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Original article

Effect of conjugates of *all-trans*-retinoic acid and shorter polyene chain analogues with amino acids on prostate cancer cell growth

Eldem Sadikoglou [a,1], George Magoulas [b,1], Christina Theodoropoulou [a], Constantinos M. Athanassopoulos [b], Efstathia Giannopoulou [a,2], Olga Theodorakopoulou [a], Denis Drainas [c], Dionysios Papaioannou [b,*], Evangelia Papadimitriou [a,**]

[a] *Laboratory of Molecular Pharmacology, Department of Pharmacy, School of Health Sciences, University of Patras, University Campus, GR-26504 Patras, Greece*
[b] *Department of Chemistry, School of Natural Sciences, University of Patras, University Campus, GR-26504 Patras, Greece*
[c] *Department of Biochemistry, School of Medicine, University of Patras, University Campus, GR-26504 Patras, Greece*

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ABSTRACT

In the present work, a series of conjugates of amino acids with *all-trans*-retinoic acid (ATRA) and shorter polyene chain analogues were rationally designed, synthesized by coupling the succinimidyl active esters of the acidic retinoids with appropriately protected amino acids or peptides followed by deprotection, and examined for their possible effect on viability of human prostate cancer LNCaP cells. In contrast to ATRA, all conjugates bearing amino acids with polar side chains showed no inhibitory effect on LNCaP cell proliferation, while conjugates with α-amino acids with lipophilic side chain, such as **7**, or linear amino acids, such as **9**, significantly decreased prostate cancer LNCaP cell number. Interestingly, while the effect of ATRA was RARα-dependent, the effect of its active analogues was not inhibited by a selective RARα antagonist. Cell cycle analysis showed no effect on cell cycle, while quantitative analysis by annexin V-propidium iodide staining revealed that neither ATRA nor its analogues affected LNCaP cell apoptosis or necrosis. These results demonstrate that compounds **7** and **9** are potentially useful agents that warrant further preclinical development for treatment of prostate cancer.

1. Introduction

Retinoids are lipophilic molecules composed of three distinct structural domains: a β-ionone ring, an isoprenoid tail and a polar end group. These small molecules exhibit multiple and diverse biological activities through their nuclear receptors, Retinoic Acid Receptors (RARs) and Retinoid X Receptors (RXRs) [1]. RARs and RXRs are encoded from different genes, have many isoforms and control the expression of a large number of genes [2]. Moreover, retinoids inhibit the expression of several genes through inhibition of transcription factor activating protein-1, which seems to be responsible for the anti-tumor effects of retinoids [3]. The induction of differentiation [1,4,5], apoptosis [5–7] and cell cycle arrest [5,7] by retinoids have made them important pharmaceutical candidates against dermal dysfunctions and certain cancer types, such as chronic leukaemia, where the use of *all-trans*-retinoic acid (ATRA) leads to 90% complete remission rate [8]. Due to problems, however, such as development of resistance [9] or toxicity [10], analogues of ATRA are widely being tested as alternatives.

Shealy et al. have reported the synthesis of a series of *N*-(*all-trans*-retinoyl)amino acids, as analogues of ATRA (**1**) with extended polyene chain and increased water solubility, and tested their capacity to reverse keratinisation in vitamin A deficient hamster trachea in culture. The amino acids used were Gly, Ala, Leu, Phe, Tyr, and Glu. All of the thus examined conjugates were less active than ATRA [11]. In that bioassay, most active amino acid was Leu, incorporating a lipophilic side chain, and least active Glu, with a polar side chain. Even between Phe and Tyr most active was Phe,

Abbreviations: Asp, aspartic acid; ATRA, *all-trans*-retinoic acid; DCC, *N,N′*-dicyclohexylcarbodiimide; DCU, *N,N′*-dicyclohexylurea; HOBt, *N*-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; βAla, β-alanine; εAca, ε-aminocaproic acid; FCC, flash column chromatography; DEAD, diethyl azodicarboxylate; Lys, lysine; RT, room temperature; Boc, *tert*-butoxycarbonyl; DIEA, ethyldiisopropylamine; Tfa, trifluoroacetyl; DMAP, 4-dimethylaminopyridine; DCM, dichloromethane; TFA, trifluoroacetic acid; Trt, triphenylmethyl (trityl); RAR, Retinoic Acid Receptor; RXR, Retinoid X Receptor.

* Corresponding author. Tel./fax: +30 2610 997156.
** Corresponding author. Tel./fax: +30 2610 969336.
E-mail addresses: dapapaio@chemistry.upatras.gr (D. Papaioannou), epapad@upatras.gr (E. Papadimitriou).

[1] These authors have equally contributed to this work.

[2] Present address: Clinical Oncology Laboratory, Division of Oncology, Department of Medicine, University Hospital of Patras, Patras Medical School, 26504 Rio, Greece.

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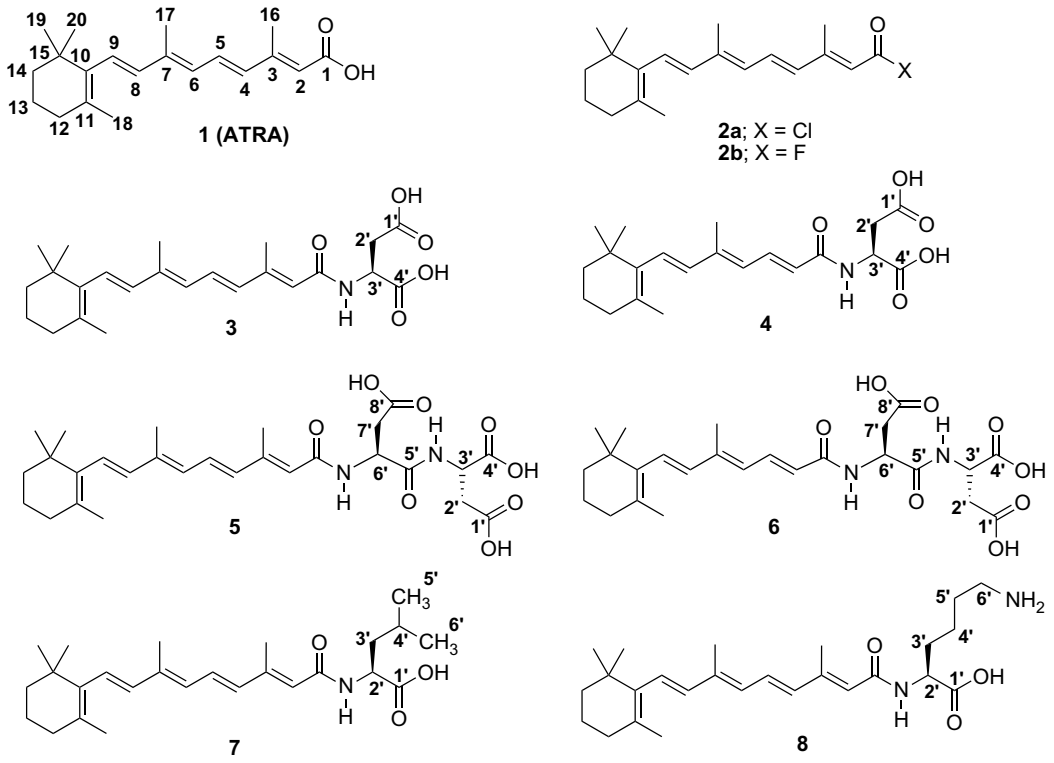


Fig. 1. Conjugates of ATRA and of its lower homologue **20** with acidic, neutral and basic α-amino acids and the dipeptide Asp–Asp.

lacking the polar hydroxyl group from position 4 of the aromatic ring of Tyr. *N*-(*all-trans*-retinoyl)-leucine and *N*-(*all-trans*-retinoyl)-glycine also inhibited the proliferation of two kinds of neoplastic cells in culture. The afore mentioned conjugates were all obtained through coupling of the *in situ* generated *all-trans*-retinoyl chloride (**2a**, Fig. 1) with methyl or ethyl esters of α-amino acids, followed by saponification [11]. In an alternative methodology, conjugates of ATRA with amino acids, such as Gly, Phe and Lys were obtained, however in low yields, by direct coupling of the isolable *all-trans*-retinoyl fluoride (**2b**) with free amino acids in aqueous solutions [12].

Taking into consideration that ATRA affects in various ways the differentiation, apoptosis and inhibition of cell proliferation in physiological and cancerous epithelial cells [5,13–15] and that retinoids have been widely tested for their effects in prostate cancer cells [16–18], we thought of interest to synthesize a series of conjugates of amino acids with ATRA, suitable for structure–activity relationship studies on the proliferation or/and apoptosis of human prostate cancer LNCaP cells. We were interested to identify alternative, more effective, ways of synthesis, as well as the possible role of (a) the nature of α-amino acid side chain, (b) the length of the chain in linear amino acids (lack of side chain), (c) the

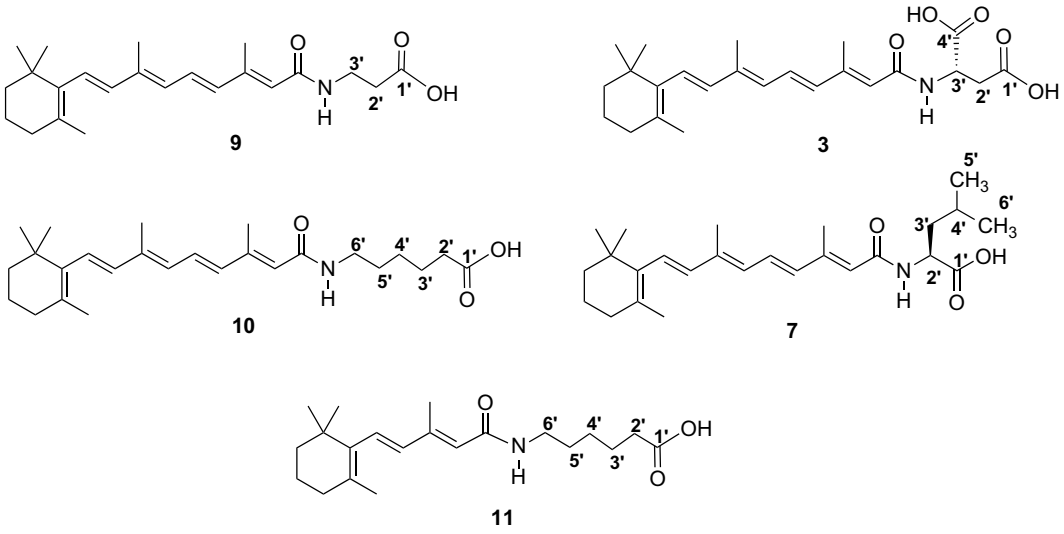
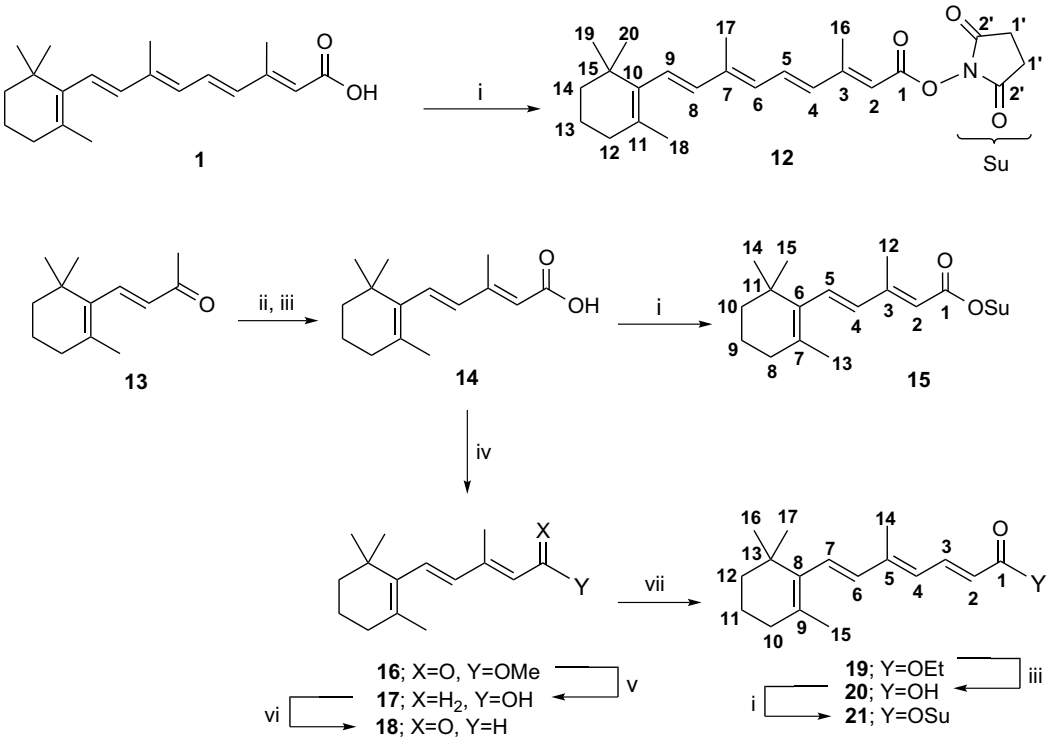


Fig. 2. Conjugates of ATRA and its lower homologue **14** with linear amino acids and their structural relationship to conjugates **3** and **7**.



Scheme 1. Synthesis of the succinimidyl 'active' esters **12**, **15** and **21**. Reagents and conditions: (i) HOSu, DCC, THF, overnight, 90% (for **12** and **15**) and 88% (for **21**); (ii) $(EtO)_2P(=O)CH_2CO_2Et$, MeONa/MeOH, PhH, 40 °C, 15 h, 89%; (iii) 8 M NaOH, MeOH, reflux, 2 h, 60% (for **14**) and 83% (for **20**); (iv) MeOH/PPh_3/DEAD, THF, 0 °C, 30 min, 90%; (v) $LiAlH_4$, THF, 0 °C to RT, 1.5 h then FCC 78%; (vi) MnO_2, Hex, 0 °C, 1 h, 88%; (vii) $Ph_3P{=}CHCO_2Et$, MeCN, reflux, 12 h, 85%.

introduction of additional acidic sites and (d) the length of the polyene chain on their activity.

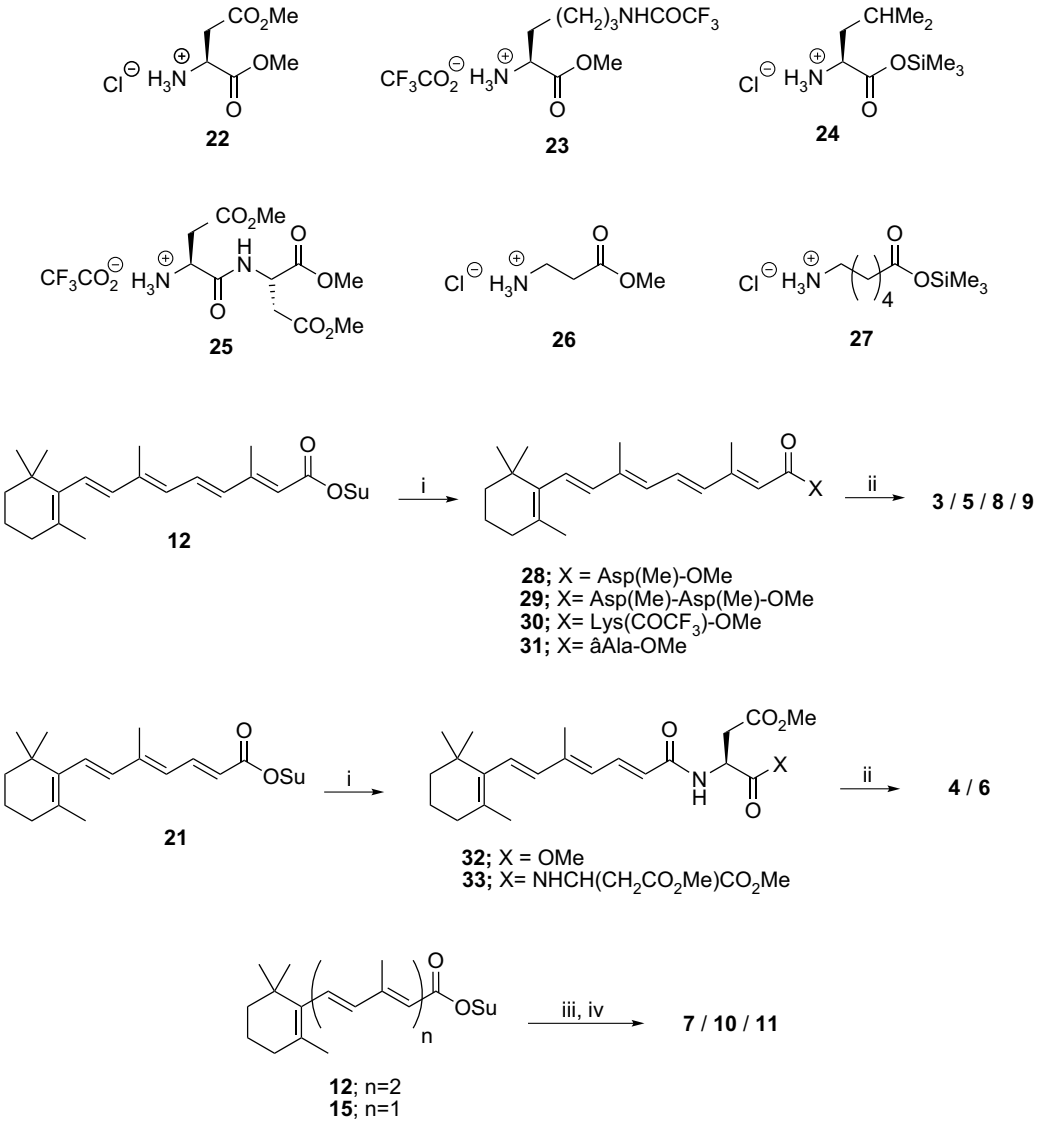
2. Results and discussion

2.1. Chemistry

The conjugates (**3**–**11**) of amino acids with ATRA and analogues with shorter polyene chain, which were synthesized in the context of the present work, are depicted in Figs. 1 and 2. With conjugates **3**, **7** and **8** (Fig. 1) incorporating amino acids with acidic, neutral and basic side chains, respectively, we anticipated to identify the role of the nature of side chain on the activity of these compounds. On the other hand, with conjugate **5** we wanted to find out the effect of additional acidic groups in the polar end of the retinoids whereas with conjugates **4** and **6** (with three double bonds) and conjugate **11** (with two double bonds, Fig. 2) we anticipated to identify the role of the length of polyene chain on the activity. Finally, conjugates **9** and **10** (Fig. 2) incorporate amino acids lacking a side chain and having different numbers of methylene groups between the amido and the carboxyl groups. In general, the conjugates **3**–**11** were readily synthesized by coupling appropriately protected α-, β- and ε-amino acids with the isolable unsaturated succinimidyl esters of the commercially available ATRA and its lower synthetic homologues **14** and **20** (Scheme 1). These esters, namely **12**, **15** and **21**, have been earlier reported to acylate selectively the primary amino functions of polyamines in the presence of unprotected secondary ones [19].

Acid **14** was readily obtained from the commercially available β-ionone (**13**) through a Wittig–Horner condensation with phosphonate ester $(EtO)_2P(=O)CH_2CO_2Et$, followed by saponification and recrystallization [20]. On the other hand, acid **20** was obtained from acid **14** as follows. Mitsunobu-type esterification with MeOH, followed by $LiAlH_4$-mediated reduction [20] produced the unsaturated (*E*,*E*)-alcohol **17** in 70% overall yield, following separation from the also formed (*E*,*Z*)-isomer by FCC. Oxidation of the (*E*,*E*)-isomer **17** with MnO_2 produced the unsaturated aldehyde **18** [21] as a mixture of geometrical isomers (only the *E*,*E*-isomer is drawn) in 88% yield, which upon Wittig reaction with the stabilized phosphorane $Ph_3P{=}CHCO_2Et$ gave ester **19** (only the *E*,*E*,*E*-isomer is drawn) in 85% as a mixture of the (*E*,*E*,*E*)- and the (*E*,*Z*,*E*)-isomers. Finally saponification of ester **19** produced the unsaturated acid **20** also as a mixture of isomers. The desirable (*E*,*E*,*E*)-acid **20** [22] (Scheme 1) was obtained pure in 83% yield by recrystallization of the mixture of isomers from EtOAc and by FCC purification of the residue from the mother liquor of recrystallization.

The required succinimidyl 'active' esters, namely **12**, **15** and **21** (Scheme 1), were readily obtained in 88–90% yields from the corresponding acids **1**, **14** and **20**, respectively, on treatment with DCC and HOSu [19]. Coupling of these esters with amino acid derivatives was realized by using two protocols (see Scheme 2). All α-amino acids and derivatives used in the present work were of the L-configuration. The first, indirect, protocol involved the coupling with suitably protected amino acids and then the desired free acidic conjugates were obtained by routine deprotection. Accordingly, the amino acids Asp and βAla were introduced into the retinoid skeleton in the form of the corresponding, commercially available, hydrochlorides of dimethyl [(H-Asp(Me)-OMe), **22**] and methyl (H-βAla-OMe, **26**) esters, respectively. Also, the commercially available compound H-Asp(Me)-OH was converted to the corresponding benzotriazolyl ester of the *N*-trityl-protected derivative [23,24] and then first coupled to H-Asp(Me)-OMe and then detritylated by TFA to give the dipeptide derivative H-Asp(Me)-Asp(Me)-OMe (**25**), as the corresponding trifluoroacetate salt (see Experimental section). This compound was suitable for the introduction of the dipeptide Asp–Asp into the retinoid skeleton. Finally, the Lys residue was



Scheme 2. General synthetic scheme for the preparation of the retinoid–amino acid conjugates **3**–**11**. Reagents and conditions: (i) **22**/**23**/**25**/**26**, DIEA, $CHCl_3$, RT, 2–12 h, 75–90%; (ii) aq. NaOH, MeOH, RT, 1–4 h, 54–64%; (iii) **24**/**27**, Et_3N, 0 °C, 15 min then RT, 1–2 d; (iv) MeOH, 65–80%.

introduced in the retinoid skeleton by using its commercially available derivative Boc-Lys($COCF_3$)-OH. This was first esterified with MeOH, using the Mitsunobu reaction, and then N^{α}-deprotected by treating with TFA to give the trifluoroacetate salt of H-Lys($COCF_3$)-OMe (**23**) (see Experimental section). Coupling of succinimidyl esters **12** and **21** with the above described methyl esters was effected in $CHCl_3$ in the presence of DIEA to give the anticipated amides **28**–**31** and **32**–**33**, respectively, in 75–90% yields. Finally, complete deprotection of these compounds by saponification gave the projected retinoid–amino acid conjugates **3**, **5**, **8** and **9** and **4** and **6**, respectively, in 54–64% yields.

The alternative direct protocol involved the temporary protection of the carboxyl function of the commercially available amino acids Leu and εAca as the corresponding trimethylsilyl esters **24** and **27** [23] (see Experimental section), followed by one-pot coupling with the succinimidyl ester **12** or **15** and carboxyl group deprotection simply by methanolysis. This, one-pot, methodology provided the conjugates **7**, **10** and **11** in 65–80% yields.

2.2. Pharmacology

2.2.1. Effect of ATRA and its conjugates on the number of LNCaP cells

We first investigated the effect of different concentrations of ATRA and its conjugates on LNCaP cell number, 48 h after the addition of the tested agents into the cell culture medium. As shown in Figs. 3 and 4, among all tested agents, only ATRA, and conjugates **7** and **9** decreased the number of LNCaP cells in a statistically significant, concentration-dependent manner, with the maximum effect being observed at the concentration of 10^{-5} M. Conjugate **10** also caused a notable but not statistically significant reduction of the number of LNCaP cells. Higher concentrations could not be used because of solubility problems. The IC_{50} values for ATRA and conjugates **7** and **9**, extrapolated from graphical data, are shown in Table 1. These results show that acidic or basic side chains of the amino acid components of the conjugates, lead to a decrease of the activity of the parent compound ATRA, as this is observed with conjugates **3**–**6** and **8**. This is observed even in the conjugates where the length of the polyene chain was decreased by

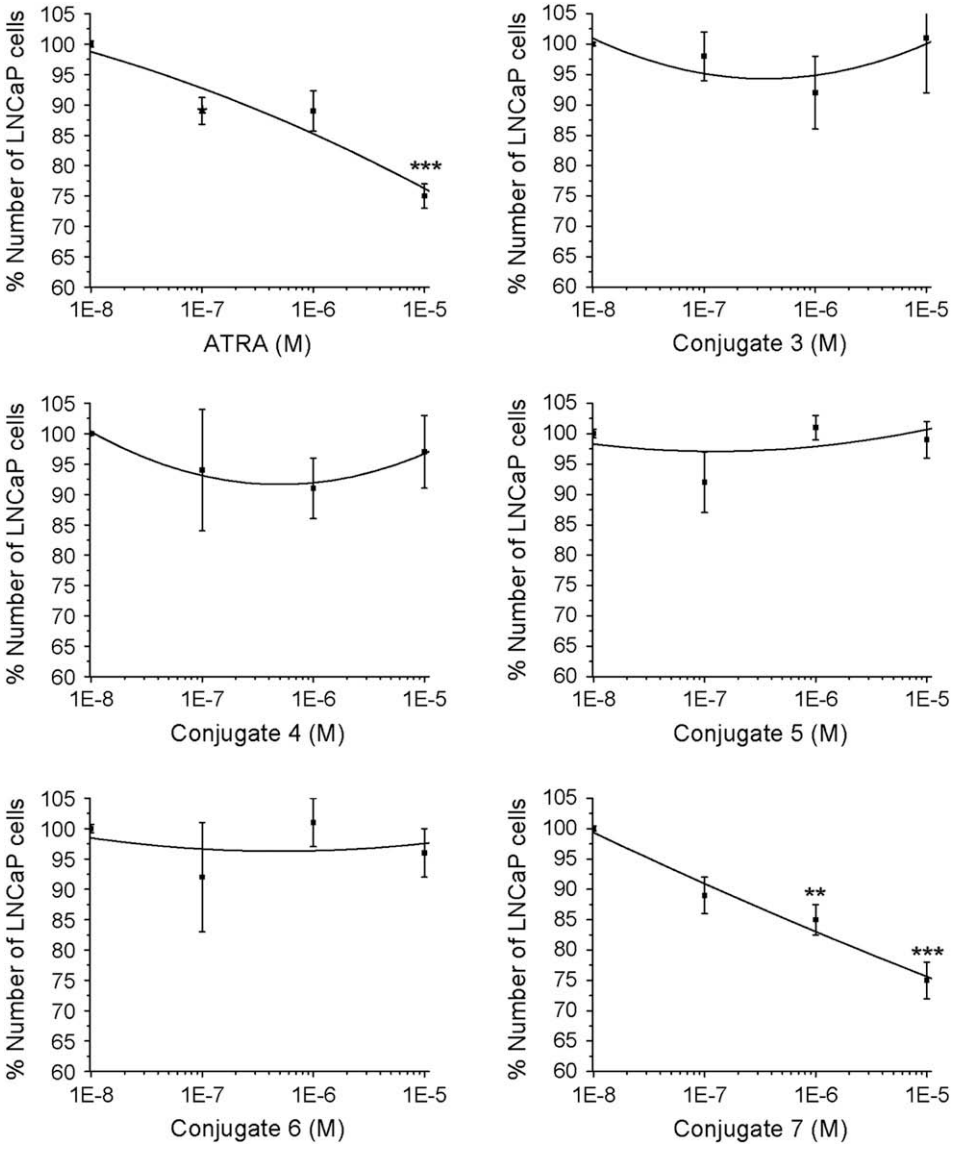


Fig. 3. Effect of ATRA and its conjugates **3**–**7** on the number of LNCaP cells 48 h after addition of the tested agents into the cell culture medium. Results are expressed as mean ± S.E.M. of the percentage number of cells in treated compared with untreated cells (set as default = 100). Asterisks denote statistical significance from untreated cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

one double bond, such as conjugate **3** compared to **4** and conjugate **5** compared to **6**. From the conjugates bearing amino acids with lipophilic chains, branched (conjugate **7**) or linear (conjugates **9**–**11**), the most active (*ca.* twice as active as ATRA) one is conjugate **7**, bearing an isobutyl side chain, followed by conjugate **9** (of *ca.* one fifth the activity of ATRA) in which there is a two methylene units distance between the amido and the carboxyl functions. Conjugate **10**, in which there is a longer distance (five methylene units) between the amido and the carboxyl functions, is much less active than conjugate **9**. Finally, decreasing the length of the polyene chain by two double bonds seems to have a significant diminishing effect, as this is apparent by the behavior of the couples of the conjugates **10** and **11**.

2.2.2. Effect of ATRA and its conjugates on LNCaP cell morphology

ATRA and its analogues **3**–**8** and **11** had no effect on the morphology of LNCaP cells (data not shown). However, LNCaP cells treated with the conjugates **9** and **10** were more elongated compared with untreated cells (Fig. 5). This change in morphology is RARα-independent and does not seem to be related to the effect of the conjugates on the number of cells; however, it may be related to other functions not addressed in the present work.

*2.2.3. Effect of ATRA and its conjugates **7** and **9** on the number of LNCaP cells in the presence of a RARa selective antagonist*

RARα has been suggested as possibly responsible for the inhibitory effect of ATRA on the growth of cells [25]. To examine whether the decrease in the number of LNCaP cells was mediated by RARα, we used the RARα selective antagonist Ro415253 and estimated its effect on the retinoid-induced decrease in the number of LNCaP cells. As shown in Fig. 6, the ATRA-induced decrease in the number of cells was totally inhibited by Ro415253 at 10^{-6} M. Higher Ro415253 concentrations were toxic to the cells. In contrast, the decrease in the number of LNCaP cells caused by conjugates **7** and **9** was not inhibited by Ro415253, suggesting that it is not mediated by the RARα receptor. These results suggest that the mechanism(s) through which conjugates **7** and **9** cause a decrease in the number of LNCaP cells may not be the same with that of ATRA.

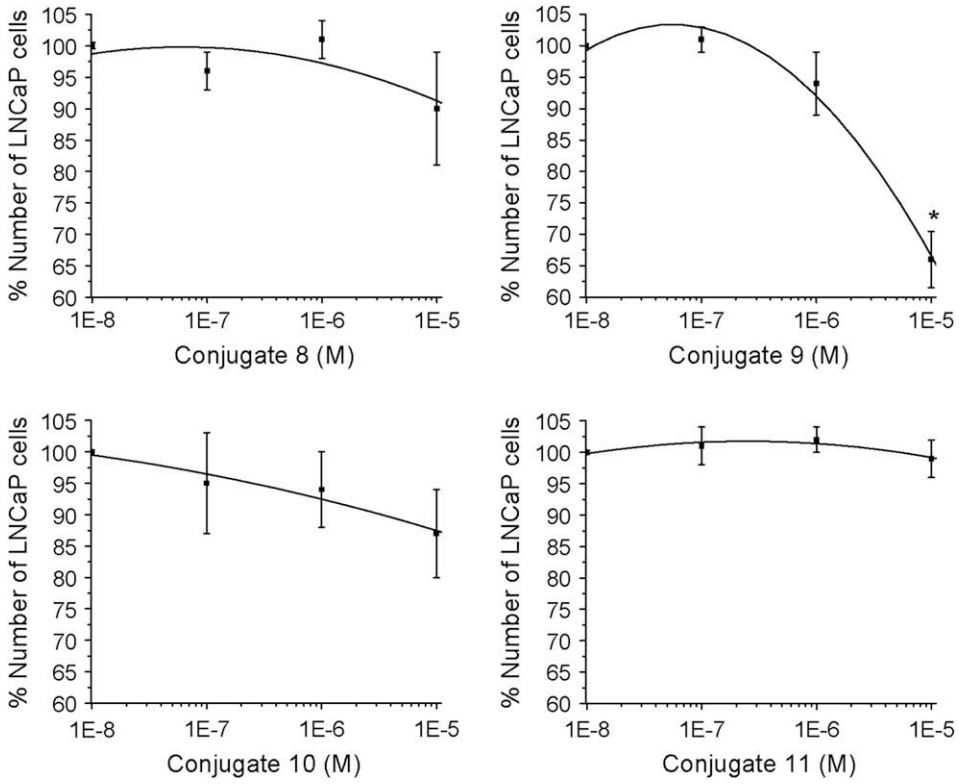


Fig. 4. Effect of ATRA conjugates **8**–**11** on the number of LNCaP cells 48 h after addition of the tested agents into the cell culture medium. Results are expressed as mean ± S.E.M. of the percentage number of cells in treated compared with untreated cells (set as default = 100). Asterisks denote statistical significance from untreated cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

*2.2.4. Effect of ATRA and its conjugates **7** and **9** on cell cycle arrest in LNCaP cells*

Since ATRA and its conjugates **7** and **9** decreased the number of LNCaP cells, we investigated whether they had any effect on the cell cycle. As shown in Table 2, none of the tested agents affected in a statistically significant manner cell cycle phase distribution of LNCaP cells.

*2.2.5. Effect of ATRA and its conjugates **7** and **9** on apoptosis of LNCaP cells*

ATRA is well known to induce apoptosis of several types of cells [5–7]. In the present study, we investigated whether the decrease in the number of LNCaP cells caused by ATRA and its conjugates **7** and **9** was, at least partly, due to induction of apoptosis. As shown in Fig. 7, neither ATRA nor its conjugates **7** and **9** had any effect on apoptosis or necrosis of LNCaP cells, as estimated by the relative amount of the annexin V and propidium iodide-stained cells in the population.

3. Conclusion

Based on the data described in the present study, the following general conclusions can be drawn: conjugates of ATRA with amino acids with acidic or basic side chains abolish the activity, while conjugates with lipophilic chain increase the effectiveness of ATRA in decreasing the number of human prostate cancer LNCaP cells. The effect of the active conjugates is not mediated by RARα, in contrast to the effect of ATRA and does not seem to be through induction of apoptosis or change in the distribution of the cells in different cell cycle phases. Further studies are in progress in order to elucidate further activities of the conjugates, as well as their possible mechanism(s) of action.

Table 1
IC_{50} values of ATRA and its most active conjugates.[a]

Retinoid	IC_{50} (M)
ATRA	5.0×10^{-7}
Conjugate **7**	2.7×10^{-7}
Conjugate **9**	2.7×10^{-6}

[a] The values were calculated from interpolations of the graphical data presented in Figs. 3 and 4 using the Microcal Origin Program. SD values for the IC_{50} values were not calculated because the latter were estimated from graphical interpolations.

4. Experimental section

4.1. General

Melting points were determined with a Buchi SMP-20 apparatus and are uncorrected. IR spectra were recorded as KBr pellets, unless otherwise stated, on a Perkin Elmer 16PC FT-IR spectrophotometer. ^{1}H NMR spectra were obtained at 400.13 MHz and ^{13}C NMR at 100.62 MHz on a Bruker Avance 400DPX spectrometer. Electron-spray ionization (ESI) mass spectra were recorded on a Micromass-Platform LC spectrometer. Mass spectra for retinoid–amino acid conjugates were recorded on a LC-UV-ESI-MS (Waters Micromass 2Q) equipped with a C-18 column (4.6 × 150 mm, 5 μm) and using MeCN (containing 0.08% TFA)/H_2O (containing 0.08% TFA) gradient from 5% to 100% MeCN in 30 min. The high-resolution MS (HR-MS) experiments were carried out in hybrid Q-Star Pulsar-i (MDS Sciex Applied Biosystems, Toronto, Canada). ATRA conjugates **7**, **8** and **10** showed the most abudant quasi-molecular ions incorporating one oxygen atom. Microanalyses were performed on a Carlo Erba EA 1108 CHNS elemental analyzer in the Laboratory of Instrumental Analysis of the University of Patras. Flash column chromatography (FCC) was performed on Merck silica gel 60 (230–400 mesh) and

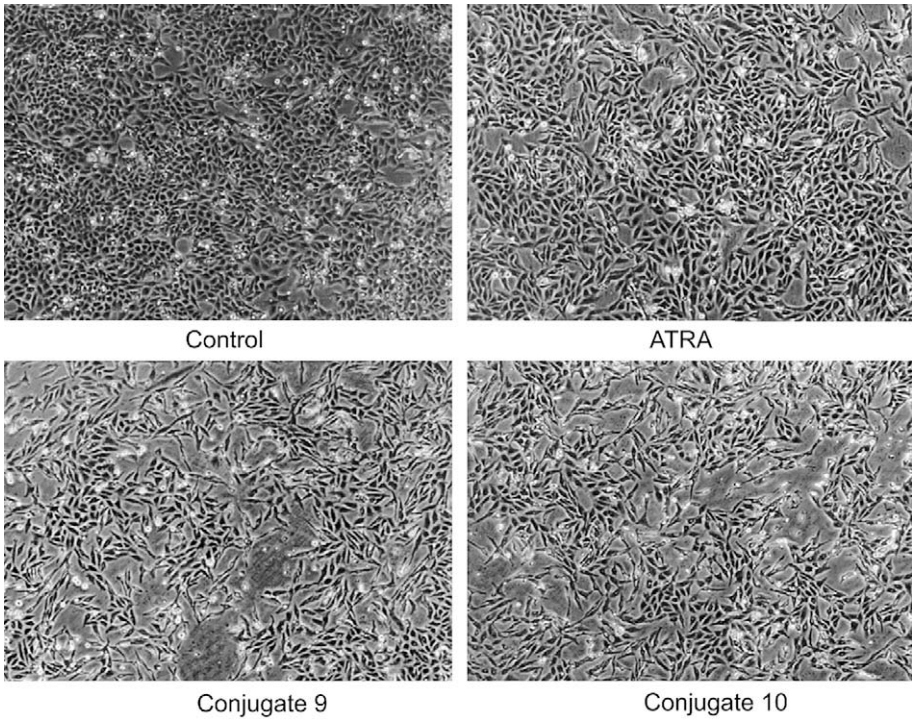


Fig. 5. Phase-contrast micrographs of LNCaP cells treated with ATRA and its conjugates **9** and **10**.

TLC on Merck 60 F254 films (0.2 mm) pre-coated on aluminium foil. The solvent systems used were: (A): $MeCN/H_2O$ (5:1), (B): $CHCl_3$/MeOH/gl.AcOH (85:10:5), (C): $CHCl_3$/MeOH/gl.AcOH (90:10:0.25), (D): $CHCl_3$/MeOH/conc.NH_3 (9:1:0.1), (E): $CHCl_3$/MeOH/conc.NH_3 (95:5:0.5), (F) $CHCl_3$/MeOH (9:1), (G): $CHCl_3$/MeOH (95:5), (H): PhMe/EtOAc (1:1), (I): PhMe/EtOAc (7:3), (J): PhMe/EtOAc (8:2), (K): PhMe/EtOAc (9:1), (L): PhMe, (M): Hex/Et_2O (1:1).

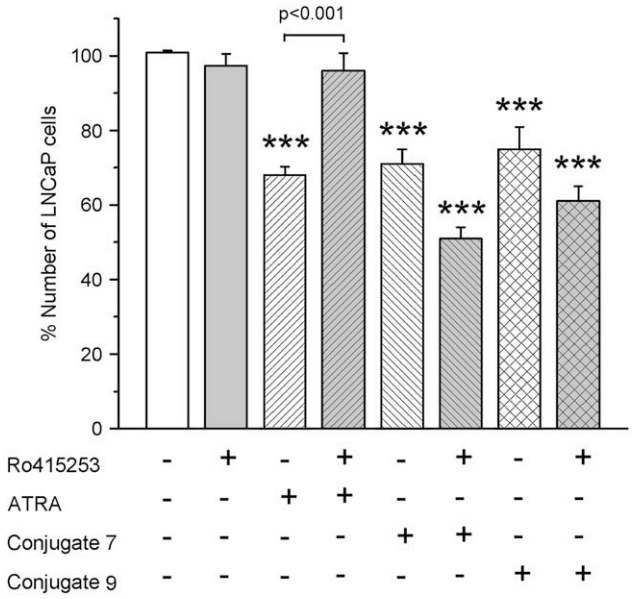


Fig. 6. Effects of RARα antagonist Ro415253 (10^{-6} M) on the decrease of the number of LNCaP cells 48 h after incubation with ATRA and its conjugates **7** and **9**. Results are expressed as mean ± S.E.M. of the percentage number of cells in treated compared with untreated cells (set as default = 100). Asterisks denote statistical significance from untreated cells. $^{***}p < 0.001$.

Spots were visualized with UV light at 254 nm or with the ninhydrin reagent (where applicable), followed by heating at 110 °C and/or the charring agent. All solvents (Merck) were dried and/or purified according to standard procedures. Solids were routinely dried under vacuo in a Chem-dry (laboratory Devices Inc. USA) apparatus. All reactions were performed under an atmosphere of Ar or under exclusion of moist air whereas those involving retinoids were also conducted in the dark. Yields of the reactions described below are not optimized.

With the exception of N^{α}-(*tert*-butoxycarbonyl)-$N^{(\omega)}$-trifluoroacetyl-L-lysine, which was purchased from BACHEM, β-ionone, *all-trans*-retinoic acid, methyl β-alaninate hydrochloride, dimethyl L-aspartate hydrochloride, β-methyl L-aspartate hydrochloride, ε-aminocaproic acid and L-leucine as well as all other necessary reagents were purchased from Sigma–Aldrich and used as such without further purification. Succinimidyl *all-trans*-retinoate was prepared according to literature procedure [19].

4.2. Synthesis of suitably protected amino acids and dipeptides

4.2.1. Methyl N^{ε}-trifluoroacetyl-L-lysinate (**23**)

To an ice-cold solution of Boc-Lys(Tfa)-Lys-OH (0.5 g, 1.46 mmol), MeOH (0.41 mL, 10.22 mmol) and a catalytic amount of DMAP in DCM (8 mL) was added DCC (0.66 g, 3.21 mmol) and the

Table 2
Flow cytometric analysis of cell cycle distribution of prostate cancer LNCaP cells treated with ATRA and its conjugates **7** and **9**. Results are expressed as mean ± S.E.M. of the percentage number of cells positive for propidium iodide.

48 h	% Cell population			
Phase	Control	ATRA	Conjugate 7	Conjugate 9
G_0/G_1	63.5 ± 2.3	66.7 ± 1.4	65.9 ± 2.6	64.0 ± 2.3
S	17.2 ± 3.6	17.6 ± 3.4	19.6 ± 3.2	14.8 ± 2.4
G_2/M	5.5 ± 2.4	4.4 ± 2.0	1.9 ± 1.0	2.2 ± 1.1

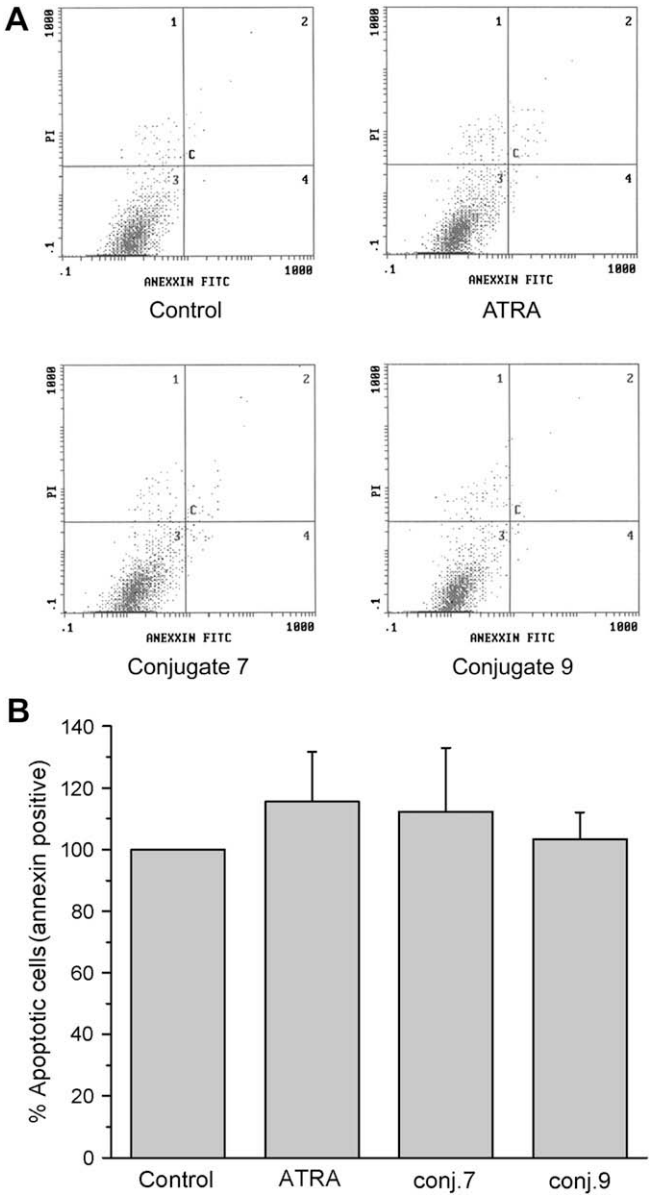


Fig. 7. Effect of ATRA and its conjugates **7** and **9** on apoptosis of LNCaP cells 48 h after addition of the tested agents into the cell culture medium. Representative pictures of FACS analysis of cells stained with annexin V and propidium iodide after treatment with the tested agents are shown in (A). Results in (B) are expressed as mean ± S.E.M. of the percentage number of annexin-positive cells in treated compared with untreated cells (set as default = 100).

resulting solution was kept at that temperature for 5 min and at ambient temperature for another 15 min. The precipitated DCU was filtered off and washed on the filter with DCM. The combined filtrates were evaporated to a minimum volume, triturated with Et_2O and refrigerated overnight. An additional amount of DCU was filtered off and the residue was subjected to FCC using as eluant the solvent system I, to give 0.49 g (94% yield) of Boc-Lys(Tfa)-OMe as a white solid. This compound had R_f (J) 0.21, m.p. 130–132 °C, MS (ESI^+): *m/z* 736.24 (2M + Na), 395.39 (M + K), 379.43 (M + Na), 357.13 (M + H), IR (KBr): cm^{-1} 3318, 1736, 1712. ^1H NMR ($CDCl_3$): δ 6.74 (1H, br s, N*H*$COCF_3$), 5.13 (1H, d, *J* 8.0 Hz, N*H*CO), 4.30 (1H, unresolved m, H-2′), 3.75 (3H, s, OCH_3), 3.37 (2H, m, H-6′), 1.71–1.58 (4H, m, H-3′ and H-5′), 1.44 (9H, s, C(CH_3)$_3$), 1.44–1.38 (2H, m, H-4′) ppm, ^{13}C NMR: δ 173.1, 157.3 (q, *J* 37 Hz, *C*OCF_3), 155.5, 115.8 (q, *J* 285 Hz, CF_3), 80.1, 52.9, 52.4, 39.6, 32.5, 28.2, 28.1, 22.4 ppm.

Boc-Lys(Tfa)-OMe (0.46 g, 1.29 mmol) was treated with a solution of TFA in DCM (3 mL, 1:1) for 45 min at 0 °C. Solvent was then evaporated to dryness. $CHCl_3$ (10 mL) was added and the resulting solution re-evaporated to dryness to leave an oily residue. Trituration with Et_2O and overnight refrigeration, followed by decantation of the solvent and drying under high vacuo, gave 0.46 g (95% yield) of CF_3CO_2H·H-Lys(Tfa)-OMe as a white solid. This compound, which had R_f (D) 0.32, MS (ESI^+): *m/z* 279.16 (M + Na), 257.18 (M + H), 227.13 (M + H-CH_2O), was used as such without further purification into the following coupling experiment.

4.2.2. *Trimethyl L-aspartyl-L-aspartate (**25**)*

This compound was obtained using the trityl methodology [23,24] as follows. To a suspension of HCl·H-Asp(Me)-OH (0.92 g, 5 mmol) in anhydrous $CHCl_3$ (8 mL) and MeCN (2 mL) was added Me_3SiCl (0.7 mL, 5.4 mmol) and the resulting mixture was refluxed for 2 h. It was then cooled to 0 °C and treated dropwise with dry Et_3N (2.4 mL, 17 mmol), followed by a solution of trityl chloride (1.42 g, 5.1 mmol) in anhydrous $CHCl_3$ (5 mL). After stirring at ambient temperature for 2 h, MeOH (3 mL) was added and the solvents were evaporated under reduced pressure to dryness. To the residue, an ice-cold 5% aqueous citric acid solution (50 mL) was added and the product was extracted twice into Et_2O. The organic phases were combined, washed twice with H_2O and once with brine, dried (Na_2SO_4) and evaporated to dryness to give 1.76 g of Trt-Asp(Me)-OH as a white foam. This compound, which was used without further purification into the next reaction, had R_f (B) 0.29, IR (KBr): cm^{-1} 3200–2400, 1712, 1691. MS (ESI^+): *m/z* 802.46 (2M + Na), 428.52 (M + K), 412.53 (M + Na), 390.53 (M + H).

To an ice-cold solution of Trt-Asp(Me)-OH (1.76 g, 4.52 mmol) and HOBt·H_2O (0.92 g, 6.78 mmol) was added DCC (0.95 g, 4.6 mmol). The reaction mixture was stirred at 0 °C for 30 min and at ambient temperature for 2 h and then a drop of gl. AcOH and two drops of H_2O were added and further stirred for 15 min. The precipitated DCU was filtered off and washed on the filter with EtOAc (4 × 15 mL). The filtrates were refrigerated overnight, filtered again, diluted with more EtOAc and washed once with an ice-cold 5% aqueous $NaHCO_3$ solution, twice with H_2O and once with brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness to afford 2.2 g of 'active' ester Trt-Asp(Me)-OBt as a white foam. This compound, which was also used without further purification into the next reaction, had R_f (I) 0.42, IR (KBr): cm^{-1} 3350, 1732, 1691. MS (ESI^+): *m/z* 1036.98 (2M + Na), 545.13 (M + K), 529.88 (M + Na), 507.36 (M + H).

To an ice-cold solution of HCl·H-Asp(Me)-OMe (0.79 g, 4 mmol) in anhydrous $CHCl_3$ (3 mL) was added dropwise DIEA (1.4 mL, 8 mmol), followed by Trt-Asp(Me)-OBt (1.6 g, 3.16 mmol). The reaction mixture was stirred at 0 °C for 30 min and at ambient temperature for 2 h and then diluted with $CHCl_3$. The organic phase was washed once with a 5% aqueous $NaHCO_3$ solution, twice with H_2O and once with brine, dried (Na_2SO_4) and evaporated to dryness to leave an oily residue. From this, pure dipeptide Trt-Asp(Me)-Asp(Me)-OMe was obtained as a white foam, after FCC purification using the solvent system I as eluant. Yield: 1.1 g (63%), R_f (I) 0.32, IR (KBr): cm^{-1} 3374, 1736, 1676, MS (ESI^+): *m/z* 1089.32 (2M + Na), 1065.25 (2M + H), 533.00 (M + H), 243.24 (Trt). ^1H NMR ($CDCl_3$): δ 8.96 (1H, d, *J* 9.2 Hz, N*H*CO), 7.55–7.53, 7.31–7.26, 7.24–7.16 (15H, three multiplets, Trt), 4.92 (1H, dt, *J* 4.8 and 8.8 Hz, H-6′), 3.84 (3H, s, OCH_3), 3.77 (3H, s, OCH_3), 3.50 (3H, s, OCH_3), 3.50–3.45 (1H, m, H-3′), 3.13 (1H, dd, *J* 4.4 and 17.6 Hz, H-7a′), 2.78 (1H, dd, *J* 4.4 and 17.2 Hz, H-2a′), 2.63 (1H, dd, *J* 3.2 and 17.2 Hz, H-2b′), 2.35 (1H, s, N*H*), 0.99 (1H, dd, *J* 5.6 and 17.2 Hz, H-7b′) ppm, ^{13}C NMR δ 173.4, 172.6, 171.6, 171.2, 145.9, 128.56, 128.22, 126.74, 71.5, 54.0, 52.9, 52.1, 51.4, 48.2, 36.0, 35.0 ppm.

The dipeptide Trt-Asp(Me)-Asp(Me)-OMe (0.8 g, 1.5 mmol) was dissolved in an ice-cold solution of 20% TFA in DCM (5 mL) and then left at ambient temperature for 1 h. Evaporation of the volatile components under reduced pressure, trituration with Et_2O and overnight refrigeration caused the precipitation of the product as a white oil. Et_2O was decanted and the oily residue was dried under high vacuo to give 0.58 g (96% yield) of CF_3CO_2H·H-Asp(Me)-Asp(Me)-OMe which had R_f (E) 0.32, MS (ESI^+): *m/z* 581.90 (2M + H), 291.31 (M + H). This compound was further used for couplings with acidic retinoids without further purification.

4.3. Synthesis of (2E,4E)-dienoic acid **14** and (2E,4E,6E)-trienoic acid **20**

4.3.1. (2E,4E)-3-Methyl-5-(2,6,6-trimethylcyclohex-1-enyl) penta-2,4-dienoic acid (**14**)

To a solution of methyl β-ionylidenacetate (12.4 g, 50 mmol), obtained as a mixture of geometric isomers (*E*/*Z*: 80:20 by GC MS) according to literature procedure [20], in MeOH (60 mL) was added a solution of NaOH (4 g, 100 mmol) in H_2O (15 mL) and the resulting mixture was refluxed for 2 h. The resulting dark red solution was concentrated under vacuo to half its volume and then was triturated at 0 °C, initially with glacial acetic acid and then with a 5% aqueous citric acid solution to pH 6. The resulting yellow precipitate was taken up in EtOAc and the organic layer was washed twice with H_2O and then a saturated aqueous solution of NaCl (brine). Drying (Na_2SO_4), evaporation to a small volume and overnight refrigeration produced a first crop of pure, crystalline, *E*,*E*-acid **14** which was washed on the filter with ice-cold EtOAc. The mother liquor and washings from crystallization were concentrated once again to a small volume and refrigerated to give a second crop of pure *E*,*E*-acid **14** as a white solid. That way, 7 g (60% yield) of pure compound **14** there were obtained. Acid **14** which had R_f (M) 0.34, m.p. 124–126 °C, MS (ESI^+): *m/z* 257.08 (M + Na), 235.68 (M + H), IR (KBr): cm^{-1} 3200–2400, 1688, 1598. ^1H NMR ($CDCl_3$): δ 11.62 (1H, br s, CO_2H), 6.63 (1H, d, *J* 16 Hz, H-5), 6.14 (1H, d, *J* 16 Hz, H-4), 5.77 (1H, s, H-2), 2.35 (3H, s, H-12), 2.03 (2H, t, *J* 6.4 Hz, H-8), 1.71 (3H, s, H-13), 1.65–1.59 (2H, m, H-9), 1.49–1.46 (2H, m, H-10), 1.03 (6H, s, H-14 and H-15) ppm, ^{13}C NMR: δ 172.5, 155.4, 137.0, 136.0, 134.7, 131.6, 117.2, 39.5, 34.2, 33.1, 28.9 (two C), 21.7, 19.1, 13.8 ppm.

4.3.2. Methyl (2E,4E)-3-methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dienoate (**16**)

To an ice-cold solution of acid **14** (4.7 g, 20 mmol), MeOH (1.6 mL, 40 mmol) and Ph_3P (5.9 g, 22 mmol), was added dropwise DEAD (3.5 mL, 22 mmol). Stirring was continued at that temperature for 30 min and the resulting solution evaporated to dryness under vacuo. Upon addition of Et_2O, overnight refrigeration and filtration, most of Ph_3PO was removed. The filtrate was evaporated to dryness and the residue was subjected to FCC using system L as eluant to give 4.5 g (90% yield) of pure ester **16** as a colourless oil. This compound had R_f (L) 0.45, IR (neat): cm^{-1} 1711, 1598, ^1H NMR ($CDCl_3$): δ 6.57 (1H, d, *J* 16 Hz, H-5), 6.14 (1H, d, *J* 16 Hz, H-4), 5.74 (1H, s, H-2), 3.71 (3H, s, OCH_3), 2.34 (3H, s, H-12), 2.02 (2H, br t, *J* 6 Hz, H-8), 1.70 (3H, d, *J* 0.8 Hz, H-13), 1.65–1.58 (2H, m, H-9), 1.49–1.45 (2H, m, H-10), 1.02 (6H, s, H-14 and H-15) ppm, ^{13}C NMR: δ 167.6, 153.1, 137.1, 136.1, 134.1, 131.1, 117.5, 50.9, 39.5, 34.2, 33.0, 28.8 (two C), 21.6, 19.1, 13.6 ppm.

4.3.3. (2E,4E)-3-Methyl-5-(2,6,6-trimethylcyclohex-1-enyl) penta-2,4-dienol (**17**)

To an ice-cold suspension of $LiAlH_4$ (0.61 g, 15.94 mmol) in THF (15 mL), ester **16** (4.4 g, 17.71 mmol) in 22 mL THF was added dropwise over a period of 30 min. The resulting mixture left at that temperature for 30 min and for an additional 1 h at ambient temperature. The excess of $LiAlH_4$ was destroyed by careful addition of a saturated aq. solution of Na_2SO_4 and the resulting white precipitate was filtered off. The filtrate was evaporated to dryness. The oily residue was dissolved in EtOAc and washed sequentially twice with H_2O and brine. 3.04 g (78% yield) of the desired alcohol **17** was finally obtained as a yellow oil through FCC using as an eluant the solvent system J. Alcohol **17** had R_f (K) 0.26, IR (neat): cm^{-1} 3326 (br OH), 1625, ^1H NMR ($CDCl_3$): δ 6.14 (1H, d, *J* 16 Hz, H-5), 6.03 (1H, d, *J* 16 Hz, H-4), 5.62 (1H, t, *J* 7.2 Hz, H-2), 4.26 (2H, t, *J* 7.2 Hz, H-1), 2.01 (2H, t, *J* 6 Hz, H-8), 1.85 (3H, s, H-12), 1.69 (3H, s, H-13), 1.64–1.57 (3H, m, H-9 and O*H*), 1.47–1.43 (2H, m, H-10), 1.02 (6H, s, H-14 and H-15) ppm, ^{13}C NMR: δ 137.5, 136.9 (two C), 128.9, 128.3, 127.1, 59.4, 39.4, 34.1, 32.8, 28.8 (two C), 21.5, 19.2, 12.4 ppm.

4.3.4. (2E,4E)-3-Methyl-5-(2,6,6-trimethylcyclohex-1-enyl) penta-2,4-dienal (**18**)

To an ice-cold solution of *E*,*E*-β-ionylidenethanol **17** (2.86 g, 13 mmol) in hexane (310 mL) was added MnO_2 (22.6 g, 260 mmol) and the resulting mixture was vigorously stirred at that temperature for 1 h. Filtration from celite and evaporation to dryness left a residue from which 2.47 g (88% yield) of aldehyde **18** (an orange-reddish oil) were obtained through FCC and using solvent L for elution. Aldehyde **18** had R_f (L) 0.17, IR (neat): cm^{-1} 2930, 1664, 1604. ^1H NMR ($CDCl_3$) of the major *E*,*E*-isomer: δ 10.13 (1H, d, *J* 8.2 Hz, H-1), 6.75 (1H, d, *J* 16 Hz, H-5), 6.22 (1H, d, *J* 16 Hz, H-4), 5.94 (1H, d, *J* 8.2 Hz, H-2), 2.32 (3H, s, H-12), 2.05 (2H, t, *J* 6 Hz, H-8), 1.73 (3H, s, H-14), 1.65–1.62 (2H, m, H-9), 1.50–1.47 (2H, m, H-10), 1.05 (6H, s, H-14 and H-15) ppm, ^{13}C NMR: δ 191.3, 155.0, 137.1, 135.7, 135.6, 128.7, 127.8, 39.8, 34.3, 33.6, 28.9 (two C), 21.7, 19.0, 12.9 ppm.

4.3.5. Ethyl (2E,4E,6E)-5-methyl-7-(2,6,6-trimethylcyclohex-1-enyl)hepta-2,4,6-trienoate (**19**)

To a solution of aldehyde **18** (2.4 g, 11 mmol) in MeCN (30 mL) was added the phosphorane $Ph_3P{=}CHCO_2Et$ (4.88 g, 14 mmol) and the resulting mixture was refluxed overnight. Evaporation to dryness, trituration with Et_2O and overnight refrigeration followed by filtration removed most of Ph_3PO. The filtrate was evaporated to dryness and subjected to FCC, using solvent L as eluant. Pure ester **19** (2.7 g, 85% yield), which was obtained as a mixture of geometric isomers (*E*/*Z*: 98:2 by GC MS) in the form of a yellowish oil, was used as such into the following saponification step. Ester **19** had R_f (L) 0.39.

4.3.6. (2E,4E,6E)-5-Methyl-7-(2,6,6-trimethylcyclohex-1-enyl)hepta-2,4,6-trienoic acid (**20**)

To a solution of ester **19** (2.5 g, 8.7 mmol) in MeOH (45 mL) was added a solution of NaOH (1.8 g, 45 mmol) in H_2O (3 mL) and the resulting mixture was refluxed for 2 h. The resulting solution was concentrated under vacuo to half its volume and triturated at 0 °C, initially with glacial acetic acid and then with a 5% aqueous citric acid solution, to pH 6. The resulting yellow precipitate was taken up in EtOAc and the organic layer was initially washed twice with H_2O and then once with a saturated aqueous solution of NaCl (brine). Drying (Na_2SO_4) and evaporation left a residue from which the first crop of pure, crystalline, *E*,*E*,*E*-acid **20**, was obtained through recrystallization from EtOAc. The crystals were washed on the filter with ice-cold Et_2O and the mother liquor and washings from filtration were evaporated to leave a residue from which a second crop of *E*,*E*,*E*-acid **20** was obtained through FCC and using solvent system J as eluant. That way, 1.9 g (83% yield) of pure compound **20** were obtained. This compound had R_f (J) 0.26, m.p. 161–163 °C, MS (ESI^+): *m/z* 781.46 (3M + H), 521.37 (2M + H), 261.08 (M + H), IR (KBr): cm^{-1} 3200–2400, 1691, 1668, ^1H NMR ($CDCl_3$): δ 7.82 (1H, dd, *J* 12 and 16 Hz, H-3), 6.44 (1H, d, *J* 16 Hz, H-7), 6.19 (1H, d, *J* 12 Hz,

H-4), 6.17 (1H, d, *J* 16 Hz, H-6), 5.88 (1H, d, *J* 16 Hz, H-2), 2.07 (3H, s, H-14), 2.03 (2H, t, *J* 6.4 Hz, H-10), 1.72 (3H, s, H-15), 1.67–1.59 (2H, m, H-11), 1.49–1.46 (2H, m, H-12), 1.04 (6H, s, H-16 and H-17) ppm, ^{13}C NMR: δ 172.7, 145.7, 142.9, 137.4, 136.6, 131.6, 131.0, 127.0, 118.9, 39.6, 34.2, 33.1, 28.9 (two C), 21.7, 19.1, 13.1 ppm.

4.4. General procedure for the preparation of succinimidyl 'active' esters

To an ice-cold solution of 10 mmol of acid **14** (2.35 g) or **20** (2.6 g) in anhydrous THF (15 mL) were sequentially added, HOSu (1.7 g, 15 mmol) and, dropwise, a solution of DCC (2.25 g, 11 mmol) in THF (5 mL). The resulting mixture was stirred at that temperature for 1 h and at RT for overnight. The precipitated DCU was filtered off and washed on the filter with ice-cold EtOAc (3×30 mL). The filtrate was sequentially washed twice with an ice-cold 5% aqueous $NaHCO_3$ solution, twice with H_2O and once with brine and dried (Na_2SO_4). Filtration and evaporation of the organic phase left a residue from which pure crystalline 'active' esters were obtained through FCC using as eluant the solvent systems indicated in the R_f values of the compounds given below.

4.4.1. Succinimidyl (2E,4E)-3-methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dienoate (**15**)

Yield: 90% (2.98 g), white crystals, R_f (K) 0.13, m.p. 110–112 °C, MS (ESI$^+$): *m*/*z* 354.30 (MNa), 332.05 (MH), IR (KBr): cm^{-1} 1762, 1732, 1604, ^{1}H NMR ($CDCl_3$): δ 6.75 (1H, d, *J* 16 Hz, H-5), 6.20 (1H, d, *J* 16 Hz, H-4), 5.94 (1H, s, H-2), 2.84 (4H, br s, H-1′), 2.37 (3H, s, H-12), 2.05 (2H, t, *J* 6 Hz, H-8), 1.71 (3H, s, H-13), 1.67-1.58 (2H, m, H-9), 1.50–1.45 (2H, m, H-10), 1.03 (6H, s, H-14 and H-15) ppm, ^{13}C NMR δ 169.7, 161.8, 160.3, 137.2, 135.3, 133.0, 129.2, 110.7, 39.7, 34.4, 33.4, 29.0 (two C), 25.8 (two C), 21.8, 19.2, 14.6 ppm, Anal. Calcd. for $C_{19}H_{25}NO_4$: C, 68.86; H, 7.60; N, 4.23. Found: C, 68.49; H, 7.48; N, 4.52.

4.4.2. Succinimidyl (2E,4E,6E)-5-methyl-7-(2,6,6-trimethylcyclohex-1-enyl)hepta-2,4,6-trienoate (**21**)

Yield: 88% (3.15 g), yellowish crystals, R_f (K) 0.28, m.p. 127–129 °C, MS (ESI$^+$): *m*/*z* 737.59 (2M + Na), 715.28 (2M + H), 358.07 (M + H), IR (KBr): cm^{-1}1748, 1614, 1588. ^{1}H NMR ($CDCl_3$): δ 7.95 (1H, dd, *J* 12 and 16 Hz, H-3), 6.52 (1H, d, *J* 16 Hz, H-7), 6.22 (1H, d, *J* 12 Hz, H-4), 6.20 (1H, d, *J* 16 Hz, H-6), 6.00 (1H, d, *J* 16 Hz, H-2), 2.86 (4H, unresolved m, H-1′), 2.08 (3H, s, H-14), 2.04 (2H, t, *J* 6.4 Hz, H-10), 1.73 (3H, s, H-15), 1.65–1.59 (2H, m, H-11), 1.50–1.45 (2H, m, H-12), 1.04 (6H, s, H-16 and H-17) ppm, ^{13}C NMR: δ 169.6, 162.5, 148.2, 145.9, 137.4, 136.3, 133.1, 131.9, 126.9, 112.5, 39.6, 34.3, 33.3, 29.0 (two C), 25.7 (two C), 21.9, 19.2, 13.3 ppm, Anal. Calcd. for $C_{21}H_{27}NO_4$: C, 70.56; H, 7.61; N, 3.92. Found: C, 70.49; H, 7.50; N, 4.12.

4.5. General procedure I; indirect method for the preparation of retinoid–amino acid conjugates

To an ice-cold solution of 1.2 mmol of HCl·H-βAla-OMe (0.17 g) or HCl·H-Asp(Me)-OMe (0.24 g) or CF_3CO_2H·H-Asp(Me)-Asp(Me)-OMe (0.49 g) or CF_3CO_2H·H-Lys(Tfa)-OMe (0.44 g) in dry $CHCl_3$ (2 mL) was added dropwise anhydrous DIEA (0.44 mL, 2.5 mmol) and, after 10 min at that temperature, 1 mmol of succinimidyl ester **20** (0.36 g) or **12** (0.4 g). The reaction mixture was stirred at that temperature for 30 min and at RT from 2–12 h. When reaction was complete (TLC), the reaction mixture was diluted with EtOAc (30 mL) and washed sequentially once with an ice-cold 5% aqueous citric acid solution, once with H_2O, once with a 5% aqueous $NaHCO_3$ solution and finally twice with H_2O. Drying and evaporation of the solvent left a residue from which pure amides were obtained following FCC and using as eluant the solvent systems indicated in the R_f values of the compounds cited below.

4.5.1. Dimethyl N-(all-trans-retinoyl)aspartate (**28**)

Yield: 75% (0.33 g), yellow foam, R_f (J) 0.24, MS (ESI$^+$): *m*/*z* 909.38 (2M + Na), 482.14 (M + K), 466.17 (M + Na), 444.36 (M + H). IR (KBr): cm^{-1} 3376, 1739, 1662. ^{1}H NMR ($CDCl_3$): δ 6.95 (1H, dd, *J* 12 and 16 Hz, H-5), 6.47 (1H, d, *J* 8.1 Hz, N*H*CO), 6.26 (1H, d, *J* 16 Hz, H-4), 6.25 (1H, d, *J* 16 Hz, H-9), 6.15 (1H, d, *J* 16 Hz, H-8), 6.12 (1H, d, *J* 12 Hz, H-6), 5.72 (1H, s, H-2), 4.94 (1H, dt, *J* 4.4 and 8.1 Hz, H-3′), 3.77 (3H, s, O*CH*$_3$), 3.69 (3H, s, O*CH*$_3$), 3.08 (1H, dd, *J* 4.4 and 16.8 Hz, H-2′a), 2.89 (1H, dd, *J* 4.4 and 16.8 Hz, H-2′b), 2.36 (3H, s, H-16), 2.04 (2H, m, H-12), 1.99 (3H, s, H-17), 1.72 (3H, s, H-18), 1.67–1.58 (2H, m, H-13), 1.48–1.45 (2H, m, H-14), 1.05 (6H, s, H-19 and H-20) ppm. ^{13}C NMR: δ 171.7, 171.4, 166.4, 149.9, 139.1, 137.7, 137.3, 135.3, 130.7, 130.3, 129.9, 129.0, 120.4, 52.8, 52.0, 48.2, 39.6, 36.3, 34.2, 33.1, 28.9 (two C), 21.7, 19.2, 13.7, 12.9 ppm.

4.5.2. Trimethyl N-(all-trans-retinoyl)aspartyl-aspartate (**29**)

Yield: 89% (0.51 g), yellow oil, R_f (H) 0.29, MS (ESI$^+$): *m*/*z* 1167.52 (2M + Na), 611.44 (M + K), 596.05 (M + Na), 574.07 (M + H). IR (KBr): cm^{-1} 3316, 1740, 1650. ^{1}H NMR ($CDCl_3$): δ 7.50 (1H, d, *J* 8.6 Hz, N*H*CO), 6.97 (1H, dd, *J* 12 and 16 Hz, H-5), 6.72 (1H, d, *J* 8.6 Hz, N*H*CO), 6.28 (1H, d, *J* 16 Hz, H-4), 6.26 (1H, d, *J* 16 Hz, H-9), 6.15 (1H, d, *J* 16 Hz, H-8), 6.15 (1H, d, *J* 12 Hz, H-6), 5.72 (1H, s, H-2), 4.94 (1H, ddd, *J* 4, 6.6 and 8.6 Hz, H-6′), 4.85 (1H, dt, *J* 4.8 and 8.6 Hz, H-3′), 3.76 (3H, s, O*CH*$_3$), 3.74 (3H, s, O*CH*$_3$), 3.70 (3H, s, O*CH*$_3$), 3.05 (1H, dd, *J* 4 and 16.8 Hz, H-7′a), 3.01 (1H, dd, *J* 4.8 and 16.4 Hz, H-2′a), 2.84 (1H, dd, *J* 4.8 and 16.8 Hz, H-2′b), 2.69 (1H, dd, *J* 6.6 and 17.2 Hz, H-7′b), 2.38 (3H, s, H-16), 2.03 (2H, t, *J* 6.4 Hz, H-12), 2.01 (3H, s, H-17), 1.73 (3H, s, H-18), 1.67-1.59 (2H, m, H-13), 1.49–1.46 (2H, m, H-14), 1.04 (6H, s, H-19 and H-20) ppm. ^{13}C NMR: δ 172.5, 171.0, 170.7, 170.6, 166.8, 150.4, 139.3, 137.7, 137.3, 135.2, 130.5, 129.9, 129.5, 128.5, 120.1, 52.8, 52.1, 52.1, 48.8, 48.7, 39.6, 35.9, 35.7, 34.2, 33.1, 28.9 (two C), 21.7, 19.2, 13.7, 12.9 ppm.

4.5.3. Methyl N$^{\alpha}$-(all-trans-retinoyl)-N$^{(\omega)}$-trifluoroacetyllysinate (**30**)

Yield: 75% (0.40 g), yellow oil, R_f (I) 0.26. MS (ESI$^+$): *m*/*z* 1101.21 (2M + Na), 561.90 (M + Na), 539.91 (M + H). IR (KBr): cm^{-1} 3312, 1735, 1715, 1650. ^{1}H NMR ($CDCl_3$): δ 6.94 (1H, dd, *J* 12 and 16 Hz, H-5), 6.82 (1H, unresolved t, N*H*COCF$_3$), 6.25 (1H, d, *J* 16 Hz, H-4), 6.23 (1H, d, *J* 16 Hz, H-9), 6.14 (1H, d, *J* 8.4 Hz, N*H*CO), 6.13 (1H, d, *J* 12 Hz, H-6), 6.12 (1H, d, *J* 16 Hz, H-8), 5.71 (1H, s, H-2), 4.67 (1H, dt, *J* 4.8 and 8.4 Hz, H-2′), 3.75 (3H, s, O*CH*$_3$), 3.36 (2H, q, *J* 6.4 Hz, H-6′), 2.33 (3H, s, H-16), 2.01 (2H, t, *J* 6.4 Hz, H-12), 1.98 (3H, s, H-17), 1.93–1.83 (1H, m, H-3′a), 1.76–1.65 (3H, m, H-13 and H-3′b), 1.70 (3H, s, H-18), 1.64–1.56 (2H, m, H-14), 1.48–1.34 (4H, m, H-4′ and H-5′), 1.01 (6H, s, H-19 and H-20) ppm. ^{13}C NMR: δ 173.1, 167.0, 150.0, 139.2, 137.7, 137.3, 135.2, 130.3, 129.9, 129.5, 129.0, 128.4, 120.3, 52.5, 51.3, 39.6, 39.5, 34.2, 33.1, 32.5, 28.9 (two C), 27.8, 22.2, 21.7, 19.2, 13.6 ppm.

4.5.4. Methyl N-(all-trans-retinoyl)-β-alaninate (**31**)

Yield: 90% (0.35 g), yellow foam, R_f (J) 0.24, MS (ESI$^+$): *m*/*z* 809.25 (2M + K), 793.32 (2M + Na), 424.14 (M + K), 408.18 (M + Na), 386.20 (M + H). IR (KBr): cm^{-1} 3379, 1734, 1666. ^{1}H NMR ($CDCl_3$): δ 6.92 (1H, dd, *J* 12 and 16 Hz, H-5), 6.25 (1H, d, *J* 16 Hz, H-4), 6.23 (1H, d, *J* 16 Hz, H-9), 6.13 (1H, d, *J* 16 Hz, H-8), 6.12 (1H, d, *J* 12 Hz, H-6), 6.08 (1H, t, *J* 6 Hz, N*H*CO), 5.64 (1H, s, H-2), 3.70 (3H, s, O*CH*$_3$), 3.58 (2H, q, *J* 6 Hz, H-3′), 2.59 (2H, t, *J* 6 Hz, H-2′), 2.36 (3H, s, H-16), 2.03 (2H, t, *J* 6.4 Hz, H-12), 1.95 (3H, s, H-17), 1.71 (3H, s, H-18), 1.65–1.58 (2H, m, H-13), 1.49–1.45 (2H, m, H-14), 1.05 (6H, s, H-19 and H-20) ppm, ^{13}C NMR: δ 173.2, 167.0, 148.8, 138.8, 137.7, 137.3, 135.5, 129.8, 129.6, 129.0, 128.2, 121.21, 51.8, 39.6, 34.7, 34.2, 33.9, 33.1, 28.9 (two C), 21.8, 19.2, 13.6, 12.9 ppm.

4.5.5. Dimethyl N-*[(2*E*,4*E*,6*E*)-5-methyl-7-(2,6,6-trimethylcyclohex-1-enyl)hepta-2,4,6-trienoyl]aspartate (**32**)*

Yield: 82% (0.33 g), yellow oil, R_f (I) 0.34, MS (ESI^+): *m*/*z* 829.38 (2M + Na), 442.20 (M + K), 426.19 (M + Na), 404.24 (M + H). IR (KBr): cm^{-1} 3288, 1740, 1658. ^1H NMR ($CDCl_3$): δ 7.70 (1H, dd, *J* 12 and 16 Hz, H-3), 6.51 (1H, d, *J* 8.2 Hz, N*H*CO), 6.36 (1H, d, *J* 16 Hz, H-7), 6.14 (1H, d, *J* 16 Hz, H-6), 6.13 (2H, d, *J* 12 Hz, H-4), 5.89 (1H, d, *J* 16 Hz, H-2), 4.97 (1H, dt, *J* 4.4 and 8.2 Hz, H-3′), 3.78 (3H, s, OC*H*$_3$), 3.70 (3H, s, OC*H*$_3$), 3.09 (1H, dd, *J* 4.4 and 17.2 Hz, H-2′a), 2.93 (1H, dd, *J* 4.4 and 17.2 Hz, H-2′b), 2.04 (3H, s, H-14), 2.05-1.95 (2H, unresolved t, H-10), 1.71 (3H, s, H-15), 1.64–1.60 (2H, m, H-11), 1.48–1.45 (2H, m, H-12), 1.03 (6H, s, H-16 and H-17) ppm, ^{13}C NMR: δ 171.7, 171.3, 166.0, 143.8, 138.2, 137.6, 136.8 (two C), 130.4, 127.1, 121.8, 52.8, 52.0, 48.6, 39.6, 36.2, 34.3, 33.1, 28.9 (two C), 21.7, 19.2, 13.0 ppm.

4.5.6. Trimethyl N-*[(2*E*,4*E*,6*E*)-5-methyl-7-(2,6,6-trimethylcyclohex-1-enyl)hepta-2,4,6-trienoyl]aspartyl-aspartate (**33**)*

Yield: 81% (0.43 g), yellow oil, R_f (H) 0.29, MS (ESI^+): *m*/*z* 1104.17 (2M + K), 1087.20 (2M + Na), 571.10 (M + K), 555.16 (M + Na), 533.20 (M + H). IR (KBr): cm^{-1} 3276, 1738, 1644. ^1H NMR ($CDCl_3$): δ 7.72 (1H, dd, *J* 12 and 16 Hz, H-3), 7.51 (1H, d, *J* 8.6 Hz, N*H*CO), 6.76 (1H, d, *J* 8 Hz, N*H*CO), 6.38 (1H, d, *J* 16 Hz, H-7), 6.15 (1H, d, *J* 16 Hz, H-6), 6.14 (1H, d, *J* 12 Hz, H-4), 5.90 (1H, d, *J* 16 Hz, H-2), 4.94 (1H, ddd, *J* 4.4, 6.8 and 8 Hz, H-6′), 4.85 (1H, dt, *J* 4.6 and 8.6 Hz, H-3′), 3.76 (3H, s, OC*H*$_3$), 3.75 (3H, s, OC*H*$_3$), 3.71 (3H, s, OC*H*$_3$), 3.07 (1H, dd, *J* 4.4 and 17.2 Hz, H-7′a), 3.01 (1H, dd, *J* 4.6 and 16.8 Hz, H-2′a), 2.84 (1H, dd, *J* 4.6 and 16.8 Hz, H-2′b), 2.71 (1H, dd, *J* 6.8 and 17.2 Hz, H-7′b), 2.05 (3H, s, H-14), 2.04 (2H, t, *J* 6.4 Hz, H-10), 1.73 (3H, s, H-15), 1.66-1.59 (2H, m, H-11), 1.51-1.46 (2H, m, H-12), 1.04 (6H, s, H-16 and H-17) ppm. ^{13}C NMR: δ 172.5, 171.0, 170.7, 170.4, 166.3, 144.1, 138.5, 137.5, 136.7, 130.6 (two C), 127.0, 121.5, 52.9, 52.2, 52.1, 49.1, 48.7, 39.6, 35.9, 35.7, 34.2, 33.1, 28.9 (two C), 21.8, 19.2, 13.0 ppm.

4.6. General method for the saponification of fully protected conjugates

To an ice-cold solution of 1.1 mmol of esters **28**–**33** in MeOH (5.4 mL) was added a solution of NaOH (0.053 g, 1.32 mmol/group to be saponified) in H_2O (0.6 mL). The resulting mixture was stirred at RT for 1 h (3–4 h for **28**–**30**), ice-cold H_2O (6 mL) was added and the pH of the solution was carefully adjusted to 7 by the dropwise addition of a 1 N HCl aqueous solution. The solvents evaporated under reduced pressure, at a bath temperature less than 40 °C, the residue was dissolved in H_2O (20 mL), cooled to 0 °C and the solution was brought to pH 3.5 by the dropwise addition of a 1 N HCl aqueous solution. The resulting yellow precipitate was filtered, washed with cold H_2O, dried under vacuo and finally recrystallized from MeCN/H_2O to give pure product as yellow solid. In the case of Lys, after the final acidification, the solution was evaporated under reduced pressure to dryness; a small volume of MeOH was added and refrigerated overnight. The precipitated NaCl was filtered off and the filtrate was re-subjected to the same procedure twice to remove NaCl. The residue was isolated, as a yellow foam, through preparative RP-HPLC using as eluant a gradient of MeCN (0.08% TFA)/H_2O (0.08% TFA), from 10% to 100% MeCN, followed by pooling and lyophilization of the fractions containing the product.

4.6.1. N-*(*all-trans-*Retinoyl)aspartic acid (**3**)*

Yield: 58% (0.27 g), yellow solid, m.p. 131–133 °C, R_f (A) 0.35, MS (ESI^+): *m*/*z* 869.39 (2M + K), 454.38 (M + K), 438.28 (M + Na), 416.25 (M + H). IR (KBr): cm^{-1} 3400–2866, 1730, 1626, 1606. ^1H NMR ($CDCl_3$): δ 6.99 (1H, dd, *J* 12 and 16 Hz, H-5), 6.34 (1H, d, *J* 16 Hz, H-4), 6.27 (1H, d, *J* 16 Hz, H-9), 6.16 (1H, d, *J* 12 Hz, H-6), 6.13 (1H, d, *J* 16 Hz, H-8), 5.90 (1H, s, H-2), 4.69 (1H, t, *J* 5.8 Hz, H-3′), 2.84 (2H, dABq, *J* 5.8 and 16.4 Hz, H-2′), 2.30 (3H, s, H-16), 2.02 (2H, t, *J* 5.2 Hz, H-12), 1.99 (3H, s, H-17), 1.70 (3H, s, H-18), 1.69–1.60 (2H, m, H-13), 1.50–1.47 (2H, m, H-14), 1.03 (6H, s, H-19 and H-20) ppm. ^{13}C NMR: δ 178.5, 177.9, 171.6, 152.3, 142.0, 141.6, 139.6, 134.3, 133.6, 133.4, 133.0, 131.5, 125.2, 53.5, 43.3, 41.0, 37.8, 36.5, 31.9 (two C), 24.5, 22.8, 16.4, 15.4 ppm. Anal. Calcd. for $C_{24}H_{33}NO_5$: C, 69.37; H, 8.00; N, 3.37. Found: C, 69.21; H, 7.88; N, 3.57.

4.6.2. N-*[(2*E*,4*E*,6*E*)-5-Methyl-7-(2,6,6-trimethylcyclohex-1-enyl)hepta-2,4,6-trienoyl]aspartic acid (**4**)*

Yield: 64% (0.26 g), yellow solid, m.p. 134–136 °C, R_f (A) 0.32, MS (ESI^+): *m*/*z* 773.65 (2M + Na), 414.62 (M + K), 398.64 (M + Na), 376.63 (M + H). IR (KBr): cm^{-1} 3400–2870, 1730, 1664. ^1H NMR ($CDCl_3$): δ 7.61 (1H, unresolved dd, H-3), 7.47 (1H, br s, N*H*CO), 6.31 (1H, d, *J* 16 Hz, H-7), 6.09 (1H, d, *J* 16 Hz, H-6), 6.07 (1H, d, *J* 12 Hz, H-4), 5.97 (1H, d, *J* 16 Hz, H-2), 4.91 (1H, br s, H-3′), 2.93 (2H, ABq, *J* 15.8 Hz, H-2′), 1.99 (2H, unresolved t, H-10), 1.94 (3H, s, H-14), 1.67 (3H, s, H-15), 1.62–1.53 (2H, m, H-11), 1.48–1.39 (2H, m, H-12), 0.99 (6H, s, H-16 and H-17) ppm, ^{13}C NMR: δ 175.0 (two C), 167.7, 144.2, 138.7, 137.4, 136.8, 130.5 (two C), 127.3, 121.6, 49.3, 39.6, 36.6, 34.2, 33.1, 28.9 (two C), 21.8, 19.2, 12.9 ppm. Anal. Calcd. for $C_{21}H_{29}NO_5$: C, 67.18; H, 7.79; N, 3.73. Found: C, 67.88; H, 7.50; N, 3.98.

4.6.3. N-*(*all-trans-*Retinoyl)aspartyl-aspartic acid (**5**)*

Yield: 57% (0.33 g), yellow solid, m.p. 118–120 °C, R_f (A) 0.29, MS (ESI^+): *m*/*z* 1083.61 (2M + Na), 569.41 (M + K), 553.42 (M + Na), 531.46 (M + H). IR (KBr): cm^{-1} 3400–2856, 1730, 1654. ^1H NMR ($CDCl_3$): δ 7.00 (1H, dd, *J* 12 and 16 Hz, H-5), 6.74 (1H, d, *J* 8.4 Hz, N*H*CO), 6.33 (1H, d, *J* 16 Hz, H-4), 6.27 (1H, d, *J* 16 Hz, H-9), 6.16 (1H, d, *J* 12 Hz, H-6), 6.13 (1H, d, *J* 16 Hz, H-8), 5.90 (1H, s, H-2), 4.80–4.73 (2H, m, H-3′ and H-6′), 2.85–2.79 (4H, m, H-2′ and H-7′), 2.29 (3H, s, H-16), 2.04 (2H, t, *J* 6 Hz, H-12), 2.01 (3H, s, H-17), 1.71 (3H, s, H-18), 1.68–1.61 (2H, m, H-13), 1.51–1.46 (2H, m, H-14), 1.03 (6H, s, H-19 and H-20) ppm. ^{13}C NMR: δ 176.5, 176.0, 174.61, 174.5, 171.9, 152.7, 142.1, 141.6, 141.5, 139.5, 133.6, 133.1, 131.6, 124.9, 124.5, 52.9, 52.7, 46.4, 43.3, 40.8, 37.8, 36.5, 31.9 (two C), 24.4, 22.8, 16.5, 15.3 ppm. Anal. Calcd. for $C_{28}H_{38}N_2O_8$: C, 63.38; H, 7.22; N, 5.28. Found: C, 63.58; H, 7.35; N, 5.01.

4.6.4. N-*[(2*E*,4*E*,6*E*)-5-Methyl-7-(2,6,6-trimethylcyclohex-1-enyl)hepta-2,4,6-trienoyl]aspartyl-aspartic acid (**6**)*

Yield: 61% (0.33 g), yellow solid, m.p. 124–126 °C, R_f (A) 0.15, MS (ESI^+): *m*/*z* 529.16 (M + K), 513.25 (M + Na), 491.24 (M + H). IR (KBr): cm^{-1} 3328, 3200–2400, 1730, 1642. ^1H NMR (CD_3OD): δ 7.64 (1H, ddd, *J* 1.6, 12 and 16 Hz, H-3), 6.39 (1H, d, *J* 16 Hz, H-7), 6.19 (1H, d, *J* 12 Hz, H-4), 6.16 (1H, d, *J* 16 Hz, H-6), 6.08 (1H, d, *J* 16 Hz, H-2), 4.83 (1H, td, *J* 2 and 5.6 Hz, H-6′), 4.74 (1H, dt, *J* 3.2 and 6 Hz, H-3′), 2.88–2.78 (4H, m, H-2′ and H-7′), 2.04 (3H, s, H-14), 2.04 (2H, t, *J* 7.6 Hz, H-10), 1.71 (3H, s, H-15), 1.68–1.61 (2H, m, H-12), 1.51–1.44 (2H, m, H-11), 1.03 (6H, s, H-16 and H-17) ppm. ^{13}C NMR: δ 176.8, 176.6, 174.6, 174.4, 171.4, 146.9, 141.4, 141.3, 141.0, 133.7, 131.3, 126.3, 126.0, 53.2, 52.8, 43.3, 40.8, 39.5, 37.8, 36.5, 31.9 (two C), 24.5, 22.8, 15.5 ppm. Anal. Calcd. for $C_{25}H_{34}N_2O_8$: C, 61.21; H, 6.99; N, 5.71. Found: C, 61.00; H, 6.75; N, 5.99.

4.6.5. Hydrochloride of N^{α}*-(*all-trans-*retinoyl)lysine (**8**)*

Yield: 54% (0.28 g), yellow foam, R_f (A) 0.15, MS (ESI^+): *m*/*z* 880.84 (2M + Na), 467.72 (M + K), 453.66 (M + Na), 429.10 (M + H). IR (KBr): cm^{-1} 3400–2882, 1725, 1638. ^1H NMR ($CDCl_3$): δ 7.71 (3H, br s, NH_3^+), 6.89 (1H, dd, *J* 12 and 16 Hz, H-5), 6.24 (1H, d, *J* 16 Hz, H-4), 6.22 (1H, d, *J* 16 Hz, H-9), 6.17 (1H, d, *J* 8.4 Hz, N*H*CO), 6.16 (1H, d, *J* 12 Hz, H-6), 6.15 (1H, d, *J* 16 Hz, H-8), 5.67 (1H, s, H-2), 4.37 (1H, dt, 4.8 and 8.4 Hz, H-2′), 2.92 (2H, m, H-6′), 2.30 (3H, s, H-16), 1.97 (2H, t, *J* 6.4 Hz, H-12), 1.94 (3H, s, H-17), 1.89–1.84 (2H, m, H-3′),

1.72–1.68 (2H, m, H-13), 1.67–1.63 (5H, m, H-14, H-18), 1.40–1.35 (2H, m, H-4′), 1.33–1.28 (2H, m, H-5′), 0.99 (6H, s, H-19 and H-20) ppm, ^{13}C NMR: δ 173.1, 167.0, 150.0, 139.1, 137.6, 137.2, 135.1, 130.3, 129.7, 129.3, 128.9, 128.2, 52.5, 39.6, 39.5, 34.2, 33.1, 32.5, 28.9 (two C), 27.8, 22.2, 21.7, 19.2, 13.6, 13.2 ppm, HR-MS (for $C_{26}H_{40}N_2O_4 + H^+$) calcd.:445.3066. Found: 445.3120.

4.6.6. N-*(*all-trans-*Retinoyl)-β-alanine (**9**)*

Yield: 61% (0.25 g), yellow solid, m.p. 137–139 °C, R_f (A) 0.48, MS (ESI$^+$): *m*/*z* 782.51 (2M + K), 766.36 (2M + Na), 410.44 (M + K), 394.46 (M + Na), 372.36 (M + H), IR (KBr): cm^{-1} 3400–2866, 1714, 1636. ^{1}H NMR ($CDCl_3$): δ 6.93 (1H, dd, *J* 12 and 16 Hz, H-5), 6.26 (1H, d, *J* 16 Hz, H-4), 6.23 (1H, d, *J* 16 Hz, H-8), 6.15 (1H, t, *J* 6 Hz, N*H*CO), 6.12 (1H, d, *J* 16 Hz, H-6), 6.11 (1H, d, *J* 12 Hz, H-9), 5.66 (1H, s, H-2), 3.59 (2H, q, 6 Hz, H-3′), 2.65 (2H, t, *J* 6 Hz, H-2′), 2.35 (3H, s, H-16), 2.02 (2H, t, *J* 5.6 Hz, H-12), 1.99 (3H, s, H-17), 1.76 (3H, s, H-18), 1.66–1.58 (2H, m, H-13), 1.48–1.45 (2H, m, H-14), 1.03 (6H, s, H-19 and H-20) ppm. ^{13}C NMR: δ 176.4, 167.5, 149.4, 139.4, 137.8, 137.3, 135.4, 130.1, 129.9, 129.6, 128.4, 120.9, 39.6, 34.7, 34.3, 34.1, 33.1, 29.0 (two C), 21.8, 19.3, 13.7, 12.9 ppm. Anal. Calcd. for $C_{23}H_{33}NO_3$: C, 74.36; H, 8.95; N, 3.77. Found: C, 74.56; H, 9.08; N, 3.51.

4.7. General procedure II; direct method for the preparation of retinoid–amino acid conjugates

To a suspension of the amino acid εACa or Leu (0.16 g, 1.2 mmol) in anhydrous $CHCl_3$ (1.5 mL) and MeCN (0.5 mL) was added Me_3SiCl (0.15 mL, 1.2 mmol) and the resulting mixture was stirred at RT for 30 min and at refluxing temperature for 1 h. The mixture was ice-cooled and treated dropwise with Et_3N (0.33 mL, 2.4 mmol) followed by the addition of 1 mmol of succinimidyl ester **12** (0.4 g) or **15** (0.33 g). The reaction mixture was stirred at 0 °C for 15 min and then for 1–2 d at RT. If necessary, the pH was readjusted to pH 9 by addition of Et_3N. Upon completion of the reaction (TLC), the reaction mixture was treated with MeOH (0.3 mL), diluted with $CHCl_3$ (20 mL) and washed sequentially twice with an ice-cold 5% aqueous citric acid solution and twice with H_2O. Drying and evaporation of the solvent left a residue from which pure compounds were obtained through FCC and using as eluant the solvent system G or C for *N*-(*all-trans*-retinoyl)leucine.

4.7.1. N-*(*all-trans-*Retinoyl)leucine (**7**)*

Yield: 65% (0.26 g), yellow foam, R_f (C) 0.46, MS (ESI$^+$): *m*/*z* 827.68 (2M + H), 452.3 (M + K), 436.4 (M + Na) 414.4 (M + H), 283.3 (M − Ala), 87.5 ($C_5H_{13}N$), IR (KBr): cm^{-1} 3316, 2956, 2920, 1724, 1632. ^{1}H NMR ($CDCl_3$): δ 6.97 (1H, dd, *J* 12 and 16 Hz, H-5), 6.27 (1H, d, *J* 16 Hz, H-4), 6.23 (1H, d, *J* 16 Hz, H-9), 6.13 (1H, d, *J* 16 Hz, H-8), 6.12 (1H, d, *J* 12 Hz, H-6), 5.83 (1H, d, *J* 7.6 Hz, N*H*CO), 5.70 (1H, s, H-2), 4.62–4.58 (1H, m, H-2′), 2.36 (3H, s, H-16), 2.04 (1H, t, *J* 6 Hz, H-12), 1.99 (3H, s, H-17), 1.82–1.72 (2H, m, H-3′), 1.71 (3H, s, H-18), 1.66–1.58 (3H, m, H-13 and H-4′), 1.49–1.45 (2H, m, H-14), 1.03 (6H, s, H-19 and H-20), 0.97 (3H, d, *J* 6.4 Hz, H-5′and H-6′), 0.96 (3H, d, *J* 6.4 Hz, H-5′) ppm. ^{13}C NMR: δ 175.5, 167.9, 150.9, 139.4, 137.7, 137.3, 135.1, 130.7, 130.0, 129.5, 128.6, 119.7, 51.2, 40.7, 39.6, 34.3, 33.1, 29.0 (two C), 24.9, 22.8, 21.9, 21.7, 19.2, 13.8, 12.9 ppm, HR-MS (for $C_{26}H_{39}NO_4 + H^+$) calcd.: 430.2957. Found: 430.3041.

4.7.2. N-*(*all-trans-*Retinoyl)-ε-aminocaproic acid (**10**)*

Yield: 79% (0.32 g), yellow foam, R_f (F) 0.37, MS (ESI$^+$): *m*/*z* 436.4 (M + Na) 414.4 (M + H). IR (KBr): cm^{-1} 3356, 3100–2500, 1707, 1628. ^{1}H NMR (DMSO-d_6): δ 11.96 (1H, br s, CO_2H), 7.89 (1H, t, *J* 5.6 Hz, N*H*CO), 6.89 (1H, dd, *J* 12 and 16 Hz, H-5), 6.28 (1H, d, *J* 16 Hz, H-4), 6.24 (1H, d, *J* 12 Hz, H-6), 6.19 (1H, d, *J* 16 Hz, H-9), 6.15 (1H, d, *J* 16 Hz, H-8), 5.81 (1H, s, H-2), 3.07 (2H, q, *J* 7 Hz, H-6′), 2.26 (3H, s, H-16), 2.19 (2H, t, *J* 7.4 Hz, H-2′), 2.00 (2H, t, *J* 6.8 Hz, H-12), 1.95 (3H, s, H-17), 1.68 (3H, s, H-18), 1.61-1.54 (2H, m, H-13), 1.50 (2H, quintet, *J* 7.4 Hz, H-3′), 1.46-1.41 (2H, m, H-14), 1.41 (2H, quintet, *J* 7 Hz, H-5′), 1.31-1.22 (2H, m, H-4′), 1.01 (6H, s, H-19 and H-20) ppm. ^{13}C NMR: δ 174.8, 166.5, 146.2, 137.9, 137.8, 137.5, 136.8, 130.6, 129.7, 129.3, 127.6, 123.6, 39.7, 38.7, 34.3, 34.1, 33.1, 29.4, 29.3 (two C), 26.5, 24.7, 21.9, 19.2, 13.6, 13.0 ppm, HR-MS (for $C_{26}H_{39}NO_4 + H^+$) calcd.: 430.2957. Found: 430.3033.

4.7.3. N-*[(2*E*,4*E*)-3-Methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dienoyl]-ε-aminocaproic acid (**11**)*

Yield: 80% (0.28 g), yellow foam, R_f (F) 0.37, MS (ESI$^+$): *m*/*z* 386.2 (M + K), 370.4 (M + Na) 348.4 (M + H). IR (KBr): cm^{-1} 3352, 3100–2500, 1712, 1626. ^{1}H NMR (DMSO-d_6): δ 11.95 (1H, br s, CO_2H), 7.85 (1H, t, *J* 6.2 Hz, N*H*CO), 6.39 (1H, d, *J* 16 Hz, H-4), 6.01 (1H, d, *J* 16 Hz, H-5), 5.77 (1H, s, H-2), 3.07 (2H, q, *J* 7.2 Hz, H-6′), 2.22 (3H, s, H-12), 2.19 (2H, t, *J* 7.4 Hz, H-2′), 2.00 (2H, t, *J* 6 Hz, H-8), 1.67 (3H, s, H-13), 1.62-1.54 (2H, m, H-9), 1.49 (2H, quintet, *J* 7.4 Hz, H-3′), 1.46–1.41 (2H, m, H-10), 1.40 (2H, quintet, *J* 7.2 Hz, H-5′), 1.31–1.22 (2H, m, H-4′), 1.00 (6H, s, H-14 and H-15) ppm. ^{13}C NMR: δ 174.9, 166.6, 145.7, 137.4, 137.0, 130.8, 130.1, 123.0, 38.6, 34.1, 32.9, 29.4, 29.2 (two C), 26.5, 25.9, 24.7, 21.8, 20.6, 19.1, 13.4 ppm, HR-MS ($C_{21}H_{33}NO_3 + H^+$) calcd.: 348.2539. Found: 348.2515.

4.8. Cell culture

LNCaP cells (from ATCC) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics, as previously described [26]. Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Cells were photographed using a digital camera-equipped Olympus reverse-phase microscope.

4.9. MTT assay

In order to measure the number of cells, the 3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide (MTT) assay was used, as previously described [26]. LNCap cells were seeded at 10×10^3 cells/well in 24-well tissue culture plates, in RPMI-1640 supplemented with 10% FBS. Twenty four hours later, cells were starved in medium supplemented with 2% FBS for 16 h. The number of cells was determined 48 h after addition of different concentrations of the tested agents. Results were always confirmed by direct counting of cells under the microscope, using a standard hemocytometer.

4.10. Apoptosis assay

LNCaP cells were seeded at 2×10^5 cells/well in 6-well plates in RPMI-1640 supplemented with 10% FBS. Twenty four hours later, cells were starved in medium supplemented with 2% FBS for 16 h and incubated with the tested agents for 48 h. At the end of the incubation periods with the tested agents, cells were resuspended in binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) to a final concentration of 5×10^5 cells/ml. The cell suspension was incubated with Annexin V-FITC in the dark at 25 °C, for 10 min. Then, 10 μl of the 20 μg/ml propidium iodide stock solution was added followed by 200 μl of binding buffer and the cells were analyzed by flow cytometry (EPICS-XL of Coulter), according to the kit manufacturer's instructions (rh Annexin V/FITC kit, Bender MedSystems).

4.11. Cell cycle analysis

The cell cycle progression was estimated with flow cytometric analysis, using Coulter DNA Prep Reagents kit (Beckman). LNCaP cells were seeded at 4×10^5 cells/well in 6-well plates in RPMI-1640

supplemented with 10% FBS. Twenty four hours later, cells were starved in medium supplemented with 2% FBS for 16 h and exposed to the tested agents for 48 h. At the end of the incubation periods with the tested agents, attached cells were collected and analyzed by flow cytometry (EPICS-XL of Coulter), according to manufacturer's instructions.

4.12. Statistical analysis

The significance of variability between the results from various groups was determined by one-way analysis of variance (ANOVA). Each experiment included triplicate measurements for each condition tested, unless otherwise indicated. All results are expressed as mean ± S.E.M. from at least three independent experiments.

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