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(+)-Usnic acid enamines with remarkable cicatrizing properties

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ABSTRACT

Wound healing is a significant concern in many pathologies (post-surgeries, burns, scars) and the search for new chemical entities is advisable. The lichen compound (+)-usnic acid (**1**) has found application in dermatological and cosmetic preparations, due to its bacteriostatic and antioxidant activities. The compound has also been shown to stimulate the wound closure of keratinocyte monolayers at subtoxic doses. Here we describe the design and synthesis of usnic acid enamines (compounds **2**–**11**), obtained through nucleophilic attack of amino acids or decarboxyamino acids at the acyl carbonyl of the enolized 1,3 diketone. The wound repair properties of these derivatives were evaluated using in vitro and in vivo assays. Compounds **8** and **9** combine low cytotoxicity with high wound healing performance, suggesting their possible use in wound healing-promoting or antiage skin preparations.

1. Introduction

Impaired tissue repair is a significant concern in many conditions (post-surgeries, burns, scars). Hence, of great interest is the search for new chemical entities to be used as cicatrizing agents (alone or in formulations with other drugs) to treat chronic wounds, burns, topical otitis, hemorrhoids, vaginal lesions, and mouth infections.

Wound healing is a complex process that can be divided into three stages: inflammation, proliferation and remodeling. These phases involve a variety of coordinated cellular activities, such as migration to the wounded area, proliferation, and deposition and remodelling of extracellular matrix, mainly the collagen lattice.[1] Various materials for wound dressing, skin substitutes, and recombinant growth factors have been shown to enhance the healing process, and some of them have been introduced into the clinical setting with therapeutic efficacy.[2] Many natural products are also known to possess wound healing properties, based on both anedoctal and scientific evidence.[3] Hence, there is a great interest to find new chemical entities that could share antibiotic, anti-inflammatory and cell remodeling properties, and to characterize their mechanism of action.

We have recently described the potential of (+)-usnic acid as a wound healing agent, due to its property to stimulate wound closure of HaCaT monolayers at subtoxic doses. The mechanism of action was correlated to its ability to stimulate cell motility.[4] Previous results indicated usnic acid sodium salt as a wound healing enhancer through the secretion of growth factor and accelerating the cell migration in a dose dependent manner.[5] In vivo experiments confirmed the property of usnic acid sodium salt to accelerate skin wound healing, but the effect was not due to stimulation of fibroblast proliferation.[6] Recent publications report the use of liposome-loaded usnic acid formulations for dermal burn healing.[7]

The most investigated lichen metabolite, usnic acid, is universally known as an antibiotic (see Merck Index) a definition that has found confirmation in the many biological data so far obtained. (+)- and (−)-usnic acid are active against Gram positive bacteria on susceptible and multidrug resistant strains.[8] Some metal complexes of usnic acid hydrazones have been found to be more active[9] and all the results so far obtained confirm a bacteriostatic rather than a bactericidal action.

Usnic acid is a potent antioxidant and has been largely used in dermatological and cosmetic products as a preservative.[10]

(+)-Usnic acid is now commercially available, easily isolated from *Usnea* or *Ramalina* species of lichens where it occurs up to 26%.[11] As a pure compound it has been formulated into creams, toothpaste, mouthwash, deodorants, antibiotic ointments and

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sunscreen products. *Usnea barbata* extracts, usnic acid, and its copper salt have been formulated as antimicrobial preparations. In most preparations, usnic acid is employed both as an active agent and a preservative.

Data concerning usnic acid toxicity, mainly deal with the systemic use of usnic acid containing preparations to promote weight loss.[12] Research on toxicity performed at the National Center for Toxicological Research (NCTR), USA, attested that *Usnea* lichen preparations containing equivalent concentrations of usnic acid produced greater toxicity than pure usnic acid.

Contact dermatitis and allergenicity of usnic acid has been reported as weak, mainly due to the (−)-enantiomer,[13] probably correlated to its general toxicity.

2. Results and discussion

2.1. Synthesis of usnic acid derivatives

(+)-Usnic acid (**1**) is a benzofurandione, phloroglucinol like, secondary metabolite from lichens, occurring in nature up to 26% in species growing in China[11] and now commercially available. The most reactive portion of the molecule is the triketone moiety of polyketide origin, which is also mainly responsible for activity.[14] But its high lipophilicity due to the strong intramolecular hydrogen bondings, probably also increases its toxicity, rendering the molecule permeable to membranes. Thus (+)-usnic acid is a good candidate to investigate factors involved in its biological behavior: it is easily affordable, it has recognized and proved in vitro activities, but it has limits in exploitation due to in vivo toxicity.[12] Toxicity drawbacks are generally overcome in cosmetic preparations through the use of complexes with liposomes or other formulations.

We have synthesized and tested some chemical derivatives obtained by nucleophilic attack of a number of amines and amino containing natural products at the acetyl carbonyl at 2-position of the enolized 1,3 diketone, selected on the basis of their intrinsic very low toxicity, commercially available at low cost. The choice fell on amino acids or decarboxyamino acids, with a particular emphasis to those that contain chemical groups involved in cell-to-cell interaction (i.e., redox systems or adhesion mechanisms). Usnic acid underwent addition of amino acids systems in neutral or basic conditions (**2**–**7**, **9**) (Fig. 1). Reaction with cysteamine occurred at low yields and limited reproducibility perhaps due to easy oxidation of the reagent and the most convenient conditions result in a two stage process, by preparing the disulfur through condensing cystamine (**10**) that was later reduced to the desired thiol (**8**) (Fig. 2).

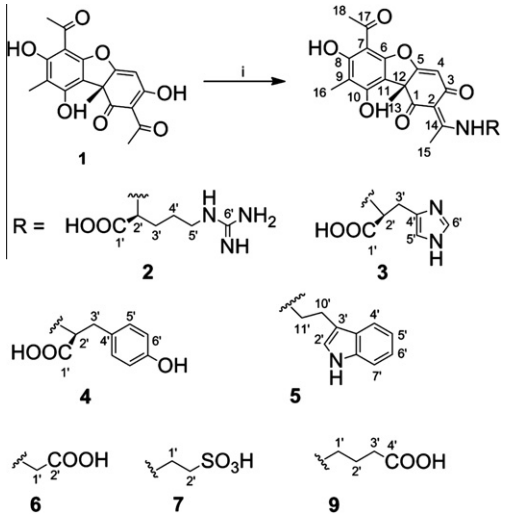

Figure 1. Reagents and conditions: (i) Amines or amino acid derivatives (1–3 equiv), abs. EtOH or abs. EtOH/triethylamine or anhydrous CH_2Cl_2/MeOH or abs. EtOH/pyridine, reflux, N_2.

The thiol (**8**) is very stable under usual preserving conditions, in any case, the two compounds were both submitted to biological testing. Enaminousnic (**11**) (Fig. 4) was also prepared as non acidic derivative by refluxing usnic acid in ammonia solution.[14c] The compounds so far obtained exhibits intrinsic wound healing properties with respect to the parent compound and, in most cases, an overall increase in water solubility.

2.2. In vitro scratch wound healing

The wound healing ability of usnic acid derivatives has been tested on an in vitro wound healing model consisting of monolayers of HaCaT keratinocytes. These cells represent an in vitro model of proliferating and migrating keratinocytes. The HaCaT cell line mimics many properties of normal epidermal keratinocytes, is not invasive and can differentiate under appropriate experimental conditions.[15,16] HaCaT have been previously used in wound healing studies by our and other laboratories as an in vitro system of re-epithelialization, a typical phase of the wound healing process.[17]

On the basis of IC_{50} values (Table 1), **4**, **5** and **6** resulted significantly more toxic than **1**, **3** had almost the same toxicity, and **2**, **7**, **8** and **9** were less toxic. These results indicate that most of the moieties attached to the usnic acid molecule have significantly modified the chemical and biological properties of the original compound. The derivatives found to be more toxic than **1** on HaCaT cells, were also tested on A431 epidermoid carcinoma cells, used as a chemoresistant model cell line. The IC_{50} (μg/mL) of **4** and **5**, 36 (32–41) and 29 (17–52), respectively, were higher or not different from that of **1**, 15 (11–21), whereas the IC_{50} of **6** was significantly lower, 4.1 (2.4–7.1), thus confirming a stronger toxicity for this latter compound also on cancer cells and suggesting its possible use as an anticancer drug.

Scratch wound analysis was performed on confluent monolayers of HaCaT, by selecting **4**, **7**, **8** and **9**, which resulted most active in preliminary tests, and comparing them with **1**. The analysis was carried out as described in Ranzato et al.[18] Compound **1** showed a significant wound healing effect, confirming our previous findings,[4] while all the derivatives displayed stronger wound healing properties (Fig. 3). The GABA–usnic acid derivative **9** induced the highest wound closure rate, almost approaching the wounding potential of the platelet lysate, a blood derivative used as reference substance (see Section 4).

2.3. In vivo assessment of wound healing activity

For the evaluation of in vivo wound healing activity, the compounds were mixed with a vehicle consisting of glycol stearate, 1,2 propylene glycol, liquid paraffin (3:6:1) in 1% concentration. Linear incision by using tensiometer and circular excision wound models were employed. Skin samples were also evaluated histopathologically.

As shown in Table 2, topical application of the ointment prepared with the compound **9** onto the incised wounds demonstrated the best wound tensile strength by the highest value of 47.6% (p <0.01) on day 10. Moreover, **1**, **7**, and **8** were found generally highly effective (31.9%, 29.3% and 35.2%, respectively) in linear incision wound model.

The contraction values of the progression healing of wounds on circular excision wound model for vehicle, negative control, compounds and reference drug treated groups were shown in Table 3. Usnic acid (**1**) and compounds **9**, **7** and **8**, were found to have wound healing potential, while the other groups showed no statistically

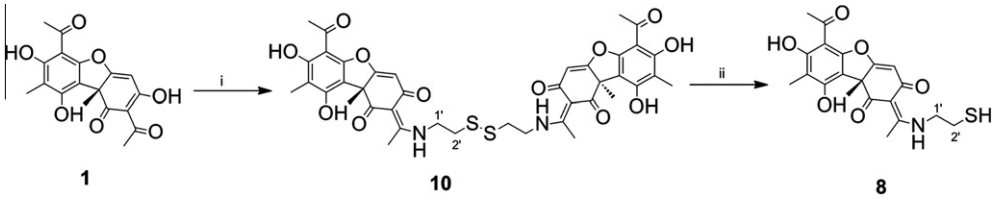

Figure 2. Reagents and conditions: (i) Cystamine dihydrochloride (0.5 equiv), abs. EtOH/pyridine, reflux, N_2, 8 h, quantitative yield; (ii) triphenylphosphine (1.5 equiv), acetone/H_2O, rt, helium, 4 days, 92%.

Table 1
In vitro toxicity of usnic acid and its derivatives to HaCaT keratinocytes, as assessed by the NRU endpoint after 24-h exposures

Compound	IC_{50} (μg/mL)	IC_{05} (μg/mL)
1	24 (18–32)	0.5 (0.2–1.3)
2	163 (108–244)	16 (8.0–30)
3	36 (22–57)	1.7 (0.5–6.5)
4	13 (9–18)	1.3 (0.5–3.5)
5	5.3 (4.7–6.3)	2.5 (1.6–4.0)
6	5.8 (3.5–9.5)	0.2 (0.04–1.1)
7	47 (39–58)	nd
8	155 (116–207)	43 (18–103)
9	150 (140–161)	67 (55–80)

IC_{50}: median effective concentration. IC_{05}: toxicity threshold. 95% confidence intervals are shown in parentheses.

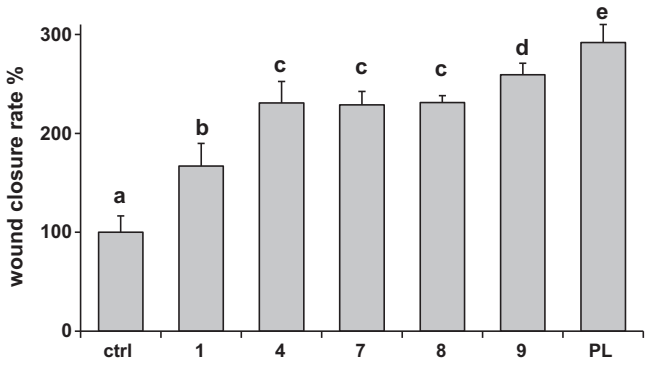

Figure 3. Scratch wound closure rates of HaCaT confluent monolayers. Cells cultured in 12-well plates were mechanically scratched and exposed to equimolar doses (5 μM) of usnic acid (**1**) (1.7 μg/mL), **4** (2.5 μg/mL), **7** (2.2 μg/mL), **8** (2.0 μg/mL), and **9** (2.1 μg/mL). One sample was exposed to 20% platelet lysate (PL) as positive control. Wound closure measurements are expressed as the difference between wound width at 0 and 24 h. Bars represent mean ± S.D. of two independent experiments, each with $n = 20$. The mean of controls was set at 100%. Different letters on bars indicate groups significantly different from each other according to the Tukey's test ($p < 0.01$).

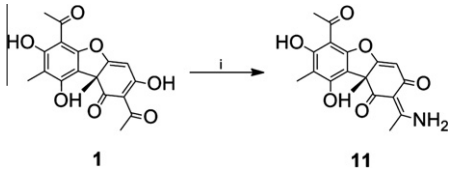

Figure 4. Reagents and conditions: (i) Ammonium hydroxide, abs. EtOH, reflux, N_2, 2 h, 77%.

significant wound healing activity. The wound contractions were 52.42% ($p < 0.01$) and 82.95% ($p < 0.001$) for **9**, 30.36% ($p < 0.05$) and 52.19% ($p < 0.01$) for **1**, 37.55% ($p < 0.05$) and 40.83% ($p < 0.01$) for **7** and 45.23% ($p < 0.01$) and 64.08% ($p < 0.01$) for **8** on day 8 and 10, respectively. These values were compared to reference drug Madecassol® [72.24% ($p < 0.01$)—100% ($p < 0.001$)].

Table 2
Effect of the compounds on linear incision wound model

Material	Mean ± S.E.M.	Tensile strength (%)
Vehicle	11.75 ± 2.48	10.6
Negative control	10.62 ± 2.63	—
1	15.50 ± 2.14	**31.9****
7	15.19 ± 2.02	**29.3***
8	15.89 ± 1.98	**35.2****
9	17.34 ± 1.92	**47.6****
10	13.62 ± 1.84	15.91
Madecassol®	18.87 ± 1.39	**60.6*****

S.E.M.: Standard error of mean. Percentage of tensile strength values: the vehicle group was compared to negative control; compounds and the reference material Madecassol® were compared to the vehicle group.
Statistically-significant values in bold.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Treated skin samples were also assessed for their hydroxyproline content, which gives an estimate of collagen concentration. Collagen is the major component of extracellular tissue. It is composed of the amino acid, hydroxyproline, which has been used as a biochemical marker for tissue collagen.[19] As shown in Table 4, high hydroxyproline content was determined for the tissues treated with the ointments of usnic acid, and compounds **7**, **8**, and **9**.

Extracellular matrix (ECM) is composed of proteoglycans and matrix proteins such as collagen and elastin. Collagen provides supportive framework to the cell, elastin maintains the skin's elasticity and hyaluronic acid keeps the moisture. All three components help wound healing.[20] Therefore, inhibition of the enzymes that break down these ECM components could improve wound healing. In the present study, we compared the hyaluronidase and collagenase enzyme inhibitory activities of usnic acid (**1**), **8**, **9**, **10**, and **11** (Tables 5 and 6), which indicate a possible mechanism of actions of the wound healing process.

Considering the low toxic effect observed on keratinocytes and the good wound healing activity, **8** and **9** seem to possess the most interesting properties.[21]

We can now draw interesting information regarding the peculiar molecular features that compounds should possess to show wound healing properties. Compound **7** bears a free thiol group which is a weak acid, **8** and **9** all bear an acid functionality, the compounds lacking such portion (like enaminousnic **11**) have no activity. A limited action on matrix protein is here reported. Compound **8** bears a free thiol group which is a light acid and also has recognized antioxidant properties. It shows wound healing properties, while the disulfur **10** has no activity. Thus acidic groups seem to be significant for the maintaining of activity.

We then synthesized the new derivatives **12** and **13** (Fig. 5) by condensing two known wound healing agents (γ-aminobutyric acid and *N*-acetylcysteine) to the most active compound in our series (**9**). Acidic groups are still present and the labile thioester bonding in **12** should be easily hydrolyzed on skin and release two therapeutic agents.[21] Future bioassays will confirm the hypothesis. These data will be reported elsewhere.

Table 3
Effect of the compounds on circular excision wound model

Material	Wound area ± S.E.M. (contraction %)					
	0 day	2 days	4 days	6 days	8 days	10 days
Vehicle	19.63 ± 2.13	18.02 ± 2.61 —	16.02 ± 2.68 —	13.32 ± 1.90 (3.41)	8.07 ± 1.11 (2.18)	3.87 ± 1.04 (6.29)
Negative control	19.55 ± 2.10	17.99 ± 2.71	15.86 ± 2.63	13.79 ± 1.76	8.25 ± 1.28	4.13 ± 1.43
1	19.59 ± 2.01	17.04 ± 2.16 (5.44)	14.10 ± 2.12 (11.99)	10.25 ± 1.84 (23.05)	5.62 ± 1.75 (**30.36**)*	1.85 ± 0.63 (**52.19**)**
7	19.33 ± 2.15	17.74 ± 2.03 (1.55)	14.52 ± 2.01 (9.36)	11.42 ± 1.38 (14.26)	5.04 ± 1.61 (**37.55**)*	2.29 ± 0.58 (**40.83**)**
8	19.46 ± 2.08	16.41 ±1.99 (8.93)	13.74 1.67 (14.23)	10.25 ±1.54 (23.05)	4.42 1.10 (**45.23**)**	1.39 ±0.61 (**64.08**)**
9	19.70 ± 2.25	16.03 ± 1.87 (11.04)	12.81 ± 1.36 (20.04)	9.01 ± 1.51 (**32.36**)*	3.84 ± 1.12 (**52.42**)**	0.66 ± 0.22 (**82.95**)***
10	19.25 ± 2.40	17.95 ± 2.36 (0.39)	15.92 ± 1.89 (0.62)	12.48 ± 1.77 (7.51)	6.48 ± 1.23 (19.70)	2.96 ± 0.74 (23.51)
Madecassol®	19.49 ± 2.11	15.21 ± 1.74 (15.59)	10.45 ± 1.17 (**34.77**)*	6.47 ± 1.29 (**51.43**)**	2.24 ± 0.52 (**72.24**)**	0.00 ± 0.00 (**100.00**)***

S.E.M.: Standard error of the mean. Percentage of contraction values: the vehicle group was compared to negative control; compounds and reference material were compared to the vehicle group.
Statistically-significant values in bold.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 4
Effect of topical ointments for 7 days on hydroxyproline content

Material	Hydroxyproline (mg/g) ± S.E.M.
Vehicle	17.1 ± 2.08
Negative control	16.9 ± 2.44
1	**36.1 ± 1.89***
7	**30.8 ± 1.92***
8	**39.8 ± 1.84***
9	**43.0 ± 1.71****
10	20.4 ± 1.99
Madecassol®	**49.7 ± 1.10*****

S.E.M.: Standard error of the mean.
Statistically-significant values in bold.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ significant from the control.

Table 5
Hyaluronidase enzyme inhibitory activity

Material	Concentration (μg/ml)	Inhibition (%) ± S.E.M.
1	50	14.45 ± 1.33
	100	33.14 ± 0.99*
8	50	10.19 ± 1.03
	100	15.64 ± 1.11
9	50	25.78 ± 1.05
	100	39.42 ± 0.78*
10	50	10.03 ± 1.21
	100	18.34 ± 1.18
11	50	35.32 ± 0.47**
	100	41.85 ± 0.34**
Tannic acid	100	76.31 ± 0.95***

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

3. Conclusion

Active principles to be used on skin in dermatological or cosmetic preparations for regenerative and antiaging purposes, should ideally possess the lowest possible cytotoxicity on skin cells, the highest antibacterial activity on infectious or opportunistic pathogens, and the strongest skin regeneration or wound healing properties. A wide number of wound healing products are currently present on the market, deriving from natural, chemical, and biomedical sources. However, in most cases these products are complex mixtures that are used on an empirical basis, while the mechanisms of action and active principles have not been clearly identified. For instance, most effective results in the healing of chronic or severe wounds are currently obtained in clinical settings by using platelet derivatives containing various growth factors and a number of low molecular-weight compounds.[22]

In contrast, in this study we have been able to attribute significant wound healing properties to single usnic acid derivatives. The results of in vitro and in vivo assays were quite consistent, showing lowest cytotoxicity combined to highest healing performance for **8** and **9**, which in most cases were preferable to their precursor usnic acid. These data suggest the possible use of these compounds in wound healing-promoting or antiage skin preparations.

4. Experimental

4.1. Chemistry

4.1.1. General

All reagents were purchased from Sigma–Aldrich and Fluka and used without further purification. Yields are given after purification, unless differently stated. When reactions were performed under anhydrous conditions, the mixtures were maintained under nitrogen. All melting points were taken on a Büchi apparatus and are uncorrected. NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C). 2D-NMR experiments (^{1}H,^{1}H COSY, ^{1}H,^{13}C HSQC, and ^{1}H,^{13}C HMBC experiments) were recorded using sequences available in Bruker software. Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration and coupling constant (Hz), whereas ^{13}C NMR analyses are reported in terms of chemical shift. Elemental analyses were performed with a Perkin–Elmer series II CHNS/O Analyzer 2400. Mass spectra were recorded on a LCQ Advantage Thermo Fisher Mass Spectrometer equipped with a ESI source (spray voltage: 4.5 kV, capillary temperature: 275.90 °C, sheat gas flow rate: 15 arbitrary units). LC–MS analysis was performed using the following equipment: HPLC Agilent 1100 with quaternary

Table 6
Collagenase and elastase enzyme inhibitory activities

Material	Concd (μg/ml)	Collagenase inhibition (%) ± S.E.M.	Elastase inhibition (%) ± S.E.M.
1	50	27.14 ± 0.99	22.08 ± 1.17
	100	51.22 ± 0.56***	36.42 ± 1.19
8	50	19.46 ± 1.27	15.28 ± 1.02
	100	27.32 ± 1.10	20.44 ± 1.18
9	50	21.42 ± 1.13	25.12 ± 1.52
	100	49.24 ± 0.68**	30.32 ± 1.21
10	50	20.82 ± 1.23	21.29 ± 1.02
	100	29.33 ± 1.20	30.15 ± 1.24
11	50	20.68 ± 1.43	13.26 ± 1.41
	100	33.10 ± 0.85*	15.32 ± 1.08
EGCG	100	41.16 ± 0.81**	88.17 ± 1.18***

S.E.M.: Standard error of the mean; EGCG: epigallocatechin gallate.
* p <0.05.
** p <0.01.
*** p <0.001.

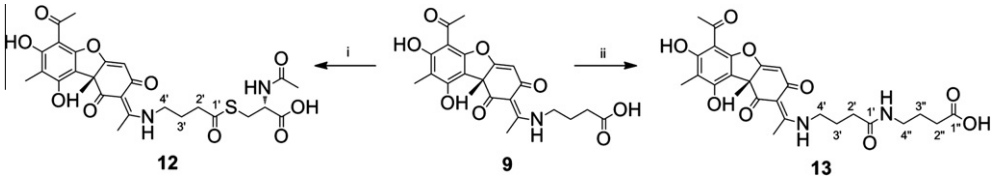

Figure 5. Reagents and conditions: (i) NMM (1.2 equiv), DMTMM (1 equiv), THF, rt, N_2, 30 min, then *N*-acetyl-L-cysteine (1 equiv), THF, rt, N_2, 3 days, 4%; (ii) NMM (1.2 equiv), DMTMM (2 equiv), THF, rt, N_2, 45 min, then 4-aminobutyric acid (1 equiv), THF, rt, N_2, 3 h and 30 min, 25%.

pump, diode array detector, autosampler, thermostated column holder, Bruker ion-trap Esquire 3000+ Mass Spectrometer with ESI; method: column Supelco Ascentis-Express 50 × 4.6 mm, 2.7 μm; phase A: Milli-Q water containing 0.05% (v/v) TFA; phase B: acetonitrile (LC–MS grade) containing 0.05% TFA; gradient: from 40% B to 100% B in 6 min, washing at 100% B for 1 min, equilibration at 5% B in the next; flow: 1 mL/min, temperature: 40 °C; UV detection at 220 and 254 nm with reference at 500 nm (40 nm bandwith); ESI+ detection in the 50–2000 m/z range with alternating MS/MS. Chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063–0.200 mm; Merck) or flash chromatography (Kieselgel 40, 0.040–0.063 mm; Merck). Reverse phase chromatography was performed on a Biotage SP1 instrument (pressure limit: 7 bar, range of flow rate: 1–100 mL/min) equipped with a UV detector (220 and 254 nm) and a Windows XP operating system control. The reactions were monitored by analytical TLC using silica gel 60 F_{254} precoated glass plates (0.25 mm thickness) and C18 reverse phase F_{254} precoated glass plates (0.25 mm thickness). Visualization was accomplished by irradiation with a UV lamp and staining with molibdic reagent ($(NH_4)_6MoO_4$, $Ce(SO_4)_2$, H_2SO_4, H_2O) or vanillin solution. Optical rotations were recorded on a JASCO P-1030 Polarimeter, using a 1 dm cell at the sodium D line (λ = 589 nm). Concentrations are expressed in g/100 mL of solvent.

4.1.2. Synthesis

4.1.2.1. (*S*)-2-(((*E*)-1-((*R*)-6-Acetyl-7,9-dihydroxy-8,9*b*-dimethyl-1,3-dioxo-1,9*b*-dihydrodibenzo[*b*,*d*]furan-2(3*H*)-ylidene)ethyl)amino)-5-guanidinopentanoic acid (2).

202 mg (1.16 mmol) of L-arginine were added to a suspension of (+)-usnic acid (400 mg, 1.16 mmol) in absolute EtOH (15 ml) and refluxed under nitrogen for 5 h, then stirred at room temperature for further 15 h. The solid which was obtained by concentration in vacuo was crystallized from diisopropylether/EtOH 9:1 giving 0.516 g (89%) of a pale yellow solid. Mp: 232–234 °C. $[\alpha]_D^{20}$ +262 (c 0.05, MeOH). ^{1}H NMR 400 MHz (DMSO-d_6) δ 1.58 (m, 5H, CH_3-13, CH_2-4′), 1.81 (m, 2H, CH_2-3′), 1.92 (s, 3H, CH_3-16), 2.57 (s, 3H, CH_3-15), 2.57 (s, 3H, CH_3-18), 3.13 (m, 2H, CH_2-5′), 4.26 (m, 1H, CH_2-2′), 5.69 (s, 1H, CH-4), 7.62 (br s, 3H), 8.89 (br s, 1H,) 12.43 (br s, 1H, OH-10), 13.28 (br s, 1H, OH-8), 13.35 (br s, 1H, NH). ^{13}C NMR 400 MHz (DMSO-d_6) δ 7.81 (C-16), 19.36 (C-15), 25.14 (C-4′), 30.45 (C-3′), 31.39 (C-18), 32.48 (C-13), 56.68 (C-12), 56.68 (C-5′), 58.87 (C-2′), 101.19 (C-7), 101.97 (C-2), 102.90 (C-4), 105.56 (C-11), 106.81 (C-9), 156.09 (C-6), 157.68 (C-6′), 158.08 (C-10), 163.08 (C-8), 172.54 (C-5), 172.77 (C-1′), 173.03 (C-14), 188.2 (C-3), 197.38 (C-1), 200.95 (C-17). HRMS (ESI), positive, m/z found: 501.1 $[M+H]^+$, Calcd for $C_{24}H_{28}N_4O_8$: 500.19. Anal. found: C 55.18%, H 5.72%, N 10.84%, Calcd for $C_{24}H_{28}N_4O_8$: C 55.59%, H 5.83%, N 10.81%.

4.1.2.2. Triethylammonium (*S*)-2-(((*E*)-1-((*R*)-6-acetyl-7,9-dihydroxy-8,9*b*-dimethyl-1,3-dioxo-1,9*b*-dihydrodibenzo[*b*,*d*]furan-2(3*H*)-ylidene)ethyl)amino)-3-(1*H*-imidazol-4-yl)propanoate (3).

L-Histidine (205 mg, 0.85 mmol) was added to a suspension of (+)-usnic acid (296 mg, 0.85 mmol), triethylamine (237 μl, 1.7 mmol) in H_2O (3 mL) and absolute EtOH (15 mL) and refluxed under nitrogen for 4 h. The mixture was stirred at room temperature for further 15 h. The solid obtained by concentration in vacuum was crystallized from diisopropyl ether/EtOH 9:1 giving 322 mg (78%) of a yellow solid. Mp: 209–211 °C. $[\alpha]_D^{20}$ +185 (c 0.05, MeOH). ^{1}H NMR 400 MHz (DMSO-d_6) δ ppm 1.16 (t, J = 8.2 Hz, 9H, CH_3-triethylamine), 1.61 (s, 3H, CH_3-13), 1.94 (s, 3H, CH_3-16), 2.50 (s, 3H, CH_3-15), 2.61 (s, 3H, CH_3-18), 2.95–3.06 (m, 7H, CH_2-triethylamine, CH_2-3′), 3.18 (m, 1H, CH_2-3′), 4.60 (m, 1H, CH-2′), 5.80 (s, 1H, CH-4), 6.83 (s, 1H, CH-5′), 7.69 (d, J = 0.8 Hz, 1H, CH-6′), 12.39 (br s, 1H, OH), 13.25 (br s, 1H, OH) 13.38 (br s, 1H, NH). ^{13}C NMR 100 MHz (DMSO-d_6) δ ppm 8.0 (C-16), 8.9 (CH_3-triethylamine), 18.9 (C-15), 31.3 (C-3′), 31.4 (C-18) 32.1 (C-13), 45.6 (CH_2-triethylamine), 56.5 (C-12), 58.8 (C-2′), 101.2 (C-7), 102.0 (C-2), 102.9 (C-4), 105.6 (C-11), 106.6 (C-9), 118.0 (C-5′), 132.8 (C-4′), 135.3 (C-6′), 156.2 (C-6), 158.2 (C-10), 162.9 (C-8), 171.8 (C-1′), 173.0 (C-5), 173.9 (C-14), 188.9 (C-3), 197.9 (C-1), 201.19 (C-17). MS (ESI), positive, m/z found: 504.1 $[M+Na]^+$, Calcd for: $C_{24}H_{23}N_3O_8$ 481.15. For the bioassays the compound was acidified and extracted with CH_2Cl_2. Anal. found: C

58.73%, H 6.41%, N 8.80%, Calcd for: $C_{24}H_{23}N_3O_8 \times 2H_2O$ C 58.24%, H 6.84%, N 9.06%.

4.1.2.3. (*S*)-2-(((*E*)-1-((*R*)-6-Acetyl-7,9-dihydroxy-8,9*b*-dimethyl-1,3-dioxo-1,9*b*-dihydrodibenzo[*b*,*d*]furan-2(3*H*)-ylidene)ethyl) amino)-3-(4-hydroxyphenyl)propanoic acid (4). L-Tyrosine (157 mg, 0.87 mmol) was added to a suspension of (+)-usnic acid (300 mg, 0.87 mmol) in absolute EtOH (10 mL) and H_2O (3 mL) and refluxed under nitrogen for 6 h, then stirred at room temperature for further 15 h. The solid which was obtained by concentration in vacuum was crystallized from diisopropyl ether/EtOH 9:1 giving 406 mg of a yellow solid (92%). Mp: 183–185 °C. $[\alpha]_D^{20}$ +95 (*c* 0.05, MeOH). ^{1}H NMR 400 MHz (DMSO-d_6) δ ppm 1.63 (s, 3H, CH_3-13), 1.96 (s, 3H, CH_3-16), 2.36 (s, 3H, CH_3-15), 2.62 (s, 3H, CH_3-18), 3.01 (dd, *J* = 7.2 Hz, 14 Hz, 1H, CH_2-3′), 3.15 (dd, *J* = 4.8 Hz, 14 Hz, 1H, CH_2-3′), 4.95 (m, 1H, CH-2′), 5.85 (s, 1H, CH-4), 6.65 (d, *J* = 8.4 Hz, 2H, CH-5′, CH-9′), 6.97 (d, *J* = 8.4 Hz, 2H, CH-6′, CH-8′), 12.11 (s, 1H, OH), 13.28 (br s, 2H, OH, NH). ^{13}C NMR 100 MHz (DMSO-d_6) δ ppm 7.7 (C-16), 18.9 (C-15), 31.4 (C-18), 32.0 (C-13), 37.9 (C-3′), 57.1 (C-12), 58.4 (C-2′), 101.2 (C-7), 102.4 (C-2), 103.0 (C-4), 105.6 (C-11), 107.2 (C-9), 116.0 (C-6′, C-8′), 126.1 (C-4′), 131.3 (C-5′, C-9′), 157.2 (C-7′), 157.3(C-6), 158.60 (C-10), 163.0 (C-8), 171.5 (C-1′), 173.4 (C-5), 174.9 (C-14), 189.2 (C-3), 198.0 (C-1), 201.4 (C-17). LC–MS (ESI), negative, *m*/*z* found: 506.14 $[M-H]^-$, Calcd for $C_{27}H_{25}NO_9$: 507.15.

4.1.2.4. (*R*,*E*)-2-(1-((2-(1*H*-Indol-3-yl)ethyl)amino)ethylidene)-6-acetyl-7,9-dihydroxy-8,9*b*-dimethyldibenzo[*b*,*d*]furan-1,3(2*H*,9*bH*)-dione (5). 0.139 g (0.87 mmol) of tryptamine were added to a suspension of (+)-usnic (0300 g, 0.87 mmol) in absolute EtOH (10 mL) and refluxed under nitrogen for 5 h, then stirred at room temperature for further 16 h. The solid which was obtained by concentration in vacuo was crystallized from diisopropylether/EtOH 9:1 giving 0.393 (93%) of a yellow solid. Mp: 218–220 °C. ^{1}H NMR 400 MHz ($CDCl_3$) δ ppm 1.69 (s, 3H, CH_3-13), 2.12 (s, 3H, CH_3-16), 2.55 (s, 3H, CH_3-15), 2.69 (s, 3H, CH_3-18), 3.22 (m, 2H, CH_2-10′), 3.82 (m, 2H, CH_2-11′), 5.78 (s, 1H, CH-4), 7.17–7.24 (m, 3H, CH-5′, CH-6′, CH-2′), 7.41 (d, *J* = 8.0 Hz, 1H, CH-7′), 7.60 (d, *J* = 7.8 Hz, 1H, CH-4′), 8.38 (s, 1H, NH-Ar), 11.98 (br s, 1H, OH), 13.36 (br s, 2H, NH, OH). ^{13}C NMR 400 MHz ($CDCl_3$) δ ppm 7.4 (C-16), 18.5 (C-15), 25.1 (C-10′), 31.1 (C-18), 32.1 (C-13), 44.46 (C-11′), 101.26 (C-7), 102.4 (C-4), 105.16 (C-2), 107.94 (C-11), 110.7 (C-9), 110.9 (C-3′), 111.6 (C-7′), 118.1 (C-6′), 119.7 (C-4′), 122.4 (C-5′), 123.0 (C-2′), 126.7 (C-3′a), 136.4 (C-7′a), 155.7 (C-6), 158.5 (C-10), 163.7 (C-8), 174.9 (C-5), 175.0 (C-14), 188.8 (C-3), 198.2 (C-1), 200.7 (C-17). HRMS (ESI), negative, *m*/*z* found: 485.17 $[M-H]^-$, Calcd for $C_{28}H_{26}N_2O_6$: 486.18.

4.1.2.5. Triethylammonium (*R*,*E*)-2-((1-(6-acetyl-7,9-dihydroxy-8,9*b*-dimethyl-1,3-dioxo-1,9*b*-dihydrodibenzo[*b*,*d*]furan-2(3*H*)-ylidene)ethyl)amino)acetate (6). L-Glycine (145 mg, 1.16 mmol) was added to a suspension of (+)-usnic acid (400 mg, 1.16 mmol), triethylamine (324 μl, 2.32 mmol) in H_2O (3 mL) and absolute EtOH (15 mL) and refluxed under nitrogen for 4 h. The mixture was stirred at room temperature for further 15 h. The solid obtained by concentration in vacuum was crystallized from diisopropyl ether/EtOH 9:1 giving 375 mg (78%) of a yellow solid.[23] Mp: 210–212 °C. $[\alpha]_D^{20}$ +207 (*c* 0.04, MeOH). ^{1}H NMR 400 MHz (DMSO-d_6) δ ppm 1.16 (t, *J* = 7.2 Hz, 9H, CH_3-triethylamine), 1.62 (s, 3H, CH_3-13), 1.94 (s, 3H, CH_3-16), 2.54 (s, 3H, CH_3-15), 2.61 (s, 3H, CH_3-18), 3.01 (q, *J* = 7.2 Hz, 6H, CH_2-triethylamine), 4.06 (m, 2H, CH-1′), 5.81 (s, 1H, CH-4), 12.43 (br s, 1H, OH), 12.94 (s, 1H, OH), 13.38 (br s, 1H, NH). ^{13}C NMR 100 MHz (DMSO-d_6) δ ppm 7.9 (C-16), 8.9 (CH_3-triethylamine), 19.7 (C-15), 31.4 (C-18), 32.23 (C-13), 45.49 (CH_2-triethylamine), 47.9 (C-1′), 56.5 (C-12), 101.2 (C-7), 102.1 (C-2), 103.0 (C-4), 105.7 (C-11), 106.7 (C-9), 156.3 (C-6), 158.2 (C-10), 162.9 (C-8), 169.6 (C-2′), 172.9 (C-5), 173.6 (C-14), 188.4 (C-3), 197.6 (C-1), 201.3 (C-17). MS (ESI), negative, *m*/*z* found: 400.10 $[M-H]^-$, Calcd for $C_{20}H_{19}NO_8$: 401.11. Anal. found: C 57.75%, H 6.75%, N 5.41%, Calcd for $C_{20}H_{19}NO_8 \times Et_3N \times H_2O$: C 59.99%, H 6.97%, N 5.38%.

4.1.2.6. (*R*,*E*)-2-((1-(6-Acetyl-7,9-dihydroxy-8,9*b*-dimethyl-1,3-dioxo-1,9*b*-dihydrodibenzo[*b*,*d*]furan-2(3*H*)-ylidene)ethyl)amino)ethanesulfonic acid (7). Procedure A: Taurine (751 mg, 6 mmol) was dissolved in aqueous EtOH (20 mL, 1:1). KOH was added to adjust pH ~9.5, and the mixture was refluxed for 1 h on water bath. Then a suspension of (+)-usnic acid (689 mg, 2 mmol) in absolute EtOH (5 mL) was added in portion over a period of 30 min, and the mixture was refluxed for 10 h. Solvent was evaporated until dryness, the crude product was diluted with CH_2Cl_2 and washed with HCl 1 N until pH 4. The aqueous layers were collected, concentrated under reduced pressure and washed with CH_3OH/H_2O 9:1 (20 mL) to remove the excess of taurine. The solution was evaporated until dryness and crystallized from diisopropylether/EtOH 9:1 to give 687 mg (1.52 mmol, 76%) of the desired product as brown solid. $[\alpha]_D^{20}$ +180 (*c* 0.11, MeOH). ^{1}H NMR 400 MHz (DMSO-d_6) δ 1.65 (s, 3H, CH_3-13), 1.98 (s, 3H, CH_3-16), 2.60 (s, 3H, CH_3-15), 2.65 (s, 3H, CH_3-18), 2.78 (t, 2H, *J* = 6.4 Hz, CH_2-2′), 3.77–3.81 (m, 2H, CH_2-1′), 5.86 (s, 1H, H-4), 7.72 (br s, 1H, SO_3H), 12.43 (s, 1H, OH-10), 12.90-12.93 (m, 1H, NH), 13.42 (s, 1H, OH-8). ^{13}C NMR 400 MHz (DMSO-d_6) δ 8.64 (C-16), 19.26 (C-15), 32.17 (C-18), 32.83 (C-13), 42.13 (CH_2-1′), 50.63 (CH_2-2′), 57.26 (C-12), 102.01 (C-7), 102.86 (C-2), 103.63 (C-4), 106.40 (C-11), 107.39 (C-9), 156.95 (C-6), 158.94 (C-10), 163.64 (C-8), 173.83 (C-5), 175.63 (C-14), 189.42 (C-3), 198.07 (C-1), 202.10 (C-17).

Procedure B (triethylammonium salt): To a solution of usnic acid (400 mg, 1.16 mmol) in 10 mL of EtOH and 6 mL of water, taurin (145 mg, 1.16 mmol) and 2 mL of triethylamine were added at room temperature. After stirring at 75 °C for 4 h and at room temperature overnight, the reaction mixture was concentrated under reduced pressure. The crude product was crystallized from diisopropylether/EtOH 9:1 to give 551 mg (1.02 mmol, 88%) of yellow solid (88%).

^{1}H NMR 400 MHz (DMSO-d_6) δ 1.17 (t, *J* = 7.3 Hz, 9H, CH_3-triethylamine), 1.63 (s, 3H, CH_3-13), 1.96 (s, 3H, CH_3-16), 2.60 (s, 3H, CH_3-15), 2.62 (s, 3H, CH_3-18), 2.79 (t, *J* = 6.4 Hz, 2H, CH_2-2′), 3.08 (q, *J* = 7.3 Hz, 6H, CH_2-triethylamine), 3.78 (m, 2H, CH_2-1′), 5.82 (s, 1H, CH-4), 12.37 (s, 1H, OH-10), 12.92 (t, *J* = 5.3 Hz, 1H, NH), 13.38 (s, 1H, OH-8). ^{13}C NMR 400 MHz (DMSO-d_6) δ 8.0 (C-16), 9.15, 18.5 (C-15), 31.5 (C-18), 32.1 (C-13), 40.7 (CH_2), 46.3 (CH_2), 50.0 (CH_2), 56.6 (C-12), 101.3 (C-7), 102.2 (C-2), 102.9 (C-4), 105.7 (C-11), 106.7 (C-9), 156.4 (C-6), 158.3 (C-10), 162.3 (C-8), 173.3 (C-5), 175.1 (C-14), 188.7 (C-3), 197.3 (C-1), 201.4 (C-17). HRMS (ESI), negative, *m*/*z* found: 450.08568 $[M-H]^-$, Calcd for $C_{20}H_{19}NO_9S$: 450.08643.

4.1.2.7. (*R*,*E*)-6-Acetyl-7,9-dihydroxy-2-(1-((2-mercaptoethyl)amino)ethylidene)-8,9*b*-dimethyldibenzo[*b*,*d*]furan-1,3(2*H*,9*bH*)-dione (8). Procedure A: To a suspension of (+)-usnic acid (1 g, 2.90 mmol) in 6 mL of anhydrous CH_2Cl_2 and 30 mL of anhydrous MeOH, a solution of cysteamine (224 mg, 2.90 mmol) was added under nitrogen. The reaction mixture was refluxed at 65 °C for 4 h, then at room temperature overnight and again refluxed at 65 °C for 2 h. The solvent was removed until dryness and the solid was purified by flash-chromatography (toluene/EtOAc 8:2) to obtain the desired product as yellow solid (511 mg, 1.27 mmol, 44%). Mp: 142–143 °C. Procedure B: To a solution of **10** (2.680 g, 3.33 mmol) in 120 mL of acetone, triphenylphosphine (1.310 g, 4.99 mmol) and 18 mL of H_2O were added

under helium and the reaction mixture was stirred at room temperature for 4 days. Solvent was evaporated until dryness, the yellow solid was dissolved in CH_2Cl_2, the organic layer was washed with brine, dried over Na_2SO_4, filtered and evaporated to dryness. The crude product was purified by flash chromatography (toluene/EtOAc 100:0 to 95:5) to give 1.233 g (3.05 mmol, 92%) of yellow solid. $[\alpha]_D^{20}$ +318 (*c* 0.11, MeOH). ^{1}H NMR 400 MHz ($CDCl_3$) δ ppm 1.63 (t, *J* = 8.6 Hz, 1H, SH), 1.71 (s, 3H, CH_3-13), 2.10 (s, 3H, CH_3-16), 2.65 (s, 3H, CH_3-15), 2.68 (s, 3H, CH_3-18), 2.83-2.88 (m, 2H, CH_2-2′), 3.69–3.73 (m, 2H, CH_2-1′), 5.80 (s, 1H, CH-4), 11.91 (br s, 1H, OH-10), 13.36 (s, 1H, OH-8), 13.74 (br s, 1H, NH). Adding D_2O, the signal at 2.83–2.88 ppm became a triplet (*J* = 6.4 Hz) and the triplet at 1.63 disappeared. ^{13}C NMR 100 MHz ($CDCl_3$) δ ppm 8.16 (C-16), 19.13 (C-15), 24.46 (C-2′), 31.95 (C-18), 32.63 (C-13), 47.38 (C-1′), 57.95 (C-12), 102.04 (C-2 + C-7), 103.00 (C-4), 105.67 (C-11), 108.69 (C-9), 156.52 (C-6), 158.88 (C-10), 164.17 (C-8), 174.97 (C-5), 175.65 (C-14), 191.54 (C-3), 199.14 (C-1), 201.33 (C-17). LC–MS (ESI), positive, *m*/*z* found: 403.9 $[M+H]^+$, 425.8 $[M+Na]^+$, Calcd for $C_{20}H_{21}NO_6S$: 403.1.

4.1.2.8. (*R*,*E*)-4-((1-(6-Acetyl-7,9-dihydroxy-8,9*b*-dimethyl-1,3-dioxo-1,9*b*-dihydrodibenzo[*b*,*d*]furan-2(3*H*)-ylidene)ethyl)amino)butanoic acid (9).

To a solution of (+)-usnic acid (1 g, 2.9 mol) in absolute EtOH(30 mL) and 2 mL of triethylamine, 4-aminobutyric acid (300 mg, 2.9 mol) was added at room temperature. After stirring at 75 °C for 5 h, the mixture was concentrated under reduced pressure, diluted with CH_2Cl_2 and washed with 0.1 N HCl. The organic layer was dried, evaporated to dryness and crystallized with diisopropyl ether/EtOH 9:1 to give the desired product (0.914 g, 73%) as a yellow solid. Mp: 202–205 °C. $[\alpha]_D^{20}$ +292 (c 0.12, MeOH). ^{1}H NMR 400 MHz (DMSO-d_6) δ ppm 1.66 (s, 3H, CH_3-13), 1.86 (m, 2H, CH_2-2′), 1.98 (s, 3H, CH_3-16), 2.36 (t, 2H, *J* = 7.3 Hz, CH_2-3′), 2.59 (s, 3H, CH_3-15), 2.65 (s, 3H, CH_3-18), 3.59 (m, 2H, CH-1′), 5.89 (s, 1H, CH-4), 12.26 (s, 1H, COOH), 12.34 (s, 1H, OH), 13.05 (t, 1H, *J* = 5.2 Hz, NH), 13.42 (s, 1H, OH). ^{13}C NMR 100 MHz (DMSO-d_6) δ ppm 8.0 (C-16), 18.6 (C-15), 24.4 (C-2′), 31.1 (C-3′), 31.5 (C-18), 32.2 (C-13), 43.3 (C-1′), 56.8 (C-12), 101.4 (C-7), 102.1 (C-2), 102.8 (C-4), 105.6 (C-11), 106.7 (C-9), 156.2 (C-6), 158.2 (C-10), 162.9 (C-8), 173.5 (C-5), 174.2 (COOH), 175.5 (C-14), 189.4 (C-3), 197.7 (C-1), 201.4 (C-17). MS (ESI), negative, *m*/*z* found: 428.0 $[M-H]^-$, Calcd for $C_{22}H_{23}NO_8$: 429.1.

4.1.2.9. (*S*,*E*)-6-Acetyl-2-(1-((2-((2-(((*E*)-1-((*R*)-6-acetyl-7,9-dihydroxy-8,9*b*-dimethyl-1,3-dioxo-1,9*b*-dihydrodibenzo[*b*,*d*]furan-2(3*H*)ylidene)ethyl)amino)ethyl)disulfanyl)ethyl)amino)ethylidene)-7,9-dihydroxy-8,9*b*-dimethyldibenzo[*b*,*d*]furan-1,3(2*H*,9*bH*)-dione (10).

24 μl of dry pyridine were added to a solution of 65 mg (0.29 mmol) of cystamine dihydrochloride in 3 mL of absolute EtOH. After stirring 30 min at room temperature, a suspension of 200 mg (0.58 mmol) of usnic acid in 3 mL of absolute EtOH was added, and dry pyridine was added until usnic acid dissolution. The reaction was refluxed under nitrogen atmosphere for 8 h. The solvent was concentrated under reduced pressure, and the crude material was acidified with 1 N HCl until pH 4, then extracted twice with EtOAc. The organic layers were collected and concentrated under reduced pressure, giving 173.2 mg of pale yellow solid, quantitative yield. ^{1}H NMR 400 MHz ($CDCl_3$) δ ppm 1.68 (s, 3H, CH_3-13), 2.07 (s, 3H, CH_3-16), 2.64 (s, 3H, CH_3-15), 2.66 (s, 3H, CH_3-18), 3.00 (t, *J* = 6.5 Hz, 2H, CH_2-2′), 3.85–3.89 (m, 2H, CH_2-1′), 5.76 (s, 1H, CH-4), 11.81 (br s, 1H, OH-10), 13.33 (s, 1H, OH-8), 13.72 (br s, 1H, NH). ^{13}C NMR 100 MHz ($CDCl_3$) δ ppm 8.18 (C-16), 19.14 (C-15), 31.94 (C-18), 32.66 (C-13), 37.26 (C-2′), 42.98 (C-1′), 58.14 (C-12), 102.04 (C-2 + C-7), 102.92 (C-4), 105.58 (C-11), 108.73 (C-9), 156.48 (C-6), 158.80 (C-10), 164.22 (C-8), 174.97 (C-5), 175.69 (C-14), 191.63 (C-3), 199.20 (C-1), 201.30 (C-17). LC–MS (ESI), positive, *m*/*z* found: 805.2 $[M+H]^+$, Calcd for $C_{40}H_{40}N_2O_{12}S_2$: 804.2.

4.1.2.10. (*R*,*E*)-6-Acetyl-2-(1-aminoethylidene)-7,9-dihydroxy-8,9*b*-dimethyldibenzo[*b*,*d*]furan-1,3(2*H*,9b*H*)-dione (11).

To a suspension of (+)-usnic acid (300 mg, 0.87 mmol) in 3 mL of absolute EtOH, 0.4 mL of concentrated ammonium hydroxide was added under nitrogen and the reaction mixture was stirred at 80 °C for 2 h. After cooling in an ice bath, the yellow solution was concentrated to approximately one third the original volume, acidified with 1 N hydrochloric acid and extracted twice with EtOAc. The combined extracts were washed with H_2O and brine solution, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was crystallized from $CHCl_3$ to produce pale yellow plates of the desired product (230 mg, 0.67 mmol, 77%). Mp 249 °C. $[\alpha]_D^{20}$ +378 (*c* 0.08, $CHCl_3$). ^{1}H NMR 400 MHz (DMSO-d_6) δ ppm 1.64 (s, 3H, CH_3-13), 1.98 (s, 3H, CH_3-16), 2.53 (s, 3H, CH_3-15), 2.65 (s, 3H, CH_3-18), 5.87 (s,1H, CH-4), 9.83 (br s, 1H, NH, D_2O exchangeable), 11.54 (br s, 1H, NH, D_2O exchangeable), 12.32 (s, 1H, OH-10, D_2O exchangeable), 13.42 (s, 1H, OH-8, D_2O exchangeable). ^{13}C NMR 100 MHz (DMSO-d_6) δ ppm 8.60 (C-16), 25.63 (C-15), 32.11 (C-18), 32.79 (C-13), 57.17 (C-12), 101.95 (C-7), 102.36 (C-2), 103.64 (C-4), 106.21 (C-11), 107.40 (C-9), 156.77 (C-6), 158.77 (C-10), 163.62 (C-8), 174.07 (C-5), 176.94 (C-14), 189.59 (C-3), 198.69 (C-1), 201.94 (C-17). MS (EI), positive, *m*/*z* found: 343 $[M^{+\cdot}]$, Calcd for $C_{18}H_{17}NO_6$: 343.

4.1.2.11. (*R*)-2-Acetamido-3-((4-(((*E*)-1-((*R*)-6-acetyl-7,9-dihydroxy-8,9*b*-dimethyl-1,3-dioxo-1,9*b*-dihydrodibenzo[*b*,*d*]furan-2(3*H*)-ylidene)ethyl)amino)butanoyl)thio)propanoic acid (12).

In an oven dried round bottomed flask, **8** (200 mg, 0.47 mmol) was dissolved in 2.5 mL of anhydrous THF (previously degassed by sparging helium). 4-Methylmorpholine (62 μl, 0.56 mmol) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)morpholine (130 mg, 0.47 mmol) were added and the reaction mixture was stirred at rt for 30 min. *N*-Acetyl-L-cysteine (77 mg, 0.47 mmol) was added and the reaction mixture was stirred at rt for 7 h, then overnight. Starting material was still visible, so 4-methylmorpholine (26 μl, 0.235 mmol) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)morpholine (65 mg, 0.235 mmol) were added and, after 30 min also *N*-acetyl-L-cysteine (38 mg, 0.235 mmol) was added. The reaction mixture was stirred for 2 days. The reaction mixture was diluted with CH_2Cl_2, then washed five times with brine. The combined organic layers were dried over Na_2SO_4, filtered and evaporated to dryness to give 234 mg of crude product as yellow solid. The crude product was purified by column chromatography (CH_2Cl_2/MeOH/EtOH 9:0.5:0.5 to 8:1:1, silica weight 50 g, volume fraction: 30 mL, fractions collected from the 43th to the 49th) and then by reverse phase using Biotage SP1 instrument (SNAP KP-C18-HS 12 g, 35–70 μm, flow rate 15 mL/min, ACN/H_2O 20:80 to 80:20) to obtain 11 mg (0.019 mmol, 4%) of the desired product as yellow solid. $[\alpha]_D^{20}$ +120 (*c* 0.14, MeOH). ^{1}H NMR 400 MHz (DMSO-d_6) δ ppm 1.66 (s, 3H, CH_3-13), 1.80 (s, 3H, *CH*$_3$CONH), 1.89-1.93 (m, 2H, CH_2-3′), 1.98 (s, 3H, CH_3-16), 2.58 (s, 3H, CH_3-15), 2.65 (s, 3H, CH_3-18), 2.64–2.69 (m, 2H, CH_2-2′), 3.04–3.09 (dd, *J* = 12.6 Hz, *J* = 6.2 Hz, 1H, S*CH*$_2$), 3.37–3.41 (dd, *J* = 12.6 Hz, *J* = 4.6 Hz, 1H, S*CH*$_2$), 3.55–3.58 (m, 2H, CH_2-4′), 4.02–4.07 (m, 1H, NH*CH*), 5.89 (s, 1H, CH-4), 7.51 (d, *J* = 6.8 Hz, 1H, *NH*CH), 12.27 (br s, 1H), 13.04 (br s, 1H, NH), 13.41 (br s, 1H), 13.45 (s, 1H). ^{13}C NMR 100 MHz (DMSO-d_6) δ ppm 8.65 (C-16), 19.26 (C-15), 23.82 (*CH*$_3$CONH), 25.57 (C-3′), 32.17 (C-18), 32.82 (C-13), 32.92 (S*CH*$_2$), 41.28 (C-2′), 43.62 (C-4′), 53.59 (NH*CH*), 57.46 (C-12), 102.01 (C-7), 102.80 (C-2), 103.49 (C-4), 106.31 (C-11), 107.44 (C-9), 156.90 (C-6), 158.84 (C-10), 163.63 (C-8), 169.79 (CH_3*C*ONH), 172.42 (COOH), 174.14 (C-5), 176.18 (C-14), 190.09 (C-3), 198.38 (C-1), 199.14 (C-1′), 202.06 (C-17). LC–MS (ESI), negative, *m*/*z* found: 572.9 $[M-H]^-$, Calcd for $C_{27}H_{30}N_2O_{10}S$: 574.2.

4.1.2.12. (*R*,*E*)-4-(4-((1-(6-Acetyl-7,9-dihydroxy-8,9*b*-dimethyl-1,3-dioxo-1,9*b*-dihydrodibenzo[*b*,*d*]furan-2(3*H*)-ylidene)ethyl) amino)butanamido)butanoic acid (13). To a solution of **9** (50 mg, 0.12 mmol) in 1.5 mL of anhydrous THF under nitrogen, *N*-methylmorpholine (15 μl, 0.14 mmol) was added and the reaction mixture was stirred at room temperature. After 30 min, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (65 mg, 0.23 mmol)was added and the reaction mixture was stirred at room temperature for 15 min. 4-Aminobutyric acid (12 mg, 0.12 mmol) was added and the reaction mixture was stirred at room temperature for 3 h and 30 min. The solvent was removed under reduced pressure and the solid obtained was diluted with CH_2Cl_2 and extracted twice with 0.1 N HCl. The combined organic layers were washed with brine, dried over Na_2SO_4 and evaporated to dryness to obtain 64 mg of orange solid. The crude product was then purified by flash-chromatography using Biotage SP1 intrument (prepacked 21X55 mm SNAP silica gel cartridge, silica weight 10 g, flow rate 10 mL/min, CH_3OH/CH_2Cl_2 0:100 to 10:90) to obtain 15 mg (0.029 mmol, 25%) of orange solid (R_f 0.31, eluent: CH_3OH/CH_2Cl_2 1:9). $[\alpha]_D^{20}$ +231 (*c* 0.09, $CHCl_3$). ^{1}H NMR 400 MHz (DMSO-d_6) δ ppm 1.58–1.62 (m, 2H, CH_2-3″), 1.66 (s, 3H, CH_3-13), 1.82-1.89 (m, 2H, CH_2-3′), 1.98 (s, 3H, CH_3-16), 2.18-2.22 (m, 4H, CH_2-2″ and CH_2-2′), 2.59 (s, 3H, CH_3-15), 2.64 (s, 3H, CH_3-18), 3.03–3.08 (m, 2H, CH_2-4″), 3.53–3.57 (m, 2H, CH_2-4′), 5.88 (s, 1H, CH-4), 7.94 (br s, 1H, NH-CO), 12.32 (s, 1H, OH-10), 13.04 (br s, 1H, NH), 13.41 (s, 1H, OH-8). ^{13}C NMR 100 MHz (DMSO-d_6) δ ppm 8.65 (C-16), 19.25 (C-15), 25.72 (C-3′), 25.80 (C-3), 32.17 (C-18), 32.51 (C-2), 32.84 (C-13), 33.22 (C-2′), 39.14 (C-4), 44.19 (C-4′), 57.45 (C-12), 102.02 (C-7), 102.76 (C-2), 103.49 (C-4), 106.31 (C-11), 107.46 (C-9), 156.92 (C-6), 158.86 (C-10), 163.66 (C-8), 172.12 (C-1′), 174.16 (C-5), 175.72 (C-1″), 176.11 (C-14), 190.08 (C-3), 198.36 (C-1), 202.07 (C-17). MS (ESI), negative, *m*/*z* found: 513.4 $[M-H]^-$, Calcd for $C_{26}H_{30}N_2O_9$: 514.2.

4.2. In vitro tests on keratinocytes

All reagents were from Sigma Chemical Co., unless otherwise indicated. Cells were maintained at 37 °C, 5% CO_2, in Dulbecco-modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS, Euroclone, Pero, Italy) and 1% antibiotic mixture. Compounds **3**, **6** and **7** were submitted to bioassays in the acid form. The corresponding triethylammonium salts were acidified and extracted with CH_2Cl_2.

4.2.1. Cell viability assay

Cell viability was assessed on HaCaT keratinocytes and on A431 human epidermoid carcinoma cells. For this analysis we used the neutral red uptake (NRU) test by following the method reported by Borenfreund et al.[24] Such a cell viability assay is based on the incorporation of neutral red dye into the lysosomes of viable cells after incubation with the test agent. Briefly, cells were seeded on 96-well plates (20,000 cells/ well), grown for 24 h prior to experiments, and then exposed for 24 h to various concentrations of usnic acid or of its derivatives. After removing the medium, a 0.05% solution of neutral red was added to each well, followed by incubation for 3 h at 37 °C. Cells were then washed with PBS, followed by the addition of a solution of 1% glacial acetic acid in 50% ethanol, in order to fix the cells and extract the neutral red dye incorporated into lysosomes. The absorption of the supernatants was measured at 540 nm by using a microplate reader. Estimates of IC_{50}, IC_{05} and of their 95% confidence intervals where obtained by using a Microsoft Excel® sheet developed by CSIRO, Australia, which makes use of a downhill logistic curve:

$$f = \frac{1}{(1 + \exp(-m \cdot (\log(D) - \log(\mathrm{IC50}))}$$

where *f* is the fraction of the endpoint affected by dose *D* and *m* is the slope shape or Hill's coefficient.[25]

4.3. Scratch wound assay

Cells were grown in multiwell plates until confluence and thereafter, cell layers were scratched by using a sterile 0.1–10 μl pipette tip. After washing away suspended cells, cultures were refed with medium and exposed to **1** or its derivatives, for 24 h. One series of samples, used as positive controls, were exposed to a dose of 20% (v/v) of a platelet lysate (PL). This preparation had been previously shown to promote scratch wound healing in HaCAT cells, and was obtained from blood samples as previously described.[17b] After exposures, cells were fixed in 3.7% formaldehyde in PBS for 30 min, and then stained with 0.1% toluidine blue at room temperature for 30 min. The width of the wound space was measured at wounding and at the end of treatments, using an inverted microscope equipped with a digital camera (Leica Microsystems, Milan, Italy), and the NIH ImageJ software. Wound closure was determined as the difference between wound width at 0 and 24 h.

4.4. In vivo bioassays

4.4.1. Animals

Male, Sprague–Dawley rats (160–180 g), and Swiss albino mice (20–25 g), were purchased from the animal breeding laboratory of Saki Yenilli (Ankara, Turkey).

Animals were left for 3 days at room conditions for acclimatization, and then maintained on standard pellet diet and water ad libitum throughout the experiment. A minimum of six animals were used in each group. The study was permitted by the Institutional Animal Ethics Committee and was performed according to the international rules considering animal experiments and biodiversity right.

4.4.2. Preparation of test samples for bioassay

Incision and excision wound models were used to evaluate the wound healing activity. For the in vivo wound models, test samples were prepared at 1% concentration in an ointment base (vehicle) consisting of glycol stearate, 1,2-propylene glycol, liquid paraffin (3:6:1). Amounts of 0.5 g of each test ointment were applied topically on the wounded site immediately after wound was created by a surgical blade. The animals of the vehicle group were treated with the ointment base only, whereas the animals of the reference drug group were treated with 0.5 g of Madecassol® (Bayer, 00001199), containing 1% extract of *Centella asiatica*.

4.4.3. Linear incision wound model

Rats, seven animals in each group, were anaesthetized with 0.15 ml Ketalar®, the hairs on the dorsal part of the rats were shaved and cleaned with 70% alcohol. Two 5 cm-length linear-paravertebral incisions were made with a sterile blade through the shaved skin at the distance of 1.5 cm from the dorsal midline on each side. Three surgical sutures were placed each 1 cm apart. The ointments prepared with test samples, the reference drug (Madecassol®), or ointment alone [glycol stearate:propylene glycol:liquid parafin (3:6:1)] were topically applied on the dorsal wounds in each group of animals once daily throughout 9 days. All the sutures were removed on the last day and tensile strength of previously wounded and treated skin was measured by using a tensiometer (Zwick/Roell Z0.5, Germany).[26]

4.4.4. Circular excision wound model

This model was used to monitor wound contraction and wound closure time. Mice, seven animals in each group, were anaesthetized by 0.01 cc Ketalar®, back hairs were depilated by shaving, and a circular wound was created on the dorsal interscapular

region by excising the skin with a 5 mm biopsy punch (Nopa instruments, Germany); wounds were left open.[27] Test samples, the reference drug (Madecassol®), and the vehicle ointment were applied topically once a day till the wound was completely healed. The progressive changes in wound area were monitored by a camera (Fuji, S20 Pro, Japan) every two days, and the wound area was then evaluated by using AutoCAD software. Wound contraction was calculated as percentage of the reduction in wounded area. A sample of tissue was isolated from the healed skin of each group of mice for histopathological examination.[28]

4.5. Hydroxyproline estimation

Tissues were dried in hot air oven at 60–70 °C until consistent weight was achieved. Afterwards, samples were hydrolyzed with 6 N HCl for 3 h at 130 °C. The hydrolyzed samples were adjusted to pH 7 and subjected to chloramin T oxidation. The colored adduct formed with Ehrlich reagent at 60 °C was read at 557 nm. Standard hydroxyproline was also run, and values were reported as μg/mg dry weight of tissue.[29]

4.6. Determination of hyaluronidase inhibitory activity

The inhibition of hyaluronidase was assessed by the measurement of the amount of *N*-acetylglucosamine released from sodium hyaluronate.[20,30] A volume of 50 μl of bovine hyaluronidase (7900 units/ml) was dissolved in 0.1 M acetate buffer (pH 3.6). This solution was then mixed with 50 μl of different concentrations of the oils dissolved in 5% DMSO. For the control group 50 μl of 5% DMSO was added instead of the oils. After 20 min incubation at 37 °C, 50 μl of calcium chloride (12.5 mM) was added to the mixture and again incubated for 20 min at 37 °C. A volume of 250 μl of sodium hyaluronate (1.2 mg/ml) was added and incubated for 40 min at 37 °C. After incubation, the mixture was treated with 50 μl of 0.4 M NaOH and 100 μl of 0.2 M sodium borate and then incubated for 3 min in boiling waterbath. A volume of 1.5 ml of *p*-dimethylaminobenzaldehyde solution was added to the reaction mixture after cooling to room temperature and was developed at 37 °C for 20 min. Absorbance was measured at 585 nm (Beckmann Dual Spectrometer; Beckman, Fullerton, CA, USA).

4.6.1. Determination of collagenase inhibitory activity

The samples were dissolved in DMSO. Sample stock solutions and *Clostridium histolyticum* (ChC) were diluted in 50 mM Tricine buffer (0.4 M NaCl, 0.01 M $CaCl_2$, pH 7.5) and preincubated at 25 °C for 5 min. Thereafter, 2 mM *N*-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) was prepared in the same buffer. Volumes of 25 μl buffer, 25 μl test sample, and 25 μl enzyme were added to each well and incubated for 15 min. 50 μl substrate was added to the mixture and the decrease of the optical density (OD) at 340 nm was promptly detected using the spectrometer. The ChC inhibition activities were calculated according to the following formula:

$$\text{ChC inhibition activity}\,(\%) = (\text{OD}_{\text{Control}} - \text{OD}_{\text{Sample}} \times 100)/\text{OD}_{\text{Control}}$$

where $OD_{control}$ and OD_{sample} represent the optical densities in the absence and presence of sample, respectively.

4.6.2. Determination of elastase inhibitory activity

The sample solution and human neutrophil elastase enzyme (HNE) (17 mU/ml) were mixed in 0.1 M Tris–HCl buffer (pH 7.5), incubated at 25 °C for 5 min, additioned with *N*-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (MAAPVN), and incubated at 37 °C for 1 h. The reaction was stopped by the addition of soybean trypsin inhibitor (1 mg/ml) and the optical density due to the formation of *p*-nitroaniline was immediately measured at 405 nm. HNE inhibition activities were calculated as reported above for the ChC inhibition activity.

4.7. Statistical analysis of data

Data were analyzed using one-way analysis of variance (ANOVA). The values of $p \leqslant 0.05$ were considered statistically significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.01.045.

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