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Towards a general synthesis of di-aza-amino acids containing peptides

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ABSTRACT:

While the incorporation of one aza-amino acid in peptides has been proved to be beneficial for inducing a structure constraint, increasing resistance towards proteolysis and improving biological activity, only very rare examples of the incorporation of two or more consecutive aza-amino acids have been reported. In this work, we demonstrated that this fact is probably due to the unsuspected difficulty to synthesize such peptide analogues, as illustrated by the synthesis of tripeptide derivatives containing two consecutive aza-amino acids. Herein, we report some general guidelines regarding the activation and the coupling of alkyl-hydrazides either mutually or with a natural amino acid, taking into account their nucleophilicity and the nature of their side chains.

KEYWORDS aza-peptides, aza-amino acids, peptides, peptidomimetics, hydrazine

1. Introduction

The number of peptide-based drugs is dramatically increasing as they generally offer greater efficacy, high potency and selectivity and a reduced risk of toxicity associated with side effects, compared to non peptide molecules.[1] However, they suffer from rapid proteolysis and suitable small modifications that increase peptide stability are highly desirable. Currently, more than 60 peptide derivatives have been approved as commercial drugs and about 140 are in clinical trials.[1e] Among the numerous modifications of peptides which include peptide side chain modifications, amino acid substitutions extensions, deletions, and the inversion of stereochemistry of the α-carbon(s), aza-peptides have attracted attention. In aza-peptides the α–CH(R) of at least one amino-acid residue is substituted by a nitrogen atom (Figure 1). This residue, called aza-amino acid (azaAA), impart special conformational properties to the parent peptide structure due to the loss of stereogenicity and reduction of flexibility.[2] Thus, the incorporated aza-amino acid can introduce some conformational constraint to the parent peptide and allow the chain to adopt a secondary structure and the proper pharmacophore orientation for activity and selectivity. Furthermore, the formed urea bond confers better resistance towards proteolytic and chemical degradation than the amide bond of natural peptides. The replacement of an α-carbon by a nitrogen in a peptide results in an additional possibility for the formation of hydrogen bonds and was also found to affect the acidity of the neighbouring amide N-H bonds, while decreasing the electrophilic character of the carbonyl group.[3,4] Application of aza-peptides in providing biologically active peptides has shown significant success.[3,4,5-7] However, we were particularly intrigued by the fact that only one aza-amino acid has been incorporated in an isolated manner and

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only very rare examples have been reported for the incorporation of two or more aza-amino acids consecutively. To our knowledge, very few groups[8] including ours [9] have reported the incorporation of two to five aza-amino acids consecutively. Remarkably, in these rare cases, aza-Gly is the most representative aza-amino acid in such aza-peptides. We suspected in a first attempt the importance of the additional NH in aza-Gly for providing supplementary hydrogen bonds and thus for increasing the binding capacity to the biological target. However, considering the scarcity of aza-peptides including consecutive aza-amino acids bearing a side chain, we also suspected a difficulty of synthesis of this kind of peptides. Thus, we decided to start by prospecting the feasibility of the synthesis of peptides containing two consecutive aza-amino acids, by varying the nature of these aza-amino acids, and we report here our attempts to find a generalizable strategy.

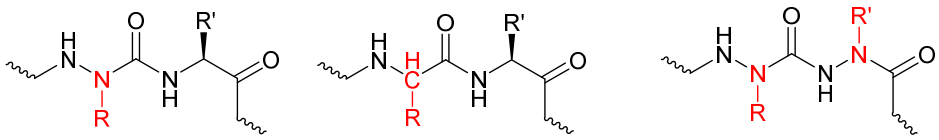

Figure 1. Comparison of aza-peptide, native peptide and azatide

In this work, the synthesis of six tripeptide analogues containing two aza-amino acids **I-VI** have been studied in order to evaluate the influence of the side chains of both the aza-amino acids and the natural amino acids on the efficiency of the synthesis. For that purpose, we considered the synthesis of Boc-azaVal-azaGly-Phe-NH_2 (**I**), Boc-azaLys-azaGly-Phe-NH_2 (**II**), Boc-azaVal-azaAla-Val-NH_2 (**III**), Boc-azaLeu-azaVal-His-OCH_3 (**IV**) and Boc-azaPhe-azaLeu-Val-OCH_3 (**V**) (Figure 2) to compare them together and also with our previously reported synthesis of Boc-azaGly-azaGly-Val-NH_2 (**VI**).[9]

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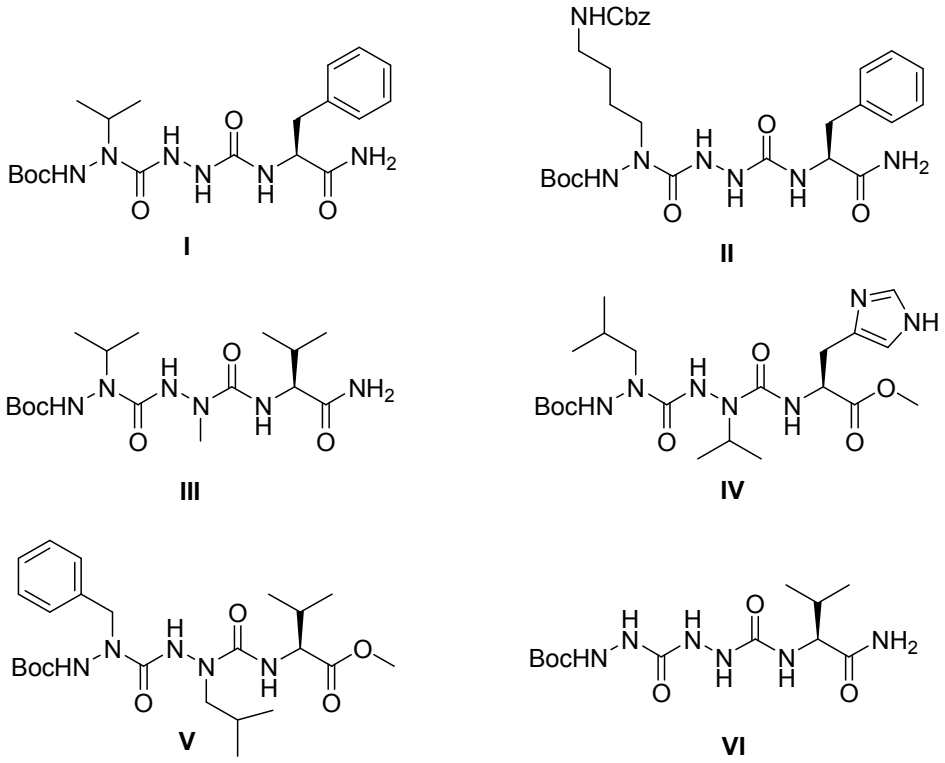

Figure 2. Structure of the tripeptide analogues containing two consecutive aza-amino acids **I-VI**.

2. Results and discussion

The synthesis of aza-peptides containing one isolated aza-amino acid has been largely reported and requires hydrazine chemistry and classical peptide synthesis, either in solution or in solid-phase. [3-9] These aza-peptides have been typically synthesized by two general approaches: either the activation of the peptide *N*-terminus (or the natural amino acid) as an isocyanate or active carbamate, followed by coupling to a hydrazide, or the activation of the hydrazide moiety, usually in the form of *N*-alkyl carbazate and the subsequent coupling with the natural amino acid. In both cases, the activation proceeds using phosgene, triphosgene, carbonyldiimidazole, bis(2,4-dinitrophenyl)carbonate, phenylchloroformate, 4-nitrophenyl chloroformate or bis(pentafluorophenyl)carbonate as carbonyl donors.[5,6,10-12] The activation and the coupling of the alkyl-hydrazides is suspected to be more challenging since they are poorer nucleophiles than simple amines.[13] The strategy for the synthesis of aza-peptides including consecutive aza-amino acids is necessarily based on the activation of protected alkyl-hydrazines. Two routes can be considered: A) the activation of the protected 2-alkyl-hydrazine **1** to obtain **2**, followed by the reaction with the protected 1-alkyl-hydrazine **3** or B) the activation of the protected 1-alkyl-hydrazine **3** to obtain **4**, followed by the reaction with the protected 2-alkyl-hydrazine **1** (Scheme 1). In our case, for the synthesis of the di-aza-amino acids containing tripeptide analogues we had also to study the formation of the urea bond between the second aza-amino acid of

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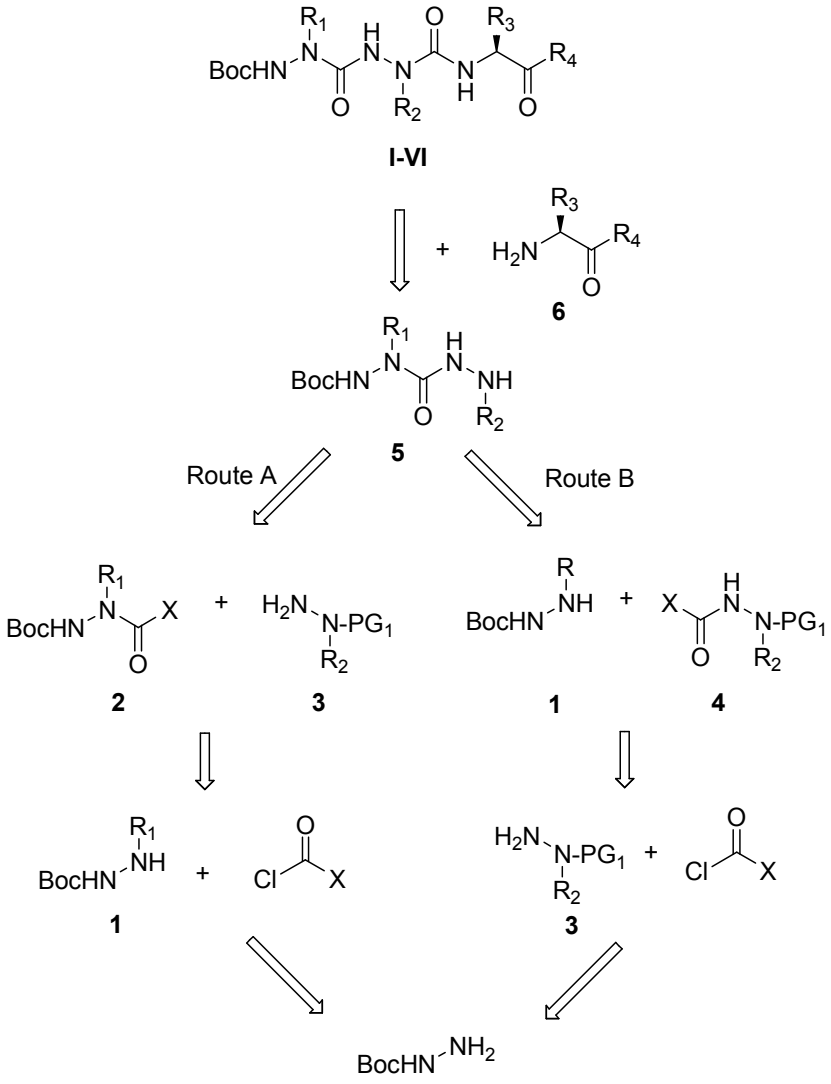
the diaza-peptide **5** and the natural amino acid **6**. For both steps, we were aware that the feasibility of the urea bond formation could be influenced by the side chain of the alkyl-hydrazine and of the amino acid residue.



Scheme 1. General strategy for the synthesis of the tripeptide analogues containing two consecutive aza-amino acids **I-VI**.

We have readily prepared Boc-azaGly-azaGly-Val-NH_2 **VI** [9] following the route A. Firstly Boc-hydrazine (*tert*-butyl carbazate) was activated using phenyl chloroformate in dry DCM in the presence of pyridine, and secondly the reaction with hydrazine hydrate **3a** (R_2=PG_1=H) at reflux of MeOH gave the intermediate **5** (where R_1= R_2= H) in good yields (97% and 72% respectively for the activation and the coupling). The activation of **5** (where R_1= R_2= H) by phenyl chloroformate in dry THF, in the presence of pyridine, and followed by reaction with *L*-Val-NH_2 afforded Boc-azaGly-azaGly-Val-NH_2

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VI in good yield (91% and 93% respectively for the activation and the coupling).[9] Janda *et al.* were able to prepare Boc-azaAA-azaAABoc (with azaGly, azaAla and azaLeu coupled to azaGly, azaAla, azaPhe, azaLeu or azaVal) in good yields (82-92%), following the route B mentioned above, i.e. activation of the protected 1-alkyl-hydrazine **3** followed the reaction with the protected 2-alkyl-hydrazine **1**.[8] They also mentioned the preparation of the tetra-aza-amino acids containing pentapeptide Tyr-azaAla-AzaGly-azaPhe-azaLeu in 50% yield.[8b] The synthesis of our new target compounds **I-V** started by the preparation of the protected 2-alkyl-hydrazine **1** and of the protected 1-alkyl-hydrazine **3**. In the literature, the condensation of *N*-protected carbazate with the appropriate aldehyde or ketone is reported to yield the hydrazone which is immediately reduced by catalytic hydrogenation or hydride addition using $NaBH_3CN$ to give protected 2-alkyl-hydrazine **1**. [10,14,15] In our hands, the Boc-protected 2-isopropyl-, Cbz-4-amino-butyl-, isobutyl- and benzyl-hydrazines **1a-d** were obtained by reaction of the commercially available *tert*-butyl carbazate with acetone, benzyl 2-hydroxypyrrolidine-1-carboxylate (prepared according to references 10, 14 and 15), butyraldehyde and benzaldehyde respectively, in dry THF, to give the corresponding hydrazone intermediates which were reduced in a one pot manner without isolation, in the presence of $NaBH_3CN$ in satisfactory yields (52%, 58%, 66% and 70% respectively). The direct protection of the commercial methylhydrazine with benzyl chloroformate yielded the Cbz-1-methyl-hydrazine **3b** in 47 % yield.[12] The Boc-2-isopropyl-hydrazine **1a** was protected with benzyl chloroformate to afford a diprotected intermediate that provided the hydrochloride salt of the Cbz-1-isopropyl hydrazine **3c** after acidic cleavage of the Boc moiety in 90% yield from **1a**. The protection of the Boc-2-isobutyl-hydrazine **1c** by the Alloc group followed by the acidic cleavage of the Boc moiety provided the hydrochloride salt of the Alloc-1-isobutyl hydrazine **3d** with a quantitative yield (Scheme 2).

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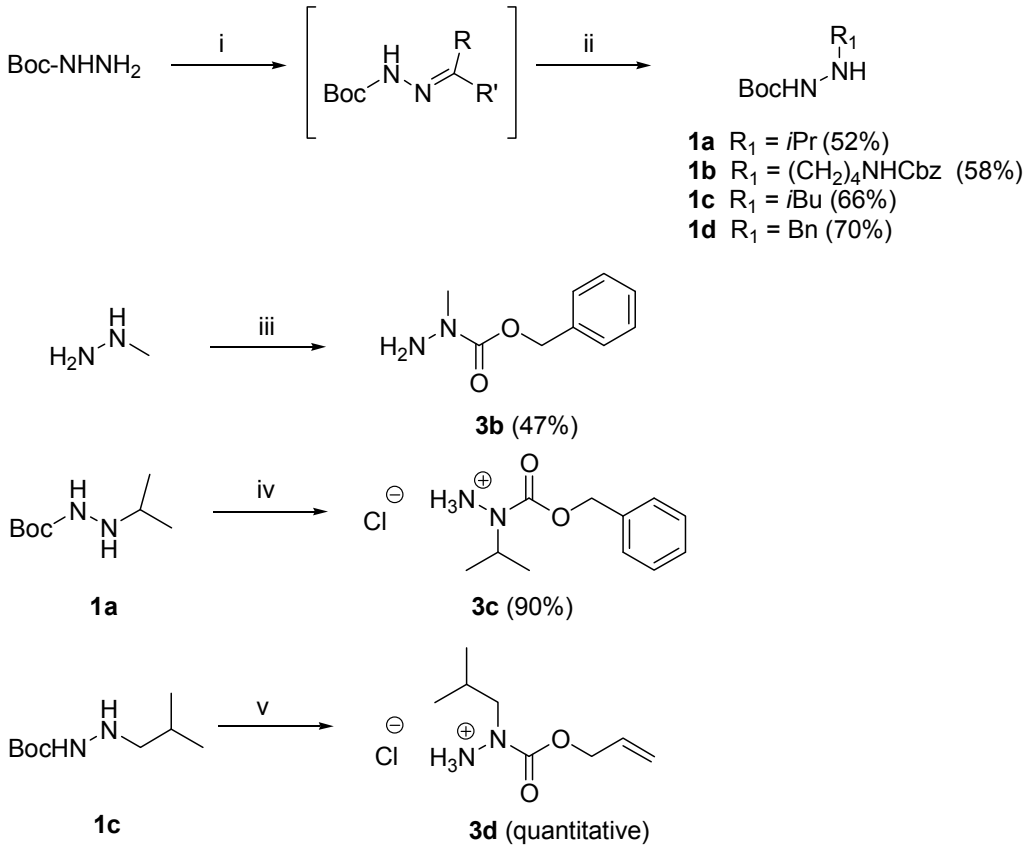

Scheme 2: Preparation of Boc-2-alkyl-hydrazine **1a-d**, Cbz-1-alkyl-hydrazine **3b** and **3c** and Alloc-1-isobutyl-hydrazine **3d**.

Reagents and conditions: i) acetone, benzyl 2-hydroxypyrrolidine-1-carboxylate [10,14] butyraldehyde, or benzyaldehyde, acetic acid, THF, r.t., 5 h to overnight ii) $NaBH_3CN$, acetic acid or PTSA THF, r.t, 1h to 24h; iii) benzyl chloroformate, NaOH, DCM, r.t, 4h, 47%; iv) a) benzyl chloroformate, TEA, DCM, r.t, overnight, b) HCl 4M dioxane, r.t, overnight; v) a) allyl chloroformate, Na_2CO_3, water, dioxane, r.t, overnight, b) HCl 4M dioxane, r.t, overnight

Having in hand the appropriate protected 2-alkyl-hydrazine **1** and 1-alkyl-hydrazine **3**, we first chose to evaluate the route A described above, i.e. the activation of the protected 2-alkyl-hydrazine **1** followed by the reaction with the protected 1-alkyl-hydrazine **3** (Scheme 3). The activation of Boc-2-alkyl-hydrazines **1a-d** in dry DCM in the presence of pyridine, using the 4-nitrophenyl chloroformate as carbonyl donor, afforded the intermediates **2a-d** in good yields (80-89%). Noteworthy, the steric hindrance of the alkyl groups of Boc-protected hydrazines **1** does not seem to prevent the nucleophilic attack of Boc-2-alkyl-hydrazines **1a-d**. The following reaction of **2a** and **2b** with hydrazine monohydrate **3a** in MeOH overnight at room temperature gave **5a** and **5b** respectively, in excellent yields (82%). To activate **5a** and **5b** we chose phenyl chloroformate, hypothesizing that the amine of the natural amino acid would be sufficiently nucleophilic to react with the carbonyl of the phenyl

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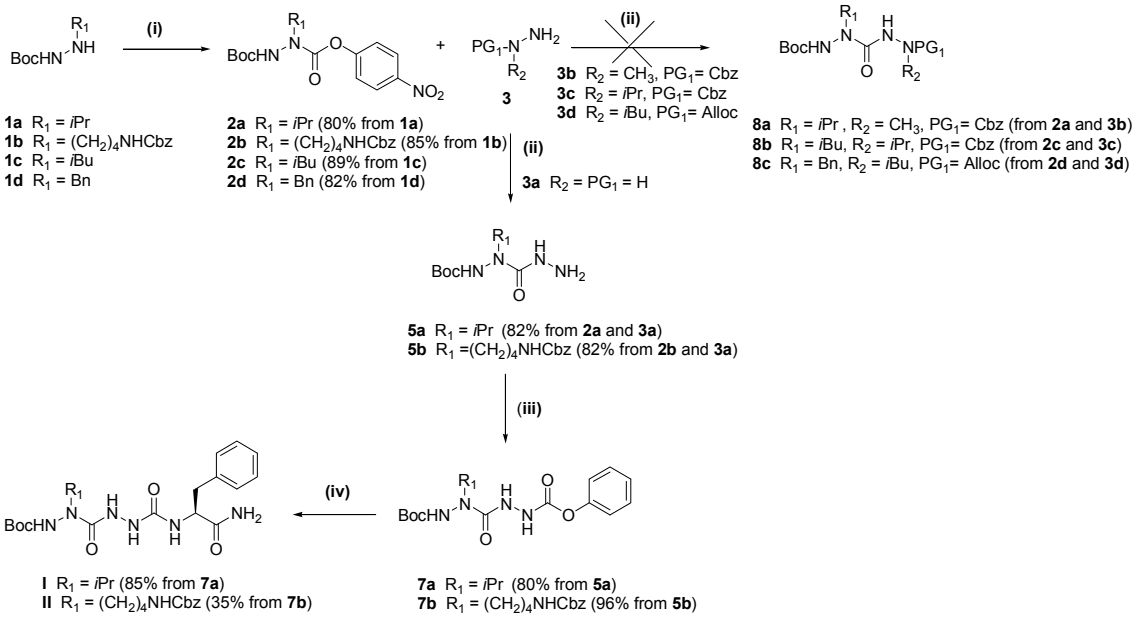
formate, as observed for the synthesis of Boc-azaGly-azaGly-Val-NH_2 **VI** (see above). However, while we obtained **7a** and **7b** in good yields (respectively 80 and 96%), the final coupling of **7a** and **7b** with *L*-Phe-NH_2 afforded **I** in good yield (85%) but **II** in modest yield (35 %). We hypothesized that the presence of the long and bulky Cbz-4-amino-butyl in **7b** may prevent the efficiency of the coupling. Unfortunately, the reaction of **2a**, **2c** and **2d** with **3b**, **3c** and **3d** respectively in order to obtain **8a, 8b** and **8c** did not occur under the conditions previously used for adding hydrazine hydrate **3a** to **2a** and **2b** (MeOH overnight at room temperature). Other conditions have been tried by changing either the solvent (DMF or DCM), or the temperature (room temperature or at 40°C), and in the presence or in the absence of DMAP. None of these conditions allowed to obtain the expected diaza-peptides **8a**-**8c**. We thus suspected that this failure could be due to the lower nucleophilicity of the protected-1-alkyl-hydrazines **3b**-**d** compared to that of hydrazine hydrate **3a** and also to the steric hindrance of their protecting and alkyl groups.



Scheme 3. Strategy A)**:** activation of the protected 2-alkyl-hydrazine **1** followed the reaction with the protected 1-alkyl-hydrazine **3**. Access to Boc-azaVal-azaGly-Phe-NH_2 (**I**) and Boc-azaLys-azaGly-Phe-NH_2 (**II**).

Reagents and conditions: i) 4-nitrophenyl chloroformate, pyridine, DCM, r.t., overnight; ii) MeOH, r.t. overnight; iii) phenyl chloroformate, pyridine, DCM, r.t., 20 min; iv) *L*-Phe-NH_2, triethylamine, ACN, r.t., 3 days.

Thus, in order to access to Boc-azaVal-azaAla-Val-NH_2 (**III**), Boc-azaLeu-azaVal-His-OCH_3 (**IV**) and Boc-azaPhe-azaLeu-Val-OCH_3 (**V**), we evaluate the second route B, i.e. the activation of the protected 1-alkyl-hydrazine **3** followed by the reaction with the protected 2-alkyl-hydrazine **1** (Scheme 4). The

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activation of the protected-1-alkyl-hydrazines **3b, 3c** and **3d** using 4-nitrophenyl chloroformate afforded the intermediates **4a**, **4b** and **4c** in good yields (93% and 82% and 73% respectively). The subsequent reaction of **4a** and **4c** with Boc-2-alkyl-hydrazines **1a** and **1d** afforded **8a** and **8c** respectively in good yields (80% and 62%, respectively). However, the yield of coupling between **4b** and **1c** to afford **8b** was very modest (28%). We decided to test the activation of **3c** with bis(pentafluorophenyl)carbonate, in order to increase the electrophilic character of the carbonyl group, but the yield of the subsequent reaction with **1c** remained similarly low. However, overall it is noteworthy that activating first the protected 1-alkyl-hydrazine **3** followed by the reaction with the protected 2-alkyl-hydrazine **1** (route B) is more convenient than the previous route A in the case of substituted and hindered alkyl hydrazines, maybe because the protected 2-alkyl-hydrazines **1** are more nucleophilic than the protected 1-alkyl-hydrazines **3**. We continued the synthesis from **8a** and **8c** to obtain the final compounds **III** and **V**. Thus, the cleavage of the Cbz group of **8a** and of the Alloc group of **8c** afforded **5c** and **5d** in satisfactory yields (95% and 51% respectively, scheme 4). Then, in a first attempt, we followed our previous strategy by activating first **5c** and reacting then with the natural amino acid *L*-Val-NH_2 **6a**. However, while the activation of **5c** using phenyl chloroformate or 4-nitrophenyl chloroformate was satisfactory, the reaction with the amino acid Val-NH_2 **6a** in the presence of TEA and in dry acetonitrile as described previously (in scheme 3 for **I** and **II**), or by adding also DMAP, did not afford the final compound **III**. Thus, we evaluated the activation firstly of *L*-Val-NH_2 **6a** with 4-nitrophenyl chloroformate in dry DMF to afford **10a** that was not isolated and directly reacted in a one pot manner with **5c** in the presence of DMAP. The final aza-tripeptide **III** was obtained with a satisfactory yield of 60%. Similarly, *L*-Val-OCH_3 **6b** was activated to afford **10c** that reacted with **5d** to give the final aza-tripeptide **V** in modest yield (35%, Scheme 4).

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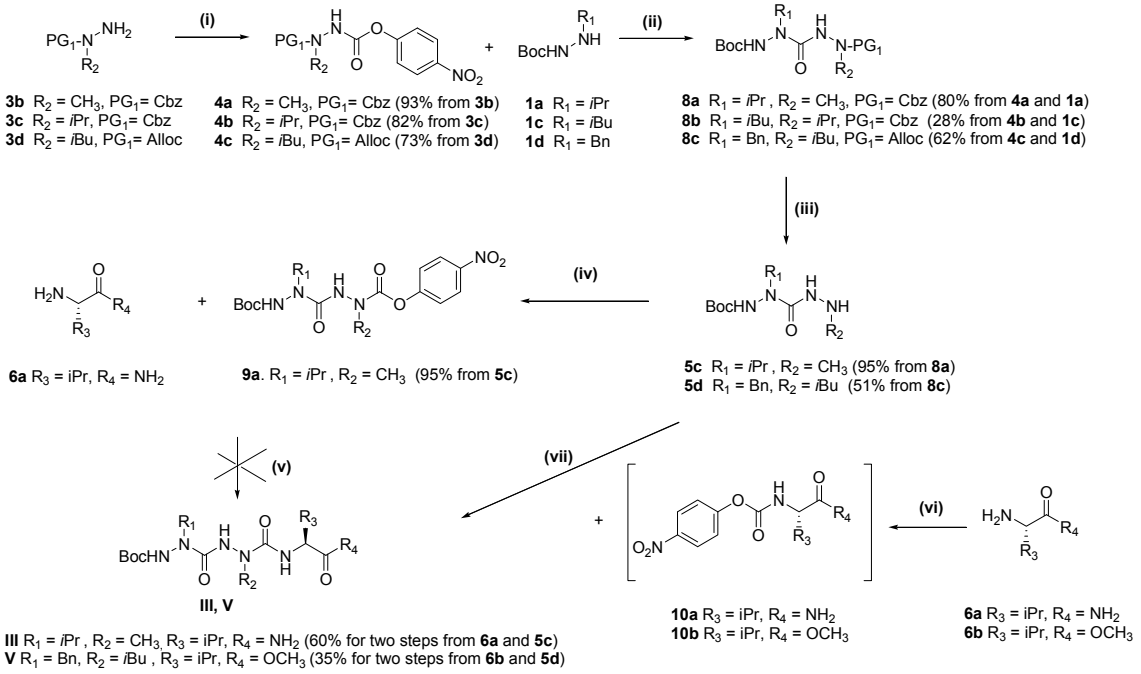

Scheme 4: Strategy B)**:** activation of the protected 1-alkyl-hydrazine followed the reaction with the protected 2-alkyl-hydrazine. Access to Boc-azaVal-azaAla-Val-NH_2 (**III**) and Boc-azaPhe-azaLeu-Val-OCH_3 (**V**).

Reagents and conditions: i) 4-nitrophenyl chloroformate, pyridine, DCM, r.t., overnight; ii) DMAP, DMF, r.t., overnight; iii) Pd/C 10%, H_2, MeOH, r.t., overnight from **8a** or $[Pd(PPh_3)_4]$/NDMBA, DCM, 35°C,4h from **8c**; iv) 4-nitrophenyl chloroformate, pyridine, DCM, r.t., overnight; v) triethylamine, ACN, r.t., 3 days; vi) 4-nitrophenyl chloroformate, pyridine, DMF, r.t., overnight; vii) DMAP, DMF, r.t., overnight

Due to the poor yield for the formation of the Boc-azaLeu-azaVal intermediate **8b**, the strategy B was given up for the synthesis of the final compound Boc-azaLeu-azaVal-His-OCH_3 **IV**. Thus, we evaluated a third and alternative route to synthesize **IV**, starting from the preparation of the *C*-terminal azaVal-His-OCH_3 dipeptide **12** and its final coupling with the *N*-terminal Boc-azaLeu **1a** (Scheme 5, strategy C).

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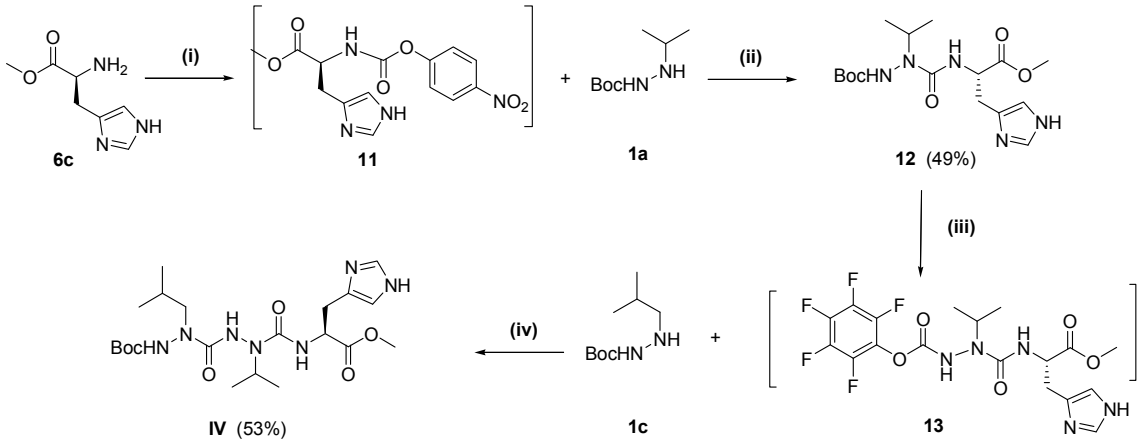

Scheme 5: Strategy C)**:** activation of the natural amino acid followed by the reaction with the protected 2-alkyl-hydrazine. Access to Boc-azaLeu-azaVal-His-OCH_3 (**IV**).
Reagents and conditions: i) 4-nitrophenyl chloroformate, pyridine, DMF, 40° C, overnight; ii) DMAP, DMF, r.t., overnight; iii) a) HCl 4M dioxane, r.t., overnight; b) bis(pentafluorophenyl)carbonate, DIPEA, DMAP, DCM, r.t., 40 min.; iv) DMAP, DCM, r.t., 4 days.

The activation of the natural amino acid *L*-His-OCH_3 **6c** was more efficient when performed at 40°C than at room temperature, and the subsequent coupling with **1a** in a one pot manner gave Boc-azaVal-His-OCH_3 **12** in 49% yield. We also tried the activation of **6c** using bis(pentafluorophenyl)carbonate, but the yield of the coupling reaction with **1a** was even lower (15%). The inverse pathway i.e. the reaction of the activated **2a** (obtained from **1a**, see scheme 3) with *L*-His-OCH_3 **6c** did not afford Boc-azaVal-His-OCH_3 **12.** After the cleavage of the Boc moiety of **12** in acidic conditions, the activation of the hydrazine moiety using bis(pentafluorophenyl)carbonate in dry DCM in the presence of DMAP and DIPEA, and the subsequent reaction with **1c** gave the final compound **IV** with a satisfactory yield of 53% (for 3 steps). The activation with 4-nitrophenyl chloroformate did not allow us to obtain the desired compound **IV**. Overall, this third strategy of synthesis, starting from the preparation of first the *C*-terminal azaVal-His-OCH_3 dipeptide and its final coupling with the *N*-terminal Boc-azaLeu was more convenient than the previous route starting from Boc-azaLeu-azaVal and coupling with the C-terminal *L*-His-OCH_3. However, it is important to note that this strategy is substrate-dependent. In fact, we tried to apply this third strategy to obtain the final compound **V**. We prepared Boc-azaLeu-Val-OCH_3 in satisfactory yield (48%) but unfortunately the last coupling with Boc-azaPhe to obtain Boc-azaPhe-azaLeu-Val-OCH_3 **V** was unsuccessful (the activation was done using either bis(pentafluorophenyl)carbonate or 4-nitrophenyl chloroformate).

3. Conclusion

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As mentioned in the introduction, before starting this study, we wondered about the scarcity of examples of the incorporation of two or more aza-amino acids consecutively in aza-peptides while the introduction of one aza-amino acid has demonstrated increased interest in biologically active peptides. As demonstrated in this work, the reactivity of alkyl-hydrazides is highly susceptible to the nature and the bulkiness of the alkyl chain. This capricious reactivity has been observed both in the case of the synthesis of azaAA-azaAA and also for the final coupling with the natural amino acid at the *C*-terminal position of the di-aza-amino acids containing tripeptides. However, from this work, we can draw some general guidelines. i) Concerning the synthesis of the diaza-peptides (**5**), the activation of the protected 1-alkyl-hydrazine **3** followed by the reaction with the protected 2-alkyl-hydrazine **1** (route B) is more efficient than the activation of the protected 2-alkyl-hydrazine **1** followed the reaction with the protected 1-alkyl-hydrazine **3** (route A), because the protected 2-alkyl-hydrazines **1** are more nucleophilic than the protected 1-alkyl-hydrazines **3**. ii) Concerning the synthesis of the final tripeptide analogues, the activation of the natural amino acid followed by the reaction with the diaza-peptide is more efficient than the activation of the diaza-peptide followed by the reaction with the amino acid. This observation indicates that unless expected, the nucleophilicity of the alkyl-hydrazides is not a real restraint. iii) Also concerning the synthesis of final tripeptide analogues, the strategy of coupling the *N*-terminal azaAA-azaAA dipeptide analogue to a *C*-terminal natural amino acid is more efficient than coupling the *N*-terminal azaAA to the *C*-terminal azaAA-AA dipeptide analogue, but this last strategy was more efficient for **IV**. The bulkiness of the alkyl-hydrazides is a real constraint, as illustrated by the easy synthesis of the two aza-Gly containing tripeptide **VI** and the easier preparation of one aza-Gly containing tripeptides **I** and **II** compared to that of **III**, **IV** and **V**. Concerning the activation, the use of 4-nitrophenyl chloroformate, in dry DMF with DMAP is efficient for the more challenging coupling reactions. However, when this strategy is not successful, the replacement of 4-nitrophenyl chloroformate by bis(pentafluorophenyl)carbonate, in dry DCM and in the presence of DMAP, can be more efficient.

4. Experimental

4.1. General Experimental Methods.

Usual dry solvents were purchased from commercial sources. 4-nitrophenyl chloroformate, hydrazine monohydrate, phenyl chloroformate, benzyl chloroformate, bis(pentafluorophenyl) carbonate, 4-aminobutyraldehyde diethylacetal, *L*-Phe-NH2 *L*-His-OMe, *L*-Val-NH_2, and *L*-Val-OCH_3 were purchased from commercial sources. Benzyl 2-hydroxypyrrolidine-1-carboxylate was prepared according to published methods.[10,14] Pure products were obtained after liquid chromatography using Merck silica gel 60 (40−63 μm). TLC analyses were performed on silica gel 60F-250 (0.26 mm thickness) plates. The plates were visualized with UV light (λ = 254 nm) or revealed with a 5% solution of phosphomolybdic acid in EtOH or with a solution of ninhydrin in EtOH. Melting points

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were determined on a Kofler melting point apparatus. Element analyses (C, H, and N) were performed on a PerkinElmer C, H, N Analyzer 2400 at the Microanalyses Service of the Faculty of Pharmacy at Châtenay-Malabry (BioCIS, France). NMR spectra were recorded on an Ultrafield Bruker AVANCE 300 (^{1}H, 300 MHz, ^{13}C, 75 MHz) or on a Bruker Avance 400 (^{1}H, 400 MHz, ^{13}C, 100 MHz). Chemical shifts δ are in ppm, and the following abbreviations are used: singlet (s), broad singlet (bs), doublet (d), doublet of triplet (dt), triplet (t), multiplet (m). IR spectra were recorded on a Bruker Vector 22 FT-IR spectrometer. Mass spectra were obtained using a Bruker Esquire electrospray ionization apparatus. HRMS were obtained using a TOF LCT Premier apparatus (Waters), with an electrospray ionization source. The purity of compounds was determined by HPLC using the 2695 Alliance system (Waters) and a Sunfire column (C_{18}, 3.5 µm, 150 mm × 2.1 mm); mobile phase, MeCN/H_2O + 0.1% formic acid from 5 to 100% in 20 min; detection at 257 nm; flow rate 0.25 mL/min.

4.2. Synthesis

Strategy A:

A1/ General procedure for the preparation of 2a-d: To a solution of the carbazate **1a, 1b**, **1c** or **1d** in dry DCM (1eq.), was added pyridine (5 eq.) at 0 °C under argon atmosphere. After 10 minutes, 4-nitrophenyl chloroformate (1.1 eq.) was introduced slowly and the reaction was stirred at room temperature overnight. After evaporation of the volatiles under vacuum, the residue obtained was taken up with EtOAc. The organic phase was successively washed with 10% aqueous citric acid, distilled water, 10% aqueous K_2CO_3, brine, dried over Na_2SO_4, filtered and concentrated. The intermediate **2a**, **2b, 2c** or **2d** could be used in the next step without any further purification.

A2/ General procedure for preparation of 5a-b: To a solution of **2a** or **2b** (1 eq.) in dry MeOH was added hydrazine monohydrate (6 eq.). The reaction mixture was stirred at room temperature for one night. After evaporation of the volatiles under vacuum, the crude material obtained was purified by column chromatography on silica gel using the appropriate solvent to yield the pure compound.

A3/ General procedure to preparation of 7a-b: To a solution of **5a** or **5b** (1eq.) in dry THF under argon atmosphere was added at 0 °C pyridine (2.5 eq.). After 10 minutes, phenyl chloroformate (1.1 eq.) was introduced slowly and the reaction was stirred at room temperature 20 minutes. After evaporation of the volatiles, the crude residue obtained was taken up with EtOAc. The organic phase was successively washed with aqueous 10% citric acid, distilled water, 10% aqueous K_2CO_3, brine, dried over Na_2SO_4, filtered and concentrated. After evaporation under vacuum, the crude residue afforded was purified by column chromatography on silica gel with the appropriate solvent to afford the pure compound.

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A4/ General procedure for preparation of I-II: To a solution of **7a** or **7b** (1eq.) in dry ACN under argon atmosphere was added the *L*-Phe-NH2 hydrochloride (1.1 eq.). Triethylamine (6 eq.) was added and the mixture was stirred at room temperature for three days. The volatiles were removed under vacuum and the crude material was purified by column chromatography on silica gel using the appropriate eluent to yield the pure compound.

Strategy B:

General procedure for preparation of 9a, 4a-c: prepared according to the general procedure A1

B1/ General procedure for preparation of 8a-c: To a solution of **4a**, **4b** or **4c** in dry DMF, were successively added at 0 °C under argon atmosphere, **1a, 1c** or **1d** (1.0 eq.) and DMAP (1.0 eq.) The reaction was stirred overnight at room temperature. After evaporation of the volatiles under vacuum, the crude residue was purified by chromatography on silica gel using the appropriate eluent to yield the pure compound.

B2/ General procedure preparation of III, V: To a solution of corresponding amino-acid **6a** or **6b** (1.1 eq.) in dry DMF at 0 °C were successively added under argon atmosphere DIPEA (2.2 eq.) and 4-nitrophenyl chloroformate (1.1 eq.). The solution was stirred at room temperature for one night and after formation of carbamate **10a**, or **10b** monitored by TLC, a solution of the previously prepared **5c** or **5d** (1.0 eq.) and DMAP (1.0 eq.) were added to the reaction mixture. After stirring overnight at room temperature, the volatiles were removed under vacuum, and the crude material obtained was purified by chromatography on silica gel using the appropriate eluent to yield the pure compound.

***Tert*-butyl 2-isopropylhydrazine-1-carboxylate (1a):** To a solution of *tert*-butyl-carbazate (2 g, 15.1 mmol, 1.0 eq.) in 20 mL of dry THF under argon atmosphere was successively added under argon atmosphere acetone (3.3 mL, 45.3 mmol, 3 eq.) and acetic acid (1.05 mL, 13.6 mmol, 0.9 eq.). After 3 h the volatiles were removed under vacuum and the remaining oil was dissolved in 100 mL of dry THF. $NaBH_3CN$ was then added with some seeds of bromocresol green. A solution of para-toluene-sulfonic acid (2.86 g, 16.6 mmol, 1.1 eq.) in 4 mL of dry THF was added dropwise and the mixture became yellow. After 1 h the resulting suspension was removed under vacuum. The remaining colourless powder was dissolved in a mixture of $EtOAc/NaCl_{aq}$, extracted with EtOAc and the organic layer washed with a mixture of aqueous $NaCl/NaHCO_3$, 1/1. The organic phase was dried over Na_2SO_4, filtered and—concentrated under reduced pressure. dried over Na_2SO_4, filtered and concentrated. The crude material obtained was then dissolved in 10 mL of MeOH and NaOH 1M (18 mL, 18.1 mmol, 1.2 eq.). At that time, the solution became successively blue and colourless. After stirring 1 hour at room temperature and evaporation of the volatiles under vacuum, the oil afforded

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was taken up in EtOAc, washed with brine, dried over Na_2SO_4 filtrated and concentrated. Purification by column chromatography on silica gel using DCM/MeOH 98:2 as eluent gave **1a** (1.37g, 7.9 mmol, 52%) as an oil which crystallized by cooling. R_f = 0.50 (DCM/MeOH 98:2); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.31 (2H, bs), 3.28 (1H, dt, *J* = 12.26 and 6.3 Hz), 1.47 (9H, s), 1.12 (6H, d, *J* = 6.4 Hz), ^{13}C NMR (75 MHz, $CDCl_3$): δ = 156.9, 80.3, 50.7, 28.3, 20.6; HRMS (TOF ESI, ion polarity positive): m/z 197.1266 calcd for $[C_8H_{18}N_2O_2 + Na]^+$, found: 197.1266.

***Tert*-butyl 2-(4-(((benzyloxy)carbonyl) amino) butyl) hydrazinecarboxylate (1b): 1b** was synthesized following the same procedure described for **1a** from *t*ert-butyl-carbazate (450 mg, 3.4 mmol, 1.0 eq.) and benzyl 2-hydroxypyrrolidine-1-carboxylate (750 mg, 3.4 mmol) to give after purification by flash chromatography on silica gel using EtOAc/hexane 6:4 as eluent a white solid (562 mg, 1.7 mmol, 49%). R_f = 0.85 (EtOAc/hexane 6:4); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.31 (5H, m), 5.74 (1H, bs), 5.21 (1H, bs), 5.07 (2H, s), 3.44 (1H, bs), 3.18 (2H, d, *J* = 5.8 Hz), 2.87 (2H, t, *J* = 6.5 Hz), 1.51 (4H, m), 1.44 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 203.5, 156.6, 136.6, 128.5, 128.1, 84.0, 66.7, 51.1, 40.3, 28.1, 26.7, 21.5; HRMS (TOF ESI, ion polarity positive): m/z 244.0950 calcd for $[C_{17}H_{27}N_3O_4 + Na]^+$: found: 244.0942.

***T*ert-butyl 2-isobutylhydrazine-1-carboxylate (1c): 1c** was synthesized following the same procedure described for **1a** from *tert*-butyl-carbazate (1 g, 7.6 mmol, 1.0 eq.) in 15 mL of dry THF and isobutyraldehyde (1.0 mL, 11.4 mmol, 1.5 eq.) to afford after purification by flash chromatography on silica gel using DCM 100% as eluent as a colourless oil (943 mg, 5.0 mmol, 66%)**.** R_f = 0.10 (DCM 100%); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.3 (1H, bs), 5.25 (1H, bs), 2.65 (2H, d, *J* = 6.8 Hz), 1.73 (1H, dd, *J* = 13.6 and 6.7 Hz), 1.44 (9H, s), 0.91 (6H, d, *J* = 6.7 Hz); ^{13}C NMR (75MHz, $CDCl_3$): δ = 157.3, 81.0, 60.4, 28.8, 27.1, 21.0; HRMS (TOF ESI, ion polarity positive): m/z 189.1603 calcd for $[C_9H_{20}N_2O_2+H]^+$, found: 189.1600

***T*ert-butyl 2-benzylhydrazine-1-carboxylate (1d): 1d** was synthesized following the same procedure described for **1a** from *tert*-butyl-carbazate (1 g, 7.6 mmol, 1 eq.) in 10 mL of dry THF and benzaldehyde (0.77 mL, 7.60 mmol, 1 eq.) to afford after purification by flash chromatography on silica gel using c-Hex/AcOEt 8:2 as eluent a colourless oil (1.19 g, 5.32 mmol, 71%). R_f = 0.35 (c-Hex /EtOAc 9:1); ^{1}H NMR (300 MHz, CD_3OD): δ = 7.36-7.26 (5H, m); 3.90 (2H, s); 1.44 (9H, s) ppm; ^{13}C NMR (75 MHz, CD_3OD): δ = 156.6, 137.5, 129.0, 128.5, 127.5, 80.6, 55.9, 28.3 ppm; HRMS (TOF ESI, ion polarity positive): m/z 245.1266 calcd. for $[C_{12}H_{18}N_2O_2 + Na]^+$, found: 245.1255.

2-(*Tert*-butyl) 1-(4-nitrophenyl)1-isopropylhydrazine-1,2-dicarboxylate (2a): 2a was obtained from **1a** (500 mg, 2.87 mmol) as a yellow powder (779 mg, 2.30 mmol, 80%) following the general

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procedure A1. R_f = 0.6 (c-Hex /EtOAc 7:3); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.25 (2H, d, *J* = 9.0 Hz), 7.30 (2H, d), 6.36 (1H, bs,), 4.5 (1H, dt, *J* = 12.0 and 6.0 Hz), 1.47 (9H, s), 1.23 (6H, d, *J* = 6.3 Hz) ; ^{13}C NMR (75 MHz, $CDCl_3$): δ = 125.7, 125.3, 122.5, 82.1, 50.4, 28.2, 19.6; MS (ESI, ion polarity positive): m/z 340.14 calcd for $[C_{15}H_{21}N_3O_6 +H]^+$, found: 340.14.

2-(*Tert*-butyl1-(4-nitrophenyl)1-(4-(((benzyloxy)carbonyl)amino)butyl)hydrazine1,2-dicarboxylate (2b): 2b was obtained from **1b** (443.1 mg, 1.31 mmol) as a white solid (560 mg, 1.12 mmol, 85%), following the general procedure A1. R_f = 0.85 (EtOAc/hexane 6:4); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.24 (2H, d, *J* = 9.0 Hz), 7.32 (7H, m),6.83 (1H, bs), 5.13 (2H, s), 4.92 (1H, bs), 3.71 (2H, m), 3.24 (2H, s), 1.68 (4H, m), 1.46 (9H, s); ^{13}C NMR (75MHz, $CDCl_3$): δ = 156.4, 155.3, 154.9, 153,8, 145.2,136.5, 128.5, 128.1, 125.1, 122.3, 122.1, 82.0, 66.8, 50.1, 40.5, 28.1, 27.0, 24.0; HRMS (TOF ESI, ion polarity positive): m/z 525.1961 calcd for $[C_{24}H_{30}N_4O_8 +Na]^+$, found: 525.1964.

2-(*Tert* -butyl) 1-(4-nitrophenyl) 1-isobutylhydrazine-1,2-dicarboxylate (2c). 2c was obtained from **1c** (200 mg, 1.06 mmol) as a colourless oil (335 mg, 0.95 mmol, 89%) following the general procedure A1. R_f = 0.3 (EtOAc/Cyclo 2:8); ^{1}H NMR (300 MHz, $CDCl_3$)**:** δ = 8.34 (2H, d, *J* =9.7 Hz), 7.49 (2H, d, *J* = 9.7 Hz), 7.30 (1H, bs), 3.46 (2H, m), 2.01 (1H, m), 1.48 (9H, s), 0.98 (6H, d, *J* = 6.4 Hz); ^{13}C NMR (75 MHz , $CDCl_3$): δ = 155.0, 150.2, 146.0, 125.7, 122.4, 77.4, 58.2, 28.31, 26.8, 20.1 ; HRMS (TOF ESI, ion polarity positive): m/z 376.1485 calcd for $[C_{16}H_{23}N_3O_6 +Na]^+$, 376.1485, found: 376.1479.

2-(*T*ert-butyl) 1-(4-nitrophenyl) 1-benzylhydrazine-1,2-dicarboxylate (2d): 2d was obtained from **1d** (67 mg, 0.30 mmol) as a yellow oil (95 mg, 0.25 mmol, 82%) following the general procedure A1. R_f = 0.55 (7:3 c-Hex /AcOEt) ; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.28 (2H, d, *J* = 9.0 Hz), 7.37 (7H, m), 6.52 (1H, bp), 4.83 (2H, m), 1.26 (9H, s); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 151.5, 129.3, 128.96, 128.7, 128.3, 127.9, 125.3, 122.4, 82.5, 52.5, 28.3.

Benzyl 1-methyl hydrazinecarboxylate (3b): To a solution of methyl hydrazine (2.0 mL, 38.0 mmol, 1.0 eq.) in DCM (20 mL) cooled at 0 °C was added slowly an aqueous solution of NaOH (30.4 mL, 30.4 mmol, 0.8 eq.). The biphasic mixture was stirred rapidly for 5 min and at that time, benzyl chloroformate (4.3 mL, 30.4 mmol, 0.8 eq.) was added dropwise. The reaction was gradually warmed to room temperature and stirred for 4h. After separation of the biphasic mixture, the isolated organic layer was successively washed with water, brine, dried over Na_2SO_4, filtered and concentrated under vacuum. Purification of the crude oil by distillation (bp 104-134 °C, 0.2 mmHg) provided pure product **3b** as colourless oil (2.56 g, 14.2 mmol, 74%); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.33 (5H, m), 5.12 (2H, s), 4.38 (2H, s), 3.13 (3H, s); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 136.4, 128.5, 128.2, 128.0, 67.7,

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38.4; HRMS (TOF ESI, ion polarity positive): m/z 181.0977 calcd for $[C_9H_{12}N_2O_2 +H]^+$, found: 181.0974; m/z 203.0796 calcd for $[C_9H_{12}N_2O_2 +Na]^+$, found: 203.0798.

Benzyl 1-isopropylhydrazinecarboxylate, HCl (3c): To a solution of **1a** (500 mg, 2.87 mmol, 1 eq.) in 10 mL of dry DCM was successively added at 0 °C TEA (0.774 mL, 5.74 mmol, 2eq.) then benzyl chloroformate (0.819 mL, 5.74 mmol, 2 eq.). A precipitate was formed. The reaction was stirred overnight at room temperature. The volatiles were evaporated under vacuum and the crude material obtained was taken up with EtOAc. The organic layer was washed successively with an aqueous saturated solution of NH_4Cl, brine, dried over Na_2SO_4, filtered and concentrated under vacuum. The resulting crude material was purified by column chromatography on silica gel using c-Hex /EtOAc 9:1 as eluent to afford a colourless oil (0.800 g, 2.60 mmol, 1 eq., 90% yield). To a solution of this oil in 5 mL of dry dioxane under argon atmosphere, was added dropwise at 0 °C, HCl 4M in dioxane (13.0 mL, 52.0 mmol, 20 eq.). The reaction was let under stirring overnight at room temperature. After removing the volatiles under vacuum and drying, the hydrochloride salt **3c** was afforded as a colourless oil (636 mg, 2.60 mmol, quantitative). R_f = 0.1 (c-Hex /EtOAc 8:2; ^{1}H NMR (400 MHz, CD_3OD): δ = 7.38 (5H, m); 5.28 (2H, s), 3.62 (1H, m), 1.32 (3H, d, J = 6.6 Hz), 1.29 (3H, d, J = 6.9 Hz); ^{13}C NMR (100 MHz, CD_3OD): δ = 155.6, 136.9, 129.5, 129.4, 129.1, 70.0, 55.4, 19.2, 17.2; HRMS (TOF ESI, ion polarity positive): m/z 209.1290 calcd for $[C_{11}H_{16}N_2O_2 +H]^+$, found: 209.1284.

Allyl 1-isobutylhydrazine-1-carboxylate, HCl (3d): To a solution of **1c** (0.300 g, 1.60 mmol, 1.0 eq.) in dioxane, was added Na_2CO_3 (450 mg, 4.25 mmol, 2.6 eq.) dissolved in 3 mL of water. At that time, allyl chloroformate (0.153 mL, 1.44 mmol, 0.9 eq.) was added dropwise at 0 °C. The mixture was stirred 30 min at this temperature and then overnight at room temperature. Water was added, and the organic phase was extracted with diethyl ether. The organic layer was dried over Na_2SO_4, filtrated and the volatiles were removed under reduced pressure. The resulting crude material was purified by column chromatography on silica gel using c-Hex /EtOAc 8:2 as eluent to afford a white powder of the Boc allyl intermediate (425 mg, 1.5 mmol, 98%). To a solution of this powder (39 mg,1.44 mmol, 1.0 eq.) in dioxane under argon atmosphere was added dropwise at 0 °C HCl 4M in dioxane (7.2 mL, 28.8 mmol, 20 eq.). The reaction was let under stirring overnight at room temperature. The volatiles were removed under reduced pressure and the powder was dried under vacuum to yield the hydrochloride salt **3d** as a colourless oil (300 mg, 1.44 mmol, quantitative). R_f = 0.1 (c-Hex /EtOAc 8:2); ^{1}H NMR (300 MHz, CD_3OD): δ = 5.97 (1H, dq, J = 10.7, 5.8 Hz), 5.32 (2H, dd, J = 27.7, 13.9 Hz), 4.71 (2H, d, J = 5.7 Hz), 3.42 (2H, d, J = 7.6 Hz), 1.97 (1H, m), 0.94 (6H, d, J = 6.6 Hz); ^{13}C NMR (75 MHz, CD_3OD): δ = 132.9, 119.5, 69.1, 57.2, 28.1, 19.8; HRMS (TOF ESI, ion polarity positive): m/z 173.1290 calcd. for $[C_8H_{16}N_2O_2 +H]^+$, found: 173.1290

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1-Benzyl 2-(4-nitrophenyl) 1-methylhydrazine-1,2-dicarboxylate (4a): 4a was obtained as a yellow oil (886.3 mg, 2.57 mmol, 93%) following the general procedure A1 from **3b** (500 mg, 2.77 mmol). R_f = 0.40 (c-Hex /EtOAc 6:4); ^{1}H NMR (300 MHz,$CDCl_3$): δ = 8.23 (2H, d, *J* = 7.9 Hz), 7.32 (8H, m and bs), 5.21 (2H, s), 3.29 (3H, s); ^{13}C NMR (75MHz, $CDCl_3$): δ = 155.1, 145.2, 135.6, 128.6, 125.2, 121.9, 128.5, 128.1, 68.6, 38.1; HRMS (TOF ESI, ion polarity positive): m/z 368.0859 calcd for $[C_{16}H_{15}N_3O_6 +Na]^+$, found: 368.0852.

1-Benzyl 2-(4-nitrophenyl) 1-isopropylhydrazine-1,2-dicarboxylate (4b): 4b was obtained as a colourless oil (133 mg, 0.35 mmol, 89%) following the general procedure A1 from **3c** (0.1 g, 0.4 mmol, 1 eq)**.** R_f = 0.2 (c-Hex /EtOAc 8:2); ^{1}H NMR (300MHz, $CDCl_3$): δ = 8.23 (2H, m), 7.36 (7H, m), 7.17 (1H, bp), 5.21 (2H, s), 4.53 (1H, m), 1.23 (6H, m); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 128.8, 128.3, 125.2, 122.0, 121.8, 68.5, 19.8; HRMS (TOF ESI, ion polarity positive): m/z 396.1172 calcd for $[C_{18}H_{19}N_3O_6 +Na]^+$, found: 369.1165.

1-Allyl 2-(4-nitrophenyl) 1-isobutylhydrazine-1,2-dicarboxylate (4c): 4c was obtained as a colourless oil (354 mg, 1.05 mmol, 73%) following the general procedure A1 from **3d** (0.3 g, 1.44 mmol, 1 eq.). R_f = 0.1 (c-Hex /EtOAc 8:2), ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.26 (2H, d, *J* = 9.0 Hz), 7.36 (2H, d, *J* = 8.7 Hz), 7.07 (1H, bp), 5.93 (1H, ddd, *J* = 22.3, 10.5, and 5.4 Hz), 5.29 (2H, dd, *J* = 25.1, 13.9 Hz), 4.67 (2H, d, *J* = 5.5 Hz), 3.42 (2H, d, *J* = 7.1 Hz), 1.68 (1H, m), 0.97 (6H, d, *J* = 6.7 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 155.3, 140.9, 132.1, 125.4, 122.0, 118.4, 67.4, 58.0, 27.0, 20.1. MS (ESI, ion polarity positive): m/z 697.26 calcd for $[2xC_{15}H_{19}N_3O_6 +Na]^+$, found: 697.26.

***Tert*-butyl-2-(hydrazinecarbonyl)-2-isopropylhydrazine-1-carboxylate (5a): 5a** was obtained as a white powder (765 mg, 3.29 mmol, 86%) following the general procedure A2 from **2a** (1.3 g, 3.83 mmol) and **3a** (1.38 ml, 22.9 mmol) after purification by column chromatography on silica gel using EtOAc/MeOH 95:5 as eluent. R_f = 0.3 (EtOAc/MeOH 96:4); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.52 (1H, bs), 6.21 (1H, bs), 4.6 (1H, dt, *J* = 13.4 and 6.7 Hz), 3.01 (2H, bs), 1.47 (9H, s), 1.11 (6H, d, *J* = 6.3 Hz; ^{13}C NMR (75 MHz, $CDCl_3$): δ = 158.2, 158.1, 80.0, 49.3, 28.3, 19.4, 19.2,; HRMS (TOF ESI, ion polarity positive): m/z 233.1614 calcd for $[C_9H_{20}N_4O_3 +H]^+$, found: 233.1604.

***Tert*-butyl 2-(4-(((benzyloxy)carbonyl) amino)butyl)-2-(hydrazinecarbonyl)hydrazinecarboxylate (5b): 5b** was obtained as a white solid (130 mg, 0.33 mmol, 82%) following the general procedure A2 from **2b** (200 mg, 0.4 mmol) and **3a** (0.12 mL, 2.4 mmol) after purification by column chromatography on silica gel using EtOAc/MeOH 9:1 as eluent. R_f = 0.60 (EtOAc/MeOH 9:1; ^{1}H NMR (300 MHz, Acetone–d_6): δ = 8.52 (2H, bs); 7.31 (5H, m), 6.37 (1H, bs), 5.01 (2H, s), 3.43 (2H, m), 3.12 (2H, s), 2.83 (2H, bs), 1.58 (4H, m), 1.44 (9H, s); ^{13}C NMR (75 Hz, Acetone–d_6): δ = 204.9,

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155.9, 155.4, 137.6, 128.2, 127.6, 80.5, 65.4, 47.3, 40.2, 27.5, 26.9, 24.4; HRMS (TOF ESI, ion polarity positive): m/z 396.2247 calcd for $[C_{18}H_{29}N_5O_5 +H]^+$, found: 396.2248.

***T*ert-butyl 2-isopropyl-2-(2-methylhydrazinecarbonyl) hydrazine carboxylate (5c): 8a** (720 mg, 1.89 mmol, 1.0 eq.) was dissolved in MeOH (15 mL). At that time, Pd/C 10% (144 mg, 20% mass) was added and the reaction mixture was kept under stirring overnight at room temperature under hydrogen atmosphere. The reaction mixture was filtered through a pad of Celite and after washing the pad of Celite several times with MeOH, the filtrate was evaporated under vacuum to afford compound **5c** (441 mg, 1.79 mmol, 95%) as white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.79 (2H, bp), 4.57 (1H, dt, *J* = 13.4, 6.7 Hz), 4.35 (1H, bp), 2.58 (3H, s), 1.45 (9H, s), 1.09 (6H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 158.2, 158.2, 81.9, 48.5, 39.7, 28.1, 19. 4, 19.2; HRMS (TOF ESI, ion polarity positive): m/z 257.1770 calcd. for $[C_{10}H_{22}N_4O_3 +H]^+$, found: 257.1775; m/z 269.1590 calcd. for $[C_{10}H_{22}N_4O_3 +Na]^+$, found: 269.1602

***Tert*-butyl 2-benzyl-2-(2-isobutylhydrazine-1-carbonyl)hydrazine-1-carboxylate (5d):** After mixing Tetrakis (triphenylphosphine) palladium (0) (40 mg, 0.034 mmol, 0.05 eq.) and *N*,*N*-dimethylbarbituric acid (323 mg, 2.07 mmol, 3 eq.) under argon atmosphere, **8c** (290 mg, 0.69 mmol, 1.0 eq.) in DCM was added dropwise. After stirring 4h at 35°C, the volatiles were removed under reduced pressure and the remaining oil was taken up in EtOAc. The organic layer was then successively washed with aqueous solution of Na_2CO_3, dried over Na_2SO_4 and filtrated. The volatiles were removed under reduced pressure and the crude material was purified by column chromatography on silica gel using c-Hex / EtOAc 6:4 as eluent to afford **5d** (119 mg, 0.35 mmol, 51%) as a colourless oil. R_f = 0.3 (c-Hex /EtOAc 6:4); ^{1}H NMR (300 MHz,$CDCl_3$): δ = 7.30 (5H, m), 4.66 (2H, bs), 3.74 (3H, m), 3.01 (2H, d, *J* = 6.5 Hz), 2.05 (1H, m), 1.38 (9H, s), 1.02 (6H, d, *J* = 6.6 Hz); ^{13}C NMR (75MHz, $CDCl_3$): δ = 129.3, 128.8, 128.2, 82.6, 59.4, 28.2, 24.8, 20.4; HRMS (TOF ESI, ion polarity positive): m/z 337.2240 calcd for $[C_{17}H_{28}N_4O_3 +H]^+$, found: 337.2244.

Phenyl N-[[(tert-butoxycarbonylamino)-isopropyl-carbamoyl]amino]carbamate (7a): 7a was obtained as a white powder (995 mg, 2.83 mmol, 90%) following the general procedure A3 from **5a** (730 mg, 3.14 mmol) after purification by column chromatography on silica gel using c-Hex /EtOAc 4:6 as eluent. R_f = 0.5 (c-Hex /EtOAc 4:6); ^{1}H NMR (300 MHz,$CDCl_3$): δ = 7.33 (2H, m), 7.18 (4H, m), 6.98 (1H, s), 6.54 (1H, bs), 4.61 (1H, dt, *J* = 13.5 and 6.9 Hz), 1.48 (9H, s), 1.14 (6H, d, *J* = 6.3 Hz); ^{13}C NMR (75MHz, $CDCl_3$): δ = 157.3, 155.7, 150.8, 129.5, 125.8, 121.6, 82.4, 49.5, 28.2, 19.2, 19.6; HRMS (TOF ESI, ion polarity positive): m/z 353.1825 calcd for $[C_{16}H_{24}N_4O_5 +H]^+$, found: 353.1816, m/z 375.1644 calcd for $[C_{16}H_{24}N_4O_5 +Na]^+$, found: 375,1649.

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Phenyl-5-((tert-butoxycarbonyl) amino)-4,11-dioxo-13-phenyl-12-oxa-2,3,5,10-tetraazatridecan-1-oate (7b): 7b was obtained as white solid (158 mg, 0.31 mmol, 96%) following the general procedure A3 from **5b** (128 mg, 0.32 mmol) after purification by column chromatography on silica gel using EtOAc/ c-Hex 6:4 as eluent. R_f = 0.35 (EtOAc/ c-Hex 6:4); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.48 (2H, bs),7.26 (10H, m), 5.31 (1H, bs), 5.08 (2H, s), 3.68 (2H, m), 3.14 (2H, s), 2.53 (1H, bs), 1.33 (13H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 157.9, 156.8, 155.7, 154.8, 150.6, 136.6, 129.3, 128.4, 128.0, 125.7, 121.4, 82.4, 65.6, 48.3, 40.6, 28.1, 26.7, 23.9 ; HRMS (TOF ESI, ion polarity positive): m/z 538.2278 calcd for $[C_{25}H_{33}N_5O_7 +Na]^+$: M/z 538.2278, found: 538.2277.

Benzyl 2-(2-(tert-butoxycarbonyl)-1-isopropylhydrazinecarbonyl)-1-methylhydrazinecarboxylate (8a): 8a was obtained as white solid (777,5 mg, 2.04 mmol, 88%) following the general procedure B1 from **1a** (404 mg, 2.32 mmol) and **4a** (800 mg, 2.32 mmol) after purification by column chromatography on silica gel using EtOAc/ c-Hex 1:1 as eluent. R_f = 0.55 (EtOAc/ c-Hex 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.33 (5H, s), 6.58 (1H, bs), 6.12 (1H, bs),5.15 (2H, s), 4.58 (1H, dt, *J* = 13.3, 6.6 Hz), 3.21 (3H, s), 1.47 (9H, s), 1.13 (6H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 163.3, 157.1, 155.3, 136.0, 128.5, 128.1, 127.9, 82.2, 68.1, 48.9, 38.5, 28.1, 19.5, 19.1; HRMS (TOF ESI, ion polarity positive): m/z 403.1957 calcd for $[C_{18}H_{28}N_4O_5 +Na]^+$, found: 403.1958.

Benzyl 2-(2-(tert-butoxy carbonyl)-1-isobutyl hydrazine-1-carbonyl)-1-isopropyl hydrazine carboxylate (8b): 8b was obtained as a colourless oil (62 mg, 0.02 mmol, 28%) following the general procedure B1 from **1c** (0.126 g, 0.67 mmol) and **4b** (0.25 g, 0.67 mmol) after purification by column chromatography on silica gel using c-Hex /EtOAc7:3 as eluent. R_f = 0.4 (EtOAc 100%); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.31 (5H, m), 5.12 (2H, s, H12), 5.12 (2H, s); 4.40 (1H, m), 3.31 (2H, m), 1.86 (1H, m), 1.48 (9H, s), 1.14 (6H, d, *J* = 6.4 Hz), 0.88 (6H, m); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.2, 156.1, 154.4, 136.6, 128.5, 128.0, 127.7, 82.1, 67.7, 56.1, 50.4, 29.6, 28.1, 26.4, 20.0; HRMS (TOF ESI, ion polarity positive): m/z 423.2607 calcd. for $[C_{21}H_{34}N_4O_5 +H]^+$, found: 423.2613, m/z 440.2873 calcd. for $[C_{21}H_{34}N_4O_5 +NH_4]^+$, found: 440.2871, m/z 445.2427 calcd. for $[C_{21}H_{34}N_4O_5 +Na]^+$: found: 445.2421.

Allyl 2-(1-benzyl-(2-(tert-butoxy carbonyl) hydrazine-1-carbonyl)-1-isobutyl hydrazine-1-carboxylate (8c): 8c was obtained as a colourless oil (94 mg, 0.02 mmol, 62%) following the general procedure B1 from **1d** (80 mg, 0.36 mmol,) and **4c** (121 mg, 0.36 mmol) after purification by column chromatography on silica gel using c-Hex /EtOAc7:3 as eluent. R_f = 0.2 (c-Hex /EtOAc 7:3); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.34 (5H, m), 5.90 (1H, ddd, *J* = 22.3, 10.5, 5.4 Hz); 5.31 (1H, d, *J* = 17.1 Hz), 5.21 (1H, d, *J* = 10.3 Hz), 4.61 (2H, m), 3.39 (2H, d, *J* = 7.0 Hz), 1.93 (1H, m), 1.48 (9H, s), 0.92 (6H, d, *J* = 6.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 156.5, 154.1, 138.4, 135.0, 132.5, 129.0, 118.0,

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82.1, 67.0, 57.9, 51.1, 28.3, 26.9, 20.1; HRMS (TOF ESI, ion polarity positive): m/z 443.2267 calcd. for $[C_{21}H_{32}N_4O_5 + Na]^+$, found: 443.2270.

Strategy C:

(S)-tert-butyl-2-((3-(1H-imidazol-5-yl)-1-methoxy-1-oxopropan-2-yl) carbamoyl)-2-isopropylhydrazinecarboxylate (12): To a solution of histidine-methyl ester (0.50 g, 2.07 mmol, 1.0 eq.) in 10 mL of dry DMF under argon atmosphere was added at 0 °C, pyridine (0.54 mL, 6.60 mmol, 3.2 eq.). The mixture was stirred 30 min at 0 °C and at this time, 4-nitrophenyl chloroformate (0.46 g, 2.28 mmol, 1.1 eq.) in 3 mL of dry DMF was added dropwise. After 5 hours stirring at 40 °C, the reaction was stopped having checked completion by TLC (8:2 EtOAc/MeOH + 0.2 $NH_4OH_{20\%Aq.}$). At this time, DMAP (0.25 g, 2.07 mmol, 1.0 eq.) was added to the previous mixture cooling to 0 °C which became immediately yellow. After stirring for 10 min, **1a** (0.36 g, 2.07 mmol, 1.0 eq.) in dry DMF was added slowly to the reaction and let stirring for 4 days at room temperature under argon atmosphere. After evaporation of the volatiles under reduced pressure, the crude residue was purified by column chromatography on silica gel using first EtOAc 100% then EtOAc/MeOH 8:2 as eluent to give **12** (381 mg, 1.03 mmol, 49%) as a white powder.

R_f = 0.44 (EtOAc/MeOH/$NH_4OH_{20\%Aq}$ 8:1.8:0.2); ^{1}H NMR (400 MHz, CD_3OD): δ = 7.58 (1H, s), 6.62 (1H, s), 4.48 (2H, m), 3.68 (3H, s), 3.06 (2H, m), 1.47 (9H, s), 1.04 (6H, m); ^{13}C NMR (100 MHz, CD_3OD): δ = 181.2, 173.6, 159.1, 136.2, 118.7, 118.2, 82.2, 54. 8, 52.8, 49.7, 30.6, 28.5, 19.9; HRMS (TOF ESI, ion polarity positive): m/z 370.2090 calcd. for $[C_{16}H_{27}N_5O_5 + H]^+$, found: 370.2092.

(1-amino-1-oxo-3-phenylpropan-2-yl)-2-(1-isopropylhydrazine-1-carbonyl)hydrazine-1-carboxamide (I): I was obtained as a white powder (850 mg, 2.01 mmol, 86%) following the general procedure A4 from **7a** (823 mg, 2.34 mmol) and L-PheNH_2 hydrochloride (474 mg, 2.36 mmol) after purification by column chromatography on silica gel using EtOAc/ c-Hex01 1:1 as eluent. R_f = 0.2 (EtOAc/MeOH 95:5); mp = 130–132 °C; ^{1}H NMR (400 MHz, DMF-d_7): 2 conformers, δ = 9.16 (0.5H, bs), 9.03 (1H, bs), 8.82 (0.5H, bs), 7.66 (2H, bs), 7.36-7.22 (5H, m), 7.13 (1H, bs), 7.04 (1H, bs), 6.50 (1H, d, *J* = 7.8 Hz), 4.52 (1H, m), 4.44 (0.5H, m), 4.34 (0.5H, m), 3.28 (0.5H, d, 13.1 Hz), 3.15 (0.5H, d, 13.1Hz), 2.92-2.81 (1H, m), 1.44 (9H, s),1.09 (6H, m); ^{13}C NMR (100 MHz, DMF-d_7): δ = 174.1, 158.9, 158.5, 156.2, 138.7, 129.2, 127.9, 125.9, 79.9, 55.1, 48.5, 37.6, 27.5, 18.6, 19.2; IR: 3255 (ν N–H); 1659 (ν C=O); 1532 (ν C=C); 1155 (ν C–O) cm^{-1}. HRMS (TOF ESI, ion polarity positive): m/z 423.2346 calcd for $[C_{19}H_{30}N_6O_5 + H]^+$, found: 423.2346, m/z 445.2175 calcd for $[C_{19}H_{30}N_6O_5 + Na]^+$, found: 445.2169. HPLC purity (XBridge C18, 3.5µm, H2O + 0.1% form. ac./ACN, gradient 5–100% in 20 min): TR =12.97 min, 100%.

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(S)-*tert*-butyl2-(2-((1-amino-1-oxo-3-phenylpropan-2-yl)carbamoyl)hydrazinecarbonyl)-2-(4-(((benzyloxy)carbonyl)amino)butyl)hydrazinecarboxylate (II): II was obtained as a white solid (143 mg, 0.25 mmol, 35%) following the general procedure A4 from **7b** (360 mg, 0.7 mmol) and L-Phe-NH_2 hydrochloride (160.5 mg, 0.8 mmol) after purification by column chromatography on silica gel using EtOAc /MeOH 95:5 as eluent. R_f = 0.70 (EtOAc/MeOH 95:5); mp = 136–138 ° C ;^{1}H NMR (400 MHz, DMSO-*d6*): δ = 9.14 (1H, bs), 8.65 (1H, bs), 7.75 (3H, bs), 7.35 (5H, m), 7.17-7.23 (5H, m), 7.10 (1H, s), 6.18 (1H, s), 5.01 (2H, m), 4.26 (1H, s), 3.35 (2H, m), 3.00 (2H, m), 2.76-3.06 (2H, m), 1.43 (2H, m), 1.42 (11H, m); ^{13}C NMR (100 MHz, DMSO-*d6*): δ = 173.7, 158.6, 157.9, 156.1, 155.0, 138.1, 129.1, 128.0, 126.1, 137.3, 128.3, 127.7, 80.1, 65.1, 54.4, 47.8, 40.1, 37.8, 28.1, 26.6, 24.0; IR: 3292 (N-H stretch), 2935 (C-H stretch), 1661 (C=O stretch), 1524 (N-H bend), 1454-1368 (C=C stretch and C-N stretch), 1249-1156 (C-C stretch) cm^{-1}; Elemental analysis: Calcd. for $C_{28}H_{39}N_7O_7 \cdot 0.75H_2O$: C 56.12, H 6.83, N 16.37; found: C 56.33, H 6.95, N 16.03. HRMS (TOF ESI, ion polarity positive): m/z 608.2809 calcd for $[C_{28}H_{39}N_7O_7 + Na]^+$, found: 608.2808. HPLC purity (XBridge C18, 3.5 µm, H_2O + 0:1% form. ac./ACN, gradient 5–100% in 20 min): TR = 14.72 min, 98%.

(S)-tert-butyl 2-(2-((1-amino-3-methyl-1-oxobutan-2-yl)carbamoyl-2-methylhydrazinecarbonyl)-2-isopropylhydrazinecarboxylate (III) : III was obtained as a white solid (95 mg, 0.24 mmol, 60%) following the general procedure B2 from **6a** (68.7 mg, 0.45 mmol) and **5c** (100 mg, 0.41 mmol) after purification by column chromatography on silica gel using EtOAc /MeOH 90:10 as eluent.) . R_f = 0.30 (EtOAc/MeOH 90:10); mp = 283–285 °C ^{1}H NMR (400 MHz, DMSO–d_6): δ = 9.14 (1H, bs), 9.01 and 8.92 (1H, 2s), 7.04 and 6.96 (2H, 2 bs), 6.05 (1H, bs), 4.36 (1H, m), 3.89 (1H, m), 2.90 (3H, s), 2.02 (1H, m),1.44 (9H, s), 1.00 (6H, m), 0.84 (6H, m); ^{13}C NMR (DMSO–d6, 100 MHz): δ = 173.8, 158.1, 157.8, 156.1, 80.0, 58.7, 48.4, 35.6, 29.6, 28.0, 19.9, 19.4, 18.8, 17.2 ppm; mp = 283–285 °C; IR: 3417-3197 (N-H stretch); 2978 (C-H stretch); 1710-1653 (C=O stretch); 1533 (N-H bend); 1393-1333 (CH_3 bend); 1275-1157 (C-N stretch) cm^{-1}; Anal.calcd. for $C_{16}H_{32}N_6O_5$: C 49.47, H 8.30, N 21.63, found: C 50.39, H 8.31, N 21.01; HRMS (TOF ESI, ion polarity positive): m/z 389.2512 calcd for $[C_{16}H_{32}N_6O_5 + H]^+$, found: 389.2505. HPLC purity (XBridge C18, 3.5 µm, H_2O + 0:1% form. ac./ACN, gradient 5–100% in 20 min): TR = 11.78 min, 100%.

***Tert*-butyl (S)-2-(2-(1H-imidazol-4-yl)-1-methoxy-1-oxopropan-2-yl)-carbamoyl)-2-isopropylhydrazine-1-carbonyl)-2-isobutylhydrazine-1-carboxylate (IV) :** To a solution of **12** (0.35 g, 0.95 mmol, 1.0 eq.) in 5 mL of dry dioxane under argon atmosphere, was added dropwise at 0 °C, HCl 4M in dioxane (4.80 mL, 19.0 mmol, 20 eq). After 3h at room temperature, the reaction was stopped having checked completion by TLC (EtOAc/MeOH 8:2). After removing the volatiles under vacuum and drying, the hydrochloride salt obtained as a white power was used immediately.

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To a solution of this salt (0.327 g, 0.96 mmol, 1.0 eq.) in dry DCM under argon atmosphere were added successively DIPEA (0.45 mL, 2.57 mmol, 3.2 eq.) and DMAP (0.117 g, 0.96 mmol,1eq.). At this time, a solution of bis(pentafluotophenyl)carbonate (0.416 g, 1.06 mmol, 1.1 eq.) in dry DCM was added dropwise. The mixture was stirred 40 min at room temperature and then a solution of compound **1c** (0.181 g, 0.96 mmol, 1.0 eq.) and DMAP (0.117 g, 0.96 mmol, 1.0 eq.) in dry DCM was added dropwise to the previous solution. The mixture was let stirring for 4 days at room temperature under argon atmosphere. After concentration of the compound under reduced pressure, the crude residue was purified by column chromatography on silica gel eluting with EtOAc 100% to afford **IV** (247 mg, 0.51 mmol, 33%) as a colorless oil. δ = 8.09 (1H, bs); 7.67 (1H, bs), 7.25-7.15 (2H, m), 4.71 (1H, m), 4.64 (1H, m), 3.71 (3H, s), 3.48 (1H, m), 3.40 (1H, m), 3.06 (2H, d, J=5.6 Hz), 2.02 (1H, m), 1.36 (9H, s), 1.07 (6H, d, J = 6.7 Hz), 0.98 (6H, d, J = 6.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 173.2, 171.2, 158.7, 158.6, 137.6, 135.4, 116.3, 83.1, 58.5, 53.0, 52.4, 45.7, 30.8, 28.2, 26.2, 20.3, 18.6; IR: 3233 (N-H stretch); 2964 (C-H stretch); 1732-1647 (C=O stretch); 1521 (N-H bend); 1438-1369 (CH_3 bend); 1260-1148 (C-N stretch) cm^{-1} HRMS (TOF ESI, ion polarity positive): m/z 484.2884 calcd for $[C_{21}H_{37}N_7O_6 + H]^+$, found: 484.2892. HPLC purity (XBridge C18, 3.5µm, H2O + 0.1% form. ac./ACN, gradient 5–100% in 20 min): tR = 13.89 min, 100%.

***Tert*-butyl (S)-2-benzyl-2-(2-isobutyl-2-((1-methoxy-3-methyl-1-oxobutan-2-yl) carbamoyl) hydrazine-1-carbonyl) hydrazine-1-carboxylate (V):** To a solution of **6b** (42 mg, 0.25 mmol, 1.0 eq.) in dry DMF under argon atmosphere was added at 0 °C pyridine (80 µL, 1.0 mmol, 4.0 eq) followed by 4-nitrophenyl chloroformate (55 mg, 0.28 mmol, 1.1 eq.). After stirring overnight at room temperature, **5d** (85 mg, 0.25 mmol, 1 eq.) and DMAP (30 mg, 0.25 mmol, 1 eq.) in dry DMF were immediately added dropwise together to the previous mixture and the reaction was kept on stirring overnight at room temperature. After evaporation of the volatiles under reduced pressure, the crude material obtained was purified by column chromatography on silica gel eluting with EtOAc/ c-Hex 4:6. to yield **V** as a colorless oil (43 mg, 0.09 mmol, 35%).

R_f = 0.4 (EtOAc/CyH 4:6); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.38-7.25 (6H, m), 6.22 (1H, s), 5.93 (1H, bs), 4.39 (1H, dd, J = 8.2, 5.4 Hz), 3.69 (3H, s), 3.57 (1H, m), 3.25 (1H, m), 2.13 (1H, m), 1.84 (1H, m), 1.45 (9H, s), 0.92 (12H, m); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.6, 158.3, 156.1, 154.5, 135.3, 129.2, 129.0, 128.3, 83.2, 58.8, 55.7, 52.0, 51.6, 31.3, 28.1, 26.9, 20.2, 19.1, 18.0; IR: 3264 (N-H stretch); 2964 (C-H stretch); 1724-1648 (C=O stretch); 1520 (N-H bend); 1468-1337 (CH_3 bend); 1254-1155 (C-N stretch) cm^{-}1; HRMS (TOF ESI, ion polarity positive): m/z 494.2979 calcd for $[C_{24}H_{39}N_5O_6 + H]^+$, found: 494.2881, m/z 516.2798 calcd for $[C_{24}H_{39}N_5O_6 + Na]^+$, found: 516.2800. HPLC purity (XBridge C18, 3.5µm, H2O + 0.1% form. ac./ACN, gradient 5–100% in 20 min): tR = 17.81 min, 63%.

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Three synthetic routes are studied and compared to introduce into peptides two consecutive aza-amino acids bearing various side chains.