Br. J. Pharmac. Chemother. (1967), **31**, 11–18.

STRUCTURE AND ANTIVIRAL ACTIVITY OF SUBSTITUTED POTASSIUM BENZYLAMINOTHIOMETHANESULPHONATES AND ALLIED COMPOUNDS*

BY

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(*Received August* 6, 1965)

Das, Kurup, Narasimha Rao & Ramaswamy (1957) demonstrated that the highest antibacterial activity was reached in benzyl isothiocyanate (AB-2) and its potassium bisulphite addition compound, potassium benzylaminothiomethanesulphonate (AB-3), among the aliphatic and unsubstituted aromatic isothiocyanates and their derivatives. Further work in this laboratory indicated that their antiviral activity was similarly manifested in *in ovo* tests. Two points emerged from these studies—viz. (1) the compounds could conceivably give rise to free isothiocyanates *in vivo* in order that they may exhibit antimicrobial activity ("isothiocyanate hypothesis"; Kurup, 1953; Das *et al.*, 1957; Narasimha Rao, 1965; (2) the highest titres are associated with the presence of a benzyl residue. It remained, therefore, to ascertain the effect of substitution in benzyl isothiocyanate and its bisulphite addition compound and the present work is mainly directed towards this end.

METHODS

The compounds shown in Table 1 were prepared by standard methods mentioned in the experimental part. Several compounds are described for the first time and the physical properties and analyses of the new compounds are included.

Viruses: vaccinia virus (Bangalore strain) and influenza virus PR_8 strain were used.

Chick embryos: embryonated white Leghorn hen eggs 10 or 11 days old were used.

The cultivation of viruses in chick embryos, the procedures for screening of compounds *in ovo* and *in vivo* for antiviral activity and other experimental details have been described earlier (Krishnamurthy, Nageswara Rao, Narasimha Rao & Praphulla, 1967).

Preparation of substituted potassium benzylaminothiomethanesulphonates and other compounds

1. Substituted potassium benzylaminothiomethanesulphonates. Water soluble derivatives (bisulphite addition compounds) of benzyl isothiocyanate and other isothiocyanates were prepared by refluxing

* Based on a thesis submitted by S. Ramanathan for the degree of Doctor of Philosophy of the Indian Institute of Science, Bangalore-12, India (1963), and Part II of the thesis submitted by K. V. Nageswara Rao for the D.Sc. Degree of the Andhra University, Waltair, India (1961).

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TABLE 1

COMPOUNDS TESTED FOR ANTIVIRAL ACTIVITY

AB No.	Compound
	(a) *Potassium benzylaminothiomethanesulphonates*
3	Potassium benzylaminothiomethanesulphonate
50	*o*-Methyl AB-3
51	*m*-Methyl AB-3
52	*p*-Methyl AB-3
53	*o*-Isopropyl AB-3
54	*o*-Methoxy AB-3
55	*m*-Methoxy AB-3
56	*p*-Methoxy AB-3
57	2:3-Dimethyl AB-3
58	2:4-Dimethyl AB-3
59	2:5-Dimethyl AB-3
60	*m*-Nitro AB-3.
61	Potassium α-phenylethylaminothiomethanesulphonate
62	Potassium α-phenylpropylaminothiomethanesulphonate
63	Potassium benzhydrylaminothiomethanesulphonate
	(b) *Potassium aminothiomethanesulphonates*
64	Potassium phenylaminothiomethanesulphonate
65	Potassium α-naphthyl-1-methylaminothiomethanesulphonate
66	Potassium β-naphthyl-1-methylaminothiomethanesulphonate
	(c) *Isothiocyanates*
2	Benzyl isothiocyanate
67	*o*-Methylbenzyl isothiocyanate
68	*m*-Methylbenzyl isothiocyanate
69	*p*-Methylbenzyl isothiocyanate
70	*o*-Methoxybenzyl isothiocyanate
71	*m*-Methoxybenzyl isothiocyanate
72	*p*-Methoxybenzyl isothiocyanate
73	α-Phenylethyl isothiocyanate
74	α-Phenylpropyl isothiocyanate
75	Phenyl isothiocyanate
76	Benzhydryl isothiocyanate
77	*o*-Isopropylbenzyl isothiocyanate
	(d) *Substituted thioureas*
78	*o*-Xylylthiourea
79	*p*-Xylylthiourea
80	Di-*p*-Methylphenylthiourea
81	1-Benzyl-3-isobutylthiourea
82	1:3-Dibenzylthiourea
83	1-Benzyl-3-acetamidophenylthiourea
84	1:3-Di-*o*-Xylylthiourea
85	1:3-Di-*p*-Xylylthiourea
	(e) 4-*Substituted thiosemicarbazides*
86	4-Methylthiosemicarbazide
87	4-Formylthiosemicarbazide
88	4-Phenylthiosemicarbazide
89	4-*o*-Methylphenylthiosemicarbazide
90	4-*p*-Methylphenylthiosemicarbazide
91	4-Benzylthiosemicarbazide
92	4-*p*-Methoxybenzylthiosemicarbazide
93	4-*o*-Xylylthiosemicarbazide
94	4-*p*-Xylylthiosemicarbazide
95	4-Benzhydrylthiosemicarbazide
	(f) *Thiosemicarbazones*
96	Benzaldehyde thiosemicarbazone
97	*o*-Hydroxybenzaldehyde thiosemicarbazone
98	*m*-Hydroxybenzaldehyde thiosemicarbazone
99	*p*-Hydroxybenzaldehyde thiosemicarbazone
100	*o*-Methoxybenzaldehyde thiosemicarbazone
101	*m*-Methoxybenzaldehyde thiosemicarbazone
102	*p*-Methoxybenzaldehyde thiosemicarbazone
103	*o*-Nitrobenzaldehyde thiosemicarbazone
104	*o*-Aminobenzaldehyde thiosemicarbazone
105	*p*-N-Dimethylaminobenzaldehyde thiosemicarbazone
106	Isatin-thiosemicarbazone
	(g) 5-*Substituted aminothiatriazole*
44	Amine-X
107	5-Aminothiatriazole
108	5-Methylaminothiatriazole
109	5-Phenylaminothiatriazole
110	5-*o*-Methylphenylaminothiatriazole
111	5-*p*-Methylphenylaminothiatriazole
112	5-Benzylaminothiatriazole
113	5-*o*-Xylylaminothiatriazole
114	5-*p*-Xylylaminothiatriazole
	(h) 1-*Substituted tetrazole-5-thiols*
115	1-Phenyltetrazole-5-thiol
116	1-Benzyltetrazole-5-thiol
	(i) *N-Alkylbenzylamines*
117	N-Methylbenzylamine
118	N-Ethylbenzylamine
119	N-Isopropylbenzylamine

the isothiocyanate (0.5 g) with potassium metabisulphite (0.5 g) for 5 hr in aqueous ethanol (50%, 10 ml.) as described by Backer, Mulder & Froentjee (1935). They generally separated in yields of 40 to 55% from hot aqueous ethanol (50 to 80%) as colourless microcrystalline powder which exhibited no definite melting point.

Although a variety of methods are available for the preparation of isothiocyanates (Assony, 1961), the substituted benzyl isothiocyanates required for the above purpose were conveniently prepared by two classical procedures involving the formation of dithiocarbamates, which are treated with mercuric chloride and steam (Kaluza, 1909) or with ethyl chloroformate followed by alkali (McKay, Garmaise, Gaudry, Baker, Paris, Kay, Just & Schwartz, 1959).

The substituted benzylamines used were, in turn, prepared by one of three methods: (a) by reduction of the benzonitriles, obtained by Sandmeyer's reaction, (b) by reduction of aldoximes and ketoximes with sodium amalgam or sodium and ethanol (Lauder & Hurd, 1921), and (c) by Leuckart's reaction—viz., using ammonium formate and formamide (Lewis, 1950). Reduction of the substituted benzonitriles was found to be advantageously carried out by hydrogenation in presence of Raney nickel at 20 and 50 atmosphere pressure (Adkins & Billica, 1948).

The compounds AB Nos. 50, 52, 53, 57, 58, 59, 61, 63, 65, 66 (see Table 1) which are described for the first time, did not show any characteristic melting point and showed the correct nitrogen content on analysis.

2. Isothiocyanates. A mixture of amine (0.05 mole) and sodium hydroxide solution (9%, 20 ml.) was treated gradually with carbon disulphide (3.8 g, 0.05 mole) with constant shaking under cooling in ice. The resulting dithiocarbamate was decomposed either by adding mercuric cholide (15 g) dissolved in water and steam-distilling the product, or by treating with ethyl chloroformate (4 g) and potassium hydroxide (18%, 10 ml.).

The new compounds AB Nos. 67 (b.p. 120°/mm), 69 (b.p. 155°/10 mm), 73 (b.p. 140°/5 mm), 74 (b.p. 160°/5 mm), 77 (b.p. 175°/25 mm) showed the correct nitrogen content on analysis.

3. Thioureas. The general procedure adopted for the preparation of the symmetrically substituted thioureas consisted in refluxing a mixture of amine (0.05 mole), carbon disulphide (0.05 mole) and ethanol (20 ml.) until the evolution of hydrogen sulphide ceased (18 hr). The mixture was cooled, and the separated crystalline material recrystallized from ethanol. The yields varied between 60 and 70%.

The new compounds AB Nos. 78 (m.p. 135°), 84 (m.p. 125°) and 85 (m.p. 145°) showed the correct nitrogen content on analysis.

4. Thiosemicarbazones. A mixture of thiosemicarbazide (0.9 g, 0.01 mole), water (30 ml.) and glacial acetic acid (2 ml.) was warmed and the clear solution added to aldehyde (0.01 mole) dissolved in absolute ethanol (25 ml.). After refluxing for 15 min, the product, which separated on cooling, was recrystallized from 95% ethanol (Sah & Daniels, 1950).

5. 4-Substituted thiosemicarbazides. A mixture of 95% hydrazine (0.1 mole) and water (5 ml.) was added gradually with stirring to a solution of isothiocyanate (0.1 mole) in ethanol (20 ml.), cooled in ice. The stirring was continued for 10 min more and the precipitate was washed with aqueous ethanol (40%) (Pillai, 1958).

The new compounds AB Nos. 92 (m.p. 170°), 93 (m.p. 149°) and 94 (m.p. 160°) showed the correct nitrogen content on analysis.

6. 5-Substituted aminothiatrizoles. Pillai's (1958) modification of Freund & Schander's (1896) procedure was adopted. To a solution of thiosemicarbazide (0.1 mole) in 3N hydrochloric acid (35 ml.) cooled in ice-salt mixture, a solution of sodium nitrite (0.1 mole) dissolved in water (15 ml.) was added over a period of 30 min with stirring. Stirring was continued for another 10 min and the product was filtered and recrystallized from 98% ethanol.

The new compounds AB Nos. 113 (m.p. 174–175°) and 114 (m.p. 115°) showed the correct nitrogen content on analysis.

7. "Amine-X." Pillai's (1958) modification of Freund & Schander's (1896) procedure for the preparation of 5-amino-1,2,3,4-thiatriazole consisted of the addition of nearly three-fourths of the theoretical amount of sodium nitrite solution to the semicarbazide and its removal before the solution turned yellow. Addition of the full amount of the reagent resulted in producing a coloured compound. Since this coloured product was active (Krishnamurthy *et al.*, 1967), the following attempts were made to isolate and characterize the active principle:

(a) The reactants were mixed in equimolar quantities and the pale yellow product dissolved in the minimum volume of ethyl acetate. By cooling to 0° C, a yellow substance separated, melting at 115–122° C (decomp.), which was raised to 125° C (decomp.) on recrystallization from ethanol.

(b) 5-Aminothiatriazole was treated with an equimolar amount of sodium nitrite in hydrochloric acid solution at 0–5° C. The product melted at 110–115° C (decomp.), which was raised to 120–122° C (decomp.). However, the substance was recovered only in small amounts.

(c) 5-Aminothiatriazole was treated with one-half of an equimolar solution of sodium nitrite as in experiment (b). The recovered material, m.p. 125–126° C (decomp.), was highly active.

(d) Thiosemicarbazide (1 mole) was mixed with 1.5 mole sodium nitrite at 0–5° C, the yellow product on recrystallization from ethyl acetate or 95% ethanol melted at 125–126° C (decomp.) (Found: N, 44.7, 44.7, 44.6%) and was recovered in a yield of 30%. Using 2 mole sodium nitrite resulted in extensive decomposition accompanied by gas evolution.

Apart from the colour, lower melting point, and antivaccinia activity, this substance did not exhibit any marked difference from 5-aminothiatriazole in ultraviolet and infrared spectral characteristics (recorded with "Infracord").

8. 1-Substituted-tetrazole-5-thiols. 1-Phenyl- and 1-benzyl-tetrazole-thiols were prepared by the isomerization of the corresponding 5-aminothiatriazoles by refluxing with aqueous sodium hydroxide (10%) as described by Pillai (1958).

9. N-Alkylbenzylamines. N-Methyl-, N-ethyl-, and N-isopropyl-benzylamines were previously reported by Surrey, Olivet & Hoppe (1954).

Preparation of the substances for screening and administration

Test substances were administered as sterile solutions in saline or buffer or as very fine suspensions or emulsions. Substituted aminothiatriazoles and AB-44 were given as solutions in 2% malic acid. Stock solutions or suspensions (1%) were freshly made or used within 24 hr. In the latter case, they were preserved in the ice-chest. Suitable dilutions of the stock solutions were made before commencement of the experiment.

RESULTS

Of the several compounds tested at near maximum tolerated dose, the antiviral effects (viz., changes in haemaglutinin production in influenza cultures, and of infectivity and mortality due to vaccinia viral inoculation) of the active compounds are indicated in Table 2. This table also includes results of variation in dosage. As can be seen from the Table, compounds 56, 57 and 91 display anti-influenza activity, while 44, 61, 93, 94 and 112 partially inhibit the growth of vaccinia virus and 3 and 50 act on both the viruses. Thus anti-influenza and anti-vaccinia activities do not seem necessarily to go together.

Antiviral activity in vivo

(a) *Vaccinia infections in rabbits.* Of the active compounds mentioned, AB-3, AB-50 and AB-44 possess sufficiently low parenteral toxicity in mice and high activity against *in ovo* vaccinia infections to facilitate *in vivo* assessment of their action. The effect of intravenous administration on 3 successive days of AB-3 (10 mg/kg/day), AB-50 (10 mg/kg/day) and AB-44 (20 and 30 mg/kg/day) to rabbits, infected intradermally with the virus, was studied using 5 animals for each experiment. The virustatic action of AB-3 in rabbits was only partial in that the viral growth was not abolished, although extensive lesion formation was prevented. In contrast, its *o*-methyl derivative, AB-50, was more effective in stemming the lesion formation, although viral growth was not entirely suppressed. Vesiculation rarely occurred in rabbits treated with AB-44, and if any did, it was no more than a slight bulging at the site of scarification which was only just visible.

(b) *Influenza infections in mice.* No study of the *in vivo* activity of AB-52 and AB-112, which show doubtful inhibitory activity *in ovo*, has been yet undertaken, since they were found to be lethal to eggs at levels higher than 0.4 and 0.7 mg/egg respectively. Even in

TABLE 2

ANTIVIRAL ACTIVITY OF SUBSTITUTED POTASSIUM BENZYLAMINOTHIOMETHANE SULPHONATES AND OTHER COMPOUNDS *IN OVO*

AB No.	Dose (mg/egg)	Influenza PR_8 virus Average haemagglutination titre		Vaccinia virus	
		Treated	Control	No. of eggs living/no. treated	Growth on membranes of treated eggs
3	0·10	192	128	0/8	Good growth
	0·25	128	256	2/8	Good growth
	0·50	4	256	7/8	Good growth
	0·70*				
50	0·20	128	128	0/6	Good growth
	0·50	16	128	0/6	Good growth
	1·00	4	128	4/6	Reduced growth
	1·50	4	128	*	
	2·00*				
56	0·30	128	128	0/6	Good growth
	0·50	32	128	0/6	Good growth
	0·70*				
57	0·30	128	128	0/6	Good growth
	0·50	32	128	0/6	Good growth
	0·70*				
61	0·30	128	128	0/6	Good growth
	0·50	64	128	0/6	Good growth
	0·70*			3/6†	Good growth
91	0·50	128	128	0/6	Good growth
	1·00	32	128	0/6; 0/6†	Good growth
	1·50*				
93	1·00	128	128	2/6; 2/6†	Good growth
94	1·00	128	128	2/6; 2/6†	Good growth
	1·50*				
44	0·50	128	128	0/6	
	1·00	128	128	1/6	
	2·00	128	128	5/6; 5/6†	Very little growth
	4·00	128	128	5/6	
	6·00*				
112	0·30	128	128	0/6	Good growth
	0·50	64	128	2/6; 2/6†	Good growth
	0·70*				

* 50 to 100% mortality was observed at this dosage; † compound administered through yolk sac.

animals, they did not seem to be well tolerated at 20 to 25 mg/kg levels in exploratory experiments.

AB-3 showed considerable *in vivo* activity when administered intraperitoneally in doses of 25 mg/kg and 50 mg/kg, affording 33% and 83% protection respectively to the treated mice. The activity of AB-50 was comparatively less, as higher doses (50 mg/kg and

TABLE 3

EFFECT OF INTRAPERITONEAL ADMINISTRATION OF AB-50 (80 mg/kg) AND AB-3 (50 mg/kg) TO MICE BEFORE OR AFTER INFECTION WITH INFLUENZA PR_8 VIRUS

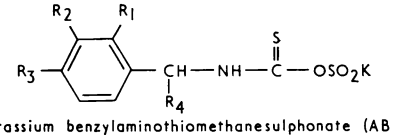
Time interval (hr.)	Administration before infection (no. of survivors/total no.)		Administration after infection (no. of survivors/total no.)	
	AB-50	AB-3	AB-50	AB-3
0·5	4/6	4/6	6/6	6/6
1·0	4/6	4/6	4/6	4/6
2·0	4/6	4/6	2/6	3/6
6·0	4/6	5/6	0/6	0/6
12·0	5/6	5/6	0/6	0/6
24·0	5/6	5/6	0/6	0/6

80 mg/kg) were required to produce the same effect. However, its low toxicity (LD_{50} 160 mg/kg intraperitoneal compared to 80 mg/kg of AB-3) is a point in its favour.

Like AB-3, AB-50 was effective when administered within 0.5 to 1 hr after, or better 24 hr earlier than, infection (vide Table 3) suggesting a prophylactic action. Further, in *in vivo* experiments the ratio of the effective doses of two compounds seemed to be maintained.

DISCUSSION

The main effects of substitution in benzyl isothiocyanate and potassium benzylaminothiomethane sulphonate residues may now be stated. Substitution at the side chain methylene group (R_4) by methyl, ethyl, or phenyl groups greatly reduces antiviral activity,



Potassium benzylaminothiomethanesulphonate (AB-3), $R_1 = R_2 = R_3 = R_4 = H$

the methyl derivative barely retaining a residual activity. The activity, however, is practically retained on introduction of a methyl group in ortho ($R_1 = CH_3$) position but considerably reduced or eliminated by substitution in para ($R_3 = CH_3$) or ortho and para ($R_1 = R_3 = CH_3$) positions. In contrast, a methoxyl group in either of these places considerably lowers the activity, producing no detectable response on vaccinia virus. Dimethyl substitution or introduction of a nitro group virtually eliminates the antiviral effects. A bulky group like isopropyl at the *o*-position ($R_1 = (CH_3)_2CH$) abolishes the activity demonstrating the significance of the size of the substituent group at this position. Replacement of the benzene by α- or β-naphthyl moiety produces inactive compounds. The contribution of the sulphur residue is apparent by comparison with the negative responses elicited by the N-alkylbenzylamines. The corresponding thioureas, thiosemicarbazones and other compounds are, in general, inactive.

In order to explain the antivaccinia and antimicrobial activities of pterygospermin and of its degradation product, AB-2, Narasimha Rao and co-workers (vide Narasimha Rao, 1965) suggested the " isothiocyanate hypothesis," according to which an active isothiocyanate moiety is formed *in situ*. The present data support this hypothesis. Despite the presence of the N–C–S sequence, the generally insignificant effects of other compounds, such as thioureas, thiosemicarbazones, etc., could be explained on the basis of the " isothiocynate hypothesis " as these compounds presumably undergo no fission to mustard oils. Further support for this view has come from other laboratories also (Winter & Ringe-Willeke, 1958 ; McKay *et al.*, 1959).

However, the activities of 5-benzylaminothiatriazole (AB-112) and AB-44 (whose precise structure is presently unknown) as well as that of isatin β-thiosemicarbazone, could possibly be exceptions to the " isothiocyanate hypothesis," since the possibility in these cases of a chemical breakdown to mustard oils seems remote, and thus they differ in their mode of action from AB-3 and AB-50. The relative antiviral activities of these compounds are reported in the subsequent communication.

SUMMARY

1. The preparation, properties, antiviral and antimicrobial activities of substituted benzylaminothiomethanesulphonates, of the related thioureas, thiosemicarbazides, thiosemicarbazones, and of substituted 5-amino-1,2,3,4-thiatriazoles and tetrazole-5-thiols are described.

2. Besides potassium benzylaminothiomethanesulphonate (AB-3), benzyl isothiocyanate (AB-2) and an impure preparation of 5-aminothiatriazole (" amine-X," AB-44) earlier reported, it has been found that the *o*-methyl derivative, AB-50 shows antiviral activity towards the two test viruses (viz., vaccinia virus (Bangalore strain) and influenza PR_8 virus) *in ovo* and *in vivo*, while the corresponding methoxy compounds inhibit only the influenza virus. 5-Benzylamino-1,2,3,4-thiatriazole (AB-112) shows significant anti-vaccinia activity.

3. AB-50 is less toxic to mice than either AB-3 or the *p*-methyl derivative, AB-52.

4. A more active preparation of " amine-X " than that reported earlier is described.

5. The present data support the isothiocyanate hypothesis—that is, the active compounds display antimicrobial activity conceivably by liberating free isothiocyanates *in situ*.

Th valuable assistance accorded by Dr. P. V. Narayana Rao, Mr. Hanumaiah and other staff members of the Vaccine Institute, as also the award of research fellowships to S.R. and H.B.P. by the Indian Institute of Science, Bangalore, and the Indian Council of Medical Research, Delhi, are duly acknowledged.

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