

Sideromimic modification of Lactivicin dramatically increases potency against extensively
drug resistant *Stenotrophomonas maltophilia* clinical isolates

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Running Title: Sideromimic Lactivicin active versus *S. maltophilia*.

Summary

1 Acetamido derivatives of the naturally antibacterial non- β -lactam Lactivicin have
2 improved activity against their penicillin binding protein targets and reduced hydrolysis by
3 β -lactamases, but penetration into Gram-negative bacteria is still relatively poor. Here we
4 report that modification of the lactivicin (LTV) lactone with a catechol-type siderophore
5 increases potency 1000-fold against *Stenotrophomonas maltophilia*, a species renowned
6 for its insusceptibility to antimicrobials. The MIC₉₀ of the modified lactone LTV17 against a
7 global collection of extensively drug resistant clinical *S. maltophilia* isolates was 0.063
8 $\mu\text{g}.\text{ml}^{-1}$. Sideromimic modification does not reduce the ability of LTVs to induce L1/L2 β -
9 lactamase production in *S. maltophilia*, and does not reduce the rate at which LTVs are
10 hydrolyzed by L1 or L2. We conclude, therefore, that lactivicin modification with a
11 siderophore known to be preferentially used by *S. maltophilia* substantially increases
12 penetration via siderophore uptake. LTV17 has the potential to be developed as a novel
13 antimicrobial for treatment of infections by *S. maltophilia*. More generally, our work
14 shows that sideromimic modification in a species-targeted manner might prove useful for
15 the development of narrow spectrum antimicrobials that have reduced collateral effects.

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19 Introduction

20 Lactivicin is highly unusual in that it is the only non β -lactam natural product known to
21 target penicillin binding proteins (PBPs). Unlike the β -lactams, which remain the most
22 important antimicrobial class, Lactivicin contains cycloserine and γ -lactam motifs, but like
23 the β -lactams, Lactivicin reacts covalently with PBPs to form a stable acyl-enzyme complex
24 (1) (Fig. 1A and 1B). However, Lactivicin has poor penetration into Gram-negative bacteria
25 and is susceptible to at least some β -lactamase enzymes (2-4). A deeper understanding of
26 the interactions between Lactivicin and its derivatives and their various enzyme targets has
27 led to the rational design of synthetic derivatives with higher potency against bacteria and
28 reduced susceptibility to β -lactamases (1,5,6), including LTV13 which has the 'ATMO' type
29 side chain (6; figure 1C). Recently, it has been shown that sideromimic modification of the
30 Lactivicin LTV γ -lactone (4) results in more favourable IC_{50} values against *Pseudomonas*
31 *aeruginosa* PBPs and improved penetration into *P. aeruginosa* strain PA01 via interaction
32 with the siderophore receptors and uptake systems of this strain. One of these Lactivicin
33 derivatives is the phthalimide-conjugated compound 17 (hereafter referred to as LTV17;
34 figure 1C) (6).

35 Here we describe the activity of Lactivicin derivatives against *Stenotrophomonas*
36 *malophilia*, which is an important nosocomial pathogen, primarily causing bloodstream and
37 respiratory tract infections in severely debilitated patients. *S. maltophilia* also causes
38 sporadic urinary tract and ocular infections and is a coloniser of the lungs of a significant
39 proportion of adult patients with Cystic Fibrosis. Clinical isolates of *S. maltophilia* are
40 notoriously resistant to antimicrobial drugs, with resistance to most β -lactams, quinolones
41 and aminoglycosides being an intrinsic property of the species (7,8). Intrinsic resistance

42 mechanisms in *S. maltophilia* include the expression of antibiotic modifying enzymes, e.g.
43 two β -lactamases, L1 and L2, which together can hydrolyse all known β -lactams (9-11); and
44 multi-drug efflux pumps, most notably SmeDEF (12,13), SmeVWX (14,15), SmeYZ and
45 SmeIJK (16).

46

47 **Materials and Methods**

48 *Bacterial isolates and materials*

49 *S. maltophilia* clinical isolates used in this study either originated from the SENTRY
50 antimicrobial resistance survey, as previously described (17), or were isolated from patients
51 being treated at the Bristol Oncology Centre (18). Isolates of other species were either
52 obtained from type strain collections, or were clinical isolates collected by SENTRY and
53 gifted by Dr Mark Toleman, Cardiff University, or have previously been described (19).
54 Growth media were from Oxoid, Chemicals were from Sigma, unless otherwise stated.
55 LTV13 was synthesized according to the literature protocol (6). LTV17 was kindly supplied by
56 Pfizer.

57

58 *β -Lactamase induction and measurement of β -lactamase activity in cell extracts*

59 MICs were determined using CLSI broth microtiter assays (20) and interpreted using
60 published breakpoints (21). For β -lactamase induction assays, an overnight culture of
61 bacteria was diluted to an optical density at 600 nm (OD_{600}) of 0.1 in nutrient broth and
62 grown at 37°C until OD_{600} was 0.4. Inducer (100 $\mu\text{g}.\text{ml}^{-1}$ ceftazidime, 10 $\mu\text{g}.\text{ml}^{-1}$ imipenem, 50

63 $\mu\text{g.ml}^{-1}$ LTV13 and $0.35 \mu\text{g.ml}^{-1}$ LTV17) was then added and incubation continued for 2 h
64 before cell extracts were prepared and β -lactamase activity in cell extracts measured as
65 described (22) using $100 \mu\text{M}$ meropenem as a substrate. Protein concentrations were
66 determined using the BioRad protein assay dye reagent concentrate, and an extinction
67 coefficient at 299 nm of 9600 AU/M/cm for meropenem was used to calculate the specific
68 meropenem hydrolysing activity in each cell extract.

69

70 *Expression and purification of L1 and L2*

71 Recombinant L1 protein was produced in *Escherichia coli* and purified as previously
72 described (23). For L2 protein production the putative signal sequence (residues 1-27) was
73 'removed' by amplifying positions 82-912 from the L2 gene of *S. maltophilia* K279a genomic
74 DNA with forward and reverse primers 5'- AAGTTCTGTTTCAGGGCCCGCGGGCAAGGCCAC-3'
75 and 5'-ATGGTCTAGAAAGCTTTATCCGATCAACCGGTCGGC-3'. Primer sequences included
76 extensions which allowed recombination into the pOPINF vector (24), resulting in a
77 construct encoding for L2 with an N-terminal hexa-His tag, with the tag being cleavable with
78 rhinovirus 3C protease. The resultant plasmid was designated pOPINF-L2 Δ 27. For protein
79 overproduction, *E. coli* SoluBL21 (DE3) cells (Genlantis) bearing pOPINF-L2 Δ 27 were grown
80 in 2xTY medium containing ampicillin ($50 \mu\text{g.mL}^{-1}$) to an OD_{600} of 0.9 at 37°C . Protein
81 production was induced with 1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) at 18°C
82 for 16 h. All subsequent purification steps were at 4°C . Cells were harvested by
83 centrifugation ($6500 \times g$, 10 min) and resuspended in 50 mM Tris pH 7.5, 150 mM NaCl,
84 1 mM tris(2-carboxyethyl)phosphine (TCEP) supplemented with EDTA-free protease
85 inhibitor (Roche). Cells were lysed by two 30,000 psi passages through a cell disruptor. After

86 centrifugation at 100,000 x *g* for 45 min, the supernatant was incubated for 1.5 h with Ni-
87 NTA resin (Qiagen). The resin was washed with buffer A (50 mM Tris pH7.5, 400 mM NaCl, 1
88 mM TCEP) plus 10 mM imidazole, then buffer A plus 0.1% v/v Triton X-100, then buffer A
89 plus 20 mM imidazole. Protein was eluted with 50 mM Tris pH 7.5, 200 mM NaCl, 400 mM
90 imidazole, 1 mM TCEP. Imidazole and NaCl concentrations were reduced to 5 mM and 150
91 mM, respectively, in an Amicon 10 kDa molecular weight cut-off (mwco) concentrator and
92 the His tag was then removed by overnight incubation with His-tagged 3C protease. The
93 digestion mixture was then incubated with Ni-NTA resin for 30 mins; the flowthrough
94 containing purified L2 was collected and concentrated to 30 mg.ml⁻¹ using a 10 kDa mwco
95 Amicon concentrator.

96

97 *Assay of lactivicin hydrolysis by NMR*

98 ¹H NMR spectra were acquired at 298 K on a Bruker AVIII 700 spectrometer with ¹H/¹³C/¹⁵N
99 TCI cryoprobe. The data were recorded employing a pulse sequence with water suppression
100 (excitation sculpting with gradients using perfect echo) (25,26). Spectra were collected with
101 11161 Hz sweep width, 2 s relaxation delay, 65536 data points, and 16 scans. For data
102 processing line broadening of 0.3 Hz was applied. The NMR samples were prepared in 50
103 mM TRIS-d₁₁ buffer pH 7.5, supplemented with 10% D₂O. LTV13 was prepared as a 100 mM
104 stock in H₂O and LTV17 as a 25 mM stock in 50% H₂O and 50% DMSO and diluted to a final
105 concentration of 0.4 mM. Final concentrations of L1 and L2 were 150 nM.

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107

108 **Results and Discussion**

109 *MICs of Lactivicin derivatives against S. maltophilia clinical isolates*

110 MICs for LTV13 and LTV17 were determined against a selection of clinical isolates
111 representing key Gram-negative species where multi-drug resistance is frequently a
112 problem. These data confirmed the antibacterial potential of LTV17; its MIC against the
113 multi-drug resistant *S. maltophilia* bloodstream isolate K279a (18) is remarkably low, and
114 more than 1000 fold more potent than LTV13, an improvement in potency greater than
115 against any other test species (Table 1).

116 According to published CLSI susceptibility testing performance standards, there are only six
117 antimicrobials that have potential for treatment of *S. maltophilia* and for which resistance
118 breakpoints have been defined (21). The current drug of choice is trimethoprim-
119 sulfamethoxazole (SXT) and there are five alternatives: ceftazidime, ticarcillin-clavulanate,
120 minocycline, levofloxacin and chloramphenicol. We used the CLSI performance standards to
121 define resistance phenotypes for a collection of 50 clinical *S. maltophilia* isolates from
122 around the world (11,13,17). Initially, the isolates were divided into two groups: 23/50 that
123 are STX resistant and 27/50 that are STX sensitive. The two groups were then sub-divided
124 based on how many alternative antimicrobials they remain sensitive to. Finally, the MICs of
125 LTV13 and LTV17 were determined against the 50 isolates using standard CLSI broth micro-
126 dilution methodology (Table 2). Clearly, LTV17 is very potent against all of these *S.*
127 *maltophilia* clinical isolates, including extensively drug resistant strains. The highest MIC
128 seen was 0.25 $\mu\text{g}.\text{ml}^{-1}$, and the MIC₉₀ for the 50 isolates was 0.063 $\mu\text{g}.\text{ml}^{-1}$.

129

130 *Induction of β -lactamase production in *S. maltophilia* by Lactivicin derivatives.*

131 One of the reasons for the initial failure of Lactivicin and its early derivatives as an antibiotic
132 was susceptibility to β -lactamases, coupled with an ability to induce β -lactamase production
133 in bacteria where inducible enzymes exist (2-4). *S. maltophilia* has two inducible β -
134 lactamases, L1 and L2, which are co-ordinately controlled by an AmpR type transcriptional
135 regulator (27). Genetic disruption of one of the main targets of Lactivicin, PBP1A, has been
136 shown to constitutively activate β -lactamase production in *S. maltophilia* (22,28). As
137 expected, we found that treatment of two well characterised *S. maltophilia* clinical isolates,
138 K279a and N531 (18), with LTV13 or LTV17 induced β -lactamase production to a similar
139 extent as the β -lactam antibiotics cefoxitin and imipenem when added to growing cells at
140 concentrations proportionate to the compounds' relative MICs (Fig. 2).

141

142 *Breakdown of Lactivicin derivatives by *S. maltophilia* β -lactamases.*

143 Since LTV13 and LTV17 both strongly induce L1 and L2 β -lactamase production, one
144 explanation for the increased potency of LTV17 versus LTV13 is that LTV17 is not such a
145 good substrate as LTV13 for the *S. maltophilia* β -lactamases. To test this hypothesis, NMR
146 spectroscopy was used to evaluate the time-dependent hydrolysis of LTV13 and LTV17 by
147 purified recombinant L1 and L2 β -lactamases. L2 was able to totally hydrolyse 400 μ M
148 ampicillin in less than 5 min (data not shown), whilst ~95% of the LTV13 and LTV17
149 remained intact. Longer incubation times confirmed that LTV13 and LTV17 are not
150 substrates for L2. They were both found to be substrates for L1 β -lactamase, however, and
151 underwent enzyme catalysed hydrolysis (Fig. 3). LTV17 was broken down faster than LTV13,

152 so reduced susceptibility to the L1 β -lactamase does not explain the increased potency of
153 LTV17 against *S. maltophilia*.

154

155 *Protection of S. maltophilia from Lactivicin derivatives by β -lactamases*

156 Whilst they are clearly substrates, the rate of LTV17 and LTV13 hydrolysis by L1 was very
157 slow compared with that of meropenem (L1 was able to totally hydrolyse 400 μ M
158 meropenem in less than 5 min using similar assay conditions when ~95 and 90% of LTV13
159 and 17 were still intact – data not shown), so we hypothesised that LTV13 and LTV17
160 actually kill *S. maltophilia* even though they induce β -lactamase production simply because
161 cellular β -lactamase hydrolysis is too slow to protect the cells. To test this hypothesis, we
162 incubated LTV17 with or without purified L1 and spotted the two mixtures onto a lawn of *S.*
163 *maltophilia* K279a. In parallel we used meropenem as a control (Fig. 4). Incubation with L1
164 does not significantly reduce the ability of LTV17 to kill *S. maltophilia* K279a (and also LTV13
165 – data not shown) where meropenem is rendered totally ineffective by L1. Pre-incubation of
166 LTV17 with L1 for 1 h prior to spotting onto K279a did reduce the zone of clearing,
167 suggestive of modest destruction of LTV17 as shown in the NMR experiments. Importantly,
168 the inhibition zone diameter for LTV17 (and LTV13 – data not shown) is the same against
169 K279a as it is for the *ampR* frameshift mutant derivative K279a::*ampR*^{FS} which cannot
170 induce β -lactamase production (27), even though the latter is far more sensitive to
171 meropenem (Fig. 4). These results imply that even though L1 can break down LTV17 at a
172 relatively modest rate (Fig. 3) its induction by LTV17 is not sufficient to protect the cell. This
173 also explains why *S. maltophilia* clinical isolates in our world-wide collection that are known
174 to express β -lactamase constitutively at high levels (11,22) are no less susceptible to the

175 Lactivicin derivatives than are isolates with normally inducible β -lactamases (Table 2).
176 Indeed, to confirm this, we tested four L1/L2 hyper-producing mutants previously derived
177 from *S. maltophilia* K279a (22,27,29) and found that the MICs of both Lactivicin derivatives
178 against K279a and these mutants are the same (32 and $\leq 0.031 \mu\text{g}\cdot\text{ml}^{-1}$ respectively for
179 LTV13 and LTV17).

180

181 *Conclusions*

182 The reason for the dramatically increased potency of LTV17 versus LTV13 against *S.*
183 *maltophilia* is not due to its relatively weak ability to induce L1/L2 β -lactamase production
184 or its relatively slow hydrolysis by either of these β -lactamases. Both LTV17 and LTV13 are
185 only slowly hydrolyzed by L1 β -lactamase, and not at a detectable level by L2, so β -
186 lactamase production by *S. maltophilia* is not actually protective against either Lactivicin
187 derivative. Accordingly, whilst we have not excluded the possibility that there is some
188 increased affinity for its PBP target(s), the 1000-fold increased potency of LTV17 over LTV13
189 is most likely to be due to an increased rate of entry into *S. maltophilia*. The major
190 difference between LTV13 and LTV17 is the presence of a catechol-type siderophore on the
191 lactone ring of LTV17 (6). Notably, all *S. maltophilia* clinical isolates previously tested,
192 including the K279a isolate used here, exclusively produce catechol-type siderophores (30).
193 Thus it is reasonable to infer that they preferentially take up this type of siderophore, and
194 the antibiotics conjugated to them. Accordingly, it would appear that the siderophore used
195 for LTV17 particularly favours uptake by *S. maltophilia*, explaining its remarkable potency
196 against this otherwise extensively drug resistant bacterium. This observation is important
197 because it implies that side chain modification of the core fused bicyclic non β -lactam ring

198 system of the Lactivicins has the potential to improve activity in the same way as it has done
199 for the β -lactams, e.g. BAL30072, which is in early phase clinical development. This can work
200 by increasing potency versus PBPs and/or reducing β -lactamase susceptibility, and in
201 addition by improving uptake (31). Moreover, the results presented in Table 1 suggest that
202 siderophore mediated uptake is not a general effect, equally seen in all species. It may be
203 that the apparent species-specificity of the effect seen is dependent on the conjugation of a
204 particular siderophore preferentially used by a particular species. In an era of improved
205 diagnostics for infection (32), the routine use of narrow-spectrum antimicrobials is
206 becoming a realistic proposition, and the benefit would be reduced collateral damage to the
207 host microbiome, and cross-selection for the acquisition of resistant isolates of other
208 species of bacteria.

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Table 1. MICs of Lactivicin derivatives against clinical Gram negative isolates.

Isolate	LTV13 MIC ($\mu\text{g}.\text{ml}^{-1}$)	LTV17 MIC ($\mu\text{g}.\text{ml}^{-1}$)
<i>Klebsiella pneumoniae</i> NCTC5055	2	0.5
<i>Citrobacter freundii</i> D571	512	16
<i>Enterobacter cloacae</i> 30950	512	16
<i>Enterobacter aerogenes</i> 15-8358A	64	4
<i>Serratia marcescens</i> NCTC1041	4	4
<i>Pseudomonas aeruginosa</i> NCTC10662	256	1
<i>Stenotrophomonas maltophilia</i> K279a	64	≤ 0.0625
<i>Acinetobacter baumannii</i> 39-4034C	256	8

Table 2. MICs of Lactivicin derivatives against *S. maltophilia* clinical isolates having different profiles of resistance to last resort antibiotics.

Resistance phenotype	Number of isolates where LTV13 MIC ($\mu\text{g}.\text{ml}^{-1}$) is:					Number of isolates where LTV17 MIC ($\mu\text{g}.\text{ml}^{-1}$) is:			
	16	32	64	128	256	≤ 0.031	0.063	0.125	0.25
SXT ^R . Sensitive to 0 to 2 alternatives	0	0	3	5	1	5	3	0	1
SXT ^R . Sensitive to 3 to 5 alternatives	1	0	7	6	0	13	1	0	0
SXT ^S . Sensitive to 1 to 3 alternatives	0	0	4	9	1	8	3	3	0
SXT ^S . Sensitive to 4 or 5 alternatives	0	5	3	5	0	12	1	0	0

217 **Figure Legends**

218 **Figure 1: Structures and mechanism of action of β - and γ -lactams.** A) Structures of β -lactam
219 antibiotics and the cycloserine derivative Lactivicin; B) Outline of mechanism of action of
220 LTV derivatives against penicillin binding proteins; C) Structures of LTV13 and LTV17 used in
221 the current study.

222

223 **Figure 2: Effect of β -lactams and Lactivicin derivatives on the production of β -lactamase by**
224 ***S. maltophilia* clinical isolates.** Bacteria were incubated in the presence of inducer (100
225 $\mu\text{g.ml}^{-1}$ ceftiofur, 10 $\mu\text{g.ml}^{-1}$ imipenem, 50 $\mu\text{g.ml}^{-1}$ LTV13 and 0.35 $\mu\text{g.ml}^{-1}$ LTV17), cell
226 extracts were prepared and β -lactamase activity in cell extracts was measured as described
227 (22) using 100 μM meropenem as a substrate. Protein concentrations were determined
228 using the BioRad protein assay dye reagent concentrate, and an extinction coefficient at 299
229 nm of 9600 AU/M/cm for meropenem was used to calculate the specific meropenem
230 hydrolysing activity in each cell extract. Data presented are means \pm standard error of the
231 mean, $n=3$.

232

233 **Figure 3** L1 catalysed turnover of Lactivicin derivatives as observed by ^1H NMR. A – Overlay
234 of ^1H NMR spectra of LTV-13 before addition of L1 and after 1.5 h of incubation with L1. B –
235 Overlay of ^1H NMR spectra of LTV17 before addition of L1 and after 1.5 h of incubation with
236 L1. C – Time course of L1 mediated hydrolysis of LTV13 and LTV17.

237

238 **Figure 4. Inhibition zone produced by LTV17 and meropenem with and without L1 β -**239 **lactamase.** A lawn of *S. maltophilia* K279a or the *ampR* frameshift mutant (*ampR*^{FS}) was240 spread as if for CLSI disc susceptibility testing (using a 10⁸ cfu/ml suspension of bacteria). In241 all cases, 2 μ L of reaction mixture (50 mM Tris pH 7.5 with meropenem (MER, 12 mM) or242 LTV17 (0.4 mM) mixed (+L1) or not (-L1) with purified L1 β -lactamase (1 μ M final) was

243 immediately spotted onto the lawn (t=0 h) or was spotted following 1 h of pre-incubation at

244 37°C (t=1 h). The plate was then incubated for 18 h at 37°C and this figure is representative

245 of three biological replicates.

Figure 1

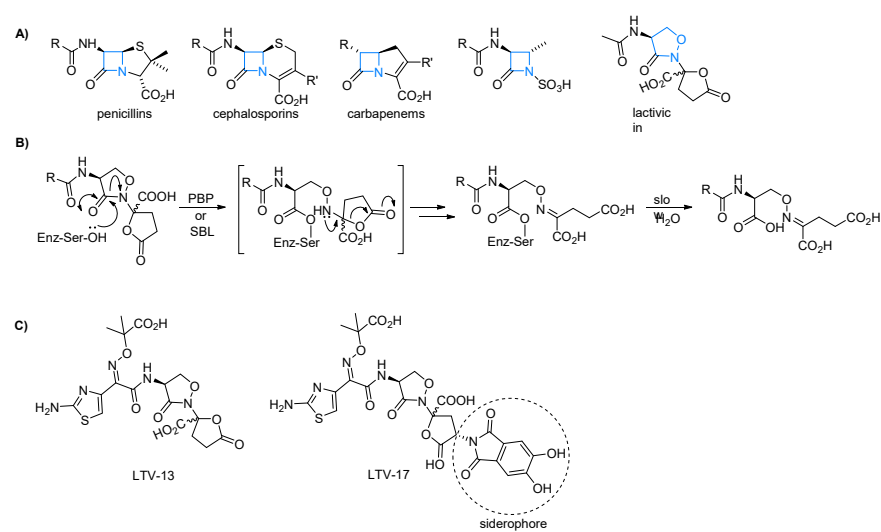


Figure 2

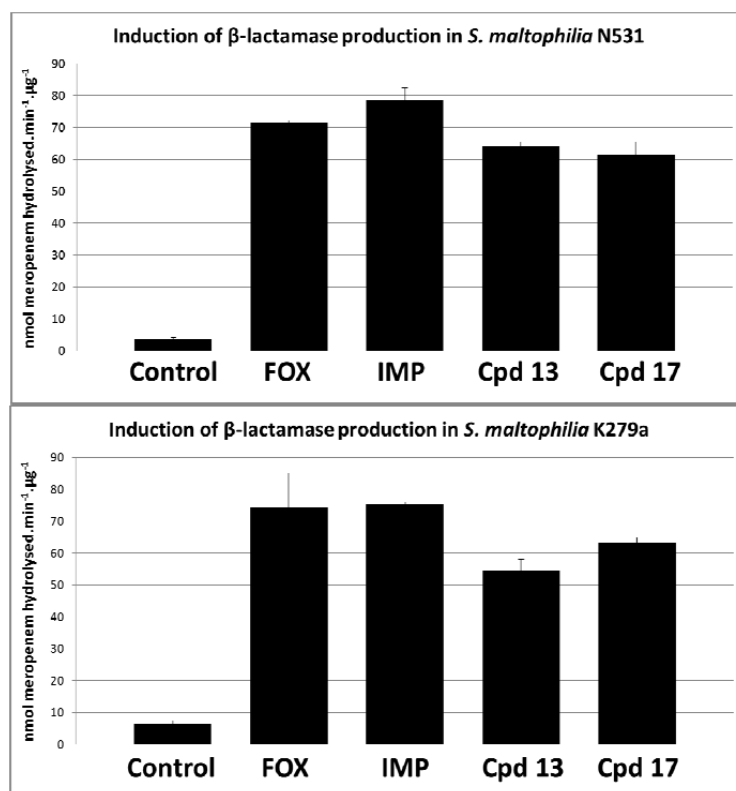


Figure 3

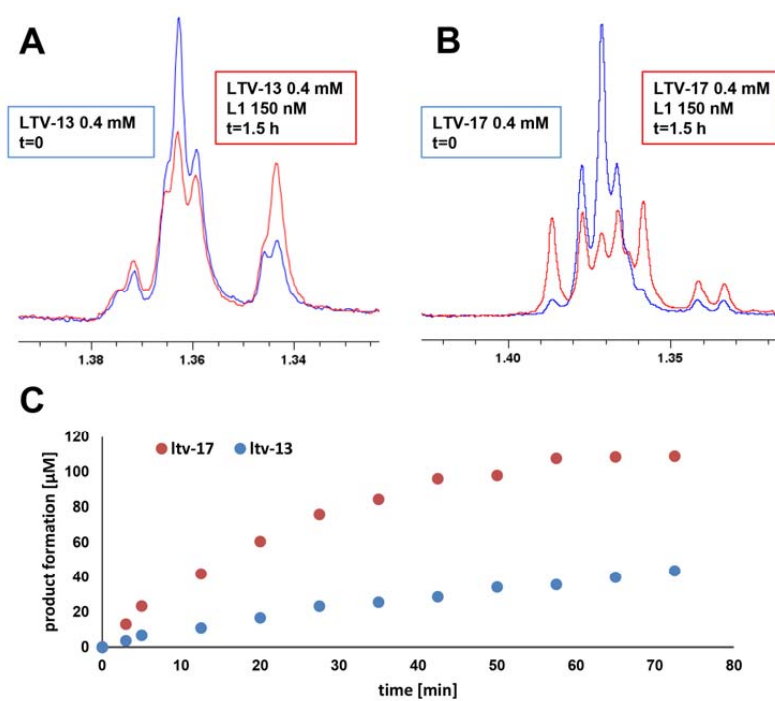


Figure 4

