SYNTHESIS AND CYTOTOXIC ACTIVITY OF MADECASSIC ACID – SILYBIN CONJUGATE COMPOUNDS IN LIVER CANCER CELLS

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Abstract

Madecassic acid – isolated from the medicinal herb *Centella asiatica* – and its derivatives exhibit cytotoxic activity against the HepG2 liver cancer cell line. Silybin, a natural compound from *Silybum marianum*, is well-known as a hepatoprotective agent. This paper describes the synthesis of madecassic acid-silybin conjugate compounds and evaluation of their cytotoxic activity against a range of liver cancer cell lines. Depending on the reaction conditions, the direct conjugation products of 2,3,23-triacetylmadecassic acid and silybin were found to be either an ester at position 7 of silybin (40%) and its 2,3-dehydrated derivative (20%), a 2,3-dehydrated ester at position 3 of silybin (42%), or an ester at position 3 of silybin with madecassic acid. We have also used linkers such as glycine, *β*-alanine and 11-aminoundecanoic acid. For further diversification, 2,3,23-triacetylmadecassic acid was dehydrated to form the $\Delta^{5,6}$-compound, and was converted to amide derivatives on reaction with glycine or *β*-alanine, and finally condensed with silybin to afford esters at position 3 of silybin. In total, sixteen new conjugates have been synthesized. The conjugates were tested *in vitro* for their cytotoxic effect on HepG2 cells using the MTT assay. Results confirmed the conjugated compounds demonstrated a stronger cytotoxic effect versus those of the parent compounds. Of these compounds, the most promising conjugate, compound **8,** was evaluated for cytotoxic activity on the Hep3B, Huh7, and Huh7R liver cancer cell lines and also for induction of apoptosis. This compound caused a rapid and significant induction of caspase 3 activity in HepG2 cells and induced cell cycle arrest in S phase,

effects distinct from the activity of madecassic acid. This is the first study on the synthesis and the cytotoxicity of the madecassic acid-silybin conjugates, and of their testing against liver cancer cell lines and provides evidence for a distinct biological profile versus madecassic acid alone.

Keywords: Madecassic acid, silybin, conjugate, HepG2, Hep3B, Huh7, Huh7R, cytotoxicity, apoptosis, cell cycle

1. Introduction

Liver cancer was one of the top five causes of cancer death in 185 countries in 2020, with an estimated 906,000 new cases and 830,000 deaths globally. The number of liver cancer deaths is precited to reach approximately 1.3 million people by 2040 (56.4% more than in 2020) [1], whilst the number of new cases of liver cancer per year is estimated to increase by 55% between 2020 and 2040, with a possible 1.4 million people diagnosed in 2040. Liver cancer is caused by viral infections including hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis D virus (HDV), and is becoming one of the most challenging and urgent problems in Vietnam today. An estimated 25,000 new cases of liver cancer were reported in 2020, making Vietnam the fifth highest country in the world for incidence [1] [2] [3]. Since 2018, liver cancer has risen above lung cancer to become the leading cause of cancer death in Vietnam [2] [3].

The herb *Centella asiatica* (L.) Urb. (Apiaceae) is extensively grown throughout Vietnam. This medicinal plant possesses a wide range of biological properties, including anti-oxidant, anticancer, memory stimulation, and wound healing [4] [5] [6]. Madecassic acid is one of the three main pentacyclic triterpenoid acids produced by the plant, together with asiatic acid and terminolic acid [7]. The compound has been reported to have valuable pharmacological activities, such as wound healing [8], antioxidant [9], anti-inflammatory [10], and antidiabetic effects [11]. Recently, madecassic acid has been showed to induce apoptosis in the mouse colon cancer cell line CT26, and much attention has been paid to increasing the anticancer activity of this molecule through chemical modification of its structure: new madecassic acid derivatives have been reported to have significantly enhanced cytotoxicity towards cancer cell lines [12] [13].

Silybins A and B are flavonolignan framework compounds isolated from the seeds of *Silybum marianum* (L.) Gaertn. (Asteraceae), and are well-known for their hepatoprotective activity [14], strong antioxidant potential and anti-lipid peroxidation action [15] [16]. Recent studies have found that silybins possess cytotoxic activity in a mouse model of prostate cancer [17], in the human non-small-cell lung carcinoma H1299, H322 and H460 cell lines [18], and the SW480 human colorectal cancer cell line [19]. In addition, they have proven cytotoxic potential in bladder, skin, prostate, colon

and lung cancers [20] [21] by a mechanism of regulating the cancer cell cycle, apoptosis and autophagy, as well as inhibiting tumor-inducing factors [22] [23]. Thus, structural modifications of silybin could provide further interesting information on silybin's applications.

Hybrid synthesis of naturally bioactive compounds into a single molecule has been regarded as an effective strategy to produce novel and better active substances for the treatment of cancer [24]. It is believed that bio-conjugated molecules could have superior efficacy in comparison with a single drug due to the minimization of the unwanted side-effects as well as the synergism of two or more active moieties in one molecule [25].

Inspired by the bio-conjugated compounds, herein we report the synthesis of madecassic acid-silybin conjugates with an ester bond between a hydroxy group of silybin and the carboxyl group of madecassic acid, or over an amino acid as a spacer, and evaluation of their antiproliferative *in vitro* potential against the HepG2 human liver cancer cell line. The most potent active compound **8** was further evaluated for cytotoxic activity on the Hep3B, Huh7, and Huh7R liver cancer cell lines together with cell cycle analysis.

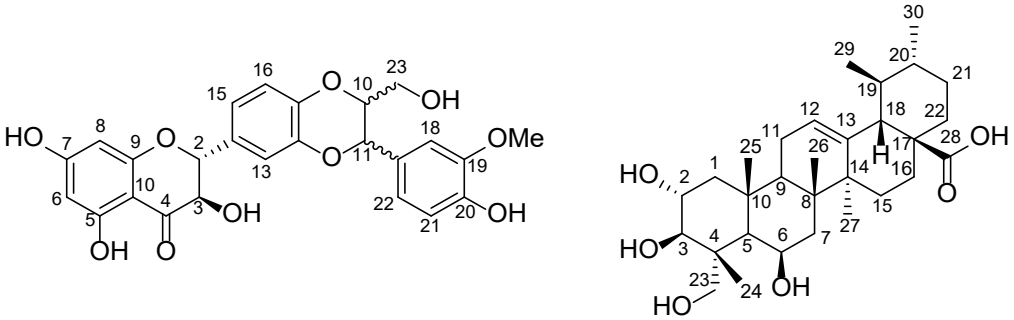
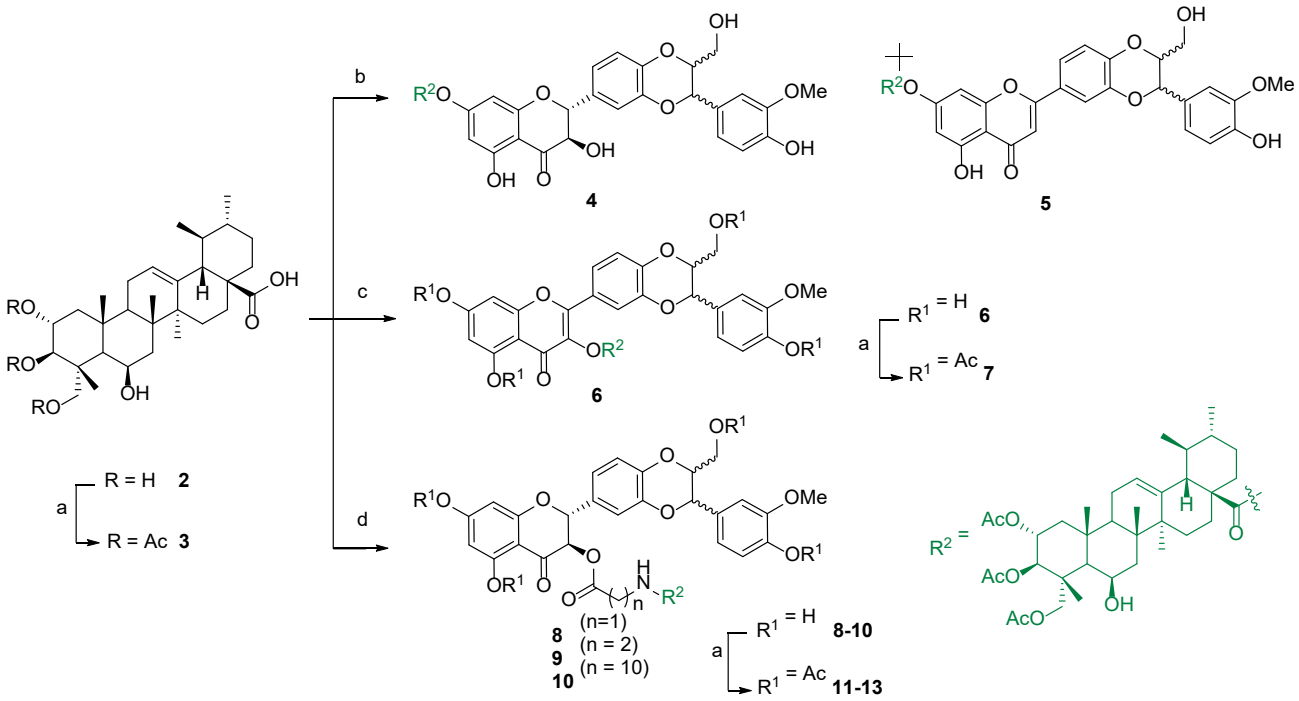


Figure 1. Chemical structures of silybin (**1**, A/B designation depends on variable stereochemistry) and madecassic acid (**2**) showing numbering of positions.

2. Results and discussion

2.1. Chemistry

Silybin (**1**) is a flavonoligan containing five functional hydroxyl groups, thus the selective esterification between the carboxylic acid group of madecassic acid (MA, **2**) and silybin moiety may be somewhat problematic (**Figure 1**). Initially, we aimed at conjugating madecassic acid with hydroxy at C-23 of silybin through an ester. Accordingly, silybin was converted into 3,5,7,20-*O*-tetraacetyl silybin as described by Armando [26], followed by reaction with 2,3,23-triacetylmadecassic acid (**3**)/or its amide derivatives, which were linked to a glycine amino acid unit. Various esterification conditions were applied, such as Staab's reagent (1,1'-carbonyldiimidazole/4-dimethylaminopyridine), Steglich esterification (DCC/DMAP/THF) [27], or the Mitsunobu reaction (DEAD/Ph_3P) [28] [29], but these attempts failed to obtain the desired ester.

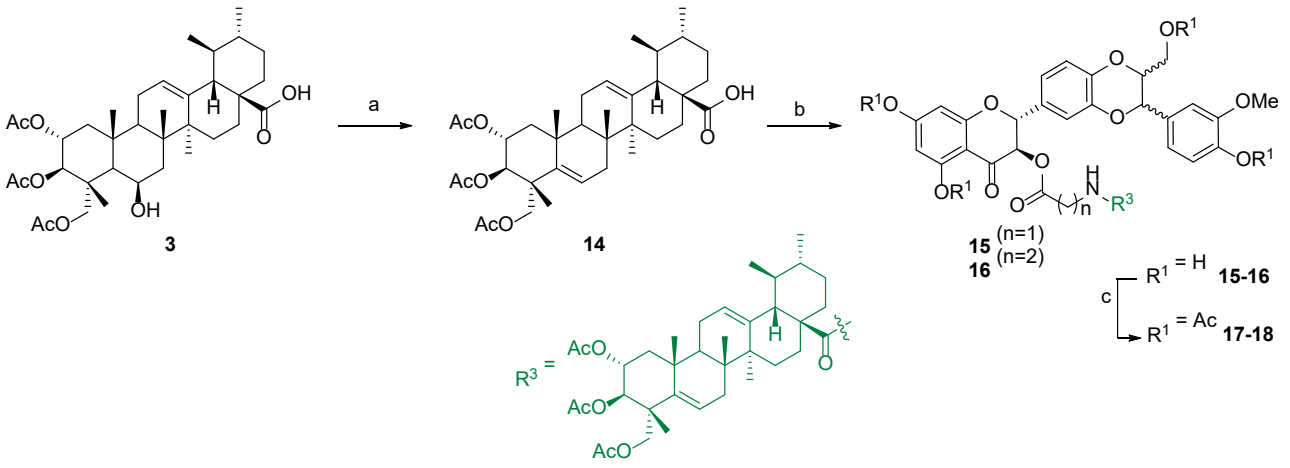


Scheme 1. Synthesis of madecassic acid conjugated with silybin. Reagents and conditions: a) Ac_2O, pyridine, rt, 12 h 84%; b) i) Oxalyl chloride, DCM, 18 h, rt, ii) TEA, DCM, Silybin, rt, **4** (40%), **5** (20%); c) Silybin, Dicyclohexylcarbodiimide (DCC), DMAP, 0 ºC, rt, 34 h, 42%; d) i) oxalyl chloride, DCM, rt, 14 h, ii) amino acid (glycine; or *β*-alanine; or 11-aminoundecanoic acid), TEA, DCM, rt, 18 h; iii) Silybin, DCC, DMAP, THF, 0 ºC, rt, 18 h; e) Pyridine, $SOCl_2$, pyridine, 2 h, rt, 47%.

Due to the failure of using 3,5,7,20-*O*-tetraacetylsilybin as precursor for the selective esterification, free silybin, without any protection was used as starting material. As reported by Li [30] and Decker [31], under Mitsunobu conditions, a regioselective 23-OH esterification of silybin moiety was formed preferentially. However, in our case the esterification did not occur when using Mitsunobu conditions (DEAD/PPh_3 or DIEA/PPh_3).

As illustrated in **Scheme 1**, treatment of acid **3** with either thionyl chloride or oxalyl chloride to produce an intermediate MA-chloride acid followed by exposure to silybin (**1**) in the presence of triethylamine (TEA) led to the formation of esters **4** and **5**, in which compound **5** showed a dehydration of hydroxyl group at C-3 on silybin by the appearance of a signal of singlet proton H-3 at δ_H 6.56 and carbon atom C-3 at δ_C 104.38 (3-CH) [28]. The selective attachment to the 7-OH group of silybin was confirmed based on NMR analysis. This result was in agreement with the report by Křen and Decker [32] [31]. On the other hand, under Steglich conditions using DCC/DMAP, esterification of acid **3** with silybin **1** provided ester **6** with a selective attachment to the 3-OH group of silybin. The NMR analysis of ester **6** confirmed an elimination of protons H-2 and H-3 on the silybin moiety, which took part in an oxidation under mild basic conditions to create 2,3-

dehydrosilybin derivatives. The conclusion was based on the disappearance of proton signals at δ_H 5.0 (d, J = 11.5 Hz, H-2) and 4.54 (dd, J = 3.5; 11.5b Hz, H-3), together with carbon signals at δ_C 84.7 (C-2) and at δ_C 73.7 (C-3) [35], accompanied by the appearance of two new carbon signals upfield at δ_C 156.7 (C-2) and 132.5 (C-3) [33] [34] (**Scheme 1**).



Scheme 2. Synthesis of dehydrated madecassic acid conjugated with silybin. Reagents and conditions: a) Pyridine, $SOCl_2$, pyridine, 2 h, rt, 47%. B) i) oxalyl chloride, DCM, rt, 14 h, ii) amino acid (glycine; or *β*-alanine), TEA, DCM, rt, 18 h; iii) Silybin, dicyclohexylcarbodiimide (DCC), DMAP, THF, 0 °C, rt, 18 h; c) Ac_2O, pyridine, rt, 12 h.

Conjugation of madecassic acid to silybin *via* an amino acid as a spacer (glycine, *β*-alanine, or 11-aminoundecanoic acid, 11-AUDA), was carried out in a two-step reaction sequence. Treatment of triacetyl madecassic acid (**3**) with oxalyl chloride to furnish the intermediate acyl chloride acid was followed by reaction with one of the three amino acids, and then subsequent coupling with silybin **1** under Steglich conditions, using DCC/DMAP in dry tetrahydrofuran (THF) at room temperature. The corresponding conjugates **8-10** were obtained in overall yields of 20-26%. Analysis of NMR spectroscopy of these conjugates **8-10** confirmed the regioselective esterification to the 3-OH group of silybin moiety. A comparison of the ^{1}H NMR spectra of silybin (**1**) with its conjugated ester exhibited distinctly that the chemical shift of proton H-3 in silybin moiety was downfield shifted from a range of 4.65-4.64 [35] up to 5.82-5.84 ppm. Only a few silybin ester derivatives in positions C-3 have been reported so far. Antoszczak et al. reported the synthesis of conjugates of silybin with the antibiotics salinomucin and monensin. The authors obtained the conjugates through an ester linkage at the 23-OH group with the yields of 43% and 35%, respectively and no conjugates at the 3-OH group of silybin [36]. However, our results are in good agreement with the results by Křen [27] who reported a selective attachment on the 3-OH group of silybin moiety under Steglich esterification conditions to form the 3-O-galloylsilybin. Acetylation of compounds **6**, **8-10** with acetic anhydride

in pyridine at room temperature provided the corresponding acetylated products (**7**, **11-13**). Their structures were evidenced by the analysis of the NMR and MS spectroscopic data.

In order to evaluate the role of the 6-OH group in madecassic acid on the cytotoxicity to HepG2 cells, a series of the conjugates **15-18** were synthesized (**Scheme 2**). Triacetyl madecassic acid (**3)** was first treated with thionyl chloride in the presence of pyridine to give the dehydrated compound **14** [12] in 47% yield after a silica gel column chromatography. In a similar two-step sequence reaction, conversion of the acid **14** into the intermediate chloride acid followed by coupling with amino acids (glycine; or *β*-alanine), and subsequently esterified with silybin (**1**) under Steglich conditions provided esters **15-16** (21-23%). Treatment of these esters with acetic anhydride provided products **17** and **18** in 68% and 58% yields, respectively, after silica gel column chromatography (Scheme 2).

2.2. Biological activities

2.2.1. Cytotoxic activity

All compounds synthesized from silybin and madecassic acid were initially screened for their anti-proliferative activity on the HepG2 cell line using the MTT assay before testing on other hepatocarcinoma cell lines. As shown in **Table 1**, the GI_{50} values for the 18 compounds on HepG2 cells was diverse. A number of compounds showed a significantly higher antiproliferative activity on HepG2 versus the original compounds silybin (**1**) and madecassic acid (**2**), whilst others exhibited weaker activity. To illustrate, compounds **3, 8, 11** and **14** showed lower GI_{50} values and therefore improved antiproliferative activity in comparison to that of the two original compounds silybin (**1**) and madecassic acid (**2**) and notably neither **3** nor **14** contain a silybin unit. In contrast, compounds **4**, **5**, **7**, **10**, **12**, **16** and **18** showed reduced antiproliferative activity (higher GI_{50} values), versus the two starting compounds and **4** and **5** (conjugated to silybin's 7-position) had GI_{50} values greater than 500 μM. The other group including compounds **6**, **9**, **13**, **15**, **17** presented improved antiproliferative activity versus silybin, but not madecassic acid.

The acetylation of the hydroxyl groups at the positions 2,3,23 of madecassic acid and its dehydrated product (**3** and **14**) significantly increased antiproliferative activity on the HepG2 cancer cell line. The conjugates between silybin or tetra-acetyl silybin with triacetyl madecassic acid (**3)** using glycine as spacer also enhanced this activity (compounds **8** and **11**), and the compound with silybin displaying free hydroxyls (**8**) had greater activity versus the equivalent acetylated version (**11**).

Table 1. Cytotoxic activity of madecassic acid (**2**), silybin (**1**) and their conjugated madecassic acid-silybin derivatives on HepG2 cancer cell lines. [a]Alcohol on the silybin unit through which conjugation is achieved. [b] +Ac = acetylation, $-H_2O$ = dehydration on 2,3 positions on silybin (S) or 5,6 positions

on madecassic acid (M). Ellipticine was used as a positive control and all values were generated using in a 96 hour MTT assay (n ≥ 3 assay repeats).

Compound	Conjugation position[a]	Linkage	Modification[b]	GI_{50} (μM)
Silybin (1)	-	-	-	285.40±1.62
Madecassic acid (2)	-	-	-	161.0±1.28
3	-	-	+Ac	44.12±2.08
4	7	Direct ester	-	>500
5	7	Direct ester	-H_2O(S)	>500
6	3	Direct ester	-	217.37±10.76
7	3	Direct ester	+Ac	471.13±17.82
8	3	Glycine	-	32.5±3.59
9	3	β-Alanine	-	141.37±39.22
10	3	11-AUDA	-	383.12±40.69
11	3	Glycine	+Ac	122.48±4.66
12	3	β-Alanine	+Ac	359.62±12.35
13	3	11-AUDA	+Ac	198.93±48.55
14	-	-	-H_2O(M)	38.47±5.78
15	3	Glycine	-H_2O(M)	173.50±96.94
16	3	β-Alanine	-H_2O(M)	343.62±19.12
17	3	Glycine	-H_2O(M) +Ac	243.41±21.96
18	3	β-Alanine	-H_2O(M) +Ac	352.08±33.11
Ellipticine	-	-	-	0.42±0.01

2.2.2. Cytotoxicity to Hep3B, Huh7 and Huh7R

Of all the compounds evaluated on HepG2 cells, compound **8** exhibited the highest cytotoxic activity and was chosen for the further evaluation on a variety of hepatocyte cancer cell lines: Hep3B, Huh7, and Huh7R, with the results compared with those of madecassic acid (**2**), using the MTT assay (**Table 2**). The antiproliferative activity of compound **8** was much greater than that of madecassic acid, with the GI_{50} decreased by multiples of 15.1 to 18.5 respectively for Hep3B, Huh7, and Huh7R versus the parent compound. Compound **8** suppressed the antiproliferative activity of the Hep3B line the most effectively versus Huh7 and Huh7R cell lines. The effectiveness of compound **8** on the these three cell lines was noted to be in the same order of ranking by GI_{50} value as for madecassic acid (**2**) i.e. Hep3B lowest and Huh7R highest GI_{50} value.

Table 2. Cytotoxic activity of madecassic acid (**2**) and compound **8** on Hep3B, Huh7, and Huh7R hepatocyte cancer cell lines (96 hour MTT assay, n ≥ 3 assay repeats).

Compound	GI_{50} (μM)		
	Hep3B	Huh7	Huh7R
Madecassic acid (2)	177.80±7.36	197.90±9.80	215.43±4.14
8	9.61±0.81	13.78±0.45	14.23±1.74

2.2.3. Apoptotic inducible activities

Based on the strong anti-proliferative activity of compound **8** on the four hepatocarcinoma cell lines, its ability to induced apoptosis was investigated. This was evaluated through measurement of Annexin V-FITC and PI staining of HepG2 cells, 24 hours after incubation with compound followed by flow cytometry for detection of these markers. The results (**Table 3, Figure 2**) showed that HepG2 cells treated with madecassic acid **(2)** at 1x GI_{50} exhibit a higher proportion of cells in early and late apoptosis when compared with the untreated control (9.00 and 4.29% respectively) and that increasing the **MA** concentration to 3x GI_{50} increased the proportion of cells in both early and late apoptosis further (13.37 and 6.16% respectively). This result is in accordance with the reported induction of apoptosis by **MA** when colon cancer cells are treated with this compound. Compound **8** at 1 x GI_{50} resulted in induction of early and late apoptosis of 10.6% and 4.92% respectively along with a strong necrotic signal of 13.32%, whilst at 3 x GI_{50} early and late apoptosis values were lower than the untreated control, whilst necrosis was still increased (13.81%)

Table 3. The effect of madecassic acid (**2**) and compound **8** on apoptosis.

	Cells (%)			
Compound	Live	Early apoptosis	Late apoptosis	necrosis
Untreated	88.62	2.98	3.06	5.34
Camptothecin	79.08	14.78	2.30	3.85
2 (1 x IC_{50})	80.48	9.00	4.29	6.23
2 (3 x IC_{50})	72.03	13.37	6.16	8.44
8 (1 x IC_{50})	71.11	10.65	4.92	13.32
8 (3 x IC_{50})	84.00	1.28	0.91	13.81

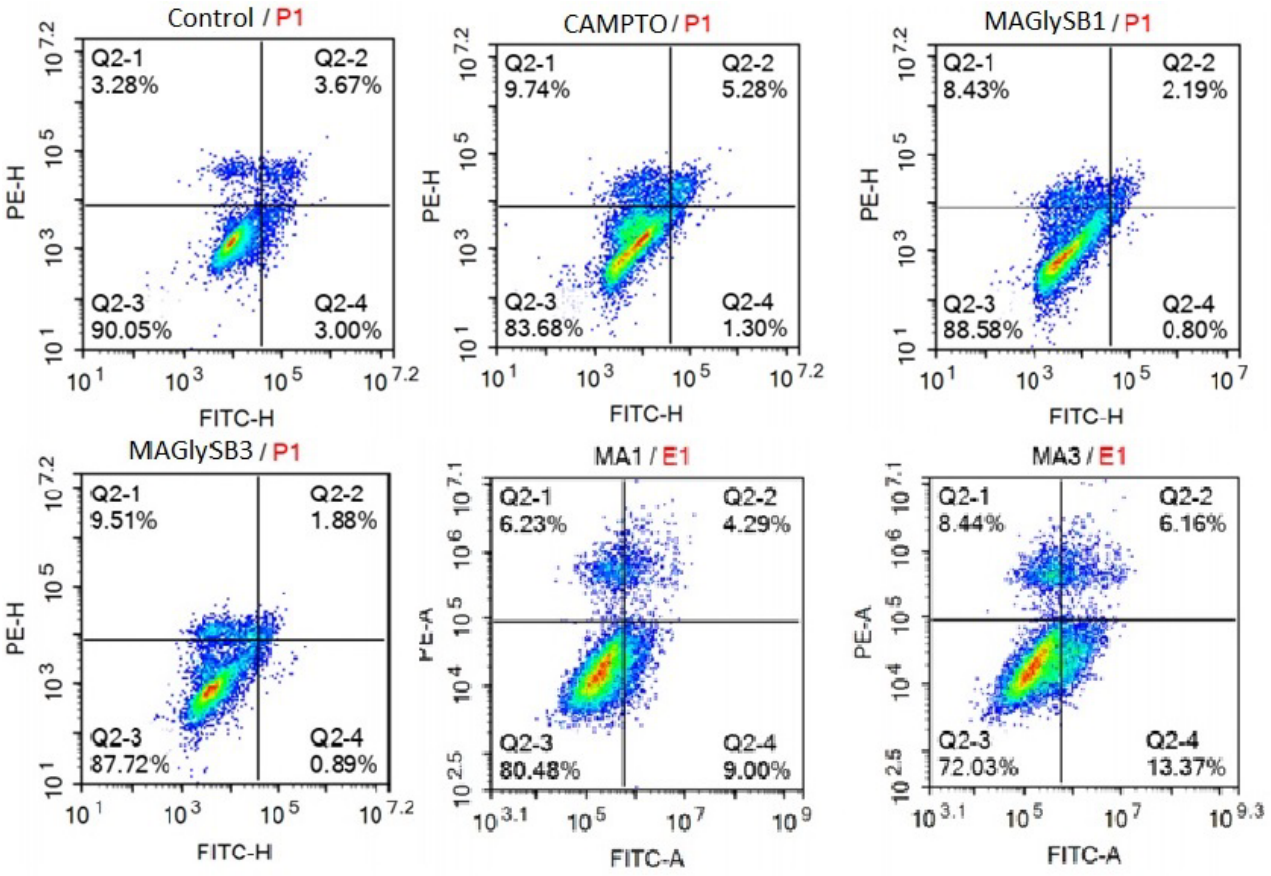


Figure 2. The effect of MA (**2**) and compound 8 on early and late apoptosis and necrosis. HepG2 cells were incubated with compounds at 1 x GI_{50} and 3 x GI_{50} for each compound for 24 hours followed by Annexin V-FITC and PI staining and analysis by flow cytometry. Camptothecin treated cells were used a positive control with untreated cells as the negative control.

Because cysteinyl aspartate specific proteinase-3 (caspase-3) plays an important role in the apoptotic pathway, the capacity of madecassic acid (**2)** and compound **8** to induce caspase 3 activity was measured at 1, 6, and 24 hours post treatment. The results in **Table 4** demonstrate that compound **2** caused a significant increase in caspase 3 activity in the HepG2 cells at 6 h post treatment, and at 3 x GI_{50}, with a fold change vs control of 1.71. Interestingly compound **8** significantly enhanced caspase 3 activity at 1, 6, and 24 hrs post treatment with 3x GI_{50}, and at 24 hours post treatment with both 0.3x and 1x GI_{50} concentrations. 24 hours post treatment.

Table 4. The effect of Madecassic acid (**2**) and compound **8** on caspase 3 activity.

Compound	Conc.	Fold change of activities					
		1h		6h		24h	
		Mean	SE	Mean	SE	Mean	SE
	0.3 x GI_{50}	0.74	0.02	0.81	0.02	0.99	0.10
2	1.0 x GI_{50}	0.94	0.10	1.16	0.05	0.68	0.04
	3.0 x GI_{50}	0.97	0.02	**1.71****	0.09	0.54	0.01
	0.3 x GI_{50}	0.59	0.03	0.76	0.04	**1.62*****	0.01
8	1.0 x GI_{50}	0.76	0.09	1.09	0.06	**1.86*****	0.05
	3.0 x GI_{50}	**1.69***	0.14	**1.27***	0.13	**1.37***	0.01
Camptothecin	0.5 µM	1.94	0.05	2.21	0.05	**2.19*****	0.02
Untreated cells		1.00	0.05	1.00	0.03	1.00	0.01
* P<0.05; ** P<0.01; *** P<0.001							

2.2.4. Cell cycle analysis

According to Pucci and college [38], there is an important linkage between the apoptosis and the cell cycle because mitosis and apoptosis express close morphological characteristics and so the effects of madecassic acid (**2**) and compound **8** on the cell cycle profile of HepG2 cells was investigated using PI staining, 24 hours post compound treatment. With 1 x GI_{50} of madecassic acid (**2**), there was a reduction in the proportion of cells in G0/G1 and G2/M with a concomitant increase in cells in S phase (**Table 5** and **Figure. 3**). The effects at 3 x GI_{50} of compound **2** were different, with the cell number at both S phase and G2/M reduced and a high number of cells in G0/G1, when compared the vehicle control (0.5% DMSO). In contrast to madecassic acid (**2**), compound **8** induced cell cycle arrest in S phase with a concomitant decrease in the number of cells in G2/M whilst the number of cells in G0/G1 phase was unchanged compared with the vehicle control at both 1 and 3x GI_{50} of compound **8**.

Table 5. Effects of compounds (**2** and **8**) on the cell cycle.

Compound	% G0/G1	% S	% G2/M
Control (DMSO 0.5%)	24.63	46.83	27.69
2 (1 x IC_{50})	19.29	58.52	21.51
2 (3 x IC_{50})	54.22	24.25	20.71
8 (1 x IC_{50})	26.72	61.09	9.78

8 (3 x IC50)	27.07	60.41	11.80

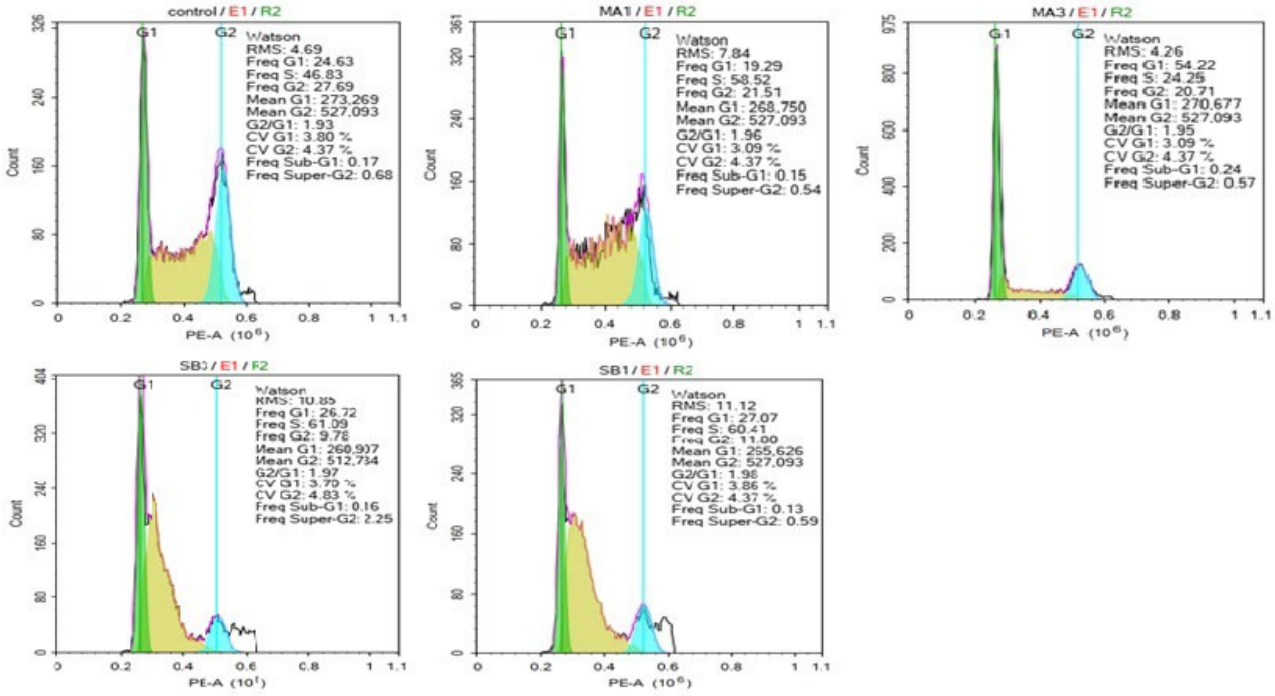


Figure 2. The cell cycle effects of madecassic acid **(2)** and compound **8**. HepG2 cells were incubated with compounds at concentration of 1 and 3 x GI_{50} for 24 hours followed by PI staining and analysis by flow cytometry. Untreated cells served as a negative control.

3. Conclusion

Sixteen novel conjugates of madecassic acid and silybin have been synthesized through esterification or using amino acids as linkers. The formation of esters at positions 3 or 7 could be controlled depending on the reaction conditions. The new conjugates were evaluated for their cytotoxicity on the HepG2 liver cancer cell lines HepG2. Nine compounds showed higher cytotoxic activity than that of silybin and five compounds exhibited greater cytotoxic activity than madecassic acid itself. Among them three compounds were very strongly active, and of these, compound **8** was taken forward for further evaluation. Compound **8** demonstrated strong cytotoxic activity against an additional three liver cancer cell lines (HepG3, Huh7 and Huh7R) along with S phase cell cycle arrest and rapid induction of caspase 3 activity in HepG2 cells, which is distinct from madecassic acid (**2).** These results provide the first evidence of a distinct biological profile of a madecassic acid-silybin conjugate versus madecassic acid (**2**) alone, and could enable the development of treatments which combine cytotoxic effects on liver cancer cells with broader hepatoprotection.

On a broader level, this work provides support for the concept of combining two bioactive compounds into a single hybrid molecule which has the potential not just for simplification of formulation and

dosage, but also giving biological properties which are enhanced or distinct from the two molecules individually.

4. Experimental section

4.1. Chemistry

Chemicals were purchased from Merck and Sigma-Aldrich and used without further purification. Madecassic acid was isolated and purified from *Centella asiatica* cultivated from Hue province by our laboratory. Solvents were redistilled before being used. NMR spectra (^{1}H, ^{13}C, HMBC, HSQC) were recorded on a Bruker AVANCE 500 MHz with tetramethylsilane (TMS) as the internal standard for 1H and solvent signals for ^{13}C NMR. Chemical shifts are reported in parts per million (δ ppm). *J* coupling constants in Hz. Proton spectra multiplicities are abbreviated as: MA madecassic acid, SB silybin, s singlet, brs broad single, d doublet, t triplet, q quartet, quin quintet, m multiplet, dd doublet of doublets, dt doublet of triplets, td triplet of doublets. Electrospray ionization (ESI) mass spectra were measured on a 1100 Agilent LC/MS ion trap. Reactions were monitored by thin-layer chromatography using silica gel G60 F254 (Merck). Silica gel 300–400 mesh (Merk) was used for column Chromatography.

Hepato-carcinoma cell lines (hepG2, hep3B, Huh and Huh7R) were the kindly gift of Prof. Chi-Ying Huang, Taiwan. DMEM (Gibco, USA) consisted of 10% fetal bovine serum (FBS, Gibco), 2mM L-Glutamine (Gibco, USA) and 1% Anti-Anti was used for growth cancer cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), ribonuclease (RNase), and propidium iodide (PI) were original from Sigma Chemical (St. Louis, MO, USA). The eBioessence^{TM} Annexin V Apoptosis Detection Kit was purchased from Invitrogen (Carlsbad, CA, USA). Caspase-3 assay kit (colorimetric) sourced from Abcam (Cambridge, UK).

4.1.1. Synthesis of madecassic acid derivatives

*4.1.1.1. 2α,3β,23-Triacetyloxy-6β-hydroxyurs-12-en-28-oic acid (**3**)*

A stirred solution of madecassica cid **2** (1 g, 2.0 mmol) in pyridine (10 mL) was treated with acetic anhydride (600 mg, 6.0 mmol) and stirred for 12 h at room temperature. Reaction mixture was concentrated under reduced pressure and ethyl acetate (200 mL) was added. The solution was washed with 1N HCl, brine solution, and dried over Na_2SO_4. Removal of solvent and purification over SiO_2 column (n-Hexane/EtOAc; 2/1) gave **3** (1.60 g, 84%) as a white powder. R_f = 0.25 (n-Hexane/EtOAc; 2/1). ESI-MS *m/z*: 629.0 $[M-H]^-$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.28 (1H, t, *J* = 3.5 Hz, H-12), 5.23 (1H, td, *J* = 11.0, 4.5 Hz, H-2), 5.01 (1H, d, *J* = 10.5 Hz, H-3), 4.34 (1H, brs, H-6), 3.94, 3.71 (each 1H, d, *J* = 12.0 Hz, H-23), 2.12 (1H, d, *J* = 11.0 Hz, H-18), 2.09 (1H, m, H-11), 2.09 (3H, s, AcO), 2.04 (1H, m, H-1), 2.05 (3H, s, AcO), 1.98 (3H, s), 1.85 (1H, m, H-11), 1.75-1.65 (5H, m), 1.53 (1H,

m, H-9), 1.49 (3H, s, H-25), 1.36-1.29 (3H, m), 1.28 (3H, s, H-24), 1.13 (1H, m, H-1), 1.05 (3H, s, H-27), 1.05 (3H, s, H-26), 0.94 (3H, d, J = 6.5 Hz, H-30), 0.86 (3H, d, J = 6.5 Hz, H-29); ^{13}C NMR (125 MHz, $CDCl_3$) δ 183.6 (C-28), 170.8 (C23-OAc), 170.4 (2xOAc), 137.2 (C-13), 125.6 (C-12), 74.9 (C-3), 69.9 (C-2), 67.9 (C-6), 65.4 (C-23), 52.4 (C-18), 48.2 (C-17), 47.9 (C-9), 47.8, 45.8 (C-1), 42.4 (C-4; C-14), 40.7, 39.1, 38.8, 38.6, 37.3, 36.6, 30.6, 27.9, 24.1, 23.5, 23.3 (C-27), 21.1 (C-30), 21.0 (AcO), 21.0 (AcO), 20.8 (AcO), 18.6 (C-25), 18.4 (C-26), 16.9 (C-29), 15.3 (C-24).

*4.1.1.2. Synthesis of esters **4** and **5***

A stirred solution of acid **3** (50 mg, 0.079 mmol) in dry DCM (5 mL) was added oxalyl chloride (0.2 mL). After being stirred at room temperature for 18 h, the reaction mixture was concentrated under reduced pressure to dryness, which was then treated with silybin (57 mg, 0.119 mmol) and triethyl amine (0.03 mL, 0.237 mmol) in dry DCM (5 mL). Reaction mixture was stirred further overnight and purified over a silica gel column chromatography (DCM/MeOH, 25/1) to obtain esters **4** (30 mg, 40%) and **5** (16 mg, 20%).

*7-O-(2α,3β,23-Triacetyloxy-6β-hydroxyurs-12-en-28-oyl)silybin (**4**)*

White amorphous powder, R_f = 0.15 (DCM/MeOH; 25/1), ESI-MS *m/z*: 1095 $[M+H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.05 (1H, s, OH), 7.18 (1H, dd, J = 1.0 Hz, 9.0 Hz), 7.05 (2H, m), 6.95 (1H, s), 6.93 (2H, m, overlap), 6.25 (1H, m), 6.20 (1H, m), 6.75 (1H, s), 6.48 (1H, s), 5.40 (1H, brs, H-12-MA), 5.23 (1H, td, J = 11.0, 4.5 Hz, H-2), 5.76 (1H, brs, OH), 5.56 (1H, m), 5.46 (1H, m), 5.29 (1H, td, J = 11.0, 4.0 Hz, H-2-SB), 5.15 (1H, m), 5.04 (1H, d, J = 12.0 Hz, H-3-SB), 4.96 (1H, d, J = 8.5 Hz), 4.58 (1H, d, J = 5.0 Hz), 4.26 (1H, s, H-6-MA), 4.06 (1H, m), 3.92 (1H, overlap, H-23-SB), 3.92 (3H, OMe), 3.79 (1H, d, J = 12.6 Hz), 3.69 (1H, d, J = 12.0 Hz, H-23-SB), 3.62 (1H, d, J = 12.6 Hz), 3.55 (1H, dd, J = 3.5 Hz, 12.5 Hz), 3.47 (1H, brs, OH), 2.21 (1H, d, J = 12.0 Hz), 2.11 (2H, overlap, H-18, H-11-MA), 2.07 (3H, s, AcO), 2.04 (3H, s, AcO), 1.98 (3H, s), 1.27 (3H, s, H-25-MA), 1.21 (3H, overlap, H-24-MA), 1.12 (3H, s, H-27-MA), 1.04 (3H, s, H-26-MA), 0.90 (3H, d, J = 6.0 Hz, H-30-MA), 0.80 (3H, d, J = 6.0 Hz, H-29-MA). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.3 (CO-SB), 174.7 (C-28), 171.0 (OAc), 170.8/170.5/170.4 (OAc), 170.3 (OAc), 162.8, 159.9, 146.9, 146.9, 144.3, 143.9, 135.1, 129.1, 127.8, 127.1, 123.8, 122.6, 121.2, 120.8, 117.4/117.4, 116.5/116.2, 114.7, 109.6, 103.9, 103.6, 102.2, 83.1, 78.3, 76.4, 74.8 (C-3-SB), 73.6, 72.6, 70.1, 69.1 (C-2-SB), 68.2 (C-6-SB), 64.9 (C-23-SB), 61.6, 56.1, 54.0, 53.4 (C-18-MA), 49.6 (C-17-MA), 49.2 (C-9-MA), 47.6, 46.5/46.3 45.0 (C-1-MA), 44.2, 43.7, 43.3, 42.3 (C-4; C-14), 41.4, 40.9, 38.8, 38.3, 36.6/36.1, 32.2, 30.5, 29.7, 24.0, 23.4 (C-27), 21.1 (C-30), 21.0 (AcO), 20.8 (AcO), 19.1 (C-25-MA), 18.6 (C-26-MA), 17.1/17.0 (C-29-MA), 14.1 (C-24-MA).

*7-O-(2α,3β,23-Triacetyloxy-6β-hydroxyurs-12-en-28-oyl)hydnocarpin D (**5**)*

White amorphous powder, R_f = 0.20 (DCM/MeOH; 25/1), ESI-MS *m/z*: 1077 $[M+H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.75 (1H, s, OH), 7.86 (1H, s), 7.80 (1H, d, *J* = 8.0 Hz), 7.09 (1H, d, *J* = 8.0 Hz), 6.96 (3H, m), 6.75 (1H, s), 6.47 (1H, s), 5.40 (1H, brs, H-12-MA), 5.23 (1H, td, *J* = 11.0, 4.5 Hz, H-2-SB), 5.01 (1H, d, *J* = 10.5 Hz), 4.99 (1H, brs), 4.36 (1H, s, H-6-MA), 4.13 (1H, brs), 3.93 (1H, overlap, H-23-SB), 3.93 (3H, OMe), 3.84 (1H, d, *J* = 12.0 Hz), 3.70 (1H, d, *J* = 12.0 Hz, H-23-SB), 3.60 (1H, d, *J* = 10.5 Hz), 2.38 (1H, d, *J* = 11.0 Hz), 2.17 (2H, overlap, H-18, H-11-MA), 2.08 (3H, s, AcO), 2.04 (3H, s, AcO), 1.98 (3H, s, OAc), 1.47 (3H, s, H-25-MA), 1.26 (3H, overlap, H-24-MA), 1.25 (3H, s, H-27-MA), 1.11 (3H, H-26-MA), 0.99 (3H, H-30-MA), 0.90 (3H, d, *J* = 6.5 Hz, H-29-MA). ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.6 (C-28), 172.9 (CO), 170.9 (OAc), 170.4 (2xOAc), 160.5, 156.6, 155.6, 147.1, 146.6, 145.5, 143.9, 136.9, 127.6, 126.2, 123.9, 121.9, 120.9, 117.3, 117.0, 114.8, 109.6, 104.3, 101.1, 78.7, 76.4, 74.9 (C-3-SB), 69.9 (C-2-SB), 67.8 (C-6-SB), 65.3 (C-23-SB), 61.6, 56.1, 52.9 (C-18-SB), 48.9 (C-17-MA), 48.2 (C-9), 47.8, 45.9 (C-1-MA), 42.8, 42.4 (C-4; C-14-MA), 41.1, 39.2, 38.9, 38.8, 37.3, 36.5, 30.6, 28.0, 24.2, 23.4 (C-27-MA), 21.1 (C-30-MA), 20.9 (AcO), 20.8 (2xAcO), 19.1 (C-25-MA), 18.6 (C-26-MA), 16.9 (C-29-MA), 15.3 (C-24-MA).

*4.1.1.3. 3-O-(2α,3β,23-Triacetyloxy-6β-hydroxyurs-12-en-28-oyl)-2,3-dehydrosilybin (**6**)*

Acid **3** (50 mg, 0.079 mmol) was treated with DCC (49 mg, 0.237 mmol), silybin (76 mg, 0.158 mmol) and DMAP (10 mg, 0.079 mmol) in dry THF (5 mL) at room temperature for 34 h. Afterward, an amount of oxalic acid (15 mg, 0.12 mmol) was added to destroy the excess of DCC. The reaction mixture was cooled to 0 ºC for 30 min, then precipitate was removed by filtration. The filtrate was concentrated under reduced pressure to dryness which was subjected over a silica gel column chromatography eluting with DCM/Acetone (5/1) to obtain ester **6** (36 mg, 42%). White amorphous powder, R_f = 0.2 (DCM/Acetone; 5/1), ESI-MS *m/z*: 1093.1 $[M+H]^+$. ^{1}H NMR (600 MHz, $CDCl_3$) δ 11.75 (1H, brs, OH), 7.87 (1H, s), 7.81 (1H, d, *J* = 8.4 Hz), 7.10 (1H, d, *J* = 8.4 Hz), 6.98 (2H, s), 6.96 (1H, overlap), 6.75 (1H, s), 6.48 (1H, s), 5.40 (1H, brs, H-12-MA), 5.23 (1H, td, *J* = 11.0, 4.5 Hz, H-2-MA), 5.01 (1H, brs, H-3-MA), 4.36 (1H, brs, H-6-MA), 4.13 (1H, m), 3.95 (1H, overlap, H-23-SB), 3.94 (3H, OMe-SB), 3.85 (1H, d, *J* = 12.6 Hz), 3.72 (1H, d, *J* = 12.0 Hz, H-23-SB), 3.61 (1H, d, *J* = 12.6 Hz), 2.12 (1H, overlap, H-18-MA), 2.10 (1H, m, H-11-MA), 2.07 (3H, s, AcO), 2.04 (1H, m, H-1-MA), 2.05 (3H, s, AcO), 1.99 (3H, s), 1.94-1.80 (10H, m), 1.47/1.46 (3H, s, H-25-MA), 1.28 (3H, overlap, H-24-MA), 1.15/1.14 (3H, s, H-27-MA), 1.11 (3H, s, H-26), 0.99 (3H, d, *J* = 6.0 Hz, H-30-MA), 0.90 (3H, d, *J* = 6.0 Hz, H-29-MA). ^{13}C NMR (150 MHz, $CDCl_3$) δ 175.6 (C-28), 174.9, 170.9 (OAc), 170.5 (2xOAc), 160.5, 156.7 (C-2-SB), 147.1/146.1, 146.6/145.5, 143.9, 136.9/136.4, 132.5 (C-3-SB), 130.9, 128.8, 127.6, 126.2, 123.9, 121.9, 120.8, 117.3, 117.0, 114.8, 109.6, 106.9, 104.3, 101.1, 78.8, 76.4, 74.9 (C-3-MA), 69.9 (C-2-MA), 68.2, 67.9 (C-6-MA), 65.3

(C-23-SB), 61.6, 56.1, 52.9 (C-18-MA), 48.9 (C-17-MA), 48.1 (C-9-MA), 47.8, 45.9 (C-1-MA), 42.9, 42.4 (C-4; C-14-MA), 41.1, 39.2, 38.9, 38.8, 37.3, 36.5, 30.6, 28.0, 24.2, 23.8, 23.4 (C-27-MA), 21.1 (C-30), 20.9 (AcO), 20.8 (AcO), 19.1 (C-25-MA), 18.6 (C-26-MA), 16.9 (C-29-MA), 15.3 (C-24-MA).

4.1.1.4. 3-O-(2α,3β,23-Triacetyloxy-6β-hydroxyurs-12-en-28-oyl)-5,7,20,23-O-tetraacetyl-2,3-dehydrosilybin (7)

A stirred solution of **6** (15 mg, 0.023 mmol) in pyridine (1 mL) was treated with acetic anhydride (0.1 mL) and stirred further 12 h at room temperature. The reaction mixture was concentrated under reduced pressure and ethyl acetate (20 mL) was added. The organic phase was washed brine solution (2x10 mL) and dried over Na_2SO_4. Removal of solvent and purification over SiO_2 column (n-Hexane-EtOAc; 2/1) gave **7** (14 g, 78%) as a grey foam. R_f = 0.15 (n-Hexane/EtOAc; 3/1), ESI-MS *m/z*: 1262 $[M+H]^+$. ^{1}H NMR (600 MHz, $CDCl_3$) δ 11.75 (1H, brs, OH), 7.50 (1H, s), 7.45 (1H, m), 7.25 (1H, d, *J* = 8.4 Hz), 7.10 (2H, m), 6.99 (2H, overlap), 6.77 (1H, s), 6.48 (1H, s), 5.41 (1H, brs, H-12-MA), 5.22 (1H, td, *J* = 11.0, 4.5 Hz, H-2-MA), 5.01 (2H, overlap, H-3-MA), 4.41 (1H, d, *J* = 12 Hz), 4.36 (1H, s, H-6-MA), 4.33 (1H, m), 4.03 (1H, m, H-23-SB), 4.39 (1H, dd, *J* = 11.4, 4.2 Hz), 3.95 (1H, overlap, H-23-SB), 3.93 (3H, OMe), 3.72 (1H, d, *J* = 12.0 Hz, H-23-MA), 2.42 (3H, s, OAc), 2.35 (1H, overlap), 2.33 (3H, s, OAc), 2.32 ((3H, s, OAc), 2.12 (2H, overlap, H-18, H-11-MA), 2.07 (3H, s, AcO), 2.05 (3H, s, OAc), 2.03 (3H, s, OAc), 1.98 (3H, s, AcO), 1.99 (3H, s), 1.94-1.80 (10H, m), 1.47/1.46 (3H, s, H-25-MA), 1.28 (3H, overlap, H-24-MA), 1.11 (3H, s, H-27-MA), 1.10 (3H, s, H-26-MA), 0.99 (3H, *J* = 6.0 Hz, H-30), 0.88 (3H, d, *J* = 6.0 Hz, H-29-MA). ^{13}C NMR (150 MHz, $CDCl_3$) δ 174.5 (C-28), 170.9 (OAc), 170.5 (2xOAc), 170.4, 170.2, 169.4, 168.8, 167.9, 156.7 (C-2-SB), 154.8, 154.7, 151.8, 150.3, 145.7, 143.5, 143.5, 140.7, 136.9, 134.1, 133.5 (C-3-SB), 130.9, 128.8, 126.2, 123.4, 119.8, 117.6, 117.4, 114.5, 113.6, 111.1, 108.7, 76.4, 75.9, 74.9 (C-3-MA), 69.9 (C-2-MA), 68.2, 67.8 (C-6-MA), 65.3 (C-23-SB), 62.5, 56.1, 52.9 (C-18-MA), 49.3 (C-17-MA), 48.0 (C-9), 47.8, 45.9 (C-1), 42.7, 42.4 (C-4; C-14-MA), 41.0, 39.1, 38.9, 38.8, 37.3, 36.4, 30.6, 28.9, 24.2, 23.8, 23.4 (C-27), 21.1 (2xOAc), 20.9 (AcO), 20.8 (AcO), 20.7 (2xOAc), 18.9 (C-25-MA), 18.6 (C-26-MA), 16.9 (C-29-MA), 15.3 (C-24-MA).

4.1.2. General synthesis of madecassic acid conjugated with silybin over linkers

A solution of *2α,3β,23*-triacetyloxy-*6β*-hydroxyurs-12-en-28-oic acid **3** (100 mg, 0.16 mmol) in dry DCM (5 mL) was treated with oxalyl chloride (0.07 mL, 0.8 mmol). After stirring for 14 h at room temperature, the solvent was removed under reduced pressure to dryness and the resulting residue was redissolved in dry DCM (5 mL) followed by addition of amino acid (glycine; or glycine methyl ester or; or *β*-alanine; or 11-aminoundecanoic acid; or methyl 11-aminoundecanoic acid methyl ester;

1.1 equiv.) in the presence of TEA (0.07 mL, 0.5 mmol). The reaction mixture was stirred for 18 h, and concentrated to dryness which was subsequently exposed to silybin (1.2 equiv.), DCC (1.5 equiv.) and DMAP (1 equiv.) in dry THF at 0 ºC. The reaction mixture was stirred at room temperature for 48 h, then an amount of oxalic acid (0.5 equiv.) was added slowly to quenched the excess amount of DCC. The reaction was stirred for 15 min and cooled to -5-0 ºC for 1 h. The precipitated solid was removed by filtration. Removal of solvent and purification over a silica gel chromatography column, using DCM/Acetone (4/1) provide product **8, 9** and **10**.

*4.1.2.1. Silybin-3-yl (N-(2α,3β,23-triacetyloxy-6β-hydroxyurs-12-en-28-oyl))aminoacetate (**8**)*

Yield 25% (46 mg), white amorphous powder, R_f = 0.18 (DCM/Acetone (4/1), ESI-MS *m/z*: 1175 $[M+Na]^+$. ^{1}H NMR (600 MHz, CD_3OD) δ 7.09 (1H, d, J = 6.6 Hz), 7.04 (1H, d, J = 4.8 Hz), 7.02 (2H, m), 6.93 (1H, m), 6.86 (1H, m), 5.98 (1H, s, H-6-SB), 5.96 (1H, s, H-8-SB), 5.84 (1H, t, J = 11.4 Hz, H-3-SB), 5.39 (2H, overlap, H-12, H-2-SB), 5.26 (1H, m, H-2), 4.97 (1H, d, J = 5.5 Hz, H-10-SB), 4.95 (1H, m, H-3), 4.29 (1H, brs, H-6), 4.11 (1H, m, H-11-SB), 3.97 and 3.92 (each 1H, overlap, CH_2-Gly), 3.92 and 3.77 (each 1H, d, J = 12.0 Hz, H-23), 3.90 (3H, s, OMe-SB), 3.73 (1H, d, J = 10.8 Hz, H-23-SB), 3.51 (1H, dd, J = 12.6, 4.8 Hz, H-23-SB), 2.11 (2H, m, overlap, H-11, H-18), 2.05 (3H, s, AcO), 2.04 (3H, s, AcO), 1.99 (3H, s, AcO), 1.87 (1H, m), 1.74 (1H, m, H-11), 1.68-1.65 (4H, m), 1.48 (3H, s, H-25), 1.36-1.29 (3H, m), 1.30 (3H, s, H-24), 1.12 (3H, s, H-27), 1.05 (3H, s, H-26), 0.98 (3H, d, J = 7.2 Hz, H-30), 0.91 (3H, d, J = 7.2 Hz, H-29). ^{13}C NMR (150 MHz, CD_3OD) δ 192.2, 180.3 (C-28), 172.6 (C23-OAc), 172.3 (OAc), 172.2 (OAc), 170.5 (COOH), 165.5, 164.0, 149.3, 148.4, 145.9, 145.4, 139.1 (C-13), 129.9, 129.4, 127.3 (C-12), 121.8, 118.3, 117.4, 116.3, 112.2, 102.1, 97.8, 96.7, 82.0 (C-2-SB), 80.1 (C-11-SB), 77.8 (C-3), 76.7 (C-10-SB), 74.5 (C-3-SB), 71.2 (C-2), 67.9 (C-6), 66.3 (C-23), 62.1 (C-23-SB), 56.6 (19-OMe-SB), 54.3 (C-18), 48.1 (C-17), 47.0 (C-9), 43.7, 41.9 (CH_2-Glycine), 41.2, 40.8, 40.2, 40.1, 38.6, 38.4, 31.9, 28.9, 25.3, 24.4, 24.1, 21.5 (C-30), 20.9 (AcO), 20.7 (2xAcO), 19.1 (C-25), 18.7 (C-26), 17.4 (C-29), 15.7 (C-24).

*4.1.2.2. Silybin-3-yl (N-(2α,3β,23-triacetyloxy-6β-hydroxyurs-12-en-28-oyl))-3-aminopropanoate (**9**)*

Yield 20% (36 mg), white amorphous powder, R_f = 0.15 (DCM/Acetone (3/1), ESI-MS *m/z*: 1167 $[M+H]^+$.^{1}H NMR (600 MHz, CD_3OD) δ 7.16-7.08 (2H, m), 7.03 (2H, m), 6.91 (1H, s), 6.85 (1H, s), 5.97/5.95 (1H, s, H-6-SB), 5.92/5.90 (1H, s, H-8-Silybin), 5.86 (1H, t, J = 12.6 Hz, H-3-SB), 5.36 (2H, overlap, H-12, H-2-SB), 5.23 (1H, m, H-2), 4.97 (1H, d, J = 10.2 Hz, H-10-SB), 4.92 (1H, m, H-3), 4.29 (1H, brs, H-6), 4.12 (1H, m, H-11-SB), 3.90 and 3.75 (each 1H, m, overlap, H-23), 3.89/3.87 (3H, s, OMe), 3.74 (1H, m, H-23-SB), 3.57 (1H, m, β-CH_2-alanine), 3.51 (1H, overlap, H-23-SB), 3.45 (1H, m, β-CH_2-alanine), 2.78 (1H, t, J = 6.0 Hz, α-CH_2-alanine), 2.49 (1H, m, α-CH_2-

alanine), 2.11 (2H, m, overlap, H-11, H-18), 2.09 (3H, s, AcO), 2.07 (3H, s, AcO), 1.99 (3H, s, AcO), 1.83 (2H, m), 1.72-1.65 (4H, m), 1.44 (3H, s, H-25), 1.29 (3H, s, H-24), 1.11 (3H, s, H-27), 1.07 (3H, s, H-26), 0.97 (3H, d, J = 7.2 Hz, H-30), 0.91 (3H, d, J = 7.2 Hz, H-29). ^{13}C NMR (150 MHz, CD_3OD) δ 178.4 (C-28), 175.5 (COOH), 170.9 (C23-OAc), 170.6 (OAc), 170.5 (OAc), 138.1 (C-13), 126.0 (C-12), 74.9 (C-3), 70.1 (C-2), 67.5 (C-6), 65.3 (C-23), 53.7 (C-18), 48.1 (C-17), 47.8 (C-9), 47.8, 45.9 (C-1), 42.9, 42.4 (C-4), 39.7, 38.9, 38.7, 37.3, 34.8, 33.8, 31.6, 30.9, 27.7, 25.3, 24.6, 23.8 (C-27), 21.2 (C-30), 20.9 (AcO), 20.8 (AcO), 20.6 (AcO), 18.6 (C-25), 18.4 (C-26), 17.1 (C-29), 15.3 (C-24).

*4.1.2.3. Silybin-3-yl (N-(2α,3β,23-triacetyloxy-6β-hydroxyurs-12-en-28-oyl))-11-amino-undecanoate (**10**)*

Yield 26% (50 mg), white amorphous powder, R_f = 0.2 (DCM/Acetone (4/1), ESI-MS *m/z*: 1279 $[M+H]^+$.^{1}H NMR (500 MHz, $CDCl_3$) δ 7.05 (3H, m), 6.95-6.89 (3H, m), 5.98 (1H, s), 5.95 (1H, s), 5.72 (1H, t, J = 12.5 Hz, H-3-SB), 5.32 (1H, brs, H-12-MA), 5.21 (1H, td, J = 11.0, 4.5 Hz, H-2-SB), 5.02 (1H, m, overlap, H-3-MA), 4.98 (1H, overlap), 4.49 (1H, m), 4.26 (1H, brs, H-6-MA), 4.18 (1H, t, J = 7.0 Hz), 4.01 (1H, brs, H-23), 3.88/3.81 (3H, s, OMe), 3.73 (1H, overlap, CH_2-23-SB), 3.72 (1H, d, J = 12.0 Hz, H-23-SB), 3.56 (1H, m, CH_2-23-SB), 3.20 (2H, m, overlap, CH_2-undecanoic acid), 2.56 (2H, t, J = 7.0 Hz, CH_2-undecanoic acid), 2.03 (3H, s, AcO), 2.00 (3H, s, AcO), 1.95 (3H, s, OAc), 1.94 (1H, m), 1.82 (2H, m), 1.74-1.57 (7H, m), 1.48-1.37 (10H, m), 1.26-1.19 (26H, m, CH_2-undecanoic acid, H-24), 1.03 (3H, s, H-27), 1.02 (3H, s, H-26), 0.92 (3H, H-30), 0.85 (3H, d, J = 6.5 Hz, H-29). ^{13}C NMR (125 MHz, $CDCl_3$) δ 185.6 (C-4-SB), 176.7 (C-28), 172.2 (COO-3-SB), 171.4, 170.9 (OAc), 139.3, 135.2, 134.3 (C-13), 132.5, 131.1, 128.9, 125.6 (C-12), 120.8, 119.9, 116.5, 110.0, 97.2, 96.1, 83.0 (C-2-SB), 78.5, 75.1, 73.9 (C-3), 73.2 (C-3-SB), 70.2 (C-2), 68.3, 67.2 (C-6), 65.3 (C-23), 61.4 (C-23-SB), 56.1 (OMe-SB), 54.2, 53.5 (C-18), 48.1 (C-17), 47.8 (C-9), 45.9 (C-1), 43.0, 42.5 (C-4), 39.9, 39.8 (CH_2-undecanoic acid), 38.8, 37.4, 34.1 (CH_2-undecanoic acid), 29.8-29.1 (8xCH_2), 28.8, 27.1, 24.8, 23.8, 23.3 (C-27), 21.2 (C-30), 21.1 (OAc), 20.9 (OAc), 20.8 (AcO), 18.6 (C-25), 18.2 (C-26), 17.2 (C-29), 15.3 (C-24).

4.1.3. General acetylation of conjugated madecassic acid derivatives

A stirred solution of ester **8**, or **9**, or **10** (20 mg) in pyridine (1 mL) was treated with acetic anhydride (0.1 mL) and stirred for 18 h at room temperature. Afterward, the reaction mixture was concentrated under reduced pressure and ethyl acetate (40 mL) was added. The solution was washed brine solution (2x10 mL), and dried over Na_2SO_4. Removal of solvent and purification over a SiO_2 column (n-Hexane/EtOAc; 2/1) yielded product **11, 12** and **13**.

*4.1.3.1. (5,7,20,23-O-Tetraacetyl)silybin-3-yl (N-(2α,3β,23-triacetyloxy-6β-hydroxyurs-12-en-28-oyl))aminoacetate (**11**)*

Yield 74% (16 mg), white amorphous powder, R_f = 0.22 (n-Hexane/EtOAc; 2/1), ESI-MS *m/z*: 1321 $[M+H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.09-7.06 (2H, m), 7.04-6.80 (4H, m), 6.78 (1H, d, J = 2.0 Hz), 6.60 (1H, d, J = 2.0 Hz), 6.45 (1H, br, NH), 5.64 (1H, t, J = 12.5 Hz, H-3-SB), 5.42 (1H, brs, H-12-MA), 5.38 (1H, dd, J = 12.5, 3.5 Hz, H-2-SB), 5.23 (1H, td, J = 11.0, 4.5 Hz, H-2-MA), 5.01 (1H, dd, J = 10.5, 3.0 Hz), 4.96 (1H, d, J = 10.5 Hz, H-3), 4.36 and 3.99 (each 1H, dd, J = 13.5, 9.0 Hz, CH_2-23-SB), 4.29 (1H, brs, H-6), 4.09 and 3.98 (1H, dd, J = 19.0, 5.5 Hz, CH_2-Gly), 3.97 (1H, overlap, H-23-MA), 3.87/3.86 (3H, s, OMe), 3.72 (1H, d, J = 12.0 Hz, H-23), 2.35 (3H, s, AcO), 2.32 (3H, s, AcO), 2.30 (3H, s, AcO), 2.12 (1H, m, overlap, H-11), 2.06 (3H, s, AcO), 2.05 (3H, s, AcO), 2.03 (3H, s, AcO), 1.90 (3H, s, OAc), 1.70-1.67 (9H, m, H-11), 1.48 (3H, s, H-25), 1.38-1.29 (6H, m), 1.26 (3H, s, H-24), 1.05 (3H, s, H-27), 1.02 (3H, s, H-26), 0.92 (3H, H-30), 0.87 (3H, d, J = 6.5 Hz, H-29). ^{13}C NMR (125 MHz, $CDCl_3$) δ 184.4 (C-4-SB), 177.9 (C-28), 170.8 (C-23-OAc), 170.4 (2xOAc), 170.3 (OAc), 169.1 (COO-3-SB), 168.8 (CO-Gly), 167.8 (2xOAc), 162.5, 156.6, 151.7, 151.5, 144.1, 143.7, 140.6, 134.4 (C-13), 130.8, 128.8, 128.1, 126.3 (C-12), 123.2, 120.7, 119.9, 117.6, 116.4, 111.3, 109.1, 80.8 (C-2-SB), 76.4, 75.7, 74.9 (C-3-MA), 74.5 (C-3-SB), 69.9 (C-2-MA), 67.6 (C-6-MA), 65.3 (C-23-MA), 62.6 (C-23-SB), 56.1 (OMe-SB), 53.6 (C-18-MA), 48.1 (C-17-MA), 47.9 (C-9-MA), 47.8, 45.9 (C-1-MA), 42.8 (C-4-MA), 41.1 (CH_2-Glycine), 39.7, 38.9, 38.8, 37.3, 36.9, 30.9, 28.9, 27.7, 24.8, 23.8, 23.4 (C-27), 21.2 (C-30), 21.7 (OAc), 20.9 (2xAcO), 20.8 (AcO), 20.7 (2xAcO), 20.6 (2xAcO), 18.6 (C-25), 18.0 (C-26), 17.1 (C-29), 15.3 (C-24).

*4.1.3.1. (5,7,20,23-O-Tetraacetyl)silybin-3-yl (N-(2α,3β,23-triacetyloxy-6β-hydroxyurs-12-en-28-oyl))-3-aminopropanoate (**12**)*

Yield 61% (13 mg), white amorphous powder, R_f = 0.25 (n-Hexane/EtOAc; 2/1), ESI-MS *m/z*: 1335 $[M+H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.14-7.09 (2H, m), 7.05-6.80 (4H, m), 6.80 (1H, d, J = 2.0 Hz), 6.60 (1H, d, J = 2.0 Hz), 6.32 (1H, t, NH), 5.74/5.73 (1H, d, J = 12.5 Hz, H-3-SB), 5.40 (1H, dd, J = 12.5, 3.5 Hz, H-2-SB), 5.36/5.31 (1H, brs, H-12-MA), 5.21 (1H, td, J = 11.0, 4.5 Hz, H-2-MA), 5.01 (1H, m, overlap), 4.98 (1H, d, J = 10.5 Hz, H-3), 4.36 and 4.03 (each 1H, dt, J = 13.5, 9.0 Hz, CH_2-23-SB), 4.29 (1H, overlap, H-6), 4.27 (1H, overlap), 3.92 (1H, dd, J = 12.0, 6.0 Hz, H-23-MA), 3.87/3.86 (3H, s, OMe), 3.70 (1H, d, J = 12.0 Hz, H-23), 3.59-3.53 (2H, m, β-CH_2-alanine), 2.53 (2H, m, α-CH_2-alanine), 2.35 (3H, s, AcO), 2.32 (3H, s, AcO), 2.30 (3H, s, AcO), 2.10 (3H, s, AcO), 2.04 (3H, s, AcO), 2.03 (3H, s, AcO), 2.0 (3H, s, OAc), 1.97 (OAc), 1.67 (9H, m, H-11-MA), 1.45 (3H, s, H-25-MA), 1.27 (3H, s, H-24-MA), 1.04 (3H, s, H-27-MA), 1.03 (3H, s, H-26-MA), 0.94 (3H, H-30-MA), 0.86 (3H, H-29-MA). ^{13}C NMR (125 MHz, $CDCl_3$) δ 185.9 (C-4-SB), 174.5 (C-28), 171.5 (COO-3-SB), 171.2, 170.9 (C-23-OAc), 170.4 (OAc), 170.1 (OAc), 168.9, 168.8 (OAc), 167.8 (OAc), 162.5, 156.8, 151.7, 151.4, 144.1, 143.9, 140.6, 134.3 (C-13), 130.8, 128.3,

126.0 (C-12), 123.3, 121.2, 119.8, 117.5/117.4, 116.5/116.4, 111.4/111.3, 109.1, 81.0/80.8 (C-2-SB), 76.4, 75.7, 74.9 (C-3), 73.6 (C-3-SB), 69.9 (C-2), 67.7 (C-6), 65.3 (C-23), 62.7/62.6 (C-23-SB), 56.1 (OMe-SB), 53.4 (C-18-MA), 48.1 (C-17-MA), 47.8 (C-9-MA), 47.7, 45.9 (C-1-MA), 42.8 (C-4-MA), 39.7, 38.9, 38.7, 37.3, 35.3 (β-CH_2-alanine), 34.3 (α-CH_2-alanine), 30.9, 29.7, 27.7, 24.6, 23.3 (C-27), 21.2 (C-30), 21.2 (OAc), 21.1 (2xAcO), 20.9 (2xAcO), 20.8(2xAcO), 20.7 (AcO), 18.6 (C-25-MA), 18.4 (C-26-MA), 17.1 (C-29-MA), 15.3/15.2 (C-24-MA).

*4.1.3.2. (5,7,20,23-O-Tetraacetyl)silybin-3-yl (N-(2α,3β,23-triacetyloxy-6β-hydroxyurs-12-en-28-oyl))-11-amino-undecanoate (**13**)*

Yield 68% (15 mg), white amorphous powder, R_f = 0.30 (n-Hexane/EtOAc; 2/1), ESI-MS *m/z*: 1445 $[M+H]^+$. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.12 (1H, brs), 7.09 (1H, m), 7.02-6.90 (4H, m), 6.78 (1H, d, J = 2.0 Hz), 6.58 (1H, d, J = 2.0 Hz), 5.84 (1H, brs, NH), 5.70/5.68 (1H, t, J = 12.5 Hz, H-3-SB), 5.38 (1H, m, overlap, H-2-SB), 5.36 (1H, overlap, H-12-MA), 5.23 (1H, td, J = 11.0, 4.5 Hz, H-2), 5.02 (1H, dd, J = 10.5, 1.5 Hz), 4.94 (1H, m, H-3), 4.37 and 4.00 (each 1H, dd, J = 13.5, 9.0 Hz, CH_2-23-SB), 4.34 (1H, overlap, H-6), 3.94 (1H, d, J = 12.0 Hz, H-23-MA), 3.87/3.86 (3H, s, OMe), 3.72 (1H, d, J = 12.0 Hz, H-23), 3.27 (1H, quin, J = 6.6 Hz, CH_2-undecanoic acid), 3.03 (1H, m, CH_2-undecanoic acid), 2.58 (1H, t, J = 7.8 Hz, CH_2-undecanoic acid), 2.37 (3H, s, AcO), 2.34 (3H, s, AcO), 2.27 (3H, s, AcO), 2.08 (1H, m, overlap, H-11), 2.06-2.03 (13H, overlap, 4xAcO), 1.94 (3H, s, OAc), 1.76-1.67 (9H, m, H-11-MA), 1.49 (3H, s, H-25-MA), 1.29-1.25 (28, m), 1.08 (3H, s, H-27-MA), 1.05 (3H, s, H-26-MA), 0.95 (3H, H-30-MA), 0.88 (3H, d, J = 6.5 Hz, H-29-MA). ^{13}C NMR (150 MHz, $CDCl_3$) δ 185.4 (C-4-SB), 177.7 (C-28), 171.9 (COO-3-SB), 171.6, 170.8 (OAc), 170.4 (2xOAc), 169.1 (OAc), 168.8 (OAc), 167.8 (2xOAc), 162.6, 156.4, 151.7, 151.7/151.4, 143.9, 143.6, 140.6, 139.2, 134.3 (C-13), 130.9, 128.8, 125.3 (C-12), 123.3, 121.3, 119.8, 117.3, 116.5, 111.3, 111.0, 108.9, 81.1/81.0 (C-2-SB), 76.4, 75.6, 74.9 (C-3-MA), 74.9, 73.2 (C-3-SB), 69.9 (C-2-MA), 67.6 (C-6), 65.3 (C-23-MA), 62.7 (C-23-SB), 56.0 (OMe-SB), 54.0, 53.4 (C-18-MA), 48.1 (C-17-MA), 47.8 (C-9), 47.7, 45.9 (C-1-MA), 42.4 (C-4), 40.8, 39.8, 39.5 (CH_2-undecanoic acid), 38.8, 37.3, 34.0 (CH_2-undecanoic acid), 30.9, 29.7-29.1 (8xCH_2-undecanoic acid), 28.9, 27.8, 24.9, 23.8, 23.4 (C-27-MA), 21.2 (C-30), 21.7 (OAc), 21.1 (2xOAc), 20.9 (2xAcO), 20.8 (AcO), 20.7 (AcO), 20.6 (AcO), 18.6 (C-25-MA), 18.3 (C-26-MA), 17.2 (C-29-MA), 15.3 (C-24-MA).

*4.1.4. Synthesis of 2α,3β,23-triacetyloxyursa-5,12-dien-28-oic acid (**14**)*

2α,3β,23-Triacetyloxy-*6β*-hydroxyurs-12-en-28-oic acid **3** (500 mg, 0.79 mmol) in pyridine (4 mL) cooled to 0 °C was added slowly thionyl chloride (0.14 mL, 2 mmol) and stirred for 1 h. The reaction mixture was concentrated to dryness and the residue was added cold water. The solid was collected and purified by a column chromatography (DCM/MeOH; 20/1) on silica gel to obtain **14** as white foam (280 mg, 47%). R_f = 0.30 (DCM/MeOH; 20/1), ESI-MS *m/z*: 612.4 $[M]^+$. ^{1}H NMR (500 MHz,

$CDCl_3$) δ 5.57 (1H, m, H-6), 5.36 (1H, m, H-12), 5.32 (1H, td, J = 11.5, 4.0 Hz, H-2), 5.14 (1H, d, J = 10.5 Hz, H-3), 4.25 and 3.68 (each 1H, d, J = 12.0 Hz, H-23), 2.37 (1H, dd, J = 19.0, 6.5 Hz, H-7), 2.22 (1H, d, J = 11.5 Hz, H-18), 2.08 (3H, s, OAc), 2.03 (3H, s, OAc), 1.98 (3H, s, OAc), 1.86 (4H, m), 1.76-1.65 (5H, m), 1.62 (1H, m, H-7b), 1.53 (1H, m, H-1), 1.35-1.28 (5H, m), 1.2 (3H, s, H-25), 1.13 (3H, s, H-24), 0.96 (3H, s, H-27), 0.93 (3H, H-30), 0.88 (3H, s, H-26), 0.84 (3H, d, J = 6.5 Hz, H-29); ^{13}C NMR (125 MHz, $CDCl_3$) δ 178.2 (C-28), 171.1 (OAc), 170.5 (OAc), 170.3 (OAc), 144.1 (C-5), 139.0 (C-13), 126.6 (C-12), 122.7 (C-6), 73.7 (C-3), 69.1 (C-2), 64.9 (C-23), 53.2 (C-18), 48.4, 46.2 (C-9), 45.1, 43.5, 42.4 (C-1), 38.8, 38.5, 38.3 (C-8, C-10), 36.5, 32.3 (C-7), 30.6, 27.3, 25.6, 23.9, (C-27), 22.9 (C-25), 22.3 (C-29), 21.9 (C-26), 21.3 (OAc), 21.1 (OAc), 20.8 (OAc), 17.1 (C-24).

4.1.5. General synthesis of dehydrated-madecassic acid conjugated with silybin

A stirred solution of *2α,3β,23*-triacetyloxyursa-5,12-dien-28-oic acid **14** (100 mg, 0.163 mmol) in dry DCM (5 mL) was treated with oxalyl chloride (0.07 mL, 0.8 mmol). After stirring for 14 h at room temperature, the solvent was removed under reduced pressure to dryness and the resulting residue was redissolved in dry DCM (5 mL) followed by addition of amino acid (glycine; or glycine methyl ester or; or *β*-alanine; or 11-aminoundecanoic acid; or methyl 11-aminoundecanoic acid methyl ester; 1.1 equiv.) in the presence of TEA (0.07 mL, 0.5 mmol). The reaction mixture was stirred for 18 h, and concentrated to dryness which was subsequently exposed to silybin (1.2 equiv.), DCC (1.5 equiv.) and DMAP (1 equiv.) in dry THF at 0 ºC. The reaction mixture was stirred at room temperature for 48 h, then an amount of oxalic acid (0.5 equiv.) was added slowly to quenched the excess amount of DCC. The reaction was stirred for 15 min and cooled to -5-0 ºC for 1 h. The precipitated solid was removed by filtration. Removal of solvent and purification over a silica gel chromatography column, eluting with DCM/Acetone (4/1) yielded products **15** and **16**.

*4.1.5.1. Silybin-3-yl (N-(2α,3β,23-triacetyloxyursa-5,12-dien-28-oyl))aminoacetate (**15**)*

Yield 23% (40 mg), white amorphous powder, R_f = 0.20 (DCM/Acetone (4/1), ESI-MS *m/z*: 1134 $[M+H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.08 (2H, m), 6.95-6.91 (4H, m), 6.57 (1H, brs, OH), 6.06 (1H, brs), 5.99 (1H, brs), 5.69 (1H, t, J = 12.5 Hz, H-3-SB), 5.53 (1H, m, H-6-MA), 5.46 (1H, m, H-12-MA), 5.29 (1H, m), 5.14 (1H, m, H-2-SB), 4.93 (1H, d, J = 10.5 Hz, H-3-MA), 4.22 and 3.69 (each 1H, d, J = 12.0 Hz, H-23-MA), 4.09 and 3.94 (1H, m, overlap, CH_2-Gly), 3.96/3.95 (3H, OMe), 3.79 (1H, m, H-23-SB), 3.52 (1H, m, H-23-SB), 2.33 (1H, dd, J = 19.0, 6.5 Hz, H-7), 2.18 (1H, d, J = 11.5 Hz, H-18), 2.08 (3H, s, OAc), 2.06 (3H, s, OAc), 1.98 (3H, s, OAc), 1.82 (4H, m), 1.76-1.60 (7H, m), 1.35-1.28 (5H, m), 1.21 (3H, s, H-25), 1.15 (3H, s, H-24), 0.98 (3H, s, H-27), 0.92 (3H, H-30), 0.89 (3H, s, H-26), 0.84 (3H, overlap, H-29). ^{13}C NMR (125 MHz, $CDCl_3$) δ 185.5 (C-4-SB),

178.8 (C-28), 171.1 (OAc), 170.8 (OAc), 170.4 (OAc), 168.8 (COO-3-SB), 166.9, 164.3, 162.3, 147.0, 146.5, 144.3 (C-5), 140.1 (C-13), 137.2,130.9, 128.8, 127.7, 127.1 (C-12), 123.7, 122.6 (C-6), 120.8, 120.5, 117.3, 116.2, 114.8, 109.7, 97.6, 96.2, 82.9 (C-2-SB), 80.5 (C-10-SB), 78.3 (C-11-SB), 76.3 (C-3-SB), 73.5 (C-3-MA), 69.1 (C-2-MA), 65.0 (C-23-MA), 61.6 (C-23-SB), 54.7 (C-18-MA), 48.1, 46.6 (C-9-MA), 45.1, 43.5, 42.3 (C-1-MA), 41.4 (CH_2-Gly), 38.9, 38.8, 38.2 (C-8, C-10-MA), 36.6, 32.3 (C-7-MA), 30.9, 27.0, 23.8 (C-27), 22.9 (C-25), 22.8 (C-29), 21.8 (C-26), 21.1 (OAc), 21.0 (OAc), 20.8 (OAc), 17.3 (C-24-MA), 16.8.

*4.1.5.2. Silybin-3-yl (N-(2α,3β,23-triacetyloxyursa-5,12-dien-28-oyl))-3-aminopropanoate (**16**)*

Yield 21% (38 mg), white amorphous powder, R_f = 0.15 (DCM/Acetone (4/1), ESI-MS *m/z*: 1149 $[M+H]^+$. ^1H NMR (500 MHz, $CDCl_3$) δ 11.42 (1H, s, OH), 7.08-7.05 (2H, m), 6.97-6.91 (4H, m), 6.68 (1H, brs, OH), 6.08 (1H, brs), 6.03 (1H, brs), 5.71 (1H, t, J = 12.5 Hz, H-3-SB), 5.54 (1H, m, H-6-MA), 5.39 (1H, m, H-12-MA), 5.29-5.26 (2H, m), 5.13 (1H, m, H-2-SB), 4.91 (1H, d, J = 10.5 Hz, H-3-MA), 4.23 and 3.68 (each 1H, d, J = 12.0 Hz, H-23), 4.05 (1H, m, H-10-SB), 3.91 (3H, OMe), 3.80 and 3.53 (each 1H, m, H-23-SB),3.59-3.53 (2H, m, β-CH_2-alanine), 2.50 (2H, m, α-CH_2-alanine), 2.33 (1H, m, H-7), 2.18 (1H, m, H-18), 2.08 (3H, s, OAc), 2.04 (3H, s, OAc), 1.99 (3H, s, OAc), 1.76 (4H, m), 1.63 (5H, m), 1.35-1.28 (6H, m), 1.18 (3H, overlap, H-25), 1.12 (3H, s, H-24), 0.96 (3H, overlap, H-27), 0.92 (3H, H-30), 0.89 (3H, s, H-26), 0.86 (3H, overlap, H-29). ^{13}C NMR (125 MHz, $CDCl_3$) δ 184.5 (C-4-SB), 178.7 (C-28), 171.3 (OAc), 170.9 (OAc), 170.4 (OAc), 167.2 (COO-3-SB), 164.3, 162.5, 147.0, 146.5, 144.3 (C-5), 144.2, 143.7, 139.9 (C-13), 137.2, 130.9, 128.8, 128.5, 127.7, 126.6 (C-12), 123.1, 122.7 (C-6), 120.8, 120.5, 117.2, 116.4, 114.8, 109.7, 101.4, 97.6, 96.3, 80.5 (C-2-SB), 78.4 (C-11-SB), 76.4 (C-3-SB), 75.9, 73.6 (C-3-MA), 69.4 (C-2-MA), 68.2, 65.0 (C-23-MA), 61.6 (C-23-SB), 56.1 (OMe), 53.8 (C-18), 48.1, 46.7 (C-9-MA), 45.1, 43.5, 42.3 (C-1-MA), 41.4 (CH_2-Gly), 38.9, 38.8, 38.5 (C-8, C-10-MA), 36.9, 35.3 (β-CH_2-alanine), 33.9 (α-CH_2-alanine), 32.5 (C-7), 30.9, 27.0, 23.8, 22.9 (C-25-MA), 22.8 (C-29), 21.8 (C-26-MA), 20.9 (OAc), 20.8 (OAc), 20.6 (OAc), 17.3 (C-24), 14.0.

*4.1.6. General acetylation of conjugated compounds **15** and **16***

A stirred solution of **15** or **16** (15 mg) in pyridine (1 mL) was treated with acetic anhydride (0.1 mL) and stirred for 14 h at room temperature. The reaction mixture was concentrated under reduced pressure and ethyl acetate (20 mL) was added. The solution was washed brine solution (2x10 mL), and dried over Na_2SO_4. Removal of solvent and purification over a SiO_2 column chromatography (n-Hexane/EtOAc; 2/1) yielded product **17** and **18**.

*4.1.6.1. (5,7,20,23-O-Tetraacetyl)silybin-3-yl (N-(2α,3β,23-triacetyloxyursa-5,12-dien-28-oyl))aminoacetate (**17**)*

Yield 61% (10 mg), white amorphous powder, R_f = 0.25 (n-Hexane/EtOAc; 2/1), ESI-MS *m/z*: 1303 $[M+H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.09 (2H, m), 7.02-6.96 (4H, m), 6.79 (1H, m), 6.59 (1H, m), 6.46 (1H, brs, NH), 5.66 (1H, t, J = 12.5 Hz, H-3-SB), 5.55 (1H, m, H-6-MA), 5.48 (1H, m, H-12-MA), 5.39 (1H, m, H-2-SB), 5.30 (1H, m, H-2-MA), 4.93 (1H, t, J = 10.5 Hz, H-3-MA), 4.37 (1H, dt, J = 9.0, 2.0 Hz, CH_2-23-SB), 4.28 (1H, m, H-10-SB), 4.22 (1H, m, H-23-MA), 4.10 (1H, m, CH_2-Gly), 3.99 (1H, dd, J = 13.5, 9.0 Hz, CH_2-23-SB), 3.92 (1H, m, CH_2-Gly), 3.86 (3H, OMe), 3.72 (1H, d, J = 12.0 Hz, H-23), 2.37 (3H, s, AcO), 2.32 (3H, s, AcO), 2.29 (3H, s, AcO), 2.08 (3H, s, AcO), 2.06 (3H, s, AcO), 2.04 (3H, s, OAc), 1.98 (3H, OAc), 1.78-1.63 (11H, m), 1.35-1.28 (5H, m), 1.22 (3H, H-25-MA), 1.12 (3H, H-24-MA), 0.98 (3H, s, H-27-MA), 0.94 (3H, H-30), 0.88 (3H, s, H-26-MA), 0.85 (3H, overlap, H-29-MA). ^{13}C NMR (125 MHz, $CDCl_3$) δ 184.5 (C-4-SB), 178.0 (C-28), 171.0 (OAc), 170.8 (OAc), 170.4 (OAc), 170.3, 169.1, 169.0 (COO-3-SB), 168.7, 167.8, 162.3, 156.6, 151.7, 151.5, 144.2 (C-5-MA), 143.8, 143.6, 140.6, 140.2 (C-13-MA), 135.1, 134.3, 132.5, 130.9, 128.8, 127.9, 126.9 (C-12-MA), 123.7, 123.3, 122.6 (C-6-MA), 120.9, 120.7, 119.9, 117.6, 116.4, 111.4, 111.2, 110.4, 109.1, 80.8 (C-2-SB), 76.4, 75.7 (C-11-SB), 74.7 (C-3-MA), 74.5 (C-3-SB), 70.1, 69.1 (C-2-MA), 68.2, 64.9 (C-23-MA), 62.6 (C-23-SB), 56.1 (OMe), 53.9 (C-18-MA), 48.3, 46.0 (C-9-MA), 45.1, 43.7, 42.4 (C-1-MA), 41.0 (CH_2-Gly), 38.9, 38.8, 38.8 (C-8, C-10-MA), 38.4, 36.6, 32.3 (C-7-MA), 30.8, 30.6, 30.4, 28.9, 27.6, 23.8, 22.9 (C-25-MA), 22.8 (C-29), 22.4, 21.2 (OAc), 21.1 (OAc), 20.9 (OAc), 20.8 (2xOAc), 20.7 (OAc), 20.6 (OAc), 17.3 (C-24-MA), 16.8.

*4.1.6.2. (5,7,20,23-O-Tetraacetyl)silybin-3-yl (N-(2α,3β,23-triacetyloxyursa-5,12-dien-28-oyl))-3-amino-propanoate (**18**)*

Yield 54% (9 mg), white amorphous powder, R_f = 0.18 (n-Hexane/EtOAc; 2/1), ESI-MS *m/z*: 1317 $[M+H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.12-7.08 (2H, m), 7.00-6.98 (4H, m), 6.80 (1H, brs), 6.41 (1H, brs), 6.46 (1H, brs, NH), 5.74 (1H, t, J = 12.5 Hz, H-3-SB), 5.54 (1H, m, H-6-MA), 5.39 (1H, m, H-12-MA), 5.34-5.32 (2H, m), 5.12 (1H, m, H-2-SB), 4.93 (1H, d, J = 10.5 Hz, H-3-MA), 4.36 and 4.00 (each 1H, d, J = 7.5Hz, CH_2-23-SB), 4.24 and 3.72 (each 1H, m, overlap, H-23-MA), 4.22 (1H, m, H-10-SB), 3.86 (3H, OMe), 3.44 and 3.33 (each 1H, m, β-CH_2-alanine), 2.47 (2H, overlap, α-CH_2-alanine), 2.37 (3H, s, AcO), 2.32 (3H, s, AcO), 2.29 (3H, s, AcO), 2.08 (3H, s, AcO), 2.03 (3H, s, AcO), 2.0 (3H, s, OAc), 1.97 (3H, OAc), 1.93 (5H, m), 1.76-1.63 (15H, m), 1.25 (3H, overlap, H-25-MA), 1.12 (3H, H-24-MA), 0.96 (3H, overlap, H-27-MA), 0.92 (3H, H-30-MA), 0.89 (3H, overlap, H-26-MA), 0.88 (3H, overlap, H-29-MA). ^{13}C NMR (125 MHz, $CDCl_3$) δ 184.5 (C-4-SB), 178.6 (C-28), 171.1 (OAc), 170.4 (2xOAc), 168.7, 167.8 (COO-3-SB), 162.6, 157.1/156.6, 151.7/151.4, 143.9, 143.5 (C-5-MA), 140.6 (C-13-MA), 134.3, 132.5, 130.9, 128.8, 126.5 (C-12-MA), 123.6, 123.3, 122.7 (C-6-MA), 119.9, 111.2, 109.1, 81.2 (C-2-SB), 78.5 (C-11-SB), 76.5 (C-3-SB), 75.6, 73.6 (C-3-MA), 69.1 (C-2-MA), 68.2, 64.9 (C-23-MA), 62.6 (C-23-SB), 56.1 (OMe), 49.3,

47.9 (C-9-MA), 46.5, 45.1, 43.7, 42.4 (C-1-MA), 40.9, 38.8, 38.5, 38.2 (C-8, C-10-MA), 37.1, 36.6 (β-CH_2-alanine), 34.9 (α-CH_2-alanine), 32.3 (C-7), 30.9, 30.4, 29.4, 29.1, 28.9, 23.8, 23.5, 22.9 (C-25-MA), 22.7 (C-29-MA), 22.3 (C-26-MA), 21.2 (OAc), 21.1 (OAc), 20.9 (2xOAc), 20.8 (2xOAc), 20.7 (OAc), 17.3 (C-24), 14.0.

4.2. Growth inhibition assay

Liver ancer cell lines HepG2, Hep3B, Huh7 and Huh7R were grown in DMEM containing 10% FBS and 1% antibiotic (Anti-Anti, Gibco, ThermoFisher Scienctific) in a humidified atmosphere at 37 ºC and 5% CO_2. In order to test the cytotoxic effect of compounds on different cell lines, cells were detached from the adherent culture surface by Trypsin – EDTA (0.05%) and then seeded into 96 wells-plate at the density of $3x10^4$ cell/mL and treated with an eight titration of each compound.. Ellipticine was used as a reference (positive) control. After 96 hours of incubation, all cell culture media was removed from wells followed by addition of 100 µL MTT solution (0.5 mg/mL solute in fresh medium) per well and incubation at 37 ºC for 4 hours. The MTT solution was then removed and DMSO (200 µL) to each well to solubilise the formazan product and mix each sample again using a pipette before determining the absorbance at 570 nm. GI_{50} values (compound concentration that reduces the MTT assay value by 50% versus untreated control) were calculated using GraphPad Prism 5.0 software.

4.3. Cell cycle determination

To evaluate cell cycle effects, HepG2 cells were seeded in T25 flasks at a density of $1x10^5$ cell/mL at 37 °C in 5% CO_2 and treated with compound at concentrations of 1 x IC_{50} and 3 x IC_{50} from the result of the cytotoxic assay. After 24 hours of treatment, cells were gently harvested with trypsin and then washed with PBS (pH 7,4). In the next step, 70% ethanol was slowly added to the cells after which they were kept in a fridge (4 ºC) for at least 2h to fix the cells. After fixation the cells were pelleted by centrifugation and the ethanol completely removed resuspension with RNase A (1 mg/mL) and incubation at 37 °C for 15 min, followed by PI staining for 45 min. Finally, at least 10,000 cells were analysed using a NovoCyte flow cytometer system with NovoExpress software (ACEA Bioscience Inc.) to generate cell cycle profiles.

4.4. Caspase 3 inducible assay

A Caspase-3 Colorimetric Assay Kit (Biovision Inc.) was used for establishing the caspase 3 activity of tested samples. Treated cells were lysed in lysis buffer for 10 minutes in ice and centrifuged at 10,000 x g for 1 minute to remove residual cellular debris (cell pellet). After determination of the protein concentration of each sample (Bradford assay), 80 µg of each sample in 50 µL assay buffer

was added to 50 µL DTT (10 mM) and 5 µL of DEVD-pNA (200 µM) in each well of a 96-well plate in triplicate. The plate was incubated at 37°C for 1 hour. The absorbance was read at 405 nm using a microplate reader (BioTek, ELx800).

4.5. Detection of apoptotic inducible activities by flow cytometry

The eBioessence™ Annexin V Apoptosis Detection Kit was used to measure the percentage of apoptotic cells after treatment with each compound for 24 hours according to the manufacturer's instructions. Briefly, after harvesting by trypsin-EDTA, cells were washed with cold PBS to completely remove trace medium. Cell pellets were resuspended in binding buffer and then stained with AnnexinV-FITC for 15 minutes at room temperature (protected from light) before being washed with the binding buffer to remove the unstained dye, resuspension in 190 µL of binding buffer and addition of 10 µL of PI solution (20 µg/mL) in a binding buffer. Analysis was performed using a NovoCyte flow cytometer system and NovoExpress software (ACEA Bioscience Inc.) to identify the proportion of apoptotic cells.

4.6. Statistical analysis

Results were analysed using Excel and GraphPad Prism 5.0 software and reported as mean ± standard error (SE). GraphPad Prism 5 software using unpaired t-test and one-way analysis of variance was used for data analysis. A value of $P < 0.05$ was considered to indicate statistical significance.

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