Bioorganic & Medicinal Chemistry 20 (2012) 583–591

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Development and characterization of improved β-lactone-based anti-virulence drugs targeting ClpP

Evelyn Zeiler [a], Vadim S. Korotkov [b], Katrin Lorenz-Baath [b], Thomas Böttcher [b,*], Stephan A. Sieber [a,*]

[a] Department Chemie, Center for Integrated Protein Science CIPSM, Technische Universität München, Institute of Advanced Studies IAS, Lichtenbergstrasse 4, 85747 Garching, Germany
[b] AVIRU, EXIST Transfer of Research, OC II, Lichtenbergstrasse 4, 85747 Garching, Germany

ARTICLE INFO

Article history:
Available online 29 July 2011

Keywords:
Beta-lactones
MRSA
Antibiotics
Virulence
Disarming pathogens

ABSTRACT

Here, we report the synthesis and in depth characterization of a second generation β-lactone derived virulence inhibitors. Based on initial results that emphasized the intriguing possibility to disarm bacteria in their virulence the present study develops this concept further and analyses the potential of this strategy for drug development. We were able to expand the collection of bioactive compounds via an efficient synthetic route. Testing of all compounds revealed several hits with anti-virulence activity. Moreover, we demonstrated that these molecules act solely by reducing virulence but not killing bacteria which is an important prerequisite for preserving the useful microbiome. Finally, incubation of lactones with eukaryotic cell lines indicated a tolerable cytotoxicity which is essential for entering animal studies.

1. Introduction

Bacterial infections pose again a serious threat for human health. Reasons for this critical development are the rapid emergence and spread of multiresistant strains and the steadily decreasing number of available drugs. As bacteria are killed by antibiotics, their excessive use exerts a paramount selective pressure favoring the development of any bypassing mechanism which increases bacterial survival. This has led to the evolution of resistances against almost any available antibiotic. However, antibiotic drug discovery declines as result of rapid resistance development which puts the commercial success of any novel antibiotic project into jeopardy. The lack of novel cellular targets and corresponding lead structures for drug development challenges the treatment of bacterial infections now and in the near future. New concepts are thus urgently needed. Recently, we discovered compounds of the β-lactone family as potent inhibitors of the protease ClpP, a central regulator of virulence in *Staphylococcus aureus*.[1,2] ClpP has been implicated in regulating the expressional levels of major virulence factors of *S. aureus* in quorum sensing dependent manner[3,4] and was thus proposed as novel drug target.[5] We demonstrated that a chemical knockout of ClpP by our β-lactones leads to a global decrease in virulence factor production. The novel concept aims to disarm pathogens instead of killing them. This is expected to decrease the risk of rapid resistance development and preserve the host's endogenous microbiome. Based on our initial inhibitor **U1** (Fig. 2A) we demonstrated its anti-virulence capacity even against aggressive and multiresistant *S. aureus* strains (MRSA).[1,2] Here, we report our efforts to expand the structural space of β-lactones, define leads for drug development and establish a GMP (good manufacturing practice) conform route for their synthesis. Active compounds were identified by a combination of enzyme inhibition and virulence factor bioassays and mechanistically supported by competitive proteomic profiling. We could confirm the hypothesis that growth of commensal bacteria of the human microbiome is not adversely affected and that the tolerable toxicity profile with eukaryotic cell lines makes our lead structures ideal candidates for a novel generation of anti-infective drugs.

2. Results and discussion

2.1. Expanding the structural space of β-lactones

One aim of the present study is to exploit the structural space for ClpP inhibition in more detail.[6,7] We used a previously established strategy to synthesize racemic *trans*-3,4-substituted β-lactones from thioesters and aldehydes in a one-pot reaction (strategy A, Fig. 1).

The five new derivatives were obtained by this strategy in low yields (5–15% for lactonization) and comprised different side groups which aimed to vary the motive of **U1** (**1**) by the hydrophobicity (**2**), the chain length (**3**), or both (**4**, **5** and **6**) (Fig. 2A). To assess the potency of the β-lactones to inhibit ClpP protease activity

* Corresponding authors. Present address: Harvard Medical School, Department of Biological Chemistry and Molecular Pharmacology, 240 Longwood Ave., Boston, MA 02115, USA (T. Böttcher). Tel.: +1 617 432 3801 (T. Böttcher), +49 89 289 13302; fax: +49 89 289 13210 (S.A. Sieber).
E-mail addresses: boettcher@aviru.de (T. Böttcher), stephan.sieber@tum.de (S.A. Sieber).

0968-0896/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2011.07.047

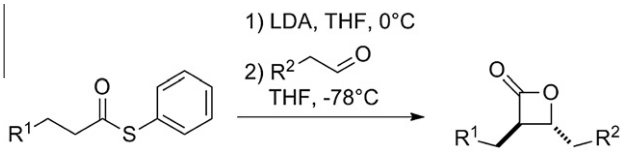

Figure 1. Synthesis of β-lactones according to strategy A.

we performed an in vitro peptidase assay with purified recombinant *S. aureus* ClpP and a fluorogenic model substrate.

Compounds **1** and **4** lead to peptidase inhibition between 30% and 70% with EC_{50} values of 3.4 and 5.4 μM, respectively (Fig. 2B). The lactone **2** was found to fully inhibit ClpP peptidase activity with an EC_{50} of 4.5 μM while all other compounds **3**, **5** and **6** had no effect (Fig. 2B and C). Although this assay is useful to demonstrate the principal inhibition of ClpP activity, it has to be considered that the natural function of ClpP is to degrade proteins rather than peptides and that ClpP requires for its full proteolytic activity interactions with many additional proteins in the cellular environment, most notably AAA+ ATPases that recognize and unfold substrate proteins and translocate them for degradation to ClpP. To investigate if ClpP is also targeted by our compounds in living cells, we performed a competitive activity based proteome profiling (ABPP) experiment (Fig. 3A).[8]

We used an ABPP probe analogue of **U1** (**U1P**) equipped with a terminal alkyne tag for click chemistry (CC) that has been previously demonstrated as selective probe for ClpP[9] and applied it to living cells of *S. aureus*. When ClpP is targeted in live cells, preincubation of the cells with a 20-fold excess of the novel β-lactones should decrease or abolish labeling by **U1P** (Fig. 3A). Indeed, only inhibitors **1**, **2** and **4** significantly reduced labeling by the ABPP probe (Fig. 3B). This demonstrates that ClpP is the preferred target of these lactones in living cells and underlines the results of the ClpP inhibition assay.

To test the efficacy of the compounds against virulence of living cells of the pathogen *S. aureus* we used agar plate and liquid medium based hemolysis assays. Production of α-toxin and β-toxin leads to rupture of sheep red blood cells (SRBCs) resulting in a clear zone around colonies on sheep blood agar.[10] These hemolysins are known to be under control of ClpP[4] and have been reported to play important roles in pathogenesis.[11–14]

It is known for *S. aureus* that the expression of virulence factors in suspension culture differs significantly from that of bacteria grown on solid medium.[15] This may also reflect the different modes of pathogenesis during systemic and tissue infections. In our assay with liquid medium we cultured the bacteria under different lactone concentrations and measured hemolysin production by the ability of sterile filtered supernatants to lyse SRBCs. Here, we obtained EC_{50} values of 11 μM for **1** and 43 μM for **2** whereas **4** had only reached approx. 20% inhibition at 100 μM (Fig. 4A). The potency of **2** (ED_{50} = 15 nmol) was also closest to **1** (ED_{50} = 7 nmol) on blood agar plates while **4** showed only incomplete inhibition of hemolysis (Fig. 4B). All other compounds were either much less active or inactive which is consistent with our ClpP peptidase assay. Differences between activities of **1**, **2** and **4** in competitive and hemolysis inhibition experiments could be attributed to different kinetics of uptake and stability in the bacterial cell. We thus identified **1** and **2** as two potent leads for further drug development.

To increase yields, avoid the diastereomeric by-product and establish an up-scalable GMP conform production route, we developed a modular synthesis strategy (strategy B) applicable for both compounds (Fig. 5).

10-Undecenoyl chloride was coupled with 2-mercaptopyridine in dichloromethane in good yields (92%). The resulting thioester **7** was deprotonated with LHMDS in THF and the enolate formed was trapped as silyl ketene acetal **8** with TBSCl. A diastereoselective tandem Mukaiyama aldol-lactonization (TMAL)[16] of **8** with the aldehydes 3-phenylpropanal or 3-(3-pyridyl)propanal **9** catalyzed by $ZnCl_2$ in dichloromethane gave **1** and **2**, respectively. The overall yield of the new synthesis was 45% for **1** and 33% for **2**, compared to 20%[2] and 15% (present study) with strategy A, respectively. The novel strategy completely avoids the use of hazardous class 1 solvents[17] and uses only Zn^{2+} as class 3 metal catalyst with minimal safety concerns as specified by the European Medicines Agency.[18] This allows GMP conform synthesis for future GLP (good laboratory practice) studies.

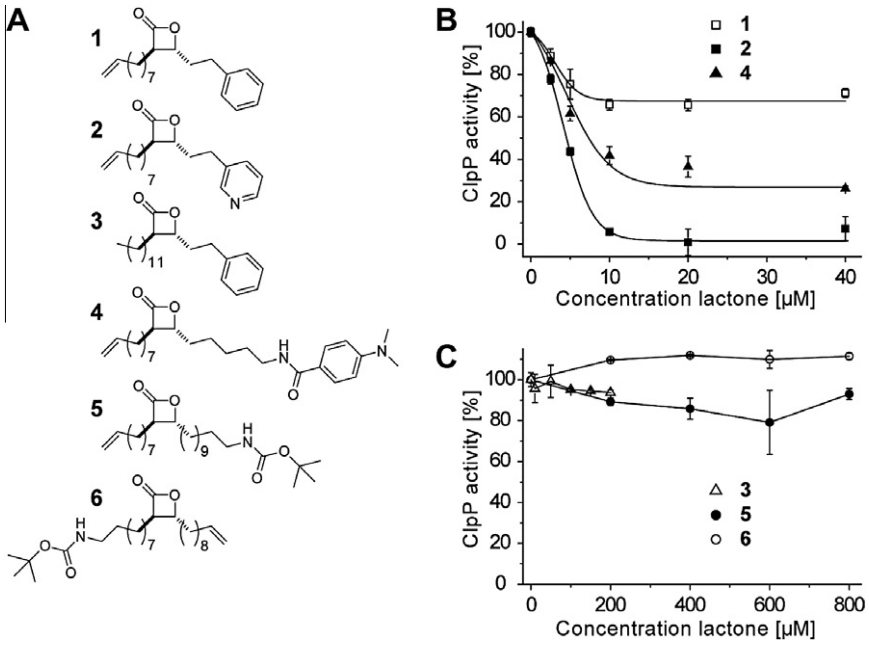

Figure 2. Second generation β-lactones as ClpP inhibitors. (A) **U1** (**1**) and a set of novel β-lactones (**2**–**6**). (B and C) peptidase activity of recombinant *S. aureus* ClpP with different β-lactones.

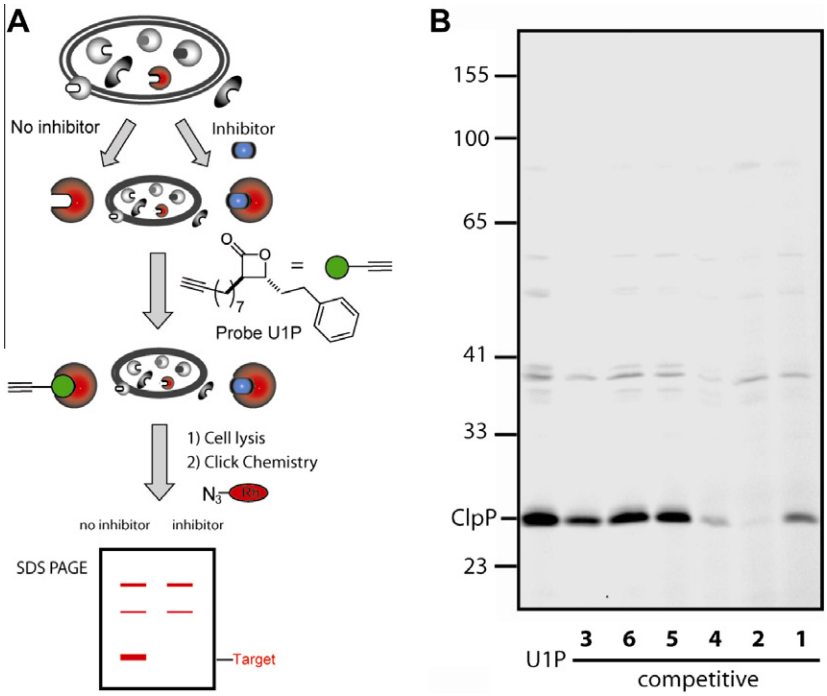

Figure 3. ClpP is targeted by certain β-lactones in living cells. (A) Principle of competitive ABPP using probe **U1P**. (B) Competitive labeling of ClpP in the proteome of live *S. aureus* cells by ABPP.

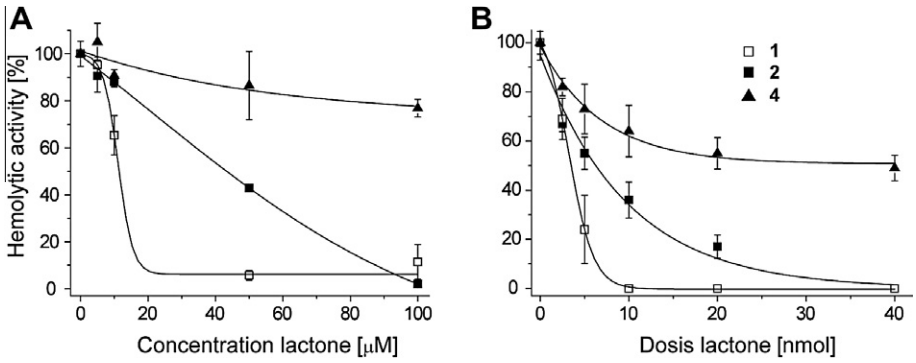

Figure 4. Hemolysis assays with sheep red blood cells. (A) Using spent culture supernatants of *S. aureus* cultures in liquid medium incubated with β-lactones and (B) agar plate based assay.

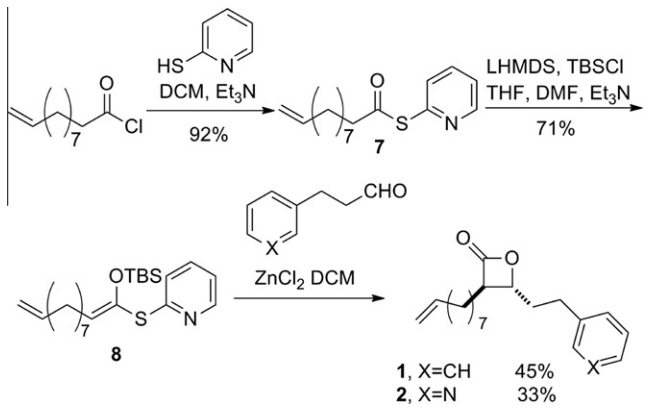

Figure 5. Improved synthesis of β-lactones (strategy B) as applied for compounds **1** and **2**.

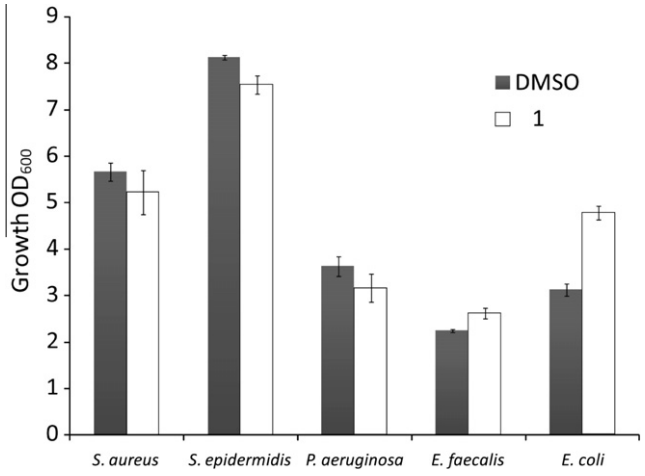

Figure 6. Growth of commensal bacteria remains unaffected upon treatment with **1** even at a high concentration of 1 mM.

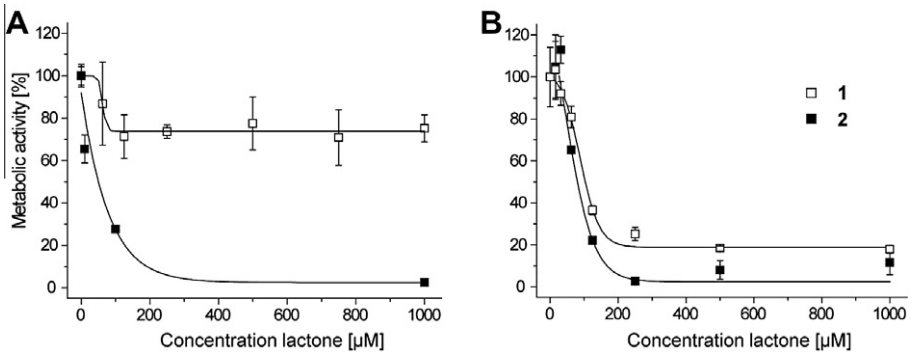

Figure 7. Cell toxicity measured by the MTT assay with (A) NIH3T3 fibroblasts and (B) HaCat keratinocytes.

We hypothesized that β-lactone based virulence inhibitors do not affect the integrity of the endogenous human microbiome. To this aim, we performed growth assays with various commensal species of the human body flora. Gram positive and negative species colonizing the skin (*S. epidermidis* and *P. aeruginosa*) and the intestinal tract (*E. coli* and *E. faecalis*) were not severely inhibited even at extremely high concentrations of 1 mM **1**, implying unprecedented advantages over existing antibiotics (Fig. 6). Although slightly increased growth of *Escherichia coli* was observed under these conditions, this effect only occurred in experiments conducted in well-plates but not in larger cultures.

Growth of *S. aureus* was not significantly reduced which is essential for our concept to avoid selective pressure on the bacterial pathogens. We may thus conclude that although compound **1** inhibits ClpP and suppresses virulence of *S. aureus*, the growth of this and other bacterial species remains unaffected.

Next we were interested to examine the cytotoxicity of our lead structures against eukaryotic cells. As primary indications of our compounds could be topical treatment of infections we used two skin cell lines. The MTT tetrazolium salt assay for metabolic activity as a measure for cytotoxicity was performed on NIH3T3 mouse fibroblasts and human HaCat keratinocytes.

The results of these assays indicate especially low cytotoxicity for **1** in NIH3T3 cells (Fig. 7A). Metabolic activity of NIH3T3 fibroblasts dropped for **1** only to approx. 80% and remained unaffected up to 1 mM while **2** exhibited its EC_{50} at 50 μM. HaCat keratinocytes were more sensitive for **1** and gave EC_{50} values of 91 and 75 μM for **1** and **2**, respectively (Fig. 7B). These data indicate moderate cytotoxicity that needs to be further evaluated in living organisms.

3. Conclusion

We here report novel β-lactones as efficient inhibitors of bacterial virulence. These were identified by ClpP target enzyme inhibition, proteomic in situ experiments and inhibition of hemolysin production in living *S. aureus* cells. We defined lead structures for future drug development and established an improved up-scaled, diastereoselective and GMP conform synthesis. While the compounds abolish virulence via inhibition of the protease ClpP, there is no adverse effect on the bacterial growth of *S. aureus* and members of the natural human body flora. Consequently, we might expect less selective pressure and thus a reduced resistance development as well as the preservation of the human microbiome. Severe side effects through the destruction of the commensal body flora and the rapid resistance development represent the most challenging issues with classical antibiotic therapy.

Our results qualify the concept of ClpP inhibition as a promising strategy for potential therapeutic intervention and demonstrate the great value of our compounds as leads for a novel generation of anti-infective drugs.

4. Experimental part

4.1. Syntheses

4.1.1. General

All chemicals were of reagent grade or better and used without further purification. Chemicals and solvents were purchased from Sigma Aldrich, Acros Organics, ABCR GmbH & Co. KG and SAFC Supply solutions. For all reactions, only commercially available solvents of purissimum grade, dried over molecular sieve and stored under argon atmosphere were used. Solvents for chromatography and workup purposes were generally of technical grade and purified before use by distillation. Temperatures were measured externally. All experiments were performed under nitrogen or argon atmosphere. Column chromatography was performed on silica gel (Merck, 0.035–0.070 mm, mesh 60 Å). For TLC analysis, Merck TLC Silica Gel 60 F254 aluminium sheets were used. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian NMR-System 600 or 300 (600 MHz, 300 MHz), a Bruker AV 500 or AV 360 (500 MHz, 360 MHz) and referenced to the residual proton and carbon signal of the deuterated solvent, respectively. Mass spectra in ESI mode were obtained by a Thermo Finnigan LTQ FT or a Thermo Finnigan LCQ classic in combination with the HPLC-System Hewlett Packard Agilent 1100 FT. EI-MS was obtained by a Thermo Finnigan MAT 8200 (70 eV). IR spectra were measured by a FT/IR-4100 instrument from JASCO.

4.1.2. Synthesis of *S*-aryl thioates

4.1.2.1. *S*-Phenyl-10-undecene thioate (10). *S*-Phenyl-10-undecene thioate (**10**) was prepared as described before.[7] To a stirring solution of thiophenol (1.02 mL, 10.0 mmol) and NEt_3 (1.40 mL, 10.0 mmol) in toluene (20 mL) was added drop wise over 30 min a solution of 10-undecenoyl chloride (2.20 mL, 10.2 mmol) in toluene (30 mL). The reaction was monitored by TLC and was completed after 20 min. The mixture was then washed with saturated $NaHCO_3$ solution (30 mL) and brine (10 mL), dried over $MgSO_4$ and the solvent was removed under reduced pressure. Flash column chromatography on silica (*iso*-hexane/ethyl acetate 25:1) yielded 2.49 g (88%) of *S*-phenyl-10-undecene thioate (**10**) as colorless oil, $R_f = 0.43$. The spectral data are identical to those published previously.[7]

4.1.2.2. *S*-Phenyl-*N*-(*tert*-butoxycarbonyl)-11-aminoundecane thioate (11). To a solution of *N*-(*tert*-butoxycarbonyl)-11-aminoundecanoic acid (**16**) (301 mg, 1.00 mmol) in CH_2Cl_2 (5 mL) was added thiophenol (153 μL, 1.50 mmol), DCC (227 mg, 1.10 mmol) and DMAP (14 mg, 0.10 mmol) and the reaction mixture was stirred for 6 h. The remaining precipitate was filtered off and the residue was washed with CH_2Cl_2 (20 mL). The solvent was removed under reduced pressure. Flash column chromatography on silica

(*iso*-hexane/ethyl acetate 8:1) afforded 270 mg (69%) of the product **11** as a white solid, R_f = 0.26. Mp 59 °C. ^{1}H NMR (599 MHz, $CDCl_3$) δ 7.40–7.38 (m, 5H, *S*-phenyl), 4.48 (s, 1H, NH), 3.09 (dd, *J* = 13.0, 6.4 Hz, 2H, CH_2N), 2.66–2.61 (m, 2H, $COCH_2$), 1.69 (dt, *J* = 15.1, 7.4 Hz, 2H, CH_2), 1.48–1.40 (m, 2H, CH_2), 1.43 (s, 9H, O*t*Bu), 1.37–1.23 (m, 12H, $(CH_2)_6$). ^{13}C NMR (151 MHz, $CDCl_3$) δ 197.52, 155.93, 134.43, 129.24, 129.10, 127.93, 78.95, 43.69, 40.60, 30.03, 29.43, 29.28, 29.22, 29.18, 28.90, 28.41. IR (film) ν = 3366 cm^{-1}, 2919, 2852, 1699, 1684, 1646, 1523, 1446, 1430, 1410, 1390, 1365, 1328, 1281, 1241, 1213, 1173, 1117, 1008, 983, 965, 908, 893, 869. HRMS (ESI) calcd for $C_{22}H_{35}NNaO_3S$ $[M+Na]^+$ 416.2235, found 416.2230.

4.1.2.3. *S*-Pyridin-2-yl-10-undecene thioate (7). To a stirred solution of 2-pyridylmercaptane (6.6 g, 60 mmol) in CH_2Cl_2 (150 mL) was added 10-undecenoyl chloride (10 g, 50 mmol) at 0 °C followed by Et_3N (15 mL, 108 mmol). The solution was stirred for 2 h at rt, quenched with 1 N HCl, phases were separated and the organic phase was washed with saturated $NaHCO_3$ solution and then dried over Na_2SO_4. Volatiles were removed under reduced pressure yielding a yellow oil that was purified by flash column chromatography (hexane/ethyl acetate 2:1, R_f = 0.76). Yield: 12.7 g (92%). ^{1}H NMR (360 MHz, $CDCl_3$) δ 8.62–8.65 (m, 1H, pyridine), 7.72–7.78 (m, 1H, pyridine), 7.61–7.65 (m, 1H, pyridine), 7.20 (ddd, *J* = 5, 7, 1 Hz, 1H, pyridine), 5.76–5.89 (m, 1H, C*H*=CH_2), 4.92–5.05 (m, 2H, CH_2=CH), 2.71 (t, *J* = 7 Hz, 2H, CH_2), 2.00 2.09 (m, 2H, CH_2), 1.68–1.79 (m, 2H, CH_2), 1.25–1.45 (m, 10H, $(CH_2)_5$). ^{13}C NMR (91 MHz, $CDCl_3$): 196.59, 151.73, 150.39, 139.15, 137.04, 130.09, 123.40, 114.17, 44.24, 33.77, 29.21, 29.17, 29.02, 28.91, 28.89, 25.41. IR (film) ν = 2925 cm^{-1}, 2855, 1627, 1575, 1560, 1449, 1417, 1253, 1081, 838, 783. MS (ESI) positive ion mode: *m*/*z* = 278 ($[M+H]^+$, 100%). HRMS (ESI) calcd for $C_{16}H_{24}NOS$ $[M+H]^+$ 278.1579, found 278.1564.

4.1.2.4. *S*-Pyridin-2-yl tetradecane thioate (12). *S*-Pyridin-2-yl tetradecane thioate (**12**) was synthesized analogous to **7** from 2-pyridylmercaptane (3.3 g, 30 mmol), tetradecanoyl chloride (8 g, 25 mmol) and Et_3N (10 mL, 80 mmol) in CH_2Cl_2 (75 mL). Yield: 6.4 g (80%). ^{1}H NMR (360 MHz, $CDCl_3$) δ 8.62–8.65 (m, 1H, pyridine), 7.72–7.78 (m, 1H, pyridine), 7.62–7.65 (m, 1H, pyridine) 7.26–7.32 (m, 1H, pyridine), 2.71 (t, *J* = 7 Hz, 2H, CH_2), 1.22–1.32 (22H, m, $(CH_2)_{11}$), 0.89 (t, *J* = 7 Hz, 3H, CH_3). ^{13}C NMR (91 MHz, $CDCl_3$) δ 196.6, 151.8, 150.4, 139.15, 137.0, 130.1, 123.4, 44.3, 31.9, 29.66, 29.63, 29.37, 29.34, 29.23, 25.4, 22.7, 14.1. HRMS (ESI) calcd for $C_{19}H_{31}NOS$ $[M+H]^+$ 321.2126, found 321.2195.

4.1.3. Synthesis of further starting compounds

4.1.3.1. 2,5-Dioxopyrrolidin-1-yl 4-(*N*,*N*-dimethylamino)benzoate (13). 4-(*N*,*N*-Dimethylamino)benzoic acid (332 mg, 2.00 mmol), *N*-hydroxysuccinimide (244 mg, 2.10 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, (EDAC) (402 mg, 2.1 mmol) were suspended in CH_2Cl_2 (10 mL). After 20 min the suspension became a clear violet solution. The reaction mixture was monitored by TLC (*iso*-hexane/ethyl acetate 1:1, 1% AcOH, R_f = 0.30) and the reaction was completed after 2.5 h. Then water (10 mL) was added and the aqueous layer was extracted with dichloromethane (2 × 10 mL). The combined organic layers were washed with brine (10 mL), dried over $MgSO_4$ and the solvent was removed under reduced pressure to yield 451 mg (86%) of **13** as a white solid. Mp 181 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.99 (d, *J* = 9.1 Hz, 2H, H*arom*), 6.67 (d, *J* = 9.2 Hz, 2H, H*arom*), 3.09 (s, 6H, $N(CH_3)_2$), 2.89 (s, 4H, $(CH_2)_2$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.83, 161.92, 154.41, 132.55, 110.81, 110.65, 39.98, 25.70. IR (film) ν = 1735 cm^{-1}, 1604, 1535, 1436, 1375, 1269, 1248, 1207, 1182, 1070, 1012, 979, 944, 825, 755. HRMS (ESI) calcd for $C_{13}H_{15}N_2O_4$ $[M+H]^+$ 263.1032, found 263.1028. The spectral data are identical to those published in literature.[19]

4.1.3.2. 4-(*N*,*N*-Dimethylamino)-*N*′-(6-hydroxyhexyl)benzamide (14). To a stirred solution of 2,5-dioxopyrrolidin-1-yl 4-(*N*,*N*-dimethylamino)benzoate (**13**) (220 mg, 0.90 mmol) in acetonitrile/water 1:1.2 (3 mL) was added NEt_3 (750 μL, 5.4 mmol) and 6-amino-1-hexanol (106 mg, 0.90 mmol). The reaction was monitored by TLC (ethyl acetate, R_f = 0.24) and was completed after 30 min. Then the mixture was extracted with ethyl acetate (2 × 20 mL), the combined organic layers were washed with brine (2 mL) and dried over $MgSO_4$ to yield 191 mg (80%) 4-(*N*,*N*-dimethylamino)-*N*′-(6-hydroxyhexyl)benzamide (**14**) as a pale yellow solid. Mp 91 °C. ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.68 (d, *J* = 8.9 Hz, 2H, H*arom*), 6.67 (d, *J* = 8.9 Hz, 2H, H*arom*), 6.14 (s, 1H, NH), 3.64 (t, *J* = 6.5 Hz, 2H, NHCH_2), 3.44 (dd, *J* = 13.1, 6.9 Hz, 2H, CH_2OH), 3.01 (s, 6H, $N(CH_3)_2$), 2.20 (br s, 1H, OH), 1.68–1.32 (m, 8H, $(CH_2)_4$). ^{13}C NMR (91 MHz, $CDCl_3$) δ 167.55, 152.39, 131.82, 128.29, 121.52, 111.09, 62.58, 45.82, 40.12, 39.62, 26.53, 8.60. IR (film) ν = 3318 cm^{-1}, 3306, 2926, 2856, 2816, 1747, 1684, 1604, 1549, 1513, 1444, 1412, 1364, 1328, 1300, 1230, 1204, 1173, 1131, 1061, 946, 925, 872, 829, 807, 768. HRMS (ESI) calcd for $C_{15}H_{25}N_2O_2$ $[M+H]^+$ 265.1916, found 265.1907.

4.1.3.3. *N*-(*tert*-Butoxycarbonyl)-12-aminododecan-1-ol (15). To a mixture of NaOH (43 mg, 1.1 mmol), in water/***tert***-BuOH 1:1 (1.0 mL) was added di-***tert***-butyl dicarbonate (225 μL, 1.05 mmol) and 12-amino-dodecan-1-ol (199 mg, 1.00 mmol). After the viscous mixture was stirred for 18 h, 0.3 M HCl solution (1.5 mL) was added and the aqueous layer was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with brine (5 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure and 294 mg (98%) of the product was obtained as white solid. Mp 75 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.51 (s, 1H, NH), 3.66 (dd, *J* = 11.1, 6.5 Hz, 2H, NHCH_2), 3.11 (dd, *J* = 13.2, 6.6 Hz, 2H, CH_2OH), 1.73–1.16 (m, 21H, $(CH_2)_{10}$, OH), 1.46 (s, 9H, O*t*Bu). ^{13}C NMR (151 MHz, $CDCl_3$) δ 155.94, 78.96, 63.03, 40.60, 32.77, 30.03, 29.53, 29.48, 29.48, 29.37, 29.23, 28.40, 26.77, 25.69. IR (film) ν = 3368 cm^{-1}, 2919, 2851, 1685, 1653, 1558, 1523, 1481, 1469, 1457, 1443, 1389, 1364, 1337, 1288, 1266, 1243, 1225, 1173, 1142, 1059, 1027, 994, 978, 867, 781, 741, 720, 679, 661. HRMS (ESI) calcd for $C_{17}H_{35}NNaO_3$ $[M+Na]^+$ 324.2515, found 324.2509.

4.1.3.4. *N*-(*tert*-Butoxycarbonyl)-11-aminoundecanoic acid (16). The product was prepared as described for **15**. To a mixture of NaOH (213 mg, 5.30 mmol), in water/***tert***-BuOH 1:1 (1.2 mL) was added di-***tert***-butyl dicarbonate (964 μL, 1.05 mmol) and 11-aminoundecanoic acid (863 mg, 4.30 mmol). After stirring for 18 h and work up 518 mg (40%) of the product (**16**) was obtained as white solid. Mp 64 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.56 (s, 1H, NH), 3.11 (dd, *J* = 12.9, 6.5 Hz, 2H, NHCH_2), 2.34 (t, *J* = 7.2 Hz, 2H, CH_2COOH), 1.70–1.57 (m, 2H, CH_2), 1.54–1.40 (m, 2H, CH_2), 1.46 (s, 9H, O*t*Bu), 1.44–1.22 (m, 12H, $(CH_2)_6$). ^{13}C NMR (151 MHz, $CDCl_3$) δ 179.30, 155.98, 79.02, 41.67, 40.60, 34.39, 34.21, 29.98, 29.39, 29.28, 29.18, 29.02, 28.40. IR (film) ν = 3365 cm^{-1}, 2976, 2917, 2852, 1698, 1682, 1523, 1471, 1443, 1428, 1410, 1388, 1364, 1328, 1280, 1239, 1213, 1186, 1171, 1116, 1072, 1058, 1044, 1025, 1008, 980, 963, 921, 902, 870, 781, 738, 719. – HRMS (ESI) calcd for $C_{16}H_{31}NNaO_4$ $[M+Na]^+$ 324.2151, found 324.2145. The spectral data are identical to those published in literature.[20]

4.1.4. General procedure for the synthesis of aldehydes from alcohols

Aldehydes were prepared from the respective alcohols by Swern oxidation.

4.1.4.1. 3-(3-Pyridyl)propanal (9). To a stirred solution of oxalyl chloride (532 μL, 6.20 mmol) in CH_2Cl_2 (15 mL) was added at −78 °C successively DMSO (881 μL, 12.4 mmol), 3-(3-pyridyl)-

1-propanol (712 mg, 5.17 mmol) in CH_2Cl_2 (1.5 mL) and NEt_3 (3.96 mL, 28.4 mmol). The reaction mixture was stirred at −78 °C and monitored by TLC (*iso*-hexane/ethyl acetate 1:1, R_f = 0.16). After 4 h it was warmed up to room temperature. Then water (15 ml) was added and the aqueous layer was extracted with diethyl ether (3 × 50 mL). The combined organic layers were washed with brine (5 mL), dried over $MgSO_4$ and the solvent was removed under reduced pressure. Purification by Kugelrohr distillation yielded 293 mg (42%) of **9** as a pale yellow oil. Bp 107 °C (0.6 mbar). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.77 (t, J = 1.1 Hz, 1H, C*H*O), 8.43 (m, 1H, 2-pyridine-C*H*), 8.40 (dd, J = 4.8, 1.7 Hz, 1H, 6-pyridine-C*H*), 7.48 (ddd, J = 7.8, 2.3, 1.7 Hz, 1H, 4-pyridine-C*H*), 7.17 (ddd, J = 7.8, 4.8, 0.8 Hz, 1H, 5-pyridine-C*H*), 2.91 (t, J = 6.9 Hz, 2H, CH_2), 2.77 (ddt, J = 6.9, 2.6, 1.1 Hz, 2H, CH_2CHO). ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.46 (*C*HO), 149.75 (pyridine-*C*H), 147.74 (pyridine-*C*H), 135.82 (pyridine-*C*H), 135.78 (pyridine-C_{quart}), 123.37 (pyridine-*C*H), 44.70 (*C*H_2), 25.11 (*C*H_2CHO). All spectral data are identical to those published in literature.[21]

4.1.4.2. 4-(*N*,*N*-Dimethylamino)-*N*′-(6-oxohexyl)benzamide (17). To a solution of oxalyl chloride (60 μL, 0.72 mmol) in CH_2Cl_2 (2 mL) was added at −78 °C DMSO (102 μL, 1.44 mmol), 4-(*N*,*N*-dimethylamino)-*N*′-(6-hydroxyhexyl)benzamide (**14**) (159 mg, 0.60 mmol) in CH_2Cl_2 (3 mL) and NEt_3 (460 μL, 3.30 mmol). The reaction mixture was stirred at −78 °C for 5 h and was then warmed up to −5 °C. After work up and flash column chromatography on silica (*iso*-hexane/ethyl acetate 1:2) 103 mg (65%) of the product **17** was obtained as white solid, R_f = 0.27. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.77 (t, J = 1.7 Hz, 1H, CHO), 8.02–7.43 (m, 2H, H*arom*), 6.71–6.65 (m, 2H, H*arom*), 6.15 (br s, 1H, NH), 3.45 (td, J = 7.0, 6.0 Hz, 2H, NHCH_2), 3.02 (s, 6H, N(CH_3)$_2$), 2.46 (td, J = 7.2, 1.7 Hz, 2H, CH_2CHO), 1.75–1.57 (m, 4H, (CH_2)$_2$), 1.49–1.35 (m, 2H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ 202.55, 167.46, 152.36, 128.30, 121.53, 111.13, 43.74, 40.16, 39.50, 29.62, 26.45, 21.65. IR (film) ν = 3335 cm^{-1}, 3309, 3276, 2922, 2859, 2818, 1717, 1685, 1607, 1542, 1513, 1444, 1411, 1363, 1328, 1299, 1229, 1203, 1173, 1134, 1064, 993, 946, 829, 768, 732. – HRMS (ESI) calcd for $C_{15}H_{23}N_2O_2$ $[M+H]^+$ 263.1760, found 263.1755.

4.1.4.3. *N*-(*tert*-Butoxycarbonyl)-12-aminododecanal (18). To a solution of oxalyl chloride (84 μL, 1.0 mmol) in CH_2Cl_2 (2.5 mL) was added at −78 °C DMSO (142 μL, 2.0 mmol), ***N***-(***tert***-butoxycarbonyl)-12-aminododecan-1-ol (**15**) (250 mg, 0.83 mmol) in CH_2Cl_2 (1.3 mL) and NEt_3 (630 μL, 4.5 mmol). The reaction mixture was stirred at −78 °C for 3 h and was then warmed up to room temperature. After work up and flash column chromatography on silica (***iso***-hexane/ethyl acetate 9:1) 182 mg (73%) of **18** was obtained as white solid, R_f = 0.19. Mp 51 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.76 (t, J = 1.9 Hz, 1H, CHO), 4.49 (s, 1H, NH), 3.09 (t, J = 7.0 Hz, 2H, NHCH_2), 2.41 (td, J = 7.4, 1.9 Hz, 2H, CH_2CHO), 1.43 (s, 9H, O*t*Bu), 1.67–1.21 (m, 18H, (CH_2)$_9$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.99, 77.19, 43.90, 30.04, 29.48, 29.44, 29.35, 29.31, 29.24, 29.13, 28.41, 26.76, 22.05. IR (film) ν = 3371 cm^{-1}, 3008, 2981, 2917, 2848, 2722, 1722, 1680, 1513, 1480, 1466, 1411, 1391, 1367, 1318, 1288, 1268, 1245, 1227, 1167, 1119, 1086, 1049, 1006, 980, 865, 784, 765, 750, 723. HRMS (ESI) calcd for $C_{17}H_{34}NO_3$ $[M+H]^+$ 300.2539, found 300.2529.

4.1.5. Synthesis of β-lactones according to strategy A

4.1.5.1. General procedure. β-Lactones were prepared according to a method developed by Danheiser and Novick[6] and as described before by Böttcher and Sieber[2,7] from various *S*-phenyl thioates and aldehydes.

4.1.5.2. (3*R,4*R**)-3-Non-8-enyl-4-(2-(3-pyridyl)ethyl)-oxetan-2-one (2).** A solution of diisopropylethylamine (88.9 μL, 0.630 mmol) in THF (6 mL) was cooled in an ice bath to 0 °C and under stirring *n*-butyllithium (252 μL, 0.630 mmol, 2.45 M in hexane) was gradually added via a syringe. The reaction mixture was stirred for 20 min at 0 °C. Then the ice bath was replaced with an acetone/dry ice bath and the mixture was cooled to −78 °C. A solution of *S*-phenyl-10-undecene thioate (**10**) (167 mg, 0.604 mmol) in THF (750 μL) was injected dropwise over 20 min. After stirring the mixture at −78 °C for 2 h a solution of 3-(3-pyridyl)propanal (**9**) (81.6 mg, 0.604 mmol) in THF (900 μL) was added drop wise over 30 min via a syringe that was externally cooled by an aluminium funnel filled with dry ice. The reaction mixture was stirred for 1.5 h at −78 °C and then gradually warmed up to −5 °C. Then half saturated NH_4Cl solution (2 ml) was added and the mixture was diluted with ethyl acetate (20 ml). The aqueous layer was extracted with ethyl acetate (2 × 20 ml) and the combined organic layers were washed with brine (5 ml) and dried over $MgSO_4$. The solvent was removed under reduced pressure to give 225 mg of crude product as yellow oil. Purification by flash column chromatography on silica (*iso*-hexane/ethyl acetate 2:1) yielded 27.1 mg (15%) of *trans*-3-non-8-enyl-4-(2-pyridin-3-yl-ethyl)-oxetan-2-one (**2**) as a pale yellow oil, R_f = 0.22 and 12.5 mg (7%) of *cis*-3-non-8-enyl-4-(2-(3-pyridyl)ethyl)-oxetan-2-one (**2b**) as a pale yellow oil contaminated with 30% of the *trans*-product, R_f = 0.18. Compound **2**: ^{1}H NMR (599 MHz, $CDCl_3$) δ 8.48–8.46 (m, 2H, 2-pyridine-C*H*, 6-pyridine-C*H*), 7.51 (ddd, J = 7.8, 2.3, 1.7 Hz, 1H, 4-pyridine-C*H*), 7.23 (ddd, J = 7.8, 4.8, 0.8 Hz, 1H, 5-pyridine-C*H*), 5.78 (ddt, J = 16.9, 10.2, 6.7 Hz, 1H, C*H*=CH_2), 4.97 (ddt, J = 17.1, 2.0, 1.6 Hz, 1H, CH=CH_2), 4.91 (ddt, J = 10.2, 2.3, 1.2 Hz, 1H, CH=CH_2), 4.20 (ddd, J = 8.6, 4.8, 4.0 Hz, 1H, 4-C*H*), 3.20 (ddd, J = 8.5, 6.8, 4.0 Hz, 1H, 3-C*H*), 2.81 (ddd, J = 14.7, 9.6, 5.4 Hz, 1H, 2-ethyl-CH^a_2), 2.70 (ddd, J = 14.2, 9.3, 7.1 Hz, 1H, 2-ethyl-CH^b_2), 2.17–2.04 (m, 2H, CH_2), 2.01 (ddt, J = 14.8, 6.7, 1.4 Hz, 2H, CH_2CH=CH_2), 1.82–1.75 (m, 1H, CH^a_2), 1.71–1.64 (m, 1 H, CH^b_2), 1.45–1.22 (m, 10H, (CH_2)$_5$). ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.91, 149.74, 147.94, 139.00, 135.80, 135.48, 123.48, 114.22, 76.67, 56.32, 35.88, 33.68, 29.16, 29.08, 28.90, 28.78, 28.63, 27.70, 26.89. IR (film) ν = 2925 cm^{-1}, 2854, 1815, 1729, 1639, 1575, 1479, 1456, 1423, 1387, 1321, 1304, 1284, 1242, 1190, 1116, 1082, 1042, 1026, 997, 956, 907, 841, 795, 714. HRMS (ESI) calcd for $C_{19}H_{28}NO_2$ $[M+H]^+$ 302.2120, found 302.2115. Compound **2b**: ^{1}H NMR (599 MHz, $CDCl_3$) δ 8.49–8.46 (m, 1H, 2-pyridine-C*H*, 6-pyridine-C*H*), 7.51 (ddd, J = 7.8, 2.2, 1.8 Hz, 1H, 4-pyridine-C*H*), 7.23 (ddd, J = 7.7, 4.8, 0.7 Hz, 1H, 5-pyridine-C*H*), 5.78 (ddt, J = 16.9, 10.1, 6.7 Hz, 1H, C*H*=CH_2), 4.99–4.94 (m, 1H, CH=CH_2), 4.93–4.89 (m, 1H, CH=CH_2), 4.52 (ddd, J = 10.6, 6.4, 3.1 Hz, 1H, 4-C*H*), 3.61 (ddd, J = 9.0, 7.3, 6.6 Hz, 1H, 3-C*H*), 2.89 (ddd, J = 14.4, 9.7, 4.9 Hz, 1H, 2-ethyl-CH^a_2), 2.71 (ddd, J = 14.1, 9.3, 7.3 Hz, 1H, 2-ethyl-CH^b_2), 2.10–1.90 (m, 2H, CH_2), 1.82–1.73 (m, 1H, CH^a_2), 1.63–1.44 (m, 2H, CH_2), 1.40–1.22 (m, 9H, (CH_2)$_4$, CH^b_2). ^{13}C NMR (151 MHz, $CDCl_3$) δ 171.65, 149.87, 147.91, 139.01, 135.96, 135.73, 123.46, 114.19, 74.14, 52.73, 33.69, 31.84, 29.25, 29.08, 28.91, 28.86, 28.79, 27.52, 23.94.

4.1.5.3. (3*R,4*R**)-4-(4-(*N*,*N*-Dimethylamino)benzamido-5-pentyl)-3-(non-8-enyl)oxetan-2-one (4).** The reaction, as described for **2**, was performed with *S*-phenyl-10-undecene thioate (**10**) (87 mg, 0.31 mmol) and 4-(*N*,*N*-dimethylamino)-*N*′-(6-oxohexyl)benzamide (**17**) (81 mg, 0.31 mmol). Standard workup and purification by flash column chromatography on silica (*iso*-hexane/ethyl acetate 2:1) yielded 16 mg (12%) of the product as colorless oil, R_f = 0.21. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.73–7.68 (m, 2H, H*arom*), 6.74 (d, J = 7.2 Hz, 2H, H*arom*), 6.10 (br s, 1H, NH), 5.82 (ddt, J = 16.9, 10.1, 6.7 Hz, 1H, C*H*=CH_2), 5.04–4.98 (m, 1H, CH=CH_2), 4.95 (ddt, J = 10.3, 2.3, 1.2 Hz, 1H, CH=CH_2), 4.23 (ddd,

J = 7.6, 5.6, 4.2 Hz, 1H, 4-C*H*), 3.46 (dd, J = 13.4, 6.1 Hz, 2H, CH_2), 3.18 (ddd, J = 8.7, 6.7, 4.0 Hz, 1H, 3-C*H*), 3.04 (s, 6H, $N(CH_3)_2$), 2.06 (dd, J = 14.5, 6.7 Hz, 2H, CH_2), 1.91–1.69 (m, 4H, $(CH_2)_2$), 1.68–1.60 (m, 2H, CH_2), 1.55–1.27 (m, 16H, $(CH_2)_8$). ^{13}C NMR (91 MHz, $CDCl_3$) δ 171.53, 167.30, 151.91, 139.04, 128.30, 114.19, 111.63, 77.97, 77.20, 56.15, 40.47, 39.60, 34.33, 33.70, 29.67, 29.20, 29.11, 28.93, 28.80, 27.81, 26.91, 26.55, 24.84. IR (film) ν = 2924 cm^{-1}, 2854, 1812, 1636, 1606, 1541, 1509, 1361, 1328, 1294, 1230, 1203, 1172, 1123, 1064, 994, 945, 908, 876, 829, 767. EI-MS, m/z (%): 428.3 (16) $[M]^+$, 384.3 (21) $[M-CO_2]^+$, 178.1 (10), 164.0 (43) $[M-C_{17}H_{28}O_2]^+$, 148.0 (100) $[M-C_{17}H_{30}NO_2]^+$, 43.9 (29). HRMS (EI) calcd for $C_{26}H_{40}N_2O_3$ $[M]^+$ 428.30389, found 428.30349.

4.1.5.4. (3*R,4*R**)-4-[*N*-(*tert*-Butoxycarbonyl)-11-aminoundecyl]-3-(non-8-enyl)-oxetan-2-one (5).** The reaction, as described for (**2**), was performed with *S*-phenyl-10-undecene thioate (**10**) (92.3 mg, 0.334 mmol) and *N*-(*tert*-butoxycarbonyl)-12-aminododecanal (**18**) (100 mg, 0.334 mmol). Standard workup and purification by flash column chromatography on silica (*iso*-hexane/ethyl acetate 8:1) yielded 8 mg (5%) of the product as white solid, R_f = 0.33. Mp 45 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.83 (ddt, J = 17.0, 10.2, 6.7 Hz, 1H, C*H*=CH_2), 5.05–4.99 (m, 1H, CH=CH_2), 4.96 (ddt, J = 10.3, 2.2, 1.2 Hz, 1H, CH=CH_2), 4.51 (s, 1H, NH), 4.23 (ddd, J = 7.0, 6.1, 4.0 Hz, 1H, 4-C*H*), 3.18 (ddd, J = 8.8, 6.6, 4.0 Hz, 1H, 3-C*H*), 3.12 (s, 2H, NHCH_2), 2.06 (dd, J = 14.2, 6.9 Hz, 2H, CH_2), 1.92–1.80 (m, 2H, CH_2), 1.80–1.19 (m, 30H, $(CH_2)_{15}$), 1.47 (s, 9H, O*t*Bu). ^{13}C NMR (91 MHz, $CDCl_3$) δ 171.65, 155.93, 139.06, 114.24, 78.16, 77.21, 56.13, 34.45, 33.74, 30.09, 29.52, 29.50, 29.45, 29.40, 29.28, 29.24, 29.15, 28.96, 28.84, 28.44, 27.88, 26.97, 26.80, 25.04. IR (film) ν = 3366 cm^{-1}, 2979, 2919, 2851, 2359, 2337, 1793, 1686, 1523, 1468, 1389, 1364, 1275, 1241, 1173, 1145, 1080, 1021, 992, 973, 910, 870, 830, 802, 782, 737, 723. EI-MS, m/z (%): 364.3 (31) $[M-C_5H_9O_2]^+$, 321.3 (39) $[M-C_6H_8O_4]^+$, 57.0 (100) $[M-C_{24}H_{42}NO_4]^+$, 41.0 (30). HRMS (ESI) calcd for $C_{28}H_{52}NO_4$ [M+H]$^+$ 466.3896, found 466.3886.

4.1.5.5. (3*R,4*R**)-3-(*N*-(*tert*-Butoxycarbonyl)-9-aminononyl)-4-(dec-9-enyl)oxetan-2-one (6).** The reaction, as described for (**2**), was performed with 10-undecenal (226 mg, 1.21 mmol) and *S*-phenyl-*N*-(*tert*-butoxycarbonyl)-11-aminoundecane thioate (**11**) (476 mg, 1.21 mmol). Standard workup and purification by flash column chromatography on silica (*iso*-hexane/ethyl acetate 8:1) yielded 38 mg (7%) of the product as white solid, R_f = 0.18. Mp 39 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.83 (ddt, J = 16.9, 10.2, 6.7 Hz, 1H, C*H*=CH_2), 5.01 (ddt, J = 17.1, 2.0, 1.6 Hz, 1H, CH=CH_2), 4.95 (ddt, J = 10.2, 2.2, 1.2 Hz, 1H, CH=CH_2), 4.51 (s, 1H, NH), 4.23 (ddd, J = 7.3, 6.0, 4.0 Hz, 1H, 4-C*H*), 3.18 (ddd, J = 8.7, 6.7, 4.0 Hz, 1H, 3-C*H*), 3.12 (t, J = 6.9 Hz, 2H, CH_2N), 2.11 2.02 (m, 2H, CH_2), 1.91 1.79 (m, 2H, CH_2), 1.78–1.68 (m, 2H, CH_2), 1.46 (s, 9H, O*t*Bu), 1.53–1.25 (m, 26H, $(CH_2)_{13}$). ^{13}C NMR (91 MHz, $CDCl_3$) δ 171.64, 156.01, 139.12, 114.18, 78.14, 77.23, 56.13, 40.65, 34.44, 33.77, 30.07, 29.40, 29.35, 29.30, 29.25, 29.22, 29.04, 28.87, 28.43, 27.88, 26.97, 26.75, 25.03. IR (film) ν = 2925 cm^{-1}, 2854, 1819, 1704, 1508, 1478, 1457, 1390, 1364, 1268, 1248, 1171, 1125, 1069, 1040, 994, 908, 870, 828, 808, 781, 745. HRMS (EI), m/z (%): calcd for $C_{23}H_{41}NO_4$ $[M-C_4H_8]^+$ 395.30356, found 395.30350 (23), 351.31296 (83) $[M-C_5H_8O_2]^+$, 307.32382 (100) $[M-C_6H_9O_4]^+$. HRMS (ESI) calcd for $C_{27}H_{49}NNaO_4$ [M+Na]$^+$ 474.3559, found 474.3553.

4.1.5.6. (3*R,4*R**)-3-Dodecyl-4-(2-phenylethyl)oxetan-2-one (3).** (3*R**,4*R**)-3-Dodecyl-4-(2-phenylethyl)oxetan-2-one (**3**) was synthesized analogous to (**2**, Strategy A) from thioester **12** (1.0 g, 3.1 mmol), 3-phenylpropanal (0.42 g, 3.1 mmol) and LHMDS (3.8 mL, 3.8 mmol, 1.0 M in THF) in THF (40 mL), yield: 96 mg (9%). R_f = 0.21 (hexane/ethyl acetate 20:1) ^{1}H NMR (360 MHz, $CDCl_3$): 7.18–7.37 (m, 5H, phenyl), 4.25 (ddd, J = 7.5, 5.4, 4.0, 1H, 4-CH), 3.20 (ddd, J = 8.5, 6.8, 3.9 Hz, 1H, 3-CH), 2.67–2.90 (m, 2H, CH_2), 2.03–2.26 (m, 2H, CH_2), 1.61–1.85 (m, 2H, CH_2), 1.35–1.46 (m, 1H, C*H*H), 1.25–1.35 (m, 19H, $(CH_2)_9$+CH*H*), 0.90 (t, J = 7 Hz, 3H, CH_3). ^{13}C NMR (91 MHz, $CDCl_3$): 171.4, 140.2, 128.6, 128.3, 126.4, 77.1, 56.3, 36.2, 31.9, 31.4, 29.64, 29.63, 29.59, 29.49, 29.3, 29.28, 29.24, 27.8, 26.9, 22.7, 14.1. IR (film) ν = 2924 cm^{-1}, 2853, 1821, 1455, 1560, 1449, 1417, 1253, 1081, 838, 783. HRMS (APCI) calcd for $C_{23}H_{37}O_2$ [M+H]$^+$ 345.2794, found 345.2800.

4.1.6. Synthesis of β-lactones according to strategy B

4.1.6.1. (*Z*)-2-((1-((*tert*-Butyldimethylsilyl)oxy)undeca-1,10-dienyl)thio)pyridine (8). LHMDS (13 mL, 13 mmol, 1 M in THF) was placed in a flask cooled to -78 °C. DMF (1.7 mL) was added via syringe followed by Et_3N (3 mL, 21 mmol) and a solution of TBSCl (1.7 g, 11 mmol) in THF (7.5 mL) and thioester **7** (3 g, 10.8 mmol) in THF (20 mL). The solution was stirred 1 h at −78 °C and then let warm up to 0 °C over 1 h and quenched with H_2O. Phases were separated; the aqueous layer was extracted with EtOAc (3 × 20 mL), and the combined organic phase was dried over Na_2SO_4. The solvent was distilled off under reduced pressure and the residue was separated by column chromatography (eluent hexane/ethyl acetate 1:10, R_f = 0.31) to give slightly yellow oil. Yield: 3 g (71%). ^{1}H NMR (360 MHz, $CDCl_3$): 8.42–8.45 (m, 1H, pyridine), 7.53–7.59 (m, 1H, pyridine), 7.33–7.37 (m, 1H, pyridine), 7.01 (ddd, J = 5, 7, 1 Hz, 1H, pyridine), 5.77–5.90 (m, 1H, C*H*=CH_2), 5.42 (t, J = 7 Hz, 1H, CH=C(OTBS), 4.92–5.05 (m, 2H, CH=CH_2), 2.16 2.24 (m, 2H, =CH-CH_2-CH_2), 2.01–2.11 (m, 2H, =CH-CH_2-CH_2), 1.25–1.49 (m, 10H, $(CH_2)_5$), 0.89 (s, 9H, *t*Bu), 0.10 (s, 6H, $Si(CH_3)_2$) ppm. ^{13}C NMR (91 MHz, $CDCl_3$): 160.6, 149.4, 139.2, 136.5, 124.1, 121.4, 119.6, 114.2, 33.8, 29.32, 29.30, 29.15, 29.11, 28.9, 26.8, 25.7, 18.1, -4.4. IR (film) ν = 2925 cm^{-1}, 2855, 1627, 1575, 1560, 1449, 1417, 1253, 1081, 838, 783. MS (ESI): m/z = 392 ([M+H]$^+$, 100). HRMS (ESI) calcd for $C_{22}H_{38}NOSSi$ [M+H]$^+$ 392.2443, found 392.2419.

4.1.6.2. (3*R,4*R**)-3-(8-Nonenyl)-4-(2-phenylethyl)oxetan-2-one (1).** To the solution of $ZnCl_2$ (0.72 g, 5.4 mmol) in CH_2Cl_2 (18 mL) was added a solution of 3-phenylpropanal (0.37 g, 2.7 mmol) in CH_2Cl_2 (5 mL) followed by ketene acetal **19** (1.5 g, 3.8 mmol). The reaction mixture was stirred for 4 days. Then the reaction was quenched with H_2O and the organic phase was separated and dried over Na_2SO_4. The solvent was distilled off under reduced pressure and the residue was separated by column chromatography (eluent hexane/ethyl acetate 20:1), R_f = 0.42. Yield: 0.36 g (45%). ^{1}H, ^{13}C NMR and mass-spectra are identical to the previously published data.[2] IR (film) ν = 2928 cm^{-1}, 2856, 1823, 1450, 1115, 907, 843, 747.

4.1.6.3. (3*R,4*R**)-3-(8-Nonenyl)-4-(2-(3-pyridyl)ethyl)oxetan-2-one (2).** (3*R**,4*R**)-3-(8-Nonenyl)-4-(2-(3-pyridyl)ethyl)oxetan-2-one was synthesized analogous to **1** (strategy B) from ketene acetal **8** (1.0 g, 2.6 mmol), 3-(3-pyridyl)propanal (**9**) (0.2 g, 1.5 mmol) and $ZnCl_2$ (0.4 g, 3.0 mmol) in CH_2Cl_2 (15 mL). Standard workup and column chromatography afforded the product (eluent *iso*-hexane/ethyl acetate 2:1, R_f = 0.22). Yield: 149 mg (33%). All spectral data were identical to the data of strategy A.

4.2. Compound preparation for biological assays

For biological and biochemical testing, stocks of the compounds were prepared in DMSO and stored at −20 °C. Dilutions for assays were prepared in DMSO and pure DMSO always was included as control.

4.3. Bacterial strains and culture

Staphylococcus aureus NCTC 8325 (Institute Pasteur, France) and the derived *Staphylococcus aureus* NCTC 8325-4 strain,

Staphylococcus epidermidis RP62A (Institute Pasteur, France), and *Enterococcus faecalis* OG1RF (ATCC, USA) were maintained in BHB (Brain Heart Broth) medium and *Pseudomonas aeruginosa* PAO1 (Institute Pasteur, France) and *Escherichia coli* K12 (DSMZ, Germany) in LB (Luria-Bertani broth) medium. All bacteria were grown at 37 °C.

4.4. Inhibition of ClpP peptidase activity

Inhibition of ClpP peptidase activity of recombinant *S. aureus* ClpP expressed in *E. coli* BL21 and purified by Strep-tag affinity chromatography was determined by cleavage of the fluorogenic model substrate SLY-AMC as described previously.[2]

EC_{50} values for the inhibition of ClpP with lactones were measured by enzymatic conversion of the fluorogenic substrate *N*-succinyl-Leu-Tyr-7-amidomethylcoumarin (SLY-AMC) that has been described as standard for ClpP activity assays.[22] Experiments were conducted in 50 μL of the activity optimized buffer (50 mM MES, 100 mM KCl, pH 7.0). In general 2 μg ClpP were incubated in activity buffer (35 μL) with varying concentrations of lactone or DMSO as control for 10 min at room temperature. Then, SLY-AMC (0.2 mM, 15 μL, 667 μM stock) was added to give a total volume of 50 μL containing 2% DMSO. Fluorescence was recorded in Greiner 96 Flat Bottom Black Polystyrol well plates by excitation at 340 nm and emission at 450 nm by a TECAN Infinite M200pro. Experiments were performed at least in triplicates. EC_{50} values were calculated from curve fittings by Microcal™ Origin 6.0.

4.5. Competitive proteome profiling

The competitive labeling experiments were performed with lactone **1**–**6** in 20-fold excess to the ABPP probe **U1P**. Cells of *S. aureus* NCTC 8325 were grown to stationary phase. For each experiment 500 μL of culture were harvested, washed with 500 μL PBS and resuspended in 100 μL PBS. The samples were then incubated 1 h at rt either with 1 μL of lactone stock (100 mM in DMSO) or with 1 μL of DMSO as labeling control. Then 1 μL of **U1P** stock (5 mM in DMSO) was directly added and the samples were incubated for 1 h at rt. The cells were washed with PBS (3 x 1 mL), resuspended in 100 μL PBS and lysed by sonication (Bandelin Sonopuls, 4 × 15 s, 80%). Lysate (43 μL) were subjected to click chemistry (CC). 100 μM rhodamine-azide (1 μL) was added as reporter followed by 1 mM TCEP (1 μL) and of 100 mM ligand (3 μL). The cycloaddition was initiated by the addition of 1 mM $CuSO_4$ (1 μL). The reactions were incubated at room temperature for 1 h, subjected to SDS PAGE and fluorescence was recorded using a Fujifilm Las-4000 Luminescent Image Analyzer with a Fujinon VRF43LMD3 Lens, an epi-green illuminator (520 nm) and a 575DF20 filter.

4.6. Hemolysis assays

4.6.1. Agar plate based assay

Hemolysis was tested as described previously.[1,2] In short, 5.5 mm Whatman® card discs on 5% sheep blood agar plates (Heipha Diagnostika, Eppelheim, Germany) were inoculated with *S. aureus* NCTC 8325 and serial dilutions of β-lactones in DMSO and incubated over night at 37 °C. Hemolysis zones were determined and normalized to the DMSO control.

4.6.2. Assay with spent culture supernatants

Hemolysis on sheep red blood cells (SRBC, ACILA Dr. Weidner GmbH, Weiterstadt, Germany) was tested with the spent supernatant of *S. aureus* NCTC 8325-4. An overnight culture of *S. aureus* NCTC 8325-4 was washed once with BHB and 4 μL were suspended in 400 μL of BHB with either 4 μL of lactone stock of various concentrations or 4 μL of DMSO. The samples were incubated under shaking at 37 °C over night. The supernatants were sterile filtered and 20 μL were incubated with 180 μL of freshly washed SRBCs (30% v/v in PBS) in a 96U well plate (Nunclon) for 30 min at 37 °C. The plate was centrifuged at 2000 g for 5 min and the absorption of the supernatant was measured at 410 nm with a plate reader (TECAN, Infinite M200pro). Experiments were performed at least in triplicates.

4.7. Effect on commensal bacteria

Overnight cultures of commensal species were diluted 1:100 in LB or BHB medium and 100 μL thereof incubated with 1 μL 100 mM **1** and DMSO as control in round bottom 96-well plates for 9 h at 37 °C under shaking. Then, OD_{600} was recorded in appropriate dilutions. Experiments were carried out in triplicates.

4.8. Determination of cytotoxicity/MTT assay

As a measure of cytotoxicity we determined metabolic activity of cell lines in response to treatment with β-lactones. The principle of the MTT assay consists in the reduction of the tetrazolium compound MTT (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan, Sigma Aldrich) by metabolically active cells to insoluble purple formazan crystals as a measure of cells viability. Two different cell lines have been used: NIH3T3 mouse fibroblasts and human HaCat keratinocytes. NIH3T3 cells were cultured in DMEM (high Glucose, L-Glutamine 4 mM, PAA) + 10% FCS-Gold (PAA). Calcium-sensitive HaCat cells were maintained in calcium-free DMEM (high-glucose, L-Glutamine 2 mM, PAA) + 10% dialyzed FCS (PAA). Cells from subconfluent cultures were used for the assay. Precisely, 4.5×10^3 HaCat cells or 1.5×10^3 NIH3T3 cells were plated in 96-well flat-bottom plates (Nunclon) in 100 μL medium and cultured for 24 h to obtain 30–40% confluent cultures. β-Lactones were diluted 1:100 from DMSO stocks in 100 μL of the appropriate culture medium and added to the cells after careful removal of the blank culture medium. After 24 h incubation 20 μL MTT substrate solution (5 mg/ml in PBS) was added. Following an incubation of 2 h, the medium was discarded and cells were lysed in 200 μL DMSO. The complete dissolution of the formazan salt was checked and the optical density measured at 570 nm (background subtraction at 630 nm). A representative assay performed in triplicates is shown.

Acknowledgments

We thank Mona Wolff for excellent technical assistance. E. Zeiler was supported by SFB749 and is a member of TUM-GS. T. Böttcher, V. Korotkov and K. Lorenz-Baath gratefully acknowledge support by an EXIST technology transfer grant of the Federal Ministry of Economics and Technology (BMWi). Stephan A. Sieber was supported by the Deutsche Forschungsgemeinschaft (Emmy Noether program), SFB749 and CIPSM.

References and notes

1. Böttcher, T.; Sieber, S. A. *J. Am. Chem. Soc.* **2008**, *130*, 14400.
2. Böttcher, T.; Sieber, S. A. *Chembiochem* **2009**, *10*, 663.
3. Frees, D.; Sorensen, K.; Ingmer, H. *Infect. Immun.* **2005**, *73*, 8100.
4. Frees, D.; Qazi, S. N.; Hill, P. J.; Ingmer, H. *Mol. Microbiol.* **2003**, *48*, 1565.
5. Michel, A.; Agerer, F.; Hauck, C. R.; Herrmann, M.; Ullrich, J.; Hacker, J.; Ohlsen, K. *J. Bacteriol.* **2006**, *188*, 5783.
6. Danheiser, R. L.; Nowick, J. S. *J. Org. Chem.* **1991**, *56*, 1176.
7. Böttcher, T.; Sieber, S. A. *Angew. Chem., Int. Ed.* **2008**, *47*, 4600.
8. Leung, D.; Hardouin, C.; Boger, D. L.; Cravatt, B. F. *Nat. Biotechnol.* **2003**, *21*, 687.
9. Rathore, S.; Sinha, D.; Asad, M.; Böttcher, T.; Afrin, F.; Chauhan, V. S.; Gupta, D.; Sieber, S. A.; Mohmmed, A. *Mol. Microbiol.* **2010**, *77*, 873.
10. Williams, R. E.; Harper, G. J. *J. Pathol. Bacteriol.* **1947**, *59*, 69.
11. Kielian, T.; Cheung, A.; Hickey, W. F. *Infect. Immun.* **2001**, *69*, 6902.

12. Nilsson, I. M.; Hartford, O.; Foster, T.; Tarkowski, A. *Infect. Immun.* **1999**, *67*, 1045.
13. Walev, I.; Weller, U.; Strauch, S.; Foster, T.; Bhakdi, S. *Infect. Immun.* **1996**, *64*, 2974.
14. O'Callaghan, R. J.; Callegan, M. C.; Moreau, J. M.; Green, L. C.; Foster, T. J.; Hartford, O. M.; Engel, L. S.; Hill, J. M. *Infect. Immun.* **1997**, *65*, 1571.
15. Elek, S. D.; Levy, E. *J. Pathol. Bacteriol.* **1954**, *68*, 31.
16. Cho, S. W.; Romo, D. *Org. Lett.* **2007**, *9*, 1537.
17. EMEA. CPMP/ICH/283/95, 2009.
18. EMEA. CHMP/SWP/4446/2000, 2008.
19. Zemski Berry, K. A.; Turner, W. W.; VanNieuwenhze, M. S.; Murphy, R. C. *Anal. Chem.* **2009**, *81*, 6633.
20. Hofmann, A.; Thierbach, S.; Semisch, A.; Hartwig, A.; Taupitz, M.; Rühl, E.; Graf, C. *J. Mater. Chem.* **2010**, *20*, 7842.
21. Voelker, T.; Xia, H.; Fandrick, K.; Johnson, R.; Janowsky, A.; Cashmana, J. R. *Bioorg. Med. Chem.* **2009**, *17*, 2047.
22. Maurizi, M. R.; Thompson, M. W.; Singh, S. K.; Kim, S. H. *Methods Enzymol.* **1994**, *244*, 314.