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Preliminary Pharmacological Studies on three Benzoyl Amides, constituents of *Aniba riparia* (Nees) Mez (Lauraceae)

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SUMMARY. Pharmacological screening of methyl ethers of *N*-benzoyltyramine (riparin I), *N*-(2-hydroxybenzoyl)-tyramine (riparin II) and *N*-(2,6-dihydroxybenzoyl)-tyramine (riparin III), obtained from *Aniba riparia* was performed. The compounds given orally (p.o.) or intraperitoneally (i.p.) in doses up to 1 g/kg caused no deaths in mice, except riparin III which was toxic by the i.p. route; lethal dose 50% (LD50) was 104.2 mg/kg. Riparin I and riparin II (500 mg/kg i.p. and p.o.), and riparin III (500 mg/kg p.o. or 35 mg/kg i.p.) were without effects in the screening for behavioural changes in mice. Riparin I, riparin II and riparin III induced nonspecific and reversible relaxation of contractions produced by acetylcholine and histamine in the guinea-pig ileum and by oxytocin and bradykinin in the rat uterus. The inhibitory concentration 50% (IC50 values) varied from 1.7-5.0 µg/ml. Riparin III was, in general, twice as potent as riparin I and riparin II, which were about equipotent. Riparin III also relaxed the guinea-pig trachea (IC50 = 1.9 µg/ml). In the rat phrenic nerve-diaphragm preparation, the compounds inhibited muscle twitches induced by either direct or indirect electrical stimulation (IC50 = 4.0 and 2.4 µg/ml, respectively, for riparin III).

RESUMEN. "Estudios Farmacológicos Preliminares sobre Tres Benzoilamidas constituyentes de *Aniba riparia* (Nees) Mez (Lauraceae)". Se realizaron estudios farmacológicos de éteres metílicos de *N*-benzoiltiramina (riparina I), *N*-(2-hidroxibenzoil)-tiramina (riparina II) y *N*-(2,6-dihidroxibenzoil)-tiramina (riparina III), obtenidos de *Aniba riparia*. Los compuestos administrados por vía oral (v.o.) o intraperitoneal (i.p.) en dosis de hasta 1g/kg no causaron la muerte de ratones, excepto riparina III, que resultó tóxico por la vía i.p. (DL50 = 104,2 mg/kg). Riparina I y riparina II (500 mg/kg i.p. y v.o.) y riparina III (500 mg/kg v.o. o 35 mg/kg i.p.) no presentaron efectos sobre cambios de comportamiento en ratones. Riparina I, riparina II y riparina III provocaron, de forma no específica y reversible, relajamiento de las contracciones producidas por acetilcolina e histamina en el íleo de ratón y por la ocitocina y bradiquinina en el útero de rata. Los valores de CI50 variaron de 1.7-5.0 µg/ml. Generalmente riparina III fue dos veces más potente que riparina I y riparina II, que fueron de igual potencia. Riparina III tambien causó relajamiento de la tráquea de ratón (CI50 = 1,9 µg/ml). Los compuestos tambien inibieron las contracciones del diafragma de ratón, producidas por estimulación eléctrica directa o indirecta (CI50 = 4,0 y 2,4 µg/ml, respectivamente, para riparina III).

INTRODUCTION

The genus *Aniba* comprises 41 species of shrubs and trees and is primarily a lowland group with its centre of diversity in Central Amazonia and Guiana. It extends, however, into the Andes, the mountains of northern Venezuela, the lesses Antilles and eastern and southern Brazil. *Aniba riparia* (Nees) Mez, a Lauraceous tree popularly known as "louro", occurs in the Humaitá region of the Amazonas state of Brazil [1].

Methyl ethers of *N*-benzoyl tyramine (riparin I), *N*-(2-hydroxybenzoyl) tyramine (riparin II) and *N*-(2,6-dihydroxybenzoyl) tyramine (riparin III) with broad spectrum antimicrobial activity have been isolated from the unripe fruit of *Aniba riparia* [1] and later synthesised by Barbosa-

KEY WORDS: *Aniba riparia*, Lauraceae, Methyl ethers of N-benzoyltyramine, Smooth and skeletal muscle relaxation.
PALABRAS CLAVE: Aniba riparia, Lauraceae, Éteres metílicos de N-bernzoitiramina, Relajamiento del músculo liso y esquelético.

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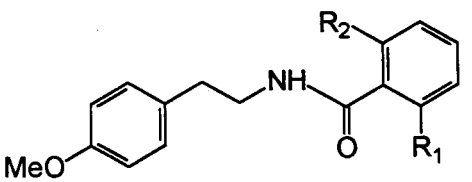
Filho *et al.* 1990 [2,3]. A number of other amides obtained from plants have also been reported to possess biological actions. Antifertility effects of amides of *Aegle marmelos* [4] and prostaglandin synthetase inhibitory properties of phenolic amides isolated from *Ipomoea aquatica* Forsk [5] are some of the examples. Reasons such as above, coupled with our interest in pursuing studies on biologically active constituents obtained from Brazilian plants, prompted us to perform a broad spectrum *in vivo* and *in vitro* pharmacological screening of riparin I, II and compare the results with that of riparin III, the smooth muscle relaxant activity of which has been reported earlier [6]. The results of these studies are presented below.



Riparin I (R_1=R_2=H)
Riparin II (R_1=OH, R_2=H)
Riparin III (R_1=R_2=OH)

MATERIALS AND METHODS

Chemicals

Acetylcholine chloride (Ach), histamine diphosphate (Hist), bradykinin acetate (Bk), carrageenan, cremophor EL, diethylstilboestrol, isoprenaline hydrochloride and oxytocin (Oxy), were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals and reagents were obtained locally and were of analytical grade.

The composition (mM) of salt solutions used for isolated tissues were as follows: a) Tyrode solution: NaCl 136.7, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, $MgSO_4.7H_2O$ 10.5, KCl 2.6, $CaCl_2$ 1.8, and glucose 5.5; b) De Jalon solution: NaCl 153.8, $NaHCO_3$ 6.0, KCl 5.5, $CaCl_2$ 0.3, and glucose 2.8; c) Kreb's solution: NaCl 113.0, $NaHCO_3$ 25.0, $MgSO_4.7H_2O$ 0.6, KCl 4.8, KH_2PO_4 1.2, $CaCl_2$ 2.5, and glucose 11.1.

Animals

Male albino mice (20-25 g) and male wistar rats (120-150 g) were selected for *in vivo* experiments except in blood pressure studies where rats weighing between 280-320 g were preferred. They had free access to water at all times eventhough food was withdrawn 16-18 h before the tests. For studies with isolated tissues male or female guinea-pigs (350-500 g) or rats (250-300 g) were used.

Methods

The amides (95-97% pure) were suspended in 0.1% Tween 80 for administration in dose volumes of 10 ml/kg for *in vivo* experiments. For *in vitro* tests the compounds were solubilized in cremophor and diluted with saline for the required concentrations. The final concentration of cremophor was always less than 0.1%. Even in cremophor the solubility was poor and the maximum concentration of the most soluble riparin III was 2 mg/ml, which hindered the use of higher concentrations of the substances in some experiments.

Acute toxicity

Mice in groups of 5 were given riparin I, II or III by oral (p.o.) or intraperitoneal (i.p.) routes. Initially, all the amides were administered up to a maximum of 1 g/kg. If deaths occurred within 48 h, further tests were made with additional doses to determine approximate lethal dose 50% (LD50) values determined graphically from log dose-probit curves.

Primary screening

Mice in groups of 3 were dosed with riparin I, II and III by p.o. and i.p. routes and were observed every 30 min for the next 4 h. While the three amides were given in doses of 500 mg/kg p.o., the i.p. doses were 500 mg/kg of riparin I and II and 35 mg/kg of riparin III which was approximately a third of its LD50 dose. A less detailed version of hippocratic screening work sheet described by Malone [7] was employed. Particular attention was paid for central nervous system activities such as behavioural changes, sedation, stimulation, tremors, loss of corneal and pinnal reflexes, respiratory alterations and analgesia, and, automomic effects such as salivation, micturation, diarrhoea, piloerection and pupil size. Additional effects looked for were alterations in skeletal muscle functions, for eg, grip strength, back plasticity, paralysis and ataxia.

Analgesic test

Analgesic activity was evaluated in rats by the method of Randall and Selitto [8] using an analgesimeter (Ugo Basile). The animals in groups of 5 were dosed with 250 or 500 mg/kg p.o. of the amides, 90 min after the subcuta-

neous (s.c.) injection of Brewer's yeast into the paws. Aspirin 300 mg/kg p.o. was given as the standard drug to one of the groups. The pressure in grams required to elicit a pain response withdrawal in the inflammed foot was measured at 30 min, 2 and 4 h after drug administration and was compared with the value at zero h to calculate the mean increase in pain threshold in each group.

Antiinflammatory test

Antiinflammatory activity was assessed in carrageenan-induced rat paw oedema as described by Winter *et al.* [9] using rat plethysmometer (Ugo Basile). The amides 250 and 500 mg/kg p.o. or aspirin 300 mg/kg p.o. were administered to groups of 5 rats, 30 min before the carrageenan. Paw volumes were measured every h for the next 4 h after carrageenan. The mean increase in paw volumes of each treated group was compared with the mean increase in the control group to calculate the percent inhibition of oedema for each h.

Isolated smooth muscle and skeletal muscle preparations

Unless otherwise stated, the techniques of preparation and recording of results of theses studies were those that are described in the *"Pharmacological experiments on isolated preparations by the staff of the Department of Pharmacology, University of Edinburgh"* [10]. Five or six tissues were used in each series of experiments.

Guinea-pig ileum preparation

Pieces of ileum were suspended in Tyrode solution at 33-34 ºC and with a 95% O_2 + 5% CO_2 gas mixture (carbogen). After a resting period of 30 min, two submaximal responses (60-75% maximum) of similar magnitude to acetylcholine or histamine were registered on kymographs using isotonic levers (6-7 fold magnification). A third response to the agonist was then obtained in the presence of a concentration of riparin I which was added 7 min before. Various concentrations of the agonist were tested and the antagonism was measured by comparing the mean contraction obtained in the absence and the response obtained in the presence of the amide. Riparin II and riparin III were also tested in the above manner.

Rat uterus preparation

Uterine horns (uterus) from rats treated with stilboestrol 100 µg/kg s.c., 24 h before, were suspended in De Jalon solution at 31-32 ºC. The inhibitory activity of the three amides on bradykinin and oxytocin induced contractions was evaluated by the procedure employed in experiments with the ileum.

Guinea-pig tracheal preparation

Zig-zag tracheal strips were prepared, as described by Emmerson and Mackay [11], suspended in Kreb's solution at 37 ºC and aerated with carbogen. The tissue was allowed to stabilize for an h. The relaxant activity of the amides was evaluated by adding cumulatively, increasing concentrations of each of the amide in separate tissues until a maximum relaxation equivalent to the response produce by the addition of 10^{-7} M isoprenaline was achieved. The responses were registered on a kymograph as before. Percent relaxation produced by the amides was calculated by considering the isoprenaline response as 100%. The participation of ß-adrenoceptors in the relaxant activity of riparin III was tested by comparing the responses to equiactive submaximal concentrations of riparin III and isoprenaline in the absence or in the presence of propranolol (10 µg/ml), a dose which was previously found to antagonize the effect of isoprenaline by 70-80%.

Rat phrenic nerve-diaphragm preparation

The preparation was suspended in Kreb's solution at 37 ºC and was aerated with carbogen. The diaphragm was stimulated directly, or indirectly through the phrenic nerve, by giving electrical shocks (6-8 v) at a rate of 8/min by rectangular wave pulses of 0.5 m sec duration. The amides were added for 20-25 min during which the nerve was stimulated and the supramaximal twitches recorded by the use of transducers coupled to a 2 channel physiograph (Beckman). When the effect of the amide had reached maximal, the muscle was stimulated directly and the responses were again registered. The preparation was washed and further concentration of amides were added when the responses had returned to pre drug levels. The effects of the amides on muscle twitches induced by direct or indirect stimulation were evaluated by comparing the amplitude of the responses before and after the addition of the agents.

Statistical analysis

Data were analysed sing student's t-test or analysis of variance and the selected level of significance for all the results was $P < 0.05$.

RESULTS

Acute toxicity

Administration of riparin I, II or III p.o. at a dose of 1 g /kg failed to cause any deaths in mice during 48 h. At similar doses, riparin I and II were also non-lethal by the i.p. route. However, riparin III caused dose dependent deaths and the LD50 value obtained graphically from the log-dose probit graph was 104.2 mg/kg i.p.

Primary screening

Riparin I and II when given in doses of 500 mg/kg p.o. or i.p. in mice produced no observable alterations in the central, autonomic, or skeletal muscle functions when compared with animals that received 0.1% Tween 80. Similarly, riparin III also failed to cause significant changes in the above functions in doses of 500 mg/kg p.o. or 35 mg/kg i.p.

Analgesic and antiinflammatory tests

The three amides in doses up to 500 mg/kg p.o. snowed no increase in pain threshold in the Randall and Selitto test and had non significant inhibitory effects in the carrageenan induced paw oedema. Thus, the highest inhibition (13.1 ± 1.9%) of oedema with riparin II obtained was not significant ($P < 0.1$). On the other hand, aspirin at 300 mg/kg p.o., significantly ($P < 0.05$) increased the pain threshold by 80.3 ± 10.7% 2 h after administration and also caused significant inhibition (39.6 ± 5.1%) of carrageenan oedema at 3 h.

Effects on isolated smooth muscle preparations

G.-pig ileum

In the ileum riparin I, II and III produced concentration dependent inhibitions of Ach and Hist induced contractions as shown in Table 1. The amides were added 7 min before the stimulants, an interval which was found to produce maximal effects in earlier studies. Comparing the IC50 values, it could be observed that the Hist responses were more sensitive to the actions of the amides. Further, while riparin I and riparin II appeared to be equipotent, riparin III was at least twice more active against Ach and about 1.9 times more effective against the Hist response. The inhibitions produced by the amides were rapidly reversible on repeated washings.

Agent	Concentration	% Inhibition		IC50 (μg/ml)	
	(μg/ml)	Ach	Hist	Ach	Hist
Riparin I	1.5	11.8 ± 1.8	19.3 ± 3.8		
Riparin I	3.0	38.0 ± 7.0 [a]	59.3 ± 5.2 [a]	4.69	3.17
Riparin I	6.0	62.0 ± 8.4 [a]	85.1 ± 6.6 [a]		
Riparin II	1.5	17.1 ± 3.0	20.5 ± 4.0 [a]		
Riparin II	3.0	44.6 ± 6.8 [a]	60.1 ± 7.2 [a]	1.27	3.20
Riparin II	6.0	65.0 ± 7.6 [a]	80.6 ± 7.4 [a]		
Riparin III	1.0	28.8 ± 5.3 [a]	30.1 ± 5.5 [a]		
Riparin III	2.0	54.2 ± 7.0 [a]	58.7 ± 6.0 [a]	1.94	1.71
Riparin III	4.0	89.5 ± 6.1 [a]	85.2 ± 6.7 [a]		

Table 1. Inhibition of acethylcholine (Ach) and histamine (Hist) induced contraction of the ileum by riparin I, II, and III. Inhibitory values are the mean ± SEM of 5 experiments.
[a] $P < 0.05$, Student's t-test.

Rat uterus

In the uterus riparin I, II and III inhibited Oxy and Bk induced contractions in a concentration dependent manner (Table 2). It appears from the Table that while riparin I and riparin II has very similar potencies riparin III is 1.61-2 times more active, depending on the agonist. The effects of the amides were easily reversible.

G.-pig trachea

In tracheal preparations additions of riparin I or riparin II in concentrations up to 50 μg/ml did not cause direct relaxation of the tissue. However, riparin III produced a concentration dependent slowly developing relaxation which was reversible only after repeated washings. Thus, riparin III in concentrations of 1, 2 and 4

Agent	Concentration (µg/ml)	% Inhibition		IC50 (µg/ml)	
		Oxy	BK	Oxy	BK
Riparin I	1.5	9.6 ± 2.0	8.1 ± 2.0		
Riparin I	3.0	36.9 ± 6.3 [a]	43.6 ± 5.9 [a]	4.98	4.68
Riparin I	6.0	58.0 ± 6.9 [a]	60.3 ± 7.8 [a]		
Riparin II	1.5	15.1 ± 3.1	24.0 ± 3.2 [a]		
Riparin II	3.0	35.5 ± 4.4 [a]	40.2 ± 5.6 a	4.55	3.82
Riparin II	6.0	65.0 ± 11.2 [a]	75.0 ± 8.0 [a]		
Riparin III	1.0	21.9 ± 2.6 [a]	11.7 ± 1.8		
Riparin III	2.0	43.4 ± 4.0 [a]	50.5 ± 4.2 [a]	2.48	2.37
Riparin III	4.0	76.9 ± 9.0 [a]	85.0 ± 8.9 [a]		

Table 2. Inhibition of ocytocin (Oxy) and bradykinin (Bk) induced contraction of the uterus by riparin I, II, and III. Inhibitory values are the mean ± SEM of 5 experiments.
[a] $P < 0.05$, Student's test.

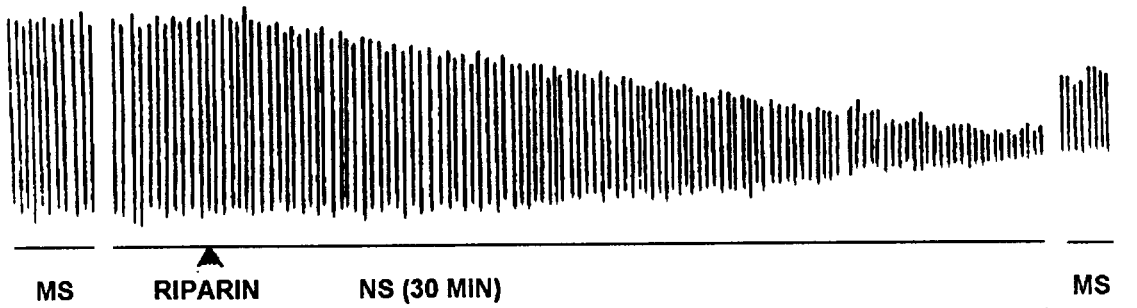


Figure 1. Inhibitory effect of riparin III (4 µg/ml) in the rat phrenic nerve-diaphragm preparation. Supramaximal twitches were induced by the electrical stimulation of the muscle, either directly (MS) or indirectly through the nerve (NS).

µg/ml relaxed the trachea significantly ($P < 0.05$) by 25.6 ± 3.0, 58.0 ± 6.1 and 92.1 ± 5.8%. The EC50 concentration calculated graphically was 1.93 µg/ml. The highest concentration of riparin III needed about an h to produce the maximum effect and the tissue required almost a similar interval to return to pre-drug level.

Effects on phrenic nerve-diaphragm preparation

A typical trace obtained with riparin III is shown in Figure 1. Riparin III at a concentration of 4 µg/ml produced a slowly developing inhibition of twitches induced by nerve stimulation. As shown, riparin also increased the tone in some preparations. When the maximum effect was obtained, the muscle was stimulated directly and these twitches were also found to be inhibited, but to a lesser extent. The effects were slowly reversible, which took almost two hours for the higher concentrations. A summary of the results obtained with riparin I, riparin II and riparin III are presented in Table 3. While an accurate relative potencies could not be determined due to solubility problems, it can be assumed that riparin III was the most active agent in suppressing both types of twitches.

DISCUSSION

The most important pharmacological actions of the three benzoyl amides were their potent relaxant effects in the isolated smooth and skeletal muscle preparations. Thus riparin I, riparin II, and riparin III inhibited in a reversible and nonspecific manner contractions induced by various agonists in the guinea-pig ileum and the rat uterus. In addition, riparin III caused a slowly developing prolonged reversible direct relaxation of the guinea-pig trachea. These effects of the compounds may be unrelated to a stimulation of the sympathetic nerves, in spite of the tyramine moiety present in the molecules. For example, none of the amides showed stimulatory activity in the rat heart or in the anaesthetized rat blood pressure [12]. Furthermore, in the present experiments only riparin III caused tracheal relaxation and this effect was not blocked by propranolol and in other experiments riparin III was inactive in stimulating cAMP levels in guinea-pig alveolar leucocytes [6].

Studies with the rat phrenic nerve-diaphragm preparation demonstrated that the three amides blocked muscle twitches induced by direct or indirect electrical stimulation, the relative poten-

Agent	Concentration (µg/ml)	% Inhibition of twitches induced by		IC50 (µg/ml)	
		MS	NS	MS	NS
Riparin I	10.0	9.0 ± 1.3	13.7 ± 1.7		
Riparin I	20.0	46.4 ± 5.5 [a]	48.9 ± 8.2 [a]		
Riparin II	5.0	0.0	14.1 ± 3.0		
Riparin II	10.0	28.5 ± 5.8 [a]	41.8 ± 9.4 [a]		11.60
Riparin II	20.0	30.4 ± 5.7 [a]	94.6 ± 3.9 a		
Riparin III	1.0	0.0	23.5 ± 9.9		
Riparin III	2.0	45.9 ± 9.2 [a]	43.5 ± 7.3 [a]	4.03	2.40
Riparin III	4.0	58.9 ± 12.4 [a]	75.3 ± 10.5 [a]		

Table 3. Effect of benzoyl amides in the rat isolated phrenic nerve diaphragm preparation. Muscle twitches were induced electrically either by direct stimulation of the muscle (MS) or indirectly through the nerve (NS). Values are mean ± SEM of 4 - 6 experiments.
[a] $P < 0.05$, Student's test.

cies being: riparin III < riparin II ≤ riparin I. The nerve mediated twitches were more sensitive to the inhibitory effects of the compounds, which indicates two sites of action for them, one at the muscle and the other on the nerve. Further studies are needed to clarify the relative importance of these two sites in the overall blockade of neuromuscular transmission by the amides.

In approximately similar concentrations, riparin III relaxed smooth muscle and skeletal muscle preparations and was atleast twice as potent as the others. However as shown earlier it was also more toxic by the i.p. route. It appears that the degree of hydroxylation of the benzoyl moiety increases both the biological activity and the toxicity. However, studies with more derivatives are needed before a clear structure-activity relationship can be established. These investigations are in progress, which will also help to determine whether the smooth and skeletal muscle effects can be separated, as well as to clarify the mechanism of action of these compounds.

It appears from their nonspecific inhibitory effects on smooth muscles, skeletal muscle and on the phrenic nerve, that these agents may act on the cell membrane, thus modifying ion channels and the metabolism of lipid derived second messengers as recently described for airway smooth muscle by Schramm and Grunstein [13].

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