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New β-strand macrocyclic peptidomimetic analogues containing α-(O-, S- or NH-)aryl substituted glycine residues: synthesis, chemical and enzymatic properties

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In so much as bis-macrocyclic peptidomimetics have been recognized as high affinity substrates for HIV-1 protease, we were interested in the design and synthesis of new bis-macrocyclic bioisosteric analogues whose general structure is displayed on Fig. 2. The structures of these new analogues are characterized by the specific replacement of the methylene of the benzyl group directly attached to the *N*-acyl glycine residue in the original molecule **1**, by its main bioisosteres, *i.e.* O-, S- and NH-aryl groups. Knowing that an intermediate in which an heteroatomic aryl group is directly linked to a free amine glycine residue is not stable, we developed an original synthetic pathway which involved the coupling of a specific side chain to the exocyclic carboxylic acid function, followed by an elegant oxidation–nucleophilic substitution Steglich-type reaction. Analogues **2a**–**d** were then submitted to chemical and enzymatic hydrolysis. We demonstrated that, as expected, the specific cleavage of the exocyclic *N*-acyl bond led to the release of aryl moieties (phenol, thiophenol and aniline species). These chemical and enzymatic stability studies brought to light the biological potential of such macrocyclic analogues in infected cells.

Introduction

Proteolytic enzymes of the four major classes (aspartyl, cysteine, metallo and serine proteases) catalyse both intracellular and extracellular peptide cleavages during numerous physiological processes. They are crucial for disease propagation and their selective inhibition appears to be a promising therapeutic route for the treatment of diseases as various as cancers, viral infections, such as HIV, or neurodegenerative disorders.[1] Recently, Fairlie *et al.* provided compelling structural evidence that aspartyl, serine, metallo and cysteine proteases all share a common conformational requirement for recognition namely an extended (β-strand) conformation for their active-site directed inhibitors and substrate analogues.[1–7] This phenomenon of conformational selection, which appears to be common for proteases, can be used for the design of protease inhibitors. Based on this conserved conformational mimicry, macrocyclic peptidomimetics **1** (Fig. 1), designed to fix inhibitors in protease-binding conformations, have been studied.[2] Taking into account the approach proposed by Fairlie *et al.*, we designed and synthesized new HIV-protease substrates **2** whose general structure is given in Fig. 2. Besides the presence of the macrocyclic ring, all the newly synthesized analogues include in their structure a glycine residue substituted on the α-carbon position by phenoxy, thiophenoxy or anilino groups. Ordinarily, a free aminal α-substituted glycine in which the α-carbon is linked to a nitrogen, oxygen or sulfur aryl group, is chemically unstable.

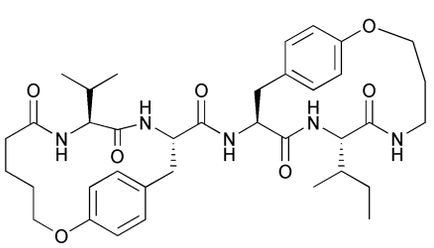

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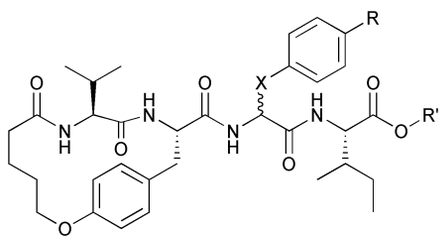
Fig. 1



2a-d

Fig. 2 General structure of new antiprotease substrates. X = O, S, NH; R = H, alkoxy groups; R′ = OH, ester group.

However, *N*-acylated α-substituted glycines of this type[8,9] or even *N*-acylated α-hydroxy glycines[10] have been described in the literature. Acylation of the amino group provides stabilization of the resulting molecule by delocalising the nitrogen electrons into the peptide bond. This synthetic approach that employs α-substitution on a glycine residue in a peptide has already been described by Kingsbury *et al.*[11,12] (Fig. 3) and previously used by our group.[13,14]

Our findings showed that if the exocyclic peptide bond is more specifically cleaved by HIV-1 protease, the result would be the release of the α-substituent. Thanks to this enzymatic specificity, selected phenols,[15] thiophenols or anilines,[16] present as α-heteroatom aryl substituents of the glycine residue could then lead to the release of toxophoric moieties which could affect HIV-1 protease processing and the fate of the infected cell.

In this paper, we report the synthesis of these new analogues, their comparative stabilities towards basic chemical hydrolysis and the HIV-1 protease substrate properties of some of them.

Results and discussion

We first synthesized the bis-macrocyclic substrate **1** as reported by Fairlie *et al.*[2] using an elegant method in which the last step is the coupling reaction between the carboxylic acid function of

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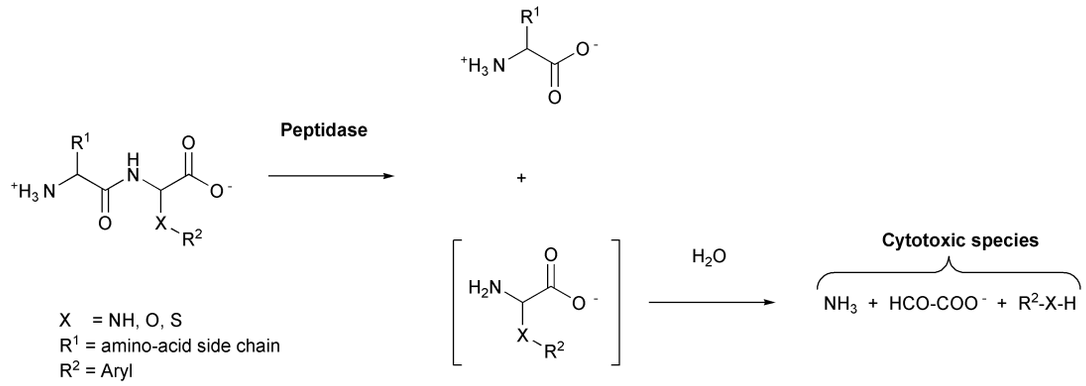
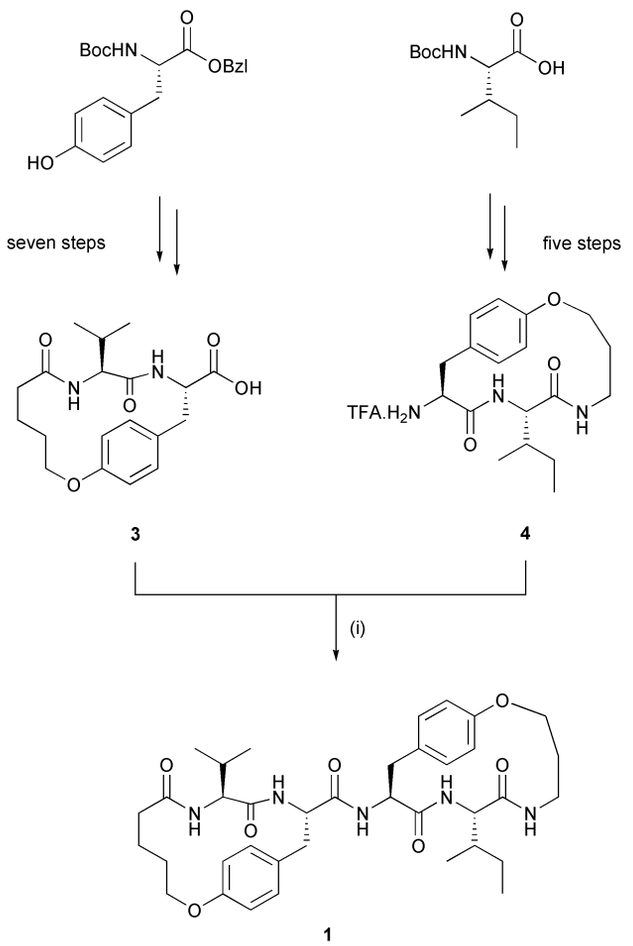


Fig. 3 Structure of α-glycine-substituted peptides and their mode of breakdown after peptidase cleavage.[11–13]



Scheme 1 Synthesis of bis-macrocycle **1**.[2,7] (i) BOP reagent, DIEA, DMF, 12 h; 57%.[2]

macrocycle **3**[2] and the amine function of macrocycle **4**[7] using BOP reagent[17] as coupling agent (Scheme 1).

The cyclic structures of both synthons were confirmed by ^{1}H NMR analysis. Due to their cyclic structures, the resulting compounds are of a rigid shape and consequently, the aromatic protons of the tyrosine residues of each cycle are different resulting in a four-different signal spectrum. We can observe this pattern on the spectrum of the protected molecule which led to the acid synthon **3**. Furthermore, the two β-protons of the tyrosine residue were not equivalent but displayed two different coupling constants with their single α-neighbouring proton: on the one hand, a *syn*-type coupling constant (J = 4.6 Hz) and, on the other hand, an *anti*-type coupling constant (J = 12.3 Hz). We were able to determine the *syn*-type coupling constant (J = 6.3 Hz) for the second macrocyclic *N*-protected species, which led to the TFA salt **4**. Unfortunately, the *anti*-type coupling constant overlapped with the characteristic peaks corresponding to the deuterated NMR solvent, *i.e.* DMSO-d_6.

This synthesis was performed in order to confirm that only the exocyclic amide bond between the two macrocyclic moieties was specifically cleaved upon incubation with HIV-1 recombinant protease. This fact was proven by HPLC analysis using both separate macrocyclic entities **3** and **4** as internal references. In contrast, this bicyclic substrate **1** was found to be very stable under alkaline hydrolysis even after several hours in the presence of a large excess of sodium hydroxide.

Unfortunately, this elegant synthetic method cannot be used for the synthesis of our new bioisosteres **2** (Fig. 2). Indeed, as already mentioned, a free aminal glycine residue bearing a good leaving group at the α-carbon position is chemically unstable, spontaneously decomposing leading to the release of the α-substituent (Fig. 3). For this reason, we cannot perform the synthesis of both separate entities, *i.e.* on the one hand the macrocyclic acid **3** and, on the other hand, the free aminal α-substituted glycine residue, followed by the coupling reaction between these two entities as the final reaction step.

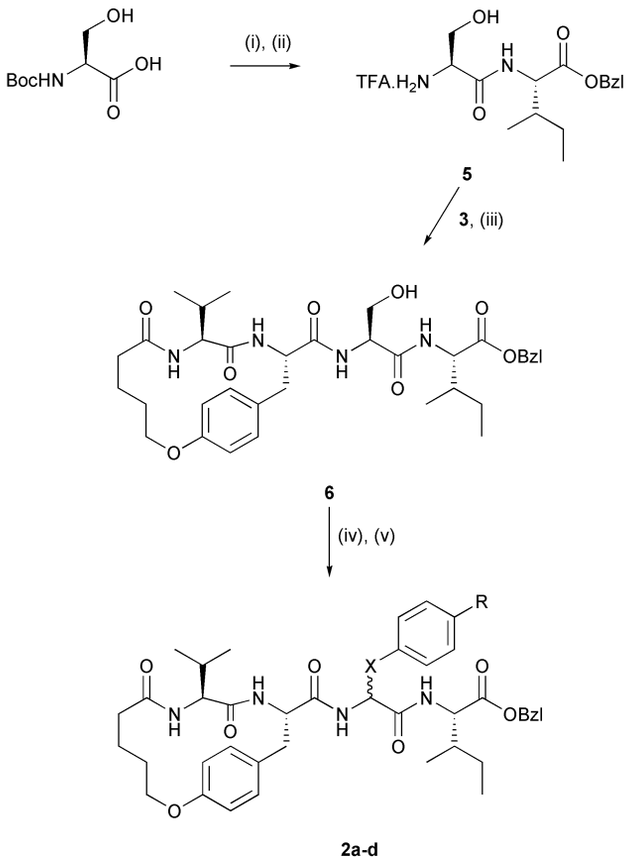
To introduce this α-substituent at this specific position, we were obliged to use a different synthetic pathway involving the synthesis of a linear peptidic side-chained analogue of the macrocyclic carboxylic acid **3**. The very important feature of this original strategy is based on the fact that this side-chain moiety can be modified on the desired position, *i.e.* on the α-carbon position linked to the macrocyclic moiety, allowing the formation of an α-branched side-chain derivative.

For this purpose, the side-chain peptide must bear a function that can be removed by a nucleophilic agent to perform the bioisosteric replacement of the methylene group of the tyrosine moiety by its bioisosteres, *i.e.* O, S and N atoms.

An analysis of the literature showed that α-acetoxyglycine peptides are good substrates for this kind of nucleophilic reactions[18–21] due to the presence of the acetoxy leaving group.

According to Steglich *et al.*,[18] a broad range of seryl peptides can be converted to α-acetoxyglycine residues by using lead tetraacetate as the oxidizing agent. The resulting acetylated derivatives could then be smoothly substituted in the presence of nucleophilic agents such as chloride,[18] thioalkyl and thioaryl derivatives,[18,19] or alcohols.[20]

The introduction of this kind of substituent on our side-chained peptidic macrocycle appears to be feasible using this methodology. For this purpose, it was necessary to have a seryl residue directly linked to the macrocyclic entity. The scaffold of the macrocyclic entity **3** was extended on its carboxylic function by a coupling reaction with a seryl-based dipeptide **6** as summarized on Scheme 2. This side-chain was then converted to a mixture[18] of its α-acetoxyglycine diastereomers by using the Steglich's $Pb(OAc)_4$ oxidation methodology. The following step consisted of the smooth elimination of the acetoxy group by treatment with the desired nucleophilic agent, *i.e.* the anilino derivative **7** which was synthesized according to a two-step procedure, leading to the final compound **2a**, as a mixture of



Scheme 2 Synthesis of new α-substituted glycine analogues **2a**–**d**. (i) *p*-tosylate.H_2N-(L)-IleOBzl, BOP reagent, DIEA, RT, 3 h; quantitative; (ii) TFA, CH_2CI_2 (1 : 1), RT, 1 h; quantitative; (iii) BOP reagent, DIEA, THF, RT, 2 h; 79%; (iv) $Pb(OAc)_4$, molecular sieves 4 Å, EtOAc, Δ, overnight; (v) a) 1-amino-4-[3-[*N*-(*tert*-butoxycarbonyl)amino]propan-1-yloxy]benzene **7**, CH_2CI_2, Et_3N, Δ, 48 h; 50% from **6**; b) 4-ethoxyphenol, CH_2CI_2, Et_3N, RT, 48 h, 38% from **6**; c) 4-methoxythiophenol, CH_2Cl_2, Et_3N, RT, 48 h, 47% from **6**; d) aniline, CH_2Cl_2, Et_3N, RT, 48 h, 15% from **6**.

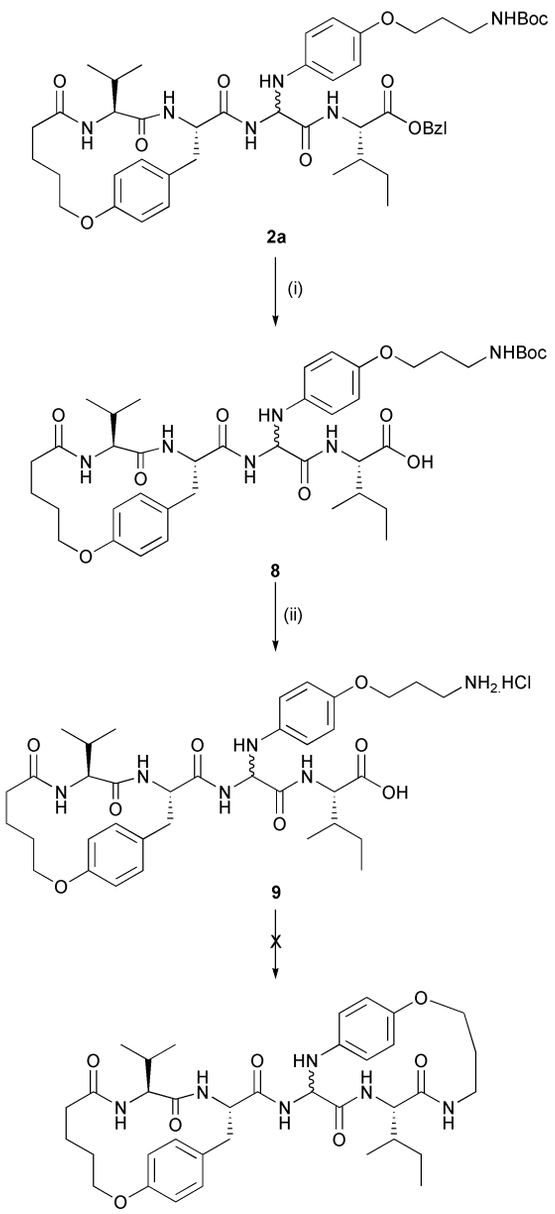
diastereomers. Separation of the diastereomeric mixture was not achieved since our objective was first to ascertain the relevance of our concept.

To complete this synthetic work, we attempted the ring-closure of the conjugate **2a** obtained from the anilino derivative **7** that was supposed to lead to the exact and original bioisostere analogue of the bis-macrocyclic substrate **1** described by Fairlie *et al.*[2]

The terminal acidic function of compound **2a** was released by hydrogenolysis of the benzyl ester protecting group to give the free acidic side-chain macrocycle **8**. The *tert*-butoxycarbonyl amino protecting group was removed by HCl treatment of this ω-*N*-protected acid **8** producing the deprotected ω-amino-acid **9** as an hydrochloride salt (Scheme 3).

Unfortunately, we were not able to accomplish the final ring-closure step to complete the desired synthesis despite various attempts including BOP, DCC–HOBt[22] or HATU–HOAt[23,24] as coupling agents in high dilution conditions.[24] The crude materials obtained from these reactions were analyzed by MS and LC-MS. Both reaction mixtures displayed no trace of the desired bicyclic system but did show a main peak which was an MS signal characteristic for the macrocyclic acid **3** (*m*/*z* 363 $(M + H)^+$).

This unfortunate chemical degradation occurring during the final ring-closure step of this synthesis is in line with our expectations and in agreement with Kingsbury *et al.*[11] Indeed, the exocyclic amide bond between the macrocyclic entity and the α-anilino substituted glycine residue was hydrolyzed, releasing an unstable *gem*-aryl diamino species which regenerates the



Scheme 3 Attempt of synthesis of the original amino bioisoster of bis-macrocyclic substrate **1**. (i) H_2 (1 atm), 10% Pd–C, THF–MeOH (1 : 3), RT, 12 h; 89%; (ii) HCl, Et_2O, RT, 6 h; 90%.

aniline derivative according to the mechanism described by Kingsbury *et al.* (Fig. 3).[11]

This cleavage reaction was not expected during the synthesis of the substrate but was supposed to be catalyzed by the HIV-1 protease in the infected cell to release toxophoric species able to affect the cell viability.

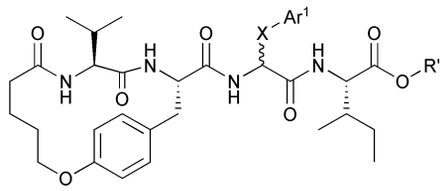
As far as we could not perform the complete synthesis of the exact bioisoster of **1**, we took advantage of the previous results to study the influence of the nature of the substitution of the glycine residue on the release of this α-substituent.

For this purpose, we elaborated the synthesis of the novel mono-cyclic substrates **2b**–**d** according to the same procedure used for the synthesis of **2a** (Scheme 2). We successively substituted the α-acetoxy leaving group by an oxygen (**2b**), a sulfur (**2c**) and a nitrogen (**2d**) atom. The nucleophilic agents were carefully selected among some known toxophoric phenols,[15] thiophenols and anilines[16] in order to release these toxophoric species as mentioned previously.

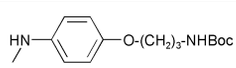
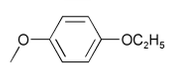
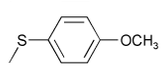
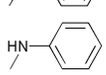
Basic hydrolysis assay

The unexpected hydrolytic process observed during the synthetic procedure led us to study the behaviour of compound **1**

Table 1 Kinetic data for the basic hydrolysis of substrates **2a**–**d**



2a-d

Compound	–X–Ar[1]	λ_{max} [a]/nm	$t_{1,2}$ [b]/min	Slope/μM^{-1} min^{-1}
2a		300	3528 ± 30	$1.2.10^{-7}$
2b		302	0.32 ± 0.07	$1.8.10^{-3}$
2c		302	98 ± 1	6.10^{-6}
2d		281	5040 ± 60	$1.7.10^{-7}$

[a] The suitable wavelength for each chromophoric substance was determined in the same conditions as for the hydrolysis reaction, *i. e.* 250 μM of the chromophoric species in the presence of 1250 μM of aqueous sodium hydroxide in EtOH solution. [b] Kinetics data, *i. e.* half-reaction time and kinetic constants, were calculated according to the mathematical rules for non-equimolar second-order rate reactions.

and our α-substituted glycine derivatives **2a**–**d** towards basic conditions. The basic hydrolysis of substrate **1** was monitored by HPLC because of the lack of a suitable chromophoric moiety for UV analysis. For **2a**–**d**, we studied the release of the chromophoric α-substituents of the side-chain glycine residue at a suitable wavelength by UV-monitoring. The substrates **2a**–**d** were hydrolyzed in ethyl alcohol in the presence of a large excess (5 equiv.) of aqueous sodium hydroxide. Experimentally, we confirmed that these basic hydrolysis reactions were non-equimolar second-order rate reactions. The quantification of the released chromophoric substances allowed the calculation of the main kinetic parameters, *i.e.* rate constants and half-reaction times (Table 1), according to the mathematical rules concerning the kinetics of this kind of reaction. The stability of our various substrates **2a**–**d** and Fairlie's bis-macrocyclic substrate **1** towards basic hydrolysis was as expected, dependent on the nature of the Ar^1-X group:

$$\underset{\mathbf{1}}{\text{-CH}_2\text{-C}_6\text{H}_4\text{-OR}} >> \underset{\mathbf{2d}}{\text{-NH-C}_6\text{H}_5} > \underset{\mathbf{2a}}{\text{-NH-C}_6\text{H}_4\text{-OR}} > \underset{\mathbf{2c}}{\text{-S-C}_6\text{H}_4\text{-O-CH}_3} >> \underset{\mathbf{2b}}{\text{-O-C}_6\text{H}_4\text{-O-C}_2\text{H}_5}$$

The effect of α-substituent groups on the rate of hydrolysis of the exocyclic amide bond is under the control of the nucleophilicity constants associated with the leaving group properties of the released moieties. Among these properties, one can list the solvation energy of the nucleophile, the strength of its bond to carbon, its effective size, the basicity properties of the substituent and the inductive and mesomeric effects. As far as all the hydrolysis reactions are carried out in the same experimental conditions of solvents and nucleophile, one can assume that only the strength of the bond which binds the heteroatom to the carbon at the α-position of the glycine residue is likely to affect the rate of hydrolysis of the new analogues.

Considering the nature of the heteroatom linked to the aliphatic carbon of the glycine residue, the phenoxy group (**2b**) drastically increases the polarisability of the C–X bond compared to the corresponding thiophenoxy group (**2c**). This polarisability is far less important in the case of an anilino substituent group (**2a** and **2d**). Consequently, the C–NH–Ar bond is more stable towards basic hydrolysis than the C–S–Ar bond, which is itself far more stable than the C–O–Ar bond.

This order of stability can be related to various factors, such as the basicity of the released species. The conjugate bases of the selected anilines that are stronger bases than the two other oxy and thio substituents, will be weaker leaving groups.

Concerning the C–S–Ar and C–O–Ar, the polarisability of the C–O–Ar bond is increased because of the higher electronegativity of the oxygen atom. Therefore, the cleavage of this bond is favoured and the rate of hydrolysis of the phenoxy analogue **2b** is enhanced compared to the thiophenoxy derivative **2c**.

Due to the presence of an electron-donating *O*-alkoxy substituent at the 4-position of the aniline ring in compound **2a**, the electronic density is enhanced on the nitrogen atom through the resonance effect. Therefore, the C–NH–Ar-(4-substituted) bond (**2a**) is weakened, and the leaving properties of this α-anilino substituent are increased compared to the unsubstituted anilino group (**2d**). The cleavage of the C–NH–Ar bond in **2a** is then favoured compared to the corresponding unsubstituted anilino derivative **2d**.

Enzymatic hydrolysis assay

Interestingly, some of these substrates lead upon enzymatic hydrolysis processing to the specific cleavage of the exocyclic amide bond. Indeed, **1** and **2a** were incubated at 37 °C for about 6 hours with the HIV-1 protease. The resulting crude reaction mixtures were then analyzed by HPLC (**1**) or LC-MS (**2a**).

In the first case, it was clearly demonstrated that the proteolytic enzyme catalyzed specifically the complete cleavage of the main compound with the release of the two entities, *i.e.* the macrocyclic acid moiety **3** and the macrocyclic free amine entity **4**. These two released species were unambiguously identified by the use of both macrocycles **3** and **4** as internal references for the HPLC analysis.

In the second case, as proven by a LC-MS method, **2a** (*m/z* 797 $(M + H)^+$) was specifically processed by the enzyme into its acidic moiety **3** (*m/z* 363 $(M + H)^+$) and the anilino compound **7** (*m/z* 267 $(M + H)^+$) according to the LC-MS traces of the different compounds used for this analytical study. In this latter case, the result confirms our expectations concerning the release of a toxophoric anilino species, and it is also in agreement with the results reported by Fairlie *et al.*[2] about the substrate characteristics of the macrocyclic derivatives. Indeed, due to this specific cleavage catalyzed by the enzyme, **2a** behaved as a substrate of the HIV-1 protease.

As demonstrated by this analysis, such reactivity can be used to deliver toxophoric substances directly into infected cells where it could selectively affect cell viability.

In conclusion, we report the synthesis of new macrocyclic modified peptides incorporating an exocyclic glycine moiety α-substituted by an heteroatomic-aryl group. As far as direct introduction of such a glycine residue is not possible due to the instability of the *gem*-heteroatom aryl aminal intermediate, we used an original retrosynthetic scheme which involved the construction of the precursor by side-chain extension of the carboxylic acid function of the first macrocyclic ring. The ring closure of the second macrocyclic moieties of **2a** was supposed to be the final step of the total convergent synthetic pathway of the original bioisostere analogue of the bismacrocyclic substrate **1** described by Fairlie *et al.*[2] An unfortunate chemical instability of the intermediate α-anilino substituted glycine residue occurring during this last synthetic step confirmed our expectations about the release of an anilino species, as shown by the cleavage of the exocyclic amide bond and the formation of an unstable *gem*-aryl diaminal species. We further demonstrated that the α-exocyclic amide bond of this new analogue **2a** enzymatically cleaved, is responsible of the release of the toxophoric anilino species. These results confirmed the substrate characteristics of this new macrocyclic peptidomimetic containing an α-anilino substituted glycine residue. Further interesting experiments will include studies on the mechanism of action of this kind of macrocyclic derivative bearing α-heteroatom aryl substituted glycine moieties to show how the release of some potent toxophoric species could affect the HIV replication cycle in infected cells or cell viability.

Experimental section

Unless otherwise noted, starting materials and reagents were obtained from commercial suppliers and were used without purification. All the protected amino acids and peptide coupling reagents were purchased from Bachem and Neosystem. Rec-HIV-1 protease kit was purchased from Bachem. Tetrahydrofuran (THF) was distilled over sodium benzophenone ketyl immediately prior to use. Methylene dichloride (CH_2Cl_2) was distilled over P_2O_5 just prior to use. Acetone was dried and distilled over calcium sulfate. Ethyl acetate (EtOAc), diethyl ether and dimethylformamide (DMF) were of anhydrous quality from commercial suppliers (Aldrich, Acros, Carlo Erba Reagents). Nuclear magnetic resonance spectra were recorded at 250 MHz for ^1H and 62.9 MHz for ^{13}C on a Bruker AC-250 spectrometer and at 300 MHz for ^1H on a Bruker Avance 300. Chemical shifts are expressed as δ units (part per million) downfield from TMS (tetramethylsilane). Electrospray mass spectral analysis and LC-MS analysis were obtained from Dr Drouot, Dr Maux and Miss Saint-Pé (Trophos, Marseille, France) on a Waters Micromass ZMD spectrometer for the ES-MS analysis by direct injection of the sample solubilized in acetonitrile. The LC-MS analysis was carried out by using a Waters model 2690 pump and a Waters C18 Symmetry column with a two-mobile phase system (0.1% formic acid in water and 0.1% formic acid in acetonitrile). Microanalyses were carried out by Service Central d'Analyses du CNRS (Venaison, France) and were within 0.4% of the theoretical values. UV spectra for basic and enzymatic hydrolysis studies were recorded on a Safas UV mc² spectrophotometer (Safas, Monaco).

Analytical thin layer chromatographies (TLC) and preparative thin layer chromatographies (PLC) were performed using silica gel plates 0.2 mm thick and 1 mm thick respectively ($60F_{254}$ Merck). Preparative flash column chromatographies were carried out on silica gel (230–240 mesh, G60 Merck). The melting points were not determined because of the amorphous character of our peptides.

(9*S*,12*S*)-12-Carboxy-7,10-dioxo-9-isopropyl-2-oxa-8,11-diazabicyclo[12.2.2]octadeca-14,16,17-triene 3[2]

The macrocyclic carboxylic acid synthon **3** was synthesized as summarized on Scheme 1, according to a 7-step reaction pathway from Boc-(L)-tyrosine benzyl ester using the procedure described by Fairlie *et al.*[2] The NMR and MS analytical data for the final compound **3** and the previous synthetic intermediates were in accordance with those described in the literature.

(11*S*,8*S*)-11-Amino-7,10-dioxo-8-(1-methylpropyl)-2-oxa-6,9-diazabicyclo[11.2.2]heptadeca-13,15,16-triene trifluoroacetic acid salt 4[7]

The trifluoroacetic acid salt **4** was synthesized according to a 5-step reaction pathway from Boc-(L)-isoleucine (Scheme 1). As previously noted for the synthesis of **3**, analytical results for each synthetic intermediate and final compound **4** were in accordance with those described by Fairlie *et al.*[7]

Bicyclic substrate 1[2]

The bicyclic substrate **1** was the result of the coupling reaction between macrocyclic carboxylic acid synthon **3** and trifluoroacetic acid salt **4** using BOP reagent as coupling agent (Scheme 1). MS and NMR analytical results were in accordance with those described by Fairlie *et al.*[2]

N-[(*S*)-Seryl]-(*S*)-isoleucine benzyl ester trifluoroacetic acid salt 5

Boc-(L)-serine (2.0 g, 9.75 mmol) was dissolved in 50 mL of anhydrous THF with BOP reagent (4.4 g, 8.86 mmol). The reaction mixture was cooled down to 0 °C and diisopropylethylamine (1.7 mL, 8.86 mmol) was added dropwise. The solution was allowed to warm up to room temperature, stirred for 30 minutes and cooled down to 0 °C. A solution of H_2N-(L)-IleOBzl *p*-tosylate salt (3.5 g, 8.86 mmol) and diisopropylethylamine (3.4 mL, 17.70 mmol) in THF (10 mL) was then added dropwise and the resulting reaction mixture was stirred at room temperature for 3 hours. The solvent was removed under reduced pressure and the residue was dissolved in EtOAc (50 mL). The organic layer was washed with HCl 1 M (5 × 20 mL), brine (20 mL), saturated $NaHCO_3$ (5 × 20 mL) and brine (2 × 20 mL), dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The crude residue was purified by flash chromatography (EtOAc–hexane 3 : 7) to give the resulting dipeptide (3.66 g, quantitative) as a white solid. R_f 0.12 (EtOAc–hexane 3 : 7). δ_H (250 MHz, $CDCl_3$) 0.62 (m, 6 H, 2 × CH_3 Ile), 1.00 (m, 1 H, CH_aH$_b$γ Ile), 1.22 (m, 10 H, 3 × CH_3 Boc + CH$_a$$H_b$γ Ile), 1.70 (m, 1 H, C$H$β Ile), 2.95 (m, 1 H, O$H$), 3.20 (m, 2 H, C$H_2$β Ser), 3.87 (m, 1 H, C$H$α Ser), 4.35 (m, 1 H, C$H$α Ile), 4.95 (AB, 2 H, *J* 12.2, –O–CH_2–C$_6$H$_5$), 5.38 (br d, 1 H, NH), 6.88 (m, 1 H, NH), 7.11 (m, 5 H, –C$_6$$H_5$). δ_C (62.9 MHz, $CDCl_3$) 11.8, 15.8, 25.1, 28.5 (3 C), 37.7, 54.9, 57.0, 63.0, 67.3, 80.7, 128.6–128.8 (5 C), 135.5, 156.4, 171.8 (2 C). MS-ES *m*/*z* 431 $(M + Na)^+$.

The previous compound (780 mg, 1.91 mmol) was dissolved in CH_2Cl_2 (1.5 mL). Trifluoroacetic acid (1.5 mL, 19.10 mmol) was added dropwise at 0 °C. The reaction mixture was stirred at room temperature for 1 hour. The solvent and excess of trifluoroacetic acid were removed under reduced pressure. The resulting oil was then cooled down in the freezer for 2 hours and allowed to warm up to room temperature to crystallize as a white solid. The solid was triturated in hexane and filtered off to give quantitatively the desired TFA salt **5** (845 mg). δ_H (250 MHz, CD_3OD) 0.70 (m, 6 H, 2 × CH_3 Ile), 1.05 (m, 1 H, CH_aH$_b$γ Ile), 1.20 (m, 1 H, CH$_a$$H_b$γ Ile), 1.72 (m, 1 H, C$H$β Ile), 3.75 (ddd + m, 3 H, *J* 11.3 and 7.2, CH_2β and CHα Ser), 4.30 (d, 1 H, *J* 5.5, CHα Ile), 4.97 (AB, 2 H, *J* 12.1, –O–CH_2–C$_6$H$_5$), 7.17 (m, 5 H, –C$_6$$H_5$). δ_C (62.9 MHz, DMSO-d$_6$) 11.0, 15.2, 24.5, 36.4, 54.1, 56.5, 60.3, 66.0, 128.0 (2 C), 128.1, 128.3

(2 C), 135.6, 167.0, 170.6. MS-ES *m/z* 331 $(M + Na)^+$ and 309 $(M + H)^+$.

N-[*N*-{(9*S*,12*S*)-7,10-Dioxo-9-isopropyl-2-oxa-8,11-diazabicyclo[12.2.2]octadeca-14,16,17-trien-12-ylcarbonyl}seryl]-(*S*)-isoleucine benzyl ester 6

The macrocyclic acid **3** (60 mg, 0.17 mmol) was dissolved in freshly distilled THF (1.2 mL). BOP reagent (75 mg, 0.17 mmol) was then added and the resulting mixture was cooled down to 0 °C. Diisopropylethylamine (30 μL, 0.17 mmol) was then added. The reaction mixture was allowed to warm up to room temperature and stirred for 1 hour. The solution was cooled down to 0 °C. A solution of the trifluoroacetic acid salt **5** (72 mg, 0.17 mmol) and diisopropylethylamine (60 μL, 0.34 mmol) in 0.5 mL of anhydrous THF was then added. The solution was allowed to warm up to room temperature and stirred for 2 hours. The solvent was evaporated under reduced pressure and the residue was dissolved in CH_2Cl_2 (10 mL). The organic layer was washed successively with HCl 1 M (2 × 2 mL), $NaHCO_3$ 5% (2 × 2 mL), brine (2 mL), dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography to yield the desired compound **6** (88 mg, 79%) as a white solid (Found: C, 64.56; H, 7.22; N, 8.73. $C_{35}H_{48}N_4O_8$ requires C, 64.39; H, 7.41; N, 8.58%). R_f 0.40 (EtOAc–MeOH 95 : 5). δ_H (250 MHz, $CDCl_3$) 0.58–0.65 (m, 9 H, CH_3 Val + 2 × CH_3 Ile), 0.80 (d, 3 H, *J* 6.7, C$H_3\gamma$ Val), 0.97–1.17 (m, 7 H, C$H\beta$ Val + –(CH_2)$_2$–CH$_2$–C(O) + C$H_2\delta$ Ile), 1.52 (m, 1 H, C$H\beta$ Ile), 1.95 (br s, 2 H, –CH_2–C(O)), 2.61 (m, 1 H, OH), 3.11 (m, 1 H, CH_aH$_b\beta$ Tyr), 3.42 (m, 1 H, CH$_a$H$_b\beta$ Tyr), 3.81–3.92 (m, 4 H, C$H\alpha$ Ser + C$H_2\beta$ Ser + C$H\alpha$ Ile), 4.13 (m, 2 H, Tyr–O–CH_2), 4.41 (m, 1 H, C$H\alpha$ Val), 4.80–5.03 (m, 4 H, –CH_2–C$_6$H$_5$ + C$H\alpha$ Tyr + NH), 6.49 (m, 1 H, ArH Tyr), 6.65 (m, 1 H, ArH Tyr), 6.98 (m, 1 H, ArH Tyr), 7.04 (m, 1 H, ArH Tyr), 7.07 (m, 5 H, –C$_6H_5$), 7.81 (br s, 1 H, NH), 7.97 (br s, 1 H, NH), 8.12 (br s, 1 H, NH). δ_C (62.9 MHz, $CDCl_3$) 11.5, 14.1, 15.6, 18.6, 19.0, 20.9, 25.0, 35.2, 37.2, 49.3, 53.4, 54.4, 57.2, 58.3, 60.4, 67.2, 71.6, 72.2, 128.3–129.3 (9 C), 135.1 (2 C), 155.9, 171.2 (2 C), 171.9, 172.2, 173.0. MS-ES *m/z* 675 $(M + Na)^+$.

1-Amino-4-[3-[*N*-(*tert*-butoxycarbonyl)amino]propan-1-yloxy]-benzene 7

3-[*N*-(*tert*-Butoxycarbonyl)amino]propan-1-ol (4.60 g, 26.3 mmol) was dissolved in freshly distilled THF (75 mL). *p*-Nitrophenol (3.65 g, 26.3 mmol) and triphenylphosphine (10.33 g, 39.4 mmol) were added. The reaction mixture was cooled down to 0 °C and DEAD (6.20 mL, 39.4 mmol) was added dropwise.[25] The reaction mixture was stirred at 0 °C for 20 minutes, then allowed to warm up to room temperature and stirred overnight. The solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (80 mL) and the organic layer was washed with saturated $NaHCO_3$ (4 × 60 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated. The residue was purified by flash chromatography to yield the desired nitro derivative (7.16 g, 92%) as a yellow solid (Found: C, 56.43; H, 6.95; N, 9.31. $C_{14}H_{20}N_2O_5$ requires C, 56.78; H, 6.81; N, 9.46%). R_f 0.30 (EtOAc–hexane 3 : 7). δ_H (250 MHz, $CDCl_3$) 1.21 (s, 9 H, 3 × CH_3 Boc), 1.80 (qt, 2 H, *J* 6.3, –CH$_2$–CH_2–CH$_2$–), 3.10 (q, 2 H, *J* 6.3, –CH_2–NH-Boc), 3.90 (t, 2 H, *J* 6.3, –O–CH_2–), 4.52 (br s, 1 H, –NH-Boc), 6.71 (dd, 2 H, *J* 7.1 and 2.1, ArH), 7.96 (dd, 2 H, *J* 7.1 and 2.1, ArH). δ_C (62.9 MHz, $CDCl_3$) 28.4 (3 C), 29.5, 37.6, 66.5, 79.4, 114.4 (2 C), 125.9 (2 C), 141.5, 156.1, 163.9. MS-ES *m/z* 319 $(M + Na)^+$.

The previous nitro compound (3.18 g, 10.8 mmol) was dissolved in MeOH (100 mL). 10% Pd-C (0.27 g) was then added and the reaction mixture was stirred 6 hours under H_2 atmospheric pressure. The solution was then filtered on Celite and concentrated under reduced pressure to give the aniline derivative **7** (2.56 g, 89%) as an yellowish solid (Found: C, 63.72; H, 8.57; N, 10.41. $C_{14}H_{22}N_2O_3$ requires C, 63.17; H, 8.33; N, 10.53%). R_f 0.36 (EtOAc–hexane 1 : 1). δ_H (250 MHz, $CDCl_3$) 1.59 (s, 9 H, 3 × CH_3 Boc), 2.07 (m, 2 H, –CH$_2$–CH_2–CH$_2$–), 3.44 (m, 2 H, –CH_2–NH-Boc), 3.58 (br s, 2 H, –NH_2), 4.09 (t, 2 H, *J* 6.0, –O–CH_2–), 4.98 (br s, 1 H, –NH-Boc), 6.79 (dd, 2 H, *J* 6.5 and 2.4, ArH), 6.88 (dd, 2 H, *J* 6.5 and 2.4, ArH). δ_C (62.9 MHz, $CDCl_3$) 28.6 (3 C), 29.8, 38.3, 66.8, 79.3, 115.8 (2 C), 116.5 (2 C), 140.4, 152.0, 156.2. MS-ES *m/z* 289 $(M + Na)^+$.

[*C*-{[4-[3-[*N*-(*tert*-Butoxycarbonyl)amino]propan-1-yloxy]-phenyl]amino}-*N*-[*N*{(9*S*,12*S*)-7,10-dioxo-9-isopropyl-2-oxa-8,11-diazabicyclo[12.2.2]octadeca-14,16,17-trien-12-ylcarbonyl}]glycyl]-(*S*)-isoleucine benzyl ester 2a

The seryl residue extended macrocyclic derivative **6** (88 mg, 0.14 mmol) was dissolved in anhydrous EtOAc (2 mL). Lead tetraacetate (146 mg, 0.34 mmol) was then added as a solid with 75 mg of molecular sieves 4 Å. The reaction mixture was stirred at 75 °C overnight. The reaction mixture was filtered off and 3 mL of citric acid 10% were added. The resulting solution was stirred until it turned clear yellow. The aqueous layer was then extracted with EtOAc (2 × 10 mL). The combined organic layers were washed successively with citric acid 5% (3 × 10 mL), H_2O (2 × 10 mL), brine (10 mL), dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure to give the desired compound as a colourless oil. This compound was used immediately without any further purification. This intermediate was dissolved in anhydrous CH_2Cl_2 (3 mL) and the solution was cooled down to 0 °C. A solution of 1-amino-4-[3-[*N*-(*tert*-butoxycarbonyl)amino]propan-l-oxy]benzene **7** (36 mg, 0.14 mmol) and triethylamine (58 μL, 0.42 mmol) in anhydrous CH_2Cl_2 (2 mL) was then added. The reaction was allowed to warm up to room temperature then was refluxed for 48 hours. The solvent was removed under reduced pressure and the crude material was purified by flash chromatography to give the desired compound **2a** (60 mg, 50%) as a pale brown solid (Found: C, 65.43; H, 7.61; N, 9.31. $C_{48}H_{66}N_6O_{10}$ requires C, 64.99; H, 7.50; N, 9.47%). R_f 0.50 (EtOAc). δ_H (300 MHz, DMSO-d$_6$) 0.58–0.70 (m, 12 H, 2 × CH_3 Val + 2 × CH_3 Ile), 1.00–1.80 (m, 19 H, C$H\beta$ Val + C$H\beta$ Ile + C$H_2\gamma$ Ile + –CH_2–CH$_2$–NHBoc + 3 × CH_3 Boc + –O–CH$_2$–(CH$_2$)$_2$–CH$_2$–), 2.01 (m, 2 H, –CH_2–C(O)–), 2.33 (m, 1 H, CH$_a$H$_b\beta$ Tyr), 2.70 (m, 1 H, CH$_a$H$_b\beta$ Tyr), 3.00 (m, 2 H, –CH_2–NHBoc), 3.58 (m, 2 H, –CH_2–O–), 3.90 (m, 2 H, –CH_2–O–), 4.17 (m, 2 H, –NH–CH–NH– + C$H\alpha$ Val), 4.50 (m, 2 H, C$H\alpha$ Tyr + C$H\alpha$ Ile), 4.91 (AB, 2 H, –CH_2–C$_6$H$_5$), 5.54 (m, 2 H, 2 NH), 5.82 (m, 1 H, NH), 6.87–6.48 (m, 8 H, ArH), 7.07 (br s, 5 H, –C$_6H_5$), 7.54 (br s, 1 H, NH), 7.80 (br s, 1 H, NH), 8.05 (br s, 1 H, NH). δ_C (62.9 MHz, DMSO-d$_6$) 11.7, 14.4, 15.9, 18.8, 19.1, 21.2, 24.3, 28.6 (3 C), 29.9, 32.0, 38.2, 38.4, 53.2, 55.6, 58.3, 60.5, 61.9, 66.0, 71.8, 73.2, 79.1, 117.5, 119.0, 128.4–129.2 (13 C), 131.8, 135.5, 140.9, 144.5, 156.2, 171.3 (2 C), 171.9, 172.2, 173.0. MS-ES *m/z* 909 $(M + Na)^+$ and 888 $(M + H)^+$.

[*C*-(4-Ethoxyphenyl)oxy-*N*-[*N*-{(9*S*,12*S*)-7,10-dioxo-9-isopropyl-2-oxa-8,11-diazabicyclo[12.2.2]octadeca-14,16,17-trien-12-ylcarbonyl}]glycyl]-(*S*)-isoleucine benzyl ester 2b

The title compound was synthesized according to the procedure described previously for the synthesis of **2a**, from seryl extended macrocyclic acid **6** (100 mg, 0.15 mmol) and 4-ethoxyphenol (21 mg, 0.15 mmol) after 48 hours at room temperature in CH_2Cl_2. **2b** was isolated after chromatography as a white solid (44 mg, 38%; Found: C, 66.15; H, 7.53; N, 7.59. $C_{42}H_{54}N_4O_9$ requires C, 66.47; H, 7.17; N, 7.38%). R_f 0.52 (EtOAc). δ_H (300 MHz, $CDCl_3$) 0.55–0.85 (m, 12 H, 2 × CH_3 Val + 2 × CH_3 Ile), 1.02–1.41 (m, 11 H, C$H\beta$ Val + C$H\beta$ Ile + C$H_2\gamma$ Ile + –O–CH$_2$–(CH_2)$_2$–CH$_2$– + –O–CH$_2$–CH_3), 2.08 (m, 2 H, –CH_2–C(O)–), 2.51 (m, 1 H, CH_aH$_b\beta$ Tyr), 2.77 (m, 1 H,

$CH_aH_b\beta$ Tyr), 3.80 (m, 2 H, –CH_2–O–), 4.32 (m, 2 H, –O–CH_2–CH_3), 4.56 (m, 2 H, –NH–CH–O– + $CH\alpha$ Val), 4.70 (m, 2 H, $CH\alpha$ Tyr + $CH\alpha$ Ile), 5.05 (m, 2 H, –CH_2–C_6H_5), 5.32 (m, 1 H, NH), 5.78 (m, 1 H, NH), 6.23 (m, 1 H, NH), 6.50–7.00 (m, 4 H, ArH Tyr), 7.00–7.30 (m, 9 H, ArH), 7.55 (m, 1 H, NH). MS-ES *m*/*z* 759 $(M + H)^+$.

[*C*-(4-Methoxyphenyl)thio-*N*-[*N*-{(9*S*,12*S*)-7,10-dioxo-9-isopropyl-2-oxa-8,11-diazabicyclo[12.2.2]octadeca-14,16,17-trien-12-ylcarbonyl}]glycyl]-(*S*)-isoleucine benzyl ester 2c

The title compound was synthesized according to the procedure described previously for the synthesis of **2a**, from seryl extended macrocyclic acid **6** (100 mg, 0.15 mmol) and 4-methoxythiophenol. **2c** was isolated after chromatography as a pale yellow solid (55 mg, 47%; Found: C, 65.06; H, 6.74; N, 7.53. $C_{41}H_{52}N_4O_8S$ requires C, 64.71; H, 6.89; N, 7.36%). R_f 0.52 (EtOAc). δ_H (300 MHz, $CDCl_3$) 0.55–0.94 (m, 12 H, 2 × CH_3 Val + 2 × CH_3 Ile), 1.07–1.43 (m, 8 H, $CH\beta$ Val + $CH\beta$ lle + $CH_2\gamma$ Ile + –O–CH_2–$(CH_2)_2$–CH_2–), 2.00 (m, 2 H, –CH_2–C(O)–), 2.41–2.75 (m, 2 H, $CH_2\beta$ Tyr), 3.02 (m, 2 H, –CH_2–O–), 3.55 (s, 3 H, –O–CH_3), 3.92–4.15 (m, 2 H, –NH–CH–S– + $CH\alpha$ Val), 4.40–4.70 (m, 2 H, $CH\alpha$ Tyr + $CH\alpha$ Ile), 5.18 (m, 2 H, –CH_2–C_6H_5), 5.36 (m, 1 H, NH), 5.72 (m, 1 H, NH), 6.38 (m, 1 H, NH), 6.57–6.95 (m, 6 H, ArH Tyr + 2 × ArH), 7.05–7.42 (m, 7 H, ArH), 7.66 (m, 1 H, NH). MS-ES *m*/*z* 761 $(M + H)^+$.

[*C*-Anilino-*N*-[*N*-{(9*S*,12*S*)-7,10-dioxo-9-isopropyl-2-oxa-8,11-diazabicyclo[12.2.2]octadeca-14,16,17-trien-12-ylcarbonyl}]-glycyl]-(*S*)-isoleucine benzyl ester 2d

The title compound was synthesized according to the procedure described previously for the synthesis of **2a**, from seryl extended macrocyclic acid **6** (104 mg, 0.16 mmol) and aniline. **2d** was isolated after chromatography as a pale brown solid (17 mg, 15%; Found: C, 67.68; H, 6.95; N, 9.73. $C_{40}H_{51}N_5O_7$ requires C, 67,30; H, 7,20; N, 9,81%). R_f 0.57 (EtOAc). δ_H (300 MHz, $CDCl_3$) 0.66–0.88 (m, 12 H, 2 × CH_3 Val + 2 × CH_3 Ile), 1.09–1.42 (m, 8 H, $CH\beta$ Val + $CH\beta$ Ile + $CH_2\gamma$ Ile + –O–CH_2–$(CH_2)_2$–CH_2–), 2.18 (m, 2 H, –CH_2–C(O)–), 2.58 (m, 1 H, $CH_aH_b\beta$ Tyr), 2.90 (m, 1 H, $CH_aH_b\beta$ Tyr), 3.07 (m, 2 H, –CH_2–O–), 4.12 (m, 2 H, –NH–CH–NH– + $CH\alpha$. Val), 4.63 (m, 2 H, $CH\alpha$ Tyr + $CH\alpha$ Ile), 5.12 (m, 2 H, –CH_2–C_6H_5), 5.25 (m, 1 H, NH), 5.66 (m, 1 H, NH), 6.27 (m, 1 H, NH), 6.62–7.16 (m, 4 H, ArH Tyr), 7.24–7.35 (m, 10 H, ArH), 7.82 (m, 1 H, NH), 8.05 (m, 1 H, NH). MS-ES *m*/*z* 714 $(M + H)^+$.

[*C*-{[4-[3-[*N*-(*tert*-Butoxycarbonyl)amino]propan-1-yloxy]-phenyl]amino}-*N*-[*N*-{(9*S*,12*S*)-7,10-dioxo-9-isopropyl-2-oxa-8,11-diazabicyclo[12.2.2]octadeca-14,16,17-trien-12-yl-carbonyl}]glycyl]-(*S*)-isoleucine 8

The benzyl ester **2a** (554 mg, 0.62 mmol) was dissolved in a mixture THF–MeOH (1 : 3) (27 mL). A suspension of 10% Pd-C (59 mg) in THF (3 mL) was then added and the reaction mixture was stirred overnight at room temperature under H_2 atmospheric pressure. The suspension was filtered and the solution was concentrated under reduced pressure. The residue was purified by flash chromatography using a gradient of MeOH in EtOAc. The acid **8** was obtained as a white solid (441 mg, 89%; Found: C, 61.51; H, 7.75; N, 10.30. $C_{41}H_{60}N_6O_{10}$ requires C, 61.83; H, 7.59; N, 10.55%). R_f 0.40 (EtOAc–MeOH 2 : 1). δ_H (250 MHz, CD_3OD) 0.53 (m, 3 H, $CH_3\delta$ Ile), 0.71 (m, 3 H, $CH_3\gamma$ Ile), 0.78 (br s, 6 H, 2 × CH_3 Val), 1.05 (m, 2 H, $CH_2\gamma$ Ile), 1.23 (m, 15 H, 3 × CH_3 Boc + –CH_2–CH_2–NHBoc + –$(CH_2)_2$–CH_2–C(O)–), 1.50–1.80 (m, 2 H, $CH\beta$ Ile + $CH\beta$ Val), 1.90 (m, 2 H, –CH_2–C(O)–), 2.30–2.53 (m, 2 H, $CH_2\beta$ Tyr), 3.00 (m, 2 H, –CH_2–NHBoc), 3.66 (m, 2 H, –CH_2–O), 3.90 (m, 4 H, –NH–CH–NH– + $CH\alpha$ Val + –CH_2–O–), 4.40–4.70 (m, 2 H, $CH\alpha$ Tyr + $CH\alpha$ Ile), 6.55 (m, 5 H, 1 × ArH cyclic peptide + 4 × ArH aniline residue), 6.71 (br s, 1 H, ArH), 6.80 (br d, 1 H, ArH), 6.98 (br d, 1 H, ArH). δ_C (62.9 MHz, CD_3OD) 11.5, 14.7, 14.9, 18.5, 19.3, 20.0, 25.6, 28.3 (3 C), 31.3, 32.0, 38.5 (2 C), 52.9, 54.9, 58.3, 62.3, 62.5, 69.4, 74.5, 81.2, 117.4, 118.5, 129.4–131.3 (10 C), 146.0, 156.6, 171.4, 171.5, 172.3, 173.0, 174.9. MS-ES *m*/*z* 819 $(M + Na)^+$ and 797 $(M + H)^+$.

Substrate 9

The previous *N*-Boc protected derivative **8** (364 mg, 0.46 mmol) was dissolved in 15 mL of anhydrous diethyl ether. The resulting solution was cooled down to 0 °C and a solution of HCl in diethyl ether (7.9 mL, 30% of HCl in weight) was then added dropwise. The reaction mixture was allowed to warm up to room temperature and was stirred for 6 hours. The reaction was monitored by TLC (EtOAc–MeOH 1 : 1 and *n*-BuOH–AcOH–H_2O 12 : 3 : 5). The solvent was evaporated to yield the final compound **9** as a brown solid (282 mg, 90%). δ_H (250 MHz, CD_3OD) 0.56–0.77 (m, 14 H, 2 × CH_3 Ile + 2 × CH_3 Val + $CH_2\beta$ Ile), 1.00–2.00 (m, 12 H, 5 × CH_2 + $CH\beta$ Val + $CH\beta$ Ile), 2.45 (m, 2 H, $CH_2\beta$ Phe), 2.90–3.40 (m, 5 H, 2 × –CH_2–O– + $CH\alpha$ Val), 3.50 (m, 1 H, $CH\alpha$ Ile), 4.22 (m, 1 H, –NH–CH–NH–), 4.54 (m, 1 H, $CH\alpha$ Phe), 6.58 (br d, 1 H, *J* 8.0, ArH), 6.67 (br d, 1 H, *J* 6.2, ArH), 6.83 (br d, 1 H, *J* 7.6, ArH), 6.91 (m, 2 H, ArH aniline), 7.03 (br d, 1 H, *J* 7.6, ArH), 7.16 (br d, 2 H, *J* 7.2, ArH). MS-ES *m*/*z* 697 $(M + H)^+$.

General method for the basic hydrolysis assay

Basic hydrolysis of a 250 μM solution of substrate **2a**–**d** was performed in ethyl alcohol in the presence of aqueous sodium hydroxide (5 equiv., 1250 μM), at 26 °C. The chromophore release was monitored by UV analysis at a suitable wavelength, *i.e.* 300 nm for 1-amino-4-[3-[*N*-(*tert*-butoxycarbonyl)amino]-propan-1-yloxy]benzene (**2a**), 302 nm for 4-ethoxyphenol (**2b**) and 4-methoxythiophenol (**2c**), and 281 nm for aniline (**2d**). The quantification curves for each chromophoric substance were performed in the same conditions in the presence of 1250 μM of aqueous sodium hydroxide. The observed results are summarized in Table 1.

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