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Bicyclic enol cyclocarbamates inhibit penicillin-binding proteins†

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Natural products form attractive leads for the development of chemical probes and drugs. The antibacterial lipopeptide Brabantamide A contains an unusual enol cyclocarbamate and we used this scaffold as inspiration for the synthesis of a panel of enol cyclocarbamate containing compounds. By equipping the scaffold with different groups, we identified structural features that are essential for antibacterial activity. Some of the derivatives block incorporation of hydroxycoumarin carboxylic acid-amino D-alanine into the newly synthesized peptidoglycan. Activity-based protein-profiling experiments revealed that the enol carbamates inhibit a specific subset of penicillin-binding proteins in *B. subtilis* and *S. pneumoniae*.

Introduction

Microorganisms produce a large variety of secondary metabolites for communication, nutrient uptake and self-defense. These structurally diverse compounds often have interesting properties, like antimicrobial and cytotoxic activity. Because of these properties, natural products and parts of their scaffold have served as starting point for the development of drugs[1] and chemical probes.[2,3] Examples of natural product based drugs include antibiotics like beta-lactams, which inhibit cell wall synthesis, and tetracyclines and aminoglycosides, which block protein synthesis. The natural products vibrolactone, epoxomicin and cyclophellitol have been converted into tools to study serine hydrolases,[4] the proteasome and glycosidases, respectively.[5] Further examples of probe molecules derived from metabolites are fluorescent penicillin and moenomycin A analogues, which have been prepared to study the transpeptidation and transglycosylation step during peptidoglycan synthesis, respectively.[3,6] The vast majority of the isolated natural products have not yet been exploited for probe and drug development, while derivatization of many of these scaffolds may also lead to interesting research tools or lead compounds. Of particular interest are natural products that display antibacterial activity. The rising resistance to commonly applied antibiotics necessitates the identification of leads that inhibit bacterial growth. A scaffold that triggered our interest was the enol cyclocarbamate. These proline-derived 5,5-fused and 5,7-fused bicyclic ring systems are found in lipopeptides that have been isolated from *Pseudomonas* extracts, such as Brabantamide A (**1a**) (Fig. 1A).[7,8] Compounds containing this scaffold, including natural product **1a** and (semi-)synthetic derivatives **1b**[9] and **2a**[10] potently inhibit lipoprotein-associated phospolipase A2 (Lp-PLA$_2$), a mammalian group VII phospholipase A2 that belongs to the serine-hydrolase superfamily.

Besides being Lp-PLA$_2$ inhibitors, enol cyclocarbamate containing lipopeptides, like **1a**, have also been shown to affect bacterial, fungal and oomycete growth by targeting specific pathways in these microorganisms.[11–13] Brabantamide A (**1a**) increases phospholipase D activity in oomycetes. Mode of action studies with reporter strains that carry a firefly luciferase gene fused to a promoter that is induced by antibiotics that block the most important biosynthetic pathways in bacteria indicated that the bactericidal activity of lipopeptide **1a** is caused by inhibition of the peptidoglycan synthesis pathway and/or cell membrane stress.[14] Although **1a** has surfactant-like properties, non-specific disruption of the cell membrane has been excluded as the sole mode of action, since lipopeptide **1a** did not permeabilize model membranes at biologically relevant concentrations.[12]

Despite the fact that it has been shown that the enol cyclocarbamate is essential to potently inhibit the mammalian Lp-PLA$_2$, it has not yet been established if the enol cyclocarbamate is required for the observed activity in bacteria and oomycetes. Neither have the molecular targets of molecules

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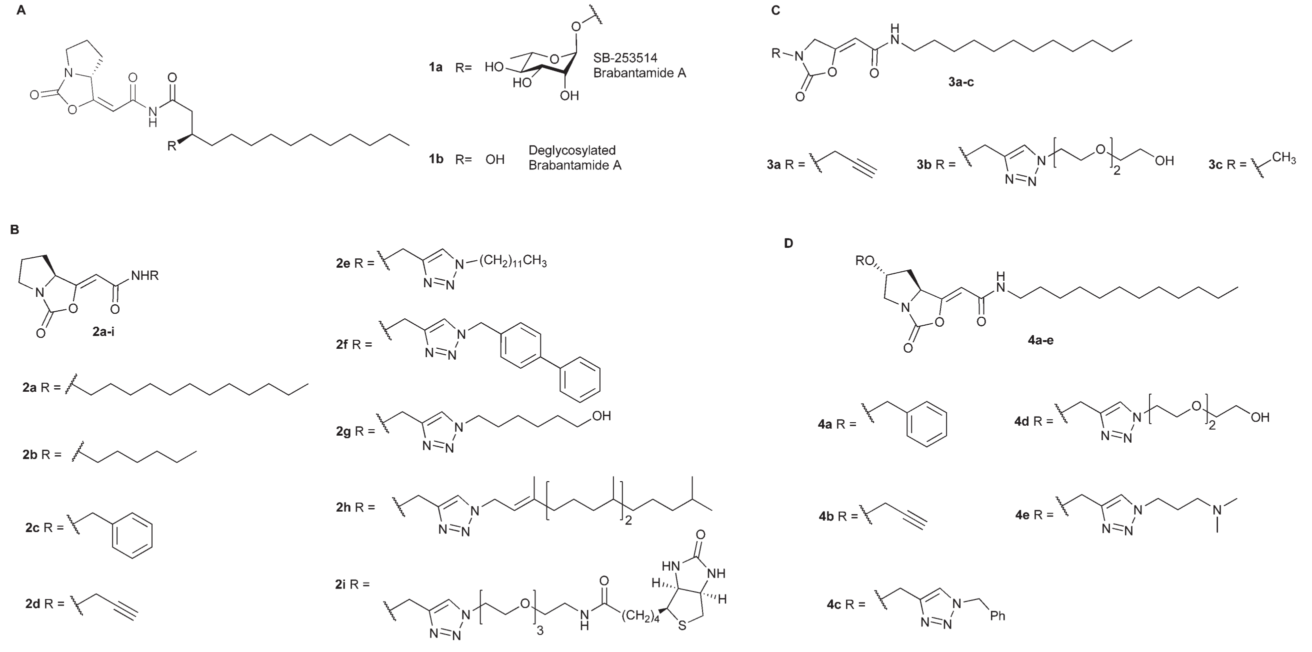


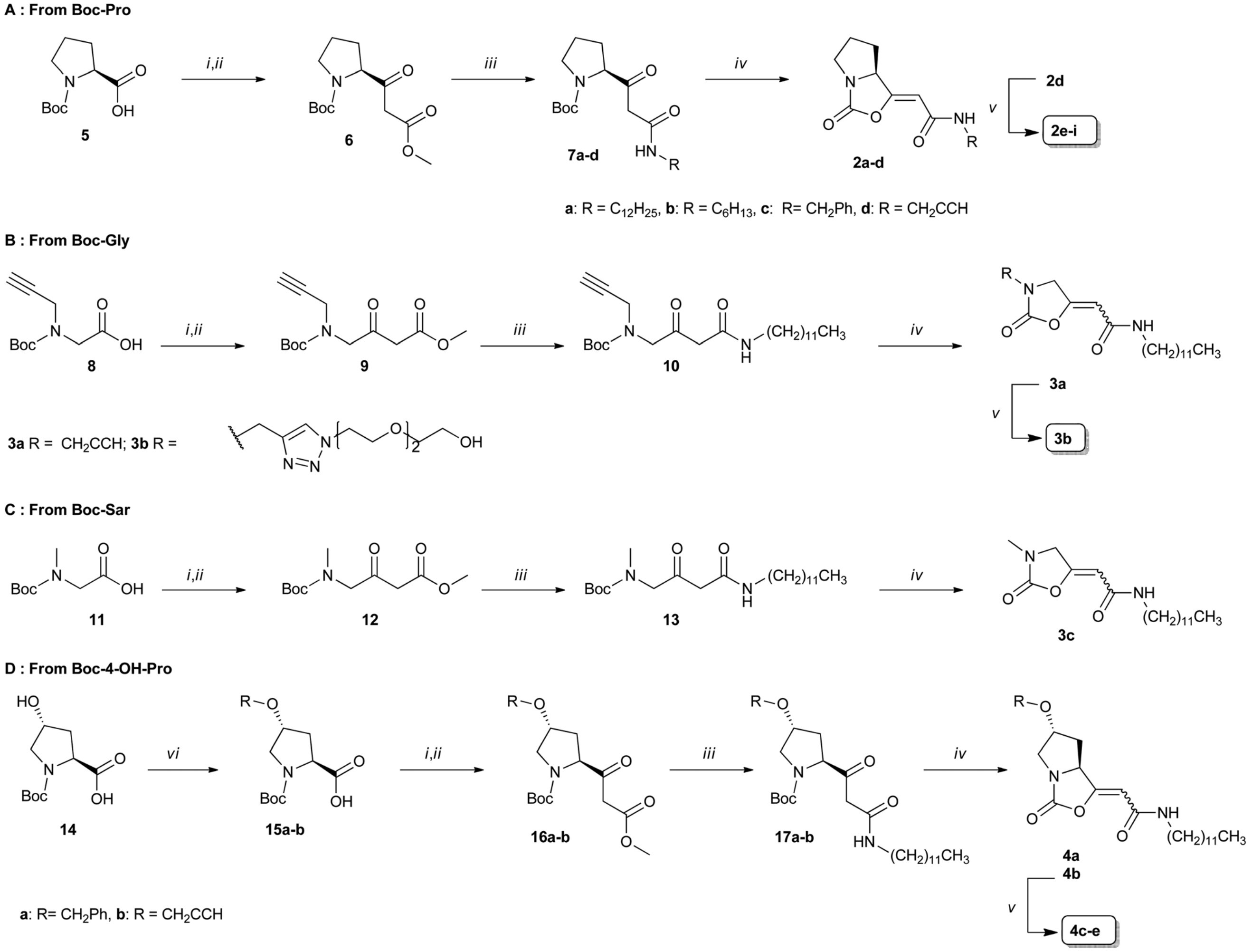
Fig. 1 (A) Structure of the natural product Brabantamide A **1a** and its deglycosylated form **1b**. The enol cyclocarbamate scaffold is depicted in grey. (B) *N*-Boc-proline derived enol cyclocarbamates **2a–i** containing various chains. (C) Monocyclic enol carbamates **3a–c** (*E* and *Z* isomers). (D) *N*-Boc-4-hydroxyproline derived enol cyclocarbamates **4a–e**.

containing this scaffold been identified in bacteria. We aimed to investigate this by preparing a series of enol cyclocarbamate derivatives **2–4** (Fig. 1B–D) that vary in the substitution pattern and by studying the antibacterial activity of the resulting compounds. To determine the role of the 5,5-fused ring system on the biological activity of the scaffold and to probe the impact of the substitution pattern, we synthesized monocyclic analogues **3a–c** (Fig. 1C) and substituted derivatives **2a–i** and **4a–e** (Fig. 1B–D), respectively. To circumvent possible decomposition of the acid and base-labile enol cyclocarbamate during synthesis,[15] we developed a novel route in which the enol cyclocarbamate is introduced at a late stage in the synthesis and that allows straightforward functionalization of this labile scaffold in the final step with copper-catalyzed click chemistry. With the panel of molecules synthesized, we identified the structural features that are required for antibacterial activity. Using metabolic labeling and activity-based protein profiling, we reveal that these enol cyclocarbamates interfere with peptidoglycan synthesis and that they inhibit Class A high molecular weight penicillin-binding proteins.

Results and discussion

Chemical synthesis

The synthesis of the panel of enol carbamate derivatives commenced with condensing Meldrum's acid to Boc-protected building blocks **5**, **8**, **11** and **15a–b** (Scheme 1A–D) using *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP). Filtration and subsequent acid–base extraction gave the crude Meldrum's acid derivative intermediates. Attempts to directly convert these into β-keto amides using five equivalents of amine in refluxing toluene resulted in complex mixtures and the β-keto amide was therefore installed using a two-step procedure. First, the Meldrum's acid intermediates were smoothly converted into the corresponding β-keto esters **6**, **9**, **12** and **16a–b** by refluxing the intermediates in anhydrous methanol.[16] In a second step, the β-keto esters were reacted with the amine of interest to form β-keto amides **7a–d**, **10**, **13** and **17a–b**. Refluxing β-keto ester **6** with hexylamine or benzylamine in THF did not result in the desired β-keto amide products **7b** and **7c**, but afforded the corresponding enamine products instead. The same, undesired products were obtained when the β-keto esters were reacted with the amine in the presence of sodium methoxide[17] or titanium isopropoxide.[18] Traces of the desired β-keto amides could be obtained by treating the amine with trimethylaluminum to generate the dimethylaluminum amide *in situ*.[19] We therefore turned our attention to DABAL-Me$_3$, the adduct of 1,4-diazabicyclo[2.2.2]octane (DABCO) and trimethylaluminum.[20,21] Activating the amine in this fashion improved the yield of the β-keto amide formation reaction and products **7a–d**, **10**, **13** and **17a–b** were obtained in moderate to reasonable yields. The final steps of the synthesis entailed the deprotection and formation of the enol cyclocarbamate. Although removal of the Boc-protecting group with hydrogen chloride in diethyl ether and subsequent cyclization of the precipitated hydrochloride salt with carbonyldiimidazole (CDI) gave the enol cyclocarbamates as separable mixture of *E* and *Z* isomers, the moderate yield prompted us to devise a novel two-step one-pot procedure. Deprotecting



Scheme 1 Synthesis of enol carbamates starting from (A) Boc-proline (B) Boc-glycine (C) Boc-sarcosine (D) *N*-Boc-4-hydroxyproline. Reagents and conditions: (i) Meldrum's acid, DCC, DMAP; (ii) MeOH, reflux (yield over 2 steps: 54–85%); (iii) DABCO, $AlMe_3$, amine (dodecylamine for **7a**, **10**, **13**, **17a** and **17b**; hexylamine for **7b**; benzylamine for **7c**; propargyl amine for **7d**) (yield: 36–73%); (iv) TMSOTf then CDI (yield over 2 steps: 34–55%); (v) $CuSO_4$, sodium ascorbate, alkyl azide (dodecyl azide for **2e**, 4-phenylbenzyl azide for **2f**, 6-azidohexanol for **2g**, phytyl azide for **2h**; biotin azide for **2i**; benzyl azide for **4c**; 2-(2-(2-azidoethoxy)ethoxy)ethanol for **3b** and **4d**; 3-azido-*N*,*N*-dimethylpropan-1-amine for **4e**) (yield: 29–68%); (vi) NaH, Br-R (benzyl bromide for **15a**; propargyl bromide for **15b**) (yield: 90%).

β-keto amides **7a–d**, **10**, **13** and **17a–b** with trimethylsilyl trifluoromethanesulfonate (TMSOTf) in CH_2Cl_2 was directly followed by cyclization with CDI affording the lipocyclocarbamates **2a–d**, **3a**, **3c** and **4a–b**. After silica gel column chromatography, the *Z*-isomers were obtained as pure products. Large-scale synthesis of **3a**, **3c** and **4b** revealed that a minor isomer was also formed and for **3c** both isomers were isolated. NOE (Nuclear Overhauser Effect) experiments with these isomers confirmed that the *Z* enol carbamate is formed preferentially. Moreover, the chemical shifts for the isomers are in accordance with the values reported in the literature for similar structures.[15] The diversity of the panel of enol cyclocarbamates was increased further *via* a copper-catalyzed click reaction. Alkyne **2d** was reacted with dodecyl azide, 6-azidohexanol, phytyl azide, 4-phenylbenzyl azide and biotin azide to obtain compounds **2e–i** that differ in chain. Reacting *Z*-lipocyclocarbamate **4b** with benzyl azide, 2-(2-(2-azidoethoxy)ethoxy)ethanol and 3-azido-*N*,*N*-dimethylpropan-1-amine led to derivatives **4c**, **4d** and **4e**, respectively. Finally, *Z*-lipocyclocarbamate **3a** was reacted with 2-(2-(2-azidoethoxy)ethoxy)ethanol to obtain monocyclic compound **3b**.

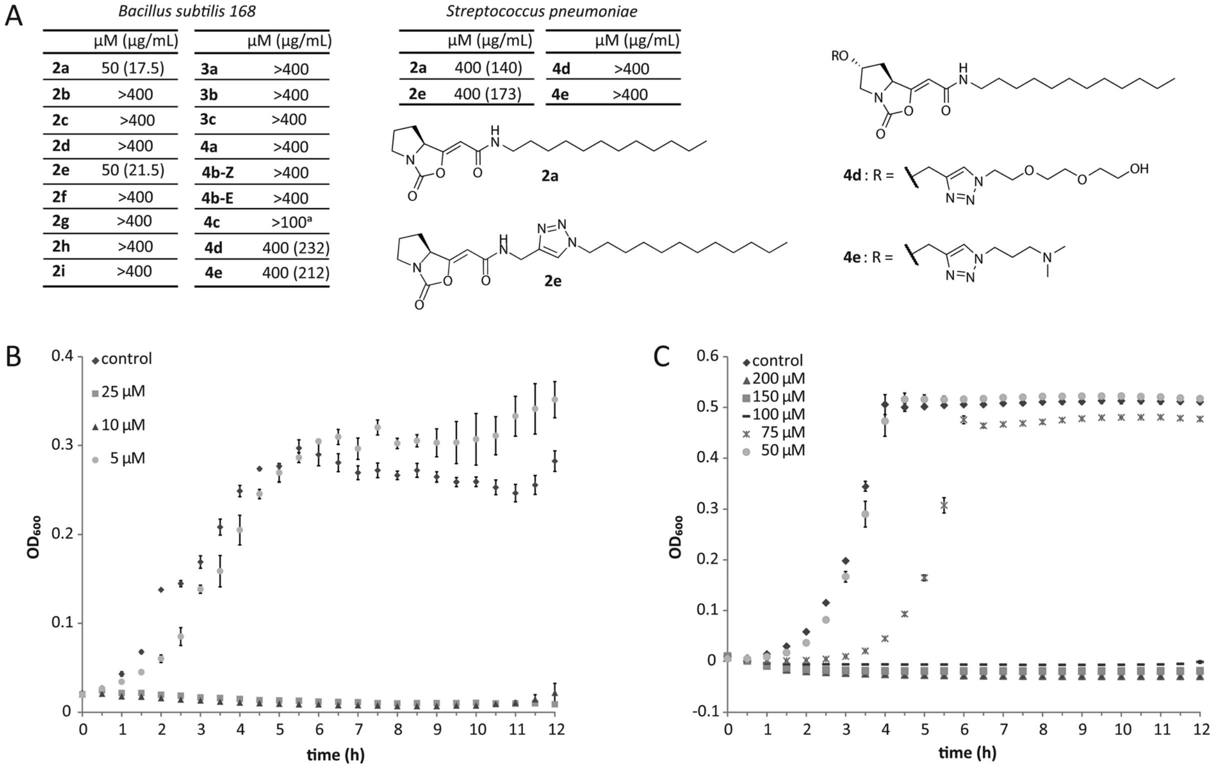
Antibacterial activity of enol carbamates

The biological activity of the panel of enol cyclocarbamates **2a–i**, **3a–c** and **4a–e** was determined using a standardized liquid-media based MIC (minimum inhibitory concentration) assay.[22] Since lipopeptide **1a** was shown to be particularly potent against Gram-positive bacteria,[12,23] we initially focused on discriminating between active and non-active compounds on the Gram-positive model bacterium *Bacillus subtilis 168*. Cells were cultured in the presence of serial dilutions of the compound for 20 hours, after which both the optical density

(OD_{600}) and resazurin were used to evaluate the activity of the compound. Metabolically active bacteria convert resazurin into highly fluorescent resorufin and the viability of the bacteria in the presence of the enol cyclocarbamates can be determined by comparing the fluorescence intensity of *B. subtilis* cells treated with compound to the fluorescence intensity of control cells treated with 0.05% DMSO (Fig. 2A and S1†).[24] Of the synthesized compounds, bicyclic enol carbamates **2a**, **2e**, **4d** and **4e** inhibit bacterial growth as judged by OD_{600} and by resazurin, with the respective MIC values being 50 μM for **2a** and **2e** and 400 μM for **4d** and **4e**.

The corresponding monocyclic analogues **3a–c** do not affect the viability of *B. subtilis*, indicating that the 5,5-fused bicyclic ring system is indispensable for potent inhibition of bacterial growth. Besides the bicyclic enol carbamate, also the substitution pattern has a large effect on the activity. Active compounds **2a**, **2e**, **4d** and **4e** all bear a long, linear alkyl chain and replacing this group by smaller and more polar substituents leads to a significant reduction in antibacterial activity. Hexyl amide derivative **2b**, benzyl amide **2c** and propargyl amide **2d** do not show any activity under the conditions used. Interestingly, the activity of propargyl amide **2d** can be restored by reacting it with dodecylazide to form derivative **2e**. However, other hydrophobic groups, such as the 4-phenylbenzyl in **2f** and the phytyl in **2h**, do not restore the activity and the introduction of more polar substituents, such as the hexylalcohol in **2g** and the biotin in **2i**, also leads to inactive compounds. Substituents at other positions of the bicyclic ring system result in remarkable behavior. Both benzyl ether **4a** and propargyl ether **4b** do not show any activity and functionalization of propargyl ether **4b** with benzyl azide, as in **4c** also leads to an inactive compound. However, equipping **4b** with a polyethylene glycol (PEG), as in **4d**, or with a tertiary amine, as in **4e**, results in compounds with moderate activity. Although the amphiphilic nature of these compounds may account for part of the activity, it is likely that the activity of **4d** is not solely caused by its surfactant-like properties. This reasoning is reinforced by the fact that PEGylated monocyclic **3b**, which is derived from inactive compound **3a** and structurally related to **4d**, does not show activity in the MIC assay, and it thus underlines the importance of the 5,5-fused bicyclic scaffold for the antibacterial activity.

We subsequently tested the activity of **2a**, **2e**, **4d** and **4e** on the Gram-positive opportunistic human pathogen *Streptococcus pneumoniae*, which is annually responsible for killing more than 1 million people and in which antibiotic resistance is on the rise. Culturing *S. pneumoniae* strain D39 with these compounds for 20 hours revealed that only



A

Bacillus subtilis 168

	μM (μg/mL)
2a	50 (17.5)
2b	>400
2c	>400
2d	>400
2e	50 (21.5)
2f	>400
2g	>400
2h	>400
2i	>400

	μM (μg/mL)
3a	>400
3b	>400
3c	>400
4a	>400
4b-Z	>400
4b-E	>400
4c	>100[a]
4d	400 (232)
4e	400 (212)

Streptococcus pneumoniae

	μM (μg/mL)
2a	400 (140)
2e	400 (173)

	μM (μg/mL)
4d	>400
4e	>400

Fig. 2 (A) MIC determination on *B. subtilis* including the full scope of lipocyclocarbamates, active compounds were also tested for MIC on *S. pneumoniae*. Structures of the most potent derivatives are depicted. Highest concentration tested was 400 μM. [a] Compound **4c** could not be tested at higher concentrations due to its poor solubility in the media. Growth curves of *B. subtilis* (B) and *S. pneumoniae* (C) in the presence of **2a**. Note: Light scattering is observed at higher concentrations of **2a** presumably due to aggregate formation. Dissociation of the aggregates over time causes a drop in optical density (OD). The depicted ODs are corrected for light scattering by adjusting the OD at the first time point with the respective OD in the absence of cells. For clarity at these low OD values, the optical density was plotted on a linear scale.

compounds **2a** or **2e** had an effect on the viability of this bacterium, with the MIC value being 400 μM.

We then studied the bacterial growth to determine whether the active compounds **2a**, **2e**, **4d** and **4e** are bacteriostatic, bactericidal or bacteriolytic. Several compounds that showed to be inactive in the MIC assay (**3c**, **4a** and **4b**) were used in these experiments as controls. Starting with an inoculum of OD_{600} 0.02 for *B. subtilis* or OD_{595} 0.04 for *S. pneumoniae* strain D39, with or without the compounds, the optical density was monitored over time. As expected, monocyclic derivative **3c** and bicyclic benzyl ether **4a**, which are inactive in the MIC assay, do not inhibit bacterial growth when evaluated at the same concentrations as the active compounds (compare growth curves Fig. 2B, C and Fig. S2 with S3†). Bicyclic propargyl ether **4b**, which was inactive in the MIC assay at 400 μM, did slow down growth at higher concentration. The cells started to grow after prolonged incubation, which corroborates the results of the MIC assays. Low concentrations of the active enol carbamates **2a**, **4d** and **4e** also cause a delay in growth of *B. subtilis* and *S. pneumoniae* (compare growth curves Fig. 2B, C and Fig. S2 with S3†), but bacterial growth is completely blocked at higher concentrations of **2a**, **4d** and **4e**. The prolonged lag phase observed in the growth curves of *S. pneumoniae* cultured with a low concentration of enol carbamate is typical for bacteriolytic agents[25] and time-lapse microscopy on *S. pneumoniae* grown on semi-solid media surfaces containing **2a** confirm the bacteriolytic activity of the enol carbamates (Fig. S4†).

Identification of the targets of the enol carbamates

Our next interest was to determine the mode of action and to identify the cellular targets of the synthesized enol carbamates. Large differences were observed when bacteria were labeled with hydroxycoumarin carboxylic acid-amino D-alanine (HADA). This fluorescent substrate analogue is incorporated into newly synthesized peptidoglycan and thus allows monitoring of *de novo* biosynthesis.[26,27] Culturing *B. subtilis* with HADA for 5 minutes results in strong fluorescent labeling of the septum, as has previously been reported (Fig. 3A). Incorporation of HADA is completely blocked when the bacteria are pre-incubated with penicillin G for 20 minutes (Fig. 3B). A similar picture was observed when we treated *B. subtilis* with bicyclic dodecylamide derivative **2a** (Fig. 3C), bicyclic PEGylated derivative **4d** (Fig. 3F), bicyclic dodecyl triazole analogue **2e** (Fig. S5†) or bicyclic dimethyl aminopropane derivative **4e** (Fig. S5†) prior to the addition of HADA. All these compounds inhibit fluorescent labeling of the septum, while inactive compounds, such as bicyclic propargylamide **2d** (Fig. 3D), and compounds that only delayed the bacterial growth, such as bicyclic propargyl ether **4b** (Fig. 3E), did not affect fluorescent labeling. Quantification of the fluorescence intensity per cell confirmed that the decrease in incorporation of HADA is significant (Fig. S6†) and live/dead cell assays[28] demonstrated that the decreased incorporation is not caused by membrane disruption and subsequent cell lysis (Fig. S7†). We obtained similar results when we cultured *S. pneumoniae D39* with HADA in the presence and absence of the compounds (Fig. 3G–L and S8†). Also in this strain, compounds **2a** and **4d** block fluorescent labeling.

In *S. pneumoniae* and *B. subtilis*, penicillin-binding proteins (PBPs) incorporate HADA[6,29] and other fluorescent D-amino acids (FDAAs)[30–32] onto the stempeptide of lipid II and peptidoglycan and the decrease in HADA labeling suggests that the enol carbamates inhibit this process. To establish which PBPs are inhibited by the enol carbamates, we performed competitive activity-based protein-profiling experiments. Cells were incubated with the enol carbamates for 60 minutes prior to cell lysis and the addition of Bocillin FL, a fluorescent penicillin derivative that labels the majority of the PBPs in *B. subtilis* (Fig. 4). Fluorescent scanning of the gels revealed that propargyl amide **2d** does not inhibit labeling of PBPs, but dodecyl amide **2a** blocks labeling of a Bocillin FL-sensitive PBP (Fig. 4A) with an approximate molecular weight of 70 kDa in concentration-dependent manner. The molecular weight of this PBP could correspond to PBP3, PBP4, or PBP4A. Complemented strains that express PBP–GFP fusions and PBP-null strains were used to determine which of these PBPs is inhibited by the enol carbamates.[33,34] The PBP–GFP fusion proteins migrate differently on the SDS-PAGE due to the increase in molecular weight and as a consequence the Bocillin FL labeling pattern will alter. In null strains, the fluorescent band of the respective PBP will be absent when labeling these cells with Bocillin FL. Labeling of PBP–GFP strains did not result in noticeable differences, but labeling of the PBP4-null strain with Bocillin FL, gave a pattern that corresponds with wild type *B. subtilis* treated with **2a**, indicating that this compound inhibits PBP4 (Fig. 4B). Profiling experiments with the complete panel of enol carbamates revealed that compounds **4d** and **4e**, and to lesser extend **2e** also block labeling of PBP4 (Fig. S9 and S10†).

We subsequently studied if the enol carbamates also target PBPs in *S. pneumoniae D39* using the same activity-based protein-profiling experiment as described for *B. subtilis*. The dodecyl amide **2a**, propargyl amide **2d** and monocyclic analogue **3c** were added to a mid-log culture of *S. pneumoniae D39*, before the cells were lysed and reacted with Bocillin FL. Incubating lysates from cells that were treated with 4% DMSO, as a control, results in three fluorescent bands with a molecular weight of 79, 73 and 45 kDa. These bands correspond with PBP1a/1b, PBP2a/2b/2x and PBP3, respectively. Addition of **2d** or **3c** to the medium does not alter the labeling profile, but incubating *S. pneumoniae* with dodecyl amide **2a** reduces the labeling intensity of the PBP1a/1b and PBP3 bands by 40 and 60%, respectively (Fig. 4C and S11†). Concomitantly, a new fluorescent band (around 40–43 kDa) appears when cells are incubated with **2a** or **4b** (Fig. 4C). It has been reported that some antibiotics increase the sensitivity of *S. pneumoniae* PBPs to proteolysis and this band therefore could be formed by proteolytic cleavage of one of the PBPs reacting with **2a**. Alternatively, the band could be caused by aberrant running of a PBP that reacted with both Bocillin FL and **2a**.[35] The addition of phenylmethylsulfonyl fluoride (PSMF), a broad-spectrum serine hydrolase inhibitor, during the incubation

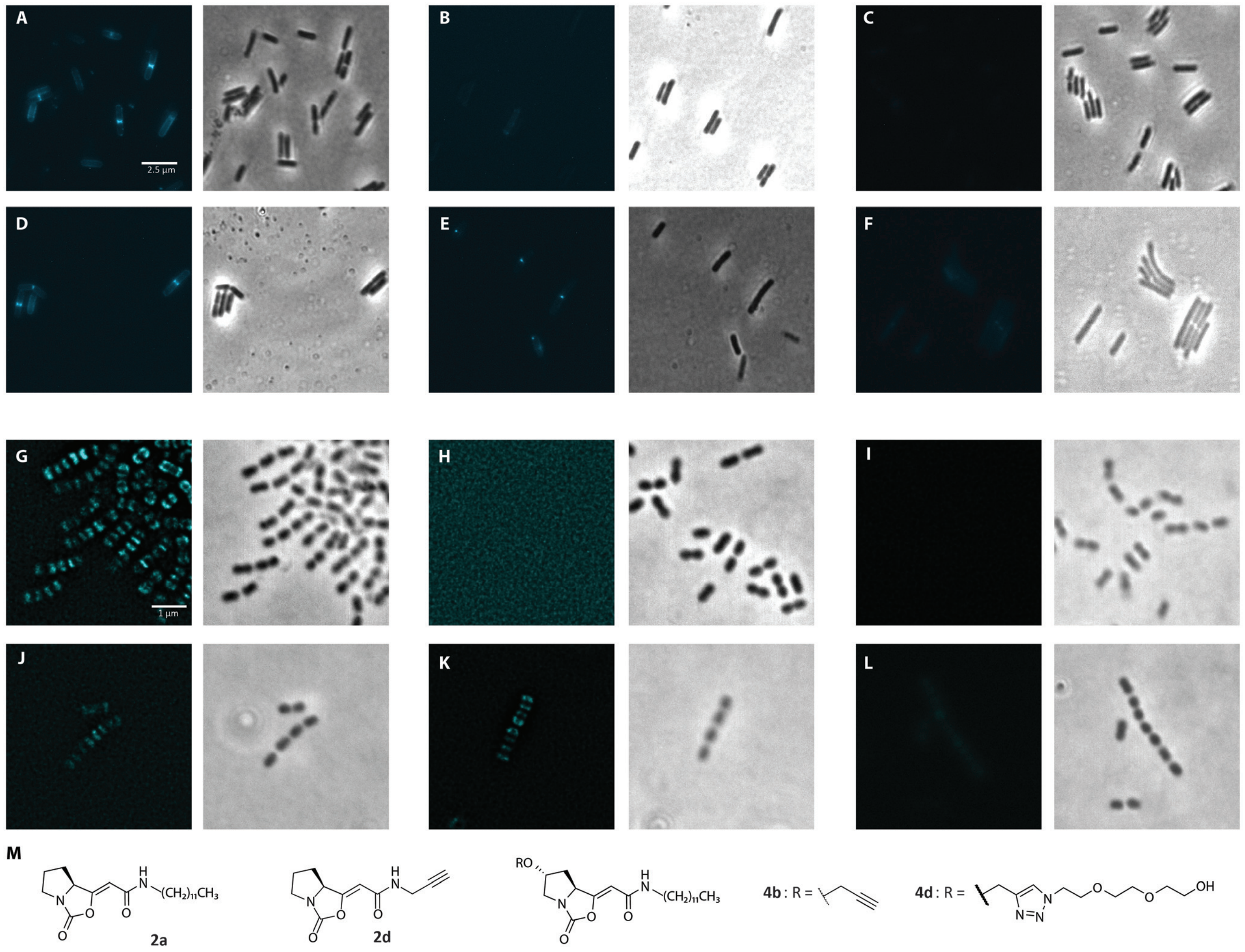


Fig. 3 HADA labeling in *B. subtilis* 168 (A–F) and *S. pneumoniae* (G–L) treated with different enol carbamates. (M) Structures of the enol cyclocarbamate derivatives used. Cells were incubated with 100 μM of enol carbamate for 20 minutes and then with HADA (0.5 mM) for 5 minutes. 0.05% DMSO/H_2O and 100 μM penicillin G were used as controls. Representative fluorescence and bright-field micrographs of *B. subtilis* cells treated with (A) 0.05% DMSO/H_2O, (B) penicillin G, (C) **2a**, (D) **2d**, (E) **4b** and (F) **4d**. For uncropped images see ESI Fig. S5.† Representative fluorescence and bright-field micrographs of *S. pneumoniae* cells treated with (G) 0.05% DMSO/H_2O, (H) penicillin G, (I) **2a**, (J) **2d**, (K) **4b** and (L) **4d**. For uncropped images see ESI Fig. S8.†

steps does not block the formation of the band (Fig. S11†). The biological relevance of this labeled protein will have to be determined.

The identified Class A high-molecular weight PBPs, which have glycosyltransferase and transpeptidase activity, are not essential for *B. subtilis* and *S. pneumoniae* viability. Deletion of PBP4 in *B. subtilis*[36] and PBP1a or PBP1b in *S. pneumoniae*[37,38] does not affect growth and cell-wall synthesis and inhibition of these PBPs by enol cyclocarbamates cannot explain the antibacterial activity of the compound class. To elucidate if inhibition of the PBPs does affect the incorporation of HADA, we treated the *B. subtilis* PBP4 null strain with HADA. PBP4 has been recently identified as one of the primary enzymes responsible for the global incorporation of unnatural D-amino acid derivatives in the peptidoglycan of *B. subtilis.*[39] Contrary to enol carbamates **2a** and **4d**, which completely block septal incorporation of HADA in wild type *B. subtilis*, septal labeling of the PBP4-null strain with HADA is not altered (Fig. S12†). The latter observation corroborates the results of Fura *et al.*, who showed that FITC-D-lysine is incorporated at the septum of a PBP4-null strain.[39] These results clearly demonstrate that the observed decrease in septal labeling in *B. subtilis* is not caused by exclusive inhibition of PBP4. This conclusion is reinforced by the observation that labeling of the septum can be inhibited by treating PBP4-null cells with **2a** (Fig. S12†).

We therefore hypothesize that enol carbamates **2a** and **4d** do not only inhibit PBP4 in *B. subtilis*, but that they also inhibit other enzymes in the peptidoglycan synthesis pathway, these being PBPs that are not labeled by Bocillin FL or other enzymes that are directly or indirectly involved in the synthesis of lipid II and the peptidoglycan. This hypothesis is supported by unpublished data from our lab, which revealed that anti-

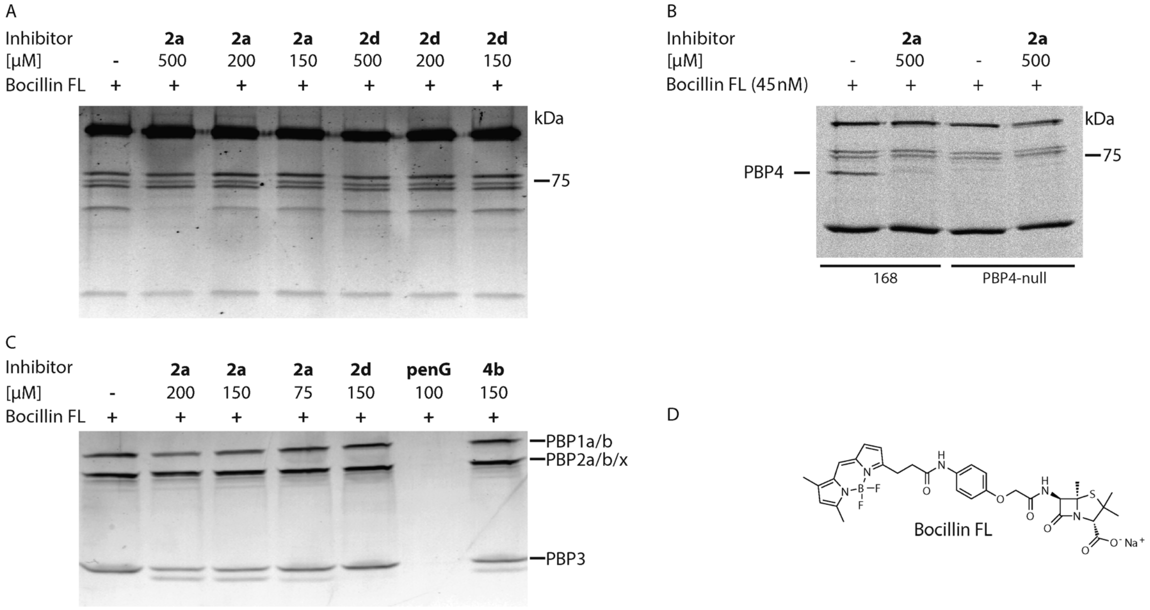


Fig. 4 Competition experiments with Bocillin FL. (A) Cells of *Bacillus subtilis* 168 were treated with indicated amount of compound for 60 min, lysed and subsequently the cell lysates were incubated with Bocillin FL (45 nM) for 30 minutes. The proteins were resolved on SDS-PAGE and analyzed by fluorescence scanning (15% SDS-PAGE gel); (B) SDS-PAGE labeling experiment using *B. subtilis* 168 wild-type and a PBP4-null strain confirms PBP4 as one of the targets of the enol cyclocarbamates; (C) cells of *Streptococcus pneumoniae* were treated with indicated amount of compound for 60 minutes, lysed and the cell lysates were subsequently incubated with Bocillin FL (45 nM) for 30 minutes. The proteins were resolved on SDS-PAGE and analyzed by fluorescence scanning (15% SDS-PAGE gel); (D) structure of Bocillin FL.

biotics that inhibit enzymes upstream of the PBPs block incorporation of HADA.

Similarly, these compounds may inhibit peptidoglycan synthesis directly or indirectly in *S. pneumoniae* and we are currently identifying these additional targets.

Conclusions

In conclusion, we developed a synthetic route that yielded a panel of enol cyclocarbamates containing compounds in four or five steps. By studying the biological activity of these compounds in a MIC assay, we showed that the bicyclic scaffold and the dodecyl alkyl chain are indispensable for antibacterial activity. Modifications on the bicyclic headgroup are tolerated, but small changes in the functional group can lead to complete loss of activity. Growth-curve experiments, time-lapse microscopy and metabolic labeling with HADA revealed that enol cyclocarbamates inhibit peptidoglycan synthesis (*i.e.*, they are bacteriolytic). Using activity-based protein-profiling experiments, we demonstrated for the first time that compounds containing an enol cyclocarbamate inhibit a specific subset of PBPs in two Gram-positive species of bacteria, *B. subtilis* and *S. pneumoniae*. While inhibition of these PBPs may contribute to the inhibition of HADA incorporation, the enol cyclocarbamates likely also target other enzymes involved in peptidoglycan synthesis. This makes the scaffold an attractive starting point in the search for novel antibiotics.

Experimental

Chemicals

Resazurin sodium salt was purchased from BD Biosciences. Syto9 and propidium iodide were purchased from Life Technologies. All the other chemicals were purchased from Sigma-Aldrich. The synthesized enol cyclocarbamate compounds were dissolved in DMSO and were stored at −20 °C.

Bacterial strains

B. subtilis strain 168 was cultured in lysogeny broth (LB). The strain was first grown in LB overnight at 37 °C before the MIC assay or the optical density (OD_{600}) assay. The MIC assay itself was performed in cation adjusted Mueller–Hinton medium.

B. subtilis PBP4-null strain was constructed by transformation of chromosomal DNA from strain PS2022 (pbpD::Erm) to *B. subtilis* 168 with selection for erythromycin resistance. Correct chromosomal insertion was verified by PCR and the absence of PBP4 in a Bocillin-FL PBP profile.[36]

S. pneumoniae D39 strain (serotype 2) was grown at 37 °C in C + Y medium with 2% acid (pH 6.8) until a mid-exponential phase (OD_{595} of 0.4).[40] The cells were centrifuged for 2 minutes at 20 800 rcf and the cell pellet was resuspended in a volume of fresh medium containing 14.5% glycerol (v/v) that would result in an OD_{595} of 0.4. The cells were then aliquoted and stored at −80 °C. These mid-exponential phase cell stocks will be referred to as T2 cells.

MIC determination

MIC determination was carried out in 96 well plate following the CLSI guidelines.[22]

B. subtilis strain 168 was grown in cation adjusted Mueller–Hinton broth at 35 °C in the presence of a serial dilution of the compound during 20 hours. The OD_{600} was measured with a BioTEK PowerWave microplate reader, before resazurin (0.015 mL of a 0.01% (wt/vol) solution water) was added. The plate was incubated at 37 °C for 20 minutes. The fluorescence emission at 585 nm (excitation 571 nm) was measured. To determine the relative viability, the observed fluorescence emission was corrected for background fluorescence and divided by the fluorescence emission observed for control cells.

S. pneumoniae was grown in cation adjusted Mueller–Hinton supplemented with 5% sheep blood for the MIC assay.

Growth curves

***B. subtilis*.** The OD curves were carried out in 96-well microtiter plates in triplicates. Cells were first cultured to mid-exponential phase (OD = 0.2 to 0.7), then diluted to OD = 0.02 and incubated in LB broth in microtiter plates with different concentrations of the compounds and the adequate controls. The suspensions were incubated at 30 °C in a microplate reader BioTEK PowerWave under agitation to favor aeration (cycles of 9 minutes agitation/1 minutes no agitation). Growth (OD_{600}) was measured every 10 minutes and the resulting values were plotted against the time to obtain the optical density curves.

***S. pneumoniae*.** T2 cells of strain D39 were diluted 100-fold and were grown until OD 0.4 in C + Y with 2% acid. For the assay, this pre-culture was diluted 100-fold in C + Y and incubated in microtiter plates with or without different concentrations of the antimicrobials. Growth (OD_{595}) was measured every 10 minutes in a Tecan Infinite F200 PRO with at least three replicates for each condition.

Microscopy

Inhibition of incorporation of the fluorescent D-amino-acid analogue HADA into peptidoglycan in live *B. subtilis* was determined using a wide-field fluorescence microscope. *B. subtilis* 168 was diluted from an overnight culture to an OD_{600} of 0.02 in casein hydrolysate medium[41] and grown at 37 °C until an OD_{600} of 0.2, at which point the compounds were added to the medium. Cells were incubated for 20 minutes with compound, followed by 5 minutes labeling with HADA (0.5 mM), after which cells were fixed with 70% ethanol. Bacteria were imaged under a Nikon Ti-E inverted microscope equipped with a CFI Plan Apochromat DM 100× oil objective, using appropriate filter sets for the dyes used. Digital images were recorded using a Hamamatsu Orca Flash 4.0 (V2) camera and prepared using Adobe Photoshop.

Activity-based protein profiling using Bocillin-FL

- *B. subtilis* 168 was diluted from an overnight culture to an OD of 0.1 and cultured until OD 0.3 (usually 2 hours), cells (4 mL per lane) were collected by centrifugation (5 minutes, 20 800 rcf) and resuspend in Mueller Hinton and incubated with or without compounds during 60 minutes (2.5% DMSO), cells were then collected by centrifugation (5 minutes, 15 000 rcf) and resuspended in cold PBS and lysed in the presence of lysozyme (0.2 mg mL^{-1}, 37 °C, 20 minutes) and DNAse (0.1 mg mL^{-1}).

- Lysates from *B. subtilis* 168 and *B. subtilis* PBP4-null strains were prepared in the following way: cells were diluted from an overnight culture to an OD of 0.1 and cultured until OD 0.3 (usually 2 hours), washed two times with PBS, lysozyme (0.5 mg mL^{-1}) was added and cells were sonicated (10 seconds pulse, 10 seconds stop (10 times) in order to control the temperature), lysates were then flash-frozen using liquid nitrogen. Lysates (19 μL, 1 mg mL^{-1} protein content) was incubated with or without compound for 60 minutes.

- *S. pneumoniae* was cultured to mid-exponential phase, cells (4 mL per lane) were collected by centrifugation (5 minutes, 20 800 rcf) and resuspend in PBS and incubated with or without compounds during 60 minutes (2.5% DMSO), cells were then lysed in the presence of lysozyme (0.2 mg mL^{-1}, 37 °C, overnight) and DNAse (0.1 mg mL^{-1}).

For both strains: lysates were then incubated with Bocillin-FL (45 nM) during 30 minutes at 37 °C. Laemmli sample buffer (SP) containing dithiothreitol (DTT) was added and the proteins were resolved on a 12% SDS-PAGE and fluorescence was visualized using a Typhoon scanner.

Synthetic procedures

General remarks. All reactions were performed using oven-dried glassware under an atmosphere of nitrogen (unless otherwise specified) using dry solvents. Reaction temperature refers to the temperature of the oil bath. Solvents were taken from a MBraun solvent purification system (SPS-800). All other reagents were purchased from Sigma Aldrich and Acros and used without further purification unless noted otherwise. Trimethylsilyl trifluoromethanesulfonate was stored under a nitrogen atmosphere in a dry Schlenk flask. TLC analysis was performed on Merck silica gel 60/Kieselguhr F254, 0.25 mm. Compounds were visualized using either ninhydrin stain (ninhydrin (1.5 g) and AcOH (3 mL) in *n*-butanol (100 mL)) or a $KMnO_4$ stain (K_2CO_3 (40 g), $KMnO_4$ (6 g), H_2O (600 mL) and 10% NaOH (5 mL)). Flash chromatography was performed using SiliCycle silica gel type SiliaFlash P60 (230–400 mesh) as obtained from Screening Devices or with automated column chromatography using a Reveleris flash purification system purchased from Grace Davison Discovery Sciences. ^1H- and ^{13}C-NMR spectra were recorded on a Varian AMX400 or a Varian 400-MR (400 and 100.59 MHz, respectively) using $CDCl_3$ or DMSO-d_6 as solvent. Chemical shift values are reported in ppm with the solvent resonance as the internal standard ($CDCl_3$: δ 7.26 for ^1H, δ 77.06 for ^{13}C, DMSO-d_6 δ 2.50 for H). Data are reported as follows: chemical shifts (δ), multiplicity (s = singlet, d = doublet, dd = double doublet, ddd = double double doublet, td = triple doublet, t = triplet, q = quartet, b = broad, m = multiplet), coupling constants *J* (Hz), and integration. High-resolution mass spectra (HRMS) were recorded on a Thermo Scientific LTQ Orbitrap XL. *Remark*: When recording the ^{13}C NMR of the Boc-protected compounds (**6**, **7a–d**, **9**, **10**, **12**, **13**, **15a–b**, **16a–b** and **17a–b**) at 25 °C in DMSO-d_6 mixtures of rotamers and enol–keto tautomers are observed in the NMR spectra and the chemical shifts of these mixtures are reported in the Experimental section. While recording the ^{13}C spectra at 75 °C does solve this issue in part, it comes at the cost of a reduced sensitivity (several carbonyl peaks are not observed in these spectra). We included both the ^{13}C NMR spectra recorded at 25 °C and at 75 °C in the ESI† for the majority of the compounds. For the ^{1}H NMR of these compounds, measuring the spectra in DMSO-d_6 at 75 °C reduced the presence of rotamers, but it resulted in a poor resolution (multiplicity of the peaks is difficult to observe). In $CDCl_3$ the multiplicity could be observed, but with presence of rotamers; for the sake of comparison and clarity, the majority is reported in DMSO-d_6.

General procedure A for *O*-alkylation of 4-hydroxy Boc-proline. A solution of 4-hydroxy Boc-proline (1 eq.) in dry THF was treated with sodium hydride (2.2 eq., 60% in mineral oil). The resulting mixture was stirred at 0 °C for 1 h and the corresponding alkyl bromide (1.1 eq.) was added. The reaction mixture was stirred until complete consumption of the starting material was observed and then acidified to pH 3 by the addition of 2 M HCl and subsequently the reaction mixture was extracted with diethyl ether (3 × 20 mL). The combined organic layers were washed with brine (2 × 20 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The crude material was purified by silica gel flash column chromatography using ethyl acetate : pentane : AcOH as eluent.

General procedure B for the synthesis of β-keto ester compounds. To a solution of *N*-Boc-protected amino-acid (1 eq.) in dry THF was added *N*,*N*′-dicyclohexylcarbodiimide (DCC) (1.1 eq.) at 0 °C. The reaction mixture was stirred for 2 minutes and 4-dimethylaminopyridine (DMAP) (1.5 eq.) and Meldrum's acid (2,2-dimethyl-1,3-dioxane-4,6-dione) (1.2 eq.) were added. The reaction mixture was stirred overnight at room temperature. Diethyl ether (30 mL) was then added to the reaction mixture and the solution was stirred for 10 minutes. After filtration over paper filter, the filtrate was washed with an aqueous Na_2CO_3 solution (60 mL), the aqueous solution was then acidified to pH 3 with 2 M HCl and the product was extracted with diethyl ether (4 × 30 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude intermediate was dissolved in MeOH (20 mL) and stirred at reflux overnight. The crude mixture was finally concentrated under reduced pressure and purified by silica gel flash column chromatography using ethyl acetate : pentane as eluent.

General procedure C for the synthesis of β-ketoamide compounds. The amidation of unactivated esters was based on the procedure described by Novak *et al.*[20] A solution of 1,4-diazabicyclo[2.2.2]octane (DABCO) (1.2 eq.) in dry toluene was cooled to 0 °C and trimethylaluminium (2.4 eq., 2 m in toluene) was added dropwise. The mixture was stirred for 4 hours at 0 °C. A solution of alkyl amine (1.2 eq.) in THF was added and the reaction mixture was heated to 40 °C and stirred for 2 h. Subsequently the corresponding β-keto ester (1 eq.) dissolved in THF was added and the reaction mixture was stirred overnight at reflux. The reaction mixture was diluted with diethyl ether (15 mL), quenched by adding 2 M HCl dropwise and washed with 2 M HCl (2 × 20 mL). The organic layer was finally dried over Na_2SO_4, concentrated under reduced pressure and purified by silica gel flash column chromatography using ethyl acetate : pentane as eluent affording the β-ketoamide compounds.

General procedure D for the synthesis of enol cyclocarbamate compounds. A solution of the corresponding β-ketoamide (1 eq.) in DCM was cooled to 0 °C before trimethylsilyl trifluoromethanesulfonate (TMSOTf) (2 eq.) was added. The reaction mixture was stirred for 3–4 hours. TLC analysis showed that all starting material had been consumed and therefore 1,1′-carbonyldiimidazole (CDI) (1.5 eq.) was added and the reaction mixture was stirred overnight. The reaction mixture was directly applied on silica gel column and flash column chromatography using ethyl acetate : pentane as eluent afforded the lipocyclocarbamate compound.

General procedure E for the synthesis of triazole compounds *via* Huisgen azide–alkyne cycloaddition. To a solution of the enol cyclocarbamate (1 eq.) and corresponding azide (1 eq.) in *tert*-butanol : MeOH : H_2O (1 : 2 : 1, v/v/v) was added sodium ascorbate (30 mol%) and $CuSO_4$ (20 mol%). The final concentration of the reaction mixture was 0.05 M. (Sodium ascorbate was added from a 40 mM stock solution in water, $CuSO_4$ was added from a 200 mM stock solution in water.) When the reaction reached completion, ethyl acetate was added and the crude mixture was washed with H_2O. The organic layer was dried over Na_2SO_4, concentrated under reduced pressure and purified by silica gel flash column chromatography using ethyl acetate : pentane and/or MeOH : DCM as eluent. The Boc-protection of *trans*-4-hydroxy-L-proline was realized following a procedure by Zhang *et al.*[42]

***N*-Boc proline β-ketoester 6.** Compound **6** was prepared according to the general procedure B using *N*-Boc-L-proline (2.15 g, 10 mmol, 1 eq.) DCC (2.25 g, 11 mmol, 1.1 eq.), DMAP (2.16 g, 15 mmol, 1.5 eq.) and Meldrum's acid (1.59 g, 11 mmol, 1.1 eq.) in THF (40 mL) to obtain the Meldrum's acid intermediate and subsequent refluxing of this intermediate in MeOH to obtain **6**. Flash chromatography using ethyl acetate : pentane (1 : 4) as eluent yielded **6** (1.9 g, 70% yield) as a yellow oil. R_f [silica, ethyl acetate : pentane (1 : 4)] = 0.30. ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C): δ = 4.31 (dd, J = 8.8, 4.9 Hz, 1H), 3.64 (s, 3H), 3.59 (d, J = 4.0 Hz, 2H), 3.47–3.27 (m, 2H), 2.29–2.03 (m, 1H), 1.93–1.70 (m, 3H), 1.37 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6, 25 °C, mixture of rotamers, 25 °C): δ = 202.96, 167.53, 167.46, 153.94, 153.02, 79.30, 64.99, 51.99, 46.71, 46.52, 45.47, 45.25, 39.52, 30.77, 29.02, 28.15, 27.90, 24.04, 23.17. IR ν_{max}/cm^{-1}: 2977, 1752, 1694, 1392, 1366, 1318, 1258, 1162, 1119, 1013, 772 cm^{-1}; HRMS: (ESI+) Calculated mass $[M + Na]^+$ $C_{13}H_{21}NO_5Na$ = 294.1312, found: 294.1315.

Dodecyl β-ketoamide 7a. β-Ketoamide **7a** was prepared according to the general procedure C by reacting DABCO (98 mg, 0.87 mmol, 1.2 eq.) with trimethylaluminium (0.87 mL, 1.74 mmol, 2.4 eq., 2 M in toluene) in toluene (2 mL) to produce DABAL *in situ*, subsequently adding dodecylamine (161 mg, 0.87 mmol, 1.2 eq.) in THF (2 mL) to activate the amine and finally adding the corresponding β-ketoester **6** (200 mg, 0.73 mmol, 1 eq.) in THF (2 mL). Flash chromatography using ethyl acetate : pentane (2 : 3) as eluent furnished **7a** (141 mg, 45% yield) as a yellow oil. R_f [silica, ethyl acetate : pentane (2 : 3)] = 0.25. ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C): δ = 7.74 (bs, 1H), 4.39–4.30 (m, 1H), 3.37–3.32 (m, 4H), 3.12–3.03 (m, 4H), 2.15–2.07 (m, 1H), 1.97–1.88 (m, 1H), 1.83–1.71 (m, 2H), 1.47–1.36 (m, 11H), 1.33–1.21 (m, 18H), 0.87 (t, J = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d$_6$, 25 °C, mixture of rotamers): δ = 204.57, 204.11, 165.87, 165.52, 154.13, 153.38, 79.37, 79.29, 65.67, 65.24, 47.64, 47.43, 47.03, 46.80, 32.36, 31.75, 30.07, 29.79, 29.44, 29.17, 28.84, 28.52, 28.30, 27.34, 26.79, 23.37, 22.54, 15.01, 14.37. IR $\nu_{\max}$/cm^{-1}: 3254, 2921, 2851, 1786, 1701, 1627, 1545, 1467, 1381, 1219, 978 cm^{-1}; HRMS: (ESI+) Calculated mass [M + Na]$^+$ $C_{24}H_{44}N_2O_4Na$ = 447.3193, found: 447.3197.

Hexyl β-ketoamide 7b. β-Ketoamide **7b** was prepared according to the general procedure C by reacting DABCO (98 mg, 0.87 mmol, 1.2 eq.) with trimethylaluminium (0.87 mL, 1.74 mmol, 2.4 eq., 2 M in toluene) in toluene (2 mL) to produce DABAL *in situ*, subsequently adding hexylamine (114 μL, 0.87 mmol, 1.2 eq.) in THF (2 mL) to activate the amine and finally adding the β-ketoester **6** (200 mg, 0.73 mmol, 1 eq.) in THF (2 mL). Flash chromatography using ethyl acetate : pentane (2 : 3) as eluent yielded **7b** (169 mg, 68% yield) as a yellow oil. R_f [silica, ethyl acetate : pentane (2 : 3)] = 0.20. ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C): δ = 7.75 (bs, 1H), 4.39–4.30 (m, 1H), 3.41–3.21 (m, 2H), 3.12–3.04 (m, 4H), 2.16–2.06 (m, 1H), 1.97–1.89 (m, 1H), 1.84–1.71 (m, 2H), 1.47–1.35 (m, 9H), 1.34–1.24 (m, 6H), 0.87 (t, J = 7.5 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d$_6$, 25 °C, mixture of rotamers): δ = 204.28, 203.87, 165.69, 165.31, 153.85, 153.12, 79.11, 64.93, 47.27, 47.11, 46.51, 40.16, 39.52, 31.09, 29.03, 28.21, 27.99, 26.13, 23.07, 22.19, 22.15, 14.03. IR $\nu_{\max}$/cm^{-1}: 2924, 2854, 1459, 1377, 754 cm^{-1}; HRMS: (ESI+) Calculated mass [M + Na]$^+$ $C_{18}H_{33}N_2O_4Na$ = 363.2254, found: 363.2254.

Benzyl β-ketoamide 7c. β-Ketoamide **7c** was prepared according to the general procedure C by reacting DABCO (98 mg, 0.87 mmol, 1.2 eq.) with trimethylaluminium (0.87 mL, 1.74 mmol, 2.4 eq., 2 M in toluene) in toluene (2 mL) to produce DABAL *in situ*, subsequently adding benzylamine (95 μL, 0.87 mmol, 1.2 eq.) in THF (2 mL) to activate the amine and finally adding β-ketoester **6** (200 mg, 0.73 mmol, 1 eq.) in THF (2 mL). Flash chromatography using ethyl acetate : pentane (1 : 1) as eluent afforded **7c** (155 mg, 62% yield) as brown oil. R_f [silica, ethyl acetate : pentane (1 : 1)] = 0.38. ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C): δ = 8.30 (bs, 1H), 7.41–7.17 (m, 5H), 4.43–4.21 (m, 3H), 3.53–3.29 (m, 4H), 2.20–2.04 (m, 1H), 2.02–1.87 (m, 1H), 1.82–1.71 (m, 2H), 1.38 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6, 25 °C, mixture of rotamers): δ = 205.56, 205.26, 167.21, 166.91, 154.42, 140.45, 129.69, 128.69, 128.26, 80.44, 66.35, 48.22, 47.82, 43.71, 30.14, 29.51, 29.28, 25.28, 24.39. IR $\nu_{\max}$/cm^{-1}: 2944, 2869, 1475, 1384, 1174 cm^{-1}; HRMS: (ESI+) Calculated mass [M + Na]$^+$ $C_{19}H_{27}N_2O_4Na$ = 347.1965, found: 347.1967.

Propargyl β-ketoamide 7d. β-Ketoamide **7d** was prepared according to the general procedure C by reacting DABCO (246 mg, 2.2 mmol, 1.2 eq.) with trimethylaluminium (2.2 mL, 4.4 mmol, 2.4 eq., 2 M in toluene) in toluene (4 mL) to produce DABAL *in situ*, subsequently adding propargyl amine (145 μL, 2.2 mmol, 1.2 eq.) in THF (3 mL) to activate the amine and finally adding β-ketoester **6** (500 mg, 1.83 mmol, 1 eq.) in THF (3 mL). Purification by flash chromatography using ethyl acetate : pentane (1 : 4) as eluent furnished **7d** (398 mg, 73% yield) as a yellow oil. R_f [silica, ethyl acetate : pentane (1 : 1)] = 0.28. ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C): δ = 8.27 (bs, 1H), 4.33 (dd, J = 8.9, 5.0 Hz, 1H), 3.96–3.84 (m, 2H), 3.41–3.30 (m, 4H), 2.97 (t, J = 2.3 Hz, 1H), 2.19–2.07 (m, 1H), 1.90 (dq, J = 12.6, 5.9 Hz, 1H), 1.86–1.73 (m, 2H), 1.38 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d$_6$, 25 °C, mixture of rotamers): δ = 204.27, 203.92, 166.01, 165.67, 154.16, 153.40, 88.79, 81.17, 81.13, 79.46, 73.65, 73.58, 65.26, 65.21, 55.28, 47.09, 47.04, 46.96, 46.82, 29.14, 28.51, 28.44, 28.30, 28.26, 24.29, 23.40. IR $\nu_{\max}$/cm^{-1}: 3294, 2977, 1684, 1539, 1399, 1366, 1163 cm^{-1}; HRMS: (ESI+) Calculated mass [M + Na]$^+$ $C_{15}H_{22}N_2O_4Na$ = 317.1472, found: 314.1474.

Dodecyl derivative 2a. Enol cyclocarbamate **2a** was prepared according to the general procedure D using the corresponding β-keto amide **7a** (100 mg, 0.24 mmol, 1 eq.), TMSOTf (86 μL, 0.47 mmol, 2 eq.) and CDI (81 mg, 0.35 mmol, 1.5 eq.) in DCM (1 mL). Flash chromatography using ethyl acetate : pentane (3 : 2) as eluent yielded **2a** (29 mg, 35% yield) as a colorless oil. R_f [silica, ethyl acetate : pentane (3 : 2)] = 0.22. $[\alpha]^{20}_{D}$ = −39.6 (c = 0.308, CHCl$_3$); ^{1}H NMR (400 MHz, CDCl$_3$): δ = 6.60 (bs, 1H), 5.18 (s, 1H), 4.49 (app t, J = 8.0 Hz, 1H), 3.73–3.61 (m, 1H), 3.36–3.26 (m, 2H), 2.29–2.20 (m, 1H), 2.20–2.12 (m, 1H), 2.12–2.02 (m, 1H), 1.77–1.65 (m, 2H), 1.58–1.50 (m, 2H), 1.34–1.23 (m, 18H), 0.87 (t, J = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, CDCl$_3$): δ = 163.40, 156.05, 154.30, 99.66, 63.14, 46.00, 39.75, 31.90, 31.43, 29.63, 29.63, 29.61, 29.58, 29.51, 29.33, 29.28, 26.94, 26.37, 22.67, 14.10. IR $\nu_{\max}$/cm^{-1}: 3257, 2921, 2851, 1786, 1701, 1627, 1545, 1467, 1381, 1219, 978 cm^{-1}; HRMS: (ESI+) Calculated mass [M + H]$^+$ $C_{20}H_{35}N_2O_3$ = 351.2642 found: 351.2645; Calculated mass [M + Na]$^+$ $C_{20}H_{34}N_2O_3Na$ = 373.2462, found: 373.2463.

Hexyl derivatve 2b. Enol cyclocarbamate **2b** was prepared according to the general procedure D using the corresponding β-keto amide (*S*)-*tert*-butyl-2-(3-(hexylamino)-3-oxopropanoyl) pyrrolidine-1-carboxylate **7b** (100 mg, 0.29 mmol, 1 eq.), TMSOTf (106 μL, 0.58 mmol, 2 eq.) and 1,1′-carbonyldiimidazole (81 mg, 0.44 mmol, 1.5 eq.) in DCM (1.5 mL). Flash chromatography using ethyl acetate : pentane (4 : 1) as eluent furnished **2b** (28 mg, 35% yield) as a yellow oil. R_f [silica, ethyl acetate : pentane (4 : 1)] = 0.20. $[\alpha]^{20}_{D}$ = −47.1 (c = 0.612, CHCl$_3$) ^{1}H NMR (400 MHz, CDCl$_3$): δ = 6.58 (bs, 1H), 5.14 (s, 1H), 4.51–4.45 (m, 1H), 3.74–3.61 (m, 1H), 3.35–3.19 (m, 3H), 2.18–2.12 (m, 1H), 2.11–2.02 (m, 1H), 2.28–2.20 (m, 1H), 1.76–1.64 (m, 1H), 1.57–1.47 (m, 2H), 1.34–1.25 (m, 6H), 0.87 (t, *J* = 5.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 163.46, 156.24, 154.32, 99.91, 63.25, 46.12, 39.81, 31.58, 31.56, 29.67, 26.73, 26.49, 22.65, 14.12. IR ν_{max}/cm^{-1}: 3293, 2929, 1792, 1694, 1535, 1463, 1391, 1217, 1030, 976 cm^{-1}; HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{14}H_{23}N_2O_3$ = 267.1703 found: 267.1710.

Benzyl derivative 2c. Enol cyclocarbamate **2c** was prepared according to the general procedure D using the corresponding β-keto amide **7c** (100 mg, 0.29 mmol, 1 eq.), TMSOTf (106 μL, 0.58 mmol, 2 eq.) and CDI (81 mg, 0.44 mmol, 1.5 eq.) in DCM (1.5 mL). Flash chromatography using ethyl acetate : pentane (4 : 1) as eluent gave **2c** (43.1 mg, 55% yield) as a yellow oil. R_f [silica, ethyl acetate : pentane (1 : 1)] = 0.15. $[\alpha]_D^{20}$ = −33.2 (*c* = 0.476, $CHCl_3$) ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.35–7.24 (m, 5H), 6.94 (bs, 1H), 5.20 (s, 1H), 4.52 (d, *J* = 5.7 Hz, 2H), 4.51–4.45 (m, 1H), 3.69–3.61 (m, 1H), 3.33–3.26 (m, 1H), 2.29–2.19 (m, 1H), 2.19–2.11 (m, 1H), 2.10–2.00 (m, 1H), 1.75–1.64 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 163.48, 156.11, 154.85, 138.42, 128.76, 127.75, 127.48, 99.57, 63.28, 46.14, 43.56, 31.54, 26.50. IR ν_{max}/cm^{-1}: 3297, 2930, 1788, 1693, 1633, 1532, 1390, 1218, 972 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{15}H_{17}N_2O_3$ = 273.1234 found: 273.1240; Calculated mass $[M + Na]^+$ $C_{15}H_{16}N_2O_3Na$ = 295.1053, found: 295.1059.

Propargyl derivative 2d. Enol cyclocarbamate **2d** was prepared according to the general procedure D using the corresponding β-keto amide **7d** (103 mg, 0.35 mmol, 1 eq.), TMSOTf (132 μL, 0.7 mmol, 2 eq.) and CDI (102 mg, 5.25 mmol, 1.5 eq.) in DCM (2 mL). Flash chromatography using ethyl acetate : pentane (2 : 3) as eluent gave **2d** (38.5 mg, 50% yield) as a white solid. R_f [silica, ethyl acetate : pentane (2 : 3)] = 0.21. $[\alpha]_D^{20}$ = −36.8 (*c* = 0.125, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.78 (bs, 1H), 5.17 (s, 1H), 4.50 (ddd, *J* = 8.9, 6.7, 1.7 Hz, 1H), 4.25–3.93 (m, 2H), 3.66 (dt, *J* = 11.4, 7.8 Hz, 1H), 3.31 (ddd, *J* = 11.3, 8.9, 4.4 Hz, 1H), 2.29–2.20 (m, 2H), 2.20–2.12 (m, 1H), 2.12–2.02 (m, 1H), 1.83–1.56 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 163.46, 156.29, 155.77, 99.36, 79.97, 71.83, 63.60, 46.47, 31.83, 29.53, 26.84. IR ν_{max}/cm^{-1}: 3286, 2924, 1795, 1693, 1636, 1529, 1217, 1029, 974 cm^{-1}; HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{11}H_{13}N_2O_3$ = 221.0921 found: 221.0921; Calculated mass $[M + Na]^+$ $C_{11}H_{12}N_2O_3Na$ = 243.0740, found: 243.0741.

Dodecyl-triazolyl derivative 2e. Triazole compound **2e** was prepared according to the general procedure E using the enol cyclocarbamate **2d** (17.9 mg, 0.08 mmol, 1 eq.) and dodecylazide (17.3 mg, 0.08 mmol, 1 eq.). Flash chromatography using ethyl acetate : pentane (7 : 3) and then methanol : DCM (1 : 24) as eluent yielded **2e** (22 mg, 65% yield) as a colorless oil. R_f [silica, methanol : DCM (1 : 24)] = 0.15. $[\alpha]_D^{20}$ = −35 (*c* = 0.5, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.61 (bs, 1H), 7.10 (bs, 1H), 5.15 (bs, 1H), 4.58 (bs, 2H), 4.47 (t, *J* = 9.2, 6.8 Hz, 1H), 4.29 (t, *J* = 7.2 Hz, 2H), 3.64 (dt, *J* = 11.6, 7.8 Hz, 1H), 3.28 (ddd, *J* = 11.6, 8.9, 4.3 Hz, 1H), 2.27–1.99 (m, 3H), 1.93–1.82 (m, 2H), 1.75–1.62 (m, 1H), 1.34–1.17 (m, 18H), 0.86 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 163.48, 155.89, 155.20, 99.04, 63.09, 50.49, 46.01, 35.15, 31.86, 31.38, 30.20, 29.56, 29.48, 29.34, 29.29, 28.97, 26.51, 26.35, 22.64, 14.08. IR ν_{max}/cm^{-1}: 3277, 2922, 2853, 1789, 1694, 1545, 1400, 1218, 1026, 845, 760 cm^{-1}; HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{23}H_{38}N_5O_3$ = 432.2969 found: 432.2961.

Biphenyl triazolyl derivative 2f. Triazole compound **2f** was prepared according to the general procedure E using the enol cyclocarbamate **2d** (20.3 mg, 0.09 mmol, 1 eq.) and 4-phenylbenzyl azide (19.35 mg, 0.09 mmol, 1 eq.). Flash chromatography using ethyl acetate : pentane (7 : 3) and then methanol : DCM (1 : 24) as eluent yielded **2f** (27 mg, 68% yield) as a colorless oil. R_f [silica, methanol : DCM (1 : 24)] = 0.17. $[\alpha]_D^{20}$ = −31 (*c* = 0.250, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.57 (t, *J* = 8.2 Hz, 4H), 7.43 (t, *J* = 7.5 Hz, 2H), 7.35 (t, *J* = 8.8 Hz, 3H), 7.11 (bs, 1H), 5.53 (s, 2H), 5.15 (bs, 1H), 4.60 (bs, 2H), 4.46 (app t, *J* = 8.0 Hz, 1H), 3.64 (dt, *J* = 11.4, 7.8 Hz, 1H), 3.29 (td, *J* = 11.4, 8.8, 4.3 Hz, 1H), 2.25–1.96 (m, 3H), 1.75–1.62 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 155.87, 155.24, 141.73, 140.23, 133.39, 128.82, 128.63, 127.78, 127.61, 127.09, 63.11, 54.14, 46.01, 35.14, 31.37, 26.3. IR ν_{max}/cm^{-1}: 3286, 1790, 1692, 1640, 1488, 1215, 1022, 909, 757, 727 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{24}H_{24}N_5O_3$ = 430.1873 found: 430.1869.

6-Hydroxyhexyl-triazolyl derivative 2g. Triazole compound **2g** was prepared according to the general procedure E using the enol cyclocarbamate **2d** (15.46 mg, 0.07 mmol, 1 eq.) and 6-azidohexanol (10 mg, 0.07 mmol, 1 eq.). Flash chromatography using methanol : DCM (1 : 24) as eluent yielded **2g** (16 mg, 63% yield) as a colorless oil. R_f [silica, methanol : DCM (1 : 24)] = 0.10. $[\alpha]_D^{20}$ = −38 (*c* = 0.450, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.56 (bs, 1H), 7.10 (bs, 1H), 5.15 (s, 1H), 4.58 (d, *J* = 5.6 Hz, 2H), 4.48 (app t, *J* = 8.1 Hz, 1H), 4.32 (t, *J* = 7.0 Hz, 2H), 3.81–3.48 (m, 3H), 3.30 (ddd, *J* = 11.9, 8.8, 4.3 Hz, 1H), 2.40–1.99 (m, 3H), 2.04–1.83 (m, 2H), 1.80–1.62 (m, 1H), 1.62–1.47 (m, 2H), 1.45–1.28 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 164.01, 156.36, 155.73, 99.48, 77.16, 63.59, 62.83, 50.61, 46.48, 35.64, 32.72, 31.85, 30.51, 26.83, 26.49, 25.4. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{17}H_{26}N_5O_4$ = 364.1972 found: 364.1979.

Phytyl triazolyl derivative 2h. Triazole compound **2h** was prepared according to the general procedure E using the enol cyclocarbamate **2d** (29 mg, 0.13 mmol, 1 eq.) and phytyl azide (42 mg, 0.13 mmol, 1 eq.). Flash chromatography using ethyl acetate : pentane (9 : 1) as eluent yielded **2h** (33 mg, 47% yield) as a colorless oil. R_f [silica, ethyl acetate : pentane (9 : 1)] = 0.09. $[\alpha]_D^{20}$ = −45.6 (*c* = 0.250, $CHCl_3$); ^{1}H NMR (400 MHz, CD_3OD): δ = 7.99 (bs, 1H), 5.50–5.44 (m, 1H), 5.35 (bs, 1H), 5.01 (t, *J* = 7.5 Hz, 2H), 4.61 (t, *J* = 8.0 Hz, 1H), 4.54 (s, 2H), 3.62 (dt, *J* = 10.9, 7.8 Hz, 2H), 3.37–3.26 (m, 1H), 2.32–2.02 (m, 4H), 1.82 (d, *J* = 6.0 Hz, 3H), 1.79–1.64 (m, 1H), 1.63–1.24 (m, 14H), 1.23–1.08 (m, 4H), 0.90 (t, *J* = 6.7 Hz, 12H). ^{13}C NMR (101 MHz, CD_3OD): δ = 159.31, 158.57, 144.99, 144.72, 118.88, 118.37, 64.80, 49.00, 47.02, 40.70, 40.54, 38.51, 38.49, 38.47, 38.45, 38.43, 38.38, 37.93, 37.85, 37.74, 37.65, 33.94, 33.75, 33.06, 32.33, 29.15, 27.36, 26.50, 26.12, 26.10, 25.91, 25.89, 25.49, 23.50, 23.10, 23.02, 20.20, 20.14, 20.08, 16.36. IR ν_{max}/cm^{-1}: 3385, 2936, 1790, 1694, 1640, 1533, 1217, 1028, 973, 761 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{31}H_{52}N_5O_3$ = 542.4064 found: 542.4053.

Biotin-PEG derivative 2i. Triazole compound **2i** was prepared according to the general procedure E using the enol cyclocarbamate **2d** (27 mg, 0.12 mmol, 1 eq.) and biotin azide (44 mg, 0.12 mmol, 1 eq.). Flash chromatography using methanol : DCM (3 : 17) as eluent yielded **2i** (15.72 mg, 20% yield) as a colorless oil. R_f [silica, methanol : DCM (3 : 17)] = 0.21. $[\alpha]_D^{20}$ = −40.1 (c = 0.5, $CHCl_3$) ^1H NMR (400 MHz, CD_3OD): δ = 7.98 (s, 1H), 7.94 (s, 1H), 5.26 (s, 1H), 4.65–4.53 (m, 4H), 4.53–4.44 (m, 3H), 4.30 (dd, J = 7.9, 4.4 Hz, 1H), 3.88 (t, J = 5.0 Hz, 2H), 3.61–3.57 (m, 10H), 3.53 (t, J = 5.5 Hz, 2H), 3.35 (q, J = 5.5 Hz, 2H), 3.24–3.14 (m, 1H), 2.92 (dd, J = 12.7, 5.0 Hz, 1H), 2.70 (d, J = 12.7 Hz, 1H), 2.34–2.04 (m, 6H), 1.82–1.53 (m, 4H), 1.54–1.36 (m, 2H). ^{13}C NMR (101 MHz, CD_3OD): δ = 176.14, 166.12, 165.96, 159.18, 158.62, 97.78, 71.56, 71.49, 71.46, 71.26, 70.57, 70.35, 68.14, 64.78, 63.37, 61.63, 57.02, 51.47, 49.64, 49.00, 47.02, 41.07, 40.36, 36.74, 35.65, 32.36, 29.76, 29.50, 27.38, 26.85. IR ν_{max}/cm^{-1}: 3364, 2936, 1799, 1691, 1632, 1512, 1078, 931, 711 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{29}H_{45}N_8O_8S$ = 665.3065 found: 665.3075.

***N*-Boc-*N*-propargyl glycine 8.** A solution of Boc-Gly-OH (1.75 g, 10 mmol, 1 eq.) in dry THF (40 mL) was treated with NaH (0.88 g, 60% in mineral oil, 22 mmol, 2.2 eq.). The resulting mixture was stirred at 0 °C for 1 hour and propargyl bromide (80 wt% in toluene, 1.23 mL, 11 mmol, 1.1 eq.) was added. After overnight stirring at room temperature, TLC analysis showed incomplete conversion and an additional amount of NaH (0.5 equiv.) and propargyl bromide (0.5 equiv.) were added at 0 °C. The reaction was stirred overnight at room temperature upon which full conversion of starting material was observed. The reaction mixture was then acidified to pH 3 by addition of 2 M HCl and subsequently was extracted with diethyl ether (3 × 50 mL). The combined organic layers were washed with brine (3 × 100 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The crude material was purified by silica gel flash chromatography using ethyl acetate : pentane : AcOH (10 : 189 : 1) as eluent to give **8** (2.12 g, 99%) as a light brown oil. R_f [silica, ethyl acetate : pentane : AcOH (10 : 189 : 1)] = 0.15. HRMS: (ESI+) Calculated mass $[M + Na]^+$ $C_{10}H_{15}NO_4Na$ = 236.0893, found: 236.0893. Spectral data were in accordance with the literature.[43]

***N*-Boc-*N*-propargyl β-ketoester 9.** β-Ketoester **9** was prepared according to the general procedure B using 2-((*tert*-butoxycarbonyl)(prop-2-yn-1-yl)amino)acetic acid **8** (2.12 g, 9.9 mmol, 1 eq.) DCC (2.22 g, 10.8 mmol, 1.1 eq.), DMAP (1.65 g, 13.5 mmol, 1.5 eq.), Meldrum's acid (1.56 g, 10.8 mmol, 1.1 eq.) in THF (50 mL) and subsequent refluxing with MeOH. Flash chromatography using ethyl acetate : pentane (1 : 11.5) as eluent gave title compound **9** (2.3 g, 85% yield) as a yellow oil. R_f [silica, ethyl acetate : pentane (1 : 11.5)] = 0.20. ^1H NMR (400 MHz, $CDCl_3$, mixture of rotamers): δ = 4.25 (s, 1H), 4.18 (s, 2H), 4.10 (s, 1H), 3.75 (s, 3H), 3.60–3.28 (app m, 2H), 2.26 (t, J = 2.5 Hz, 1H), 1.64–1.36 (m, 9H). ^{13}C NMR (101 MHz, DMSO-d_6, 25 °C, mixture of rotamers): δ = 199.45, 171.29, 167.77, 154.72, 80.58, 80.42, 80.24, 80.05, 79.97, 75.15, 74.90, 56.05, 55.75, 52.42, 48.12, 46.29, 37.75, 37.54, 37.05, 36.92, 28.36, 28.27, 28.16. IR ν_{max}/cm^{-1}: 3281, 2978, 1698, 1453, 1394, 1368, 1248, 1161, 872, 774 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + Na]^+$ $C_{13}H_{19}NO_5Na$ = 292.1155, found: 292.1159.

***N*-Boc-*N*-propargyl β-ketoamide 10.** β-Ketoamide **10** was prepared according to the general procedure C by reacting 1,4-DABCO (60 mg, 0.53 mmol, 1.2 eq.) with trimethylaluminium (0.53 mL, 1.06 mmol, 2.4 eq., 2 M in toluene) in toluene (1.5 mL) to produce DABAL *in situ*, subsequently adding dodecylamine (98 mg, 0.53 mmol, 1.2 eq.) in THF (1 mL) to activate the amine and finally adding the corresponding β-keto ester **9** (120 mg, 0.44 mmol, 1 eq.) in THF (1 mL). Flash chromatography using ethyl acetate : pentane (2 : 3) as eluent afforded **10** (68 mg, 36% yield) as a yellow oil. R_f [silica, ethyl acetate : pentane (2 : 3)] = 0.26. ^1H NMR (400 MHz, $CDCl_3$, mixture of rotamers): δ = 6.99–6.73 (m, 1H), 4.60–3.96 (m, 4H), 3.39 (d, J = 10.7 Hz, 2H), 3.31–3.17 (m, 2H), 2.25 (m, 1H), 1.58–1.33 (m, 11H), 1.31–1.05 (m, 18H), 0.86 (t, J = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6, 25 °C, mixture of rotamers): δ = 201.10, 200.58, 166.11, 165.80, 154.74, 80.69, 80.43, 79.95, 77.10, 74.83, 74.57, 69.05, 57.67, 55.84, 55.53, 55.08, 48.14, 48.07, 36.87, 31.66, 29.39, 29.32, 29.18, 29.06, 28.25, 28.07, 26.64, 25.62, 22.47, 14.32. IR ν_{max}/cm^{-1}: 3312, 1925, 1854, 1704, 1454, 1367, 1247, 1163, 945 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + Na]^+$ $C_{24}H_{42}N_2O_4Na$ = 445.3037, found: 445.3039.

Propargyl functionalized monocyclic derivative 3a. Enol cyclocarbamate **3a** was prepared according to the general procedure D by reacting the appropriate β-keto amide **10** (51 mg, 0.12 mmol, 1 eq.), TMSOTf (45 μL, 0.24 mmol, 2 eq.) and CDI (35 mg, 0.18 mmol, 1.5 eq.) in DCM (0.8 mL). Flash chromatography using ethyl acetate : pentane (3 : 7) gave ***Z*-3a** (10.4 mg, 25% yield) and ***E*-3a** (4.2 mg, 10% yield) as colorless oil. *Z*-Isomer: R_f [silica, ethyl acetate : pentane (3 : 7)] = 0.19. ^1H NMR (400 MHz, $CDCl_3$): δ = 6.57 (bs, 1H), 5.22 (t, J = 2.0 Hz, 1H), 4.38 (d, J = 2.1 Hz, 2H), 4.19 (d, J = 2.5 Hz, 2H), 3.54–3.17 (m, 2H), 2.40 (t, J = 2.5 Hz, 1H), 1.66–1.46 (m, 2H), 1.43–1.10 (m, 18H), 0.88 (t, J = 6.5 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 183.74, 162.99, 148.20, 100.37, 74.74, 47.55, 39.72, 33.65, 31.90, 29.61, 29.57, 29.52, 29.33, 29.28, 26.94, 22.67, 14.10. IR ν_{max}/cm^{-1}: 2924, 2854, 1801, 1693, 1623, 1454, 1154, 1054 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{20}H_{33}N_2O_3$ = 349.2486 found: 349.2482. *E*-Isomer: R_f [silica, ethyl acetate : pentane (3 : 7)] = 0.55. ^1H NMR (400 MHz, $CDCl_3$): δ = 5.57 (s, 1H), 5.42 (t, J = 2.3 Hz, 1H), 4.79 (d, J = 2.6 Hz, 2H), 4.17 (d, J = 2.5 Hz, 2H), 3.28 (q, J = 6.7 Hz, 2H), 2.34 (t, J = 2.6 Hz, 1H), 1.55–1.45 (m, 2H), 1.35–1.20 (m, 18H), 0.88 (t, J = 6.7 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 165.45, 157.91, 153.58, 97.33, 74.35, 49.35, 39.65, 33.67, 29.78, 29.50, 29.43, 27.09, 22.84, 14.27. IR ν_{max}/cm^{-1}: 2923, 2852, 1803, 1691, 1619, 1055 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{20}H_{33}N_2O_3$ = 349.2486 found: 349.2474.

PEG-functionalized monocyclic derivative 3b. Triazole compound **3b** was prepared according to the general procedure E using the enol cyclocarbamate **3a-*Z*** (4 mg, 0.012 mmol, 1 eq.) and polyethylene glycol azide (3 mg, 0.017 mmol, 1.4 eq.). Flash chromatography using ethyl acetate : pentane (3 : 2) then methanol : DCM (1 : 199) as eluent yielded **3b** (3.6 mg, 60% yield) as a yellow oil. R_f [silica, methanol : DCM (1 : 199)] = 0.11. ^{1}H NMR (400 MHz, CDCl$_3$): δ = 7.95 (s, 1H), 6.55 (t, J = 6.1 Hz, 1H), 5.14 (s, 1H), 4.62 (s, 2H), 4.57 (t, J = 4.9 Hz, 2H), 4.40 (s, 2H), 3.88 (t, J = 4.9 Hz, 2H), 3.76 (t, J = 4.5 Hz, 2H), 3.64 (s, 4H), 3.59 (t, J = 4.5 Hz, 2H), 3.30 (q, J = 6.8 Hz, 2H), 1.59–1.47 (m, 2H), 1.31–1.20 (m, 18H), 0.88 (t, J = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, CDCl$_3$): δ = 163.29, 153.61, 148.99, 100.11, 77.16, 72.58, 70.58, 70.34, 69.26, 61.85, 50.49, 48.64, 39.83, 38.96, 32.07, 29.80, 29.76, 29.70, 29.51, 29.47, 27.11, 22.85, 14.28. IR ν_{max}/cm^{-1}: 3286, 2911, 2836, 1761, 1654, 1621, 1512, 1442, 1367, 1211, 1021 cm^{-1}. HRMS: (ESI+) Calculated mass [M + H]$^+$ $C_{26}H_{46}N_5O_6$ = 524.3443 found: 524.3434; Calculated mass [M + Na]$^+$ $C_{26}H_{46}N_5O_6Na$ = 546.3262, found: 546.3252.

***N*-Boc sarcosine β-ketoester 12.** β-Ketoester **12** was prepared according to the general procedure B using *N*-(*tert*-butoxycarbonyl)-sarcosine **11** (1.89 g, 10 mmol, 1 eq.) DCC (2.26 g, 11 mmol, 1.1 eq.), DMAP (1.83 g, 15 mmol, 1.5 eq.), Meldrum's acid (1.59 g, 11 mmol, 1.1 eq.) in THF (50 mL) and subsequent refluxing with MeOH. Flash chromatography using ethyl acetate : pentane (2 : 3) as eluent gave title compound **12** (2.45 g, 55% yield) as a yellow oil. R_f [silica, ethyl acetate : pentane (2 : 3)] = 0.25. ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C): δ = 4.12 (s, 2H), 3.66 (s, 3H), 3.55 (s, 2H), 2.79 (s, 3H), 1.38 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d$_6$, 25 °C, mixture of rotamers): δ = 199.72, 199.54, 167.35, 167.28, 155.21, 154.78, 79.07, 78.90, 58.14, 57.51, 51.90, 51.87, 45.91, 45.79, 39.52, 35.17, 35.10, 27.95, 27.77. IR ν_{max}/cm^{-1}: 2979, 1692, 1459, 1391, 1362, 1241, 1169, 871 cm^{-1}. HRMS: (ESI+) Calculated mass [M + Na]$^+$ $C_{11}H_{19}NO_5Na$ = 268.1155, found: 268.1155.

***N*-Boc sarcosine β-ketoamide 13.** β-Ketoamide **13** was prepared according to the general procedure C by reacting 1,4-DABCO (660 mg, 5.83 mmol, 1.2 eq.) with trimethylaluminium (5.83 mL, 11.66 mmol, 2.4 eq., 2 M in toluene) in toluene (10 mL) to produce DABAL *in situ*, subsequently adding dodecylamine (1.07 g, 5.83 mmol, 1.2 eq.) in THF (4 mL) to activate the amine and finally adding the corresponding β-keto ester **12** (1.19 g, 4.85 mmol, 1 eq.) in THF (3 mL). Flash chromatography using ethyl acetate : pentane (1 : 3) as eluent afforded **13** (972.5 mg, 50% yield) as a yellow oil. R_f [silica, ethyl acetate : pentane (1 : 3)] = 0.21. ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C): δ = 7.79 (bs, 1H), 4.11 (s, 2H), 3.28 (s, 2H), 3.19–2.98 (m, 2H), 2.77 (s, 3H), 1.45–1.34 (m, 11H), 1.33–1.13 (m, 18H), 0.87 (t, J = 6.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d$_6$, 25 °C, mixture of rotamers): δ = 201.04, 200.69, 165.27, 165.02, 155.15, 154.85, 78.87, 78.62, 58.12, 57.56, 47.83, 47.79, 39.52, 38.67, 35.08, 35.00, 31.32, 29.09, 29.04, 28.96, 28.77, 28.75, 27.95, 27.78, 26.37, 22.11, 13.89. IR ν_{max}/cm^{-1}: 1928, 1851, 1709, 1458, 1371, 1245, 1169, 940 cm^{-1}. HRMS: (ESI+) Calculated mass [M + Na]$^+$ $C_{22}H_{42}N_2O_4Na$ = 445.3037, found: 445.3039.

Methyl functionalized monocyclic derivative 3c. Enol cyclocarbamate **3c** was prepared according to the general procedure D by reacting the β-keto amide **13** (209 mg, 0.52 mmol, 1 eq.), TMSOTf (193 µL, 1.02 mmol, 2 eq.) and CDI (145 mg, 0.75 mmol, 1.5 eq.) in DCM (3 mL). Flash chromatography using ethyl acetate : pentane (7 : 3) then ethyl acetate : pentane (7 : 3) gave ***Z*-3c** (81.34 mg, 48% yield) and ***E*-3c** (34.86 mg, 21% yield) as colorless oil. *Z*-Isomer: R_f [silica, ethyl acetate : pentane (7 : 3)] = 0.19. ^{1}H NMR (400 MHz, CDCl$_3$): δ = 6.63 (bs, 1H), 5.15 (s, 1H), 4.28 (s, 2H), 3.41–3.24 (m, 2H), 2.99 (s, 3H), 1.62–1.45 (m, 2H), 1.46–1.03 (m, 18H), 0.87 (t, J = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, CDCl$_3$): δ = 163.45, 153.72, 148.92, 99.73, 77.16, 50.54, 40.15, 39.84, 34.34, 32.04, 30.72, 29.78, 29.76, 29.72, 29.67, 29.65, 29.56, 29.48, 29.44, 29.39, 27.09, 27.00, 22.82, 14.25. IR ν_{max}/cm^{-1}: 2931, 2849, 1807, 1699, 1621, 1450, 1159, 1034 cm^{-1}. HRMS: (ESI+) Calculated mass [M + H]$^+$ $C_{18}H_{33}N_2O_3$ = 325.2485 found: 325.2482. *E*-Isomer: R_f [silica, ethyl acetate : pentane (7 : 3)] = 0.58. ^{1}H NMR (400 MHz, CDCl$_3$): δ = 5.55 (s, 1H), 4.66 (d, J = 2.5 Hz, 2H), 3.36–3.18 (m, 2H), 2.97 (s, 3H), 1.58–1.43 (m, 2H), 1.39–1.10 (m, 18H), 0.87 (t, J = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, CDCl$_3$): δ = 165.65, 158.07, 154.38, 96.77, 77.16, 52.04, 39.59, 32.05, 30.57, 29.82, 29.77, 29.76, 29.71, 29.67, 29.48, 29.42, 27.08, 22.82, 14.25. IR ν_{max}/cm^{-1}: 2934, 2851, 1805, 1689, 1629, 1459, 1151, 1046 cm^{-1}. HRMS: (ESI+) Calculated mass [M + H]$^+$ $C_{18}H_{33}N_2O_3$ = 325.2485 found: 325.2481; *Remark*: NOE experiment confirmed the *E* and *Z* isomers (with a typical chemical shift of the alkene proton for each isomer).

***N*-Boc-*trans*-4-benzyloxy-L-proline 15a.** The title compound **15a** was prepared according to general procedure A using *N*-Boc-*trans*-4-hydroxy-L-proline (500 mg, 2.16 mmol, 1 eq.), NaH (190 mg, 4.75 mmol, 2.2 eq., 60% in mineral oil) and benzyl bromide (283 µL, 3.6 mmol, 1.1 eq.) in THF (12 mL). Flash chromatography using ethyl acetate : pentane : AcOH (120 : 79 : 1) as eluent gave **15a** (619 mg, 90% yield) as a yellow oil. R_f [silica, ethyl acetate : pentane : AcOH (120 : 79 : 1)] = 0.15. ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C): δ = 7.59–7.09 (m, 5H), 4.65–4.40 (m, 2H), 4.33–4.09 (m, 2H), 3.64–3.36 (m, 2H), 2.42–2.28 (m, 1H), 2.18–1.98 (m, 1H), 1.38 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6, 25 °C, mixture of rotamers): δ = 174.55, 174.07, 154.11, 153.59, 138.73, 138.67, 128.71, 128.04, 127.93, 79.46, 76.90, 76.13, 70.45, 70.37, 58.11, 57.84, 52.23, 51.90, 36.19, 35.46, 28.52, 28.33. HRMS: (ESI+) Calculated mass [M + H]$^+$ $C_{17}H_{24}NO_5$ = 322.1649 found: 322.1651. Spectral data were in accordance with the literature.[42,43]

***N*-Boc-*trans*-4-propargyloxy-L-proline 15b.** The title compound **15b** was prepared according to general procedure A using *N*-Boc-*trans*-4-hydroxy-L-proline (755 mg, 3.27 mmol, 1 eq.), NaH (288 mg, 7.19 mmol, 2.2 eq., 60% in mineral oil) and propargyl bromide (535 µL, 3.6 mmol, 1.1 eq., 80 wt% in toluene) in THF (18 mL). Flash chromatography using ethyl acetate : pentane : AcOH (80 : 119 : 1) as eluent afforded **15b** (781 mg, 90% yield) as a slightly yellow oil. R_f [silica, ethyl acetate : pentane : AcOH (80 : 119 : 1)] = 0.19. ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C): δ = 12.26 (bs, 1H), 4.25 (app p, 1H), 4.17 (d, J = 2.4 Hz, 2H), 4.12 (t, J = 7.7 Hz, 1H), 3.51–3.40

(m, 2H), 3.28 (t, J = 2.4 Hz, 1H), 2.36–2.27 (m, 1H), 2.09–1.97 (m, 1H), 1.39 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6, 25 °C, mixture of rotamers): δ = 174.53, 174.07, 154.05, 153.54, 80.74, 79.51, 77.55, 76.66, 75.89, 58.00, 57.72, 56.19, 56.13, 52.03, 51.69, 35.91, 35.17, 28.49, 28.30. HRMS: (ESI+) Calculated mass $[M + Na]^+$ $C_{13}H_{19}NO_5Na$ = 292.1155, found: 292.1162. Spectral data were in accordance with the literature.[44]

***N*-Boc-*trans*-4-benzyloxy-L-proline β-ketoester 16a.** β-Ketoester **16a** was prepared according to the general procedure B using (2*S*,4*R*)-4-(benzyloxy)-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid **15a** (544 mg, 1.69 mmol, 1 eq.) DCC (380 mg, 1.86 mmol, 1.1 eq.), DMAP (367 mg, 2.55 mmol, 1.5 eq.), Meldrum's acid (269 mg, 1.86 mmol, 1.1 eq.) in THF (6.5 mL) to obtain the Meldrum's acid intermediate, which was converted into **16a** by subsequent refluxing in MeOH. Flash chromatography using ethyl acetate : pentane (3 : 17) as eluent furnished **16a** (345 mg, 54% yield) as a brownish oil. R_f [silica, ethyl acetate : pentane (3 : 17)] = 0.17. ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C): δ = 7.40–7.24 (m, 5H), 4.53 (d, J = 12.2 Hz, 2H), 4.48 (d, J = 12.0 Hz, 2H), 4.40 (t, J = 8.2 Hz, 1H), 4.18–4.12 (m, 1H), 3.65 (s, 3H), 3.61–3.54 (m, 1H), 3.49–3.41 (m, 1H), 2.38–2.28 (m, 1H), 2.06–1.99 (m, 1H), 1.38 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6, 25 °C, mixture of rotamers): δ = 203.59, 168.39, 153.94, 139.23, 129.31, 129.23, 128.50, 128.34, 80.62, 80.49, 77.55, 76.66, 70.84, 70.76, 64.89, 64.60, 52.95, 52.87, 52.74, 46.06, 46.05, 35.45, 28.81, 28.80. IR ν_{max}/cm^{-1}: 2924, 2854, 1743, 1611, 1455, 1377, 1240, 1030, 861, 755 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + Na]^+$ $C_{20}H_{28}NO_6Na$ = 378.1911, found: 378.1911.

***N*-Boc-*trans*-4-propargyloxy-L-proline β-ketoester 16b.** *N*-Boc-*trans*-4-propargyloxy-L-proline β-ketoester **16b** was prepared according to the general procedure B using (2*S*,4*R*)-1-(*tert*-butoxycarbonyl)-4-(prop-2-yn-1-yloxy)pyrrolidine-2-carboxylic acid **16b** (781 mg, 2.9 mmol, 1 eq.) DCC (642 mg, 3.19 mmol, 1.1 eq.), DMAP (532 mg, 4.36 mmol, 1.5 eq.) and Meldrum's acid (460 mg, 3.19 mmol, 1.1 eq.) in THF (16 mL) to obtain the Meldrum's acid intermediate and subsequent refluxing of this intermediate in MeOH to obtain **16b**. Flash chromatography using ethyl acetate : pentane (1 : 4) as eluent gave **16b** (669 mg, 71% yield) as a yellow oil. R_f [silica, ethyl acetate : pentane (1 : 4)] = 0.22. ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C) δ = 4.35 (t, J = 8.2 Hz, 1H), 4.23–4.19 (m, 1H), 4.17 (d, J = 2.4 Hz, 2H), 3.65 (s, 3H), 3.61–3.49 (m, 2H), 3.49–3.42 (m, 2H), 3.29 (t, J = 2.1 Hz, 1H), 2.36–2.22 (m, 1H), 2.13–1.86 (m, 1H), 1.38 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6, 25 °C, mixture of rotamers): δ = 203.06, 167.82, 167.75, 154.47, 153.36, 80.72, 80.68, 80.13, 80.06, 77.67, 77.64, 76.78, 75.89, 64.29, 64.10, 63.90, 56.04, 52.50, 52.45, 52.34, 52.19, 52.15, 49.03, 45.61, 45.38, 34.73, 34.68, 33.90, 33.80, 28.47, 28.43, 28.28, 28.21, 28.18, 25.86, 25.82. IR ν_{max}/cm^{-1}: 2978, 1695, 1396, 1367, 1162, 1255, 1086, 772 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + Na]^+$ $C_{16}H_{23}NO_6Na$ = 348.1418, found: 348.1419.

***N*-Boc-*trans*-4-benzyloxy-L-proline β-ketoamide 17a.** β-Ketoamide **17a** was prepared according to the general procedure C by reacting DABCO (116 mg, 1.04 mmol, 1.2 eq.) with trimethylaluminium (1.05 mL, 2.08 mmol, 2.4 eq., 2 M in toluene) in toluene (3 mL) to produce DABAL *in situ*, subsequently adding dodecylamine (192 mg, 1.04 mmol, 1.2 eq.) in THF (3 mL) to activate amine and finally adding the corresponding β-keto ester **16a** (328 mg, 0.87 mmol, 1 eq.) in THF (3 mL). Flash chromatography using ethyl acetate : pentane (1 : 1) as eluent yielded **17a** (304 mg, 60% yield) as a yellow oil. R_f [silica, ethyl acetate : pentane (1 : 1)] = 0.15. ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C): δ = 7.77 (s, 1H), 7.41–7.18 (m, 5H), 4.54–4.46 (m, 2H), 4.46–4.38 (m, 1H), 4.17–4.10 (m, 1H), 3.58–3.41 (m, 2H), 3.39–3.27 (m, 2H), 2.34–2.17 (m, 1H), 2.11–1.99 (m, 1H), 1.37 (s, 9H), 1.31–1.22 (m, 18H), 0.92–0.82 (m, 3H). ^{13}C NMR (101 MHz, DMSO-d_6, 25 °C, mixture of rotamers): δ = 204.49, 204.03, 165.91, 165.52, 153.55, 138.63, 128.69, 127.97, 79.80, 77.12, 76.19, 70.28, 64.11, 63.90, 52.26, 47.43, 35.11, 34.28, 31.73, 29.48, 29.42, 29.14, 28.46, 28.25, 26.78, 22.53, 14.38. IR ν_{max}/cm^{-1}: 3245, 2927, 1696, 1401, 1161, 1094, 739 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + Na]^+$ $C_{31}H_{50}N_2O_5Na$ = 553.3612, found: 553.3611.

***N*-Boc-*trans*-4-propargyloxy-L-proline β-ketoamide 17b.** β-Ketoamide **17b** was prepared according to the general procedure C by reacting DABCO (489 mg, 4.36 mmol, 1.3 eq.) with trimethylaluminium (4.36 mL, 8.72 mmol, 2.6 eq., 2 M in toluene) in toluene (8 mL) to produce DABAL *in situ*, subsequently adding dodecylamine (808 mg, 4.36 mmol, 1.3 eq.) in THF (4 mL) to activate the amine and finally adding the corresponding β-ketoester **13b** (1.09 g, 3.09 mmol, 1 eq.) in THF (4 mL). Flash chromatography using ethyl acetate : pentane (3 : 17) as eluent gave **17b** (391 mg, 63% yield) as a yellow oil. R_f [silica, ethyl acetate : pentane (3 : 17)] = 0.28. ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C): δ = 7.77 (s, 1H), 4.48–4.34 (m, 1H), 4.24–4.17 (m, 1H), 4.16 (s, 2H), 3.57–3.50 (m, 1H), 3.48–3.39 (m, 1H), 3.35 (d, J = 8.6 Hz, 2H), 3.31–3.26 (m, 1H), 3.13–3.03 (m, 2H), 2.32–2.21 (m, 1H), 2.15–1.94 (m, 1H), 1.42–1.35 (m, 11H), 1.31–1.21 (m, 18H), 0.87 (t, J = 6.5 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6, 25 °C, mixture of rotamers): δ = 204.42, 203.93, 165.85, 165.47, 154.35, 153.41, 80.71, 80.67, 79.80, 77.65, 77.61, 76.84, 75.88, 64.00, 63.80, 56.07, 52.48, 52.12, 47.56, 47.31, 34.79, 33.96, 31.75, 29.51, 29.45, 29.20, 29.17, 28.47, 28.25, 26.80, 22.55, 14.39. IR ν_{max}/cm^{-1}: 3311, 2923, 2853, 1649, 1547, 1394, 1162, 1130, 975 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + Na]^+$ $C_{27}H_{46}N_2O_5Na$ = 501.3299, found: 501.3299.

***trans*-4-Benzyloxy-L-proline derivative 4a.** Enol cyclocarbamate **4a** was prepared according to the general procedure D by reacting the appropriate β-keto amide **17a** (112.5 mg, 0.19 mmol, 1 eq.), TMSOTf (71 μL, 0.39 mmol, 2 eq.) and CDI (54 mg, 0.29 mmol, 1.5 eq.) in DCM (1 mL). Flash chromatography using ethyl acetate : pentane (3 : 7) as eluent afforded **4a** (39 mg, 44% yield) as a colorless oil. R_f [silica, ethyl acetate : pentane (1 : 1)] = 0.55. $[\alpha]_D^{20}$ = −55 (c = 0.040, CHCl$_3$); ^{1}H NMR (400 MHz, CDCl$_3$): δ = 7.40–7.27 (m, 5H), 6.56 (bs, 1H), 5.16 (s, 1H), 4.75 (dd, J = 10.7, 6.0 Hz, 1H), 4.52 (s, 2H), 4.38 (t, J = 4.9 Hz, 1H), 3.86 (dd, J = 12.5, 4.9 Hz, 1H), 3.40 (d, J = 12.5 Hz, 1H), 3.35–3.25 (m, 2H), 2.39 (dd, J = 13.3, 6.0 Hz, 1H), 1.72 (dd, J = 13.3, 4.7 Hz, 1H), 1.58–1.47 (m, 2H), 1.27–1.22 (m, 18H), 0.86 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, CDCl$_3$): δ = 163.18, 155.87, 153.49, 136.98, 128.63, 128.16, 127.68, 100.13, 79.62, 71.57, 61.84, 52.68, 39.73, 38.08, 31.90, 29.63, 29.61, 29.58, 29.51, 29.33, 29.28, 26.94, 22.67, 14.10. IR $\nu_{\max}$/cm^{-1}: 3245, 2922, 2853, 1790, 1694, 1632, 1535, 1455, 1374, 1215, 1094, 1026, 979 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{27}H_{41}N_2O_4$ = 457.3061 found: 457.3063.

***trans*-4-Propargyloxy-L-proline derivative 4b.** Enol cyclocarbamate **4b** was prepared according to the general procedure D by reacting the appropriate β-keto amide **17b** (130 mg, 0.27 mmol, 1 eq.), TMSOTf (98.8 µL, 0.54 mmol, 2 eq.) and CDI (75.4 mg, 0.4 mmol, 1.5 eq.) in DCM (1.5 mL). Flash chromatography using ethyl acetate : pentane (from 1 : 4 to 3 : 2) as eluent gave ***E*-4b** (9.8 mg, 8.8% yield) and ***Z*-4b** (27.8 mg, 25.2% yield) as a colorless oil. The chemical shifts of the protons that are characteristic for the *E* and *Z* isomer are in correspondence with those that have been reported for the isomers of lipocyclocarbamates intermediates.[15] *Z*-Isomer: R_f [silica, ethyl acetate : pentane (3 : 2)] = 0.20. $[\alpha]_D^{20}$ = −39.3 (c = 0.214, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.56 (bs, 1H), 5.19 (s, 1H), 4.77–4.67 (m, 1H), 4.52 (t, J = 5.4 Hz, 1H), 4.19 (d, J = 2.4 Hz, 2H), 3.90 (dd, J = 12.7, 5.5 Hz, 1H), 3.39 (d, J = 12.7 Hz, 1H), 3.36–3.26 (m, 2H), 2.49 (t, J = 2.4 Hz, 1H), 2.42 (dd, J = 13.3, 6.0 Hz, 1H), 1.75 (ddd, J = 13.3, 10.6, 5.3 Hz, 1H), 1.58–1.48 (m, 2H), 1.32–1.23 (m, 18H), 0.87 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 163.30, 155.98, 153.47, 114.05, 100.34, 79.55, 75.54, 61.87, 56.95, 52.75, 39.86, 38.01, 32.03, 29.76, 29.74, 29.71, 29.65, 29.46, 29.42, 27.07, 22.80, 14.24. IR $\nu_{\max}$/cm^{-1}: 3310, 2924, 2854, 1800, 1693, 1626, 1551, 1365, 1167, 1091, 981 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{23}H_{37}N_2O_4$ = 405.2748 found: 405.2748; Calculated mass $[M + Na]^+$ $C_{23}H_{36}N_2O_4Na$ = 427.2567, found: 427.2563. *E*-Isomer: R_f [silica, ethyl acetate : pentane (3 : 2)] = 0.51. $[\alpha]_D^{20}$ = −9.7 (c = 0.064, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.55 (s, 1H), 5.44 (bs, 1H), 5.20 (dd, J = 10.6, 6.0 Hz, 1H), 4.50 (t, J = 5.9 Hz, 1H), 4.25 (dt, J = 15.8, 1.6 Hz, 1H), 4.14 (dt, J = 16.1, 1.8 Hz, 1H), 3.93 (dd, J = 12.7, 5.9 Hz, 1H), 3.46–3.14 (m, 3H), 2.88 (dd, J = 13.7, 6.0 Hz, 1H), 2.44 (app q, J = 2.2 Hz, 1H), 1.61 (ddd, J = 13.7, 10.2, 5.5 Hz, 1H), 1.60–1.46 (m, 2H), 1.44–1.20 (m, 18H), 0.88 (t, J = 6.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 164.63, 161.65, 156.83, 97.95, 78.74, 77.31, 74.94, 62.71, 56.42, 52.80, 39.54, 36.49, 31.89, 29.62, 29.55, 29.52, 29.32, 29.26, 26.93, 22.66, 14.10. IR $\nu_{\max}$/cm^{-1}: 3288, 2922, 2853, 1792, 1696, 1633, 1545, 1465, 1375, 1216, 1092, 1032, 980 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{23}H_{37}N_2O_4$ = 405.2748 found: 405.2742.

Benzyl triazole derivative 4c. Triazole compound **4c** was prepared according to the general procedure E using the enol cyclocarbamate **4b** (5 mg, 0.012 mmol, 1 eq.) and benzylazide (1.53 µL, 0.012 mmol, 1 eq.). Flash chromatography using methanol : DCM (1 : 24) as eluent yielded **4c** (8 mg, 50% yield) as a colorless oil. R_f [silica, methanol : DCM (1 : 24)] = 0.18. $[\alpha]_D^{20}$ = −3.8 (c = 0.313, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.45 (bs, 1H), 7.41–7.35 (m, 3H), 7.34–7.26 (m, 2H), 6.61–6.48 (m, 1H), 5.53 (s, 2H), 5.13 (s, 1H), 4.69 (dd, J = 10.7, 5.7 Hz, 1H), 4.60 (s, 2H), 4.45 (t, J = 5.4 Hz, 1H), 3.86 (dd, J = 12.5, 5.3 Hz, 1H), 3.46–3.21 (m, 3H), 2.37 (dd, J = 13.3, 5.7 Hz, 1H), 1.84–1.64 (m, 1H), 1.60–1.44 (m, 2H), 1.37–1.12 (m, 18H), 0.87 (t, J = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 163.41, 155.94, 153.72, 134.33, 129.39, 129.16, 128.38, 100.17, 80.11, 62.86, 61.98, 54.62, 52.86, 39.97, 38.12, 32.06, 29.79, 29.74, 29.68, 29.49, 29.44, 27.10, 22.84. IR $\nu_{\max}$/cm^{-1}: 3241, 2924, 2853, 1793, 1696, 1635, 1535, 1466, 1390, 1218, 1090, 1049, 984 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{30}H_{44}N_5O_{34}$ = 538.3388 found: 538.3384.

PEG-triazole derivative 4d. Triazole compound **4d** was prepared according to the general procedure E using the enol cyclocarbamate **4b** (11 mg, 0.027 mmol, 1 eq.) and polyethylene glycol azide (6.1 mg, 0.035 mmol, 1.3 eq.). Flash chromatography using methanol : DCM (1 : 199) as eluent yielded **4d** (8.3 mg, 52% yield) as a yellow oil. R_f [silica, methanol : DCM (1 : 199)] = 0.11. $[\alpha]_D^{20}$ = −10 (c = 0.180, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.80 (s, 1H), 6.63–6.48 (m, 1H), 5.16 (s, 1H), 4.73 (dd, J = 10.6, 5.9 Hz, 1H), 4.66 (d, J = 12.4 Hz, 1H), 4.62 (d, J = 12.4 Hz, 1H), 4.56 (t, J = 5.0 Hz, 2H), 4.48 (t, J = 5.4 Hz, 1H), 3.94–3.81 (m, 3H), 3.72 (t, J = 4.4 Hz, 2H), 3.62 (s, 4H), 3.57 (t, J = 4.5 Hz, 2H), 3.36 (d, J = 12.6 Hz, 1H), 3.29 (q, J = 6.7 Hz, 2H), 2.41 (dd, J = 13.2, 6.0 Hz, 1H), 1.72 (td, J = 13.2, 10.6, 5.1 Hz, 1H), 1.58–1.46 (m, 2H), 1.33–1.21 (m, 18H), 0.87 (t, J = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 163.24, 155.86, 153.54, 124.22, 100.03, 79.89, 72.39, 70.49, 70.19, 69.24, 62.67, 61.81, 61.69, 52.73, 50.38, 39.75, 37.95, 31.89, 29.63, 29.60, 29.57, 29.51, 29.32, 29.28, 26.94, 22.66, 14.10. IR $\nu_{\max}$/cm^{-1}: 3299, 2922, 2853, 1789, 1694, 1631, 1537, 1465, 1375, 1218, 1032 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{29}H_{50}N_5O_7$ = 580.3705 found: 580.3670.

1-(3-(Dimethylamino)propyl)-triazole derivative 4e. Triazole compound **4e** was prepared according to the general procedure E using the enol cyclocarbamate **4b** (8 mg, 0.020 mmol, 1 eq.) and 3-azido-*N*,*N*-dimethylpropan-1-amine (3 mg, 0.022 mmol, 1.4 eq.). Flash chromatography using methanol : DCM (1 : 9) as eluent yielded **4e** (3 mg, 29% yield) as a yellow oil. R_f [silica, methanol : DCM (1 : 9)] = 0.15. $[\alpha]_D^{20}$ = −27.4 (c = 0.073, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.65 (s, 1H), 6.54 (bs, 1H), 5.16 (s, 1H), 4.73 (dd, J = 10.8, 6.1 Hz, 1H), 4.66 (d, J = 12.6 Hz, 1H), 4.62 (d, J = 12.8 Hz, 1H), 4.56–4.36 (m, 2H), 3.88 (dd, J = 12.6, 5.5 Hz, 1H), 3.37 (d, J = 12.6 Hz, 1H), 3.33–3.26 (m, 2H), 2.51–2.31 (m, 9H), 2.25–2.14 (m, 2H), 1.80–1.67 (m, 1H), 1.61–1.47 (m, 2H), 1.32–1.17 (m, 18H), 0.88 (t, J = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 163.09, 155.89, 153.28, 144.14, 123.66, 100.28, 79.98, 62.71, 61.76, 55.33, 52.70, 47.36, 43.97, 39.70, 37.99, 31.89, 29.61, 29.52, 29.33, 29.29, 26.95, 22.67, 14.10. IR $\nu_{\max}$/cm^{-1}: 3287, 2922, 2853, 1788, 1694, 1632, 1544, 1465, 1391, 1219, 1091, 1034, 837 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{28}H_{49}N_6O_4$ = 533.3810 found: 533.3809.

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Notes and references

1 J. Clardy, M. A. Fischbach and C. T. Walsh, *Nat. Biotechnol.*, 2006, **24**, 1541–1550.

2 M. P. Baggelaar, F. J. Janssen, A. C. M. van Esbroeck, H. den Dulk, M. Allarà, S. Hoogendoorn, R. McGuire, B. I. Florea, N. Meeuwenoord, H. van den Elst, G. A. van der Marel, J. Brouwer, V. Di Marzo, H. S. Overkleeft and M. van der Stelt, *Angew. Chem., Int. Ed.*, 2013, **52**, 12081–12085.

3 G. Zhao, T. I. Meier, S. D. Kahl, K. R. Gee and L. C. Blaszczak, *Antimicrob. Agents Chemother.*, 1999, **43**, 1124–1128.

4 E. Zeiler, N. Braun, T. Böttcher, A. Kastenmüller, S. Weinkauf and S. A. Sieber, *Angew. Chem., Int. Ed.*, 2011, **50**, 11001–11004.

5 M. D. Witte, M. T. C. Walvoort, K.-Y. Li, W. W. Kallemeijn, W. E. Donker-Koopman, R. G. Boot, J. M. F. G. Aerts, J. D. C. Codée, G. A. van der Marel and H. S. Overkleeft, *ChemBioChem*, 2011, **12**, 1263–1269.

6 M. D. Lebar, J. M. May, A. J. Meeske, S. A. Leiman, T. J. Lupoli, H. Tsukamoto, R. Losick, D. Z. Rudner, S. Walker and D. Kahne, *J. Am. Chem. Soc.*, 2014, **136**, 10874–10877.

7 J. Thirkettle, E. Alvarez, H. Boyd, M. Brown, E. Diez, J. Hueso, S. Elson, M. Fulston, C. Gershater, M. L. Morata, P. Perez, S. Ready, J. M. Sanchez-Puelles, R. Sheridan, A. Stefanska and S. Warr, *J. Antibiot.*, 2000, **53**, 664–669.

8 C. W. Johnston, R. Zvanych, N. Khyzha and N. A. Magarvey, *ChemBioChem*, 2013, **14**, 431–435.

9 J. Thirkettle, *J. Antibiot.*, 2000, **53**, 733–735.

10 I. L. Pinto, H. F. Boyd and D. M. Hickey, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 2015–2017.

11 P. F. Andersson, J. Levenfors and A. Broberg, *BioControl*, 2012, **57**, 463–469.

12 K. Reder-Christ, Y. Schmidt, M. Dörr, H.-G. Sahl, M. Josten, J. M. Raaijmakers, H. Gross and G. Bendas, *Biochim. Biophys. Acta, Biomembr.*, 2012, **1818**, 566–573.

13 J. M. Raaijmakers, I. De Bruijn, O. Nybroe and M. Ongena, *FEMS Microbiol. Rev.*, 2010, **34**, 1037–1062.

14 Y. Schmidt, PhD Thesis, University of Bonn, 2013.

15 J. R. Duvall, F. Wu and B. B. Snider, *J. Org. Chem.*, 2006, **71**, 8579–8590.

16 Y. Oikawa, K. Sugano and O. Yonemitsu, *J. Org. Chem.*, 1978, **43**, 2087–2088.

17 T. Ohshima, Y. Hayashi, K. Agura, Y. Fujii, A. Yoshiyama and K. Mashima, *Chem. Commun.*, 2012, **48**, 5434–5433.

18 C. Han, J. P. Lee, E. Lobkovsky and J. A. Porco, *J. Am. Chem. Soc.*, 2005, **127**, 10039–10044.

19 J. I. Levin, E. Turos and S. M. Weinreb, *Synth. Commun.*, 1982, **12**, 989–993.

20 A. Novak, L. D. Humphreys, M. D. Walker and S. Woodward, *Tetrahedron Lett.*, 2006, **47**, 5767–5769.

21 D. Glynn, D. Bernier and S. Woodward, *Tetrahedron Lett.*, 2008, **49**, 5687–5688.

22 M. Sadiki and S. K. Ibnsouda, *J. Pharm. Anal.*, 2016, **6**, 71–79.

23 Y. Schmidt, M. van der Voort, M. Crüsemann, J. Piel, M. Josten, H.-G. Sahl, H. Miess, J. M. Raaijmakers and H. Gross, *ChemBioChem*, 2014, **15**, 259–266.

24 L. P. Candeias, D. P. S. MacFarlane, S. L. W. McWhinnie, N. L. Maidwell, C. A. Roeschlaub, P. G. Sammes and R. Whittlesey, *J. Chem. Soc., Perkin Trans. 2*, 1998, 2333–2334.

25 R. A. Sorg and J. W. Veening, *Nat. Commun.*, 2015, **6**, 8773.

26 E. Kuru, H. V. Hughes, P. J. Brown, E. Hall, S. Tekkam, F. Cava, M. A. de Pedro, Y. V. Brun and M. S. VanNieuwenhze, *Angew. Chem., Int. Ed.*, 2012, **51**, 12519–12523.

27 E. Kuru, S. Tekkam, E. Hall, Y. V. Brun and M. S. Van Nieuwenhze, *Nat. Protoc.*, 2015, **10**, 33–52.

28 W. K. Jung, H. C. Koo, K. W. Kim, S. Shin, S. H. Kim and Y. H. Park, *Appl. Environ. Microbiol.*, 2008, **74**, 2171–2178.

29 F. Cava, M. A. de Pedro, H. Lam, B. M. Davis and M. K. Waldor, *EMBO J.*, 2011, **30**, 3442–3453.

30 S. E. Pidgeon, J. M. Fura, W. Leon, M. Birabaharan, D. Vezenov and M. M. Pires, *Angew. Chem., Int. Ed.*, 2015, **54**, 6158–6162.

31 M. S. Siegrist, B. M. Swarts, D. M. Fox, S. A. Lim and C. R. Bertozzi, *FEMS Microbiol. Rev.*, 2015, **39**, 184–202.

32 M. J. Boersma, E. Kuru, J. T. Rittichier, M. S. VanNieuwenhze, Y. V. Brun and M. E. Winkler, *J. Bacteriol.*, 2015, **197**, 3472–3485.

33 D.-J. Scheffers, L. J. F. Jones and J. Errington, *Mol. Microbiol.*, 2003, **51**, 749–764.

34 D. J. Scheffers, *Microbiology*, 2005, **151**, 999–1012.

35 A. L. Lovering, L. De Castro and D. Lim, *Protein Sci.*, 2006, **15**, 1701–1709.

36 D. L. Popham and P. Setlow, *J. Bacteriol.*, 1994, **176**, 7197–7205.

37 J. Paik, I. Kern, R. Lurz and R. Hakenbeck, *J. Bacteriol.*, 1999, **181**, 3852–3856.

38 J. Hoskins, P. Matsushima, D. L. Mullen, J. Tang, G. Zhao, T. I. Meier, T. I. Nicas and S. R. Jaskunas, *J. Bacteriol.*, 1999, **181**, 6552–6555.

39 J. M. Fura, D. Kearns and M. M. Pires, *J. Biol. Chem.*, 2015, **290**, 30540–30550.

40 A. Minnen, L. Attaiech, M. Thon, S. Gruber and J. W. Veening, *Mol. Microbiol.*, 2011, **81**, 676–688.

41 M. E. Sharpe, P. M. Hauser, R. G. Sharpe and J. Errington, *J. Bacteriol.*, 1998, **180**, 547–555.

42 S.-P. Zhang, X.-K. Fu and S.-D. Fu, *Tetrahedron Lett.*, 2009, **50**, 1173–1176.

43 V. Mihali, F. Foschi, M. Penso and G. Pozzi, *Eur. J. Org. Chem.*, 2014, 5351–5355.

44 M. P. Winters, C. A. Teleha and Z. Sui, *Tetrahedron Lett.*, 2014, **55**, 2150–2153.