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Original article

Competition between the β-hydroxylation of a primary and a tertiary carbon atom in rats

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Abstract – In order to study the effect of steric hindrance on competition between two kinds of β-hydroxylation, a compound bearing on a pyrimidinetrione nucleus both a branched side chain with a tertiary carbon atom in position β (isobutyl group) and a linear side chain (ethyl group), was selected and administered to rats. Urine and faeces were collected and extracted. Hydroxymetabolites and their derivatives were isolated and then identified. The β-hydroxylation of the linear chain was more important than the β-hydroxylation of the branched chain. Steric hindrance plays a decisive role in this regioselectivity. © 2000 Éditions scientifiques et médicales Elsevier SAS

hydroxylation / regioselectivity / metabolism / pyrimidinetriones

1. Introduction

Metabolic hydroxylation usually happens on sites able to give stabilized radicals.

It is the reason why tertiary carbon atoms are especially sensitive to this biodegradation process. However, despite its primary structure, the methyl group situated at the end of an ethyl chain is easily oxidized because of its weak steric hindrance.

In order to study the competition between these two oxidation pathways, both leading to β-hydroxymetabolites, we used a model bearing an isobutyl group and an ethyl group on a pyrimidinetrione cycle which was selected because it is well known for its in vivo stability [1] (*figure 1*).

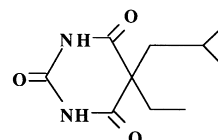

Figure 1.

2. Chemistry

2.1. Determination of potential metabolites

Before administration of $\mathbf{A_1}$ to animals, it was necessary to prepare models of potential metabolites in order to have standards to identify the metabolites. According to the literature data and to previous work [2–5], the potential metabolites could result from various oxidation processes.

If oxidation of the β tertiary carbon atom took place it would lead to an alcohol, $\mathbf{A_2}$, which could then undergo an alcoholysis leading to an allophanyl-γ-lactone, $\mathbf{A_7}$ (*figure 2*).

If oxidation of the primary β carbon atom took place, it would lead to alcohol $\mathbf{A_3}$ (*figure 3*).

Two different evolutions could then occur from this intermediate. First, the primary alcohol $\mathbf{A_3}$ could be oxidized into an unstable aldehyde $\mathbf{A_4}$, which could itself be oxidized into a carboxylic acid $\mathbf{A_5}$ (*figure 4*).

The second possible evolution consists of alcoholysis of $\mathbf{A_3}$, leading to the allophanyl-γ-lactone $\mathbf{A_6}$ (*figure 5*).

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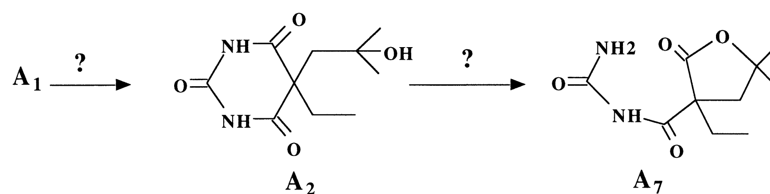
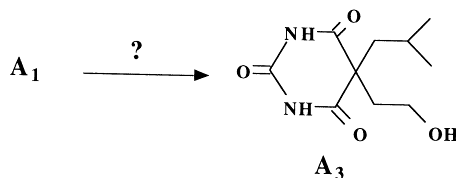


Figure 2.



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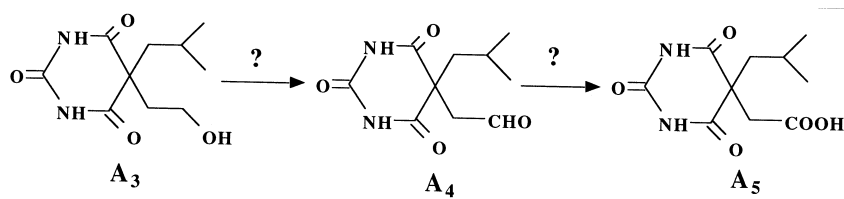

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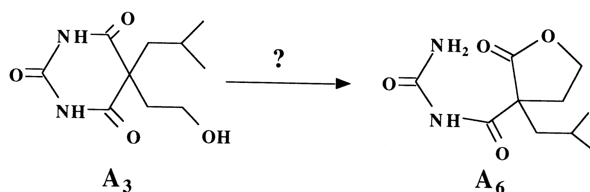


Figure 5.

2.2. Syntheses of potential metabolites

2.2.1. Synthesis of hydroxybarbiturate $\mathbf{A_2}$

Chromic oxidation of a branched side chain is usually easier than the oxidation of a linear chain and proved to give good yields in the barbiturate series [6]. But, in the case of $\mathbf{A_1}$, the hydroxybarbiturate $\mathbf{A_2}$ was only obtained with low yields using chromic acid in acetic acid medium (*figure 6*).

Another method in three steps then had to be used: synthesis of an alkenyl compound, cyclisation and then hydration of the double bond. First, the diethyl 2-ethylmalonate was alkylated in alkaline medium by 3-bromo-2-methylpropene (*figure 7*).

Then, the dialkylmalonate was condensed with urea in alkaline medium (*figure 8*).

Finally, the hydration of the double bond was obtained in acidic medium (*figure 9*).

2.2.2. Synthesis of hydroxybarbiturate $\mathbf{A_3}$

The hydroxybarbiturate $\mathbf{A_3}$ was obtained in three steps: introduction of the alcohol function protected as a tetrahydropyranylether, condensation of the malonyl ester with urea and deprotection of the alcohol function. Diethyl isobutylmalonate was alkylated in position two with 2-bromoethanol pyranylether in alkaline medium (*figure 10*).

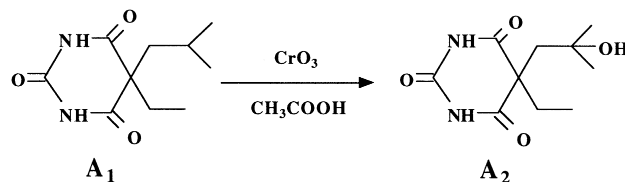
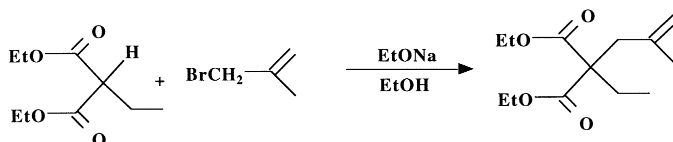


Figure 6.



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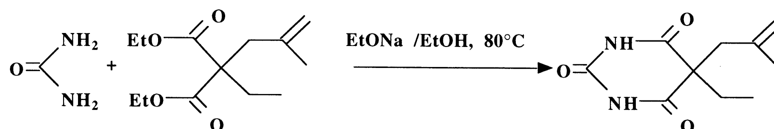

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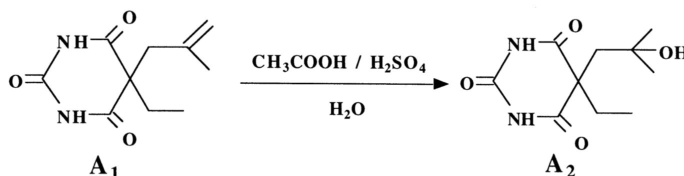


Figure 9.

The diethyl dialkylmalonate was then condensed with urea in alkaline medium (*figure 11*).

The protective group was removed in acidic medium (*figure 12*).

2.2.3. Synthesis of allophanyl-γ-lactones $\mathbf{A_6}$ *and* $\mathbf{A_7}$

β-Hydroxybarbiturates, compounds in which the alcohol function is situated in a favourable position, usually undergo an intramolecular alcoholysis of the lactame function leading to corresponding γ-lactone [7–9] (*figure 13*).

The opposite aminolysis of the lactone group leads back to the corresponding β-hydroxybarbiturates. In order to avoid the aminolysis, it is necessary to add chloroform to the medium in order to extract the γ-lactone as soon as it is formed.

Allophanyl-γ-lactones $\mathbf{A_6}$ and $\mathbf{A_7}$ were obtained from $\mathbf{A_3}$ and $\mathbf{A_2}$, respectively, at pH 10 in the presence of chloroform at 80 °C (*figure 14*).

2.2.4. Synthesis of potential metabolites at higher oxidation rates

2.2.4.1. Synthesis of aldehyde $\mathbf{A_4}$

Aldehyde $\mathbf{A_4}$ cannot be obtained via direct oxidation of the corresponding alcohol because it is too sensitive to oxidation to be isolated under the usual conditions. It was therefore necessary to introduce a protected aldehyde function on diethyl alkylmalonate and then to create the heterocycle and finally to deprotect the function. The aldehyde function was protected as a dioxolane. Diethyl 2-isobutylmalonate was alkylated in alkaline medium with 2-bromomethyl-1,3-dioxolane (*figure 15*).

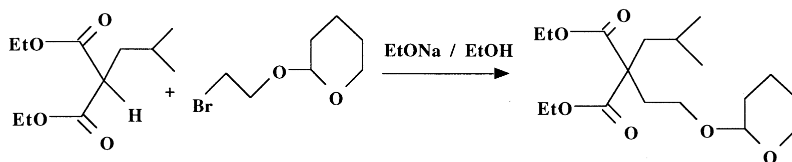
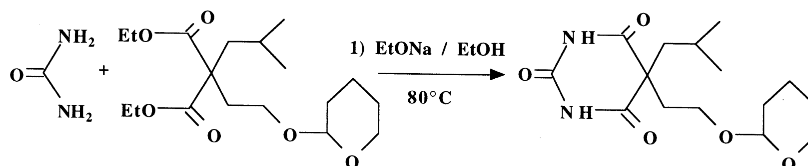


Figure 10.



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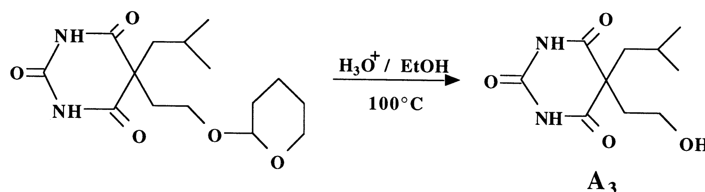


Figure 12.

The disubstituted malonate was then condensed with urea in alkaline medium (*figure 16*).

In acidic medium the dioxolane was destroyed, leading to a free aldehyde function (*figure 17*).

2.2.4.2. Synthesis of carboxylic acid $\mathbf{A_5}$

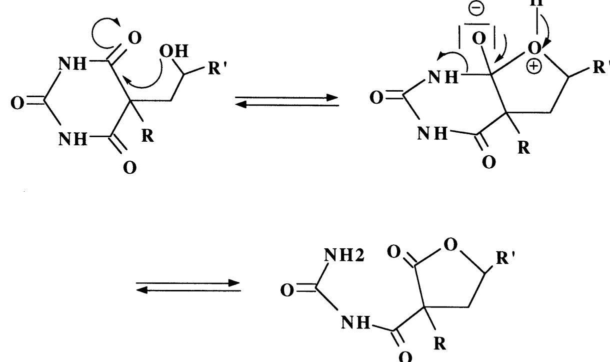
Direct chromic oxidation of alcohol $\mathbf{A_3}$ led to bad yields caused by side degradation reactions. A three step synthesis of $\mathbf{A_5}$ was chosen. First, the diethyl isobutylmalonate was alkylated by a bromoalkene: allyl bromide (*figure 18*).

Disubstituted malonate was then condensed with urea in alkaline medium (*figure 19*).

For the third step, two oxidation methods were alternatively used:

1) Chromic oxidation gave the carboxylic acid, but with low yields (*figure 20*).

2) Oxidation by potassium permanganate and magnesium dichloride gave better yields (*figure 21*).



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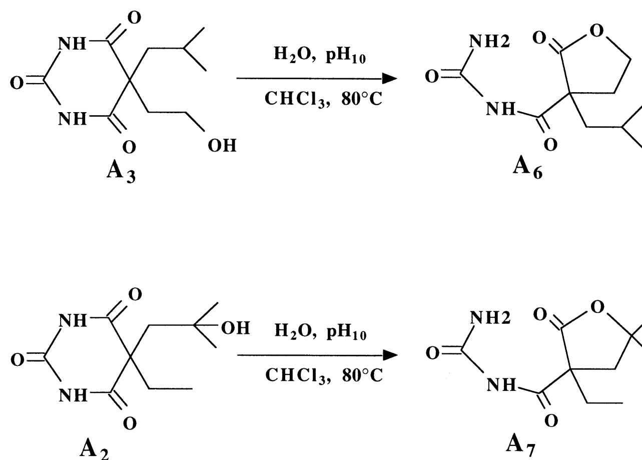

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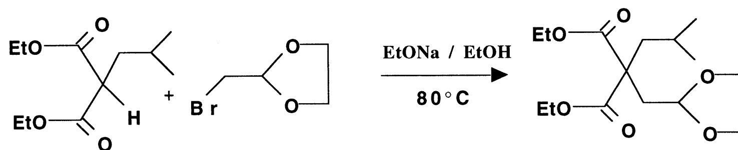

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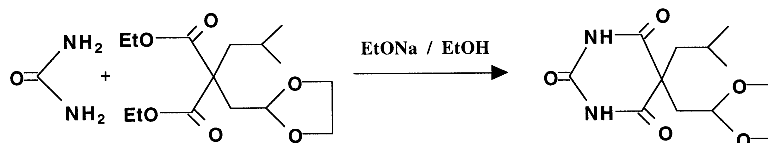


Figure 16.

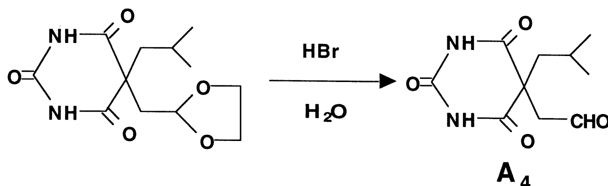


Figure 17.

3. Biology

Compound $\mathbf{A_1}$ was administered *per os* to rats at a dose of 20 mg/kg/day. Urine and faeces were extracted with ethyl acetate and the isolated compounds were identified.

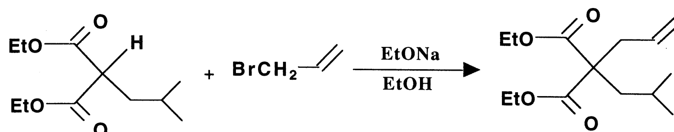
The results of these experiments are summarized in *table I*.

Carboxylic acid $\mathbf{A_5}$ and aldehyde $\mathbf{A_4}$ were not recovered in urine and faeces under the extraction conditions.

Untransformed compound $\mathbf{A_1}$ was recovered in both urine (8.2 ± 0.26% of the dose) and faeces (2.24 ± 0.25% of the dose). Tertiary alcohol $\mathbf{A_2}$ was recovered both in urine (10.29 ± 0.54% of the dose) and in faeces (3.51 ± 0.31% of the dose). The primary alcohol $\mathbf{A_3}$ was present in urine (3.5 ± 0.34% of the dose) but not in the faeces. The lactonic metabolite $\mathbf{A_7}$ resulting from the alcoholysis of $\mathbf{A_2}$ was only recovered in urine (2.34 ± 0.14% of the dose). The major metabolite was the allophanyl-γ-lactone $\mathbf{A_6}$ resulting from alcoholysis of $\mathbf{A_3}$ (30.13 ± 1.36% were recovered in urine but not any in the faeces).

Table I. Administration conditions and isolated metabolites.

Rat numbers	Total weight (kg)	Sex	Duration (days)		Administered compound (g)	Compounds isolated from faeces (mg)		Compounds isolated from urine (mg)				
			Administration	Collection		A1	A2	A1	A2	A3	A6	A7
32	14.50	F	10	12	2.901	60	100	230	300	96	904	65
30	13.81	M	11	13	3.038	65	125	250	355	122	1 045	70
30	15.81	M	10	12	3.162	80	120	270	360	130	1 090	80



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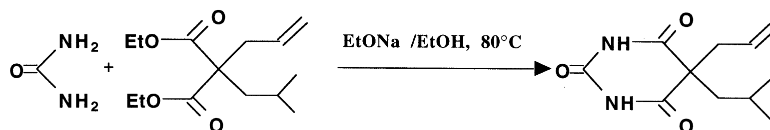


Figure 19.

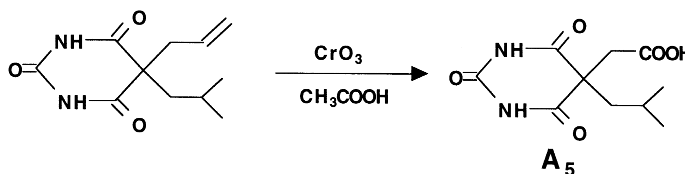


Figure 20.

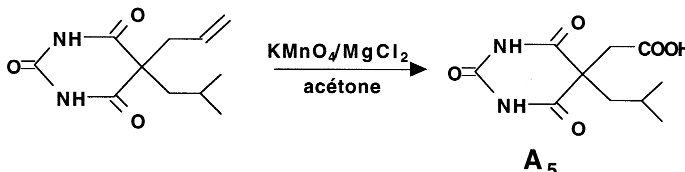


Figure 21.

4. Discussion and conclusion

Metabolites isolated from the 5-ethyl-5-isobutyl-barbituric acid correspond either to a hydroxylation of a tertiary carbon (16%) or an oxidation of a linear chain (33.5).

Competition at the hepatic level between oxidation of an isobutyl group and an ethyl group is not selective in favour of hydroxylation of the branched side chain. Although the latter is usually easier because of:

1) Stabilization of tertiary radicals.

2) The regioselectivity for metabolic hydroxylation of a carbon in position ω-1.

In fact, the results show that metabolic hydroxylation is mainly observed in the linear side chain and in position ω.

The tertiary carbon atom of the isobutyl group is sensitized to chemical oxidation, but it is also hindered, while the primary atom on the ethyl chain, which is not a priori sensitized to oxidation, is easily accessible by oxidation enzymes.

Another point in favour of β-hydroxylation of the ethyl chain is that the alcoholysis, which takes place in the second phase of the metabolism of both $\mathbf{A_2}$ and $\mathbf{A_3}$, is easier in the case of $\mathbf{A_3}$ because of less steric hindrance than in the case of $\mathbf{A_2}$. Steric hindrance appears to have a more efficient influence on regioselectivity of oxidation than electronic effects have.

5. Experimental protocols

^{1}H-NMR spectra were recorded on a Varian T60 spectrometer using $(CH_3)_4Si$ as a reference. Melting points were recorded on a Kofler apparatus and are

uncorrected. Elemental analysis indicated by the symbols of the elements were within ± 0.4% of the theoretical values.

5.1. Chemistry

Diethyl 2-ethylmalonate, urea, transcutol (diethyleneglycol monoethylether), ethyl acetate, petroleum ether, diethylether and chloroform were commercial products.

5.1.1. 5-Ethyl-5-isobutylbarbituric acid $\mathbf{A_1}$

5-Ethyl-5-isobutylbarbituric acid $\mathbf{A_1}$ was prepared according to the standard method [10]: condensation of urea with diethyl 2-ethyl-2-isobutylmalonate in alkaline medium. Yield: 64%. Anal. (C, H, N) $C_{10}O_3H_{16}N_2$. ^{1}H-NMR (DMSO-d_6) δ ppm: 0.75 (m, 9H, $2CH_3$ isobutyl and CH_3 ethyl); 1.5 (m, 1H, CH isobutyl); 1.75 (q, 4H, $2CH_2$); 11.5 (s, 2H exch. D_2O, 2NH).

5.1.2. 5-Ethyl-5-(2-hydroxy-2-methylpropyl)-barbituric acid $\mathbf{A_2}$

Method 1: chromic oxidation of $\mathbf{A_1}$:

5-Ethyl-5-(2-hydroxy-2-methylpropyl)barbituric acid $\mathbf{A_2}$ was prepared from $\mathbf{A_1}$ by chromic oxidation in acetic medium according to the method described in [6]. Yield: 20%. Anal. (C, H, N) $C_{10}H_{16}O_4N_2$. ^{1}H-NMR (DMSO-d_6) δ ppm: 0.7 (t, 3H, CH_3); 1.05 (s, 6H, $2CH_3$); 1.65 (q, 2H, CH_2); 2.1 (s, 2H, CH_2); 4.5 (s, 1H, OH); 11.15 (s, 2H exch. D_2O, 2NH).

Method 2: oxidation of 5-ethyl-5-(2-methylpropenyl)-barbituric acid:

5-Ethyl-5-(2-methylpropenyl)barbituric acid was prepared according to the standard method: condensation of urea with diethyl 2-ethyl-2-methylpropenyl malonate in alkaline medium [10].

Diethyl 2-ethyl-2-methylpropenyl malonate (40 g, 0.165 mol) was added to (13.22 g, 0.220 mol) of urea in the reactant obtained by action of sodium (9.4 g, 0.408 mol) on dry ethanol (260 mL) [11]. The mixture was stirred at 80 °C for 16 h. After cooling, the solvent was evaporated and the residue was dissolved in water and hydrochloric acid was added dropwise. The precipitate was filtered and crystallized from water. Yield: 87%. Anal. (C, H, N) $C_{10}H_{14}O_3N_2$. ^{1}H-NMR (DMSO-d_6) δ ppm: 0.75 (t, 3H, CH_3 ethyl); 1.55 (s, 3H, CH_3–C=); 1.8 (q, 2H, CH_2 ethyl); 3.25 (s, 2H, –CH_2–C=); 4.6 (s, 1H, H–C=); 4.8 (s, 1H, H–C=); 11.5 (s, 2H exch. D_2O, 2NH).

5-Ethyl-5-(2-methylpropenyl)barbituric acid (8.61 g, 0.041 mol) was dissolved in 25 mL acetic acid (95%). The flask was placed in a boiling water bath. Sulfuric acid (5mL) was added, and the mixture stirred for 30 min. After cooling, the solid residue was filtered and crystallized from water/ethanol. Yield: 70%.

5.1.3. Hydroxymetabolite 5-(2-hydroxyethyl)-5-isobutylbarbituric acid $\mathbf{A_3}$

This synthesis was performed in four steps.

2-bromoethyltetrahydropyranylether:

Bromoethanol (125 g, 1 mol) was dissolved into dihydropyran (84 g, 1 mol). Two drops of hydrochloric acid were added. The mixture was stirred at 20 °C for 3 h. Sodium hydroxide (10%) 100 mL was added. The organic solution was extracted with diethylether and dried (sodium sulfate). The solvent was evaporated and the residue was purified by distillation under reduced pressure. Yield: 80%.

Alkylation of diethyl 2-isobutyl malonate:

Sodium (9.2 g, 0.4 mol) was dissolved in 300 mL of dry ethanol [11]. After cooling, diethyl 2-isobuyl malonate (54 g, 0.25 mol) was added. The mixture was stirred at 80 °C for 18 h. After cooling, the solvent was evaporated. The residue was dissolved in water. The organic phase was extracted with diethylether and dried (sodium sulfate). The solvent was evaporated and the residue was purified by column chromatography (silica: Kieselgel 60 Merck; solvent: diethylether/petroleum ether 10:100). Yield: 70%.

Condensation of alkylmalonate with urea:

The tetrahydropyranylether of 5-(2-hydroxyethyl)-5-isobutylbarbituric acid was prepared according to the standard method [10]. Yield: 52%. Anal. (C, H, N) $C_{15}H_{24}O_5N_2$. ^{1}H-NMR (DMSO-d_6) δ ppm: 0.9 (d, 6H, $2CH_3$ isobutyl); 1 (m, 1H, CH isobutyl); 1.5 (m, 6H, $3CH_2$); 1.8 (d, 2H, CH_2); 2.1 (d, 2H, CH_2); 3.3 (m, 2H, CH_2); 3.6 (t, 2H, CH_2); 4.5 (s, 1H, CH); 11.4 (s, 2H exch. D_2O, 2NH).

Hydrolysis of the tetrahydropyranylether of 5-(2-hydroxyethyl)-5-isobutylbarbituric acid:

Pyranylether (4.84 g, 0.0155 mol) was dissolved into 300 mL dry ethanol [11]. Sulfuric acid (128 mL) was then added to the mixture, which was heated (20 °C) without stirring for 24 h. Then 100 mL of water were added. The mixture was cooled at 4 °C for 48 h. The compound was filtered and then dried. Yield: 70%. Anal. (C, H, N) $C_{10}H_{16}O_4N_2$. ^{1}H-NMR (DMSO-d_6) δ ppm: 0.8 (d, 6H, $2CH_3$); 1.5 (m, 1H, CH); 1.8 (d, 2H, CH_2); 2 (t, 2H, CH_2); 3.3 (t, 2H, CH_2O); 4.5 (s, 1H exch. D_2O, OH); 11.4 (s, 2H exch. D_2O, 2NH).

5.1.4. 5-(2-Oxoethyl)-5-isobutylbarbituric acid $\mathbf{A_4}$

This synthesis was performed in three steps.

Alkylation of diethyl 2-(1,3-dioxolyl)-2-isobutylmalonate:

Sodium (18.4 g, 0.8 mol) was dissolved in 450 mL dry ethanol [11]. After cooling, diethyl 2-isobutylmalonate (108 g, 0.5 mol) was added. The mixture was stirred at

room temperature for 1 h. 2-Bromomethyl-1,3-dioxolane (83.5 g, 0.5 mol) was introduced. The mixture was stirred at 80 °C for 14 h. After cooling, the solvent was evaporated. The residue was dissolved in water. The organic phase was extracted with diethylether and dried (sodium sulfate). The solvent was evaporated and the solid residue was purified by column chromatography (silica: Kieselgel 60 Merck; solvent: diethylether/petroleum ether 10/100). Yield: 60%.

Synthesis of 5-(1,3-dioxolyl)-5-isobutylbarbituric acid:

Diethyl 2-[methyl-2-(1,3-dioxolyl)]-2-isobutylmalonate (78.64 g, 0.257 mol) was added to urea (20.6 g, 0.343 mol) in 400 mL of dry ethanol [11] containing 14.6 g, 0.634 mol of sodium. The mixture was stirred at 80 °C for 16 h. After cooling, the solvent was evaporated. The residue was dissolved in water. Hydrochloric acid was added dropwise until pH = 5. The precipitate was filtered and crystallized from water. Yield: 45%. Anal. (C, H, N) $C_{12}H_{18}O_5N_2$. ^{1}H-NMR (DMSO-d_6) δ ppm: 0.85 (d, 6H, 2CH_3 isobutyl); 1.5 (q, 1H, CH isobutyl); 1.75 (d, 2H, CH_2); 3.7 (s, 4H, 2CH_2–O); 4.75 (t, 1H, CH–O); 11.0 (s, 2H exch. D_2O, 2NH).

Hydrolysis of 5-(1,3-dioxolyl)-5-isobutylbarbituric acid:

5-(1,3-dioxolyl)-5-isobutylbarbituric acid (2.16 g, 8 mmol) was dissolved into 30 mL of hydrobromic acid (47%). The mixture was stirred at room temperature for 24 h. The solid was filtered and washed with water and dried. Yield: 47%. Anal. (C, H, N) $C_{10}H_{14}O_4N_2$. ^{1}H-NMR (DMSO-d_6) δ ppm: 0.9 (d, 6H, 2CH_3 isobutyl); 1.55 (m, 1H, CH isobutyl); 1.7 (d, 2H, CH_2); 2.4 (d, 2H, CH_2); 9.6 (s, 1H, CHO); 11.5 (s, 2H exch. D_20, 2NH).

5.1.5. 5-(2-Carboxyethyl)-5-isobutylbarbituric acid **A_5**

This synthesis was performed in two steps.

Synyhesis of 5-isobutyl-5-propenylbarbituric acid:

It was prepared according to the standard method: condensation of urea with diethyl 2-isobutyl-2-propenylmalonate in alkaline medium (sodium ethylate in ethanol) [10]. Yield: 53%. Anal. (C, H, N) $C_{11}H_{16}O_3N_2$. ^{1}H-NMR (DMSO-d_6) δ ppm: 0.8 (d, 6H, 2CH_3); 1.4 (m, 1H, CH); 1.8 (d, 2H, CH_2); 2.5 (d, 2H, CH_2); 5–5.6 (m, 3H ethylenic); 11.5 (s, 2H exch. D_2O, 2NH).

Oxidation of 5-isobutyl-5-propenylbarbituric acid. Method 1:

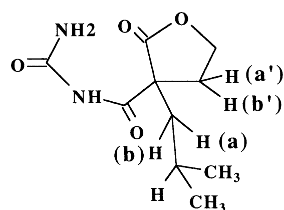
5-(2-Carboxyethyl)-5-isobutylbarbituric acid was prepared from 5-isobutyl-5-propenylbarbituric acid by chromic oxidation according to the method described in [6]. Yield: 16%.

Method 2:

5-isobutyl-5-propenylbarbituric acid was added to potassium permanganate (2.65 g, 16.8 mmol) and magnesium dichloride (2.8 g, 29.4 mmol) in 84 mL acetone (66%). The mixture was stirred at room temperature for 3 h. The solvent was filtered and evaporated, hydrochloric acid (70%) was added dropwise until pH 1. The organic phase was extracted with 50 mL of diethylether and dried (sodium sulfate). The solvent was evaporated and the residue was crystallized. Yield: 47%. Anal. (C, H, N) $C_{10}H_{14}O_5N_2$. ^{1}H-NMR (DMSO-d_6) δ ppm: 0.9 (d, 6H, 2CH_3 isobutyl); 1.5 (m, 1H, CH isobutyl); 1.7 (d, 2H, CH_2); 2.9 (s, 2H, CH_2); 11.45 (s, 2H, 2NH).

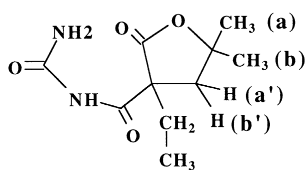
5.1.6. 2-Allophanyl-2-isobutyl-γ-butyrolactone **A_6**

Compound **A_3** (2.28 g, 0.001 mol) was dissolved in 500 mL of water, pH 7.5. Chloroform (200 mL) was added. The medium was stirred at 80 °C for 0.5 h, this operation was repeated for 5 h. After cooling, the organic phase was dried (sodium sulfate) and the solvent was evaporated. The compound was filtered and crystallized with diethylether. Yield: 11%. Anal. (C, H, N) $C_{10}H_{16}O_4N_2$. ^{1}H-NMR (DMSO-d_6) δ ppm: 0.9 (d, 6H, 2CH_3 isobutyl); 1.7 (m, 2H, CH isobutyl and H (b)–CH_2); 2.1 (m, 1H, H (a)–CH_2); 2.25 (m, 1H, H (a′)–CH_2); 3 (m, 1H, H (b′)–CH_2); 4.3 (m, 2H, CH_2–O); 5.15 (s, 1H, NH_2–CO); 7.9 (s, 1H, NH_2–CO); 8.75 (s, 1H, CO–N-H–CO).



5.1.7. 2-Allophanyl-4-4-dimethyl-2-ethyl-γ-butyrolactone **A_7**

2-Allophanyl-4-4-dimethyl-2-ethyl-γ-butyrolactone was prepared according to the method described before. Yield: 7%. Anal. (C, H, N) $C_{10}H_{16}O_4N_2$. ^{1}H-NMR (DMSO-d_6) δ ppm: 0.9 (t, 3H, CH_3 ethyl); 1.3 (s, 3H, CH_3 (a)); 1.5 (s, 3H, CH_3 (b)); 1.9–2.1 (m, 2H, CH_2 ethyl and H (a′) CH_2); 3 (d, 1H, H (b′) CH_2); 5.8 (s, 1H, NH_2–C=O); 8 (s, 1H, NH_2–C=O); 8.8 (s, 1H, C=O (NH)–C=O).



5.2. Biology

5-ethyl-5-isobutylbarbituric acid $\mathbf{A_1}$ was administered.

5.2.1. Formulation

A suspension of $\mathbf{A_1}$ was prepared according to the following procedure. In n mL of solution (30:70 transcutol/water) (n = number of rats × number of days) (*table I*) were added n′ mg of compound $\mathbf{A_1}$ (n′ = total weight of rats (kg) × daily dose (20 mg/kg) (*table I*). The medium was homogenized for 15 min using a mixer (Silverson R). A few drops of Tween 80 were then added. Homogenization was again performed for 10 min. The suspension was stored at 4 °C for the entire experiment.

5.2.2. Animals

30–32 rats (Sprague–Dawley) were placed in Pajon metabolism cages with free access to food and water.

5.2.3. Administration

A dose of 1 mL of the suspension was administered daily to each rat directly into the stomach using a curved canula 60/10 (Carrieri). Data concerning the conditions of the experiments are summarized in *table I*.

5.2.4. Collection of urine and faeces samples

Urine and faeces, for all experiments, were collected and frozen (–18 °C) during each administration period and for the following 2 days.

5.2.5. Extraction procedure

Urine samples were allowed to warm at room temperature. The pH was then adjusted to 5 with hydrochloric acid. The samples were divided into 1 L aliquots. Each aliquot was extracted 3 times with ethyl acetate (1 L). Organic solutions were then dried over anhydrous sodium sulfate and filtered. The solvent was then evaporated under reduced pressure. The pH of each urine sample was then lowered to 0.5 and the procedure repeated.

Faeces samples were allowed to warm at room temperature in 5 L ethyl acetate for 12 h. The medium was mixed and the liquid phase was separated. Hydrochloric acid was added to the residue until pH 5. The medium was then extracted with ethyl acetate (5 L). The new residue was stirred and refluxed with ethyl acetate (5 L) for 1 h and the liquid phase was separated. The three organic solutions were collected and dried over anhydrous sodium sulfate and filtered. The solvent was then evaporated under reduced pressure.

5.2.6. Isolation

The solid residues obtained after extraction were treated by column chromatography (silica: Kieselgel 60 H Merck; solvent chloroform/ethyl acetate 70:30). Collected fractions were then studied by thin-layer chromatography (silica: Kieselgel 60F 254R Merck; solvent: chloroform/ethyl acetate 80:20). All similar fractions were then assembled and the solvents were evaporated under reduced pressure.

5.2.7. Identification and quantification

Compounds isolated from urine or feaces were identified by comparaison of their ^{1}H-NMR spectra to those of synthesized models. Quantification was performed by gravimetry.

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