Published in final edited form as:
J Med Chem. 2012 November 26; 55(22): 9541–9548. doi:10.1021/jm300805x.

Nitric oxide synthases activation and inhibition by metallacarborane cluster-based isoform-specific affectors

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Abstract

A small library of boron cluster and metallacarborane cluster-based ligands was designed, prepared and tested for isoform-selective activation or inhibition of the three nitric oxide synthase isoforms. Based on the concept of creating a hydrophobic analog of a natural substrate, a stable and non-toxic basic boron cluster system, previously used for boron neutron capture therapy, was modified by the addition of positively charged moieties to its periphery, providing hydrophobic and non-classical hydrogen bonding interactions with the protein. Several of these compounds show efficacy for inhibition of NO synthesis with differential effects on the various nitric oxide synthase isoforms.

Keywords

Nitric oxide synthase; Isoform-specific affectors; Boron clusters; Metallacarboranes; Cobalt bis(dicarbollide)

Introduction

Nitric oxide (NO) is a biological messenger, produced by the nitric oxide synthases (NOSs), which is involved in many physiological processes serving a variety of regulatory and immunological functions. The mammalian NOS family consists of three isoforms, neuronal NOS (nNOS or NOSI), inducible NOS (iNOS or NOSII), and endothelial cell NOS (eNOS or NOSIII). All isoforms are homodimers that catalyze the NADPH-dependent oxidation of L-arginine to L-citruline and nitric oxide. The three NOS isoforms play unique, separate, and characteristic roles in various tissues and cell types; the NO produced has been implicated in such varied processes as hemodynamic control, neurotransmission, and the immune response (for review, see refs. [1–8]).

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Overproduction of NO by nNOS and iNOS has been implicated in ischemic injury following strokes, migraine headaches, autoimmune diseases such as rheumatoid arthritis and ulcerative colitis, Parkinson's and Alzheimer's diseases, liver cirrhosis, inflammation, and hypotension and vascular leakage during septic shock. Underproduction of NO by eNOS has been implicated in hypertension, atherosclerosis, and endothelial dysfunction. Clearly, the ability to selectively inhibit nNOS and iNOS, but not eNOS, has great potential in developing treatments for these diseases.

Regulation of NO production, with an emphasis on inhibition, is one of the leading themes in NOS biochemistry and structural biology. To date, many NOS inhibitors have been described, but from a structural point of view, only several tectons have been used for their construction, *i.e.* arginine and several other amino acid derivatives, amidines, pyridines, urea and thiourea derivatives, indazole derivatives and oligopeptides [9–20]. Applied structural motifs for the construction of NOS inhibitors have recently been reviewed [21].

From a physiological point of view, the final goal is to achieve isoform-specific inhibition; the challenge is to come up with a design for a protein-specific compound and the synthetic methodology to create it. Based on x-ray-structural analysis of individual NOS isoforms, it is evident that the design and the construction of isoform-specific inhibitors should rely on the molecular recognition of a region of the protein proximate to the binding site of its natural substrate. A search for other types of unconventional chemical structures that would fit into the NOS binding site, be biologically stable, and enable facile chemical modification identified several groups of inorganic compounds, icosahedral boranes, carboranes and metallacarboranes, as promising frameworks for a novel class of non-peptide protein inhibitors [22, 23]. These boron clusters are polyhedra based on a three-dimensional skeleton with triangular facets [22, 23]. Boron cluster derivatives (boranes, carboranes, metallacarboranes) are of interest as they have been rigorously studied because of their use in boron neutron capture therapy [24–29] and in radioimaging [28–31]. There are now a few examples in the literature of the use of carboranes as stable hydrophobic pharmacophores [22, 23, 32], namely enzyme inhibitors (HIV-1 protease [33–35], cyclooxygenase [36, 37], serine protease [38] or protein kinase C [28, 39,40]). Structurally, the variety of the known types of boranes, heteraboranes and metallacarboranes provide an interesting alternative to organic compounds, particularly aromatics. The icosahedral cage is slightly larger than the space occupied by a rotating phenyl ring; metallacarboranes consisting of two eleven vertex dicarbollide sub-clusters sandwiching the central metal atom [41] occupy approximately the same volume as a rotating anthracene ring. Among metallacarboranes, the cobalt bis(1,2-dicarbollide) ion is unique due to its synthetic availability, wide possibilities of *exo*-skeletal modifications, high stability, charge delocalization, low nucleophilicity, strong acidity of conjugated acids, high hydrophobicity, unique solution properties, and ion-pairing behavior [41–52].

The great potential seen with boron clusters as pharmacophores promoted our venture to this area in search of NOS inhibitors. Many new molecular systems based on two or more hydrophobic anions covalently bound together by simple or more sophisticated organic bridging moieties have been prepared and tested over the last several years.

Here we report a novel approach towards the development of isoform-specific inhibitors of NOS based on the application of properly substituted boron clusters. This non-natural, resistant-to-catabolism, non-toxic class of compounds represents a very interesting scaffold for the construction of a novel class of NOS inhibitors.

The structures of the designed inhibitors are shown in Fig. 1. This design allows variation of size, shape and proper peripheral structural modification for the construction of novel

compounds for specific NOS interaction. The intention was to attach known NOS binding motifs, like guanidine, urea, thiourea, aminoguanidine, imidazole or substituted amine binding groups to the boron cluster periphery, allowing for creation of variety of compounds with the potential to inhibit NOS. The compounds generated were variations of the boron cluster type, metallacarborane clusters, with a positive charge on the periphery (protonated amino group, quaternary ammonium, guanidinium, aminoguanidinium, thiouronium salts) with various lengths of spacer between the positively charged peripheral functionality (primary interaction site) and the 3D boron cluster.

Synthetic results will be discussed along with spectroscopic results, solubility and other physicochemical properties of the new compounds. The results obtained indicate high efficacy of several lead structure types as inhibitors of NOS, with IC_{50} values from *in vitro* experiments lying in the 1–5 μM range.

Results and Discussion

Compounds **15**–**30** were prepared by nucleophilic dioxane ring opening reaction of 8-dioxane-3-cobalt bis(dicarbollide) (**14)** with various nucleophiles. This general procedure has already become a routine method for attachment of the cobalt bis(dicarbollide) moiety to substance via a diethylene glycol spacer [33–35, 43,48–52]. If the nucleophile is anion, the reaction produced anionic derivative (compounds **15** and **18**); in the case of uncharged or thiourea-type nucleophiles betain-type zwitterion are formed (compounds **16**, **17**, **19**–**30**). Synthesis of compounds **15**–**30** are shown on Scheme 1.

Recent quantum chemical studies showed that boron clusters exhibit specific and well-characterized non-classical dihydrogen bonding interactions with peptide backbones and functionalities [32, 53]. Metallacarborane derivatives usually display very low aqueous solubility as well as spontaneous self-assembling that are caused by their hydrophobicity. Self-assembling of metallacarboranes in aqueous solution is time-dependent. Fortunately, self-assembly can be easily suppressed by use of suitable biocompatible excipients or by dilution of their DMSO solution, which can also serve as solubility enhancing agents [51,52]. For this reason, solutions of metallacarboranes were used immediately after preparation. We also used their mixtures with heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DIMEB), which are stable in time, and boron cluster do not aggregate and/or precipitate [52]. In biological system, serum albumin can serve as an excipient [52].

The high degree of similarity between the arginine-binding sites of the NOS isoforms [54–58] makes design of highly selective inhibitors targeted to this region difficult. Our approach is based on the concept of affinity variations in the NOS isoforms outside of the binding cavity, where x-ray structure analysis reveals the most differences, rather than within the substrate-binding site. To this end, a library of compounds was generated, where the same basic binding motif targeted to the arginine binding site is combined with a variety of boron cluster structural motifs based on borane, carborane and metallacarborane compounds that would presumably interact with regions in close proximity to, but outside of, the substrate-binding site, where real differences between the NOS isoforms may exist. The same or modified binding groups as in the natural substrate were used, but arginine was avoided as the core structure in most of the compounds to prevent unwanted interactions with other arginine-utilizing enzymes. Arginine transport or metabolizing proteins, like dimethylarginine dimethylaminohydrolase (DDAH), arg-gly amidiniotransferase, arginase, argininosuccinate synthase and peptide deiminases, might also recognize inhibitors with close structural similarity to arginine.

NO synthesis assays were performed under direct, competitive or non-competitive conditions without benefit of pre-incubation. This experimental protocol was designed to detect interactions with the heme- and/or flavin-binding domains. The parent compounds **10** and **11** (structures shown on Fig. 1) displayed no inhibition of NOS activity; when conjugated with a guanidine group (compounds **12** and **13**) as a substrate analog, however, these compounds (at 10 RM) completely inhibited all NOS activity, albeit in a non-specific manner. Thus, preliminary exploration of these structures has produced compounds that show potential for being modified even further to produce potent, highly specific inhibitors of the NOS isoforms. The IC_{50}s of several of these compounds containing such further modifications obtained with nNOS, eNOS and iNOS using the hemoglobin capture assay to measure NO production are listed in Table 1 (for compounds **20**, **22**, **23** and **26**). Other compounds (**1**–**11**, **15**–**19**, **21**, **24**, **25** and **27**–**30**) display no significant inhibition of NOS activity.

These data clearly show the differential effects of compounds **20** and **23** on the catalysis of NO formation by the three NOS isoforms. As can be seen by comparison with the unsubstituted boron-cage compounds, these substituted structures hold promise for inhibition of NOS activity as appropriate linkers and functional groups (in these cases, thiourea and aminoguanidine groups, respectively) are added to the basic cages.

To confirm that binding of these metallacarborane compounds was actually occurring with binding affinities approximating the catalytic IC_{50} values, fluorescence binding experiments were performed. The representative data shown in Fig. 2 illustrate that binding of **22** by iNOS is biphasic and confirm that it is in the same concentration range as that obtained in the IC_{50} determinations, 3.66 and 1.8 μM, respectively (data fit to Equation 1 in Experimental Methods).

These studies are preliminary and these “proof-of-principle” experiments show promise for leading to efficacious differential inhibitors. We have demonstrated that boron clusters represent convenient building blocks that can create important interactions with hydrophobic patches of the NOS binding site. *Exo*-skeletal substitution of the parent metallacarborane compounds introduces additional non-covalent interactions leading to dramatic improvement in inhibition efficacy and selectivity. The combination of the hydrophobic interactions of the scaffold with substitutions allowing for specific dihydrogen bonding and coulombic interactions might further increase the potency of this novel class of non-peptide NOS inhibitors based on an inorganic framework. As these compounds are very stable, exhibit low toxicity, and enable the introduction of modified side groups, they are extremely attractive prospects for development into much more specific inhibitors of the NOS isoforms.

Conclusions

We have identified metallacarboranes (cobalt bis(dicarbollide) derivatives) as a novel structural motif for the construction of isoform-specific NOS activators and/or inhibitors. The potential of these compounds relies on their stability, easy exo-skeletal substitution and spectroscopic properties. The boron cluster compounds thus represent a novel class of NOS activators and/or inhibitors depending on peripheral substitution of a boron cluster compound, which differ substantially from the organic ones in their constructions and structures.

Experimental Methods

Chemicals

Chemical used are as follows: (6R)-5,6,7,8-tetrahydrobiopterin was from Research Biochemicals International (Natick, MA). Sodium chloride was from EMD Chemicals, Inc. (Darmstadt, Germany). 8-dioxane-3-cobalt bis(1,2-dicarbollide) (14) were purchased from Katchem Ltd, Czech Rep. All other chemicals and solvents were purchased from Sigma-Aldrich and were of the highest grade available.

Characterization of compounds

^{1}H and ^{11}B NMR spectroscopy were performed at Varian Gemini 500 HC instrument using DMSO-d6 as solvent and tetramethyl silane as internal standard for ^{1}H NMR and BF_3*Et_2O as external standard for 11B NMR, respectively. Chemical shifts are quoted in parts per million (δ scale; s = singlet, d = doublet, m = multiplet), coupling constants J in Hz. Mass spectrometry measurements were performed on a Bruker Esquire 3000 instrument using Electrospray Ionization (MS-ESI). Negative ions were detected. The purity of the prepared compounds was determined by elemental analysis and liquid chromatography (coupled with MS-ESI). All compounds have purity ≥97%.

Enzymes

The neuronal, endothelial, and inducible isoforms of NOS were expressed and purified as previously described [59–62]. Calmodulin was prepared by the method of Zhang and Vogel [63].

Inhibition assays

The generation of nitric oxide was measured at 23°C by the Hb capture method [64] on a Molecular Dynamics VERSAmax microplate reader in flat bottom 96 well plates with a path length of 0.72 cm. Inhibitors (10 RM) plus 100 to 200 nM nNOS, iNOS, or eNOS were assayed in 8 RM oxy Hb, 25 RM L-arginine, 50 RM Ca^{++}, 1 RM CaM, 5 RM BH_4, and 100 RM NADPH, in 50 mM TRIS/HCl, 100 mM NaCl, pH 7.5, in a total volume of 0.3 ml. A mixture of oxy Hb, L-arginine, Ca^{++}, and CaM was added to each well with a multichannel pipette, while inhibitor, NOS, and BH_4 were added individually from a single container in an effort to prevent degradation. The reaction was started with the addition of 100 μM NADPH, and the change in aborbance at 401 nm was measured every 3 seconds for 1 minute. The extinction coefficient used to calculate the rates was 60 mM^{-1}. Only compounds that showed a differential inhibition of NOS isoforms were chosen for determination of IC_{50} values

Fluorescence study

The fluorescence spectra of iNOS were recorded at 25°C using a Fluorolog-3 (Jobin Yvon-Spex, Edison, NJ) spectrofluorometer. Excitation was at 280 nm (path length = 0.5 mm; slit = 2 nm band pass) and emission was recorded in the range 250–450 nm (path length 0.2 mm; 2 nm band pass). Buffer (50 mM TrisHCl, 100 mM NaCl, pH 7.5 at 25 °C) was filtered using 0.2 micron Corning filters and degassed. Intensities at the emission maximum of 330nm did not show any observable shifts as the concentration of the compound was increased by titrating in Rl volumes of concentrated stock solution into the protein solution. The final volume of the added solution was very small and did not cause any significant changes in fluorescence intensities.

After trying a variety of models for binding constants [65], the data fitted best to a two-part equation (Eqn. 2), with the first term similar to a Hill type equation for binding constants for specific binding and the second part consisting of a straight line.

$$F_{obs} = \frac{F_{min} * [S]^n}{K + [S]^n} + (Slope * [S] + Intercept) \quad \text{Equation 1}$$

Where, F_{obs} are the observed fluorescence intensities of iNOS in the presence of varying concentrations of the compound (0–50 RM range); F_{min} is the theoretical minimum of the observed fluorescence intensity; S is the compound concentration; n is the Hill Coefficient related to the number of binding sites; and K is the apparent binding constant.

Chemical syntheses

Derivatives **1**–**11**, **15**, **20**, **22** and **25** (structures shown in Fig. 1) were prepared as described in literature: **2** and **3** [45], **4** [66], **5** [67], **6** [68], **7** [69], **8** [46], **9** [70], **10** [71], **11** [72, 73], **15** [74], **20**, **22** and **25** [51]. Guanidinium derivatives **12** and **13** were prepared from corresponding aminoderivatives (**10** and **11** resp.) by standard guanylation reactions [75–77].

The synthetic protocol for the preparation of the library of boron cluster inhibitors is shown in Scheme 1, where 8-dioxane-3-cobalt bis(dicarbollide) (**14)** is opened by series of S- and N-nucleophiles. All compounds were verified by elementary analysis, mass spectrometry, ^{1}H NMR and ^{11}B NMR spectroscopy.

General method of preparation of compounds 15–30—To a stirred slurry of sodium hydride (60% susp. in oil) in anhydrous THF was added S-; O- or salt of N-nucleophile. Reaction mixture was stirred at r.t. for 30–60 min. Then was added a solution of 8-dioxane-3-cobalt bis(dicarbollide) (**14**) in toluene-THF mixture (3:1 v/v) (molar ratio nucleophile : **14** = 2:1). In the case of neat N-nucleophiles, solution of **14** was added directly to the solution of N-nucleophile. The reaction mixture was heated to 70 °C for 15–60 min (TLC monitoring; eluent: dichloromethane). After cooling down, water was added and aqueous phase was extracted with dichloromethane (5x 50 mL). The combined organic layers were washed with water (20 mL) and brine (20 mL) and dried ($MgSO_4$). After evaporation, the resulting glassy solid was dissolved in CH_2Cl_2 (5 ml) and precipitated by addition of hexane (30 ml) or purified by column chromatography on silica (eluent: dichloromethane followed by dichloromethane/acetone 3:1 to 1:1 v/v). Orange solid product was dried in vacuum at 50°C.

^{1}H and ^{11}B NMR spectra of compounds 15–30 are similar—^{1}H NMR (DMSO-d_6) δ: 0.90–4.15 (m; BH and OCH_2, NCH_2, $SCH_2^{(if\ present)}$, $CH_2^{(if\ present)}$ and NH); 4.14 (s; $CH_{carborane}$); compound **26** additional signal: 6.98 (s; 2H; imidazole-*H*), compound **18** additional signal: 2.32 (s; 6H; NMe_2), compound **19** additional signal: 3.31 (s; 9H; N^+Me_3), compound **27** additional signal: 7.11 (s; 2H; imidazole-*H*).

^{11}B NMR (DMSO-d6): δ = −28.3 (d; 1B; B6; $^1J_{(B,H)}$ = 140 Hz); −21.6 (d; 1B; B6'; $^1J_{(B,H)}$ = 171 Hz); −20.4 (d; 2B; B5, B11; $^1J_{(B,H)}$ = 153 Hz); −17.3 (d; 2B; B5', B11'; $^1J_{(B,H)}$ = 145 Hz); −7.8 – −8.2 (m; 6B; B4, B7, B9, B12, B9', B12'), −4.3 (d; 2B; B4', B7'; $^1J_{(B,H)}$ = 153 Hz); −2.5 (d; 1B; B10; $^1J_{(B,H}$ = 143 Hz); 0.4 (d; 1B; B10'; $^1J_{(B,H)}$ = 141 Hz); 3.9 (d; 1B; B8'; $^1J_{(B,H)}$ = 142 Hz); 23.3 (s; 1B; B8)

Compound 18: NaH (48 mg, 60% in oil, 1.2 mmol), 2-(dimethylamino)ethanethiol hydrochloride (85 mg, 0.6 mmol), THF (4 mL), then **14** (165 mg, 0.4 mmol), toluene-THF

(3:1 v/v, 6 mL). Yield of **18**: 204 mg (95%). Anal. Calcd for $C_{12}H_{39}B_{18}CoNNaO_2S$: C, 26.79; H, 7.31; N, 2.60. Found: C, 26.70; H, 7.40; N, 2.55. MS-ESI m/e: 538.37 (100.0%), 537.38 (100.0%), 539.37 (90.2%), 536.38 (63.3%), 540.36 (34.6%), 535.38 (33.6%), 540.37 (15.4%), 534.39 (15.0%), 538.38 (12.7%), 541.36 (11.4%), 541.37 (6.2%), 533.39 (4.9%), 536.39 (4.8%), 542.36 (2.7%), 535.39 (2.3%), 539.38 (2.2%), 532.39 (1.3%), 540.38 (1.3%), 542.37 (1.0%)

Compound 19: Acethydrazide trimethylammonium chloride (Girard's reagent T; 135 mg, 0.8 mmol), **14** (165 mg, 0.4 mmol), THF (12 mL). Yield of **19**: 201 mg (93%). Anal. Calcd for $C_{13}H_{42}B_{18}CoN_3O_3$: C, 28.81; H, 7.81; N, 7.75. Found: C, 28.75; H, 7.90; N, 7.73. MS-ESI m/e: 541.44 (100.0%), 542.43 (98.1%), 543.43 (74.4%), 540.44 (63.3%), 544.43 (46.1%), 539.45 (36.1%), 543.44 (15.8%), 538.45 (14.5%), 542.44 (13.4%), 545.42 (8.1%), 545.43 (5.7%), 540.45 (5.1%), 537.45 (4.9%), 544.44 (1.9%), 546.43 (1.7%), 536.46 (1.4%), 542.45 (1.1%)

Compound 20: NaH (16 mg, 60% in oil, 0.4 mmol), thiourea (31 mg, 0.4 mmol), THF (2 mL), then **14** (83 mg, 0.2 mmol), toluene-THF (3:1 v/v, 3 mL). Yield of **20**: 80 mg (82%). Anal. Calcd for $C_9H_{33}B_{18}CoN_2O_2S$: C, 22.20; H, 6.83; N, 5.75. Found: C, 22.14; H, 6.79; N, 5.77. MS-ESI m/e: 487.34 (100.0%), 486.34 (84.4%), 488.33 (67.2%), 485.35 (61.2%), 489.33 (36.5%), 484.35 (31.2%), 488.34 (13.8%), 490.33 (13.7%), 483.35 (13.1%), 489.34 (8.0%), 486.35 (6.1%), 482.36 (4.6%), 491.33 (2.7%), 484.36 (1.4%), 481.36 (1.2%), 490.34 (1.2%), 487.35 (1.1%)

Compound 21: NaH (16 mg, 60% in oil, 0.4 mmol), thiosemicarbazide (37 mg, 0.4 mmol), THF (2 mL), then **14** (83 mg, 0.2 mmol), toluene-THF (3:1 v/v, 3 mL). Yield of **21**: 93 mg (92%). Anal. Calcd for $C_9H_{34}B_{18}CoN_3O_2S$: C, 21.53; H, 6.83; N, 8.37. Found: C, 21.55; H, 6.80; N, 8.35. MS-ESI m/e: 502.35 (100.0%), 501.35 (89.6%), 503.35 (87.4%), 500.36 (66.4%), 504.34 (39.8%), 499.36 (33.8%), 505.34 (14.9%), 498.36 (14.2%), 502.36 (9.9%), 501.36 (8.6%), 504.35 (8.4%), 497.37 (5.0%), 506.34 (2.8%), 503.36 (1.6%), 499.37 (1.5%), 505.35 (1.4%), 496.37 (1.3%)

Compound 22: NaH (400 mg, 60% in oil, 10 mmol), guanidine hydrochloride (955 mg, 10 mmol), THF (10 mL), then **14** (410 mg, 1 mmol), toluene-THF (3:1 v/v, 3 mL). Yield of **22**: 452 mg (95%).

For guanidine carbonate (900 mg, 5 mmol), THF (20 mL), then 14 (821 mg, 2 mmol). Yield of 22: 549 mg (58%). Anal. Calcd for $C_9H_{34}B_{18}CoN_3O_2$: C, 23.00; H, 7.29; N, 8.94. Found: C, 22.96; H, 7.32; N, 9.00. MS-ESI m/e: 470.38 (100.0%), 469.38 (85.2%), 471.37 (69.5%), 468.38 (59.1%), 472.37 (33.2%), 467.39 (33.1%), 466.39 (13.5%), 473.37 (10.9%), 471.38 (9.7%), 472.38 (7.9%), 469.39 (6.3%), 465.39 (4.6%), 468.39 (3.2%), 464.40 (1.3%)

Compound 23: NaH (240 mg, 60% in oil, 6 mmol), aminoguanidine hemisulphate (738 mg, 6 mmol), THF (10 mL), then **14** (410 mg, 1 mmol), toluene-THF (3:1 v/v, 10 mL). Yield of **23**: 482 mg (98%). Anal. Calcd for $C_9H_{35}B_{18}CoN_4O_2$: C, 22.29; H, 7.27; N, 11.55. Found: C, 22.31; H, 7.30; N, 11.56. MS-ESI m/e: 485.39 (100.0%), 484.39 (85.2%), 486.38 (69.7%), 483.39 (58.7%), 487.38 (33.4%), 482.40 (31.8%), 481.40 (13.5%), 488.38 (11.0%), 486.39 (9.4%), 487.39 (7.9%), 484.40 (6.2%), 480.41 (4.7%), 483.40 (3.7%), 482.41 (1.4%), 479.41 (1.3%), 485.40 (1.0%)

Compound 24: NaH (16 mg, 60% in oil, 0.4 mmol), *N,N´*-diaminoguanidine hydrochloride (50 mg, 0.4 mmol), THF (2 mL), then **14** (83 mg, 0.2 mmol), toluene-THF (3:1 v/v, 4 mL). Yield of **24**: 94 mg (93%). Anal. Calcd for $C_9H_{36}B_{18}CoN_5O_2$: C, 21.62; H, 7.26; N, 14.01.

Found: C, 21.68; H, 7.33; N, 13.98. MS-ESI m/e: 500.40 (100.0%), 499.40 (92.8%), 501.39 (74.2%), 498.41 (67.7%), 502.39 (36.5%), 497.41 (34.6%), 496.41 (14.7%), 501.40 (12.0%), 503.39 (11.9%), 500.41 (10.0%), 502.40 (8.2%), 499.41 (6.7%), 495.42 (5.2%), 497.42 (1.5%), 494.42 (1.4%)

Compound 26: NaH (48 mg, 60% in oil, 1.2 mmol), 2-aminoimidazole hemisulphate (80 mg, 0.6 mmol), THF (4 mL), then **14** (165 mg, 0.4 mmol), toluene-THF (3:1 v/v, 6 mL). Yield of **26**: 172 mg (87%). Anal. Calcd for $C_{11}H_{34}B_{18}CoN_3O_2$: C, 26.75; H, 6.94; N, 8.51. Found: C, 26.68; H, 6.93; N, 8.45. MS-ESI m/e: 494.38 (100.0%), 493.38 (83.6%), 495.37 (68.2%), 492.38 (58.1%), 491.39 (32.8%), 496.37 (32.6%), 490.39 (13.3%), 495.38 (11.5%), 497.37 (11.4%), 496.38 (9.4%), 493.39 (7.5%), 489.39 (4.5%), 492.39 (3.9%), 488.40 (1.3%), 498.37 (1.1%)

Compound 27: NaH (48 mg, 60% in oil, 1.2 mmol), 2-(1*H*-imidazol-2-yl)-ethylamine hydrochloride (89 mg, 0.6 mmol), THF (4 mL), then **14** (165 mg, 0.4 mmol), toluene-THF (3:1 v/v, 8 mL). Yield of **27**: 177 mg (85%). Anal. Calcd for $C_{13}H_{38}B_{18}CoN_3O_2$: C, 29.91; H, 7.34; N, 8.05. Found: C, 29.83; H, 7.38; N, 7.43. MS-ESI m/e: 522.41 (100.0%), 521.41 (82.3%), 523.40 (66.1%), 520.42 (61.5%), 524.40 (32.0%), 519.42 (30.7%), 523.41 (13.9%), 518.42 (13.1%), 525.40 (11.9%), 524.41 (10.9%), 521.42 (8.5%), 517.43 (4.6%), 519.43 (2.0%), 522.42 (1.3%), 516.43 (1.2%), 526.40 (1.2%), 523.42 (1.2%), 525.41 (1.1%)

Compound 28: L-arginine (70 mg, 0.4 mmol) in THF (4 mL), then **14** (83 mg, 0.2 mmol), toluene-THF (3:1 v/v, 6 mL). Yield of **28**: 110 mg (94%). Anal. Calcd for $C_{15}H_{45}B_{18}CoN_4O_4$: C, 30.07; H, 7.57; N, 9.35. Found: C, 29.97; H, 7.64; N, 9.40. MS-ESI m/e: 585.44 (100.0%), 584.44 (92.8%), 586.44 (91.2%), 583.45 (69.7%), 587.43 (36.4%), 582.45 (34.6%), 585.45 (15.9%), 581.45 (14.7%), 587.44 (12.8%), 584.45 (10.2%), 588.43 (8.4%), 588.44 (7.2%), 580.46 (5.0%), 582.46 (2.4%), 586.45 (2.4%), 589.43 (1.4%), 579.46 (1.4%), 587.45 (1.3%)

Compound 29: D-arginine (52 mg, 0.3 mmol) in THF (3 mL), then **14** (109 mg, 0.26 mmol), toluene (4 mL). Yield of **29**: 133 mg (86%). Anal. Calcd for $C_{15}H_{45}B_{18}CoN_4O_4$: C, 30.07; H, 7.57; N, 9.35. Found: C, 29.96; H, 7.66; N, 9.36. MS-ESI m/e: 585.44 (100.0%), 584.44 (92.8%), 586.44 (91.2%), 583.45 (69.7%), 587.43 (36.4%), 582.45 (34.6%), 585.45 (15.9%), 581.45 (14.7%), 587.44 (12.8%), 584.45 (10.2%), 588.43 (8.4%), 588.44 (7.2%), 580.46 (5.0%), 582.46 (2.4%), 586.45 (2.4%), 589.43 (1.4%), 579.46 (1.4%), 587.45 (1.3%)

Compound 30: NaH (48 mg, 60% in oil, 1.2 mmol), acetamidine hydrochloride (57 mg, 0.6 mmol), THF (4 mL), then **14** (165 mg, 0.4 mmol), toluene-THF (3:1 v/v, 6 mL). Yield of **30**: 166 mg (88%). Anal. Calcd for $C_{10}H_{35}B_{18}CoN_2O_2$: C, 25.61; H, 7.52; N, 5.97. Found: C, 25.57; H, 7.59; N, 5.93. MS-ESI m/e: 468.39 (100.0%), 469.38 (99.7%), 470.38 (86.2%), 467.39 (64.4%), 471.37 (35.9%), 466.39 (34.7%), 465.40 (15.3%), 469.39 (10.7%), 471.38 (8.5%), 472.37 (8.2%), 464.40 (5.0%), 472.38 (4.8%), 467.40 (4.1%), 466.40 (1.7%), 470.39 (1.5%), 463.40 (1.4%), 471.39 (1.0%)

Acknowledgments

This work was supported by NIH Grant GM52419 (to LJR and BSSM), the Grant Agency of the Czech Republic (grant No. P303/11/1291), a MSMT grant (MSM0021620849), and grants from Charles University, Prague (PRVOUK P24/LF1/3 and UNCE 204011). BSSM holds the Robert A. Welch Distinguished Chair in Chemistry (AQ-0012).

Abbreviations: The abbreviations used are as follows

NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
NO	nitric oxide
CaM	calmodulin

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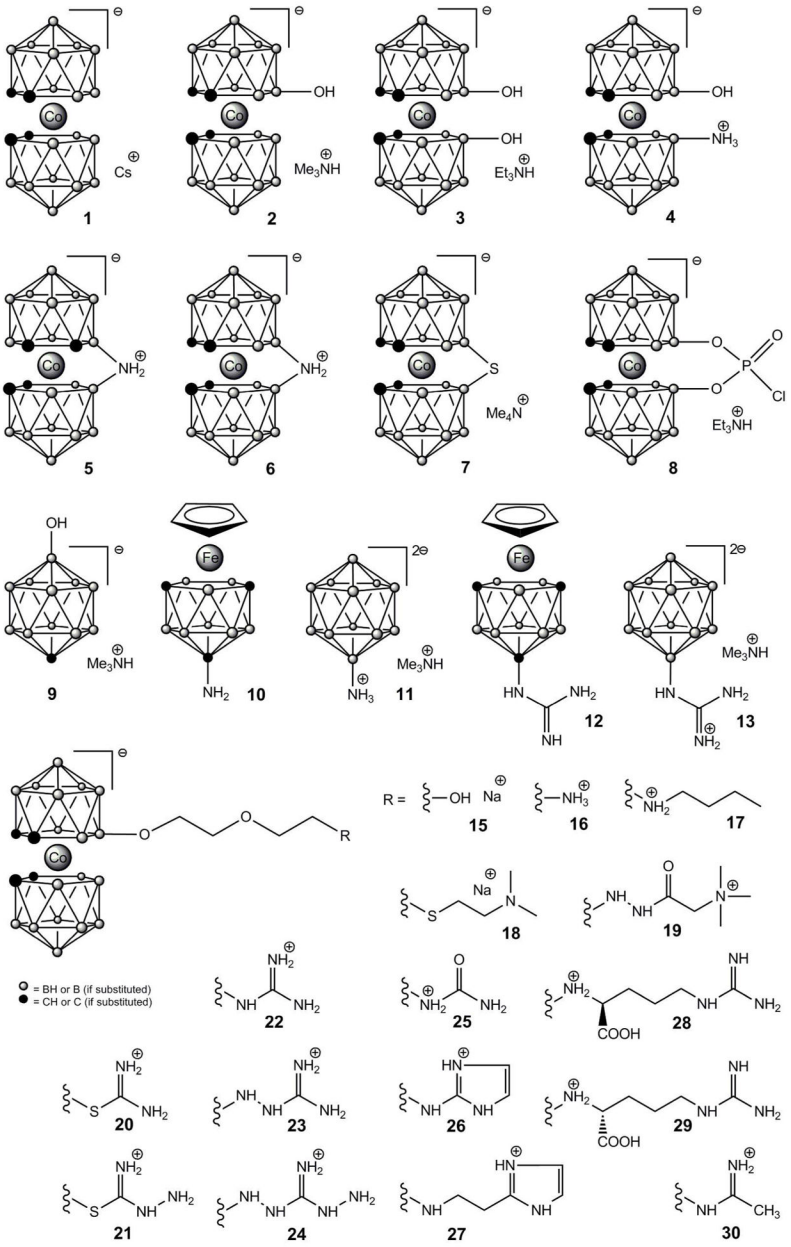


Figure 1.
Structures of tested boron cluster derivatives

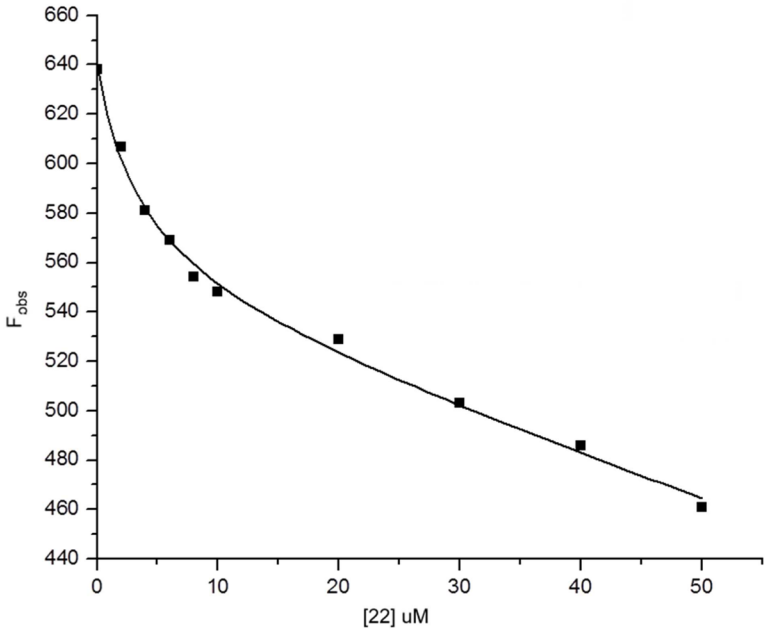
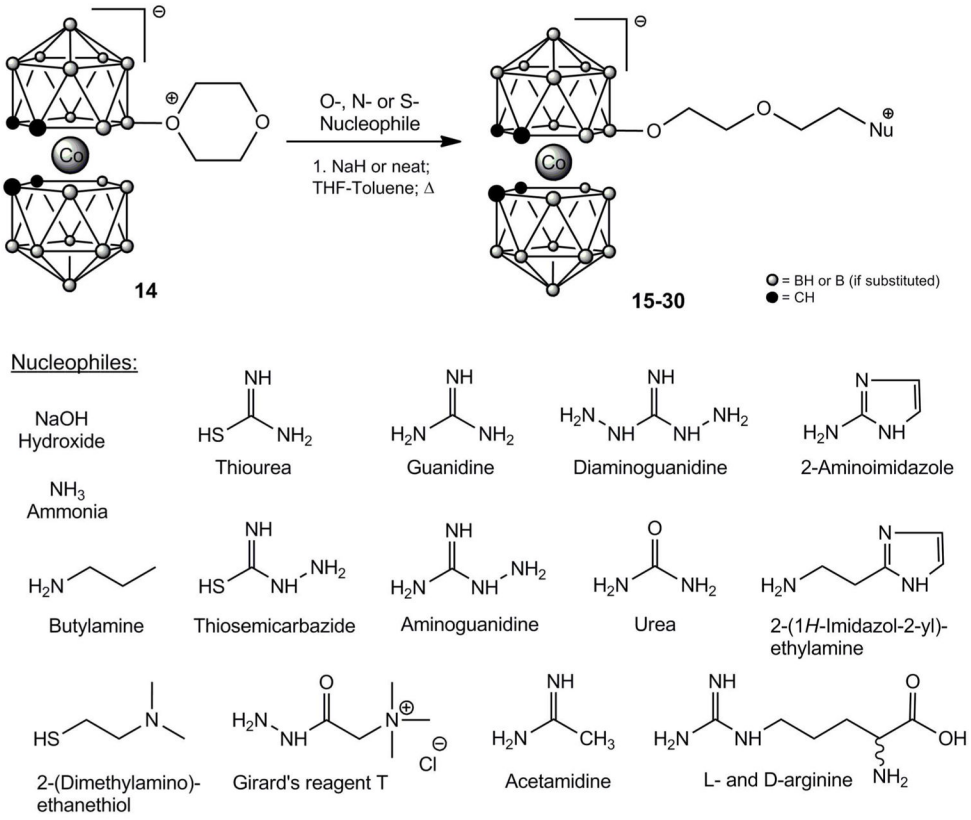


Figure 2.

Quenching of the intrinsic fluorescence of iNOS by **22**.

The [**22**] was varied from 0–50 RM. The experimental conditions were as follows: excitation = 280 nm; emission = 330 nm; [TrisHCl] = 50 mM; NaCl = 100 mM, pH = 7.5 at 25°C. The fitted line passing through the data is from Eqn. 1 as described in the Experimental Methods. Other reaction conditions are also described.



Scheme 1.
Preparation of derivatives **15**–**30** via nucleopholic dioxane ring-opening reaction of **14**

Table 1

Inhibition of NO synthesis by **20, 22, 23** and **26**: IC_{50} values (RM)

	iNOS	nNOS	eNOS	eNOS/iNOS	eNOS/nNOS
20	4.6 ± 0.33	34.6 ± 9.9	39.2 ± 9.2	8.5	1.1
22	1.8 ± 0.18	19.9 ± 7.6	9.1 ± 3.8	5.1	0.46
23	2.6 ± 0.18	13.7 ± 3.3	22.6 ± 3.6	8.7	1.6
26	4.7 ± 0.70	13.8 ± 2.1	38.2 ± 10.6	8.1	2.8

The reported IC_{50} values are +/− SE; selectivity ratios of eNOS to the other isoforms are also reported.