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Synthesis of all-*trans* arachidonic acid and its effect on rabbit platelet aggregation

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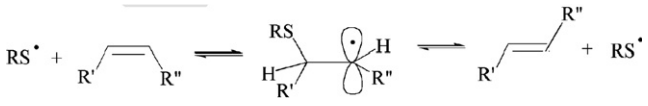
Abstract—A simple and high-yielding method to convert natural all-*cis* PUFA derivatives to the corresponding all-trans geometrical isomers is described. The method is based on the thiyl radical-catalyzed cis–trans isomerization. The all-trans isomer of arachidonic acid was found to cause rabbit platelet aggregation at concentrations higher than 0.1 mM and inhibition of PAF-induced platelet aggregation in a concentration dependent manner with an IC_{50} in the micromolar range.

1. Introduction

Lipids are central to the cell membrane composition due to their amphiphilic nature, and to regulation and control of cellular functions through their signaling activity.[1,2] There is currently much research interest in lipid signaling cascades, which mediate diverse physiological and pathological responses. In this context, polyunsaturated fatty acid (PUFA) structures with methylene-interrupted double bonds in the cis configuration are known to be crucial for enzyme binding.[3] In recent years, the role of the naturally occurring *cis* lipid geometry has attracted attention, in particular for the cis–trans isomerization process catalyzed by free radical species.[4]

Scheme 1 shows the isomerization mechanism catalyzed by thiyl radicals attacking an isolated double bond. The fact that the conversion of a cis to the more stable trans configuration is very efficient also under biomimetic conditions, has given a multidisciplinary meaning to the formation of *trans* lipids.[4–6]



Scheme 1. Mechanism of cis–trans isomerization catalyzed by thiyl radicals.

It is worth recalling that *trans* lipids are studied as dietary components, which can exert harmful effects on human health.[7] In this view, *trans* lipid metabolism and biological activity have been examined and the incorporation of these isomers in different lipid structures, including membrane phospholipids, is well documented.[8,9] The total trans content in a sample of PUFA can be easily determined from its infrared spectrum, because the unconjugated trans double bonds show a specific band.[10] On the other hand, the access to each trans isomer is a difficult task. Eicosenoates that have a 20 carbon atom chain with a high number of unsaturations (up to five double bonds) are one of the most important classes of lipid mediators, and each isomer can have a biological activity. In fact, two isomers of arachidonic acid (AA), namely the mono-trans isomers in positions 5 and 14 prepared by total synthesis, were tested in lipid enzymatic cascades.[11,12]

The 14-trans isomer decreased platelet aggregation and inhibited prostaglandin biosynthesis,[13,14] whereas the 5-trans isomer did not cause inhibition and could be transformed to 5-*trans*-PGE_2.[11] Each of the four possible mono-trans isomers of arachidonic acid has

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been recently prepared by a multistep stereospecific protocol, and their interaction with enzymes was demonstrated.[14,15]

Based on our studies of thiyl radical-catalyzed isomerization process with formation of mono- and polyunsaturated trans fatty acid derivatives,[4–6] we thought that the synthesis of all-*trans* PUFA could be of interest, since a single geometrical isomer is produced, and is expected to have pharmacological applications. From the known reaction profile of arachidonic acid isomerization,[6] it is derived that several cis/trans isomeric compositions can be obtained at different reaction times and a straightforward synthesis of all-trans isomers could be envisaged, by an exhaustive isomerization process.

In this letter, we on report the preparation of all-trans isomer of arachidonic acid (*t*-AA) and its corresponding methyl ester (*t*-AAME) and the results of biological activity assays for rabbit platelet aggregation (Scheme 2).

2. Synthesis of *t*-AA and *t*-AAME

Among the possible isomerization protocols, the radical-catalyzed methodology was found to convert specifically the cis double bonds into their geometrical trans counterparts.[16] A 2.2 mL solution of 15 mM AA in *i*-PrOH was placed in a quartz photochemical reactor and bubbled with nitrogen for 20 min. The thiol (2-mercaptoethanol) in a 50% mole equivalent with respect to AA was added and the mixture was exposed to low-pressure mercury lamp (5.5 W) at 25 °C. A TLC monitoring of the reaction under appropriate conditions, showed that *t*-AA was formed as the major product in 40 min, by a step-by-step conversion of the mono-, di-, and tri-trans isomers progressively formed in the reaction mixture.[6b,17] After workup and purification, *t*-AA was obtained in an 85% yield (purity >97%). The acid was also converted to the corresponding methyl ester (*t*-AAME) by diazomethane for GC/MS analysis. Similarly, arachidonic acid methyl ester (AAME) was transformed to all-trans isomer (*t*-AAME). Figure 1 shows the different retention times of AAME (run A) and *t*-AAME (run B) in GC, the reference being methyl stearate (peak 1).

NMR analysis provides significant information on the differences between the two isomeric structures. Indeed,

Scheme 2. Structure and abbreviation of arachidonic acid (AA) and its all-trans isomer (*t*-AA).

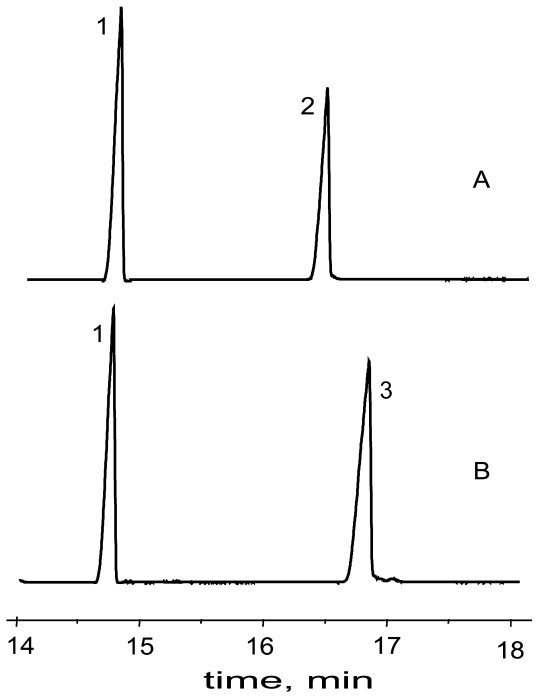

Figure 1. GC runs: (A) before and (B) after isomerization. Peak labels: (1) methyl stearate (18:0) as reference; (2) AAME; (3) *t*-AAME.

it is known that NMR spectra of cis and trans isomers have significant differences in the ethylenic carbon atom resonances, and bisallylic and allylic hydrogen chemical shifts.[19,20]

In Figure 2, the comparison of ^{13}C NMR regions between AAME (spectrum A) and *t*-AAME (spectrum B) is given and all ethylenic carbon atom resonances of the trans isomer appeared at lower fields than the corresponding cis isomer.[19] In Figure 3, an enlargement of

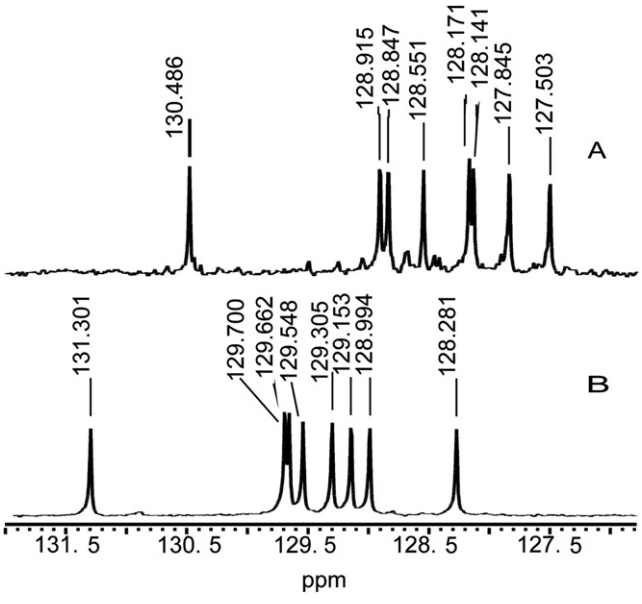

Figure 2. Ethylenic carbon atom region of the ^{13}C NMR spectra of AAME (A) and *t*-AAME (B).

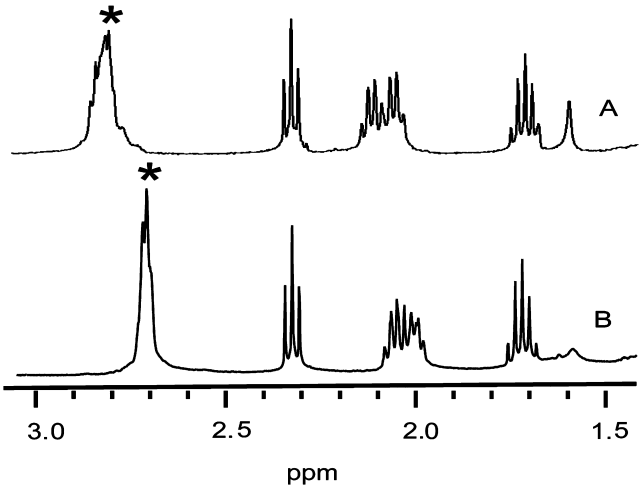

Figure 3. ^{1}H NMR spectra of AAME (A) and *t*-AAME (B) in the region 1.5–3.0 ppm. Bisallylic hydrogens are indicated with an asterisk.

^{1}H NMR spectrum in the region of 1.5–3.0 ppm of AAME (A) and *t*-AAME (B) is shown, and the difference for the two isomers is considerable, being the *trans* bisallylic hydrogen signal at higher fields, as expected.[20]

From a mechanistic point of view, thiyl radicals were generated by means of direct photolysis of 2-mercaptoethanol in deoxygenated *i*-PrOH solutions.[6a] In this condition, the generation of thiyl radicals and H atoms occurred as shown in Eq. 1, and the H atoms were efficiently quenched by the solvent. The $(CH_3)_2C(^{\bullet})OH$ radical in turn reacted with the thiol to give thiyl radicals (Eq. 3). It is worth underlining that reaction (3) is reversible with an equilibrium constant of $K = 1 \times 10^4$.

$$HOCH_2CH_2SH \xrightarrow{h\nu} HOCH_2CH_2S^{\bullet} + H^{\bullet} \quad (1)$$

$$H^{\bullet} + (CH_3)_2CHOH \rightarrow H_2 + (CH_3)_2C(^{\bullet})OH \quad (2)$$

$$(CH_3)_2C(^{\bullet})OH + HOCH_2CH_2SH \rightleftharpoons (CH_3)_2CHOH + HOCH_2CH_2S^{\bullet} \quad (3)$$

Under our experimental conditions, that is, [*i*-PrOH] = 13.06 M and $[HOCH_2CH_2SH] = 0.007$ M, the equilibrium is still shifted to the right but the forward reaction is only 5–6 times faster than the reverse reaction.[6a]

3. Biological activity of *t*-AA and *t*-AAME

The physiological role of AA in the aggregation cascade is well known and some of its mono-trans isomers were found to be inhibitors of this process.[21,22] Although mono-*trans* arachidonic acid isomers have been detected in human plasma,[23] there is no evidence and it is highly improbable that all-trans isomer could be generated in the blood. We tested the effect of *t*-AA and *t*-AAME on rabbit platelet aggregation. We also focused on PAF-induced platelet aggregation since *cis* fatty acids, but not saturated and *trans* fatty acids, were reported

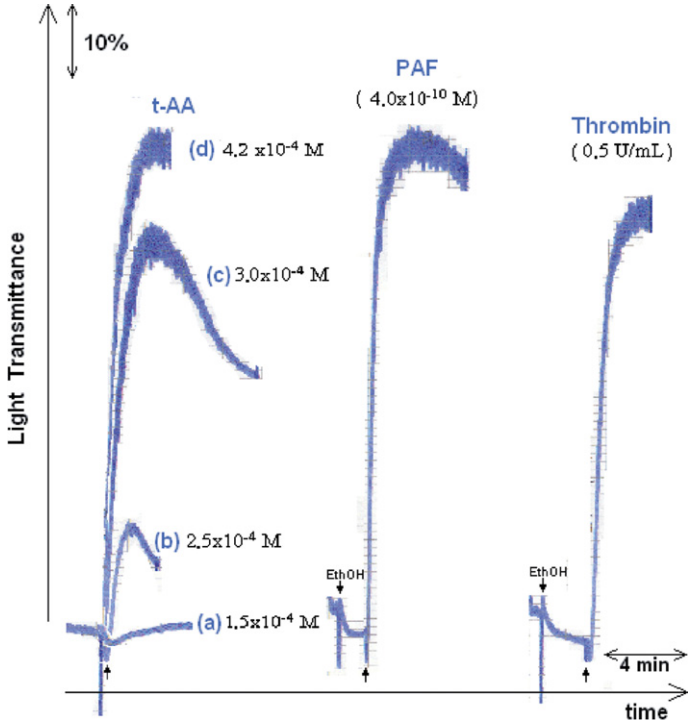

Figure 4. Aggregation effect of *t*-AA on rabbit platelets. *t*-AA (in ethanol) at concentrations indicated, was added to cuvettes containing 0.5 mL of prewarmed (37 °C) platelet suspension (2.5×10^8 cells/mL). Control aggregation curves with PAF and thrombin were obtained after preincubation with ethanol.

as inhibitors of this path.[24] *t*-AA was added at several concentrations to the platelet suspension (2.5×10^8 cells/mL) and the results are shown in Figure 4. Control aggregation curves with platelet aggregation factor PAF (4.0×10^{-10} M) and thrombin (0.5 U/mL) were also obtained after preincubation with ethanol for 1 min. *t*-AA induced rabbit platelet aggregation in a concentration higher than 2.5×10^{-4} M. At high, non-physiological concentrations, cell viability was always checked without detecting any toxicity or lysis of platelets. In Figure 4, runs b, c, and d correspond to 18%, 63%, and 78% aggregation, respectively. At concentration of 1.5×10^{-4} M (run a), it could be also observed that *t*-AA induces a shape change on rabbit platelets. Aggregation was not 100% reversible even at concentrations that induce a small aggregation (e.g., 2.5×10^{-4} M). When thrombin (0.5 U/mL) was added after desaggregation, platelets were activated again and aggregated irreversibly, suggesting that the high concentrations of *t*-AA were not toxic. For comparison, under the same experimental conditions, 4.0×10^{-10} M PAF and 0.5 U/mL thrombin induced 78% and 67% platelet aggregation, respectively, whereas AA induced platelet aggregation at a 10 times lower concentration (not shown). *t*-AA activity was also assayed on the PAF-induced aggregation of rabbit platelets and Table 1 shows the results of different concentrations. *t*-AA caused inhibition of a 4×10^{-10} M PAF-induced aggregation in a concentration dependent manner, with $IC_{50} \sim 6 \times 10^{-5}$ M. *t*-AA at 2.1×10^{-4} M concentration caused 100% inhibition of PAF 8×10^{-10} M.

It should be recalled that AA at micromolar concentrations caused platelet aggregation. CP/CPK caused inhibition (100%) of the aggregation induced by *t*-AA (2.8×10^{-4} M), suggesting the involvement of ADP release. This hypothesis needs further investigation.

The effect of *t*-AAME on rabbit platelet aggregation was not found to be significant. In fact, it did not cause rabbit platelet aggregation at concentration between 0.2×10^{-4} M and 6.0×10^{-4} M indicating that a free form of acid is necessary for the aggregation effect.

Furthermore, it did not cause inhibition of PAF–induced aggregation at any of the concentrations tested in the range 2.0–4.0×10^{-4} M. It should be also added that the respective saturated fatty acid (arachidic acid) was found to be inactive under the same experimental conditions (not shown).

Table 1. Effect of *t*-AA on rabbit platelet aggregation induced by 4×10^{-10} M PAF

t-AA M	Inhibition %
1.5×10^{-6}	0
1.5×10^{-5}	6
3.0×10^{-5}	24 (±5)
7.5×10^{-5}	61 (±8)
2.1×10^{-4}	100

Values are means of three experiments. Standard deviation is given in parentheses. Aggregation given by 4×10^{-10} M PAF is considered 100%.

In conclusion, we have described a straightforward and simple access to all-*trans* PUFA molecules and in particular, *t*-AA was prepared and tested for rabbit platelet aggregation showing to be active only at high concentrations (>10^{-4} M). More interestingly, at micromolar concentrations it caused inhibition of aggregation induced by the strong platelet agonist PAF, which was not predictable from the known behavior of other *trans*-monounsaturated fatty acids, and apparently it could be due to the unique geometry of the polyunsaturated molecule. Indeed, fatty acid structural modifications can lead to a change in the biological activity and the presence of trans double bonds is known to confer a more linear molecular shape, compared to the cis configuration, which usually forms a kink in the carbon atom chain. All-*trans* fatty acids appear to be interesting candidates for a chemical biology approach investigating the molecular mechanism of PAF-induced platelet aggregation, which is currently under study.

Acknowledgements

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