Bioorganic & Medicinal Chemistry Letters xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

An efficient synthetic route for preparation of antimycobacterial wollamides and evaluation of their *in vitro* and *in vivo* efficacy

Henok Asfaw[a], Thomas Wetzlar[a], Maria Santos Martinez-Martinez[b], Peter Imming[a,*]

[a] Institut für Pharmazie, Martin-Luther-Universität Halle-Wittenberg, 06120 Halle, Germany
[b] Diseases of the Developing World, Medicines Development Campus, GlaxoSmithKline, 28760 Tres Cantos, Madrid, Spain

ARTICLE INFO

Keywords:
Wollamides
Cyclic peptides
Mycobacteria
SPPS
ADME

ABSTRACT

A convenient solid phase peptide synthetic (SPPS) route is reported for the preparation of antimycobacterial wollamides. The method is based on on-resin head-to-tail cyclization and is fast, efficient and amenable to automation. The *in vitro* antimycobacterial activities of the newly synthesized wollamides were evaluated against *M. tuberculosis* H37Rv (Mtb H37Rv). To assess their drug-likeness, *in vitro* pharmacokinetic (ADME) profiling was also performed. For wollamides with potent extracellular potency, intracellular activities and *in vivo* efficacy were determined. The results disclose the potent antimycobacterial ($MIC_{Mtb\ H37Rv}$ = 1.1 μM) and suitable drug-like properties of wollamide A (**4b**). Out of the synthesized wollamides, four compounds (**4b–e**) exhibited potent intracellular activities against *Mtb* H37Rv infected human macrophages (IC_{50} = 0.2–1.3 μM). Results of *in vivo* blood exposure and efficacy assays for **4d** and **4e** are discussed.

The rapid emergence and spread of multidrug resistant (MDR) mycobacterial strains is worsening the incumbent threat posed by tuberculosis (TB).[1] To tackle the problem, there is a need to develop new anti-TB drugs with novel mechanisms of action that are less vulnerable to the classical resistance mechanisms that microbes have already acquired. In this regard cyclic peptides, with a molecular size in the "middle space" (molecular mass in the range of 500–2000), offer great potential as they are ideally suited for targeting new drug targets that are non-druggable with traditional small molecules, such as protein-protein interaction.[2–5] The cyclic hexapeptide wollamides represent one such class of compounds with great potential to be the next anti-TB agents. Wollamide A and B were isolated from Australian soil derived *Streptomyces* spp. and reported to exert antimycobacterial activity against *M. bovis* (BCG) with an IC_{50} of 2.8 and 3.1 μM respectively. In addition, wollamide B exerted intracellular activity against BCG infected murine macrophages.[6]

Recently we reported for the first time the anti-TB potential of wollamide B and its analogues by synthesizing and testing their activities against *M. tuberculosis* (Mtb).[7] Wollamide B and analogues demonstrated very good extracellular antimycobacterial potency, safety and some important drug-like attributes such as water solubility, plasma and microsomal stability. The major feature of the class that needed improvement was their poor membrane permeability. Initial attempts to improve membrane permeability through introduction of more lipophilic amino acids in different parts of wollamide B have caused reduced microsomal stability or potency. Hence, to effectively transform the class into successful anti-TB lead candidates, more work remained to be done to design and synthesize analogues with the right balance of potency, membrane permeability and microsomal stability. This in turn necessitated development of suitable synthetic routes.

In the present work we report a fast, more efficient and simple synthetic route for the preparation of wollamides. The *in vitro* antimycobacterial activities and pharmacokinetics of the newly synthesized wollamides will be presented. Moreover, the intracellular and *in vivo* antimycobacterial activities of the most active wollamides will be discussed.

The synthetic route previously developed for wollamides involved three steps: (1) on-resin construction of the linear hexapeptide

Abbreviations: AUC, area under curve; DNAUC, dose normalized area under curve; 2-CT, 2-chlorotrtityl chloride; CFU, colony forming unit; DCM, dichloromethane; DIPEA, *N,N*-diisopropylethylamine; DMF, dimethylformamide; ADME, drug metabolism and pharmacokinetics; DMSO, dimethyl sulfoxide; Fmoc-, fluorenylmethyloxycarbonyl; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; HFIP, hexafluoro-2-propanol; HOBt, hydroxybenzotriazole; IC_{50}, half maximal inhibitory concentration; MIC, minimum inhibitory concentration; Mtb, *Mycobacterium tuberculosis*; NMP, *N*-methyl-2-pyrrolidone; Pbf-, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium-hexafluorophosphat; SPPS, solid phase peptide synthesis:; *t*-Bu, *tert*-butyl; TFA, trifluoroacetic acid; TIPS, triisopropylsilane

* Corresponding author.
E-mail address: peter.imming@pharmazie.uni-halle.de (P. Imming).

https://doi.org/10.1016/j.bmcl.2018.07.021
Received 16 April 2018; Received in revised form 18 June 2018; Accepted 11 July 2018

precursors using Fmoc based solid phase peptide synthesis strategy, (2) cyclization of the hexapeptide precursors in solution phase under highly diluted environment and (3) removal of the reactive amino acid side chain protecting groups.[7,8] Though this method was relatively simple and workable, it had some drawbacks. The first limitation was the slow and discontinuous cyclization step in solution phase, which not only makes automation difficult but also increased the synthesis cost as it required large volumes of solvent for cyclization. The other drawback was the need to purify each intermediate cyclic peptide precursor which consumed both time and materials.

The new synthetic route was designed to deal with the above limitations. The general principle involved solid phase assembly of protected linear peptidyl-resin precursors by anchoring a trifunctionalized amino acid (Fmoc-Asp-OAllyl) *via* its side chain. Following this, the carboxy and amino protecting group of the peptidyl-resin precursors were removed and subjected to on-resin head-to-tail cyclization.[9] The final cyclic peptides and the side chain protecting groups were then released from the resins using the appropriate cleaving conditions.[10,11]

Preparations of the linear peptidyl-resins were initiated through attachment of Fmoc-Asp-OAllyl, *via* its side chain, to 2-chlorotrityl chloride (2-CT) or TantaGel (TAG S RAM) resins (Scheme 1). Loading of the amino acid to 2-CT resin (loading capacity = 1.6 mmol/g) involved direct esterification of Fmoc-Asp-OAllyl to the resin with DIPEA (1.8 eq) in DCM. The efficiency of the loading was calculated to be 90% from the increase in the mass of the resin after loading.

To load Fmoc-Asp-OAllyl on the TantaGel resin (loading capacity = 0.24 mmol/g), the Fmoc protecting group of the resin-linker was first removed with 20% piperidine in DMF, and the amino acid was coupled using HATU/HOBt/DIPEA (3:3:6 eq) in NMP.

For subsequent elongation of the peptides (**1a–e**), a standard Fmoc SPPS strategy that involved HATU/DIPEA/NMP coupling was used.[12] Finally, prior to the removal of the last Fmoc group, the allyl protecting group was selectively deprotected using $Pd(Ph_3P)_4$ (0.1 eq) and the non-basic borane dimethylamine complex (4 eq) as scavenger[13,14] (Scheme 2).

To assess identity and purity, the synthesized linear hexapeptides (**2a–e**) were cleaved from a small portion of the resin and analyzed using analytical RP HPLC and ESI-MS. While the ESI-MS spectra showed the formation of the expected product, RP HPLC analysis confirmed that the peptides had purity level of more than 98% (Supplementary file).

Once the linear hexapeptidyl-resin precursors were constructed, the next step was on-resin cyclization (**3a–e**). On both types of resins, the cyclizations were successfully performed using either HATU/HOBt/DIPEA (3:3:6 eq) or PyBOP/HOBt/DIPEA (3:3:6 eq) in NMP.

To optimize the best conditions, cyclizations were performed by changing different variables such as time of cyclization, type of the cyclizing/coupling agent and volume of the solvent used for cyclization.

The first optimization was done to find the best cyclization time. This was done through cyclizing the linear peptidyl-resin (**2a**) for 1 h, 3 h, 5 h and 18 h using HATU/HOBt/DIPEA in NMP. The percent conversion of the starting material to the desired cyclic peptide was then assessed by analytical RP HPLC. The result of this assessment (Table 1) showed 63% conversion after 1 h of cyclization. There was no significant change in the percent of conversion when the cyclization continued for the next 5 h (67%). Complete conversion occurred only after 18 h which could be related to the length of incubation time of the resin with the solvent (Supplementary file). The more the resin stays in the solvent, the better it swells and hence allows penetration of the cyclizing agent deep in the resin matrix. This provides the peptide terminal groups to come in contact with the cyclizing agent.[15]

Similarly, to check if the time of cyclization changed with the type of resin used, cyclization of the linear peptidyl-resin (**2b**) was done for 5 h and 18 h using the same conditions as described above. ESI-MS analysis after cleavage of the peptides showed full conversion at both time points. This could be due to the nature of TantaGel resin (and the linker) where unlike 2-CT, the attached peptidyl chain projected into the solution rather than being anchored in the proximity of the polymer backbone.[16–18] This apparently gave easy access of cyclizing reagents to the cyclizing ends.

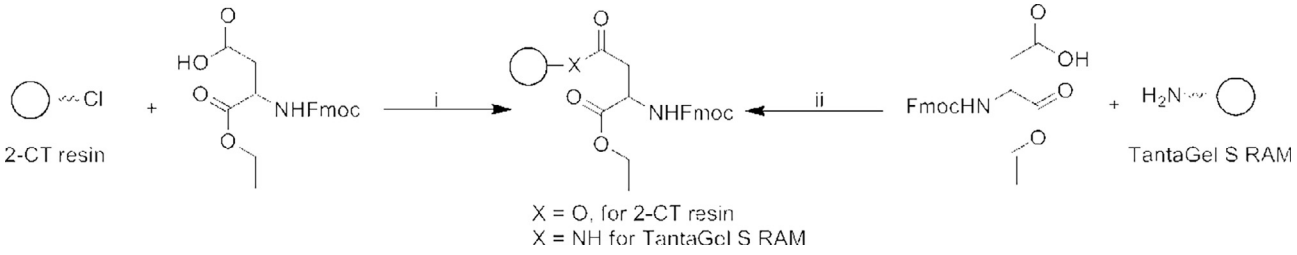
To assess the impact of changing the type of cyclizing agent on the time of cyclization, PyBOP was used instead of HATU when cyclizing the peptidyl-resin **2a**. Cyclization with PyBOP/HOBt/DIPEA was done at two different time points (5 and 18 h). The extent of cyclization after 5 h (69% conversion) was nearly similar with what was achieved by HATU/HOBT/DIPEA (67% conversion). However, unlike the other method, cyclization could not be completed after 18 h (82% conversion only).

The ESI-MS spectra of the cyclic peptide that was synthesized on 2-CT resin (**4a**) showed formation of a dimeric side product. The extent of dimerization was found to be 8% when calculated from the isolated dimer during column purification. This was also corroborated by the HPLC purity assay where the cyclized peptide showed around 95% level of purity.

Macrocyclization under high dilution is a common technique that is used to reduce the formation of oligomers.[5,19,20] Accordingly, hoping to minimize the dimerized side product, cyclization was performed at different levels of dilution (0.1 mM and 1.0 mM) by suspending the peptidyl-resin bead in different volumes of the cyclizing solvent (Table 1). However, as confirmed by HPLC analysis, this did not alter the extent of dimerization. In such circumstances, the extent of dimerization is assumed to be related to the loading capacity of the resin bead rather than the concentration of the peptide loaded bead in the solvent. The more the loading capacity of the resin type, the higher the number of the peptides per bead and hence more chance of dimerization.[21] This was also proved by the total absence of dimerized product when cyclization was done on the TantaGel resin (**4b**) which has very low loading capacity (0.24 mmol/g) compared to the 2-CT resin (1.6 mmol/g).[5]

Cleavage of the formed cyclic peptides from the resins was achieved by using either 20% TFIP/DCM for peptide assembled on 2-CT resin (**4a**) or TFA/TIPS/H_2O (95:2.5:2.5) for peptides synthesized on TantaGel resin (**4b—e**). While the cleavage of the peptides from the 2-CT resin was orthogonal to the side chain protecting groups, it caused simultaneous removal of the side chain protecting groups when done from the TantaGel resin (Scheme 3). After cleavage from the resin, purification of the final cyclic peptides was done either by column chromatography or a cation exchange cartridge.

Syntheses of the cyclic hexapeptides on TantaGel resin were



Scheme 1. Loading of 2-chlorotrityl chloride and TantaGel S RAM resins. **Reagent & conditions**: i) DIPEA, DCM, 2 h, rt; ii) HATU, HOBt, DIPEA, NMP, 1 h, rt.