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Mechanism of the formation *in vivo* of α-phenyl-γ-lactones in the glutethimide series

Y Al-Tarakji-Khalfh[1], C Cavé[1], S Ménager[2], Y Legras[1], C Combet Farnoux[1], O Lafont[2*]

[1]*BIOCIS ERS OO45 and Laboratoire de Chimie Organique, Faculté de Pharmacie, rue JB Clément, 92296 Châtenay Malabry Cedex;*
[2]*Laboratoire de Pharmacochimie et Biopharmacie, Faculté de Médecine et de Pharmacie de Rouen, BP 97, 76803 Saint Etienne du Rouvray Cedex, France*

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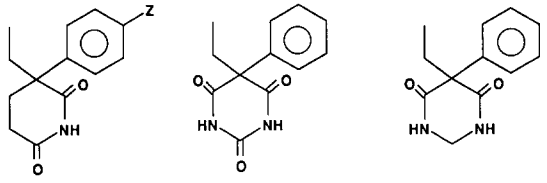
Summary — The isolation of γ-lactones from the urines of humans or animals treated with glutethimide or aminoglutethimide required an explanation. The question was studied on the glutethimide model. Potential metabolites such as β-hydroxymetabolite, γ-lactone amide, and γ-lactone acid were synthesized. Glutethimide was first administered to rats at high and normal doses. The corresponding γ-lactone amide, resulting from alcoholysis of β-hydroxyglutethimide, was also administered to rats. The amounts of the various γ-lactone derivatives isolated from the urine were discussed and it was concluded that the α-phenyl-γ-lactone was formed *in vivo via* β-hydroxylation of glutethimide followed by intramolecular alcoholysis. The lactone amide was then oxidized and dealkylated *via* an intramolecular mechanism leading to the corresponding α-phenyl-γ-lactone.

glutethimide series / metabolism / alcoholysis / γ-lactone formation

Introduction

Breast cancer is one of the medicinal chemists' major concerns, and one of the research directions consists of the design of new aromatase inhibitors [1–4]. However, aminoglutethimide, **1** (scheme 1) is still the only antiaromatase drug on the market. This compound is a piperidine-2,6-dione substituted in position 3 by an ethyl rest and an aromatic nucleus bearing an amino group.

This structure is close to that of antiepileptic drugs such as phenobarbital **2**, and primidone **3** (scheme 1), which contain a more complex heterocycle, respectively pyrimidine-2,4,6-trione or pyrimidine-4,6-dione, but both of these bear a phenyl and an ethyl group in the same position as aminoglutethimide.



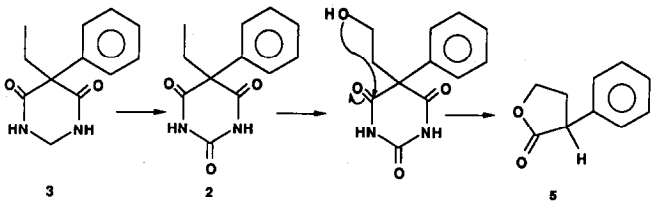
4 : Z=H, Glutethimide **2** : Phenobarbital **3** : Primidone

1 : Z=NH_2, Aminoglutethimide

Scheme 1.

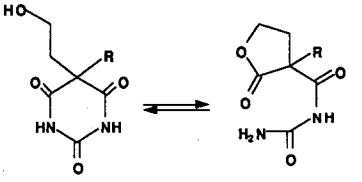
The 2 pyrimidine derivatives **2** and **3** undergo *in vivo* biodegradation, leading to α-phenyl-γ-butyrolactone [5]. In the case of phenobarbital, the ethyl group is β-hydroxylated, then alcoholysis opens the heterocycle and forms a γ-lactone [6]. In the case of primidone, the compound has first to be converted into phenobarbital before undergoing the same transformation [7] (scheme 2).

* Correspondence and reprints



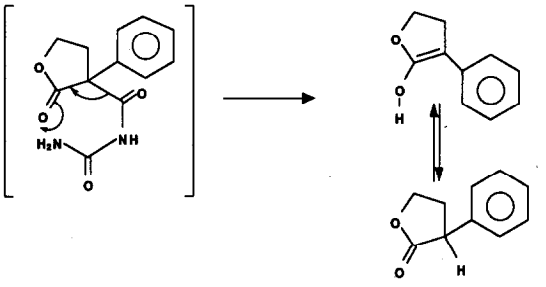
Scheme 2.

Usually β-hydroxybarbiturates formed during the metabolism of barbiturates undergo an alcoholysis–aminolysis equilibrium, which allows the isolation of an allophanyl-γ-lactone [8–11] (scheme 3).



Scheme 3.

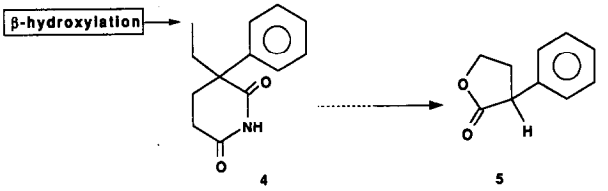
In the case of phenyl substituted compounds such as **2** and **3**, the allophanyl-γ-lactone cannot be isolated and is spontaneously transformed into the corresponding γ-lactone **5** [6, 7] (scheme 4).



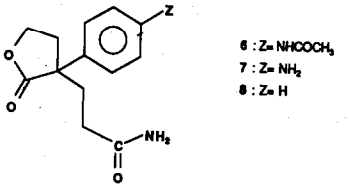
Scheme 4.

The same γ-lactone **5** was also detected in the urine of humans who had received toxic doses of glutethimide **4** [12]. This observation allowed us to make the hypothesis that this compound **5** was formed *in vivo* through β-hydroxylation of the ethyl side chain of glutethimide, followed by alcoholysis of the heterocycle, similar to that which occurs for phenobarbital [6] (scheme 5).

On the other hand, in the case of aminoglutethimide a γ-lactonic compound **6** was also detected in the urine of rats [13] (scheme 6).



Scheme 5.



Scheme 6.

The authors of this observation did not give any interpretation regarding formation of this compound but, later on, in a book, it was stated that "this metabolite might be formed *via* the hydroxylated compound by an intramolecular transesterification reaction", without any experimental support being provided [14].

The above observations needed to be interpreted because the mechanism of the bioformation of α-phenyl-γ-butyrolactones in the glutethimide series still remained unknown.

Our purpose was to experimentally establish that β-hydroxy metabolites could be the precursors of these lactones, and to confirm the role of intermediates *in vivo* of polysubstituted-γ-lactones like **7** or **8** (scheme 6) in the formation of α-phenyl-γ-butyrolactones (type **5**).

In order to avoid the difficulties caused by the *N*-acylation of the aminoglutethimide amino group, it was decided to establish this mechanism on models of glutethimide metabolites, which is the best simplified structure corresponding to aminoglutethimide.

Chemistry

First of all, it was necessary to synthesize the models of glutethimide metabolites in order to study their stability and to show that a complex γ-lactone, **8** (devoid of a carbonyl group in the α position), could be an intermediate for the chemical formation of α-phenyl-γ-butyrolactone **5**.

Synthesis of models of metabolites

4-(2-Tetrahydropyranyloxy)-2-phenylbutanenitrile **9** was used as a turntable for the synthesis of the various models of glutethimide metabolites.

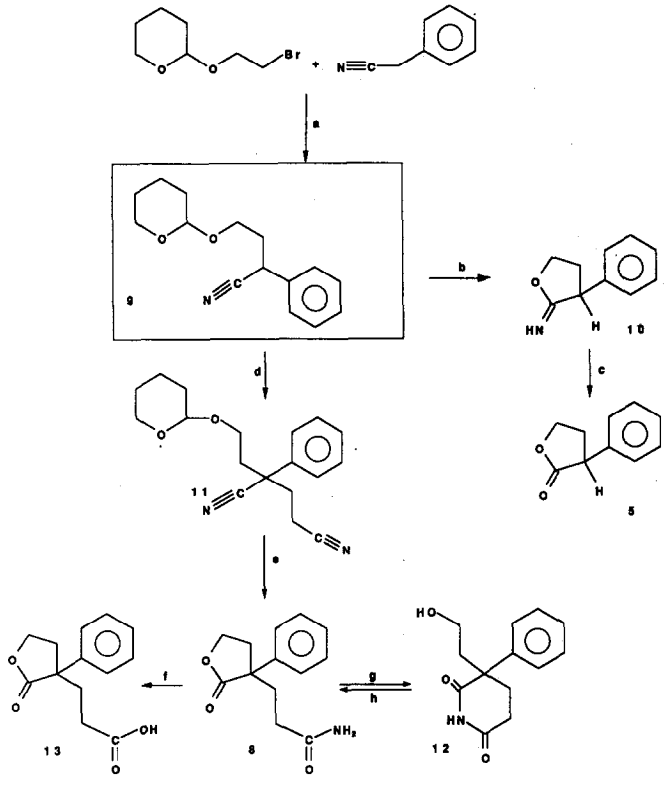
Heating the nitrile **9** gave a cyclic compound **10**. The iminogroup of **10** was then hydrolyzed in acidic medium into α-phenyl-γ-butyrolactone **5**.

The same compound **9** treated by acrylonitrile and triton B led to a dinitrile **11**. Pentane-1,5-dinitriles treated, first by acetic acid and sulfuric acid at a temperature of 80°C for 4 h and then by disodium carbonate usually cyclize in dihydropyrimidine-2,6-diones [1]. But under the same conditions, the dinitrile **11** underwent lactonization leading to the lactone amide **8**. The reverse transformation of **8** into the β-hydroxy derivative of glutethimide **12** was possible at pH 3.5. The lactone carboxylic acid **13** was synthesized by alkaline hydrolysis of the lactone amide **8**.

All these syntheses are summarized in scheme 7.

Stability of 8 and 12

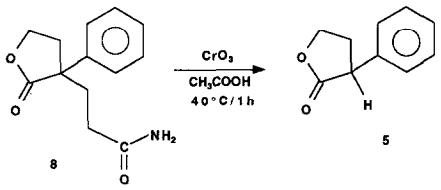
The transformation of one form into the other proved to be pH-dependent. In strongly acidic medium, pH < 3, the β-hydroxyglutethimide **12** was converted into the lactone amide **8**. When pH was > 4, the hydroxy-derivative **12** was also transformed into the lactone **8**, (scheme 6). **12** was then only stable in a narrow pH range (3 < pH < 4). Anyway, the stable form in aqueous medium at pH 7.4 was **8**. On the other hand, the lactone amide **8** was converted into β-hydroxy-glutethimide **12** at a pH between 3 and 4.



Scheme 7. a) $NaNH_2$, toluene; b) 160°C 0.017b; c) H_2O, H^+; d) triton B, acrylonitrile; e) 1) CH_3COOH/H_2SO_4, 80°C, 4h, 2) Na_2CO_3; f) 1)NaOH, H_2O, 2)H^+; g) 3<pH<4; h) pH>4 or pH<3.

Chemical transformation of 8 into 5

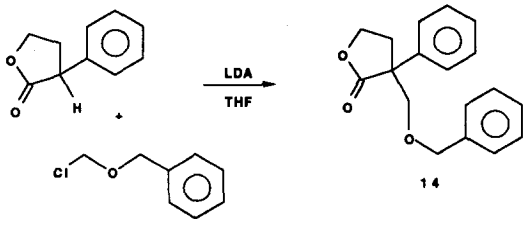
The stability of the lactone amide **8** contrasts with the spontaneous degradation of allophanyl-γ-lactones which appeared during the course of the metabolism of β-hydroxybarbiturates bearing a phenyl group in position 5 (scheme 4). It was then necessary to demonstrate that this plurisubstituted γ-lactone **8** devoid of a carbonyl group in the α-position, could be a chemical precursor of α-phenyl-γ-butyrolactone **5**. This synthesis was performed using CrO_3 in acetic acid as oxidation reagent (scheme 8).



Scheme 8.

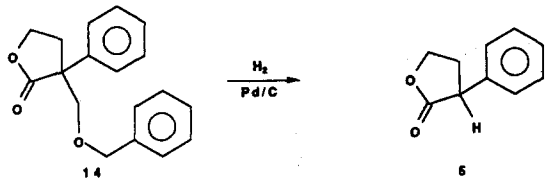
The direct transformation of the lactone amide **8** into the dealkylated lactone **5** suggested the synthesis of an intermediate, hydroxylated in the α-position, and undergoing an intramolecular dealkylation during chemical oxidation. This hypothesis was confirmed by the following experiments.

α-Phenyl-γ-butyrolactone **5** was first substituted using chloromethylbenzylether as alkylating agent, LDA (lithium diisopropylamide) as basic agent in THF which yielded the benzylether **14** (scheme 9).



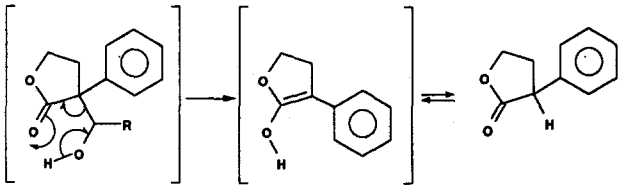
Scheme 9.

Usually the benzylethers can be cleaved by catalytic hydrogenation into the corresponding primary alcohols. In the case of **14**, the primary alcohol could not be isolated and the dealkylated lactone **5** was obtained (scheme 10).



Scheme 10.

The lack of stability of the potential α-hydroxy intermediates can be interpreted as an intramolecular process leading to the corresponding conjugated enol form of **5** (scheme 11).

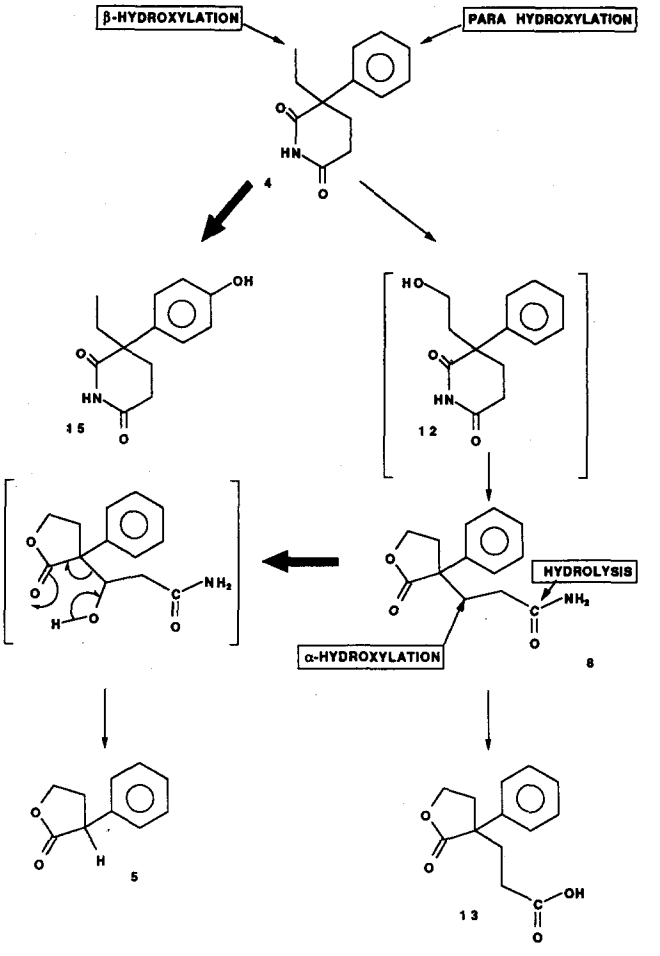


Scheme 11.

Biology

First of all, in order to establish the presence of the lactone amide **8** in the urine of animals treated with glutethimide **4**, an observation which had never been made, the latter compound was administered to rats at a dose of 20 mg/kg/d (9.2 10^{-5} mol/kg/d). The results of these experiments are summarized in table I.

10.94 ± 1.49 % of the administered glutethimide **4** were recovered unchanged in the urine. The main metabolite (scheme 12) was, as usual [15], parahydroxygluthethimide **15** (59.20 ± 6.29% of the dose) (these values were adjusted according to the molecular weight of the compounds). A small quantity of the lactone amide **8** corresponding to 0.71 ± 0.02% of administered drug, was also isolated and characterized. No lactone **5** was found in the urine of rats.



Scheme 12.

In order to confirm the formation of the lactone amide **8** at high doses, conditions under which the γ-lactone **5** was habitually present in urine, glutethimide **4** was administered to rats at a single dose of 200 mg/kg (9.2 10^{-4} mol/kg). The results of these experiments are presented in table II.

Table I. Compounds isolated from urine of rats treated with glutethimide at normal doses.

Experiment	*Total administered glutethimide* ***4*** *(mg)*	*Compounds isolated from urine*		
		Glutethimide ***4*** *(mg)*	*p-Hydroxyglutethimide* ***15*** *(mg)*	*Lactone amide* ***8*** *(mg)*
1	3580	412	2480	28
2	2700	250	1515	20
3	3030	366	1980	23

Table II. Compounds isolated from urine of rats treated with glutethimide at high doses.

Experiment	*Total administered glutethimide* ***4*** *(mg)*	*Compounds isolated from urine*			
		Glutethimide ***4*** *(mg)*	*p-Hydroxyglutethimide* ***15*** *(mg)*	*Lactone amide* ***8*** *(mg)*	*γ-Lactone* ***5*** *(mg)*
4	3000	712	1350	25	177
5	2380	619	976	24	151
6	2910	733	1350	28	210

With high doses, the amount of glutethimide recovered unchanged increased up to 24.97 ± 1.15 % of the administered drug. Parahydroxyglutethimide **15** was still the main metabolite, with 41.10 ± 2.60% of the dose (values were adjusted according to the molecular weight of the compounds). The lactone amide **8** corresponded to 0.88 ± 0.05% of the dose. The lactone **5** was present in relatively large amounts (8.69 ± 0.89%).

In order to establish the role of 2-phenyl-2(3-propanamidyl)-γ-butyrolactone **8** in the formation of the γ-lactone **5** *in vivo*, compound **8** was administered to rats at a dose of 20 mg/kg/d (8.6 10^{-5} mol/kg/d). The results of these experiments are summarized in table III.

The amount of lactone amide **8** recovered untransformed in the urine was 22.71 ± 1.57 % of the administered dose. The γ-lactone **5** was the main biodegradation product, at 43.79 ± 1.30% of the dose (values were adjusted according to the molecular weight of the compounds). A new metabolite appeared, the lactone acid **13**, at 14.31 ± 0.66% of the dose.

Discussion and conclusion

The chemical data show that the stable form for the potential metabolite formed *via* β-hydroxylation of the ethyl side chain of glutethimide is not the β-hydroxy form **12**, but the lactone amide **8**. This compound proved to be a chemical precursor of the γ-lactone **5**, because the chemical oxidation of the γ-lactone amide **8**, leads to the γ-lactone **5**.

The biological experiments established that the lactone amide **8** was present in the urine of rats administered glutethimide **4**, both at normal and high doses (respectively 20 mg/kg/d and 200 mg/kg). The γ-lactone **5** was only isolated from the urine of animals treated with a single high dose of glutethimide (200 mg/kg).

All these data suggested that **8** was also the precursor of **5** *in vivo*. This hypothesis was confirmed by the administration of the lactone amide **8** to rats. A new metabolite appeared, the lactone acid **13**, formed by hydrolysis of the amide function of **8**. The main biodegradation product was then the lactone **5**. The mechanism of the transformation of **8** into **5** can be interpreted as an intramolecular dealkylation of the α-hydroxyderivative of **8**, which can be generated both during the course of chemical oxidation by CrO_3 and *via* biological oxidation in the rats.

The various biodegradation pathways of glutethimide have been summarized in scheme 12. The main route always consists of parahydroxylation. β-Hydroxylation becomes important when the doses are high. The lactone amide which is then the intermediate is mainly transformed into α-phenyl-γ-butyrolactone **5**, of which the carboxy derivative **13** is only a minor metabolite of **8**.

These observations made on a simplified glutethimide model confirm the hypothesis that in the glutethimide series the ring opening is caused by

Table III. Compounds isolated from urine of rats treated with compound **8**.

Experiment	*Drug (mg)*	*Lactone amide* ***8*** *(mg)*	*Lactone acid* ***13*** *(mg)*	*Lactone* ***5*** *(mg)*
7	2763	615	385	868
8	2664	652	375	789
9	2656	569	402	805

β-hydroxylation followed by an intramolecular alcoholysis, and that the corresponding lactone amides are the precursors of α-phenyl-γ-butyrolactones.

Experimental protocols

^{1}H-NMR spectra were recorded on a Varian T60 Spectrometer using $(CH_3)_4$ Si as internal standard. Melting points were recordered on a Kofler apparatus and are uncorrected. Analyses indicated by the symbols of the elements were within ± 0.4% of theoretical values. Thin-layer chromatograms (TLC) were run on pre-coated silicagel 60F 254 plates using various mobile phases.

Chemicals

Glutethimide **4** was prepared according to the synthesis previously described [16].

Phenylacetonitrile, bromoethanol and tetrahydropyran were obtained commercially.

Parahydroxyglutethimide **15** was prepared according to the method described in [17].

All these compounds were fully characterized spectroscopically and controlled by microanalysis; C, H, N.

Chemistry

Synthesis of models of metabolites

*Synthesis of 4-(2-tetrahydropyranyloxyethyl)-2-phenylbutanenitrile **9***

Phenylacetonitrile (0.1 mol) dissolved in dry toluene (30 ml) was slowly added to a suspension of sodium amide (0.1 mol) in dry toluene (100 ml). The medium was heated (80°C) for 1 h, 2-(2-bromoethoxy)tetrahydropyran (0.1 mol) was then introduced dropwise and the medium refluxed for 3 h. After cooling, water was added and the organic phase was isolated and extracted with water. The organic phase was then dried over sodium sulfate. The solvent was evaporated under reduced pressure and the residue purified by column chromatography (silica: Kieselgel 60 Merck; solvent: diethylether/cyclohexane 50:50).

Bp: 150–160°C; yield: 40%. ^{1}H-NMR, DMSO-d_6 δ ppm = 1.6 (m, 6H, $3CH_2$ cycle); 2.1 (q, 2H, CH_2CH-CN); 3.6 (m, 4H, $2CH_2O$); 4.0 (m, 1H, CH-CN); 4.6 (m, 1H, OCHO); 7.3 (m, 5H, C_6H_5). Microanalysis; CHN: $C_{15}H_{19}NO_2$.

*Synthesis of 2-imino-3-phenyltetrahydrofuran **10***

Phenylacetonitrile (0.1 mol) dissolved in dry toluene (30 ml) was slowly added to a suspension of sodium amide (0.1 mol) in dry toluene (100 ml). The medium was heated (80°C) for 1 h, 2-(2-bromoethoxy)tetrahydropyran (0.1mol) was then introduced dropwise and the medium refluxed for 3 h. After cooling, toluene was evaporated under reduced pressure. The oily residue was distilled at 160°C under a pressure of 0.017 bar, yielding **10** (30%). ^{1}H-NMR $CDCl_3$, δ ppm = 2.1–3.0 (m, 2H, CH_2-CH_2O); 2.4 (ex D_2O, 1H, NH); 3.2–3.6 (m, 1H, CH); 3.9–4.3 (m, 2H, CH_2-O); 7.3–7.9 (m, 5H, C_6H_5). Microanalysis; C, H, N: $C_{10}H_{11}NO$.

*Synthesis of α-phenyl-γ-butyrolactone **5** from **10***

Compound **10** (0.0125 mol) was introduced into water (100 ml) at pH 1 (HCl). The medium was heated for 2 h (80°C). After cooling, dichloromethane (50 ml) was added and the organic phase was isolated and treated with a saturated solution of sodium hydrogenocarbonate (10 ml) and then with water. The organic phase was then dried over sodium sulfate, and the solvent was evaporated under reduced pressure yielding **5** (88%).

*Synthesis of 2-(2-tetrahydropyranyloxyethyl)-2-phenylpentane-1,5-dinitrile **11***

Acrylonitrile (0.1 mol) and compound **9** (0.1 mol) were dissolved in anhydrous tetrahydrofuran (100 ml). The medium was heated (100°C) and benzyltrimethylammonium hydroxide (Triton B) (0.1 mol) in solution in methanol was added dropwise. It was then stirred and refluxed for 15 h. The solvent was then evaporated under reduced pressure and the residue dissolved in a mixture of diethylether and water. The organic solution was then separated and treated with water. The organic phase was then dried over sodium sulfate, the solvent evaporated under reduced pressure and the residue purified by column chromatography (silica: Kieselgel 60 Merck; solvent: dichloromethane/ethylacetate 95:5). Yield: 24%; ^{1}H-NMR $CDCl_3$, δ ppm = 1.6 (m, 6H, $3CH_2$ cycle); 2.4 (m, 6H, C-CH_2-CH_2-CN and CH_2-CH_2-O); 3.8 (m, 4H, $2CH_2O$); 4.3 (m, 1H, CH-O); 7.3 (m, 5H, C_6H_5). Microanalysis; C,H,N: $C_{18}H_{22}N_2O_2$.

*Synthesis of 3-phenyl-3(3-propanamidyl)-2-oxotetrahydrofuran **8***

Compound **11** (0.1 mol) was introduced into a mixture of sulfuric acid (150 ml) and acetic acid (500 ml). The medium was heated (80°C) for 6 h, poured on ice and extracted with dichloromethane. The organic solution was treated with sodium carbonate (saturated aqueous solution) and dried over sodium sulfate. The solvent was evaporated under reduced pressure and the residue purified by column chromatography (silica: Kieselgel 60 Merck; solvent: dichloromethane/ethylacetate 80:20). Mp: 186°C; yield: 22%; ^{1}H-NMR $CDCl_3$, δ ppm = 2.2–2.7 (m, 4H, CH_2-CH_2O and CH_2-C=O); 4.0–4.6 (m, 4H, CH_2O and CH_2 α cycle); 6.0 (m, 2H, ex D_2O, NH_2); 7.3–7.7 (m, 5H, C_6H_5). Microanalysis; C,H,N: $C_{13}H_{15}NO_3$.

*Synthesis of 3-(2-hydroxyethyl)-3-phenylpiperidine-2,6-dione **12***

Compound **8** (0.1 mol) was introduced into 200 ml water (3 < pH < 4, HCl). The medium was stirred for 24 h at 20°C, and extracted with ethylacetate (3 x 50 ml). The organic phase was separated and dried over sodium sulfate. The solvent was evaporated under reduced pressure. The residue was purified by column chromatography (silica: Kieselgel 60 Merck; solvent: ethylacetate/cyclohexane 80:20). Mp: 212°C; yield: 74%; ^{1}H-NMR $CDCl_3$, δ ppm = 2.3–2.8 (m, 6H, CH_2-CH_2O and 2 CH_2 cycle); 3.6 (t, 2H, CH_2O); 4.8 (1H, ex D_2O, OH); 7.3–7.7 (m, 5H, C_6H_5); 8.5 (1H, ex D_2O, NH). Microanalysis; CHN: $C_{13}H_{15}NO_3$.

*Synthesis of 3-phenyl-3(3-ethanecarboxylic)-2-oxotetrahydrofuran **13***

Compound **8** (0.0043 mol) was introduced into an aqueous solution of sodium hydroxide (10%). The medium was stirred for 24 h at 20°C, and then filtered. Then the medium was acidified (pH 2, HCl). The carboxylic acid **13** was then filtered and treated with 50 ml water (5°C). The crystals were then dried. Mp: 135°C; yield: 81%; ^{1}H-NMR $CDCl_3$, δ ppm = 1.9–2.7 (m, 6H, 3 CH_2); 3.9–4.3 (m, 2H, CH_2O); 7.1–7.4 (m, 5H, C_6H_5); 10.4 (1H, ex D_2O, COOH).Microanalysis; C,H,N: $C_{13}H_{14}O_4$.

Table IV. Experimental conditions for gluthethimide administration and collection of urines.

Experiment	*Rats*			*Administration*		*Collection*
	Number	*Sex*	*Total weight (kg)*	*Dose (mg/kg/d)*	*Duration (d)*	*Duration (d)*
1	37	Female	17.91	20	10	12
2	32	Male	13.50	20	10	12
3	56	Female	15.15	20	10	12
4	33	Male	15.00	200	1	4
5	44	Female	11.92	200	1	4
6	52	Female	14.55	200	1	4

Table V. Experimental conditions for administration of the lactone amide and collection of urines.

Experiment	*Rats*			*Administration*		*Collection*
	Number	*Sex*	*Total weight (kg)*	*Dose (mg/kg/d)*	*Duration (d)*	*Duration (d)*
7	56	Female	17.27	20	8	10
8	33	Male	14.80	20	9	11
9	36	Male	16.60	20	8	10

*Stability of **8** and **12***

β-Hydroxyglutethimide **12** (500 mg) was dissolved in various buffers ($1 \leq pH \leq 6$). The medium was stirred for 24 h at 37°C, and extracted with ethyl acetate. The organic phase was separated and dried over sodium sulfate. The solvent was evaporated under reduced pressure. The residue was weighed and identified by ^{1}H-NMR.

At most of the pH values (*ie* 1,2,3,4,5 and 6) the lactone amide **8** was obtained in 100% yield. All the β-hydroxycompound **12** was transformed into **8**.

At pH 3.5 only, a different result was obtained: compound **12** was not transformed into **8** at all and 100% of **12** was then isolated.

In the same manner lactone amide **8** (500 mg) was dissolved in buffer (pH 3.5). The medium was stirred for 24 h at 37°C and extracted with ethyl acetate. The organic phase was separated and dried over sodium sulfate. The solvent was evaporated under reduced pressure. The residue was weighed and identified by ^{1}H-NMR. Compound **12** was obtained in 100% yield.

The same experiment performed at pH 2 or 5 gave different results because **8** was not transformed into **12**.

*Chemical transformation of **8** into **5***

Direct oxidation of **8** with CrO_3 in acidic medium was performed according to the method described in [7] and yielded **5** (35%).

*Synthesis of 3-benzyloxymethyl-3-phenyl-2-oxotetrahydrofuran **14***

Under a nitrogen flow diisopropylamine (0.0334 mol) was added to THF (70 ml). The medium was cooled at 0°C and a suspension of butyllithium (0.0313 mol) in hexane was added. The medium was stirred for 15 min, cooled at –78°C in a dry-ice/acetone bath and α-phenyl-γ-butyrolactone **5** (0.024 mol) was then added dropwise. The medium was stirred for 2 h and chloromethylbenzylether (0.036 mol) was added. Then the medium was stirred for 12 h and the temperature slowly increased to 20°C. A saturated solution of NaCl (7 ml) was then added. The solvent was evaporated under reduced pressure. The residue was dissolved in diethylether (50 ml). The organic phase was treated with water (10 ml) and dried over sodium sulfate. The solvent was then evaporated under reduced pressure. The compound **14** was purified by column chromatography (silica: Kieselgel 60 Merck; solvent: heptane/ethyl acetate 80:20). Yield: 46%; ^{1}H-NMR $CDCl_3$, δ ppm = 2.4–3.4 (m, 2H, CH_2-CH_2O); 4.1–4.5 (m, 2H, CH_2-O-C=O); 4.8 (s, 2H, C-CH_2O); 5.1 (s, 2H, -O-CH_2-C_6H_5); 7.4–7.9 (m, 10H, 2 C_6H_5). Microanalysis; C,H,N: $C_{18}H_{18}O_3$.

*Catalytic hydrogenation of **14** leading to **5***

In a Parr bomb, compound **14** (0.0035 mol) was dissolved in methanol (20 ml). Pd/C (5% Pd) (50 mg) were then added. Hydrogen was introduced under a pressure of 3 bars. The medium was stirred for 2 h at room temperature. The medium was then filtered and the solvent evaporated under reduced pressure without any heating. The lactone **5** was isolated. Yield: 80%.

Biology

Administered compounds

Glutethimide **4** was administered in experiments 1–6 and the lactone amide **8** in experiments 7–9.

Formulation
A suspension of glutethimide **4** or lactone amide **8** was prepared according to the following procedure.

In 500 ml water, 1 g carboxymethylcellulose 12 H (Hercules, France) was introduced part after part. The medium was homogenized for 10 min using a mixer (Silverson R). In *n* ml of this pseudosolution (*n* = number of rats x number of days), were added *n'* g of compound **4** or **8** (*n'* = total weight of rats (kg) x daily dose (g/kg). In the case of high doses of **4** (200 mg/kg) the total amount of glutethimide (*n'*g) was dissolved in 5 ml ethanol before addition. The medium was homogenized for 15 min using the same apparatus as previously. A few drops of Tween 80 were then added. Homogenization was again performed for 10 min. Then the suspension was treated in an ultrasonic vat for a further 10 min in order to release the dissolved gas. The suspension was then stored at 4°C for the entire experiment.

Animals
n Rats were placed in Pajon metabolism cages, 3 rats per cage, and with free access to food and water.

Administration
Every day 1 ml of the suspension was administered to each rat directly in the stomach, using a curved cannula 60/10 (Carrieri).

Collection of urine samples
Urine was collected every day and allowed to freeze (–18°C), during the administration period and for the following 2 or 3 days.

Extraction procedure
Urine samples from one experiment were allowed to warm to room temperature without any heating, and collected. The pH was then adjusted to 1. The samples were divided into 1.5-l aliquots. Each aliquot was extracted 3 times with ethyl acetate (500 ml). Organic solutions were then dried over anhydrous sodium sulfate (300 g) and filtered. The solvent was then evaporated *in vacuo* at room temperature.

Isolation

All the residues corresponding to the same experiment were collected and treated by column chromatography (silica: Kieselgel 60 H ; pressure: 4 bars; pump: Duramat; solvent: ethyl acetate/cyclohexane 20:80). Collected fractions were then studied by thin-layer chromatography (silica: Kieselgel 60 F 254; solvent: ethyl acetate) in order to identify those fractions which contained the same compounds. All similar fractions were then assembled, solvents were evaporated under reduced pressure and residues were dried.

Identification and quantification
A sample of each isolated product was dissolved in the appropriate deuterated solvent $CDCl_3$ or DMSO–d_6 and studied by ^{1}H–NMR. Spectra of isolated compounds were identical to those of synthesized models. Identified compounds were then weighed.

Experimental conditions are summarized in tables IV and V.

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