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

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Received 12th January 2010, Accepted 15th March 2010
First published as an Advance Article on the web 25th March 2010
DOI: 10.1039/c000789g

The application of microwaves as an efficient form of volumetric heating to promote organic reactions was recognized in the mid-1980 s. It has a much longer history in the food research and industry where microwave irradiation was studied in depth to optimize food browning and the development of desirable flavours from Maillard reactions. The microwave-promoted Maillard reaction is a challenging synthetic method to generate molecular diversity in a straightforward way. In this paper we present a new rapid and efficient one-pot procedure for the preparation of pentosidine and other AGEs under microwave irradiation.

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 the Maillard reaction.[1]

Although the pioneer investigations on Maillard reactions under microwave (MW) heating[2] go back about 30 years[3] surprisingly the interesting findings in food research,[4] so far have not been fully exploited in organic synthesis. The Maillard reaction, under controlled experimental conditions, is a straightforward route for the preparation of a vast number of complex compounds that would, otherwise, have required time-consuming multistep synthetic protocols. A broad spectrum of heterocyclic, aromatic, carbocyclic, and bicyclic flavor active compounds are known to be formed during Maillard reactions.[5] Yaylayan and associates introduced for the Maillard reaction the definition of "combinatorial flavor chemistry".[6] They also investigated the feasibility of simultaneous MW-assisted synthesis and the extraction of selected Maillard reaction products from sugar-amino acid mixtures, by irradiating at atmospheric pressure.[7] Comparitive studies between MW and conductive heating in food processing, showed several differences in the composition of Maillard products.

Besides food preparation,[8] this reaction occurs in biological systems and in several diseases. The preponderance of evidence gathered over the past decades suggests 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 signalling by peptide hormones, activation of AGE-specific receptors, crosslinking of structural proteins, impairment of protein recycling *etc.* The etiopathology of chronic diseases such as diabetes, neuropathy, arteriosclerosis and neurodegenerative diseases (Alzheimer's and Parkinson's diseases and amyotrophic lateral sclerosis) has been related to the products of the Maillard reaction.[9] A number of AGEs have been found in physiological systems and have different biological functions: some are protein cross-linkers (pentosidine, MOLD and GOLD, Fig. 1), others are recognition factors for specific AGE-binding cell-surface receptors (CML, methyl glyoxal-derived hydroimidazolone) and markers or risk predictors of diseases processes (GLAP).[10] 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 cross linked by reaction with carbonyls. In 1989 Monnier was the first, to isolate pentosidine from *dura mater* and characterize the structure also obtained by synthesis.[11] So far, pentosidine has been obtained in a very low overall yield (0.83%) by mixing ribose, lysine and arginine for 6 days at 65 °C in PBS (pH 9).[12] Yokokawa *et al.*[13] described the total synthesis of pentosidine (1 g) which requires 18–19 steps and an enormous effort to purify it.

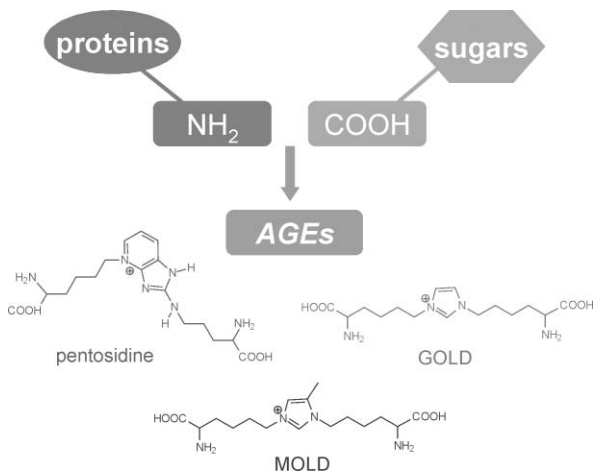

Fig. 1 Structures of some AGE derivatives.

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† Electronic supplementary information (ESI) available: Additional spectra. See DOI: 10.1039/c000789g

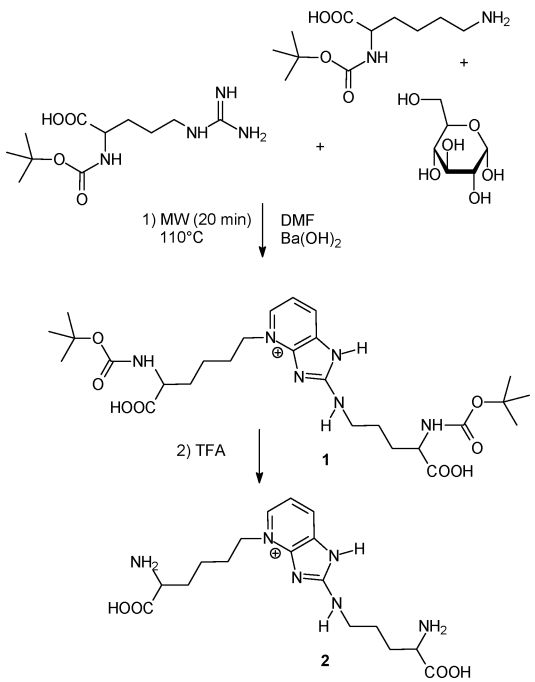
‡ This paper is part of an *Organic & Biomolecular Chemistry* web theme issue on enabling technologies for organic synthesis.

In this paper, we report a fast and reliable one-pot MW-assisted synthesis of pentosidine, MOLD[9] and GOLD[9] that affords relatively clean products in much better yields compared to previous procedures. The reaction progress was followed by HPLC-HRMS, which allows the monitoring of reagents consumption and the products formation on the basis of their accurate mass.

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 such 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). When using unmodified amino acids for the synthesis, we did not obtain pentosidine. This fact can be explained by the high reactivity of α-amino groups, which can only condense with ribose in very low quantities. This problem is solved by blocking α-amino groups with butyloxycarbonyl (BOC) residues.



Scheme 1 Synthesis of pentosidine (2).

Thus, with the aim of synthesizing 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). Few trials in a monomode reactor (Discover, CEM) gave substantially the same results. Table 1 summarize reaction conditions and yields of free pentosidine.

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 (Fig. 2).

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

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

[a] Conv.: conductive heating (oil bath) under magnetic stirring. [b] Published procedure (ref. 9). [c] MW: irradiation 300 W × 5 min repeated 3 times, with interposed pauses of 3 min. [d] Not formed.

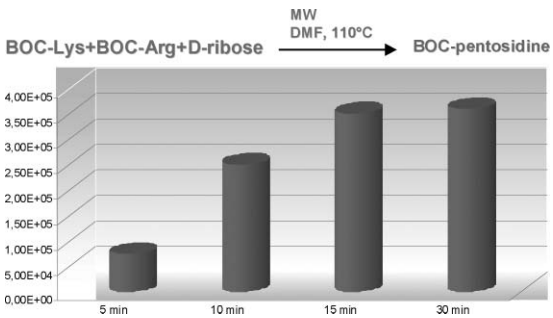


Fig. 2 Analysis of the areas obtained by LC-ESI MS4 chromatograms of 579 m/z ion of BOC-pentosidine measured after 5, 10, 15 and 30 min.

Although the formation of the Schiff 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 lies close to 9. Temperature is a crucial parameter in the Maillard reaction;[12] we found that the best reaction rates were achieved at 110 °C, while only 10 degrees Celsius more led to side reactions and partial degradation. Besides pH and temperature, a major role is played by the solvent. As shown in Table 1, the 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 min compared to 6 h under conductive heating.

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

Liquid chromatography-mass spectrometry

Reactions were monitored by HPLC-UV-HRMS using a hybrid MS analyzer (LTQ-Orbitrap, high mass resolution m/Δm = 10,000). Chromatographic separation of BOC derivatives was carried out on an RP-C18 column with aqueous trifluoroacetic acid (TFA)/acetonitrile as eluent, while unprotected products with aqueous heptafluorobutanoic acid (HFBA)/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 product structure were determined by high resolution multistage mass spectrometry (Fig. 2) Di-*N*-Boc-pentosidine was analyzed using a MS^4 method, following the

successive fragmentations: 579 $m/z \rightarrow$ 479 $m/z \rightarrow$ 379 $m/z \rightarrow$ typical pentosidine spectrum (Fig. 3).

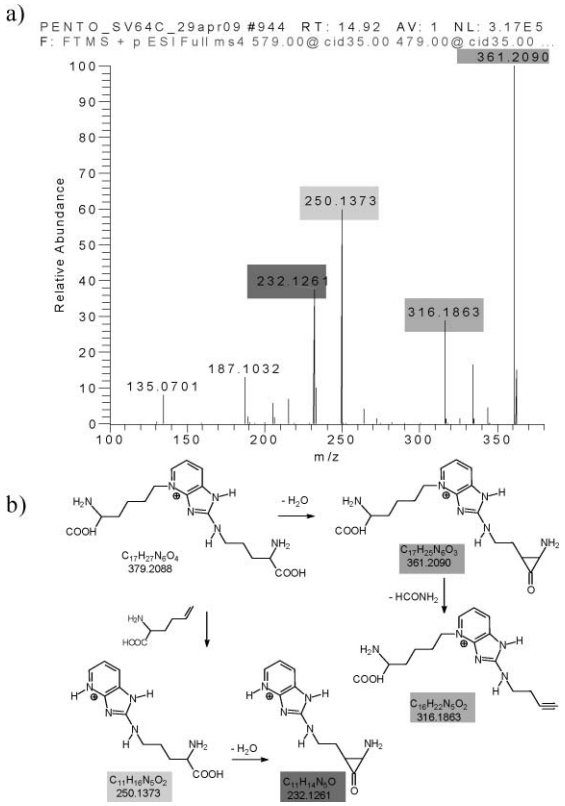


Fig. 3 BOC-pentosidine LC-ESI MS^4 (579 m/z) spectrum (a) and main fragmentation pathway (b).

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 aqueous HFBA)/acetonitrile 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 the direct injection of the reaction mixture after solvent evaporation obtaining the pure product in a few minutes. Our protocol might pave the road of high throughput applications.

MOLD and GOLD have been well described in literature[14–18] and they were obtained with good yields. But our method offers a good alternative, being biomimetic and fast.

Glyoxal-lysine dimer is formed by the reaction of glyoxal and two molecules of BOC-protected lysine. Methylglyoxal reacts analogously to give MOLD (Scheme 2).

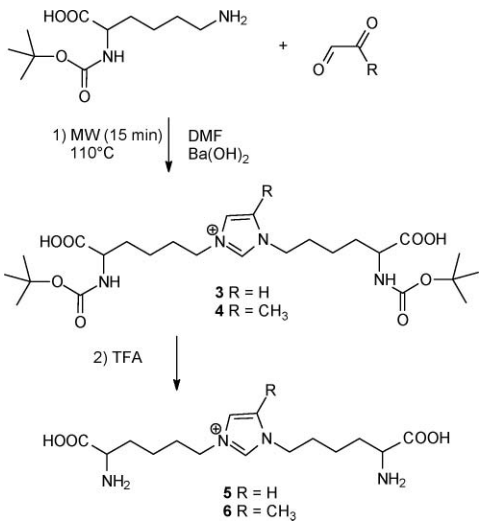
Table 2 summarizes reaction conditions and yields of BOC-protected MOLD and GOLD.

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 $m/z \rightarrow$ 427 $m/z \rightarrow$ 327 $m/z \rightarrow$ GOLD spectrum and 541 $m/z \rightarrow$ 441 $m/z \rightarrow$ 341 $m/z \rightarrow$ GOLD spectrum. The first two MH-100 steps are again due to the loss of the *BOC* originated neutral fragments. Furthermore 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 Fig. 3. FTMS^4 Di-*N*-*BOC*-MOLD spectrum is shown in Fig. 4.

Table 2 Synthesis of diBOC- MOLD and diBOC-GOLD: conventional methods (Conv) *vs.* MW-assisted reactions

Entry	Method	Solvent	Time	T/°C	Yield (%)
diBOC- MOLD	Conv.[a]	DMF	6 h	110	8
diBOC- MOLD	MW[b]	DMF	15 min	110	17
diBOC- GOLD	Conv.[a]	DMF	6 h	110	10
diBOC- GOLD	MW[b]	DMF	15 min	110	16

[a] Conv.: conductive heating (oil bath) under magnetic stirring. [b] MW: irradiation 300 W × 5 min repeated 3 times, with interposed pauses of 3 min.



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

Our results showed that reactions were fast giving relatively clean products. Fig. 3, shows the chromatograms of the crude reaction mixtures and AGE product yields (values calculated by HPLC-HRMS peak areas).

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

Conclusions

In conclusion, we have developed a simple, fast and efficient method for the one-pot synthesis 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 should pave the way to the synthesis of other AGEs. The availability of pentosidine and other AGE biomarkers in gram scale, at a reasonable price, will surely lead to a plethora of new biological and medical investigations.

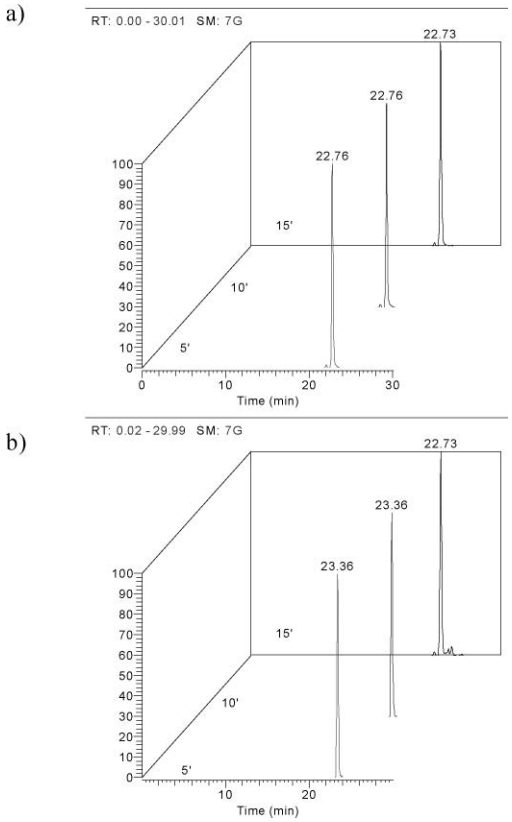


Fig. 4 LC-ESI MS^4 chromatograms of 527 and 541m/z ions of diBOC–GOLD (a) and diBOC-MOLD (b) after 5, 10 and 15 min.

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 MicroSYNTH oven (Milestone, Bergamo - Italy). Reactions were monitored by HPLC-PDA UV-HR MS using a 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. NMR spectra were recorded on a Bruker Avance 300 operating at 7 T.

Synthetic procedure

Synthesis of Pentosidine. In a 50 mL three-necked round-bottomed flask, *BOC*-L-lysine (1 g, 4.06 mmol), BOC-L-arginine (1 g, 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 h. The hydrolyzed sample was purified by preparative HPLC-MS spectrometry. NMR spectra were in accordance to those reported in literature.[10]

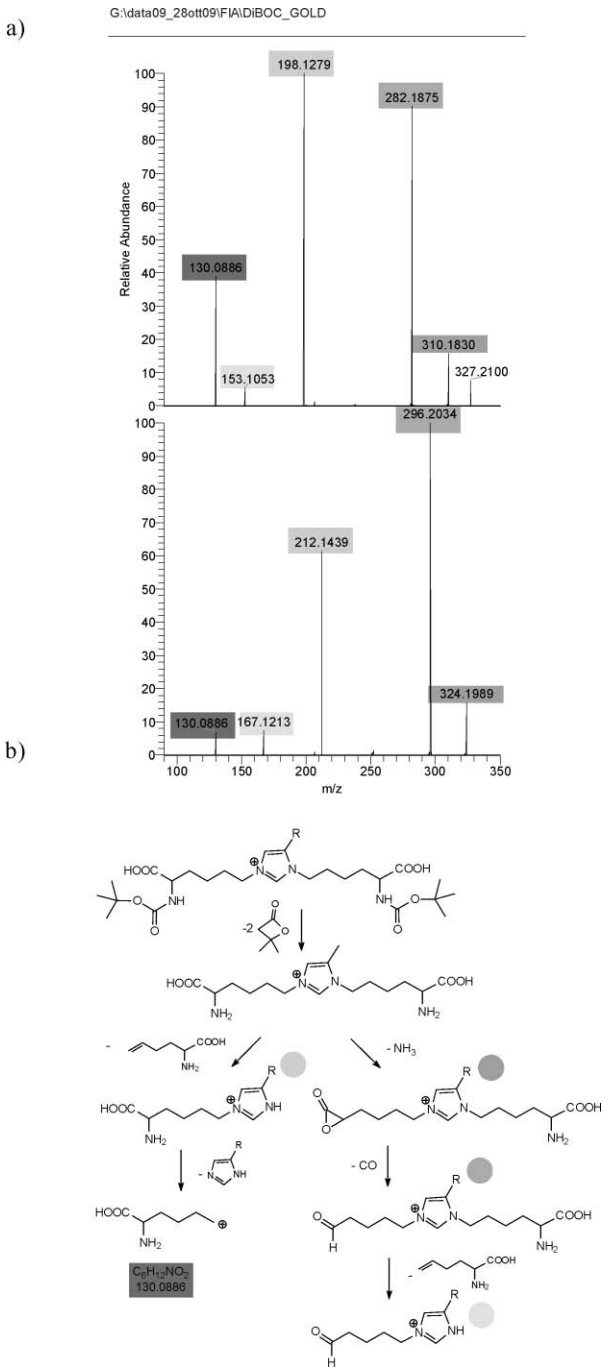


Fig. 5 BOC-GOLD and BOC-MOLD LC-ESI MS^4 (527 and 541 m/z) spectrum (a) and main fragmentation pathways (b).

Synthesis of diBOC-GOLD. BOC-L-lysine (0.2 g, 0.8 mmol), and glyoxal (0.13 g, 2.4 mmol) were dissolved in DMF (20 mL) In a 50 mL three-necked 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. The solvent was evaporated under vacuum to afford a residue that was purified by preparative HPLC-MS.

Synthesis of diBOC-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-necked 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. The solvent was evaporated under vacuum to afford a residue that was purified by preparative HPLC-MS.

Synthesis of GOLD and MOLD. The cleavage of diBOC protected compounds was achieved after two hours stirring at room temperature with concentrated TFA. The hydrolyzed samples were further purified by preparative HPLC-MS. HRMS and NMR spectra were in accordance to those reported in literature.[14–18]

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^{-1}. Gradient mobile phase composition was adopted: 0/100 to 30/70 in 25 min. TFA/acetonitrile 0.1% or HFBA 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 separations were made on a Waters Fraction Link autopurification system equipped with a Waters 2996 diode array detector and a Waters Micromass ZQ ESCI MS detector on a XTerra RP C18, 19/50 5 µm column.

BOC-protected AGEs purification

Typical injection volume was 500 µL and flow rate 20 mL min^{-1}. Gradient mobile phase composition: from 75/15 to 0/100 in 11 min. of TFA 0.1% in water/TFA 0.1% in methanol. 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^{-1}. Gradient mobile phase composition: from 75/15 to 60/40 in 7 min. of HFBA 5mM in water–methanol. ESI source conditions: capillary voltage 2.00 KV, cone voltage 26.00V.

Acknowledgements

Financial support from the Università di Torino and MIUR (PRIN, prot. 2008M3Y5WX) is gratefully acknowledged.

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