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Assignment of Absolute Configuration of the α,β-Unsaturated γ-Methyl-γ-Lactone of the Annonaceous Acetogenins by a Simple Enzymatic Method[1]

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Abstract: The absolute configuration of the C-36 or C-34 chiral centers of Annonaceous acetogenins has been determined by simple enzymatic method focusing on the HPLC detection of NADH issued from the enzymatic oxidation of L- or D-lactic acid (obtained by oxidative cleavage of the terminal α,β-unsaturated γ-methyl-γ-lactone) to pyruvic acid by lactate dehydrogenase.

Annonaceous acetogenins are polyketides recently discovered up to now only from the tropical and subtropical plants of the Annonaceae family. They are characterized by a long alkyl chain of 35-37 carbon atoms possessing one or two tetrahydrofuran rings, several oxygenated functions (hydroxyl, ketone, acetoxyl) and a terminal γ-methyl-γ-lactone (Figure 1). In addition to these natural biologically active products, some natural precursors have been isolated which bear only the terminal γ-lactone and either hydroxyl groups and/or 1,2-epoxides with double bonds on the alkyl chain. Most of these compounds exhibit interesting parasiticide, insecticide, immunomodulating properties and cytotoxic activities with promising antitumoral potential.[2,3]

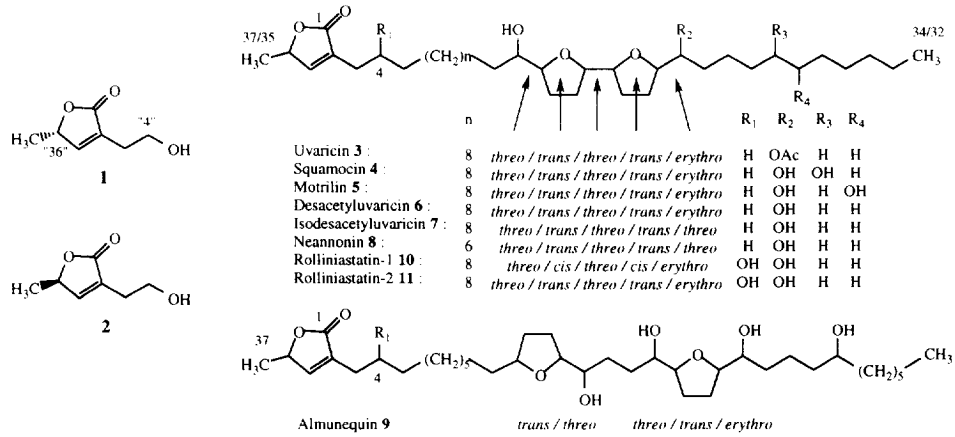

Fig. 1

General methods for determining the relative configuration of the tetrahydrofuranyl groups of these compounds and for assigning the absolute configuration of carbinol centers adjacent to the THF rings, as well as at C-4, have been previously described.[4,5] Moreover Hoye's group[6] has recently adapted Mosher's ester method to define the absolute configuration at C-36 or C-34 in acetogenins which possess an hydroxyl group in position 4 (subtype-1b) as in rolliniastatin-2 (= bullatacin) **11**. The absolute configuration of the γ-methyl-γ-lactones of subtype-1a (without 4-OH) representing more than 80 natural acetogenins, as in motrilin **5** (Figure 1), remained to be established and was only unambiguously determined as 36 *S* for uvaricin **1**[7] and squamocin **4**[8]. Although a similar biosynthetic pathway for all Annonaceous acetogenins may be assumed, it would be excessive to conclude that all such products possess the same absolute configuration. Optical rotary dispersion methods have also been used to determine the configuration of this stereogenic center.[5,9] At present these methods cannot be considered as reliable because of the lack of studies on both configurations.[2] We report here a simple method for assigning the absolute configuration at C-34 (36) of the γ-methyl-γ-lactone of subtypes-1a/1b of the Annonaceous acetogenins. This is based on a classical enzymatic end-point method usually employed in biochemistry, clinical chemistry, microbiology and foodstuff chemistry for the detection and quantitative analysis of L- and D-lactic acid.[10]

The first step of this straightforward protocol is illustrated in Figure 2. It consists of an oxidative cleavage of the α,β-unsaturated γ-methyl-γ-lactone which can be performed on a 1 to 5 mg sample. This crucial reaction was readily achieved in two hours at 70°C in the presence of an excess of periodic acid (15 eq.) with a catalytic amount of ruthenium (III) chloride in the ternary mixture of $CCl_4/CH_3CN/H_2O$ to give lactic acid as a byproduct which was identified by gas chromatography. As indicated in Figure 2, the latter degradative product[11] was then separately incubated in turn with stereospecific L(+) and D(-)-lactate dehydrogenase (L-LDH from rabbit muscles and D-LDH from *Lactobacillus leichmannii*, Boehringer-Mannheim) and nicotinamide-adenine-dinucleotide (NAD, Boehringer-Mannheim) as a coenzyme in a buffered medium (aqueous solution of 0.16M tris-(hydroxymethyl)-aminomethane/1N HCl pH=9/hydrazine : [80:15:5] v/v/v).

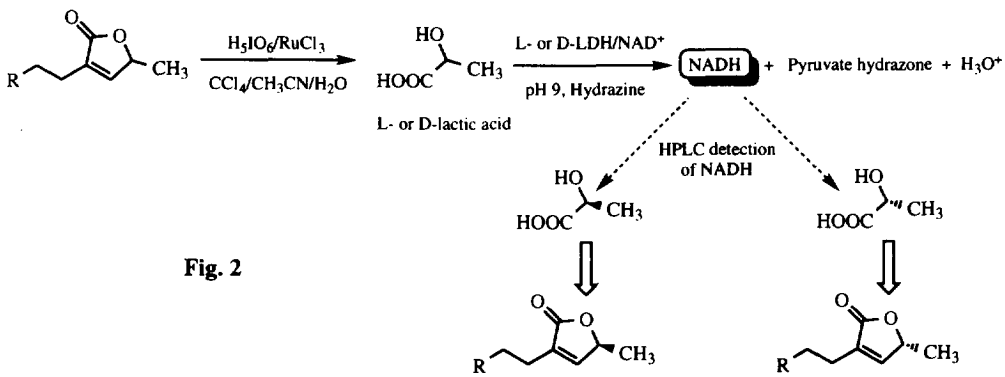

Fig. 2

The absolute configuration of lactic acid was then determined in a third step which required chromatographic means.[12] Lactic acid was oxidized by lactate dehydrogenase leading to pyruvic acid which was converted *in situ* to its hydrazone derivative, and coenzyme NADH.

NADH was then detected by HPLC against blanks.[13] Stereospecific formation of NADH in the L- or D-LDH incubation medium was thus directly correlated to the presence of either L- or D-lactic acid in the degradative products. Therefore the absolute configuration of the stereogenic center of the γ-lactone of the parent molecule can easily be deduced.

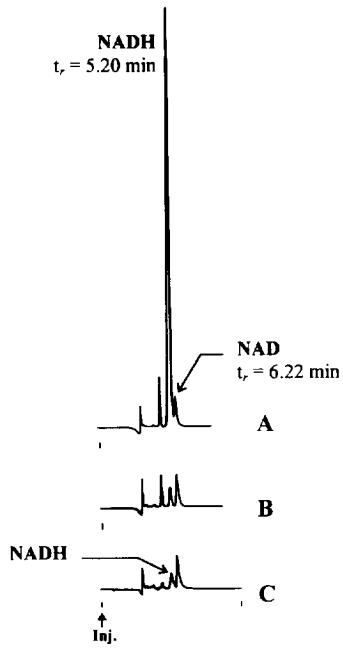

Fig. 3

HPLC detection of NADH in the LDH incubation media of degradative product of squamocine **4**.
A : L-LDH incubation medium.
B : D-LDH incubation medium.
C : L-LDH blank.

Table.

Compounds	HPLC detection of NADH (Δ%)[a]		Configuration of the γ-methyl-γ-lactone
	L-LDH medium	D-LDH medium	
L(+)-lactic acid	+	-	*S*
D(-)-lactic acid	-	+	*R*
Butenolide **1**	+	-	"36"*S*
Butenolide **2**	-	+	"36"*R*
Squamocin **4**	+	-	36*S*
Motrilin **5**	+	-	36*S*
Neoannonin **6**	+	-	34*S*
Desacetyluvaricin **7**	+	-	36*S*
Isodesacetyluvaricin **8**	+	-	36*S*
Almunequin **9**	+	-	36*S*
Rolliniastatin-1 **10**	+	-	36*S*
Rolliniastatin-2 **11**	+	-	36*S*

[a]All experiments showed a Δ% (HPLC "L(D)-LDH medium" area/ HPLC "D(L)-LDH medium" area) up to 98% = (+) (**2**), whereas when Δ% < 2% = (-).

Standards of L- and D-lactic acid have been first tested to study cross reactions between L- or D-lactic acid and D- or L-LDH verifying the stereospecificity of these enzymatic systems. We applied next this procedure to the case of two synthetic model butenolides **1-2** of known opposite configurations[14] and to eight acetogenins (Figure 1) belonging to subtype-1a (squamocin **4**, motrilin **5**, neoannonin **6**, desacetyluvaricin **7**, isodesacetyluvaricin **8**, almunequin **9**) and -1b (rolliniastatin-1 **10** and rollinastatin-2 **11**). Results are compiled in the table, showing that all the acetogenins examined in the present study have the same C-34 (36) *S* absolute configuration. In conclusion, this procedure is well adapted for the absolute configuration determination of these natural products, since it requires only little material (10^{-6} moles). It is very sensitive, and can be generalized to any α,β-unsaturated γ-methyl-γ-lactone as in ancepsenolides.[15]

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11. Procedure : Periodic acid H_5IO_6 (15 eq.) and a catalytic amount of $RuCl_3$ were added to a biphasic solution of acetogenin (1-5 mg, 1.6-8 µmol) in ternary mixture $CCl_4/CH_3CN/H_2O$ [57:57:86] v/v/v. The mixture was stirred two hours at 70°C before being filtered and concentrated. Water (0.5-1 ml) was then added to the residue and the solution was extracted with 3x0.5 ml AcOEt. The organic phases were evaporated and the residue was solubilized in (100-500 µl) buffered solution of 0.1 M TRIS/1N HCl pH=9/hydrazine [80:15:5] v/v/v. 20-50 µl of this alkaline solution were separately uncubated in turn with D- or L-LDH (10 µl) and 20-50 µl of 1% aqueous solution of NAD. Enzymatic preparations were incubated during 20 min at 40°C before HPLC analyses.
12. HPLC experiments : Column (Sup-Rs Spherisorb S5ODS2, 4.6 x 250 mm, Prolabo, France); mobile phase : aqueous solution of 0.2M TRIS/0.1N HCl pH=8/0.2M aqueous solution of 0.425 mM EDTA/MeOH [23.75:26.5:44.75:5] v/v/v/v; flow rate : 1 ml/min; specific UV detection of NADH at 340 nm (sensitivity could be increased using fluorimetric detection with 260 and 460 nm as excitation and emission wavelenghts respectively); sample volume injected : 20-50 µl.
13. Perspiration of the hands containing large amounts of L(+)-lactic acid, care must be taken to ensure that any parts that come in contact with the test solutions, *e.g.* pipette tips, are not touched with the fingers. Blank reactions were determined for the enzymatic preparations and compared with experimental results.
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