Microwave-assisted Maillard reactions for the preparation of advanced glycation end products (AGEs)

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Sonja Visentin,[a] Claudio Medana,[b] Alessandro Barge,[a] Valeria Giancotti[b] and Giancarlo Cravotto*[a]

A new rapid and efficient synthetic approach for the preparation of pentosidine and other AGEs is presented.

Introduction

Primary and secondary aliphatic amino groups of aminoacids and carbonyls of reducing sugars as well as lipids and ascorbic acid, are the typical substrates of Maillard reaction.[1] It occurs in food preparation,[2] in biological systems and in several diseases. Preponderance of evidence gathered over the past decades suggest that uncontrolled Maillard reaction is detrimental to the function and integrity of biological systems. These adverse effects can be caused by the early glycation intermediates (EGPs), or the final advanced glycation endproducts (AGEs) through a variety of mechanisms. These include, among others: production of oxygen free radicals from EGPs, impairment of enzyme functions, perturbations of signaling by peptide hormones, activation of AGEs specific receptors, crosslinking of structural proteins, impairment of protein recycling etc. Etiopathology of chronic diseases such as diabetes, neuropathy, arteriosclerosis and neurodegenerative diseases (Alzheimer's disease, Parkinson and amyotrophic lateral sclerosis) has been related to the products of Maillard reaction.[3] A number of AGEs have been found in physiological systems and have different biological functions: some are protein cross-linkers (pentosidine, MOLD and GOLD), other are recognition factors for specific AGE-binding cell-surface receptors (CML, methyl glyoxal-derived hydroimidazolone) and markers or risk predictors of diseases processes (GLAP).[4] One of the most interesting product from a physiological point of view is pentosidine. The former is an AGE product which is formed when arginine and lysine residues in proteins are crosslinked by reaction with carbonyls. In 1989 Monnier, as first, isolated pentosidine from *dura mater* and characterized the structure also obtained by synthesis.[5] So far, pentosidine was obtained in a very low overall yield (0.23%) by mixing ribose, lysine and arginine for 6 days at 65°C in PBS (pH 9).[6] Yokokawa *et al*.[7] described the total synthesis of pentosidine (1 g) which requires 18-19 steps and an enormous effort for the purification.

In this paper, we report a fast and reliable one-pot microwave-assisted synthesis of pentosidine, that affords relatively clean products in much better yields compared to previous procedures.

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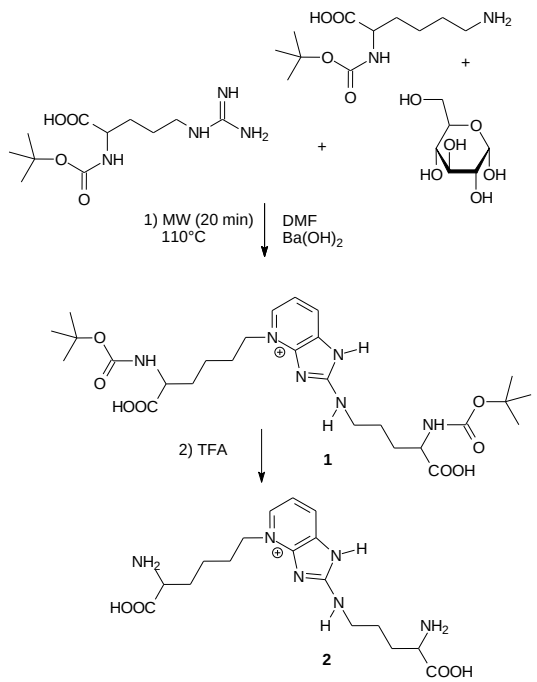
Results and discussion

Synthesis

Results showed that our procedure is extremely versatile. It is in fact well suited for the preparation of any type of AGEs as MOLD[9] and GOLD.[9] Pentosidine is obtained by condensation of BOC-*L*-Lysine and BOC-*L*-arginine with D-ribose followed by the cleavage of BOC groups (scheme 1). Using unmodified amino acids for the synthesis, we didn't obtain pentosidine. This fact can be explained to the high reactivity of α-amino groups, which can only condense with ribose in very low quantities. This problem is solved by blocking Nα-amino groups with butyloxycarbonyl (BOC) residues.



Scheme 1 Synthesis of pentosidine (2)

Thus, aiming to synthesized this AGE derivative, we experimented several conditions, solvents and techniques. Each protocol was performed both under conventional heating

with vigorous stirring and under MW irradiation. MW-assisted reactions were performed in a multimode professional oven (MicroSYNTH, Milestone). A couple of trials in a monomode reactor (Discover, CEM) gave substantially the same results. Table 1 summarize reaction conditions and yields of free pentosidine.

Table 1 Synthesis of pentosidine: conventional methods (Conv) vs MW-assisted reactions.

Entry	Method	Solvent	Time	Temp (°C)	Yield (%)
1	Conv[b]	PBS	7 d	65	0.2
3	Conv[a]	DMF	6 h	110	4
4	Conv[b]	CH_3CN	6h	110	traces
5	MW[c]	PBS	30 min	110	traces
6	MW[c]	DMF	15 min	110	8
7	MW[c]	CH_3CN	15min	110	traces

[a] Conv: heating in oil bath under magnetic stirring;
[b] Published procedure (rif. 9);
[c] MW: irradiation 300W x 5 min repeated 3 times, with interposed pauses of 3 min.

To determine the optimal duration of synthesis, the reaction mixture was sampled at regular intervals; synthesis was continued until the concentration of pentosidine measured by LC-MS reached a plateau (figure 2).

Although the formation of the Shiff base requires weak acidic conditions, basic pH values are necessary to enhance the formation of Amadori compounds.[11] Under our conditions the optimal pH values lie close to 9. Temperature is a crucial parameter in Maillard reaction;[12] we found that best reaction rates were achieved at 110°C, while only 10 Celsius degrees over, promoted side reactions and partial degradation. Besides pH and temperature, a major role is played by the solvent. As shown in Table 1, highest yields were achieved in DMF both under conventional and dielectric heating. MW dramatically accelerated the synthesis of pentosidine that went to completion in 15 minutes compared to 6 hours under conventional heating.

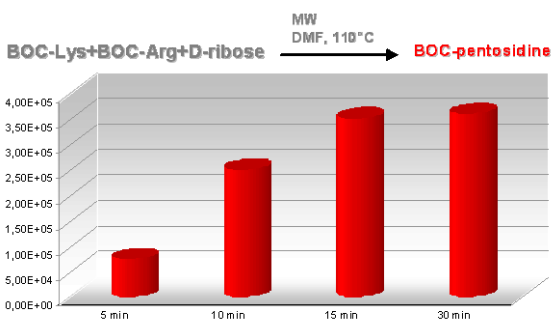


Figure 2 Analysis of the areas obtained by LC-ESI MS4 chromatograms of 579 m/z ion of BOC-pentosidine measured after 5, 10 and 15 minutes.

On the contrary using other solvents like acetonitrile or PBS buffer, the reaction does not proceeds even under MW in closed vessel at 110°C. In aqueous solvents though closer to biological conditions, it is clearly inhibited the dehydration step.

Liquid chromatography-mass spectrometry

Reactions were monitored by HPLC-UV-HRMS using an hybrid MS analyzer (LTQ-Orbitrap, high mass resolution m/Δm=10,000). Chromatographic separation of BOC derivatives was carried out on a RP-C18 column with aqueous trifluoroacetic acid/acetonitrile as eluent, while unprotected products with aqueous heptafluorobutanoic acid/acetonitrile. Each species was followed by MS^n analysis acquiring $[MH]^+ \rightarrow [MH-100]^+ \rightarrow$ transitions (BOC derivative fragmentation gives a characteristic neutral loss of 100 Da).

The reaction kinetics and products structure were determined by high resolution multistage mass spectrometry (Figure 2)

Di-*N*-Boc-pentosidine was analyzed using a MS^4 method, following the successive fragmentations: 579 m/z → 479 m/z → 379 m/z → typical pentosidine spectrum (Figure 3).

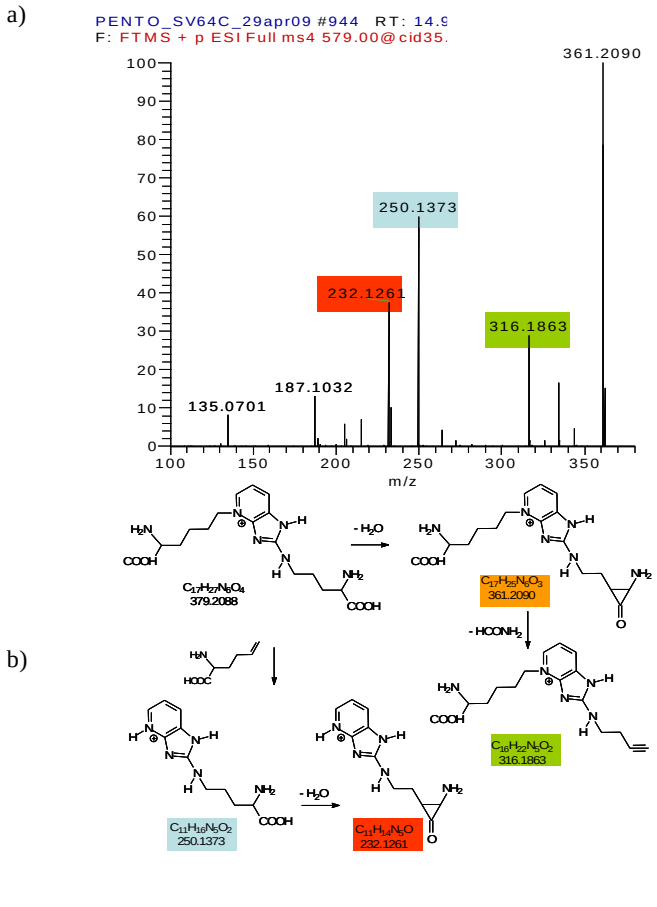


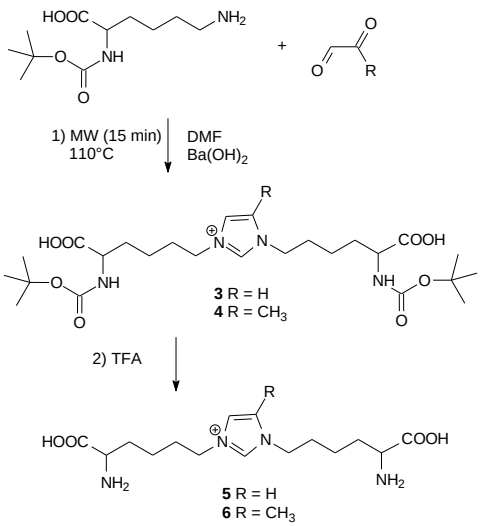
Figure 3 BOC-pentosidine LC-ESI MS^4 (579 *m/z*) spectrum (a) and main fragmentation pathway (b).

Apart from MH-18 (water) and MH-17 (ammonia) at 361 and 362 m/z, the most abundant ion characterizing pentosidine

spectrum is represented by the signal at 250 m/z, originated by the elimination of the 2-amino-5-hexenoic acid deriving from lysine moiety. Pure free pentosidine was obtained by semi-preparative HPLC-MS using heptafluorobutanoic acid as eluent.

It is worthy of mention that in the syntheses reported in literature, the purification of pentosidine requires multiple steps including desalting, preconcentration and finally semi-preparative purification. Our procedure permits to inject directly the reaction mixture after solvent evaporation obtaining the pure product in few minutes. Our protocol might pave the road of high throughput applications.

This promising method was succesfully applied to the synthesis of several AGE compounds, namely MOLD and GOLD, starting from suitable protected aminoacids and carbonyl substrates.



Scheme 2 Synthesis of GOLD (**5**) and MOLD (**6**)

Table 1 Synthesis of BOC- MOLD and BOC-GOLD: conventional methods (Conv) *vs* MW-assisted reactions.

Entry	Method	Solvent	Time	Temp (°C)	Yield (%)
MOLD	Conv[a]	DMF	6 h	110	8
MOLD	MW[c]	DMF	15 min	110	17
GOLD	Conv[c]	DMF	6h	110	10
GOLD	MW[c]	DMF	15min	110	16

[a] Conv: heating in oil bath under magnetic stirring;
[b] MW: irradiation 300W x 5 min repeated 3 times, with interposed pauses of 3 min.

Di-N-*BOC*-GOLD and Di-N-*BOC*-MOLD were monitored using a MS^4 method analogously to Di-N-*BOC*-pentosidine. The successive fragmentations were: 527 *m/z* → 427 *m/z* → 327 *m/z* → GOLD spectrum and 541 *m/z* → 441 *m/z* → 341 *m/z* → GOLD spectrum. The first two MH-100 steps are due again to the loss of the *BOC* originated neutral fragments. Also GOLD and MOLD derivatives eliminate ammonia and carbon monoxide or, alternatively, the deaminated-unsaturated lysine chain. GOLD and MOLD spectra show the common product ion 130 *m/z* after imidazole (methylimidazole) elimination. FTMS[4] Di-N-*BOC*-GOLD spectrum is shown in figure 3. FTMS[4] Di-N-*BOC*-MOLD spectrum is shown in figure 4.

Although work is still in progress, our results showed that reactions were fast giving relatively clean products. Their full characterization will be soon completed. Figure 3, shows the chromatograms of the crude reaction mixtures and AGE products yields (approximated values calculated by HPLC-HRMS peak areas).

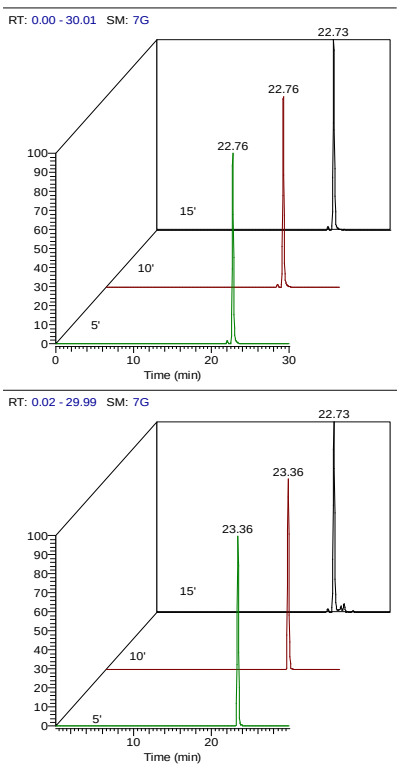


Figure 4. LC-ESI MS^4 chromatograms of 527 and 541*m/z* ions of BOC-GOLD and BOC-MOLD after 5, 10 and 15 minutes.

As shown in figures 2 and 4, for protected pentosidine reaction progress reaches a plateau in 15 min, while for BOC-GOLD/MOLD reaction could be considered completed in 5 min.

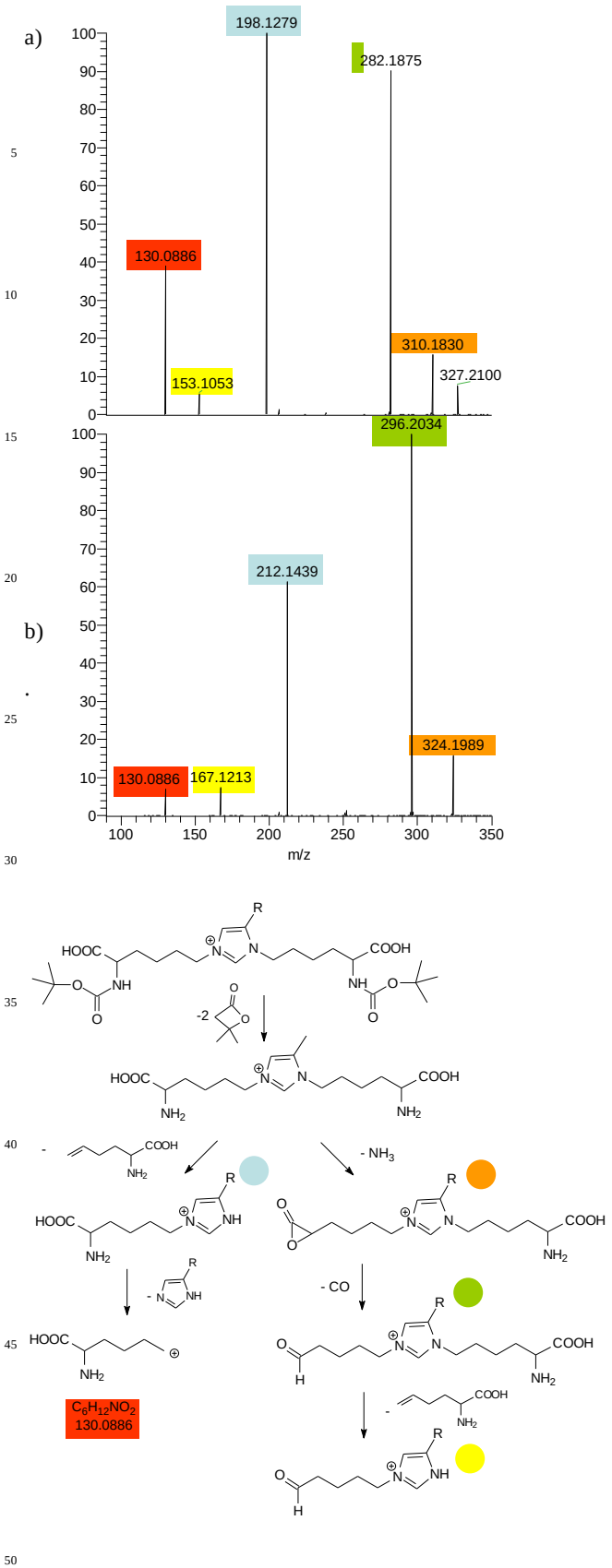


Figure 5 BOC-GOLD and BOC-MOLD LC-ESI MS^4 (527 and 541 *m/z*) spectrum (a) and main fragmentation pathways (b).

Conclusions

In conclusion, we have developed a simple, fast and efficient method for the preparation of pentosidine and other AGE derivatives under MW irradiation. This method has several advantages such as ease of manipulation, short reaction and purification times. The versatility and reproducibility of this methodology shall pave the road to the synthesis of other AGEs. The availability of pentosidine and other AGE biomarkers in gram scale, at a reasonable price, surely will blossom into a plethora of new biological and medical investigations.

Experimental

Methods and materials.

Solvents and starting materials were of the highest commercially available purity (Sigma-Aldrich, Milan, Italy) and used as received. MW promoted reactions were carried out in a MILSTONE ATC-FO MicroSYNTH oven. Reactions were monitored by HPLC-PDA UV-HRMS using an hybrid MS analyzer (LTQ-Orbitrap). Semi preparative HPLC–MS were carried out on a Waters FractionLynx autopurification system equipped with Waters 2996 diode array and Micromass ZQ (ESCI ionization mode) detectors.

Synthetic procedure

Synthesis of 2-ammonio-6-{2-[4-ammonio-5-oxido-5-oxopentyl)-amino]-3H-imidazo[4,5-b]pyridine-4-ium-4-yl}hexanoate (pentosidine)

In a 50 mL three-nacked round-bottomed flask, *BOC*-L-lysine (1 g, 4.06 mmol), *BOC*-L arginine (1g, 3.64 mmol) and D-ribose (1.25 g, 8.33 mmol) were dissolved in DMF (150 mL). $Ba(OH)_2$ (1.5 g, 8.77 mmol) was added to the mixture. The reaction was irradiated by MW at 110°C (3 times for 5 min, 300 W, with interposed pauses of 3 min) and monitored by LC-MS spectrometry. The solvent was evaporated under vacuum to afford a residue that was purified by preparative HPLC-MS. To remove *BOC* groups, protected pentosidine was incubated at room temperature with concentrated TFA for 2 hours. The hydrolyzed sample was purified by preparative HPLC-MS spectrometry. NMR spectra were in accordance to that reported in literature.

Synthesis of 1,3-bis-(5-Boc-amino -carboxypentyl)-3H-imidazolium (Boc-GOLD)

BOC-L-lysine (0.2 g, 0.8 mmol), and glyoxal (0.13g, 2.4 mmol) were dissolved in DMF (20 mL) In a 50 mL three-nacked round-bottomed flask. $Ba(OH)_2$ (0.15 g, 0.9 mmol) was added to the mixture. The reaction was irradiated by MW at 110°C (3 times for 5 min, 300 W, with interposed pauses of 3 min) and monitored by LC-MS spectrometry. HRMS $C_{25}H_{43}N_4O_8$ calc. 527.3075 found 527.3056.

Synthesis of 1,3-bis-(5-Boc-amino-5-carboxypentyl-4-methyl-3H-imidazolium (Di-Boc-MOLD)

BOC-L-lysine (0.2 g, 0.8 mmol), and methylglyoxale (0.18 g, 2.4 mmol) were dissolved in DMF (20 mL) In a 50 mL three-nacked round-bottomed flask. $Ba(OH)_2$ (0.15 g, 0.9 mmol) was added to the mixture. The reaction was irradiated by MW at 110°C (3 times for 5 min, 300 W, with interposed pauses of 3 min) and monitored by LC-MS spectrometry. HRMS $C_{26}H_{45}N_4O_8$ calc. 541.3232 found 541.3214.

Analytical procedures

The chromatographic separations monitored using an MS analyzer were run on a C18 column Phenomenex (Torrance, CA, USA) Synergi, 150 × 2.0 mm using an Ultimate 3000 HPLC instrument (Dionex, Milan, Italy). Injection volume was 20 µL and flow rate 200 µL/min. Gradient mobile phase composition was adopted: 0/100 to 30/70 in 25 min. acetonitrile/trifluoro acetic acid 0.1% or heptafluorobutanoic acid 5 mM for deprotected pentosidine.

Mass Spectrometry

A LTQ Orbitrap mass spectrometer (ThermoFisher, Rodano, Italy) equipped with an atmospheric pressure interface and an ESI ion source was used. The LC column effluent was delivered into the ion source using nitrogen as sheath and auxiliary gas. The source voltage was set to 4.1 kV. The heated capillary temperature was maintained at 275°C. The main tuning parameters adopted for ESI source were: capillary voltage 13.00 V, tube lens 70 V. Mass accuracy of recorded ions (vs calculated) was ± 15 ppm (without internal calibration).

Semipreparative purifications

All chromatographic separation were made on Waters Fraction Link autopurification system equipped with Waters 2996 diode array detector and Waters Micromass ZQ ESCI MS detector on a XTerra RP C18, 19/50 5 □m column.

BOC-protected AGEs purification. Typical injection volume was 500 µL and flow rate 20 mL/min. Gradient mobile phase composition: 75/15 to 0/100 in 11 min. % water TFA 0.1% /methanolic TFA 0.1%. ESI source conditions: capillary voltage 2.00 KV, cone voltage 26.00V.

Unprotected AGEs purification. Injection volume 300 µL and flow rate 20 mL/min. Gradient mobile phase composition: 75/15 to 60/40 in 7 min. water and HFBA 5mM /methanol.

ESI source conditions: capillary voltage 2.00 KV, cone voltage 26.00V.

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