New NO-Donors with Antithrombotic and Vasodilating Activities, Part 17

Arylazoamidoximes and 3-Arylazo-1,2,4-oxadiazol-5-ones☆

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Summary

Seven arylazoamidoximes (**3**), six phenoxycarbonyl derivatives (**4**), and six 1,2,4-oxadiazol-5-ones (**5**) have been prepared and their structure and purity etablished by spectroscopy and elemental analysis. In the EI mass spectra ready elimination of NO from the title amidoximes was observed. A new addition reaction of **3a** with hydrochloric acid to 4-chlorophenylhydroazoamidoxime **7** is described. The compounds were tested for nitric oxide dependent biological properties, i.e. platelet aggregation, antithrombotic effects, and decrease in blood pressure. In arterioles of rats 5/19 compounds inhibited the formation of thrombi with a laser beam by ≥ 20% 2 h after oral administration of 60 mg/kg. Among these are three amidoximes (**3a**, **3e**, **3f**), one phenoxycarbonyl derivative (**4a**), and one oxadiazolone (**5a**). With the 4-chlorophenylazoamidoxime **3c** a long lasting (24 h) decrease of blood pressure in spontaneously hypertensive rats was observed. Microsomal fractions of rat liver oxidize arylazoamidoximes and generate nitric oxide (e.g. **3a** and **3b**). NO was measured by the oxyhemoglobin assay. The influence of SOD, pretreatment of the rats with dexamethasone, as well as kinetic parameters were determined. Type **3** compounds, therefore, are a new class of NO donors. Type **4** and **5** compounds function as their prodrugs.

Introduction

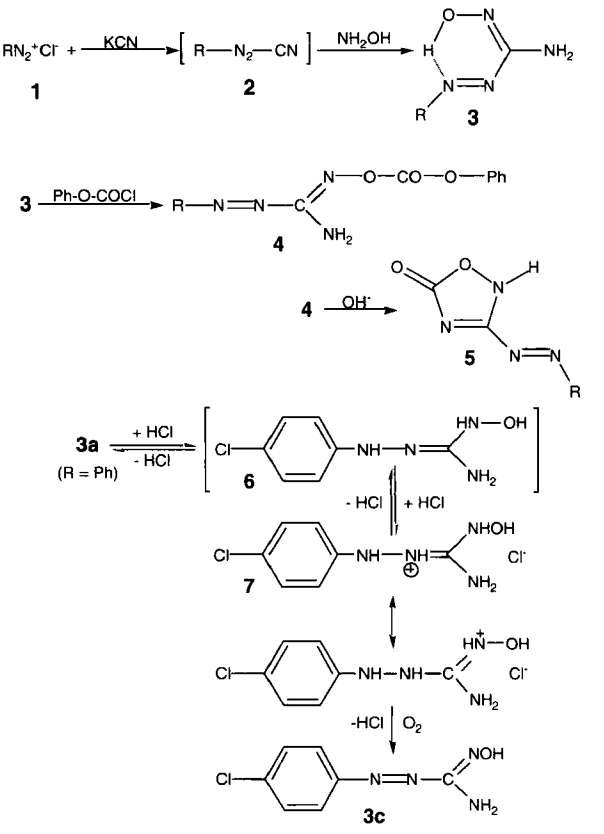
During the biosynthesis of nitric oxide L-arginine is oxidized by molecular oxygen to N^{ω}-hydroxyarginine and further to NO. The reaction is catalyzed by nitric oxide synthases and specific for L-arginine[1]. More recently it has been shown that the hydroxylation of guanidine moieties other than L-arginine can be mediated by cytochromes CYP 450[2]. These catalysts are furthermore able to support the formation of NO from N^{ω}-hydroxyarginine[3] and other *N*-hydroxyguanidines or amidoximes, respectively[4].

The title arylazoamidoximes are structurally related to both types of compounds and in principle should be suitable as substrates for the CYP 450 catalyzed formation of NO.

For the long term prevention of thrombus formation the oral administration of suitable drugs is obligatory. As arylazoamidoximes are very polar compounds we designed the title oxadiazolones as prodrugs. This might improve the absorption from the gastrointestinal tract and hence the bioavailability of the arylazoamidoximes.

Chemistry

For the preparation of type **3** arylazoamidoximes (see Scheme 1) the method of Longo[5, 5a] was used. The reaction has to be carried out in a two phase water/ether system, with immediate removal of the azonitriles **2** from the aqueous layer. The ethereal layer is then stirred with an alkaline solution of hydroxylamine to form **3**. The oxime proton is found in the ^{1}H-NMR spectrum as a sharp signal at ≈11 ppm as well (e.g. 11.25 ppm in **3a**). The amino group forms a sharp singlet at ≈6 ppm as well (e.g. 5.90 ppm in **3a**). Both are exchangeable with D_2O. In principle four configurational



Scheme 1: Synthesis of azoamidoximes **3** and their corresponding oxadiazolones **5** and unexpected formation of **3c** from **3a** with diluted hydrochloric acid in the presence of oxygen.

[1] Part of the PhD thesis S. Bade, FU Berlin, **1995**.

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isomers of **3** are possible. The NMR spectrum indicates that only one isomer is present. Hydrogen bond formation via a six membered ring suggests that the *E*(azo)/Z(oxime) configuration is dominant. In the mass spectra a strong M^+–30 peak (intensity of 62% for **3a**) is present. Precise mass measurements indicate that this fragment is due to the expulsion of nitric oxide. Reaction of type **3** compounds with phenyl chloroformate afforded type **4** compounds, which underwent unusual cyclisation in alkaline medium to the oxadiazolones **5**. In alkaline solution the anion is formed and **5** thereby protected against hydrolysis. In acidic media the hydrolysis of **5** proceeds much faster.

In another series of experiments we tried to synthesize arylazo-*N*-hydroxyamidoximes according to the method of Armand[6]. These compounds should be especially prone to the release of NO. As ammonia is more basic than hydroxylamine the exchange reaction can only take place when the released ammonia is bound by acid. Surprisingly we could not detect any exchange of the amino group against hydroxylamine. Instead we observed the addition of hydrochloric acid to yield **7**. Evidence of the chlorine substitution at position 4 of the ring is given by the typical AA'XX' spin system at 6.77 (2,6-H) and 7.28 (3,5-H) ppm. The coupling constant is 9 Hz. The signals for the five exchangeable protons are found at 7.93 (NH_2), 8.32 (1H), 9.88 (1H), 9.99 (1H), and 10.93 (NOH) ppm. Interestingly the reaction is reversible. A rise in pH regenerates **3a**. If **7** is kept in solution in the presence of oxygen **3c** is formed.

Pharmacological Experiments

Antithrombotic effects

Nitric oxide donors inhibit platelet aggregation and hence are able to show inhibition of thrombosis[7]. Therefore we submitted **3a–g**, **4a–f**, and **5a–f** to an in vivo thrombosis model[8]. Briefly the formation of thrombi in mesenteric vessels of rats is induced by the beam of an argon laser via a microscope (35 mW, 50 ms). The number of exposures ("shots") necessary to form a thrombus of defined size is counted. From the average shot number the percentage of inhibition of thrombosis is calculated. The results are compiled in Table 1. The results show that antithrombotic effects occur with all three classes of compounds. In the arterioles 13/19 substances inhibit thrombus formation by 10% or more. Five of these even exhibit a reduction of at least 20%. Three of them belong to the amidoximes **3** (**3a**, **3c**, **3f**), one is a phenoxyderivative (**4a**) and one the oxadiazolone **5a**. Three compounds did not influence the thrombus formation in arterioles. Two of them surprisingly belong to the amidoximes **3** (**3b**, **3d**). One is the phenoxy derivative **4d**, while **all** oxadiazolones exhibited antithrombotic properties. The inactive amidoximes are interestingly those with the strongest electron donating (**3b**) or electron withdrawing (**3d**) properties. In the first case this will result in a less acidic NOH group and a more basic amidoximo group. In the 4-fluoro derivative **3d** it is just the other way round. In either case this might lead to an unfavourable dissociation status, namely protonation or dissociation of the hydroxyimino group, respectivly. Both effects would decrease the membrane passage, i.e. the absorption from the gastrointestinal tract. This view is backed by the fact that in both cases the oxadiazolone prodrugs **5b** and **5d** are active compounds. This makes sure that they have had to be absorbed from the g.i. tract. As prodrug formation obviously enhances the absorption one should expect in general an increase of activity in the series 3 ⇒ 4 ⇒ 5. Table 1 however shows that this is not the case. At best an equal activity in the pairs **3a / 5a**, **3c / 5c**, **3e / 5e** is seen. In the pair **3f / 5f** the activity even decreases. These results suggest that in type **4** and **5** compounds the formation of nitric oxide is decreased compared to the type **3** amidoximes. This is presumably due to a rather slow cleavage of the prodrug **5** to the drug **3**.

It is known that *N*-hydroxyguanidines and similar *N*-hydroxylated structures can be reduced to guanidines and amidines *in vitro*[2,9,10] and *in vivo*[11]. Thus it could be possible that the extent of the reduction of the investigated arylazoamidoximes to inactive arylazoamidines is different *in vivo* thus leading to differences in activities. Studies are in progress to answer this question.

Effects on Blood Pressure

Principle and device used for blood pressure measurements in spontaneously hypertensive rats have been described in detail recently[12]. Briefly the animal is warmed up (10 ± 5 min) in a perspex tube to 37 °C. The rat tail which is out of the tube is surrounded by a pressure cuff and more distally by

Table 1: Antithrombotic effect at type **3–5** compounds in rats 2 h after p.o. administration of 60 mg/kg. (molsidomine: 30 mg/kg) Statistics: Man-Whitney U-test; n.s. = not significant.

R	Compound	Inhibition of thrombus formation in arterioles % ± σ ($p \leq$)	venules% % ± σ ($p \leq$)
phenyl	**3a**	21 ± 3 (0.002)	15 ± 6 (0.01)
	4a	24 ± 6 (0.002)	22 ± 6 (0.002)
	5a	20 ± 2 (0.002)	7 ± 5 (0.01)
4-methoxyphenyl	**3b**	0 ± 2 (n.s.)	0 ± 2 (n.s.)
	4b	9 ± 5 (0.02)	0 ± 3 (n.s.)
	5b	11 ± 7 (0.02)	7 ± 5 (0.01)
4-chlorophenyl	**3c**	22 ± 2 (0.002)	13 ± 3 (0.01)
	4c	18 ± 2 (0.002)	5 ± 3 (0.1)
	5c	18 ± 6 (0.002)	7 ± 2 (0.01)
4-fluorophenyl	**3d**	0 ± 3 (n.s.)	0 ± 2 (n.s.)
	4d	0 ± 3 (n.s.)	1 ± 2 (n.s.)
	5d	14 ± 3 (0.002)	7 ± 2 (0.01)
2,4-dichlorophenyl	**3e**	19 ± 2 (0.002)	6 ± 6 (0.01)
	4e	7 ± 3 (0.01)	3 ± 2 (n.s.)
	5e	17 ± 3 (0.002)	12 ± 3 (0.002)
1-naphthyl	**3f**	20 ± 2 (0.002)	6 ± 4 (0.01)
	4f	4 ± 2 (0.05)	3 ± 2 (n.s.)
	5f	13 ± 2 (0.002)	11 ± 2 (0.002)
2-hydroxycarbamoyl-phenyl	**3g**	11 ± 3 (0.002)	10 ± 4 (0.002)
	molsidomine	62 ± 15 (0.002)	31 ± 9 (0.002)

a piezo pulse wave transducer. The measurement begins when the correct opening of the tail arteries is indicated by a light signal for constant pulse. The pressure in the cuff is raised until the pulsations have ceased.

Then the pressure is decreased continuously until the pulse returns. As pressure and pulse are recorded simultaneously the arterial blood pressure can be determined directly from the chart of the recorder. The heart rate is recorded digitally.

The results obtained with six compounds are summarized in Table 2. The series **3b–5b** was chosen as example for inactive or moderately antithrombotic compounds while **3c–5c** represent compounds with maximum antithrombotic effects. In the first series only **5b** after 4 h significantly decreased the arterial blood pressure by 9%. In type **c** compounds **3c** rather strongly decreased the blood pressure for 6 h. After 24 h a smaller effect which still is significant was observed. If **3c** were a nitric oxide donor (see blow) this would mean that the endothelial and/or smoth muscle cells of the arterial vessels can use **3c** as source of nitric oxide. The prodrug **4c** obviously is not suitable for this purpose. In contrast **5c** exhibited a smaller but long lasting effect. This might be due to the (rather slow) formation of **3c** from the prodrug **5c**. In so far this result corresponds to the conclusion drawn from the respective antithrombotic effects.

Table 2: Time dependend lowering of the arterial blood pressure in spontaneously hypertensive rats after p.o. administration of 60 mg/kg of the compounds stated below. (n.s. = not significant). Minoxidil: 2 mg/kg.

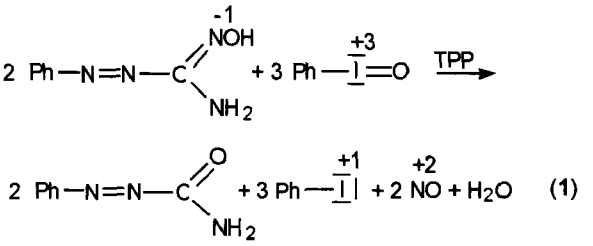
Cpd	% lowering ± σ after ($p \leq$) 2 h	4 h	6 h	24 h
3b	4 ± 12 (n.s.)	4 ± 10 (n.s.)	3 ± 12 (n.s.)	0 ± 8 (n.s.)
4b	0 ± 5 (n.s.)	0 ± 8 (n.s.)	0 ± 8 (n.s.)	0 ± 11 (n.s.)
5b	0 ± 10 (n.s.)	9 ± 5 (0.05)	8 ± 10 (n.s.)	0 ± 4 (n.s.)
3c	14 ± 7 (0.01)	16 ± 10 (0.01)	13 ± 8 (0.01)	5 ± 3 (0.01)
4c	3 ± 4 (n.s.)	3 ± 5 (n.s.)	0 ± 5 (n.s.)	0 ± 3 (n.s.)
5c	8 ± 3 (0.01)	9 ± 7 (0.1)	7 ± 2 (0.01)	9 ± 3 (0.01)
min-oxidil	18 ± 4 (0.01)	20 ± 6 (0.01)	23 ± 6 (0.01)	22 ± 6 (0.01)

In vitro Formation of Nitric Oxide from Phenyl Azoamidoxime 3a and the Corresponding Oxadiazolone 5a

Reaction of 3a and 5a with Fe^{III}-tetraphenyl-porphyrine (TPP) and I,I-bis-trifluoroacetoxy-iodobenzene (IB)

In nature the oxidation of N^{ω}-hydroxyarginine to nitric oxide is performed by molecular oxygen and catalyzed by hemoproteins, i.e. nitric oxide synthases. As stated in the introduction[2,3] in amidoximes cytochromes CYP 450 function as catalysts.

Recently Duchstein[13] has suggested a simple model for mimicking such cytochrome CYP 450 mediated oxidation reactions. Briefly the reaction between the potential NO donor e.g. **3a** and an oxygen donor e.g. IB which is an bisacylated iodosobenzene, should occur according to equation (1). TPP replaces CYP 450 as catalyst.



(1)

The reaction is carried out under anaerobic conditions in an argon atmosphere. The detection of nitric oxide is performed by the usual chemoluminescence method[13].

When using these reagents we observed that with IB alone after a few minutes about 3% of **3a** had already been oxidized to NO. Neither longer reaction times nor the addition of TPP improve the yield. So we could only draw the conclusion that iodosobenzene is able to oxidize **3a** to nitric oxide.

Generation of Nitric Oxide from Compounds 3a and 3b by Microsomal Oxidation

Subcellular distribution and cofactor requirements

The reaction rates of the generation of nitric oxide by microsomal rat liver enzymes under differing incubation conditions are listed in Table 3. In the absence of protein, cosubstrate, substrate or with denaturated microsomes, the conversion rates were low and significantly higher in the presence of all components. Microsomes from rats which were not pretreated with dexamethasone (control microsomes) converted compound **3b** but not **3a** to nitric oxide.

Table 3: Rates of the formation of nitric oxide by microsomal transformation of compound **3a** and **3b**.

Incubation mixture	Formation of NO Compound **3a** pmol/min/nmol P450	Formation of NO Compound **3b** pmol/min/nmol P450
complete	269.23	225.34
	SD = 8.45	SD = 11.99
microsomes heated at 100°C for 5 min	80.38 + SD = 46.20	119.67 SD = 45.26
– NADPH	37.40 *	81.27*
	SD = 25.67	SD = 9.09
– protein	24.56 *	75.67*
	SD = 4.59	SD = 3.90
– substrate	69.45 +	69.45 +
	SD = 18.59	SD = 18.59
control microsomes	n.D	171.06
	–	SD = 24.72
– NADPH	n.D	n.D
– protein	8.24	14.96 +
	SD = 2.14	SD = 3.62

Rates ± SD were calculated from three determinations with microsomes from 10 with dexamethasone pretreated rats and 10 control rats. Incubations were carried out as described in Materials and Methods. *Statistically different from control (complete incubation mixture) $P < 0.001$ or $+P < 0.01$ (Student's *t*-test).

The formation of nitric oxide was found to be proportional to the protein concentration up to 0.86 nmol cytochrome P 450/0.6 mL incubation mixture. This concentration proved to be optimal for the analysis procedure. Investigations on the dependence of the reaction rate on cofactor requirement at this amount of cytochrome P 450 revealed that concentrations of 0.4 mM NADPH were sufficient for optimal rates.

Kinetics of the formation of nitric oxide

The reaction rate of the enzymatic formation of nitric oxide was linear up to an incubation time of 12 minutes. The oxidation of both substrates followed Michaelis-Menten kinetics; the kinetic parameters are shown in Fig. 1 and 2.

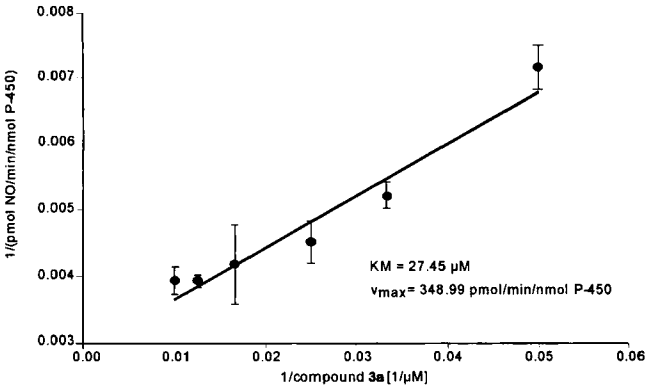


Fig.1: Lineweaver-Burk plot of the oxidation of compound **3a**. Each point is the mean of three different experiments (with an enzyme preparation from 10 livers of rats, pretreated with dexamethasone) ± SD. Incubations were carried out as described in the Experimental Part.

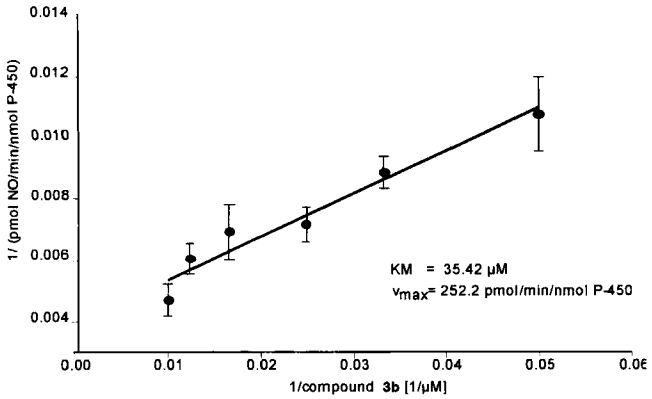


Fig.2: Lineweaver-Burk plot of the oxidation of compound **3b**. Each point is the mean of three different experiments (with an enzyme preparation from 10 livers of rats, pretreated with dexamethasone) ± SD. Incubations were carried out as described in the Experimental Part.

Effect of superoxide dismutase

The addition of superoxide dismutase to normal incubation mixtures with compound **3a** as substrate showed no influence on the formation of nitric oxide as shown in Table 4. In contrast the formation of nitric oxide with compound **3b** was significantly suppressed by the addition of superoxide dismutase. This effect was proportional up to an amount of 1000 U superoxide dismutase/mL incubation mixture as shown in Fig. 3.

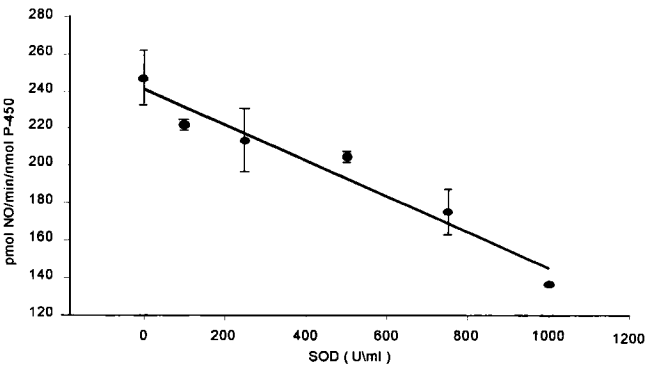


Fig. 3: Effect of superoxide dismutase on reaction rates with compound **3b**. Rates ± SD were calculated from three determinations with microsomes from 10 with dexamethasone pretreated rats and 10 control rats. Incubations were carried out as described in the Experimental Part.

Table 4: Effect of superoxide dismutase on the microsomal formation of NO from compound **3a** and **3b**.

Incubation mixture + SOD (U/ml)	Formation of nitric oxide Compound **3a** pmol/min/nmol P450	Formation of nitric oxide Compound **3b** pmol/min/nmol P450
0	200.82	247.12
	SD = 14.66	SD = 14.68
100	232.25	221.86
	SD = 23.42	SD = 3.01
250	199.40	195.75
	SD = 21.63	SD = 32.95
500	183.84	187.55
	SD = 37.79	SD = 30.57
750	203.31	169.94+
	SD = 11.24	SD = 13.09
1000	183.64	136.63*
	SD = 12.69	SD = 36.60

Rates ± SD were calculated from three determinations with microsomes from 10 rats pretreated with dexamethasone and 10 control rats. Incubations were carried out as described in Materials and Methods. *Statistically different from control (complete incubation mixture) $P < 0.001$ or $+P < 0.01$ (Student's *t*-test).

Discussion

It is known that *N*-hydroxylated structures, such as *N*-hydroxyguanidines can be transformed to nitric oxide by P 450 isoenzymes comparable to the formation of NO from *N*-hydroxy-L-arginine by NO-synthases[2–4]. It is quite obvious that for arylazoamidoximes different P 450 isoenzymes are capable of oxidizing **3a** and **3b**. Whereas for the conversion of **3a** dexamethasone inducible isoenzymes (3A) seem to be involved, the transformation of **3b** is catalysed by constitutive P 450 isoenzymes as no inducing effect was observed (Table 3). The effect of superoxide dismutase on the reaction rates supports this hypothesis. As incubation mixtures with compound **3a** showed no significant decrease in reaction rates on addition of superoxide dismutase up to a concentration of

1000 U/ml (Table 4), the rates with compound **3b** showed a linear dependence on SOD concentration (Fig. 3).

On considering reactions with cytochrome P 450, not only the monooxygenase activity but also its oxidase and peroxidase activity have to be taken in account[14]. If the substrate is bound to the enzyme but then uncouples the catalytic cycle, the peroxidase activities occur and the compound finally undergoes an indirect oxidation by the oxygen species formed in the NADPH reduction[15]. Comparable to numerous similar compounds[2,4] this mechanism seems to play a major role in the transformation of **3b**, and the release of $O_2^{\bullet-}$ is responsible for the oxidation [4,16]. The biotransformation of **3a** not influenced by SOD could be performed by a P 450-Fe(III)-$OO^{\bullet}$ species[4,16]. This means **3a** is a high affinity substrate for the liver P 450 enzymes involved. It is oxidized before decomposition of P 450-Fe(III)-$OO^{\bullet}$ to $O_2^{\bullet-}$ occurs. In this respect **3a** is an exceptional compound in comparison to similar *N*-hydroxylated compounds [4,16].

Inhibition of platelet aggregation

If NO is generated in platelet rich plasma (PRP) from NO donating compounds an inhibition of the platelet aggregation (e.g. induced by collagen) is observed. The reason for this is that activation of guanylate cyclase, and hence formation of guanosine triphosphate (GTP) which decreases the concentration of platelet cytosolic calcium ions, occurs. In so far the Born test is a simple bioassay for nitric oxide formation.

In the series of the arylazoamidoximes **3** a half-maximal inhibition (IC_{50}) of human platelet was observed at the following concentrations: 37 µmol/L (**3c**), 25 µmol/L (**3d**), and 31 µmol/L (**3f**). This has to be classified as a poor effect. In all other compounds including type **4** and **5** the IC_{50} was >62.5 µmol/L which we define as lack of activity. We conclude that in PRP at best small amounts of NO are generated during the incubation period of 6 min. Obviously the enzyme pattern of the platelet is not very suitable for the oxidation of **3** to nitric oxide.

Experimental Part

Chemistry[1)]

Devices: Mp (uncorrected) Lintström.– Elemental analysis: Perkin-Elmer element analyzer 240 C and Elementar Vario EL.– IR: Perkin-Elmer 1420 and ATI Mattson, Serie Genesis FTIR.– ^{1}H-NMR: Bruker AC 300 and DPX 400.– MS: Varian MAT CH 7A, Kratos MS 25 RF (EI), Varian MAT CH 5-DF (FAB).

General procedure for the synthesis of arylazoamidoximes **3**[5]

20 mmol of the aromatic amine are dissolved in 50 mL of 10% hydrochloric acid, cooled to 0–5 °C and 20 mmol sodium nitrite are added slowly. The mixture is neutralized with 20% sodium hydrogencarbonate solution until pH 8 is reached. The same volume of ether and 18 mmol potassium cyanide are added. The two phases are stirred vigorously for 30 min and separated. The ethereal layer containing the arylazonitrile **2** is shaken with an aqueous solution from 40 mmol hydroxylamine hydrochloride and 70 mmol sodium hydroxide. The phases are separated when no more change in colour is observed. The aqueous layer is acidified with glacial acetic acid. The precipitate (**3**) is sucked off and recrystallized.

1) The full set of data is in the PhD thesis S. Bade, FU Berlin, **1995.**

Phenylazomethaneamidoxime (**3a**)

From 7.30 g (78.38 mmol) aniline. Long orange needles (ethyl acetate/hexane), mp 122 °C (dec.), ref.[4] 125–126 °C, yield 3.09 g (24%).– Anal. $C_7H_8N_4O$.– IR (KBr): 3472 cm^{-1} (NH_2), 3354 (NH_2), 2812 (OH), 1678 (C=N), 1462 (N=N).– UV (methanol): λ_{max} (log ε): 226 (3.933), 294 (3.964), 352 (4.019) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 5.90 (s, 2H, D_2O exchange, NH_2), 7.57 (m, 3H, aromatic 3-H, 4H, 5-H), 7.83 ("dd", *J* = 8/2 Hz, aromatic 2-H, 6-H), 11.25 (s, 1H, D_2O exchange, NOH).– MS (70 eV): *m/z* (%) = 164 (9) [M^+], 134 (62) [M^+-NO], 77 (100) [$C_6H_5^+$].

4-Methoxyphenylazo-methanamidoxime (**3b**)

From 3.57 g (28.98 mmol) 4-methoxyaniline. Yellow orange crystals (methanol), mp 152 °C (dec.), yield 2.81 g (50%).– Anal. $C_8H_{10}N_4O_2$.– IR (KBr): 3360 cm^{-1} (NH_2), 3246 (NH_2), 1673 (C=N).– UV (methanol): λ_{max} (log ε) = 242 (3.960), 354 (4.321) nm.– ^{1}H-NMR ([D$_6$]DMSO: δ = 3.86 (s, 3H, CH_3), 5.81 (s, 2H, D_2O exchange, NH_2), 7.13 ("d", *J* = 9 Hz, 2H, aromatic 3-H, 5-H), 7.82 ("d", *J* = 9 Hz, aromatic 2-H, 6-H), 11.01 (s, 1H, D_2O exchange, NOH).– MS (70 eV): *m/z* (%) = 194 (44) [M^+], 164 (32 [M^+-NO], 122 (100), 107 (87) [4-CH_3O-ph$^+$].

4-Chlorophenylazo-methanamidoxime (**3c**)

From 5.30 g (41.55 mmol) 4-chloroaniline. Yellow needles (ethyl acetate/hexane), mp 202 °C (dec.), yield 4.54 g (55%).– Anal. $C_7H_7ClN_4O$.– IR (KBr): 3471 cm^{-1} (NH_2), 3352 (NH_2), 2802 (OH), 1676 (C=N).– UV (methanol): λ_{max} (log ε): 228 (3.964), 308 (4.050), 358 (4.087) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 5.93 (s, 2H, D_2O exchange, NH_2), 7.65 ("d", *J* = 8 Hz, 2H, aromatic 3-H, 5-H), 7.82 ("d", *J* = 8 Hz, aromatic 2-H, 6-H), 11.35 (s, 1H, D_2O exchange, NOH).– MS (70 eV): *m/z* (%) = 200 (9), 198 (28) [M^+], 170 (27), 168 (86) [M^+-NO], 113 (37), 111 (100) [4-Cl-ph].

4-Fluorophenylazo-methanamidoxime (**3d**)

From 15.00 g (135.50 mmol) 4-fluoroaniline. Yellow crystals (water), mp 158–166 °C (dec.), yield 1.23 g (5%). Anal. $C_7H_7FN_4O$.– IR (KBr): 3472 cm^{-1} (NH_2) 3354 (NH_2), 1677 (C=N).– UV (methanol): λ_{max} (log ε): 226 (3.990), 298 (3.994), 352 (4.053) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 5.90 (s, 2H, D_2O exchange, NH_2), 7.43 (m, 2H, aromatic 3-H, 5-H), 7.89 (m, 2H, aromatic 2-H, 6-H), 11.25 (s, 1H, D_2O exchange, NOH).– MS (70 eV): *m/z* (%) = 182 (15) [M^+], 152 (51) [M^+-NO], 95 (100) [4-F-ph].

2,4-Dichlorophenylazo-methanamidoxime (**3e**)

From 10.80 g (66.66 mmol) 2,4-dichloroaniline. Yellow orange crystals (ethanol/water), mp 176 °C (dec.), yield 7.46 g (48%).– Anal. $C_7H_6Cl_2N_4O$.– IR (KBr): 3484 cm^{-1}, 3453, 3370, 3291 (NH_2), 1660 (C=N).– UV (methanol): λ_{max} (log ε): 208 (4.011), 244 (3.821), 302 (3.901), 368 (4.006) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 5.88 (s, 2H, D_2O exchange, NH_2), 7.57 (dd, *J* = 9/2 Hz, 1H, aromatic 5-H), 7.65 (d, *J* = 9 Hz, 1H, aromatic 6-H), 7.92 (d, *J* = 2 Hz, 1H, aromatic 3-H), 11.67 (s, 1H, D_2O exchange, NOH).– MS (70 eV): *m/z* (%) = 236 (3), 234 (16), 232 (25) [M^+], 204 (39), 202 (58) [M^+-NO], 149 (13), 145 (100) [2,4-Cl_2-ph].

1-Naphthylazo-methanamidoxime (**3f**)

From 11.50 g (80.31 mmol) 1-naphthylamine. Red orange leaves (methanol), mp 200 °C (dec.), yield 4.30 g (25%).– Anal. $C_{11}H_{10}N_4O$.– IR (KBr): 3415 cm^{-1} (NH_2), 3304, 3147 (NH_2), 1642 (C=N).– UV (methanol): λ_{max} (log ε): 212 (4.656), 260 (4.101), 384 (4.193) nm.– ^{1}H-NMR ([D$_6$]acetone): δ = 5.91 (s, 2H, D_2O exchange, NH_2), 7.62 - 7.72 (m, 3H, aromatic 3-H, 6-H, 7-H), 7.87 (dd, *J* = 7/1 Hz, 1H, aromatic 4-H), 8.05 (m, 1H, aromatic 5-H), 8.15 (d, *J* = 8 Hz, 1H, aromatic 2-H), 8.97 (m, 1H, aromatic 8-H).– MS (70 eV): *m/z* (%) = 214 (8) [M^+], 184 (15) [M^+-NO], 127 (100) [naphthyl].

2-(Amino-hydroxyimino-methyl-azo)-benzene-hydroxamic acid (**3g**)

From 7.35 g (44.49 mmol) 2-amino-benzoic acid ethyl ester. Orange crystals (ethanol/water), mp 130 °C (dec.), yield 2.88 g (29%).– Anal. $C_8H_9N_5O_3$.– IR (KBr): 3367 cm^{-1}, 3167 (NH_2), 2842 (OH), 1643 (C=N, C=O).– UV (methanol): λ_{max} (log ε) = 294 (3.865), 360 (3.967) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 5.87 (s, 2H, D_2O exchange, NH_2), 7.60 (s, 4H, aromatic H), 9.25 (s, 1H, D_2O exchange, N*H*OH), 10.87 (s, 1H, D_2O exchange, = NOH), 11.37 (s, 1H, D_2O exchange, NH-O*H*).– MS ([+]-FAB, DMSO/glycerol): *m/z* (%) = 224 (46) [M+H$^+$], 193 (24) [M$^+$-NO], 191 (100) [M$^+$-NHOH].

O-Acylderivatives (**4**) *of arylazoamidoximes* (**3**)

10 mmol amidoxime are dissolved or suspended in 50 mL ether. 10 mmol triethylamine and 10 mmol chloroformic acid phenyl ester are added. The mixture is stirred until the crystalline educt (**3**) has been converted (≈ 30 min) to an amorphous product (**4**). This is sucked off, dried, and recrystallized. It should be stored protected from light.

0-(Phenoxycarbonyl)-phenylazo-methanamidoxime (**4a**)

From 3.50 g (21.32 mmol) **3a**. Thin yellow needles (ethyl acetate/hexane), mp 141 °C, yield 2.06 g (34%).– Anal. $C_{14}H_{11}N_4O_3$.– IR (KBr): 3458 cm^{-1}, 3361 (NH_2) 1766 (C=O), 1652 (C=N).– UV (methanol): λ_{max} (log ε) = 308 (4.106) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 7.23 (s, 2H, CF_3COOD exchange, NH_2), 7.36 (m, 3H, aromatic 2′-H, 4′-H, 6′-H), 7.51 (m, 2H, aromatic 3′-H, 5′-H), 7.67 (m, 3H, aromatic 3-H, 4-H, 5-H), 7.94 (dd, *J* = 7/2 Hz, 2H, aromatic 2-H, 6-H).– MS ([+]-FAB, DMSO/glycerol): *m/z* (%) = 285 (14) [M+H$^+$], 77 (100) [phenyl].

4-Methoxyphenylazo-O-(phenoxycarbonyl)-methanamidoxime (**4b**)

From 2.80 g (14.42 mmol) **3b**. Yellow orange crystals (DMF/water), mp 173 °C (dec.), yield 3.22 g (71%).– Anal. $C_{15}H_{14}N_4O_4$.– IR (KBr): 3481 cm^{-1}, 3341 (NH_2), 1778 (C=O), 1656 (C=N).– UV (methanol): λ_{max} (log ε) = 244 (3.792), 350 (4.131) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 3.90 (s, 3H, OCH_3), 7.10 (s, 2H, CF_3COOD exchange, NH_2), 7.27 ("d", *J* = 9 Hz, 2H, aromatic 3-H, 5-H), 7.35 (m, 3H, aromatic 2′-H, 4′-H, 6′-H), 7.50 (m, 2H, aromatic 3′-H, 5′-H), 7.95 ("d", *J* = 9 Hz, 2H, aromatic 2-H, 6-H).– MS ([+]FAB, DMSO/glycerol): *m/z* (%) = 315 (12) [M+H$^+$].

4-Chlorophenylazo-O-phenoxycarbonyl-methanamidoxime (**4c**)

From 8.20 g (41.29 mmol) **3c**. Yellow orange leaves (ethyl acetate/hexane), mp 172 °C (dec.), yield 8.03 g (61%).– Anal. $C_{14}H_{11}ClN_4O_3$.– IR (KBr): 3472 cm^{-1}, 3331 (NH_2), 1779 (C=O), 1658 (C=N).– UV (methanol): λ_{max} (log ε) = 318 (4.150) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 7.24 (s, 2H, CF_3COOD exchange, NH_2), 7.34 (m, 3H, aromatic 2′-H, 4′-H, 6′-H), 7.50 (m, 2H, aromatic 3′-H, 5′-H), 7.74 ("dd", *J* = 8/2 Hz, 2H, aromatic 3-H, 5-H), 7.86 ("dd", *J* = 8/2 Hz, aromatic 2-H, 6-H).– MS ([+]-FAB, DMSO/glycerol): *m/z* (%) = 321 (10), 319 (24) [M+H$^+$], 113 (33), 111 (100) [4-Cl-ph].

4-Fluorophenylazo-O-(phenoxycarbonyl)-methanamidoxime (**4d**)

From 1.75 g (9.60 mmol) **3d**. Yellow orange needles (ethanol), mp 151 °C (dec.), yield 1.42 g (49%).– Anal. $C_{14}H_{11}FN_4O_3$.– IR (KBr): 3473 cm^{-1}, 3328 (NH_2), 1782 (C=O), 1658 (C=N).– UV (methanol): λ_{max} (log ε) = 314 (4.176) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 7.21 (s, 2H, CF_3COOD exchange, NH_2), 7.35 (m, 3H, aromatic 2′-H,, 4′-H,, 5′-H), 7.51 (m, 4H, aromatic 3-H, 5-H, 3′-H, 5′-H), 8.02 ("dd", *J* = 9/5 Hz, 2H, aromatic 2-H, 6-H).– MS ([+]-FAB, DMSO/3-nitrobenzyl alcohol): *m/z* (%) = 303 (58) [M+H$^+$], 95 (100) [4-F-ph].

2,4-Dichlorophenylazo-O-(phenoxycarbonyl)-methanamidoxime (**4e**)

From 1.20 g (5.19 mmol) **3e**. Small yellow crystals (ethanol/water), mp 171 °C (dec.), yield 1.25 g (69%).– Anal. $C_{14}H_{10}Cl_2N_4O_3$.– IR (KBr): 3450 cm^{-1}, 3344 (NH_2) 1767 (C=O), 1655 (C=N).– UV (methanol): λ_{max} (log ε) = 242 (3.836), 330 (4.095).– ^{1}H-NMR ([D$_6$]DMSO): δ = 7.15 (s, 2H, CF_3COOD exchange, NH_2), 7.37 (m, 3H, aromatic 1′-H, 4′-H, 6′-H), 7.51 (m, 2H, aromatic 3′-H, 5′-H), 7.62 (dd, *J* = 9/2 Hz, 1H, aromatic 5-H), 7.72 (d, *J* = 9 Hz, 1H, aromatic 6-H), 8.00 (d, *J* = 2 Hz, 1H, aromatic 3-H).– MS ([+]-FAB, DMSO/glycerol): *m/z* (%) = 355 (13), 353 (18) [M+H$^+$], 147 (72), 145 (100) [2,4-Cl_2-ph].

1-Naphthylazo-0-(phenoxycarbonyl)-methanamidoxime (**4f**)

From 2.00 g (9.34 mmol) **3f**. Red brown crystal (ethanol/water), mp 153 °C. (dec.), yield 1.81 g (58%).– Anal. $C_{18}H_{14}N_4O_3$.– IR (KBr): 3456 cm^{-1}, 3341 (NH_2), 1773 (C=O), 1646 (C=N).– UV (methanol): λ_{max} (log ε) = 264 (4.035), 384 (3.937) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 7.37 (s, 2H, CF_3COOD exchange, NH_2), 7.40 (m, 3H, aromatic 2′-H, 4′-H, 6′-H), 7.52 (m, 2H, aromatic 3′-H, 5′-H), 7.68–7.90 (m, 3H, aromatic 3-H, 6-H, 7-H), 7.92 (d, *J* = 7 Hz, 1H, aromatic 4-H), 8.11 (d, *J* = 8 Hz, 1H, aromatic 5-H), 8.29 (d, *J* = 8 Hz, 1H, aromatic 2-H), 9.17 (d, *J* = 8 Hz, 1H, aromatic 8-H).– MS ([+]-FAB, DMSO/glycerol): *m/z* (%) = 335 (6) [M+H$^+$], 127 (100) [naphthyl].

General procedure for the preparation of 3-arylazo-1,2,4-oxadiazol-5-ones (**5**)

10 mmol of the diester **4** are suspended in 100 mL of 5% sodium hydroxide solution. The suspension is warmed to 100 °C and stirred until a solution is obtained. After cooling to room temp. the solution is acidified with 20% hydrochloric acid. The precipitate is sucked off, washed with hydrochloric acid (20%) and then with water until pH 7 is reached. The residue is recrystallized.

3-Phenylazo-1,2,4-oxadiazol-5-one (**5a**)

From 3.50 g (12.31 mmol) **4a**. Yellow crystals (acetone/ water), mp 155 °C (dec.), yield 1.64 g (70%).– Anal. $C_8H_6N_4O_2$.– IR (KBr): 3116 cm^{-1} (NH), 1779, 1762 (C=O).– UV (methanol): λ_{max} (log ε) = 322 (4.107) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 7.70 - 7.91 (m, 3H, aromatic 3-H, 4-H, 5-H), 7.99 (d, *J* = 7 Hz, 2H, aromatic 2-H, 6-H), 13.43 (s, 1H, D_2O exchange, NH).

3-(4-Methoxyphenylazo)-1,2,4-oxadiazol-5-one (**5b**)

From 4.50 g (14.32 mmol) **4b**. Orange neddles (DMF/water), mp 165 °C (dec.), yield 1.67 g (53%).– Anal. $C_9H_8N_4O_3$.– IR (KBr): 3414 cm^{-1} (NH), 1770 (C=O).– UV (methanol): λ_{max} (log ε) = 246 (3.832), 360 (4.250) nm.– ^{1}H-NMR ([D$_6$]acetone): δ = 3.99 (s, 3H, OCH_3), 7.21 (m, 2H, aromatic 3-H, 5-H), 8.00 (m, 2 Hz, aromatic 2-H, 6-H).– MS (70 eV): *m/z* (%) = 220 (100) [M$^+$].

3-(4-Chlorophenylazo)-1,2,4-oxadiazol-5-one (**5c**)

From 4.00 g (12.55 mmol) **4c**. Yellow crystals (acetone/water), mp 185 °C (dec.), yield 1.18 g (42%).– Anal. $C_8H_5ClN_4O_2$.– IR (KBr): 3415 cm^{-1} (NH_2), 1820, 1788 (C=O).– UV (methanol): λ_{max} (log ε) = 232 (3.832), 328 (4.088) nm.– ^{1}H-NMR([D$_6$]DMSO): δ = 7.77 ("d", *J* = 9 Hz, 2H, aromatic 3-H, 5-H), 7.99 ("d", *J* = 9 Hz, 2H, aromatic 2-H, 6-H).– MS (70 eV): *m/z* (%) = 226 (8), 224 (23) [M$^+$], 113 (34), 111 (100) [4-Cl-ph].

3-(4-Fluorophenylazo)-1,2,4-oxadiazol-5-one (**5d**)

From 1.10 g (3.64 mmol) **4d**. Yellow crystals (ethanol/water) mp 187–195 °C (dec.), yield 0.39 g (51%).– Anal. $C_8H_5FN_4O_2$.– IR (KBr) = 3434 cm^{-1} (NH), 1801, 1787 (C=O).– UV (methanol): λ_{max} (log ε) = 226 (4.135), 304 (4.189) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 7.54 (m, 2H, aromatic 3-H, 5-H), 8.07 (m, 2H, aromatic 2H, 6-H).– MS (70 eV): *m/z* (%) = 208 (33) [M$^+$], 95 (100) [4-F-ph].

3-(2,4-Dichlorophenylazo)-1,2,4-oxadiazol-5-one (**5e**)

From 1.25 g (3.54 mmol) **4e**. Beige compound (dioxane/water), mp 205 °C (dec.), yield 0.50 g (54%).– Anal. $C_8H_4Cl_2N_4O_2$.– IR (KBr): 3225 cm^{-1} (NH), 1805 (C=O).– UV (methanol): λ_{max} (log ε) = 244 (3.869), 336 (4.139) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 7.64 (dd, *J* = 7/2 Hz, 1H, aromatic 5-H), 7.78 (d, *J* = 9 Hz, 1H, aromatic 6-H), 8.03 (d, *J* = 2 H, 1H, aromatic 3-H).– MS (70 eV): *m/z* (%) = 262 (4), 260 (13), 258 (34) [M$^+$], 149 (13), 147 (66), 145 (100) [2,4-Cl_2-ph].

3-(1-Naphthylazo)-1,2,4-oxadiazol-5-one (**5f**)

From 4.95 g (14.81 mmol) **4f**. Orange crystals (DMF/water), mp 190 °C (dec.), yield 0.14 g (4%).– Anal. $C_{12}H_8N_4O_2$.– IR (KBr): 3227 cm^{-1} (NH), 1790, 1774 (C=O).– UV (methanol): λ_{max} (log ε) = 266 (4.020), 392 (3.956) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 7.71 - 7.83 (m. 3H, aromatic 3-H, 6-H, 7-H), 8.03 (d, *J* = 8 Hz, 1H, aromatic 4-H), 8.13 (d, *J* = 8 Hz, 1H, aromatic 5-H), 8.38 (d, *J* = 8 Hz, 1H, aromatic 2-H), 9.13 (d, *J* = 8 Hz, 1H, aromatic 8-H), 13.50 (s, 1H, D_2O exchange, NH).– MS (70 eV): *m/z* (%) = 240 (11) [M^+], 127 (100) [naphthyl].

Reaction of phenylazoamidoxime **3a** *with hydrochloric acid*

Compound **3a** is dissolved in little diluted hydrochloric acid and kept in the refrigerator. After a few minutes the formation of crystals begins. The precipitate **7** is sucked off after 5 h. The crystals need no further purification.

1-(4-Chlorophenyl)-2-methanamidoximyl)-hydrazine hydrochloride (**7**)

From 0.30 g (1.83 mmol) **3a**. Crystals, mp 158–170 °C (dec.), yield 0.34 g (78%).– Anal. $C_7H_{10}Cl_2N_4O$.– IR (KBr): 3500–2800 cm^{-1} (NH, OH), 1647 (C=N).– UV (methanol): λ_{max} (log ε) = 238 (4.217), 290 (3.317) nm.– ^{1}H-NMR ([D$_6$]DMSO): δ = 6.77 ("d", *J* = 9 Hz, aromatic 2-H, 6-H), 7.28 ("d", *J* = 9 Hz, 2H, aromatic 3-H, 5-H), 7.93 (s, 2H, D_2O exchange, NH_2), 8.32 (s, 1H, D_2O exchange, NH), 9.88 (s, 1H, D_2O exchange, NH), 9.99 (s, 1H, D_2O exchange, NH), 10.93 (s, 1H, D_2O exchange, NOH).– MS ([+]-FAB, DMSO/glycerol): *m/z* = (%) = 203 (33), 201 (100) [$M+H^+$].

Biology

Platelet aggregation

The platelet aggregation experiments (Born test) were carried out as usual[17].

Thrombosis model

The thrombosis experiment were performed as already described in detail[8].

Blood pressure measurements

The decrease of blood pressure in SHR animals were described quite recently[12].

Experiments with rat liver microsomes
Reagents and biochemicals

Catalase, superoxide dismutase and human hemoglobin were obtained from Sigma Chemical Co. (Deisenhofen, FRG). Oxyhemoglobin was prepared from hemoglobin as described before [18]. NADPH (tetrasodium salt) as well as all other chemicals and solvents were from Merck (Darmstadt, FRG) unless otherwise stated.

Preparation of microsomal fractions

Livers from control rats and rats pretreated with dexamethasone were obtained as described previously[2].

Analytical procedures

The cytochrome P450 content was analysed using the method of Omura and Sato [19]. The protein content was determined by the method of Gornall et al. [20] (reagent kit, Merck, Darmstadt, FRG). Bovine serum albumin was used as standard.

Oxyhemoglobin assay

The incubation mixture (0.6mL) contained the following components: 50mM phosphate buffer (pH7.4), 100 µM substrate, 8.7µM oxyhemoglobin and 0.4 mM NADPH.

The formation of NO was measured using a difference-spectrophotometric method [21]. It is based on the quantitative oxidation of oxyhemoglobin by nitric oxide to methemoglobin, with resulting shift in wavelength in the Soret region. The NO formation was quantified by measuring the decrease in absorbance at 576 nm, because at the usual wavelength of 401 or 420 nm, the influence of NADPH and the substrate were too high. The assay was carried out with a Beckman DU 7000 Diode array spectrophotometer with temperature control at 37 °C.

Incubations

All components without microsomes were mixed in a vessel, pipetted into the cuvettes and kept at at 37 °C. The reaction was started by the addition of microsomes equivalent to 0.86 nmol P-450 final concentration and was continued over 12 minutes. The blank consisted of the normal incubation mixture with denaturated microsomes.

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☆ Dedicated to Prof Dr. W. Wiegrebe on the occasion of his 65th birthday.

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