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Bioactive Constituents, Metabolites, and Functions

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Metabolization of the advanced glycation end product *N*-ε-carboxymethyllysine (CML) by different probiotic *E. coli* strains

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Abstract

N-ε-carboxymethyllysine (CML) is formed during glycation reactions (synonym, Maillard reaction). CML is degraded by the human colonic microbiota, but nothing is known about the formation of particular metabolites. In the present study, six probiotic *E. coli* strains were incubated with CML in the presence or absence of oxygen either in minimal or nutrient-rich medium. CML was degraded by all strains only in the presence of oxygen. HPLC-MS/MS was applied for identification of metabolites of CML. For the first time, three bacterial metabolites of CML have been identified, namely *N*-carboxymethylcadaverine (CM-CAD), *N*-carboxymethylaminopentanoic acid (CM-APA) and the *N*-carboxymethyl-Δ^1-piperideinium ion. During 48 h of incubation of CML with five different *E. coli* strains in minimal medium in the presence of oxygen, 37–66% of CML was degraded, while CM-CAD (1.5–8.4% of the initial CML dose) and CM-APA (0.04–0.11% of the initial CML dose) were formed linearly. Formation of the metabolites is enhanced when dipeptide-bound CML is applied, indicating that transport phenomena may play an important role in the "handling" of the compound by microorganisms.

Keywords

Maillard reaction; *N*-ε-Carboxymethyllysine (CML); *E. coli*; metabolism; biogenic amine; probiotic strain

Introduction

N-ε-carboxymethyllysine (CML) is an advanced glycation end product formed during the Maillard reaction (synonyms—non-enzymatic browning, glycation) in food, but also under physiological conditions. In the Maillard reaction, reducing sugars first react with amino and imino groups of free amino acids, peptides, and proteins to form the so-called Amadori rearrangement products (ARPs). By this reaction, *N*-ε-fructosyllysine is formed at the ε-amino group of lysine. "Blockage" by this compound can afford for the modification of up to 30% of the side-chains of the essential amino acid lysine in milk products and up to 80% in bakery products.[1,2] ARPs degrade in the second step of the reaction, what gives rise to 1,2-dicarbonyl compounds such as 3-deoxyglucosone, methylglyoxal, and glyoxal. These compounds can also be formed directly from reducing sugars.[3,4] In the last stage of the Maillard reaction, reactive 1,2-dicarbonyl compounds react with the nucleophilic side-chains of protein-bound amino acids to form the so-called "advanced glycation end products (AGEs)", mainly alkylated lysine derivatives, pyrrole and pyridinyl compounds that include the ε-amino group of lysine and hydroimidazolones at the guanidino group of arginine.[1,5] The alkylated lysine derivative *N*-ε-carboxymethyllysine (CML, Figure 1) has first been described as an unusual metabolite in children's urine and only later as to result from the Maillard reaction.[6,7] Different routes of formation have been described for this compound: The reaction of glyoxal with lysine including an intramolecular Cannizzaro reaction, the oxidative degradation of intermediate Schiff bases (Namiki pathway) and ARPs, respectively, which is facilitated in alkaline environments.[8,9,10,11]

The most reliable quantitative data for CML in food are based on chromatographic means such as high-pressure liquid chromatography with tandem mass-spectrometric detection (HPLC-MS/MS). CML is most abundant in bakery products such as bread and biscuits (1.3–46 mg/kg), high-heat processed meat products (0.9–48 mg/kg), evaporated milk (4.7–46 mg/kg), processed nuts (2.1–48 mg/kg), pasta products (3–16 mg/kg), and chocolate (5–37 mg/kg).[12,13,14,15,16] A

daily dietary intake between 2.1 and 11.3 mg CML was estimated.[17,18,19] Reliability of quantitation of CML by an immunological method, which counterintuitively detected the highest quantities of CML in fat-rich and virtually protein-free foods and nearly none in bakery products,[20] is more and more put into question.[21,22,23] However, based on this latter methodology, many *in vivo* studies resulted in the detection of adverse effects of CML intake such as inflammatory processes, endothelial dysfunction, insulin resistance, and oxidative stress. Most of these studies are not considered conclusive due to methodological shortcomings (for reviews, see references 24,25,26).

The metabolic fate of CML has been a subject of several works in the recent decade. Predominantly protein-bound in foods,[27] CML is released from proteins during intestinal luminal digestion into absorbable peptides.[28] Works on Caco-2 cells suggest that CML is taken up into enterocytes when it is bound in dipeptides and then may get into circulation to a small extent.[29] Between 15 and 25% of dietary CML is excreted via the urine in humans; the higher the ingested amount, the lower the fraction that is excreted renally.[18] Incorporation of dietary CML into plasma and tissue proteins, especially the heart, has been suggested but is still a matter of controversy.[17,30,31] The predominant part of dietary CML is transferred into the large intestine because it is either not absorbed or released from desquamated enterocytes. Between 20 and 30% of ingested CML is excreted via the feces in humans; the higher the ingested amount, the higher the fraction that is excreted with the feces.[18] Human and animal studies consistently show that dietary CML is not fully recovered in urine and feces.[18,32,33,34]

It was shown that CML is degraded by the human colonic microbiota with strong interindividual differences.[35] Positive effects of heat-treated foods and especially of CML on intestinal dysbiosis have been shown in colitic mice.[36,37] Biological effects of dietary constituents in an organism may not only be evoked by the compounds themselves, but also by their metabolites, especially when the microbiota is involved. As there are no studies available on metabolites of

CML, we intended to elucidate possible metabolization pathways of this compound in the present study by using well-characterized probiotic *E. coli* strains as model microorganisms.[38,39,40]

Materials and Methods

Chemicals. Luria-Bertani agar (Miller) and M9 minimal salts (5×) were purchased from Becton Dickinson (Heidelberg, Germany). Glacial acetic acid and D-glucose were from Roth (Karlsruhe, Germany). Nonafluoropentanoic acid (NFPA), *o*-dianisidine, *N*-Boc-cadaverine, palladium on activated charcoal (10%), 5-aminopentanoic acid and brain heart infusion (BHI) broth were obtained from Sigma-Aldrich (Steinheim, Germany). Calcium chloride dihydrate, L-cysteine, sodium citrate dihydrate and magnesium sulfate were obtained from Merck Millipore (Darmstadt, Germany) and [$^2H_2$]CML from PolyPeptide (Strasbourg, France). Acetonitrile was from Fisher Scientific (Schwerte, Germany), and methanol and hydrochloric acid were from VWR (Darmstadt, Germany). Resazurin sodium salt, glyoxylic acid monohydrate and 5-aminopentanol were purchased from Alfa Aesar (Karlsruhe, Germany). The cation exchange resin DOWEX 50WX8 and formic acid were obtained from Acros Organics (Geel, Belgium). Sodium hydroxide and potassium iodide were from Grüssing (Filsum, Germany), and ninhydrin from Serva (Heidelberg, Germany). The syntheses of CML and alanyl-(*N*-ε-carboxymethyl)-lysine (Ala-CML) were performed according to the literature.[29]

Preparation of strains for metabolic experiments. *E. coli* strains (Table 1) were supplied by SymbioPharm (Herborn-Hörbach, Germany). The strains were routinely precultured using Luria-Bertani agar (LB agar) with concentrations of 15.0 g/L agar, 5.0 g/L yeast extract, 10.0

106 g/L sodium chloride, and 10.0 g/L tryptone, which were dissolved in doubly distilled water (pH
107 = 7.0 ± 0.2) followed by autoclaving (122 °C, 20 min). Liquid agar was poured into Petri dishes
108 (d = 9.4 cm) after cooling to 50 °C. BHI medium for aerobic experiments was prepared by
109 dissolving 7.40 g of BHI broth in 200 mL of doubly distilled water (pH = 7.4 ± 0.2) followed
110 by autoclaving (122 °C, 20 min). Minimal medium for aerobic experiments was prepared as
111 follows: An autoclaved stock solution of M9 minimal salts was diluted with autoclaved water
112 and added with a sterile filtered glucose-salt mix to yield final concentrations of 5 g/L
113 ammonium chloride, 0.01 g/L calcium chloride, 33.9 g/L disodium hydrogen phosphate, 3.91
114 g/L D-glucose, 0.24 g/L magnesium sulphate, 15.0 g/L potassium dihydrogen phosphate, and
115 2.5 g/L sodium chloride (pH = 6.8 ± 0.2). For anaerobic experiments, 0.4 g/L L-cysteine and
116 0.001 g/L resazurin were added to the media. Media were aliquoted into Hungate tubes (Ochs,
117 Bovenden-Lenglern, Germany) and autoclaved (122 °C, 20 min) after three cycles of
118 alternating vacuum application (0.04 bar) and nitrogen overpressure (0.4 bar).[35]

119 Cryocultures of *E. coli* strains in glycerol medium were spread on LB agar plates by use of an
120 inoculating loop and incubated at 37 °C overnight in an incubator (TH30, Edmund Bühler,
121 Bodelshausen, Germany). Single colonies were transferred into 5 mL of autoclaved liquid
122 medium in aerobic culture tubes (12 mL) and the suspensions were incubated overnight at 37
123 °C in a table-top incubator under agitation (225 rpm). On the next day, the OD_{600} of the bacterial
124 suspension was measured after appropriate dilution with PBS buffer using the spectrometer
125 Evolution 201 UV (Thermo Scientific, Dreieich, Germany) in cuvettes (V = 1.5 mL) at
126 extinctions not exceeding 0.3. In order to perform the incubation of test substrates in the
127 exponential growth phase, the overnight cultures were diluted with the respective media to yield
128 OD_{600} values of 0.06 in BHI medium, or 0.3 in minimal medium, respectively. After 80 min of
129 incubation under exclusion of light (37 °C, 225 rpm), the OD_{600} was checked again, and

incubations with MRPs were only started if the optical density had reached values between 0.5 and 0.7.

For the preparation of anaerobic cultures in BHI medium, single colonies of *E. coli* were inoculated in 100 µL of BHI medium and transferred into a Hungate tube containing 5.7 mL of anaerobic BHI medium. After overnight incubation, 100 µL of the suspension was transferred to a new Hungate tube containing 5.7 mL of anaerobic BHI medium. The suspensions were preincubated for 80 min (37 °C, 225 rpm) before the addition of MRP solution.

For anaerobic cultures in minimal medium, each of two cultures of single colonies of *E. coli* was inoculated in 124 µL of glucose-salt mix (1 M glucose, 0.1 M $MgSO_4$, 4.5 mM $CaCl_2$), added to 5.7 mL anaerobic minimal medium in a Hungate tube by means of a sterile syringe and grown overnight. Both overnight cultures were combined and after centrifugation and removal of the supernatant, the residue was suspended in 0.4 mL of minimal medium. Then, 0.1 mL was mixed with 124 µL of glucose-salt mix and transferred to 5.6 mL of anaerobic minimal medium in a Hungate tube by means of a sterile syringe. The suspensions were preincubated for 80 min (37 °C, 225 rpm) before the addition of MRP solution.

Incubation of MRPs in the presence or absence of *E. coli* strains. The following substances were subjected to incubations with *E. coli*: CML, Ala-CML, *N*-carboxymethylcadaverine (CM-CAD), *N*-carboxymethylaminopentanoic acid (CM-APA), and *N*-carboxymethylaminopentanol (CM-APO). Sterile-filtered (0.2 µm) MRP solutions were added to the preincubated bacterial suspensions in order to yield a final concentration of 250 µM as in our previous study.[35] Two additional control samples were run in parallel: In order to monitor the stability of the MRPs in the absence of bacteria, MRPs were incubated in the medium alone at a concentration of 250 µM. Metabolization of medium components was monitored by

incubating bacterial suspensions without adding MRPs. The mixtures were incubated at 37 °C for up to 72 h under constant agitation (225 rpm).

Samples (200 µL) were taken directly after mixing (0 h), and after 4 h, 24 h, 48 h, and 72 h and stored at -22 °C until analysis. The optical density of the suspensions was measured in parallel. When incubations were performed in Hungate tubes, 200 µL samples were taken by means of a sterile syringe.

Amino acid analysis. CML, Ala-CML, and lysine were quantitated with the amino acid analyser S4300 (Sykam, Fürstenfeldbruck, Germany) using a PEEK column filled with the cation exchange resin LCA K07/Li (150 × 4.6 mm, 7 µm). The respective lithium buffers were also obtained from Sykam and employed for custom gradient programs utilized previously.[28,29] Post-column derivatization with ninhydrin was performed, and the absorbance of the eluate was monitored with a two-channel photometer (λ = 440 nm, 570 nm). For amino acid analysis, 50 µL of the samples taken during the incubations was diluted with 50 µL of methanol. After lyophilization, 100 µL of 10 mM NFPA was added, and 50 µL of the solution was mixed with 150 µL of 0.12 N lithium citrate buffer, pH 2.20. External calibration was performed with the standards of CML and Ala-CML synthesized previously.[29] The injection volume was 50 µL.

High-Pressure Liquid Chromatography with Mass Spectrometric Detection. This was performed using the Agilent system 1200 Series (Agilent Technologies, Böblingen, Germany), consisting of a binary pump, an online degasser, a column oven and an autosampler. The column used was a stainless steel column (Zorbax 300 SB-C18, 50 mm × 2.1 mm, 3.5 µm; Agilent). Solutions of 10 mM NFPA in doubly distilled water (solvent A) and of 10 mM NFPA in acetonitrile (solvent B) were used as the eluents. The chromatographic system was connected

to the mass spectrometer 6410 Triple Quad (Agilent), working in the positive mode with a capillary voltage of 4000 V and a source temperature of 350 °C. The gas flow was 11 L/min (N_2), and the nebulizer pressure was set at 35 psi. Data acquisition and evaluation were performed with the software Mass Hunter B.02.00 (Agilent).

System 1 was utilized for qualitative screening for potential metabolites. A linear gradient from 2% B to 47% B in 11 minutes was applied at a flow rate of 0.25 mL/min and a column temperature of 35 °C. During 3 to 11 min, the detector was operated in the SCAN mode (*m/z* 80–400; dwell time, 500 ms; fragmentor voltage, 100 V). Samples taken during incubation (100 µL) were mixed with 100 µL of methanol. The mixtures were lyophilized and taken up in 200 µL of 10 mM NFPA. After centrifugation (10,621 × *g*, 20 °C, 10 min), the supernatants were transferred to HPLC vials, and 5 µL was injected. The same method was used for recording product ion scans by modifying fragmentor voltage (60–135 V) and collision energy (0–30 eV) at preset *m/z* ratios.

System 2 was utilized for the quantitation of CML by a stable-isotope dilution assay.[35] A linear gradient from 10% B to 34% B in 8 minutes was applied at a flow rate of 0.25 mL/min and a column temperature of 35 °C. The transitions *m/z* 205→130 (Fragmentor voltage, 100 V; collision energy, 9 eV) and *m/z* 207→130 (80 V; 5 eV) were recorded for quantitation of CML and [$^2H_2$]CML, respectively. Simultaneously, the transitions *m/z* 205→142 (100 V; 9 eV) and *m/z* 207→144 (80 V; 12 eV) were recorded for confirming the presence of the analytes. Transitions were recorded between 3 and 9 min (dwell time, 250 ms). External calibration was performed with a standard of CML between 0.9 and 26.8 pmol per injection. Prior to HPLC-MS/MS analysis, 50 µL of the samples taken during incubation was mixed with 50 µL of methanol. Then, 900 µL of HPLC eluent A (10 mM NFPA) was added. After mixing and centrifugation (16.060 × *g*, 10 min, 4 °C), 100 µL of the supernatant was diluted with 60 µL of

eluent A and 40 µL of a 0.25 mg/L solution of [$^2H_2$]CML in water. After mixing, the solution was transferred to an HPLC vial, and 5 µL was injected.

System 3 was utilized for the analysis of metabolites of CML. The same device and eluents were used as described above, but a gradient from 2% B to 66% B was formed during 15 min. The transitions used for the quantitation of the analytes (quantifier, Q) and for the confirmation of the presence of the analytes (qualifier, q) are compiled in Table 2. Standard addition was performed for quantitation as follows: For the first sample, 100 µL of the supernatant was mixed with 20 µL of water. For the second sample, 100 µL of the supernatant was mixed with 10 µL of water and 10 µL of a standard solution. For the third sample, 100 µL of the supernatant was mixed with 20 µL of the standard solution. In the standard solution, the CML metabolites had the following concentrations: CM-CAD (132.4 µg/L), CM-APA (105.1 µg/L), CM-APO (93.7 µg/L). Different addition standard solutions were utilized when the metabolites were incubated in the presence of *E. coli* strain $G_{1/2}$. The concentration of the incubated metabolite was increased by a factor of 100, while the concentrations of the other compounds were left as stated above.

Semi-preparative ion exchange chromatography – general procedures. All separations of synthesis mixtures were accomplished by using a column (1.5 cm × 50 cm) filled with 80 mL of the strong cation-exchange resin DOWEX 50WX8 in the sodium form. The column was reconditioned before each fractionation by rinsing first with 250 mL of 6 M HCl, 250 mL of water, 250 mL of 1 M NaOH, 250 mL of water, and lastly with 250 mL of 0.1 N sodium citrate buffer, pH 3.0 (loading buffer). Samples were prepared in loading buffer and membrane filtered (0.45 µm). After adjustment of the pH value (≤ 3.0), samples were applied by gravity. The column was first rinsed with 40 mL of loading buffer and then with the buffers as mentioned below. Starting from sample application, the eluate was collected in 6-mL fractions using a

fraction collector (Model 2110, Bio-Rad Laboratories, München, Germany). The presence of products in the samples was monitored by a spotting test. Aliquots (1 µL) of each fraction were transferred to a TLC plate and sprayed with ninhydrin or modified Reindel-Hoppe reagent[41] after drying. For preparation of the ninhydrin reagent, 100 mg ninhydrin was dissolved in 100 mL ethanol, and 3 mL glacial acetic acid was added. Prior to detection with the second reagent, plates were stored in a chlorine atmosphere for 30 min and then air-dried for 30 min. The reagent was prepared by dissolving 16 mg *o*-dianisidine in a mixture of 50 mL water and 3 mL glacial acetic acid, to which 100 mg potassium iodide was added. Fractions containing primary or secondary amines showed characteristic black spots. Target fractions were then pooled, and the pH of the solution was adjusted to 2.0 with 6 M HCl. Desalting was performed with a column (2.5 cm × 15 cm) filled with 70 mL of the strong cation-exchange resin DOWEX 50WX8. The column was reconditioned by rinsing first with 250 mL of 6 M HCl and then 250 mL of water. After application of the sample, the column was rinsed with 250 mL of water, 250 mL of 1 M HCl, and 250 mL of 4 M HCl. The fraction eluting with 4 M HCl was evaporated to dryness, taken up in water and lyophilized.

Characterization of synthesized compounds. Proton NMR spectra were recorded on an AVANCE III HD Nanobay 400 MHz UltraSield device from Bruker (Rheinstetten, Germany) at 400.13 MHz. Chemical shifts are given in parts per million (ppm), relative to the internal HOD signal (4.70 ppm). Coupling constants (*J*) are reported in Hz. Elemental analysis data were obtained on a Vario Micro Cube CHNS elemental analyser (Elementar, Hanau, Germany). For calculating the product content of synthesized compounds, the percentage of nitrogen determined in the preparation was divided by the theoretical percentage of nitrogen and the content expressed in per cent by weight. HPLC-MS/MS system 1 was employed for the determination of molecular mass and fragmentation behaviour of the synthesized compounds.

Synthesis of *N*-carboxymethylcadaverine (CM-CAD) 2. Boc-cadaverine (809 mg, 4.0 mmol) and glyoxylic acid monohydrate (369.2 mg, 4.0 mmol) were dissolved in 30 mL of water, and the pH was adjusted to 8.75 with conc. NaOH. After addition of 50.0 mg palladium on activated charcoal, the mixture was stirred under a hydrogen atmosphere overnight. Then, the catalyst was filtered off, water was added to the mixture to a final volume of 50 mL, and 50 mL 6 M HCl was added. The solution was stirred at room temperature for 1 h to remove the Boc protecting group. The mixture was evaporated to dryness using a rotary evaporator and taken up in 30 mL 0.1 N sodium citrate buffer, pH 3.0 before chromatographic separation. Elution was performed first with 300 mL of 0.3 N sodium citrate buffer, pH 5.2, and then with 600 mL of 0.3 N sodium citrate buffer, pH 5.5. The product eluted between 230 mL and 550 mL of the latter buffer. After desalting of the respective fractions and lyophilization, **2** was obtained as a light yellow product and stored at -18 °C.

Analytical data: ^{1}H-NMR (400 MHz, D_2O), δ [ppm]: 1.42 (m, 2H, H-3); 1.69–1.75 (m, 4H, H-2, H-4); 2.96 (t, 2H, J = 6.9 Hz, H-1); 3.07 (t, 2H, J = 7.6 Hz, H-5); 3.83 (s, 2H, H-6). HPLC-MS/MS (system 1): t_R, 9.5 min; fragmentation (100 V, 10 eV) of $[M + H]^+$ (*m/z* 161): 86 (100), 144 (44), 98 (42), 161 (6). Elemental analysis: $C_7H_{16}N_2O_2$ (MW = 160.21), calculated, C 52.48%, H 10.07%, N 17.48%; found, C 35.53%, H 7.48%, N 11.78%; content = 67.4%, based on nitrogen. Yield: 446.9 mg (47%).

Synthesis of *N*-carboxymethylaminopentanoic acid (CM-APA) 3. Aminopentanoic acid (468.5 mg, 4.0 mmol) and glyoxylic acid monohydrate (368.0 mg, 4.0 mmol) were dissolved in 30 mL of water, and the pH was adjusted to 8.75 with conc. NaOH. After addition of 50.0 mg palladium on activated charcoal, the mixture was stirred under a hydrogen atmosphere

overnight. Then, the catalyst was filtered off, and water was added to the mixture to a final volume of 75 mL. After addition of 500 mg sodium citrate dihydrate, the pH was adjusted to 3.0 with 6 M HCl before chromatographic separation. Elution was performed first with 250 mL of 0.15 N sodium citrate buffer, pH 3.25, and then with 250 mL of 0.15 N sodium citrate buffer, pH 3.5. The product eluted between 10 mL and 100 mL of the latter buffer. After desalting of the respective fractions and lyophilization, 49.2 mg of a white product was obtained, which was stored at -18 °C.

Analytical data: ^{1}H-NMR (400 MHz, D_2O), δ [ppm]: 1.59–1.75 (m, 4H, H-3, H-4); 2.40 (t, 2H, J = 7.0 Hz, H-2); 3.07 (t, 2H, J = 7.6 Hz, H-5); 3.81 (s, 2H, H-6). HPLC-MS/MS (system 1): t_R, 4.8 min; fragmentation (100 V, 10 eV) of $[M + H]^+$ (*m/z* 176): 112 (100), 101 (58), 55 (33), 84 (33), 176 (23), 83 (20), 76 (12), 140 (10), 130 (8), 59 (8), 158 (3). Elemental analysis: $C_7H_{13}NO_4$ (MW = 175.18), calculated, C 47.99%, H 7.48%, N 8.00%; found, C 35.85%, H 6.09%, N 6.19%; content = 77.4%, based on nitrogen. Yield: 49.2 mg (5%).

Synthesis of *N*-carboxymethylaminopentanol (CM-APO) 4. 5-Aminopentanol (416.0 mg, 4.0 mmol) and glyoxylic acid monohydrate (370.9 mg, 4.0 mmol) were dissolved in 30 mL of water, and the pH was adjusted to 8.75 with conc. NaOH. After addition of 50.0 mg palladium on activated charcoal, the mixture was stirred under a hydrogen atmosphere overnight. Then, the catalyst was filtered off, and water was added to the mixture to a final volume of 50 mL. After addition of 500 mg sodium citrate dihydrate, the pH was adjusted to 3.0 with 6 M HCl before chromatographic separation. Elution was performed with 350 mL of 0.2 N sodium citrate buffer, pH 3.5. The product eluted between 80 mL and 200 mL of the elution buffer. After desalting of the respective fractions and lyophilization, **4** was obtained as a white product and stored at -18 °C.

Analytical data: ^{1}H-NMR (400 MHz, D_2O), δ [ppm]: 1.38 (m, 2H, H-3); 1.53 (m, 2H, H-2); 1.69 (m, 2H, H-4); 3.06 (m, 2H, H-5); 3.65 (t, 2H, J = 6.4 Hz, H-1); 3.83 (s, 2H, H-6). HPLC-MS/MS (system 1): t_R, 5.4 min; fragmentation (100 V, 10 eV) of $[M + H]^+$ (*m/z* 162): 69 (100), 162 (76), 116 (68), 98 (68), 76 (59), 144 (18), 87 (18). Elemental analysis: $C_7H_{15}NO_3$ (MW = 161.20), calculated, C 52.16%, H 9.38%, N 8.69%; found, C 41.32%, H 7.48%, N 7.12%; content = 81.9%, based on nitrogen. Yield: 146.4 mg (19%).

Results and discussion

Identification of metabolites of CML formed by *E. coli*. As a model organism for our studies on CML metabolism, *E. coli* was chosen due to its quantitative importance in the gut microbiome and its ability to grow both under aerobic and anaerobic conditions. The strains were the six probiotic strains that constitute the probiotic formulation Symbioflor 2.[38,42] Properties of individual strains are compiled in Table 1. Initially, strain $G_{1/2}$ was regarded as a representative of the Symbioflor strains, and growth and CML metabolizing activity of this strain were investigated under different conditions. The strain was able to grow both under aerobic and anaerobic conditions in BHI and minimal media (Figures 2A and 2B). Under the experimental conditions used, the stationary growth phase was reached at the latest after 24 h. CML was fully stable in the utilized media when they were not inoculated with bacteria as determined by the stable-isotope dilution assay. Degradation of CML was observed only in the presence of bacteria under aerobic conditions and was more pronounced in minimal medium than in BHI medium (Figures 2C and 2D). The apparent stability of CML in M9 medium under anaerobic conditions cannot be ascribed to metabolic inactivity of the strain, since degradation of 250 μM lysine was observed to be complete after 24 h (data not shown). In BHI medium, CML degradation was delayed as compared to minimal medium and started only after 24 h when the stationary growth phase had been reached. This shows that *E. coli* $G_{1/2}$ does not revert to "unusual" substrates unless it faces inadequate nutrient conditions after consumption of the medium. The same effect was observed with the other strains (data not shown). Considering all strains except G_5 which did not grow in minimal medium, 96 ± 7% of CML had remained after 24 h when strains were grown in BHI medium under aerobic conditions, but significantly less CML (89 ± 6%, $P < 0.05$) had remained after 24 h in the presence of strains in minimal medium.

As BHI medium is very rich in amino acids that may interfere with the detection of specific metabolites from CML, all following experiments were performed with the use of minimal

medium under aerobic conditions. First, strain $G_{1/2}$ was incubated with CML for 24 h, and LC-MS/MS analyses were performed in the scan mode (*m/z* 100–250) in comparison to a $G_{1/2}$ culture that had been incubated in parallel without CML. Mass-by-mass chromatogram extraction revealed differences in the peak patterns of the extracted ion chromatograms for *m/z* 161 and *m/z* 176. The respective peaks were present in the cultures that had been incubated with CML, but not in those incubated without CML. They were formed during incubation, because they were not present in the cultures at the beginning of the experiment. Since mainly protonated monoisotopic molecular ions are detected by ESI-MS under the experimental conditions chosen in this study, we concluded that the substances causing these peaks should have molecular masses of 160 Da and 175 Da, respectively, and should be equivalent to the biogenic amine CM-CAD **2** and the acid CM-APA **3**, respectively (Figure 1). The potential metabolites were synthesized from the respective 5-aminopentane derivatives by reductive alkylation in the presence of glyoxylic acid.[29] In addition to the acid **3** and the amine **2**, CM-APO **4** as a further possible metabolite from the Ehrlich pathway of amino acids was synthesized.[43,44] This compound has been used as a linker in the course of studies concerning RNA backbone modification.[45] CM-CAD **2** was used as a linker in the synthesis of antibiotic conjugates.[46] The acid **3** had been detected as an acid hydrolysis product of an enzymatic metabolite of the alkali-induced amino acid L-D-lysinoalanine (LAL).[47] None of these compounds has yet been discussed as a metabolite of CML. In order to analyze these metabolites, a method was established based on HPLC-MS/MS working in the MRM mode with quantitation by standard addition. The limits of detection and quantitation as well as the conversion rate of CML to the respective metabolite at the concentration of the LOD are compiled in Table 3.

In parallel to the decrease of CML as measured by amino acid analysis (Figure 3A), the formation of CM-CAD **2** was observed by HPLC-MS/MS (Figure 3B). The formation of **2** was

already observable after 4 hours and linearly increased during the incubation time to reach 2.5 ± 0.4% of the initial CML dose after 48 h of incubation. Regarding the decrease in the CML concentration of 34.5 ± 6.1% in the same time, apparently not more than 7% of the decline can be explained by the formation of this metabolite by this strain. CM-APA **3** as a further metabolite was not observed before 24 h of incubation and accounted for only 0.1% of the initial CML dose thus explaining ca. 0.3% of the degradation of CML. CM-APO **4** was not formed. We conclude that further metabolites must exist which were not discovered under the experimental conditions used in this study. The formation of CM-APA **3** requires the intermediate formation of the aldehyde **5**, which unites a secondary amine group and an aldehyde group in the same molecule in close proximity. A chemical degradation of such an intermediate through formation of the piperidine derivative **6** and further dehydration to the *N*-carboxymethyl-Δ^1-piperideinium cation **7** may not be excluded (Figure 4). This cation was searched for by comparison of extracted ion chromatograms recorded after incubation of CML with the strain $G_{4/9}$. The respective peak of a substance showing an *m/z* of 142 was detected only when CML had been incubated in the presence of *E. coli*. It was neither produced in the absence of CML nor in the absence of *E. coli*, nor was it present at the beginning of the experiment (Figure 5A). Moreover, it was detected when the lyophilization step which could theoretically invoke dehydration of **6**, was omitted. The product ion spectrum of *m/z* 142 recorded at the retention time of the peak revealed that the main fragments of the ion *m/z* 142 are *m/z* 114 and *m/z* 96 (Figure 5B). The transition *m/z* 142 → 96 can be explained by elimination of HCOOH as proposed in Figure 5C. Loss of elements of HCOOH is common to amino acid fragmentation reactions.[48] The transition *m/z* 142 → 114 may be due to retro-Diels-Alder reaction under elimination of ethylene.[49] The formation of *N*-substituted piperideine derivatives, which necessarily are cations, may be a unique feature of *N*-ε-alkylated lysine derivatives during microbial metabolism. Piperideine as such has been detected as a metabolite of lysine in the biosynthesis of piperidine alkaloids in *Nicotiana* species.[50]

The detection of a metabolic pathway of an unusual amino acid in a whole organism may be precluded by the inappropriate choice of the substrate. In *S. cerevisiae*, metabolization of glycated amino acids became visible only after application of glycated dipeptides instead of glycated amino acids.[43] Therefore, alanyl-(*N*-ε-carboxymethyl-)-lysine (Ala-CML) was applied to *E. coli* strain $G_{1/2}$ as a glycated dipeptide in minimal medium under aerobic conditions. The dipeptide was degraded very fast under formation mainly of CML (Figures 6A and 6B). A certain chemical instability of Ala-CML was observed when it was incubated in M9 medium in the absence of *E. coli* strains: After 24 h, 84 ± 8% of the compound was left, but the metabolites **2** and **3** were not detected. As the majority of *E. coli* peptidases are located in the cytoplasm,[51] we postulate that Ala-CML is transported into the cell and cleaved to alanine and CML. Then, CML is either metabolized or removed from the cells by (an) efflux system(s). This postulate is substantiated by the observation that metabolite formation turns into a biphasic course as compared to the application of free CML (Figure 6C). The initial strong increase of the metabolite concentrations may be ascribed to fast peptide transport providing high amounts of substrates intracellularly. From 7 h on, the rate of metabolite formation is approximating the time course observed during the incubation of free CML, because extracellular peptide is expended and now free CML must be internalized by (less effective) amino acid transport systems. Transport of unusual amino acids and peptides by *E. coli*, but also generally by intestinal microorganisms, needs to be explored more intensely.

Further, we investigated whether *E. coli* strain $G_{1/2}$ is able to metabolize the compounds **2**, **3**, and **4**, because literature data indicated that some *E. coli* strains can degrade biogenic amines.[52] When strain $G_{1/2}$ was incubated with 250 µM of CM-CAD **2** or CM-APA **3**, no significant change in the concentrations was detected during 48 h of incubation. However, the concentration of CM-APO **4** decreased by 15.4 ± 0.7% during 48 h. CM-APA **3** and CM-CAD **2** as potential metabolites were not detectable. We conclude that CM-CAD **2** and CM-APA **3**

are stable end products of CML metabolism of the *E. coli* strains investigated in this study. A possible pathway of metabolization of CM-APO **4** may be the oxidation to the aldehyde **5** and the further degradation of the compound (Figure 4).

Phenotypic comparison of six *E. coli* strains. The probiotic formulation Symbioflor is constituted by a mixture of six *E. coli* strains (Table 1) that slightly differ in their genome.[38] In a preliminary experiment, we wanted to elucidate as to whether these differences have an influence on the degradation of CML and on the formation of metabolites. Strain G_5 was not culturable in minimal medium and had to be excluded from these investigations. The other strains were cultivated in the same way as described for strain $G_{1/2}$.

During 48 h of incubation with or without addition of CML to the minimal medium, all strains reached optical densities between 1.0 and 1.3. As becomes apparent from the calculation of the relative OD, all strains reproducibly grew better when CML had been added to minimal medium than in the absence of the compound (Table 4). This may be indicative of a use of CML as a nitrogen and/or carbon source by the strains. Differences were detected in the ability of the strains to degrade CML and to produce the metabolites CM-CAD **2** and CM-APA **3**. These differences were qualitatively, but not quantitatively reproducible. Therefore, Table 4 presents the data of one experiment with two biological replicates from the same stock culture. The genetically closely related strains $G_{1/2}$, $G_{6/7}$, and G_8 behaved very similarly in degrading CML and in producing both CM-APA **3** and CM-CAD **2**.[38] In the presence of strain $G_{4/9}$, the highest proportion of CML was removed and the formation of CM-CAD **2** was most pronounced. Strain $G_{3/10}$ was least able to degrade CML and to produce the metabolites, although it grew as well as strain $G_{4/9}$. Higher production of CM-CAD **2** correlated with a higher production of CM-APA **3**. This may indicate that the pathways of metabolization have a similar activity relative to another, and that metabolization of free CML in general should depend on the ability of the

compound to get into the bacterial cell. Thus, differences in the metabolization may be due to differences in amino acid membrane transport. Differences in intracellular metabolic activity resulting from potential differences in gene expression, enzyme activity or substrate affinity may also not be excluded. Further insight into the biochemical background of our findings may be gained by metatranscriptomic analysis of the strains. This would allow the identification of genes that are potentially involved in CML transport or metabolism. Such genes later could be searched for in the genomes of other intestinal bacteria. Together with the incubation experiments as described in the present study, this would help to identify further species that are able to metabolize CML.

Discussion. CML **1** is a polyfunctional amino acid that may undergo metabolic reactions at different molecular sites (Figure 1). The biogenic amine **2** was identified as the main metabolite of CML in this study. Decarboxylation of amino acids with the formation of biogenic amines is a common metabolic pathway in many gut bacterial species, among them *Clostridia*, *Bifidobacteria*, and *Enterobacteriaceae* such as *E. coli* and *Lactobacilli*.[53,54] The production of biogenic amines from amino acids was described as to provide cellular protection for *Enterobactericeae* in an acidic environment, which prevails in the large intestine (pH 5.5–6.8).[55] The ability to produce biogenic amines is widespread among intestinal bacteria with pronounced inter-species variations in the individual amines that are formed (e.g., putrescine, cadaverine, tyramine, spermine, spermidine).[52] The acid **3** was identified as a further CML metabolite. Similarly to other glycated amino acids,[43] CML **1** may be subject to the Ehrlich pathway, a metabolic route that is present not only in yeasts, but also in lactic acid bacteria.[44] The Ehrlich pathway involves first the transamination of an amino acid to an α-keto acid **8**, which is then oxidatively decarboxylated to an aldehyde **5**. In the case of CML, the aldehyde **5** may be reduced to the alcohol CM-APO **4** or oxidized to the fatty acid CM-APA **3**.[44] The α-

keto acid **8** may also be formed through oxidation by L-amino acid oxidases, which can be present in bacteria.[56] Lastly, CM-CAD **2** may be a precursor of **3**, because biogenic amines can be oxidized to aldehydes via amine oxidases that also have been described in several microorganisms.[57]

In summary, the present study shows for the first time that *E. coli* strains are able to degrade CML to defined metabolites with the biogenic amine CM-CAD **2** as the main product. Metabolization may be oxygen-dependent at least for the six strains studied. Despite the quantitative abundance of *E. coli* in feces, other (facultatively) anaerobic bacteria than *E. coli* must have been responsible for the degradation of CML in the presence of the human intestinal microbiota in our previous study.[35] Provided that the metabolites are also formed by the complex human gut microbiota, further studies need to be performed concerning the physiological relevance of these compounds: Do the metabolites pass into the circulation and can they be detected in urine? Does **2** address trace amine-associated receptors?[58] Does **2** have an influence on digestive processes as described for other intestinally produced amines?[59] The present study lays the foundation for a new view on structure-activity relationships of MRPs in human physiology. Not only glycated amino acids as such need to be investigated, but also the physiological effects of their metabolites.

Abbreviations Used

AGE, advanced glycation end product; Ala-CML, alanyl-(*N*-ε-carboxymethyl)-lysine; ARP, Amadori rearrangement product; BHI, brain heart infusion; CM-APA, *N*-carboxymethylaminopentanoic acid; CM-APO, *N*-carboxymethylaminopentanol; CM-CAD, *N*-carboxymethylcadaverine; CML, *N*-ε-Carboxymethyllysine; LOD, limit of detection; LOQ,

481 limit of quantitation; MRM, multiple reaction monitoring; MRP, Maillard reaction product;
482 NFPA, nonafluoropentanoic acid; OD, optical density.

483

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489

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Figure captions

Figure 1. Putative pathways of CML metabolization by microorganisms (Literature see text). Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; DEC, decarboxylase; MAO, monoamine oxidase; TAM, transaminase.

Figure 2. Time course of the optical density (OD_{600}) of inocula of *E. coli* strain $G_{1/2}$ in (A) nutrient-rich medium (BHI broth) and (B) minimal medium (M9) under aerobic or anaerobic conditions. Time course of the concentration of CML in (C) nutrient-rich medium and (D) minimal medium under aerobic or anaerobic conditions. Initial concentration, 250 µM. Open signs, minimal medium; closed signs, BHI medium; black squares, aerobic conditions; grey circles, anaerobic conditions. Data are given as means ± S.D. (n = 2–6).

Figure 3. Incubation of CML in the presence of *E. coli* strain $G_{1/2}$ in minimal medium under aerobic conditions. (A) Amino acid analysis chromatograms with focus on the CML peak. (B) HPLC-MS/MS chromatograms recorded in the MRM mode at the most intense transition of *N*-carboxymethylcadaverine (CM-CAD). (C) Time course of the formation of the metabolites CM-CAD and *N*-carboxymethylaminopentanoic acid (CM-APA). The concentrations are given in percent relative to the initial CML concentration (250 µM). Data are given as means ± S.D. (n = 2).

Figure 4. Proposed pathway of the chemical degradation of *N*-carboxymethylaminopentanal **5** as a possible intermediate in the formation of CM-APA.

522

523 **Figure 5.** (A) Extracted ion chromatograms (*m/z* = 142) of cultures of *E. coli* strain $G_{4/9}$ at (a)
524 0 h of incubation in the absence of CML, (b) 48 h of incubation in the absence of CML, (c) 0 h
525 of incubation in the presence of CML, (d) 48 h of incubation in the presence of CML. (B)
526 Product ion spectrum of the peak eluting at 3.4 min (fragmentor voltage, 100 V; collision
527 energy, 10 eV). (C) Proposed fragmentation reactions leading to the formation of the abundant
528 fragments in the product ion spectrum.

529

530 **Figure 6.** Incubation of Ala-CML in the presence of *E. coli* strain $G_{1/2}$ in minimal medium
531 under aerobic conditions. (A) Amino acid analysis chromatograms of Ala-CML and CML. (B)
532 HPLC-MS/MS chromatograms recorded in the MRM mode at the most intense transition of *N*-
533 carboxymethylaminopentanoic acid (CM-APA). (C) Time course of the formation of the
534 metabolites CM-CAD and CM-APA. The concentrations are given in percent relative to the
535 initial CML concentration (250 μM). Data are given as means ± S.D. (*n* = 2).

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References

[1] Hellwig, M.; Henle, T. Baking, ageing, diabetes: a short history of the Maillard reaction. *Angew. Chem. Int. Ed. Engl.* **2014**, *53*, 10316–10329.

[2] Mehta, B.M.; Deeth, H.C. Blocked lysine in dairy products: Formation, occurrence, analysis and nutritional implications. *Comp. Rev. Food Sci. Food Safety* **2016**, *15*, 206–218.

[3] Thornalley, P.J.; Langborg, A.; Minhas, H.S. Formation of glyoxal, methylglyoxal, and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem. J.* **1999**, *344*, 109–116.

[4] Glomb, M.A.; Tschirnich, R. Detection of α-dicarbonyl compounds in Maillard reaction systems and in vivo. *J. Agric. Food Chem.* **2001**, *49*, 5543–5550.

[5] Henning, C.; Glomb, M.A. Pathways of Maillard reaction under physiological conditions. *Glycoconjugate J.* **2016**, *33*, 499–512.

[6] Wadman, S.K.; Debree, P.K., Vansprang, F.J.; Kamerling, J.P.; Haverkamp, J.; Vliegenthart, J.F.G. N-ε-(Carboxymethyl)lysine, a constituent of human urine. *Clin. Chim. Acta* **1975**, *59*, 313–320.

[7] Ahmed, M.U.; Thorpe, S.R.; Baynes, J.W. Identification of N^{ε}-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J. Biol. Chem.* **1986**, *261*, 4889–4894.

[8] Glomb, M.A.; Monnier, V.M. Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. *J. Biol. Chem.* **1995**, *270*, 10017–10026.

[9] Nagai, R.; Ikeda, K.; Kawasaki, Y.; Sano, H.; Yoshida, M.; Araki, T.; Ueda, S.; Horiuchi, S. Conversion of Amadori product of Maillard reaction to N-ε-(carboxymethyl)lysine in alkaline condition. *FEBS Lett.* **1998**, *425*, 355–360.

[10] Kasper, M.; Schieberle, P. Labeling studies on the formation pathway of N^{ε}-Carboxymethyllysine in Maillard-type reactions. *Ann. N.Y. Acad. Sci.* **2005**, *1043*, 59–62.

[11] Ferreira, A.E.N.; Ponces Freire, A.M.J.; Voit, E.O. A quantitative model of the generation of N-ε-(carboxymethyl)lysine in the Maillard reaction between collagen and glucose. *Biochem. J.* **2003**, *376*, 109–121.

[12] Assar, S.H.; Moloney, C.; Lima, M.; Magee, R.; Ames, J.M. Determination of N-ε-(carboxymethyl)lysine in food systems by ultra-performance liquid chromatography-mass spectrometry. *Amino Acids* **2009**, *36*, 317–326.

[13] Wellner, A.; Nußpickel, L.; Henle, T. Glycation compounds in peanuts. *Eur. Food Res. Technol.* **2012**, *234*, 423–429.

[14] Hull, G.L.J.; Woodside, J.V.; Ames, J.M.; Cuskelly, G.J. N^{ε}-(carboxymethyl)lysine content of foods commonly consumed in a Western style diet. *Food Chem.* **2012**, *131*, 170–174.

[15] Scheijen, J.L.J.M.; Clevers, E.; Engelen, L.; Dagnelie, P.C.; Brouns, F.; Stehouwer, C.D.A.; Schalkwijk, C.G. Analysis of advanced glycation endproducts in selected food items by ultra-performance liquid chromatography tandem mass spectrometry: Presentation of a dietary AGE database. *Food Chem.* **2016**, *190*, 1145–1150.

[16] Hellwig, M.; Kühn, L.; Henle, T. Individual Maillard reaction products as indicators of heat treatment of pasta—a survey of commercial products. *J. Food Comp. Anal.* **2018**, *72*, 83–92.

[17] Birlouez-Aragon, I.; Saavedra, G.; Tessier, F. J.; Galinier, A.; Ait-Ameur, L.; Lacoste, F.; Niamba, C.-N.; Alt, N.; Somoza, V.; Lecerf, J.-M. A diet based on high-heat-treated foods promotes risk factors for diabetes mellitus and cardiovascular diseases. *Am. J. Clin. Nutr.* **2010**, *91*, 1220−1226.

[18] Delgado-Andrade, C.; Tessier, F.J.; Niquet-Léridon, C.; Seiquer, I.; Navarro, M.P. Study of the urinary and faecal excretion on N^{ε}-carboxymethyllysine in young human volunteers. *Amino Acids* **2012**, *43*, 595–602.

[19] Scheijen, J.L.J.M.; Hanssen, N.M.J.; van Greevenbroek, M.M.; van der Kallen, C.; Feskens, E.J.M.; Stehouwer, C.D.A.; Schalkwijk, C.G. Dietary intake of advanced glycation endproducts is associated with higher levels of advanced glycation endproducts in plasma and urine: The CODAM study. *Clin. Nutr.* **2018**, *37*, 919–925.

[20] Uribarri, J.; Woodruff, S.; Goodman, S.; Cai, W.; Chen, X.; Pyzik, R.; Yong, A.; Striker, G.E.; Vlassara, H. Advanced glycation end products in foods and a practical guide to their reduction in the diet. *J. Am. Diet. Assoc.* **2010**, *110*, 911–916.

[21] Buetler, T.M.; Leclerc, E.; Baumeyer, A.; Latado, H.; Newell, J.; Adolfsson, O.; Parisod, V.; Richoz, J.; Maurer, S.; Foata, F.; Piguet, D.; Junod, S.; Heizmann, C.W.; Delatour, T. N^{ε}-carboxymethyllysine-modified proteins are unable to bind to RAGE and activate an inflammatory response. *Mol. Nutr. Food Res.* **2008**, *52*, 370–378.

[22] Calder, P.C.; Ahluwalia, N.; Brouns, F.; Buetler, T.; Clement, K.; Cunningham, K.; Esposito, K.; Jönsson, L.S.; Kolb, H.; Lansink, M.; Marcos, A.; Margioris, A.; Matusheski, N.; Nordmann, H.; O'Brien, J.; Pugliese, G.; Rizkalla, S.; Schalkwijk, C.; Tuomilehto, J.; Wärnberg, J.; Watzl, B.; Winklhofer-Roob, B.M. Dietary factors and low-grade inflammation in relation to overweight and obesity. *Br. J. Nutr.* **2011**, *106*, S5–S78.

[23] Lund, M.N.; Ray, C.A. Control of Maillard reactions in foods: Strategies and chemical mechanisms. *J. Agric. Food Chem.* **2017**, *65*, 4537–4552.

[24] Kellow, N.J.; Savige, G.S. Dietary advanced glycation end-product restriction for the attenuation of insulin resistance, oxidative stress and endothelial dysfunction: a systematic review. *Eur. J. Clin. Nutr.* **2013**, *67*, 239–248.

[25] Kellow, N.J.; Coughlan, M.T. Effect of diet-derived advanced glycation end products on inflammation. *Nutr. Rev.* **2015**, *73*, 737–759.

[26] Clarke, R.E.; Dordevic, A.L.; Tan, S.M.; Ryan, L.; Coughlan, M.T. Dietary advanced glycation end products and risk factors for chronic disease: A systematic review of randomized control trials. *Nutrients* **2016**, *8*, 125.

[27] Hegele, J.; Buetler, T.; Delatour, T. Comparative LC-MS/MS profiling of free and protein-bound early and advanced glycation-induced lysine modifications in dairy products. *Anal. Chim. Acta* **2008**, *617*, 85–96.

[28] Hellwig, M.; Matthes, R.; Peto, A.; Löbner, J.; Henle, T. N-ε-fructosyllysine and N-ε-carboxymethyllysine, but not lysinoalanine, are available for absorption after simulated gastrointestinal digestion. *Amino Acids* **2014**, *46*, 289–299.

[29] Hellwig, M.; Geissler, S.; Matthes, R.; Peto, A.; Silow, C.; Brandsch, M.; Henle, T. Transport of free and peptide-bound glycated amino acids: Synthesis, transepithelial flux at Caco-2 cell monolayers, and interaction with apical membrane transport proteins. *ChemBioChem* **2011**, *12*, 1270–1279.

[30] Li, M.; Zeng, M.; He, Z.; Zheng, Z.; Qin, F.; Tao, G.; Zhang, S.; Chen, J. Effect of long-term exposure to free N^{ε}-(Carboxymetyhl)lysine on rats fed a high-fat diet. *J. Agric. Food Chem.* **2015**, *63*, 10995–11001.

[31] Tessier, F.J.; Niquet-Léridon, C.; Jacolot, P.; Jouquand, C.; Genin, M.; Schmidt, A.-M.; Grossin, N.; Boulanger, E. Quantitative assessment of organ distribution of dietary protein-bound ^{13}C-labeled N^{ε}-carboxymethyllysine after a chronic oral exposure in mice. *Mol. Nutr. Food Res.* **2016**, *60*, 2446–2456.

[32] Alamir, I.; Niquet-Léridon, C.; Jacolot, P.; Rodriguez, C.; Orosco, M.; Anton, P.M.; Tessier, F.J. Digestibility of extruded proteins and metabolic transit of N^{ε}-carboxymethyllysine in rats. *Amino Acids* **2013**, *44*, 1441–1449.

[33] Roncero-Ramos, I.; Delgado-Andrade, C.; Tessier, F.J.; Niquet-Léridon, C.; Strauch, C.; Monnier, V.M.; Navarro, M.P. Metabolic transit of *N*ε-carboxymethyl-lysine after consumption of AGEs from bread crust. *Food Funct.* **2013**, *4*, 1032–1039.

[34] Roncero-Ramos, I.; Niquet-Léridon, C.; Strauch, C.; Monnier, V.M.; Tessier, F.J.; Navarro, M.P.; Delgado-Andrade, C. An advanced glycation end product (AGE)-rich diet promotes Nε-carboxymethyl-lysine accumulation in the cardiac tissue and tendons of rats. *J. Agric. Food Chem.* **2014**, *62*, 6001–6006.

[35] Hellwig, M.; Bunzel, D.; Huch, M.; Franz, C.M.A.P.; Kulling, S.E.; Henle, T. Stability of individual Maillard reaction products in the presence of the human colonic microbiota. *J. Agric. Food Chem.* **2015**, *63*, 6723–6730.

[36] Anton, P.M.; Craus, A.; Niquet-Léridon, C.; Tessier, F.J. Highly heated food rich in Maillard reaction products limit an experimental colitis in mice. *Food Funct.* **2012**, *3*, 941–949.

[37] Al-Jahdali, N.; Gadonna-Widehem, P.; Delayre-Orthez, C.; Marier, D.; Garnier, B.; Carbonero, F.; Anton, P.M. Repeated oral exposure to *N*ε-carboxymethyllysine, a Maillard reaction product, alleviates gut microbiota dysbiosis in colitic mice. *Dig. Dis. Sci.* **2017**, *62*, 3370–3384.

[38] Zschüttig, A.; Auerbach, C.; Meltke, S.; Eichhorn, C.; Brandt, M.; Blom, J.; Goesmann, A.; Jarek, M.; Scharfe, M.; Zimmermann, K.; Wassenaar, T.M.; Gunzer, F. Complete sequence of probiotic Symbioflor 2 Escherichia coli strain G3/10 and draft sequences of Symbioflor 2 *E. coli* strains G1/2, G4/9, G5, G6/7, and G8. *Genome Announc.* **2015**, *3*, e01330-14.

[39] Zschüttig, A.; Zimmermann, K.; Blom, J.; Goesmann, A.; Pöhlmann, C.; Gunzer, F. Identification and characterization of microcin, a new antibacterial peptide produced by probiotic *Escherichia coli* G3/10. *Plos One* **2012**, *7*, e33351.

[40] Wassenaar, T.M.; Zschüttig, A.; Beimfohr, C.; Geske, T.; Auerbach, C.; Cook, H.; Zimmermann, K.; Gunzer, F. Virulence genes in a probiotic E. coli product with a recorded long history of safe use. *Eur. J. Microbiol. Immunol.* **2015**, *5*, 81–93.

[41] Reindel, F.; Hoppe, W. Über eine Färbemethode zum Anfärben von Aminosäuren, peptide und Proteinen auf Papierchromatogrammen und Papierelektropherogrammen. *Chem. Ber.* **1954**, *87*, 1103–1107.

[42] Wassenaar, T.M. Insights from 100 years of research with probiotic *E. coli. Eur. J. Microbiol. Immunol.* **2016**, *6*, 147–161.

[43] Hellwig, M.; Börner, M.; Beer, F.; Van Pée, K.-H.; Henle, T. Transformation of free and dipeptide-bound glycated amino acids by two strains of *Saccharomyces cerevisiae*. *ChemBioChem* **2017**, *18*, 266–275.

[44] Hazelwood, L.A.; Daran, J.-M.; Van Maris, A.J.A.; Pronk, J.T.; Dickinson, J.R. The Ehrlich pathway for fusel alcohol production: A century of research on *Saccharomyces cerevisiae* metabolism. *Appl. Environ. Microbiol.* **2008**, *74*, 2259–2266.

[45] Greiner, B.; Breipohl, G.; Uhlmann, E. (2‘-*O*-Methyl-RNA)-3‘-PNA chimeras: A new class of mixed backbone oligonucleotide analogues with high binding affinity to RNA. *Helv. Chim. Acta* **2002**, *86*, 2619–2626.

[46] Heinisch, L.; Wittmann, S.; Stoiber, T.; Berg, A.; Ankal-Fuchs, D.; Möllmann, U. Highly antibacterial active aminoacyl penicillin conjugates with acylated bis-catecholate siderophores based on secondary diamino acids and related compounds. *J. Med. Chem.* **2002**, *45*, 3032–3040.

[47] Engelsma, J.W.; Van der Meulen, J.D., Slump, P.; Haagsma, N. The oxidation of lysinoalanine by L-amino acid oxidase. Identification of products. *Lebensm. Wiss. Technol.* **1979**, *12*, 203–207.

[48] Langrock, T.; Czihal, P.; Hoffmann, R. Amino acid analysis by hydrophilic interaction chromatography coupled on-line to electrospray ionization mass spectrometry. *Amino Acids* **2007**, *30*, 291–297.

[49] Demarque, D.P.; Crotti, A.E.M.; Vessecchi, R.; Lopes, J.L.C.; Lopes, N.P. Fragmentation reactions using electrospray ionization mass spectrometry: in important tool for the structural elucidation and characterization of synthetic and natural products. *Nat. Prod. Rep.* **2016**, *33*, 432–455.

[50] Leete, E.; Chedekel, M.R. The aberrant formation of (–)-*N*-methylanabasine from *N*-methyl-Δ^1-piperideinium chloride in *Nicotiana tabacum* and *N. glauca*. *Phytochemistry* **1972**, *11*, 2751–2756.

[51] Lazdunski, A.M. Peptidases and proteases of *Escherichia coli* and *Salmonella typhimurium*. *FEMS Microbiol. Rev.* **1989**, *63*, 265–276.

[52] Pugin, B.; Barcik, W.; Westermann, P.; Heider, A.; Wawrzyniak, M.; Hellings, P.; Akdis, C.A.; O'Mahony, L. A wide diversity of bacteria from the human gut produces and degrades biogenic amines. *Microb. Ecol. Health Dis.* **2017**, *28*, 1353881.

[53] Pircher, A.; Bauer, F.; Paulsen, P. Formation of cadaverine, histamine, putrescine and tyramine by bacteria isolated from meat, fermented sausages and cheeses. *Eur. Food Res. Technol.* **2007**, *226*, 225–231.

[54] Smith, E.A.; Macfarlane, G.T. Studies on amine production in the human colon: Enumeration of amine forming bacteria and physiological effects of carbohydrate and pH. *Anaerobe* **1996**, *2*, 285–297.

[55] Bearson, B.; Bearson, S.; Foster, J.W. Acid stress responses in enterobacteria. *FEMS Microbiol. Lett.* **1997**, *147*, 173–180.

[56] Hossain, G.S.; Li, J.; Shin, H.; Du, G.; Liu, L.; Chen, J. L-Amino acid oxidases from microbial sources: types, properties, functions, and applications. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 1507–1515.

[57] Yagodina, O.V.; Nikol'skaya, E.B.; Khovanskikh, A.E.; Kormilitsyn, B.N. Amine oxidases of microorganisms. *J. Evol. Biochem. Physiol.* **2002**, *38*, 251–258.

[58] Pei, Y.; Asif-Malik, A.; Canales, J.J. Trace amines and the trace amine-associated receptor 1: Pharmacology, neurochemistry, and clinical implications. *Front. Neurosci.* **2016**, *10*, 148.

[59] Sánchez, M.; Suárez, L.; Andrés, M.T.; Flórez, B.H.; Bordallo, J.; Riestra, S.; Cantabrana, B. Modulatory effect of intestinal polyamines and trace amines on the spontaneous phasic contractions of the isolated ileum and colon rings of mice. *Food Nutr. Res.* **2017**, *61*, 1321948.

Table 1. Strains of Probiotic *E. coli* Investigated in this Study.

E. coli strain[a]	DSMZ number	Chromosome size (bp)	Characteristics[b]
$G_{1/2}$	DSM 16441	5,090,326	*
$G_{3/10}$	DSM 16443	4,999,267	Encodes an extrachromosomal microcin S synthesis operon[39]
$G_{4/9}$	DSM 16444	4,545,818	
G_5	DSM 16445	4,787,583	
$G_{6/7}$	DSM 16446	5,236,262	*, **
G_8	DSM 16448	5,160,208	*, **

[a] These strains constitute the probiotic preparation ”Symbioflor 2“ (DSM 17252).

[b] Strains marked with * share huge genomic similarity. Strains marked with ** share the same extrachromosomal plasmids and are considered closely related due to a huge genomic homology.[38,40]

Table 2. Transitions Recorded during MRM Measurement of CML Metabolites.[a]

Compound	Time frame [min]	Precursor ion [*m/z*]	Product ion [*m/z*]	Collision energy [eV]	Dwell time [ms]	Q/q[b]
CM-APA	3.0–7.0	176	112	10	160	q
			101	10		Q
			84	20		q
CM-APO		162	116	10		q
			98	10		Q
			69	10		q
CM-CAD	7.0–11.0	161	98	10	200	q
			86	10		Q
			69	20		q

[a] General conditions: Fragmentor voltage, 100 V; polarity, positive.

[b] Q, transition used for quantitation; q, transition used to confirm the presence of the analyte.

Table 3. Performance Parameters of the HPLC-MS/MS Method for the Determination of Metabolites of CML in Bacterial Inocula.

Compound	LOD[a]	LOQ[a]	Conversion rate[b]	Recovery[c]
	[µg/L]	[µg/L]	[%]	[%]
CM-APA	0.5	1.7	0.01	103 ± 5
CM-APO	0.5	1.5	0.01	106 ± 8
CM-CAD	1.8	6.1	0.04	103 ± 13

[a] At the limits of detection (LOD) and quantitation (LOQ), respectively, the analytes show peaks with signal-to-noise ratios of 3 and 10, respectively.

[b] Lowest detectable conversion rates are calculated from the LOQs taking as a basis a starting concentration of 250 µM CML.

[c] Recovery was calculated from spiking experiments of analyte-free inocula.

Table 4. Comparison of Growth and Metabolic Performance of Six Probiotic *E. coli* Strains after 48 h of Incubation in Minimal Medium in the Presence of Air.

E. coli strain[a]	$\Delta(OD_{600})$[b]	Relative OD[c]	CML[d]	CM-APA[d]	CM-CAD[d]
			[%]	[%]	[%]
$G_{1/2}$	1.3 ± 0.3	1.5	38 ± 7	0.09 ± 0.01	3.2 ± 0.8
$G_{3/10}$	1.0 ± 0.2	1.3	63 ± 8	0.04 ± 0.01	1.5 ± 0.3
$G_{4/9}$	1.0 ± 0.1	1.3	34 ± 1	0.10 ± 0.02	8.4 ± 0.4
$G_{6/7}$	1.2 ± 0.1	1.4	50 ± 8	0.09 ± 0.01	3.4 ± 0.3
G_8	1.1 ± 0.1	1.7	48 ± 8	0.11 ± 0.02	5.4 ± 0.7

[a] For strain characteristics cf. Table 1.

[b] $\Delta(OD_{600})$ refers to the change in optical density during 48 h of incubation.

[c] Relative OD_{600} is calculated by dividing the changes in OD_{600} of individual *E. coli* strains incubated in the presence of CML by the OD_{600} change of the same strain in the absence of CML.

[d] Concentrations are given relative to the initial CML amount.

Figures

Figure 1

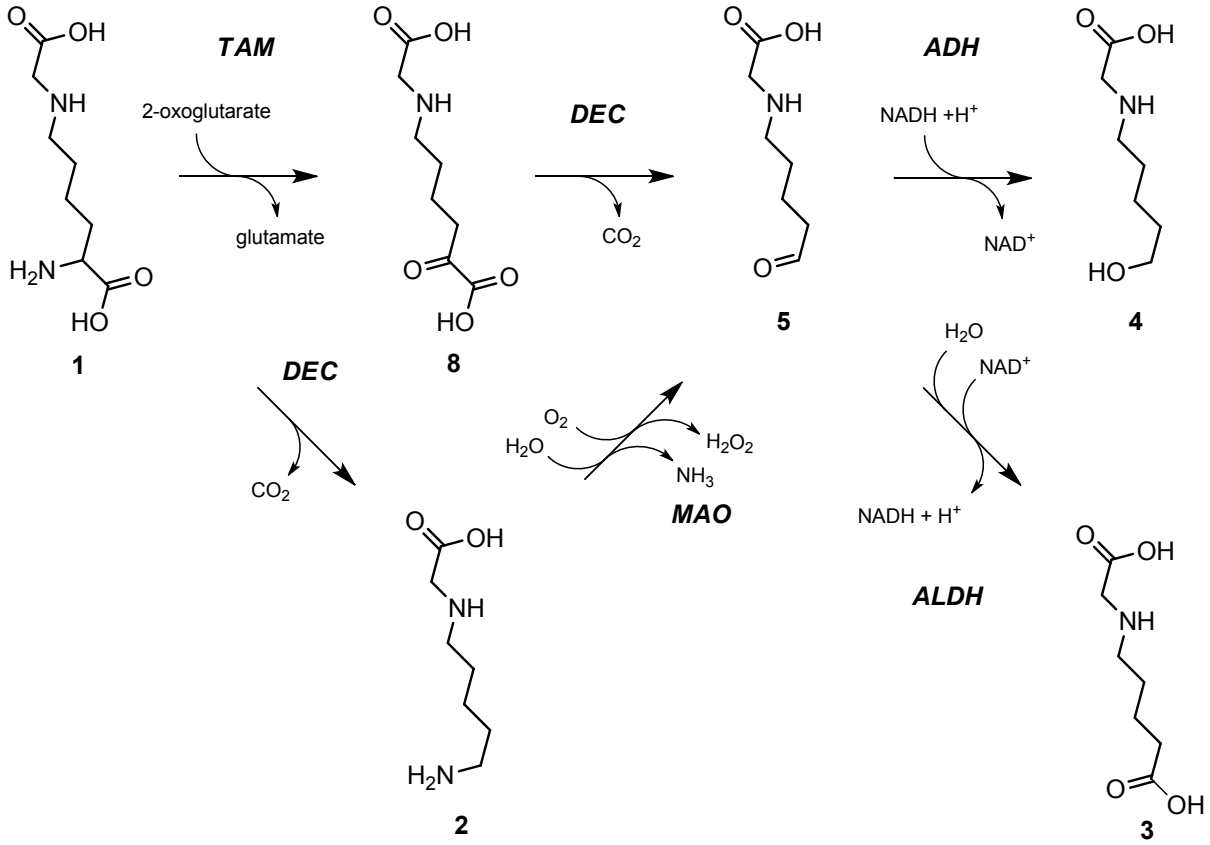


Figure 2

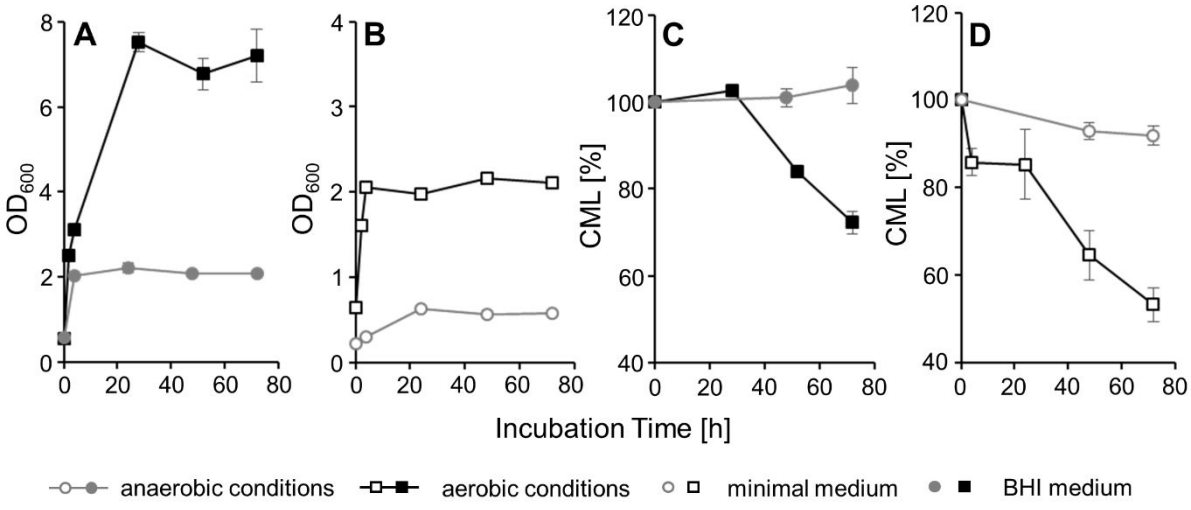

A
B
C
D
OD600
CML [%]
Incubation Time [h]
anaerobic conditions
aerobic conditions
minimal medium
BHI medium

Figure 3

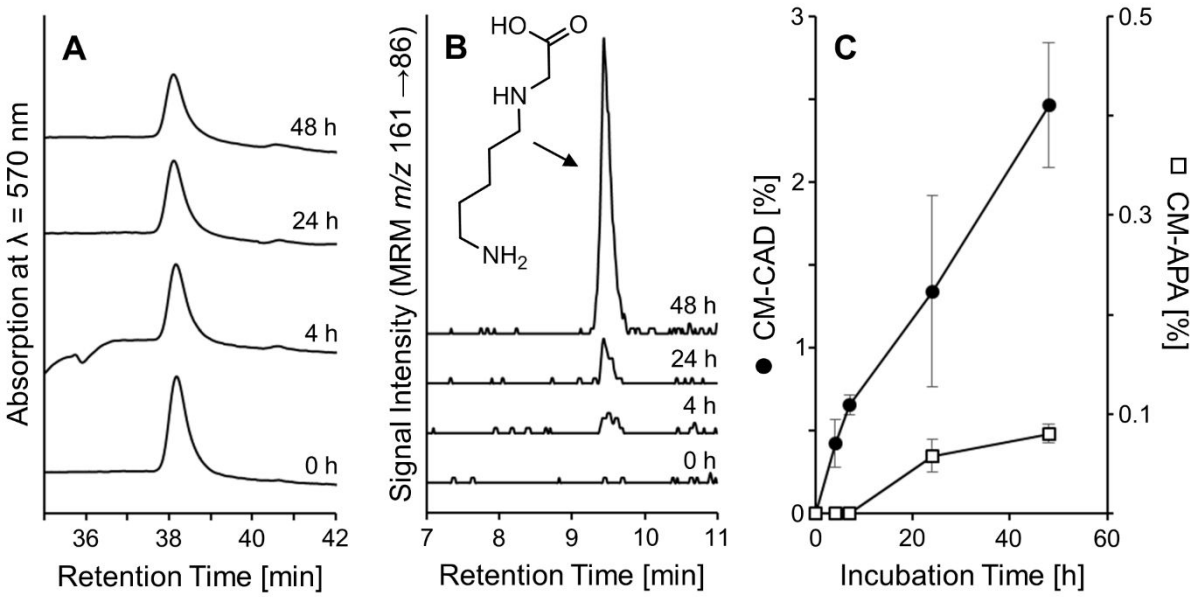

A
Absorption at λ = 570 nm
48 h
24 h
4 h
0 h
36
38
40
42
Retention Time [min]
B
HO
O
HN
NH2
Signal Intensity (MRM m/z 161 →86)
48 h
24 h
4 h
0 h
7
8
9
10
11
Retention Time [min]
C
CM-CAD [%]
CM-APA [%]
0
1
2
3
0.1
0.3
0.5
0
20
40
60
Incubation Time [h]

Figure 4

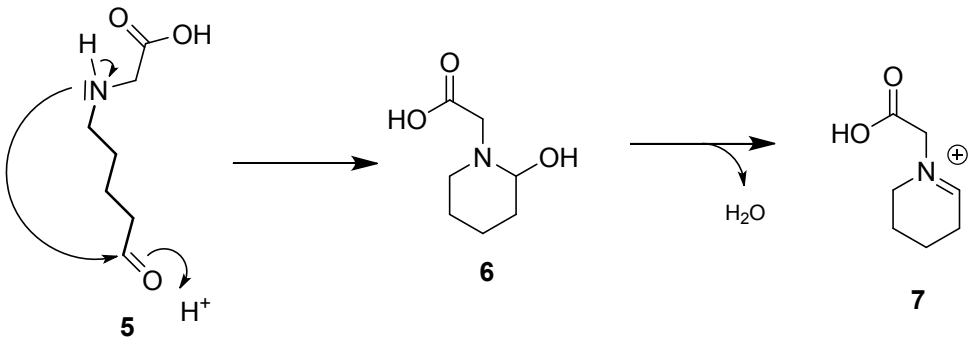


Figure 5

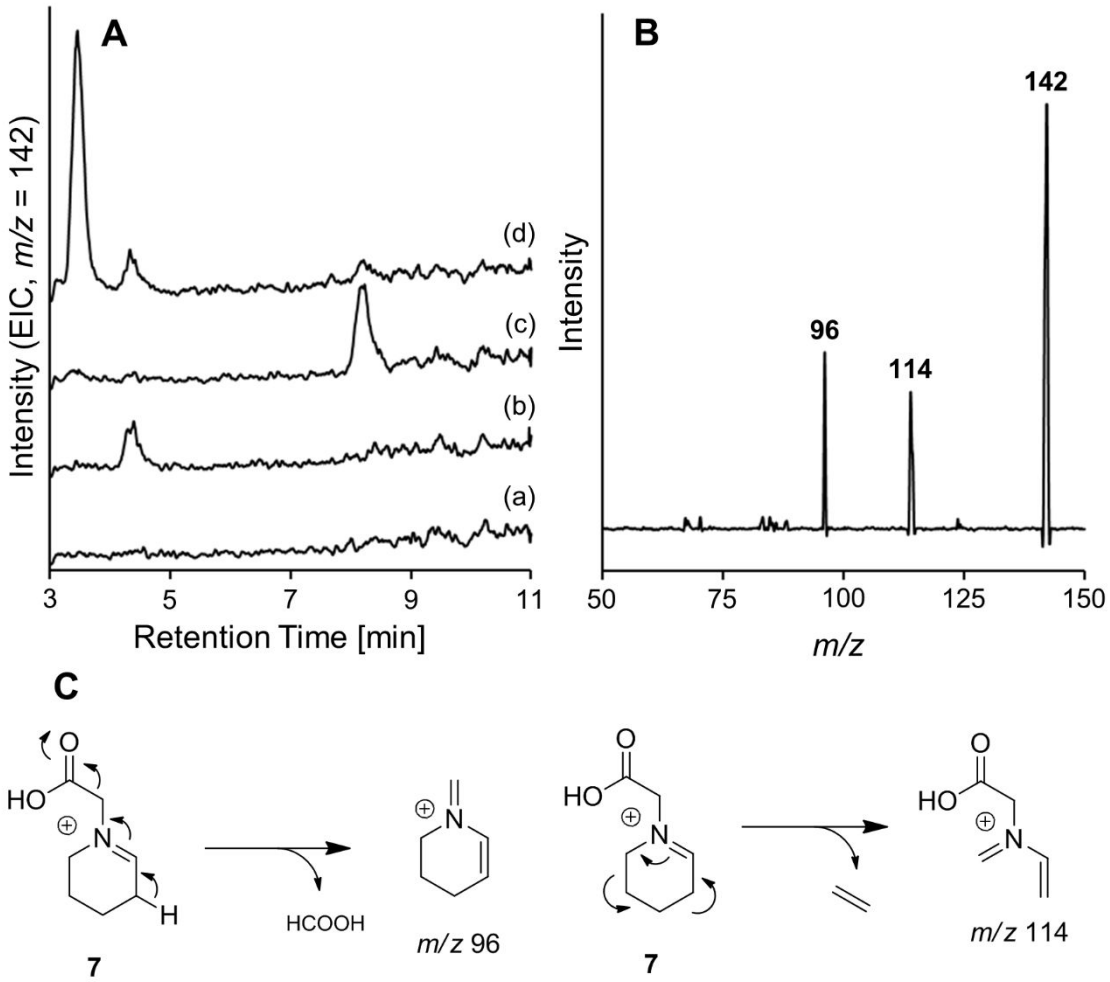
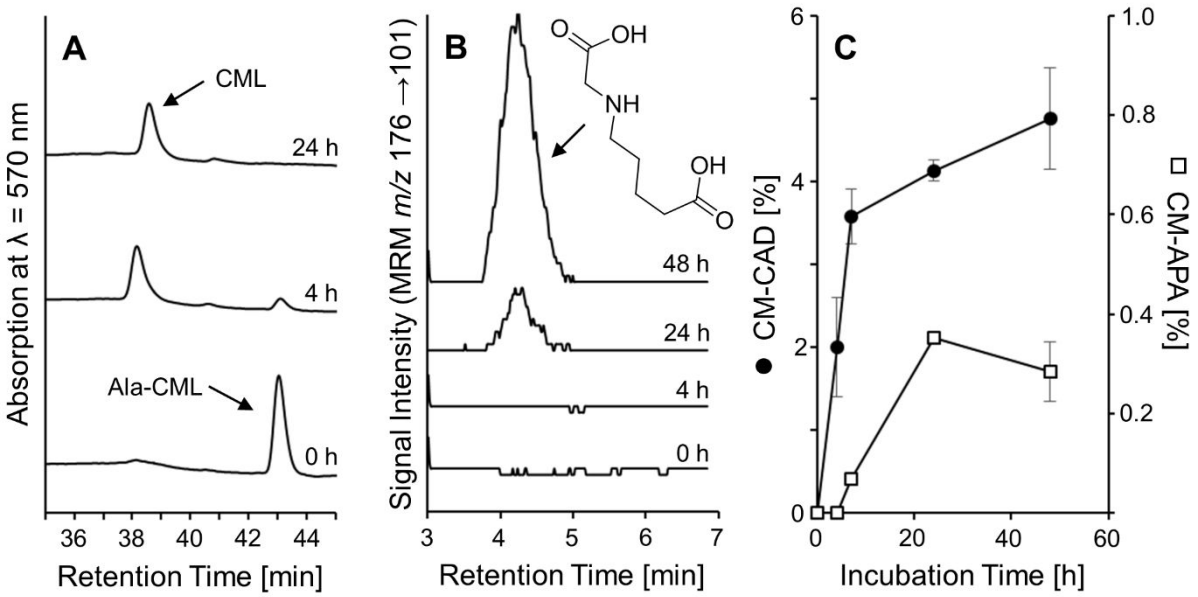

A
Intensity (EIC, m/z = 142)
(d)
(c)
(b)
(a)
3
5
7
9
11
Retention Time [min]
B
Intensity
142
96
114
50
75
100
125
150
m/z
C
HO
O
N
H
7
HCOOH
m/z 96
HO
O
N
7
HO
O
N
m/z 114

Figure 6


A
CML
24 h
4 h
Ala-CML
0 h
Absorption at λ = 570 nm
36 38 40 42 44
Retention Time [min]
B
OH
NH
OH
O
48 h
24 h
4 h
0 h
Signal Intensity (MRM m/z 176 →101)
3 4 5 6 7
Retention Time [min]
C
● CM-CAD [%]
□ CM-APA [%]
0 2 4 6
0 0.2 0.4 0.6 0.8 1.0
0 20 40 60
Incubation Time [h]

TOC graphic