# Biotransformation of Desoxypeganine by Microsomal Enzymes of the Rabbit Liver

**Sascha Illgner and Rudolf Matusch**

Department für Pharmazie – Zentrum für Pharmaforschung, Philipps-Universität Marburg, Marburg, Germany

The biotransformation of the anticholinergic quinazoline alkaloid Desoxypeganine is studied by means of aerobic incubation with the non-induced supernatant obtained at 9000*g* from rabbit liver homogenates as enzyme source followed by an admixture of NADPH. The metabolites were identified by high-performance liquid chromatography, chemical ionisation mass spectrometry (LC-CI MS) and electron impact mass spectrometry (LC-EI MS) in comparison with synthetic reference compounds and typical ion fragments taken from literature data. C-oxidation of Desoxypeganine to the major metabolite Pegenone was observed as well as the hydroxylation of the alicyclic ring. The incubation mixture followed Michaelis-Menten kinetics characterised by $K_m = 5.8 \times 10^{-5}$ mol $L^{-1}$ and $V_{max} = 4.32$ nmol Pegenone/min per mg protein or 3.37 nmol Pegenone/min per nmol CYP 450, respectively. These in vitro results demonstrate that the bioactive substance Desoxypeganine is easily oxidised to its ineffective metabolite Pegenone. This provokes a problem for correct dosage finding in formulations for the treatment of Alzheimer's disease and in the therapy of alcoholism and nicotine dependence.

**Keywords**: Desoxypeganine; Microsomes; Metabolism; Michaelis-Menten kinetic

Received: July 13, 2004; Accepted: September 1, 2004 [FP921]

## Introduction

Desoxypeganine 1 (1,2,3,9-tetrahydropyrrolo[2,1-*b*]-quinazoline; Scheme 1) is an alkaloid isolated from Peganum harmala L. / Zygophyllaceae [1], a perennial, glabrous herbal plant which grows in semi-arid rangeland of the Middle East and North Africa [2]. The substance can also be synthesized in two steps via the intermediate Pegenone 2 [3, 4, 5]. Desoxypeganine 1 causes depression of monoaminoxidase-A-activity and inhibition of acetylcholinesterase two times superior than galanthamine in spite of being less toxic [6]. These effects allow a large variety of therapeutic uses such as nicotine dependence as well as alcoholism. Desoxypeganine 1 can also slow down the progression of Alzheimer's disease and improve mental cognitive capabilities.

In vitro studies with microsomes from different species like rabbits or pigs are usually carried out at an early stage of drug development in order to get preliminary information on metabolic routes of a new drug. The advantage of these studies is to detect also unstable and perhaps toxic metabolites as ultra-short-lived or short lived intermediates which never enter the blood circulation and therefore are not excreted into bile or urine [7]. In addition, animal experiments can be reduced. The biotransformation of Desoxypeganine 1 was previously examined by in vivo studies with animals. In all cases stable, long-lived urinary and biliary metabolites were found [8, 9, 10]. Pegenone 2, Vasicinone 3 and Isovasicinone 4 were described several times to appear in the urine of rats receiving Desoxypeganine 1 (Scheme 1).

In this study the Desoxypeganine 1 metabolites from aerobic incubation of 9000*g* supernatant of rabbit liver homogenates as enzyme source shall be characterized, using LC-MS in the electron impact (EI) and positive chemical ionisation ($CI^+$) modes in comparison with reference compounds and literature data. Since samples of microsomal preparations were utilised under the admixture of NADPH as coenzyme, only oxidative metabolism took place. For further characterization the kinetic parameters of the conversion of Desoxypeganine 1 to Pegenone 2 (1,2,3,9-tetrahydropyrrolo[2,1-*b*]-quinazoline-9-one) are reported.

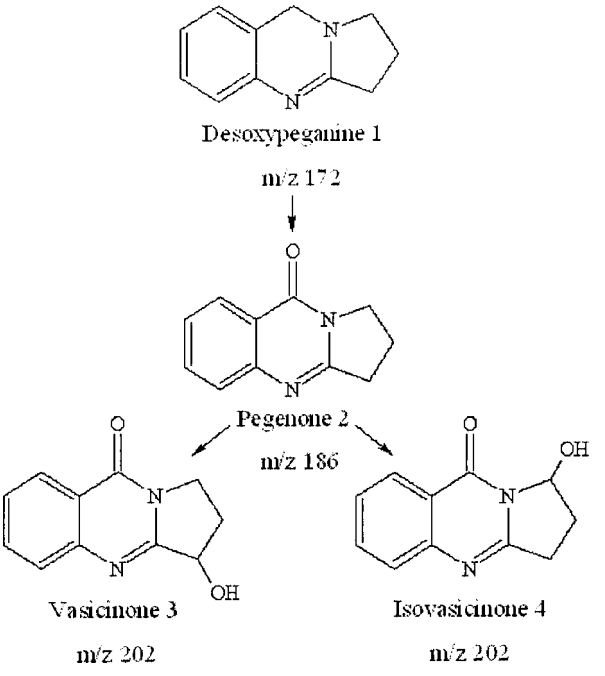
**Correspondence**: Sascha Illgner, Institut fuer Pharmazeutische Chemie, Philipps-Universitaet Marburg, Marbacher Weg 6, D-35037 Marburg, Germany. Phone: +49 6421 28-25899, Fax: +49 6421 28-25899, illgner@staff.uni-marburg.de

## Results

### Identification of Desoxypeganine metabolites

The incubation mixtures were first characterised by using LC-MS in the positive chemical ionisation ($CI^+$) mode in order to detect unstable metabolites like *N*-oxides of Desoxypeganine 1 and Pegenone 2. These *N*-oxides were described as compounds in plants like Nitraria komarovii L./ Zygophyllaceae [11, 12]. Although they appear as possible

Full Paper




**Scheme 1.** Proposed main metabolic pathway for Desoxypeganine 1 by microsomal enzymes.

metabolites in our case none were detected in any of the measured samples. It is well known that Desoxypeganine 1 is primarily metabolized in rats to the main metabolite Pegenone 2 followed by hydroxylation of the alicyclic ring system to Vasicinone 3 and Isovasicinone 4. Figure 1 shows a typical total ion chromatogram and extracted mass chromatograms obtained from an incubation mixture with Desoxypeganine 1. Retention times, mass spectra and typical ion fragments (EI mode) of each peak were compared with those of standards and literature data, peaks 1-3 were identified as Vasicinone 3, Isovasicinone 4 and Pegenone 2, respectively.

All incubation samples containing Desoxypeganine 1 or Pegenone 2 as substrate together with rabbit liver microsomes in two different buffer solutions (pH 7.4 and pH 8.6), delivered the metabolites as described before without deviations. The main metabolic pathway for Desoxypeganine 1 by microsomal enzymes is summarized in scheme 1.

## Quantitative analysis of Pegenone formed by C-oxidation of Desoxypeganine

After 40 min of incubation time at 37°C 78% of Desoxypeganine 1 were converted to Pegenone 2 by C-oxidation considering the recovery rate. This C-oxidation also took place in the control samples without coenzyme NADPH (yield 42% Pegenone 2) or enzyme source (yield 19% Pegenone 2). The hydroxylated metabolites 3 and 4 in the total amounted 10-15%. No mass peak of m/z 202 was found, however, in the control samples without enzyme source.

## Kinetics of the C-oxidation of Desoxypeganine

The formation of Pegenone 2 by incubating Desoxypeganine 1 with the 9000*g* supernatant and NADPH followed Michaelis-Menten kinetics. The $K_m$ and $V_{max}$ values were calculated using the Lineweaver-Burk diagram resulting from samples of liver preparations from three different rabbits (Figure 2). The $K_m$ value was $5.8 \times 10^{-5}$ mol $L^{-1}$ and the $V_{max}$ value was 4.32 nmol Pegenone/min per mg protein or 3.37 nmol Pegenone/min per nmol CYP 450, respectively.

## Discussion and conclusions

The aim of the present study was to investigate the in vitro metabolism of Desoxypeganine 1 by the 9000*g* supernatant of rabbit liver homogenate as cytochrome P-450 enzyme source. Our results were compared with in vivo studies on rats and we found the same three metabolites as described in literature. The value of $V_{max}$ that we calculated fits into the range between 1 and 15 nmol product per minute per milligram of microsomal protein typical also for other organic substrates metabolised by cytochrome P-450 enzymes. The determined $K_m$ value of $5.8 \times 10^{-5}$ mol $l^{-1}$ reveals that Desoxypeganine 1 belongs to a group of substrates with high affinity to the cytochrome P-450 enzyme family [13, 14]. The fact that 78% of Desoxypeganine 1 were oxidised to Pegenone 2 which has no anticholinergic activity (unpublished data) under described conditions, and even 19% of Desoxypeganine 1 were oxidised without any enzyme source solely under aerobic conditions, provokes a serious problem for pharmaceutical formulations of Desoxypeganine 1. Since a central active compound at least should survive as it gets over the blood-brain barrier, the quick metabolism of Desoxypeganine 1 possibly could be the limiting factor for the dosage in the treatment of Alzheimer's disease or therapy of alcoholism and nicotine dependence. The aim therefore should be the development of suitable pharmaceutical formulations.

## Experimental

### Materials and methods

NADPH (tetrasodiumsalt) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Boric acid, sodium hydroxide, hydrochloric acid, potassium dihydrogen phosphate, sodium monohydrogenphosphatedihydrate were obtained from Merck AG (Darmstadt, Germany). Sodium chloride and potassium chloride were purchased from Roth GmbH + Co (Karlsruhe, Germany). Acetonitrile was HPLC-gradient grade from Merck AG (Darmstadt, Germany). All other solvents and chemicals were obtained from Sigma-Aldrich GmbH (Seelze, Germany).

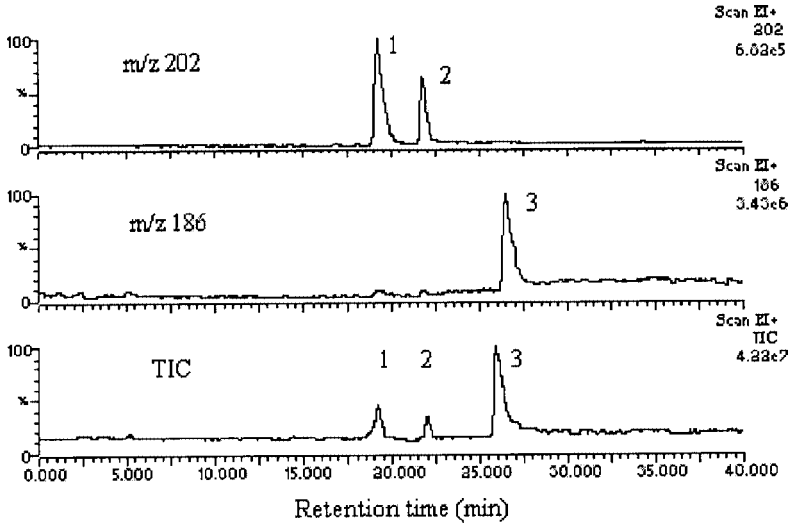



**Figure 1.** Total ion chromatogram and extracted mass chromatograms obtained from an incubation mixture with Desoxypeganine 1. Peak 1 Vasicinone 3, Peak 2 Isovasicinone 4, Peak 3 Pegenone 2.

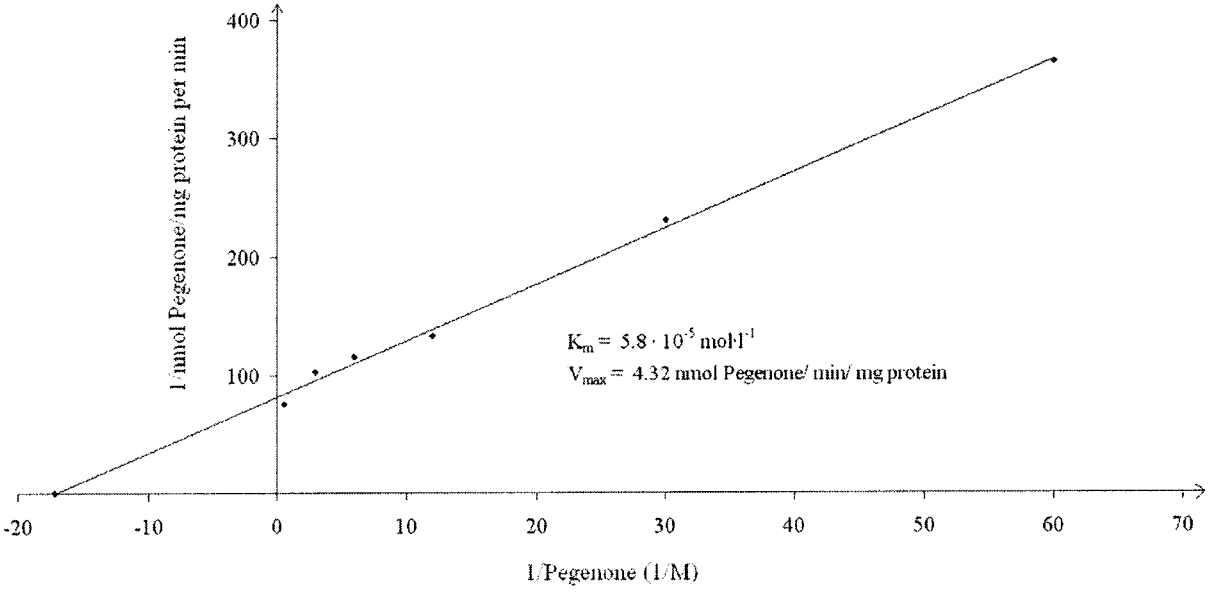



**Figure 2.** Lineweaver-Burk plot of *C*-oxidation of Desoxypeganine 1 measured by Pegenone 2 formation in the incubation mixtures containing the components described under Materials and methods. Mean values are presented from three determinations.

### Chemistry

Desoxypeganine 1 and Pegenone 2 are known compounds which were synthesized by reported methods. Pegenone-*N*-oxide (1,2,3,9-tetrahydropyrrolo[2,1-b]-quinazoline-9-one-4-oxide) was prepared by treating Pegenone with 30% hydrogenperoxide, and purified by preparative HPLC. All compounds were analyzed in the usual way.

### Biological assay

*Preparation of the 9000g supernatant of rabbit liver*

All the following operations were carried out at 0−4°CC. The 9000*g* supernatant preparations were produced in most cases as described previously [15]. The removed livers from rabbits were washed four times with phosphate buffer pH 7.4, dried with a towel, weighed and minced with a scalpel. The minced livers were homogenised with 2 parts of 1.15% (w/v) KCl solution using an Ultra-Turrax homogeniser (Ultra-Turrax type T45, Janke Kunkel GmbH Co KG Staufen, Germany) four times for 30 s under permanent cooling the round-bottomed flask in an ice bath. The homogenate was transferred into plastic tubes and centrifuged for 30 min at 9000*g* and 2°C. The 9000*g* supernatant was carefully decanted and used as enzyme source. The incubation test series and the kinetic experiments were realised on one day storing the enzymes permanently on ice. Protein concentration was determined as described by Gornall et al. [16] and the content of cytochrome P 450 in the supernatant was measured by using the method of Omura and Sato

[17]. The kinetic experiments were carried out with an UV-VIS Scanning Spectrophotometer Shimadzu UV-2101 PC (Shimadzu Corporation, Kyoto, Japan).

*In vitro incubation*

Incubations were carried out in 20 mL Erlenmeyer flasks at 37°C in a shaking water bath. The incubation samples contained 1 or 2 mL of the 9000g supernatant, 1 mL cofactor solution (containing 2 mg NADPH tetrasodium salt, 0.2 mL $MgCl_2$ 0.1 M and distilled water to a final volume of 1.0 mL), 1 mL of the substrate solution (Desoxypeganine 1 10 μmol $mL^{-1}$ or Pegenone 2 10 μmol $mL^{-1}$) and 3 mL of phosphate buffer pH 7.4 (13 mM $KH_2PO_4$, 53 mM $Na_2HPO_4 \cdot 2\ H_2O$) or 3 mL of borax buffer pH 8.6 (30.4 mM $H_3BO_3$, 60.8 mM NaOH, 33.1 mM HCl). Control samples were carried out without substrate or enzyme or cofactor in order to compare the results with respect to the dependence between enzyme, coenzyme and substrate. The reaction was started after preincubation at 37°C by addition of the 9000*g* supernatant. After 40 min incubation time the reaction was stopped by cooling the flasks in ice.

*Extraction of the incubation samples*

Incubation samples were transferred to capped centrifuge tubes followed by the addition of 4 g NaCl and 3 mL ice-chilled diethyl ether, and shaken for 30 min. The organic layer of each sample was removed with a Pasteur pipette and concentrated by evaporation under reduced pressure.

**HPLC analysis and mass spectrometry**

The residue was dissolved under initial conditions of the mobile phase (water-acetonitrile (9:1, v/v)) and aliquots (20−100 μL) were analysed using a HPLC system with autosampler (WatersTM LC Module I plus, Waters Associates, Eschborn, Germany), photodiode array detector (WatersTM 996) and a mass spectrometer Fisons MD 800 (Fisons instruments, Mainz, Germany) with an EI source or $CI^+$ source using anhydrous ammonia as reactand gas and nitrogen as nebulising gas. For evaluation and quantification of the fractions Waters MilleniumTM Chromatography Manager Version 2.15 software was used and MassLab Version 1.3 software for mass spectra analysis. The separation was performed at room temperature on a prepacked RP18-SH column (Prontosil 120-5-C 18-SH, particle size 5 μm, length 250 mm × 4.6 mm i.d., Bischoff Chromatography, Leonberg, Germany). The initial conditions of the mobile phase with a flow rate of 1.0 $mL \cdot min^{-1}$ acetonitrile-water (1:9, v/v) were kept for 10 min, then the acetonitrile-water ratio was increased in a linear gradient up to 9:1 (v/v) within 40 min. A calibration curve (area under the curve = AUC) of reference incubation samples at 0°C with 1, 2.5, 5, 10, 25 and 50 $\mu mol \cdot mL^{-1}$ Pegenone 2 was performed under the same conditions as described before. The correlation coefficient was 0.995. It was possible to determine the concentrations of Pegenone 2 in unknown incubation samples directly from this reference curve. In comparison to a calibration curve with the same concentrations of Pegenone 2 without incubation mixture a recovery rate of 81.4% was observed. Depending on the detection limits of the HPLC analysis or mass spectrometry systems aliquots up to 100 μl were used.

## References

[1] Kh. N. Khashimov, M.V. Telezhenetskaya, S. Yususov, *Khim. Prir. Soedin.* **1969**, *5*, 456.

[2] M. Mahmoudian, H. Jalilpour, P. Salehian, *Iran. J. Pharm. Ther.* **2002**, *1*, 1−4.

[3] R. C. Morris, W. E. Hanford, R. Adams, *J. Am. Chem. Soc.* **1935**, *57*, 951−954.

[4] Kh. Shakhidayatov, A. Irisbaev, Ch. Kadyrov, *Khim. Prir. Soedin.* **1974**, *5*, 681−682.

[5] K. D. Sargazakov, Kh. N. Aripov, L. V. Molchanov, V. N. Plugar, *Khim. Prir. Soedin.* **1990**, *4*, 506−507.

[6] N. Tulyaganov, F. Sadritdinov, G. A. Suleimanova, *Farmakol. i Toksikol.* **1986**, *49*, 37−40.

[7] J. R. Gillete, S. S. Lau, T. J. Monks, *Biochem. Soc.Trans.* **1984**, *12*, 4−7.

[8] V. N. Plugar, N. D. Abdullaev, Y. V. Rashkes, M. R. Yagudaev, N. Tulyaganov, *Khim. Prir. Soedin.* **1983**, *6*, 758−766.

[9] V. N. Plugar, Y. V. Rashkes, N. Tulyaganov, *Khim. Prir. Soedin.* **1981**, *2*, 201−206.

[10] S. C. Sharma, M. A. Siddiqi, U. Zutshi, C. K. Atal, *Ind. Drugs* **1983**, *8*, 431−434.

[11] T. S. Tulyaganov, *Khim. Prir. Soedin.* **1993**, *1*, 87−88.

[12] T. S. Tulyaganov, P. Atabshanov, N. D. Abdullaev, O. Kristallovich, S. Osmanov, *Khim. Prir. Soedin.* **1993**, *4*, 580−583.

[13] C. Sams, G. D. Loizou, J. Cocker, M. Lennard, *Toxicol. Lett.* **2004**, *147*, 253−260.

[14] Y. Lin, P. Lu, C. Tang, Q. Mei, G. Sandig, A. D. Rodrigues, T. H. Rushmore, M. Shou, *Drug Meta. Disp.* **2001**, *29*, 368−374.

[15] B. Clement, M. Zimmermann, *Biochem. Pharmacol.* **1987**, *36*, 3127−3133.

[16] A. G. Gornall, C. J. Bardawill, M. M. David, *J. Biol. Chem.* **1949**, *177*, 751−766.

[17] T. Omura, R. Sato, *J. Biol. Chem.* **1964**, *239*, 2379−2385.