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Solution- and solid-phase synthesis and anti-HIV activity of maslinic acid derivatives containing amino acids and peptides

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ABSTRACT

Maslinic acid (**1**) has been coupled at C-28 with several α- and ω-amino acids by using solution- and solid-phase synthetic procedures. Twelve derivatives (**2**–**13**) with a single amino acid residue were prepared in solution phase, whereas a dipeptide (**14**), a tripeptide (**15**), and a series of conjugate dipeptides (**16**–**24**) were synthesized in solid phase. The anti-HIV activity of these compounds was assessed on MT-2 cells infected with viral clones carrying the luciferase gene as a reporter. While in maslinic acid (**1**) were present both cytotoxic and antiviral activities, only the derivatives **13** and **24** showed anti-HIV-1 activity and therefore represent a novel class of anti-HIV-1 compounds.

1. Introduction

Natural products have played an important role in drug development and chemical biology.[1] They also represent promising scaffolds for diversification by using combinatorial procedures to compile different libraries of compounds with worthwhile potential biological activities.[2] Moreover, solid-phase combinatorial chemistry plays a significant part in the generation of molecular diversity to accelerate the pharmaceutical lead generation and lead optimization process.[3]

Triterpenoids are a large family of natural products biosynthetically derived from squalene and widely found in nature. Pentacyclic triterpenes and saponins exhibit a wide range of biological activities, some of which may be used in medicine. Maslinic acid (**1**, MA) (Scheme 1) is an oleanene triterpene acid, firstly isolated from the whole plant of *Geum japonicum*,[4] and other natural sources,[5] that inhibit the human immunodeficiency virus (HIV-1) protease. Other structural analogues of MA (**1**) exhibit interesting pharmacological profiles as antioxidant,[6] anti-cancer,[7] and anti-HIV agents.

During recent decades the number of natural products exhibiting anti-HIV activity has increased considerably. These natural anti-HIV compounds can be classified into different groups according to their activity on the replicating cycle of HIV.[8] The HIV cycle can be divided into ten different steps: virus-cell adsorption, virus-cell fusion, uncoating, reverse transcription, integration, DNA replication, transcription, translation, budding and maturation. Many anti-HIV compounds isolated from natural resources are clinical candidates as drugs for treating HIV infection and some of them are currently in different phases of preclinical or clinical studies.

In this sense, a group of natural pentacyclic triterpene acid compounds such as betulinic, platonic, oleanolic, pomolic, glycyrrhetinic, ursolic, moronic, and other structurally related triterpenoids were identified as potent and selective inhibitors of human immunodeficiency virus type 1 replication.[9] Mechanistic studies revealed that this class of compounds interferes with HIV entry in the cells at a post-binding step. In these compounds the C-3 hydroxyl and C-17 carboxylic acid groups appear to be responsible for the anti-HIV activity. Thus, a series of ω-undecanoic amides and ω-amino alkanoic acid derivatives of betulinic acid were synthesized on C-28 carboxylic acid.[10] Some of these derivatives presented more potent anti-HIV activity.

On the basis of these findings, the modification of several lead triterpene compounds led to the discovery of more potent anti-HIV agents. The next functional modification was the introduction of an acyl group at the C-3 hydroxyl group of betulinic acid, betulin and ursolic acid.[11] On these derivatives, several structure–activity relationship studies were carried out and, among them, 3-*O*-dimethylsuccinyl and 3-*O*-dimethylglutaryl derivatives demonstrated extremely potent anti-HIV activities. Several studies of action mechanisms have suggested that there are two classes of triterpene

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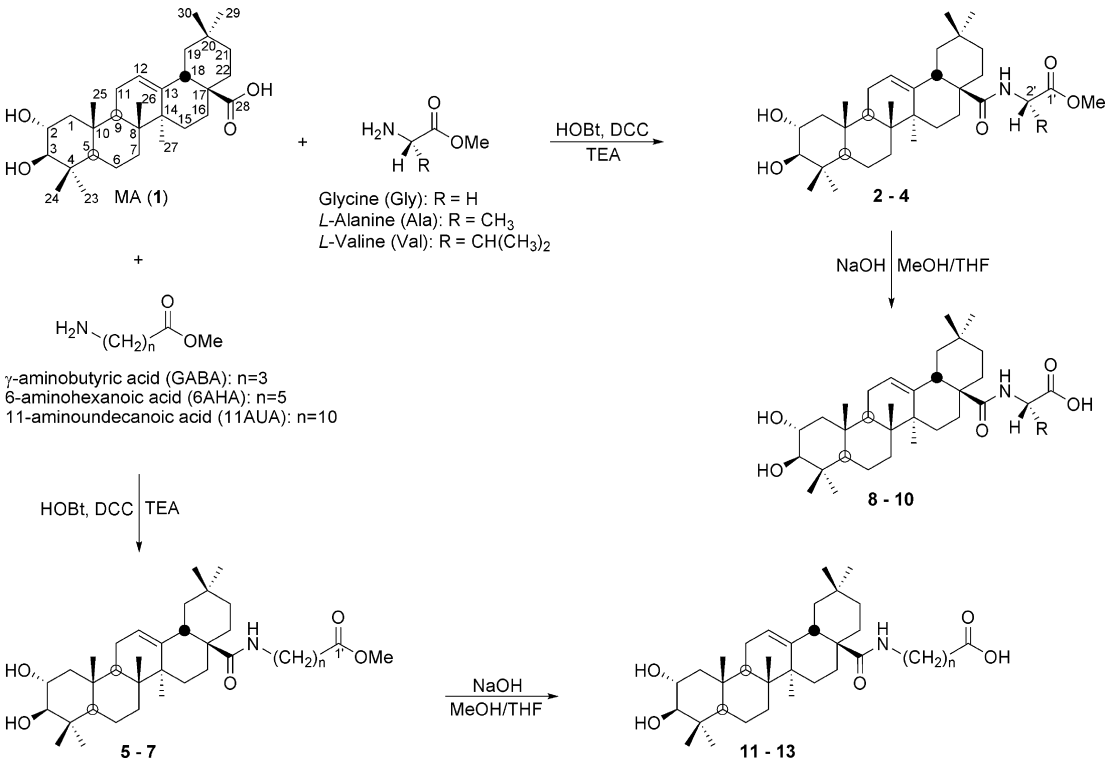


Scheme 1. Coupling of MA (**1**) with several α- and ω-amino acids in solution-phase.

anti-HIV inhibitors.[12] Derivatives with a side chain at C-3 inhibit HIV maturation, and derivatives with a side chain at C-28 can block HIV-1 entry. Therefore, a design that incorporates both C-3 and C-28 pharmacophores can lead to bi-functional triterpene derivatives[13] with enhanced anti-HIV activities. In this sense, new several studies of structure–activity relationships (SAR) were carried out. SAR studies indicated an important influence of the C-3 and C-28 substituents and the scaffold type on the anti-HIV activity of these triterpene derivatives. On the other hand, these studies demonstrated that these products have distinct mechanisms of action from clinically available anti-HIV therapeutics, actuating on two clinical steps in the HIV replication cycle, entry and maturation.

A method to obtain large quantities of MA (**1**) from the solid wastes of olive-oil pressing has been reported by our group,[14] and also we have described several reactions on the methyl maslinate modifying the A- and C-rings or hydroxylating some methyl groups of the molecule.[15] Moreover, we have recently reported the solid-phase synthesis of analogs of the cyclodepsipeptide Kahalalide F with oleanolic and maslinic acids using *p*-nitrobenzoyl (*p*NZ) as a permanent protecting group.[16]

In the present paper, we carry out the solution-phase semi-syntheses of 12 derivatives (**2**–**13**), with a single amino acid residue, from six different α- and ω-amino acids with MA (**1**) through its carboxylic group (C-28). Moreover, using solid-phase techniques, we have also prepared a dipeptide (**14**), a tripeptide (**15**), and a series of dipeptide conjugate derivatives (**16**–**24**). Among them, the non-cytototoxic compounds 13 and 24 are endowed with potent anti-HIV-1 activity and therefore show a suitable profile for drug development.

2. Results and discussion

2.1. Chemistry

The one-residue derivatives (**2**–**13**) were prepared by a general solution-phase procedure condensing MA (**1**) and the corresponding amino acid (α-amino acids: glycine (Gly), L-alanine (Ala) and L-valine (Val); or ω-amino acids: γ-aminobutyric acid (GABA), 6-aminohexanoic acid (6AHA) and 11-aminoundecanoic acid (11AUA)) with the carboxylic group protected as methyl ester (Scheme 1). The condensations were carried out at rt in anhyd dichloromethane (DCM) in presence of *N*-hydroxybenzotriazole (HOBt), *N*,*N*′-dicyclohexylcarbodiimide (DCC) and triethylamine (TEA). Thus, the maslinic acid derivatives (**2**–**7**) with the corresponding amino acid methyl ester fragments were obtained (Scheme 1). Saponification of compounds **2**–**7**, with NaOH/MeOH/THF at rt afforded the corresponding carboxylic acid derivatives (**8**–**13**). The yields of all these compounds (**2**–**13**) were satisfactorily high (80–95%) (Scheme 1).

Structures of compounds **2**–**13** can be easily deduced from their ^{1}H and ^{13}C NMR data. Thus, in their ^{1}H NMR spectra, the signal of the proton of the new amide group appears between δ 7.01 and 7.76 as a doublet or double doublet, depending on the substituent on the adjacent carbon atom. Moreover, in their ^{13}C NMR spectra, the quaternary carbon at C-28 is noticeably more deshielded in the amino acid conjugated compounds (δ 175.0–177.0) than in MA (**1**) (δ 180.6), because of the new amide group formed at this position.

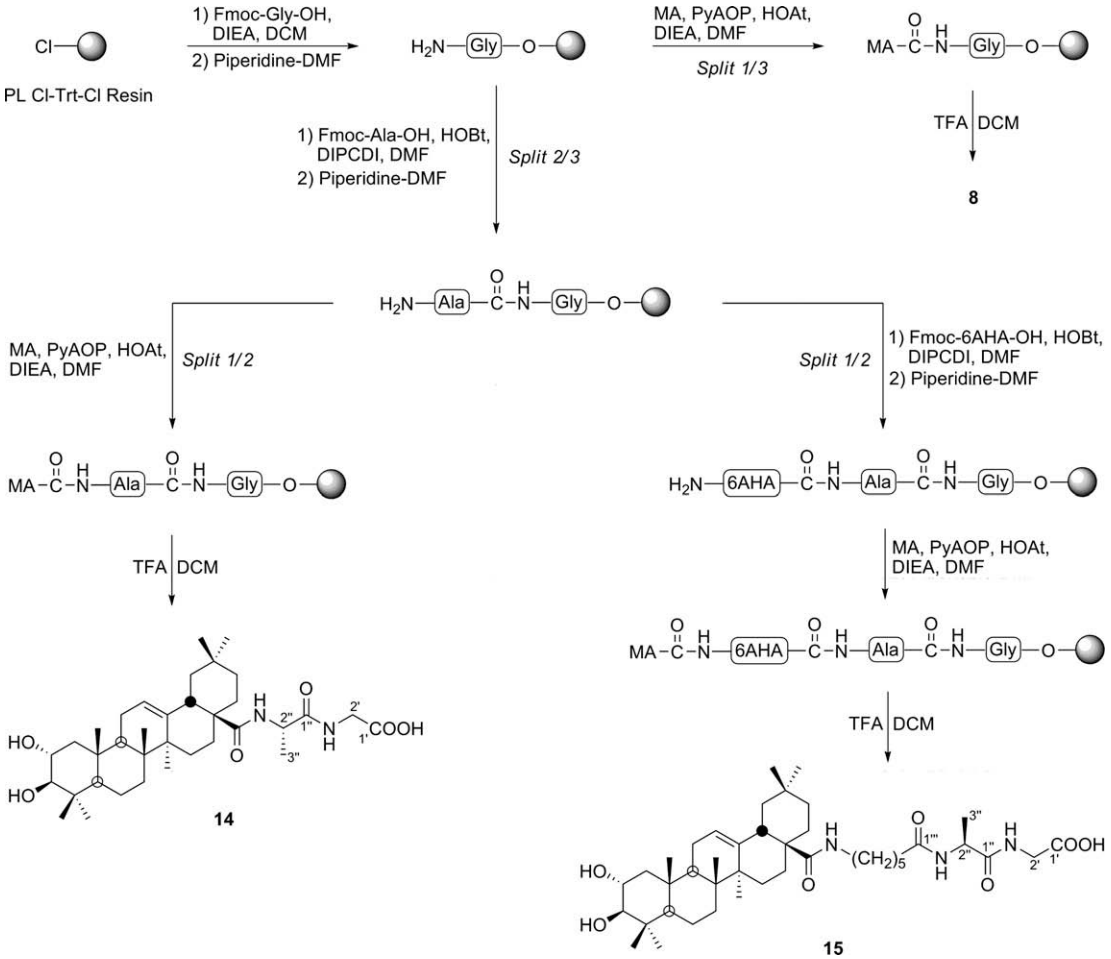
However, the solution-phase synthesis is not the most effective strategy when a large number of compounds must be prepared. Furthermore, although MA (**1**) can be manipulated in solution, its high hydrophobicity makes it very suitable for being used in solid-phase. Therefore, we have used a more efficient solid-phase synthetic procedure. Our strategy with this technique was initially focused on the coupling of one, two, or three amino acid fragments with the carboxyl group at C-28 of MA (**1**). This initial approximation enabled us to optimize different parameters and condition reactions, and also to obtain three amino acid conjugate compounds (**8**, **14** and **15**) in which the first amino acid of the chain was always glycine (Scheme 2).

Thus, after an adequate solvation of the resin with DCM, Fmoc-Gly-OH was first coupled onto the 2-chlorotrityl chloride polymer resin (PL Cl-Trt-Cl resin) (loading 1.27 mmol/g). This resin, which

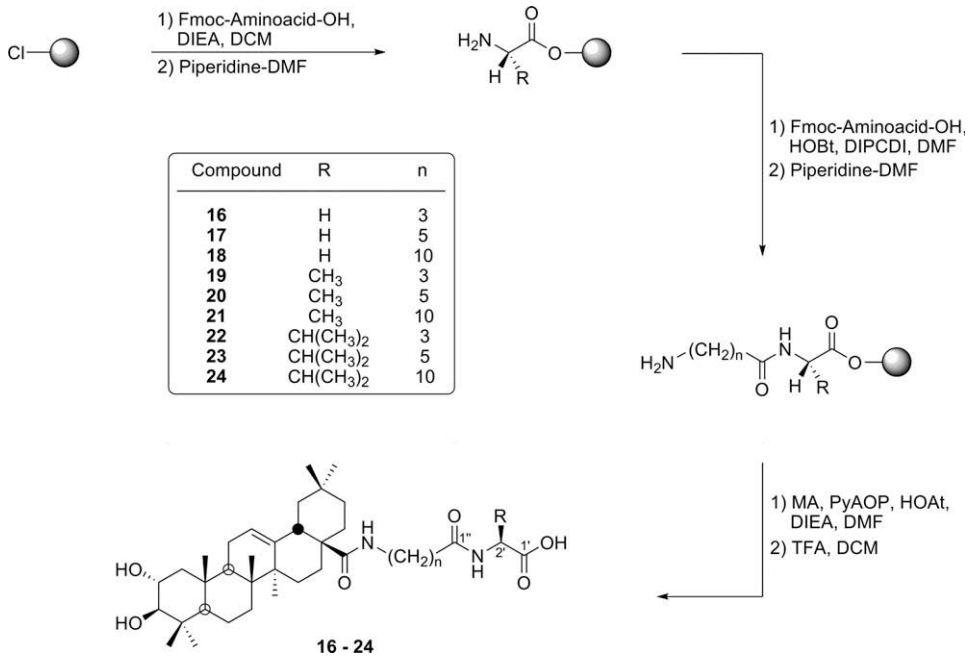
allows the release of the compounds by treatment with low concentrations of acid, is the only one suitable for the preparation of maslinic acid derivatives, because this triterpenic acid is not totally stable at high TFA concentrations. The Fmoc group was removed with piperidine/DMF (1:4) and the coupled glycine-resin residue was split into 1/3- and 2/3-portions (Scheme 2). MA (**1**) was anchored onto the first portion (1/3), with 7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP), 1-hydroxy-7-azabenzotriazole (HOAt), and *N*,*N*-diisopropylethylamine (DIEA), following the optimized general procedure, and then the coupled MA-glycine was cleaved from the resin with TFA/DCM (1:99) (3 × 30 s). This residue was analyzed by HPLC (>95% of purity), chromatographed in a silica-gel column, and then compound **8** was characterized by its physical and spectroscopic properties.

For attachment of the second amino acid, the larger aliquot (2/3) was treated with Fmoc-L-Ala-OH, HOBt and *N*,*N*′-diisopropylcarbodiimide (DIPCDI), according to the general procedure. The Fmoc-group was removed, and the residue divided into two identical portions. One of these portions was treated with MA (**1**) under the above-described conditions and the crude residue of dipeptidyl-MA (MA-Ala-Gly) was separated from the resin with TFA/DCM to obtain compound **14** (>90% of purity). The other portion (1/2) of H-Ala-Gly-resin was treated with Fmoc-6AHA-OH, deprotected with piperidine/DMF, coupled with MA (**1**), and cleaved with TFA/DCM from the resin according with the aforementioned conditions, obtaining compound **15** (>85% of purity). Compound **14** had a molecular formula of $C_{35}H_{56}N_2O_6$ and its ^{1}H NMR spectrum presented the signals of the proton of two amide groups at δ 7.25 (alanine) and δ 8.05 (glycine), along with the H-2″ of alanine (δ 4.20), the methylene of glycine (δ 3.78), the methyl group of alanine (δ 1.22), and also the signals of the triterpene skeleton of MA (**1**). Finally, its ^{13}C NMR spectrum showed, besides the signals of maslinic acid skeleton, two carbonyl groups at 172.5 and 170.8 ppm, C-2″ of alanine (δ 47.8), C-2′ of glycine (δ 40.5), and C-3″ of alanine (δ 18.41). Compound **15** presented a molecular formula of $C_{41}H_{67}N_3O_7$, and three amide groups were detected in its ^{1}H NMR spectrum (δ 8.11, 7.95, and 7.21), and in its ^{13}C NMR spectrum (δ 175.9, 172.5 and 171.7), together with a signal of the corresponding carboxyl group (δ 171.0). The completion of all coupling was confirmed with a negative ninhydrin test result (Scheme 2).

Subsequently, our approach to generate a series of nine dipeptide/maslinic acid derivatives via solid-phase synthesis is shown in Scheme 3. The synthetic procedure began with the coupling of three α-amino acids (Gly, L-Ala or L-Val) with PL Cl-Trt-Cl resin according to the above-described methodology. The amine group of the different amino acids was deprotected with piperidine/DMF (1:4), and each portion of resin, coupled with the three different α-amino acids, split into three identical aliquots. The nine fractions thus obtained were now treated (in groups of three) with the three ω-amino acids (GABA, 6AHA or 11AUA) using as coupling agents HOBt/DIPCDI. Finally, after the Fmoc group was removed, MA (**1**) was anchored on the different dipeptidyl-resins. The cleavage with TFA/DCM from the resin provided a series of nine dipeptidyl-MA derivatives (**16**–**24**) (>75% of purity) which had several long-chain ω-amino acids attached to the C-28 carboxylic group



Scheme 2. Coupling of MA (**1**) with several α- and ω-amino acids in solid-phase.



Compound	R	n
16	H	3
17	H	5
18	H	10
19	CH_3	3
20	CH_3	5
21	CH_3	10
22	$CH(CH_3)_2$	3
23	$CH(CH_3)_2$	5
24	$CH(CH_3)_2$	10

Scheme 3. Semi-synthesis of a series of conjugate dipeptides (**16**–**24**) in solid-phase.

of MA (**1**) and after them, various short α-amino acids. In the ^1H NMR spectra of all these compounds (**16**–**24**), were present the signals of MA (**1**), such as the geminal hydrogen atom of the hydroxyl groups at C-2 and C-3, along with signals of the protons of two amide groups of the amino acids attached. In the respective ^{13}C NMR spectra the signals of the three carbonyl groups were significant, as were the signals of the different carbons atoms of the amino acid fragments present in the molecule.

2.2. Biological evaluation

To study the anti-HIV activity of these maslinic acid derivatives containing amino acids and peptides, we infected MT-2 cells with the pNL4-3 HIV-1 clone pseudotyped with the VSV envelope, which bypasses the natural mode of HIV-1 entry into these cells supporting robust HIV-1 replication.[17] Upon integration into host chromosomes, this recombinant virus expresses the firefly luciferase gene and consequently luciferase activity in infected cells correlates with the rate of viral replication. Thus, high luciferase activity levels were detected 24 h after cellular infection with the VSV-pseudotyped HIV-1 clone, and pretreatment of MT-2 cells 30 min prior infection with MA (**1**) and derivatives resulted in a dose-dependent inhibition of the luciferase activity, with compounds **1**, **5**, **13**, and **24** being the most effective inhibitors of HIV-1 replication (Table 1).

Since MA (**1**) have been shown to exert pro-apoptotic activities[7c,18] we analyzed the effects of MA (**1**) and the most potent anti-HIV-1 derivatives in the different phases of the cell cycle in HIV-1-infected MT-2 cells. After treatment for 24 h, the different phases of the cell cycle were analyzed using propidium iodide staining and flow cytometry. Control and infected MT-2 cells were full cycling and progressed through the S, G2 and M phases of the cell cycle (35.1 and 38.9% of the cells, respectively) without significant hypodiploidy (loss of fragmented DNA) as a marker for apoptosis (3.5% and 2.1%). It bears noting that compounds **13** and **24**, which are potent HIV-1 inhibitors, did not affect any phase of the cell cycle in MT-2 infected cells. By contrast, MA (**1**), at the concentration tested, clearly increased the percentage of apoptotic cells (13.5% and 51.8%) (Table 2). Compound **5** also induced apoptosis in MT-2 cells (data not shown).

3. Conclusions

In this report, a convenient solid-phase method for the preparation of maslinic acid derivatives has been described. This method is fast and overcomes the problems of solubility and stability associated with MA (**1**), and should represent the base for the construction

Table 1
Effects of MA (**1**) and the derivatives **2**–**24** on recombinant virus replication

Compd	Dose 10 μM	Dose 25 μM
% Infection (untreated cells = 100% infection)		
1	56.3	6.7
2	75.5	56.3
3	63.6	8.9
4	72.0	11.1
5	32.5	12.0
6	69.6	36.8
7	66.9	35.6
8	162.1	130.7
9	124.2	60.9
10	102.8	121.2
11	126.4	93.8
12	146.4	79.3
13	56.7	21.5
14	134.5	184.8
15	170.2	89.6
16	112.3	143.3
17	125.7	122.1
18	85.6	49.8
19	83.7	50.1
20	107.0	70.2
21	71.7	27.6
22	66.2	130.2
23	83.8	96.5
24	44.9	21.2

MT-2 cells (106/mL) were pretreated with the indicated compounds for 30 min and then infected with the VSV-pseudotyped-HIV recombinant virus for 24 h. Luciferase activity in cell extracts was determined and results represented as % of activation ± S.D. compared to untreated infected cells (100% activation).

Table 2
Effects of MA (**1**) and compounds **13** and **24** on cellular apoptosis

	Control	RV Infected	**1** + RV		**13** + RV		**24** + RV	
			10 μM	25 μM	10 μM	25 μM	10 μM	25 μM
Apoptosis	3.5	2.1	13.5	51.8	3.5	5.2	2.1	4.6
G0/G1	61.6	59.0	47.9	23.1	62.6	53.7	54.6	59.2
S	18.4	18.1	20.6	17.7	18.8	22.2	21.4	20.5
G2/M	16.7	20.8	18.4	7.5	15.1	18.9	21.6	15.5

MT2 cells (106/mL) were pretreated with the indicated compounds for 30 min and then infected with the VSV-pseudotyped-HIV recombinant virus (RV) for 24 h, harvested and the percentage of cycling and hypodiploid cells was determined by flow cytometry analysis.

of large libraries of these compounds with the objective of developing anti-HIV, anti-cancer, and anti-inflammatory therapeutic agents. Here we report that some of the semi-synthesized triterpene derivatives are not cytotoxic compared to MA (**1**) and showed potent anti-HIV activity.

4. Experimental

4.1. General experimental procedures

Measurements of NMR spectra (300.13 MHz ^{1}H and 75.47 MHz ^{13}C) were made in $CDCl_3$ (which also provided the lock signal) with 300 MHz spectrometers. The assignments of ^{13}C chemical shifts were made with the aid of distortionless enhancement by polarization transfer (DEPT) using a flip angle of 135°. High-resolution mass spectra were made by LSIMS ionization mode. Silica gel (40–60 μm) was used for flash chromatography. CH_2Cl_2 or hexane, containing increasing amounts of Me_2CO, MeOH or AcOEt, were used as eluents. Analytical plates (silica gel) were rendered visible by spraying with H_2SO_4–HOAc, followed by heating to 120 °C.

PL Cl-Trt-Cl resin (1.27 mmol/g), protected ester and Fmoc-amino acid derivatives, HOAt, HOBt, PyAOP, were purchased from different sources as well as DIEA, DIPCDI, piperidine, DMF, DCM, methanol, TFA, and CH_3CN. RP-HPLC analyses were carried out with a C_{18} reverse-phase column (0.4 μm diameter of particle; 150 mm × 3.9 mm) with a flow rate of 1 mL/min. A wavelength of 220 nm was selected for the purity analysis. The analysis was performed using a linear gradient of 0–100% of B in 10 min, where A is H_2O containing 1% TFA and B is CH_3CN containing 1% TFA.

4.1.1. Isolation of MA (1)

MA (2α,3β-dihydroxy-12-oleanen-28-oic acid)[19] (**1**) was isolated from solid wastes resulting from olive-oil production, which were extracted in a Soxhlet with hexane and EtOAc successively. Hexane extracts were a mixture of oleanolic acid and MA (80:20), whereas this relationship was (20:80) for the EtOAc extracts. Both products were purified from these mixtures by column chromatography over silica gel, eluting with a $CHCl_3$/MeOH or CH_2Cl_2/acetone mixtures of increasing polarity.

4.2. Solution-phase general procedures

4.2.1. Coupling of amino acids with MA (1)

A solution of MA (**1**) (200 mg, 0.42 mmol) in DCM (20 mL), were successively treated with the corresponding Me or Et ester hydrochloride amino acid (0.50 mmol), HOBt (0.42 mmol), DCC (0.42 mmol), and TEA (0.1 mL). The resulting mixture was stirred at rt for 8 h, diluted with water (20 mL), and the organic layer was washed twice with water, dried over anhyd Na_2SO_4, filtered, and evaporated in vacuum. The residue was purified by chromatography column on silica gel, eluting with mixtures of DCM/acetone. Working as indicated above, compounds **2**–**7** were obtained as syrup, respectively.

4.2.2. Saponification of the methyl or ethyl ester amino acid derivatives

An aqueous solution of NaOH (4 N, 1 mL) was added to a solution of the methyl (or ethyl) ester derivative (**2**–**7**) (0.25 mmol) in MeOH/THF (1:1.5) (5 mL). After stirring at rt for 24 h and concentrating the solution in vacuum, the residue was suspended in distilled water (15 mL). The suspension was treated with aqueous 5 N HCl (0.75 mL). After 20 min, the suspension was filtered, washed with distilled water to pH 7, and dried over anhyd Na_2SO_4, filtered and evaporated in vacuum. The residue was purified by chromatography column on silica gel, eluting with mixtures of DCM/acetone. Following the work-up above described, products **8**–**13** were formed, respectively.

4.3. Solid-phase synthesis of 8, 14 and 15

4.3.1. *N*′-[*N*-(2α,3β-Dihydroxy-12-oleanen-28-oyl)-L-alanyl]-glycine (14)

PL Cl-Trt-Cl resin (300 mg, 1.27 mmol/g) was placed in a 10 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was washed with DCM (4 mL) for 20 min and drained. A solution of Fmoc-Gly-OH (225 mg, 2 equiv), DIEA (1.35 mL, 10 equiv) and DCM (1.5 mL) was added, and the mixture was stirred for 1.5 h. The reaction was terminated by addition of MeOH (0.75 mL), after stirring for 15 min. The Fmoc-Gly-O-Trt-Cl-resin was subjected to the above-described washings/deprotecting treatments. The H-Gly-O-Trt-Cl-resin was split into 1/3- and 2/3-portions.

MA (**1**) (180 mg, 3 equiv), PyAOP (600 mg, 3 equiv), HOAt (0.19 mL, 3 equiv), DIEA (0.60 mL, 9 equiv) in 0.7 mL of DMF was added to the 1/3 sample of H-Gly-O-Trt-Cl-resin. The mixture was stirred at rt for 24 h, and after this time, the ninhydrin test was negative. The MA-Gly-OH formed was separated from the resin as the above-described method, and the crude obtained after evaporation showed a purity of 90% of compound **8**, identical to that obtained by the solution-phase procedure.

A mixture of Fmoc-L-Ala-OH (312 mg, 4 equiv), HOBt (620 mg, 4 equiv), DIPCDI (0.64 mL, 4 equiv) in 1.4 mL of DMF was added to the 2/3-portion of H-Gly-O-Trt-Cl-resin. The mixture was stirred for 1.5 h, and after this time, the ninhydrin test was negative. The Fmoc-L-Ala-Gly-O-Trt-Cl-resin was subjected to the above-described washings/deprotecting treatments. The H-L-Ala-Gly-O-Trt-Cl-resin was split in two identical portions.

MA (**1**) (180 mg, 3 equiv), PyAOP (600 mg, 3 equiv), HOAt (0.19 mL, 3 equiv), DIEA (0.60 mL, 9 equiv) in 0.7 mL of DMF were added to the first sample (1/2) of H-L-Ala-Gly-O-Trt-Cl-resin. The mixture was stirred at rt for 24 h, and after this time, the ninhydrin test was negative. The MA-L-Ala-Gly-OH formed was separated from the resin as the above-described method, and the crude obtained after evaporation showed a purity of 90% of compound **14**. Syrup; ^{1}H NMR (DMSO-d_6) δ 8.05 (dd, 1H, $J_1 = J_2 = 5.7$ Hz, NH Gly), 7.25 (d, 1H, $J = 6.9$ Hz, NH Ala), 5.23 (dd, 1H, $J_1 = J_2 = 3.2$ Hz, H-12), 4.20 (dq, 1H, $J_1 = J_2 = 7.1$ Hz,

H-2″), 3.78 (dd, 2H, J_1 = 5.5 Hz, J_2 = 17.3 Hz, 2H-2′), 3.41 (ddd, 1H, J_1 = 4.3 Hz, J_2 = 10.5 Hz, J_3 = 12.2 Hz, H-2), 2.77 (m, 2H, H-18 and H-3), 1.22 (d, 3H, J = 6.9 Hz, 3H-3″), 1.10, 0.93, 0.89, 0.89, 0.89, 0.71, 0.62 (s, 3H each, CH_3); ^{13}C NMR (DMSO-d_6) δ 175.7, 172.5, 170.8, 143.6, 121.6, 82.1, 67.0, 54.6, 47.8, 47.0, 46.8, 45.7, 45.0, 41.1, 40.7, 40.5, 38.8, 38.6, 37.5, 33.2, 32.8, 32.2, 32.2, 30.3, 28.7, 26.7, 25.5, 23.4, 22.9, 22.2, 18.4, 17.9, 17.0, 16.5, 16.2; HRMS m/z calcd for $C_{35}H_{56}N_2O_6Na$ 623.4036 $[M+Na]^+$, found 623.4034.

4.3.2. *N*″-[*N*′-[*N*-(2α,3β-Dihydroxy-12-oleanen-28-oyl)-6-aminohexanoyl]-L-alanyl]-glycine (15)

A mixture of Fmoc-6AHA-OH (180 mg, 4 equiv), HOBt (620 mg, 4 equiv), DIPCDI (0.64 mL, 4 equiv) in 1.4 mL of DMF was added to the second portion (1/2) of H-L-Ala-Gly-O-Trt-Cl-resin. The mixture was stirred for 1.5 h and after this time, the ninhydrin test was negative. The Fmoc-6AHA-L-Ala-Gly-O-Trt-Cl-resin was subjected to the above-described washings/deprotecting treatments.

MA (**1**) (180 mg, 3 equiv), PyAOP (600 mg, 3 equiv), HOAt (0.19 mL, 3 equiv), DIEA (0.60 mL, 9 equiv) in 0.7 mL of DMF were added to H-6AHA-L-Ala-Gly-O-Trt-Cl-resin. The mixture was stirred at rt for 24 h, and after this time, the ninhydrin test was negative. The MA-6AHA-L-Ala-Gly-OH formed was separated from the resin as the above-described method, and the crude obtained after evaporation showed a purity of 80% of compound **15**. Syrup; ^{1}H NMR (DMSO-d_6) δ 8.11 (dd, 1H, $J_1 = J_2$ = 5.8 Hz, NH Gly), 7.95 (d, 1H, J = 7.8 Hz, NH Ala), 7.21 (dd, 1H, $J_1 = J_2$ = 5.1 Hz, NH 6AHA), 5.23 (dd, 1H, $J_1 = J_2$ = 3.2 Hz, H-12), 4.32 (dq, 1H, $J_1 = J_2$ = 7.8 Hz, H-2″), 3.75 (m, 2H, 2H-2′), 3.41 (ddd, 1H, J_1 = 4.3 Hz, J_2 = 10.5 Hz, J_3 = 12.2 Hz, H-2), 2.99 (m, 2H, 2H-6‴), 2.77 (dd, 1H, J_1 = 3.4 Hz, J_2 = 12.5 Hz, H-18), 2.73 (d, 1H, J = 10.5 Hz, H-3), 2.11 (t, 2H, J = 7.4 Hz, 2H-2‴), 1.20 (d, 3H, J = 7.2 Hz, 3H-3″), 1.10, 0.93, 0.91, 0.89, 0.88, 0.71, 0.67 (s, 3H each, CH_3); ^{13}C NMR (DMSO-d_6) δ 175.9, 172.5, 171.7, 171.0, 144.1, 121.2, 82.1, 67.0, 54.7, 47.6, 47.0, 46.7, 45.9, 45.0, 41.2, 40.5, 40.3, 38.6, 38.8, 38.6, 37.5, 35.0, 33.5, 32.8, 32.7, 32.3, 30.3, 28.8, 28.7, 26.7, 26.2, 25.6, 24.8, 23.5, 22.9, 22.1, 18.2, 17.9, 17.0, 16.7, 16.2; HRMS m/z calcd for $C_{41}H_{67}N_3O_7Na$ 736.4876 $[M+Na]^+$, found 736.4872.

4.4. Solid-phase synthesis of conjugates of MA (1) with α-amino and ω-amino acids (16–24)

Three samples of PL Cl-Trt-Cl resin (300 mg each) were placed in three 10 mL polypropylene syringes, and the resin was washed with DCM for 20 min and drained. A solution of Fmoc-xxx-OH (α-amino acid: xxx = Gly, Ala or Val) (2 equiv), DIEA (10 equiv) and DCM (1.5 mL) was added, respectively, to each syringe, and the mixtures were stirred for 1.5 h. The reaction was finished by addition of MeOH (0.50 mL), after stirring for 15 min. The Fmoc-xxx-O-Trt-Cl-resin was subjected to the above-described washings/deprotecting treatments.

The content of each syringe was distributed into other three syringes and a mixture of 4 equiv of the Fmoc-xxx-OH (ω-amino acid: xxx = GABA, 6AHA or 11AUA), 4 equiv of HOBt, 4 equiv of DIPCDI and 0.7 mL of DMF was added to each syringe containing the PL Cl-Trt-Cl resin with the corresponding N-deprotected α-amino acid. The mixture was stirred at rt for 1.5 h. Each α- and ω-amino acid dipeptidyl-resin was subjected to the above-described washings/deprotecting treatments.

A mixture of 3 equiv of MA (**1**), 3 equiv of PyAOP, 3 equiv of HOAt, 9 equiv of DIEA and 0.7 mL of DMF was added to each syringe containing the N-deprotected α- and ω-amino acid dipeptidyl-resin. The mixture was stirred at rt for 24 h. The resin was successively washed with DMF, piperidine–DMF, DMSO and DCM, treated with DCM/TFA (1% in TFA in DCM) and filtrated. The solvent was evaporated and the residues were analyzed by RP-HPLC, LC–MS, and NMR. In all cases after 90 min of coupling, the ninhydrin test was negative. The dates of purity (RP-HPLC), ^{1}H and ^{13}C NMR and HRMS of compounds **16–24** are given as complementary material.

4.5. Biological methods

4.5.1. Cell lines and reagents

MT-2 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium (Lonza, Belgium) containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (50 units/mL), and streptomycin (50 μg/mL), maintained at 37 °C in a 5% CO_2 humidified atm. 293 T cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (Lonza) supplemented with fetal bovine serum and antibiotics at 37 °C in a 5% CO_2 humidified atm.

4.5.2. Production of VSV-pseudo-typed recombinant viruses and infection assays

High-titer VSV-pseudo-typed recombinant virus stocks were produced in 293T cells as previously described.[20] Briefly, the cells were co-transfected with the pNL4-3.Luc.R$^-$ E$^-$ plasmid along with the pcDNA$_3$-VSV plasmid by the calcium phosphate transfection method. Supernatants, containing virus stocks, were harvested 48 h post-transfection and were centrifuged 5 min at 500 g to remove cell debris, and stored at −80 °C until use. Cell-free viral stock was tested using an enzyme-linked immunoassay for antigen HIV-1 p24 and cultures were infected with 200 ng of HIV-1 gag p24 protein as follow; MT2 cells (10^6/mL in 24-well plates) were pre-treated with the compounds for 30 min and then inoculated with the recombinant virus stocks. 20 h later the cells were washed twice in PBS and lysed in 25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol during 15 min at rt. Then the lysates were spun down and the supernatants were used to measure luciferase activity using an Autolumat LB 9510 (Berthold, Bad Wildbad, Germany) following the instructions of the luciferase assay kit (Promega, Madison, WI, USA). The results are represented as the percentage of activation (considering the infected and untreated cells 100% activation).

4.5.3. Cytofluorimetric analyses of cell cycle

For DNA profile analyses, MT2 cells were washed in PBS, fixed in EtOH (70%, for 24 h at 4 °C), followed by RNA digestion (RNAase-A, 50 U/mL) and propidium iodide (PI, 20 μg/mL) staining, and analyzed by cytofluorimetry. Ten thousand gated events were collected per sample and the percentage of cells in every phase of the cell cycle was determined. The frequency of cells having undergone chromatinolysis was calculated by determining the sub G_0/G_1 fraction.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.12.041.

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