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Antibacterial and Mutagenic Activities of New Isothiocyanate Derivatives

A. ŠOVČÍKOVÁ[a], M. MIKULÁŠOVÁ[a], K. HORÁKOVÁ[a], Ľ. FLOCH[b]

[a]*Department of Biochemistry and Microbiology and* [b]*Department of Organic Chemistry, Faculty of Chemical Technology, Slovak University of Technology, 812 37 Bratislava, Slovakia*

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ABSTRACT. Nine newly synthetized isothiocyanate derivatives were demonstrated to posses antibacterial and genotoxic activities *in vitro*. 4-Hydroxybutyl isothiocyanate exhibited a broad antibacterial effect, with MIC values of 762 μmol/L for *Staphylococcus aureus* and *Escherichia coli*. Ethyl 4-methylsulfoxidobutanoate had the highest antibacterial activity in Gram-positive bacteria, the MIC value being 425 μmol/L for *S. aureus*. The highest tested concentrations of ethyl 4-isothiocyanatobutanoate and 4-hydroxybutyl isothiocyanate produced a bacteriocidal effect in Gram-positive bacteria. The compounds showed no mutagenic effects on *Salmonella typhimurium* tester strains TA 98 and TA 100, either in the absence or in the presence of a metabolically active microsomal S9 fraction from rat liver using standard Ames test.

More than one-hundred isothiocyanates (ITCs) and glucosinolates (GLTs), and their precursors, have been isolated from plants, many of which belong to the family *Cruciferae* and more specifically to the genera *Brassica* and *Raphanus* (Fenwick *et al.* 1983).

Naturally occurring and synthetic ITCs are among the most effective chemopreventive agents known (Hecht 1995; Talalay and Zhang 1996). A number of ITCs block chemical carcinogenesis in a variety of animal models by inhibiting phase I enzymes involved in carcinogen activation and by inducing phase II enzymes that accelerate the inactivation of carcinogens (Zhang and Talalay 1998). Moreover, these substances are widely and often abundantly distributed in edible plants and studies carried out in humans using high, but still realistic human consumption levels of brassica vegetables have shown putative positive effects on health (Verhoeven *et al.* 1996).

ITCs are also known as natural or synthetic antifungal (Drobnica *et al.* 1967; Saksena 1985; Tajima *et al.* 1998), antibacterial (Ono *et al.* 1998; Tajima *et al.* 1998) and cytotoxic agents (Horáková *et al.* 1971). Various isothiocyanates have been reported as antimutagens in *Escherichia coli*, where they participate in inactivating enzymes relevant to the metabolic activation of mutagens, resulting in a decrease in the frequency of chemically induced mutagenesis (Kawazoe and Kato 1982). The study of the mutagenic effects of some isothiocyanates *in Salmonella typhimurium* using standard assay produced negative results (Horáková *et al.* 1989).

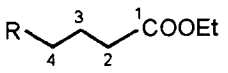
The aim of the our study was to determine the antibacterial effects of nine newly synthesized isothiocyanate derivatives (**1–9**), as the first step in the complex investigation of their biological activities. Moreover, their mutagenic activities toward *S. typhimurium* tester strains TA 98 and TA 100 were evaluated in the absence or in the presence of a microsomal fraction from rat liver (S9) using the Ames test.

MATERIALS AND METHODS

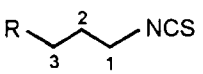
Compounds. Ethyl 4-isothiocyanatobutanoate (**1**), formally a derivative of 4-aminobutyric acid (γAbu), was synthesized from ethyl 4-aminobutyrate hydrochloride with thiophosgene in a chloroform–water system (Floch and Kováč 1975). Compounds **2–9** were also synthesized according to an unpublished procedure. Full physico-chemical characterization will be the subject of a separate publication (Floch 2001). The synthesis of derivative **6** has already been described (Tajima and Li 1997). Substances **1–8** are liquids, whereas derivative **9** is solid with a low melting point. The purity of the tested compounds was analyzed by HPLC and mass spectrometry before testing their antibacterial and mutagenic effects. All other chemicals and solvents were of the highest quality available and were obtained from *Merck* (Germany). For testing the antibacterial and mutagenic potency, the compounds were dissolved in dimethyl sulfoxide in various concentrations.

Microorganisms. Bacillus subtilis strain CCM 1718, *Staphylococcus aureus* strain CCM 3953, *Proteus mirabilis* strain CCM 1941, *Escherichia coli* strain CM 3988, obtained from the *Collection of Microorganisms of the Department of Biochemistry and Microbiology, Faculty of Chemical Technology,*

Slovak University of Technology, were used in antibacterial assays. *Salmonella typhimurium* strains TA 98 and TA 100 provided by the *Czech Collection of Microorganisms* (Brno, Czechia), were used in the Ames test.

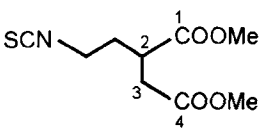


1, 2



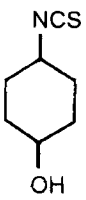
3, 4

	R			R	
1	NCS	ethyl 4-isothiocyanatobutanoate	**3**	CH_2OH	4-hydroxybutyl isothiocyanate
2	MeSO	ethyl 4-methylsulfoxidobutanoate	**4**	NMe_2	3-dimethylaminopropyl isothiocyanate



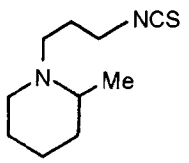
5

dimethyl 2-(2-isothiocyanato-ethyl)succinate



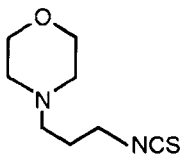
6

4-hydroxycyclohexyl isothiocyanate



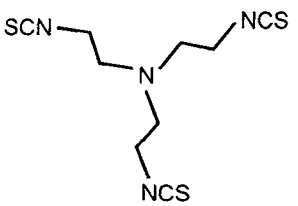
7

1-(3-isothiocyanato-propyl)-2-pipecoline



8

4-(3-isothiocyanatopropyl)morpholine



9

tris-(2-isothiocyanatoethyl)amine

The *antibacterial effect* of isothiocyanate derivatives was determined by a microdilution method on 96-well microtitration plates (Jantová *et al.* 1995). Cultures of bacteria were grown overnight (16 h before the test) in Nutrient broth no. 2 at 30 °C with rapid shaking. After filtration of the growing inoculum a 2 % suspension of bacteria was prepared; 180 μL of this suspension was added to 20 μL of the appropriate concentration of the tested compounds and cultured for 10 h on a reciprocal shaker in a thermostat at 30 °C. The time course of absorbancy (A_{630}) was determined in three parallels. Ampicillin at concentrations of 715, 286, 143, 28.6, 2.9 and 0.3 μmol/L was used as a positive control. The bacteria were inoculated onto a solid culturing medium after 8 h of their cultivation with the tested derivatives and the microbistatic or microbicidal concentration after 1 d of cultivation was determined. Antibacterial effects were characterized by the concentration of a substance which inhibits bacterial growth by 50 % relative to the control (IC_{50}) and by the minimum inhibitory concentration of a substance which inhibits bacterial growth by 100 % (MIC).

Preparation of the liver S9 fractions. Wistar rats (200–250 g) were treated with Delor 103 following the standard procedures (Maron and Ames 1983). This S9 fraction was routinely included in an S9-mix, which contained also (in mol/L) KCl 1.65, $MgCl_2 \cdot 6H_2O$ 0.4, sodium phosphate 0.2, glucose 6-phosphate 0.1, Na-NADP 0.1 (pH 7.4).

The *Ames test* was carried out by the standard method (Maron and Ames 1983). 0.1 mL cell suspension (16 h overnight culture, approximate cell concentration 200–500/nL, *i.e.* 2–5 × 10^8 cells per mL) was pipetted into melted top agar containing 50 μmol/L L-histidine and 50 μmol/L biotin; 0.1 mL of the tested compounds and 0.5 mL S9 mixture (in tests with metabolic activation) were added. The mixture was poured into plates containing minimal medium and incubated for 2 d at 37 °C; then numbers of revertant colonies were counted. The positive control was 5-nitro-2-furylacrylic acid (48.8 μmol per plate) in the

absence of metabolic activation and 2-aminoathracene (25.9 μmol per plate) in the presence of the S9 fraction. A positive response was defined as a reproducible two-fold increase of revertants with dose-response relationship and statistical evaluation using Student's *t*-test.

RESULTS AND DISCUSSION

IC_{50} and MIC values against two Gram-positive (*B. subtilis* and *S. aureus*) and two Gram-negative (*P. mirabilis* and *E. coli*) bacteria are summarized in Table I. The effects against bacterial strains were markedly lower than the effect of ampicillin. Derivative **2** was most effective in Gram-positive bacteria, but the growth of Gram-negative bacteria was not affected by it. *S. aureus* was relatively sensitive also to derivatives **1**, **3** and **9** (IC_{50} = 31.7–140 μmol/L). The most effective isothiothiocyanates in Gram-negative bacteria were derivatives **1** and **3**, the IC_{50} values ranging from 86.6 μmol/L to 191 μmol/L, except derivative **1** in *P. mirabilis*. Moreover, inhibition of growth of the Gram-negative bacteria by derivatives **6** and **7** was observed. The highest concentrations of derivatives **1** and **3** produced a bacteriocidal effect in both Gram-positive bacteria; this effect at 1342 μmol/L of derivative **7** in *B. subtilis* was found. The other isothiocyanate derivatives at all tested concentrations had only bacteriostatic effects.

Table I. Antibacterial activity[a] of isothiocyanate derivatives

Compound	*M* g/mol	*B. subtilis*		*S. aureus*		*P. mirabilis*		*E. coli*	
		IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
1	173.23	1039	>1443	**31.7**	1443	693	>1443	*86.6*	1443
2	235.33	*102*	>2125	***25.5***	425	>2125	>2125	467	>1062
3	131.20	534	3811	**114**	762	*191*	1906	122	762
4	144.24	1248	3466	**499**	>1733	1040	3466	693	>1733
5	231.27	778	2162	**151**	>1081	>2162	>2162	454	1081
6	157.23	1018	3180	369	1590	572	3180	**204**	1590
7	272.46	483	>1342	311	>1342	403	>1342	**140**	537
8	186.31	807	>1260	**227**	>1260	857	>1260	353	>1260
9	349.40	**110**	>537	140	>537	734	>1342	264	>1342
Ampicillin	–	11.4	143	0.46	286	11.4	286	5.7	28.6

[a]μmol/L; the lowest value is shown in **boldface** characters (in each line); the most effective compounds toward the individual microorganisms are shown in *italics* (in each column).

Mutagenic activities of isothiocyanate derivatives in *S. typhimurium* strains TA 98 and TA 100, in the absence or in the presence of a microsomal fraction, are shown in Table II. The tested isothiocyanate compounds showed no mutagenic effect in both *Salmonella* tester strains with or without metabolic activating system. The frequencies of spontaneous revertants of *S. typhimurium* TA 98 were 26 ± 6 *his*$^+$ revertant colonies per plate in the absence of liver S9 fraction and 32 ± 6 *his*$^+$ revertant colonies per plate in the presence of liver S9 fraction. Despite the absence of any detectable mutagenicity of isothiocyanate derivatives, toxic effects toward the cells of the TA 98 strain were slightly higher in the presence of the S9 fraction in derivatives **1**, **3**, **5** and **6**. In contrast, the toxic effects of derivatives **2** and **9** were slightly reduced by the microsomal enzyme activity of the S9 fraction.

The frequencies of spontaneous revertant of *S. typhimurium* strain TA 100 were 169 ± 28 *his*$^+$ revertant colonies per plate in the absence of liver S9 fraction and 182 ± 26 in the presence of liver S9 fraction. A slightly dose-dependent increase of mutant numbers of the *Salmonella* tester strain TA 100 in the presence of metabolically active microsomal S9 fraction was induced by derivative **2**, but the two-fold increase of revertants was not reached.

Some isothiocyanates are reported to have mutagenic effects in *S. typhimurium* (Neudecker and Henschler 1985) or *E. coli* (Říhová 1982) after longer, non-standard preincubation times greater than 20 min in the presence of metabolically active microsomal S9 fraction. Our isothiocyanate derivatives had no mutagenic activity toward *S. typhimurium* TA 98 and TA 100 in the Ames test, either with or without the rat liver S9 fraction. These findings are similar to the results of other investigators who reported that aliphatic isothiocyanates are mostly not mutagenic to strains TA 98 and TA 100 as well as to other *Salmonella* tester strains, either with or without the liver S9 fraction when using the Ames test (Horáková *et al.* 1989).

Table II. Mutagenic effects of isothiocyanate derivatives in *Salmonella typhimurium* tester strains TA 98 and TA 100 with (+S9) or without (–S9) the microsomal fraction from rat liver using the Ames test

Compound	Dosage μmol per plate	Number of revertants per plate[a]			
		TA 98		TA 100	
		+S9	–S9	+S9	–S9
1	0	25 ± 6	22 ± 6	142 ± 20	129 ± 24
	57.7	15 ± 2	17 ± 0	134 ± 4	–
	144	4 ± 2	19 ± 2	120 ± 0	119 ± 24
	289	0†	13 ± 4	132 ± 11	107 ± 16
	577	0†	6 ± 4	113 ± 21	95 ± 32
	1443	0†	0†	0†	0†
2	0	25 ± 6	22 ± 6	142 ± 20	129 ± 24
	42.5	24 ± 11	19 ± 5	–	–
	213	22 ± 1	16 ± 5	132 ± 2	–
	425	25 ± 0	7 ± 4	167 ± 5	117 ± 26
	1062	16 ± 0	0†	186 ± 22*	107 ± 27
	2125	0†	0†	188 ± 20†*	78 ± 17
3	0	25 ± 6	22 ± 6	142 ± 20	129 ± 24
	76.2	7 ± 2	24 ± 0	–	–
	191	0†	21 ± 3	53 ± 11	142 ± 15
	381	0†	17 ± 7	32 ± 0†	143 ± 28
	762	0†	14 ± 1†	0†	124 ± 20
	1906	0†	0†	0†	51 ± 5†
4	0	25 ± 6	22 ± 6	142 ± 20	129 ± 24
	69.3	18 ± 2	16 ± 1	–	147 ± 23
	347	12 ± 1	14 ± 1	132 ± 6	140 ± 18
	693	10 ± 1	15 ± 4	100 ± 13	127 ± 25
	1733	0†	15 ± 1†	84 ± 9	122 ± 27
	3466	0†	0†	75 ± 0	86 ± 12†
5	0	25 ± 6	22 ± 6	–	129 ± 24
	43.2	23 ± 4	23 ± 4	–	105 ± 5
	108	5 ± 1	11 ± 0	–	135 ± 6
	216	1 ± 1†	10 ± 3	–	112 ± 0
	432	0†	5 ± 2	–	88 ± 0
	1081	0†	0†	–	0†
6	0	25 ± 6	22 ± 6	142 ± 20	129 ± 24
	63.6	14 ± 1	20 ± 1	–	–
	159	5 ± 1	15 ± 4	72 ± 14	–
	318	3 ± 4†	14 ± 4	109 ± 10	112 ± 17
	636	0†	11 ± 6	84 ± 23	107 ± 25
	1590	0†	5 ± 1	71 ± 11	53 ± 11
7	0	25 ± 6	21 ± 3	142 ± 20	129 ± 24
	53.7	16 ± 7	29 ± 16	–	99 ± 26
	134	6 ± 4	5 ± 1	82 ± 14	46 ± 8
	268	0†	0†	37 ± 6	26 ± 9
	537	0†	0†	0†	0†
	1342	0†	0†	0†	0†
8	0	25 ± 6	21 ± 3	142 ± 20	129 ± 24
	50.4	20 ± 4	19 ± 5	–	144 ± 2
	126	8 ± 4	5 ± 1	80 ± 3	104 ± 27
	252	0†	3 ± 0†	30 ± 1	43 ± 5
	504	0†	0†	9 ± 3	25 ± 0
	1260	0†	0†	0†	0†
Control[b]	–	32 ± 6	26 ± 6	182 ± 26	169 ± 28
Nfa[c]	48.8	–	330 ± 79*	–	1629 ± 382*
Aan[c]	25.9	548 ± 68*	–	1425 ± 134*	–

[a]±SD; * – $p = 0.05$; † – toxic effects. [b]Spontaneous revertants.
[c]Nfa – 5-Nitro-2-furylacrylic acid. [d]Aan – 2-Aminoanthracene.

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REFERENCES

DROBNICA Ľ., ZEMANOVÁ M., NEMEC P., ANTOŠ K., KRISTIÁN P., STULLEROVÁ A., KNOPPOVÁ V., NEMEC P. Jr.: Antifungal activity of isothiocyanates and related compounds. I. Naturally occurring isothiocyanates and their analogues. *Appl.Microbiol.* **15**, 701–709 (1967).

FENWICK G.R., HEANEY R.K., MULLIN W.J.: Glucosinolates and their breakdown products in food and food plants. *Crit.Rev.Food Sci. Nutr.* **18**, 123–201 (1983).

FLOCH Ľ., *will be published* (2001).

FLOCH Ľ., KOVÁČ S.: Synthesis and properties of alkyl isothiocyanatocarboxylates. *Collect.Czechosl.Chem.Commun.* **40**, 2845–2854 (1975).

HECHT S.S.: Chemoprevention by isothiocyanates. *J.Cell Biochem.* **22** (Suppl.), 195–209 (1995).

HORÁKOVÁ K., DROBNICA Ľ., NEMEC P.: Cytotoxic and cancerostatic effect of isothiocyanates and related compounds. VII. Effect of isothiocyanate chalkones on HeLa cells. *Neoplasma* **18**, 355–359 (1971).

HORÁKOVÁ K., STRAKOVÁ Z., JANTOVÁ S., MÚČKOVÁ M., FLOCH Ľ.: Biological effects of 2-isothiocyanate carboxylic acid esters. I. Cytotoxic, antimicrobial and genotoxic screening of amino acid isothiocyanate derivatives. *Biologia* **44**, 637–646 (1989).

JANTOVÁ S., HUDECOVÁ D., STANKOVSKÝ S., ŠPIRKOVÁ K., RUŽEKOVÁ Ľ.: Antibacterial effect of substituted 4-quinazolylhydrazines and their arylhydrazones determined by modified microdilution method. *Folia Microbiol.* **40**, 611–614 (1995).

KAWAZOE Y., KATO M.: Antimutagenic effect of isocyanates and related compounds in *Escherichia coli*. *Gann* **73**, 255–263 (1982).

MARON D.M., AMES B.N.: Revised methods for the *Salmonella* mutagenicity test. *Mutat.Res.* **113**, 173–215 (1983).

NEUDECKER T., HENSCHLER D.: Allyl isothiocyanate is mutagenic in *Salmonella typhimurium*. *Mutat.Res.* **156**, 33–37 (1985).

ONO H., TESAKI S., TANABE S., WATANABE M.: 6-Methylsulfinylhexyl isothiocyanate and its homologues as food-originated compounds with antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. *Biosci.Biotechnol.Biochem.* **62**, 363–365 (1998).

ŘÍHOVÁ E.: Mutagenic effects of allylisothiocyanate in *Escherichia coli* WP 67. *Folia Microbiol.* **27**, 25–31 (1982).

SAKSENA N.K.: Fungicidal action and structure correlation of monosubstituted phenyl isothiocyanates. *Folia Microbiol.* **30**, 359–362 (1985).

TAJIMA H., LI G.: Synthesis of hydroxyallyl isothiocyanates. *Synlett* **7**, 773–774 (1997).

TAJIMA H., KIMOTO H., TAKETO Y., TAKETO A.: Effects of synthetic hydroxy isothiocyanates on microbial systems. *Biosci.Biotechnol. Biochem.* **62**, 491–495 (1998).

TALALAY P., ZHANG Y.: Chemoprotection against cancer by isothiocyanates and glucosinolates. *Biochem.Soc.Trans.* **24**, 806–810 (1996).

VERHOEVEN D.T., GOLDBOHM R.A., VAN POPPEL G., VERHAGEN H., VAN DEN BRANDT P.A.: Epidemiological studies on brassica vegetables and cancer risk. *Cancer Epidemiol.Biomark.Prev.* **5**, 733–748 (1996).

ZHANG Y., TALALAY P.: Mechanism of differential potencies of isothiocyanates as inducers of anticarcinogenic Phase 2 enzymes. *Cancer Res.* **58**, 4632–4639 (1998).