Synthesis and antiviral activity of bis-spirocyclic derivatives of rhodanine*

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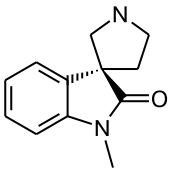
Bis-spiro heterocycles containing spiro units at the 1,3-positions of the pyrrolizidine (isothiapyrrolizidine) moiety were synthesized by the reaction of unstabilized azomethine ylides, which were generated *in situ* from isatin and proline (isothiaproline), with hetarylidene-substituted rhodanines. Quantum chemical calculations of potential energy surface sections and descriptors controlling the regioselectivity of cycloaddition were carried out. For a number of compounds *in vitro* activity against the influenza virus A/California/07/09 (H1N1)pdm2009 was experimentally established.

Key words: spiro cycle, rhodanine, azomethine ylide, 1,3-dipolar cycloaddition, influenza, antiviral drugs, chemotherapy, AM1, DFT B3LYP/6-31G**.

Natural alkaloids containing the spiro-pyrrolidine-oxindole moiety of type **1** (for example, horsfiline, pteropodine, mitraphylline, and rhynchophylline) show a broad spectrum of biological activities.[1] Due to this fact, the derivatives and analogs of such alkaloids, which are of interest for medicinal chemistry, have attracted attention. Main approaches to the synthesis of compounds with the spiro-pyrrolidine-oxindole ring system are covered in the reviews[2,3] and references cited therein. In recent years numerous experimental studies showed that the use of scaffolds not only of type **1** but also of type **2** hold promise for the design of structures exhibiting antitumor,[4—6] antimicrobial,[7—9] antifungal,[10] or antituberculosis activity[11—13] and capable of inhibiting acetylcholinesterase[14,15] and the MDM2 oncogene.[16]



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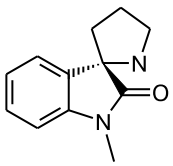

2

One of the most efficient methods for the formation of the spiro-oxindole core is based on the 1,3-dipolar addition of unstabilized azomethine ylides **3** (generated *in situ* from isatins and *N*-alkylamino acids) to the carbon-carbon double bond of type **4** bearing an electron-withdrawing group.[17] One of efficient methods for the construction of such bonds, which are readily involved in the [3+2]-cycloaddition, is based on the reaction of heterocycles containing an active endocyclic methylene group (for example, of thiazolidinones,[18] thiazolotriazines,[19] and oxindoles[20]) with carbonyl compounds (for example, with isatins[21] or 2,3-dioxopyrrolo[2,1-*a*]isoquinolines[22]) (Scheme 1). The regioselectivity of the reaction of asymmetric azomethine ylide with an asymmetric dipolarophile is an important problem, the solution of which requires the analysis of the mechanisms of polar [3+2]-cycloaddition.

Earlier,[23] we have synthesized bis-spirocyclic compounds **8** by the reactions of isatin **5**, thiaproline **6**, and arylidene derivatives of rhodanine **7**, and studied these compounds by NMR spectroscopy and X-ray diffraction (Scheme 2). The evaluation of their biological activity showed that compounds **8** are promising agents for the fight against influenza viruses, as exemplified by the pandemic influenza virus A/California/07/09 (H1N1)pdm2009.

The aim of the present work is to study the mechanism of the formation of bis-spirocyclic structural analogs and isomers of **8** and to evaluate their antiviral activity.

We performed the reactions of arylidene derivatives of rhodanine **9a—d** with azomethine ylides generated *in situ* from isatin and isothiaproline (**10a**) and from isatin and proline (**10b**). In all cases, the reactions giving bis-spiro-

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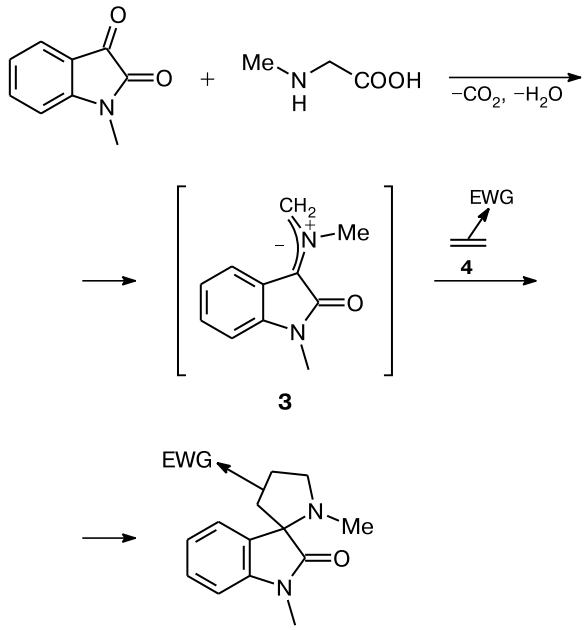
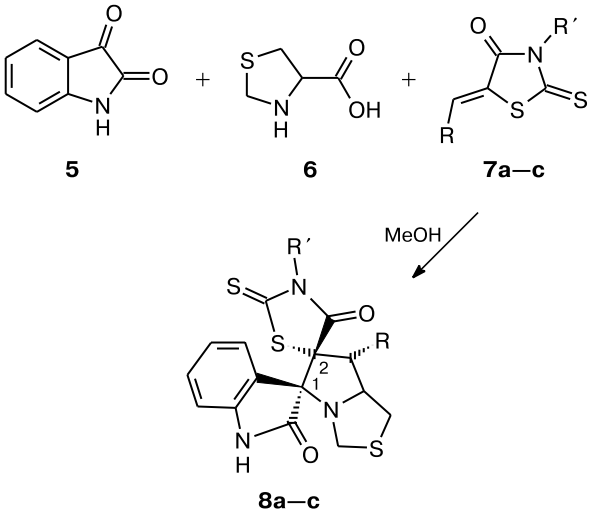
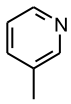
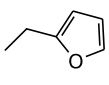
Scheme 1



Scheme 2

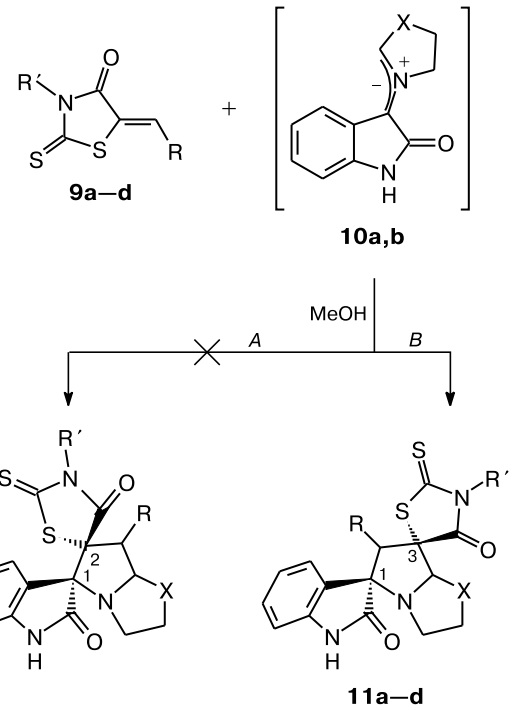


R =  (**a**, **b**), Ph (**c**); R′ =  (**a**), Bn (**b**, **c**)

cyclic compounds **11a**—**d** occur with perfect diastereoselectivity and regioselectivity. It should be emphasized that, unlike bis-spirocyclic compounds **8** prepared with the use of thiaproline, bis-spirocyclic compounds **11**, which were synthesized with the participation of isothiaproline and proline, contain spiro units in the 1,3-arrangement rather than in the 1,2-arrangement (Scheme 3).

This is evidence that the cycloaddition follows exclusively the pathway *B* and not the pathway *A*. Figure 1 and

Scheme 3



10: X = S (**a**), CH_2 (**b**)

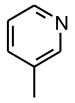
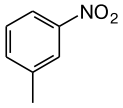
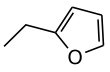
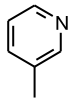
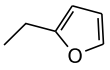
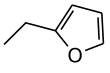
11	R	R′	X
a		Bn	S
b			S
c			CH_2
d			CH_2

Table 1 present the characteristics of the most informative ^1H, ^{13}C, and ^{15}N signals of compound **11c** obtained using one- and two-dimensional NMR experiments. The ^1H NMR spectrum shows a singlet of the proton 2′, and the spin-spin coupling between the protons 2′ and 7a′ is absent. These facts attest to the 1,3-arrangement of the spiro units.

In order to explain this unexpected reversal of the regioselectivity of the cycloaddition in going from thiaproline to isothiaproline, we studied the mechanism of the reaction of azomethine ylide based on isothiaproline **10a** with dipolarophile **12** by quantum chemical methods.

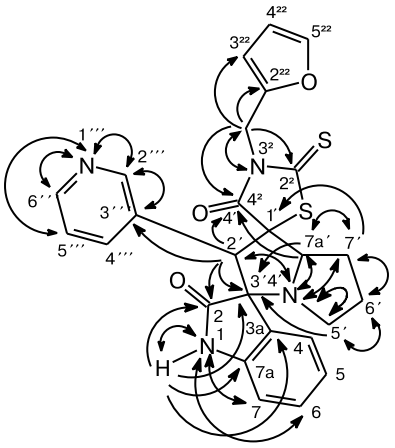


Fig. 1. Correlations between protons and the corresponding nuclei in the HMBC ($^1H \leftrightarrow ^{15}N$; $^1H \rightarrow ^{13}C$) and COSY ($^1H \leftrightarrow ^1H$) NMR spectra.

A dipolarophile containing only hydrocarbon substituents (R = Ph, R′ = CH_2Ph) was used as the model.

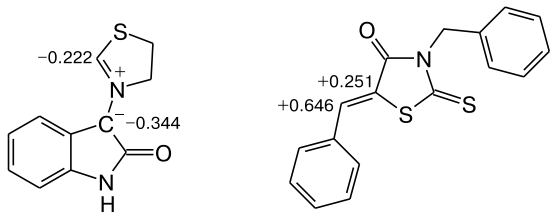
On the one hand, perturbations of the reaction centers introduced by bulky peripheral substituents inevitably complicate quantum chemical calculations. On the other hand, it was experimentally shown that peripheral substituents of different electronic nature (π-excessive or π-deficient heterocycles) have no effect on the regioselectivity of the process. Thus, the pathways *A* and *B* in Scheme 3 are independent of the nature of the substituents in the rhodanine component of the reaction.

Table 1. Assignment of signals for bis-spirocyclic compound **11c**

Atom, group	NMR (δ, *J*/Hz)		
	^1H	^{15}N	^{13}C
HN(1)	10.78 s	141.94	—
C(2)	—	—	177.88
C(1′)	—	—	69.92
HC(2′)	4.73 s	—	63.40
C(3′)	—	—	74.97
N(4′)	—	64.43	—
H_2C(5′)	2.52—2.58 m 2.58—2.66 m	—	46.83
H_2C(6′)	1.79—1.87 m 2.02—2.13 m	—	27.60
H_2C(7′)	1.87—1.95 m 2.02—2.13 m	—	29.51
HC(7a′)	4.51 (dd, *J* = 6.4, *J* = 8.2)	—	74.45
C(2″)	—	—	200.71
N(3″)	—	191.81	—
C(4″)	—	—	176.65
H_2CN(3″)	4.92, 5.05 (both d, *J* = 15.55)	—	40.54
N(1‴)	—	317.34	—

In the first step, we calculated (DFT/B3LYP/6-31G**) the Parr global and local electrophilicity and nucleophilicity indices, which have earlier been shown to be efficient in the prediction of the regioselectivity of a series of polar cycloaddition reactions.[24]

An analysis of these reactivity descriptors shows that the oxindole carbon atom of azomethine ylide based on isothiaproline **10a**, which corresponds to the initial coordination site of the electron-deficient carbon atom of the ylidene moiety in dipolarophile **12**, has the highest nucleophilicity.



10a **12**

Since the compounds under study have complex structures, in the next step we evaluated the pathways *A* and *B* by the semiempirical AM1 method taking into account the solvent effects.[25] The results presented in Fig. 2 as sections of the minimum-energy pathways (MEPs *A* and *B*) of the reactions are indicative, firstly, of the formation of the pre-reactive complexes $\mathbf{PRC_A}$ and $\mathbf{PRC_B}$ in the initial step of both processes, secondly, of the concerted (one-step) mechanism of the reactions under study, and, thirdly, of the thermodynamic favorability of the pathway *B*, which is in complete agreement with the experimental data.

For the energetically favorable pathway *B*, we performed detailed DFT calculations both in the gas phase and solution. According to the results of these calculations, the cycloaddition starts with (as shown in Fig. 2) the barrierless formation of the pre-reactive complex **PRC**, which is more stable than the infinitely separated reactants, by 7.0 kcal mol^{-1} in the gas phase and by 3.5 kcal mol^{-1} in methanol. Then MEP of the reaction follows through the transition state **TS** (the principal interatomic distances for **TS** are given in Fig. 3). The activation barrier for the

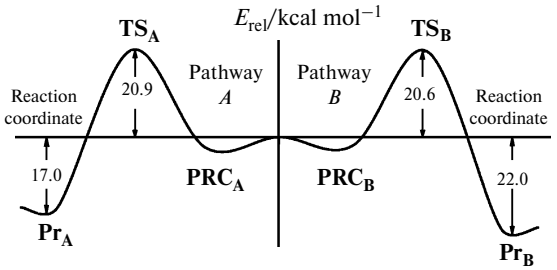


Fig. 2. Minimum-energy pathways *A* and *B* of the [3+2]-cycloaddition in methanol calculated by the AM1 method.

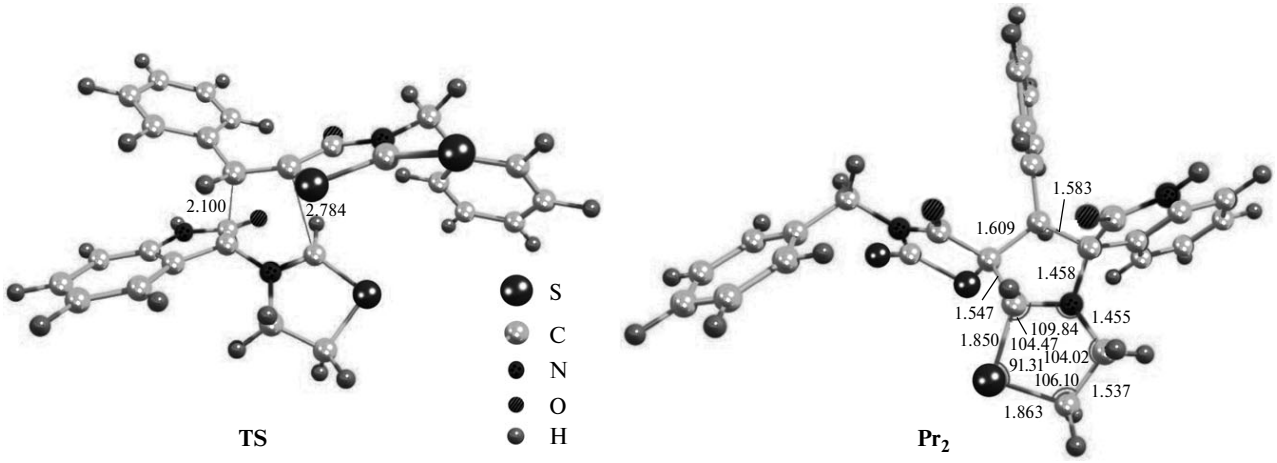


Fig. 3. Structures of the transition state **TS** and the final product $\mathbf{Pr_2}$ calculated by the DFT method taking into account the solvent effects. The interatomic distances are given in angströms and the angles in degrees.

process in the solvent is lower compared with that in the gas phase.

This gives rise to the system $\mathbf{Pr_1}$ with the expected 1,3-arrangement of the spiro units corresponding to the local minimum in the PES. Then, according to the results of DFT calculations, the low-barrier inversion of the thiazolidine ring of the thiapyrrolizidine moiety occurs in this intermediate through the transition state $\mathbf{TS}_i$ (the activation barrier is lower than 1 kcal mol^{-1}) to form the final reaction product $\mathbf{Pr_2}$ (see Figs 3 and 4).

Therefore, the quantum chemical calculations showed that the experimentally observed regioselectivity of cycloaddition is not an artifact and depends only on the electronic parameters of azomethine ylide.

It is particularly important to find descriptors for the reliable prediction of the regio- and stereoselectivity of cycloaddition because some of the compounds under consideration exhibit considerable antiviral activity. The further synthetic optimization of the leading structure can be substantially accelerated by means of the preliminary virtual screening (using, for example, molecular docking)[26] of a set of variable structures of similar type, and the correct construction of this set requires the reliable prediction of the structures.

At the present time, compounds of two chemical groups active against different viral targets are employed for the treatment of influenza infections. Compounds of one group (adamantane derivatives) inhibit the virus-specific M2 proton channel. The domestic drug remantidine and its analog, amantadine,[27] may be referred to as examples of this group. Another group of compounds is targeted against the viral enzyme neuraminidase necessary for budding of progeny influenza virions. There are two drugs, namely oseltamivir (trade mark Tamiflu) and zanamivir (Relenza),[28] approved for the use in Russia, as well as two drugs, namely peramivir (Rapiacta)[29] and laninamivir (Inavir),[30] approved for the influenza treatment in foreign countries. Due to a high rate of viral replication and the ability to rapidly mutate, the virus can develop strains resistant to both classes of approved drugs.[31,32] This reduces the efficiency of chemotherapy of influenza infections. Therefore, there is a continuing need for the search for and design of new antiviral drugs having alternative targets and, probably, a broader spectrum of activities.

Spiro derivatives of pyrrolidine, which are structurally similar to bis-spirans **8** and **11** prepared in the present study, showed activity against *Mycobacterium tuberculosis*.[33] The antiviral activity of this class of compounds has not been described earlier.

The antiviral activity of bis-spirocyclic derivatives of rhodanine, which we have described previously[23] (**8a—c**) and synthesized for the first time (**11a—d**), was evaluated against the influenza virus A/California/07/09 (H1N1)pdm2009. The results are summarized in Table 2.

The statistically reliable estimation of the contribution of structural factors to the biological activity cannot be made because of the insufficient number of the synthesized and characterized compounds. Nevertheless, based on the results of the present study, it can be concluded that

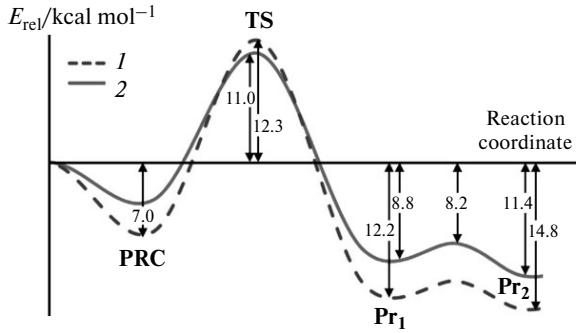


Fig. 4. PES sections along the MEP of the [3+2]-cycloaddition in the gas phase (*1*) and in methanol (*2*). The calculations were carried out by the DFT method.

Table 2. Biological activity characteristics of bis-spirocyclic compounds **8a—c** and **11a—d**

Compound	Biological activity characteristics		
	CTD_{50}	EC_{50}	SI
	μg mL^{-1}		
8a	121	33	4
8b	401	380	1
8c	415	26	16
11a	169	15	11
11b	286	25	11
11c	37	2	19
11d	62	30	2

Note. CTD_{50} is the concentration of the compound causing the death of 50% of cells in the culture, EC_{50} is the concentration of the compound required to reduce the virus yield by 50%; SI is the selectivity index, the CTD_{50} to EC_{50} ratio.

compounds containing a benzyl substituent at the position 3″ are less toxic compared with compounds bearing a furylmethyl group at this position (compounds **8b**, **8c** and **11a**, **11b)**. In compounds containing the benzyl substituent at the position 3″, the replacement of the phenyl substituent in the thiapyrrolizidine ring by the pyridine substituent completely eliminates antiviral activity (compounds **8c** and **8b**). The movement of the sulfur atom from the position 6′ to 7′ and the change from the 1,2- to 1,3-arrangement of the spiro atoms resulted in the restoration of the activity; however, this was accompanied by approximately a twofold increase in toxicity (compounds **8b** and **11a**). The removal of the sulfur atom, *i.e.*, in going from thiapyrrolizidine derivatives to pyrrolizidine derivatives, unexpectedly resulted in a sharp increase in toxicity of the compounds (compounds **8a**, **11c**, and **11d)**.

Therefore, the substituents at the position 3″ of the pyrrolizidine ring, as well as the presence and the position of the sulfur atom in this ring, play an important role in the antiviral activity of the group of compounds under study. Compound **11c** exhibits the optimal characteristics. Despite high toxicity (CTD_{50} = 37 μg mL^{-1}), this compound efficiently inhibits viral replication (EC_{50} = = 1.9 μg mL^{-1}, SI = 19.2).

The target and the mechanism of antiviral activity of bis-spirocyclic derivatives of rhodanine cannot be ultimately determined based on the published data and our results. The chemical structures of this class of compounds basically differ from the structures of all other anti-influenza drugs. Hence, it can be expected that the virus-inhibitory activity of these compounds differs from that of the drugs used in the clinical practice and that these compounds can provide the basis for the further structure optimization and the design of a radically new class of anti-influenza drugs.

Experimental

The NMR spectra of compounds **11a—b** and **11d** were recorded on a Bruker AVANCE DPX-250 spectrometer (^{1}H, 250 MHz); of compound **11c**, on a Bruker AVANCE III 600 spectrometer (^{1}H, 600 MHz; ^{13}C, 151 MHz; ^{15}N, 61 MHz). The ^{15}N NMR chemical shifts of compound **11c** were assigned using ^{1}H—^{15}N HMBC experiments. *N*-Benzylrhodanine[34] and *N*-furfurylrhodanine[35] were synthesized according to known methods. Arylidene- and heteroarylidene derivatives of rhodanine **9a—d** were prepared according to the method, which we have described earlier.[23] The synthesis of bis-spirooxyindolorhodanines **8a—c** has been described earlier.[23]

Synthesis of bis-spirooxyindolorhodanines 11a—d (general procedure). Dipolarophile **9a—d** (1 mmol), isatin (147 mg, 1 mmol), and the corresponding cyclic α-amino acid (1 mmol) were refluxed in methanol (30 mL) for 12 h. Then the reaction mixture was cooled, the solvent was distilled off under reduced pressure, and the residue was recrystallized from methanol.

3″-Benzyl-6′-(pyridin-3-yl)-2″-thioxo-2′,3′-dihydro-4″*H*-dispiro[indole-3,5′-pyrrolo[2,1-*b*][1,3]thiazole-7′,5″-[1,3]thiazolidine]-2,4″(1*H*)-dione (11a). Colorless crystals. Yield 41%. M.p. 244—246 °C. Found (%): C, 61.18; H, 4.25; N, 10.55. $C_{27}H_{22}N_4O_2S_3$. Calculated (%): C, 61.11; H, 4.18; N, 10.56. ^{1}H NMR (DMSO-d_6), δ: 2.70—3.23 (m, 4 H, C(2′)H$_2$, C(3′)H$_2$); 4.66 (s, 1 H, H(6′)); 4.90 and 5.07 (both d, 1 H each, N(3″)CH$_2$, *J* = 15.5 Hz); 5.72 (s, 1 H, H(7′a)); 6.56—6.75 (m, 2 H, H(5), H(7)); 6.92—7.36 (m, 7 H, H(5″′), H(6), Ph); 7.54 (d, 1 H, H(4), *J* = 7.0 Hz); 7.64 (d, 1 H, H(4″′), *J* = 7.9 Hz); 8.20 (d, 1 H, H(2″′), *J* = 1.9 Hz); 8.43 (d, 1 H, H(6″′), *J* = 4.4 Hz); 10.84 (s, 1 H, N(1)H).

3″-(2-Furylmethyl)-6′-(3-nitrophenyl)-2″-thioxo-2′,3′-dihydro-4″*H*-dispiro[indole-3,5′-pyrrolo[2,1-*b*][1,3]thiazole-7′,5″-[1,3]thiazolidine]-2,4″(1*H*)-dione (11b). Colorless crystals. Yield 51%. M.p. 198—200 °C. Found (%): C, 55.39; H, 3.54; N, 9.83. $C_{26}H_{20}N_4O_5S_3$. Calculated (%): C, 55.31; H, 3.57; N, 9.92. ^{1}H NMR ($CDCl_3$), δ: 2.76—2.90 (m, 1 H, C(2′)H$_2$); 2.94—3.10 (m, 1 H, C(2′)H$_2$); 3.12—3.38 (m, 2 H, C(3′)H$_2$); 4.79 (s, 1 H, H(6′)); 4.92 and 5.15 (both d, 1 H each, N(3″)CH$_2$, *J* = 14.9 Hz); 5.85 (s, 1 H, H(7a′)); 6.04 (d, 1 H, H(3‴), *J* = 2.9 Hz); 6.12 (dd, 1 H, H(4‴), *J* = 2.9 Hz, *J* = 1.9 Hz); 6.69 (d, 1 H, H(7), *J* = 7.6 Hz); 7.03—7.24 (m, 4 H, H(5), H(6), H(5′′), H(5‴)); 7.52 (d, 1 H, H(4), *J* = 7.6 Hz); 7.61 (d, 1 H, H(4′′), *J* = 8.5 Hz); 7.80 (s, 1 H, N(1)H); 7.92 (d, 1 H, H(2′′), *J* = 1.6 Hz); 7.98 (d, 1 H, H(6′′), *J* = 7.4 Hz).

3″-(2-Furylmethyl)-2′-(pyridin-3-yl)-2″-thioxo-5′,6′,7′,7a′-tetrahydro-4″*H*-dispiro[indole-3,3′-pyrrolizine-1′,5″-[1,3]thiazolidine]-2,4″(1*H*)-dione (11c). The product was additionally purified by column chromatography (Al_2O_3, CH_2Cl_2)/EtOAc (5 : 1)). Colorless crystals. Yield 50%. M.p. 234—236 °C. Found (%): C, 62.20; H, 4.47; N, 11.05. $C_{26}H_{22}N_4O_3S_2$. Calculated (%): C, 62.13; H, 4.41; N, 11.15. ^{1}H NMR (DMSO-d_6), δ: 1.79—1.87 (m, 1 H, C(6′)H$_2$); 1.87—1.95 (m, 1 H, C(7′)H$_2$); 2.02—2.13 (m, 2 H, C(6′)H$_2$, C(7′)H$_2$); 2.52—2.58 (m, 1 H, C(5′)H$_2$); 2.58—2.66 (m, 1 H, C(5′)H$_2$); 4.51 (dd, 1 H, H(7a′), *J* = 6.4 Hz, *J* = 8.2 Hz); 4.73 (s, 1 H, H(2′)); 4.92 and 5.05 (both d, 1 H each, N(3″)CH$_2$, *J* = 15.6 Hz); 5.84 (d, 1 H, H(3‴), *J* = 3.2 Hz); 6.24 (dd, 1 H, H(4‴), *J* = 1.9 Hz, *J* = 3.2 Hz); 6.64 (d, 1 H, H(7), *J* = 7.7 Hz); 6.97 (dd, 1 H, H(5), *J* = 7.4 Hz, *J* = 7.5 Hz); 7.09 (dd, 1 H, H(6), *J* = 7.5 Hz, *J* = 7.7 Hz); 7.16 (dd, 1 H, H(5′′), *J* = 4.8 Hz, *J* = 8.0 Hz); 7.37 (d, 1 H, H(5‴), *J* = 1.4 Hz);

7.47 (d, 1 H, H(4), J = 7.4 Hz); 7.54 (ddd, 1 H, H(4˝˝), J = 1.5 Hz, J = 2.3 Hz, J = 8.0 Hz); 8.21 (d, 1 H, H(2˝˝), J = 2.3 Hz); 8.34 (dd, 1 H, H(6˝˝), J = 1.5 Hz, J = 4.8 Hz); 10.78 (s, 1 H, N(1)H). ^{13}C NMR (DMSO-d_6), δ: 27.59 (C(6′)); 29.50 (C(7′)); 40.54 (N(3″)$\underline{C}H_2$); 46.83 (C(5′)); 63.40 (C(2′)); 69.92 (C(1′)); 74.45 (C(7a′)); 74.97 (C(3′)); 108.26 (C(3‴)); 109.90 (C(7)); 110.22 (C(4‴)); 122.63 (C(5)); 123.10 (C(5˝˝)); 123.80 (C(4)); 126.73 (C(3˝˝)); 128.62 (C(3a)); 129.39 (C(6)); 137.02 (C(2˝˝)); 141.25 (C(7a)); 142.59 (C(5‴)); 147.37 (C(2‴)); 149.68 (C(6˝˝)); 150.39 (C(2˝˝)); 176.65 (C(4″)); 177.88 (C(2)); 200.71 (C(2″)). ^{15}N NMR (DMSO-d_6), δ: 64.43 (N(4′)); 141.94 (N(1)); 191.81 (N(3″)); 317.34 (N(1˝˝)).

2′-(2-Furyl)-3″-(2-furylmethyl)-2″-thioxo-5′,6′,7′,7a′-tetrahydro-4″*H*-dispiro[indole-3,3′-pyrrolizine-1′,5″-[1,3]thiazolidine]-2,4″(1*H*)-dione (11d). Colorless crystals. Yield 55%. M.p. 244—246 °C. Found (%): C, 61.15; H, 4.26; N, 8.44. $C_{25}H_{21}N_3O_4S_2$. Calculated (%): C, 61.08; H, 4.31; N, 8.55. ^{1}H NMR ($CDCl_3$), δ: 1.76—2.01 (m, 2 H, C(6′)H_2, C(7′)H_2); 2.03—2.30 (m, 2 H, C(6′)H_2, C(7′)H_2); 2.56—2.86 (m, 2 H, C(5′)H_2); 4.58 (dd, 1 H, H(7a′), J = 6.0 Hz, J = 7.8 Hz); 4.98 (s, 1 H, H(2′)); 5.10 and 5.30 (both d, 1 H each, N(3″)CH_2, J = 14.9 Hz); 5.93 (d, 1 H, H(3‴), J = 3.2 Hz); 5.96—6.03 (m, 1 H, H(3˝˝)), 6.23—6.34 (m, 2 H, H(4‴), H(4˝˝)); 6.76 (d, 1 H, H(7), J = 7.0 Hz); 6.85 (d, 1 H, H(5˝˝), J = 1.2 Hz); 7.06 (dd, 1 H, H(5), J = 7.5 Hz, J = 7.6 Hz); 7.19 (dd, 1 H, H(6), J = 7.0 Hz, J = 7.6 Hz); 7.31 (d, 1 H, H(5‴), J = 1.3 Hz); 7.45 (d, 1 H, H(4), J = 7.5 Hz); 7.56 (s, 1 H, N(1)H).

Quantum chemical calculations by AM1 and DFT methods using the Gaussian 03 program package[25] and calculations of the Parr reactivity indices were described in detail in the study.[24]

Viruses and cells. The antiviral activity of the tested compounds was evaluated using the influenza virus A/California/07/09 (H1N1)pdm09 accumulated for 48 h (influenza A virus) in allantoic cavity of 10—12-day-old chicken embryos from the collection of virus strains of the Research Institute of Influenza (St. Petersburg, Russia). Experiments in cell cultures were performed using MDCK cells (ATCC # CCL-34).

Investigation of toxicity of agents. The toxicity of the products was preliminary studied in MDCK cells. The cells were seeded in 96-well plates and cultured at 37 °C in the MEM medium containing 10% cattle serum under 5% CO_2 until a monolayer formed. The mother liquor of the compounds in dimethyl sulfoxide at a concentration of 10 mg mL^{-1} was prepared, and then twofold serial dilutions of the samples in the MEM medium from 1000 to 3.75 μg mL^{-1} were obtained. Dissolved samples were placed into wells of the plates and incubated at 37 °C for 2 days. Then the cells were washed twice for 5 min with a phosphate saline buffer, and the number of surviving cells was estimated by the tetrazolium MTT test. For this purpose, the solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (ICN Biochemicals Inc., Aurora, Ohio) (5 mg mL^{-1}) in a physiological solution was added into wells of plates (100 μL per well). The cells were incubated at 37 °C under 5% CO_2 for 2 h and then washed with a phosphate saline buffer for 5 min. The precipitate was dissolved in DMSO (100 μL per well). Then the absorbance in the wells of the plates was measured using a Perkin Elmer Victor 1420 multi-function reader at a wavelength of 535 nm. Based on the results of the test, the 50% cytotoxic dose (CTD_{50}), *i.e.*, the concentration of the agent causing the death 50% of cells in a culture, was determined for each product.

Virus titration. To evaluate the virus-inhibitory activity of the compounds, tenfold serial dilutions (10^{-1}—10^{-7}) were prepared from the starting virus-containing material. The samples at the specified concentrations were placed into wells of a plate containing cells and incubated for 1 h. Then the cells were infected with tenfold serial dilutions of the viral material and incubated in a thermostat for 48 h. After the completion of the incubation, the culture liquid was transferred into wells of a plate for immunological reactions, and then an equal volume of 1% chicken embryos in a physiological solution was added.

The level of viral replication in the wells of the plate was estimated using the hemagglutination reaction (HAR) with erythrocytes. The value of the inverse common logarithm of the maximum virus dilution that can cause the positive hemagglutination reaction was taken as the viral titer, and the latter was expressed as the logarithms of the 50% experimental infectious dose of the virus (log EID_{50}). The antiviral activity of compounds was evaluated from their ability to decrease the infectious virus titer. From these data, the 50% inhibitory concentration (EC_{50}), *i.e.*, the concentration of the compound that causes a twofold decrease in the virus yield (by 0.3 log EID_{50}), and the selectivity index (SI), *i.e.*, the CTD_{50} to EC_{50} ratio, were calculated.

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