Article

Antibiotic discovery with synthetic fermentation: library assembly, phenotypic screening, and mechanism of action of beta-peptides targeting penicillin-binding proteins

Iain A. Stepek, Trung Cao, Anika Koetemann, Satomi Shimura, Bernd Wollscheid, and Jeffrey W. Bode

ACS Chem. Biol., **Just Accepted Manuscript** • DOI: 10.1021/acschembio.9b00227 • Publication Date (Web): 16 Apr 2019

Just Accepted

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Antibiotic discovery with synthetic fermentation: library assembly, phenotypic screening, and mechanism of action of beta-peptides targeting penicillin-binding proteins

Iain A. Stepek[1], Trung Cao[1], Anika Koetemann[2], Satomi Shimura[1], Bernd Wollscheid[2*] and Jeffrey W. Bode[1, 3*]

[1]Laboratorium für Organische Chemie, Department of Chemistry and Applied Biosciences, ETH-Zürich, 8093 Zürich, Switzerland

[2] Department of Health Sciences and Technology & Institute of Molecular Systems Biology & BioMedical Proteomics Platform (BMPP), ETH Zurich, Zurich, Switzerland
*e-mail: wbernd@ethz.ch

[3] Institute of Transformative Bio-Molecules (WPI-ITbM), Nagoya University, Chikusa, Nagoya 464-8602, Japan
*e-mail: bode@org.chem.ethz.ch

Abstract

In analogy to biosynthetic pathways leading to bioactive natural products, synthetic fermentation generates mixtures of molecules from simple building blocks under aqueous, biocompatible conditions, allowing for the resulting cultures to be directly screened for biological activity. In this work, a novel β-peptide antibiotic was successfully identified using the synthetic fermentation platform. Phenotypic screening was carried out in an initially random fashion, allowing for simple identification of active cultures. Subsequent deconvolution, focused screening and structure-activity relationship studies led to the identification of a potent antimicrobial peptide, showing strong selectivity for our model system *Bacillus subtilis* over human HEK293 cells. To determine the antibacterial mechanism of action, a peptide probe bearing a photoaffinity tag was readily synthesized through the use of appropriate synthetic fermentation building blocks and utilized for target identification using a quantitative mass spectrometry-based strategy. The chemoproteomic approach led to the identification of a number of bacterial membrane proteins as prospective targets. These findings were validated through binding affinity studies with penicillin-binding

protein 4 using microscale thermophoresis, with the bioactive peptide showing a dissociation constant (K_d) in the nanomolar range. Through these efforts, we provide a proof of concept for the synthetic fermentation approach presented here as a new strategy for the phenotypic discovery of novel bioactive compounds.

Introduction

Synthetic fermentation allows for the rapid, operationally simple preparation of libraries of bioactive molecules by covalent bond forming reactions that occur simply upon mixing the constituent building blocks in aqueous buffer. This process is based on chemoselective couplings that do not require reagents or produce toxic byproducts, allowing the resulting mixtures to be screened directly in a biological assay.[1, 2] The active components can be identified by straightforward deconvolution strategies and – in analogy to natural products isolation [3] – the most potent compounds purified by chromatographic methods and characterized. In contrast to most other library-based approaches, synthetic fermentation hits can be rapidly resynthesized from the constituent building blocks without the need for traditional multi-step organic synthesis. By adjustment of building block selection, hit optimization and structure activity relationship studies can be initiated even before the active component is conclusively identified. [2] The potential of synthetic fermentation using the α-ketoacid–hydroxylamine (KAHA) amide formation as a platform for the identification of bioactive molecules was first demonstrated with the discovery of a β-peptide HCV NS3/4A protease inhibitor with an IC_{50} of 1.0 μM (Figure 1). [1]

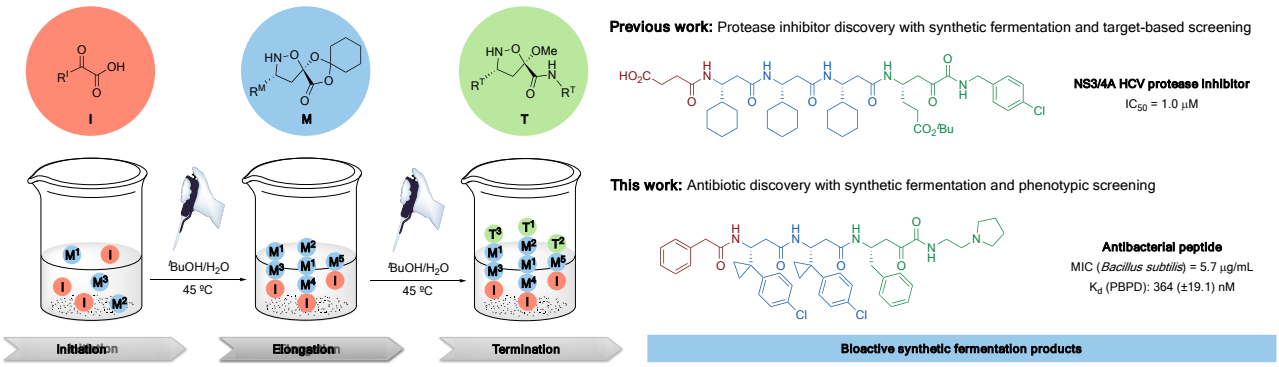


Figure 1: Identification of bioactive compounds through screening with the synthetic fermentation platform. [1]

Although synthetic fermentation produces mixtures of compounds that must eventually be deconvoluted, it has the advantage of being ideally suited for phenotypic screening. The

leading display technologies, including DNA-encoded libraries [4, 5] and mRNA-display [6, 7], can produce far larger numbers of molecules but the minuscule amounts employed and large tags present preclude screening on living systems. As alternatives medium-throughput approaches including diversity-oriented synthesis [8, 9], multicomponent reactions forming sequence-controlled peptides [10], sequence-defined cation oligomers [11, 12], synthetic macrocycles [13, 14] , and even entirely random peptide cocktails [15] have been employed for the discovery of new pharmaceutical lead structures to great success. With synthetic fermentation, the quantities of compounds produced, the approximate numbers of distinct molecules, and their general sequences can be controlled by the initial setup. Upon completion of the assembly, the aqueous mixtures are diluted and used directly for screening in cell-based or other phenotypic assays. With fifty initiators, monomers and terminators, a prospective fermentation library of up to 300 million chemically distinct products is accessible. [1]

As a first demonstration of synthetic fermentation for the phenotypic screening of readily prepared compound libraries, we sought to identify new antibiotics simply by combining a small set of monomeric building blocks with basic liquid handling techniques. In this report, we document the successful identification of a peptide targeting penicillin-binding proteins using synthetic fermentation for hit identification and optimization, target identification, and initial binding studies. The entire process can be conducted in a safe and reproducible manner without any technical knowledge or practical experience in organic chemistry. Using just 50 building blocks, we successfully identified a β-peptide α-ketoamide that exhibits excellent antibacterial activity against an indicator strain, prepared a derivative for a chemical proteomics approach to identify the molecular target, and confirmed nanomolar binding to a penicillin binding protein. This work therefore not only establishes a practical approach to preparing and screening thousands of molecules on a live organism, but also uncovers a new antibiotic and its protein target.

RESULTS AND DISCUSSION

Library Design: At the outset of our studies, we sought to employ our robust conditions for the formation of β-peptide oligomers with KAHA ligation [16] toward the preparation of short sequences that inhibit the growth of a microbial target. Based on our success in identifying protease inhibitors in our first report [1] and previous reports from Gellman and others showcasing β-peptides as antimicrobal agents, [17,18,19] we elected to maintain the basic approach of combining α-ketoacid initiators, isoxazolidine monomers, and terminators. The

greatest diversity and increase in compound numbers arises from the elongation monomers, and for this project we sought to complement our established library with new monomer building blocks containing a variety of side chain structures.

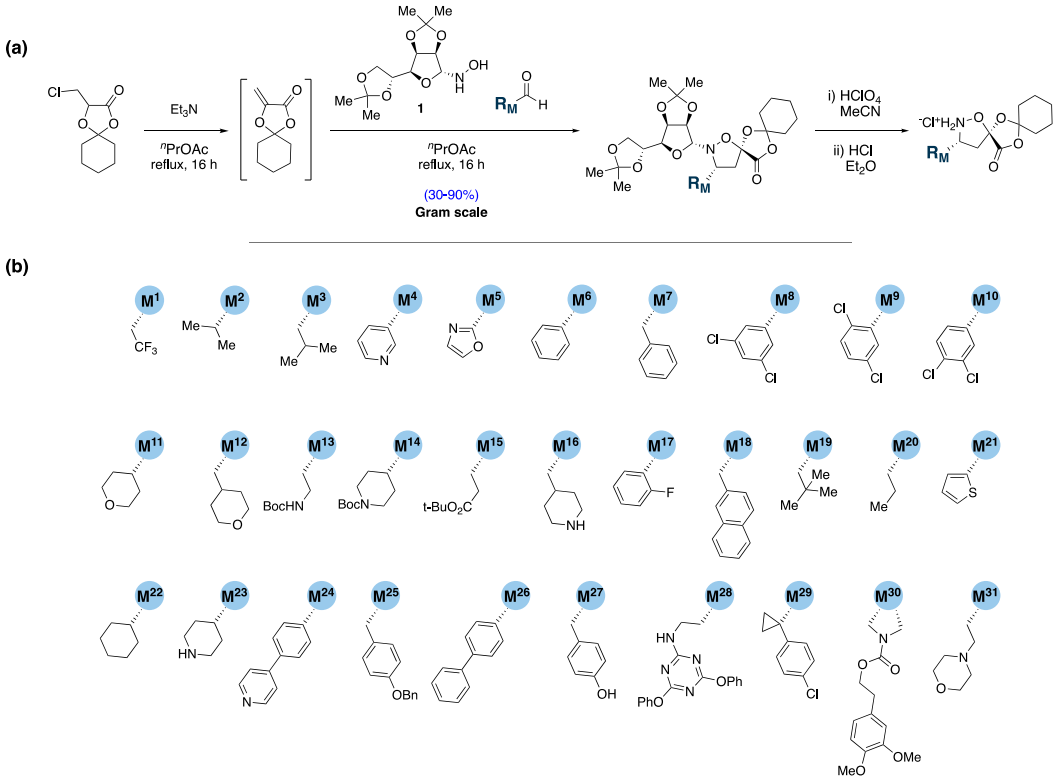


Figure 2. Elongation monomer building blocks for synthetic fermentation. **(a)** Synthetic route to isoxazolidine elongation monomers. **(b)** Monomer side-chains synthesized for phenotypic screening against *Bacillus subtilis.*

Using our previously developed unified route to isoxazolidine elongation monomers (Figure 2a), [1, 20, 21] we prepared the compounds shown in Figure 2b by nitrone cycloadditions of aldehydes with gulose-derived hydroxylamine **1**. Following crystallization to afford diastereomerically pure material and auxiliary removal with $HClO_4$, the isoxazolidine monomers were converted into hydrochloride salts for long-term storage and ease of use. The resulting salts are bench-stable solids, and are typically stored at -20 °C; the vast majority of these compounds remain stable for years under these conditions, without any significant precautions required. This two step protocol is suitable for both aliphatic and aromatic aldehydes; the enantiomeric monomers can be produced simply by using the readily available (L)-gulose derived auxiliary. [22]

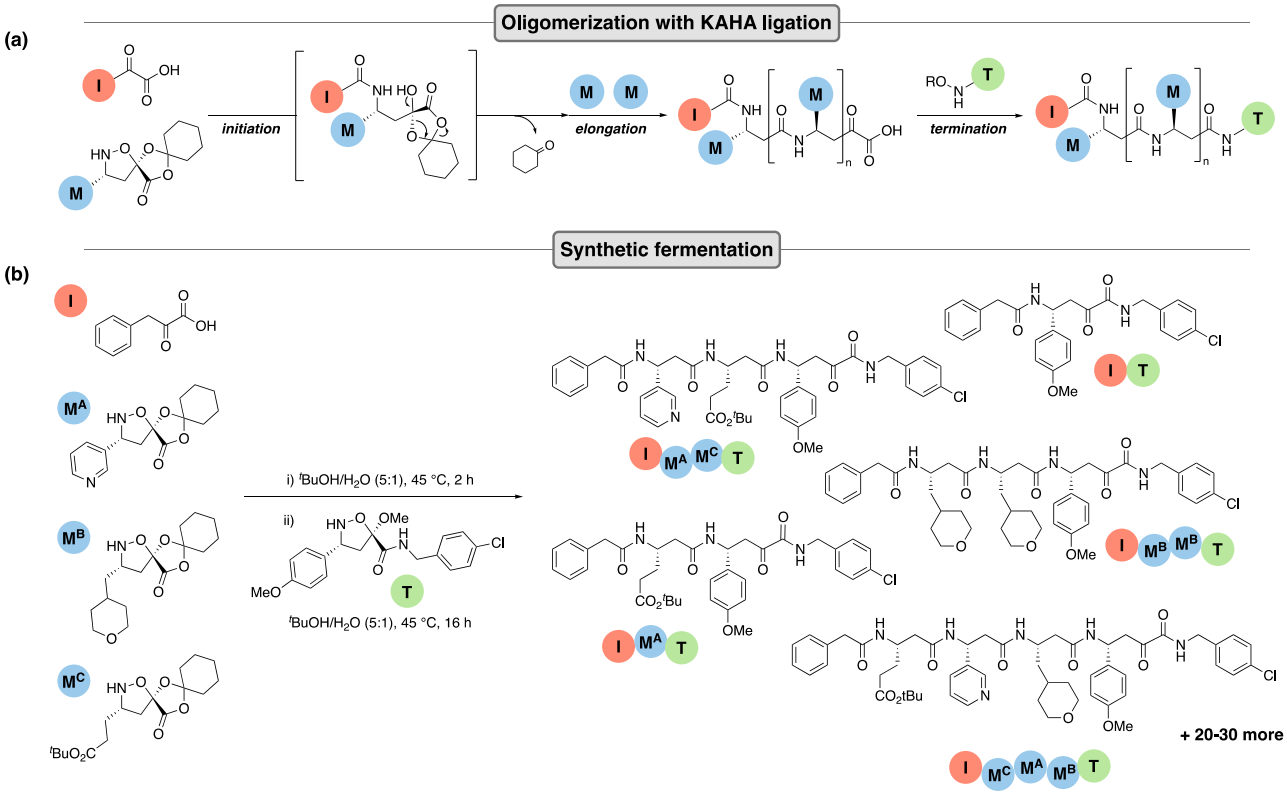


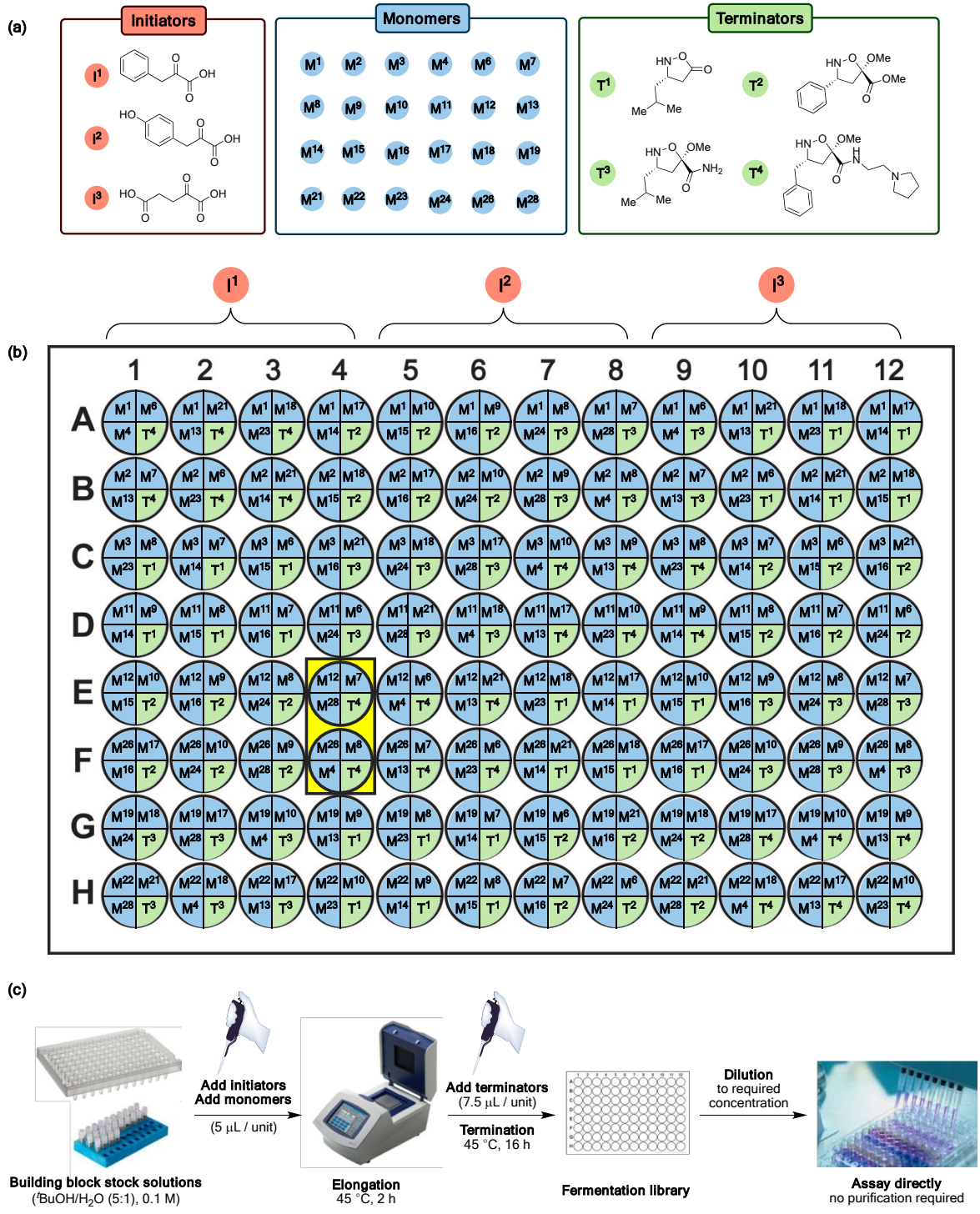
Figure 3: Synthetic fermentation of β-peptide oligomers by KAHA ligation. **(a)** The coupling of the initiator and elongation monomer unveils another α-ketoacid, which can react with further monomer units or a hydroxylamine terminator. **(b)** Reaction conditions and sample products generated in a typical three-monomer synthetic fermentation used in this study. 1 equivalent of initiator and each monomer was used, while terminators were added in slight excess (1.5 equiv) to ensure complete capping. [1, 20]

We began by preparing a modest number of compounds by using one initiator, one terminator, and three elongation monomers per well, in a 96-well plate format (see Figure 3 for a representative example). Initiators were selected from the large range of commercially available α-ketoacids. Monomers incorporating natural and non-natural alkyl and aryl amino acid side-chains were included, as were monomers bearing side-chain esters, amines, and aromatic and saturated heterocycles. Terminators capable of generating α-ketoamides – well known pharmacophores for enzyme inhibitors [23, 24] – as well as α-ketoesters, aldehydes and carboxylic acid functionalities in the final products were utilized. We hypothesized that the modular nature of the synthetic fermentation process would allow for facile modification and optimization of any prospective leads, aiding in the identification of pharmacophore regions and enabling rapid and efficient structure-activity relationship studies.

Lead Identification: The first culture prepared in this study (Culture 1) consisted of a randomized, broad-ranging screen of three initiators, 24 monomers and four terminators (Figure 4a). The monomers were divided into three general categories – aliphatic, aromatic, and hydrophilic – based on the chemical properties of their side chains; one from each group was added to each well (please see the Supporting Information for specific plate design). Each well therefore contained one initiator, three monomers and one terminator. If only products of type I-M_n-T (where n = 0-3) are considered, mixtures of this composition generate around 40 compounds per well. A 96-well plate prepared in this manner from less than 50 milligrams of total starting material allows for screening of around 4000 compounds for biological activity – the total time required for the synthetic fermentation and anti-bacterial assay is less than 48 hours.

The fermentation reactions were conducted by the addition of 5 μL each of initiator and monomer stock solutions (^{t}BuOH/H_2O, 5:1, 0.1 M) to the desired wells of a 96-well plate. The plate was sealed and heated to 45 °C for two hours in a PCR thermal cycler. Termination was achieved by the addition of 7.5 μL of a 0.1 M terminator stock solution to each well and heating the plate to 45 °C for a further 16 h. The gram-positive bacterium *Bacillus subtilis* was chosen as our model system at the outset of our study due to its ease of handling, fast growth and recognized utility as an indicator strain for antibiotic screening. [25] To determine antibacterial activity, aliquots from each well were transferred to an assay plate containing a sample of *Bacillus subtilis* in bacterial growth medium, and the assay plates were incubated at 37 °C for 24 hours. Bacterial growth inhibition was determined by optical density measurements at 600 nm using a plate reader. [26] In this initial screen, wells which were visibly clear and showed an optical density of < 0.2 were considered to be active. Positive controls were provided by 2.5 μL and 5 μL of a 10 μg/mL tetracycline solution, while a negative control was provided by 2.5 μL ^{t}BuOH/H_2O (5:1). A blank well containing no additional material was also measured for reference (see Supporting Information for full assay setup).

Culture 1


(a)
Initiators
Monomers
Terminators
(b)
(c)
Add initiators
Add monomers
(5 µL / unit)
Elongation
45 °C, 2 h
Add terminators
(7.5 µL / unit)
Termination
45 °C, 16 h
Dilution
to required
concentration
Building block stock solutions
(tBuOH/H2O (5:1), 0.1 M)
Fermentation library
Assay directly
no purification required

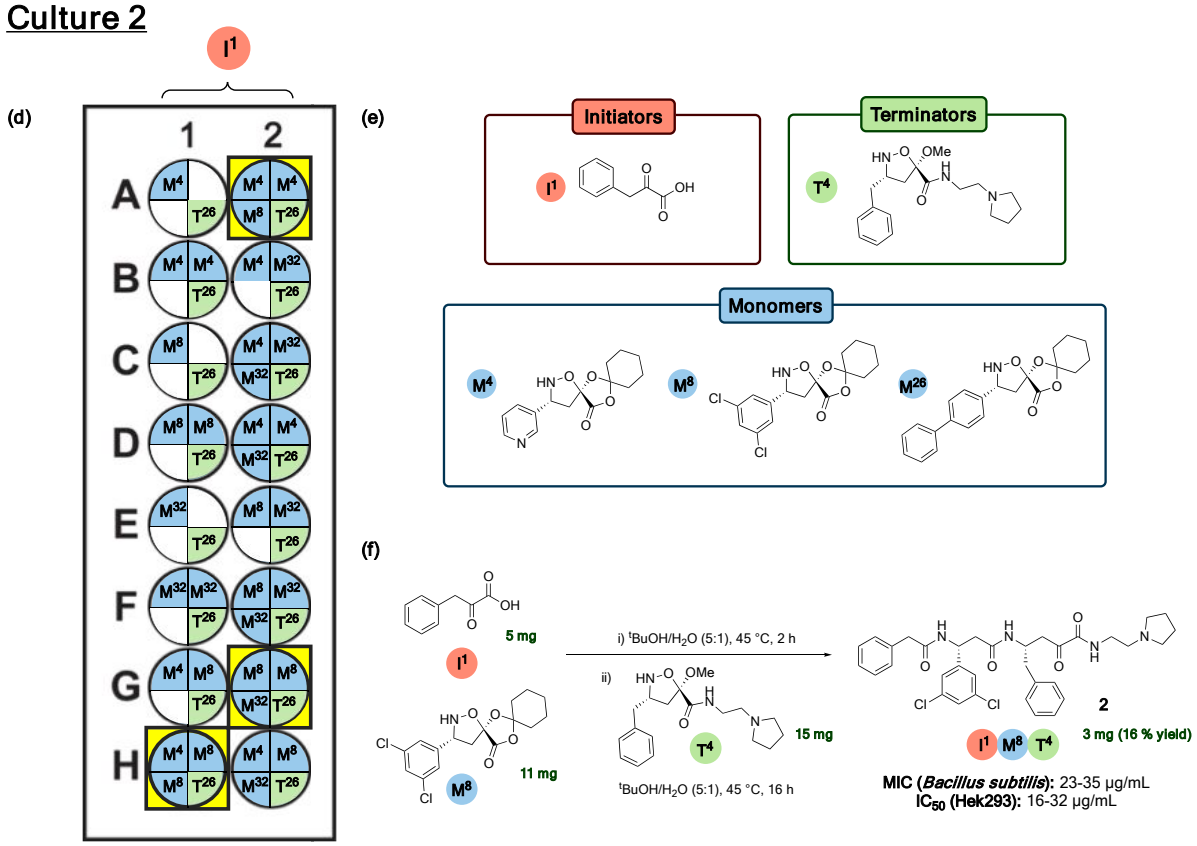


Figure 4. Design of Culture 1 and Culture 2 and results of the coupled antibacterial assays. **(a)** Synthetic fermentation building blocks utilized in Culture 1 (See Figure 2b for monomer side-chains). Three initiators, 24 monomers and four terminators were used. **(b)** Well design of Culture 1. The building blocks were distributed throughout the plate; one initiator, three monomers, and one terminator were used per well. Two active wells (OD_{600} < 0.2) were identified (highlighted in yellow). **(c)** Typical synthetic fermentation workflow. Building block stock solutions were transferred to a 96-well plate using standard liquid handling procedures, and the reaction mixtures were incubated at 45 °C to induce elongation. Terminators were added using the same procedure. The synthetic fermentation cultures can be directly assayed after dilution to appropriate concentration. **(d)** Well design of Culture 2. Building blocks found in biologically active well F4 from Culture 1 (Figure 4b) were identified and distributed in every logical permutation. The effects of varying stoichiometries were also tested. Each monomer listed corresponds to one equivalent of building block. Wells showing growth inhibition (OD_{600} < 0.35) are highlighted in yellow. **(e)** Synthetic fermentation building blocks utilized in Culture 2. One initiator, three monomers and one terminator were used. **(f)** Milligram scale production and biological properties of lead molecule **2**.

From this initial screen of 96 wells, two cultures showing significant growth inhibition on *Bacillus subtilis* were identified: one containing I^1, M^7, M^{12}, M^{28} and T^4, and one containing I^1, M^4, M^8, M^{26}, and T^4 (Figure 4 a, b, c). A second round of screening was carried out on the constituents of these wells to determine the active components. These deconvolution cultures were designed so that each new well would contain a fraction of the possible products from the active mother well (Figure 4d, e). This was achieved by oligomerization using each monomer individually, as well as all possible two-monomers-per-well

permutations. Monomer stoichiometry was also varied to probe the effect of peptide length on biological activity – for example, when three equivalents of elongation monomers are used, products containing two to four monomer units will be most prominently observed.

Wells showing bacterial growth inhibition in this focused library were analyzed by LC/MS, revealing that the two most active mixtures prominently featured peptide I^1-M^8-T^4 (**2**). As peptide **2** was also observed in the active mother well in Culture 1, we postulated that this was the active compound. Synthesis of **2** on milligram scale was achieved simply by mixing phenylpyruvic acid (**I^1**) and **M^8** in ^{t}BuOH / H_2O (5:1) and heating to 45 °C for 2 h, followed by addition of **T^4** and allowing the reaction to stand at 45 °C for 16 h; 3 mg of the desired peptide was successfully isolated after purification by HPLC (16 % yield, Figure 4f). The ability to rapidly prepare adequate quantities of the hit compound simply by mixing the components is a key feature of synthetic fermentation. Quantitative testing of **2** for bacterial growth inhibition against *Bacillus subtilis* using optical density measurement revealed a minimum inhibitory concentration (MIC) of 23–35 μg/mL. However, cytotoxicity tests carried out against human HEK293 cells showed an IC_{50} of 16–32 μg/mL, indicating a lack of selectivity for bacterial cells. Nonetheless, **2** was considered as a promising lead compound for further investigation.

Using hit compound **2** as a starting point, we carried out a broader screening of monomer and terminator building blocks. A terminator screen (Culture 3) was prepared in which each well contained I^1, M^8, and one of 16 different terminators, including several that had not been previously employed (Figure 5a, c). The assay results obtained from this screen showed that while some of these new terminator types displayed some inhibitory activity, the well containing T^4 remained the most active.

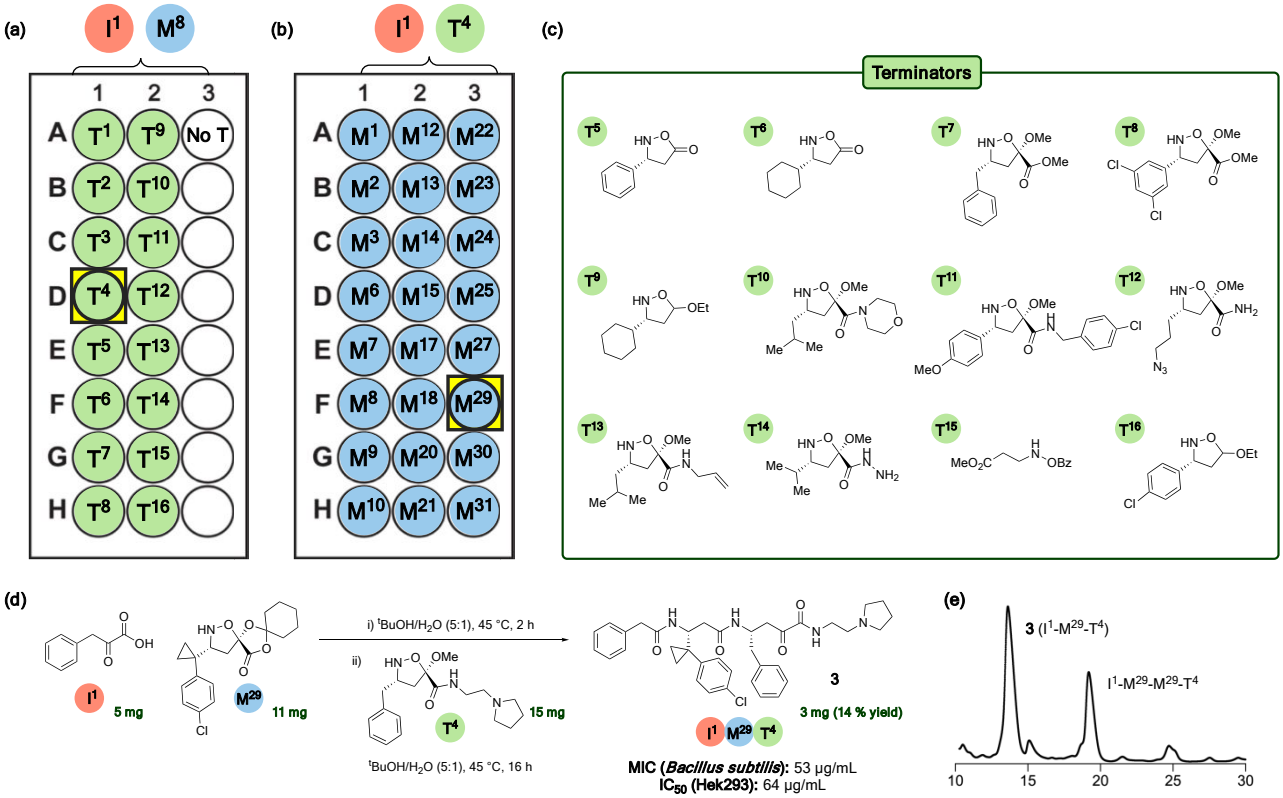


Figure 5. Design of Culture 3 and Culture 4 and results of the antibacterial assays. **(a)** Well design of Culture 3. I^1 and M^8 were kept constant and tested alongside 16 different terminators. The most active well is highlighted in yellow. **(b)** Well design of Culture 4. I^1 and T^4 were kept constant and tested alongside 24 monomers. The most active well is highlighted in yellow. **(c)** New terminator structures utilized in Culture 3. **(d)** Milligram scale production and biological properties of lead molecule **3**. **(e)** Preparative HPLC trace from the synthetic fermentation of **3**.

A conceptually analogous monomer screen (Culture 4, Figure 5a, b) was also carried out, in which 24 monomer types were tested in the presence of I^1 and T^4. The previously untested monomer M^{29} stood out as a promising candidate, and I^1-M^{29}-T^4 (**3**) was quickly synthesized by the same method described for **2** (Figure 5d, e). The preparative HPLC trace shown in Figure 5e highlights the clean nature of these scale-up processes. Desired I-M-T peptide **3** was easily isolated as the major peak; the only other significant product observed was the analogous I-M-M-T peptide incorporating two monomer units. Encouragingly, peptide **3** displayed antibacterial properties (MIC = 53 µg/mL) as well as lowered cytotoxicity against HEK293 cells (IC_{50} = 64 µg/mL). However, this peptide showed reduced inhibition of *Bacillus subtilis* compared to lead compound **2** (Figure 5d).

Structure-Activity Relationship Studies: With these preliminary results in hand, we embarked upon structure-activity relationship (SAR) studies. These efforts had two goals: to

identify a potent antibiotic peptide with improved selectivity for *Bacillus subtilis* over human HEK293 cells, and to elucidate the relationship between the structure of lead compound **2** and its biological function. The compounds investigated were synthesized by scaled up synthetic fermentation procedures and isolated in milligram quantities after purification by HPLC.

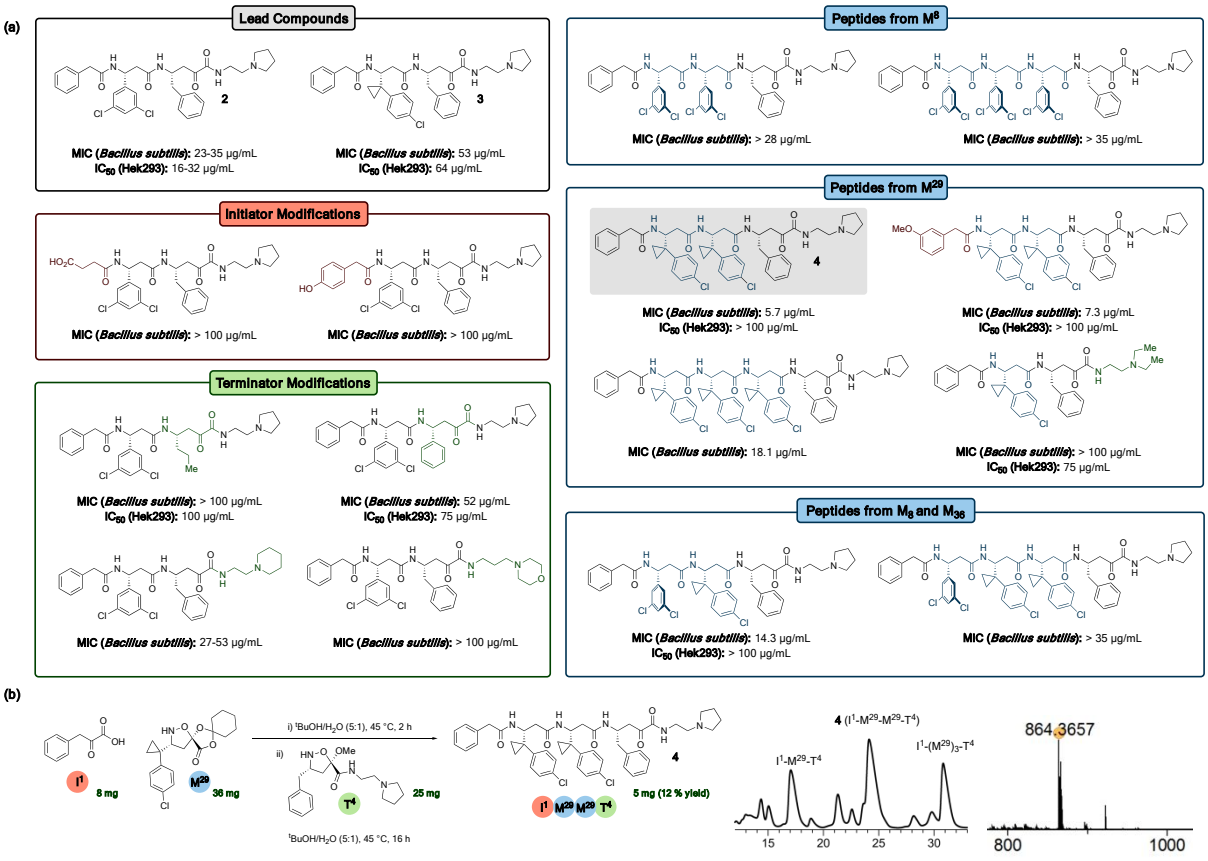


Figure 6. Chemical structure and biological properties of peptides synthesized in structure-activity relationship studies. **(a)** A tight SAR profile was observed when modulating building blocks and peptide length. The most active peptide identified (**4**) is highlighted in grey. **(b)** Synthesis and isolation of active peptide **4**. Preparative HPLC trace and mass spectrum of the purified compound shown.

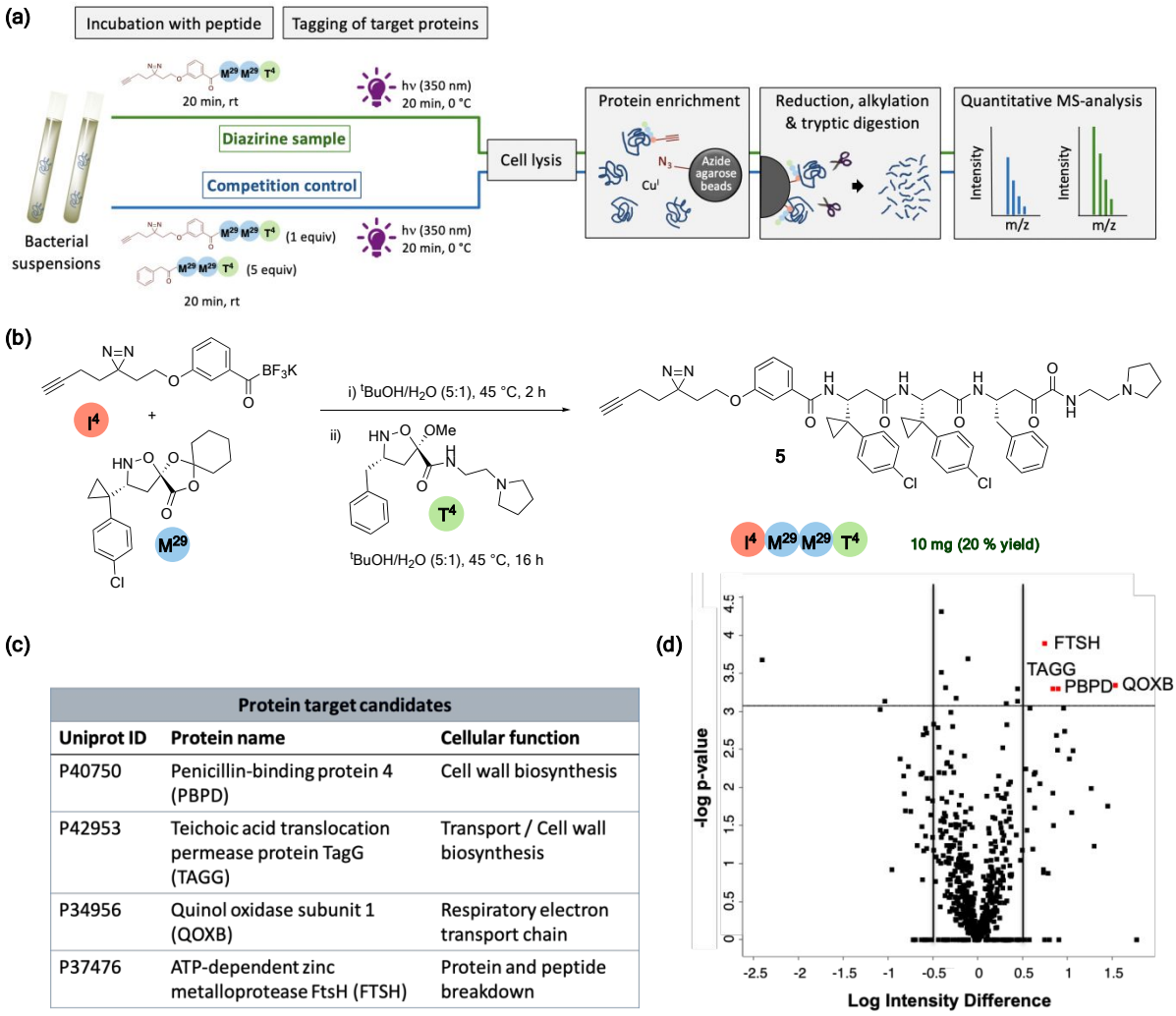
We initiated a systematic study of lead compound **2** through the sequential modification of each region of the peptide (Figure 6a). Replacement of the benzyl subunit of the initiator with either its phenol analogue or an alkyl carboxylic acid was not found to improve inhibition of *Bacillus subtilis* growth. A tight structure-activity relationship was also seen in the terminator region of the molecule, with the biological activity proving sensitive to even minor modulation of the tertiary amine. Substitution of the pyrrolidine tail with other saturated N-heterocycles significantly impaired the biological activity, as did replacement of the benzyl side-chain with phenyl or *n*-propyl groups. We therefore concluded that the use of terminator T^4 was

important for the activity of the lead peptide. Interestingly, these observations echo the findings of Hergenrother, who proposed that amphiphilic small molecules bearing basic amines are best suited to penetrate bacterial cell membranes and serve as broad-spectrum antibiotics. [27]

Further SAR studies were carried out investigating the effect of peptide elongation on biological activity (Figure 6). For this study, peptides of type I-M-M-T and I-M-M-M-T were synthesized. Our initial studies in this area focused on the impact of peptide elongation through the incorporation of additional M^8 subunits. While these compounds retained some biological activity, no significant improvement in comparison to lead compound **2** was observed. Exploration of longer peptides containing M^{29} units proved more effective. Peptides I^1-M^{29}-M^{29}-T^4 and I^1-M^{29}-M^{29}-M^{29}-T^4 exhibited a clear increase in inhibition compared to the lead compound, as did mixed monomer compound I^1-M^8-M^{29}-T^4. Moreover, peptides of this type showed significantly lowered cytotoxicity against the human HEK293 cell line. The most potent of these, I^1-M^{29}- M^{29}-T^4 (**4**), showed an almost tenfold increase in bacterial growth inhibition (MIC = 5.7 μg/mL) while displaying no measurable toxicity against HEK293 cells (IC_{50} > 100 μg/mL, see Supporting Information for details) (Figure 6b).

These studies identified a tight SAR profile in many areas of the molecule, supporting our confidence in synthetic fermentation as an effective medium-throughput screening technique. Our goals for the investigation were further met by the discovery of **4**, a potent inhibitor of *Bacillus subtilis* growth bearing no apparent toxicity towards our human HEK293 cell model system.

Target Identification: Although we initially anticipated that the antimicrobial activity might be due to simple membrane disruption, the tight structure-activity relationships observed in **4** implied a discrete molecular target in bacteria. To investigate the mechanism of action, we prepared a variant **5** suitable for photoaffinity labeling of receptor proteins and their subsequent identification by a quantitative mass spectrometry (MS)-based strategy (Figure 7a). [28, 29, 30] For the synthetic fermentation of probe **5**, an initiator (I^4) was employed containing both a diazirine as a photolabile group and an alkyne for the enrichment of target proteins (Figure 7b), where the small size of these groups should ensure minimal interference with target binding. [31] The ability to quickly prepare a molecule for target identification is another advantage of this approach.



Protein target candidates		
Uniprot ID	**Protein name**	**Cellular function**
P40750	Penicillin-binding protein 4 (PBPD)	Cell wall biosynthesis
P42953	Teichoic acid translocation permease protein TagG (TAGG)	Transport / Cell wall biosynthesis
P34956	Quinol oxidase subunit 1 (QOXB)	Respiratory electron transport chain
P37476	ATP-dependent zinc metalloprotease FtsH (FTSH)	Protein and peptide breakdown

Figure 7: Target identification for antibacterial oligopeptide **4** using a photoaffinity labeling approach. **(a)** Workflow followed for target identification studies. Photoaffinity probe **5** was incubated with bacterial suspensions, with and without the presence of a competition control. After light-induced covalent bond formation between the probe and its target proteins, the bacterial cells were lysed and the captured proteins were enriched by click chemistry to agarose beads and subjected to on-bead tryptic digestion. The resulting purified peptides were identified by mass spectrometry. **(b)** Synthetic fermentation of peptide **5** bearing a photoaffinity probe. **(c)** List of identified protein target candidates and their cellular functions. **(d)** Enrichment of protein target candidates relative to a competition control (n=3, FDR=0.01, log intensity difference ≥0.5).

We incubated probe conjugate **5** with suspensions of *Bacillus subtilis* in PBS for 20 minutes to allow target binding, followed by irradiation at 350 nm for 20 minutes on ice. The reactive carbene generated from the diazirine upon UV irradiation mediates covalent linkages with adjacent biomolecules. [31] In a control sample, the bacterial suspensions underwent simultaneous treatment with a five-fold excess of unconjugated peptide **4** to compete with binding of diazirine probe **5** at specific binding partners. After cell lysis, proteins trapped by the probe were enriched by click reaction of the alkyne with azide-bearing agarose beads.

Following on-bead tryptic digestion and purification of resulting peptides, proteins were identified and quantified relative to the competition control by MS-analysis.

Using stringent cut-offs for statistical significance and enrichment over the control, we identified four high confidence protein candidates for binding partners, all of which are located in the plasma membrane of the bacterial cell (Figure 7c, d). Among these candidates, penicillin binding protein 4 (pbp4) seemed a promising molecular target to mediate the antibiotic activity of **4**, as it is involved in the essential biosynthesis of the bacterial cell wall, and a known target of β-lactam antibiotics such as penicillin. [32]

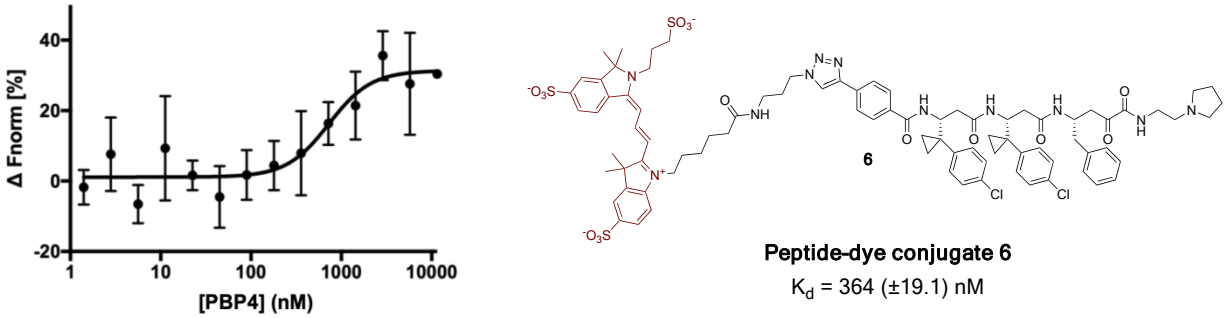


Figure 8: Dose-response curve and dissociation constant obtained from binding studies between peptide conjugate **6** and penicillin-binding protein 4 in three independent measurements using microscale thermophoresis.

To validate these findings, we sought to determine the binding affinity of **4** with recombinant, purified penicillin-binding protein 4. Initial binding studies using surface-based methods such as surface plasmon resonance [33] and bio-layer interferometry [34] proved challenging due to a high level of non-specific binding between the hydrophobic membrane protein and the surface. However, solution-based microscale thermophoresis using peptide-dye conjugate **6** proved to be suitable. [35] A dose response curve for **6** was obtained, allowing determination of a dissociation constant (K_d) of 364 ± 19.1 nM, while a negative control peptide also bearing a cyanine-3 dye unit showed no binding (Figure 8, see Supporting Information for details). This value compares favorably with members of the 4-quinolone family that have been shown to bind PBPs but show no antibacterial activity against *Bacillus subtilis*. [36] Notably, penicillin follows a two-step serine acylation mechanism with penicillin-binding proteins and has a surprisingly low dissociation constant prior to the formation of the covalent bond, independently delineated as 0.9 mM by Lu [37] and 20 mM by Chesnel [38] for PBP2x.

Conclusions

The discovery of antibacterial peptide **4** provides a proof of concept of synthetic fermentation as a platform for rapid and effective phenotypic screening. The robust synthesis of monomer and terminator precursors has allowed the accumulation of a diverse building block library capable of the efficient generation and screening of large numbers of compounds. Upon the discovery of a hit, the modular nature of synthetic fermentation enables facile preparation and modification of promising compounds, allowing for rapid investigation of pharmacophore regions and structure-activity relationships. Probe materials – including fluorescent variants and photoaffinity trapping reagents – for target identification studies are readily generated through the use of bifunctional building blocks, faciliting the often tedious process of preparing such derivatives. In this manner, we discovered that the peptide identified by rapid molecular assembly and phenotypic screnning is a nanomolar binder of penicillin-binding protein 4, a known target of beta-lactam antibiotics.

The appeal of synthetic fermentation is the ability to construct relatively complex, non-natural molecules simply upon mixing the building blocks in aqueous media. The resulting mixtures can be directly screened for biological activity in both biochemical and phenotypic assays. Although small mixtures are produced – requiring some deconvolution steps and HPLC isolation of the active compounds – the ease of preparing new, focused libraries and synthesizing derivatives suitable for target identification simplifies the overall approach to drug discovery. Using this technique, we envision that simple kits of the constituent building blocks will allow researchers with little experience with synthetic organic chemistry to prepare and screen new molecules for specific biological activities.

METHODS

Synthetic Fermentation: The syntheses of cultures were carried out in 96-well plates. 0.1 M stock solutions of initiators and monomers in ^{t}BuOH/H_2O (5:1) were prepared, and the required volumes were added to their respective wells using a micropipette. After addition of initiators and monomers, the 96-well plate was capped, shaken and placed in a PCR thermal cycler. The PCR machine (equipped with a heated lid to prevent solvent condensation on the cap) was then heated to 45°C for 6 h. The plate was then removed and allowed to cool. 0.1 M stock solutions of terminators in ^{t}BuOH/H_2O (5:1) were then added to each well using a micropipette. The plate was capped, stirred and heated at 45°C for 16 h.

***Bacillus subtilis* preparation:** *Bacillus subtilis* bacteria were stored at –80°C in a Luria-Broth (LB) medium containing 15% glycerol. A trace amount of bacteria was placed into a

Falcon tube using a toothpick and diluted with 10 mL LB media solution. LB media solution was prepared from 25 g LB media in 1 L MQ-H_2O. The bacterial solution was incubated and allowed to shake at 37 °C overnight, generating a bacterial preculture. 100 μL of this preculture was transferred to a Falcon tube, diluted with 10 mL LB media, and incubated and shaken at 37 °C. Bacterial growth was monitored by spectrophotometric optical density determination. Once optical density at 600 nm had reached 0.6, the bacterial solution was stored at 0°C and used in the assay within 1 h. All steps were carried out under sterile conditions using pre-sterilized equipment.

Growth inhibition assay: 66 μL of bacterial solution was transferred into a pre-sterilized Falcon tube, and diluted with 10 mL LB media solution to generate a bacterial stock solution. 2.5 μL of crude mixture from each synthetic fermentation well was added to the respective wells of an assay plate, and 150 μL of the bacterial stock solution was added to each well. The assay plate was allowed to shake gently at 37 °C for 24 h. Positive controls were provided by 2.5 μL and 5 μL of a 10 μg/mL tetracycline solution. Negative control was provided by 2.5 μL ^{t}BuOH/H_2O (5:1). Assay results were measured by inhibition of bacterial growth using a plate reader, based on spectrophotometric optical density determination of each well at 600 nm after 24 h incubation. This assay was always performed in triplicate, with the final results given by the average value of the three measurements.

Minimum inhibitory concentration determination: Desired peptide was synthesized on a milligram scale and purified by reverse phase HPLC. The peptide was dissolved in DMSO to generate a 25 mM solution. 5 μL of this solution was added to 620 μL LB media, generating a 200 μM stock solution. Different volumes of this solution were added to 50 μL *Bacillus subtilis* solution in LB media, generating a gradient of final concentrations (e.g. 100, 75, 50, 37.5, 25, 10 and 5 μM) for measurement. The assay plate was incubated at 37 °C for 24 hours and analyzed as described above. Assay results were converted into μg/mL units for final publication.

Cytotoxicity determination: [39] [40] Cytotoxicity tests were carried out on HEK293 cells using the commercial product Cell Counting Kitmg8 (CCK-8), and experiments were performed according to the manual provided. 100 μL of cell suspension (5000 cells/well) was dispensed in a 96-well plate, which was incubated for 24 h at 37 °C. The media was then removed. 100 μL of seven different concentrations (200, 150, 100, 75, 50, 25, 10 μM) of I_1-M_{36}-M_{36}-T_{26} in DMSO were added to the plate, which was then incubated for a further 24 h at 37 °C. 10 μL of CCK-8 solution was added to each well of the plate, which was incubated at 37 °C for a

final 2 h. The absorbance was measured directly. Assay results were converted into μg/mL units for final publication.

Target identification using quantitative mass spectrometry: Cultures of *Bacillus subtilis* (in log phase) were harvested by centrifugation (6000 xg for 10 min at room temperature) and washed in phosphate buffered saline (PBS, Gibco). 200 mg bacterial pellet per sample was resuspended in 10 mL PBS in thin-walled glass tubes and treated with 100 µg of the peptide-diazirine-alkyne construct (0.07% DMSO end concentration) for 20 min at room temperature. In a competition control, cells were simultaneously treated with 100 µg of the peptide-diazirine-alkyne conjugate and 500 µg of the unconjugated peptide. Experiments were conducted in triplicates per condition (n=3). Samples were cooled on ice and irradiated for 20 min at 350 nm using a Rayonet RPR-200 photoreactor with Hitachi FL8BL-B light tubes. Cells were transferred to 15 ml Falcon tubes, collected by centrifugation and resuspended in 2 ml PBS supplemented with protease inhibitors (cOmplete ULTRA Tablets, EDTA-free, Roche) on ice. Cells were lysed by three rounds in a French pressure cell press (SLM Aminco). For protein solubilisation, samples were sonicated in a VialTweeter (Hielscher, 30 s at 80% amplitude and 80% cycle time) with 0.1% RapiGest surfactant (Waters) at 4 °C, and cell debris were removed by centrifugation (16.000 xg for 10 min at 4 °C).

For enrichment of alkyne-labeled target proteins, lysates were incubated with 100 µL azide beads (Jena Biosciences), 1 mM $CuSO_4$, 5 mM THPTA, and 100 mM sodium ascorbate, slowly rotating overnight at room temperature. Beads were washed in Biospin columns (BioRad) connected to a Vac-Man Laboratory Vacuum Manifold (Promega) with 5 x 1 mL 1% sodium dodecyl sulfate (SDS) in 100 mM Tris-base, pH 8, 5 x 1 mL 80% isopropanol, 5 x 1 mL 8M urea in 100 mM ammonium bicarbonate with 5 mM EDTA, 5 x 1 mL 5 M sodium chloride and 5 x 1 mL 50 mM ammonium bicarbonate. Bead-bound proteins were reduced for 30 min with 5 mM Tris(2-carboxyethyl)phosphine (TCEP, Pierce) in 50 mM ammonium bicarbonate, 0.1% RapiGest, alkylated for 30 min with 5 mM iodoacetamide in 50 mM ammonium bicarbonate in the dark, and digested overnight, rotating at 37 °C, with 1 µg trypsin (Sequence grade modified trypsin, Promega) in 500 µL 50 mM ammonium bicarbonate, 0.1% RapiGest. Supernatants were acidified to pH 2 by the addition of formic acid, subjected to C18 purification using 3–30 µg UltraMicroSpin Columns (The Nest Group) according to the manufacturer's instructions and dried in a speed-vac (Thermo Scientific).

Peptides resuspended in 5% MeCN in H_2O with 0.1% formic acid (FA) were separated by reversed-phase chromatography on a high-pressure liquid chromatography (HPLC) column (75-µm inner diameter, New Objective) that was packed in-house with a 15-cm stationary phase (ReproSil-Pur C18-AQ, 1.9 µm) and connected to a nano-flow HPLC combined with an autosampler (EASY-nLC II, Proxeon). The HPLC was coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific) equipped with a nano-electrospray ion source (Thermo Scientific). Peptides were loaded onto the column with 100% buffer A (99.9% H_2O, 0.1% FA) and eluted at a constant flow rate of 300 nl/min with a 50 min linear gradient from 5–35% buffer B (99.9% MeCN, 0.1% FA), 5 min 35-50% and 5 min 50-90% buffer B, with a subsequent washing step with 90% buffer B. In between batches of runs, the column was cleaned with two steep consecutive gradients of MeCN (10-98%). Mass spectra were acquired in a data-dependent manner (TopSpeed, charge state 2-6, dynamic exclusion duration 30 s). High-resolution MS1-spectra were acquired at 120,000 resolution (automatic gain control target value 2.0*10e5) to monitor peptide ions in the mass range of 395–1,500 m/z, followed by HCD MS/MS-scans (Quadrupole isolation, HCD 30%, Detector IonTrap, Scan rate turbo, automatic gain control target value 1.0*10e2, Maximum injection time 250 ms, centroid).

RAW data were analysed using the MaxQuant software (version 1.6.0.16). [41] Spectra were searched against a Uniprot-based database of *Bacillus subtilis* proteins, and label-free quantitation was performed using standard settings. Statistical analysis and data visualization was done using the Perseus software (version 1.6.0.7). [42] Data were filtered for contaminants, decoys (reverse sequences) and proteins that were only identified by a modification site, and statistical significance was determined by a two-sample t-test (with at least two valid values in at least one group). Proteins were considered candidates if they showed statistical significance with a stringent false discovery rate cut-off of 0.01, and a difference between log intensity values of at least 0.5.

Determination of protein binding affinity with microscale thermophoresis: A trace amount of peptide-dye conjugate **6** or control conjugate **7** was dissolved in NanoTemper MST buffer (200 µL) and diluted fivefold until no visible colour was observed. A 11.5 µM solution of recombinant *Bacillus subtilus* penicillin binding protein 4 (Biomatik) in NanoTemper MST buffer (20 µL) was prepared, and used as the source for a 1:1 dilution series of sixteen samples. To each resulting 10 µL sample was added 10 µL of peptide-dye conjugate solution. Capillaries were used to take up a small amount of each solution, and these were ordered sequentially by concentration and placed inside a NanoTemper Monolith

NT.115. A cap scan was performed to determine optimal LED power, and the samples were subjected to binding affinity testing by thermophoresis + T-Jump experiments. Experiments were performed in triplicate, and K_d calculations were performed by NanoTemper NTAnalysis software based on the resulting calibration curves. Error bars = standard deviation from three independent measurements.

Associated Content

Supporting Information

Experimental procedures and spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

Author Information

Corresponding Authors

*E-mail: bode@org.chem.ethz.ch

*E-mail: wbernd@ethz.ch

Notes

The authors declare no competing financial interest.

Acknowledgements. This work was supported by the ETH Research Commission (ETH 06 15-2) and Swiss National Science Foundation Agora Funding (CRAGP2_171667) to J.B. and from the Swiss National Science Foundation SNSF #31003A_160259 to B.W.. We would like to thank J. Hubert and Y. Stöferle for contributions to monomer synthesis, J. Sobek and S. Oishi for assistance with the target validation studies, V. Pattabiraman for sourcing starting materials, and R. Hofmann for helpful discussions.

References

(1) Huang, Y.-L., and Bode, J. W. (2014) Synthetic fermentation of bioactive non-ribosomal peptides without organisms, enzymes or reagents *Nat Chem. 6*, 877–884.

(2) Stepek, I. A., and Bode, J. W. (2018) Synthetic fermentation of bioactive molecules *Curr. Opin. Chem. Biol. 46*, 18–24.

(3) Sattely, E. S., Fischbach, M. A., and Walsh, C. T. (2008) Total biosynthesis: in vitro reconstitution of polyketide and nonribosomal peptide pathways. *Nat. Prod. Rep. 25*, 757–793.

(4) Zimmermann, G., and Neri, D. (2016) DNA-encoded chemical libraries: foundations and applications in lead discovery *Drug Discov. Today 21*, 1828–1834.

(5) Goodnow Jr., R. A., Dumelin, C. E., and Keefe, A. D. (2017) DNA-encoded chemistry: enabling the deeper sampling of chemical space *Nat. Rev. Drug Discov.* 16, 131–147.

(6) Obexer, R., Walport, L. J., and Suga, H. (2017) Exploring sequence space: harnessing chemical and biological diversity towards new peptide leads *Curr. Opin. Chem. Biol. 38*, 52–61.

(7) Roberts, R. W., and Szostak, J. W. (1997) RNA–peptide fusions for the in vitro selection of peptides and proteins. *Proc. Natl Acad. Sci. USA 94*, 12297–12302.

(8) Cordier, C., Morton, D., Murrison, S., Nelson, A., and O'Leary-Steele, C. (2008) Natural products as an inspiration in the diversity-oriented synthesis of bioactive compound libraries *Nat. Prod. Rep. 25*, 719–737.

(9) Chow, S., Liver, S., and Nelson, A. (2018) Streamlining bioactive molecular discovery through integration and automation *Nature Rev. Chem.* 2, 174–183.

(10) Wang, S., Tao, Y., Wang, J., Tao, Y., and Wang, X. (2019) A versatile strategy for the synthesis of sequence- defined peptoids with side-chain and backbone diversity via amino acid building blocks *Chem. Sci. 10*, 1531–1538.

(11) Porel, M., Thornlow, D. N., Artim, C. M., and Alabi, C. A. (2017) Sequence-Defined Backbone Modifications Regulate Antibacterial Activity of OligoTEAs *ACS Chem. Biol. 12*, 715–723.

(12) Artim, C. M., Phan, N. N., and Alabi, C. A. (2018) Effect of Composition on Antibacterial Activity of Sequence-Defined Cationic Oligothioetheramides *ACS Infect. Dis. 4*, 1257–1263.

(13) Porel, M., Thornlow, D. N., Phan, N. N., and Alabi, C. A. (2016) Sequence-defined bioactive macrocycles via an acid-catalysed cascade reaction *Nature Chem. 8*, 590–596.

(14) Dow, M., Marchetti, F., Abrahams, K. A., Vaz, L., Besra, G. S., Warriner, S., and Nelson, A. (2017). Modular Synthesis of Diverse Natural Product-Like Macrocycles: Discovery of Hits with Antimycobacterial Activity. *Chemistry 23*, 7207–7211.

(15) Amsoa, Z., and Hayouka, Z. (2019) Antimicrobial random peptide cocktails: a new approach to fight pathogenic bacteria *Chem. Commun.*, Advance Article (DOI: 10.1039/C8CC09961H)

(16) Bode, J. W. (2017) Chemical Protein Synthesis with the α-Ketoacid–Hydroxylamine Ligation *Acc. Chem. Res. 50*, 2104–2115

(17) Porter, E. A., Weisblum, B., Gellman, S. H. (2002) Mimicry of Host-Defense Peptides by Unnatural Oligomers: Antimicrobial β-Peptides *J. Am. Chem. Soc. 124*, 7324–7330

(18) Godballe, T.,Nilsson, L. L., Petersen, P. D., Jenssen, H. (2011) Antimicrobial β-Peptides and α-Peptoids *Chem. Biol. Drug Des. 77*, 107–116

(19) Epand, R. F., Raguse, T. L., Gellman, S. H., Epand, R. M. (2004) Antimicrobial 14-Helical β-Peptides: Potent Bilayer Disrupting Agents *Biochemistry 43*, 9527–9535

(20) Chiang, Y.-L., Russak, J. A., Carrillo, N, and Bode, J. W. (2012) Synthesis of Enantiopure Isoxazolidine Monomers for the Preparation of β3-Oligopeptides by Iterative α-Ketoacid–Hydroxylamine (KAHA) Ligations *Helv. Chim. Acta 95*, 2481–2501.

(21) Gerfaud, T., Chiang, Y.-L., Kreituss, I., Russak, J., and Bode, J. W. (2016) Enantioselective, chromatography-free synthesis of β3-amino acids with natural and unnatural side chains *Org. Process Res. Dev. 16*, 687–696.

(22) Yu, S.; Ishida, H.; Juarez-Garcia, M. E.; Bode, J. W. (2010) Unified synthesis of enantiopure β2h, β3h and β2,3-amino acids *Chem. Sci. 1*, 637–641.

(23) Han, W., Hu, Z., Jiang, X., and Decicco, C. P. (2000) α-Ketoamides, α-ketoesters and α-diketones as HCV NS3 protease inhibitors *Bioorg. Med. Chem. Lett. 10*, 711–713.

(24) Zhou, J., Mock, E. D., Martella, A., Kantae, V., Di, X., Burggraaff, L., Baggelaar, M. P., Al-Ayed, K., Bakker, A., Florea, B. I., et al (2019) Activity-Based Protein Profiling Identifies α-Ketoamides as Inhibitors for Phospholipase A2 Group XVI *ACS Chem. Biol.* Article ASAP (DOI: 10.1021/acschembio.8b00969)

(25) Zeigler, D. R., Prágai, Z., Rodriguez, S., Chevreux, B., Muffler, A., Albert, T., Bai, R., Wyss, M. and Perkins, J. B. **(**2008) The origins of 168, W23, and other *Bacillus subtilis* legacy strains. *J. Bacteriol. 190*, 6983–6995.

(26) Campbell, J. (2012) High-Throughput Assessment of Bacterial Growth Inhibition by Optical Density Measurements *Curr. Protoc. Chem. Biol. 2*, 195–208.

(27) Richter, M. F., Drown, B. S., Riley, A. P., Garcia, A., Shirai, T., Svec, R. L., and Hergenrother, P. J. (2017) Predictive Compound Accumulation Rules Yield a Broad-Spectrum Antibiotic *Nature 545*, 299–304.

(28) Koh, M., Park. J., Koo, J. Y., Lim, D., Cha, M. Y., Jo, A., Choi, J. H., and Park, S. B. (2014) Phenotypic Screening to Identify Small-Molecule Enhancers for Glucose Uptake: Target Identification and Rational Optimization of Their Efficacy *Angew. Chem. Int. Ed. 53*, 5102–5106.

(29) Burdine, L., and Kodadek, T. (2004) Target identification in chemical genetics: the (often) missing link *Chem. Biol. 11*, 593–597.

(30) Parker, C. G., Galmozzi, A., Wang, Y., Correia, B. E., Sasaki, K., Joslyn, C. M., Kim, A. S., Cavallaro, C. L., Lawrence, R. M., Johnson, S. R. et al. (2017) Ligand and Target Discovery by Fragment-Based Screening in Human Cells *Cell 168*, 527–541.

(31) Li, Z., Hao, P., Li, L., Tan, C. Y. J., Cheng, X. Chen, G. Y. J., Sze, S. K., Shen, H.-M., and Yao, S. Q. (2013) Design and Synthesis of Minimalist Terminal Alkyne-Containing Diazirine Photo-Crosslinkers and Their Incorporation into Kinase Inhibitors for Cell- and Tissue-Based Proteome Profiling *Angew. Chem. Int. Ed. 52*, 8551–8556.

(32) Fisher, J. F., Meroueh, S. O., and Mobashery, S. (2005) Bacterial Resistance to β-Lactam Antibiotics: Compelling Opportunism, Compelling Opportunity! *Chem. Rev*. *105*, 395–424.

(33) Schuck, P. (1997) Use of surface plasmon resonance to probe the equilibrium and dynamic aspects of interactions between biological macromolecules. *Annu. Rev. Biophys. Biomol. Struct. 26*, 541–566.

(34) Abdiche, Y., Malashock, D., Pinkerton, A., and Pons, J. (2008) Determining kinetics and affinities of protein interactions using a parallel real-time label-free biosensor, the Octet *Anal. Biochem.* 377, 209–217.

(35) Seidel, S. A. I., Wienken, C. J., Geissler, S., Jerabek-Willemsen, M., Duhr, S., Reiter, A., Trauner, D., Braun, D., and Baaske, P. (2012) Label-Free Microscale Thermophoresis Discriminates Sites and Affinity of Protein-Ligand Binding *Angew. Chem. Int. Ed. 51*, 10656–10659.

(36) Shilabin, A. G., Dzhekieva, L., Misra, P., Jayaram, B., and Pratt, R. F. (2012) 4-Quinolones as Noncovalent Inhibitors of High Molecular Mass Penicillin-Binding Proteins *ACS Med. Chem. Lett. 3*, 592−595.

(37) Lu, W. P., Kincaid, E., Sun, Y., and Bauer M. D. (2001) Kinetics of beta-lactam interactions with penicillin-susceptible and -resistant penicillin-binding protein 2x proteins from Streptococcus pneumoniae. Involvement of acylation and deacylation in beta-lactam resistance. *J. Biol. Chem. 276*, 31494–31501.

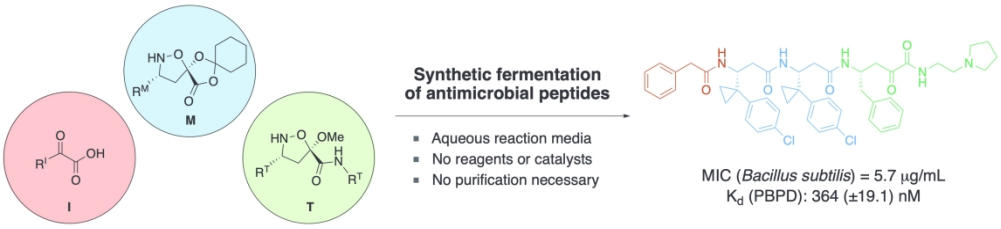
(38) Chesnel, L., Pernot, L., Lemaire, D., Champelovier, D., Croize, J., Dideberg, O., Vernet, T., and Zapun, A. (2003) The structural modifications induced by the M339F substitution in PBP2x from Streptococcus pneumoniae further decreases the susceptibility to beta-lactams of resistant strains. *J. Biol. Chem. 278*, 44448–44456.

(39) Ishiyama, M., Miyazono, Y., Sasamoto, K., Ohkura, Y., and Ueno, K. (1997) A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. *Talanta 44*, 1299–1305.

(40) Tominaga, H., Ishiyama, M., Ohseto, F., Sasamoto, K., Hamamoto, T., Suzuki, K., and Watanabe, M. (1999) A water-soluble tetrazolium salt useful for colorimetric cell viability assay. *Anal. Commun. 36*, 47–50.

(41) Cox, J. and Mann, M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b. range mass accuracies and proteome-wide protein quantification, *Nat. Biotechnol.* **26**, 1367–1372.

(42) Tyanova, S.; Temu, T.; Sinitcyn, P.; Carlson, A.; Hein, M. Y.; Geiger, T.; Mann, M. and Cox, J. (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data, *Nat. Methods* **13**, 731–740.



282x63mm (300 x 300 DPI)