5-Substituted pyridylisoxazoles as effective inhibitors of platelet aggregation

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A series of 5-substituted 3-pyridylisoxazoles were synthesized using [3+2] cycloaddition of nitrile oxides to alkynes with variation of substituents at position 5 of the isoxazole ring without additional synthetic stages and with retention of 2-pyridyl-, 3-pyridyl, and 4-pyridyl substituents at position 3 of the isoxazole ring. Substituted pyridylisoxazoles are the potential antiaggregatory agents showing *in vitro* activity in the concentration range from $1 \cdot 10^{-6}$ mol L^{-1} to $1 \cdot 10^{-4}$ mol L^{-1} toward the human platelet rich blood plasma with arachidonic acid being used as the inductor of aggregation. These compounds do not act as cyclooxygenase or thromboxane synthase inhibitors, nor as thrombin inhibitors.

Key words: thromboxane A_2 receptor antagonists, antiaggregatory activity, 3,5-disubstituted isoxazoles, platelets, [3+2] cycloaddition.

The search for and development of new antiaggregatory agents have been highly topical during the last 50 years, as the mortality caused by disorders in the blood clotting at cardiovascular diseases is the number one in the world. Currently, much attention is given to the design and synthesis of novel chemical structures exhibiting this type of biological activity upon the interaction with either known protein targets or newly discovered protein receptors and enzymes or their isoforms involved in blood coagulation.[1—10]

The targeted regulation of the human hemostasis system is a highly topical task of fundamental and applied science. The study of the possibilities of this regulation is an extremely challenging problem comprising the investigation of possible ways for preventing a pathological thrombus formation, resulting in an infarction, a stroke, and the death. A key unit of hemostasis is represented by platelets, which are able to aggregate and participate in the white thrombus formation. At the current stage of research, several signal transduction pathways have been found in a platelet, resulting in the aggregation of these cells to give a white thrombus as the final biological response to the action of various platelet membrane receptor agonists.[2,5,8—29] The interaction of the blood vessel endothelium with platelets takes a special place in the thrombus formation. A number of peculiar features of this process at drug therapy have been revealed.[29,30] Especially important signaling pathways of this interaction are those initiated by prostanoids, namely, thromboxane A_2 (TxA_2) and prostacyclin (PGI_2), the former being synthesized in the platelets as the major arachidonic acid metabolite with a chemical half-life of 30 s and the other being synthesized by endothelial cells upon triggering the arachidonic acid cascade *via* activation of phospholipase A_2.[20,30—33] During the last 15 years, eight prostanoid receptors, assigned to the family of rhodopsin-like receptors conjugated with G-proteins starting from thromboxane A_2 receptor (TP-receptor), were discovered and studied, the existence of two TP-receptor isoforms (TPα (in platelets) and TPβ (in blood vessel edothelial cells)) was proven, the major G-proteins interacting with these isoforms were determined, and the possibilities for heterodimerization of prostanoid receptors, explaining to some extent the unusual action of nonsteroidal antiinflammatory drugs, were elucidated. Despite all achievements, the interactions of already discovered signaling pathways with other signaling pathways involved in this processes in the platelets that would induce complete or partial cell response are still unknown.[1]

Heterocylic compounds of various chemical classes possess broad ranges of biological activity, and the synthesis of libraries of various classes of heterocyclic compounds with a large diversity of substituents exhibiting a specified biological activity is a topical task of combinatorial organ-

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ic chemistry.[34—36] Among these are quite a number of compounds affecting the arachidonic acid cascade involved in the metabolic pathways of signal transduction in the cells.[5,21,37—44] Although a number of known medicaments are used for the treatment of cardiovascular diseases, the development of new nonsteroidal antiaggregatory agents with less side effects based on heterocycles is of interest.[2,45] The development of both selective TP-receptor antagonists and dual-action antiaggregatory compounds affecting both the TP-receptor and the thromboxane synthase are most important. A minor number of thromboxane synthase inhibitors and TP-receptor antagonists are currently clinically used to prevent a number of cardiovascular disorders.[1]

After the analysis of published data, we chose a combination of pyridine and isoxazole moieties as the basic structure for the synthesis of antiaggregatory agents.[45] We synthesized a number of new 3,5-disubstituted isoxazoles and revealed the presence of antiaggregatory activity.[45—49] Relying on analysis of published data, we assumed that these compounds could act as thromboxane synthase inhibitors or TP-receptor antagonists. Prostanoids possess a very broad spectrum of activity;[1,50—53] therefore, earlier, it was believed that substances with this type of activity are needed only for fundamental research of signaling pathways. Currently, the development of selective TxA_2 antagonists and thromboxane synthase inhibitors or substances with dual mode of action affecting simultaneously the TP-receptor and thromboxane synthase or leukotriene synthase is needed for both fundamental research and clinical applications including the therapy of thrombosis and asthma.[1,2]

This study is devoted to the synthesis of a series of 5-substituted 3-pyridylisoxazoles, the kinetics of human platelet aggregation under the action of some compounds of this series, and the elucidation of the possible mechanism of their action upon interaction with platelets.

Results and Discussion

Synthesis of 5-substituted 3-pyridylisoxazoles

The last decade has witnessed high interest in the search for new biologically active isoxazole derivatives and development of new synthetic routes to them.[36,54—57] During the last seventy years the following two most general methods have been used to prepare substituted isoxazoles: (1) the reaction of [3+2] cycloaddition of nitrile oxides with terminal alkynes and (2) the synthesis from traditional synthons, namely, 1,3-diketones and hydroxylamine.[56]

A series of 5-substituted 3-pyridylisoxazoles was synthesized using the [3+2]-cycloaddition reaction of nitrile oxides to terminal alkynes, which allowed us to vary substituents in position 5 of the isoxazole ring without additional synthetic steps and with the retention of 2-, 3-, or 4-pyridine moiety as the substituent in position 3 of the isoxazole ring. An advantage of this approach consists in the regioselectivity of reaction in the preparation of 5-substituted 3-pyridylisoxazoles, because it is known that a mixture of 3,5- and 5,3-disubstituted regioisomeric isoxazoles is obtained by using unsymmetrical 1,3-diketones and hydroxylamine, the separation of which is a complicated task in some cases.[46,58,59]

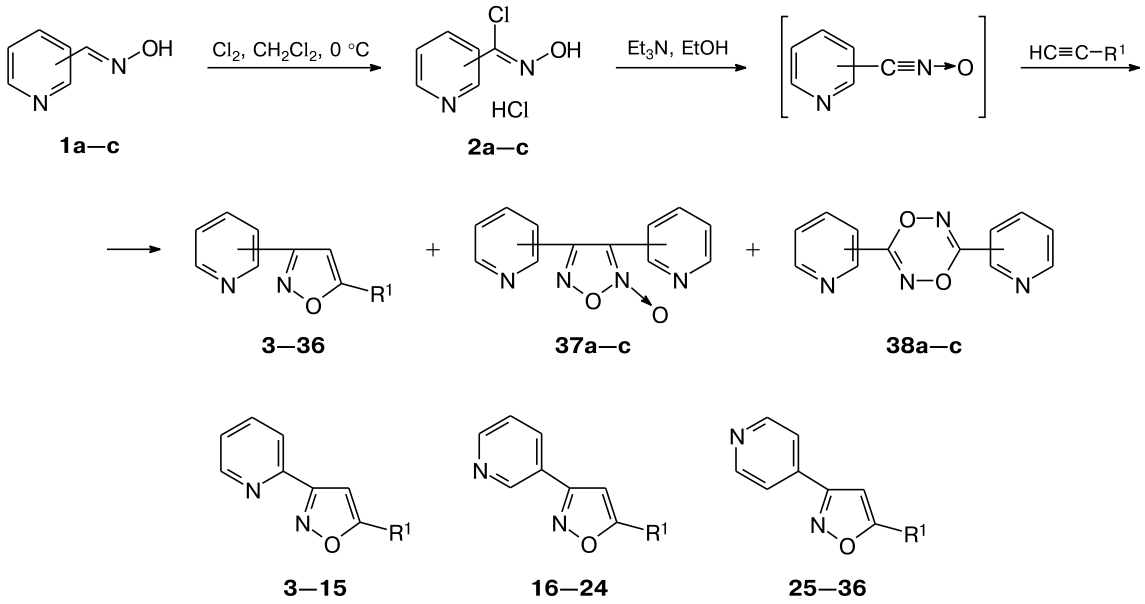
The synthesis of 5-substituted 3-pyridylisoxazoles from the initial compounds, oximes of 2-, 3-, and 4-pyridinecarbaldehydes **1a—c** and terminal alkynes, is shown in Scheme 1.

Commercially available 2-, 3-, and 4-pyridinecarbaldehyde oximes **1a—c** were chlorinated with chlorine gas to give pyridine-2-, pyridine-3-, and pyridine-4-hydroximoyl chlorides hydrochlorides **2a—c** in 92—99% yields.[59] Note that crystalline compounds **2a—c** are stable on the prolonged storage at room temperature under dry and dark conditions. Then pyridinehydroximoyl chloride hydrochloride **2a—c** and alkyne were dissolved in dry ethanol at a ratio of 1 : (3—5), respectively. The reaction was carried out in the temperature range from −5 to 5 °C with slow addition of a triethylamine solution in ethanol to the resulting suspension over a 30—60 min period with intense stirring and subsequent maintenance of the reaction mixture for 24—48 h. This gave 5-substituted 3-pyridylisoxazoles **3—36** (see Scheme 1).

The attempt to prepare pyridinehydroximoyl chlorides **2a—c** from commercially available pyridinecarbaldehyde oximes **1a—c** using *tert*-butyl hypochlorite in dichloromethane as the chlorinating agent instead of gaseous chlorine[60,61] proved unsuccessful, as the reaction gave a product mixture that could not be separated.

The [3+2] cycloaddition occurs regioselectively at temperatures of −5—5 °C; the yields of 3,5-disubstituted isoxazoles **3—36** are 15 to 60% depending on the nature of substituents and the ratio of pyridinehydroximoyl chlorides **2a—c** to terminal alkynes. When pyridinehydroximoyl chlorides are used, apart from lowering the reaction temperature, it is necessary to control the rate of formation of the intermediate nitrile oxide by using slow dropwise addition of triethylamine in anhydrous EtOH to the initial suspension of pyridinehydroximoyl chloride hydrochloride **2** and terminal acetylene in order to shift the equilibrium toward the formation of 3,5-disubstituted isoxazoles and to diminish the formation rate of by-products, most of all, furoxan **37**. We found that a decrease in the amount of terminal alkyne from 5 to 3 equiv. *versus* 1 equiv. of compound **2** does not affect the yields of products **3—36**. Further decrease in the amount of terminal alkyne to 2.3—2.5 equiv. reduces the product yield by 20—30%. It is noteworthy that the greatest amount of the furoxan by-product **37** was formed when 4-pyridylhydroximoyl chloride hyrochloride (**2c**) served as the initial com-

Scheme 1



1, **2**, **37**, **38:** 2-pyridyl (**a**), 3-pyridyl (**b**), 4-pyridyl (**c**)

R^1 = H (**3**, **16**, **25**), CH_2OH (**4**, **17**, **26**), CH_2Br (**5**), Pr (**6**, **27**), Bu (**7**, **28**), C_5H_{11} (**8**, **29**), Ph (**9**, **19**, **30**), C_6H_{13} (**10**, **31**), C_7H_{15} (**11**, **32**), C_8H_{17} (**12**, **33**), $CONH_2$ (**13**, **18**, **34**), COOH (**14**, **21**, **35**), COOEt (**15**, **22**, **36**), $C(OH)Me_2$ (**20**), $CH_2OC(O)CF_2CF_2CF_3$ (**23**), C(O)NHBn (**24**)

pound; in this case, another by-product, compound **38**, with molecular weight equal to that of furoxan, was also formed (up to 5%). No by-product **38** was detected or isolated when the reactions were carried out with 2- (**2a**) and 3-pyridylhydroximoyl (**2b**) chlorides hydrochlorides. According to mass spectrometry and NMR spectroscopy, by-product **38**, like furoxan **37**, is formed from two 4-pyridylnitrile oxide molecules and contains a six-membered ring, which is consistent with published data.[62] The use of 2-pyridylhydroximoyl chloride hydrochloride (**2a**) resulted in the minimum amount of furoxan **37**.

The highest yields (up to 60%) were attained for 5-substituted 3-(2-pyridyl)isoxazoles **3**—**15**, moderate yields (up to 40—45%) were obtained for 5-substituted 3-(3-pyridyl)isoxazoles **16**—**24**, and 5-substituted 3-(4-pyridyl)-isoxazoles **25—36** were formed in low yields (up to 30%). Compounds **3**, **4**, **6**—**8**, **10**—**12**, **16**, **17**, **20**, **23**, **25**—**29**, and **31**—**33** were synthesized in this work for the first time. Compounds **29**, **30**, and **35** were obtained in moderate yields when 2.3 equiv. (instead of 3 equiv.) of terminal alkynes were introduced in the reaction. 3-(2-Pyridyl)- (**9**), 3-(3-pyridyl)- (**19**), and 3-(4-pyridyl)-5-phenylisoxazoles (**30**) were first prepared in our study according to Scheme 1.[46] It is known that these compounds have been obtained[63] from 1,3-diketones and hydroxylamine, the yields being not higher than those achieved in our work. The same publication[63] describes an unsuccessful attempt to prepare these compounds from pyridinehydroximoyl chlorides.

5-Substituted 3-pyridylisoxazoles containing carboxy group in position 5 were prepared by hydrolysis of the corresponding ethyl or methyl esters, since the reaction may be accompanied by side processes at the unprotected carboxy group of the propiolic acid, resulting in a sharp decrease in the yield of the desired product down to traces.

The structures of the obtained compounds were confirmed by advanced techniques of physicochemical analysis: ^1H and ^{13}C NMR spectroscopy and mass spectrometry (electron impact ionization). The yields, melting points, and ^1H NMR and mass spectral data for all of the synthesized compounds are summarized in Table 1.

The isolation of some 3,5-disubstituted isoxazoles from the reaction mixture as individual substances proved to be rather complicated because the by-products formed in the reaction (unsaturated compounds, furoxans) had R_f values close to R_f of the major products ($\Delta R_f < 0.01$). Alkyl derivatives of 3-pyridylisoxazoles were formed as oils or low-melting compounds, which resisted isolation in a pure state by either recrystallization or column chromatography without too large consumption of eluents (~2—2.5 L). These compounds were isolated by flash chromatography using the Biotage+ system or by HPLC using the Smart-Line 1000 system (Knauer, Germany). Flash chromatography with the Biotage+ system provided substances of ≥98% purity with a minimum consumption of the eluent; however, sample preparation was rather labor-consuming and took 3 to 6 h.[58] The use of HPLC allowed complete

Table 1. Physicochemical and spectral data for 5-substituted 3-pyridylisoxazoles

Compound	M.p. /°C	R_f* (eluent)	Yield (%)	^{1}H NMR spectrum ($CDCl_3$,δ, *J*/Hz)**	MS (EI, 70 eV), *m/z*, $[M]^+$
3	Oil	0.73 (A)	88	6.77 (d, 1 H, H(4), isox., J = 2.0); 7.03 (ddd, 1 H, H(5), pyridyl, J = 8.0, J = 5.0, J = 1.5); 7.48 (td, 1 H, H(4), pyridyl, J = 8.0, J = 2.0); 7.80 (d, 1 H, H(3), pyridyl, J = 8.0); 8.29 (d, 1 H, H(5), isox., J = 1.8); 8.38 (d, 1 H, H(6), pyridyl, J = 5.0)	146
4	101—102	0.31 (A)	53	4.10 (br.s, 1 H, OH); 4.82 (s, 2 H, CH_2O); 6.90 (s, 1 H, H(4), isox.); 7.36 (ddd, 1 H, H(5), pyridyl, J = 8.0, J = 5.0, J = 1.3); 7.82 (td, 1 H, H(4), pyridyl, J = 8.0, J = 2.0); 8.08 (ddd, 1 H, H(3), pyridyl, J = 8.0, J = 1.8, J = 0.7); 8.68 (ddd, 1 H, H(6), pyridyl, J = 5.0, J = 2.0, J = 0.7)	176
5	64—65	0.32 (A)	21	5.06 (s, 2 H, CH_2Br); 7.14 (s, 1 H, H(4), isox.); 7.58 (m, 1 H, H(5), pyridyl); 8.06 (m, 2 H, H(3), H(4), pyridyl); 8.76 (m, 1 H, H(6), pyridyl)	239
6	Oil	0.57 (A)	49	1.00 (t, 3 H, C(3″)H_3, J = 7.4); 1.77 (m, 2 H, C(2″)H_2); 2.78 (t, 2 H, C(1″)H_2, J = 8.0); 6.63 (s, 1 H, H(4), isox.); 7.31 (ddd, 1 H, H(5), pyridyl, J = 7.6, J = 4.9, J = 1.2); 7.76 (td, 1 H, H(4), pyridyl, J = 7.6, J = 1.8); 8.04 (dt, 1 H, H(3), pyridyl, J = 7.6, J = 1.0); 8.65 (ddd, 1 H, H(6), pyridyl, J = 4.9, J = 1.8, J = 1.0)	188
7	Oil	0.58 (A)	64	0.93 (t, 3 H, C(4″)H_3, J = 7.3); 1.40 (m, 2 H, C(3″)H_2); 1.72 (t, 2 H, C(2″)H_2, J = 7.5); 2.80 (t, 2 H,C(1″)H_2, J = 7.3); 6.67 (s, 1 H, H(4), isox.); 7.33 (ddd, 1 H, H(5), pyridyl, J = 7.5, J = 4.9, J = 1.2); 7.78 (td, 1 H, H(4), pyridyl, J = 7.8, J = 1.8); 8.05 (dt, 1 H, H(3), pyridyl, J = 7.9, J = 1.0); 8.66 (ddd, 1 H, H(6), pyridyl, J = 4.9, J = 1.8, J = 1.0)	202
8	Oil	0.73 (A)	50	0.89 (t, 3 H, C(5″)H_3, J = 7.0); 1.36 (m, 4 H, C(3″)H_2, C(4″)H_2); 1.75 (m, 2 H, C(2″)H_2); 2.80 (t, 2 H, C(1″)H_2, J = 7.1); 6.63 (s, 1 H, H(4), isox.); 7.31 (ddd, 1 H, H(5), pyridyl, J = 7.6, J = 4.9, J = 1.3); 7.77 (td, 1 H, H(4), pyridyl, J = 7.6, J = 1.8); 8.05 (dt, 1 H, H(3), pyridyl, J = 7.9, J = 1.1); 8.66 (ddd, 1 H, H(6), pyridyl, J = 4.9, J = 1.8, J = 0.9)	216
9	129—130	0.81 (A)	51	7.14 (s, 1 H, H(4), isox.); 7.27 (d.dt, 1 H, H(5), pyridyl, J = 8.0, J = 5.0, J = 1.0); 7.40 (m, 3 H, H_m, H_p, Ph); 7.72 (tdd, 1 H, H(4), pyridyl, J = 8.0, J = 2.0, J = 1.0); 7.77 (m, 2 H, H_o, Ph); 8.06 (dq, 1 H, H(3), pyridyl, J = 8.0, J = 1.0); 8.64 (dd, 1 H, H(6), pyridyl, J = 5.0, J = 1.0)	222
10	Oil	0.78 (A)	57	0.83 (m, 3 H, C(6″)H_3); 1.26 (t, 6 H, C(3″)H_2, C(4″)H_2, C(5″)H_2, J = 7.0); 1.69 (quint, 2 H, C(2″)H_2, J = 8.0); 2.76 (t, 2 H, C(1″)H_2, J = 7.5); 6.58 (s, 1 H, H(4), isox.); 7.27 (ddd, 1 H, H(5), pyridyl, J = 8.0, J = 5.0, J = 1.2); 7.72 (td, 1 H, H(4), pyridyl, J = 8.0, J = 2.0); 8.01 (dq, 1 H, H(3), pyridyl, J = 8.0, J = 1.0); 8.62 (ddd, 1 H, H(6), pyridyl, J = 5.0, J = 2.0, J = 1.0)	230
11	Oil	0.62 (B)	47	0.87 (m, 3 H, C(7″)H_3); 1.27—1.30 (m, 8 H, C(3″)H_2, C(4″)H_2, C(5″)H_2, C(6″)H_2); 1.74 (m, 2 H, C(2″)H_2); 2.76 (t, 2 H, C(1″)H_2, J = 7.5); 6.63 (s, 1 H, H(4), isox.); 7.32 (ddd, 1 H, H(5), pyridyl, J = 7.5, J = 4.9, J = 1.2); 7.77 (td, 1 H, H(4), pyridyl, J = 8.0, J = 2.0); 8.05 (dt, 1 H, H(3), pyridyl, J = 8.0, J = 1.1); 8.66 (ddd, 1 H, H(6), pyridyl, J = 4.9, J = 1.8, J = 1.0)	244
12	Oil	0.67 (B), 0.57 (A)	49.5	0.73 (t, 3 H, C(8″)H_3, J = 7.0); 1.12 (m, 10 H, C(3″)H_2, C(4″)H_2, C(5″)H_2, C(6″)H_2, C(7″)H_2); 1.59 (m, 2 H, C(2″)H_2); 2.64 (t, 2 H, C(1″)H_2, J = 7.5); 6.51 (s, 1 H, H(4), isox.); 7.15 (ddd, 1 H, H(5), pyridyl, J = 7.5, J = 4.9, J = 1.2); 7.60 (td, 1 H, H(4), pyridyl, J = 7.8, J = 1.8); 7.91 (d, 1 H, H(3), pyridyl, J = 8.0, J = 1.1); 8.51 (d, 1 H, H(6), pyridyl, J = 4.1)	258

(*to be continued*)

Table 1 (*continued*)

Compound	M.p. /°C	R_f* (eluent)	Yield (%)	^{1}H NMR spectrum ($CDCl_3$,δ, *J*/Hz)**	MS (EI, 70 eV), *m/z*, $[M]^+$
13	214—216	0.05 (A)	69	7.58 (s, 1 H, H(4), isox.); 7.55 (ddd, 1 H, H(5), pyridyl, *J* = 7.4, *J* = 4.8, *J* = 1.3); 7.99 (td, 1 H, H(4), pyridyl, *J* = 7.8, *J* = 1.8); 8.03 (br.s, 1 H, NH_2); 8.07 (dt, 1 H, H(3), pyridyl, *J* = 7.9, *J* = 1.1); 8.42 (br.s, 1 H, NH_2); 8.74 (ddd, 1 H, H(6), pyridyl, *J* = 4.8, *J* = 1.7, *J* = 1.0)	189
14	230—232 (dec.)	0.0 (A), 0.41 (D)	97	7.56 (s, 1 H, H(4), isox.); 7.55 (ddd, 1 H, H(5), pyridyl, *J* = 7.8, *J* = 4.9, *J* = 1.3); 7.99 (td, 1 H, H(4), pyridyl, *J* = 7.8, *J* = 1.8); 8.08 (dt, 1.H, H(3), pyridyl, *J* = 7.8, *J* = 1.0); 8.74 (ddd, 1 H, H(6), pyridyl, *J* = 4.8, *J* = 1.7, *J* = 1.0)	190
15	55—58	0.90 (C)	67	1.39 (t, 3 H, CH_3, *J* = 7.1); 4.42 (q, 2 H, OCH_2, *J* = 7.1); 7.34 (ddd, 1 H, H(5), pyridyl, *J* = 7.8, *J* = 4.9, *J* = 1.1); 7.54 (s, 1 H, H(4), isox.); 7.78 (td, 1 H, H(4), pyridyl, *J* = 7.8, *J* = 1.7); 8.08 (dt, 1 H, H(3), pyridyl, *J* = 7.8, *J* = 0.9); 8.66 (d, 1 H, H(6), pyridyl, *J* = 4.6)	218
16	44—47	0.59 (A)	84	6.63 (d, 1 H, H(4), isox., *J* = 2.0); 7.30 (dd, 1 H, H(5), pyridyl, *J* = 8.0, *J* = 5.0); 8.05 (dt, 1 H, H(4), pyridyl, *J* = 8.0, *J* = 1.5); 8.44 (d, 1 H, H(5), isox., *J* = 1.5); 8.56 (dd, 1 H, H(6), pyridyl, *J* = 5.0, *J* = 1.5); 8.93 (d, 1 H, H(2), pyridyl, *J* = 2.0)	146
17	139—140	0.18 (A)	40	2.95 (br.s, 1 H, OH); 4.82 (s, 2 H, CH_2O); 6.61 (s, 1 H, H(4), isox.); 7.41 (ddd, 1 H, H(5), pyridyl, *J* = 8.0, *J* = 5.0, *J* = 0.7); 8.14 (dt, 1 H, H(4), pyridyl, *J* = 8.0, *J* = 2.0); 8.66 (dd, 1 H, H(6), pyridyl, *J* = 5.0, *J* = 2.0); 8.97 (d, 1 H, H(2), pyridyl, *J* = 2.0)	176
18	>240 (dec.)	0.0 (A)	97	7.58 (ddd, 1 H, H(5), pyridyl, *J* = 8.0, *J* = 4.9, *J* = 0.8); 7.70 (s, 1 H, H(4), isox.); 8.05 (br.s, 1 H, NH_2); 8.30 (dt, 1 H, H(4), pyridyl, *J* = 8.0, *J* = 1.7); 8.43 (br.s, 1 H, NH_2); 8.72 (dd, 1 H, H(6), pyridyl, *J* = 4.8, *J* = 1.6); 9.10 (dd, 1 H, H(2), pyridyl, *J* = 2.2, *J* = 0.8)	189
19	142—143	0.57 (A)	38	6.83 (s, 1 H, H(4), isox.); 7.37 (dd, 1 H, H(5), pyridyl, *J* = 8.0, *J* = 5.0); 7.43 (m, 3 H, H_m, H_p, Ph); 7.79 (m, 2 H, H_o, Ph); 8.14 (dt, 1 H, H(4), pyridyl, *J* = 8.0, *J* = 2.0); 8.65 (dd, 1 H, H(6), pyridyl, *J* = 5.0, *J* = 1.8); 9.03 (d, 1 H, H(2), pyridyl, *J* = 2.0)	222
20	89—91	0.25 (A)	58	1.5 (s, 6 H, CH_3); 5.72 (s, 1 H, OH); 6.97 (s, 1 H, H(4), isox.); 7.53 (ddd, 1 H, H(5), pyridyl, *J* = 8.0, *J* = 5.0, *J* = 0.9); 8.25 (ddd, 1 H, H(4), pyridyl, *J* = 8.0, *J* = 2.3, *J* = 1.8); 8.67 (dd, 1 H, H(6), pyridyl, *J* = 5.0, *J* = 1.8); 9.06 (dd, 1 H, H(2), pyridyl, *J* = 2.3, *J* = 0.8)	204
21	>240	0.0 (A), 0.35 (D)	94	7.58 (ddd, 1 H, H(5), pyridyl, *J* = 8.0, *J* = 4.9, *J* = 0.8); 7.90 (s, 1 H, H(4), isox.); 8.35 (dt, 1 H, H(4), pyridyl, *J* = 8.0, *J* = 1.6); 8.73 (dd, 1 H, H(6), pyridyl, *J* = 4.8, *J* = 1.6); 9.15 (dd, 1 H, H(2), pyridyl, *J* = 2.1, *J* = 0.8)	190
22	75—78	0.85 (A)	64	1.44 (t, 3 H, CH_3, *J* = 7.1); 4.46 (q, 2 H, OCH_2, *J* = 7.1); 7.29 (s, 1 H, H(4), isox.); 7.43 (ddd, 1 H, H(5), pyridyl, *J* = 7.9, *J* = 5.6, *J* = 0.7); 8.18 (dt, 1 H, H(4), pyridyl, *J* = 7.9, *J* = 1.8); 8.72 (dd, 1 H, H(6), pyridyl, *J* = 4.8, *J* = 1.6); 9.03 (d, 1H, H(2), pyridyl, *J* = 2.3)	218
23	Oil	0.6 (A)	27	5.27 (s, 2 H, CH_2); 6.71 (s, 1 H, H(4), isox.); 7.39 (dd, 1 H, H(5), pyridyl, *J* = 7.8, *J* = 4.9); 8.12 (dt, 1 H, H(4), pyridyl, *J* = 8.0, *J* = 2.0); 8.67 (d, 1 H, H(6), pyridyl, *J* = 4.0); 8.98 (s, 1 H, H(2), pyridyl)	372
24	199—201	0.0 (A)	86	4.49 (d, 2 H, CH_2, *J* = 6.1); 7.27, 7.34 (m, 5 H, Ph); 7.58 (ddd, 1 H, H(5), pyridyl, *J* = 8.0, *J* = 4.8, *J* = 0.8); 7.76 (s, 1 H, H(4), isox.); 8.31 (ddd, 1 H, H(4), pyridyl, *J* = 8.0, *J* = 2.3, *J* = 1.7); 8.72 (dd, 1 H, H(6), pyridyl, *J* = 4.8, *J* = 1.7); 9.12 (dd, 1 H, H(2), pyridyl, *J* = 2.2, *J* = 0.8); 9.62 (t, 1 H, NH, *J* = 6.0)	279

(*to be continued*)

Table 1 (*continued*)

Compound	M.p. /°C	R_f* (eluent)	Yield (%)	^{1}H NMR spectrum ($CDCl_3$,δ, *J*/Hz)**	MS (EI, 70 eV), *m/z*, $[M]^+$
25	76—79	0.43 (A)	17	6.71 (d, 1 H, H(4), isox., J = 1.8); 7.68 (dd, 2 H, H(3), H(5), pyridyl, J = 6.0, J = 1.8); 8.51 (d, 1 H, H(5), isox., J = 2.0); 8.72 (dd, 2 H, H(2), H(6), pyridyl, J = 6.0, J = 0.8)	146
26	132—132	0.15 (A)	41	4.64 (d, 2 H, CH_2O, J = 6.0); 5.77 (t, 1 H, OH, J = 6.0); 7.07 (s, 1 H, H(4), isox.); 7.83 (m, 2 H, H(3), H(5), pyridyl); 8.79 (m, 2 H, H(2), H(6), pyridyl)	176
27	40—41	0.56 (A)	12	1.01 (t, 3 H, C(3″)H_3, J = 7.3); 1.77 (m, 2 H, C(2″)H_2); 2.79 (t, 2 H, C(1″)H_2, J = 7.4); 6.34 (s, 1 H, H(4), isox.); 7.65 (dd, 2 H, H(3), H(5), pyridyl, J = 4.5, J = 1.6); 8.70 (dd, 2 H, H(2), H(6), pyridyl, J = 4.5, J = 1.6)	188
28	44—45	0.57 (A)	11	0.95 (t, 3 H, C(4″)H_3, J = 7.3); 1.42 (m, 2 H, C(3″)H_2); 1.73 (m, 2 H, C(2″)H_2); 2.81 (t, 2 H, C(1″)H_2, J = 7.8); 6.33 (s, 1 H, H(4), isox.); 7.66 (dd, 2 H, H(3), H(5), pyridyl, J = 4.5, J = 1.7); 8.70 (dd, 2 H, H(2), H(6), pyridyl, J = 4.5, J = 1.7)	202
29	<35 (amorph.)	0.63 (A)	18.2	0.89 (t, 3 H, C(5″)H_3, J = 7.0); 1.36 (m, 4 H, C(3″)H_2, C(4″)H_2); 1.75 (m, 2 H, C(2″)H_2); 2.80 (t, 2 H, C(1″)H_2, J = 7.0); 6.33 (s, 1 H, H(4), isox.); 7.65 (dd, 2 H, H(3), H(5), pyridyl, J = 4.5, J = 1.7); 8.71 (dd, 2 H, H(2), H(6), pyridyl, J = 4.5, J = 1.7)	216
30	164—165	0.49 (A)	27	6.85 (s, 1 H, H(4), isox.); 7.46 (m, 3 H, H_m, H_p, Ph); 7.72 (d, 2 H, H(3), H(5), pyridyl, J = 6.0); 7.80 (m, 2 H, H_o, Ph); 8.72 (d, 2 H, H(2), H(6), pyridyl, J = 6.0)	222
31	<31 (amorph.)	0.63 (A)	35	0.83 (m, 3 H, C(6″)H_3); 1.31 (t, 6 H, C(3″)H_2, C(4″)H_2, C(5″)H_2, J = 7.0); 1.70 (quint, 2 H, C(2″)H_2, J = 8.0); 2.77 (t, 2 H, C(1″)H_2, J = 7.5); 6.33 (s, 1 H, H(4), isox.); 7.65 (dd, 2 H, H(3), H(5), pyridyl, J = 4.5, J = 1.7); 8.71 (dd, 2 H, H(2), H(6), pyridyl, J = 4.5, J = 1.7)	230
32	<30 (amorph.)	0.45 (A)	11	0.89 (m, 3 H, C(7″)H_3); 1.27—1.30 (m, 8 H, C(3″)H_2, C(4″)H_2, C(5″)H_2, C(6″)H_2); 1.78 (m, 2 H, C(2″)H_2); 2.76 (t, 2 H, C(1″)H_2, J = 7.5); 6.35 (s, 1 H, H(4), isox.); 7.66 (dd, 2 H, H(3), H(5), pyridyl, J = 4.5, J = 1.7); 8.74 (dd, 2 H, H(2), H(6), pyridyl, J = 4.5, J = 1.7)	244
33	<35 (amorph.)	0.44 (B)	17	0.83 (t, 3 H, C(8″)H_3, J = 7.0); 1.22 (m, 10 H, C(3″)H_2, C(4″)H_2, C(5″)H_2, C(6″)H_2, C(7″)H_2); 1.68 (m, 2 H, C(2″)H_2); 2.76 (t, 2 H, C(1″)H_2, J = 7.5); 6.31 (s, 1 H, H(4), isox.); 7.63 (d, 2 H, H(3), H(5), pyridyl, J = 6.1); 8.66 (d, 2 H, H(2), H(6), pyridyl, J = 6.1)	258
34	>240	0.0 (A)	64	7.74 (s, 1 H, H(4), isox.); 7.88 (d, 2 H, H(3), H(5), pyridyl, J = 6.1); 8.07 (br.s, 1 H, NH_2); 8.45 (br.s, 1 H, NH_2); 8.76 (d, 2 H, H(2), H(6), pyridyl, J = 6.1)	189
35	> 230 (dec.)	0.30 (D)	84	7.99 (s, 1 H, H(4), isox.); 8.05 (d, 2 H, H(3), H(5), pyridyl, J = 6.2); 8.82 (d, 2 H, H(2), H(6), pyridyl, J = 6.2)	190
36	124—126	0.60 (A)	20	1.35 (t, 3 H, CH_3, J = 7.1); 4.41 (q, 2 H, OCH_2, J = 7.1); 7.95 (d, 2 H, H(3), H(5), pyridyl, J = 6.2); 8.06 (s, 1 H, H(4), isox.); 8.76 (d, 2 H, H(2), H(6), pyridyl, J = 6.2)	218
39	141—142	0.81 (A)	21—46	6.86 (s, 1 H, H(4), isox.); 7.45 (m, 6 H, H_m, H_p, Ph); 7.81 (m, 4 H, H_o, Ph)	221

* For TLC, the following eluents were used: chloroform—methanol, 10 : 1 (A), dichloromethane—methanol, 10 : 1 (B), 20 : 1 (C), ethanol—water, 7 : 3 (D); the spots were visualized in the UV light or iodine vapor.

** The ^{1}H NMR spectra of compounds **3**, **4**, **6**—**12**, **15**—**17**, **19**, **22**, **23**, **25**, and **27**—**33** were recorded in $CDCl_3$, and the spectra of compounds **5**, **13**, **14**, **18**, **20**, **21**, **24**, **26**, and **34**—**36** were measured in DMSO-d_6.

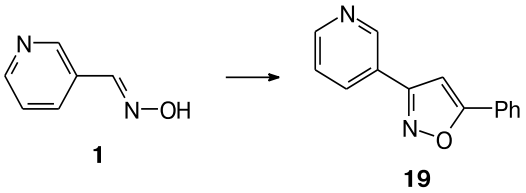
separation of impurities to give the product with a more than 99.5% purity with minimum eluent consumption and without additional sample preparation except for the separation of resinous components by column chromatography on silica gel or recrystallization. The least pure compounds were obtained by using only column chroma-

tography (97—98% purity) for eluent consumption of not more than 1.5—2 L.

5-Hydroxymethyl-3-pyridylisoxazoles and 3-pyridyl-5-phenylisoxazoles, which have rather high melting points, were purified by recrystallization from hexane.[46]

Since the synthesis according to Scheme 1 uses the chlorination by chlorine gas, we attempted to find an alternative, chlorine-free route to 3,5-disubstituted isoxazole preparation. We demonstrated the possibility to prepare 5-substituted 3-pyridylisoxazoles using phase transfer catalysis (Scheme 2).[58] The preparation of 3,5-disubstituted isoxazoles from benzaldehyde oxime analogs containing substituents in the phenyl moiety and terminal alkenes or alkynes by means of phase transfer catalysis has been described.[57] As initial compounds, we took 3-pyridinecarboxaldoxime and phenylacetylene in 1 : 1 ratio, and the Belizna (Whiteness) product was used as the chlorinating agent (see Scheme 2). The reaction was catalyzed by Aliquat 336. After 48 h at room temperature, 3-(3-pyridyl)-5-phenylisoxazole (**19**) was obtained in 15.7% yield. When the amounts of the catalyst and the chlorinating agent were doubled, the yield of the final product increased to 25—29%, while further addition of the catalyst and the Belizna product did not affect the yield of the desired compound **19**. Thus, we demonstrated that the alternative preparation of 3,5-disubstituted isoxazoles from commercially available reactants without chlorination with the chlorine gas is, in principle, possible.

Scheme 2



Reagents and conditions: HC≡CPh, NaOCl (Belizna product), Aliquat 336, CH_2Cl_2, 20 °C, 48 h.

Antiaggregatory activity of 5-substituted 3-pyridylisoxazoles. Kinetics of the process

A study of the interaction of potential antiaggregatory agents of various chemical nature with platelets using a number of natural inductors of aggregation provide the understanding of the mechanism of action of these compounds on the signaling pathways of a platelet and defines the pharmacophoric fragments of molecules for the design of drugs with minimized side effects. Possible targets for the antiaggregatory agents are G-protein conjugated membrane platelet receptors (GPCRs) and the enzymes involved in signal transduction: tyrosine kinases, phospholipases, and arachidonic acid (AA) cascade enzymes.[5,12,13,15] The molecular recognition between the protein target and the ligand (activator, inhibitor, or promoter) is the initial stage of virtually any process occurring in biological systems. The geometrical and chemical complementarity of small ligand molecules to the macromolecular biological structures such as protein targets (receptors, enzymes) determines the signal transduction pathways in the cells or metabolic routes, thus inducing physiological response.[7] The initial stage of creation of target-oriented libraries is the primary search for the lead structure that would be the ligand with a particular affinity for the given protein (receptor, enzyme), and this is followed by optimization of the revealed chemical structure. The knowledge of the exact three-dimensional structure of the protein target is a necessary condition for further research, which reduces the research cost and time. Unfortunately, a great part of the membrane receptor proteins and proteolytic enzymes connected to the membrane have not been obtained in the crystalline state due to the complexity of the crystallization. If the presumptive protein targets have not been obtained in the crystalline state and preliminary computer simulation is impossible, it is necessary to perform experimental biochemical studies to elucidate the true target or targets, which was actually done in this work.

The effect of 5-substituted 3-pyridylisoxazoles on the platelet aggregation induced by aggregation inductors, *viz.*, arachidonic acid, adenosine diphosphate (ADP), and adrenaline (EPI), was studied by the turbidimetric method based on the continuous detection of relative variation of the light transmission coefficient of the human platelet rich plasma (PRP) or a human platelet suspension after the addition of an aggregation inductor using a Biola aggregometer (Russia).[45] While investigating the effect of the test compounds on the platelet aggregation, we determined the following kinetic parameters: the maximum rate of aggregation (V_{max}) and the maximum degree of aggregation (A_{max}) (Fig. 1, *a*). The dependences for these data on the inhibitor concentration [I] were plotted — $V_{max}/[I]$ and $A_{max}/[I]$, and the IC_{50} values for each of the test compounds were determined. As the reference compound, we chose indomethacin, a competitive inhibitor of cyclooxygenase (the COX-1 isoform in the platelets), the first enzyme of the arachidonic acid cascade.

The ADP- or EPI-induced platelet aggregation is known to comprise two aggregation waves, the first one being independent of the AA cascade, whereas the second one being determined by the AA cascade. The ADP and EPI concentrations for these experiments were selected in the ranges of 5—15 μmol L^{-1} and 1—4 μmol L^{-1}, respectively, to obtain an inflection point of the aggregation curves (see Fig. 1, *b* and *c*). In the case of using AA with the concentration of 500 μmol L^{-1}, only one aggregation wave was observed for the test sample (see Fig. 1, *a*).

First, a number of compounds were tested on PRP samples using AA to induce the aggregation. Compounds

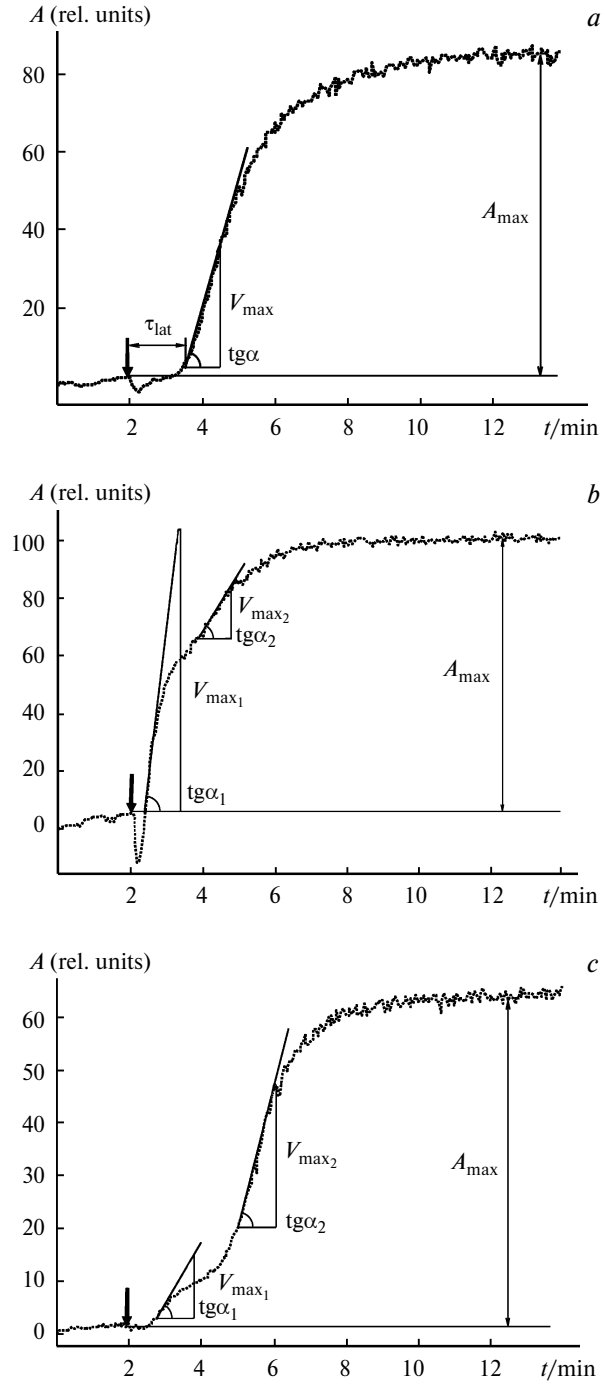


Fig. 1. Agregation patterns obtained experimentally using the following inductors in platelet rich human blood plasma samples: (*a*) arachidonic acid (500 μmol L^{-1}); (*b*), ADP (15 μmol L^{-1}); (*c*), adrenaline (1 μmol L^{-1}). The instant of addition of the inductor is marked by arrow; τ_{lat} is the latent period.

containing hydroxymethyl (**4**, **17**, **26**), phenyl (**9**, **19**, **30**), and 2-hydroxy-2-propyl (**20**) moieties in position 5 of the isoxazole ring were tested. The testing demonstrated that all of the compounds completely inhibited the human platelet aggregation in the dose-dependent mode. The V_{max}/[I] and A_{max}/[I] curves of all compounds show a threshold dependence: the A_{max} and V_{max} values sharply decrease in the narrow inhibitor concentration range (the concentration difference was 0.5—2 μmol L^{-1}). The curves V_{max}/[I] and A_{max}/[I] were identical in shape almost in all cases, and IC_{50} values found from these curves coincided. As an example, Figs 2—4 present the dependences V_{max}/[I] and A_{max}/[I] for 3-(3-pyridyl)-5-phenylisoxazole (**19**) and indomethacin (see Fig. 2), for **19** and 5-hydroxymethyl-3-(3-pyridyl)isoxazole (**17**) (see Fig. 3), and for 3-pyridylisoxazoles **4**, **17**, and **26** containing a hydroxymethyl group in position 5 of the isoxazole ring and indomethacin (see Fig. 4). Note that the comparisons of biological activities for pairs or groups of compounds in each of the experiments presented in Figs 2—4 were performed for one plasma sample, which made the comparison of the biological activities for two or more compounds legitimate.

The experimental curves and the IC_{50} values calculated from the curves provided the conclusion that 5-hydroxymethyl-3-(3-pyridyl)isoxazole (**17**) is more active than 5-hydroxymethyl-3-(4-pyridyl)- (**26**) or 5-hydroxymethyl-3-(2-pyridyl)isoxazoles (**4**) but is inferior to indomethacin (see Figs. 2 and 4). In the testing of 3-pyr-

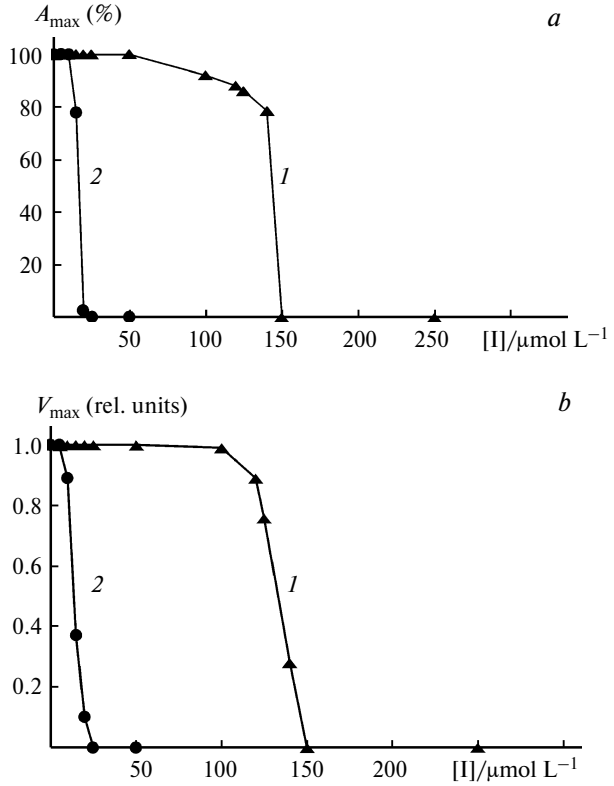


Fig. 2. Maximum degree of aggregation (*a*) and maximum rate of aggregation (*b*) *vs.* inhibitor concentration for 3-(3-pyridyl)-5-phenylisoxazole (**19**) (*1*) and indomethacin (*2*) found for the same human PRP sample with arachidonic acid (500 μmol L^{-1}) as the aggregation inductor.

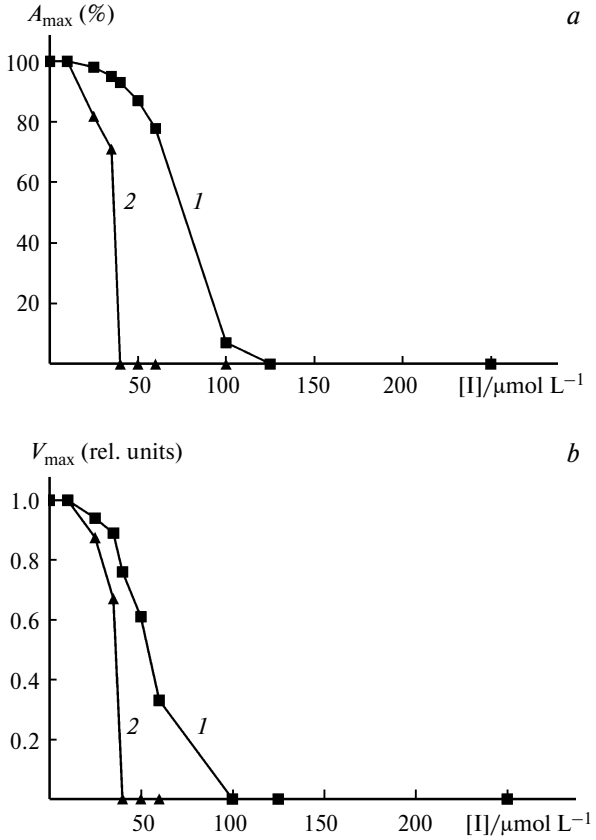


Fig. 3. Maximum degree of aggregation (*a*) and maximum rate of aggregation (*b*) *vs.* the inhibitor concentration for 3,5-disubstituted isoxazoles**17** (*1*) and **19** (*2*) found for the same human PRP sample with arachidonic acid (500 μmol L^{-1}) as the aggregation inductor.

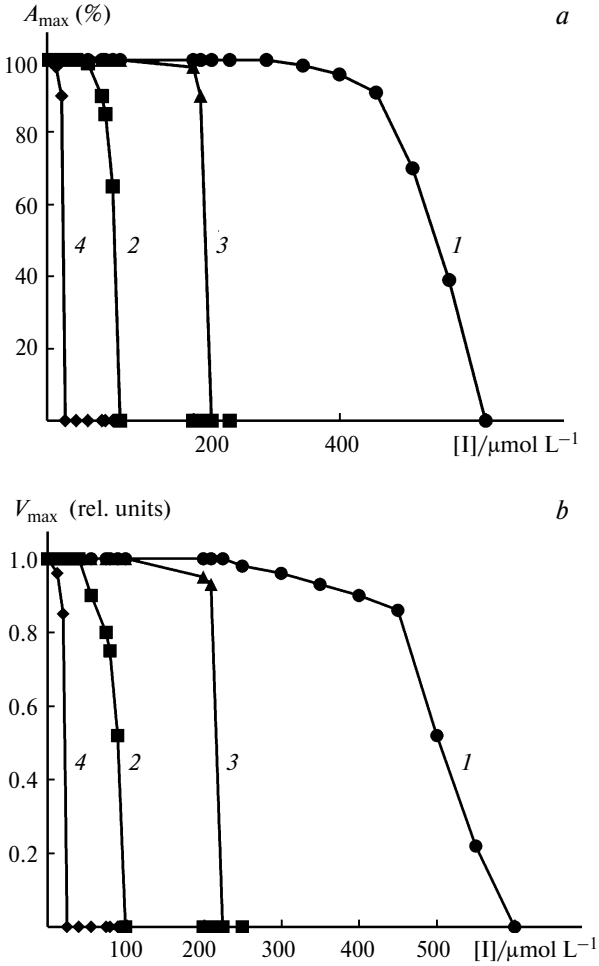


Fig. 4. Maximum degree of aggregation (*a*) and maximum rate of aggregation (*b*) *vs.* the inhibitor concentration for compounds **4** (*1*), **17** (*2*), **26** (*3*), and indomethacin (*4*) found for the same human PRP sample with arachidonic acid (500 μmol L^{-1}) as the aggregation inductor.

idylisoxazoles **6**, **19**, and **30** containing a phenyl group in position 5 of the isoxazole ring, the curves V_{max}/[I] and A_{max}/[I] were similar to the curves obtained for 3-pyridylisoxazoles **4**, **17**, and **26** (see Fig. 4). A comparison of the best samples of these two series demonstrated that 3-(3-pyridyl)-5-phenylisoxazole (**19**) is more active than 5-hydroxymethyl-3-(3-pyridyl)isoxazole (**17**) (see Fig. 3). 5-(2-Hydroxy-2-propyl)-3-(3-pyridyl)isoxazole (**20**) was found to be inferior to both compound **19** and compound **17** (Table 2). All of the test compounds inhibited the platelet aggregation in human PRP samples in the dose-dependent mode in the concentration range of $1 \cdot 10^{-6}$—$1 \cdot 10^{-3}$ mol L^{-1} (see Table 2), the curves V_{max}/[I] and A_{max}/[I] for these cases being S-shaped with a clear-cut threshold dependence, showing the sharp drop of V_{max} and A_{max} values in narrow concentration ranges. Indomethacin also tends to show scattered IC_{50} values and a threshold dependence for the curves V_{max}/[I] and A_{max}/[I].

When one and the same compound was tested on 5—15 or more human PRP samples, the IC_{50} values were scattered due to individual features of the PRP samples. This might be due to different amounts of particular plasma proteins that bind the liphophlic compounds being tested.[32] In order to level off this scatter and analyze the experimental data in more detail, the relative values for the maximum degree of aggregation and the maximum rate of aggregation were plotted against the ratio of the current inhibitor concentration to IC_{50} for 3-pyridylisoxazoles **17** and **19** (Fig. 5). These curves were plotted (see Fig. 5) using the experimental data shown in Fig. 3. The relative values of A_{max} and V_{max} were defined as A_{max}/A_{max_0} and V_{max}/V_{max_0}, where A_{max} and V_{max} are the maximum degree of aggregation and the maximum rate of aggregation derived from the experimental aggregation patterns obtained using an aggregation inhibitor and AA to induce the aggregation, and A_{max_0} and V_{max_0} are the maximum degree of aggregation and the maximum rate of aggregation derived from the aggregation patterns obtained under the influence of AA only (see Fig. 1). The plotted dependences V_{max}/[I] or A_{max}/[I] can be used to compare the

Table 2. Experimental data on the antiaggregatory activity of 5-substituted 3-pyridylisoxazoles

Compound	IC_{50}/μmol L^{-1} Aggregation inductor			K_i*/mol L^{-1}
	Arachidonic acid	Adenosin diphosphate	Adrenaline	
4	100±40	420±40 (2nd wave); >1000 (1st wave)	—	$1.6 \cdot 10^{-4}$—$9 \cdot 10^{-4}$
9	100±20	380±50 (2nd wave); >1000 (1st wave)	—	$(2—7) \cdot 10^{-4}$
16	75±40	>1000 (1st wave)	—	$5.2 \cdot 10^{-5}$
17	80±30	320±40 (2nd wave); >1000 (1st wave)	150±50 (2nd wave)	$(6.3—7.3) \cdot 10^{-5}$—$1.7 \cdot 10^{-4}$
19	60±30 (8—46 (best data))	70±40 (1st wave) >1000 (1st wave)	70±40 (1st wave)	$3.8 \cdot 10^{-6}$—$(4.5 \pm 2.2) \cdot 10^{-5}$
20	190±60	>1000 (1st wave)	—	—
26	90±40	340±50 (2nd wave) >1000 (1st wave)	—	$6.3 \cdot 10^{-5}$—$2.3 \cdot 10^{-4}$
30	75±40	240±60 (2nd wave); >1000 (1st wave)	—	$7 \cdot 10^{-5}$—$3 \cdot 10^{-4}$
39	85±30	>1000 (1st wave)	—	$(1.72—1.82) \cdot 10^{-5}$
Indomethacin	15±10	—	—	$(1.4—2.5) \cdot 10^{-5}$

* For arachidonic acid-induced aggregation.

experimental data for one and the same 3,5-disubstituted isoxazole and different PRP samples, thus leveling off the scatter of IC_{50} for a large number of experiments.

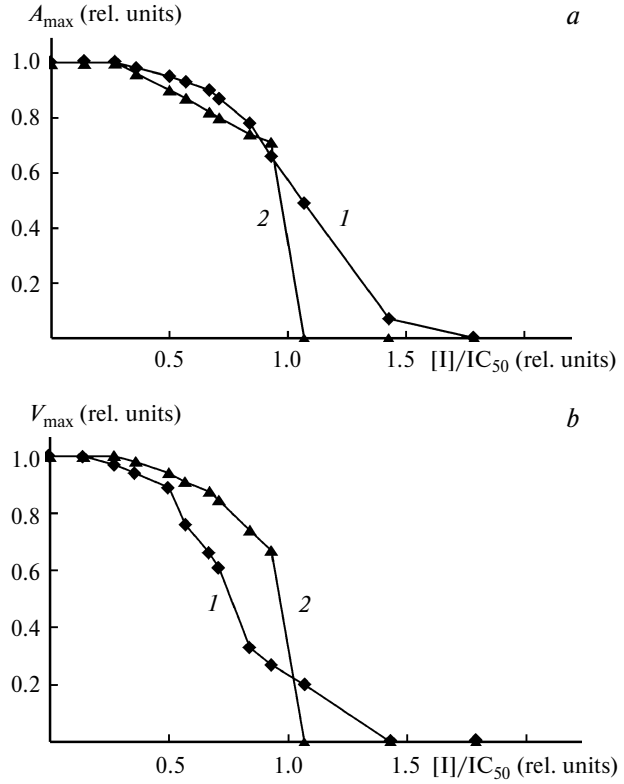


Fig. 5. Relative maximum degree of aggregation (*a*) and maximum rate of aggregation (*b*) *vs.* the ratio of current inhibitor concentration to the inhibitor IC_{50} for 3,5-disubstituted isoxazoles **17** (*1*) and **19** (*2*) obtained from the experimental data shown in Fig. 3.

We found experimentally that all compounds indicated above did not suppress the first aggregation wave but did suppress the second wave when the aggregation was induced by ADP or EPI in the concentration range of up to $1 \cdot 10^{-3}$ mol L^{-1}. The experimental data for compounds **17** and **19** obtained for the same PRP sample for one experiment are shown in Figs 6 and 7. Both compounds do not suppress the aggregation completely, which is

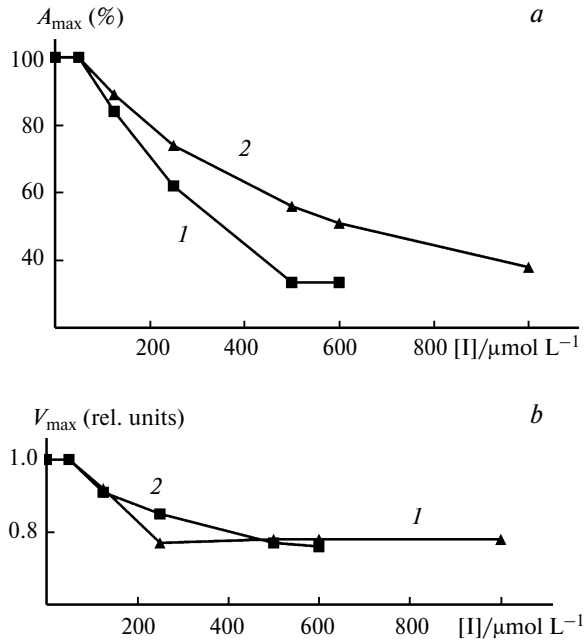


Fig. 6. Maximum degree of aggregation (*a*) and maximum rate of aggregation (*b*) *vs.* the inhibitor concentration for 3,5-disubstituted isoxazoles **17** (*1*) and **19** (*2*) found for the same human PRP sample with ADP (10 μmol L^{-1}) as the aggregation inductor.

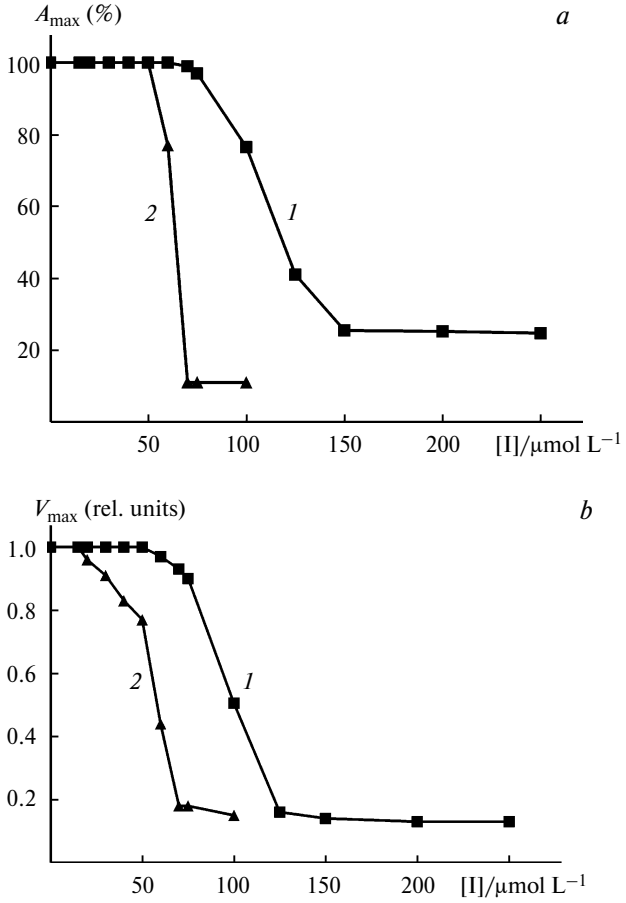


Fig. 7. Maximum degree of aggregation (*a*) and maximum rate of aggregation (*b*) *vs.* the inhibitor concentration for 3,5-disubstituted isoxazoles **17** (*1*) and **19** (*2*) found for the same human PRP sample with adrenaline (2 μmol L^{-1}) as the aggregation inductor.

demonstrated most clearly by the curve V_{max}/[I], which runs parallel to the abscissa axis after suppression of the second aggregation wave (see Figs. 6, *b* and 7, *b*).

The AA-initiated human platelet aggregation has so-called latent period (τ_{lat}), which lasts for 15 s to 1.5 min without any visible initial platelet aggregation in the aggregation pattern. If the initial τ_{lat} for a particular PRP sample is 30 s or more, it can increase upon the increase in the inhibitor concentration.

The dependence of the τ_{lat} values on the concentration of the inhibitor, compound **17**, which is, in the first approximation, linear in the concentration range from $1 \cdot 10^{-6}$ to $1 \cdot 10^{-3}$ mol L^{-1}, is shown in Fig. 8. The data of experiments for all tested compounds demonstrated that this type of dependence was observed for every compound. It is also noteworthy that in some experiments, the initial τ_{lat} remained constant throughout the whole experiment and its value did not exceed 15—20 s.

The experimental data were approximated by the Hill equation[64]

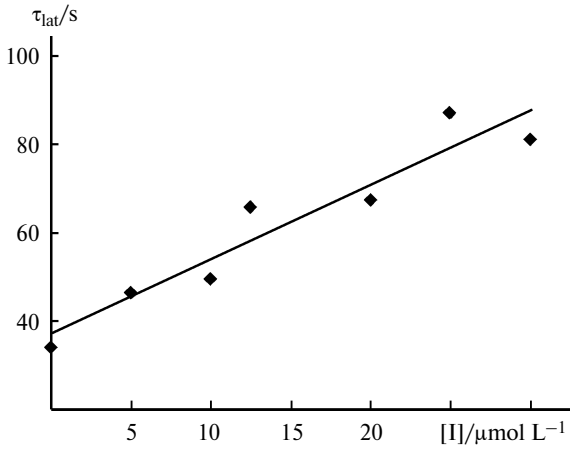


Fig. 8. Duration of the latent period (τ_{lat}) *vs.* the concentration of inhibitor, compound **17**.

$$V_{max}/V_0 = 1/[1 + ([I]/K_i)^{n_i}], \quad (1)$$

where n_i is the Hill coefficient, V_0 and V_{max} are the initial and current relative rates of the change of PRP light transmission, [I] is the inhibitor concentration, K_i is the inhibition constant, which is numerically equal to the inhibitor concentration at which $V_0/V_{max} = 0.5$, *i.e.*, IC_{50} found from the plot V_{max}/[I].

The n_i and K_i values were calculated from the experimental data on the linear interpolation in the coordinates $\log[(V_0 - V)/V]/\log[I]$. The attempt to use the Scatchard coordinates for K_i value determination failed, because the dependence was nonlinear. The approximation of the experimental dependence of the maximum rate of aggregation on the inhibitor (compound **17**) concentration (AA as the aggregation inductor, 500 μmol L^{-1}) by the Hill equation with the adjustment of Hill coefficients is shown in Fig. 9.

From the experimental data, the following values were obtained: $n_i = 4$ and $K_i = 63.1$ μmol L^{-1}. The theoretical curves constructed using $n_i = 4$, 5, 5.5, and 6 coincide well with the experimental curve in the upper and medium part but then continue as classical S-shaped curves without reproducing the threshold dependence of the experimental curve. At $n_i = 10$, the theoretical curve does show some threshold dependence, although it is not so sharp as in the experiment, but the upper part of this curve does not coincide with experimental data (see Fig. 9).

For the array of experimental data obtained in testing of compounds **17** (Fig. 10, *a*, 10 experiments) and **19** (Fig. 10, *b*, 15 experiments) on different human PRP samples, a series of theoretical curves were constructed with different Hill coefficient values and with superimposition onto the experimental data in the relative coordinates $(V/V_{max})/([I]/IC_{50})$, where V and [I] are the current values for the maximum rate of aggregation and the inhibitor concentration, respectively, while V_{max} is the maximum rate of aggregation at a zero inhibitor concentration.

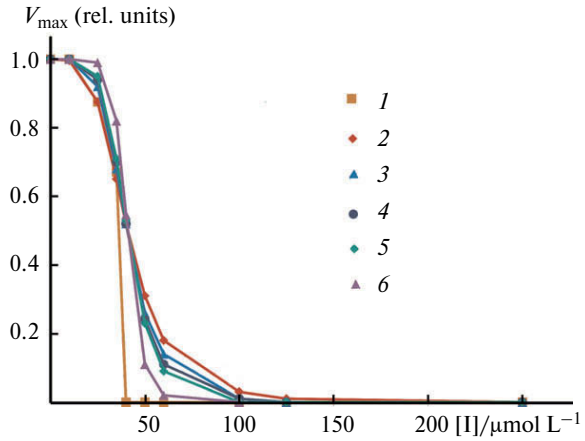


Fig. 9. Approximation of the experimental (*1*) dependence of the maximum rate of aggregation on the concentration of inhibitor, compound **17** (arachidonic acid, 500 μmol L^{-1} as the aggregation inductor) by the Hill equation with adjustment of the Hill coefficient: n_i = 4 (*2*), 5 (*3*), 5.5 (*4*), 6 (*5*), and 10 (*6*).
Note. Figures 9 and 10 are available in full color on the web-page of the Journal (www.link.springer.com).

It is noteworthy that the theoretical curves with n_i from 6.5 to 15 are most optimal for our substances. These approximations can describe rather accurately the upper and the medium parts of the curve; in this case, the n_i value should be above 10 or equal 15. However, the lower part of the curve is described poorly, showing smooth rather than sharply terminating attenuation of aggregation. At higher Hill coefficients ($10 \le n_i \le 30$), the threshold dependence and the lower part of the curve can be described very accurately; however, the upper part of the theoretical curve almost does not coincide with experimental data. Despite this fact, the Hill equation (1) is now the only way for approximating our experimental data.

This type of normalization was used in the comparison of a large array of experimental data for leveling off the scattering of IC_{50} and allowed us to make sure that there is threshold dependence of A_{max} and V_{max} on the inhibitor concentration for each compound and that the shapes of the curves are similar. This suggests that the compounds being tested act by the same mechanism.[65]

The considerable scatter of the IC_{50} values obtained in a series of experiments for different human PRP samples, which is found for all of the test compounds, may be attributable to differences in the biochemical composition of blood samples from different donors.[32] The experimental data were used to plot the dependences of the IC_{50} values for pairs of compounds: **17** and **19** (Fig. 11, *a*) and **4** and **17** (Fig. 11, *b*). It was shown that there is a linear correlation between the IC_{50} values for these pairs of compounds. For the pair of isoxazoles **17** and **19** containing a hydroxymethyl or phenyl group in position 5 of the isoxazole ring, this correlation is described by the equation

$$IC_{50(\mathbf{19})} = 0.3 \cdot IC_{50(\mathbf{17})}, \quad (2)$$

while for the pair of isoxazoles **4** and **17**, also containing the hydroxymethyl group in isoxazole position 5, the correlation is described by the equation

$$IC_{50(\mathbf{17})} = 0.13 \cdot IC_{50(\mathbf{4})}. \quad (3)$$

In both cases, the relationship between the IC_{50} values for each pair of compounds can be described by a linear function. This also suggests that these compounds affect the aggregation process by the same mechanism.

While starting the design of our library, we analyzed a large array of published data and suggested that the combination of two pharmacophores, pyridine and isoxazole, into one molecule will be important for the antiaggregatory activity to be manifested. To confirm this assumption

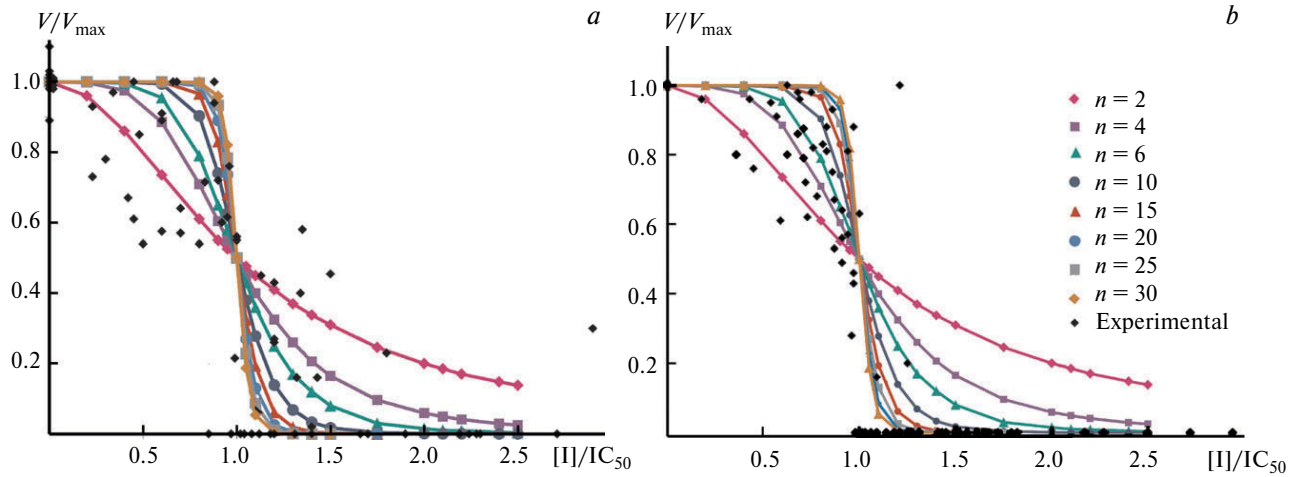


Fig. 10. Theoretical dependences of the maximum rate of aggregation on the concentration of inhibitors, compounds **17** (*a*) and **19** (*b*) (arachidonic acid, 500 μmol L^{-1} as the aggregation inductor) constructed using the Hill equation with superimposition onto experimental data of 10 (compound **17**) and 15 (compound **19**) experiments; *n* are the Hill coefficients.

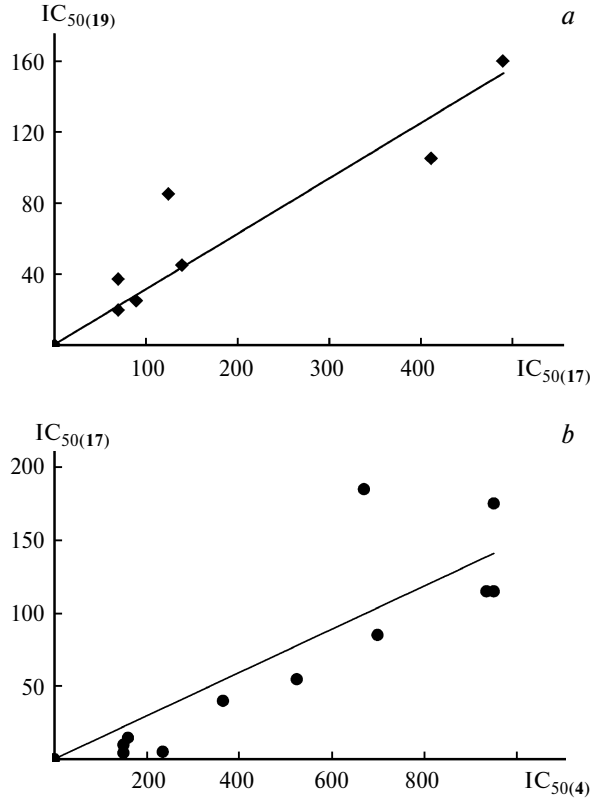


Fig. 11. Dependences for IC_{50} for pairs of compounds: 5-hydroxymethyl-3-(3-pyridyl)isoxazole (**17**)—3-(3-pyridyl)-5-phenylisoxazole (**19**) (*a*) and 5-hydroxymethyl-3-(3-pyridyl)isoxazole (**17**)—5-hydroxymethyl-3-(2-pyridyl)isoxazole (**4**) (*b*).

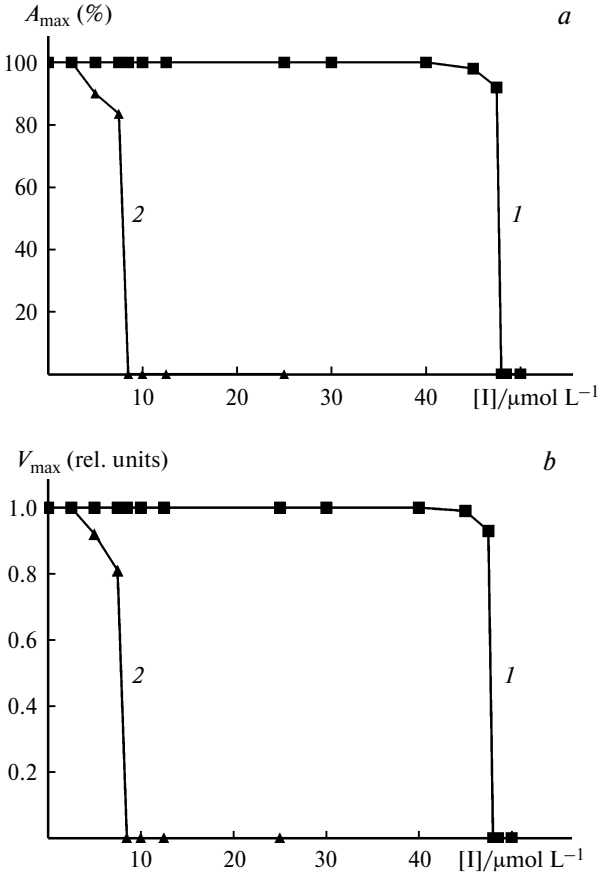
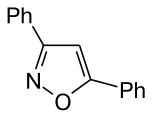


Fig. 12. Maximum degree of aggregation (*a*) *vs* the inhibitor concentration for 3,5-disubstituted isoxazoles **16** (*1*) and **19** (*2*) found for the same human PRP sample with arachidonic acid (500 μmol L^{-1}) as the aggregation inductor.

and to determine the possible pharmacophore moiety in this series of biologically active compounds, we selected, for further biological tests, 3-(3-pyridyl)-5-phenylisoxazole (**19**) as the most active compound, 5-unsubstituted 3-(3-pyridyl)isoxazole (**16**), and 3,5-diphenylisoxazole (**39**), which were also tested for antiaggregatory activity using AA to induce the aggregation.



39

According to experimental data, compound **19** is 1.4—2.6 times more active than 5-unsubstituted compound **16** (Fig. 12) and 1.1—1.3 times more active than 3,5-diphenylisoxazole (**39**) (Fig. 13, Table 2). Thus, our assumption that the pharmacophore moiety comprises both the pyridine and isoxazole rings proved valid.

The experimental IC_{50} and K_i values for the compounds tested for antiaggregatory activity are summarized in Table 2. All experimental data were obtained in 5—10 or more experiments.

Study of the effect of test compounds on the ADP- or EPI-induced human platelet aggregation demonstrated that in the initial testing of a PRP sample for a biological response, *viz.* platelet aggregation under the action of each of the three aggregation inductors without the addition of aggregation inhibitor, the most pronounced scatter of the IC_{50} values was found for PRP samples that have not aggregated under the action of one or two aggregation inductors. The results of these experiments with abnormally small or the largest possible IC_{50} values were neglected when determining the averaged IC_{50} and K_i values.

According to the data given in Table 2, compound **19** was most active among the test 3,5-disubstituted isoxazoles. Compounds **16** and **39** were 1.3—6 and 1.12—1.3 times less active than 3-(3-pyridyl)-5-phenylisoxazole (**19**), respectively, but indomethacin was 2—8 times superior in antiaggregatory activity than **19**. The following antiaggregatory activity sequence can be composed for the compounds in question: 3-(3-pyridyl)-5-phenylisoxazole (**19**) > 5-(hydroxymethyl)-3-(3-pyridyl)isoxazole (**17**) > > 3-(4-pyridyl)-5-phenylisoxazole (**30**) ≥ 5-(hydroxymethyl)-3-(4-pyridyl)isoxazole (**26**) > 3-(2-pyridyl)-5-phenylisoxazole (**9**) > 5-(hydroxymethyl)-3-(2-pyridyl)-isoxazole (**4**) ≥ 5-(2-hydroxy-2-propyl)-3-(3-pyridyl)-isoxazole (**20**).

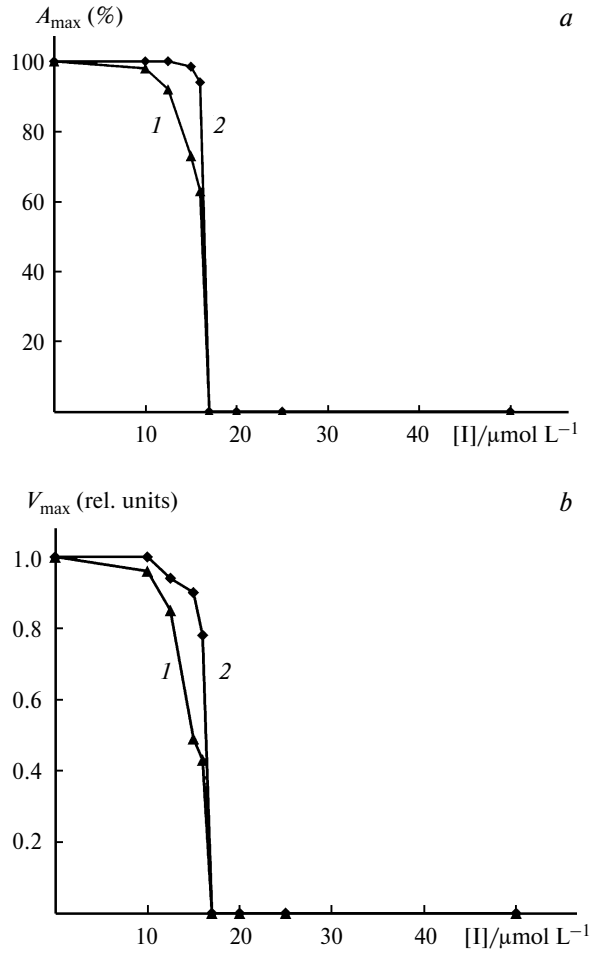


Fig. 13. Maximum degree of aggregation (*a*) and maximum rate of aggregation (*b*) *vs.* the inhibitor concentration for 3,5-disubstituted isoxazoles **19** (*1*) and **39** (*2*) found for the same human PRP sample with arachidonic acid (500 μmol L^{-1}) as the aggregation inductor.

Study of the mechanism of action of 3,5-disubstituted isoxazoles

Since all of the studied compounds suppressed completely the platelet aggregation induced by AA and the second waves of aggregation induced by ADP or EPI, without suppressing the first aggregation waves, we suggested that these compounds could behave as cyclooxygenase inhibitors (by analogy with indomethacin) or thromboxane synthase inhibitors, or thromboxane A_2 receptor (TP-receptor) antagonists.

To study the mechanism of action of 3,5-disubstituted isoxazole, we chose the most active compound, namely, compound **19**. We studied the effect of compound **19** on the activity of the first enzyme of the AA cascade, *viz.*, cyclooxygenase and on the activity of thromboxane synthase. As the source of these enzymes, we used the microsomal fraction of the sheep vesicular glands (cyclooxygenase) and the microsomal fraction of the human platelets (a complexes of cyclooxygenase and thromboxane synthase). The cyclooxygenase (PGH-synthase) inhibition was studied by polarography using the Clark electrode and the following experimental conditions: 150 μmol L^{-1} of AA, a standard potassium phosphate buffer (pH 7.8), and 25 °C.[66,67] The PGH-synthase activity was defined as the ratio of the oxygen absorption rate in the presence of an inhibitor to the oxygen absorption rate without an inhibitor (V/V_0) expressed in percent. The experimental data are summarized in Table 3. It follows from the data that compound **19** in the concentration range of $3 \cdot 10^{-7}$—$3 \cdot 10^{-4}$ mol L^{-1} does not suppress the cyclooxygenase activity, whereas the reference compound, indomethacin, suppresses the cyclooxygenase activity at $3 \cdot 10^{-5}$ mol L^{-1}.

The activity of thromboxane synthase from human platelet microsomes was determined by the procedure similar to that reported previously.[68] To elucidate the effect of compound **19** on thromboxane synthase, the transformation of radioactively labeled AA into thromboxane A_2 was measured based on its inactive metabolite, thromboxane B_2 (Table 4). For every sample indicated in Table 4, five measurements were performed; the values for thromboxane B_2 and AA (in percent of the total radioactivity) were averaged over the five measurements. The obtained data (see Table 4) indicate that 3-(3-pyridyl)-5-phenylisoxazole (**19**) in concentration of $1 \cdot 10^{-3}$ mol L^{-1} does not inhibit thromboxane synthase, as the amounts of thromboxane B_2 were nearly equal in the presence and in the absence of this compound. Indomethacin in the concentration of $1 \cdot 10^{-3}$ mol L^{-1} completely suppresses the synthesis of thromboxane A_2 by inhibiting cyclooxygenase but does not act as a thromboxane synthase inhibitor.

The antithrombin activity of compound **19** was studied at the Laboratory of Physical Biochemistry of the National Research Center for Hematology of the Ministry of Health of the Russian Federation. To study the possible

Table 3. Effect of indomethacin and 3-(3-pyridyl)-5-phenylisoxazole (**19**) on the cyclooxygenase activity*

Inhibitor concentration/mol L^{-1}	V/V_0	
	Indomethacin	Compound **19**
0	1	1
$3 \cdot 10^{-7}$	1	—
$1 \cdot 10^{-6}$	0.58	1
$3 \cdot 10^{-6}$	0.12	—
$3 \cdot 10^{-5}$	—	1
$3 \cdot 10^{-4}$	—	1

* Experimental conditions: 150 μmol L^{-1} of arachidonic acid, standard potassium phosphate buffer (pH 7.8), 25 °C. The average values of three experiments are given.

Table 4. Effect of indomethacin and 3-(3-pyridyl)-5-phenylisoxazole (**19**) on the thromboxane synthase activity*

Inhibitor	TxB_2**	Unreacted AA**
None***	8.7±2.2	46.4±3.9
Indomethacin	0	90.2±0.9
19	8.8±1.3	65.5±1.6

* Experimental conditions: enzyme, the microsomal fraction of human platelets; 200 μmol L^{-1} of arachidonic acid, 1 μmol L^{-1} of the inhibitor; standard potassium phosphate buffer (pH 7.4), 25 °C. The average values of five experiments are given.

** In percent of the total radioactivity.

*** Blank experiments.

antithrombin action of compound **19**, the rate of thrombin hydrolysis of the specific low-molecular chromogenic substrate Chromozyme TH (CTH) in the presence of various concentrations of compound **19** (5, 10, 25, 50, 100, and 125 μmol L^{-1}) and in the absence of the test compound was measured. Compound **19** did not show an antithrombin activity in the performed four experiments. It follows from the results that compound **19** in a concentration below 125 μmol L^{-1} is not a thrombin inhibitor.[45]

To verify the possible mechanism of action of 3-(3-pyridyl)-5-phenylisoxazole (**19**) as the TP-receptor antagonist, experiments of the radioactively labeled 3-(3-pyridyl)-5-phenylisoxazole ([^{3}H]-**19**) binding to human platelets were carried out. The tritium labeled [^{3}H]-**19** was prepared by tritium exchange.[69] The total radioactivity of the solution of [^{3}H]-**19** was 4.6 mCi, while the specific activity was 0.24 Ci mol^{-1}.

The experiment of labeled compound binding to native platelets was carried out by a reported procedure[68] using human PRP; the duration of incubation was 2 and 30 min (see Experimental). The total binding was determined from the amount of [^{3}H]-**19** that has bound to the platelets; the nonspecific binding was determined from the amount of [^{3}H]-**19** that has bound to platelets in the presence of a 100-fold excess of unlabeled compound **19**. The specific binding was found as the difference between the total and nonspecific bindings. Measurement of the sample radioactivity in counts min^{-1} (cpm) was performed on a Delta scintillation counter, the count duration was 1 min with the use of the ZhS-8 liquid scintillator.

Compound [^{3}H]-**19** was used in concentrations of 0.34 and 34 μmol L^{-1}. The difference between the sample preparation times was 2—5 min. For the same concentration of [^{3}H]-**19**, several samples were prepared (Table 5). The platelet source was human PRP, the incubation was carried out at 37 °C. During the incubation, no visible changes in the PRP properties were detected (no spontaneous Brownian aggregation or gravitational deposition of platelets).

The data for [^{3}H]-**19** concentration of 0.34 μmol L^{-1} are presented in the radioactivity diagram (Fig. 14). By comparing the diagrams obtained at different incubation times, one can see that the nonspecific binding decreases upon increase in the incubation period, although the total binding increases insignificantly.

It follows from analysis of experimental data that at the [^{3}H]-**19** concentration of 0.34 μmol L^{-1}, a specific binding takes place. Thus, presumably, we observe the ligand—receptor specific interaction: there are ~$3 \cdot 10^3$ reversibly bound molecules per platelet, which corresponds to the number of thromboxane A_2 receptors on each platelet[15] ((2500—3000 TP-receptors) (platelet)$^{-1}$).

Thus, our investigations provide the conclusion that 3-(3-pyridyl)-5-phenylisoxazole (**19**) is the TP-receptor antagonist, and the other compounds that we studied are also TP-receptor antagonists.

Previously, we suggested[65] that the Hill coefficient of 10, 20, and more indicated that the substance operates in the cell up to the supercooperativity stage, which accounts for the threshold dependence in the curves of platelet aggregation degree and rate: the substance operates at the stage of signal transduction prior to the stage of sharp

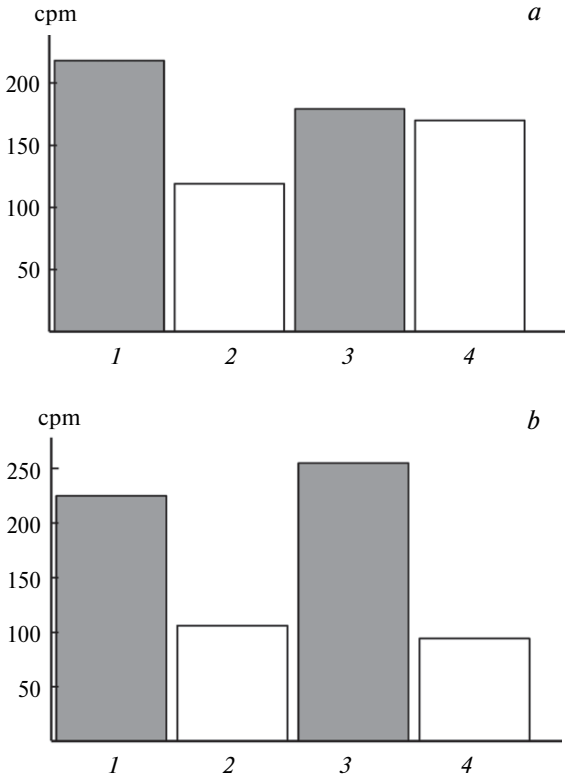


Fig. 14. Radioactivity diagram for the incubation of samples for 2 (*a*) and 30 min (*b*). Data for samples *1* and *2* were obtained after treatment of the samples containing 200 μL of PRP, 0.34 μmol L^{-1} of compound [^{3}H]-**19** and control (solvent for the unlabeled compound). The data for samples *3* and *4* were obtained after treatment of the sample containing 200 μL of PRP, 0.34 μmol L^{-1} of compound [^{3}H]-**19**, and 34 μmol L^{-1} of unlabeled compound **19**. The overall binding is shown in gray and the non-specific binding is in white.

Table 5. Data on binding of [^{3}H]-3-(3-pyridyl)-5-phenylisoxazole ([^{3}H]-**19**) with human platelets

Sample	Sample composition before formation of the precipitate*	Sampling time/min	Counting (cpm)	Radioactivity /10^{-9} Ci
1	200 μL of PRP + solvent compound **19**	2	396	23.76
2	200 μL of PRP + solvent compound **19**	30	90	5.4
3	200 μL of PRP + [^{3}H]-**19** (34 μmol L^{-1}) + solvent compound **19**	2	2903	174.18
4	200 μL of PRP + [^{3}H]-**19** (34 μmol L^{-1}) + + solvent compound **19**	30	3516	210.96
5	200 μL of PRP + [^{3}H]-**19** (34 μmol L^{-1}) + + **19** (3.4 mmol L^{-1})	2	13112	786.72
6	200 μL of PRP + [^{3}H]-**19** (34 μmol L^{-1}) + + **19** (3.4 mmol L^{-1})	30	5142	308.52
7	200 μL of PRP + [^{3}H]-**19** (34 μmol L^{-1}) + + **19** (3.4 mmol L^{-1})	2	14111	846.66
8	200 μL of PRP + [^{3}H]-**19** (34 μmol L^{-1}) + + **19** (3.4 mmol L^{-1})	30	24460	1467.6
9	200 μL of PRP + [^{3}H]-**19** (0.34 μmol L^{-1}) + + solvent compound **19**	2	218	13.08
10	200 μL of PRP + [^{3}H]-**19** (0.34 μmol L^{-1}) + + solvent compound **19**	30	225	13.5
11	200 μL of PRP + [^{3}H]-**19** (0.34 μmol L^{-1}) + + solvent compound **19**	2	179	10.74
12	200 μL of PRP + [^{3}H]-**19** (0.34 μmol L^{-1}) + + solvent compound **19**	30	255	15.3
13	200 μL of PRP + [^{3}H]-**19** (0.34 μmol L^{-1}) + + **19** (34 μmol L^{-1})	2	119	7.14
14	200 μL of PRP + [^{3}H]-**19** (0.34 μmol L^{-1}) + + **19** (34 μmol L^{-1})	30	106	6.36
15	200 μL of PRP + [^{3}H]-**19** (0.34 μmol L^{-1}) + + **19** (34 μmol L^{-1})	2	170	10.2
16	200 μL of PRP + [^{3}H]-**19** (0.34 μmol L^{-1}) + + **19** (34 μmol L^{-1})	30	94	5.64
17	200 μL of PRP	2	119	7.14
18	200 μL of PRP	30	56	3.36
19	200 μL of PRP + EtOH	2	38	2.28
20	200 μL of PRP+ EtOH	30	110	6.6
21	Microcentrifugal tube tip + + [^{3}H]-**19** (0.34 μmol L^{-11})	—	18703	1122.18

* Sample composition for determination of radioactivity: 1 mL of sodium dodecyl sulfate + scintillation liquid ZhS-8 + + precipitate of the sample (platelets and [^{3}H]-**19** bound to them), the initial composition of which is given in this Table.

avalanche-like increase in the number of secondary messengers involved in the subsequent signal transduction by the signaling pathway. The comparison of the biological activities of compound **19** and the selective TP-receptor antagonist GB32191B demonstrated that the dependences of the platelet degree of aggregation and the aggregation rate on the inhibitor concentration for these two compounds are very similar: they tend to exhibit a threshold pattern in a narrow concentration range and a high Hill coefficient. The TP-receptor antagonist GB32191B did not suppress the first wave of ADP- and adrenaline-induced aggregation either.[45] High Hill coefficients may also be typical for the receptor antagonists involved in the signal transduction at the beginning or in the middle of signal pathway, apart from elucidation of the supercooperativity stage (as in the case of indomethacin).

Thus, we obtained a library of 5-substituted 3-pyridylisoxazoles using [3+2] cycloaddition of nitrile oxides to alkynes by varying substituents in position 5 of the isoxazole ring without additional synthetic stages and with retention of 2-, 3-, or 4-pyridine moiety in position 3 of the isoxazole ring. Unlike the synthesis *via* 1,3-diketones, the approach we used allows one to prepare only 3,5-disubstituted isoxazoles in moderate yields without additional separation of a mixture of regioisomeric isoxazoles.

We studied the kinetics of the platelet aggregation process under the action of 5-substituted 3-pyridylisoxazoles and demonstrated that the dependences of the maximum platelet aggregation rate and degree of aggregation on the inhibitor concentration are S-shaped curves with a threshold pattern. It was shown that these dependences can be described in terms of the Hill equation taking account of the effect of inhibitor; however, this approximation describes adequately only the upper and the medium part of the curve if the Hill coefficient is equal to or is greater than 6.5. The threshold dependence as well as the lower part of the curve can be described rather accurately by using higher Hill coefficients ($10 \le n_i \le 30$) but in this case, the upper part of the theoretical curve almost does not coincide with the experimental curve. For our compounds, the theoretical curves with n_i of 6.5 to 15 are optimal. The latent period of the arachidonic acid-induced platelet aggregation was found to obey a linear dependence on the inhibitor concentration.

5-Substituted 3-pyridylisoxazoles were found to be potential antiaggregatory agents, exhibiting *in vitro* activity in the concentration range of $1 \cdot 10^{-6}$—$1 \cdot 10^{-4}$ mol L^{-1} on the platelet-enriched human blood plasma with arachidonic acid being used as the inductor. These compounds completely suppress the arachidonic acid-induced platelet aggregation and the second wave of ADP- or adrenaline-induced aggregation but do not affect the first wave of the ADP- or adrenaline-induced aggregation. For all of the studied compounds, the IC_{50} values characterizing the antiaggregatory activity were determined; according to the results, 3-(3-pyridyl)-5-phenylisoxazole was found to be most active.

A study of the possible mechanism of action of this class of compounds in relation to 3-(3-pyridyl)-5-phenylisoxazole demonstrated that this compound is not a cyclooxygenase or thromboxane synthase inhibitor, nor thrombin inhibitor. The results of experiment of [^{3}H]-3-(3-pyridyl)-5-phenylisoxazole binding to human platelet provide the assumption that it acts as a thromboxane A_2 receptor antagonist.

Experimental

The following commercial chemicals were used: ADP (Reanal, Hungary), adrenaline (Fluka, Germany), arachidonic acid (Acros Organics, MP Biomedicals, USA) and [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid (Amersham, USA), acetylene (Russia), hemin (Serva, Switzerland), 1-hexyne and 1-heptyne (Sigma-Aldrich, USA), sodium hydroxide (Russia), 1-decyne (Sigma-Aldrich, USA), sodium dodecyl sulfate (Serva, Germany), 0.05 *M* and 0.2 *M* potassium phosphate buffers (pH 7.8), citric acid (Russia), 1-nonyne (MP Biomedicals, USA), 1-octyne and 1-pentyne (Sigma-Aldrich, USA), 2-pyridinecarbaldoxime, 3-pyridinecarbaldoxime, and 4-pyridinecarbaldoxime (Merck, Germany and Acros Organics, USA), propargyl alcohol (Ferak, Germany), propiolic acid (Acros Organics, USA), saccharose (MP Biomedicals, USA), hydrochloric acid (Russia), ZhS-8 scintillation liquid (Russia), thrombin (Sigma, USA), triethylamine (Russia), phenylacetylene (Russia), HEPES (Sigma, USA), Chromozyme TH (CTH) (Sigma, USA), sodium citrate (Fluka, Switzerland), egg albumin (Sigma, Germany), and distilled chemical pure grade solvents (Russia): dichloromethane, ethanol, hexane, methanol, and diethyl ether. Phenylacetylene was distilled under argon immediately prior to use.

The sheep vesicular glands (Russia) were used as the source of cyclooxygenase (PGH-synthase).

The experiments were carried out using Eppendorf type 1.7 mL microcentrifuge tubes, 10-mL plastic vials for radioactive compounds, 15 and 25 mL polypropylene centrifuge tubes with plugs, and 200 and 1000 μL Eppendorf polypropylene pipette tips.

^{1}H NMR spectra were recorded on a Bruker DPX-300 spectrometer (Germany) operating at 300 MHz. The chemical shifts are given in the δ scale with respect to Me_4Si (internal standard). Mass spectra (EI, 70 eV) were recorded on a Finnigan-4021 gas chromatograph/mass spectrometer (USA) with direct sample injection.

The melting points were determined on a Koeffler hot stage to an accuracy of 1—2 °C.

Thin layer chromatography was carried out on Silufol UV-254 plates (Kavalier, Czechia), Kieselgel $60F_{254}$ (Merck, Germany), and Sorbfil (Russia) in chloroform—methanol (10 : 1) (A), dichloromethane—methanol (10 : 1) (B), dichloromethane—methanol (20 : 1) (C), and ethanol—water (7 : 3) (D) solvent systems; the spots were visualized in the UV light and in iodine vapor.

Column chromatography was performed on Kieselgel 60 (0.063—0.200 μm) (Merck, Germany) using a chloroform—ethanol or dichloromethane—ethanol mixture with ethanol gradient from 0 to 5% (v/v) as an eluent.

Individual compounds were isolated by HPLC using the SmartLine chromatographic system (Knauer, Germany) on a Knauer column (250×20 mm, Eurospher 100-10 Si, flow rate 7 mL min^{-1}, detection wavelength 254 nm) using elution with 1.5% EtOH in CH_2Cl_2 (for compounds **3**—**15**), 2% EtOH in CH_2Cl_2 (for compounds **16**—**24**), and 2.5% EtOH in CH_2Cl_2 (for compounds **25**—**36**).

The pyridinehydroximoyl chloride hydrochlorides **2a**—**c** were synthesized from the corresponding pyridinecarbaldehydes **1a**—**c** by a known procedure.[46]

Pyridine-3-hydroximoyl chloride hydrochloride (2b). A strong flow of dry chlorine was passed through a stirred solution of commercially available 3-pyridinecarboxaldoxime (**1b**) (6.32 g, 0.052 mol) in 150 mL of freshly distilled dichloromethane at a temperature of −5—0 °C for 3 h until persistent yellow-green color of the reaction mixture appeared and the pyridine-3-hydroximoyl chloride hydrochloride precipitate formed. Then the reaction mixture was diluted with 250 mL of dry diethyl ether, and the precipitate was separated by filtration, washed with dry diethyl ether (3×100 mL), and dried *in vacuo* (0.01 Torr). Yield 9.5 g (95%), white crystals, m.p. 192—193 °C. ^{1}H NMR (DMSO-d_6), δ: 7.98 (dd, 1 H, H(5), pyridyl, J = 8.2 Hz, J = 5.0 Hz); 8.70 (ddd, 1 H, H(4), pyridyl, J = 8.2 Hz, J = 2.0 Hz, J = 1.2 Hz); 8.94 (dd, 1 H, H(6), pyridyl, J = 5.0 Hz, J = 1.2 Hz); 9.10 (d, 1 H, H(2), pyridyl, J = 2.0 Hz); 9.64 (br.s, 1 H, NH); 13.26 (br.s, 1 H, =NOH). Found (%): C, 36.95; H, 2.98; N, 13.72; Cl, 35.87.

$C_6H_6N_2Cl_2O$. Calculated (%): C, 37.33; H, 3.13; N, 14.51; Cl, 36.73.

Pyridine-2-hydroximoyl chloride hydrochloride (2a) was prepared similarly to **2b** from 2-pyridinecarbaldoxime (**1a**) (4.00 g, 0.033 mol) in 100 mL of freshly distilled dichloromethane. Yield 5.91 g (93%), white crystals, m.p. 143—145 °C. ^{1}H NMR (DMSO-d_6), δ: 7.60 (ddd, 1 H, H(5), pyridyl, J = 7.0 Hz, J = 5.0 Hz, J = 1.3 Hz); 7.94 (d, 1 H, H(3), pyridyl, J = 7.0 Hz); 8.00 (td, 1 H, H(4), pyridyl, J = 7.0 Hz, J = 1.5 Hz); 8.70 (d, 1 H, H(6), pyridyl, J = 5.0 Hz); 10.04 (br.s, 1 H, NH); 12.98 (br.s, 1 H, NOH). ^{1}H NMR ($CDCl_3$—CD_3OD (1 : 2)), δ: 7.60 (dd, 1 H, H(5), pyridyl, J = 8.0 Hz, J = 6.0 Hz, J = 1.3 Hz); 8.11 (d, 1 H, H(3), pyridyl, J = 8.0 Hz); 8.33 (td, 1 H, H(4), pyridyl, J = 8.0 Hz, J = 1.5 Hz); 8.53 (dd, 1 H, H(6), pyridyl, J = 6.0 Hz). Found (%): C, 37.17; H, 3.02; N, 13.51; Cl, 35.14. $C_6H_6N_2Cl_2O$. Calculated (%): C, 37.33; H, 3.13; N, 14.51; Cl, 36.73.

Pyridine-4-hydroximoyl chloride hydrochloride (2c) was prepared similarly to **2b** from 4-pyridinecarbaldoxime (**1c**) (9.40 g, 0.077 mol) in 150 mL of freshly distilled dichloromethane. Yield 14.14 g (95%), white crystals, m.p. 226—228 °C. ^{1}H NMR (DMSO-d_6), δ: 8.17 (m, 2 H, H(3), H(5), pyridyl); 8.91 (m, 2 H, H(2), H(6), pyridyl); 13.67 (s, 1 H, NOH). Found (%): C, 36.79; H, 2.85; N, 14.01; Cl, 35.03. $C_6H_6N_2Cl_2O$. Calculated (%): C, 37.33; H, 3.13; N, 14.51; Cl, 36.73.

Synthesis of 5-substituted 3-pyridylisoxazoles 3—36 (general procedure).[46,58] A solution of triethylamine (1.62 g, 16.0 mmol) in 30 mL of ethanol was added dropwise at 0—5 °C to a stirred solution of pyridine-2-, 3-, or 4-hydroximoyl chloride hydrochlorides **2a—c** (1.56 g, 8.0 mmol) and terminal alkyne (24.0—40.0 mmol) in 80 mL of anhydrous ethanol. The course of the reaction was monitored by TLC. The reaction mixture was kept for 24—48 h at 20 °C and then the solvent was removed *in vacuo*. The residue was suspended in 50 mL of diethyl ether, the precipitated triethylamine hydrochloride was separated by filtration and washed with diethyl ether (3×10 mL), and the filtrates were combined. After the solvent from the combined filtrate had been removed *in vacuo*, the residue thus obtained was separated by column chromatography to obtain several fractions containing the product and a minor portion of impurities (elution with dichloromethane—ethanol with ethanol gradient from 0 to 4% v/v). The individual product was isolated by purification of the fractions by HPLC. The yields, melting points, and the data of ^{1}H NMR and mass spectra for all of the products are summarized in Table 1.

3-(2-Pyridyl)- (3), 3-(3-pyridyl)- (16), and 3-(4-pyridyl)-isoxazoles (25) were prepared from the appropriate pyridine-2-, 3-, or 4-hydroximoyl chloride hydrochloride **2a—c** (1.0 g, 5.2 mmol), a saturated solution of acetylene in 30 mL of dry methanol, and a solution of triethylamine (1.1 g, 1.52 mL, 10.9 mmol) in 15 mL of methanol at −(10—5) °C. The reaction mixture was stirred for 20—24 h at 20 °C. After workup of the reaction mixture, the product was isolated by column chromatography (elution with $CHCl_3$—MeOH with the MeOH gradient from 0 to 5% v/v).

3-(2-Pyridyl)- (9), 3-(3-pyridyl)- (19), and 3-(4-pyridyl)-5-phenylisoxazoles (30)[46,47] were prepared from the appropriate pyridine-2, 3-, or 4-hydroximoyl chloride hydrochloride **2a—c** (1.0 g, 5.2 mmol) and phenylacetylene (2.65 g, 26.0 mmol). The workup of the reaction mixture and recrystallization from hexane (compound **9**), benzene (compound **19**), or a benzene—hexane mixture (compound **30**) afforded the purified product. Instead of recrystallization, the individual product can be isolated by HPLC (see above).

5-Hydroxymethyl-3-(2-pyridyl)- (4), 5-hydroxymethyl-3-(3-pyridyl)- (17), and 5-hydroxymethyl-3-(4-pyridyl)isoxazoles (26)[46,48] were prepared from the appropriate pyridine-2-, 3-, or 4-hydroximoyl chloride hydrochloride **2a—c** and freshly distilled 1-propyn-3-ol. The workup of the reaction mixture and recrystallization from benzene (compounds **4** and **26**) or ethanol (compound **17**) afforded the purified product. Instead of recrystallization, the individual product can be isolated by HPLC (see above).

5-(2-Hydroxy-2-propyl)-3-(3-pyridyl)isoxazole (20)[46] was prepared from the pyridine-3-hydroximoyl chloride (**2b**) and freshly distilled 2-hydroxy-2-methyl-3-butyne. The workup of the reaction mixture and recrystallization from an ethanol—water mixture (10 : 1) afforded the purified product.

5-Bromomethyl-3-(2-pyridyl)isoxazole (5)[46] was prepared from the pyridine-2-hydroximoyl chloride hydrochloride (**2a**) (0.62 g, 3.2 mmol), propargyl bromide (0.96 mL, 12.8 mmol) in 35 mL of ethanol, and triethylamine (0.65 g, 6.4 mmol) in ethanol (5 mL). The workup of the reaction mixture and three recrystallizations from a hexane—benzene mixture afforded the purified product.

5-Alkyl-3-(2-pyridyl)- (6—8, 10—12) and 5-alkyl-3-(4-pyridyl)isoxazoles (27—29, 31—33) were prepared from the appropriate pyridinehydroximoyl chloride hydrochloride **2a—c** (1.0 g, 5.2 mmol) and terminal alkyne (1-pentyne, 1-hexyne, 1-heptyne, 1-octyne, 1-nonyne, and 1-decyne) (15.6 mmol). The individual product was isolated by HPLC (see above).

Ethyl 3-(2-pyridyl)- (15), 3-(3-pyridyl)- (22), and 3-(4-pyridyl)-5-isoxazolecarboxylates (36) were prepared from the appropriate pyridinehydroximoyl chloride hydrochloride **2a—c** (1.0 g, 5.2 mmol) and ethyl propiolate (1 mL, 9.87 mmol). Compounds **15**, **21**, and **36** were isolated by recrystallization from hexane.

3-(2-Pyridyl)- (14), 3-(3-pyridyl)- (21), and 3-(4-pyridyl)-5-isoxazolecarboxylic acids (35) were prepared by hydrolysis of their ethyl esters (0.3 g, 1.38 mmol) in methanol with a 10% solution of KOH in methanol (0.2 mL). The mixture is stirred for 15 min, water (2 mL) was added, and the resulting water—methanol mixture was neutralized with 5% HCl, and the precipitate, which was the individual product, was filtered off and dried *in vacuo*.

3-(2-Pyridyl)- (13), 3-(3-pyridyl)- (18), and 3-(4-pyridyl)-5-isoxazolecarboxamides (34) were prepared by amination of the corresponding ethyl esters. Ammonia-saturated methanol (2 mL) was added to compound **15**, **22**, or **36** (0.3 g, 1.38 mmol) and the mixture was stirred for 15—30 min. The white precipitate, which was the individual product, was filtered off and dried *in vacuo*.

***N*-Benzyl-3-(3-pyridyl)-5-isoxazolecarboxamide (24).** Benzylamine (0.15 mL, 1.45 mmol) was added with stirring to ethyl 3-(3-pyridyl)-5-isoxazolecarboxylate (0.3 g, 1.38 mmol) in 10 mL of ethanol, the mixture was heated to reflux and refluxed for 3 h. The white precipitate formed after cooling of the reaction mixture was filtered off and dried *in vacuo*.

5-(2,2,3,3,4,4,4-Heptafluorobutanoyloxymethyl)-(3-(3-pyridyl)isoxazole (23) was prepared in 27% yield by acylation of 5-hydroxymethyl-3-(3-pyridyl)isoxazole (**17**) by the method of mixed anhydrides in the presence of 4-(dimethylamino)pyridine at 0 °C.

3,5-Diphenylisoxazole (39). The starting compound, benzaldehyde oxime, was prepared from benzaldehyde (12.72 g, 0.12 mol) and hydroxylamine hydrochloride (8.43 g, 0.12 mol). Benzaldehyde and 30% aqueous NaOH (to pH 12) were slowly added at 0—2 °C to a solution of hydroxylamine hydrochloride in 20 mL of water. The product was extracted with diethyl ether (3×100 mL), the combined extract was dried over anhydrous sodium sulfate, and the solvent was removed *in vacuo*. The yield of benzaldoxime was 13.6 g (95%), m.p. 62—63 °C. Benzaldoxime (1.7 g, 14.0 mmol), phenylacetylene (1.6 g, 15.6 mmol), and 100 μL of Aliquat 336 was added with stirring at 0—2 °C to 50 mL of a 7% aqueous solution of sodium hypochlorite (3.5 g, 47.0 mmol) . The reaction mixture was kept for 20—24 at 20 °C. 3,5-Diphenylisoxazole was filtered off and washed with cold water (3×10 mL), the precipitate was triturated with diethyl ether, filtered off, washed with cooled diethyl ether, and twice recrystallized from $CHCl_3$.

3-(3-Pyridyl)-5-phenylisoxazole (19) was prepared by phase transfer catalysis procedure. A solution of the Belizna agent (15.3 mL, 8.2 mmol) was added dropwise with stirring at a temperature of 20 °C to a solution containing 3-pyridinecarbaldoxime (**1b**) (8.2 mmol), phenylacetylene (12.7 mmol), and 100 μL of Aliquat 336 in 60 mL of dichloromethane. The reaction mixture was stirred for 48 h at 20 °C. The reaction was monitored by TLC. The organic layer was separated, the aqueous layer was extracted with dichloromethane (3×20 mL), the combined organic layer was dried with sodium sulfate and filtered, and the solvent was removed *in vacuo*. The individual product was isolated by column chromatography (elution with CH_2Cl_2—EtOH with the EtOH gradient from 0 to 20% v/v). Yield 2.38 mmol (29%).

Liquid-phase method for the introduction of a tritium label into the 3-(3-pyridyl)-5-phenylisoxazole molecule.[69] A tube containing 3-(3-pyridyl)-5-phenylisoxazole (**19**) (4 mg), the 5% $Pd/BaSO_4$ catalyst (12 mg), and 0.2 mL of dioxane was frozen with liquid nitrogen, evacuated, and filled with tritium gas up to a pressure of 350 GPa. After thawing, the content of the tube was stirred for 1 h at 25 °C. Then the excess tritium was removed: the catalyst was filtered off and washed with methanol (1 mL), the filtrate was evaporated to dryness, the residue was re-dissolved three times in methanol (3 mL), and the resulting solution was concentrated. The labeled product was isolated by preparative HPLC (Knauer chromatograph, Smart Line 1000 model, a Silasorb 10×250 mm column, 7 μm, elution with 90% aqueous methanol, radioactivity detector, flow rate 3 mL min^{-1}). This gave an indefinitely tritium-labeled 3-(3-pyridyl)-5-phenylisoxazole [^{3}H]-**19** in 50—60% yield and with a molar radioactivity of 0.24 Ci mol^{-1} (8.88 GBq mol^{-1}). The most probable sites of tritium substitution for protium are in the phenyl group (mainly the *ortho*-positions).

Testing of compounds for antiaggregatory activity *in vitro*.[45] The effect of the potential antiaggregatory agents on the platelet aggregation in human blood plasma samples was studied by a previously elaborated procedure using the turbidimetric method on a Biola aggregometer (Russia). As the natural inductors of human platelet aggregation, arachidonic acid, adrenaline, or adenosine diphosphate were used.

Platelet-rich plasma (PRP) was obtained in the laboratory of physical biochemistry of the National Research Center for Hematology of the Ministry of Health of the Russian Federation by the generally accepted procedure using blood samples freshly taken at the blood transfusion station from male volunteers below 40 years of age using a 3.8% solution of sodium citrate (pH 7.4). The blood to anticoagulant ratio was 9 : 1. The blood was centrifuged for 15 min at 200*g* at room temperature on a MLW K70D centrifuge (GDR), then PRP was separated. Platelet poor plasma (PPP) was prepared by additional centrifuging of PRP at 1000*g* for 15 min at room temperature on a OP_N-8-U 4.2 centrifuge (USSR). The initial concentration of platelets in PRP was determined on an ABX—MICROS counter (Austria); then the concentration was brought to a standard value of $2 \cdot 10^5$ cells μL^{-1} by diluting PPP. The instrument was calibrated using both PRP and PPP. Platelet rich plasma (235—249 μL) was placed into an agregometer cell, incubated for 2 min at 37 °C and at continuous stirring (1000 rpm), then the aggregation inductor was added, and the measurements were carried out over a period of 10 min after its addition.

In the study of potential platelet aggregation inhibitors, an inhibitor was added to the cell after introduction of the PRP, the mixture was incubated for 2 min, the aggregation inductor was added (the final concentration of the inductor in the cell was 500 μmol L^{-1} for arachidonic acid, 10—15 μmol L^{-1} for adenosine diphosphate, and 1—4 μmol L^{-1} for adrenaline), and the measurements were carried out. The total volume of cell content was 250 μL.

For comparing the antiaggregatory properties of the test compounds, the following kinetic parameters were determined in each experiment: the maximum degree of aggregation (A_{max}), the maximum rate of aggregation (V_{max}), and the inhibition constant K_i (by the Hill equation, see Ref. 65). The antiaggregatory activity was quantitatively characterized by the concentration of the compound providing a 50% inhibition of platelet aggregation (IC_{50}). This value was determined from the S-shaped curves for A_{max} and V_{max} plotted *vs.* the test compound concentration (A_{max}/[I]) and V_{max}/[I]).

Testing of compounds for the antithrombin activity.[70] The possible antithrombin action of the test compounds was studied by measuring the kinetics of thrombin-induced cleavage of the specific low-molecular-weight chromogenic substrate Chromozyme TH (CTH) in the presence and in the absence of these compounds.

A buffer containing 140 m*M* NaCl, 20 m*M* HEPES, and 0.1% polyethylene glycol (6000) (pH 8.0) was introduced into the wells of a 96-well plate. The substrate, Chromozyme TH (final concentration in the well of 100 μmol L^{-1}), thrombin (final concentration in the well of $1.9 \cdot 10^{-10}$ mol L^{-1}), and a thrombin inhibitor in various concentrations (5, 10, 25, 50, and 125 μmol L^{-1}) were added. The accumulation of the colored reaction product, *p*-nitroaniline, was determined on a Molecular Devices spectrophotometric microplate reader (Thermomax, USA) by measuring the increase in the absorption at 405 nm. The initial reaction rate was determined as the slope of the straight segment of the kinetic curve (the first 10—15 min of measurement). The reaction rate without an inhibitor was taken as 100%. Each result was the average of four repeated measurements with a scatter between them of no more than 2—5%.

Study of the effect of 3-(3-pyridyl)-5-phenylisoxazole (19) on the activity of cyclooxygenase from the sheep vesicular glands.[66] The cyclooxygenase specimen, the microsomal fraction from the sheep vesicular glands, was prepared by a reported procedure.[67] The protein content in the specimen was determined by

the Lowry method. The cyclooxygenase activity and the effect of two inhibitors on the enzyme were studied by polarography according to a reported procedure.[66] The reaction using the enzyme specimen was performed with continuous stirring at 25 °C in a 0.82 mL cell equipped with a platinum silver Clark electrode with a hole of diameter 0.8 mm for reactant injection, maintained in a thermostat. The reaction mixture contained 0.05 *M* potassium phosphate buffer (pH 7.8), 10 μL of an enzyme solution, $3.3 \cdot 10^{-6}$ mol L^{-1} of hemin, and the inhibitor (the inhibitor concentrations are given in Table 3). The mixture was incubated for 2 min, then 150 μmol L^{-1} of arachidonic acid was added. The oxygen concentration in the system was continuously recorded by an OH-105 polarograph (Radelkis, Hungary) based on the diffusion current at 600 mV at the working platinum electrode. The initial rate of the cyclooxygenase reaction was determined by the slope of the tangent to the kinetic curve at the initial instant of the reaction. The inhibiting action of the studied compounds was characterized by the ratio V/V_0, where V and V_0 are the oxygen absorption rates in the reaction in the presence and in the absence of the inhibitor, respectively.

Study of the effect of 3-(3-pyridyl)-5-phenylisoxazole (19) on the thromboxane synthase activity.[68] Adrenaline (5 mmol L^{-1}), hemin (2 μmol L^{-1}, and an inhibitor solution in ethanol ($1 \cdot 10^{-3}$ mol L^{-1}) were added to a suspension of platelet microsomes (50 μg of the protein), and the mixture was incubated for 5 min at 25 °C. Then the volume of the mixture was brought to 200 μL by adding 20 μL of 0.2 *M* potassium phosphate buffer and distilled water. A mixture of 2.0 μCi [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid and unlabeled arachidonic acid (12 mg, 200 μmol L^{-1}) in ethanol was placed in a 1.7 mL Eppendorf type microcentrifuge tube. Ethanol was removed, and 200 μL of the prepared incubated medium was added, as described above, to the resulting mixture of labeled and unlabeled arachidonic acids. The resulting specimen was incubated for 1 h at 25 °C and at continuous stirring, then acidified with 0.5 *M* citric acid to pH 3 and extracted with ethyl acetate (3×700 μL). The solvent from the combined organic extract was removed *in vacuo*, the residue was dissolved in 50 μL of methanol and applied onto a 20×20 cm Silufol plate. After chromatography in the chloroform—methanol—acetic acid system (90 : 90 : 1), the distribution of radioactive products was determined using a Berthold radioactivity scanner (Germany). The amount of thromboxane B_2 was calculated from the peak area in percent of the total radioactivity. The data of the experiment are summarized in Table 4.

Binding of [^{3}H]-3-(3-pyridyl)-5-phenylisoxazole to receptors of human platelet membrane receptors.[68] The binding of tritium-labeled 3-(3-pyridyl)-5-phenylisoxazole [^{3}H]-**19** was studied by a known procedure using, as the source of platelets, human platelet rich plasma with a platelet concentration of 200000 cells μL^{-1} obtained from a blood sample of a healthy donor at the National Research Center for Hematology of The Ministry of Health of the Russian Federation. The total activity of the [^{3}H]-**19** solution was 4.6 mCi, and the specific radioactivity was 0.24 Ci mol^{-1}. For each sample, the PRP volume was 0.5 mL. For samples, concentrations of 34 and 0.34 μmol L^{-1} of [^{3}H]-**19** were taken, and concentrations of 3.4 mmol L^{-1} and 34 μmol L^{-1}, respectively, were taken for unlabeled compound **19**; the times of incubation were 2 and 30 min.

For each concentration of labeled [^{3}H]-**19**, several samples were prepared in microcentrifuge tubes (see Table 5) pretreated with an egg albumin solution (1 mg L^{-1}) and dried at room temperature for 1 h. The pretreated samples were incubated at 37 °C.

After incubation (2 or 30 min), 200 μL samples were taken and layered on a cooled (4 °C) saccharose solution (200 g L^{-1}) in solution 1 (137 m*M* NaCl, 2.68 m*M* KCl, 1.19 m*M* $NaHCO_3$, 0.417 m*M* NaH_2PO_4 in distilled water, pH 7.0) of 1 mL volume in microcentrifuge tubes pretreated with albumin, and centrifuged for 2 min on a 320a microcentrifuge (Poland) at 8000 rpm. The tip of the tube together with the precipitate was cut off by a dedicated device and, after removal of the remaining solution, placed into a plastic vial where 1 mL of a 1% solution of sodium dodecyl sulfate was then added. The vials were shaken for 3 h on a WU-3 shaker (Poland) at room temperature, then the ZhS-8 liquid scintillator (15 mL) was added, the mixture was stirred for 30 min, and the radioactivity was measured using a Delta liquid scintillaion counter, the radioactivity being determined in cpm (counts per minute). The cpm values were converted into Ci in the following way: 1 Ci = $37 \cdot 10^9$ Bq, where 1 Bq is equal to the number of decays per second. Since cpm = $\alpha \cdot$dpm, where dpm is decays per minute, $\alpha = 0.3$ is the counting efficiency, we have that 1 Ci = $667 \cdot 10^9$ cpm. All data are presented in Table 5.

The total binding was determined from the amount of [^{3}H]-**19** that has bound to platelets; nonspecific binding was found from the amount of [^{3}H]-**19** that has bound to platelets in the presence of unlabeled compound. The specific binding was determined as the difference between the total and non-specific binding.

Samples *1*, *2*, and *17—24* were taken as the controls (see Table 5).

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