Biomimetic Oxidation of Denaverine Hydrochloride

Kai Smolinka[a] and Berthold Göber*[a]

Dedicated to Professor Dr. Siegfried Ebel on the occasion of his 65th birthday

Keywords: Biomimetic oxidations / Cytochrome P450 / Metalloporphyrins / Denaverine / Metabolism

The behavior of denaverine (**1**) in various chemical model systems based on metalloporphyrins as catalysts was investigated to determine the possible oxidative metabolism. The resulting derivatives were compared to a biological model system and in vivo metabolism in rat and human.

Cytochrome P450 isoenzymes (Cyt P450) play an important role in the metabolism of xenobiotics. Many biological and chemical models have been studied mimicking such enzymatic reactions.[1] In recent years model systems based on metalloporphyrins as catalysts have been established successfully mimicking monooxygenations such as alkane hydroxylations, epoxidations of alkenes, and aromatic hydroxylations.[2] They show similar reactivity and selectivity to Cyt P450, whereas more simple models, for example Udenfriend's (Fe^{2+}, ascorbic acid, O_2) or Fenton's reagent (Fe^{2+}, H_2O_2) yield more varied results.[3] There are some reports of application of metalloporphyrin-based model systems to drug metabolism studies. These systems are composed of a metalloporphyrin as a catalyst, an N-base as a co-catalyst, and an O-donor such as peroxides, peracids, iodosylbenzene and derivatives, sodium hypochlorite, and others.[4] In a previous study we proved the ability of such biomimetic systems for selective aromatic oxygenations by comparing the reaction profile of propiverine, a drug not metabolized by aromatic oxygenation, with that of clemastine, which is favourably metabolized on the aromatic rings. In both in vivo and aqueous media reactions, these biomimetic systems were able to reveal differences in metabolic pathways.[3b,5]

The spasmolytic drug denaverine hydrochloride (**1**-HCl) is used in therapy for more than 25 years. Data is available on its in vivo metabolism in rat, whilst metabolism in human is not well documented. The aim of our investigations was to determine possible oxidative metabolic pathways of **1** by biomimetic reactions. In order to compare the chemical model systems with a biological model system we examined the reaction of **1** in a 10,000 *g* supernatant fraction of rat liver homogenate, a simple biological model for microsomal oxidations.

1. Investigations with (Porphyrin)iron or -manganese Compounds in Nonaqueous Medium

Table 1 shows different chemical model systems used in our investigations. [5,10,15,20-Tetrakis(pentafluorophenyl)-porphyrin]manganese(III) chloride (MnTPFPPCl) and the corresponding iron compound FeTPFPPCl are resistant towards oxidative degradation and give good yields of aromatic oxygenation products in combination with imidazole or pyridine as co-catalysts.[6] Iodosylbenzene was used to compare reaction profile with that of hydrogen peroxide.

Table 1. Biomimetic systems used

Catalyst	Co-catalyst	O-donor	Medium
MnTPFPPCl	imidazole	hydrogen peroxide	nonaqueous
MnTPFPPCl	imidazole	iodosylbenzene	nonaqueous
MnTPFPPCl	pyridine	hydrogen peroxide	nonaqueous
MnTPFPPCl	imidazole	hydrogen peroxide	nonaqueous[a]
FeTPFPPCl	pyridine	hydrogen peroxide	nonaqueous
–	pyridine	hydrogen peroxide	nonaqueous
$MnTPFPS_4PCl$	imidazole	hydrogen peroxide	aqueous, pH = 1
$MnTPFPS_4PCl$	imidazole	hydrogen peroxide	aqueous, pH = 6–8
$MnTPFPS_4PCl$	–	–	aqueous, pH = 1
$FeTPFPS_4PCl$	imidazole	hydrogen peroxide	aqueous, pH = 1
$FeTPFPS_4PCl$	imidazole	hydrogen peroxide	aqueous, pH = 6–8
–	imidazole	hydrogen peroxide	aqueous, pH = 1
–	imidazole	hydrogen peroxide	aqueous, pH = 6–8
–	–	hydrogen peroxide	aqueous, pH = 6–8

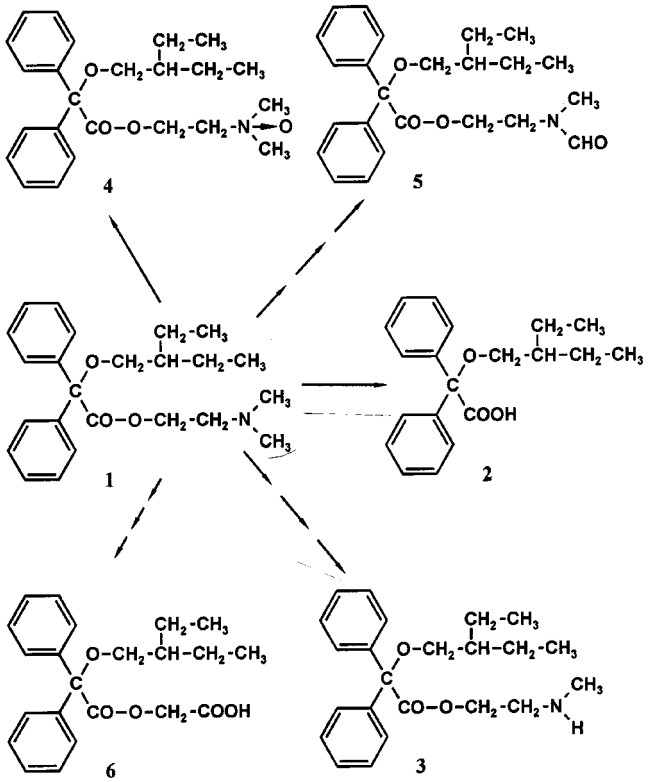
[a] Dichloromethane/methanol (1:1).

Generally, in addition to unchanged **1**, nonaqueous biomimetic systems produce five potential metabolites, **2**–**6** which are products of cleavage of the ester bond (**2**), of *N*-demethylation (**3**), of *N*-oxidation (**4**), and of *C*-oxidation (**5**, **6**) (see Scheme 1). The main products are **5**, **2**, and **4**.

Without a catalyst only cleavage of the ester bond and *N*-oxidation take place leading to **2** and **4** in very low amounts. The use of iodosylbenzene as an O-donor prevents any reaction. The use of methanol instead of aceto-

[a] Institut für Pharmazie, Humboldt-Universität zu Berlin, Goethestr. 54, D-13086 Berlin, Germany

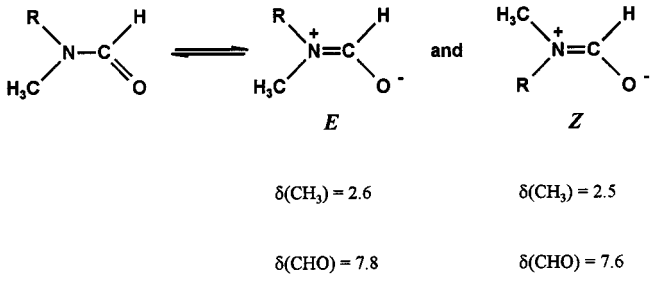
  1434–193X/99/0303–0679 $ 17.50+.50/0



Scheme 1. Reactions in nonaqueous system

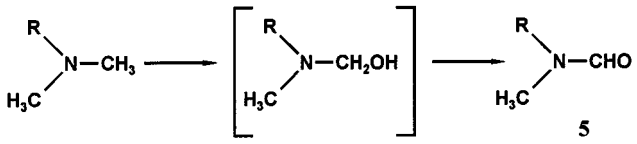
nitrile does not lead to a different reaction profile. No methyl ester of **2** was found in contrast to the aqueous system (see 2.). The use of (porphyrin)iron as catalyst yields lower levels of products, particulary those produced by ester bond cleavage and *N*-demethylation. The main product is **5**. Furthermore, use of pyridine instead of imidazole increases yields, but due to an easier handling imidazole was primarily used.

Compound **5** is the *N*-formyl derivative of **1**. The two conformational forms (see Scheme 2) occur in a *Z*/*E* ratio of 2:1 up to 1:1 estimated from integrals of formyl and methyl proton signals in the ^{1}H-NMR spectrum.



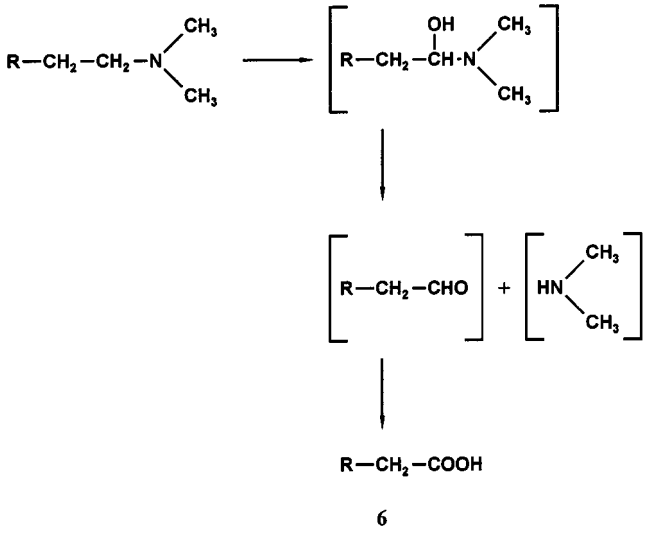
Scheme 2. Conformational isomerism of **5**

A likely mechanism of formation of **5** is a further oxidation of the intermediate carbinolamine formed by *C*-oxidation of one *N*-methyl group (see Scheme 3).



Scheme 3. Proposed formation of **5**

Compound **6**, 2-[2-(2-ethylbutoxy)-2,2-diphenylacetoxy]-acetic acid, results from oxidative deamination followed by oxidation of the intermediate aldehyde, formed by the hypothetic α-carbinolamine (see Scheme 4).



Scheme 4. Proposed formation of **6**

The formation of α-carbinolamine could be initiated by hydrogen atom abstraction or electron transfer followed by proton abstraction.[7]

2. Investigations with (Porphyrin)iron or -manganese Compounds in Aqueous Solutions

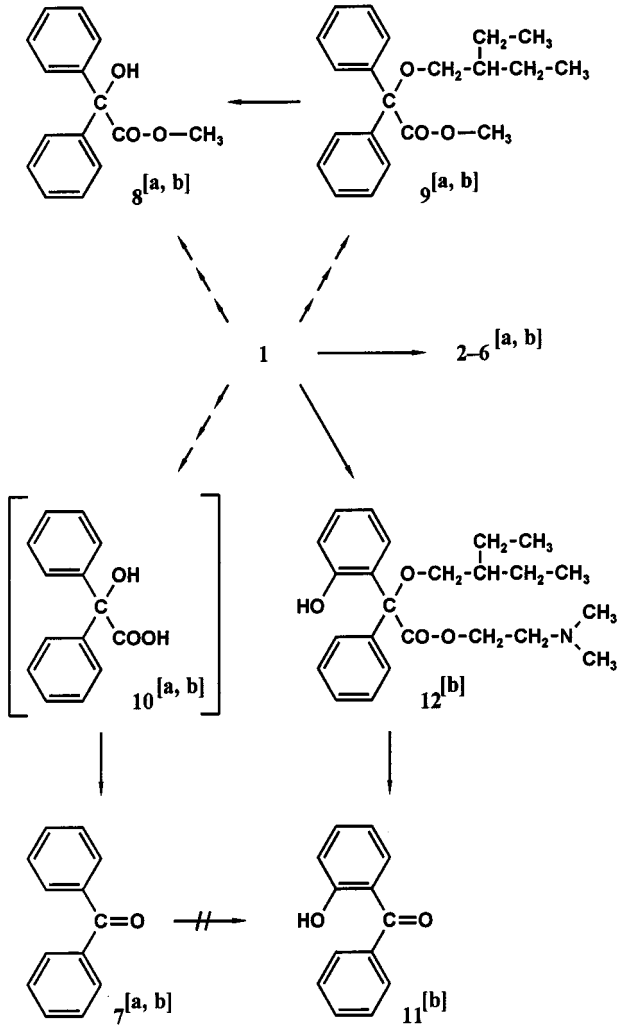
Biomimetic oxidations of **1** in aqueous media were performed with (porphyrin)iron or -manganese/imidazole/hydrogen peroxide in water (pH = 6−8) or at pH = 1 by adding hydrochloric acid (6 mol/L). The influence of higher pH values on the metabolic spectrum could not be investigated due to the insolubility of **1**.

Generally, all known derivatives were found in higher yields in the aqueous system. The most potent system was [5,10,15,20-tetrakis(pentafluorophenyl)-β-tetrasulfonatoporphyrin]manganese(III) chloride ($MnTPFPS_4PCl$)/imidazole/hydrogen peroxide at pH = 6−8. Other products were detected depending on the catalyst used and/or the pH value. Various systems are shown in Table 1.

Benzophenone (**7**) and the methyl esters of benzilic acid (**8**) and of *O*-(2-ethylbutyl)benzilic acid (**9**) were found in addition to the known metabolites **2**−**6** with (porphy-

rin)manganese at pH = 1 or pH = 6−8 (see Scheme 5). Compounds **8** and **9** are artifacts formed by transesterification which is caused by working with methanol. Aqueous solutions of **1** with manganese catalyst and without O-donors produced no derivatives. A reaction with molecular oxygen from air could be excluded.

We were unable to confirm the occurrence of benzilic acid (**10**). However, the formation of benzophenones from benzilic acids is well known, so detection of **7** suggests earlier benzilic acid.



Scheme 5. Reactions in aqueous system: [a] MnTPFPS$_4$PCl/imidazole/H_2O_2 at pH = 6−8 or 1; [b] FeTPFPS$_4$PCl/imidazole/H_2O_2 at pH = 6−8

These derivatives are also known from the reaction of **1** with the chemical model system FeTPFPS$_4$PCl/imidazole/hydrogen peroxide. In addition, two phenolic compounds were detected in very small amounts. 2-Hydroxybenzophenone (**11**) could be elucidated by comparison with an authentic sample. The formation of **11** from **7** could be excluded by biomimetic oxidation of **7** leading to the 3- and 4-hydroxy derivatives.[6c]

The second phenolic metabolite (**12**) results from **1** by monohydroxylation at one aromatic ring. The 2-position of the phenolic group is inferable from the occurrence of **11** and the absence of any other benzophenone derivative. 2-Hydroxybenzilic acid was not observed. For all detected derivatives see Scheme 5.

In contrast to the wide metabolic spectrum at pH = 6−8 reaction of FeTPFPS$_4$PCl/imidazole/H_2O_2 with **1** generates no products in acidic solution.

3. Investigations with a 10,000 *g* Supernatant Fraction of Rat Liver Homogenate

To compare the chemical system with a biological system a 10,000 *g* supernatant fraction of rat liver homogenate was used. In this fraction many microsomal Cyt P450 occur. Moreover, this fraction is relatively easy to prepare and to handle.

2−**4** were isolated from an incubation mixture with **1**-HCl. Further products were found in trace amounts. They do not correspond with products known from our biomimetic investigations. Compound **2** occurs in similar amount in the blank value. It is assumed to be an artifact caused by the working procedure and not a product of an enzymatic catalysis.

4. Discussion

Table 2 shows the results of biomimetic studies in comparison with the in vitro method and metabolism studies in rat and in human. Eleven metabolites have been observed in rat studies as well as intact **1**. The identified metabolites are products of cleavage of the ester and ether bond (**10**), of the oxidative *N*-demethylation (**3**), of cleavage of the ether bond and a further ring closure giving 3,3-diphenylmorpholin-2-one (**13**), of reductive cleavage of the ether bond resulting in 2-(dimethylamino)ethyl 2,2-diphenylacetic acid (**14**) and 2,2-diphenylacetic acid (**15**), and of transesterifications generating ethyl benzilate, methyl and ethyl *O*-(2-ethylbutyl)benzilate. Three metabolites could not be identified. The main metabolites are **10** and **13**.[8] Products of single ether bond cleavage were not found in either in the biomimetic way or in the 10,000 *g* supernatant fraction, or in the metabolism in rat or human. Compound **10** and its methyl ester found in the biomimetic system only in very low yields. This proves the possibility of *O*-dealkylations with the biomimetic method, but the ether bond cleavage is not favoured in this study. In contrast to **1** the biomimetic oxidation of propiverine[5] or clemastine[3b] affords several products of *O*-dealkylation in moderate yields.

The absence of **14** and **15** in the biomimetic reaction is not surprising, because they are products of reducing reactions. The described biomimetic system mimics oxygenations, reduced metabolites were never observed. Compound **3**, another metabolite discovered in rat and human, was found in moderate yields. *N*-Dealkylations are well-known reactions mimicked by chemical model systems.

Furthermore, **2** and its methyl ester **9** were obtained in biomimetic studies. From metabolism in rat only **9** and the

Table 2. Results in comparison

Metabolite	Biomimetic method	Metabolism in rat	In vitro method	Metabolism in human
2	+	+[a]	+[b]	−
3	+	+	+	+
4	+	−	+	−
10	+	+	−	+
13		+		
14	−	+	−	−
15	−	+	−	−
further	**5**, **6**, **7**, **8**, **9**, **11**, **12**	detected, not identified[8]	detected, not identified	not found[9]

[a] Ethyl and methyl ester of **2**. − [b] No result of enzyme activity.

ethyl ester of **2** are known. They are artifacts like ethyl benzilate formed by isolating procedures using methanol or ethanol.[8] The possible primary metabolite is **2**. The formation of **2** shows conformity with our biomimetic investigations.

Less than 1% of the applied dose of **1**-HCl could be detected in metabolism studies in human.[9] Besides unchanged **1**, compounds **3** and **10** were quantified. They are generated in chemical model systems, too. Thus, all metabolites formed by oxidations and known from metabolism in rat or human were found in biomimetic studies, with the exception of **13**. A comparison of quantities of different methods was not indicated. Elaboration of validated quantification does not seem beneficial because of the wide product spectrum in chemical model system, no published quantities of metabolites in rat, and the recovery of less than 1% of applied dose in metabolism studies in human. Total **1** is oxidized to 10−20% in the biomimetic system as estimated from isolated derivatives.

With the incubation of the 10,000 *g* supernatant fraction as an in vitro method only **3** was identified corresponding with metabolism in rat and human, whilst **2** is not a product of enzymatic activity. The main in vivo metabolites **10** and **13** were not found, cleavage of the ether bond was not observed. Most derivatives were obtained with the described biomimetic method. Mainly, metabolic reactions are supposed to occur on the ester side chain. Aromatic oxygenations are not expected due to their minor role in biomimetic studies. The minor role of the ether bond cleveage is noteworthy. The importance of this fact will be seen in further metabolism studies. The new derivatives **5**, **6** could be useful as reference substances for additional screening in future studies on the metabolism of **1** in human.

Now, the application of such biomimetic systems to the evaluation of analytical procedures in order to isolate and elucidate derivatives of new drug substances for metabolism studies is in progress.

Experimental Section

Equipment: NMR: Bruker DPX 300 (300 MHz and 75 MHz, for ^1H and ^{13}C, respectively), $CDCl_3$ as solvent, TMS as internal standard. − MS: Hewlett Packard 5995 A (EIMS), 70 eV. For ESI MS, Triple-Quadrupol-MS TSQ 700, Finnigan MAT Bremen. For FAB MS, Autospect, Micromass, magic bullet as matrix. For CI MS, MAT Finnigan 95 ST, isobutane as reactant gas. − HPLC: Hewlett Packard 1090, using a LiChroCart column (125 × 4 mm) with precolumn LiChroCart (4 × 4 mm) containing LiChroSpher RP select-B (5 μm), Merck, diode-array detector, monitoring at 230 nm, flow 1 mL/min, linear gradient: 60−98% or 70−98% methanol in disodium hydrogen phosphate solution 0.002 mol/L, 0−20 min. − TLC: Analytical TLC; HPTLC plates, silica gel $60F_{254}$, 5 × 7.5 cm, Merck, saturation time 15 min, front 6 cm. Preparative TLC; PSC plates, silica gel $60F_{254}$, 2 mm, 20 × 20 cm, Merck. Eluents used; dichloromethane/methanol (9:1), dichloromethane/cyclohexane/methanol/25% ammonia solution (5:3.5:1.5:0.05), cyclohexane/dioxane/ethyl acetate/ethanol/25% ammonia solution (1.5:1.5:4:2:1), toluen/dioxane/glacial acetic acid (7:2.7:0.3), and toluene/ethyl acetate (8:2). Detection reagents; concentrated sulphuric acid, Dragendorff reagent by AB-DDR 87, 3-methyl-2-benzothiazolinone hydrazone (MBTH) reagent, after saturation of plates with ammonia vapour, coupling with MBTH solution (2% in methanol) and following oxidation with potassium hexacyanoferrate(III) solution (8% in water).

Materials: Commercial reagents were purchased from standard chemical suppliers and were used without further purification. **1**-HCl and metabolites **2**−**4**, and **10** were gifts from Apogepha Arzneimittel GmbH, Dresden (Germany). The methyl esters **8** and **9** were prepared according to ref.[8] MnTPFPPCl and FeTPFPPCl were prepared according to ref.[6], $MnTPFPS_4PCl$ and $FeTPFPS_4PCl$ according to ref.[10]

2-(*N*-Formyl-*N*-methylamino)ethyl *O*-(2-Ethylbutyl)benzilate (5): ^1H NMR: δ = 0.7 (6 H, CH_3), 1.27 (5 H, $CH-CH_2$), 2.5 and 2.6 [1 H and 1 H, (*Z*)- and (*E*)-$N-CH_3$], 3.0 (2 H, $O-CH_2$), 3.3 and 3.4 (2 H and 2 H, $N-CH_2$), 4.19 and 4.25 (2 H and 2 H, $CO-O-CH_2$), 7.2−7.4 (10 H, aromatic H), 7.6 and 7.8 (1 H and 1 H, *CH*O). − ^{13}C NMR: δ = 11.54 and 11.56, 23.84 and 23.86, 30.0 and 35.6, 42.1, 43.6 and 48.5, 62.0 and 63.2, 86.5, 128.19, 128.29, 128.41, 128.80, 128.83, 141.2 and 141.5, 163.0 and 163.1, 172.1. − EI MS; *m*/*z* (%): 297 (< 1), 267 (17), 183 (100), 165 (17), 105 (77), 86 (23), 77 (22), 72 (8). − FAB MS; *m*/*z*: 420 [M^+ + Na]. − ESI MS; *m*/*z*: 398 [M^+ + H], 420 [M^+ + Na].

2-[2-(2-Ethylbutoxy)-2,2-diphenylacetoxy]acetic Acid (6): ^1H NMR: δ = 0.8 (6 H, CH_3), 1.2 (5 H, $CH-CH_2$), 3.1 (2 H, $O-CH_2$), 4.2 (2 H, $CO-O-CH_2$), 7.1−7.4 (10 H, aromatic H). − ^{13}C NMR: δ = 11.0, 23.1, 41.2, 64.9, 65.2, 85.2, 127.42, 127.46, 128.4, 142.1, 168.9, 170.9. − EI MS; *m*/*z* (%): 267 (24), 183 (100), 165 (23), 105 (75), 77 (25). − FAB MS; *m*/*z*: 393 [M^+ + Na], 357 [M^+ + H − H_2O]. − CI MS; *m*/*z*: 357 [M^+ + H − H_2O].

2-(Dimethylamino)ethyl *O*-(2-Ethylbutyl)-2-hydroxybenzilate (12): EI MS; *m*/*z* (%): 299 (1), 283 (< 1), 199 (16), 181 (4), 121 (9), 105

(26), 93 (3), 77 (9), 71 (50), 58 (100). − FAB MS; *m/z*: 400 [M^+ + H], 422 [M^+ + Na].

Methods: Chemical model system: − (i) Nonaqueous system: 1 mmol of (porphyrin)iron or -manganese, 10 mmol of N-base, and 700 mmol of **1**-HCl were dissolved in 10 mL of dichloromethane/acetonitrile (1:1). After addition of 1500 mmol of oxidant, the mixture was stirred at room temperature for 24 h. Blank values were carried out without catalyst or oxidant. − (ii) Aqueous system: Compounds were dissolved in the same way and ratio in 10 mL of water, if necessary hydrochloric acid (6 mol/L) was added. − (iii) Analytical procedure: Products were separated by preparative layer chromatography and purified by TLC. Aqueous systems were freeze-dried initially and then dissolved in 10 mL of methanol. Isolated derivatives were characterized by TLC, HPLC, MS, and NMR data, if possible they were compared with authentic samples.

Biological model system: − (i) Preparation of the 10,000 *g* supernatant fraction: Rat livers of male Wistar rats were homogenized in ice-cold phosphate buffer (0.01 mol/L, pH = 7.6) with 1.15% potassium chloride in a Potter-Elvehjem-homogenizer. Homogenate was centrifuged at 10,000 *g* for 10 min at 4°C. The resulting supernatant fraction was used immediately. 2 mL of the supernatant fraction corresponded to 0.5 g of liver. − (ii) Reaction conditions: 1 mL of **1**-HCl solution (2.6 mmol/L), 3 mL of cofactor, and 2 mL of the 10,000 *g* supernatant fraction were incubated for 1 h at 37°C. One blank value contained 1 mL of water instead of **1**-HCl solution and the other one 2 mL of phosphate buffer (0.01 mol/L, pH = 7.6) with 1.15% potassium chloride instead of the 10,000 *g* supernatant fraction. 3 mL of cofactor solution contained 0.65 µmol of NADP, 10 µmol of glucose-6-phosphate, 50 µmol of magnesium chloride in phosphate buffer (0.5 mol/L, pH = 7.6). − (iii) Analytical procedure: Incubated fractions were freeze-dried, extracted with a mixture of dichloromethane and 2-propanol (3:1), three times and concentrated to dryness in vacuum. The residue was dissolved in methanol. Isolation of single metabolites was carried out analogeous to the chemical system by layer chromatography.

Acknowledgments

We would like to thank the Apogepha Arzneimittel GmbH, Dresden (Germany) for financial support and Dr. Bartoszek from the Institut für Angewandte Chemie Adlershof e.V., Berlin (Germany) for FAB MS.

[1] A. Langner, *Pharm. Unserer Zeit* **1995**, *24*, 207−218.
[2] [2a] D. Mansuy, P. Battioni, J.-P. Battioni, *Eur. J. Biochem.* **1989**, *184*, 267−285. − [2b] D. Mansuy, *Pure Appl. Chem.* **1994**, *66*, 737−744. − [2c] B. Meunier, *Bull. Soc. Chim. Fr.* **1986**, 578−594.
[3] [3a] H. Masumoto, K. Takeuchi, S. Ohta, M. Hirobe, *Chem. Pharm. Bull.* **1989**, *37*, 1788−1794. − [3b] L. Fröhlich, B. Pietzyk, K. Smolinka, B. Göber, *Pharmazie* **1996**, *51*, 409−414.
[4] [4a] M. S. Chorghade, D. R. Hill, E. C. Lee, R. J. Pariza, D. H. Dolphin, F. Hino, L. Zhang, *Pure Appl. Chem.* **1996**, *68*, 753−756. − [4b] H. Masumoto, S. Ohta, M. Hirobe, *Drug Metab. Dispos.* **1991**, *19*, 768−780. − [4c] Y. Nagatsu, T. Higuchi, M. Hirobe, *Chem. Pharm. Bull.* **1989**, *37*, 1410−1412. − [4d] Y. Nagatsu, T. Higuchi, M. Hirobe, *Chem. Pharm. Bull.* **1990**, *38*, 400−403. − [4e] M. N. Carrier, P. Battioni, D. Mansuy, *Bull. Soc. Chim. Fr.* **1993**, *130*, 405−416. − [4f] M. Hirobe, *Pure Appl. Chem.* **1994**, *66*, 729−736. − [4g] M. Komuro, Y. Nagatsu, T. Higuchi, M. Hirobe, *Tetrahedron Lett.* **1992**, *33*, 4949−4952. − [4h] M. Vidal, M. Bonnafous, S. Defrance, P. Loiseau, J. Bernadou, B. Meunier, *Drug Metab. Dispos.* **1993**, *21*, 811−817. − [4i] L. Fröhlich, B. Göber, *Pharm. Ind.* **1997**, *59*, 803−810.
[5] [5a] L. Fröhlich, B. Pietzyk, B. Göber, *Pharmazie* **1995**, *50*, 736−740. − [5b] L. Fröhlich, B. Pietzyk, K. Smolinka, B. Göber, *Pharmazie* **1996**, *51*, 745−747.
[6] [6a] B. Pietzyk, L. Fröhlich, B. Göber, *Pharmazie* **1995**, *50*, 747−750. − [6b] B. Pietzyk, L. Fröhlich, B. Göber, *Pharmazie* **1996**, *51*, 654−660. − [6c] L. Fröhlich, B. Pietzyk, B. Göber, *Arch. Pharm.* **1995**, *328*, 389−390.
[7] E. Baciocci, O. Lanzalunga, A. Lapi, L. Manduchi, *J. Am. Chem. Soc.* **1998**, *120*, 5783−5787.
[8] B. Göber, H. Lisowski, D. Friese, P. Franke, *Pharmazie* **1988**, *43*, 493−495.
[9] A. Staab, *Pharmacokinetic and Bioavailability Investigations of the Neurotropic-Musculotropic Spasmolytic Agent Denaverine*, Shaker Verlag GmbH, Aachen−Maastricht, **1998**.
[10] I. Artaud, K. Ben-Aziza, D. Mansuy, *J. Org. Chem.* **1993**, *58*, 3373−3380.

Received September 10, 1998
[O98412]