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Original article

Synthesis and biological evaluation of a new derivative of bevirimat that targets the Gag CA-SP1 cleavage site



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ABSTRACT

Bevirimat (**2**), the first-in-class HIV-1 maturation inhibitor, shows a low efficacy due essentially to the natural polymorphism of its target, the CA-SP1 junction. Moreover, its low hydrosolubility makes it difficult to study its interaction with the CA-SP1 junction. We have synthesized new derivatives of bevirimat by adding different hydrophilic substituents at the C-28 position to improve their hydrosolubility and perform the structural study of a complex by NMR. Synthesis of the new derivatives, the effect of substituents at the C-28 position and their hydrosolubility are discussed. The ability of these molecules to inhibit viral infection and their cytotoxicity is assessed. Compared to the well-known bevirimat (**2**), one of our compounds (**16**) shows a higher hydrosolubility associated with a 2.5 fold increase in activity, a higher selectivity index and a better antiviral profile. Moreover, for the first time a direct interaction between a derivative of bevirimat (**16**) and the domain CA-SP1–NC is shown by NMR. Information from this study should allow us to decipher the mechanism by which bevirimat inhibits HIV-1 maturation and how the natural polymorphism of the spacer peptide SP1 triggers resistance to inhibitors.

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1. Introduction

Most of the anti-HIV-1 drug inhibitors target the reverse transcriptase (RT) [1,2], the integrase (IN) [3,4] or the protease (PR) [1,5] and cause the emergence of multidrug-resistant HIV strains. Gag precursor (Pr55Gag) assembly and maturation are important steps of the HIV-1 replication and constitute attractive targets for the design of molecules competent to inhibit the retroviral cycle. Pr55Gag is necessary and sufficient for HIV-1 particle assembly and is cleaved during or after the assembly of the entire particle by PR in four mature proteins, matrix (MA), capsid (CA), nucleocapsid (NC),

p6 and two extra spacer peptides, SP1 and SP2 [6,7]. The first cleavage occurs between SP1 and NC leading to the intermediate MA-CA-SP1, which is cleaved to MA and precursor CA-SP1 (p25). The cleavage of p25, critical for the proper formation of mature and infectious viral particles [8], occurs late in the virus life cycle to generate the mature CA (p24) and SP1.

The region surrounding the CA-SP1 junction plays an important role in the morphogenesis of the virion [9–14]. The mutation of the residues within the CA-SP1 junction results in the production of defective and noninfectious viral particles with aberrant cores [12,15] demonstrating that the SP1 peptide is crucial both for HIV-1 assembly and maturation. Moreover, it has been shown that the presence of the SP1 domain at the C-terminus of CA allows Pr55Gag to multimerize [16]. Previous studies have shown that the CA-SP1 junction adopts an α -helical conformation [17] confirmed recently by electron cryotomography studies of immature HIV-1 virions [18]. The ability of SP1 to undergo a concentration-dependent transition from a nearly unstructured conformation to an α -helical one has suggested that SP1 behaves as a molecular switch to prime assembly of HIV-1 Pr55Gag in the immature lattice [19].

Abbreviations: Ac₂O, acetic anhydride; DEPT135, Distortionless Enhancement by Polarization Transfer; DIEA, *N,N*-diisopropylethylamine; DMAP, 4-(dimethylamino) pyridine; DQF-COSY, double quantum filtered-correlation spectroscopy; HMBC, Heteronuclear Multiple Bond Correlation; HSQC, Heteronuclear Single Quantum Correlation; *n*-Bu₄NF, tetrabutylammonium fluoride; PyBOP, (benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate; *t*-BuMe₂SiOTf, *tert*-butyldimethylsilyltrifluoromethanesulfonate; TOCSY, Total Correlation Spectroscopy.

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Derivatives of betulinic acid (BA, **1**) (Fig. 1) retain antiviral properties against HIV-1 [20–22]. The 3-*O*-(3',3'-dimethylsuccinyl)-betulinic acid derivative, also referred to as DSB, PA-457 or bevirimat (**2**) (Fig. 1) exhibits an anti-HIV activity in acutely infected H9 lymphocytes with EC₅₀ values greater than 3.5×10^{-4} μ M [20,23] and blocks HIV-1 replication by interfering with the processing of CA-SP1 [15,24–26]. *In vivo*, bevirimat inhibits Pr55Gag assembly and VLP release in various cellular models, including mammalian cells as well as baculovirus-infected cells (Sf9) [27]. Recent results using cryo-electron tomography to examine the structures of HIV-1 viral particles isolated from DSB-treated cells suggest that DSB inhibits viral infectivity not only by blocking the CA-SP1 cleavage site but also by stabilizing the immature lattice [27]. Recently, it was shown that a molecule, PF-46396, structurally different from DSB had a similar mode of action and that these two molecules interact differently with the putative maturation inhibitor binding pocket [28].

Derivatives of bevirimat having a side chain at the C-28 position, have been reported for their potent activity against HIV-1 replication and their interference with the processing of p25 [29–32]. Some of them block HIV-1 entry and constitute a new class of bi-functional inhibitors with the potential to block both entry and maturation. The introduction of basic substituents at the C-28 position of bevirimat results in compounds with high anti-HIV activity [33]. Molecules derived from betulin that target the late stage of the replication have been proposed as anti-HIV agents [34,35].

However, the nature of the interaction between Pr55Gag and bevirimat remains unclear and the characterization of those interactions may help to understand the mechanism underlying the inhibitory effect of bevirimat. Moreover, the design of new maturation inhibitors, derived from bevirimat, with improved efficiency against both assembly and maturation may also throw light on the mechanism. Such studies may also help to understand how the natural polymorphism of the CA-SP1 junction enables the virus to escape the effect of bevirimat. To address these queries and apprehend the mechanism by which bevirimat disrupts the maturation of the virion, we have initiated structural studies of the complex by NMR. Unfortunately, the structural study of both partners together was complicated by the lack of solubility of bevirimat in water as well as in the presence of organic solvents such as acetonitrile or trifluoroethanol. The C-3 ester group within bevirimat is essential to interact with its target during HIV-1 maturation [36]. Consequently, novel bevirimat derivatives maintaining the dimethylsuccinyl group at the C-3 position and various substituents at the C-28 position, were synthesized to gain in hydrosolubility (Fig. 1): *N*-[(3 β)-3-(3-carboxy-3-methylbutanoyloxy)lup-20(29)-en-28-oyl]-glycine (**12**), *N*-[(3 β)-3-(3-carboxy-3-methylbutanoyloxy)lup-20(29)-en-28-oyl]- β -alanine (**13**), *N*⁶-[(1,1-dimethylethoxy)carbonyl, *N*²-[(3 β)-3-(3-carboxy-3-methylbutanoyloxy)-lup-20(29)-en-28-oyl]-L-lysine (**15**), 4-[(28-[(2-aminoethyl)amino]-28-oxolup-20,29-en-3 β -yl)oxy]-2,2-dimethyl-4-oxobutanoic acid (**16**) and *N*²-[(3 β)-3-(3-carboxy-3-methylbutanoyloxy)lup-20(29)-en-28-oyl]-L-lysine (**17**). Our goal was not to obtain new derivatives of bevirimat with higher activity against HIV replication but rather to get more hydrosoluble molecules with the same activity against HIV replication. Thus, we focused on the hydrosolubility of our compounds and various hydrophilic functions were added at the C-28 position of bevirimat to make them amenable to NMR structural studies within a complex formed with peptides containing the CA-SP1 junction.

This paper describes the synthesis, the characterization by chemical analysis, NMR and Mass Spectroscopy (MS) of a new series of compounds. The polarity of these products was compared by HPLC analysis, their antiviral activity assessed and their IC₅₀ value was determined. Their toxicity was controlled using models of mammalian cells infected with the NL4.3 HIV-1 laboratory strain and compared to bevirimat (**2**). One of our molecules (**16**) showed a better polarity evaluated by HPLC and an antiviral profile with a higher selectivity index. Moreover, for the first time, a direct interaction between compound **16** and the peptide CA-SP1–NC was proved by NMR. More recently, we showed that compound **16** was able to generate an aberrant assembly of VLP, resulting in non-enveloped, morula-like particles of 100 nm in diameter [37]. All together, these data constitute a first step in the understanding of the mechanism by which bevirimat inhibits HIV-1 assembly and maturation as proposed in a previous model [27,38]. Several patents have been filed describing derivatives of bevirimat at the C-28 position. None of them corresponds to the new molecules that we designed and synthesized except for one of them (WO 2006/053255 A2) that is quoted but has never been synthesized and its biological activity never tested against HIV-1.

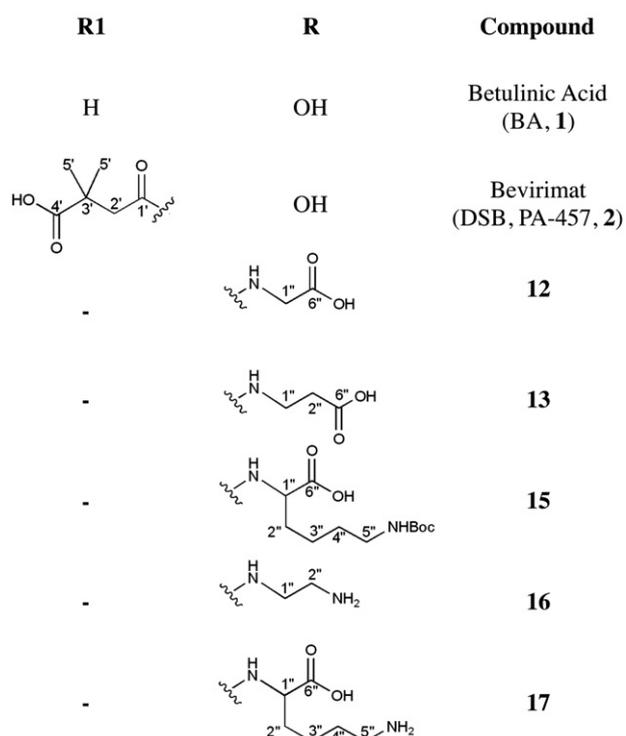
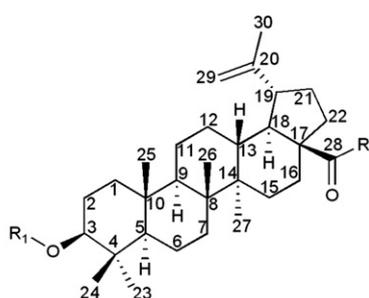


Fig. 1. The structure of the betulinic acid (BA, **1**), bevirimat (**2**) and derivatives containing a dimethyl group at the C-3 position and various hydrophilic substituents at the C-28 position. These molecules were synthesized to gain in hydrosolubility: *N*-[(3 β)-3-(3-carboxy-3-methylbutanoyloxy)lup-20(29)-en-28-oyl]-glycine (**12**), *N*-[(3 β)-3-(3-carboxy-3-methylbutanoyloxy)lup-20(29)-en-28-oyl]- β -alanine (**13**), *N*⁶-[(1,1-dimethylethoxy)carbonyl, *N*²-[(3 β)-3-(3-carboxy-3-methylbutanoyloxy)-lup-20(29)-en-28-oyl]-L-lysine (**15**), 4-[(28-[(2-aminoethyl)amino]-28-oxolup-20,29-en-3 β -yl)oxy]-2,2-dimethyl-4-oxobutanoic acid (**16**) and *N*²-[(3 β)-3-(3-carboxy-3-methylbutanoyloxy)lup-20(29)-en-28-oyl]-L-lysine (**17**).

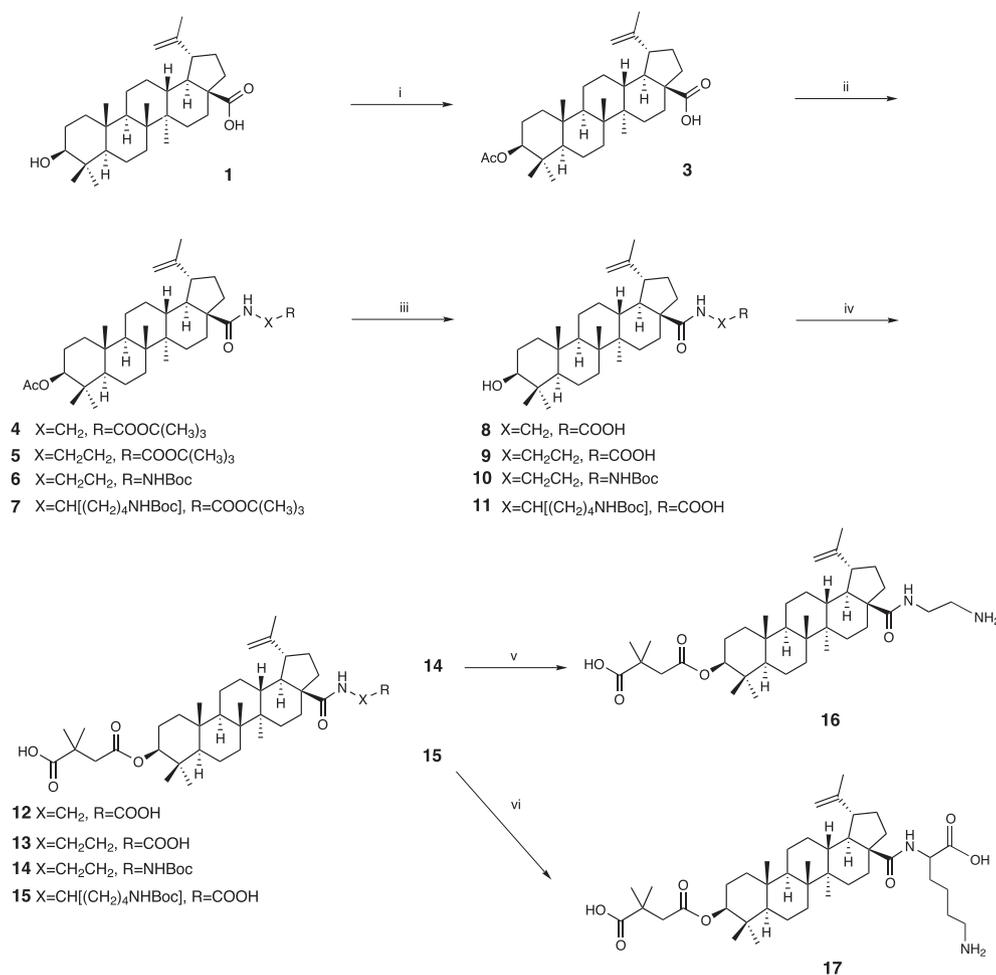
2. Results

2.1. Chemistry

In the last fifteen years, many BA derivatives have been synthesized with modifications at the C-3 hydroxyl [39], at the C-28 carboxylic acid [29,33–35] or at the C-19 isopropenyl groups along with their resulting functional properties [20,23,40,41]. Two distinct classes of BA derivatives exhibit different activities against HIV-1 according to the substitution. The BA derivatives in which the C-3 hydroxyl group is acylated are efficient compounds against the cleavage of the CA-SP1 junction leading to maturation inhibition. Bevirimat (**2**) is the lead product of this derivative family (Fig. 1) developed by the pharmaceutical company Panacos [39,40]. We have synthesized this molecule, as previously described [20], with slight modifications [41]. The molecules with a C-3 hydroxyl group acylation have also been described to lead to assembly inhibition [41,42]. A second class of compounds produced by a modification of the C-28 carboxylic acid amide obtained by introducing a hydrophobic side chain was also studied. This class of BA derivatives, including the molecule named IC9564 was reported to inhibit HIV-1 replication by blocking the viral entry step [21,22,32,43,44]. More recently, a comprehensive study of the structure–activity relationship of several BA derivatives has been achieved by using

3D-QSAR and docking methods and made it possible to propose new BA derivatives [45]. In the light of those results, the aim of our work was, starting from the bevirimat drug, to propose a series of more hydrosoluble molecules, by modifying the C-28 carboxylic acid function, while conserving intact the C-3 dimethylsuccinyl group required for HIV-1 maturation inhibition. Therefore, the side chain at the C-28 position was modified by coupling different substituents. As an example, the free acid glycine conjugate of BA exhibits an increased hydrosolubility [48].

First, the C-3 hydroxyl group (**1**, Scheme 1) was protected by the acetyl group (**3**, Scheme 1), then various substituents were introduced by peptide coupling with PyBOP and DIEA at the C-28 carboxylic acid. After the purification of the intermediates by flash chromatography (**4–7**, Scheme 1), the saponification of the ester by a 4 M sodium hydroxide solution was performed to furnish the compounds with free hydroxyl group at the C-3 position (**8–11**, Scheme 1). For compounds **8**, **9** and **11** (Scheme 1) the saponification was accompanied by a non-classical *tert*-butyl ester cleavage of the coupled amino acid. The free hydroxyl derivatives (**8–11**, Scheme 1) reacted with the 2,2-dimethylsuccinic anhydride in pyridine, in the presence of DMAP, and provided a mixture of 3-*O*-(2',2'-dimethylsuccinyl)- and 3-*O*-(3',3'-dimethylsuccinyl) BA derivatives. The desired products (**12–15**, Scheme 1) were separated by flash chromatography. Data from the literature show that



Scheme 1. Synthesis of the derivatives of bevirimat.

the 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid was a more potent anti-HIV-1 inhibitor than the 3-*O*-(2',2'-dimethylsuccinyl) betulinic acid [20].

The *N*-Boc protection of product **14** (Scheme 1) was first removed with *tert*-butyldimethylsilyltrifluoromethanesulfonate in the presence of 2,6-lutidine to produce *tert*-butyldimethylsilyl carbamates [46]. The removal of the *N*-*tert*-butyldimethylsilyloxycarbonyl group was then achieved by treatment with *n*-Bu₄NF in THF leading to compound **16**. The *N*-Boc protection of product **15** (Scheme 1) was cleaved with 6 M HCl in THF and compound **17** was obtained after purification by flash chromatography.

2.2. Analysis of the polarity of the compounds by HPLC

As substitutions at the C28 position were performed to increase the polarity of bevirimat and subsequently its hydrosolubility, while keeping intact the function at the C-3 position, we assessed their hydrosolubility by HPLC after purification. The compounds were analyzed with an isocratic HPLC mode using MeOH (90%) in water as solvent and a C18 column. In these conditions it was possible to correlate the polarity of the compounds with their retention time (Fig. 2). As an example, a glycine residue introduced at the C28 position in bevirimat (retention time: 17.890 min) divided by two the retention time in compound **12** (retention time: 8.352 min). These results are in keeping with those obtained previously on a derivative of the betulinic acid [47]. The most interesting compounds were found to be **16** and **17** with respectively an ethylenediamine and a lysine group as substituent at the C28 position and a retention time of 3.428 min and 2.295 min respectively determined by HPLC analysis. The compounds were classified according to their retention time (or polarity) from the most hydrophilic compound to the least hydrophilic one (**17** > **16** > **12** > **13** > **1** > **2**).

2.3. NMR

Our compounds were characterized by High Resolution Mass Spectroscopy (HRMS) and by NMR. The complete assignment of the ¹H (Table 1) and ¹³C (Table 2) resonances was achieved by standard 1D and 2D NMR experiments, the IUPAC convention was used to number the carbon atoms. ¹H–¹H COSY (CORrelations Spectroscopy), TOCSY (TOTAL Correlation Spectroscopy), 2D (¹H–¹³C)-HSQC (Heteronuclear Single Quantum Correlation) and 2D (¹H–¹³C)-HMBC (Heteronuclear Multi Bond Correlation). The ¹³C spectra of the six products (**2**, **12**, **13**, **15**, **16** and **17**) revealed 36 to 48 carbon signals out of which 6 were assigned to the C-3 esterification (1' to 4', two 5' methyl), 6 to the C-28 amide modifications (1'' to 6'') and 30 to the triterpenoid skeleton (Figures S1 to S5 in supplementary data).

Significant variations were observed when comparing the carbon chemical shifts of bevirimat and its derivatives with those reported for the BA [48,49], mainly for the carbons C-2 ($\Delta\delta = -4$ ppm), C-3 ($\Delta\delta = +3$ ppm) and C-4 ($\Delta\delta = -1$ ppm) resulting from C-3 esterification and C-16 ($\Delta\delta = +0.4$ to $+1$ ppm), C-18 ($\Delta\delta = +0.3$ to $+1$ ppm), C-20 ($\Delta\delta = +0.5$ to $+1$ ppm), C-22 ($\Delta\delta = +0.4$ to $+1.5$ ppm), C-24 ($\Delta\delta = +1$ ppm) and C-28 ($\Delta\delta = -1.6$ ppm to $+0.5$) consecutive to the C-28 amide modification. Moreover, the carbon signal of C-24 was more perturbed than that of C-23 because it is located above the plane of the first ring A, as is the hydroxyl group at the C-3 position. All together, these data support that the configuration of C-3 was well conserved during the synthesis.

The opening of the 2,2-dimethylsuccinic anhydride during the C-3 esterification synthesis leads to the formation of two compounds, which can be differentiated, on the 2D (¹H–¹³C)-HMBC experiment (data not shown). We observed that the C-4' carbon

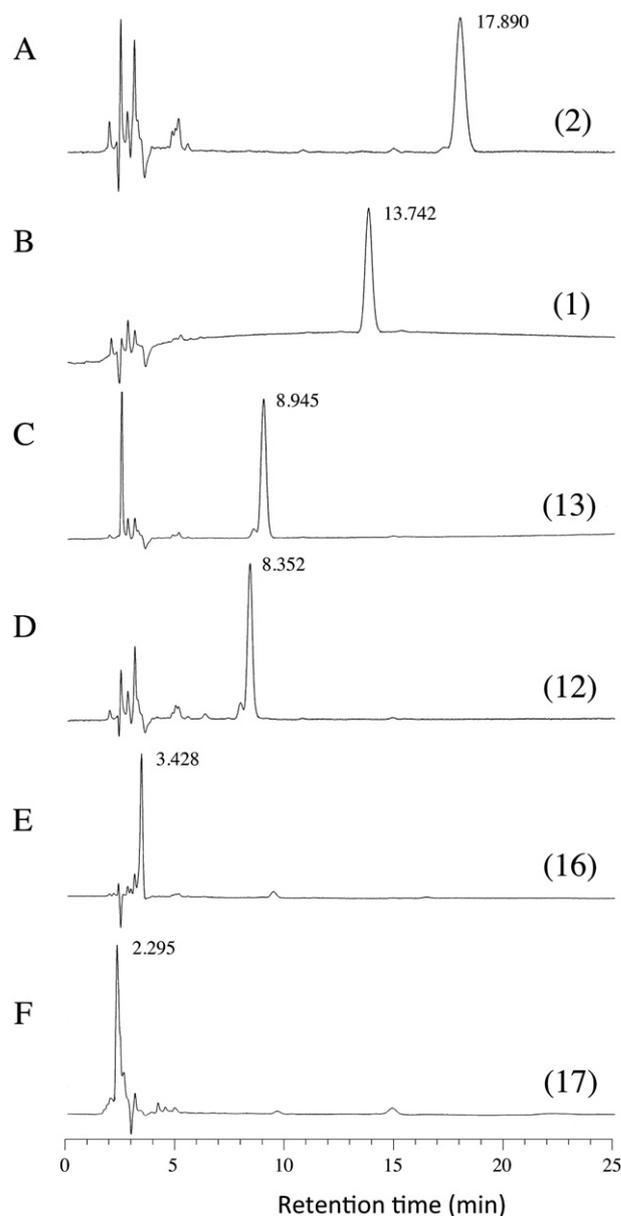


Fig. 2. The evaluation of the retention times of the different compounds (**1**, **2**, **12**, **13**, **16** and **17**) by HPLC analysis conducted on a Shimadzu LC-10ADvp with a Nucleodur 4.6 mm × 250 mm C18 HTec 5 μm column and a Shimadzu SPD-M10Avp detector at 210 nm wavelength. The solvent system used was MeOH(0.1%TFA):water(0.1% TFA) = 90:10 with an isocratic HPLC mode. In these conditions it was possible to compare the water solubility of the compounds and classify them according to their retention time from the most hydrophilic compound to the least hydrophilic one **17** > **16** > **12** > **13** > **1** > **2**. Compound **15** has not been tested by HPLC since it corresponds to compound **17** with a *N*-Boc protection of the HNe of lysine. It is then thought to be less hydrosoluble than **17**.

signal strongly correlates with the H-5' proton signal for the 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid derivatives while the same H-5' proton strongly correlates with the C-1' carbon signal for the 2-*O*-(2',2'-dimethylsuccinyl) betulinic acid derivatives. Additionally, the 2D (¹H–¹³C)-HMBC spectrum shows that the H-3 proton signal correlates with the C-1' carbon signal and not with the C-4' one. These data validate the correct incorporation of the chain at the C-3 position of the purified compounds. The correlations between the C-28 carbon signal and the NH and H-1'' proton signals for the five bevirimat derivatives prove the correct formation of the amide.

Table 1¹H NMR spectral data for **2**, **12**, **13**, **15**, **16** in pyridine-d₅ and **17** in methanol-d₄. o, overlapped signals.

	2	12	13	15	16	17
	δ_{H}	δ_{H}	δ_{H}	δ_{H}	δ_{H}	δ_{H}
1	0.91 1.56 (o)	0.89 1.52 (o)	0.91 1.58 (o)	0.9 1.59 (o)	0.86 1.54 (o)	0.99 1.72 (o)
2	1.66 1.8 (o)	1.67 1.77 (o)	1.7 1.82 (o)	1.7 1.78 (o)	1.67 1.77 (o)	1.6 1.68 (o)
3	4.76 (d) (o) <i>J</i> = 5.5 Hz	4.77 (d) <i>J</i> = 5 Hz	4.78 (o)	4.78 (dd) <i>J</i> = 5 Hz, 11.5 Hz	4.77 (o) d	4.44 (dd) <i>J</i> = 5 Hz, 10 Hz
4	—	—	—	—	—	—
5	0.77 (d) <i>J</i> = 11 Hz	0.77 (o)	0.74 (o)	0.82 (o)	0.77 (o)	0.82 (o)
6	1.42 (o)	1.3 1.4 (o)	1.3 1.4 (o)	1.36 1.46 (o)	1.3 1.4 (o)	1.44 1.52 (o)
7	1.32 (o)	1.37 (o)	1.33 (o)	1.44 (o)	1.30 (o)	1.38 1.45 (o)
8	—	—	—	—	—	—
9	1.29 (o)	1.3 (o)	1.3 (o)	1.30 (o)	1.28 (o)	1.36 (o)
10	—	—	—	—	—	—
11	1.2 1.3 (o)	1.15 1.3 (o)	1.17 1.31 (o)	1.15 1.33 (o)	1.13 1.3 (o)	1.26 1.43 (o)
12	1.15 1.94 (o)	1.15(o) 1.93(m)	1.16 (o) 1.96 (m)	1.15 1.95 (o)	1.12(o) 1.92 (m)	1.04 1.7 (o)
13	2.73 (m)	3.08 (m)	3.04 (m)	3.07 (m)	3.03 (m)	2.53 (o)
14	—	—	—	—	—	—
15	1.22 1.84 (o)	1.26 2.03 (m)	1.2 1.85 (o)	1.32 1.89 (o)	1.17 1.7 (o)	1.18 1.49 (o)
16	1.55(o) 2.63 (m)	1.67(o) 2.59(m)	1.58 (o) 2.44 (d) <i>J</i> = 13 Hz	1.67 (o) 2.58 (m)	1.64 (o) 2.67 (d) <i>J</i> = 12 Hz	1.56 (o) 2.17 (m)
17	—	—	—	—	—	—
18	1.75 (o)	1.74 (o)	1.7 (o)	1.73 (o)	1.7 (o)	1.64 (o)
19	3.55 (m)	3.66 (m)	3.64 (m)	3.64 (m)	3.61 (m)	3.06 (m)
20	—	—	—	—	—	—
21	1.56 2.26 (o)	1.54 2.38 (o)	1.5 (o) 2.24 (m)	1.53 (o) 2.34 (m)	1.48 2.14 (m)	1.35 1.9 (o)
22	1.56 2.25 (o)	1.59 2.39 (o)	1.52 (o) 2.14 (m)	1.54 (o) 2.43 (m)	1.55 2.25 (m)	1.47 1.93 (o)
23	0.97 (s)	0.96 (s)	0.97 (s)	0.97 (s)	0.97 (s)	0.87 (s)
24	0.92 (s)	0.92 (s)	0.94 (s)	0.96 (s)	0.93 (s)	0.85 (s)
25	0.73 (s)	0.73 (s)	0.74 (s)	0.76 (s)	0.75 (s)	0.92 (s)
26	1.02 (s)	1.12 (s)	1.08 (s)	1.09 (s)	1.03 (o)	0.96 (s)
27	1.05 (s)	1.06 (s)	1.04 (s)	1.08 (s)	1.03 (o)	1.01 (s)
28	—	—	—	—	—	—
29	4.78 (s) 4.95(s)	4.75 (s) 4.96(s)	4.77 (s) 4.94 (o)	4.77 (s) 4.92 (s)	4.75 (s) 4.93(s)	4.57 (s) 4.7 (s)
30	1.78 (s)	1.78 (s)	1.81 (s)	1.78 (s)	1.77 (s)	1.7 (s)
1'	—	—	—	—	—	—
2'	2.89 2.97 (2d) <i>J</i> = 15 Hz	2.91 2.95(2d) <i>J</i> = 15.5 Hz	2.94 3.00 (2d)(o) <i>J</i> = 15.5 Hz	2.90 2.98 (2d) <i>J</i> = 15.5 Hz	2.9 2.96 (2d) <i>J</i> = 15.5 Hz	2.55 2.62 (2d) <i>J</i> = 15.5 Hz
3'	—	—	—	—	—	—
4'	—	—	—	—	—	—
5'	1.55 (o)	1.55 (o)	1.55 (o)	1.55 (o)	1.55 (o)	1.25 (o)
1''	—	4.44 4.51(dd) <i>J</i> = 6 Hz, 17.5 Hz	3.88 4.00 (m)	5.17 (m)	3.87 3.81 (m)	4.3 (m)
2''	—	—	3.00 (mo)	2.06 2.31 (m)	3.28 (m)	1.75 1.89 (m)
3''	—	—	—	1.80 (o)	—	1.46 (o)
4''	—	—	—	1.74 (o) 1.83	—	1.7
5''	—	—	—	3.43 (m)	—	2.9 (t) <i>J</i> = 8 Hz
6''	—	—	—	—	—	—
NH	—	8.76 (t) <i>J</i> = 5.5 Hz	8.33 (t) <i>J</i> = 5.5 Hz	8.27 (d) <i>J</i> = 8 Hz	8.74 (o)	7.1

Several remarks regarding the proton chemical shifts (Fig. 3) can be made. The most important variation concerns the H-3 α that is less shielded (+1.3 ppm) after the esterification of the β -hydroxyl group. The chemical shifts of the H-1 protons (α and β), H-6 β , H-9, 3H-23 and the 3H-25 differ by 0.1–0.2 ppm. The supplementary changes resulting from the C-28 amide modifications concern the H-13 proton (+0.3 ppm).

The most active compound (**16**) was tested by NMR in interaction with a wild type CA-SP1–NC peptide containing 48 amino acids [17] and derived from the HIV-1 Pr55Gag sequence (Fig. 4A). NOESY experiments were performed on the peptide in the presence or not of compound **16** and perturbation of the chemical shifts of the junction CA-SP1–NC has been analyzed, demonstrating that an interaction takes place between compound **16** and the wild type junction (Fig. 4B). The amino acids involved in the interaction with compound **16** were identified within SP1-(A366, M367, Q369, V370, N372 and I376) and in the NC (M378). Some amino acids showed their chemical shifts perturbed after the addition of compound **16** but they were located

in the unstructured N- and the C-terminal extremities of the peptide and were very sensitive to the solvent conditions. The perturbation of the chemical shifts of the wild type junction is represented as a histogram (Fig. 4C).

2.4. Bio assays

Novel bevirimat derivatives with higher solubility, containing the dimethyl group at the C-3 position and various modifications at the C-28 amide position were tested for their capacity to inhibit HIV-1 assembly and/or maturation and consequences on viral infectivity were determined. To this end, viruses produced by transfection of the HIV-1 pNL-4-3 molecular clones in 293T cells maintained in the presence of newly synthesized compounds **12**, **13**, **15**, **16** and **17** were analyzed for their capacity to infect MAGIC-5B cells in parallel with BA (**1**) and bevirimat (**2**). All compounds displayed an IC₅₀ value lower than that of BA, with compound **16** being more efficient than bevirimat at inhibiting HIV-1 infectivity. The bioassay data are summarized in Table 3.

Table 2
¹³C spectral data for **2**, **12**, **13**, **15**, **16** in pyridine-d₅ and **17** in methanol-d₄.

	2	12	13	15	16	17
	δ _C					
1	39.0	39.0	39.0	39.0	39.1	39.8
2	24.6	24.6	24.6	24.6	24.6	24.8
3	81.3	81.4	81.4	81.4	81.3	82.8
4	38.5	38.5	38.5	38.5	38.5	39
5	56.1	56.0	56.1	56.1	56.1	57
6	18.85	18.9	18.9	19.0	19.0	19.4
7	35.0	35.0	35.0	35.15	35.0	35.7
8	41.45	41.6	41.6	41.6	41.5	42.2
9	51.05	51.2	51.2	51.2	51.3	52.1
10	37.7	37.7	37.75	37.75	37.7	38.4
11	21.6	21.6	21.7	21.7	21.6	22.3
12	26.4	26.6	26.6	26.6	26.5	27.2
13	39.0	38.1	38.2	38.1	38.1	39.2
14	43.25	43.2	43.2	43.2	43.15	43.7
15	30.7	30.3	30.3	30.3	30.3	30.9
16	33.3	34.0	34.0	34.2	34.0	34.4
17	57.0	56.4	56.3	56.5	56.5	57.4
18	50.1	51.0	51.1	51.1	51.2	51.4
19	48.2	47.7	47.7	47.7	47.7	48.3
20	151.8	152.2	152.2	152.2	152.2	152.4
21	31.6	31.8	31.8	31.9	31.8	32.1
22	38.0	39.1	39.0	38.8	39.0	39.3
23	28.5	28.5	28.6	28.6	28.5	28.7
24	17.35	17.3	17.4	17.4	17.4	17.2
25	16.65	16.7	16.7	16.7	16.75	17.0
26	16.8	17.0	16.9	17.0	16.9	17.1
27	15.3	15.3	15.2	15.3	15.2	15.2
28	179.3	177.9	177.2	177.7	177.7	178.9
29	110.5	110.3	110.2	110.2	110.2	110.2
30	19.9	20.0	20.0	20.0	20.0	19.8
1'	172	172.1	172.0	172.1	172.1	173.1
2'	45.7	45.7	45.7	45.7	45.7	45.8
3'	41.3	41.3	41.3	41.3	41.4	41.5
4'	179.8	179.8	179.8	179.8	179.9	180.8
5'	26.7	26.7	26.7	26.4	26.7	26.2
1''		42.4	36.7	53.0	41.05	54.0
2''			35.7	32.4	42.2	32.7
3''				24.5		24.05
4''				30.6		28.2
5''				41.4		40.7
6''		174.0	175.4	176.6		177.3

To further elucidate the mechanism of action of these compounds, viral samples used for infection assays were subjected to biochemical characterization (Fig. 5). The analysis of the conversion of uncleaved p25 (CA-SP1) to mature p24 (CA) revealed that compounds **2**, **12**, **13**, **15** and **16** partially inhibit the CA-SP1 cleavage while compounds **1** and **17** do not. However, this biochemical approach is dependent on the threshold of sensitivity of Western blot and we can not exclude that the cleavage is inhibited for few molecules of CA-SP1.

To determine whether defect in p25 to p24 process induced by **1**, **2**, **12**, **13**, **15**, **16** and **17** affected virus morphology, an analysis by EM was performed. In the absence of compounds, cells transfected with pNL-4-3 produced virus particles with the classical mature morphology characterized by the presence of condensed, conical cores (Fig. 6A). In contrast, viruses from cells treated with **2**, **12**, **13**, **15** and **16** displayed different phenotype with various distribution from aberrant, spherical and acentric core to aberrant immature virions (Chazal, N et al., in preparation) (Fig. 6C–G). Compounds **1** and **17** had almost no capacity to inhibit viral maturation. Taken together, these results indicate that **2**, **12**, **13**, **15** and **16** positively block proper virion maturation.

3. Discussion

Very recently different modifications at the C-3 position of anti-HIV triterpene-derived agents have been proposed and showed

that the C-3 dimethyl substitution of the succinyl side chain as well as the carboxylic acid, were necessary for antiviral activity [50–52]. Although it is efficient in *ex vivo* bio assays, the use of bevirimat (**2**) as an anti-HIV drug is hindered by its low efficacy due to the wide natural polymorphism of the spacer peptide SP1 [53–58]. Moreover, bevirimat (**2**) was shown to have a low solubility in physiological solvents as well as in organic solvent mixtures, preventing any structural study by NMR of a complex with the CA-SP1–NC domain previously characterized as an amphipathic helix [17].

We optimized the synthesis of triterpene derivatives by adding different hydrophilic substituents at the C-28 position (**12**, **13**, **15**, **16** and **17**). The new molecules showing a higher polarity according to their retention time evaluated by HPLC, were characterized by NMR and by HRMS, they were tested for their capacity to inhibit viral infectivity and their cytotoxicity was evaluated (Table 3). We showed that the essential maturation inhibitory activity of our molecules, due to the dimethylsuccinyl group at the C-3 position, was conserved for all the molecules and therefore our compounds remained fully active against maturation and replication.

Moreover, the most active compound (**16**) was shown to interact with the junction CA-SP1-NC by chemical shift perturbation NMR experiments (Fig. 4B). The amino acids involved in this interaction were identified in the domain SP1 (A366, M367, Q369, V370, N372, I376) and in NC (M378). Our data confirm the results obtained recently with photoaffinity analogs of bevirimat that were found to crosslink to sequences overlapping the CA-SP1 junction and are consistent with previous biochemical data on the effect of bevirimat on Pr55 Gag processing [59]. Thus, introduction of a constituent containing an amino group at the C-28 position of bevirimat made it possible to demonstrate by NMR for the first time a direct interaction between a maturation inhibitor and its target within the CA-SP1-NC junction. This NMR study was impossible to realize with bevirimat because of its weak solubility in water.

The newly synthesized bevirimat derivatives were evaluated for their capacity to inhibit HIV-1 replication by blocking the protease (PR)-mediated cleavage at the CA-SP1 junction. Only derivative **17** showed a moderate anti-HIV-1 activity with a selectivity index (SI) of 11 and an IC₅₀ of 4.33 μM. However, we found that all compounds **12**, **13**, **15** and **16** exhibited a good anti-HIV-1 efficacy with an IC₅₀ values of 0.160 μM, 0.118 μM, 0.170 μM and 0.016 μM and a SI of 309, 411, 199 and 2118 respectively. These results should be compared to those obtained in a very recent publication by Qian et al. [31] in which a product with an IC₅₀ of 0.0059 μM was studied.

To elucidate the mechanism of action of our series of compounds, HIV-1_{NL4.3} particles produced by transfection of 293T cells maintained in the presence of the newly synthesized molecules were subjected to characterization. Biochemical analysis of cell free viral particles revealed that, derivatives **12**, **15** and **16** function as maturation inhibitors and partially inhibit the conversion of p25 (CA-SP1) to p24 (CA) as previously reported for BA and bevirimat. These compounds result in a defective Pr55Gag processing and to the production of morphologically abnormal and noninfectious particles.

Among all tested molecules, compound **16**, displayed the most potent anti-HIV-1 activity. It is important to note that this molecule, unlike other derivatives designed in this work, is the only one that does not come from the condensation with an amino acid at the C-28 position. It seems that the presence of the carboxylic group, introduced by the condensation of an amino acid at the C-28 position, decreases the activity of our compounds (**12** and **13**), compared to the introduction of an amino group (**16**). Moreover the distance between the triterpenic skeleton and the carboxylic function may modulate this activity as shown by the comparison of the results obtained with compounds **2**, **12** and **13** (Table 3). Compared to the well-known bevirimat, compound **16** showed an

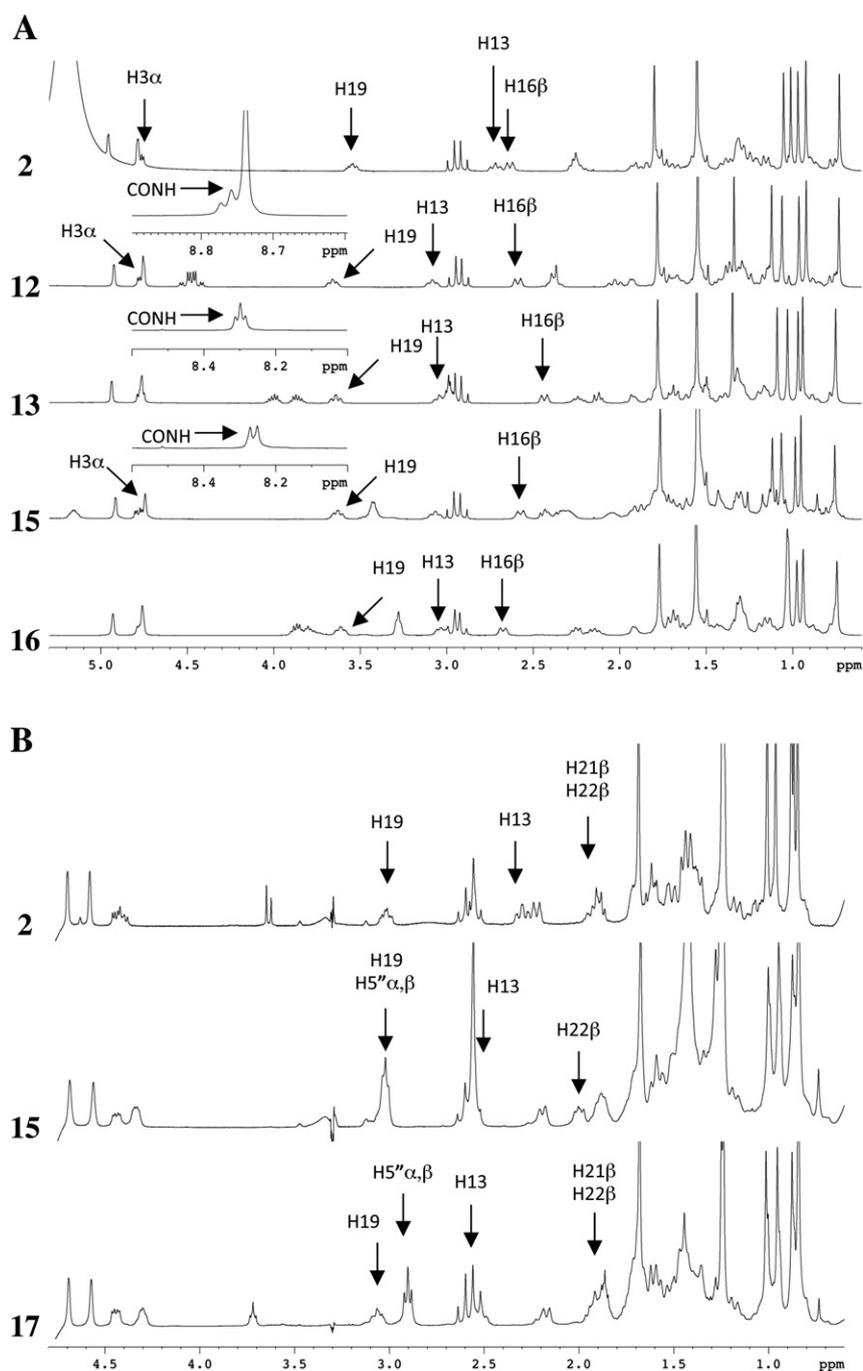


Fig. 3. One-dimensional ¹H NMR spectra of bevirimat (**2**) and derivatives. A. Derivatives **2**, **12**, **13**, **15** and **16** in pyridine -d₅. B. Derivatives **2**, **15** and **17** in MeOH-d₄.

improved capacity to inhibit p25 maturation with a selectivity index which is twice that of bevirimat and represents an attractive promising HIV-1 inhibitor lead for future clinical trials. The *in vitro* efficacy of compound **16** against HIV-1 seems to be related to its polarity and we assume that it is possible to improve the activity of these molecules by increasing their polarity and subsequently their hydrosolubility.

Since the solubility of our molecules has been increased by adding hydrophilic functions at the C-28 position, the resolution of a complex between the junction CA-SP1-NC and one of these new molecules becomes feasible. The elucidation of such complexes could help to elucidate the molecular bases required for the inhibition of HIV-1 maturation by bevirimat and its derivatives. The

structure of that complex may help to explain how the natural polymorphism and mutations in the spacer peptide SP1 lead to resistance to maturation inhibitors [53–58].

4. Conclusions

Bevirimat derived compounds reported in the present study display an increased hydrosolubility generated by the addition of various hydrophilic substituents at the C-28 position. All the new compounds still behave as maturation inhibitors but compound **16** showed a significant improvement in anti-HIV-1 activity compared to the original bevirimat. Moreover, compound **16** was shown to interact with the peptide CA-SP1-NC by NMR. This direct

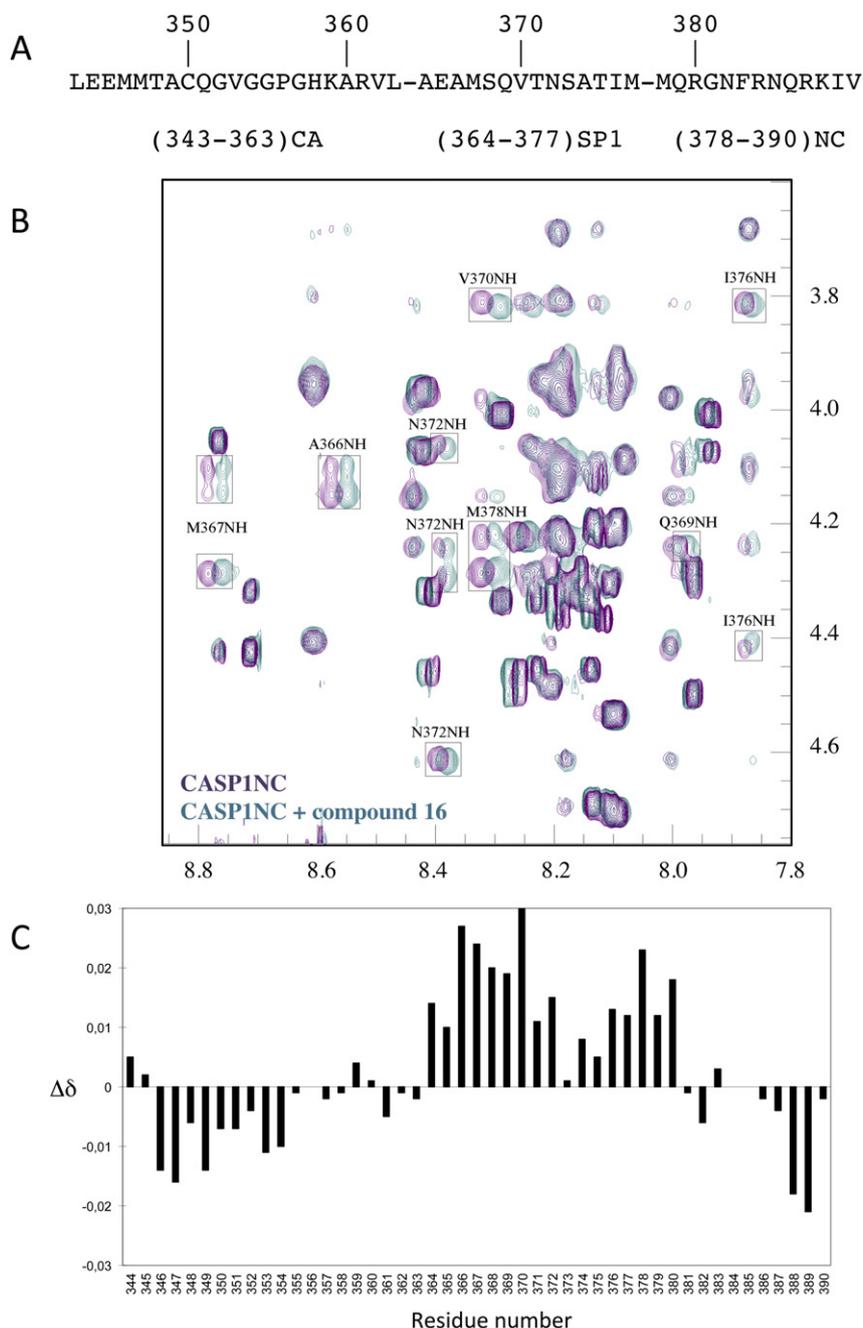


Fig. 4. Highlight of the interaction of the most active compound (**16**) with the wild type CA-SP1–NC domain. (A) Amino acid sequence of the wild type CA-SP1–NC domain used in this study to test the interaction with compound **16**. (B) 2D ^1H – ^1H NOESY spectra showing the superimposition of the HN–H α regions of the wild type CA-SP1–NC peptide in the absence (blue) and presence (magenta) of compound **16**. The perturbation of the chemical shifts of the peptide by adding compound **16** are boxed and numbered on the spectra and shows the strength of the interaction. Spectra were recorded at a pH of 3.8, in $\text{H}_2\text{O}/\text{TFE}$ (70/30) and $\text{DMSO}-d_6$ (1%) at 293 K. The amino acids undergoing the most important perturbation of their chemical shifts were identified in the wild type domain and the variation of their perturbation of their chemical shifts has been reported on a histogram (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

interaction between a maturation inhibitor and its target is revealed for the first time by NMR and launches the field to the design of new molecules based on the substitution at the C-28 position by hydrophilic groups. Beside the undeniable interest of this property for future use as anti-HIV-1 molecules, it paves the way to the resolution of complexes formed between derivatives of bevirimat and CA-SP1–NC. The elucidation of such complexes will help clarifying the mechanism accounting for HIV-1 maturation inhibition by this class of compounds and will help to explain how the natural polymorphism of the SP1 domain leads to the resistance

to maturation inhibitors. Bevirimat was launched by Panacos Pharmaceuticals, Inc. [39,40] and later by Myriad Pharmaceuticals. Recently, bevirimat succeeded in Phase IIa clinical trials and despite the encouraging results obtained in Phase IIb clinical trials, the development on this new class of anti-HIV drug was interrupted principally because of the natural polymorphism of the CA-SP1 junction that leads to a natural resistance of the virus to maturation inhibitors. The new compounds reported herein, represent attractive and promising leads for future development of the next generation of HIV-1 maturation inhibitors. Once their interaction

Table 3
Efficiency of HIV-1 infection inhibition by betulinic acid derivatives.^a

Compound	IC ₅₀ (μM)	CC ₅₀ (μM)	SI ^b
(1)	5.315	4.52	0.85
(2)	0.040	31.0	775.00
(12)	0.160	49.5	309.37
(13)	0.118	48.5	411.01
(15)	0.170	33.9	199.41
(16)	0.016	33.9	2118.75
(17)	4.330	48.7	11.24

^a The mean values (m) for the 50% inhibitory activity (IC₅₀) of infection were given as μM (m). The mean values (m) for cytotoxicity (CC₅₀) were given as μM (m).

^b The selectivity index (SI) represented the CC₅₀/IC₅₀ ratio.

with the CA-SP1 junction is fully characterized, these inhibitors will serve as model to build new molecules conserving important functions but not necessarily based on a triterpenic skeleton.

5. Material and methods

5.1. Chemistry

5.1.1. Generals

BA, acetic anhydride, PyBOP, DIEA, DMAP, 2,2-dimethylsuccinic anhydride, 2,6-lutidine, *tert*-butyldimethylsilyltrifluoromethanesulfonate were purchased from Aldrich. All amino acids, from Bachem were *O*-*t*Bu protected and the lysine side chain was protected as N-Boc. The final compounds were characterized by ¹H and ¹³C NMR and high resolution mass spectrometry experiments (Bruker MicrO-Tof-Q 2 system). Their purity was determined to be ≥ 95% by elemental analyses (C, H, N).

5.1.2. General procedure for the synthesis of derivatives 4–7

PyBOP (0.31–0.47 g, 0.6–0.9 mmol) and the appropriate amine (0.6–0.9 mmol) were successively added to a mixture of 3-*O*-Ac-BA (3) (200–300 mg, 0.4–0.6 mmol) and DIEA (0.28–0.42 mL, 1.6–2.4 mmol) in DMF (2–3 mL). The mixture was stirred for 72 h and then diluted with CH₂Cl₂ (50 mL), washed with aqueous citric acid solution (10%), water, brine and dried over MgSO₄. The organic layer was concentrated under vacuum and the residue was purified by silica gel chromatography to provide the corresponding C28 intermediates.

5.1.2.1. Glycine, *N*-[(3β)-3-(acetyloxy)-28-oxolup-20(29)-en-28-yl]-, 1-(1,1-dimethylethyl) ester (4). *R*_f: 0.38 (toluene/acetone 10/0.5); Yield: 64% (158 mg); ¹H NMR (300 MHz, CDCl₃): δ 0.75 (m, 1H, -CH in 5), 0.82 (s, 3H, -CH₃), 0.83 (s, 3H, -CH₃), 0.91 (s, 3H, -CH₃), 0.95 (s, 3H, -CH₃), 1.09 (m, 2H), 1.10–1.45 (m, 11H), 1.47 (s, 12H, -CH₃, *t*-Bu), 1.50–1.65 (m, 6H), 1.67 (s, 3H, -CH₃), 1.75–2.0 (m, 3H), 2.03 (s, 3H, -COCH₃), 2.45 (dt, 1H, *J* = 3.5 Hz, *J* = 13 Hz), 3.10 (dt, 1H, *J* = 3.5 Hz, *J* = 11 Hz), 3.89 (m, 2H, -CH₂), 4.45 (t, 1H, *J* = 7.5 Hz), 4.58 (s, 1H, =CH), 4.73 (s, 1H, =CH), 6.10 (t, 1H, *J* = 5.2 Hz). ¹³C NMR (300 MHz, CDCl₃): δ 14.6; 16.0; 16.2; 16.5; 18.2; 19.4; 20.9; 21.3;

23.7; 25.5; 27.9; 28.0; 29.4; 30.8; 33.6; 34.3; 36.5; 37.1; 37.6; 37.8; 38.3; 38.4; 40.7; 41.9; 42.5; 45.9; 46.7; 50.0; 50.5; 55.4; 55.7; 80.9; 82.0; 109.4; 150.9; 169.6; 171.0; 176.4.

5.1.2.2. β-Alanine, *N*-[(3β)-3-(acetyloxy)-28-oxolup-20(29)-en-28-yl]-, 1-(1,1-dimethylethyl) ester (5). *R*_f: 0.45 (toluene/acetone 10/0.5); Yield: 80% (300 mg); ¹H NMR (400 MHz, C₅D₅N): δ 0.80 (m, 4H, -CH in 5, -CH₃ in 25), 0.87–0.9 (m, 7H, -CH₃ in 23 and 24, -CH in 1), 1.08 (1s, 3H, -CH₃ in 27), 1.12 (1s, 3H, -CH₃ in 26), 1.18 (m, 1H, -CH in 12), 1.19 (m, 1H, -CH in 11), 1.21 (m, 1H, -CH in 15), 1.33 (m, 1H, -CH in 9), 1.34 (m, 1H, -CH in 11), 1.35 (m, 1H, -CH in 6), 1.42 (m, 2H, -CH in 7), 1.45 (m, 1H, -CH in 6), 1.49 (s, 9H, -COOtBu), 1.5 (m, 1H, -CH in 21), 1.54 (m, 1H, -CH in 22), 1.6 (m, 2H, -CH in 1 and 16), 1.65 (m, 1H, -CH in 2), 1.7 (m, 1H, -CH in 2), 1.74 (m, 1H, -CH in 15), 1.77 (s, 3H, -CH₃ in 30), 1.94 (m, 1H, -CH in 12), 2.08 (s, 3H, -COCH₃), 2.13 (m, 1H, -CH in 22), 2.21 (m, 1H, -CH in 21), 2.4 (m, 1H, -CH in 16), 2.77 (m, 1H, -CH in 2''), 2.85 (m, 1H, -CH in 2''), 3.08 (m, 1H, -CH in 13), 3.62 (m, 1H, -CH in 19), 3.7 (m, 1H, -CH in 1''), 3.85 (m, 1H, -CH in 1''), 4.7 (dd, 1H, -CH in 3, *J* = 4.5 Hz and 11.5 Hz), 4.78 (s, 1H, =CH), 4.94 (s, 1H, =CH), 8.35 (d, 1H, -CO-NH-, *J* = 7 Hz). ¹³C NMR (400 MHz, C₅D₅N): δ 15.2 (C27); 16.8 (C25); 16.9 (C26); 17.2 (C24); 18.9 (C6); 20 (C30); 21.6 (C2'); 21.66 (C11); 24.5 (C2); 26.6 (C12); 28.5 (3C, *t*-Bu and C23); 30.3 (C15); 31.8 (C21); 34 (C16); 35.1 (C7); 36.3 (C2''); 36.5 (C1''); 37.7 (C10); 38 (C13); 38.5 (C4); 39 (C22 and C1); 41.6 (C8); 43.2 (C14); 47.65 (C19); 51.1 (C18); 51.3 (C9); 56.1 (C5); 56.3 (C17); 80.7 (C*t*-Bu); 81.2 (C3); 110.2 (C29); 152.1 (C20); 171.1 (C1'); 172.4 (C COOt-Bu); 177.4 (C28).

5.1.2.3. *N*-[(3β)-3-acetyloxy-lup-20(29)-en-28-oyl]-1,1-dimethylethyl-(2-aminoethyl)carbamate (6). *R*_f: 0.48 (toluene/acetone 90/10); Yield: 86% (330 mg); ¹H NMR (300 MHz, CDCl₃): δ 0.77 (m, 1H), 0.83 (s, 3H, -CH₃), 0.84 (s, 6H, 2-CH₃), 0.94 (s, 3H, -CH₃), 0.96 (s, 3H, -CH₃), 1.09 (m, 1H), 1.10–1.41 (m, 8H), 1.47 (s, 9H, *t*-Bu), 1.49–1.65 (m, 8H), 1.68 (s, 3H, -CH₃), 1.70–1.80 (m, 3H), 1.98 (m, 3H), 2.04 (s, 3H, -COCH₃), 2.45 (dt, 1H, *J* = 3.1 Hz, *J* = 12.5 Hz), 3.13 (dt, 1H, *J* = 4.4 Hz, *J* = 11 Hz), 3.27 (m, 2H, -CH₂), 3.34 (m, 2H, -CH₂), 4.46 (t, 1H, *J* = 7.0 Hz), 4.59 (s, 1H, =CH), 4.74 (s, 1H, =CH), 5.04 (m, 1H, -NH-CO-), 6.33 (m, 1H, -CO-NH-).

5.1.2.4. *l*-Lysine, *N*⁶-[(1,1-dimethylethoxy)carbonyl]-, *N*²-[(3β)-3-(acetyloxy)-28-oxolup-20(29)-en-28-yl]-, 1-(1,1-dimethylethyl) ester (7). *R*_f: 0.28 (toluene/acetone 95/5); Yield: 73% (410 mg); ¹H NMR (400 MHz, C₅D₅N): δ 0.80 (m, 4H, -CH in 5, -CH₃ in 25), 0.90 (m, 7H, -CH₃ in 23 and 24, -CH in 1), 1.09 (2s, 6H, -CH₃ in 27 and 26), 1.16 (m, 1H, -CH in 11), 1.17 (m, 1H, -CH in 12), 1.29 (m, 1H, -CH in 15), 1.32 (m, 1H, -CH in 11), 1.33 (m, 1H, -CH in 9), 1.42 (m, 3H, -CH in 6 and 7), 1.48 (s, 9H, -COOtBu and m, 1H, -CH in 6), 1.52 (m, 2H, -CH in 21 and 22), 1.55 (s, 9H, -NHBOc), 1.58 (m, 2H, -CH in 1 and 2), 1.69 (m, 2H, -CH₂, Lys), 1.70 (m, 3H, -CH in 18, -CH₂, Lys), 1.71 (m, 1H, -CH in 2), 1.78 (s, 3H, -CH₃ in 30), 1.80 (m, 1H, -CH in 15), 1.86 (m, 1H, -CH_β Lys), 1.92 (m, 1H, -CH in 12), 2.07 (m, 1H, -CH_β Lys), 2.08 (s, 3H, -COCH₃), 2.30 (m, 1H, -CH in 21), 2.33 (m, 1H, -CH in 22), 2.54 (m, 1H, -CH in 16), 3.03 (m, 1H, -CH in 13), 3.40 (m, 2H, -CH₂, Lys), 3.58 (m, 1H, -CH in 19), 4.7 (dd, 1H, -CH in 3, *J* = 5 Hz and 11.5 Hz), 4.75 (s, 1H, =CH), 4.91 (m, 2H, =CH and -CH_γ Lys), 7.55 (t, 1H, -NH-CO- *J* = 5 Hz), 8.22 (d, 1H, -CO-NH-, *J* = 8 Hz). ¹³C NMR (400 MHz, C₅D₅N): δ 15.3 (C27); 16.7 (C25); 17 (C26); 17.2 (C24); 19 (C6); 20 (C30); 21.6 (C2'); 21.7 (C11); 24.3 (C_γLys); 24.5 (C2); 26.6 (C12); 28.4 (3C, *t*-Bu); 28.5 (C23); 29 (3C, Boc); 30.3 (C15); 30.5 (C_δLys); 31.9 (C21); 32 (C_βLys); 34 (C16); 35.1 (C7); 37.8 (C10); 38.1 (C13); 38.5 (C4); 38.65 (C22); 39 (C1); 41.3 (C_εLys); 41.6 (C8); 43.2 (C14); 47.6 (C19); 51.1 (C18); 51.3 (C9); 53.4 (C_αLys); 56.1 (C5); 56.4 (C17); 78.4 (CBoc); 81.2 (C3); 81.3 (C*t*-Bu); 110.2 (C29); 152.1 (C20); 157.25 (C NHCOOt-Bu); 171.1 (C1'); 173.4 (C COOt-Bu); 177.3 (C28).

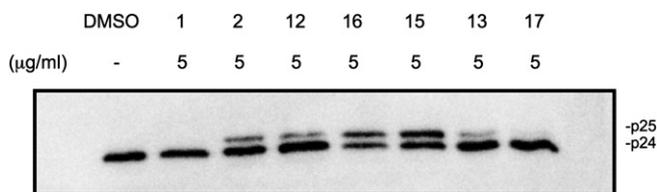


Fig. 5. Effect of the compounds on the virus particle production and Pr55Gag processing was analysed by western blot analysis. Note the accumulation of p25 in the presence of 2, 12, 16, 15, 13 and 17.

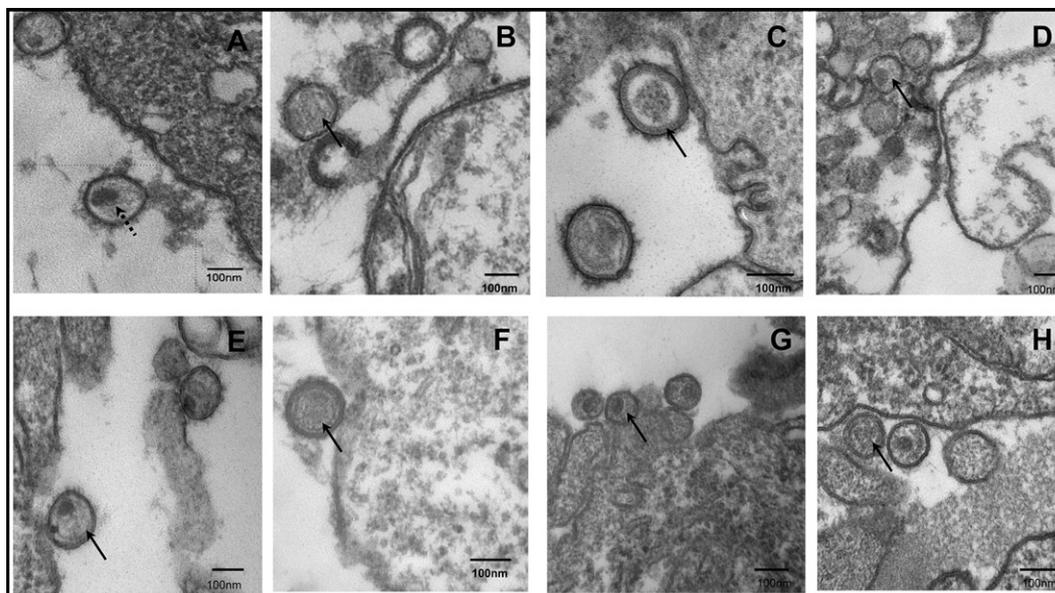


Fig. 6. Thin-section EM analysis of virions produced from BA, **2**, **12**, **16**, **15**, **13**, **17**-treated cells. 293T cells were transfected with pNL-4-3 and were not treated (A) or treated (B, C, D, E, F, G, H) with BA, **2**, **12**, **16**, **15**, **13**, **17**. Two days post transfection, cells were fixed and analyzed by thin-section EM. Dashed arrow in A indicate mature, conical cores; arrows in B, C, D, E, F, G, H indicate the crescent-shaped, electron-dense layer inside the viral membrane that results from inhibition of p25 processing. (Bar: \approx 100 nm).

5.1.3. General procedure for the synthesis of derivative **8–11**

An aqueous NaOH (4 M, 0.75 mL) was added to the solution of the above intermediates (0.3 mmol) in THF/MeOH (1/1.4 mL). After stirring for 12 h at room temperature, the mixture was acidified with 1 M HCl. The resulting precipitate was collected, washed with water and dried over vacuum to yield the corresponding compounds **8** and **9**. For compounds **10** and **11**, the mixture was extracted with CH_2Cl_2 (50 mL), the organic layer was washed with water, brine and dried over MgSO_4 and then concentrated under vacuum. The residue was purified by silica gel chromatography to provide the corresponding derivatives.

5.1.3.1. *N*-[(3 β)-3-hydroxy-lup-20(29)-en-28-oyl]-glycine (8**).** Yield: 83% (128 mg); ^1H NMR (CDCl_3): δ 0.70 (m, 1H, $-\text{CH}$ in 5), 0.77 (s, 3H, $-\text{CH}_3$), 0.83 (s, 3H, $-\text{CH}_3$), 0.93 (s, 3H, $-\text{CH}_3$), 0.98 (s, 3H, $-\text{CH}_3$), 0.99 (s, 3H, $-\text{CH}_3$), 1.09 (m, 1H), 1.10–1.65 (m, 20H), 1.70 (s, 3H, $-\text{CH}_3$), 1.90 (m, 3H), 2.40 (dt, 1H, $J = 3.5$ Hz, $J = 13$ Hz), 3.10 (dt, 1H, $J = 3.5$ Hz, $J = 11$ Hz), 3.20 (m, 1H), 4.05 (t, 2H, $J = 7.5$ Hz), 4.61 (s, 1H, $=\text{CH}$), 4.75 (s, 1H, $=\text{CH}$), 6.14 (t, 1H, $J = 5.2$ Hz).

5.1.3.2. *N*-[(3 β)-3-hydroxy-lup-20(29)-en-28-oyl]- β -alanine (9**).** Yield: 89% (141 mg); ^1H NMR (400 MHz, $\text{C}_5\text{D}_5\text{N}$): δ 0.86 (m, 1H, $-\text{CH}$ in 5), 0.88 (m, 3H, $-\text{CH}_3$ in 25), 0.99 (m, 1H, $-\text{CH}$ in 1), 1.04 (s, 3H, $-\text{CH}_3$ in 24), 1.08 (s, 3H, $-\text{CH}_3$ in 27), 1.16 (s, 3H, $-\text{CH}_3$ in 26), 1.2 (m, 1H, $-\text{CH}$ in 12), 1.24 (m, 2H, $-\text{CH}$ in 11 and $-\text{CH}$ in 15), 1.27 (m, 3H, $-\text{CH}_3$ in 23), 1.41 (m, 3H, $-\text{CH}$ in 6 and $-\text{CH}$ in 7), 1.42 (m, 1H, $-\text{CH}$ in 9), 1.44 (m, 1H, $-\text{CH}$ in 11), 1.5 (m, 2H, $-\text{CH}$ in 21 and $-\text{CH}$ in 22), 1.58 (m, 2H, $-\text{CH}$ in 6 and $-\text{CH}$ in 16), 1.68 (m, 1H, $-\text{CH}$ in 1), 1.72 (m, 1H, $-\text{CH}$ in 18), 1.8 (s, 3H, $-\text{CH}_3$ in 30), 1.86 (m, 1H, $-\text{CH}$ in 15), 1.89 (m, 2H, $-\text{CH}$ in 2), 1.98 (m, 1H, $-\text{CH}$ in 12), 2.13 (m, 1H, $-\text{CH}$ in 22), 2.28 (m, 1H, $-\text{CH}$ in 21), 2.44 (m, 1H, $-\text{CH}$ in 16), 2.96 (m, 2H, $-\text{CH}$ in 2''), 3.05 (m, 1H, $-\text{CH}$ in 13), 3.46 (m, 1H, $-\text{CH}$ in 3), 3.65 (m, 1H, $-\text{CH}$ in 19), 3.93 (m, 1H, $-\text{CH}$ in 1''), 3.98 (m, 1H, $-\text{CH}$ in 1''), 4.77 (s, 1H, $=\text{CH}$), 4.93 (s, 1H, $=\text{CH}$), 8.36 (d, 1H, $-\text{CO}-\text{NH}-$, $J = 7$ Hz). ^{13}C NMR (400 MHz, $\text{C}_5\text{D}_5\text{N}$): δ 16.2 (C27); 17.8 (C24); 17.86 (C25); 17.92 (C26); 20.2 (C6); 21 (C30); 22.6 (C11); 27.6 (C12); 29.7 (C2); 30 (C23); 31.2 (C15); 32.8 (C21); 35 (C16); 36.2 (C7); 37.4 (C2''); 37.9 (C1''); 38.9 (C10); 39.2 (C13); 40.1 (C22); 40.6 (C1); 40.9

(C4); 42.6 (C8); 44.1 (C14); 48.7 (C19); 52.1 (C18); 52.4 (C9); 57.28 (C17); 57.34 (C5); 79.5 (C3); 110.1 (C29); 153.1 (C20); 177.5 (C3''); 178.1 (C28).

5.1.3.3. *N*-[(3 β)-3-hydroxy-lup-20(29)-en-28-oyl]-1,1-dimethylethyl-(2-aminoethyl)carbamate (10**).** R_f : 0.48 (toluene/acetone 80/20); Yield 67% (210 mg); ^1H NMR (300 MHz, CDCl_3): δ 0.70 (m, 1H, $-\text{CH}$ in 5), 0.77 (s, 3H, $-\text{CH}_3$), 0.83 (s, 3H, $-\text{CH}_3$), 0.94 (s, 3H, $-\text{CH}_3$), 0.97 (s, 6H, 2- CH_3), 1.09 (m, 1H), 1.10–1.40 (m, 9H), 1.45 (s, 9H, $t\text{-Bu}$), 1.50–1.65 (m, 7H), 1.69 (s, 3H, $-\text{CH}_3$), 1.72 (m, 3H), 1.99 (m, 2H), 2.46 (dt, 1H, $J = 3.5$ Hz, $J = 13$ Hz), 3.20 (m, 6H), 3.20 (m, 1H), 4.60 (s, 1H, $=\text{CH}$), 4.74 (s, 1H, $=\text{CH}$), 5.01 (m, 1H, $-\text{NH}-\text{CO}-$), 6.33 (m, 1H, $-\text{CO}-\text{NH}-$).

5.1.3.4. *N*⁶-[(1,1-dimethylethoxy)carbonyl], *N*²-[(3 β)-3-hydroxy-lup-20(29)-en-28-oyl]-*L*-lysine (11**).** R_f : 0.32 (*n*-heptane/ethyl acetate/acetic acid 10/10/0.25); Yield 97% (277 mg); ^1H NMR (400 MHz, $\text{C}_5\text{D}_5\text{N}$): δ 0.86 (m, 4H, $-\text{CH}$ in 5, $-\text{CH}_3$ in 25), 1.02 (m, 1H, $-\text{CH}$ in 1), 1.06 (s, 3H, $-\text{CH}_3$ in 24), 1.10 (s, 3H, $-\text{CH}_3$ in 27), 1.18 (s, 3H, $-\text{CH}_3$ in 26), 1.20 (m, 1H, $-\text{CH}$ in 12), 1.22 (m, 1H, $-\text{CH}$ in 11), 1.26 (s, 3H, $-\text{CH}_3$ in 23), 1.35 (m, 1H, $-\text{CH}$ in 15), 1.42 (m, 1H, $-\text{CH}$ in 11), 1.43 (m, 1H, $-\text{CH}$ in 9), 1.48 (m, 1H, $-\text{CH}$ in 6), 1.50 (m, 2H, $-\text{CH}_2$ in 7), 1.52 (m, 1H, $-\text{CH}$ in 21), 1.53 (m, 1H, $-\text{CH}$ in 22), 1.55 (s, 9H, $-\text{NH}(\text{Boc})$), 1.61 (m, 1H, $-\text{CH}$ in 6), 1.67 (m, 1H, $-\text{CH}$ in 16), 1.70 (m, 1H, $-\text{CH}$ in 1), 1.75 (m, 1H, $-\text{CH}$ in 18), 1.77 (s, 3H, $-\text{CH}_3$ in 30), 1.80 (m, 2H, $-\text{CH}_2$ Lys), 1.81 (m, 2H, $-\text{CH}_2$ Lys), 1.88 (m, 2H, $-\text{CH}_2$ in 2), 1.91 (m, 1H, $-\text{CH}$ in 15), 1.96 (m, 1H, $-\text{CH}$ in 12), 2.05 (m, 1H, $-\text{CH}_\beta$ Lys), 2.30 (m, 1H, $-\text{CH}_\beta$ Lys), 2.36 (m, 1H, $-\text{CH}$ in 21), 2.45 (m, 1H, $-\text{CH}$ in 22), 2.58 (m, 1H, $-\text{CH}$ in 16), 3.09 (m, 1H, $-\text{CH}$ in 13), 3.43 (m, 2H, $-\text{CH}_2$ Lys), 3.49 (m, 1H, $-\text{CH}$ in 3), 3.65 (m, 1H, $-\text{CH}$ in 19), 4.77 (s, 1H, $=\text{CH}$ in 29), 4.92 (s, 1H, $=\text{CH}$ in 29), 5.16 (m, 1H, $-\text{CH}_\alpha$ Lys), 7.58 (m, 1H, $-\text{NH}-\text{CO}-$), 8.25 (d, 1H, $-\text{CO}-\text{NH}-$, $J = 8$ Hz). ^{13}C NMR (400 MHz, $\text{C}_5\text{D}_5\text{N}$): δ 15.3 (C27); 16.85 (C24); 17 (C25); 17.1 (C26); 19.3 (C6); 20 (C30); 21.7 (C11); 24.5 (C_γ Lys); 26.7 (C12); 28.8 (C2); 29.1 (3C, Boc); 29.2 (C23); 30.4 (C15); 30.6 (C δ Lys); 31.9 (C21); 32.5 (C β Lys); 34.2 (C16); 35.4 (C7); 38 (C10); 38.2 (C13); 38.8 (C22); 39.75 (C1); 40 (C4); 41.4 (C ϵ Lys); 41.7 (C8); 43.2 (C14); 47.7 (C19); 51.2 (C18); 51.6 (C9); 53 (C α Lys); 56.4 (C5); 56.6 (C17); 78.4 (3CBoc);

78.6 (C3); 110.1 (C29); 152.2 (C20); 157.3 (C, NHCOO⁻-Bu); 176.6 (C, COOH); 177.4 (C28).

5.1.4. General procedure for the synthesis of derivatives **12–15**

A mixture of the above intermediates (**8**, **9**, **10** or **11**), 2,2-dimethylsuccinic anhydride (10 equiv) and DMAP (1 equiv) in anhydrous pyridine (20 mL/mmol) was refluxed overnight. The mixture was then concentrated under vacuum and the residue was chromatographed on silica gel to yield the derivatives **12**, **13**, **14** and **15**.

5.1.4.1. N-[(3β)-3-(3-carboxy-3-methylbutanoyloxy)lup-20(29)-en-28-oyl]-glycine (12**).** *R_f*: 0.23 dichloromethane/methanol/acetic acid (10/0.1/0.02); Yield 58% (80 mg); HRMS (ESI) calcd for C₃₈H₅₈N₇ [M – H]⁻ 640.4219 found 640.4232; ¹H NMR (400 MHz, C₅D₅N): δ 0.73 (s, 3H, –CH₃ in 25), 0.77 (m, 1H, –CH in 5), 0.89 (m, 1H, –CH in 1), 0.92 (s, 3H, –CH₃ in 24), 0.96 (s, 3H, –CH₃ in 23), 1.06 (s, 3H, –CH₃ in 27), 1.12 (s, 3H, –CH₃ in 26), 1.15 (m, 1H, –CH in 12), 1.15 (m, 1H, –CH in 11), 1.26 (m, 1H, –CH in 15), 1.30 (m, 2H, –CH in 9, –CH in 11), 1.30–1.40 (m, 4H, –CH₂ in 6, –CH₂ in 7), 1.52 (m, 1H, –CH in 1), 1.54 (m, 1H, –CH in 21), 1.55 (s, 3H, –CH₃ in 5'), 1.59 (m, 1H, –CH in 22), 1.67 (m, 2H, –CH in 2, –CH in 16), 1.74 (m, 1H, –CH in 18), 1.77 (m, 1H, –CH in 2), 1.78 (s, 3H, –CH₃ in 30), 1.93 (m, 1H, –CH in 12), 2.03 (m, 1H, –CH in 15), 2.38 (m, 1H, –CH in 21), 2.39 (m, 1H, –CH in 22), 2.59 (m, 1H, –CH in 16), 2.91 (d, 1H, –CH in 2', *J* = 15.5 Hz), 2.95 (d, 1H, –CH in 2', *J* = 15.5 Hz), 3.08 (m, 1H, –CH in 13), 3.66 (m, 1H, –CH in 19), 4.44–4.51 (dd, 2H, –CH₂ in 1'', *J* = 6 Hz, *J* = 17.5 Hz), 4.75 (s, 1H, –CH in 29), 4.77 (d, 1H, –CH in 3, *J* = 5 Hz), 4.96 (s, 1H, –CH in 29), 8.76 (t, 1H, –NH–CO–, *J* = 5.5 Hz). ¹³C NMR (400 MHz, C₅D₅N): δ 15.3 (C27); 16.7 (C25); 17 (C26); 17.3 (C24); 18.9 (C6); 20 (C30); 21.6 (C11); 24.6 (C2); 26.6 (C12); 26.7 (C5'); 28.5 (C23); 30.3 (C15); 31.8 (C21); 34 (C16); 35 (C7); 37.7 (C10); 38.1 (C13); 38.5 (C4); 39 (C1); 39.1 (C22); 41.3 (C3'); 41.6 (C8); 42.4 (C1''); 43.2 (C14); 45.6 (C2'); 47.7 (C19); 51.0 (C18); 51.2 (C9); 56.0 (C5); 56.4 (C17); 81.4 (C3); 110.3 (C29); 152.2 (C20); 172.1 (C1'); 174 (C6''); 177.9 (C28); 179.8 (C4'). Anal. (C₃₈H₅₉NO₇·0.5H₂O) C, H, N.

5.1.4.2. N-[(3β)-3-(3-carboxy-3-methylbutanoyloxy)lup-20(29)-en-28-oyl]-β-alanine (13**).** *R_f*: 0.27 *n*-heptane/ethyl acetate/acetic acid (10/10/0.5); Yield 2.62% (166 mg); HRMS (ESI) calcd for C₃₉H₆₀N₇ [M – H]⁻ 654.4375 found 654.4375, calcd for C₃₉H₅₉N₇Na [M – 2H + Na]⁻ 676.4195 found 676.4186; ¹H NMR (400 MHz, C₅D₅N): δ 0.74 (m, 1H, –CH in 5 and s, 3H, –CH₃ in 25), 0.91 (m, 1H, –CH in 1), 0.94 (s, 3H, –CH₃ in 24), 0.97 (s, 3H, –CH₃ in 23), 1.04 (s, 3H, –CH₃ in 27), 1.08 (s, 3H, –CH₃ in 26), 1.17 (m, 2H, –CH in 11 and –CH in 12), 1.2 (m, 1H, –CH in 15), 1.30 (m, 2H, –CH in 9, –CH in 6), 1.31 (m, 1H, –CH in 11), 1.33 (m, 2H, –CH in 7), 1.4 (m, 1H, –CH in 6), 1.52 (m, 2H, –CH in 21 and –CH in 22), 1.55 (s, 3H, –CH₃ in 5'), 1.58 (m, 2H, –CH in 1 and –CH in 16), 1.7 (m, 2H, –CH in 18 and –CH in 2), 1.81 (s, 3H, –CH₃ in 30), 1.82 (m, 1H, –CH in 2), 1.85 (m, 1H, –CH in 15), 1.96 (m, 1H, –CH in 12), 2.16 (m, 1H, –CH in 22), 2.27 (m, 1H, –CH in 21), 2.47 (m, 1H, –CH in 16), 2.94 (d, 1H, –CH in 2', *J* = 15.5 Hz), 3.00 (d, 1H, –CH in 2', *J* = 15.5 Hz), 3.01 (m, 1H, –CH in 2''), 3.07 (m, 1H, –CH in 13), 3.67 (m, 1H, –CH in 19), 3.9 (m, 1H, –CH₂ in 1''), 4.03 (m, 1H, –CH₂ in 1'') 4.79 (m, 2H, –CH in 3 and –CH in 29), 4.97 (s, 1H, –CH in 29), 8.33 (t, 1H, –NH–CO–, *J* = 7 Hz). ¹³C NMR (400 MHz, C₅D₅N): δ 15.2 (C27); 16.7 (C25); 16.9 (C26); 17.4 (C24); 18.9 (C6); 20 (C30); 21.7 (C11); 24.6 (C2); 26.6 (C12); 26.7 (C5'); 28.6 (C23); 30.3 (C15); 31.8 (C21); 34 (C16); 35 (C7); 35.7 (C2''); 36.7 (C1''); 37.7 (C10); 38.2 (C13); 38.5 (C4); 39 (C1); 39.1 (C22); 41.3 (C3'); 41.6 (C8); 43.2 (C14); 45.7 (C2'); 47.7 (C19); 51.1 (C18); 51.2 (C9); 56.1 (C5); 56.3 (C17); 81.4 (C3); 110.2 (C29); 152.2 (C20); 172 (C1'); 175.4 (C6''); 177.2 (C28); 179.8 (C4'). Anal. (C₃₉H₆₁NO₇·0.5H₂O) C, H, N.

5.1.4.3. 4-([28-[1,1-dimethylethyl-(2-aminoethyl)carbamate]-28-oxolup-20,29-en-3β-yl]oxy)-2,2-dimethyl-4-oxobutanoic acid (14**).** *R_f*: 0.25 dichloromethane/methanol/acetic acid (10/0.2/0.01); Yield 82% (190 mg); ¹H NMR (300 MHz, CDCl₃): δ 0.72 (m, 1H, –CH in 5), 0.77 (s, 3H, –CH₃), 0.78 (s, 3H, –CH₃), 0.79 (s, 3H, –CH₃), 0.89 (s, 3H, –CH₃), 0.92 (s, 3H, –CH₃), 1.10 (m, 1H), 1.26 (s, 6H), 1.27–1.40 (m, 6H), 1.42 (s, 9H, *t*-Bu), 1.42–1.63 (m, 7H), 1.69 (s, 3H, –CH₃), 1.72 (m, 2H), 1.99 (m, 2H), 2.46 (m, 1H), 2.57 (m, 2H), 3.08 (m, 1H), 3.31 (m, 9H), 4.42 (m, 1H), 4.55 (s, 1H, =CH), 4.69 (s, 1H, =CH), 5.17 (m, 1H, –NH–CO–), 5.28 (m, 1H, –CO–NH–).

5.1.4.4. N⁶-[(1,1-dimethylethoxy)carbonyl, N²-[(3β)-3-(3-carboxy-3-methylbutanoyloxy)lup-20(29)-en-28-oyl]-L-lysine (15**).** *R_f*: 0.31 *n*-heptane/ethyl acetate/acetic acid (10/10/0.25); Yield 36% (106 mg); HRMS (ESI) calcd for C₄₇H₇₅N₂O₇ [M – H]⁻ 811.5478 found 811.5476; ¹H NMR (400 MHz, C₅D₅N): δ 0.76 (s, 3H, –CH₃ in 25), 0.82 (m, 1H, –CH in 5), 0.9 (m, 1H, –CH in 1), 0.96 (s, 3H, –CH₃ in 24), 0.97 (s, 3H, –CH₃ in 23), 1.08 (s, 3H, –CH₃ in 27), 1.09 (s, 3H, –CH₃ in 26), 1.15 (m, 2H, –CH in 12 and –CH in 11), 1.3 (m, 1H, –CH in 9), 1.32 (m, 1H, –CH in 15), 1.33 (m, 1H, –CH in 11), 1.36–1.46 (m, 4H, –CH₂ in 6, –CH₂ in 7), 1.53 (m, 1H, –CH in 21), 1.54 (m, 1H, –CH in 22), 1.55–1.59 (s, 3H, –CH₃ in 5' and s, 9H, –NH₂Boc and m, 1H, –CH in 1), 1.67–1.70 (m, 2H, –CH in 2, –CH in 16), 1.70, 1.73 (m, 1H, –CH in 18), 1.78 (m, 1H, –CH in 2), 1.78 (s, 3H, –CH₃ in 30), 1.8 (m, 2H, –CH_{2γ} Lys), 1.83 (m, 1H, –CH_{2δ} Lys), 1.89 (m, 1H, –CH in 15), 1.95 (m, 1H, –CH in 12), 2.06 (m, 1H, –CH_{2β} Lys), 2.3 (m, 1H, –CH_{2β} Lys), 2.34 (m, 1H, –CH in 21), 2.43 (m, 1H, –CH in 22), 2.58 (m, 1H, –CH in 16), 2.9 (d, 1H, –CH in 2', *J* = 15.5 Hz), 2.98 (d, 1H, –CH in 2', *J* = 15.5 Hz), 3.07 (m, 1H, –CH in 13), 3.43 (m, 2H, –CH_{2ε} Lys), 3.63 (m, 1H, –CH in 19), 4.77 (s, 1H, –CH in 29), 4.78 (dd, 1H, –CH in 3, *J* = 5 Hz and *J* = 11.5 Hz), 4.92 (s, 1H, –CH in 29), 5.17 (m, 1H, –CH_α Lys), 7.58 (m, 1H, –NH–CO–O), 8.26 (d, 1H, –CO–NH–, *J* = 8 Hz). ¹³C NMR (400 MHz, C₅D₅N): δ 15.3 (C27); 16.7 (C25); 17 (C26); 17.4 (C24); 19 (C6); 20.0 (C30); 21.7 (C11); 24.5 (C_γLys); 24.6 (C2); 26.4 (C5'); 26.6 (C12); 28.6 (C23); 29.1 (3C, Boc); 30.3 (C15); 30.6 (C_δLys); 31.9 (C21); 32.4 (C_βLys); 34.2 (C16); 35.1 (C7); 37.75 (C10); 38.1 (C13); 38.5 (C4); 38.8 (C22); 39 (C1); 41.3 (C3'); 41.4 (C_εLys); 41.6 (C8); 43.2 (C14); 45.7 (C2'); 47.7 (C19); 51.1 (C18); 51.2 (C9); 53 (C_αLys) 56.1 (C5); 56.5 (C17); 78.4 (COBoc); 81.4 (C3); 110.2 (C29); 152.2 (C20); 172.1 (C1'); 176.6 (C6''); 177.7 (C28); 179.8 (C4'). Anal. (C₄₇H₇₆N₂O₉·2H₂O) C, H, N.

5.1.5. Procedure for synthesis of compound 4-([28-[(2-aminoethyl)amino]-28-oxolup-20,29-en-3β-yl]oxy)-2,2-dimethyl-4-oxobutanoic acid (**16**)

tert-Butyldimethylsilyltrifluoromethanesulfonate (*t*-BuMe₂-SiOTf, 150 μL, 0.65 mmol) was added dropwise to a stirred solution under argon of *N*-*tert*-Boc derivatives **14** (0.19 mg, 0.26 mmol) and 2,6-lutidine (91 μL, 0.78 mmol) in dry CH₂Cl₂ (1 mL). The reaction mixture was stirred 30 min, quenched with saturated aqueous ammonium chloride solution (2 mL). The mixture was diluted with CH₂Cl₂ (5 mL), the organic layer was washed with water, brine and dried over MgSO₄. The solvent was concentrated under vacuum and the residue was used in the next step without purification. Tetra-butylammonium fluoride (260 μL, 1 M solution in THF, 0.26 mmol) was added to a stirred solution of the *N*-(*tert*-butyldimethylsilyloxycarbonyl) derivatives in dry THF (0.5 mL) at room temperature. The reaction mixture was stirred for 1 h, quenched with saturated aqueous ammonium chloride solution (2 mL). The resulting precipitate was collected, washed with water and dried under vacuum to yield the corresponding compound **16** (Refer to Supporting data). Yield: 26% (45 mg); HRMS (ESI) calcd for C₃₈H₆₁N₂O₅ [M – H]⁻ 625.4586 found 625.4569; ¹H NMR (400 MHz, C₅D₅N): δ 0.75 (s, 3H, –CH₃ in 25), 0.77 (m, 1H, –CH in 5), 0.86 (m, 1H, –CH in 1), 0.93 (s, 3H, –CH₃ in 24), 0.97 (s, 3H, –CH₃ in 23), 1.03 (s, 3H, –CH₃ in 27), 1.03 (s, 3H, –CH₃ in 26), 1.12 (m, 1H, –CH in 12), 1.13 (m, 1H, –CH in 11),

1.17 (m, 1H, –CH in 15), 1.30 (m, 2H, –CH in 9, –CH in 11), 1.30–1.40 (m, 4H, –CH₂ in 6, –CH₂ in 7), 1.48 (m, 1H, –CH in 21), 1.54 (m, 1H, –CH in 1), 1.55 (s, 3H, –CH₃ in 5'), 1.55 (m, 1H, –CH in 22), 1.64–1.67 (m, 2H, –CH in 2, –CH in 16), 1.70 (m, 2H, –CH in 15, –CH in 18), 1.77 (m, 1H, –CH in 2), 1.77 (s, 3H, –CH₃ in 30), 1.92 (m, 1H, –CH in 12), 2.14 (m, 1H, –CH in 21), 2.25 (m, 1H, –CH in 22), 2.67 (m, 1H, –CH in 16), 2.90 (d, 1H, –CH in 2', $J = 15.5$ Hz), 2.96 (d, 1H, –CH in 2', $J = 15.5$ Hz), 3.03 (m, 1H, –CH in 13), 3.28 (m, 2H, –CH₂ in 2''), 3.61 (m, 1H, –CH in 19), 3.87–3.81 (m, 2H, –CH₂ in 1''), 4.75 (s, 1H, –CH in 29), 4.77 (d, 1H, –CH in 3, $J = 5$ Hz), 4.93 (s, 1H, –CH in 29), 8.74 (m, 1H, –NH–CO–). ¹³C NMR (400 MHz, C₅D₅N): δ 15.2 (C27); 16.7 (C25); 16.9 (C26); 17.4 (C24); 19 (C6); 20.0 (C30); 21.6 (C11); 24.6 (C2); 26.5 (C12); 26.7 (C5'); 28.5 (C23); 30.3 (C15); 31.8 (C21); 34.0 (C16); 35.0 (C7); 37.7 (C10); 38.1 (C13); 38.5 (C4); 39.0 (C22); 39.1 (C1); 41.0 (C1''); 41.4 (C3'); 41.5 (C8); 42.2 (C2''); 43.1 (C14); 45.7 (C2'); 47.7 (C19); 51.2 (C18); 51.3 (C9); 56.1 (C5); 56.5 (C17); 81.3 (C3); 110.2 (C29); 152.2 (C20); 172.1 (C1'); 177.7 (C28); 179.9 (C4'). Anal. (C₃₈H₆₂N₂O₅·3H₂O) C, H, N.

5.1.6. Procedure for synthesis of compound N²-[(3 β)-3-(3-carboxy-3-methylbutanoyloxy)lup-20(29)-en-28-oyl]-L-lysine (**17**)

The *N*-Boc protection of product **15** was cleaved with 6 M HCl in THF for 1 h. Compound **17** was obtained after purification by flash chromatography. Yield: 45% (20 mg); HRMS (ESI) calcd for C₄₂H₆₇N₂O₇ [M – H][–] 711.4954 found 711.4960; ¹H NMR (400 MHz, CD₃OD): δ 0.82 (m, 1H, –CH in 5), 0.85 (s, 3H, –CH₃ in 24), 0.87 (s, 3H, –CH₃ in 23), 0.92 (s, 3H, –CH₃ in 25), 0.96 (s, 3H, –CH₃ in 26), 0.99 (m, 1H, –CH in 1), 1.01 (s, 3H, –CH₃ in 27), 1.04 (m, 1H, –CH in 12), 1.18 (m, 1H, –CH in 15), 1.25 (s, 3H, –CH₃ in 5'), 1.26 (m, 1H, –CH in 11), 1.35 (m, 1H, –CH in 21), 1.36 (m, 1H, –CH in 9), 1.38–1.52 (m, 9H, –CH₂ in 6, –CH₂ in 7, –CH in 11, –CH in 15, –CH in 22, –CH₂ in 3''), 1.56 (m, 1H, –CH in 16), 1.6 (m, 1H, –CH in 2), 1.64 (m, 1H, –CH in 18), 1.68 (m, 1H, –CH in 2), 1.7 (m, 6H, –CH₃ in 30, –CH in 12, 2H, –CH₂ in 4''), 1.72 (m, 1H, –CH in 1), 1.75 (m, 1H, –CH in 2''), 1.89 (m, 1H, –CH in 2''), 1.9 (m, 1H, –CH in 21), 1.93 (m, 1H, –CH in 22), 2.17 (m, 1H, –CH in 16), 2.53 (m, 1H, –CH in 13), 2.55 (d, 1H, –CH in 2', $J = 15.5$ Hz), 2.62 (d, 1H, –CH in 2', $J = 15.5$ Hz), 2.90 (t, 2H, –CH in 5'', $J = 8$ Hz), 3.06 (m, 1H, –CH in 19), 4.3 (m, 1H, –CH in 1''), 4.44 (dd, 1H, –CH in 3, $J = 5$ Hz and $J = 10$ Hz), 4.57 (s, 1H, –CH in 29), 4.7 (s, 1H, –CH in 29), 7.1 (m, 1H, –NH–CO–). ¹³C NMR (400 MHz, CD₃OD): δ 15.2 (C27); 17 (C25); 17.1 (C26); 17.2 (C24); 19.4 (C6); 19.8 (C30); 22.3 (C11); 24.05 (C3''); 24.8 (C2); 26.2 (C5'); 27.2 (C12); 28.2 (C4''); 28.7 (C23); 30.9 (C15); 32.1 (C21); 32.7 (C2''); 34.4 (C16); 35.7 (C7); 38.4 (C10); 39 (C4); 39.2 (C13); 39.3 (C22); 39.8 (C1); 40.7 (C5''); 41.5 (C3'); 42.2 (C8); 43.7 (C14); 45.8 (C2'); 48.3 (C19); 51.4 (C18); 52.1 (C9); 54 (C1''); 57 (C5); 57.4 (C17); 82.8 (C3); 110.2 (C29); 152.4 (C20); 173.1 (C1'); 177.3 (C6''); 178.9 (C28); 180.8 (C4'). Anal. (C₄₂H₆₈N₂O₇·2H₂O) C, H, N.

5.2. HPLC analysis

HPLC analysis were conducted using a Shimadzu LC-10ADvp with a Nucleodur 4.6 mm × 250 mm C18 HTec 5 μ m column and a Shimadzu SPD-M10Avp detector at 210 nm wavelength. The solvent system used was MeOH(0.1%TFA):water (0.1%TFA) = 90:10. The isocratic HPLC mode was used and the flow rate was set to 1 mL/min.

5.3. NMR

¹H NMR, ¹³C NMR, DEPT135, DQF-COSY, TOCSY, HSQC and HMBC spectra of compounds **2**, **12**, **13**, **15**, **16** and **17** (Fig. 1) were recorded on a Bruker AVANCE 400 NMR spectrometer operating at 400.13 MHz (SF) for ¹H and 100.623 MHz (SF) for ¹³C with a 5 mm inverse probe at room temperature. The concentration of the different compounds was 10 mM in 0.5 mL C₅D₅N, in 5 mm NMR

tubes. All chemical shifts are given in ppm (Fig. 3) and (Tables 1 and 2 and Figures S1 to S5 in supplementary data). The NMR spectra were registered with a spectral width of 4084.97 Hz and 22,522.52 Hz for the ¹H and ¹³C dimensions respectively. WALTZ [60,61] pulse sequence was used for ¹H decoupling. The DEPT135 experiment (Distortionless Enhancement by Polarization Transfer) using polarization transfer from proton to other nuclei via one covalent bond allowed the determination of the CH_{*n*} multiplicity. All 2D experiments were performed using standard pulse sequences with the Bruker software program XWIN-NMR vs. 3.0. DQF-COSY and TOCSY spectra were recorded with 2048 points in the *F2* dimension and 512 increments in the *F1* dimension. Each increment was obtained with 40 scans, a spectral width of 4084.97 Hz, a recycling delay of 2 s and a mixing time of 80 ms were used for the TOCSY experiments. The HSQC spectra were obtained with the one-bond ¹H–¹³C coupling constant set to 145 Hz, a GARP ¹³C decoupling WALTZ [60,62], 2048 points in the *F2* dimension and 256 increments in the *F1* dimension, with a relaxation delay of 2 s. The spectral width was set to 10,964.91 Hz and 4084.97 Hz in the *F2* and *F1* dimensions respectively. The 2D (¹H–¹³C)-HMBC experiments were performed with 40 scans for each of 256 *F1* increments and 2048 data points in *F2*. Spectral widths of 4084.97 Hz and 22,522.52 Hz were used in the ¹H and ¹³C dimensions respectively.

2D NMR spectra demonstrating the interaction between the CA-SP1–NC junction and compound **16** were recorded at 293 K on a 600 MHz Bruker Avance spectrometer equipped with a 5 mm triple resonance inverse probe and a XYZ gradient unit. 2D ¹H–¹H NOESY spectra were recorded for the peptide CA-SP1–NC (250 μ M) in the absence and presence of compound **16** (550 μ M) and superimposed, at a pH of 3.8, in H₂O/TFE (70/30) mixture to prevent aggregation, DMSO-*d*₆ (1%) and a spectral width of 5296.6 Hz in both dimensions. Water suppression was achieved by using excitation sculpting with a 2 ms selective Gaussian pulse at the water signal frequency and a 250 ms NOE mixing time (Fig. 4B).

5.4. HIV-1 infection inhibition assays

Infectivity of HIV-1 particles produced in the presence of inhibitors was carried out using MAGIC-5B indicator cells, which stably express the β -galactosidase reporter gene cloned downstream of the HIV-1 LTR promoter. Cells were plated at 8×10^4 cells per well, in 24-well plates and exposed to virus-containing supernatants produced by transfection of the HIV-1 molecular clone (pNL-4.3) in 293T cells maintained in the presence or absence of compounds **1**, **2**, **12**, **13**, **15**, **16** and **17**. Forty-eight hours post-infection, viral infectivity was monitored by quantification of *o*-nitrophenyl β -D-galactopyranoside hydrolysis from cell lysates (kit). β -galactosidase activity was normalized according to the total protein content in the cell lysate. The control wells containing the virus and cells only (no virus or drug) were also prepared. The 50% infection inhibition (IC₅₀) was defined as the concentration of the compound that reduced the HIV-1 infection level by 50% compared to the untreated controls (Table 3).

5.5. Electron microscopy analysis

293T cells were transfected with HIV-1 molecular clones (pNL-4-3) using the JetPei transfection reagent (QBiogen) and maintained in the presence or absence of **1**, **2**, **12**, **13**, **15**, **16** and **17** (5 μ g/ml). Two days after transfection, the cells were processed for thin-layer electron microscopy as follows: the cells were fixed *in situ* with 2.5% glutaraldehyde in cacodylate buffer (pH 7.4) for 60 min at 4 °C. They were then post-fixed with 2% osmium tetroxide, washed in cacodylate buffer containing 0.5% tannic acid, and embedded in epon (Embed-812, Electron Microscopy Sciences Inc.). The sections were

counterstained with uranyl acetate and lead citrate and examined with an Hitachi H7100 transmission electron microscope.

5.6. Cytotoxicity

The cellular toxicity of the different compounds was evaluated using the MTT assays (CellTiter 96 aqueous one solution proliferation assay system, Promega). The 50% cytotoxic concentration (CC₅₀) was defined as the concentration of the compounds that reduced the cell viability by 50% compared to that for the untreated controls. The selectivity index (SI) was defined as the ratio CC₅₀:IC₅₀ (Table 3).

5.7. HIV-1 maturation inhibition assay

Viruses were produced by transfection of HIV-1 molecular clones (pNL-4-3) in 293T cells maintained in the presence or absence of compounds **1**, **2**, **12**, **13**, **15**, **16** and **17** in DMSO using the JetPei transfection reagent (QBiogen). Two days after the transfection, virus-containing supernatants were concentrated by ultracentrifugation through a layer of 20% sucrose at 25,000 rpm for 2 h 30 min. Viral pellets were then suspended in RIPA lysis buffer [10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.25% sodium deoxycholate, 0.2% phenylmethylsulfonyl fluoride (PMSF)]. Viral proteins were loaded on a 12.5% SDS–PAGE gel. The proteins transferred to the PVDF membrane (Millipore) were revealed using a goat anti-HIV CAp24 serum (AbD Serotec). Secondary antibody conjugated to horseradish peroxidase was revealed by enhanced chemiluminescent detection (Pierce Biotechnology, Inc).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejmech.2013.01.013>.

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