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N-Terminal 2,3-diaminopropionic acid (Dap) peptides as efficient methylglyoxal scavengers to inhibit advanced glycation endproduct (AGE) formation

N. André Sasaki [a,b,*], Maria Concepcion Garcia-Alvarez [a], Qian Wang [a], Ludmila Ermolenko [a], Gisèle Franck [a], Naïma Nhiri [a], Marie-Thérèse Martin [a], Nicolas Audic [b], Pierre Potier [✠]

[a] Institut de Chimie des Substances Naturelles, CNRS, 91198 Gif-sur-Yvette Cedex, France
[b] Pharmamens, Laboratoire de Chimie Thérapeutique, Faculté de Pharmacie, 80037 Amiens Cedex 1, France

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This paper is dedicated to the memory of Professor Pierre Potier, an outstanding international figure in medicinal chemistry

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ABSTRACT

2,3-Diaminopropionic acid (Dap) and N-terminal Dap peptides have been found to inhibit in vitro protein-modifications by methylglyoxal (MG), one of the highly reactive α-dicarbonyl compounds. MG scavenging potency of the newly synthesized N-terminal Dap peptides is demonstrated by RP-HPLC, SDS–PAGE and non-denaturing PAGE analysis, assays for enzymatic activity and cell viability study and was compared with that of known AGE inhibitors, such as aminoguanidine, pyridoxamine, metformin and carnosine. Two addition products of MG and L-Dap-L-Leu are separated by HPLC and their chemical structures are characterized by ^{1}H and ^{13}C NMR spectroscopy to indicate that both of them are pyrazines derived from 2 molecules of MG and 1 molecule of L-Dap-L-Leu. Mutagenic activities of L-Dap-L-Leu and L-Dap-L-Val and their metabolites according to the Ames assay are found to be negative.

1. Introduction

Non-enzymatic glycation of proteins, known as the Maillard reaction, is initiated by the condensation of amino groups present in proteins with compounds containing reactive carbonyl groups. Early-stage of this reaction leads to the formation of the early glycation adduct, fructosyl-lysine, via Schiff base and concomitant Amadori rearrangement. Later stage Maillard reaction is the formation of advanced glycation endproducts (AGEs) by the reaction of amino, guanido and thiol groups of proteins with α-dicarbonyl compounds derived from the degradation of glycated proteins. The most notable highly reactive α-dicarbonyls are glyoxal (GO), methylglyoxal (MG) and 3-deoxyglucosone (3-DG) that are originated from non-enzymatic dephosphorylation of triosedihydroxyacetone phosphate and glyceraldehyde-3-phosphate as well as the lipid peroxidation, the glucose auto-oxidation and degradation of glycated proteins.

These α-dicarbonyl compounds lead to the non-enzymatic formation of toxic and immunogenic AGEs that are cross-linking and non-cross-linking of proteins. Many of these cross-links include heterocyclic structures such as MOLD (methylglyoxal lysine dimer), GOLD (glyoxal lysine dimer), GODIC (glyoxal lysine arginine dimer), MODIC (methylglyoxal lysine arginine dimer), pentosidine and glucosepane. Examples of non-cross-linking AGEs are G-H1, MG-H1, 3DG-H1 (hydroimidazolone adducts of arginine with GO, MG and 3-DG, respectively), CML (N^{ε}-carboxymethyl lysine), CEL (N^{ε}-carboxyethyl lysine) and Argpyrimidine.[1–4] Glycation reactions take place endogenously in all tissues and body fluids under physiological conditions.

The consequence of AGE formation is the reticulation of proteins. This phenomenon has been observed in long-lived proteins such as tissue collagen, lens crystallin, fibronectin, tubulin, laminin, actin, hemoglobin, albumin and lipids associated on low-density lipoproteins. Protein modification in the form of AGEs progressively increases with aging and it is considered to contribute to the modification of normal tissues. The formation and accumulation of increased AGEs has been implicated in the development of cataracts, uremia, atherosclerosis, Alzheimer's disease, Parkinson's disease and above all, clinical complications of

* Corresponding author. Tel.: +33 1 69823101; fax: +33 1 69077247.
E-mail address: andre.sasaki@icsn.cnrs-gif.fr (N.A. Sasaki).
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type 2 diabetes, such as micro- and macroangiopathy, retinopathy, nephropathy, neuropathy and ulcer.[5–13] It is to be noted that the concentration of MG was found to be 2–6-fold higher in patients with both insulin-dependent and non-dependent diabetes.[14] Normally, the glutathione-dependent glyoxylase system (glyoxalase I and glyoxalase II) catalyzes the detoxification of the α-dicarbonyls by transforming them into D-lactate, glycolate and acetol.[15,16] However, dysfunction of this metabolism of detoxification stimulates the accumulation of the AGEs in the body leading to protein damage during general aging and several pathological conditions.[17]

In view of the wide occurrence and their deleterious consequences of AGEs, numerous agents have been reported as potent scavengers of the reactive α-dicarbonyls. These are aminoguanidine (Pimagedine®), pyridoxamine (Pyridorin®), L-arginine, metformin, carnosine, tenilsetam, thiamine, OPB-9195, D-penicillamine, and LR-90.[18–28] Although aminoguanidine has shown to be an excellent anti-AGE drug, it has been abandoned due to adverse side effects that were observed in phase three clinical trials in patients with diabetes. On the other hand, thiamine and pyridoxamine are considered as highly promising AGE inhibitors. Despite interesting scavenging effects, studies on the chemical mechanisms of many of these scavengers with respect to the reactive α-dicarbonyls have yet to be confirmed except for aminoguanidine, metformin, and D-penicillamine. Taking into account of the 1,2-vicinal carbonyl groups in the molecule, it seems rational to postulate 1,2-diamino compounds for trapping α-dicarbonyls by forming six-membered ring piperazine and/or pyrazine derivatives. Validity of this approach has been exploited in the use of *o*-phenylenediamine or 1,2-diamino-4,5-dimethoxybenzene for the quantification of plasmic dicarbonyls.[29,30] However, surprisingly, no work has been reported on the therapeutic use of 1,2-diamino systems as α-dicarbonyl scavengers presumably due to toxic properties of the aromatic diamines. We deemed that it would be worthy to evaluate therapeutic potential of 1,2-vicinal diaminoalkyl compounds as sequestrating agents of the highly reactive α-dicarbonyls. To this end, introduction of an acidic functional group such as CO_2H or SO_3H into the 1,2-diamino containing molecule is considered a primary requirement for facilitating the ready excretion of the resultant condensation products by kidney. In this context, dipeptides with 2,3-diaminopropionic acid (Dap) moiety in its N-terminal appears to be an interesting model since side chain functional group of the C-terminal amino acid can serve as a site for enhancing bioavailability or therapeutic effects of the molecule. We report here our studies on Dap and N-terminal Dap dipeptides to demonstrate their potential as reactive α-dicarbonyl compound scavengers.

2. Results and discussion

2.1. Synthesis

N-Terminal Dap dipeptides were prepared by coupling *N*,*N*′-di-Boc-2,3-Dap-OH[31] with the methyl ester of amino acids in the presence of EDC and HOBt. After alkaline hydrolysis of the methyl ester with LiOH, *N*,*N*′-di-Boc groups were removed by the treatment with 3 M HCl–dioxane (or THF) to obtain dipeptides as dihydrochloride salts (Scheme 1). While yields of the coupling reaction were in the range of 80–98%, those of the following two-step reaction were almost quantitative.

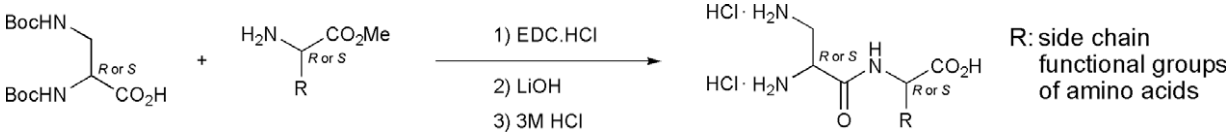
2.2. Dicarbonyl-scavenging activity of Dap and N-terminal Dap dipeptides

2.2.1. Evaluation by RP-HPLC

On the assumption that the nucleophilic trapping of α-dicarbonyl compounds by a scavenger is much more rapid than that of the basic functional groups of polypeptide or protein, monitoring the quantity or quality of the unmodified polypeptide or protein can serve as a means to evaluate the sequestering capacity of a tested scavenger.

Efficacy of each scavenger in this study was first assessed on RP-HPLC by comparative measurement of the remaining intact insulin vis-à-vis the control when it is incubated with MG in phosphate buffer of pH 7.4 at 37 °C for 24 h in the presence of a scavenger compound (1.1 mol equiv with respect to MG). In this work, we have chosen insulin (51 amino acids with 1 Arg and 1 Lys) as model polypeptide because of its easy access and well documented HPLC studies thereupon.[32] Krause and his colleagues have reported that the retention times of native bovine insulin and those of GO- and MG-modified insulin differ from each other on HPLC analysis.[33] As a scavenger, we have arbitrarily tested D-Dap-L-Leu. When incubated with MG alone, insulin peak on HPLC chromatogram diminishes in proportion to the increasing amount of MG in concentration dependent manner until its complete disappearance at 100 equiv level as shown in Figure 1a. However, under the same conditions, incubation of insulin with MG in the presence of D-Dap-L-Leu (dipeptide/MG = 1.1 equiv) significantly reduces this trend (Fig. 1b). In case insulin is incubated with 10 equiv of MG, it takes about 7 days to observe complete degradation (Fig. 2b). In order to shorten the incubation time to 24 h and thus facilitate the analysis, 100 and 110 M equiv of MG and MG-scavenger, respectively, with respect to insulin, were used throughout this study.

Quantification of the protected insulin is performed by comparing the insulin incubated with MG in the presence of a scavenger with that of the control. When insulin is incubated in 10 mM phosphate buffer of pH 7.4 (containing 0.1 M NaCl) with MG in the presence of D-Dap-L-Leu at 37 °C and pH 7.4 for 24 h, 83% of insulin is found to be protected from the MG-modification (Fig. 1b). Among the products formed under these reaction conditions, the one with retention time of 10 min in Figure 1b corresponds to Adduct I or II formed by 2 mol of MG and 1 mol of D-Dap-L-Leu (vide post). From co-injection and mass spectrometry analysis, we confirmed that the peak with retention time of 14.8 min in Figure 1b belongs to that of unmodified human insulin. Then, we investigated the scavenging potency of the different N-terminal Dap-dipeptides that were prepared for this study. Thus, insulin was incubated in the same conditions as described above. Figure 3 illustrates that N-terminal Dap-dipeptides, such as Dap-Leu, Dap-Nle, Dap-Pro and Dap-Ile possess excellent capacity to prevent the degradation of insulin by MG. For the comparison, efficacy of other known scavengers such as aminoguanidine, pyridoxamine, penicillamine, cysteine, metformin and carnosine was also investigated under the same conditions. Whereas aminoguanidine, penicillamine and cysteine are potent



Scheme 1. Synthesis of Dap dipeptides.

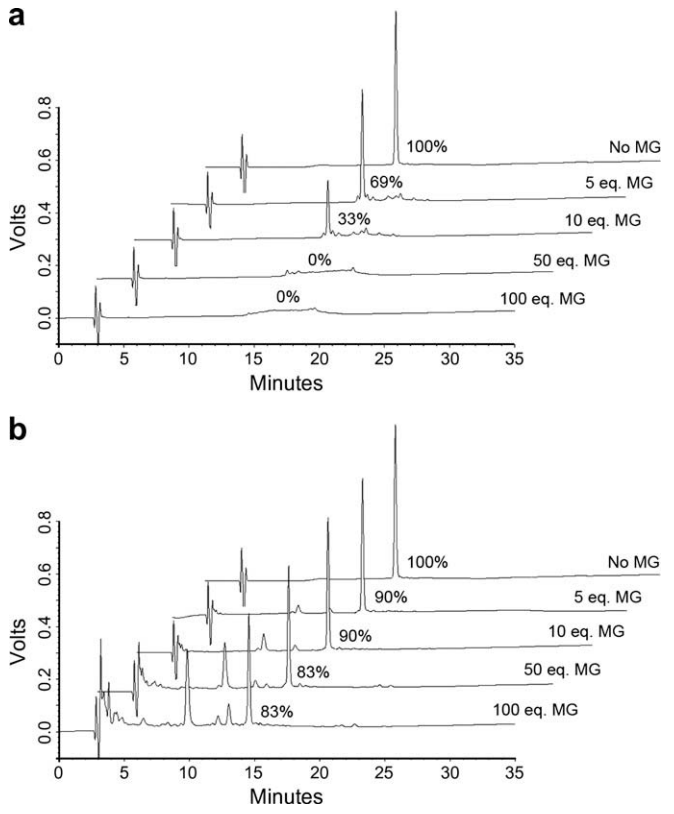


Figure 1. (a) Degradation of insulin incubated with different quantity of MG in 0.1 M NaCl and 10 mM phosphate buffer of pH 7.4 at 37 °C for 24 h. (b) Protection of insulin by D-Dap-L-Leu against different quantity of MG under the same conditions as described above; D-Dap-L-Leu/MG = 1.1. RP-HPLC conditions: Column: Grace-Vydac Protein and Peptides C18 218TP54 (4.6 × 250 mm); injection volume 20 μL of the reaction mixture; flow rate: 1 mL/min; temperature 25 °C; eluent A: H_2O + 0.1% TFA; eluent B: CH_3CN/H_2O 60/40 + 0.1% TFA; linear gradient: 40% B to 100% B in 35 min; detection: PDA (chromatograms extracted at 220 nm).

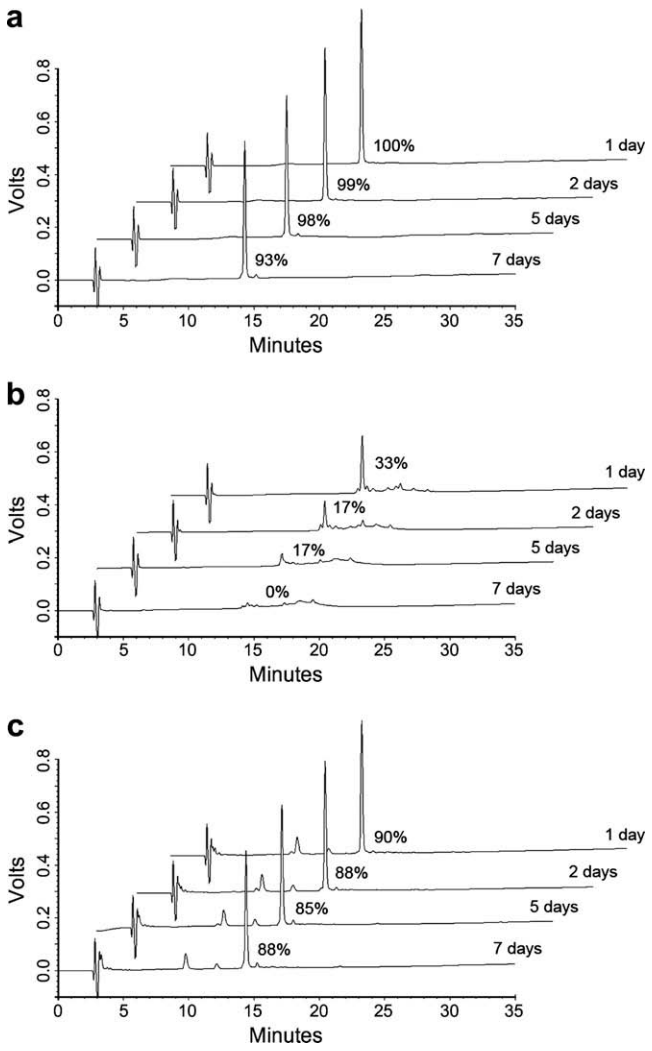


Figure 2. (a) Stability of insulin in a 0.1 M NaCl and 10 mM phosphate buffer of pH 7.4 at 37 °C for 24 h. (b) Degradation of insulin in the presence of 10 equiv of MG under the same conditions as described for Figure 1a. (c) Protection of insulin by D-Dap-L-Leu against 10 equiv of MG (D-Dap-L-Leu/MG = 1.1) under the same conditions as described for Figure 1a. RP-HPLC conditions: the same as those described for Figure 1.

scavengers of MG, the sequestration capacity of pyridoxamine and metformin was shown to be significantly low and that of carnosine was undetectable under the studied conditions. It is noticeable that the α-dicarbonyl scavenging potency of 1,2-diamino moiety of Dap is much higher than that of 1,3-analogue of 2, 4-diaminobutyric acid.

2.2.2. Evaluation by electrophoresis

2.2.2.1. Test on RNase A. We next turned our attention to evaluate the scavenging potency of N-terminal Dap-dipeptides by standard SDS–PAGE electrophoresis. Bovine pancreatic ribonuclease A (RNase A, 124 amino acids with 10 Lys and 4 Arg) has been extensively used as a model protein for cross-linking studies.[3,34] We have selected L-Dap-L-Leu and L-Dap-L-Val as cross-linking inhibitors for the similar study. The ratio of MG to RNase A is 1:1 since incubation with equimolar amount of MG is found enough to observe the formation of RNase A dimer and trimer. Consequently, 1.1:1 mole ratio of scavenger with respect to MG was employed. After 48 h of incubation of RNase A with MG at 37 °C, protein cross-linking is observed. However, addition of L-Dap-L-Leu to the co-incubation inhibits this process. Similarly, L-Dap-L-Val protects the protein against the structural modification by MG (Fig. 4).

2.2.2.2. Test on chicken lysozyme. SDS–PAGE electrophoresis was also used for demonstrating the inhibitory efficacy of tenisletam against lysozyme modification by 3-DG.[24] In our study, it is found that incubation of chicken lysozyme (129 amino acids with 6 Lys and 11 Arg) with MG modifies the protein by forming the dimer and trimer as shown in Figure 5. On the contrary, the same treatment in the presence of L-Dap-L-Leu or L-L-Dap-L-Val inhibits the protein modification. The test was carried out under the same conditions as those for RNase A. It is to be noted that in both cases of RNase A and lysozyme, N-terminal Dap peptides are much more efficient MG scavenger than AG that leads to the formation of a dimer.

2.2.2.3. Test on glyoxalase I. A third electrophoresis study was carried on human glyoxalase I (184 amino acids with 18 Lys and 5 Arg), a key protein for the detoxification system of the reactive α-dicarbonyl compounds.[16] Since the basic functional groups in glyoxalase I are much more abundant than in RNase and in lysozyme, formation of dimer or trimer might not be observed under previous SDS–PAGE conditions when glyoxalase I was incubated with equimolar amount of MG. PAGE of glyoxalase I was then performed under native conditions to demonstrate the change in the electrophoretic mobility of the MG-treated protein. Figure 6 demonstrates that exposure to 10 mM of MG for 24 h markedly increases the mobility of the protein toward the anode, a change consistent with the loss of positive charges from ε-amino and

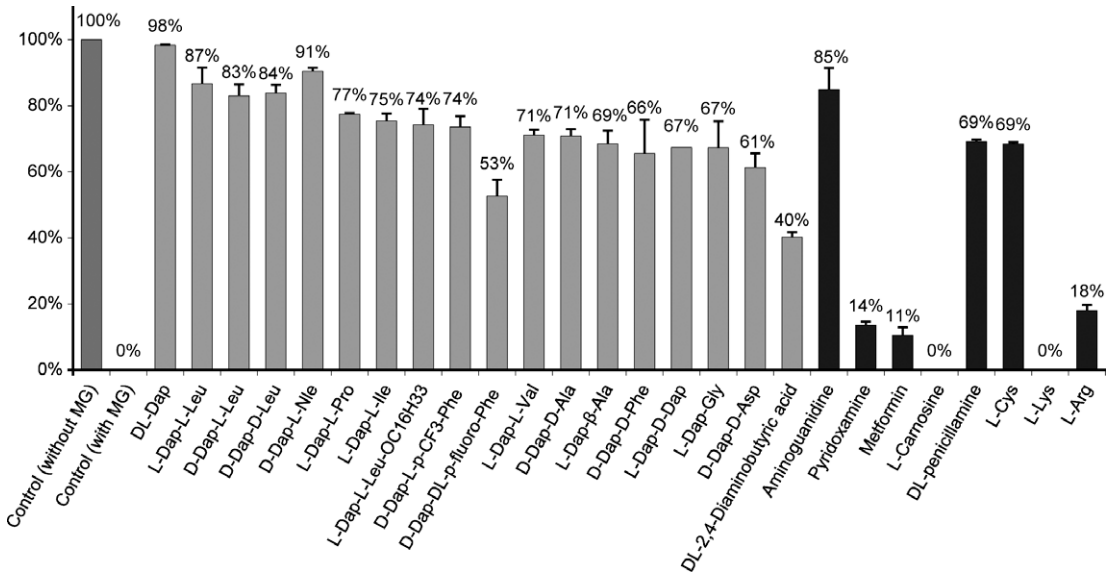


Figure 3. Efficiency of Dap and N-terminal Dap-dipeptides to prevent the insulin modification by MG in comparison with that of known α-dicarbonyl scavengers.

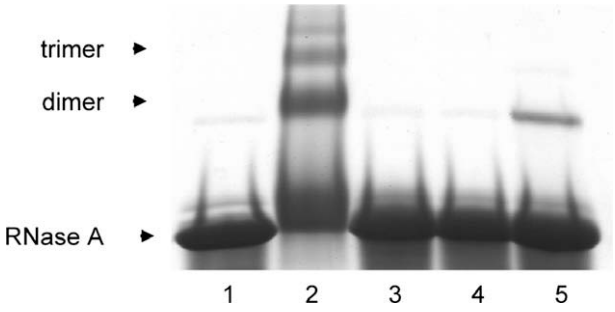


Figure 4. SDS–PAGE of RNase A incubated with MG in the presence and absence of N-terminal Dap-dipeptides at 37 °C and pH 7.4 for 48 h: (1) incubated control RNase A, (2) RNase A + MG (RNase A/MG = 1/1), (3) RNase A + MG + L-Dap-L-Leu (1/1/1.1), (4) RNase A + MG + L-Dap-L-Val (1/1/1.1), (5) RNase A + MG + AG (1/1/1.1).

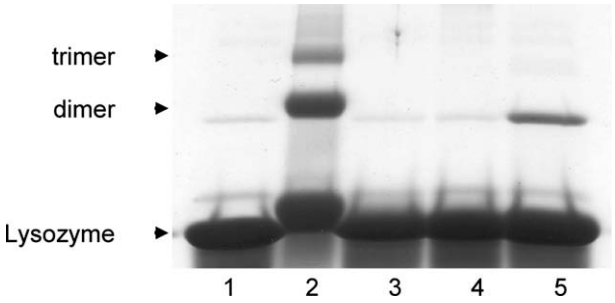


Figure 5. SDS–PAGE of lysozyme incubated with MG in the presence and absence of N-terminal Dap-dipeptides at 37 °C and pH 7.4 for 48 h: (1) incubated control lysozyme, (2) lysozyme + MG (lysozyme/MG = 1/1), (3) lysozyme + MG + L-Dap-L-Leu (1/1/1.1), (4) lysozyme + MG + L-Dap-L-Val (1/1/1.1), 5) lysozyme + MG + AG (1/1/1.1).

guanidine groups and gain of negative charges, presumably via formation of CEL and MG-H1 moieties. Incubation of glyoxalase I with MG in the presence of L-Dap-L-Leu inhibits this process.

2.2.3. Evaluation by enzymatic activity

Measurement of a remaining enzymatic activity of an enzyme incubated with equimolar amount of MG in the presence of a MG-trapping agent is one way to evaluate its scavenging potency.

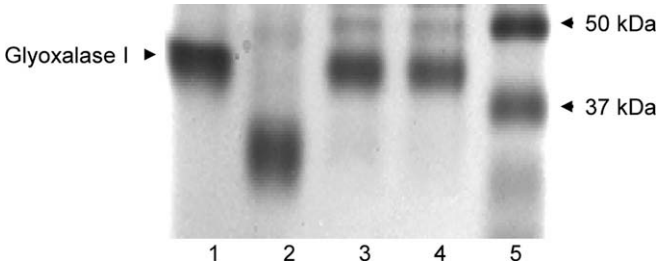


Figure 6. Non-denaturing PAGE of glyoxalase I incubated with MG in the presence and absence of L-Dap-L-Leu at 37 °C and pH 7.4 for 24 h: (1) incubated control glyoxalase I, (2) glyoxalase I + MG (glyoxalase I/MG = 1/1), (3) glyoxalase I + MG + L-Dap-L-Leu (1/1/1.1), (4) glyoxalase I + MG + L-Dap-L-Leu (1/1/2.2), (5) molecular weight standard.

2.2.3.1. Test on RNase A. The enzymatic activity of RNase A was measured according to the method developed by Greiner–Stoeffelle and his colleagues.[35] When incubated with equimolar amount of MG at 37 °C for 24 h, RNase A loses 90% of its enzymatic activity under the studied conditions. On the other hand, it retains its activity from 70% to 94% upon incubation with MG in the presence of Dap peptides. Although AG is also a good inhibitor, the superiority of the dipeptides with alkyl side chains, L-L-Dap-L-Leu, in particular, is significant as shown in Figure 7. To our surprise, Dap alone turned out to be much less effective agent for the conservation of the enzymatic activity.

2.2.3.2. Test on glyoxalase I. The enzymatic activity of glyoxalase I was assayed by measuring the rate of formation of *S*-D-lactoylglutathione from the hemithioacetal that is in turn formed non-enzymatically from MG and reduced glutathione. Thus, the hemithioacetal formed from 1/1 mixture of MG and reduced glutathione, after allowing 15 min for the non-enzymatic reaction, was incubated with glyoxalase I in the absence or presence of L-Dap-L-Leu and the formation of *S*-D-lactoylglutathione was followed by measuring the absorbance at 240 nm. Incubation of human glyoxalase I with equimolar amount of MG modifies the protein. This modification induces a diminution of the enzymatic activity to 50% in comparison with the control. Simultaneous incubation of the enzyme with MG in the presence of the dipeptide L-Dap-L-

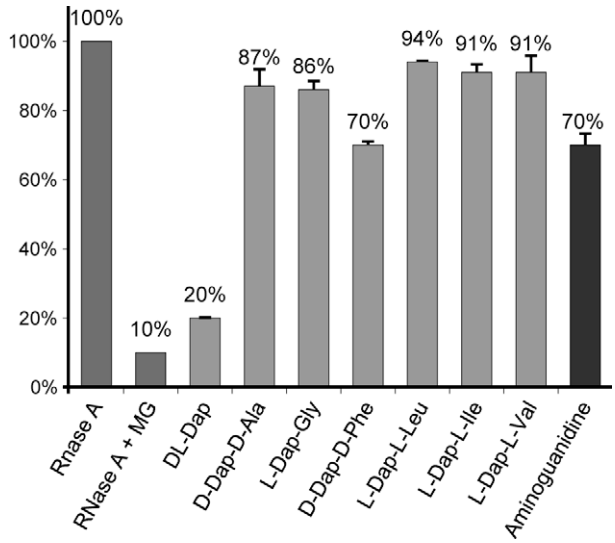


Figure 7. Effect of conserving RNase A enzymatic activity by N-terminal Dap-dipeptides after incubation with MG at 37 °C and pH 7.4 for 48 h.

Leu protects the polypeptide from the structural modifications and preserves the enzymatic activity up to 96%.

2.2.4. Evaluation by cell viability

It is suggested that carbonyl stress-induced loss of mitochondrial integrity could contribute to the cytotoxicity of MG. The MG-induced effects such as ATP depletion and mitochondrial dysfunction could be prevented by pre-incubation of the cells with the carbonyl scavengers.[36]

As evident from Figure 8, MG suppresses cell growth up to about 33% under the conditions of this study. D-Dap alone has turned out to be slightly cytotoxic. The known AGE inhibitors such as aminoguanidine, metformin, pyridoxamine, carnosine and penicillamine can be considered non-toxic. However, the viability of the cell incubated with MG in the presence of these compounds considerably diminishes except in the case of AG and penicillamine, to a lesser extent. On the contrary, the N-terminal Dap-dipeptides with alkyl side chains on the C-terminal amino acids such as L-Dap-L-Ile, L-Dap-L-Leu and L-Dap-L-Nle, and L-Dap-L-Val, in particular, are excellent cell protectors to counter the MG induced effects. We are intrigued to find out that Dap-containing dipeptide ester with 1-hexadecanol is a cytotoxic compound.

2.3. NMR studies on addition products of MG with dipeptide scavenger D-Dap-D-Leu

It is easily conceivable that nucleophilic addition of 1,2-diamines to MG can lead to piperazine and/or pyrazine derivatives. Indeed, the addition reaction of MG and D-Dap-D-Leu under incubation conditions (pH 7.4 at 37 °C) provides many products as depicted in Figure 9. In order to find out the nature of some major addition products, we have separated two of them, Adduct I and Adduct II, by preparative HPLC for the NMR studies. From ^{1}H NMR, ^{13}C NMR (Table 1) and mass spectroscopic analyses, we tentatively postulate the structures of Adduct I and Adduct II as pyrazine derivatives. For Adduct I, it is speculated that the monohydrate form of MG[37] is involved in the formation of piperazine ring system. As for Adduct II, the formation of dihydropyrazine ring by the condensation of MG and 1,2-diamine functional groups of dipeptide followed by the addition–condensation reactions. It is clear that 1 mol of 1,2-diamine-containing peptide scavenges 2 mol of MG in both cases.

2.4. Evaluation of the mutagenic activity of L-Dap-L-Leu

Mutagenic activity of L-Dap-L-Leu and its metabolites (produced by S9 human liver fraction) was evaluated on the *Salmonella typhimurium* TA7001, TA7002, TA7003, TA7004, TA7005, TA7006 (these six strains are mixed in the assay) and TA98 according to the Ames assay. The test substances and positive controls (2-aminoanthracene, 4-nitroquinoline N-oxide and 2-nitrofluorene) were incubated with different strains ± S9 human fraction at 37 °C for 90 min and then incubated with Indicator medium for 60 h before the analysis. The experiments were performed in duplicate and each point in 48 wells. The results are expressed as mutagenicity ratio in comparison to solvent control (sterile water).

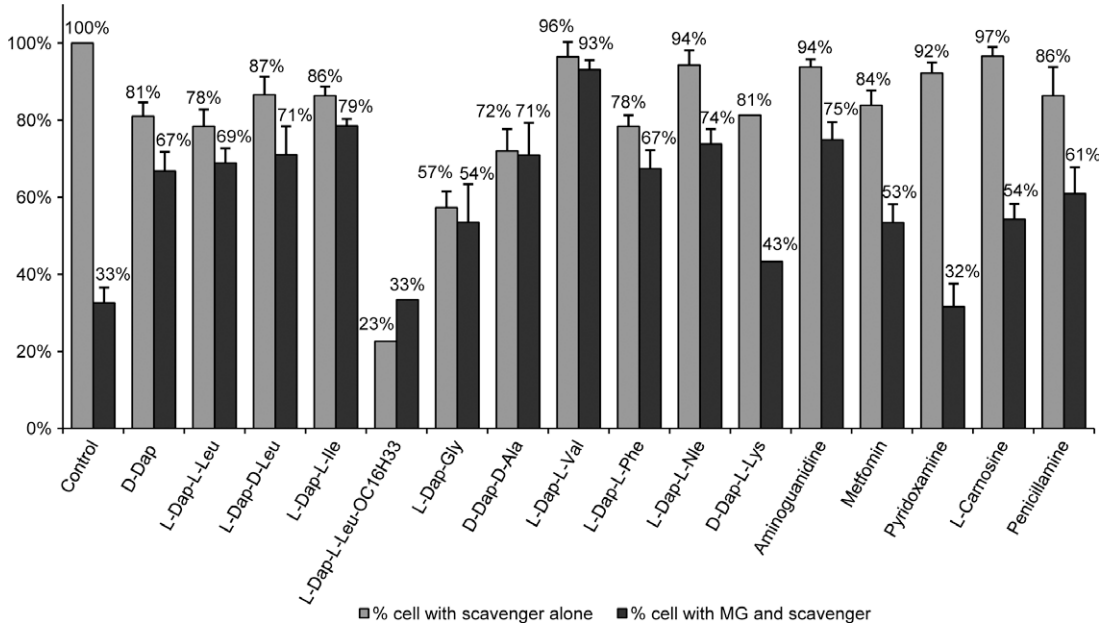


Figure 8. MG scavenging effect of N-terminal-Dap-dipeptides and known AGE inhibitors in the protection of EAhy926 endothelial cells.

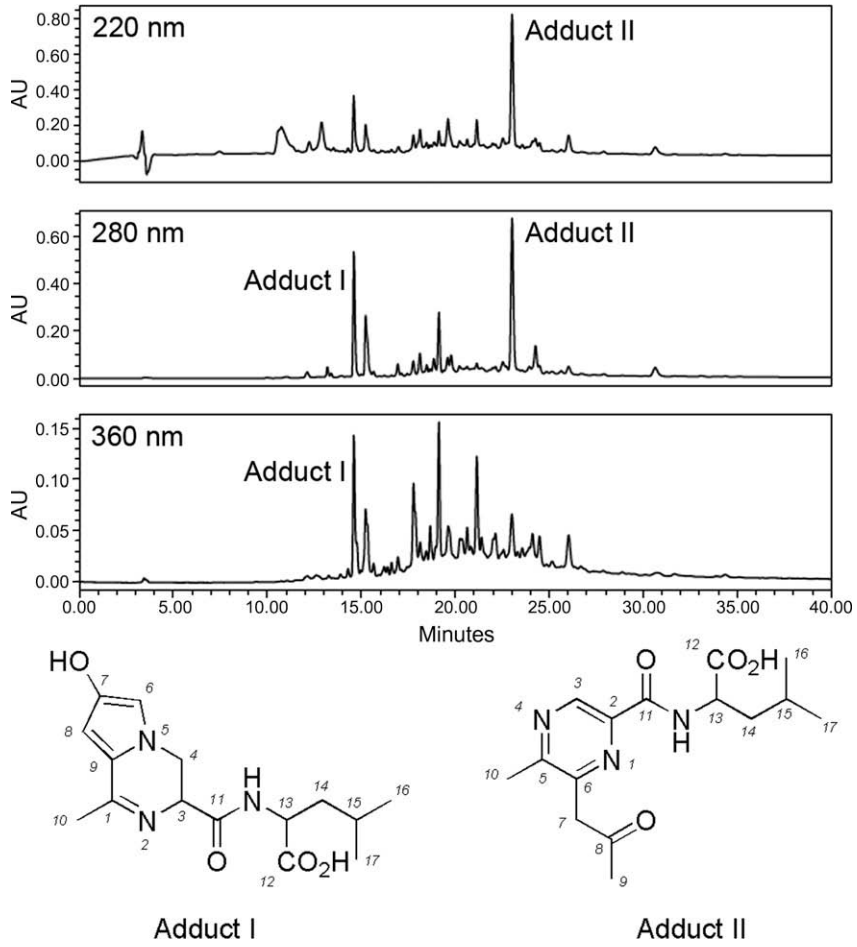


Figure 9. RP-HPLC chromatograms of reaction products of MG and L-Dap-L-Leu. Incubation conditions: L-Dap-L-Leu·2HCl (3.7 mM) and MG (3.4 mM) in 0.1 M NaCl and 10 mM phosphate buffer of pH 7.4 at 37 °C for 24 h. RP-HPLC conditions: column: symmetry 300 C-18 (4.6 × 250 mm); injection volume: 100 μL of the reaction mixture; flow rate: 1 mL/min; temperature 40 °C; eluent A: H_2O + 0.1% TFA, eluent B: CH_3CN/H_2O 60/40 + 0.1% TFA; linear gradient: 10% B to 50% B in 15 min; detection: PDA (chromatograms extracted at 220, 280 and 360 nm).

Concentrations of the Ames test were chosen as follows: L-Dap-L-Leu alone: 10 μM, 1 μM and 0.1 μM; mixture of L-Dap-L-Leu/MG: 10 μM, 1 μM and 0.1 μM; MG: 10 μM.

As seen from Table 2, the test substance alone (L-Dap-L-Leu) or in combination with MG is neither mutagenic against the 6 mixed and TA98 strains nor on the S9 fraction of human liver in the tested concentrations.

Although D-Dap and L-Dap alone are excellent scavengers of MG, D-Dap has been reported to induce renal tubular necrosis.[38] D- and L-Dap undergo transamination to form pyruvate and ammonia by widely occurring 1,2-diaminopropionate ammonia-lyase in bacteria, notably in *S. typhimurium*.[39] Moreover, D-Dap showed a mutagenic activity with S9 mix, in *S. typhimurium* TA 100 strain.[40] For these reasons, we chose the N-terminal Dap-containing dipeptides for further assessments of the therapeutic potency of the new MG scavengers.

It has been generally accepted that transition metal ions may play an important role during AGE formation in vivo by catalyzing the generation of superoxide and α-dicarbonyls.[41,42] Several research groups have reported the reduction of AGE formation by trapping Cu(II) with chelating agents such as trientine,[43] D-penicillamine[27] pyridoxamine, 6-diaminopyridoxamine, trolox, aminoguanidine,[44] carnosine, tenilsetam, OPB-9195.[45] Recent progress in the so-called metal-chelation therapy in Alzheimer's and other CNS diseases may further promote the development of rational strategies for the treatment of these AGE related neurodegenerative diseases.[46–48] In this regard, it is interesting to know the Cu(II) chelating property of the N-terminal Dap containing peptides. Our preliminary results obtained from the measurement of Cu(II) in ultrafiltrate of human serum by atomic absorption spectrophotometry indicates that D-Dap-L-Nle and D-Dap-L-Leu are found to be excellent Cu(II) chelators, as potent as trientine and carnosine, significantly better than AG and metformin but less efficient than D-penicillamine in the 1-15 mM range.[49]

Throughout the present in vitro studies, we have demonstrated that the N-terminal Dap is the essential moiety for the MG-trapping capacity regardless of its stereochemistry. As shown in Figure 3, insulin is equally well protected against MG modification both by D-Dap-L-Leu and L-Dap-L-Leu. While the former is an excellent insulin protector, the latter significantly contributes to conserve enzymatic activities of RNase A and glyoxalase 1 in confrontation with MG. With respect to the stereochemistry of the C-terminal amino acid, it seems to play little role for the scavenging potency of the dipeptides as evidenced in Figure 3 for the Dap-Leu series. As to the functional group on the side chain of the C-terminal amino acid, longer alkyl groups in general give satisfactory results compared with the polar or aromatic ones (Fig. 3).

3. Conclusions

In vitro efficacy of N-terminal Dap containing dipeptides as scavengers of reactive α-dicarbonyl compounds such as MG is

Table 1
^{1}H and ^{13}C NMR chemical shifts of Adduct I and Adduct II. Hydrogens and carbons were attributed from detailed NMR spectroscopic analyses

Atom numbering	Adduct I	
	δ_H/ppm (600 MHz, $(CD_3)_2SO$)	δ_C/ppm (500 MHz, CD_3OD)
Pyrazine- moiety		
11		168.21
1		163.68
9		122.00
OH	11.64 (s, 1H)	
6	7.26 (d, *J* = 1.5 Hz, 1H]	124.40
7		150.20
8	6.79 (d. *J* = 1.5 Hz, 1H)	110.10
3	4.87 (t, *J* = 5.4 Hz, 1H)	55.97
4	4.45 (dd, *J* = 5.4 Hz, *J* = 13.5, 1H)	47.02
	4.37 (dd, *J* = 5.4 Hz, *J* = 13.5, 1H)	
10	2.55 (s. 3H)	18.14
Leucine-moiety		
NH	8.76 (d, *J* = 8 0 Hz, 1H)	
12		175.31
13	4,25–422 (m, 1H)	52.64
14	1.57–1.52 (m, 3H)	41.23
15		26.14
16	0.87 (d, *J* = 5.9 Hz, 3H)	23.34
17	0.80 (d, *J* = 5.9 Hz, 3H)	21.60
	Adduct II	
	δ_H/ppm (600 MHz, $(CD_3)_2SO$)	δ_C/ppm (500 MHz, CD_3DO)
Pyrazine-moiety		
8		206.52
11		165.29
5		158.55
6		150.56
2		142.85
3	8.92 (s, 1H)	141.95
7	4 24 (s, 2H)	50.10
9	2.29 (s, 3H)	30.16
10	2.46 (s, 3H)	22.06
Leucine-moiety		
12		175.90
13	4.33 (br s, 1H)	52.30
14	1.64–1.62 (m, 3H)	42.06
15		26.23
16		23.38
17	0.88 (br s, 6H)	22.06

For atom numbering, see Figure 9.

Table 2
Mutagenicity of L-Dap-L-Leu alone and in combination with MG against 7 *Salmonella* strains

Test substance	Concentrations (μM)	Human S9	6 Mixed strains	TA98 Strain
L-Dap-L-Leu	10/1/0.1	No	–	
L-Dap-L-Leu (metabolites)	10/1/0.1	Yes	–	–
L-Dap-L-Leu + MG	10/1/0.1	No	–	–
L-Dap-L-Leu + MG (metabolites)	10/1/0.1	Yes		–
MG	10	No		–
MG (metabolites)	10	Yes	–	–
Positive control (4NQO)	2.6	No	+	+
Positive control (2AA) (metabolites)	51.7	Yes	+	+
Negative control (solvent)	–	No	–	–
Negative control (solvent)		Yes		

demonstrated. In a physiological solution, insulin, RNase A, lysozyme and glyoxalase I are highly susceptible to modifications by addition of MG. However, the presence of the new N-terminal Dap containing dipeptides in the tested medium efficiently inhibits this trend. Among the MG scavengers known in the literature, only AG matches to the new type of compounds presented here in terms of the efficacy to inhibit the modification of proteins. Despite the seemingly excellent bifunctional characters of Dap-dipeptides as α-dicarbonyl scavenger and Cu(II) chelator, further studies are needed to confirm their efficiency by measuring, for example, the formation of AGEs of BSA incubated with D-(+)-glucose in the absence or presence of the peptides and metal ions. For the inhibition of intracellular AGE formation, these scavengers must possess high physiological stability and excellent bioavailability. Indeed, our preliminary results suggest that selection of D-configuration in one or both of the constituting amino acids in dipeptides seems to be very important factor to circumvent the problem arising from proteolysis. Pharmacokinetic studies of these dipeptides are currently under investigation in our laboratory. In view of the increasing evidences that protein-AGE formation is closely associated with aging and diabetes complications, some of N-terminal Dap dipeptides de-

scribed in this paper may provide an effective means to decelerate and treat these insidious processes.

4. Experimental

4.1. Materials and methods

Methylglyoxal, aminoguanidine hydrochloride, 1,1-dimethylbiguanide hydrochloride (metformin), pyridoxamine dihydrochloride, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-2*H*-tetrazolium bromide (MTT), 2-aminoanthracene, 4-nitroquinoline N-oxide and 2-nitrofluorene, human insulin, bovine pancreatic RNase A, chicken lysozyme, yeast RNA were purchased from Sigma–Aldrich. L-carnosine and DL-penicillamine were purchased from Fluka. DL-Diaminobutyric acid, 2,3-diaminopropionic acid (D-, L- and DL-), methyl esters of amino acids, *N*-Cbz-L-Leu, 1-hexadecanol, 1-[3-(dimethylamino)propy]-3-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) were from Acros Organics. Tris-glycine SDS–PAGE gel and prestained SDS–PAGE standards were obtained from Bio-Rad (Marnes-la-Coquette, France). Thymidine was purchased from Invitrogen (Cergy-Pontoise, France). Trypsin/EDTA was from Invitrogen (France). Human glyoxalase I was obtained from Protein'eXpert SA (Grenoble, France). All the *S. typhimurium* strains are obtained from Biogenic (France) and the S9 human liver fraction was purchased from BD Biosciences (France).

4.1.1. RP-HPLC

Control experiments were realized by incubating insulin (34 μM) in phosphate buffer (10 mM, pH 7.4 + 0.1 M NaCl) at 37 °C for 24 h in absence or presence of MG (3.4 mM). Efficacy of each scavenger in this study was assessed by comparative measurement of the remaining intact insulin vis-à-vis the control (insulin alone) when it is incubated under the same conditions with MG (3.4 mM) in the presence of a scavenger compound (3.75 mM, 1.1 mol equiv with respect to MG). Each compound was assessed for three times and RP-HPLC analyses were realized under the following conditions: column: Symmetry 300 C-18 (4.6 × 250 mm); injection volume: 100 μL of the reaction mixture; flow rate: 1 mL/min; temperature 40 °C; eluent A: H_2O + 0.1% TFA, eluent B: CH_3CN/H_2O 60/40 + 0.1% TFA; linear gradient: 50% B to 55% B in 15 min; detection: PDA (chromatograms extracted at 220 nm).

4.1.2. SDS–PAGE and non-denaturing PAGE analysis

Stock solution of a model protein was prepared by solubilizing the protein in phosphate buffer to give a final concentration of 10–15 mg/mL. The resultant solution was transferred into Eppendorf tubes and incubated with MG in the presence or absence of α-dicarbonyl scavenger for 24 h (glyoxalase I) or 48 h (RNase A and lysozyme) at 37 °C then stored at −20 °C prior to analysis. PAGE electrophoresis was performed by loading 10 μL of the incubated protein on a 8–16% gradient Tris-glycine SDS–PAGE gel. The glyoxalase I samples were analyzed on 12% Tris-glycine PAGE gel. L-Dap-L-Leu, L-Dap-L-Val and AG have been used as scavengers.

4.1.3. Assay for enzymatic activity

4.1.3.1. RNase A. Enzymatic activity of RNase A was assayed as described in the literature.[35] Thus, 600 μg/mL of yeast RNA was employed as working concentration. 10 μL of RNAse (concentration of 10 μg/mL) that has been subjected to glycation by MG in the absence or presence of MG-scavenger was added to 1 mL of methylene blue-RNA solution. The difference between the absorbance value of the methylene blue-RNA complex at 688 nm and the one obtained after the enzymatic digestion of the complex is taken as control (100%). The subsequent cleavage of RNA with glycated RNase A was measured at 688 nm. The scavenging potency of AG, DL-Dap, D-Dap-D-Ala, L-Dap-Gly, D-Dap-D-Phe, L-Dap-L-Leu, L-Dap-L-Ile, L-Dap-L-Val were assessed for this study.

4.1.3.2. Glyoxalase I. The activity of glyoxalase I was assayed by measuring the rate of formation of *S*-D-lactoylglutathione from the hemithioacetal formed non-enzymatically from MG and reduced glutathione. Hemithioacetal was prepared by incubating 2 mM MG with 2 mM reduced glutathione in 50 mM sodium phosphate, pH 6.6, for 20 min at 37 °C. The normal hemimercaptal concentration was 600 μM, assuming the equilibrium constant for the hemithioacetal formation was 3.33 M^{-1}. The reaction mixture was allowed to stand for 15 min to ensure the equilibration of hemithioacetal formation. An aliquot of enzyme of control or that of co-incubation with MG in the absence or presence of L-Dap-L-Leu was added to the hemithioacetal solution and the formation of *S*-D-lactoylglutathione was monitored by following absorbance at 240 nm; $\Delta\varepsilon_{240} = 2.86\ mM^{-1}\ cm^{-1}$.

The activity of glyoxalase I was calculated in unit per ml where unit is the amount of enzyme required to catalyze the formation of 1 μmol of *S*-D-lactoylglutathione per minute under assay conditions.

4.1.4. Cell culture

A continuous cell line of immortalized EAhy926 endothelial cells derived from fusion of human umbilical vein endothelial cells (HUVECs) with A549 lung carcinoma cells was routinely cultured in 75 mL flasks in moist air containing 5% CO_2 at 37 °C using Dulbecco minimal essential medium (DMEM) containing 4.5 g/L glucose and supplemented with 10% fetal calf serum, 1% glutamine, HAT (100 μM hypoxantine, 0.4 μM aminopterin, 16 μM thymidine. The cultured cell line was routinely harvested by trypsin treatment (0.5 g/L trypsin/EDTA from Invitrogen) when cells EAhy926 attained 90% confluence. All experiments were carried out on 12-well dishes where cells EAhy926 were seeded at 1×10^5 cells per well in 2 mL of DMEM. Cells were left overnight to attach to the plate, and the appropriate carbonyl scavenger (1 mM) was then added 30 min prior to the addition of MG (600 μM). Following a 48 h exposure to carbonyl scavenger and/or MG, cell viability was evaluated on the reduction of 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazoliumbromide (MTT).[50] Results are expressed as the ratio (%) of the number of cells with treatment to the number of cells without treatment (control) and is the mean ± S.E (n = 3 triplicates). The detection is carried out by UV spectrometry at 570 nm.

4.2. Chemical synthesis

4.2.1. *N,N′*-Di-Boc-2,3-L-Dap-L-Leu-OMe

To a solution of *N,N′*-Di-Boc-2,3-L-Dap-OH (9.39 g, 30.88 mmol) and L-Leu-OMe hydrochloride (6.17 g, 33.97 mmol) in CH_2Cl_2 (150 mL) were added Et_3N (4.74 mL, 33.97 mmol), EDC (7.10 g, 37.05 mmol) and HOBt (4.59 g, 33.97 mmol) and the reaction mixture was stirred at room temperature overnight. Water was added and the aqueous phase was extracted three times with EtOAc. The combined organic layers were washed successively with 1 N HCl, H_2O, saturated $NaHCO_3$ and brine, dried over Na_2SO_4 and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography on silica gel with heptane–EtOAc (3/1 then 1/1) to give 13.30 g of *N,N′*-Di-Boc-L-2,3-Dap-L-Leu-OMe (quantitative yield).

$[\alpha]_D$ −44.6 (*c* 2.0, $CHCl_3$).

^1H NMR (300 MHz, CD_3CN) δ 7.05 (d, J = 7.9 Hz, 1H), 5.94 (br s, 1H), 5.57 (br s, 1H), 4.42 (ddd, J = 8.6, 8.3, 5.9 Hz, 1H), 4.11–4.09

(m, 1H), 3.66 (s, 3H), 3.41–3.22 (m, 2H), 1.66–1.55 (m, 3H), 1.42, 1.41 (two s, 18H), 0.92 (d, J = 6.4 Hz, 3H), 0.89 (d, J = 6.3 Hz, 3H); ^{13}C NMR (75 MHz, CD_3CN) δ 174.0, 171.6, 157.8, 156.7, 80.3, 80.0, 56.5, 52.9, 51.7, 43.0, 41.2, 28.7, 25.5, 23.3, 21.8;

MS (ESI) m/z 454 $[M+Na]^+$.

4.2.2. *N,N′*-Di-Boc-L-2,3-Dap-L-Leu

To a solution of *N,N′*-Di-Boc-L-2,3-Dap-L-Leu-OMe (12.05 g, 27.96 mmol) in THF/MeOH/H_2O (1/1/1, 140 mL) at 0 °C was added LiOH·H_2O (1.17 g, 27.96 mmol) and the reaction mixture was stirred at room temperature until all of the starting methyl ester disappeared (about overnight, followed by TLC). The reaction mixture was acidified with aqueous $KHSO_4$ solution to pH 4, and then extracted five times with CH_2Cl_2. The organic extracts were dried over Na_2SO_4, filtered through Celite and evaporated under reduced pressure to give 11.70 g (100%) of the crude 2,3-di-Boc-L-Dap-L-Leu that was used directly to the next reaction without further purification.

$[\alpha]_D$ −19.7 (c 2.0, $CHCl_3$).

^{1}H NMR (300 MHz, CD_3OD) δ 4.37 (dd, J = 8.7, 5.6 Hz, 1H), 4.21 (br t, J = 6.2 Hz, 1H), 3.44 (dd, J = 14.1, 4.8 Hz, 1H) , 3.27 (dd, J = 14.1, 8.3 Hz, 1H), 1.78–1.59 (m, 3H), 1.44, 1.44 (two s, 18H), 0.95 (d, J = 6.5 Hz, 3H), 0.93 (d, J = 6.9 Hz, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 178.1, 172.8, 158.8, 157.8, 80.8, 80.6, 56.4, 53.5, 43.2, 42.2, 28.9, 26.1, 23.8, 22.2; MS (ESI) m/z 440 $[M+Na]^+$; HRMS calcd for $C_{19}H_{35}N_3O_7Na$ (M+Na) 440.2373; Found: 440.2380.

4.2.3. L-Dap-L-Leu·2HCl

A solution of 2,3-di-Boc-L-Dap-L-Leu (11.00 g, 26.38 mmol) in 3 M HCl–dioxane (130 mL) was stirred at room temperature for 6 h. The volatile was removed under reduced pressure to give 7.65 g of L-Dap-L-Leu·2HCl in quantitative yield.

$[\alpha]_D$ +3.4 (c 1.0, 6 N HCl).

^{1}H NMR (300 MHz, CD_3OD) δ 4.37 (t, J = 5.8 Hz, 1H), 4.30 (dd, J = 9.4, 5.6 Hz, 1H), 3.45 (dd, J = 13.9, 6.0 Hz Hz, 1H), 3.34 (dd, J = 13.9, 5.3 Hz, 1H), 1.64–1.49 (m, 3H), 0.78 (d, J = 6.4, 3H), 0.74 (d, J = 6.4 Hz, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 176.6, 167.3, 53.0, 51.7, 41.4, 40.6, 26.1, 23.4, 21.5; MS (ESI) m/z 218 $[M+H]^+$; HRMS calcd for $C_9H_{20}N_3O_3$ (M+H) 218.1505, found 218.1512.

4.2.4. L-Dap-D-Leu·2HCl

$[\alpha]_D$ +7.8. (c 1, H_2O).

^{1}H NMR (300 MHz, CD_3OD) δ 4.38 (dd, J = 6.7, 5.0 Hz, 1H), 4.30 (t, J = 7.8Hz, 1H), 3.60-3.43 (m, 2H), 1.67–1.56 (m, 3H), 0.86 (d, J = 6.0 Hz, 3H), 0.83 (d, J = 6.0 Hz, 3H); ^{13}C NMR (75 MHz, D_2O) δ 175.9, 166.0, 52.3, 50.7, 39.6, 39.1, 24.5, 22.0, 20.9; MS (ESI) m/z 218 $[M+H]^+$; HRMS calcd for $C_9H_{20}N_3O_3$ (M+H) 218.1505, found 218.1552.

4.2.5. D-Dap-D-Leu·2HCl

$[\alpha]_D$ −11.7 (c 1.0, H_2O).

^{1}H NMR (300 MHz, D_2O) δ 4.46 (m, 2H), 3.58 (dd, J = 6.1, 0.9 Hz, 2H), 1.68 (m, 3H), 0.87 (m, 6H); ^{13}C NMR (62.5 MHz, D_2O) δ 176.0, 166.1, 52.0, 50.4, 39.6, 38.9, 24.4, 22.1; MS (ESI) m/z 218 $[M+H]^+$; HRMS calcd for $C_9H_{20}N_3O_3$ (M+H) 218.1505, found 218.1537.

4.2.6. D-Dap-L-Nle·2HCl

$[\alpha]_D$ −43.0 (c 1.4, H_2O).

^{1}H NMR (300 MHz, D_2O) δ 4.43 (dd, J = 6.6, 5.1 Hz, 1H), 4.33 (dd, J = 8.1, 5.7 Hz, 1H), 3.60 (dd, J = 14.5, 5.1 Hz, 1H), 3.53 (dd, J = 14.5, 6.6 Hz, 1H), 1.82 (m, 2H), 1.34 (m, 4H), 0.86 (t, J = 7.2 Hz, 3H); ^{13}C NMR (75 MHz, D_2O) δ 175.6, 166.0, 53.8, 50.6, 39.4, 29.9, 27.1, 21.6, 13.0; MS (ESI) m/z 218 $[M+H]^+$; HRMS calcd for $C_9H_{20}N_3O_3$ (M+H) 218.1505, found 218.1522.

4.2.7. L-Dap-L-Pro·2HCl

$[\alpha]_D$ −72.2 (c 1.3, H_2O).

^{1}H NMR (300 MHz, D_2O) δ 4.62 (m, 1H), 4.39 (m, 1H), 3.62 (d, J = 7.6 Hz, 1H), 3.57 (m, 2H), 3.42 (dd, J = 13.6, 6.1 Hz, 1H), 2.05 (m, 2H), 1.95 (m, 2H); ^{13}C NMR (62.5 MHz, D_2O) δ 173.1, 164.9, 59.6, 52.6, 46.0, 39.2, 28.2, 22.3; MS (ESI) m/z 202 $[M+H]^+$; HRMS calcd for $C_9H_{20}N_3O_3$ (M+H) 202.1192, found 202.1196.

4.2.8. L-Dap-L-Ile·2HCl

$[\alpha]_D$ +22.4 (c 1.2, H_2O).

^{1}H NMR (300 MHz, D_2O) δ 4.52 (t, J = 5.9 Hz, 1H), 4.46 (d, J = 4.9 Hz, 1H), 3.60 (d, J = 5.9 Hz, 2H), 2.05 (m, 1H), 1.82 (m, 2H), 1.45 (m, 1H), 1.27 (m, 1H), 0.97 (d, J = 6.9 Hz, 3H), 0.90 (t, J = 7.3 Hz, 3H); ^{13}C NMR (62.5 MHz, D_2O) δ 175.5, 166.8, 58.8, 51.1, 40.3, 37.0, 25.3, 15.7, 11.6; MS (ESI) m/z 218 $[M+H]^+$; HRMS calcd for $C_9H_{20}N_3O_3$ (M+H) 218.1505, found 218.1537.

4.2.9. *N*-Cbz-L-Leu-$OC_{16}H_{33}$

To a solution of L-Cbz-Leu-OH (2.85 g, 10.75 mmol) and 1-hexadecanol (2.60 g, 10.75 mmol) in CH_2Cl_2 (100 mL) were added EDC (2.27 g, 11.83 mmol) and DMAP (131 mg, 1.075 mmol) and the reaction mixture was stirred at room temperature overnight. Water was added and the aqueous phase was extracted three times with EtOAc. The combined organic layers were washed successively with 1 N HCl, H_2O, saturated $NaHCO_3$ and brine, dried over Na_2CO_3 and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography on silica gel with heptane–EtOAc (30/1 then 20/1) to give 2.69 g of *N*-Cbz-L-Leu-$OC_{16}H_{33}$ (51% unoptimized yield).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34–7.25 (m 5H), 5.31 (br s, 1H), 4.38 (dt, J = 5.3, 8.7 Hz, 1H), 4.11 (t, J = 6.7 Hz, 2H), 1.74–1.47 (m, 5H), 1.35–1.26 (m, 26H), 0.96-0.86 (m, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.3, 156.1, 136.5, 128.2, 128.2, 67.0, 65.6, 52.7, 41.0, 32.1, 29.8, 29.7, 29.7, 29.5, 29.3, 28.6, 26.0, 24.9, 22.9, 22.8, 22.1, 14.3; MS (ESI) m/z 512 $[M+Na]^+$.

4.2.10. L-Leu-$OC_{16}H_{33}$·HCl

A suspension of *N*-Cbz-L-Leu-$OC_{16}H_{33}$ (2.4 g, 4.91 mmol) and 10% Pd–C (240 mg) in EtOAc was hydrogenated under atmospheric pressure for 4 h. The catalyst was removed by filtration through Celite. The filtrate was evaporated. The residue was treated with 0.1 M HCl in dioxane and evaporated to dryness to give 1.83 g of L-Leu-$OC_{16}H_{33}$·HCl (95% yield).

^{1}H NMR (300 MHz, CD_3OD) δ 4.87 (br s, 1H), 4.31–4.19 (m, 2H), 4.03 (t, J = 7.0 Hz, IH), 1.86–1.66 (m, 5H), 1.43-1.29 (m, 26H), 1.11 (d, J = 6.2 Hz, 3H), 1.02 (d, J = 6.0 Hz, 3H), 1.00 (t, J = 6.8 Hz, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 171.2, 67.7, 52.6, 40.9, 33.2, 30.9, 30.8, 30.8, 30.6, 30.4, 29.7, 27.0, 25.8, 23.9, 22.7, 22.6, 14.5; MS (ESI) m/z 356 $[M+H]^+$.

4.2.11. *N,N′*-Di-Boc-L-Dap-L-Leu-$OC_{16}H_{33}$

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.18 (br s, 1H), 5.85 (br s, 1H), 5.36 (br s, 1H), 4.58–4.51 (m, 1H), 4.24–4.22 (m, 1H), 4.11 (t, J = 6.8 Hz, 2H), 3.52–3.43 (m, 2H), 1.67–1.54 (m, 5H), 1.45–1.44 (two s, 18H), 1.35–1.26 (m, 26H), 0.93 (br d, J = 4.9 Hz, 6H), 0.88 (t, J = 6.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.7, 170.7, 157.2, 156.3, 80.3, 80.0, 65.6, 55.5, 51.1, 42.6, 41.4, 32.1, 29.8, 29.7, 29.6, 29.5, 29.3, 28.6, 28.5, 28.4, 26.0, 24.9, 23.0, 22.8, 22.0, 14.3; MS (ESI) m/z 664 $[M+Na]^+$.

4.2.12. L-Dap-L-Ile-$OC_{16}H_{33}$·2HCl

$[\alpha]_D$ +13.1 (c 2.0, MeOH).

^{1}H NMR (300 MHz, CD_3OD δ *4.34–4.28* (m 2H), 4.01–3.86 (m, 2H), 3.41–3.29 (m, 2H), 1.61–1.39 (m, 5H), 1.16–1.05 (m, 26H), 0.76 (d, J = 6.4 Hz, 3H), 0.72 (d, J = 6.4 Hz, 3H), 0.66 (t, J = 6.7 Hz, 3H), 0.90 (t, J = 7.3 Hz, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 174.6, 167.2, 58.8,

67.2, 53.0, 51.8, 41.4, 40.9, 33.2, 30.9, 30.6, 30.6, 30.6, 30.3, 29.7, 27.0, 26.1, 23.8, 23.4, 21.8, 14.6; MS (ESI) *m/z* 442 $[M+H]^+$; HRMS calcd for $C_{25}H_{52}N_3O_3$ (M+H) 442.4009, found 442.3983.

4.2.13. L-Dap-L-*p*-CF_3-Phe·2HCl

^{1}H NMR (300 MHz, D_2O) δ 7.58 (d, J = 7.9 Hz, 2H), 7.35 (d, J = 8.1 Hz, 2H), 4.69 (m, 1H), 4.25 (t, J = 6.0 Hz 1H), 3.27 (dd, J = 13.9, 5.8 Hz, 1H), 3.17 (d, J = 6.0 Hz, 2H), 3.04 (dd, J = 14.0, 9.3 Hz, 1H); ^{13}C NMR (75 MHz, D_2O) δ 174.1, 165.7, 140.7, 130.0, 129.6 (q, J = 32.9 Hz), 125.5, 125.0 (q, J = 271.0 Hz) 54.3, 50.7, 39.5, 36.3; MS (ESI) *m/z* 320 $[M+H]^+$; HRMS calcd for $C_{13}H_{17}F_3N_3O_3$ (M+H) 320.1222, found 320.1236.

4.2.14. L-Dap-DL-*p*-F-Phe·2HCl

^{1}H NMR (300 MHz, D_2O) δ 7.20–7.15 (m, 2H), 7.03–6.95 (m, 2H), 4.66–4.59 (m, 1H), 4.27 (t, J = 5.7 Hz, 0.5H), 4.25 (t, J = 5.8 Hz, 0.5H), 3.43 (d, J = 6.2 Hz, 0.5H), 3.42 (d, J = 5.7 Hz, 0.5H), 3.20–3.10 (m, 1H), 3.17 (d, J = 6.0 Hz, 1H), 2.97 (dd, J = 14.1, 8.6 Hz, 0.5H), 2.94 (dd, J = 14.1, 9.2 Hz, 0.5H); ^{13}C NMR (75 MHz, D_2O) δ 174.3, 174.2, 165.8, 165.7, 162.8 (d, J = 243.7 Hz), 133.0, 132.8, 131.6, 116.3, 116.0, 54.7, 54.5, 50.7, 50.4, 39.5, 35.7, 35.5; MS (ESI) *m/z* 270 $[M+H]^+$; HRMS calcd for $C_{12}H_{17}FN_3O_3$ (M+H) 270.1254, found 270.1255.

4.2.15. L-Dap-L-Val·2HCl

$[\alpha]_D$ +22.2 (*c* 2.0, H_2O).

^{1}H NMR (300 MHz, D_2O) δ 4.54 (t, J = 5.9 Hz, 1H), 4.42 (d, J = 5.0 Hz, 1H), 3.60 (d, J = 5.9 Hz, 2H), 2.29 (m, 1H), 0.97 (t, J = 6.7 Hz, 6H); ^{13}C NMR (62.5 MHz, D_2O) δ 175.5, 166.9, 59.5, 51.1, 40.3, 30.4, 19.0, 17.6; MS (ESI) *m/z* 204 $[M+H]^+$; HRMS calcd for $C_8H_{18}N_3O_3$ (M+H) 204.1348, found 204.1365.

4.2.16. D-Dap-D-Ala·2HCl

$[\alpha]_D$ −21.9 (*c* 1.0, MeOH).

^{1}H NMR (300 MHz, D_2O) δ 4.53 (q, J = 7.4 Hz, 1H), 4.48 (t, J = 6.0 Hz, 1H), 3.64 (d, J = 6.0 Hz, 2H), 1.50 (d, J = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, D_2O) δ 176.7, 166.5, 51.2, 49.9, 40.3, 16.6; MS (ESI) *m/z* 176 $[M+H]^+$; HRMS calcd for $C_6H_{14}N_3O_3$ (M+H) 176.1035, found 176.1037.

4.2.17. L-Dap-β-Ala·2HCl

^{1}H NMR (300 MHz, CD_3OD) δ 4.40 (t, J = 5.8 Hz, 1H), 3.54 (m, 4H), 2.64 (m, 3H); ^{13}C NMR (62.5 MHz, CD_3OD) δ 174.0, 166.6, 52.1, 41.1, 36.9, 34.2; MS (ESI) *m/z* 176 $[M+H]^+$.

4.2.18. L-Dap-L-Phe·2HCl

^{1}H NMR (300 MHz, CD_3OD) δ 7.32 (m, 5H), 4.79 (dd, J = 9.9, 4.4 Hz, 1H), 4.48 (t, J = 5.9 Hz, 1H), 3.61 (dd, J = 13.9, 6.1 Hz, 1H), 3.51 (dd, J = 13.9, 5.7 Hz, 1H), 3.35 (dd, J = 14.2, 3.4 Hz, 1H), 3.08 (dd, J = 14.2, 9.9 Hz, 1H); ^{13}C NMR (62.5 MHz, CD_3OD) δ 175.1, 167.2, 138.2, 130.3, 129.7, 128.1,68.2, 56.2, 51.7, 41.3, 37.5; MS (ESI) *m/z* 252 $[M+H]^+$, 269 $[M+H_2O]^+$.

4.2.19. D-Dap-D-Phe·2HCl

$[\alpha]_D$ −38.0 (*c* 1.9, H_2O).

^{1}H NMR (300 MHz, CD_3OD) δ 7.32 (m, 5H), 4.79 (dd, J = 9.9, 4.4 Hz, 1H), 4.48 (t, J = 5.9 Hz, 1H), 3.61 (dd, J = 13.9, 6.1 Hz, 1H), 3.51 (dd, J = 13.9, 5.7 Hz, 1H), 3.35 (dd, J = 14.2, 3.4 Hz, 1H), 3.08 (dd, J = 14.2, 9.9 Hz, 1H); ^{13}C NMR (62.5 MHz, CD_3OD) δ 175.1, 167.2, 138.2, 130.3, 129.7, 128.1, 68.2, 56.2, 51.7, 41.3, 37.5; MS (ESI) *m/z* 252 $[M+H]^+$, 269 $[M+H_2O]^+$.

4.2.20. L-Dap-D-Dap·3HCl

$[\alpha]_D$ −43.4 (*c* 0.4, H_2O).

^{1}H NMR (300 MHz, D_2O) δ 4.60 (m, 1H), 4.55 (dd, J = 6.5, 4.9 Hz, 1H), 3.65 (m, 3H), 3.45 (dd, J = 13.5, 7.7 Hz, 1H); ^{13}C NMR (62.5 MHz, D_2O) δ 71.6, 167.6, 51.8, 51.5, 40.2, 40.1; MS (ESI) *m/z* 191 $[M+H]^+$; HRMS calcd for $C_6H_{15}N_4O_3$ 191.1144, found 191.1146.

4.2.21. D-Dap-Gly·2HCl

$[\alpha]_D$ +38 (*c* 0.2, MeOH).

^{1}H NMR (300 MHz, D_2O) δ 4.53 (t, J = 5.9 Hz, 1H), 4.08 (d, J = 2.6 Hz, 2H), 3.63 (d, J = 5.9 Hz, 2H); ^{13}C NMR (75 MHz, D_2O) δ 176.3, 167.6, 51.1, 42.6, 39.9; MS (ESI) *m/z* 161 $[M+H]^+$; HRMS calcd for $C_5H_{13}N_3O_3$ 161.1039, found 161.1042.

4.2.22. D-Dap-D-Asp·2HCl

$[\alpha]_D$ −32.4 (*c* 0.7, H_2O).

^{1}H NMR (300 MHz, D_2O) δ 4.75 (m, 1H), 4.38 (dd, J = 6.3, 5.3 Hz, 1H), 3.50 (dd, J = 14.4, 5.3 Hz, 1H), 3.42 (dd, J = 14.4, 6.4 Hz, 1H), 2.93 (d, J = 6.3 Hz, 2H); ^{13}C NMR (75 MHz, D_2O) δ 174.2, 173.2, 165.9, 50.6, 49.4, 39.4, 35.1; MS (ESI) *m/z* 220 $[M+H]^+$; HRMS calcd for $C_7H_{14}N_3O_5$ 220.0933, found 220.0903.

4.2.23. D-Dap-L-Lys·3HCl

^{1}H NMR (300 MHz, D_2O) δ 4.42 (dd, J = 6.6, 5.1 Hz, 1H), 4.28 (dd, J = 8.0, 5.8 Hz, 1H), 3.52 (m, 2H), 2.90 (t, J = 7.6 Hz, 2H), 1.82 (m, 2H), 1.60 (m, 2H), 1.37 (m, 2H); ^{13}C NMR (75 MHz, D_2O) δ 174.9, 166.1, 53.5, 50.7, 39.5, 39.2, 29.7, 26.3, 22.1; MS (ESI) *m/z* 233 $[M+H]^+$.

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