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Preliminary communication

Synthesis and biological evaluation of indazole derivatives

Rosa M. Claramunt [a,*], Concepción López [a], Ana López [c,d], Carlos Pérez-Medina [a], Marta Pérez-Torralba [a], Ibon Alkorta [b], José Elguero [b], Germaine Escames [c,d], Darío Acuña-Castroviejo [c,d,**]

[a] Departamento de Química Orgánica y Bio-Orgánica, Facultad de Ciencias, UNED, Senda del Rey 9, E-28040 Madrid, Spain
[b] Instituto de Química Médica, CSIC, Centro de Química Orgánica Manuel Lora-Tamayo, Juan de la Cierva 3, E-28006 Madrid, Spain
[c] Centro de Investigación Biomédica, Parque Tecnológico de Ciencias de la Salud, Universidad de Granada, Avenida del Conocimiento s/n, E-18100 Armilla, Granada, Spain
[d] Departamento de Fisiología, Facultad de Medicina, Avenida de Madrid 11, 18012 Granada, Spain

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In memoriam of our friend Professor Concepción Foces-Foces.

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ABSTRACT

The inhibition of neuronal and inducible nitric oxide synthases (nNOS and iNOS) by a series of 36 indazoles has been evaluated, showing that most of the assayed derivatives are better iNOS than nNOS inhibitors. A parabolic model relating the iNOS inhibition percentage with the difference, E_{rel}, between stacking and apical interaction energies of indazoles with the active site of the NOS enzyme has been established.

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1. Introduction

A family of enzymes known as nitric oxide synthases (NOS) catalyzes the oxidation of L-arginine to L-citrulline and nitric oxide (NO), a molecule that plays an important role in the regulation of blood pressure, neurotransmission, and the immune response. Three isoforms of nitric oxide synthase have been identified in different tissues. Neuronal (nNOS) and endothelial (eNOS) nitric oxide synthases are expressed constitutively and are calcium/calmodulin dependent; inducible nitric oxide synthase (iNOS) is expressed in response to inflammatory or immunologic stimuli and its activity is independent of calmodulin. All NOS isoforms are protoporphyrin IX heme enzymes and require NADPH, FAD, FMN and tetrahydrobiopterin (H_4B) as cofactors [1–4].

We present here the results of our investigation on the selectivity of inducible iNOS compared to constitutive nNOS of a series of indazoles depicted in Fig. 1, in a similar approach to that of the Pharmacia & Upjohn group with U-19451A [5] where they explored the iNOS/nNOS selectivity. Save the first three 7-nitro derivatives, **1**–**3**, 3-methylindazole (**5**), the three 3-bromo-7-nitro derivatives, **15**–**17**, 3,7-dinitroindazole (**18**), 3-hydroxyindazole (**19**) and 3-hydroxy-7-nitroindazole (**23**), the remaining 26 indazoles contain fluorine substituents. To date, only our group has explored the inhibitory activity of fluoroindazoles against iNOS.

This is our third paper on the inhibitory properties of indazoles against NOS. In the first one [6], we reported the percentage of nNOS and iNOS inhibition of eleven indazoles at 1 mM concentration: **1**, **2**, **3**, **8**, **12**, **13**, **14**, **15**, **16**, **17** and **22**, whose values will be used in this paper. In the second one we proposed a theoretical model based on the stacking of indazoles above the heme [7].

2. Results and discussion

2.1. In vitro NOS inhibition

The 36 indazoles studied in this article have been tested for their inhibitory properties employing the method reported by Bredt et al. for the determination of NOS activity (Section 4.1). 25 of these indazoles are studied for the first time; each value reported in Table 1 is the average of three measurements.

* Corresponding author.
** Corresponding author. Departamento de Fisiología, Facultad de Medicina, Avenida de Madrid 11, 18012 Granada, Spain.
E-mail addresses: rclaramunt@ccia.uned.es (R.M. Claramunt), dacuna@ugr.es (D. Acuña-Castroviejo).

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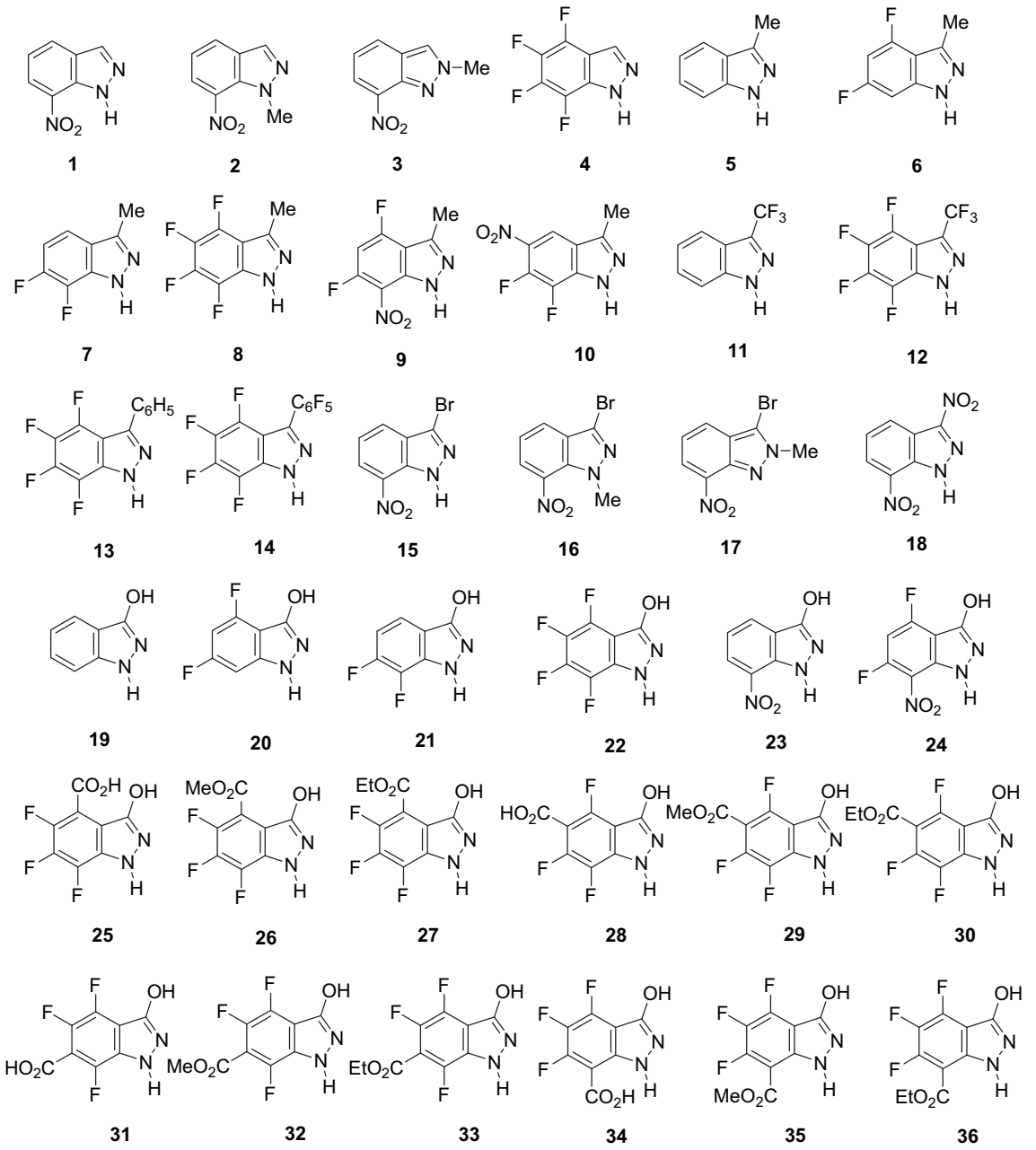


Fig. 1. The indazoles series.

Some values of iNOS and nNOS percentages of inhibition (marked ~) for compounds **37**, **38** and **39** (Fig. 2) have been estimated. Bland-Ward and Moore described that the iNOS inhibitory properties of indazole itself (**37**) should be weak [8], and consequently we have used a 45% value. The IC_{50} mM values of 7-methoxyindazole (**38**) were established by Boucher et al.: nNOS = 1.5 and iNOS = 5.5 [11], and since the percentage of inhibition against nNOS was known (80.0), [9] we have estimated the effect on iNOS to be similar but slightly lower, 70%. Vichard et al. [12] have reported the IC_{50} mM values for 7-cyanoindazole (**39**): nNOS = 25 and iNOS = 110 (these much larger values determined by a different technique should not be compared with those of other authors) and also for 7-nitroindazole (**1**), nNOS = 25 and iNOS = 50. Therefore, we have used the same value for nNOS (88**%,** as for **1**) and reduced the value for iNOS to 80%.

A perusal of Table 1 shows that the most potent iNOS inhibitors are, in decreasing order, **4** (98.7) > **15** (97.0) > **10** (95.6) > **9** (92.0) > **24** (91.0) > **18** (89.3) > **21** (89.1) > **1** (84.0) > **8** (83.0) (Fig. 3). Meanwhile the nNOS inhibitory potency decreases as follows: **15** (95.0) > **9** (90.0) > **1** (88.0) > **10** (85.4) (Fig. 4).

Regarding selectivity we can say that the majority of the assayed indazoles are better iNOS than nNOS inhibitors. At a concentration of 1 mM, compounds **18** (40-fold selective), **14** (10-fold), **24** (6-fold) and **4** (2-fold) show good promise as possible iNOS selective inhibitors. At the same concentration, the other potent iNOS inhibitors (compounds **1**, **9** and **15**) also show high potency vs. nNOS and thus poor selectivity between the isoforms. However, these compounds deserve to be studied more in depth before ruling out selectivity.

All indazoles bearing a carboxylic or an ester substituent, **25**–**36**, proved to be moderate iNOS inhibitors and very weak nNOS ones,

Table 1
iNOS and nNOS % inhibition in the presence of indazoles. Values from Ref. [6] are underlined. Except when indicated, the concentration of these compounds used in the NOS assays was 1 mM.

Compound	iNOS	nNOS
1 (7-Nitro-1*H*-indazole)	84.0	88.0
2 (1-Methyl-7-nitro-1*H*-indazole)	25.0	27.3
3 (2-Methyl-7-nitro-2*H*-indazole)	36.0	29.0
4 (4,5,6,7-Tetrafluoro-1*H*-indazole)	98.7	43.3
5 (3-Methyl-1*H*-indazole)	77.4	33.7[a]
6 (3-Methyl-4,6-difluoro-1*H*-indazole)	74.3	52.1
7 (3-Methyl-6,7-difluoro-1*H*-indazole)	69.2	58.4
8 (3-Methyl-4,5,6,7-tetrafluoro-1*H*-indazole)	83.0	63.0
9 (3-Methyl-4,6-difluoro-7-nitro-1*H*-indazole)	92.0	90.0
10 (3-Methyl-6,7-difluoro-5-nitro-1*H*-indazole)	95.6	85.4
11 (3-Trifluoromethyl-1*H*-indazole)	80.1	69.9
12 (3-Trifluoromethyl-4,5,6,7-tetrafluoro-1*H*-indazole)	21.0	41.0
13 (3-Phenyl-4,5,6,7-tetrafluoro-1*H*-indazole)	37.0	11.0
14 (3-Pentafluorophenyl-4,5,6,7-tetrafluoro-1*H*-indazole)	80.0	8.0
15 (3-Bromo-7-nitro-1*H*-indazole)	97.0	95.0
16 (1-Methyl-3-bromo-7-nitro-1*H*-indazole)	10.0	22.0
17 (2-Methyl-3-bromo-7-nitro-2*H*-indazole)	7.0	29.0
18 (3,7-Dinitro-1*H*-indazole)	89.3	2.1
19 (3-Hydroxy-1*H*-indazole)	55.1	31.4[b]
20 (3-Hydroxy-4,6-difluoro-1*H*-indazole)	48.1	11.1
21 (3-Hydroxy-6,7-difluoro-1*H*-indazole)	89.1	57.1
22 (3-Hydroxy-4,5,6,7-tetrafluoro-1*H*-indazole)	6.0	23.0
23 (3-Hydroxy-7-nitro-1*H*-indazole)	8.2	18.9
24 (3-Hydroxy-4,6-difluoro-7-nitro-1*H*-indazole)	91.0	14.9
25 (3-Hydroxy-5,6,7-trifluoro-1*H*-indazole-4-carboxylic acid)	62.6	1.8
26 (3-Hydroxy-5,6,7-trifluoro-1*H*-indazole-4-carboxylic acid methyl ester)	22.6	6.3
27 (3-Hydroxy-5,6,7-trifluoro-1*H*-indazole-4-carboxylic acid ethyl ester)	38.7	1.2
28 (3-Hydroxy-4,6,7-trifluoro-1*H*-indazole-5-carboxylic acid)	16.3	12.1
29 (3-Hydroxy-4,6,7-trifluoro-1*H*-indazole-5-carboxylic acid methyl ester)	48.9	13.3
30 (3-Hydroxy-4,6,7-trifluoro-1*H*-indazole-5-carboxylic acid ethyl ester)	51.8	2.5
31 (3-Hydroxy-4,5,7-trifluoro-1*H*-indazole-6-carboxylic acid)	42.0	5.4
32 (3-Hydroxy-4,5,7-trifluoro-1*H*-indazole-6-carboxylic acid methyl ester)	46.2	19.7
33 (3-Hydroxy-4,5,7-trifluoro-1*H*-indazole-6-carboxylic acid ethyl ester)	53.5	10.2
34 (3-Hydroxy-4,5,6-trifluoro-1*H*-indazole-7-carboxylic acid)	40.7	11.1
35 (3-Hydroxy-4,5,6-trifluoro-1*H*-indazole-7-carboxylic acid methyl ester)	49.6	5.1
36 (3-Hydroxy-4,5,6-trifluoro-1*H*-indazole-7-carboxylic acid ethyl ester)	43.7	5.6
37 (1*H*-indazole) [8]	~45	11.2
38 (7-Methoxy-1*H*-indazole) [9]	~70	80.0
39 (7-Cyano-1*H*-indazole) [12]	~80	~88

[a] The related 3-ethyl-1*H*-indazole has a % of nNOS inhibition of 18.8 [8].
[b] 3-Hydroxy-1*H*-indazole (**19**) is inactive towards nNOS (IC_{50} (μM) > 1000) [6,10].

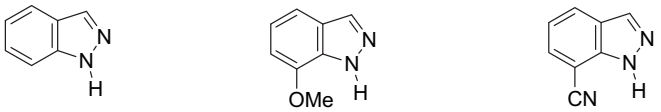
being 3-hydroxy-5,6,7-trifluoroindazole-4-carboxylic acid (**25**) (iNOS = 62.6%, nNOS = 1.8% – 35-fold selective) the most promising candidate. A Free–Wilson analysis using presence–absence matrices [13,14] of these 12 indazoles affords the values gathered in Table 2.

Coefficients in Table 2 indicate small sensitivity to the nature of R (H, CH_3, C_2H_5) and the position (4, 5, 6, 7) of the substituent, but are always larger for iNOS than for nNOS.

If one wants to exclude nitroaromatic compounds to avoid toxicity problems [15–17], then the most interesting indazole is **4** distantly followed by **21** and **8**. In fact 7-cyanoindazole (**39**) and its 3-bromo derivative were prepared [12] to by-pass the aforementioned problems shown by the nitroindazoles **1** and **15**.

2.2. Chemistry

We have already reported the preparation and physicochemical properties of the 36 assayed compounds save those of **4**, **18**, **20**, **21** and **24**. 7-Nitroindazole (**1**) is commercially available and was used without further purification. Compounds **5**, **8**, **11** and **12** can be found in Ref. [18] whereas indazoles **2**, **3**, **13**, **14**, **16**, **17** and **22** in Ref. [6]. Difluoro-3-methylindazoles **6**, **7**, **9** and **10** are reported in Ref. [19] and 3-hydroxyindazole (**19**) and 3-hydroxy-7-nitroindazole (**23**) in Ref. [20]. Finally, trifluoro-3-hydroxy-indazolecarboxylic acids and esters, **25**–**36**, are described in Ref. [21].



37 **38** **39**

Fig. 2. Three literature indazoles.

In order to prioritize comparability over correct nomenclature, we have named all the compounds as substituted indazoles even in the case of the 3-hydroxy groups that in accordance with IUPAC rules are indazol-3-ols. Also, for the sake of clarity we avoided the use of the prefix 1*H* for unsubstituted indazoles in the text. See Table 1 for the full name of all derivatives.

4,5,6,7-Tetrafluoroindazole (**4**) was prepared, not without difficulty, according to Scheme 1.

When pentafluorobenzaldehyde (**40**) reacted with hydrazine in the standard conditions to prepare indazoles, only azine **42** was isolated [22]. To obtain hydrazone **41** the reaction was carried out in ethanol/water at low temperature and high-dilution. When **41** was heated in toluene or *N*,*N*′-dimethylformamide the only product isolated was **42**. These failed attempts are in agreement with the results reported by Lukin et al. [23] about the difficulty to cyclize hydrazones derived from benzaldehydes when compared to those derived from acetophenones, which could be due to an unfavorable conformation of the former [24].

However, according to Lukin, the formation of indazoles from hydrazones occurs through a hydrazine–hydrazone intermediate like **43** (in the original article without fluorine substituents) that cyclizes into 3-hydrazineindazoline **44** to afford, after elimination of a molecule of hydrazine, the desired indazole **4**. Therefore, we carried out the reaction of **40** with an excess of hydrazine (1:5) and, although we obtained the desired product **4** in 13% yield, we also isolated a large amount of the isomeric hydrazine–hydrazone **45**.

3,7-Dinitroindazole (**18**) has been described in the literature by two different groups both using the same synthetic approach (Scheme 2) involving the nitration of 7-nitroindazole (**1**) to afford 2,7-dinitroindazole (**46**) that on heating in anisole [25] or amyl alcohol [26] solution was converted into **18**. We have followed the method described in Ref. [25], with similar results to those reported.

3-Hydroxy-4,6-difluoroindazole (**20**) and 3-hydroxy-6,7-difluoroindazole (**21**) were prepared in a similar way to other 3-hydroxyindazoles described in a previous paper [21] (Scheme 3). Secondary products resulting from the nucleophilic replacement of the fluorine atoms in *para*-position to the ester group were also detected.

Indazoles **20** and **21** have CAS Registry numbers (887567-77-3 and 1000343-93-0, respectively) but only the first one appears to have been described in a patent [27]. Finally, nitration of compound **20** affords 3-hydroxy-4,6-difluoro-7-nitroindazole (**24**) in good yield (Scheme 3).

2.3. Modeling studies

In our previous work we advocated the use of the difference E_{rel} of stacking and apical interaction energies between indazoles and

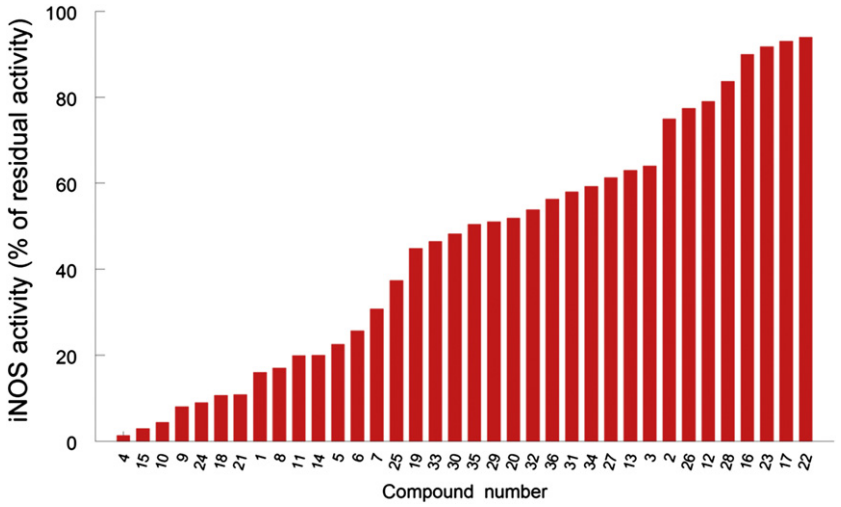


Fig. 3. Percentage of residual activity of iNOS in the presence of the tested compounds.

porphyrines to estimate the inhibitory potency of these compounds [7]. The model was based on crystallographic studies [28–30], replacing the Fe by Zn for computational ease. The NOS inhibitory activity (in percentage) was related, in an exponential relationship, with the difference, E_{rel}, between the stacking and the apical (lone-pair) interaction energies as follows: % inhibition = $104.0 - 2.21 \times e^{(E_{rel}/8.54)}$. A similar result was obtained using stacking energies instead of E_{rel}. Compound **14**, $C_{13}HF_9N_2$, due to its excessive size was excluded from the calculations.

The interaction of indazoles with the active site of the enzyme involves not only stacking but hydrogen bonding (HB) of the N–H (indazole)···O=C and N–H···N(indazole) type. Then, we explored a model including N–H HB acidity and N: HB basicity using the thermodynamic acidity (ΔH) and basicity (PA or proton affinity), since it is known that they are proportional for structurally related compounds [7,31,32]. Table 3 contains all the information we have gathered on this problem, including data for different heterocycles (pyridine, imidazole and pyrazole) of known experimental basicity (PA) and acidity (ΔH).

In order to check the validity of the calculated values before using them for modelization purposes, we have carried out two comparative analyses making use of the values we found in the NIST database [33]: pyridine basicity 930, imidazole basicity 942.8 and acidity 1466, pyrazole basicity 894.1 and acidity 1480, indazole basicity 900.8 and acidity 1457 kJ mol^{-1}. Using these values, the following trendlines can be calculated:

$$\begin{aligned} PA &= (75 \pm 38) + (0.92 \pm 0.04)\ \text{basicity},\ \ n = 4, R^2 \\ &= 0.996\ \left(\text{differences } \pm 2.7\ \text{ kJ mol}^{-1}\right) \end{aligned} \quad (1)$$

$$\begin{aligned} \Delta H &= (506 \pm 81) + (0.64 \pm 0.05)\ \text{acidity},\ \ n = 3, R^2 \\ &= 0.993\ \left(\text{differences} - 26\ \text{ kJ mol}^{-1}\right) \end{aligned} \quad (2)$$

Although the experimental gas-phase acidities are systematically underestimated by the calculations, they are proportional (Eq. (2)) and therefore can be used in the models of activity.

In a previous report, we related iNOS activity with the E_{rel} for a total of eleven indazoles, suggesting the existence of an asymptotic model [6]. Now, with a larger number of compounds available, we consider that a parabolic model is a better fit than the former. Parabolic relationships (corresponding to polynomial models of

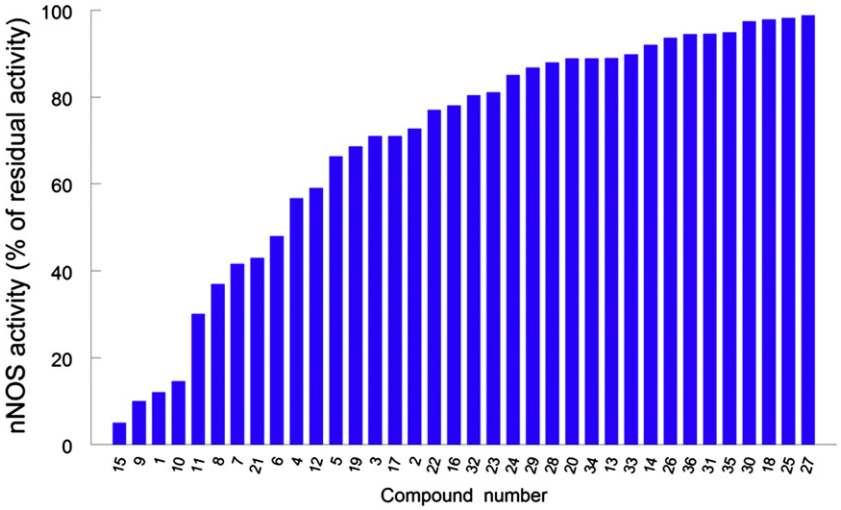


Fig. 4. Percentage of residual activity of nNOS in the presence of the tested compounds.

Table 2
Contribution of the different substituents and positions to the activity of the 12 indazoles bearing a CO_2R substituent.

Substituent	iNOS	nNOS
CO_2H	40 ± 7	8 ± 3
CO_2CH_3	42 ± 7	11 ± 3
$CO_2C_2H_5$	47 ± 7	5 ± 3
R^2	0.93	0.76
4-CO_2R	41 ± 8	3 ± 3
5-CO_2R	39 ± 8	9 ± 3
6-CO_2R	47 ± 8	12 ± 3
7-CO_2R	45 ± 8	7 ± 3
R^2	0.93	0.80

order 2) were introduced by Hansch and soon became classical in medicinal chemistry [14,34,35].

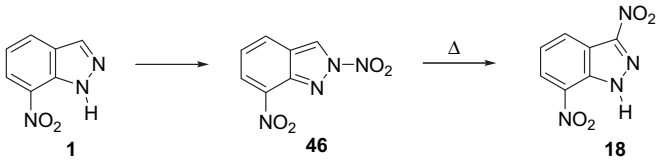
Fig. 5 represents the variation of iNOS inhibition percentage with E_{rel} after excluding compounds **12** (3-trifluoromethyl-4,5,6,7-tetrafluoroindazole) and **21** (3-hydroxy-6,7-difluoroindazole). The trendline corresponds to:

$$\begin{aligned}\text{iNOS \% inhibition} &= (95 \pm 3) - (0.24 \pm 0.12)E_{rel} \\ &\quad - (0.036 \pm 0.005)E_{rel}^2, \ \text{n} \\ &= 13, R^2 = 0.81 \end{aligned} \quad (3)$$

The curve reaches its maximum (100%) for values of E_{rel} close to 0 (no experimental data correspond to it).

The only other property that appears to have an effect on some inhibition data is the thermodynamic basicity, PA. The acidity does not appear to have an influence of the inhibitory properties. Eqs. (4)–(6) can be calculated from the data reported in Tables 1 and 3, and although only the first one is significant, the other two suggest a necessary criterion for selectivity iNOS vs. nNOS: the indazole must have low basicity.

$$\begin{aligned}\%\,\text{iNOS} &= (189 \pm 67) - (0.22 \pm 0.11)E_{rel} \\ &\quad - (0.30 \pm 0.07)\left(E_{rel}^2\right)/10 - (0.11 \pm 0.08)\text{PA, n} \\ &= 13, R^2 = 0.85 \end{aligned} \quad (4)$$



Scheme 2. Synthesis of indazole **18**.

$$\begin{aligned}\%\,\text{nNOS} &= -(325 \pm 284) - (0.62 \pm 0.26)\left(E_{rel}^2\right)/10 \\ &\quad + (0.50 \pm 0.32)\text{PA, n} \\ &= 13, R^2 = 0.35 \end{aligned} \quad (5)$$

$$\begin{aligned}\text{Selectivity } (\Delta &= \%\,\text{iNOS} - \%\,\text{nNOS}) \\ &= (365 \pm 205) - (0.72 \pm 0.42)E_{rel} \\ &\quad - (0.55 \pm 0.28)\text{PA, n} \\ &= 13, R^2 = 0.48 \end{aligned} \quad (6)$$

3. Conclusion

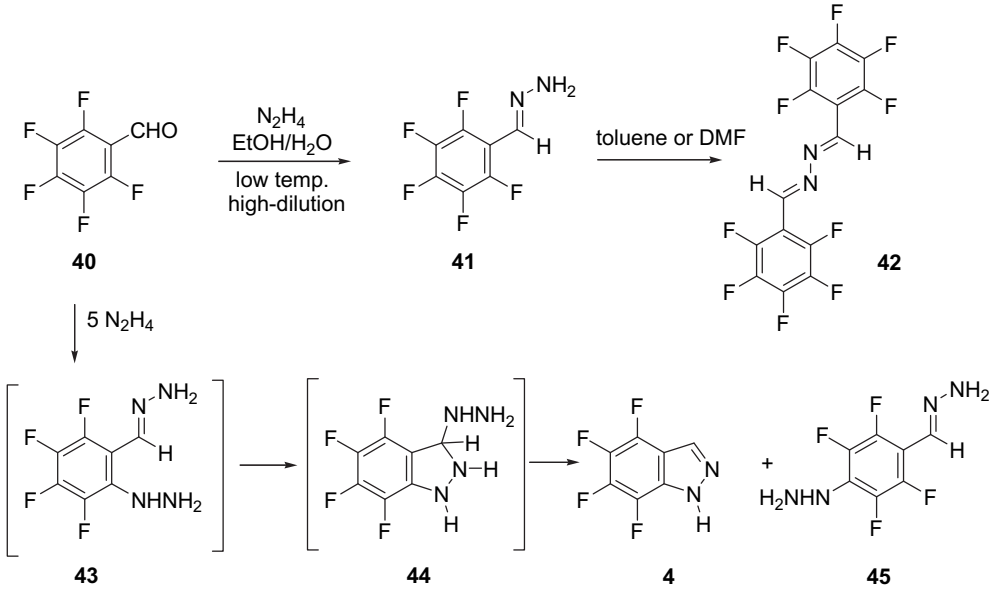
For the first time the inhibitory properties of fluorinated indazoles against iNOS have been evaluated. From a library of 34 indazole derivatives we have identified a very potent iNOS inhibitor, 4,5,6,7-tetrafluoroindazole (**4**), a non-nitro derivative that constitutes a promising candidate for future developments.

We advocate the use of computational modeling of the interaction between a large series of indazole candidates with Zn-porphyrins and proceed to synthesize and test only those that the model predicts to be active and selective. Even though these predictions are not entirely precise they suffice to know where to address the research.

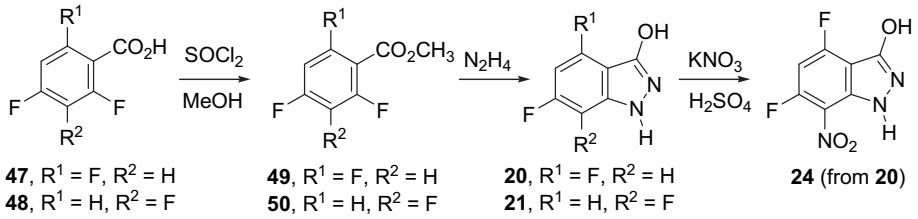
4. Experimental

4.1. Assay of iNOS/nNOS activities

L-Arginine, L-citruline, *N*-(2-hydroxymethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), DL-dithiothreitol (DTT), leupeptin,



Scheme 1. Synthesis of indazole **4**.



47, R^1 = F, R^2 = H
48, R^1 = H, R^2 = F

49, R^1 = F, R^2 = H
50, R^1 = H, R^2 = F

20, R^1 = F, R^2 = H
21, R^1 = H, R^2 = F

24 (from **20**)

Scheme 3. Synthesis of indazoles **20**, **21** and **24**.

aprotinin, pepstatin, phenylmethylsulfonyl-fluoride (PMSF), hypoxantine-9-β-D-ribofuranosid (inosine), ethylene glycol-bis-(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), bovine serum albumin (BSA), Dowex-50W (50 × 8–200), FAD, NADPH and 5,6,7,8-tetrahydro-L-biopterin dihydrocloride (H_4-biopterin) were obtained from Sigma–Aldrich Química (Spain). L-[^{3}H]-arginine (58 Ci/mmol) was obtained from Amersham (Amersham Biosciences, Spain). Tris(hydroxymethyl)-aminometane (Tris–HCl) and calcium chloride were obtained from Merck (Spain).

For iNOS activity determination, male Wistar rats (3-months old, 220–250 g) were breed and kept in the University 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 [36]. Briefly, three days before the experiment the jugular vein was cannulated under i.p. equithesin anesthesia (1 mL/kg) for LPS administration. Rats were i.v. injected with 10 mg/kg b.w. 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 [37]. The crude homogenate was 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 [38] or used immediately for NOS activity measurement.

For nNOS activity determination, untreated male Wistar rats (200–250 g) were killed by cervical dislocation and striata were quickly collected and immediately used to measure nNOS 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 homogenates were centrifuged 5 min at 1000*g*, and an aliquot of the supernatant was frozen at −80 °C for total protein determination [38].

NOS activity was measured by the method of Bredt et al. [39], monitoring the conversion of L-^{3}H-arginine to L-^{3}H-citrulline. Enzyme activity was referred as pmol L-^{3}H-citrulline/min/mg prot. 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-^{3}H-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 iNOS was measured in the presence or absence of 10 mM EDTA. EDTA removes calcium from the medium preventing the activation of nNOS; thus, in the presence of EDTA only iNOS activity is detected. The reaction was stopped adding 400 μL of cold 0.1 M HEPES containing 10 mM EGTA, 175 mM L-^{3}H-citrulline, pH 5.5. The mixture was decanted onto a 2 mL column packed with Dowex-50W ion exchanger resin (Na^+ form) and eluted with 1.2 mL of water. L-^{3}H-citrulline was quantified by liquid scintillation counting in a Beckman LS-6000 system (Beckman Coulter, Fullerton, CA, USA). The retention of L-^{3}H-arginine in this process was greater then 98%.

A total of 25 compounds were assayed for nNOS/iNOS activities: 4,5,6,7-tetrafluoro-1*H*-indazole (**4**); 3-methyl-1*H*-indazole (**5**); 3-methyl-4,6-difluoro-1*H*-indazole (**6**); 3-methyl-6,7-difluoro-1*H*-indazole (**7**); 3-methyl-4,6-difluoro-7-nitro-1*H*-indazole (**9**); 3-methyl-6,7-difluoro-5-nitro-1*H*-indazole (**10**); 3-trifluoromethyl-1*H*-indazole (**11**); 3,7-dinitro-1*H*-indazole (**18**); 3-hydroxy-1*H*-indazole (**19**); 3-hydroxy-4,6-difluoro-1*H*-indazole (**20**); 3-hydroxy-6,7-difluoro-1*H*-indazole (**21**); 3-hydroxy-7-nitro-1*H*-indazole (**23**); 3-hydroxy-4,6-difluoro-7-nitro-1*H*-indazole (**24**); 3-hydroxy-5,6,7-trifluoro-1*H*-indazole-4-carboxylic acid (**25**); 3-hydroxy-5,6,7-trifluoro-1*H*-indazole-4-carboxylic acid methyl ester (**26**); 3-hydroxy-5,6,7-trifluoro-1*H*-indazole-4-carboxylic acid ethyl ester (**27**); 3-hydroxy-4,6,7-trifluoro-1*H*-indazole-5-carboxylic acid (**28**); 3-hydroxy-4,6,7-trifluoro-1*H*-indazole-5-carboxylic acid methyl ester (**29**); 3-hydroxy-4,6,7-trifluoro-1*H*-indazole-5-carboxylic acid ethyl ester (**30**); 3-hydroxy-4,5,7-trifluoro-1*H*-indazole-6-carboxylic acid (**31**); 3-hydroxy-4,5,7-trifluoro-1*H*-indazole-6-carboxylic acid methyl ester (**32**); 3-hydroxy-4,5,7-trifluoro-1*H*-indazole-6-carboxylic acid ethyl ester (**33**); 3-hydroxy-4,5,6-trifluoro-1*H*-indazole-7-carboxylic acid (**34**); 3-hydroxy-4,5,6-trifluoro-1*H*-indazole-7-carboxylic acid methyl ester (**35**); 3-hydroxy-4,5,6-trifluoro-1*H*-indazole-7-carboxylic acid ethyl ester (**36**). Except when indicated, the concentration of these compounds used in the NOS assays was 1 mM.

4.2. Chemistry

Melting points for compounds **4**, **20**, **21** and **24** were determined by DSC on a Seiko DSC 220C connected to a Model SSC5200H Disk

Table 3
PA (basicity), ΔH (acidity), E_i stacking energies, E_i apical energies, E_{rel} (E_i stacking − E_i apical). M05-2x/6-311+G(d) calculations including BSSE. All values in kJ mol^{-1}.

Ligand	PA	ΔH	Stacking	Apical	E_{rel}
Pyridine	927.8	–	–	–	–
Imidazole	945.5	1489.0	–	–	–
Pyrazole	891.4	1513.0	–	–	–
1	860.2	1446.8	−41.1	−60.0	18.9
4	839.2	1409.0	−63.6	−73.1	9.5
5	918.3	1482.2	−82.7	−56.7	−26.0
8	862.7	1416.2	−45.4	−66.6	21.2
9	858.8	1424.5	−75.3	−74.4	−0.9
10	844.4	1382.5	−78.8	−78.4	−0.4
12	803.9	1358.5	−45.0	−49.7	4.7
15	843.3	1408.1	−44.9	−55.7	10.8
18	793.7	1358.4	−54.4	−43.5	−10.9
19	901.4	1477.0	−92.7	−55.1	−37.6
21	872.0	1444.8	−61.4	−91.2	29.8
24	849.4	1417.0	−88.4	−75.1	−13.3
37	898.7	1477.9	−36.7	−68.5	31.8
38	911.2	1487.8	−62.8	−76.2	13.4
39	859.2	1428.4	−57.5	−72.8	15.3

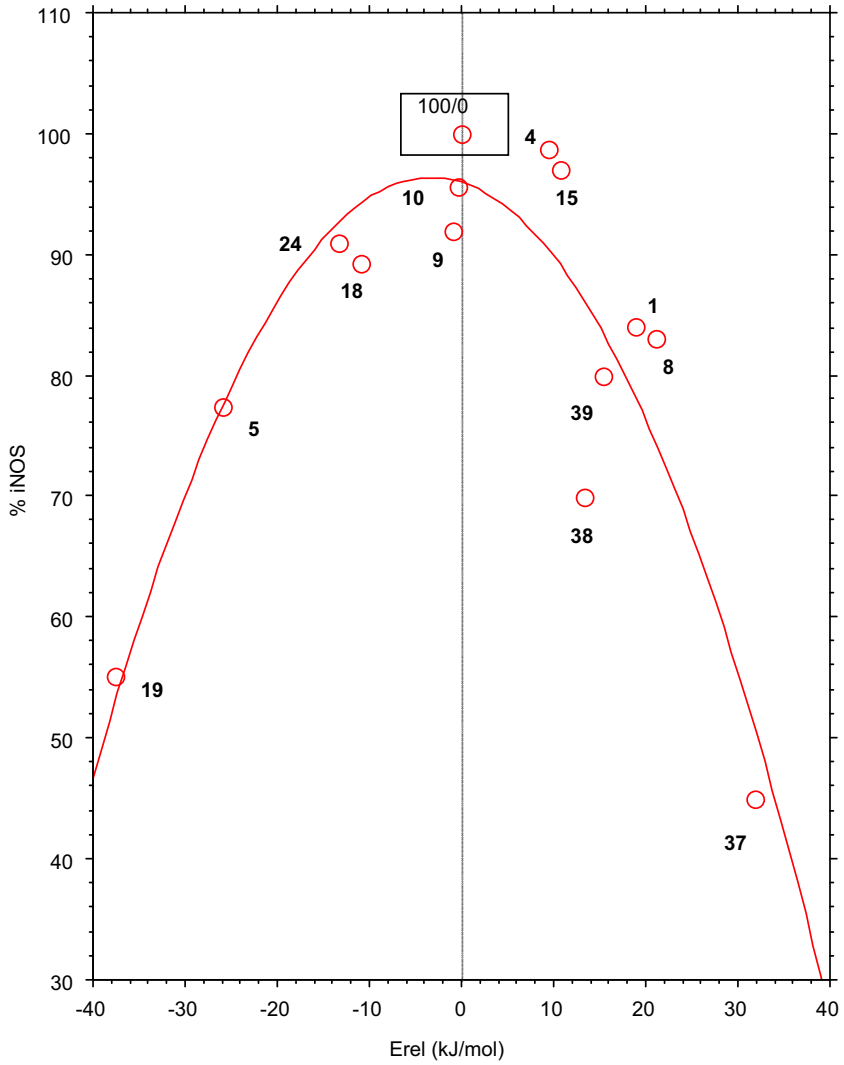


Fig. 5. Variation of the percentage of iNOS inhibition with E_{rel}.

Station while for compounds **18**, **41**, **42** and **45** a ThermoGalen hot stage microscope was used. Thermograms (sample size 0.003–0.0010 g) were recorded at a scanning rate of 2.0–5.0 °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 on a Perkin–Elmer 240 analyzer. Exact mass was determined on a Q-TOF LC/MS 6520 (Agilent Technologies) equipment using ESI+.

Solution spectra were recorded, at 300 K save specified, on a Bruker DRX 400 (9.4 Tesla, 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), THF-d_8 (3.58) and CD_3CN (1.93) for ^{1}H and $CDCl_3$ (77.0), DMSO-d_6 (39.5), THF-d_8 (67.4) and CD_3CN (1.4 and 118.7) for ^{13}C. And 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 [40]. 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.

4.2.1. 4,5,6,7-Tetrafluoro-1H-indazole (**4**)

In a three-neck round-bottom flask equipped with a reflux condenser, 98% hydrazine monohydrate (0.70 g, 14 mmol) was dissolved in THF (200 mL). The flask was placed in a bath of ice/water and 2,3,4,5,6-pentafluorobenzaldehyde (**40**) (0.55 g, 2.8 mmol) dissolved in THF (150 mL) was added dropwise. The mixture was then stirred at 70 °C for 48 h. The solution was decanted and solvent removed under reduced pressure to obtain a yellowish solid which was suspended in hexane. The solid was filtered, washed with chloroform and discarded (0.29 g). In the mother liquor the product together with 2,3,5,6-tetrafluoro-4-(hydrazonomethyl)phenylhydrazine (**45**) was present. The separation of these two components was carried out by column chromatography on silica gel with hexane/diethyl ether 20:1 to afford **4** (70 mg, 13%) as a white solid. Mp 191.5 °C (DSC). Anal. Calcd

for $C_7H_2F_4N_2$: C, 44.23; H, 1.06; N, 14,74. Found: C, 44.34; H, 1.17; N, 14.62. ^{1}H NMR ($CDCl_3$) δ 10.79 (br s, 1H, NH), 8.24 (d, $^5J_{F7} = 3.1$, 1H, H3); ^{19}F NMR ($CDCl_3$) δ −145.5 (dd, $^3J_{F5} = 18.9$, $^5J_{F7} = 18.9$, F4), −165.5 (ddd, $^3J_{F4} = 18.9$, $^3J_{F6} = 18.9$, $^4J_{F7} = 2.2$, F5), −156.9 (dd, $^3J_{F5} = 18.9$, $^3J_{F7} = 18.9$, F6), −159.0 (dd, $^3J_{F6} = 18.9$, $^5J_{F4} = 18.9$, F7); ^{13}C NMR (THF-d_8) δ 132.2 (d, $^1J = 194.9$, C3), 111.4 (dd, $^2J = 20.8$, $^3J = 3.2$, C3a), 139.6 (dddd, $^1J = 250.1$, $^2J = 11.4$, $^3J = {}^4J = 3.8$, C4), 135.8 (ddd, $^1J = 242.7$, $^2J = {}^2J = 15.5$, C5), 139.8 (dddd, $^1J = 247.6$, $^2J = {}^2J = 15.1$, $^3J = 2.5$, C6), 132.7 (ddd, $^1J = 250.9$, $^2J = 14.7$, $^3J = 4.1$, C7), 126.3 (ddd, $^2J = 15.4$, $^3J = 6.6$, $^3J = 3.5$, C7a); ^{15}N NMR (THF-d_8, T = 207 K) δ −201.1 (N1), −58.6 (N2).

2,3,5,6-Tetrafluoro-4-(hydrazonomethyl)phenylhydrazine (**45**) was obtained as a yellow solid. Mp > 300 °C. Exact mass (ESI+) calc for $C_7H_6F_4N_4$ 222.0529, found 222.0527. ^{1}H NMR (DMSO-d_6) δ 7.62 (s, 1H, −CH═N−), 7.11 (s, 2H, H_2N−NH−C1), 6.94 (s, 1H, −NH−), 4.46 (br s, 2H, ═N−NH_2); ^{19}F NMR (DMSO-d_6) δ −147.9 (m, F3 and F5), −159.3 (m, F2 and F6); ^{13}C NMR (DMSO-d_6) δ 143.9 (ddd, $^1J = 246.6$, $^2J = 12.7$, $^3J = 3.4$, C3 and C5), 136.8 (ddd, $^1J = 239.2$, $^2J = 15.3$, $^3J = 3.8$, C2 and C6), 129.8 (dd, $^2J = {}^2J = 10.0$, C1), 126.1 (−CH═), 103.5 (dd, $^2J = {}^2J = 13.2$, C4).

Reaction of 2,3,4,5,6-pentafluorobenzaldehyde (**40**) with hydrazine monohydrate in toluene leads to 2-bis(perfluorobenzylidene) hydrazine (**42**), obtained after purification by column chromatography on silica gel with hexane/ethyl acetate 40:1 and crystallization in chloroform as yellow needles (62%). Mp 124–130 °C (lit. [22] 131 °C). ^{1}H NMR ($CDCl_3$) δ 8.76 (s, 1H, −CH═N−); ^{13}C NMR ($CDCl_3$) δ 108.9 (m, C1), 137.9 (dm, C3 and C5), 142.9 (dm, C2 and C6), 146.0 (dm, C4), 152.3 (d, −CH═N−); ^{19}F NMR ($CDCl_3$) δ −161.5 (m, F3, F3′, F5 and F5′), −148,9 (m, F4 and F4′), −139,0 (m, F2, F2′, F6 and F6′). If the reaction of 2,3,4,5,6-pentafluorobenzaldehyde (**40**) was carried out with hydrazine monohydrate in ethanol/water at low temperature and high-dilution, perfluorobenzylidenehydrazine (**41**) was obtained as a white solid (89%). Mp 68–69 °C (lit. [22] 68 °C). ^{1}H NMR ($CDCl_3$) δ 6.00 (s, 2H, NH_2), 7.70 (s, 1H, −CH═N−); ^{13}C NMR ($CDCl_3$) δ 110.4 (m, C1), 128.7 (d, −CH═N−), 137.8 (dm, C3 and C5), 140.5 (dm, C2 and C6), 144.5 (dm, C4); ^{19}F NMR ($CDCl_3$) δ −163.2 (m, F3 and F5), −155.7 (m, 1F, F4), −144.2 (m, F2 and F6).

4.2.2. 3,7-Dinitro-1H-indazole (**18**)

This compound was prepared according to Ref. [25] and obtained as a yellow solid. Mp 220–222 °C (lit. [25] 220 °C). ^{1}H NMR (CD_3CN) δ 12.83 (br s, 1H, NH), 7.65 (t, 1H, $^3J = 8.0$, H5), 8.48 (d, 1H, $^3J = 7.9$, H6), 8.62 (d, 1H, $^3J = 8.1$, H4); ^{13}C NMR (CD_3CN) δ 151.5 (m, C3), 120.2 (d, $^2J = 9.5$, C3a), 130.3 (dd, $^1J = 171.7$, $^3J = 8.6$, C4), 126.4 (d, $^1J = 168.7$, C5), 126.8 (ddd, $^1J = 168.5$, $^2J = 2.3$, $^3J = 8.6$, C6), 134.9 (m, C7), 135.4 (dd, $^3J = {}^3J = 6.7$, C7a); ^{15}N NMR (CD_3CN) δ −14.2 (NO_2), the other nitrogen atoms were not detected.

4.2.3. General procedure for esterifications

A solution of 2,4,6-trifluorobenzoic acid (**47**) or 2,3,4-trifluorobenzoic acid (**48**) (0.3 g, 1.7 mmol) in dry methanol (10 mL) was prepared in a three-necked, round-bottom flask fitted with a reflux condenser and a calcium chloride tube. The flask was placed in an ice/water bath, thionyl chloride (1.4 mL) was then added dropwise, and the mixture was heated at 60–65 °C for three days under argon. After removal of solvent, the resulting residue was purified by extraction with pentane in the case of **49** and using column chromatography on silica gel (hexane/diethyl ether 6:1) for **50**.

Methyl 2,4,6-trifluorobenzoate (***49***): Colorless oil, yield 85% (lit. [41] Bp 105–110 °C/15 mmHg). ^{1}H NMR ($CDCl_3$) δ 3.94 (s, 3H, OCH_3), 6.72 (dd, $^3J = {}^3J = 8.3$, 2H, H3 and H5).

Methyl 2,3,4-trifluorobenzoate (***50***): Colorless oil, yield 81%. ^{1}H NMR ($CDCl_3$) δ 3.95 (s, 3H, OCH_3), 7.03 (m, 1H, H6), 7.74 (m, 1H, H5).

4.2.4. 3-Hydroxy-4,6-difluoro-1H-indazole (**20**)

In a three-neck round-bottom flask equipped with a reflux condenser, methyl 2,4,6-trifluorobenzoate (**49**) (0.20 g, 1.1 mmol) was dissolved in tetrahydrofuran (20 mL); then, a solution of 98% hydrazine monohydrate (0.15 g, 3.0 mmol) in ethanol (10 mL) was added dropwise. The mixture was heated at 70 °C for 24 h. After cooling at room temperature, the solution was decanted and the organic solvent evaporated under vacuum. The solid residue was washed with dichloromethane and dried to afford **20** (0.13 g, 76%) as a white solid. Mp 293.5 °C. Anal. Calcd for $C_7H_4F_2N_2O$: C, 49.42; H, 2.37; N, 16.47. Found: C, 49.44; H, 2.49; N, 16.27. ^{1}H NMR (DMSO-d_6) δ 11.8 (br s, 1H, NH), 10.9 (br s, 1H, OH), 6.71 (ddd, $^3J_{F4} = {}^3J_{F6} = 10.3$, $^4J_{H7} = 1.9$, 1H, H5), 7.09 (dd, $^3J_{F6} = 9.2$, $^4J_{H5} = 1.9$, 1H, H7); ^{19}F NMR (DMSO-d_6) δ −115.9 (dd, $^3J_{H5} = 9.6$, $^4J_{F6} = 8.4$, F4), −111.6 (br s, F6); ^{13}C NMR (DMSO-d_6) δ 153.6 (s, C3), 98.9 (d, $^2J = 20.2$, C3a), 155.7 (dd, $^1J = 253.3$, $^3J = 16.7$, C4), 94.4 (dd, $^2J = 30.5$, $^2J = 23.3$, C5), 161.8 (dd, $^1J = 242.7$, $^3J = 11.7$, C6), 92.0 (dd, $^2J = 26.1$, $^4J = 4.7$, C7), 143.6 (dd, $^3J = 15.1$, $^3J = 10.3$, C7a); ^{15}N NMR (THF-d_8, T = 207 K) δ −224.1 (N1, $^1J = 107.1$), −117.9 (N2).

4.2.5. 3-Hydroxy-6,7-difluoro-1H-indazole (**21**)

In a three-neck round-bottom flask equipped with a reflux condenser, methyl 2,3,4-trifluorobenzoate (**50**) (0.21 g, 1.1 mmol) was dissolved in toluene (10 mL), then, a solution of 98% hydrazine monohydrate (0.24 g, 4.7 mmol) in ethanol (10 mL) was added dropwise. The mixture was heated at 80 °C for 24 h. After cooling at room temperature 5 mL of toluene were added, the solution was decanted and the organic solvent evaporated under vacuum. The solid residue was washed with pentane and 0.16 g of a crude solid was obtained. After silica gel chromatography with (hexane/ethyl acetate 3:1), **21** was obtained (0.11 g, 60%) as a white solid. Mp 237.3 °C (DSC). Anal. Calcd for $C_7H_4F_2N_2O$: C, 49.42; H, 2.37; N, 16.47. Found: C, 49.43; H, 2.61; N, 16.01. ^{1}H NMR (DMSO-d_6) δ 12.1 (br s, 1H, NH), 11.0 (br s, 1H, OH), 7,43 (ddd, $^3J_{H5} = 8.8$, $^4J_{F6} = 4.4$, $^5J_{F7} = 0.7$, 1H, H4), 6.99 (ddd, $^3J_{F6} = 11.0$, $^3J_{H4} = 8.8$, $^4J_{F7} = 6.6$, 1H, H5); ^{19}F NMR (DMSO-d_6) δ −145.2 (ddd, $^3J_{F7} = 20.3$, $^3J_{H5} = 10.9$, $^4J_{H4} = 3.1$, F6), −158.7 (dd, $^3J_{F6} = 20.3$, $^4J_{H5} = 6.5$, F7); ^{13}C NMR (DMSO-d_6) δ 155.9 (s, C3), 112.7 (d, $^3J = 4.3$, C3a), 116.5 (dd, $^2J = 9.6$, $^3J = 4.5$, C4), 109.1 (d, $^2J = 21.0$, C5), 148.2 (dd, $^1J = 241.2$, $^3J = 9.0$, C6), 135.0 (dd, $^1J = 247.3$, $^3J = 16.3$, C7), 131.7 (dd, $^2J = 11.6$, $^3J = 3.6$, C7a); ^{15}N NMR (THF-d_8, T = 207 K) δ −234.4 (N1), −115.1 (N2).

4.2.6. 3-Hydroxy-4,6-difluoro-7-nitro-1H-indazole (**24**)

A round-bottomed flask containing 3-hydroxy-4,6-difluoro-1*H*-indazole (**20**) (0.06 g, 0.35 mmol) and potassium nitrate (0.04 g, 0.4 mmol) was placed in an ice–water bath. Then, concentrated sulphuric acid 95–98% conc. (0.8 mL, d = 1.84 g/mL) was added dropwise, and the mixture kept at 0–4 °C for 1 h. The mixture was stirred at room temperature for 24 h and poured into ice–water (5 mL). The solution was extracted with ethyl acetate (3 × 20 mL), dried with anhydrous sodium sulfate and solvent was removed under reduced pressure to afford a yellow solid. The product was purified by silica gel chromatography with (hexane/ethyl ether 4:1), to afford **24** (0.045 g, 60%) as a yellow solid. Mp 217.4 °C (DSC). Anal. Calcd for $C_8H_6F_2N_3O_2$: C, 39.08; H, 1.41; N, 19.53; Found: C, 39.36; H, 1.61; N, 19.00. ^{1}H NMR (DMSO-d_6) δ 12.8 (br s, 1H, NH), 11.7 (br s, 1H, OH), 7.13 (dd, $^3J_{F6} = 12.4$, $^3J_{F4} = 9.9$, 1H, H5); ^{19}F NMR (DMSO-d_6) δ −101.2 (dd, $^4J_{F6} = 17.7$, $^3J_{H5} = 9.8$, F4), −110.3 (br s, F6); ^{13}C NMR (DMSO-d_6) δ 154.6 (s, C3), 101.5 (d, $^2J = 20.1$, C3a), 159.2 (dd, $^1J = 265.4$, $^3J = 16.8$, C4), 96.4 (dd, $^2J = 28.7$, $^2J = 25.5$, C5), 158.4 (dd, $^1J = 265.9$, $^3J = 14.4$, C6), 118.6 (dd, $^2J = 8.8$, $^4J = 5.0$, C7), 136.2 (dd, $^3J = 11.3$, $^3J = 2.5$, C7a); ^{15}N NMR (THF-d_8, T = 207 K) δ −216.3 ($^1J = 112.3$, N1), −112.4 (N2), the NO_2 signal was not detected.

4.3. Statistical analysis

Data are expressed as 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.

4.4. Computational details

The geometry of the systems was initially optimized at the M05-2x/6-311+G(d) computational level [42,43]. This functional has shown to provide a good description for a large variety of molecular interaction complexes [44,45]. Frequency calculations at this computational level were performed to confirm that the structures obtained correspond to energetic minima. All these calculations were carried out within the Gaussian 03 package [46].

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