Neuraminidase Inhibition and Viral Chemotherapy

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Two neuraminidase inhibition assay procedures are described. The rationale for utilizing enzyme inhibitors as potential chemotherapeutic agents in myxoviral infections is discussed. An acidic class of inhibitors consists of substituted β-aryl-α-mercaptoacrylic acids. New types of basic inhibitors containing an *o*-aminophenyl moiety removed by one carbon from a basic nitrogen heteroatom are described. Structure–activity relationships are discussed and results of *in vitro* and *in vivo* antiviral tests are reported for the most active inhibitors in each class.

The sialic or neuraminic acids are 9-carbon amino sugar substances which are synthesized chemically and biologically *via* an aldol condensation between pyruvic acid or a potential pyruvic moiety and *N*-acetyl or glycolyl D-mannosamine.[1] These acidic sugars are α-ketosidically linked to other sugars in the terminal position of oligosaccharide chains in glyco and mucoproteins.[2] Virtually all of the serum proteins except albumin contain sialic acid. The mucoproteins found in submaxillary glands, connective tissue, cartilage, and mucus of the epithelial surfaces in the respiratory, digestive, and urinary tract are rich in sialic acid. They occur in animal cell membranes and are reported to be partly responsible for the electrical charge and ion transport through these membranes.[3] The hormones involved in rapid growth and fertility such as erythropoietin,[4] human chorionic gonadotropin,[5] follicle stimulating and luteinizing hormones[6] all contain sialic acids. Removal of these residues enzymatically or chemically completely inactivates these hormones. It has been postulated that terminal sialic acid moieties serve as viscosity enhancing agents which tend to preserve the conformational structures of proteins.

The neuraminidases or sialidases are enzymes of diverse biological origin which selectively cleave the terminal sialic acid moieties from its neighboring sugar. The enzyme was first observed in the influenza virus where its role in the sorption and desorption from erythrocytes was evolved.[7] It has been shown to be associated with other viruses of the myxo group[8] as well as with a number of microorganisms.[9]

Hirst[7] first demonstrated that the influenza virus becomes attached to the red blood cell through the sialic acid residues imbedded in the cell membrane. Such an adsorption is accompanied by agglutination of the red cell. The virus, in order to become desorbed from the red cell, must rely on its neuraminidase. Such liberated virus is fully infective and can hemagglutinate fresh red cells. The agglutinated red cells however can no longer adsorb the influenza virus due to the loss of sialic acid residues. Neuraminidase has therefore been called the virus receptor destroying enzyme.

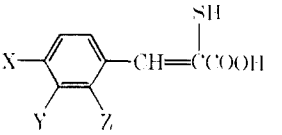
It has been clearly demonstrated that glycoproteins[10] and mucoproteins[11] which contain high levels of sialic acid can compete with the red cell for adsorption of the virus and are therefore hemagglutination inhibitors. Such proteins can therefore be considered as one of the body's primary defense mechanisms against such infections. Viral and bacterial neuraminidase on the other hand can be considered as the microbe's answer to these host cell defense mechanisms. A neuraminidase inhibitor would theoretically prevent a myxo virus from becoming desorbed from the protective mucoproteins found in the epithelial surfaces of the respiratory tract and could be envisioned as an enhancer of the body's defense mechanism. Such enzyme inhibitors might therefore serve as myxo viral chemotherapeutic agents which would not necessarily affect a key metabolic step in the viral life cycle but serve instead as an inhibitor to viral transport to specific target sites within the host.

Recent developments in this area tend to corroborate the above rationale. Campbell[12] has observed that ovine α_1 glycoprotein inhibited the hemagglutination properties of Newcastles Disease Virus and was active in plaque reduction tests. This activity was lost on treatment with neuraminidase. Roberts[13] reported that virulent Mycoplasma strains contain neuraminidase while avirulent organisms did not. Schmidt[14] protected mice against lethal influenza virus challenge by the intravenous administration of sialomucoids isolated from human and cows milk. Treatment with neuraminidase reduced the protective effect. Pepper[15] has recently shown that the α_2 macroglobulin from horse serum inhibits the A2 strain of influenza virus and that the determinant group is 4-*O*-acetyl-*N*-acetylsialic acid. Schulman[16] reported that antibody directed towards neuraminidase protected mice against influenza virus challenge. The utilization of neuraminidase inhibition as a potential method for antiviral screening is not unique although when our studies were initiated no report existed in the literature. Rafel-

(1) D. G. Comb and S. Roseman, *J. Biol. Chem.*, **235**, 2529, (1960).
(2) For a comprehensive review see A. Gottschalk, "The Chemistry and Biology of Sialic Acids and Related Substances," Cambridge University Press, Cambridge, England, 1960.
(3) J. L. Glick and S. Githens III, *Nature*, **208**, 88 (1965).
(4) P. Lowy, G. Keighley, and H. Borsook, *ibid.*, **185**, 102 (1960).
(5) R. Brossmer and K. Walter, *Klin. Wochenschr.*, **36**, 925 (1958).
(6) A. Gottschalk, W. Whitten, and F. Graham, *Biochim. Biophys. Acta*, **38**, 183 (1959).
(7) G. K. Hirst, *J. Exp. Med.*, **76**, 195 (1942).
(8) G. L. Ada and J. D. Stone, *Brit. J. Exp. Pathol.*, **31**, 263, 275 (1960).
(9) R. Heimer and K. Meyer, *Proc. Nat. Acad. Sci. U. S.*, **42**, 728 (1956).
(10) J. F. McCrea, *Aust. J. Exp. Biol. Med. Sci.*, **26**, 355 (1948).
(11) J. F. McCrea, *Biochem. J.*, **55**, 132 (1953).
(12) B. J. Campbell, A. Schneider, D. Howe, and D. Durand, *Biochim. Biophys. Acta*, **148**, 137 (1967).
(13) D. H. Roberts, *Nature* **213**, 87 (1967).
(14) E. Schmidt and M. Janssen, *Z. Kinderheilk.*, **98**, 307 (1966).
(15) D. S. Pepper, *Biochim. Biophys Acta*, **156**, 317 (1968).
(16) J. L. Schulman, M. Khakpour, and E. D. Kilbourne, *J. Virol.*, **2**, 778 (1968).

TABLE I

INHIBITION OF BACTERIAL NEURAMINIDASE BY SUBSTITUTED β-PHENYL-α-MERCAPTOACRYLIC ACIDS



No.	X	Y	Z	Ref	Mp, °C	Formula	Analyses	% inhibition, 100 μg/ml	I_{50}, μml/ml
1	H	H	H	*a*	125	$C_9H_8O_2S$		11	
2	Cl	H	Cl		150–155	$C_9H_6Cl_2O_2S$	S, Cl	95	0.26
3	Cl	Cl	H	*b*	168–172	$C_9H_6Cl_2O_2S$		97	0.22
4	NO_2	H	H	*c*	*ca.* 150 dec	$C_9H_7NO_4S$	N, S	97	0.14
5	COOH	H	H		>260 dec	$C_{10}H_8O_4S$	C, H, S	16	
6	F	H	H		164–166	$C_9H_7FO_2S$	C, H, S	11	
7	OH	H	H	*d*	185–186	$C_9H_8O_3S$		9	
8	CH_3O	H	H	*d*	168–170	$C_{10}H_{10}O_3S$		8	
9	$(CH_3)_2N$	H	H	*e*	>150 dec	$C_{11}H_{13}NO_2S$		5	
10	H	NO_2	H	*c*	255 dec	$C_9H_7NO_4S$		98	0.16
11	H	H	Cl	*f*	146–147	$C_9H_7ClO_2S$		11	
12	Disulfide of 11			*f*	205–206	$C_{18}H_{12}Cl_2O_4S_2$		15	

[a] See ref 21. [b] Rohm & Haas Co. [c] See Experimental Section. [d] D. G. Bew and G. R. Clemo, *J. Chem. Soc.*, 1150 (1954). [e] P. N. Bhargava, K. N. Permeswaran, and S. Venkataramani, *J. Indian Chem. Soc.*, **35**, 161 (1958). [f] D. Libermann, J. Himbert, and L. Hengl, *Bull. Soc. Chim. Fr.*, 1120 (1948).

son[17] first reported the inhibitory effects of some common chemicals on this enzyme and Edmond[18] described the inhibitory effects of some simple N-substituted oxanic acids. Wagner and coworkers[19] utilized antineuraminidase activity as a viral screening procedure and finally Brammer, *et al.*,[20] reported the antineuraminidase and antiviral activity of some 3,4-dihydroisoquinolines which they claimed were active against influenza infections in mice and humans.

We wish to report two sialidase inhibition assay procedures which have been utilized in our laboratories and which have produced two new classes of potent enzyme inhibitors. The acidic class of inhibitors have structural similarities to the substrate neuraminic acid in that they are sulfur analogs of pyruvic acid. These compounds may be represented as thiopyruvic acids I in equilibrium with their enol or mercapto

forms II. Campaigne and Cline[21] prepared a series of β-aryl-α-mercaptoarylic acids and showed by uv absorption studies that these compounds are correctly represented as ene-thiols II rather than thiones I. The acids prepared and tested for bacterial enzyme inhibitor power are listed in Tables I and II. Inhibition studies utilizing the viral enzyme showed good correlation with the bacterial enzyme. In addition, **4** and **10** were active in the plaque reduction assay using the HA-1 parainfluenza 3 virus.[22]

(17) M. E. Rafelson, *Exposes Annu. Biochim. Med.*, **24**, 121 (1963).

(18) J. D. Edmond, R. G. Johnston, D. Kidd, H. L. Rylance, and R. C. Sommerville, *Brit. J. Pharmacol. Chemother.* **27**, 415 (1966).

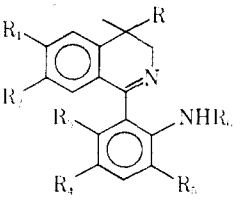
(19) R. B. Wagner, B. A. Steinberg and I. Ruchman, *Appl. Microbiol.*, **15**, 239 (1967).

(20) K. W. Brammer, C. R. McDonald, and M. S. Tute, *Nature*, **219**, 515 (1968).

(21) E. Campaigne and R. Cline, *J. Org. Chem.*, **21**, 32 (1956).

(22) The authors are indebted to the Virus Chemotherapy Group, Parke, Davis and Company for these assays.

The basic class of inhibitors consisted of two chemical types. The first of these types consisted of 2-(*o*-aminophenyl)benzimidazole analogs which are listed in Table III. The other class consisted of 1-(*o*-aminophenyl)-3,4-dihydroisoquinolines which are listed in Table V.



None of the basic type inhibitors were found active in the viral plaque reduction test. Other types of imidazoles are listed in Table IV.

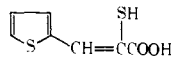
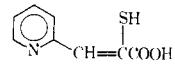
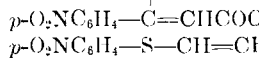
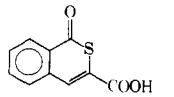
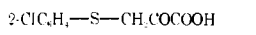
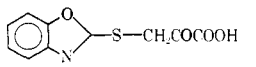
Two enzymic assay methods were used. The procedure used for routine screening utilized a bacteria enzyme from *Clostridium perfringens* and human α_1 glycoprotein as the substrate. Enzyme studies were conducted at pH 4.5 which is optimal for this enzyme and the liberated sialic acid determined colorimetrically.[23] The second method employed influenza virus (Strain B mass/3/66) as the source of enzyme and the aforementioned glycoprotein as substrate. Incubations were done at pH 6.5 and liberated sialic acid determined as above. In most instances, good correlation in enzyme inhibitory power was observed with the two methods.

Inhibition Results.—The α-mercaptoacrylic acid analogs represent some of the most active enzyme inhibitors found to date. The 50% inhibitory end point (I_{50}) of the most active analog **4** was in the range of 0.14 μmol/ml. Structural requirements for optimal activity include the presence of an acid function, a potential α-thio ketone and an aromatic or conjugated substituent in the β position. Electronegative sub-

(23) D. Aminoff, *Biochem. J.*, **81**, 384 (1961).

TABLE II

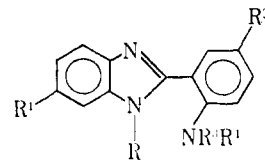
INHIBITION OF BACTERIAL NEURAMINIDASE BY SULFUR-CONTAINING ACIDS

No.	Structure	Ref	Mp, °C	Formula	Analyses	% inhibition, 100 μg/ml	I_{50} μmol/ml
13		*a*	188	$C_7H_6O_2S_2$		95	0.26
14	Disulfide of 13	*b*	214–215	$C_{14}H_{10}O_4S_4$		73	0.18
15		*b*	154–155	$C_{11}H_{10}O_2S$		91	0.32
16		*c*	123–124	$C_9H_7NO_4S$	C, H, S	7	
17		*d,e*	126–129	$C_9H_7NO_4S$		19	
18		*f*	218–219	$C_9H_7NO_4S$		4	
19		*g*	262–263	$C_{10}H_6O_3S$		4	
20		*h*	140–143	$C_9H_7ClO_5S$		40	
21		*i*	180–185	$C_{10}H_7NO_4S$	CHN	0	

[a] See footnote *f*, Table I. [b] See ref 21. [c] B. M. Ferrier and N. Campbell, *Chem. Ind.*, 1089 (1958). [d] Z. Reyes and R. M. Silverstein, *J. Amer. Chem. Soc.* **80**, 6373 (1958). [e] Prepared by 1 *N* NaOH hydrolysis of Et ester (20-min reflux). [f] H. Hogeveen, *Rec. Trav. Chim. Pays-Bas*, **83**, 813 (1964). [g] D. J. Dyksman and G. T. Newbold, *J. Chem. Soc.*, 1213 (1951). [h] Eastman Kodak Company. [i] Prepared from mercaptan and bromopyruvic acid in DMF.

TABLE III

NEURAMONIDASE INHIBITION BY 2-(*o*-AMINOPHENYL)BENZIMIDAZOLES



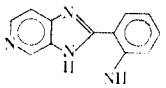
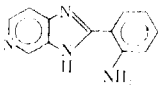
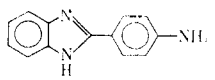
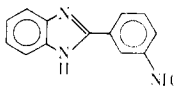
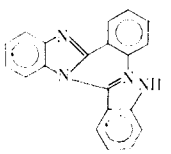
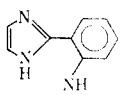
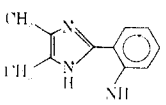
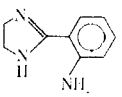
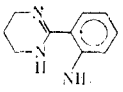
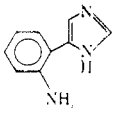
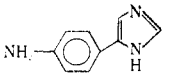
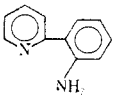
No.	R	R^1	R^2	R^3	R^4	Ref	Mp, °C	Formula	Analyses	% inhibition 100 μg/ml	I_{50} μmol/ml	% viral inhibition, 100 μg/ml
22	H	H	H	H	H	*a*	213–214	$C_{13}H_{11}N_3$		62	0.48	51
23	H	H	Cl	H	H	*b*	244–245	$C_{17}H_{10}ClN_3$		23		63
24	H	H	CH_3	H	H	*b*	230–232	$C_{14}H_{13}N_3$		41		21
25	H	CH_3	H	H	H	*b*	190–191	$C_{14}H_{13}N_3$		80	0.31	58
26	CH_3	H	H	H	H	*c*	140–141	$C_{14}H_{13}N_3$		20		16
27	C_6H_5	H	H	H	H	*d*	141–142	$C_{19}H_{15}N_3$	C, H, N	13		
28	H	CH_3O	H	H	H	*e*	143–144	$C_{14}H_{13}N_3O$		60	0.36	
29	H	CO_2H	H	H	H	*f*	260–265 dec	$C_{14}H_{11}N_3O_2 \cdot 2HCl$	C, H, N, Cl	12		
30	H	NH_2	H	H	H	*f*	225–226	$C_{13}H_{12}N_4$	C, H, N	58	0.40	10
31	H	H	H	CH_3	H	*d*	208–209	$C_{14}H_{13}N_3$	C, H, N	1		
32	H	H	H	CH_3	CH_3	*d*	230–231	$C_{15}H_{15}N_3$	C, H, N	0		

[a] See ref 30. [b] See ref 31. [c] F. Montanari and R. Passerinai, *Boll. Sci. Fac. Chim. Ind. Univ. Bologna*, **11**, 42 (1953); *Chem. Abstr.*, **48**, 6436*g* (1954). [d] Prepared by method A under benzimidazoles. [e] A. L. L. Poot, J. F. Williams, and F. C. Hengebaert, *Bull. Soc. Chim. Belg.* **72**, 365 (1963). [f] Prepared by method B under benzimidazoles—see Experimental Section.

stitution in the *meta* and *para* positions of the aromatic moiety enhanced the potency. Basic substituents in the phenyl nucleus or aromatic bases in the β position destroyed the activity. In the *p*-nitrophenyl series of acids introduction of S into positions other than α lowered the inhibitory effect. Poor activity was also noted with substituted β-thiopyruvic acids **20–21**. Oxidation of the α-mercapto to its disulfide analog did not alter the activity appreciably (**12**, **14**). These compounds were all less inhibitory when assayed in 0.1 *M* phosphate buffer, pH 7.0 than at 4.5 suggesting a dependency of acid strength with activity.

Activity prerequisites for the basic type inhibitors appears to be an *o*-aminophenyl moiety removed by one carbon from a basic N heteroatom. Substitutions on the primary amine by acylation or alkylation destroyed activity. Amino groups in the *meta* or *para* positions also abolished activity. In the benzimidazole

TABLE IV
NEURAMINIDASE INHIBITION BY SUBSTITUTED IMIDAZOLES

No.	Structure	Ref	Mp, °C	Formula	Analyses	% inhibition, 100 μg/ml	% viral inhibition, 100 μg/ml
33		*a*	208–209	$C_{12}H_{10}N_4$	C, H, N	38	10
34		*b*	321–323	$C_{12}H_{10}N_4$		33	17
35		*c*	246–247	$C_{13}H_{11}N_3$		0	
36		*c*	265–267	$C_{13}H_{11}N_3$		0	
37		*d*	270–271	$C_{20}H_{14}N_4$		0	
38		*e*	134–135	$C_9H_9N_3$		43	
39		*a*	175–176	$C_{11}H_{13}N_3 \cdot 0.5H_2O$	C, H,[b] N	42	
40		*g*	253–255	$C_9H_{11}N_3 \cdot 2HCl \cdot H_2O$	C, H, N	3	
41		*a*	193–199	$C_{10}H_{13}N_3 \cdot 2HCl$	C, H, N, Cl	0	
42		*e*	130–131	$C_9H_9N_3$		18	20
43		*e*	72–74	$C_9H_9N_3 \cdot H_2O$		4	
44		*h*	bp 119–120 (0.3 mm)	$C_{11}H_{10}N_2$		30	36

[a] See Experimental Section. [b] D. L. Garmaise and J. Komlossy, *J. Org. Chem.*, **29**, 3403 (1964). [c] B. Miklaszewski and St. von Niementowsky, *Ber*, **34**, 2959 (1901). [d] M. Davis and F. G. Mann, *J. Chem. Soc.*, 945 (1925). [e] I. E. Balaban and H. King, *ibid.*, 2701 (1925). [f] H: calcd, 7.19; found, 6.72. [g] Obtained from nitro compound[e] by catalytic reduction. [h] P. Oxley and W. F. Short, *ibid.*, 497 (1947); J. W. Haworth, I. M. Heilbron, and D. H. Hey, *ibid.*, 349 (1940).

series lowering of resonance energy by substitution on the imidazole N reduced activity. The presence of electron rich or donating groups in the phenyl ring had little effect. On the other hand a halogen *para* to the primary amine function increased inhibitory potency toward the viral enzyme and had the reverse effect on the enzyme of bacterial origin. Substituted imidazoles **38**, **39**, **42** were not as effective as benzimidazoles and their dihydro analogs **40**, **41** were inert. Other ring systems such as oxazoles and thiazoles, **66**, **67**, were inactive.

The same skeletal requirements for activity were observed in the 3,4-dihydroisoquinoline series. Substituting on the primary arylamine or increasing the active skeletal chain III by one C (**68**) destroyed the activity. Substitutions in the arylamine ring lowered the inhibitory effect while full saturation or aromatization of the N containing ring resulted in complete inactivation. It was interesting to note that only one **49** of the substituted dihydroisoquinolines was more active than the parent base **45**. For comparative purposes the *p*-methoxyphenoxy[20] analog **60** was included

TABLE V

NEURAMINIDASE INHIBITION BY SUBSTITUTED 1-(*o*-AMINOPHENYL)-3,4-DIHYDROISOQUINOLINES[a]

No.	R	R_1	R_2	R_3	R_4	R_5	R_6	Ref	Mp, °C	Formula	Analyses	% inhibition 100 μg/ml	μmol/ml	% viral inhibition, 100 μg/ml
45	H	H	H	H	H	H	H	*a*	97–98	$C_{15}H_{14}N_2$		66	0.32	40
46			CH_3O					*b*	118–119	$C_{16}H_{16}N_2O$	C, H, N	66	0.35	42
47		CH_3O	CH_3O						149–150	$C_{17}H_{18}N_2O_2$	C,[c] H, N	23		30
48			CH_3						110–112	$C_{16}H_{16}N_2$	C, H, N	59	0.36	7
49	(*dl*)± CH_3								210–212	$C_{16}H_{16}N_2 \cdot 2HCl$	C, H, N, Cl	64	0.23	44
50			CH_3O		CH_3				180–183	$C_{17}H_{18}N_2O \cdot 2HCl$	C,[d] H, N	45		27
51					CH_3				238–242	$C_{16}H_{16}N_2 \cdot 2HCl$	C, H, N, Cl	49	0.32	20
52					CH_2O				180–190 dec	$C_{16}H_{16}N_2O \cdot 2HCl$	C, H, N, Cl	12		8
53				CH_3					255–257	$C_{16}H_{16}N_2 \cdot HCl$	C, H, N, Cl	8		6
54						CH_3			187–189	$C_{16}H_{16}N_2 \cdot HCl \cdot H_2O$	C, H,[e] N, Cl	2		6
55			Cl						100–101	$C_{15}H_{15}ClN_2$	C, H, N, Cl	52	0.39	13
56					CH_3			*a*	75–77	$C_{16}H_{18}N_2$	C, H, N	9		
57					$CH_3C{=}O$			*b*	123	$C_{17}H_{16}N_2O \cdot 0.5H_2O$	C, H, N	0		

[a] H. Ott, G. E. Hardtmann, M. Denzer, A. J. Frey, J. H. Gogerty, G. H. Leslie, and J. H. Trapold, *J. Med. Chem.*, **11**, 777 (1968). [b] See Experimental Section. [c] Calcd: C, 72.32. Found: C, 71.83. [d] Calcd: C, 60.18. Found: C, 59.70. [e] Calcd: H, 6.59. Found H, 6.00. [f] Prepared by N-acetylation of **45** with pyridine–Ac_2O. [g] Structure given in text.

Figure 1.—Effect of inhibitors on neuraminidase from *Cl. perfringens* at pH 4.5. Enzyme was preincubated 5 min either in the absence of inhibitors (●); in the presence of compound **22** at 0.43 m*M* (×); with compound **45** at 0.31 m*M* (○); or with compound **4** at 0.13 m*M* (▲). Incubation period was 10 min at 37°. Human α_1 glycoprotein concentration was varied from 0.2 to 1.0 mg/ml while enzyme concentration was maintained at 0.0056 unit/ml.

and showed very weak inhibitory properties in our system.

The structural requirements for enzyme inhibition appear to be rather restrictive for basic type molecules. For example, certain triazoles, oxadiazoles, and triazoliums (sydnones) having an *o*-aminophenyl group in similar orientation to the basic heteroatom were inactive. It is possible that these heterocyclic systems should be fused to a planar aromatic ring for demonstrable activity. One may therefore envisage an orientation of C and N atoms as depicted in III which

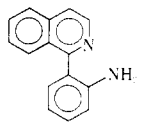
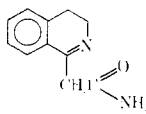
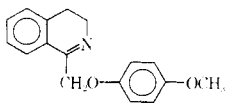
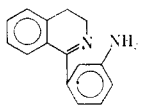
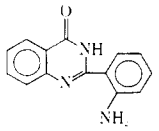
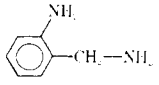
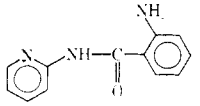
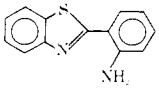
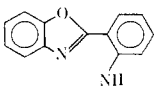
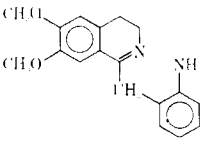
III

may complex with the neuraminidase enzyme at or near the active site. A fused ring system may be desirable to ensure adsorption to the enzyme surface and could explain the superior inhibiting properties of the benzimidazoles over the imidazoles, triazoles, etc. Metal chelating agents such as EDTA, citric and ascorbic acids showed no inhibitory action on the bacterial enzyme system.

The effect of **4**, **22**, and **45** on the initial reaction velocity and glycoprotein substrate concentration was examined with the *Cl. perfringens* enzyme at pH 4.5. A Lineweaver and Burk reciprocal plot[24] of the enzyme–substrate reaction with and without inhibitor were nonlinear. However substitution of the square of the substrate concentration in the reciprocal plots yielded essentially linear relationships as illustrated in Figure 1.

(24) H. Lineweaver and D. Burk, *J. Amer. Chem. Soc.*, **56**, 658 (1934).

TABLE VI
NEURAMINIDASE INHIBITION BY MISCELLANEOUS BASES

No.	Structure	Ref	Mp, °C	Formula	Analyses	% inhibition, 100 μg/ml	% viral inhibition, 100 μg/ml
58		*a*	153–154	$C_{15}H_{12}N_2$	C, H, N	4	
59		*b*	198–200	$C_{11}H_{12}N_2O$		0	13
60		*c*	173–185	$C_{17}H_{17}NO_2 \cdot HCl$		14	0
61		*d*	240–255 dec	$C_{15}H_{14}N_2 \cdot 2HCl$		0	0
62		*e*	245–246	$C_{14}H_{11}N_3O$		29	37
63	1-Adamantanamine	*f*	108–109	$C_{10}H_{17}N$		14	5
64			214–218	$C_7H_{10}N_2 \cdot 2HCl$		0	
65		*g*	132–133	$C_{12}H_{11}N_3O$		0	
66		*h*	213–214	$C_{13}H_{10}N_2S$		4	
67		*i*	105	$C_{13}H_{10}N_2O$	C,[j] H, N	3	
68		*k*	163–164	$C_{18}H_{20}N_2O_2$	C, H, N	0	

[a] See Experimental Section. [b] E. Grumberg and H. N. Prince, *Proc. Soc. Exp. Biol. Med.* **129**, 422 (1968). [c] See ref 20. [d] V. M. Rodionov and E. V. Yavorskaya, *J. Gen. Chem. (USSR)*, **13**, 491 (1943). [e] K. Butler and M. W. Partridge, *J. Chem. Soc.*, 2396 (1959). [f] Purchased from Aldrich Chemicals. [g] P. A. Petyunin and Y. V. Kozhevnikov, *J. Gen. Chem. (USSR)* **30**, 2333 (1960). [h] See ref 30. [i] Prepared by the polyphosphoric acid method given in the Experimental Section. [j] Calcd: C, 74.27. Found: C, 73.77. [k] Prepared by Raney Ni reduction of the corresponding nitro compound [J. M. Gulland and R. D. Haworth, *J. Chem. Soc.*, 581 (1928)].

The characteristics of the linear reciprocal plots indicated that **22** and **45** inhibited neuraminidase competitively. The K_m of the uninhibited reaction occurred at a substrate concentration of about 0.37 mg/ml. The plot obtained with **4** failed to intercept the $1/v$ axis at high substrate concentration. An inhibition analysis as described by Ackermann and Potter[25] plotting the reaction velocity *vs.* enzyme concentration is shown in Figure 2. A concave upward curve at low enzyme concentration indicated reversible inhibition. In the absence of inhibitor a slower reaction rate at higher enzyme concentrations suggested product inhibition. Extrapolation of the linear portion of the curve of the inhibited reaction to the abscissa gave the amount of enzyme bound to the inhibitor which calculated to be 18 μmoles of **4** per unit of neuraminidase.

The chemotherapeutic effect of the most potent enzyme inhibitors of each class proved to be disappointing in influenza viral challenge experiments in mice. Compound **4**, the most active inhibitor of the acidic class, failed to protect mice at the 500 mg/kg level when administered by the oral and ip route using a PR-8/A challenge.[26] Similar results were obtained with

(25) W. W. Ackermann and V. R. Potter, *Proc. Soc. Exp. Biol. Med.*, **72**, 1 (1949).

(26) The authors are indebted to Dr. Frank Brandon for these assays.

22, **25**, **30**, **45**, **49**, and **62**. Compounds **45** and **46** when premixed with Jap 305 strain of influenza virus and administered to mice intranasally showed a slight increase in survival time over virus treated controls.[27] No increase in the number of survivors was observed. Kendal[28] has recently classified the neuraminidase enzymes of 23 virus strains into 5 kinetic and 10 antigenic groups. He postulated that the molecular configurations forming the enzyme active site and the antigenic sites of these viral enzymes were different. The possibility exists that these enzyme inhibitors might elicit *in vivo* activity against other viral strains than those described herein. However, in view of the success enjoyed by vaccines, the primary target for influenza chemotherapy should be broad activity against the majority of prevalent strains.

Experimental Section

Enzyme Assay. Materials.—Purified neuraminidase from *C. perfringens* was obtained from Worthington Biochemical Corp. Human α_1 glycoprotein was obtained from Pentex Co. This preparation was made up to a concentration of 2 mg/ml and adjusted to pH 11 with alkali for 15 min to cleave *o*-acetyl residues. The solution was then adjusted to the proper pH for test purposes. Purified influenza virus strain B mass/3/66 was obtained from Dept. of Microbiology, Parke, Davis and Company and contained 7900 CCA units/ml.

Bacterial Enzyme Test.—Test compounds were incubated at a concentration of 100 μg/ml at 37° with 0.0028 unit of neuraminidase and 200 μg of substrate in a total volume of 0.5 ml of 0.1 *M* KOAc buffer pH 4.5. After 2-hr incubation the enzyme reaction was terminated by the addition of periodic acid (30 min at 37°) followed by the determination of free neuraminic acid released from the glycoprotein substrate by the thiobarbituric acid procedure.[23] Color readings of the acid *n*-BuOH extracts at 549 mμ were corrected for substrate and sample color by appropriate blanks. The values expressed as micrograms of free neuraminic acid released per 0.5 ml of enzyme reaction mixture compared with 99% pure synthetic neuraminic acid were corrected to percentage inhibition with respect to color controls. Compounds which inhibited the enzyme reaction >50% were tested at lower concentrations to determine by a simple plot the concentration at 50% inhibition (I_{50}).

Viral Enzyme Test.—Incubation mixtures contained 200 μg of glycoprotein, 100 μg of test substance, 8 CCA units of influenza virus, 20 μl of 0.01 *M* $CaCl_2$ in 0.01 *M* Tris-maleate buffer, pH 6.5, in a total vol of 0.4 ml. Incubations were conducted at 37° for 2 hr and the liberated neuraminic acid determined as above. Control tubes for each inhibitor were included by adding 100 μl of test substance after the 2-hr incubation. Tubes containing enzyme and substrate were run with each set of analyses to test enzyme potency.

Chemistry.[29] **α-Mercapto β-Substituted Acrylic Acids.**—Acids were prepared by alkaline hydrolysis of the corresponding rhodanine[21] and purified either by recrystn or pptn from $NaHCO_3$ solutions.

α-Mercapto-β-(*p*-nitrophenyl)acrylic Acid (4).—A solution of 12.8 g (0.045 mol) of 5-(*p*-nitrobenzylidene)rhodanine in 600 ml of 0.33 *N* NaOH was heated at 70° for 20 min on a steam bath. The solution was cooled in ice and acidified with 6 *N* HCl. After stirring at 0° for 1 hr, the mixture was filtered and washed with cold H_2O. The solid was dissolved in 360 ml of H_2O containing 0.045 mol of $NaHCO_3$ and clarified by centrifugation and filtration through Celite. The filtrate was added dropwise to 60 ml of 2 *N* HCl and the brown solid was filtered, washed with H_2O, and dried *in vacuo*; yield 7.5 g (74%): $\lambda_{max}^{0.1\ N\ NaOH}$ 281 mμ (ϵ 9100).

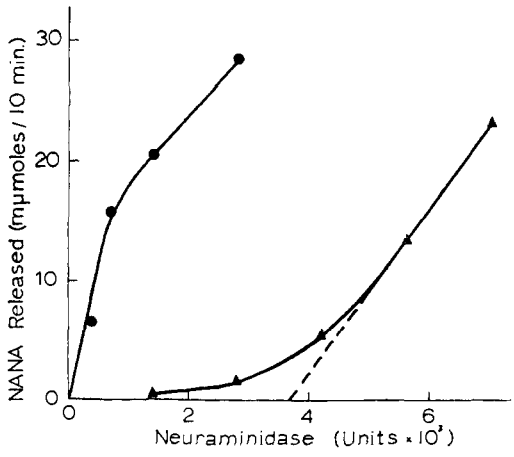


Figure 2.—Effect of acidic type inhibitor on neuraminidase at pH 4.5. Enzyme was preincubated for 5 min either in the absence of inhibitor (●) or in the presence of compound **4** at 0.13 m*M* (▲). Substrate concentration was maintained at 0.4 mg/ml.

Benzimidazoles. A. Polyphosphoric Acid Method.[30]—Equimolar quantities of diamine and anthranilic acid were stirred in polyphosphoric acid at 250–260° for 2–4 hr.

B. *o*-Nitrobenzaldehyde Method.[31]—The diamine and *o*-nitrobenzaldehyde were allowed to react at room temperature in AcOH and then refluxed for 1.5 hr. Alternatively, the intermediate imine was preformed in EtOH solution and then cyclized in refluxing $PhNO_2$.

2-(*o*-Aminophenyl)-1*H*-imidazo[4,5-*c*]pyridine (33).—A solution of 3 g (0.0275 mol) of 3,4-diaminopyridine and 3.77 g (0.0275 mol) of anthranilic acid in 40 g of polyphosphoric acid was stirred at 250° for 2 hr. The cooled solution was poured into 500 ml of H_2O and the solid collected by filtration. The solid was stirred with 100 ml of 10% Na_2CO_3 solution, filtered, and washed with H_2O, affording 3.2 g of crude product. The solid was digested with hot $CHCl_3$ and filtered and the insoluble product was recrystd (EtOH–H_2O): yield 2.6 g (45%), mp 123–130°, resolidified and melted at 208–209°.

2-(*o*-Aminophenyl)-5-benzimidazolecarboxylic Acid (29).—A solution of 7.0 g (0.046 mol) of 3,4-diaminobenzoic acid and 7.0 g (0.046 mol) of *o*-nitrobenzaldehyde in 150 ml of AcOH was refluxed 1 hr, evaporated *in vacuo* to 50 ml, and poured into H_2O. The solid was filtered and dissolved in 500 ml of dil $NaHCO_3$ solution, decolorized with Darco, acidified, and filtered. The dried solid (5.15 g) was dissolved in 300 ml of AcOH and hydrogenated at atmospheric pressure with 0.5 g of 20% Pd–C for 2 hr. The catalyst was filtered and the filtrate concd to dryness *in vacuo*. The residue was dissolved in hot 6 *N* HCl and decolorized with Darco affording off-white crystals, yield 0.5 g (3.3%) mp 260–265° dec.

2-(*o*-Aminophenyl)-4,5-dimethylimidazole (39).—A solution of 4.3 g (0.050 mol) of 2,3-butanedione, 7.5 g (0.050 mol) of *o*-nitrobenzaldehyde, and 20 g of NH_4OAc in 7 ml of AcOH was refluxed 1 hr and diluted to 600 ml with H_2O. The solution was made alkaline with NH_4OH and a small amount of solid removed by filtration. The filtrate was extracted with EtOAc, back extracted into 2 *N* H_2SO_4 which was then made alkaline with K_2CO_3, and extracted with EtOAc. The dried solution was evapd; yield 3.2 g (30%), mp 182–184°. The nitro product was dissolved in MeOH and hydrogenated with 1.5 g of 10% Pd–C for 3.5 hr at atmospheric pressure. Filtration and evaporation afforded 1.77 g (55%), recrystd (MeOH–H_2O); mp 175–176°.

2-(*o*-Aminophenyl)-1,4,5,6-tetrahydropyrimidine (41).—The monotosylate salt of 1,3-diaminopropane was prepared from 3.7 g of amine (0.050 mol) and 9.5 g of toluenesulfonic acid·H_2O in EtOH. After evaporation to dryness, 7.4 g (0.050 mol) of *o*-nitrobenzonitrile was added and the mixture slurried in hot C_6H_6 and evapd to dryness. The residue was refluxed at 155–160° for 1.5 hr. The cooled solid was dissolved in 100 ml H_2O and decolorized with Darco. After removal of unreacted nitrile by filtration and $CHCl_3$ extraction, the aq layer was made alkaline with NaOH and extracted with $CHCl_3$. After drying and re-

(27) The authors are indebted to Dr. Robert Sidwell, Southern Research Institute, Birmingham, Ala., for these assays.

(28) A. P. Kendal and C. R. Madeley, *Biochim. Biophys. Acta*, **185**, 163 (1969).

(29) Melting points (uncorrected) were taken on a Thomas-Hoover capillary apparatus.

(30) D. W. Hein, R. J. Alheim, and J. J. Leavitt, *J. Amer. Chem. Soc.*, **79**, 427 (1957).

(31) L. L. Zaika and M. M. Joullié, *J. Heterocycl. Chem.*, **3**, 289 (1966).

moval of solvent the solid was recryst (C_6H_6–hexane); yield 4.2 g (41%), mp 151–152°. Hydrogenation of 1.0 g with 20% Pd–C gave the amino compound isolated as the HCl salt from EtOH–Et_2O, mp 193–199°; yield 0.83 g (69%).

3,4-Dihydroisoquinolines.—The amino-substituted dihydroisoquinolines were prepared by a slight modification of the Bischler–Napieralski reaction[32] using the corresponding *N*-phenethylnitrobenzamides followed by hydrogenation. Noncrystalline bases were converted into crystalline HCl salts.

7-Methoxy-1-(*o*-aminophenyl)-3,4-dihydroisoquinoline (46).—To a solution of 18 g (0.060 mol) of *N*-(*p*-methoxyphenethyl)-*o*-nitrobenzamide in 60 ml of $(CHCl_2)_2$ and 40 ml $POCl_3$ was added 14 g of P_2O_5 with rapid stirring. The solution was stirred under reflux 1 hr and most of the solvent removed *in vacuo*. The residue was poured into ice–H_2O and extracted 3 times with Et_2O. The aq layer, after filtration, was made alkaline with NaOH and extracted with CH_2Cl_2. After drying and evaporation *in vacuo* the nitro compound was obtained as a yellow gum (13.5 g). The product was dissolved in MeOH and hydrogenated with 0.5 g of Raney Ni at 3.5 kg/cm². Filtration and concentration afforded 8.7 g (58%) of product, mp 118–119° (MeOH–H_2O).

1-(*o*-Aminophenyl)isoquinoline (58).—A suspension of 5.3 g (0.024 mol) of 1-(*o*-aminophenyl)-3,4-dihydroisoquinoline and 0.3 g of 20% Pd–C in 50 ml of decalin was stirred at reflux for 20 hr. The cooled solution was diluted with C_6H_6, filtered, and concd *in vacuo*. The solid was filtered and recrystd twice (C_6H_6–hexane): yield 2.5 g (47%); mp 153–154°; λ_{max}^{MeOH} 322 mμ (ε 5100), 310 (4530), 283 (6500), 273 (6820), and 22 (66700).

(32) *Org. React.*, **6**, 74 (1951).

Acknowledgment.—The authors wish to express their appreciation to Dr. Frank Brandon for aid in the viral assays, Dr. J. M. Vandenbelt for spectral analyses, Mr. C. E. Childs for microanalyses, and Mr. W. Pearlman for hydrogenations.

Synthesis and Pharmacological Evaluation of Some Pyridylmethyl Substituted Ethylenediamines

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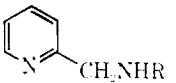
A series of new 2-pyridylmethyl (picolyl) substituted ethylenediamines has been prepared. Comparative pharmacological screening of the series included tests for antihistaminic and antispasmodic activity. *N,N*-Dimethyl-*N'*-phenyl-*N'*-(2-picolyl)ethylenediamine was the most active antihistaminic and had about one-third the activity of tripelennamine. *N*-(2-Picolyl)-*N*-phenyl-*N*-(2-piperidinoethyl)amine was the most active antitussive compound, being somewhat more potent in this respect than codeine. Comparison of structure–activity relationships in the series supports the hypothesis of Kasé and Yuizono that a piperidino group in the molecule enhances antitussive activity.

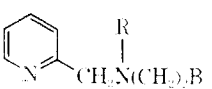
The ready preparation[1,2] of a number of *N*-(2-picolyl)anilines (I) and *N*-(2-picolyl)-2-aminopyridines (II) in our laboratory prompted us to attempt the preparation of a series of new ethylenediamine-type antihistaminic with a 2-picolyl group attached to N and to ascertain whether the introduction of a 2-picolyl group may provide desirable pharmacological activities.



I, R = phenyl, substituted phenyl
II, R = 2-pyridyl

The favorable effect of the replacement of benzyl by a thenyl group in the ethylenediamine series has been reported by previous workers.[3–5] However, no synthesis of the isosteres in which a 2-picolyl group is introduced in place of benzyl has been reported.

In this paper the synthesis of a series of 2-picolyl substituted ethylenediamines which are represented as a general formula (III) in which R is phenyl, substituted phenyl, or 2-pyridyl, and B is Me_2N, Et_2N, pyrrolidino, or piperidino, and their pharmacological screening results are recorded. Compound **1** (Table I) is an isomer of the known antihistamine, tripelennamine, since the position of two groups, phenyl and 2-pyridyl, are reversed.



III

The syntheses of these antihistaminic tertiary amines were carried out by alkylating the *N*-picolyl secondary amines with dialkylaminoethyl chloride. The condensation was conducted by refluxing the reactants in toluene in the presence of $NaNH_2$. The intermediate secondary amines were prepared according to the general procedure we recently reported.[1,2] One *N*-(4-picolyl) analog **10** was also prepared.

Pharmacological screening included studies of acute toxicity, antihistaminic, antispasmodic, and antitussive activities.

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