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Diacetyldiamidoximeester of Pentamidine, a Prodrug for Treatment of Protozoal Diseases: Synthesis, in vitro and in vivo Biotransformation

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Pentamidine is an effective antimicrobial agent. To increase its poor oral bioavailability due to the strong basic amidine functionality, the less basic O-acetylamidoxime prodrug, the diacetyldiamidoximeester, was used, which has greatly improved lipophilicity. The objectives of this investigation were the synthesis of all potential metabolites of the double prodrug, the conformational analysis of its structure, and to study the in vitro and in vivo biotransformation by ester cleavage and N-reduction to pentamidine via four intermediate metabolites. The biotransformation of diacetyldiamidoximeester to pentamidine involving the reduction of the amidoxime function and the ester cleavage could be demonstrated. The kinetic parameters were determined. Amidoximes were efficiently metabolized by several enzyme systems located in microsomes and mitochondria of different organs including the final formation of the active metabolite pentamidine. The formation of pentamidine after oral administration of the diacetyldiamidoximeester to rats could be demonstrated as well.

Introduction

Protozoans cause several diseases such as leishmaniasis, malaria, toxoplasmosis, and trypanosomiasis, known as sleeping sickness, in tropical and subtropical countries. Pentamidine **1**, an aromatic diamidine, is used in the treatment of leishmaniasis and the first, hemolymphatic stage of trypanosomiasis. The second stage of trypanosomiasis begins when the parasite invades the central nervous system (CNS). The neurological phase of trypanosomiasis is medicated by melarsoprol, an arsenical derivative, with several side effects.[1] Eflornithine is the alternative to melarsoprol treatment, which is effective against *Trypanosoma brucei gambiense* infections at the second stage.[1] A cerebral course of disease is also known for toxoplasmosis.[2] Lindsay et al.[3] demonstrated the activity of pentamidine **1** against *Toxoplasma gondii* in in vitro studies. Furthermore, pentamidine **1** is established in the treatment of *Pneumocystis* pneumonia (PcP). HIV infected persons are commonly afflicted with this opportunistic infection.[4] Bell et al.[5] demonstrated in vivo the activity of pentamidine **1** against *Plasmodium falciparum*, so diamidines are also suitable for novel antimalarial compounds.

Amidines are protonated, highly mesomerically stabilized and very hydrophilic under physiological conditions because of their strong basicity,[6] resulting in poor oral bioavailability.[7] Application of pentamidine **1** for the treatment of African sleeping sickness at the second stage and toxoplasmosis, failed so far because of its inability to cross the blood brain barrier (BBB). However, the amidine functional group is essential for activity against the protozoans as it interacts with the parasite's DNA to inhibit its reproduction.[8]

The in vitro and in vivo reduction of amidoximes to amidines was independently demonstrated by Hauptmann et al.[9] and Clement et al.[10] in 1988. Hence the prodrug principle amidoximes were developed instead of amidines.[11,12] This is possible because N-hydroxylated amidines are less basic ($pK_a = 5$), are unprotonated under physiological conditions, and can be absorbed as the free base from the gastrointestinal tract. The enzymatic basis of this reduction which is observed in every organ and cell organelle of all species studied to date, has been the subject of numerous studies.[12,13] So far no drug–drug interactions or toxic effects can be related to this unusual transformation of amidoximes (*N*-hydroxyamidines) to amidines. Thus, the prodrug principle amidoximes instead of amidines has been applied to drug candidates such as sibrafiban[14] and ximelagatran,[15,16] the first orally available thrombin inhibitor on the market. Tidwell and his group transferred the prodrug principle onto 2,5-bis(4-amidinophenyl)furan DB75, a pentamidine analogue, and developed *O*-alkoxyamidine prodrugs such as DB289.[17] DB75 and DB289 exhibit a broad antimicrobial spectrum,[5,18,19] similar to pentamidine **1**. Clement and Raether demonstrated the activity of pentamidine **1** and its amidoximes (**2, 3**) against *Trypanosoma* spp. and *Leishmania donovani*.[20] Pentamidine derivatives are the first *N*-hydroxyamidine prodrugs of amidines[11] described.

Esterifications of diamidoximes forms the diacetyldiamidoximeester **4**, a double prodrug of pentamidine **1**. The diacetyldiamidoximeester **4** is also unprotonated under physiological conditions. The activity of **4** in vivo against a *Pneumocystis jiroveci* infection of rats after oral administration, and

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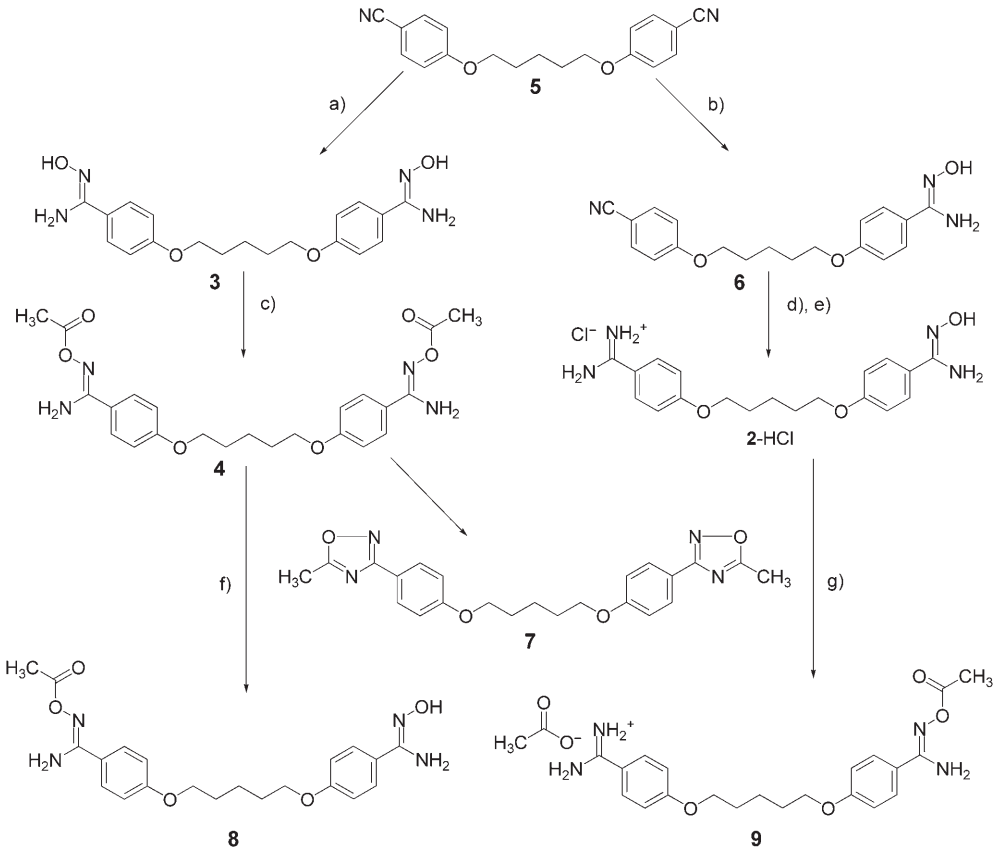
against *Trypanosoma* spp. in mice and *Leishmania donovani* in hamster after s.c. application could be demonstrated.[11] The activity of the diacetyldiamidoximeester **4** in rats, infected with PcP, was comparable to parenteral administrated pentamidine **1**.[11] So the oral availability of pentamidine **1** after administration of this prodrug **4** has been demonstrated.[11] In addition, lipophilicity of the diacetyldiamidoximeester **4** is increased compared to the diamidoxime **3**. Therefore, crossing of the BBB by the diacetyldiamidoximeester **4** could be possible and the double prodrug is developed to be used in the treatment of *Trypanosoma* spp. infections at second stage.

The objectives of this study were the synthesis of the potential metabolites of **4**, and the characterization of the double prodrug's structure. A further aim was to explain the ester cleavage and N-reduction of the diacetyldiamidoximeester **4** by in vitro biotransformation studies with several enzyme sources, including the final formation of the active metabolite pentamidine **1**. Additionally the oral absorption of the prodrug **4**, its activation to pentamidine **1**, and the distribution were determined by an in vivo study in rats.



Scheme 1. Synthesis of 1,5-Bis(4′-acetoxyamidinophenoxy)pentane **4** and metabolites: a) 2 NH_2OH; b) NH_2OH; c) Ac_2O; d) EtOH/HCl; e) NH_3; f) lipase; g) Ac_2O.

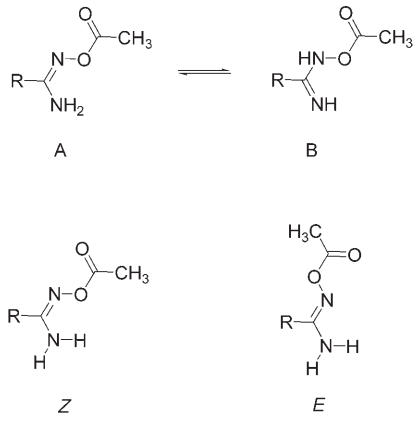
Results and Discussion

Chemistry

The diacetyldiamidoximeester **4** and its metabolites (**2**, **3**, **7**, **8**, **9**) can be synthesized according to the reaction route displayed in Scheme 1. The start of the synthesis is the dinitrile **5**,[21] which reacts with the hydroxylamine base in anhydrous ethanol.[20] Depending on the amount of hydroxylamine base the diamidoxime **3** or amidoximenitrile **6** is obtained.[20] The acetanhydride and **3** form the diacetyldiamidoximeester **4** at room temperature, which can cyclize[22,23] to dioxadiazole **7** in hot ethanol. One of the functional ester groups of **4** can be removed selectively in the presence of lipase from pig pancreas[24,25] to isolate the monoester **8**. The monoamidoxime hydrochloride **2**-HCl is synthesized from amidoximenitrile **6**[20] by Pinner synthesis.[26] Addition of acetanhydride to **2**-HCl results in the formation of the acetate of **9**.

Configurational analysis of diacetyldiamidoxime

O-acetylamidoximes may exist in the two tautomeric forms A and B (Scheme 2). Tautomer A is claimed to be the preferred form[27] with the two possible configurations *E* and *Z*. Srivastava et al.[28] demonstrated for other amidoximeesters that the *Z* configuration of tautomer A is the energetically favoured configuration with an antiperiplanar conformation for the N–O bond. The diacetyldiamidoximeester **4** was characterized by IR



Scheme 2. Tautomeric (A and B) and *E* and *Z* forms of *O*-acetylamidoximes.

and ^{1}H, ^{13}C, and ^{15}N NMR spectroscopy to confirm that **4** also prefers this conformation and configuration. The IR spectrum shows a broad signal at 1744 cm^{-1} which belongs to a carbonal of an ester group. Signals with upper wave numbers indicate asymmetric ($\nu_{as} = 3490\ cm^{-1}$) and symmetric stretching motions ($\nu_{sym} = 3342\ cm^{-1}$) of a primary amine. According to Bell et al.[27] a signal at 1614 cm^{-1} represents C=N stretching motions. Consequently the IR spectrum proves the formation of an *O*-acetylamidoxime from diamidoxime and acetanhydride. Furthermore, the preferred tautomeric form A could be demonstrated. The ^{1}H NMR spectrum confirmed these results. A singlet with a chemical shift of 2.15 ppm, assigned to three protons is consistent with the existence of an acetylester. A signal at 6.71 ppm belongs to an NH_2 group of the amidoximeester,[28] proving the absence of tautomeric structures. The ^{13}C NMR spectrum shows a signal at 167.4 ppm, for the ester carbon and for benzamidoximes which also exclusively exists in the oxime type tautomeric form. The signal at 154.96 ppm can be attributed to an oxime type carbon. In accordance with tautomer A the ^{15}N NMR spectrum is characterized by a singlet at 88.3 ppm and a triplet at 305.3 ppm, excluding the tautomer B. The coupling constant of the NH_2 group with 90 Hz is in the range of sp^2 nitrogen atoms which makes electron delocalization possible. The ^{15}N NMR spectrum does not provide any evidence for the existence of geometrical isomers (*E* or *Z*) at the carbon–nitrogen double bond. The data of this spectrum are in good agreement with the spectrum of N,N-unsubstituted amidoximes and N-monosubstituted amidoximes which all exist in the *Z* configuration.[29,30]

Biotransformation studies

Pentamidine **1** plays a prominent role in the treatment of protozoal diseases. Because of its physicochemical characteristics, especially its strong basicity, pentamidine is protonated under physiological conditions, resulting in poor oral bioavailability. N-hydroxylated derivatives of amidines (amidoximes) can improve the absorption rate from the gastrointestinal tract.[11] Amidoximes are less basic and therefore unprotonated under physiological conditions, allowing the transport of the drug across the mucosal membrane by diffusion.

This in vitro biotransformation study was directed towards the explanation of bioactivation of diacetyldiamidoximeester **4** by cleavage of the ester and N-reduction of an amidoxime function. The aim of this study was to demonstrate the formation of the active metabolite pentamidine **1** by different intermediates (**2, 3, 8, 9**). Carboxyl esterases from human and porcine microsomes, and mitochondria from liver and kidney were used. A new HPLC analytical method was developed to separate and quantify all metabolites of diacetyldiamidoximeester **4**. A representative chromatogram after the incubation of **4** with pig liver microsomes is shown in Figure 1. The retention times of the metabolites are in accordance with those of the synthetic standards.

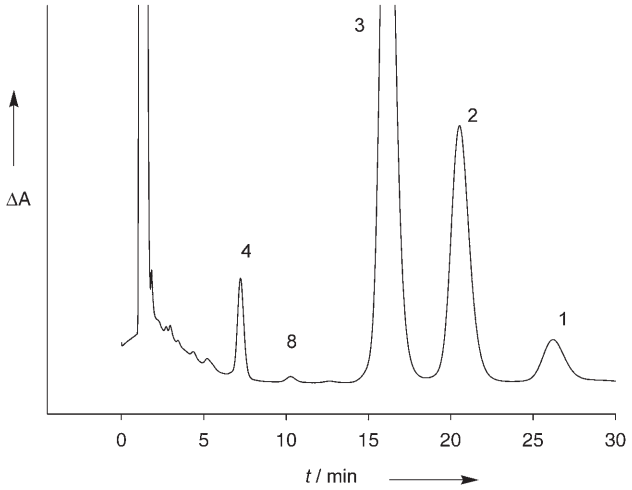


Figure 1. Representative HPLC trace after incubation of diacetyldiamidoximeester **4** with microsomal preparations from pig liver homogenate; monoester **8**, diamidoxime **3**, monoamidoxime **2**, pentamidine **1**. The incubation mixture consisted of microsomal protein (0.25 mg mL^{-1}) from pig liver, diacetyldiamidoximeester **4** (0.5 mM) as substrate, and NADH (1 mM) as cosubstrate in 100 mM potassium phosphate buffer pH 6.3. For incubation conditions and HPLC analysis, see Experimental Section.

In vitro ester cleavage was demonstrated with unspecific carboxyl esterases from pig liver. Conversions of diacetyldiamidoximeester **4** and monoester **8** to diamidoxime **3** were determined as well as the conversion of amidineamidoximeester **9** to monoamidoxime **2**. Ester cleavages were linear for more than 60 min and followed Michaelis–Menten kinetics (Table 1). Optimized concentrations for esterases (0.5 U) and for substrates (2 mM) were determined. At high pH values product formation increased slightly, and finally a physiological pH of 7.4 was selected for further studies. Ester cleavages by unspecific carboxyl esterases were not dependent on cosubstrates such as NADH, NADPH, and $MgCl_2$. The diacetyldiamidoximeester **4** was easily metabolized to the diamidoxime **3** by a two-step ester cleavage reaction (data not shown). The monoester **8** was formed by a single ester cleavage reaction, but was barely detectable.

Table 1. Kinetic parameters of the ester cleavages and reductions.

	K_m [mM]	V_{max} [nmol min^{-1} (mg protein)$^{-1}$]	V_{max}/K_m^{-1} [min (mg protein)$^{-1}$]
Ester Cleavages			
diacetyldiamidoximeester	27.93	1250.0	4.48×10^{-5}
monoester	1.85	256.41	1.39×10^{-4}
amidineamidoximeester	6.57	1428.57	2.17×10^{-4}
N-Reductions			
diamidoxime	0.295	0.2	6.78×10^{-7}
monoamidoxime	0.551	0.83	1.51×10^{-6}

Rather **8** was immediately hydrolyzed to the diamidoxime **3** after its formation. Ester cleavages could even be observed in the absence of carboxyl esterases, caused by chemical hydrolysis in aqueous medium. Incubation of **4** in plasma demonstrated the fast conversion into **8** and **3** within a period of 120 min (Figure 2). 30% of the diacetyldiamidoximeester **4** remained after 7 h, while the formation of the diamidoxime **3** increased to 80%, based on the initial concentration of **4**. The ester cleavage of the diacetyldiamidoximeester **4** was found to be even higher when plasma was replaced by 1 N HCl.

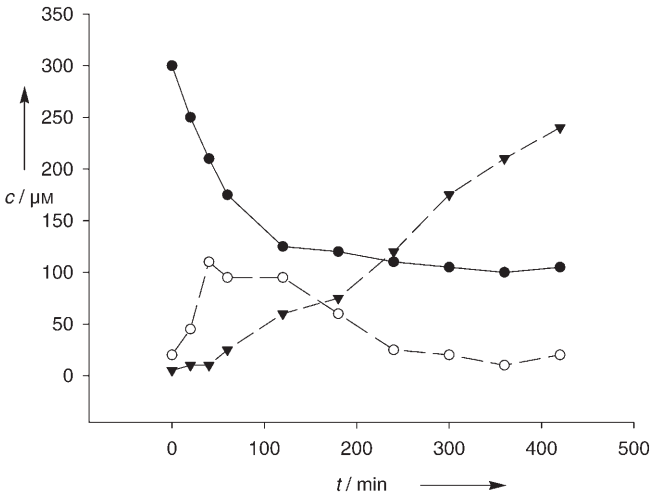


Figure 2. Stability of diacetyldiamidoximeester **4** in human plasma. The incubation mixture consisted of the diacetyldiamidoximeester **4** (0.3 mM) added to a total volume of 100 μL of human plasma. For incubation conditions and HPLC analysis, see Experimental Section; diacetyldiamidoximeester ●, monoester ○, diamidoxime ▼.

N-reduction of diamidoxime **3** and monoamidoxime **2** to pentamidine **1** was demonstrated by microsomes and mitochondria of liver and kidney from human and pig (Table 2, 3). Conversions were linear up to 60 min and followed Michaelis–Menten kinetics (Table 1). N-reductions required NADH or NADPH as cosubstrate, but NADH increased conversion rates compared to NADPH (Table 4). The optimized pH value for N-reductions was pH 6.3. Formation of pentamidine **1** from monoamidoxime **2** showed high conversion rates, especially with preparations from pig kidney. The N-reduction of diamidoxime **3** to pentamidine **1** was incomplete when using human proteins, remaining at the level of monoamidoxime **2** (Table 2). The ability of human tissue proteins to reduce amidoximes could be demonstrated by the formation of pentamidine **1** from **2**, shown in Table 3. A lack in quality of human tissues, from which microsomes and mitochondria are extracted, could be a reason for lower conversion rates. Tissues were obtained from patients suffering from cancer or hepatitis, resulting in lower or modified enzyme levels in comparison with healthy persons.

The N-reduction of the monoester **8** is possible. However, no amidineamidoximeester **9** with a retention time of 16.2 ± 0.5 min was detectable when incubating **8** with microsomes and mitochondria from human or pig. The monoester **8** was hydrolyzed rapidly to the diamidoxime **3** by carboxyl esterases or chemical hydrolysis, and the N-reduction of **8** could take place. If the amidineamidoximeester **9** were to be formed as an intermediate during the incubation with microsomes or mitochondria it would be hydrolyzed quickly. This explains why **9** was not observed during any of the incubations.

The complete activation of the double prodrug **4** via four intermediates to pentamidine **1** was also examined. The activation of the double prodrug diacetyldiamidoximeester **4** up to

Table 2. In vitro reduction of diamidoxime **3** to monoamidoxime **2** and pentamidine **1** by liver and kidney microsomes and mitochondria of human and pig.

Enzyme Sources	Monoamidoxime	Pentamidine
	[nmol min^{-1} (mg protein)$^{-1}$][a]	
Pig liver microsomes	0.69 ± 0.28	0.97 ± 0.07
Human liver microsomes	0.25 ± 0.05	ND
Pig liver mitochondria	0.88 ± 0.07	5.08 ± 0.17
Human liver mitochondria	0.55 ± 0.15	ND
Pig kidney microsomes	0.82 ± 0.26	6.43 ± 0.40
Human kidney microsomes	0.54 ± 0.08	ND
Pig kidney mitochondria	0.73 ± 0.05	4.78 ± 0.16
Human kidney mitochondria	0.37 ± 0.04	ND

[a] Conversation rates are means ± SD of three determinations; ND: not detectable.

Table 3. In vitro reduction of monoamidoxime **2** to pentamidine **1** by liver and kidney microsomes, and mitochondria of human and pig.

Enzyme Sources	Pentamidine
	[nmol min^{-1} (mg protein)$^{-1}$][a]
Pig liver microsomes	6.67 ± 0.48
Human liver microsomes	1.24 ± 0.34
Pig liver mitochondria	13.5 ± 1.8
Human liver mitochondria	1.59 ± 0.20
Pig kidney microsomes	20.2 ± 3.0
Human kidney microsomes	5.2 ± 1.5
Pig kidney mitochondria	22.8 ± 4.9
Human kidney mitochondria	2.07 ± 0.38

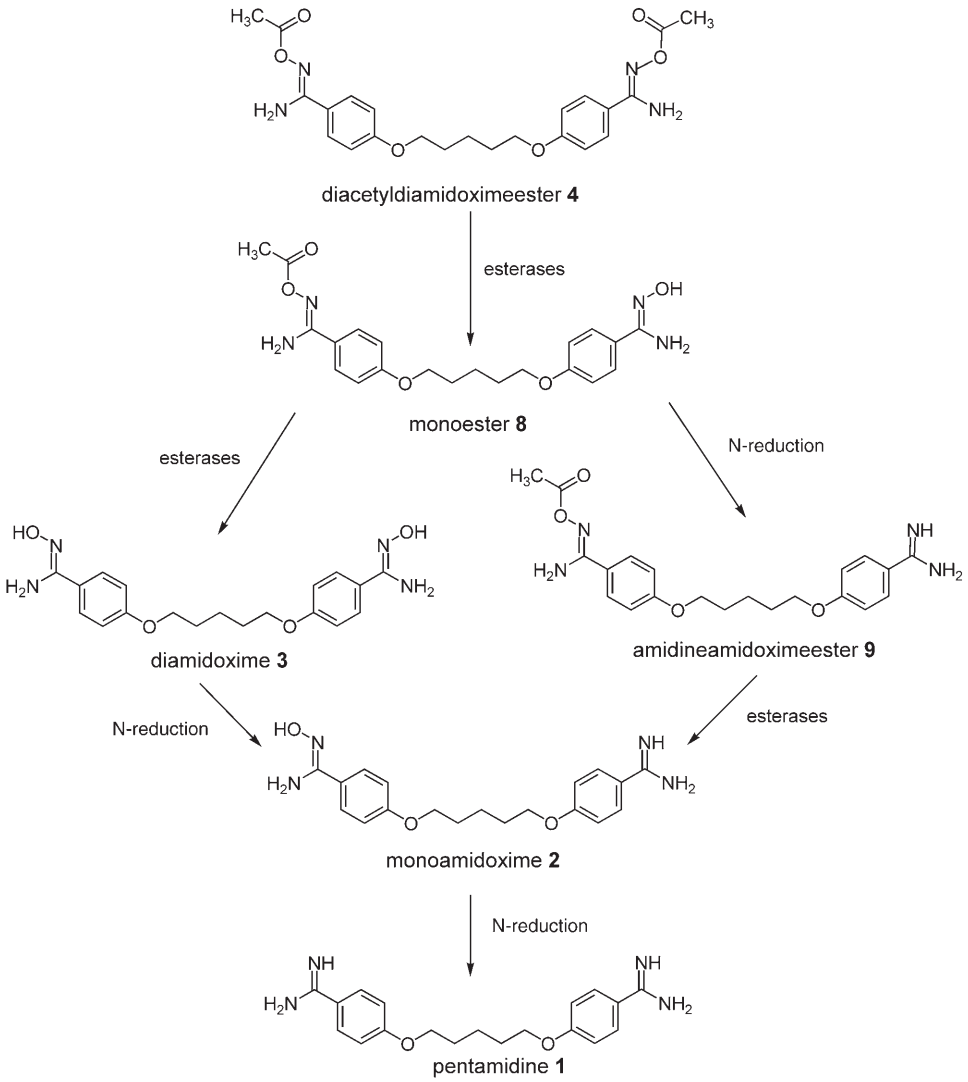
[a] Conversation rates are means ± SD of three determinations.

Table 4. Characterization of the reduction of diamidoxime **3** and monoamidoxime **4** depending on cosubstrates NADH and NADPH.

	Monoamidoxime	Pentamidine
	[nmol min^{-1} (mg protein)$^{-1}$][a]	
Diamidoxime		
standard incubation mixture	0.18 ± 0.01	
without NADH	ND	
without NADH/with NADPH	0.12 ± 0.01	
Monoamidoxime		
standard incubation mixture		0.40 ± 0.01
without NADH		ND

[a] Conversation rates are means ± SD of four determinations; ND: not detectable.

pentamidine **1** was catalyzed by all enzyme sources from the pig (Figure 1, Table 5). Pentamidine **1** could not be detected after incubations of **4** with some human enzyme preparations (liver microsomes and mitochondria, kidney mitochondria), but was shown by human kidney microsomes. Mitochondria might be unable to activate the double prodrug as esterases have not been described to occur in mitochondria. In summary the biotransformation of diacetyldiamidoximeester **4** to pentamidine **1** involving the reduction of the amidoxime function and the ester cleavage could be demonstrated in particular by microsomal preparations, as shown in Scheme 3. The double prodrug **4** could easily be activated by enzymes present in several organs and cell organelles. Currently several enzyme systems reducing N-hydroxylated amidines have been reported. One is located in adipose tissue,[31] one is a two-component system composed of cytochrome b_5 and cytochrome b_5 reductase,[32,33] and one is a mitochondrial and microsomal three-component system, which was presented in preliminary studies.[13] This third component exhibits increased conversion rates in comparison to the two-component system, and it is supposed to be a CYP450 enzyme in microsomal fractions[13] and a desaturase in fat tissue.[31] However, in mitochondria the third component was found not to be a CYP450 enzyme or a desaturase.[34,35]



Scheme 3. Metabolic pathway of diacetyldiamidoximeester **4** to pentamidine **1**.

Boykin et al.[17] developed the 2,5-bis(4-amidinophenyl)furan-bis-*O*-methylamidoxime (DB289), a *N*-methoxy prodrug of 2,5-bis(4-amidinophenyl)furan (DB75), a pentamidine analogue. The activation of the double prodrug DB289 is complex, an oxidative O-demethylation is followed by a N-reduction via sever-

Table 5. In vitro bioactivation of the diacetyldiamidoximeester **4** via monoester **8**, diamidoxime **3**, and monoamidoxime **2** to pentamidine **1** by liver and kidney microsomes and mitochondria of human and pig.

Enzyme Source	Monoester	Diamidoxime	Monoamidoxime	Pentamidine
		[nmol min^{-1} (mg protein)$^{-1}$][a]		
Pig liver microsomes	0.09 ± 0.04	1.69 ± 0.10	1.39 ± 0.09	1.161 ± 0.005
Human liver microsomes	ND	3.33 ± 0.66	0.59 ± 0.10	ND
Pig liver mitochondria	ND	0.38 ± 0.02	0.05 ± 0.01	0.54 ± 0.10
Human liver mitochondria	ND	2.29 ± 0.64	0.63 ± 0.09	ND
Pig kidney microsomes	0.002 ± 0.015	0.62 ± 0.05	0.05 ± 0.01	2.05 ± 0.13
Human kidney microsomes	ND	1.08 ± 0.06	0.81 ± 0.03	0.47 ± 0.02
Pig kidney mitochondria	0.47 ± 0.15	0.31 ± 0.08	0.04 ± 0.01	0.10 ± 0.02
Human kidney mitochondria	0.28 ± 0.03	0.160 ± 0.001	0.096 ± 0.008	ND

[a] Conversation rates are means ± SD of three determinations; ND: not detectable.

al intermediates.[36,37,38] The O-demethylation of DB289 is catalyzed by CYP1A2 and CYP3A4.[32] Consequently relevant drug–drug interactions can be expected, for example in combination with HIV Protease Inhibitors. In contrast the initial activation of diacetyldiamidoximeester **4** by ester cleavage proceeds quickly and in great quantities. Furthermore, the occurrence of drug–drug interactions is highly unlikely and has never been reported for esterase catalyzed reactions.

The aim of the in vivo biotransformation study was to demonstrate the oral absorption of **4** and to determine plasma, tissue, and urine levels of oral prodrug-derived pentamidine **1** in rats. In addition, pentamidine **1** was applied intraperitoneally (i.p., 5 mg kg^{-1}) to determine the bioavailability of **4**. After oral administration of 20 and 50 mg kg^{-1} of the diacetyldiamidoximeester **4** neither pentamidine **1** nor other intermediates (**2, 3, 8, 9**) were detectable in plasma. In contrast, small quantities of pentamidine **1** could be detected after i.p. administration, as shown in Figure 3. The highest mean plasma concentration (C_{max}) of pentamidine **1** was 1.15 ± 0.04 nmol mL^{-1} pentamidine after 30 min and the half life was 242 min, which is consistent with Terlinden and Römer[39] and Conte et al.[40] Plasma concentrations fell below detection within 24 h. The mean plasma clearance was 2.86 L kg^{-1} h^{-1}. In contrast, the active metabolite was detected in tissue homogenate after p.o. and i.p. administration of the diacetyldiamidoximeester **4** and pentamidine **1**, respectively (Table 6). Furthermore, pentamidine **1** was found in great quantities in urine samples (data not shown). In summary the oral bioavailability of the prodrug **4** and its biotransformation into the active drug pentamidine **1** could be demonstrated, confirming the efficacy of **4** against *Pneumocystis jiroveci* infections of rats after oral administration.[11] After formation in lung, liver, and kidney, pentamidine **1** is either accumulated in these tissues or shows high renal elimination rates, resulting in low plasma levels. Relative oral bioavailability can be specified for kidneys, liver, and lung (7.5 %, 28.5 %, and 9.2 %, respectively).

In addition to oral bioavailability, it would be a great advantage if pentamidine **1** could be delivered to the brain to treat the second stage of sleeping sickness. At that stage the parasites are present within the central nervous system (CNS), causing characteristic signs and symptoms: confusion, sensory disturbances, poor coordination, and disturbance of the sleep cycle.[41] The diacetyldiamidoximeester **4** exhibits an increased lipophilicity in comparison to the diamidoxime **3**, making penetration through the blood brain barrier possible. The N-reduction of benzamidoxime as a model compound was demonstrated with brain microsomes.[34] Esterases are also present in the brain.[42] So the activation of **4** is possible in the brain. Although most of **4** will be transformed into pentamidine **1**, sufficient amounts for penetration into the brain may be left.

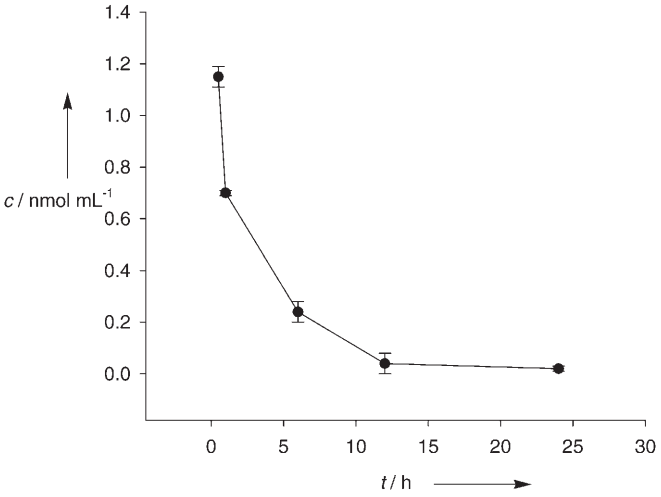


Figure 3. Plasma concentrations of pentamidine **1** following a single intraperitoneal dose (5 mg kg^{-1}).

Table 6. Pentamidine **1** concentrations detected in different tissue homogenates after oral administration of the diacetyldiamidoximeester **4** and intraperitoneal administration of pentamidine **1**.

	Diacetyldiamidoximeester[a]	Diacetyldiamidoximeester[b] [nmol (g tissue homogenate)$^{-1}$][d]	Pentamidine[c]
liver	37.5 ± 20.0	62.66 ± 29.13	27.5 ± 15.0
kidney	52.5 ± 15.0	102.4 ± 52.3	155.0 ± 22.5
lung	7.5 ± 2.5	18.13 ± 8.94	20.0 ± 15.0

[a] 20 mg kg^{-1} p.o. [b] 50 mg kg^{-1} p.o. [c] 5 mg kg^{-1} i.p. [d] Concentrations are means ± SD of three determinations of tissue homogenates from five rats.

Experimental Section

Synthesis: Uncorrected melting points were measured on a Büchi 510 Melting Point apparatus (Büchi Labortechnik AG, Flawil, Switzerland). IR spectra were obtained by an FTIR 1600 PC spectrophotometer (PerkinElmer, Überlingen, Germany). ^{1}H, ^{13}C, and ^{15}N NMR spectra were recorded on a AM 400 spectrometer (Bruker Biospin, Rheinstetten, Germany) using the following frequencies: ^{1}H: 400.134 MHz, ^{13}C: 100.614 MHz, ^{15}N: 40.55 MHz. Chemical shifts (δ) are in ppm relative to the internal standard TMS (^{1}H NMR), and nitromethane used as external standard (^{15}N NMR).

1,5-Bis(4′-acetoxyamidinophenoxy)pentane (diacetyldiamidoximeester) (4): 1,5-Bis(4′-hydroxyamidinophenoxy)pentane **3** (1 g) in anhydrous acetanhydride was stirred for 30 min. Residual acetanhydride was then hydrolyzed by adding water. The residue was washed thoroughly with ether, followed by recrystallization from acetonitrile.

Yield: 86.5 %; mp 154 °C; IR: ν = 3490 (NH), 3342 (NH), 1744 (COOR), 1614 cm^{-1} (C=N); ^{1}H NMR: δ = 1.61 (quint, 2H, CH_2), 1.82 (q, 4H, CH_2), 2.15 (s, 6H, CH_3), 4.06 (t, 4H, CH_2O), 6.71 (bs, 4H, NH_2), 7.34 ppm (mc, AA′, BB′, 8H, Ar-H); ^{13}C NMR: δ = 167.44 (COOR), 159.27 (C-1′), 154.96 (C=N), 126.99 (C-3′/3″), 122.47 (C-4′), 113.00 (C-2′/2″), 66.41 (CH_2-O), 27.21 (CH_2), 21.05 (CH_2), 18.75 ppm (CH_3); ^{15}N NMR: δ = −88.29 (s, N-O), −305.29 ppm (t, $^1J(^{15}N,^1H)$ = 90.3 Hz, NH).

4-[5-(4′-acetoxyamidinophenoxy)-pentyloxy]benzamidoximium-hydrogenoxalate (monoester) (8): A

mixture of 1,5-Bis(4′-acetoxyamidinophenoxy)pentane **4** (1.2 g) and lipase (20 g) in a solution of chloroforme/methanol (120 mL, 9:1, *v/v*) was stirred under reflux at 40 °C for 24 h. The enzyme was separated by filtration and washed with chloroforme/methanol (9:1, *v/v*). Filtrates were united and oxalic acid (500 mg) was added. The resulting colorless precipitate was isolated and discarded. The filtrate was poured to the same volume of ether. A colorless solid was obtained overnight, which was filtrated and washed with 20% acetic acid. While stirring and cooling with ice, 3 N NH_3 was slowly added dropwise to the filtrate. A colorless precipitate was collected by filtration and washed with water and ether.
Yield: 9.5%; mp 137 °C; IR: $\nu = 3492$ (NH_2), 3348 (NH_2), 3190 (OH, NH), 1740 (C=O), 1666 (COO^-), 1612 cm^{-1} (C=N-O); ^1H NMR: $\delta =$ 1.62 (quint, 2H, CH_2), 1.84 (quint, 4H, CH_2), 2.17 (s, 3H, CH_3), 4.08 (t, 4H, CH_2O), 6.72 (s, 4H, NH_2), 7.35 (mc, AA′, BB′, 4H, Ar-H), 7.37 ppm (mc, AA′, BB′, 4H, Ar-H); ^{13}C NMR: $\delta = 169.21$ (COO^-), 163.52 (COOH), 161.54 (C-1), 161.35 (C-1′), 156.99 (C=N), 153.72 (C=N), 128.79 (C-2/2′), 128.25 (C-2″/2‴), 124.78 (C-4′), 124.33 (C-4), 114.92 (C-2″/2‴), 114.84 (C-2/2′), 68.50 (CH_2-O), 29.39 (CH_2), 23.10 (CH_2), 19.91 ppm (CH_3).

4-[5-(4′-acetoxyamidinophenoxy)pentyloxy]benzamidinium-acetate (amidineamidoximeester) (9): 4-[5-(4′-hydroxyamidinophenoxy)pentyloxy]-benzamidinium-chloride **2** (140 mg) and anhydrous acetanhydride (2 mL) were stirred at room temperature for 1 h. Residual acetanhydride was hydrolyzed by adding water (20 mL). Subsequent NH_3 conc. was slowly added dropwise while stirring and cooling with ice. The resulting colorless precipitate was then filtrated and washed with ether.
Yield: 36%; mp 221 °C; IR: $\nu = 3482$ (NH_2), 3340 (NH_2), 3300–3100 (OH, NH), 1738 (C=O), 1696 (COO^-), 1614 cm^{-1} (C=N-O); ^1H NMR: $\delta = 1.60$ (quint, 2H, CH_2), 1.83 (quint, 4H, $2CH_2$), 1.73 (s, 3H, CH_3), 2.15 (s, 3H, CH_3), 4.04 (t, 2H, CH_2O), 4.10 (t, 2H, CH_2O), 7.27 (mc, AA′, BB′, 4H, Ar-H), 7.47 (mc, AA′, BB′, Ar-H), 10.20 ppm (bs, 4H, C=NH_2^+(NH_2)).

In vitro biotransformation assays: Human liver and kidney samples were obtained from the medicinal department of the University of Kiel. Tissue came from patients, suffering from secondary liver and kidney tumors, who had to undergo a partial hemihepatectomy or heminephrotectomy. Prior consent of the local medical ethics committee and from the donors was obtained for these studies. NADH, unspecific carboxyl esterases from pig liver and lipase from pig pancreas were purchased from Sigma Aldrich (Deisenhofen, Germany). All other chemicals and solvents were commercially available and of analytical grade, except methanol, which was of HPLC grade.

Enzyme preparation: Human and pig liver microsomes were prepared according to Clement et al.[43] by ultracentrifugation. Human and pig mitochondria were obtained by differential centrifugation as described previously[34] with modifications, Beattie[44] and Kline et al.[45] Proteins of microsomes and mitochondria were quantified as described previously by Smith[46] (BCA reagent kit; Pierce Chemical, Rockford, IL, USA). Cytochrome P450 concentrations were obtained by determination of the carbon monoxide difference spectra after reduction with dithionite according to Omura and Sato.[47]

Incubation assays: The complete activation of diacetyldiamidoximeester **4** to pentamidine **1** via four metabolites was carried out in an incubation mixture, containing microsomal or mitochondrial protein (0.25 mg) and diacetyldiamidoximeester **4** (0.5 mM) as substrate in 100 mM potassium phosphate buffer pH 6.3. The reaction was linear up to 120 min. After a preincubation time of 5 min at 37 °C under aerobic conditions the reaction was started by adding NADH (1 mM) to a total volume of 250 μL. The reaction was stopped after 120 min by the addition of ice-cold methanol (250 μL), followed by centrifugation at 10000 rpm (Mikroliterzentrifuge Hettich, Tuttlingen, Germany). 10 μL of the supernatant was quantified by HPLC. Reductions of diamidoxime **3** and monoamidoxime **2** to pentamidine **1** and of monoester **8** to amidineamidoximeester **9** were assayed analogously, with differences in the use of protein concentration (0.3 mg), substrate concentration (2 mM), and incubation time (30 min), respectively. Studies of the ester cleavages of diacetyldiamidoximeester **4** to monoester **8** and diamidoxime **3**, and amidineamidoximeester **9** to monoamidoxime **2** were performed in a 250 μL incubation mixture, containing carboxylic esterases (0.5 U) from pig liver, with 2 mM diacetyldiamidoximeester **4**, monoester **8**, or amidineamidoximeester **9** as substrate in 50 mM potassium phosphate buffer pH 7.4. After a 5 min preincubation time at 37 °C under aerobic conditions the reaction was started by adding the esterases equilibrated at the same temperature. The reaction was terminated after 20 min by the addition of ice-cold methanol (250 μL) and the samples were centrifuged at 10000 rpm (Mikroliterzentrifuge Hettich, Tuttlingen, Germany). 10 μL of the supernatant was analyzed by HPLC.

Stability of diacetyldiamidoximeester: To determine substrate stability, the diacetyldiamidoximeester **4** (0.3 mM) was added to a total volume of 100 μL of human plasma or 1 N hydrochloric acid. After an incubation time of 0 to 5 h at 37 °C the reaction was terminated by the addition of acetonitrile/methanol (300 μL, 1:1, *v/v*). Samples were centrifuged at 10000 rpm and 10 μL of the supernatant was analyzed by HPLC.

In vivo biotransformation study: 20 adult male Spague–Dawley rats were used in the in vivo study, which was performed by the medicinal department of the University of Munich. Prior consent of the local medical ethics committee was obtained for these studies. Solid phase extraction cartridges, C8 were purchased from Restek (Bad Homburg, Germany). All other chemicals and solvents were commercially available and of analytical grade, except methanol, which was of HPLC grade. Pharmacokinetic parameters were estimated using Pharsight WinNonlin Professional 4.1, Mountain View, CA.

Animal experiment: The diacetyldiamidoximeester **4** (20 and 50 mg kg^{-1}) was administered daily by gavage over a period of 10 days. For intraperitoneal treatments pentamidine **1** (5 mg kg^{-1}) was applied over the same period. Substances were dissolved in sterile water (1 mL) before the treatment. Plasma samples were taken by cardiac puncture 0.5, 1, 6, 12 and 24 h after application. Urine samples were also collected. After euthanasia by decapitation, tissues (liver, kidneys, and lung) were removed and frozen immediately on dry ice.

Tissue homogenization and compound extraction: Tissue samples (5 g) were homogenized in 50 mM potassium phosphate buffer pH 7.4 (2.0 mL). Prior to HPLC injection, the analytes were extracted from plasma and tissue homogenate by solid phase extraction and evaporation to dryness according to Lin et al.[48] The residue was reconstituted in 1000 μL (liver, kidney) or 100 μL (lung, plasma) HPLC solvent. Urine samples were centrifuged at 10000 rpm for 10 min and 10 μL of the supernatant was analyzed by HPLC.

HPLC analyses: The HPLC system consisted of Waters 1525 pump, autosampler 717 plus, 2487 UV detector and Breeze Software (Ver-

sion 3.20). The mobile phases were filtrated through a 0.45 μm Sartolon membrane filter (Sartorius AG, Göttingen, Germany) and degassed. The separation was carried out at room temperature by a LiChrospher RP-select B column (125×4 mm; Merck, Darmstadt, Germany) and a RP select B guard column (4×4 mm; Merck). The detection wavelength was 260 nm. Fluorescence excitation was set at 275 nm and emission at 340 nm. The mobile phase was made up of octylsulfonate (30 mM, tetramethylammoniumchloride 20 mM, pH 3.0) and methanol (52:48, *v/v*). The injection volume for both standards and samples was 10 μL per run. The diacetyldiamidoximeester **4** was eluted with a retention time of 8.0±0.5 min, monoester **8** at 10.3±0.6 min, amidineamidoximeester **9** at 12.5±0.5 min, diamidoxime **3** at 16.1±0.6 min, monoamidoxime **2** at 20.5±0.7 min, and pentamidine **1** at 26.2±0.5 min. Linearity was verified for each metabolite with standard curves (correlation coefficients >0.99). Precision and accuracy of the assays were assessed by adding different concentrations (0–500 μM) of the metabolite to the standard incubation mixture (without NADH), tissue homogenate or plasma. Standard curves were determined by linear regression. The recovery of the metabolites from the incubation mixtures and samples which contained the same amount of each metabolite dissolved in phosphate buffer was found to be (100.8±8.0)% (standard incubation mixture), (41.4±1.5)% (rat liver homogenate), (57.6±5.2)% (rat lung homogenate), (55.6±0.2)% (rat kidney homogenate), (109.3±7.2)% (urine) and (93.4±11.6)% (plasma). The detection limits were approximately <1 μM for all metabolites detected.

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