Notes

Chem. Pharm. Bull. 22(10)2444—2447(1974)

UDC 615.28.011.5 : 547.574.3.057.09

Antiviral Compounds. IV.[1] Synthesis and Anti-influenza Virus Activity of Amidinohydrazones

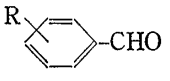
Tamio Nishimura, Chiji Yamazaki, Hiroshige Toku, Shin Yoshii, Katsushige Hasegawa, Mitsuji Saito and Daizo Nagaki

School of Hygienic Sciences, Kitasato University[2]

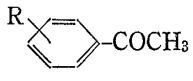
(Received December 11, 1973)

In our laboratory, a systematic investigation has been made of the inhibitory activities of thiosemicarbazones from benzaldehydes (BA), acetophenones (AP), cinnamaldehydes and benzalacetones (BZ) against the multiplication of influenza virus in the membrane culture.[3] As these thiosemicarbazones are, in general, difficultly soluble in water, these carbonyl compounds were converted into water-soluble amidinohydrazone acid salts with the expectation of the preservation of the antiviral activity. Although there is no report concerning the antiviral activity of amidinohydrazones, guanidine which constitutes a part of amidinohydrazones has been known to be active against polio,[4] measles,[5] and coxsakie[6] viruses. Development of 1,1-anhydrobis(2-hydroxyethyl)biguanide (ABOB)[7] that is clinically effective, stimulated us to introduce 1,1-anhydrobis(2-hydroxyethyl) moiety of ABOB into the amidine part of the amidinohydrazone.

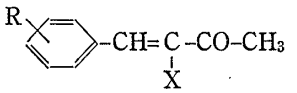
In this paper, amidinohydrazones (AH), 1,1-anhydrobis(2-hydroxyethyl)amidinohydrazones (MH) and 1,1-tetramethyleneamidinohydrazones (TH) of BA, AP and BZ series shown below were prepared and tested for their antiviral activity *in vitro*.



BA



AP



	BZ	M-BZ	C-BZ	B-BZ
X:	H	CH_3	Cl	Br

	0	1	2	3	4	5
R:	H	*p*-NO_2	*m*-NO_2	*p*-Cl	*p*-CH_3CONH	*p*-$(CH_3)_2N$

The antiviral activity of amidinohydrazones were preliminarily tested by method B at one concentration (100 or 50 mcg/ml). Benzalacetones have previously been shown to be slightly active,[8] but the three aminoguanidine were all inactive. Most of amidinohydrazones of BA, AP and BZ were active except those having *p*-acetylamino group. The antiviral activity was remarkably reduced by replacement of morpholino or pyrrolidino group for NH_2 of the amidine moiety. The positive result in method B means that an active

1) Part III: K. Hasegawa, F. Taguchi, D. Nagaki, C. Yamazaki, H. Sakaguchi and T. Nishimura, *Kitasato Arch. of Exp. Med.*, **46**, Nos. 3—4, 31 (1973).
2) Location: *Asamizodai, Sagamihara-shi.*
3) K. Iwasaki, T. Nishimura, Y. Igarashi and D. Nagaki, *Kitasato Arch. of Exp. Med.*, **28**, 31 (1955).
4) T. Ueda, S. Toyoshima, T. Tsuji, Y. Seto and J. Nomoto, *Keio J. Med.*, **10**, 257 (1961).
5) T. Ueda, S. Toyoshima, T. Tsuji, Y. Seto and J. Nomoto, *Antibiot. Chemotherapy*, **12**, 330 (1962).
6) I. Tamm and H.J. Eggers, *Virology*, **18**, 439 (1962).
7) B. Melander, *Antibiot. Chemotherapy*, **10**, 34 (1960).
8) T. Nishimura, F. Taguchi, M. Saito, K. Hasegawa and D. Nagaki, *Kitasato Arch. of Exp. Med.*, **45**, 105 (1972).

TABLE I. Antiviral Potencies of Amidinohydrazones by Method A and B

Notation	Antiviral activity (γ/ml) Method A	Method B	Toxicity (γ/ml) to CAM	Index A[a)]	Index B[a)]
BA-AH					
1	100	50	>100	>1	>2
2	100	100	>100	>1	>1
3	50	50	100	2	2
5	25	25	100	4	4
AP-AH					
1	50	25	>100	>2	>4
2	100	100	100	1	1
3	100	50	100	1	2
BZ-AH					
1	50	50	50	1	1
2	25	25	25	1	1
3	25	25	25	1	1
M-BZ-AH					
0	12.5	12.5	12.5	1	1
1	50	25	100	2	4
2	50	25	25	0.5	1
3	25	12.5	12.5	0.5	1
4	100	50	100	1	2
5	100	100	>100	>1	>1
C-BZ-AH					
0	100	50	50	0.5	1
1	50	50	50	1	1
2	25	25	25	1	1
3	25	25	25	1	1
4	100	25	50	0.5	2
5	50	25	50	1	2
B-BZ-AH					
0	50	50	50	1	1
1	50	25	50	1	2
2	50	25	50	1	2
3	50	25	25	0.5	1
4	100	50	100	1	2
BZ-MH					
1	50	50	>100	>2	>2
3	100	100	>100	>1	>1
M-BZ-MH					
1	100	50	>100	>1	>2
3	50	50	100	2	2
B-BZ-MH					
4	>100	100	>100		≧1
BA-TH					
1	100	100	100	1	1
M-BZ-TH					
0	100	100	100	1	1

a) index A: toxicity(γ/ml) to CAM/antiviral activity(γ/ml) by method A
index B: toxicity(γ/ml) to CAM/antiviral activity(γ/ml) by method B

compound may be inhibitory against the virus or toxic to chorio-allantoic membranes (CAM) at a concentration indicated or be virucidal at 5 times higher concentration.

The active compounds selected by the above screening test were further examined by methods A and B at final concentrations of 100, 50, 25 and 12.5 mcg/ml (Table I). The result of the toxicity test of the active compounds to CAM is also shown in Table I. Compounds which showed $\geqq 4$ of inhibitory index (index A) or virucidal index (index B) expressed by the ratio of toxicity to antiviral activity were BA-AH-5 in method A, and BA-AH-5, AP-AH-1 and M-BZ-AH-1 in method B. Therefore, BA-AH-5 is inhibitory at a concentration of 25 mcg/ml, and AP-AH-1 and M-BZ-AH-1 are virucidal at a concentration of 125 mcg/ml. However, the indices of these active compounds are not high enough to suggest further investigation.

Experimental[9]

Materials and Methods

Infectivity Titration——Infected allantoic fluid of 80th and 81st egg-passages of influenza A_2 virus Adachi strain was ampuled in an amount of 1 ml each and stored in a dry-ice box until use. One-tenth ml of serial ten-fold dilution of the stock virus was inoculated into a culture tube (14×120 mm) containing a pice (5×10 mm) of CAM and a pice (5×5 mm) of egg-shell, and 0.9 ml of Hanks' balanced salt solution (Hanks' BSS, pH 7.2—7.4). After shaking the cultures of 4 tubes per each dilution for 48 hr at 36°, hemagglutination (HA) titer of each culture fluid was determined by the HA test. The maximal dilution showing positive HA in all 4 tubes was detected. The exact titer of the stock virus was determined by further serial dilution between this maximal dilution and the next higher dilution. The maximal dilution thus determined is named one MID_{100} (100% membrane infective dose).

Hemagglutination Test——Two-fold serial dilution of the culture fluid were made with phosphate buffer saline (PBS) on a plastic plate. Two-tenths ml of 0.5% chiken red cell suspension was added to 0.2 ml each of the dilution and the mixture was allowed to hemagglutinate at room temperature for 60 min.

Inhibitory Test (Method A)——One-tenth ml of the virus suspension of 10 MID_{100}/0.1 ml was inoculated into the culture tube (0.8 ml of Hanks' BSS and 0.1 ml of a sample solution in distilled water or 50% glycerol). The tube was rubber-stoppered and incubated on a shaking machine (stroke length 120 mm, 110 strokes per min) for 48 hr at 36°. The minimum concentration of a compound at which all 4 tubes showed negative HA was taken as the minimum inhibitory concentration.

Virucidal Test (Method B)——The procedure is the same as that of method A, except that a mixture of 0.5 ml of a sample solution and 0.5 ml of the virus suspension was allowed to stand at room temperature for 30 min and 0.2 ml of the mixture was inoculated.

Toxicity Test in Chorio-allantoic Membranes——A piece of CAM was incubated in 0.9 ml of Hanks' BSS with 0.1 ml of an appropriately diluted compound solution for 18 hr at 36° on a shaking machine. The incubated membrane was washed twice with 5 ml of PBS, and used to cultivate the virus in 1 ml of Hanks' BSS. After 48 hr incubation at 36°, the hemagglutinin titer of the culture fluid was determined by the HA test. The maximal concentration of the compound at which the same degree of hemagglutinin production as that of the control was observed, was taken as the maximal non-toxic concentration.

Preparation of Chemicals

Benzaldehydes (BA-0—5) and acetophenones (AP-0—4) were purchased. *p*-Dimethylaminoacetophenone (AP-5),[10] unsubstituted (BZ-0),[11] *p*-nitro (BZ-1)-,[12] *p*-chloro (BZ-3)-,[13] *p*-acetylamino (BZ-4)-,[12] and *p*-dimethylamino (BZ-5)-[13] benzalacetones, and unsubstituted (M-BZ-0)-[14] and *m*-nitro (M-BZ-2)-[14] α-methylbenzalacetones, unsubstituted (B-BZ-0)-[15] and *p*-nitro (B-BZ-1)-[12] α-bromobenzalacetones were prepared according to the known methods.

***m*-Nitrobenzalacetone (BZ-2)**——To an ice-cooled mixture of 1.5 g (0.01 mole) of *m*-nitrobenzaldehyde, 12 ml acetone and 12 ml of water were added 6 drops of 10% NaOH and the mixture was stirred at room temperature for 30 min. The mixture was then neutralized with 10% HCl, extracted with ether, and the ether evaporated. To the residue were added 2 ml of 10% HCl and 3 ml of EtOH, and the mixture was

9) Melting points are uncorrected. All the new compounds gave satisfactory analyses.
10) A.W. Nineham, *J. Chem. Soc.*, **1952**, 636.
11) N.L. Drake and P. Allen, Jr., *Organic Syntheses*, Coll. Vol. I, 77 (1948).
12) T. Nishimura, *Bull. Chem. Soc. Japan*, **26**, 253 (1953).
13) T. Nishimura, A. Konishi and A. Matsumae, *Kitasato Arch. of Exp. Med.*, **30**, 51 (1957).
14) C. Harries and G.H. Mueller, *Ber.*, **35**, 966 (1902).
15) N.H. Cromwell, *J. Am. Chem. Soc.*, **65**, 305 (1943); *idem*, *Organic Syntheses*, **27**, 9 (1947).

refluxed for 1 hr to give 1.8 g (95%) of the desired product, slightly yellow needles, mp 96—98°. Recrystallization from EtOH–H_2O (5: 3) raised the mp to 97—99°.

α-Chlorobenzalacetone (C-BZ-0)——To a solution of 13.3 g (0.091 mole) of benzalacetone in 80 ml of glacial acetic acid was absorbed with ice-cooling 7.1 g (0.1 mole) of chlorine. Then, 10 g (0.094 mole) of anhydrous Na_2CO_3 was added, and the mixture was heated at 90° for 15 min. After cooling, the mixture was diluted with 200 ml of water, and separated oil was subjected to distillation under reduced pressure. bp 119—120° (6 mmHg); oxime, mp 132—133° (lit.[16] mp 132—133°). The following compounds (mp (°C), yield (%)) were similarly synthesized: C-BZ-1, 113—115 (lit.[17] 113—115°), 69; C-BZ-2, 90, 44; C-BZ-3, 88—89, 78; C-BZ-4, 207—208 (decomp.), 40; C-BZ-5, 115—116 (lit.[18] 115°), 18.

α-Bromo-*p*-chlorobenzalacetone (B-BZ-3)——*p*-Chlorobenzalacetone (7.7 g, 0.043 mole) and 7.0 g (0.44 mole) of bromine was treated in the same manner as in C-BZ-0 to yield 8.5 g of the desired product, mp 94—100°, slightly yellow prisms. Three recrystallizations from C_6H_6–*n*-hexane (1: 2) raised the mp to 99.5—100.5°. The following compounds (mp (°C), yield (%)) were obtained similarly: B-BZ-2, 76—77, 66; B-BZ-4, 218—218.5, 78; B-BZ-5, 104—105, 20.

α-Methyl-*p*-chlorobenzalacetone (M-BZ-3)——A mixture of 7.0 g (0.05 mole) of *p*-chlorobenzaldehyde, 7.0 g (0.097 mole) of butanone and 10 ml of concentrated HCl was stirred at room temperature for 7.5 hr. Separated oil was extracted with chloroform, the extract was washed with 5% $NaHCO_3$, and dried over anhydrous Na_2SO_4. The residue obtained by evaporation of the solvent was recrystallized from petroleum ether to yield the desired product, mp 50—51°, pale yellow needles, yield 44%. In the same manner, M-BZ-1, mp 94.5—95.5° (lit.[19] 94—95°), 82% and M-BZ-4, 189—191°, 24% were prepared by the reaction at 40° and room temperature, respectively. Crude M-BZ-5, mp 113—116° was obtained in 100% yield by the reaction at refluxing temperature for 20 min. Column chromatography of this product on silica gel with C_6H_6 gave yellow crystals (from ligroin), mp 122—123°. Oxidation of this product with alkaline sodium hypochlorite solution gave pale yellow needles, mp 203—204°. Mixed mp of this product with authentic α-methyl-*p*-dimethylaminocinnamic acid[20] did not show depression.

Amidinohydrazones (AH)·HCl——A mixture of a carbonyl compound, aminoguanidine bicarbonate and EtOH was acidified with concentrated hydrochloric acid and refluxed for 15—30 min. After cooling, precipitated crystals were recrystallized from 95% EtOH containing a small amount of concentrated hydrochloric acid. The following AH·HCl were obtained: carbonyl compd., mp (°C) of AH·HCl; BA-0, 178—179; BA-1, 246—247 (decomp.); BA-2, 254—255 (decomp.); BA-3, 170—172; BA-4, 259; BA-5, 233 (decomp.). AP-0, 202—203; AP-1, 280; AP-2, 230—231; AP-3, 246—247; AP-4, 271; AP-5, 229—230. BZ-0, 239; BZ-1, 270; BZ-2, 233—234; BZ-3, 226; BZ-4, 233; BZ-5, 210—211. M-BZ-0, 181—182; M-BZ-1, 244; M-BZ-2, 242—243; M-BZ-3, 213.5; M-BZ-4, 232 (decomp.); M-BZ-5, 209—210. C-BZ-0, 217.5—218; C-BZ-1, 251—252; C-BZ-2, 253; C-BZ-3, 231—232; C-BZ-4, 236 (decomp.); C-BZ-5, 175—176. B-BZ-0, 214—215; B-BZ-1, 249—250; B-BZ-2, 243—244; B-BZ-3, 219; B-BZ-4, 230; B-BZ-5, 198.

1,1-Anhydrobis(2-hydroxyethyl)amidinohydrazones(MH)·HI——They were similarly prepared from 1,1-anhydrobis(2-hydroxyethyl)-3-aminoguanidine·HI[21] and hydriodic acid (heated for 5—60 min). The following MH–HI were obtained: carbonyl compd., mp (°C) of MH·HI; BA-0, 209—212; BA-1, 221 (decomp.); BA-2, 226—229; BA-3, 230—231; BA-4, 259—260 (decomp.); BA-5, 207—209. AP-0, 197—199; AP-1, 194—195 (decomp.); AP-2, 210—211; AP-3, 214—215; AP-4, 246—247 (decomp.); AP-5, 184—185 (decomp.). BZ-0, 197 (decomp.); BZ-1, 243.5 (decomp.); BZ-2, 230; BZ-3, 222; BZ-4, 233 (decomp.); BZ-5, 183—184. M-BZ-0, 151; M-BZ-1, 197 (decomp.); M-BZ-2, 213—213.5; M-BZ-3, 164—165; M-BZ-4, 219 (decomp.); M-BZ-5, 176 (decomp.). B-BZ-0, 145—147; B-BZ-1, 196 (decomp.); B-BZ-2, 181.5 (decomp.); B-BZ-3, 169 (decomp.); B-BZ-4, 225—225.5.

1,1-Tetramethyleneamidinohydrazones(TH)·HI——They were prepared from 1,1-tetramethylene-3-aminoguanidine–HI[21] and hydriodic acid in the same manner as MH·HI. The following TH·HI were obtained: carbonyl compd., mp (°C) of TH·HI; BA-0, 276—276.5; BA-1, 266. AP-0, 263—263.5; AP-1, 233—233.5. BZ-0, 265—266; BZ-1, 213—213.5. M-BZ-0, 238—239.5.

16) G.H. Coleman and D. Craig, *J. Am. Chem. Soc.*, **50**, 1820 (1928).
17) G. Maerkl, *Chem. Ber.*, **95**, 3003 (1962).
18) K.H. Bauer and F. Werner, *Ber.*, **55**, 2494 (1922).
19) D.S. Noyce and L.R. Synder, *J. Am. Chem. Soc.*, **81**, 620 (1959).
20) W.J. Gensler and E. Berman, *J. Am. Chem. Soc.*, **80**, 4949 (1958).
21) H. Toku, I. Watanabe, S. Nakada and T. Nishimura, *Yuki Gosei Kagaku Kyokaishi*, **28**, 237 (1970).