ISOLATION OF A NEW ATRACTYLOSIDE TYPE COMPOUND

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1. Introduction

Recently a homologue of atractyloside (ATR) was identified by Danieli et al. [1] in extracts from *Atractylis gummifera*. The structure of this compound was established as 4-carboxy-atractyloside (CAT) (fig. 1,I). CAT can be assumed to be the native compound of the plant as it can be decarboxylated to ATR by heating [1]. Subsequently it was shown by Defaye et al. [2] that the compound "gummiferin" also isolated from this plant and defined as an inhibitor of adenine nucleotide transport in mitochondria, could also be converted to ATR. They were thus able to confirm the identity of gummiferin with CAT as first suggested by Luciani et al. [3].

In principle the decarboxylation of CAT should result in the formation of stereoisomers II and III (fig. 1,II and III).

Piozzi et al. [4] pointed already out, using NMR-data that the aglycone atractyligenin is probably identical with isomer II where the carboxyl group is axial to the ring. Isomer II of the aglycone could be converted to isomer III with the carboxyl group in the equatorial position by drastic conditions. The converted form was called epi-atractyligenin. The existence of epi-atractyloside was not yet reported.

In our laboratory since some time during the production of ^{35}S-labelled CAT and ATR, the occurrence of a third ^{35}S-labelled compound with ATR-like properties was noted during the isolation of the ^{35}S-labelled inhibitors from extracts derived out of *Atractylis gummifera* grown on ^{35}S-sulfate. The present report describes the isolation of this third ATR-like compound which was called by us ATR′ and which we believe is identical with epi-ATR and its properties as an inhibitor of adenine nucleotide exchange.

Abbreviations:
ATR, atractyloside; CAT, carboxy-atractyloside.

2. Experimental

2.1. *Isolation of ATR-type compounds*

Plants of *Atractylis gummifera* grown from seeds were brought at an age of 2–20 weeks into a liquid culture supplied with carrier-free ^{35}S-sulfate. The plants are harvested after two weeks exposure to ^{35}S-

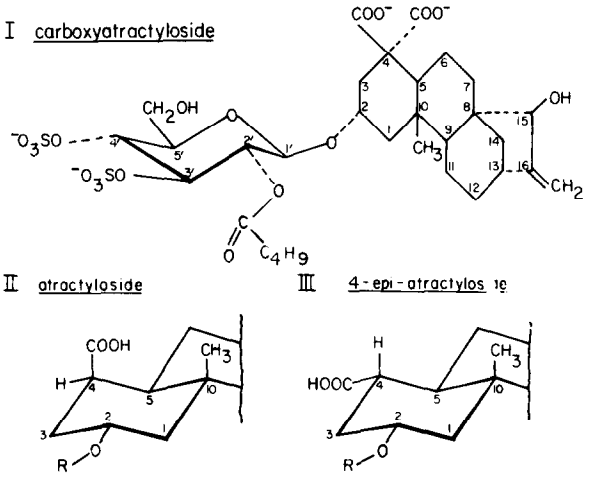

Fig. 1. I) Structure of carboxy-atractyloside. II) Configuration of carboxyl group in atractyloside. III) Configuration of carboxyl group in 4-epi-atractyloside.

sulfate. The roots are homogenized in 98% ethanol and the homogenate extracted up to 3 hr at temperatures ranging from 4 to 92° in different preparations. Then the homogenate was filtered and the filtrate evaporated at room temp.

For the separation and purification of the ^{35}S-labelled compounds the extract was first chromatographed on thin-layer using silica-gel and as elution system, $CHCl_3$:MeOH:HAC:H_2O, 55:25:8:4.

The thin-layer plates were scanned in two dimensions with an automatic thin-layer radioactivity scanner. Strips were scratched from the plate at radioactive peaks with appropriate R_f's corresponding to the various atractyloside type compounds. The powder was extracted with 50% ethanol.

The extract was evaporated and purified on a DEAE-cellulose column by elution with a gradient of triethylamin hydrogen carbonate up to 0.4 M. The radioactivity appeared in the middle range and about 30% of the fractions (out of 100 fractions) were pooled and lyophilized.

For the third step of purification the extract was brought on cellulose plates with the elution system of n-butanol, acetic acid, H_2O at a ratio 4:1:2. Again after scanning, the peaks were extracted and as a fourth purification step, another thin-layer chromatography with silica-gel was applied.

After these procedures the purity was about 98–99% for the three ATR type compounds according to appropriate binding studies on mitochondrial membranes. The specific activity of ATR′ ranged in various preparations from 7×10^4 to 10×10^4 dpm/nmole. The specific activity was taken to be the same as that of isolated ATR and CAT from the same preparations or was determined by titrating the adenine nucleotide exchange under comparison with authentic ATR or CAT.

The identity of two of the purified compounds to commercial ATR and CAT was assessed by thin-layer chromatography in the two systems using silica-gel and cellulose plates as shown in fig. 2. The purity of all the compounds is demonstrated by the symmetrical peaks showing no satellite bands.

[^{35}S]ATR runs close to ATR on silica-gel with a somewhat higher R_f. On cellulose the separation of ATR′ from ATR is favored (fig. 2b). This is unexpected in view of the assumption that ATR and ATR′ are stereo-isomers. However, a discrimination according

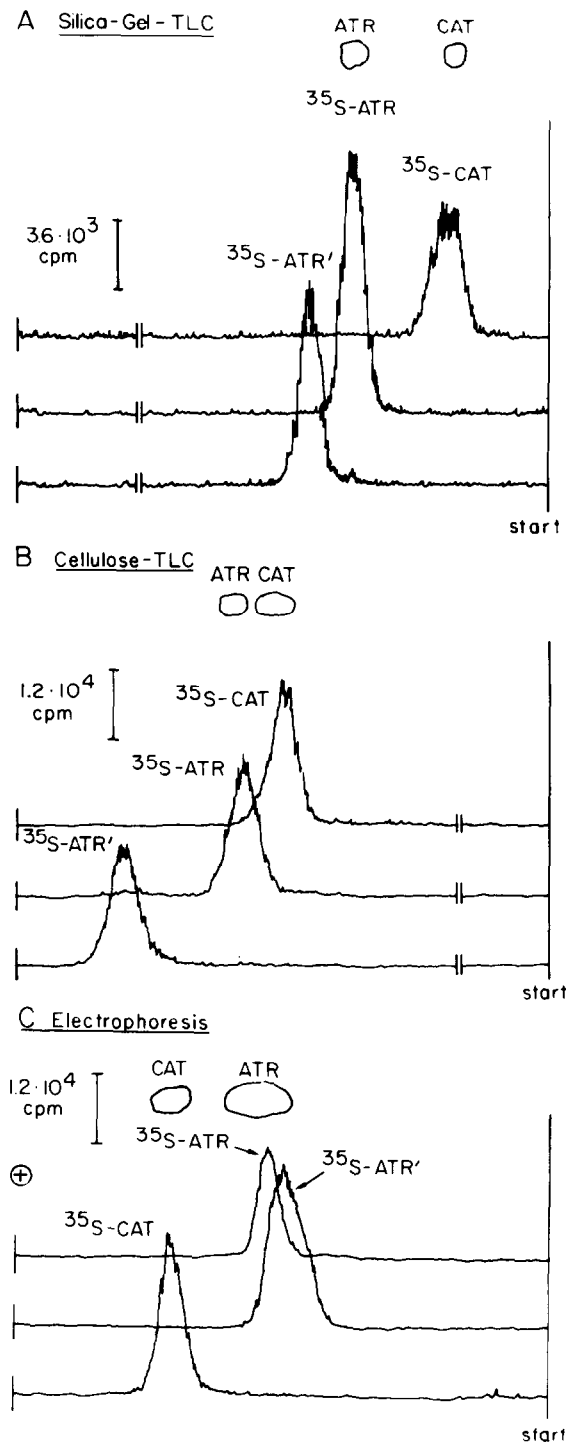

Fig. 2. Separation and identification procedure by thin-layer chromatography and electrophoresis.

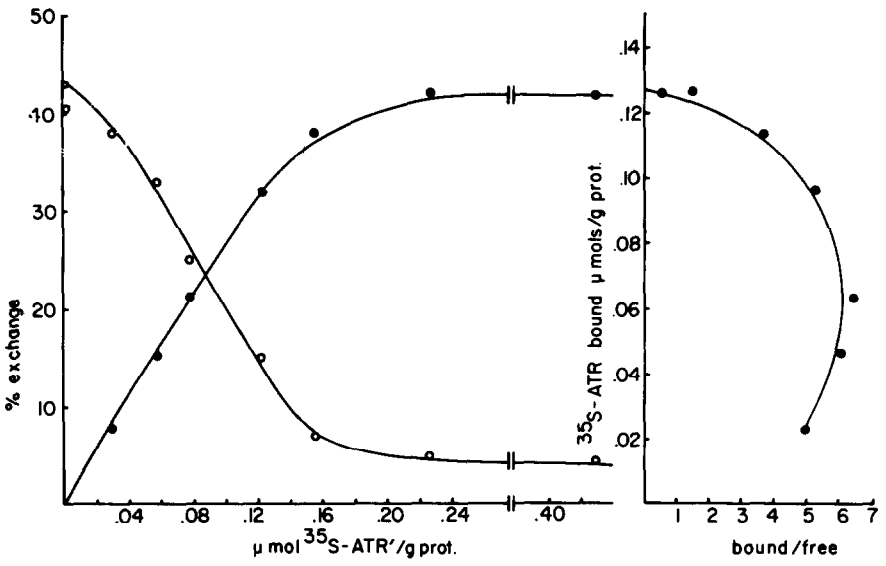

Fig. 3. Inhibition of [^{3}H]ADP exchange by [^{35}S]ATR' in rat liver mitochondria (RLM) and correlation to [^{35}S]ATR' binding. Rat liver mitochondria loaded with [^{3}H]ADP by preincubation for 30 min and washing according to [5]. 0.11 mg protein/0.5 ml was incubated in 0.25 M sucrose, 10 mM TRAP, 1 mM EDTA, pH 7.2 at 10°. [^{35}S]ATR' was added in increasing concentrations. After 1 min 200 μM ADP were added and the [^{3}H]ADP exchange was stopped by addition of 20 μM CAT after 10 sec. After centrifugation sediments were extracted for [^{35}S]ATR' content with 2% Lubrol.

to the anionic charge should be more critical by electrophoresis. Cellulose thin-layer electrophoresis: 800 V, 90 min, 3.5 mA; electrode buffer: pyridine, acetic acid, 0.1 mM EDTA; 0.35:0.125:625. Spots were made visible by spraying the plates with 0.5% vanillin (w/v) in 50% phosphoric acid.

On cellulose thin-layer electrophoresis (fig. 2c) ATR and ATR' migrate closely together, whereas CAT has advanced to a considerable higher extent towards the anode. This implicates that the charge differences between ATR and ATR' may be minor (or none) as compared to CAT and supports the conclusion that ATR' is identical to epi-ATR.

2.2. *Inhibition of adenine nucleotide translocation by ATR' and binding of ATR' to rat liver mitochondria*

ATR is an inhibitor of the adenine nucleotide exchange similar as ATR and CAT. This is shown in an experiment with rat liver mitochondria, titrating the inhibition with increasing amounts of ATR' (fig. 3). An approximately linear decrease of the exchange is obtained with nearly full inhibition at about 0.14 μmole ATR'/g protein. In the sediments of the samples the binding of [^{35}S]ATR' was determined and a close correlation of binding and inhibition is demonstrated. When nearly full inhibition is reached, the binding is also saturated. In a mass action plot the binding gives a strongly nonlinear curve similar as with CAT [6] which does not permit evaluation of the K_d. It can be concluded that the new ^{35}S-labelled compound ATR' is an ATR-type compound since it inhibits the adenine nucleotide translocation and also binds to the membrane with about the same titer as [^{35}S]ATR or [^{35}S]CAT [7].

3. Conclusions

There are now three ATP-type compounds which can be isolated from the roots of *Atractylis gummifera* and which all three inhibit the adenine nucleotide exchange in mitochondria. The assumption that ATR' is identical with epi-ATR is based mainly on the chromatographic and electrophoretic behaviour. Both on silica-gel chromatography and electrophoresis ATR' runs close to ATR. The existence of a third ATR-type

compound appears logical assuming that it is epi-ATR since in principle by decarboxylation of CAT two isomers may be produced according to whether the axial or equatorial CO_2H-group is removed. Therefore, the family of ATR-type homologues based on the CO_2H-group in 4-position should be complete now. It is however not known whether ATR and ATR′ (= epi-ATR) are in fact derived from CAT in the plant by decarboxylation.

The question may also arise whether ATR and epi-ATR are derived from CAT by the isolation or extraction procedure. It appears well possible that both ATR and epi-ATR occur already in the root since both are obtained on extraction at low temperature. It is possible to convert CAT to ATR by heating [1, 2], but no significant amounts of epi-ATR are formed (unpublished observation). This is explained by Danieli et al. [1] in a mechanism according to which the formation of ATR is kinetically favored. The formation of epi-ATR in the plant may be the result of enzymatic decarboxylation of CAT or an isomerization of ATR to epi-ATR.

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