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ORIGINAL ARTICLE

# Design and synthesis of Acyldepsipeptide-1 analogues: Antibacterial activity and cytotoxicity screening

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**Abstract** Acyldepsipeptides (ADEPs) are receiving more attention as prospective antimicrobial agents due to their unique mode of action and chemical properties. However, their therapeutic potential is limited by their poor pharmacokinetic properties. Chemical modifications have been successful in improving the biocompatibility and bioavailability of ADEPs. In the current study, ADEP1 was modified by introducing a disulphide linkage, replacement of the octa-2,4,6-trienoic acid (OTEA) with either adamantane (Ada) or palmitic acid (Pal), and lastly, comparing the use of D versus L amino acids. The antibacterial effects of the ADEP1 analogues were investigated in Gram-positive and Gram-negative strains using agar well diffusion and microdilution assays. Cytotoxicity was evaluated in human embryonic kidney (HEK)-293 and colon cancer (Caco-2) cells by the MTS assay. Using solid phase peptide synthesis (SPPS), the percentage yield of the synthetic peptides was increased to > 37% with > 96% purity. The anionic ADEP1 analogues demonstrated

*Abbreviations*: ADEP, Acyldepsipeptide; AMP, Antimicrobial peptide; ClpP, Caseinolytic protease; MIC, Minimum inhibitory concentration; MBC, Minimum bactericidal concentration; SPPS, Solid phase peptide synthesis; OTEA, Octa-2,4,6-trienoic acid; Ada, Adamantane; Pal, Palmitate

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a broad-spectrum antibacterial activity. Although the ADEP1 analogues did not display the expected antibacterial activity in relation to the parent structure, they were not cytotoxic against the tested cell lines. This study proved that biocompatibility of natural ADEPs can be improved by modifying some of its chemical groups. Further studies are required to enhance the antibacterial activity of the ADEP1 analogues either by structural modifications or through supplementation of these anionic antimicrobial peptides (AMPs) with divalent metal ions.



## 1. Introduction

The rapid increase in antimicrobial resistance remains a major concern in public health, which warrants the development of new and effective antimicrobial agents. Acyldepsipeptides (ADEPs) have gained attention as potential antimicrobial agents as they have a different mode of action to current drugs and might be able to overcome drug resistance (Cobongela et al., 2022). ADEPs are cyclic antimicrobial peptides (AMPs) produced by *Streptomyces hawaiiensis* (Michel & Kastner, 1985) as part of their innate immune response against resident bacteria, and were reported to be mostly active against Gram-positive bacterial strains (Culp & Wright, 2017). ADEPs were reported to bind and deregulate the bacterial caseinolytic protease (ClpP) (Brötz-Oesterhelt et al., 2005), a cytosolic protease responsible for homeostatic degradation of misfolded and aggregated proteins and peptides (Chandu & Nandi, 2004). Under controlled homeostatic conditions, ClpP is regulated by AAA + chaperone-like ATPases, which include the ClpC1, ClpX, and ClpA. These Clp-ATPases specifically bind to the hydrophobic pockets of the ClpP, resulting in an active proteolytic conformation (Brötz-Oesterhelt et al., 2005). ADEPs act by mimicking the binding of the Clp-ATPases to ClpP, and this inhibits the binding of Clp-ATPases while inducing uncontrollable proteolysis (Kim et al., 2001; Lee et al., 2010; Li et al., 2010). The increased proteolytic activity of ClpP rapidly degrades both essential and nonessential bacterial proteins leading to bacterial cell death (Cobongela et al., 2022).

There is a number of natural and synthetic ADEP analogues, which have been reported in literature. The first reported ADEP, ADEP1 also called ADEP Factor A (A54556A), was isolated from cultured *Streptomyces hawaiiensis*. The antibacterial activity of ADEP1 against Gram-positive bacteria (Brötz-Oesterhelt et al., 2005; Goodreid et al., 2016), Gram-negative bacteria (Goodreid et al., 2014), as well as its bactericidal action against a number of antibiotic-resistant strains (Goodreid et al., 2016) made this compound an attractive component in antibiotic research. As a result, several synthetic derivatives of the peptide have been developed over the past few years. However, a major drawback associated with this class of novel antibiotics, and other therapeutic AMPs, are their unfavorable pharmacokinetic properties and cytotoxic traits (Brunetti et al., 2016; Lewis & Richard, 2015). This therefore raises a need to improve these natural peptides to drug-like molecules with minimal to no cytotoxicity, high selectivity, good solubility, increased proteolytic stability, etc. This in turn should increase the bioavailability of these peptides and consequently improve their therapeutic activity.

Several strategies, such as chemical modification and use of drug carriers, have been successful in increasing the bioavailability and efficacy of AMPs in the past (Fadaka et al., 2021). A number of synthetic AMP analogues have been modified to produce derivatives with improved physicochemical properties compared to their natural counterparts. A change even in a single amino acid in the peptide sequence can have significant effects on the AMP's behavior within the biological environment as well as in their bioactivity (Zai et al, 2021; Zhou et al, 2020). In the case of Temporin-PF, deletion or substitution of amino acids resulted in analogues with reduced hemolytic activity, but lesser or no antibacterial activity (Zai et al, 2021). The use of cationic and hydrophobic amino acids in Dermaseptin-PS3 (Tan et al., 2018) and AcrAP1 (Ma et al., 2019) improved their interaction with the negatively charged cellular membrane, resulting in higher potency. Some of these factors were taken into consideration when derivatizing the ADEP1 in this study. The modifications done on ADEP1 include the incorporation of D amino acids, cyclization via disulphide bond between two cysteine side chains, and ADEP1 tail replacement with lipophilic molecules. Therefore, the study was aimed to design and synthesize ADEP1 analogues and investigate their antibacterial activity and cytotoxicity effects.

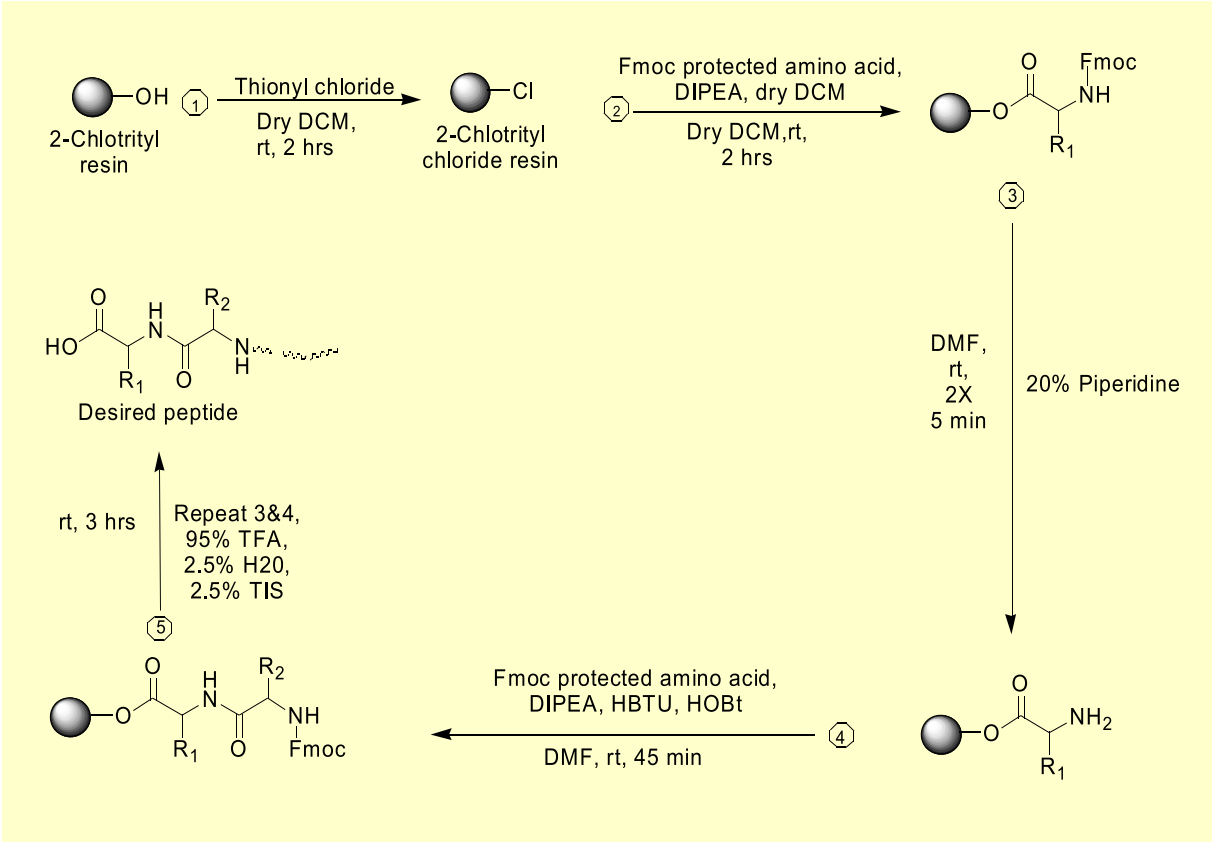
## 2. Materials and methods

### 2.1. Materials

2-Chlorotrityl chloride resin, N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU), Fluorenylmethyloxycarbonyl chloride (Fmoc) amino acids, Triisopropylsilane (TIS), and Hydroxybenzotriazole (HOBt) were purchased from GL Biochem Ltd., Shanghai, China. N, N'−Dimethylformamide (DMF) Dichloromethane (DCM), Diisopropylethylamine (DIPEA), Acetonitrile, and Dimethyl sulfoxide (DMSO) were purchased from Radchem (PTY) LTD., Alberton, South Africa. Palmitic acid (Pal), 1-Adamantanecaboxylic acid (Ada), Trifluoroacetic acid (TFA), Thionyl chloride, Formic acid, Iodine, Dulbecco's modified Eagle Medium (DMEM), phosphate, Trypsin, Penicillin-Streptomycin, Fetal bovine serum (FBS), and trypan blue stain dye were purchased from Sigma Aldrich (St Louis, MO, USA). Human embryonic kidney (HEK)-293 and Colorectal adenocarcinoma (Caco-2) were purchased from Cellonex (Randburg, Gauteng Province, South Africa).

### 2.2. Solid phase synthesis of ADEP1 analogues

The ADEP1 analogues were synthesized *via* a modified solid phase peptide synthesis (SPPS) using Fmoc-amino acids, following a previously reported method as depicted in Scheme 1 (Merrifield, 1963). The 2-chlorotrityl chloride resin was used as a solid structure and activated for 2 h by 20% thionyl chloride in distilled (dry) DCM solution. The first amino acids in all ADEP1 analogues were loaded at four equivalence to the activated resin with five equivalence of the DIPEA. The concentration of reagents used during coupling cycles of the peptides were 0.5 M DIPEA, 0.19 M HBTU, 0.095 M HOBt and 0.2 M amino acids. Each amino acid was double-coupled while Pal or Ada were triple-coupled. The peptide sequence, stereochemistry and net charge are summarized in Table 1.




**Scheme 1** Schematic representation of a solid phase peptide synthesis of ADEP1 analogues.

**Table 1** Peptide sequences, stereochemistry, and net charge.

| Peptide code | Peptide sequence | Stereochemistry | Net charge (pH 7.0) |
|---|---|---|---|
| ADEP1 | OTEA-FSA(m)AA(m)P | L | 0 |
| SC005 | Pal-FCPAAPC | D | −1 |
| SC006 | Ada-FCPAAPC | D | −1 |
| SC007 | Ada-FCPAAPC | L | −1 |
| SC008 | Pal-FCPAAPC | L | −1 |

Note: m denotes methyl.

#### 2.2.1. Loading capacity and yield of ADEP1 analogues

The yield of the peptides was determined by adding duplicate samples of 5 to 10 mg into 1 mL of 20% piperidine in DMF. The reaction was left to shake for 20 min at room temperature, and then centrifuged at 6000 rpm for 10 min. This concentration of piperidine cleaves off the Fmoc from the amino acids. Piperidine and Fmoc forms a dibenzofulvene-piperidine byproduct that is measurable at 301 nm. The supernatant was diluted 1:100 in DMF and read at 301 nm using Ultraviolet visual (UV-viz) spectrophotometer (SpectraMax Plus 384 Microplate Reader, Molecular Devices, California, United States). The loading capacity, theoretical yield, expected yield and percentage yield were calculated using these equations (EQ):

$$L = V(A)/\varepsilon(w) \tag{1}$$

$$Y_t = L\ x\ m\ x\ MM \tag{2}$$

$$Y_e = 0.97^{mer}\ x\ Y_t \tag{3}$$

$$Y_e = 0.97^{mer}\ x\ Y_t\ Yield\ = m_p/\ Y_e$$

Where L is loading capacity, V is final volume, A is absorbance at 301 nm, $\varepsilon$ is extinction coefficient of the dibenzofulvene-piperidine, w is mass of the loaded resin in mg, $Y_t$ is theoretical yield, m is mass of the total resin, MM is molecular weight of the peptide, $Y_e$ is the expected mass, $m_p$ is mass of the peptide.

### 2.3. Cyclization of ADEP1 analogues

#### 2.3.1. Iodine-mediated oxidation

Cyclization of the Ada-containing peptides (SC006 and SC007) was performed as previously described using 10 equivalence iodine in DMF: water in a 4:1 ratio (Pohl et al., 1993). The reaction was carried out on resin bound peptides, left shaking at room temperature for 40 min and the reaction was stopped by adding 1 M L-ascorbic acid. The resin was washed thoroughly by 1 M L-ascorbic acid followed by DMF and then DCM. The peptides were then cleaved from the resin using a cleavage solution containing 95% TFA, 2.5% $H_2O$, and 2.5% TIS. The solution was filtered and solvents were removed using rotary evaporator (Rotavapor R-210, Buchi Laboratory Equipment, St Gallen, Switzerland)

set at 60-65 ℃. Toluene:TFA solution at a 10:1 ratio was added during the evaporation process to aid the removal of TFA, which usually binds on the free carboxylic end of the peptides. A complete removal of the solvents left an oily and golden brown residual on the round bottom flask which was dissolved in acetonitrile containing 5% dimethyl sulfoxide (DMSO) for further analysis.

#### 2.3.2. DMSO-mediated oxidation

The Pal-containing peptides (SC005 and SC008) were cleaved using the TFA cleavage solution described above, and subsequently cyclized using DMSO. Briefly, SC005 and SC008 cyclization was carried out in acetonitrile containing 20% DMSO. The reaction was left shaking overnight at room temperature. The solvents were removed at 60-65 ℃ using rotary evaporator. The DMSO residues were removed by a lyopholyzer (LyoQuest, Telstar, Terrassa, Barcelona). The peptides were also re-suspended in acetonitrile containing 5% DMSO for further analysis.

### 2.4. ADEP1 analogues purification by HPLC

The peptides were purified using Preparative High-pressure liquid chromatography (prep-HPLC) (Shimadzu, Kyoto, Japan) with a prep-HPLC c-18 column with 30 × 250 mm, 10 µm dimensions (Phenomenex, California, United States) with UV detection at 215 and 245 nm. The purification method was a 20 min running time with 20 mL/min flow rate. The gradient elution was 5–95% B (0.1% FA in acetonitrile) in A (0.1% FA in water) for 12 min and 95% B in A from 12 to 20 min. Molecular weight of the peptides were confirmed using Thermo Scientific Ultimate 3000 (Bruker, Massachusetts, United States). The HR-LCMS also was also performed on a c-18 column (5 µm, 100 Å, 4.60 mm × 150 mm) with a two-buffer solvent system, solvent A and B at a flow rate of 0.3 mL/min. The expected peptide molecular weight confirmed by HR-LCMS eluted between 13 and 14 min on the prep-HPLC. The corresponding prep-HPLC peaks were therefore collected and solvent removed using rotary evaporator at 60-65 ℃. The residuals were lyophilized using a freeze dryer for further biochemical analysis.

### 2.5. Antibacterial activity tests of the ADEP1 analogues

The antibacterial activity of the ADEP1 analogues was tested against three Gram-positive (*Bacillus subtilis* ATCC 11774, *Staphylococcus aureus* ATCC 25923 and Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300) and two Gram-negative (*Escherichia coli* ATCC 33876 and *Pseudomonas aeruginosa* ATCC 15442) bacterial strains using agar well diffusion and microdilution assays according to previously reported protocols (Adeyemi et al., 2018). The bacterial strains were purchased from the American Type Culture Collection (Manassas, Virginia, USA).

#### 2.5.1. Agar well diffusion method

Antibacterial activity of the ADEP1 analogues were first screened using an agar well diffusion assay, a modified Kirby-Bauer disc diffusion method (Tan & Ng, 2006). The bacterial strains were cultured at 37 °C (250 rpm) for overnight, and bacterial cultures (200 µL) were then spread on LB agar plates followed by addition of 100 µL of the ADEP1 analogues at 100, 500, and 1000 µM (ADEP1 analogues stock solutions were prepared in 5% DMSO in PBS) in 8 mm agar wells. Ampicillin (11.5 mM) was used as a positive control against *E. coli*, while gentamicin (0.84 mM) was used as positive control against *B. subtilis*, *S. aureus*, MRSA and *P. aeruginosa*. Negative controls contained 2% DMSO (without the antibiotics/test compounds) in LB broth. The plates were then incubated overnight at 37 °C and the diameter of the zones of inhibition were measured using vernier caliper.

#### 2.5.2. Minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values of the ADEP1 analogues against the selected bacterial strains were determined using the microdilution assay. Briefly, the tested strains were cultured at 37 °C overnight and the concentrations were adjusted to $\sim 1.5 \times 10^8$ CFU/mL prior to use. Two-fold serial dilutions of the peptides, ranging from 2000 to 15,625 µM, were added to the wells at 100 µL followed by 100 µL of the diluted bacterial cultures, which yielded the following final peptide concentrations: 1000, 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 µM 3.9. The peptides were tested alongside ampicillin and gentamycin as positive controls. The plates were incubated overnight at 37 °C. The MIC values were recorded as the lowest concentration of peptides at which there was no visible bacterial growth. Three independent experiments were performed for each bacterial strain.

For MBC, 1 µL of the samples from the MIC assay were transferred to fresh LB agar plates and incubated overnight at 37 °C. MBC was represented as the lowest concentration at which there was no observed bacterial growth. The test was performed in triplicates for each ADEP1 analogue.

### 2.6. Cell viability studies

The cytotoxicity of the ADEP1 analogues was investigated on HEK-293 and Caco-2 cell lines using the MTS cell proliferation assay. The cells were purchased from Cellonex (South Africa) and cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C in a 5% $CO_2$ humidified incubator. For the cell viability assay, the cells were seeded at a density of $1x10^5$ cells/mL in 96 well plates (100 µL/well). The plates were incubated at 37 °C overnight, after which the ADEP1 analogues at 15.6 µM to 2 mM were added. The plates were incubated at 37 °C for an additional 48 h. Auranofin (0.78125 - 100 µM) was used as a positive control and 2% DMSO was used as a negative control. The MTS dye reagent (10%) was added to the wells and incubated for 4 h. The absorbance was read at 490 nm wavelength using SpectraMax Plus 384 Microplate Reader. The percentage cell viability was calculated using the equation below.

$$\%\ Cell\ viability = \left(\frac{OD\ of\ test\ sample}{OD\ of\ control}\right) x100\%$$

### 2.7. Statistical analysis

Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by two-tailed *t*-test analysis of variance using the GraphPad Prism 9.510. Results with $p < 0.05$ were considered statistically significant and were expressed as the mean ± SEM of $n = 6$.

## 3. Results and discussion

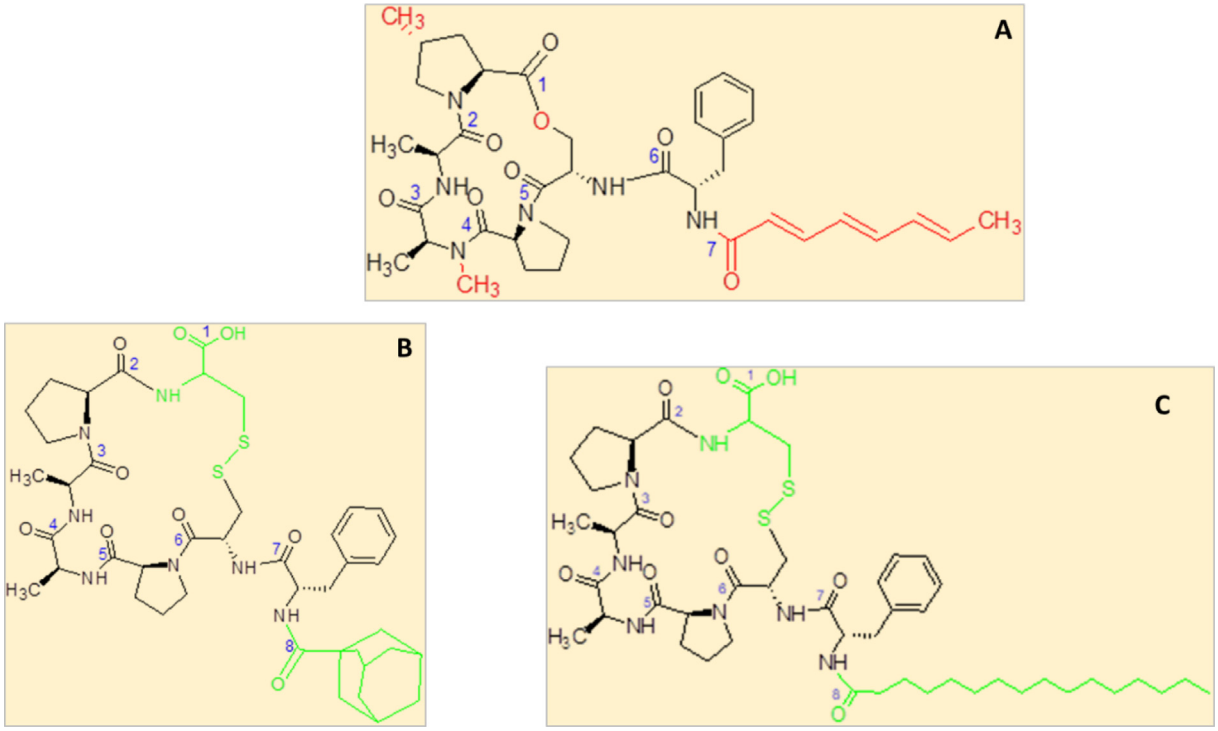
### 3.1. Synthesis and characterization of ADEP1 analogues

ADEPs have potential to overcome microbial resistance to antimicrobial agents as they follow a different mode of action, however, their bioactivity is limited by metabolic instability and side effects, among others. There were several strategies that were previously used to overcome these challenges. In the current study, the modifications introduced to the ADEP1 structure (Scheme 2A) were motivated by: 1. The need to produce low cost drug candidates in high yield by developing a shorter synthetic route and use of natural peptides. Therefore, 4-methyl proline and N-methylated alanine were substituted with natural amino acids (Pro2 and Ala4) (Scheme 1B and C). 2. To improve lipophilicity and biocompatability by replacing OTEA with Ada in SC006 and SC007 (Scheme 2B) or Pal in SC005 and SC008 (Scheme 2C). 3. Incorporation of D-amino acids to improve metabolic stability and prevent biodegradation. SC005 and SC006 contained D-amino acids while SC007 and SC008 were made of L-amino acids.

The structure of ADEP1 is cyclized by an ester bond between the carboxylic end of Pro1 and the hydroxyl functional side chain of Ser5. The ester bond linkage was replaced by a disulphide bond by adding Cys1 and substituting Ser5 with Cys6. Substitution of amino acid residues with Cys produce disulphide bridges and increase stability of the peptides. For example, mutations in subtilisin E that resulted in replacement of Gly61 and Ser98 with Cys residues increased its activity, thermal stability and shelf-life (Liu et al., 2016; Takagi et al., 1990; Zabetakis et al., 2014).

The synthesis of ADEP analogues via SPPS generally pose difficulties in cyclization with the ester bond (Wen et al., 2008; White & Yudin, 2011). The natural ADEPs synthesis in biological systems easily form the ester bond linkage through bacterial depsipeptide synthases (Alonzo & Schmeing, 2020). However, chemical synthesis make use of esterification techniques, which result in poor (~ 6%) yields (Eyermann et al., 2020). Although fragment convergence synthesis have better yields, this synthesis route does not follow the normal peptide synthesis. It uses different protecting groups, prone to intermolecular reaction, and it also comes with numerous coupling and purification steps (Goodreid et al., 2014, 2015; Li et al., 2017). This synthesis approach disassembles the ADEPs into three fragments namely tripeptide, linkage, and Phe-heptenamide (Li et al., 2017). The incorporation of disulphide cyclization allows for a simple SPPS using one protecting group (Fmoc), with single cleavage and purification steps.

HOBt was used as a coupling additive to suppress racemization. It is also a nucleophilic catalyst, which helps accelerate the rate of the coupling reaction time (Al-Warhi et al., 2012). HOBt considerably reduced coupling reaction times from 1 h to 45 min while reducing the presence of impurities. The expected molecular mass (MM) of the analogues was confirmed by MS. Figs. A1, A3, A5, and A7 shows MM [M + 1] of the peptides before cyclization. After cyclization with iodine or 20% DMSO, the MM observed on the MS




**Scheme 2** ADEP1 analogues in relation to ADEP1 parent structure (A). The ADEP analogues were modified with either Ada (SC006 and SC007) (B) or Pal (SC005 and SC008) (C).

(Figs. A4, A6, and A8) suggested the loss of the two hydrogen molecules during the formation of the disulphide bond. This confirmed the synthesis of cyclic analogues.

With the analysis on both prep-HPLC and LCMS, the peptides presented a higher retention time (Figs. A9-A12). This was attributed to the hydrophobic and nonpolar nature of the peptides. Out of seven amino acids in their sequence, five of those (Pro, Ala, and Phe) possess nonpolar and hydrophobic side chains. In addition, the formation of the disulphide bond reduced the polarity and hydrophilicity of the Cys residues. The peptides containing Pal were analysed with a longer LC method (16 min) compared to the Ada-containing peptides (14 min). The LCMS chromatogram and spectra of the four ADEP1 analogues are presented in Table 2. The analogues presented high purity (≥96%) with improved yields.

The anionic nature of all the analogues is due to the ionisable -COOH of Cys at neutral pH range to give -$COO^-$. This was confirmed by their negative zeta potential values and the net charges of the ADEP1 analogues reported in Table 2. As a consequence of using the Cys1 and Cys6 thionyl groups to cyclize the peptides, the -$COO^-$ of Cys1 was exposed and therefore giving the analogues a negative net charge. The addition of the two Cys also enlarged the ring, which can allow for slight conformational freedom.

### 3.2. Antibacterial activity of the ADEP analogues

The potential antibacterial activity of the ADEP1 analogues was screened against the benign (*B. subtilis* and *E. coli*) and the pathogenic (*P. aeruginosa, S. aureus* and MRSA) strains. Table 3 shows that the Pal-based ADEP1 analogues had higher activity compared to Ada-based ADEP1 analogues, in the order SC008 > 5 > 6 > 7. The Pal-based ADEP1 analogues were active against all the tested bacterial strains with SC008 showing more potency compared to its D-enantiomer, SC005. The Ada-based ADEP1 analogues showed selective activity, SC006 did not show any activity on *P. aeruginosa* while SC007 showed no activity on *S. aureus* and *E.coli*. Pal is a long chain of hydrocarbons (saturated fatty acid) that better mimics the OTEA than the Ada. Pal has been used to increase lipophilicity of drug-like compounds (Carta et al., 2017; Irby et al., 2017), and its conjugation to ADEP1 analogues increased their lipophilicity, which enhanced their interaction with the bacterial cell and increased cellular uptake. Both Ada and Pal are lipophilic molecules and incorporated in the ADEP1 analogues to increase their cell permeation ability. Fatty acids (linoleic, oleic acid, Pal, and Ada) are exploited in pharmaceuticals to serve as chemical cell permeability enhancers (Cizinauskas et al., 2017). They are widely used as drug delivery systems and targeted therapies in biomedical applications (Spilovska et al., 2016; Schleyer, 1957; Stimac et al., 2017). However, agar diffusion methods have been reported to lack sensitivity for non-polar molecules (Eloff, 2019) and highly dependent on their ability to diffuse in the agar. The microdilution assay was thus used to further evaluate the activity of the peptides against all the bacterial strains, their MIC and MBC values are presented in Table 4.

All the ADEP1 analogues showed a broad-spectrum antibacterial activity on the selected bacterial species at concentration range of 125 – 500 μM (MIC) and 125 – 750 μM (MBC). The mode of action of these ADEP1 analogues has not been determined yet. However, previous reports have highlighted the specific mechanism of ADEP by introducing ClpP mutations in *B. subtilis*, *S. aureus*, and *E. coli*. The bacterial strains with ClpP mutation showed high resistance to ADEP treatment (Brotz-Oesterhelt & Sass, 2014; Malik et al., 2020). This suggested that it is unlikely for the ADEPs to target any bacterial component than ClpP, thus making their mode of action target specific. The ADEPs binds to the ClpP, which then degrade proteins necessary for whole cell metabolism and survival (Frees et al., 2014; Sass et al., 2011). Therefore, the ADEP1 analogues might follow mode of action similar to the one for ADEPs due to their structural similarities.

All the ADEP1 analogues were able to inhibit growth of the Gram-positive (MIC = 63–125 μM) and Gram-negative (MIC = 250 μM) bacterial strains. The Gram-negative bacteria showed less susceptibility towards the ADEP1 analogues. *E. coli* presented the highest resistance with an MIC of 500 μM for all the analogues while *P. aeruginosa* was inhibited by an MIC of 250 μM for SC0006 – 8 and more susceptible to SC005 (MIC = 125 μM). This is in line with what has been reported about ADEPs or anionic AMPs which have higher potency on Gram-positive than Gram-negative bacteria (Culp & Wright, 2017). Overall, the peptides showed similar antibacterial trends despite different antimicrobial activities previously discussed between the D- and L-enantiomers. In literature, D-enantiomer peptides possess higher antimicrobial activity compared to the L-enantiomer (Ye & Aparicio, 2022), this might be due to their (D-enantiomer) proteolytic stability leading to increased bioavailability *in vivo*. Natural peptides, ADEPs included, consist of L-amino acids, which are susceptible to enzymatic degradation (Vlieghe et al., 2010), while peptides composed of D-amino acids may escape protease degradation thereby increasing their bioavailability (Garton et al., 2018; Uppalapati et al., 2016). Similarly, disulphide bonds also play a major role in peptide/protein structure stability by increasing resistance to proteolysis while also increasing activity (Siddiqui et al., 2005; Trivedi et al., 2009).

**Table 2** Peptide analysis by HR-LCMS and Zetasizer.

| Peptide code | Retention time (minutes) | Calculated mass (MM = g/mol) | Chemical formula | Found $[M + H]^+$ m/z[a] | % purity | % yield | **Zeta potential (mV)** |
|---|---|---|---|---|---|---|---|
| SC005 | 8.8 | 943.51 | $C_{47}H_{73}N_7O_9S_2$ | 944.4973 | 99.9 | 46 | −7.2 ± 1.6 |
| SC006 | 9.7 | 867.37 | $C_{42}H_{57}N_7O_9S_2$ | 868.3730 | 98 | 37 | −3.8 ± 1.1 |
| SC007 | 10.2 | 867.37 | $C_{42}H_{57}N_7O_9S_2$ | 868.3747 | 96 | 39 | −3.9 ± 0.8 |
| SC008 | 8.5 | 943.51 | $C_{47}H_{73}N_7O_9S_2$ | 944.4621 | 99.9 | 45 | −6.9 ± 1.5 |

**Table 3** Diameter of zones of inhibition of CC ADEP analogues.

| Microbes | [Peptide] μM | SC005 (mm) | SC006 (mm) | SC007 (mm) | SC008 (mm) | 0.84 mM Gentamicin (mm) | 11.5 mM Ampicilin (mm) |
|---|---|---|---|---|---|---|---|
| *B. subtilis* | 100 | 6.5 ± 0.14 | 0 | 0 | 8.5 ± 0.14 | 12 ± 0.16 | NT |
| | 500 | 8.5 ± 0.32 | 6.5 ± 0.08 | 6.5 ± 0.16 | 8.5 ± 0.32 | | |
| | 1000 | 10.5 ± 0.25 | 6.5 ± 0.18 | 8.5 ± 0.16 | 8.5 ± 0.25 | | |
| *S. aureus* | 100 | 0 | 0 | 0 | 0 | 12 ± 0.08 | NT |
| | 500 | 0 | 0 | 0 | 0 | | |
| | 1000 | 0 | 0 | 4.5 ± 0.180 | 0 | | |
| *MRSA* | 100 | 0 | 0 | 0 | 24.5 ± 0.14 | 8.8 ± 0.16 | NT |
| | 500 | 10.5 ± 0.17 | 0 | 0 | 24.5 ± 0.32 | | |
| | 1000 | 10.5 ± 0.16 | 6.5 ± 0.14 | 0 | 24.5 ± 0.25 | | |
| *P. aeruginosa* | 100 | 0 | 0 | 0 | 6.5 ± 0.14 | 12 ± 0.16 | NT |
| | 500 | 0 | 0 | 4.5 ± 0.39 | 8.5 ± 0.32 | | |
| | 1000 | 6.5 ± 0.31 | 0 | 10.5 ± 0.39 | 8.5 ± 0.25 | | |
| *E. coli* | 100 | 0 | 6.5 ± 0.20 | 0 | 8.5 ± 0.14 | NT | 11.5 ± 0.16 |
| | 500 | 10.5 ± 0.22 | 6.5 ± 0.17 | 0 | 8.5 ± 0.32 | | |
| | 1000 | 10.5 ± 0.13 | 6.5 ± 0.18 | 0 | 8.5 ± 0.25 | | |

NT = Not tested.

**Table 4** MIC and MBC of the ADEP analogues on bacteria.

| ADEP1 analogues | *B. subtilis* | | *S. aureus* | | *MRSA* | | *P. aeruginosa* | | *E. coli* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MIC (μM) | MBC (μM) | MIC (μM) | MBC (μM) | MIC (μM) | MBC (μM) | MIC (μM) | MBC (μM) | MIC (μM) | MBC (μM) |
| SC005 | 125 | 125 | 63 | 125 | 125 | 250 | 125 | 250 | 500 | 750 |
| SC006 | 250 | 250 | 63 | 125 | 250 | 250 | 125 | 250 | 500 | 750 |
| SC007 | 125 | 125 | 63 | 125 | 250 | 500 | 125 | 250 | 500 | 750 |
| SC008 | 125 | 125 | 63 | 125 | 250 | 500 | 125 | 250 | 500 | 750 |
| Gentamicin | 26.1 | 52.3 | 0.5 | 1.0 | 26.2 | 52.4 | 418.8 | 837.5 | NT | NT |
| Ampicillin | NT | NT | NT | NT | NT | NT | NT | NT | 2862 | 5724 |

All four ADEP1 analogues were cyclized with a disulphide bond to replace the ester bond linkage that has been reported to be susceptible to enzymatic degradation (Finer & Santerre, 2004; Yourtee et al., 2001).

The naturally occurring ester bond has not yet been reported to play a vital role in molecular conformation, interaction with ClpP, and biological activity of the ADEPs. However, a study by Li and colleagues (Li et al., 2017) made strides in comparing the effect of using different linkage motifs on the conformational behavior and bioactivity of the ADEP analogues. Their study showed a significant decline in *in vitro* biochemical activity when the ester bond was substituted with amide bond or *N*-methyl amide bond (Li et al., 2017). The same phenomenon was observed in this study, where the MIC values for ADEP1 analogues were multiple folds higher than that reported for ADEP1. Another study presented challenges of incorporating 4-methylated Pro and N-methyl-Ala during synthesis of ADEPs, and removing the methyl groups in the amino acids resulted in complete loss of antibacterial activity (Hinzen et al., 2006). This then highlighted the importance of methyl amino acids in the antimicrobial potency of ADEPs.

Although the ADEP1 analogues had antibacterial activity in the micromolar range, they showed great bactericidal effects. Bactericidal effects (MBC) of potential drugs are vital as they represent a concentration that completely eradicates bacteria rather than inhibiting their growth. For example, ADEPs has shown inhibition of cell division in *B. subtilis*, however, bacterial cells recover upon incubation on a fresh media (Mayer et al., 2019). This shows that even though the MICs of most ADEPs are low, they lack the bactericidal effect. In some cases, their bactericidal effect is multiple folds higher than the MIC (Sass et al., 2011). In this study, all four tested analogues had the MIC that was at the same concentration as the MBC against *B. subtilis*. Usually, the MBC of antimicrobial drugs is greater or equals to two fold of the MIC (Levison & Levison, 2009), as observed in the other three bacterial strains where MBC was twice the concentration of MIC.

### 3.3. Cytotoxicity

It is imperative that antimicrobial agents are selective in their actions to prevent or reduce off-target effects. However, the biocompatibility of natural ADEPs is challenged by their non-selectivity and toxicity to mammalian cells. Similar to microorganisms, human cells also express ClpP (hClpP) which is structurally similar to its bacterial counterparts and might

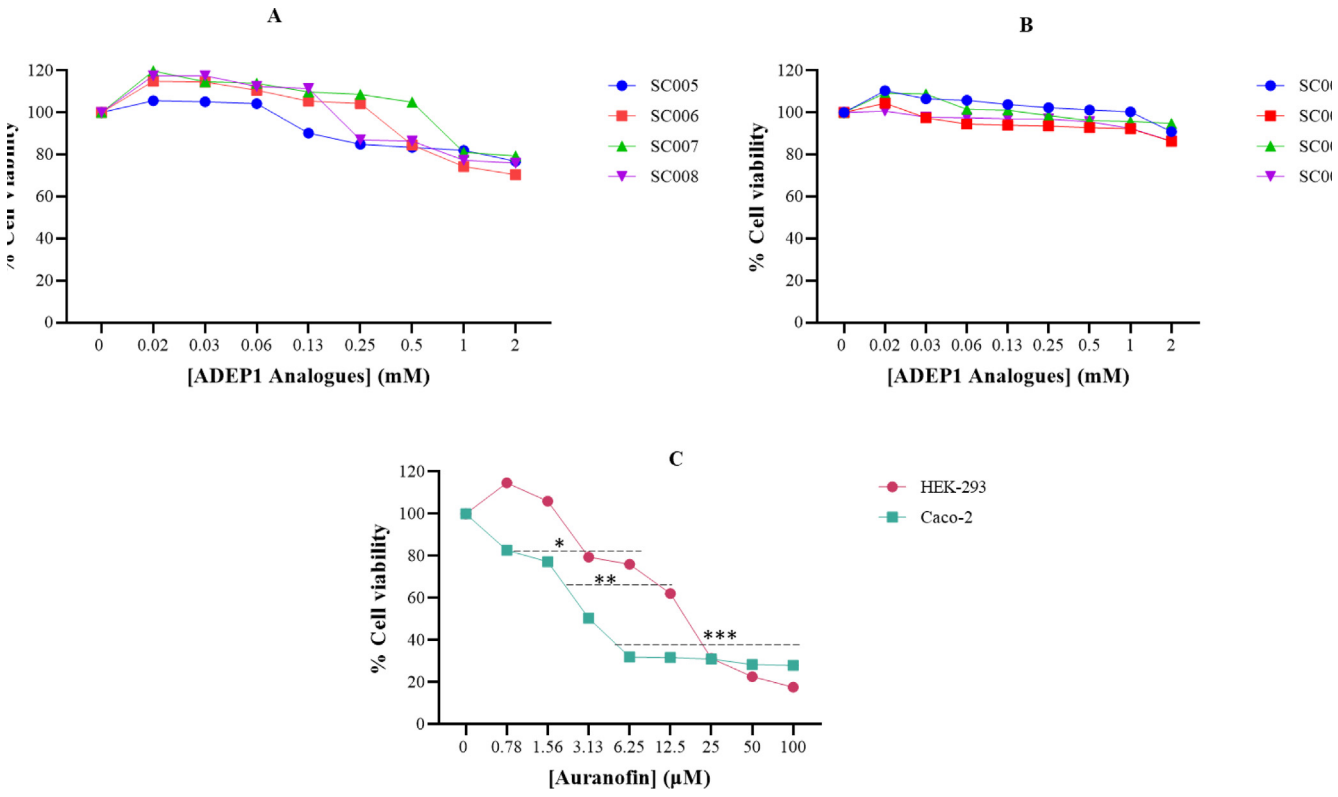



**Fig. 1** Effect of ADEP analogues on the viability of HEK-293 (A) Caco-2 (B) cells, and Auranofin treatment on HEK-293 and Cac-2 (C). Cell viability was assessed by MTS assay, where $p < 0.05$ was considered statistically significant. *$= p < 0.05$, **$= p < 0.01$, and***$= p < 0.001$.

respond the same way to the ADEPs. Human proteostasis is also coordinated by a network of chaperones and proteases including hClpP (Kang et al., 2005). The activity of hClpP is regulated by ClpX, an ATP-dependent protease, and dysregulated by ADEPs (Cole et al., 2015). Thus, the effects of ADEP1 analogues were evaluated on HEK-293 and Caco-2 cells at $\leq 2$ mM using MTS assay. There was no significant reduction in cell viability of HEK-293 (Fig. 1A) and Caco-2 (Fig. 1B) cells in response to increasing concentrations of ADEP1 analogues. Fig. 1C shows that Auranofin, a food and drug administration (FDA) approved antirheumatic drug (Boullosa et al., 2021), was cytotoxic to all the cells at $\geq 0.125$ mM.

The mode of action involved in the dysregulation of hClpP and the physiological consequences have not been thoroughly investigated. However, in 2018, Wong and colleagues reported that the dysregulation of hClpP by ADEPs induced cytotoxicity to various human cell types including HEK-293 cells (Wong et al., 2018). The concentration dependent toxicity effect of ADEPs on hClpP was demonstrated *in vitro* and *in vivo* using different ADEPs and ADEP analogues (Wong et al., 2018). Toxicity has been the major hindrance in the development of ADEPs as active pharmaceutical ingredient. The ADEP1 analogues under study showed no significant toxicity at a dose multiple folds higher (2 mM) than their MICs. The ADEP1 analogues in this study showed an essential improvement in their biocompatibility compared to the cytotoxicity of ADEP analogues in literature. Generally, ADEPs start showing toxicity at concentrations from $\geq 0.215$ μM (Wong et al., 2018). The ADEP1 analogues showed insignificant activity up to 2 mM, and implied that these synthetic peptides are not cytotoxic at their MICs and therefore has potential for clinical applications.

## 4. Conclusion

ADEPs are potent antimicrobial agents; unfortunately, they possess bystander toxicity at their bioactive concentrations. Synthetic ADEP analogues have shown potential as alternative antimicrobial agents compared to their parent structures. The analogues can be tailored to have a different characteristics, enhanced efficacy and lower cytotoxicity. The current study demonstrated that the use of enantiomers, amino acid replacement, change in cyclization and replacement of OTEA in the parent ADEP1 structure drastically reduced cytotoxicity. Our findings also indicated that the changes made on the parent structure significantly altered and compromised the antibacterial activity exhibited by ADEP1. Despite the limitations, the study has demonstrated that these peptides showed a broad spectrum antibacterial activity. This is contrary to what has been reported about anionic peptides and their selective activity against Gram-positive bacteria. For future studies, addition of cationic amino acids or use of a resin yielding a positive C-terminal to improve interaction with the negatively charged bacterial membrane would be beneficial. Structural enhancement would improve the potency of the ADEP1 analogues. In addition, the supplementation of these anionic AMPs with divalent metal ions such as zinc may increase their antimicrobial activitie. In addition, evaluation of synergistic effects evaluation of ADEP analogues with known antibacterial drugs would be beneficial.

## Declaration of conflict of interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2023.105000.

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