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Carbohydrate Research 301 (1997) 111–121

CARBOHYDRATE RESEARCH

Amadori products from model reactions of D-glucose with phosphatidyl ethanolamine—Independent synthesis and identification of 1-deoxy-1-(2-hydroxyethylamino)-D-fructose derivatives

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Received 12 August 1996; accepted in revised form 10 February 1997

Abstract

Nonenzymatic glycosylation of aminophospholipids is supposed to play an important role for lipid oxidation in vivo. Investigations are reported on how the Amadori products 1-deoxy-1-[2-(1,2-ditetradecanoyl-*sn*-glycero-3-phosphooxy)ethylamino]-D-fructose (**4**) and 1-deoxy-1-[2-(1,2-dihexadecanoyl-*sn*-glycero-3-phosphooxy)ethylamino]-D-fructose (**5**) can be identified from model reactions of D-glucose and phosphatidyl ethanolamine. Independent syntheses and unequivocal structural characterization are given for the *E/Z*-1-deoxy-1-(2-hydroxyethylamino)-D-fructose (3-methylbenzothiazolin-2-ylidene)hydrazone (**12a,b**) and the peracetylated *E/Z*-1-deoxy-1-(2-hydroxyethylamino)-D-fructose *O*-methyloxime (**13a,b**). Chromatographic and spectroscopic data for these 1-deoxy-1-(2-hydroxyethylamino)-D-fructose derivatives were established by either GLC–MS or HPLC with diode array detection (DAD). Phosphatidyl ethanolamine and D-glucose were incubated at 37 °C, pH 7.4, in neat buffer or ethanol–buffer mixtures for four weeks, and the phospholipid fraction was purified on a C18 solid-phase extraction column. The phosphatidic acid was cleaved with phospholipase D and the free 1-deoxy-1-(2-hydroxyethylamino)-D-fructose derivatized to give **12a,b** or **13a,b**, respectively. Both these derivatives could be identified from all incubations by GLC–MS and HPLC–DAD analyses, respectively. Formation of **4** and **5** is favored in ethanol–buffer reaction mixtures relative to those in buffer solution only. © 1997 Elsevier Science Ltd.

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PII S0008-6215(97)00090-6

Keywords: Maillard reaction; Amadori compound; 1-Deoxy-1-(2-hydroxyethylamino)-D-fructose derivatives; Phosphatidyl ethanolamine

1. Introduction

Carbonyl–amine reactions are ubiquitous in nature. They manifest themselves, inter alia, in the nonenzymatic reaction of reducing carbohydrates, such as D-glucose, with free amino acids and protein-bound amino acid moieties, known as the Maillard reaction [1]. The initial phase of the reaction is triggered by an amine adding to the carbonyl function of a reducing sugar, glycosylamines **1** (Fig. 1) being formed. With aliphatic amines, these intermediates as a rule quickly rearrange, via the aminoenoles **2**, into the aminoketoses **3** (Amadori products) [2]. Aminoketoses have been identified in a number of foodstuffs [3] and also in the human organism [4]. They are further converted into deoxyosones which enter into a complex series of reactions, involving e.g. dehydration, cyclization, oxidation, and fragmentation, resulting in an abundance of secondary products [1]. The so-called Advanced Glycosylation Endproducts (AGEs) represent the terminal stage of the overall reaction scheme; little is known, though, about their chemical nature [5–7]. Nonenzymatic reaction of amines with D-glucose leads to structural/functional changes in proteins. The increased glycosylation level for various proteins in diabetics links the characteristic hyperglycemia of this metabolic disease with the long-term complications involved [8].

Recently, Cerami and co-workers [9] have shown phosphatidyl ethanolamine (PE), a lipid component of low density lipoprotein (LDL), to likewise react with glucose and so to initiate advanced glycosylation reactions, forming lipid-linked AGEs. The AGE level, detected by ELISA, shows significant correlation with the level of oxidized LDL, thus supporting the hypothesis that AGE-initiated oxidation plays an important role for lipid oxidation in vivo. The enhanced endocytosis of oxidized LDL by vascular wall macrophages transforms them into lipid-laden foam cells which are characteristic for the early stage of arteriosclerotic lesion.

Knowledge about both primary and secondary products from the reaction of D-glucose with aminophospholipids could provide a deeper insight into the mechanism of AGE-initiated lipid oxidation. Pamplona et al. [10] have detected 5-hydroxymethylfurfural, a stable reaction product of Amadori compounds, which is obtained upon treatment of glucose–aminophospholipid adducts with acid. Ravandi et al. [11,12] detected glucosylation products of PE and phosphatidyl serine by HPLC and on-line mass spectrometry with electrospray ionization (ESI–MS). The authors ascribed a Schiff-base structure to these adducts without, however, giving explicit structural proof.

We now report the independent syntheses of 1-deoxy-1-(2-hydroxyethylamino)-D-fructose derivatives

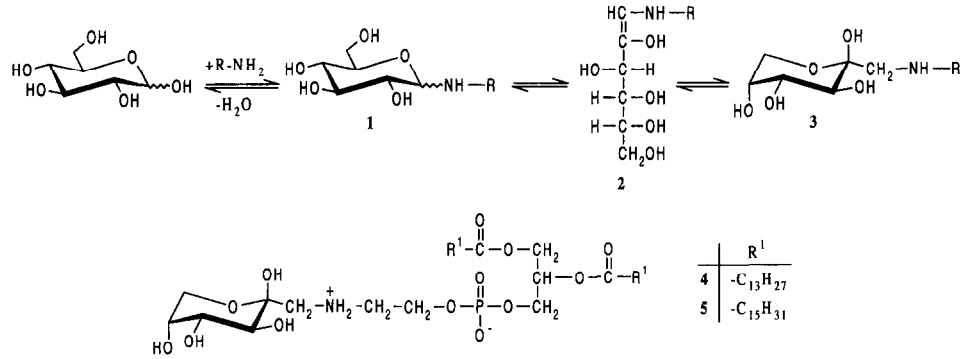


Fig. 1. Formation of 1-alkylamino-1-deoxy-D-fructose (**3**) from the reaction of D-glucose with primary aliphatic amines via glucosylamine **1** and aminoenol **2**; structure of the Amadori products 1-deoxy-1-[2-(1,2-ditetradecanoyl-*sn*-glycero-3-phosphooxy)ethylamino]-D-fructose (**4**) and 1-deoxy-1-[2-(1,2-dihexadecanoyl-*sn*-glycero-3-phosphooxy)ethylamino]-D-fructose (**5**).

and their spectroscopic and chromatographic characterization. With these data sets, it could be unequivocally proven that the Amadori products **4** and **5** (see Fig. 1) are in fact formed from D-glucose and phosphatidyl ethanolamine in an in vitro model system.

2. Results and discussion

1-Deoxy-1-(2-hydroxyethylamino)-D-fructose (**14**, Fig. 3) itself is not reported in the literature; we therefore attempted a synthesis of **14** from native D-glucose and ethanolamine, following a procedure given by Nedvidek for 1-deoxy-1-propylamino-D-fructose [13] (basically according to a method of Micheel et al. [14]). Under these conditions, however, not even traces of **14** are obtained. This might be due to the fact that the *N*-(2-hydroxyethyl)-D-glucosylamine, formed in the first step, resists the Amadori rearrangement initiated by oxalic acid, as has been shown for some other aliphatic N-glycosides. Micheel and Frowein [15] have demonstrated how to overcome this basic problem by employing

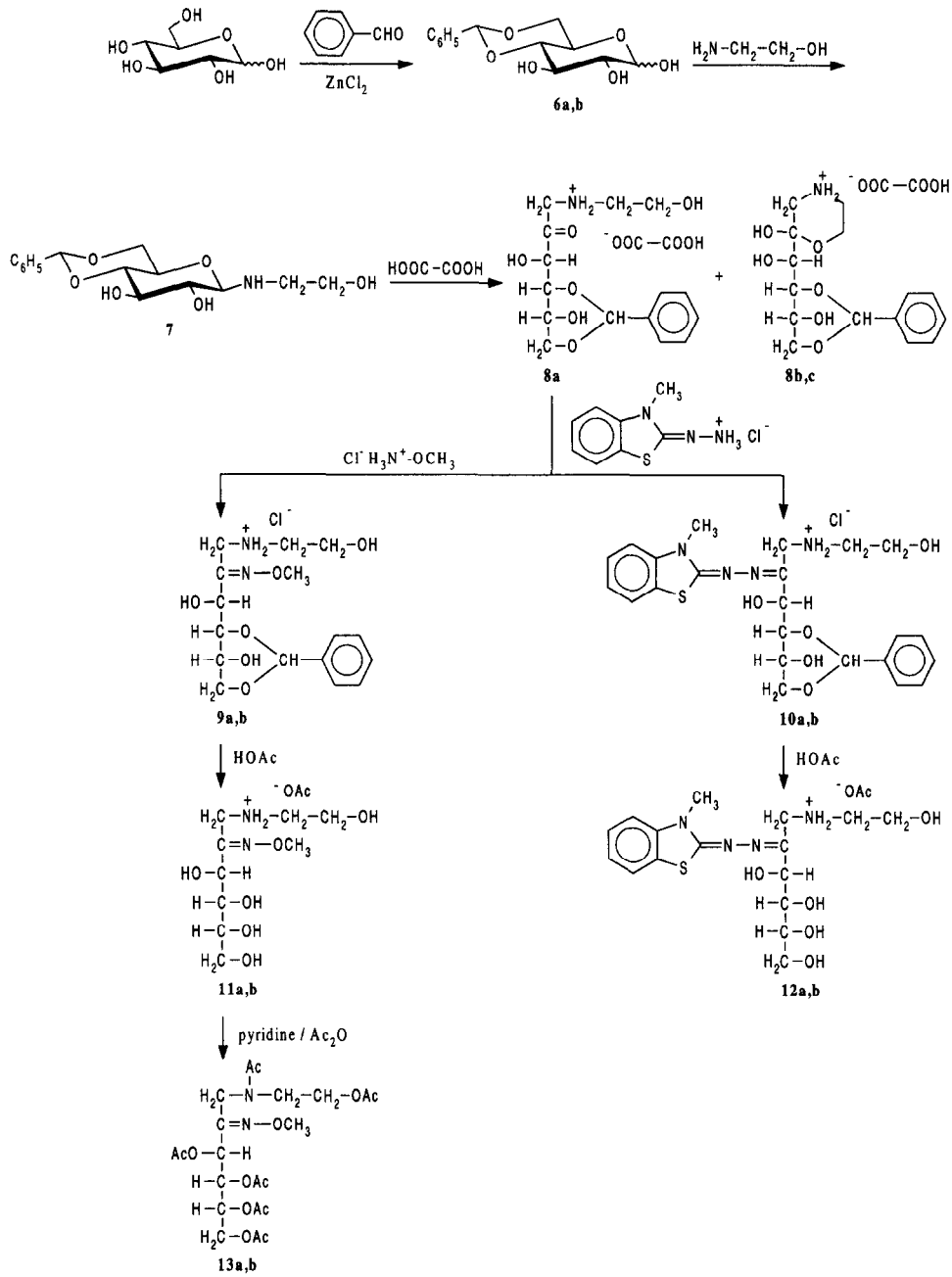


Fig. 2. Reaction pathways for the synthesis of *E/Z*-1-deoxy-1-(2-hydroxyethylamino)-D-fructose (3-methylbenzothiazolin-2-ylidene)hydrazone (**12a,b**) and peracetylated *E/Z*-1-deoxy-1-(2-hydroxyethylamino)-D-fructose *O*-methyloxime (**13a,b**).

4,6-*O*-benzylidene-D-glucopyranose (**6a,b**) in lieu of unprotected D-glucose.

Fig. 2 outlines our synthetic pathway, based on this strategy, to the 1-deoxy-1-(2-hydroxyethylamino)-D-fructose derivatives **12a,b** and **13a,b**. The benzylidenated glucose **6a,b** was obtained with benzaldehyde–anhydrous $ZnCl_2$ as reported by Zervas [16]. Upon addition of ethanolamine to a methanolic solution of **6a,b**, 4,6-*O*-benzylidene-*N*-(2-hydroxyethyl)-β-D-glucopyranosylamine (**7**) crystallizes spontaneously. The precipitate has been structurally characterized by NMR and MS techniques (see below). It is smoothly transformed into 4,6-*O*-benzylidene-1-deoxy-1-(2-hydroxyethylamino)-D-fructose (**8**) in the presence of anhydrous oxalic acid. The ^{1}H and ^{13}C NMR data show three isomeric forms of **8** coexisting in dimethylsulfoxide: an open chain structure (**8a**, ca. 50%) with a free carbonyl function (δ C(2)=O 207.3) and two anomeric hemiketals **8b,c** (δ C-2 94.8, 94.0) formed by nucleophilic attack of the OH function of ethanolamine on the C(2)=O function. The exclusive addition of this hydroxyl group, rather than C(5)–OH, corresponds with the observation by Micheel and Frowein that the C(5)–OH group in 4,6-*O*-benzylidene-protected Amadori products is not available for hemiketal formation [15].

For GLC analysis, the Amadori-product–isomer mixture **8a–c** is transformed by *O*-methylhydroxylammonium chloride in pyridine to the respective *E/Z*-*O*-methyloximes **9a,b**. The 4,6-*O*-benzylidene-protecting group is cleaved (70% acetic acid, 75 °C, nitrogen atmosphere), the solvent evaporated, and the residue acetylated by pyridine–acetic anhydride in the presence of catalytic amounts of 4-dimethylamino–pyridine to insure peracetylation. Coupled GLC–CIMS of the peracetylated *E/Z*-1-deoxy-1-(2-hydroxyethylamino)-D-fructose *O*-methyloxime (**13a,b**) shows a characteristic twin peak in the total ion chromatogram for the *E/Z*-isomer pair. The mass of the quasimolecular ion $[M+H]^+$ is 505 Da in each case, and the fragmentation pattern is almost identical.

HPLC with diode array detection (DAD) requires a chromophore to be introduced into **8**. Dansyl hydrazine is the most widely used reagent for aldose derivatization; it has been reported, though, as less reactive towards 2-amino-2-deoxy sugars and ketoses [17]. We therefore decided in favor of the more nucleophilic (3-methyl-2-benzothiazolinone)hydrazone · HCl (MBTH) to transform the **8a–c** isomeric mixture into the corresponding hydrazones, which show a characteristic UV absorption. Optimum reaction parameters for coupling MBTH to Amadori products were worked out with 1-deoxy-1-propylamino-D-fructose as a model substrate. The carbonyl activity of this compound is expected to be similar to that of compounds **4**, **5**, and **14** (see Figs. 1 and 3); it also can easily form hemiketals involving the C(6)–OH function. 4,6-*O*-Benzylidene-1-deoxy-1-(2-hydroxyethylamino)-D-fructose (**8**) should be more reactive since the major isomer has a free carbonyl group. Reaction of **8a–c** with MBTH under the optimized conditions gave *E/Z*-4,6-*O*-benzylidene-1-deoxy-1-(2-hydroxyethylamino)-D-fructose (3-methylbenzothiazolin-2-ylidene)hydrazone (**10a,b**) which was separated from excess reagent by preparative HPLC. Cleavage of the protecting group, as described for **9a,b**, was monitored by HPLC. The azine function was also partially hydrolyzed under these conditions; thus, after two hours, only 40% of the starting material was isolated in form of the *E/Z*-1-deoxy-1-(2-hydroxyethylamino)-D-fructose (3-

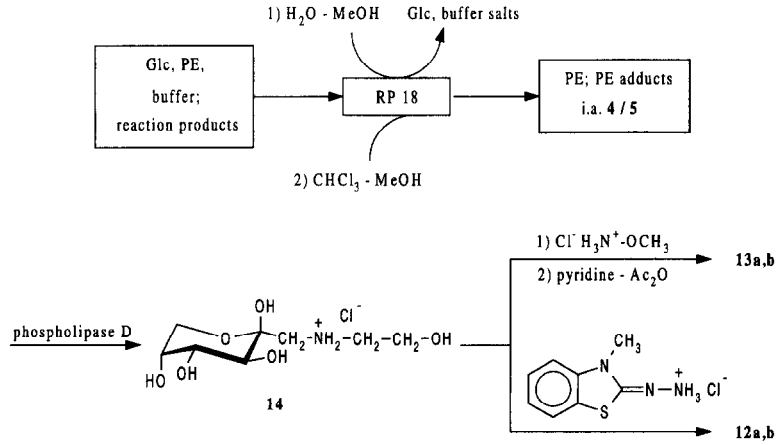


Fig. 3. Schematic workup procedure for glucose (Glc)–phosphatidyl ethanolamine (PE) incubations.

methylbenzothiazolin-2-ylidene)hydrazone (**12a,b**). Removing the benzylidene group hydrogenolytically, however, is no suitable alternative since this would destroy the chromophore. Compounds **12a,b** were purified by preparative HPLC and unequivocally characterized, as all precursors, by ^{1}H and ^{13}C NMR as well as by FABMS and ESI–MS. The spectroscopic data are discussed in detail below.

Once chromatographic and spectroscopic data have been established for compounds **12a,b** and **13a,b**, reaction mixtures of phosphatidyl ethanolamine and D-glucose can be tested for Amadori-product formation. Two model phospholipids, 1,2-ditetradecanoyl- and 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (16 mM each) were incubated with D-glucose (500 mM) for four weeks, respectively, at physiological pH and temperature. The medium was either 0.1 M phosphate buffer or a 3:2 ethanol–buffer mixture, the ethanol improving the stability of the phospholipid suspension. The workup of these reactions is outlined schematically in Fig. 3. The suspensions were transferred to reversed phase cartridges (RP18); both unreacted D-glucose and buffer salts were eluted first with 1:1 methanol–water, followed by methanol. Elution of the phospholipids and their reaction products required 2:1 chloroform–methanol; the respective fractions were evaporated to dryness and the residue was treated with phospholipase D to cleave the phosphatidic acid. The lyophilized reaction mixture, containing free 1-deoxy-1-(2-hydroxyethylamino)-D-fructose (**14**), was split in two portions. One aliquot was derivatized for GLC–CIMS analysis with *O*-methylhydroxylammonium chloride and acetic anhydride in pyridine, the other was reacted with MBTH for HPLC analysis. For all incubations, peracetylated *E/Z*-1-deoxy-1-(2-hydroxyethylamino)-D-fructose *O*-methyloxime (**13a,b**) and *E/Z*-1-deoxy-1-(2-hydroxyethylamino)-D-fructose (3-methylbenzothiazolin-2-ylidene)hydrazone (**12a,b**) could be identified. This result unequivocally proves, for the first time, the formation of Amadori products (such as **4** and **5**, Fig. 1) from the reaction of glucose with phosphatidyl ethanolamine.

Typical HPLC chromatograms for identifying compounds **12a,b** in the reaction mixtures are given in Fig. 4 (225 and 318 nm DAD traces, respectively). The hydrazones **12a,b** have their UV absorption maxima at 225 and 318 nm. (3-Methyl-2-benzothiazolinone)hydrazone · HCl shows virtually no absorption at 318 nm; the 225 nm monitoring wavelength, however, lies still far within the main absorption band (215 nm). Chromatogram (a) was obtained directly from the MBTH derivatization mixture of the lyophilized residue after phospholipase D cleavage. The 318 nm DAD trace shows the signals for the *E/Z*-isomers **12a,b** with the overlying MBTH peak. At 225 nm, the UV response is completely dominated by the derivatizing agent; no UV spectra can be obtained to ascertain the identity of the **12a,b** twin peak. Adding acetone to the reaction mixture transforms excess MBTH quantitatively into 3-methyl-2-benzothiazolinone isopropylidenehydrazone (**15**). Since **15** is retained significantly longer on RP18 than MBTH, the **12a,b** signals can now be unequivocally characterized by their UV spectra [chromatogram (b)]. Under the given reaction conditions (16 h, room temperature), acetone does not release MBTH from **12a,b** as experiments with authentic synthetic material have shown. Nevertheless, we made no attempts to quantify **12a,b** because it is not possible to

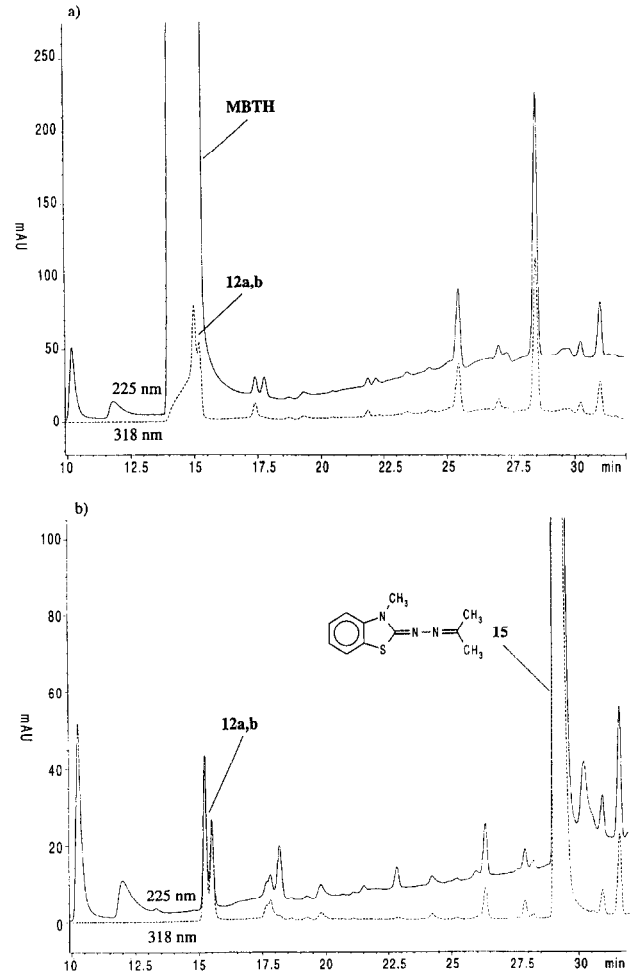
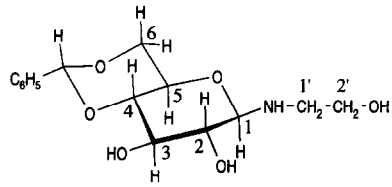
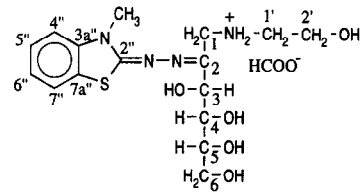


Fig. 4. HPLC chromatograms for identifying *E/Z*-1-deoxy-1-(2-hydroxyethylamino)-D-fructose (3-methylbenzothiazolin-2-ylidene)hydrazone (**12a,b**): (a) obtained directly from the reaction mixture; (b) after addition of acetone for chemically binding of the excess of MBTH.

Table 1
^{1}H and ^{13}C NMR spectroscopic data of compounds **7** and **12a,b** (in Me_2SO-d_6). 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 measurements





	7	**12a**	**12b**
^{1}H NMR/Protons	δ [ppm]		
H-1	3.91	–	–
H-1a	–	3.74	3.87
H-1b	–	3.39	3.54
H-2	3.03	–	–
H-3	3.42	5.45	4.53
H-4	3.33	3.56	3.42
H-5	3.30	3.56	3.55
H-6a	3.63	3.41	3.41
H-6b	4.16	3.61	3.62
H-1'a	2.63	2.72	2.65
H-1'b	2.83	2.74	2.65
H-2'	3.42	3.54	3.52
HN-1	2.53	–	–
HO-2	4.85	–	–
HO-3	5.22	–	–
HO-2'	4.43	–	–
CHPh	5.56	–	–
Ph	7.44 (2 H, *o*); 7.36 (3 H, *m,p*)	–	–
H_3C-3''	–	3.50	3.55
H-(4''–7'')	–	7.57; 7.30; 7.24; 7.07	7.57; 7.30; 7.24; 7.07
$HCOO^-$	–	8.29	8.29
	J [Hz]		
$^{2}J_{1a,1b}$	–	(–)14.2	(–)12.8
$^{2}J_{6a,6b}$	(–)10.2	(–)10.8	(–)11.0
$^{2}J_{1'a,1'b}$	(–)12.0	(–)12.0	–
$^{3}J_{1,2}$	8.6	–	–
$^{3}J_{2,3}$	8.6	–	–
$^{3}J_{3,4}$	9.0	1.3	1.8
$^{3}J_{4,5}$	9.0	[a]	9.0
$^{3}J_{5,6a}$	4.7	3.3	3.3
$^{3}J_{5,6b}$	9.8	5.8	5.8
$^{3}J_{1'a,2'}$	5.5	5.5	5.5
$^{3}J_{1'b,2'}$	7.0	5.5	5.5
$^{3}J_{HO\text{-}2,2}$	4.2	–	–
$^{3}J_{HO\text{-}3,3}$	4.9	–	–
$^{3}J_{HO\text{-}2',2'}$	5.6	–	–
^{13}C NMR/Carbons	δ [ppm]		
C-1	91.7	49.2	43.8
C-2	74.3	164.7 [b]	165.8 [c]
C-3	73.3	68.2	72.4
C-4	81.0	71.8 [d]	74.1
C-5	66.8	71.2 [d]	70.8

Table 1 (continued)

	7	12a	12b
^{13}C NMR/Carbons	δ [ppm]		
C-6	68.1	63.5	63.5
C-1′	47.9	50.0	50.8
C-2′	60.9	58.6	58.8
CHPh	100.5	–	–
Ph	137.8; 128.7; 127.9; 126.2	–	–
C-2″	–	163.2 [b]	163.0 [c]
H_3C-3″	–	30.6	30.8
C-3a″	–	140.6	140.6
C-(4″–7″)	–	126.3; 122.2; 121.5; 109.8	126.3; 122.2; 121.6; 109.9
C-7a″	–	123.1	123.2
$HCOO^-$	–	164.5	164.5

[a] A coupling constant could not be determined, due to overlapping signals of the *E/Z*-isomers.
[b,c,d] Assignment for the respective carbons may have to be reversed.

estimate the degree of conversion for the phospholipase D cleavage. Thus, the amount of **12a,b** cannot be extrapolated, in principle, to how much of the Amadori products **4** and **5** were formed primarily. The relative intensities of the HPLC peaks clearly prove, though, that formation of **4** and **5** is favored in ethanol–buffer incubations compared to those in neat buffer solutions. As mentioned above, this may be due to the phospholipids forming a more stable suspension in the presence of ethanol.

With Amadori-product formation from glucose and phosphatidyl ethanolamine now established definitively, further investigations are in progress on whether and how Amadori compounds influence lipid oxidation.

3. Structural assignments

In the literature, dealing with aminophospholipid–glucose reactions, no explicit and unequivocal structural proof has been given so far for the products obtained. We therefore invested special effort in definitively establishing the structure of compounds **7** and **12a,b**. The ^{1}H and ^{13}C NMR data (chemical shifts, δ; coupling constants, *J*) are collected in Table 1.

NMR data for glycosylamines with aliphatic amine moieties are scarce since only those obtained from aromatic and heterocyclic amines, e.g. purines, are sufficiently stable. As mentioned above, reaction of 4,6-*O*-benzylidene-D-glucopyranose (**6a,b**), instead of unprotected D-glucose, with aliphatic amines gives N-glucosides which crystallize spontaneously. They can thus be rapidly removed from the reaction mixture and subjected to NMR analysis. The large vicinal coupling constant (^3J 8.6 Hz) between H-1 and H-2 can be reconciled only with a diaxial orientation of these protons and thus clearly proves the β configuration for the N-glucoside **7**. For the α anomer, formed as minor constituent, a ^3J value of 3–4 Hz is expected. The predominant formation of the β anomer may be rationalized also by a straightforward stereochemical argument: in a six-membered ring, with chair conformation, an equatorial position for the bulky 2-hydroxyethylamino substituent is preferred. Additionally, the polar medium methanol, in which the synthesis was carried out, and the equatorial orientation of the 2-OH group in **7** favor the β anomer due to the anomeric effect [18].

The Amadori product **8** exists in three isomeric forms as proven by the ^{1}H and ^{13}C NMR data (see above); the complex set of overlying signals cannot be analyzed in detail. The 1-deoxy-1-(2-hydroxyethylamino)-D-fructose derivative **12** likewise represents a mixture of isomers. Both the NMR spectra and the HPLC chromatogram (Fig. 4b) show the presence of two isomers in a 2:1 ratio (**12a,b**; *E/Z*). One NMR signal set can be assigned on the basis of the relative intensities for each isomer as shown in Table 1. It is not possible, though, to differentiate between **12a** and **12b** from the NMR data. The HPLC–ESI mass spectra give an indication, however, how to resolve this problem. The fragmentation pattern for the respective isomers is almost identical except for the signal at 324 Da (relative intensity: 80% base peak for **12a**; 29% for **12b**). A plausible mechanism for the favored cleavage of ethanolamine from the $[M + H]^+$ ion of **12a** is outlined in Fig. 5. Nucleophilic attack of the sulfur at C-1 of the carbohydrate chain is possible only for the (*E*) configuration of the C(2)=N double bond. The stereochemistry of the C(2″)=N double

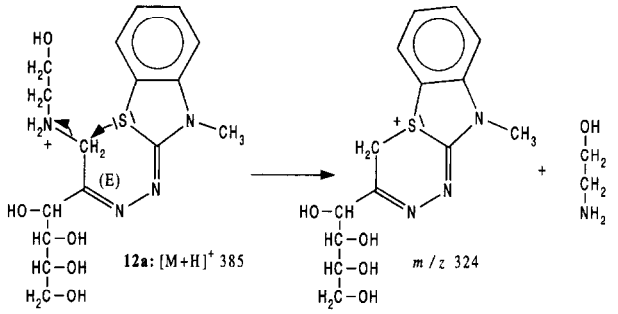


Fig. 5. Hypothetical fragmentation pathway for the formation of *m/z* 324 from the *E*-1-deoxy-1-(2-hydroxyethylamino)-D-fructose (3-methylbenzothiazolin-2-ylidene)hydrazone (**12a**) $[M+H]^+$ ion in ESI–MS.

bond (for the numbering, see Table 1) is not fixed since this bond is incorporated in a thioguanidino partial structure.

4. Experimental

General methods.—Melting points were determined on an Electrothermal 8600 apparatus and are not corrected. UV spectra were measured with a Perkin–Elmer Lambda 2 instrument (Überlingen, Germany). ^1H and ^{13}C NMR spectra were recorded on Bruker (Karlsruhe, Germany) AC-250/ARX-500 spectrometers at 250/500 and 63/126 MHz, respectively. Chemical shifts (δ) are given in ppm relative to internal Me_4Si. Liquid secondary-ion mass spectra (SIMS-MS, analogous to FABMS) were obtained on a Finnigan (Bremen, Germany) MAT 95 instrument. The analytical HPLC system comprised an HP1050 autosampler, an HP1050 gradient pump, and an HP1050 diode array detector (DAD) module (Hewlett–Packard, Waldbronn, Germany). For data acquisition and processing, an HP3D Chem Station (Rev. A. 03.01) software system was used. Column (Bischoff, Leonberg, Germany): Nucleosil RP18, 5 μm, 250 × 4 mm; flow rate, 0.8 mL/min; MeOH–0.01 M phosphate buffer (pH 4.0) gradient: % MeOH [*t* (min)], 5 [0]–95 [30]–95 [40]–5 [45]–5 [55]; DAD detection wavelengths: 225 and 318 nm; spectral band width (SBW), 4 nm; reference 500 nm (SBW 100 nm). HPLC–ESI–MS spectra were obtained on an HP1050 system (for chromatographic conditions, see above; a NH_4OAc buffer (0.01 M, pH 4.0) was used instead of the phosphate buffer coupled to a Micromass (Manchester, UK) VG platform 2 quadrupole mass spectrometer equipped with an ESI interface, operating with a 20–80 V cone voltage ramp. A Knauer (Berlin, Germany) 64 liquid chromatograph, combined with an A0293 variable wavelength detector and a Kronlab (Sinsheim, Germany) HPLC column (guard column 20 × 50 mm, column 20 × 250 mm; Nucleosil RP18, 7 μm; flow rate, 10 mL/min; injection volume, 1 mL; detection, 225 nm) was employed for preparative HPLC. GLC–MS was performed using a Finnigan MAT Ion Trap 800 equipped with a Perkin–Elmer 8420 gas chromatograph. Injector and transfer line temperatures were set at 290 °C. Injection was in the split mode into a fused-silica capillary column (0.25 mm × 30 m) wall-coated with PVMS 54 (0.3 μm film thickness, Perkin–Elmer), programmed from 200 to 290 °C at 10 °C/min with 10 min isothermal at 290 °C. Helium was used as the carrier gas at a linear velocity of 21 cm/s (determined by injection of methane, oven temperature 200 °C). Column effluents were analyzed by CIMS using MeOH as the reagent gas. Silica Gel 60 F_{254} (E. Merck, Darmstadt, Germany) was used for TLC, Chromabond (C18, 1000 mg, 6 mL) from Macherey–Nagel (Düren, Germany) for solid-phase extraction (SPE).

Materials.—Ethanolamine, anhyd oxalic acid, *O*-methylhydroxylamine hydrochloride, and (3-methyl-2-benzothiazolinone)hydrazone · HCl were obtained from Fluka Chemie AG (Buchs, Switzerland); 1,2-ditetradecanoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, and phospholipase D from *Streptomyces chromofucus* from Sigma (St. Louis, USA); and 4-dimethylamino–pyridine and sodium dodecyl sulfate (SDS) from E. Merck (Darmstadt, Germany).

Preparation of 4,6-O-benzylidene-D-glucopyranose (**6a,b**) [16].—Compound **6a,b** was prepared following a procedure by Zervas: mp 183–184 °C, lit. 188 °C for the α anomer [16]. ^1H NMR analysis showed the anomeric ratio of **6a** and **6b** to be 1:2 (α:β). Morales et al. [19] have already listed ^1H NMR data for the anomeric mixture (in CD_3OD) without signal assignment. NMR of the α anomer (Me_2SO-d_6): ^1H, δ 7.44 (m, 2 H, *o*-Ph), 7.36 (m, 3 H, *m,p*-Ph), 6.55 (d, 1 H, $J_{HO\text{-}1,1}$ 4.8 Hz, HO-1), 5.56 (s, 1 H, CHPh), 5.10 (d, 1 H, $J_{HO\text{-}3,3}$ 5.1 Hz, HO-3), 4.99 (dd, 1 H, $J_{1,2}$ 3.7 Hz, H-1), 4.81 (d, 1 H, $J_{HO\text{-}2,2}$ 7.0 Hz, HO-2), 4.10 (dd, 1 H, $J_{5,6b}$ 4.9, $J_{6a,6b}$ (−)10.0 Hz, H-6b), 3.80 (ddd, 1 H, $J_{5,6a}$ 9.0, $J_{4,5}$ 9.5 Hz, H-5), 3.65 (dd, 1 H, H-6a), 3.63 (dt, 1 H, $J_{2,3} = J_{3,4} = 9$ Hz, H-3), 3.34 (t, 1 H, H-4), 3.28 (ddd, 1 H, H-2); ^{13}C, δ 137.8 (C-1, Ph), 128.7 (C-4, Ph), 127.9 and 126.3 (C-2,3/5,6, Ph), 100.7 (CHPh), 93.1 (C-1), 81.6 (C-4), 72.8 (C-2), 69.6 (C-3), 68.3 (C-6), 61.9

(C-5); NMR of the β anomer (Me_2SO-d_6): ^1H, δ 7.44 (m, 2 H, *o*-Ph), 7.36 (m, 3 H, *m,p*-Ph), 6.84 (d, 1 H, $J_{HO\text{-}1,1}$ 6.6 Hz, HO-1), 5.57 (s, 1 H, CHPh), 5.23 (d, 1 H, $J_{HO\text{-}3,3}$ 4.9 Hz, HO-3), 5.17 (d, 1 H, $J_{HO\text{-}2,2}$ 5.1 Hz, HO-2), 4.46 (dd, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.16 (dd, 1 H, $J_{5,6b}$ 4.6, $J_{6a,6b}$ (−)10.3 Hz, H-6b), 3.67 (dd, 1 H, $J_{5,6a}$ 6.3 Hz, H-6a), 3.40 (ddd, 1 H, $J_{2,3}$ 8.5, $J_{3,4}$ 9.4 Hz, H-3), 3.33 (t, 1 H, $J_{4,5}$ 9.4 Hz, H-4), 3.30 (ddd, 1 H, H-5), 3.03 (ddd, 1 H, H-2); ^{13}C, δ 137.8 (C-1, Ph), 128.7 (C-4, Ph), 127.9 and 126.3 (C-2,3/5,6, Ph), 100.6 (CHPh), 97.5 (C-1), 80.8 (C-4), 75.7 (C-2), 72.8 (C-3), 67.9 (C-6), 65.7 (C-5).

4, 6 - O - Benzylidene - N - (2 - hydroxyethyl) - β - D - glucopyranosylamine (**7**).—To a soln of **6** (2.2 g, 8.2 mmol) in MeOH (8.2 mL) at 60 °C was added ethanolamine (630 μL, 10.5 mmol). When the mixture was allowed to cool to room temperature, **7** precipitated spontaneously. The precipitate was filtered off, washed with 1:1 ether–petroleum ether (2 × 10 mL), and dried in vacuo (5 Pa), yielding **7** (1.77 g, 70%); ^1H and ^{13}C NMR (Me_2SO-d_6): see Table 1 ($\beta:\alpha \approx 80:20$); FABMS (*m*-nitrobenzyl alcohol): *m/z* 312 $[M + H]^+$.

4,6-O-Benzylidene-1-deoxy-1-(2-hydroxyethylamino)-D-fructose (**8a–c**).—To a suspension of **7** (1.77 g, 5.7 mmol) in MeOH (35 mL) was added anhyd oxalic acid (512 mg, 5.7 mmol) in MeOH (0.5 mL). When the mixture had turned to a clear soln, this was concd until it became opalescent, and kept for 1 h at 4 °C. The precipitate was filtered off, washed with ether (2 × 10 mL), and air-dried. The three isomeric products **8a–c** were obtained as the respective oxalates in the form of colorless crystals (0.9 g, 39%); mp 135–137 °C under decomposition; ^{13}C NMR (Me_2SO-d_6): the relative intensity of the C(2)=O signal at δ 207.2 shows about 50% of **8** to have a free carbonyl function (isomer **8a**), δ 94.8 and 94.0 (C-2, hemiketals **8b,c**); FABMS (*m*-nitrobenzyl alcohol): *m/z* 312 $[M + H]^+$.

Peracetylated E/Z-1-deoxy-1-(2-hydroxyethylamino)-D-fructose O-methyloxime (**13a, b**).—A mixture of **8** (50 mg, 0.13 mmol) and *O*-methylhydroxylamine hydrochloride (250 mg, 3 mmol) in pyridine (2 mL) was kept for 30 min at 60 °C. The solvent was removed in vacuo (5 Pa), the residue dissolved in 70% HOAc (4 mL), then heated for 90 min at 75 °C under N_2. After concn (5 Pa), the residue was acetylated with pyridine–Ac_2O in the presence of a catalytic amount of 4-dimethylamino-pyridine. The mixture was poured on ice, the resulting slurry stirred for 1 h and extracted with CH_2Cl_2 (3 × 10 mL). The extracts were combined, washed with 0.5 M H_2SO_4 (20 mL) and water (3 × 10 mL), and dried with anhyd Na_2SO_4. Compound **13a,b** was obtained as a yellow oil (49 mg, 78%); GLC–CIMS: **13a** (t_R 12.58 min), *m/z* 505 (1) $[M + H]^+$, 474 (100), 445 (60), 414 (6), 295 (6), 116 (7), 87 (8); **13b** (t_R 12.92 min), *m/z* 505 (3) $[M + H]^+$, 474 (100), 445 (34), 414 (5), 295 (5), 116 (7), 87 (6).

E/Z-4,6-O-Benzylidene-1-deoxy-1-(2-hydroxyethylamino)-D-fructose (3-methylbenzothiazolin-2-ylidene)hydrazone (**10a,b**).—Compound **8** (100 mg, 0.25 mmol) and (3-methyl-2-benzothiazolinone)hydrazone · HCl (MBTH) (111 mg, 0.47 mmol) were dissolved in MeOH (15 mL), and the soln was kept for 20 h at room temperature. The formation of **10a,b** was monitored by TLC (5:4:1 CH_2Cl_2– *tert*-BuOH–HOAc): R_f (MBTH) 0.21, R_f (**10a,b**) 0.29; detection at 254 nm. The soln was concd and the residue was taken up in 63:37 MeOH–0.01 M HCO_2NH_4 buffer (pH 4.0) (5 mL), then subjected to preparative HPLC, using this solvent mixture as eluent. Fractions with t_R 12.0 min were combined, and yielded, after lyophilization, the formates of **10a,b** (48 mg, 37%); HPLC: t_R 23.68 and 24.30 min; UV(H_2O): λ_{max} [nm] (lg ε) 225 (4.39), 318 (4.36); NMR (Me_2SO-d_6): **10a** (numbering as for **12a,b**, Table 1) ^1H, δ 7.57, 7.30, 7.24, and 7.07 (4 m, each 1 H, H-4″–7″), 7.42 (m, 2 H, *o*-Ph), 7.33 (m, 3 H, *m,p*-Ph), 5.51 (s, 1 H, CHPh), 4.65 (d, 1 H, $J_{3,4}$ 2.2 Hz, H-3), 4.15 (dd, 1 H, $J_{5,6b}$ 5.3, $J_{6a,6b}$ (−)10.5 Hz, H-6b), 3.84 (dt, 1 H, $J_{4,5} = J_{5,6a}$ = 9.7 Hz, H-5), 3.82 (d, 1 H, $J_{1a,1b}$ (−)14.0 Hz, H-1b), 3.78 (d, 1 H, H-1a), 3.77 (dd, 1 H, H-4), 3.55 (dd, 1 H, H-6a), 3.54 (s, 3 H, H_3C-3″), 3.48 (t, 2 H, $J_{1',2'}$ 5.5 Hz, H-2′), 2.67 (t, 2 H, H-1′); ^{13}C, δ 166.3 and 160.7 (C-2,2″), 140.6 (C-3a″), 138.1 (C-1, Ph), 128.5 (C-4, Ph), 127.8 and 126.0 (C-2,3/5,6, Ph), 126.2, 122.2, 121.7, and 110.0 (C-4″–7″), 123.2 (C-7a″), 100.0 (CHPh), 84.3 (C-4), 70.9 (C-6), 70.7 (C-3), 59.7 (C-5), 58.7 (C-2′), 50.4 (C-1′), 45.1 (C-1), 30.8 (H_3C-3″); NMR (Me_2SO-d_6): **10b** (numbering as for **12a,b**, Table 1) ^1H, δ 7.57, 7.30, 7.24, and 7.07 (4 m, each 1 H, H-4″–7″), 7.42 (m, 2 H, *o*-Ph), 7.33 (m, 3 H, *m,p*-Ph), 5.55 (d, 1 H, $J_{3,4}$ 1.6 Hz, H-3), 5.43 (s, 1 H, CHPh), 4.13 (dd, 1 H, $J_{5,6b}$ 5.0, $J_{6a,6b}$ (−)10.5 Hz, H-6b), 3.92 (dd, 1 H, $J_{4,5}$ 9.5 Hz, H-4), 3.89 (dt, 1 H, $J_{5,6a}$ 9.5 Hz, H-5), 3.83 (d, 1 H, $J_{1a,1b}$ (−)16.2 Hz, H-1b), 3.63 (d, 1 H, H-1a), 3.55 (dd, 1 H, H-6a), 3.54 (s, 3 H, H_3C-3″), 3.52 (dd, 2 H, $J_{1'a,2'}$ 5.8, $J_{1'b,2'}$ 5.5 Hz, H-2′), 2.82 (dt, 1 H, $J_{1'a,1'b}$ (−)12.2 Hz, H-1′b), 2.77 (dt, 1 H, H-1′a); ^{13}C, δ 165.2 and 161.3 (C-2,2″), 140.6 (C-3a″), 138.1 (C-1, Ph), 128.4 (C-4, Ph),

Table 2
Composition of the glucose–phosphatidyl ethanolamine (PE) incubation mixtures

Phosphatidyl ethanolamine	PE [mg/mmol]	Glucose [mg/mmol]	Solvent/[mL]
TD-PE	21.0/0.033	179.6/0.998	buffer/2.0
HD-PE	22.0/0.032	181.6/1.009	buffer/2.0
TD-PE	20.4/0.032	182.0/1.011	buffer–EtOH/2.0
HD-PE	21.5/0.031	180.3/1.002	buffer–EtOH/2.0

127.8 and 126.0 (C-2,3/5,6, Ph), 126.2, 122.2, 121.6, and 109.8 (C-4″–7″), 123.1 (C-7a″), 99.7 (CHPh), 82.5 (C-4), 70.7 (C-6), 66.4 (C-3), 59.4 (C-5), 58.5 (C-2′), 50.2 and 50.0 (C-1,1′), 30.7 (H_3C-3″); high resolution FABMS (*m*-nitrobenzyl alcohol): *m/z* 473.1881 $[M + H]^+$ (473.1859, Calcd for $C_{23}H_{29}N_4O_5S$).

E/Z-*1-Deoxy-1-(2-hydroxyethylamino)*-D-*fructose (3-methylbenzothiazolin-2-ylidene)hydrazone* (**12a,b**). —A soln of **10a,b** (20 mg, 0.039 mmol) in 70% HOAc (4 mL) was heated for 90 min at 75 °C under N_2. The progress of the reaction was monitored by HPLC–DAD. After concn, the residue was taken up in 35:65 MeOH–0.01 M HCO_2NH_4 buffer (pH 4.0) (5 mL), and subjected to preparative HPLC, using this solvent mixture as eluent. Fractions with t_R 16.0 min were combined and yielded, after lyophilization, the formates of **12a,b** (7 mg, 41%); UV data are identical to those of **10a,b**; HPLC: t_R 15.10 and 15.34 min; ^1H and ^{13}C NMR (Me_2SO-d_6): see Table 1; HPLC–ESI–MS: **12a** (t_R 15.10), *m/z* 791 (5) $[M + Na + M]^+$, 407 (25) $[M + Na]^+$, 385 (100) $[M + H]^+$, 324 (80), 306 (12); **12b** (t_R 15.34), *m/z* 791 (3) $[M + Na + M]^+$, 407 (26) $[M + Na]^+$, 385 (100) $[M + H]^+$, 324 (29), 306 (7); high resolution FABMS (*m*-nitrobenzyl alcohol): *m/z* 385.1543 $[M + H]^+$ (385.1545, Calcd for $C_{16}H_{25}N_4O_5S$).

D-*Glucose–phosphatidyl ethanolamine incubations*. —1,2-Ditetradecanoyl- (TD-PE) and 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (HD-PE) were incubated, respectively, with D-glucose at pH 7.4, 37 °C in 0.1 M phosphate buffer or 2:3 buffer–EtOH for four weeks (see Table 2).

Workup of incubation mixtures and enzymatic cleavage of the phospholipids.—The C18 SPE cartridge was attached to a vacuum manifold, fitted with an adapter and 20-mL reservoir, and conditioned with 1:1 MeOH–H_2O (5 mL). The incubation mixture was transferred to the SPE column, vacuum applied, and the eluent discarded. The cartridge was rinsed with 1:1 MeOH–H_2O (2 × 2 mL) and MeOH (1.5 mL); the eluent was discarded. The phospholipid fraction was eluted with 2:1 $CHCl_3$–MeOH (8 mL) at 30–35 °C, the soln concd to 1 mL, and the solvent finally removed under a gentle stream of N_2. To the residue were added H_2O (8.8 mL), Tris buffer (0.8 M, pH 8.0, 1.35 mL), and SDS soln (50 mM, 0.7 mL). The resulting suspension was stirred at 30 °C, 0.5 M $CaCl_2$ (1.36 mL) and phospholipase D soln (40 units/mL, 1 mL) were added, and the mixture kept for 90 min at 30 °C. After filtration (membrane filter, 0.45 μm), the soln was lyophilized and divided into two portions.

General derivatization procedures for GLC – MS and HPLC – DAD analyses.—One aliquot of the enzymatic cleavage product was dissolved in pyridine (1 mL), *O*-methylhydroxylamine hydrochloride (30 mg, 0.36 mmol) was added, and the mixture was heated for 30 min at 60 °C. Upon addition of Ac_2O (1 mL), the reaction mixture was kept for 4 h at room temperature. Further workup followed the procedure given for **13a,b**. The CH_2Cl_2 extract was concd to 200 μL, and 1-μL aliquots were applied to the GLC–MS system.

To the other aliquot, dissolved in MeOH (1 mL) were added MBTH (2 mg, 0.008 mmol) and HOAc (50 μL), and the mixture was kept for 16 h at room temperature in the dark. Aliquots of 10 μL were injected for HPLC–DAD analysis. After addition of acetone (10 μL), the soln was kept at room temperature for another 16 h.

Acknowledgements

We want to thank Prof. Dr. Th. Severin, Institut für Pharmazie und Lebensmittelchemie, Universität München, and Priv.-Doz. Dr. P. Fischer, Institut für Organische Chemie, Universität Stuttgart, for many helpful discussions. To Prof. Dr. W. Schwack, Institut für Lebensmittelchemie, we are grateful for the excellent working conditions at his Institute.

References

[1] F. Ledl and E. Schleicher, *Angew. Chem. Int. Ed. Engl.*, 29 (1990) 565–594.
[2] M. Amadori, *Atti. Acad. Naz. Lincei, Cl. Sci. Fis., Mat. Nat., Rend.*, 13 (1931) 72–195.

[3] R. Wittmann and K. Eichner, *Z. Lebensm. Unters. Forsch.*, 188 (1989) 212–220.
[4] J.F. Day, S.R. Thorpe, and J.W. Baynes, *J. Biol. Chem.*, 254 (1979) 595–597.
[5] R. Bucala and A. Cerami, *Adv. Pharmacol.*, 23 (1992) 1–34.
[6] F.G. Njoroge and V.M. Monnier, *Prog. Clin. Biol. Res.*, 304 (1989) 85–107.
[7] M.A. Glomb and V.M. Monnier, *J. Biol. Chem.*, 270 (1995) 10017–10026.
[8] J.W. Baynes and V.M. Monnier (Eds), *The Maillard Reaction in Aging, Diabetes, and Nutrition*, Liss, New York, 1989, pp 1–22.
[9] R. Bucala, Z. Makita, T. Koschinsky, A. Cerami, and H. Vlassara, *Proc. Natl. Acad. Sci. USA*, 90 (1993) 6434–6438.
[10] R. Pamplona, M.J. Bellmunt, M. Portero, D. Riba, and J. Prat, *Life Sciences*, 57 (1995) 873–879.
[11] A. Ravandi, A. Kuksis, L. Marai, and J.J. Myher, *Lipids*, 30 (1995) 885–891.
[12] A. Ravandi, A. Kuksis, L. Marai, J.J. Myher, G. Steiner, G. Lewisa, and H. Kamido, *FEBS Lett.*, 381 (1996) 77–81.
[13] W. Nedvidek, PhD thesis (1992), Universität Stuttgart, Germany.
[14] F. Micheel and G. Hagemann, *Chem. Ber.*, 92 (1959) 2836–2840.
[15] F. Micheel and A. Frowein, *Chem. Ber.*, 90 (1957) 1599–1605.
[16] L. Zervas, *Ber. Dtsch. Chem. Ges.*, 64 (1931) 2289–2296.
[17] W.F. Alpenfels, *Anal. Biochem.*, 114 (1981) 153–157.
[18] S.J. Angyal, *Conformations of Sugars*, in W. Pigman and D. Horton (Eds), *The Carbohydrates, Chemistry and Biochemistry*, 2nd ed., Vol. 1A, Academic Press, New York, 1972, pp 204–205.
[19] E.Q. Morales, J.I. Padron, M. Trujillo, and J.T. Vazquez, *J. Org. Chem.*, 60 (1995) 2537–2548.