Reactions of Dehydroascorbic Acid with Primary Aliphatic Amines Including N^{α}-Acetyllysine

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The reaction of L-dehydroascorbic acid (DHA) with primary aliphatic amines including N^{α}-acetyllysine was examined by HPLC. A new aminoreductone, 2-deoxy-2-(propylamino)ascorbic acid **(1)** was isolated and the structure elucidated by spectroscopic data. Furthermore oxalic acid mono- **(2)** and diamides **(3)** were identified as important degradation products of DHA under Maillard conditions. 3-Deoxy-3-(alkylamino)ascorbic acids (**4**), typical condensation products of L-ascorbic acid and amines, were also detected in the reaction mixtures of DHA. All products are formed under oxidative and nonoxidative conditions from DHA.

Keywords: *Maillard reaction; L-dehydroascorbic acid; L-ascorbic acid; oxalic acid amides; aminoreductones; protein glycation; protein cross link*

INTRODUCTION

L-Ascorbic acid (AA) which is also known as vitamin C is an important naturally occurring component of our nutrition. Additionally it is added to a wide variety of food stuffs as an antioxidant or for other technological purposes [e.g., Borenstein (1987)]. During food processing or heating AA degrades, leading not only to loss of vitamin but also to the formation of browning products or melanoidines (Löschner et al., 1990; Löschner et al., 1991; Rogacheva et al., 1995). It was shown that the nonenzymatic browning of AA can be responsible for the discoloration of fruit juices (Nagy et al., 1990), dried products (Kirk, 1981), and other food stuffs (Liao and Seib, 1987). As a consequence, the stability of AA during storage and processing of food is a major problem for food technology and nutrition.

The degradation of AA has been thoroughly investigated during the past decades. In a reversible process, AA is oxidized to give L-dehydroascorbic acid (DHA), which further breaks down to a wide variety of products, including 2,3-diketogulonic acid (DKG), threonic acid, threose, xylosone, and 3-deoxyxylosone (Lopéz and Feather, 1992; Niemelä, 1987; Shin and Feather, 1990; Velisek et al., 1976).

However, little is known about the reaction of AA or its degradation product DHA with amines, amino acids, or proteins, although it is proposed that this type of reaction is responsible for browning reactions (Bensch et al., 1985; Clegg, 1964; Ranganna and Setty, 1968, 1974). Moreover the reaction of AA and DHA with amines, amino acids, or proteins gains interest in medical biochemistry. It has been demonstrated that incubation of eye lens protein with AA, DHA, or their degradation products leads to the formation of colored and fluorescent compounds and protein cross-links, similar to the conditions in cataractous lenses. Thus it was concluded that, in diabetic patients, because of a metabolic disorder, AA reacts with side chains of lens crystalline, resulting in cataract (Bensch et al., 1985; Ortwerth and Olesen, 1988; Ortwerth et al., 1988).

The reaction of AA with amines shows similarities to the Maillard reaction of sugars, but only few degradation products of L-ascorbic acid in which nitrogen is incorporated have been isolated so far. It was shown, that DHA initiates Strecker degradation of α-amino acids resulting in the formation of scorbamic acid (SCA). SCA can condense with another molecule of DHA to give 2,2′-nitrilodi-2(2′)-deoxyascorbic acid, a red pigment (Koppanyi et al., 1945; Kurata et al., 1973). On the other hand, AA can react with primary amines such as N^{α}-acetyllysine to give aminoreductones of general structure **4** (Pischetsrieder et al., 1995).

In this paper we report on more detailed investigations of the reaction of DHA with primary amines and amino acids, which led to the isolation and identification of new amine-containing degradation products.

MATERIALS AND METHODS

Apparatus. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), DEPT (distortionsless enhancement by polarization transfer), COSY (correlated spectroscopy), and COLOC (correlated spectroscopy via long-range coupling) spectra were recorded with a Jeol 400 GSX spectrometer with $(CH_3)_4Si$ as internal standard. Chemical shifts are reported in ppm. Mass spectral analyses were obtained with a Varian MAT CH7 (EI: 70 eV, CI with CH_4). Elemental analyses were obtained with a Heraeus Rapid instrument. For UV spectra a Beckman spectrometer 33, Uvikon 810 (Kontron) was used or the UV spectra were directly taken from the diode array detector where mentioned. Melting points were determined in open capillaries using a Buechi apparatus and are uncorrected.

Reagents. The water used for HPLC was distilled and filtered through a nylon membrane of 0.45 μm. HPLC grade methanol was used without further purification. All solvents were degassed with helium. DHA was purchased from Sigma-Aldrich (Steinheim, Germany).

High-Performance Liquid Chromatography (HPLC). Analytical HPLC was performed with a Merck L-7100 gradient pump and a Merck L-7450 photodiode array detector including Merck-Hitachi Model D-7000 Chromatography Data Station software. A column packed with LiChroSorb (RP 18, 280 × 4 mm i.d., 5 μm particle size) was used. The column was protected with a guard cartridge (25 × 4 mm) packed with the same material as the column. The eluents used were water, 10 mmol of KH_2PO_4, pH 3.0, adjusted with H_3PO_4, (solvent A), and methanol (solvent B) with a gradient of 0−75% B in 25 min and then continuing with 100% B from 25 to 40 min. The substances were detected with a diode array detector from 200 to 400 nm.

For preparative chromatography, a Merck L-6250 pump, a Merck L-4000 UV detector, and a Merck D-2500 chromatoin-

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tegrator were used. Preparative HPLC was performed on a HiBar column packed with LiChroSorb (RP 18, 250 × 20 mm i.d., 7 μm particle size).

Reactions of DHA with Propylamine and N^{α}-Acetyllysine in Phosphate Buffer in the Presence and Absence of Oxygen. To a solution of DHA (17.4 mg/mL, 0.1 mmol/mL) in phosphate buffer (pH 7) was added propylamine or N^{α}-acetyllysine in different ratios. The mixture was adjusted to pH 7 and heated for different times at 40, 70, or 100 °C. Diluted samples were injected into the analytical HPLC system (Figures 1 and 2). For experiments in the absence of oxygen, diethylentriaminepentaacetic acid (DTPA) was added (39.3 mg/mL, 0.1 mmol/mL) and nitrogen was bubbled through the solution during heating.

2-Deoxy-2-(propylamino)ascorbic Acid (1). L-Dehydroascorbic acid (400 mg, 2.3 mmol) was suspended in 8 mL of THF and propylamine (300 mg, 5.1 mmol) was added. The mixture was kept at 40 °C for 1 h in a closed vessel. After the solvent was removed under reduced pressure, the residue was dissolved in a mixture of water and methanol (8:2, 4 mL) and filtered through a Durapore poly(vinylidene fluoride) membrane (0.45 μm). Isolation of **1** was achieved by preparative HPLC [eluent, water−methanol (95:5); flow rate, 12 mL/min; UV detection at 246 nm; Injection volume, 0.8 mL]. The fraction between 13 and 15 min was collected and the aqueous solution was lyophilized. **1** was obtained as a colorless solid which rapidly turned red under atmospheric conditions (yield after purification, 4%): ^{1}H NMR (CD_3OD, COSY) δ 0.89 (t, 3H, CH_2CH_3), 1.57 (m, 2H, CH_2CH_3), 2.95 (m, 2H, NCH_2), 3.58 (m, 2H, CH_2O), 3.85 (dt, 1H, CH_2OCHO), 4.40 (d, 1H, CHOC=); ^{13}C NMR (CD_3OD, COSY, DEPT) δ 11.2 (CH_2CH_3), 20.5 (CH_2CH_3), 51.8 (NCH_2), 63.9 (CH_2O), 71.5 (CH_2OCHO), 81.3 (CHOC=), 94.3 ($^+NH_2CH_2$), 175.9 ($^-$OC=), 184.2 (C=O); MS (*m*/*z*, CI) 218 (M + 1); (*m*/*z*, EI) 217 (M), 170, 156, 100; UV λ_{max} (H_2O) = 245.5 (pH 3), 247 (pH 7), 271 nm (pH 11).

Isolation of Oxalic Acid Monopropylamide (2) from a Reaction Mixture of Dehydroascorbic Acid and Propylamine. L-Dehydroascorbic acid (160 mg, 0.92 mmol) was dissolved in phosphate buffer (pH 7.0, 8 mL) and propylamine (271 mg, 2.6 mmol) was added. The mixture was adjusted to pH 7.0 and heated at 100 °C in a closed vessel. After 2 h the reaction was stopped by freezing the solution. The frozen reaction mixture was lyophilized to remove the solvent and 1 g of the residue was dissolved in 4 mL of the HPLC eluent. Each time, 1.0 mL was injected into the preparative HPLC system [eluent, phosphate buffer (10 mmol KH_2PO_4, pH 3.0, adjusted with H_3PO_4); flow rate, 12 mL/min; UV detection at 220 nm]. The fraction between 23 and 26 min was collected and lyophilized. To remove the phosphate buffer, the residue was dissolved in methanol and filtered. The methanol was evaporated. This procedure was repeated several times. **2** was obtained as a colorless solid (yield, 25%): mp 113 °C (dec); ^{1}H NMR ($CDCl_3$) δ 0.90 (t, J = 7.4 Hz, 3H, CH_2CH_3), 1.56 (sex, J = 7.4 Hz, 2H, CH_2CH_3), 3.28 (m, J = 7.4 Hz, 2H, NCH_2), 7.24 (NH); ^{13}C NMR ($CDCl_3$) δ 11.3 (CH_2CH_3), 22.4 (CH_2CH_3), 42.3 (NCH_2), 157.6 (OC=O), 160.0 (HNC=O); MS (*m*/*z*, CI) 132 (M + 1).

Isolation of Oxalic Acid Dipropylamide (3) from a Reaction Mixture of Dehydroascorbic Acid and Propylamine. L-Dehydroascorbic acid (600 mg, 3.4 mmol) was suspended in 6 mL of THF and propylamine (1116 mg, 18.9 mmol) was added. The mixture was allowed to stand for 1 h at 40 °C in a closed vessel. After removal of the solvent under reduced pressure, the residue was dissolved in 100 mL of water. The aqueous solution was extracted with ethyl acetate (3 × 40 mL). The collected organic layers were dried over anhydrous sodium sulfate. After evaporation of ethyl acetate, the residue was dissolved in a mixture of water and methanol (8:2, 3 mL). For purification, this solution was injected into the preparative HPLC system [eluent, water−methanol (45:55); flow rate, 9 mL/min; UV detection at 220 nm; injection volume, 1.0 mL]. The fraction between 20 and 23 min was collected. Methanol was removed under reduced pressure and the aqueous solution was lyophilized. **3** was obtained as a colorless solid (yield after purification, 15%): mp 162 °C (dec); ^{1}H NMR ($CDCl_3$) δ 0.95 (t, J = 7.4 Hz, 3H, CH_2CH_3), 1.61 (m, 2H, J = 7.4 Hz, CH_2CH_3), 3.27 (m, J = 7.4 Hz, 2H, NCH_2), 7.51 (NH); ^{13}C NMR ($CDCl_3$) δ 11.7 (CH_2CH_3), 22.9 (CH_2CH_3), 41.8 (NCH_2), 160.4 (C=O); MS (*m*/*z*, CI) 173 (M + 1); (*m*/*z*, EI) 172, 144, 143, 131, 115; UV λ_{max} = 217 nm (UV spectrum was directly taken from the diode array detector).

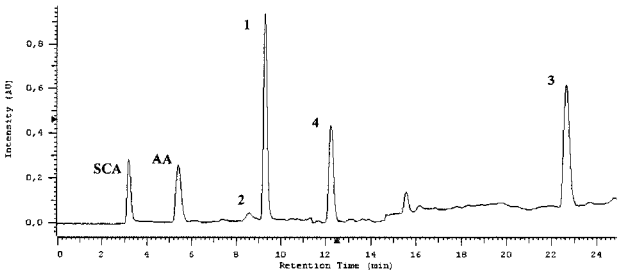

Figure 1. HPL chromatogram of a reaction mixture of DHA (50 mg, 0.29 mmol) and propylamine (33.9 mg, 0.57 mmol) in THF (2.0 mL) heated for 1.5 h at 40 °C: UV detection: 0 min, 246 nm; 11.4 min, 278 nm; 15.0 min, 219 nm. Numbers on top of peaks refer to structures in Figure 6.

Syntheses of Reference Oxalic Acid Amides. *Oxalic Acid Monopropylamide (**2**).* Oxalyl chloride (4.3 mL, 50 mmol) was stirred at −17 °C while ice-cold propylamine (4.1 mL, 50 mmol) was added (very carefully) during 2 h. After an additional 1 h, 50 mL of ice water was added slowly and the reaction mixture was adjusted to pH 13 with NaOH (1 N). The aqueous solution was extracted with ethyl acetate to remove the oxalic acid diamide and adjusted to pH 1.5 with HCl (2 N). After extraction of the solution with ethyl acetate, the combined organic layers were dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and **2** was obtained as a slightly yellow solid which recrystallizes from $CHCl_3$ in needles. The spectral data were identical with those of **2** described above (yield, 10%): Anal. Calcd for $C_5H_9O_3N$: C, 45.78; H, 6.92; N, 10.68. Found: C, 45.82; H, 6.81; N, 10.55.

*Oxalic Acid Dipropylamide (**3**).* Propylamine (1 mL, 12.2 mmol) was stirred at −17 °C. Oxalyl chloride (0.5 mL, 5.8 mmol) was added in small portions during 1 h. The mixture was stirred for an additional 1 h, and 20 mL of ice water was added. The resulting suspension was extracted three times with ethyl acetate (20 mL). The combined organic layers were reextracted with HCl (0.1 N, 20 mL) and NaOH (0.1 N, 20 mL) and dried over anhydrous sodium sulfate. After removal of ethyl acetate under reduced pressure, a colorless solid was obtained (yield, 90%). The spectral data were identical with those of **3** described above. Anal. Calcd for $C_8H_{16}O_2N_2$: C, 55.77; H, 9.37; N, 16.27. Found: C, 55.58, H, 9.67; N, 16.19.

RESULTS AND DISCUSSION

When DHA is reacted with primary aliphatic amines or N^{α}-acetyllysine, several reaction products can be separated by HPLC and observed using UV detection (Figures 1 and 2). Major products are 3-deoxy-3-(alkylamino)ascorbic acids (**4**) with a characteristic absorption maximum at 278 nm. The propylamine and N^{α}-acetyllysine derivatives were identified by comparing retention times and UV spectra with those of authentic samples. **4** was previously isolated from reaction mixtures of AA and aliphatic amines (Pischetsrieder et al., 1995). It was proposed that the 3-hydroxyl group of AA is substituted by the amine leading to **4**. If DHA is the educt, however, another reaction mechanism can be envisaged. A Schiff base of the 3-keto group of DHA can be formed, which is then reduced to give the product **4** (Figure 3). It can be assumed that reductones or aminoreductones, which are formed during the degradation of DHA [e.g., pentose reductone (Wisser et al., 1968)], are the compounds that reduce the intermediate Schiff base. Another possible pathway is the reaction

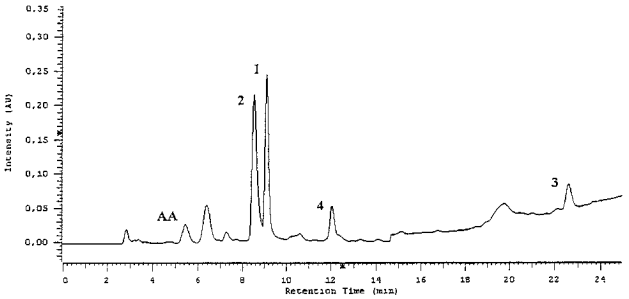

Figure 2. HPL chromatogram of a reaction mixture of DHA (50 mg, 0.29 mmol) and propylamine (33.9 mg, 0.57 mmol) in phosphate buffer (pH 7, 2.0 mL) heated for 1 h at 70 °C. UV detection: 0 min, 246 nm; 11.4 min, 278 nm; 15.0 min, 219 nm. Numbers on top of peaks refer to structures in Figure 6.

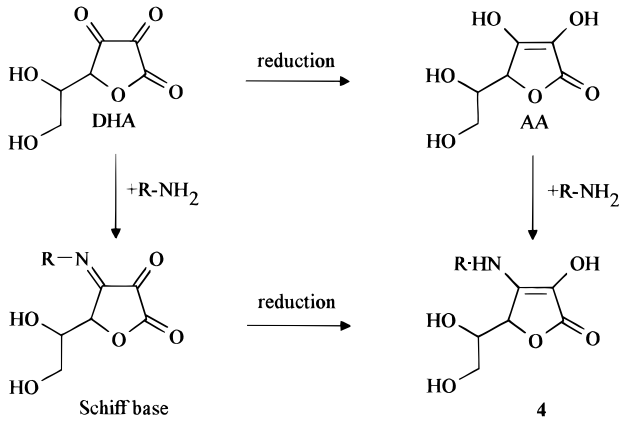

Figure 3. Two possible reaction mechanisms for the formation of **4** from DHA. R = C_3H_7; C_4H_9; $(CH_2)_4CHNH(COCH_3)$-COOH.

of amine with regenerated ascorbic acid. In Maillard reaction mixtures, AA is reversibly formed from DHA and can be detected by HPLC (Figures 1 and 2).

The formation of **4** from AA and DHA as well shows the importance of this product for the first steps of the Maillard reaction of ascorbic acid, but it must be considered as an unstable intermediate.

The main product of the reaction of DHA with propylamine, which appears in the HPLC chromatogram, does not show a characteristic UV maximum, but only an end absorption below 220 nm. Despite the difficulties of detection, the substance could be isolated and identified by the spectral data as oxalic acid monopropylamide (**2**). To confirm the structure, the reference compound was synthesized.

Another compound, which can be detected as a byproduct in the aqueous reaction mixtures of DHA and propylamine, was isolated. Spectral analyses and synthesis revealed the formation of oxalic acid dipropylamide (**3**). The yield of **3** is highly dependent on the amine concentration. In a model reaction mixture of DHA with a 5-fold excess of propylamine in THF, oxalic acid dipropylamide is almost the only product that can be detected.

Parrod has discovered that oxalic acid diamides are formed from AA in alkaline, highly concentrated, aqueous solutions of amine (50%). He proposed that DHA reacts to **3** only, if H_2O_2 is added (Parrod, 1938 and 1939). In contrast, our experiments show that both oxalic acid mono- and diamides are generated in neutral phosphate-buffered solution of DHA and equimolar amounts of amine, conditions that can be compared to those of food processing or in vivo.

Several investigations have shown that there is strong

^{13}C-NMR		^{1}H-NMR	
carbons	ppm	hydrogens	ppm
C-1	184.2		
C-2	94.3		
C-3	175.9		
C-4	81.3	H-4	4.40
C-5	71.5	H-5	3.85
C-6	63.9	H-6	3.58
C-7	51.8	H-7	2.95
C-8	20.5	H-8	1.57
C-9	11.2	H-9	0.89

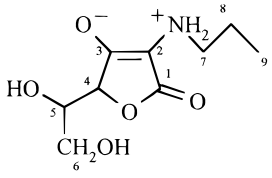

Figure 4. NMR data of 2-deoxy-2-(propylamino)ascorbic acid (**1**).

evidence that AA is responsible for protein cross-links which occur in vivo and in vitro under physiological conditions (Ortwerth and Olesen, 1988; Slight et al., 1990). It was demonstrated that oxygen is necessary for the crosslinks, if the reaction starts from AA. However, when DHA is used as substrate, protein dimerization occurs to the same extent under aerobic and anaerobic conditions (Prabhakaram and Ortwerth, 1991). It can be assumed that in a first step AA is oxidized to DHA, which then reacts with protein without requiring oxygen. So far a crosslink product which is derived from AA and protein could not be identified.

Assuming that lysine side chains of proteins react in a similar way as primary aliphatic amines like propylamine, oxalic acid diamides represent model compounds which show how two molecules of protein can be connected. In accordance with the above-mentioned results we found that **3** is formed to the same extent in the presence as well as in the absence of oxygen.

Another important product, which can be detected in the reaction mixtures, was purified by preparative HPLC. It is formed after heating in phosphate buffer at 40 °C, but the yield is highly increased when DHA is reacted in THF with a 2-fold excess of amine. The assignment of the structure is based on spectroscopic data.

The ^{1}H NMR shows the signals of an ascorbic acid derivative in which propylamine is incorporated. The molecule peak in the mass spectrum appears at 217 (EI, *m*/*z*), referring to a structure in which one hydroxy group of AA is replaced by propylamine. A COLOC experiment (5 Hz) shows the long-range coupling of the hydrogens at NCH_2 (2.95 ppm) and the carbon C-2 (94.3 ppm) over three bonds (Figure 4). Thus, the substitution pattern in which the propylamino group is linked to C-2 is proven. The ^{13}C NMR and the UV spectra reveal the betaine structure of the new product. The signals for C-1 and C-3 are both located in the same region at 184.2 (C-1) and 175.9 ppm (C-3) while C-2 is shifted high-field to 94.3 ppm, a rather low value for olefinic N-substituted carbons, but characteristic for betaines. C-1 and C-3 differ only with respect to their chemical environment. C-2 is rich in electrons due to the mesomeric effect of the enolate. The UV absorbency at different pH values confirms the betaine structure. The UV maximum in neutral solution is at 247 nm (H_2O, pH 7). It is hardly influenced by addition of acid (λ_{max} = 245.5 nm, pH 2) but shifts to higher wavelengths in alkaline solution (λ_{max} = 271.5 nm, pH 11). The amino group is protonated in acidic and neutral solution. The proton is abstracted by the solvent only under alkaline conditions. Scorbamic acid, which has the same substitution pattern (amino group at C-2, hydroxyl group at C-3), displays a similar UV maximum at 246.5 nm (H_2O) (Kurata et al., 1973). The ^{13}C NMR and UV spectral data are in accordance with literature values

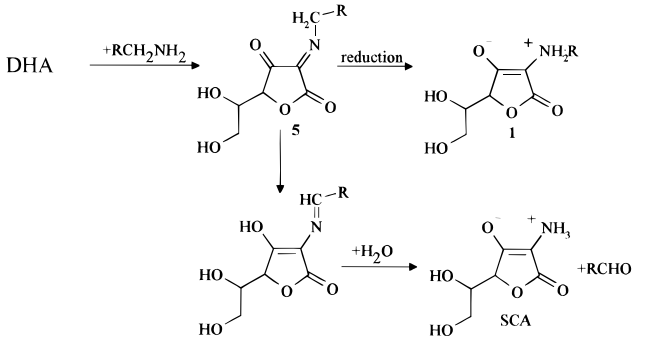

Figure 5. Reactions of intermediate **5** to give SCA or **1**. R = C_3H_7; C_4H_9; $(CH_2)_4CHNH(COCH_3)COOH$.

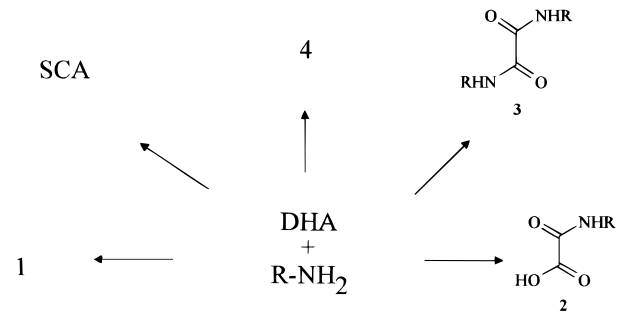

Figure 6. Degradation pathways of dehydroascorbic acid in the presence of amines. R = C_3H_7; C_4H_9; $(CH_2)_4CHNH(COCH_3)COOH$.

of betaines (Knerr et al., 1994). This leads to the conclusion that the previously unknown Maillard reaction product of DHA is **1**.

It can be assumed that the formation of **1** is initiated by the reaction of amine with the C-2 carbonyl group of DHA to give a Schiff base **5** (Figure 5). In the next step, the intermediate is reduced to give **1**. Redox reactions are generally observed in Maillard mixtures of DHA, involving oxidative and antioxidative degradation products (e.g., pentose reductone). On the other hand, it is known that **5** can also undergo deprotonation, a reaction comparable to the Strecker degradation which gives rise to SCA (Namiki et al., 1986) (Figure 5). Both products SCA and **1** are formed in reaction mixtures of DHA and amines. The ratio depends on the heating conditions. In neutral phosphate buffer, reduction of **5** is favored and **1** is a major product whereas SCA cannot be detected (Figure 2). However, when THF is used as solvent, **1** is still the major product, but additionally SCA is formed (Figure 1). It can be concluded that SCA is an important product when free α-amino acids are reacted with DHA or AA. In contrast, when the α-carboxyl group in the amine component is missing or not accessible for decarboxylation, as it is the case in protein bound lysine, reduction of the Schiff base **5** is favored and **1** is by far the more important product. This is of significance, since SCA is the precursor of colored compounds, but the reaction mechanism includes the cleavage of the C−N bond from the amine chain (Figure 5). In contrast, in **1** the aminoreductone moiety is still connected to the unchanged carbon chain of the amine. Provided that propylamine and N^{α}-acetyllysine are accepted as model compounds which represent lysine side chains in proteins, it can be assumed that **1**, but not SCA, can be responsible for AA-induced protein glycation resulting in the formation of cross-link, browning, and modification of physical and physiological properties of the proteins.

Thus we were able to isolate and identify several products of DHA and aliphatic amines, which are formed under Maillard conditions (Figure 6). A compound that is derived from AA and amines could be found in DHA reaction mixtures as well. It was shown that the products can be formed from a variety of amines and amino acids, such as propylamine, butylamine, or N^{α}-acetyllysine. Moreover **1**−**4** can be detected at a wide range of reaction temperature. At higher temperatures, such as 100 °C, they arise during a short time of heating, but they could also be found after several days, when the reaction mixture was incubated at 40 °C. It can be concluded that products similar to **1**−**4** may be responsible for protein glycation which is caused by AA during food processing and under physiological conditions in vivo. These results are in accordance with experiments, which show that amino acid analyses after incubation of proteins with AA indicate mainly a loss of lysine and the formation of so far unidentified products (Dunn et al., 1990; Ortwerth and Olesen, 1988). Investigations to confirm the assumption, that compounds **1**−**4** are also formed as derivatives of lysine side chains of proteins are in progress.

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