Thermal degradation of ceramides as studied by mass spectrometry and vibrational spectroscopy

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The degradation of ceramides type IV induced by thermal treatment up to 140 °C was studied by using electrospray mass spectrometry, Fourier transform infrared photoacoustic spectroscopy and Fourier transform Raman spectrocopy. At temperatures above 125 °C structural changes such as cleavage of the C=C bond, destruction of the amide structure and variations of the hydrogen bonds were observed in the head group of ceramides type IV. The mass spectrometry studies revealed the formation of free α-hydroxy fatty acids due to heat treatment. Degradation of ceramide III was not evident in the temperature range studied.

Ceramides are an essential fraction of the lipid matrix of the *stratum corneum* (SC), the outermost layer of mammalian skin. It is supposed that ceramides play an important role in the water holding properties of the SC[1] and in the epidermal barrier function.[2] Although numerous studies on the structure and function of ceramides have been documented,[3–8] no investigations concerning their stability against exposure to sunlight or thermal treatment have been done.

In our efforts to elucidate the thermotropic phase behaviour of binary mixtures of SC lipids, we have chosen ceramides type IV as a model substance for the ceramides fraction.[7,9] In the case of the system ceramides type IV–cholesterol, we found that the differential scanning calorimetry traces up to temperatures of 150 °C (mp of cholesterol) were not reproducible in repeated scans. This experimental finding has prompted us to examine the thermal stability of the ceramide samples. In the present study, we monitored the heat-induced degradation of ceramides type IV using mass spectrometry, which was recently used for structural elucidation of ceramides.[10] Fourier transform infrared photoacoustic spectroscopy (FTIR-PAS) and FT Raman spectroscopy were also employed to monitor the degradation of ceramides after heat treatment. Stearic acid and ceramide III served as reference substances.

Materials and methods

Sample and heat treatment

Ceramides type IV (CER IV) and stearic acid were purchased from Sigma Chemical (St. Louis, MO, USA) and were used as received. Ceramide III (*N*-stearoylphytosphingosine) was kindly delivered by Cosmoferm, Delft, The Netherlands. CER IV originates in bovine brain and consists of sphingosine as long chain base and α-hydroxy fatty acids. Ceramide III, a fermentation derived lipid, contains phytosphingosine as long chain base (see Fig. 1).

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The samples were melted in a brass cup (5 mm diameter and 0.5 mm depth) which fits into both the PAS and Raman sample holders. In this manner IR as well as Raman spectra of identical probes could be registered. The sample was thermally treated in the following sequence: 1 h at 120 °C, 1 h at 120 °C + 1 h at 125 °C, 1 h at 120 °C + 1 h at 125 °C + 1 h at 130 °C, and finally 1 h at 120 °C + 1 h at 125 °C + 1 h at 130 °C +1 h at 140 °C. After each treatment the mass spectrum and the vibrational spectra at room temperature were taken.

FTIR photoacoustic spectroscopy

The IR spectra were collected on a Bruker FTIR spectrometer IFS 28 (Karlsruhe, Germany) equipped with an MTEC Photoacoustics model 200 photoacoustic cell (Ames, IA, USA). Using the Bruker OPUS software package, step-scan experiments were conducted by applying a sinusoidal phase modulation technique with a modulation frequency of 195 Hz and a modulation amplitude of 2 λ_{HeNe} (1.25 μm) and 10 coadditions. In this version with digital signal processing electronics, the demodulation of the photoacoustic (PA) signal with reference to the IR beam modulation is provided by the acquisition processor and results in two components, namely the 'in-phase' (I) and 'in-quadrature' (Q) signals. From the two signal components the magnitude spectrum $M = (I^2 + Q^2)^{1/2}$ was calculated. All spectra were normalized by ratioing the sample spectrum with a carbon black spectrum. The PA spectra were acquired at a resolution of 12 cm^{-1} using the strong Beer–Norton apodization function. The PA cell was purged with helium prior to measuring each sample.

Fourier transform Raman spectroscopy

Vibrational Raman spectra were recorded with a Bruker FT-Raman spectrometer RFS 100/S (Karlsruhe, Germany) using a diode pumped Nd:YAG laser at an operating wavelength of 1064 nm. The measurements were performed with 180° angle scattering geometry with 400 scans and a laser power of 300 mW at the sample location. The interferograms were apodized

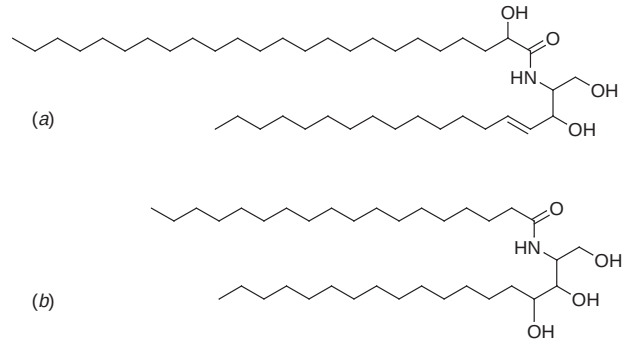

Fig. 1 Structures of (*a*) ceramides type IV (main component *N*-α-hydroxylignoceroylsphingosine) and (*b*) ceramide III (*N*-stearoyl-phytosphingosine).

with the Blackman–Harris four-term function and Fourier transformed to give spectra with a resolution of 4 cm^{-1}.

Mass spectrometry

Electrospray mass spectrometric studies were carried out using a Finnigan ion trap mass spectrometer LCQ (Thermo Quest, Egelsbach, Germany). The ceramides were dissolved in methanol. The sample was injected *via* an integrated syringe pump at a flow rate of 15 μl min^{-1}. In order to obtain complementary information, the electrospray ionization was performed in the positive as well as in the negative mode with voltages of +4.5 and −4.5 kV, respectively. The temperature of the heated capillary was 200 °C. The collision induced dissociation (CID) takes place in the octapole region of the ion source. The relative collision energy (0 to 100%) corresponds to 0 to +100 V dc octapole offset voltage for negative ionization. The use of a CID voltage of 25% improved the resolution without significant decrease of the signal intensity.

Results and discussion

For stearic acid and ceramide III thermal degradation was not observed in the temperature range up to 140 °C.

The FTIR PA and Raman spectra for the CER IV sample annealed at various temperatures are shown in Figs. 2 and 3, respectively. The vibrational bands observed for untreated CER IV at room temperature and the assignments according to literature data[11,12] are summarized in Table 1. The spectra of the sample annealed at 120 °C are almost identical to those of the untreated sample. The first hints of spectral changes become evident when the temperature is increased to 125 °C. These changes become more pronounced after annealing at tem-

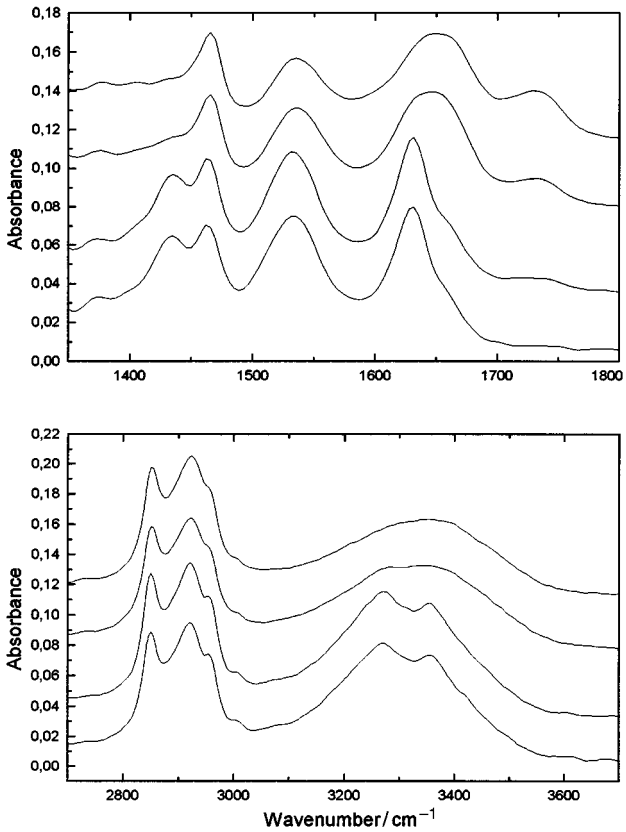

Fig. 2 FTIR photoacoustic spectra of ceramides type IV annealed under various conditions. From bottom to top: (*a*) untreated; (*b*) 120 °C for 1 h; (*c*) 120 °C for 1 h + 125 °C for 1 h + 130 °C for 1 h; (*d*) 120 °C for 1 h + 125 °C for 1 h + 130 °C for 1 h + 140 °C for 1 h.

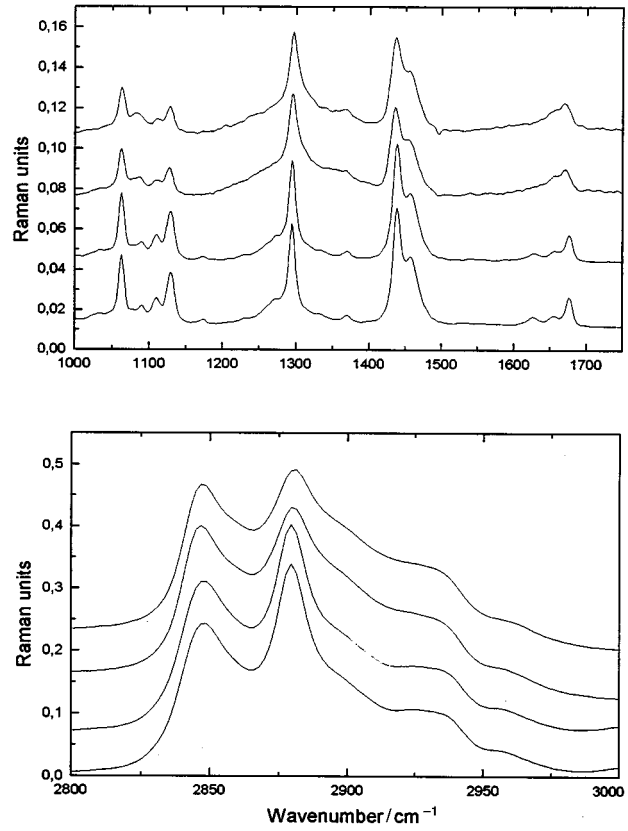

Fig. 3 FT Raman spectra of ceramides type IV annealed under various conditions. From bottom to top: (*a*) untreated; (*b*) 120 °C for 1 h; (*c*) 120 °C for 1 h + 125 °C for 1 h + 130 °C for 1 h; (*d*) 120 °C for 1 h + 125 °C for 1 h + 130 °C for 1 h + 140 °C for 1 h.

Table 1 Relevant vibrational bands for ceramides type IV (Sigma) at room temperature and assignments according to literature data[11,12]

Infrared	
Band position/cm^{-1}	Assignment
1435	CH_2 scissoring (-CH_2-CH=CH-R)
1463	CH_2 scissoring
1534	δ(C-N-H) in plane bend + ν(C-N): amide II
1630	ν(C=O) + ν(C-N) + ν(N-H): amide I
2850	$\nu_s(CH_2)$
2921	$\nu_{as}(CH_2)$
2954	$\nu_{as}(CH_3)$
3271	ν(O-H) hydrogen-bonded
3363	ν(N-H)
3417	ν(O-H) hydrogen-bonded
Raman	
Band position/cm^{-1}	Assignment
1063	ν_{as}(C-C) ordered chain
1090	ν(C-C) chain with gauche units
1100	ν_s(C-C) progression
1130	ν_s(C-C] ordered chain
1296	CH_2 twisting
1369	CH_2 wagging
1438; 1459	CH_2 scissoring
1628; 1654	Amide I
1678	ν(C=C)
2847	$\nu_s(CH_2)$
2880	$\nu_{as}(CH_2)$

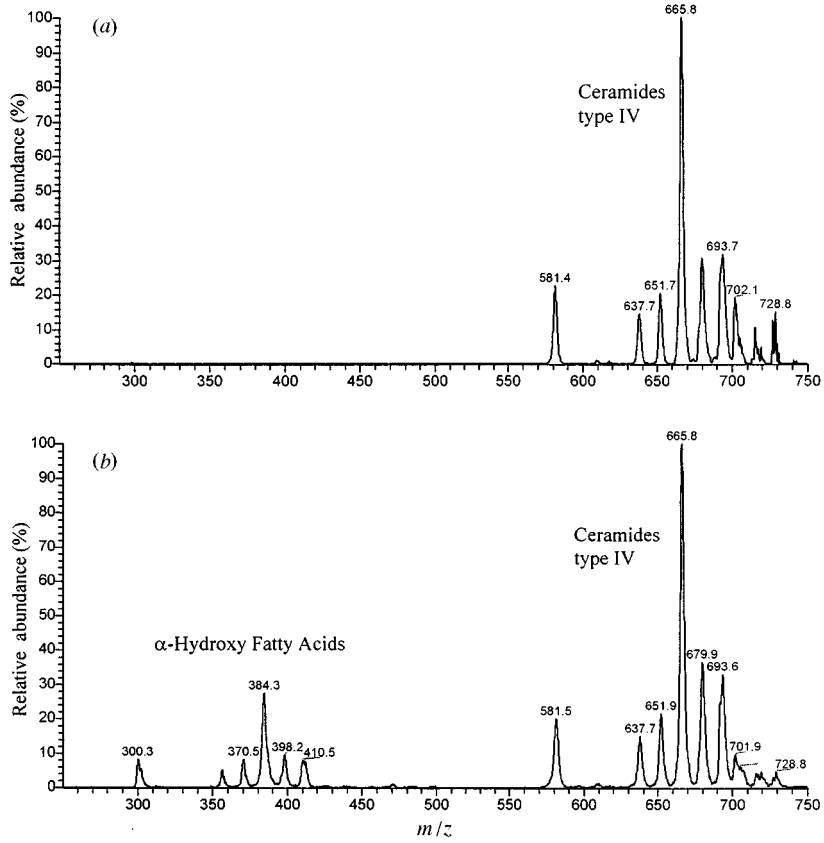

Fig. 4 Electrospray mass spectra of ceramides type IV, recorded in the negative ionization mode (electrospray ionization voltage −4.7 kV; 25% source CID): (*a*) untreated sample; (*b*) after thermal degradation. The mass-to-charge ratios (*m*/*z*) 581, 637, 651, 665, 679 and 693 refer to the *N*-(α-hydroxy)acylsphingosines which contain the α-hydroxy fatty acids with chain lengths of 18, 22, 23, 24, 25 and 26 carbon atoms. The spectrum of the degraded sample shows additionally the *m*/*z* corresponding to the free α-hydroxy fatty acids.

peratures above 125 °C. The following major observations were made:

(a) The intensities of all vibrational bands associated with the amide residue decreased continuously in the course of the degradation process. (b) The relatively sharp N–H stretching IR band superimposed on the broad hydrogen bonded O–H stretching band vanished. (c) A relatively broad IR band rose at 1651 cm^{-1} and a new ν(C=O) IR band appeared at 1733 cm^{-1}. (d) The intensity of the C=C stretching Raman band at 1678 cm^{-1} decreased. After the treatment of the sample at 140 °C, a broad and ill-defined Raman band occurred in the range between 1600 and 1700 cm^{-1}. (e) The CH_2 scissoring IR mode of the (–CH_2–CH=CH–) structure disappeared in the course of the treatment. (f) The intensity of the sharp stretching Raman bands $\nu_{as}(CH_2)$, ν_s(C–C) and ν_{as}(C–C), which belong to the alkyl chain residue in the *trans* conformation (three or more *trans* bonds in sequence), decreased with proceeding degradation.

The mass spectrum of the untreated CER IV is shown in Fig. 4(*a*). It was found that CER IV contains α-hydroxy fatty acids with chain lengths of C18, C22, C23, C24, C25 and C26, but not those with chain lengths of C19, C20 and C21.

For the heat-treated sample, the mass spectra recorded in the negative mode [see Fig. 4(*b*)] indicate an increase in the amount of free α-hydroxy fatty acids. All peaks mentioned were confirmed using zoom scans which result in an improved mass resolution. Unfortunately, the remainder is not detectable because it cannot be negatively ionized.

The positive ionization spectra of the untreated CER IV show a normal pattern of $[M + H]^+$ and $[M + Na]^+$ peaks (data not shown). Another series of peaks exhibits mass-to-charge ratios (*m*/*z*) smaller by 40, attributed to $[M + Na]^+$ and by 18, attributed to $[M + H]^+$. The latter indicates that these are $[M + H_2O + H]^+$ peaks. The occurrence of these peaks is influenced by instrumental parameters like temperature of the heated capillary and CID voltage. Therefore, the amount of dehydrated ions does not correlate with the degradation process.

The experimental findings demonstrate that the thermal degradation of CER IV results in (i) cleavage of the C=C bond, (ii) destruction of the amide structure, (iii) changes of the hydrogen bond system in the headgroup and (iv) a decrease of the ordered structure of the alkyl chain residues. The mass spectra clearly show the formation of free α-hydroxy fatty acids. Unfortunately, other cleavage products could not be identified owing to experimental reasons. In the case of the vibrational spectra, it was not possible to interpret the superposition of the spectra, resulting from several species in the final system.

Finally, we emphasize that it should be borne in mind that degradation effects can appear in conjunction with studies on the thermotropic phase behaviour of lipid systems.

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