

# Toxicity of *Palicourea marcgravii*: Combined Effects of Fluoroacetate, N-methyltyramine and 2-Methyltetrahydro- $\beta$ -carboline

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*Palicourea marcgravii*, N-methyltyramine, 2-Methyltetrahydro- $\beta$ -carboline,  $\omega$ -Fluorofatty Acid, Fluoroacetate

Feeding experiments carried out with cattle and horses could prove the toxic effects of *P. marcgravii* (Rubiaceae) in all cases. The typical symptoms of “sudden death”, however, are observed in ruminants only. This difference could not be explained so far.

Apart from fluoroacetate, two more substances also have influence the toxic effects and have been isolated from *P. marcgravii* for the first time: N-methyltyramine and 2-methyltetrahydro- $\beta$ -carboline (2-Me THBC). Structure elucidation of these compounds is mainly accomplished by  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$  and MS techniques.

Due to the small quantity of fluoroacetate (5.4  $\mu\text{g/g}$  plant), the main toxic effect obviously lies in the two discovered substances. In contrast to the slow death of horses (monogastrics), the “sudden death syndrome” of cattle (ruminants) can be explained as a result of the higher resorbability of these two substances in the gastro-intestinal system.

Given orally, both substances influence the monoamine oxidase type A (MAO-A): N-methyltyramine acts as a competitive substrate, and 2-Me THBC is one of the most effective MAO-A-inhibitors.

Thus, the decomposition of the specific MAO-A-substrates noradrenaline and adrenaline as well as of N-methyltyramine itself is inhibited. The  $\alpha$ - and  $\beta$ -receptors of the sympathetic system are stimulated more strongly, which leads to a drastic rise in blood pressure and thereby to a more rapid distribution of fluoroacetate in the body. This results in a reinforced input of fluoroacetate in the cells of especially active organs of the body (heart etc.). Thus, even smaller quantities of fluoroacetate are lethal.

## Introduction

*P. marcgravii* (Rubiaceae) is a poisonous plant which is endemic in the Amazonian area. The plant causes the “sudden death” of cattle (Döbereiner and Tokarnia, 1986), which is described as follows: it takes only 1–10 min from the appearance of the first symptoms until death takes place. There are signs of cardiac insufficiency such as positive venous pulse and muscle tremor. The animals lie down or fall on their belly or side. Tachypnoe, rowing movements with their legs, opisthotonus and finally death occur.

Until now, fluoroacetate has been considered to be the only cause. The mechanism is well-known: In the organism fluoroacetate is transferred to fluoro-citrate which is irreversibly bound to aconitase.

Thereby the citrate cycle is blocked (Metzler, 1977).

The quantities of fluoroacetate are relatively low, amounting to 5.4  $\mu\text{g}$  per g dried plant (Krebs, Kemmerling and Habermehl, 1994). Subcutaneous injection of sodium fluoroacetate yields a  $\text{LD}_{50}$  value of 200  $\mu\text{g/kg}$  in rats (Gribble, 1973).

Experimental poisoning of horses (Tokarnia *et al.*, 1993) does not lead to the symptoms of “sudden death” as in the case of cattle. Main symptoms are nervous manifestations as well as intensive sweating, restlessness, abrupt involuntary movements of the head or the whole body. The clinical course of the poisoning ranged from 10 to 43 hours. It may be concluded that more substances must be responsible for the toxic effects.

## Material and Methods

The mass spectra were taken on a Finnigan spectrometer MAT 312. Measurements were

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taken with an ionization potential of 70 eV. The signal intensities were given as percentages of the basal peaks. The temperature is given in degrees centigrade.

The  $^1\text{H}$ -NMR spectra were taken on a Bruker spectrometer AM 300 in combination with tetramethylsilane as internal standard.  $\text{CDCl}_3$  or  $\text{CD}_3\text{OD}$  respectively served as solvent. The signals were characterized by their multiplicity (s = singlet, d = doublet, t = triplet), coupling pattern ( $J$  in Herz) and integration.

The  $^{13}\text{C}$ -NMR spectra were taken on a Bruker spectrometer AM 300 using tetramethylsilane as internal standard.  $\text{CDCl}_3$  or  $\text{CD}_3\text{OD}$  respectively served as solvent. Measurements were taken according to the DEPT-method. The chemical shifts are given as  $\delta$ -values in ppm.

For the analytic thin-layer chromatography, pre-fabricated foils by MERCK (Kieselgel 60 F 254) were used.

Dragendorff-reagent was used as a spray reagent for the thin-layer chromatography.

The column chromatography was performed with silica gel (Flashgel) by Baker, grain diameter 0.02–0.036 mm, with low pressure ("flash"). The solvent mixtures are given with the data.

The solvents were applied dried and distilled.

#### *Plant material*

The plants (leaves) were collected in Brazil, State of Para. The collecting date wasn't given. The leaves were dried and ground.

#### *Extraction of the leaves from P. marcgravii*

400 g leaves were extracted with 4 litres of petrol-ether (four days) and subsequently 4 litres of methylene chloride (four days), by aid of a heating device, a 6 litre round beaker with 2000 ml soxhlet and reflux cooler. The PE and  $\text{CH}_2\text{Cl}_2$  extracts gained were put aside. The plant material was dried (removing rests of  $\text{CH}_2\text{Cl}_2$ ) and then extracted with 4 litres of methanol for four days. In this way, 1200 g leaves were extracted altogether. The methanol was removed by evaporation and 160 g of brown, thick extract were yielded.

#### *Production of alkaloid crude extract*

160 g MeOH-extract were suspended in 1000 ml 1 N  $\text{H}_2\text{SO}_4$  (with ultra sound). Extraction was performed twice with 500 ml diethylether each.

The combined diethylether phases were reextracted twice with 300 ml fresh 1 N  $\text{H}_2\text{SO}_4$  each. The ether phases were set aside and the acid aqueous phases were combined.

The acid aqueous phases (1.4 l approx.) were stirred with 5 g of zinc powder in a glass beaker for 24 hours with a magnetic stirring device.

The surplus of zinc powder was removed by filtering, and the acid phase was adjusted to pH 12 with  $\text{NH}_3(\text{conc.})$ . 15 g NaCl were dissolved per 100 ml extract. The alkaline extract was extracted four times with 700 ml  $\text{CH}_2\text{Cl}_2$ .

The combined  $\text{CH}_2\text{Cl}_2$ -phases were dried over  $\text{Na}_2\text{SO}_4$  and the solvent was removed in a rotation evaporator. The extraction yielded 1.4 g dark red-brown, thick alkaloid crude extract.

#### *Enrichment of the substances from the alkaloid crude extract*

The alkaloid crude extract (1.4 g) was chromatographed on a silica gel column (diameter 45 mm, 140 g silica gel), with  $\text{CHCl}_3$  : MeOH :  $\text{NH}_3(\text{conc.})$  80 : 10 : 1. The fractions in the range of  $R_F$  0.23 (N-methyltyramine) and  $R_F$  0.68 (2-Me THBC) were collected separately. The thin-layer control was performed by Dragendorff spray reagent. The alkaloids showed an intensive yellow colour on TL. This process yielded crude extracts of 0.62 g N-methyltyramine and 0.21 g 2-Me THBC.

#### *Isolation of N-methyltyramine*

The crude extract of N-methyltyramine (0.62 g) was chromatographed on a silica gel column (diameter 25 mm, 40 g silica gel) with  $\text{CHCl}_3$  : MeOH 80 : 10. It was eluated until all nonalkaloid substances were removed (TLC control). Subsequently N-methyltyramine was eluated with  $\text{CHCl}_3$  : MeOH :  $\text{NH}_3(\text{conc.})$  80 : 10 : 1. This process yielded 520 mg crystalline substance.

#### *Isolation of 2-Me THBC*

The crude extract of 2-Me THBC (0.21 g) was chromatographed on a silica gel column (diameter 25 mm, 20 g silica gel) with  $\text{CHCl}_3$  : MeOH 80 : 5 until all non-alkaloid substances were removed (TLC control). Subsequently, 2-Me THBC was eluated with  $\text{CHCl}_3$  : MeOH :  $\text{NH}_3(\text{conc.})$  80 : 10 : 1. This procedure yielded 51 mg crystalline substance.

All in all the yields of the alkaloids were 42.5 µg 2-Me THBC and 433.3 µg N-methyltyramine per g dried leaves.

The isolation was performed several times. It could be observed that the amount of N-methyltyramine in the plant material was swaying. The highest amount was 433.3 µg.

### Spectroscopic data

#### 2-Me THBC

**<sup>13</sup>C-NMR** (see Figs 1 and 2);

**<sup>1</sup>H-NMR**, 300 MHz, CDCl<sub>3</sub>: 7.47 (d, 1H-H<sub>a</sub>, *J* = 8 Hz), 7.3 (d, 1H-H<sub>b</sub>, *J* = 8 Hz), 7.12 (m, 2H-H<sub>c</sub>), 3.57 (s, 2H, Ar-CH<sub>2</sub>-N<sup>+</sup>), 2.81 (m, 4H, Ar-CH<sub>2</sub>-CH<sub>2</sub>-N<sup>+</sup>), 2.48 (s, 3H, N-CH<sub>3</sub>);

**MS**, 110°C: M<sup>+</sup> = 186 (20.5), 143 (100), 128 (5), 116 (10.2), 115 (15.4), 77 (7) (see Fig. 2).

#### N-Methyltyramine

**<sup>13</sup>C-NMR** (see Figs 1 and 3);

**<sup>1</sup>H-NMR**, 300 MHz, CDCl<sub>3</sub>: 7.0 (d, 2H-2,6, *J* = 9 Hz), 6.7 (d, 2H-3,5, *J* = 9 Hz), 2.75 (t, 2H, *J* = 6 Hz) and 2.85 (t, 2H, *J* = 6 Hz; -CH<sub>2</sub>-CH<sub>2</sub>-), 2.4 (s, 3H; N-CH<sub>3</sub>), CD<sub>3</sub>OD two exchangeable H (-OH, -NH-CH<sub>3</sub>);

**MS**, 120°C: M<sup>+</sup> = 151 (100), 135 (5.1), 121 (13.1), 120 (11.4), 108 (38), 107 (59.3), 91 (16.3), 77 (47.8) (see Fig. 3).

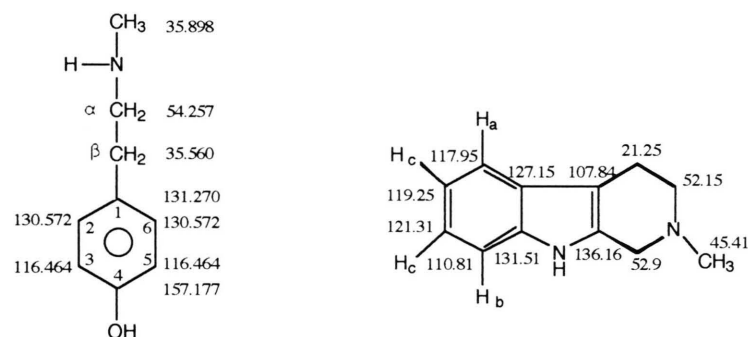
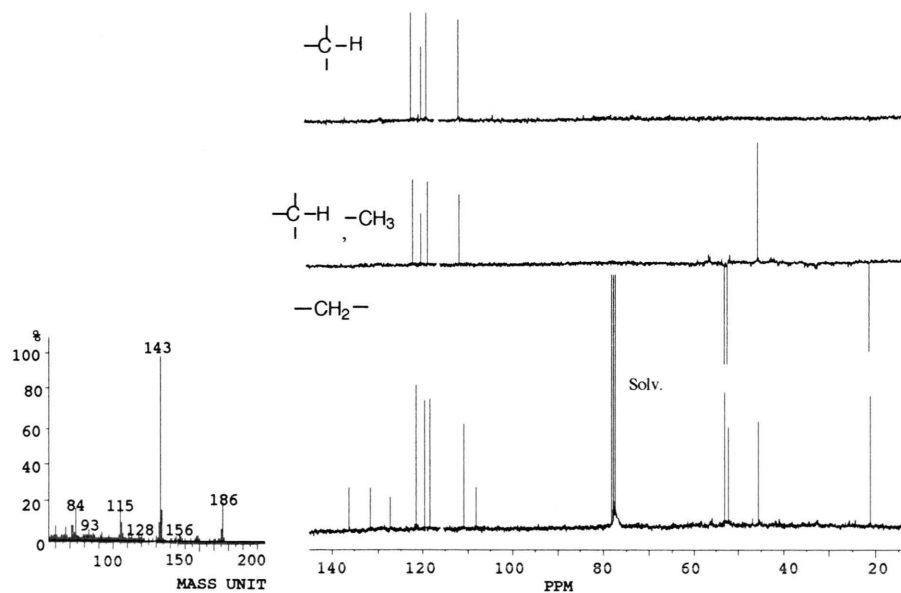


Fig. 1. <sup>13</sup>C-NMR N-methyltyramine. 2-Methyltetrahydro-β-carboline (2-Me THBC). The numbers next to the C-atoms show the chemical shift in ppm (75 MHz, DEPT, 2-Me THBC in CDCl<sub>3</sub>, N-methyltyramine in CD<sub>3</sub>OD).



Mass Spectrum (110°C)

<sup>13</sup>C-NMR (75 MHz, DEPT, Solvent CDCl<sub>3</sub>)

Fig. 2. Spectra of 2-methyltetrahydro-β-carboline (2-Me THBC).

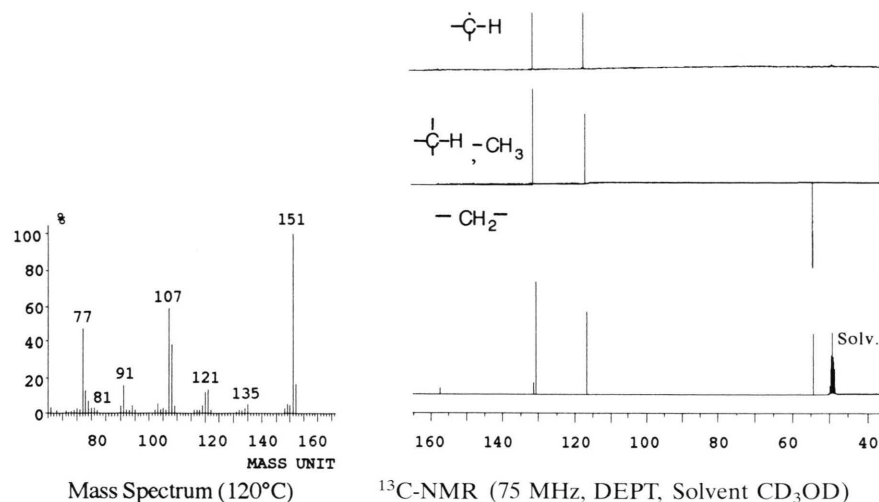


Fig. 3. Spectra of N-methyltyramine.

## Results and Discussion

The plant material is freed from unpolar parts by the extraction with petrol-ether and methylene chloride. Afterwards, extraction is continued with methanol. The extracts gained by this procedure also contain the alkaloids.

The enrichment of the two alkaloids is based on acid-base separation. In the acid medium they exist as cations showing a high polarity.

The MeOH extract of the plants suspended in 1 N  $H_2SO_4$  can be separated without any difficulty from the more unpolar parts of the extract by aid of diethylether. The alkaloids stay in the acid aqueous phase. The reduction with zinc sets the protonated alkaloids free again from the partly formed N-oxides.

Now the pH is adjusted to 12 approximately, by means of  $NH_3$ (conc.). The N-methylated amino groups of the alkaloids are then uncharged. In addition, the enrichment of the aqueous phase with NaCl leads to a higher volatility of the substances. Therefore, they can be extracted with methylene chloride.

The isolation of the substances from the alkaloid crude extract must be carried out in two steps by column chromatography. The two alkaloid fractions of the first column chromatography also contain nonalkaloidal substances (apart from the alkaloids) with the same  $R_F$ -value.

Therefore in the second column chromatography no ammonia is used for isolation purposes. Because of the weak acidity of the silica gel the

nonalkaloidal compounds can be eluated, whereas the alkaloids stay bound.

Afterwards the alkaloids are isolated in pure form with a solvent containing ammonia.

N-methyltyramine causes, among other effects, positive venous pulse and tachypnoe (guinea pigs) and "wobbling legs" (sheep and goats). These symptoms found by Evans *et al.* (1979) are the same like those described in cattle by Tokarnia *et al.* (1986).

*P. marcgravii* also contains 2-Me THBC in addition to N-methyltyramine. Both alkaloids have enormous effects on the physiology of an animal organism and potentiate the poisonous effect (Fig. 4).

The isolated 2-Me THBC is one of the most effective MAO-A inhibitors (Meller *et al.*, 1977), i.e. the decomposition of adrenaline and noradrenaline is inhibited. The MAO catalyzes the oxidative desamination of primary and secondary amines. There are two types of MAO: Type A and type B have different substrate specificity. The neurotransmitters adrenaline and noradrenaline are specific type A substrates. This has been proved by Singer *et al.* (1979) with enzyme models.

The second isolated substance, N-methyltyramine, is also a MAO-A substrate (Singer *et al.*, 1979). It competes with adrenaline and noradrenaline for the active centre of the MAO-A. Thus, the activity of the MAO-A is "blocked" in two ways:

1) The MAO-A inhibitor 2-Me THBC lowers the activity.

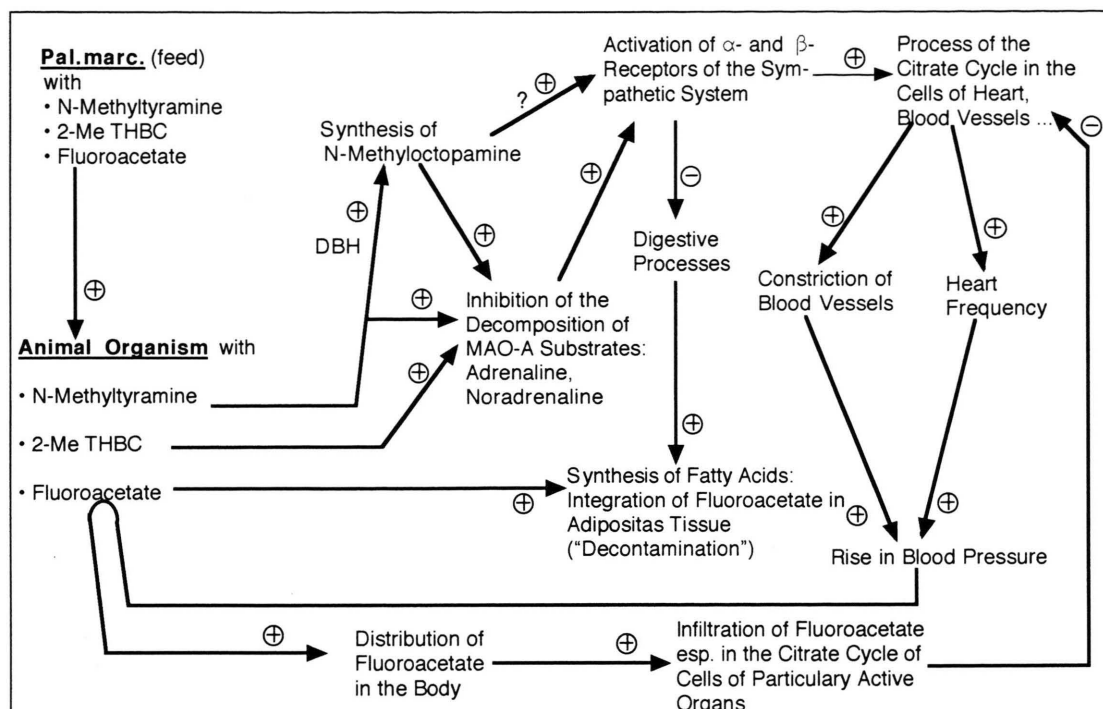


Fig. 4. Pattern of the physiology of the poisonous effect.

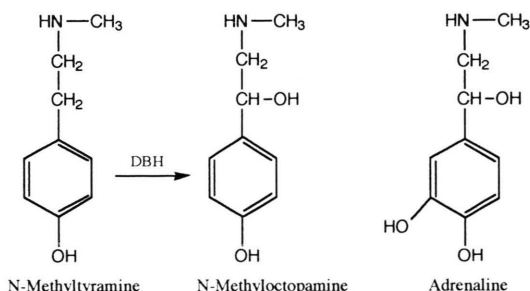
2) N-methyltyramine serves as a competitive substrate.

Both effects result in a strong rise of the concentrations of the MAO-A substrates adrenaline and noradrenaline in the blood. This leads to a stronger stimulation of the  $\alpha$ - and  $\beta$ -receptors of the sympathetic system and thereby to a dramatic rise in blood pressure and decrease of the digestive activity. Thus, N-methyltyramine works as an indirect sympathomimetic. This is proved by tests performed with heart cells culture (Kemmerling, 1995).

But it is also conceivable that it works as a direct sympathomimetic. N-methyltyramine was transformed from dopamine- $\beta$ -hydroxylase (DBH) to N-methyloctopamine with 25 % of the dopamine activity (Fujita *et al.*, 1971) (see Fig. 5).

N-methyloctopamine differs from adrenaline only in one hydroxyl group in position 3, and it possibly has the same effect.

The rise in blood pressure causes an increase in the energy demand in the cells of heart and blood vessels. The adrenergic stimulation of the citrate cycle is opposed by the inhibition caused by fluoracetate. Under these conditions the latter is distributed rapidly in the body and transformed to aconitase-blocking fluorocitrate. This preferably happens in places of the highest consumption of

Fig. 5. Reaction of dopamine- $\beta$ -hydroxylase (DBH).

energy, i.e. for example in the heart. Whether death is caused by high blood pressure, inhibition of the citrate cycle or a combination of both, depends on the respective doses of the substances in the plant and their resorbability in the gastro-intestinal system of the grazing animals.

The isolated alkaloids allow to explain the differences in the poisonous effect on ruminants (cattle) and monogastrics (horses) for the first time. In this way, cattle die of "sudden death" (Tokarnia, Döbereiner, 1986) whereas horses die after 10–43 hours (Tokarnia *et al.*, 1993). When considering similar doses, this difference can only be explained by the different resorbability in the gastro-intestinal systems.

The ruminant system causes a better digestion of the food. Furthermore in the rumen, reticulum and omasum the alkaloids are mostly deproton-

ized, i.e. they are more unpolar and therefore resorption is better. The presence of alkaloids is responsible for the lethal effect of even smaller amounts of fluoroacetate in cattle.

The strong acid pH in the stomach of horses is responsible for a protonisation of the alkaloids which are therefore resorbed to a lesser degree. That is why death is delayed.

A fourth substance group participating in poisonous effect are  $\omega$ -fluorofatty acids. This could be proved by a new extraction method followed by a  $^{19}\text{F}$ -NMR-examination (Kemmerling, 1995).

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