, 2 H, 1 H exchanges in D_2O, H-6, NH), 3.5–2.8 [complex, including 2 quartets (J = 7 Hz) at δ 3.20 and 3.01 (6 H, $2CH_2CH_3$, H-4,4′)], 1.85 (m, 2 H, H-5,5′), 1.15 (t, J = 7 Hz, 6 H, 2Me); ^{19}F NMR data ($CHCl_3$ + C_6F_6) 82.1 ppm downfield from C_6F_6 (d, J = 7 Hz); mass spectrum *m/e* 260 (15%, $M^{\cdot+}$), 245 (100, $[M - CH_3]^+$), 188 (3, $[M - N(C_2H_5)_2]^+$). Anal. ($C_8H_{16}F_3N_2O_2P$) C, H, N.

Diastereoisomer B (0.76 g, 15%, R_f 0.39, TLC, EtOH–$CHCl_3$, 1:9) of **6**, eluted by 400–800 ml of solvent, had mp 65–80°: ^1H NMR data ($CDCl_3$) δ 4.85 (m, 1 H, H-6), 3.8–2.6 [complex including 2 quartets, (J = 7 Hz) at δ 3.25 and 3.05 (7 H, $2CH_2CH_3$, H-4,4′, NH)], 2.0 (m, 2 H, H-5,5′), 1.1 (t, J = 7 Hz, 6 H, 2Me); ^{19}F NMR data ($CHCl_3$ + C_6F_6) 81.9 ppm downfield from C_6F_6 (d, J = 6 Hz); mass spectrum *m/e* 260 (40%, $M^{\cdot+}$), 245 (100, $[M - CH_3]^+$), 188 (4, $[M - N(C_2H_5)_2]^+$). Anal. ($C_8H_{16}F_3N_2O_2P$) C, H, N.

N,N-Diethylamino-*N′,N″*-di(3-hydroxy-4,4,4-trifluorobut-1-yl)phosphorotriamidate (**15**) (0.59 g, 15%, R_f 0.37, TLC, EtOH–$CHCl_3$, 1:9), eluted by 800–1600 ml of solvent, had mp 91–95°: ^1H NMR data ($CDCl_3$) δ 5.79 (t, J = 7 Hz, 2 H, exchanges in D_2O, 2-OH), 4.15 (m, 2 H, 2H-3), 3.4–2.4 [complex including 2 quartets (J = 7 Hz) at δ 3.14 and 2.94 (10 H, $2CH_2CH_3$, 2H-1,1′,2NH)], 1.7 (m, 4 H, 2H-2,2′), 1.08 (t, J = 7 Hz, 6 H, 2Me); mass spectrum *m/e* 403 (77%, $M^{\cdot+}$), 388 (10,$[M - CH_3]^+$), 384 (10, $[M - F]^+$), 331 (100, $[M - N(C_2H_5)_2]^+$), 261 (15, $[M - HOCH(CF_3)CH_2CH_2NH]^+$). Anal. ($C_{12}H_{24}F_6N_3O_3P$) C, H, N.

2-(Diethylamino)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-Oxide (18). A solution of 3-aminopropan-1-ol (**19**) (1.76 g, 23.5 mmol) and dry Et_3N (4.74 g, 46.9 mmol) in dry CH_2Cl_2 (80 ml) was added during 40 min to a stirred solution of *N,N*-diethylphosphoramidic dichloride (**14**, 4.44 g, 23.5 mmol) in dry CH_2Cl_2 (80 ml) at 0–5°. The mixture was stirred at room temperature for 18 hr and the product (R_f 0.39, TLC, EtOH–$CHCl_3$, 1:9) was then isolated as described above for 6-trifluoromethylcyclophosphamide and was further purified by distillation giving **18** as a colorless oil (1.73 g, 38%), bp 140–142° (1.0 mm). Anal. ($C_7H_{17}N_2O_2P$) H, N, P; C: calcd, 43.74; found, 43.25.

Metabolism. 6-Trifluoromethylcyclophosphamide was incubated at 200 μg/ml for 45 min at 37° with washed microsomes from the livers of male Wistar rats (pretreated with sodium phenobarbital) and the appropriate cofactors, as previously described.[5] Under these conditions, no reactions due to soluble enzymes can occur. Protein was precipitated by addition of 4 vol of redistilled EtOH and was removed by centrifugation at $1200g_{av}$. Most of the EtOH was removed by evaporation at reduced pressure and the resulting solution was acidified to pH 4 (1 *N* HCl) and extracted (3 times) with $CHCl_3$. The extract was dried (Na_2SO_4) and part was subjected to TLC in EtOH–$CHCl_3$ (1:9). A component at R_f 0.7, reactive toward an acidic 2,4-dinitrophenylhydrazine spray reagent, was isolated and subjected to mass spectrometry. The mass spectral features [*m/e* 372 (0.35%, $M^{\cdot+}$), 327 (9, $[M - C_2H_5O]^+$), 323 (32, $[M - CH_2Cl]^+$), 277 (100, $[M - C_2H_5OH - CH_2Cl]^+$)] were consistent with the structure of 4-ethoxy-6-trifluoromethylcyclophosphamide (**16**) (cf. mass spectra of corresponding cyclophosphamide[5] and 6-methylcyclophosphamide[15] derivatives).

A second portion of the extract was treated with a saturated solution of 2,4-dinitrophenylhydrazine in 2 *N* HCl for 10 min and then adjusted to pH 9 (2 *N* NaOH) and extracted (3 times) with $CHCl_3$. TLC (C_6H_6) of the concentrated extract revealed a major yellow component at R_f 0.43 with mass spectrum [*m/e* 304 (100%, $M^{\cdot+}$), 285 (10, $[M - F]^+$), 235 (65, $[M - CF_3]^+$)] consistent with the structure of 4,4,4-trifluorocrotonaldehyde 2,4-dinitrophenylhydrazone (**17**) (cf. mass spectrum of crotonaldehyde 2,4-dinitrophenylhydrazone[16]). This compound was also formed when the entire incubation mixture reacted directly with acidic 2,4-dinitrophenylhydrazine (2 vol) without prior extraction or protein precipitation. The 2 *N* HCl precipitated the protein, and the supernatant, after centrifugation ($1200g_{av}$) and extraction, yielded **17** as the major product.

The same derivative was similarly isolated following the metabolism of each diastereoisomer of the diethylamino analog (**6**) of 6-trifluoromethylcyclophosphamide; under the same conditions cyclophosphamide (**1**) and its diethylamino analog **18** gave acrolein 2,4-dinitrophenylhydrazone (R_f 0.39, TLC, C_6H_6) having the previously reported[5] mass spectrum.

The extent of metabolism of 6-trifluoromethylcyclophosphamide was quantitatively similar to that of cyclophosphamide as assessed by the yield of product from parallel incubations.

Bioassays and in Vivo Testing. Compounds were tested in vitro against Walker ascites tumor cells as previously described.[5] When the effect of metabolism was being studied the incubation system used for the production and isolation of metabolites was utilized on a smaller scale and under semisterile conditions.

Testing of compounds in vivo against the advanced ADJ/PC6 solid tumor in mice was essentially by the method of Connors et al.[17] All compounds were dissolved in water for ip injection.

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† Analyst unable to determine F and P simultaneously.

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Potential Antitumor Agents. 16. 4′-(Acridin-9-ylamino)methanesulfonanilides

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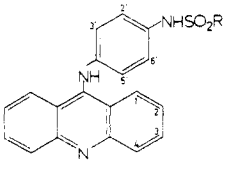
The structure–antileukemic activity (L1210) relationships for sulfonanilide ring-substituted variants of 4′-(acridin-9-ylamino)methanesulfonanilides have been investigated. Electron-donor substituents are necessary for antileukemic activity and it is suggested that high electron density at the 6′ position is associated with high activity. A 3′-OCH_3 function markedly increases (2–8-fold) potency with a variety of acceptable acridine ring substituents. Further variants with hydrophobic acridine 3-substituents have been shown to be more active than expected on the basis of overall molecular hydrophilic–lipophilic balance. There is a size limit to 3-substituents which may acceptably be as large as an iodine atom but should be smaller than an isopropyl function.

In a recent publication[1] we analyzed the structure–antileukemic activity relationships in a series of acridine ring-substituted 4′-(acridin-9-ylamino)alkanesulfonanilides **1**. The present work examines the effect on antileukemic activity (L1210) of substituents appended to the sulfonanilide ring of **1** and probes our earlier conclusion that lipophilic substituents at the 3 position of the acridine can increase activity.



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Chemistry. There was a common reaction step involved in the preparation of the bulk of the congeners described (Table I), viz., acid-catalyzed coupling of a 9-chloroacridine with a 4-aminosulfonanilide component. Necessary aminosulfonanilide components were prepared by either of two methods. In method A a substituted 4-nitroaniline was acylated with a sulfonyl chloride and the nitro function in the resulting 4-nitrosulfonanilide reduced (Fe/H^+). Alternatively (method B), the same substituted 4-nitroaniline intermediate was first converted to the 4-nitroacetanilide, the nitro group reduced, and then the amine function so generated acylated with a sulfonyl chloride. Hydrolytic removal of the protecting acetyl function from the resulting 4-acetamidosulfonanilide then provided a 4-aminosulfonanilide having the isomeric substitution pattern to that produced by application of method A.

Isolation and further purification of the aminosulfonanilides prepared by method B were unnecessary and indeed wasteful. Direct coupling of 9-chloroacridine with acid hydrolysates of 4-acetamidosulfonanilides provided higher overall yields of product (method C).

The 2′- and 3′-amino-substituted variants **7** and **16** were prepared by reduction (Fe/H^+) of the corresponding nitro compounds **6** and **15**, respectively.

The 4″-aminobenzenesulfonanilide congeners (e.g., **25**) were obtained by mild acidic hydrolysis of the acetamido analogs **24** (method D).

The nitrosulfonanilide required as the precursor to the 4″-methylsulfonyl analog **28** was obtained by oxidation ($KMnO_4/OH^-$) of 4-methylthiobenzenesulfon-*p*-nitroanilide.

While the 2′-azalog **21** could be readily prepared by application of method A, attempted synthesis of the 3′ isomer **22** by similar means failed. Reaction of 9-chloroacridine and 2-amino-5-nitropyridine provided 2-(acridin-9-ylamino)-5-nitropyridine and reduction of the nitro function in this molecule to amino and a following acylation with mesyl chloride provided the desired 3′-azalog **22.** Similarly, the 3′-NO_2 analog **15** was most readily prepared via 4′-(acridin-9-ylamino)-3′-nitroacetanilide in turn prepared by coupling 9-chloroacridine and 4-amino-3-nitroacetanilide. Hydrolytic removal of the acetyl function from this acetanilide and mesylation of the resulting amine provided the required **15.**

The use of a 2-halobenzoic acid and the readily available 3-trifluoromethylaniline in the Jourdan–Ullmann reaction provides *N*-(3-trifluoromethylphenyl)anthranilic acid.[2] Ring closure of this acid ($POCl_3$) provides a mixture of 1- and 3-trifluoromethyl-9-chloroacridines but the required 3 isomer, necessary for the preparation of variants **31** and **32,** is that produced in lower yield. Necessary trifluoromethyl-substituted intermediates that allow unambiguous preparation of the required 3-trifluoromethyl-9-chloroacridine in reasonable yield are relatively inaccessible. In seeking al-