Thioholgamides - thioamide-containing cytotoxic RiPPs natural products

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Abstract

Thioviridamide is a structurally unique ribosomally synthesized and post-translationally modified peptide (RiPP), which contains several thioamide bonds and is active against a number of cancer cell lines. In the search for naturally occurring thioviridamide analogues, we employed genome mining that led to the identification of several related gene clusters. Chemical screening followed by cultivation and isolation yielded thioholgamide A and B, two new additions to the thioviridamide family, with several amino acid substitutions, a different N-capping moiety and with one less thioamide bond. Thioholgamides display improved cytotoxicity in the sub-µM range against a range of cell lines and an IC_{50} of 30 nM for Thioholgamide A on HCT-116 cells. Herein, we report the isolation and structure elucidation of thioholgamide A and B, a proposed biosynthetic cluster for their production and their bioactivities against a larger panel of microorganisms and cancer cell lines.

Introduction

Cancer is among the leading causes of death worldwide. The number of new cases is steadily increasing and is expected to continue to do so in the coming years *(1)*. Therefore, there is an omnipresent need to develop new chemotherapeutics, preferably with fewer side effects and high target specificity.

As much as 75% of anticancer drugs and 69% of anti-infectives are derived from or inspired by natural products, which remain one of the most promising sources of drug lead molecules due to their inherently privileged structures *(2,3)*. Microorganisms are the most practical producers of secondary metabolites for drug development because they are relatively easy to grow and upscale. Especially the gram-positive streptomycetes are a major source of antibiotics used in the clinic (e.g. chloramphenicol, fosfomycin, daptomycin, and streptomycin) *(4,5)*, but also produce secondary metabolites with other activities *(6)*, including the immunosuppressant rapamycin *(7–9)* and the chemotherapeutics

doxorubicin *(10)* and dactinomycin *(11)*. Despite many years of mining for biologically active molecules from *Streptomyces* species, there is still much potential hidden under this genus *(12)*.

Ribosomally synthesized and posttranslationally modified peptides (RiPPs) are a relatively recent addition to the major classes of natural products, including the well-known alkaloids, polyketides, non-ribosomal peptides, and terpenoids *(13,14)*. By modifications of a precursor peptide, the RiPPs biosynthetic machinery can produce various intriguing compound families, from the lantibiotics to the thiopeptides (or thiazolyl peptides) containing thiazoles and other heterocycles. Two articles from 2006 by Hayakawa et al. described the isolation and characterization of thioviridamide from *Streptomyces olivoviridis (15,16)*, a RiPP with five thioamide bonds and a permanent positive charge. In 2013, the biosynthetic gene cluster was identified *(17)*, and in 2015 a derivative was identified (resulting from heterologous expression) with lactic acid as N-terminal modification *(18)*. By definition, the thiopeptide RiPPs family does not include any peptides with thioamide bonds (containing N-C=S, like thioviridamide), which are extremely rare in natural products. To date, only three compound families of bacterial origin contain this type of thioamide (N-C=S) bonds: thioviridamide and its analogue JBIR-140, the methanobactins (also RiPPs) *(19)*, and closthioamide *(20)*. In addition, the small molecule cycasthioamide from the plant *Cycas revolute* contains a single thioamide bond *(21)*. Thioamides generally have higher stability towards hydrolysis than standard amides, and isosteric replacement has been a tool for medicinal chemists to change degradation and ADME properties of biologically active small molecules *(22)*. In some cases this can lead to new or enhanced bioactivity *(19)*, but it can also completely abolish the activity, as was seen for closthioamide when its hexaoxo analogue closamide was synthesized and found inactive *(20)*.

Results and Discussion

Fascinated by the chemistry, biosynthesis and bioactivity of thioviridamide-like molecules we set out to identify novel members of this compound class by genome mining. Searching publically available genome sequences of actinomycetes and other microbes for homologous biosynthetic gene clusters (see SI), we identified 13 genomes (Figure 1) with loci that closely resemble the biosynthetic gene cluster published for thioviridamide *(17)*. All genome sequences but one belonged to the Actinobacteria (*Streptomyces* spp., *Amycolatopsis alba*, *Micromonospora eburnea*, and *Nocardiopsis potens*) and one strain was a cyanobacterium (*Mastigocladus laminosus*). The newly found biosynthetic gene clusters showed varying gene synteny. Of all identified, 5 biosynthetic gene clusters had nearly-identical gene compositions. The others showed varying degrees of gene rearrangements, insertions, and deletions (Figure 1). Homologs of the core thioviridamide biosynthetic proteins TvaA, TvaC, TvaD, TvaE, TvaF, TvaG, TvaH, and TvaI were found in all 13 genomic loci, while homologs of TvaJ, TvaK, and TvaL were located in 12 of 13 loci. Genes *tvaB*, *tvaM*, *tvaN*, and *tvaO* had no

homologs in examined sequences, suggesting that they are either not involved in, not crucial for biosynthesis, or are located elsewhere in the respective genomes. Of the examined biosynthetic loci, several showed the existence of additional proteins, which were not present in the query sequences but nevertheless might be involved in the biosynthesis of respective compounds: A putative SDR family oxidoreductase (designated as X-homolog) and a putative methyltransferase (designated as Y-homolog) (Figure 1). The variability of biosynthetic loci prompted us to obtain and cultivate four strains (DSM 100712, DSM 45234, DSM 44262, and NRRL S-87). Subsequent LCMS analysis of the secondary metabolite profiles led us to identify *Streptomyces malaysiense* MUSC 136 57 (DSM 100712) as a prolific producer of a series of thioviridamide-like compounds, even though pairwise sequence identity to thioviridamide biosynthetic genes was merely between 23 and 53%. These compounds with masses in the same range as thioviridamide (~1,300 Da) also contained a similarly high number of sulfur atoms, as seen from the isotopic patterns, and fractions containing these compounds showed strong cytotoxicity against the human cervix carcinoma cell line KB-3.1. Upscaling, isolation, and analysis by LCMS and NMR spectroscopy, revealed new peptides in the thioviridamide family, with structural changes in N-terminal modification, amino acid sequence, and the number of thioamide bonds. Extraction and fractionation of combined 150 ml cultures amounting to a total cultivation volume of 6 liters, allowed the purification and structural characterization of thioholgamide A (**1**, 11.3 mg) and B (**2**, 4.3 mg) (see details in SI), while the putative thioholgamide C and D were merely observed by LCMS and partially characterized based on MS^2 fragmentation patterns.

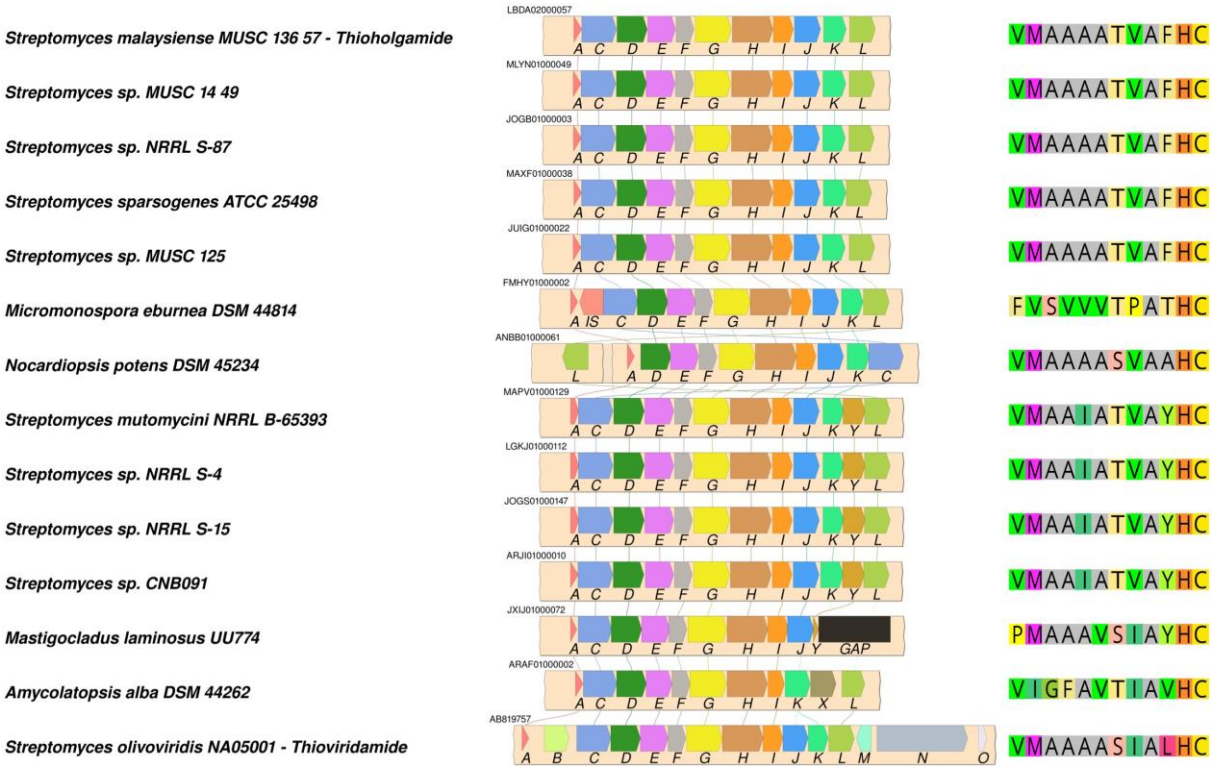


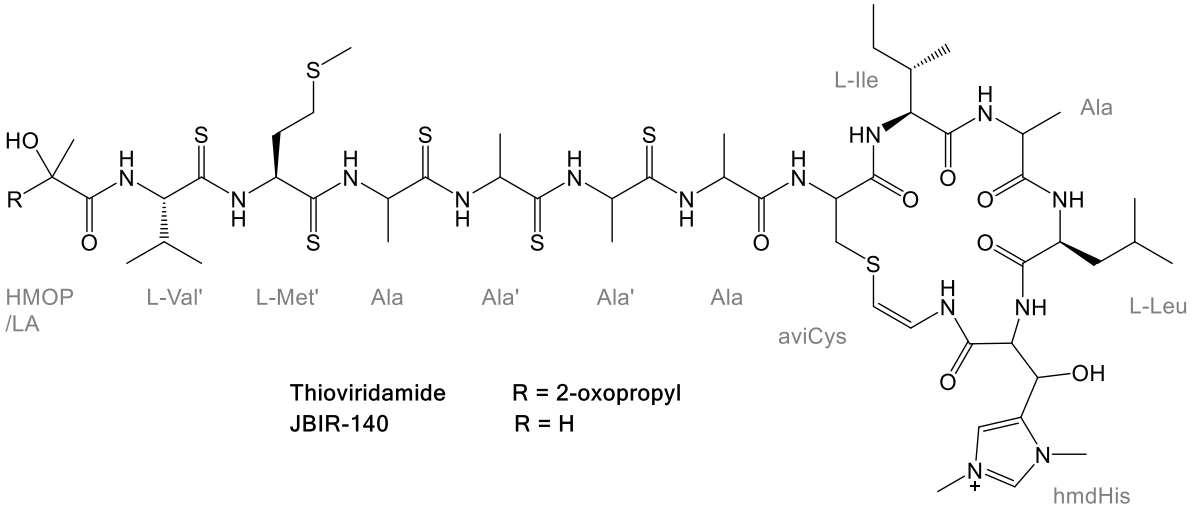
Figure 1 Proposed thioholgamide biosynthetic gene cluster (top) from *Streptomyces malaysiense* MUSC 136 57 compared to homologous gene clusters from other organisms by the SimpleSynteny program. A – precursor peptide, B – putative SARP family regulator, C – hypothetical protein, D – hypothetical protein, E – hypothetical protein, F – putative flavoprotein decarboxylase, G – putative methyltransferase, H – hypothetical protein, I – TfuA-like core domain-containing protein, J – putative phytanoyl-CoA dioxygenase family protein, K – putative proteinase, L – hypothetical protein, M – putative regulatory protein, N – putative LuxR family transcriptional regulator, O – hypothetical protein, X – putative SDR family oxidoreductase, Y – putative methyltransferase, IS – putative IS5/IS1182 family transposase, GAP – sequencing gap. The peptide sequence encoded in the precursor peptide is shown on the right.

HRESIMS analysis of **1** displayed the molecular ion $[M]^+$ at m/z 1305.4901 consistent with the molecular formula $C_{56}H_{85}N_{14}O_{10}S_6^+$ (Δ = 0.7 ppm), which underlined the connection to thioviridamide (exact mass 1345.5244 and formula $C_{56}H_{93}N_{14}O_{10}S_7^+$) *(16)* but also suggested that there must be some difference between the structures, based on the number of hydrogen and sulfur atoms. This was confirmed by analysis of the 2D NMR data (DQF-COSY, HSQC, HMBC, and ROESY) of **1** (see NMR tables in SI). Whereas NMR data for thioviridamide and JBIR-140 were acquired in methanol-d_4 *(16,18)*, **1** displayed limited solubility and broad resonances in and close to the backbone of the macrocyclic ring system. Data in DMSO-d_6 showed less structural flexibility, but the best results were obtained in D_2O (both datasets are reported in the SI). Proton spin systems of the individual amino acids of **1** were connected via DQF-COSY correlations, and ^{13}C connectivities were determined from the HSQC spectrum. Spin systems were then connected via HMBC and ROESY NMR data, and it was soon clear that the alanine residue next to methionine had a standard peptide bond (173 ppm), and not a thioamide bond as for thioviridamide. Key correlations supporting this claim are given in detail in the SI. The remaining sulfur atoms were placed as in thioviridamide, as seen from the four ^{13}C=S resonances around 205 ppm, the methionine residue (e.g. singlet from the -S-Me at 2.07 and 14.9

ppm), and the cysteine-based bridge to Thr giving an [(*Z*)-2-aminovinyl]-3-methylcysteine (aviMeCys) moiety to form the macrocyclic ring. From the gene sequences coding for the precursor peptide (VMAAAATVAFHC), two further amino acids in the macrocyclic ring were expected to be exchanged (from isoleucine to leucine, and from leucine to phenylalanine), and this was corroborated by the NMR data. Furthermore, the N-terminal modification of the peptide was identified as pyruvate, as seen from an acetate-like methyl group (2.42 and 25.0 ppm) with correlations to two carbonyls at 198.3 and 162.6 ppm (the latter also correlating to H_α of Met). Altogether, this resulted in the structure of **1** as seen below (Figure 2).

HRESIMS analysis of **2** displayed a molecular ion at m/z 1321.4862 ($[M]^+$) consistent with the molecular formula $C_{56}H_{85}N_{14}O_{11}S_6^+$ (Δ = 1.2 ppm), and thus just one additional oxygen compared to **1**. This was found in the methionine residue, where oxidation to methionine sulfoxide resulted in higher chemical shifts of the adjacent C_γ and C_ε resonances (Me here at 2.68 and 37.2 ppm). This sulfoxide analogue was observed in the raw extract of *Streptomyces malaysiense* MUSC 136 57 (DSM 100712) and therefore we expect it to be a genuine secondary metabolite and not a product of the work-up procedure.

Thioviridamide precursor peptide: V M A A A A S I A L H C



Thioholgamide precursor peptide: V M A A A A T V A F H C

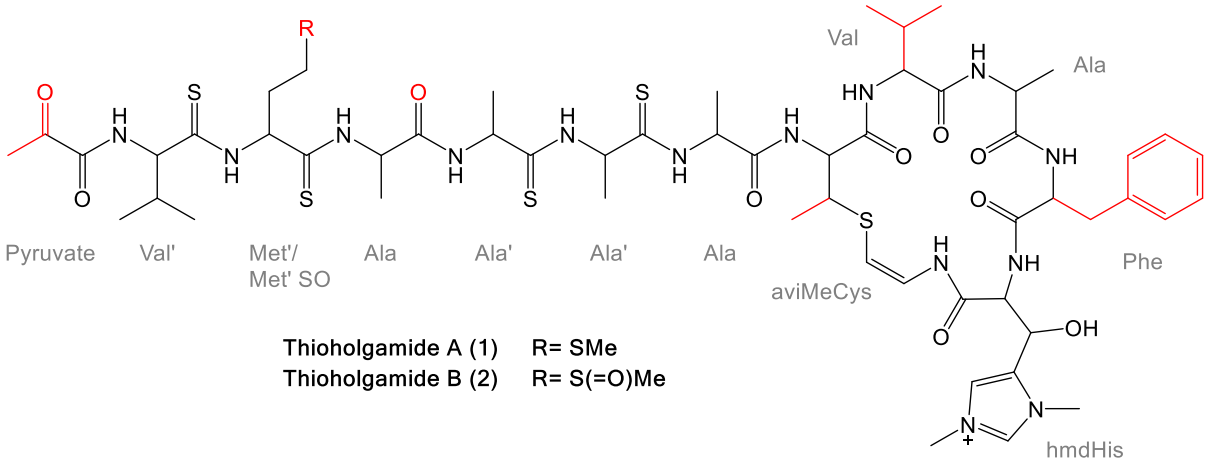


Figure 2 Structures of thioviridamide, JBIR-140, and thioholgamide A (1) and B (2). The methionine in 1 is oxidized to methionine sulfoxide in 2. Differences in the thioholgamides compared to thioviridamide are highlighted in red.

Two further analogues at 1289.4963 ($C_{56}H_{85}N_{14}O_9S_6^+$, Δ = 0.9 ppm) and m/z 1307.5077 ($C_{56}H_{87}N_{14}O_{10}S_6^+$, Δ = 1.3 ppm) contain one less oxygen and one less double bond equivalent, respectively. Based on MS^2 fragmentation the missing oxygen stems from loss of the hydroxy group in the hdmHis, and the compound with m/z 1307 is believed to be an analogue with a different N-capping group, as MS^2 fragmentation for the molecule is identical from the macrocycle to (excl.) the methionine moiety (see MS^2 spectra and interpretation in SI).

Amino acid analysis of **1** was attempted by Marfey's method and showed the presence of L-Val, L-Phe, and both L- and D-Ala. The methionine appeared as two very small peaks of both configurations, which, along with the relative amounts observed for Ala, Val, and Phe, suggests that the thioamide bonds are not readily hydrolyzed, even when heated for prolonged periods in hydrochloric acid.

Crude extracts of *Streptomyces malaysiense* MUSC 136 57 displayed strong cytotoxic activity on KB-3.1 cells and additionally inhibited the growth of some bacteria such as *Staphylococcus aureus*. We therefore assessed the biological activity of thioholgamides A and B with a panel of cell lines and microorganisms. Thioholgamide A showed markedly increased activity compared to thioviridamide and JBIR-140 (both being active on cancer cell lines in the two-digit µM range *(18)*), with IC_{50} values against human SW480 colon carcinoma, Jurkat acute T cell leukemia and KB-3.1 cervix carcinoma cell lines of 0.11, 0.53 and 0.54 µM, respectively. Human SKOV-3 ovarian adenocarcinoma and U937 histiocytic lymphoma cells were somewhat less sensitive. The most promising results were obtained with the human colon carcinoma cell line HCT-116 with IC_{50} values for thioholgamides A and B of 30 and 510 nM, respectively (Table 1). In most assays, thioholgamide B displayed approximately ten-fold lower activity compared to thioholgamide A.

Besides their sub-µM activity on different cancer cell lines, thioholgamides A and B showed only moderate inhibitory activity against Gram-positive bacteria at 4-32 µg ml^{-1} and were particularly active on *Mycobacterium smegmatis* (minimum inhibitory concentration, MIC 1-2 µg ml^{-1}). The compounds displayed no activity against Gram-negative bacteria and eukaryotic microorganisms (Table 2). In contrast to their activity on human cancer cell lines, both compounds were almost equipotent in the antibacterial testing, which might hint towards a different mechanism of action in bacteria and cancer cells.

Table 1 Cytotoxic activity of thioholgamides A (1) and B (2).

	1	**2**
Cell line	**IC_{50} [µM]**	
HCT-116	0.03	0.51
Jurkat	0.53	5.28
KB-3.1	0.54	4.49
SKOV-3	2.49	20.89
SW480	0.11	12.17
U937	2.19	16.94

Table 2 Minimum inhibitory concentrations (MIC) of thioholgamides A (1) and B (2) on a panel of microorganisms.

	1	**2**
Indicator strain	**MIC [µg/ml]**	
Bacillus subtilis DSM 10	4	4
Micrococcus luteus DSM 1790	4-8	4-8
Mycobacterium smegmatis mc^2155	1-2	1-2
Staphylococcus aureus Newman	8	32
Chromobacterium violaceum DSM 30191	> 64	> 64
Escherichia coli DSM 1116	> 64	> 64
Escherichia coli (TolC-deficient)	> 64	> 64
Pseudomonas aeruginosa PA14	> 64	> 64
Candida albicans DSM 1665	> 64	> 64
Mucor hiemalis DSM 2656	> 64	> 64
Wickerhamomyces anomalus DSM 6766	> 64	> 64

In summary, the thioholgamides were described as new members of a RiPP family of thiopeptides also containing thioviridamide and JBIR-140 *(15,18)*, with multiple structural differences and enhanced cytotoxic activities. In addition to the difference in their amino acid make-up, which is the direct result of precursor peptide sequence variations, the additional structural differences are likely the result of the divergent enzymes involved in biosynthesis. Based on the constructs used for the production of thioviridamides in a heterologous host, it has been suggested that genes *tvaA* – *tvaO* are necessary for thioviridamide production. Since we observe the same types of chemical modifications in thioholgamides, we propose that genes "*A*" to "*L*" are sufficient. A list of their pairwise sequence identity on a protein level and putative functions can be found in Table S7.2. We did not find homologs of *tvaM*, *tvaN*, or *tvaO* in the genome of the thioholgamide producer. Their absence in all other gene clusters, which could be identified as potential producers of thioviridamide-like compounds, is a further indicator that they are not required for biosynthesis (Figure 1). Thioholgamide A was approximately 10-fold more active than thioholgamide B, thioviridamide, and JBIR-140 *(18)*. Further characterization of their biological potential will include a more comprehensive profiling with a larger number of cell lines of different origin. Studies towards understanding the molecular basis of thioholgamide are underway. The sequence of the proposed thioholgamide biosynthetic gene cluster has been deposited in GenBank database under accession id MF593843.

Methods

General experimental procedures as well as cultivation and isolation protocols are available in the Supplementary Information. Biological assay methods as previously described *(23)*.

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Supporting information available, which contains experimental procedures, spectroscopic and spectrometric data as well as genome mining supplementary. This material is available free of charge via the internet at http://pubs.acs.org.

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