Characterization and Synthesis of Selected Secondary Metabolites produced by *Xenorhabdus* and *Photorhabdus* spp

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Datum der Disputation:

There is no answer as big as the question, there is no victory as big as the lesson, you go on and you see where your detours will take you to, there is no power like understanding.

Tina Dico

To my friends and my family who were neglected now and then in the process of this work but nevertheless helped to make it happen.

Acknowledgement

Anybody who has been seriously engaged in scientific work of any kind realizes that over the entrance to the gates of the temple of science are written the words: “Ye must have faith.”

Max Planck

With these words I would like to thank all the people who were involved in this work and had to restore my faith in sciences from time to time, namely

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Abstract

Natural products have been a rich source of biological active compounds to the point of pharmaceutical lead structures and drugs. *Photorhabdus* and *Xenorhabdus* spp are considered as interesting and promising producers of natural products, since 6.0 - 7.5% of their genome is assigned to secondary metabolite clusters. This thesis focuses on the synthesis and characterization of natural products from these entomopathogenic bacteria. The synthesized and characterized molecules can be categorized in four mayor classes: small molecules (phurealipids), cyclic macrolactams (xenotetrapeptide, GameXPeptides and ambactin), cylic macrolactons (szentiamide, xentrivalpeptides and xenephematide) and backbone methylated linear peptides (rhabdopeptides and rhabdopeptide-like structures).

The first part of this thesis describes the identification and characterization of the phurealipids which were initially found in *P. luminescens* TTO1. Phylogenetic analysis in combination with LC-MS/MS data revealed that these alkylated urea derivatives not only occur in *P. luminescens* TTO1 but are widely distributed amongst the *Photorhabdus* species. Though, their actual biological activity remains in the shades until now, this suggests an important function of these molecules for *Photorhabdus* spp. Another compound class comprised of similarly small molecules, are the so called cytotoxic fatty acid amides.[1] These small amides showed cytotoxicity against insect cell lines in *in vitro* assays. Although, their biological activity is known, their actual target molecules have not been identified yet. Hence, photo-activatable derivatives were synthesized. The synthesis proceeded in good yields and purities. Unfortunately, the target molecule(s) could not be identified in the subsequent analyses.

The second publication focuses on the optimization of the structure elucidation of peptidic natural products based on an LC-MS/MS approach in combination with stable isotope labeling in transaminase deficient mutants.[2] In order to underline this method and proof the proposed structure of the here presented xenotetrapeptide, this cyclic tetrapeptide was synthesized. The third publication concentrates on the cylic GameXPeptide E-H, which are produced by *P. luminescens* TTO1 exclusively within the insect.[3] Upon entering the insect the bacteria start to produce the non-proteinogenic amino acid 4-methylamino-phenylalanine (MMPAPA) which is then incorporated in the GameXPeptides instead of the standard D-phenylalanine. It has been shown that the cluster responsible for the production of MMPAPA is not inactive at the standarded growth conditions in the laboratory (such as *Luria Bertani* medium at 30°C upon shaking). Hence, this compound class is an interesting example for the fact that microorganisms grown at these standardized conditions not inevitably

produce the whole palette of naturally occurring secondary metabolites. A further representative of cyclic macrolactams produced by *Photorhabdus* and *Xenorhabdus* spp. is the so called ambactin. In order to synthesize this peptide, which was initially identified and characterized by genome mining and heterologous expression,[4] the standard cyclization conditions had to be modified and optimized. The best results were obtained by lowering the reaction temperature and adding 1-hydroxy-7-azabenzotriazole hydrate to the reaction mixture.

The fourth publication presents the successful synthesis of the cyclic depsipeptide szentiamide. Subsequent testing of this peptide which was initially identified in *X. szentirmaii* DSM16338[5] revealed an activity against *Plasmodium falciparum* NF54. This activity against the causative agent of malaria probably relies on a cross activity against amoeba which live in the soil and are commonly considered as food predators. Other successfully synthesized depsipeptides are the xentrivalpeptides and the xenephematide.

The last compound class which was covered in this thesis consists of backbone methylated linear peptides. *In vivo expression technology* (IVET) analyses indicated the possible function of these peptides as virulence factors.[6] Unfortunately, only a few of these compounds have been isolated. This is due to their low solubility and their diversity which often results in inseparable compound mixtures. Backbone methylated peptides are rarely easily accessible by organic synthesis. The smaller peptides were obtained in relatively good yields upon synthesis in solution. Unfortunately, longer sequences could not be synthesized in solution due to their heightened insolubility. Hence, these peptides were synthesized on the solid phase using optimized permethylation conditions employing Li*t*OBu and CH_3I.

Zusammenfassung

Die hier vorliegende Dissertation befasst sich mit der Synthese von Naturstoffen aus *Xenorhabdus* und *Photorhabdus* spp. Da 6,0 - 7,5% ihres Genoms Sekundärmetabolit Clustern zuzuordnen sind, gelten diese entomopathogenen Bakterien als vielversprechende Naturstoffproduzenten. Die Palette der von ihnen produzierten Naturstoffe reicht von Antibiotika über Insektizide bis hin zu potentiellen Zytostatika. Die im Rahmen dieser Arbeit synthetisierten und charakterisierten Substanzen lassen sich in vier Kategorien einteilen: kleine Sekundärmetabolite (Phurealipide), zyklische Makrolaktame (Xenotetrapeptide, GameXPeptide und Ambactin), zyklische Makrolaktone (Szentiamide, Xentrivalpeptide und Xenephematide) und methylierte lineare Peptide (Rhabdopeptide und Rhabdopeptid-ähnliche Moleküle).

Im Rahmen des ersten Manuskripts dieser kumulativen Dissertation wurden die Phurealipide in *Photorhabdus luminescens* TTO1 identifiziert und charakterisiert. Hierbei handelt es sich um kleine Sekundärmetabolite, die sich aus einer Harnstoffentität und verschiedenen Alkylketten zusammensetzen. Der Strukturvorschlag der einzelnen Substanzen wurde auf Basis von Informationen erstellt, die in LC-MS/MS und Markierungsexperimenten *(stable isotope labeling)* im Wildtyp und einer *pliB*-Deletionsmutante gesammelt wurden. Zum Beweis dieser Strukturvorschläge wurden Phurealipide A-F und einige nicht-natürliche isobare Harnstofflipide sowie Glycinamide synthetisiert und nachfolgend in LC-MS/MS Analysen verglichen. Die Synthese der substituierten Harnstofflipide erfolgte durch eine Curtius Umlagerung. Die Glycinamide wurden durch eine Kondensationsreaktion in Lösung erhalten. Des Weiteren zeigte eine phylogenetische LC-MS/MS Analyse, dass eine Vielzahl von *Photorhabdus* und auch einige wenige *Xenorhabdus* Stämme diese Harnstofflipide produzieren. Dies weist darauf hin, dass es sich bei den Phurealipiden um wichtige (Virulenz-) Faktoren insbesondere für *Photorhabdus* spp. handeln könnte. Darüber hinaus ähneln die Phurealipid strukturell stark bekannten Inhibitoren der Epoxidase des juvenilen Hormons III (*juvenile hormone III epoxidase*), was das gehäufte Vorkommen in diesen entomopathogenen Bakterien erklären könnte.

Die zweite Publikation[7] befasste sich mit der Optimierung MS-basierter Strukturaufklärung von Naturstoffen, die eine zeitaufwendige Reinigung der einzelnen Moleküle obsolet macht. Die vorhergehende Arbeit von Helge B. Bode *et al* zeigte, dass es möglich ist mit Hilfe von Mutanten, die keine Transaminase aufweisen (*transaminase-deficient mutants)*, in Kombination mit Markierungsexperimenten auch Informationen zur Stereochemie

peptidischer Naturstoffe zu erhalten und die Strukturaufklärung zu vereinfachen.[8] Zur Verbesserung der stereochemischen Information wurde von Carsten Kegler *et al* ein *Escherichia coli* Stamm erstellt, der keine Transaminasen mehr aufwies.[7] Die Stereochemie der peptidischen Produkte von Clustern, die heterolog in diesem Stamm in Anwesenheit von markierten Bausteinen exprimiert wurden, wie z. B. das Xenotetrapeptid, konnte massenspektrometrisch eindeutig bestimmt werden. Um diese Methode und den dadurch erhaltenen Strukturvorschlag für das Xenotetrapeptid zu bekräftigen bzw. zu beweisen wurde das zyklische Makrolaktam mit Hilfe von Festphasenpeptidsynthese und nachfolgender Zyklisierung in Lösung hergestellt. Eine Isolierung des Naturstoffes aus der Bakterienkultur war auf Grund der geringen Produktion bzw. Löslichkeit des Produktes nicht möglich. Allerdings zeigte ein direkter Vergleich der LC-MS/MS Analysen, dass das synthetisierte Peptid mit dem Naturstoff von *X. nematophila* HGB081 übereinstimmte.

Die dritte Publikation beschäftigte sich mit den GameXPeptiden E-H, die von *Photorhabdus luminescens* TTO1 ausschließlich im Insekt produziert werden.[3] Die GxpS-Synthase, welche verantwortlich für die Produktion der zyklischen Pentapeptide ist, trägt unter diesen natürlicheren Bedingungen die „unnatürliche“ Aminosäure 4-Methylamino-D-phenylalanin (MMPAPA) an Stelle des „ursprünglichen“ D-Phenylalanins. Bei näheren Untersuchungen des Genoms konnte ein Cluster identifiziert werden, der verantwortlich für die Produktion des MMPAPAs ist. Allerdings ist dieser Cluster inaktiv unter den üblichen Laborbedingungen (z. B. die Anzucht in *Luria Bertani* Medium bei 30°C unter Schütteln). Die Substanzklasse der GameXPeptide ist deshalb ein hervorragendes Beispiel dafür, dass möglicherweise eine Vielzahl relevanter Naturstoffe bei der Anzucht unter Laborbedingungen nicht produziert werden und damit nicht identifiziert bzw. charakterisiert werden können.

Als ein weiterer Vertreter von natürlichen zyklischen Makrolaktamen wurde das Ambactin aus *X. miraniensis* DSM17902 synthetisiert.[4] Im Gegensatz zu dem Xenotetrapeptid und den GameXPeptiden, weist dieses zyklische Hexapeptid nicht nur hydrophobe sondern auch hydrophile Aminosäure auf. Deshalb mussten die Bedingungen der Zyklisierung in Lösung sowie die nachfolgende Aufarbeitung angepasst werden. Es stellte sich heraus, dass eine Senkung der Temperatur von 70°C auf 55°C unter Verwendung von *N*-(3-Dimethylamino-propyl)-*N′*-ethylcarbodiimide Hydrochlorid/1-Hydroxy-7-azabenzotriazole Hydrat (EDC/HOAt) oder *N*-[(Dimethylamino)-1*H*-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide /1-Hydroxy-7-azabenzotriazole hydrate (HATU/HOAt) als Aktivierungsreagenzien das gewünschte Produkt lieferte. Der Vergleich

zwischen synthetischen und natürlichen Produkt mit Hilfe von LC-MS/MS Analysen zeigte die Übereinstimmung der beiden Substanzen.

Die vierte Publikation stellte die Synthese und Charakterisierung des in *X. szentirmaii* DSM16338 vorkommenden Szentiamids vor.[9] Nach der erfolgreichen Synthese und weiterführenden biologischen Tests, konnte diesem zyklischen Depsipeptid, welches ursprünglich von Ohlendorf *et al* identifiziert wurde,[5] eine Aktivität gegen *Plasmodium falciparum* NF54, dem Erreger von Malaria, zugeordnet werden. Hierbei handelt es sich vermutlich um eine Kreuzaktivität, da *P. falciparum* ebenso wie im Boden lebende Amöben, die als Fressfeinde der Bakterien gelten, zu den einzelligen Protisten zählt.

Ein weiteres Beispiel zyklischer Depsipeptide stellen die Xentrivalpeptide dar, die von Qiuqin Zhou *et al* in *X. stokiae* DSM17904 identifiziert wurden.[10] Diese Substanzklasse zeichnet sich durch eine große Diversität aus, bis jetzt konnten siebzehn strukturell engverwandter Substanzen identifiziert werden, für deren Produktion ein und derselbe NRPS-Cluster verantwortlich ist. Diese Diversität in Kombination mit teilweise sehr niedrigen Produktionskonzentrationen verhinderte allerdings, dass ein Großteil der Peptide isoliert und damit genauer charakterisiert werden konnte. Deshalb wurden drei Vertreter, das Hauptprodukt, ein Ring-kontrahiertes Derivat sowie ein Produkt, welches nur in Spuren produziert wurde, für die Synthese ausgewählt. Im Zuge dieser Synthese wurden unterschiedliche Methoden zur Depsipeptid-Bindungsknüpfung untersucht. Hierbei stellte sich heraus, dass die optimalen Bedingungen für die Esterbildung stark abhängig sowohl von der Sequenz des linearen Precursors (des „Alkohols“) als auch von der Struktur der Carbonsäure sind. Im Falle der Xentrivalpeptide zeigte eine Prä-Aktivierung der Aminosäure mit 1-(Mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazol in Anwesenheit einer Base die besten Ergebnisse.

Mit den hier optimierten Methoden wurden weitere Depsipeptide, u. a. das kürzlich charakterisierte Xenephematide (Florian Grundmann, unpublizierte Ergebnisse), synthetisiert. Darüber hinaus wurde dieses Peptid als Modell-Substanz für das intrinsische Markieren auf Basis eines Fluoreszenz-Farbstoffes verwendet. Hierzu wurde an Stelle der Phenylessigsäure zur *N*-terminalen Derivatisierung die synthetisch leicht zugängliche 7-Dimethylamino-coumarin-4-essigsäure eingesetzt. Die so Floureszenz-markierten Peptide zeigten sowohl bei der Inkubation mit Lipidvesikeln als auch mit Insektenzellen eine Interaktion mit der Membranoberfläche.

Eine weitere Substanzklasse, die im Rahmen dieser Dissertation, bearbeitet wurde, bestand aus am Peptidrückgrad methylierten linearen Peptiden. *In vivo expression technology* (IVET)-Analysen wiesen darauf hin, dass diese Rhabdopeptide[6] und Rhabdopeptid-ähnlichen Strukturen *Photorhabdus* and *Xenorhabdus* spp. als Virulenzfaktoren dienen. Allerdings konnten bislang nur wenige dieser Peptide isoliert und charakterisiert werden, auf Grund ihrer geringen Löslichkeit und der großen Substanzvielfalt, die häufig in untrennbaren Gemischen resultierte. Methylierte Peptide sind i. d. R. schwer synthetisch zugänglich und traditionelle Synthesestrategien, insbesondere an der festen Phase, stoßen häufig an ihre Grenzen. Deshalb wurden die kleineren Peptide in Lösung hergestellt. Bei längeren Sequenzen wurde allerdings die Löslichkeit durch die Inkorporation weiterer hydrophober Aminosäure neben den Methylgruppen am Amidrückgrad soweit herabgesetzt, dass eine Synthese in Lösung nur noch schwer möglich war. Infolgedessen wurde die Synthese an der festen Phase durchgeführt. Hierzu musste insbesondere die Methylierungsreaktion optimiert werden. Eine Kombination von Li*t*OBu und CH_3I ergab die besten Permethylierungsergebnisse. Allerdings konnte im Zuge dieser Optimierung auch festgestellt werden, dass eine β-Verzweigung der Seitenkette die Methylierung der Amide graduell erschweren kann.

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10. Zhou, Q. *et al.* Xentrivalpeptides A–Q: Depsipeptide Diversification in *Xenorhabdus*. *J. Nat. Prod.* **75,** 1717-22 (2012).

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Friederike I. Nollmann

Chapters of this thesis have been published preliminary with the approval of the faculty which is represented by the mentor.

List of Selected Publications

Nollmann F. I., Dowling A, *et al*. Synthesis of szentiamide, a depsipeptide from entomopathogenic with activity against. *Beilstein J. Org. Chem.* **8,** 528-533 (2012).

Fuchs S. W., Sachs C. C., Kegler C., Nollmann F. I., Karas M, Bode HB. Neutral loss fragmentation pattern based screening for arginine-rich natural products in *Xenorhabdus* and *Photorhabdus*. *Anal. Chem*. **84,** 6948-55 (2012).

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Nollmann F. I., Heinrich A. K., Brachmann A. O., Morisseau C., Mukherjee K., Casanova-Torres A. M., Strobl F., Kleinhans D., Kinski S., Schultz K., Beeton M. L., Kaiser M., Chu Y. Y., Phan Ke L., Thanwisai A., Bozhüyük K. A., Chantratita N., Götz F., Waterfield N. R., Vilcinskas A., Stelzer E. H., Goodrich-Blair H., Hammock B. D., Bode H. B. *ChemBioChem*. **16,** 766-71 (2015).

List of Selected Contribution to Conferences

Nollmann, F. I., Dauth, C., Reimer, D., Bode, H. B. *European Peptide Symposium* **2012** "Peptides in *Xenorhabdus* and *Photorhabdus* spp." (Poster)

Nollmann, F. I., Bode, H. B. *American Peptide Symposium* **2013** "Peptides produced by *Xenorhabdus* and *Photorhabdus* spp., the insect killers" (Poster, aaptec Travel Grant Awardee)

Nollmann, F. I., Bode, H. B. *German Peptide Symposium* **2013** "Killing two birds with one stone: a new total synthesis of xenematide A through D" (Talk)

Nollmann, F. I., Reimer, D., Bode, H. B. *German Peptide Symposium* **2013** "Synthesis of the highly methylated rhabdopeptides found in *Xenorhabdus* spp." (Poster)

Nollmann, F. I., Reimer, D., Cowles, K. N., Goodrich-Blair, H., Bode, H. B. *25. Irseer Naturstofftage* **2013** "Insect-specific production of natural products" (Poster + short talk)

Nollmann, F. I., Bode, H. B. 8th Status Seminar Chemical Biology **2013** "Looking for the target: Synthesis of peptides found in *Photorhabdus* and *Xenorhabdus* spp." (Poster + short talk)

INTRODUCTION

The most exciting phrase to hear in science, the one that heralds the most discoveries, is not “Eureka!” (I found it!) but “That's funny...”.

Isaac Asimov

1 Introduction

With the discovery of penicillin by Alexander Fleming, which marks a milestone in medical treatment and the beginning of rational and widespread screening of microorganisms, civilization became aware of its dependence on drugs, which heal, help and prolong life (Figure 1).

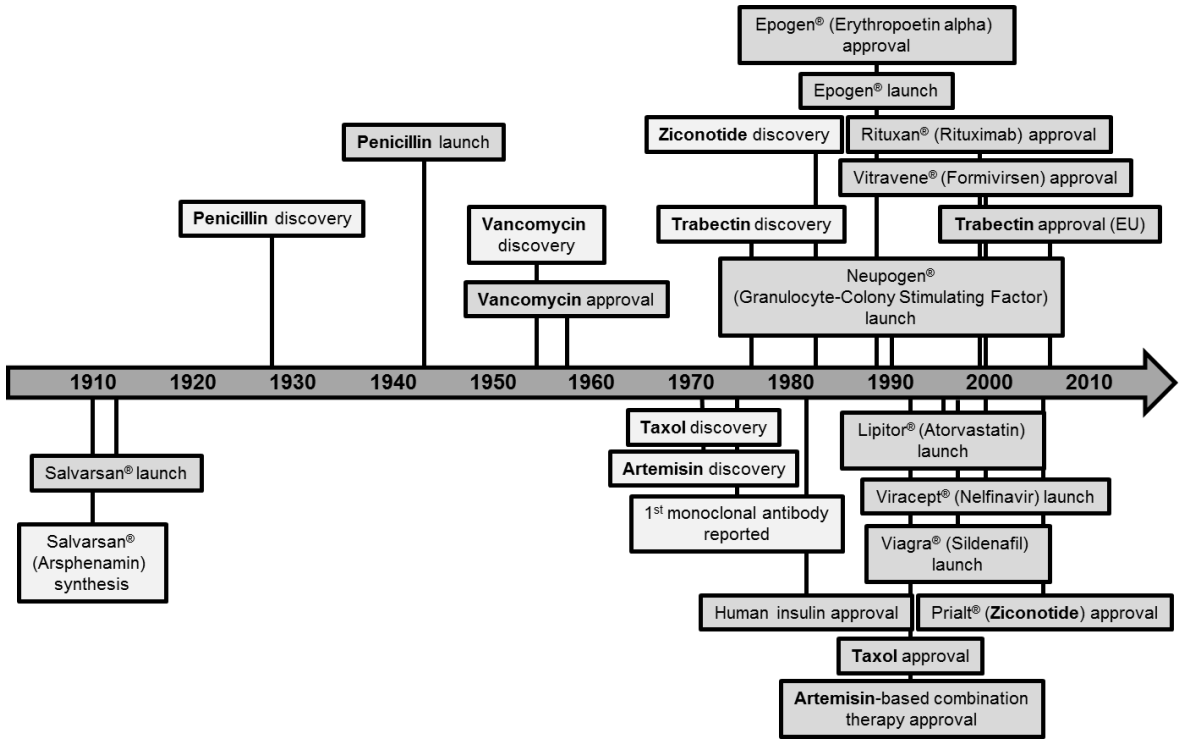

Figure 1: Overview on drug development between 1910 and 2010, secondary metabolites are bold.[1-3]

Nevertheless, the alarmingly development of bacterial resistances as well as diseases, which can be treated but cannot yet be cured or only at high costs to the patient, motivated and still motivates research. Apart from the combinatorial approach,[4-6] which is based on a known target, the search for new natural products as lead structures currently experiences a renaissance. In 2010 an analysis of the drugs approved by the FDA stated that approximately 10% were natural, not altered drugs, 29% were semi-synthetic natural product derivatives and over 61% were of a synthetic origin.[7] In the 1990s the share of natural or semi-synthetic drugs only accounted for 34%.[8] Most of these substances, e. g. taxol or artemisinin (Figure 1), are derived from secondary metabolites of higher plants. All in all, 160,000 natural products have already been structurally elucidated and characterized, a value growing by 10,000 per year. Approximately 100,000 of these are produced by plants, which amounts to nearly 20% of the probable total number of herbal secondary metabolites (estimated to be between 500,000 and 600,000). Though plants remain the most potential source of biological active substances until

today, several interesting compounds, especially toxins and neurotoxins, e. g. trabectedin or ziconotide (Figure 1) as well as (tetracycline) antibiotics, have been discovered after screening marine organisms and microrganisms.[9] Nevertheless, only a little more than 20,000 microbial secondary metabolites are known, most of them being produced by *Streptomyces* spp..[10] Considering the vast number of the different microbial classes and families in addition to the unidentified ones, the potential of microorganisms as source for bioactive natural compounds still remains untapped.[10,11]

1.1 Xenorhabdus and Photorhabdus spp.

Bacteria of the genus of *Xenorhabdus* or *Photorhabdus* belong to the class of Gram-negative γ-proteobacteria, which live in a unique mutualistic symbiosis with nematodes of the genus *Steinernema* and *Heterorhabditidae*, respectively. With the help of phylogenetic analysis three species of *Photorhabdus* (*P. luminescens*, *P. temperata* and *P. asymbiotica*)[12] and twenty highly diverse species of *Xenorhabdus* (to name a few: *X. nematophila, X. szentirmaii* and *X. indica*) have been determined so far. Both, *Photorhabdus* and *Xenorhabdus* spp., display rod shaped, asporogenous and peritrichous flagellated cells. All so far characterized strains belong to the class of mesophilic bacteria with an optimal growth temperature between 28°C and 30°C, only some strains exhibiting an even broader temperature range between 16°C and 38°C. During their mutually beneficial symbiosis, nematodes and bacteria undergo a complex life cycle (Figure 2).

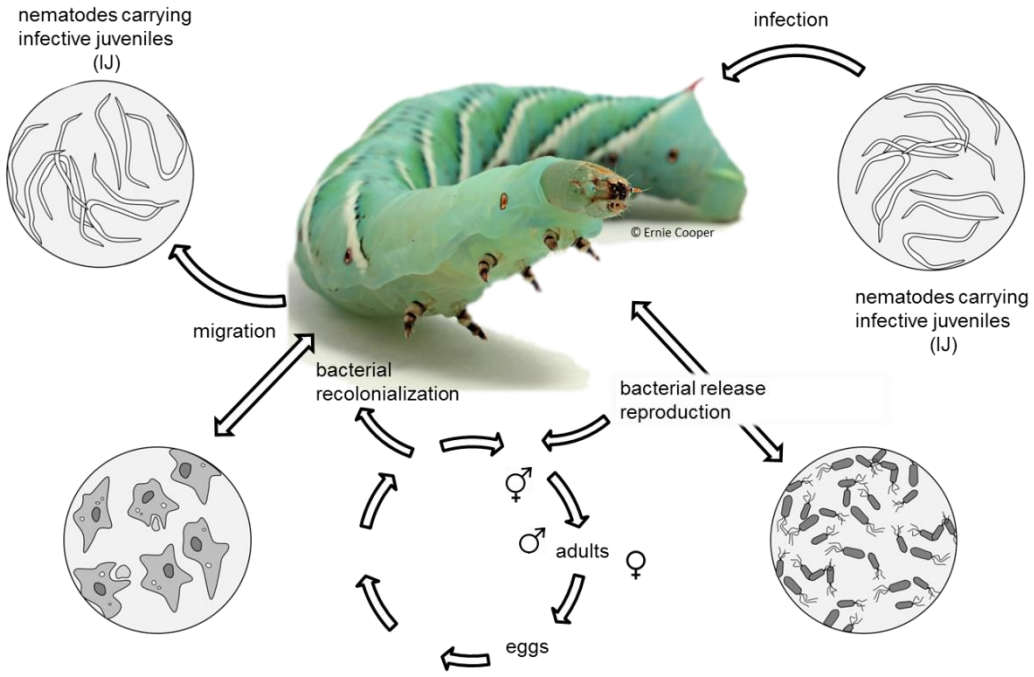

Figure 2: Lifecycle of the nematode-bacteria association, adapted from Herbert *et al.*[13]

For the association between bacteria and nematode the infective juvenile (IJ) stage is vital. In this stage the nematode carries between approximately 50 and 200 colony-forming units of the bacteria. These IJs can survive for long periods of time in the soil before locating and entering an insect host. Once the nematodes have entered an insect larvae through its natural openings (*Steinernema* and *Heterorhabditidis* spp.) or even directly by penetration (only *Heterorhabditidis* spp.), they migrate to the insect blood cavity. There they begin to ingest the blood which triggers the release of the bacteria into the hemocoel. The bacteria start to multiply and produce several different factors that ensure the survival of their nematode host. First they produce compounds that influence or even suppress the insect's immune response and eventually kill the larvae. Then, after the death of the prey, they release compounds that will protect their food source from other opportunistic infections, e. g. other bacteria, amoeba or fungi, as well as natural predators, e. g. ants. Moreover, the bacteria seem to be involved in the conversion of the cadaver into biomass, which bears substrates that support and influence the nematodes' development. Within the cadaver the nematodes undergo four different juvenile stages until adulthood and mating, followed by egg deposition. Hundreds of thousands of these eggs will eventually develop into the pre-infective stage and be re-colonized by the bacteria. Then these colonized IJs will emerge from the larvae, thus completing the life cycle.[14,15] Undergoing their complex and partly entomopathogenic life cycle,[13,14,16] the bacteria not only produce highly active antibiotics and insecticides[17] but also a great variety of different small molecular weight compounds and peptides.[15]

1.2 Secondary metabolites

Secondary metabolites are generally defined as organic compounds which are not directly connected to reproduction, development or growth of an organism. In contrast to primary metabolites their absence does not result in an immediate death, but rather leads to long-time impairment of the organism or does not have any influence at all. They usually display great diversity of chemical structural elements and biological activities. Moreover, secondary metabolites are often derived from complex biosynthetic pathways and they are strain specific in many cases. Hardly surprising is that their production is directed by organized sets of genes which are connected to special control mechanisms that are well incorporated into the organism's physiology.[18] The majority of microbial metabolites can be classified roughly as derivatized (amino) acids, polyketides, peptides or polyketide-peptide hybrids based on their structure and the underlying biochemistry (Figure 3). For example, chloramphenicol, a well-known bacteriostatic antibiotic, was isolated from a *Streptomyces* strain in 1947.[19,20] The

biosynthesis of this very effective wide range antibiotic originates in the shikimate pathway for assembling aromatic metabolites.[21] The chorismic acid is eventually converted into *p*-amino phenylalanine, which then serves as a precursor of the *p*-nitrophenylserinol component of chloramphenicol. Another class of low molecular weight compounds, which had a big impact on human welfare, is without doubt penicillin and its derivatives (**3**). Penicillins are based on the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine. This tripeptide is then enzymatically converted into isopenicillin N, which bears the typical β-lactam thiazolidine ring structure and already displays antibacterial activity. The final step is the exchange of the α-aminoadipyl side chain by a (derivatized) carboxylic acid.[22,23]

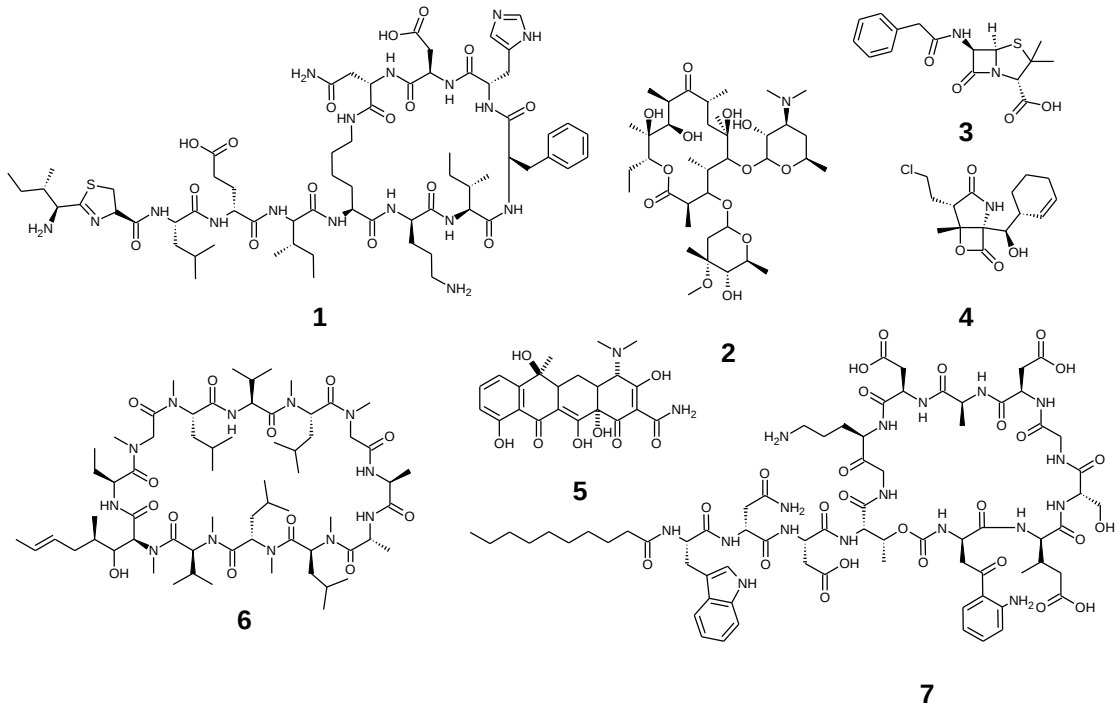

Figure 3: Well-known prokaryotic secondary metabolites; bacitracin (**1**; from *Bacillus subtilis*),[24] erythromycin A (**2**; from *Streptomyces erythreus*),[25] penicillin G (**3**; from *Penicillium notatum*), salinosporamide A (**4**; from *Salinispora tropica*),[26] tetracycline (**5**; from *Streptomyces aureofaciens*),[27,28] cyclosporine A (**6**; from *Cylindrocarpon lucidum*)[29] and daptomycin (**7**; from *Streptomyces roseosporus*).[10,11,30,31]

Other well-known representatives for peptidic structures with biological activities are for example bacitracin (**1**)[24,29], cyclosporine (**6**)[32] and daptomycin (**7**).[33-35] These peptides highlight the structural diversity of compounds produced by nonribosomal peptide synthetases (NRPSs). They bear different ring systems, backbone methylations and highly unusual amino acids. Typical polyketidesynthase (PKS) derived compounds, which was found to have a pharmaceutical purpose, are for example the erythromycin A (**2**)[25,31] and the class of

tetracycline antibiotics (represented by **5**).[27,28] These polyketides are produced by several *Streptomyces* strains and have been used widely to treat bacterial infections.

Photorhabdus and *Xenorhabdus* spp. are unique sources for novel natural products (Figure 4), because of their specific adaption to their ecological niche. Moreover, they are part of an exceptional yet well-defined system, which can be reduced to the nematode, as the symbiont, the insect, as the prey, other organisms, as food predators, and the bacteria itself. Research has been able to shed some light on mechanisms underlying this complex interaction of friend and foe. Firstly, the bacteria produce several different substances that are important for the interaction with their nematode-host and its development, e. g. the crystalline inclusion proteins CipA and CipB and IP1 and IP2, respectively, produced by *Photorhabdus* and *Xenorhabdus*, respectively, which contribute to the nematode's growth.[15,16]

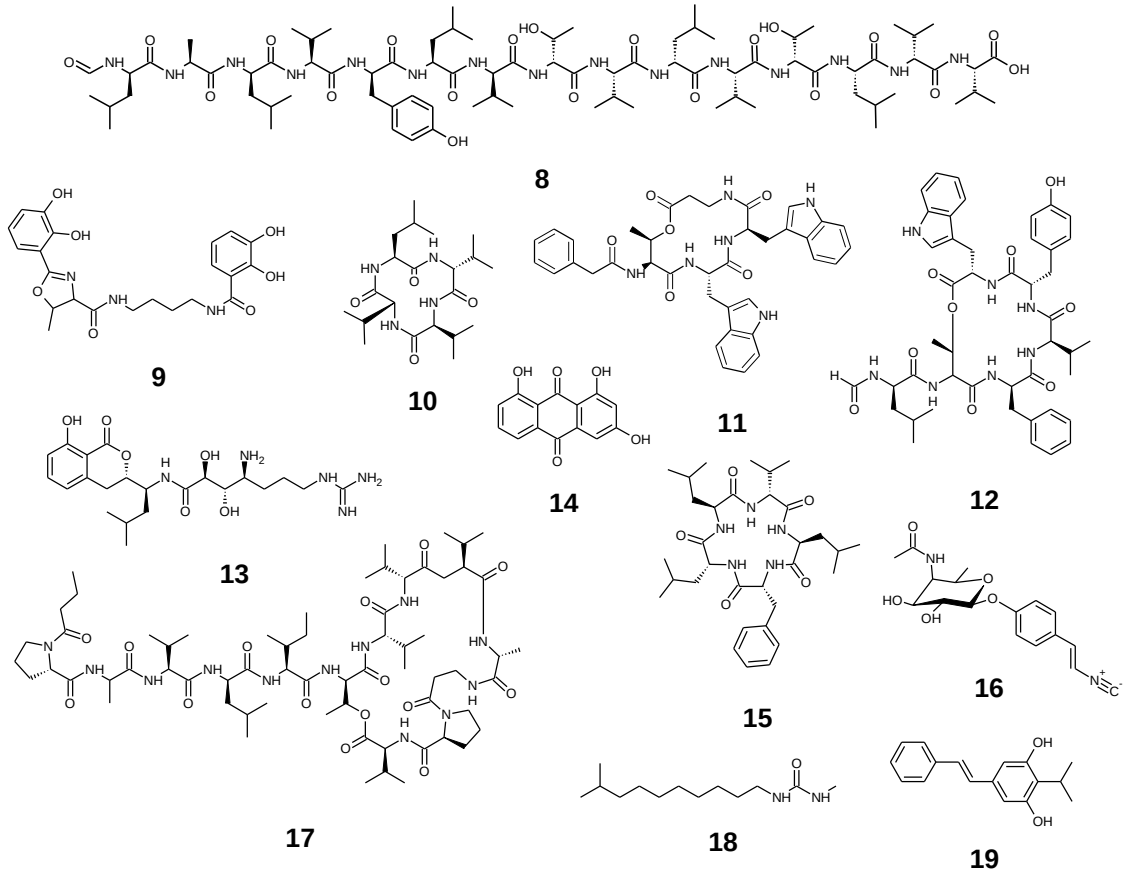

Figure 4: Some examples for secondary metabolites produced by *Xenorhabdus* and *Photo¬rhabdus* spp.; kolossin (**8**; from *P. luminescens*, unpublished data), photobactin (**9**; from *P. luminescens*),[36] tetrapeptide (**10**; from *X. nematophila*),[37] xenematide A (**11**; from *X. nematophila*),[38] szentiamide (**12**; from *X. szentirmaii*),[39] xenocoumacin (**13**; from *X. nematophila*),[40] anthraquinone (**14**; from *P. luminescens*),[41] GameXPeptide A (**15**; from *P. luminescens*, however found in several other strains as well), rhabduscin (**16**; from *X. nematophila* and *P. luminescens*),[42] xenoamicin A[43] (**17**; from *X. doucetiae* and *X. mauleonii*), phurealipid A (**18**; from *P. luminescens*)[44]and the hydroxy stilbene (**19**; from *P. luminescens*).[41,45]

Secondly, there are compounds like rhabduscin (**16**) and the phurealipids (**18**), which actively influence the prey's immune response. The first inhibits the phenoloxidase (PO), which is responsible for the melanization triggered by humoral activation.[13] The latter probably inhibits the juvenile hormone III epoxide hydrolase and as a result modulates the production of antimicrobial peptides.[46] To fight off predators and food competitors, they also produce several different compounds. Anthraquinones (**14**; Figure 4) are known to have antiplasmodial activity and are commonly described as bird repellent.[47-49] The cyclic depsipeptide szentiamide (**12**) displays an antiplasmodial activity.[50] The xenematides (**11**) were published as being insecticidal in addition to an antibacterial activity against *Bacillus subtilis*, a common soil bacterium.[38] Another potent antibacterial compound class consists of the so called xenocoumacins (**13**). The products of the corresponding PKS-NPRS hybrid display a unique pro-drug activation mechanism which can be found throughout different bacterial taxa. Another interesting compound class are the stilbenes (**19**). They are *Photorhabdus'* jack of all trades, since they have antibacterial and antioxidative as well as PO-inhibitory activity. However, their major purpose lays in their function during the advancement from the IJ stage to another stage in the life cycle of the nematode.[45] Nevertheless, the purpose of several secondary metabolites, which are produced at high energy cost to the bacterium, is still unclear. Some examples for compounds, whose activities remain in the shades, are kolossin (**8**; Alexander Brachmann, unpublished data), the xentrivalpeptides,[51] the tetrapeptide (**10**)[37] and the xenoamicins (**17**).[43]

1.2.1 Nonribosomal peptide synthetase-derived secondary metabolites

Nonribosomal peptides (NRPs) often display big pharmaceutical potential as they are usually bioactive. They are assembled by NRPSs, which are composed of large multienzyme complexes that catalyze stepwise amino acid condensations.[52,53] These ribosome-independent processes manage highly regio- and stereospecific reactions which in turn lead to structurally as well as functionally diverse peptide scaffolds. During these processes the catalytic domains select, activate and modify the covalently bound intermediates in order to control the chain elongation and release of the product (Figure 5). Moreover, this machinery is not limited by the twenty proteinogenic amino acids as substrates, but hundreds of different building blocks.[52,54] Three domains are quintessential in NRP synthesis, the adenylation (A) domain, responsible for the activation of amino acids (building blocks), the peptidyl carrier protein (PCP), responsible for the thiolation and therefore, propagation of the growing peptide chain, and the condensation (C) domain, responsible for the condensation of amino acids (building

blocks). A fourth commonly found domain, which is associated with the product release, is the thioesterase (TE) domain. Usually a C- and an A-domain form together with the PCP a module, which can be classified according to its role in the biochemical pathway as initiation, elongation or termination module. The initiation module often lacks a condensation domain in its beginning and its solely purpose is providing the first amino acid. Then the condensation reaction is carried out by the C-domain of the succeeding module. All of the following modules are called elongation modules, except for the last module, which is known as a termination module. In this module the TE domain is usually situated and triggers the release of the product via hydrolysis or macrocyclization.

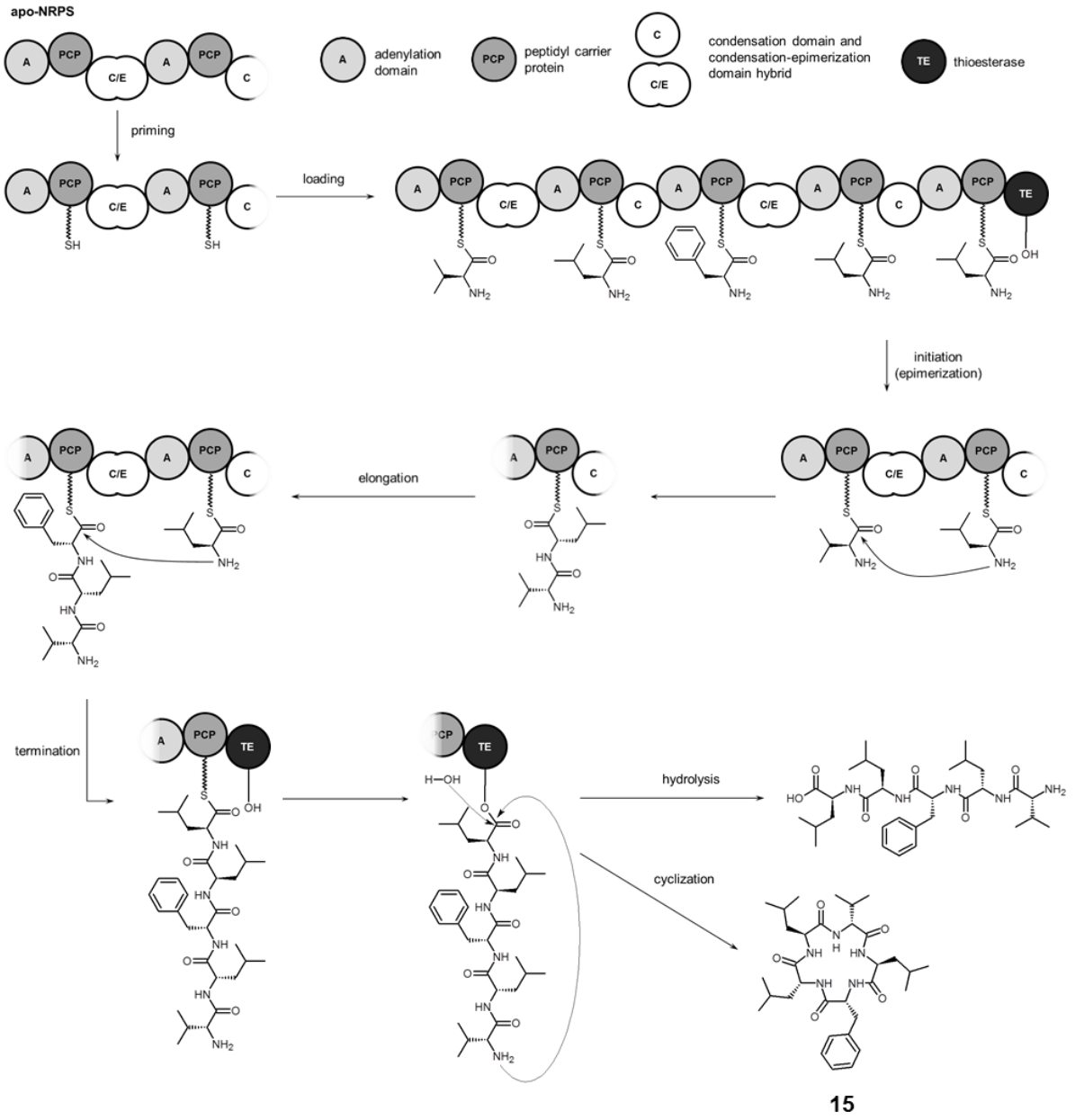

Figure 5: NRPS assembly line, exemplified by the synthesis of GameXPeptide A.[55]

The final products of the NRPS can be divided into linear, cyclic, branched and complex structures, which consist of more than one cycle and sometimes display additional branching.[29] The vast majority of NRPs display a cyclic or linear structure (Figure 6). Additionally, they are heavily modified through epimerization, methylation and acylation as well as hydroxylation or heterocyclization. Additionally, complex structures usually bear sugar groups and/or oxidative cyclized phenolic side chains, e. g. vancomycin.[56] As seen before, NRPS-derived secondary metabolites display unique features resulting from their biosynthesis, like D-amino acids, macro- and heterocycles (e. g. the PAX peptide (**21**) or the FABclavine (**22**)), *N*-/*O*-methylation (e. g. rhabdopeptides such as **20**) or ester bonds, as well as the incorporation of fatty acids or non-proteinogenic amino acids. Some of these chemical features lead to challenging problems for chemists in the attempt to synthesize or modify these natural products.

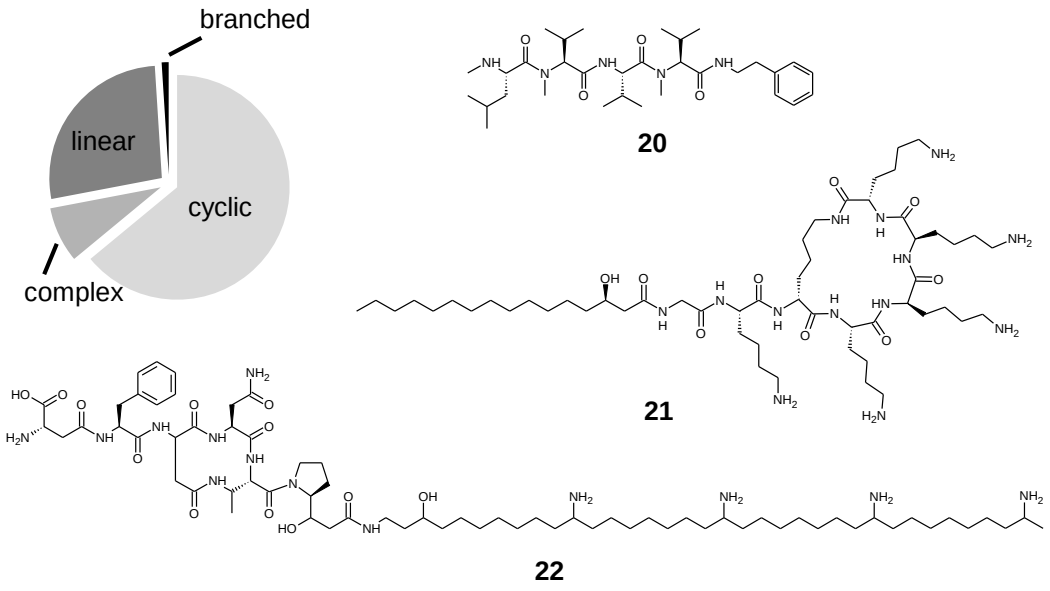

Figure 6: Structural classification of NRPs exemplified by peptides found in *Xenorhabdus* and *Photorhabdus* spp.[29] Rhabdopeptides, for example **20**,[57] belong to the class of linear natural product. Moreover, they display a uniquely methylated backbone. The PAX peptide (**21**)[58] as well as the FABclavine[59] can be categorized into the class of cyclic natural products.

1.3 Organic Peptide Synthesis and its Limitations

More than one hundred years ago, in 1901,[60] the first coupling of two amino acids to form a free dipeptide was achieved by Emil Fisher using acyl chlorides (the protected analogue, benzoylglycylglycine, had already been prepared a decade earlier by Theodor Curtius).[61] But until 1932 it was inconceivable to synthesize longer sequences due to the unavailability of suitable *N*-terminal protecting groups and activating agents. Then the development of the first

protecting groups by Leonidas Zervas and Max Bergmann made directed peptide synthesis possible.[62] The development of novel and orthogonal protecting groups, e. g. the Boc-group by Louis A. Carpino[63] and Frank C. McKay and Noel F. Albertson,[64,65] was accompanied by an intensive search for new coupling reagents. In the course of this research the development of carbodiimide-based activation reagents[66] has to be mentioned as a major achievement. However, the approach to synthesize on a solid support by Robert B. Merrifield[67] several years later truly revolutionized peptide chemistry.[68]

1.3.1 Solution Phase Synthesis

Until the late seventies of the last century solution phase synthesis of peptides was one of the most important tools in the investigation of biochemical coherence.[68,69] Though solution phase synthesis is burdened with time consuming procedures which require certain experience and knowledge, it has been possible to synthesize several challenging sequences in the past decades such as human insulin or the neurohormone oxytocin (by Vincent du Vigneaud in 1953, awarded with a Nobel prize two years later). Although solid phase synthesis has shifted more and more into focus, synthesis in solution still has its eligibility. Even today solution phase synthesis is fancied when difficult and complex structures or subunits have to be mastered. Especially highly methylated peptides are difficult to synthesize on the solid support and therefore, are preferably obtained by synthesis in solution. Recently, cyclosporine A has been synthesized entirely in solution employing several subsequent four component Ugi reactions and paving the road for more detailed mapping of its structure-activity relationship (SAR).[32] Although, entire peptides still are synthesized in solution, the bigger share of reactions in solution is employed to prepare building blocks, e. g. *N*-methylated/alkylated amino acids, non proteinogenic amino acids or dipeptide building blocks or bigger segments of difficult sequences, which then in turn are used during the synthesis on the solid support.

1.3.2 Solid Phase Synthesis

Though a vast amount of literature addressed the preparation and modification of polymers, the use of insoluble polymers linked reversible to a growing peptide chain was without chemical precedent when Bruce Merrifield started working on this matter in 1959.[68] In contrast to naturally occurring peptide synthesis, the sequence elongation starts out with the *C*-terminal amino acid immobilized on the insoluble support via a cleavable linker. Amino acids and coupling reagents have to be applied in excess, but can be washed away thus minimizing purification issues.[69] Moreover, at today's state-of-art of science and technology

the individual reaction steps can be easily automized, leading to better time management. These principles make solid phase peptide synthesis simple but yet charming.

Nevertheless, some peptide sequences, e. g. β-sheet structures or hydrophobic peptides, are categorized as difficult sequences due to the problems that might arise during their synthesis. For one, a high abundance of hydrophobic side chains or side chain protection groups[70] together with the van der Waals forces within the peptide backbone[71] can lead to intra- and intermolecular aggregation.[72] These aggregation processes then result in low solvation of the solid support, which implies low accessibility of the reagents and a varying degree of sterical hinderance of the *N*-terminus.[73] This suffers the consequence of incomplete reaction or loss of the *N*-terminal protection group, which then leads to chain termination or deficient sequences. But not only hydrophobic peptides but also sequences that incorporate polar or aromatic and especially alkylated amino acids bear the risks of side reactions. For example, non-specific intramolecular ringformations can occur and lead to e. g. aspartimide or diketopiperazine formation.[74] Moreover, some amino acids can make the synthesis more challenging, e. g. tryptophan, which is prone to be alkylated during synthesis and/or cleaved from the resin, and cysteine, which is readily oxidized and has to be protected in a complex manner in order to prevent the undirected formation of disulfide bonds, as well as arginine which tends to hinder the coupling of the subsequent amino acid due to its bulky side chain protecting groups.

1.3.3 Limitations and how to Circumvent them

Peptide synthesis neither in solution nor on the solid phase is flawless. In contrast to the enzymatic catalyzed biosynthesis, depending on the amino acid sequence and content, difficulties may arise, that have to be overcome. During the peptide synthesis in solution the solubility of fully protected peptide fragments drops significantly with the growing of the peptide chain. These solubility issues can be answered by a so called soluble-tag assisted liquid phase method, which has been successfully employed to synthesize several different natural or natural derived peptides.[75-77] One example for this method is the total synthesis of mahafacyclin B, a cyclic hexapeptide with antimalarial activity found in the latex of *Jatropha mahafalensis*.[78,79] Though the hydrophilic tail adds to the solubility of the intermediate, it probably also helps the cyclization reaction changing the peptide's conformation and making the ring closure more favorable. Another side reaction not only in solution but also on the solid support is the formation of diketopiperazines which often occurs during the synthesis of methylated or alkylated peptides. This undesired cyclization is facilated by *N*-alkylation and a *C*-terminal leaving group in form of an ester.[80-83] It can be suppressed to a certain extent by

employing spatial demanding linker[80] or certain coupling reagents with the synthesis on the solid support.[81,83]

Without doubt the occurrence of unwanted side reactions is one of the mayor problems of the solid phase peptide synthesis, because they will eventually lead to a massive decrease in yield and purity of the final product. To circumvent some of these side reactions, e. g. aggregation, miss coupling or truncation, different auxiliaries can be incorporated in the growing peptide chain or different resin matrices can be employed. The most prominent auxiliaries are turn inducers, like pseudoprolines based on threonine, serine and cysteine or derivatives thereof, and isoacyl dipeptides as well as *N*-2-hydroxy-4-methoxybenzyl- (Hmb-)[84] or hydroxyl-substituted nitrobenzyl- (Hnb-)[85,86] derivatized amino acids which take advantage of the *N*-*O* shift at elevated pH. To obtain peptides with longer chain lengths or even entire proteins, ligation methods have to be employed. Today the most commonly known ones are the native chemical and KAHA ligation as well as traceless Staudinger reactions.[87]

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OBJECTIVES

If we knew what it was we were doing, it would not be called research, would it?

Albert Einstein

2 Objectives

The aim of this work was to gain new insights on the purpose and biological function of secondary metabolites produced by *Xenorhabdus* and *Photorhabdus* spp. Therefore, some of these natural products were synthesized and submitted to continuative testing or derivatization.

The research focused on the characterization and synthesis of peptides, to be divided in cyclic hydrophobic peptides, cyclic depsipeptides and linear highly methylated peptides as well as small weight molecules, represented by the urea lipids and phenylethylamides. In order to be able to determine the biological function and in some cases the specific biological target strategies had to be developed to meet the needs and requirements of the synthesis or assay.

The peptides, which are found in *Photorhabdus* or *Xenorhabdus* and already have been characterized, usually are products formed by non-ribosomal peptide synthetases. Due to the fact that they are not ribosomally made these peptides show some unique features, e. g. incorporation of D-amino acids or unusual building blocks as well as methylation or cyclization. Therefore, the standard peptide synthesis strategies on the solid support had to be modified or replaced by hybrid synthesis, which combines solution phase and solid phase peptide synthesis. In the case of smaller peptides (up to three building blocks) it was resigned from solid phase synthesis and these reactions were conducted in solution.

In order to identify the biological target, three different approaches were employed, light-induced labeling of the target proteins followed by reaction with a fluorophor (i), direct labeling of the probe (ii) or biotinylation followed by an binding affinity extraction (iii).

SMALL MOLECULES

[Science is] an imaginative adventure of the mind seeking truth in a world of mystery.

Sir Cyril Herman Hinshelwood

A *Photorhabdus* natural product inhibits insect juvenile hormone epoxide hydrolase

Friederike I. Nollmann, Antje K. Heinrich, Alexander O. Brachmann, Christophe Morisseau, Krishnendu Mukherjee, Ángel M. Casanova-Torres, Frederic Strobl, David Kleinhans, Sebastian Kinski, Katharina Schultz, Mike Beeton, Marcel Kaiser, Ya-Yun Chu, Long Phan Ke, Aunchalee Thanwisai, Kenan A. J. Bozhüyük, Narisara Chantratita, Friedrich Götz, Nick R. Waterfield, Andreas Vilcinskas, Ernst Stelzer, Heidi Goodrich-Blair, Bruce D. Hammock, Helge B. Bode

Abstract

Simple urea compounds named phurealipids have been identified from entomopathogenic *Photorhabdus luminescens* bacteria. Very similar analogs of these compounds have been previously developed as inhibitors of juvenile hormone epoxide hydrolase (JHEH), a key enzyme in insect development and growth and indeed phurealipids also inhibit JHEH. Manipulation of JHEH activity by phurealipids during a normal infection can also serve to modulate the insect immune system and therefore contribute to bacterial virulence. Our findings illustrate the principle of convergent evolution, although unexpectedly in this case, between human technology and a bacterial natural product.

Author's Effort

The author synthesized the phurealipids, collected and analyzed the obtained analytical data. Moreover, the author wrote this publication together with Helge B. Bode.

CYCLIC PEPTIDES

Science is simply common sense at its best.

Thomas Huxley

Synthesis of the Xenotetrapeptide produced by *Xenorhabdus nematophila*

This synthesis has been published in shorter length in

Rapid Determination of the Amino Acid Configuration of Xenotetrapeptide

Carsten Kegler, Friederike I. Nollmann, Tilman Ahrendt, Florian Fleischhacker, Edna Bode and Helge B. Bode

Abstract

An *E. coli* strain with deletions in five transaminases (Δ*aspC* Δ*ilvE* Δ*tyrB* Δ*avtA* Δ*ybfQ*) was constructed to be unable to degrade several amino acids. This strain was used as an expression host for the analysis of the amino acid configuration of nonribosomally synthesized peptides, including the novel peptide "xenotetrapeptide" from *Xenorhabdus nematophila*, by using a combination of labeling experiments and mass spectrometry. Additionally, the number of D-amino acids in the produced peptide was assigned following simple cultivation of the expression strain in D_2O.

Author's Effort

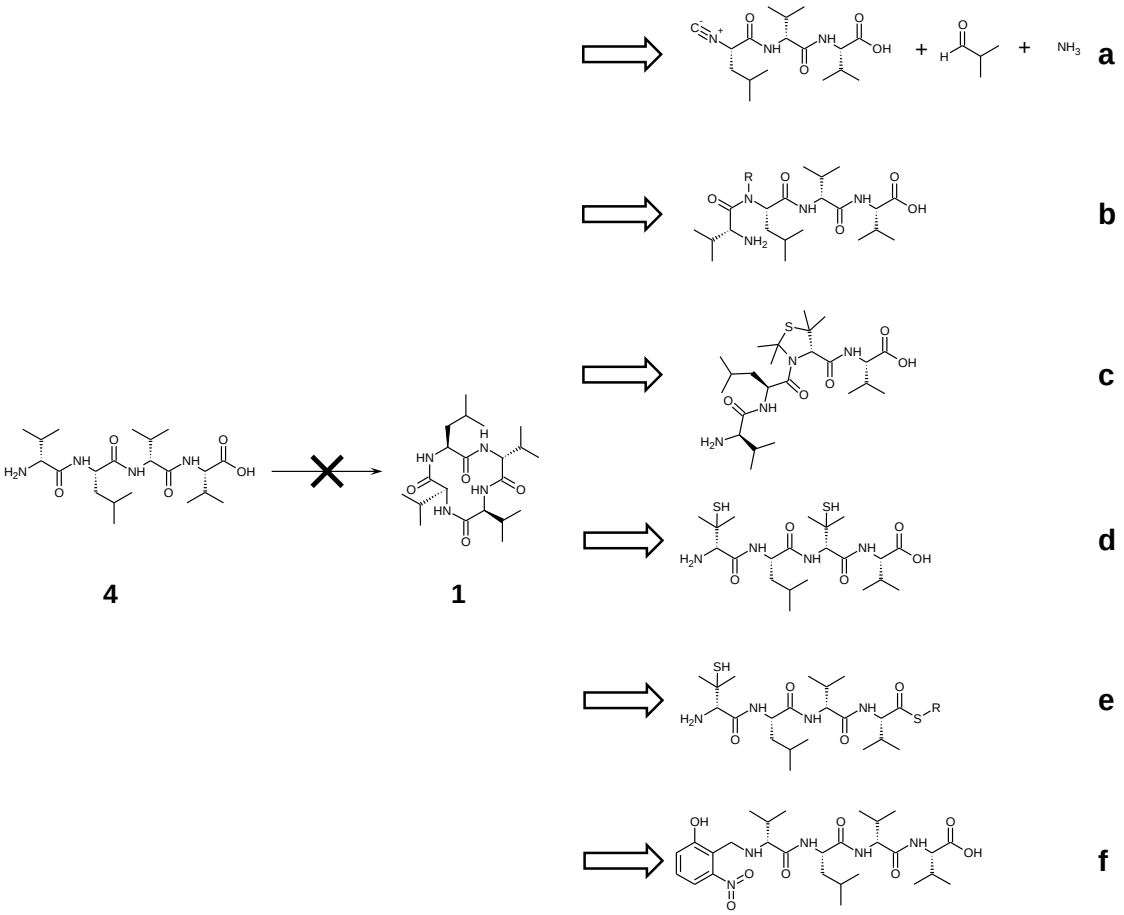
The author designed and conducted the synthesis of the Xenotetrapeptide leading to the proof of the structural proposal.

4.1 Synthesis of the Xenotetrapeptide produced by Xenorhabdus nematophila

Natural head-to-tail cyclized tetrapeptides are usually characterized as highly potent and selective substances which cover a diverse range of therapeutic areas. Their very rigid 12-membered ring makes them unique and probably plays a big role in their target specificity. However, this rigidity is also responsible for difficulties arising during their synthesis. These so-called difficult cyclic sequences favor during the cyclization reaction the formation of linear and cyclic oligomers over the cyclic monomer even at high dilution conditions.[1] Thus, these compounds, which are usually bioactive and promising candidates in drug discovery, are hardly ever taken into consideration for further development.

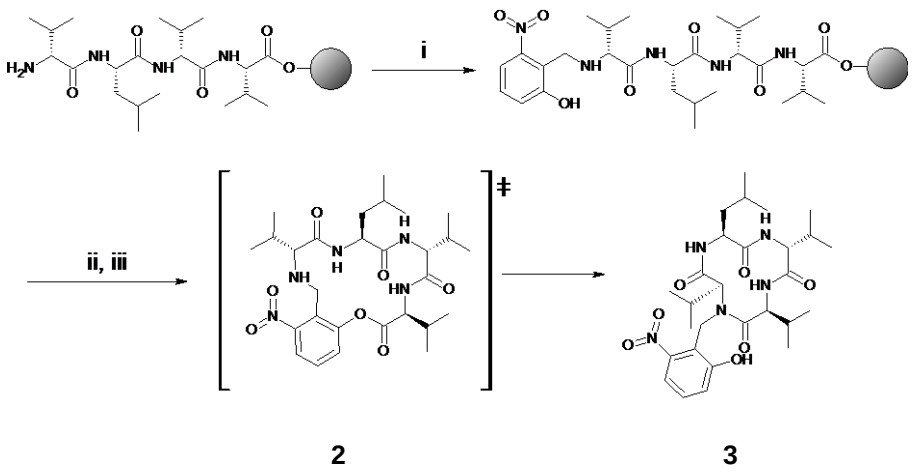
Xenorhabdus nematophila HGB081 produces, amongst others, a tetrapeptide consisting of D-/L-valine and L-leucine (Scheme 1), the so-called xenotetrapeptide (**1**). Since this compound is only produced in minute amounts in addition to a low solubility in aqueous media, it is difficult to purify it. Therefore, it had to be made accessible to further testing by organic synthesis. Using standard Fmoc/^{t}Bu-strategy the linear sequence was assembled on resin, cleaved (**2**) and cyclized in solution. Unfortunately, neither the cyclic monomer nor a dimeric side product nor the linear starting material was detected in the HPLC analysis of the crude mixture (Christina Dauth, Veronica Dill; data not shown). Thus, a different synthesis approach had to be found. Since the cyclization occurs head-to-tail via a macrolactam formation, different synthesis strategies that involve and/or facilitate establishing this amide bond were taken into consideration. One possibility is the Ugi reaction (Scheme 1a), a four component reaction, which has been employed widely for the generation of peptoid libraries and synthesis of sterically hindered building blocks.[2,3] Unfortunately, the Ugi reaction leads to a racemic mixture of the newly formed chiral center (varying in the proportion depending on temperature, solvent and reactants). Another possibility is the reversible alkylation of the amide backbone (Scheme 1b) in order to lower the flexibility of the peptide chain and force the termini in a closer proximity. The major disadvantages of this technique are the facilitation of diketopiperazine formation and harsh coupling conditions that have to be employed, because of the steric hindrance of the *N*-terminus by the bulky protection group and spatial valine residue. A recently developed approach to make cyclic peptides (which do not contain threonine or serine residues) more accessible is the incorporation of pseudoprolines on the basis of cysteine or its derivatives (Scheme 1c). More intensive studies have shown that turn inducers of this kind can elevate the cyclization yield, but they may also give rise to epimerization (depending on the sequence and the cyclization conditions). [4] Based

on the possibilities in sulfur chemistry, the incorporation of penicillamines instead of valines could be an option (Scheme 1d).[4,5] These sulfur bearing amino acids might lead to a better solubility of the cyclized intermediate and would allow purifying it more easily prior to the desulfurization. Another possibility, which takes advantage of the attachment of sulfur containing structures, is the native chemical ligation (Scheme 1e). This technique is usually used to synthesize larger structures, e. g. proteins, but has also been reported as being helpful in cyclization reactions.[6,7]



Scheme 1: Xenotetrapeptide (**1**) found in *X. nematophila* and possible synthetic strategies to obtain it; Ugi reaction (a) leading to a racemic mixture, reversible *N*-alkylation (b) using commonly known backbone protection groups in order to rigidify the flexible amide bond, incorporation of pseudoprolines (c) as turn inducers or penicillamines (d) in order to elevate the solubility and proton affinity of the intermediate, native chemical ligation (e) based on an *N*-terminal penicillamine and a *C*-terminal thioester, ring contraction strategy using the auxiliary 2-hydroxy-6-nitrobenzaldehyde (f).

Though, a different approach, which is based on the auxiliary 2-hydroxy-6-nitrobenzaldehyde (Hnb; Scheme 1f) and an *N*-*O*-acyl shift occurring at elevated pH, was chosen for the synthesis of the tetrapeptide. In contrast to the other methods, which are presented here, the use of the auxiliary not only facilitates the ring formation via a less constrained intermediate (**2**), but also allows purifying it more easily due to the incorporation of a chromophore (**3**).



Scheme 2: Ring contraction strategy employed to synthesis the photolabile tetrapeptide precursor. First the free *N*-terminus is reductively alkylated with the auxiliary (i, 10eq 2-hydroxy-6-nitrobenzaldehyde, 20eq $NaBH_3CN$ in a mixture of methanol and dimethylformamide (1:1) at room temperature overnight), then the peptide is cleaved from the resin (ii, 20% trifluoroacetic acid in dichloromethane at room temperature for 20 min) and subsequently cyclized (iii, 1.5eq *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate in dimethylformamide with 10eq *N,N*-diisopropylethylamine for 20 min at 75°C and 25W).

However, since this method is prone to racemization at the *C*-terminus, we employed the same cyclization conditions to the linear non-derivatized peptide (for the detailed synthesis see supplementary information). Surprisingly, at these modified conditions the cyclization of the non-derivatized peptide yielded the product as a colorless precipitate (16.3 mg, 40%). Upon comparison with the natural product, both compounds displayed the same retention time and the same fragmentation pattern when submitted to analytical HPLC-MS (Figure 7).

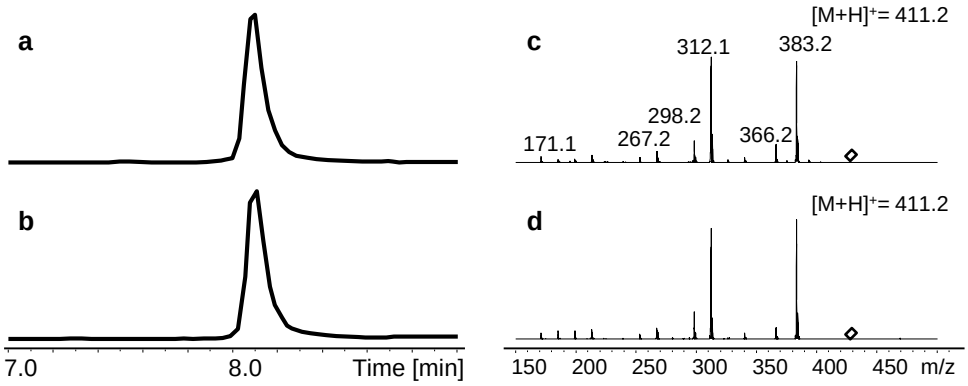


Figure 7: Comparison of extracted ion chromatograms of the natural tetrapeptide (a, *E. coli* DH10B pCK_mtaA^{p15A} pCOLA_xtp) and the synthetic one (b) and their fragmentation pattern (c, natural and d, synthetic) adapted from Carsten Kegler *et al.*[8]

Although, this small cyclic peptide is characterized by its insolubility, we were able to determine its structure and purity with NMR analysis (for detailed NMR analysis see supplementary information).

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Insect-specific production of novel GameXPeptides, widespread natural products in entomopathogenic *Photorhabdus* and *Xenorhabdus* bacteria

Friederike I. Nollmann, Christina Dauth, Geraldine Mulley, Carsten Kegler, Marcel Kaiser, Nick R. Waterfield and Helge B. Bode

Abstract

Discovery of new natural products by heterologous expression reaches its limits especially when specific building blocks are missing in the heterologous host or the production medium. Here, we describe the insect-specific production of the novel GameXPeptides E-H (**5**-**8**) from *Photorhabdus luminescens* which can be produced heterologously only upon coexpression of an additional operon or supplementation of the production media with the missing building blocks and thus must be regarded as the true natural products under natural conditions.

Author's Effort

The author performed the synthesis of several GameXPeptides as well as unusual building blocks and analyzed the NMR spectra of all of them. Additionally, the author collected and evaluated the data needed for the Heatmap in connection with the phylogenetic tree. Moreover, the author wrote the publication together with Helge B. Bode.

Synthesis of Ambactin, a Cyclic Peptide from *Xenorhabdus miraniensis*

ADDITIONAL RESULTS

This synthesis has been published in shorter length in

Yeast Homologous Recombination Cloning Leading to the Novel Peptides Ambactin and Xenolindicin

Olivia Schimming, Florian Fleischhacker, Friederike I. Nollmann and Helge B. Bode

Abstract

Heterologous production of GameXPeptide A (**1**), as well as of the novel peptide natural products ambactin (**2**) and xenolindicins A–C (**3 a**–**c**), was achieved by using the "overlap extension PCR-yeast homologous recombination" (ExRec) method. ExRec cloning is based on the ability of yeast to assemble overlapping DNA fragments into functional plasmids. Here we used this technique to clone a total of 15 biosynthesis gene clusters from *Photorhabdus* and *Xenorhabdus* with sizes of up to 45 kb. The structures of the novel compounds **2** and **3 a**, which were produced in *Escherichia coli*, were elucidated by detailed MS and bioinformatics analysis, and additionally confirmed by their chemical synthesis.

Author's Effort

The author established and optimized the synthesis of Ambactin leading to the proof of the structural proposal.

4.3 Synthesis of Ambactin, a cyclic peptide from Xenorhabdus miraniensis

By now, more than 1000 genomes of microorganisms are completely sequenced and disposable.[1-3] Amongst others this ready availability of genomic sequences paved the way for extensive genome mining as a powerful tool in the discovery of novel potent natural products. Both *Photorhabdus* and *Xenorhabdus* spp. display a high amount of different biosynthetic gene clusters in their genome, which are responsible for the production of secondary metabolites, ranging from 6 - 7.5% percent of the whole genome (e. g. *X. bovienii* 6.1%,[4] *X. nematophila* 7.5%[4] or *P. luminescens* TTO1 5.9%[5,6]) This is even more than the amount found in *Streptomyces* spp. *(S. coelicolor* 4.5%[7] and *S. avermitilis* 6.6%[8]), which in the past have been considered valuable sources of secondary metabolites. Though there are various approaches for the discovery of metabolic products from unfamiliar or cryptic gene clusters, they can be summarized as follows:[9] (i) genome scanning, (ii) prediction of the putative function of the found cluster, (iii) identification of expression conditions and/or genetic manipulations (of regulatory genes or promoter regions), (iv) structural prediction, (v) proof of this prediction by continuative testing (e. g. gene knock-out or heterologous expression as well as stable isotope labelling together with MS^2 methods etc.) or if needed or rather if possible by synthesis.

Based on this general approach (after genetic manipulations followed by inverse feeding experiments[10] and database mining) a new compound from *X. miraniensis* DSM17902, the so called ambactin (**1**, Figure 8a), was elucidated.[11] In order to confirm the proposed structure the peptide had to be synthesized. Therefore, the linear peptide sequence was subsequently build up on the solid support and afterwards cleaved from the resin while preserving the side chain protecting groups. Then the linear peptide was cyclized in solution and the remaining protecting groups were detached with a cleavage cocktail. Unfortunately, under the standard microwave assisted cyclization conditions (1.5eq *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetra-methyluronium hexafluorophosphate (HATU) in dimethylformamide with 2eq *N,N*-diisopropylethylamine (DIEA) for 20 min at 75°C and 25W) the desired product was only obtained as a minor fraction of the reaction mixture. Therefore, the cyclization process had to be optimized. First the temperature was lowered to 55°C, while maintaining the same conditions as before, without any significant improvement. However, upon addition of 1-hydroxy-7-azabenzotriazole (HOAt; 0.5eq) to the reaction mixture, almost no side product was detected in the subsequent HPLC-MS analysis. The substitution of HATU by *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC)[12] in the presence of HOAt

led to the slightly better results (for the more detailed synthesis see supplementary information) yielding 60% of **1**.

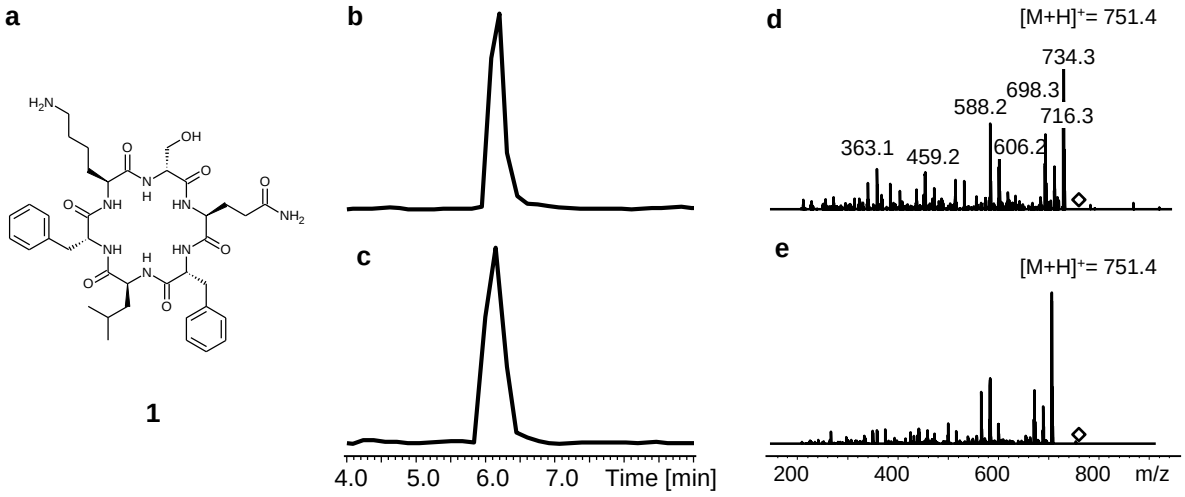

Figure 8: Structure of the cyclic peptide ambactin found in *X. miraniensis* DSM17902 (a) and comparison of extracted ion chromatograms of the natural (b, produced in *E. coli* DH10B after heterologous expression of the corresponding gene cluster) and the synthetic one (c) and their fragmentation pattern (d, natural and e, synthetic).

The purity of the cyclized product was analyzed by NMR (for detailed NMR analysis see supplementary information) and the compound was subsequently submitted to bioactivity testing against the causative agents of neglected tropical diseases.

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CYCLIC DEPSIPEPTIDES

Observations always involve theory.

Edwin Hubble

Synthesis of szentiamide, a depsipeptide from entomopathogenic *Xenorhabdus szentirmaii* with activity against *Plasmodium falciparum*

Friederike I. Nollmann, Andrea Dowling, Marcel Kaiser, Klaus Deckmann, Sabine Grösch, Richard ffrench-Constant, Helge B. Bode

Abstract

The synthesis of the recently characterized depsipeptide szentiamide (**1**), which is produced by the entomopathogenic bacterium *Xenorhabdus szentirmaii*, is described. Whereas no biological activity was previously identified for **1**, the material derived from the efficient synthesis enabled additional bioactivity tests leading to a notable activity against insect cells and *Plasmodium falciparum*, the causative agent of malaria.

Author's Effort

The author performed the fermentation of *X. szentirmaii*, isolated the szentiamide and established the synthesis thereof. Additionally, she conducted the disc diffusion assay. Moreover, the author wrote this publication together with Helge B. Bode.

Synthesis of Selected Xentrivalpeptides

ADDITIONAL RESULTS

Author's Effort

The author established and optimized the synthesis of cyclic depsipeptides produced by *Xenorhabdus stockiae*, the so-called xentrivalpeptides.

5.2 Synthesis of Selected Xentrivalpeptides

Recently, a new depsipeptide compound class produced by *Xenorhabdus stokiae* (*Xenorhabdus* sp 85816, Monsanto Company) was characterized.[1] This compound class is comprised of seventeen structural closely related cyclic depsipeptides (Figure 9a), which differ in the *N*-terminal fatty acid, amino acid composition or ring size. The main component of the mixture, xentrival A (**1**), was isolated from a culture of *Xenorhabdus* sp 85816 (4.4 mg per liter of production culture), characterized completely and subsequently submitted to testing.[1] Unfortunately, the other derivatives were only produced in minute amounts by *X. stockiae* and could not be isolated from the production culture. In order to gain more insight into the biological purpose of this compound class, other members in addition to **1** were synthesized and submitted to continuative testing (Figure 9b; for detailed information concerning the synthesis and NMR analysis see supplementary information). **1** and xentrival P (**2**) were chosen in order to determine the influences of the *N*-terminal fatty acid. Xentrival O (**3**) was also synthesized, since this is the only ring contracted derivative.

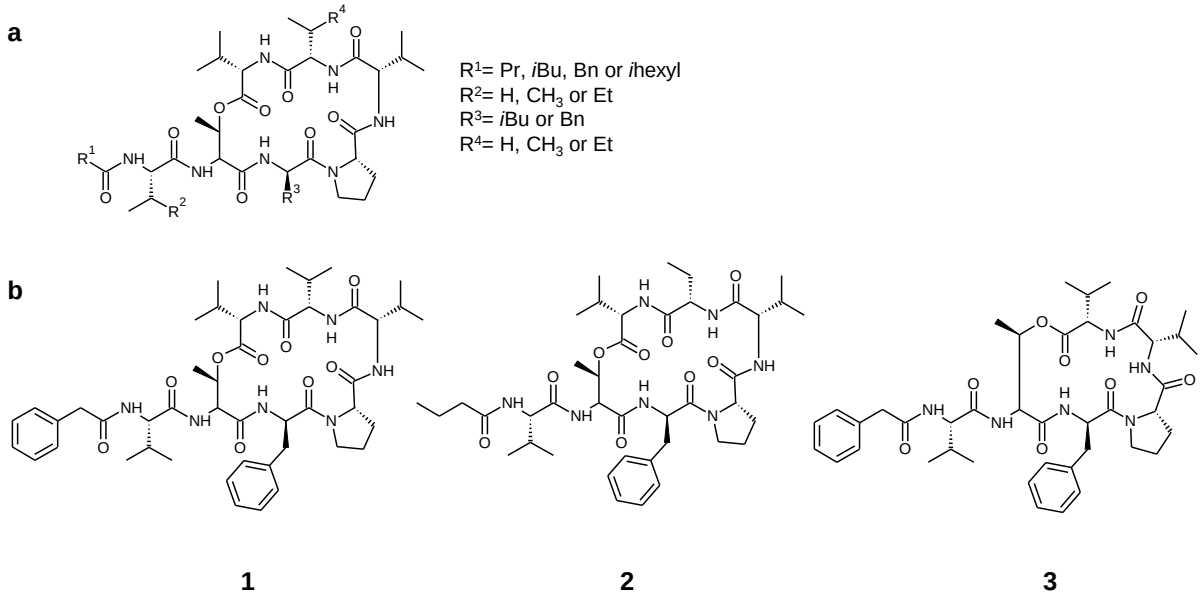
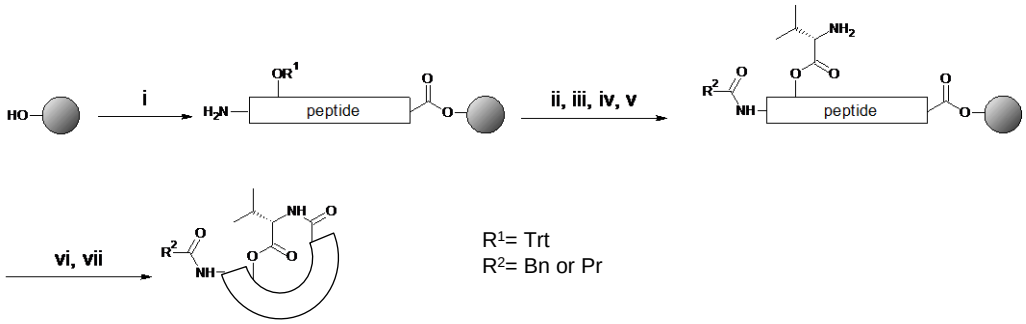


Figure 9: General structure of the xentrivalpeptides (a)[1] and the selected synthesized candidates **1-3** (b).

Since lactamization is preferred over lactonization, as previous syntheses of depsipeptides have shown,[2,3] the preparation of the xentrivalpeptides was conducted as follows: (i) assembly of the linear sequence, (ii) *N*-terminal acylation (iii) selective deprotection of the threonine residue, (iv) ester bond formation on resin, (v) deprotection of the *N*-terminus of the branched peptide, (vi) cleavage of the precursor peptide and (vii) cyclization in solution (Scheme 3). The first reaction steps, such as the assembly of the linear peptide sequence and the *N*-

terminal acylation, proceed usually in very good yields. Unfortunately, after the formation of the ester bond and the subsequent cyclization in solution the yield usually decreases significantly. Forming the ester bond on resin can considerably improve the reaction yield, but it usually remains the most difficult step during the preparation of natural depsipeptides.[4]



Scheme 3: General scheme for the synthesis of the xentrivalpeptides, adapted from Nollmann *et al*.[5] Sequential synthesis of the linear precursor employing standard fluorenylmethyloxycarbonyl/*tert*-butyl chemistry using a wang polysterene resin (i, *N*-terminal protecting group cleaved upon incubation with 40% and 20% piperidine in dimethylformamide (DMF), amino acid coupling was achieved by incubation with 5.0 eq amino acid in the presence of 5.0 eq *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate and 10.0 eq *N,N*-diisopropylethylamine (DIEA) in DMF for 45 min at room temperature); ii, acylation of the *N*-terminus by the corresponding *N*-hydroxysuccinimide preactivated acids; iii, selective removal of the trityl protecting group of the threonine side chain upon incubation with 2% of trifluoroacetic acid (TFA) in dichloromethane; iv, ester bond formation employing different conditions (see table 1); v, deprotection of the branched peptide (dependent on the building block used); vi, cleavage of the branched precursor by incubation with 95% TFA, 2.5% triisopropylsilane and 2.5% water for 90 min at room temperature followed by precipitation with a mixture of petrol ether and diethyl ether; vii, cyclization using 1.5 eq 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate and 2.0 eq DIEA in DMF upon microwave irradiation at 50°C for 20 min).

In the case of the xentrivalpeptides the ester bond is especially hindered since it connects two β-branched amino acids with each other. The ester bond formation was attempted using *N,N′*-diisopropylcarbodiimide (DIC) and 4-(dimethylamino)-pyridin (DMAP) and its hydrochloride (DMAP-HCl). Unfortunately, even after reaction times of eighteen hours no product was observed (estimation based on LC-MS traces; Table 1, entry 6). In comparison, the above mentioned conditions were successful for the syntheses of taxlllaid A[6] and fatflabet A (Table 1, entry 10 and 11; Max Kronenwerth, unpublished data). The employment of modified Yamaguchi conditions, which have successfully been used by Kuo-yuan Hung *et al* synthesizing xenematide A[7] (Table 1, entry 2) and by Nollmann *et al* synthesizing szentiamide[5] (Table 1, entry 5), yielded only moderate amounts of the product (estimation based on LC-MS traces; Table 1, entry 7). The best yields were observed using a 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) pre-activated *tert*-butyloxycarbonyl

Table 1: Ester bond formation depending on the sequence and the amino acid used of cyclic depsipeptides produced by *Xenorhabdus* spp.; polystyrol resin (PS), phenyl acetic acid (PhAc), formyl (For), *tert*-butyl oxycarbonyl (Boc), fluorenylmethoxycarbonyl (Fmoc), *o*-nitrobenzenesulfonyl (*o*NBS), 4-(dimethylamino)-pyridin (DMAP), *N*,*N*'-diisopropylcarbodiimide (DIC), benzoyl chloride (BzCl), 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT), *N*,*N*-dimethylformamid (DMF), methylene chloride (CH_2Cl_2), triethylamine (Et_3N), *N*-methyl imidazole (NMI), *N*,*N*-Diisopropylethylamine (DIEA), amino acid (AA).

		Linear Sequence	Amino Acid	Conditions	Comment
1	xenematide A	PhAc-TYy-PS	Boc-β-Ala-OH	3eq AA, 3eq DMAP, 3eq DIC in DMF, Δ	no product observed[7]
2				20eq AA, 20eq BzCl, 40eq Et_3N in CH_2Cl_2, rt, 18h	quantitative conversion[7]
3				6eq AA. 8eq MSNT, 4eq NMI, 12eq DIEA, in CH_2Cl_2, rt, 16h	quantitative conversion
4	szentiamide	For-lTfvY-PS	Fmoc-Trp-OH	3eq AA, 1.5eq DMAP-HCl, 1.5eq DMAP, 3eq DIC in CH_2Cl_2, rt, 18h	no product observed[5]
5				20eq AA, 20eq BzCl, 40eq Et_3N in CH_2Cl_2, rt, 18h	quantitative conversion[5]
6	xentrivalpeptides	PhAc/ProAc-VTfPV-PS	Boc-Val-OH	3eq AA, 1.5eq DMAP-HCl, 1.5eq DMAP, 3eq DIC in CH_2Cl_2, rt, 18h	no product observed
7			Fmoc-Val-OH	20eq AA, 20eq BzCl, 40eq Et_3N in CH_2Cl_2, rt, 18h	moderate amounts of product observed
8			Boc-Val-OH	6eq AA. 8eq MSNT, 4eq NMI, 12eq DIEA, in CH_2Cl_2, rt, 16h	quantitative conversion
9			*o*NBS-Val-OH	6eq AA. 8eq MSNT, 4eq NMI, 12eq DIEA, in CH_2Cl_2, rt, 16h	quantitative conversion
10	taxlllaid A	*i*HepAc-TALllL-PS	Boc-Ala-OH	3eq AA, 3eq DMAP, 3eq DIC in DMF, rt, 18h	quantitative conversion[6]
11	fatflabet A	*i*HepAc-TFl-PS	Boc-β-Ala-OH	3eq AA, 3eq DMAP, 3eq DIC in DMF, rt, 18h	quantitative conversion (Max Kronenwerth; unpublished data)

(Boc) or *o*-nitrobenzenesulfonyl (*o*NBS) protected valine (estimation based on LC-MS traces; Table 1, entry 8 and 9). In contrast to Boc, *o*NBS allows the continuation of the branched peptide sequence, since its deprotection conditions are considered orthogonal to the Fmoc/*t*Bu strategy. Moreover, the *o*NBS protecting group displays an additional activating effect on the carbonyl group of the amino acid, due its electron withdrawing properties, allowing challenging couplings to proceed more easily.[8]

When the cyclization was conducted upon microwave irradiation and at 70°C, the LC-MS analysis revealed two peaks corresponding to the cyclic product (data not shown). However, when the temperature was lowered to and maintained at 50°C, only a single product peak was detected in the crude mixture (Figure S1). The purification yielded **1**, **2** and **3** in 18%, 57% and 26%, respectively. Yet again, two peaks were observed in the LC-MS traces of the purified products (Figure 10c, f and i). These might be attributed to a cis/trans isomerism of the proline residue.[9,10] Moreover, this isomerism is also detected in the purified natural product **1** (Figure S2). Nevertheless, in order to prove this hypothesis, extensive NMR studies at different pH values and different temperatures would have to be conducted. In addition to these experiments circular dichroism could shed light on the three-dimensional structure of these cyclic depsipeptides as a function of the surrounding environment.

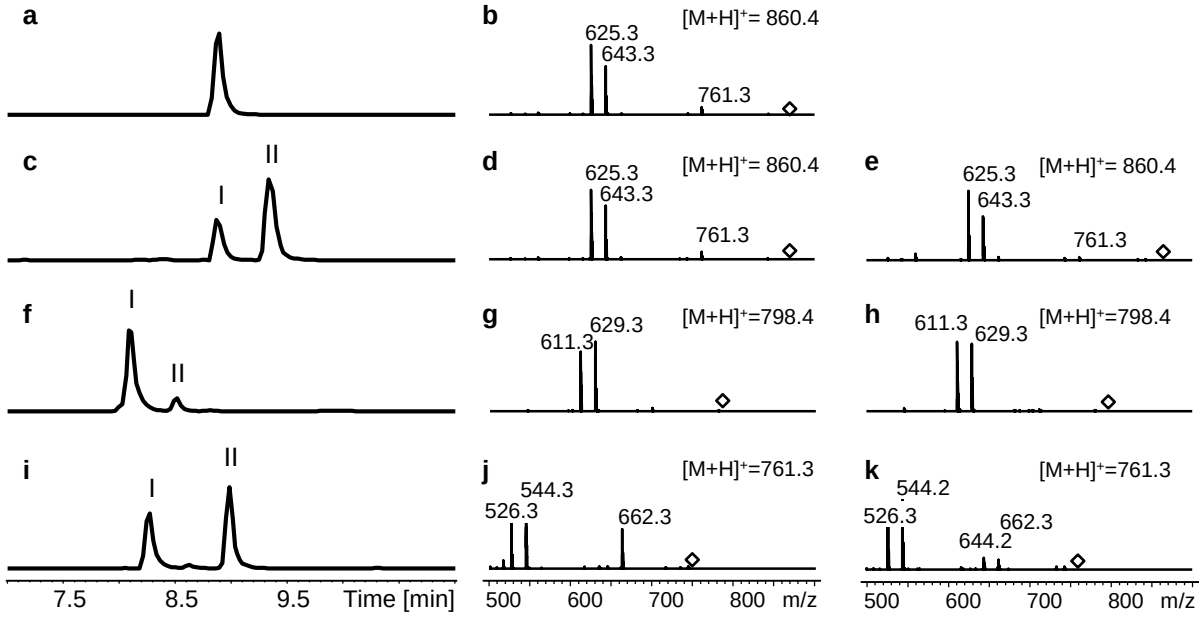


Figure 10: Extracted ion chromatograms and MS/MS data of the natural **1** (a, extracted ion chromatogram; b, MS/MS data) and the synthetic **1** (c, extracted ion chromatogram; d, MS/MS data of peak I; e, MS/MS data of peak II), **2** (f, extracted ion chromatogram; g, MS/MS data of peak I; h, MS/MS data of peak II) and **3** (i, extracted ion chromatogram; j, MS/MS data of peak I; k, MS/MS data of peak II).

In conclusion, syntheses of several natural occurring depsipeptides produced by *Xenorhabus* spp. showed that the ideal reaction conditions and yields of the esterification may vary considerably depending on the peptide sequence and the used protecting groups (estimated by LC-MS, Table 1).[4]

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METHYLATED PEPTIDES

Science may set limits to knowledge, but should not set limits to imagination.

Bertrand Russel

6 Methylated peptides

Biologically active natural products often display special features, like macrocycles, ester bonds, D-amino acids or backbone *N*-methylation which lead to highly interesting therapeutic profiles.[1,2] Since the methylation of the backbone plays a crucial role in the peptide's conformational state and physical properties, it may enhance pharmacokinetic key characteristics.[3] Especially peptides with multiple *N*-methylations of the backbone display high pharmaceutical potential.[4,5] The antineoplastic antibiotic actinomycin (**1**), the antitumoral IB-01212 (**2**)[6,7] the immune¬suppressant cyclosporine (**3**) and the potent antineoplastic dolastatin (**4**)[8,9] are success stories of highly methylated compounds as they or derivatives thereof are in concluding clinical trials or already have been approved as drugs. However, many highly methylated natural compounds are not yet accessible to testing or further optimization, due to problems which arise during their synthesis and/or purification.

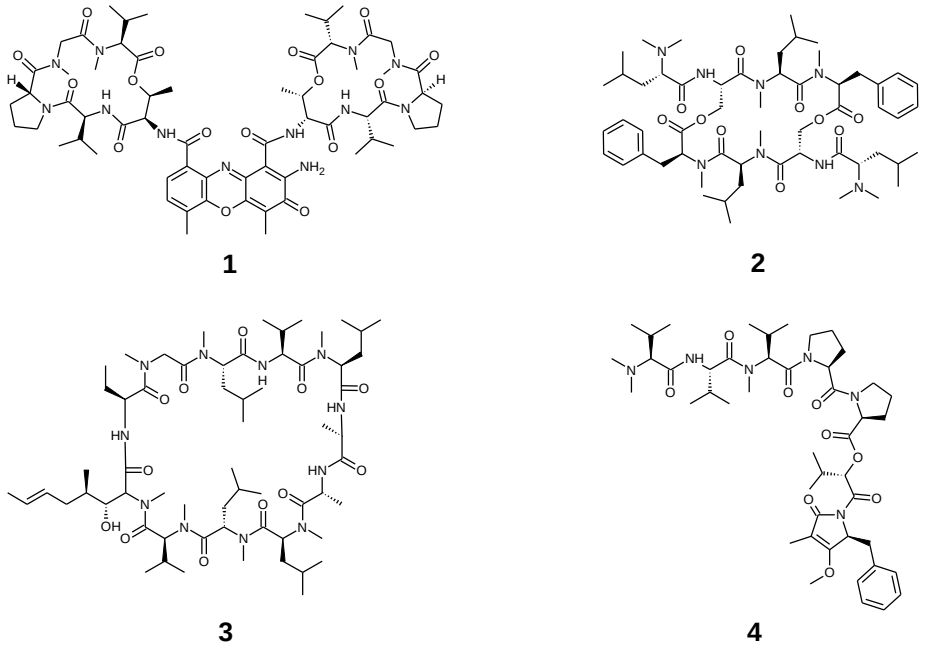


Figure 11: Highly methylated natural products with interesting biological activities, actinomycin (**1**), cyclosporine (**2**), IB-01212 (**3**), dolastatin (**4**).

Photorhabus and *Xenorhabdus* spp. produce several different highly methylated peptides, e. g. the rhabdopeptides (**5-6**, Scheme 4) found in *X. nematophila* HGB081[10] and rhabdopeptide-like structures, such as **7**, which are found in several different *Xenorhabdus* strains (Daniela Reimer, unpublished data), or the mevalagmapeptide (**8**, Scheme 4) from *P. luminescens* TT01.[11,12] Due to their structural diversity, which results from minor changes in the amino acid sequence or composition as well as the methylation pattern, the isolation and further characterization is nearly impossible. Nevertheless, detailed *in vivo* expression

technology (IVET) analyses revealed that the rhabdopeptides are insect-specific virulence factors in HGB081.[10] Hence, some of these back-bone methylated peptides were synthesized making them available to thorough characterization and testing.

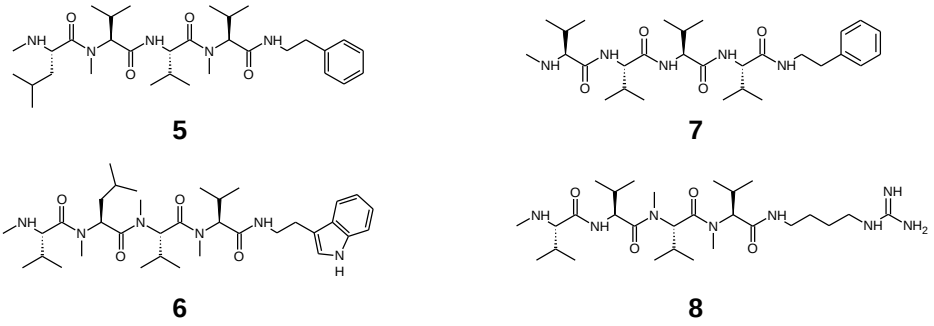


Figure 12: Structures of natural occurring backbone methylated peptides found in *Photorhabdus* and *Xenorhabdus* spp. [10,11]

6.1 Synthesis of short partly methylated sequences in solution

The genome of *Xenorhabdus* PB62.4 contains a gene cluster, which displays a high similarity to one found in *X. nematophila* HGB081 (**Figure 13**a and b) which amongst others is responsible for the production of xenortides C (**9a**)[13] and D (**9b**)[14] (Figure 13c).

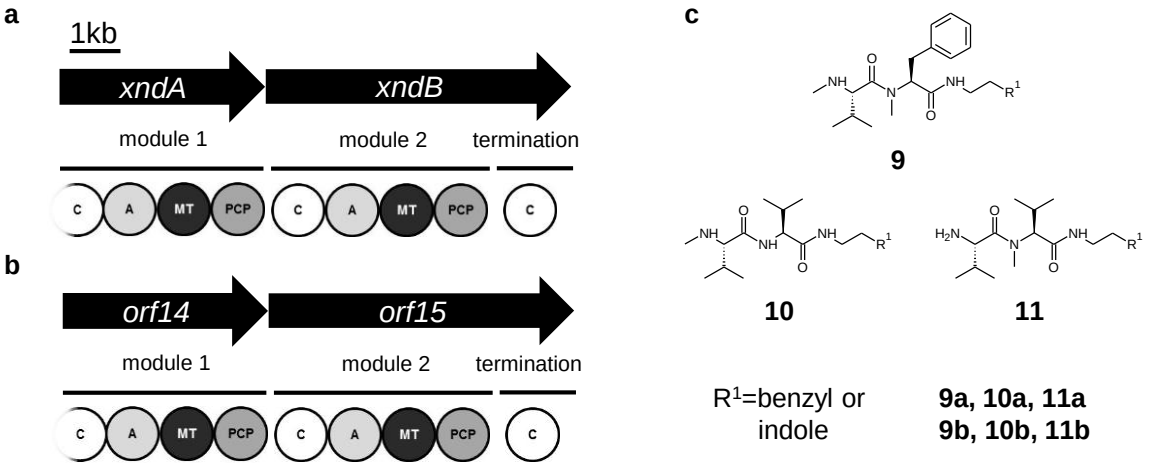
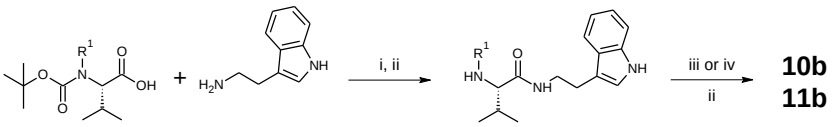


Figure 13: The cluster (comparison of the gene cluster responsible for the production of the xenortides[14] (a) and short rhabdopeptides (b); Daniela Reimer, unpublished data) and HPLC-MS/MS analysis indicated a methylated structure similar to xenortide C (**9a**) and D (**9b**) for the short rhabdopeptides (**10a, b** and **11a, b**; c).

The xenortides are permethylated peptides which consist of a phenylalanine and a leucine (xenortide A and B)[13,15] or a valine (xenortide C and D), respectively. Moreover, they display an amidated *C*-terminus (with phenylethylamine or tryptamine, respectively). The analysis of the gene cluster in *Xenorhabdus* PB62.4 suggested a permethylated *C*-terminal amidated dipeptide consisting of two valines. Unfortunately, continuative LC-MS analysis of PB62.4 did not reveal any such peptide. It was then postulated that one of the methylation domains in

this gene cluster is not functional (Daniela Reimer, unpublished data). The LC-MS analysis of *Xenorhabdus* PB62.4 revealed two natural products which are 1Da smaller than the proposed methylated peptides. Though the fragmentation pattern of these natural products (Figure 14a-d) displays similarities to other methylated peptides found in *Xenorhabdus* spp., it has to be assumed that these compounds are not pure peptides but rather acylated tryptamine or phenylethylamine derivatives. Nevertheless, the two possible peptide core sequences in combination with a *C*-terminal phenylethylamine (**10a** and **11a**) or tryptamine (**10b** and **11b**) were synthesized in order to underline this assumption as well as tools for the identification of so far unidentified rhabdopeptides (Figure 13c).

Unfortunately, methylated peptides are prone to form diketopiperazines (DKPs) especially when synthesized on a commonly used resin. Thus, the syntheses of these small methylated peptides were conducted in solution (Scheme 4).



Scheme 4: Synthese of short rhabdopeptides exemplified by the synthesis of **10b** and **11b** (i, condensation of 1.0 eq amino acid (R^1 = H, CH_3) with 1.5 eq amine in the presence of 1.1 eq 1-hydroxybenzotriazole (HOBt) and 1.0 eq *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC); ii, boc-deprotection with 4.0 eq trifluoroacetic acid (TFA) in dichloromethane (CH_2Cl_2); iii, condensation of 1.0 eq amino acid with 1.0 eq secondary amine in the presence of *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TBTU) and *N,N*-diisopropylethylamine (DIEA); iv, condensation of 1.0 eq amino acid with 1.0 eq primary amine in the presence of 1.1 eq HOBt and 1.0 eq EDC).

The *C*-terminal amine was attached to the *tert*-butyl oxycarbonyl protected amino acid (Boc-Val-OH and Boc-*N*Me-Val-OH, respectively), followed by the cleavage of the protection group employing 4 eq trifluoroacetic acid (TFA) in dichloromethane (CH_2Cl_2; Scheme 4, i and ii). The next amino acid (Boc-*N*Me-Val-OH and Boc-Val-OH, respectively) was coupled with 1-hydroxybenzotriazole (HOBt) and *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC) or in the case of the less reactive secondary amine with *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TBTU) and *N,N*-diisopropylethylamine (DIEA; Scheme 4, iii or iv). Afterwards, the *N*-terminal protection group was cleaved at the conditions mentioned above (Scheme 4, ii). The final products were purified by normal phase flash chromatography and analyzed by NMR spectroscopy (for detailed information see supplementary information S6.1). The LC-MS analysis shows subtle distinctions in the retention time of *N*-terminal and internal methyl groups (Figure 14c-f). Unfortunately, upon evaluation of LC-MS analyses of any other currently available *Xenorhabdus* and *Photorhabdus* strain neither naturally produced **10a** and **10b** nor **11a** and

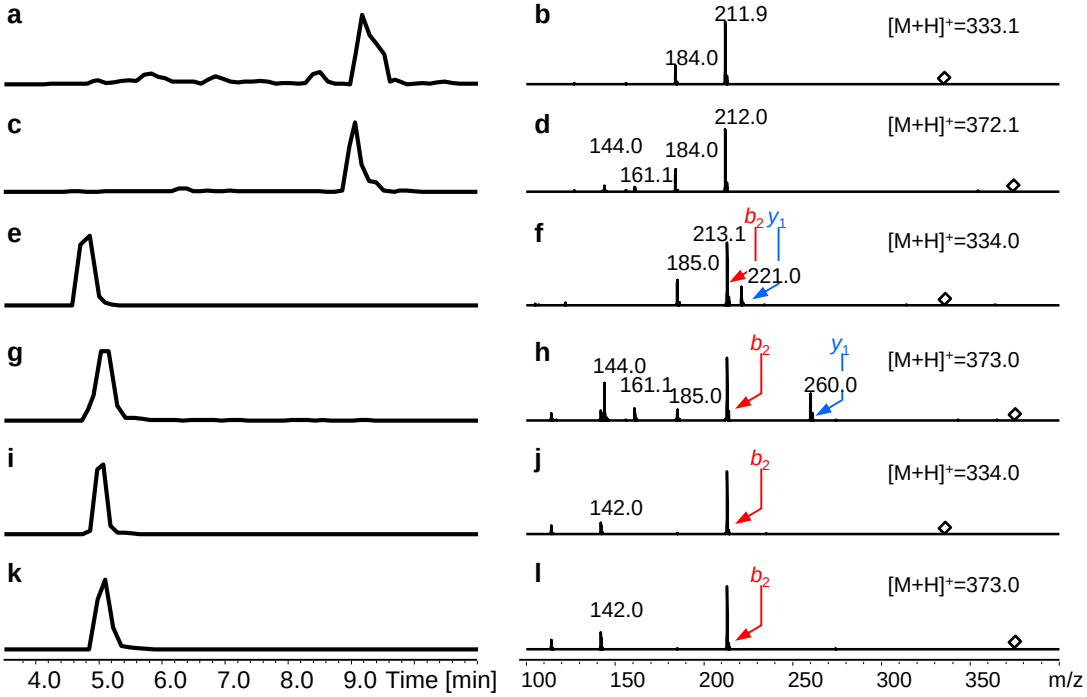
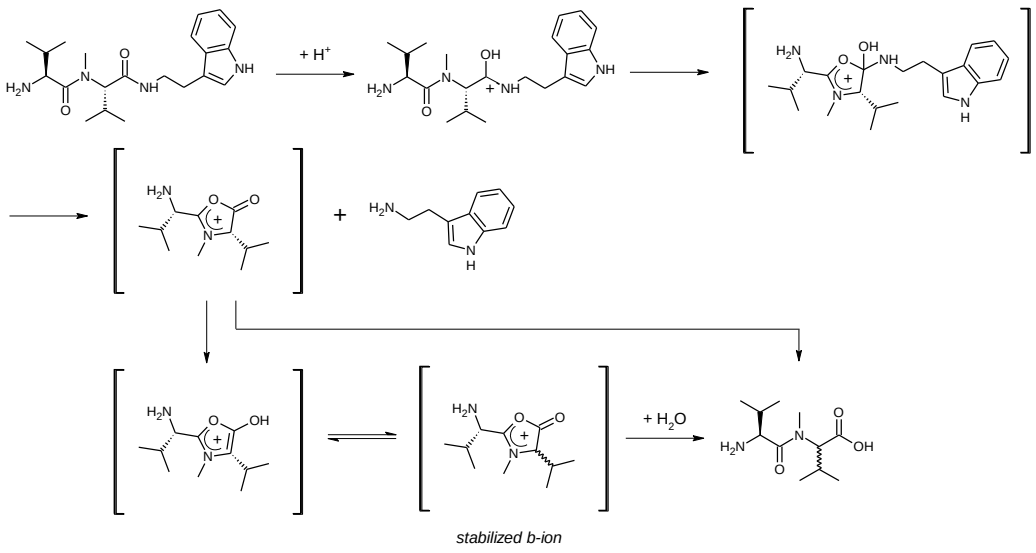


Figure 14: Comparison of the extracted ion chromatograms and MS/MS fragmentation patterns of the natural products (a-d) and the synthetic rhabdopeptides **10a** (e and f) and **10b** (g and h) as well rhabdopeptides **11a** (i and j) and **11b** (k and l); red arrows indicate *b*-type ions, blue arrows indicate *y*-type ions.

11b were detected. Taking a closer look at the MS/MS analysis of the synthetic compounds revealed a significant difference in the fragmentation behavior based on their methylation pattern. **10a** and **10b**, which bear an *N*-terminal methyl group, show *b*-type (Figure 14e-h; red arrows) as well as *y*-type ions (Figure 14e-h; blue arrows). In contrast, **11a** and **11b**, which bear an internal methyl group, show only *b*-type ions (Figure 14i-l; red arrows).



Scheme 5: Mechanism that leads to a stabilized *b*-type ion formation due to an internal (and isolated) methyl group exemplified by **11b** according to the solution phase acid-catalyzed analogy model by Vaisar and Urban.[16]

This is in good accordance with a solution phase acid-catalyzed analogy model developed by Tomas Vaisar and Jan Urban.[15] This model postulates that an isolated and internal backbone methylation activates the adjacent amide bond towards the *C*-terminus resulting in the formation of a stabilized *b*-type ion with the structure of an *N*-methyl oxazolone (**Scheme 5**). Upon formation of this protonated *N*-methyl oxazolone species the *y*-type ion is suppressed and only the *b*-type ion is detected. Based on this characteristic fragmentation pattern and upon reevaluation of certain *Xenorhabdus* strains a short rhabdopeptide-like molecule (**12b**) was identified in *X. indica* DSM17382 (Figure 15a-c). Additional stable isotope labeling experiments verified the incorporation of tryptamine (Figure 16g-i), three valines (Figure 16j-l) and indicated a backbone methylation (Figure 16m-o), leucine and isoleucine, however, were not incorporated (data not shown).

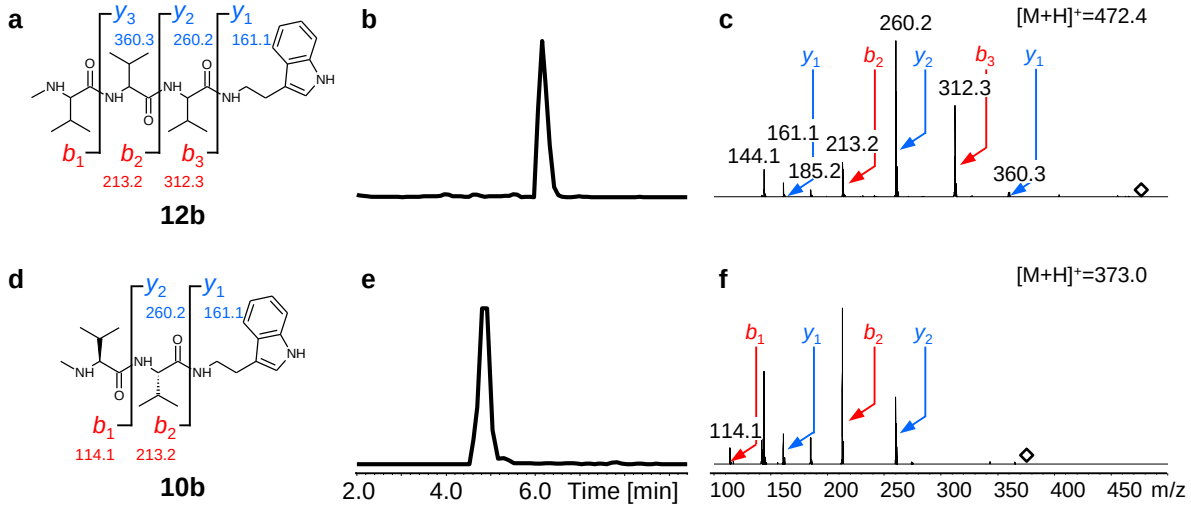


Figure 15: Extracted ion chromatograms (b and e) in addition to MS/MS spectra (c and f) of the here identified rhabdopeptide-like substance **12b** (proposed structure based on stable isotope labeling experiments and MS/MS fragmentation) in *Xenorhabdus indica* DSM17382 (a) and the synthetic **10b** (d); red arrows indicate *b*-type ions, blue arrows indicate *y*-type ions.

Here, the bacteria were cultivated in a ^{13}C-enriched ISOGRO media and supplemented with ^{12}C amino acids or building blocks. Upon incorporation of the building block the monoisotopic mass of the natural products decreases in respect to the entirely ^{13}C-labeled ones.[11,17] Taking then a closer look at the obtained MS/MS data, **12b** displays not only *b*-type ions (Figure 15c; red arrows) but also *y*-type ions (Figure 15c; blue arrows), which suggest either an *N*-terminal methyl group or no methylation at all.

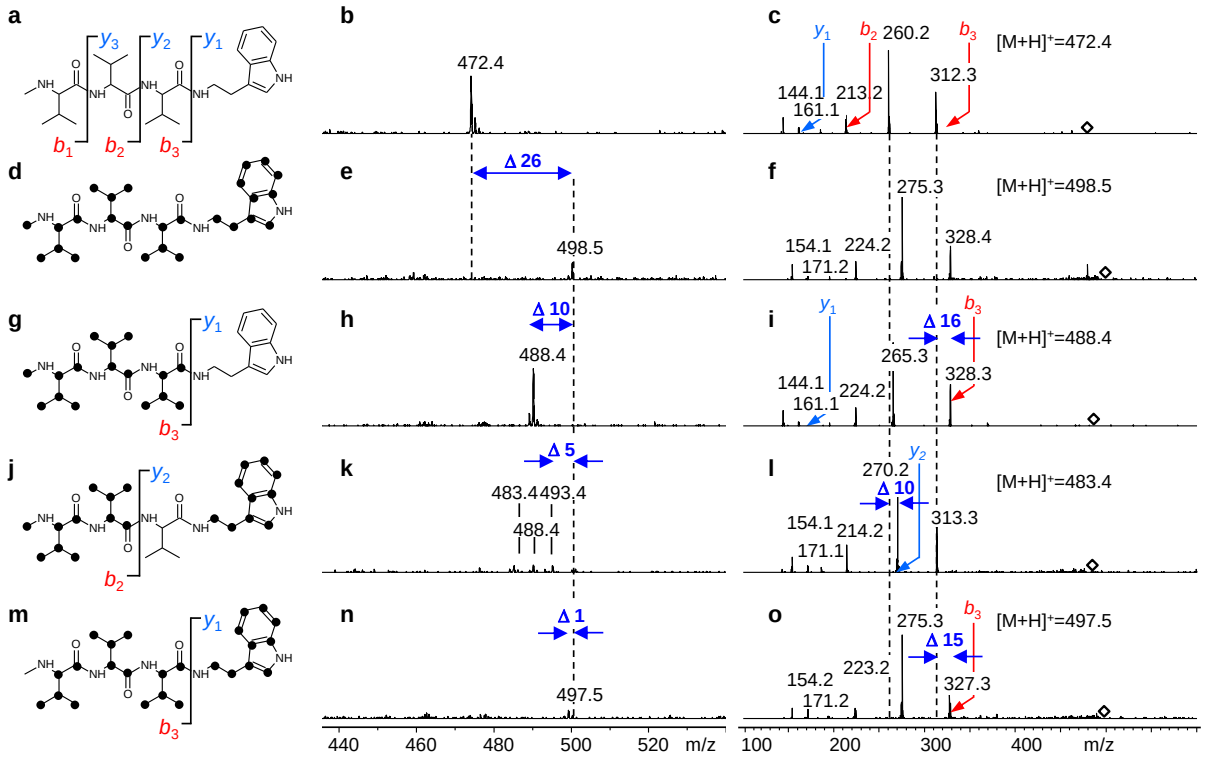


Figure 16: Stable isotope labeling experiments in *Xenorhabdus indica* DSM17382; determination of the number of carbon atoms by growing *X. indica* in standard growth media (a-c) and fully labeled ^{13}C-media (d-f). In order to determine the building blocks of **12b** *X. indica* was also grown in fully labeled ^{13}C-media upon addition of tryptamine (g-i), valine (j-l) and methionine (m-o). Moreover, the MS/MS data of the corresponding labeled compound is shown. Based on the obtained results (indicated by the dark blue arrows; only exemplary for valine) the ^{13}C labeled building blocks were highlighted with dots in the proposed structure (a, d, g, j and m; in the case of valine and methione only exemplary positions are indicated). The *y*-type ions are labeled with blue and the *b*-type ions with red arrows.

Moreover, in comparison with **10b**, **12b** not only shows the *b*-type ion 213, which corresponds to 3-methyl-2-{[3-methyl-2-(methylamino)butanoyl]amino}butanoic acid or 2-[(2-amino-3-methylbutanoyl)(methyl)amino]-3-methylbutanoic acid, respectively (Figure 16c), but also the *b*-type ion 312 (Figure 16c), which is built up like 213 but elongated by a single valine. This is further underlined by the MS/MS data of ^{13}C-labeled **12b** bearing a ^{12}C-valine (Figure 16l) and the methyl group of a ^{12}C-methionine (Figure 16o). The *y*-type ion 260, which was found in MS/MS experiments of **12b** and **10b**, corresponds to 2-amino-*N*-[2-(1*H*-indol-3-yl)ethyl]-3-methylbutanamide. This is confirmed by the MS/MS data of ^{13}C-labeled **12b** upon incorporation of ^{12}C-tryptamine (Figure 16f). The fragment ions 161 and 144 correspond to tryptamine and a tryptamine-fragment formed by an α-cleavage process,[18] which involves the characteristic loss of the primary amino group (Figure 16c, f and i). The subsequently synthesized **12b** is in good accordance with the natural product in retention time and fragmentation pattern (Figure 17; for additional information see chapter 6.2 and supplementary information).

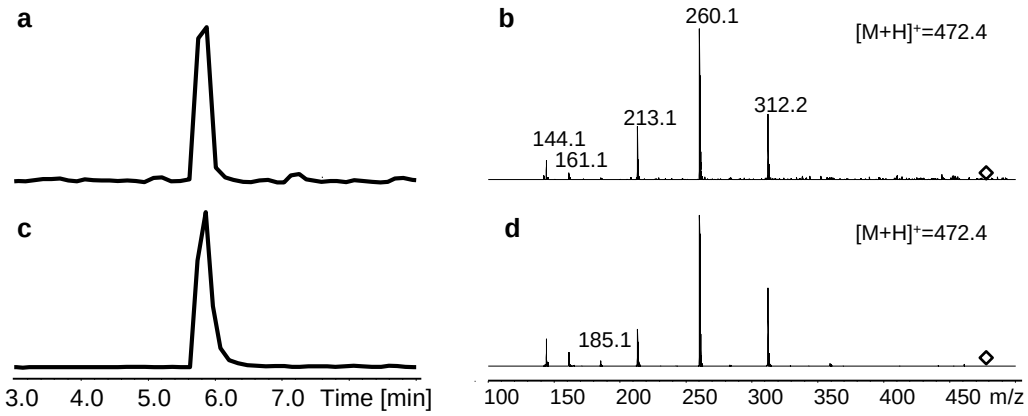


Figure 17: Extracted ion chromatograms and MS/MS data of the natural **12b** produced by *Xenorhabdus indica* (a and b) and the synthesized **12b** (c and d).

6.2 Synthesis of partly methylated sequences on resin

An induced increased expression of the corresponding gene clusters leads to the production of artificial rhabdopeptides which amongst others display only isolated methyl groups (Daniela Reimer, unpublished data). The synthesis of the partly methylated peptides (**12a**, **12b**, **13a** and **13b**; Figure 18) was achieved by incorporating the corresponding building block or by methylation of individual amino acids on resin,[5,19-21] followed by the condensation with tryptamine or phenylethylamine using HOBt and EDC in solution (for detailed information concerning the synthesis and NMR analysis of **12a** see supplementary information S5.1). There was no significant difference detected in the final yield and purity of the peptide core sequence (determined by LC-MS/MS; data not shown) whether commercially available building blocks were used or the methylation was conducted on resin.

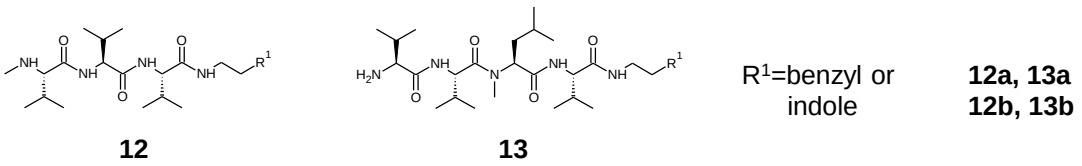


Figure 18: General structure of partly methylated rhabdopeptide-like structures which occur naturally (**12b**) or upon induced expression of the corresponding gene cluster (**13a**; Daniela Reimer, unpublished data). As well as their synthetic phenylethylamine (**12a**) and tryptamine derivatives (**13b**).

12b is not only detected upon increased expression, but also occurs naturally (Figure 19a, b (natural), i and j (synthetic); see also chapter 6.1) in *Xenorhabdus indica* DSM17382. **13a** can be found in trace when *X. nematophila* HGB081 is injected into insect larvae (Figure 19c and d) or upon artificially overexpression of the *rdpABC*[10] cluster (Figure 19e and f; Carsten Kegler, Daniela Reimer; unpublished data).

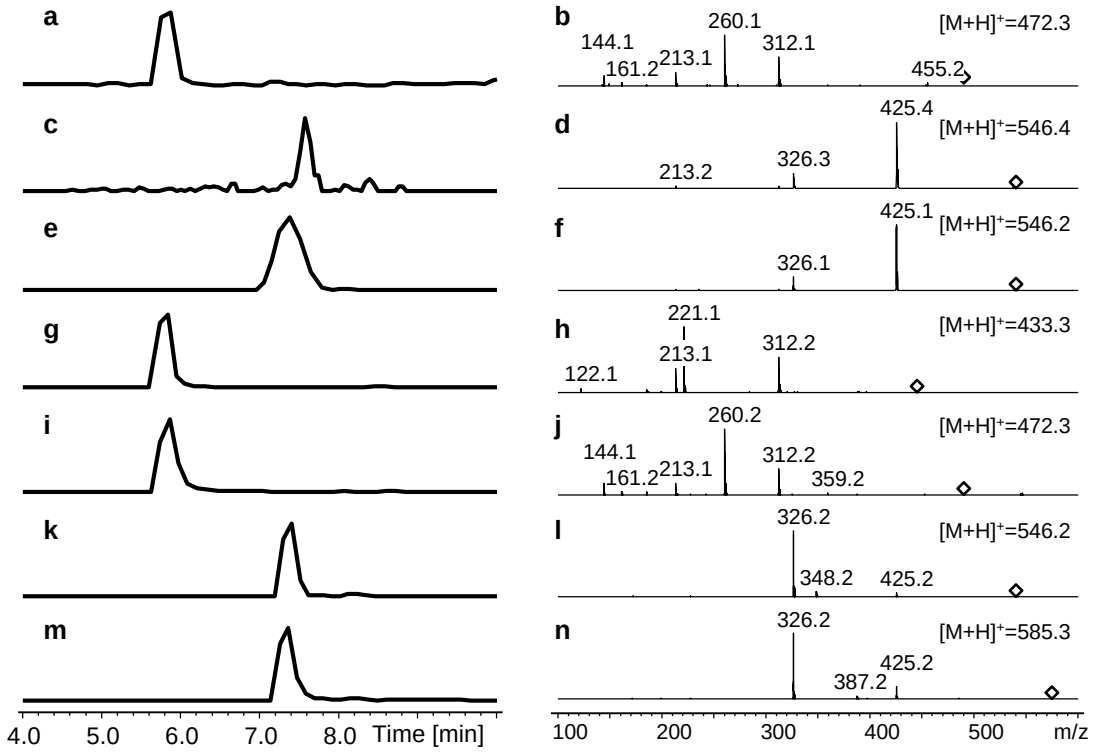


Figure 19: Extracted ion chromatograms and MS/MS data of the natural **12b** produced by *Xenorhabdus indica* (a and b), **13a** produced by *X. nematophila* HGB081 upon injection into *Galleria mellonella* larvae (c and d, Daniela Reimer; unpublished data) or heightened expression of the *rdpABC* cluster (e and f; Daniela Reimer and Carsten Kegler; unpublished data) in comparison to the synthetized **12b** (i and j) and **13a** (k and l), as well as their synthetic counterparts **12a** (g and h) and **13b** (m and n).

Here, a significant peak broadening in the extracted ion chromatogram (Figure 19e) can be observed indicating a mixture of different methylation sites in the peptide backbone. Peptides **12a** (Figure 19g and h) and **13b** (Figure 19m and n) are their synthetic counterparts.

6.3 Synthesis of permethylated sequences

As mentioned before not only isolated backbone methylations are observed in these non-ribosomally synthesized peptides. Some rhabdopeptides or rhabdopeptide-like structures (**Figure 12**) display even a permethylated peptide backbone. Permethylation of peptides can be achieved in solution employing 6.3 eq per amide sodium hydride (NaH) and 10.0 eq per amide iodomethane (CH_3I).[22] However, these conditions were only successful in the case of shorter sequences such as the peptide core of **6.**[10] This is probably due to the augmenting insolubility of larger peptide fragments (data not shown). Hence an approach on resin was established and optimized based on previous works by Tina White *et al.*[4] Here, lithium *tert*-butoxide (Li*t*OBu) and CH_3I were used as the base and the methylation reagent, respectively. Variation of the permethylation conditions by using different bases such as "dry" sodium hydroxide (NaOH),[23] *n*-buthyllithium (*n*-BuLi)[24,25] or the above mentioned NaH led only to

inferior results (Table 2; estimated by HPLC-MS analysis, peaks that could be sufficiently separated are combined).

Table 2: Estimation of the approximate reaction yield based on the area of the extracted ion chromatograms of the corresponding masses after incubating BocVVVV-ClTrt-PS with 6.3 eq base/amide and 10.0 eq CH_3I/amide in a mixture of tetrahydrofuran and dimethylsulfoxide twice for two hours. Experiments were performed in a 10 µmolar scale. Results from mass signals that could not be separated well enough from each other were combined in one cell.

	conditions	percentage of methylated peptide [%]				
		no CH_3	1x CH_3	2x CH_3	3x CH_3	4x CH_3
1	6.3 eq/NH Li*t*BuO, 10.0 eq CH_3I/NH	17.3	22.1	18.2		42.4
2	6.3 eq/NH "dry" NaOH, 10.0 eq CH_3I/NH	76.0	15.2		8.8	
3	6.3 eq/NH *n*-BuLi, 10.0 eq CH_3I/NH	84.5	9.4		6.1	
4	6.3 eq/NH NaH, 10.0 eq CH_3I/NH	98.5	1.5	0.0	0.0	0.0

Moreover, reducing the amount of reagents by half resulted in a diminished amount of backbone methylations or no methylation at all (data not shown). Increasing the reagent amount or prolonging the incubation time longer than four hours did not have any effect on the methylation degree of the peptides (data not shown). Incubation of Li*t*OBu and CH_3I with different *tert*-butyl oxycarbonyl (Boc) protected tetrapeptides (attached to a 2-chloro trityl chloride polystyrol resin (ClTrt-PS); Table 3 entries 1 to 6) revealed a sequence dependent efficacy of the methylation reaction. Especially peptides consisting of β-branched amino acids, such as aminobutyric acid (Abu, Table 3 entry 1), valine (Table 3 entry 2) and isoleucine (Table 3 entry 5), were difficult to permethylate.

The permethylation of BocAbu-Abu-Abu-Abu-ClTrt-PS yielded approximately 50% of the product. The permethylation of BocVal-Val-Val-Val-ClTrt-PS yielded 42% of the product and the permethylation of BocIle-Ile-Ile-Ile-ClTrt-PS gave less than 19% yield. In contrast, the permethylation of BocLeu-Leu-Leu-Leu-ClTrt-PS proceeded to 88% (Table 3 entry 4). Even the permethylation of Boc-Phe-Phe-Phe-Phe-ClTrt-PS resulted in more than 70% product (Table 3 entry 6). Surprisingly, the permethylation of a tetrapeptide consisting of aminobutyric acid and valine (BocAbu-Val-Abu-Val-ClTrt-PS; Table 3 entry 3) yielded results similar to BocLeu-Leu-Leu-Leu-ClTrt-PS or BocPhe-Phe-Phe-Phe-ClTrt-PS. A similar effect can be observed upon incorporation of leucine (BocVal-Leu-Leu-Val-ClTrt-PS and BocVal-Leu-Val-Val-ClTrt-PS; Table 3 entries 8 and 9). Moreover, there seems to be an optimum of sequence length as a comparison of BocVal-Leu-Val-Val-Val-ClTrt-PS,

BocVal-Leu-Val-Val-Val-Val-ClTrt-PS and BocVal-Leu-Val-Val-Val-Val-Val-ClTrt-PS (Table 3 entries 9 to 11) reveals. Unfortunately, the incubation of Li*O*tBu and CH_3I with BocLeu-Leu-Leu-ClTrt-PS (Table 3 entry 7) in a 100 µmolar scale yielded only 54% of the permethylated product. This in comparison with BocLeu-Leu-Leu-Leu-ClTrt-PS reduced yield can probably be attributed to the scale up from 10 µmol to 100 µmol.

Table 3: Estimation of the approximate reaction yield based on the area of the extracted ion chromatograms of the corresponding masses after incubation of the immobilized peptides with 6.3 eq Li*t*OBu/amide and 10.0 eq CH_3I/amide in a mixture of tetrahydrofuran and dimethylsulfoxide twice for two hours. Results from mass signals that could not be separated well enough from each other were combined in one cell. Amino butyric acid (Abu; X); *tert*-butyl oxycarbonyl (Boc); polystyrol resin (PS). Experiments were conducted in a 10 µmolar scale (entries 1 to 6) and 100 µmolar scale (entries 7 to 11), respectively.

	sequence	percentage of methylated peptide [%]						
		no CH_3	1x CH_3	2x CH_3	3x CH_3	4x CH_3	5xCH_3	6x CH_3
1	Boc-XXXX-ClTrt-PS	1.6	13.5	31.7	53.4			
2	Boc-VVVV-ClTrt-PS	17.3	22.1	18.2		42.4		
3	Boc-XVXV-ClTrt-PS	2.0	1.1	22.0	4.6	70.3		
4	Boc-LLLL-ClTrt-PS	0.7	0.7	0.3	10.2	88.1		
5	Boc-IIII-ClTrt-PS	50.0	52.3		19.1			
6	Boc-FFFF-ClTrt-PS	3.0	1.0	0.0	18.4	77.7		
7	Boc-LLL-ClTrt-PS	11.2		34.7	54.0			
8	Boc-VLLV-ClTrt-PS	4.7	11.8	22.3	33.7	27.5		
9	Boc-VLVV-ClTrt-PS	75.8	5.5	5.9	3.4	9.4		
10	Boc-VLVVV-ClTrt-PS	12.3	15.9	31.7		25.3	14.9	
11	Boc-VLVVVV-ClTrt-PS	14.2	14.6	20.7	28.5		22.0	

In conclusion, the incubation with Li*t*OBu and CH_3I gave the best results, but the methylation success was highly sequence dependent. Especially, peptides containing β-branched amino acids were difficult to methylate (Abu > Val >> Ile). The most promising candidates (**Table 3**, entry 7 and 8; 100 µmol each), based on their permethylation degree (estimated by LC-MS), were condensed with the corresponding amines in solution (Figure 20). Unfortunately, the ^{1}H-NMR spectra of the resulting peptides revealed major impurities, resulting from an insufficient *C*-terminal amidation as well as incomplete methylation of the backbone, which could not be removed during normal phase purification (see supplementary information S6.3 and Figure S24).

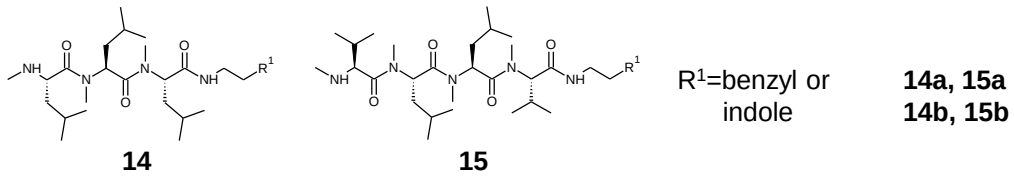


Figure 20: General structure of the permethylated rhabdopeptide-like structures (**14a**, **14b**, **15a** and **15b**), which were synthesized after permethylation on resin.

Nevertheless, they can be used to a certain extent for structure elucidation purposes, e. g. for comparison of LC-MS traces and fragmentation behavior. A natural product with the same fragmentation pattern of **14a**, was found in *X. vietnamiensis* DSM22392 (Figure 21a and b). *X. griffiniae* DSM17911 produces a natural product with the mass of 542.4. Unfortunately, its fragmentation pattern does not coincide with the synthesized **14b** (Figure 21c and d). Moreover, a natural product with the same mass like **15a** was found upon injection of *X. nematophila* HGB081 into *G. mellonella* larvae (Daniela Reimer, unpublished data).

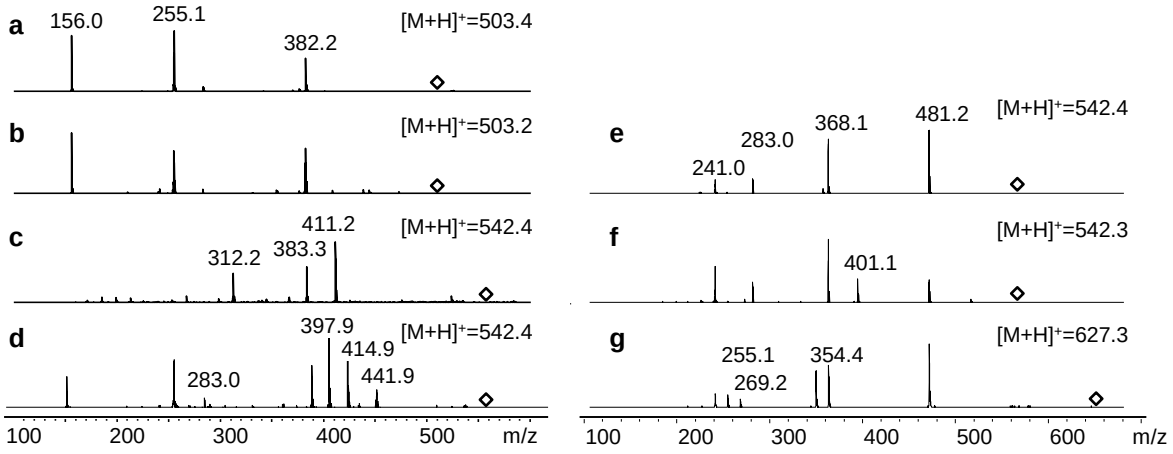


Figure 21: Fragmentation pattern of the natural **14a** produced by *Xenorhabdus vietnamiensis* DSM22392 (a) in and the synthetic **14a** (b). A peptide produced by *Xenorhabdus griffiniae* DSM17911 (c) and in comparison the synthetic **14b** (d). Unfortunately, the fragmentation pattern of **15a** (f) does not entirely coincide entirely with the rhabdopeptide produced upon injection of HGB081 into *Galleria mellonella* (e). **15b** is the synthetic tryptamine counterpart of **15a** (g).

Yet again, the fragmentation pattern of the natural product detected under these conditions (Figure 21e) did not entirely coincide with the fragmentation pattern of the synthetic **15a** (Figure 21f). **15b** is a purely synthetic derivative and has not been found so far in a natural environment (Figure 21g).

6.4 Synthesis of highly methylated sequences

The class of rhabdopeptides not only consists of sequences with isolated methylations or permethylated ones, but also sequences that bear one single non-methylated amide (Figure 22a; free amide is indicated by the red arrow). These peptides pose a synthetic obstacle, since

they are prone to form cyclic side products (Figure 22b), the so-called diketopiperazines (DKPs), during the synthesis. This side reaction occurs more frequently during the synthesis of alternating backbone methylations and is induced upon fluorenylmethoxycarbonyl (Fmoc)-deprotection with piperidine (Figure 22b).[6] Moreover, it is promoted by a *C*-terminal ester bond, which acts as a leaving group and drives the reaction. Additionally, these highly methylated peptides characteristically display low coupling rates and low fragment solubility.

The suppression of DKP formation can be achieved to a certain extent by using a 2-chloro trityl chloride linker.[26] Its steric demand reduces the likeliness of the cyclic side reaction and is known to prevent the racemization of the primarily immobilized amino acid.[27] Unfortunately, even upon using the above described linker the synthesis cannot start with a backbone methylated amino acid because of the spontaneous DKP formation after coupling and deprotecting of the second amino acid (Figure 22b).[6,28,29]

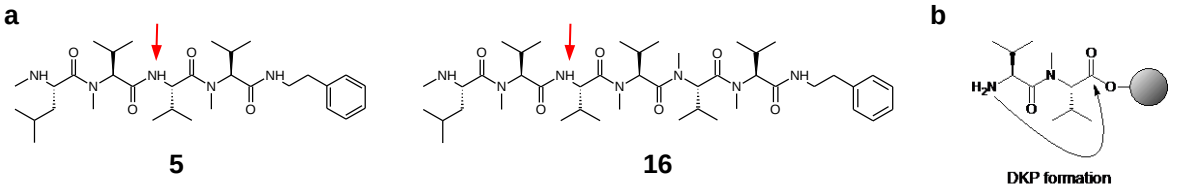


Figure 22: General structure (a) of naturally occurring highly methylated rhabdopeptides (**5** and **16**) and the mayor side reaction in their synthesis (b), spontanous diketopiperazine (DKP) formation upon fluorenylmethoxycarbonyl-deprotection (Fmoc).

6.5 Fragment condensation approach

Taking all the above mentioned difficulties into consideration, rhabdopeptide **5** (Figure 22a) was attempted to be synthesized in two fragments, one on the solid phase, the other one in solution, which were then condensed in solution. The fragmentation pattern of the natural **5** (Figure 23a) and the Boc-protected synthetic **5** (Figure 23b) coincide (the Boc-protection group is lost at the ionization conditions and therefore only the mass of the free peptide is observed). Unfortunately, this synthesis approach was prone to side reactions in addition to the degradation of the product upon final Boc-deprotection with trifluoroacetic acid (Figure 23c). Regrettably, the degradation/decomposition of alkylated especially methylated peptides upon incubation in acetic conditions is a common process.[30,31] In order to make these kind of peptides accessible for further testing, different synthetic approach has to be found.

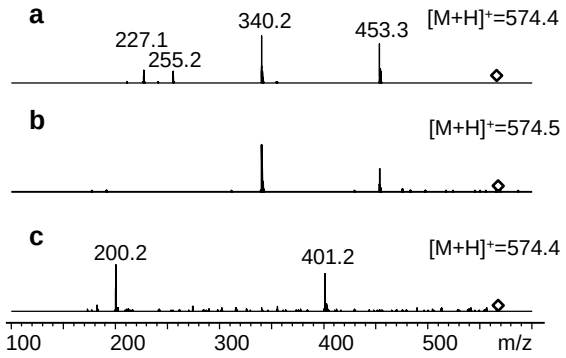


Figure 23: MS/MS data of the natural **5** (a) and the synthetic **5** prior to Boc-deprotection (b) and after Boc-deprotection (c). Upon incubation of the Boc-protected peptide with trifluoroacetic acid in CH_2Cl_2 the peptide decomposes.

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TARGET IDENTIFICATION

PRELIMINARY WORK

The scientist is not a person who gives the right answers; he is one who asks the right questions.

Claude Lévi-Strauss

7 Target Identification

Natural products are interesting lead structures since they usually specifically target complex biosynthetic pathways. However, at first the natural target has to be identified. In order to do so, several different approaches can be employed, e. g. label transfer chemistry, so-called pull down assays or (photoactivated) crosslinking with intrinsically or subsequently labeled probes.

7.1 Biotinylated GameXPeptides

In 1999 Belofsky *et al* found a potent cytotoxic agent, called sansalvamide (**1**), in a marine fungus of the genus *Fusarium* (Figure 24a).[1] After having synthesized **1** in high yields (67%, >95% pure) by using a polymer tethered phenylalanine building block in 2000,[2] Silverman and co-workers employed a similar strategy on the synthesis of the cyclic macrolactam analogue (sansalvamide A peptide or san A amide, **2**).[3] Even though, the replacement of an ester bond in natural depsipeptides usually leads to a mayor decrease in the biological activity,[4] they observed a 10-fold increase in activity against HCT-116 colon cancer cells for **2** in comparison to **1**.

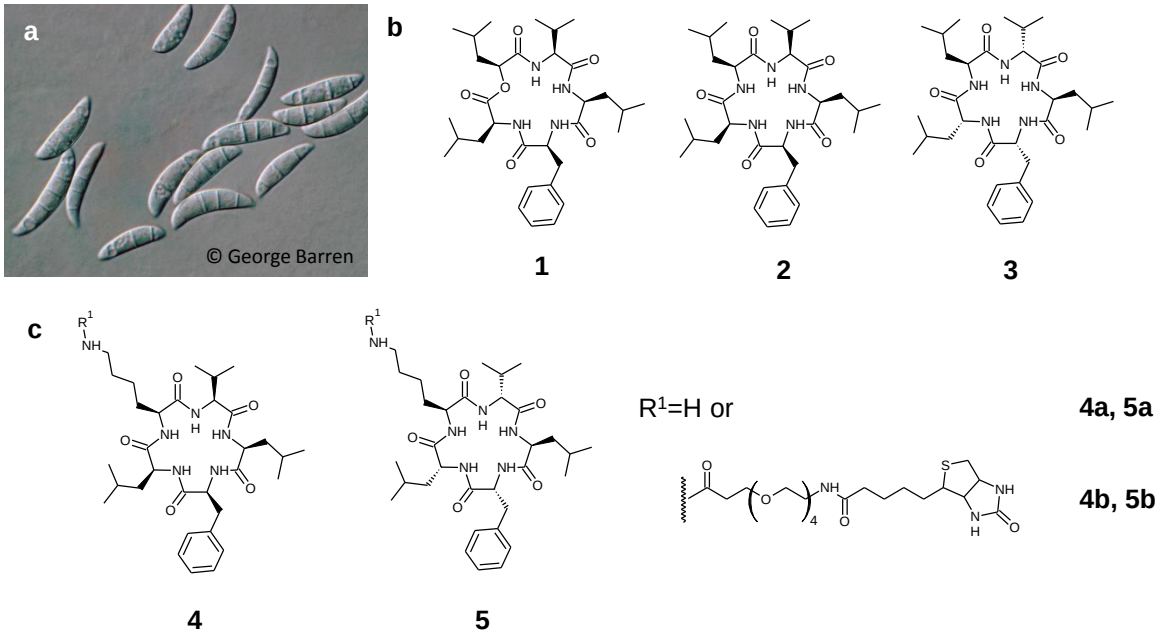
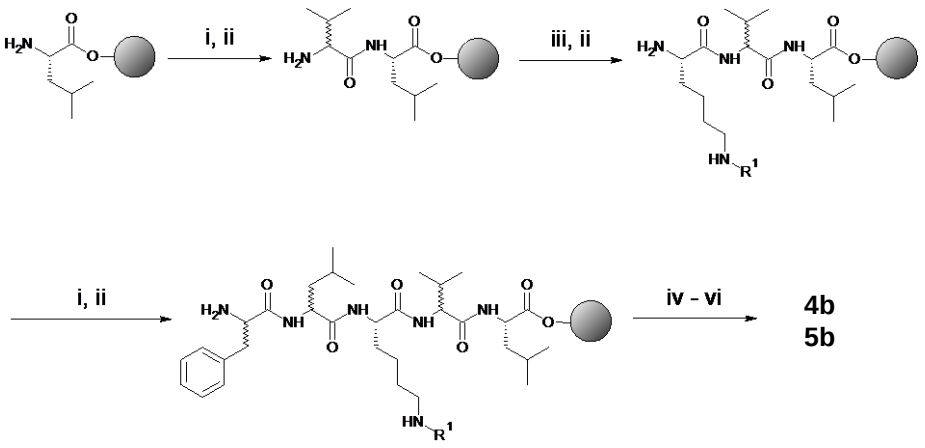


Figure 24: *Fusarium* spp. (a, spores of *F. culmorum*) produce amongst others the cytotoxic depsipeptide sansalvamide (**1**).[1] Comparision (b) of the natural **1** with the synthetic derivative sansalvamide A peptide (**2**)[3] and GameXPeptide A (**3**), found in *Photorhabdus luminescens.*[5] Overview on (c) the derivatized sansalvamide A peptide (**4a** and **4b**) as well as the derivatized GameXPeptide A (**5a** and **5b**).

Later on, the group of Shelli R. McAlpine extensively studied the structure-activity relationship (SAR) of **2** by making different modifications along its backbone, leading to derivatives with cytotoxic activity at low micro molar concentrations against several different

cancer cell lines.[6-8] Interestingly, the GameXPeptide A (**3**), which is produced by *P. luminescens* TTO1,[5] shows a high accordance to the natural product **1** and the synthetic **2**. Thus, we wanted to explore whether **3** has the same or a similar target like **1** and **2**. Based on SAR studies by Rodríguez *et al*, which indicated that the leucine in position 5 can be varied without mayor changes in the activity,[7] compounds **4** and **5** (Figure 24) were synthesized in each case with a free lysine (**4a** and **5a**) and a biotinylated lysine residue (**4b** and **5b**). In 2010, Vasko *et al* were able show in a pulldown assay that **4b** specifically bound to the *N*-middledomain of Hsp90 and as a result inhibited the binding of the protein, which is supposedly interacting with the *C*-terminal subunit of Hsp90 (Figure 25).[9] Thus, we wanted to explore whether or not the GameXPeptides, in particular GameXPeptide A, also bind to Hsp90 and this region. For the synthesis of the derivatives solid phase peptide synthesis was employed using an acid sensitive resin and standard Fmoc/^{t}Bu strategy. For **4b** and **5b** a prebiotinylated lysine building block (Iris Biotech, Markredwitz) was incorporated in the growing peptide chain using modified coupling conditions (Scheme 6). The slight excess of coupling reagent leads to a guanidylation of the free *N*-terminus, which is usually considered a side reaction of aminium/uronium-based coupling reagents.[10]



Scheme 6: Overview on the synthesis of **4b** and **5b**, amino acid coupling was performed according to standard procedures (i, 5.0 eq *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), 6.0 eq amino acid, 10.0 eq *N*,*N*-diisopropylethylamine (DIEA) in *N*,*N*-dimethylformamide (DMF) at room temperature for 45 min; ii, 40% piperidine in DMF at room temperature for 2 min followed by an incubation with 20% piperidine in DMF at room temperature for 10 min; iii, 1.4 eq *N*-alpha-(9-fluorenylmethyloxy-carbonyl)-*N*-ε-[15-(biotinamido)-4,7,10,13-tetraoxa-pentadecanoyl]-L-lysine (Fmoc-L-Lys(dPEG™(4)-biotin)-OH), 1.5 eq *O*-(7-aza¬benzo¬triazol-1-yl)-*N,N,N′,N′*-tetra¬methyl¬uronium hexafluorophosphate (HATU), 1.5 eq 1-hydroxy-7-azabenzotriazole (HOAt), 1.65 eq 4-methylmorpholine (NMM) in 1-methyl-2-pyrrolidinone (NMP) at room temperature overnight; iv, 95% aqueous trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS) for 90 min at room temperature, precipitated with cold diethylether (Et_2O); v, (1.5eq HATU in DMF with 2 eq DIEA for 20 min at 75°C and 25W) ; vi, resuspended in 1mL TFA precipitated with 40mL cold Et_2O).

Since this side reaction proceeds more slowly than the actual amino acid coupling, it was used as an irreversible capping method and therefore, prevented the synthesis of biotin-deficient peptides. After cleavage from the resin and precipitation of the product with cold diethyl ether these truncated peptides eventually remained in solution and a preliminary purification was achieved (for more synthesis details see supplementary information S6.1). The linear precursor peptides were cyclized in solution, washed several times and then submitted to testing. Upon incubation of the **2**, **3**, **4a** and **5a** with murine embryonic fibroblasts (100 µM) no significant activity referring to the induction of Hsp70 and smallHsp was observed in comparison to the positive control 17-AAG (10 µM). However, upon incubation with HeLa cells (under the same conditions), a human cervical cancer cell line, the peptides showed a similar activity like the positive control 17-AAG (Prof. Martin Vabulas, unpublished data).

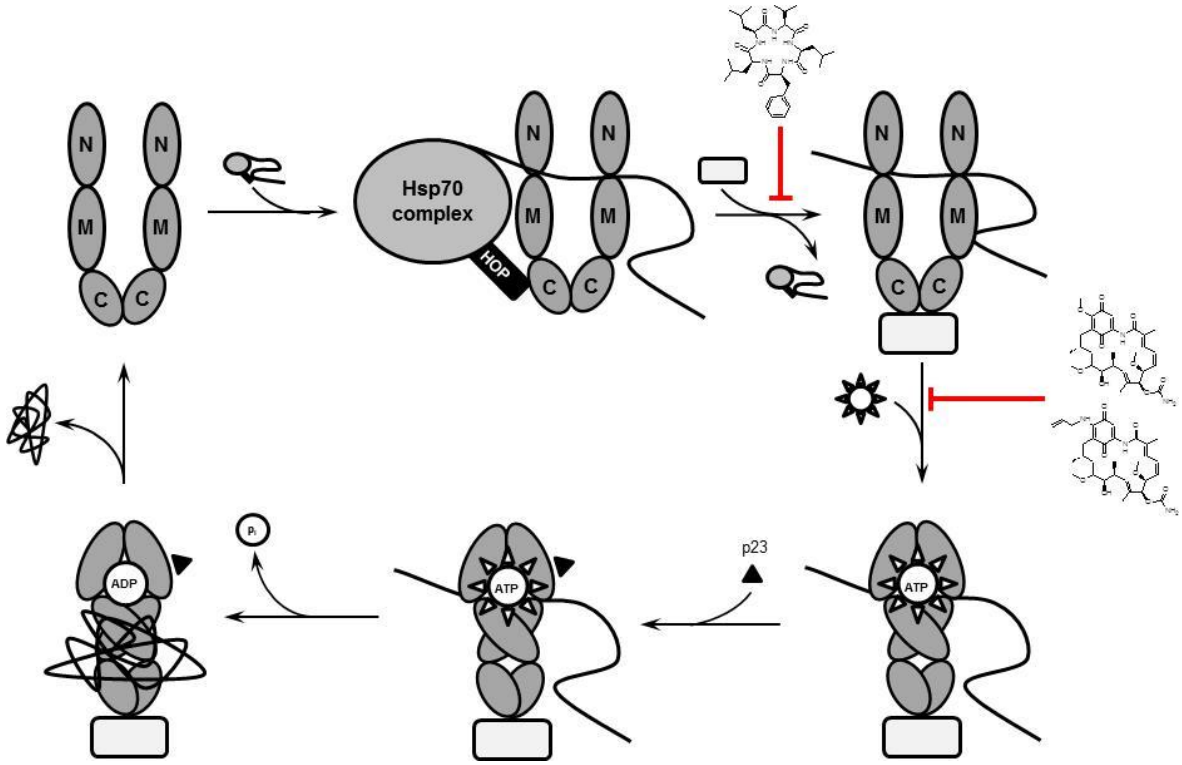


Figure 25: Currently proposed mechanism of protein folding involving the Hsp90 dimers and known inhibitors sansalvamide A, geldanamycin and 17-AAG adapted from Veronica C. Ardi *et al.*[11]

Then **4b** and **5b**, respectively, were incubated with recombinantly expressed and purified human Hsp90. Unfortunately, in these initial experiments only **4b** bound to the chaperone (Prof. Martin Vabulas, unpublished data). Nevertheless, the GameXPeptides[12] as well as their derivative **5a** (IC_{50} = 5.8 µg/mL; chloroquine was used as a positive control) show a significant activity against *P. falciparum* NF54. The Hsp90 protein sequences are highly conserved across all species, however, minor differences cluster them into three different

clades (prokaryotic, eukaryotic ER/chloroplast and cytosolic Hsp90 homologues) upon phylogenetic analysis.[13] The Hsp90 sequence of *Plasmodium falciparum* and the human α-Hsp90 are closely related, however, not entirely identical (they both belong to the class of cytosolic expressed Hsp90 homologues). These minor changes might be addressed by the variation in the amino acid configuration of **3** (in respect to **2**). This could also explain the increased activity of **3** (IC_{50} = 1.4 µg/mL) compared to that of **2** (IC_{50} = 4.1 µg/mL) against the causative agent of malaria.

7.2 Intrinsically labelled Xenephematides

Recently, a new depsipeptide, named xenephematide, was found in *Xenorhabdus* PB30.3 (Florian Grundmann, unpublished data). Since the peptide contained a D-phenylalanine as well as an L-phenylalanine according to results of Marfey's experiments, the possible diastereomers **6a** and **7a** (Figure 26) had to be synthesized in order to determine the right structure. The synthesis followed the established synthesis route of depispeptides (see 4.2).

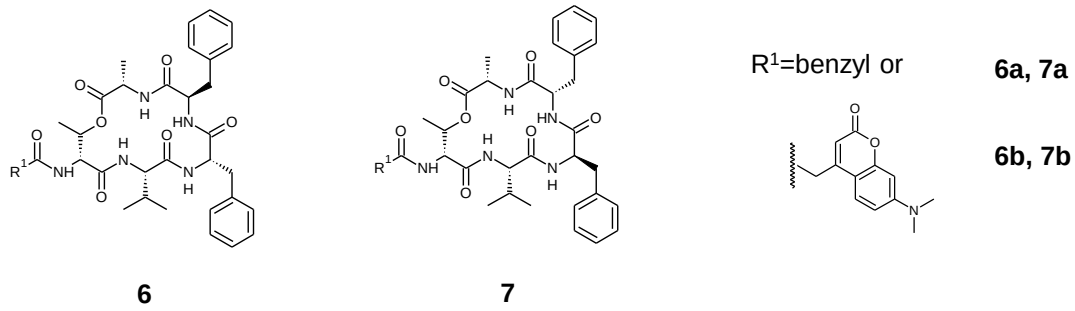


Figure 26: Structure of the natural (**6a**) and the synthetic xenephematide (**7a**) as well as their coumarin labelled derivatives (**6b** and **7b**).

Unfortunately, the right stereochemistry of the natural product could not be established by comparing HPLC-MS anaylses of the purified natural product (Figure 27a) and the synthetized peptides (Figure 27c and e) due to racemization during cyclization. However, extensive NMR studies indicated that the xenephematide contains the peptide sequence PA-L-[Thr-D-Val-L-Phe-D-Phe-L-Ala] (Florian Grundmann, unpublished data).

Moreover, these HPLC analyses also illustrated that the sequence of **6** favors the ring closure rather than the sequence of **7**, which results in the racemization of the *C*-terminal amino acid (Figure 27e-g, Peak I and II). These findings stressed the observation that cyclization reactions generally proceed faster between D- and L-amino acids rather than between α-stereocenters with the same configuration.[14,15]

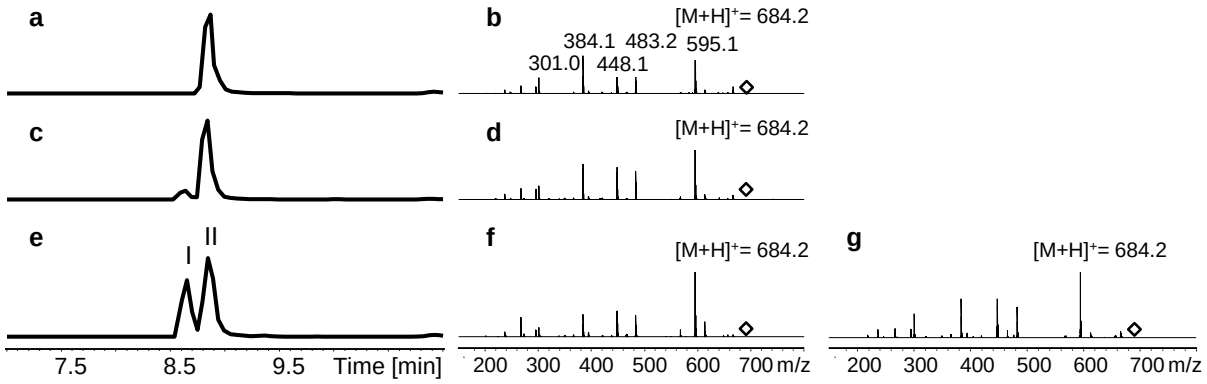


Figure 27: HPLC-MS analysis of the natural occurring xenephematide (a, extracted ion chromatogram; b, MS/MS data) in comparison to the synthesized diastereomers **6a** (c, extracted ion chromatogram; d, MS/MS data) and **7a** (e, extracted ion chromatogram; f, MS/MS data of peak I; g, MS/MS data of peak II). During the cyclization reaction of **7a** racemization occurred at the *C*-terminal amino acid. The resulting peaks displayed the same mass and fragmentation pattern, but slightly different retention times.

In addition to the xenephematide isolated from *Xenorhabdus* P30.3 the synthetic derivatives were submitted to continuative testing against the causative agents of neglected tropical diseases. The activity of the natural xenephematide (Table 4, entry 1) and **6a** (Table 4, entry 2) were comparable for *Trypanosoma brucei rhodesiense* and *Leishmania donovani*, the IC_{50} determined for **7a** were at least three times higher (Table 4, entry 3). In the case of *Trypanosoma cruzi* and *Plasmodium falciparum* NF54 the IC_{50} values obtained for **6a** were slightly elevated. Surprisingly, neither **6a** nor **7a** nor the natural xenephematide showed any significant cytotoxicity against L6 cells (Table 4). The xenephematide was then used as a

Table 4: Overview on the biological activity of natural xenephematide as well as its synthetic derivatives. The half maximal inhibitory concentration (IC_{50}) against *Trypanosoma brucei rhodesiense* (positive control melarsoprol IC_{50}=0.002 µg/mL), *Trypanosoma cruzi* (positive control benznidazole IC_{50}=0.45 µg/mL), *Leishmania donovani* (positive control miltefosine IC_{50}=0.174 µg/mL), and *Plasmodium falciparum* NF54 (positive control chloroquine IC_{50}=0.002 µg/mL) were determined as described previously. Moreover, the cytotoxity in L6 cells was evaluated (positive control podophyllotoxin IC_{50}=0.008 µg/mL). [16]

	compound	IC_{50} [µg/mL] / IC_{50} [µM]				
		T. b. rhod.	*T. cruzi*	*L. don. ax.*	*P. falc. NF54*	*L6 cells*
1	natural xenephematide	13.6 / 19.9	19.6 / 28.7	11.7 / 17.1	5.8 / 8.5	51.2 / 74.9
2	**6a**	14.5 / 21.3	40.9 / 59.8	15.4 / 22.5	17.6 / 25.8	>100
3	**7a**	47.4 / 69.4	65.1 / 95.3	76.1 / 111.4	26.5 / 38.8	90.9 / 133.1
4	**6b**	34.0 / 42.8	42.8 / 53.8	25.5 / 32.1	24.3 / 30.6	>100
5	**7b**	35.0 / 44.0	77.8 / 97.9	>100	24.0 / 30.2	>100

model for intrinsic labelling with a coumarin entity (**6b** and **7b**) displacing the phenylacetic acid at the *N*-terminus, which was readily synthesized starting from diethyl-1,3-acetonedicarboxylate and *m*-dimethylaminophenol (68% yield).[17,18] **6a**, **6b**, **7a** and **7b** were incubated with bacteria and insect cells as well as lipid vesicles filled with fluorescein. Upon incubation of **6a** and **6b** with the lipid vesicles a tube formation was observed (Figure 28a-f). This did not occur during the incubation with the unnatural derivatives **7a** and **7b** (see supplementary information S7.2 Figure xxx). Previously, this phenomenon of tube formation or budding process has been observed e. g. melittin[19] and penetratin.[20] Upon incubation of **6b** with insect cells a blue fluorescence localized at the cell membrane was detected (Figure 28g and h). Currently, these compounds are undergoing quartz crystal microbalance (QMC) measurements and further testing.

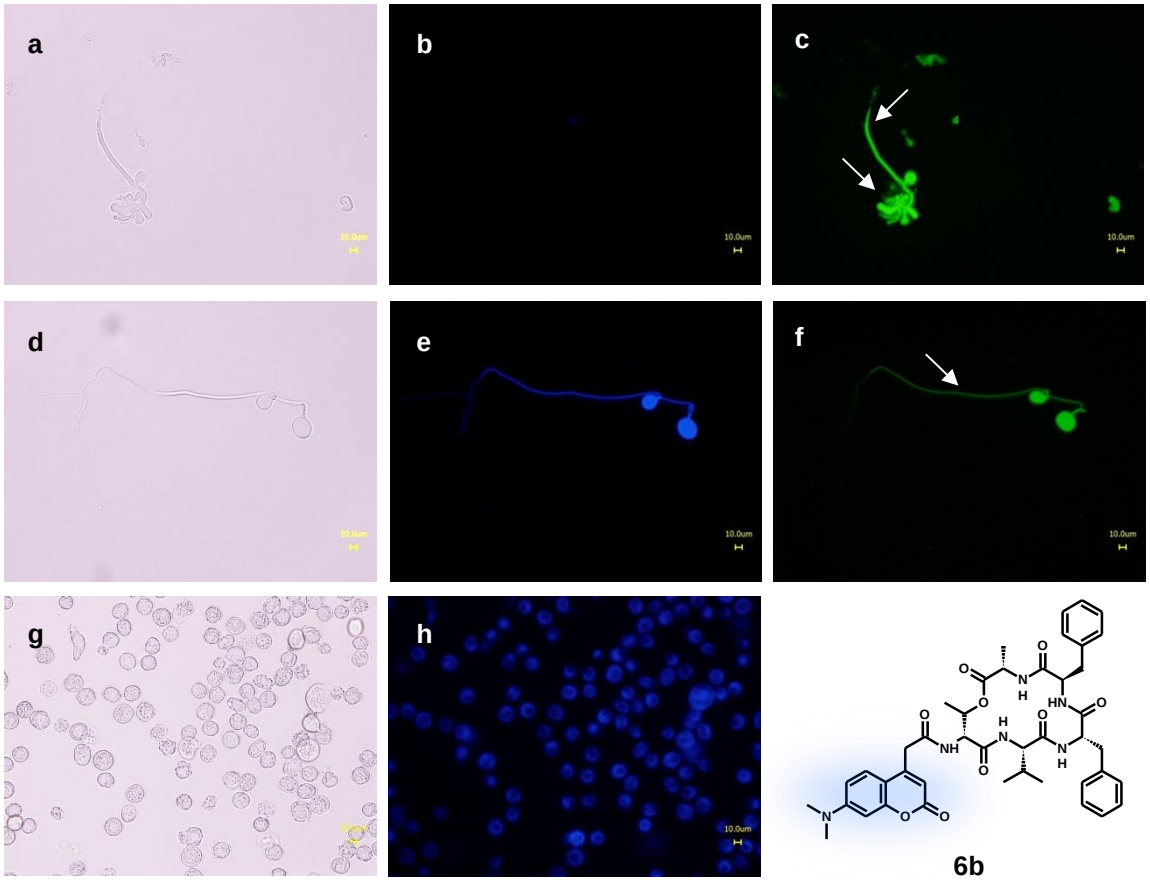


Figure 28: The non-labeled xenephematide (**6a**) was incubated with fluorescein-filled small unilamellar vesicles (bright field (a; exposure time 1/20s), DAPI (b; exposure time 1/20s) and GFP filter (c; exposure time 1/20s)), in comparison to that the labelled xenephematide (**6b**; bright field (d; exposure time 1/20s), DAPI (e; exposure time 1/20s) and GFP filter (f; exposure time 1/20s)). Additionally, **6b** was incubated with Sf9 insect cells (bright field (g; exposure time 1/50s) and DAPI filter (h; exposure time 1/20s)).

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DISCUSSION

Every so often, you have to unlearn what you thought you already knew, and replace it by something more subtle. This process is what science is all about, and it never stops.

Terry Pratchett

8 Discussion

In the following the different compounds, which are generally classified as small molecules, cyclic (depsi)peptides and methylated peptides, are discussed in detail regarding their synthesis and/or their potential biological function. The group of small molecules is represented by the phurealipids (see chapter 2). The cyclic peptides comprise the xenotetrapeptide (see chapter 3.1), GameXPeptides (see chapter 3.2 and 6.1) and the ambactin (see chapter 3.3). The szentiamide (see chapter 4.1), xentrivalpeptides (see chapter 4.2) and the xenephematide (see chapter 6.2) belong to the class of cyclic depsipeptides. The last class consists of several different backbone methylated peptides which are recapitulated and discussed in the last subitem (see chapter 5).

8.1 Small Molecules

Low molecular weight natural products have played a crucial role in the advance of medical treatment and human expectancy of life, showing e. g. antimicrobial or cytostatic properties. However, the natural producers not only use them as antibiotics or cytostatics but also for a variety of different applications perfectly adapted to their ecological niche.[1,2] Hence, these small molecules perform a wide scope of important tasks, amongst others as signaling molecules or virulence factors. However, the genuine biological purpose often remains unknown even for the majority of already fully characterized natural products.[1,3]

8.1.1 Phurealipids

Resulting probably from the complex interaction network of the bacterium with its nematode host and the insect prey as well as additional environmental factors, *Photorhabus luminiscens* TTO1 is a multiproducer of natural product libraries.[4-6] One of these libraries is comprised of urea lipids.[7] These so called phurealipids inhibit the juvenile hormone epoxide hydrolase (JHEH), a key enzyme in the metamorphosis and immune response of insect larvae. Surprisingly, already a decade ago synthetic inhibitors of the JHEH, which are structurally related to the natural phurealipids, have been developed as insecticides.[8-10] The structure of the natural occurring urea derivatives was determined by detailed stable isotope labeling experiments in the wild type as well as a knock out mutant of the biosynthetically important methyltransferase PliB aided by LC-MS/MS analysis. Here, three methylated phurealipids and their desmethyl counterparts, one being produced naturally and the others upon rendering PliB inactive, were characterized and subsequently synthesized. Based on the characteristic neutral loss during the collision induced dissociation (CID) of 57 Da (methyl urea moiety) and 43 Da (desmethyl urea moiety), respectively, several phurealipid producing strains were

identified which mostly belong to the species of *P. luminiscens*. The highly clustered production within different *P. luminescens* strains with the exception of a few *Xenorhabdus* strains from Vietnam indicates a species specific function of these small molecules. Recently, Chu *et al* identified similar molecules, the Yayueras, as inhibitors of quorum sensing in γ-proteobacteria, for example, which are produced by *Staphylococcus* spp.[11] These small molecules belong just like the phurealipids to the class of substituted urea. Unfortunately, the phurealipids exhibited neither antibacterial nor antifungal nor quorum quenching properties in continuative testing. Nevertheless, this similarity suggests an involvement in signalling processes within the *P. luminescens* species in addition to the JHEH and JHE modulatory/inhibitory properties. Interestingly, the desmethyl phurealipids exhibited an additional activity against *Leishmania donovani*, which is responsible for a severe form leishmaniasis, also known as kala-azar, black fever or Dumdum fever,[12] being 10-times more active than their methylated counterparts. So far JH-like regulatory pathways have not been identified in *L. donovani*. However, studies concerning the effects of JH and JH analogues on metabolic processes of *trypanosomatids*, e. g. *Crithidia* spp.[13] and *Trypanosoma cruzi*[14] revealed that JH itself as well as its analogues are capable of interfering with growth and reproduction of the parasites. Here, the obtained data indicate that the (reversible) inhibition rather takes places on a transcription than a translation level. Thus, it seems reasonable to assume that the desmethyl phurealipids display a similar mode of action, especially when the structural similarities are taken into consideration.

8.2 Cyclic Peptides

Natural cyclic peptides are associated with a whole scope of different biological activities.[15] Hence, they are interesting candidates for target identification and in depth characterization. Nowadays, the structure elucidation can be aided not only by advanced mass spectrometric techniques but also by extended genome mining and other tools from molecular biology.[16-18] Nevertheless, the cyclic structure complicates its elucidation and it has often to be ensured by synthesis. *Xenorhabdus* and *Photorhabdus* spp. produce several different cyclic peptides, ranging from hydrophilic to hydrophobic sequences with different biological activities.[19,20]

8.2.1 Xenotetrapeptide

Recently, Bode *et al* developed a stable isotope labeling approach allowing the structure elucidation of peptidic natural products including the absolute configuration.[16] Here, detailed MS/MS analyses were aided by the use of transaminase deficient mutants (of *Photorhabdus luminescens* TTO1 and *Xenorhabdus nematophila* HBG081), enabling the structure

elucidation in crude extracts without having to isolate or purify the natural products. This technique was then refined and further developed by Kegler *et al* leading amongst others to the structural elucidation of the xenotetrapeptide produced by *X. nematophila* HGB081.[17,18] The isolation and characterization of this particular compound was complicated by its insolubility in aqueous media as well as organic solvents. In order to confirm the structural proposal and substantiate the above mentioned approach it had to be synthesized. Prior syntheses on resin and in solution (Veronica Dill and Christina Dauth, unpublished data) using standardized protocols were not successful. Hence, an auxiliary was incorporated in the peptide chain in order to facilitate the macrolactamization via a less constrained intermediate.[21,22] Unfortunately, this approach often leads to the racemization of the *C*-terminal amino acids. In order to evaluate the influence of the cyclization conditions, such as temperature, energy/power or base equivalents, on the amount of racemization, a linear non-derivatized peptide was incubated at identical conditions. Surprisingly, during said evaluation process, the product formed as a colorless precipitate from *N*,*N*-dimethyl-formamide (DMF) in the presence of Hünig's base. Cyclic tetrapeptides are characterized by a very rigid 12-membered ring which makes them unique while playing a big role in their target specificity. However, this rigidity is also responsible for difficulties which arise during their synthesis. These so-called difficult cyclic sequences usually favor the formation of linear and cyclic oligomers over the cyclic monomer even at high dilution conditions.[23] Intensive research all over the world led to several different synthetic techniques, which allowed to even synthesize all-L tetrapeptides devoid of proline or other turn inducers.[21,22,24-29] Yet, in the case of the xenotetrapeptide its synthesis was not impeded by its rigidity but rather by its insolubility. Here, the ring formation is probably facilitated by the spatial valine residues as well as the alternating conformation of the incorporated stereo centers.

8.2.2 GameXPeptides

The general structure of GameXPeptides was initially elucidated based on the beforehand mentioned stable isotope labeling approach.[16] Interestingly, this compound class is highly dependent on environmental factors.[30] When *Photorhabdus luminescens* TTO1 is grown within insect larvae another subset of derivatives (showing 4-amino-D-phenylalanine (PAPA) or 4-methylamino-D-phenylalanine (MMPAPA)) is predominantly produced due to the insect-specific production of certain building blocks and a relaxed substrate-specificity of the involved synthetase. Consequently, these derivatives will not be produced upon heterologous expression of the *gxpS* gene on its own, but only upon supplementation with the specific

building blocks or co-expression with the auxiliary PAPA gene cluster. Interestingly, only the genomes of *P. luminescens* TTO1, *P. temperata* NC19 and *P. asymbiotica* bear in addition to the *gxpS* cluster a PAPA gene cluster. None of the *Xenorhabdus* strains which possess *gxpS* homologues appear to have incorporated the PAPA gene cluster into their genome (analyses were restricted to accessible genomes). The widespread occurrence of the GameXPeptides (based on LC-MS analyses of over two hundred *Photorhabdus* and *Xenorhabdus* strains) indicates an important function in the entomopathogenic sister taxa, yet the actual biological function remains unknown. GameXPeptide A bears a striking resemblance with the sansalvamide A peptide,[31-34] which displays cytotoxic activity in several different cell lines due to Hsp90 modulatory properties.[35] Thus, it seems plausible that the GameXPeptide A also targets Hsp90 or similar proteins. Unfortunately, in initial experiments with human Hsp90 only the sansalvamide A peptide displayed specific binding affinities. Nevertheless, the GameXPeptides show a significant activity against *Plasmodium falciparum* NF54.[30] On the one hand, this might be explained by Hsp90 protein sequences being highly conserved with minor variations across all species.[36] The Hsp90 sequence of *Plasmodium falciparum* and the human α-Hsp90 are indeed closely related, but not identical. These minor changes might be addressed by the variation in the amino acid configuration of the GameXPeptides in comparison to the sansalvamide A peptide. This hypothesis is supported by the increased activity of GameXPeptide A against the causative agent of malaria. On the other hand, several bacteria are known to hijack and feed on protozoa,[37,38] which are the natural predator of bacteria. Hence, the GameXPeptides might be a first defense lines against amoeba and the activity against *P. falciparum* is actually a "cross-activity".

8.2.3 Ambactin

Another representative for cyclic macrolactams produced by these entomopathogenic bacteria is the ambactin. This cyclic hexapeptide is produced by *X. miraniensis* DSM17902 and was identified upon genome mining in addition to stable isotope labeling after heterologous expression of the corresponding gene cluster.[39] In contrast to the xenotetrapeptide and the GameXPeptides, ambactin displays not only hydrophobic but also hydrophilic amino acids. Hence, the cyclization conditions had to be adapted. Though, cyclization reactions are still characterized by a "trial and error" approach, studies have shown that the use of 1-hydroxy-7-azabenzotriazole (HOAt)-based coupling reagents is beneficial concerning the yield and the retention of chiral integrity. Due to the electron withdrawing properties of the nitrogen atom at position seven, which affects its stability and its leaving group properties, in addition to a

classical neighboring effect, HOAt display a higher reactivity than "normal" benzotriazoles.[40] *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU), which is the tetramethyluronium derivative of HOAt, combines the advantages of azabenzotriazoles with even higher reaction velocities.[41,42] Hence, it is usually superior to benzotriazole-based coupling reagents.[43,44] Nevertheless, its major side reaction is the slow and irreversible capping of the *N*-terminus. In the case of the ambactin the use of HATU (at 70°C as well as 55°C) on its own led to major side products. Upon lowering the reaction temperature to 55°C and addition of HOAt or the use of a combination of *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC)[15] and HOAt instead of HATU the yield and purity of the resultant product improved significantly.

8.3 Cyclic Depsipeptides

Similar to natural cyclic peptides, the bioactivities associated with cyclic depsipeptides are as diverse as their structures.[15] Here, the ester bond is often responsible for precisely these activities and its substitution by a lactam frequently results in a total inactivity of the compound. Unfortunately, the incorporation of an ester bond into the peptide sequence may impose a serious obstacle depending on the peptide sequence and structure.

8.3.1 Szentiamide

Usually, cyclic depsipeptides are associated with marine microorganisms and actinomycetes providing several (pharmaceutical) interesting molecules.[45-47] Unsurprisingly, the in 2011 newly characterized szentiamide represented only the second cyclic depsipeptide isolated and elucidated from *Xenorhabdus* spp besides the well-known xenematide. By now, several other more complex depsipeptide families have been associated with these entomopathogenic bacteria.[48-50] The szentiamide itself is a unique peptide due to its *N*-terminal formylation, which is commonly known for the initiation of the prokaryotic ribosomal protein biosynthesis but is exceptional for the nonribosomal peptide synthesis.[51] Moreover, this cyclic depsipeptide displays antiparasitic properties with a particular good activity against *Plasmodium falciparum* (IC_{50} = 1.2 µM).[20] Several natural depsipeptides have been reported to show antimalarial activity[52] with IC_{50} values ranging from nM to low µM concentrations.[53,54] Unfortunately, the actual biological target and mode of action mostly remain unknown. Just as the szentiamide's natural purpose remains subject to speculation.

8.3.2 Xentrivalpeptides

Though, from a stereo-chemical point of view macrolactonization at nonepimerizable sites is preferred over macrolactamization at epimerizable sites,[55] lactamization usually proceeds more efficiently resulting in higher yield and purity.[22,56-58] Forming the ester bond on resin can considerably improve the reaction yield, but it usually remains the most difficult step during the preparation of natural depsipeptides.[15] Nevertheless, after the formation of the ester bond and the subsequent cyclization in solution the yield typically decreases significantly. In the course of exploring *Photorhabdus* and *Xenorhabdus* spp. as natural product producers several cyclic depsipeptides have been characterized.[48-50,59-61] Several different routes to form the depside bond have been employed during their synthesis. Esterification reactions employing carbodiimides in the presence of 4-(dimethylamino)-pyridin (DMAP; Steglich esterification) or rather DMAP salts (Boden-Keck conditions)[62] only worked rarely, e. g. in the case of the taxlllaids[63] or the fatflabets. The modified Yamaguchi conditions have successfully been used for the synthesis of xenematide A[64] and the szentiamide.[20] Unfortunately, the beforehand listed reaction conditions worked poorly or even not at all for the synthesis of the xentrivalpeptides. Here, the best yields were observed using a 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) pre-activated *tert*-butyloxycarbonyl (Boc) or *o*-nitrobenzenesulfonyl (*o*NBS) protected valine. The activation of the amino acid occurs via an aromatic mixed anhydride which is characterized by a good leaving group and therefore higher reactivity. In contrast to Boc, *o*NBS allows the continuation of the branched peptide sequence and displays an additional activating effect on the carbonyl group of the amino acid allowing challenging couplings to succeed even more easily.[65] This is advantageous in the case of the xentrivalpeptides, since the coupling has to proceed between the sterical hindered amino acids threonine and valine. Exemplary for the comprehension that the ideal reaction condition and yield of the esterification may vary considerably depending on the peptide sequence and the used protecting groups.[15]

8.3.3 Xenephematide

The recently characterized Xenephematide (Florian Grundmann, unpublished data) was used as a model compound for intrinsic fluorescent labeling. Ethyl-7-dimethylaminocoumarin-4-acetate was chosen as a dye substituting the *N*-terminal phenylacetic acid, because of its relatively small size, easy synthetic availability and photostability.[66,67] The major disadvantage of intrinsic modifications to natural products is the potential loss of the activity. Fortunately, the activity of the labeled xenephematide against protozoa did not drop

significantly. Upon incubation of the natural xenephematide as well as its labeled derivative with insect cells a blue fluorescence localized at membrane surface was detected. When incubated with giant unilamillar vesicles tube formation was observed. This did not occur with the unnatural enantiomers. The phenomenon of tube formation or budding process has been previously observed with membrane active peptides.[68-73] However, in order to further characterize the xenephematide's membrane destabilizing properties additional experiments have to be conducted.

8.4 Methylated Peptides

The entomopathogenic bacteria *Photorhabus* and *Xenorhabdus* spp. produce several different highly methylated peptides.[16,74] Due to their structural diversity resulting from minor changes in the amino acid sequence or composition as well as the methylation pattern, the isolation and further characterization is challenging. Detailed *in vivo* expression technology analyses indicate that these are insect-specific virulence factors in particular.[74] Therefore, these peptides are interesting candidates for further investigation, which have to be made available by synthesis. Four monomethylated dipeptides bearing a *C*-terminal tryptamine or phenylethylamine were synthesized in solution. Upon CID fragmentation they displayed a characteristic fragmentation pattern, which led to the identification of a short rhabdopeptide-like molecule (MeVal-Val-Val-TRA) in *X. indica* DSM17382. Additional stable isotope labeling experiments as well as the subsequent synthesis of the peptide verified its structure. Upon genome mining aided by LC-MS analysis another monomethylated peptide (Val-Val-MeLeu-Val-PEA) was identified in *X. nematophila* HGB081 (Daniela Reimer, unpublished data). The subsequent synthesized peptide corresponded to a rhabdopeptide which was produced upon injection of *X. nematophila* into *Galleria mellonella* or upon heightened expression of the associated *rdpABC* cluster (Daniela Reimer and Carsten Kegler, unpublished data). Not only monomethylated but also highly methylated or even permethylated peptides are produced by the entomopathogenic bacteria. In order to obtain these molecules, which are increasing insoluble with increasing sequence length, a resin-based permethylation was established. The data obtained in the experiments showed that incubation with Li*t*OBu and CH_3I gave the best results, but that the methylation success was highly sequence dependent. Especially, peptides containing β-branched amino acids were difficult to methylate. Employing the optimized methylation conditions for synthesis the structure of two rhabdopeptides naturally occurring in different *Xenorhabdus* strains was verified. Based on the experience gained during the synthesis of several of highly methylated

peptides on the solid support, some guiding principles can be established: An immobilization to the solid support via an amide bond is preferred over an ester bond. Moreover, alternating *N*-methylated amide bonds increasingly induce diketopiperazine (DKP) formation. If an *N*-methylated amino acid is located at the *C*-terminus, the Fmoc-deprotection of the subsequent amino acid leads to an immediate DKP formation. Furthermore, TFA mediated deprotection and cleavage from the solid support may lead to major side reactions,[75] here, hydrochloric acid in hexafluoroisopropanol turned out to be a good alternative. Unfortunately, the solubility of these highly methylated or permethylated rhabdopeptide precursors decreases immensely with the length of the initial peptide chain. This impairs the amidation in solution which worked quite nicely for shorter sequences. Hence, a different synthesis approach has to be found such as immobilizing the *C*-terminal amine via a back bone linker (such as the PAL or BAL linker) onto the resin followed by the synthesis of the peptide by coupling preformed building blocks.

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APPENDIX

General Procedures

I High Performance Liquid Chromatography Coupled to Electron Spray Ionisation Mass Spectrometry

For the analytical characterization of the synthetic and natural products amongst others a Dionex UltiMate 3000 System coupled to a Bruker AmaZon X mass spectrometer is used. The chromatographic separation is achieved by using a RP C18 BEH Acquity UPLC column (Waters Corporation, USA; 50 mm × 2,1 mm × 1,7 µl; 30°C oven temperature; 5µL inject volume; 0.6 mL/min) with a gradual elution beginning at 5% MeCN (0.1% FA) and ending at 95% MeCN (0.1% FA) within twenty minutes. The analytes are detected by a diode array detector (λ=272 nm) and a secondary electron multiplier detector after ionization by electron spray and separation by an ion trap.

II Nuclear Magnetic Resonance

^{1}H, COSY, HSQC and HMBC spectra were recorded on a Bruker AV400 [400 MHz (^{1}H) and 100 MHz (^{13}C)] spectrometer. ^{13}C-NMR spectra were recorded on a Bruker AV300 [300 MHz (^{1}H) and 75 MHz (^{13}C)]. Chemical shifts are reported in ppm (δ) with respect to the solvent signal ($CDCl_3$; ^{1}H-NMR: δ = 7.27 ppm, ^{13}C-NMR: δ = 77.2 ppm, CD_3OD; ^{1}H-NMR: δ = 3.14 ppm, ^{13}C-NMR: δ = 49.2 ppm, CD_3COOD; ^{1}H-NMR: δ = 2.04 ppm, ^{13}C-NMR: δ = 20.0 ppm, [D5]-pyridine ^{1}H-NMR: δ = 7.22 ppm; ^{13}C-NMR: δ = 123.9 ppm, [D6]-DMSO; ^{1}H-NMR: δ = 2.50 ppm; ^{13}C-NMR: δ = 39.51 ppm).

III Solid Phase Synthesis

In order to perform solid phase synthesis the resins have to be pre-incubated with an appropriated solvent (usually DMF or NMP, but CH_2Cl_2 or CH_3OH can also be used depending on the resin type used).

III.I Immobilization of the First Amino Acid on the Solid Support

There are several different ways to immobilize the *C*-terminal amino acid on the solid phase depending on the linker type and building block used. The conditions listed below are the ones which were commonly used to obtain the molecules described herein.

III.I.I Wang resin

The immobilization of the *C*-terminal amino acid on the Wang linker is achieved by incubation of 5.0 eq amino acid (in DMF or NMP, 0.5 M) in the presence of 8.25 eq pyridine and 5.0 eq 2,6-dichlorobenzoylchloride for 18 hours at room temperature.[1] After washing

several times with DMF and CH_2Cl_2, the approximate loading is determined with the DBU-Fulven test.[2]

III.I.II 2-Chloro Trityl Chloride resin

The immobilization of the *C*-terminal amino acid on the 2-chloro trityl chloride linker is achieved by incubation of 1.3 eq amino acid (in CH_2Cl_2, 0.15 M) in the presence of 3.0 eq DIEA for 18 hours at room temperature. The remaining free binding sites are capped upon incubating twice with a mixture of 80% CH_2Cl_2 (v/v), 15% CH_3OH (v/v) and 5% DIEA (v/v) for ten minutes at room temperature. After washing several times with DMF, CH_3OH and CH_2Cl_2, the approximate loading is determined with the DBU-Fulven test.[2]

III.I.III Rink Acid Resin

The immobilization of the *C*-terminal amino acid on the Rink acid linker is achieved by incubating twice with 50.0 eq TFAA (in 2,4,6-lutidine, 1M) for thirty minutes at room temperature. After this preactivation step, the resin is incubated with 1.3 eq amino acid (in CH_2Cl_2, 0.15 M) in the presence of 3.0 eq DIEA for 18 hours at room temperature.

III.II Deprotection

III.II.I Orthogonal Deprotection of Fmoc-protected Amines

Fmoc-deprotection is achieved by incubating the resin with 40% piperidine (v/v) in DMF or NMP for three minutes, followed by a second incubation with 20% piperidine (v/v) in DMF or NMP for ten minutes. The resin is then washed several times with DMF or NMP in order to remove any remaining piperidine.

III.II.II Selective Deprotection of Trityl-protected Alcohols

Trityl-deprotection of alcohols is achieved by adding 2% TFA (v/v) in CH_2Cl_2 to the resin (0.02 mL/µmol). The yellow color of the cleavage mixture indicates the liberation of the tritylium ions, which is decolorized upon immediate addition of 5.0 eq of triisopropylsilane. This has to be repeated until the cleavage mixture remains colorless. Then the resin is washed several times with CH_2Cl_2.

III.III Protection of Amines

III.III.I Carbamate-based Protection Groups

Carbamate-based protection groups, e. g. Fmoc or Boc, are introduced at the *N*-terminus upon incubation with 20.0 eq of the corresponding chloride, anhydride or succinimid ester (in DMF or NMP, 0.5 M) in the presence of 10.0 eq DIEA for two hours at 40°C. The resin then

washed several times with DMF or NMP. The progress of the reaction is monitored with the Kaiser test.[3]

III.III.II Nosyl-based protection groups

Nosyl-based protection groups, e. g. *o*-NBS, are introduced at the *N*-terminus upon incubation twice with 4.0 eq of the corresponding sulfonyl chloride (in DMF or NMP, 0.14 M) in the presence of 10.0 eq of *sym*-collidine for fifteen minutes at room temperature. Then the resin is washed several times with DMF or NMP.[4]

III.III.II.I Selective N-methylation (not suitable for cysteine, histidine or its derivatives)

For the selective *N*-methylation of individual amide bonds an *o*-NBS protection group is introduced at the transient *N*-terminus. The resin is then incubated with 3.0 eq DBU (in DMF or NMP, 0.21 M) for at least three minutes at room temperature. Afterwards 10.0 eq DMS (in DMF or NMP, 0.7 M) are added to the suspension. The incubation is prolonged by two minutes at room temperature vigorously agitated. The above described procedure is repeated once and then the resin is washed several times with DMF or NMP.[4]

III.III.II.II Deprotection of o-NBS Protected Primary Amines

The deprotection of *o*-NBS protected primary amines is achieved by incubating at least twice with a freshly prepared mixture of 5% thiophenol in DMF containing 2.0 eq of aqueous K_2CO_3 for thirty minutes at room temperature. Then the resin is washed repeatedly with DMF and CH_3OH.[5]

III.III.II.III Deprotection of o-NBS Protected Secondary Amines

The deprotection of *o*-NBS protected secondary amines is achieved by incubating at least twice with 10.0 eq 2-mercaptoethanol (in DMF or NMP, 0.35 M) in the presence of 5.0 eq DBU at room temperature for five minutes. Then the resin is washed repeatedly with DMF or NMP.[4]

III.III.III Aldehyde-based Protection Groups

Aldehyde-based protection groups, e. g. Hnb, MIM or EDOTn, are introduced at the *N*-terminus via a reductive alkylation. Therefore, the resin was incubated with 10.0 eq of the corresponding aldehyde (in 50% CH_3OH (v/v) and 50% DMF (v/v), 0.5 M) for twenty minutes at room temperature. Upon removal of the aldehyde solution the resin is washed once and immediately incubated with 20.0 eq $NaBH_3CN$ (in 45% CH_3OH (v/v), 45% DMF (v/v) and 10% AcOH (v/v), 1 M) for thirty minutes at room temperature. Then the resin is washed

several times with CH_3OH and DMF.[6,7] The progress of the reaction is monitored by the Kaiser[3] and chloranil test.[8]

III.IV Amino Acid Coupling

There are several different ways to achieve an amino acid coupling on the solid phase. The conditions listed below are the ones which were commonly used to obtain the molecules described herein. [9]

III.IV.I Primary Amines

Coupling of primary amines is generally achieved upon incubation of the resin with 5.0 eq of the corresponding amino acid (in DMF or NMP, 0.5 M) in the presence of 5.0 eq of the uronium-based coupling reagents HBTU or HCTU (in DMF or NMP, 0.5 M) and 10.0 eq of DIEA for an hour at room temperature while shaking. Then the resin is washed several times. The progress of the reaction is monitored by the Ninhydrin-based Kaiser test[3] or a test cleavage.

III.IV.II Secondary Amines

The coupling of secondary amines is achieved by incubating the resin twice with 3.0 q amino acid (in DMF or NMP, 0.5 M) in the presence of 3.0 eq HATU (in DMF or NMP, 1M), 3.0 eq HOAt (in DMF or NMP, 1M) and 3.3 eq NMI for three hours at room temperature while shaking.[4] The progress of the reaction is monitored by the chloranil test[8] or a test cleavage.

III.IV.III Acylation

The acylation of the *N*-terminus with e. g. fatty acids is usually achieved by a pre-activation step. 1.5 eq of acid is incubated with 1.5 eq DCC and 1.5 eq NHS or PFP in DMF (0.5 M) at room temperature. The pre-activation is considered complete when the precipitation of 1,3-dicyclohexylurea occurs. Then the resin is incubated with 1.5eq of the pre-activated species for 90 min at room temperature. In the case of acid-labile linkers or protecting groups 3.0eq of base, such as DIEA or NMI, are added.

III.V Cleavage

As there are several different ways to couple an amino acid, the conditions to separate a peptide from the solid support also vary depending on the determining factors and requirements.[10] Below the conditions are listed which are commonly used to obtain the molecules described herein. Test cleavages are performed like full cleavages on a smaller scale.

III.V.I Protected Peptide Fragments

When protected peptide fragments are needed the synthesis has to be conducted using acid-labile resins, such as 2-chloro trityl chloride or Rink acid resin. The cleavage of these fully protected peptide fragments is then achieved by incubating twice with 2% TFA (v/v) or 5-20% hexafluoroisopropanol (v/v) in CH_2Cl_2 for five minutes and one hour, respectively. The cleavage mixture is then evaporated at reduced pressure and the evaporate is washed several times with CH_2Cl_2 and ethyl acetate or CH_3OH.

III.V.II Unprotected Peptide Fragments

In order to liberate the peptide from the resin and its side chain protection groups the resin is incubated twice with 95% TFA (v/v), 2.5% triisopropylsilane (v/v) and 2.5% water (v/v) for 90 minutes at room temperature while shaking. If possible the peptide is then precipitated with cold diethyl ether or a mixture thereof with petrol ether. In the case of hydrophobic sequences the cleavage mixture is evaporated at reduced pressure and the evaporate is washed several times with CH_2Cl_2 and ethyl acetate or CH_3OH.

IV Solution Phase Synthesis

IV.I Boc-Deprotection

In order to cleave the Boc-protection group from the *N*-terminus the peptides are stirred in the presence of TFA (3 mL/mmol) diluted by CH_2Cl_2 (6 mL/mmol) at 4°C. The temperature is then slowly raised to room temperature. The progress of the reaction is monitiored by TLC. Upon completion, the solvents are removed at reduced pressure and the target molecule is isolated as the corresponding TFA-salt. In the case of molecules prone to form diketopiperazines or other acid-induced side products only 4.0 eq (or less) TFA (in CH_2Cl_2; 6 mL/mmol) are used.

In other cases the Boc-protecting group is cleaved by addition of HCl in dioxane (4.0 M or less) at 4°C. Then the temperature is slowly raised to room temperature and the progress of the reaction is monitored by TLC. Upon completion, the solvents are removed at reduced pressure and the target molecule is isolated as the corresponding HCl-salt.

IV.II Ester Saponification

Usually the removal of ester-based protection groups is achieved by refluxing in the presence of 2.0 eq of NaOH or 1.5 eq of LiOH (in a mixture of THF and water). Upon completion of the reaction (monitored by TLC) the reaction mixture is diluted with water and neutralized

with 1M HCl. Then the aqueous phase is extracted with CH_2Cl_2. The organic phase is dried with Na_2SO_4 and the solvent removed at reduced pressure.

IV.III Amino Acid Coupling

IV.III.I Primary Amines

The free acid is dissolved in dry CH_2Cl_2 (5 mL/mmol) and cooled to 4°C. Then 1.1 eq HOBt and 1.0 eq EDC are added to the reaction mixture. Finally, 1.5 eq amine is added and the reaction mixture is slowly warmed to room temperature and stirred for 18 hours. After the completion of the reaction, which is monitored with TLC, it is diluted with CH_2Cl_2, washed with water, 5% $NaHCO_3$ solution (m/v) and 1M $NaHSO_4$. Then the combined organic phases are dried over $NaSO_4$ and evaporated at reduced pressure.

IV.III.II Secondary Amines

The free acid is dissolved in dry CH_2Cl_2 (5 mL/mmol) and cooled to 4°C. Then 1.1 eq TBTU are added followed by 4.0 eq DIEA. This reaction mixture is stirred thirty minutes at 4°C, then 1.0 eq of the amine dissolved in CH_2Cl_2 (5 mL/mmol) are added. The reaction mixture is slowly warmed to room temperature and stirred for 18 hours. After the completion of the reaction, which is monitored with TLC, it is diluted with CH_2Cl_2, washed with saturated NH_4Cl solution. The aqueous phase is extracted three times with CH_2Cl_2 and the combined organic phases are dried over $MgSO_4$ and evaporated at reduced pressure. The product is obtained after normal phase chromatography.

IV.III.III Macrolactamization

Macrolactamization is conducted in solution with the help of microwave irradiation (20 min, 25 W; 75°C) by using 1.5 eq HATU and 3.0 eq DIEA in DMF (c = 4 mmol/L). The solvents are evaporated at reduced pressure and the precipitate is washed several times with ethyl acetate and water. If racemization occurred the cyclization conditions are adjusted accordingly.

V Small Unilamellar Vesicles

The fluorescein filled small unilamellar vesicles are prepared according to Sybille Rex.[11] Briefly, 3.9 mg of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholin (DMPC) are dissolved in 1.147 mL $CHCl_3$ and 2.9 mg of 1,2-dimyristoyl-*sn*-glycero-rac-(1-glycerol) (DMPG) in 40% CH_3OH (v/v) and 60% $CHCl_3$ (v/v). Then 500µL of DMPC are added to 250µL of DMPG in a glass test tube. During constant rotation the lipid mixture is dried in a steady N_2-stream, followed by further desiccation at reduced pressure over night. The lipid film is then detached

upon incubation with 16.61 mg fluorescein dissolved in 2mL 5% CH_3OH in phosphate buffered saline (PBS, pH 7.4) by slow continuous rotation. Then the formation of small vesicles is induced by sonification at room temperature for ten minutes followed by sterile filtration (0.2 µm pore size). In order to remove the excessive fluorescein the small vesicles are repeatedly washed with PBS.

VI Cell Culture

VI.I Bacteria

The nonpathogenic strains *E. coli* BL21, *M. luteus*, *B. subtilis* as well as the different *Photorhabdus* and *Xenorhabdus* strain are inoculated from an Luria Bertani agar plate or directly from the cryo culture and cultivated in Luria Bertani medium overnight prior to every experiment.

VI.II Yeast

S. cerevisae PK113 (a kind gift of Prof. Dr. E. Boles) is inoculated from an YPD agar plate and cultivated in YPD medium for 24h prior to every experiment.

VI.III Insect Cell lines

Insect cells are cultivated according to the Thermo Scientific protocol. The Sf21 cells can immediately be cultivated in the bottom-baffled flasks in suspension. The Sf9 cells have to be cultivated as an adherent culture. The cells are thawed, the DMSO removed and the cells are directly transferred to tissue culture flasks in 10 mL growth medium. The growth medium is exchanged daily and after three days the cells are transferred to the cultivation in suspension in 40mL Sf900-II SFM medium, supplemented with 5% fetal bovine serum in 250 mL bottom-baffled flasks with membrane screw caps for gas exchange at 28°C while shaking 150 rpm in an ambient air-regulated incubator. Every three days the number of cells is checked and the cells are subcultured into fresh medium. In order to prepare cryo stocks, the medium is prepared and sterilized by filtration directly before use and cooled to 4 °C. The cell suspension is dispensed into cryovials and is frozen in isopropanol at 1°C/min to -80 °C. After 48h the vials are transferred into the vapor phase of liquid nitrogen .The Sf9 and Sf21 cells are stored in the vapor phase of liquid nitrogen until needed. Then they are thawed at 30°C while shaking gently.

VII Media and Buffer

Media and buffer are prepared according to established protocols.

References

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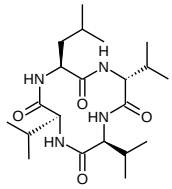
SUPPLEMENTARY INFORMATION

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.

William Lawrence Bragg

S4.1 Synthesis of the Xenotetrapeptide produced by Xenorhabdus nematophila



3

The linear precursor (**4**) was synthesized using standard Fmoc/^{t}Bu chemistry on a preloaded 2-chlorotrityl resin in a 100 µmol scale with the SyroWave® System at room temperature (Biotage, Sweden; see general procedures). **4** was cleaved from the solid support with 90% TFA in water, the solvents were removed under reduced pressure and the precipitate was washed several times with CH_2Cl_2. The cyclization of the linear precursor in solution with 1.5 eq HATU and 10.0 eq DIEA (4 mM in DMF, 20 min, 75°C, 25W) yielded **3** as a colorless precipitate (16.3 mg, 40% yield, 97.9%), which was washed several times with ice cold water and ethyl acetate prior to drying overnight. Then **3** was dissolved in CD_3COOD and submitted to NMR analysis. Due to its low solubility, only strong signals were detected even after extended measurements.

Table S1: NMR data of the xenotetrapeptid (**4**). [a]signal sets are interchangeable

subunit	position	δ_C	δ_H, mult. (*J* in Hz)	COSY	HMBC
D-Val^{1a}	1 2 3 4 5 NH	174.3 59.9 26.4 17.5 17.5	 4.10 2.13-2.08, m 0.92, m 0.92, m n. d.	 0.92 2.13-2.08 2.13-2.08	 174.3
L-Leu^{2}	1 2 3 4 5 6 NH	n. d. 51.7 36.6 24.6 17.9 17.9	 4.60 1.56-1.68, m 1.59, m 0.91-0.87, m 0.91-0.87, m n. d.	 1.56, 1.68 4.60 4.60 1.59 1.59	
D-Val^{3a}	1 2 3 4 5 NH	174.8 61.6 26.4 18.0 18.0	 4.20 2.13-2.08, m 0.97-0.95 0.97-0.95 n. d.	 2.13-2.08 2.13-2.08 2.13-2.08	 174.8
L-Val^{4a}	1 2 3 4 5 NH	n. d. 58.8 n. d. 17.5 18.2	 4.25 1.88 0.92 1.04, t (6.5) n. d.	 1.88 0.92, 1.04 1.04	

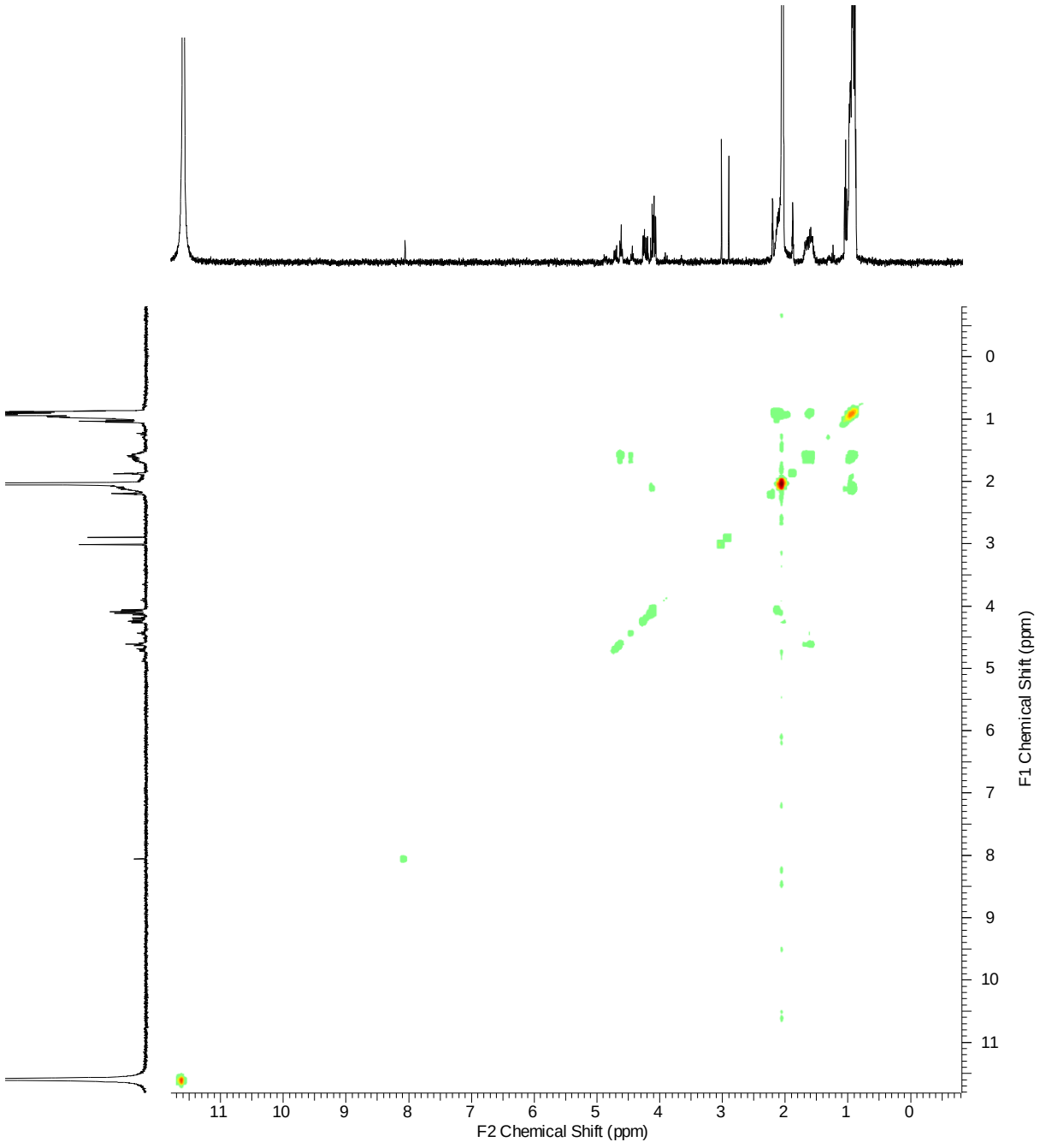


Figure S1: ^{1}H,^{1}H-COSY spectra of the xenotetrapeptide (**3**).

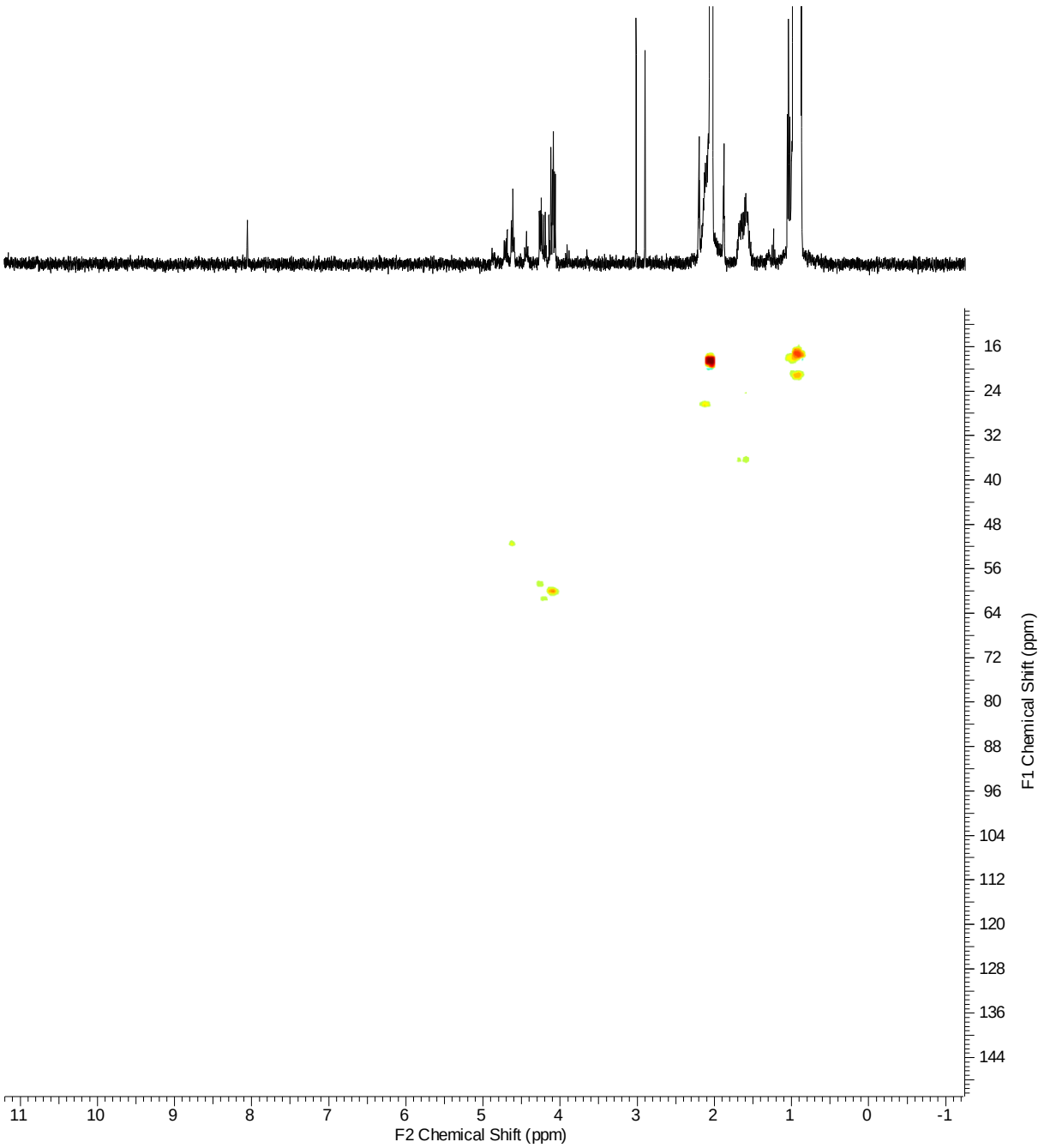


Figure S2: $^{1}H,^{13}C$-HSQC spectra of the xenotetrapeptide (**3**).

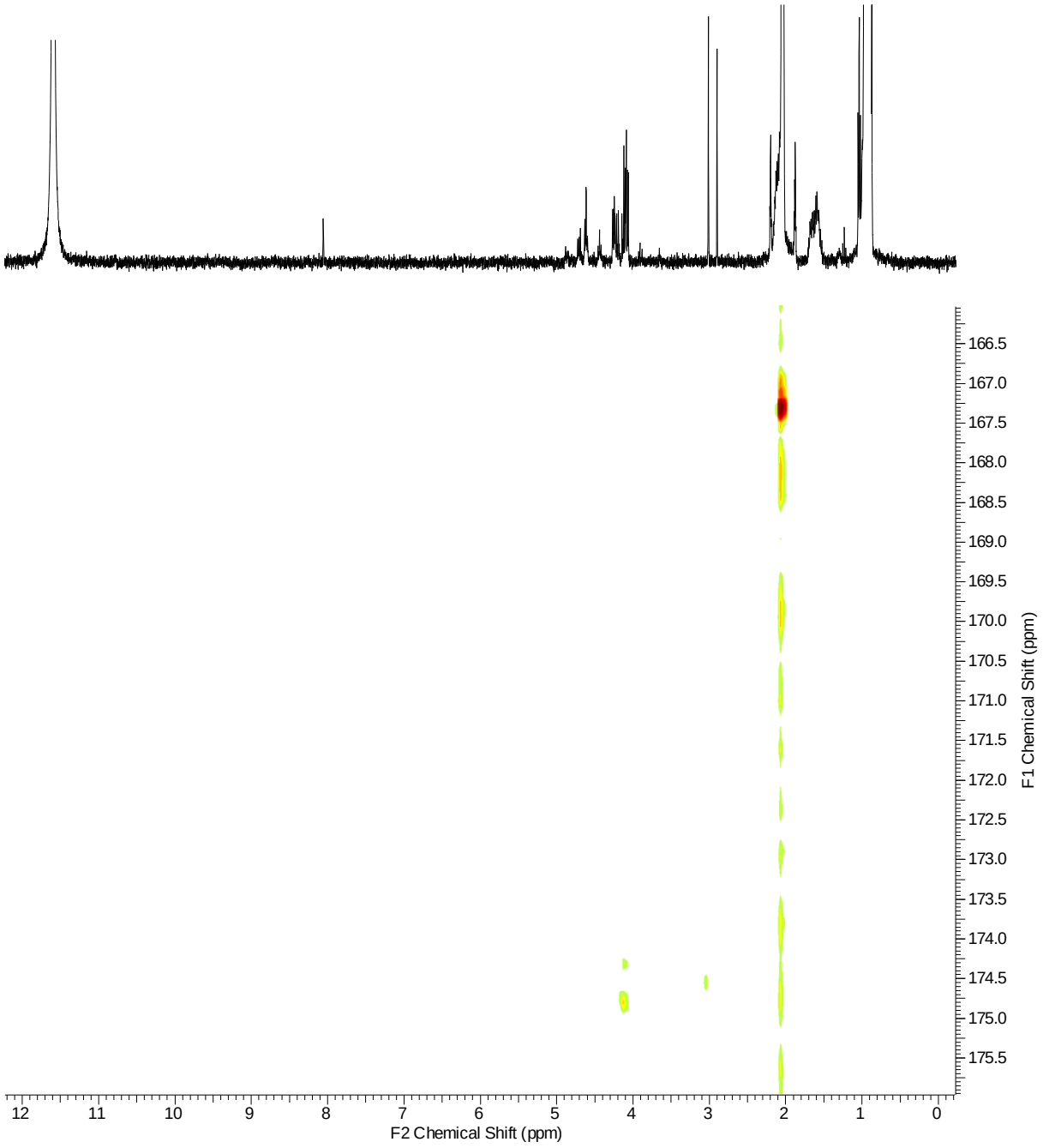
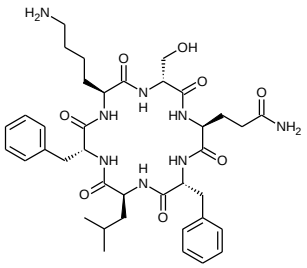


Figure S3: $^{1}H,^{13}C$-HMBC spectra of the xenotetrapeptide (**3**).

S4.3 Synthesis of Ambactin, a cyclic peptide from Xenorhabdus miraniensis



1

The linear precursor (**2**, H_2N-ser(^{t}Bu)-Gln(Trt)-phe-Leu-phe-Lys(Boc)-OH) was manually synthesized using standard Fmoc/^{t}Bu chemistry on a preloaded 2-chlorotrityl resin (see general procedures) in a 50 µmol scale at room temperature. **2** was cleaved from the solid support with 2% TFA in CH_2Cl_2, the solvents were removed under reduced pressure and the precipitate was washed several times with CH_2Cl_2. The cyclization was achieved using high dilution conditions (4 mM in DMF, 20 min, 55°C, 25W) and upon activation with 1.5 eq EDC in the presence of 0.5 eq HOAt and 2.0 eq DIEA. The solvents were removed and the remaining protecting groups were then detached upon incubation with TFA containing 2.5% deionized water and 2.5% TIS for 2 h at room temperature. **1** was then precipitated with cold diethyl ether and washed several times (22.4 mg, 60%). The purity of the crude peptide was estimated using LC-MS and NMR analysis (in deuterated DMSO, 95.2% pure).

Table S2: NMR data of the ambactin (**1**). [a]signal sets are interchangeable [b]overlayed by a residual water peak

subunit	position	δ_C	δ_H, mult. (*J* in Hz)	COSY
L-Lys[1]	1	n. d.		
	2	53.6	4.56	2.95
	3	26.4	1.43	2.71, 4.56
	4	38.3	2.71	2.87
	5	37.8	2.87	2.71, 4.56
	6	n. d.	2.95	
	ε-NH_2		1.55	
	NH		8.39	4.56
D-Phe[2a]	1	n. d.		
	2	51.2	4.41	
	3	n.d	3.69	
			3.79	
	4	n. d.		
	5/9	128.3	7.39	7.29
	6/8	129.5	7.29	7.11, 7.39
	7	126.5	7.11	7.29
	NH		8.00	4.41
L-Leu[3]	1	n. d.		
	2	n. d.	4.17	1.08
	3	26.9	1.08	0.66
	4	n. d.	0.94	0.66
	5	21.5	0.66	0.68, 0.94, 1.08
	6	22.6	0.68	0.66, 0.94, 1.08
	NH		8.08	4.17
D-Phe[4a]	1	n. d.		
	2	48.8	4.86	3.54
	3	n. d.	3.54	
			3.83	
	4	n. d.		
	5/9	128.3	7.39	7.29
	6/8	129.5	7.29	7.11, 7.39
	7	126.5	7.11	7.29
	NH		8.45	
L-Gln[5]	1	n. d.		
	2	n. d.	4.61	2.68, 2.89
	3	n. d.	2.89	2.68
	4	37.5	2.68	2.89
	δ-NH_2		6.70	
	NH		8.33	4.61
D-Ser[6]	1	n. d.		
	2	53.3	4.47	2.87
	3	37.5	2.87	
	OH		n. d.[b]	
	NH		8.36	

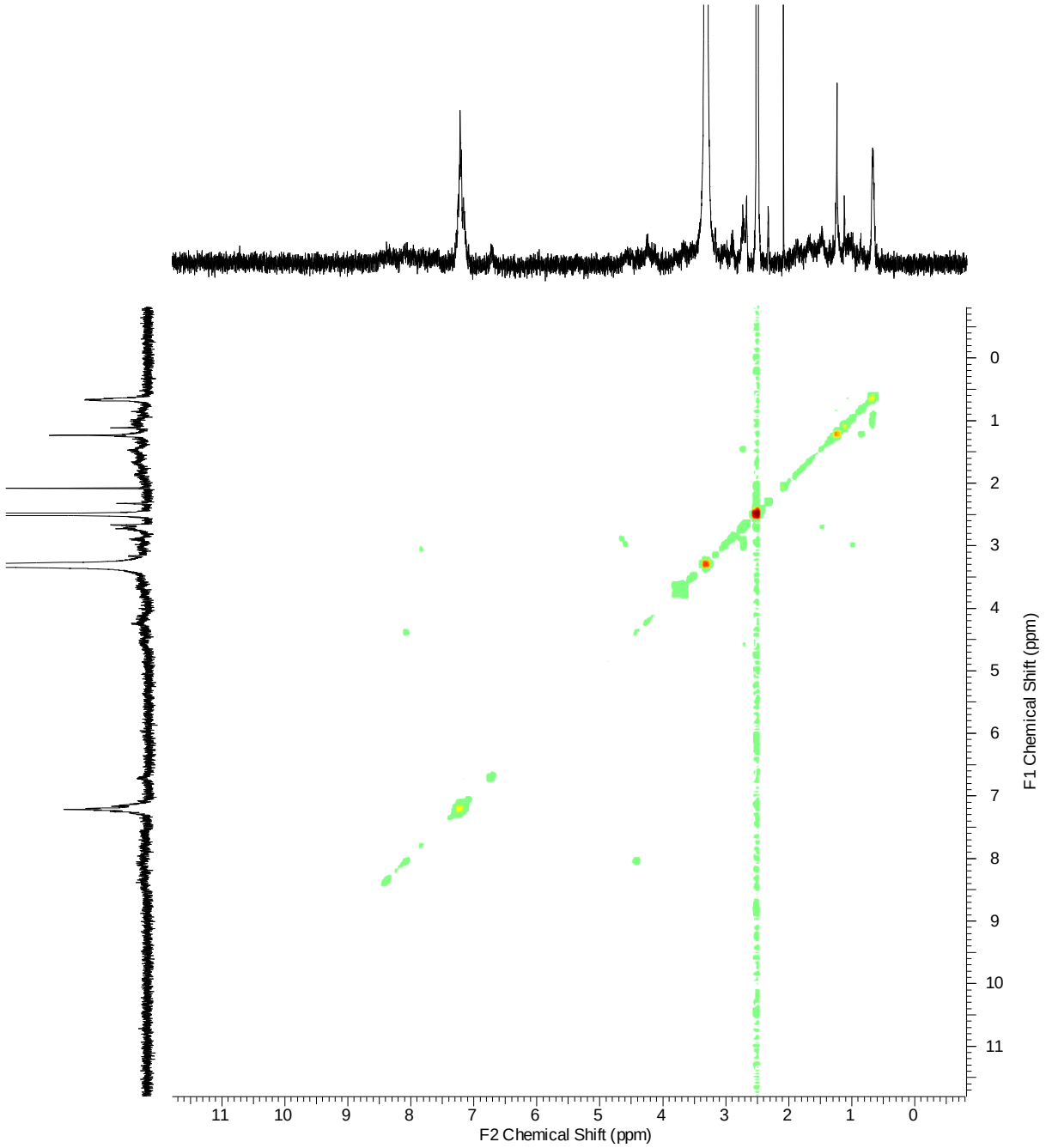


Figure S4: ^{1}H,^{1}H-COSY spectra of the ambactin (**1**).

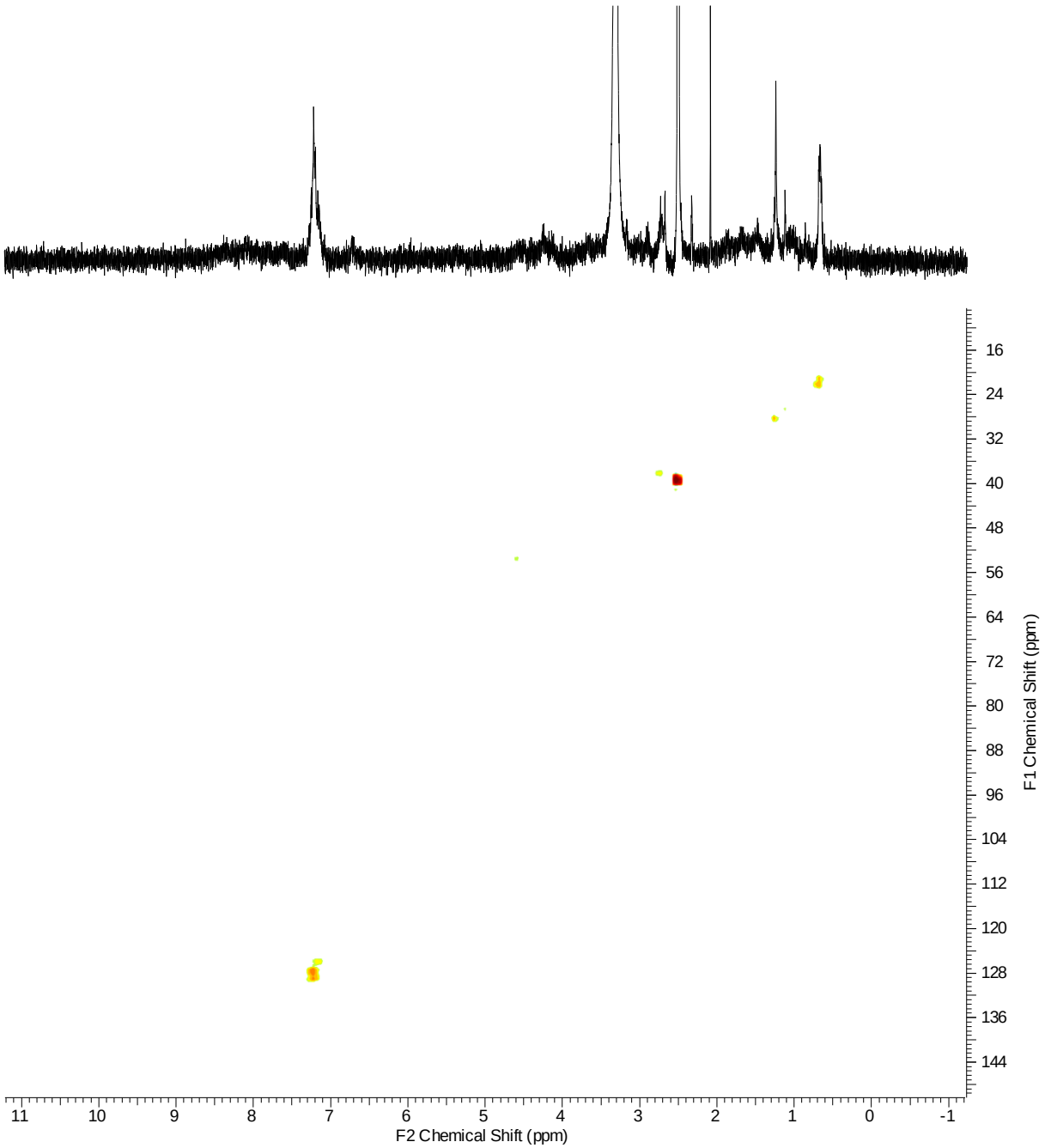


Figure S5: $^{1}H,^{13}C$-HSQC spectra of the ambactin (**1**).

S5.2 Synthesis of Selected Xentrivalpeptides

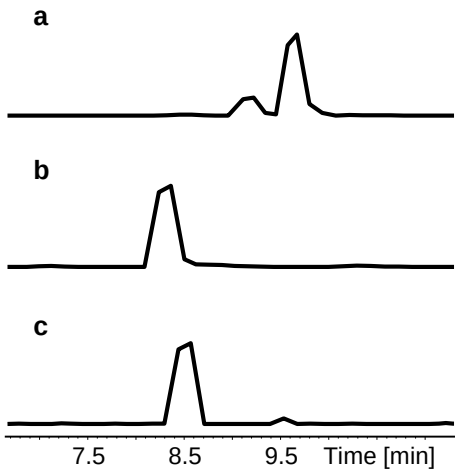


Figure S6: Extracted ion chromatograms of the crude reaction mixture of xentrivalpeptides A (**1**; a), P (**2**; b) and O (**3**; c).

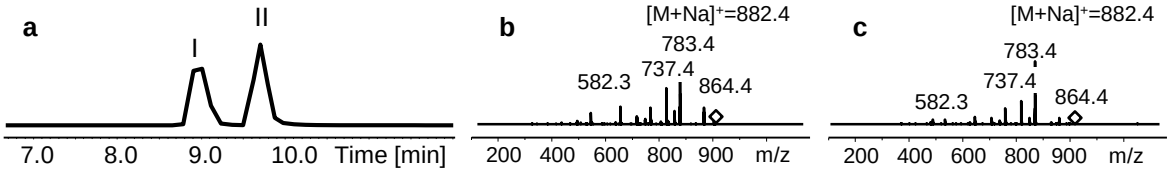
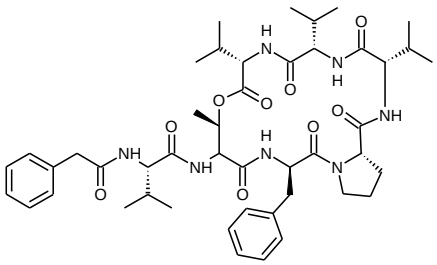


Figure S7: Extracted ion chromatogram (a) and MS/MS data (b, Peak I and c, Peak II) of the purified natural xentrivalpeptide A (**1**; courtesy of Florian Grundmann; unpublished data).



1

The linear precursor was manually synthesized using standard Fmoc/^{t}Bu chemistry on a commercially available preloaded Wang resin (Iris Biotech, Germany) in a 50 µmol scale at room temperature (see general procedures and chapter 4). Then the *N*-terminus was acylated (see general procedures), followed by the selective deprotection of the threonine (see general procedures) and the introduction of an *o*NBS-protected valine via an ester bond using MSNT in the presence of NMI and DIEA (see general procedures and chapter 4). The *o*NBS protecting group (see general procedures) was removed and a Boc-protected valine was coupled using HBTU and DIEA. The resultant branched peptide precursor was cleaved from the resin (see general procedures) with TFA, the cleavage cocktail was removed under reduced pressure and the precipitate was washed several times with CH_2Cl_2 and ethyl acetate.Then the cyclization was achieved using high dilution conditions (4 mM in DMF, 20 min, 50°C, 25W) and upon activation with 1.5 eq HATU in the presence of 2.0 eq DIEA. The solvents were removed and the precipitate was washed several times. The crude peptide was submitted to normal phase purification (ethyl acetate in hexane (0% to 100% in eight column volumes; using pre-packed silica flash cartridges and the Biotage Flash SP Purification System) yielding 7.7 mg (9 µmol, 18% yield) of the title compound **1**. The purity of the peptide was estimated by LC-MS and NMR analysis (in CD_3OD, 88.4% pure).

Table S3: NMR data of the xentrivalpeptide A (**1**). [a]signal sets are interchangeable [b]signals are interchangeable

subunit	position	δ_C	δ_H, mult. (*J* in Hz)	COSY
PhAc[1]	1	n. d.		
	2	41.9	3.67, m	
	3	n. d.		
	4/8	126.6	7.23, m	3.67, 7.32
	5/7	128.2	7.32, m	7.23, 7.25
	6	128.9	7.25, m	7.32
L-Val[2a]	1	n. d.		
	2	59.9	3.86, d (6.4)	
	3	30.1	2.02, m	3.86
	4	18.1	0.94	2.02
	5	18.1	0.94	2.02
	NH		n. d.	
L-Thr[3]	1	n. d.		
	2	53.1	4.78, d (2.6)	5.11
	3	n. d.	5.11, m	1.15, 4.78
	4	16.9	1.15	
	NH		n. d.	
D-Phe[4]	1	n. d.		
	2		4.73	
	3	37.4	2.79	3.37, 4.73
			3.37	2.79
	4	n. d.		
	5/9	126.6	7.23, m	7.32
	6/8	128.2	7.32, m	7.23, 7.25
	7	128.9	7.25, m	7.32
	NH		n. d.	
L-Pro[5]	1	n. d.		
	2	52.9	4.82	
	3		3.02	
	4		2.92	3.02, 3.27, 4.82
	5		3.27	
	NH		n. d.	
L-Val[6a]	1	n. d.		
	2	59.0	4.07	4.07
	3	30.3	2.08	0.92-0.98
	4	17.3[b]	0.92-0.98, m	2.08
	5	18.0[b]	0.92-0.98, m	2.08
	NH		n. d.	
L-Val[7a]	1	n. d.		
	2	59.1	4.30	2.14
	3	30.0	2.14	0.92-0.98, 4.30
	4	17.3[b]	0.92-0.98, m	2.14
	5	18.0[b]	0.92-0.98, m	2.14
	NH		n. d.	
L-Val[8a]	1	n. d.		
	2	57.8	4.31	2.11
	3	30.2	2.11	4.31
	4	18.0	0.94, m	1.04, 2.11
	5	20.1	1.04, m	0.94, 2.11
	NH		n. d.	

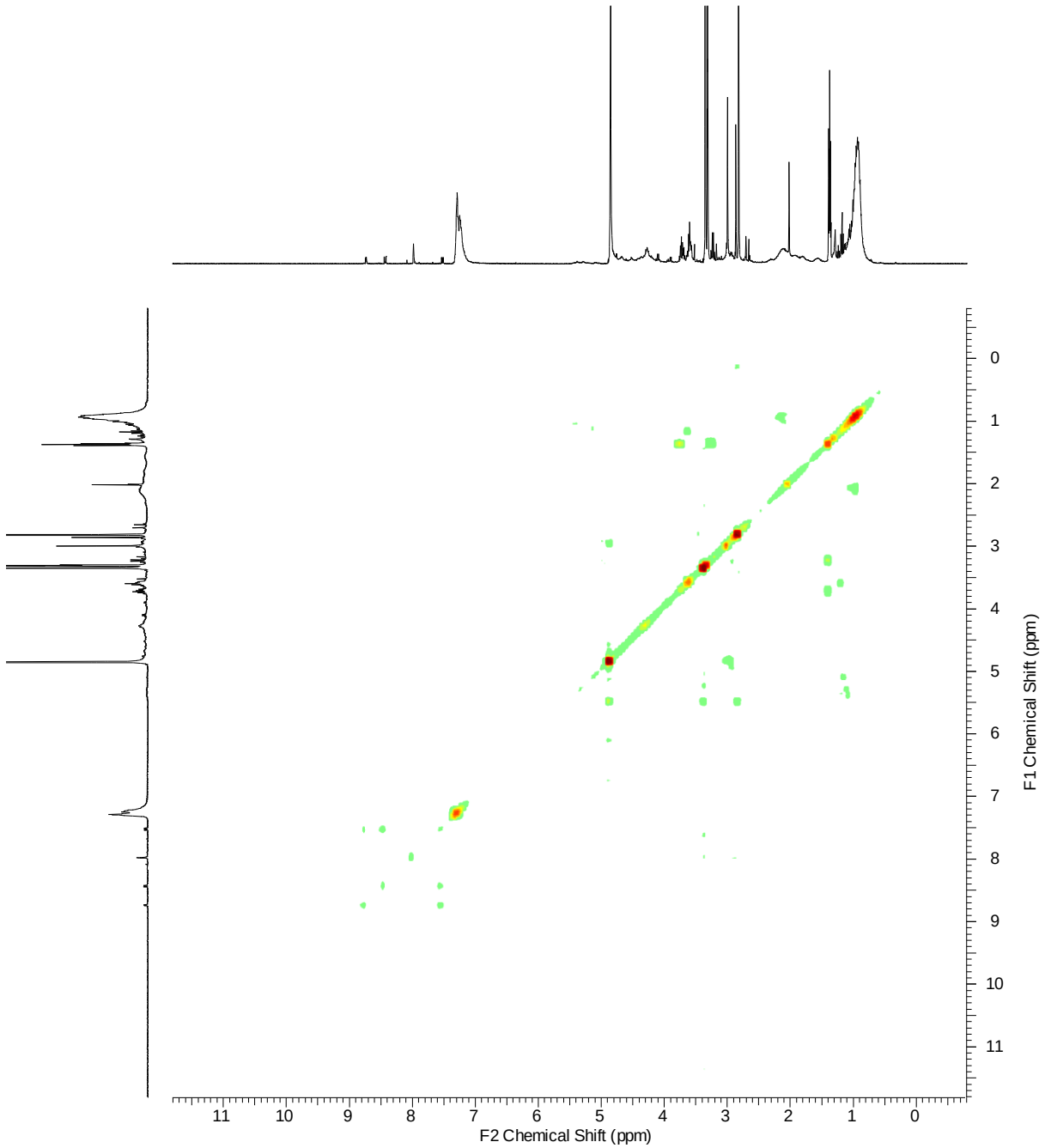


Figure S8: ^{1}H,^{1}H-COSY spectra of xentrivalpeptide A (**1**).

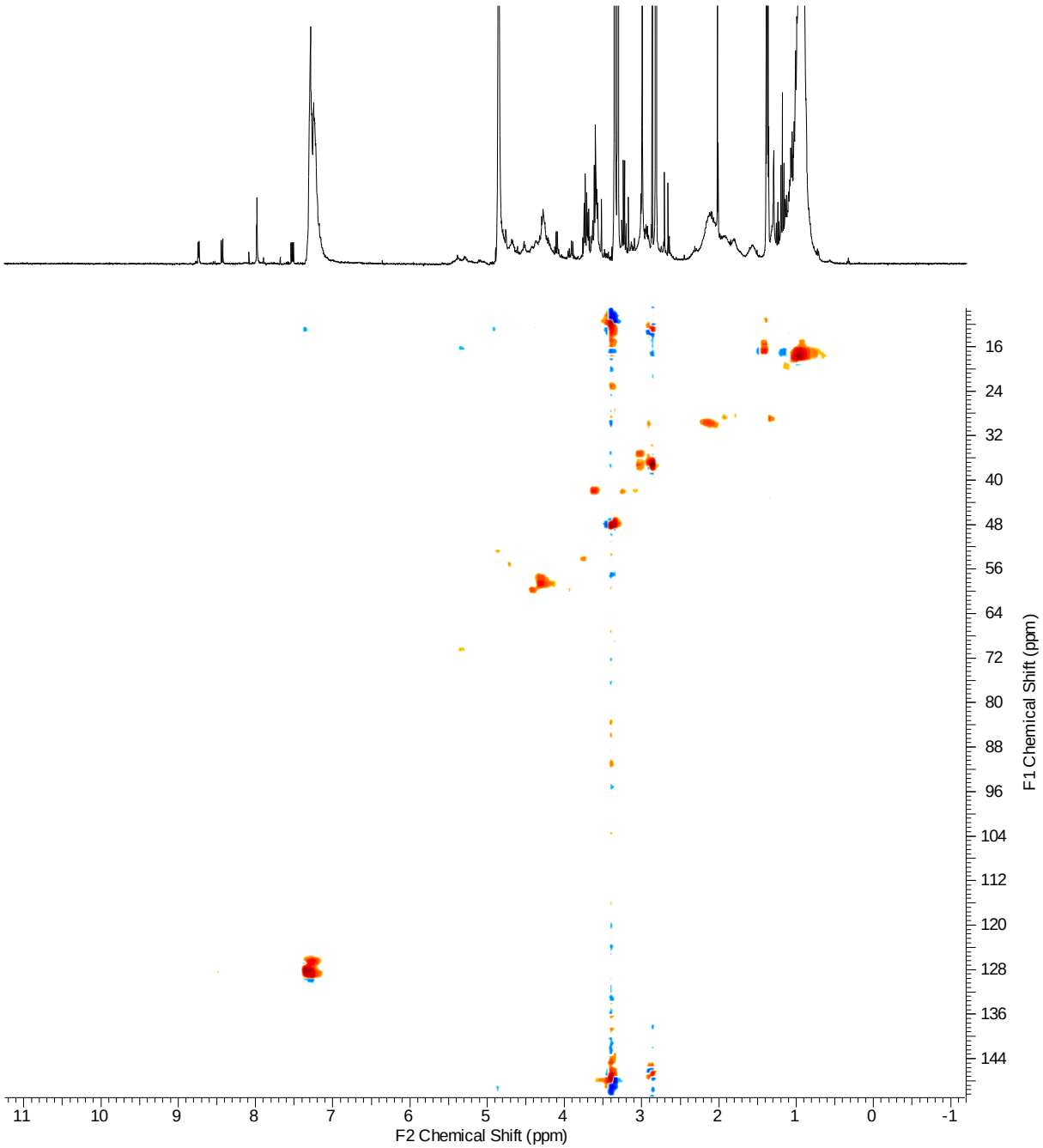
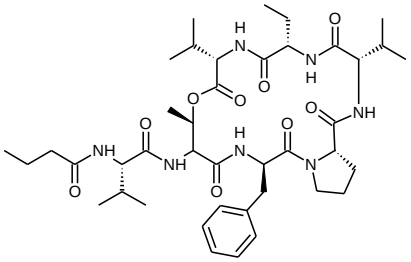


Figure S9: $^{1}H,^{13}C$-HSQC spectra of xentrivalpeptide A (**1**).



2

The linear precursor was manually synthesized using standard Fmoc/^{t}Bu chemistry on a commercially available preloaded Wang resin (Iris Biotech, Germany) in a 50 µmol scale at room temperature (see general procedures and chapter 4). Then the *N*-terminus was acylated (see general procedures), followed by the selective deprotection of the threonine (see general procedures) and the introduction of an *o*NBS-protected valine via an ester bond using MSNT in the presence of NMI and DIEA (see general procedures and chapter 4). The *o*NBS protecting group (see general procedures) was removed and a Boc-protected amino butyric acid was coupled using HBTU and DIEA. The resultant branched peptide precursor was cleaved from the resin (see general procedures) with TFA, the cleavage cocktail was removed under reduced pressure and the precipitate was washed several times with CH_2Cl_2 and ethyl acetate. Then the cyclization was achieved using high dilution conditions (4 mM in DMF, 20 min, 50°C, 25W) and upon activation with 1.5 eq HATU in the presence of 2.0 eq DIEA. The solvents were removed and the precipitate was washed several times. The crude peptide was submitted to normal phase purification (ethyl acetate in hexane (0% to 100% in eight column volumes; using pre-packed silica flash cartridges and the Biotage Flash SP Purification System) yielding 22.7 mg (29 µmol, 57% yield) of the title compound **2**. The purity of the peptide was estimated by LC-MS and NMR analysis (in CD_3OD, 92.3% pure).

Table S4: NMR data of the xentrivalpeptide O (**2**). [a]signal sets are interchangeable

subunit	position	δ_C	δ_H, mult. (*J* in Hz)	COSY
n-BuAc[1]	1	n. d.		
	2	37.2	2.26, m	0.94, 1.65
	3	18.8	1.65, m	0.94, 2.26
	4	10.6	0.94, m	1.65, 2.26
L-Val[2a]	1	n. d.		
	2	59.9	4.15	1.77
	3	28.2	1.77	0.93, 4.15
	4	17.4	0.93, m	1.77
	5	17.4	0.93, m	1.77
	NH		n. d.	
L-Thr[3]	1	n. d.		
	2	n. d.	3.61, q (14.1, 6.9)	
	3	n. d.	5.19, m	1.19
	4	n. d.	1.19, m	3.61, 5.19
	NH		n. d.	
D-Phe[4]	1	n. d.		
	2	n. d.	5.02, m	
	3	37.5	2.98, m	
			3.17, m	
	4	n. d.		
	5/9	128.0	7.28, m	7.19, 7.25
	6/8	128.9	7.25, m	7.19, 7.28
	7	128.1	7.19, m	7.25
	NH		n. d.	
L-Pro[5]	1	n. d.		
	2	n. d.	4.81, m	2.01
	3	37.6	2.99, m	3.17, 4.81
	4	n. d.	3.17, m	2.99, 3.13, 4.81
	5	n.d.	3.13, m	3.17
	NH		n. d.	
L-Val[6a]	1	n. d.		
	2	59.1	4.23	
	3	n. d.	2.08 m	4.23
	4	17.1	0.95, m	1.64
	5	17.1	0.95, m	1.64
	NH		n. d.	
L-Abu[7]	1	n. d.		
	2	n. d.	5.44	1.29
	3	16.1	1.29	0.88, 5.44
	4	12.7	0.88, m	
	NH		n. d.	
L-Val[8a]	1	n. d.		
	2	57.7	4.27, m	
	3	30.1	2.14, m	0.98, 1.03, 4.27
	4	17.3	0.98	1.03, 2.14
	5	17.5	1.03	0.98, 2.14
	NH		n. d.	

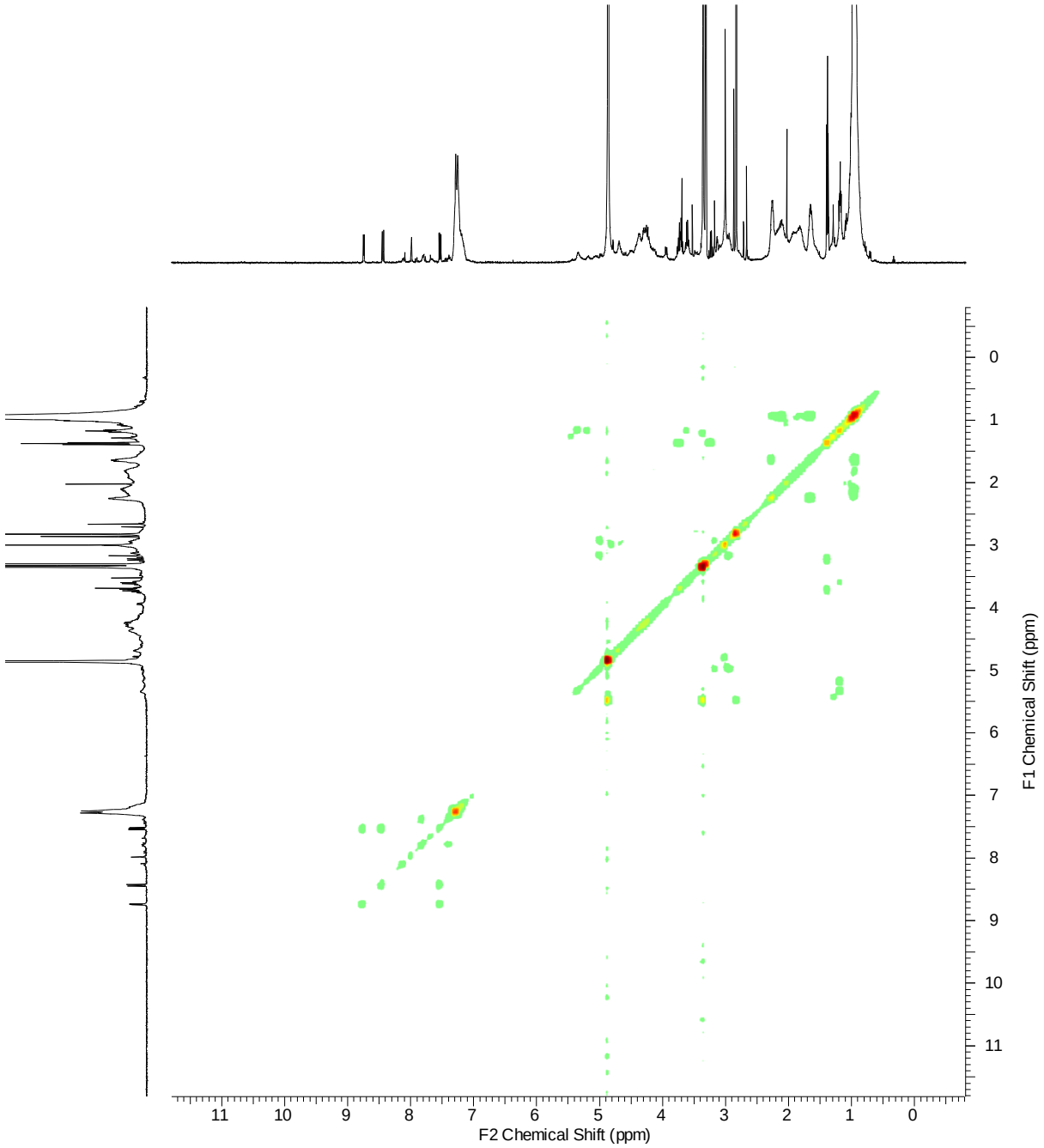


Figure S 10: ^{1}H,^{1}H-COSY spectra of xentrivalpeptide O (**2**).

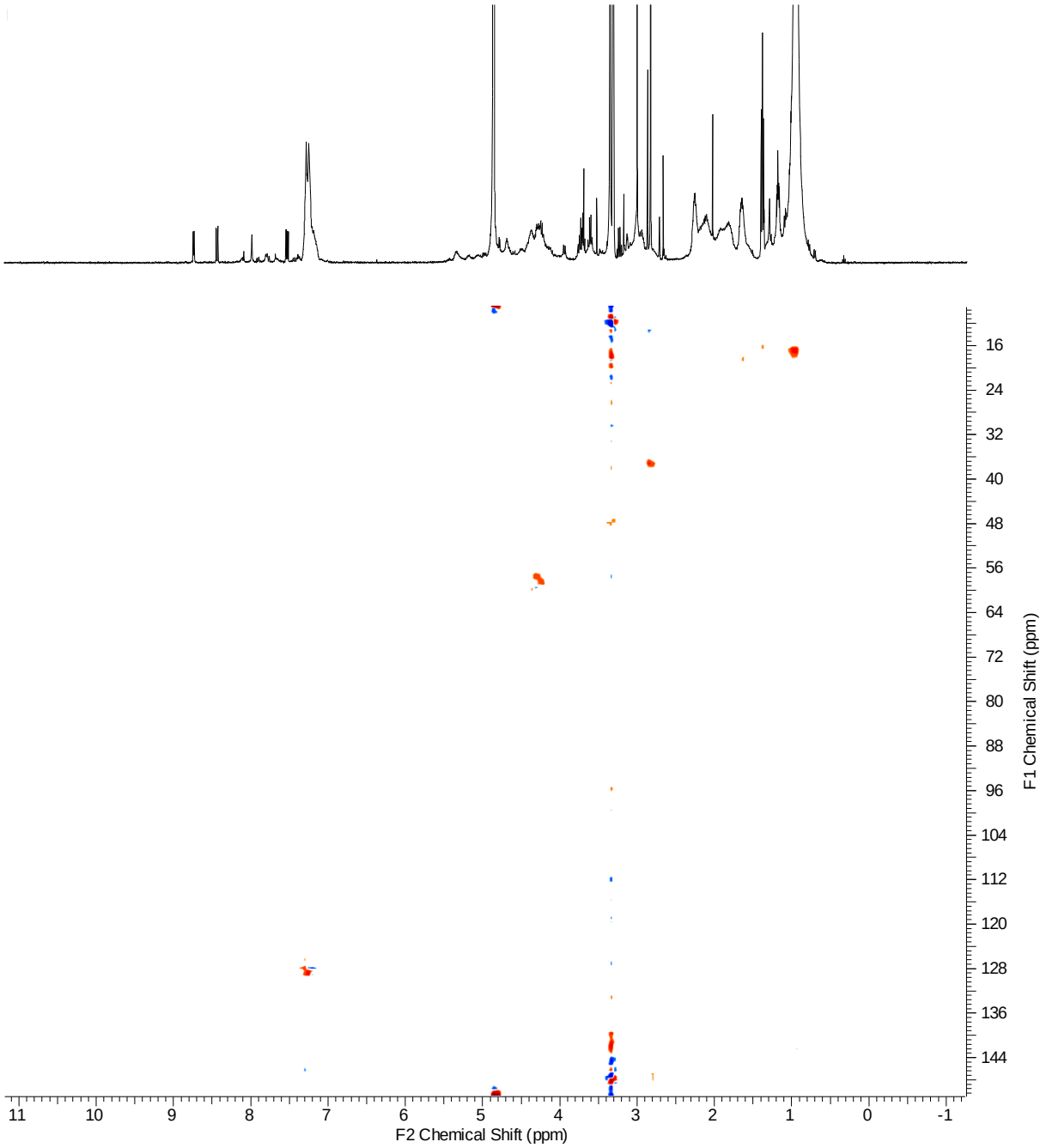
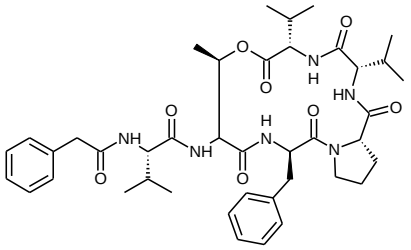


Figure S11: $^{1}H,^{13}C$-HSQC spectra of xentrivalpeptide O (**2**).



3

The linear precursor was manually synthesized using standard Fmoc/^{t}Bu chemistry on a commercially available preloaded Wang resin (Iris Biotech, Germany) in a 50 µmol scale at room temperature (see general procedures and chapter 4). Then the *N*-terminus was acylated (see general procedures), followed by the selective deprotection of the threonine (see general procedures) and the introduction of an oNBS-protected valine via an ester bond using MSNT in the presence of NMI and DIEA (see general procedures and chapter 4). After removing the oNBS protecting group (see general procedures), this branched peptide precursor was cleaved from the resin (see general procedures), the cleavage cocktail was removed under reduced pressure and the precipitate was washed several times with CH_2Cl_2 and ethyl acetate. Then the cyclization was achieved using high dilution conditions (4 mM in DMF, 20 min, 50°C, 25W) and upon activation with 1.5 eq HATU in the presence of 2.0 eq DIEA. The solvents were removed and the precipitate was washed several times. The crude peptide was submitted to normal phase purification (ethyl acetate in hexane (0% to 100% in eight column volumes; using pre-packed silica flash cartridges and the Biotage Flash SP Purification System) yielding 9.8 mg (13 µmol, 26% yield) of the title compound **3**. The purity of the peptide was estimated by LC-MS and NMR analysis (in CD_3OD, 87.9% pure).

Table S5: NMR data of the xentrivalpeptide P (**3**). [a]signal sets are interchangeable

subunit	position	δ_C	δ_H, mult. (*J* in Hz)	COSY
PhAc[1]	1	n. d.		
	2	n. d.	3.61	
	3	n. d.		
	4/8	129.6	7.22, m	3.61, 7.10
	5/7	129.6	7.10, m	7.15, 7.28
	6	129.6	7.15, m	7.10
L-Val[2a]	1	n. d.		
	2	n. d.	3.91	
	3	n. d.	1.82, m	0.82, 0.92, 1.82, 3.91
	4	17.2	0.82	1.82
	5	17.2	0.92	1.82
	NH		n. d.	
L-Thr[3]	1	n. d.		
	2	n. d.	4.05	1.92, 4.15
	3	n. d.	4.15	1.92
	4	n. d.	1.92	4.05, 4.15
	NH		n. d.	
D-Phe[4]	1	n. d.		
	2	n. d.	4.26	
	3	n. d.	2.25	3.71, 4.26
			3.71	2.25
	4	n. d.		
	5/9	129.6	7.25, m	3.71, 7.10
	6/8	129.6	7.10, m	7.15, 7.28
	7	129.6	7.15, m	7.10
	NH		n. d.	
L-Pro[5]	1	n. d.		
	2	n. d.	4.60	2.92, 3.11
	3		3.11	
	4		2.92	3.11
	5		3.02	2.92
	NH		n. d.	
L-Val[6a]	1	n. d.		
	2	n. d.	4.05	0.91-0.99, 1.92
	3	n. d.	1.92	0.91-0.99, 4.05
	4	17.5	0.91-0.99, m	
	5	17.5	0.91-0.99, m	
	NH		n. d.	
L-Val[7a]	1	n. d.		
	2	n. d.	4.89	
	3	n. d.	2.15, m	0.93, 3.89
	4	17.2	0.93	2.15
	5	17.2	0.93	2.15
	NH		n. d.	

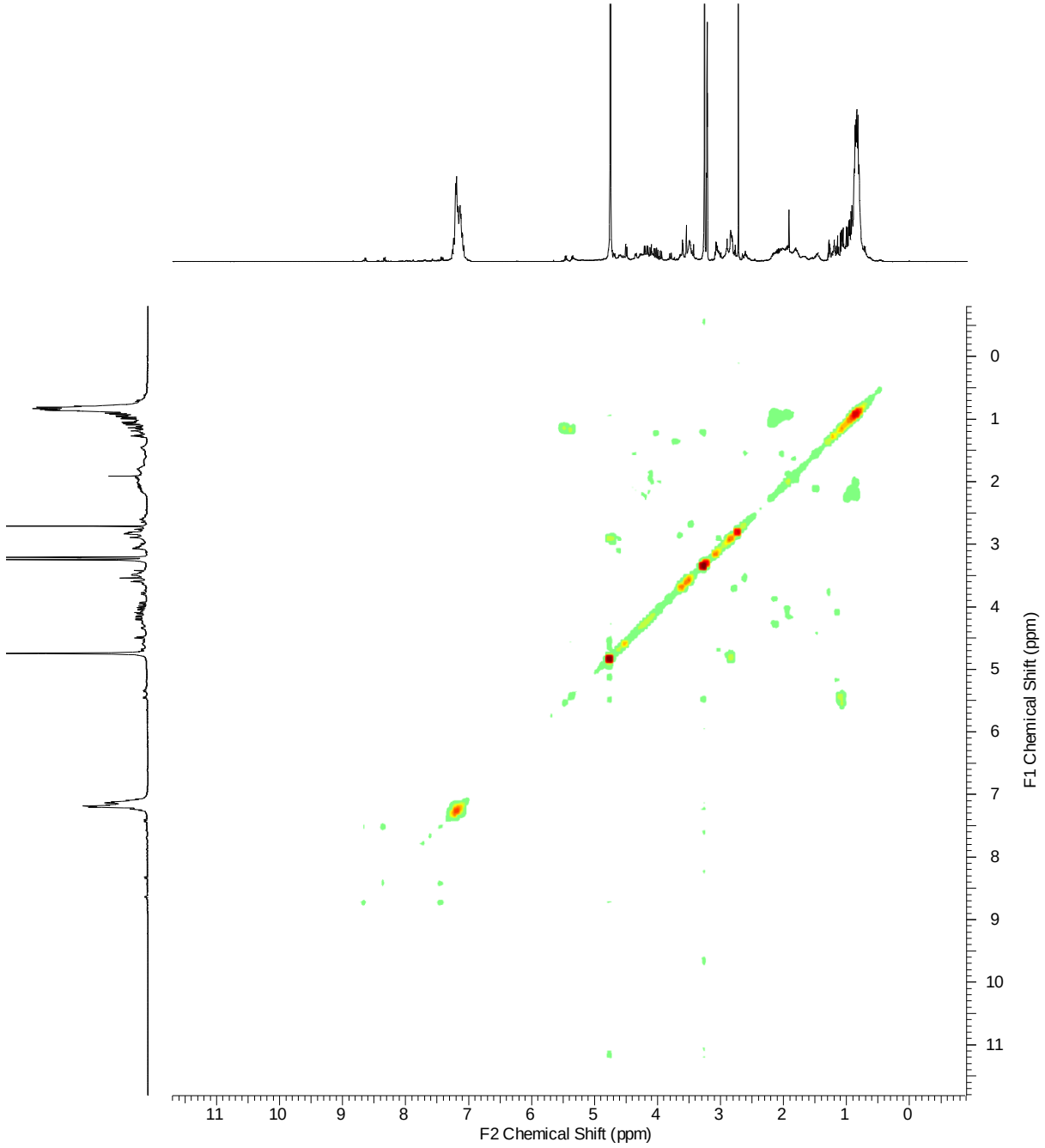


Figure S12: ^{1}H,^{1}H-COSY spectra of xentrivalpeptide P (**3**).

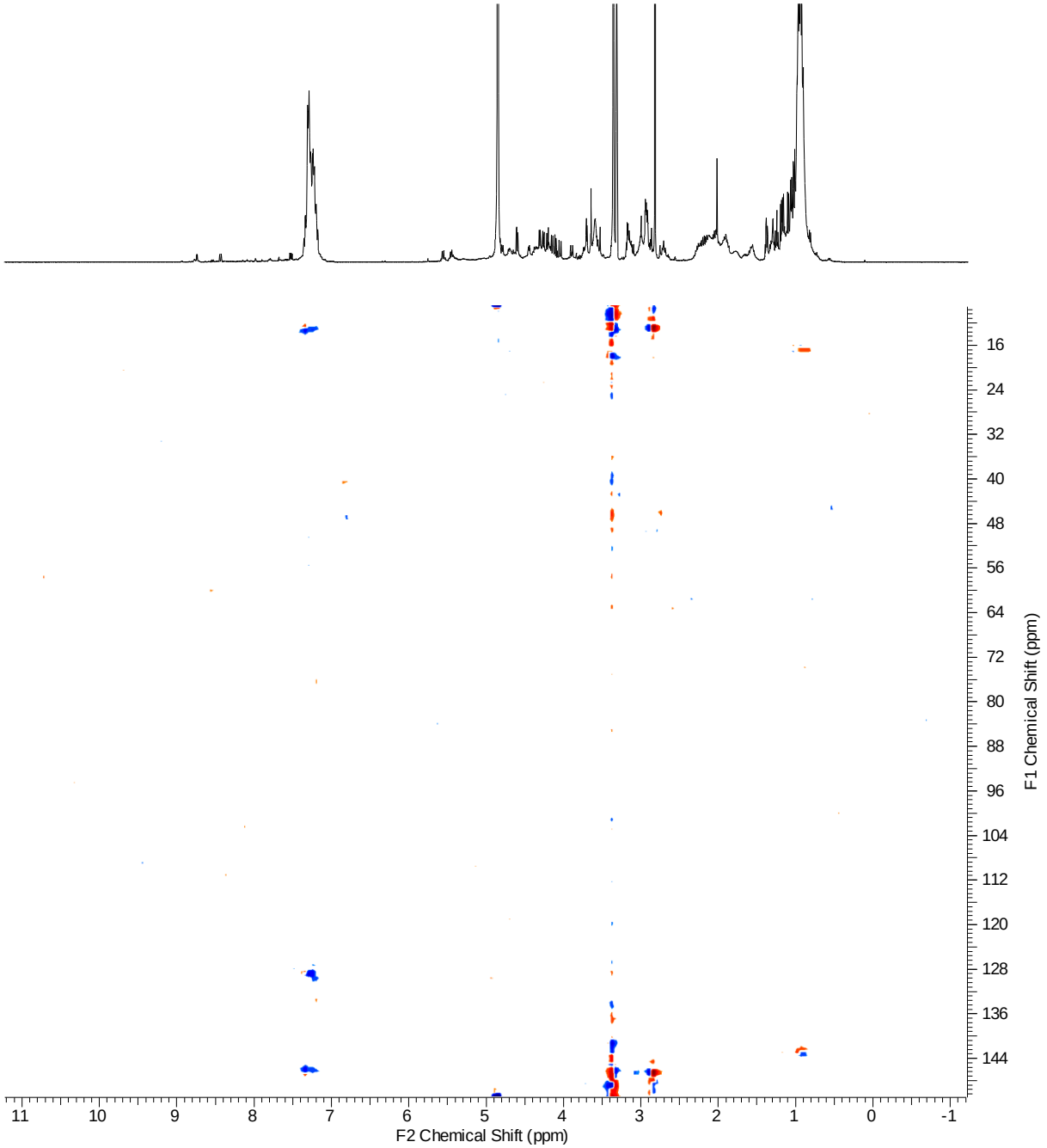
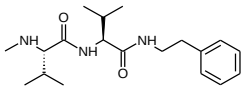


Figure S4: $^{1}H,^{13}C$-HSQC spectra of xentrivalpeptide P (**3**).

S6.1 Synthesis of short partly methylated sequences in solution



10a

The synthesis of compound **10a** was conducted according to the general procedures by coupling **21b** with Boc-MeVal-OH in a 100 µmol scale. The following Boc-deprotection (used amount of acid was lowered to 4.0 eq) proceeded quantitatively (monitored by TLC) yielding 25.5 mg of **10a** (76.5 µmol, 77% yield, 89.5% pure).

^{1}H-NMR (500 MHz, $CDCl_3$, **10a**): δ 8.01 (t, J = 3.5 Hz, 1H), 7.24 (d, J = 7.1 Hz, 2H), 7.18 (d, J = 7.3 Hz, 1H), 7.14 (d, J = 7.1 Hz, 2H), 3.87 (d, J = 4.7 Hz, 1H), 3.57 (m, 1H), 3.45 (s, 3H), 3.32 (m, 1H), 2.87 (m, 2H), 2.12 (m, 1H), 1.11 (d, J = 6.9 Hz, 3H), 1.00 (d, J = 6.9 Hz, 3H), 0.94 (d, J = 6.7 Hz, 3H), 0.91 (d, J = 6.7 Hz, 3H)

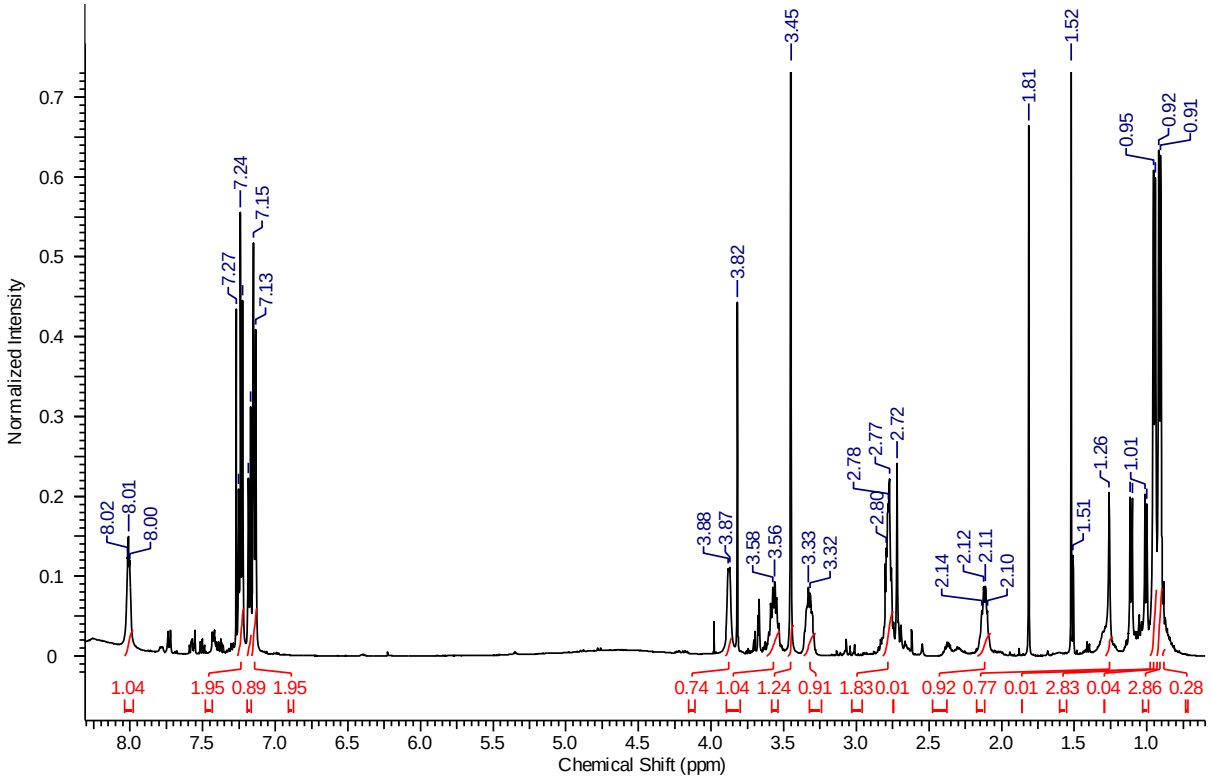
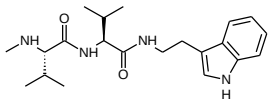


Figure S13: ^{1}H-NMR of rhabdopeptide **10a**.



10b

The synthesis of compound **10b** was conducted according to the general procedures by coupling **22b** with Boc-MeVal-OH in a 100 µmol scale. The following Boc-deprotection (used amount of acid was lowered to 4.0 eq) proceeded quantitatively (monitored by TLC) yielding 29 mg of **10b** (77.9 µmol, 78% yield, 74.8% pure).

^{1}H-NMR (500 MHz, $CDCl_3$, **10b**): δ 8.77 (br, 1H), 7.55 (d, J = 7.9 Hz, 1H), 7.33 (d, J = 8.1 Hz, 1H), 7.16 (t, J = 8.0 Hz, 1H), 7.08 (m, 1H), 7.03 (d, 1H), 6.59 (t, J = 5.4 Hz, 1H), 4.43 (d, J = 11.2 Hz, 1H), 4.09 (d, J = 5.1 Hz, 1H), 3.55 (m, 1H), 3.49 (m, 1H), 3.01 (br, 2H), 2.91 (m, 2H), 2.81 (s, 3H), 2.27 (m, 1H), 1.92 (m, 1H), 0.89 (d, J = 6.5 Hz, 3H), 0.84 (d, J = 6.9 Hz, 3H), 0.79 (d, J = 6.9 Hz, 3H), 0.76 (d, J = 6.6 Hz, 3H)

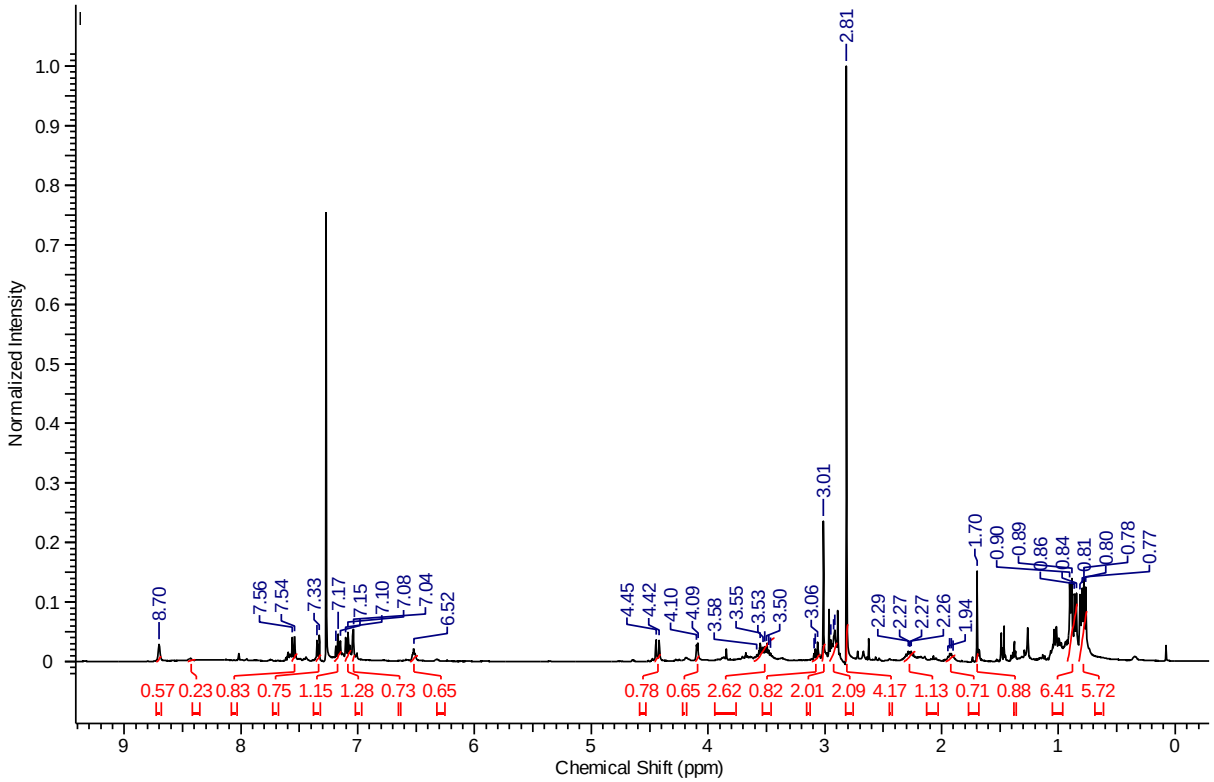
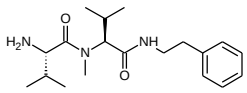


Figure S14: ^{1}H-NMR of rhabdopeptide **10b**.



11a

The synthesis of compound **11a** was conducted according to the general procedures by coupling **23b** with Boc-Val-OH in a 100 µmol scale. The following Boc-deprotection (used amount of acid was lowered to 4.0 eq) proceeded quantitatively (monitored by TLC) yielding 31.6 mg of **11a** (94.9 µmol, 95% yield, 82.7% pure).

^{1}H-NMR (500 MHz, $CDCl_3$, **11a**): δ 8.77 (t, J = 5.3 Hz, 2H), 7.31 (m, 2H), 7.24 (m, 3H), 3.64 (q, J = 13.2 Hz, J = 7.1 Hz, 2H), 2.91 (m, 2H), 2.85 (s, 3H), 2.41 (m, 1H), 2.22 (m, 1H), 1.14 (d, J = 6.9 Hz, 3H), 1.11 (d, J = 6.9 Hz, 3H), 1.04 (d, J = 6.9 Hz, 3H), 0.98 (d, J = 6.7 Hz, 3H)

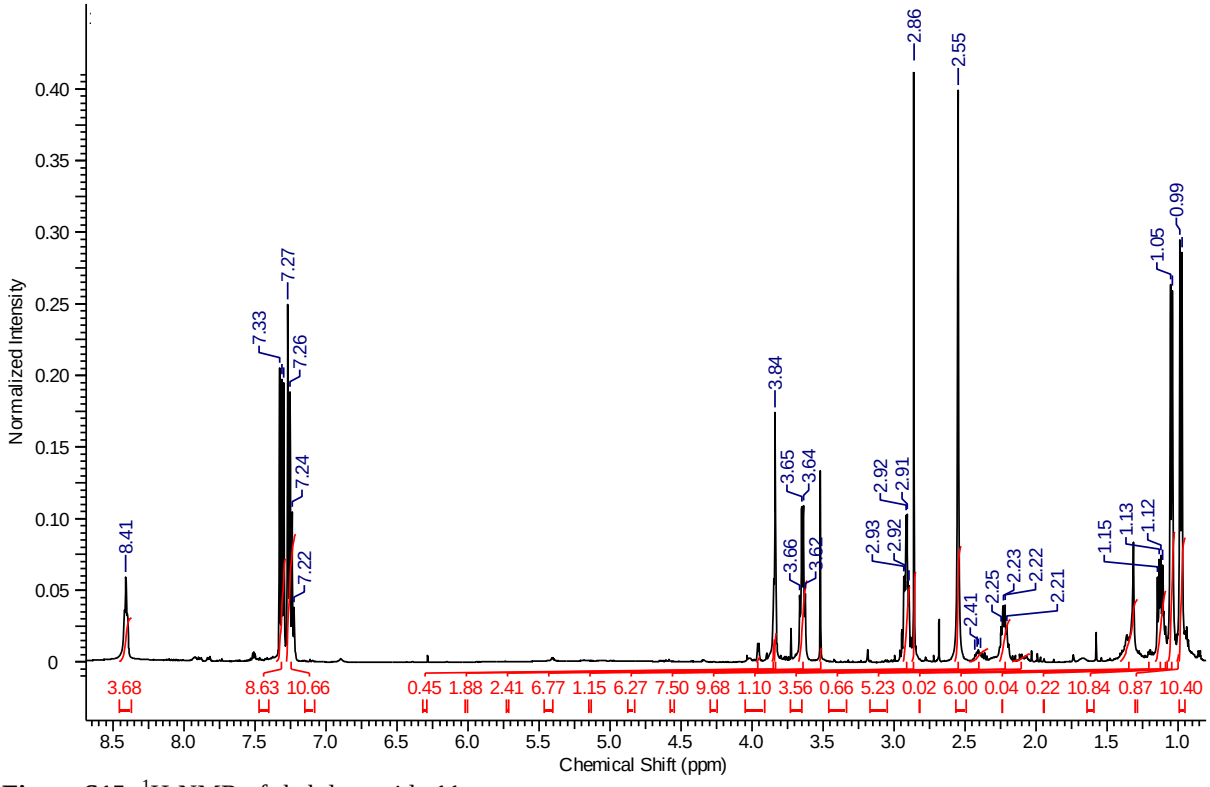
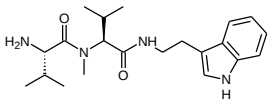


Figure S15: ^{1}H-NMR of rhabdopeptide **11a**.



11b

The synthesis of compound **11b** was conducted according to the general procedures by coupling **24b** with Boc-Val-OH in a 100 µmol scale. The following Boc-deprotection (used amount of acid was lowered to 4.0 eq) proceeded quantitatively (monitored by TLC) yielding 29.9 mg of **11b** (80.1 µmol, 80% yield, 84.9% pure).

^{1}H-NMR (500 MHz, $CDCl_3$, **10b**): δ 8.77 (br, 1H), 7.54 (d, *J* = 7.9 Hz, 1H), 7.34 (d, *J* = 8.1 Hz, 1H), 7.17 (t, *J* = 7.1 Hz, 1H), 7.08 (t, *J* = 7.8 Hz, 1H), 7.04 (br, 1H), 6.52 (t, *J* = 4.7 Hz, 1H), 4.43 (d, *J* = 11.2 Hz, 1H), 4.09 (d, *J* = 4.9 Hz, 1H), 3.51 (m, 2H), 3.49 (m, 1H), 3.01 (br, 2H), 2.92 (m, 2H), 2.81 (s, 3H), 2.27 (m, 1H), 1.92 (m, 1H), 0.89 (d, *J* = 6.4 Hz, 3H), 0.85 (d, *J* = 6.9 Hz, 3H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.77 (d, *J* = 6.6 Hz, 3H)

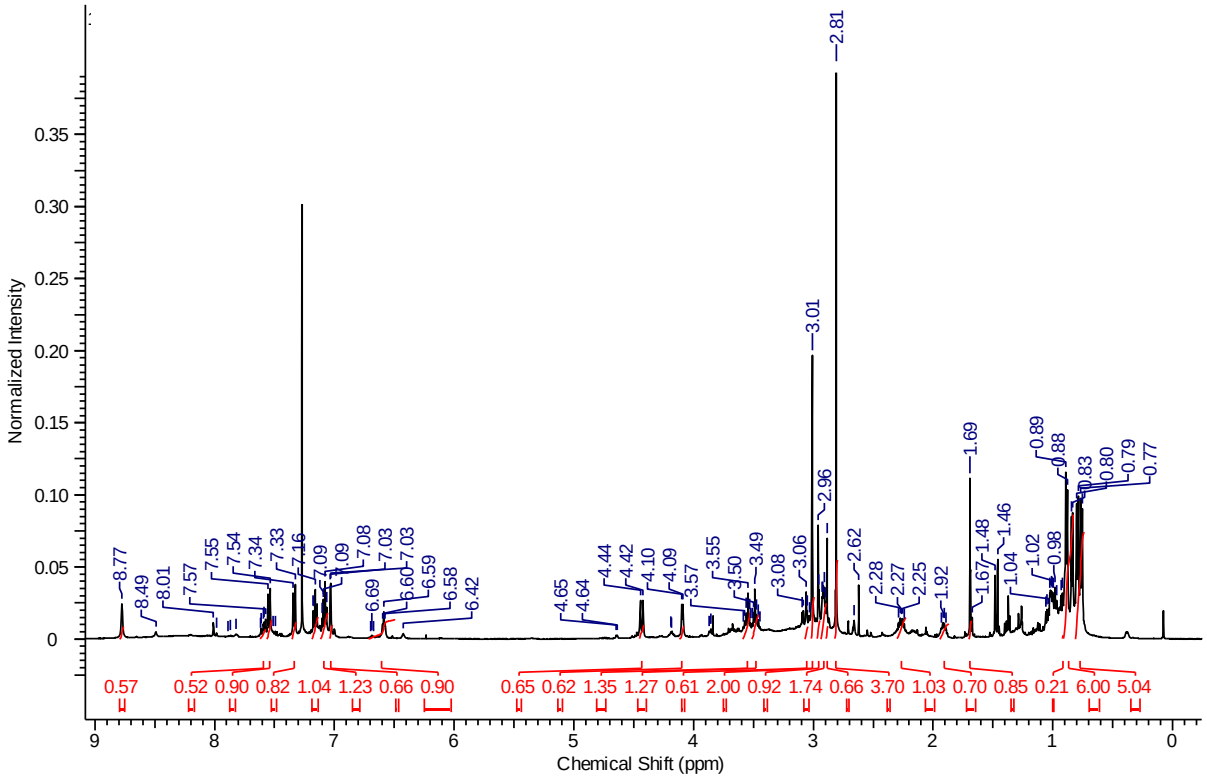
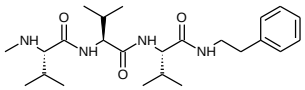


Figure S16: 1H-NMR of rhabdopeptide **11b**.



12a

The precursor (Boc*N*MeVal-Val-Val-OH) was manually synthesized using standard Fmoc/^{t}Bu chemistry on a preloaded 2-chlorotrityl resin (see general procedures) in a 50 µmol scale at room temperature while activating the building blocks including Boc-*N*MeVal-OH with HBTU in the presence of DIEA (the success of the synthesis was evaluated by LC-MS). Then the Boc-protected peptide, which was obtained upon incubation with HFIP in CH_2Cl_2 (see general procedures), was condensed with phenylethylamine in solution using EDC and HOBt (see general procedure), followed by normal phase purification (ethyl acetate in hexane (0% to 70% in five column volumes; using pre-packed silica flash cartridges and the Biotage Flash SP Purification System) and a final acidic deprotection step (see general procedures). The purity of the resultant peptide (5.3 mg, 12.3 µmol, 25% yield) was estimated by HPLC-MS and NMR (in $CDCl_3$, 77.4% pure).

Table S6: NMR data of the rhabdopeptide (**12a**).

subunit	position	δ_C	δ_H, mult. (*J* in Hz)	COSY	HMBC
L-*N*MeVal[1]	1	167.2			
	2	n. d.	2.08	0.94, 1.35	30.8
	3	29.3	1.35	2.08	
	4	18.9	0.94, s	2.08	
	5	18.9	0.94, s	2.08	
	CH_3	30.8	2.18, s		167.2
	NH		n. d.		
L-Val[2]	1	n. d.			
	2	n. d.	2.04, m	0.89, 0.91	30.8
	3	31.6	1.27, m	0.89, 0.91	
	4	18.8	0.91, s		
	5	22.5	0.89, s	2.04	
	NH		n. d.		
L-Val[3]	1	n. d.			
	2	27.2	2.02, m	1.32, 5.39	
	3	22.7	1.32, m	0.86, 0.91, 2.02	
	4	18.8	0.86, s		22.7
	5	18.8	0.91, s		
	NH		n. d.		
PEA[4]	NH		5.39	2.83	
	1	40.8	3.62	2.90	
	2	35.2	2.83		
			2.90	3.62	
	3	n. d.			
	4/8	128.7	7.33	7.21	
	5/7	128.6	7.21	7.17, 7.33	
	6	126.3	7.17	7.21, 7.33	

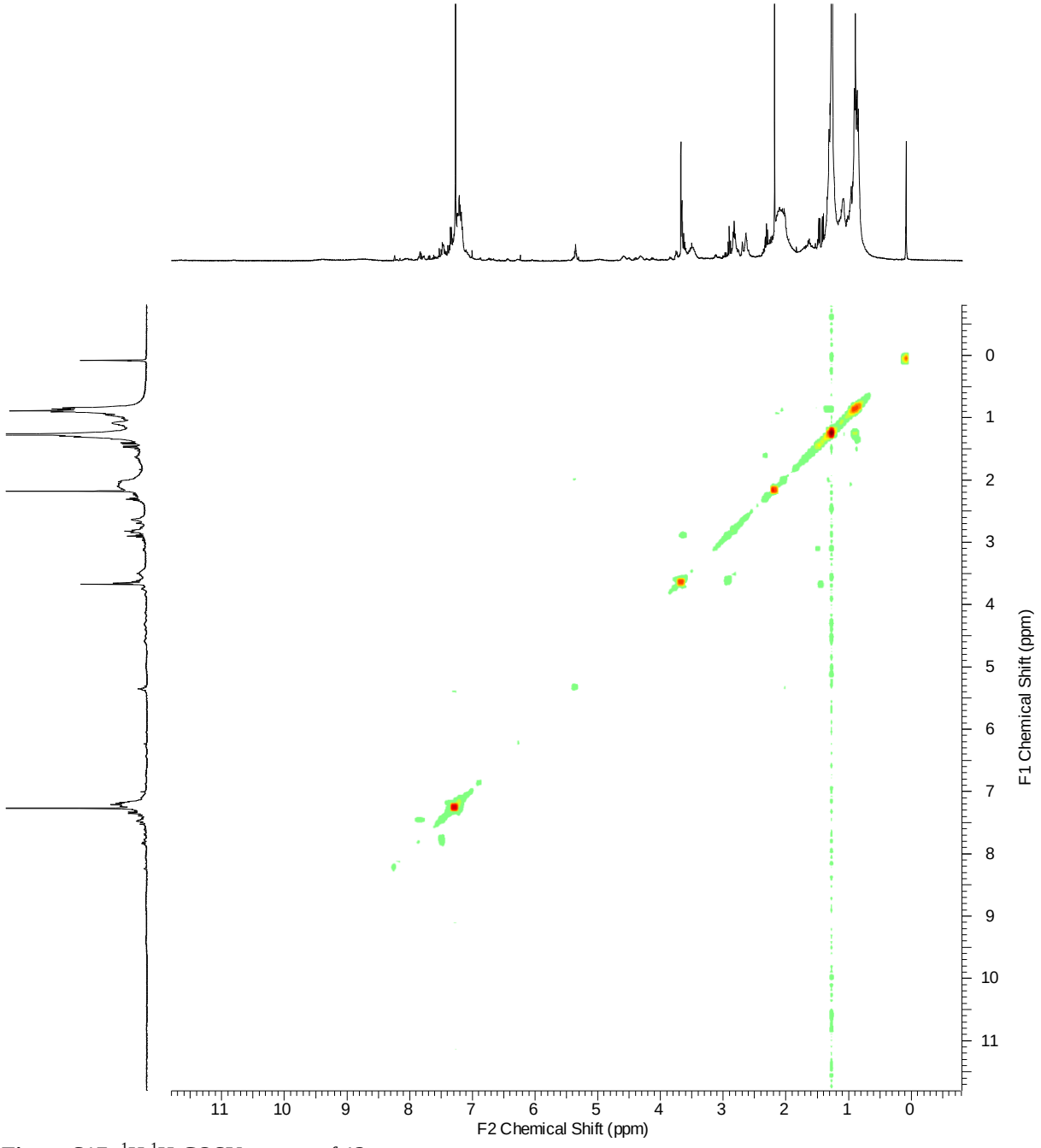


Figure S17: ^{1}H,^{1}H-COSY spectra of **12a.**

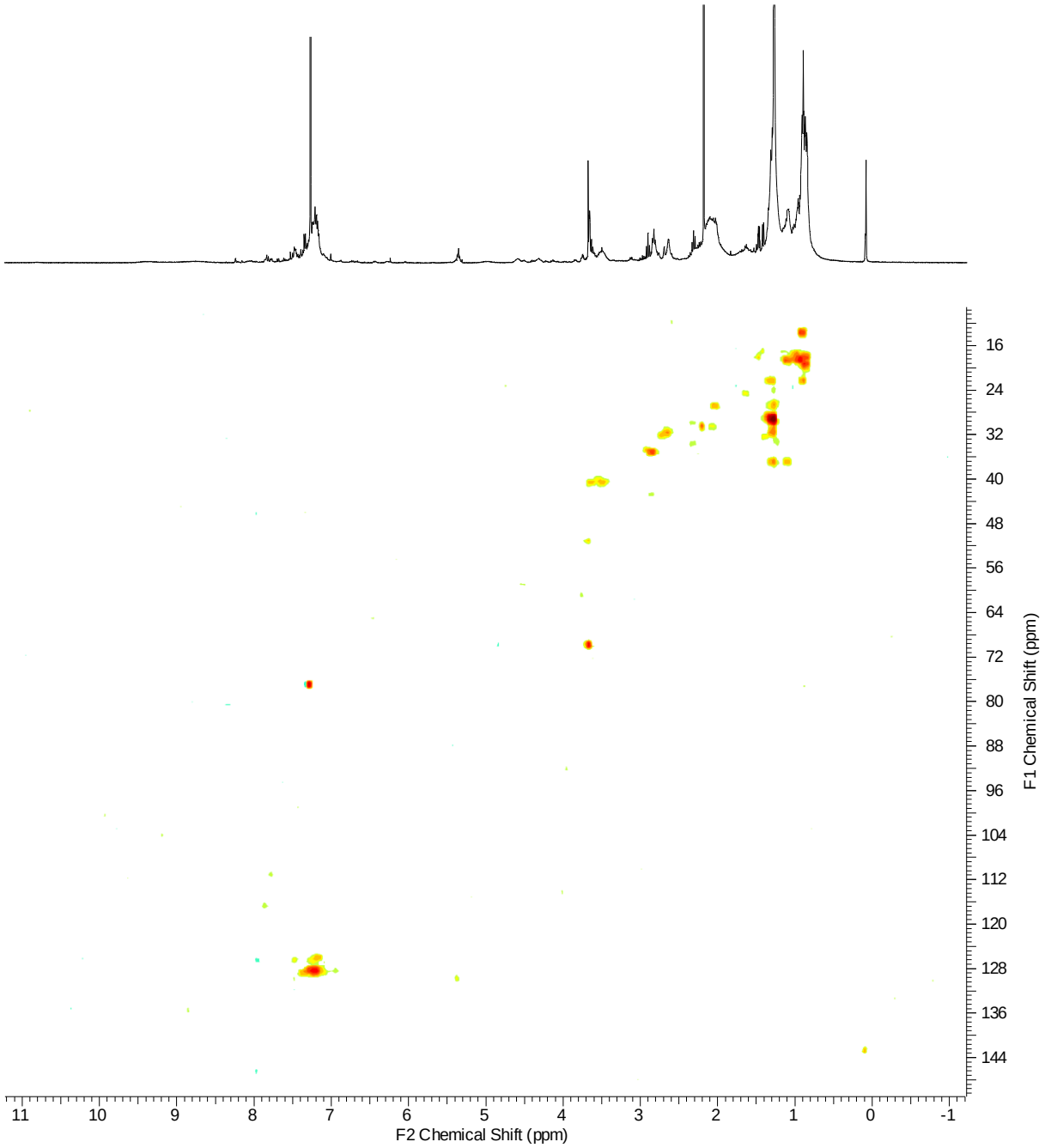


Figure S18: $^{1}H,^{13}C$-HSQC spectra of **12a**.

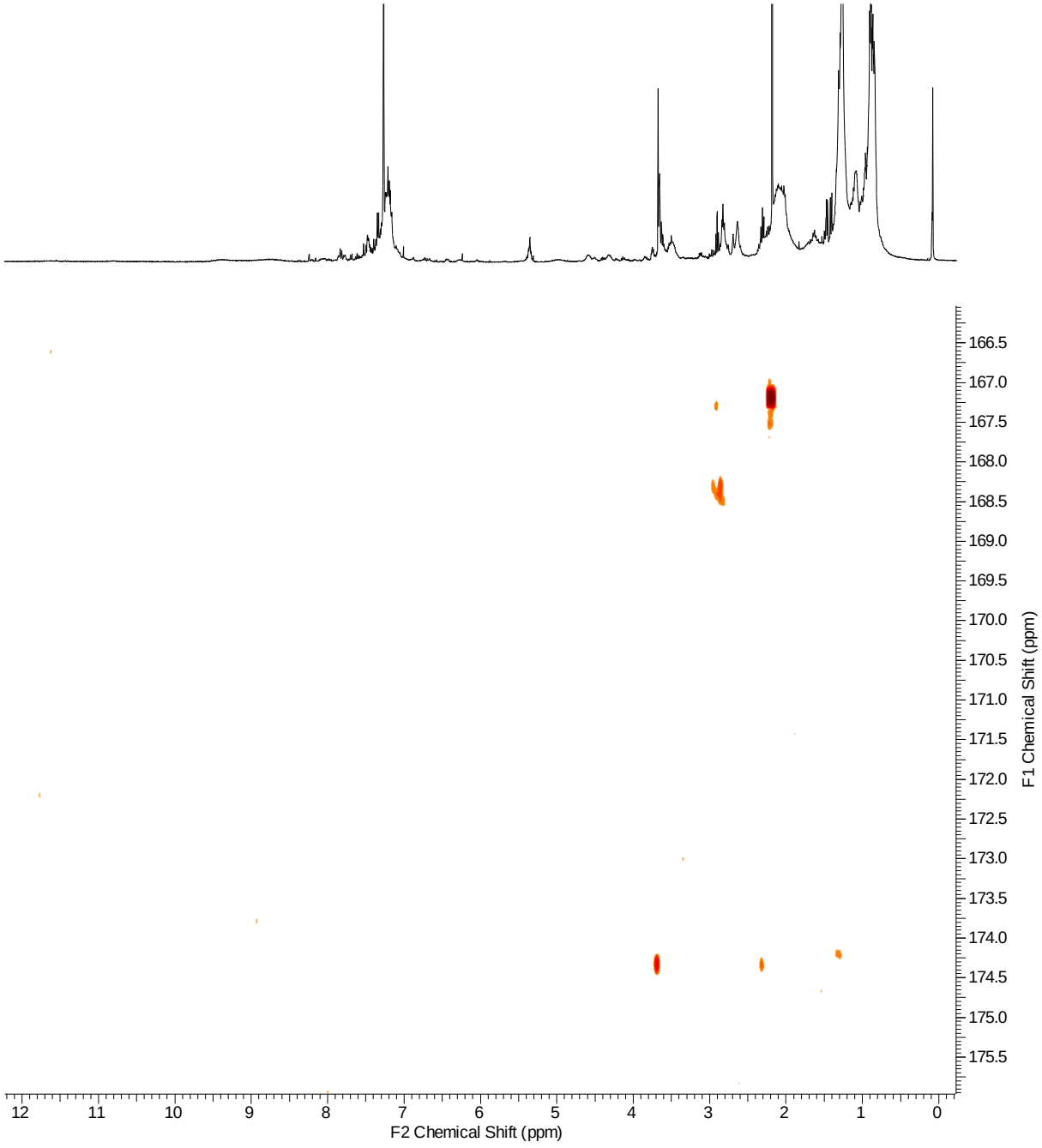
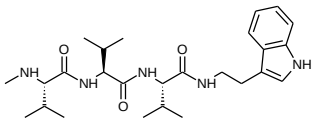
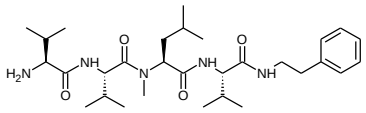


Figure S19: $^{1}H,^{13}C$-HMBC spectra of **12a**.



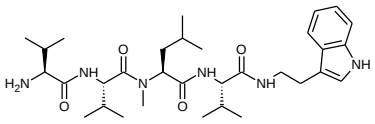
12b

The precursor (Boc*N*MeVal-Val-Val-OH) was manually synthesized using standard Fmoc/^{t}Bu chemistry on a preloaded 2-chlorotrityl resin (see general procedures) in a 50 µmol scale at room temperature while activating the building blocks including Boc-*N*MeVal-OH with HBTU in the presence of DIEA (the success of the synthesis was evaluated by LC-MS). Then the Boc-protected peptide, which was obtained upon incubation with HFIP in CH_2Cl_2 (see general procedures), was condensed with tryptamine in solution using EDC and HOBt (see general procedure), followed by normal phase purification (ethyl acetate in hexane (0% to 70% in five column volumes; using pre-packed silica flash cartridges and the Biotage Flash SP Purification System) and a final acidic deprotection step (see general procedures). Unfortunately, due to major impurities the resultant peptide was only submitted to HPLC-MS comparing the synthetic product with the natural one.



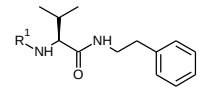
13a

The precursor (BocVal-Val-*N*MeLeu-Val-OH) was manually synthesized using standard Fmoc/^{t}Bu chemistry on a preloaded 2-chlorotrityl resin (see general procedures) in a 50 µmol scale at room temperature while activating the building blocks with HBTU in the presence of DIEA or in the case of the secondary amine of the methylated leucine with HATU and HOAt in the presence of NMM (see general procedures; the success of the synthesis was evaluated by LC-MS). Then the Boc-protected peptide, which was obtained upon incubation with HFIP in CH_2Cl_2 (see general procedures), was condensed with phenylethylamine in solution using EDC and HOBt (see general procedure), followed by normal phase purification (ethyl acetate in hexane (0% to 70% in five column volumes; using pre-packed silica flash cartridges and the Biotage Flash SP Purification System) and a final acidic deprotection step (see general procedures). Unfortunately, due to major impurities the resultant peptide was only submitted to HPLC-MS comparing the synthetic product with the natural one.



13b

The precursor (BocVal-Val-*N*MeLeu-Val-OH) was manually synthesized using standard Fmoc/^{t}Bu chemistry on a preloaded 2-chlorotrityl resin (see general procedures) in a 50 µmol scale at room temperature while activating the building blocks with HBTU in the presence of DIEA or in the case of the secondary amine of the methylated leucine with HATU and HOAt in the presence of NMM (see general procedures; the success of the synthesis was evaluated by LC-MS). Then the Boc-protected peptide, which was obtained upon incubation with HFIP in CH_2Cl_2 (see general procedures), was condensed with tryptamine in solution using EDC and HOBt (see general procedure), followed by normal phase purification (ethyl acetate in hexane (0% to 70% in five column volumes; using pre-packed silica flash cartridges and the Biotage Flash SP Purification System) and a final acidic deprotection step (see general procedures). Unfortunately, due to major impurities the resultant peptide was only submitted to HPLC-MS comparing the synthetic product with the natural one.



17a R^1 = Boc
17b R^1 = H

To obtain compound **17a** phenylethylamine was coupled to Boc-Val-OH (according to the general procedure) in a 200 µmol scale yielded 45 mg of **17a** (140 µmol, 70% yield, 98.8% pure). The following Boc-deprotection (used amount of acid was lowered to 4.0 eq) proceeded quantitatively (**17b**, monitored by TLC).

^{1}H-NMR (400 MHz, $CDCl_3$, **17a**): δ 7.34-7.23 (m, 5H), 5.77 (d, J = 8.9 Hz, 1H), 4.10 (t, J = 7.9 Hz, 1H), 3.64 (m, 1H), 3.47 (m, 1H), 2.89 (t, J = 7.3 Hz, 2H), 2.13 (m, 1H), 1.52 (s, 9H), 1.02 (d, J = 7.2 Hz, 3H), 1.00 (d, J = 7.1 Hz, 3H)

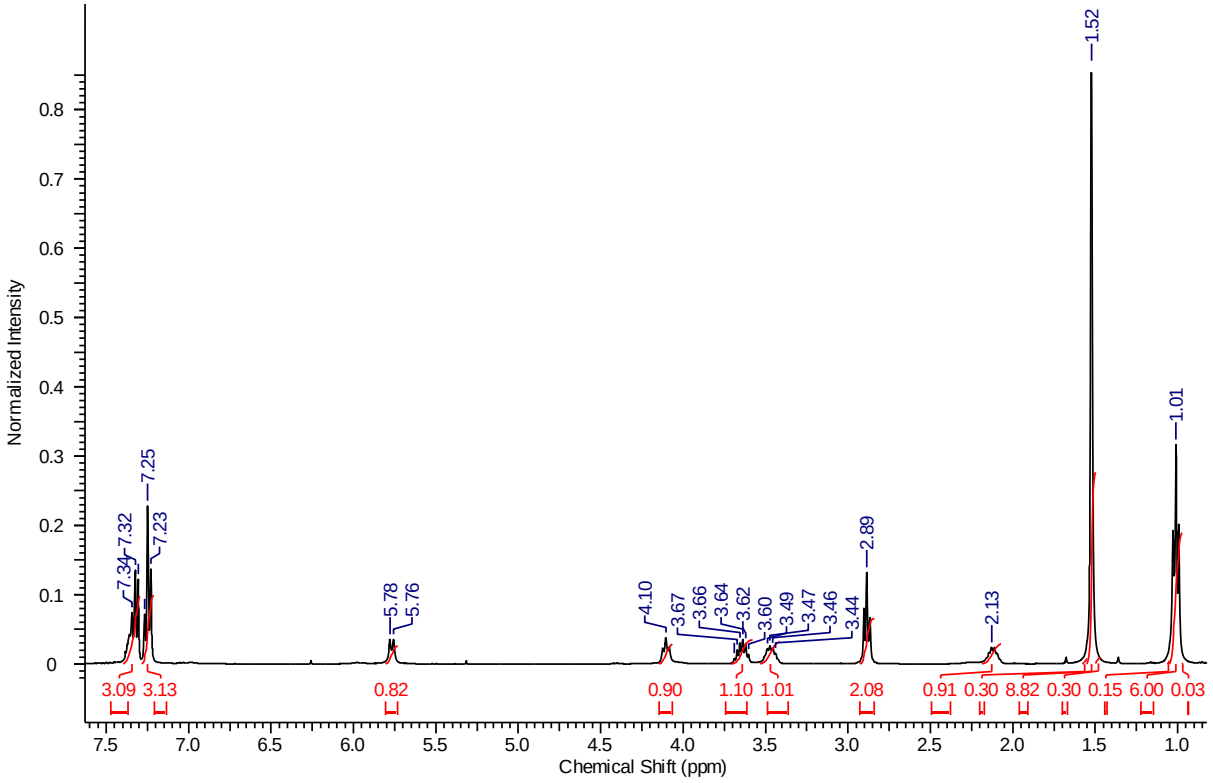
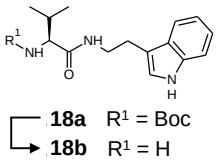


Figure S20: ^{1}H-NMR of rhabdopeptide precursor **17a**.



To obtain compound **18a** phenylethylamine was coupled to Boc-Val-OH (according to the general procedure) in a 200 µmol scale yielding 46.7 mg of the product (129.7 µmol, 65% yield, 92.3% pure). The following Boc-deprotection (used amount of acid was lowered to 4.0 eq) proceeded quantitatively (**18b**, monitored by TLC).

^{1}H-NMR (400 MHz, $CDCl_3$, **18a**): δ 9.02 (s, 1H), 7.58 (d, J = 7.8 Hz, 1H), 7.35 (d, J = 8.1 Hz, 1H), 7.19 (t, J = 7.3 Hz, 1H), 7.10 (t, J = 7.5 Hz, 1H), 6.90 (br, 1H), 5.60 (d, J = 8.7 Hz, 1H), 4.08 (t, J = 7.3 Hz, 1H), 3.59 (m, 1H), 3.50 (m, 1H), 2.94 (t, J = 6.5 Hz, 2H), 2.10 (m, 1H), 1.47 (s, 9H), 0.98 (d, J = 6.6 Hz, 3H), 0.94 (d, J = 6.9 Hz, 3H)

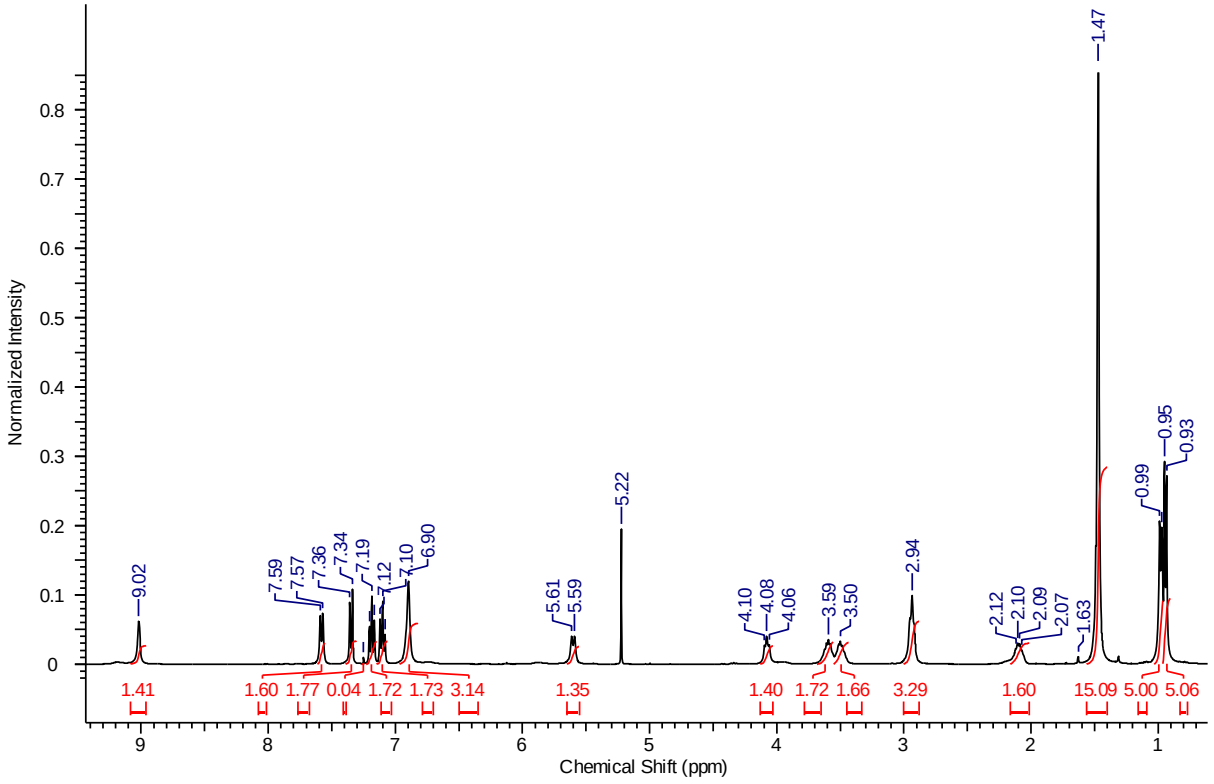
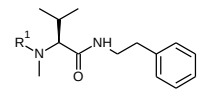


Figure S21: ^{1}H-NMR of rhabdopeptide precursor **18a**.



19a R^1 = Boc
19b R^1 = H

To obtain compound **19a** phenylethylamine was coupled to Boc-NMeVal-OH in a 200 µmol scale (57.8 mg, 86% yield, 95.1% pure). The following Boc-deprotection (used amount of acid was lowered to 4.0 eq; see general procedures) proceeded quantitatively (**19b**, monitored by TLC).

^{1}H-NMR (400 MHz, $CDCl_3$, **19a**): δ 8.10 (br, 1H), 7.37-7.19 (m, 5H), 6.35 (br, 1H), 4.03 (d, J = 11.0 Hz, 1H), 3.56 (m, 2H), 2.85 (t, J = 6.9 Hz, 2H), 2.83 (s, 3H), 2.31 (m, 1H), 1.51 (s, 9H), 0.96 (d, J = 6.2 Hz, 3H), 0.90 (d, J = 6.7 Hz, 3H)

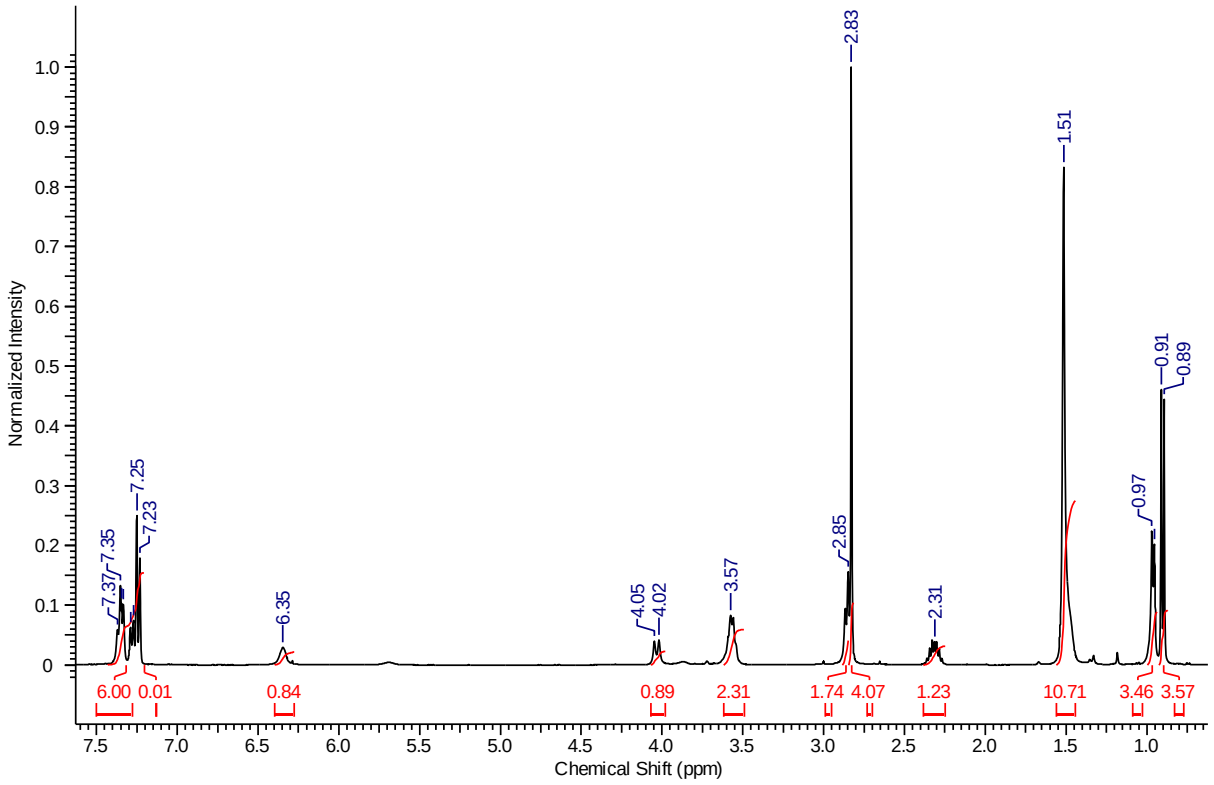
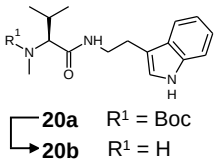


Figure S22: ^{1}H-NMR of rhabdopeptide precursor **19a**.



To obtain compound **20a** tryptamine was coupled to Boc-NMeVal-OH in a 200 µmol scale (65.8 mg, 88% yield, 93.9% pure). The following Boc-deprotection (used amount of acid was lowered to 4.0 eq; see general procedures) proceeded quantitatively (monitored by TLC).

^{1}H-NMR (400 MHz, $CDCl_3$, **20a**): δ 8.43 (br, 1H), 7.59 (d, J = 7.8 Hz, 1H), 7.33 (d, J = 8.1 Hz, 1H), 7.17 (t, J = 7.3 Hz, 1H), 7.10 (t, J = 7.5 Hz, 1H), 6.97 (d, J = 1.71 Hz, 1H), 6.26 (br, 1H), 3.99 (d, J = 10.9 Hz, 1H), 3.56 (m, 2H), 2.91 (m, 2H), 2.79 (s, 3H), 2.27 (m, 1H), 1.43 (s, 9H), 0.90 (d, J = 6.1 Hz, 3H), 0.84 (d, J = 6.7 Hz, 3H)

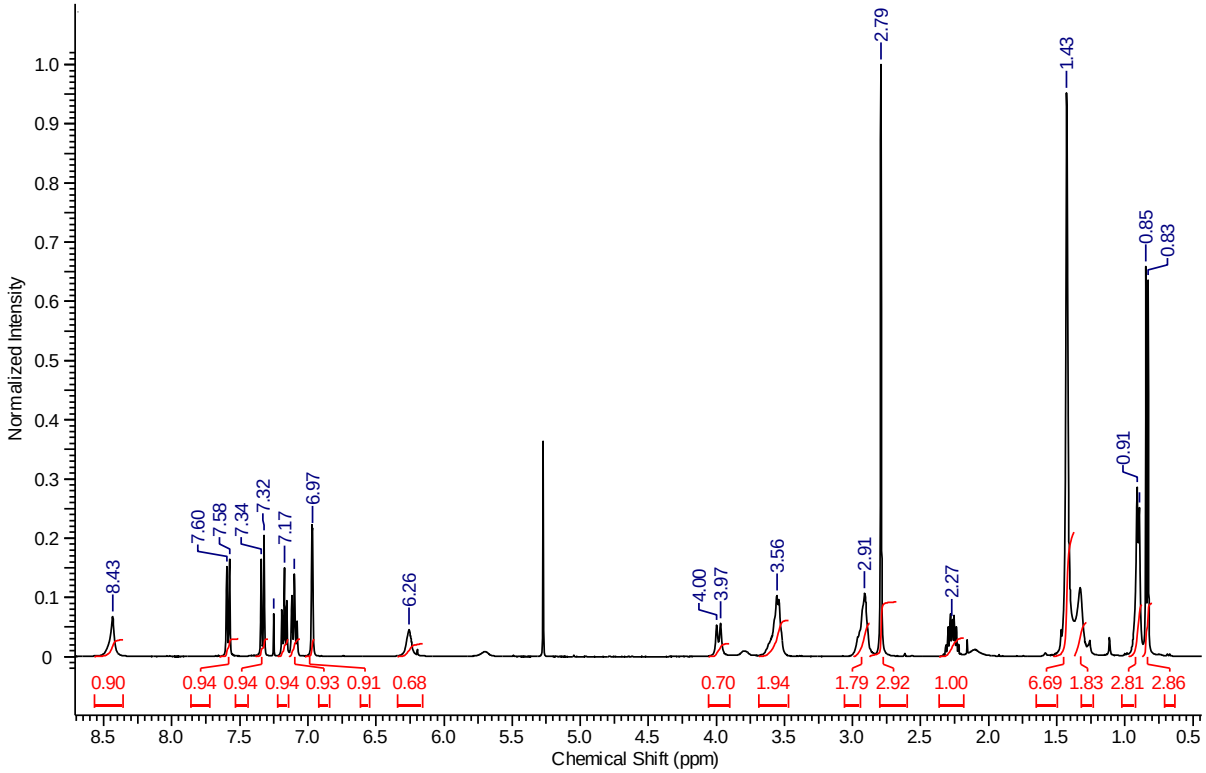


Figure S23: 1H-NMR of rhabdopeptide precursor **20a**.

S6.3 Synthesis of permethylated sequences

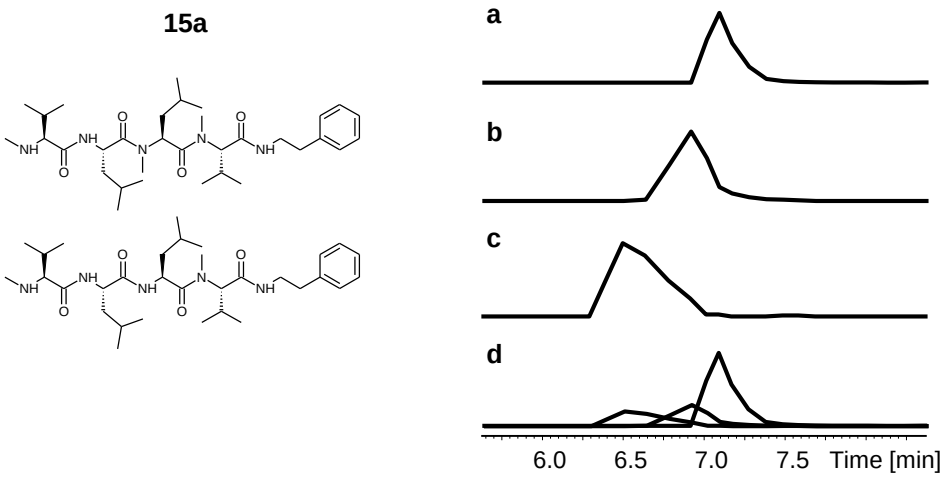
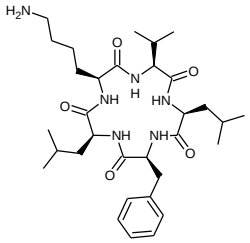


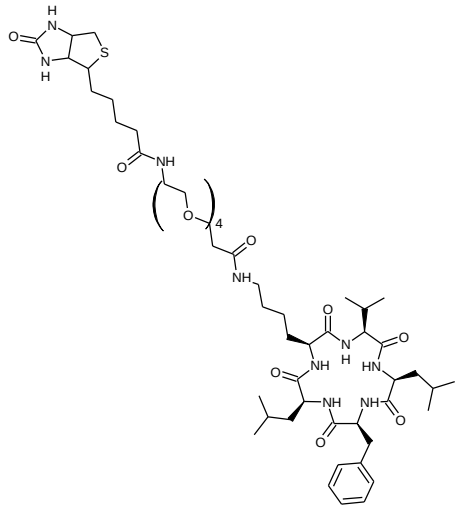
Figure S24: Extracted ion chromatograms of **15a** (a) and the two major impurities derived from an incomplete backbone methylation (b, -1xCH_3 and c, -2x CH_3; d, overlay of the three different species) after normal phase purification.

S7.1 Biotinylated GameXPeptides



4a

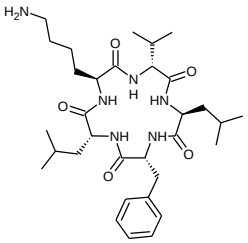
The linear precursor of compound **4a** was synthesized in a 25 µmol scale on a 2-chloro trityl resin employing a standard Fmoc/tBu strategy at room temperature using a SyroWave® system (Biotage, Sweden). Then this precursor was cleaved while sustaining the Boc-protecting group at the lysine. The obtained solid was washed several times with CH_2Cl_2 and ethyl acetate followed by cyclization in solution using microwave irradiation (see general procedures). After removing the solvent and the excess base, the precipitate was resuspended in 1mL 95% aqueous trifluoro acetic acid and precipitated with 40mL cold diethylether. It was then submitted to normal phase purification methanol in $CHCl_3$ (0% to 10% in six column volumes; using pre-packed silica flash cartridges and the Biotage Flash SP Purification System) 6.3 mg of the product (10.5 µmol, 42%).



4b

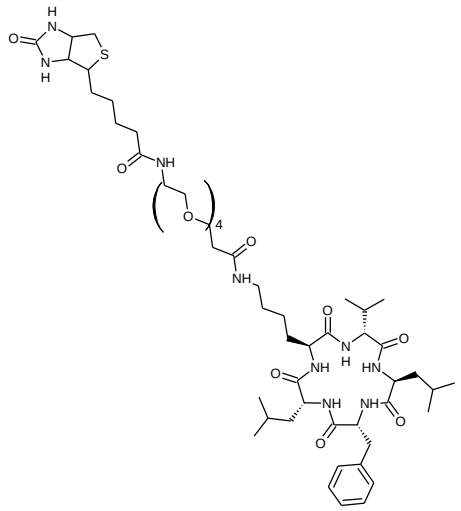
The linear precursor of compound **4b** was synthesized in a 50 µmol scale on a Wang resin employing a standard Fmoc/tBu strategy at room temperature using a SyroWave® system

(Biotage, Sweden). 1.4 eq of the commercially available building block *N*-alpha-(9-fluorenyl-methyloxycarbonyl)-*N*-ε-[15-(biotinamido)-4,7,10,13-tetraoxa-pentadecanoyl]-L-lysine (Fmoc-L-Lys-(dPEG™(4)-biotin)-OH; Iris Biotech, Germany) was coupled with a slight excess of 1.5 eq *O*-(7-aza¬benzo¬triazol-1-yl)-*N,N,N′,N′*-tetra¬methyl¬uronium hexafluoro-phosphate (HATU) in the presence of 1.5 eq 1-hydroxy-7-azabenzotriazole (HOAt) and 1.65 eq 4-methylmorpholine (NMM) in 1-methyl-2-pyrrolidinone (NMP) at room temperature overnight. Then the peptide sequence was elongated by Fmoc-D-Leu-OH and Fmoc-D-Phe-OH using standard procedures (see general procedures). The so obtained linear precursor was cleaved and the obtained solid was washed several times with CH_2Cl_2 and ethyl acetate followed by cyclization in solution using microwave irradiation (see general procedures). After removing the solvent and the excess base, the precipitate was resuspended in 1mL 95% aqueous trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS) for 90 min at room temperature, precipitated with cold diethylether, yielding 21.4 mg of **4b** (20.2 µmol, 40%).



5a

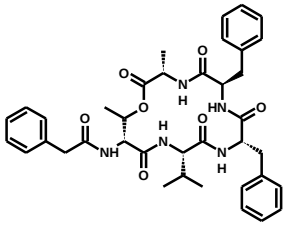
The linear precursor of compound **5a** was synthesized in a 25 µmol scale on a 2-chloro trityl resin employing a standard Fmoc/tBu strategy at room temperature using a SyroWave® system (Biotage, Sweden). Then this precursor was cleaved while sustaining the Boc-protecting group at the lysine. The obtained solid was washed several times with CH_2Cl_2 and ethyl acetate followed by cyclization in solution using microwave irradiation (see general procedures). After removing the solvent and the excess base, the precipitate was resuspended in 1mL trifluoro acetic acid and precipitated with 40mL cold diethyl ether. It was then submitted to normal phase purification methanol in $CHCl_3$ (0% to 10% in six column volumes; using pre-packed silica flash cartridges and the Biotage Flash SP Purification System) yielding 8.2 mg of the product (13.6 µmol, 54%).



5b

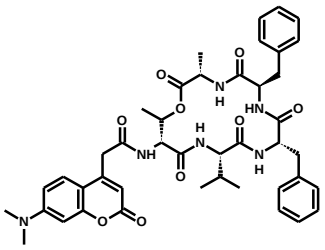
The linear precursor of compound **5b** was synthesized in a 50 µmol scale on a Wang resin employing a standard Fmoc/tBu strategy at room temperature using a SyroWave® system (Biotage, Sweden). 1.4 eq of the commercially available building block *N*-alpha-(9-fluorenyl-methyloxycarbonyl)-*N*-ε-[15-(biotinamido)-4,7,10,13-tetraoxa-pentadecanoyl]-L-lysine (Fmoc-L-Lys-(dPEG™(4)-biotin)-OH; Iris Biotech, Germany) was coupled with a slight excess of 1.5 eq *O*-(7-aza¬benzo¬triazol-1-yl)-*N,N,N′,N′*-tetra¬methyl¬uronium hexafluoro-phosphate (HATU) in the presence of 1.5 eq 1-hydroxy-7-azabenzotriazole (HOAt) and 1.65 eq 4-methylmorpholine (NMM) in 1-methyl-2-pyrrolidinone (NMP) at room temperature overnight. Then the peptide sequence was elongated by Fmoc-D-Leu-OH and Fmoc-D-Phe-OH using standard procedures (see general procedures). The so obtained linear precursor was cleaved and the obtained solid was washed several times with CH_2Cl_2 and ethyl acetate followed by cyclization in solution using microwave irradiation (see general procedures). After removing the solvent and the excess base, the precipitate was resuspended in 1mL 95% aqueous trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS) for 90 min at room temperature, precipitated with cold diethylether, yielding 31.4 mg of **5b** (29.7 µmol, 59%).

S7.2 Intrinsically labelled Xenephematides



6a

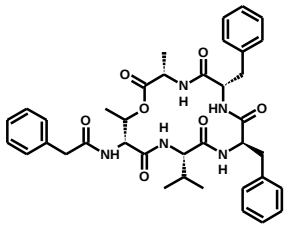
The linear precursor was manually synthesized using standard Fmoc/^{t}Bu chemistry on a preloaded Wang resin in a 50 µmol scale at room temperature (see general procedures and chapter 4). Then the *N*-terminus was acylated (see general procedures), followed by the selective deprotection of the threonine (see general procedures) and the introduction of an Boc-protected alanine via an ester bond using MSNT in the presence of NMI and DIEA (see general procedures and chapter 4). The branched peptide precursor was cleaved from the resin (see general procedures), the cleavage cocktail was removed under reduced pressure and the precipitate was washed several times with CH_2Cl_2 and ethyl acetate. Then the cyclization was achieved using high dilution conditions (4 mM in DMF, 20 min, 50°C, 25W) and upon activation with 1.5 eq HATU in the presence of 2.0 eq DIEA. The solvents were removed and the precipitate was washed several times. The crude peptide was submitted to normal phase purification (ethyl acetate in hexane (0% to 100% in eight column volumes; using pre-packed silica flash cartridges and the Biotage Flash SP Purification System) yielding 10.8 mg (15.8 µmol, 32% yield) of compound **6a**.



6b

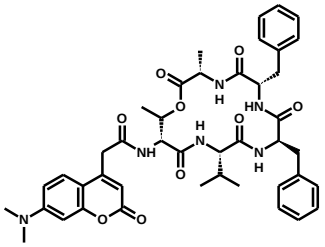
The linear precursor was manually synthesized using standard Fmoc/^{t}Bu chemistry on a preloaded Wang resin in a 50 µmol scale at room temperature (see general procedures and chapter 4). Then the *N*-terminus was acylated with the previously obtain 7-(dimethyl-amino)coumarin-4-acetic acid (which was synthesized according to Portonovo *et al.*),

followed by the selective deprotection of the threonine (see general procedures) and the introduction of an Boc-protected alanine via an ester bond using MSNT in the presence of NMI and DIEA (see general procedures and chapter 4). The branched peptide precursor was cleaved from the resin (see general procedures), the cleavage cocktail was removed under reduced pressure and the precipitate was washed several times with CH_2Cl_2 and ethyl acetate. Then the cyclization was achieved using high dilution conditions (4 mM in DMF, 20 min, 50°C, 25W) and upon activation with 1.5 eq HATU in the presence of 2.0 eq DIEA. The solvents were removed and the precipitate was washed several times. The crude peptide was submitted to normal phase purification (ethyl acetate in hexane (0% to 100% in eight column volumes; using pre-packed silica flash cartridges and the Biotage Flash SP Purification System) yielding 7.3 mg (9.2 µmol, 18% yield) of compound **6b**.



7a

The linear precursor was manually synthesized using standard Fmoc/^{t}Bu chemistry on a preloaded Wang resin in a 50 µmol scale at room temperature (see general procedures and chapter 4). Then the *N*-terminus was acylated (see general procedures), followed by the selective deprotection of the threonine (see general procedures) and the introduction of an Boc-protected alanine via an ester bond using MSNT in the presence of NMI and DIEA (see general procedures and chapter 4). The branched peptide precursor was cleaved from the resin (see general procedures), the cleavage cocktail was removed under reduced pressure and the precipitate was washed several times with CH_2Cl_2 and ethyl acetate. Then the cyclization was achieved using high dilution conditions (4 mM in DMF, 20 min, 50°C, 25W) and upon activation with 1.5 eq HATU in the presence of 2.0 eq DIEA. The solvents were removed and the precipitate was washed several times. The crude peptide was submitted to normal phase purification (ethyl acetate in hexane (0% to 100% in eight column volumes; using pre-packed silica flash cartridges and the Biotage Flash SP Purification System) yielding 9.85 mg (14.4 µmol, 29% yield) of a racemic mixture.



7b

The linear precursor was manually synthesized using standard Fmoc/^{t}Bu chemistry on a preloaded Wang resin in a 50 µmol scale at room temperature (see general procedures and chapter 4). Then the *N*-terminus was acylated with the previously obtain 7-(dimethyl-amino)coumarin-4-acetic acid (which was synthesized according to Portonovo *et al.*), followed by the selective deprotection of the threonine (see general procedures) and the introduction of an Boc-protected alanine via an ester bond using MSNT in the presence of NMI and DIEA (see general procedures and chapter 4). The branched peptide precursor was cleaved from the resin (see general procedures), the cleavage cocktail was removed under reduced pressure and the precipitate was washed several times with CH_2Cl_2 and ethyl acetate. Then the cyclization was achieved using high dilution conditions (4 mM in DMF, 20 min, 50°C, 25W) and upon activation with 1.5 eq HATU in the presence of 2.0 eq DIEA. The solvents were removed and the precipitate was washed several times. The crude peptide was submitted to normal phase purification (ethyl acetate in hexane (0% to 100% in eight column volumes; using pre-packed silica flash cartridges and the Biotage Flash SP Purification System) yielding 7.8 mg (9.8 µmol, 20% yield) of a racemic mixture.

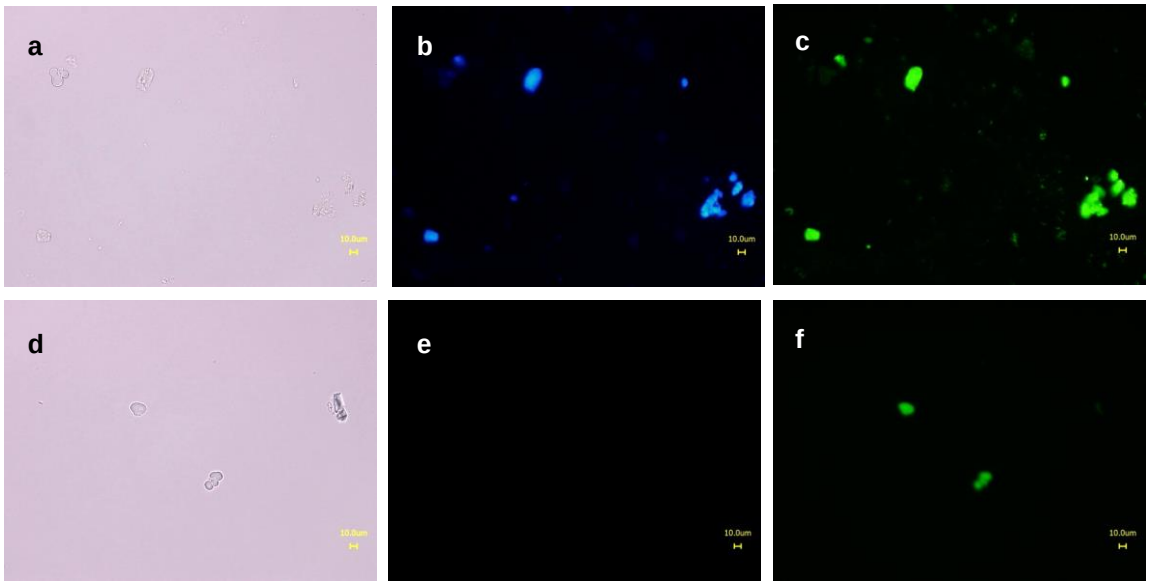


Figure S25: The non-labeled xenephematide (**7a**) was incubated with fluorescein-filled small unilamellar vesicles (bright field (a; exposure time 1/20s), DAPI (b; exposure time 1/20s) and GFP filter (c; exposure time 1/20s)), in comparison to that the labelled xenephematide (**7b**; bright field (d; exposure time 1/20s), DAPI (e; exposure time 1/20s) and GFP filter (f; exposure time 1/20s)).

Photorhabdus natural product inhibits insect juvenile hormone epoxide hydrolase

F. I. Nollmann, A. K. Heinrich, A. O. Brachmann, C. Morisseau, K. Mukherjee, A. M. Casanova-Torres, F. Strobl, D. Kleinhans, S. Kinski, K. Schultz, M. L Beeton, M. Kaiser, Y. Y. Chu, L. Phan Ke, A. Thanwisai, K. A. Bozhüyük, N. Chantratita, F.Götz, N. R. Waterfield, A. Vilcinskas, E. H. Stelzer, H. Goodrich-Blair, B. D. Hammock, H. B. Bode

Project Development	
	60% HBB, 40% FIN
Realization of the Individual Experiments	
	The synthesis of the different phurealipids was conducted by FIN (initial synthetic strategies were developed by KS, who also prepared the corresponding glycine amides) involving LC-MS and NMR analysis. FIN and AOB prepared the growth kinetic study of *P. luminescens* TTO1. AKH generated the knock-out mutants *pliA* and *pliB* and prepared the stable isotope labeling experiments.
Compilation of Data and Preparation of Figures	
	FIN prepared most of the data and figures of the supplementary information (with the exception of *Tribolium castaneum* data and the data concerning the construction of mutants). FIN also prepared the figures included in the main article under the guidance of HBB.
Analysis and Interpretation of the Obtained Data	
	FIN analyzed the HPLC-MS data derived from the stable isotope labeling experiments and proposed/revised the final structures. KAJB compiled the phylogenetic tree based on the *recA* sequence. FIN analyzed the production spectra by HPLC-MS of all the strains.
Introduction, Results and Discussion	
	100% HBB

Frankfurt, 3rd of June 2015

Friederike I. Nollmann	Helge B. Bode

Rapid Determination of the Amino Acid Configuration of Xenotetrapeptide

C. Kegler, F. I. Nollmann, T. Ahrendt, F. Fleischhacker, E. Bode and H. B. Bode

Project Development	
	40% HBB, 30% CK 30% FIN
Realization of the Individual Experiments	
	CK oversaw and supervised the molecular biological experiments leading to this publication. FIN exploited the different synthesis strategies leading to the synthesis of the xenotetrapeptide underlining the observation made in the biological experiments.
Compilation of Data and Preparation of Figures	
	CK and HBB prepared the data and figures of the manuscript. FIN contributed the part related to the synthesis.
Analysis and Interpretation of the Obtained Data	
	CK and HBB prepared the manuscript.
Introduction, Results and Discussion	
	60% HBB, 40% CK

Frankfurt, 3rd of June 2015

Friederike I. Nollmann

Helge B. Bode

Insect-specific production of novel GameXPeptides, widespread natural products in entomopathogenic Photorhabdus and Xenorhabdus bacteria

F. I. Nollmann, C. Dauth, G. Mulley, C. Kegler, M. Kaiser, N. R. Waterfield, H. B. Bode

Project Development	
	40% HBB, 30% FIN, 30% CD
Realization of the Individual Experiments	
	CD developed the synthesis strategy; FIN synthesized the GameXPeptides and their derivatives verifying the compounds by detailed NMR experiments and LC-MS.
Compilation of Data and Preparation of Figures	
	FIN prepared the data and figures for this publication (with exception of the phylogenetic tree).
Analysis and Interpretation of the Obtained Data	
	KAJB compiled the phylogenetic tree based on the *recA* sequence. FIN analyzed the production spectra by HPLC-MS of all the- strains.
Introduction, Results and Discussion	
	40% HBB, 60% FIN

Frankfurt, 3rd of June 2015

Friederike I. Nollmann

Helge B. Bode

Synthesis of szentiamide, a depsipeptide from entomopathogenic Xenorhabdus szentirmaii with activity against Plasmodium falciparum

F. I. Nollmann, A. Dowling, M. Kaiser, K. Deckmann, S. Grösch, R. ffrench-Constant, H. B. Bode

Project Development	
	30% HBB, 70% FIN
Realization of the Individual Experiments	
	FIN developed and established the synthesis strategy for depsipeptides, in this case the szentiamide. The final characterization involved detailed NMR analysis and LC-MS comparing the synthetic peptide with the natural one (prior isolated from *X. szentirmaii* by FIN). MK tested the natural product against the causative agents of neglected tropical disease.
Compilation of Data and Preparation of Figures	
	FIN prepared the data and figures for this publication.
Analysis and Interpretation of the Obtained Data	
	FIN analyzed and interpreted the data presented in this publication.
Introduction, Results and Discussion	
	40% HBB, 60% FIN

Frankfurt, 3rd of June 2015

Friederike I. Nollmann

Helge B. Bode