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Fluorinated indazoles as novel selective inhibitors of nitric oxide synthase (NOS): Synthesis and biological evaluation

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To our friend Dr. Sergio Erill of the Dr. Antonio Esteve Foundation

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ABSTRACT

In order to find new compounds with neuroprotective activity and NOS-I/NOS-II selectivity, we have designed, synthesized, and characterized 14 new NOS inhibitors with an indazole structure. The first group corresponds to 4,5,6,7-tetrahydroindazoles (**4**–**8**), the second to the *N*-methyl derivatives (**9**–**12**) of 7-nitro-1*H*-indazole (**1**) and 3-bromo-7-nitro-1*H*-indazole (**2**), and the latter to 4,5,6,7-tetrafluoroindazoles (**13**–**17**). Compound **13** (4,5,6,7-tetrafluoro-3-methyl-1*H*-indazole) inhibited NOS-I by 63% and NOS-II by 83%. Interestingly, compound **16** (4,5,6,7-tetrafluoro-3-perfluorophenyl-1*H*-indazole) inhibited NOS-II activity by 80%, but it did not affect to NOS-I activity. Structural comparison between these new indazoles further supports the importance of the aromatic indazole skeleton for NOS inhibition and indicate that bulky groups or N-methylation of **1** and **2** diminish their effect on NOS activity. The fluorination of the aromatic ring increased the inhibitory potency and NOS-II selectivity, suggesting that this is a promising strategy for NOS selective inhibitors.

1. Introduction

Nitric oxide synthases (NOS) constitute an enzyme family comprising three isoforms: neuronal (NOS-I or Type I), inducible (NOS-II or Type II), and endothelial (NOS-III or Type III).[1–6] NOS isoforms are homodimers and catalyze a two-step NADPH-dependent oxidation of L-arginine to NO and L-citrulline. NOS monomers consist of a reductase domain and an oxygenase domain. The first one is homologous to cytochrome P-450 reductase and contains binding sites for NADPH, FAD, FMN, and calmodulin (CaM).[7–10] The oxygenase domain binds L-arginine, the iron protoporphyrin IX (heme prosthetic group) and tetrahydrobiopterin (H_4B). The formation of stable NOS homodimers is an H_4B-substrate and heme-dependent process. Dimerization of NOS is critical for the flavin-to-heme electron transfer step, as the flow of electrons during catalysis occurs in *trans* from the reductase domain of one monomer subunit to the oxygenase domain of the other monomer. There is a high degree of structural similarity within the critical catalytic center and dimer interface regions between NOS isoforms.[11–16]

Nitric oxide plays a key role in the physiology and pathophysiology of the cardiovascular, central nervous, and immune systems. NO reacts with molecular oxygen, thiols, transition metal centers and other biological targets allowing it to function as a rapidly reversible, specific, and local signal transduction molecule as well as a nonspecific mediator of tissue damage.[17–23]

In view of the pivotal function of NO, many efforts have been devoted to selectively inhibit any of the three NOS isoforms, and from the several structures investigated moving away from the amino acid arginine-like template, indazole derivatives deserve special attention by their potency. So 7-nitro-1*H*-indazole (7-NI, **1**) and 3-bromo-7-nitro-1*H*-indazole (NIBr, **2**) at 10 μM concentration suppress NOS-I activity by 87% and 96%, respectively, the latter compound being one of the most potent inhibitors known until now.[24] In vitro inhibition of the three NOS isoforms occurs, but in vivo the inhibition is selective for the type I NOS and the mechanism of such action is very well established.[25–29] IC_{50} values for these and other indazole derivatives are presented in Scheme 1.

The most important contribution to the understanding of the mechanism of action of nitroindazoles is that of Getzoff and coworkers[29] that reported the X-ray structures of inducible (NOS-II) and endothelial (NOS-III) NOS oxygenase domains cocrystallized with **1**, **2**, 5- and 6-nitroindazoles (that of **2** cocrystallized with NOS-III was previously reported by Raman et al.[15]). Their main

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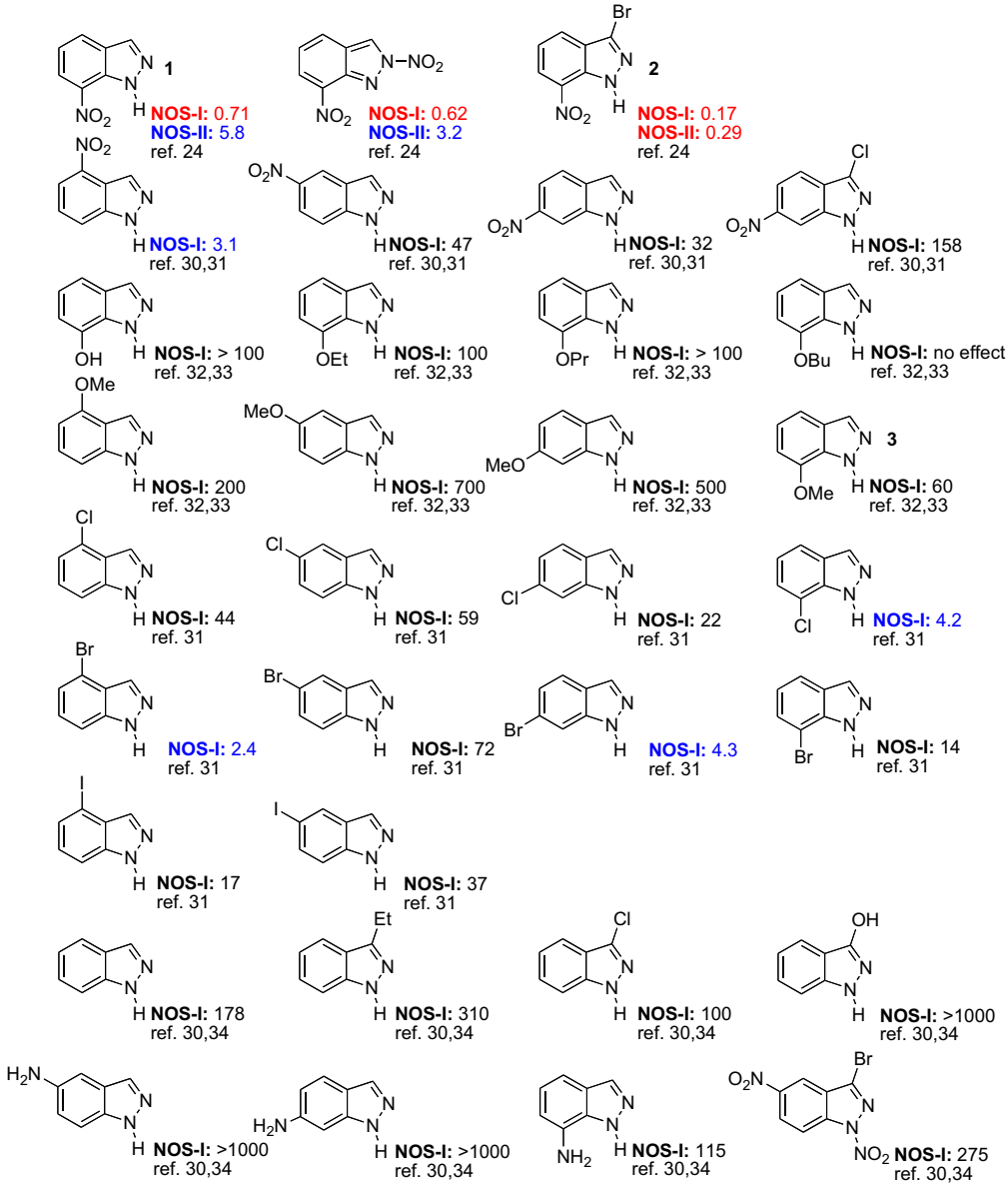
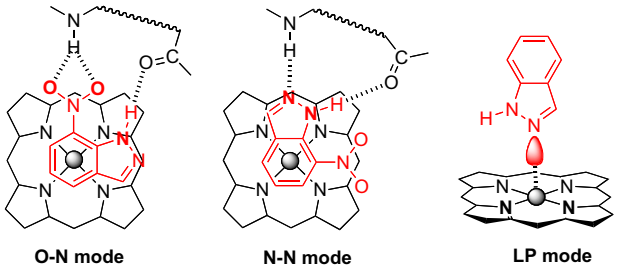


Scheme 1. IC_{50} (μM) of literature indazoles.

conclusion is that there are two modes of binding named (according to their hydrogen bonds) **O–N** and **N–N** (Scheme 2). Compound **2** adopts the **O–N** conformation while 6- and 7-nitroindazoles prefer the **N–N** one. Compound **1** crystallizes in both conformations.

Porubsky et al. have reported the structure of indazole coordinated through its lone pair (**LP** model) to the heme iron (Scheme 2)



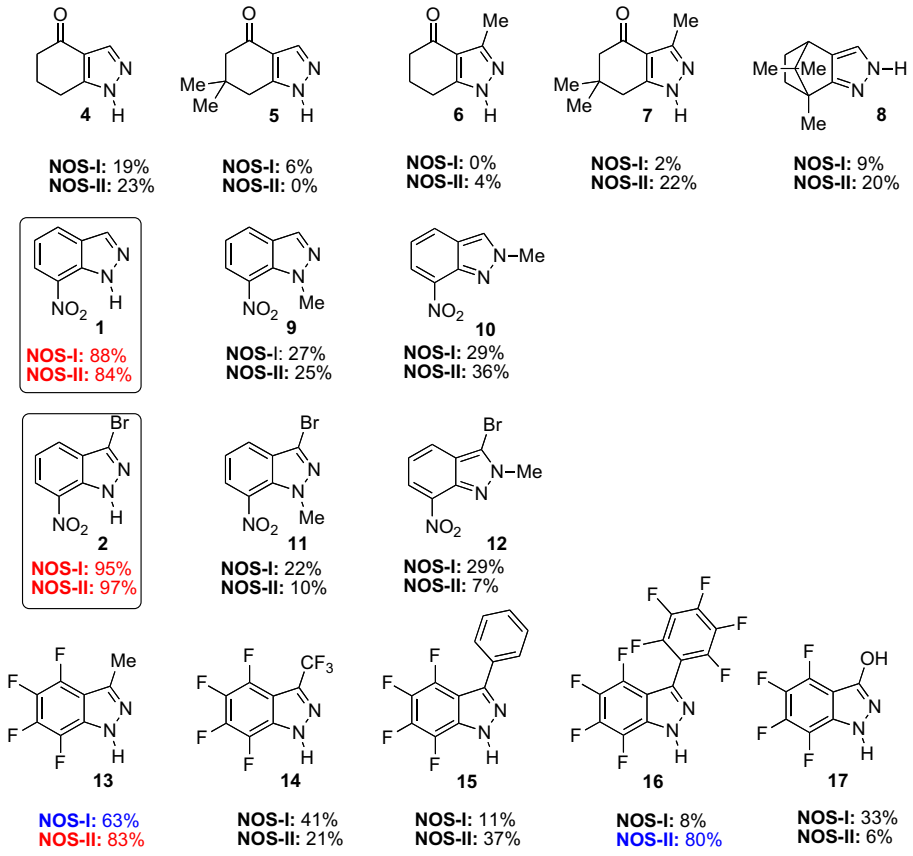
Scheme 2. The different binding modes of indazoles to heme.

of cytochrome P-450 2E1 like 4-methylpyrazole as well as histidine residues.[30]

Other indazoles (nitro, chloro, bromo, and iodo) with different substitution patterns were also found to be active but with higher IC_{50} than **1** and **2**. Amino and alkoxy indazoles afforded poor inhibition results, save 7-methoxy-1*H*-indazole (7-MI, **3**) that showed unexpected activity.[31–36] We have summarized in Scheme 1 all the available information concerning indazoles and NOS previous to the present work.

From all these data, it appears that: (i) the aromatic indazole skeleton with both hydrogen acceptor and donor nitrogen atoms, seems to be crucial for the inhibitory action, (ii) hydrogen bonding acceptor groups (electron withdrawing and electron donating) in the benzo ring increase the activity in the order 7 > 4 > 6 > 5 positions, (iii) substitution at position 3 by an electronegative group such as bromine increases the potency too, but its effect is weaker than substitution at position 7, and (iv) bulky groups diminish the activity due to steric constraints in the enzyme active center.

In this report we describe our studies on three groups of indazoles, a total of 14 derivatives depicted in Scheme 3. The first cor-



Scheme 3. Structures of the studied compounds with% of inhibition (1 mM).

responds to 4,5,6,7-tetrahydroindazoles (**4**–**8**), the second to the *N*-methyl derivatives (**9**–**12**) of 7-nitro-1*H*-indazole (**1**) and 3-bromo-7-nitro-1*H*-indazole (**2**), and the latter to 4,5,6,7-tetrafluoroindazoles (**13**–**17**).

These different structural types provide insights on the influence of steric effects, suppression of hydrogen bond donors (N*H*), and charge distribution inversion[37] of the benzo ring in the fluorinated indazoles resulting in the discovery of a new class of potent and selective inhibitors of NOS-II activity.

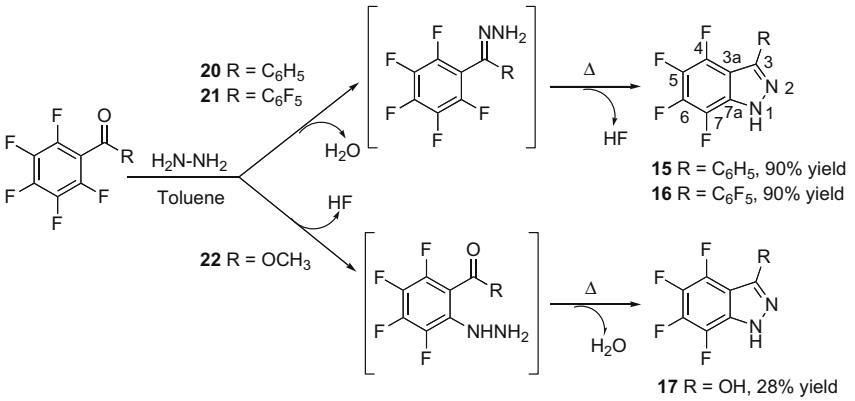
2. Results and discussion

2.1. Chemistry

Indazoles are synthesized by formation of one or two bonds of the pyrazole ring and the most common route uses the reaction of hydrazines with cyclohexanones or mono or bifunctional arenes.[38–42]

1(2),5,6,7-Tetrahydro-4*H*-indazol-4-ones (**4**–**7**) were obtained from 1,3-cyclohexanediones instead of cyclohexanone and fully described by us in a structural paper.[43] (±)-4,5,6,7-Tetrahydro-7,8,8-trimethyl-4,7-menthane-2*H*-indazole (**8**) was prepared by the same method starting from (±)-camphor (**18**) via (±)-(*Z*)-3-(hydroxymethylene)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-one (**19**).

In what concerns fluorinated indazoles, 3-methyl-4,5,6,7-tetrafluoro-1*H*-indazole (**13**) and 3-trifluoromethyl-4,5,6,7-tetrafluoro-1*H*-indazole (**14**) were prepared by ring-closure reaction of octafluoro- and pentafluoro-acetophenone with hydrazine and published already.[37,44] The same method, as shown in Scheme 4, has been applied in this study to the synthesis of indazoles (**15**–**17**) but using pentafluorobenzophenone (**20**), decafluorobenzophenone (**21**), and the methyl ester of 2,3,4,5,6-pentafluorobenzoic



Scheme 4. General synthetic pathway followed in the preparation of fluoroindazoles **15**–**17**.

acid (**22**), respectively. The low yield in the latter example is due to the fact that substitution of the fluorine by hydrazine occurs in *ortho* and *para* position with respect to the ester group,[45] so besides 4,5,6,7-tetrafluoro-1*H*-indazole-3-ol (**17**), the methyl 2,3,5,6-tetrafluoro-4-hydrazinylbenzoate is formed.

Finally, derivatives **9**–**12** have been synthesized from the lead indazole scaffolds, 7-nitro-1*H*-indazole (**1**) and 3-bromo-7-nitro-1*H*-indazole (**2**), by methylation in dry methanol and sodium methoxide with methyl iodide as alkylating agent.[46]

2.2. In vitro NOS inhibition

Table 1 and Figure 1 illustrate the NOS-I and NOS-II inhibition in the presence of 1 mM of the four tetrahydroindazolones [1(2),5,6,7-tetrahydro-4*H*-indazole-4-one (**4**), 6,6-dimethyl-1(2),5,6,7-tetrahydro-4*H*-indazole-4-one (**5**), 3-methyl-1(2),5,6,7-tetrahydro-4*H*-indazole-4-one (**6**), 3,6,6-trimethyl-1(2),5,6,7-tetrahydro-4*H*-indazole-4-one (**7**)], and (±)-4,5,6,7-tetrahydro-7,8,8-trimethyl-4,7-methane-2*H*-indazole (**8**). It can be seen that none of these compounds show an important inhibitory activity, with 1(2),5,6,7-tetrahydro-4*H*-indazole-4-one (**4**) the most active of them, showing only a 20% of NOS inhibition. These results further support the importance of the aromatic indazole (benzo[*c*]pyrazole) skeleton for NOS inhibition, and suggest that bulky groups diminish their activity due to steric constraints in the enzyme active center.

Table 2 and Figure 2 represent the NOS-I and NOS-II inhibition in the presence of 1 mM of the known inhibitors 7-nitroindazole (**1**) and 3-bromo-7-nitroindazole (**2**), and their N-methylated derivatives 1-methyl-7-nitro-1*H*-indazole (**9**), 2-methyl-7-nitro-2*H*-indazole (**10**), 3-bromo-1-methyl-7-nitro-1*H*-indazole (**11**), and 3-bromo-2-methyl-7-nitro-2*H*-indazole (**12**). Compared with **1** and **2**, their methylated derivatives show relatively weak activity. Compounds **9** and **10** inhibit both NOS-I and NOS-II by about 25%, whereas compounds **11** and **12** display a slight selective inhibition of NOS-I. Anyway, the low inhibitory ability of these compounds suggests that N-methylation of **1** and **2** prevents their effect on NOS activity.

Table 3 and Figure 3 show the NOS-I and NOS-II inhibition in the presence of 1 mM of the tetrafluoroindazoles 4,5,6,7-tetrafluoro-3-methyl-1*H*-indazole (**13**), 4,5,6,7-tetrafluoro-3-trifluoromethyl-1*H*-indazole (**14**), 3-phenyl-4,5,6,7-tetrafluoro-1*H*-indazole (**15**), 4,5,6,7-tetrafluoro-3-perfluorophenyl-1*H*-indazole (**16**), and 4,5,6,7-tetrafluoro-1*H*-indazole-3-ol (**17**). This group of compounds was the most active against NOS activity. Specifically, compound **13** inhibited NOS-I by 63% and NOS-II by 83%, respectively, suggesting a slight selectivity for NOS-II. Interestingly, compound **16** inhibited NOS-II activity by 80%, but it did not affect to NOS-I

Table 1
NOS-I and NOS-II inhibition in the presence of the assayed 4,5,6,7-tetrahydroindazoles (compounds **4**–**8**)

Compound	% Inhibition NOS-I	% Inhibition NOS-II
4	19 ± 1	23 ± 1
5	6 ± 3	2 ± 1
6	2 ± 2	4 ± 1
7	2 ± 2	22 ± 7
8	9 ± 1	20 ± 2

Data represent the mean ± SEM of the percentage of NOS-I and NOS-II inhibition produced by 1 mM of each compound. Each value is the mean of three experiments performed by triplicate in homogenates of rat lung samples.

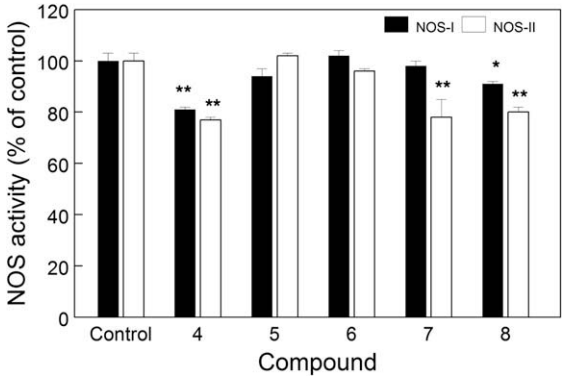


Figure 1. Percent of NOS-I and NOS-II activities in the presence of 1 mM concentration of the 4,5,6,7-tetrahydroindazoles assayed (compounds **4**–**8**), as compared with those of untreated samples (control). Each value is the mean of three experiments performed by triplicate in homogenates of rat lung samples. $^{*}P$ <0.05 and $^{**}P$ <0.01 versus control.

Table 2
NOS-I and NOS-II inhibition in the presence of the nitroindazoles assayed (compounds **9**–**12**) and 7-nitro-1*H*-indazole and 3-bromo-7-nitro-1*H*-indazole (compounds **1** and **2**, respectively)

Compound	% Inhibition NOS-I	% Inhibition NOS-II
1	88 ± 4	16 ± 4
9	27.3 ± 0.3	25 ± 3
10	29 ± 5	36 ± 1
2	95 ± 1	97 ± 1
11	22 ± 1	10 ± 2
12	29 ± 1	7 ± 1

Data represent the mean ± SEM of the percentage of NOS-I and NOS-II inhibition produced by 1 mM of each compound. Each value is the mean of three experiments performed by triplicate in homogenates of rat lung samples.

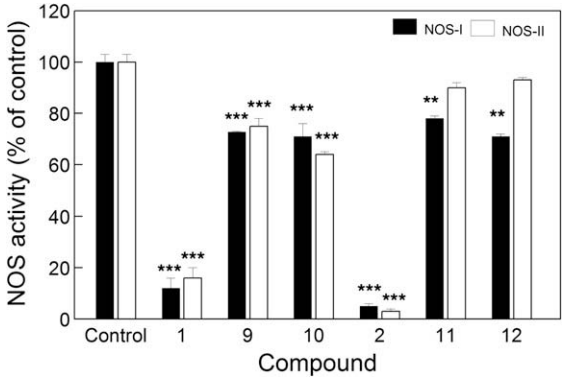


Figure 2. Percent of NOS-I and NOS-II activities in the presence of 1 mM concentration of the nitroindazoles assayed (compounds **9–12**) and 7-nitro-1*H*-indazole (compound **1**) and 3-bromo-7-nitro-1*H*-indazole (compound **2**), as compared with those of untreated samples (control). Each value is the mean of three experiments performed by triplicate in homogenates of rat lung samples. $^{**}P$ <0.01 and $^{***}P$ <0.001 versus control.

Table 3
NOS-I and NOS-II inhibition in the presence of the 4,5,6,7-tetrafluoro-1*H*-indazoles assayed (Compounds **13**-**17**).

Compound	% Inhibition NOS-I	% Inhibition NOS-II
13	63 ± 7	83 ± 1
14	41 ± 2	21 ± 1
15	11 ± 2	37 ± 2
16	8 ± 3	80 ± 1
17	23 ± 3	6 ± 7

Data represent the mean ± SEM of the percentage of NOS-I and NOS-II inhibition produced by 1 mM of each compound. Each value is the mean of three experiments performed by triplicate in homogenates of rat lung samples.

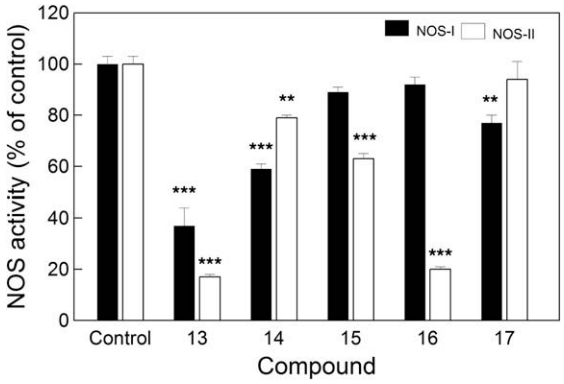


Figure 3. Percent of NOS-I and NOS-II activities in the presence of 1 mM concentration of the 4,5,6,7-tetrafluoro-1*H*-indazoles assayed (compounds **13**–**17**), as compared with those of untreated samples (control). Each value is the mean of three experiments performed by triplicate in homogenates of rat lung samples. $^{**}P$ <0.01 and $^{***}P$ <0.001 versus control.

activity. So, this compound **16** show an important selectivity for NOS-II inhibition. Compounds **14** and **15** behave as partial selective inhibitors of NOS-I and NOS-II, respectively, whereas compound **17** was the least active of all tetrafluoroindazoles assayed. Because of the selective activity compound **16**, the inhibition of NOS-II activity was studied in the presence of different concentrations of this compound. Table 4 shows the results of these experiments. Compound **16** inhibits the activity of NOS-II in a dose-dependent manner with an IC_{50} of 0.4 mM. The fact that fluorination of the benzene ring could improve the aromatic stacking, together with the possibility that fluorine atoms may form weak hydrogen bounds in the active center, may account for the activity of these fluorinated molecules.

3. Conclusions

A major challenge of modern medicine is to design compounds that modulate specific enzymes while leaving related isozymes unaffected. Very few studies report both the NOS-I and NOS-II inhibitory properties of indazoles (Scheme 1).[24] The most interesting data reported in Schemes 1 and 3 can be summarized as follows:

NOS-I:
Less than 1 μM (in red): **1**, **2** and 2,7-dinitroindazole.
Between 1 and 10 μM (in blue): 4-nitro, 7-chloro, 4-bromo, and 6-bromoindazole.
Percentages of inhibition higher than 80% (in red): **1** and **2**.
Percentages of inhibition higher than 60% (in blue): **13**.
NOS-II:
Less than 1 μM: **2**.
Between 1 and 10 μM: **1** and 2,7-dinitroindazole.
Percentages of inhibition higher than 80% (in red): **1**, **2**, and **13**.
Percentages of inhibition higher than 60% (in blue): **16**.

Table 4
Dose–response curve for NOS-II activity in the presence of compound **16**

Concentration (mM)	NOS-II activity
1	20 ± 1
0.5	58 ± 3
0.1	90 ± 3

Data represent the mean ± SEM of the percentage of NOS-I and NOS-II inhibition produced by 1 mM of each compound. Each value is the mean of three experiments performed by triplicate in homogenates of rat lung samples.

Of the 14 indazole derivatives studied, 4,5,6,7-tetrafluoro-1*H*-indazoles **13** and **16** show good NOS inhibition although with different selectivity, the latter being a selective inhibitor of the NOS-II isoform. Selective inhibition of NOS-II is of great interest from a therapeutic point of view being potentially useful for treating sepsis, neurodegenerative disorders, diabetes, and arthritis. In this regard, the comparative study of the 14 compounds assayed strongly supports that fluorination of the benzene moiety of the indazoles is a promising strategy in searching for potent and selective NOS inhibitors. Their binding mode to heme (**O–N** vs **N–N**, Scheme 2) is under study. The **LP** mode is probably reserved to basic indazoles, like alkoxy-substituted ones (Scheme 1).

4. Experimental

4.1. Chemistry

Melting points for compounds **8**–**12** and **17** were determined by DSC on a Seiko DSC 220C connected to a Model SSC5200H Disk Station and for compounds **15** and **16**, a ThermoGalen hot stage microscope was used. Thermograms (sample size 0.003–0.0010 g) were recorded at the scanning rate of 2.0 °C min^{-1}. Thin-layer chromatography (TLC) was performed with Merck Silica Gel (60 F_{254}). Compounds were detected with a 254-nm UV lamp. Silica gel (60–320 mesh) was employed for routine column chromatography separations with the indicated eluent. Elemental analyses for carbon, hydrogen, and nitrogen were carried out by the Microanalytical Service of the Universidad Complutense of Madrid on a Perkin–Elmer 240 analyzer.

Solution spectra were recorded, at 300 K save specified, on a Bruker DRX 400 (9.4 T, 400.13 MHz for ^{1}H, 376.50 for ^{19}F, 100.62 MHz for ^{13}C and 40.56 MHz for ^{15}N) spectrometer with a 5-mm inverse detection H–X probe equipped with a z-gradient coil for ^{1}H, ^{13}C, and ^{15}N. For ^{19}F NMR experiments, a 5-mm inverse detection QNP probe equipped with a z-gradient coil was used. Chemical shifts (δ in ppm) are given from internal solvents, $CDCl_3$ (7.26), DMSO-d_6 (2.49), for ^{1}H and $CDCl_3$ (77.0), DMSO-d_6 (39.5) for ^{13}C. External references, $CFCl_3$ (0.00) for ^{19}F and CH_3NO_2 (0.00) for ^{15}N NMR were used. 2D (^{1}H–^{1}H) gs-COSY and inverse proton detected heteronuclear shift correlation spectra, (^{1}H–^{13}C) gs-HMQC, (^{1}H–^{13}C) gs-HMBC, (^{1}H–^{15}N) gs-HMQC, and (^{1}H–^{15}N) gs-HMBC, were acquired and processed using standard Bruker NMR software and in non-phase-sensitive mode.[47] Gradient selection was achieved through a 5% sine truncated shaped pulse gradient of 1 ms. Variable temperature experiments were recorded on the same spectrometer. A Bruker BVT3000 temperature unit was used to control the temperature of the cooling gas stream and an exchanger to achieve low temperatures.

7-Nitro-1*H*-indazole (**1**) is commercially available and used without further purification. The following compounds were prepared according to published procedures: 3-bromo-7-nitro-1*H*-indazole (**2**),[46] 1(2),5,6,7-tetrahydro-4*H*-indazol-4-one (**4**),[43] 6,6-dimethyl-1(2),5,6,7-tetrahydro-4*H*-indazol-4-one (**5**),[43] 3-methyl-1(2),5,6,7-tetrahydro-4*H*-indazol-4-one (**6**),[43] 3,6,6-trimethyl-1(2),5,6,7-tetrahydro-4*H*-indazol-4-one (**7**),[43] 3-methyl-4,5,6,7-tetrafluoro-1*H*-indazole (**13**),[44] and 3-trifluoromethyl-4,5,6,7-tetrafluoro-1*H*-indazole (**14**).[37]

4.2. (±)-4,5,6,7-Tetrahydro-7,8,8-trimehyl-4,7-menthane-2*H*-indazole (8)

In a round-bottomed flask equipped with reflux condenser and magnetic stirrer a slurry of NaH (3.26 g, 0.14 mol) in THF (60 mL) was prepared, (±)-camphor (**18**) (4.0 g, 26 mmol) was added and gas evolution commenced immediately. The mixture was refluxed

for 25 min until gas evolution ceased and was then cooled to room temperature. Ethyl formate (8.4 mL, 0.1 mol) was added dropwise over 1 h, and the mixture was then stirred for an additional 16 h. After quenching (2 mL of 2-propanol and 60 mL of water), the mixture was treated with Et_2O (3 × 25 mL) and then acidified to pH 1 with concentrated HCl. The oil was extracted with Et_2O (3 × 25 mL), the extracts were dried with anhydrous sodium sulfate and solvent was removed under reduced pressure to afford (±)-(*Z*)-3-(hydroxymethylene)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-one (**19**) as a brown solid (2.5 g, 55%). ^{1}H NMR (DMSO-d_6) δ 0,71 (s, 3H, CH_3-b), 0,79 (s, 3H, CH_3-a), 0.86 (s, 3H, CH_3 (C1)), 1.22 (m, 2H, H5), 1.60 (m, 1H, H6^{ax}), 1.89 (m, 1H, H6^{ec}), 2.74 (d, ^3J = 3.8 Hz, 1H, H4), 7.18 (d, ^3J = 4.9 Hz, 1H, =C*H*(OH)), 10.17 (d, ^3J = 6.1 Hz, 1H, OH).

A solution of **19** (2.5 g, 14 mmol) and hydrazine hydrate 55% (0.89 g, 15 mmol) in MeOH (150 mL) was refluxed for 41 h. Solvent was removed under reduced pressure to afford **8** as a yellow solid (2.4 g, 95%) The product was purified by sublimation and crystallization in water; mp. 139.1 °C (DSC),[48] enantiomer (+) 144–145 °C]. ^{1}H NMR ($CDCl_3$) δ 10.10 (br s, 1H, NH), 7.08 (s, 1H, H3), 2.77 (d, ^3J = 4.0, 1H, H4); 1.15 and 2.07 (ddd, 2H, H5^{ax} and H5^{eq}), 1.29 and 1.84 (ddd, 2H, H6^{ax} and H6^{eq}), 1.15 (s, 3H, CH_3-7), 0.95 (s, 3H, CH_3-a), 0.65 (s, 3H, CH_3-b); ^{13}C NMR ($CDCl_3$) δ 119.8 (d, ^1J = 184.5, C3), 125.7 (s, C3a), 47.1 (d, ^1J = 145.0, C4), 27.8 (t, ^1J = 132.2, C5), 33.7 (t, ^1J = 132.4, C6), 49.9 (s, C7), 165.8 (s, C7a), 61.0 (s, C8) 20.4 (q, ^1J = 124.7, CH_3-b), 19.3 (q, ^1J = 124.9, CH_3-a), 10.8 (q, ^1J = 125.2, CH_3-7).

4.3. 1-Methyl-7-nitro-1*H*-indazole (9) and 2-methyl-7-nitro-2*H*-indazole (10)

In a round-bottomed flask equipped with reflux condenser, 7-nitro-1*H*-indazole (**1**) (1.0 g, 6.1 mmol) was dissolved in dry methanol (40 mL). Then, sodium methoxyde (0.42 g, 7.4 mmol) and 1.75 g of methyl iodide (0.77 mL, 12.3 mmol) were added. The mixture was heated to reflux for 2 days and then the solvent was removed under reduced pressure. Water (10 mL) was added and the residue was extracted with chloroform (3 × 15 mL). The organic layers were combined, dried (Na_2SO_4), and concentrated to afford a crude formed by the two isomers and the starting material. After silica gel chromatography with hexane/ethyl acetate 10:1, **9** was obtained first (0.21, 19%) and reaching 1:1 to finally afford **10** (0.32, 29%). Mp (**9**): 100.5 °C (DSC) (98–100 °C).[45,49] Mp (**10**): 144.9 °C (DSC) (145 °C).[46] Anal. Calcd for $C_8H_7N_3O_2$ (**9** and **10**): C, 54.11; H, 4.20; N, 23.17. Found: C, 54.06; H, 4.10; N, 22.45.

Compound **9** ^{1}H NMR (DMSO-d_6) δ 8.37 (s, 1H, H3), 8.23 (dd, ^3J = 7.8, ^4J = 1.0, 1H, H4), 7.32 (dd, ^3J = ^3J = 7.8, 1H, H5), 8.17 (dd, ^3J = 7.8, ^4J = 1.0, 1H, H6), 4.13 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 134.3 (dd, ^1J = 193.2, ^3J = 2.4, C3), 128.5 (ddd, ^2J = ^2J = 9.9, ^3J = 2.5, C3a), 128.6 (dd, ^1J = 165.8, ^3J = 8.4, C4), 120.1 (d, ^1J = 167.2, C5), 124.5 (ddd, ^1J = 166.5, ^2J = 2.8, ^3J = 8.1, C6), 134.8 (m, C7), 130.5 (m, C7a), 40.9 (q, ^1J = 141.3, CH_3); ^{15}N NMR (DMSO-d_6) δ −200.3 (N1), −46.3 (N2). (**10**) ^{1}H NMR (DMSO-d_6) δ 8.73 (s, 1H, H3), 8.25 (dd, ^3J = 7.9, ^4J = 0.9, 1H, H4), 7.23 (dd, ^3J = ^3J = 7.9,1H, H5), 8.28 (dd, ^3J = 7.9, ^4J = 0.9, 1H, H6), 4.27 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 127.7 (d, ^1J = 195.3, ^3J = 2.4, C3), 125.4 (d, ^2J = 9.2, C3a), 129.9 (dd, ^1J = 165.4, ^3J = 8.1, C4), 119.6 (d, ^1J = 166.8, C5), 124.6 (ddd, ^1J = 165.4, ^2J = 2.7, ^3J = 9.1, C6), 136.4 (m, C7), 139.6 (ddd, ^2J = ^3J = ^3J = 6.7, C7a), 40.6 (q, ^1J = 156.6, CH_3); ^{15}N NMR (DMSO-d_6) δ −92.3 (N1), −152.5 (N2).

4.4. 3-Bromo-1-methyl-7-nitro-1*H*-indazole (11) and 3-bromo-2-methyl-7-nitro-2*H*-indazole (12)

In a round-bottomed flask equipped with reflux condenser, 3-bromo-7-nitro-1*H*-indazole (**2**) (0.25 g, 1.0 mmol) was dissolved in dry methanol (10 mL). Then, sodium methoxyde (0.07 g, 1.3 mmol) and 0.25 g of methyl iodide (0.11 mL, 1.8 mmol) were added. The mixture was heated to reflux for 2 days and then the solvent was removed under reduced pressure. Water (10 mL) was added and the residue was extracted with chloroform (3 × 15 mL). The organic layers were combined, dried (Na_2SO_4), and concentrated to afford a crude formed mainly by the two isomers. After silica gel chromatography with (hexane/ethyl acetate 30:1), **11** was obtained first (0.24, 37%) and increasing to 1:1 to afford **12** (0.29, 44%). Mp (**11**): 161.1 °C (DSC) (160–162 °C).[46] Mp (**12**): 200.5 °C (DSC) (194–196 °C).[46] Anal. Calcd for $C_8H_6BrN_3O_2$ (**11** and **12**): C, 37.25; H, 2.51; N, 16.32. Found: C, 37.37; H, 2.47; N, 16.26. (**11**) ^{1}H NMR (DMSO-d_6) δ 8.03 (dd, ^3J = 7.9, ^4J = 0.7, 1H, H4), 7.43 (dd, ^3J = ^3J = 7.9, 1H, H5), 8.28 (dd, ^{3} J = 7.8, ^4J = 0.7, 1H, H6), 4.13 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 121.1 (d, ^3J = 4.0, C3), 127.3 (d, ^2J = 9.4, C3a), 127.2 (dd, ^1J = 167.0, ^3J = 8.5, C4), 121.2 (d, ^1J = 168.8, C5), 126.0 (ddd, ^1J = 167.2, ^2J = 2.9, ^3J = 7.7, C6), 135.0 (d, ^3J = 9.2, C7), 131.9 (dd, ^3J = ^3J = 6.9, C7a), 40.9 (q, ^1J = 142.5, CH_3); ^{15}N NMR (DMSO-d_6) δ −198.9 (N1), −53.6 (N2). (**12**) ^{1}H NMR (DMSO-d_6) δ 8.02 (dd, ^3J = 7.9, ^4J = 0.8, 1H, H4), 7.31 (dd, ^3J = ^3J = 7.9, 1H, H5), 8.35 (dd, ^3J = 7.9, ^4J = 0.8, 1H, H6), 4.24 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 111.0 (m, C3), 124.9 (d, ^2J = 9.2, C3a), 128.3 (dd, ^1J = 166.5, ^3J = 8.1, C4), 120.8 (d, ^1J = 167.4, C5), 125.9 (ddd, ^1J = 167.4, ^2J = 2.9, ^3J = 9.5, C6), 136.4 (d, ^3J = 7.7, C7), 139.3 (dd, ^3J = ^3J = 6.9, C7a), 39.5 (q, ^1J = 142.0, CH_3); ^{15}N NMR (DMSO-d_6) δ −87.1 (N1), −155.2 (N2).

4.5. 3-Phenyl-4,5,6,7-tetrafluoro-1*H*-indazole (15)

In a round-bottomed flask equipped with reflux condenser, pentafluorobenzophenone (**20**) (0.70 g, 2.5 mmol) was dissolved in toluene (30 mL), hydrazine monohydrate (0.40 g, 8.0 mmol) was added and the mixture refluxed during 6 h. After this period, the mixture was cooled and the solvent was removed under reduced pressure. The product was purified by silica gel chromatography (hexane/ethyl ether 15:1) to afford **15** (0.67, 90%) as a white solid. Mp 164–165 °C; Anal. Calcd for $C_{13}H_6F_4N_2$: C, 58.66; H, 2.27; N, 10.52. Found: C, 58.58; H, 2.45; N, 10.40. ^{1}H NMR ($CDCl_3$) δ 11.48 (br s, 1H, NH), 7.57 (m, 2H, Hm), 7.45 (m, 3H, Ho, Hp); ^{19}F NMR ($CDCl_3$) δ −140.9 (dd, $^3J_{F5}$ = 19.3, $^5J_{F7}$ = 19.3, F4), −165.2 (ddd, $^3J_{F4}$ = 19.3, $^3J_{F6}$ = 19.3, $^4J_{F7}$ = 2.6, F5), −156.8 (dd, $^3J_{F5}$ = 19.3, $^5J_{F7}$ = 19.3, F6), −159.6 (ddd, $^3J_{F6}$ = 19.3, $^5J_{F4}$ = 19.3, $^4J_{F5}$ = 2.6, F7); ^{13}C NMR ($CDCl_3$) δ 130.8 (C3), 107.8 (dd, ^2J = 18.8, ^3J = 3.8, C3a), 139.5 (dddd, ^1J = 253.1, ^2J = 12.0, ^3J = ^4J = 3.8, C4), 135.7 (ddd, ^1J = 246.2, ^2J = ^2J = 16.3, C5), 139.6 (ddd, ^1J = 252.4, ^2J = ^2J = 14.1, C6), 132.3 (dddd, ^1J = 249.2, ^2J = 12.6, ^3J = 5.0, ^4J = 2.5, C7), 128.3 (overlapped, C7a), 146.3 (m, C_i), 129.2 (dt, ^1J = 161.0, ^2J = 7.7, C_p), 128.7 (dd, ^1J = 161.0, ^2J = 7.7, C_o), 128.4 (dm, ^1J = 160.5); ^{15}N NMR ($CDCl_3$, T = 213 K) δ −205.1 (N1).

4.6. 4,5,6,7-Tetrafluoro-3-perfluorophenyl-1*H*-indazole (16)

In a round-bottomed flask equipped with reflux condenser, decafluorobenzophenone (**21**) (0.50 g, 1.4 mmol) was dissolved in toluene (20 mL), hydrazine monohydrate (0.10 g, 2.0 mmol) was added and the reaction was refluxed for 2 h. After cooling, the solvent was removed under reduced pressure and the product was purified by silica gel chromatography (hexane/ethyl ether, 10:1) to afford **16** (0.45 g, 90%) as a white solid. Mp 114–116 °C; Anal. Calcd for $C_{13}HF_9N_2$: C, 43.84; H, 0.28; N, 7.87. Found: C, 43.84; H, 0.41; N, 8.19. 1H NMR ($CDCl_3$) δ 11.38 (br s, 1H, NH); ^{19}F NMR ($CDCl_3$) δ −151.8 (dd, $^3J_{F5}$ = 19.2, $^5J_{F7}$ = 19.2, F4), −163.2 (ddd, $^3J_{F4}$ = 19.2, $^3J_{F6}$ = 19.2, $^4J_{F7}$ = 2.6, F5), −155.1 (dd, $^3J_{F5}$ = 19.2, $^3J_{F7}$ = 19.2, F6), −158.6 (dd, $^3J_{F6}$ = 19.2, $^5J_{F4}$ = 19.2, F7), −161.7 (m, C_6F_5, F_m), −148.2 (m, C_6F_5, F_p), −140.7 (m, C_6F_5, F_o); ^{13}C NMR ($CDCl_3$) δ 131.5 (C3), 109.9 (dd, ^2J = 18.8, ^3J= 2.5, C3a), 139.0 (dddd,

^1J = 256.2, ^2J = 12.6, ^3J = ^4J = 4.4, C4), 136.4 (ddd, ^1J = 249.1, ^2J = ^2J = 15.7, C5), 140.2 (dddd, ^1J = 254.9, ^2J = ^2J = 14.4, C6), 132.6 (dddd, ^1J = 251.2, ^2J = 16.3, ^3J = 5.0, ^4J = 2.5, C7), 127.7 (ddd, ^2J = 13.9, ^3J = 7.5, ^3J = 3.8, C7a), 145.3 (dddd, ^1J = 251.2, ^2J =15.7, ^3J = 6.6, ^3J = 3.8, C_o), 142.3 (dtt, ^1J = 257.5, ^2J = 13.2, ^3J = 5.0, C_p), 138.0 (dtd, ^1J = 250.9, ^2J = 14.7, ^3J = 4.5, C_m), 106.5 (td, ^2J = 17.2, ^3J = 3.8, C_i); ^{15}N NMR ($CDCl_3$, T = 213 K) δ −201.6 (N1).

4.7. 4,5,6,7-Tetrafluoro-1*H*-indazole-3-ol (17)

In a round-bottomed flask equipped with reflux condenser, methyl 2,3,4,5,6-pentafluorobenzoate (**22**) (1.0 g, 4.4 mmol) was dissolved in toluene (10 mL), hydrazine monohydrate (0.27 g, 5.3 mmol) was added and the reaction was heated and stirred at 70 °C for 29 h. The solvent was removed under reduced pressure and the product was purified by silica gel chromatography (dichloromethane/methanol, 30:1) and crystallized from CH_2Cl_2/CH_3OH to afford **17** (0.26 g, 28%) as a white solid. Mp 198.0 °C (DSC). Anal. Calcd for $C_7H_2F_4N_2O$: C, 40.79; H, 0.98; N, 13.59. Found: C, 40.87; H, 1.24; N, 13.62. 1H NMR (DMSO-d_6) δ 12.71 (br s, 1H, NH), 11.32 (br s, 1H, OH); ^{19}F NMR (DMSO-d_6) δ −148.8 (dd, $^3J_{F5}$ = 19.5, $^5J_{F7}$ = 19.5, F4), −170.7 (dd, $^3J_{F4}$ = 19.5, $^3J_{F6}$ = 19.5, F5), −159.1 (br s, F6), −160.5 (ddd, $^3J_{F6}$ = 19.5, $^5J_{F4}$ = 19.5, $^4J_{F5}$ = 4.5, F7; ^{13}C NMR (DMSO-d_6) δ 154.7 (d, ^3J= 2.4, C3), 98.8 (dd, ^2J = 18.5, ^3J = 4.8, C3a), 138.6 (dddd, ^1J = 250.6, ^2J = 11.8, ^3J = ^4J = 3.9, C4), 132.7 (ddd, ^1J = 239.5, ^2J = ^2J = 15.5, C5), 138.9 (dddd, ^1J = 246.7, ^2J = ^2J = 15.4, ^3J= 1.8, C6), 131.8 (dddd, ^1J = 248.2, ^2J = 13.8, ^3J = 5.0, ^4J = 2.2, C7), 127.6 (ddd, ^2J = 13.9, ^3J = 7.8, ^3J = 3.9, C7a);^{15}N NMR (THF-d_8, T = 207 K) δ −233.4 (N1), −111.9 (N2).

4.8. Assay of NOS-I/NOS-II activities

L-Arginine, L-citrulline, *N*-(2-hydroxymethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), DL-dithiothreitol (DTT), leupeptin, aprotinin, pepstatin, phenylmethylsulfonylfluoride (PMSF), hypoxanthine-9-β-D-ribofuranosid (NOS-IIine), ethylene glycol-bis-(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), bovine serum albumin (BSA), Dowex-50 W (50 × 8-200), FAD, NADPH and 5,6,7,8-tetrahydro-L-biopterin dihydrochloride were obtained from Sigma–Aldrich Química (Spain). L-3H-arginine (58 Ci/mmol) was obtained from Amersham (Amersham Biosciences, Spain). Tris(hydroxymethyl)aminomethane (Tris–HCl) and calcium chloride were obtained from Merck (Spain).

For NOS-II activity determination, male Wistar rats (3-months old, 220–250 g) were bred and kept in the University's animal facility on a 12 h light/12 h dark cycle at 22 ± 2 °C, on regular chow and tap water until the day of the experiment. All experiments were performed according to the Spanish Government Guide and the European Community Guide for animal care. The experimental paradigm was published elsewhere.[50] Briefly, three days before the experiment the jugular vein was cannulated under ip equithesin anesthesia (1 mL/kg) for LPS administration. Rats were iv injected with 10 mg/kg bw LPS (*E. coli* 0127:B8, Sigma–Aldrich, Madrid, Spain) dissolved in 0.3 mL of saline. Six hours after LPS injection, animals were killed by decapitation. Lungs were quickly collected, washed, and frozen to −80 °C in liquid nitrogen. Pieces of lungs were homogenized (0.1 g/mL) in ice-cold buffer (25 mM Tris, 0.5 mM DTT, 10 μg/mL pepstatin, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM PMSF, pH 7.6) at 0–4 °C.[51] The crude homogenate were centrifuged at 2500*g* at 4 °C for 5 min, and aliquots of the supernatant were either stored at −80 °C for total protein determination[52] or used immediately for NOS activity measurement.

For NOS-I activity determination, untreated male Wistar rats (200–250 g) were killed by cervical dislocation and striata were quickly collected and immediately used to measure NOS-I activity. Upon removal, tissues were cooled in an ice-cold buffer (25 mM Tris, 0.5 mM DTT, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 10 μg/mL aprotinin, 1 mM PMSF, pH 7.6). Two striata were placed in 1.25 mL of the same buffer and sonicated (10 s × 6). The crude homogenate was centrifuged 5 min at 1000*g*, and an aliquot of the supernatant was frozen at −80 °C for total protein determination.[52]

NOS activity was measured by the method of Bredt et al.,[53] monitoring the conversion of L-3H-arginine to L-3H-citrulline. The final incubation volume was 100 μL and consisted of 10 μL sample added to prewarmed (37 °C) buffer to give a final concentration of 25 mM Tris, 1 mM DTT, 30 μM H_4-biopterin, 10 μM FAD, 0.5 mM inosine, 0.5 mg/mL BSA, 0.1 mM $CaCl_2$, 10 μM L-arginine and 40 nM L-3H-arginine, pH 7.6. The reaction was started by the addition of 10 μL NADPH (0.75 mM final concentration) and continued for 30 min at 37 °C. Control incubations were performed in absence of NADPH. The activity of NOS-II was measured in the presence or absence of 10 mM EDTA. EDTA removes calcium from the medium preventing the activation of NOS-I; thus, in the presence of EDTA only NOS-II is detected. The reaction was stopped adding 400 μL of cold 0.1 M HEPES containing 10 mM EGTA, 175 mM L-3H-citrulline, pH 5.5. The mixture was decanted onto a 2 mL column packed with Dowex-50 W ion exchanger resin (Na^+ form) and eluted with 1.2 mL of water. L-3H-citrulline was quantified by liquid scintillation counting in a Beckman LS-6000 system (Beckman Coulter, Fullerton, CA, USA). The retention of L-3H-arginine in this process was greater then 98%. NOS activity was measured by monitoring the conversion of L-3H-arginine to L-^{3}H-citrulline. Enzyme activity was referred as pmol L-3H-citrulline/min/mg prot.

A total of 16 compounds were assayed for NOS-I/NOS-II activities: 1(2),5,6,7-tetrahydro-4*H*-indazole-4-one (**4**), 6,6-dimethyl-1(2),5,6,7-tetrahydro-4*H*-indazole-4-one (**5**), 3-methyl-1(2),5,6,7-tetrahydro-4*H*-indazole-4-one (**6**), 3,6,6-trimethyl-1(2),5,6,7-tetrahydro-4*H*-indazole-4-one (**7**), (±)-4,5,6,7-tetrahydro-7,8,8-trimethyl-4,7-methane-2*H*-indazole (**8**), 7-nitro-1*H*-indazole (7NI, **1**), 3-bromo-7-nitro-1*H*-indazole (NIBr, **2**), 1-methyl-7-nitro-1*H*-indazole (**9**), 2-methyl-7-nitro-2*H*-indazole (**10**), 3-bromo-1-methyl-7-nitro-1*H*-indazole (**11**), 3-bromo-2-methyl-7-nitro-2*H*-indazole (**12**), 4,5,6,7-tetrafluoro-3-methyl-1*H*-indazole (**13**), 4,5,6,7-tetrafluoro-3-trifluoromethyl-1*H*-indazole (**14**), 3-phenyl-4,5,6,7-tetrafluoro-1*H*-indazole (**15**), 4,5,6,7-tetrafluoro-3-perfluorophenyl-1*H*-indazole (**16**) y 4,5,6,7-tetrafluoro-1*H*-indazole-3-ol (**17**). Except when indicated, the concentration of these compounds used in the NOS assays was 1 mM.

4.9. Statistical analysis

Data are expressed at the mean ± SEM. One-way analysis of variance, followed by the Newman–Keuls multiple range test was used. A P <0.05 value was considered statistically significant.

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