Acknowledgment. Crystallographic computations were performed at the University of Connecticut Computer Center.

Supplementary Material Available: Figure 3, torsion angles, bond distances, angles, and pertinent bond distances; Table II, atomic positional parameters; and Table III, a listing of the structure-factor amplitudes and the atomic thermal parameters (29 pages). Ordering information is given on any current masthead page.

Notes

Structure-Activity Relationship of Diphenylthiourea Antivirals

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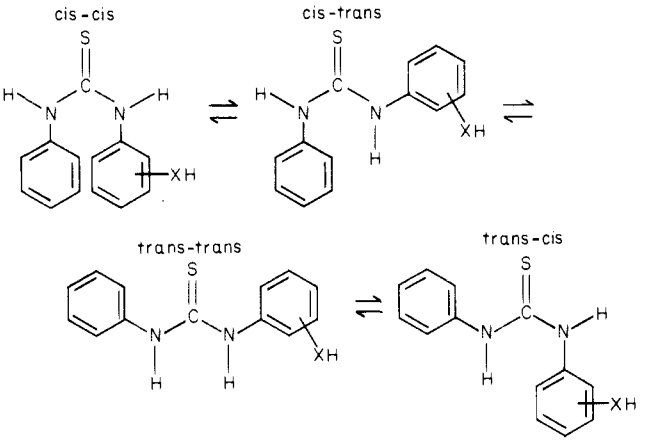
The dependence between chemical structure and antiviral activity of *N,N'*-diphenylthioureas is studied by synthesis and testing of model compounds and use of conformational data. The analysis revealed a number of structural features as essential for the antiviral effect: (1) the presence of an intact -NHC(=S)NH- grouping; (2) the presence of a substituent of the XH type (X = O, NH) in the aromatic ring; (3) the distance between these substituents and the sulfur atom in the 6.68–6.75 Å range for the active compounds; (4) a trans conformation of the -C(=S)NH- group bound to the substituted phenyl ring. The directed synthesis of compounds satisfying the above requirements yielded the derivative *N*-phenyl-*N'*-(*m*-aminophenyl)thiourea exhibiting a very high antipicornavirus activity in vitro and several other active analogues (four out of seven synthesized). The possible mechanism of interaction between the active diphenylthiourea derivatives and the viral target is discussed.

N,N'-Disubstituted thioureas were reported to show a strong antipicornavirus activity both in vitro and in vivo.[1-6] The structure–activity analysis of 54 *N*-phenyl-*N'*-aryl- or -alkylthiourea derivatives on poliovirus 1 (Mahoney) growth in cell cultures revealed a marked antiviral effect in compounds containing a hydroxyl group in the phenyl or alkyl radical bound to one of the nitrogen atoms, the spatial location of the hydroxyl group being of critical importance. The molecular geometry of these compounds shows that the most active among them have one common feature: the distance between the oxygen atom of the hydroxyl group and the nitrogen atom attached to the same ring is within the 4.73–4.81 Å range. Besides, it was found that the electronic effect of the substituents is not significant. Thus, for example, the corresponding methoxy derivatives which have a similar electronic effect are inactive.[1] The governing role of the geometric factor in antiviral activity made us study the conformation of those derivatives, since the -NHC(=S)NH- grouping offers several possible conformations. IR and NMR spectral analysis of diphenylthioureas in neutral medium reveals a state of equilibrium between several conformational forms[7] (Scheme I). Obviously, a systematic structure–activity relationship study should cover all conformational possibilities.

Scheme I



Results and Discussion

For the purpose of a more detailed study of the structure–activity relationship of diphenylthioureas, we carried out the synthesis of a series of model compounds and diphenylthiourea derivatives expected to exhibit an antiviral effect on the basis of data accumulated until now. Antiviral activity was tested in vitro against three human enteroviruses—poliovirus 1 (Mahoney), Coxsackie B1, and ECHO19—using the agar-diffusion plaque-inhibition screening method of Rada and Zâvada,[8,10] which in the case of poliovirus 1 was supplemented with the one-step growth cycle setup (two-stage system).[1,9,10] The two most active

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(10) Details of the methods used for antiviral testing are given under Experimental Section.

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Table I. Effect of *N,N'*-Diphenylthiourea Derivatives and Model Compounds on Human Enteroviruses Growth in FL Cells

	effect in the agar-diffusion plaque-inhibition test[a]												effect on poliovirus 1 reproduction in the 1-step growth cycle setup, % of inhibn[b]	
	poliovirus 1				Coxsackie B1				ECHO19					
no.	inhibn zone: ϕ, mm	toxicity zone: ϕ, mm	$\Delta\phi$, mm	antiviral effect[c]	inhibn zone: ϕ, mm	toxicity zone: ϕ, mm	$\Delta\phi$, mm	antiviral effect	inhibn zone: ϕ, mm	toxicity zone: ϕ, mm	$\Delta\phi$, mm	antiviral effect	0.1 mM	0.2 mM
1	0	0	0	–										
2	33.0	15.8	17.2	+									99.42	99.94
3	36.1	17.9	18.2	+										99.92[d]
4	26.7	11.4	15.2	+	39.0	33.3	5.7	±	37.0	29.0	8.0	±	73.47	89.05
5	0	0	0	–										
6	28.0	9.0	19.0	+	44.0	41.0	3.0	–	39.0	37.0	2.0	–	80.61	98.67
7	28.3	18.0	10.3	+	36.5	16.7	19.8	+	40.0	20.0	20.0	++		99.98
8	0	0	0	–										
9	53.7	39.4	14.3	+	40.0	34.0	6.0	±	0	43.5	0	–		
10	0	0	0	–										
11	36.2	32.0	4.2	–										
12	13.3	7.3	6.0	±	9.2	6.5	2.7	–	11.2	6.5	4.7	–	66.33	96.63
13	0	0	0	–										
14	0	0	0	–										
15	0	0	0	–										
16	0	0	0	–										
17	26.0	22.0	4.0	–										
18	0	0	0	–										
19	0	0	0	–										

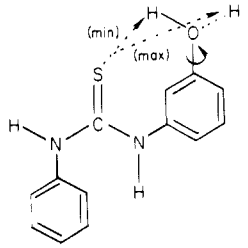
[a] The compounds were applied as 4% solution in ethanol. [b] The concentrations used were preliminarily determined as well-tolerated by the cells. [c] Criteria for antiviral effect in the agar-diffusion plaque-inhibition test (ϕ = diameter): –, $\Delta\phi \leq 5$ mm; ±, $\Delta\phi = 6$–10 mm; +, $\Delta\phi = 11$–20 mm; ++, $\Delta\phi \geq 20$ mm. [d] The concentration of 3 used was 0.15 mM, since a concentration of 0.20 mM is a slightly toxic.

Table II. Geometrical Characteristics and Antiviral Activity of *N,N'*-Diphenylthiourea Molecules Containing Substituents of the XH Type (X = O, NH)

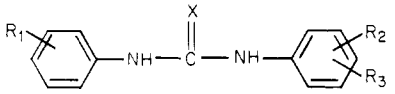
	trans[a]		cis[a]		antiviral act.[b]		
substit	$r_{S\rightarrow H}$,[c] Å	$r_{S\rightarrow X}$,[d] Å	$r_{S\rightarrow H}$,[c] Å	$r_{S\rightarrow X}$,[d] Å	poliovirus 1 (Mahoney)	Coxsackie virus B1	ECHO virus 19
o-OH	4.68, 5.97	5.05	4.27, 6.05	5.07	–[e]	–[e]	–[e]
o-NH_2	4.75, 6.06	5.14	4.23, 6.10	5.08	+	–	–
o-COOH	4.19, 6.75	4.53, 6.55	3.41, 6.94	4.34, 6.48	+[e]	–[e]	++[e]
m-OH	6.68, 6.94	6.42	7.14, 8.06	7.31	++	++[e]	++[e]
m-NH_2	6.78, 7.07	6.50	7.20, 8.17	7.34	+	+	++
m-COOH	6.70, 7.05	7.20, 7.47	6.53, 8.65	7.40, 8.62	–[e]	–[e]	–[e]
p-OH	5.95, 6.74	6.02	8.19, 8.52	7.87	±[e]	++[e]	–[e]
p-NH_2	6.04, 6.79	6.10	8.36, 8.65	7.97	–	–	–
p-COOH	5.66, 7.30	6.36, 7.30	8.14, 8.67	8.62, 8.95	–[e]	–[e]	–[e]

[a] The two possible end values for the planar structure of the molecules are given. [b] In the agar-diffusion plaque-inhibition test[8] and the gradient plaque-inhibition test.[1] [c] H = active hydrogen atom in substituents OH, NH_2, COOH. [d] X = O, N. [e] Antiviral activity data from ref 1 and 2.

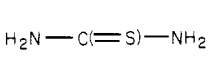
Chart I



antivirals from the previous screening, *N*-phenyl-*N'*-(*m*-hydroxyphenyl)thiourea (**2**) and *N*-phenyl-*N'*-(*m*-hydroxy-*p*-carboxyphenyl)thiourea (**3**), were also tested for comparison (Table I).



	R_1	R_2	R_3	X
1	H	H	H	S
2	H	*m*-OH	H	S
3	H	*m*-OH	*p*-COOH	S
4	*m*-OH	*m*-OH	H	S
5	*p*-OH	*p*-OH	H	S
6	H	*o*-NH_2	H	S
7	H	*m*-NH_2	H	S
8	H	*p*-NH_2	H	S
9	*o*-NH_2	*o*-NH_2	H	S
10	*p*-NH_2	*p*-NH_2	H	S
11	H	*o*-OH	H	O
12	H	*m*-OH	H	O
13	H	*p*-OH	H	O
14	H	*o*-NH_2	H	O
15	H	*m*-NH_2	H	O
16	H	*p*-NH_2	H	O



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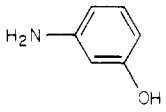

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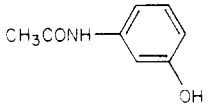

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Some of the studied compounds resemble fragments of the molecule of *N,N'*-diphenylthioureas. Their study was aimed at determining more precisely the lead structure responsible for the antiviral effect of this group of compounds. *m*-Aminophenol (**18**) and *m*-hydroxyacetanilide (**19**) contain a $-NHC_6H_4$(*m*-OH) grouping with a suitable distance between the NH and OH groups, which was expected to account for the antiviral effect. The lack of effect, however, shows that this combination of substituents in the aromatic ring is not of decisive importance.

N,N'-Diphenylurea derivatives (**11** and **13–16**), structural analogues of active thioureas, also lack an antiviral effect. It is of interest to note that only *N*-phenyl-*N'*-(*m*-hydroxyphenyl)urea (**12**) shows some antiviral activity, being a complete structural analogue of the most active thiourea, **2**. Obviously, the sulfur atom in the thiocarbonyl group is of essential importance for antiviral activity. The difference in the effects of ureas and thioureas could be attributed to the stronger electron-donor properties of the sulfur atom, facilitating the formation of associative, coordinative, and other intermolecular bonds. Unsubstituted thiourea (**17**) and diphenylthiourea (**1**) are not active.

The next step in the structure–activity relationship analysis was the synthesis and study of *N,N'*-diphenylthioureas in which the hydroxyl group is replaced with an amino group having similar properties. These properties are expressed in the ability of those groups to form hydrogen bonds, on the one hand, and to coordinate various atoms by their free electron pairs, on the other hand. A

Chart II

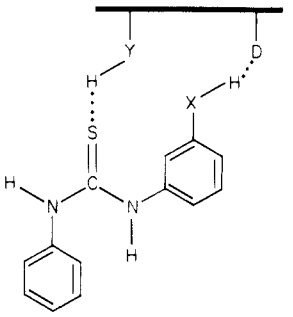
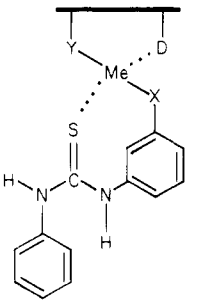


Chart III



number of these type of compounds (**6**, **7**, and **9**) possess marked antiviral activity. Of particular interest among them is **7** [*N*-phenyl-*N'*-(*m*-aminophenyl)thiourea], which shows an extremely high activity.

Symmetric substitution by an hydroxyl or amino group at the ortho or meta position in the two rings results in partially retained activity (**4** and **9**), whereas substitution at the para position, as expected, makes the compounds inactive (**5** and **10**).

Further analysis of the structure–activity relationship required a more detailed examination of the role of the geometric factor for the compounds effect. New data on the conformation of the thioamide group[7] show that several possible conformational forms should necessarily be considered. Attention was predominantly focused on the determination of the mutual position of those groups, which were shown above to be of importance for antiviral activity. Table II shows the calculated distances between the thiocarbonyl sulfur atom and the atoms of the functional groups XH (OH, NH_2, and OH in COOH) located at the ortho, meta, and para position of the aromatic ring. Interatomic distances were calculated using standard values for the geometrical parameters of the molecules.[11] The value for the C=S bond distance used was 1.64 Å.[12] Due to distance variations as a result of rotation around single bonds, two end values (the minimal and the maximal) are shown for all the distances S → H and S → X in Chart I, except for the cases when, because of symmetry, distances do not vary in rotation. Rotational variations are especially great with the carboxyl group as a substituent. It should be noted, however, that the conjugation between the carbonyl group in COOH and the benzene ring will favor the flat conformations which correspond to the two end values of the intervals.

The above presented data reveal some common structural characteristics for the active compounds (*m*-OH, *m*-NH_2, *o*-COOH). In the trans conformation, the distance $r_{S \to H}$ for all three compounds is within the 6.68–6.75 Å

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(12) A. Yamaguchi, R. B. Penland, S. Mizushima, P. J. Lane, C. Curran, and J. V. Kuagliano, *J. Am. Chem. Soc.*, **80**, 521 (1958).

range, and the distance $r_{S \rightarrow X}$ (X = NH, O) is in the interval 6.42–6.55 Å. In the cis conformation, the correlation of data is lower. If we assume that the sulfur atom and the oxygen and nitrogen atoms are the two active centers which bind the corresponding viral receptors, the trans conformation is obviously more favorable, as shown in Chart II. This is partially confirmed by the above-cited data for the distance $r_{S \rightarrow H}$, which show consistency only for the trans conformation.

The following two hypotheses are possible with regard to the mechanism of interaction between the diphenylthioureas and the viral target: (1) Simultaneous participation of the S atom and the H atom in hydrogen bond formation (Chart II), where D is an electron-donor atom, Y could be O, S, or NH, and X is O or NH. (2) An alternative is the participation of thioureas in chelate complexes with metal ions bound to virus-specific proteins (Chart III). The presence of heavy metal ions was reported for several virus-specific proteins (enzymes).[13-16]

Formation of an eight-membered chelate ring might not seem highly probable. Similar chelate complexes, however, were reported.[17-19] We also have experimental evidence to support such a chelating hypothesis. Addition of copper or zinc ions to the culture medium markedly increases the antiviral effect of **2** on poliovirus growth.[20] We think the most probable reason for this increase is the easier chelate formation between the tested compound and the virus-specific target. Of course, one should also consider the possibility for a synergistic effect of compound **2** and the heavy metal ions, although the latter were applied in concentrations which do not affect poliovirus reproduction.[20]

Both mechanisms suppose favorable spatial location of the sulfur atom and the proton-donor substituents (OH, NH_2, COOH). This requires, on the one hand, a trans –C(=S)NH– conformation for the NH bond in the substituted phenol ring and, on the other hand, sets limits on the possible distance between the groups taking part in the complex formation.

Conclusion

Studies of the relationship between the chemical structure and the antiviral activity of the *N,N'*-diphenylthioureas reveal a number of structural features as essential for the effect of these compounds: (1) The presence of an intact –NHC(=S)NH– grouping; the substitution of the sulfur atom for oxygen leads to a loss or considerable decrease in the effect. (2) The presence of a substituent of the XH type, where X = O or NH (in OH, COOH, and NH_2 groups) in the aromatic ring; a simultaneous substitution of both aromatic rings results in a certain decrease of activity. (3) The spatial location of these functional groups to the –NHC(=S)NH– grouping is of essential importance for the antiviral activity. The distance between the sulfur atom and the substituents, $r_{S \rightarrow X}$, and the hydrogen atom of the substituent, $r_{S \rightarrow H}$, shows values which for the most active compounds are in a very narrow range, 6.68–6.75 and 6.42–6.55 Å, respectively. (4) The most probable active form of the compounds is in the trans conformation, which is more favorable, both for hydrogen bond formation and chelation with the participation of two active centers in the molecule.

The directed synthesis of compounds which would meet the requirements 1–4 yielded the derivative **7**, which shows the highest antipicornavirus activity in vitro of all studied thioureas. The synthesis of similar structural analogues led to a series of active derivatives (four out of seven).

Experimental Section

The compounds studied were synthesized using known methods:[21] (a) from phenylisocyanate and the corresponding aromatic amines (compounds **13**,[22] **11** and **15**,[23] **14** and **16**),[24] (b) from CS_2 and *p*-phenylenediamine (**10**)[25] or *m*-aminophenol (**4**),[26] (c) from phenyl isothiocyanate and the respective aromatic amine (**6–8**),[24] (d) from $CSCl_2$ and *p*-aminophenol (**5**),[27] (e) from *m*-hydroxyphenylurea and aniline (**12**).[25]

Agar-Diffusion Plaque-Inhibition Test. Monolayer FL cell cultures in Anumbra (Czechoslovakia) petri dishes (90 mm diameter) were inoculated (adsorption: 60 min at 20 °C) with 1.88 mL of a virus dose, giving semiconfluent plaques in 24–48 h of incubation at 37 °C. A 12.5-mL agar overlay (1% agar in MM Eagle Difco containing 10% heated calf serum, 1.65 mg/mL sodium bicarbonate, 100 units/mL each of penicillin and sterptomycin) was placed on the dish. One to three glass cylinders (6 mm diameter) were fixed in the agar overlay, in which 0.1 mL of each tested compound was added (usually in 2–4% solution). A second overlay was added following incubation, containing 1.5% agar and 0.02% neutral red in physiological saline. The antiviral effect of a given compound was recorded on the basis of the size [diameter (ϕ), in mm] of the zone of plaque inhibition and the zone of cytotoxicity (three to five cylinders per compound in a minimum of two petri dishes) and designated as follows: –, $\Delta\phi \leq 5$ mm; ±, $\Delta\phi$ = 6–10 mm; +, $\Delta\phi$ = 11–20 mm; ++, $\Delta\phi \geq 20$ mm.

One-Step Growth Cycle Setup. Monolayer FL cell cultures in Brosse 60-mL flasks (2.4×10^6 cells/flask) were inoculated at multiplicity above 90. After virus adsorption (60 min at 4 °C), the inoculum was removed and the cells were washed three times with Hanks' saline. The tested compounds were added to the maintenance medium immediately after virus inoculation in maximal tolerated concentration (MTC), preliminarily defined by testing the effect of various concentrations of a given compound on the cell growth curve and cell morphology and metabolism. Maintenance medium (5 mL per flask): Eagle's MBE Eutroph with 5% calf serum and antibiotics. At the end of the growth cycle (8th h) the cultures were frozen and thawed three times, and the total infectious virus produced was determined (in pfu/mL). The percentage of inhibition was calculated in relation to the untreated control.

Acknowledgment. The authors thank Lilly Goranova for her excellent technical assistance.

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