not obtained. A soln of 7.88 g of the CF_3COOH salt in 350 ml of H_2O required filtration to remove a small amt of insol material. The pH of this clear soln was raised from 2 to 5 by gradually adding about 50 g of Amberlite IR4B resin that had been washed several times with H_2O. Good agitation was necessary, and the time required for conversion was about 10 min. Darco (1 g) was added to the soln after filtration and mixed in for 5 min. The light yellow soln resulting from this treatment was concd on a rotary evaporator using a vacuum pump. The concn was stopped when crystn of the product began, at a vol of approx 25 ml. Storage in the cold for several hours, followed by the addition of 150 ml of EtOH with strong agitation, led to the formation of small particles that were easily filtered. The product was washed with EtOH and dried *in vacuo* at 45–50°. The anhyd product absorbed 1 mole of H_2O from the atm. The yield was 3.56 g (58%) of colorless crystals; iodometric assay, 97.9%, as monohydrate. A second crop (0.77 g) was obtained by concn of the mother liquor. *Anal.* ($C_{16}H_{19}N_3O_4S \cdot H_2O$) C, H, N.

7-[D-2-Amino-2-(1,4-cyclohexadienyl)acetamido]cephalosporanic acid (II) was prepd in the same manner as III using VIII and isolated as light tan crystals: mp 265–270° dec. *Anal.* ($C_{18}H_{21}N_3O_6S \cdot H_2O$) C, H, N.

Acknowledgment.—We are indebted to Joseph Alicino and Joseph Hydro and to Mrs. Mary Young for analytical data, to Dr. Allen Cohen and Miss Barbara Keeler for spectroscopic studies, and to Dr. Harold Jacobson for p*K* determinations.

Irreversible Enzyme Inhibitors. 180.[1,2] Irreversible Inhibitors of the C′1a Component of Complement[3] Derived from *m*-(Phenoxypropoxy)benzamidine and Phenoxyacetamide

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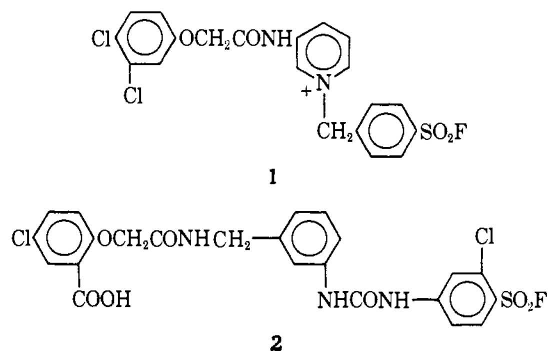
A new assay for irreversible inhibition of the C′1a component of complement has been established. A series of substituted pyridines quaternized with fluorosulfonylbenzyl bromide related to 1 in structure were previously shown to be good inhibitors of whole guinea pig complement; many of these compounds are excellent irreversible inhibitors of the C′1a component of complement. The good correlation in irreversible inhibition of C′1a and inhibition of whole complement by analogs of 1 strongly suggests that the main site of action by compounds of type 1 is inhibition of C′1. In contrast, the lack of correlation of irreversible inhibition of C′1a and inhibition of whole complement by benzamidines of type **5** strongly suggests that the main site of action of the benzamidines is one of the other 8 components of complement. *m*-[*m*-(*p*-Fluorosulfonylphenylureido)phenoxypropoxy]benzamidine (**5**) is the most potent inhibitor of guinea pig complement yet observed; **5** is about 1000 times as potent as benzamidine and 3000 times as potent as *N*-tosyl-L-arginine Me ester (TAME).

Inhibitors of the serum complement system could have medicinal utility for organ transplantation and in treatment of some arthritic states.[4,5] The serum complement system is a mixture of 11 distinct proteins.[5–7] One of the functions of complement is to kill foreign cells such as bacteria and protozoa; however, it can also lyse foreign mammalian cells and causes rejection of organ transplants.[5–7] Some of the proteins of the complement system are proteases with "tryptic" or "chymotrypic" properties;[5–7] therefore it is not surprising that complement is inhibited by certain inhibitors of trypsin[4] or chymotrypsin[8,9] when measured by the lysis of sheep red blood cells (RBC) by guinea pig complement and antibody.[4,10]

Two types of chymotryptic inhibitors of complement have emerged from this laboratory as exemplified by 1[8]



and **2**;[9] in both cases, removal of the SO_2F moiety resulted in loss of their activity, indicating that the SO_2F group was necessary for activity, presumably by irreversible inhibition[11] of one of the complement enzymes. That **1** was an irreversible inhibitor of the C′1a component of complement was shown by Becker.[8]

Benzamidine, a strong trypsin inhibitor,[12,13] is a weak inhibitor of complement;[4] inhibition is enhanced 6-fold by introduction of a *m*-phenoxypropoxy substituent (**3**)[4] which is further enhanced to 400-fold by substitution of *m*-(*p*-nitrophenylurea) on the phenoxy moiety (**4**)[3] (Table III). Notable is the fact that **3** and **4**, which are most probably reversible inhibitors in con-

(1) This work was generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper in this series see B. R. Baker and W. T. Ashton, *J. Med. Chem.*, **13**, 1165 (1970).

(3) For the previous paper on complement see B. R. Baker and M. Cory, *ibid.*, **12**, 1053 (1969), paper 165 of this series.

(4) B. R. Baker and E. H. Erickson, *ibid.*, **12**, 408 (1969), paper 152 of this series.

(5) H. J. Müller-Eberhard, *Advan. Immunol.*, **8**, 1 (1968).

(6) P. H. Schur and K. F. Austen, *Annu. Rev. Med.*, **19**, 1 (1968).

(7) Ciba Foundation Symposium, "Complement," G. E. W. Wolstenholme and J. Knight, Ed., Little, Brown and Co., Boston, Mass., 1965.

(8) (a) B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **12**, 677 (1969), paper 156 of this series. (b) B. R. Baker and J. A. Hurlbut, *ibid.*, **12**, 902 (1969), paper 161 of this series.

(9) B. R. Baker and J. A. Hurlbut, *ibid.*, **12**, 415 (1969), paper 153 of this series.

(10) E. A. Kabat and M. M. Mayer, "Experimental Immunochemistry," 2nd ed, C. C. Thomas Co., Springfield, Ill., 1967, pp 149–153.

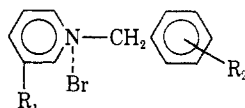
(11) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," Wiley, New York, N. Y., 1967.

(12) M. Mares-Guia and E. Shaw, *J. Biol. Chem.*, **240**, 1579 (1964).

(13) B. R. Baker and E. H. Erickson, *J. Med. Chem.*, **10**, 1123 (1967), paper 106 of this series.

TABLE I

IRREVERSIBLE INHIBITION[a] OF THE C′1A COMPONENT OF COMPLEMENT BY

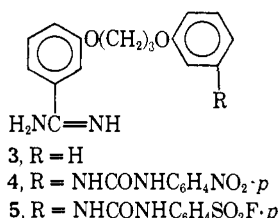


No.	R_1	R_2	mM inhibr	C′1a,[b] % inactvn	Whole complement, % inhibn[c]
1	3,4-$Cl_2C_6H_3OCH_2CONH$	4-SO_2F	0.50	67	45
6	3,4-$Cl_2C_6H_3OCH_2CONH$	3-SO_2F	0.50	70	58
			0.25	6	
7	3,4-$Cl_2C_6H_3OCH_2CONH$	2-SO_2F	0.50	95	93
			0.25	96	89
			0.125	70	91
			0.062	37	63
			0.031		27
8	3,4-$Cl_2C_6H_3OCH_2CONH$	2-Cl-4-SO_2F	0.25	76	71
			0.125	28	37
9	3,4-$Cl_2C_6H_3OCH_2CONH$	3-Cl-4-SO_2F	0.125[d]	7	28
10	3,4-$Cl_2C_6H_3OCH_2CONH$	4-Cl-3-SO_2F	0.125[d]	0	30
11	3,4-$Cl_2C_6H_3OCH_2CONH$	3-Cl-2-SO_2F	0.125	82	82
			0.062	33	49
12	3,4-$Cl_2C_6H_3OCH_2CONH$	4-Cl-2-SO_2F	0.125[d]	96	96
			0.062	94	79
			0.031	36	17
13	3,4-$Cl_2C_6H_3OCH_2CONH$	5-Cl-2-SO_2F	0.25	91	94
			0.125	94	90
			0.062	64	68
14	3,4-$Cl_2C_6H_3OCH_2CONH$	6-Cl-2-SO_2F	0.50	98	94
			0.25	98	96
			0.125	73	96
			0.062	55	84
			0.031		45
15	3,4-$Cl_2C_6H_3OCH_2CONHCH_2$	4-SO_2F	1.0	88	56
			0.5	68	19
			0.25	42	
16	3,4-$Cl_2C_6H_3OCH_2CONHCH_2$	2-SO_2F	0.50	96	92
			0.25	79	91
			0.125	53	89
			0.062	25	68
17	3,4-$Cl_2C_6H_3OCH_2CONHCH_2$	2-Cl-4-SO_2F	0.50	95	68
			0.25	51	33
			0.125	29	
18	3,4-$Cl_2C_6H_3OCH_2CONHCH_2$	3-Cl-4-SO_2F	0.50	38	42
19	3,4-$Cl_2C_6H_3OCH_2CONHCH_2$	4-Cl-3-SO_2F	0.5	10	51
20	3,4-$Cl_2C_6H_3OCH_2CONHCH_2$	3-Cl-2-SO_2F	0.50	98	92
			0.25	71	88
			0.125	44	67
21	3,4-$Cl_2C_6H_3OCH_2CONHCH_2$	4-Cl-2-SO_2F	0.50	92	90
			0.25	100	95
			0.125	98	90
			0.062	78	46
			0.031	32	12
22	3,4-$Cl_2C_6H_3OCH_2CONHCH_2$	5-Cl-2-SO_2F	0.50	100	95
			0.125	93	93
			0.062	55	65
23	3,4-$Cl_2C_6H_3OCH_2CONHCH_2$	6-Cl-2-SO_2F	0.25	99	88
			0.125	87	90
			0.062	48	56

[a] The technical assistance of Julie Leseman, Diane Shea, and Pauline Minton is acknowledged. [b] Inhibitor incubated 10 min at 37° with C′1a, then whole complement restored; see Experimental Section. [c] Data from ref 8. [d] Maximum solubility in incubation mixture.

trast to 1 and **2**, are more potent than 1 and **2**; therefore it is possible that compounds of the benzamidine series bearing a terminal SO_2F group (such as **5**) could be extremely potent inhibitors of the complement system if they were irreversible inhibitors.

In order to determine if a compound is an irreversible inhibitor of one of the components of complement, it is necessary to separate the one component from all the others. The lysis assay can then be made rate limiting upon the concentration of the one component to be tested. After preincubation of the component with the potential irreversible inhibitors, the whole complement

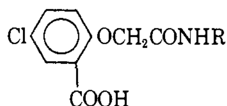


system is reconstituted with the other components and lysis of RBC measured. If the component has been partially or totally irreversibly inactivated, then lysis is slowed or stopped since the rate in this assay depends upon the concentration of the isolated component.

The easiest component to separate is the first one, C'1; since C'1 is insoluble at low ionic strength, it is only necessary to dialyze guinea pig complement and C'1 separates.[14a,b] The other components remain in the supernatant. The system is readily reconstituted to make the C'1 component rate limiting. The C'1 component, a zymogen form, is readily activated to C'1a by 10-min incubation at 37°.[14c] The candidate irreversible inhibitor is then added and incubation continued 10 min more before reconstituting the whole complement system (see Experimental Section).[14,15] If irreversible inhibition of the C'1a component has taken place, the rate of lysis is decreased. Such data are presented in Tables I–III with compounds of types 1, **2**, and **5** and constitute the subject of this paper.

TABLE II

IRREVERSIBLE INHIBITION[a] OF THE C'1A COMPONENT OF COMPLEMENT BY



No.	R	mM inhibr	C'1a,[b] % inactvn	Whole complement, % inhibn[c]
2	m-$CH_2C_6H_4NHCONHC_6H_3$-3-Cl-4-SO_2F	0.25[d]	30	82
		0.12		24
24	m-$CH_2C_6H_4NHCONHC_6H_4$-4-SO_2F	0.50[d]	20	70
		0.25		18
25	m-$CH_2C_6H_4NHCONHC_6H_4$-3-SO_2F	0.50[d]	48	50
		0.25		2
26	m-$CH_2C_6H_4NHCONHC_6H_3$-4-Me-3-SO_2F	0.50[d]	32	86
		0.25		39
27	m-$CH_2C_6H_4NHCONHC_6H_3$-6-Cl-3-SO_2F	0.5	65	89
		0.25	39	54
28	p-$CH_2C_6H_4NHCONHC_6H_4$-4-SO_2F	0.50[d]	31	70
		0.25		26
29	p-$CH_2C_6H_4NHCONHC_6H_4$-3-SO_2F	0.50[d]	18	55
		0.33		26
30	p-$CH_2C_6H_4NHCONHC_6H_3$-4-Me-3-SO_2F	0.25[d]	12	37
		0.125		0
31	m-$C_6H_4NHCONHC_6H_4$-4-SO_2F	0.50	0	15
32	m-$C_6H_4NHCONHC_6H_4$-3-SO_2F	0.50	57	82
		0.25		43
33	m-$C_6H_4NHCONHC_6H_4$-4-Me-3-SO_2F	0.25[d]	33	80
		0.10		17
34	m-$C_6H_4NHCONHC_6H_4$-6-Cl-3-SO_2F	0.125[d]	34	54

[a] The technical assistance of Julie Leseman, Diane Shea, and Pauline Minton is acknowledged. [b] Inhibitor incubated 10 min at 37° with C'1a, then whole complement restored; see Experimental Section. [c] Data from ref 9. [d] Maximum solubility in assay medium.

(14) (a) See ref 10, pp 163–165; (b) E. E. Ecker and S. Seifter, *Proc. Soc. Exp. Biol. Med.*, **47**, 18 (1943). (c) I. H. Lepow, O. D. Ratnoff, F. S. Rosen, and L. Pillemer, *ibid.*, **92**, 32 (1956).

(15) A different assay method for irreversible inhibition of C'1a has been described by E. L. Becker and K. F. Austen, *J. Exp. Med.*, **120**, 491 (1964).

Biological Results.—An important point in interpreting the amount of inactivation of C'1a should now be made. Note that as the concentration of an inhibitor such as **7** in Table I is reduced, the amount of inactivation stays constant over a fourfold range, then drops precipitously at 0.062 mM. The failure to achieve > 90% inactivation at 0.062 mM can be due to one of a number of factors previously observed in this laboratory. For example, the inhibitor concentration may be less than the enzyme concentration;[16] this is not the case with C'1a since the original concentration of C'1 in guinea pig complement is about 0.5 μM[5], and no concentrations of inhibitor below 31 μM were employed. The second possibility is that the SO_2F group is being hydrolyzed enzymatically to the irreversibly inert SO_3H group by C'1a itself[17] or by a serum "sulfonyl fluoridase."[18] The third and most likely possibility is a kinetic one. The rate of irreversible inhibition by an active-site-directed irreversible inhibitor is dependent upon the concentration of reversible complex between enzyme and inhibitor and upon the relative rate constant of reaction expressed by k.[19] In the case of **7** at 0.125–0.50 mM, the enzyme is probably reversibly saturated with **7** and the reaction is essentially complete in 10 min; below this concentration the enzyme is probably not saturated and reaction is incomplete in the arbitrary 10-min incubation time.

All of these factors can be lumped into one simple number in order to compare compounds in their ability to irreversibly inhibit C'1a, namely, the concentration of inhibitor necessary to give 50% inactivation of C'1a in 10 min. On this basis, the two best active-site-directed irreversible inhibitors of C'1a of the pyridine quaternary type (1) in Table I are **14** and **21**; also noteworthy is that **14** and **21** are the two best inhibitors of whole complement in Table I. Furthermore it should be noted that good inhibition of whole complement parallels irreversible inhibition of C'1a within a factor of two; exceptions are poor inhibitors of whole complement such as **9**, **10**, **18**, and **19**. Thus it is highly probable that the main site of inhibition of whole complement by inhibitors in Table I is the irreversible inhibition of C'1a as it is formed in the whole complement assay.

In Table II are presented data on analogs of **2**; here irreversible inhibition of C'1a at a given concentration is always considerably less than the inhibition of whole complement—indicating that C'1a is not the primary site of action of compounds of the type in Table II.

Of the highly potent benzamidines in Table III, the most potent benzamidine reported to date[3] on inhibition of whole complement was the m-nitrophenylurea derivative **4** of m-phenoxypropoxybenzamidine. Replacement of the NO_2 of **4** with SO_2F (**5**) increased the potency about twofold against complement; however, **5** showed no irreversible inhibition of C'1a. Eight structural variants (**35–42**) of **5** were then synthesized where the substituent on the amino group of m-(m-aminophenoxypropoxy)benzamidine was varied. All were less effective than **5** on inhibition of whole complement,

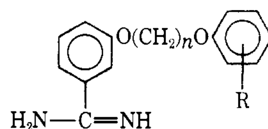
(16) (a) B. R. Baker and E. H. Erickson, *J. Med. Chem.*, **12**, 112 (1969), paper 144 of this series; (b) B. R. Baker and J. A. Hurlbut, *ibid.*, **12**, 118 (1969), paper 145 of this series.

(17) B. R. Baker and J. A. Hurlbut, *ibid.*, **11**, 233 (1968), paper 113 of this series.

(18) E. F. Fölsch and J. R. Bertino, *Mol. Pharmacol.*, **6**, 93 (1970).

(19) See ref 11, p 122.

TABLE III
IRREVERSIBLE INHIBITION[a] OF THE C′1A COMPONENT OF COMPLEMENT BY



No.	*n*	R	m*M* inhibr	C′1a,[b] % inactvn	Whole complement % inhibn	Whole complement % lysis[c]
4[d]	3	*m*-$NHCONHC_6H_4NO_2$-*p*	0.062[e]		95	
			0.031		78	
			0.015		60	
			0.0078		32	
5	3	*m*-$NHCONHC_6H_4SO_2F$-*p*	0.125		5	+
			0.062	0	57	21
			0.031	0	77	
			0.015		77	
			0.0077		54	0
			0.0038		43	
			0.0019		29	
35	3	*m*-$NHCONHC_6H_4SO_2F$-*m*	0.125		21	+
			0.062	20	66	14
			0.031		51	0
			0.015		17	
36	3	*m*-$NHCOC_6H_4SO_2F$-*p*	0.125	10	87	2
			0.062	0	73	
			0.031		54	
			0.015		40	
37	3	*m*-$NHCOC_6H_4SO_2F$-*m*	0.125	33	78	0
			0.062	17	62	
			0.031		40	
			0.015		11	
38	3	4-Me-3-$NHCONHC_6H_4SO_2F$-*p*	0.062[e]		36	30
			0.031	0	60	3
			0.015		58	
			0.0077		23	
39	3	4-Me-3-$NHCOC_6H_4SO_2F$-*p*	0.125	4	83	1
			0.062		63	
			0.031		42	
40	3	*m*-$NHCOCH_2C_6H_4SO_2F$-*p*	0.125	60	74	32
			0.062	21	64	2
			0.031		52	
			0.015		33	
41	3	*m*-$NHCOCH_2OC_6H_4SO_2F$-*p*	0.062[e]		71	9
			0.031	0	60	0
			0.015		34	
42	3	*m*-$NHCONH(CH_2)_2C_6H_4SO_2F$-*p*	0.062[e]	88	86	1
			0.031	60	85	
			0.015	38	72	
			0.0077		55	
			0.0039		35	
43	3	*o*-$NHCONHC_6H_4SO_2F$-*p*	0.25	40	61	21
			0.125	15	61	9
			0.062		51	
			0.031		26	
44	3	*o*-$NHCOC_6H_4SO_2F$-*p*	0.125	16	38	6
			0.062		23	
45	3	*o*-$NHCOC_6H_4SO_2F$-*m*	0.25	89	80	9
			0.125	84	64	
			0.062	70		
			0.031	29		
46	3	*o*-$NHCOC_6H_5$	0.5	−13	90	10
			0.25	−8	83	
			0.125		32	
47	4	*m*-$NHCONHC_6H_4SO_2F$-*p*	0.062[e]	0		41
			0.031	0	22	34
			0.015	0	35	5
			0.0077	0	23	
48	4	*m*-$NHCOC_6H_4SO_2F$-*p*	0.125	−2		+
			0.062	3	53	3
			0.031	−5	26	

TABLE III (*Continued*)

No.	*n*	R	m*M* inhibr	C'1a,[b] % inactvn	Whole complement % inhibn	Whole complement % lysis[c]
49	4	*p*-NHCONHC$_6$H$_3$-2-OCH$_3$-5-SO$_2$F	0.062[e]	−10	59[d]	0[d]
50	2	*m*-NHCONHC$_6$H$_4$SO$_2$F-*p*	0.125[e]	0	85	5
			0.062	0	76	
			0.031		55	
51	2	*m*-NHCONHC$_6$H$_4$SO$_2$F-*m*	0.125	40		+
			0.062	35	58	3
			0.031	12	35	
52	2	*m*-NHCOC$_6$H$_4$SO$_2$F-*p*	0.5	72	87	8
			0.25	57	85	1
			0.125		80	
			0.062		74	
			0.031		64	
			0.015		41	
53	2	*m*-NHCOC$_6$H$_4$SO$_2$F-*m*	0.5	72	77	11
			0.25	35	81	1
			0.125		74	
			0.062		67	
			0.031		40	

[a] The technical assistance of Diane Shea and Pauline Minton is acknowledged. [b] Inhibitor incubated 10 min at 37° with C'1a, then whole complement restored; see Experimental Section. [c] Lysis by the compound in the absence of the complement expressed as per cent of total lysis possible; see ref 4 for assay method. [d] Data from ref 3. [e] Maximum solubility.

except **42** which was about equivalent. Four of the compounds (**35, 37, 40, 42**) showed some irreversible inhibition of C'1a. However to intrepret the amount of irreversible inhibition, the 1 → 10 dilution of the irreversible incubation mixture for assay must be considered; for example, when C'1a is preincubated with 0.062 m*M* **42**, then diluted 1 → 10 for assay, about 45–50% of the 88% inhibition observed is due to reversible inhibition of whole complement.

Four derivatives (**43–46**) of *m*-(*o*-aminophenoxypropoxy)benzamidine were then synthesized for evaluation; none were as good as **5** on whole complement. However, **45** was an excellent irreversible inhibitor of C'1a. That the inhibition of whole complement by **45** was *not* due to inhibition of C'1a was indicated by the fact that **45** without an SO$_2$F group, namely **46**, was as effective an inhibitor of whole complement as **45**.

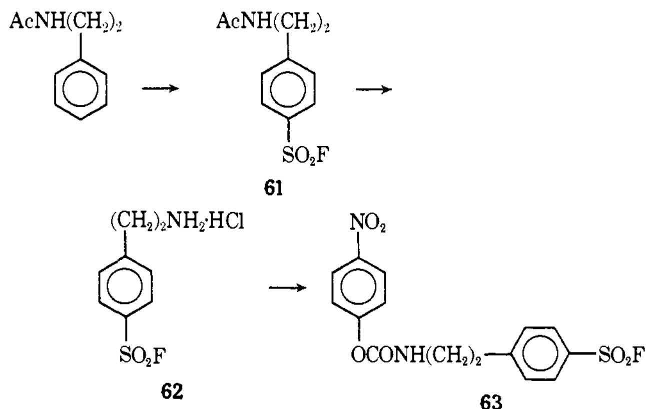
Some structural variants of **5** were then synthesized where the PrO bridge was changed to EtO (**50–53**) or BuO (**47–49**). The BuO derivatives were not as good as **5** on inhibition of whole complement and did not show irreversible inhibition of C'1a. The apparent irreversible inhibition of C'1a by **52** and **53** is due to reversible inhibition of whole complement when the 1 → 10 dilution in the assay is taken into account; however, **51** definitely showed some irreversible inhibition of C'1a, even though it was only about as effective as **50, 52,** and **53** on whole complement. It was clear that **50–53** were fairly potent inhibitors of whole complement, but were not as good as **5**.

From the data presented in Tables I–III, it is probable that the main blockade of complement by the compounds in Table I is C'1 and that the main blockade by the compounds in Tables II and III is one of the other components of complement between C'2 and C'9. Whether or not more potent irreversible inhibitors of C'1a related in structure to **45** can be found is under active investigation.

The most potent inhibitor of whole complements yet observed is the substituted benzamidine **5**; **5** is about 1000 times as potent as the parent benzamidine and 3000 times as potent as *N*-tosyl-L-arginine methyl ester (TAME).[9]

Chemistry.—The intermediate substituted *m*-(phenoxyalkoxy)benzonitriles were prepared by the previously described alkylation of *m*-cyanophenol (method A);[13] these were converted into the amidines through the imino ether hydrochlorides (method B).[20] Catalytic reduction of the NO$_2$ group with 5% Pd–C (method C)[20] gave crystalline aminoamidine salts which could be acylated to the desired amides or ureas (methods D and E).

Fluorosulfonation of *N*-phenethylacetamide at −10 to −20° gave **61**. Hydrolysis with 6 *N* HCl gave **62** which was reacted with *p*-nitrophenylchloroformate to give **63**, then used in method E.



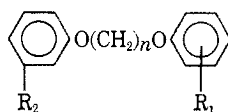
Experimental Section

Melting points were determined in capillary tubes on a Mel-Temp block and are uncorrected. All analytical samples had ir spectra compatable with their assigned structures and moved as a single spot on tlc on Brinkmann silica gel GF or polyamide MN$_{254}$; each gave combustion values for C, H, and N, or F within 0.4% of theory.

***m*-[*m*-(*p*-Fluorosulfonylbenzamido)phenoxypropoxy]benzamidine Toluenesulfonate (36) (Method D).**—To a soln of 0.358 g (1.0 mmole) of *m*-(*m*-aminophenoxypropoxy)benzamidine·2HCl[3]

(20) B. R. Baker and E. H. Erickson, *J. Med. Chem.*, **11**, 245 (1968), paper 115 of this series.

TABLE IV
PHYSICAL CONSTANTS OF



No.	n	R_1	R_2	Method[a]	% yield	Mp, °C	Formula[b]
5	3	*m*-$NHCONHC_6H_4SO_2F$-*p*	$C(NH_2)$=NH·picrate	E	35[c]	199–202	$C_{29}H_{26}FN_7O_{12}S$[d]
5	3	*m*-$NHCONHC_6H_4SO_2F$-*p*	$C(NH_2)$=NH·HCl	E	78[e]	153–155	$C_{23}H_{24}ClFN_4O_5S \cdot H_2O$
35	3	*m*-$NHCONHC_6H_4SO_2F$-*m*	$C(NH_2)$=NH·picrate	E	30[f]	216–218	$C_{29}H_{26}FN_7O_{12}S$[d]
36	3	*m*-$NHCOC_6H_4SO_2F$-*p*	$C(NH_2)$=NH·TsOH	D	45[g]	229–232	$C_{30}H_{30}FN_3O_8S_2 \cdot 0.5H_2O$
37	3	*m*-$NHCOC_6H_4SO_2F$-*m*	$C(NH_2)$=NH·TsOH	D	44[c]	215–217	$C_{30}H_{30}FN_3O_8S_2$[d]
38	3	4-Me-3-$NHCONHC_6H_4SO_2F$-*p*	$C(NH_2)$=NH·TsOH	E	14[e]	135–137	$C_{31}H_{33}FN_4O_8S_2 \cdot H_2O$[d]
39	3	4-Me-3-$NHCOC_6H_4SO_2F$-*p*	$C(NH_2)$=NH·picrate	D	25[e]	173–175	$C_{30}H_{27}FN_6O_{12}S$
40	3	*m*-$NHCOCH_2C_6H_4SO_2F$-*p*	$C(NH_2)$=NH·picrate	D	52[c]	125–127	$C_{30}H_{27}FN_6O_{12}S$
41	3	*m*-$NHCOCH_2OC_6H_4SO_2F$-*p*	$C(NH_2)$=NH·TsOH	D	38[e]	214–216	$C_{31}H_{32}FN_3O_9S_2$
42	3	*m*-$NHCONH(CH_2)_2C_6H_4SO_2F$-*p*	$C(NH_2)$=NH·TsOH	E[h]	51[e]	209–211	$C_{32}H_{35}FN_4O_8S_2 \cdot 0.5H_2O$
43	3	*o*-$NHCONHC_6H_4SO_2F$-*p*	$C(NH_2)$=NH·TsOH	E	31[c]	135–136	$C_{30}H_{31}FN_4O_8S_2 \cdot C_2H_5OH$[d]
44	3	*o*-$NHCOC_6H_4SO_2F$-*p*	$C(NH_2)$=NH·TsOH	D	38[c]	206–208	$C_{30}H_{30}FN_3O_8S_2$[d]
45	3	*o*-$NHCOC_6H_4SO_2F$-*m*	$C(NH_2)$=NH·picrate	D	18[i]	229–230	$C_{29}H_{25}FN_6O_{12}S$[d]
46	3	*o*-$NHCOC_6H_5$	$C(NH_2)$=NH·picrate	D	20[i]	224–226	$C_{29}H_{26}N_6O_{10}$
47	4	*m*-$NHCONHC_6H_4SO_2F$-*p*	$C(NH_2)$=NH·picrate	E	42[c]	130–133	$C_{30}H_{28}FN_7O_{12}S \cdot 0.5H_2O$
48	4	*m*-$NHCOC_6H_4SO_2F$-*p*	$C(NH_2)$=NH·TsOH	D	41[c]	210–213	$C_{31}H_{32}FN_3O_8S_2$
50	2	*m*-$NHCONHC_6H_4SO_2F$-*p*	$C(NH_2)$=NH·TsOH	E	58[e]	220–221	$C_{29}H_{29}FN_4O_8S_2$
51	2	*m*-$NHCONHC_6H_4SO_2F$-*m*	$C(NH_2)$=NH·picrate	E	48[e]	231–233	$C_{28}H_{24}FN_7O_{12}S$
52	2	*m*-$NHCOC_6H_4SO_2F$-*p*	$C(NH_2)$=NH·TsOH	D	27[e]	130–133	$C_{29}H_{28}FN_3O_8S_2$
53	2	*m*-$NHCOC_6H_4SO_2F$-*m*	$C(NH_2)$=NH·TsOH	D	37[c]	125–128	$C_{29}H_{28}FN_3O_8S_2 \cdot H_2O$
54	4	*m*-NO_2	CN	A	74[j]	103–107	$C_{17}H_{16}N_2O_4$
55	4	*m*-NO_2	$C(NH_2)$=NH·TsOH	B	36[f]	128–130	$C_{24}H_{27}N_3O_7S$
56	4	*m*-NH_2	$C(NH_2)$=NH·2TsOH	C	59[f]	128–130	$C_{31}H_{37}N_3O_8S_2$
57	2	*m*-NO_2	CN	A	35[j]	116–119	$C_{15}H_{12}N_2O_4$
58	2	*m*-NO_2	$C(NH_2)$=NH·TsOH	B	55[c]	179–181	$C_{22}H_{23}N_3O_7S$
59	2	*m*-NH_2	$C(NH_2)$=NH·2TsOH	C	57[c]	155–158	$C_{29}H_{33}N_3O_8S_2$
60	3	3-NH_2-4-CH_3	$C(NH_2)$=NH·2TsOH	C	78[k]	120–121	$C_{31}H_{37}N_3O_8S_2 \cdot H_2O$

[a] Methods: A,[13] alkylation of *m*-cyanophenol with dibromoalkane then further alkylation of the bromoalkyl ether; B,[20] CN → imino ether → amidine; C,[20] reduction of NO_2; D and E, see Experimental Section. [b] Anal. for C, H, N unless otherwise indicated. [c] Recrystd from abs EtOH. [d] Anal. for C, H, F. [e] Recrystn from 50% aq EtOH. [f] Recrystd from H_2O. [g] Recrystd from 95% EtOH. [h] Method E with reaction temp of 60° for 40 hr. [i] Recrystd from 50% Me_2CO. [j] Recrystd from C_6H_6. [k] Recrystd from 25% aq EtOH.

in 2 ml of DMF protected from moisture was added 1 g of 3 A Molecular Sieves (Linde) followed by 2.0 ml of 1 *M* pyridine in DMF. The resulting mixture was stirred 1 hr at ambient temp, and a soln of 0.252 g (1.1 mmoles) of *p*-fluorosulfonylbenzoyl chloride in 2 ml of DMF was added. The mixture was stirred 1 hr at ambient temp, then poured into 75 ml of Et_2O. The resulting mixture was chilled, and the Et_2O was decanted. The residue was dissolved in hot 95% aq EtOH, and excess *p*-TsOH was added; upon cooling a cryst product was obtained. Recrystn from 95% EtOH afforded 0.297 g (45%), mp 229–232°. See Table IV for additional data.

m-[*m*-(*p*-**Fluorosulfonylphenylureido)phenoxypropoxy]benzamidine Picrate (5) (Method E).**—To a soln of 1.79 g (5.0 mmoles) of *m*-(*m*-aminophenoxypropoxy)benzamidine·2HCl[3] in 10 ml of DMF protected from moisture was added 5 g of 3 A Molecular Sieves (Linde) followed by 10 ml of 1 *M* pyridine in DMF. The resulting mixture was stirred 1 hr at ambient temp, then a soln of 1.93 g (5.5 mmoles) of *O*-(*p*-nitrophenyl) *N*-(*p*-fluorosulfonylphenyl)carbamate[21] in 10 ml of DMF was added. The mixture was stirred 24 hr at ambient temp, then poured into 300 ml of Et_2O. The resulting mixture was chilled, and the Et_2O was decanted. The residue was dissolved in EtOH, and excess picric acid was added; upon cooling a cryst product was obtained. Two recrystals from EtOH gave 1.25 g (35%), mp 199–202°. See Table IV for additional data.

m-[*m*-(*p*-**Fluorosulfonylphenylureido)phenoxypropoxy]benzamidine·HCl (5).**—Repetition of the preceding reaction gave 1.80 g (50%) of yellow crystals, mp 195–198°. This picrate was dissolved in 100 ml of EtOH, 36 g (wet weight) of Dowex 2-X8 ion-exchange resin (Cl^- form) was added, and then the mixture was stirred 24 hr. The pale yellow soln was decanted and poured slowly through a chromatography column contg an additional 10.0 g (wet weight) of resin. The clear colorless soln was evapd *in vacuo*, and the crystal residue was recrystd from 50% EtOH; yield, 1.2 g (87% conversion), mp 153–155°. Prepn of the HCl salt directly using the above product for seed crystals and method E gave 78% yield, mp 153–155°. See Table IV for additional data.

N-(*p*-**Fluorosulfonyl)phenylethylacetamide (61).**—To 150 ml of FSO_3H in a Teflon beaker at −10° was added 30.0 g (0.184 moles) of *N*-phenylethylacetamide.[22] The resulting mixture was stirred for 4 hr, keeping the temp between −10° and −20°, then poured slowly into 1.5 l. of ice, and extd with C_6H_6 (5 × 300 ml). The combined exts were washed with H_2O until the washings were neutral. The soln was dried, treated with Norite A, then concd to 100 ml. Cooling gave 25.0 g (50%) of white crystals, mp 148–150°. The nmr spectrum was consistant with a para-substituted product. *Anal.* ($C_{10}H_{12}FNO_3S$) C, H, N.

p-**Fluorosulfonylphenethylamine Hydrochloride (62).**—A mixture of 5.0 g (20.4 mmoles) of **61** and 10 ml of 6 *N* HCl was stirred at reflux for 2 hr. The cooled mixture was evapd *in vacuo* to dryness. The residue was dissolved in 100 ml of EtOH, filtered, then concd to 20 ml. The product was collected to yield 2.5 g (53%), mp 238–240°. *Anal.* ($C_8H_{10}FNO_2S \cdot HCl$) C, H, N.

O-(*p*-**Nitrophenyl)** *N*-(*p*-**Fluorosulfonylphenylethyl)carbamate (63).**—To a stirred suspension 2.39 g (10 mmoles) of **62,** 30 ml of C_6H_6, and 1.99 g (10 mmoles) of *p*-nitrophenyl chloroformate was added dropwise 2.8 ml (20 mmoles) of Et_3N. The mixture was stirred 24 hr at ambient temp and filtered, and then the filtrate was concd to 10 ml and cooled. The product was collected and recrystd from EtOAc; yield, 1.32 g (36%) of white crystals, mp 156–159°. *Anal.* ($C_{15}H_{13}FN_2O_6S$) C, H, N.

Assay of Irreversible Inhibition of the C′1a Component of Com-

(21) B. R. Baker and N. M. J. Vermeulen, *ibid.*, **12**, 74 (1969), paper 134 of this series.

(22) N. D. Cheronis and J. B. Entrikin "Semimicro Qualitative Organic Analysis" 2nd ed., Interscience Publishers, New York, N. Y., 1957, p 409.

plement. **Materials.**—Sheep red blood cells (RBC) suspended in Alsever's soln, guinea pig hemolysin (antibody), and lyophilized guinea pig complement were purchased from Grand Island Biologicals, Oakland, Calif., or Hyland Laboratories, Costa Mesa, Calif. RBC were standardized to 10^9 cells/ml of buffer A as previously described.[4] Hemolysin was diluted 1:800 with buffer A and was stable at 3° indefinitely; as used in the assay below, this gives 5 times the amt needed for max velocity.

Solutions.—Buffer A was 5 m*M* Tris hydrochloride containing 0.5 m*M* $MgCl_2$–0.15 m*M* $CaCl_2$–0.15 *M* NaCl–0.1% gelatin. Buffer B was pH 5.4 K phosphate buffer of 0.02 ionic strength. Citrate–saline was a 1:4 mixture of 0.075 *M* Na citrate and 0.15 *M* NaCl.

Separation of C'1.—The lyophilized complement was dissolved in restoring soln at 0°, then 10 ml was dialyzed at 3° against 3 l. of buffer B by continuous flow for 10–15 hr. The dialyzed suspension was centrifuged at 3° at 20,000 rpm in a No. 40 head of a Spinco L centrifuge. The pellet contained C'1 and the supernatant, the remainder of the components of complement,[14,15] and is called the R fraction.

The supernatant R fraction was adjusted to pH 7 with 0.1 *N* NaOH, then brought to 0.15 ionic strength by addition of the appropriate amt of 1.71 *M* NaCl. The soln was kept frozen at −20° in 1-ml aliquots.

The pellet of C'1 was rinsed twice with 2-ml portions of buffer B. The solid was resuspended in 30 ml of 0.15 *M* NaCl and stirred 1 hr at 0°. This mixture was then dialyzed and centrifuged as described above. The supernatant was discarded and the pellet of C'1 stirred 1 hr at 0° with 30 ml of 0.15 *M* NaCl. The soln was clarified by centrifugation and the supernatant containing C'1 was frozen in 1-ml aliquots.

Recombination Assay and Standardization.—The soln of C'1 and R were dild 1:2 with buffer A. In a centrifuge tube was placed 0.25 ml of RBC (10^9/ml) and 0.25 of hemolysin (1:800) in a bath at 37°. After 15 min the contents were treated with 0.30 ml of buffer A and 100 μl of C'1; after 7 min, 100 μl of R was added. After 14 min the lysis reaction was stopped by addition of 2.75 ml of ice-cold citrate–saline. The tube was centrifuged for 3 min in a clinical centrifuge, then the OD of the hemoglobin in the supernatant was read at 541 mμ in a glass cuvette.

When either the C'1 or the R fraction is replaced by buffer, there should be no lysis above a control where both the C'1 and R fractions are replaced by buffer; no lysis shows that separation of C'1 and R was sufficient for the recombination assay. The R soln is further dild to a concn 1:3 and 1:4. If the rate is the same as 1:2, then the 1:3 or 1:4 diln is used with buffer A.

The amount of C'1 is then decreased by further diln with buffer A until the above conditions given an OD reading of 0.30–0.42; the C'1 is usually dild in the range of 1:5 to 1:7. This assay is then rate limited by the concn of C'1.

Irreversible Inhibition of C'1a.—In 2 tubes are placed 323 μl of C'1 at the proper diln; these are then placed in a bath at 37° for 10 min to activate C'1 to C'1a.[15] To one tube is added 10 μl of inhibitor in MeOEtOH, and to the other tube is added 10 μl of MeOEtOH containing no inhibitor. Meanwhile two tubes containing 0.25 ml of RBC (10^9/ml) and 0.25 ml of 1:800 hemolysin was fixed for 15 min at 37°. To each RBC tube was added 0.30 ml of buffer A and 100 μl of C'1a incubation mixture plus or minus inhibitor. After 7 min, 100 μl of R fraction was added, and the RBC were allowed to lyse a predetermined time (7–15 min), then quenched by addition of 2.75 ml of ice-cold citrate–saline. The OD for hemoglobin was read after centrifugation as described in the previous paragraph; the per cent irreversible inhibition was determined by comparing the OD of the control tube *vs.* the OD of the inhibitor tube.

In practice 5–6 tubes were run simultaneously, one control tube, 3–4 tubes contg an inhibitor, and one lysis control where the C'1 and R fractions of the control tube were replaced by buffer. The amount of lysis of RBC caused by the compd after 1:10 diln in this assay has usually already been determined in the whole complement assay of the compd.

Folic Acid Analogs. Modifications in the Benzene-Ring Region. 1. 2'- and 3'-Azafolic Acids[1]

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2'- and 3'-azafolic acids were synthesized as part of a program to design and obtain folic acid analogs that might have antineoplastic activity because of their altered ability to function as one-carbon transfer agents. The reductive condensation of 2-acetamido-6-formylpteridin-4(3*H*)-one (**2**) with diethyl *N*-(5-aminopicolinoyl)-glutamate (**7b**) followed by blocking group hydrolysis afforded 2'-azafolic acid (**10**). Alternatively, **2** was condensed with **7b** nonreductively, and the product anil was isolated and subsequently converted into **10**. Acetylation of the hydrolyzed reductive condensation product of **2** and ethyl 6-aminonicotinate (**1b**) afforded N^2,N^{10}-diacetyl-3'-azapteroic acid (**3c**). Coupling of **3c** with diethyl glutamate gave diethyl N^2,N^{10}-diacetyl-3'-azafolate (**5b**); acetylation of the reductive condensation product of **2** and diethyl *N*-(6-aminonicotinoyl)-glutamate (**4**) also yielded **5b**. Blocking group hydrolysis of **5b** then afforded 3'-azafolic acid (**6**). Compounds **4**, **6**, **7b**, and **10** were found to be inactive in initial tests against leukemia L1210 in mice; they were also found to be noncytotoxic when tested *vs.* HEp-2 cells in culture. Analogs **6** and **10** were observed to be growth supporting when tested *vs. Streptococcus faecalis*.

N^{10}-Formyltetrahydrofolate, N^5,N^{10}-methenyltetrahydrofolate, and N^5,N^{10}-methylenetetrahydrofolate are formed enzymatically from tetrahydrofolate and formate or formaldehyde donors. These tetrahydrofolate derivatives function as agents in the transfer of one-carbon units.[2] Structural alterations between the pteridine and the glutamate portions of folic acid type molecules could alter the capacity of such structures to accept or to donate one-carbon units. For example, the potential of folic acid type molecules for forming one-carbon transfer agents may be lessened by reducing electron availability at position 10 (N^{10}). Reduction of electron availability might be effected either by introducing electron-withdrawing substituents on the benzene ring or by replacing the benzene ring with certain heterocyclic rings. Conversely, the presence of electron-donating groups on the benzene ring may, by increasing electron availability at the N^{10} position, stabilize one-carbon transfer agents and thereby render more difficult the transfer of a one-carbon unit from the folic acid type cofactor to the substrate. These struc-

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(2) M. Friedkin, *Annu. Rev. Biochem.*, **32**, 185 (1963); F. M. Huennekens in "Biological Oxidations," T. P. Singer, Ed., Wiley, New York, N. Y., 1968, pp 439–513.