# Reactivity of Lysine Moieties toward $\gamma$-Hydroxy-$\alpha,\beta$-unsaturated Epoxides: A Model Study on Protein–Lipid Oxidation Product Interaction

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Epoxyols are generally accepted as crucial intermediates in lipid oxidation. The reactivity of $\gamma$-hydroxy-$\alpha,\beta$-unsaturated epoxides toward lysine moieties is investigated, employing *trans*-4,5-epoxy-*trans*-2-hexen-1-ol (EH) as a model substrate and propylamine or $N^2$-acetyl-L-lysine 4-methylcoumar-7-ylamide (AcLys-MCA) as model reagents for protein-bound lysine. Independent syntheses are reported for EH and AcLys-MCA. Reaction of the amine components with EH in THF yields 4-(propylamino)-*trans*-2-hexene-1,5-diol and $N^2$-acetyl-$N^6$-(1,5-dihydroxy-*trans*-2-hexen-4-yl)-L-lysine 4-methylcoumaryl-7-amide, respectively. Unequivocal structural characterization of the products prove the epoxy ring cleavage to proceed by a true $S_N2$ mechanism. Incubation of EH with propylamine and AcLys-MCA in aqueous medium at 37 °C shows the turnover to decrease with lower pHs. From reaction of EH (100 mM) with AcLys-MCA (50 mM) under physiological conditions (pH 7.4), 3% of the lysine moieties can be identified in the form of $N^2$-acetyl-$N^6$-(1,5-dihydroxy-*trans*-2-hexen-4-yl)-L-lysine 4-methylcoumar-7-ylamide after 24 h.

**Keywords:** *Lipid oxidation; lysine model reactions; $\gamma$-hydroxy-$\alpha,\beta$-unsaturated epoxides; epoxide ring cleavage; aminol product formation*

## INTRODUCTION

Polyunsaturated fatty acids, incorporating a 1,4-*cis,cis*-pentadiene partial structure, may be oxidized either *via* autoxidation processes or enzymatically; the respective lipoxygenase is present in various plants as well as in certain mammalian tissues. The *cis*–*trans*-conjugated hydroperoxides, resulting from such oxidation, are further converted into an abundance of secondary products. Epoxyols, for instance, are formed under the action of various iron-containing catalysts, such as a $FeCl_3$/cysteine couple (Gardner et al., 1974), hemoglobin (Hamberg, 1975), hematin (Dix et al., 1985), or enzyme systems. Successive action of a lipoperoxidase and an isomerase from oat transfers the 9-hydroperoxyoctadecadienoic acid (9-LHPO) predominantly into 9-hydroxy-*cis*-12,13-epoxy-*trans*-10-octadecenoic acid (Heimann et al., 1973). $\gamma$-Hydroxy-$\alpha,\beta$-unsaturated epoxides have also been characterized as metabolites of linoleic acid hydroperoxides, generated by a prostaglandin endoperoxide synthase from adult or fetal blood vessels (Powell et al., 1983). Analogous structures have been isolated as decomposition products of 13-hydroperoxyoctadecadienoic acid (13-LHPO) from a reaction with carp intestinal acetone powder (Kazuhiko et al., 1986). Incubation of arachidonic acid and its 12-hydroperoxy derivative with a rat lung preparation (Pace-Asciak et al., 1983) also affords $\gamma$-hydroxy-$\alpha,\beta$-unsaturated epoxides.

These epoxides thus represent crucial intermediates in the polyunsaturated fatty acid metabolism, both in foodstuffs and under physiological conditions. Since an epoxy function vicinal to an olefinic double bond is susceptible to hydrolysis, nucleophilic attack by, e.g., the $\epsilon$-amino function of a lysine moiety may likewise be envisaged. In this context, Pokorny et al. (1966) have reported that both 9,10-epoxystearic acid and the respective methyl ester are bonded to albumin without, however, characterizing the structure of the addition product.

Interaction between a lipid hydroperoxide and proteins and/or amino acids constitutes a complex reaction scheme; this complexity stems from both the lipid hydroperoxide itself and the secondary products derived therefrom. Lysine and histidine are the moieties most susceptible to attack by the lipid oxidation products. Nielsen et al. (1985) report a significant loss of lysine (up to 71%) and histidine (up to 57%) in a whey protein/methyl linolenate ($C_{18:3}$)/water model system. Steinbrecher (1987) has found that 32% of the lysine residues of apolipoprotein B are modified by lipid peroxide decomposition products from human low-density lipoprotein (LDL). Due to the innate complexity of these processes, few structures have so far been established unequivocally for the products formed from lipid oxidation derivatives and amino acid moieties.

For studying the reactivity of a given lipid oxidation product, model systems should be employed which facilitate isolation, purification, and structural assignment. We here report on reactions of *trans*-4,5-epoxy-*trans*-2-hexen-1-ol (**3**), a model substrate for the $\gamma$-hydroxy-$\alpha,\beta$-unsaturated epoxides **4**–**6** of linoleic and arachidonic acids (see Figure 1), with propylamine (**1**) and $N^2$-acetyl-L-lysine 4-methylcoumar-7-ylamide (**2**) as model reagents for protein-bound lysine moieties.

## EXPERIMENTAL PROCEDURES

**Materials.** *m*-Chloroperbenzoic acid (25800), propylamine (82100), 7-amino-4-methylcoumarin (08440), 1,1′-carbonyldiimidazole (21860), and di-*tert*-butyl dicarbonate (34660) were obtained from Fluka Chemie AG (Buchs, Switzerland); L-lysine monohydrochloride (5700) and 4-(dimethylamino)pyridine

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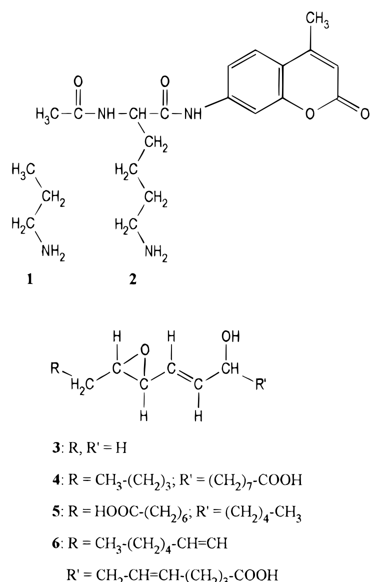



**Figure 1.** Chemical structure of propylamine (**1**), $N^2$-acetyl-L-lysine 4-methylcoumar-7-ylamide (**2**), *trans*-4,5-epoxy-*trans*-2-hexen-1-ol (**3**), 9-hydroxy-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid (**4**), 13-hydroxy-*trans*-9,10-epoxy-*trans*-11-octadecenoic acid (**5**), and 8-hydroxy-*cis*-11,12-epoxy-*all-cis*-5,9,-14-eicosatrienoic acid (**6**).

(820499) were from Merck (Darmstadt, Germany); *trans*-2-*trans*-4-hexadien-1-ol (18,305-9) was from Aldrich Chemical Co. (Milwaukee, WI).

**Synthesis of *trans*-4,5-Epoxy-*trans*-2-hexen-1-ol (3).** Acetylation of 3.0 g (30.5 mmol) of *trans*-2-*trans*-4-hexadien-1-ol (**7**) in a pyridine/acetic anhydride mixture yielded 4.2 g (30 mmol) of *trans*-2-*trans*-4-hexadien-1-ol acetate. A solution of this acetate in 50 mL diethyl ether was cooled to 0 °C in an ice bath, 9.4 g of *m*-chloroperbenzoic acid (≈55%, 30 mmol) added, and the reaction mixture stirred for 24 h at 0 °C. The reaction mixture was extracted three times with 40 mL of 10% aqueous NaOH each and washed three times with 30 mL of water each (negative starch iodine reaction in the washing water indicating complete removal of peroxides). The solvent was stripped off and the residue purified on a silica gel column (hexane/ethyl acetate, 9 + 1, v:v). Fractions of 10 mL were collected and tested for *trans*-4,5-epoxy-*trans*-2-hexen-1-ol acetate (**10**) by TLC ($R_f$ 0.18; solvent mixture as above; detection, spray reagent 5% $H_2SO_4$ in anhydrous ethanol/10 min at 120 °C). All fractions containing **10** were combined and evaporated to dryness. The colorless oily residue (1.6 g, 10.3 mmol, 34%) was dissolved in 21 mL of methanol, 1.5 g of potassium carbonate in 9 mL of water added, and the mixture stirred for 1 h at room temperature. The solution was diluted with 50 mL of water and extracted five times with 70 mL of ether each. The combined ether layers were washed twice with 30 mL of water each (the aqueous phase was tested for neutral reaction), dried with anhydrous $Na_2SO_4$, filtered, and concentrated. *trans*-4,5-Epoxy-*trans*-2-hexen-1-ol (**3**) was obtained as a colorless oil: 0.7 g (6.1 mmol, 20%). GLC: $t_R$ = 7.3 min. CI-GLC/MS: *m/z* 115 ($MH^+$, 1), 97 (100), 83 (10), 79 (25), 75 (7). $^1$H-NMR, $^{13}$C-NMR ($CDCl_3$): see Table 1.

**Synthesis of 4-(*N*-Acetyl-*N*-propylamino)-1,5-diacetoxy-*trans*-2-hexene (12).** A mixture of 55 mg (0.5 mmol) of *trans*-4,5-epoxy-*trans*-2-hexen-1-ol (**3**) and 59 mg (1 mmol) of propylamine in 0.5 mL of anhydrous THF was heated for 16 h to 100°C in a tightly sealed tube. The solvent was stripped off, and unreacted starting material removed *in vacuo* (5 Pa, 2 h). The residue was acetylated with pyridine/acetic anhydride in the presence of catalytic amounts of 4-(dimethylamino)pyridine, yielding 90 mg (0.30 mmol, 62%) of 4-(*N*-acetyl-*N*-propylamino)-1,5-diacetoxy-*trans*-2-hexene (**12**). GLC: $t_R$ = 27.6 min. CI-GLC/MS: m/z 300($MH^+$, 1), 240 (100), 212 (2), 199 (2), 180 (4), 138 (3), 110 (4). $^1$H-NMR, $^{13}$C-NMR ($CDCl_3$): see Table 1.

**Incubation of Propylamine with 3 in Aqueous Medium at 37 °C.** A solution of propylamine in 0.8 mL of 0.2 N HCl (14.7 mg/mL) and 1 mL of the respective Sörensen phosphate buffer (0.33 M, pH 9.0, 8.0, 7.0, 6.0) were combined, and 46 mg (0.4 mmol) of **3** was added. The pH was adjusted to the respective buffer value and the volume filled up to 2 mL. This mixture was incubated for 24 h at 37 °C, lyophilized, acetylated as described for **12**, and analyzed by GLC/MS.

**Reaction of Propylamine with 3 under Acid Conditions.** To 23 mg (0.2 mmol) of **3** in 0.8 mL of ethyl acetate was added a solution of 47 mg (0.8 mmol) of propylamine in 0.2 mL of water (acidified with hydrochloric acid to pH 1−2). The resulting emulsion was stirred for 3 h at room temperature and evaporated to dryness, and the residue was acetylated as described for **12** and analyzed by GLC/MS. The TIC showed an additional peak with the same $MH^+$ ion as **12** and the following data. GLC: $t_R$ = 26.1 min. CI-GLC/MS: m/z 300 ($MH^+$; 2), 240 (100), 199 (24), 169 (7), 149 (17), 112 (7), 97 (34).

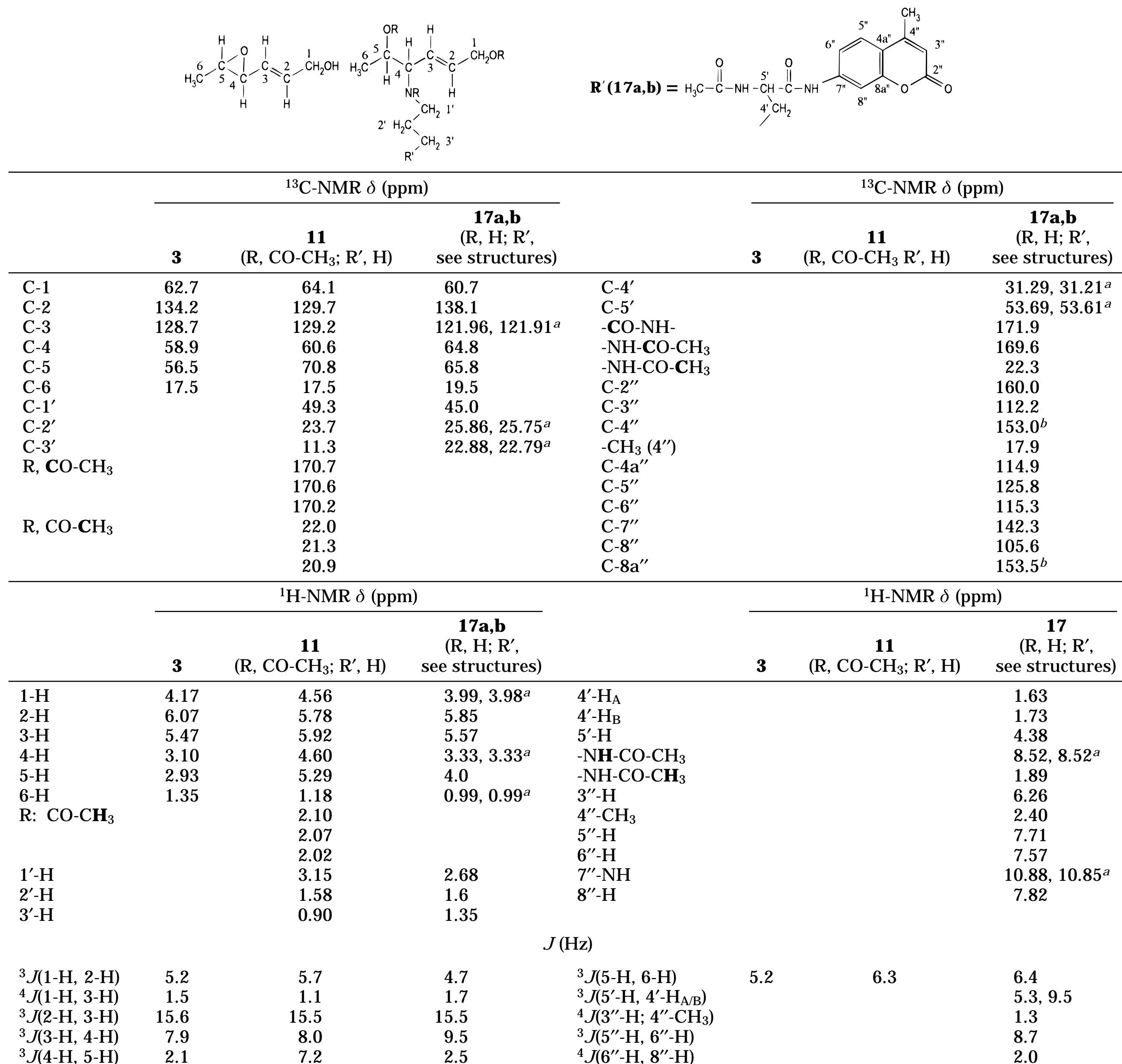
**Synthesis of $N^2$-Acetyl-L-lysine 4-Methylcoumar-7-ylamide (2).** *$N^6$-(tert-Butyloxycarbonyl)-L-lysine (**13**).* **13** was synthesized according to the procedures of Moroder et al. (1976) and Bayer et al. (1973).

*$N^2$-Acetyl-$N^6$-(tert-butyloxycarbonyl)-L-lysine (**14**).* A solution of 8.4 g (34 mmol) of **13** in 43 mL of 1 N NaOH was cooled to 0 °C in an ice bath. Every 3 min, 0.65 mL of acetic anhydride and 8.7 mL of 1 N NaOH were added alternately for 0.5 h. The reaction mixture was stirred for 20 min at room temperature, adjusted to pH 7.0, and extracted three times with 90 mL ether each. The organic layer was discarded, and the pH of the aqueous phase was adjusted to 3.0 with 2 N HCl. The solution was extracted seven times with 90 mL of $CH_2Cl_2$ each; the $CH_2Cl_2$ phase was washed twice with 50 mL of water each and dried with anhydrous $Na_2SO_4$. The solvent was stripped off and the crude product dried *in vacuo* (5 Pa) over KOH. The $^1$H-NMR analysis of the $N^2$-acetyl-$N^6$-(*tert*-butyloxycarbonyl)-L-lysine (**14**) thus obtained (5.7 g, 19.8 mmol, 58%) showed the product to still contain 2% acetic acid.

FAB-MS (*m*-nitrobenzyl alcohol): 599 ($[M_2Na]^+$, 11) 577 ($[M_2H]^+$, 31), 311 ($[MNa]^+$, 51), 289 ($[MH]^+$, 98), 233 (79), 189 (100), 128 (15), 84 (20), 57(9). $^1$H-NMR (DMSO-$d_6$): $\delta$ 12.41 (s, 1H), 8.09 (d, 1H, $J$ = 7.8 Hz), 6.79 (t, 1H, $J$ = 5.5 Hz), 4.11 (ddd, 1H, $J$ = 4.9, 7.8, 8.9 Hz), 2.90 (ddt, 1H, $J$ = 5.5, 7.5, (−)14.8 Hz), 2.87 (ddt, 1H, $J$ = 5.5, 7.5, (−)14.8 Hz), 1.84 (s, 3H), 1.65 (dddd, 1H, $J$ = 5.0, 6.7, 9.5, (−)13.2 Hz), 1.54 (m, 1H, $J$ = 5.5, 8.9, (−)13.2 Hz), 1.37 (s, 9H), 1.35 (m, 2H), 1.27 (m, 2H). $^{13}$C-NMR (DMSO-$d_6$): $\delta$ 173.8, 169.2, 155.5, 77.3, 51.7, 39.5, 30.7, 29.1, 28.2, 22.7, 22.2.

*$N^2$-Acetyl-$N^6$-(tert-butyloxycarbonyl)-L-lysine 4-Methylcoumar-7-ylamide (**16**).* A mixture of 5.4 g (18.8 mmol) of $N^2$-acetyl-$N^6$-(*tert*-butyloxycarbonyl)-L-lysine (**14**) and 3.25 g (20.6 mmol) of 1,1′-carbonyldiimidazole in 150 mL of anhydrous THF was kept for 1 h at room temperature (until $CO_2$ formation had subsided); 2.5 g (14.3 mmol) of 7-amino-4-methylcoumarin (**15**) was added and the reaction mixture incubated for 9 days at 50 °C in a tightly closed tube. Progress of the reaction was monitored by HPLC equipped with a diode array detector (DAD). A methanol/0.01 M phosphate buffer (pH 4.0) gradient was employed (% MeOH($t$ min)): 5(0)−95(30)−95(40)−5(45)−5(55); $t_R$(**15**) = 18.3 min, $t_R$(**16**) = 25.2 min. Transformation of **15** to **16** had reached 66% after 9 days. The solvent was stripped off and the residue taken up in 400 mL of ethyl acetate and extracted twice with 80 mL of 5% aqueous $NaHCO_3$ each (**16** already precipitated during the extraction). The aqueous layer was discarded and the organic phase reduced to 150 mL, and kept at 4°C for 24 h. The precipitate was filtered off, washed twice with 10 mL of ethyl acetate each, and dried *in vacuo* (5 Pa), yielding 3.8 g (8.5 mmol, 60%) of $N^2$-acetyl-$N^6$-(*tert*-butyloxycarbonyl)-L-lysine 4-methylcoumar-7-ylamide (**16**). FAB-MS (*m*-nitrobenzyl alcohol): 446 ($[MH]^+$, 33), 445($M^{\bullet+}$; 21), 346 (100), 57 (59). $^1$H-NMR (DMSO-$d_6$): $\delta$ 10.55 (s, 1H), 8.28 (d, 1H, $J$ = 7.5 Hz), 7.80 (d, 1H, $J$ = 2.1 Hz), 7.71 (d, 1H, $J$ = 8.7 Hz), 7.50 (dd, 1H, $J$ = 2.1, 8.7 Hz), 6.80 (t, 1H, $J$ = 5.8 Hz), 6.27 (q, 1H, $J$ = 1.3 Hz), 4.38 (ddd, 1H, $J$ = 5.5, 7.5, 8.9 Hz), 2.40 (d, 3H, $J$ = 1.3 Hz), 1.90 (s, 3H), 2.91 (m, 2H), 1.69 (m, 1H), 1.62 (m, 1H), 1.38 (m, 2H), 1.36 (s,

**Table 1. $^{1}$H- and $^{13}$C-NMR Spectroscopic Data of Compounds 3 and 11 (0.1 M, in $CDCl_3$) and 17a,b (0.1 M, in DMSO-$d_6$)**



| | $^{13}$C-NMR δ (ppm) | | | | $^{13}$C-NMR δ (ppm) | | |
|---|---|---|---|---|---|---|---|
| | **3** | **11** (R, CO-$CH_3$; R′, H) | **17a,b** (R, H; R′, see structures) | | **3** | **11** (R, CO-$CH_3$ R′, H) | **17a,b** (R, H; R′, see structures) |
| C-1 | 62.7 | 64.1 | 60.7 | C-4′ | | | 31.29, 31.21[a] |
| C-2 | 134.2 | 129.7 | 138.1 | C-5′ | | | 53.69, 53.61[a] |
| C-3 | 128.7 | 129.2 | 121.96, 121.91[a] | -**C**O-NH- | | | 171.9 |
| C-4 | 58.9 | 60.6 | 64.8 | -NH-**C**O-$CH_3$ | | | 169.6 |
| C-5 | 56.5 | 70.8 | 65.8 | -NH-CO-**C**$H_3$ | | | 22.3 |
| C-6 | 17.5 | 17.5 | 19.5 | C-2″ | | | 160.0 |
| C-1′ | | 49.3 | 45.0 | C-3″ | | | 112.2 |
| C-2′ | | 23.7 | 25.86, 25.75[a] | C-4″ | | | 153.0[b] |
| C-3′ | | 11.3 | 22.88, 22.79[a] | -$CH_3$ (4″) | | | 17.9 |
| R, **C**O-$CH_3$ | | 170.7 | | C-4a″ | | | 114.9 |
| | | 170.6 | | C-5″ | | | 125.8 |
| | | 170.2 | | C-6″ | | | 115.3 |
| R, CO-**C**$H_3$ | | 22.0 | | C-7″ | | | 142.3 |
| | | 21.3 | | C-8″ | | | 105.6 |
| | | 20.9 | | C-8a″ | | | 153.5[b] |

| | $^{1}$H-NMR δ (ppm) | | | | $^{1}$H-NMR δ (ppm) | | |
|---|---|---|---|---|---|---|---|
| | **3** | **11** (R, CO-$CH_3$; R′, H) | **17a,b** (R, H; R′, see structures) | | **3** | **11** (R, CO-$CH_3$; R′, H) | **17** (R, H; R′, see structures) |
| 1-H | 4.17 | 4.56 | 3.99, 3.98[a] | 4′-$H_A$ | | | 1.63 |
| 2-H | 6.07 | 5.78 | 5.85 | 4′-$H_B$ | | | 1.73 |
| 3-H | 5.47 | 5.92 | 5.57 | 5′-H | | | 4.38 |
| 4-H | 3.10 | 4.60 | 3.33, 3.33[a] | -N**H**-CO-$CH_3$ | | | 8.52, 8.52[a] |
| 5-H | 2.93 | 5.29 | 4.0 | -NH-CO-C**H**$_3$ | | | 1.89 |
| 6-H | 1.35 | 1.18 | 0.99, 0.99[a] | 3″-H | | | 6.26 |
| R: CO-C**H**$_3$ | | 2.10 | | 4″-$CH_3$ | | | 2.40 |
| | | 2.07 | | 5″-H | | | 7.71 |
| | | 2.02 | | 6″-H | | | 7.57 |
| 1′-H | | 3.15 | 2.68 | 7″-NH | | | 10.88, 10.85[a] |
| 2′-H | | 1.58 | 1.6 | 8″-H | | | 7.82 |
| 3′-H | | 0.90 | 1.35 | | | | |
| ***J*** **(Hz)** | | | | | | | |
| $^{3}J$(1-H, 2-H) | 5.2 | 5.7 | 4.7 | $^{3}J$(5-H, 6-H) | 5.2 | 6.3 | 6.4 |
| $^{4}J$(1-H, 3-H) | 1.5 | 1.1 | 1.7 | $^{3}J$(5′-H, 4′-$H_{A/B}$) | | | 5.3, 9.5 |
| $^{3}J$(2-H, 3-H) | 15.6 | 15.5 | 15.5 | $^{4}J$(3″-H; 4″-$CH_3$) | | | 1.3 |
| $^{3}J$(3-H, 4-H) | 7.9 | 8.0 | 9.5 | $^{3}J$(5″-H, 6″-H) | | | 8.7 |
| $^{3}J$(4-H, 5-H) | 2.1 | 7.2 | 2.5 | $^{4}J$(6″-H, 8″-H) | | | 2.0 |

[a] Hydrogen and/or carbon chemical shifts for the two diastereoisomers 17a,b. [b] Assignment for these two carbons may have been reversed. δ (ppm), chemical shift for the indicated hydrogen/carbon; *J* (Hz), coupling constant between the indicated protons. Hydrogen/carbon assignment is validated by $^{1}$H,$^{1}$H-COSY, $^{1}$H,$^{13}$C-COSY, and $^{13}$C-DEPT technique.

9H), 1.37−1.22 (m, 2H). $^{13}$C-NMR (DMSO-$d_6$): δ 171.8, 169.5, 160.0, 155.5, 153.6, 153.0, 142.2, 125.8, 115.2, 115.0, 112.2, 105.6, 77.3, 53.6, 39.6, 31.5, 29.2, 28.2, 22.8, 22.3, 17.9.

*$N^2$-Acetyl-L-lysine 4-Methylcoumar-7-ylamide (**2**).* $N^2$-Acetyl-$N^6$-(*tert*-butyloxycarbonyl)-L-lysine 4-methylcoumar-7-ylamide (**16**) (3.7 g, 8.3 mmol) was suspended in an ethyl acetate/hydrochloric acid (37%) mixture (88 + 12, v:v) and stirred for 30 min at room temperature. Water was added (200 mL), the pH adjusted to 3.0 with 10% aqueous NaOH, and the solution extracted three times with 100 mL of $CH_2Cl_2$ each; the organic layer was discarded. The pH of the aqueous phase was adjusted to 12.5 by further addition of NaOH and the solution extracted five times with 100 mL of $CH_2Cl_2$ each. The organic solvent was dried with anhydrous $Na_2SO_4$ and concentrated. $N^2$-Acetyl-L-lysine 4-methylcoumar-7-ylamide (**2**) was obtained as colorless crystals (1.7 g, 4.9 mmol, 59%): mp 200−202 °C. FAB-MS (*m*-nitrobenzyl alcohol): 346 ([MH]$^+$, 100). $^{1}$H-NMR (DMSO-$d_6$): δ 8.25 (d, 1H, $J$ = 7.6 Hz), 7.80 (d, 1H, $J$ = 2.0 Hz), 7.72 (d, 1H, $J$ = 8.7 Hz), 7.50 (dd, 1H, $J$ = 2.0, 8.7 Hz), 6.27 (q, 1H, $J$ = 1.2 Hz), 4.38 (ddd, 1H, $J$ = 5.4, 7.6, 8.8 Hz), 2.53 (m, 2H), 2.40 (d, 3H, $J$ = 1.2 Hz), 1.88 (s, 3H), 1.68 (m, 1H), 1.61 (m, 1H), 1.43−1.27 (m, 4H). $^{13}$C-NMR (DMSO-$d_6$): δ 171.9, 169.4, 159.9, 153.6, 153.0, 142.2, 125.8, 115.2, 115.0, 112.2, 105.6, 53.7, 41.4, 32.9, 31.7, 22.9, 22.3, 17.9. UV ($H_2O$): $\lambda_{max}$ (nm) (log ε) 326 (4.25), 296 (4.06), 228 (4.27), 204 (4.54). Fluorescence ($H_2O$): $\lambda_{ex}$ (nm) 326, $\lambda_{em}$ (nm) 390.

**Synthesis of $N^2$-Acetyl-$N^6$-(1,5-dihydroxy-*trans*-2-hexen-4-yl)-L-lysine 4-Methylcoumar-7-ylamide (17a,b).** A suspension of 86 mg (0.25 mmol) of $N^2$-acetyl-L-lysine-4-methylcoumar-7-ylamide (**2**) and 79 mg (0.69 mmol) of *trans*-4,5-epoxy-*trans*-2-hexen-1-ol (**3**) in 0.5 mL of anhydrous THF was heated for 12 h to 100 °C. Progress of the reaction was monitored by HPLC/DAD. A methanol/0.01 M phosphate buffer (pH 4.0) gradient was employed (% MeOH(*t*, min)): 5(0)−60(35)−95(40)−95(50)−5(55)−5(65); $t_R$(**2**) = 23.0 min, $t_R$(**17a,b**) = 24.4 min. The solvent was stripped off and the residue taken up in 5 mL of the proper eluent and subjected to preparative HPLC. Fractions with $t_R$ (preparative HPLC) of 10.2 min were combined and yielded, after lyophilization, 70 mg (0.14 mmol, 55%) of compounds **17a,b** as the respective

formiates. FAB-MS (*m*-nitrobenzyl alcohol): 460 ([MH]$^+$; 100), 414 (19), 176 (7), 84(8). $^1$H-NMR, $^{13}$C-NMR (DMSO-$d_6$): see Table 1. UV absorbance and fluorescence data are identical with those of compound **2**.

**Incubation of $N^2$-Acetyl-L-lysine 4-Methylcoumar-7-ylamide (2) with 3 in Aqueous Medium at 37 °C.** To a solution of 34.5 mg (0.1 mmol) of **2** in 0.8 mL of 0.1 N HCl was added 1 mL of the respective Sörensen phosphate buffer (0.33 M, pH 9.0, 7.4, 6.0) and 23 mg (0.2 mmol) of **3**. The pH was adjusted to the respective buffer value and the volume filled up to 2 mL. The reaction mixture was incubated for 48 h at 37 °C; after 6, 24, and 48 h, respectively, an aliquot (20 $\mu$L) was taken from each tube, diluted with water to 5 mL, and subjected to HPLC.

**Melting points** were determined on an electrothermal apparatus (8600) and are not corrected.

**Spectra.** Ultraviolet (UV) spectra were measured with a Perkin-Elmer Lambda 2 instrument (Überlingen, Germany) and fluorescence spectra with a Spex Fluorolog 2 instrument (Edison, NJ). $^1$H- and $^{13}$C-nuclear magnetic resonance (NMR) spectra were recorded on Bruker (Karlsruhe, Germany) AC-250/ARX-500 spectrometers at 250/500 and 63/126 MHz nominal frequency, respectively. Chemical shifts are given in $\delta$ (ppm) relative to TMS (tetramethylsilane) as internal standard; coupling constants $J$ (Hz): s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, with dd, dt, ddd, ddt, and dddd denoting a combination of the respective multiplicities. Liquid secondary-ion mass spectra (SIMS, analogous to FAB-MS) were obtained on a Finnigan MAT 95 instrument (Bremen, Germany).

**Analytical High-Performance Liquid Chromatography (HPLC).** The HPLC system consisted of an HP1050 autosampler, an HP1050 gradient pump, an HP1050 DAD module (Hewlett Packard, Waldbronn, Germany), and a Shimadzu (Kyoto, Japan) RF 535 fluorescence monitor. For data aquisition and processing, an HP 3D Chem Station (Revision A. 03.01) software system was used. Column (Bischoff, Leonberg, Germany): Nucleosil RP 18, 5 $\mu$m, 250 × 4 mm; flow rate 0.8 mL·min$^{-1}$; detection wavelengths for DAD at 230, 326, and 348 nm with a spectral band width (SBW) of 4 nm, reference 500 nm (SBW 100 nm); fluorescence excitation wavelength 326 nm, emission wavelength 390 nm.

**Preparative HPLC Purification.** A Knauer (Berlin, Germany) 64 liquid chromatograph was employed, combined with a A0293 variable wavelength detector and a Kronlab (Sinsheim, Germany) HPLC column (guard column 20 × 50 mm, column 20 × 250 mm; Nucleosil RP 18, 7 $\mu$m): flow rate 10 mL·min$^{-1}$; MeOH/0.01 M $NH_4HCOO$ buffer (pH 4) 45 + 55, v:v); injection volume 1.5 mL; detection at 326 nm.

**Capillary Gas-Liquid Chromatograms (GLC/FID).** These were run on a Perkin-Elmer (Überlingen, Germany) 8600 instrument: flame-ionization detector (FID); quartz capillary column (25 m, i.d. 0.32 mm, PVMS 54, 1 $\mu$m, He, 80 kPa, 33 cm·s$^{-1}$); injection and detection ports 270 °C; temperature program 80 °C → 270 °C/6 °C·min$^{-1}$, 10 min isothermal 270 °C.

**Gas-Liquid Chromatography/Mass Spectrometry Analysis.** GLC/MS was performed on a Finnigan MAT Ion Trap 800, 70 eV EI and positive $CH_3OH$-CI mode, coupled to a Perkin-Elmer 8420 gas chromatograph: quartz capillary column (25 m, i.d. 0.25 mm, PVMS 54, 0.3 $\mu$m, He, 80 kPa, 21 cm·s$^{-1}$); injection port 270 °C; temperature program as given above.

**Liquid Chromatography.** Silica gel 60 $F_{254}$ (Merck 5554 and 5717, Darmstadt, Germany) was used for thin-layer chromatography (TLC), and silica gel (63-200 $\mu$m) (Baker 0253, Gross-Gerau, Germany) for column chromatography.

**Lyophilization.** A Leybold-Heraeus (Köln, Germany) Lyovac GT 2 instrument was applied.

## RESULTS AND DISCUSSION

For studying the reactivity of $\gamma$-hydroxy-$\alpha$,$\beta$-unsaturated epoxides toward protein-bound lysine $\epsilon$-amino functions, *trans*-4,5-epoxy-*trans*-2-hexen-1-ol (**3**) was

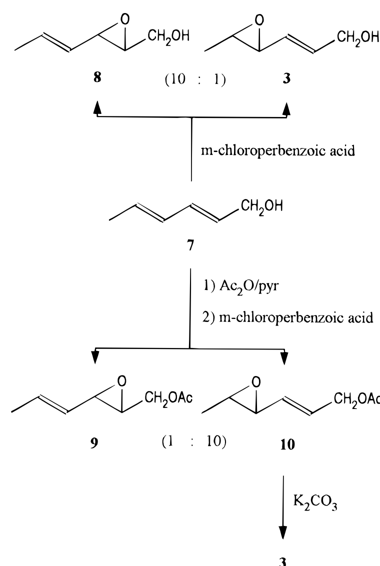



**Figure 2.** Reaction pathway for the synthesis of *trans*-4,5-epoxy-*trans*-2-hexen-1-ol (**3**).

considered a suitable model substrate. Since this compound has not been reported in the literature, we have devised an independent synthesis of **3**, starting from *trans*-2-*trans*-4-hexadien-1-ol (**7**) (Figure 2). Direct epoxidation with *m*-chloroperbenzoic acid yields *trans*-2,3-epoxy-*trans*-4-hexen-1-ol (**8**) and the desired isomeric epoxide **3** in a 10:1 ratio, as shown by gas-liquid chromatography (GLC) and nuclear magnetic resonance (NMR) analysis. If **7** is transformed, however, into the acetate and then subjected to epoxidation, the ratio of the 2,3- and 4,5-epoxides **9** and **10** is inverted. From the crude reaction mixture, pure **10** may be obtained by column chromatography on silica gel. The acetate group is cleaved with potassium carbonate in a methanol/water mixture, and the structure of the product is proven unequivocally as *trans*-4,5-epoxy-*trans*-2-hexen-1-ol (**3**) by the $^1$H- and $^{13}$C-NMR and mass spectrometry (MS) data (see below).

Propylamine (**1**) represents a simple but efficient model for protein-bound lysine and has thus been successfully employed in Maillard chemistry for years (Ledl et al., 1990). First reactions of **1** and **3** were carried out in a non-nucleophilic, aprotic solvent to avoid nucleophilic attack of solvent molecules. A concentrated solution of **1** and **3** in THF was incubated for 16 h at 100°C, the solvent evaporated to dryness, and the residue acetylated by pyridine/acetic anhydride with catalytic amounts of 4-(dimethylamino)pyridine added to ensure peracetylation. Coupled GLC/mass spectrometry analysis in CI mode (CI-GLC/MS) shows one main peak, besides traces of **10**, in the total ion chromatogram (TIC). The mass of the quasimolecular ion for this peak (MH$^+$, 300 Da) proves 1:1 addition of **1** and **3**. Since only this one compound is formed in nearly quantitative yield, the crude product may be employed for NMR analyses which definitively establish the 4-(*N*-acetyl-*N*-propylamino)-1,5-diacetoxy-*trans*-2-hexene (**12**) structure (Figure 3). Formation of the precursor 4-(propylamino)-*trans*-2-hexene-1,5-diol (**11**) as the sole product from the reaction of **1** and **3** clearly proves that, under the given conditions, nucleophilic attack of the amine occurs exclusively in the allylic position at C-4 and that the reaction strictly follows an $S_N2$ mechanism. An $S_N1$ process as a mechanistic alternative would proceed *via* the allylic carbocation which then could be attacked, by the respective nucleophile, at either C-2 or C-4. Such

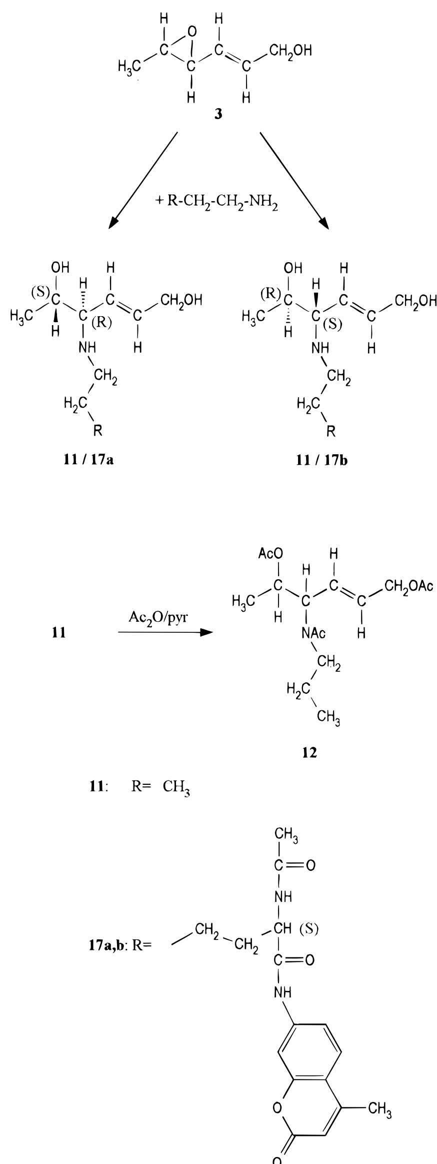


**Figure 3.** Reaction scheme for the formation of 4-(propylamino)-*trans*-2-hexene-1,5-diol (**11**) and $N^2$-acetyl-$N^6$-(1,5-dihydroxy-*trans*-2-hexen-4-yl)-L-lysine 4-methylcoumar-7-yl-amide (**17a,b**) from **3**. Acetylation of **11** yields 4-(*N*-acetyl-*N*-*n*-propylamino)-1,5-diacetoxy-*trans*-2-hexene (**12**).

a mechanism would by necessity lead to formation of two regioisomers, probably with a ratio far from 1:1, each of these, in turn, being formed as a pair of diastereoisomers. An $S_N2'$ mechanism as a third possible route can also be ruled out since not even traces of a compound with allylic shift are formed. The preference for nucleophilic attack at C-4 rather than at C-5 is due probably to the resonance stabilization of the $S_N2$ process transition state vicinal to a double bond.

To test whether **11** is also formed under milder reaction conditions and in the presence of water, compounds **1** (100 mM) and **3** (200 mM) were incubated in various phosphate buffers (0.17 M, pH 9.0, 8.0, 7.0, 6.0) for 48 h at 37 °C. The lyophilized reaction mixtures were analyzed, after acetylation, by GLC and GLC/MS. The FID and TIC chromatograms show formation of **11** to decrease with lower pHs, hydrolysis becoming the dominant reaction. Reaction at pH 7.0 already affords only traces of **12**; at pH 6.0, no **12** can be detected anymore. It seems to be a reasonable assumption that at lower pH values protonation of the epoxide becomes

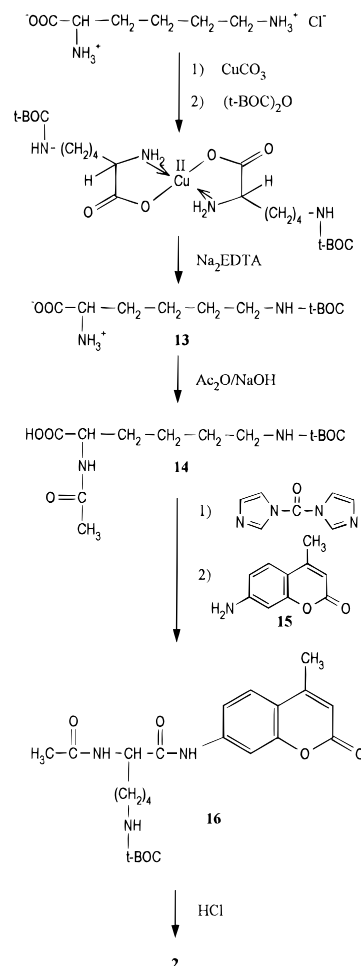


**Figure 4.** Reaction pathway for the synthesis of $N^2$-acetyl-L-lysine 4-methylcoumar-7-yl amide (**2**).

more and more favored, with the reaction thus changing over to $S_N1$. The CI-GLC/MS analysis of a concentrated emulsion of **1** and **3** in an ethyl acetate/water system, acidified with hydrochloric acid, indeed shows another small peak with the same $MH^+$ ion as **12**. Since only this GLC/MS trace was available, the structure could not be definitely resolved; a scrupulous structural elucidation would have to be based on a $^1H$-NMR analysis.

For an in-depth study of the reaction of **3** with lysine moieties, we decided to synthesize a lysine derivative which incorporates a fluorescent chromophore, and thus allows direct and sensitive high-performance liquid chromatographic (HPLC) monitoring of the reaction. In addition, labeling of the amine component enables a straightforward correlation between the HPLC peak areas of the lysine educt and eventual reaction products. Figure 4 outlines the synthetic pathway for such a derivative: $N^6$-(*tert*-butyloxycarbonyl)-L-lysine (**13**), although commercially available, is easily synthesized from L-lysine following procedures reported by Moroder et al. (1976) and Bayer et al. (1973) and acetylated in sodium hydroxide solution to yield $N^2$-acetyl-$N^6$-(*tert*-butyloxycarbonyl)-L-lysine (**14**). In the next step, the 7-amino-4-methylcoumarin fluorophore (**15**) is introduced. The resulting amide exhibits a characteristic ultraviolet (UV) spectrum and shows a high fluorescence quantum yield. Gratuitously, it is relatively inert toward oxidation, hydrolysis, and aminolysis. Also, the fluorophore bears no nucleophilic groups which might interfere in the epoxide cleavage. The optimum reaction

parameters for coupling **15** with an amino acid carboxyl group are discussed controversially in the literature (Zimmerman et al., 1977; Khammungkhune et al., 1980; Kanaoka et al., 1977). We have therefore tested dicyclohexylcarbodiimide (DCC), isobutyl chloroformate, and 1,1′-carbonyldiimidazole in anhydrous THF for activating the carboxyl group, employing *N*-acetylleucine as model substrate. 1,1′-Carbonyldiimidazole gave the best yield and allowed for the most simple workup, although the rather long reaction time represents a tedious aspect. Coupling of $N^2$-acetyl-$N^6$-(*tert*-butyloxycarbonyl)-L-lysine (**14**) with **15** under the optimized reaction parameters yields 60% of $N^2$-acetyl-$N^6$-(*tert*-butyloxycarbonyl)-L-lysine 4-methylcoumaryl-7-amide (**16**). The protecting group is cleaved off with hydrochloric acid, and the resulting $N^2$-acetyl-L-lysine 4-methylcoumar-7-ylamide (**2**) which so far has not been described in the literature characterized, as all precursors, by $^1$H- and $^{13}$C-NMR as well as by fast-atom-bombardment mass spectrometry (FAB-MS).

For the synthesis of $N^2$-acetyl-$N^6$-(1,5-dihydroxy-*trans*-2-hexen-4-yl)-L-lysine 4-methylcoumar-7-ylamide (**17a,b**) (Figure 3), *trans*-4,5-epoxy-*trans*-2-hexen-1-ol (**3**) is heated with **2** in THF. The progress of the reaction is monitored by HPLC, turnover being almost quantitative within 12 h. The solvent is stripped off and the residue taken up in the proper solvent mixture for preparative HPLC. The structure of the isolated products was proven unequivocally as **17a,b** (Figure 3) from their spectroscopic data; we did not succeed in separating the two diastereoisomers chromatographically.

The fact that reaction of **2** and **3** indeed affords these two diastereoisomers represents an independent proof for the epoxide ring being cleaved by an $S_N2$ mechanism. With the stereochemistry of the oxirane **3** established as *trans*, the absolute configuration consequently must be 4-*S*/5-*S* or 4-*R*/5-*R*, respectively (for the numbering, see Table 1). The ring cleavage product **17a,b** then is formed, in the course of an $S_N2$ process with Walden inversion, as 4-*R*/5-*S* and 4-*S*/5-*R*; these two configurations are enantiotopic. Since the added nucleophile, however, *i.e.,* the (*S*)-lysine derivative **2**, is by itself chiral, the addition product is formed as a pair of diastereoisomers. In contrast, addition of the achiral propylamine as nucleophile generates a pair of enantiomers. If the cleavage of the epoxide ring in **3** by the amine **2** proceeded *via* an $S_N1$ process, the stereochemical correlation at C-4 and C-5 would be lost in the intermediate allylic carbocation. Thus, at least four diastereoisomers would be expected for an $S_N1$ attack (for a detailed discussion of the spectroscopic data, see below).

In analogy to incubation of **1** with *trans*-4,5-epoxy-*trans*-2-hexen-1-ol (**3**) in aqueous media, phosphate-buffered solutions of **2** (50 mM) and **3** (100 mM) were kept at 37 °C, progress of the reaction being monitored by HPLC equipped with both a DAD and a fluorescence detector. As outlined above, the reaction is strongly pH dependent. At pH 9.0, 33% of **2** is transformed, within 6 h, into $N^2$-acetyl-$N^6$-(1,5-dihydroxy-*trans*-2-hexen-4-yl)-L-lysine 4-methylcoumar-7-ylamide (**17a,b**); conversion mounts to 50% within 24 h. As expected, formation of **17a,b** is reduced under physiological conditions (pH 7.4), approximately 2% of lysine residues being detectable as **17a,b** after 6 h and 3%, respectively, after 24 h. The HPLC chromatogram shows an additional peak (2% after 24 h), eluted directly after **17a,b**, the UV and fluorescence characteristics of which are identical with those of **2** and **17a,b**. Under these conditions, the reaction may no longer strictly proceed *via* $S_N2$ attack at C-4, further regio- and/or diastereoisomers perhaps being formed besides **17a,b**. At pH 6.0, compound **2** does not react anymore with **3**. For all reaction conditions, disappearance of **3** is observed by thin layer chromatography (TLC), supporting the observation (obtained by GLC analysis) from the propylamine incubations that hydrolysis of the epoxide **3** becomes the major reaction pathway at lower pH. At pH 7.4, traces of **3** can still be found after 24 h, whereas at pH 6.0, **3** is completely hydrolyzed within 6 h. The severely reduced nucleophilicity of the lysine $\epsilon$-amino function at lower pH may well be the decisive cause for the decreasing formation of **17a,b**. A similar pH dependence has been observed by Zamora et al. (1995) for reaction of protein-bound lysine with *trans*-4,5-epoxy-*trans*-2-heptenal.

The results of the model study reported above clearly show that lysine moieties, incorporated in a protein, may react with $\gamma$-hydroxy-$\alpha,\beta$-unsaturated epoxides generated in the course of lipid oxidation. Formation of aminol structures such as compounds **11** and **17a,b** (Figure 3) competes with attack by, e.g., water or other nucleophiles. Under almost anhydrous conditions, for instance in foodstuffs with a low water content, aminolysis of $\alpha,\beta$-unsaturated epoxides by lysine moieties may therefore be supposed to be an important mode of interaction between proteins and lipid oxidation products. Since formation of aminols also takes place under physiological conditions (pH 7.4, 37 °C), lipoproteins may be expected to be especially susceptible to such transformations, due to the proximity of the respective reaction partners and the low water activity in the hydrophobic regions of such globular proteins. Further investigations are in progress to transfer the experiences of the model study to biological systems and to either verify or disprove these hypotheses.

## STRUCTURAL ASSIGNMENT

Special effort was invested in unequivocally establishing the structure of compounds **3**, **11**, and **17a,b** since the mechanistic arguments rest on the respective product structures. The $^1$H- and $^{13}$C-NMR chemical shifts ($\delta$) and coupling constants ($J$) are collected in Table 1.

The *trans* configuration of the epoxide ring in **3** is proven definitely by the value of the vicinal coupling constant between 4-H and 5-H ($^3J$ = 2.1 Hz); for the alternative *cis* orientation, a $^3J$ value of at least 5−6 Hz is expected. The absolute configuration at C-4 and C-5 therefore can only be *SS* or *RR*, and **3** thus exists as a pair of enantiomers. From the structure of the individual products obtained, the mechanism of the nucleophilic cleavage of the epoxide ring in **3** can be derived in a straightforward manner. The 4-H and 5-H resonances in the reaction products **11** and **17a,b** were assigned on the basis of the respective multiplicities and by correlation spectroscopy ($^1$H,$^1$H-COSY). In both product structures **11** and **17a,b**, the resonances of 5-H (5.29 and 4.00 ppm, respectively) appear shifted to lower field by 0.7 ppm relative to those of the proton at C-4 (4.60 and 3.33 ppm), clearly establishing the OR-function to have been introduced at C-5 ($OCO$-$CH_3$, **11**; OH, **17a,b**) and the amine moiety at C-4. An allylic shift, *via* an $S_N2'$ mechanism, is likewise ruled out definitely by the spectroscopic evidence. Both olefinic protons, 2-H and 3-H, appear split into triplets in **11** and **17a,b**: $^3J$ = 5.7 and 4.7 Hz, $^4J$ = 1.1 and 1.7 Hz,

respectively. This is possible only with the $CH_2OH$ function adjacent to the double bond. This assignment is further confirmed by the $^1H,^1H$-COSY spectrum. The $^1H$- and $^{13}C$-NMR spectra of compound **11** each show one set of signals only; thus, one of the two possible diastereoisomers has been formed exclusively. In contrast, a number of resonances in the spectra of compound **17**, marked with a superscript index *a* in Table 1, appear doubled (1:1 relative intensity), indicating **17** to exist as a pair of diastereoisomers, **17a**,**17b**.

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