

ISOACETOGENINS, ARTIFACTS ISSUED FROM TRANSLACTONIZATION
FROM ANNONACEOUS ACETOGENINS¹

Philippe Duret ^a, Alain Laurens ^a, Reynald Hocquemiller ^a, Diego Cortes ^b, and
André Cavé ^{a*}

^a Laboratoire de Pharmacognosie, URA 1843 CNRS (BIOCIS), Faculté de
Pharmacie, Université Paris XI, 92296 Châtenay-Malabry Cedex, France

^b Departamento de Farmacologia, Facultad de Farmacia, Universidad de
Valencia, 46010 Burjasot, España

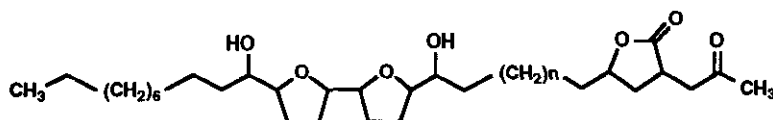
Abstract- Extraction of isoacetogenins from *Annona cherimolia* roots led us to consider these compounds as artifacts obtained by translactonization of 4-hydroxyacetogenins. To confirm this hypothesis, extraction and characterization of initial acetogenins from fresh crude material and effect of alkaloids, basic medium, and alcohol on the kinetic of the translactonization are performed. A mechanism is discussed for this reaction. Cytotoxic activities of isoacetogenins and corresponding acetogenins are evaluated.

Over the past six years, studies of several Annonaceae species led to the characterization of 13 biologically active acetogenins belonging to subtype 2 (isoacetogenins).² As part of our continuing investigation of Annonaceae as a source of bioactive agents, we have succeeded in isolation of four isoacetogenins from *Annona cherimolia* roots.³

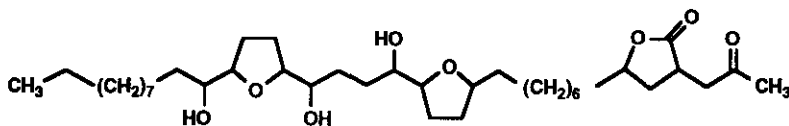
We present in this paper arguments and experimental data proving that these compounds as well as the previously described isoacetogenins must not be considered as natural products but as artifacts of purification derived from the corresponding 4-hydroxyacetogenins by translactonization⁴; this would be consecutive to the formation of cyclic orthoesters followed by tautomerization involving the hydroxyl group at C-4.

Through partitioning of the crude methanolic extract of *Annona cherimolia* roots, with solvents (hexane/H₂O; methylene chloride) and several chromatographic steps of purification, three isoacetogenins of types B2 and one of C2 type were isolated, isorolliniastatin-2 (**1**), isomolvizarin-1 (**2**), isomolvizarin-2 (**3**), and isocherimolin-1 (**4**), respectively. Two known compounds belonging to types B1 and C1 (unsaturated γ -lactone acetogenins with two adjacent or not adjacent tetrahydrofuran) have been also identified as squamocin and almunequin respectively.³

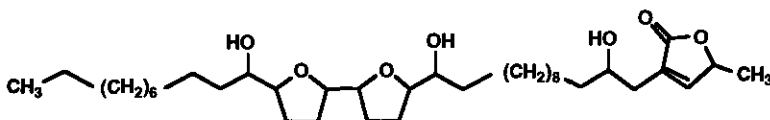
Examination of ir and nmr spectra of **1**, **2**, **3** and **4** revealed the characteristic pattern for Annonaceous isoacetogenins: a saturated α -acetyl- γ -lactone fragment. The distinctive double signals on their ¹H and ¹³C-nmr spectra suggested mixtures of C-2/C-4 cis and trans diastereomers as typical for all described isoacetogenins.²



Isomolvizarin-1 (**2**): threo/trans/threo/trans/erythro n=6
 Isomolvizarin-2 (**3**): threo/trans/threo/trans/threo n=6
 Isorolliniastatin-1 (**6**): threo/cis/threo/cis/erythro n=8
 Isorolliniastatin-2 (**1**): threo/trans/threo/trans/erythro n=8



Isocherimolin-1 (**4**): threo-threo/trans/erythro



Rolliniastatin-1 (**5**): threo/cis/threo/cis/erythro

Unusual in nature, such a mixture induced us to consider isoacetogenins as artifacts which appear when 4-hydroxyacetogenins are subjected to alkaline medium. Indeed these acetogenins are easily converted to the corresponding epimeric mixture of isoacetogenins after treatment by a strong base like 2% KOH in *tert*-BuOH with a yield of 50%.⁵⁻⁷ A weak base such as diethylamine is sufficient to convert a 4-hydroxyacetogenin into

the corresponding cis and trans isomers at room temperature with a better yield. For example, the translactonization of 4-hydroxyacetogenins is accomplished quantitatively after treatment with a methanolic solution of 10% diethylamine during 18 h at 25°C. Moreover, simple elution from silica gel column with a solvent mixture as CH₂Cl₂ - 2% diethylamine is sufficient to transform acetogenins into isoacetogenins in about 60% yield. However, it is not evident that acetogenins are subjected to alkaline medium during their extraction or isolation.

Four pairs of cis and trans isoacetogenins (**1-4**), and two unsaturated γ -lactone acetogenins without hydroxyl group at C-4, have been isolated from the methanolic extract of Annona cherimolia dried roots without any special precaution. Therefore, some experiments have been performed to confirm the transformation of 4-hydroxyacetogenins into isoacetogenins during the extraction and purification processes.

Extraction of acetogenins from fresh crude material, avoiding all the possible causes of rearrangement was firstly carried out. Fresh ground roots were firstly macerated with 0.5N hydrochloric acid in order to eliminate alkaloids and the dried plant material was extracted with methylene chloride. Then, the CH₂Cl₂ extract was analysed by tlc with samples of isoacetogenins and corresponding acetogenins as references. None of isoacetogenins could be evidenced by this analytical method and their absence was confirmed by ¹H and ¹³C-nmr analyses. This allows us to conclude that, in the first extraction, isoacetogenins were formed during the drying or the extraction processes and that they do not exist in the fresh material.

Then, the effect of alkaloids existing in the crude material on acetogenins was studied. This study has been performed on a 4-hydroxyacetogenin, rolliniastatin-1 (**5**), isolated from Rollinia membranacea⁸ in large amount.

The capability of several alkaloids, particularly those existing in the roots of Annona cherimolia, to transform rolliniastatin-1 (**5**) into isorolliniastatin-1 (**6**), was firstly envisaged. Indeed, alkaloids of Annona cherimolia could assist the rearrangement according to their more or less basic nature. Moreover, all described isoacetogenins have been isolated from Annonaceae materials (leaves, barks or roots) containing alkaloids.

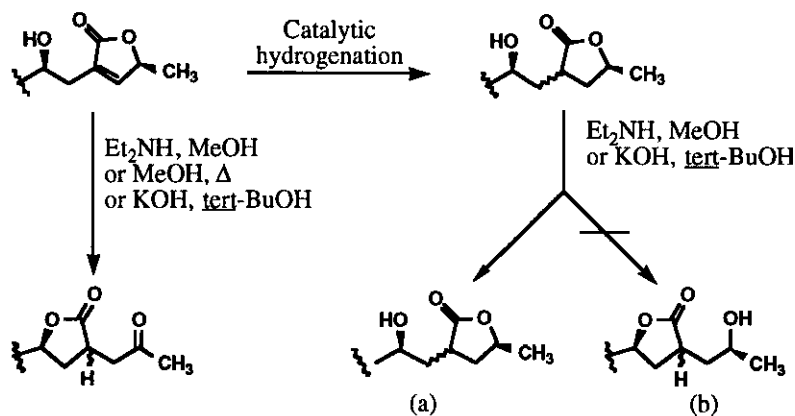
Four alkaloids, as salts or bases, have been used, namely anonaine, anonaine hydrochloride, coclaurine, coclaurine tartrate, berberine sulfate and nuciferine. They were separately dissolved in aqueous methanol with rolliniastatin-1 at room temperature. The solutions so obtained were regularly tested by tlc comparatively to samples of rolliniastatin-1 and isorolliniastatin-1. Under these conditions, with anonaine base, **5** was transformed into **6** in 50% in a month and completely in three months, but no transformation was observed with

anonaine hydrochloride. Translactonization was also observed with nuciferine and coclaurine base as well as with coclaurine tartrate however more slowly. With berberine sulfate, no rearrangement occurred.

It looks like that isoacetogenins are formed in the presence of alkaloids as bases or as weak acid salts. The kinetic of transformation is very depending on the nature of the alkaloid and it is strongly increased with heating.

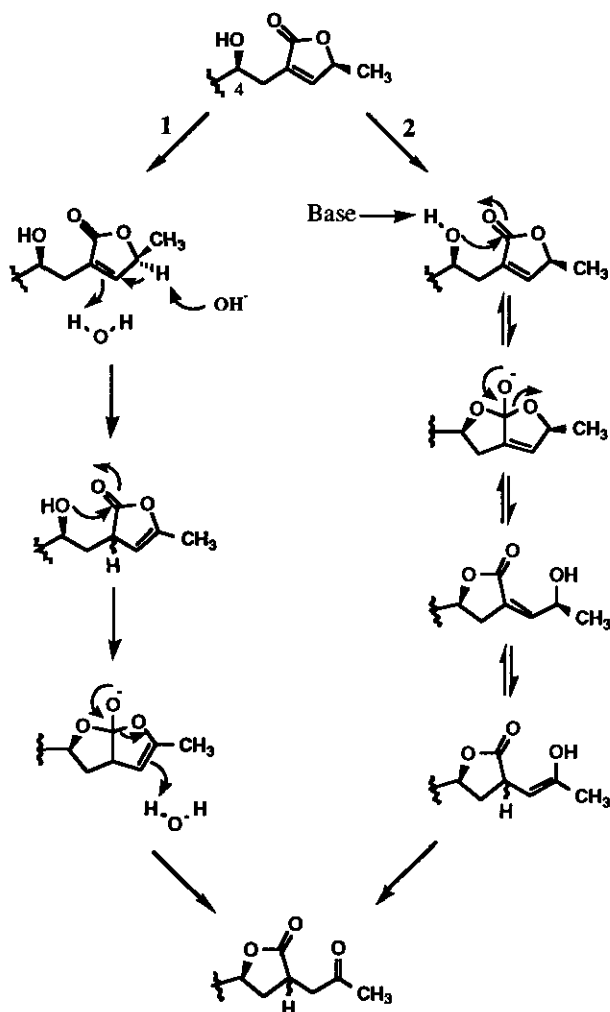
Recently, isolation of isoacetogenins from seeds of *Annona muricata* in which no alkaloids were isolated so far, was described.⁹ We did not found any isoacetogenins in seeds of the same species.^{10,11} Therefore, to study influence of the solvent used during the extraction processes, we have subjected rolliniastatin-1 to heating in methanolic solution. Such a treatment is sufficient to give rise to a translactonization. After heating for 4 h, a notable quantity of isorolliniastatin-1 was detected and after 48 h, rolliniasatatin-1 was converted to **6**, in a 50% yield. Therefore, we can conclude that isoacetogenins can take rise from the corresponding 4-hydroxyacetogenins after heating in an alcohol.

The prime influence of the double bond in the lactone ring on translactonization has been finally investigated. This study demonstrates that conversion of acetogenins into the iso forms is complete when treated with diethylamine or a weak base. On the other hand, when the dihydroacetogenins obtained after hydrogenation of the double bond (dihydroannonacin and dihydrorolliniastatin-1) are treated in the same conditions as above, the starting material is recovered unchanged. It is worth of note that treatment of dihydroacetogenins by a strong base, led to the potassium salt according to Born and coll.¹² After acidification, the extraction affords only the dihydroacetogenin (a) when a mixture of (a) and (b) was expected (Scheme 1).



Scheme 1

Two mechanisms of transesterification could be proposed (Scheme 2) : the first one, route 1, involving the hydrogen atom in γ position of the lactone⁶ and the second one, route 2, the hydrogen of C-4 hydroxyl. The key step in route 1, is the formation of a cyclic orthoester after deconjugation of the double bond, followed by ring opening leading to the formation of an enol in equilibrium with its keto form. In route 2, the cyclic orthoester is formed first, followed by ring opening leading to the formation of the exocyclic double bond conjugated to the lactone. Then an antarafacial thermal [1,3] H sigmatropic rearrangement could occur to afford the acetyl function. As it is well known, intramolecular transesterification can be catalysed by heating and/or by weak bases. It is likely that the catalyst functions by assisting removal of the hydroxyl proton in the rate-determining step. At this point, it is difficult to favour one of these two routes in view of the experimental data available.



Scheme 2

Henceforth, we must avoid during extraction and purification excessive temperature and/or alkaline media. Acetogenins exhibit a broad range of potent biological activities specially antitumoral, cytotoxic and pesticidal properties. Translactonization of 4-hydroxyacetogenins seems to release these activities. The results of cytotoxic activity assay against KB, HeLa and Vero cell lines are reported in Table 1. Acetogenins and their corresponding isoacetogenins were significantly toxic to the three cell lines, in the range of 10^{-1} to 10^{-3} $\mu\text{g/ml}$ for Vero and $3 \cdot 10^{-3}$ to 10^{-5} $\mu\text{g/ml}$ for KB and HeLa cells. As for most acetogenins, iso forms are more toxic against KB or HeLa than against VERO cells on which there is not great differences in activity between the two forms. It is worth noting that for KB and HeLa cells lines, isoacetogenins are either as active as their acetogenins counterpart or more active (see isorolliniastatin-2 and rolliniastatin-2). Furthermore it is interesting to notice that rolliniastatin-2 which differs from rolliniastatin-1 by the relative configuration across the bis-tetrahydrofuran rings, threo/trans/threo/trans/erythro and threo/cis/threo/cis/erythro respectively, is 300 times less cytotoxic on KB or HeLa cells; this shows the importance of stereochemical relationships across the THF skeleton. Moreover in our laboratory we have submitted rolliniastatin-1 and isorolliniastatin-1 to a toxicological trial on mouse by subcutaneous route. The results show that isorolliniastatin-1 with a LD_{50} of 200 mg/kg is considerably less toxic than rolliniastatin-1 (LD_{50} : 5 mg/kg) even though the cytotoxic activity on cell lines is the same. So, these artifacts called isoacetogenins should be considered as a new tool in the search for antitumoral compounds with a broader therapeutic index than the corresponding acetogenins.

Table 1. Cytotoxic activity of acetogenins/isoacetogenins (EC_{50} in $\mu\text{g/ml}$)

Acetogenins / isoacetogenins	KB	HeLa	VERO
Cherimolin-1/ isocherimolin-1	$10^{-4}/3 \cdot 10^{-4}$	$10^{-4}/3 \cdot 10^{-5}$	ND
Molvizarin / isomolvizarin	$10^{-5}/3 \cdot 10^{-4}$	$10^{-5}/10^{-4}$	$10^{-3}/10^{-3}$
Rolliniastatin-2 / isorolliniastatin-2	$3 \cdot 10^{-3}/10^{-5}$	$10^{-2}/10^{-5}$	$10^{-1}/1.5 \cdot 10^{-3}$
Rolliniastatin-1 / isorolliniastatin-1	$10^{-5}/3 \cdot 10^{-5}$	$3 \cdot 10^{-6}/3 \cdot 10^{-5}$	$1.5 \cdot 10^{-3}/10^{-3}$
Vinblastine	10^{-3}	ND	$>0,3$

EXPERIMENTAL

General Experimental Procedures. The ^1H and ^{13}C -nmr spectra were obtained with a Bruker AM-400 spectrophotometer at 200 and 50 MHz, respectively. Tlc was performed on Alufolien kieselgel 60F254 Merck and the visualization was accomplished using Kedde's reagent and sulfuric acid in 50% methanol.

Plant material. Fresh roots of *Annona cherimolia* were collected in February 1993 at Barrancos de las Zorras, Molvizar in "cherimoyo vale" Granada coast. A voucher specimen is deposited in the herbarium of the Department of Botany, University of Valencia (Spain).

Extraction procedures. Fresh ground roots (100 g) were extracted with normal hydrochloric acid (4 x 1.5 l) for 1 h at room temperature, and extraction was carried until the Mayer reaction was negative. The plant material was dried and extracted with methylene chloride (4 x 1.25 l) for 1 h; the organic solution was concentrated to 150 ml and washed successively by acidic aqueous solution and water. After drying over anhydrous sodium sulfate, the organic solvent was evaporated leaving a residue (990 mg). This was firstly analysed by tlc, AcOEt/MeOH [10:0.2] with reference samples of **1**, **2**, **3** and **4** and corresponding acetogenins, rolliniastatin-2, molvizarin and cherimolin-1, previously isolated from *Annona cherimolia* seeds,¹³ and then by ^1H and ^{13}C -nmr.

Isorolliniastatin-1 (6). - Translactonization by alkaloids : six solutions of rolliniastatin-1 (**5**) (10.3 to 16.4 mg, 0.016 to 0.026 mmol) in MeOH/H₂O (95/5) (3 ml) were prepared and in each separately was added : anonaine (40.0 mg, 0.15 mmol), or anonaine hydrochloride (39.5 mg, 0.13 mmol), or coclaurine (43.5 mg, 0.15 mmol), or coclaurine tartrate (64.5 mg, 0.18 mmol), or berberine sulfate (56.0 mg, 0.13 mmol) or nuciferine (55.0 mg, 0.19 mmol). The reaction mixtures were allowed to stand at room temperature and regularly analysed by tlc. When isorolliniastatin-1 was visualized in a sample by tlc, this one was purified by flash chromatography and identified by ^1H -nmr; CDCl_3 , δ : 0.86 (3H, t, J = 7Hz, CH₃-34), 1.20-1.98 (46H, 3m, H-5-14, H-17-18, H-21-22, and H-25-33), 1.98 (1H, m, H-3b), 2.20 (3H, s, CH₃-37), 2.54 (1H, m, H-3a), 2.62 (1H, m, H-35a), 3.05 (2H, m, H-2 and H-35b), 3.38 (1H, m, H-24), 3.78 (5H, m, H- 15-16, H- 19-20, and H-23), 4.45 (1H, m, H-4).
- Translactonization by a weak base : 11 mg of **5** were treated with 10% diethylamine (0.3 ml) in MeOH/H₂O (95/5) (2.7 ml) at room temperature for 18 h; the solution was dried under vacuum. The unique product is the 36-oxo saturated γ -lactone (**6**) (11 mg), identified by ^1H -nmr.
- Translactonization in alcohol : 14 mg of **5** were dissolved in 5 ml of MeOH/H₂O (95/5) and the mixture was heated at reflux for 48 h. The mixture was separated by flash chromatography. Rolliniastatin-1 (**5**) (4.7 mg), and isorolliniastatin-1 (**6**) (6.8 mg) were obtained and identified by ^1H -nmr.

Dihydorolliniastatin-1 and dihydroannonacin : Catalytic hydrogenations of rolliniastatin-1 (80 mg) and annonacin (50 mg) were performed in MeOH (2.5 ml) with 10% Pd/C (11 mg) at room temperature under an atmospheric pressure of H₂ for 15 h. According to our own results¹⁴ mixtures of two cis and trans diastereomers have been obtained and identified by ¹H and ¹³C-nmr as dihydorolliniastatin-1 (76 mg) and dihydroannonacin (44 mg).

Dihydorolliniastatin-1 (20 mg) and dihydroannonacin (20 mg) were separately treated with 2% KOH in *tert*-BuOH (1.5 ml) at room temperature for 24 h; the solutions were acidified with 5% HCl, and partitioned between CH₂Cl₂/H₂O. The only products in organic extracts were identified as the initial compounds by tlc, ¹H and ¹³C-nmr analyses. Same result have been obtained after treatment of dihydroannonacin (10 mg) with 10% diethylamine in MeOH (2.7 ml) at room temperature for 24 h.

Cytotoxic activity. Assays were performed in 96-well cultures plates. Serial three fold dilutions of products were added to a 24 hours old monolayer of KB (human epidermoid carcinoma cells), HeLa (human carcinoma of the cervix cells) or VERO (Africa green monkey kidney cells), and the plates incubated for three days at 37°C in a humidified 5% CO₂ atmosphere. Cytotoxicity was evaluated by microscopic examination of the cell culture and by a photometric method at 650 nm with methylene blue. Cytotoxicity was expressed as concentration of product which caused 50% loss of cells (EC₅₀).

ACKNOWLEDGMENTS

This work was sponsored by CNRS (URA 1843, BIOCIS) and "Direction de la Recherche et des Etudes Doctorales" through a biennial contract with "Réseau de la Recherche en Pharmacochimie". The authors expressed their gratitude to Dr. B. Figadère for helpful discussion and to Dr. J. Cotte-Lafitte for the cytotoxicity results, Laboratoire de Virologie-Immunologie, Châtenay-Malabry.

REFERENCES AND NOTES

1. Part 34 in the series of Acetogenins of Annonaceae. For part 33 see : V.T. Tam, P.Q.C. Hieu, B. Chappe, F. Roblot, O. Laprévote, B. Figadère, and A. Cavé.
2. X.-P. Fang, M.J. Rieser, Z.-M. Gu, G.-X. Zhao, and J.L. McLaughlin, *Phytochem., Anal.*, 1993, 4, 27.
3. Ph. Duret, D. Gromek, R. Hocquemiller, D. Cortes, and A. Cavé, *J. Nat. Prod.*, in press.

4. A. Cavé, D. Cortes, B. Figadère, R. Hocquemiller, O. Laprévote, A. Laurens, and M. Lebœuf, in : "Recent Advances in Phytochemistry : Phytochemical Potential of Tropical Plants." Vol. 27, ed. by K.R. Downum, J.T. Romeo, and H.A. Stafford, Plenum Press, New York, 1993, pp. 167-202.
5. Y.-H. Hui, J.K. Rupprecht, Y.M. Liu, J.E. Anderson, D.L. Smith, C.-J. Chang, and J.L. McLaughlin, *J. Nat. Prod.*, 1989, **52**, 463.
6. J.M. Cassady, W.M. Baird, and C.-J. Chang, *J. Nat. Prod.*, 1990, **53**, 23.
7. S.H. Myint, A. Laurens, R. Hocquemiller, A. Cavé, D. Davoust, and D. Cortes, *Heterocycles*, 1990, **31**, 861.
8. J. Saez, S. Sahpaz, L. Villaescusa, R. Hocquemiller, A. Cavé, and D. Cortes, *J. Nat. Prod.*, 1993, **56**, 351.
9. M.J. Rieser, X.-P. Fang, J.K. Rupprecht, Y.-H. Hui, D.L. Smith, and J.L. McLaughlin, *Planta Med.*, 1993, **59**, 91.
10. S.H. Myint, D. Cortes, A. Laurens, R. Hocquemiller, M. Lebœuf, A. Cavé, J. Cotte, and A.-M. Quéro, *Phytochemistry*, 1991, **30**, 3335.
11. D. Cortes, S.H. Myint, A. Laurens, R. Hocquemiller, M. Lebœuf, and A. Cavé, *Can. J. Chem.*, 1991, **69**, 8.
12. L. Born, F. Lieb, J.P. Lorentzen, H. Moeschler, M. Nonfon, R. Söllner, and D. Wendisch, *Planta Med.*, 1990, **56**, 312.
13. D. Cortes, S.H. Myint, B. Dupont, and D. Davoust, *Phytochemistry*, 1993, **32**, 1475.
14. D. Cortes, S.H. Myint, J.C. Harmange, S. Sahpaz, and B. Figadère, *Tetrahedron Lett.*, 1992, **33**, 5225.

Received, 30th March, 1994