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Research paper

New derivatives of dehydroabietic acid target planktonic and biofilm bacteria in *Staphylococcus aureus* and effectively disrupt bacterial membrane integrity

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ABSTRACT

The combination of the dehydroabietic acid scaffold with different amino acids resulted in the discovery of a new class of hybrid compounds that targets both planktonic and biofilms bacteria in *Staphylococcus aureus* strains and are far more potent anti-biofilm agents than conventional antibiotics. Unlike dehydroabietic acid, these compounds can disrupt biofilms within a short time period and compromise the integrity of the bacterial membrane. Two of the compounds identified in our study are the most potent abietane-type anti-biofilm agents reported so far and display robust activity against pre-formed biofilms at concentrations only 3–6-fold higher than those required to inhibit biofilm formation. Their easy preparation based on proteolysis-resistant D- and unusual amino acids makes them useful chemical probes to gain a deeper understanding of bacterial biofilms and outstanding candidates for further development into new drugs to fight infections.

1. Introduction

Bacterial biofilms are multicellular structured communities of bacteria embedded in a self-produced extracellular matrix [1,2]. This collective lifestyle typically occurs when bacteria attach to surfaces, and allows them to be highly resilient to different environmental and chemical stresses, when compared to single-cells [3]. The burden of biofilms in human health derives from the fact that they are highly tolerant to immune responses as well as to conventional antibiotics. Indeed, in comparison to the non-biofilm (planktonic) lifestyle, biofilms have been proven to withstand 10 to 1000-fold higher concentrations of antibiotics, which often exceed their highest deliverable clinical concentrations. It has been estimated that over 65% of all bacterial infections tolerant to chemotherapy are biofilms-related [4]. A challenging problem is caused by biofilm involvement in nosocomial infections which will continue rising due to the increasing use of indwelling medical devices [4].

Staphylococcus aureus is a Gram-positive bacterium and a common cause of skin and respiratory tract infections, medical devices-associated infections, and food contamination [5]. A great deal of literature has been dedicated to methicillin-resistant *S. aureus* (MRSA) strains and the threat they pose, but an even more alarming fact is that also methicillin-susceptible *S. aureus* strains can switch to the biofilm state and become remarkably tolerant to antibiotics [6]. Therefore, biofilm formation by *S. aureus* poses a major challenge in the treatment of infections caused by this microorganism. This scenario calls for the development of new compounds with broader antimicrobial effects, against both single-cells and multicellular bacterial communities. The screening for promising biofilm targets, such as signaling pathways, and the use of high-throughput screening have significantly contributed towards identifying relevant chemical scaffolds for the development of anti-biofilm agents [7–9]. However, there are currently no approved drugs targeting bacterial biofilms and only a limited

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number of compounds can selectively act on biofilms and eradicate them at low concentrations, especially in the case of *S. aureus* [10–13].

Abietanes are a family of naturally occurring diterpenoids with interesting biological activities [14]. We have recently shown that dehydroabietic acid **1**, an abietane-type diterpenoid abundant in the resin of coniferous trees, prevents *S. aureus* biofilm formation in the low micromolar range, and only 2–4-fold higher concentrations of **1** are needed to significantly reduce the viability and biomass of existing *S. aureus* biofilms [15]. In pursuit of more potent anti-biofilm agents, our attention was drawn by reports on the ability of D-amino acids to trigger biofilm disassembly in some bacterial species [16–18]. Eradication of wild-type and multi-resistant *Pseudomonas aeruginosa* biofilms has also been reported recently with D-enantiomeric peptides [19]. Thus, we decided to combine **1** with several amino acids and investigate the antimicrobial and anti-biofilm properties of the resulting hybrid compounds [20].

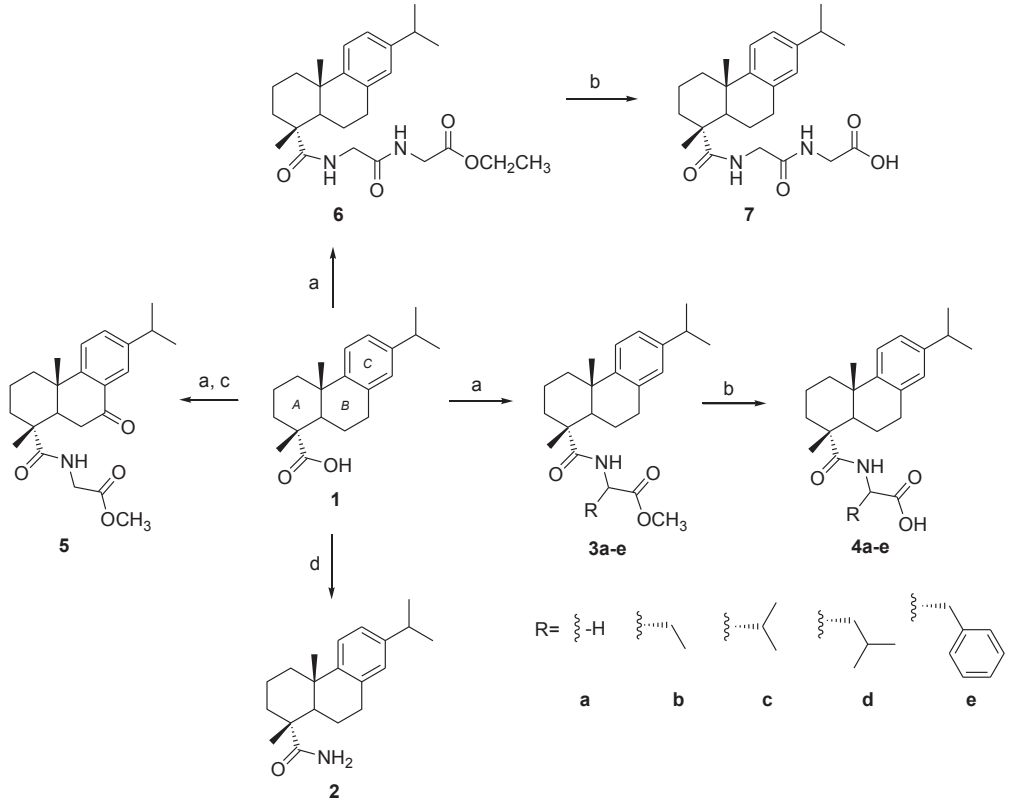
2. Results and discussion

2.1. Design and synthesis

Our design strategy focused on generating a library of dehydroabietic acid **1** derivatives by chemical modification of rings A and B of its diterpenoid core. The synthesis was supported by bioactivity testing and the best compounds were selected for further mechanistic studies. A first set of compounds was synthesized from **1** and ammonia **2**, or the methyl ester hydrochlorides of glycine **3a** and the L-amino acids homoalanine **3b**, valine **3c**, leucine **3d**, and phenylalanine **3e**, using carbodiimide-mediated couplings (Scheme 1).

Derivatives with free carboxyl groups **4a-e** were prepared from the corresponding esters by alkaline hydrolysis. Oxidation of the ring B position 7 of the glycine derivative **3a** with chromium(VI) trioxide gave compound **5**. Compounds **6** and **7**, bearing an extended side chain, were also prepared from **1** (Scheme 1). Screening for anti-biofilm activity of these compounds was done using a phenotypic assay that measures cellular viability of *S. aureus* biofilms with a redox dye [21]. The effects of the compounds prior to and after biofilm formation were measured (pre- and post-exposure assays, respectively) against two different *S. aureus* strains (ATCC 25923 and Newman; Supplementary Tables 1 and 2) to exclude strain-specific effects. Compound **1** was used as a reference, together with penicillin G and vancomycin. We tested the compounds first at a high concentration of 400 μM, and the active ones were re-tested at 100 μM. Compounds displaying more than 50% inhibitory activity against biofilms at 100 μM in both pre- and post-exposure modes were studied further. Their antimicrobial activities (minimum inhibitory concentration, MIC) and anti-biofilm potencies (IC_{50}) are depicted in Table 1.

From our initial set of compounds we first identified the single amino acid-bearing **4d** and **4e** as good anti-biofilm agents against *S. aureus* ATCC 25923, with potencies comparable to that of **1**. Like **1**, these compounds not only prevent biofilm formation (in the pre-exposure assay) but also significantly reduce the viability of existing biofilms (in the post-exposure assay). As expected, the activity of penicillin G and vancomycin on pre-formed biofilms was modest even at the highest concentration tested (400 μM). We found that the free carboxyl group is important for the anti-biofilm activity of our compounds as the methyl esters in the set **3a-e** were inactive (Supplementary Tables 1 and 2). In the pre-exposure assay,



Scheme 1. Synthesis of compounds **2**, **3a**–**e**, **4a**–**e**, and **5**–**7**. a) EDC, HOBt, DIPEA, amino acid/dipeptide alkyl ester hydrochloride, DMF, r.t. b) NaOH (4 M, aq.), THF:MeOH, 0 °C to r.t. c) CrO_3, EtOAc, HOAc, 50 °C d) EDC, HOBt, NH_3 (25% aq.), DMF, r.t. DIPEA = *N*,*N*-diisopropylethylamine; DMF = *N*,*N*-dimethylformamide; EDC = 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; HOBt = *N*-hydroxybenzotriazole; MeOH = methanol; THF = tetrahydrofuran.

Table 1
Antimicrobial activity and anti-biofilm potencies of the seven most active compounds.

Cmpd	*S. aureus* ATCC 25923				*S. aureus* Newman			
	Biofilms (IC_{50}, μM)			Planktonic bacteria	Biofilms (IC_{50}, μM)			Planktonic bacteria
	PRE	POST	Fold[b]	MIC (μM)	PRE	POST	Fold[b]	MIC (μM)
1	27.8	112.8	4	70	31.6	72.4	2	70
4d	62.2	121.3	2	80	51.8	134.5	3	90
4e	35.5	108.7	3	50	35.9	83.7	2	40
9a	37.4	98.2	3	70	36.2	110.3	3	80
9b	33.2	86.1	3	60	20.9	71.7	3	60
11	9.4	27.9	3	15	7.9	48.2	6	20
13	60.6	145.3	2	100	63.5	93.1	1	100
Pen G	0.13	57%[a]	–	0.12	0.27	73%[a]	–	0.25
Van	0.71	25%[a]	–	2.7	1.3	37.9	29	2.5

[a] Inhibitory activity determined at 400 μM.
[b] Fold = IC_{50} (POST)/IC_{50} (PRE).

inhibition of biofilm formation can be at least partially attributed to killing of planktonic cells. Both compounds **4d** and **4e** indeed have antimicrobial activity against single-cells on both staphylococcal strains, as revealed by their MIC values.

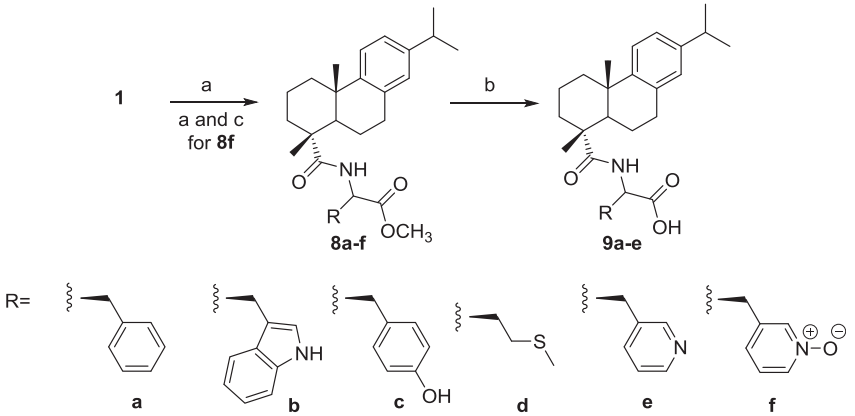
We next prepared compound **9a** (Scheme 2) as the D-amino acid counterpart of compound **4e**. We also used the unusual D-amino acid 3-pyridylalanine to study the effect of introducing a nitrogen atom into the aromatic ring (**8e** and **9e**), and further oxidized it to produce the *N*-oxide **8f**. In addition, other D-amino acids namely tryptophan, tyrosine, and methionine were used as building blocks in our synthesis (**8b-d**) as these have also been reported to affect bacterial biofilms [16,17].

As before, the methyl esters were hydrolyzed to yield compounds **9b-d**. The presence of a 3-pyridyl side chain in compound **8e** and the oxidation to the corresponding *N*-oxide **8f** did not contribute towards an overall improvement of the anti-biofilm activity of the compounds. However, we found that like **4e**, the D-phenyl derivative **9a** is a good anti-biofilm and antimicrobial agent, with similar potency to **1** (Table 1). We also identified the D-tryptophan derivative **9b** as a potent anti-biofilm and antimicrobial agent with a modest activity improvement, particularly in pre-formed biofilms of *S. aureus* ATCC 25923, when compared to **1** (Table 1).

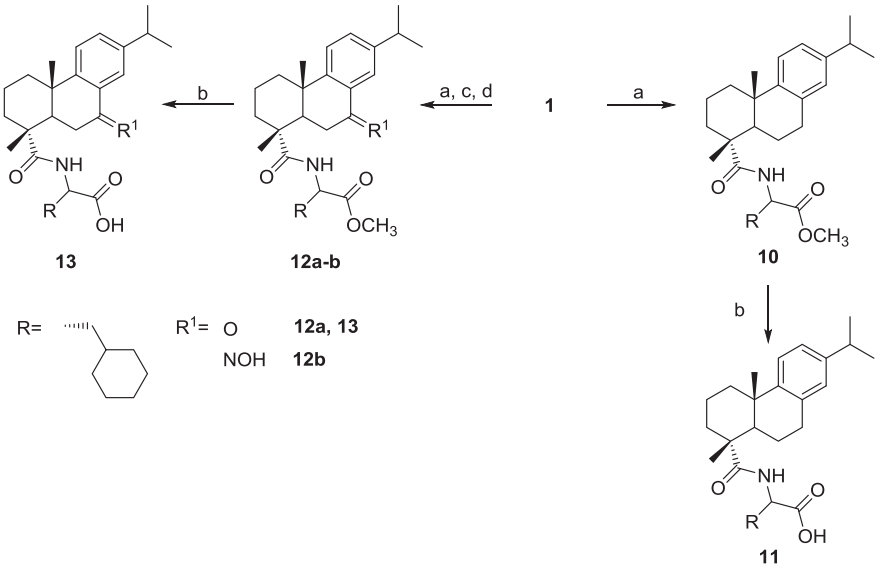
Next we investigated the importance of the aromaticity of the amino acid side chain of compound **4e** for its activity by using the unusual amino acid cyclohexyl-L-alanine for the synthesis of compound **11** (Scheme 3). A set of derivatives of this compound was also prepared by introducing a carbonyl group at position 7 as before (**12a**, **13**) and producing an oxime derivative (**12b**). As with most of the compounds in our previous sets, those bearing the free carboxyl groups were the most active anti-biofilm agents (**11** and **13**).

Compound **11** was identified as the most potent anti-biofilm and antimicrobial derivative in comparison to the parent compound **1**. It potently prevented biofilm formation in both staphylococcal strains and only 3–6-fold higher concentrations of compound were needed to reduce the viability and biomass of pre-formed biofilms, indicating that biofilms are less tolerant to **11** than to the control antibiotics which cannot achieve such effect even at impractical millimolar concentrations. Compound **11** is also the best antimicrobial agent in our study, with significantly low MIC values (<10 μg/mL, Supplementary Table 3) [22] for both the staphylococcal strains tested. The improved antimicrobial and anti-biofilm potencies of this compound when compared to those of the derivatives **4d**, **4e** and **9a** indicate that the size and the aromaticity of the side chain are important factors governing the observed activity. Although a few other abietane-type diterpenoids namely, salvipisone and 4-*epi*-pimaric acid, have been reported to prevent biofilm formation on staphylococcal strains, their activities are modest and characterized in much less detail [23,24]. Our design strategy starting from compound **1** gave compounds **9b** and **11**, the most potent abietane-type anti-biofilm agents reported so far [20]. These two compounds and also compounds **3b**, **4b**, **4d**, **5**–**7**, **8b**, **8d**–**f**, **9a**–**c**, **10**, **11**, **12a**–**b**, and **13** are new.

The most active compounds identified from our sets were followed-up in selectivity studies, by determining their inhibitory



Scheme 2. Synthesis of compounds **8a**–**f** and **9a**–**e**. a) EDC, HOBt, DIPEA, amino acid alkyl ester hydrochloride, DMF, r.t. b) NaOH (4 M, aq.), THF:MeOH, 0 °C to r.t. c) *m*-CPBA, $CHCl_3$, 0 °C to r.t. *m*-CPBA = *m*-chloroperoxybenzoic acid.



Scheme 3. Synthesis of cyclohexyl-L-alanine derivatives **10**, **11**, **12a**–**b**, and **13**. a) EDC, HOBt, DIPEA, cyclohexyl-L-alanine·HCl, DMF, r.t. b) NaOH (4 M, aq.), THF:MeOH, 0 °C to r.t. c) CrO_3, EtOAc, HOAc, 50 °C d) $H_2NOH·HCl$, pyridine, EtOH, 100 °C.

activities against three other biofilm-forming strains. For this study, we tested the compounds at a concentration corresponding to their IC_{50} values, earlier determined on *S. aureus* ATCC 25923 biofilms (Table 2, pre-exposure assay). Like compound **1**, the selected compounds were overall inactive against the Gram-negative *Escherichia coli* showing less than 15% inhibition at the concentration tested. For the other staphylococcal strains tested, the inhibition values were comparable to those determined on the *S. aureus* ATCC 25923 biofilms, indicating that all compounds act on a broader representation of Gram-positive bacterial biofilms.

2.2. Studies of the mechanistic action

To shed light into the possible mechanism of actions of our compounds, we first followed the changes in cellular viability after the exposure of pre-formed *S. aureus* ATCC 25923 biofilms to compounds **1**, **9b** and **11**, for a short period of time (1 h). The viability of the biofilms remained unaltered upon exposure to **1**, in agreement with our previous findings that its anti-biofilm effects are preferentially elicited upon binding to intracellular targets and require longer response times [15]. However, in sharp contrast, a very rapid and sudden drop in the viable biofilm core (more than 50% of inhibition of viable biofilms) was induced by both compounds **9b** and **11**, with **9b** being particularly effective and fast-acting, reaching nearly 70% inhibition (Fig. 1A) within the first hour. As the action of the compounds was found to involve rapid events, we reasoned that one plausible target would be the bacterial membrane or the peptidoglycan (PG) layer.

Thus, we next measured the changes occurring in the bacterial membrane potential after 1 h of treatment with **1**, **9b** and **11**. Whereas **1** did not alter the membrane potential of pre-formed *S. aureus* biofilms, a statistically significantly higher fluorescence emission of the potential sensitive probe $DiBAC_{4(3)}$ was detected in **9b** and **11**-treated cells, when compared to control (untreated) cells, clearly correlating with a dissipation of the membrane potential (Fig. 1B). This prompted us to further investigate the occurrence of ATP leakage. Using a luciferase-based assay, efflux of ATP from **9b** and **11**-treated biofilms to the extracellular medium was shown to be significantly higher than in untreated biofilms (Fig. 1C), confirming that both compounds are able to destabilize the biofilm integrity leading to a lethal metabolic collapse. It is known that ATP efflux correlates with rapid changes in cellular viability, and typically it occurs as result of the formation of large and stable membrane pores (>1.5 nm in diameter) [25]. We found that unlike the parent dehydrobietic acid **1**, the amino acid-containing derivatives **9b** and **11** were shown to evoke a fast and very effective destabilization of the membrane integrity [25].

Furthermore, membrane perturbation was directly followed by

Table 2
Anti-biofilm activity of the seven most active compounds against three other biofilm forming strains.

Cmpd[a]	% Inhibition		
	E. coli XL1 Blue	*S. epidermidis* ATCC 12228	*S. epidermidis* ATCC 35984
1	7.4 (±3.3)	49.4 (±1.0)	23.2 (±8.0)
4d	14.2 (±4.5)	62.4 (±2.2)	53.4 (±4.8)
4e	7.4 (±2.7)	57.3 (±4.6)	58.1 (±4.1)
9a	N.A.	92.3 (±1.3)	55.2 (±1.5)
9b	8.7 (±5.6)	70.3 (±5.6)	66.9 (±0.9)
11	5.2 (±11.3)	42.3 (±9.5)	48.8 (±4.8)
13	N.A.	66.6 (±12.6)	52.9 (±6.5)
Pen G	9.6 (±6.2)	28.0 (±2.3)	15.5 (±15.0)
Van	3.2 (±4.1)	N.A.[b]	N.A.[b]

[a] IC_{50} value determined on *S. aureus* ATCC 25923.
[b] N.A. = Not active.

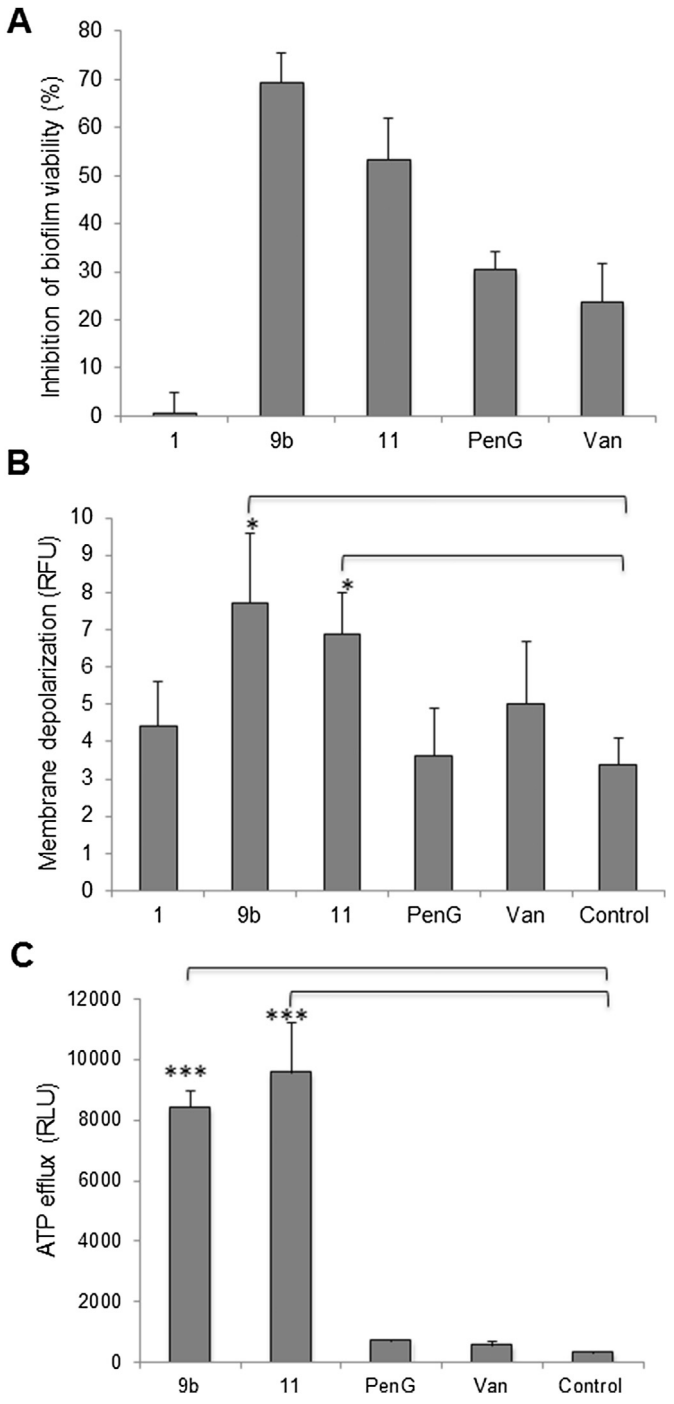


Fig. 1. Changes occurring in biofilms exposed to **1**, **9b** and **11** (100 μM) during 1 h $^{*}p < 0.05$; $^{***}p < 0.001$ indicate statistically significant differences when compared to untreated biofilms (unpaired t-test with Welch's correction).

fluorescence imaging using Syto9 and propidium iodide (PI) staining (Fig. 2). After 1 h of treatment with **9b** and **11**, the green fluorescence of Syto9 was quenched by the red fluorescence of PI (B and C images), in comparison to intact biofilms (A). Since PI can only enter cells with damaged membranes, this confirms that biofilms exposure to **9b** or **11** indeed results in a rapid loss of membrane integrity. The fact that the membrane-targeting effect is observed in **9b** and **11**, but it is absent in the parent compound **1**, lacking the amino acid motif, is a noticeable mechanistic aspect. Various bacterial species, among them *S. aureus*, produce D-amino acids in the stationary phase of growth. In the best study species (*Bacillus subtilis*), these amino acids have been thought to play regulatory functions by modulating the synthesis of the PG layer (acting as substrates of the PG synthesis and causing structural changes on it) as well as by regulating periplasmic enzymes, such as penicillin-binding proteins (PBP) [26]. It has been demonstrated that treatment of *S. aureus* biofilms with D-amino acids, among them D-tryptophan, causes disorganization of several proteins that surround the cell wall, although the exact proteins involved have not yet been identified [17]. It seems thus plausible to hypothesize that a short-period exposure to the compounds may trigger a similar disruption of the PG layer, ultimately compromising the membrane integrity and overall architectural stability of the *S. aureus* biofilms, as we have demonstrated here. A common concern related to antibacterial and anti-biofilm compounds is unspecific cytotoxicity. We tested the activity of **9b** against HL cells (originating from the human respiratory tract) and found that it causes unspecific cytotoxicity as only 23% (±5.9) of the cells remained viable upon exposure to 100 μM of this compound (Supplementary Table 4). However, no such effect was observed for compound **11** as no statistically significant reduction in the viability of HL cells was observed at any of the tested concentrations (up to 100 μM).

3. Conclusion

This study highlights the value of abietane-type diterpenoids such as **1** as starting materials for the development of new antimicrobial and anti-biofilm agents useful against staphylococcal infections. New, potent compounds were prepared using not only the naturally occurring L-amino acids as side chains but also D- and unusual amino acids, which have the competitive advantage of resistance to enzymatic proteolysis. The most active compounds identified in our study, **9b** and **11**, are the most potent abietane-type anti-biofilm agents reported so far and display robust activity against pre-formed biofilms at concentrations only 3–6-fold higher than those required to inhibit biofilm formation. Thus, our design strategy rendered faster-acting anti-biofilm agents, in comparison to the parent compound **1**, and more importantly, towards which biofilms are less tolerant than to standard antibiotics. Our compounds seem to act preferentially on Gram-positive species and effectively disrupt bacterial biofilms with a mode of action that resembles that of many antimicrobial peptides (AMPs). However, while practical applications of many AMPs are hampered by their large sizes, susceptibility to enzymatic degradation, and high production costs, our compounds overcome these limitations, as they can easily be prepared in high yield and purity, in few reaction steps. In addition, their fast-acting kinetics can be exploited for the preparation of more active biocides, cleaning agents or other microbicidal products requiring short contact times with pathogenic microorganisms. Taken together, our findings provide a very promising role for the abietane-type diterpenoids in the search of new antimicrobial and anti-biofilm agents against Gram-positive bacteria. We are currently working towards the synthesis of additional amino acid derivatives of **1** to explore the full potential of our hybrid design strategy against several other relevant biofilm-forming bacterial strains.

4. Experimental procedures

4.1. Chemistry

All reagents were obtained from Sigma Aldrich Co or TCI Europe. Dehydroabietic acid (**1**, 90% purity) was obtained from Pfaltz & Bauer, USA. (−)-2-Aminobutyric acid methyl ester hydrochloride, H-Gly-Gly-OEt·HCl, and H-Ala-Ala-Ala-OMe acetate salt were

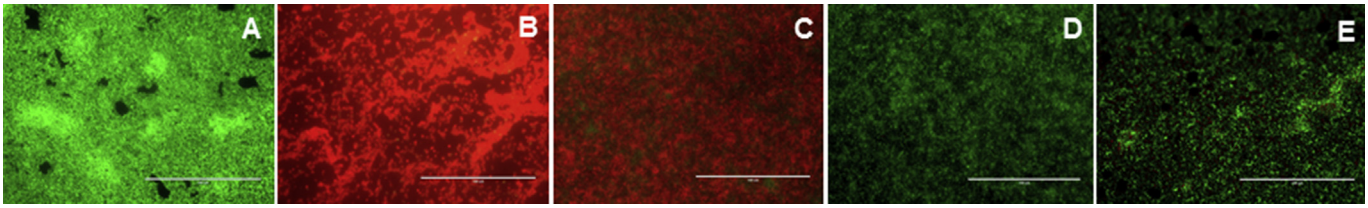


Fig. 2. Membrane permeability studies in biofilms exposed to the following treatments for 1 h. A: untreated biofilms; B: biofilms +**9b** (100 μM); C: biofilms +**11** (100 μM); D: biofilms + PenG (400 μM); E: biofilms + Van (400 μM). Scale bars correspond to 100 μm.

obtained from Bachem (Weil am Rhein, Germany). β-Cyclohexyl-L-alanine methyl ester hydrochloride was obtained from Novabiochem (Läufelfingen, Switzerland). For thin layer chromatography (TLC) analysis, Kieselgel 60HF254/Kieselgel 60G was used (Merck). Flash column chromatography (FCC) was made with a Biotage High-Performance Flash Chromatography Sp4-system (Uppsala, Sweden) using a 0.1-mm pathlength flow cell UV-detector/recorder module (fixed wavelength: 254 nm), and 12-mm or 25-mm flash cartridges. Melting points were recorded with an Electrothermal capillary tube melting point apparatus and are uncorrected. IR spectra were obtained using a Vertex 70 (Bruker Optics Inc., MA, USA) FTIR instrument. The FTIR measurements were made directly in solids with a horizontal attenuated total reflectance (ATR) accessory (MIRacle, Pike Technology, Inc, WI, USA). The transmittance spectra were recorded at a 4 cm^{-1} resolution between 4000 and 600 cm^{-1} using the OPUS 5.5 software (Bruker Optics Inc., MA, USA). NMR spectra were obtained using a Varian Mercury Plus 300 spectrometer, in $CDCl_3$ or DMSO-d_6, with tetramethylsilane (TMS) as the internal standard. The chemical shifts were reported in parts per million (ppm) and on the δ scale from TMS as an internal standard. The coupling constants J are quoted in Hertz (Hz). ESI-MS was performed by direct injection using a Synapt G2 HDMS (Waters, MA, USA) instrument.

4.1.1. Compound synthesis

*4.1.1.1. Dehydroabietyl amide (**2**).* Compound **1** (0.500 g, 1.66 mmol) was dissolved in DMF (5 mL) at room temperature. EDC hydrochloride (351 mg, 1.83 mmol) and HOBt monohydrate (247 mg, 1.83 mmol) were added and the mixture was stirred for 1 h before the dropwise addition of a 25% aqueous ammonia solution (1.2 mL). After 48 h the reaction was completed. The reaction mixture was suspended by addition of diethyl ether (100 mL) and water (20 mL). The aqueous phase was further extracted with diethyl ether (2 × 100 mL). The resulting organic phase was washed with a 1 M aqueous solution of HCl (2 × 50 mL), and water (50 mL), dried with Na_2SO_4, filtered, and evaporated to dryness to give a white solid (440 mg) which was purified by FCC using ethyl acetate:*n*-hexane (2:1) to give **2** as a white solid (357 mg, 72%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.21 (s, 3H), 1.23 (s, 6H), 1.29 (s, 3H), 2.11 (m, 1H), 2.32 (m, 1H), 2.85 (m, 3H), 5.73 (brs, 2H), 6.87 (s, 1H), 6.99 (d, J = 8.2 Hz, 1H), 7.16 (d, J = 8.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.8, 18.9, 21.3, 24.1, 24.1, 25.3, 30.1, 33.6, 37.2, 37.5, 38.2, 45.7, 47.5, 124.0, 124.2, 127.0, 134.7, 145.9, 147.1, 181.4. IR (ATR) 3433, 3362, 1628, 1606, 823, 632 cm^{-1}. HRMS *m*/*z*: calcd. for $C_{20}H_{30}NO$ 300.2327 $[M+H]^+$, found 300.2329.

*4.1.1.2. Methyl N-(abiet-8,11,13-trien-18-oyl) glycinate (**3a**).* Compound **1** (1.00 g, 3.33 mmol) was dissolved in DMF (10 mL), at room temperature. EDC hydrochloride (956 mg, 4.99 mmol) and HOBt monohydrate (676 mg, 5.00 mmol) were added and the mixture was left to agitate for 1 h. Glycine methyl ester hydrochloride (628 mg, 5.00 mmol) and DIPEA (1.76 mL, 10.1 mmol) were then added and the mixture was left to agitate for another hour after which the reaction was completed. The reaction mixture was suspended by addition of diethyl ether (200 mL) and water (40 mL). The aqueous phase was further extracted with diethyl ether (2 × 100 mL). The resulting organic phase was washed with a 1 M aqueous solution of HCl (50 mL), a saturated solution of $NaHCO_3$ (50 mL), water (50 mL), and brine (50 mL), dried with Na_2SO_4, filtered, and evaporated to dryness to give **3a** as a white solid (1.2 g, 97%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.21 (s, 3H), 1.23 (s, 6H), 1.32 (s, 3H), 2.14 (dd, J = 12.4, 2.1 Hz, 1H), 2.31 (d, J = 12.9 Hz, 1H), 2.86 (m, 3H), 3.76 (s, 3H), 4.04 (d, J = 5.1 Hz, 2H), 6.32 (brs, 1H), 6.86 (s, 1H), 6.98 (d, J = 8.1 Hz, 1H), 7.16 (d, J = 8.1 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.6, 18.8, 21.2, 24.1, 24.1, 25.4, 30.1, 33.6, 37.2, 37.4, 38.1, 41.7, 45.7, 47.5, 52.4, 124.0, 124.2, 127.0, 134.8, 145.8, 147.0, 170.9, 178.9. IR (ATR) 3376, 1758, 1640, 1519, 1205, 1179, 820 cm^{-1}. HRMS *m*/*z*: calcd. for $C_{23}H_{34}NO_3$ 372.2539 $[M+H]^+$, found 372.2538.

*4.1.1.3. Methyl N-(abiet-8,11,13-trien-18-oyl) L-ethylglycinate (**3b**).* Following the procedure for compound **3a**, compound **3b** was prepared from **1** (250 mg, 0.83 mmol), EDC hydrochloride (239 mg, 1.25 mmol), HOBt monohydrate (169 mg, 1.25 mmol), (−)-2-aminobutyric acid methyl ester hydrochloride (276 mg, 1.79 mmol), and DIPEA (0.44 mL, 2.5 mmol) in DMF (2.5 mL). Compound **3b**: white solid (307 mg, 92%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.92 (t, J = 7.5 Hz, 3H), 1.21 (s, 3H), 1.23 (s, 3H), 1.24 (s, 3H), 1.31 (s, 3H), 2.11 (dd, J = 12.4, 1.8 Hz, 1H), 2.32 (d, J = 12.9 Hz, 1H), 2.86 (m, 3H), 3.74 (s, 3H), 4.58 (m, 1H), 6.25 (d, J = 7.5 Hz, 1H), 6.88 (s, 1H), 7.00 (d, J = 8.1 Hz, 1H), 7.17 (d, J = 8.1 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 9.8, 16.6, 18.9, 21.3, 24.1, 24.1, 25.4, 25.7, 30.2, 33.6, 37.3, 37.6, 38.2, 45.9, 47.5, 52.4, 53.5, 124.0, 124.2, 127.1, 134.8, 145.9, 147.1, 173.4, 178.2. IR (ATR) 3361, 1743, 1637, 1517, 1207, 821 cm^{-1}. HRMS *m*/*z*: calcd. for $C_{25}H_{38}NO_3$ 400.2852 $[M+H]^+$, found 400.2853.

*4.1.1.4. Methyl N-(abiet-8,11,13-trien-18-oyl) L-valinate (**3c**).* Following the procedure for compound **3a**, compound **3c** was prepared from **1** (250 mg, 0.83 mmol), EDC hydrochloride (239 mg, 1.25 mmol), HOBt monohydrate (169 mg, 1.25 mmol), valine methyl ester hydrochloride (209 mg, 1.25 mmol), and DIPEA (0.44 mL, 2.5 mmol) in DMF (2.5 mL). Compound **3c**: white solid (309 mg, 90%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.93 (d, J = 7.0 Hz, 3H), 0.95 (d, J = 6.4 Hz, 3H), 1.21 (s, 3H), 1.23 (s, 3H), 1.24 (s, 3H), 1.31 (s, 3H), 2.11 (dd, J = 12.4, 2.0 Hz, 1H), 2.18 (m, 1H), 2.33 (d, J = 13 Hz, 1H), 2.88 (m, 3H), 3.74 (s, 3H), 4.58 (dd, J = 8.4, 4.8 Hz, 1H), 6.23 (d, J = 8.4 Hz, 1H), 6.89 (s, 1H), 7.00 (dd, J = 8.1, 1.8 Hz, 1H), 7.17 (d, J = 8.1 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.6, 18.1, 18.9, 19.2, 21.4, 24.1, 24.1, 25.5, 30.2, 31.4, 33.6, 37.4, 37.8, 38.2, 45.9, 47.7, 52.2, 57.3, 124.0, 124.3, 127.1, 134.9, 145.9, 147.0, 173.0, 178.4. IR (ATR) 3454, 1737, 1658, 1498, 1305, 821 cm^{-1}. HRMS *m*/*z*: calcd. for $C_{26}H_{40}NO_3$ 414.3008 $[M+H]^+$, found 414.3009.

*4.1.1.5. Methyl N-(abiet-8,11,13-trien-18-oyl) L-leucinate (**3d**).* Following the procedure for compound **3a**, compound **3d** was prepared from **1** (250 mg, 0.83 mmol), EDC hydrochloride (239 mg,

1.25 mmol), HOBt monohydrate (169 mg, 1.25 mmol), leucine methyl ester hydrochloride (226 mg, 1.24 mmol), and DIPEA (0.44 mL, 2.5 mmol) in DMF (2.5 mL). Compound **3d**: white solid (305 mg, 86%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.95 (d, J = 5 Hz, 6H), 1.21 (s, 3H), 1.23 (s, 3H), 1.24 (s, 3H), 1.30 (s, 3H), 2.07 (dd, J = 12.4, 2.0 Hz, 1H), 2.33 (d, J = 13.3 Hz, 1H), 2.87 (m, 3H), 3.73 (s, 3H), 4.63 (m, 1H), 6.07 (d, J = 8 Hz, 1H), 6.89 (s, 1H), 7.0 (dd, J = 8.2, 1.8 Hz, 1H), 7.17 (d, J = 8.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.5, 18.9, 21.3, 22.1, 23.0, 24.1, 24.1, 25.2, 25.4, 30.1, 33.6, 37.3, 37.6, 38.2, 41.7, 46.0, 47.4, 51.0, 52.3, 124.0, 124.2, 127.1, 134.8, 145.9, 147.1, 173.9, 178.3. IR (ATR) 3346, 1755, 1629, 1527, 1155, 821 cm^{-1}. HRMS m/z: calcd. for $C_{27}H_{42}NO_3$ 428.3165 $[M+H]^+$, found 428.3169.

*4.1.1.6. Methyl N-(abiet-8,11,13-trien-18-oyl) L-phenylalaninate (**3e**).* Following the procedure for compound **3a**, compound **3e** was prepared from **1** (250 mg, 0.83 mmol), EDC hydrochloride (239 mg, 1.25 mmol), HOBt monohydrate (169 mg, 1.25 mmol), phenylalanine methyl ester hydrochloride (269 mg, 1.25 mmol), and DIPEA (0.44 mL, 2.5 mmol) in DMF (2.5 mL). Compound **3e**: white solid (372 mg, 97%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.19 (s, 3H), 1.20 (s, 6H), 1.22 (s, 3H), 2.01 (d, J = 12.2 Hz, 1H), 2.27 (d, J = 12.8 Hz, 1H), 2.78 (m, 3H), 3.05 (dd, J = 14.1, 7.0 Hz, 1H), 3.17 (dd, J = 13.5, 5.9 Hz, 1H), 3.71 (s, 3H), 4.90 (m, 1H), 6.12 (d, J = 7.6 Hz, 1H), 6.84 (s, 1H), 6.97 (d, J = 8.2 Hz, 1H), 7.12 (m, 3H), 7.25 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.5, 18.9, 21.1, 24.1, 25.4, 30.1, 33.6, 37.2, 37.4, 38.1, 45.7, 47.4, 52.4, 53.2, 124.0, 124.2, 127.0, 127.2, 128.7, 129.3, 134.8, 136.2, 145.8, 147.0, 172.5, 178. IR (ATR) 3388, 1743, 1639, 1517, 1515, 1215, 821 cm^{-1}. HRMS m/z: calcd. for $C_{30}H_{40}NO_3$ 462.3008 $[M+H]^+$, found 462.3004.

*4.1.1.7. N-(Abiet-8,11,13-trien-18-oyl) glycine (**4a**).* Compound **2** (0.200 g, 0.54 mmol) was dissolved in THF:MeOH 1:1 (4.8 mL), at 0 °C, under magnetic stirring. A 4 M aqueous solution of NaOH (4.4 mL) was added dropwise and after the addition the mixture was left to agitate at room temperature for 1 h, after which the reaction was suspended by careful addition of a 4 M aqueous solution of HCl dropwise until the pH reached 6–7. The mixture was concentrated and extracted with diethyl ether (3 × 75 mL) after the addition of water (25 mL). The resulting organic phase was washed with a 1 M aqueous solution of HCl (50 mL), water (50 mL), and brine (50 mL), dried with Na_2SO_4, filtered, and evaporated to dryness to give **4a** as a white solid (183 mg, 95%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.20 (s, 3H), 1.23 (s, 6H), 1.32 (s, 3H), 2.13 (dd, J_1 = 12.4, 2.1 Hz, 1H), 2.31 (d, J = 12.6 Hz, 1H), 2.84 (m, 3H), 4.06 (d, J_1 = 5.0, 2.9 Hz, 2H), 6.45 (brs, 1H), 6.87 (s, 1H), 6.99 (d, J = 8.2, 1.8 Hz, 1H), 7.15 (d, J = 8.2 Hz, 1H), 8.06 (bs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.6, 18.8, 21.2, 24.1, 25.4, 30.0, 33.6, 37.2, 37.3, 38.1, 42.0, 45.7, 47.6, 124.1, 124.2, 127.1, 134.8, 145.9, 146.9, 173.3, 180. IR (ATR) 3380, 1730, 1641, 1522, 1197, 820 cm^{-1}. HRMS m/z: calcd. for $C_{22}H_{32}NO_3$ 358.2382 $[M+H]^+$, found 358.2383.

*4.1.1.8. N-(Abiet-8,11,13-trien-18-oyl) L-ethylglycine (**4b**).* Following the procedure for compound **4a**, compound **4b** was prepared from **3b** (150 mg, 0.38 mmol), using THF:MeOH 1:1 (3.7 mL), and 4 M NaOH (3 mL). Compound **4b**: white solid (133 mg, 92%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.97 (t, J = 7.4 Hz, 3H), 1.21 (s, 3H), 1.23 (s, 3H), 1.24 (s, 3H), 1.31 (s, 3H), 2.11 (dd, J_1 = 12.4, 2 Hz, 1H), 2.33 (d, J = 12.1 Hz, 1H), 2.85 (m, 3H), 4.55 (dd, J = 7.2, 5.5 Hz, 1H), 6.29 (d, J = 7.2 Hz, 1H), 6.88 (s, 1H), 7.00 (dd, J = 8.2, 1.8 Hz, 1H), 7.17 (d, J = 8.2 Hz, 1H), 10.5 (bs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 9.9, 16.5, 18.9, 21.3, 24.1, 24.1, 25.2, 25.4, 30.1, 33.6, 37.3, 37.5, 38.1, 45.8, 47.6, 53.9, 124.1, 124.2, 127.1, 134.8, 146.0, 147.0, 176.3, 179.3. IR (ATR) 3435, 1716, 1623, 1529, 1224, 821 cm^{-1}. HRMS m/z: calcd. for $C_{24}H_{36}NO_3$ 386.2695 $[M+H]^+$, found 386.2691.

*4.1.1.9. N-(Abiet-8,11,13-trien-18-oyl) L-valine (**4c**).* Following the procedure for compound **4a**, compound **4c** was prepared from **3c** (118 mg, 0.29 mmol), using THF:MeOH 1:1 (4 mL), and 4 M NaOH (2.4 mL). Compound **4c**: white solid (108 mg, 94%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.96 (d, J = 7.0 Hz, 3H) 0.99 (d, J = 6.4 Hz, 3H), 1.21 (s, 3H), 1.24 (s, 3H), 1.25 (s, 3H), 1.32 (s, 3H), 2.11 (d, J = 12.3 Hz, 1H), 2.28 (m, 2H), 2.85 (m, 3H), 4.58 (m, 1H), 6.27 (d, J = 8.2 Hz, 1H), 6.88 (s, 1H), 7.0 (d, J = 8.2 Hz, 1H), 7.17 (d, J = 8.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.6, 18.0, 18.9, 19.3, 21.4, 24.1, 24.1, 25.5, 30.1, 31.0, 33.6, 37.4, 37.7, 38.2, 45.9, 47.8, 57.5, 124.1, 124.3, 127.1, 134.8, 145.9, 147.0, 176.2, 179.2. IR (ATR) 3435, 3076, 1724, 1637, 1525, 1406, 1207, 821 cm^{-1}. HRMS m/z: calcd. for $C_{25}H_{38}NO_3$ 400.2852 $[M+H]^+$, found 400.2852.

*4.1.1.10. N-(Abiet-8,11,13-trien-18-oyl) L-leucine (**4d**).* Following the procedure for compound **4a**, compound **4d** was prepared from **3d** (150 mg, 0.35 mmol), using THF:MeOH 1:1 (3.7 mL), and 4 M NaOH (3 mL). Compound **4d**: white solid (120 mg, 83%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.96 (m, 6H), 1.21 (s, 3H), 1.24 (s, 6H), 1.30 (s, 3H), 2.09 (dd, J = 12.3, 1.7 Hz, 1H), 2.33 (d, J = 12.8 Hz, 1H), 2.85 (m, 3H), 4.60 (m, 1H), 6.12 (d, J = 7.7 Hz, 1H), 6.88 (s, 1H), 7.00 (d, J = 8.2 Hz, 1H), 7.17 (d, J = 8.2 Hz, 1H), 10.49 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.5, 18.9, 21.3, 21.9, 24.1, 24.1, 25.2, 25.4, 30.0, 33.6, 37.3, 37.5, 38.2, 41.0, 45.9, 47.5, 51.3, 124.1, 124.2, 127.1, 134.8, 146.0, 147.0, 177.3, 179.3. IR (ATR) 3359, 1733, 1627, 1523, 1232, 819 cm^{-1}. HRMS m/z: calcd. for $C_{26}H_{40}NO_3$ 414.3008 $[M+H]^+$, found 414.3007.

*4.1.1.11. N-(Abiet-8,11,13-trien-18-oyl) L-phenylalanine (**4e**).* Following the procedure for compound **4a**, compound **4e** was prepared from **3e** (150 mg, 0.32 mmol), using THF:MeOH 1:1 (3.7 mL), and 4 M NaOH (3 mL). Compound **4e**: white solid (135 mg, 93%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.10 (s, 3H), 1.15 (s, 3H), 1.20 (s, 3H), 1.22 (s, 3H), 2.08 (d, J = 10.9 Hz, 1H), 2.28 (d, J = 12.3 Hz, 1H), 2.80 (m, 3H), 2.94 (dd, J = 13.5, 10.5 Hz, 1H), 3.08 (dd, J = 13.5, 4.1 Hz, 1H) 4.54 (m, 1H), 6.87 (d, J = 1.2 Hz, 1H), 7.01 (d, J_1 = 8.2 Hz, 1H), 7.26 (m, 6H), 7.80 (m, 1H), 12.55 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 16.2, 18.5, 20.0, 23.9, 24.0, 24.9, 29.2, 32.9, 35.8, 36.2, 36.6, 37.3, 44.4, 46.3, 53.5, 123.4, 123.9, 126.1, 126.3, 127.9, 129.1, 134.6, 138.2, 145.0, 147.1, 173.4, 177.3. IR (ATR) 3442, 1755, 1598, 1537, 1261, 1091, 1020, 798 cm^{-1}. HRMS m/z: calcd. for $C_{29}H_{38}NO_3$ 448.2852 $[M+H]^+$, found 448.2851.

*4.1.1.12. Methyl N-(7-oxoabiet-8,11,13-trien-18-oyl) glycinate (**5**).* Compound **3a** (0.20 g, 0.54 mmol) was dissolved in glacial acetic acid (3.1 mL) and added dropwise to a cooled (0 °C) solution of chromium (VI) oxide (0.060 g, 0.60 mmol) in glacial acetic acid (0.8 mL) and ethyl acetate (1.7 mL), over a period of about 10 min. The reaction mixture was then warmed to 50 °C, under argon. After 4 h more chromium (VI) oxide (0.060 g, 0.60 mmol) was added and after 1 h the reaction was completed. The reaction mixture was suspended by cooling in an ice bath and adding ice and dichloromethane (150 mL). The aqueous phase was further extracted with dichloromethane (2 × 75 mL). The combined organic phases were washed with water (50 mL), a saturated aqueous solution of $NaHCO_3$ (50 mL), water (50 mL), and brine (50 mL), dried with Na_2SO_4, filtered, and evaporated to dryness. Purification by FCC using ethyl acetate:*n*-hexane (2:1) gave **5** as a white solid (116 mg, 56%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.22 (d, J = 1 Hz, 3H), 1.24 (d, J = 1 Hz, 3H), 1.26 (s, 3H), 1.39 (s, 3H), 2.35 (d, J = 11.7 Hz, 1H), 2.58 (m, 3H), 2.91 (m, 1H), 3.76 (s, 3H), 4.01 (m, 2H), 6.33 (brs, 1H), 7.27 (d, J = 8.2 Hz, 1H), 7.39 (dd, J = 8.2 Hz, 1.2 Hz, 1H), 7.83 (d, J = 1.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.5, 18.4, 23.9, 24.0, 33.7, 36.9, 37.1, 37.4, 37.4, 41.8, 44.2, 46.6, 52.6, 123.5, 125.2, 130.9, 132.7, 147.0, 153.1, 170.6, 177.5, 198.7. IR (ATR) 3392, 1755, 1678, 1645, 1526, 1198 cm^{-1}.

HRMS *m/z*: calcd. for $C_{23}H_{32}NO_4$ 386.2331 $[M+H]^+$, found 386.2333.

*4.1.1.13. Ethyl N-(abiet-8,11,13-trien-18-oyl) glycyl-glycinate (**6**).* Following the procedure for compound **3a**, compound **6** was prepared from **1** (0.500 g, 1.66 mmol), EDC hydrochloride (478 mg, 2.49 mmol), HOBt monohydrate (338 mg, 2.49 mmol), H-Gly-Gly-OEt•HCl (472 mg, 2.40 mmol), and DIPEA (0.88 mL, 5.0 mmol) in DMF (5 mL). Compound **6**: white solid (600 mg, 81%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.20 (s, 3H), 1.21 (s, 3H), 1.22 (s, 3H), 1.27 (m, 3H), 1.31 (s, 3H), 2.15 (m, 1H), 2.29 (m, 1H), 2.83 (m, 3H), 4.01 (m, 4H), 4.18 (m, 2H), 6.75 (m, 1H), 6.85 (s, 1H), 6.98 (m, 2H), 7.14 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.3, 16.6, 18.8, 21.4, 24.1, 24.1, 25.3, 30.1, 33.6, 37.2, 37.4, 38.1, 41.5, 43.6, 45.5, 47.5, 61.6, 124.0, 124.1, 127.0, 134.7, 145.8, 147.0, 169.6, 169.7, 179.5. IR (ATR) 3335, 1747, 1637, 1520, 1196, 1024. HRMS *m/z*: calcd. for $C_{26}H_{39}N_2O_4$ 443.2910 $[M+H]^+$, found 443.2910.

*4.1.1.14. N-(Abiet-8,11,13-trien-18-oyl) glycyl-glycine (**7**).* Following the procedure for compound **4a**, compound **7** was prepared from **6** (0.600 g, 1.35 mmol), using THF:MeOH 1:1 (12 mL), and 4 M NaOH (11 mL). Compound **7**: white solid (270 mg, 48%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.19 (s, 6H), 1.22 (s, 3H), 1.28 (s, 3H), 2.12 (d, J = 12.4 Hz, 1H), 2.27 (m, 1H), 2.81 (m, 3H), 3.99 (m, 4H), 6.85 (m, 1H), 6.98 (m, 1H), 7.13 (m, 2H), 7.29 (s, 1H), 8.86 (bs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.5, 18.8, 21.3, 24.1, 25.3, 30.0, 33.6, 37.2, 37.2, 38.0, 41.5, 43.5, 45.4, 47.6, 124.0, 124.2, 127.0, 134.7, 145.9, 147.0, 170.4, 172.4, 180.4. IR (ATR) 3358, 1733, 1634, 1520, 1209, 820 cm^{-1}. HRMS *m/z*: calcd. for $C_{24}H_{35}N_2O_4$ 415.2597 $[M+H]^+$, found 415.2597.

*4.1.1.15. Methyl N-(abiet-8,11,13-trien-18-oyl) D-phenylalaninate (**8a**).* Compound **1** (1.00 g, 3.33 mmol), D-phenylalanine methyl ester hydrochloride (1.08 g, 5.00 mmol), EDC (0.96 g, 5.0 mmol), HOBt (0.68 g, 5.0 mmol) were dissolved in dry DMF (11 mL). The reaction mixture was stirred until all the solids were dissolved. DIPEA (1.74 mL, 10.0 mmol) was added. After stirring the mixture at room temperature for 105 min, it was poured into water (100 mL). The resulting precipitate was filtered and purified by FCC (silica column, 15 → 25% EtOAc in *n*-hexane). Compound **8a**: white solid (0.65 g, 42%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.19 (m, 9H), 1.22 (m, 3H), 2.04 (d, J = 12.4 Hz, 1H), 2.28 (d, J = 13.5 Hz, 1H), 2.76 (m, 3H), 3.05 (dd, J = 14.1, 6.4 Hz, 1H), 3.17 (dd, J = 14.1, 5.3 Hz, 1H), 3.73 (s, 3H), 4.88 (m, 1H), 6.15 (d, J = 7.6 Hz, 1H), 6.83 (s, 1H), 6.97 (d, J = 8.2 Hz, 1H), 7.10 (m, 3H), 7.25 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.5, 18.9, 21.2, 24.1, 24.1, 25.4, 30.0, 33.6, 37.2, 37.4, 38.0, 38.1, 45.6, 47.5, 52.4, 53.3, 124.0, 124.2, 127.0, 127.3, 128.7, 129.3, 134.8, 136.2, 145.8, 147.1, 172.6, 178.1. IR (ATR) 3360, 1742, 1639, 1497, 1213, 700 cm^{-1}. HRMS *m/z*: calcd. for $C_{30}H_{40}NO_3$ $[M+H]^+$ 462.3008, found 462.3009.

*4.1.1.16. Methyl N-(abiet-8,11,13-trien-18-oyl) D-tryptophanate (**8b**).* Following the procedure for compound **3a**, compound **8b** was prepared from **1** (0.500 g, 1.66 mmol), EDC hydrochloride (478 mg, 2.49 mmol), HOBt monohydrate (338 mg, 2.49 mmol), D-tryptophan methyl ester hydrochloride (634 mg, 2.49 mmol), and DIPEA (0.88 mL, 5.0 mmol) in DMF (5 mL). Compound **8b**: white solid (655 mg, 79%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15 (s, 3H), 1.18 (s, 3H), 1.22 (s, 3H), 1.24 (s, 3H), 2.06 (m, 1H), 2.29 (m, 1H), 2.70 (m, 1H), 2.83 (m, 1H), 3.33 (d, J = 5.8 Hz, 2H), 3.72 (s, 3H), 4.96 (m, 1H), 6.31 (d, J = 7.4 Hz, 1H), 6.84 (d, J = 1.3 Hz, 1H), 6.97 (m, 1H), 7.04 (m, 1H), 7.08 (m, 1H), 7.17 (m, 2H), 7.35 (d, J = 8.2 Hz, 1H), 7.55 (d, J = 8.2 Hz, 1H), 8.31 (bs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.4, 18.8, 21.0, 24.1, 24.1, 25.3, 27.6, 29.9, 33.6, 37.1, 38.1, 45.6, 47.4, 52.4, 53.3, 110.3, 111.4, 118.7, 119.8, 122.4, 122.7, 123.9, 124.1, 127.0, 127.7, 134.8, 136.3, 145.7, 147.0, 172.9, 178.4. IR (ATR) 3315, 1743, 1635, 1498, 1213, 738 cm^{-1}. HRMS *m/z*: calcd. for $C_{32}H_{41}N_2O_3$ 501.3117 $[M+H]^+$, found 501.3115.

*4.1.1.17. Methyl N-(abiet-8,11,13-trien-18-oyl) D-tyrosinate (**8c**).* Following the procedure for compound **3a**, compound **8c** was prepared from **1** (0.500 g, 1.66 mmol), EDC hydrochloride (478 mg, 2.49 mmol), HOBt monohydrate (338 mg, 2.49 mmol), D-tyrosine methyl ester hydrochloride (578 mg, 2.49 mmol), and DIPEA (0.88 mL, 5.0 mmol) in DMF (5 mL). Compound **8c**: white solid (623 mg, 79%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.19 (s, 3H), 1.20 (s, 3H), 1.21 (s, 3H), 1.22 (s, 3H), 2.07 (dd, J = 12.4, 2 Hz, 1H), 2.28 (d, J = 12.9 Hz, 1H), 2.79 (m, 3H), 2.96 (m, 1H), 3.09 (dd, J = 13.8, 5.9 Hz, 1H), 3.72 (s, 3H), 4.82 (m, 1H), 6.21 (d, J = 7.6 Hz, 1H), 6.71 (m, 2H), 6.84 (s, 1H), 6.92 (m, 2H), 6.97 (dd, J = 8.2, 1.7 Hz, 1H), 7.13 (d, J = 8.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.5, 18.8, 21.1, 24.1, 25.3, 30.0, 33.6, 37.2, 37.2, 38.0, 45.6, 47.5, 52.5, 53.5, 115.8, 124.0, 124.1, 127.0, 127.2, 130.3, 134.7, 145.8, 146.9, 155.7, 172.7, 178.7. IR (ATR) 3354, 1743, 1633, 1514, 1220, 821 cm^{-1}. HRMS *m/z*: calcd. for $C_{30}H_{40}NO_4$ 478.2957 $[M+H]^+$, found 478.2954.

*4.1.1.18. Methyl N-(abiet-8,11,13-trien-18-oyl) D-methioninate (**8d**).* Following the procedure for compound **3a**, compound **8d** was prepared from **1** (0.500 g, 1.66 mmol), EDC hydrochloride (478 mg, 2.49 mmol), HOBt monohydrate (338 mg, 2.49 mmol), D-methionine methyl ester hydrochloride (497 mg, 2.49 mmol), and DIPEA (0.88 mL, 5.0 mmol) in DMF (5 mL). Compound **8d**: white solid (620 mg, 84%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.21 (s, 3H), 1.23 (s, 6H), 1.31 (s, 3H), 2.00 (m, 1H), 2.09 (s, 3H), 2.17 (m, 2H), 2.32 (d, J = 12.7 Hz, 1H), 2.49 (m, 2H), 2.85 (m, 3H), 3.76 (s, 3H), 4.72 (m, 1H), 6.49 (d, J = 7.5 Hz, 1H), 6.87 (s, 1H), 6.99 (dd, J = 8.2, 1.8 Hz, 1H), 7.16 (d, J = 8.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 15.7, 16.6, 18.9, 21.3, 24.1, 25.4, 30.2, 30.2, 31.7, 33.6, 37.3, 37.5, 38.1, 45.9, 47.5, 51.9, 52.6, 124.1, 124.2, 127.0, 134.7, 145.9, 147.0, 172.9, 178.5. IR (ATR) 3356, 1743, 1523, 1213, 823 cm^{-1}. HRMS *m/z*: calcd. for $C_{26}H_{40}NO_3S$ 446.2729 $[M+H]^+$, found 446.2727.

*4.1.1.19. Methyl N-(abiet-8,11,13-trien-18-oyl) H-β-(3-pyridyl)-D-alaninate (**8e**).* Compound **1** (540 mg, 1.80 mmol), *H*-β-(3-pyridyl)-D-Ala-OMe hydrochloride (0.500 g, 1.98 mmol), EDC (380 mg, 1.98 mmol), HOBt (270 mg, 1.98 mmol) were dissolved in dry DMF (11 mL). DIPEA (1.74 mL, 10.0 mmol) was added. After stirring the mixture at room temperature for 16 h, it was poured into cold H_2O (80 mL). The precipitated solid was filtered and purified by FCC (silica column, 50% EtOAc in *n*-hexane) Compound **8e**: white solid (0.70 g, 84%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.20 (s, 6H), 1.23 (s, 3H), 1.23 (s, 3H), 2.09 (dd, J = 12.3, 2.3 Hz, 1H), 2.29 (d, J = 11.7 Hz, 1H), 2.80 (m, 3H), 3.07 (dd, J = 14.1, 6.5 Hz, 1H), 3.24 (dd, J = 14.1, 5.3 Hz, 1H), 3.77 (s, 3H), 4.93 (m, 1H), 6.30 (d, J = 7.0 Hz, 1H), 6.86 (d, J = 1.8 Hz, 1H), 6.99 (dd, J = 8.2, 2.3 Hz, 1H), 7.15 (d, J = 8.2 Hz, 1H), 7.24 (m, 1H), 7.49 (dt, J = 8.2, 2.3 Hz, 1H), 8.35 (d, J = 1.8 Hz, 1H), 8.51 (dd, J = 5.3, 1.8 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.5, 18.8, 21.3, 24.1, 25.4, 30.0, 33.6, 35.3, 37.2, 37.5, 38.0, 45.4, 47.5, 52.7, 53.0, 123.6, 124.0, 124.2, 127.0, 132.1, 134.7, 137.1), 145.9), 146.9, 148.3, 150.2, 172.1, 178.4. IR (ATR) 3252, 1732, 1653, 1539, 1234, 708 cm^{-1}. HRMS *m/z*: calcd. for $C_{29}H_{39}N_2O_3$ $[M+H]^+$ 463.2961, found 463.2957.

*4.1.1.20. Methyl N-(abiet-8,11,13-trien-18-oyl) H-β-(3-pyridyl-N-oxide)-D-alaninate (**8f**).* Compound **8e** (0.10 g, 0.22 mmol) was dissolved in $CHCl_3$ (2 mL). This solution was cooled on an ice bath and *m*-CPBA (0.10 g, 0.45 mmol) was added in small portions. The reaction mixture was stirred at room temperature for 17 h. It was transferred to a silica gel column and purified by FCC (10% MeOH in EtOAc). Compound **8f**: white solid (50 mg, 47%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.20 (s, 3H), 1.20 (s, 3H), 1.22 (s, 3H), 1.26 (s, 3H), 2.11 (dd,

J = 12.3, 2.1 Hz, 1H), 2.29 (d, J = 11.7 Hz, 1H), 2.80 (m, 3H), 3.03 (dd, J = 14.4, 6.2 Hz, 1H), 3.21 (dd, J = 14.4, 5.6 Hz, 1H), 3.79 (m, 3H), 4.88 (q, J = 6.5 Hz, 1H), 6.48 (d, J = 7.0 Hz, 1H), 6.86 (s, 1H), 6.99 (dd, J = 8.2, 1.8 Hz, 1H), 7.15 (d, J = 8.2 Hz, 2H), 7.24 (m, 1H), 8.05 (s, 1H), 8.14 (d, J = 6.5 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.6, 18.8, 21.4, 24.1, 24.1, 25.4, 30.0, 33.6, 35.0, 37.2, 37.5, 38.0, 45.4, 47.6, 52.7, 53.0, 124.1, 124.2, 125.8, 127.0, 127.6, 134.6, 136.3, 137.9, 139.8, 145.9, 146.9, 171.6, 178.8. IR (ATR) 3254, 1742, 1649, 1261, 1213, 1159, 681 cm^{-1}. HRMS m/z: calcd. for $C_{29}H_{38}N_2O_4Na$ $[M+Na]^+$ 501.2729, found 501.2731.

*4.1.1.21. N-(Abiet-8,11,13-trien-18-oyl) D-phenylalanine (**9a**).* Compound **8a** (550 mg, 1.2 mmol) was dissolved in 1:1 THF/MeOH (15 mL). A 4 M aqueous solution of NaOH (13 mL) was added. After stirring the mixture at room temperature for 3 h, it was cooled on an ice bath and acidified with 4 M HCl. The precipitate was filtered and dried *in vacuo*. Compound **9a**: white solid (490 mg, 91%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.19 (s, 6H), 1.22 (s, 3H), 1.24 (s, 3H), 2.03 (d, J = 12.3 Hz, 1H), 2.29 (d, J = 12.3 Hz, 1H), 2.78 (m, 3H), 3.11 (dd, J = 14.1, 7.0 Hz, 1H), 3.29 (dd, J = 14.1, 5.8 Hz, 1H), 4.87 (q, J = 6.4 Hz, 1H), 6.17 (d, J = 7.0 Hz, 1H), 6.85 (s, 1H), 6.99 (d, J = 8.2 Hz, 1H), 7.16 (m, 3H), 7.27 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.4, 18.8, 21.2, 24.2, 25.3, 29.9, 33.6, 37.2, 37.2, 37.3, 38.0, 45.6, 47.6, 53.5, 124.0, 124.1, 127.1, 127.4, 128.9, 129.4, 134.8, 135.9, 145.9, 146.9, 175.1, 179.5. IR (ATR) 3445, 1747, 1600, 1539, 1205, 700 cm^{-1}. HRMS m/z: calcd. for $C_{29}H_{38}NO_3$ $[M+H]^+$ 448.2852, found 448.2856.

*4.1.1.22. N-(Abiet-8,11,13-trien-18-oyl) D-tryptophan (**9b**).* Following the procedure for compound **4a**, compound **9b** was prepared from **8b** (250 mg, 0.50 mmol), using THF:MeOH 1:1 (5.0 mL), and 4 M NaOH (4.2 mL). Compound **9b**: white solid (226 mg, 93%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.05 (s, 3H), 1.13 (s, 3H), 1.22 (s, 3H), 1.24 (s, 3H), 1.89 (d, J = 12.3 Hz, 1H), 2.23 (d, J = 12.6 Hz, 1H), 2.81 (m, 3H), 3.36 (m, 2H), 4.83 (m, 1H), 6.25 (m, 1H), 6.79 (m, 1H), 7.07 (m, 5H), 7.29 (m, 2H), 7.56 (d, J = 7.8 Hz), 8.11 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.3, 18.7, 21.0, 24.1, 24.1, 25.3, 26.9, 29.8, 31.0 33.6, 37.0, 37.1, 38.0, 45.7, 47.5, 53.8, 109.7, 111.6, 118.5, 119.9, 122.4, 123.2, 123.9, 124.1, 127.0, 127.7, 134.8, 136.3, 145.8, 146.9, 175.5, 179.8. IR (ATR) 3402, 3257, 1728, 1629, 1529, 740 cm^{-1}. HRMS m/z: calcd. for $C_{31}H_{39}N_2O_3$ 487.2961 $[M+H]^+$, found 487.2961.

*4.1.1.23. N-(Abiet-8,11,13-trien-18-oyl) D-tyrosine (**9c**).* Following the procedure for compound **4a**, compound **9c** was prepared from **8c** (250 mg, 0.52 mmol), using THF:MeOH 1:1 (7.3 mL), and 4 M NaOH (4.4 mL). Compound **9c**: white solid (217 mg, 89%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.20 (s, 3H), 1.21 (s, 3H), 1.24 (s, 6H), 2.08 (dd, J = 11.1, 2.0 Hz, 1H), 2.30 (d, J = 12.6 Hz, 1H), 2.80 (m, 3H), 3.00 (dd, J = 13.9, 6.6 Hz, 1H), 3.14 (dd, J = 13.9, 5.1 Hz, 1H), 4.87 (m, 1H), 6.37 (d, J = 7.5 Hz, 1H), 6.72 (d, 1H, J = 8.2 Hz), 6.86 (s, 1H), 6.98 (m, 3H), 7.15 (d, J = 8.2 Hz, 1H), 7.36 (bs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.4, 18.8, 21.2, 24.1, 24.1, 25.4, 30.0, 33.6, 36.8, 37.2, 37.2, 38.0, 45.3, 47.6, 53.7, 115.9, 124.0, 124.2, 127.1, 127.3, 130.4, 134.7, 145.9, 146.9, 155.4, 175.3, 179.7. IR (ATR) 3280, 1718, 1616, 1515, 1220, 821 cm^{-1}. HRMS m/z: calcd. for $C_{29}H_{38}NO_4$ 464.2801 $[M+H]^+$, found 464.2801.

*4.1.1.24. N-(Abiet-8,11,13-trien-18-oyl) D-methionine (**9d**).* Following the procedure for compound **4a**, compound **9d** was prepared from **8d** (250 mg, 0.56 mmol), using THF:MeOH 1:1 (7.9 mL), and 4 M NaOH (4.7 mL). Compound **9d**: white solid (234 mg, 96%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.21 (s, 3H), 1.24 (s, 6H), 1.32 (s, 3H), 2.14 (m, 3H), 2.10 (s, 3H), 2.33, (m, 1H), 2.57 (t, 2H, J = 7.3 Hz), 2.85 (m, 3H), 4.72 (m, 1H), 6.70 (d, J = 7.2 Hz, 1H), 6.88 (d, J = 1.6 Hz, 1H), 7.00 (dd, J = 8.2, 1.8 Hz, 1H), 7.17 (d, J = 8.2 Hz, 1H), 9.66 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 15.6, 16.5, 18.8, 21.3, 24.1, 25.4, 30.1, 30.2, 30.8, 33.6, 37.2, 37.4, 38.1, 45.9, 47.6, 52.3, 124.1, 124.2, 127.1, 134.7, 145.9, 146.9, 175.6, 179.7. IR (ATR) 3373, 1737, 1631, 1541, 1228, 821 cm^{-1}. HRMS m/z: calcd. for $C_{25}H_{38}NO_3S$ 432.2572 $[M+H]^+$, found 432.2573.

*4.1.1.25. Methyl N-(abiet-8,11,13-trien-18-oyl) H-β-(3-pyridyl)-D-alanine (**9e**).* Following the procedure for compound **4a**, compound **9e** was prepared from **8e** (240 mg, 0.52 mmol), using THF:MeOH 1:1 (4.8 mL) and 4 M NaOH (4.3 mL). The reaction mixture was acidified with 1 M HCl and the water phase was extracted with diethyl ether. The organic phase was dried with anhydrous Na_2SO_4 and evaporated. The crude product was purified by FCC (DCM/MeOH). Compound **9e**: white solid (102 mg, 44%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.08 (s, 3H), 1.09 (s, 3H), 1.13 (s, 3H), 1.16 (s, 3H), 1.88 (d, J = 11.7 Hz, 1H), 2.25 (d, J = 12.3 Hz, 1H), 2.70 (m, 3H), 3.03 (dd, J = 13.5, 5.3 Hz, 1H), 3.14 (dd, J = 13.5, 5.3 Hz, 1H), 4.14 (m, 1H), 6.79 (m, 1H), 6.94 (d, J = 8.21 Hz, 1H), 7.12 (m, 2H), 7.21 (dd, J = 7.62, 4.69 Hz, 1H), 7.49 (d, J = 7.62 Hz, 1H), 8.30 (m, 1H), 8.34 (dd, J = 4.69, 1.76 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 16.2, 18.3, 20.4, 23.9, 24.9, 29.3, 32.8, 34.0, 36.5, 36.7, 37.7, 44.8, 46.2, 54.5, 122.7, 123.6, 123.9, 126.3, 134.2, 134.2, 136.8, 144.9, 146.9, 150.4, 173.3, 176.1. IR (ATR) 3316, 1594, 1497, 1415, 821, 712 cm^{-1}. HRMS m/z: calcd. for $C_{28}H_{37}N_2O_3$ 449.2804 $[M+H]^+$, found 449.2805.

*4.1.1.26. Methyl N-(abiet-8,11,13-trien-18-oyl) cyclohexyl-L-alaninate (**10**).* Following the procedure for compound **3a**, compound **10** was prepared from **1** (250 mg, 0.83 mmol), EDC hydrochloride (239 mg, 1.25 mmol), HOBt monohydrate (169 mg, 1.25 mmol), β-cyclohexyl-L-alanine methyl ester hydrochloride (277 mg, 1.25 mmol), and DIPEA (0.44 mL, 2.5 mmol) in DMF (2.5 mL). Compound **10**: white solid (374 mg, 96%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.21 (s, 3H), 1.23 (s, 3H), 1.24 (s, 3H), 1.30 (s, 3H), 2.09 (dd, J = 12.4, 2.1 Hz, 1H), 2.32 (d, J = 12.9 Hz, 1H), 2.88 (m, 3H), 3.72 (s, 3H), 4.67 (m, 1H), 6.07 (d, J = 8.2 Hz, 1H), 6.89 (s, 1H), 7.0 (dd, J = 8.2, 1.8 Hz, 1H), 7.17 (d, J = 8.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.4, 18.7, 21.1, 23.9, 24.0, 25.3, 26.1, 26.3, 26.4, 30.0, 32.4, 33.4, 33.6, 34.4, 37.2, 37.3, 38.0, 40.0, 45.8, 47.3, 50.1, 52.2, 123.9, 124.1, 126.9, 134.7, 145.7, 146.9, 173.9, 178.1. IR (ATR) 3344, 1751, 1627, 1525, 1448, 1172, 819 cm^{-1}. HRMS m/z: calcd. for $C_{30}H_{46}NO_3$ 468.3478 $[M+H]^+$, found 468.3477.

*4.1.1.27. N-(Abiet-8,11,13-trien-18-oyl) cyclohexyl-L-alanine (**11**).* Following the procedure for compound **4a**, compound **11** was prepared from **10** (0.10 g, 0.21 mmol), using THF:MeOH 1:1 (3 mL), and 4 M NaOH (1.8 mL). Compound **11**: white solid (95 mg, 98%). ^{1}H NMR (300 MH, $CDCl_3$) δ 1.21 (s, 3H), 1.24 (s, 6H), 1.31 (s, 3H), 2.10 (m, 1H), 2.33 (d, J = 11.8 Hz, 1H), 2.86 (m, 3H), 4.64 (m, 1H), 6.11 (d, J = 7.7 Hz, 1H), 6.89 (s, 1H), 7.00 (dd, J = 8.2, 1.7 Hz, 1H), 7.17 (d, J = 8.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.5, 18.9, 21.3, 24.1, 24.1, 25.4, 26.2, 26.4, 26.5, 30.1, 32.5, 33.6, 33.7, 34.6, 37.3, 37.4, 38.2, 39.5, 45.9, 47.5, 50.6, 124.1, 124.2, 127.1, 134.8, 145.9, 147.0, 177.1, 179.3. IR (ATR) 3346, 1732, 1625, 1525, 1232, 819 cm^{-1}. HRMS m/z: calcd. for $C_{29}H_{44}NO_3$ 454.3321 $[M+H]^+$, found 454.3322.

*4.1.1.28. Methyl N-(7-oxoabiet-8,11,13-trien-18-oyl) cyclohexyl-L-alaninate (**12a**).* Following the procedure for compound **5**, compound **12a** was prepared from **10** (0.20 g, 0.43 mmol), chromium(VI) oxide (133 mg, 1.34 mmol), glacial acetic acid (3.9 mL), and ethyl acetate (1.7 mL). Compound **12a**: white solid (98 mg, 47%). ^{1}H NMR ($CDCl_3$) δ 1.23 (s, 3H), 1.25 (s, 3H), 1.27 (s, 3H), 1.39 (s, 3H), 2.38 (m, 2H), 2.67 (m, 2H), 2.92 (sept. J = 7 Hz, 1H), 3.72 (s, 3H), 4.64 (m, 1H), 6.10 (d, J = 8.1 Hz, 1H), 7.28 (d, J = 8.1 Hz, 1H), 7.39 (d, J = 8.1 Hz), 7.85 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.6, 18.5, 23.9, 23.9, 24.0, 26.1, 26.3, 26.5, 32.7, 33.6, 33.7, 34.6, 37.1, 37.2, 37.4, 37.5, 40.2, 44.0, 46.5, 50.6, 52.4, 123.4, 125.2, 131.0, 132.6, 147.0, 153.1, 173.8, 176.9, 198.4. IR (ATR) 3394, 1745, 1681, 1676, 1521, 1450, 1251,

1197, 835 cm^{-1}. HRMS *m/z*: calcd. for $C_{30}H_{44}NO_4$ 482.3270 $[M+H]^+$, found 482.3271.

*4.1.1.29. Methyl N-(7-hydroxyiminoabiet-8,11,13-trien-18-oyl) cyclohexyl-L-alaninate (**12b**).* Compound **12a** (250 mg, 0.52 mmol) and hydroxylamine hydrochloride (0.060 g, 0.88 mmol) were dissolved in EtOH (1.5 mL) and pyridine (63 μL) was added. The reaction mixture was stirred in a closed vial at 100 °C for 3 h. Solvents were evaporated and the residue was purified by FCC (silica column, 15 → 50% EtOAc in *n*-hexane). Compound **12b**: white solid (200 mg, 78%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.12 (s, 3H), 1.23 (s, 3H), 1.25 (s, 3H), 1.42 (s, 3H), 2.27 (m, 2H), 2.67 (m, 2H), 2.88 (sept, *J* = 6.9 Hz, 1H), 3.71 (s, 3H), 4.66 (m, 1H), 6.13 (d, *J* = 8.2 Hz, 1H), 7.20 (m, 2H), 7.68 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.6, 18.4, 23.1, 23.5, 23.9, 24.2, 26.0, 26.3, 26.5, 32.5, 33.7, 33.8, 34.4, 36.7, 37.3, 37.4, 40.1, 42.1, 46.5, 50.5, 52.3, 122.4, 122.9, 128.0, 129.0, 146.6, 148.8, 155.6, 174.0, 177.4. IR (ATR) 3360, 1738, 1641, 1508, 1447, 1204, 951, 729 cm^{-1}. HRMS *m/z*: calcd. for $C_{30}H_{45}N_2O_4$ $[M+H]^+$ 497.3379, found 497.3379.

*4.1.1.30. N-(7-Oxoabiet-8,11,13-trien-18-oyl) cyclohexyl-L-alanine (**13**).* Compound **12a** (350 mg, 0.73 mmol) was dissolved in THF/MeOH 1:1 (10 mL) and a 4 M aqueous solution of NaOH (8.5 mL) was added. The color of the reaction mixture changed to yellow. The reaction mixture was then stirred at room temperature for 2 h 45 min. The mixture was acidified with a 4 M aqueous solution of HCl and concentrated. Water was added and the mixture was extracted three times with ethyl acetate. The organic phase was then washed with a 1 M aqueous solution of HCl, water and brine and dried with Na_2SO_4 and evaporated. The crude product was purified by FCC (silica column, 50% EtOAc in *n*-hexane and 2% acetic acid). Compound **13**: white solid (0.23 g, 68%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.23 (s, 3H), 1.25 (s, 3H), 1.26 (s, 3H), 1.38 (s, 3H), 2.40 (m, 2H), 2.65 (m, 2H), 2.92 (sept, *J* = 6.9 Hz, 1H), 4.62 (m, 1H), 6.15 (d, *J* = 7.6 Hz, 1H), 7.28 (d, *J* = 9.4 Hz, 1H), 7.40 (dd, *J* = 8.2, 1.8 Hz, 1H), 7.85 (d, *J* = 2.3 Hz, 1H), 9.21 (bs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.5, 18.4, 23.9, 24.0, 24.0, 26.1, 26.3, 26.5, 32.6, 33.7, 33.7, 34.6, 37.0, 37.1, 37.4, 39.6, 43.9, 46.6, 50.6, 123.5, 125.3, 130.9, 132.8, 147.1, 153.2, 176.7, 177.8, 198.8. IR (ATR) 3348, 1732, 1681, 1636, 1531,1232, 1194, 833 cm^{-1}. HRMS *m/z*: calcd. for $C_{29}H_{41}NO_4Na$ $[M+Na]^+$ 490.2933, found 490.2932.

4.2. Biology

4.2.1. Bacterial strains and growth conditions

Biofilm-producing, clinical strains of *S. aureus* (ATCC 25923 and Newman), *Staphylococcus epidermidis* (ATCC 12228, non-clinical and ATCC 35984, clinical) and *E. coli* (XL1 Blue, non-clinical) were provided by the Faculty of Pharmacy, University of Helsinki, Finland. Freezer stocks of bacterial strains containing 20% glycerol were maintained at −70 °C. Bacteria were pre-cultured in 30 g/L tryptic soy broth (TSB, Fluka Biochemika, Buchs, Switzerland) overnight in 37 °C with 220 rpm shaking. Cultures were prepared by diluting the overnight cultures 1000 times (*S. aureus*, *E. coli*) and 100 times (*S. epidermidis*) in TSB and bacteria were grown under aerobic conditions in 37 °C, 200 rpm shaking approximately for 4 h to an OD_{595} of ~0.4 which corresponds to ~10^8 colony forming units per milliliter (CFU/mL).

4.2.2. Biofilm formation

Biofilms were formed according to the conditions previously optimized and described by Refs. [21,27]. Fresh, exponentially grown cultures were diluted to be 10^6 CFU/mL and added to flat-bottomed 96-well plates (Nunclon™ Δ surface, Nunc, Roskilde, Denmark), 200 μL per well, or flat-bottomed 6-well plates (Costar®, Corning Incorporated, NY, USA), 5 mL per well depending on the type of assay and plates were incubated in 37 °C, 200 rpm for 18 h.

4.2.3. Exposure to compounds and biofilm quantification

Anti-biofilm activity of the compounds in DMSO was assessed against both *S. aureus* strains and in two exposure conditions, prior-to and post-biofilm formation, as recently described [15,28]. In the pre-exposure mode of the assay, compounds and bacterial suspensions were added simultaneously prior to biofilm formation and plates were incubated for 18 h in 37 °C with shaking (200 rpm). During the post-exposure mode, the compounds were added after biofilms were first formed as described earlier (37 °C, 200 rpm for 18 h) and plates were incubated for additional 24 h at 37 °C, 200 rpm. Penicillin G (potassium salt, Fluka Biochemika, Buchs, Switzerland) and vancomycin (hydrochloride hydrate, Sigma Aldrich, St. Louis, MO, USA) prepared in Mueller Hinton Broth (MHB, Fluka Biochemika, Buchs, Switzerland) were used as control antibiotics and tested always at 400 μM. Untreated biofilms, TSB-media controls (cell-free) and wells containing biofilms and 2% DMSO were included as controls. Initial screening of the compound collection was performed at final compound concentrations of 400 and 100 μM. Resazurin assay was used for the quantification of biofilms viability as previously described [21,29]. Planktonic suspension was carefully removed from plates and biofilms were stained with 20 μM resazurin (Sigma Aldrich, St. Louis, MO, USA) solution prepared in sterile 0.01 M phosphate buffered saline PBS (pH = 7.3–7.5, Lonza, Verviers, Belgium) and incubated at room temperature (200 rpm shaking, darkness, 20 min) followed by fluorescence measurements (λex = 560 nm, λem = 590 nm) using Varioskan Flash Multimode Plate Reader operated with SkanIt® software, version 2.4.3 (ThermoFisher Scientific, Vantaa, Finland).

4.2.4. Determination of anti-biofilm potencies and effects on planktonic bacteria

Anti-biofilm potencies (IC_{50} and IC_{90}) of the most active compounds **1**, **4d**, **4e**, **9a**, **9b**, **11**, **13** and reference antibiotics were determined in both exposure modes on both strains of *S. aureus*. At least 12 concentrations ranging from 0.001 to 400 μM were tested in duplicates for the inhibitory activity using the resazurin-based viability assay. Potencies were calculated via a non-linear regression analysis (sigmoidal dose–response with variable slope) using Microsoft Excel 2010 and GraphPad Prism v. 4.0 software (GraphPad software Inc., US). To estimate compounds effects on planktonic bacteria minimal inhibitory concentrations (MIC) of the most active compounds were determined. Minimal concentrations causing no turbidity on suspended bacteria were measured. For that purpose, after exposing biofilms to the compounds (37 °C, 200 rpm, 18 h), planktonic phase was transferred to a sterile 96-microtiter well plate and quantitative readouts were detected at λ = 620 nm using Varioskan Plate Reader (2.4.3 software) [15,28].

4.2.5. Anti-biofilm activity against other strains

The most active compounds (**1**, **4d**, **4e**, **9a**, **9b**, **11**, **13**) were further tested for the anti-biofilm activity against two strains of *S. epidermidis* and *E. coli*. Control antibiotics at a concentration of 400 μM were also included. Compounds were tested in four biological replicates at concentrations at which they caused 50% inhibition of biofilm viability in pre-exposure mode of assay on *S. aureus* ATCC 25923. Biofilm viability was quantified using resazurin staining assay, as described above, but in case of *E. coli*, the incubation period was prolonged to 35 min [29].

4.2.6. Time-killing studies

Kinetics of the anti-biofilm activity of the two most active compounds (**9b** and **11**) and reference compound **1** in the post-

exposure mode of the assay were monitored on *S. aureus* ATCC 25923 biofilms. Biofilms were formed as described above and after an incubation period of 18 h, planktonic suspension was removed and biofilms were exposed to the compounds at a final concentration of 100 μM and fresh TSB was added into the control wells. Penicillin G and vancomycin were included as control antibiotics and tested at a concentration of 400 μM. Plates were further incubated in 37 °C, 200 rpm and biofilms were quantified with resazurin staining assay after 1 h incubation, as previously described.

4.2.7. Determination of membrane depolarization

Membrane depolarization caused by compounds **1**, **9b** and **11** was detected using a membrane-potential-sensitive probe, bis(1,3-dibutylbarbituric acid) trimethine oxonol, $DiBAC_{4(3)}$ (Molecular Probes®, Invitrogen, CA, US) according to the protocol originally described [25] with some modifications. *S. aureus* ATCC 25923 biofilms were formed on flat bottomed 96-well plates (Nunclon™ Δ surface, Nunc, Roskilde, Denmark) for 18 h. After finishing the 18 h incubation, planktonic suspension was removed and biofilm were washed with PBS (0.01 M, pH = 7.3–7.5, Lonza, Verviers, Belgium) followed by pre-incubation with 5 μM $DiBAC_{4(3)}$ solution prepared in PBS (100 μL) at room temperature for 30 min. Then, solution was removed and biofilms were exposed to solutions containing $DiBAC_{4(3)}$ at a concentration of 5 μM and compounds at a final concentration of 100 μM. Control antibiotics, penicillin G and vancomycin were included at a concentration of 400 μM. Plates were incubated at room temperature for 1 h. Biofilms were washed twice with PBS and followed by $DiBAC_{4(3)}$ fluorescence was measured at λex = 485 nm and λem = 535 nm using Varioskan Flash Multimode Plate Reader (2.4.3 software, ThermoFisher, Vantaa, Finland).

4.2.8. Measurement of ATP efflux from biofilms

ATP efflux from the *S. aureus* ATCC 25923 biofilms after exposure to compounds **9b** and **11** was quantified. *S. aureus* ATCC 25923 biofilms were formed on flat bottomed 6-well plates (Corning® Costar®, NY, US) in 37 °C with 200 rpm shaking for 18 h. Negative control wells containing 5 mL of cell-free TSB were also included. After the incubation period, the planktonic suspension was removed from the wells and biofilms were incubated with 1.25 mL of compound solutions **9b** and **11**, prepared in sterile PBS at a final concentration of 100 μM. Control antibiotics penicillin G and vancomycin were tested at 400 μM. Untreated biofilms and negative controls wells were incubated only with PBS. After incubation in 37 °C for 1 h suspensions were filtered using 0.22 μm centrifugal filter (Jet Biofil, China) and diluted 10-fold with sterile PBS in Eppendorf tubes. Then, 100 μL of each filtrate in duplicates was transferred to a clear-bottom 96-well plate (Isoplate-TC) (Perkin Elmer, Waltham, MA, US). CellTiter-Glo® reagent (Promega, Madison, WI, US) was prepared, used according to the manufacturer's protocol and luciferin luminescence was measured at 560 nm using Varioskan Flash Multimode Plate Reader (2.4.3 software, ThermoFisher, Vantaa, Finland).

4.2.9. Membrane permeability studies using fluorescence microscopy

Membrane permeability on *S. aureus* ATCC 25923 biofilms upon exposure to compounds **9b** and **11** for 1 h was also measured. *S. aureus* ATCC 25923 biofilms were first formed on flat-bottomed 24-well plates (Nunc, Roskilde, Denmark) in which coverslips have been placed on the bottom (total growth area was estimated to be 1.9 cm^2) in the same manner as described earlier (exponentially grown cultures of 10^6 CFU/mL incubated at 37 °C with 200 rpm shaking for 18 h). After the incubation period, the planktonic suspension was removed from the wells and biofilms were incubated with 1 mL of compound solutions **9b** and **11**, prepared in sterile PBS at a final concentration of 100 μM. Control antibiotics penicillin G and vancomycin were tested at 400 μM. Untreated biofilms and negative controls wells were incubated only with sterile PBS. After 1 h, the solutions were then removed and a mixture of probes (34 μL) containing 5 μM of Syto9 and 30 μM of propidium iodide (LIVE/DEAD® BacLight™, Molecular Probes, Life Technologies, Eugene, OR, US) was added and incubated for 15 min at room temperature in the darkness. Afterwards, coverslips were flipped and mounted with a drop of Mounting Oil (LIVE/DEAD® BacLight™, Molecular Probes, Life Technologies, Eugene, OR, US) into slides that were imaged using an Inverted Evos FL microscope with a 40× coverslip-corrected objective. Images were obtained using cubes for GFP and RFP (for Syto9 and PI fluorescence, respectively) and directly acquired with the microscope; overlapping of the green and red fluorescence was done directly on the microscope while taking the pictures.

4.2.10. Cytotoxicity studies

Human lung (HL) epithelial cells [30] were grown in RPMI 1640 (Biowhittaker, Lonza, Walkersville, USA) supplemented with inactivated FBS 7%, L-glutamine 2 mM and gentamicin 20 μg/mL. Cells were routinely cultured in 75 cm^2 cell culture flasks at 37 °C in 5% CO_2 in an air-ventilated humidified incubator to around 90% confluence. The harvesting was done by adding 0.25% trypsin in PBS. Cell suspensions (200 μL) of 4×10^5 cells/ml were added into plates and incubated at 37 °C for 24 h. After that, 20 μL of culture media was removed and replaced with a similar volume of compounds solutions (**9b** and **11**) at different concentrations (1–100 μM) and plates were incubated for additional 24 h at 37 °C. Then, culture media was replaced with a solution of resazurin in PBS at a concentration of 20 μM and maintained for 2 h at 37 °C in the incubator conditions [31]. Reduced resazurin signal as indication of cell viability was measured using a Varioskan Flash multimode plate reader (λex = 570 nm and λem = 590 nm) and results expressed as relative fluorescence units (RFUs). Cells without test compounds were included as positive controls, wells containing only media as negative controls and 0.5% DMSO as solvent controls. Percentages of cell viability were calculated in relation to the untreated cells.

4.2.11. Statistical analysis

Assay performance was evaluated using statistical parameters: screening window coefficient Z'-factor, signal-to-noise (S/N) and signal-to-background (S/B) –ratios [27]. In each assay, control wells containing only bacteria and TSB were included to establish maximal and minimum signals, respectively. For the analysis of data from ATP efflux and membrane depolarization experiments unpaired t-test with Welch's correction was applied using GraphPad Prism v. 4.0 –software (GraphPad software Inc., US). $p < 0.05$ was considered in all cases as statistically significant.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.07.038.

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