Determination of 4Hydroxy2,5-dimethyl-3(2 H)-furanone and 2(or 5)Ethyl4-hydroxy-5(or 2)-methyl-3(2 H)-furanone in Pentose Sugar-Based Maillard Model Systems by Isotope Dilution A...

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Determination of 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone and 2(or 5)-Ethyl-4-hydroxy-5(or 2)-methyl-3(2*H*)-furanone in Pentose Sugar-Based Maillard Model Systems by Isotope Dilution Assays

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The formation of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) and 2(or 5)-ethyl-4-hydroxy-5(or 2)-methyl-3(2*H*)-furanone (EHMF) from pentose sugars was studied in Maillard model systems. The amounts generated at 90 °C for 1 h were determined by isotope dilution assay (IDA). The internal standards used for IDA, i.e., [$^{13}C_2$]HDMF and [$^{2}H_3$]EHMF, were prepared in good overall yields in three steps: addition of labeled acetaldehyde or propionaldehyde to *tert*-butyloxycarbonyl (Boc)-protected and lithiated 3-butyn-2-ol; oxidation of the Boc-protected diol with permanganate to 1,2-dione; and finally cyclization to the target molecules after removal of the protective groups under acidic conditions. Quantitative data confirmed previous findings that HDMF and EHMF are preferentially formed in the presence of glycine and L-alanine, respectively. The yields obtained were 2.6−5.1 μg of HDMF and 6.8−10 μg of EHMF per mmol pentose. Formation of both furanones was favored in phosphate-buffered solutions at pH 7 compared to pH 5, particularly in the presence of an excess of amino acid. These data are well in agreement with the previously proposed formation mechanism of HDMF and EHMF via Strecker-assisted chain elongation of the pentose moiety. However, both furanones were also produced to a lesser extent by sugar fragmentation−condensation reactions.

Keywords: *Maillard reaction; pentose model system; 4-hydroxy-2,5-dimethyl-3(2H)-furanone; Furaneol; 2(or 5)-ethyl-4-hydroxy-5(or 2)-methyl-3(2H)-furanone; Homofuraneol; synthesis; alcohol protection; alkyne oxidation; 1,2-dione; stable-isotope label; isotope dilution assay; GC−MS*

INTRODUCTION

4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF, Furaneol, a registered trademark of Firmenich S.A., Geneva, Switzerland) and 2(or 5)-ethyl-4-hydroxy-5(or 2)-methyl-3(2*H*)-furanone (EHMF, Homofuraneol) are important naturally occurring food flavor compounds with a caramel-like note. HDMF is especially prevalent, and it has been found in fruits, fermented products, and thermally processed foods (Blank and Fay, 1996, and references cited therein).

The thermally induced formation of 3(2*H*)-furanones from sugars is explained to occur via 2,3-enolization in the Maillard reaction leading to 1-deoxyosones as intermediates (Hodge et al., 1972; Ledl and Schleicher, 1990). In general, the carbon skeleton of the sugar is determinant for the furanone formed, i.e., HDMF is produced from hexoses. As recently shown in model experiments using ^{13}C-labeled precursors, HDMF and EHMF can also be formed from pentose sugars in the presence of amino acids such as glycine and L-alanine, i.e., by Strecker-assisted chain elongation of the sugar moiety (Blank and Fay, 1996) and by sugar fragmentation−condensation reactions (Blank et al., 1996a). However, no quantitative data have yet been published on the amounts of HDMF and EHMF formed.

The quantitative analysis of dihydrofuranones is a challenging task because of their rather fragile nature, water miscibility, and delicate gas chromatographic behavior (Pickenhagen et al., 1981; Blank et al., 1992). The isotope dilution assay (IDA) has been shown to be a sensitive, accurate, and reliable quantification technique in flavor research (Schieberle and Grosch, 1987; Guth and Grosch, 1990) and has also been applied to HDMF and EHMF in strawberries (Sen et al., 1991), cheese (Preininger and Grosch, 1994), and coffee (Semmelroch et al., 1995). This method involves spiking food materials with known amounts of a labeled substance prior to sample preparation and analysis by GC−MS. In this way, losses can be accounted for because whatever changes occur in the natural substance also occur in the labeled version.

As multiply labeled standards simplify mass spectrometric analyses, labeled HDMF and EHMF having at least two stable isotopes per molecule are required. While a number of preparations exist for the unlabeled furanones (Mazenod et al., 1992; Huber, 1992), few are well suited for multiply labeled versions. HDMF in which both methyl groups were ^{13}C-labeled has previously been synthesized (Sen et al., 1991). Deuterium-labeled EHMF was similarly prepared, but in considerably reduced yield, and complicated purifications of intermediates were required (Preininger and Grosch, 1994).

In the present study we report on quantification of HDMF and EHMF generated from pentose sugars in Maillard model reactions. In addition, we present an improved preparation mode of the labeled internal standards, [$^{13}C_2$]HDMF and [$^{2}H_3$]EHMF, required for IDA.

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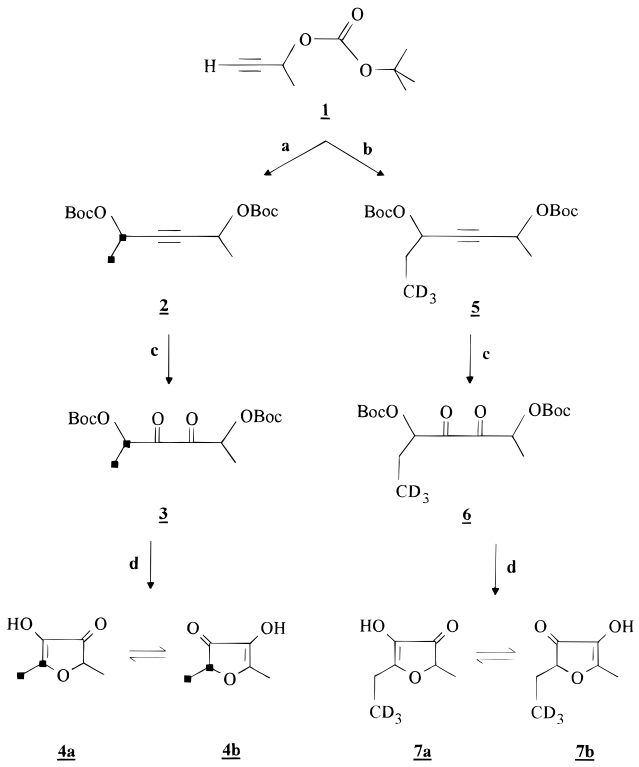

Figure 1. Schematic procedure to synthesize 4-hydroxy-2(or 5)-[^{13}C]methyl-5(or 2)-methyl-3(2*H*)-[2(or 5)-^{13}C]furanone([$^{13}C_2$]-HDMF, ***4ab***) and 2(or 5)-([2,2,2-$^{2}H_3$]ethyl)-4-hydroxy-5(or 2)-methyl-3(2*H*)-furanone ([$^{2}H_3$]EHMF, ***7a*** and ***7b***). Conditions: **a**, lithium diisopropyl amide (LIDA), $^{13}CH_3{}^{13}CHO$, (*t*-BuOCO)$_2$O, THF; **b**, LIDA, CD_3CH_2CHO, (*t*-BuOCO)$_2$O, THF; **c**, $KMnO_4$, acetone/H_2O/CH_3COOH; **d**, oxalic acid, H_2O.

EXPERIMENTAL PROCEDURES

General. D-Xylose, D-ribose, D-arabinose, glycine, L-alanine, and 4-aminobutyric acid of highest purity (≥99%) were from Fluka (Buchs, Switzerland) as well as reagents and solvents for synthesis which were used as received. Butyllithium was stored in a Schlenk tube. [1,2-$^{13}C_2$]Ethanal and [2,2,2-$^{2}H_3$]-ethyl bromide were from Dr. Glaser AG (Basel, Switzerland). Tetrahydrofuran (THF) was distilled from benzophenone/sodium prior to use.

^{1}H-NMR spectra were recorded with a Bruker AC-250 spectrometer at 250 MHz with $CDCl_3$ as solvent and the $CHCl_3$ resonance set at 7.27 ppm. Proton-decoupled ^{13}C-NMR spectra were recorded with a Bruker AMX-400 spectrometer at 100 MHz with $CDCl_3$ solvent set at 77.1 ppm. Infrared spectroscopy was performed with a Perkin-Elmer 1420. The sample was applied to NaCl disks as a thin film.

Qualitative mass spectrometry (MS) was performed on a Finnigan MAT 8430 mass spectrometer (Bremen, Germany). Electron impact (EI) mass spectra were generated at 70 eV, and positive chemical ionization (CI) was at 150 eV with ammonia as the reagent gas. Nonvolatile samples were introduced directly into the ion source held at 200 °C. Volatile components were sampled via a Hewlett-Packard HP-5890 gas chromatograph (Geneva, Switzerland) using the following conditions: cold on-column injector; fused silica capillary column [DB-FFAP (J&W Scientific), 30 m × 0.25 mm; film thickness, 0.25 μm; carrier gas, helium (90 kPa)]; temperature program, 50 °C (2 min), 4 °C/min to 180 °C, 10 °C/min to 240 °C (10 min).

Syntheses. Labeled Furaneol ([$^{13}C_2$]HDMF, **4ab**) and Homofuraneol ([$^{2}H_3$]EHMF, **7ab**) were prepared in three steps from labeled acetaldehyde or propionaldehyde (Figure 1). Aldehyde addition to *tert*-butyloxycarbonyl (Boc) protected and lithiated 3-butyn-2-ol followed by trapping with di-*tert*-butyl dicarbonate led to di-*tert*-butyloxycarbonyl-protected diol intermediates. These were oxidized by acidic aqueous permanganate to give 1,2-diones. The protective groups were removed under acidic conditions while cyclization occurred as the final step.

*3-Butyn-2-yl tert-Butyl Carbonate (**1**).* This was prepared from 3-butyn-2-ol and di-*tert*-butyl dicarbonate according to the method of Eren and Keinan (1988). **1** was distilled (36−38 °C/0.3 mmHg) in 92% yield. $n_D{}^{20}$ = 1.4195. ^{1}H-NMR δ ($CDCl_3$): 1.49 (s, 9H), 1.52 (d, J = 6.9 Hz, 3H), 2.47 (d, J = 2.0 Hz, 1H), 5.23 (dq, J = 6.9, 2.0 Hz, 1H). IR (cm^{-1}): 3260, 2960, 1730, 1260, 1085. MS (EI) *m*/*z* (% relative abundance): 155 (8), 115 (25), 114 (100), 93 (15), 57 (8); the molecular ion was confirmed by CI, ammonia *m*/*z* (% relative abundance): 188 (43, $[M + NH_4]^+$).

*[1,2-$^{13}C_2$]-3-Hexyn-2,5-diyl Bis(tert-butyl carbonate) (**2**).* A lithium diisopropyl amide (LIDA) solution was prepared by adding butyllithium (15.2 mL, 1.43 M in hexane, 21.7 mmol) to 25 mL of THF containing diisopropylamine (3.08 mL, 21.7 mmol) at −30 °C. After 30 min, it was added over 20 min by cannula to the Boc-protected butynol **1** (3.90 mL, 3.70 g, 21.7 mmol) in 30 mL of THF and kept at −78 °C for 30 min. [1,2-$^{13}C_2$]Ethanal (1.00 g, 21.7 mmol) was then added, stirring continued for 1 h, and di-*tert*-butyl dicarbonate (5.68 g, 26.0 mmol) in 20 mL of THF was added by an addition funnel over 20 min. The reaction was kept at −78 °C for 1 h before it was warmed to 25 °C, diluted with 100 mL of Et_2O, and worked up sequentially with 2 N NaOH, H_2O, and brine. Drying (Na_2SO_4) and evaporation of the solvent provided a product in high purity and quantitative yield (7.18 g). An equimolar mixture of diastereomers was obtained as an oil. The resonances of the corresponding nuclei of both diastereomers were isochronous, that is, had coincidentally the same chemical shifts. ^{1}H-NMR δ ($CDCl_3$): 1.48 (s, 18H), 1.52 (ddd, $^1J_{CH}$ = 129 Hz, $^2J_{CH}$ = 4.5 Hz, $^3J_{HH}$ = 6.8 Hz, 3H), 1.52 (d, ∼6.8 Hz, 3H), 5.28 (dm, $^1J_{CH}$ = 153 Hz, 1H), 5.3 (m, 1H). ^{13}C-NMR δ ($CDCl_3$): 21.2 (d, J = 39 Hz), 63.0 (d, J = 39 Hz). IR (cm^{-1}): 2960, 1730, 1260, 1240, 1150. MS (EI) *m*/*z* (% relative abundance): 261 (1), 205 (3), 143 (100), 116 (32), 98 (58), 81 (93), 57 (83); the molecular ion was confirmed by CI, ammonia *m*/*z* (% relative abundance): 334 (100, $[M + NH_4]^+$).

The corresponding unlabeled compound, 3-hexyn-2,5-diyl bis(*tert*-butyl carbonate), was prepared in the same manner as the labeled material, but by substituting unlabeled acetaldehyde. ^{1}H-NMR δ ($CDCl_3$): 1.48 (s, 18H), 1.50 (d, ∼6.5 Hz, 6H), 5.28 (q, J = 6.5 Hz, 2H). ^{13}C-NMR δ ($CDCl_3$): 21.2, 27.9, 63.0, 82.5, 83.4, 152.4. IR (cm^{-1}): 2980, 1745, 1265, 1165. MS (EI) *m*/*z* (% relative abundance): 259 (2), 203 (3), 141 (97), 114 (30), 96 (77), 79 (78), 57 (100); the molecular ion was confirmed by CI, ammonia *m*/*z* (% relative abundance): 332 (100, $[M + NH_4]^+$).

*[1,2-$^{13}C_2$]-3,4-Hexanedione-2,5-diyl Bis(tert-butyl carbonate) (**3**).* Potassium permanganate (4.87 g, 30.8 mmol) was dissolved in a solvent mixture consisting of 140 mL of acetone, 28 mL of H_2O, and 4.2 mL of acetic acid chilled in an ice bath. Alkyne **2** (7.15 g) was dissolved in 20 mL of acetone and poured into the purple solution, resulting in an exothermic reaction and precipitation of brown MnO_4. After 3.5 h at ice temperature, the mixture was filtered through Celite, and excess permanganate was destroyed with a dilute solution of $NaHSO_3$. After a second filtration and evaporation of acetone, the aqueous remainder was extracted with CH_2Cl_2 (3 × 70 mL). The combined organic layers were extracted with saturated $NaHCO_3$ and dried (Na_2SO_4), and the solvent was stripped to give 6.38 g (84% from $^{13}CH_3{}^{13}CHO$) of a yellow solid. ^{1}H-NMR δ ($CDCl_3$): (both diastereomers) 1.48 (s, 18H), 1.49 (s, 18H), 1.50 (d, ∼7.0 Hz, 6H), 1.50 (ddd, $^1J_{CH}$ = 130 Hz, $^2J_{CH}$ = 4.0 Hz, $^3J_{HH}$ = 7.0 Hz, 6H), 5.50 (q, J = 7.0 Hz, 1H), 5.57 (q, J = 7.0 Hz, 1H), 5.53 (dm, $^1J_{CH}$ = 154 Hz, 2H). ^{13}C-NMR δ ($CDCl_3$): (both diastereomers) 15.5 (d, J = 36 Hz), 15.6 (d, J = 36 Hz), 72.5 (d, J = 36 Hz), 73.6 (d, J = 36 Hz). IR (cm^{-1}): 2960, 1730, 1720, 1265, 1242. MS (EI) *m*/*z* (% relative abundance): 277 (8), 175 (16), 141 (13), 130 (3), 113 (4), 57 (100); the molecular ion was confirmed by CI, ammonia *m*/*z* (% relative abundance): 366 (35, $[M + NH_4]^+$).

The corresponding unlabeled compound, 3,4-hexanedione-2,5-diyl bis(*tert*-butyl carbonate), was prepared by substituting

unlabeled alkyne. The yield of the yellow solid formed was 88% from acetaldehyde. ^{1}H-NMR δ ($CDCl_3$): (both diastereomers) 1.46 (s, 18H), 1.48 (d, ~7.0 Hz, 12H), 5.48 (q, J = 7.0 Hz, 2H), 5.54 (q, J = 7.0 Hz, 2H); ^{13}C-NMR δ ($CDCl_3$): (both diastereomers) 15.8, 15.9, 27.8, 72.5, 73.5, 83.3, 152.6, 152.8, 193.9, 194.2. IR (cm^{-1}): 2960, 1733, 1722, 1265, 1245. MS (EI) *m/z* (% relative abundance): 275 (48), 173 (94), 141 (60), 128 (19), 111 (17), 57 (100); the molecular ion was confirmed by CI, ammonia *m/z* (% relative abundance): 364 (65, $[M + NH_4]^+$).

*4-Hydroxy-2(or 5)-[^{13}C]methyl-5(or 2)-methyl-3(2H)-[2(or 5)-^{13}C]furanone ([$^{13}C_2$]HDMF) (**4ab**).* Labeled dione **3** (6.35 g, 18.2 mmol) was refluxed in 38 mL of H_2O with 3.5 g anhydrous oxalic acid for 5 h under nitrogen. After cooling, the reaction mixture was diluted with 60 mL of saturated NaCl and extracted with CH_2Cl_2 (5 × 50 mL). The combined organic layers were extracted with saturated $NaHCO_3$, dried (Na_2SO_4), and the solvent was stripped to give 1.30 g of pale yellow crystals. Further purification was performed by sublimation (50 °C/0.3 mmHg) providing 1.02 g (43%) of colorless crystals, mp 77−79.5 °C (Re et al., 1973: 77−79 °C). ^{1}H-NMR δ ($CDCl_3$): (both tautomers) 1.45 (d, J = 7.0 Hz, 3H), 1.45 (ddd, $^1J_{CH}$ = 130 Hz, $^2J_{CH}$ = 4.5 Hz, $^3J_{HH}$ = 7.0 Hz, 3H), 2.26 (d, J = 1.0 Hz, 3H), 2.26 (ddd, $^1J_{CH}$ = 130 Hz, $^2J_{CH}$ = 7.0 Hz, $^6J_{HH}$ = 1.0 Hz, 3H), 4.5 (m, 1H), 4.48 (dm, $^1J_{CH}$ = 150 Hz, 1H). ^{13}C-NMR δ ($CDCl_3$): (both tautomers) 13.6 (d, J = 48 Hz), 16.5 (d, J = 39 Hz), 80.5 (d, J = 39 Hz), 174.5 (d, J = 48 Hz). MS (EI) *m/z* (% relative abundance): 130 (100), 87 (12), 85 (13), 59 (32), 57 (41), 45 (47), 43 (45), 29 (18); the molecular ion was confirmed by MS (CI), ammonia *m/z* (% relative abundance): 148 (100, $[M + NH_4]^+$).

The corresponding unlabeled compound, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF), was prepared by substituting unlabeled dione. Yield of crude isolated product, 85%. ^{1}H-NMR δ ($CDCl_3$): 1.45 (d, J = 7.0 Hz, 3H), 2.27 (d, J = 1Hz, 3H), 4.50 (dq, J = 7.0, 1.0 Hz, 1H). ^{13}C-NMR δ ($CDCl_3$): 13.6, 16.5, 80.5, 134.0, 175.2, 199.2. IR (cm^{-1}): 3300 (br), 1680, 1600, 1295, 1185. MS (EI) *m/z* (% relative abundance): 128 (100), 85 (23), 57 (67), 43 (82), 29 (22); the molecular ion was confirmed by MS (CI), ammonia *m/z* (% relative abundance): 146 (100, $[M + NH_4]^+$).

[7,7,7-$^2H_3$]-3-Heptyn-2,5-diyl Bis(tert-butyl carbonate) (**5**). Proceeded as for ^{13}C-labeled alkyne **2**, but using CD_3CH_2CHO (1.43 g) prepared from CD_3CH_2Br, magnesium, and *N*-formylpiperidine in THF according to the method of Olah and Arvanaghi (1981). The aldehyde was codistilled with solvent, dried with molecular sieves (3 Å), and used as such [^{1}H-NMR δ ($CDCl_3$): 2.41 (2H), 9.78 (1H)]. Concentration was determined by ^{1}H-NMR using benzene as internal standard and integrating against the aldehydic hydrogen (23.4 mmol in 50 mL THF). **5** was obtained as a pale yellow oil (8.26 g) consisting of a mixture of two diastereomers which was taken directly to the next step. The diastereomers showed identical NMR spectra. ^{1}H-NMR δ ($CDCl_3$): 1.50 (s, 18H), 1.5 (m, 3H), 1.80 (br d, J = 6.5 Hz, 2H), 5.16 (t, J = 6.5, 1.0 Hz, 1H), 5.3 (m, 1H). ^{13}C-NMR δ ($CDCl_3$): 21.2, 27.6, 63.0, 68.0, 82.1, 82.6, 84.0, 152.3, 152.5. IR (cm^{-1}): 2960, 1730, 1260, 1240, 1150. MS (EI) *m/z* (% relative abundance): 175 (10), 158 (100), 131 (30), 127 (20), 116 (20), 113 (85), 96 (80), 70 (15), 57 (75); the molecular ion was confirmed by CI, ammonia *m/z* (% relative abundance): 349 (100, $[M + NH_4]^+$).

The corresponding unlabeled compound, 3-heptyn-2,5-diyl bis(*tert*-butyl carbonate), was obtained by substituting unlabeled propanal. It quantitatively yielded a pale yellow oil consisting of a mixture of diastereomers showing identical NMR spectra. ^{1}H-NMR δ ($CDCl_3$): 0.96 (t, J = 7.2 Hz, 3H), 1.45 (s, 18H), 1.5 (m, 3H), 1.78 (pentet, J = 7.0 Hz, 2H), 5.10 (t, J = 7.0 Hz, 1H), 5.2 (m, 1H). ^{13}C-NMR δ ($CDCl_3$): 9.2, 21.2, 27.7, 28.0, 63.0, 68.0, 82.0, 82.6, 84.0, 152.3, 152.5. IR (cm^{-1}): 2960, 1730, 1260, 1240, 1150. MS (EI) *m/z* (% relative abundance): 172 (5), 155 (75), 128 (15), 126 (10), 113 (10), 110 (40), 93 (25), 67 (10), 57 (100); the molecular ion was confirmed by CI, ammonia *m/z* (% relative abundance): 346 (100, $[M + NH_4]^+$).

[7,7,7-$^2H_3$]-3,4-Heptanedione-2,5-diyl Bis(tert-butyl carbonate) (**6**). Proceeded as for ^{13}C-labeled dione except that oxidation took place for 1.5 h at ice temperature and an additional 1.0 h at 25 °C. The product was a bright yellow oil (7.43 g, 87% from propanal-d_3). ^{1}H-NMR δ ($CDCl_3$): (both diastereomers) 1.47 (s, 18H), 1.48 (s, 18H), 1.5 (m, 6H), 1.8 (m, 4H), 5.36 (dd, J = 8.0, 4.5 Hz, 1H), 5.44 (dd, J = 8.0, 4.5 Hz, 1H), 5.54 (q, J = 7.0, 1.0 Hz, 1H). ^{13}C-NMR δ ($CDCl_3$): (both diastereomers) 15.8, 23.2, 27.6, 72.4, 73.5, 77.1, 78.4, 83.3, 152.4, 152.7, 152.9, 153.1, 193.7, 193.8, 194.0, 194.1. IR (cm^{-1}): 2960, 1730, 1720, 1270, 1240, 1150. MS (EI) *m/z* (% relative abundance): 292 (43), 252 (20), 234 (15), 190 (95), 158 (64), 146 (45), 128 (30), 113 (30), 108 (35), 91 (50), 57 (100); the molecular ion was confirmed by CI, ammonia *m/z* (% relative abundance): 381 (25, $[M + NH_4]^+$).

The unlabeled compound, 3,4-heptanedione-2,5-diyl bis(*tert*-butyl carbonate), was prepared by substituting unlabeled alkyne. Yield of bright yellow oil was 85%. ^{1}H-NMR δ ($CDCl_3$): (both diastereomers) 0.99 (t, J = 7.0 Hz, 6H), 1.43 (s, 18H), 1.45 (s, 18H), 1.5 (m, 6H), 1.8 (m, 4H), 5.32 (dd, J = 7.0, 4.2 Hz, 1H), 5.40 (dd, J = 7.0, 4.5 Hz, 1H), 5.50 (q, J = 7.0 Hz, 1H), 5.54 (q, J = 7.0 Hz, 1H). ^{13}C-NMR δ ($CDCl_3$): (both diastereomers) 9.4, 9.7, 15.6, 23.4, 23.6, 27.5, 31.4, 72.5, 73.5, 77.0, 78.4, 83.1, 152.4, 152.7, 152.9, 153.1, 193.7, 193.8, 194.0. IR (cm^{-1}): 2960, 1730, 1720, 1270, 1240, 1150. MS (EI) *m/z* (% relative abundance): 289 (11), 249 (5), 231 (5), 187 (33), 155 (10), 143 (21), 125 (15), 110 (5), 105 (10), 91 (10), 86 (35), 84 (50), 57 (100); the molecular ion was confirmed by CI, ammonia *m/z* (% relative abundance): 378 (60, $[M + NH_4]^+$).

*2(or 5)-([2,2,2-$^2H_3$]Ethyl)-4-hydroxy-5(or 2)-methyl-3(2H)-furanone ([$^2H_3$]EHMF) (**7ab**).* [$^2H_3$]Dione (**6**) (7.40 g) was treated similarly to ^{13}C-labeled dione (**3**). Reflux was maintained for 6 h. The crude product was purified by flash chromatography (silica gel; 1:1 hexane/ethyl acetate) providing 2.02 g (74%) of a pale yellow oil. ^{1}H-NMR δ ($CDCl_3$): (major tautomer) 1.70 (dd, J = 15.0, 7.0 Hz, 1H), 1.95 (dd, J = 15.0, 4.5 Hz, 1H), 2.26 (d, J = 1.0 H, 3H), 4.38 (ddd, J = 7.0, 4.5, 1.0, Hz, 1H); (minor tautomer) 1.44 (d, J = 7.0 Hz, 3H), 2.62 (s, 2H), 4.48 (q, J = 7.0 Hz, 1H). ^{13}C-NMR δ ($CDCl_3$): (both tautomers) 13.5, 16.5, 21.0, 24.4, 80.0, 84.9, 133.1, 135.0, 176.0, 179.1, 198.1, 199.2. IR (cm^{-1}): 3250 (br), 2205, 1680, 1605, 1185. MS (EI) *m/z* (% relative abundance): (minor tautomer) 145 (71), 89 (10), 85 (26), 60 (100), 57 (48), 29 (15); (major tautomer) 145 (96), 127 (41), 102 (37), 73 (77), 55 (28), 43 (100); both molecular ions were confirmed by MS (CI), ammonia *m/z* (% relative abundance): 163 (100, $[M + NH_4]^+$).

The unlabeled compound, 2(or 5)-ethyl-4-hydroxy-5(or 2)-methyl-3(2*H*)-furanone (EHMF), was prepared by using unlabeled dione. Yield of crude isolated product was 88%. ^{1}H-NMR δ ($CDCl_3$): (major tautomer) 0.98 (t, J = 7.2 Hz, 3H), 1.73 (ddq, J = 14.3, 7.2, 7.1 Hz, 1H), 1.98 (ddq, J = 14.3, 7.2, 4.5 Hz, 1H), 2.27 (d, J = 1.0 Hz, 3H), 4.38 (ddd, J = 7.1, 4.5, 1.0 Hz, 1H); (minor tautomer) 1.25 (t, J = 7.5 Hz, 3H), 1.45 (d, J = 7.2 Hz, 3H), 2.65 (q, J = 7.5 Hz, 2H), 4.49 (q, J = 7.2 Hz, 1H). ^{13}C-NMR δ ($CDCl_3$): (both tautomers) 8.5, 10.0, 13.4, 16.5, 21.0, 24.5, 80.0, 84.9, 133.1, 134.9, 175.5, 179.0, 198.1, 199.4. IR (cm^{-1}): 3250 (br), 1680, 1605, 1185. MS (EI) *m/z* (% relative abundance): (minor tautomer) 142 (54), 85 (12), 57 (100); (major tautomer) 142 (87), 127 (34), 102 (32), 71 (64), 55 (25), 43 (100); both molecular ions were confirmed by MS (CI), ammonia *m/z* (% relative abundance): 160 (100, $[M + NH_4]^+$).

Quantification Experiments. Sample preparation was performed as recently described (Blank and Fay, 1996) with some modifications. The precursors were allowed to react in water and phosphate- and malonate-buffered solutions (0.2 M, Na_2HPO_4, $Na_2H_2C_3O_4$) at different pHs (5, 6, 7) at 90 °C for 1 h. After the reaction mixture was cooled rapidly, water (100 mL) and the labeled internal standards (9.6−48.2 μg of [$^{13}C_2$]-HDMF and 10.0−50.2 μg of [$^2H_3$]EHMF) were added. The solution was saturated with NaCl, and the pH was adjusted to 4.0 (aqueous HCl, 2 M) which is the pH optimum for the stability of HDMF in aqueous solutions (Hirvi et al., 1980). Neutral compounds were continuously extracted with Et_2O overnight using a rotation perforator. The organic phase was dried over Na_2SO_4 at +4 °C and concentrated to 0.5−1 mL. All experiments were performed in duplicate.

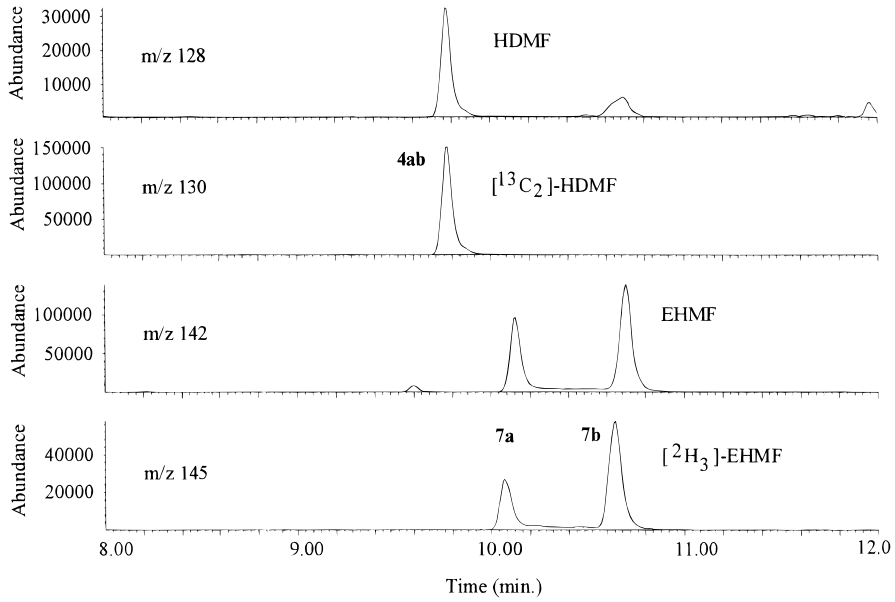

Figure 2. Quantification of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) and 2(or 5)-ethyl-4-hydroxy-5(or 2)-methyl-3(2*H*)-furanone (EHMF) by isotope dilution assay using [$^{13}C_2$]HDMF (***4ab***) and [$^{2}H_3$]EHMF (***7a*** and ***7b***) as internal standards. The mass chromatograms were recorded by GC-MS operating in the selected ion monitoring mode to measure the molecular ions *m/z* 128 of HDMF, *m/z* 142 of EHMF (two tautomers), *m/z* 130 of [$^{13}C_2$]HDMF, and *m/z* 145 of [$^{2}H_3$]EHMF (two tautomers).

GC−MS analyses were performed on an HP-5971 mass spectrometer connected to an HP-5890 gas chromatograph equipped with an HP-7673 autosampler. The interface was kept at 220 °C, and the ion source working in EI mode at 70 eV was held at about 180 °C. The samples were injected via a splitless injector onto a Carbowax capillary column using the chromatographic conditions as previously described (Blank et al., 1996b). As shown in Figure 2, unlabeled HDMF and EHMF and the corresponding labeled internal standards were detected by selected ion monitoring (SIM) of their molecular ions, i.e., *m/z* 128 (HDMF), *m/z* 142 (EHMF, two tautomers), *m/z* 130 ([$^{13}C_2$]HDMF), and *m/z* 145 ([$^{2}H_3$]EHMF, two tautomers).

The calibration curves were established with standard mixtures containing defined amounts of labeled and unlabeled compound in different ratios following the procedure described by Guth and Grosch (1990). A good linearity was found in the concentration range of 3−50 μg/mL ($r^2 = 0.999$). Samples for establishing the calibration curves and for quantifying HDMF and EHMF in the Maillard model reactions were injected twice.

RESULTS AND DISCUSSION

Synthesis of the Internal Standards [$^{13}C_2$]HDMF and [$^{2}H_3$]EHMF. A simple three-step preparation has been devised allowing labeled HDMF and EHMF to be produced from a common unlabeled starting material merely by substitution of one aldehyde reactant for another (Figure 1). Yields were generally excellent, and purification steps were not required until the final products. In the case of HDMF, ^{13}C-labeling was indispensable to avoid exchange of hydrogen and oxygen atoms during sample preparation (Sen et al., 1991). On the contrary, EHMF could be deuterated in the terminal position of the ethyl group.

The *tert*-butyloxycarbonyl (Boc) moiety is a frequent choice for amine protection (Greene and Wuts, 1991) but served perfectly well for our alcohol protection. It was easily administered, withstood lithiation/addition and oxidation steps, and was smoothly removed during the final cyclization step. Thus, beginning with Boc-protected alkynol **1**, deprotonation with lithium diisopropyl amide and addition of [1,2-$^{13}C_2$]ethanal produced an alkoxide which was trapped *in situ* with di-*tert*-butyl dicarbonate to give doubly-protected alkyne **2** as a 1:1 mixture of diastereomers. The crude product was sufficiently pure to be directly oxidized with potassium permanganate in aqueous acetone. The success of this reaction depended upon low temperature and the presence of acetic acid. Dione **3** was then cyclized according to the procedure of Re at al. (1973) resulting in tautomers **4a** and **4b** (1:1 ratio). These conditions proved efficacious for the concomitant removal of both Boc protective groups, the prerequisite for cyclization. The MS (EI) of **4ab** is shown in Figure 3.

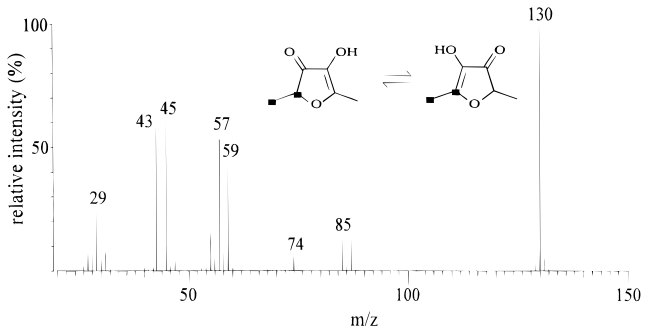

Figure 3. Electron impact mass spectrum of a tautomeric mixture (1:1) of 4-hydroxy-2-[^{13}C]methyl-5-methyl-3(2*H*)-[2-^{13}C]furanone and 4-hydroxy-5-[^{13}C]methyl-2-methyl-3(2*H*)-[5-^{13}C]furanone ([$^{13}C_2$]HDMF) obtained by synthesis. Symbol ■ indicates the labeling position.

Similarly, the lithium acetylide derived from alkyne **1** was treated with [3,3,3-$^{2}H_3$]propanal followed by di-*tert*-butyl dicarbonate to give doubly-protected alkyne **5**. Oxidation to dione **6** and cyclization afforded EHMF (**7a**/**7b** = 1:3). The MS (EI) of **7a** and **7b** reported in Figure 4 are in good agreement with those reported by Preininger and Grosch (1994).

Quantification of HDMF and EHMF in Maillard Model Reactions. As recently shown in model experiments, HDMF and EHMF can be generated from pentose sugars in the presence of glycine and L-alanine (Blank and Fay, 1996; Blank et al., 1996a). This, however, is a side reaction leading to relatively low amounts of HDMF and EHMF. Therefore, isotope

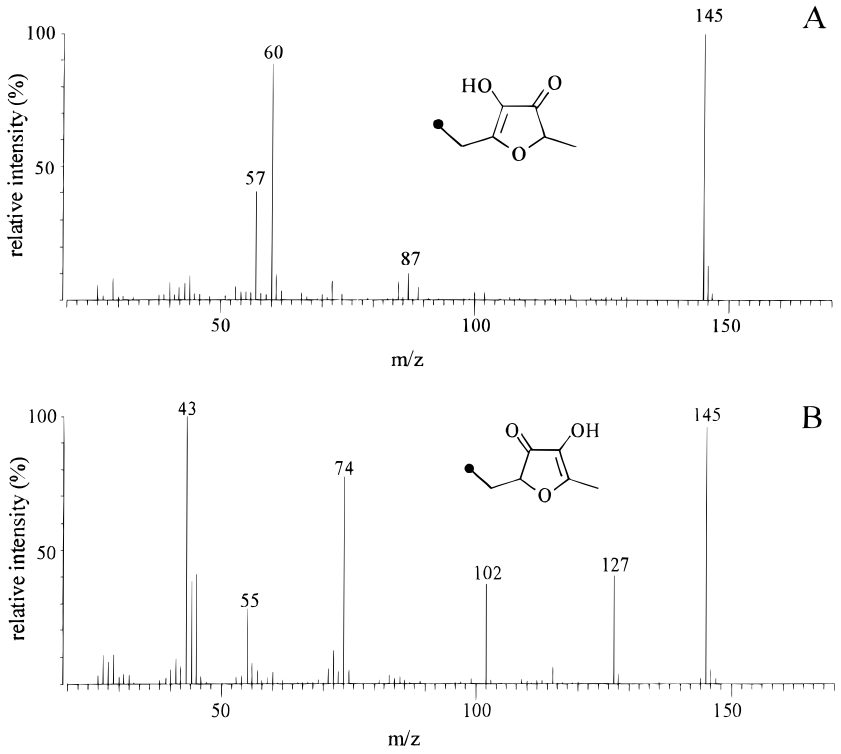

Figure 4. Electron impact mass spectra of (A) 5-([2,2,2-$^2H_3$]ethyl)-4-hydroxy-2-methyl-3(2*H*)-furanone ([$^2H_3$]EHMF, **7a**) (B) and 2-([2,2,2-$^2H_3$]eth-1-yl)-4-hydroxy-5-methyl-3(2*H*)-furanone ([$^2H_3$]EHMF, **7b**) obtained by synthesis. Symbol ● indicates the labeling position.

Table 1. Formation of HDMF and EHMF from Pentose Sugars in Maillard Model Systems Containing Glycine or L-Alanine[a]

model system	HDMF[b]	EHMF[b]
arabinose/glycine	5.1	1.3
xylose/glycine[c]	2.6	0.3
ribose/glycine	3.6	0.7
arabinose/L-alanine	1.2	6.8
xylose/L-alanine[c]	0.9	7.5
ribose/L-alanine	1.6	10.0

[a] Equimolar amounts of precursors were used. Reaction conditions: phosphate buffer (0.2 M, pH 6), 90 °C, 1 h. [b] Data are means of at least two assays, each of them injected twice; maximum SD ≤ 10% (in μg/mmol of sugar). [c] The control sample (without amino acid) resulted in less than 0.01 μg of HDMF and EHMF per mmol of sugar.

dilution assays were applied to obtain reliable data about the absolute concentrations of HDMF and EHMF formed and the parameters influencing the reaction.

Formation from Pentose Sugars. The formation of HDMF from pentose sugars was favored in the presence of glycine (Table 1) resulting in about 2−5 μg of HDMF. On the contrary, EHMF was preferentially generated in the presence of L-alanine (6.8−10 μg). The quantitative results support the hypothesis (Blank and Fay, 1996) that HDMF and EHMF are mainly formed by Strecker-assisted chain elongation of pentoses, i.e., through combination of the latter with aldehydes produced by Strecker deamination of glycine and alanine. In other terms, formaldehyde (active C_1) liberated from glycine is required for the formation of HDMF as is acetaldehyde (active C_2 from L-alanine) for EHMF. However, in all samples analyzed, 4-hydroxy-5-methyl-3(2*H*)-furanone was the main reaction product (data not shown) directly formed from pentose sugars (Feather, 1981).

As indicated in Table 1, both furanones were also generated by sugar fragmentation, i.e., 0.9−1.6 μg of HDMF and 0.3−1.3 μg of EHMF detected in the model reactions pentose/L-alanine and pentose/glycine, respectively. This, however, is a minor reaction pathway where the amino acid indirectly contributes to the formation of these furanones by accelerating the Maillard reaction, i.e., by favoring sugar fragmentation and condensation of the corresponding sugar fragments. Several reactive sugar fragments have been reported in the literature (review by Ledl and Schleicher, 1990). First trials have shown that some of the well-known C_3 fragments do generate HDMF, e.g., methylglyoxal, acetol, and dihydroxyacetone (data not shown). These results will be reported elsewhere.

Table 2. Formation of HDMF and EHMF in Maillard Model Systems Containing Xylose and Different Amino Acids[a]

model system	HDMF[b]	EHMF[b]
xylose/4-aminobutyric acid	0.4	0.1
xylose/4-aminobutyric acid/glycine	1.5	0.2
xylose/4-aminobutyric acid/L-alanine	0.7	3.2

[a] Equimolar amounts of precursors were used. Reaction conditions: phosphate buffer (0.2 M, pH 6.0), 90 °C, 1 h. [b] Data are means of at least two assays, each of them injected twice; maximum SD ≤ 10% (in μg/mmol of sugar).

4-Aminobutyric acid was employed to study the formation of 3(2*H*)-furanones by sugar fragmentation. As a γ-amino acid, it does not decompose by Strecker deamination. The amounts of HDMF and EHMF formed were relatively low, i.e., 0.4 and 0.1 μg, respectively (Table 2). The data indicate that the generation of HDMF by sugar fragmentation is favored compared to that of EHMF, most likely due to higher amounts of reactive C_3 fragments formed, whereas EHMF requires both reactive C_3 and C_4 fragments. As shown in Table 2, the amount of HDMF was significantly increased in the presence glycine (1.5 μg). Similarly, addition of L-alanine resulted in higher yields of EHMF (3.2 μg).

Table 3. Effect of Reaction Medium on Formation of HDMF and EHMF from Xylose in Maillard Model Systems[a]

model system	reaction medium	HDMF[b]	EHMF[b]
xylose	water[c]	<0.01	<0.01
xylose	phosphate	<0.01	<0.01
xylose/glycine	water[c]	0.06	<0.01
xylose/glycine	phosphate	2.6	0.3
xylose/glycine	malonate	0.6	0.1
xylose/L-alanine	water[c]	0.02	0.05
xylose/L-alanine	phosphate	0.9	7.5
xylose/L-alanine	malonate	0.3	0.8

[a] Equimolar amounts of precursors were used. Reaction conditions: pH 6, 90 °C, 1 h. [b] Data are means of at least two assays, each of them injected twice; maximum SD ≤ 10% (in μg/mmol of sugar). [c] The pH was not controlled during the reaction.

Table 4. Effect of pH on Formation of HDMF and EHMF from Xylose in Maillard Model Systems Containing Glycine or L-Alanine[a]

model system	pH	HDMF[b]	EHMF[b]
xylose/glycine	5	2.6	0.3
xylose/glycine	6	2.6	0.3
xylose/glycine	7	3.1	0.7
xylose/L-alanine	5	0.3	2.0
xylose/L-alanine	6	0.9	7.5
xylose/L-alanine	7	2.5	13.5

[a] Equimolar amounts of precursors were used. Reaction conditions: phosphate buffer (0.2 mol/L), 90 °C, 1 h. [b] Data are means of at least two assays, each of them injected twice; maximum SD ≤ 10% (in μg/mmol of sugar).

Parameters Affecting the Formation of HDMF and EHMF. Xylose was selected to study the formation of HDMF and EHMF from pentose sugars in more detail. As shown in Table 3 the furanones were preferentially formed in the phosphate-buffered systems, i.e., 2.6 μg of HDMF in xylose/glycine and 7.5 μg of EHMF in xylose/alanine, thus indicating a catalytic effect of phosphate, particularly in the presence of the amino acids. The effect of malonate was less pronounced, yielding 0.6 μg of HDMF in xylose/glycine and 0.8 μg of EHMF in xylose/alanine. The model systems without buffer gave rise to less than 0.1 μg of HDMF and EHMF per mmol of pentose.

The formation of both furanones was favored at pH 7 resulting in 3.1 μg of HDMF and 13.5 μg of EHMF (Table 4). This is in good agreement with the more intense caramel-like overall flavor of these samples compared to those prepared at pH 5. In general, 3(2*H*)-furanones are formed from 1-deoxyosones which are reactive intermediates in the Maillard reaction generated by degradation of Amadori compounds via 2,3-enolization (Hodge et al., 1972; Ledl and Schleicher, 1990). This reaction is favored under neutral and slightly alkaline conditions as shown by Beck et al. (1988), i.e., the ratio 1-deoxyglucosone to 3-deoxyglucosone (formed via 1,2-enolization) was 20:1 at pH 7 and 8:5 at pH 4.5.

The amounts of HDMF and EHMF can also be influenced by the ratio of the precursors (Table 5). In general, increasing concentrations of the amino acids favor the formation the furanones, e.g., a 4-fold increase of alanine resulted in 2.7-fold more EHMF. One possible explanation is that elevated concentrations of amino acids give higher levels of aldehydes, the Strecker deamination products, required for the formation of HDMF and EHMF from pentose sugars (Blank and Fay, 1996). Alternatively, higher amounts of amino acids

Table 5. Effect of Amino Acid Concentration on the Formation of HDMF and EHMF from Xylose in Maillard Model Systems[a]

model system	molar ratio xylose/amino acid	HDMF[b]	EHMF[b]
xylose/glycine	1:1	2.6	0.3
xylose/glycine	1:2	3.2	0.4
xylose/glycine	1:4	4.2	0.5
xylose/L-alanine	1:1	0.9	7.5
xylose/L-alanine	1:2	1.2	12.5
xylose/L-alanine	1:4	1.6	20.0

[a] Reaction conditions: phosphate buffer (0.2 M, pH 6.0), 90 °C, 1 h. [b] Data are means of at least two assays, each of them injected twice; maximum SD ≤ 10% (in μg/mmol sugar).

may accelerate the Maillard reaction, producing reactive intermediates such as 1-deoxyosones which may decompose to smaller fragments and so generate furanones by condensation reactions (Blank et al., 1996a).

CONCLUSION

The formation of HDMF and EHMF from pentose sugars via Strecker-assisted chain elongation has been substantiated. Quantitative data support the hypothesis that HDMF is preferably formed in the presence of glycine and EHMF in the presence of alanine. However, sugar fragmentation/condensation reactions represent an alternative formation pathway, particularly for HDMF, which will be studied in more detail.

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