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Production of the novel lipopeptide antibiotic trifluorosurfactin via precursor-directed biosynthesis

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ABSTRACT

Incorporation of fluorine into antibiotics can moderate their biological activity, lipophilicity and metabolic stability. The introduction of fluorine into an antimicrobial lipopeptide produced by *Bacillus* sp. CS93 via precursor-directed biosynthesis is described. The lipopeptide surfactin is synthesised non-ribosomally by various *Bacillus* species and is known for its biological activity. Administering 4,4,4-trifluoro-DL-valine to cultures of *Bacillus* sp. CS93 results in the formation of trifluorosurfactin in quantities sufficient for detection by LC–MS/MS. ^{19}F NMR analysis of the culture supernatant revealed that the bulk of the fluorinated amino acid was transformed and thus was unavailable for incorporation into surfactin. Detection of ammonia, and MS analysis indicated that the transformation proceeds with deamination and reduction of the keto acid, yielding 4,4,4-trifluoro-2-hydroxy-3-methylbutanoic acid.
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1. Introduction

Surfactin is a cyclic lipopeptide composed of a 3-hydroxy acid acyl chain of varying lengths (C10–C16) and heptapeptide with a primary sequence of L-Leu-D-Leu-L-Asp-L-Val-D-Leu-L-Leu-L-Glu. It is produced by *Bacillus subtilis*, and is an effective biosurfactant with a range of interesting properties and potential applications, including anti-mycoplasma, anti-clotting and anti-inflammatory activity, and it has been shown to improve biodegradation of pesticides in soil [1]. The biosynthesis of surfactin is via non-ribosomal peptide synthesis, and the genes of the surfactin synthase cluster have been sequenced and characterized [2]. The NRPS is composed of three subunits, SrfA, SrfB and SrfC, and the seven modules required for the synthesis of the heptapeptide are distributed across the subunits in a 3/3/1 fashion. Thus SrfA is responsible for the synthesis of Glu-Leu-D-Leu and SrfB for Val-Asp-D-Leu; SrfC incorporates the final Glu residue and contains a thioesterase domain (SrfTE) that is responsible for the macro-lactonisation of the 3-(*R*)-3-hydroxyacyl heptapeptide [3].

Modification of bioactive compounds is important for changing their physicochemical characteristics or improving their biological activity. One conceptually straightforward method to achieve structural diversity is precursor-directed biosynthesis, which involves supplementing the culture with a modified precursor of the natural product of interest. The modified precursor must be accepted as a substrate by the biosynthetic enzymes in order for it to be eventually incorporated into the product [4]. The incorporation of fluorine into biologically active compounds is of particular importance [5]; fluorine's small size means that its presence in a molecule has minimal steric consequences, but its electro negativity and the strength of the carbon–fluorine bond can have dramatic effects on, for example, receptor binding and metabolic stability. While de novo biosynthesis of organofluorine compounds is rare and fluorination of natural products using synthetic approaches can be challenging, precursor-directed biosynthesis has enabled the production of many natural products containing one or more fluorine atoms [6]. In this paper we describe our efforts to produce a trifluorinated derivative of surfactin using the biosynthetic approach, in the bacterium *Bacillus* sp. CS93, which was originally isolated from Pozol and produces a number of peptide antibiotics [7,8].

2. Results and discussion

2.1. Trifluorosurfactin production

Following the method of Moran et al. [9] 4,4,4-trifluoro-DL-valine (10 mg) was added to growing cultures of *Bacillus* sp. CS93, with little or no inhibition of growth observed. Upon direct analysis of the culture supernatants using electrospray ionisation mass spectrometry, $[M-H]^-$ ion m/z 1074.7, which is the expected mass of C-13 trifluorosurfactin, was observed in low

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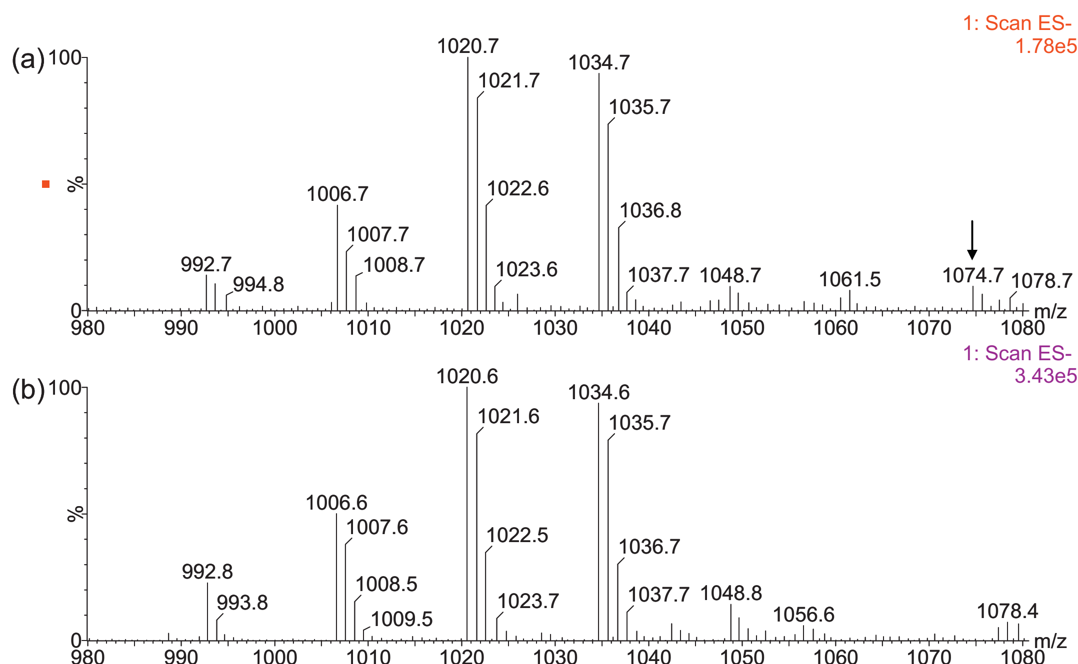


Fig. 1. Low resolution mass spectra (negative ion) of lipopeptide fractions from *Bacillus* sp. CS93. When the cultures were supplemented with trifluorovaline new ions were observed indicated by an arrow (a) compared with a control culture to which no fluoroamino acids was added (b).

abundance and was not present in supernatants of unsupplemented control cultures. This result suggested that incorporation of the precursor into surfactin had been achieved. When the amount of the amino acid added was doubled to 20 mg this ion was more prominent, suggesting increased production of C-13 trifluorosurfactin, and there was a new $[M-H]^-$ ion *m/z* 1061.5, which was possibly due to production of C-12 trifluorosurfactin. However, further additions of amino acid, either at the start of the incubation period, or made throughout growth, did not result in increased abundance (Fig. 1).

Accurate mass measurement of $[M-H]^-$ ion *m/z* 1074.7 using high resolution mass spectrometry determined an exact mass of 1074.6342, which is within the accuracy range for $C_{52}H_{87}N_7O_{13}F_3$, which is consistent with the empirical formula of C-13 trifluorosurfactin. Additional tandem mass spectrometry (MS/MS) experiments were then carried out on the ion *m/z* 1074.7 (Fig. 2b) and the fragmentation compared with that of the corresponding non-fluorinated surfactin (*m/z* 1020.7) (Fig. 2a). These results showed a common product ion *m/z* 339.2 formed due to the loss of Leu-Leu-Asp residues (*m/z* 339.2), while complementary lipopeptide fragments showed a difference of +54 Da illustrating the incorporation of trifluorovaline (*m/z* 732.5 vs. *m/z* 678.5). The loss of the β-hydroxy fatty acid from the resulting lipopeptide chain was shown by the fragment ion *m/z* 452.3 (-Glu-Leu-Leu-Val-), and a further loss of glutamate from the N-terminus was illustrated by the product ion *m/z* 323.2 (-Leu-Leu-Val-). Corresponding losses of these peptides containing trifluorovaline were observed at *m/z* 506.3 and *m/z* 377.2 respectively (Fig. 2a and b). Further MS/MS experiments on the sodiated ions of the same species (Fig. 2c and d) were carried out in the positive ion mode which showed sequence specific product ions following a ring-opening of the cyclic peptide at the ester site as proposed by Hue et al. [10]. The MS/MS data showed 54 Da heavier ions for b_5 and higher b ions and same mass change was reflected for y''_3 and higher y''-ions confirming the presence of triflurovaline (Table 1; Fig. 2c and e).

2.2. Biotransformation of 4,4,4-trifluoro-DL-valine

GC–MS analysis demonstrated that approximately 50% of the trifluorovaline remained in the culture after incubation. ^{19}F NMR analysis of culture supernatants revealed that in addition to 4,4,4-trifluorovaline (δ −69.1 and −70.9 ppm), there were two new resonances seen at δ −67.8 and −70.4 ppm (Fig. 3). However, these are unlikely to be trifluoro-surfactin, given the comparative insensitivity of NMR with MS. Since L-valine is incorporated into the growing peptide by surfactin synthase [12], trifluoro-L-valine incorporation is most likely; therefore the remaining trifluorovaline in the cultures is the D-*erythro*/*threo* isomers. The other fluorinated compounds observed probably arise from transformation of the bulk of the trifluoro-L-valine via amino acid metabolising enzyme(s). This would result in the bioavailability of the fluorinated amino acid for incorporation into surfactin being significantly diminished, and might account for the poor yields observed. The most probable biotransformation of an amino acid is transamination/deamination to yield its corresponding α-keto

Table 1
Expected product ions for the sodiated C-13 surfactins: M = C13-surfactin; M* = trifluorinated surfactin.

Product ions	*m/z*	$[(M-H)+Na]^+$ *m/z*	$[(M^*-H)+Na]^+$ *m/z*
b_1	338.2	360.2	360.2
b_2	451.3	473.3	473.3
b_3	564.4	586.4	586.4
b_4	679.4	701.4	701.4
b_5	778.5	800.5	854.5
b_6	891.5	913.5	967.5
$b_6 + H_2O$	909.5	931.5	985.5
y''_1	132.1	154.1	154.1
y''_2	245.2	267.2	267.2
y''_3	344.2	366.2	420.2
y''_4	459.3	481.3	535.3
y''_4-H_2O	441.3	463.3	517.3
y''_5	572.4	594.4	648.4
y''_6	685.4	707.4	761.4

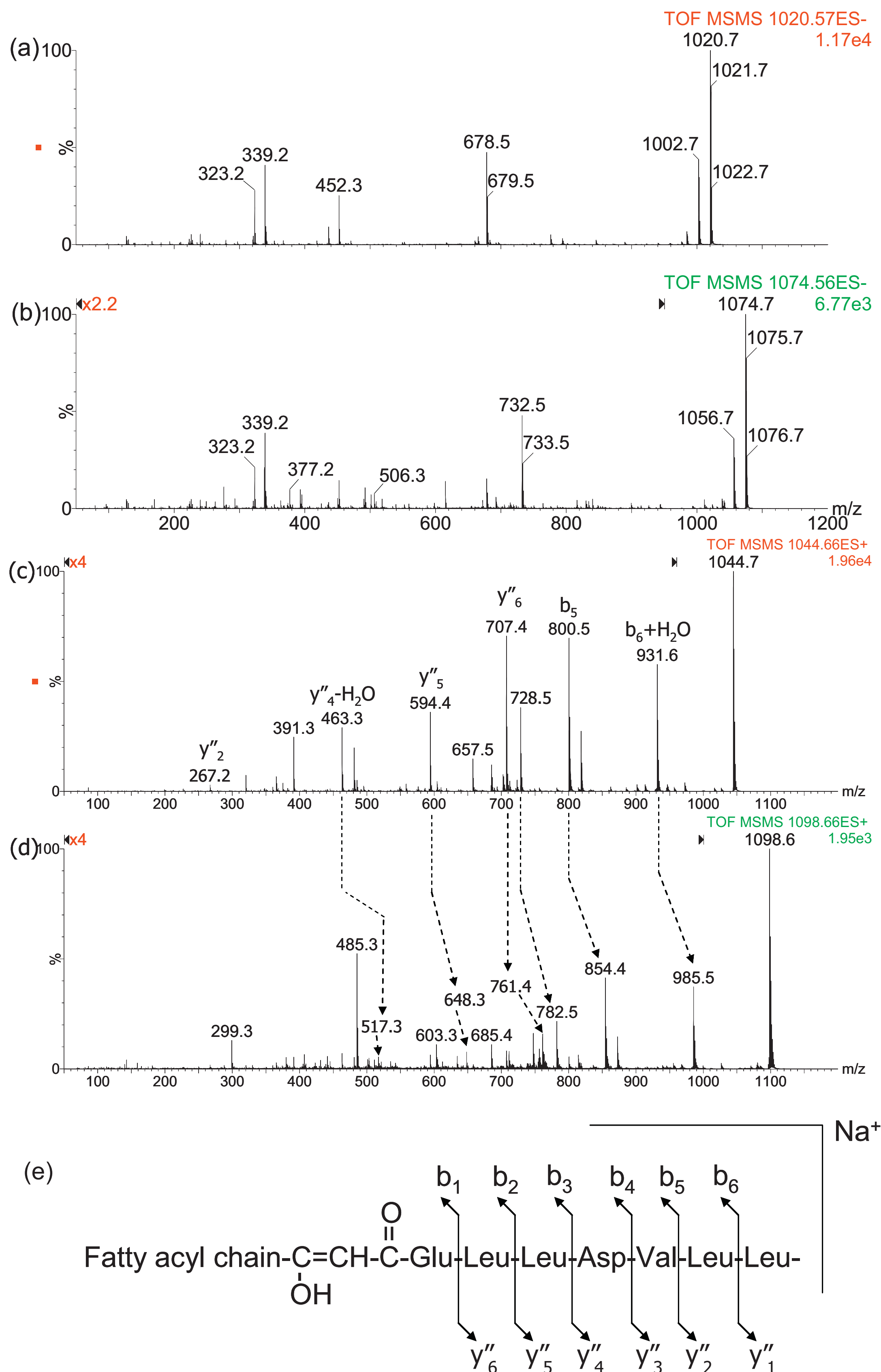


Fig. 2. Collision induced dissociation mass spectra of deprotonated C-13 surfactins and trifluorinated C-13 surfactins in negative ion mode [(a) and (b)]; MS/MS spectra of sodiated C-13 surfactins and trifluorinated C-13 surfactins in positive ion mode are shown in (c) and (d); the gain of 54 Da due to trifluorinated valine is shown with dotted arrows in (d); (e) shows the fragmentation pathway in positive ion mode where the incorporation of trifluorinated valine is identified by b_5 and/or higher b-ions and complemented by y_n-ions ($n \geq 3$).

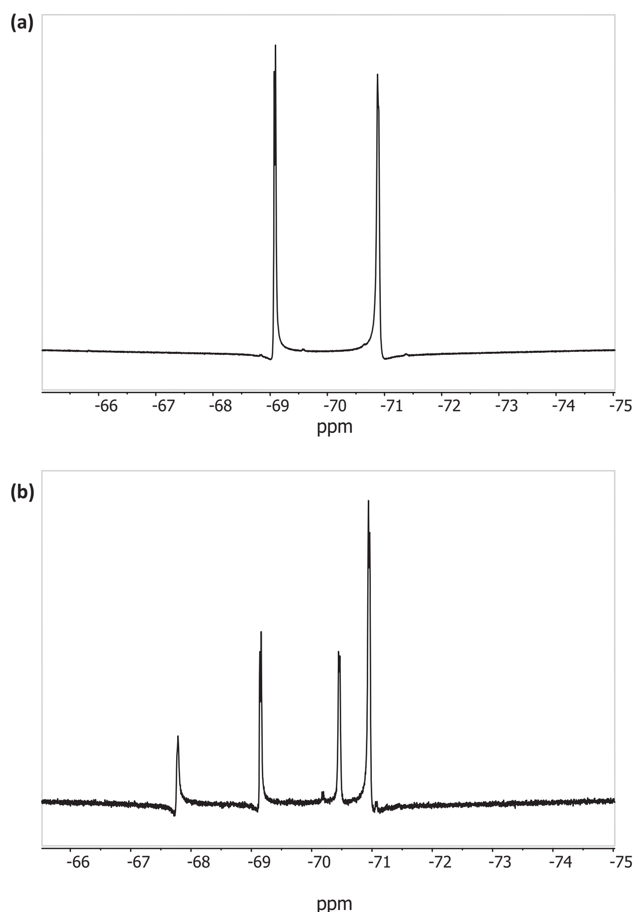


Fig. 3. ^{19}F NMR spectra of 4,4,4-trifluoro-DL-valine (a) and lipopeptide extract of CS93 culture after incubation with the fluorinated amino acid (b). The two signals seen in (a) are the *erythro* and *threo* diastereomers of 4,4,4-trifluoro-DL-valine [11].

acid. According to the Kyoto Encyclopedia of Genes and Genomes (KEGG), *B. subtilis* degrades L-valine to give the corresponding α-keto-acid via transamination catalysed by a branched-chain amino acid aminotransferase.

4,4,4-Trifluoro-DL-valine was fed to several different surfactin-producing strains of *B. subtilis* (NCTC 3610, NCIMB 8872, DSMZ 3256, ATCC 39096), two strains of *Staphylococcus aureus* and one strain of *Escherichia coli*, to determine if this biotransformation is unique to the CS93 strain. ^{19}F NMR analysis proved that the transformation seen in *Bacillus* sp. CS93 growing cultures also occurs in other surfactin-producing *Bacillus* species. However, the same products were not observed in the *E. coli* or *S. aureus* cultures, suggesting that the trifluorovaline transformation is restricted to *Bacillus* spp. There was a minor resonance (δ – 70.2 ppm) in the *S. aureus* and *E. coli* cultures that was most likely the result of an abiotic reaction as the same resonance was present in the supernatants of autoclaved cells after incubation with trifluorovaline.

Attempts were made to identify the enzyme(s) responsible for the biotransformation of trifluoro-valine so that an inhibition strategy could be developed, thereby increasing precursor supply for trifluorosurfactin synthesis. Commercially available branched chain amino-acid aminotransferase and L-amino acid oxidase were incubated with 4,4,4-trifluoro-DL-valine following assay conditions detailed by Taylor and Jenkins [13] and Schadewaldt et al. [14]. However, there was almost no change in the ^{19}F NMR spectra after enzyme treatment; in addition to the starting material a minor resonance at −120 ppm was observed, indicating a small degree of defluorination of the 4,4,4-trifluorovaline.

To qualitatively determine if deamination of the 4,4,4-trifluorovaline had occurred when the amino acid was incubated with *Bacillus* sp. CS93, culture supernatant was mixed with Nessler's reagent, resulting in a sharp yellow precipitate indicating the presence of ammonia (Figure S1). No precipitate was observed when supernatant from cultures without trifluorovaline was mixed with Nessler's reagent. Further analysis of the supernatant

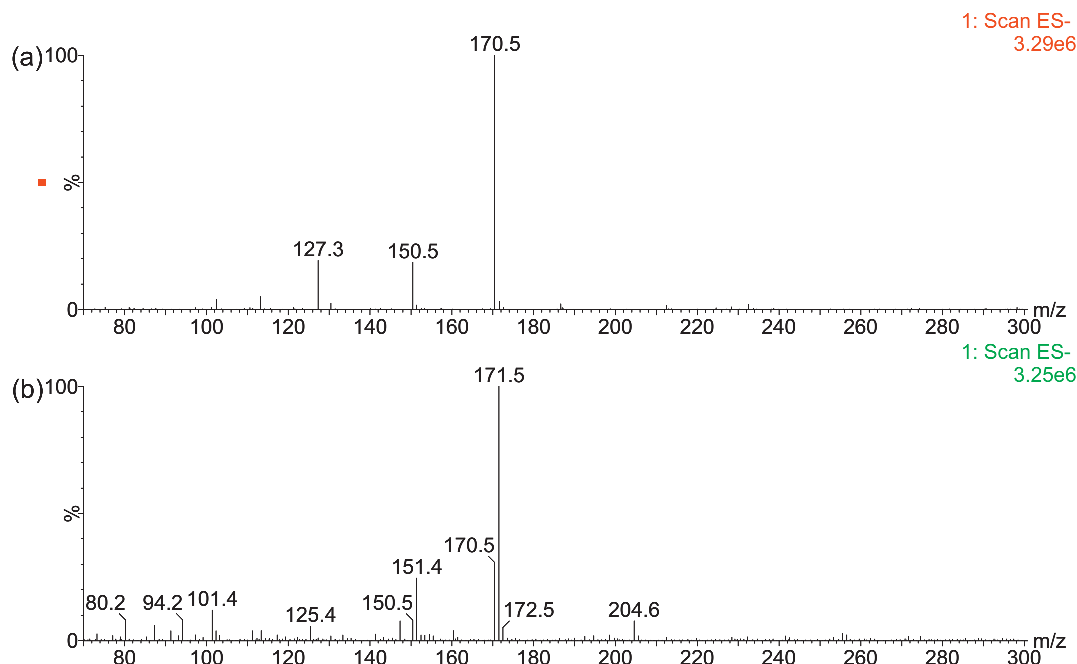
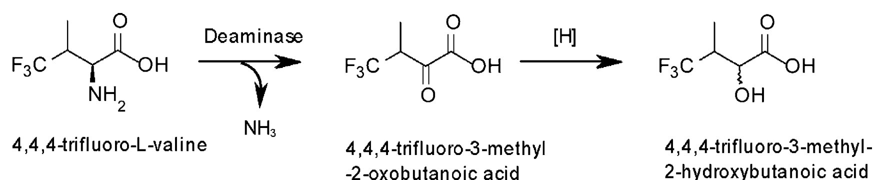


Fig. 4. Low resolution MS (negative mode) of authentic 4,4,4-trifluoro-DL-valine (a) and *Bacillus* sp. CS93 supernatant from culture supplemented with trifluorovaline (b). Ion m/z 170.5 diminished substantially after incubation with the bacterium, while there is a concomitant increase in ion m/z 171.5.



Scheme 1. Proposed biotransformation of 4,4,4-trifluoro-DL-valine in growing cultures of *Bacillus* sp. CS93.

was conducted via mass spectrometry to determine the masses of any new products that were formed. It was initially expected that ion m/z 169.5 would be apparent from the corresponding α-keto acid arising from deamination. However, this ion was not visible, but ion m/z 171.5 was easily detected that was not present either in controls from cultures in which no trifluorovaline was added, or a trifluorovaline standard (Fig. 4). This mass is that expected for the corresponding α-hydroxy acid; high resolution mass spectrometry determined the exact mass to be m/z 171.0261 ($C_5H_6O_3F_3$ gives 171.0269). Additionally, GC–MS analysis of the silylated supernatant revealed an analyte eluting after 2.16 min that was not present in unsupplemented controls (Figure S2). The fragment ions were one mass unit greater than those of silylated trifluorovaline. Therefore, the most likely transformation of trifluorovaline by *Bacillus* sp. CS93 is deamination to the keto acid followed by rapid reduction yielding 4,4,4-trifluoro-2-hydroxy-3-methylbutanoic acid (Scheme 1), consequently very little of the amino acid is incorporated into surfactin.

3. Conclusion

Trifluoro-containing amino acids have been incorporated into proteins in vivo [15,16], but not into any lipopeptide antibiotic. Here, we have demonstrated for the first time the production of small amounts of trifluorosurfactin by cultures of *Bacillus* sp. CS93 upon inclusion of 4,4,4-trifluoro-DL-valine in the culture medium. Thus the surfactin synthase must accept the trifluorinated amino acid as a substrate. However, the bulk of the trifluorovaline was transformed to 4,4,4-trifluoro-2-hydroxy-3-methylbutanoic acid, probably through the actions of a deaminase and reductase, thus only a small proportion of it was available for trifluorosurfactin synthesis, and the product was formed in such small amounts that no biological testing was possible. One possible strategy for improving the incorporation of trifluorovaline into the lipopeptide is to identify the enzyme responsible for the deamination step and either inhibit it or generate a knock-out mutant. However, the possibility exists that the enzyme is key for the primary metabolism in *Bacillus* sp. CS93, and its inhibition or knock out might be catastrophic for the cell.

4. Experimental

4.1. Chemicals and micro organisms

4,4,4-Trifluoro-DL-valine was purchased from Apollo Scientific. D_2O and Nessler's reagent were purchased from Sigma–Aldrich. *Bacillus* sp. CS93 was obtained from the Microbial Genomics and Bioprocessing Research Unit, National Centre for Agricultural Utilization Research, Peoria, IL. *S. aureus* IMD26 and IMD261 and *E. coli* were all obtained from the culture collection of the School of Biomolecular and Biomedical Science, University College Dublin.

4.2. Culture conditions

Bacillus spp. were cultivated on tryptic soya agar plates. Microorganisms were inoculated into 250 ml Erlenmeyer flasks containing 50 ml Fred Waksman Basic 77 medium, prepared according to the procedure described by Phister et al. [7] and incubated for 72 h with rotary agitation (200 rpm) at 37 °C. *E. coli* and *S. aureus* were cultivated using tryptic soy broth and incubated for 72 h with rotary agitation (200 rpm) at 37 °C.

4.3. Precursor directed biosynthesis and lipopeptide extraction

4,4,4-Trifluoro-DL-valine (10–30 mg) was added to 50 ml cultures of *Bacillus* spp. both immediately after inoculation and at intervals of 24 h (5 mg). Cultures were centrifuged (10,000 rpm, 15 min), the supernatant was acidified to pH 2 and allowed to stand at 4 °C for 12 h. The precipitate was collected by centrifugation and extracted with methanol. The methanol extracts were filtered after a further 12 h, dried and resuspended in water. Further purification was achieved by solid-phase extraction using a C-18 octadecyl bonded, silica-based column (Agilent Technologies). The lipopeptides were eluted with a stepwise water–methanol gradient, and the lipopeptides eluted with 90% methanol.

4.4. Analysis of lipopeptides and transformation products

Fluorine-19 nuclear magnetic resonance spectroscopy was used to analyse supernatants and lipopeptide extracts. All ^{19}F NMR analyses were conducted using a Varian Inova 400 MHz NMR spectrometer. Samples were dissolved in D_2O to provide a lock and 4098 scans were performed for each sample. Low resolution mass spectra were acquired on a Waters Quattro Micro tandem quadrupole mass spectrometer while the high resolution mass spectra were recorded with a Waters Q-Tof Premier coupled to Waters Alliance 2695 HPLC system. Quantification of trifluorovaline in cultures was carried out by gas chromatography–mass spectrometry (GC–MS) after forming the *N*-ethoxycarbonyl ethyl ester derivative [17]. Trimethylsilyl derivatives were also analysed by GC–MS (*N*-methy-*N*-(trimethylsilyl)trifluoroacetamide, 100 °C, 1 h); 4,4,4-trifluoro-DL-valine eluted at 2.44 min and had fragment ions m/z 198 (100%), 130 (5%), 272 (3.75%), 100 (3.5%), 300 (1.25%), 117 (1.25%). 4,4,4-Trifluoro-2-hydroxy-3-methylbutanoic acid eluted at 2.16 min had fragment ions of values m/z 131 (100%); 199 (44%); 301 (28%); 273 (14%); 117 (11%); 101 (9%).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jfluchem.2012.06.033.

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