

Biosynthesis of Two Dihydropyrrole-Polyketides from a Marine-Derived *Penicillium citrinum*

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Experimentos de incorporação com precursores marcados com ¹³C foram realizados objetivando-se estabelecer a biossíntese de dois dihidropirróis *N*-acilados, (8*E*)-1-(2,3-dihidro-1*H*-pirrol-1-il)-2-metildec-8-eno-1,3-diona (**1**) e 1-(2,3-dihidro-1*H*-pirrol-1-il)-2-metildecano-1,3-diona (**2**), isolados de culturas de *Penicillium citrinum* de origem marinha. A biossíntese de ambos, **1** e **2**, procede através da incorporação de acetato, metionina e ornitina.

Feeding experiments with ¹³C-labeled precursors were performed in order to establish the biosynthesis of two *N*-acylated dihydropyrroles, (8*E*)-1-(2,3-dihydro-1*H*-pyrrol-1-yl)-2-methyldec-8-ene-1,3-dione (**1**) and 1-(2,3-dihydro-1*H*-pyrrol-1-yl)-2-methyldecane-1,3-dione (**2**), isolated from the cultures of a marine-derived *Penicillium citrinum*. The biosynthesis of both, **1** and **2**, involves the incorporation of acetate, methionine and ornithine.

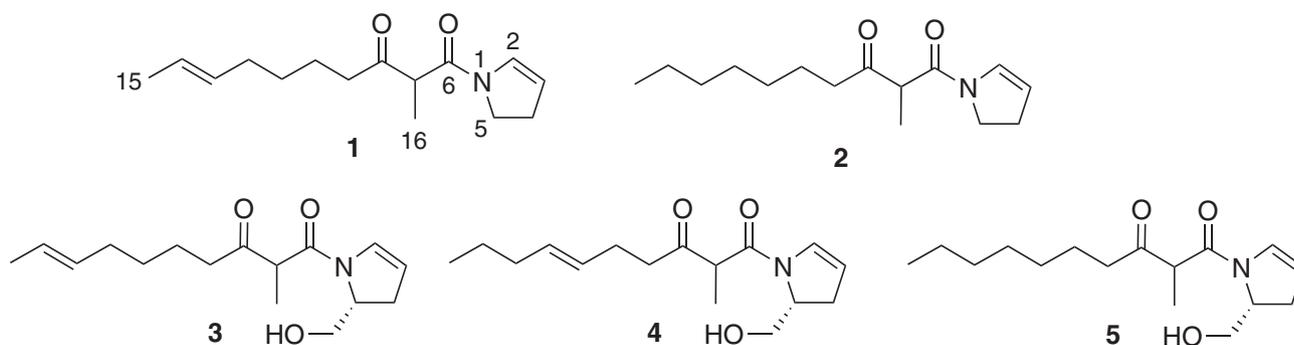
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Introduction

Pyrrole, dihydropyrrole (pyrroline) and tetrahydropyrrole (pyrrolidine) alkaloids constitute a unique class of secondary metabolites, which include several bioactive members such as the tropane alkaloids,¹ tambjamines²⁻⁴ and prodigiosin,⁵ anatoxin-a,⁶ as well as 2-alkyl or 2,5-dialkyl-branched pyrrolidine and pyrroline ant chemical defenses.⁷ However, such alkaloids are extremely rare in fungi, the only examples found in literature⁸ include the dihydropyrrole-bearing peptide brevigellin isolated from

Penicillium brevicompactum,⁹ the dihydropyrrole β-keto amides **1** and **2** also isolated from *P. brevicompactum*,¹⁰⁻¹² the mildly antibiotic scalusamides A–C (**3-5**), which have been isolated from a marine-derived *P. citrinum*,¹³ as well as the tetrahydropyrrole-citrinin conjugate perinadine A, isolated from *P. citrinum* of marine origin.¹⁴

Compounds **1** and **2** display insecticidal, fungicidal activities and induce precocious metamorphosis in the milkweed bug *Oncopeltus fasciatus* (Insecta).¹² We have recently isolated the same compounds **1** and **2** from a marine-derived *P. citrinum*,¹⁵ and became interested to



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establish the actual biosynthetic pathway leading to **1** and **2**. This is because while pyrrole, dihydropyrrole or pyrrolidine alkaloids isolated from non-fungal sources are typically derived from proline,^{16,17} ornithine,¹ or even from acetate,¹⁶ the biosynthesis of the above mentioned dihydropyrroles isolated from fungi have not yet been investigated. Particularly, we aimed to verify if the dihydropyrrole moiety in **1** and **2** is derived from proline or ornithine, and if the branched moiety is derived from propionate or from acetate *plus* methionine. To the best of our knowledge, this is the first report on the biosynthesis of fungal dihydropyrrole-bearing secondary metabolites.

Results and Discussion

After toxicity evaluation with unlabeled sodium acetate, added to the growth medium of *P. citrinum* (up to 5 g L⁻¹), for which no morphology alterations of the fungus in the growth medium or even alterations in the production yield of **1** and **2** were observed, experiments with sodium[1-¹³C]acetate, sodium[1,2-¹³C₂]acetate, sodium[2,3-¹³C₂]propionate, [*methyl*-¹³C]methionine, [U-¹³C₅]ornithine and [U-¹³C₅]proline were performed. ¹³C-Labeled precursors were added at the fourth day of growth, at a final concentration of 1 mg mL⁻¹ for each precursor, using previously optimized growth conditions.¹⁵ Separate growth experiments were performed without the addition of labeled precursors as control. Cultures were harvested after 14 days for each growth experiment using distinct ¹³C-labeled precursors. After growth media filtration and solid phase extraction, fractions were evaporated and subjected to HPLC-UV-ESI-MS analysis.

Preliminary small-scale experiments in 50 mL of growth media for each ¹³C-labeled precursor gave, after the growth media clean-up, fractions containing both **1** and **2** which clearly showed the incorporation of sodium[1-¹³C]acetate, sodium[1,2-¹³C₂]acetate, [*methyl*-¹³C]methionine and of [U-¹³C₅]ornithine by HPLC-UV-MS analyses. Clusters at *m/z* 250 for the [M+H]⁺ ion and at *m/z* 272 for the [M+Na]⁺ ion were observed (see Supplementary Information). The preliminary experiments with sodium[2,3-¹³C₂]propionate and [U-¹³C₅]proline showed no incorporation in both **1** and **2**. Therefore, large-scale feeding experiments with ¹³C-labeled acetate, ornithine and methionine were performed in order to obtain sufficient amounts of labeled **1** and **2** for ¹³C NMR analyses.

Separate *P. citrinum* growth experiments in a total volume of approximately 1.4 L were performed using sodium[1-¹³C]acetate, sodium[1,2-¹³C₂]acetate, [*methyl*-¹³C]methionine and [U-¹³C₅]ornithine (see Experimental). After growth in the presence of the labeled

precursors, solid-phase extraction of the growth media and extensive purification of both **1** and **2** by HPLC, ¹³C NMR analyses clearly showed the incorporation of the labeled precursors. *P. citrinum* grown in medium enriched with sodium[1-¹³C]acetate gave, after isolation, compound **1** with significant incorporation at C-6 (δ 166.2), C-8 (δ 206.3), C-10 (δ 22.5), C-12 (δ 31.7) and C-14 (δ 124.5), in agreement with a pattern expected for an acetate-derived polyketide chain (Table 1 and Figure S3 in Supplementary Information). An additional feeding experiment with sodium[1,2-¹³C₂]acetate confirmed this result, indicating that the β -keto-amide chains in **1** and **2** are derived from acetate. The incorporation ratio of sodium[1,2-¹³C₂]acetate into **1** was calculated as previously proposed by Kubanek and Andersen.¹⁸ We measured the incorporation rate at the amide carbonyl group (C-6) and at the terminal methyl group of the alkyl chain (C-15) as 12.7 and 11.7%, respectively (Table 1; see also Figures S6 to S10).

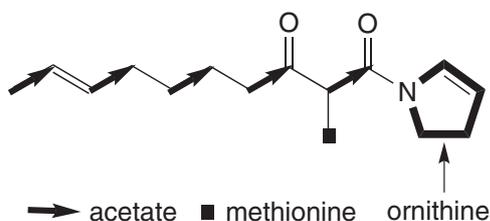
Although propionate is considered as toxic for fungi,¹⁹⁻²¹ in few instances it has been demonstrated that labeled propionate was incorporated into fungal polyketides.^{22,23} Therefore, in principle the incorporation of propionate into **1** and **2** could not be ruled out. We have not verified sodium[2,3-¹³C₂]propionate incorporation into **1** and **2** (data not shown). The C-16 methyl group was clearly methionine-derived, confirmed after a feeding experiment with [*methyl*-¹³C]methionine that showed a very significant incorporation at C-16 (δ 12.7; see Table 1 and Figure S12). The feeding experiment with [U-¹³C₅]proline showed no incorporation into **1** and **2**. On the other hand, feeding with [U-¹³C₅]ornithine showed marked incorporation at the dihydropyrrole group, for which ¹³C-¹³C coupling constants of the dihydropyrrole moiety could be unambiguously measured: 74 Hz for C-2/C-3, 36 for C-3/C-4, 36 for C-4/C-5 and 7 for C-2/C-5 (Figures S14 to S16). Since compound **2** has been isolated as a minor derivative,¹⁵ some of the feeding experiments gave insufficient amounts of labeled **2** to obtain reliable ¹³C NMR spectra to measure the incorporation rates of [1,2-¹³C₂]acetate and of [U-¹³C₅]ornithine. The results obtained evidenced that both **1** and **2** are derived from acetate, ornithine and methionine.

The dihydropyrrole moiety of **1** and **2** did not show incorporation of labeled acetate. This result was surprising, considering that ornithine is biosynthetically derived from arginine, itself originary from α -ketoglutarate via glutamate in the urea cycle.²⁴ An additional growth experiment with *P. citrinum* in starch and unlabeled sodium acetate was performed in order to double check this result. No production of **1** and **2** was observed, as expected, since fungi require exogenous nitrogen sources.²⁴ Therefore, we

Table 1. Incorporation ratios of ^{13}C -labeled precursors into dihydropyrrole **1**

Position	$^{13}\text{C}^a$	$[1-^{13}\text{C}_1]\text{AcONa}$	$[1,2-^{13}\text{C}_2]\text{AcONa}$	$[\text{methyl-}^{13}\text{C}_1]\text{methionine}$	$[\text{U-}^{13}\text{C}_5]\text{ornithine}$
1	-	-	-	-	-
2	129.4	1.15	1.25	0.94	10.18
3	111.9	1.1	1.1	1.1	15.02
4	27.7	0.92	0.7	0.77	13.67
5	44.9	1.48	0.69	0.84	13.14
6	166.2	10.55	15.35	0.84	1.3
7	50.5	0.79	7.39	0.38	0.76
8	206.3	8.87	13.17	1.1	1.37
9	39.9	nd	nd	nd	nd
10	22.5	8.34	10.28	0.65	0.56
11	28.3	0.88	8.5	0.62	0.47
12	31.7	8.46	9.24	0.68	0.55
13	131	1.25	7.83	0.57	0.52
14	124.5	8.06	7.54	0.61	0.62
15	17.7	1.13	7.56	0.56	0.44
16	12.7	0.89	0.66	28.24	1.29

^a ^{13}C NMR spectra recorded in $\text{DMSO-}d_6$ at 100.1 MHz; nd: not detected.



Scheme 1. Biosynthetic precursors incorporated into (8E)-1-(2,3-dihydro-1H-pyrrol-1-yl)-2-methyldec-8-ene-1,3-dione (**1**).

assumed that the biosynthesis of the dihydropyrrole moiety in **1** and **2** needs ornithine, but not proline, as precursor.

Conclusions

We have unambiguously established the biosynthetic origin of dihydropyrroles **1** and **2** as derived from acetate, methionine and ornithine. However, the sequence of events leading to compounds **1** and **2** in *Penicillium citrinum* still has to be established.

Experimental

General procedures

NMR spectra were recorded on a Bruker ARX 9.4T instrument, operating at 100.10 MHz for ^{13}C channels, at 25 °C using TMS as internal reference. Solvents used for extraction and chromatography were HPLC-grade solvents. HPLC separations were performed either with a Waters quaternary pump 600, double beam UV detector 2487, and

data module 746, or with a Waters 600E system controller liquid chromatography attached to a Waters 996 photodiode array detector, on which the UV spectra have been recorded as well. HPLC-UV-MS analysis were performed using a Waters Alliance 2695 coupled on-line with a Waters 2996 photodiode array detector, followed by a Micromass ZQ2000 mass spectrometry detector with an electrospray interface. The photodiode array scanned the samples at λ_{max} 230 and 254 nm. The mass spectrometer detector was optimized using the following conditions: capillary voltage, 3.00 kV; source block temperature, 100 °C; desolvation temperature, 350 °C; voltage cone, 25 V; electrospray, positive mode; detection range, 200-550 Da with total ion count extracting acquisition. Cone and desolvation gas flow were at 50 and 350 L h⁻¹, respectively, using a Nitrogen Peak Scientific N110DR as a nitrogen source. Data acquisition and processing were performed using Empower 2.0.

Microbial strain

The *P. citrinum* strain was isolated from a seaweed (*Caulerpa* sp.).¹¹ *P. citrinum* F53 strain have been deposited in the CBMAI collection under the accession number CBMAI 1186.

Small scale incorporation experiments with ^{13}C -labeled precursors

Fifty mL of sterilized and modified MF fermentation media (12 g L⁻¹ glucose; 6.0 g L⁻¹ starch; 12.0 g L⁻¹ soytone; 3.0 g L⁻¹ peptone; 0.18 g L⁻¹ meat extract; 3.0 g L⁻¹ yeast

extract) in artificial sea water (ASW: 0.27 g L⁻¹ CaCl₂; 1.94 g L⁻¹ MgCl₂; 0.12 g L⁻¹ KCl; 6.0 g L⁻¹ NaCl; 0.03 g L⁻¹ Na₂HPO₄; 0.64 g L⁻¹ NaSO₄; 0.034 g L⁻¹ NaHCO₃; 0.020 g L⁻¹ KBr; 0.008 g L⁻¹ SrCl₂; 0.006 g L⁻¹ H₃BO₃) were prepared in 250 mL Schott flasks, in duplicates for each incorporation experiment. Then, 10⁵ spores of a *P. citrinum* inoculum were inoculated in each Schott flask, followed by incubation at 30 °C for 14 days. ¹³C-labeled precursors (sodium[1-¹³C]acetate, sodium[1,2-¹³C₂]acetate, sodium[2,3-¹³C₂]propionate, [U-¹³C₅]proline, [U-¹³C₅]ornithine and [methyl-¹³C]methionine) were added in a final concentration of 1 mg mL⁻¹ on the fourth day of microbial growth. A separate culture without the addition of any precursor was incubated as a control. Cultures were harvested after 14 days of growth and filtered through celite. The liquid media of each growth experiment using distinct ¹³C-labeled precursors were separately subjected to a reversed-phase solid phase extraction using a C₁₈ cartridge eluted with 100% H₂O (fraction 1, discarded), 1:1 MeOH/H₂O (fraction 2, F2) and 100% MeOH (fraction 3, F3). Fractions F2 and F3 were collected, dried *in vacuo* and weighed. Aliquots of 1 mg of each F2 and F3 were analyzed by HPLC-UV-MS using a reversed-phase C₁₈ column (X-Terra® MS, 50 × 2.1 mm, 3.5 μm), eluted with a MeOH/H₂O gradient. Fractions collected from the growth experiments using sodium[1-¹³C]acetate, sodium[1,2-¹³C₂]acetate, [U-¹³C₅]ornithine and [methyl-¹³C]methionine showed incorporation. These experiments were selected to be repeated in a larger scale.

Large scale incorporation experiments with ¹³C-labeled precursors

A total volume of 1.4 L of culture media distributed in 14 Schott flasks with 100 mL each were inoculated with 10⁵ spores of *P. citrinum*, followed by incubation at 30 °C during 14 days. Growth experiments were performed using ¹³C-labeled precursors (sodium[1-¹³C]acetate, sodium[1,2-¹³C₂]acetate, [U-¹³C₅]ornithine and [methyl-¹³C]methionine), added at a final concentration of 1 mg mL⁻¹ on the fourth day of microbial growth. Cultures were harvested after 14 days of growth and filtered through celite. The liquid media of identical growth experiments were pooled and subjected to a reversed-phase solid phase extraction using a C₁₈ cartridge eluted with 100% H₂O (fraction 1, discarded), 1:1 MeOH/H₂O (fraction 2, F2) and 100% MeOH (fraction 3, F3). Fractions F2 and F3 were collected, dried *in vacuo* and weighed. A total amount of crude F3 fractions of each ¹³C-labeled precursor experiments were obtained: 78.3 mg of experiment using sodium[1-¹³C]acetate; 40.4 mg of experiment using

sodium[1,2-¹³C₂]acetate; 79.2 mg of experiment using [U-¹³C₅]ornithine; 89.7 mg of experiment using [methyl-¹³C]methionine. Each of these crude fractions was purified by HPLC-UV-MS using a reversed-phase C₁₈ column (Inertsil ODS-3, 250 × 4.6 mm, 5 μm), eluted with 7:3 MeOH/H₂O. The following amounts of **1** and **2** obtained after each incorporation experiment were, respectively: 5.4 and 1.1 mg using sodium[1-¹³C]acetate; 4.3 and 1.0 mg using sodium[1,2-¹³C₂]acetate; 7.5 and 2.1 mg using [U-¹³C₅]ornithine; 4.0 and 1.0 mg using [methyl-¹³C]methionine.

Small scale experiment with soluble starch and acetate

Two growth experiments with a total volume of 50 mL of culture media each (6 g L⁻¹ soluble starch; 2 g L⁻¹ unlabeled sodium acetate; in ASW (0.27 g L⁻¹ CaCl₂; 1.94 g L⁻¹ MgCl₂; 0.12 g L⁻¹ KCl; 6.0 g L⁻¹ NaCl; 0.03 g L⁻¹ Na₂HPO₄; 0.64 g L⁻¹ NaSO₄; 0.034 g L⁻¹ NaHCO₃; 0.020 g L⁻¹ KBr; 0.008 g L⁻¹ SrCl₂; 0.006 g L⁻¹ H₃BO₃) were inoculated with 10⁵ spores of *P. citrinum*, followed by incubation at 30 °C during 7, 14 and 28 days, at an initial pH 8.0. At the end of growth, cultures were harvested and filtered through celite. The liquid media of identical growth experiments were pooled and subjected to a reversed-phase solid phase extraction using a C₁₈ cartridge eluted with 100% H₂O (fraction 1, discarded), 1:1 MeOH/H₂O (fraction 2, F2) and 100% MeOH (fraction 3, F3). Fractions F2 and F3 were collected, dried *in vacuo* and weighed. Aliquots of 1 mg of each F2 and F3 were analyzed by HPLC-UV-MS using a reversed-phase C₁₈ column (X-Terra® MS, 50 × 2.1 mm, 3.5 μm), eluted with a MeOH/H₂O gradient.

[¹³C-6, ¹³C-8, ¹³C-10, ¹³C-12, ¹³C-14](8E)-1-(2,3-dihydro-1H-pyrrol-1-yl)-2-methyldec-8-ene-1,3-dione (**1**)

Incolor gum. 5.4 mg. Seven relevant ions were detected in the positive mode ESI mass spectrum with *m/z* 250.0, 251.0, 252.0, 253.0, 254.0, 255.0 and 272.0, of which *m/z* 250.0 corresponded to the [M+H]⁺ ion of unlabeled **1** and peaks at *m/z* 251.0, 252.0, 253.0, 254.0, 255.0 corresponded to the protonated molecular ions of sodium[1-¹³C]acetate labeled **1**. An additional ion detected at *m/z* 272.0 corresponded to sodium adduct ion of unlabeled **1**. See Figures S2 to S4 (Supplementary Information).

[¹³C-6, ¹³C-8, ¹³C-10, ¹³C-12, ¹³C-14]-1-(2,3-dihydro-1H-pyrrol-1-yl)-2-methyldecane-1,3-dione (**2**)

Incolor gum. 1.1 mg. Seven relevant ions were detected in the positive mode ESI mass spectrum with *m/z* 252.0, 253.0, 254.0, 255.0, 256.0, 257.0 and 274.0,

of which m/z 252.0 corresponded to the $[M+H]^+$ ion of unlabeled **2** and peaks at m/z 253.0, 254.0, 255.0, 256.0, 257.0 corresponded to the protonated molecular ions of sodium $[1-^{13}C]$ acetate labeled **2**. An additional ion detected at m/z 274.0 corresponded to the $[M+Na]^+$ ion of unlabeled **2**.

$[^{13}C-6, ^{13}C-7, ^{13}C-8, ^{13}C-9, ^{13}C-10, ^{13}C-11, ^{13}C-12, ^{13}C-13, ^{13}C-14, ^{13}C-15](8E)-1-(2,3-dihydro-1H-pyrrol-1-yl)-2-methyldec-8-ene-1,3-dione$ (**1**)

Incolor gum. 4.3 mg. Seven relevant ions were detected in the positive mode ESI mass spectrum with m/z 250.0, 252.0, 254.0, 256.0, 258.0, 260.0 and 272.0, of which m/z 250.0 corresponded to the $[M+H]^+$ ion of unlabeled **1** and m/z 252.0, 254.0, 256.0, 258.0, 260.0 corresponded to the protonated molecular ions of sodium $[1,2-^{13}C_2]$ acetate labeled **1**. An ion detected at m/z 272.0 corresponded to the $[M+Na]^+$ ion of unlabeled **1**. See Figures S5 to S10.

$[^{13}C-6, ^{13}C-7, ^{13}C-8, ^{13}C-9, ^{13}C-10, ^{13}C-11, ^{13}C-12, ^{13}C-13, ^{13}C-14, ^{13}C-15]1-(2,3-dihydro-1H-pyrrol-1-yl)-2-methyldecane-1,3-dione$ (**2**)

Incolor gum. 1.0 mg. Seven relevant ions were detected in the positive mode ESI mass spectrum with m/z 252.0, 254.0, 256.0, 258.0, 260.0, 262.0 and 274.0, of which m/z 252.0 corresponded to the $[M+H]^+$ ion of unlabeled **2** while ions at m/z 254.0, 256.0, 258.0, 260.0, 262.0 corresponded to the protonated molecular ions of sodium $[1,2-^{13}C_2]$ acetate labeled **2**. An ion detected at m/z 274.0 corresponded to the sodium adduct of unlabeled **2**.

$[^{13}C-16](8E)-1-(2,3-dihydro-1H-pyrrol-1-yl)-2-methyldec-8-ene-1,3-dione$ (**1**)

Incolor gum. 4.0 mg. Four relevant ions were detected in the positive mode ESI mass spectrum with m/z 250.0, 272.0, 251.0 and 273.0, of which m/z 250 and 272.0 corresponded to the unlabeled $[M+H]^+$ and $[M+Na]^+$ ions of **1**, respectively. Ions detected at m/z 251.0 and 273.0 corresponded to the protonated molecular ion and to the sodium adduct ion of $[methyl-^{13}C]$ methionine labeled **1**, respectively. See Figures S11 and S12.

$[^{13}C-16]1-(2,3-dihydro-1H-pyrrol-1-yl)-2-methyldecane-1,3-dione$ (**2**)

Incolor gum. 1.0 mg. Four relevant ions were detected in the positive mode ESI mass spectrum with m/z 252.0, 274.0, 253.0 and 275.0, of which m/z 252.0 and 274.0 corresponded to the unlabeled $[M+H]^+$ and $[M+Na]^+$ ions of **2**, respectively. Ions detected at m/z 253.0 and 275.0 corresponded to $[M+H]^+$ and $[M+Na]^+$ ions of $[methyl-^{13}C]$ methionine labeled **2**.

$[^{13}C-2, ^{13}C-3, ^{13}C-4, ^{13}C-5](8E)-1-(2,3-dihydro-1H-pyrrol-1-yl)-2-methyldec-8-ene-1,3-dione$ (**1**)

Incolor gum. 7.5 mg. Four relevant ions were detected in the positive mode ESI mass spectrum with m/z 250.0, 272.0, 254.0 and 276.0, of which m/z 250.0 and 272.0 corresponded to the $[M+H]^+$ and $[M+Na]^+$ ions of unlabeled **1**, and the ions at m/z 254.0 and 276.0 corresponded to the $[M+H]^+$ and $[M+Na]^+$ ions of $[U-^{13}C_5]$ ornithine labeled **1**. See Figures S13 to S16.

$[^{13}C-2, ^{13}C-3, ^{13}C-4, ^{13}C-5]1-(2,3-dihydro-1H-pyrrol-1-yl)-2-methyldecane-1,3-dione$ (**2**)

Incolor gum. 2.1 mg. Four relevant ions were detected in the positive mode ESI mass spectrum with m/z 252.0, 274.0, 256.0 and 278.0, of which m/z 252.0 and 274.0 corresponded to the $[M+H]^+$ and $[M+Na]^+$ ions of unlabeled **2**, while m/z 256.0 and 278.0 corresponded to the $[M+H]^+$ and $[M+Na]^+$ ions of $[U-^{13}C_5]$ ornithine labeled **2**.

Supplementary Information

ESI-MS and ^{13}C NMR spectra of all ^{13}C -labeled compounds are available free of charge at <http://jbcbs.sbq.org.br> as a PDF file.

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