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Original article

Modelling, synthesis and biological evaluation of an ethidium–arginine conjugate linked to a ribonuclease mimic directed against TAR RNA of HIV-1

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Abstract

Using molecular modelling studies, an active anti-HIV ethidium–arginine conjugate targeted against the viral TAR RNA sequence has been linked to an artificial ribonuclease, with the aim to obtain an irreversible inhibitor. The ribonuclease moiety consists of an *N*-[*N*-(3-aminopropyl)-3-aminopropyl] glycine and has been constructed via two successive *N*-alkylations following the Fukuyama procedure. © 2002 Published by Éditions scientifiques et médicales Elsevier SAS.

Keywords: HIV-1; TAR; RNA; Ribonuclease; Fukuyama; *N*-alkylation

1. Introduction

The remarkable efficacy of protease and reverse transcriptase inhibitor associations for the treatment of HIV-1 infection has been clearly established [1]. However, due to the rapid development of viral resistance against these drugs [2], it is necessary to elaborate new chemotherapeutic agents directed against other viral targets. The interaction of the viral Tat protein with the TAR RNA region of HIV-1 is essential for the regulation of gene expression [3–5] and represents an attractive target for inhibiting HIV replication. Recently, we rationally designed bifunctional molecules capable of binding to two close sites of TAR which are involved in the interaction with Tat [6]. These compounds were constituted by an arginine residue covalently linked, via a polyamide spacer, to an ethidium-intercalating moiety. During the last decade, several arginine-containing molecules directed against TAR RNA have been developed, including Tat-derived peptides and peptoids [7], as well as arginine conjugates of aminoglycosides [7,8] or of polyamide nucleic acids [9]. In our case, the most promising conjugate of the ethidium–arginine series (compound **1**, Fig. 1) was shown (i) to inhibit HIV-1 replication ($IC_{50} = 1$ μM, without any toxicity up to 100 μM), and (ii) to interact strongly with the TAR RNA. Furthermore, melting temperature and Rnase protection experiments suggested that the ethidium moiety of **1** was inserted next to the A_{17} residue while the arginine side chain occupied the pyrimidine bulge of TAR (Fig. 2), in agreement with molecular modelling studies.

To make compound **1** a more potent inhibitor of the HIV replication, we envisaged to link it to an artificial ribonuclease, which could induce an irreversible hydrolysis of the TAR RNA. The glycine aminoacid was selected as ribonuclease mimic rather than well known polyamines [10–12] or imidazole moiety [13–16] because glycine–anthraquinone conjugates were shown to hydrolyse efficiently and selectively 5′-cytidine(C)–adenosine(A)-3′ sites of tRNA, via an intramolecular cooperation of carboxylate and ammonium ions [17]. As shown in Fig. 2, such a 5′-CA-3′ sequence is found in close proximity of the postulated intercalation site on TAR RNA of the ethidium moiety of compound **1**

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(C_{19}–A_{20}). Thus, using molecular modelling studies, compound **2** was designed in which the active ethidium–arginine conjugate **1** is covalently attached, via a polyamine linker, to a glycine residue (Fig. 1). The nature and the optimal length of the linker was established to allow the location of the glycine residue in front of the potentially hydrolysable 5′-C_{19}–A_{20}-3′ base pair of TAR (Fig. 2). This spacer consists of two

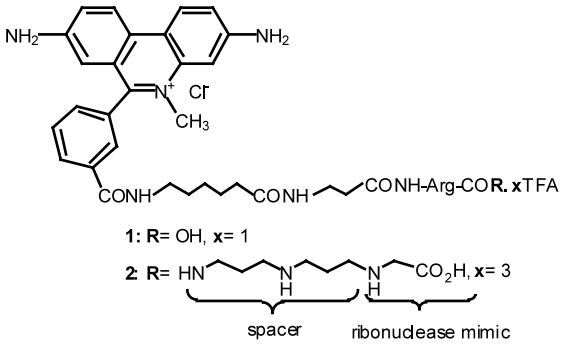

Fig. 1. Structures of ethidium–arginine conjugates **1** and **2**.

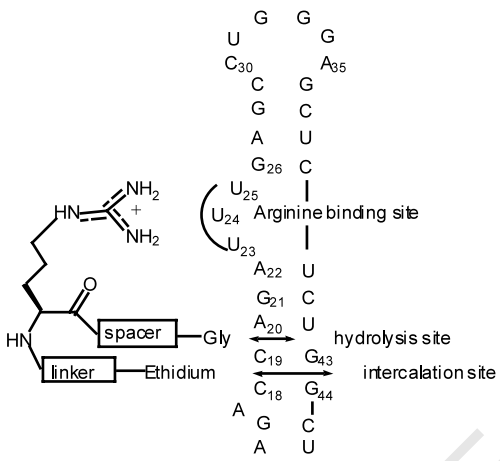

Fig. 2. Schematic potential interaction of ethidium–arginine conjugate **2** with the TAR RNA of HIV-1.

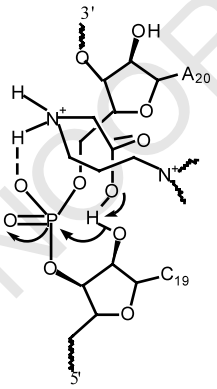

Fig. 3. Mechanism proposed by Endo for the hydrolysis of $tRNA^{Phe}$ by a glycine moiety.

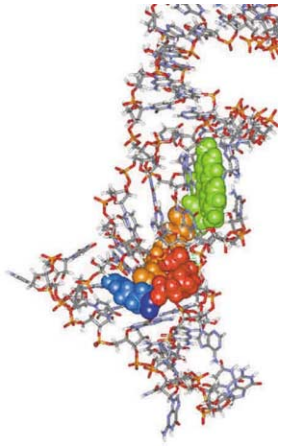

Fig. 4. Molecular model of the interaction between compound **2** and TAR RNA of HIV-1. The ethidium moiety is in green, the arginine residue in blue, the linker in yellow and the nucleasic moiety in red.

consecutive aminopropyl units in which the central ammonium ion is expected to enhance the binding of **2** to RNA through electrostatic interactions.

In this paper, we report on the synthesis of compound **2** following the Fukuyama procedure [18,19] as well as its biological evaluation. In this context, an unusual issue of the Fukuyama *N*-alkylation is also reported.

2. Chemistry

2.1. Dynamic molecular modelling studies

A model for the interaction of TAR RNA and compound **2** was built starting from the previous one established for compound **1** [4], and according to the proposed mechanism for the 5′-CA-3′ hydrolysis by a glycine residue [17] (Fig. 3). First, the nucleasic moiety was positioned in front of the phosphate group of the potentially hydrolysable 5′-C_{19}–A_{20}-3′ site of TAR. Two distance constraints were defined: the first one between an oxygen atom of the carboxylate moiety of the glycine residue and an hydrogen atom of the 2′-OH group of C_{19}, the second one between an hydrogen of the ammonium ion of the glycine and an oxygen of the targeted phosphate group. A linker was then designed in order to connect the nucleasic moiety to the carboxylic group of the arginine residue of compound **1** and its length was geometrically optimised. Molecular dynamic simulations were performed in order to evaluate the stability of the resulting hypothetical compound **2**–TAR complex (Fig. 4). The means of the energy interaction, mainly electrostatic, was found of -1023 kCal mol^{-1}. The interaction mechanism was investigated and its analysis showed that compound **2** first interacts by its arginine domain. In a second step, the

main linker between arginine and ethidium penetrates in the minor groove of TAR. Then, the ethidium moiety intercalates between the base pairs C_{18}–G_{44} and C_{19}–G_{43}. Lastly, the nucleasic domain interacts with the accessible phosphate group of the C_{19}–A_{20} base pair.

2.2. Synthesis

As the amide bond formation from suitably protected compound **1** and the ribonuclease moiety would most probably lead to substantial epimerisation of the arginine residue in **1**, the synthesis of compound **2** was alternatively performed from the ethidium derivative **3** and the arginine–ribonuclease containing moiety **4**, as outlined in Fig. 5. The synthesis of the ethidium derivative **3** was previously described [6]. Compound **4** was constructed starting from the linear *N*-Fmoc aminoacid chain (**5**), the commercially available *N*-Fmoc–Arg(Pmc)–OH (**6**), and the suitably protected polyamine key synthon **7** (see below).

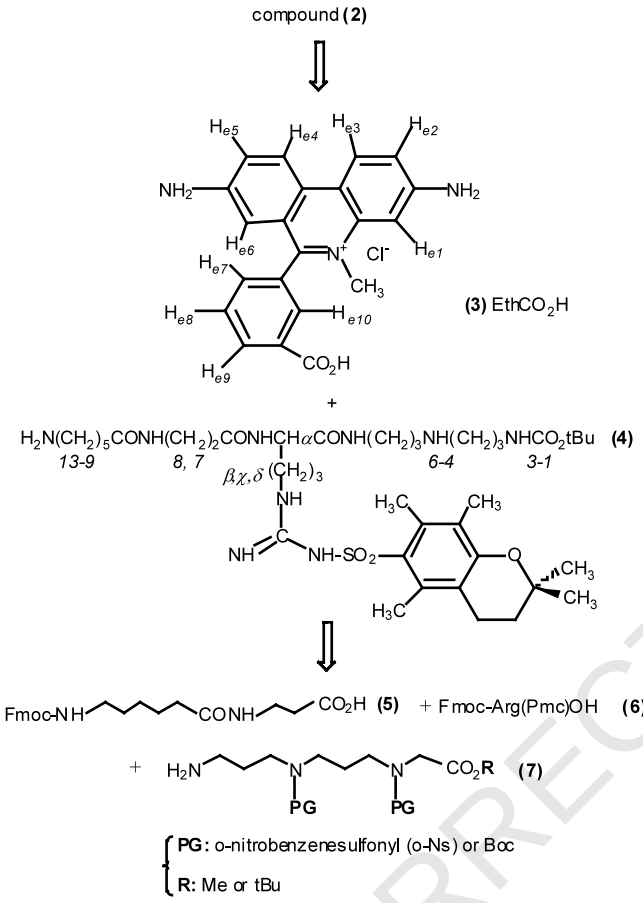

Fig. 5. Retrosynthesic scheme of compound **2**. (atom numbering corresponds to NMR assignments).

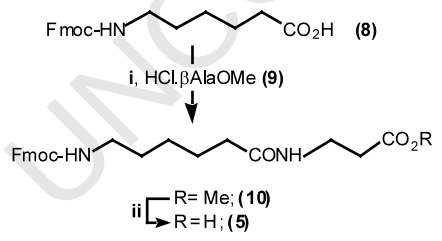

Fig. 6. Synthesis of compound **5**. Reagents and conditions: (i) Bop, DIEA, CH_2Cl_2. (ii) 1/1 4 N HCl–dioxane.

The synthesis of compound **5** was carried out in two steps starting from the commercially available *N*-Fmoc-6-aminocaproic acid (**8**) and β-alanine methyl ester (**9**) (Fig. 6). Coupling of **8** with **9** using Bop reagent led to **10** (91%) which was then hydrolysed with HCl to yield **5** (85%).

The major difficulty in synthesising compound **4** lay in the construction of the ribonuclease-containing polyamine moiety **7** (Fig. 5). Various synthetic approaches have been described for the synthesis of selectively N-functionalised polyamines [20]. These strategies require either orthogonal protections of the amino groups incorporated into an available polyamine skeleton, or stepwise construction of the desired polyamine chain using reactions, such as nucleophilic substitution [21], reductive alkylation [22] or, more recently, *N*-alkylation of sulfonamides [18,19,23]. More particularly, the Fukuyama's 2-nitrobenzenesulfonyl (*o*-Ns) group has been extensively used, both in solution and solid phase, for the selective preparation of N-protected primary amines [25], N-substituted amino acid derivatives [26–30], *N*-alkylated peptides [31], oligoureas [32], polyamides [33] and PNA [34]. Moreover, its utility in organic chemistry as an amino protecting group is most promising, as its removal occurs under mild conditions (PhS^-) which are highly selective as they do not affect the most commonly used amino protecting groups (i.e. Boc, Z, Alloc).

To explore the potential of the *o*-Ns group in multistep syntheses, and in particular as a permanent amino protecting group, we first elaborated strategy A to prepare compound **2**, as outlined in Fig. 7. Although the key di-*o*-Ns-protected compound **21** could be synthesised, our attempts to remove the two *o*-Ns groups by a conventional treatment (PhSH–DIEA) were unsuccessful, as we obtained a complex mixture from which our target derivative **2** could not be isolated. Moreover the *N*-alkylation of the di-*o*-Ns-protected compounds **15a**,**b** applying the Fukuyama's procedure, was found to be hampered by the presence of the *o*-Ns group on the amino function of the glycine residue. Indeed, and even under mild conditions (i.e. 1 equiv. of Cs_2CO_3 and room temperature (r.t.)), compounds **16a**,**b** were obtained from the di-*o*-Ns-protected **15a**,**b** and from the N-protected aminopropyl bromide **11a**,**b** with only low to medium yields (25 and 50%, respectively) together with the unexpected by-products **17a**,**b** (about 20%) and **18a** (10%), thus complicating substantially their purification. The structures of **17a**,**b** and **18a**,

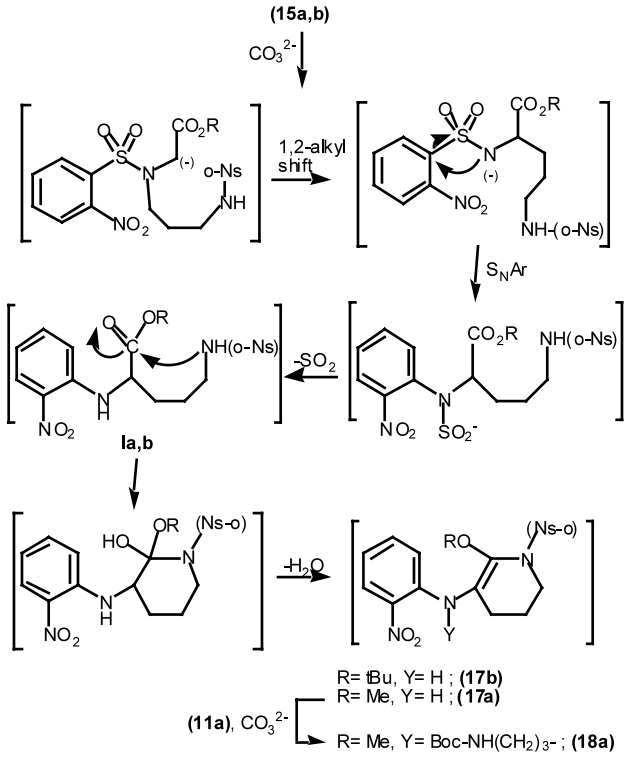

Fig. 7. Attempt in the synthesis of compound **2** following the strategy A Reagents and conditions: (i) for **11a**: $(Boc)_2O$, TEA, CH_2Cl_2 or for **11b**: Mmt–Cl, TEA, CH_2Cl_2; (ii) *o*-Ns–Cl, NMM, CH_2Cl_2, 0 °C; (iii) Cs_2CO_3, DMF; (iv) for **11a**: 50% TFA–CH_2Cl_2 or for **11b**: 1% TFA–CH_2Cl_2; (v) Bop, DIEA, CH_2Cl_2; (vi) DEA, CH_2Cl_2; (vii) Fmoc-deprotected **19**, HOSu, DCC, DMF; (viii) Fmoc deprotected **20**, Bop, HOBt, DIEA, DMF.

derivating from 2-nitro aniline, were unambiguously attested by mass spectrometry and ^{1}H-NMR. Furthermore, the formation of such by-products is consistent with the reported formation of 2-nitroaniline derivatives, which occurs during the Fukuyama *N*-alkylation of *o*-Ns-protected valine methyl ester [28]. The mass spectrum of **17a** confirmed, with respect to structure **16a**, the loss of SO_2 and of H_2O, indicating that, as displayed in Fig. 8 (see discussion below), rearrangement, cyclisation and subsequent water elimination producing **17a**, have occurred. Concerning the mass spectrum of **18a**, it confirmed, with respect to **17a**, the presence of a *N*-Boc–aminopropyl group (also attested by ^{1}H-NMR) and consequently, supported alkylation of **17a** with bromide **11a**. That this alkylation involved the amine group of aniline in **17a** was suggested by the absence, in the ^{1}H-NMR spectrum of **18a**, of the NH signal, measured at 6.9 ppm for **17a**. One should underscore that, excepted the specific NH and Boc–NH$(CH_2)_3$ signals for **17a** and **18a**, respectively, these two derivatives display a very similar ^{1}H pattern, indicating a very close chemical structure. Furthermore, their ^{1}H-NMR spectra exhibited a methoxy singlet at 4.05 ppm that is no more characteristic of a methyl ester function (for the glycine derivatives **11a** to **16a**, this signal was measured within a 3.65–3.80 ppm range). Moreover, a ^{1}H signal corresponding either to the two Hα of the glycine residue, as in the starting material, or to a single Hα, as in intermediate **Ia** (see Fig. 8) was not detected in the ^{1}H-NMR spectrum of neither **17a** nor **18a**, confirming that rearrangement, cyclisation and water elimination involved the glycine methyl ester residue, following the mechanism proposed in Fig. 8. This mechanism consists in an unusual based-catalysed rearrangement of **15a**,**b** to give the nitroaniline intermediates **Ia**,**b**, which then undergo an intramolecular cyclisation, producing the six-member ring derivatives **17a**,**b**. The formation of **18a** results from *N*-alkylation of **17a** with bromide **11a**. The complex rearrangement of **15a**,**b** into intermediates **Ia**,**b** is in line with the proposed one in the literature [28]. It is initiated by the deprotonation of the glycine methylene followed by a 1,2-alkyl shift, probably by a Stevens rearrangement whose mechanism is not fully understood [35]. A S_NAr rearrangement, then a loss of SO_2 leads to the 2-nitroaniline derivatives **Ia**–**b**. Concerning the conversion of **I** into **17**, it is noteworthy that replacement of the glycine methyl ester group of **15a** by

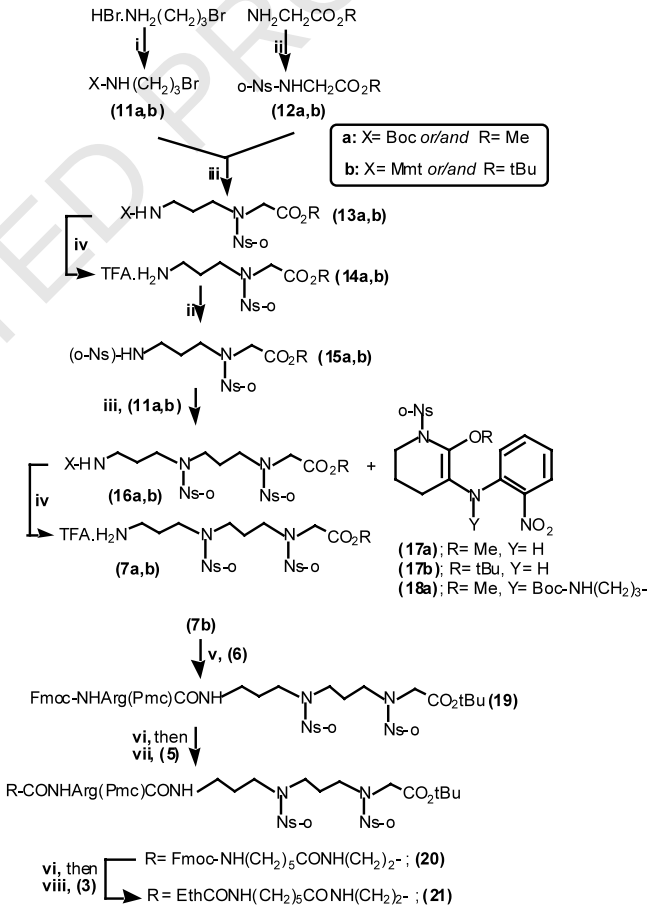

Fig. 8. Putative mechanism for the formation of by-products **17a**,**b** and **18a**.

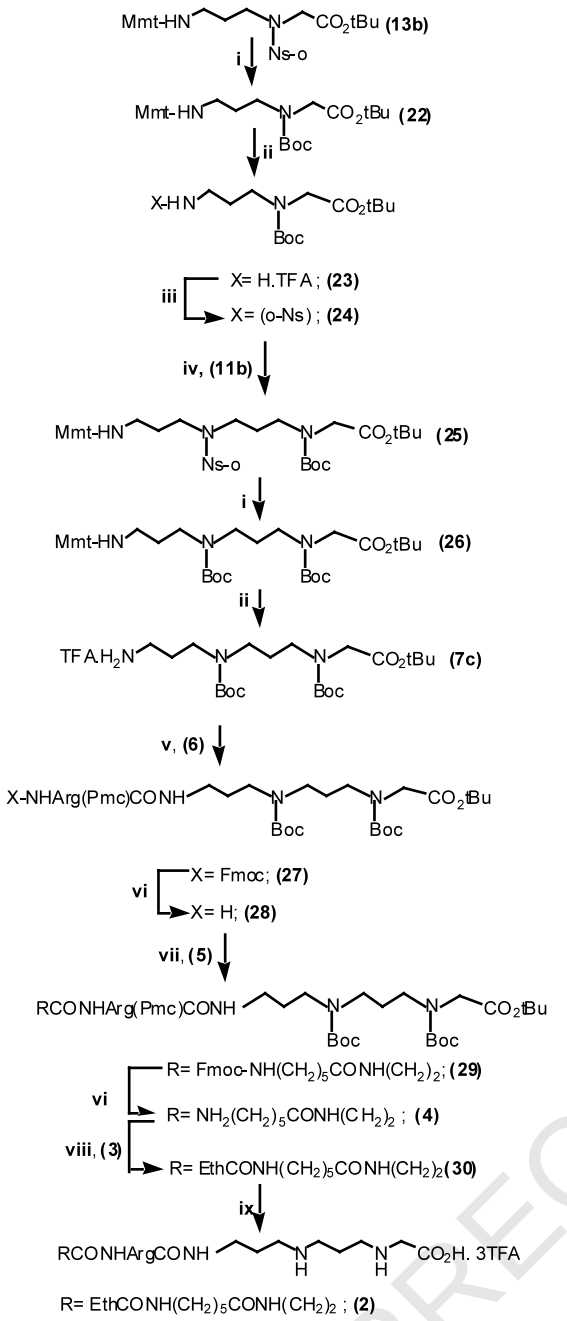

Fig. 9. Synthesis of compound **2** following the strategy B. Reagents and conditions: (i) PhSH, DIEA, DMF, 2–6 h then $(Boc)_2O$; (ii) 1% TFA–CH_2Cl_2; (iii) *o*-Ns–Cl, NMM, CH_2Cl_2; (iv) Cs_2CO_3, DMF; (v) Bop, DIEA, CH_2Cl_2; (vi) DEA, CH_2Cl_2; (vii) DCC, HOSu, DMF, 12 h then **29**, NMM; (viii) Bop, HOBt, DIEA, DMF; (ix) 9/1 TFA–H_2O.

the bulky tertiobutyl one, as in **15b**, did not prevent the intramolecular cyclisation of **Ib**.

This side reaction highlights the enhanced acidic character of the α protons in the *N*-alkylated *N*-sulfonylated glycine residue of **15a**,**b** which, in this context, consistently limits the potential of the *o*-Ns group as a permanent amino protecting group of the glycine.

The difficulties to obtain the di-(*o*-Ns)-protected compounds **16a**,**b** and our unsuccessful attempts to deprotect the two *o*-Ns groups at the last stages of the synthesis of **2** led us to elaborate the strategy B shown in Fig. 9. It consisted in replacing the troublesome *o*-Ns groups by the Boc amino protecting group after each *N*-alkylation step. This replacement allowed us to obtain our target compound **2** after classical deprotection of **30** (i.e. the Boc analog of the *o*-Ns derivative **21**). Moreover, this substitution was found to be more efficient for the preparation of **25** (i.e. the Boc analog of the *o*-Ns derivative **16**), as the key Fukuyama *N*-alkylation of **24** proceeded smoothly and cleanly.

Thus, starting from **13b**, compound **22** was isolated in 70% yield after the cleavage of the *o*-Ns group with PhSH and DIEA, and its replacement by the Boc group, by means of Boc_2O. These two steps were performed without isolation of the deprotected intermediate, thus shortening substantially this synthetic pathway. The selective cleavage of the Mmt protecting group of the Mmt-, Boc and *O*-*t*-Bu-protected compound **22** was achieved almost quantitatively by means of 1% TFA–CH_2Cl_2, affording the TFA salt **23**. Reaction between 2-nitro-benzenesulfonyl chloride and **23** gave the corresponding sulfonamide **24** in 86% yield, which was then alkylated with *N*-Mmt aminopropyl bromide **11b**, affording compound **25** in high yield (95%). The replacement, 'in one pot', of the *o*-Ns group of **25** by the Boc one led to **26** in 85% yield. Finally, the cleavage of the Mmt protecting group in **26** was achieved selectively with 1% TFA–CH_2Cl_2, giving quasi quantitatively the key synthon **7c**.

The next step consisted in the preparation of **27**, by coupling **7c** with **6** using Bop reagent (90%). Removal of the Fmoc protecting group in **27** by means of diethylamine yielded **28** (95%), which was then condensed with the *N*-Fmoc aminoacid **5**, via a DCC–HOSu preactivation, to lead the fully protected compound **29** (60%). At last, Fmoc cleavage with diethylamine afforded compound **4** in 95% yield.

Finally, condensation of the ethidium derivative **3** onto the free terminal amino group of **4** (Bop–HOBt activation), afforded the polyprotected conjugate **30** in 65% yield. Simultaneous cleavage of the Pmc, Boc and *tert*-butyl protecting groups of the various amine and acid functions was performed quantitatively using TFA–H_2O. At last, compound **2** was purified by semi-preparative HPLC. Its purity was demonstrated by HPLC analyses and its structure confirmed by HRMS experiments.

3. Pharmacology

The binding of compound **2** to TAR RNA was evaluated by melting temperature experiments, by mea-

suring its ability to alter the thermal denaturation profile of the TAR RNA sequence. The capacity of **2** to hydrolysis the TAR RNA sequences was evaluated by polyacrylamide gel electrophoresis. Finally, the anti-HIV-1 activity and cytotoxicity of compound **2** were evaluated in infected CEM-SS, MT4 and PBMC cells.

4. Results and discussion

Melting temperature studies clearly indicated that the ligand **2** maintained a high affinity for the TAR RNA sequence, which was further comparable with that of **1**. Indeed, in the presence of conjugate **2**, at a conjugate/RNA ratio of 0.4, the ΔTm of the TAR RNA (ΔTm = Tm complex − Tm RNA) was increased by 10 °C whereas at the same ratio, the ΔTm increase was only of 7.5 °C in the presence of ethidium bromide, and of 11.5 °C in the presence of conjugate **1** [6]. Disappointingly, however, no hydrolysis of the TAR RNA by **2** was observed at 37 °C and pH 7, as attested by polyacrylamide gel electrophoresis, suggesting an inappropriate positioning of the cleaver towards the potential scission site.

Moreover, unlike conjugate **1**, compound **2** was devoid of any antiviral activity nor of toxicity (IC_{50} and $CC_{50} > 10^{-5}$ M). As melting temperature experiments showed that compound **2** was able to bind to TAR in vitro as efficiently as compound **1**, the lack of any anti-HIV activity for **2** could be due to a poor intracellular uptake.

5. Conclusion

An efficient procedure has been developed to synthesise a designed ethidium–arginine–ribonuclease conjugate targeting the HIV-1 TAR RNA sequence. In the context of the Fukuyama *N*-alkylation of the *N*-(3-aminopropyl) *N*-(2-nitrobenzenesulfonyl) glycine moiety, the 2-nitrobenzenesulfonyl group was found to be unsuitable as permanent amino protecting group of the glycine residue. Nevertheless, it could be used provisionally for the *N*-alkylation and then efficiently replaced in a 'one pot' procedure by the Boc urethane-protecting group.

Biological studies have shown that although the ethidium–arginine–ribonuclease conjugate **2** strongly interacted with TAR RNA, it did not induce its cleavage nor displayed an antiviral activity on HIV-infected cells. Changes in both the length and the nature of the spacer linking the glycine and arginine residue in view of inducing TAR–RNA hydrolysis are currently under consideration. Moreover, a prodrug approach involving conversion of the carboxylic acid into an ester must be considered, in view of increasing the cell membrane permeability.

6. Experimental

Unless otherwise stated, all reagents were obtained from commercial suppliers and were used without further purification. All solvents were freshly distilled. The following abbreviations are employed: Ethidium (Eth), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (HOSu), 1-hydroxybenzotriazole (HOBt), benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium-hexafluorophosphate (Bop), 2-nitrobenzenesulfonyl chloride (*o*-Ns–Cl), monomethoxytrityl chloride (Mmt–Cl), *tert*-butyloxycarbonyl (Boc), allyloxycarbonyl (Alloc), benzyloxycarbonyl (Z), 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc), *N*-α-9-fluorenylmethoxycarbonyl-N^G-(2,2,5,7,8-pentamethylchroman-6-sulfonyl) arginine [Fmoc–Arg(Pmc)OH], *N*-methylmorpholine (NMM), diethylamine (DEA), diisopropylethylamine (DIEA), triethylamine (TEA), trifluoroacetic acid (TFA), acetic acid (AcOH), dimethylformamide (DMF), hexane (Hex). Melting points were determined using an electrothermal digital melting point apparatus IA900 Series and were uncorrected. ^{1}H-NMR spectra were recorded on a Brucker AC 200 (200 MHz) Fourier Transform spectrometer. Thin-layer chromatographies (TLC) were run on precoated silica gel plates (Merck, 60F 254). Column chromatographies were carried out on silica gel 60 (Merck 230–400 mesh ASTM) or on LH20 Sephadex (Sigma, 25–100 μm). HPLC analyses and purifications were performed on a HP1100 equipped with UV detector (at 254 nm), using a C_{18} Merck Interchrom column as support. Elution gradients of H_2O (0.1% TFA)–CH_3CN (0.1% TFA) were used with a flow of 1 mL min^{-1}. Optical rotations were evaluated on a Bellingham and Stanley polarimeter (ADP220) and were at $\pm 1°$. Mass spectrometry analyses were carried out on a TSQ 7000 FINNIGAN MAT (ESIMS) instrument. FAB–HRMS was performed by the Service Central de Microanalyses, CNRS (Vernaison-France).

6.1. Dynamic molecular modelling studies

All molecular modelling studies were performed using the Insight II/Discover molecular modelling package. Simulations were performed for a duration of 200 ps at constant energy using a dielectric continuum to simulate the solvent, and the co-ordinates of the different atoms of TAR RNA were frozen. Energy was computed with CFF93 as force field.

6.2. Chemistry

6.2.1. Fmoc–$NH(CH_2)_5CONH(CH_2)_2CO_2Me$ (**10**)

Bop (442 mg, 1.0 mmol) was added at 0 °C to a stirred solution of *N*-Fmoc-6-aminocaproic acid (**8**) (353 mg, 1.0 mmol), HCl·β-alanine methyl ester (**9**) (168

mg, 1.2 mmol), DIEA (0.7 mL, 4.0 mmol) in CH_2Cl_2 (4 mL). After 2 h stirring, the mixture was diluted with CH_2Cl_2 (20 mL). The organic layer was washed successively with a 1 M aqueous $KHSO_4$ solution, a 10% aqueous $NaHCO_3$ solution, brine and dried over $MgSO_4$. The solvent was evaporated in vacuo. Compound **10** was obtained as an amorphous powder (91%) and used without further purification. TLC (7/3 Hex–EtOAc, UV) $R_f = 0.45$; ^{1}H-NMR ($CDCl_3$) δ: 7.9–7.2 (8H, Fmoc, m), 6.1 (1H, NH amide, m), 5.25 (1H, NH (Fmoc), m), 4.25 (2H, CH_2 (Fmoc), d), 4.15 (1H, CH (Fmoc), t), 3.7 (3H, OCH_3, s), 3.5 (2H, CH_2-8, q), 3.2 (2H, CH_2-13, q), 2.55 (2H, CH_2-7, t), 2.15 (2H, CH_2-9, t), 1.8–1.2 (6H, CH_2-10,11,12, m); MS $m/z = 439.2$ $[M + H]^+$.

*6.2.2. Fmoc–NH(CH$_2$)$_5$CONH(CH$_2$)$_2$CO$_2$H (**5**)*

To a solution of compound **10** (438 mg, 1.0 mmol) in dioxane (10 mL) was added 4N aqueous solution HCl (10 mL). The mixture was heated at 50 °C for 1 h EtOAc (50 mL) was then added. The organic layer was washed with water until pH 7, with brine then dried over $MgSO_4$. The solvent was evaporated in vacuo and the residue was triturated with Et_2O. The white amorphous powder was filtered off giving **5** in 85% yield. TLC (99/1 EtOAc–AcOH, UV) $R_f = 0.30$; ^{1}H-NMR (CD_3OD) δ: 7.75 (2H, Fmoc, d), 7.65 (2H, 2H, Fmoc, d), 7.4 (4H, Fmoc, m), 4.35 (2H, CH_2 Fmoc, d), 4.25 (1H, CH Fmoc, t), 3.5 (2H, CH_2-8, t), 3.1 (2H, CH_2-13, t), 2.5 (2H, CH_2-7, t), 2.25 (2H, CH_2-9, t), 1.6–1.4 (6H, CH_2-10,11,12, m); MS $m/z = 425.1$ $[M + H]^+$.

*6.3. Synthesis of compounds **11a**–**16a**, by-products **17a** and **18a***

*6.3.1. N-Boc-3-bromopropylamine (**11a**)*

TEA (1.40 mL, 10 mmol) was slowly added to a cold solution (0 °C) of HBr·3-bromopropylamine (1.1 g, 5.0 mmol) and Boc_2O (1.09 g, 5.0 mmol) in 30 mL of CH_2Cl_2. The solution was stirred 2 h at r.t., then washed with water. The organic layer was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (Hex 100% to 1/1 Hex–AcOEt). Compound **11a** was obtained as an oil in 82% yield. TLC R_f (CH_2Cl_2, ninhydrine) $R_f = 0.39$; ^{1}H-NMR ($CDCl_3$) δ: 4.9 (1H, NH, m), 3.4 (2H, CH_2Br, t), 3.15 (2H, Boc–NHCH_2, m), 2.0 (2H, C–CH_2–C, m), 1.5 (9H, Boc, s); MS $m/z = 238.3$, 240.4 $[M + H]^+$.

*6.3.2. N-(o-Ns) methyl glycinate (**12a**)*

This compound was previously described in the literature [19]. NMM (1.1 mL, 10 mmol) was added to a cold solution of CH_2Cl_2 (30 mL) containing HCl·Gly–OMe (502 mg, 4.0 mmol) and *o*-Ns–Cl (886.5 mg, 4.0 mmol). The solution was stirred 2 h at r.t., then washed with an aqueous 10% $NaHCO_3$ solution, with an aqueous 10% citric acid solution and finally with H_2O. The solution was dried over $MgSO_4$ and evaporated to dryness. Et_2O was added to precipitate compound **12a**, which was then filtered off. Compound **12a** was obtained in 84% yield, as a white amorphous powder. TLC (3/7 Hex–AcOEt, UV) $R_f = 0.54$; ^{1}H-NMR ($CDCl_3$) δ: 8.15 (1H, *o*-Ns, dd), 8.0 (1H, *o*-Ns, dd), 7.8 (2H, *o*-Ns, td), 6.15 (1H, NH, s), 4.1 (2H, CH_2 α (Gly), s), 3.65 (3H, OCH_3, s); MS $m/z = 275.1$ $[M + H]^+$.

*6.3.3. Boc–NH(CH$_2$)$_3$N–(o-Ns)CH$_2$CO$_2$CH$_3$ (**13a**)*

Compound **12a** (274 mg, 1.0 mmol) was dissolved in DMF (2 mL) then Cs_2CO_3 was added (358 mg, 1.1 mmol). The solution was stirred 30 min at r.t., then compound **11a** (357 mg, 1.5 mmol) was added. Stirring was continued for 18 h. DMF was removed under reduced pressure and the residue was dissolved in EtOAc. The organic layer was washed with H_2O, dried over $MgSO_4$ and evaporated to dryness. The product was purified on a silica gel column (7/3–1/1 Hex–EtOAc) and compound **13a** was obtained as a colourless oil in 80% yield. TLC (1/1 Hex–AcOEt, UV) $R_f = 0.67$; ^{1}H-NMR ($CDCl_3$) δ: 8.15 (1H, *o*-Ns, m), 7.85 (3H, *o*-Ns, m), 5.0 (1H, Boc–NH, m), 4.3 (2H, CH_2 α (Gly), s), 3.8 (3H, OCH_3, s), 3.5 (2H, CH_2-1, t), 3.25 (2H, CH_2-3, m), 1.8 (2H, CH_2-2, m), 1.5 (9H, Boc, s); MS $m/z = 432.1$ $[M + H]^+$.

*6.3.4. TFA·NH$_2$(CH$_2$)$_3$N–(o-Ns)CH$_2$CO$_2$CH$_3$ **14a***

Compound **13a** (1.1 g, 2.55 mmol) was dissolved in 6 mL of a solution of 1/1 TFA–CH_2Cl_2. After stirring for 1 h at 0 °C, the solvent was evaporated in vacuo and the residue was taken up in Et_2O. The white solid that precipitated was filtered off, giving the TFA salt **14a** in 95% yield. TLC (1/1 EtOAc–MeOH, ninhydrine) $R_f = 0.30$; ^{1}H-NMR ($CDCl_3$) δ: 8.1 (1H, *o*-Ns, m), 7.85 (3H, *o*-Ns, m), 4.3 (2H, CH_2α (Gly), s), 3.8 (3H, OCH_3, s), 3.6 (2H, CH_2-1, m), 3.3 (2H, CH_2-3, m), 2.1 (2H, CH_2-2, m); MS $m/z = 332.2$ $[M + H]^+$.

*6.3.5. o-Ns–NH(CH$_2$)$_3$N–(o-Ns)CH$_2$CO$_2$CH$_3$ (**15a**)*

Compound **15a** was obtained from **14a** (445 mg, 1.0 mmol) and *o*-Ns–Cl (221 mg, 1.0 mmol), following the above procedure for the preparation of **12a**. Purification by silica gel column chromatography (7/3–1/1 Hex–EtOAc) afforded **15a** in 75% yield, as an amorphous solid. TLC (1/1 Hex–EtOAc, UV) $R_f = 0.42$; ^{1}H-NMR ($CDCl_3$) δ: 8.2 (1H, *o*-Ns, m), 8.1 (1H, *o*-Ns, m), 8.0–7.6 (6H, *o*-Ns, m), 5.85 (1H, NH, t), 4.1 (2H, CH_2α (Gly), s), 3.8 (3H, OCH_3, s), 3.55 (2H, CH_2-1, t), 3.3 (2H, CH_2-3, q), 1.9 (2H, CH_2-2, m); MS $m/z = 517.4$ $[M + H]^+$.

*6.3.6. Boc–NH[(CH$_2$)$_3$N–(o-Ns)]$_2$CH$_2$CO$_2$CH$_3$ (**16a**), by-products **17a** and **18a***

Cs_2CO_3 (391 mg, 1.2 mmol) was added to a solution of **15a** (598.5 mg, 1.16 mmol) and **11a** (552 mg, 2.32 mmol) in DMF (2 mL). The mixture was stirred for 4 h at r.t. then the solvent was evaporated in vacuo. The residue was taken up with EtOAc and the organic layer was successively washed with 10% aqueous $NaHCO_3$ solution, 10% aqueous citric acid solution and water. After drying over $MgSO_4$, the solution was evaporated to dryness. Purification by silica gel column chromatography (1/1–0/1 Hex–EtOAc) gave **16a**, **17a** and **18a** in 25, 11 and 18% yield, respectively.

*6.3.7. Compound **16a***

TLC (3/7 Hex–EtOAc, UV) $R_f = 0.65$; ^{1}H-NMR ($CDCl_3$) δ: 8.0 (2H, *o*-Ns, m), 7.75–7.5 (6H, *o*-Ns, m), 4.8 (1H, NH, m), 4.15 (2H, $CH_2\alpha$ (Gly), s), 3.65 (3H, OCH_3, s), 3.3 (6H, CH_2-1,3,4, m), 3.1 (2H, CH_2-6, m), 1.85 (2H, CH_2-2, m), 1.7 (2H, CH_2-5, m), 1.4 (9H, Boc, s); MS $m/z = 696.3$ $[M + Na]^+$.

*6.3.8. Compound **17a***

TLC (3/7 Hex–EtOAc, UV) $R_f = 0.53$; ^{1}H-NMR ($CDCl_3$) δ: 8.1 (1H, *o*-Ns, m), 8.0 (1H, Ar, m), 7.9–7.5 (4H, Ar and *o*-Ns, m), 7.3 (2H, Ar, m), 6.9 (1H, NH, bs), 5.0 (2H, C*H*$_2$N–(*o*-Ns), t), 4.05 (3H, OCH_3, s), 3.1 (2H, C*H*$_2$–(C=C), m), 2.1 (2H, C–C*H*$_2$–C, m);); MS $m/z = 457.0$ $[M + Na]^+$.

*6.3.9. Compound **18a***

TLC (3/7 Hex–EtOAc, UV) $R_f = 0.49$; ^{1}H-NMR ($CDCl_3$) δ: 8.0 (1H, *o*-Ns, m), 7.9 (1H, Ar, m), 7.89–7.5 (4H, Ar and *o*-Ns, m), 7.3 (2H, Ar, m), 5.0 (1H, Boc–NH, m), 4.80 (2H, C*H*$_2$N–(*o*-Ns), t), 4.0 (3H, OCH_3, s), 3.6 (2H, Boc–NHC*H*$_2$, m), 3.4 (2H, C*H*$_2$N–, m), 3.15 (2H, C*H*$_2$–C=C), m), 2.1 (2H, C–C*H*$_2$–C (cycl), m), 1.75 (2H, C–C*H*$_2$–C, m), 1.4 (9H, Boc, s); MS $m/z = 614.2$ $[M + Na]^+$.

*6.4. Synthesis of compounds **11b–17b***

*6.4.1. N-Mmt-3-bromopropylamine (**11b**)*

TEA (2.675 mL, 19 mmol) was slowly added to a cold solution (0 °C) of HBr·3-bromopropylamine (2.0 g, 9.13 mmol) and Mmt–Cl (2.81 g, 9.13 mmol) in 10 mL of CH_2Cl_2. The solution was stirred for 2 h at r.t., then washed with water. The organic layer was evaporated under reduced pressure. The residue was purified by chromatography on silica gel column (1/0–9/1 Hex–AcOEt). Compound **11b** was obtained as a yellow oil in 95% yield. TLC (9/1 Hex–AcOEt, UV) $R_f = 0.72$; ^{1}H-NMR ($CDCl_3$) δ: 7.6–7.1 (12H, Mmt, m), 6.9 (2H, Mmt, dd), 3.85 (3H, OCH_3, s), 3.65 (2H, CH_2Br, t, *J* 6.7 Hz), 2.4 (2H, CH_2NH, t, *J* 6.5 Hz), 2.1 (2H, $CH_2CH_2CH_2$, m), 1.7 (1H, NH, s); MS $m/z = 410.2$, 412.1 $[M + H]^+$.

*6.4.2. N-(o-Ns) tertiobutyl glycinate (**12b**)*

This compound was prepared from AcOH·Gly–O *t*-Bu (1.05 g, 5.35 mmol) and *o*-Ns–Cl (1.3 g, 5.50 mmol), following the above procedure for the preparation of **12a**. Compound **12b** was obtained in 90% yield, as a white amorphous powder. TLC (7/3 Hexane–AcOEt, UV) $R_f = 0.42$; ^{1}H-NMR ($CDCl_3$) δ: 8.20 (1H, *o*-Ns, dd), 8.0 (1H, *o*-Ns, dd), 7.8 (2H, *o*-Ns, td), 6.1 (1H, NH, s), 4.0 (2H, Hα (Gly), s), 1.4 (9H, O*t*-Bu, s); MS $m/z = 317.0$ $[M + H]^+$.

*6.4.3. Mmt–NH(CH$_2$)$_3$N–(o-Ns)CH$_2$CO$_2$t-Bu (**13b**)*

This compound was prepared from **12b** (3.0 g, 9.5 mmol), **11b** (7.75 g, 18.9 mmol) and Cs_2CO_3 (3.4 g, 10.4 mmol), following the above procedure for the preparation of **13a**. The crude product was purified on silica gel column (7/3–1/1 Hex–EtOAc) and compound **13b** was obtained as a colourless oil in 95% yield. TLC (7/3 Hex–EtOAc, UV) $R_f = 0.52$; ^{1}H-NMR ($CDCl_3$) δ: 8.0 (1H, *o*-Ns, m), 7.7–7.1 (15H, *o*-Ns, Mmt, m), 6.85 (2H, Mmt, d), 4.1 (2H, Hα (Gly), s), 3.8 (3H, OCH_3, s), 3.5 (2H, CH_2-1, m), 2.2 (2H, CH_2-3, m), 1.9–1.6 (3H, NH, CH_2-2, m), 1.4 (9H, O*t*-Bu, bs); MS $m/z = 668.45$ $[M + Na]^+$.

*6.4.4. TFA·NH$_2$(CH$_2$)$_3$N–(o-Ns)CH$_2$CO$_2$t-Bu (**14b**)*

Compound **13b** (600 mg, 0.93 mmol) was dissolved in 15 mL of 1/99 TFA–CH_2Cl_2. The mixture was stirred for 2 h then the solvent was evaporated in vacuo. The residue was purified by silica gel column chromatography (1/0–1/1 EtOAc–MeOH), giving **14b** (92%) as a colourless resin. TLC (8/2 EtOAc–MeOH, ninhydrine) $R_f = 0.33$; ^{1}H-NMR ($CDCl_3$) δ: 8.1 (2H, *o*-Ns, m), 7.85 (2H, *o*-Ns, m), 4.1 (2H, Hα (Gly), s), 3.6 (2H, CH_2-1, m), 3.3 (2H, CH_2-3, m), 2.1 (2H, CH_2-2, m), 1.4 (9H, O*t*-Bu, s); MS $m/z = 374.2$ $[M + H]^+$.

*6.4.5. o-Ns–NH(CH$_2$)$_3$N–(o-Ns)CH$_2$CO$_2$t-Bu (**15b**)*

Compound **15b** was obtained from **14b** (487 mg, 1.0 mmol) and *o*-Ns–Cl (222 mg, 1.0 mmol) following the above procedure for the preparation of **15a**. Purification by silica gel column chromatography (7/3–1/1 Hex–EtOAc) afforded **15b** in 77% yield, as a yellow amorphous solid. TLC (1/1 Hex–EtOAc, UV) $R_f = 0.53$; ^{1}H-NMR ($CDCl_3$) δ: 8.2–8.1 (2H, *o*-Ns, m), 8.0–7.6 (6H, *o*-Ns, m), 5.85 (1H, NH, t), 4.1 (2H, Hα (Gly), s), 3.55 (2H, CH_2-1, t), 3.3 (2H, CH_2-3, q), 1.9 (2H, CH_2-2, m), 1.4 (9H, O*t*-Bu, s); MS $m/z = 559.1$ $[M + H]^+$.

*6.4.6. Mmt–NH[(CH$_2$)$_3$N–(o-Ns)]$_2$CH$_2$CO$_2$t-Bu (**16b**), by-product **17b***

Cs_2CO_3 (358 mg, 1.1 mmol) was added to a solution of **15b** (603 mg, 1.08 mmol) and **11b** (886 mg, 2.16 mmol) in DMF (1.5 mL). The mixture was stirred for 18 h at r.t. then the solvent was evaporated in vacuo.

The residue was taken up in EtOAc and the organic layer was washed with 10% aqueous $NaHCO_3$ solution then with water. The solution was dried over $MgSO_4$, then evaporated to dryness. Purification by silica gel column chromatography (7/3–1/1 Hex–EtOAc) gave **16b** and **17b** in 50 and 25% yield, respectively.

*6.4.7. Compound **16b***

TLC (1/1 Hex–EtOAc, UV) $R_f = 0.64$; ^{1}H-NMR ($CDCl_3$) δ: 8.0 (2H, *o*-Ns, m), 7.6–7.0 (18H, Ar and *o*-Ns, m), 6.7 (2H, Mmt, d), 4.0 (2H, Hα (Gly), s), 3.7 (3H, OCH_3, s), 3.3 (6H, CH_2-1,3,4, m), 2.0 (2H, CH_2-6, m), 1.7 (5H, CH_2-2,5, NH, m), 1.4 (9H, O*t*-Bu, s); MS $m/z = 887.9$ $[M + H]^+$.

*6.4.8. Compound **17b***

TLC (1/1 Hex–EtOAc, UV) $R_f = 0.52$; ^{1}H-NMR ($CDCl_3$) δ: 8.0 (2H, Ar and *o*-Ns, m), 7.7 (2H, Ar and *o*-Ns, m), 7.3 (2H, Ar, m), 6.9 (1H, NH, m), 4.95 (2H, CH_2N–(*o*-Ns), t), 3.1 (2H, CH_2–(C=C), m), 2.1 (2H, C–CH_2–C, m), 1.65 (9H, O*t*-Bu, s); MS $m/z = 477.2$ $[M + H]^+$.

*6.4.9. TFA·NH_2[$(CH_2)_3$N–(o-Ns)]$_2CH_2CO_2$t-Bu (**7b**)*

Compound **16b** (220 mg, 0.25 mmol) dissolved in 4 mL of 1/99 TFA–CH_2Cl_2 was stirred for 2 h at 0 °C, then the solvent was evaporated in vacuo. The residue was purified by silica gel column chromatography (1/0–1/1 EtOAc–MeOH), giving **7b** (93%) as a colourless resin. ^{1}H-NMR ($CDCl_3$) δ: 8.15 (4H, *o*-Ns, m), 7.8–7.6 (4H, *o*-Ns, m), 4.0 (2H, Hα (Gly), s), 3.4 (6H, CH_2-1, 3, 4, m), 3.3 (2H, CH_2-6, m), 2.1 (4H, CH_2-2, 5, m), 1.4 (9H, O*t*-Bu, s); MS $m/z = 616.2$ $[M + H]^+$.

*6.4.10. Fmoc–Arg(Pmc)CONH[$(CH_2)_3$N–(o-Ns)]$_2CH_2$-CO_2t-Bu (**19**)*

FmocArg(Pmc)OH (**6**) (378 mg, 0.57 mmol), **7b** (415.5 mg, 0.57 mmol) and DIEA (0.4 mL, 2.28 mmol) were placed at 0 °C in CH_2Cl_2. Bop (252 mg, 0.57 mmol) was then added. The mixture was stirred for 2 h at r.t. then CH_2Cl_2 (20 mL) was added. The organic layer was washed with a 10% aqueous $NaHCO_3$ solution, brine and dried over $MgSO_4$. The solvent was evaporated in vacuo. Purification by silica gel column chromatography (1/0–1/1 EtOAc–MeOH) gave **19** as a colourless resin, in 88% yield. TLC (EtOAc, UV) $R_f =$ 0.42; ^{1}H-NMR ($CDCl_3$) δ: 7.9 (2H, *o*-Ns, m), 7.8–7.4 (10H, *o*-Ns and Fmoc, m), 6.7 (1H, NH, m), 6.25 (3H, guanidino, bs), 5.3 (1H, NH, m), 4.3 (1H, Hα (Arg), m), 4.25 (2H, CH_2 (Fmoc), d), 4.15 (1H, CH (Fmoc), m), 4.0 (2H, CH_2α (Gly), s), 3.5–3.0 (10H, $CH_2\delta$ (Arg), CH_2-1,3,4,6, m), 2.6–2.4 (8H, $2CH_3$ (Pmc), CH_2 (Pmc), m), 2.05 (3H, CH_3 (Pmc), s), 1.8 (4H, CH_2-2,5, m), 1.65–1.5 (6H, CH_2β-γ (Arg), CH_2 (Pmc)), 1.4 (9H, O*t*-Bu, s), 1.4 (18H, Boc, m), 1.15 (6H, $2CH_3$ (Pmc), s); MS $m/z = 1261.2$ $[M + H]^+$.

*6.4.11. Compound **20***

DEA (2 mL) was added to a solution of **19** (340 mg, 0.27 mmol) in CH_2Cl_2 (2 mL). After stirring for 2 h, the solvent was evacuated in vacuo. The residue was purified by silica gel column chromatography (1/0–1/1 EtOAc–MeOH), giving the free amino compound in 93% yield. This one (260 mg, 0.25 mmol), compound **5** (106 mg, 0.25 mmol), HOSu (43 mg, 0.375 mmol) and NMM (28 μL, 0.25 mmol) were then placed in DMF (2 mL) at 0 °C. DCC (57.5 mg, 0.28 mmol) was added and the mixture was stirred for 20 h at r.t. The solvent was evaporated under reduced pressure. The residue was taken up in EtOAc, DCU was filtered off and washed with EtOAc. The filtrate was washed with a 10% aqueous $NaHCO_3$ solution, brine and finally dried over $MgSO_4$. The solvent was evaporated in vacuo. Purification by silica gel column chromatography (1/0–8/2 EtOAc–MeOH) gave **20** as a colourless resin, in 70% yield. TLC (8/2 EtOAc–MeOH, UV) $R_f = 0.46$; ^{1}H-NMR ($CDCl_3$) δ: 7.9 (2H, *o*-Ns, m), 7.8–7.4 (10H, *o*-Ns and Fmoc, m), 7.3 (4H, Fmoc, m), 7.1 (1H, NH amide, m), 6.7 (1H, NH, m), 6.4–6.1 (3H, NH guanidinium, m), 5.3 (1H, NH (Fmoc), m), 4.45 (1H, Hα (Arg), m), 4.25 (2H, CH_2 (Fmoc), d), 4.15 (1H, CH (Fmoc), t), 3.95 (2H, Hα (Gly), s), 3.45 (CH_2-8, m), 3.4–3.0 (12H, $CH_2\delta$ (Arg), CH_2-1,3,4,6,13, m), 2.50 (10H, $2CH_3$ (Pmc), CH_2 (Pmc), CH_2-7, m), 2.1 (2H, CH_2-9, m), 2.05 (3H, CH_3m (Pmc), s), 1.9–1.65 (10H, CH_2-2,5,10,11,12, m), 1.6–1.4 (6H, CH_2-β,γ (Arg), CH_2 (Pmc), m), 1.3 (9H, O*t*-Bu, m), 1.15 (6H, $2CH_3$ (Pmc), s); MS $m/z = 1467.1$ $[M + Na]^+$.

*6.4.12. Compound **21***

DEA (1 mL) was added to a solution of **20** (274 mg, 0.19 mmol) in CH_2Cl_2 (1 mL). After stirring for 2 h, the solvent was evacuated in vacuo. The residue was purified by silica gel column chromatography (1/0–1/1 EtOAc–MeOH) to give the free amino compound in 90% yield. This one (208 mg, 0.17 mmol), compound **3** (65 mg, 0.17 mmol), HOBt (24 mg, 0.17 mmol) and DIEA (0.12 mL, 0.68 mmol) were then placed in DMF (0.5 mL). Bop (75.1 mg, 0.17 mmol) was added at 0 °C and the mixture was stirred for 2 h. Then, CH_2Cl_2 (10 mL) was added and the organic layer was washed with a 10% aqueous $NaHCO_3$ solution, water, dried over MgSO4 and evaporated in vacuo. A LH 20 column chromatography (1/0–1/1 H_2O–MeOH) afforded compound **21** as a purple resin in 50% yield. HPLC [8/2–0/1 H_2O (1/1000 TFA)–CH_3CN (1/1000 TFA) in 30 min, 288 nm]: Rt = 23.1 min; ^{1}H-NMR (DMSO) δ: 8.6 (2H, H-e3, H-e4, m), 8.2 (2H, H-e9, H-e10, m), 7.9 (2H, *o*-Ns, m), 7.8 (3H, H-e5, H-e7, H-e8, m), 7.6–7.1 (11H, H-e1, H-e2, m), 7.0 (2H, NH_2, bs), 6.6 (2H, NH_2, bs), 6.35 (1H, H-e6, s), 4.2 (1H, Hα (Arg), m), 4.0 (3H, N^+(Me), s), 3.9 (2H, Hα (Gly), s), 3.4–3.0 (14H, CH_2-1,3,4,6,8,13, CH_2-δ (Arg), m), 2.5–2.35 (10H,

2CH_3 (Pmc), CH_2 (Pmc), CH_2-7, m), 2.15 (2H, CH_2-9, m), 2.05 (3H, CH_3m (Pmc), s), 1.8–1.6 (10H, CH_2-2,5,10,11,12, m), 1.6–1.4 (6H, CH_2-β,γ (Arg), CH_2 (Pmc), m), 1.4 (9H, O*t*-Bu, s), 1.15 (6H, 2CH_3 (Pmc), s); MS $m/z = 1548.3$ $[M]^+$.

*6.4.13. Mmt–NH(CH_2)$_3$N–(Boc)CH_2CO_2t-Bu (**22**)*

PhSH (421 μL, 4.11 mmol) and DIEA (573 μL, 3.29 mmol) were added to a solution of compound **13b** (529 mg, 0.82 mmol) in 3 mL of DMF. After 2 h of stirring, Boc_2O (218 mg, 1 mmol) was added. The mixture was stirred two additional hours then DMF was evaporated in vacuo. The residue was taken up with EtOAc. The organic layer was washed with 10% aqueous $NaHCO_3$ solution then with water and dried over $MgSO_4$. The solvent was evaporated in vacuo. The crude residue was purified by silica gel column chromatography (CH_2Cl_2 then 7/3 Hex–EtOAc) to yield **22** in 75% yield. TLC (9/1 Hex–EtOAc, UV) $R_f = 0.27$; ^{1}H-NMR ($CDCl_3$) δ: 7.6–7.1 (12H, Mmt, m), 6.85 (2H, Mmt, d), 3.8 (3H, OCH_3, s), 3.75 (2H, Hα (Gly), d), 3.35 (2H, CH_2-1, m), 2.15 (2H, CH_2-3, m), 1.9–1.6 (3H, NH, CH_2-2, m), 1.5 (9H, O*t*-Bu, s), 1.4 (9H, Boc, d); MS $m/z = 561.3$ $[M + H]^+$.

*6.4.14. TFA·NH_2(CH_2)$_3$N–(Boc)CH_2CO_2t-Bu (**23**)*

Compound **22** (605 mg, 1.08 mmol), dissolved in 15 mL of 1/99 TFA–CH_2Cl_2, was stirred at r.t. for 3 h. The solvent was evaporated under reduced pressure and the crude residue purified by silica gel column chromatography (1/0–0/1 EtOAc–MeOH). Compound **23** was obtained as a colourless resin (84%). TLC (8/2 EtOAc–MeOH, ninhydrine) $R_f = 0.47$; ^{1}H-NMR ($CDCl_3$) δ: 3.8 (2H, Hα (Gly), d), 3.45 (2H, CH_2-1, m), 3.25 (2H, CH_2-3, m), 1.9 (3H, NH, CH_2-2, m), 1.5 (9H, O*t*-Bu, s), 1.4 (9H, Boc, d); MS $m/z = 289.3$ $[M + H]^+$.

*6.4.15. o-Ns–NH(CH_2)$_3$N–(Boc)CH_2CO_2t-Bu (**24**)*

Compound **23** (462 mg, 1.15 mmol) and *o*-Ns–Cl (255 mg, 1.15 mmol) were placed at 0 °C in 6 mL of CH_2Cl_2. NMM (380 μL, 3.45 mmol) was slowly added. The mixture was stirred at r.t. for 2 h. The solvent was then evaporated in vacuo. The residue was taken up in EtOAc and the organic layer was washed with 10% aqueous $NaHCO_3$ solution, with brine and finally dried over $MgSO_4$. Evaporation of the solvent under reduced pressure led to an oil that was purified by silica gel column chromatography (1/1 Hex–EtOAc), giving **24** (86%) as a colourless resin. TLC (1/1 Hex–EtOAc, UV) $R_f = 0.57$; ^{1}H-NMR ($CDCl_3$) δ: 8.1 (1H, *o*-Ns, m), 7.85–7.6 (3H, *o*-Ns, m), 6.35 (1H, NH, m), 3.65 (2H, Hα (Gly), d), 3.35 (2H, CH_2-3, m), 3.15 (2H, CH_2-1, m), 1.65 (2H, CH_2-2, m), 1.5–1.3 (18H, O*t*-Bu and Boc, m); MS $m/z = 474.3$ $[M + H]^+$.

*6.4.16. Mmt–NH–(CH_2)$_3$–N(o-Ns)–(CH_2)$_3$–N(Boc)–CH_2CO_2t-Bu (**25**)*

Compound **24** (331 mg, 0.7 mmol) was dissolved in DMF (2 mL) then Cs_2CO_3 was added (342 mg, 1.05 mmol). After stirring for 30 min at r.t., compound **11b** was added (570 mg, 1.4 mmol). Stirring was continued at r.t. for 18 h. DMF was removed under reduced pressure and the residue was dissolved in EtOAc. The organic layer was washed with water, brine then dried over $MgSO_4$ and evaporated to dryness. The product was purified by silica gel column chromatography (7/3–1/1 Hex–EtOAc) and compound **25** was obtained as an yellow oil in 95% yield. TLC (7/3 Hex–EtOAc, UV) $R_f = 0.31$; ^{1}H-NMR ($CDCl_3$) δ: 8.0 (1H, *o*-Ns, m), 7.7–7.1 (15H, *o*-Ns and Mmt, m), 6.75 (2H, Mmt, d), 3.75 (3H, OCH_3, s), 3.7 (2H, Hα (Gly), d) 3.1–3.4 (6H, CH_2-1,3,4, m), 2.05 (2H, CH_2-6, m), 1.85–1.55 (5H, NH, CH_2-2,5, NH, m), 1.4 (9H, O*t*-Bu, s), 1.35 (9H, Boc, d); MS $m/z = 825.7$ $[M + Na]^+$.

*6.4.17. Mmt–NH–[(CH_2)$_3$–N(Boc)]$_2$–CH_2CO_2t-Bu (**26**)*

PhSH (338 μL, 3.3 mmol) and DIEA (460 μL, 2.64 mmol) were added to a solution of compound **25** (529 mg, 0.66 mmol) in 3 mL of DMF. After disappearance of the starting material (about 6 h, TLC monitoring), Boc_2O (0.218 g, 1 mmol) was added. The mixture was stirred for 4 h additional then DMF was evaporated in vacuo. The residue was taken up with EtOAc. The organic layer was washed with water and dried over $MgSO_4$. The solvent was evaporated in vacuo and the crude residue was purified by silica gel column chromatography (CH_2Cl_2 then 7/3 Hex–EtOAc) to yield **26** in 85% yield. TLC (7/3 Hex–EtOAc, UV) $R_f = 0.67$; ^{1}H-NMR ($CDCl_3$) 7.5–7.0 (12H, Mmt, m), 6.7 (2H, Mmt, d), 3.75 (2H, Hα Gly), d), 3.7 (3H, OCH_3, s), 3.35–3.0 (6H, CH_2-1,3,4, m), 2.05 (2H, CH_2-6, m), 1.75–1.55 (5H, NH, CH_2-2,5, m), 1.4 (9H, O*t*-Bu, s), 1.45 (18H, Boc, m); MS $m/z = 740.7$ $[M + Na]^+$.

*6.4.18. TFA·NH_2–[(CH_2)$_3$–N(Boc)]$_2$–CH_2CO_2t-Bu (**7c**)*

Compound **26** (351 mg, 0.49 mmol) was dissolved in 8 mL of 1/99 TFA–CH_2Cl_2.The mixture was stirred at r.t. for 3 h. The solvent was then evaporated under reduced pressure and the crude residue purified by silica gel column chromatography (EtOAc 100% to 8/2 EtOAc–MeOH), allowing the isolation of **7c** as an oil (85%). TLC (8/2 EtOAc–MeOH, ninhydrine) $R_f = 0.60$; ^{1}H-NMR ($CDCl_3$) δ: 3.75 (2H, Hα (Gly), d), 3.35–3.0 (6H, CH_2-1,3,4, m), 3.3 (2H, CH_2-6, m), 1.9–1.7 (4H, CH_2-2,5, m), 1.4 (9H, O*t*-Bu, s), 1.45 (18H, Boc, m); MS $m/z = 445.5$ $[M + H]^+$.

*6.4.19. Fmoc–NHArg(Pmc)CONH–[(CH_2)$_3$–N(Boc)]$_2$–CH_2CO_2t-Bu (**27**)*

This compound was synthesised following the same protocol as for compound **19**, starting from

Fmoc–Arg(Pmc)OH (**6**) (332 mg, 0.5 mmol) and compound **7c** (280 mg, 0.5 mmol). Purification by silica gel column chromatography (EtOAc) gave **27** as a colourless resin (92%). TLC (EtOAc, UV) $R_f = 0.38$; ^{1}H-NMR ($CDCl_3$) δ: 7.7–7.1 (8H, Fmoc, m), 6.4–6.0 (3H, guanidino, bs), 5.3 (1H, NH, m), 4.3 (1H, Hα (Arg), m), 4.25 (2H, CH_2 (Fmoc), d), 4.15 (1H, CH (Fmoc), m); 3.7 (2H, Hα (Gly), d), 3.5–2.9 (10H, CH_2δ (Arg), CH_2-1,3,4,6, m), 2.65–2.40 (8H, $2CH_3$ (Pmc), CH_2 (Pmc), m), 2.0 (3H, CH_3 (Pmc), s), 1.85–1.45 (10H, CH_2-β,γ (Arg), CH_2 (Pmc), CH_2-2,5, m), 1.4 (9H, O*t*-Bu, s), 1.35 (18H, Boc, m), 1.15 (6H, $2CH_3$ (Pmc), s); MS $m/z = 1113.2$ $[M + Na]^+$.

*6.4.20. H_2N–Arg(Pmc)CONH–[$(CH_2)_3$–N(Boc)]$_2$–CH_2CO_2t-Bu (**28**)*

DEA (2 mL) was added to a solution of **27** (185 mg, 0.17 mmol) in CH_2Cl_2 (2 mL). After stirring for 2 h, the solvent was evaporated in vacuo. The residue was purified by silica gel column chromatography (from EtOAc to 1/1 EtOAc–MeOH) to give **28** as an oil in 93% yield. TLC (EtOAc–MeOH 1:1, ninhydrine) $R_f =$ 0.35; ^{1}H-NMR ($CDCl_3$) δ: 6.25 (3H, guanidinium, bs), 4.5 (1H, Hα (Arg), m), 3.7 (2H, Hα (Gly), d), 3.4–3.0 (10H, CH_2δ (Arg), CH_2-1,3,4,6, m), 2.65–2.40 (8H, $2CH_3$ (Pmc), CH_2 (Pmc), m), 2.0 (3H, CH_3 (Pmc), s), 1.85–1.45 (10H, CH_2-β,γ (Arg), CH_2 (Pmc), CH_2-2,5, m), 1.45–1.3 (27H, O*t*-Bu, 2 Boc, 2 s), 1.15 (6H, $2CH_3$ (Pmc), s); MS $m/z = 891.2$ $[M + Na]^+$.

*6.4.21. Compound **29***

Compound **5** (153 mg, 0.36 mmol) and HOSu (62 mg, 0.54 mmol) were placed in DMF (2 mL) at 0 °C. DCC (82 mg, 0.4 mmol) was then added. The reaction mixture was stirred for 12 h at r.t. then compound **28** (312.5 mg, 0.36 mmol) and NMM (60 μL, 0.54 mmol) were added. The solution was stirred for 4 h, then the solvent was evaporated under reduced pressure. The residue was taken up in EtOAc, DCU was filtered off and washed with EtOAc. The filtrate was washed with a 10% aqueous $NaHCO_3$ solution, brine and finally dried over $MgSO_4$. The solvent was evaporated in vacuo. The residue was purified by silica gel column chromatography (EtOAc to 8/2 EtOAc–MeOH), giving **29** in 60% yield. TLC (EtOAc, UV) $R_f = 0.25$; ^{1}H-NMR ($CDCl_3$) δ: 7.8–7.15 (8H, Fmoc, m), 7.1 (1H, NH amide, m), 6.45–6.15 (3H, NH guanidinium, m), 5.5 (1H, NH (Fmoc), m), 4.45 (1H, Hα (Arg), m), 4.30 (2H, CH_2 (Fmoc), d), 4.15 (1H, CH (Fmoc), t), 3.75 (2H, Hα (Gly), d), 3.45 (CH_2-8, m), 3.15 (12H, CH_2-δ (Arg), CH_2-1,3,4,6,13, m), 2.50 (10H, $2CH_3$ (Pmc), CH_2 (Pmc), CH_2-7, m), 2.1 (2H, CH_2-9, m), 2.05 (3H, CH_3m (Pmc), s), 1.9–1.2 (16H, CH_2-2,5,10,11,12, CH_2-β,γ (Arg), CH_2 (Pmc), m), 1.45–1.3 (27H, O*t*-Bu, 2 Boc, 2 s), 1.15 (6H, $2CH_3$ (Pmc), s); MS $m/z = 1297.25$ $[M + Na]^+$.

*6.4.22. Compound **4***

To a solution of **29** (128 mg, 0.1 mmol) in CH_2Cl_2 (1 mL) was added DEA (1 mL). After stirring for 3 h, the solvent and DEA were removed under reduced pressure. The residue was triturated three times with a 1/1 Et_2O–Hex mixture, allowing the isolation of **4** in 85% yield. Compound **4** was used in the next step without further purification. HPLC [H_2O (1/1000 TFA)–CH_3CN (1/1000 TFA): from 80/20 to 0/100% in 30 min, 254 nm] Rt = 20.2 min; ^{1}H-NMR ($CDCl_3$) δ: 6.3 (3H, NH guanidinium, m), 4.45 (1H, Hα (Arg), m), 3.75 (2H, Hα (Gly), d), 3.45 (2H, CH_2-8, m), 3.15 (10H, CH_2-δ (Arg), CH_2-1,3,4,6, m), 2.95 (2H, CH_2-13, m), 2.7–2.4 (10H, $2CH_3$ (Pmc), CH_2 (Pmc), CH_2-7, m), 2.15 (2H, CH_2-9, m), 2.05 (3H, CH_3m (Pmc), s), 1.9–1.2 (16H, CH_2-2,5,10,11,12, CH_2-β,γ (Arg), CH_2 (Pmc), m), 1.4 (27H, O*t*-Bu, 2 Boc, 2 s), 1.2 (6H, $2CH_3$ (Pmc), s); MS $m/z = 1074.6$ $[M + Na]^+$

*6.4.23. Compound **30***

Bop (42 mg, 95 μmol) was added to a solution of ethidium derivative **3** (36 mg, 95 μmol), compound **4** (100 mg, 95 μmol), HOBt (13 mg, 95 μmol) and DIEA (70 μL, 0.4 mmol) in DMF (0.5 mL). The mixture was stirred for 2 h, then CH_2Cl_2 (10 mL) was added. The organic layer was washed with a 10% aqueous $NaHCO_3$ solution, water then dried over $MgSO_4$ and evaporated in vacuo. A LH 20 column chromatography (from H_2O to 1/1 H_2O–MeOH) afforded compound **30** as a purple resin in 70% yield. HPLC [H_2O (1/1000 TFA)–CH_3CN (1/1000 TFA): from 80/20 to 0/100% in 30 min, 288 nm]: Rt = 22.2 min; $[\alpha]_D = -87°$ (MeOH, c = 1,15 g l^{-1}, 24 °C); ^{1}H-NMR (CD_3OD) δ: 8.6 (2H, H-e3, H-e4, m), 8.3 (1H, H-e9, m), 8.2 (1H, H-e10, s), 7.9 (2H, H-e8, H-e7, m), 7.6 (1H, H-e5, m), 7.4 (2H, H-e1, H-e2, m), 6.6 (1H, H-e6, s), 4.3 (1H, Hα (Arg), m), 4.15 (3H, N^+(Me), s), 3.9 (2H, Hα (Gly), s), 3.55–3.1 (14H, CH_2-8,13, 1,3,4,6, CH_2-δ (Arg), m), 2.8–2.4 (10H, $2CH_3$ (Pmc), CH_2 (Pmc), CH_2-7, m), 2.15 (2H, CH_2-9, m), 2.05 (3H, CH_3m (Pmc), s), 1.9–1.3 (16H, CH_2-2,5,10,11,12, CH_2-β,γ (Arg), CH_2 (Pmc), m), 1.45 (27H, O*t*-Bu, 2 Boc, 2 s), 1.35 (6H, $2CH_3$ (Pmc), s); MS $m/z = 1377.6$ $[M]^+$

*6.4.24. Compound **2***

Compound **30** (69 mg, 50 μmol) was dissolved in 1 mL of a 90/10 TFA–H_2O solution. After 30 min of stirring, the solvent was evaporated in vacuo. The crude product was purified by reverse-phase semi-preparative HPLC, giving 40 mg of pure compound **2**. HPLC [H_2O (1/1000 TFA)–CH_3CN (1/1000 TFA): from 80/20 to 0/100% in 30 min, 288 nm]: Rt = 8.2 min. $[\alpha]_D = +10°$ (H_2O, $c = 1.03$ g l^{-1}, 24 °C); FAB–HRMS calc. for $C_{44}H_{63}N_{12}O_6$ m/z: 855.4993, Found: m/z: 855.4982% $[M^+]$.

6.5. Pharmacology

Hydrolysis assays were realised as described by Endo et al., using polyacrylamide gel electrophoresis [17]. Melting temperature studies were performed as described for compound **1** [6]. In vitro anti-HIV-1 activity and cytotoxicity of **2** were evaluated on infected CEM-SS, MT4 and PBMC cells, using well-described procedures [6,36].

7. Uncited reference

[24,37].

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