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Formation of a Phospholipid-Linked Pyrrolecarbaldehyde from Model Reactions of D-Glucose and 3-Deoxyglucosone with Phosphatidyl Ethanolamine

Markus O. Lederer* and Markus Baumann

Institut für Lebensmittelchemie (170), Universität Hohenheim, Garbenstr. 28, D-70593 Stuttgart, Germany

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Abstract—Phospholipid-linked 'advanced glycation end products' (AGEs) are supposed to play an important role for lipid oxidation in vivo. The identification of the pyrrolecarbaldehyde 1-[2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl]-4,10-dioxo-7-(tetradecanoyloxy)-3,5,9-trioxa-4λ^5-phosphatricosan-4-olate (**7**) from model reactions of D-glucose or 3-deoxyglucosone (**4**, 3-DG) with phosphatidyl ethanolamine (PE) is described. A preparation method is given for 1-(2-hydroxyethyl)-5-(hydroxymethyl)-1*H*-pyrrole-2-carbaldehyde (**8**). Independent syntheses as well as unequivocal structural characterization are reported for the substitution products of **8** 1-(2-hydroxyethyl)-5-(methoxymethyl)-1*H*-pyrrole-2-carbaldehyde (**9a**) and 5-(ethoxymethyl)-1-(2-hydroxyethyl)-1*H*-pyrrole-2-carbaldehyde (**9b**). For all these compounds, chromatographic and spectroscopic data were established by GLC–MS and HPLC with diode array detection (DAD). PE and D-glucose or 3-DG **4** were either incubated at pH 7.4, 100 °C for 3 h or at pH 7.4, 37 °C for 5 weeks in neat buffer or ethanol–buffer mixtures. The phospholipid fraction was purified on a C18 solid-phase extraction column and cleaved with ethanolic potassium hydroxide. The carbaldehyde **8**, released in this process, was identified by GLC–MS and quantified by HPLC–DAD. Formation of **7** is favored in the ethanol–buffer reactions relative to those in buffer solution only although the amounts determined from the 37 °C incubations generally are very low. It seems likely, therefore, that phospholipid-linked pyrrolecarbaldehydes, such as **7**, are biomarkers rather than effectors of membrane damage in vivo. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Carbonyl-amine reactions are ubiquitous in nature, manifesting themselves, inter alia, in the nonenzymatic reaction of reducing carbohydrates, such as D-glucose, with free amino acids and protein-bound amino acid moieties.[1,2] These processes, known as Maillard reactions, have been shown to proceed in vitro as well as in vivo. 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 aminoenols **2**, into the aminoketoses **3** (Amadori products).[3]

These are slowly degraded, in complex reaction pathways, to a large number of compounds summarized as 'advanced glycation end products' (AGEs). Dicarbonyl compounds, such as the 3-deoxyglucosone (**4**, 3-DG), represent key intermediates in this overall reaction sequence. Pyrrolecarbaldehydes of general structure **5** (AGE) have been detected in foodstuffs and in tissue proteins;[4–6] their formation from **4** may be formulated via enolization, dehydration, and subsequent Michael addition of an amine to C-5 of the carbohydrate chain.[7] In vivo, such chemical changes are considered to cause gradual deterioration in structure and function of tissue proteins, and thus to contribute to the pathophysiology of the normal aging process.[8–10] The accelerated Maillard reaction in diabetics and the subsequent increased glycation level for various proteins link the characteristic hyperglycemia of this metabolic disease with the long-term complications involved.

Several recent reports indicate that aminophospholipids may be targets for Maillard reactions just as proteins. Bucala et al.[11,12] have shown phosphatidyl ethanolamine (PE), a lipid component of low density lipoprotein (LDL), to likewise react with glucose and so to initiate advanced glycation reactions, forming phospholipid-linked AGEs. The AGE levels, detected by ELISA,

Keywords: Maillard reaction; phosphatidyl ethanolamine; pyrrolecarbaldehyde; D-glucose.

*Corresponding author. Tel.: +49-711-459-4095; fax: +49-711-459-4096; e-mail: ledererm@uni-hohenheim.de

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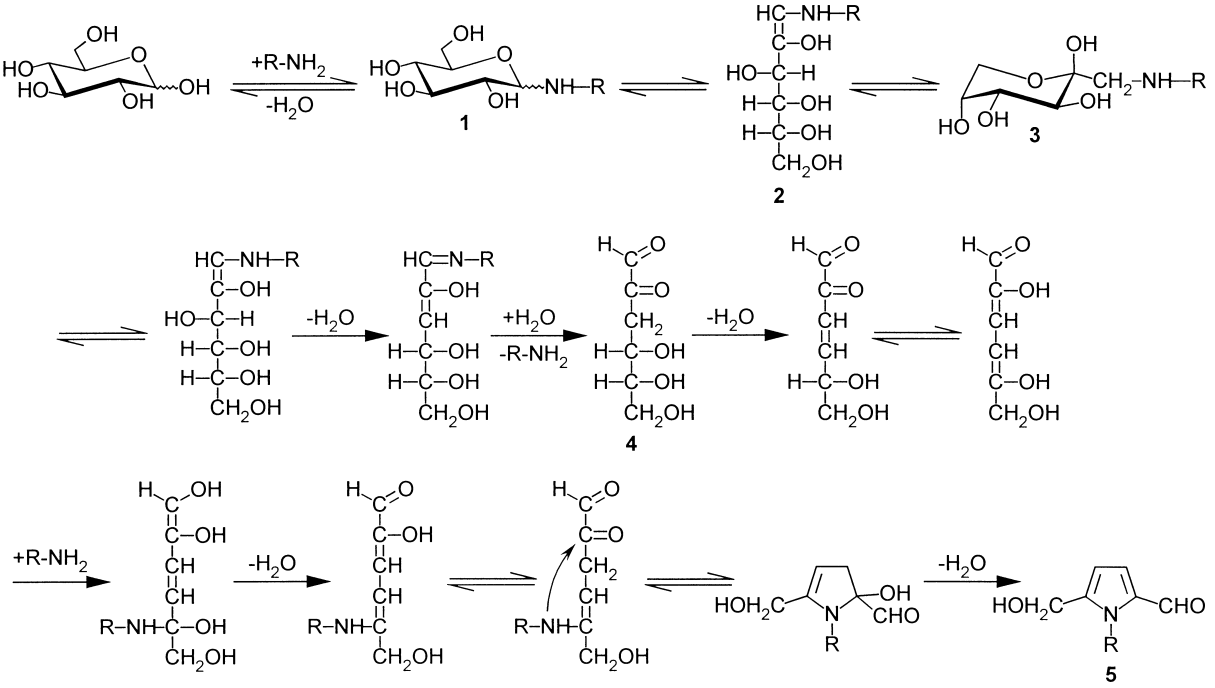


Figure 1. Hypothetic reaction pathway for the formation of 1-alkyl-5-(hydroxymethyl)-1*H*-pyrrole-2-carbaldehyde (**5**) from the reaction of D-glucose with primary aliphatic amines via glucosylamine **1**, aminoenol **2**, Amadori product **3**, and 3-deoxyglucosone (**4**, 3-DG).

show significant correlation with the level of oxidized LDL; this finding supports 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, 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.[13] have obtained 5-hydroxymethylfurfural as a stable follow-up product of Amadori compounds upon treatment of glucose-aminophospholipid adducts with acid; they found increased levels of glycated aminophospholipids in the liver of rats with streptozotocin-induced diabetes. Ravandi et al.[14,15] further documented the existence of glycation products of PE in human red blood cells. The analytical techniques employed by both groups could not distinguish, however, between glycosylamines **1** and aminoketoses **3**. Recently, we have definitely established the formation of the Amadori product 1-deoxy-1-[2-(1,2-ditetradecanoyl-*sn*-glycero-3-phosphooxy)ethylamino]-D-fructose from model reactions of D-glucose with PE.[16] Finally, carboxymethyl-PE, originating from either glycoxidation or lipoxidation processes, has been detected in red blood cells[17] and mitochondrial membranes.[18]

We now report on the spectroscopic and chromatographic characterization of 1-(2-hydroxyethyl)-5-(hydroxymethyl)-1*H*-pyrrole-2-carbaldehyde (**8**; see Fig. 2) and its alkoxy derivatives. On the basis of these data sets, it could be unequivocally proven that the phospholipid-linked AGE 1-[2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl]-4,10-dioxo-7-(tetradecanoyloxy)-3,5,9-trioxa-4λ^5-phosphatricosan-4-olate (**7**) is in fact formed from PE and D-glucose or 3-DG in model systems.

Results and Discussion

Following a preparation given by Kato and Fujimaki,[19] we first synthesized 1-(2-hydroxyethyl)-5-(hydroxymethyl)-1*H*-pyrrole-2-carbaldehyde (**8**) from D-glucose and ethanolamine in methanolic solution.

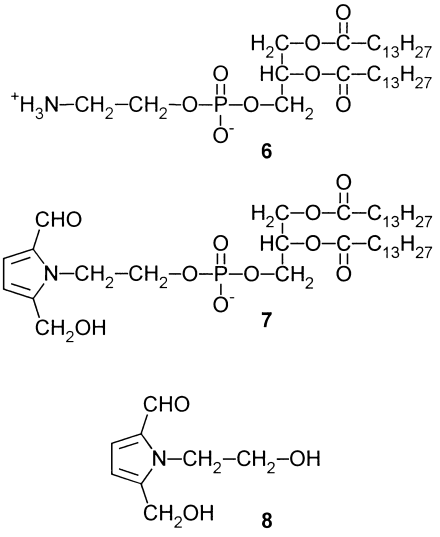


Figure 2. Structural formulae of 1,2-ditetradecanoyl-*sn*-glycero-3-phosphoethanolamine (**6**), 1-[2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl]-4,10-dioxo-7-(tetradecanoyloxy)-3,5,9-trioxa-4λ^5-phosphatricosan-4-olate (**7**), and 1-(2-hydroxyethyl)-5-(hydroxymethyl)-1*H*-pyrrole-2-carbaldehyde (**8**).

As described, the diethyl ether extract of the reaction mixture yields **8** as the main product and a minor constituent to which the authors assigned a 5-(hydroxymethyl)-1-(2-methoxyethyl)-1*H*-pyrrole-2-carbaldehyde (**10**) structure; the postulated formation pathway is given in Figure 3. However, it has been shown that especially the hydroxy group at the α-carbon of a pyrrole alkyl side chain is readily substituted by an alcoholic OR moiety.[20] 1-(2-Hydroxyethyl)-5-(methoxymethyl)-1*H*-pyrrole-2-carbaldehyde (**9a**), therefore, seemed to be a more plausible structure for the by-product. In the context of the present investigation, clearing up this problem was an absolute prerequisite. If the pathway of Kato and Fujimaki was correct, at least partial hydrolysis or alcoholysis of compound **7** would be expected in the course of incubation and work up since the phosphoric acid diester function in this compound is a potent leaving group; no accurate quantification of **7** thus would have been feasible.

^{1}H and ^{13}C NMR data of **8** and **9a** are compiled in Table 1, and the ^{1}H data of **8** are fully consistent with those given in the literature.[19] The numbering of the carbon atoms 1–6 follows that of the native carbohydrate. Differentiation between the two potential structures **9a** and **10** rests on a ^{1}H-coupled ^{13}C NMR spectrum. It clearly displays a triplet ($^1J_{C\text{-}6,H_2\text{-}6}$ = 143.5 Hz) of quartets ($^3J_{C\text{-}6,H_3\text{-}1''}$ = 5.0 Hz) at 65.6 ppm

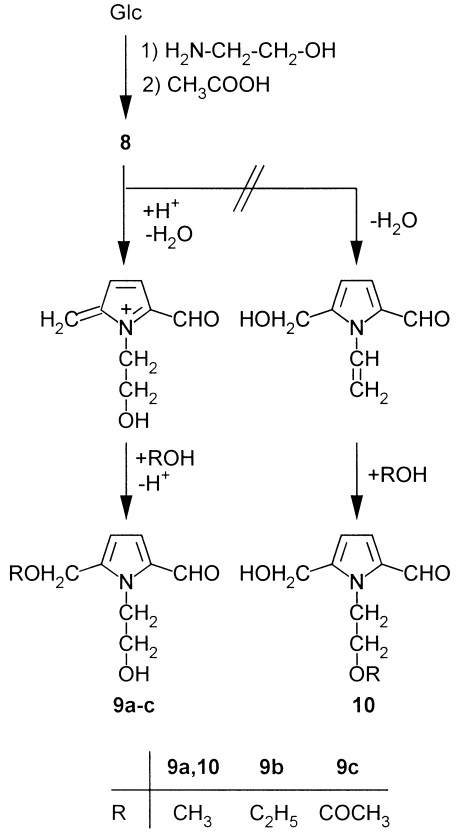
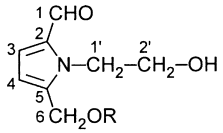


Figure 3. Reaction pathway for the formation of 1-(2-hydroxyethyl)-5-(methoxymethyl)-1*H*-pyrrole-2-carbaldehyde (**9a**), 5-(ethoxymethyl)-1-(2-hydroxyethyl)-1*H*-pyrrole-2-carbaldehyde (**9b**), and [5-formyl-1-(2-hydroxyethyl)-1*H*-pyrrol-2-yl]methyl acetate (**9c**). 5-(Hydroxymethyl)-1-(2-methoxyethyl)-1*H*-pyrrole-2-carbaldehyde (**10**) was assigned by Kato and Fujimaki[19] for the methoxy derivative of **8**.

for C-6. The C-2′ resonance (62.8 ppm), in contrast, only shows a triplet ($^1J_{C\text{-}2';H_2\text{-}2'}$ = 144.5 Hz) with line broadening, characteristic for unresolved OH coupling and a very small $^2J_{C\text{-}2';H_2\text{-}1'}$ coupling constant. The quartet multiplicity of the C-6 signal which is due to the methoxy substituent definitely proves structure **9a** for the minor constituent, and confirms the susceptibility of the 6-OH group in **8** to nucleophilic substitution.

Our previous experiments with PE have shown glycation of the phospholipid to be favored in ethanol–buffer

Table 1. ^{1}H and ^{13}C NMR spectroscopic data of **8** and **9a**,**b** (in $CDCl_3$)[a]



	8	**9a**	**9b**
R	H	1″ CH_3	1″ 2″ CH_2CH_3
^{1}H NMR		δ (ppm)	
H-1	9.46	9.49	9.50
H-3	6.93	6.93	6.93
H-4	6.25	6.27	6.26
H_2-6	4.57	4.48	4.52
H_2-1′	4.47	4.50	4.52
H_2-2′	3.91	3.90	3.93
H_3-1″	—	3.39	—
H_2-1″	—	—	3.58
H_3-2″	—	—	1.24
J(H,H) [Hz]			
$^2J_{3,4}$	4.0	3.9	4.0
$^3J_{1',2'}$	4.5	5.2	5.2
$^3J_{1'',2''}$	—	—	7.0
^{13}C NMR		δ (ppm)	
C-1	179.9	180.0	180.0
C-2	132.1	132.8	132.8
C-3	125.3	124.8	124.8
C-4	111.0	111.9	111.7
C-5	142.9	139.3	139.6
C-6	55.1	65.6	63.7
C-1′	47.5	48.0	48.1
C-2′	62.2	62.8	62.9
C-1″	—	58.1	66.2
C-2″	—	—	14.9
J(C,H) [Hz]			
$^1J_{C\text{-}6,H_2\text{-}6}$	142.4	143.5	143.1
$^3J_{C\text{-}6,H_3\text{-}1''}$	—	5.0	—
$^1J_{C\text{-}1'',H_3\text{-}1''}$	—	141.5	—
$^1J_{C\text{-}1'',H_2\text{-}1''}$	—	—	141.3
$^3J_{C\text{-}1'',H_2\text{-}6}$	—	3.8	3.6
$^3J_{C\text{-}6,H_2\text{-}1''}$	—	—	3.4
$^1J_{C\text{-}1',H_2\text{-}1'}$	140.2	140.0	139.8
$^2J_{C\text{-}1',H_2\text{-}2'}$	(−)2.5	(−)3.0	< 1
$^1J_{C\text{-}2',H_2\text{-}2'}$	141.9	144.5	144.5
$^2J_{C\text{-}2',H_2\text{-}1'}$	(−)3.0	< 1	(−)2.7

[a]δ (ppm), chemical shift for the indicated hydrogen/carbon; *J* [Hz], coupling constant between the indicated nuclei. Hydrogen/carbon assignment has been validated by ^{1}H, ^{1}H-COSY and ^{1}H, ^{13}C-COSY measurements.

mixtures relative to those in neat buffer.[16] Hence, analogous to the reaction of **8** to **9a** in methanolic solution, 1-[2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl]-4,10-dioxo-7-(tetradecanoyloxy)-3,5,9-trioxa-4λ^5-phosphatricosan-4-olate (**7**) may in part be transformed into its ethoxy derivative in the presence of ethanol. To clarify whether this product is actually formed from PE and D-glucose or 3-DG in ethanol-buffer mixtures, we also synthesized 5-(ethoxymethyl)-1-(2-hydroxyethyl)-1*H*-pyrrole-2-carbaldehyde (**9b**) by carrying out the procedure of Kato and Fujimaki in ethanolic solution. Unfortunately, the **9b** formed shows coelution on silica gel with another minor constituent, [5-formyl-1-(2-hydroxyethyl)-1*H*-pyrrol-2-yl]methyl acetate (**9c**, see Fig. 3), which represents the substitution product of **8** by acetate. No such reaction between pyrrolecarbaldehydes and carboxylates has yet been described in the literature. This process represents an interesting new aspect for the cross-linking of proteins since lysine-bound pyrrolecarbaldehydes (e.g. pyrraline), incorporated in a protein, may be expected to react with glutamic or aspartic acid moieties.

Products **9b**,**c** were isolated by preparative reversed phase HPLC. As a welcome side effect, the yield of unsubstituted **8** increases 3-fold upon changing the solvent from methanol to ethanol. The NMR data of **9b** are also included in Table 1; the ^{1}H-coupled ^{13}C NMR spectrum again proves that nucleophilic substitution has occurred at C-6.

With the chromatographic and spectroscopic data of compounds **8**,**9b** firmly established, reaction mixtures of PE and D-glucose or 3-DG **4** in buffer as well as in ethanol–buffer mixtures can be probed for the formation of **7** and its ethoxy derivative. 3-Deoxyglucosone (**4**) was synthesized following a procedure given in the literature.[21] The model phospholipid, 1,2-ditetradecanoyl-*sn*-glycero-3-phosphoethanolamine (**6**) (16 mM) was incubated with 500 mM D-glucose or **4**. The medium was either 0.1 M phosphate buffer (pH 7.4) or a 3:2 ethanol-buffer mixture, the ethanol improving solubilization of the phospholipid. The chosen reaction conditions are summarized in Table 2; the work up of these incubation mixtures is outlined schematically in Figure 4.

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 neat methanol. Elution of the phospholipids and their reaction products required 2/1 chloroform/methanol; the respective fractions were evaporated to dryness,

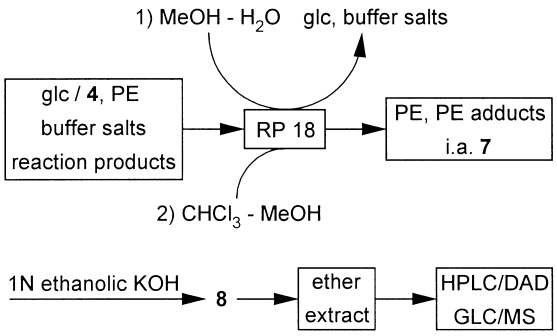


Figure 4. Schematic work up procedure for the D-glucose/3-DG-phosphatidyl ethanolamine (PE) incubations.

and the residue treated with 1 N ethanolic potassium hydroxide solution. The alcoholic solvents, employed for the work up procedure, again might transform 1-[2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl]-4,10-dioxo-7-(tetradecanoyloxy)-3,5,9-trioxa-4λ^5-phosphatricosan-4-olate (**7**) into its respective OR derivatives.

Determination of the phospholipid-linked Amadori product requires enzymatic cleavage with phospholipase D.[16] Since **8**, contrary to the Amadori compound, is perfectly stable in alkaline medium we have rather employed ethanolic potassium hydroxide for hydrolysis. Under these conditions, PE **6** quantitatively yields free fatty acids, glycerol-1-phosphate, and ethanolamine (not detailed in the experimental section), that is, exclusively the ethanolamine C-phosphate bond of the phosphoric acid diester is cleaved. This procedure thus allows **7** to be properly quantified in form of the 1-(2-hydroxyethyl)-5-(hydroxymethyl)-1*H*-pyrrole-2-carbaldehyde (**8**). The specificity of phospholipase D cleavage of **7**, in contrast, has not yet been ascertained. The hydrolyzates were extracted with diethyl ether, and the organic phase analyzed by GLC–MS and HPLC–DAD for unequivocal product identification. From all incubations, except C (see Table 2), compound **8** was detected by both analytical techniques. This result definitely proves, for the first time, formation of **7** in the course of the Maillard reaction of PE with D-glucose or 3-DG **4**. The substitution products **9a**,**b** could not be identified, though, in any of the hydrolyzates. This may be due to the fact that formation of alkoxyalkylpyrrole ethers very likely proceeds via an S_N1 mechanism, and thus is favored in an acidic reaction medium as employed for the synthesis of **8**.[22] The PE incubations described above and the work up were carried out in mildly or strongly alkaline media which fortunately precluded any nucleophilic substitution. Hence, for the quantification

Table 2. Reaction conditions for the D-glucose/3-DG-phosphatidyl ethanolamine (PE) incubations and conversion rate of PE to 1-[2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl]-4,10-dioxo-7-(tetradecanoyloxy)-3,5,9-trioxa-4λ^5-phosphatricosan-4-olate (**7**)

Incubation	Glycating agent	Solvent	Incubation time	Temperature (°C)	mmol **7**/mol PE
A	glc	Buffer	3 h	100	0.46
B	glc	EtOH–buffer	3 h	100	2.52
C	glc	Buffer	35 days	37	n.d.
D	glc	EtOH–buffer	35 days	37	0.08
E	3-DG	Buffer	35 days	37	0.03
F	3-DG	EtOH–buffer	35 days	37	0.17

of **7**, the HPLC–DAD system had to be calibrated only with **8** in the range of 0.162–5.185 mg **8**/L, using a detection wavelength of 298 nm. The linear calibration graph is given by the equation area = (0.21 ± 0.55) + (105.82 ± 0.23) L/mg × c(**8**), the values in brackets representing means ± confidence intervals ($p = 95\%$). The standard error was determined as 0.80. Limit of detection (LOD) 0.04 mg/L and limit of quantitation (LOQ) 0.06 mg/L were calculated according to the 'Recommendations of the Deutsche Forschungsgemeinschaft' (DFG).[23] In this special case, the GLC–MS system, equipped only with a split injector is not suitable for quantification, since **8** shows partial loss of water, presumably from the 1-(2-hydroxyethyl) substituent, during vaporization; the signal for the respective elimination product is characterized by a molecular ion ($M^{+\cdot}$) at m/z 151.

The amounts of **8**, determined by HPLC–DAD for the incubations A–F, can be directly correlated with the PE derivatization rate as given in Table 2. The data clearly show that formation of **7** is favored in the presence of ethanol, due probably to a better solubilization of PE. Only in incubation B, where the phospholipid is completely dissolved during the reaction at 100 °C, the yield of **7** is close to that of **8**, obtained from the synthesis in ethanol. For incubation C at 37 °C in pure buffer, not even traces of **8** could be detected; additionally, this suspension is the only one which shows no browning at all. Comparison of the reaction mixtures with 3-DG **4** to those with D-glucose also suggests that the solubilization of PE indeed is an important rate limiting criterion for the conversion to **7**. Since **4** represents a reactive intermediate in the formation of pyrrolecarbaldehydes **5** (see Fig. 1), a much higher yield of **7** would have to be expected for these incubations. Though, the rather low concentrations, determined in mixtures E and F, imply that 3-DG **4** is prone to other pathways like reverse aldol and/or condensation reactions being also responsible for the extensive browning of both suspensions.

Conclusion

The data presented above clearly demonstrate that 1-[2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl]-4,10-dioxo-7-(tetradecanoyloxy)-3,5,9-trioxa-4λ^5-phosphatricosan-4-olate (**7**) is formed in model reactions of PE with D-glucose or 3-DG **4**. Even though, the amounts determined for the 37 °C incubations are very low. The extremely poor solubilization of PE, especially in pure aqueous solution, probably is the one decisive factor for this result. In biological systems, however, the polar head groups of phospholipids, as main constituents of membranes, are well-solvated and thus should be more susceptible to glycation reactions. We now have independently synthesized phospholipid-linked pyrrolecarbaldehydes similar to **7**, and elaborated an LC–MS method for their quantification. Presently, red cell membranes of diabetics and control subjects as well as phospholipids from foodstuffs are investigated for such structures. The results from the in vivo study will then show whether PE-derivatives, such as **7**, can be important effectors of membrane damage or whether they may only serve as biomarkers for AGE modified phospholipids.

Experimental

General methods

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker (Karlsruhe, Germany) AC 250/ARX 500 spectrometers at 250/500 and 63/126 MHz, respectively, in $CDCl_3$. Chemical shifts (δ) are given in ppm relative to internal Me_4Si, coupling constants (J) in Hz. For the 2-D NMR experiments ^{1}H, ^{1}H-COSY[24] and ^{1}H, ^{13}C-COSY,[25] Bruker standard software (X-WIN-NMR 2.0) was employed. UV spectra were measured with a Perkin–Elmer Lambda 2 instrument. The analytical high performance liquid chromatography (HPLC) system comprised an HP1100 autosampler, HP1100 gradient pump, and HP1100 diode array detector (DAD) module (Hewlett Packard, Waldbronn, Germany). For data acquisition and processing, an HP Chem Station (Rev. A. 04.02) software was used. Column (Bischoff, Leonberg, Germany): Nucleosil C18, 5 μm, 100 Å (column 250×4 mm, guard column 10×4 mm); flow rate 0.8 mL/min; injection volume 20 μL; water-acetonitrile gradient: % MeCN(t [min]) 2(0)-8(15)-50(35)-95(40-50)-2(55-65); DAD detection wavelengths 254 and 298 nm, spectral band width (SBW) 4 nm, reference 500 nm (SBW 100 nm). The preparative HPLC system consisted of a Knauer (Berlin, Germany) 64 liquid chromatograph combined with an A0293 variable wavelength detector and a Kronlab HPLC column (Nucleosil C18, 7 μm, 100 Å, column 250×20 mm, guard column 50×20 mm); flow rate 10 mL/min; eluent: Water/MeOH 55:45 (v/v); injection volume 1.0 mL; detection wavelength 254 nm. Solutions were filtered (membrane filter, 0.45 μm) before preparative HPLC. Silica gel 60 F_{254}, 0.2 mm (Merck, Darmstadt, Germany) was used for thin-layer chromatography (TLC), silica gel, 63–200 μm (Baker, Gross-Gerau, Germany) for column chromatography, and Chromabond C18, 1000 mg-6 mL cartridges (Macherey-Nagel, Düren, Germany) for solid phase extraction (SPE). GLC–MS was performed using a Finnigan MAT (Bremen, Germany) Ion Trap 800 equipped with a Perkin–Elmer (Überlingen, Germany) 8420 gas-liquid chromatograph. Injector and transfer line temperatures were set at 270 °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 80 to 270 °C at 8 °C/min with 10 min isothermal at 270 °C. Helium was used as the carrier gas at a linear velocity of 27 cm/s (determined by injection of methane, oven temperature 100 °C). Column effluents were analyzed in the 70 eV EI mode.

Materials

Ultrapure water, obtained from a Milli-Q 185 Plus apparatus (Millipore, Eschborn, Germany), HPLC grade methanol, and HPLC grade acetonitrile were used for LC. For preparative HPLC solvents were degassed by flushing with helium. Ethanolamine was purchased

from Fluka (Neu-Ulm, Germany), and 1,2-ditetradecanoyl-*sn*-glycero-3-phosphoethanolamine from Sigma (Steinheim, Germany).

Preparation of 1-(2-hydroxyethyl)-5-hydroxymethyl-1*H*-pyrrole-2-carbaldehyde (8) and 1-(2-hydroxyethyl)-5-(methoxymethyl)-1*H*-pyrrole-2-carbaldehyde (9a).[19] Compounds **8** and **9a** were prepared according to a procedure given by Kato and Fujimaki.[19] The amounts for reagents and MeOH were doubled, and isolation of the products was modified as follows: water (100 mL) was added to the reaction mixture, the solution extracted with diethyl ether (3×100 mL), the organic layer washed with a saturated solution of $NaHCO_3$, and the washing liquid extracted with diethyl ether (100 mL). The combined organic layers were dried with anhydrous Na_2SO_4. The solution was evaporated to dryness and the residue purified on a silica gel column (2×15 cm; ethyl acetate/hexane, 2/1, v/v, 300 mL). Fractions (7 mL each) were collected and tested for **8** and **9a** by TLC (R_f 0.23 and 0.43, respectively, solvent mixture as above). From the respective fractions, containing **8** or **9a**, the solvent was stripped off and the residues were dried in high vacuum, yielding **8** (74 mg, 0.44 mmol, 0.11%) and **9a** (30 mg, 0.16 mmol, 0.04%); ^1H and ^{13}C NMR ($CDCl_3$): see Table 1; ^1H NMR and UV data are fully consistent with those in ref 19; HPLC–DAD: t_R 16.8 min (**8**), 24.7 min (**9a**); GLC–MS: t_R 15.2 min (**8**), *m/z* 169 (9, $M^{+\cdot}$), 151 (50), 136 (13), 123 (46), 108 (100), 94 (41), 80 (83) 68 (51), 53 (47); t_R 13.7 min (**9a**), *m/z* 183 (30, $M^{+\cdot}$), 151 (53), 122 (61), 108 (71), 96 (100), 80 (70), 65 (38), 53 (61).

5-(Ethoxymethyl)-1-(2-hydroxyethyl)-1*H*-pyrrole-2-carbaldehyde (9b). The reaction was carried out in ethanol instead of methanol, following the procedure described above. Fractions from the silica gel column (7 mL each) were collected and tested for **8** and **9b** by TLC (R_f 0.23 and 0.50, respectively). From the respective fractions, containing **8** or **9b**, the solvent was stripped off and the residues were dried in vacuo yielding **8** (225 mg, 1.33 mmol, 0.33%) and crude **9b** (70 mg, with [5-formyl-1-(2-hydroxyethyl)-1*H*-pyrrol-2-yl]methyl acetate (**9c**) as by-product). Compounds **9b**,**c** were purified by preparative HPLC. Fractions at t_R 13.6 and 16.7 min were collected, the methanol was removed in vacuo, and the aqueous layer extracted with CH_2Cl_2 (3×20 mL). The organic phase was dried with anhydrous Na_2SO_4 and evaporated to dryness, yielding **9c** (3 mg, 0.014 mmol, 0.004%) and **9b** (19 mg, 0.096 mmol, 0.02%), respectively; **9b**: ^1H and ^{13}C NMR ($CDCl_3$): see Table 1; UV (ethanol): λ_{max} [nm] (lg ε) 294 (4.19); HPLC–DAD: t_R 27.60 min; GLC–MS: t_R 14.78 min, *m/z* 197 (33, $M^{+\cdot}$), 180 (1), 168 (12), 152 (62), 122 (53), 108 (67), 96 (100), 80 (66) 65 (30), 53 (53); **9c**: ^1H NMR ($CDCl_3$) δ 9.52 (s, H-1), 6.95 (d, $J_{3,4}$ = 4.1 Hz, H-3), 6.35 (d, H-4), 5.18 (s, H_2-6), 4.52 (t, $J_{1',2'}$ = 5.3 Hz, H_2-1′), 3.94 (t, H_2-2′), 2.10 (s, H_3-2″) (for the numbering see Table 1); HPLC–DAD: t_R 26.71 min; GLC–MS: t_R 16.40 min, *m/z* 211 (22, $M^{+\cdot}$), 182 (7), 169 (25), 151 (37), 122 (45), 108 (100), 96 (77), 80 (59) 65 (35), 53 (52).

D-Glucose/3-DG-phosphatidyl ethanolamine incubations. D-Glucose (180 mg, 1 mmol) or **4** (240 mg of a product containing 67% 3-DG, 1 mmol, synthesized as in ref 21) and 1,2-ditetradecanoyl-*sn*-glycero-3-phosphoethanolamine (**6**, 21 mg, 0.032 mmol) were dissolved in phosphate buffer (0.1 M, pH 7.4) or in 3:2 EtOH:buffer (2 mL each) (see Table 2). The vials were sonicated in an ice bath for 30 min to produce lipid suspension, and incubated in the dark either for 3 h at 100 °C or for 5 weeks at 37 °C.[26]

Work up of incubation mixtures and cleavage of the phospholipid. 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 again discarded. The phospholipid fraction was eluted with 2/1 $CHCl_3$/MeOH (8 mL) at 30–35 °C, the solution concentrated to 1 mL, and the solvent finally removed under a gentle stream of nitrogen. To the residue was added 1 N ethanolic potassium hydroxide (2 mL) and the solution kept at 80 °C for 45 min. After addition of water (15 mL), the mixture was extracted with diethyl ether (10×15 mL), the organic layer dried with anhydrous Na_2SO_4, concentrated to about 0.5 mL, and 1 μL injected into the GLC–MS system. From the remaining solution the solvent was stripped off under a gentle stream of nitrogen, the residue redissolved in a water:MeOH mixture (1/1, v/v, 0.5 mL), and analyzed by HPLC–DAD.

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References

1. Ledl, F.; Schleicher, E. *Angew. Chem.* **1990**, *102*, 597.
2. Friedman, M. *J. Agric. Food Chem.* **1996**, *44*, 631.
3. Amadori, M. *Atti R. Acad. Naz. Lincei Mem. Cl. Sci. Fis. Mat. Nat.* **1931**, *13*, 72.
4. Jurch, G. R.; Tatum, J. H. *Carbohydr. Res.* **1970**, *15*, 233.
5. Chiang, G. H. *J. Agric. Food Chem.* **1988**, *36*, 506.
6. Hayase, F.; Nagaraj, R. H.; Miyata, S.; Njoroge, F. G.; Monnier, V. M. *J. Biol. Chem.* **1989**, *264*, 3758.
7. Ledl, F. In *The Maillard Reaction in Food Processing, Human Nutrition and Physiology*; Finot, P. A.; Aeschbacher, H. U.; Hurrell, R. F.; Liardon, R., Eds.; Birkhaeuser: Basel, 1990; pp 19–42.
8. Dyer, D. G.; Blackledge, J. A.; Katz, B. M.; Hull, C. J.; Adkisson, H. D.; Thorpe, S. R.; Lyons, T. J.; Baynes, J. W. *Z. Ernaehrungswiss.* **1991**, *30*, 29.
9. Monnier, V. M. *Prog. Clin. Biol. Res.* **1989**, *304*, 1.
10. Sell, D. R.; Lane, M. A.; Johnson, W. A.; Masoro, E. J.; Mock, O. B.; Reiser, K. M.; Fogarty, J. F.; Cutler, R. G.;

Ingram, D. K.; Roth, G. S.; Monnier, V. M. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 485.
11. Bucala, R.; Makita, Z.; Vega, G.; Grundy, S.; Koschinsky, T.; Cerami, A.; Vlassara, H. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 9441.
12. Bucala, R.; Makita, Z.; Koschinsky, T.; Cerami, A.; Vlassara, H. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 6434.
13. Pamplona, R.; Bellmunt, M. J.; Portero, M.; Riba, D.; Prat, J. *Life Sci.* **1995**, *57*, 873.
14. Ravandi, A.; Kuksis, A.; Marai, L.; Myher, J. J.; Steiner, G.; Lewisa, G.; Kamido, H. *FEBS Lett.* **1996**, *381*, 77.
15. Ravandi, A.; Kuksis, A.; Marai, L.; Myher, J. *J. Lipids* **1995**, *30*, 885.
16. Lederer, M. O.; Dreisbusch, C. M.; Bundschuh, R. M. *Carbohydr. Res.* **1997**, *301*, 111.
17. Requena, J. R.; Ahmed, M. U.; Fountain, C. W.; Degenhardt, T. P.; Reddy, S.; Perez, C.; Lyons, T. J.; Jenkins, A. J.; Baynes, J. W.; Thorpe, S. R. *J. Biol. Chem.* **1997**, *272*, 17473.
18. Pamplona, R.; Requena, J. R.; Porterootin, M.; Prat, J.; Thorpe, S. R.; Bellmunt, M. J. *Eur. J. Biochem.* **1998**, *255*, 685.
19. Kato, H.; Fujimaki, M. *Lebensm.-Wiss. Technol.* **1972**, *5*, 172.
20. Klein, E.; Ledl, F.; Bergmueller, W.; Severin, T. Z. *Lebensm. Unters. Forsch.* **1992**, *194*, 556.
21. Madson, M. A.; Feather, M. S. *Carbohydr. Res.* **1981**, *94*, 183.
22. Sonnet, P. E. *J. Heterocycl. Chem.* **1970**, *7*, 1101.
23. Walter, H.-F.; Holtz, K.-H.; Frehse, H.; Gorbach, S. G.; Thier, H.-P. In *Rückstandsanalytik von Pflanzenschutzmitteln - Mitteilung VI der Senatskommission für Pflanzenschutz-, Pflanzenbehandlungs-, und Vorratsschutzmittel - Methodensammlung der Arbeitsgruppe 'Analytik'*; Senatskommission der Deutschen Forschungsgemeinschaft, Ed.; VCH-Verlag: Weinheim, 1991, chapter XI-A, pp 1–22.
24. Braun, S.; Kalinowski, H.-O.; Berger, S. 150 and more basic NMR experiments: a practical course, Wiley-VCH: Weinheim, 1998, 2nd ed., pp. 353–355.
25. Braun, S.; Kalinowski, H.-O.; Berger, S. 150 and more basic NMR experiments: a practical course, Wiley-VCH: Weinheim, 1998, 2nd ed., pp. 375–377.
26. London, E.; Feigenson, G. W. *Anal. Biochem.* **1978**, *88*, 203.