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Antimicrobial activity of N-glucosylrhodanines and N-glucosylthioureas

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Synopsis

Rhodanine and rhodanine derivatives have shown significant inhibitory activity against bacteria, fungi, and some parasites, and have potential as preservative agents. No glycosylated derivatives of rhodanine or its derivatives have previously been evaluated as antimicrobial agents; the presence of a sugar moiety should provide compounds of low toxicity. A series of N-(2,3,4,6-tetra-0-acetyl-β-D-glucopyranosyl)-5-aralkylidenerhodanines has been prepared and their antimicrobial activities against several organisms determined. Attempts to remove the acetyl groups by ammonia in methanol provided a synthetic method for obtaining N-glycosylthiourea. Use of other amines was found to give the substituted N-glycosylthioureas. Antimicrobial activities of these compounds against several microorganisms were found in several cases to be equal to or slightly better than those from rhodanine or glycosylated rhodanine derivatives.

INTRODUCTION

Many preservatives are available for use in cosmetics, but the actual options which face the cosmetic chemist and microbiologist, when they are dealing with a specific formula, may be very narrow. The difficulty in choosing a proper compound for a preservative is also the case for the synthesis of those compounds which should meet primary criteria such as water solubility and chemical and physical stability as well as biological activity. It was considered that N-glucosylthioureas and N-glucosylrhodanines might prove suitable as preservatives, and accordingly this study was undertaken.

A large number of thioureas have been synthesized and found to be biologically active as antiviral (1,2) and antibacterial-antifungal (3,4) agents. Also, many rhodanine derivatives have been reported with significant inhibitory activities against virus (5,6), bacteria (7,8), fungi (9-11), and parasites (12,13). However, these compounds have not been used in cosmetics as preservatives because of their toxicities (14-16), and because of changes in color, viscosity, and pH in aqueous solution. In attempts to achieve

lower toxicities, it has been demonstrated that the presence of a sugar group decreases the toxicity of some antibacterial agents (17,18) and some anticancer agents (19,20).

N-glucosylthioureas and N-glucosylrhodanine derivatives have therefore been synthesized, and their antimicrobial activities were determined by the agar plate streak method and by the broth dilution method for determining minimal inhibitory concentrations (MIC).

SYNTHESIS

As early as 1914, Fischer (21) synthesized tetra-0-acetyl-glucopyranosylthiourea from tetra-0-acetylglucopyranosylisothiocyanate and ammonia, but he was unable to get the deacetylated product. Other investigators (22,23) have prepared glycosylthioureas by using acylated glycosylisothiocyanates and amines, but have generally been unsuccessful in hydrolyzing the acyl groups. Foye and Tovivich (24) synthesized N-glucosylthiourea from the condensation product of α-acetobromo-D-glucose and 5-aralkylidenerhodanines followed by a successful hydrolysis using a methanol-ammonia system to remove acetyls and cleave the rhodanine ring.

The N-glucosylthiourea derivatives were synthesized by hydrolyzing N-(2,3,4,6-tetra-0-acetylglucopyranosyl)-5-benzylidenerhodanine in methanolic amine solutions. N-β-D-Glucopyranosyl-5-aralkylidenerhodanine derivatives were prepared by hydrolyzing their tetraacetyl derivatives with methanolic hydrogen chloride without cleavage of the rhodanine ring structure. Representative syntheses for the N-glucosylthioureas and N-glucosylrhodanines are described in the Experimental section.

ANTIMICROBIAL EVALUATION

The term "antimicrobial agent" (25) may be used to designate any substance of natural, semi-synthetic, or synthetic origin that inhibits or kills free living, commensal, or pathogenic microorganisms while causing little or no injury to the host. There are several currently used methods for testing antimicrobial susceptibility (26). The agar diffusion test and antimicrobial dilution test have been the most widely employed.

For the quantitative estimate of antimicrobial activity, the minimal inhibitory concentration (MIC) is determined as the lowest concentration that will inhibit growth of the microorganism. In our experiments, the 10-fold dilution method was used instead of the doubling dilution method, and the MIC was expressed in μg/ml.

In our antimicrobial test, five different types of organisms were selected. The inclusion of *Aspergillus niger* and *Pseudomonas aeruginosa* in the inocula is recommended (27) to ensure a wide range of protection, because these ubiquitous organisms grow in a large variety of formulations and are relatively resistant to antimicrobial agents. *Staphylococcus aureus* and *Escherichia coli* were also included as representative of Gram-positive and Gram-negative organisms, and *Candida albicans* was included to provide a yeast. The incidence of contaminated cosmetics has been the subject of several investigations (28,29); *Pseudomonas* was the predominant organism found. Microbial spoilage of cosmetic preparations was a major concern of manufacturers in former years (30) as well. Spoilage in this context was defined as microbial growth that results in various deleterious effects such as noxious odors and gases, changes in pH, viscosity, and

color, and destruction of emulsions. The presence of a visible means of growth or bacterial slime on the surface of a product obviously renders it unsuitable for marketing.

EXPERIMENTAL

2,3,4,6-TETRA-0-ACETYL-β-D-GLUCOPYRANOSYL BROMIDE

This was prepared by the method of Lemieux (31) in 89% yield; mp 88-89° (lit. mp 88-89°) (31).

5-BENZYLIDENERHODANINE

Rhodanine (13.32 g, 0.1 mole) was dissolved in 100 ml of glacial acetic acid, and to this was added 24.6 g (0.3 mole) of fused sodium acetate. After the solution became clear, 10.61 g (0.1 mole) of benzaldehyde was added and the reaction mixture was refluxed for 45 min. The whole mixture was poured into 500 ml of water, and the crude product was separated by filtration to give 19.88 g (90% yield) of yellowish brown crystals with mp 204-208°. Recrystallization from toluene improved the mp to 208-209° (lit. mp 204-205°) (32).

N-(2,3,4,6-TETRA-0-ACETYL-β-D-GLUCOPYRANOSYL)-5-BENZYLIDENERHODANINE

To a solution of 22.13 g (0.1 mole) of 5-benzylidenerhodanine in 700 ml of acetone, 40 ml of 10% sodium hydroxide solution was added, followed by 44.12 g (0.1 mole) of acetobromoglucose. The mixture was stirred at room temperature for 22 hr. The reaction mixture was evaporated to dryness in vacuo. The crude product was recrystallized from hot methanol, yielding 42.3 g (82%); mp 192-193°, $[\alpha]_D^{25} = -168°$ (C = 3.1 pyridine) (lit. mp 193-194°) (33).

N-β-D-GLUCOPYRANOSYLTHIOUREAS

N-(2,3,4,6-Tetra-0-acetyl-β-D-glucopyranosyl)-5-benzylidenerhodanine (III) (5.52 g, 0.01 mole) was added to a solution of diethylamine (5.85 g, 0.08 mole) in 70 ml of methanol in a pressure bottle. The reaction mixture was stirred at room temperature for 20 hr with the stopper well closed. The reaction mixture was stirred for 1 hr with 70 ml of DOWEX-50WX8 (cation exchange resin) to remove the excess amine and α-mercaptocinnamyl amide, and filtered. The filtrate was dehydrated with anhydrous sodium sulfate and evaporated to a syrupy material. The residue was crystallized with acetone-methanol (2:1) mixture, yielding 2.41 g (81.5%); mp 106-109° dec., $[\alpha]_D^{25} = -11.0°$ (C = 1.1).

IR(KBr): 3520 (OH), 3100 (NH), 1080 (C = S), 890 (β-form)cm^{-1}. NMR (dimethylsulfoxide-d6): δ 5.40 (d, 1H, NH), 3.21 (m, 4H, $N(CH_2)_2$), 1.17 (t, 6H, $2CH_3$) ppm.

Analysis-Calculated for $C_{11}H_{22}N_2O_5S \cdot H_2O$: C, 42.29; H, 7.74; N, 8.96; S, 10.26.

Found: C, 42.57; H, 7.66; N, 8.87; S, 10.34.

Other glucosylthioureas were prepared by the same method.

N-β-D-GLUCOPYRANOSYL-5-ARALKYLIDENERHODANINES

N-(2,3,4,6-Tetra-0-acetyl-β-D-glucopyranosyl)-benzylidenerhodanine (2.56 g, 0.0046 mole) was suspended in 700 ml of methanol, and 1.9 M hydrochloric acid (10.5 ml) was added. The reaction mixture was stirred at room temperature for 3 d. The reaction solution was filtered and evaporated to a semisolid material at a temperature under 30° in vacuo. The semisolid was crystallized from ethanol and water, yielding 1.7 g (96.5%); mp 104-110°, $[\alpha]_D^{20} = -78.0°$ (C = 0.8, pyridine) (lit. mp 104-110°) (24).

The same method was applied for the preparation of the other glucosylrhodanine derivatives.

TEST ORGANISMS

One Gram-positive and two Gram-negative bacteria, one yeast and one mold were selected for the test. These were, respectively, *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 11229), *Pseudomonas aeruginosa* (ATCC 15442), *Candida albicans* (ATCC 10259), and *Aspergillus niger* (ATCC 1015).

PREPARATION OF INOCULUM

Portions of four or five discrete colonies representative of the organisms to be tested were inoculated into 10.0 ml of a suitable broth medium and marked accordingly. Bacterial cultures were incubated at 37°, while fungal cultures were incubated at 25° for 18-24 hr.

AGAR PLATE STREAK METHOD FOR QUALITATIVE EVALUATION

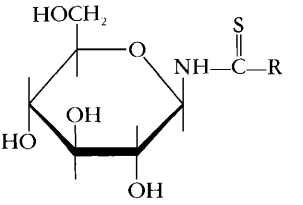
Trypticase Soy Agar (BBL) was used for bacteria and Sabouraud Maltose Agar (BBL) for the yeast and mold. A 10-cm round plastic plate was used for the preparation of the medium. Each plate was divided into four areas by marking on the bottom of the plate. The agar plate was streaked by the appropriate inoculating loop. Three thin loopfuls of inocula were applied and covered the agar plate completely. Approximately 10-15 mg of compound to be tested was placed on the corresponding area of each inoculated plate. The plates inoculated with *S. aureus, E. coli,* and *P. aeruginosa* were incubated at 37° for 24-48 hr, and those inoculated with *C. albicans* and *A. niger* were incubated at 25° for 48-72 hr.

After incubation, if a zone of inhibition was visible around the compound, the susceptibility of the organism to the compound was considered to be positive. The degree of inhibition of the compound was decided by the size of the zone. The results of the qualitative evaluation of the compounds are shown in Tables I and II.

BROTH DILUTION METHOD FOR THE DETERMINATION OF THE MINIMAL INHIBITORY CONCENTRATION (MIC)

Quantitative evaluation was made for the compounds which showed some inhibitory activity in the preliminary agar screening test against any of the five organisms. The broth dilution method was chosen for the determination of the MIC. A 10^{-1} molar stock solution was prepared by dissolving or suspending the required amount of

Table I
Agar Plate Screening Test of Antimicrobial Activity of Glucosylthioureas

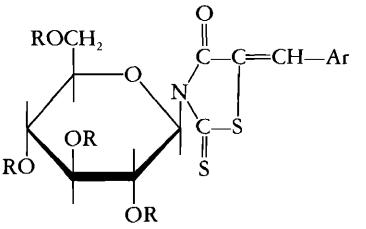


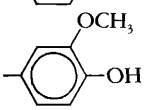
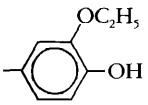
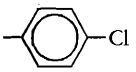
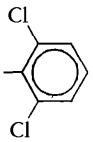
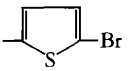
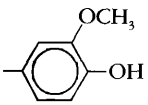
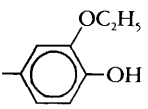
Compounds tested R	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. niger*
$-N(CH_3)_2$	+	−	−	−	−
$-NH(CH_2)_3CH_3$	++	−	−	+	+
$-NHCH_2-C_6H_4-OCH_3$	+	−	−	+	+
$-NHCH_2CH_2-C_6H_5$	+	−	−	−	−
$-NHCH_2-C_6H_5$	−	+	+	−	−
$-N(C_2H_5)_2$	−	−	−	−	−
$-N$(piperazine)$-CH_3$	−	−	−	−	−
$-N$(morpholine)	−	−	−	−	−
$-N$(thiomorpholine)	−	−	−	−	−
$-NHCH_2CH{=}CH_2$	++	−	−	−	−
$-NHN(CH_3)_2$	+	−	−	+	−
$-N$(pyrrolidine)	+	−	+	−	−
$-NH_2$	++	−	−	+	+
Rhodanine	+++	+++	+++	+++	+++

+++ Very active; ++ moderately active; + slightly active; − no activity.

compound in sterilized distilled water. The desired number of sterile test tubes were aseptically filled with 9.0 ml of Trypticase Soy Broth (BBL). One ml of the stock solution was added to the first tube and mixed thoroughly. With a sterile pipette, 1.0 ml was transferred from the first tube to the second tube, 1.0 ml was transferred with a separate pipette to the third tube. This process was continued through the penultimate tube, from which 1.0 ml was removed and discarded. Eventually, a serial dilution of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} molar concentrations was made. The sixth tube without any antimicrobial agent served as a growth control, and also a blank tube was prepared, separately. Except for the blank tube, the inoculum was prepared by adding 0.05 ml of 24 hours-old broth culture without any dilution. The tubes were incubated for 24-48 hr at 37° for bacteria and 48-72 hr at 25° for the yeast and mold. The lowest concentration of antimicrobial compound resulting in complete inhibition of visible growth represented the MIC; a very faint haziness or a small clump of possible growth

Table II
Agar Plate Screening Test of Antimicrobial Activity of N-β-D-Glycosyl-5-ylidene Rhodanines



Compounds tested		Organisms tested				
Ar	R	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. niger*
	H	–	+	–	–	–
	H	–	–	–	–	–
	H	–	–	–	–	–
	H	+	–	–	–	–
	H	++	–	–	+	+
	H	–	–	–	–	–
	Acetyl	–	–	–	–	–
	Acetyl	–	–	–	–	–
Rhodanine		+++	+++	+++	+++	+++

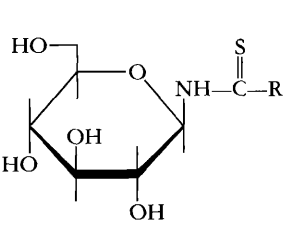
+++ Very active; ++ moderately active; + slightly active; – no activity.

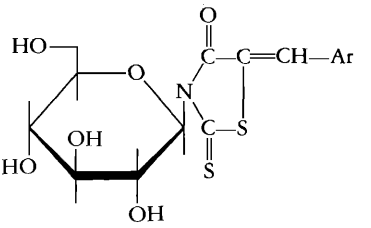
was disregarded, whereas a large cluster or growth or definite turbidity was considered evidence that the compound had failed to inhibit growth completely at that concentration (26). The minimal inhibitory concentrations for the compounds tested are shown in Table III.

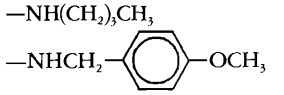
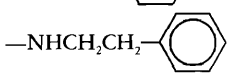
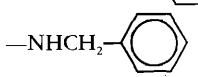
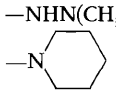
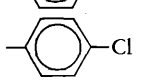
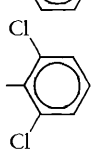
ACUTE TOXICITY DETERMINATIONS

Compounds II, V, VIII, and IX were dissolved or suspended in normal saline and administered intraperitoneally to 20-25 g Charles River CD-1 male mice. Three mice

Table III
Minimal Inhibitory Concentrations of Compounds Tested by the Broth Dilution Method





No.	Compounds tested R or Ar	M.I.C. (μg/ml) *S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. niger*
I	$-N(CH_3)_2$	2,633				
II	$-NH(CH_2)_3CH_3$	333			33	333
III		3,584			358	3,584
IV		342				
V			3,864	3,864		
VI	$-NHCH_2CH{=}CH_2$	28				
VII	$-NHN(CH_3)_2$	2,813			2,813	
VIII		3,064		306		
IX	$-NH_2$	24			238	2,383
X			383			
XI		418				
XII		453			453	4,533
XIII	Rhodanine	133	133	133	133	1,332

were used for each compound, two animals receiving a dose of 1000 mg/kg and one animal receiving 2000 mg/kg. The mice were observed periodically for a week, at which time all animals appeared normal (34).

RESULTS AND CONCLUSIONS

A number of the compounds (Table III) showed inhibitory activities against *Staphylococcus aureus;* N-β-D-glucopyranosyl-N′-allylthiourea (VI) and N-β-D-glucopyranosylthiourea (IX) proved to be the most active. All of the glycopyranosylthioureas which exhibited inhibitory activities were derivatives of primary amines except compounds I and VIII. This indicates that N′, N′-disubstituted thioureas will in general show decreased antimicrobial activities.

A smaller number of compounds exhibited inhibitory activity against *Candida albicans* and *Aspergillus niger*. N-β-D-Glucopyranosyl-N'-butylthiourea (II) was found to be most active and N-β-D-glucopyranosyl-N'-dimethylaminothiourea (VII) was least active against *Candida albicans*. Against *Aspergillus niger*, also, compound II demonstrated the highest activity. The rest of the active compounds were found to be slightly active.

N-β-D-Glucopyranosyl-N'-benzylthiourea (V) exhibited slight activity against *Escherichia coli* and *Pseudomonas aeruginosa*. N-β-D-Glycopyranosyl-1-piperidinethiocarboxamide (VIII) showed activity against *Pseudomonas aeruginosa*, and N-β-D-glycopyranosyl-5-benzylidenerhodanine (X) was active against *E. coli*. Other glucosylrhodanine derivatives did not show activity against either *E. coli* or *P. aeruginosa*, the Gram-negative organisms.

Among the N-β-D-glucopyranosyl-5-aralkylidenerhodanine derivatives, compound XII, having two chloro groups, was found to be active against three organisms: *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus niger*. The monochloro derivative XI showed activity only against *Staphylococcus aureus*. None of the N-(2,3,4,6-tetra-0-acetyl-β-D-glucopyranosyl)-5-aralkylidenerhodanine derivatives exhibited activity against any of the five organisms.

Diethylamine, N-methylpiperazine, morpholine and thiomorpholine derivatives of glucosylthiourea had no antimicrobial activity. Also, methoxy, ethoxy, and hydroxy groups on the benzene ring of the glucosylrhodanine derivatives did not seem to confer any antimicrobial activity. However, the presence of chloro groups provided some antimicrobial activity, even though these derivatives were very poorly soluble in water.

None of the tested compounds showed activity against all five organisms in comparison with rhodanine itself, the precursor of these compounds. However, II, III, IX, and XII were active against three organisms and V, VII and VIII exhibited activity against two of the organisms.

It can be concluded that glycosylthioureas possess antimicrobial activities which tend to decrease with greater N-substitution on the NH_2 group. In other words, the primary amine derivatives of glycosylthioureas show higher activities than derivatives of secondary amines. The presence of chloro groups in the benzene ring of glucosyl-5-aralkylidenerhodanines appears to be necessary to provide antimicrobial activity with this type of compound. Glucosylthioureas showed relatively good activities against a Gram-positive organism, *Staphylococcus aureus*, and a yeast, *Candida albicans*, but poor activities against the Gram-negative organisms, *Escherichia coli* and *Pseudomonas aeruginosa*, and a mold, *Aspergillus niger*. Glucosyl-5-aralkylidenerhodanines exhibited a limited profile of antimicrobial activities.

Toxicity levels of several of the glucosylthioureas were determined in mice, and the compounds were found to be remarkably non-toxic. Compounds II, V, VIII and IX were administered intraperitoneally up to a dose of 2000 mg/kg, and no deaths resulted. All animals survived the acute effects and appeared normal after a week. Soon after administration of the compounds, all animals showed decreased activity and labored respiration with the effects more pronounced at the higher dose. These effects disappeared within three hours. In comparison, rhodanine showed an LD_{50} of 164 mg/kg in mice (15).

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