

PYROLYTIC CONVERSION OF GUMMIFERIN INTO ATRACTYLOSIDE. CHEMICAL AND BIOLOGICAL EVIDENCE

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1. Introduction

The thistle *Atractylis gummifera* L. contains two toxic glucosides which can crystallize as potassium salts. The first one, named by Lefranc [1] atractylic acid, is now currently called atractyloside. The second one, isolated for the first time in 1964 and called gummiferin [2] has been identified with 4-carboxy-atractyloside [3] (fig. 1); the presence of such a compound in extracts of *Atractylis gummifera* L. has been independently established by another group [4].

While atractyloside (ATR) and gummiferin (GUM) are both specific inhibitors of adenine nucleotide translocation in mitochondria, striking differences in their inhibitory effects are encountered: competitiveness (ATR) and non competitiveness (GUM) with ADP, saturation of sites and strong homotropic interaction exclusively in the case of GUM binding [5]. A single difference in the genin structure (a supplementary carboxyl group in GUM) is responsible for these different properties, substantiating our earlier conclusion

[6] that the atractyligenin moiety of ATR was responsible for the inhibition of ADP translocation and could play a role of allosteric effector.

In this note, the biological effects of decarboxy-GUM are compared with those of authentic ATR and GUM. Two possible isomeric decarboxylated compounds could be expected: one with the carboxyl axial like the methyl on C₁₀ (fig. 2a), the other one with the carboxyl equatorial (fig. 2b). The two corresponding aglycones have been described and called by Piozzi et al. [7] atractyligenin and 4-epi-atractyligenin, respectively. The chemical and biological tests presented below show that ATR and decarboxy-GUM are identical. This finding confirms the structure I for GUM. Work is in progress to isolate the C₄-stereoisomer of ATR; this compound should be a new supplementary tool to analyze the topography of the binding site in the inner mitochondrial membrane.

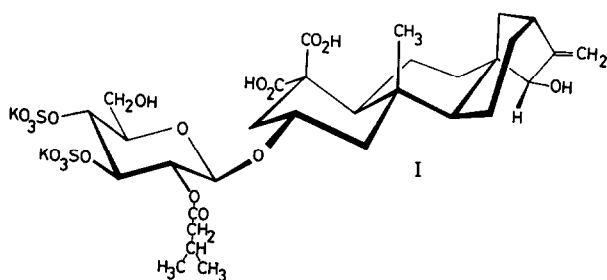


Fig. 1. Structure of gummiferin.

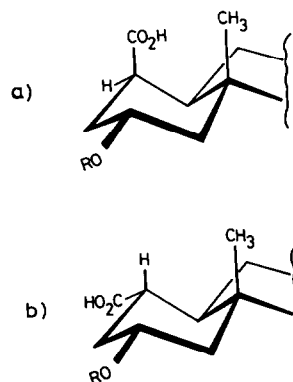


Fig. 2. a) Configuration found in atractyligenin. b) Configuration in 4-epi-atractyligenin [7].

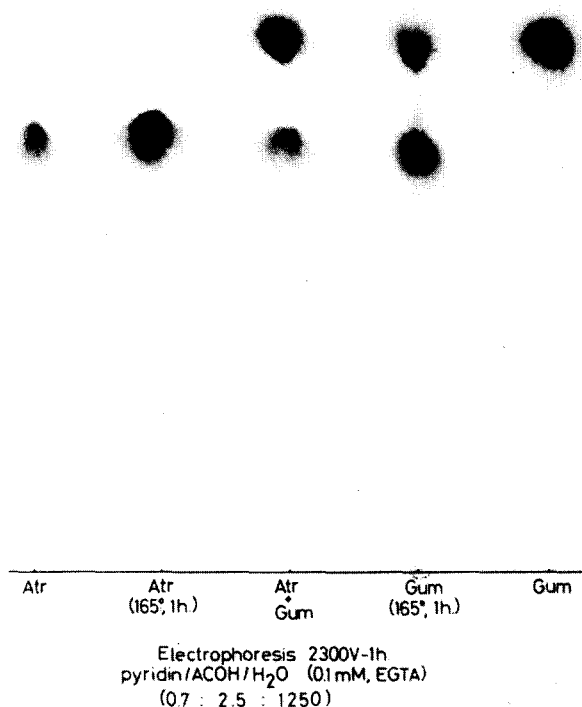


Fig. 3. Electrophoretic migration of [^{35}S]ATR and [^{35}S]GUM before and after pyrolysis. Autoradiogram.

2. Criteria of identification of gummiferin structure

The experimental pieces of evidence are the following:

1) GUM is slightly more polar and negatively charged than ATR, these properties allowing for the separation of the two compounds by chromatography [2, 5] and electrophoresis (fig. 3).

2) Since the acetylated and silylated derivatives of desulfated GUM and ATR give the same mass spectra [5], the different migrating behaviour could be explained by isomerism or by the presence of an additional, labile, weakly acidic group.

3) The tritylation of the primary alcohol gave two distinct tritylated derivatives for ATR and GUM, respectively [3], indicating that the C_6' alcohol on glucose was free in both compounds.

4) From the 100 MHz spectra of the methyl ester derivatives in both desulfated ATR and desulfated GUM, the genin moiety is linked by a β -osidic linkage [8] (a result at variance with the conclusion of Poizzi et al. [9] based on a lack of hydrolysis by a β -glycosidase).

5) The NMR spectra of the methyl esters of ATR and GUM indicated the presence of two carboxylic groups in GUM and only one in ATR [3, 10].

6) Consideration of diterpene biosynthetic pathways led us to postulate [3] a location at C_4 of the atractylenin for the supplementary carboxyl group in GUM, producing a malonic structure in that part of the molecule.

7) Heating [^{35}S]GUM powder at 165° , a treatment able to produce the decarboxylation of malonic acid, gave a compound migrating like authentic ATR by chromatography and electrophoresis (fig. 3) confirming the position of the extra carboxyl group at the C_4 position of the atractylenin moiety. Heating at 165° does not significantly destroy [^{35}S]GUM but progressively transforms it into [^{35}S]ATR.

3. Criteria of identification of decarboxy-GUM with ATR

3.1. Chemical evidence

After heating [^{35}S]GUM powder for 1 hr at 165° , two radioactive spots could be resolved by high voltage electrophoresis and detected by autoradiography (fig. 3), the faster one migrating like the original [^{35}S]GUM sample and the second moving like authentic [^{35}S]ATR. The two spots were eluted and derivatives of either the glucosides or of their corresponding aglycones [9] were prepared and analyzed either by nuclear magnetic resonance spectrometry (NMR) or gas liquid chromatography (GLC).

3.1.1. NMR analysis of the glucoside derivatives

The glucosides were desulfated, their hydroxyl groups acetylated and their carboxylic acids esterified by diazomethane. The NMR spectrum corresponding to the slow moving spot (fig. 3) taken at 100 MHz in CDCl_3 , was found identical to the NMR spectrum of the corresponding ATR-derivative.

Table 1
Chemical shift (δ) of tertiary methyl group.

	Chemical shift (δ) (ppm)
Atractyligenin Me ester	0.92
4-Epi-atractyligenin Me ester	1.0
Genin from decarboxylated-GUM Me ester	0.92

Chemical shift of the tertiary methyl of genin methyl esters obtained at 100 MHz in CDCl_3 with a Varian HA-100 spectrometer.

Table 2
Retention time of the silylated aglycones analyzed by GLC.

Compound	Relative retention time (MU)*	
	OV 1	OV 17
Atractyligenin	27.00	28.56
4-Epi-atractyligenin	26.39	28.29
Genin from decarboxylated-GUM	27.00	28.56

* MU = methylene unit

The genins were silylated with bis (trimethylsilyl) trifluoroacetamide (BSTFA) in pyridine. Retention time values are referred, as methylene units, to the retention time of saturated hydrocarbons (C_{24} – C_{30}) [11]. Conditions: separation in an Intersmat DFL 15 model gas chromatograph, 6 feet column packed with 1% OV 1 (methylsiloxane polymer) (or OV 17, phenylsiloxane polymer) on Gaschrom Q; temperature programmed at $2^\circ/\text{min}$ from 205 to 290° .

3.1.2. The NMR analysis of the methyl esters of the aglycones indicated that the genin of decarboxy- ^{35}S GUM behaved like atractyligenin and not like 4-epi-atractyligenin (table 1).

3.1.3. GLC analysis was performed on the silylated derivatives of the aglycones. Table 2 shows that the genin of decarboxy-GUM has the same retention time as that of atractyligenin and is different from that of 4-epi-atractyligenin.

3.2. Biological evidence

The two compounds contained in the ^{35}S GUM sample which had been submitted to pyrolysis, isolated as shown in fig. 3, located by autoradiography then eluted, were tested in parallel assays, in order to check

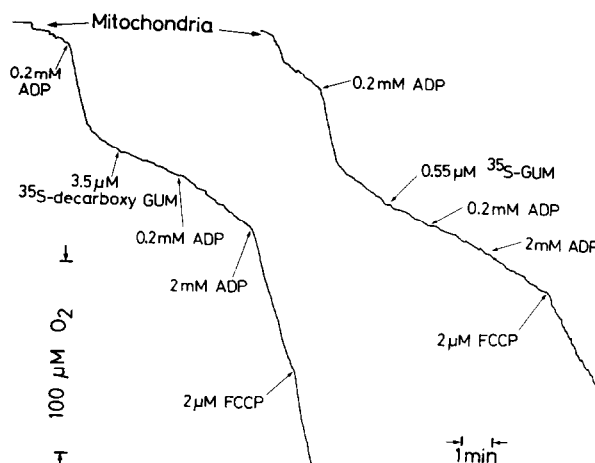


Fig. 4. Effect of ^{35}S -GUM and decarboxylated- ^{35}S -GUM on the ADP-stimulated respiration of rat liver mitochondria. The reaction medium contained 110 mM KCl, 16 mM phosphate buffer, pH 7.4, 6 mM MgCl_2 , 10 mM glutamate and 4 mg of mitochondrial protein. FCCP: carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazine. Temperature 25° .

whether the fast moving spot was really unaltered ^{35}S GUM and whether the slow moving one behaved like ^{35}S ATR or iso- ^{35}S ATR.

3.2.1. Oxygraphic assays had shown that both GUM and ATR inhibit the stimulation by ADP of mitochondrial respiration. This inhibition is reversed by excess ADP in the case of ATR, but not in the case of GUM [2, 5]. Fig. 4 shows that the inhibition by decarboxylated- ^{35}S GUM is reversed by excess ADP and that the compound which has resisted pyrolytic decomposition behaves like GUM.

3.2.2. The binding properties of the two compounds (^{35}S GUM and decarboxy- ^{35}S GUM) to mitochondria and the sensitivity of that binding to externally added ADP are illustrated in fig. 5. While binding of ^{35}S GUM was not altered by addition of 0.2 mM ADP, the affinity of decarboxy- ^{35}S GUM for mitochondria became 40 times smaller in the presence of 0.2 mM ADP (K_d increased from $0.03 \mu\text{M}$ up to $1.2 \mu\text{M}$). The same lowering of affinity upon ADP addition has been observed for ^{35}S ATR binding [12].

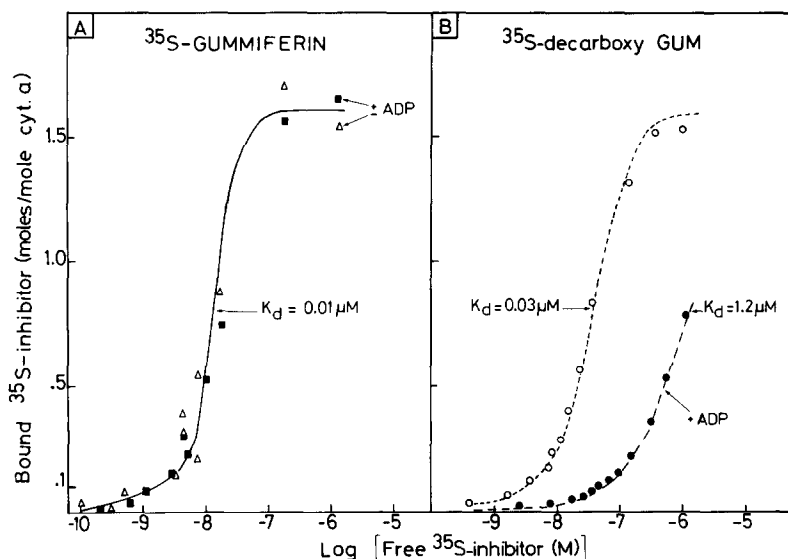


Fig. 5. Binding of [^{35}S]GUM and decarboxy- ^{35}S]GUM to mitochondria. Effect of ADP. Rat liver mitochondria (9.4 mg protein) were incubated for 45 min at 2° with different concentrations of ^{35}S -inhibitors in series of tubes containing 10 ml of 110 mM KCl, 6 mM MgCl_2 , 10 mM Tris-sulfate, pH 7.2, and where indicated 200 μM ADP. The incubation was ended by centrifugation. The mitochondrial pellets were dissolved in 1 ml of formamide at 180° and their radioactivity was determined by liquid scintillation.

These two sets of data shown in fig. 4 and fig. 5 correlated with one another. They indicate that, like ATR, decarboxy- ^{35}S]GUM is a competitive inhibitor of adenine nucleotide transport in mitochondria.

Both from chemical studies (NMR and GLC analysis of atractyligenin, 4-epi-atractyligenin and decarboxylated-gummiferin genin) and from biological studies (competitive inhibition of ADP-stimulated respiration, binding properties) it is concluded that decarboxylated- ^{35}S]GUM obtained by pyrolysis of ^{35}S]GUM is identical with ATR.

Acknowledgements

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