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4-Substituted indazoles as new inhibitors of neuronal nitric oxide synthase

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Abstract—A series of halo-1-*H*-indazoles has been synthesized and evaluated for its inhibitory activity on neuronal nitric oxide synthase. Introduction of bromine at the C4 position of the indazole ring system provided a compound almost as potent as the reference compound, that is, 7-nitroindazole (7-NI). The importance of position 4 is further demonstrated by the synthesis and pharmacological evaluation of the 4-nitroindazole which was also a potent inhibitor of NOS activity. These compounds also exhibited in vivo NOS inhibitory activity, as attested by potent antinociceptive effects following systemic administration.

The importance of nitric oxide (NO) as a biological messenger in numerous physiological processes has been demonstrated in a growing fashion over the last decades. This molecule is indeed involved in various fundamental functions such as neurotransmission,[1] blood pressure and blood flow regulation,[2] platelet aggregation and inflammation.[3] On the other hand, overproduction of NO plays a role in a variety of disorders, such as septic shock, pain,[4] ischaemia[5] and several neurodegenerative diseases.[6] NO is synthesized in several cell types from L-arginine by different isoforms of nitric oxide synthase (NOS). To date, three isoforms have been cloned: neuronal (nNOS) and endothelial (eNOS) types, which are both constitutive and calcium dependent, and an inducible, calcium independent one (iNOS).[7] Since these isoforms possess a distinct cellular localisation and are differentially regulated, they represent specific targets for potential therapeutical approaches. Development of selective inhibitors of one of these isoforms is therefore of considerable interest, both for a therapeutical purpose and for their use as specific pharmacological tools. For example, NO of neuronal origin is involved in pain transmission[8,9] and constitutes thus a potential target for antinociceptive drugs. Likewise, nNOS and/or iNOS[10] should be selectively targeted for neuroprotection against ischaemia, while the eNOS isoform, which has beneficial cerebrovascular effects, should be leaved unaffected.[11] Numerous nNOS inhibitors have therefore been developed over the last decade but only few present both potency and a clear selectivity towards this isoform.[5] The first developed inhibitors belong to the L-arginine analogues family[12] and are mostly not selective for the neuronal isoform. Another series of inhibitors is constituted by heterocycles such as substituted indazoles or imidazoles. The nitroindazole family (with 7-nitroindazole, 7-NI, as the lead compound) are potent nNOS inhibitors but their selectivity over the other isoforms remains low, at least in vitro.[8,13] We already partially performed the pharmacomodulation of the indazole nucleus, especially on the 7 position, in order to develop novel potent and specific inhibitors of nNOS and we showed that the nitro-substitution was not absolutely necessary for the biological activity of the indazole ring.[14] We rather demonstrated the inhibitory properties against NOS activity of the 7-methoxy-substituted indazole, which presents strong similarities with the mechanism of inhibition of 7-NI.[15] In order to improve our knowledge of the structure–activity relationships of the indazole derivatives, we aimed here to pharmacomodulate more extensively the indazole ring

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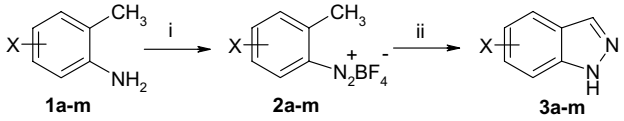
by exploring systematically all positions from 4 to 7. To this end, we synthesized and evaluated the NOS inhibitory properties of 4- to 7-halogeno-substituted indazoles. Due to its difficult chemical access, substitution at the position 4 had indeed been until now poorly investigated. Our present results reveal that substitution of the indazole nucleus on the latter position led to active compounds, we investigated this position in more detail with a reference substituent, that is, a nitro-group.

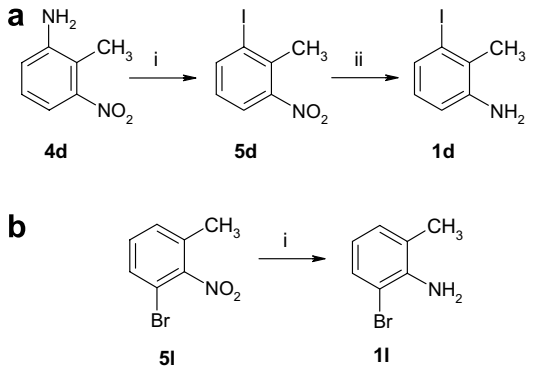
Most of the syntheses of the indazoles substituted on the six-membered ring reported in the literature proceed from benzene precursors in which the pyrazole moiety was generated by ring closure starting from isatines, phenylhydrazones or *o*-toluidines.[16–19] Amongst these reactions, we found that the most efficient proceeded by a phase transfer catalyzed reaction from *o*-methylbenzenediazonium tetrafluoroborates[20] as presented in Scheme 1. Indeed, in our laboratory, the successful method for the preparation of indazoles substituted by electrondonating or electronwithdrawing[14,21] groups prompted us to apply it to the synthesis of indazoles bearing halogen atoms in 4, 5, 6 or 7 position (Scheme 1).

Our first concern was to prepare commercially unavailable aniline precursors **1d** and **1l**. Following classical Sandmeyer procedure and reduction we were able to prepare according to Scheme 2 **1c** and **1k**, respectively, in 85% and 83% overall yields.

Indazoles **3a**–**m** were obtained by diazotization of 2-methylanilines **1a**–**m** with aqueous sodium nitrite solution in fluoroboric acid (50% solution in water) to give



Scheme 1. Synthesis of halo-1-*H*-indazoles **3a**–**m**. Reagents and conditions: (i) 1—HBF_4 aq 50%; 2—$NaNO_2$ aq, 0 °C; (ii) AcOK, 18-crown-6, $CHCl_3$, rt.



Scheme 2. Synthesis of **1d** and **1l**. Regeants and conditions: (a) (i) 1—$NaNO_2$, H_2SO_4 concd, 0 °C to rt, 2 h; 2—aq KI, rt, 12 h then $Na_2S_2O_3$ aq, 80 °C (91%); (ii) HCl concd, $SnCl_2$, 0 °C then 50 °C, 2 h (93%); (b) (i) HCl concd, $SnCl_2$, 0 °C then 50 °C, 2 h (83%).

the corresponding diazonium tetrafluoroborate salt **2a**–**m** in excellent yields. Cyclization of these salts, promoted by potassium acetate and 18-crown-6 in dry chloroform, gave indazoles **3a**–**m** in good yields (Scheme 1 and Table 1).[22]

6-Iodoindazole **3n** was obtained according to Scheme 3.[23] Unfortunately, the same pathway applied to 7-aminoindazole did not allow access to the corresponding 7-iodoindazole.

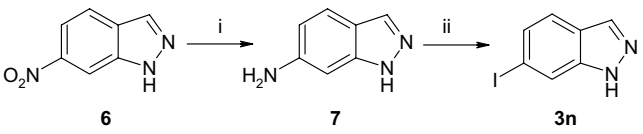
4-Nitroindazole **3o** was obtained starting from the corresponding *o*-toluidine according to Scheme 1 in 63% overall yield.

Effects of the derivatives on nNOS activity were evaluated in vitro on rat cerebellum homogenates. IC_{50} were determined from the NOS inhibition curves constructed with four concentrations (0.1, 1, 10 and 100 μM). Enzymatic activities were assayed by monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H] citrulline according to a previously described method[12] (1 mM $CaCl_2$, 200 μM β-NADPH, 0.88 μM L-arginine, 0.12 μM L-[^{3}H]arginine, 15 min at 37 °C). Basal activity represented 120 ± 15 pmol citrulline formed/mg protein/h and the already described lead compound, 7-NI (dissolved in DMSO 0.4%), exhibited an IC_{50} value of 0.6 ± 0.2 μM (mean ± SEM; $n = 5$ experiments).

As shown in Table 2, the 7-substituted tested indazoles displayed a consistent inhibitory effect against the NOS activity (albeit lower than the reference compound 7-nitroindazole, 7-NI), confirming the already known importance of this position on the indazole nucleus,[13,14] on condition that the substitution is not a bulky one.[24,25] Interestingly, our results revealed the importance of the

Table 1. Formation of halo-1-*H*-indazoles **3a**–**m**

X	Diazonium **2a**–**m** (%)		X	Indazole **3a**–**m** (%)	
3-Br	**2a**	99	4-Br	**3a**	58
3-Cl	**2b**	92	4-Cl	**3b**	57
3-F	**2c**	80	4-F	**3c**	69
3-I	**2d**	97	4-I	**3d**	49
4-Br	**2e**	98	5-Br	**3e**	80
4-Cl	**2f**	68	5-Cl	**3f**	51
4-F	**2g**	92	5-F	**3g**	70
4-I	**2h**	84	5-I	**3h**	74
5-Br	**2i**	99	6-Br	**3i**	41
5-Cl	**2j**	99	6-Cl	**3j**	98
5-F	**2k**	78	6-F	**3k**	50
6-Br	**2l**	92	7-Br	**3l**	91
6-Cl	**2m**	80	7-Cl	**3m**	64



Scheme 3. Synthesis of 6-iodoindazole **3n**. Reagents and conditions: (i) H_2 Pd/C, EtOH (92%); (ii) 1—$NaNO_2$, HCl concd, 0 °C to rt, 2 h; 2—aq KI, 50 °C then rt, 12 h, then NaOH, $NaHSO_3$ aq (28%).

Table 2. IC_{50} values of the 4- to 7-substituted halo-indazoles and 4-nitroindazole against rat cerebellar nNOS (NS, non-soluble)

Compound	IC_{50}, μM mean ± SEM	% inhibition at 100 μM	Vehicle (%)	Number of experiments
3a	2.4 ±0.5	100	DMSO 0.4	3
3e	72 ± 2	58	DMSO 0.4	3
3i	4.3 ± 1.5	100	DMSO 0.4	3
3l	14.3 ± 2.9	68	DMSO 0.4	3
3b	44.3 ± 3.2	57	DMSO 0.4	3
3f	59.3 ± 2.2	61	Ethanol 0.4	3
3j	22 ± 4.2	68	DMSO 0.4	3
3m	4.2 ± 0.5	100	DMSO 0.4	3
3d	17 ± 3.1	74	PEG 0.4	3
3h	37 ± 3.7	72	DMSO 0.4	3
3n	NS			
3c	>100	38	DMSO 0.4	3
3g	>100	35	DMSO 0.4	3
3k	>100	45	DMSO 0.4	3
3o	3.1 ± 1.1	100	DMSO 0.4	3

position 4 to obtain inhibitory properties against the NOS activity (Table 2). Indeed, whereas the 4-chloro- and 4-fluoroindazoles are almost ineffective to inhibit NOS activity ($IC_{50} > 50\mu M$), the 4-iodoindazole is a weak inhibitor and the 4-bromoindazole is the most active compound of this series, almost as potent as the reference compound, that is, 7-NI.

The importance of this position 4 is further demonstrated by the synthesis and pharmacological evaluation of 4-nitroindazole **3o** (Table 2). We found that this compound bearing an electronwithdrawing group in position 4 was also a potent inhibitor of NOS activity.

In order to characterise the in vivo pharmacology of the most potent 4-substituted indazoles, that is, the 4-nitro- and 4-bromoindazoles, as well as the 7-chloroindazole, these drugs were tested for their antinociceptive properties in a mouse model of nociception, the writhing test.[26] These compounds (12.5, 25, 50 mg/kg, dissolved in saline), as well as two reference drugs, 7-nitroindazole (50 mg/kg, dissolved in arachis oil) and acetylsalicylic acid (15 mg/kg, dissolved in saline), were administered ip, 15 min before an ip injection of 0.6% acetic acid aqueous solution. The number of writhes was then counted during 10 min, 10 min after acetic acid administration.

In these conditions, the three tested compounds produced a strong antinociceptive effect, statistically significant from the weakest dose and with, at the higher dose, a maximal effect close to that of acetylsalicylic acid (15 mg/kg) and 7-NI (50 mg/kg, ip) (Table 3).

The antinociceptive effect of the three tested indazoles (50 mg/kg, ip) was reversed by pretreatment with L-arginine (50 mg/kg, ip administered 5 min before corresponding indazoles) (Table 4), suggesting that the analgesic effect was effectively mediated by the inhibition of NOS activity.

We report here the synthesis and pharmacological evaluation of 4- to 7-halogeno-substituted indazoles as

Table 3. Antinociceptive activity of selected 4-substituted indazole and 7-chloroindazole new derivatives, compared to 7-nitroindazole and acetylsalicylic acid in the writhing test in mice ($n = 8$ per group)

Compound	Dose (mg/kg)	Number of writhes (mean ± SEM)
Saline (vehicle)		24.6 ± 1.1
3a	12.5	11 ± 2.1**
	25	8.8 ± 1.9***
	50	9.3 ± 2.4***
3o	12.5	18 ± 2.2**
	25	9.9 ± 2.1***
	50	9.3 ± 1.2***
3m	12.5	15 ± 2.2**
	25	12.9 ± 2.3**
	50	7.9 ± 12.3***
Acetylsalicylic acid	12.5	7.6 ± 1***
Arachis oil (vehicle)		22.1 ± 1
7-NI	50	6.8 ± 1.5***

** $p < 0.01$.
*** $p < 0.001$ versus control (saline for 4-bromo-, 4-nitro-, 7-chloroindazole and acetylsalicylic groups; arachis oil for 7-nitroindazole) (ANOVA and PLSD of Fischer).

Table 4. Effect of L-arginine pretreatment on antinociceptive activity of 4-bromo-, 4-nitro- and 7-chloroindazoles ($n = 8$ per group)

Compound	Dose (mg/kg)	Number of writhes (mean ± SEM)
Saline (vehicle)		23.9 ± 1.4
L-Arginine	50	22.9 ± 2.1
3a	50	10.8 ± 0.3***
L-Arginine + **3a**	50	24.3 ± 1.9###
3o	50	11.8 ± 0.3***
L-Arginine + **3o**	50	23.8 ± 1.4###
3m	50	12.1 ± 1.3***
L-Arginine + **3m**	50	23 ± 1.1###

*** $p < 0.001$ versus saline.
$p < 0.001$ versus corresponding 4- and 7-substituted indazoles (ANOVA and PLSD of Fischer).

inhibitors of neuronal NOS. Our results show that the position 4 seems as important as the position 7 to confer inhibitory properties against NOS activity. The importance of this position is further demonstrated by the potency of the 4-nitro-substituted indazole to inhibit NOS activity. The in vitro properties of these 4-substituted compounds are associated with an in vivo efficiency, as attested by potent antinociceptive effects following systemic administration. These results open novel avenues for a better understanding of the structure–activity relationships of the indazole derivatives as NOS inhibitors. We currently aim to compare the interactions of 4-bromoindazole, 4-nitroindazole and 7-NI with the enzyme NOS, by modelling studies and to investigate their selectivity against the three isoforms on adapted in vitro enzymatic assays.

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22. 4-Iodoindazole (**3d**): A cooled aqueous solution of sodium nitrite (1.22 g in 2.45 mL H_2O, 1.79 mmol) was added at 0 °C dropwise to a ice cooled solution of 3-iodo-2-methylaniline (4.17 g, 17.9 mmol) dissolved in fluoroboric acid (50% solution in water) (7.35 mL). After the end of the addition, the mixture was stirred for 1 h without cooling. The resulting precipitate was filtered and washed with Et_2O (3× 100 mL) to obtain 3-iodo-2-methylphenyldiazonium tetrafluoroborate salt **2d** as a beige solid (5.76 g, 97%): mp 178 °C; IR (KBr): 3122, 2286, 1580, 1552, 1448, 1055 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.75 (s, 3H), 7.52 (t, 1H, J = 8.3 Hz), 8.62–8.68 (m, 2H). The diazonium tetrafluoroborate salt **2d** (1 g, 3.03 mmol) was added under nitrogen in one portion to a stirred mixture of dried and powdered potassium acetate (0.61 g, 6.06 mmol) and 18-crown-6 (0.04 g, 0.05 mmol) in chloroform (40 mL). After 1 h, the resulting precipitate was filtered, washed with H_2O (3× 50 mL), dried over $CaCl_2$ and evaporated in vacuo. The crude gum was purified by column chromatography on silica gel (EtOAc/cyclohexane 1:3) to give **3d** as a brown solid (0.36 g, 49%) mp 208 °C. IR (KBr): 3114, 2853, 1615, 1347, 1181, 958 cm^{-1};^1H NMR ($CDCl_3$) δ 7.13 (t, 1H, J = 8.3 Hz), 7.48 (d, 1H, J = 7.6 Hz), 7.57 (d, 1H, J = 7.6 Hz), 7.98 (s, 1H), 10.19 (br s, 1H). HRMS/EI Calcd for $C_7H_5IN_2$ $[M]^+$ 243.9498; found: 243.9486.
23. Spectral data for **3n**: mp 204 °C. IR (KBr): 3193, 1658, 1610, 1426, 1341, 1074, 944 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.46 (d, 1H, J = 8.4 Hz), 7.52 (d, 1H, J = 8.4 Hz), 7.92 (s, 1H), 8.04 (s, 1H), 10.09 (br s, 1H). HRMS/EI Calcd for $C_7H_5IN_2$ $[M]^+$ 243.9498; found: 243.9488.
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