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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Biosynthesis of 2-aminooctanoic acid and its use to terminally modify a lactoferricin B peptide derivative for improved antimicrobial activity

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Abstract

Terminal modification of peptides is frequently used to improve their hydrophobicity. While N-terminal modification with fatty acids (lipidation) has been reported previously, C-terminal lipidation is limited as it requires the use of linkers. Here we report the use of a biocatalyst for the production of an unnatural fatty amino acid, (*S*)-2-aminooctanoic acid (2-AOA) with enantiomeric excess > 98% *ee* and the subsequent use of 2-AOA to modify and improve the activity of an antimicrobial peptide. A transaminase originating from *Chromobacterium violaceum* was employed with a conversion efficiency 52–80% depending on the ratio of amino group donor to acceptor. 2-AOA is a fatty acid with amino functionality, which allowed direct C- and N-terminal conjugation respectively to an antimicrobial peptide (AMP) derived from lactoferricin B. The antibacterial activity of the modified peptides was improved by up to 16-fold. Furthermore, minimal inhibitory concentrations (MIC) of C-terminally modified peptide were always lower than N-terminally conjugated peptides. The C-terminally modified peptide exhibited MIC values of 25 μg/ml for *Escherichia coli*, 50 μg/ml for *Bacillus subtilis*, 100 μg/ml for *Salmonella typhimurium*, 200 μg/ml for *Pseudomonas aeruginosa* and 400 μg/ml for *Staphylococcus aureus*. The C-terminally modified peptide was the only peptide tested that showed complete inhibition of growth of *S. aureus*.

Keywords ω-Transaminase · *Chromobacterium violaceum* DSM30191 · Unnatural amino acids · 2-aminooctanoic acid · Antimicrobial peptide

Introduction

During reductive amination, a transaminase (TA) transfers an amino group from a donor to an acceptor via a pyridoxal-5`-phosphate (PLP)-dependent mechanism to generate a chiral amine (Ghislieri and Turner 2013; Humble et al. 2012). TAs are widely used in the production of chiral amino compounds such as the antidiabetic drug sitagliptin (Savile et al. 2010). Enantiopure amino compounds can have bioactivity, or they can be used as building blocks for the synthesis of bioactive compounds (Breuer et al. 2004; Panke et al. 2004). Approximately 80% of the pharmaceuticals currently produced are chiral amines (Breuer et al. 2004).

Unnatural (nonproteinogenic) amino acids are receiving increasing attention as building blocks for pharmaceuticals, agrochemicals and other value added products (Mathew and Yun 2012). Biocatalysts such as TAs offer an option for the "green" production of these chiral compounds. The unnatural amino acids are structurally and functionally diverse, and therefore can have multiple different applications. L-*tert*-

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leucine, a building block of a protease inhibitor acting on the HIV virus, has been synthesised in a process employing a TA from *Escherichia coli* (Seo 2011). The enantiomerically pure unnatural amino acid L-homoalanine was asymmetrically synthesised using an ω-TA, and it is a key intermediate for the production of pharmaceutical compounds such as antiepileptic and antituberculosis drugs (Shin and Kim 2009; Zhang et al. 2010). One of the applications of unnatural amino acids is the synthesis of antimicrobial peptides (AMPs). The incorporation of unnatural amino acids in AMPs leads to increased bioactivity and stability when compared with naturally occurring peptides (Bhonsle et al. 2013).

AMPs are biomaterials evolved in virtually every class of organisms as a host defence mechanism (Bhonsle et al. 2013; Yeaman and Yount 2003). Increased interest in AMPs in recent years is due to their activity towards a wide range of bacteria, including some antibiotic-resistant strains, different fungi, viruses and parasites (Ageitos et al. 2017; Hancock 2001). AMPs are usually selective and efficient (Fosgerau and Hoffmann 2015) and unlike conventional antibiotics, they exhibit dual activity: disrupt targeted cell membranes and modulate the immune system (Hancock and Sahl 2006). Bacterial resistance to AMP would require severe rearrangements of the membrane structure, which would significantly delay the emergence of the resistance (Aoki and Ueda 2013; Chen et al. 2014). Thus, AMPs are promising candidates for the treatment of resistant infections.

The modification of naturally occurring peptide sequences to develop synthetic AMPs with desired properties has previously been reported (Bhonsle et al. 2013; Goodwin et al. 2012; Malina and Shai 2005; Reinhardt and Neundorf 2016). Terminal modification of peptides frequently adds hydrophobic moieties to increase the hydrophobicity of peptides (Chu-Kung et al. 2010). It was demonstrated that increasing hydrophobicity of a peptide via lipidation, either internally (Lee et al. 2002) or terminally (Chen et al. 2016), increased the antimicrobial activity. The modification of the N-terminus by fatty acids has been reported previously (Chicharro et al. 2001). However, the C-terminal modification of peptides is limited due to the need for an amino function which is absent in fatty acids. The synthesis of unnatural amino acids with the hydrophobic tail of fatty acids allows the N- and C-terminal modification of peptides offering a greater diversity of modifications.

Here we demonstrate the production of an unnatural amino acid, 2-aminooctanoic acid (2-AOA), which was subsequently used for the modification of N- and C-termini of a lactoferricin-like peptide. An ω-TA from *Chromobacterium violaceum* DSM3019 was used for the production of 2-AOA. The product was purified from the biotransformation reaction using Fmoc-Cl derivatisation, which demonstrates the possibility to directly apply the derivatised amino acid for peptide synthesis. Furthermore, we demonstrated improved antimicrobial activity of a lactoferricin-like AMP modified with 2-AOA, with the highest improvement (16-fold for *E. coli*) though C-terminal modification.

Materials and methods

Reagents The amino group donor (*S*)-(-)1-phenylethylamine (1-PEA) and the amino group acceptor 2-oxooctanoic acid (2-OOA), racemic (*R*, *S*)-2-aminooctanoic acid (AOA) and the cofactor PLP were purchased from Sigma-Aldrich (Dublin, Ireland). (*S*)-Fmoc-2-AOA was obtained from Eurogentec (Liege, Belgium). Trifluoroacetic acid (TFA), isopropyl-b-D-thiogalactopyranoside (IPTG) and imidazole were obtained from Fisher Scientific (UK). Restriction enzymes, BamHI and HindIII, were obtained from Thermo Scientific (UK). The oligonucleotide primers were synthesised by Sigma Genosys (Dublin, Ireland). BugBuster® was purchased from Merck Chemicals (Nottingham, UK). All other chemicals were from Sigma-Aldrich (Dublin, Ireland).

Cloning and expression of the CV_TA Standard recombinant DNA techniques were performed as previously described (Sambrook et al. 1989). In brief, the CV_2025 gene (kegg.jp) was amplified by PCR from the genomic DNA of *Chromobacterium violaceum* DSM30191 using the primers listed in Table 1 and ligated into an expression vector pET-45b(+) (Table 1).

The CV_TA construct was introduced into *E. coli* BL21 (DE3) to give *E. coli* CV_TA. The recombinant strain was grown in LB medium supplemented with carbenicillin (50 μg/ml). For CV_TA expression, 1 ml of overnight culture was inoculated in 400 ml LB medium with carbenicillin (50 μg/ml) and grown at 37 °C, 200 rpm until an OD_{600} of 0.5 was reached. The culture was then cooled for 30 min at 4 °C, followed by the induction with 0.5 mM IPTG and the CV_TA was expressed for an additional 4 h. The cells were harvested by centrifugation 6000 rpm (Sorvall RC-5, refrigerated floor centrifuge) for 10 min at 4 °C and the pellet was washed twice with 400 ml of 100 mM potassium phosphate buffer (pH 7). The protein was purified from the cells as described previously (Hume et al. 2009) using HisTrap FF 1 mL column (Amersham Biosciences/GE Healthcare, England) and ÄKTAprime chromatography system (Amersham Biosciences/GE Healthcare, England). The fractions (2 ml volume) were collected and analysed by 8% SDS-PAGE under denaturing conditions (Laemmli 1970). The purified protein concentration was determined by bicinchoninic acid (BCA) assay (Smith et al. 1985).

Analysis of the activity of CV_TA towards 2-OOA The activity of purified CV_TA was tested by a spectrophotometric assay (Schatzle et al. 2009). The biotransformation was carried out in 200 μl total reaction volume in microtiter plate (Greiner UV-Star® 96 well plates, Sparks) and contained in 100 mM

Table 1 Oligonucleotides, bacterial strains and plasmids used in this study

Strain/Primer/Plasmid	Relevant characteristics	Reference
CV_2025_BamHI	ACGGATCCACAGATCTATGCAGAAGCAACGTACGA	This work
CV_2025_HindIII	TCTAAGCTTTCTAAGCTTCTAAGCCAGCCCGCGCGCCT	This work
C. violaceum	DSM 30191 (ATCC 12472); source of gene CV_2025	DSMZ
E. coli BL21 (DE3)	F-; *omp*T; high level expression regulated by T7 promoter	Novagen
E. coli CV_TA	Expressing CV_2025	This work
pET-45b(+)	Expression under T7 promoter, amp^R	Novagen
CV_TA	pET-45b(+) containing 1380 bp fragment encoding CV_2025	This work

phosphate buffer pH 7: 2.5 mM 1-PEA, 2.5 mM 2-OOA or pyruvate, 0.25% DMSO, 22 μg/ml (0.43 μM) CV_TA pre-incubated with 0.2 mM PLP for 10 min at 4 °C. The conversion of 1-PEA to acetophenone was monitored over 60 min at 30 °C spectrophotometrically at 245 nm (SPECTROstar Nano, BMG LABTECH). CV_TA activity was expressed as nmoles/min/mg. When CV_TA activity between two different substrates was compared, relative activity (%) was used, and it was calculated as the percentage of the activity with its native substrate, pyruvate.

The activity of CV_TA was characterised using 10 mM 1-PEA and 2-OOA concentrations ranging from 0.1 to 20 mM. The course of the reaction was followed spectrophotometrically as described in the previous paragraph, and the activity was calculated from the initial rates using the molar extinction coefficient of acetophenone ($\varepsilon = 7.03\ mM^{-1}\ cm^{-1}$). The kinetic parameters were determined by non-linear regression analysis, (Enzfitter for Windows 2.0.18.0 (Elsevier, Biosoft®, UK)).

Optimum pH and temperature for the synthesis of 2-AOA

To determine the optimal conditions for the production of 2-AOA, the reaction was tested in 100 mM phosphate buffers pH range 6 to 8. The temperature optimum was determined in 100 mM phosphate buffer pH 7 at a temperature range from 25 to 55 °C.

Optimisation of the production of 2-AOA Different donor:acceptor ratios (mol:mol) 1-PEA:2-OOA = 4:1, 1.7:1, 1.5:1 and 1.3:1 were employed to find the optimal ratio for the production of 2-AOA. The reaction was performed in 10 ml of 100 mM potassium phosphate buffer pH 7, at 45 °C and with 44 μg/ml (0.86 μM) of CV_TA pre-incubated with 0.2 mM PLP. Aliquots (0.5 ml) were sampled at times 0 and then every 15 min until the end of the reaction (180 min) and mixed with the same volume of 0.2% TFA to stop the reaction, followed by tenfold dilution with potassium phosphate buffer (100 mM, pH 7) and subjected to HPLC analysis after derivatisation.

A sequential addition of the donor and acceptor was attempted in order to improve the yield of 2-AOA. The reaction was performed in 10 ml total volume for 6 h at 45 °C using the donor:acceptor ratio of 4:1. The samples (0.5 ml) were retrieved at 90, 180 and 270 min of the reaction. The concentration of the produced AP was measured spectrophotometrically as described in section 2.3. Based on the estimated 1-PEA and 2-OOA consumption, the donor and the acceptor were added to reach 10 and 2.5 mM concentration while maintaining the reaction volume (10 ml). Finally, using a donor:acceptor ratio of 1.7:1, the production of 2-AOA was tested in a 100 ml reaction volume at optimal conditions.

Derivatisation of the amino compounds and HPLC analysis The biotransformation mixture and standard solutions of 1-PEA and 2-AOA were derivatised with 9-fluorenylmethyloxycarbonyl chloride (Fmoc-Cl) by mixing 150 μl of a standard/reaction mixture with 150 μl of 400 mM borate buffer pH 9 and 300 μl of 20 mM Fmoc-Cl dissolved in acetonitrile (Jambor and Molnar-Perl 2009). The reaction was incubated at room temperature for 20 min and stopped by the addition of 25 μl of 100 mM heptylamine (HEPA). The mixture was incubated for additional 3 min at room temperature, centrifuged at 13000 rpm (Centrifuge 5430 R, Eppendorf) for 1 min, followed by the filtration of the supernatant through 0.45 μm filter (Mini-UniPrep, GE Healthcare).

The supernatants were analysed by a modified HPLC method (Fabiani et al. 2002), using a C18 ACE 5 reverse phase column (150 mm × 4.6 mm, particle size 5 μm; Advance Chromatography Technologies, Aberdeen, UK) with the detector set at 263 nm. The samples (20 μl) were injected at a flow rate of 1 ml/min using 50 mM acetate buffer (pH 4.2) as eluent A and acetonitrile as eluent B. The amino compounds were separated with the following linear gradient elution: (min/A%): 0/40, 10/0, 13/0, 14/40, 18/40.

Purification and characterisation of 2-AOA To purify Fmoc-2-AOA, the fractions eluted between 6.4 and 7.2 min were collected using the program described above, and the purity of samples was verified by HPLC. Accurate mass measurements were obtained with a LCT time-of-flight mass spectrometer (Waters Corp., Dublin, Ireland).

Chiral HPLC To determine the enantiomeric excess of the produced 2-AOA, the commercial (*S*)-Fmoc-2-AOA (Eurogentec, Belgium) and purified Fmoc-2-AOA were analysed by normal phase HPLC (Agilent 1200 Series) using a CHIRALPACK IC column and with the detector set at 254 nm. The sample (5 μl) was injected at a flow rate of 1 ml/min using 95% heptane to 5% ethanol.

Optical activity The 1 mg/ml solutions of the standard (*S*)-Fmoc-2-AOA (Eurogentec, Belgium) and Fmoc-2-AOA purified from the biotransformation mixture were prepared in methanol (Fisher Chemical, HPLC grade, Ireland). The rotation of polarised light [α]D was recorded using a single wavelength polarimeter (Polarimeter Model 343, PerkinElmer, USA).

Antimicrobial peptides (AMP) synthesis and antimicrobial activity testing The core sequence of lactoferricin B (LFcin B) RRWQWRMKK (Wakabayashi et al. 1999) and an acylated derivative (2-AOA)-RRWQWRMKK and amidated derivative RRWQWRMKK-(2-AOA) were chemically synthesised in Eurogentec (Liege, Belgium).

The antimicrobial activity of the synthesised peptides was assayed with two Gram-positive bacteria, *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 25923), and three Gram-negative bacteria, *Escherichia coli* (NCTC 9001), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella typhimurium* (NCTC 12023). The minimal inhibitory concentrations (MIC) of the peptides were determined using the standard microdilution method (EUCAST 2003). The bacterial strains were grown for 16 h in LB broth with decreased NaCl (0.1%), followed by dilution with LB (0.1% NaCl). A dilution series of synthesised AMPs were prepared in 40% DMSO to give peptide stock concentrations from 0.125 to 4 mg/ml. The assay was performed in 96-well polypropylene microtiter plate (96 well cell culture cluster, Costar) and it consisted of 20 μl of AMP at the appropriate concentration, 100 μl of diluted bacterial cultures (5×10^5 CFU/ml) and 80 μl of LB. The assay allowed bacterial growth for 18 h at 35 °C and 200 rpm, and the growth was measured as A_{600} (JENWAY 6300 spectrophotometer). The appropriate controls were included: growth of the strains in LB (0.1% NaCl), LB (0.1% NaCl) with the addition of 4% DMSO, as well as non-inoculated LB. MIC was defined as the lowest concentration of AMP at which no bacterial growth was observed. All samples were assayed in triplicates.

Results

Gene cloning, expression and purification of CV_2025

The N-terminally histidine tagged protein CV_TA, expressed in *E. coli* BL21 (DE3), was purified to homogeneity using a nickel-chelating column and an imidazole step gradient from 0 to 100%. The purity of CV_TA was analysed using SDS-PAGE 8% denaturing gel (Fig. 1a). The majority of CV_TA eluted in three fractions (Fig. 1a), which combined, gave 2.5 mg/ml of pure protein.

CV_TA activity towards non-native amino group acceptor 2-OOA

We have tested the production of 2-aminooctanoic acid (2-AOA) using the purified CV_TA as a biocatalyst, (*S*)-(-)1-phenylethylamine (1-PEA) as an amino donor and 2-oxooctanoic acid (2-OOA) as an amino group acceptor using a donor:acceptor ratio of 1:1. Using 1-PEA as a donor allows monitoring of the reaction spectrophotometrically, as the deamination product, acetophenone (AP), absorbs in the UV spectrum (A_{245}).

The CV_TA produced the desired product, 2-AOA, which was confirmed by HPLC. However, the rate of the reaction with 2-OOA was 1.7-fold lower compared with pyruvate (Fig. 1b), considered the native substrate of CV_TA (Sayer et al. 2007).

The affinity of CV_TA towards 2-OOA is 14-fold lower than the affinity towards pyruvate, the native substrate of the enzyme (Table 2). This contributes significantly to a 29-fold lower catalytic efficiency towards 2-OOA compared to pyruvate. Despite this lower catalytic efficiency, 45% conversion of the acceptor into 2-AOA was observed in 60 min.

The optimal pH for the conversion of 2-OOA into 2-AOA is pH 7 (Fig. 2a) while the optimal temperature is 45 °C (Fig. 2b).

Optimisation of 2-AOA production through alteration of the amino donor to recipient ratio

An excess of the amino group donor is usually supplied to a transaminase catalysed reaction, in order to drive the reaction towards product formation (Koszelewski et al. 2010). Different ratios of the donor, 1-PEA and the acceptor, 2-OOA were applied in order to find the optimal donor:acceptor ratio for the production of 2-AOA (Table 3). The biotransformation was performed in a total reaction volume of 10 ml with 44 μg/ml (0.86 μM) of CV_TA at 45 °C and pH 7. The consumption of 1-PEA and formation of 2-AOA were quantified by HPLC, which required a derivatisation of the amino compounds using Fmoc-Cl (see section 2.6).

While the highest conversion efficiency of 2-OOA into 2-AOA (84%) was achieved when a donor:acceptor ratio of 4:1 was applied (Table 3), the conversion of 2-OOA into 2-AOA at a ratio of 1.7:1 (entry 2) resulted in twice as much product as the reaction with 4:1 ratio (Table 3). A further decrease in donor:acceptor ratio decreased product yield further (Table 3). Increasing the concentration of 1-PEA (donor) to

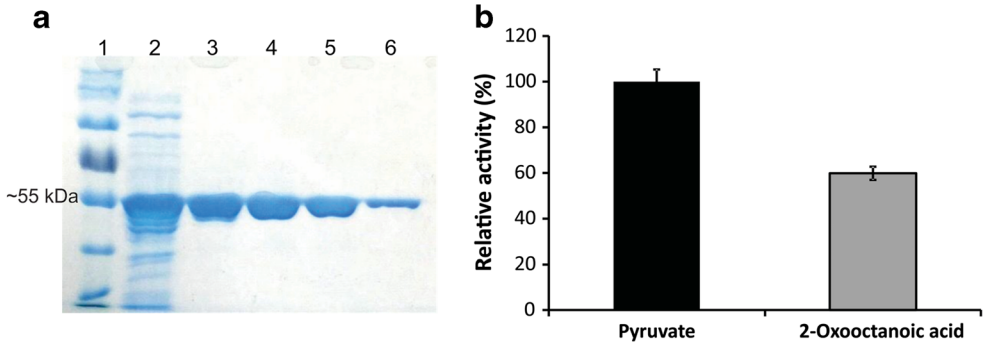

Fig. 1 **a** SDS-PAGE analysis of His-tagged CV_TA as it appears at ~ 50 kDa. Lane 1: PageRuler Pre-stained Protein Ladder (Fisher BioReagent); lane 2: cell free extract; lanes 3–6: fractions containing pure protein after affinity chromatography purification. **b** Comparison of the reaction rates of CV_TA towards 2.5 mM 2-OOA and pyruvate as amino acceptors and 2.5 mM of 1-PEA as amino donor with 22 μg purified protein/ml at 30 °C

15 mM and 2-OOA (recipient) to 10 mM resulted in the highest concentration of 2-AOA but at a lower efficiency of conversion compared to the same ratio but with 10 mM donor and 6 mM acceptor. We therefore scaled-up the reaction to 100 ml employing the donor:acceptor ratio of 1.7:1 with 10 mM donor and 6 mM acceptor, which produced 62.2 mg (4.1 mM) of 2-AOA (Supplemental information, Fig. S1).

A sequential addition of the substrates was investigated in order to further improve the reaction (Supplemental information, Fig. S2). The acceptor and the donor were added to the reaction to maintain the initial concentrations, i.e. 10 and 2.5 mM while maintaining the volume of the reaction. However, the highest achieved concentration of 2-AOA was only 2.8 mM (Supplemental information, Fig. S2), most likely due the inhibition of CV_TA (Kaulmann et al. 2007; Park et al. 2013b).

Purification and characterisation of Fmoc-2-AOA

After the optimal donor:acceptor ratio for the conversion of 2-OOA into 2-AOA was determined, the biotransformation was performed under optimal conditions in order to allow product purification and characterisation. After 180 min of the biotransformation, the reaction was stopped with 0.25% TFA and subjected to derivatisation with Fmoc-Cl. The derivatised amino compounds were separated from the rest of the reaction mixture by HPLC and the fraction containing the derivatised Fmoc-2-AOA collected. The collected fraction was lyophilised, resuspended in water and re-run on HPLC to verify the purity (Supplemental information, Fig. S3). High-resolution mass spectrometry confirmed the presence of purified Fmoc-2-AOA (ESI-TOF) m/z: $[M + Na]^+$ Calcd for $C_{23}H_{27}NO_4Na$ 404.1838; Found 404.1848. The sample of Fmoc-2-AOA purified from the biotransformation mixture was then analysed using a chiral HPLC column (CHIRALPACK IC). A standard for (*S*)-Fmoc-2-AOA (Eurogentec, Belgium) is > 98% *ee* and elutes from the column at 12.035 min (Fig. 3b). The Fmoc-2-AOA purified from the biotransformation mixture eluted at time 11.985 (Fig. 3a). A trace of dibenzofulvene, Fmoc cleavage product is observed in the purified sample, suggesting a low level of cleavage (Fig. 3a, c). The observed rotation of the commercial and produced and purified Fmoc-2-AOA was − 22°. Chiral analysis and specific rotation strongly suggest that the produced compound is the *S*-enantiomer with > 98% *ee*.

Table 2 Kinetic constants of purified CV_TA towards pyruvate and 2-aminooxanoic acid (2-OOA) with 10 mM of (*S*)-(-)-1-phenylethylamine (1-PEA) as amino donor. CV_TA exists as a homodimer with two active sites. The K_{cat} is therefore expressed per active site

Acceptor	K_{cat} (min^{-1})	K_m (mM)	K_{cat}/K_m (min^{-1} mM^{-1})
Pyruvate	52.3 ± 6.1	0.03 ± 0.01	1743.3
2-OOA	24.7 ± 4.4	0.41 ± 0.15	60.2

C- and N-terminal modification of a nine amino acid core of lactoferricin B with 2-AOA and testing of antimicrobial activity

The antimicrobial activity of LFcin B against a range of Gram-positive and Gram-negative bacteria is known (Ulvatne et al. 2001). This peptide likely exhibits its antimicrobial activity via membrane perturbation (Haukland et al. 2001); however, the mechanism is not completely understood. A region of the LFcin B rich with cationic amino acids, RRWQWRMKK (Wakabayashi et al. 1999), is important for the antimicrobial activity of LFcin B (Ulvatne et al. 2001) and was modified by the addition of 2-AOA on either N- or C- terminus. The antibacterial activities of the modified peptides were tested against Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative bacteria (*E. coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*) using the standard microdilution method (EUCAST 2003). The MIC values, which are defined as the lowest concentration that inhibits the growth of microorganisms after 18 h of incubation with a peptide, are summarised in Table 4.

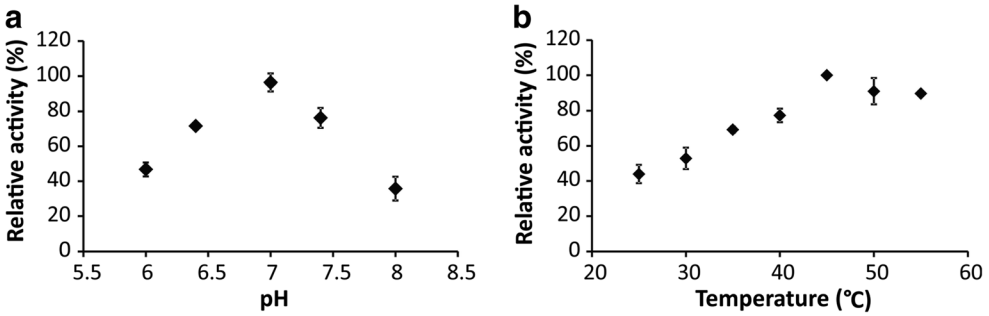

Fig. 2 Effect of pH (**a**) and temperature (**b**) on the activity of purified CV_TA. The optimum pH and temperature were determined in 100 mM potassium phosphate buffer. Activity was tested across the pH range at 30 °C and across the temperature range at pH 7.0. CV_TA activity was determined by measuring acetophenone formation at A_{245}. Data is the average of triplicates (SD < 5%)

Both N- and C-terminal modifications of the nonameric peptide showed improved antimicrobial activity towards the tested bacterial strains (Table 4, Fig. 4). The most profound effect was observed with RRWQWRMKK-2-AOA, with MICs determined for all tested strains, while unmodified peptide inhibited only the growth of *E. coli* at 400 μg/ml (Table 4). Only the C-terminally modified peptide exhibited a bactericidal effect on *S. aureus* at 400 μg/ml (Table 4). The N-terminal modified peptide inhibited growth of *E. coli*, *S. typhymirium* and *B. subtilis* at 100, 200 and 400 μg/ml, respectively (Table 4). Furthermore, even concentrations below the MIC for C- and N-terminally modified peptides reduced growth of the tested strains up to fourfold when compared to the growth in the presence of the unmodified peptide (Fig. 4).

Discussion

For the first time, we demonstrate the process encompassing the synthesis of an enantiomerically pure unnatural amino acid 2-aminooctanoic acid (2-AOA), its subsequent purification by HPLC and use to terminally modify and improve the activity of an antimicrobial peptide. Unnatural amino acids are a valuable toolbox for drug creation, particularly in peptide research. Microbial transaminases (TAs) are well established for the synthesis of chiral amines such as amino acids (Mathew and Yun 2012; Park et al. 2013a). We have demonstrated here the potential to use an ω-TA form *Chromobacterium violaceum* to synthesise a lipophilic unnatural amino acid, 2-AOA using 2-oxooctanoic acid (2-OOA) as an acceptor in a reductive amination reaction. The transamination reaction is reversible, and the desired product formation is therefore determined by the thermodynamic equilibrium constant and the initial concentrations of substrates (Tufvesson et al. 2011). The kinetics of a TA-catalysed reaction could be changed by employing an amino donor such as 1-phenyethylamine (1-PEA) in excess (Busto et al. 2014; Tufvesson et al. 2011). The optimal donor:acceptor ratio seems to be dependent on the transaminase and substrates used. Recently identified transaminases named pQR118 and pQR1114 showed the best conversion yield at 1-PEA:cyclohexanecarboxaldehyde 5:1 ratio, while pQR1113 showed the same conversion yield at 2:1, 3:1 and 5:1 ratios (Baud et al. 2017). The best conversion of the acceptor into 2-AOA employing CV_TA was obtained with a 1-PEA:2-OOA ratio of 4:1 (Table 3). However, to reduce the amount of unused donor, we have tested the effect of decreasing 1-PEA:2-OOA ratio. The highest production of 2-AOA with 46% conversion of the donor into acetophenone and 68% efficiency in conversion of 2-OOA to 2-AOA was observed with 1-PEA:2-OOA 1.7:1 ratio (Table 3, entry 2). Increasing the concentration of both donor and acceptor while maintaining the 1.7:1 ratio however resulted in decreased conversion yield for both 1-PEA and 2-OOA to 42 and 62%, respectively

Table 3 The effect of the ratio of the amino donor (1-PEA) to the amino acceptor (2-OOA) in the production of 2-AOA

Starting concentration			After 180 min of the reaction		
Ratio	1-PEA (mM)	2-OOA (mM)	Consumed 1-PEA (mM)	Produced 2-AOA (mM)	Conversion into 2-AOA (%)
4:1	10	2.5	2.3*	2.1	84
1.7:1	10	6	4.6	4.1	68
1.5:1	10	6.6	4.5	3.7	56
1.3:1	10	7.5	4.7	3.9	52
1.7:1	15	9	6.3	6.2	62

*All values are an average of three independent biological replicates with SD < 5%

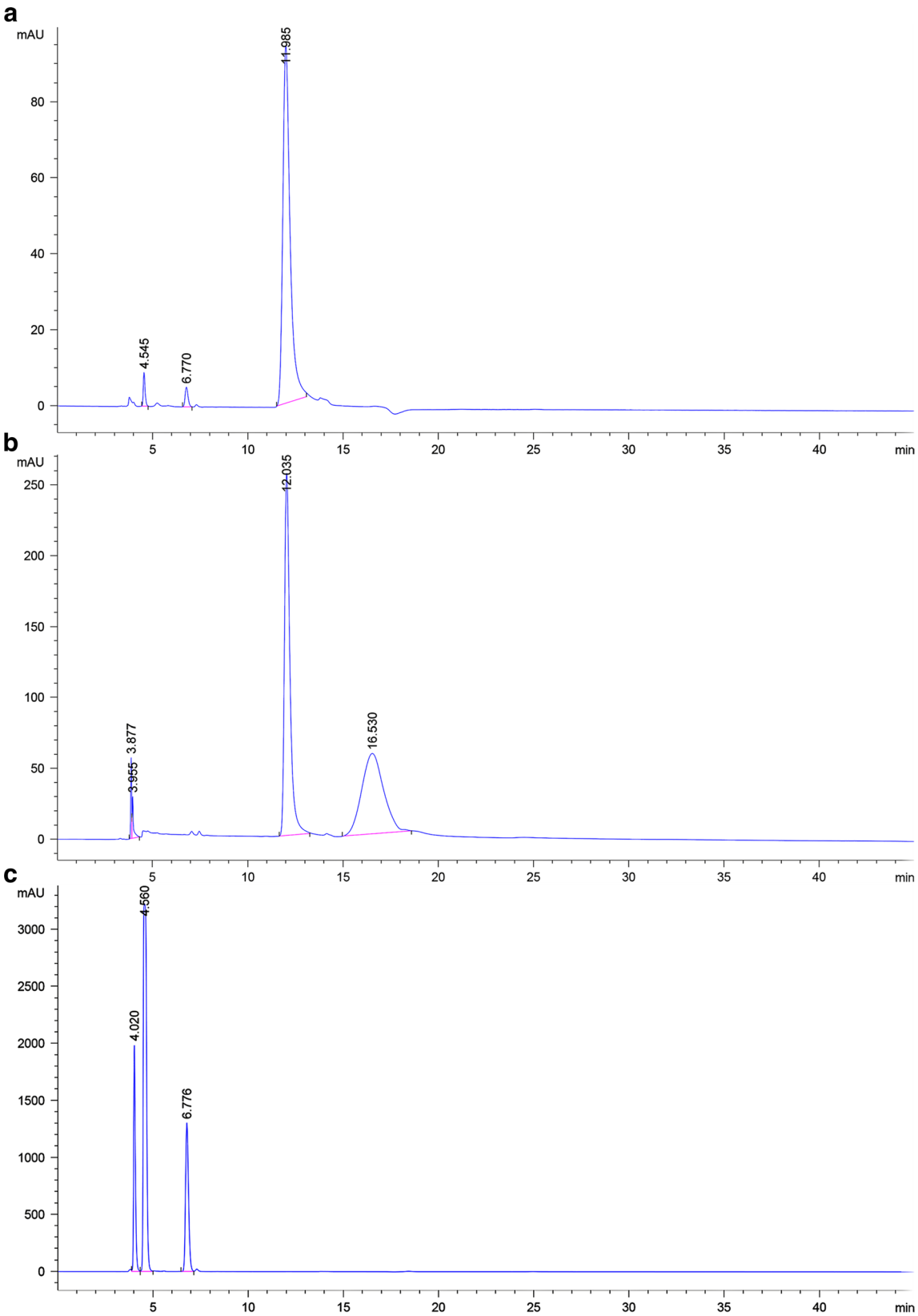

Fig. 3 Chiral HPLC analysis of Fmoc-2AOA purified from the biotransformation mixture (**a**), standard (*S*)-Fmoc-2-AOA (98% *ee*; (**b**)) and Fmoc-Cl (**c**)

Table 4 Antimicrobial activity of core nonameric region of LFcin B and its derivatives against different bacteria

AMP	MIC* (μg/ml)				
	E. coli	*P. aeruginosa*	*B. subtilis*	*S. typhimirium*	*S. aureus*
LFcin B (4-12) RRWQWRMKK	400	> 400	> 400	> 400	> 400
C-terminal modification RRWQWRMKK-(2-AOA)	25	200	50	100	400
N-terminal modification(2-AOA)-RRWQWRMKK	100	> 400	400	200	> 400

*MIC: Minimal inhibitory concentration was defined as the lowest concentration that inhibits the growth of microorganisms after 18 h of incubation with a peptide

(Table 3, entry 5). It has been reported that high concentration of substrates and products could inhibit the TA activity (Malik et al. 2012; Shin and Kim 2002). To avoid the potential inhibition of CV_TA by high starting concentrations of 1-PEA and 2-OOA, we have tested the sequential addition of the substrates. However, this strategy still resulted in the

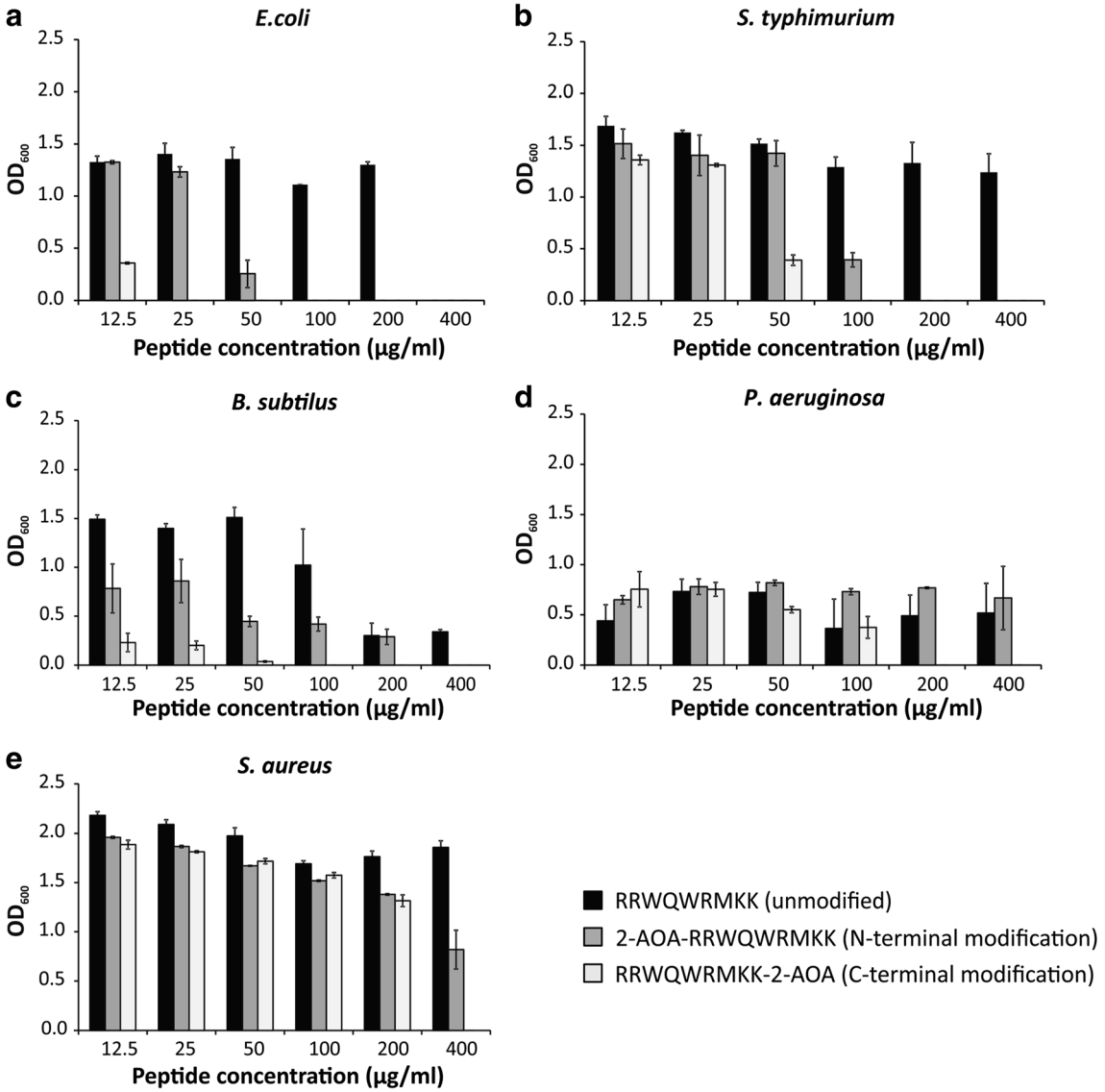

Fig. 4 The inhibitory effect of different concentrations of LFcin B (RRWQWRMKK), C-terminally modified LFcin B (RRWQWRMKK-2-AOA) and N-terminally modified LFicin B (2-AOA-RRWQWRMKK) on growth of *Escherichia coli* (**a**), *Salmonella typhimirium* (**b**), *Bacillus subtilis* (**c**), *Pseudomonas aeruginosa* (**d**) and *Staphylococcus aureus* (**e**). The controls (LB with 4% DMSO) reached OD_{600} of 1.7 for *E. coli*, 1.9 for *S. typhimirium*, 1.9 for *B. subtilis*, 2.1 for *P. aeruginosa*, and 2.1 for *S. aureus*

inhibition of CV_TA activity by substrates/products (Supplemental information, Fig. S2).

Derivatisation of amino compounds present in the reaction mixture with Fmoc-Cl allowed separation and purification of Fmoc-2-AOA, which could subsequently be used for peptide synthesis (O'Connor et al. 2013). The incorporation of unnatural amino acids in the sequence of an antimicrobial peptides (AMP) can alter the physicochemical properties of the peptide, increasing its activity, metabolic stability and selectivity (Bhonsle et al. 2013). AMPs are attractive as they offer a viable alternative to antibiotics due to their broad spectrum antimicrobial activity and the mechanism of action which reduces the possibility of acquired resistance (Aoki and Ueda 2013; Chen et al. 2014). The net positive charge and amphipathicity of AMPs are the most important characteristics determining the nature of AMP—bacterial cell interaction (Bhonsle et al. 2013). The molecular interactions between AMP and the cell are important for binding selectivity and the outcome of an antibacterial treatment (Ciumac et al. 2017). Cationic AMPs are positively charged peptides which show specific folding with the positively charged residues grouped on one side of the molecule and hydrophobic residues on the opposite side (Oren and Shai 1998).

N-terminal lipidation of peptides has been usually employed to modify the activity and selectivity of the peptide (Chu-Kung et al. 2010; Krishnakumari and Nagaraj 2015; Wakabayashi et al. 1999). Lipidation of a C-terminus however is rarely reported. C-terminal lipidation requires a linker, such as lysine (Albada et al. 2012), which has to be additionally modified. Our biocatalytic platform for the production of amino fatty acids offers the possibility to directly introduce a lipid tail at the C-terminus without the need for a linker, using standard techniques of peptide synthesis. Hydrophobicity is viewed as a highly desirable feature of AMPs, as it improves the interaction with bacterial membranes (Albada et al. 2012; Findlay et al. 2010; Wenzel et al. 2016).

Lactoferricin B (LFcin B) is a 25-mer formed by pepsin digestion of bovine lactoferrin and it has demonstrated antimicrobial activity against a range of bacteria (Bellamy et al. 1992; Gifford et al. 2005; Wakabayashi et al. 1999). The cationic region of LFcin B corresponding to residues R4-K12 is part of an α-helix (Hwang et al. 1998) and was modified with 2-AOA. This core nonamer of LFcin B is particularly interesting as it shows good anti-bacterial activity and low haemolytic activity (Liu et al. 2011). In the current study, C-terminal modification of the cationic region of LFcin B inhibited growth of all tested bacterial strains, showing the highest activity towards *E. coli* (MIC 25 μg/ml; Table 4). C-terminal modification of LFcin B has not been reported previously. Interestingly, N-terminally modified peptide exhibited lower activity compared with the C-terminally modified peptide but the N-terminal modification was not inhibitory as the latter exhibited better activity than the unmodified peptide (Table 4). For example, N-terminally modified peptide inhibited growth of *E. coli* at fourfold lower concentration compared with the unmodified peptide, and it also inhibited growth of *S. typhimirium* and *B. subtilis* at 200 and 400 μg/ml, respectively. *S. aureus* was the most resistant strain among tested bacteria, with only C-terminally modified peptide completely inhibiting its growth at 400 μg/ml (Table 4). The hydrophobic interactions between the peptides modified with 2-AOA and bacterial membranes were the likely reason for their increased antimicrobial activity (Dathe and Wieprecht 1999; Dathe et al. 1997; Wieprecht et al. 1997). Albada and co-workers reported that no significant difference in antimicrobial activity was observed when a short peptide was modified on either C- or N-terminus via acylated lysine residue (Albada et al. 2012). Our results demonstrate that C-terminal modification has a more profound effect on the antimicrobial activity but the reasons for this are not known. Increasing the hydrophobicity at C- rather than N-terminus has been reported to improve peptide and peptidomimetic selectivity (Dorner and Lienkamp 2013; Kragol et al. 2002).

In conclusion, CV_TA can be employed as a biocatalyst for the synthesis of an unnatural amino acid, 2-aminooctanoic acid. Both C- and N-terminal modifications of a cationic region of a known antimicrobial peptide lactoferricin B significantly improved the activity against Gram-positive and Gram-negative strains tested in this study, with C-terminal modification having a greater positive impact on antibacterial activity of the peptide. This study demonstrates the potential of the product of a transaminase catalysed reaction to be directly used for lipidation of C- and N-termini for the improvement of the activity of antimicrobial peptides.

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