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Zelkovamycin is an OXPHOS inhibitory member of the argyrin natural product family

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Abstract

Natural products (NPs) are an important inspirational source for developing drugs and chemical probes. In 1999, the group of Ōmura reported the constitutional elucidation of Zelkovamycin. Although largely unrecognized so far, this NP displays structural similarities as well as differences to the argyirin NP family, a class of peptidic NPs with promising anticancer activities and diverse mode-of-action at the molecular level. By a combination of structure elucidation experiments, the first total synthesis of Zelkovamycin and bioassays, we determined the configurational and revised the previously proposed molecular structure of Zelkovamycin. The full structure assignment proves Zelkovamycin as an additional member of the argyrins with however unique OXPHOS inhibitory properties. Zelkovamycin may therefore not only serve as a new starting point for chemical inhibitors of the OXPHOS system, but also guide customized argyirin NP isolation and biosynthesis studies.

Main Text

Natural products (NPs) represent an exceptional source of inspirations in drug discovery and frequently serve as valuable starting points in chemical probe synthesis.¹ To harness the full potential of NPs, a profound characterization of their chemical structure as well as inherent bioactivities is however essential.²

In 1999, the group of Ōmura reported the isolation of the NP Zelkovamycin as a moderately potent antibacterial cycloheptapeptide from a fermentation broth of *Streptomyces* sp. K96-0670 (**Fig. 1a**).³ In 2002, the argyrins, a class of currently fifteen cycloheptapeptide NPs, were discovered which besides antibiotic properties display very potent and promising anticancer activities (**Fig. 1b**).⁴ Although largely unrecognized to date, Zelkovamycin obviously displays pronounced structural similarities with the argyrin NP family, suggesting that also Zelkovamycin may harbor so far unexplored bioactivities (**Fig. 1a** and **Fig. 1b**).

In addition to the lack of knowledge of its bioactivities, the exact molecular structure of Zelkovamycin is however still elusive. The Ōmura group reported Zelkovamycin as a cycloheptapeptide built up in the given order from the amino acids glycine (Gly), 2-amino butyric acid (Abu), (*Z*)-dehydrobutyric acid ((*Z*)-Dhb), sarcosine (Sar), an alanine-thiazole amino acid (AlaThz), 7-methoxy tryptophan ((7-MeO)Trp) and 2-methyl dehydro threonine ((2-Me) Δ Thr), a so far unprecedented amino acid in a peptide NP (**Fig. 1a**); the stereochemistry of the different amino acids however was not reported. Moreover, despite the striking structural similarities between Zelkovamycin and the argyrins, there are also distinct differences: Most obviously, argyrins typically feature a 4-methoxy tryptophan ((4-MeO)Trp) or an unsubstituted Trp, but never a (7-MeO)Trp residue, as reported for Zelkovamycin.^{4a-c} Moreover, the position of the (4-MeO)Trp residue in the argyrins is occupied by the unusual (2-Me) Δ Thr moiety in Zelkovamycin, while the (7-MeO)Trp residue of Zelkovamycin is at the site of an unmodified Trp residue in the argyrins. Therefore, more detailed structural studies that enable stereochemical assignment and a confirmation of its overall structure are required.

Chemical synthesis is a well-established strategy to unambiguously elucidate the molecular structure of a NP, including cyclopeptides.⁵ So far, chemical synthesis of Zelvovamycin has not yet been achieved which contrasts with the argyirin NP family for which a few total syntheses have already been established.⁶ In order to unambiguously establish the molecular structure of Zelvovamycin and to characterize its biological activities, we describe in this study a set of structure elucidation experiments, a total synthesis and bioassays that i) resulted in a structural revision and full stereochemical assignment of Zelvovamycin, ii) characterized Zelvovamycin as a member of the argyirin NP family, and iii) revealed Zelvovamycin as an inhibitor of oxidative phosphorylation (OXPHOS).

We started our investigations by re-analyzing Zelvovamycin's overall amino acid sequence. With a small sample of isolated Zelvovamycin at hand, we performed an MS² experiment and annotated the resulting spectrum with the software mMass using either the originally reported amino acid sequence (AlaThz|(MeO)Trp|(2-Me) Δ Thr|Gly|Abu|Dhb|Sar; cyclic) or a more argyirin-like sequence where the (MeO)Trp group was placed on the position of the (2-Me) Δ Thr moiety (AlaThz|(2-Me) Δ Thr|(MeO)Trp|Gly|Abu|Dhb|Sar; cyclic).⁷ As the published sequence enabled assignment of 66% vs. 57% of the total intensity (**Supporting Fig. 1** and **Supporting File 1**) and most of the unassigned peaks were intense b₂-b₄ ions containing the motif AlaThz|(MeO)Trp and Sar|AlaThz|(MeO)Trp (Δ mass smaller than 0.001 ppm; **Supporting Table 1**), this analysis strongly supported the reported sequence connectivity (**Fig. 1c**).

Nevertheless, these experiments did not allow to differentiate between (4-MeO)Trp and (7-MeO)Trp residues due to their identical *m/z* value or to define the stereochemistry of the different amino acids. We thus performed a LC-MS-based Marfey's analysis after HCl hydrolysis of which enabled detection and stereochemical assignment of the amino acids Gly, (D)-Abu, Sar and (D)-AlaThz (**Fig. 1c** and **Supporting Fig. 2 & 3**); of note, the stereochemical assignments were thereby verified by direct comparison with corresponding chemically synthesized Marfey's modified amino acids (**Supporting Fig. 3 & 4**). The other amino acids

of Zelkovamycin however could not be detected, neither as a Marfey derivative nor in an unmodified form. Detection of the (MeO)Trp and Z-Dhb residue is hampered by their limited chemical stability during hydrolysis conditions.⁸ The failed identification of a Marfey's-modified (2-Me) Δ Thr residue however might be caused by its low reactivity due to its quaternary sp³-center at the α -position. For defining the so far missing exact constitution and configuration of the (MeO)Trp residue, we thus resorted to a milder hydrolysis protocol and treated isolated Zelkovamycin with 3 N TsOH in presence of 2% thioglycolic acid at 110 °C for 18 h.⁸ Subsequent LC-MS analysis of this hydrolysate in conjunction with a co-injection experiments with chemically synthesized (L)-(4-MeO)Trp or (L)-(7-MeO)Trp revealed the presence of a (4-MeO)Trp residue (as found in all other argyrins known so far, **Supporting Fig. 5**). A subsequent Marfey's analysis, using also a chemically synthesized Marf-(L)-(4-MeO)Trp as a control, then demonstrated that the (4-MeO)Trp moiety has the (L)-configuration (**Supporting Fig. 6**).

Altogether, these experiments thus led to a refined and partially revised proposal for the chemical structure of Zelkovamycin in which all amino acids displayed 'argyrin-like' features (constitution and stereochemistry, **Fig. 1d**). Unfortunately, the stereochemistry of the (2-Me) Δ Thr residue however remained elusive. To obtain a full assignment and proof of the chemical structure of Zelkovamycin and to provide further material for subsequent biological assays, we thus arbitrarily assigned a (L)-configuration to this amino acid, *inter alia* because the argyrins feature an (L)-amino acid on this position (**Fig 1d**). We then embarked for a total synthesis using a flexible, modular synthesis approach. Accordingly, Zelkovamycin was formally divided into three peptidic fragments (fragments A – C) with comparable structural complexity and with a C-terminal amino acid resistant to racemization during fragment coupling conditions (**Fig. 2**). As we suspected that the carbonyl moiety of the (2-Me) Δ Thr residue might cause problems during the synthesis, we decided to install it at the last stage of the synthesis *via* oxidation from the corresponding alcohol.

For synthesis of fragment A, we first generated Boc-(D)-AlaThz-OMe via the Arndt methodology (**Supporting Fig. 7**).⁹ Cleavage of the Boc protecting group with 4 M HCl in dioxane, followed by a coupling of Boc-Sar-OH using EDC/HOBt activation and another Boc deprotection step then finalized fragment A (**Fig. 3a**). Fragment B was obtained from H-(D)-Dab-OH that was Boc-protected, followed by an EDC/HOBt-mediated coupling with H-Thr-OMe (**Fig. 3b**). This dipeptide was then converted into Boc-(D)-Dab-(Z)-Dhb-OH via an EDC/Cu(I)Cl-mediated water elimination using a modified protocol from the Albericio group,¹⁰ followed by a methyl ester hydrolysis with LiOH. For the synthesis of fragment C, the generation of the (2-Me)Thr and (4-MeO)Trp building block was first required. As the retrosynthetic plan involved a late-stage oxidation of the hydroxyl residue of the (2-Me)Thr moiety, the stereochemistry at this site can be freely chosen. We therefore decided to synthesize *allo*-(L)-(2-Me)Thr-OMe due to an available synthesis protocol from the Goodman group that was slightly adapted to our needs (**Supporting Fig. 8**).¹¹ The synthesis of the required (L)-Boc-(4-MeO)Trp-OH building block was carried out according to a Pd-catalyzed heteroannulation approach from the Zhu group (**Supporting Fig. 9**).¹² With these compounds in hands, we then synthesized fragment C by first coupling Boc-(L)-(4-MeO)Trp-OH with *allo*-(L)-(2-Me)Thr-OMe (**Fig. 3c**) via pre-treatment of *allo*-(L)-H-(2-Me)Thr-OMe with trimethyl silyl chloride, followed by a treatment with HATU/HOAt. Saponification of the methyl ester with LiOH, a subsequent coupling with H-Gly-OMe and Boc-deprotection with TFA in DCM then completed the synthesis of fragment C. Assembly of the different fragments into Zelkovamycin started with fragment A (H-Sar-(D)-AlaThz-OMe) that was coupled to fragment B (Boc-(D)-Abu-(Z)-Dhb-OH) with EDC/HOBt (**Fig. 4**). The C-terminal ester of the resulting linear polypeptide was hydrolyzed and this intermediate was coupled to fragment C ((L)-H-(4-MeO)Trp-*allo*-(L)-(2-Me)Thr-Gly-OH) featuring the same coupling conditions. Cyclization was then achieved by a sequence of reactions consisting of methyl ester hydrolysis, acidic cleavage of the N-terminal Boc-protecting group and EDC/HOBt-mediated intramolecular peptide coupling under high dilution conditions. After Dess-Martin periodinane oxidation, the desired final product **1** was obtained. A comparison of the spectral data of synthesized

Zelkovamycin with those reported for isolated Zelkovamycin as well as MS² fragmentation, LC-MS co-injection and NMR co-mixture experiments with isolated Zelkovamycin revealed that our structure assignment, including the (L)-stereochemistry of the (2-Me) Δ Thr residue, was correct (**Supporting Fig. 1, 10 and 11**).³ Zelkovamycin thus has the chemical structure shown in **Fig. 1d**.

We next started to evaluate the bioactivity of Zelkovamycin in a HeLa cell culture and noted an apparent color change of the medium pH indicator (Phenol red) due to an unexpected extracellular acidification after 48 h treatment with 20 μ M Zelkovamycin (**Fig. 5a**). We could mimic these effects by an alternative addition of 2.5 μ M Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) or 1 μ M Antimycin A (AMA), both inhibitors of oxidative phosphorylation (OXPHOS) in mitochondria.¹³ As extracellular acidification may be caused by increased secretion of lactate,¹⁴ we measured the medium lactate concentrations after 48 h treatment with 20 μ M Zelkovamycin and found increased levels in HeLa and an even stronger rise in SH-SY5Y cells in a time-dependent manner (**Fig. 5b**).

These experiments thus indicate a Zelkovamycin-induced metabolic switch towards a more glycolytic phenotype. To corroborate this with an unbiased approach, we performed a full proteome analysis of HeLa cells after a 16 h treatment with 20 μ M Zelkovamycin. The comparison of the resulting label-free quantification (LFQ) profile with the corresponding DMSO treatment resulted in the identification of 2424 proteins of which 101 were significantly less and 5 more abundant after Zelkovamycin treatment (**Supporting Table 2**). Intriguingly, 60 (59%) of the less abundant proteins are annotated to be localized in mitochondria or linked to mitochondrial function (**Fig. 5c**). A gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of these proteins furthermore indicates that most of these proteins are associated with ATP biosynthesis, suggesting that Zelkovamycin affects mitochondrial function in eukaryotic cells (**Fig 5d**).^{14, 15}

All performed assays so far thus indicate that long-term Zelkovamycin treatment leads to an impairment of mitochondrial function. To deduce if Zelkovamycin however also directly

affects mitochondria, we next tested its impact on cellular OXPHOS levels that can be displayed by real-time cell measurements of the mitochondrial oxygen consumption rate (OCR, 'seahorse assay'). Zelvovamycin application to HeLa cells and the more OXPHOS-dependent SH-SY5Y and WM3734 melanoma cells resulted in an immediate concentration-dependent OCR reduction (**Fig. 6a** and **Supporting Fig. 12**).¹⁶ Zelvovamycin has a unique chemical structure which is distinct from the related argyrins by the presence of the (2-Me) Δ Thr and the position of the (4-MeO)Trp moiety in the macrocycle. We therefore wondered if the observed OXPHOS effects are Zelvovamycin-specific and depend on these unusual moieties. Accordingly, we repeated the OCR measurements with Argyrin B as an argyirin representative with a potential mitochondrial target (elongation factor G) and two additional Zelvovamycin analogues **13** ((2-Me) Δ Thr \rightarrow (2-Me)Thr) and **14** ((4-MeO)Trp \rightarrow Trp, **Fig. 6b**).^{49-h} In contrast to Zelvovamycin, none of them displayed significant OXPHOS reduction at the tested 10 μ M concentration (**Fig. 6c**) nor led to higher lactate levels in cell medium (**Supporting Fig. 13**).

We thus showed that Zelvovamycin is a direct OXPHOS inhibitor. As impairment of mitochondrial function should also lead to reduced cell viability, we tested the effect of Zelvovamycin on 293T, HepG2, HeLa Kyoto, HeLa, HCT116 cells that were only weakly affected after 48 h treatment, and SH-SY5Y neuroblastoma cells, for which cell viability was reduced by more than 50% already at 20 μ M Zelvovamycin (**Supporting Fig. 14**). This finding is in accordance with the different metabolic needs of SH-SY5Y cells that gain their cellular energy mostly by mitochondrial OXPHOS, thereby also explaining the observed higher lactate levels (**Fig. 5b**), whereas the other five cancer cells rely on cytosol-located glycolysis.¹⁷ Accordingly, Zelvovamycin treatment of HeLa cells in a glucose-deprived and thus glycolysis-restrained cell culture medium (4.5 mM instead of 25 mM glucose) resulted in a > 100-fold reduction of cell viability (from an IC₅₀ > 200 μ M in glucose-rich medium to 0.5 μ M in glucose-deprived medium, **Fig. 6d**). Of note, SH-SY5Y cells, in accordance with their stronger dependence on OXPHOS, displayed an IC₅₀ of 19.5 μ M in glucose-rich medium while they did not grow at all on glucose-deprived medium. Finally, cell viability after 72 h of the OXPHOS-

dependent melanoma cell line WM3734 was decreased by 75% at 10 μ M Zelkovamycin or 60% at 5 μ M Zelkovamycin, while no effect was observed on MeWo melanoma control cells that can metabolize glutamine as a mitochondrial-alternative energy source (**Fig. 6e**).¹⁶

Overall, our studies therefore clarified and revised the chemical structure of Zelkovamycin, demonstrating that it is a so far unrecognized member of the argyirin NP family. Moreover, we established its first chemical synthesis, thereby allowing the future chemical synthesis of customized analogues, e.g. for structure-activity relationship studies. Finally, we demonstrated that Zelkovamycin is an OXPHOS inhibitor which may offer possibilities for developing compounds with potential chemical biology or clinical use.¹⁸ We anticipate that these findings demonstrate Zelkovamycin as a promising argyirin NP for future research, e.g. of its direct molecular target or unusual biosynthesis.^{4c, 19}

Conflict of Interest

There are no conflicts to declare.

Acknowledgement

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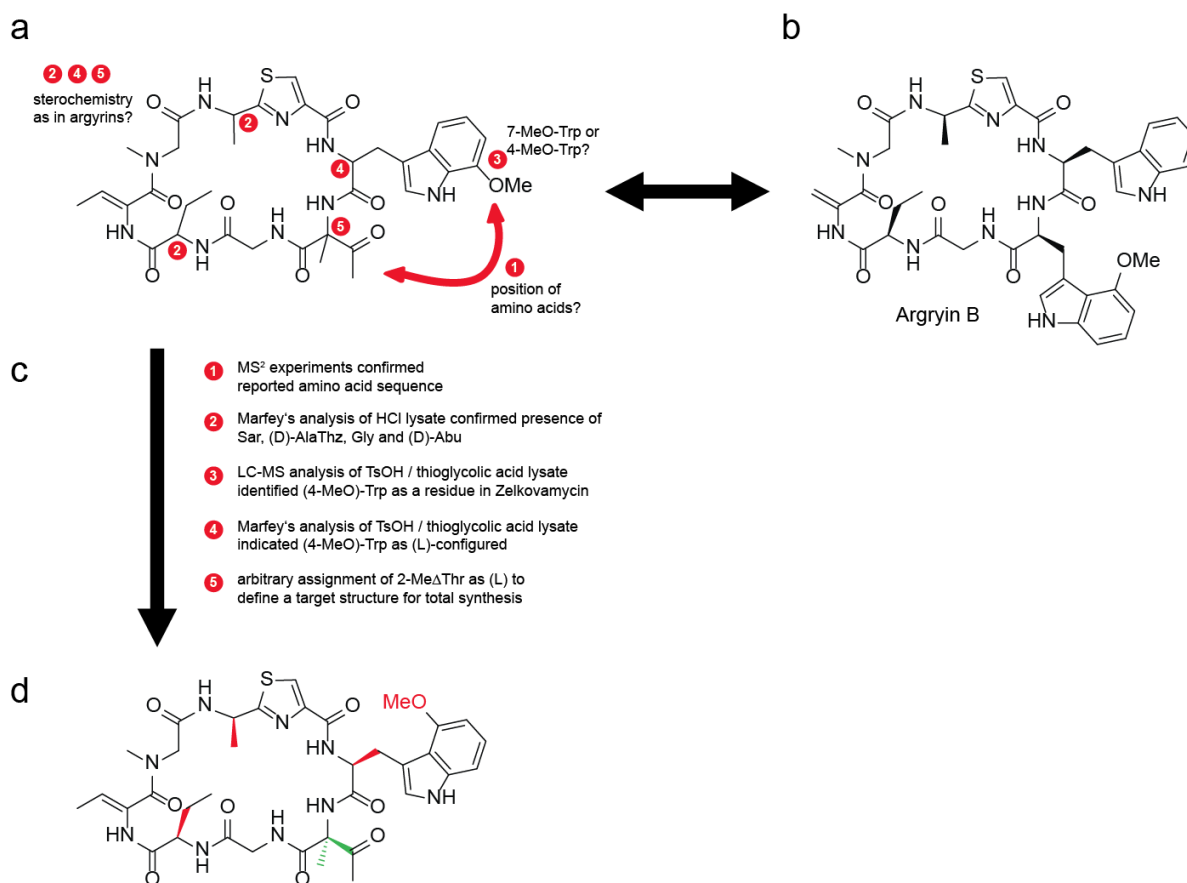


Figure 1. Structural similarities of Zelvovamycin and the argyrins and workflow for structure elucidation. **(a)** Chemical structure of Zelvovamycin as published in the original structure elucidation study³. The numbered bullet points indicate positions in the molecule where the connectivity or stereochemistry was uncertain. Comparison of this structure with Argyrin B **(b)** as a representative member of the argyirin natural product family reveal substantial structural similarities but also differences that needed to be clarified for a full assignment and confirmation of the chemical structure of Zelvovamycin.^{4a-c} **(c)** Overview of the different experiments that were carried out to define the structure and stereochemistry of Zelvovamycin. **(d)** Revised structure of Zelvovamycin (**1**) with elucidated stereochemistry as deduced from the above experiments. The configuration of the (2-Me) Δ Thr residue (green color) could however not be established and was therefore arbitrarily set to (L) to define a target structure for the subsequent total synthesis. Later chemical synthesis then confirmed that this proposed structure is the correct chemical structure of Zelvovamycin.

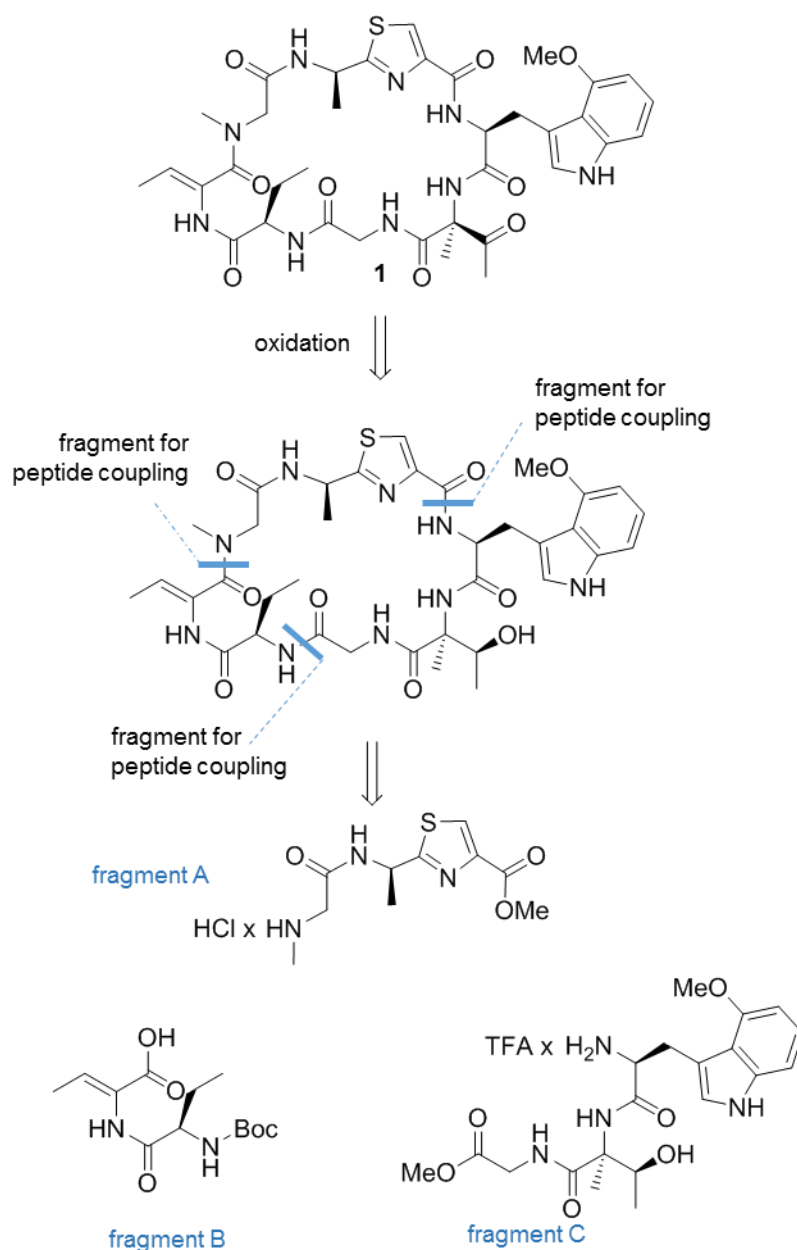


Figure 2. Overview on the envisaged synthesis route to Zelkovamycin. Zelkovamycin was retrosynthetically divided into three fragments A – C with comparable structural complexity and a C-terminal amino acid insusceptible to racemization upon activation during peptide couplings.

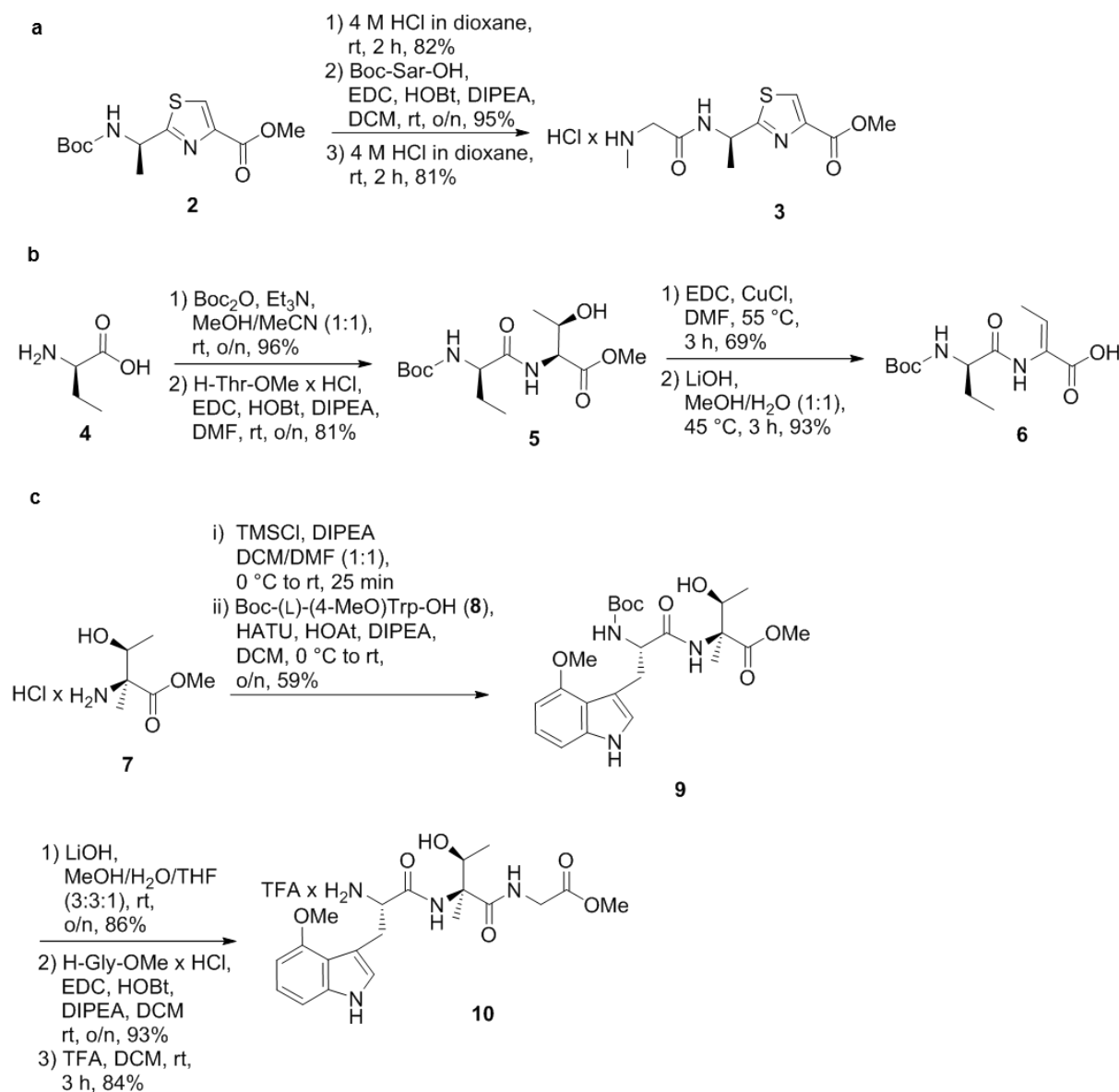


Figure 3. Synthesis of the three fragments A (a), B (b) and C (c) for assembly of the Zelkovamycin core ring system.

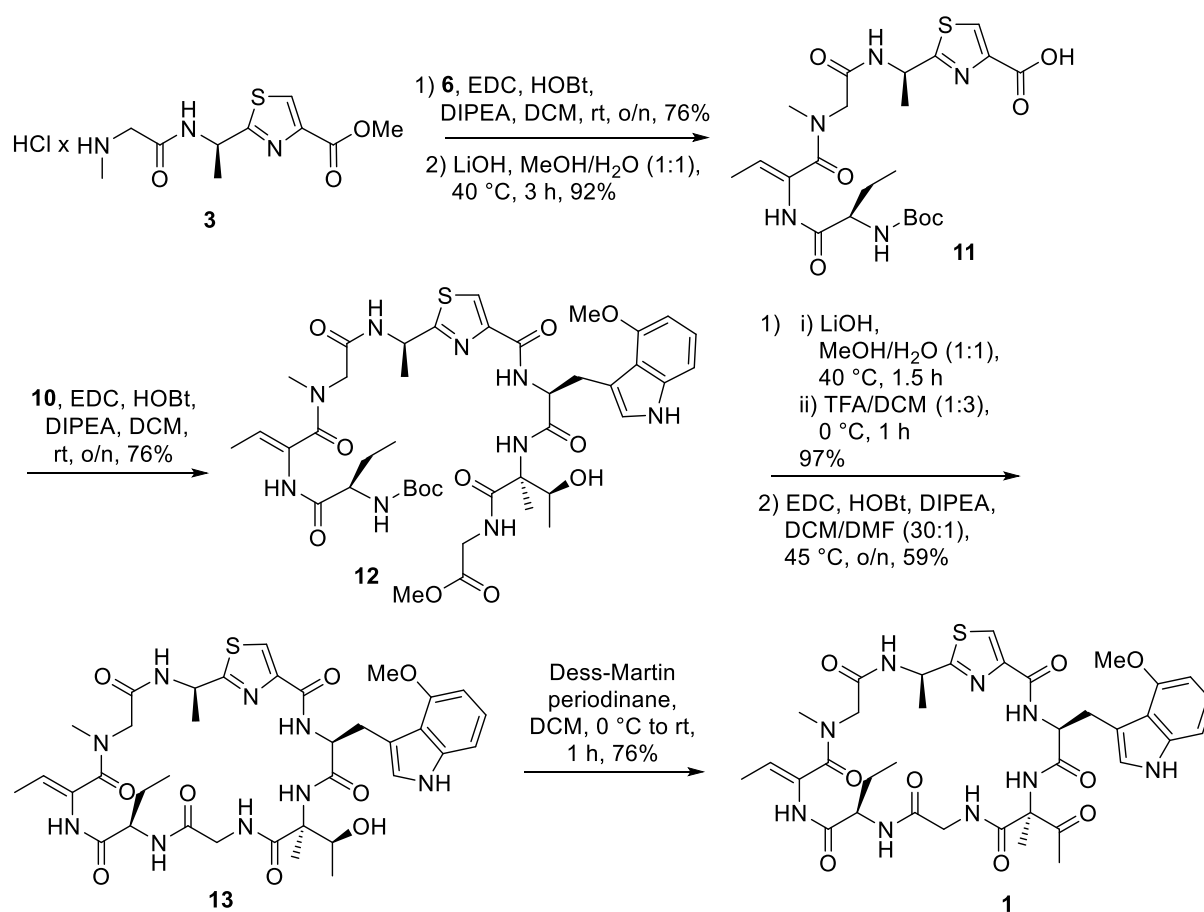


Figure 4. Assembly of the fragments to the final natural product Zelkovamycin (**1**).

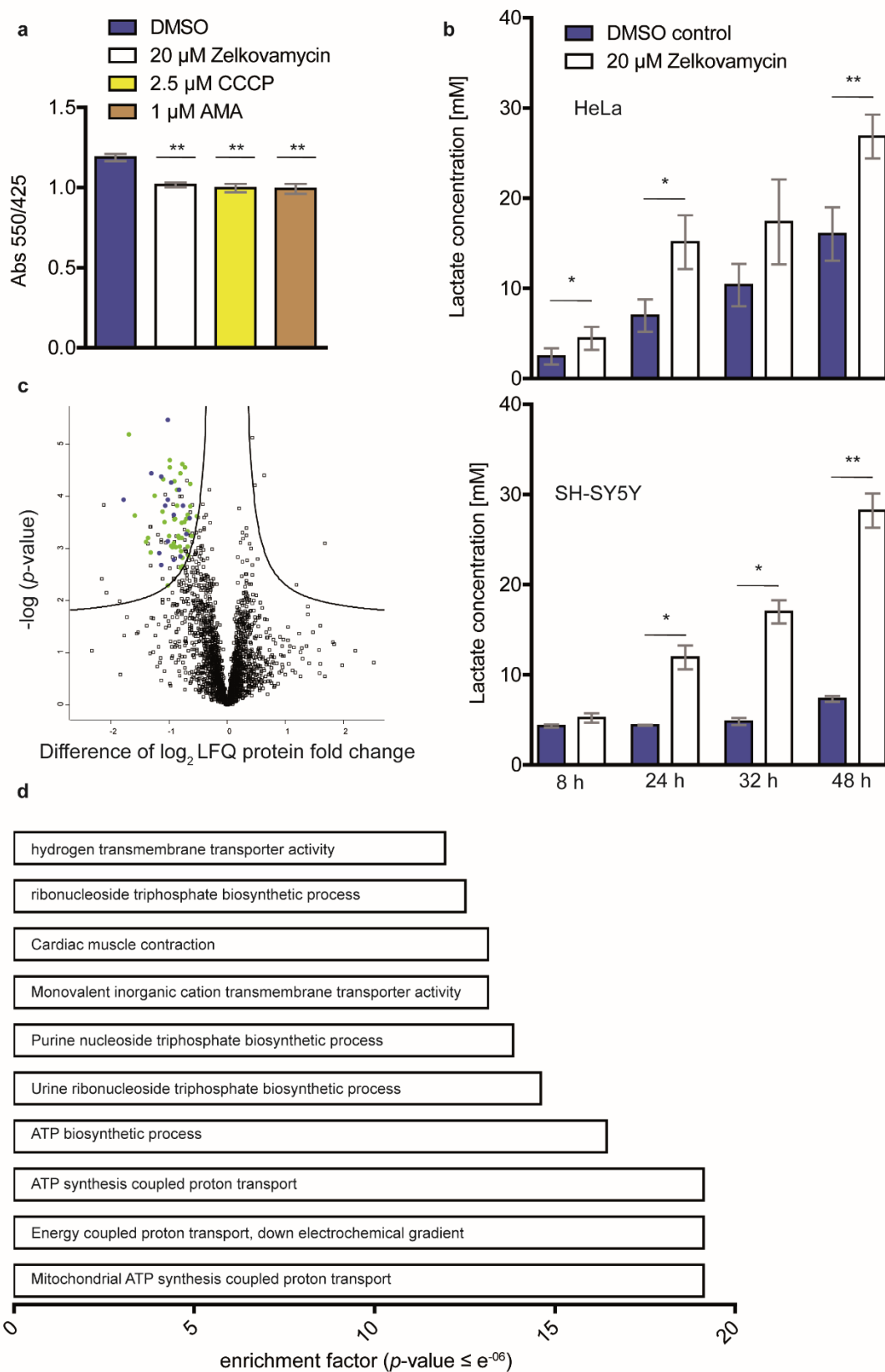


Figure 5. Zelkovamycin induces lactate reflux and lower abundances of mitochondrial proteins. (a) Zelkovamycin leads to extracellular medium acidification, which can be mimicked

by the OXPHOS inhibitors CCCP and AMA.¹³ HeLa cells were treated with indicated compounds for 48 h. Acidification was measured by absorbance (Abs) of phenol red (550 nm, 425 nm = reference). Error bars indicate standard deviation from three replicates and significance was tested by student's t-test. ** indicates a p -value < 0.01. **(b)** Zelkovamycin increases extracellular medium lactate levels in a time-dependent manner. Lactate levels of culture medium (DMEM: +10 % FCS and 1 % Penicillin/Streptomycin) were measured by an (L)-Lactate Assay Kit after cells (upper panel: HeLa, lower panel: SH-SY5Y) were grown for 48 h and an aliquot of 100 μ L was analyzed at the indicated time points. Error bars indicate standard deviation from three replicates and significance was tested by student's t-test. * and ** indicates a p -value < 0.05 or 0.01, respectively. **(c)** Zelkovamycin treatment results in lower protein abundance of mitochondrial proteins after label-free MS-based proteome analysis. HeLa cells were treated with 20 μ M Zelkovamycin for 16 h and applied to MS-based proteome analysis. The black line indicates significantly (permutation-based FDR = 0.01, s_0 = 0.1, n = 6) regulated proteins (squares). Y-axis indicates the $-\log p$ -value, while x-axis indicates the protein abundance difference in \log_2 -fold change (LFQ). Proteins on the upper left of the significance line are down-regulated, while proteins on the upper right of the significance line are up-regulated. Proteins colored in green are annotated to be localized in mitochondria or interact with mitochondrial function. Blue colored proteins are related with ATP biosynthesis. **(d)** Enrichment analysis of all significantly down-regulated proteins. For enrichment analysis, Gene Names were annotated by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms. P -value was calculated by Benjamini-Hochberg-FDR. Enrichment factor indicates increased occurrence of GO or KEGG term compared to approximate occurrence. Displayed are top ten enriched GO or KEGG terms.

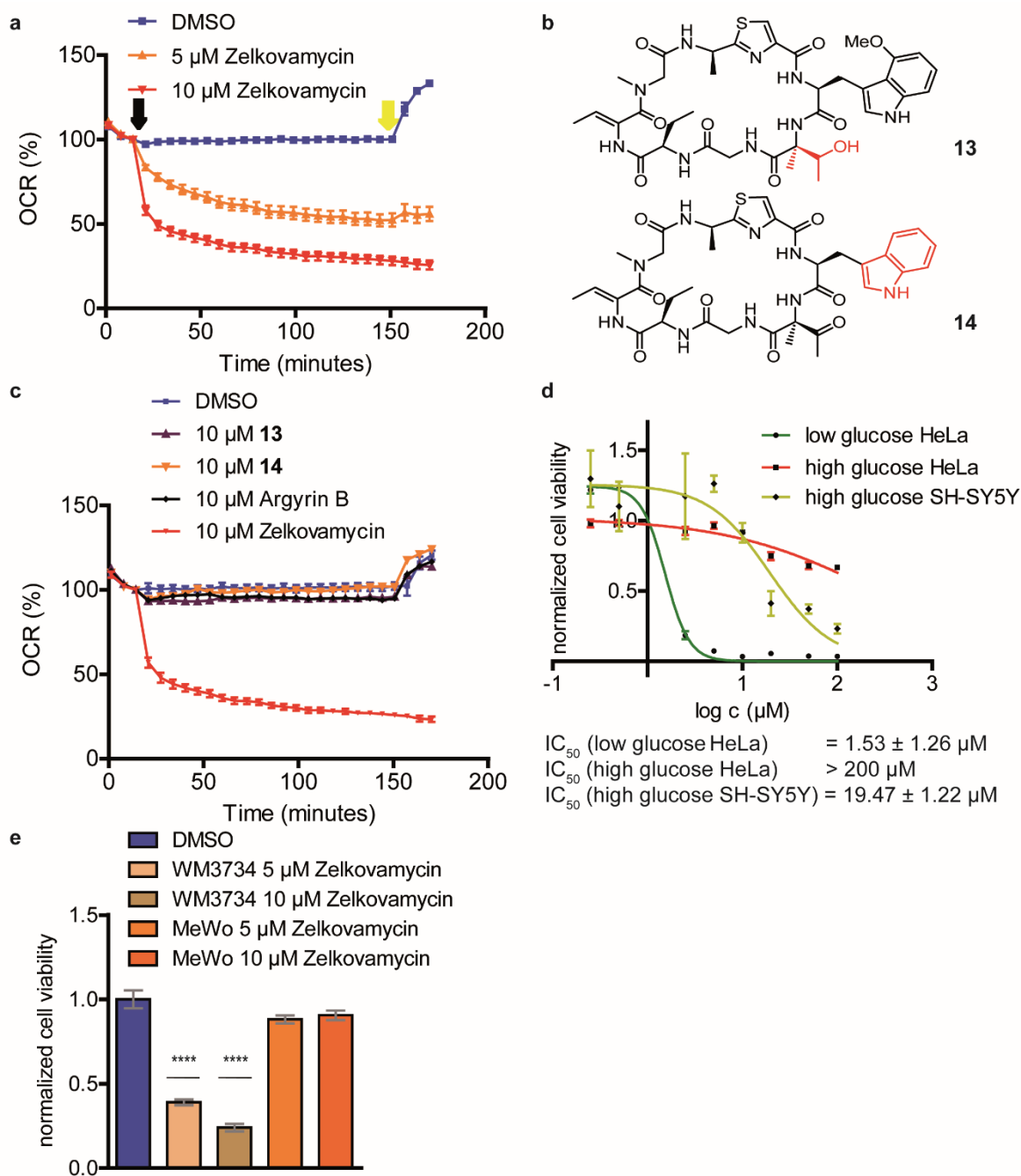
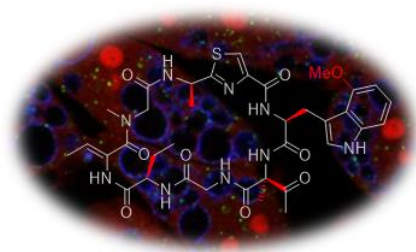


Figure 6. Zelkovamycin inhibits OXPHOS in different cell lines. **(a)** Zelkovamycin inhibits OCR in a concentration-dependent manner. OCR of HeLa cells was measured after application of Zelkovamycin (black arrow). To depict RRC, FCCP was applied (yellow arrow). Error bars indicate standard deviation from 6 - 14 replicates. **(b)** Chemical structure of two Zelkovamycin derivatives (**13** & **14**) used for structure-activity relationship studies. Red colored moieties indicate structural differences to Zelkovamycin. **(c)** Argirin B or Zelkovamycin derivatives **13** & **14** do not affect OCR. OCR of HeLa cells was measured after application of the compounds

(black arrow). To depict RRC, FCCP was applied (yellow arrow). Error bars indicate standard deviation from 6 – 14 replicates. **(d)** Cell viability of SH-SY5Y and HeLa cells at high or low glucose concentrations determined by a MTT assay in the concentration range of 0.25 - 100 μ M Zelkovamycin and normalized to DMSO control. Error bars indicate standard deviation from four replicates. **(e)** Cell viability of the OXPHOS-dependent melanoma WM3734 and non-susceptible MeWo melanoma cell line determined by a MTT assay and normalized to DMSO. Error bars indicate standard deviation from four replicates and significance was tested by student's t-test. **** indicates a p -value < 0.0001.

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Zelkovamycin is an argyrian NP. By a combination of structural elucidation methodologies, total synthesis and bioassays, the exact molecular structure of Zelkovamycin was assigned and revised and its OXHPOS inhibitory properties revealed.