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Nitrocatechols versus Nitrocatecholamines as Novel Competitive Inhibitors of Neuronal Nitric Oxide Synthase: Lack of the Aminoethyl Side Chain Determines Loss of Tetrahydrobiopterin-Antagonizing Properties

Anna Palumbo,[a] Alessandra Napolitano[b] and Marco d'Ischia[b,*]

[a]*Laboratory of Biochemistry, Zoological Station "Anton Dohrn", Villa Comunale I-80121 Naples, Italy*
[b]*Department of Organic Chemistry and Biochemistry, University of Naples "Federico II", Via Cinthia 4, I-80126 Naples, Italy*

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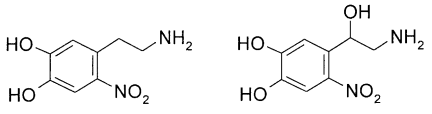
Abstract—6-Nitrocatecholamines were recently described as novel neuronal nitric oxide synthase inhibitors competing with both L-arginine and tetrahydrobiopterin (BH_4). We report now that simple nitrocatechols are also competitive inhibitors, lacking however BH_4-antagonizing properties. It is argued that 6-nitrocatecholamines interact with the L-arginine- and BH_4-binding sites through the nitrocatechol and aminoethyl moieties, respectively.

6-Nitrodopamine (**1**) and 6-nitronorepinephrine (**2**), novel catecholamine metabolites arising by nitric oxide (NO)-induced nitration under oxidative stress conditions,[1–4] have recently been shown to be effective inhibitors of nitric oxide synthase (NOS) with a significant selectivity for the neuronal isoform (nNOS or type I NOS).[5] Both **1** and **2** proved competitive with respect to the substrate L-arginine ($K_i = 45$ and 52 μM, respectively) and displayed antagonizing properties toward tetrahydrobiopterin (BH_4)-dependent enzyme activation and dimerization. This peculiar action profile, which is reminiscent of that of the antinociceptive drug 7-nitroindazole,[6] hinted at 6-nitrocatecholamines as novel lead structures for NOS inhibitors of potential investigative value to unravel intriguing mechanistic aspects relating to the role of BH_4.

In the various NOS isoforms, the BH_4-binding site is located proximally to the L-arginine-binding site near the heme. This arrangement entails a cross-talk between BH_4 and substrate binding which seems to be critical for the catalytic activity.[6] Therefore, an investigation of the mode of interaction of 6-nitrocatecholamines with the substrate- and BH_4-binding sites of nNOS was expected to yield new insights into the functional relationships between these sites and the possible implications for substrate conversion.

*Corresponding author. Fax: +39-081-674393; e-mail: dischia@unina.it

As an initial approach, we therefore investigated the effects of nitrocatechols on the enzyme activity with a view to dissecting the specific roles of the 4-nitrocatechol and 2-aminoethyl substructures of 6-nitrocatecholamines in nNOS inhibition. The results revealed an interesting, unprecedented example of nNOS inhibitors where modification or omission of a residue caused loss of BH_4 antagonizing properties without affecting the competitive pattern of inhibition.



1 **2**

Chemistry

Besides 4-nitrocatechol (**3**), other nitrocatechols were prepared to assess possible relationships between the position of the nitro group and the electronic properties of substituents, and the inhibitory effects. This goal was pursued through careful choice of reagents and experimental conditions, since nitration of catechols with conventional HNO_3-based reagents[7] normally faces problems relating to the susceptibility of the substrates to oxidation, polymerization and polynitration. 3-Nitrocatechol (**4**) was obtained in 20% yield by mild nitration of catechol with HNO_3 in ether,[8] the remainder

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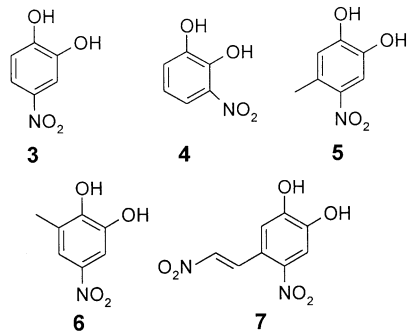
being 4-nitrocatechol (40%) and other uncharacterized material. The $NaNO_2/H_2SO_4$ system, on the other hand, proved the most convenient option for nitration of methylcatechols, yielding the corresponding nitrocatechols in satisfactory yields (60–90%)[9] and with high *para* regioselectivity with respect to the *o*-dihydroxy functionality.[10] Thus, 4-methylcatechol gave 80% of 4-methyl-5-nitrocatechol (**5**),[11] and 3-methylcatechol gave 60% of 3-methyl-5-nitrocatechol (**6**).[12] Notably, in the latter case only one regioisomer was obtained, due probably to steric effects.

Attempts to extend the $NaNO_2/H_2SO_4$ reaction or HNO_3-based procedures to catechols bearing electron-withdrawing groups proved unrewarding: 3,4-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid gave mixtures of nitration products in small amounts not amenable to chromatographic separation; 3,4-dihydroxycinnamic acid (caffeic acid) suffered extensive decomposition with only traces of ring nitration products; 3,4-dihydroxybenzaldehyde was recovered unchanged; (*E*)-3,4-dihydroxy-β-nitrostyrene gave exceedingly low yields of the desired 2-nitro-4,5-dihydroxy-β-nitrostyrene (**7**) with the $NaNO_2/H_2SO_4$ system or other nitrating agents, and the product was preferably obtained by a reported ad hoc procedure.[13] *O*-Methyl catechols, for example 1,2-dimethoxybenzene, proved virtually unreactive to the $NaNO_2/H_2SO_4$ system.

Overall, these results supported the validity of the $NaNO_2/H_2SO_4$ nitration as a practical access to 4-nitrocatechols. In close analogy to the case of phenols,[15] this reaction appears to involve one-electron oxidation of catechols. Regioselectivity, then, would be dictated by the relatively high spin density on the *para* position of semiquinone intermediates,[1] orienting radical coupling with NO_2 or related species produced by acidic decomposition of NO_2^-.[3,14,15] As alternate mechanism, only a HOMO-controlled reaction with electrophilic nitrating species could be considered, since ionic-type interactions would predictably proceed with *ortho* selectivity,[16] at variance with experimental data. An electrophilic substitution mechanism, however, seemed unlikely in view of (a) the high reduction potential of the $NO_2 + e^- \rightarrow NO_2^-$ semireaction ($E^\circ = 0.99$ V), making catechol oxidation the favored route, and (b) the lack of reactivity of 1,2-dimethoxybenzene, an electronically activated aromatic substrate to which, however, mild oxidative routes are precluded.[17]



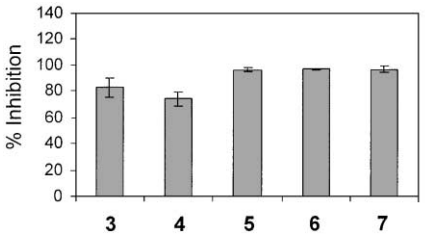
Inhibitory Properties of Nitrocatechols on nNOS

Figure 1 reports the effects of the various nitrocatechols on the activity of recombinant nNOS, as determined with radioactive L-arginine/L-citrulline conversion assay.[5]

The results indicated that all nitrocatechols were effective inhibitors of the enzyme with comparable potencies when used at 500 μM concentration. At the same concentration, the parent catechols affected nNOS activity to a lesser extent, not exceeding 60% of the inhibitory effect of the nitrated derivative (not shown). The IC_{50} values were also determined for comparison with the nitrocatecholamines **1** and **2**. On this basis, **3** was selected as a representative member of the series to assess the mechanism of nNOS inhibition in comparison with 6-nitrocatecholamines. Figure 2 shows the inhibitory effects of **3** on the various NOS isoforms, that is type II NOS (inducible NOS, iNOS) and type III NOS (endothelial NOS, eNOS).

Although the inhibition was more pronounced in the case of the neuronal isoform, a substantial effect was observed also on iNOS.

Kinetic data were consistent with a competitive pattern of inhibition of nNOS by **3** (Fig. 3), with $K_i = 28$ μM. Notably, unlike 6-nitrocatecholamines, **3** increased K_m for L-arginine without affecting V_{max} (Table 1), a behavior that is consistent with that of a competitive inhibitor. In the presence of **3**, L-citrulline formation was linear with time, ruling out any irreversible inhibition during the 15 min time course examined (not shown).



Cmpd	IC_{50} (μM)
1	71±1[5]
2	78±1[5]
3	33±1
4	94±5
5	35±2
6	28±2
7	55±2

Figure 1. Effect of nitrocatechols on the activity of recombinant nNOS. nNOS activity was measured as the production of L-[^{14}C]-citrulline from L-[^{14}C]arginine, using 1.57 μM L-arginine and nitrocatechols **3**–**7** at 500 μM concentration. For comparison IC_{50} values of nitrocatechols **3**–**7** are reported as determined under the same conditions described for nitrocatecholamines **1** and **2**.[5]

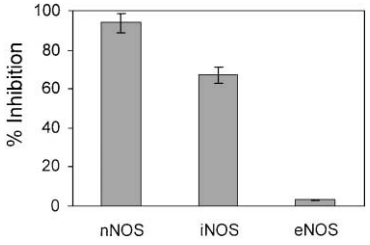


Figure 2. Inhibition of NOS isoforms by **3**. NOS activity of the different isoforms was measured as the production of L-[^{14}C]citrulline from L-[^{14}C]arginine, under the same experimental conditions and using comparable enzyme activities.[5] Compound **3** was tested at 500 μM. Each point represents the mean of three experiments±S.D.

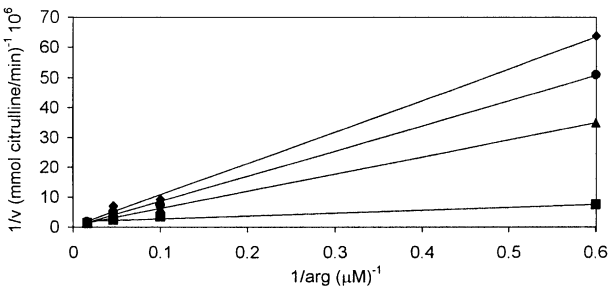


Figure 3. Double reciprocal plot of nNOS activity as a function of L-arginine concentrations. The concentration of L-[^{14}C]arginine was varied over the range shown (1.57–61.57 μM). Compound **3** was at concentration 0 μM (■), 100 μM (△), 250 μM (●) or 500 μM (◆). The plot shown is representative of three separate experiments, with S.D. not exceeding 5%.

Table 1. Effect of **3** on the kinetics of L-citrulline formation from L-arginine catalyzed by nNOS[a]

Inhibitor	V_{max} (μmol mg^{-1} min^{-1})	K_m (μM)
None	0.59±0.03	5.6±0.8
3[b]	0.57±0.04	16±1.8

[a]Kinetic experiments were performed with 1.57–61.57 μM L-arginine and 1 μM BH_4. Data were obtained from double reciprocal plots and are means±S.E. of five experiments.
[b]At concentration of 100 μM.

The possible interaction of **3** with the reductase and oxygenase domains of nNOS was systematically investigated by assessing the effects on relevant activities, including cytochrome c reduction, H_2O_2 production and BH_4-induced enzyme activation and dimerization. At 0.1 mM, **3** did not affect cytochrome c reduction in the absence of Ca^{2+}/calmodulin, although it induced about 50% inhibition of H_2O_2 production in the absence of L-arginine and BH_4. The inhibitory effect of **3** on L-citrulline formation did not depend on the various cofactors of the enzyme, including FAD, FMN and, notably, BH_4. In particular, nNOS inhibition by **3** was not reversed by up to 10 μM BH_4 which, by contrast, completely suppressed enzyme inhibition by **1** and **2**. Consistent with this observation, **3** did not affect BH_4-induced nNOS dimerization as monitored by low temperature SDS-PAGE. Similar results were obtained with **5** (data not shown).

Mechanistic Issues and Conclusions

Control experiments (UV, HPLC) showed that all nitrocatechols were fairly stable under the assay conditions for nNOS, whereas a slight oxidation (ca. 5%) was observed in the case of catechol and methylcatechols, suggesting for the latter compounds the intervention of spurious factors (e.g., quinone formation) in the inhibition mechanism. In fact, the present data definitely support the role of the nitro group as the structural component determining inhibition of nNOS by nitrocatechols. Since the inhibitory properties did not depend to a significant degree on the position of the nitro group on the aromatic ring, it could be argued that this group acts mainly by increasing the acidity of the *o*-diphenolic functionality. Determination of pK_a values for the nitrocatechols tested gave values around 6.4, implying ca. 90% ionization at pH 7.4. 4-Nitrocatechols display potent chelating properties toward Fe^{3+} which account, inter alia, for the established inhibitory effects of **3** on 5-lipoxygenase, an iron-containing enzyme.[18] On this basis, it may be tempting to speculate that nitrocatechols efficiently fit within the L-arginine binding site near the heme and occupy the open axial position of heme iron possibly acting as sixth ligand. This interaction would engage the ionized hydroxyl group *para* to the nitro group and would perturb the spin state of the metal center that is critical for catalytic activity. Unfortunately the strong absorption features of the nitrocatechol group prevented conclusive demonstration of this point by spectrophotometric techniques. However, the involvement of a nitrocatechol-heme interaction is supported by the inhibitory effect on hydrogen peroxide production in the absence of L-arginine and BH_4.

The most outstanding finding of this study was the lack of effect of 4-nitrocatechol on BH_4-dependent nNOS activation and dimerization. In view of the purely competitive pattern of nNOS inhibition, this finding would allow to conclude that the 4-nitrocatechol ring is the active moiety of 6-nitrocatecholamines interfering with L-arginine binding, whereas the aminoethyl side chain specifically affects BH_4-dependent enzyme activation. Given the reported affinity of the BH_4 binding site for positively charged groups,[19] and considering that the flexible aminoethyl group is largely protonated at physiologically relevant pH,[20] it can be argued that the folding of the catecholamine necessary to accommodate the nitrocatechol ring within the L-arginine binding site orients the side chain toward the adjacent BH_4 binding site to target a negatively charged residue. An attractive candidate in this respect is the heme propionate, a residue that seems to be critical for both L-arginine and BH_4 binding.[21] Brief inspection of geometry-optimized structure of **1** [22] indicated a distance between the oxygen atom at C-3 and the amino nitrogen of about 7 Å which seems compatible with the proposed interaction. This is schematically illustrated in Figure 4 where binding of the nitrocatechol moiety to the heme dictates proper positioning of the aminoethyl side chain in sufficiently close proximity to the carboxylate group. This mechanism would provide a convincing explanation for the

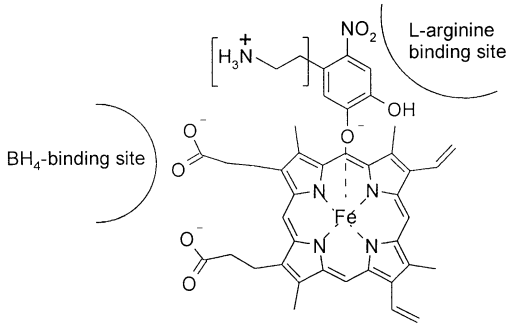


Figure 4. Schematic view of the interaction of nitrocatechols/nitrocatecolamines with the heme group at the nNOS L-arginine binding site.

small, yet significant non-competitive component in the inhibitory action of 6-nitrocatecholamines[5] which is not observable in the case of **3**. In fact, the proposed interaction of the amino side chain of **1** should increase binding to the enzyme with respect to **3** which does not seem to be supported by experimental data. Available evidence does not allow to assess this point, which merits further investigation. It can be speculated that in the case of nitrocatecholamines the gain of energy derived from interaction of the side chain with the negatively charged residue is partially vained by a less efficient binding to the heme iron center due to molecular distortion (Fig. 4).

In summary, the results of this study provide evidence that the aminoethyl side chain is the structural element of 6-nitrocatecholamines specifically determining BH_4-antagonizing properties. This finding offers new leads for the shaping of innovative, nitrocatechol-based pharmacophores of potential therapeutic interest. Further insights await the availability of a broader range of nitrocatechols, whose preparation was beyond the restricted scope of the present study and was deferred to a more detailed investigation of quantitative structure–activity relationships.

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8. Catechol nitration with HNO_3: To a solution of catechol (10 g) in ethyl ether (500 mL) in a ice bath fuming nitric acid (4 mL) is added, and the mixture is left for 24 h at rt. After extraction with water (3×100 mL), the organic layer is dried and the residue is triturated with boiling light petroleum to give, after recrystallization, 3-nitrocatechol (**4**) as yellow needles (2.7 g, 19% yield). Column purification of the light petroleum unsoluble fraction with chloroform/ethyl acetate 8:2 gave 4-nitrocatechol (**3**) in 40% yield. **4**: mp 87 °C; ^{1}H NMR (acetone-d_6) δ (ppm) 6.84 (1H, dd, J=8 Hz, 8 Hz), 7.12 (1H, dd, J=8 Hz, 1.6 Hz), 7.53 (1H, dd, J=8, 1.6 Hz); ^{13}C NMR δ (ppm) 116.96 (CH), 121.10 (CH), 123.42 (CH), 137.2 (C), 146.2 (C), 150.0 (C); EI-MS: m/z 155 (M^+). Anal. calcd for $C_6H_5NO_4$: C, 46.45; H, 3.22; N, 9.03. Found: C,46.40; H, 3.15; N, 9.12.
9. Catechol nitration with nitrite in acid media. General procedure. A solution of the appropriate catechol (1 mmol) in water (6 mL) is added with sodium nitrite (4 mmol) and 96% sulfuric acid (0.5 mL) in an ice-bath under stirring. After 30 min the reaction product is obtained either by filtration or by column chromatography purification (silica gel, eluant chloroform/ethyl acetate).
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12. ^{1}H NMR (acetone-d_6) δ (ppm) 2.29 (3H, s), 7.60 (1H, d, J=2.4 Hz), 7.64 (1H, d, J=2.4 Hz); ^{13}C NMR δ (ppm) 108.6 (CH), 118.5 (CH), 125.4 (CH), 140.3 (C), 145.1 (C), 151.33 (C). EI-MS: m/z 169 (M^+). Anal. calcd for $C_7H_7NO_4$: C, 58.00; H, 4.14; N, 8.28. Found: C, 58.08; H, 4.32; N, 8.20.
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