UDC 577.15.08+577.158

Inhibitors of Nitric Oxide Synthases: the Chemical Aspect of the Problem

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(Received February 19, 2010; revised October 10, 2010)

Abstract

Different classes of compounds exhibiting the properties of inhibitors for NO synthases are considered in the review. Literature data on natural and synthetic agents published between the years 2003 and 2009 are analyzed and systematized.

Key words: nitrogen oxide, inhibitors of nitrogen oxide, NO synthases, selectivity

Contents

INTRODUCTION

The discovery of the physiological and pathophysiological role of nitric oxide NO in the 80es of the past century became one of the most outstanding discoveries in the area of biological sciences [1–3]. It was demonstrated in the studies dealing with the role of NO in neurotransmission [4, 5] and in the formation of responses of the immune system [6] that NO is a messenger which is unique in its nature and the mechanisms of action in the majority of cells in an organism. It participates in regulating many physiologically essential processes such as vasodilation, thrombocyte aggregation, memory state, and some pathological processes [7, 8]. A deep resonance of the important biological role of NO in an organism and a large number of publications in the world scientific literature served as a basis for the journal Science to declare NO to be "The Molecule of Year" in 1992 [9]. American scientists R. Furchgott, F. Murad and L. Ignarro were awarded Nobel Prize in physiology and medicine in 1998 for the discovery of nitric oxide as a signal molecule in the cardiovascular system. The review proposed by us now deals with the search and synthesis of the substances possess-

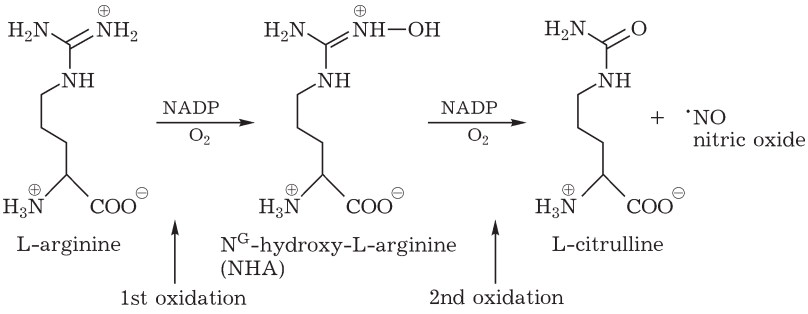
ing the properties of inhibitors of NO synthases, it embraces chemical and biological studies since 2003 till 2009. Research in this area published before 2003 was thoroughly reviewed in [10].

In the chemical sense, NO is uncharged paramagnetic molecule. Its chemical and physiological properties are due to the tendency to stabilize the unpaired electrode which is present in the molecule [11, 12]. Unlike for oxygen radicals, the half-decay time for NO can reach several second depending on tissue type and physiological conditions [13]. Due to this fact, NO can freely penetrate through biological membranes, interact with intra- and extracellular structures located relatively far from the site of the formation of this molecule, and easily react with other compounds [14]. For example, NO is able to form stable S-nitrosothiols [15] and to add to the central heme of heme-containing proteins thus forming methemoglobin, which can be considered as the transport form of nitrogen oxide [16]. The role of NO in the organism is dual: on the one hand, it diffuses into parasitic cells, inhibits enzymes that are necessary for the cells, and destroy them thus playing the protective part. On the other hand, NO being a strong cytostatic agent causes tangible hazard to the organism under the conditions of oxidative stress and the formation of active oxygen forms (AOF), mainly peroxynitrites [17]. Peroxynitrites cause damage of proteins and lipids in cell membranes, destroy vascular endothelium, increase thrombocyte aggregation. When inside a cell, NO damages the DNA of the target cell through deamination, as well as due to the inhibition of ribonucleotide reductase which controls the rate of DNA replication.

Almost the whole amount of endogenous NO is synthesized from L-arginine in the process of catabolism of L-arginine into L-citrulline by the family of cytochrome P450-like hemoproteins – NO synthases (NOS) [18] (Scheme 1). NO synthases are multidomain proteins composed of the heme oxidative domain, calmodulin binding linker and nicotinamide adenine dinucleotide phosphate (NADP) reductive domain, which catalyzes the formation of NO [19, 20]. At present, three isoforms of NOS are distinguished; they were called according to the type of cells in which they were discovered for the first time [21]: NOS-1 – neuronal (nNOS) or brain (bNOS); NOS-2 – inducible (iNOS) or macrophage (mNOS); NOS-3 – endothelial (eNOS). Isoforms of NOS are the products of different genes. Before 1994, different forms of NOS were identified according to several characteristics [22, 23] but at present they are not so evident:

1. nNOS and eNOS are constitutive, iNOS is inducible. In resting cells NOS-2 (iNOS) usually is not determined. For its expression, induction by liposaccharides (LPS) or cytokines, such as interleukine-1, interleukine-2, interferon-γ (IFN-g), tumour necrosis factor α (TNF-a) and others, is necessary. It should be noted that the constitutive forms promote liberation of a small amount of NO (picomoles); the amount of NO formed under the action of iNOS may vary and reach large amounts (nanomoles). In this situation, the production of NO is conserved for a longer period of time. It is now evident that all the three isoforms can be induced, though by different stimulators, and all of them can be permanently present in some cells and tissues [24].

2. nNOS and eNOS are calmodulin dependent, iNOS is calmodulin independent. This relates first of all to iNOS of humans and ro-



Scheme 1. Formation of NO with the help of NOS.

dents. Another regularity was revealed for iNOS of cavies [25].

3. nNOS and iNOS are localized exclusively in cytosol, while eNOS is usually bound with cell membranes. It has been established by present that all three isoforms can be present either in dissolved state or in the bound one [26].

Nevertheless, all the three NOS isoforms require coenzymes for the transformation of L-arginine into NO and L-citrulline: the reduced form of nicotinamide adenine dinucleotide phosphate (NADP), tetrahydrobiopterin (H_4B), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) [27]. The mechanism of NO formation from L-arginine is similar for all the three isoforms: it is a two-step oxidation reaction. The electrons rendered by NADP are transferred through FAD and FMN to the heme group, which leads at the second stage to the formation of L-citrulline and to the evolution of NO.

The mechanism of the «second oxidation» was under verification for a long time [28–30]. For instance, the authors of newer works [31, 32] suppose that after the rupture of N–H bond by heme iron-peroxo, an intermediate cation radical is formed, which promotes the rupture of O–H bond with the formation of iminoxy radical. The nucleophilic attack of the heme iron-peroxo at the iminoxy radical leads to the tetrahedral intermediate which decomposes with the formation of NO and citrulline (Scheme 2).

Three isoforms of NOS are involved in various pathological processes including Alzheimer disease and stroke (NOS-1 or nNOS), septic shock, arthritis and inflammatory processes (NOS-2 or iNOS), the formation of edema and endothelial disorders (NOS-3 or eNOS). This defines the need for selective inhibitors of various NOS isoforms [33]. In addition, an ideal NOS inhibitor should not inhibit the enzyme irreversibly because this can cause undesirable consequences.

It should be stressed that NOS can be divided into the following groups according to the mechanism of their action:

1. Compounds those are able to prevent admission of L-arginine to the active centre of the enzyme.

2. Substances that inactivate cofactors which are necessary for catalyzed NOS oxidation of L-arginine.

3. Inhibitors of electron transfer with the participation of NADP and flavins, as well as the agents able to interfere in heme functions.

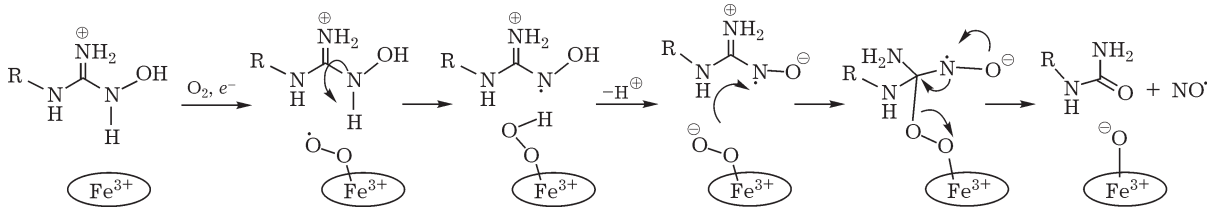
4. Compounds inhibiting NOS expression.

5. Compounds that decrease the concentration of nitric oxide formed [10].

According to their origin, inhibitors of NO synthases can be divided into natural, semisynthetic and synthetic. By present, search for selective inhibitors of NOS is carried out in all these three directions.

NOS INHIBITORS OF NATURAL ORIGIN

Plant raw material is the most important source of biologically active compounds, so one of the main directions of the search for selective NOS inhibitors of natural origin is testing of plant extracts or individual compounds isolated from them. When choosing the plant raw material, one often keeps in mind the area of the application of this plant in folk medicine and the possibility to collect it in nature or to cultivate it. Primary biological screening of plant extracts, isolation of active agents, tests of individual compounds and their further modification for the purpose of increasing their activity, improvement of solubility, and accordingly their biological availability form a standard scheme of the search for potential inhibitors of NOS. Limitations of this approach are connected with the fact that the extraction of



Scheme 2. Mechanism of the second oxidation.

natural compounds from plant raw material in sufficient amounts is possible not always because of the low content of the required compound in the plant, labour-intensive isolation procedure, inaccessibility of a plant itself. In this connection, phytochemical investigations of this kind are rarely performed according to the full scheme. Most frequently researchers limit their efforts to the extraction of major biologically active compounds from plants.

Alkaloids, isoprenoids, steroids

It was demonstrated during the tests of 80 % water/methanol extract of the bulbs of *Crinum yemense* (amaryllis family) that the extract inhibits the release of NO in LPS activated macrophage medium. Alkaloids isolated from this extract – yemenine A **1**, (+)-crinamine **2**, (+)-6-hydroxycrinamine **3** and (–)-lycorine **4** (Fig. 1) – exhibited the properties of iNOS inhibitors [34]. According to the mechanism of action, these compounds were related by the authors of that work to the agents suppressing the expression of iNOS.

Sesquiterpene lactones **1–13** (see Fig. 1) extracted from *Artemisia sylvatica* [35] exhibited inhibiting activity against LPS induced expression of NF-kB. The latter, which is a nuclear factor, a protein of k group discovered for the first time in b-lymphocytes, transformed into the active state, for example, by the action of lipopolysaccharides, triggers transcription with the formation of corresponding *m*-RNA. The latter thens get liberated into the cytoplasm, "and the synthesis of corresponding proteins including iNOS is triggered at the ribosomes. The mechanism of the action of above-indicated sesquiterpene lactones on the inhibition of LPS induced expression of nuclear factor NB-kB was not elucidated by the authors but the consequence of this process is the inhibi-

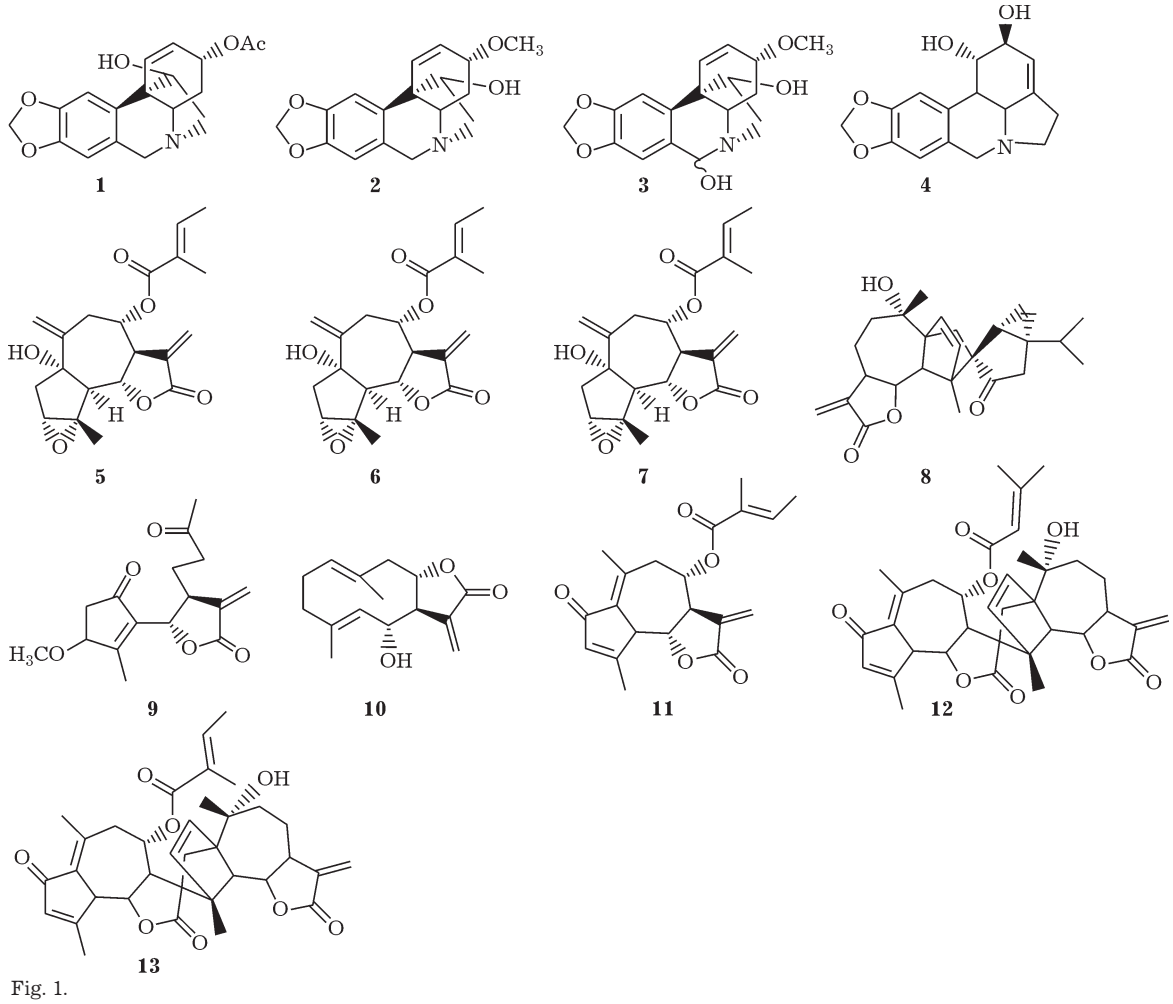


Fig. 1.

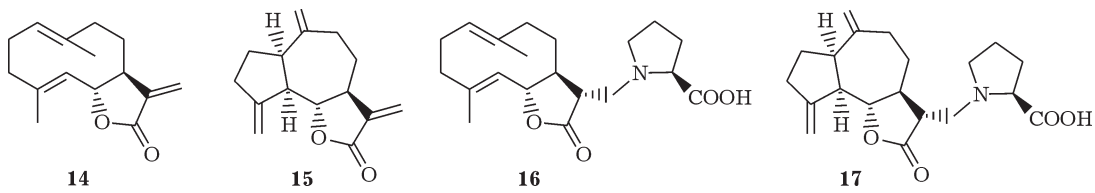


Fig. 2.

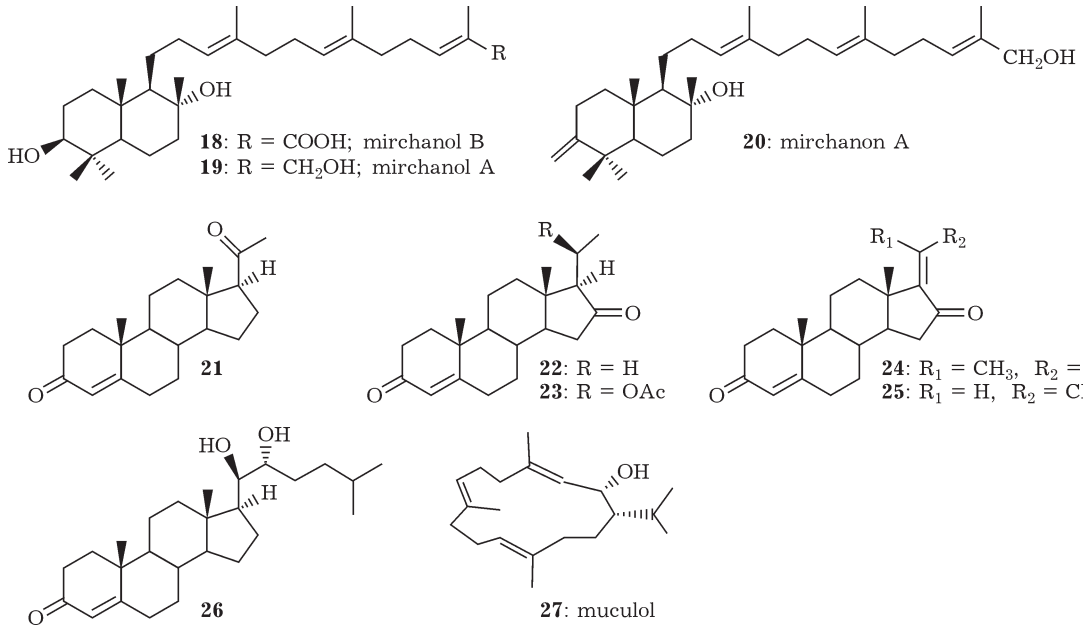


Fig. 3.

tion of iNOS expression. In this connection, the authors stress the importance of using the components of the extract in medicine to treat inflammations of any ethiology.

Similar properties are exhibited by the major components of the methanol extract of the roots of *Saussurea lappa* Clarke – lactones **14**, **15** (Fig. 2) and conjugates of these lactones with amino acids **16**, **17**, described in [36]. They cause a decrease in the production of NO in LPS activated peritoneal macrophages of mice suppressing the expression of nuclear factor NF-kB.

The authors of [37] studied medicinal plant *Balsamodendron mukul* Hook growing in India, Sri Lanka and northern Africa. Water/methanol extract of the galipot of this plant also inhibited the liberation of NO in LPS activated peritoneal macrophages of mice. Compounds of different structures that were isolated from the extract – diterpenoids, triterpenoids, and steroids **18**–**27** (Fig. 3) exhibited inhibiting activity without any cytostatic effect. In particular, the most active compounds **19**, **27** showed themselves as selective dose-dependent inhibitors of iNOS expression. Possibly it is the presence of these components that determines the therapeutic action of this plant.

Stilbenes, bibenzyls and bisbibenzyls

It was shown in [38] that pterostilbene **28** (Fig. 4) – *trans*-3,5-dimethoxy-4′-hydroxystilbene, dimethyl analogy of resveratrol **29**, extracted from *Vaccinium ashei*, *Vaccinium stamineum* possess anti-inflammatory activity similar to that exhibited by compound **29**, and causes apoptosis of different types of cancer cells. It was demonstrated with the help of PCR analysis that pterostilbene **28** blocks the synthesis of protein and *m*-RNA iNOS, and cyclooxygenase-2 (COX-2) in LPS induced macrophages.

Dihydrostilbenes (bibenzyls) **30**–**35** (Fig. 5) isolated from *Dendrobium nobile* also exhibit iNOS inhibiting activity [39] (Table 1).

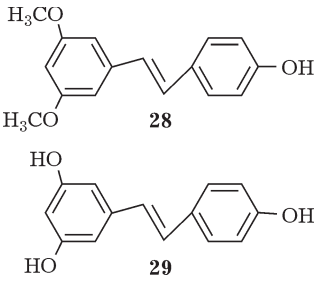
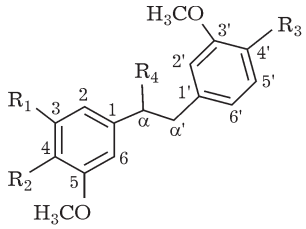


Fig. 4.



30: $R_1 = OCH_3$, $R_2 = R_3 = R_4 = OH$ nobilin D
31: $R_1 = R_2 = OCH_3$, $R_3 = OH$, $R_4 = H$
32: $R_1 = R_2 = R_3 = OCH_3$, $R_4 = H$
33: $R_1 = R_3 = OCH_3$, $R_2 = OH$, $R_4 = H$
34: $R_1 = OCH_3$, $R_2 = R_3 = OH$, $R_4 = H$
35: $R_1 = R_3 = H$, $R_2 = R_4 = OH$

Fig. 5.

Compounds **30**, **32**, **34**, **35** inhibit the production of NO without cytostatic effect, while the values of IC_{50} for compounds **31**, **33** are within the range of cytostatic concentrations. It should be noted that nobilin D **30** exhibits higher NO inhibiting activity in comparison with the reference preparation resveratrol **29** and exhibits two times higher activity than that of dihydrostilbene **34**. The latter fact is likely to be explained by the presence of hydroxyl group in α position (R_4) of nobilin D **30**, which forms the difference of this compound from bibenzyls **31**–**35**.

TABLE 1

Values of IC_{50} for bibenzyls **30**–**35** and resveratrol **29**

Compounds	IC_{50}, μmol
30	15.3
31	Cytotoxicity*
32	48.2
33	Cytotoxicity*
34	36.8
35	32.9
29	23.5

* The value of IC_{50} is within the range of cytostatic concentrations.

Biogenetically bound with dihydrostilbenes are macrocyclic dimer bibenzyls – bisbibenzyls **36**–**54** (Fig. 6). Their natural source is liverwort moss [40]. The inhibiting activity of 19 bisbibenzyls was evaluated in that work (Table 2). One can see that the strongest inhibitor marchantin A **36** (IC_{50} = 1.44 μmol); the introduction of hydroxyl group in position C-12 (as one can see for the example of marchantin B **37**) somehow decreases the inhibiting capacity of bisbibenzyl **37** (IC_{50} = 4.1 μmol). Additional hydroxyl group in C-7′ position decreases the activity of compound **39** by a factor of 7 (IC_{50} = 10.18 μmol), while its methylation (similarly to the case of compound **40**) causes a drop of activity by a factor of 40 (IC_{50} = 62.16 μmol) in comparison with marchantin A **36**. Perrotetin F **48** exhibits not very high inhibiting activity (IC_{50} = 7.4 μmol); the substituents in rings A and C are similar to the substituents present in marchantin A **36**. The IC_{50} values for marchantins A and B, methylated at hydroxyl groups, – compounds **53**, **54** – are equal to 42.5 and 42.45 μmol, respectively, which is an evidence of the essential role of NO non-substituted hydroxyl groups in inhibition. Nevertheless, rickardines A and F **49**, **51** having one methoxy group in position 11 and 1′ demonstrate high inhibiting activity (2.5 and 5 μmol, respectively). This may be explained by the entire set of structural features of rickardines: free hydroxyl group in position C-13′, C14–C12′ biphenyl bond and methoxy group in position C-11 or C-1′.

The data presented in Table 2 confirm the correctness of these considerations for the example of weak inhibiting activity of rickardine C **50** for which the IC_{50} value exceeds 100 μmol. It should be stressed that the inhibiting activity of isoplagiochine D **45** is three times lower than that of rickardine F **51**, though the rings B and D of them are substituted identically. A possible reason of this fact may be the biphenyl bond C6–C2′ and hydroxyl groups in rings A and C which provide the rigid conformation of compound **45**.

Diarylheptanoids and flavonoids

Compounds **55**–**60** were isolated from 80 % water/acetone extract of *Alpinia officinarum*,

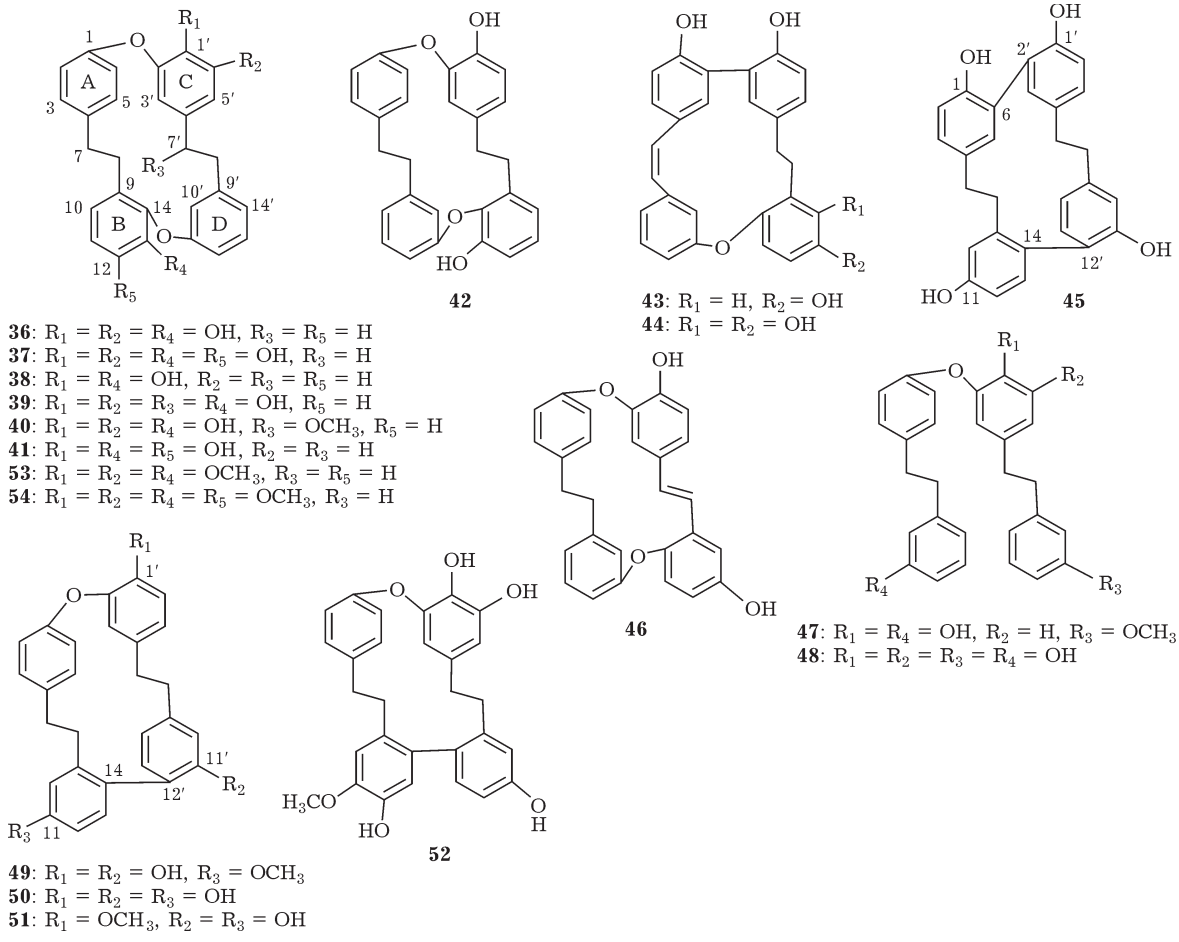


Fig. 6.

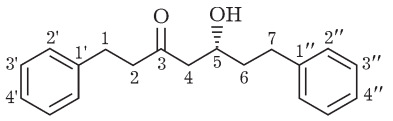
TABLE 2

Values of IC_{50} for compounds 36–54, μmol

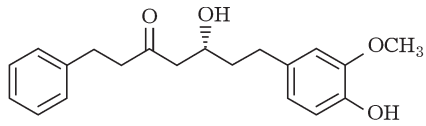
Compounds	Title	IC_{50}
36	Marchantin A	1.44
37	Marchantin B	4.1
38	Marchantin C	13.28
39	Marchantin D	10.18
40	Marchantin E	62.16
41	MarchantinH	15.34
42	Isomarchantin C	>100
43	Isomarchantin A	>100
44	Isoplagiochin B	>100
45	Isoplagiochin D	14.32
46	Ptichantol A	>100
47	11′-Methyl ester of perrotetin E	49.85
48	Perrotetin F	7.42
49	Rickardine A	2.5
50	Rickardine C	>100
51	Rickardine F	5
52	Plagiochin A	9.07
53	Trimethyl ester of marchantin A	42.5
54	Tetramethyl ester of marchantin B	42.45

a Chinese medicinal plant. Among these compounds, **57–59** exhibit the ability to inhibit iNOS [41] (Fig. 7). The values of IC_{50} for these compounds were 33, 62 and 55 μmol, respectively. Pinobaxine **60** exhibited low activity because at the concentration of 100 μmol it inhibited 43.3 % of NO production. To estimate the effect of the framework of compounds **57** on its NO inhibiting activity, tests with similar compounds **61–67** including curcumine **61** and bisdemethoxycurcumine **63** isolated from *Curcuma zedoaria* were carried out. It was demonstrated as a result of investigations that the inhibition of the production of nitrogen oxide by compounds **55** (without hydroxy and methoxy substituents in the aromatic rings), **56** (with one substituted ring) and **65** (in which both aromatic rings have substituents) only slightly differ from each other. They inhibit 31, 32 and 36 % of nitrogen oxide production, respectively.

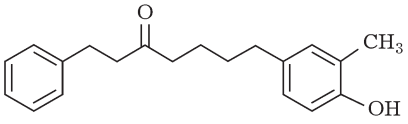
The values of IC_{50} for compounds **61**, **63** differ insignificantly – 11 and 14 μmol, respec-



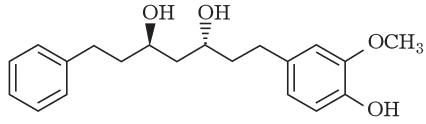
55: 5-hydroxy-1,7-diphenyl-3-heptanone



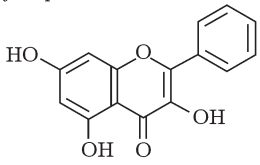
56: 5-hydroxy-7-(4''-hydroxy-3''-methoxyphenyl)-1-phenyl-3-heptanone



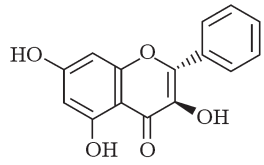
57: 7-(4''-hydroxy-3''-methoxyphenyl)-1-phenylhept-4-en-3-one



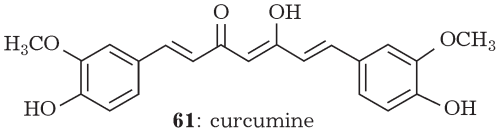
58: 3,5-dihydroxy-1,7-diphenylheptane



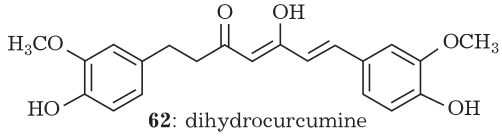
59: galangin



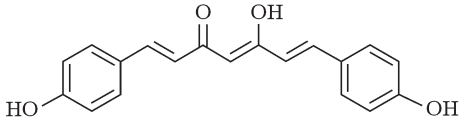
60: pinobaxine



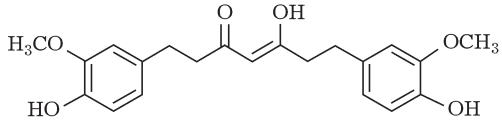
61: curcumine



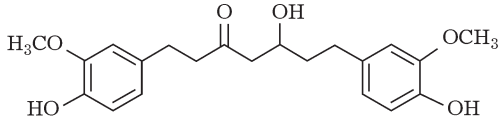
62: dihydrocurcumine



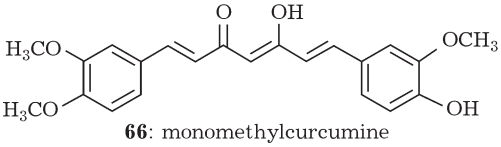
63: bis-demetoxycurcumine



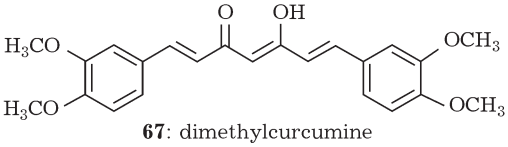
64: tetrahydrocurcumine



65: (+/−)hexahydrocurcumine



66: monomethylcurcumine



67: dimethylcurcumine

Fig. 7.

tively. This allows one to conclude that 3′,3″-methoxy- and 4′,4″-hydroxy substitution in aromatic rings does not have any decisive importance for the manifestation of NO inhibiting activity by the compounds of this type. Nevertheless, methylation of 4′,4″-hydroxy groups leads to insignificant decrease in activity. The value of IC_{50} for compounds **61**, **66** and **67** is 11, 14 and 18 μmol, respectively. Analysis of the structure of diarylheptanoids (see Fig. 7) allows one to conclude that the number of double bonds in the aliphatic fragment binding the aromatic rings affects the inhibiting activity of the compounds of this type. Thus, with a decrease in the number of double bonds passing from curcumine **61** to compounds **62**, **64** and **65** their inhibiting ability decreases: IC_{50} is 11, 25, 90 and more than 100 μmol, respectively. Authors assume that the mechanism of action of these compounds is blockage of the activation of NF-kB.

As shown in [42], the ethanolic extract of the leaves of *Argimonia pilosa* Ledeb. containing a large amount of polyphenol compounds inhibited the production of NO in LPS induced macrophages. Authors isolated five compounds

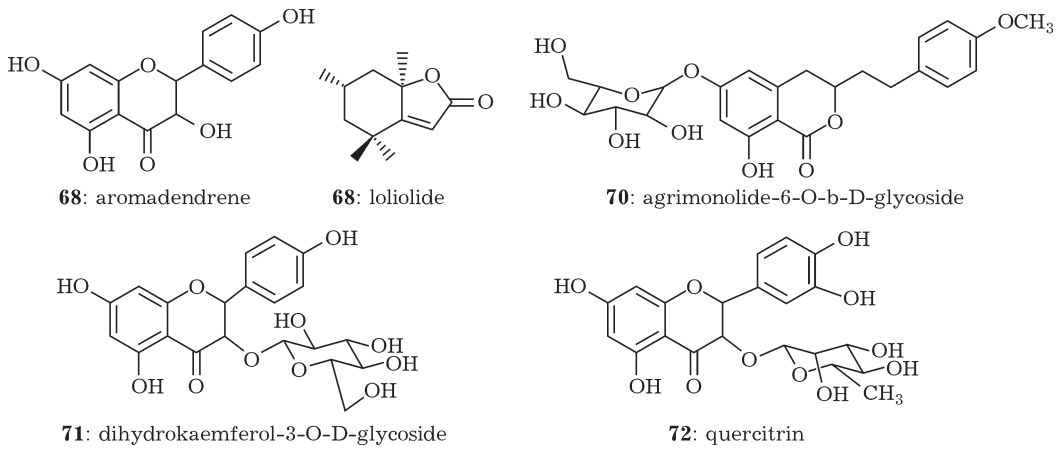


Fig. 8.

(**68–72**, Fig. 8) including three flavonoids (**68**, **71** and **72**) in the free and glycolyzed forms. As experiments showed, all these isolated substances suppress the production of NO and do not exhibit cytostatic properties in the concentrations tested. Repeating several times the generally acknowledged fact that phenol compounds are good traps for radicals and thus exhibit antioxidant properties, the authors of [42] in order to reveal the mechanism of action for compounds **68–72** carried out experiments both with LPS induced macrophage cells and in the presence of NO donor, 4-ethyl-2-hydroxyamino-5-nitro-3-hexenamide (NOR3). A decrease in the concentration of nitrogen oxide was observed in all the experiments. On the basis of the latter facts, authors assume that compounds **68–72** can act both as inhibitors of iNOS and as traps for the formed NO.

The authors of [43] isolated four glycosides of flavonol kaempferol – compounds **73–76** (Fig. 9) – from the methanol extract of the leaves of *Cinnamomum osmorphloemum* Kaneh. These compounds exhibited dose dependent inhibition of NO, in LPS and IFN-g activated macrophage cells. Among the tested compounds, the strongest inhibitor turned out to be compound **74** – kaempferol-3-O-β-D-apiofuranosyl-(1→2)-α-L-arabinofuranosyl-7-O-α-L-ramnopyranoside. For comparison, in the same concentration (20 μmol), compound **74** inhibits the production of NO by 69 %, while compound **75** – only by 9 %. The value of IC_{50} was 40, 15 and 20 μmol for compounds **73**, **74** and **76**, respectively. According to the results of investigations, the isolated glycosides can be ranged as their ability to inhibit NO production decreases: **74** > **76** > **73** > **75**. Authors of the paper do not discuss the correlation between structure and properties. However, comparing compounds **73–76** that differ from each other by the structure of glycoside residue R in position 3, one may note that the most active compound **74** contains two furanose cycles in the residue, compound **76** – one furanose cycle (apifuranosyl, similarly to compound **74**), while the least active compounds of this series **73**, **75** contain only pyranose residues.

More than 50 compounds were isolated from the extracts of *Angelica furcijuga*, a known medicinal plant widely used in the folk medicine of Japan as a hepatoprotective, anti-in-

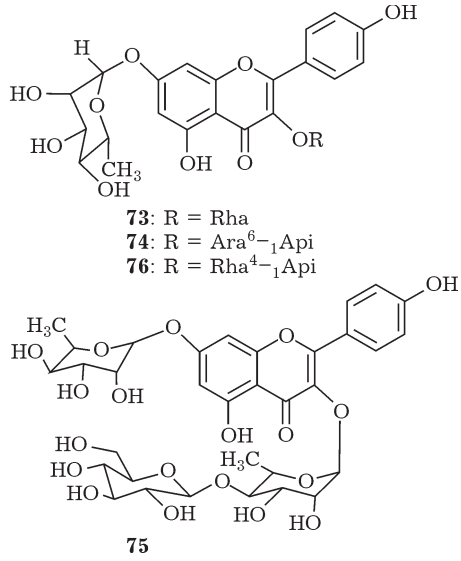
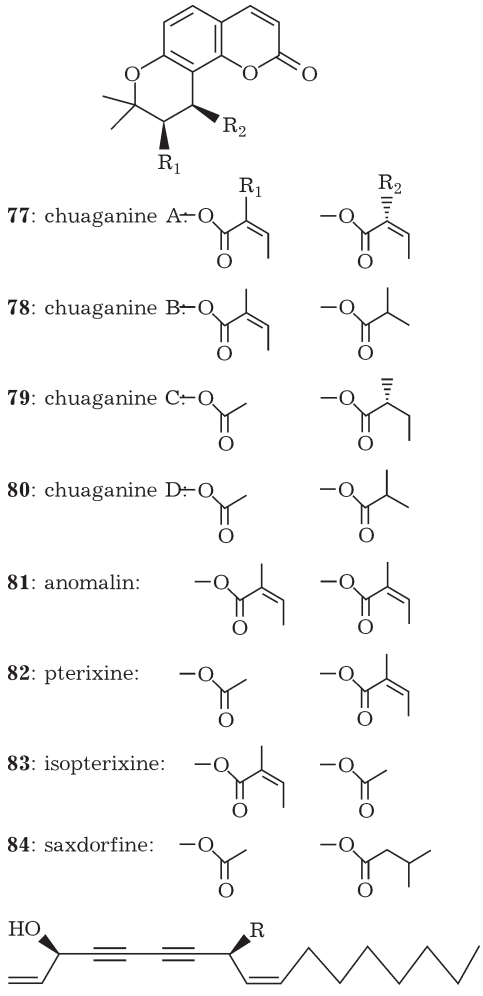


Fig. 9.

flammatory, antiallergic and hypotensive agent. Some metabolites with coumarin framework (**77–84**) and diacetylenic derivatives **85**, **86** (Fig. 10) were tested for iNOS inhibiting activity in LPS activated macrophages [44].

The inhibiting activity of compounds **77–81**, **83** is rather high: $IC_{50} < 10$ μmol without cytotoxic effect (Table 3). Exceptions are pterixine **82** and saxdorfine **84** for which the values of IC_{50} are 20 and 11 μmol, respectively. The preparation for comparison was L-N^G-monomethylarginine (L-NMMA) which exhibited lower NO inhibiting activity.



85: (−)-falcarinol R = H
86: (−)-falcarindiol R = OH

Fig. 10.

FUNCTIONALIZED NATURAL AGENTS

Synthetic transformations of natural metabolites are the leading world direction of the development of new pharmaceutical agents and biologically active substances. Directed modification of a natural compound, as a rule, changes its native properties and increases the biological activity. In the context of the search for selective inhibitors of iNOS, the examples of this kind may be the transformations of amino acids, flavonoids, triterpenic acids.

Derivatives of amino acids

The use of different forms of inhibitor NOS compounds structurally similar to arginine appears very reasonable because the similarity with arginite (substrate) determines the competitive mechanism of inhibition between the substrate and the inhibitor for the active centre of the enzyme. L-arginine itself in the concentration above 100 μmol is an inhibitor of constitutive eNOS [10]. Many its substituted analogs exhibit high inhibiting ability in smaller concentrations (Table 4).

TABLE 3

NO inhibiting effect of the components of methanol extract of the roots of *Angelica furcijuga* **77–86** with respect to L-NMMA

Compounds	Inhibition, %			IC_{50}, μmol
	1 μmol	10 μmol	30 μmol	
77	25.7±3.1	62.6±3.5	78.4±0.3	5.1
78	3.7±3.9	47.4±2.5	85.2±1.8	8.8
79	32.4±3.1	43.3±2.4	74.4±1.5	8.2
80	35.2±5.3	54.1±2.6	68.9±4.3	4.2
81	23.0±5.2	62.7±3.4	96.8±1.6	3.4
82	14.0±2.2	32.9±6.4	59.0±1.2	20
83	20.4±2.1	47.8±3.4	75.5±2.2	8.8
84	1.9±6.5	50.4±3.3	71.7±3.5	11
85	29.4±6.2	70.0±7.4	96.1±0.8	4.8
86	24.4±4.5	65.4±2.6	102.9±0.2	4.4
L-NMMA	4.4±2.0	17.7±2.8	52.3±1.5	28

In general, nitrogen-substituted derivatives of arginine are weakly selective with respect to NOS isoforms and cause blocking effect for constitutive NOS and iNOS when introduced into isolated tissues or organs, or *in vivo* (Table 5).

The values of IC_{50} and/or K_i for the inhibitors of two or three isoforms of NOS obtained by the authors of [48] are presented in Table 5. According to these values, a series of potential inhibitors or iNOS was obtained: L-N^G-aminoarginine **91** > L-N^G-methylarginine (L-

TABLE 4

Amino acid inhibitors of different NOS forms **87–99**

Compounds	*n*	R_1	R_2	Title
87	3	NH	NH_2	L-N^G-arginine (substrate)
88	3	NOH	NH_2	L-N^G-hydroxyarginine (intermediate)
89	3	NH	$NHCH_3$	L-N^G-monomethylarginine (L-NMMA)
90	3	NH	$NHNO_2$	L-N^G-nitroarginine (L-NNA)
91	3	NH	$NHNH_2$	L-N^G-aminoarginine
92	3	NH	$NHCH_2CH_3$	L-N^G-ethylarginine
93	3	NH	$NHCH_2CHCH_2$	L-N^G-allylarginine
94	3	NH	$NHOCH_3$	L-N^G-methoxyarginine
95	3	O	NH_2	L-citrulline (product)
96	3	NH	CH_3	L-iminoethylornithine (L-NIO) [45]
97	3	NH	SCH_3	S-methyl-L-thiocitrulline
98	3	NH	SCH_2CH_3	S-ethyl-L-thiocitrulline
99	4	NH	CH_3	L-iminoethyllysine (L-NIL) [46, 47]

NMMA) **89** > L-N^G-hydroxyarginine > L-N^G-allylarginine **93** = L-N^G-nitroarginine (L-NNA) **90** >> L-N^G-methoxyarginine **94** > L-N^G-ethylarginine **92**. The sequence of nNOS looks like follows: L-N^G-nitroarginine (L-NNA) **90** > L-N^G-methylarginine (L-NMA) **89** > L-N^G-allylarginine **93** > L-N^G-methoxyarginine **94** >> L-N^G-hydroxyarginine **88** > L-N^G-ethylarginine **92** [48]. This clearly demonstrates the problem of selectivity of the inhibitors for different forms of NOS.

Different NOS inhibiting activity can be demonstrated by different stereoisomers of the same compound. For example, the authors of [49] synthesized an analogue of compound **99** – 6-(iminoethyl)-5-fluoro-D,L-lysine **100** (Fig. 11).

One can see in Table 6 that the inhibition of iNOS by compound **99** and the racemic mixture of compounds **100** are comparable. At the same time, the results of the tests of individual stereoisomers **101–104** showed that the strongest inhibitor for iNOS is (2S,5S)-stereoisomer of **101**. Other stereoisomers of amidine **100** and their racemic mixture exhibited much lower activity and selectivity. In the case of optically inactive compound **105** and optically pure difluoroamidine **106**, the latter turned out to be more active in NOS inhibition in combination with insignificant selectivity with respect to different isoforms.

The authors of [50] synthesized four optically pure analogues of L-arginine – syn-(**107**)

TABLE 5

Values of IC_{50} and inhibition constant (K_i) for amino acid inhibitors **89–99**

Compounds	IC_{50}, μmol			K_i, μmol		
	iNOS	nNOS	eNOS	iNOS	nNOS	eNOS
89	14	10	5.9	2.5	7.5	
90	7.6	0.52	0.5	8.7	0.2	
91				1.7	1.2	
92				~81	~66	
93				8.5	0.85	
94				20.5	6.0	
95	2.2	3.9				
96				34	1.2	11
97				17	0.5	24
98	5	61	138			
99	12	150	8420			

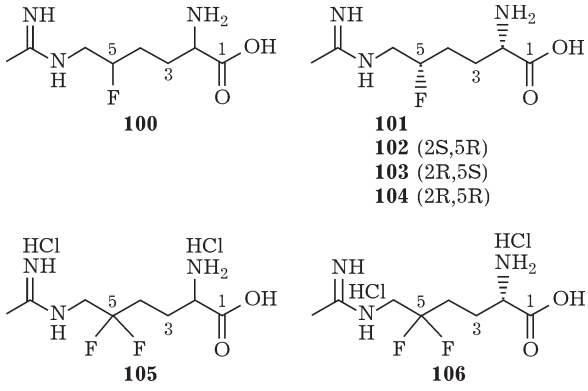


Fig. 11.

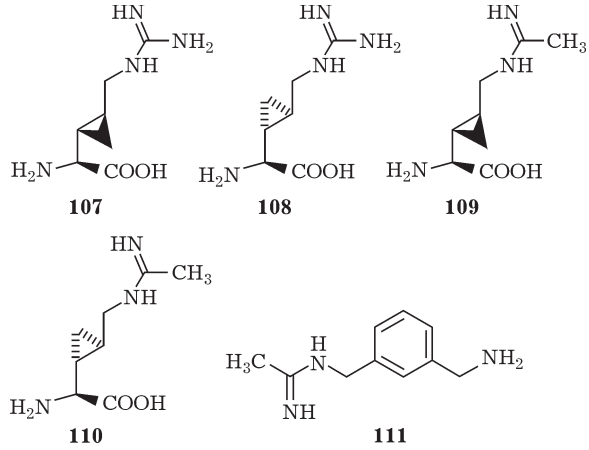


Fig. 12.

and anti-(**108**)-3,4-cyclopropyl-L-arginine, syn-(**109**) and anti-(**110**)-3,4-cyclopropyl-N-(L-iminoethyl)-L-ornithine (Fig. 12). Tests for NOS inhibiting activity showed that compound **107** does not inhibit any isoform of NOS but appears to be a weak substrate for NOS with K_M (Michaelis–Menthen constant) exceeding K_M for L-arginine (8.5±1 and 2.8±1.6 μmol, respectively). Compound **108** exhibits weak inhibiting ability towards all the three NOS isoforms without any isoform selectivity. Syn-3,4-cyclopropyl-N-(L-iminoethyl)-L-ornithine **109** proved itself as a competitive inhibitor of all NOS isoforms; the degree of iNOS inhibition increased with incubation time. At the same time, the selectivity of compound **109** towards nNOS turned out to be approximately five times higher in comparison with the selectivity of the known inhibitor of iNOS 1400 W ([N-(3-aminomethyl)benzyl]acetamidine) **111** [51] (Table 7). Anti-3,4-cyclopropyl-N-(L-iminoethyl)-L-ornithine **110** turned out to be weak competitive inhibitor of the constitutive isoforms of NOS and a weak binding inhibitor of iNOS. Analysis of the structure–activity dependence shows that passing from compounds **107**, **108** to substances **109**, **110** with the replacement of amino group in the guanidine fragment by methyl an increase in NOS inhibiting ability occurs (the sample structural fragment is present in the known NOS inhibitor **111**). In addition, the syn-isomer **109** with respect to the anti-isomer **110** exhibits higher activity and selectivity towards NOS.

Arginine and its analogues can be considered as the derivatives of guanidine **113** (Fig. 13). The guanidine fragment of arginine is necessary to bind the substrate and enzyme, so the compounds having composition **112–120** are potential inhibitors of NOS isoforms.

TABLE 6

Values of IC_{50} for compounds **99–106**

Compounds	Inhibition of NOS (IC_{50}, μmol)			Selectivity	
	iNOS	eNOS	nNOS	iNOS/eNOS	iNOS/ nNOS
99	4.9±1.65	138	35	28	7
100	7.84	182	70.4	23	9
101 (2S,5S)	2.36	81	21.7	34	9
102 (2S,5R)	4.0*	28.2*	0.00*		
103 (2R,5S)	47.8	374	311	8	7
104 (2R,5R)	8.9*	6.52*	18*		
105	21.4	139	140	7	7
106	8.75	57.5	33.8	7	4

* In per cent of inhibition at 10 μmol.

TABLE 7

Selectivity of compounds **109**, **110** and amidine **111**

Compounds	Selectivity	
	nNOS/iNOS	eNOS/iNOS
109	109	59
110	19	13
1400 W (**111**)	22	382

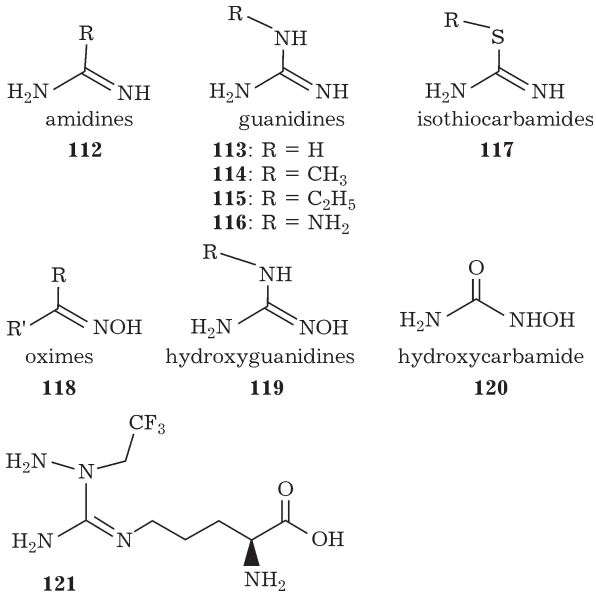


Fig. 13.

Guanidine itself **113** is neither inhibitor nor substrate for different NOS forms, while methyl- (**114**) and ethylguanidine **115** exhibit the inhibiting ability. Among guanidine derivatives, the best known compound is aminoguanidine **116** as one of the first compounds for which selectivity with respect to iNOS inhibition was demonstrated. In the majority of *in vitro* systems aminoguanidine **116** and L-N^G-monomethylarginine (L-NMMA) **89** are similarly efficient in the inhibition of the inducible isoform, but the former compound is an order of magnitude less active towards constitutive forms. Many N-aminoguanidines suppress the activity of NOS [52]. Among them, N^G-amino-L-arginine **91** should be marked. This compound is a widely known irreversible inhibitor of NOS. It inactivates NOS due to covalent interactions with the heme cofactor [53]. As shown in [54], this mechanism is not general for all NOS inhibitors with N-aminoguanidine framework. For instance, N^G-amino-N^G-2,2,2-trifluoroethyl-L-arginine **121** (see Fig. 13) prevents the oxidation of NADP thus preventing the catalytic oxidation of L-arginine.

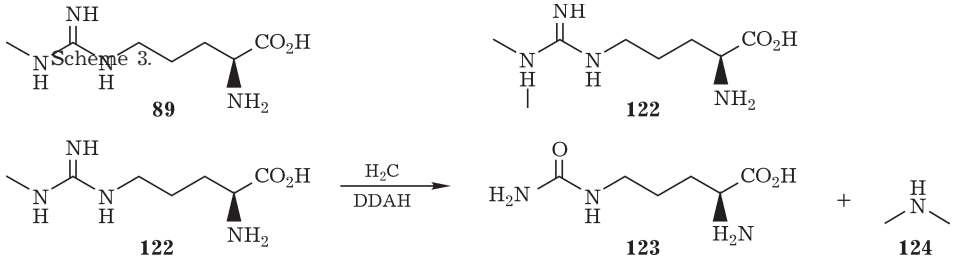
Another approach to the inhibition of NOS was demonstrated in [55]. As the authors of that paper stress, methylarginines – L-N^G-methylarginine (L-NMMA) **89** and asymmetric dimethylarginine (ADMA) **122** (Scheme 3) are non-selective endogenous inhibitors of NOS. Dimethylarginine dimethylaminohydrolase (DDAH) participates in the hydrolysis of methylarginines **89** and **122** [56, 57] transforming inhibitors, for example compound **122** into L-citrulline **123** and dimethylamine **124** (see Scheme 3). So, having chosen the inhibitor for DDAH, one can govern the production of NO.

The authors of [55] synthesized DDAH inhibitors based on 2-amino-4-(N^G-methylguanidino)-butanoic acid **125** (Fig. 14). It should be noted that all the compounds synthesized in that work have almost negligible direct action on different forms of NOS. According to the mechanism of action, this approach relates to the synthesis of agents inhibiting the expression of NOS.

By varying the substituents at nitrogen atom (R_1 and R_2) in compounds **125–130** (Table 8), compound **127** was selected as the most promising one in the sense of DDAH inhibition (IC_{50} for it was 189 μmol).

In turn, on the basis of (S)-2-amino-4-(N′-(2-methoxyethyl)guanidine)butanoic acid **127**,

Scheme 3.



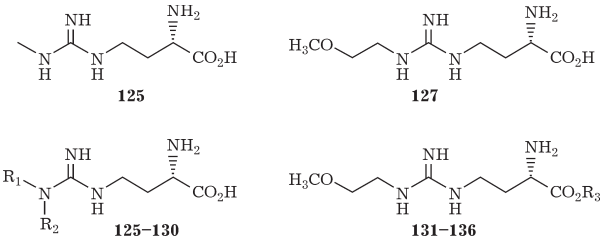


Fig. 14.

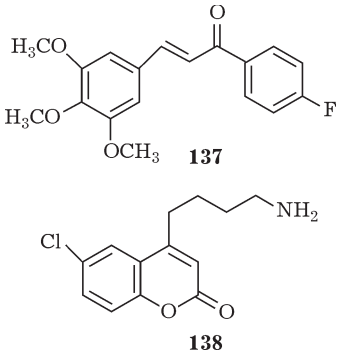


Fig. 15.

TABLE 8

Values of IC_{50} for compounds **125–130**, μmol

Compounds	R_1	R_2	Inhibition at 1 μmol, %	IC_{50}
125	Me	H	48	1510
126	Et	H	100	300
127	2-Methoxyethyl	H	90	189
128	2-Isopropoxyethyl	H	97	301
129	Me	Me	85	325
130	Piperidinyl		74	264

TABLE 9

Values of IC_{50} for compounds **131–136**, μmol

Compounds	R_3	Inhibition at 100 μmol, %	IC_{50}
131	Me	75	96
132	Et	44	159
133	*n*-Pr	64	111
134	*n*-Bu	40	113
135	Bz	96	27
136	*i*-Pr	30	189

its esters **131–136** were synthesized; the most active inhibitor of DDAH among them turned out to be benzyl ester **135** (Table 9).

At present, due to the insufficient selectivity of amino acid inhibitors, the majority of researchers reject the amino acid template [58] and propose the inhibitors of different structure [59].

Chalcones and coumarins

The authors of [60] synthesized 3,4,5-trimethoxy-4′-fluorochalcone **137** (Fig. 15) that is a dose-dependent inhibitor of NO production *in vivo*, possessing rather low cytotoxicity (IC_{50} = 0.033 μmol). The authors of that paper assume that the evolution of NO decreases as a consequence of the inhibition of iNOS expression. Experiments *in vitro* showed that chalcone **137** is the inhibitor of the nuclear factor NF-kB. Results of the tests *in vitro* and *in vivo* correlate with each other. A known inhibitor of iNOS 1400W (**111**) and dexamethazone (Dx) were used as a reference.

The authors of [61] synthesized a number of compounds based on coumarin framework and studied the dependence between structure and NOS inhibiting activity. As a result, the best selectivity in iNOS inhibition was demonstrated by coumarin **138** (see Fig. 15) with IC_{50} = 0.095 μmol.

Triterpenic acids, phenol compounds

The authors of [62] obtained 20 derivatives of 18β-glycyrrhetic acid; these compounds were tested for anti-inflammatory and antioxidant activity. Seco derivative **139** exhibited the highest activity as the inhibitor of iNOS expression (Fig. 16).

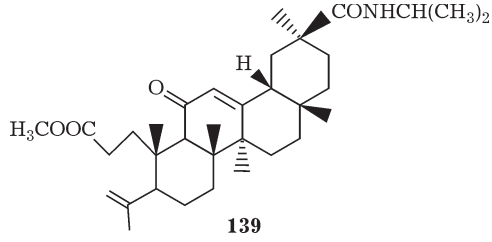


Fig. 16.

Prunioside A **140** was isolated from the methanol extract of *Spiraea prunifolia* [63]. This compound itself does not exhibit NOS inhibiting activity but its acylated derivative **141** is iNOS inhibitor. While establishing the structure of compound **140**, absolute configuration of asymmetric carbon atoms the authors of that work performed hydrolysis of the initial compound into products **142** and **144**. Analysis of the biological activity of compounds **142**, **144** and the corresponding acetylated derivatives **143** and **145** was performed by the authors of [63]; they showed that it is acylated derivatives that exhibit iNOS inhibiting activity in LPS induced macrophages (Fig. 17).

The authors of [64] synthesized 16 new triterpenoids with oleanolic and ursolic frameworks; these compounds were tested as iNOS inhibitors. Among the synthesized compounds, the most efficient ones were 2-cyano-3,12-dioxoolean-1,9(11)-diene-28-carboxylic acid (CDDO) **146** (Fig. 18) and its methyl ester (CDDO-Me) **147**. The value of IC_{50} for these compounds was 0.0001 μmol. As demonstrated in [65], according to the mechanism of action compound **147** relates to the inhibitors of electron transfer.

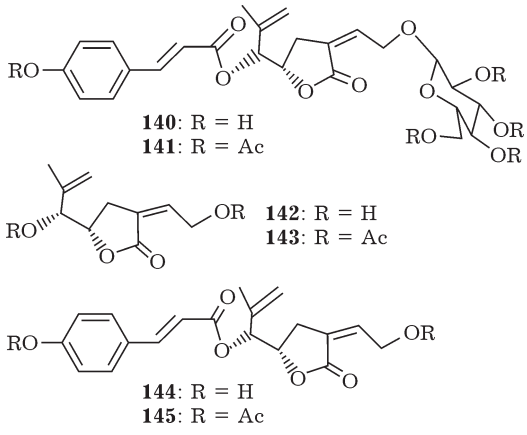


Fig. 17.

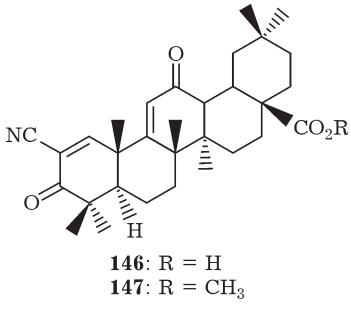


Fig. 18.

SYNTHETIC INHIBITORS OF INOS

Amidines

Among amidines, it is worth mentioning N-[3-(aminomethyl)benzyl]acetamidine (1400W) **111**, which is a highly selective inhibitor of iNOS *in vitro* and *in vivo* [51]. Previous studies [45] showed that it is regenerated after iNOS completely lost its activity; other isoforms of NOS were not detected. In addition, it was shown in [66] that the selectivity of acetamidine 1400W **111** is higher than that of N-5-(L-iminoethyl)-L-ornithine (L-NIO) **96**. In [67], analogues of acetamidine **111** were synthesized among which the highest activity was exhibited by N-benzylacetamidine **148** (Fig. 19). Though for compound **148** IC_{50} = 0.20 μmol (for 1400W **111** the value is 0.33 μmol), its selectivity with respect to eNOS turned out to be lower than that of acetamidine **111** (1750 and 3300, respectively). The mechanism of inhibition by amidine inactivators is explained by primary competitive binding with enzyme instead of substrate L-arginine but due to methyl group which is present in the amidine fragment peroxo intermediate of heme is not protonated thus preventing its transformation into the oxo intermediate. Thus, the mechanism of the action of amidines is connected with their ability to interfere with the functions of heme.

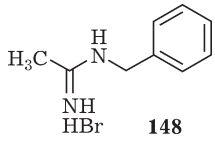


Fig. 19.

TABLE 10

Values of IC_{50} for aminopyridines **149–154**

Compounds	R_1	R_2	R_3	IC_{50}, μmol		
				iNOS	eNOS	nNOS
149	Me	H	H	0.12	0.30	0.11
150	Me	Me	H	0.045	0.17	0.098
151	Me	Pr	H	0.01	0.05	0.01
152	Me	Me	Me	86	30	>100d
153	Et	H	H	1.1	4.3	2.2
154	MeO	H	H	1.0	1.0	1.0

Nitrogen-containing heterocyclic compounds

It was demonstrated [58] that the substantial role in the oxidative domain of iNOS is played by amino acid residue Glu^{371}, which takes part in the formation of hydrogen bonds with guanidine fragment. In this connection, the most widespread way of synthesis of potential iNOS inhibitors is the introduction of cis-amidine fragment into synthetic agents. This fragment provides bidentate interaction with the carboxylic group of Glu^{371}. Taking into account this pharmacophore group, screening of the activity of some 2-aminopyridines **149–154** based on the framework of 4-substituted aminopyridine was carried out (Table 10).

One can see that alkylation at C-6 atom (R_2) increases the inhibiting capacity (methylaminopyridines **150, 151**); the isoform selectivity of compounds increases, too. N-methylation leads to the loss of inhibiting activity (compound **152**). An increase in the size of substituent R_1 (aminopyridines **153**, **154**) also causes a decrease in the activity of corresponding agents. So, 4-methylsubstitution is optimal for the pyridine ring. The introduction of 4-piperidinyl carbamate fragment into the framework causes the formation of compound **155**; its inhibiting activity only slightly differs from that of aminopyridine **149**, while the isoform selectivity of N-piperidinylaminopyridine is higher (Table 11). With the substitution of R_1 in N-piperidinylaminopyridine **155** by hydrogen (compound **156**) the activity of the inhibiting agent drops sharply, which confirms the assumption concerning the necessity of the presence of a substituent in position 4 of pyridine ring. It should be noted that, unlike for the observed structure – activity dependence for the series of aminopyridines **149–154**, an increase in the size of substituent R_1 in the series of N-substituted aminopyridines **151–157** does not always lead to a drop of activity. For example, in compound **157** the substitution of methyl by methoxy group causes a fourfold increase in isoform selectivity towards iNOS with respect to aminopyridine **155**. In addition, the selectivity towards eNOS and nNOS increases (by a factor of 450 and 350, respectively). Further increase in the size of R_1 (compound **158**) causes

TABLE 11

Values of IC_{50} for compounds **155–161**

Compounds	R_1	R_2	IC_{50}, μmol		
			iNOS	eNOS	nNOS
155	Me	OEt	0.35	50	21
156	H	OEt	13	>100	>100
157	MeO	OEt	0.089	41	32
158	EtO	OEt	3.8	>100	96
159	MeO	Ph	0.53	100	28
160	MeO	4-ClPh	0.053	36	7.8
161	MeO	4-CNPh	0.071	>100	6.6

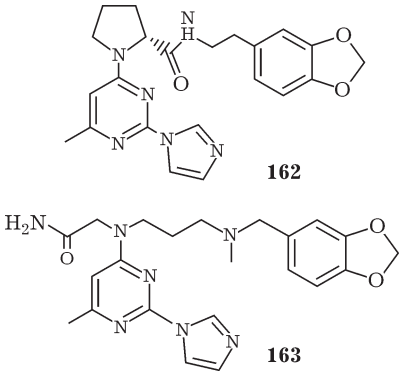


Fig. 20.

a 40-fold decrease in activity with respect to aminopyridine **157**, thus marking the limiting size of the substituent.

Further modification of compound **157** was carried out by varying the substituents in carbamate ring and led to the formation of aminopyridines **159–161**. Among them, compound **161** should be marked. It is distinguished by the high inhibiting activity towards iNOS and the best isoform selectivity.

The authors of [68] carried out screening of the combinatory library for possible inhibitors of NOS that would directly interact with heme, and discovered 2-(imidazole-1-yl)-pyrimidines. They inhibit dimerization of NOS monomers preventing the formation of the active form of enzyme. Imidazolylpyrimidine **162** (Fig. 20) turned out to be the most selective one among the tested pyrimidines.

Compound with conventional title PH-302 **163** (see Fig. 20) also inhibits iNOS coordinating with the heme of the monomeric (inactive) form of enzyme and preventing the formation of the active dimer of iNOS [69]. The mechanism of inhibition in this case is inhibition of cytochrome P450 3A4.

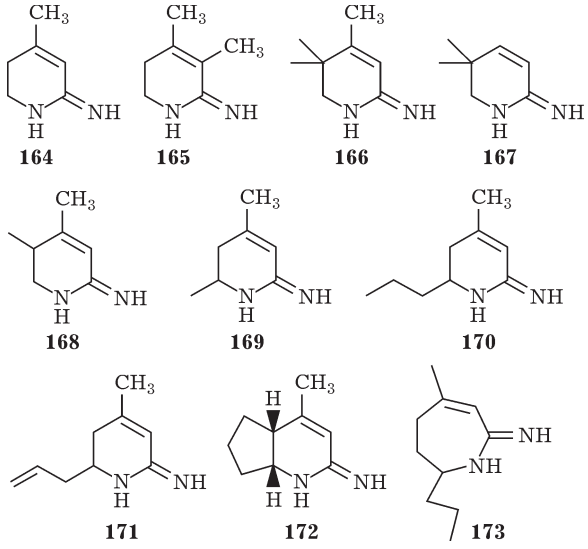


Fig. 21.

In [70], α,β-unsaturated cyclic amidines were presented: 4-methyl-5,6-dihydropyridine-2(1H)-imine **164** and its derivatives **165–173** (Fig. 21). It was demonstrated in experiments with mice that compounds **166**, **167**, **170**, **171** and **173** exhibited isoform selectivity eNOS/iNOS within the range 5 to 19.

Attempts to unite the properties of inhibitors of several enzymes in one agent are frequent in literature. For example, iNOS togeth-

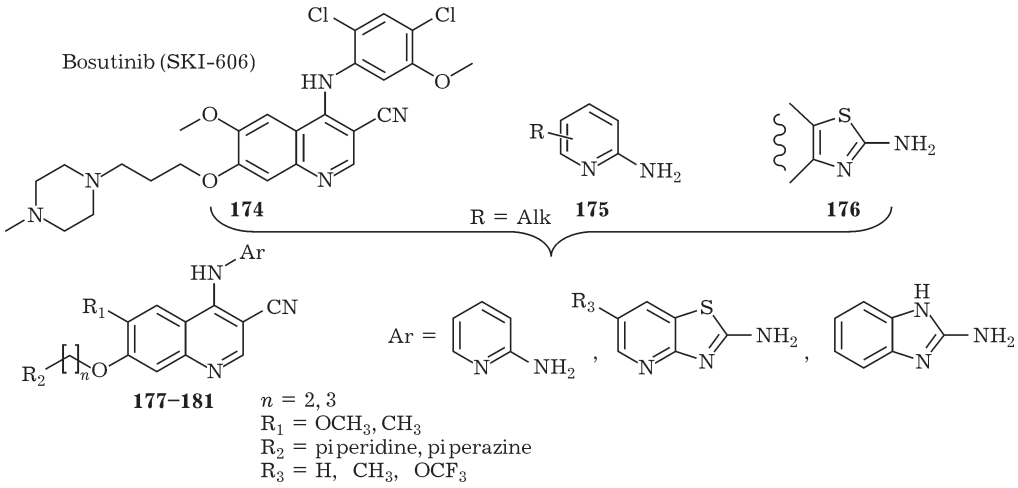


Fig. 22.

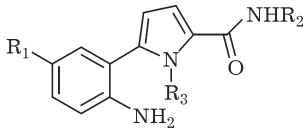
TABLE 12

Values of IC_{50} for compounds **174–181** [71]

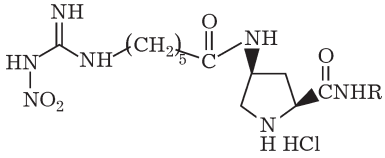
Compounds	R_1	*n*	R_2	Ar	c-Src, IC_{50}, nmol	iNOS, IC_{50}, μmol
177	Me	3	N-Me-piperazine	2-Pyridine	75.9	18.6
178	Me	3	N-Me-piperazine	2-(6-Me-benzothiazole)	646	12.7
179	Me	3	Piperidine	2-(6-Me-benzothiazole)	15.4	313
180	Me	3	N-Me-piperazine	2-(6-Trifluoromethoxy-benzothiazole)	111	16.3
181	OMe	2	N-Me-piperazine	2-Benzothiazole	9.23	2.18
Bosutinib **174**					1.2	–
L-canavanine **175**					–	60
2-aminothiazole **176**					–	18

er with c-Src (the family of proto-oncogene tyrosine kinases) play the part of major regulators in tumour formation processes. The idea of making one agent which would be able to inhibit both these enzymes is of course very attractive. In this connection, the authors of [71] synthesized a series of compounds based on the known anticancer agent bosutinib (SKI-606, 4-aniline-3-quinolinecarbonitrile **174**) which is at the II stage of clinical tests, as well as 2-aminopyridine **175** and 2-aminothiazole **176**.

On the basis of these templates, a number of compounds **177–181** was synthesized (Fig. 22). These compounds were tested for the inhibition of c-Src (the reference was bosutinib **174**) and iNOS (by comparing with commercially available L-canavanine and 2-aminothiazole **176**). Unfortunately, the majority of synthesized compounds turned out to be active only towards one of the enzymes though some of them were able to inhibit both though to different extent (Table 12).



182: $R_1 = R_3 = H$, $R_2 = Me$
183: $R_1 = R_3 = H$, $R_2 = cyclo\text{-}C_5H_9$



184: $R = -C_6H_5$
185: $R = 3,4\text{-}F_2\text{-}C_6H_3$
186: $R = 3\text{-}Cl\text{-}4\text{-}CH_3\text{-}C_6H_3$

Fig. 23.

Thus, compound **181** exhibits the properties of a strong inhibitor of both c-Src and iNOS, while agents **177**, **178**, **180** inhibit only iNOS; IC_{50} values for c-Src are rather high and equal to 0.076, 0.646 and 0.111 μmol, respectively. The reverse situation is observed for compound **179**: it is a good inhibitor of c-Src but its IC_{50} for iNOS is 313 μmol.

The authors of [72] synthesized a series of new 5-phenyl-1H-pyrrole-2-carboxamide derivatives. These compounds demonstrated medium activity *in vitro* as inhibitors of nNOS and iNOS, in some cases (compounds **182**, **183**, Fig. 23) the data on some selectivity for iNOS were obtained. These compounds in *in vivo* experiments promoted a sharp decrease in the amount of NO in cytosol and mitochondria.

A definite conformation of the pyrrolidine ring was used as the main unit by the authors of [73] to obtain selective pyrrolidine-containing inhibitors of iNOS. All the compounds were synthesized from commercially available trans-4-hydroxy-L-proline. To optimize the pyrrolidine unit, the following transformations were carried out:

1) the fragment of (3-nitroguanidine)hexanoic acid (for structural similarity with L-NNA **90**) was attached to the 4th carbon atom of pyrrolidine fragment;

2) the secondary amino group in position 1 of pyrrolidine ring was conserved;

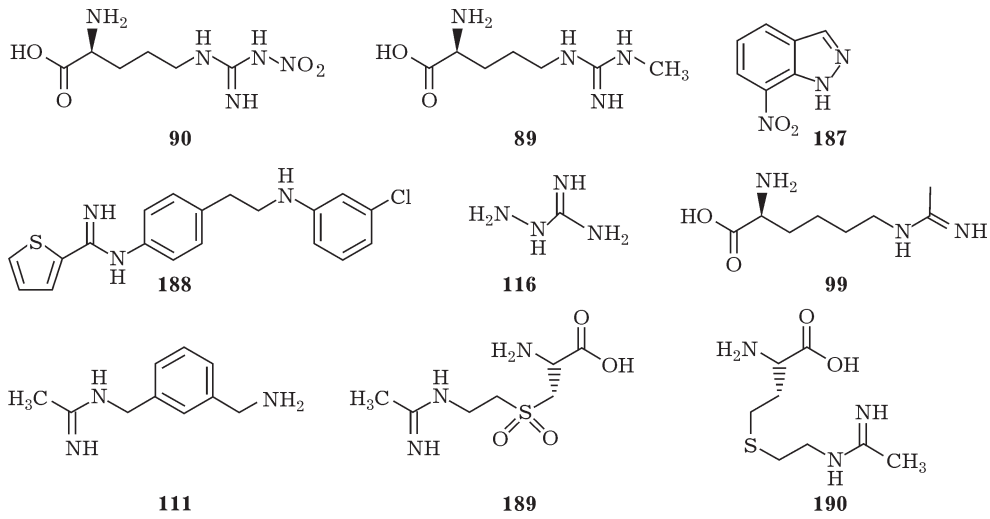


Fig. 24.

3) through the carboxamide group, aromatic rings with different substituents were attached.

As a result, a number of compounds were obtained; among them, pyrrolidines **184–186** are to be stressed (see Fig. 23) as promising inhibitors of iNOS: along with the high inhibiting activity (2.36, 2.68, 2.5 μmol, respectively), they exhibit low cytotoxicity; for L-NNA **90** under the same conditions IC_{50} = 14.74 μmol.

BEST KNOWN INHIBITORS OF INOS

It should be noted that in the majority of papers under analysis there are definite alternative versions hindering interpretation of results; they refer to the following aspects:

1. Selectivity with respect to different NOS forms: what is to be considered as selectivity – the activity towards one form exceeds the activity towards the other by a factor of 2, 10 or 100?

2. The units of selectivity measurement (IC_{50}, K_i, or pharmacologically efficient dose).

3. The subject of investigation (isolated enzymes, cells or *in vivo*) [26].

In this connection, the authors of [26] propose to determine the isoform selectivity of potential inhibitors and separate them into nonselective (activity to one isoform is less than 10 times higher than that to another isoform), partially selective (10–50 times excess) and selective (more than 50 times). According to this approach, the authors tested the best known inhibitors of different NOS forms (Fig. 24) under the same conditions (Table 13).

According to the data of Table 13 and inhibitor classification according to Alderton, com-

TABLE 13

Comparison of isoform selectivity of the best known iNOS inhibitors

Inhibitors	IC_{50}, μmol			Selectivity		
	iNOS	nNOS	eNOS	iNOS/nNOS	iNOS/eNOS	nNOS/eNOS
L-NNA **90**	3.1	0.29	0.35	0.09	0.11	1.2
L-NMMA **89**	6.6	4.9	3.5	0.7	0.5	0.7
7-NI **187**	9.7	8.3	11.8	0.9	1.2	1.4
ARL-17477 **188** [74]	0.33	0.07	1.6	0.2	5	23
Aminoguanidine **116**	31	170	330	5.5	11	1.9
L-NIL **99**	1.6	37	49	23	49	1.3
1400W **111**	0.23	7.3	1000	32	>4000	>130
GW273629 **189** [75]	8.0	630	1000	78	>125	>1.6
GW274150 **190**	1.4	145	466	104	333	32

pounds **89**, **90** and **187** are non-selective inhibitors; agent **188**, aminoguanidine **116** and acetamide analogue of arginine L-NIL **99** can be related to partially selective inhibitors of NOS isoforms. The high degree of selectivity towards iNOS is exhibited by inhibitor 1400W **111**, but in view of its toxicity in high concentrations, it is unacceptable as a therapeutic agent for humans, though it is admissible for animal models. Quite contrary to 1400W **111**, sulphur-containing acetamide amino acids **189** and **190** do not exhibit high toxicity but they are highly efficient inhibitors of iNOS, though their IC_{50} values are rather high.

CONCLUSIONS

On the basis of the analysis of the modern state of the problem connected with the search for selective iNOS inhibitors, the following conclusions can be made:

1. A large number of different classes of compounds were tested for the inhibiting ability towards iNOS.

2. Amino acid inhibitors were studied in most detail due to their similarity with the substrate (L-arginine) and possible competitive inhibition with the substrate.

3. Modification of potential inhibitors is carried out using the methods of combinatory chemistry followed by tests of the results obtained.

4. In the case of natural compounds, these are often data on the activity of only natural objects themselves because their chemical modification may be hindered due to poor availability of these compounds and multistage total synthesis of them.

5. The most promising inhibitors of iNOS are semi-synthetic and synthetic compounds.

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