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Short communication

Thermodynamic and structural characterization of *cis–trans* isomerization of 12-(*S*)-hydroxy-(5*Z*, 8*E*,10*E*)-heptadecatrienoic acid by high-performance liquid chromatography and gaschromatography-mass spectrometry

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Abstract

It is shown that 12-(*S*)-hydroxy-(5*Z*,8*E*,10*E*)-heptadecatrienoic acid (5-*cis*-HHT)—a physiological metabolite of arachidonic acid—is acid-catalyzed converted into a less polar substance with its maximum UV-absorption at $\lambda_{max} = 232$ nm and a molar absorptivity of about $\varepsilon = 26600 \pm 200$ M^{-1} cm^{-1}. Using a reversed-phase high-performance liquid chromatographic (HPLC) method this equilibrium reaction ($K_c = 1.78 \pm 0.05$ at pH 1.10 and 298 K) could be thermodynamicly characterized as a pH dependent, exergonic and exothermic reaction according to kinetics of a first order reaction (at pH 1.10 and 298 K: $\Delta_R G° = -1.42 \pm 0.07$ kJ mol^{-1}, $\Delta_R H° = -3.50 \pm 0.9$ kJ mol^{-1}, $\Delta_R S° = -7.0 \pm 3.0$ J mol^{-1}*K, $\Delta_R H_f^{\#} = 100.0 \pm 4.0$ kJ mol^{-1}). Kinetic data for several pH-values and temperatures are presented. These data and structural characterization by gaschromatography-mass spectrometry (GC/MS) lead to the conclusion that 5-*cis*-HHT is isomerized to 12-(*S*)-hydroxy-(5*E*,8*E*,10*E*)-heptadecatrienoic acid (5-*trans*-HHT).

Keywords: 12-(*S*)-Hydroxyheptadecatrienoic acid (HHT); *Cis–trans* isomerization; High-performance liquid chromatography; Gas chromatography-mass spectrometry

1. Introduction

Arachidonic acid (AA) is an ubiquitous essential unsaturated fatty acid. AA is converted

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prostaglandin H_2 (PGH_2) by the cyclooxygenase enzyme complex (Hamberg and Samuelsson, 1974; Hamberg et al., 1974). PGH_2 is metabolized via thromboxane synthase to thromboxane A_2, malondialdehyde and 12-(*S*)-hydroxyheptadecatrienoic acid (HHT). This synthesis is stereospecific for the 5*Z*,8*E*,10*E*-HHT isomer (5-*cis*-HHT) and appears predominantely in lung microsomes (Wlodawer and Hammarström, 1978) and platelets (Yoshimoto et al., 1977). There is still little information available about the physiological role of HHT, its enzymatic metabolization or chemical derivatization in vivo (John et al., 1998). In order to clarify the physiological function of HHT, fundamental knowledge of its chemical properties and behaviour is required. The relevance of chemical transformation might be discussed with respect to the physiological function of HHT. Furthermore, these properties may be important for the determination of HHT. When analyzing HHT, this substance is often extracted under acid conditions, since analyses of prostaglandins and HHT are done after acidification of the samples. In addition HPLC and thinlayer chromatographic measurements of underivatized fatty acids are performed with acid solvents in order to protonate the carboxylic functions of the analytes (Pruimboom et al., 1992; McKinnon et al., 1993). Following these procedures HHT is also stored and treated in acid solutions for variable periods of time and at different temperatures. Therefore, this report describes the chemical behaviour of the physiological 5-*cis*-HHT especially under acid conditions in vitro. The appearance of a HHT-derivative—defined as HHT-D—could be observed in a time and pH dependent manner. The acid-catalyzed conversion of 5-*cis*-HHT into HHT-D—not discussed in the literature so far—is illustrated with respect to kinetic and thermodynamic data. The physiological potency of HHT remains to be investigated, however.

2. Materials and methods

2.1. Materials

12-(*S*)-Hydroxy-(5*Z*,8*E*,10*E*)-heptadecatrienoic acid was obtained from Cayman (Ann Arbor, MI). Acetonitrile (ultragradient grade) and water (HPLC grade) were obtained from J.T. Baker (Deventer, The Netherlands). Tri-sodium citrate 5.5 hydrate, trifluoracetic acid (TFA), ethanol and petrolether were provided by Merck (Darmstadt, Germany) and were of reagent grade. Solid-phase extraction (SPE) columns (Chromabond C18 ec, 500 mg in glass) were obtained from Macherey-Nagel (Düren, Germany).

2.2. Kinetic studies on 5-cis-HHT conversion

Aliquots of ethanolic solutions of physiological 5-*cis*-HHT (10 μg) were dried in glass tubes under a gentle stream of nitrogen. The residues were redissolved in temperature adjusted mixtures comprising of 60% v/v acetonitrile and 40% v/v 0.061 M citrate buffer. These solutions were stored at 11°C (pH 1.10), at 25°C (pH 1.10, 1.24 and 1.56) and at 38°C (pH 1.10) in waterbathes. After several periods of reaction time (0–18 h, dependent on pH and temperature) aliquots of 20 μl were chromatographed using the HPLC method described below and peak areas were integrated.

2.3. Purification of 5-cis-HHT and HHT-D

HPLC eluates corresponding to 5-*cis*-HHT and HHT-D were collected in glass tubes, diluted with water (resulting in 30% v/v acetonitrile) and extracted using solid-phase extraction on C18 ec material (John et al., 1998). SPE eluates were evaporated to dryness and used for GC/MS measurements.

2.4. Chromatographic methods

The HPLC equipment consisted of a Rheodyne 7725i syringe loading injector (Cotati, CA, USA), a pump system 322, a diode-array detector 440 (DAD) and the Kroma 2000 HPLC software from Kontron Instruments (Neufahrn, Germany).

The isocratic HPLC analyses (CH_3CN–H_2O, pH 3.5, 55:45, v/v) were carried out at 30°C on a 125 × 4.6 mm I.D. column packed with Nucleosil 120 C18 (particle size 5 μm), and a pre-column (26 × 6.0 mm I.D.) of the same material which was obtained from Bischoff (Leonberg, Germany). The temperature of the RP column was adjusted to 30°C in a Precitherm PFV water bath (Boehringer, Mannheim, Germany). The flow rate was 1.0 ml min^{-1}. HHT and HHT-D were detected at 232 nm. For GC/MS analyses purified 5-*cis*-HHT and HHT-D were converted into their bis-(trimethylsilyl)-derivatives. The GC (Varian 3400, Varian, Walnut Creek, CA, USA) equipment consisted of a cold inlet system, Gerstel 502 with a 25 m HPU two capillary column (Hewlett-Packard, Waldbronn, Germany) which terminates at the MS-EI source, 70 eV, (Finnigan MAT 8230, Finnigan, San Jose, CA). Helium was used as carrier gas. The injection temperature rose from 60 up to 310 °C and the oven temperature rose from 80 up to 300°C at 8°C min^{-1} for 24 min.

3. Results and discussion

The investigation of the conversion of physiological 5-*cis*-HHT under acid conditions requires complete dissolution of HHT in the solvent. As acid buffer solutions effect a partition equilibrium of HHT between the vial surface and the aqueous solution the addition of acetonitrile was used to avoid this phenomenon (John, H., unpublished data). In addition the sample matrices comprising of 60% v/v acetonitrile allowed direct chromatographic analyses. 5-*cis*-HHT was detected at 232 nm according to its conjugated double-bonds and its α-hydroxy-group (Fig. 3).

When analyzing the HHT-solutions by HPLC-technology after increasing periods of reaction time decreasing 5-*cis*-HHT concentrations (t_R = 5.98 ± 0.02 min) could be observed for all tested pH-values and temperatures corresponding to increasing concentrations of a new product (t_R = 6.98 ± 0.02 min)—defined as HHT-D (Fig. 1). As indicated by its higher retention time HHT-D is less polar than 5-*cis*-HHT. The UV-spectrum of HHT-D—measured from 190 to 300 nm by DAD—was identical to that one of HHT and its UV-absorption maximum was detected at 232 nm as well (results not shown). These spectroscopic data suggest the existance of the same chromophoric group for 5-*cis*-HHT and HHT-D.

In order to characterize the kinetics of this conversion concentration-time curves were measured for all combinations of pH and temperatures. A typical curvature is shown in Fig. 2a. These concentration profiles are typical for an equilibrium reaction. As demonstrated by the total peak area, ΣA, sum of peak area of 5-*cis*-HHT, A(HHT), and peak area of HHT-D, A(HHT-D), the molar absortivity of HHT-D at 232 nm, ε(HHT-D), is smaller than that one of 5-*cis*-HHT, ε(HHT) = 33000 M^{-1} cm^{-1} (Hecker and Ullrich, 1988). ε(HHT-D) was determined by plotting ΣA-A(HHT) versus A_0(HHT)-A(HHT) resulting in straight lines with the slope α (results not shown). A_0(HHT) is the initial peak area of 5-*cis*-HHT. α was defined as:

$$\alpha = \varepsilon(\text{HHT} - \text{D})/\varepsilon(\text{HHT}) \quad \text{at 232 nm.} \qquad (1)$$

The ratio of molar absorptivities, α, was calculated by the mean and standard deviation of all measurements to be 0.806 ± 0.006 resulting in ε(HHT-D) = 26600 ± 200 M^{-1} cm^{-1}.

Furthermore, Fig. 2, a shows the period of half change, $\tau_{1/2}$, that is defined as the period of reaction time producing half the amount of the equilibrium concentration of HHT-D. $\tau_{1/2}$ was determined graphically.

The influence of pH and temperature on the kinetics were measured using the following combinations: at 298 K and close to 10^6 Pa (chemical standard conditions) for pH 1.10, 1.24 and 1.56 and at pH 1.10 for 284, 298 and 311 K, respectively. As shown in Table 1 the period of half change decreased strongly with the increasing reaction temperature and the decreasing pH-values.

The reaction order and the rate constants for the conversion of 5-*cis*-HHT were calculated using kinetic data on the hypothesis of a first order equilibrium reaction. Plots of $\ln\{(A_0(\text{aHHT})-A_{eq}(\text{HHT}))/(A(\text{HHT})-A_{eq}(\text{HHT}))\}$ versus the reaction time yielded in straight lines (regressions of first order) proving the correctness of the hypoth-

Table 1
Thermodynamic data of the conversion of 5-*cis*-HHT

pH	T (K)	K_c (–)	m (h^{-1})	k_1 (h−1)	k_{-1} (h−1)	$\tau_{1/2}$ (h)	$\Delta_R G$ (kJ*mol^{-1})	$\Delta_R H°$ (kJ*mol^{-1})	$\Delta_R S°$ (J*mol^{-1}*K^{-1})	$\Delta_R H_f^{\#}$ (kJ*mol^{-1})
1.56	298	2.03 ± 0.06	0.347 ± 0.009	0.233 ± 0.009	0.115 ± 0.005	2.80 ± 0.05	−1.75	−3.72	−6.61	103.2
1.24	298	1.93 ± 0.05	0.754 ± 0.007	0.50 ± 0.01	0.26 ± 0.01	1.15 ± 0.05	−1.63 ± 0.07	−3.60 ± 8.2	−6.61 ± 27	101.4.1
1.10	298	1.78 ± 0.05	1.31 ± 0.02	0.84 ± 0.03	0.47 ± 0.02	0.62 ± 0.05	−1.42 ± 0.07	−3.50 ± 0.09	−6.99 ± 3.0	100.0 ± 4.0
1.10	284	1.86 ± 0.05	0.192 ± 0.001	0.125 ± 0.004	0.065 ± 0.003	4.15 ± 0.05	−1.46 ± 0.07			
1.10	311	1.63 ± 0.05	8.00+0.60	4.9 ± 0.40	3.00 ± 0.2	0.14 ± 0.05	−1.25 ± 0.07			

pH, pH of reaction mixture; T, reaction temperature; K_c, equilibrium constant; m, slope of straight lines according to Fig. 2,b; k_1,rate constant of forward reaction; k_{-1}, rate constant of reverse reaction; $\tau_{1/2}$, period of half change; $\Delta_R G$, Gibbs free energy of reaction; $\Delta_R H°$, standard enthalpy of reaction; $\Delta_R S°$, standard entropy of reaction; $\Delta_R H_f^{\#}$,heat of activation of the forward reaction. Deviations of m, $\Delta_R H°$ and $\Delta_R H_f^{\#}$ were calculated by statistical treatment of regressions of first order ($P = 0.95$).

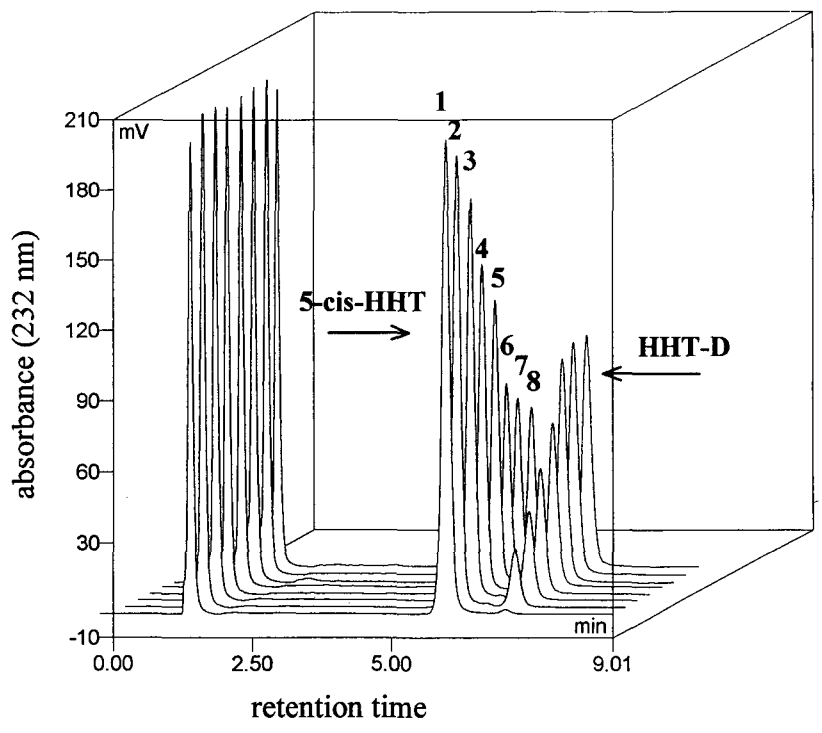

Fig. 1. HPLC analyses of reaction time dependent conversion of 5-*cis*-HHT into HHT-D. Nucleosil 120 C18, 125 × 4.6 mm I.D., 5 μm particle size, CH_3CN-H_2O 55:45 v/v, pH 3.5, 1 ml min^{-1}, 30°C, 232 nm, t_R(5-*cis*-HHT) = 5.98 ± 0.02 min, t_R(HHT-D) = 6.98 ± 0.02 min, reaction times: 1: 0.013 h, 2: 1.5 h, 3: 2.5 h, 4: 4.5 h, 5: 6.5 h, 6: 13 h, 7: 16 h and 8: 18.5 h at 284 K and pH 1.10

esis (Fig. 2,b). Data of Table 1 present the slopes, m, of these plots and underline the high accuracies of regressions. The slopes, m, of these plots are described by:

$$m = (1 + K_c^{-1}) * k_1 \quad (2)$$

with

$$K_c = k_1/k_{-1}. \quad (3)$$

K_c is the equilibrium constant of the conversion of 5-*cis*-HHT and k_1 and k_{-1} are the rate constants of the forward and reverse reaction, respectively. According to these results the conversion of 5-*cis*-HHT is described by the Eqs. (4) and (5):

$$5 - cis - HHT \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HHT - D \quad (4)$$

$$K_c = [HHT - D]/[5 - cis - HHT]$$
$$= A_{eq}(HHT - D)/A_{eq}(HHT) * \alpha \quad (5)$$

With respect to these equations the equilibrium constant, K_c, the Gibbs free energy of reaction, $\Delta_R G$, and the rate constants could be calculated. The results are summarized in Table 1. K_c rose from 1.63 ± 0.05 at 311 K (pH 1.10) to 1.86 ± 0.05 at 284 K (pH 1.10) indicating an exothermic reaction. The equilibrium concentration of HHT-D is about 60% higher than that one of 5-*cis*-HHT indicating a thermodynamic more stable product under the test conditions. The negative standard Gibbs free energy of reaction, $\Delta_R G° = -1.42 \pm 0.07$ kJ mol^{-1} (298 K, pH 1.10), indicates an exergonic process.

An Arrhenius plot was used to calculate the heat of activation, $\Delta_R H^{\#}$, and the frequency factors, **A**, for the forward (f) and reverse (r) reactions (pH 1.10). Frequency factors and heats of activation were obtained as follows: $\mathbf{A}_f = 2.89 * 10^{17}$ h^{-1}, $\Delta_R H_f^{\#} = 100 \pm 4.0$ kJ mol^{-1} (forward reaction) and $\mathbf{A}_r = 6.70 * 10^{17}$ h^{-1}, $\Delta_R H_r^{\#} = 103.4 \pm 4.0$ kJ mol^{-1} (reverse reaction). The standard entropy of reaction, $\Delta_R S°$, of the 5-*cis*-HHT conversion was calculated using Eq. (6):

$$\Delta_R S° = R \ln(A_f/A_r) \quad (6)$$

resulting in -7.0 ± 3.0 J mo^{-1}*K (pH 1.10). The standard enthalpy of reaction, $\Delta_R H°$, was obtained using Eq. (7):

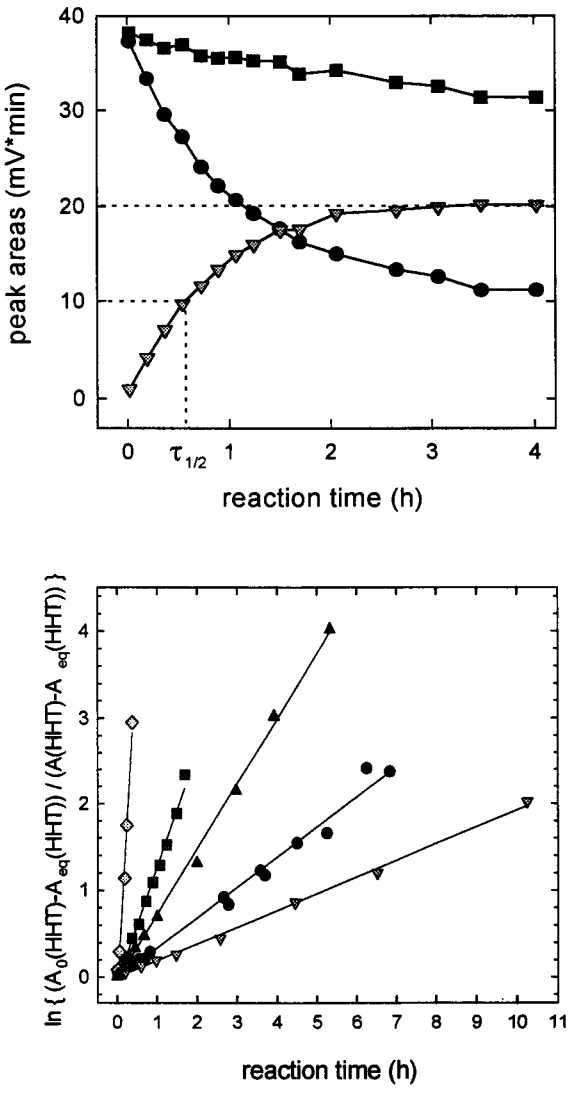

Fig. 2. (a) Peak area-time curves of the conversion of 5-*cis*-HHT into HHT-D measured by HPLC (298 K, pH 1.10) –●– 5-*cis*-HHT, –▽– HHT-D, –■– total of preak areas $\tau_{1/2}$: period of half change, Nucleosil 120 C18, 125 × 4.6 mm I.D., 5 μm particle size, CH_3CN-H_2O 55:45 v/v, pH 3.5, 1 ml min^{-1}, 30°C, 232 nm. (b) Determination of rate constants for the conversion of 5-*cis*-HHT Slopes, m, are defined by: $m = (1 + K_c^{-1*})k_1$. ◇311 K, pH 1.10; ■ 298 K, pH 1.10; ▲ 298 K, pH 1.24; ● 298 K, pH 1.56; ▼ 284 K, pH 1.10.

$$\Delta_R H° = \Delta_R G° + T\Delta_R S° \quad (7)$$

$\Delta_R H°$ was negative (-3.50 ± 0.9 kJ mol^{-1} at pH 1.10) proving an exothermic reaction. Assuming that the frequency factors are independent on the pH-values the heats of activation for the forward and reverse reaction for all tested pH-values (pH_i) were calculated according to Eqs. (8) and (9):

$$R\ T \ln\{k(pH_i)/k(pH\ 1.10)\} = \Delta\Delta_R H^{\#} \quad (8)$$

$$\Delta_R H^{\#}(pH_i) = \Delta_R H^{\#}(pH\ 1.10) + \Delta\Delta_R H^{\#} \quad (9)$$

The standard enthalpies of reaction for tested pH-values were characterized by Eq. (10):

$$\Delta_R H°(pH_i) = \Delta_R H_f^{\#}(pH_i) - \Delta_R H_b^{\#}(pH_i) \quad (10)$$

The results are listed in Table 1. Using these data gives the standard entropies of reaction:

$$\Delta_R S° = (\Delta_R G° - \Delta_R H°)/T \quad (11)$$

as demonstated in Table 1.

With respect to the quantity and negative value of $\Delta_R H°$ the cyclization of 5-*cis*-HHT between C5 and C9 to a cyclopentene-derivative ($\Delta_R H° \approx +84$ kJ mol^{-1}) must be excluded. Our findings show equal values when compared with the general isomerizations of *cis* (*c*) double-bonds to the more stable trans (*t*) conformations. The standard enthalpies and standard entropies are also in the same range: *c*-2-pentene to *t*-2-pentene: $\Delta_R H° = -4$ kJ mol^{-1}, $\Delta_R S° = -6$ J mol^{-1}*K; *c*-2-hexene to *t*-2-hexene: $\Delta_R H° = -2$ kJ mol^{-1}, $\Delta_R S° = -6$ J mol^{-1}*K and *c*-3-hexene to *t*-3-hexene: $\Delta_R H° = -6$ kJ mol^{-1}, $\Delta_R S° = -5$ J mol^{-1}*K (Aylward and Finlay, 1974).

The thermodynamic results of our study may also point to a *cis*-trans isomerization of 5-*cis*-HHT to 5-*trans*-HHT under physiological conditions. In order to evaluate the structure of HHT-D this substance was isolated and purified by HPLC and concentrated by solid-phase extraction. GC/MS measurements were carried out after trimethylsilylation of HHT and HHT-D using *N*-methyl-*N*-(trimethylsilyl-)-2,2,2 trifluoroacetamide (MSTFA). The mass spectrum of the HHT-D derivative is shown in Fig. 3. The molecular peak at $m/z = 424$ proved that HHT-D has been derivatized to its bis-(trimethylsilyl)-derivative indicating a free hydroxy- and a free car-

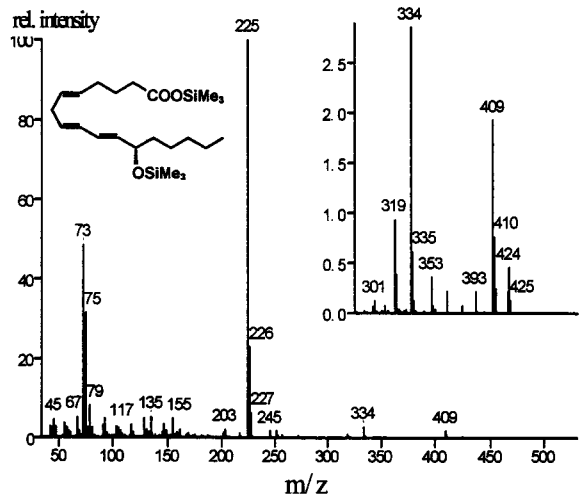

Fig. 3. Mass spectrum (70 eV) of bis-(trimethylsilyl)-derivative of HHT-D; molecular weight, 424. GC/MS analysis was done with a 25 m HPU 2 capillary column, MS-EI, 70 eV. Conditions for GC: carrier gas: He, injection volume: 1 μl, injection temperature: 60–310°C, oven temperature, 80–300°C at 8°C min^{-1} for 24 min m/z 424 (M^+), 409 (M^+-15, loss of CH_3), 353 (M^+-71, loss of C_5H_{11}), 334 (M^+-90, loss of Me_3SiOH), 225 (M^+-199, loss of CH_2–CH=CH–$(CH_2)_3$–COO–$SiMe_3$), 73 (Me_3Si^+) and 75 (Me_2SiOH^+).

boxylic group. The data of the MS profile exclude hydration, dehydration, esterification or other reaction processes that could change the molecular weight or functionality of 5-*cis*-HHT. In comparison to the mass spectrum of 5-*cis*-HHT (John et al., 1998) the same fragments differing in their relative intensity can be noticed. These facts indicate very similar structures of 5-*cis*-HHT and HHT-D.

In consequence of the discussed thermodynamic ($\Delta_R H°$ and $\Delta_R S°$), kinetic (first order reaction), spectroscopic (λ_{max}, ε, UV-spectrum) and structural (free –OH and –COOH groups, less polarity) data, it can be concluded that 5-*cis*-HHT is acid-catalyzed converted into 5-*trans*-HHT.

Therefore, longer treatment or storage of 5-*cis*-HHT under acid conditions have to be avoided in order to prevent false analyses. Furthermore, our findings may be relevant under physiological conditions. Geometric isomers are known for their different physiological potencies as described for pheromones or cis-trans retinal—necessary for the eye-function—(Stryer, 1995) and it has been reported that *cis*-trans isomers are metabolized differently by the same enzymes (Lawson and Holman, 1981; Berdeaux et al., 1996). The conversion of *cis*- into *trans*-isomers can be achieved by isomerases (Chen et al., 1994). At least it has been shown that enzymes inducing peroxidation of polyunsaturated fatty acids are able to produce both the *cis*- and the *trans*-isomers of hydroperoxides (Lodge et al., 1995). Therefore, further studies on the physiological power of 5-*trans*-HHT might be useful to clarify the physiological role of HHT.

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