# Re and $^{99m}$Tc organometallic complexes containing pendant L-arginine derivatives as potential probes of inducible nitric oxide synthase†

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***Received 9th April 2008, Accepted 27th August 2008***
***First published as an Advance Article on the web 23rd October 2008***
**DOI: 10.1039/b805986a**

Aiming to design radioactive compounds based on the core "$^{99m}$Tc(CO)$_3$" for probing inducible nitric oxide synthase (iNOS) levels *in vivo*, we have synthesized conjugates containing a pyrazolyl-diamine chelating unit and pendant L-arginine analogues (substrates and inhibitors of NOS). Reaction of the conjugates with *fac*-[M(CO)$_3$]$^+$ (M = Re, $^{99m}$Tc) gave bioorganometallic complexes of the type *fac*-[M(CO)$_3$(k$^3$-L)] in good yield. After *in vitro* testing using the oxyhemoglobin NO capture assay, we concluded that the affinity of the inhibitor-containing conjugates to iNOS seems to be less affected upon metallation with rhenium than the substrate-containing conjugates. The complexes bearing guanidino substituted analogues of L-arginine still present considerable inhibitory action ($N^{\omega}$-monomethyl-L-arginine, $K_i$ = 36 μM; $N^{\omega}$-nitro-L-arginine, $K_i$ = 84 μM), being the first examples of organometallic complexes able to inhibit the iNOS. These results seem to indicate that $^{99m}$Tc(CO)$_3$-labeled L-argininine analogues, namely NOS inhibitors, may hold potential for monitoring increased levels of iNOS *in vivo.*

## Introduction

Medicinal organometallic chemistry has become one of the most important sub-topics within bioorganometallic chemistry, which aims at the development of new metal complexes with M–C bonds for biological or medicinal applications.[1]

The development of bioactive radioactive complexes based on the "MCO$_3$" core (M = Re, $^{99m}$Tc), a particular type of organometallic pharmaceuticals, for probing complex biochemical processes is a paradigmatic example of the potential application of organometallics in the diagnosis (M = $^{99m}$Tc) or therapy ($^{186/188}$Re) of different diseases.[1a,2] Despite the great variety of targeting biomolecules which have been labeled with the *fac*-[$^{99m}$Tc(CO)$_3$]$^+$ moiety,[2a] one of the most intense areas of research is currently focused on the $^{99m}$Tc(CO)$_3$-labeling of receptor-targeting peptides for tumor imaging *in vivo.*[3]

The importance of enzymes as natural targets for metal complexes is well recognized, and the use of inorganic/organometallic drugs holds promising diagnostic/therapeutic potential for pathologies associated to abnormal enzymatic activity.[1b,4] Most recently, it has been described that Re-complexes in the oxidation states V and I present inhibition capacity towards specific enzymes, which may open the possibility of probing enzyme levels *in vivo* with the surrogate $^{99m}$Tc-complexes.[5]

The visualization of enzyme activity in both brain and peripheral organs in Humans using nuclear imaging techniques (positron emission tomography—PET, and single photon emission computed tomography—SPECT) may not only be useful for diagnostic purposes but also for therapeutic monitoring of the effects of existing drugs or of those in development. Probing those low-capacity enzyme systems *in vivo* is a challenging task, being much dependent not only on the target selective uptake, but also on an high affinity of the radioprobe to the enzyme. Thus, the use of highly potent inhibitors and/or substrates of enzymes is mandatory for probing enzyme levels *in vivo.* Most of the research efforts have been directed toward the development of radiolabeled enzyme inhibitors and substrates with positron emitters for PET.[6] Despite the strict prerequisites needed, the activity of several enzymes such as cerebral acetylcholinesterase, cyclooxygenase, monoamine oxidase and dopamine decarboxylase, among others, have been successfully probed *in vivo* with PET, using either the radiollabeled inhibitor or the radiolabelled substrate approach.[6]

Nitric oxide synthase (NOS) is the enzyme responsible for the endogenous production of nitric oxide (NO), a very important key signaling mammalian mediator in several physiological processes (*e.g.* vasodilation, neurotransmission, host-defence and platelet aggregation).[7] NO is produced endogenously by conversion of L-arginine to L-citrulline by NOS. This enzyme presents three structurally distinct isoforms. Two of the them are constitutively expressed, being $Ca^{+2}$-dependent (nNOS [neuronal NOS, NOS1] and eNOS [endothelial NOS, NOS3]).[7] The third isoform is $Ca^{+2}$-independent (iNOS, NOS2) and is inducible. The targeting of NOS expression *in vivo* by a non-invasive technique would be particularly desirable since it could provide insight into a wide variety of pathophysiologic processes. In fact, and despite being still a controversial issue, previous research suggests that enhanced expression of NOS (mainly eNOS and iNOS) is observed in certain tumours, namely in melanoma, breast cancer and gastric cancers.[8] Therefore, the use of labeled substrates or inhibitors of the NOS

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† Electronic supplementary information (ESI) available: $^1$H-$^1$H COSY spectra; $^1$H-$^{13}$C HSQC spectra; $^1$H/$^{13}$C-NMR spectra. See DOI: 10.1039/b805986a

with γ- or β$^+$-emitting radionuclides is a challenging approach for *in vivo* imaging of NOS expression. The inhibitor $N^{\omega}$-nitro-L-arginine [$^{11}$C]methyl ester has been precluded as PET tracer for NOS distribution due to its *in vivo* instability.[9a] The assessment of $^{11}$C-Labeled thiopheneamidines analogues as nNOS-selective imaging agents for the evaluation of regional brain uptake in rodents and primates, revealed an high degree of nonspecific binding.[9b] The specificity of $^{125}$I-labeled diphenyleneiodonium bisulfate (NOS inhibitor), unique example of a radioprobe containing a γ-emitter for NOS targeting, in animals was negligible.[9c] So far, the β$^+$-emitting iNOS selective inhibitors S-[$^{11}$C]methylisothiourea and S-(2-[$^{18}$F]fluoroethyl)isothiourea were the only compounds that revealed promising properties for assessing induction of iNOS with PET.[9d] In fact, using an LPS-pretreated rat model with widespread tissue expression of iNOS, enhanced uptake of both radioprobes was observed in some of the organs that are reported to possess increased iNOS levels in such animal model.[9d] These experiments, together with the cell uptake studies, suggested for the first time that it was possible to probe increased levels of iNOS *in vivo* using radioactive compounds. To the best of our knowledge, no examples of NOS inhibitors or substrates labeled with the *d*-transition metal $^{99m}$Tc, have ever been reported.

Owing to its physico-chemical properties and widespread availability, $^{99m}$Tc is the radionuclide of choice for imaging. Thus, we aim at exploring the possibility of labeling well established NOS-substrates or -inhibitors with the organometallic core *fac*-[$^{99m}$Tc(CO)$_3$]$^+$ using a bifunctional chelator, and to use those radioactive probes for targeting NOS expression *in vivo*.

In this paper we report the synthesis and characterization of "bioactive conjugates" containing a pyrazolyl-diamine chelating unit and pendant L-arginine analogues, and of the corresponding bioorganometallic complexes of the type *fac*-[M(CO)$_3$(k$^3$-L)] (M = $^{99m}$Tc, Re; L = conjugates containing L-arginine analogues). The kinetic parameters of iNOS (mouse recombinant enzyme) in the presence of both the bioactive conjugates and the Re-complexes were determined.

## Results and discussion

### Synthesis and characterization of the bioactive conjugates L$^1$–L$^5$

The new conjugates **L**$^1$–**L**$^5$ comprise a pyrazolyl-diamine chelating unit for stabilization of the "M(CO)$_3$" core (M = Re, $^{99m}$Tc) and "bioactive" pendant L-arginine analogues for NOS recognition, which have been selected as well established substrates/inhibitors of NOS with minimal selectivity among the NOS isoforms. The conjugates were synthesized by functionalization of the Boc-protected bifunctional chelating ligand 4-{(2-aminoethyl)[2-(3,5-dimethyl-1*H*-pyrazol-1-yl)ethyl]amino}butanoic acid (**pz-COOH**) with substrates (L-arginine methyl ester, **L**$^1$; L-arginine, **L**$^2$) and inhibitors ($N^{\omega}$-nitro-L-arginine methyl ester, **L**$^3$; $N^{\omega}$-nitro-L-arginine, **L**$^4$; $N^{\omega}$-methyl-L-arginine, **L**$^5$) of nitric oxide synthase. The synthetic pathway involved the activation of the carboxylic acid group in **pz-COOH(Boc)** with *N*-hydroxysuccinimide (*ca.* 98% yield), followed by reaction with the corresponding L-arginine analogues, with formation of an amide bond (Scheme 1). Deprotection of the Boc group with trifluoroacetic acid (TFA) gave, in high yield, the bioactive conjugates **L**$^1$, **L**$^3$ and **L**$^5$ as air-stable colorless oils after purification by preparative RP-HPLC.

Conjugate **L**$^2$ was obtained in good yield by alkaline hydrolysis of the methyl ester group of **L**$^1$-Boc, yielding **L**$^2$-Boc, followed by Boc-deprotection with TFA. Surprisingly, if the same treatment was applied to **L**$^3$-Boc in order to obtain **L**$^4$, only undesirable species, tentatively assigned as lactam-derived products, were detected (results not presented). Thus, **L**$^4$ was obtained in low yield (*ca.* 36%) directly from **L**$^3$-Boc using a 3 N HCl solution to promote simultaneous hydrolysis of the methyl ester group and Boc-deprotection. Both **L**$^2$ and **L**$^4$, obtained as air-stable colorless oils, were also purified by RP-HPLC. The purity (≥ 98%) of all isolated bioactive conjugates was ascertained by RP-HPLC.

All conjugates were thoroughly characterized by $^1$H/$^{13}$C-NMR and IR spectroscopy, and electrospray ionization mass spectrometry (ESI-MS). The use of 2D-NMR experiments ($^1$H-$^1$H COSY and $^1$H-$^{13}$C HSQC) was crucial for peak assignment in the NMR spectra, and for full characterization of the compounds described in the experimental part. The $^1$H-NMR spectra ($D_2O$) of the conjugates presented the typical sharp singlet peaks for the H(4) proton ($\delta$ 5.95, **L**$^1$; $\delta$ 5.78, **L**$^2$; $\delta$ 5.81, **L**$^3$; $\delta$ 5.85, **L**$^4$; $\delta$ 5.89, **L**$^5$) and the methyl groups of the azolyl ring ($\delta$ 2.15/2.07, **L**$^1$; $\delta$ 2.08/1.98, **L**$^2$; $\delta$ 2.08/1.99, **L**$^3$; $\delta$ 2.09/2.02, **L**$^4$; $\delta$ 2.13/2.04, **L**$^5$). Resonances for the methylenic protons of the pyrazolyl-diamine backbone and L-arginine analogue pendant unit were also found. Singlet peaks for the methyl ester groups ($\delta$ 3.59, **L**$^1$; $\delta$ 3.57, **L**$^3$), and $N^{\omega}$-methyl group ($\delta$ 2.67, **L**$^5$) were also observed in the $^1$H-NMR spectra. The $^{13}$C spectra presented signals corresponding to all the carbon nuclei of the molecules.

### Synthesis and characterization of the Re(I) complexes *fac*-[Re(CO)$_3$(k$^3$-L)]$^+$ (1, L = L$^1$; 2, L = L$^2$; 3, L = L$^3$; 4, L = L$^4$; 5, L = L$^5$)

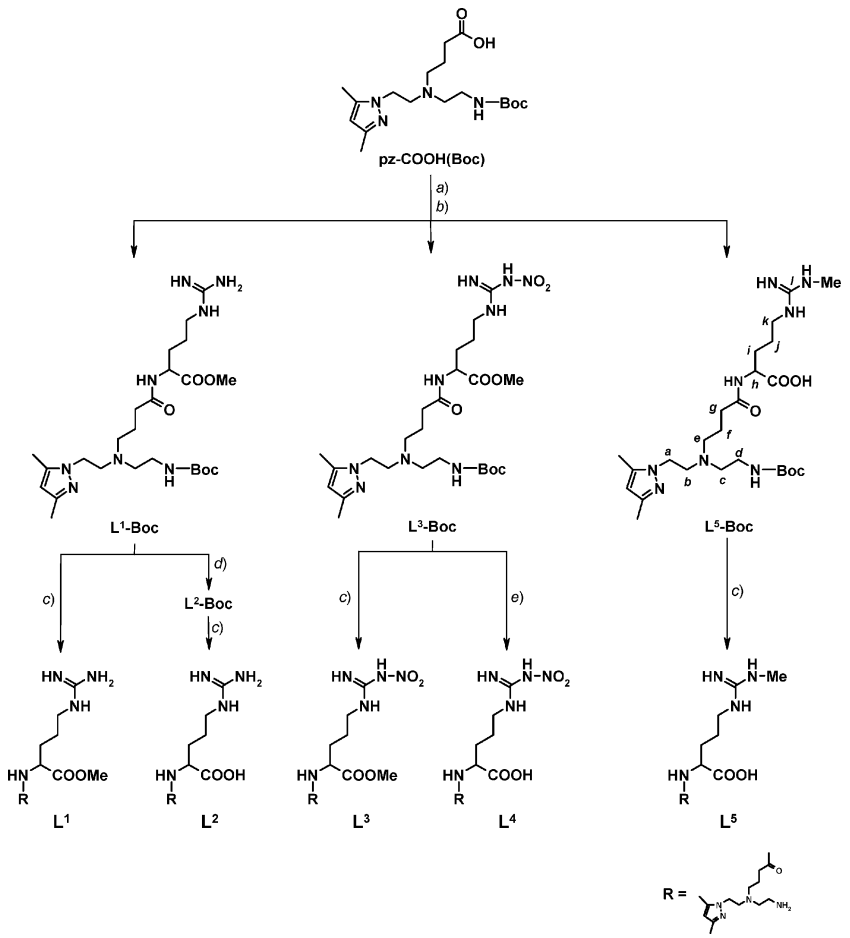
Reactions of the "bioactive" conjugates with the precursor (NEt$_4$)$_2$[ReBr$_3$(CO)$_3$] in refluxing methanol (**L**$^1$ and **L**$^3$) or water (**L**$^2$, **L**$^4$ and **L**$^5$), in the 1 : 1 molar ratio, afforded the cationic rhenium organometallic complexes **1–5** (Scheme 2). After evaporation of the solvents, the obtained residues were washed with $CHCl_3$ in order to remove NEt$_4$Br, and further purified by preparative RP-HPLC. The complexes were obtained as air-stable colorless viscous oils in moderate yields (50–80%). These compounds were synthesized as "non radioactive" analogues of the corresponding $^{99m}$Tc-organometallic complexes (**1a–5a**).

Complexes **1–5** were characterized by RP-HPLC, ESI-MS and NMR spectroscopy ($^1$H/$^{13}$C-NMR, $^1$H-$^1$H COSY, and $^1$H-$^{13}$C HSQC).

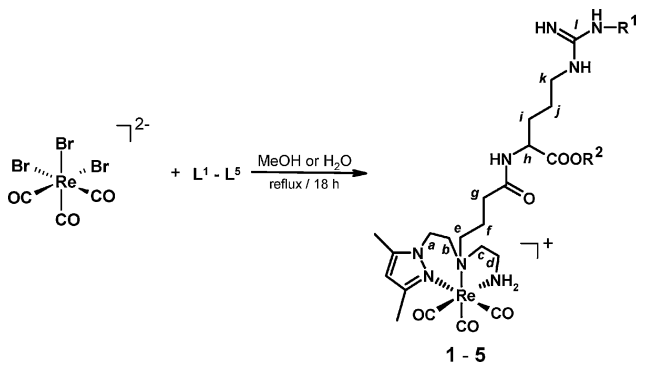
The RP-HPLC chromatograms of complexes **2**, **4** and **5**, which present terminal –COOH groups, presented two close sharp peaks, assigned as isomers. For the sake of an example, we show in Fig. 1A the RP-HPLC chromatogram of **2**, which clearly displays the presence of two isomers (***isomer a***: ret. time 25.0 min, ***isomer b***: ret. time 25.2 min) in the ratio 1 : 1.

The existence of two isomers arises from the presence of two chiral centers in the compounds, namely the single chiral centre constituted by the rhenium complex and the tertiary amine, and the α-carbon of the pendant amino acid derivative.

The isomers of complex **2** (identified as ***isomers a*** and ***b***) have been separated by semi-preparative RP-HPLC (Fig. 1B),



**Scheme 1** Synthesis of the bioactive conjugates $\mathbf{L^1}$–$\mathbf{L^5}$ (identification system for NMR assignments is displayed for $\mathbf{L^5}$-Boc as an example). (a) NHS, DCC, $CH_2Cl_2$, r.t., 18h. (b) L-Arginine analogue, $H_2O$–THF, $NEt_3$, r.t., 18 h. (c) $CF_3COOH$–$CH_2Cl_2$ (3 : 1), 3 h, r.t. (d) NaOH 2 N, r.t., 22 h. (e) HCl 3 N, r.t., 72 h.



**Scheme 2** Preparation of the rhenium tricarbonyl complexes **1–5** (**1**, $R^1$ = H, $R^2$ = Me; **2**, $R^1 = R^2$ = H; **3**, $R^1 = NO_2$, $R^2$ = Me; **4**, $R^1 = NO_2$, $R^2$ = H; **5**, $R^1$ = Me, $R^2$ = H). Identification system for NMR assignments is displayed.

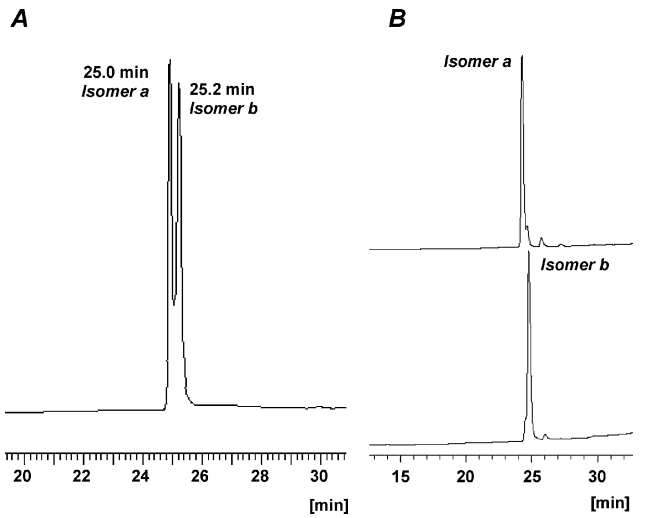



**Fig. 1** RP-HPLC analytical chromatograms (UV detection) of **2** before (A) and after separation (B) of the ***isomers a*** and ***b*** by semi-preparative RP-HPLC.

and independently characterized. Interestingly, no interconversion and/or decomposition was observed, even after 5 days in aqueous solution at room temperature. Despite the different experimental conditions tested (*e.g.* column type, gradient, and solvents), we were not able to separate the corresponding isomers of complexes **4** and **5** by semi-preparative RP-HPLC. In fact, the single isomers were never obtained in sufficient amount and enough purity for further evaluation.

The $^{1}$H-NMR spectra of **1–5** in $D_2O$ presented the typical singlet peaks assigned to the H(4) of the azole ring ($\delta$ 5.98–6.00) and the two characteristic singlet peaks attributed to the methyl groups of the pyrazolyl ring ($\delta$ 2.21–2.24 and $\delta$ 2.11–2.13). Despite some occasional overlapping of resonances, the chemical shifts and splitting pattern of the diastereotopic $NH_2$ ($\delta$ 5.03–5.07 and $\delta$ 3.61–3.67) and methylenic protons of the ligand backbone were comparable to those found for other previously reported complexes of the same type.[10] The NMR data obtained for all the complexes is consistent with the tridentate coordination mode of the pyrazole–diamine backbone of the conjugates $\mathbf{L}^1$–$\mathbf{L}^5$.

No significant differences in chemical shifts, intensity and multiplicity were observed in the $^{1}$H-NMR spectra of each isolated isomer (***a*** and ***b***) of complex **2**. As expected, the mass spectrometry data (ESI-MS) were also similar for both species.

The $^{13}$C-NMR spectra of complexes **2**, **4** and **5** allowed the identification of the corresponding two isomers, as could be ascertained by the duplication of certain signals of the methylenic carbon atoms (see Experimental section).

To ascertain the coordination mode of the bioactive conjugates towards the organometallic core *fac*-$[M(CO)_3]^+$ (M = $^{99m}$Tc, Re), complex **2** was synthesized by an alternative synthetic pathway, in which the intermediate complex $[Re(CO)_3(k^3\text{-pz-COOH})]^+$, prepared according to a published method,[10a] was coupled to L-arginine as outlined in Scheme 3.

The complex **2**, obtained *via* the direct (Scheme 2) and indirect method (Scheme 3), was independently characterized by RP-HPLC, ESI-MS and NMR spectroscopy. In both cases, the same chemical species was present (**2**), and no significant differences in the $^{1}$H/$^{13}$C NMR spectra and analytical RP-HPLC chromatograms were found. These results confirm that the stabilization of the *fac*-$[M(CO)_3]^+$ unit is done only through the pyrazolyl–diamine backbone of the conjugate, and that no nonspecific interactions between the guanidine group and the metal centre are present.

### Assay of iNOS activity

To get insight into the way the enzymatic activity of NOS in the presence of well established substrates (L-arginine methyl ester and L-arginine) and inhibitors ($N^{\omega}$-nitro-L-arginine methyl ester, $N^{\omega}$-nitro-L-arginine and $N^{\omega}$-methyl-L-arginine) was affected by their attachment to a pyrazolyl-diamine chelating unit, and further to the organometallic fragment "$M(CO)_3$" (M = Re, $^{99m}$Tc), the conjugates $\mathbf{L}^1$–$\mathbf{L}^5$, and the rhenium complexes **1–5** were tested as substrates or competitive inhibitors of iNOS (mouse recombinant enzyme). The assessment of the enzymatic activity was performed both in the presence of complex **2** as an isomeric mixture, and of the corresponding isolated ***isomers a*** and ***b***. In the case of complexes **4** and **5**, only the complete isomeric mixtures were assayed.

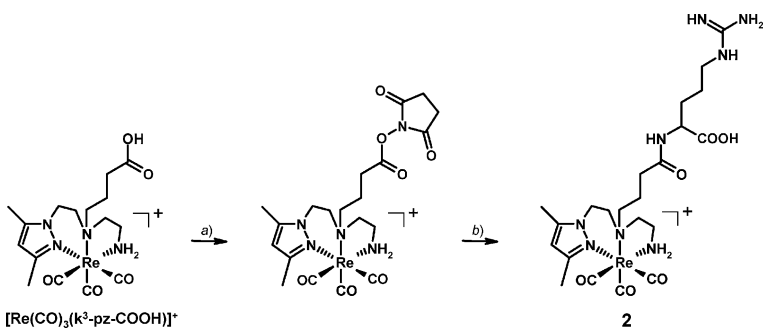
**Table 1** $K_m$ and $K_i$ values for substrates ($\mathbf{L}^1$, $\mathbf{L}^2$, **1** and **2**) and competitive inhibitors ($\mathbf{L}^3$, $\mathbf{L}^4$, $\mathbf{L}^5$, **3**, **4** and **5**) determined experimentally using the oxyhemoglobin NO capture assay

| Substrate | $K_m$ or $K_i$ values[a]/μM | Reference |
|---|---|---|
| L-Arginine methyl ester | 13 | This work |
| L-Arginine | 6 | This work |
| | 2–16 | 11*a* |
| $\mathbf{L}^1$ | 72 | This work |
| $\mathbf{L}^2$ | 57 | This work |
| **1** | 377 | This work |
| **2** (complete mixture) | 245 | This work |
| ***Isomer a*** | 123 | |
| ***Isomer b*** | 104 | |
| Inhibitor | | |
| $N^{\omega}$-Nitro-L-arginine | 3 | This work |
| | 8 | 11*c* |
| $N^{\omega}$-Methyl-L-arginine | 8 | This work |
| | 4 | 11*c* |
| $\mathbf{L}^3$ | > 10 000 | This work |
| $\mathbf{L}^4$ | 178 | This work |
| $\mathbf{L}^5$ | 18 | This work |
| **3** | 1094 | This work |
| **4** | 84 | This work |
| **5** | 36 | This work |

[a] Results are given as a mean of more than or equal to three independent experiments. Standard deviations of ± 5–10% were observed.

iNOS activity was determined spectrophotometrically by monitoring the NO-mediated conversion of oxyhemoglobin to methemoglobin at two wavelengths (401 and 421 nm).[11] The kinetic parameters ($K_m$ and $K_i$) for the compounds under study were determined by the method of Eisenthal and Cornish-Bowden, being summarized in Table 1.

The derivatization of the NOS substrates L-arginine methyl ester and L-arginine with the pyrazolyl–diamine chelating unit affected negatively their binding to the active site of iNOS, as confirmed by the significantly higher $K_m$ values found for $\mathbf{L}^1$ (72 μM) and $\mathbf{L}^2$ (57 μM) when compared with the $K_m$ values determined for the corresponding free substrates (L-arginine methyl ester:




**Scheme 3** Preparation of complex **2** *via* an indirect method. (a) NHS, DCC, DMF, r.t., 24 h. (b) L-ArgOH, $NEt_3$, $H_2O$, r.t., 16 h.

13 μM, L-arginine: 6 μM). Metallation of both conjugates, yielding complexes **1** ($K_m = 377$ μM) and **2** ($K_m = 245$ μM), respectively, led again to a considerable loss of affinity for the enzyme. Complex **2** presents a smaller $K_m$ value than the analogue compound **1**, which possesses a terminal methyl ester group. The same tendency was also observed when the $K_m$ values of the compounds containing a $-CO_2H$ group (L-arginine: $K_m = 6$ μM; $\mathbf{L}^2$: 57 μM) were compared with the equivalent compounds bearing a $-CO_2Me$ function (L-arginine methyl ester: 13 μM, $\mathbf{L}^1$: 72 μM). These results underline the importance of the interaction between the free carboxylic acid and the active site of NOS, through hydrogen bonding, for enzyme activity.[12]

Assessment of the enzymatic activity of iNOS in the presence of the isolated ***isomers a*** ($K_m = 123$ μM) and ***b*** ($K_m = 104$ μM) of complex **2** revealed that the isomers had comparable affinities to the enzyme, which are higher than that presented by the initial isomeric mixture.

The inhibitory potency of the well established NOS inhibitor $N^\omega$-nitro-L-arginine methyl ester was completely lost upon conjugation to the pyrazolyl chelator, as confirmed by the fact that the resulting conjugate $\mathbf{L}^3$ was inactive ($K_i > 10\,000$ μM). A less drastic effect was observed in the case of the conjugates containing the $N^\omega$-nitro-L-arginine and $N^\omega$-methyl-L-arginine pendant inhibitors, which showed decreased potencies on iNOS ($\mathbf{L}^4$, $K_i = 178$ μM and $\mathbf{L}^5$, $K_i = 18$ μM), when compared with the original inhibitors ($N^\omega$-nitro-L-arginine, $K_i = 3$ μM and $N^\omega$-methyl-L-arginine, $K_i = 8$ μM). However, the inhibitory potency of $N^\omega$-methyl-L-arginine was less affected upon attachment to the pyrazolyl-diamine chelating unit than the analogue $N^\omega$-nitro-L-arginine.

Interestingly, the reaction of the inactive compound $\mathbf{L}^3$ with the organometallic core *fac*-$[Re(CO)_3]^+$ yielded a complex (**3**) with a slight inhibitory action ($K_i = 1094$ μM). This tendency became even more evident in the case of the conjugate $\mathbf{L}^4$ ($K_i = 178$ μM), which gave a complex with increased inhibitory potency (**4**, $K_i = 84$ μM). Such type of effect have already been observed with other metal complexes that presented higher inhibitory potency than the corresponding metal-free inhibitors.[5b,13] The striking differences observed in the kinetic parameters of iNOS in the presence of complex **3** ($K_i = 1094$ μM) and **4** ($K_i = 84$ μM) highlighted again the importance of the interaction between the carboxylic acid and the active site of NOS for the inhibitory effect of L-arginine derived inhibitors.[12]

Unlike the $N^\omega$-nitro-containing conjugates $\mathbf{L}^3$ and $\mathbf{L}^4$, which upon metallation with "$Re(CO)_3$" led to complexes with increased inhibitory potency (**3** and **4**), $\mathbf{L}^5$ ($K_i = 18$ μM) gave an organometallic complex that was two-fold less potent (**5**, $K_i = 36$ μM) than the free conjugate.

Nevertheless, As far as we are aware, compounds **4** and **5** are the first examples of organometallic complexes able to inhibit iNOS *in vitro*.

Taken together these results indicated that the affinity of the conjugates containing inhibitors of NOS is less affected upon coordination to the metal ($\mathbf{L}^4$ *versus* **4**, and $\mathbf{L}^5$ *versus* **5**) than the conjugates with substrates ($\mathbf{L}^1$ *versus* **1**, and $\mathbf{L}^2$ *versus* **2**). Moreover, there is still room for improving the substrate (**1** and **2**)/inhibitor (**4** and **5**) recognition by NOS using different chelating systems and/or spacers in order to avoid unspecific interactions between the chelator and the arginine carboxylic acid group (fundamental for active site recognition).

**Radiosynthesis and characterization of *fac*-$[^{99m}Tc(CO)_3(k^3\text{-L})]^+$ (1a, L = $L^1$; 2a, L = $L^2$; 3a, L = $L^3$; 4a, L = $L^4$; 5a, L = $L^5$)**

The conjugates $\mathbf{L}^1$ and $\mathbf{L}^3$ reacted with the organometallic precursor *fac*-$[^{99m}Tc(CO)_3(H_2O)_3]^+$ giving the corresponding complexes in very low yields (**1a**: < 1%; **3a**: ≈ 17%) due to hydrolysis of the methyl ester groups under the tested labeling conditions. In fact, the main reaction products were **2a** (98%) and **4a** (81%). Even after a through optimization of the reaction conditions (temperature, pH, reaction time, conjugate final concentration), it was never possible to prepare **1a** and **3a** as single species in acceptable yields.

The compounds $\mathbf{L}^2$, $\mathbf{L}^4$ and $\mathbf{L}^5$ reacted with the organometallic precursor giving in high yields (>90%) the complexes *fac*-$[^{99m}Tc(CO)_3(\kappa^3\text{-}\mathbf{L}^2)]^+$ (**2a**), *fac*-$[^{99m}Tc(CO)_3(\kappa^3\text{-}\mathbf{L}^4)]^+$ (**4a**) and *fac*-$[^{99m}Tc(CO)_3(\kappa^3\text{-}\mathbf{L}^5)]^+$ (**5a**) as single well-defined species by RP-HPLC, respectively.

The chemical identity of complexes **1a–5a** was confirmed by comparing their retention times in the RP-HPLC chromatograms (γ detection) with those of the corresponding rhenium complexes **1–5** (UV detection). No evidence for the formation of isomers of the complexes **2a**, **4a** and **5a** was found under the same analytical chromatographic conditions used to discriminate the isomers of the rhenium compounds **2**, **4** and **5**. However, using simultaneously a more discriminating gradient (45 min - 10% MeOH/90% 0.1% TFA aqueous solution ⇒ 100% MeOH) and a smaller flow cell volume in the radiometric detector (5 μl), it was possible to detect the presence of the radioactive isomers by RP-HPLC. As an example, we present in Fig. 2 a comparison of the RP-HPLC profiles of complexes **2** and **2a**.

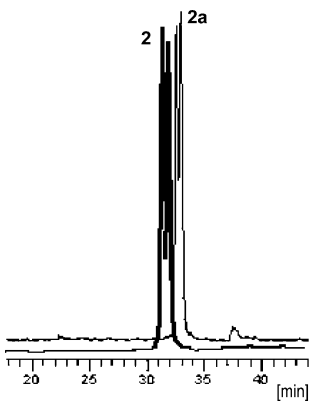



**Fig. 2** RP-HPLC analytical chromatograms of **2** (UV detection) and **2a** (γ-detection).

Determination of the partition coefficient for the radioconjugates **2a**, **4a** and **5a** were measured by octanol–0.1 M PBS extraction at pH 7.4. The average log $P_{o/w}$ values for the three trials performed were $-1.837 \pm 0.002$, $-1.409 \pm 0.008$ and $-2.190 \pm 0.002$ for **2a**, **4a** and **5a**, respectively. These values reveal the highly hydrophilic character of all synthesized radioactive complexes.

As expected from previous studies performed with other complexes with the same pyrazolyl-diamine chelating unit,[10] complexes **2a**, **4a** and **5a** were stable *in vitro* under the tested conditions. In fact, no decomposition or reoxidation products were observed by RP-HPLC analysis, even after incubation of the radioactive

complexes in PBS (pH 7.4, 37 °C, 24 h) or in human plasma (37 °C, 4 h).

## Conclusion

Herein we have introduced a new family of "bioactive" conjugates comprising a pyrazolyl-diamine backbone for stabilization of the *fac*-$[M(CO)_3]^+$ core (M = Re, $^{99m}$Tc) and pendant L-arginine analogues (iNOS substrates and inhibitors) for recognition of the active site of nitric oxide synthase (**L**$^1$–**L**$^5$). These conjugates reacted with the corresponding organometallic precursors yielding general compounds of the type *fac*-$[M(CO)_3(k^3\text{-L})]^+$ (M = $^{99m}$Tc, Re; L = **L**$^1$–**L**$^5$), which were thoroughly characterized by the usual analytical techniques in chemistry and radiochemistry. The biological affinity of the "bioactive" conjugates and rhenium complexes was tested *in vitro* with iNOS (oxyhemoglobin NO capture assay). The affinity of the conjugates with pendant inhibitors seemed to be less affected upon metallation with rhenium than the conjugates containing pendant substrates. The complexes bearing guanidine-substituted analogues of L-arginine still presented considerable inhibitory action (**4**, $K_i$ = 84 μM, $N^\omega$-nitro-L-arginine; **5**, 36 μM, $N^\omega$-methyl-L-arginine). These compounds are the first examples of organometallic complexes able to inhibit the iNOS. Taken together, the enzymatic studies and the high stability shown by the radioactive complexes revealed that "$^{99m}$Tc(CO)$_3$-labeled" L-argininine analogues, mainly NOS inhibitors, may hold great potential for monitoring increased levels of iNOS *in vivo.* Biological assessment of these compounds in specific cell lines with localized iNOS expression (*e.g.* LPS-activated macrophages) and specific animal models is underway.

## Experimental

### General procedures and materials

All chemicals and solvents were of reagent grade and were used without purification unless stated otherwise. The BOC-protected precursor 4-{(2-aminoethyl)[2-(3,5-dimethyl-1*H*-pyrazol-1-yl)ethyl]amino}butanoic acid (**pz-COOH(Boc)**) and $(Et_4N)_2[Re(CO)_3Br_3]$ were prepared according to published methods.[10b,14] All other chemicals not specified above were purchased from Aldrich. Na[$^{99m}$TcO$_4$] was eluted from a $^{99}$Mo/$^{99m}$Tc generator, using 0.9% saline. The radioactive precursor *fac*-$[^{99m}Tc(CO)_3(H_2O)_3]^+$ was prepared using a IsoLink® kit (Malinckrodt, Inc.). $^1$H and $^{11}$C NMR spectra were recorded at room temperature on a Varian Unity 300 MHz spectrometer. $^1$H and $^{11}$C chemical shifts were referenced with the residual solvent resonances relatively to tetramethylsilane. The spectra were assigned with the help of 2D experiments ($^1$H–$^1$H correlation spectroscopy, COSY and $^1$H–$^{13}$C heteronuclear single quantum coherence, HSQC). Assignments of the $^1$H and $^{13}$C NMR resonances are given in accordance with the identification system shown in Schemes 1 and 2. Infrared spectra were recorded as KBr pellets on a Bruker Tensor 27 spectrometer. All conjugates (**L**$^1$–**L**$^5$) and complexes (**1–5**) were characterized by electrospray ionization mass spectrometry (ESI-MS) using a Bruker model Esquire 3000 plus. Aliquots of ~ 5 mg of pure compounds (≥98% ascertained by RP-HPLC) were lyophilized in Eppendorf tubes and used for radioactive labelling and *in vitro* studies. HPLC analyses were performed on a Perkin Elmer LC pump 200 coupled to a Shimadzu SPD 10AV UV/Vis and to a Berthold-LB 509 radiometric detector, using an analytic Macherey-Nagel C18 reversed-phase column (Nucleosil 100–5, 250 × 3 mm) with a flow rate of 0.5 cm$^3$ min$^{-1}$. Purification of the inactive compounds were achieved on a semi-preparative Macherey-Nagel C18 reversed-phase column (Nucleosil 100–7, 250 × 8 mm) or on a preparative Waters μ Bondapak C18 (150 × 19 mm) with a flow rate of 2.0 cm$^3$ min$^{-1}$ and 5.5 cm$^3$ min$^{-1}$, respectively. UV detection: 254 or 220 nm. Eluents: aqueous 0.1% $CF_3COOH$–MeOH. **Gradient:** $t$ = 0–5 min: 10% MeOH; 5–30 min: 10→100% MeOH; 30–34 min: 100% MeOH; 34–35 min: 100→10% MeOH; 35–40 min: 10% MeOH.

NOS assays were recorded on an Agilent Technologies 8453 UV-Vis diode array spectrophotometer with a thermostated multicuvette holder with stirring. The iNOS (mouse recombinant enzyme), bovine hemoglobin, NADPH, $BH_4$ (Tetrahydrobiopterin), HEPES and DTT were purchased from Sigma Chemical Co.

**Synthesis of the intermediate activated ester pz-COOSuccinimide(Boc).** To a solution of **pz-COOH(Boc)** (0.200 g, 0.543 mmol) and *N*-hydoxysuccinimide (NHS, 0.075 g, 0.651 mmol) in dry dichloromethane (7 cm$^3$) was added dropwise a solution of *N*,*N*′-dicyclohexylcarbodiimide (DCC, 0.134 g, 0.651 mmol) in the same solvent (3 cm$^3$). The reaction solution was stirred at room temperature for 18 h. After removing the DCC–urea (DCU) by filtration through a pad of celite, hexane was added to the filtrate. After cooling to 4 °C, the precipitate thus formed was filtered and the filtrate evaporated to dryness. The yellow pale oil obtained was used in the next steps without further purification (98%, calculated from $^1$H-NMR spectrum). Attempts to purify the activated ester by silica gel column chromatography were unsuccessful, and only **pz-COOH(Boc)** precursor was isolated. $\delta_H$ (300MHz; $CD_3OD$) 5.83 (1H, s, H(4)pz), 4.06 (2H, br t, $CH_2$); 3.12 (2H, br t, $CH_2$), 2.90 (2H, br m, $CH_2$); 2.83 (4H, s, $CH_2$), 2.65 (2H, br d, $CH_2$), 2.56 (2H, t, $CH_2$), 2.27 (3H, s, $CH_3$pz), 2.16 (3H, s, $CH_3$pz), 1.86–1.68 (4H, m, $CH_2$), 1.42 (9H, s, $CH_3$-Boc). Others signals in the $^1$H-NMR spectrum: 2.78 (s, unreacted NHS), 1.49 (s, $CH_3$-Boc, unreacted **pz-COOH(Boc)**), 1.37–1.11 (DCU). $R_f$ (TLC silica-gel, $CH_3OH$ 15%/$CH_2Cl_2$) = 0.81.

### Synthesis of L$^1$.

*Preparation of the intermediate **L1-Boc**.* To a solution of **pz-COOSuccinimide(Boc)** (0.250 g, 0.536 mmol) in THF (10 cm$^3$) were added L-arginine methyl ester (0.160 g, 0.616 mmol) and triethylamine (0.379 g, 3.752 mmol) dissolved in the minimum amount of water. After a few seconds under stirring the suspension became clear. Additional triethylamine (0.065mg, 0.643 mmol) was added and the reaction mixture stirred for 18 h at room temperature. After completion, the solvent was removed in vacuum. The residue was washed with ethyl acetate and suspended in water for precipitation of the remaining DCU. After centrifugation, the supernatant was concentrated and purified by RP-HPLC using a preparative Waters μ Bondapak C18 column at a flow rate of 5.5 cm$^3$ min$^{-1}$ with a gradient of aqueous 0.1% $CF_3COOH$ and MeOH. Chromatograms were obtained by monitoring the UV absorption at 254 nm. Evaporation of the solvent from the collected fractions yielded the Boc-protected intermediate **L1-Boc·TFA** (0.195 g, 56%, calcd. for $C_{25}H_{46}N_8O_5$·TFA) as a colorless

oil. $\delta_H$ (300 MHz; $D_2O$) 5.89 (1H, s, H(4)pz), 4.32 (2H, t, $CH_2^a$), 4.20 (1H, q, $CH^h$), 3.56 (3H, s, $OCH_3$), 3.49 (2H, t, $CH_2^b$), 3.27 (2H, d, $CH_2^{c/d}$), 3.23 (2H, d, $CH_2^{c/d}$), 3.11 (2H, t, $CH_2^g$), 3.03 (2H, t, $CH_2^k$), 2.28 (2H, t, $CH_2^e$), 2.12 (3H, s, $CH_3$pz), 2.04 (3H, s, $CH_3$pz) 1.82 (2H, m, $CH_2^f$), 1.70 (1H, m, $CH^i$), 1.54 (1H, m, $CH^{i'}$), 1.44 (2H, m, $CH_2^j$), 1.20 (9H, s, $CH_3$); $\delta_C$ (75.5 MHz; $D_2O$) 177.4 (CO), 176.7 (CO), 165.4 (q, $CF_3COO^-$), 160.2 (CO), 158.7 ($C^l$-guanidine-L-ArgOMe), 150.9 (C(3/5)pz), 146.1 (C(3/5)pz), 118.7 (q, $CF_3COO^-$), 108.9 (C(4)pz), 83.7 ($C(CH_3)_3$, Boc), 55.4, 55.1, 54.6, 54.0, 51.9, 50.8, 43.8, 42.4, 37.2, 33.8, 29.5($C(CH_3)_3$), 26.5, 21.1, 13.6 ($CH_3$pz), 12.0 ($CH_3$pz). Retention time (analytic RP-HPLC): 25.1 min. $R_f$ (TLC silica-gel, $CHCl_3$ 49%/$CH_3OH$ 49%/$NH_4OH$ 2%) = 0.75. See identification system for NMR assignment in Scheme 1.

*Preparation of **L1***. **L**$^1$ was obtained directly by dissolving **L**$^1$-Boc·TFA (0.100 g, 0.153 mmol) in a mixture dichloromethane–TFA (1 $cm^3$–3 $cm^3$) and allowed to react for 3 h at room temperature with stirring. The residue obtained after evaporation of the solvents was dissolved in water, filtered through a 0.45 μm Millipore® filter, and purified by preparative RP-HPLC. The fractions containing **L**$^1$·2TFA were collected and the solvent removed to provide a clear viscous oil (0.050 g, 49%, calcd. for $C_{20}H_{38}N_8O_3$·2TFA). $\nu_{max}$(KBr)/$cm^{-1}$ ~ 1678 (C=O), 1400, 1203, 1135, 836, 801 and 722; $\delta_H$ (300 MHz; $D_2O$) 5.95 (1H, s, H(4)pz), 4.35 (2H, t, $CH_2^a$), 4.21 (1H, q, $CH^h$), 3.59 (3H, s, $OCH_3$), 3.54 (2H, t, $CH_2^b$), 3.43 (2H, t, $CH_2^{c/d}$), 3.28 (2H, t, $CH_2^{c/d}$), 3.12 (2H, t, $CH_2^g$), 3.04 (2H, t, $CH_2^k$), 2.28 (2H, t, $CH_2^e$), 2.15 (3H, s, $CH_3$pz), 2.07 (3H, s, $CH_3$pz), 1.82 (2H, m, $CH_2^f$), 1.70 (1H, m, $CH^i$), 1.54 (1H, m, $CH^{i'}$), 1.44 (2H, m, $CH_2^j$); $\delta_C$ (75.5 MHz; $D_2O$) 174.0 (CO), 173.4 (CO), 165.2 (q, $CF_3COO^-$), 157.5 ($C^l$-guanidine-L-ArgOMe), 148.5 (C(3/5)pz), 142.8 (C(3/5)pz), 118.2 (q, $CF_3COO^-$), 106.1 (C(4)pz), 52.6, 52.3, 51.9, 51.4, 49.5, 41.5, 39.7, 33.1, 30.8, 26.8, 23.7, 18.2, 11.0 ($CH_3$pz), 9.3 ($CH_3$pz); *m/z* (ESI +) 439.3 $[M + H]^+$ (100%) ($C_{20}H_{38}N_8O_3$ requires 438.3). Retention time (analytical RP-HPLC): 18.9 min.

**Synthesis of L$^2$.**

*Preparation of the intermediate **L**$^2$-Boc·TFA.* The Boc-protected intermediate **L**$^1$-Boc·TFA (0.140 g, 0.214 mmol) was dissolved in a 2 N NaOH solution (3.5 $cm^3$, 3.564 mmol). After 22 h at room temperature under stirring, the solution was neutralized with 2 N HCl and the solvent evaporated. After extraction of the residue with MeOH the solvent was vacuum-dried. The residue was dissolved in water, filtered through a 0.45 μm Millipore® filter and purified by preparative RP-HPLC. The intermediate compound **L**$^2$-Boc·TFA was obtained as a colorless oil (0.100 g, 73%, calcd. for $C_{24}H_{44}N_8O_5$·TFA). $\delta_H$ (300 MHz; $D_2O$) 5.99 (1H, s, H(4)pz), 4.38 (2H, t, $CH_2^a$), 4.15 (1H, q, $CH^h$), 3.54 (2H, t, $CH_2^b$), 3.29 (2H, br d, $CH_2^{c/d}$), 3.23 (2H, br d, $CH_2^{c/d}$), 3.15 (2H, t, $CH_2^g$), 3.02 (2H, t, $CH_2^k$), 2.30 (2H, t, $CH_2^e$), 2.16 (3H, s, $CH_3$pz), 1.88 (3H, s, $CH_3$pz), 1.83 (2H, m, $CH_2^f$), 1.73 (1H, m, $CH^i$), 1.57 (1H, m, $CH^{i'}$), 1.47 (2H, m, $CH_2^j$), 1.21 (9H, s, $CH_3$). Retention time (analytical RP-HPLC): 24.3 min.

*Preparation of **L**$^2$*. To a solution of **L**$^2$-Boc·TFA (0.100 g, 0.156 mmol) in dichloromethane was added TFA (1:3 $cm^3$). After stirring at room temperature for 3 h, the solvent was removed under vacuum, the residue dissolved in water and purified by preparative RP-HPLC. Evaporation of the solvents from the collected fractions yielded the compound **L**$^2$·2TFA as a colorless oil (0.080 g, 79%, calcd. for $C_{19}H_{36}N_8O_3$·2TFA). $\nu_{max}$(KBr)/$cm^{-1}$ ~1676 (C=O), 1430, 1204 and 1137. $\delta_H$ (300 MHz; $D_2O$) 5.78 (1H, s, H(4)pz), 3.97 (1H, q, $CH^h$), 3.91 (2H, t, $CH_2^a$), 3.01 (2H, t, $CH_2^k$), 2.82 (2H, t, $CH_2^e$), 2.73 (2H, t, $CH_2^b$), 2.62 (2H, t, $CH_2^d$), 2.40 (2H, t, $CH_2^g$), 2.08 (3H, s, $CH_3$pz), 2.02 (2H, t, $CH_2^e$), 1.98 (3H, s, $CH_3$pz), 1.64 (1H, m, $CH^i$), 1.51 (3H, m, $CH_2^f$ + $CH^{i'}$), 1.44 (2H, m, $CH_2^j$); $\delta_C$ (75.5 MHz; $D_2O$) 180.7 (CO), 177.5 (CO), 165.4 (q, $CF_3COO^-$), 158.7 ($C^l$-guanidine-L-Arg), 150.6 (C(3/5)pz), 143.5 (C(3/5)pz), 118.3 (q, $CF_3COO^-$), 107.3 (C(4)pz), 56.6 ($C^h$), 54.4 ($C^b$), 54.3 ($C^g$), 52.2 ($C^d$), 47.5 ($C^a$), 42.6 ($C^k$), 38.9 ($C^e$), 34.9 ($C^e$), 30.8 ($C^i$), 26.6 ($C^j$), 24.1 ($C^f$), 12.3 ($CH_3$pz), 12.0 ($CH_3$pz); *m/z* (ESI +) 425.3 $[M + H]^+$ (100%)($C_{19}H_{36}N_8O_3$ requires 424.3). Retention time (analytical RP-HPLC): 17.8 min.

**Synthesis of L$^3$.**

*Preparation of the intermediate **L**$^3$-Boc.*

*Method 1*. **L**$^3$-Boc was prepared by the same procedure as described above for **L**$^1$-Boc, starting from 0.107 g (0.230 mmol) of the activated ester **pz-COOSuccinimide(Boc)**, and from 0.077 g (0.288 mmol) of the $N^\omega$-nitro-L-arginine methyl ester. The Boc-protected intermediate **L**$^3$-Boc was obtained as a colorless oil after purification by preparative RP-HPLC (0.018 g, 13%, calcd. for $C_{25}H_{45}N_9O_7$).

*Method 2*. To a solution of **pz-COOH(Boc)** (0.200 g, 0.543 mmol) in dry dimetilformamide/acetonitrile (3:1) were added dry triethylamine (0.164 g, 1.628 mmol) and *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU, 0.380 g, 3.751 mmol). After a few minutes $N^\omega$-nitro-L-arginine methyl ester was added, and the reaction mixture stirred at room temperature under a nitrogen atmosphere. The analytical RP-HPLC chromatogram revealed that the reaction was completed after 2 h. The solvent was then removed under vacuum, and the residue purified by preparative RP-HPLC. **L**$^3$-Boc was obtained as a colorless oil (0.296 g, 93%, calcd. for $C_{25}H_{45}N_9O_7$). $\delta_H$ (300 MHz; $D_2O$) 6.07 (1H, s, H(4)pz), 4.44 (2H, t, $CH_2^a$), 4.24 (1H, q, $CH^h$), 3.57 (3H, s, $OCH_3$), 3.54 (2H, t, $CH_2^b$), 3.31 (2H, br d, $CH_2^{c/d}$), 3.25 (2H, br d, $CH_2^{c/d}$), 3.17 (4H, m, $CH_2^g$ + $CH_2^k$), 2.38 (2H, t, $CH_2^e$), 2.19 (3H, s, $CH_3$pz), 2.13 (3H, s, $CH_3$pz), 1.83 (2H, m, $CH_2^f$), 1.74 (1H, m, $CH^i$), 1.61 (1H, m, $CH^{i'}$), 1.50 (2H, m, $CH_2^j$), 1.21 (9H, s, $CH_3$). Retention time (analytical RP-HPLC): 28.8 min. $R_f$ (silica-gel, $CHCl_3$ 49%/$CH_3OH$ 49%/$NH_4OH$ 2%) = 0.90.

*Preparation of **L**$^3$*. The Boc-deprotection of the intermediate **L**$^3$-Boc (0.215 g, 0.368 mmol) with dichloromethane–TFA (1 $cm^3$–3 $cm^3$), as above described for **L**$^1$, gave a colorless oil after purification by preparative RP-HPLC (0.128 g, 58%, calcd. for $C_{20}H_{37}N_9O_5$·TFA). $\nu_{max}$(KBr)/$cm^{-1}$ ~1680 (C=O), 1427, 1279 ($NO_2$), 1203 and 1134; $\delta_H$ (300 MHz; $D_2O$) 5.81 (1H, s, H(4)pz), 4.18 (3H, m, $CH_2^a$ + $CH^h$), 3.57 (3H, s, $OCH_3$), 3.34 (2H, t, $CH_2^b$), 3.25 (2H, br t, $CH_2^{c/d}$), 3.22 (2H, br t, $CH_2^{c/d}$), 3.10 (2H, br t, $CH_2^k$), 2.94 (2H, t, $CH_2^g$), 2.21 (2H, t, $CH_2^e$), 2.08 (3H, s, $CH_3$pz), 1.99 (3H, s, $CH_3$pz), 1.76 (3H, m, $CH_2^f$ + $CH^i$), 1.60 (1H, m, $CH^{i'}$), 1.49 (2H, m, $CH_2^j$); $\delta_C$ (75.5 MHz; $D_2O$) 176.9 (CO), 176.0 (CO), 165.6 (q, $CF_3COO^-$), 160.9 ($C^l$-guanidine-$N^\omega$-nitro-L-ArgOMe), 151.4 (C(3/5)pz), 144.1 (C(3/5)pz), 118.5 (q, $CF_3COO^-$), 108.2

(C(4)pz), 55.3 ($C^b$), 55.0 ($OCH_3$), 54.6 ($C^g$), 51.9 ($C^h$), 50.9 ($C^c$), 44.9 ($C^a$), 42.6 ($C^k$), 36.6 ($C^d$), 33.9 ($C^e$), 29.8 ($C^i$), 26.0 ($C^j$), 21.6 ($C^f$), 14.2 ($CH_3$pz), 12.0 ($CH_3$pz). *m/z* (ESI +): 484.3 $[M + H]^+$ (100%)($C_{20}H_{37}N_9O_5$ requires 483.3). Retention time (analytical RP-HPLC): 21.9 min.

**Synthesis of $L^4$.** $L^4$ was obtained directly by hydrolysis of the methyl ester group and Boc-deprotection of the intermediate $L^3$-Boc (0.081 g, 0.139 mmol) with a 3 N HCl solution (5 $cm^3$, 15 mmol) at room temperature, for 72 h. After neutralization of the reaction mixture with a 1 N NaOH solution, the water was evaporated and the obtained residue extracted with methanol. The solvent was evaporated and the residue dissolved again in water. Purification by RP-HPLC gave a colorless oil (0.029 g, 36%, calcd. for $C_{19}H_{35}N_9O_5$·TFA). $\nu_{max}$(KBr)/$cm^{-1}$ ~1654 (C=O), 1396, 1275 ($NO_2$), 1203 and 1133. $\delta_H$ (300 MHz; $D_2O$) 5.85 (1H, s, H(4)pz), 4.44 (2H, t, $CH_2{}^a$), 4.00 (1H, q, $CH^h$), 3.45 (2H, t, $CH_2{}^b$), 3.36 (2H, t, $CH_2{}^{c/d}$), 3.17 (2H, t, $CH_2{}^{c/d}$), 3.10 (2H, t, $CH_2{}^k$), 2.95 (2H, t, $CH_2{}^g$), 2.21 (2H, t, $CH_2{}^e$), 2.09 (3H, s, $CH_3$pz), 2.02 (3H, s, $CH_3$pz), 1.72 (2H, m, $CH_2{}^f$), 1.56 (1H, m, $CH^i$), 1.45 (1H, m, $CH^{i'}$), 1.36 (2H, m, $CH_2{}^j$); $\delta_C$ (75.5 MHz; $D_2O$) 177.5 (CO), 176.8 (CO), 164.9 (q, $CF_3COO^-$), 160.9 ($C^l$-guanidine-$N^\omega$-nitro-L-Arg), 151.1 (C(3/5)pz), 145.9 (C(3/5)pz), 118.4 (q, $CF_3COO^-$), 109.1 (C(4)pz), 55.5 ($C^h$); 54.7($C^b$), 54.1($C^g$); 51.8($C^d$), 44.2 ($C^a$), 42.6 ($C^k$), 36.0 ($C^c$), 33.8 ($C^e$), 29.8 ($C^i$), 26.0 ($C^j$), 21.0 ($C^f$), 13.6 ($CH_3$pz), 12.0 ($CH_3$pz). *m/z* (ESI +) 470.3 $[M + H]^+$ (100%)($C_{19}H_{35}N_9O_5$ requires 469.5). Retention time (analytical RP-HPLC): 20.5 min.

**Synthesis of $L^5$.**

*Preparation of the intermediate $L^5$-Boc.* This compound was prepared as described for $L^1$. Starting with 0.130 g of **pz-COOSuccinimide(Boc)** (0.279 mmol) and 0.025 g of $N^\omega$-$CH_3$-L-arginine ($N^\omega$-methyl-L-Arg, 0.101 mmol) in THF–water a colourless oil was obtained after purification by RP-HPLC (0.030 g, 55%, calcd. for $C_{25}H_{46}N_8O_5$); $\delta_H$ (300 MHz; $D_2O$) 6.07 (1H, s, H(4)pz), 4.44 (2H, br t, $CH_2{}^a$), 4.18 (1H, q, $CH^h$), 3.55 (2H, br t, $CH_2{}^b$), 3.31 (2H, br d, $CH_2{}^{c/b}$), 3.27 (2H, br d, $CH_2{}^{c/b}$), 3.17 (2H, br t, $CH_2{}^g$), 3.00 (2H, t, $CH_2{}^k$), 2.63 (3H, s, $NHCH_3$), 2.36 (2H, t, $CH_2{}^e$), 2.20 (3H, s, $CH_3$pz), 2.13 (3H, s, $CH_3$pz), 1.84 (2H, m, $CH_2{}^f$), 1.75 (1H, m, $CH^i$), 1.60 (1H, m, $CH^{i'}$), 1.47 (2H, m, $CH_2{}^j$), 1.22 (9H, s, $CH_3$). Retention time (analytical RP-HPLC): 27.2 min. $R_f$ (silica-gel, $CHCl_3$ 49%/$CH_3OH$ 49%/$NH_4OH$ 2%) = 0.80.

*Preparation of* $L^5$. $L^5$-Boc (0.030 g, 0.056 mmol) was treated with an excess of TFA in dichloromethane (3 : 1) as previously described. After purification by RP-HPLC, $L^5$ was obtained as a colorless oil (0.024 g, 77%, calcd. for $C_{20}H_{38}N_8O_3$·TFA). $\delta_H$ (300 MHz; $D_2O$) 5.89 (1H, s, H(4)pz), 4.30 (2H, t, $CH_2{}^a$), 4.11 (1H, q, $CH^h$), 3.53 (2H, t, $CH_2{}^b$), 3.44 (2H, t, $CH_2{}^c$), 3.29 (2H, t, $CH_2{}^d$), 3.09 (4H, m, $CH_2{}^g$ + $CH_2{}^k$), 2.67 (3H, s, $NHCH_3$), 2.30 (2H, t, $CH_2{}^e$), 2.13 (3H, s, $CH_3$pz), 2.04 (3H, s, $CH_3$pz), 1.85 (2H, t, $CH_2{}^f$), 1.77 (1H, m, $CH^i$), 1.62 (1H, m, $CH^{i'}$), 1.50 (2H, m, $CH_2{}^j$); $\delta_C$ (75.5 MHz; $D_2O$) 178.1 (CO), 176.8 (CO), 164.9 (q, $CF_3COO^-$), 158.5 ($C^l$-guanidine-$N^\omega$-$CH_3$-L-Arg), 151.7 (C(3/5)pz), 144.6 (C(3/5)pz), 120.4 (q, $CF_3COO^-$), 108.5 (C(4)pz), 55.6 ($C^h$), 55.1 ($C^b$), 54.6 ($C^g$), 51.9 ($C^d$), 44.4 ($C^a$), 42.5 ($C^k$), 36.1 ($C^c$), 33.9 ($C^e$), 29.8 ($NHCH_3$), 29.4 ($C^i$), 26.7 ($C^j$), 21.2 ($C^f$), 14.1 ($CH_3$pz), 12.0 ($CH_3$pz). *m/z* (ESI+) 439.4 $[M + H]^+$ (100%) ($C_{20}H_{38}N_8O_3$ requires 438.5). Retention time (analytical RP-HPLC): 19.5 min.

**General procedure for the preparation of the Re complexes *fac*-[Re(CO)$_3$($k^3$-L)] (1, L = $L^1$; 2, L = $L^2$; 3, L = $L^3$; 4, L = $L^4$; 5, L = $L^5$)**

$(NEt_4)_2[ReBr_3(CO)_3]$ was reacted with equimolar amounts of the compounds $L^1$–$L^5$ in refluxing water ($L^2$, $L^4$ and $L^5$) or methanol ($L^1$ and $L^3$) for 18 h. After this time, the solvent was removed under vacuum, and the resulting residue washed with $CHCl_3$ to remove excess $[NEt_4]Br$. The resulting residue was dissolved in water, the precipitate formed eliminated by centrifugation and the supernatant purified by preparative RP-HPLC.

**Synthesis of fac-[*Re(CO)$_3$*($k^3$-$L^1$)]$^+$(1).** Starting from 0.013 g (0.020 mmol) of $L^1$·2TFA, a colorless oil formulated as **1** was obtained (0.010 g, 55%, calcd. for $C_{23}H_{38}N_8O_6Re$·2TFA). $\delta_H$ (300 MHz; $D_2O$) 6.00 (1H, s, H(4)pz), 5.07 (1H, br s, $NH_2$), 4.33 (1H, br m, $CH^a$), 4.26 (1H, br m, $CH^h$), 4.04 (1H, br m, $CH^{a'}$), 3.66 (1H, br s, $NH_2$), 3.59 (3H, s, $OCH_3$), 3.46 (1H, br t, $CH^g$), 3.28 (2H, br m, $CH^{g'}$ + $CH^b$), 3.02 (3H, t, $CH_2{}^k$ + $CH^d$), 2.70 (2H, br m, $CH_2{}^c$), 2.55 (1H, br t, $CH^{b'}$), 2.37 (1H, br m, $CH^{d'}$), 2.25 (2H, br m, $CH_2{}^e$), 2.24 (3H, s, $CH_3$pz), 2.13 (3H, s, $CH_3$pz), 2.05 (1H, br m, $CH^f$), 1.90 (1H, br m, $CH^{f'}$), 1.75 (1H, m, $CH^i$), 1.59 (1H, m, $CH^{i'}$), 1.50 (2H, br m, $CH_2{}^j$); $\delta_C$ (75.5 MHz; $D_2O$) 3 × ~ 196.2 (C≡O), 177.6 (CO), 176.3 (CO), 165.0 (q, $CF_3COO^-$), 159.3 ($C^l$-guanidine-L-ArgOMe), 155.8 (C(3/5)pz), 146.3 (C(3/5)pz), 120.4 (q, $CF_3COO^-$), 109.5 (C(4)Pz), 67.6 ($C^g$), 63.1 ($C^c$), 55.0 ($OCH_3$), 54.8 ($C^h$), 54.6 ($C^b$), 48.9 ($C^a$), 44.0 ($C^d$), 42.4 ($C^k$), 34.3 ($C^e$), 29.7 ($C^i$), 26.7 ($C^j$), 22.1 ($C^f$), 17.5 ($CH_3$pz), 13.0 ($CH_3$pz); *m/z* (ESI +) 709.4 $[M + H]^+$ (100%)($C_{23}H_{38}N_8O_6Re$ requires 708.8). Retention time (analytical RP-HPLC): 26.2 min.

**Synthesis of *fac*-[Re(CO)$_3$($k^3$-$L^2$)]$^+$(2).**

*Direct method.* Starting from 0.010 g (0.015 mmol) of $L^2$·2TFA, a colorless oil formulated as **2** was obtained. Yield (calcd. for $C_{22}H_{36}N_8O_6Re$·2TFA): 73% (0.010 g, 0.011 mmol).

*Indirect method.* To a solution of [Re(CO)$_3$($k^3$-pz-COOH)]$^+$ (0.030 g, 0.056 mmol), prepared according to a published method,[10a] and NHS (0.008 g, 0.064 mmol) in dry DMF (2 $cm^3$) was added DCC (0.013 g, 0.064 mmol) in the same solvent (0.5 $cm^3$), and the mixture was stirred for 24 h at room temperature. After addition of L-arginine (0.011 g, 0.064 mmol) and $NEt_3$ (0.019 g, 0.192 mmol) dissolved in the minimum amount of water (approximately 0.5 $cm^3$), stirring was continued at room temperature for 16 h. The DCC–urea precipitate was removed by centrifugation and the resulting solution evaporated. The crude residue was dissolved in water, filtered to discard the remaining DCC–urea and purified by preparative RP-HPLC. Evaporation of the solvent of the collected fractions gave complex **2** as a colorless clear oil (0.016 g, 30%, calcd. for $C_{22}H_{36}N_8O_6Re$·2TFA). $\nu_{max}$(KBr)/$cm^{-1}$ 2029 and 1915 (C≡O), 1676 (C=O), 1427, 1203 and 1135. $\delta_H$ (300 MHz; $D_2O$) 5.98 (1H, s, H(4)pz), 5.03 (1H, br s, $NH_2$), 4.30 (1H, br m, $CH^a$), 4.22 (1H, br m, $CH^h$), 4.02 (1H, br m, $CH^{a'}$), 3.62 (1H, br s, $NH_2$), 3.45 (1H, br t, $CH^g$), 3.24 (2H, br m, $CH^{g'}$ + $CH^b$), 3.03 (3H, t, $CH_2{}^k$ + $CH^d$), 2.69 (2H, br m, $CH_2{}^c$), 2.52 (1H, br t, $CH^{b'}$), 2.31 (1H, br m, $CH^{d'}$), 2.23

(2H, br m, $CH_2^e$), 2.21 (3H, s, $CH_3$pz), 2.11 (3H, s, $CH_3$pz), 2.02 (1H, br m, $CH^f$), 1.89 (1H, br m, $CH^{f'}$), 1.77 (1H, m, $CH^i$), 1.62 (1H, m, $CH^{i'}$), 1.53 (2H, br m, $CH_2^j$); $\delta_C$ (75.5 MHz; $D_2O$) 194.7 (C≡O), 194.3 (C≡O), 193.1 (C≡O), 175.8 (CO), 175.6 (CO), 156.9 ($C^l$-guanidine-L-Arg), 153.8 (C(3/5)pz), 144.4 (C(3/5)pz), 107.8 (C(4)Pz), 65.8 and 65.7 ($C^g$, *isomers*), 61.3 and 61.2 ($C^c$, *isomers*), 52.9, 52.8, 52.7 and 52.6 (m, $C^h$, $C^b$, *isomers*), 49.1 ($C^a$), 42.3 ($C^d$), 40.7 ($C^k$), 32.5 ($C^e$), 28.0 and 27.9 ($C^i$, *isomers*), 24.8 ($C^j$), 20.3 and 20.2 ($C^f$, *isomers*), 15.4 ($CH_3$pz), 10.9 ($CH_3$pz). *m/z* (ESI +) 695.4 $[M + H]^+$ (100%) ($C_{22}H_{36}N_8O_6Re$ requires 694.7). Retention time (analytical RP-HPLC): ***isomer a***—25.0 min; ***isomer b***—25.2 min.

**Synthesis of *fac*-[Re(CO)$_3$(k$^3$-L$^3$)]$^+$(3).** Starting from 0.040 g (0.067 mmol) of **L**$^3$·TFA, a colorless oil formulated as **3** was obtained (0.038 g, 66%, calcd. for $C_{23}H_{37}N_9O_8Re$·TFA). $\nu_{max}$(KBr)/cm$^{-1}$ 2028 and 1911 (C≡O), 1678 (C=O), 1650 ($NO_2$), 1427, 1272 ($NO_2$), 1203 and 1135. $\delta_H$ (300 MHz; $D_2O$) 6.00 (1H, s, H(4)pz), 5.02 (1H, br s, $NH_2$), 4.31 (1H, br m, $CH^a$), 4.26 (1H, br m, $CH^h$), 4.04 (1H, br m, $CH^{a'}$), 3.61 (1H, br s, $NH_2$), 3.59 (3H, s, $OCH_3$), 3.45 (1H, br t, $CH^g$), 3.25 (2H, br m, $CH^{g'}$ + $CH^b$), 3.12 (2H, br t, $CH_2^k$), 3.01 (1H, br m, $CH^d$), 2.71 (2H, br m, $CH_2^c$), 2.53 (1H, br t, $CH^{b'}$), 2.34 (1H, br m, $CH^{d'}$), 2.25 (2H, br m, $CH_2^e$), 2.23 (3H, s, $CH_3$pz), 2.12 (3H, s, $CH_3$pz), 2.03 (1H, br m, $CH^f$), 1.90 (1H, br m, $CHf'$), 1.76 (1H, br m, $CH^i$), 1.61 (1H, br m, $CH^{i'}$), 1.55 (2H, br m, $CH_2^j$); $\delta_C$ (75.5 MHz; $D_2O$) 3 × ~ 196.1 (C≡O), 177.6 (CO), 176.2 (CO), 161.0 ($C^l$-guanidine-$N^\omega$-nitro-L-ArgOMe), 155.7 (C(3/5)pz), 146.2 (C(3/5)pz), 109.8 (C(4)Pz), 67.5 ($C^g$), 63.3 ($C^c$), 55.1 (OMe), 54.8 ($C^h$), 54.6 ($C^b$), 48.9 ($C^a$), 44.2 ($C^d$), 42.6 ($C^k$), 34.4 ($C^e$), 29.8 ($C^i$), 26.3 ($C^j$), 22.2 ($C^f$), 17.3 ($CH_3$pz), 12.9 ($CH_3$pz); *m/z* (ESI +) 754.4 $[M + H]^+$ (100%) ($C_{23}H_{37}N_9O_8Re$ requires 753.8). Retention time (analytical RP-HPLC): 29.8 min.

**Synthesis of *fac*-[Re(CO)$_3$(k$^3$-L$^4$)]$^+$(4).** Starting from 0.016 g (0.027 mmol) of **L**$^4$·TFA, a colourless oil formulated as **4** was obtained (0.010 g, 44%, calcd. for $C_{22}H_{35}N_9O_8Re$·TFA). $\delta_H$ (300 MHz; $D_2O$) 5.98 (1H, s, H(4)pz), 5.06 (1H, br s, $NH_2$), 4.33 (1H, br m, $CH^a$), 4.25 (1H, br m, $CH^h$), 4.00 (1H, br m, $CH^{a'}$), 3.63 (1H, br s, $NH_2$), 3.44 (1H, br t, $CH^g$), 3.23 (2H, br m, $CH^{g'}$ + $CH^b$), 3.12 (2H, br t, $CH_2^k$), 3.00 (1H, br m, $CH^d$), 2.70 (2H, br m, $CH_2^c$), 2.49 (1H, br t, $CH^{b'}$), 2.30 (1H, br m, $CH^{d'}$), 2.24 (2H, br m, $CH_2^e$), 2.21 (3H, s, $CH_3$pz), 2.11 (3H, s, $CH_3$pz), 2.05 (1H, br m, $CH^f$), 1.90 (1H, br m, $CH^{f'}$), 1.75 (1H, br m, $CH^i$), 1.65 (1H, br m, $CH^{i'}$), 1.58 (2H, br m, $CH_2^j$); $\delta_C$ (75.5 MHz; $D_2O$) 3 × ~195.6 (C≡O), 177.4, 177.3 (CO), 160.8 ($C^l$-guanidine-$N^\omega$-nitro-L-Arg), 155.6 (C(3/5)pz), 146.1 (C(3/5)pz), 109.6 (C(4)Pz), 67.4 and 67.6 ($C^g$, *isomers*), 63.1 and 62.9 ($C^c$, *isomers*), 54.5 ($C^h$, *isomers*), 54.4 ($C^b$), 48.8 ($C^a$), 44.1 ($C^d$), 42.5 ($C^k$), 34.4 and 34.3 ($C^e$, *isomers*), 29.8 and 29.7 ($C^i$, *isomers*), 26.3 ($C^j$), 22.1 and 21.9 ($C^f$), 17.2 ($CH_3$pz), 12.7 ($CH_3$pz); *m/z* (ESI +) 740.4 $[M + H]^+$ (100%) ($C_{22}H_{35}N_9O_8Re$ requires 739.7). Retention time (analytic RP-HPLC): ***isomer a***—27.2 min; ***isomer b***—27.4 min.

**Synthesis of *fac*-[Re(CO)$_3$(k$^3$-L$^5$)]$^+$(5).** Starting from 0.010 g (0.018 mmol) of **L**$^5$·TFA, a colorless oil formulated as **5** was obtained (0.010 g, 67%, calcd. for $C_{23}H_{38}N_8O_6Re$·TFA). $\delta_H$ (300 MHz; $D_2O$) 6.00 (s, 1H, H(4)pz), 5.03 (1H, br s, $NH_2$), 4.32 (1H, br m, $CH^a$), 4.21 (1H, br m, $CH^h$), 4.04 (1H, br m, $CH^{a'}$), 3.67 (1H, br s, $NH_2$), 3.47 (1H, br t, $CH^g$), 3.26 (2H, br m, $CH^{g'}$ + $CH^b$), 3.05 (3H, t, $CH_2^k$ + $CH^d$), 2.73 (2H, br m, $CH_2^c$), 2.63 (3H, s, $N^\omega$-methyl-L-Arg), 2.54 (1H, br m, $CH^{b'}$), 2.35 (1H, br m, $CH^{d'}$), 2.24 (2H, br m, $CH_2^e$), 2.24 (3H, s, $CH_3$pz), 2.13 (3H, s, $CH_3$pz), 2.03 (1H, br m, $CH^f$), 1.90 (1H, br m, $CH^{f'}$), 1.74 (1H, m, $CH^i$), 1.62 (1H, m, $CH^{i'}$), 1.51 (2H, br m, $CH_2^j$); $\delta_C$ (75.5 MHz; $D_2O$) 3 × ~195.6, 177.6 (CO), 160.0 ($C^l$-guanidine-$N^\omega$-methyl-L-Arg), 155.7 (C(3/5)pz), 146.2 (C(3/5)pz), 109.7 (C(4)Pz), 67.5 and 67.4 ($C^g$, *isomers*), 63.1 and 62.9 ($C^c$, *isomers*), 54.7 and 54.6 ($C^h$, *isomers*), 54.5–54.3 ($C^b$, *isomers*), 48.9 ($C^a$), 44.2 ($C^d$), 42.5 ($C^k$), 34.3 ($C^e$), 29.7 and 29.6 ($C^i$, *isomers*), 29.4 ($N^\omega$-$CH_3$), 26.8 ($C^j$), 22.1 and 21.9 ($C^f$, *isomers*), 17.3 ($CH_3$pz), 12.8 ($CH_3$pz); *m/z* (ESI +) 709.6 $[M + H]^+$ (100%) ($C_{23}H_{38}N_8O_6Re$ requires 708.8). Retention time (analytical RP-HPLC): ***isomer a***—25.9 min; ***isomer b***—26.2 min.

### Synthesis of the $^{99m}$Tc(I) Complexes (1a–5a).

*General method.* In a nitrogen-purged glass vial, 100 μL of a $10^{-3}$ or $10^{-4}$ M aqueous solution of the compounds **L**$^1$–**L**$^5$ were added to 900 μL of a solution of the organometallic precursor *fac*-[$^{99m}$Tc(CO)$_3$(H$_2$O)$_3$]$^+$ (1–2 mCi) in saline or phosphate buffer pH 7.4 (**3a** and **4a**). The reaction mixture was then heated to 100 °C for 30–60 min, cooled on an ice bath and the final solution analyzed by RP-HPLC. Retention times: 29.1 min (**1a**), 26.6 min (**2a**), 29.2 min (**3a**), 27.4 min (**4a**) and 26.4 min (**5a**).

### Plasma stability *in vitro*

100 μl of **2a**, **4a** and **5a** were added to 500 μl of human plasma and incubated at 37 °C. After incubation (24 h), aliquots (100 μl) were taken and the plasmatic proteins precipitated with ethanol (200 μl). The plasma was centrifuged at 3000 rpm for 15 min at 4°C and the supernatant (protein free plasma) filtered trough a Millipore filter (0.22 μm), and analyzed by RP-HPLC.

### Partition coefficient

Was evaluated by the "shakeflask" method.[15] The radioconjugate was added to a mixture of octanol (1 cm$^3$) and 0.1 M PBS pH = 7.4 (1 cm$^3$), previously saturated in each other by stirring the mixture. This mixture was vortexed and centrifuged (3000 rpm, 10 min, room temperature) to allow phase separation. Aliquots of both octanol and PBS were counted in a gamma counter. The partition coefficient ($P_{o/w}$) was calculated by dividing the counts in the octanol phase by those in the buffer, and the results expressed as log $P_{o/w}$.

### Enzyme kinetic assays

The iNOS activity assay was based on the method of hemoglobin assay previously described by Hevel and Marletta with slight modifications.[11a,b] The kinetics parameters for iNOS were determined using initial rate analysis. Initial rate data were fitted to irreversible single substrate Michaelis–Menten models. The kinetic parameters were determined using the direct linear plot of Eisenthal and Cornish-Bowden and the Hyper software (J.S. Easterby, University of Liverpool, UK; http://www.liv.ac.uk/~jse/software.html).[11e] This method was chosen primarily because of its robustness.[11f] The $K_m$ and $K_i$ values represent a mean of triplicate measurements. Standard deviations of ± 5 to 10% were observed.

### Hemoglobin capture assay

**Preparation of oxyhemoglobin.** Oxyhemoglobin was prepared using a previously described protocol with some modifications.[11d] Briefly, bovine hemoglobin in 50 mM HEPES pH 7.4 was reduced to oxyhemoglobin with 10-fold molar excess of sodium dithionite. The sodium dithionite was later removed by dialysis against 50 volumes of HEPES buffer for 18 hours at 4 °C. The buffer was replaced 3 times. The concentration of oxyhemoglobin was determined spectrophotometrically using $\varepsilon_{415\,nm} = 131\ mM^{-1}cm^{-1}$. Oxyhemoglobin was stored at –80°C before use.

### Determination of kinetic parameters

**Determination of $K_m$ values.** All initial velocity measurements were recorded at 37 °C. Total reaction volumes were 1500 μL and contained 50 mM HEPES pH 7.4, 6 μM oxyhemoglobin, 200 μM NADPH, 10 μM $BH_4$, 100 μM DTT and at least three concentrations of substrate (20–500 μM; L-ArgOH, L-ArgOMe, **L**[2], **1** and **2**). Magnetic stirring in the spectrophotometer cuvette was essential to maintain isotropic conditions. Reactions were initiated by the addition of iNOS enzyme (~1 U) to the prewarmed cuvette (~5 min). The NO-mediated conversion of oxyhemoglobin to methemoglobin was followed by monitoring the increase in absorbance at dual wavelength (401 and 421 nm) for 10 min.[11g] Controls were performed in the some conditions without iNOS enzyme.

**Determination of $K_i$ values.** The $K_i$ values were obtained by measuring inhibition with at least three concentrations of L-arginine (20–150 μM) in the presence of 150 μM of the inhibitor, in the same conditions previously described. The formula used to calculate the $K_i$ is: $K_i = [I]/((K_m^{app}/K_m) - 1)$ where [I] is the inhibitor concentration, $K_m$ is the Michaelis–Menten constant of the substrate L-arginine and $K_m^{app}$ is the apparent value of $K_m$ for a substrate in the presence of the inhibitor.[11e] $K_m$ value for L-arginine was determined as 6 μM.

## Acknowledgements

This work has been supported by the Fundação para a Ciência e Tecnologia (FCT) through the project POCI/SAU-FCF/58855/2004. We thank Mallinkrodt-Tyco Inc. for providing the IsoLink® kits. Bruno L. Oliveira would like to thank FCT for a BI grant. We wish to acknowledge the Mass Spectrometry Laboratory for performing the ESI-MS determinations at the Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal.

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