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Anthelmintic activity of 3,6-dibenzyl-2,5-dioxopiperazine, cyclo(L-Phe-L-Phe)

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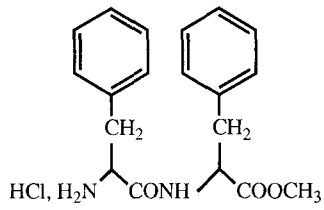
Summary. After oral administration, dipeptide Phe-Phe-OMe **1** exhibits anthelmintic activity against *Echinococcus multilocularis* larvae, cestoda, in mongolian gerbils (intraperitoneal localization), but not against *Hymenolepis nana*, cestoda, in fasted mice (gastro-intestinal localization). This compound rapidly provides its cyclization product dioxopiperazine **2** in pH 7.4 buffer at 37°C, but was stable at pH 2.4 during 16h at 30°C. It was postulated that dipeptide **1** could act as a prodrug of **2**. Initial pharmacokinetics studies were carried out in mice and dogs. After oral administration, biotransformation of **1** into **2** occurred to some extent in mice but not in fasted dogs. Results of these studies did not allow to ascertain that **1** is a prodrug of **2**. Compound **2** has been tested in mice against *H. nana* and *Schistosoma mansoni*, a gastro-intestinal trematoda. Albeit less active than the reference compound praziquantel, **2** has shown a good activity against both worms. 2,5-dioxopiperazines represent therefore a new class of anthelmintics.

Keywords: Amino acids – 2,5-piperazine dione – Dipeptide – Anthelmintics – *Hymenolepis nana* – *Schistosoma mansoni* – Cyclo(Phe-Phe)

In an earlier report (Walchshofer et al., 1993), we described the anthelmintic activity of the dipeptide L-Phe-L-Phe-OMe, HCl **1** (Fig. 1) administered *per os* in Mongolian gerbils against *Echinococcus multilocularis* larvae (intraperitoneal localization). The larval stage of this cestoda causes alveolar echinococcosis, a lethal disease in man (Amman et al., 1988) with primarily liver injury. Until now, no drug has proved to be definitively effective in humans. Dipeptide **1** was tested initially as a better absorbed derivative of L-phenylalanine (Bodor et al., 1977), a well-known inhibitor of alkaline



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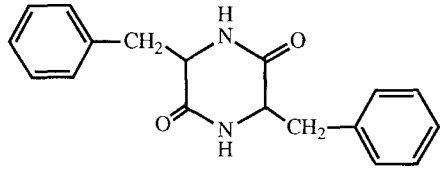

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Fig. 1. Molecular formula of dipeptide **1** and dioxopiperazine **2**

phosphatases (Onica et al., 1990) which have been designed as a target for drugs active against *E. multilocularis* (Sarciron et al., 1991). However, as described herein, a chemical stability study of Phe-Phe-OMe **1** performed at pH 7.4 has shown a rapid cyclization of **1** into 3,6-dibenzyl-2,5-dioxopiperazine **2**. It was then postulated that **1** could act as a prodrug of **2**. These considerations led us to investigate the anthelmintic activity of **2** in mice, in first attempts against a gastro-intestinal cestoda, *Hymenolepis nana*, and a gastro-intestinal trematoda, *Schistosoma mansoni*. Schistosomiasis is the second worldwide endemy after malaria: this parasite disease occurs in about 2 millions humans and runs at the present time (WHO expert committee on the control of schistosomiasis, 1993).

Compounds **1** and **2** were tested *versus* praziquantel as reference compound. Praziquantel is effective against cestoda and trematoda (Groll, 1984) and is the drug of choice of human schistosomiasis (Anonymous, 1993).

The effects of dipeptide **1** and compound **2** against *H. nana* and the effects of **2** against *S. mansoni* are detailed in this paper. Preliminary plasma profile studies of **1** and **2** after oral administration in mice and dogs are also reported.

Materials and methods

Synthesis

3,6-Dibenzyl-2,5-dioxopiperazine **2**

To a solution of L-Phe-L-Phe, HCl **1** (Walchshofer et al., 1993) (1.5 g, 4.14 mmol) in methanol (50 ml) was added triethylamine (1.67 g, 16.5 mmol). The solution was stirring at reflux during 12 h. After evaporation, the residue was washed with minimal cold propan-2-ol to give **2**. 70% yield. mp > 300°C (Mettler FP1). ^{1}H NMR (DMSO d_6, TMS) δ 2.18 and 2.25 (2d, 4H, C$\underline{H}_2$CH, J = 6.3 Hz), 3.92–4.02 (m, 2H, C$\underline{H}$CH$_2$), 7.0–7.32 (m, 10H,

ArH), 7.94 (broad s, 2H, NH), spectra recorded using a Brücker BZH, 200 MHz spectrometer.

Chemical stability of Phe-Phe-OMe, HCl **1**

Chromatographic analyses were performed on a Kontron high performance liquid chromatograph equiped with a 420 pump, a 423 wavelength UV detector and a Schimadzu integrator. The column was a C_{18} Vydac 250 mm × 4.6 mm. The mobile phase consisted of acetonitrile/water (50:50) at a flow rate of 1 ml/min, detection λ = 220 nm. Molar extinctions of **1** and **2** were determined at λ = 220 nm with a Beckman UV spectrophotometer.

The following buffer solutions were prepared: potassium dihydrogen phosphate – disodium hydrogen phosphate, pH 7.46. Potassium hydrogenophtalate – hydrochloric acid 0.1 N, pH 2.4.

A solution of 1.1×10^{-4} M of dipeptide **1** in methanol/buffer pH 7.46 (20:80) was equilibrated in a water bath at 37°C. At appropriate time intervals 20 μl of the reaction mixture were analysed by isocratic HPLC. Dioxopiperazine **2**/dipeptide **1** ratios were calculated based on the peak areas and molar extinctions then plotted *versus* time (Fig. 2).

On an other hand, 1 g of dipeptide **1** was stirred in 30 ml of buffer solution pH 7.46 during 10 h at 37°C. The formed precipitate was filtered, washed with water to afford a white powder which was identified as dibenzodioxopiperazine **2** (80% yield). After stirring in 30 ml of buffer solution pH 2.4 during 16 h at 30°C, **1** was recovered in 90% yield.

Anthelmintic activity

Drugs: Praziquantel PZQ, purchased from BAYER, dipeptide **1** and dioxopiperazine **2** were suspended in 0.2 ml of a solution of cremophor EL 1% (Sigma, St Louis M.O., USA) in glycerol 25% (Delgado et al., 1992). The latter solution without any drug was used as placebo.

Animals: mice were purchased from IFFA-CREDO (France).

S. mansoni: 20 BALB/C male mice, 4 weeks old, were infected by subcutaneous injection of 120 cercariae/mouse. After fifty days, a control of the infection was realized by examinating feces of each mouse, then the mice were divided into 3 groups (Table 1): group 1, controls (6 animals, infected but untreated, received once 0.2 ml of the placebo *per os*); group 2 (7 animals infected and treated with PZQ at the single oral dose of 500 mg/kg); group 3 (7 animals infected and treated with compound **2** at the single oral dose of 500 mg/kg). The selected oral single dose was 500 mg/kg, the ED_{95} of praziquantel in mice being 685 mg/kg (Andrews, 1981). The rate of *Schistosoma* egg excretion in the feces of mice was estimated by the Ritchie technique (formol/ether) (Rousset, 1989) at days 24 and 28 after treatment. Degenerated (died) and viable eggs present in 200 mg of feces were counted. Percent removal of eggs was determined as follows: % removal = $[(N - n)/N] \times 100$. N: average number of eggs per untreated mouse, n: average number of eggs per treated mouse.

H. nana: 40 NMRI male mice, 4 weeks old, were infected by gavage with *H. nana* cysticercoïdes. After 3 weeks, the infection was controlled. The preceding day of treatment, the animals were deprived of solid food. The mice received only 5% sucrose solution *ad libitum* and were divided into 4 groups (Table 1), which received usual doses (Walchshofer et al., 1987): group 1, controls (10 animals infected but untreated received once 0.2 ml of the placebo *per os*); group 2 (10 animals infected and treated with PZQ at the single oral dose of 30 mg/kg); group 3 (10 animals infected and treated with dipeptide **1** at the single oral dose of 200 mg/kg); group 4 (10 animals infected and treated with dioxopiperazine **2** at the single oral dose of 30 mg/kg). Autopsy was carried out one day

Table 1. Protocols of treatment and anthelmintic activity of dipeptide **1** and dioxopiperazine **2** *versus* praziquantel

Helminthe	Group	N	Treatment (compound, oral single dose)	Activity
Schistosoma mansoni	1	6	placebo	0
	2	7	PZQ, 500 mg/kg	++
	3	7	**2**, 500 mg/kg	+
Hymenolepis nana	1	10	placebo	0
	2	10	PZQ, 30 mg/kg	++
	3	10	**1**, 200 mg/kg	0
	4	10	**2**, 30 mg/kg	+

N number of mice.

after dosage and worms were counted. Percent removal of worms was determined as percent removal of eggs above.

Disposition study in mice and dogs

Specific pathogen free male CD mice, weighing 20 g b.w. were purchased from IFFA-CREDO (France). After reception, they were maintained for one week in the laboratory under conventional conditions. Three healthy male Beagle fasted dogs from the indoor colony, 12 to 14 kg b.w., were used.

Compounds **1** or **2** were administered at the oral dose of 40 mg/kg as a 0.25% suspension in carboxymethylcellulose. After dosage, blood samples were collected from mice by decapitation and from dogs by jugular puncture at standardized intervals. Blood samples were immediatly centrifuged, plasma was separated and frozen at −20°C until analysis. **1** and **2** were extracted from plasma by a liquid-solid procedure using C18 precolumns (Vac Elut System Varian) which were activated by methanol and rinced by Analar R water before percolation of 200 μl of plasma and subsequent cleaning by 750 μl water and 1,000 μl of the mixture methanol-water (30:70).

HPLC analysis used an Automated Analytical Sample Processor (AASP) Varian, two 110b pumps and a 421 controller (Beckman), an UV 2,050 detector (Varian) and a 3,393 integrator (HP). The column used was a Kromasil RP18 (Interchim) 5 μm particle size, 250 mm length and 4.6 mm internal diameter. Flow rate was 2 ml/min. Solvent A was acetonitrile Chromosorb and solvent B was Analar R water (BDH) + 1% phosphoric acid. The elution gradient shifted from 85% B to 65% B within 15 min. UV detection was set at 220 nm. In these conditions, retention times were 11.2 min for **1** and 13.1 min for **2**. Using spiked plasma samples, extraction rates were 100% for **1** and 83% for **2**.

Results and discussion

*Cyclization of dipeptide **1** into dioxopiperazine **2***

The *in vitro* studies show that dipeptide **1** undergoes a rapid cyclization into the dioxopiperazine **2** (Fig. 2) at pH 7.4 and 37°C. **2** is the only formed product and the half-life of **1** was determinated as 2h 36 min. Dipeptide **1** was found stable at pH 2.4 and 30°C during at less 16 h. Rate of cyclization depends greatly from the pH conditions as also reported for other dipeptides (Gaines and Bada, 1987).

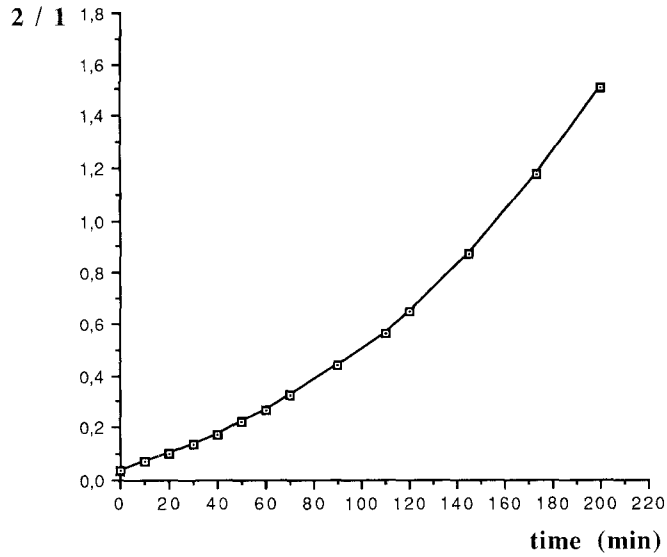

Fig. 2. Cyclization of **1** at pH 7.4. Concentration ratios of **1** and **2** formed from it *versus* time

In mice, after oral administration of **1**, this compound was never detected in plasma. On the contrary, the cyclization product **2** was shown (0.24μg/ml) as early as 30min after dosage, culminated at 0.92μg/ml at 1h and declined to 0.05μg/ml 4h after administration of **1**.

In dogs, after oral administration of **1**, no significant concentrations of both **1** and **2** were detected. After administration of **2**, this compound was evidenced in plasma of a single dog at a very low concentration (0.2μg/ml) at 1h after dosage.

The results of these preliminary studies *in vivo* indicate that dioxopiperazine **2** is a metabolite of the dipeptide **1** in mice, but certainly not the only one formed. Compound **2** was not found in dogs after administration of **1**. However, dogs were fasted contrary to mice. The gastric pH of fasted animals is more acidic, which may preclude a possible cyclization of **1** in the stomach. Further studies should consider the influence of gastric pH on cyclisation and the fact that dipeptide methyl ester **1** could be hydrolyzed to some extent in the gut.

*Effect of compounds **1** and **2** against Hymenolepis nana, cestoda, in fasted mice* (Table 1)

Less active than praziquantel (worm reduction 100% at 30mg/kg), compound **2** shows however an interesting activity against *H. nana* (worm reduction 43% at 30mg/kg). On the other hand, the dipeptide **1** was found inactive against this gastro-intestinal cestoda, even at the high dose of 200mg/kg. It is therefore unlikely that the dipeptide **1** will be cyclized to dioxopiperazine **2** in the stomach of fasted mice or even in the gut. Whether activity of **1** in not fasted

Table 2. Percent removal and average number of *S. mansoni* eggs ± SD per mouse, found in infected animals treated with dioxopiperazine **2** or praziquantel PZQ (500mg/kg) *versus* placebo, at days 24 and 28 after treatment

Compound	Egg number mean values ± SD		Removal of viable eggs	
	Day 24	Day 28	Day 24	Day 28
placebo	105.2 ± 19.1*	146.2 ± 18.4*	–	–
2	28.7 ± 14.5* 23.7 ± 12.3**	28.1 ± 18.1* 21.4 ± 14.8**	72%	81%
PZQ	2.1 ± 3.7*	2.3 ± 3.6*	98%	98.5%

*viable eggs, **degenerated eggs.

gerbils (Walchshofer et al., 1993) against the tissue cestoda *E. multilocularis* proceeds from **2** or not cannot be determined without more extensive studies.

*Effect of compound **2** against Schistosoma mansoni (trematoda) in mice* (Tables 1 and 2)

If we compare the total number of eggs found in the feces of control animals with those found in the feces of animals treated with compound **2** (Table 2), we observe a 66% decrease at day 28 after treatment. However, about 50% of the total egg amount were degenerated and percent removal of viable eggs was 81%. This percentage was 98 to 99% for animals treated with praziquantel and no degenerated eggs were noted. Compound **2** is less active than the latter, but possesses a schistosomicide activity still at day 28. When the activity of drugs is moderate, the oogram may return to normal in a few days (Pellegrino and Faria, 1965).

Conclusions

We have described in this report the anthelmintic properties of 3,6-dibenzo-2,5-dioxopiperazine, cyclo(Phe-Phe). Witiak and Wei (1990) have reviewed the chemistry and biology of 2,5-dioxopiperazines. These compounds have shown e.g. antitumor, cholinesterase inhibitor or platelet-activating factor inhibitor properties, but antiparasitic activity has not yet been described on these derivatives. They represent therefore a new class of anthelmintics (Walchshofer et al., 1994). Furthermore, 2,5-dioxopiperazine derivatives are less costly than other molecules like praziquantel, whose high cost represents a major constraint in achieving control of schistosomiasis (WHO expert committee on the control of schistosomiasis, 1993). Further investigation in this class of anthelmintics is in progress.

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