TABLE I

N-CHLOROETHYL DERIVATIVES

$ZNCH_2CH_2Cl$

No.	ZN	Mp, °C	Eluent	Yield, %	Formula[f]
1	2-Chlorophenothiazinyl[c,3]	118–120	*a*	47	$C_{14}H_{11}Cl_2NS$[g]
2	2-(Trifluoromethyl)phenothiazinyl[d]	80–81	*b*	50	$C_{15}H_{11}ClF_3NS$[h]
3	9-Methylbenzo[*a*]phenothiazinyl	88–90	*a*	35	$C_{19}H_{16}ClNS$
4	10-Methylbenzo[*a*]phenothiazinyl	87–88	*a*	45	$C_{19}H_{16}ClNS$
5	Benzo[*b*]phenothiazinyl[e]	163–164	...	37	$C_{18}H_{14}ClNS$

[a] 3:1 ligroin (bp 70–90°)–C_6H_6. [b] Ligroin (bp 60–90°). [c] Woelm neutral alumina, activity grade 1. [d] Kindly supplied by Dr. Harry L. Yale of the Squibb Institute for Medical Research, New Brunswick, N. J. [e] Recrystallized from $CHCl_3$. [f] All compounds showed proper analytical values for C, H, N unless otherwise noted. [g] Not analyzed. [h] C: calcd, 54.62; found, 55.11.

TABLE II

2-[BIS(2-HYDROXYETHYL)AMINO]ETHYL DERIVATIVES

$ZNCH_2CH_2N(CH_2CH_2OH)_2$

No.	ZN	Mp, °C	Eluent	Yield, %	Formula[d]
1	2-Chlorophenothiazinyl	Oil	*a*	82	$C_{18}H_{21}ClN_2O_2S$
2	2-(Trifluoromethyl)phenothiazinyl	Oil	*b*	79	$C_{19}H_{21}F_3N_2O_2S$
3	9-Methylbenzo[*a*]phenothiazinyl	Oil	*a*	97	$C_{23}H_{26}N_2O_2S$
4	10-Methylbenzo[*a*]phenothiazinyl	Oil	*a*	90	$C_{23}H_{26}N_2O_2S$
5	Benzo[*b*]phenothiazinyl	94–95	*c*	72	$C_{22}H_{24}N_2O_2S$

[a] 4:1 C_6H_6–Me_2CO. [b] 9:1 C_6H_6–Me_2CO. [c] 7:3 C_6H_6–Me_2CO. [d] Difficulty was experienced in securing proper elementary analytical values on the bis(2-hydroxyethyl)aminoethyl derivatives. However, the bis(2-chloroethyl)aminoethyl derivatives and the hydrochlorides gave proper analytical values.

TABLE III

2-[BIS(2-CHLOROETHYL)AMINO]ETHYL DERIVATIVES[a]

$ZNCH_2CH_2N(CH_2CH_2Cl)_2$

No.	ZN	Yield, %	Formula[b]
1	2-Chlorophenothiazinyl	43	$C_{18}H_{19}Cl_3N_2S$
2	2-(Trifluoromethyl)phenothiazinyl	60	$C_{19}H_{19}Cl_2F_3N_2S$
3	9-Methylbenzo[*a*]phenothiazinyl	33	$C_{23}H_{24}Cl_2N_2S$
4	10-Methylbenzo[*a*]phenothiazinyl	67	$C_{23}H_{24}Cl_2N_2S$
5	Benzo[*b*]phenothiazinyl	59	$C_{22}H_{22}Cl_2N_2S$

[a] All compounds in this table were oils. [b] C, H, N analyses.

TABLE IV

2-[BIS(2-CHLOROETHYL)AMINO]ETHYL HYDROCHLORIDES

$ZNCH_2CH_2N(CH_2CH_2Cl)_2 \cdot HCl$

No.	ZN	Mp, °C	Yield, %	Formula[a]
1	2-Chlorophenothiazinyl	140–142	83	$C_{18}H_{20}Cl_4N_2S$
2	2-(Trifluoromethyl)phenothiazinyl	140–141	97	$C_{19}H_{20}Cl_3F_3N_2S$
3	9-Methylbenzo[*a*]phenothiazinyl	176–177	94	$C_{23}H_{26}Cl_3N_2S$
4	10-Methylbenzo[*a*]phenothiazinyl	160–161	96	$C_{23}H_{26}Cl_3N_2S$
5	Benzo[*b*]phenothiazinyl	151–153	88	$C_{22}H_{22}Cl_3N_2S$

[a] C, H, N analyses.

stirred at ice-bath temperature for 1 hr and at room temperature for 18 hr. An almost colorless precipitate formed in the course of the reaction. C_6H_6 and H_2O were added to the reaction mixture. The C_6H_6–Et_2O layer was separated and concentrated by evaporation. Ligroin (bp 70–90°) was added to produce a 3:1 mixture by volume of ligroin and benzene. Chromatographic separation of the solution over Alcoa grade F-20 alumina, using a 3:1 mixture of ligroin (bp 70–90°) and benzene as eluent, yielded 2.5 g (67%) of white solid, mp 82–83°.

10-{2-[Bis(2-hydroxyethyl)amino]ethyl}-3-methoxyphenothiazine.—A solution of 2.0 g (6.9 mmoles) of 10-(2-chloroethyl)-3-methoxyphenothiazine in 30 ml of diethanolamine was stirred at 140–150° for 30 hr and cooled to room temperature, and 50 ml of cold H_2O was added. The suspension was extracted ($CHCl_3$) and the extract was washed (H_2O). The $CHCl_3$ was evaporated and the resulting oil was dissolved in C_6H_6. The solution was chromatographed over 60–100 mesh Florisil. Elution with C_6H_6 yielded a small amount of 10-(2-chloroethyl)-3-methoxyphenothiazine. The product, an oil, was eluted with a 1:4 mixture of acetone and benzene. The yield was 1.8 g (73%). *Anal.* ($C_{19}H_{24}N_2O_3S$) H, N; C: calcd, 63.30; found, 63.77, 63.80.

10-{2-[Bis(2-chloroethyl)amino]ethyl}-3-methoxyphenothiazine.—$POCl_3$ (10 ml) was added slowly to 2.0 g (6.8 mmoles) of the corresponding hydroxyethyl compound at ice temperature. The mixture was allowed to warm slowly to room temperature and then heated on a steam bath for 1 hr. Excess $POCl_3$ was removed under reduced pressure and the residual oil was dissolved in acetone. The solution was poured over crushed ice and neutralized (Na_2CO_3). The resulting solution was extracted several times ($CHCl_3$). The combined extracts were washed (H_2O), concentrated, and placed on a chromatographic column of Florisil. The product was eluted with C_6H_6 to yield 0.5 g (19%) of oil. Conversion to the **hydrochloride** gave, after crystallization from $CHCl_3$–Et_2O, 94% yield of colorless crystals, mp 108–110°. *Anal.* ($C_{19}H_{23}Cl_3N_2OS$) C, H, N.

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Cysteine Analogs as Potential Amino Acid Antagonists in Bacteria

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Relatively few compounds have been reported to be cysteine antagonists in microorganisms. Allylglycine[1] inhibits in part utilization of cysteine in bacteria and yeast. Klubes and Schultze[2] found that S-(1,2-dichlorovinyl)cysteine inhibited growth of *Escherichia coli* and showed that the L enantiomer was the more active isomer. Cysteine thioethers[3] from chloroethylenes have also been reported to inhibit growth of fungi and algae.

In the present studies, 21 cysteine or cystine analogs were tested as inhibitors of cysteine or cystine utiliza-

(1) K. Dittmer, H. L. Goering, I. Goodman, and S. J. Cristol, *J. Am. Chem. Soc.*, **70**, 2499 (1948).

(2) P. Klubes and M. O. Schultze, *Biochim. Biophys. Acta*, **78**, 114 (1963).

(3) L. L. McKinney, A. C. Eldridge, and J. C. Cowan, *J. Am. Chem. Soc.*, **81**, 1423 (1959).

tion by *Leuconostoc mesenteroides*, a cysteine–cystine-dependent bacterium, and by *E. coli*, an organism able to synthesize all its amino acid requirements.

The data in Table I list the seven compounds of the 21 tested which at a final concentration of 800 μg/ml inhibited growth of *L. mesenteroides* by at least 50%. L-Cysteine hydantoin and L-cystine hydantoin were the most active and inhibited growth by 70–80% at 10-μg/ml levels. Both compounds also effectively inhibited growth of *E. coli* in a chemically defined medium and caused complete growth inhibition at 20 μg/ml. Of the remaining compounds tested in *E. coli*, marginal growth inhibition was found only with S-carbamoyl-L-cysteine whose minimal inhibitory concentration (MIC) for complete inhibition was 400 μg/ml and N-succinoyl-L-cysteine with a MIC of 800 μg/ml. S-Carbamoyl-L-cysteine[4] has been described as a noncompetitive antagonist of glutamine in bacteria.

TABLE I

COMPARATIVE GROWTH INHIBITION IN *Leuconostoc mesenteroides* BY VARIOUS CYSTEINE AND CYSTINE ANALOGS

	Inhib of growth, %, Concn, μg/ml					
Test compound[a]	25	50	100	200	400	800
N-Acetyl-L-cysteine[b]	10	10	35	75	85	95
N-Propionyl-L-cysteine[b]	25	35	45	85	90	100
L-Cysteine hydantoin	100					
S-Benzyl-L-cysteine methyl ester·HCl[c]	10	40	50	55	60	65
S-Benzyl-N-(10-undecenoyl)-L-cysteine	45	80	95	100		
N,N′-Di(10-undecenoyl)-L-cystinepiperazinium salt	10	15	40	50	60	70
L-Cystine hydantoin	100					

[a] Many of the compounds listed are described elsewhere: T. A. Martin, D. H. Causey, and J. R. Corrigan, *J. Med. Chem.*, 11, 625 (1968). Other cysteines tested which required concentrations >800 μg/ml for 50% growth inhibition were L-N-formyl, D-N-acetyl-β,β-dimethyl, DL-N-acetyl-S-ethyl, DL-N-acetyl-S-benzyl, DL-N-acetyl-S-guanyl, L-N,S-diacetyl, L-N-succinoyl,[b] DL-α-methyl·HCl,[d] L-S-diphenylmethyl-N-formyl,[e] L-S-ethyl,[f] L-S-carbamoyl·0.5H_2O,[g] and L-S-diphenylmethyl;[e] also two L-cystines: N,N′-bis(dichloroacetyl) and N,N′-bis(trichloroacetyl). [b] T. A. Martin, J. R. Corrigan, and C. W. Waller, *J. Org. Chem.*, **30**, 2839 (1965). [c] R. A. Boissonnas, St. Guttmann, P. A. Jaquenoud, and J. P. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955). [d] T. A. Connors and C. W. J. Ross, *Chem. Ind.* (London), 366 (1958). [e] L. Zervas and I. Photaki, *J. Am. Chem. Soc.*, **84**, 3887 (1962). [f] W. O. Foye and M. Verderame, *Pharm. Sci.*, **46**, 273 (1957). [g] T. J. McCord and C. G. Skinner, *Biochem. Prepn.*, **10**, 19 (1963).

Five of the most active compounds (Table II) were tested at equimolar concentrations in the presence of several levels of L-cysteine hydrochloride. Whereas the inhibitory activities of the compounds were equivalent at 1 μg/ml of L-cysteine, the inhibition of N-acetyl-L-cysteine and N-propionyl-L-cysteine was markedly decreased at 10 μg/ml of cysteine and virtually abolished at 100-μg/ml additions.

In contrast, the growth inhibition observed with L-cysteine hydantoin or L-cystine hydantoin was reversible with added L-cysteine or L-cystine but only at lower levels of inhibitor. Concentrations of either inhibitor at 10–40 μg/ml in the presence of 1 μg/ml of L-cysteine·HCl caused an 80–90% inhibition of growth with *L. mesenteroides*. This growth inhibition was completely reversed by the addition of 1000 μg/ml of L-cysteine or L-cystine. Intermediate levels of L-cysteine brought about partial neutralization of the inhibitory activity. Similarly, in *E. coli*, growth was completely inhibited by 20 μg/ml of L-cysteine hydantoin or L-cystine hydantoin in the absence of L-cysteine or L-cystine. In order to maintain this level of growth inhibition with 100-μg/ml additions of L-cysteine or L-cystine, it was necessary to increase the inhibitor levels to 80–160 μg/ml.

TABLE II

GROWTH INHIBITION OF *L. mesenteroides* BY AMINO ACID ANALOGS[a]

L-Cysteine·HCl, μg/ml	Inhib of growth, % A	B	C	D	E
1	91	84	97	100	98
10	57	53	100	99	100
100	0	5	99	98	96
1000	0	7	98	100	98

[a] All test compounds were added at 0.003 *M*. A = N-acetyl-L-cysteine, B = N-propionyl-L-cysteine, C = S-benzyl-N-(10-undecenoyl)-L-cysteine, D = L-cysteine hydantoin, and E = L-cystine hydantoin.

The growth inhibition observed with S-benzyl-N-(10-undecenoyl)-L-cysteine in *L. mesenteroides* appears to be nonspecific and not readily amenable to reversal by cysteine–cystine even at lower levels of inhibitor.

In view of the inability of D-cysteine or D-cystine to substitute for the L isomers in the growth of *L. mesenteroides*, it was not possible to determine the relative effectiveness of the D and DL isomers in antagonizing the growth inhibition of some of the inhibitors described in Table II. Interestingly, the DL isomer of N-acetylcysteine was equivalent to the L isomer in growth inhibitory activity in *L. mesenteroides*. Thus, these data suggest that the inhibition observed does not reside exclusively with only one of the isomers in this particular compound. Furthermore, the growth inhibitory activity associated with N-acetylcysteine itself was completely negated by the introduction of substituents. These compounds, all of which showed no significant growth inhibition for *L. mesenteroides* at concentrations up to 800 μg/ml, were N,S-diacetyl-L-cysteine, N-acetyl-S-benzyl-L-cysteine, N-acetyl-S-ethyl-DL-cysteine, and N-acetyl-S-guanyl-DL-cysteine.

None of the 21 compounds tested showed any significant cysteine–cystine replacement activity for growth of *L. mesenteroides*.

In summary, of the 21 compounds tested as possible antagonists of cysteine–cystine utilization in bacteria, seven compounds showed significant growth inhibition in *L. mesenteroides* and *E. coli*. In most cases, this inhibition was readily reversed by the addition of cysteine–cystine.

Experimental Section

Microbiological Assay Procedures. *L. mesenteroides.*—The procedures used were similar to those previously described[5] with the exceptions that cystine assay medium (Difco) and final culture volumes of 10 ml were employed. Solutions of L-cysteine hydrochloride and most test compounds were sterilized by Seitz filtration. L-Cystine and those test compounds not completely water soluble were suspended in sterile, distilled water, neutral-

(4) J. M. Ravel, T. J. McCord, C. K. Skinner, and W. Shive, *J. Biol. Chem.*, **232**, 159 (1958).

(5) W. A. Zygmunt, R. L. Evans, and H. E. Stavely, *Arch. Biochem. Biophys.*, **102**, 270 (1963).

ized, and sterilized by free flowing steam for 10 min. Cells for the inoculum were grown in Henderson and Snell[6] basal medium and harvested after 6–8 hr at 35°.

E. coli.—The methods used were similar to those described[5] with the exceptions that total volumes of 10 ml/50-ml erlenmeyer flask and incubation on a reciprocating shaker at 28° were employed.

Testing Procedures.—Initially, all compounds were tested at final concentrations of 100, 200, 400, and 800 μg/ml, both in the absence and presence of exogenous L-cysteine hydrochloride (1.5 μg/ml, a level required for about half-maximal growth) with *L. mesenteroides*. With *E. coli* similar levels of compound were evaluated but only in the absence of cysteine.

(6) L. M. Henderson and E. E. Snell, *J. Biol. Chem.*, **172**, 15 (1948).

N-Acylcysteines

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As a continuation of work on N-acylcysteines,[1,2] a number of cysteine and cystine derivatives, including several N-acylcysteine salts, were synthesized. The mucolytic effects of representative compounds have been reported.[3a] The biological activities of many of these derivatives as amino acid antagonists in bacteria are presented elsewhere.[3b]

The selective N-acylation method,[1,2] involving the reaction of a mercaptoamino acid with an equivalent of acetic anhydride in the presence of an acid acceptor or buffering agent, *e.g.*, sodium acetate, was employed for the preparation of N-acetyl-DL-cysteine (**9**) and N-acetyl-D-penicillamine (**10**). The previously unreported **9** was also obtained from inactive cystine[4] and from S-benzyl-DL-cysteine.[5]

N-Formyl-L-cysteine (**1**), prepared[6] originally by the reduction of N,N′-diformyl-L-cystine, was more readily obtained in crystalline form by heating[7] S-diphenylmethyl-N-formyl-L-cysteine with trifluoroacetic acid and phenol.

N-Acetyl-S-guanyl-DL-cysteine (**18**) was prepared by the addition of thiourea to 2-acetamidoacrylic acid.

The compounds not described in the Experimental Section are listed in Table I.

Experimental Section[8]

Examples of Preparative Methods. A. N-Acetyl-S-benzyl-DL-cysteine (**15**) was prepared in 37% yield according to the procedure of Eiger and Greenstein[9] and in 95% yield by the following method.

To a cold (0–3°) solution of 21 g (0.1 mole) of S-benzyl-DL-cysteine[5] and 10 g of NaOH in 100 ml of H_2O was added slowly 15 ml (16.2 g, 0.16 mole) of Ac_2O. The resulting suspension was warmed to 45–50° at which point a dark solution resulted. After 0.5 hr, the reaction mixture was cooled to 0–10° and treated with 75 ml of 4 *N* HCl to precipitate 24 g (95%) of white solid of mp 155–157°. *Anal.* ($C_{12}H_{15}NO_3S$) N, S.

B. N-Acetyl-DL-cysteine (**9**). (**a**) Debenzylation of **15** (21 g, 0.083 mole) was achieved in liquid NH_3 with metallic Na (*ca.* 2.3 equiv). The white sodio derivative was suspended in THF and treated slowly with 17 ml of concentrated HCl to give 8.5 g (63%) of crude product in two crops. Recrystallization from 2-PrOH gave the purified product, mp 127–129°. *Anal.* ($C_5H_9NO_3S$) N, SH.

(**b**) Debenzylation of 42.3 g of S-benzyl-DL-cysteine[5] to the sodio derivative and then selective acetylation[1,2] with 1 equiv of Ac_2O, in 6 ml of HOAc and 100 ml of 80% THF, gave the product in an over-all yield of 60%.

(**c**) DL-Cystine[4] (0.1 mole) in 135 ml of 2 *N* NaOH was acetylated in the cold with 40 ml of Ac_2O to N,N′-diacetylcystine, which, without isolation, was reduced with excess Zn dust. After filtering to remove excess Zn, the filtrate was passed through a Dowex 50W-X8 (200–400 mesh, H^+ form) column and concentrated to give the product in an over-all yield of 23%.

C. N-Acetyl-D-penicillamine (**10**).[10]—Selective acetylation of D-penicillamine·HCl[11] gave this derivative in 77% crude yield. Recrystallization from H_2O gave the purified product of mp 178–179° dec, $[\alpha]^{25}D$ +22° (*c* 1, 50% EtOH), lit.[10] $[\alpha]^{25}D$ +18°. *Anal.* ($C_7H_{13}NO_3S$) N, SH.

D. Piperazinium N-Acetyl-L-cysteine Hydrate (**4**).—To a solution of 16.3 g (0.1 mole) of N-acetyl-L-cysteine (NAC) in 50 ml of MeOH was added a solution of 9.7 g (0.05 mole) of piperazine hexahydrate in *ca.* 50 ml of MeOH. The reaction temperature increased from 20 to 31°. After cooling, the precipitated solid was collected, washed with MeOH, and dried; yield 14.3 g (66%). Recrystallization of 5 g from 150 ml of MeOH–EtOH (1:2) gave 3.1 g of white solid, mp 152.5–154.5° dec. *Anal.* $[(C_5H_9NO_3S)_2 \cdot C_4H_{10}N_2 \cdot H_2O]$ C, H, SH.

E. N,N′-Dichloroacetyl-L-cystine (**23**).—A solution of 32.4 g (0.22 mole) of $CHCl_2COCl$ in 100 ml of anhydrous Et_2O and 110 ml of 2 *N* NaOH were added simultaneously over 1 hr to a cold solution (4–8°) of 24 g (0.1 mole) of L-cystine in 103 ml of 2 *N* NaOH. The reaction mixture was stirred for 4 hr, allowed to stand overnight, and acidified with 6 *N* HCl. Extraction with EtOAc yielded 13 g (30%) of product. It was recrystallized from H_2O in 67% yield, mp 166.5–168.5° dec, $[\alpha]^{25}D$ −53.6° (*c* 2.5, 1 *N* NaOH). *Anal.* ($C_{10}H_{12}Cl_4N_2O_6S_2$) N, S, Cl.

F. L-Cysteine Hydantoin (**11**).[12]—Fifty grams of dried Dowex 50W-X8 (200–400 mesh, H^+ form) was added slowly to a warm (50–60°) mixture of 20 g (0.0688 mole) of L-cystine hydantoin (**25**),[13] 10 g of Zn dust, and 125 ml of H_2O. After holding 2.5 hr at 55–60°, the mixture was filtered to remove the resin (Zn^{2+} form) and excess Zn dust. The filter cake was washed with hot H_2O and the total filtrate was cooled to give 15.6 g (77%) of product in three crops. Recrystallization from H_2O gave 10.5 g (53%) of pure product of mp 147.5–149°, $[\alpha]^{25}D$ −97.8° (*c* 1, DMSO). *Anal.* ($C_4H_6N_2O_2S$) C, H, N.

G. N-Acetyl-S-guanyl-DL-cysteine (**18**).—A stirred suspension of 8.1 g (0.106 mole) of thiourea, 20 ml of 5.4 *N* HCl, 40 ml of CH_3OH, and 12.9 g (0.1 mole) of 2-acetamidoacrylic acid[14] was warmed at 38–40° for 5 hr. At the end of this time the slightly turbid solution was filtered. The filtrate was concentrated to a semisolid which was dissolved in 15 ml of H_2O and treated with 8.4 g of $NaHCO_3$ to bring the pH of the solution to *ca.* 6. Addition of 20 ml of MeOH precipitated the crude product which was recrystallized from 75% MeOH to give 4 g (19%) of pure product, mp 195.5–196.5° dec. The nmr spectrum was consistent with the structure. *Anal.* ($C_6H_{11}N_3O_3S$) C, H, N.

H. L-Cysteinium N-Acetyl-L-cysteinate (**2**).—To a solution of 48.4 g (0.4 mole) of L-cysteine and 500 ml of H_2O was added

(1) T. A. Martin and C. W. Waller, U. S. Patent, 3,184,505 (1965).

(2) T. A. Martin, J. R. Corrigan, and C. W. Waller, *J. Org. Chem.*, **30**, 2839 (1965).

(3) (a) A. L. Sheffner, *Ann. N. Y. Acad. Sci.*, **106**, 298 (1963); U. S. Patent 3,091,569 (1963); *Pharmacotherapeutica*, **1**, 46 (1965); (b) W. A. Zygmunt and T. A. Martin, *J. Med. Chem.*, **11**, 623 (1968).

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(8) We are grateful to Messrs. John G. Schmidt, Clarence Kennedy, and Charles M. Combs of our Control Laboratories for the analytical and instrumental data. The infrared spectra of all the described compounds were consistent with the assigned structures. The melting points are corrected (Thomas–Hoover capillary apparatus). In general, all preparative operations involving sulfhydryl compounds were carried out in an atmosphere of nitrogen, using deionized water. Where analyses are indicated only by symbols of the elements analytical results obtained for those elements were within ±0.4% of the theoretical values.

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