PERKIN 1

Structure–function studies on nucleoside antibiotic mureidomycin A: synthesis of 5′-functionalised uridine models

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The importance of functional groups in nucleoside antibiotic mureidomycin A (MRD A) for biological activity has been examined by derivatisation of samples of the natural product, and by synthesis of uridine-containing analogues. *N*-Succinyl and di- and tri-acetyl derivatives MRD A have been prepared, and were found to have reduced activity as inhibitors of *E. coli* translocase I. The enamide alkene of MRD A was found to be extremely resistant towards hydrogenation by a variety of reagents. Several 5′-functionalised uridine derivatives were synthesised from N^3-*p*-methoxybenzyl-2′,3′-isopropylideneuridine. A series of 5′-aminoacyl derivatives were prepared, and the 3-aminopropionyl (IC_{50} 260 μM) and 7-aminoheptanoyl (IC_{50} 1.5 mM) derivatives were found to act as reversible inhibitors. An analogue mimicking the carboxy terminus of MRD A was synthesised, and also acted as an inhibitor of translocase I (IC_{50} 1.9 mM). A phosphonate analogue designed as a possible suicide inhibitor showed modest inhibition (IC_{50} 3.7 mM), which was shown to be irreversible.

Introduction

Inhibition of bacterial cell wall peptidoglycan biosynthesis is the site of action of many clinically important antibiotics, including the penicillins and the glycopeptide antibiotics.[1] The emergence of widespread clinical resistance to penicillins, and the recent emergence of bacterial resistance to vancomycin,[2] threatens the efficacy of antibiotics in current use and necessitates the exploration of new targets for antibacterial chemotherapy.

We have previously reported that the nucleoside antibiotic mureidomycin A acts as a potent slow-binding inhibitor (K_i 36 nM, K_i* 2 nM) for phospho-MurNAc-pentapeptide† translocase (translocase I) from *Escherichia coli*.[3] This enzyme is an integral membrane protein which catalyses the first step of the intramembrane cycle of reactions involved in peptidoglycan assembly.[1] This enzyme is encoded by the *mraY* gene,[4] and has only recently been overexpressed and solubilised.[3] It is therefore of great interest to elucidate which functional groups in the mureidomycin A skeleton are important for its biological activity.

Mureidomycin A consists of a 3′-deoxyuridine nucleoside linked *via* an unusual enamide linkage to a modified peptide chain, which contains two *m*-tyrosine residues, one methionine residue, and one 3-aminomethyl-3-deoxythreonine residue.[5] Two closely related families of natural products, namely the pacidamycins[6] and the napsamycins,[7] share the overall skeleton but show small differences in the amino acid composition (see Fig. 1). All contain the uridine nucleoside, the enamide linkage, a free amino terminus, and a carboxy-terminal aromatic amino acid.

In the previous paper we examined the role of the enamide linkage *via* synthesis of enamide-containing analogues, concluding that although reactive in simpler model systems the enamide linkage in MRD A and a uridine model showed low chemical reactivity.[8] In this paper we examine the importance of other functional groups in the mureidomycin A skeleton by derivatisation of the natural product, and by synthesis of 5′-functionalised uridine models.

† MurNAc = *N*-acetylmuramyl.

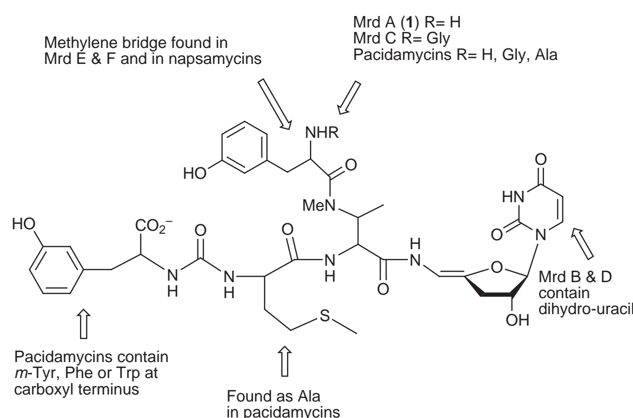


Fig. 1 Structures of the mureidomycin, pacidamycin, and napsamycin families of nucleoside antibiotics.

Results

Derivatisation of mureidomycin A

Mureidomycin A contains several nucleophilic groups which can be derivatised chemically, as shown in Fig. 2. A series of reactions were carried out on a milligram scale using samples of mureidomycin A. The reaction products were desalted by gel filtration, then analysed by reversed phase HPLC/electrospray mass spectrometry. New derivatives were isolated by reversed phase HPLC and tested as inhibitors of solubilised *E. coli* translocase I. The data is summarised in Table 1.

The amino terminus was selectively modified by treatment with succinic anhydride.[9] HPLC-MS analysis showed a new peak corresponding to the *N*-succinyl derivative (MH^+ 941), which was purified by semi-prep HPLC. Reaction of this product with Sanger's reagent (2,4-dinitrofluorobenzene) gave no characteristic yellow colour or UV absorbance at 340 nm, whereas a control sample of MRD A gave a positive result, verifying that the free amino group had been modified. Assays with solubilised translocase I verified that the *N*-succinyl derivative was still an effective inhibitor (IC_{50} 7 μM), but with reduced potency compared with MRD A (IC_{50} < 0.1 μM).

Table 1 HPLC-MS Analysis of synthetic derivatives of mureidomycin A. Reversed phase HPLC retention times (R_t), molecular masses (MH^+) observed by positive ion electrospray mass spectrometry, and IC_{50} values for inhibition of solubilised *E. coli* translocase I in radiochemical assay are given

Reaction	Product	RP-HPLC R_t/min	ES-MS MH^+	IC_{50} (μM)
Control	MRD A	30.0	841.0	<0.1
Succinylation: succinic anhydride, H_2O	*N*-succinyl	27.0	940.9	7.0
Acetylation: Ac_2O, NaOAc	Diacetyl	31.5	925.3	56
	Diacetyl	32.5	925.3	132
	Triacetyl	34.0	967.1	⩾100
Methylation: CH_2N_2	Cyclised, +42 amu	—	865.3[a]	ND[b]
Reduction:				
$NaBH_3CN$, pH 4	MRD A only			
H_2/cat (Pd/C; $Rh(PPh_3)_3Cl$)	MRD A only			
HN=NH, pH 4	MRD A only			
Et_3SiH, 10% CF_3COOH	−uracil − H_2O + 2H, 6H	32.4, 34.2	717.3, 713.1	
Et_3SiH, AcOH	MRD A only			

[a] Derivative analysed as mixture of desalted products by electrospray MS. [b] ND not determined.

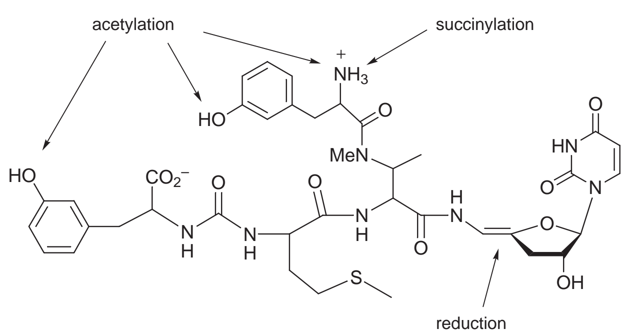


Fig. 2 Derivatisation of mureidomycin A.

Treatment of MRD A with acetic anhydride in the presence of sodium acetate gave rise to two diacetylated derivatives (MH^+ 925), and a triacetylated derivative (MH^+ 967). None of the acetylated derivatives gave a positive reaction with Sanger's reagent, suggesting that the amino group had been modified in each case. Assays showed that the diacetyl derivatives were inhibitors with reduced potency (IC_{50} 56 μM and 132 μM), while the triacetyl derivative was a weak inhibitor ($IC_{50} \geqslant 100$ μM). The data suggests that each of these groups forms a significant interaction with the enzyme, but that none is absolutely essential for inhibition.

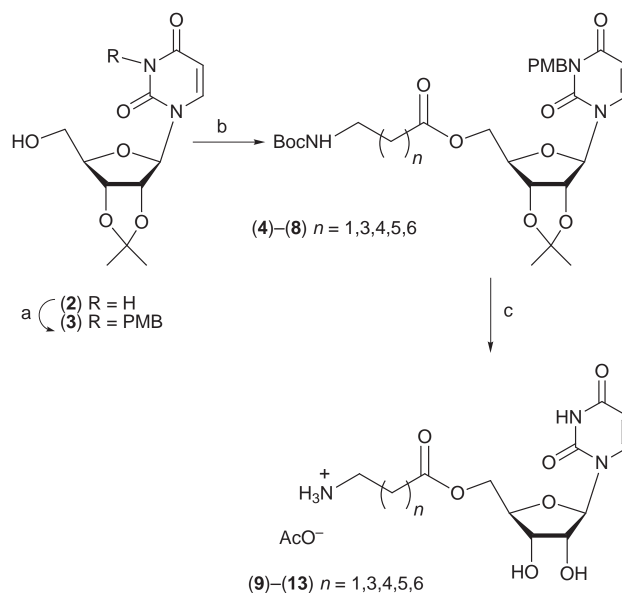
Treatment with diazomethane, in an attempt to modify the carboxy terminus, gave none of the desired methyl ester, but gave a major product of mass MH^+ 865. This product corresponds to loss of H_2O, perhaps due to cyclisation of amino and carboxy termini, followed by three successive methylations (peaks with MH^+ 837 and 851 were also observed).

Attempts were made to hydrogenate the enamide alkene of MRD A. Catalytic hydrogenation using palladium catalysts, or using Wilkinson's catalyst [$(PPh_3)_3RhCl$], failed to give any new products. Reduction with borohydrides [$NaBH_3CN$, $NaBH_4$, $Me_4N(AcO)_3BH$] under mildly acidic conditions, a reaction which is precedented for reduction of enamides[10] and which worked in the case of an earlier model,[8] also failed to give any new product. Treatment with triethylsilane in 10% trifluoroacetic acid gave new products corresponding to MH^+ 713, 715 and 717, suggesting that the uracil base had been cleaved, followed by elimination of water and addition of 2H, 4H, and 6H. There is chemical precedent for the reduction of glycosidic linkages to ethers by triethylsilane, in the presence of Lewis acids.[11] Thus it seems plausible that in this case the 3′-deoxyribose ring is reduced eventually to a tetrahydrofuran. However, treatment with triethylsilane under less forcing conditions (AcOH) gave no new products. Treatment with diimide, prepared either from potassium azodicarboxylate[12] or from toluene-*p*-sulfonyl hydrazide,[13] also gave only recovered MRD A. These observations imply that the enamide alkene is highly resistant to hydrogenation, and is only reduced after the uracil base is cleaved. The reasons for this extremely low reactivity are not clear, but it appears most likely that approach to the alkene is sterically hindered.

Synthesis of 5′-aminoacyluridine analogues

In order to assess further the role of the free amino terminus for biological activity, a series of 5′-aminoacyluridine derivatives were prepared, containing a variable length alkyl spacer. Preliminary synthetic work indicated a need to protect N-3 of the uracil base in order to achieve selective reaction at the 5′ position of the ribose ring. Accordingly, a number of protecting groups were investigated using 2′,3′-isopropylideneuridine. In our hands the protecting group of choice was *p*-methoxybenzyl (PMB),[14] which was inserted in 93% yield, and could be deprotected under mild conditions using cerium(IV) ammonium nitrate, which also removed the isopropylidene group.

The synthetic route, shown in Scheme 1, involved initial Boc protection of C-3, C-5, C-6, C-7 and C-8 ω-aminocarboxylic acids. The Boc-protected acids were then coupled onto the PMB-protected uridine **3** *via* DCC couplings, in 65–86% yield, to give the 5′-acylated products **4**–**8**. Deprotection of the PMB,



Scheme 1 Synthesis of ω-(aminoacyl)uridine analogues. a, *p*-Methoxybenzyl chloride, DBU, MeCN, 70 °C, 93%; b. $BocNHCH_2(CH_2)_nCO_2H$, DCC, DMAP, CH_2Cl_2, 65–86%; c, $(NH_4)_2Ce(NO_3)_6$, MeCN–H_2O, 50 °C.

Table 2 Inhibition data (IC_{50}) for uridine-based analogues *versus* solubilised *E. coli* translocase I in a radiochemical assay

Compound	5′-Sidechain	IC_{50} (mM)
9	$–CO(CH_2)_2NH_2$	0.26
10	$–CO(CH_2)_4NH_2$	⩾10
11	$–CO(CH_2)_5NH_2$	⩾10
12	$–CO(CH_2)_6NH_2$	1.5
13	$–CO(CH_2)_7NH_2$	⩾10
C-terminal analogue **16**	–β-Ala-succ-L-Phe-CO_2H	1.9
Phosphonate diester **18**	$–(CH_2)_3PO(OEt)_2$	⩾10
Phosphonate monoester **19**	$–(CH_2)_3P(OEt)O_2Na$	3.7

Boc and isopropylidine groups was then achieved by heating in the presence of aqueous cerium(IV) ammonium nitrate. Final products **9**–**13** were purified by G10 gel filtration, followed by reversed phase HPLC, and characterised by NMR spectroscopy and electrospray mass spectrometry.

The 5′-aminoacyluridine analogues were tested as inhibitors of solublised *E. coli* translocase, and the data is summarised in Table 2. The 5-aminopentanoyl **10**, 6-aminohexanoyl **11** and 8-aminooctanoyl **13** analogues showed little or no inhibition at 5 mM concentration, whereas the 3-aminopropionyl **9** (IC_{50} 0.26 mM) and 7-aminoheptanoyl **12** (IC_{50} 1.5 mM) analogues showed effective enzyme inhibition. This somewhat surprising pattern of inhibition will be discussed below.

Synthesis of a carboxy terminal analogue

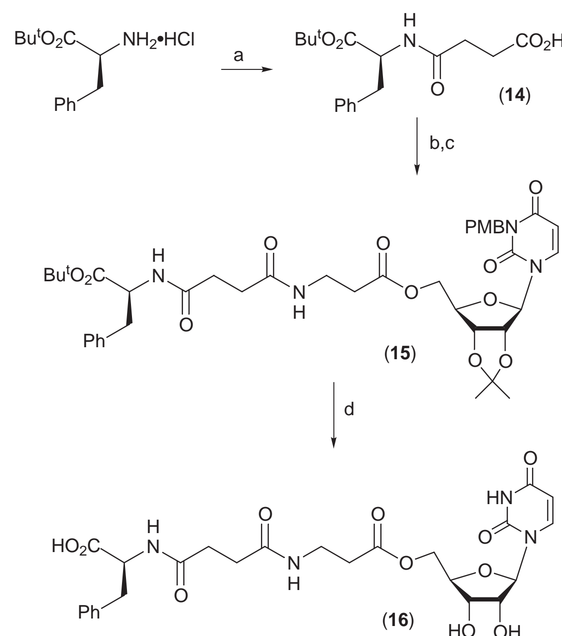
As mentioned above, all of the members of the mureidomycin, pacidamycin and the napsamycin families contain an aromatic amino acid at their carboxy termini. In the mureidomycins this residue is always *m*-tyrosine, but in the pacidamycins it is found as phenylalanine, *m*-tyrosine, or tryptophan (see Fig. 1). In order to probe the role of this group, an analogue **16** containing L-phenylalanine-COOH attached *via* an 8-carbon spacer to the 5′-position of uridine was synthesised, as illustrated in Scheme 2. The L-isomer of phenylalanine was used, although the configuration of the amino acid residues in mureidomycin A has not been determined.

L-Phenylalanine *tert*-butyl ester was reacted with succinic anhydride to give the succinyl derivative **14** in 90% yield. This acid was coupled to β-alanine *via* a mixed anhydride coupling, and the crude product was coupled onto the PMB-protected uridine **3** to give the fully protected derivative **15** in 38% yield overall. Deprotection with cerium(IV) ammonium nitrate under acidic conditions, followed by gel filtration and HPLC purification gave the target compound **16**. Enzyme assays (see Table 2) revealed that **16** was an inhibitor of translocase I (IC_{50} 1.9 mM).

Synthesis of a phosphonate suicide inhibitor

The catalytic mechanism of the translocase I reaction is thought to involve nucleophilic attack of an active site nucleophile onto the β-phosphate of the substrate uridine-diphospho-MurNAc-pentapeptide (see Fig. 3A,B), followed by attack of undecaprenyl phosphate on the covalent intermediate.[15] It was therefore envisaged a phosphonate analogue **19** might act as a suicide inhibitor of translocase I by attack of the active site nucleophile to give a covalently bound species which could not dissociate from the enzyme active site, as illustrated in Fig. 4C.

The synthetic route to the desired phosphonate is shown in Scheme 3. Diethyl [3-(*p*-tolylsulfonyl)oxypropyl]phosphonate[16] was used to alkylate the PMB-protected uridine **3**, using sodium hydride as base, in 52% yield, to give the protected phosphonate **17**. The PMB and isopropylidene groups were removed using cerium(IV) ammonium nitrate to give the deprotected phosphonate diester **18**. Half of this material was then converted to the phosphonate monoester **19** by partial hydrolysis using sodium hydroxide. The phosphonates **18** and **19** were tested as inhibitors of solubilised translocase I (see Table 2). The phosphonate diester **18** showed no inhibition at 13 mM concentration, however the monoester **19** showed modest inhibition (IC_{50} 3.7 mM).



Scheme 2 Synthesis of carboxy-terminal analogue **16** of mureidomycin A. a, Succinic anhydride, *N*-methylmorpholine, THF–DMF, 90%; b, isobutyl chloroformate, NEt_3, $H_2N(CH_2)_2CO_2H$, THF; c, (**3**), DCC, DMAP, CH_2Cl_2, 38% overall; d, $(NH_4)_2Ce(NO_3)_6$, MeCN–H_2O, 50 °C.

Characterisation of enzyme inhibition

In order to examine the type of enzyme inhibition being shown by phosphonate **19** and the 3-aminopropionyl analogue **9**, the time dependence of enzyme inhibition was examined. Incubations of enzyme and inhibitor were set up, and aliquots examined for activity at time points over 2 hours. The results, shown in Table 3, imply that phosphonate **19** is exhibiting time-dependent inhibition, whereas amine **9** is not. At the end of this experiment, the incubations were dialysed overnight, and the residual enzyme activity measured and compared with controls. The results are shown in Table 3. The incubation containing enzyme and phosphonate **19** showed only 18% residual activity, and no recovery of activity, implying that this inhibitor has resulted in an irreversible loss of enzyme activity; whereas in the case of amine **9** the enzyme has recovered almost full activity after dialysis. These data imply that phosphonate **21** is acting as a time-dependent irreversible inhibitor, consistent with the suicide inhibition mechanism, although its affinity for the enzyme is modest. Amine **9** appears to be acting as a reversible inhibitor, whose inhibition is reduced in the presence of the natural substrate, consistent with binding to the enzyme active site.

Conclusions

In view of the potency of translocase I inhibition by mureidomycin A, it is of considerable interest to elucidate the structural features within MRD A which are responsible for potent slow-binding inhibition. In the previous paper we have examined the role of the unusual enamide linkage found in mureidomycin A, and we have found that, although reactive in simpler model systems, in mureidomycin A and a uridine model it displays low reactivity.[8] The uridine nucleoside itself appears to be essential for activity, since all known inhibitors of this

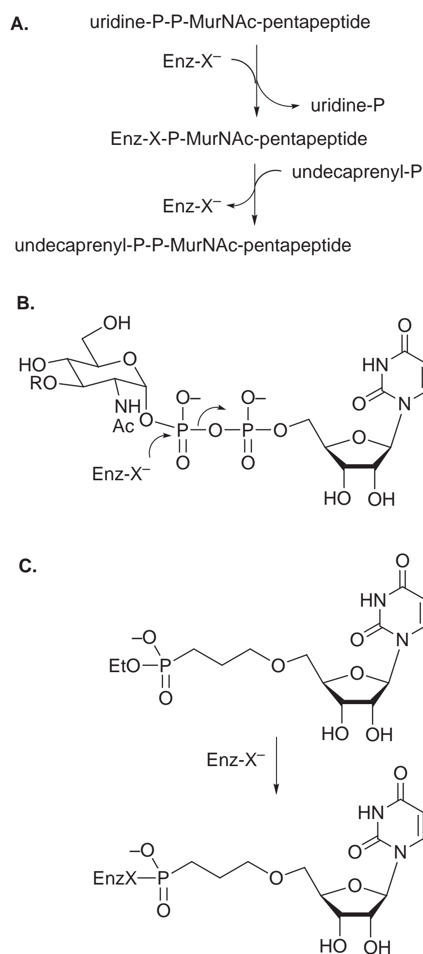
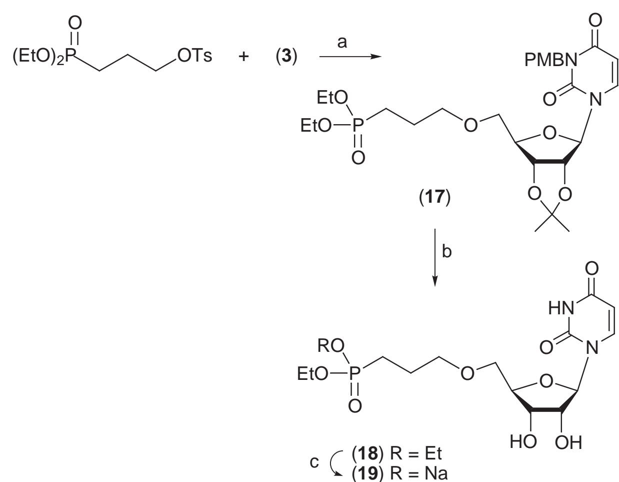


Fig. 3 A. Two-step mechanism for translocase I involving an active site nucleophile. B. Attack of enzyme active site nucleophile on β-phosphate of UDPMurNAc-pentapeptide. C. Anticipated mechanism of suicide inhibition by phosphonate analogue **19**.



Scheme 3 Synthesis of phosphonate inhibitor (**19**). a, NaH, DMF, 52%; b, $(NH_4)_2Ce(NO_3)_6$, $MeCN–H_2O$, 50 °C; c, NaOH, H_2O.

enzyme, including tunicamycin and the liposidomycins, contain a uridine nucleoside.[1] The activity shown by the chemical derivatives of MRD A reported in this work suggest that the amino terminus and the phenolic hydroxy groups of MRD A form significant binding interactions with the enzyme active site, although no single functional group is essential for inhibition.

The pattern of inhibition shown by the aminoacyluridine analogues is intriguing, and merits discussion. Only the 3-aminopropionyl **9** and the 7-aminoheptanoyl **12** analogues showed significant enzyme inhibition, none being observed for the 5-aminopentanoyl or 6-aminohexanoyl analogues, suggesting that there are different explanations for the inhibition observed by **9** and **12**. It is known that translocase I requires Mg^{2+} for activity, maximum activity being observed at 5–10 mM concentration.[3] Analysis of an amino acid sequence alignment has revealed a conserved Asp-Asp-Xaa-Xaa-Asp/Asn at position 115–119 of the *E. coli* sequence, which is a known sequence motif found in pyrophosphate-utilising enzymes such as terpene cyclases.[17] An X-ray crystal structure of farnesyl pyrophosphate synthase has revealed that the Asp–Asp pair co-ordinates a Mg^{2+} cofactor, which in turn co-ordinates the pyrophosphate linkage.[18] It is therefore a reasonable hypothesis that such a Mg^{2+} binding site exists in the translocase I active site, as shown in Fig. 4A.

Table 3 A. Time-dependent inhibition of translocase I by phosphonate **19** and amine **9**. B. Regain of translocase I activity following treatment with phosphonate **19** and 3-aminopropionyl analogue **9** and overnight dialysis

Table A

Time/min	% Translocase activity	
	+1.5 mM **19**	+85 μM **9**
0	90	42
15	76	—
30	—	54
60	—	48
120	60	52

Table B

Incubation	% Activity before dialysis	% Activity after dialysis
Enzyme only (control)	100	100
Enzyme + 11 μM UDPMurNAc-pentapeptide	92	96
Enzyme + 1.7 mM phosphonate **19**	27	18
Enzyme + 2.2 mM amine **9**	37	84
Enzyme + 2.2 mM amine **9** + 11 μM substrate	51	121
Enzyme + 1.2 mM amine **13**	61	114
Enzyme + 2.4 mM C-terminal analogue **16**	38	121

The question then arises: why should amine sidechains inhibit an enzyme which would normally bind a pyrophosphate bridge? Since the Mg^{2+} cofactor is bound relatively weakly by the enzyme, it is reasonable to suppose that inhibitors could bind to the active site in the absence of Mg^{2+}, in which case there would be a vacant cation binding site which could be occupied by the ammonium sidechain. We therefore tentatively suggest that the inhibition by the 3-aminopropionyl analogue **9** is due to the inhibitor binding with a *syn* ester conformation, as shown in Fig. 4B, whereas the 7-aminoheptanoyl analogue **12** binds with an *anti* ester conformation, shown in Fig. 4C. According to this model, the 5- and 6-carbon spacers would be too long to bind in the *syn* conformation, but not long enough for the chain to extend around when adopting the *anti* conformation. It is interesting to note that in MRD A there is a 7-atom spacer between the uridine C-5′ position and the amino terminus, consistent with this model (see Fig. 4D). Further experiments are needed to confirm this hypothesis.

The modest inhibition shown by the carboxy terminal analogue **16** shows that there is some binding interaction for the C-terminal part of MRD A, but that it is not strong by itself. It therefore seems likely that the potency of inhibition by MRD A is due to a number of binding interactions from different parts of the molecule, and that more complex synthetic analogues

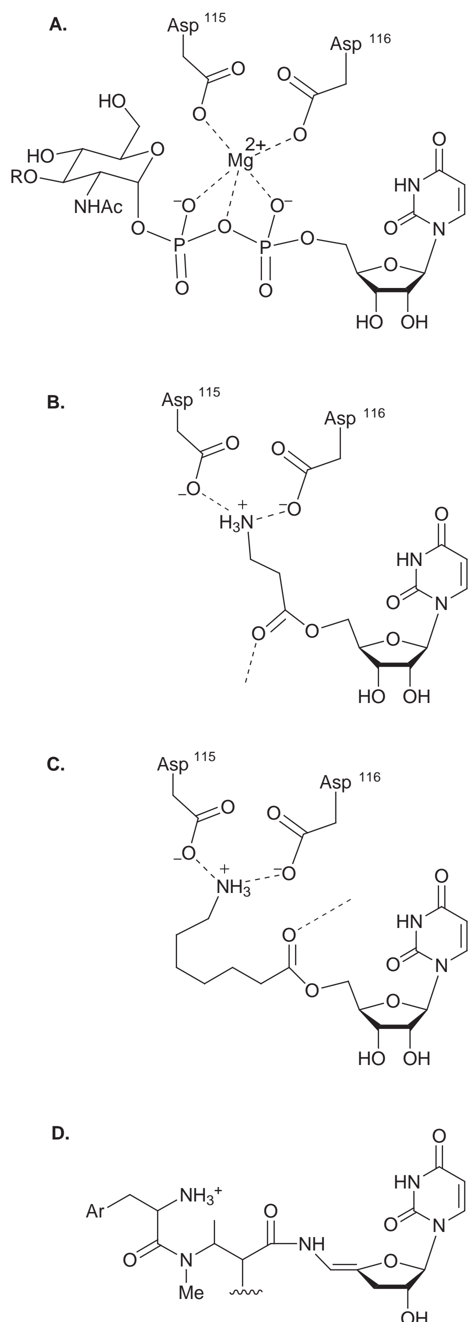


Fig. 4 Model for rationalisation of enzyme inhibition data for aminoacyl-uridine analogues. A. Proposed role of Mg^{2+} cofactor and Asp–Asp pair. B. Mode of inhibition of 3-aminopropionyl analogue (**9**). C. Mode of inhibition of 7-aminoheptanoyl analogue **12**. D. Structure of mureidomycin A, illustrating similarity in structure of the amino-terminal portion.

will be needed in order to realise tight binding to the enzyme active site. It is of interest that phosphonate **19** was shown to act as an irreversible inhibitor for translocase I. Although not a potent inhibitor, this result verifies that it is feasible in principle to design an irreversible inhibitor for this enzyme. These data may be valuable in the design of more potent translocase I inhibitors.

Regarding the mechanism of slow-binding inhibition of translocase I by mureidomycin A, our observations suggest that both N-terminal and C-terminal ends of the peptide chain of MRD A contribute towards active site binding. We tentatively suggest that the N-terminal part of MRD A binds in the Mg^{2+} binding site of the enzyme, which would bring the amino-terminal *m*-tyrosine residue close to the MurNAc binding site of the enzyme. The C-terminal *m*-tyrosine may bind in place of the co-substrate undecaprenyl phosphate, which is also hydrophobic and negatively charged. It is possible that the slow-binding inhibition is caused by a conformational change from the EI to the EI* complex. Since the enzyme must acquire the co-substrate undecaprenyl phosphate from the lipid bilayer, and release the lipid-linked product into the bilayer, it is quite likely that there is a protein conformational change which opens the active site to the lipid bilayer. Investigation of an enzyme conformational change awaits the purification to homogeneity of translocase I.

Experimental

General

Infra-red spectra were recorded on a 1600 series Perkin-Elmer FTIR spectrometer. 300 MHz NMR spectra were recorded on a Bruker AC300 Fourier Transform Spectrometer. Mass spectra were recorded on a VG platform quadruple E.S.I. mass spectrometer. Analytical RP-HPLC was performed on a Hewlett-Packard HP1100 ChemStation Chromatograph using a Phenomenex Prodigy ODS3 column, 3 mm × 150 mm, 0.1% TFA–H_2O→0.04% TFA–MeCN, 20 min gradient, 0.5 mL min^{-1}, observing at 254 nm, unless stated otherwise. Semi-prep RP-HPLC was performed on a Waters Associates Chromatograph using a Zorbax ODS C18 column, 10 mm × 250 mm, 0.1 M NH_4OAc–H_2O→0.1 M NH_4OAc–1 : 1 H_2O–MeCN, 30 min gradient, 2.5 mL min^{-1}, observing at 260 nm. UV absorbances were recorded on a Cary 1 UV spectrometer. Radioactivity was measured by mixing sample with 3.5 mL Optiphase Hi-Safe 3 scintillation fluid and counting in a Beckman LS 6500 multipurpose scintillation counter.

2′,3′-*O*-Isopropylideneuridine **2** was prepared from uridine using 2-methoxypropene–TsOH in 70–85% yield.[19] *N*-Boc-ω-aminocarboxylic acids were prepared by the method of Jorgenson.[20] Diethyl [3-(*p*-tolylsulfonyl)oxypropyl]phosphonate was prepared from 3-bromopropan-1-ol using the method of Capson *et al.*[16] ^{14}C-UDPMurNAc-pentapeptide (specific activity 160 μCi μmol^{-1}) was a gift from SmithKline Beecham Pharmaceuticals.

Succinylation of mureidomycin A

Mureidomycin A **1** (5 mg, 5.9 μmol, 1 equiv.) was dissolved in water (1 mL) and adjusted to pH 9.0 by addition of 0.07 M NaOH. Succinic anhyhdride (3 mg, 30 mol, 5 equiv.) was dissolved in water (100 μL) and added to the ice-cooled reaction mixture in 10 μL aliquots over 30 min. After a total of 1 h the mixture was lyophilised to yield a white solid. This was taken up in minimum water and applied to a Sephadex G-10 gel filtration column (12 mm × 600 mm), eluting with water. Fractions showing a UV absorbance maximum at 260 nm were combined and lyophilised to yield an off-white solid. The solid was taken up in water to give $A_{260\,nm} \approx 2.0$, and purified by semi-prep RP-HPLC to yield the title compound as a white solid after lyophilisation, 2 mg, 36%, R_t 27.0 min: LR-ESMS (+ve ion) MH^+ 940.9.

Acetylation of mureidomycin A

Mureidomycin A **1** (5 mg, 5.9 μmol, 1 equiv.) was dissolved in water (0.8 mL) and an equal volume of half-saturated sodium acetate solution added. The mixture was cooled in an ice-bath and treated with acetic anhydride (5 × 5 μL, 270 μmol, ~46 equiv.) over 3 h. Lyophilisation yielded a white solid which was taken up in minimum water and applied to a Sephadex G-10 gel filtration column, eluting with water. Fractions showing a UV absorbance maximum at 260 nm were combined and lyophilised to yield a white solid. The solid was taken up in water to give $A_{260\,nm} \approx 2.0$ and purified by semi-prep RP-HPLC to give three products (after lyophilisation): (1) diacetylated MRD A, 1.8 mg, R_t 31.5 min, LR-ESMS (+ve ion) MH^+ 925; (2)

diacetylated MRD A, 1.7 mg, R_t 32.5 min, LR-ESMS (+ve ion) MH^+ 925; (3) triacetylated MRD A, 1.2 mg, R_t 34.0 min, LR-ESMS (+ve ion) MH^+ 967.

N^3-(*p*-Methoxybenzyl)-2′,3′-*O*-isopropylideneuridine 3

A solution of 2′,3′-*O*-isopropylideneuridine **2** (5.04 g, 17.8 mmol, 1 equiv.) in dry acetonitrile (150 mL) was treated with DBU (5.3 mL, 35.4 mmol, 2 equiv.), resulting in a transient colour change from colourless to green, and with *p*-methoxybenzyl chloride (5.00 g, 31.9 mmol, 1.8 equiv.) The reaction mixture was heated to reflux under an inert atmosphere. After 24 h the solvent was removed *in vacuo* and the residue partitioned between water (50 mL) and ethyl acetate (50 mL). The aqueous layer was acidified with 5% $KHSO_4$ solution and re-extracted with ethyl acetate (2 × 50 mL). The combined organics were washed with saturated brine and dried over $MgSO_4$. Removal of the solvent *in vacuo* yielded a yellow oil which was purified by column chromatography (EtOAc) to yield the title compound as a white solid, 6.65 g, 93%, R_f 0.51 (EtOAc): mp 101–102 °C; LR-ESMS (+ve ion) MH^+ 405.2, MNa^+ 427.2; IR (nujol mull) 3400–3500 (br, OH), 1702 (C=O), 1650 (C=O) cm^{-1}; δ_H (300 MHz, $CDCl_3$) 7.44 (2H, d, $J = 9$ Hz), 7.33 (1H, d, $J = 8$ Hz, uracil 6-H), 6.83 (2H, d, $J = 9$ Hz), 5.76 (1H, d, $J = 8$ Hz, uracil 5-H), 5.56 (1H, d, $J = 3$ Hz, 1′-H), 5.07–4.96 (4H, m), 4.31 (1H, q, $J = 4$ Hz, 4′-H), 3.92 and 3.85 (2H, 5′-H AB system), 3.75 (3H, s) (OMe), 1.58 and 1.37 (2 × 3H, s, CH_3); δ_C (75.42 MHz, $CDCl_3$) 162.64, 159.32, 151.19, 140.72, 131.04, 128.78, 114.46, 113.88, 102.38, 97.08, 87.07, 83.93, 80.44, 62.86, 55.40, 43.78, 27.42, 25.43.

Preparation of *N*-Boc-ω-aminoacyl 2′,3′-*O*-isopropylidene-N^3-(*p*-methoxybenzyl)uridines 4–8

Procedure for 5-aminopropionyl analogue (**5**): A solution of *N*-*tert*-butyloxycarbonyl-5-aminopentanoic acid[18] (326 mg, 1.5 mmol, 1 equiv.) in dry dichloromethane (10 mL) was cooled to 0 °C and treated with dimethylaminopyridine (20 mg) and DCC (340 mg, 1.7 mmol, 1.1 equiv.). After 30 min N^3-(*p*-methoxybenzyl)-2′,3′-*O*-isopropylideneuridene (**3**) (607 mg, 1.5 mmol, 1 equiv.) was added and the reaction stirred at room temperature. After 18 h the reaction mixture was filtered and the filtrate washed with water (20 mL). The aqueous layer was re-extracted with dichloromethane (20 mL) and the combined organic extracts washed with saturated $NaHCO_3$ (2 × 20 mL) and saturated brine, and dried over $MgSO_4$. Removal of the solvent *in vacuo* afforded a yellow oil. Column chromatography (2:1 EtOAc–cyclohexane) allowed the isolation of the title compound as a viscous, colourless oil, 778 mg, 86%, R_f 0.57 (2:1 EtOAc–cyclohexane): LR-ESMS (+ve ion) MH^+ 604.5, MNa^+ 626.5; IR (nujol mull) 3391 (NH), 1653 (very br, C=O) cm^{-1}; δ_H (300 MHz, $CDCl_3$) 7.45 (2H, d, $J = 9$ Hz), 7.22 (1H, d, $J = 8$ Hz, uracil 6-H), 6.84 (2H, d, $J = 9$ Hz), 5.77 (1H, d, $J = 8$ Hz, uracil 5-H), 5.63 (1H, d, $J = 2$ Hz, 1′-H), 5.03 (2H, ABq, $J = 14$ Hz, $PhCH_2$), 4.97 (1H, dd, $J = 2$, 6 Hz, 2′-H), 4.82 (1H, dd, $J = 4$, 6 Hz, 3′-H), 4.63 (1H, br s, NH), 4.41–4.23 (3H, m), 3.81 (3H, s, OMe), 3.11 (2H, q, $J = 6$ Hz, 2.32 (2H, td, $J = 2$, 7 Hz), 1.68–1.58 (2H, m), 1.52–1.43 (2H, m), 1.58 (3H, s, CH_3), 1.44 (9H, s, $C\{CH_3\}_3$), 1.37 (3H, s, CH_3); δ_C (75.42 MHz, $CDCl_3$) 172.97, 162.61, 159.28, 155.97, 150.77, 139.88, 130.97, 128.87, 114.63, 113.87, 102.27, 96.05, 85.63, 85.06, 81.46, 79.20, 64.32, 55.39, 43.67, 40.18, 33.65, 29.57, 28.56, 27.28, 25.48, 22.04. Other analogues were prepared by the same method, using the appropriate *N*-Boc-ω-aminocarboxylic acid. Yields were as follows: (**4**) 96%; (**6**) 84%; (**7**) 65%, (**8**) 78%.

Preparation of 5′-ω-aminoacyluridines (9–13)

Procedure for 3-aminopropionyl analogue **9** as follows: A solution of protected 5′-aminopropionyluridine **4** (100 mg, 0.17 mmol, 1 equiv.) in acetonitrile (5 mL) was treated with a solution of ammonium cerium(IV) nitrate (190 mg, 0.35 mmol, 2 equiv.) in water (2 mL), and the resulting yellow solution was heated to 50 °C under nitrogen for 48 h. Water (10 mL) was added, and the aqueous layer was washed twice with ethyl acetate (5 mL). The aqueous phase was neutralised to pH 7.0 by addition of aqueous sodium bicarbonate solution, filtered, and lyophilised. The resultant brown solid was dissolved in water (2 mL) and desalted by Sephadex G10 gel filtration (12 mm × 600 mm). Fractions which showed UV absorbance at 260 nm were combined and lyophilised. The crude product was purified by semi-prep RP-HPLC to yield, after lyophilisation to constant mass, the title compound as a white solid, 30 mg, 55% yield: analytical RP-HPLC R_t 4.4 min; LR-ESMS (+ve ion) MH^+ 316.3; δ_H (300 MHz, D_2O) 7.71 (1H, d, $J = 8$ Hz, uracil 6-H), 5.88 (1H, d, $J = 8$ Hz, uracil 5-H), 5.81 (1H, d, $J = 4$ Hz, 1′-H), 4.47–4.25 (5H, m, 2′-H, 3′-H, 4′-H, 5′-H), 3.29 (2H, t, $J = 6$ Hz, H_2NCH_2), 2.88 (2H, t, $J = 6$ Hz, NCH_2CH_2CO). Analogues **10**, **11**, **12**, and **13** were prepared using the same method, on a 400 mg scale. In these cases only a portion of the final product was purified by HPLC. Crude yields after gel filtration were in the range 60–100% (including some inorganic contaminants). LR-ESMS and HPLC retention times for (**9**–**13**) were as shown in Table 4.

Table 4 LR-ESMS and HPLC retention times for (**9**–**13**)

5′-Sidechain of product	LR-ESMS (+ve ion) MH^+	HPLC retention time/min
$H_2N(CH_2)_2CO$– (**9**)	316.3	4.4
$H_2N(CH_2)_4CO$– (**10**)	344.4	6.5
$H_2N(CH_2)_5CO$– (**11**)	358.3	6.9
$H_2N(CH_2)_6CO$– (**12**)	372.3	7.4
$H_2N(CH_2)_7CO$– (**13**)	386.3	7.9

(*S*)-4-[(1-Butoxycarbonyl-2-phenylethyl)amino]-4-oxobutanoic acid 14

A suspension of L-phenylalanine *tert*-butyl ester·HCl (500 mg, 1.9 mmol, 1 equiv.) in THF (8 mL) was cooled to 0 °C and *N*-methylmorpholine (469 µL, 432 mg, 4.3 mmol, 2.2 equiv.) was added dropwise. A solution of succinic anhydride (213 mg, 2.1 mmol, 1.1 equiv.) in THF (2 mL) was added, followed by DMF (40 mL) until dissolution was complete. The reaction was allowed to warm to room temperature and stirred for 18 h. The THF was removed *in vacuo* and the residue partitioned between ethyl acetate (100 mL) and 1 M HCl (200 mL). The aqueous layer was re-extracted with ethyl acetate (40 mL) and the combined organics dried over $MgSO_4$. Removal of the solvent *in vacuo* afforded the title compound as a white solid, 560 mg, 90%, R_f 0.36 (EtOAc): LR-ESMS (+ve ion) MH^+ 322.3, MNa^+ 344.3; IR (nujol mull) 3436 (br, OH), 1695 (C=O), 1648 (C=O) cm^{-1}; δ_H (300 MHz, $CDCl_3$) 7.32–7.20 (3H, m), 7.15 (2H, d, $J = 7$ Hz), 6.38 (1H, d, $J = 8$ Hz, NH), 4.76 (1H, dt, $J = 6$, 8 Hz, Phe α-H), 3.10 (2H, d, $J = 6$ Hz, $PhCH_2$), 2.68 (2H, ABq, $J = 6$ Hz, succinyl CH_2), 2.52 (2H, t, $J = 6$ Hz, succinyl CH_2), 1.43 (9H, s, $C\{CH_3\}_3$); δ_C (75.42 MHz, $CDCl_3$) 177.02, 171.49, 170.96, 136.14, 129.67, 128.53, 127.15, 82.84, 53.86, 38.13, 30.68, 29.50, 28.08.

5′-*O*-(*N*-{4-[(Butoxycarbonyl-2-phenylethyl)amino]-4-oxo-butanoyl}aminopropanoyl)-N^3-(*p*-methoxybenzyl)-2′,3′-*O*-isopropylideneuridine 15

A solution of *N*-succinyl derivative **14** (236 mg, 0.73 mmol, 1 equiv.) in THF (5 mL) was treated with triethylamine (206 µL, 1.5 mmol, 2 equiv.) and cooled to −20 °C. A solution of isobutyl chloroform (105 µL, 0.81 mmol, 1.1 equiv.) in dry THF (2 mL) was then added dropwise causing the formation of a white precipitate. The mixture was stirred at −20 °C for 30 min

then a solution of β-alanine (72.0 mg, 0.81 mmol, 1.1 equiv.) in 1:1 H_2O–THF (4 mL) was added dropwise. The reaction was allowed to warm to room temperature and stirred for 18 h. The THF was removed *in vacuo* and the residue partitioned between ethyl acetate (50 mL) and 10% citric acid (50 mL). The aqueous layer was re-extracted with ethyl acetate (50 mL) and the combined organic extracts washed with 10% citric acid and dried over $MgSO_4$. Removal of the solvent *in vacuo* afforded a 14:4 mixture of the β-alanine-linked product–starting acid (by ^1H NMR analysis) as a colourless oil, 225 mg, 81% (R_f 0.16 (EtOAc), LR-ESMS (−ve ion) $\{M + TFA - H\}^-$ 505.7), which was used directly for the next step. A solution of this material (194 mg) in dry dichloromethane (5 mL) was cooled to 0 °C. DCC (112 mg, 0.54 mmol, 1.1 equiv.) and DMAP (10 mg) were added and the mixture stirred for 30 min. N^3-(*p*-Methoxybenzyl)-2′,3′-*O*-isopropylideneuridine **3** (200 mg, 0.49 mmol, 1 equiv.) was added and the reaction allowed to warm to room temperature. After 18 h the reaction was filtered and concentrated *in vacuo*. The residue was partitioned between dichloromethane (25 mL) and water (30 mL) and the aqueous portion re-extracted with dichloromethane (25 mL). The combined organics were washed with saturated $NaHCO_3$ and saturated brine, then dried over $MgSO_4$ and concentrated *in vacuo*. Column chromatography (2:1 EtOAc–cyclohexane) allowed the isolation of the title compound as a yellow foam, 221 mg, 38% over two steps, R_f 0.37 (EtOAc): LR-ESMS (+ve ion) MH^+ 779.9, MNa^+ 801.9; IR (nujol mull) 3415 (NH), 1731 (C=O), 1712 (C=O), 1665 (C=O) cm^{-1}; δ_H (300 MHz, $CDCl_3$) 7.48 (2H, d, $J = 9$ Hz), 7.24–7.05 (6H, m), 6.77 (2H, d, $J = 9$ Hz), 6.42 (1H, t, $J = 6$ Hz, N*H*CH_2), 6.35 (1H, d, $J = 8$ Hz, Phe NH), 5.72 (1H, d, $J = 8$ Hz, uracil 5-H), 5.56 (1H, d, $J = 2$ Hz, 1′-H), 5.00–4.88 (3H, m), 4.78 (1H, dd, $J = 4$, 6 Hz, 3′-H), 4.63 (1H, dt, $J = 6$, 8 Hz, Phe α-H), 4.32–4.19 (3H, m, 4′-H and 5′-H), 3.70 (3H, s, OMe), 3.41 (2H, q, $J = 6$ Hz, NHCH_2), 3.01 (2H, d, $J = 6$ Hz, Phe PhCH_2), 2.49–2.32 (6H, m), 1.49 (3H, s), 1.32 (9H, s), 1.29 (3H, s); δ_C (75.42 MHz, $CDCl_3$) 171.99, 171.70, 171.48, 170.58, 162.44, 159.18, 150.66, 140.16, 136.24, 130.70, 129.48, 128.74, 128.37, 126.92, 114.55, 113.78, 102.26, 96.02, 85.38, 84.74, 81.37, 82.27, 64.32, 55.24, 53.72, 43.56, 38.05, 34.98, 34.07, 31.40, 29.67, 27.94, 27.15, 25.34.

5′-*O*-(*N*-{4-[(1-Carboxy-2-phenylethyl)amino]-4-oxobutanoyl}-aminopropanoyl)uridine 16

A solution of the protected derivative **15** (220 mg, 0.28 mmol, 1 equiv.) in acetonitrile (4 mL) was treated with a solution of ammonium cerium(IV) nitrate (170 mg, 0.31 mmol, 1.1 equiv.) in water (1 mL), and the resulting yellow solution heated to 50 °C under an inert atmosphere. After 18 h the solvent was removed *in vacuo* and the residue neutralised with 1 M NaOH and partitioned between water (10 mL) and ethyl acetate (10 mL). The organic layer was re-extracted with water (10 mL) and the combined aqueous portions lyophilised. The resultant brown solid was dissolved in water (3 mL) and desalted by Sephadex G-10 gel filtration. Fractions which showed UV absorbance at 260 nm were combined and lyophilised. A portion of the crude product was purified by semi-prep RP-HPLC to yield, after lyophilisation to constant mass, the title compound as a white solid, 2.0 mg: analytical RP-HPLC R_t 9.3 min (84%); LR-ESMS (+ve ion) MH^+ 563.5, MNa^+ 585.4; HR-FABMS (+ve ion) 563.1996 (calc. 563.1989); δ_H (300 MHz, D_2O) 7.50 (1H, d, $J = 8$ Hz, uracil 6-H), 7.18–7.02 (5H, m), 5.66 (1H, d, $J = 8$ Hz, uracil 5-H), 5.64 (1H, d, $J = 4$ Hz, 1′-H), 4.32–4.01 (6H, m), 3.22 (2H, t, $J = 6$ Hz, NHCH_2), 2.73 (2H, dd, $J = 8$, 14 Hz, PhCH_2), 2.42 (2H, t, $J = 7$ Hz), 2.31–2.13 (4H, m).

N^3-(*p*-Methoxybenzyl)-5′-*O*-{3-[(diethoxy)phosphoryl]propyl}-2′,3′-*O*-isopropylideneuridine 17

N^3-(*p*-Methoxybenzyl)-2′,3′-*O*-isopropylideneuridine (**3**) (250 mg, 0.62 mmol, 1 equiv.) was added to a suspension of sodium hydride (60% in mineral oil, 36 mg, 0.93 mmol, 1.5 equiv.) in dry DMF (12 mL). The mixture was stirred at room temperature for 15 min, with a concurrent colour change to orange, before the addition of diethyl {3-[(*p*-tolylsulfonyl)oxo]propyl}-phosphonate[16] (650 mg, 1.85 mmol, 3 equiv.). After 6 h TLC (EtOAc) showed starting uridine (R_f 0.46) remaining so a further 1 equiv. of tosylate was added. After a total of 24 h the reaction mixture was partitioned between water (70 mL) and ethyl acetate (3 × 50 mL). The combined organics were washed with saturated brine, dried over $MgSO_4$ and concentrated *in vacuo* to yield a yellow oil. Column chromatography (EtOAc→20% MeOH–EtOAc) allowed the isolation of the title compound as a yellow oil, 186 mg, 52%, R_f 0.13 (EtOAc): LR-ESMS (+ve ion) MH^+ 583.2, MNa^+ 605.2; IR (liquid film) 3453, 2984, 2935, 1708, 1665, 1512 cm^{-1}; δ_H (300 MHz, $CDCl_3$) 7.48 (1H, d, $J = 8$ Hz, uracil 6-H), 7.44 (2H, d, $J = 9$ Hz), 6.80 (2H, d, $J = 9$ Hz), 5.84 (1H, d, $J = 3$ Hz, 1′-H), 5.73 (1H, d, $J = 8$ Hz, uracil 5-H), 5.03 (2H, ABq, $J = 15$ Hz, PhCH_2), 4.76 (1H, dd, $J = 6$, 3 Hz) and 4.73 (1H, br d, $J = 5$ Hz) (2′ and 3′-H), 4.36 (1H, $J = 4$ Hz, 4′-H), 4.16–4.01 (4H, m, POC$H_2$$CH_3$), 3.77 (3H, s, OMe), 3.69 (1H, dd, $J = 12$, 2 Hz) and 3.57 (1H, dd, $J = 12$, 2 Hz) (5′-H in AB system), 3.50 (2H, t, $J = 7$ Hz, PCH_2CH_2CH_2), 1.97–1.65 (4H, m, PCH_2CH_2), 1.58 (3H, s, CH_3), 1.37 (3H, s, CH_3), 1.32 (6H, t, $J = 7$ Hz, $POCH_2$CH_3); δ_C (75.42 MHz, $CDCl_3$) 162.74, 159.22, 150.95, 138.76, 130.94, 129.09, 114.26, 113.79, 101.73, 98.98, 85.97, 85.63, 80.93, 71.29 (d, $J = 16$ Hz, PO*C*H_2CH_3), 70.84, 61.76 (d, $J = 6$ Hz, PCH_2CH_2*C*H_2), 55.36, 43.65, 27.36, 25.48, 23.42, 22.92, 22.85, 21.52 (m, P*C*H_2*C*H_2), 16.63 (d, $J = 6$ Hz, $POCH_2$*C*H_3).

5′-*O*-{3-[(Diethoxy)phosphoryl]propyl}uridine 18

A solution of the protected phosphonate **17** (230 mg, 0.39 mmol, 1 equiv.) in acetonitrile (5 mL) was treated with an aqueous solution (2 mL) of cerium(IV) ammonium nitrate (CAN, 102 mg, 185 µmol, 4 equiv.) and stirred at 50 °C. After 4 h TLC showed starting material remaining (10:1 EtOAc–MeOH, R_f 0.60) so a further 2 equiv. of CAN were added and stirring continued. After a further 2 h another 2 equiv. of CAN were added and the reaction was cooled and stirred at room temperature overnight. Water (20 mL) was added and the mixture washed with ethyl acetate (2 × 20 mL). The organic fractions were washed with water (30 mL) and the combined aqueous portions lyophilised to yield a yellow solid. The solid was taken up in the minimum amount of water and applied to a Sephadex G-10 gel filtration column. Fractions showing UV absorbance at 260 nm were analysed by LR-ESMS (+ve ion) and fractions containing a major signal of MH^+ 423.2 were combined and lyophilised to yield an off-white solid (280 mg). Half of this product was carried on to the monohydrolysis step; the remainder was purified by semi-prep RP-HPLC. Lyophilisation to constant mass yielded the title compound as a white solid, 5.1 mg, 6% yield, R_t 28.4 min; LR-ESMS (+ve ion) MH^+ 423.3, MNa^+ 445.3; δ_H (300 MHz, D_2O), 7.78 (1H, d, $J = 8$ Hz, uracil 6-H), 5.81 (1H, d, $J = 4$ Hz, 1′-H), 5.79 (1H, d, $J = 8$ Hz, uracil 5-H), 4.28–4.10 (3H, m), 4.03 (4H, quintet, $J = 7$ Hz, CH_3CH_2OP), 3.75 (1H, dd, $J = 12$, 2 Hz) and 3.61 (1H, dd, $J = 12$, 4 Hz) (5′-CH_2 in AB system), 3.60–3.45 (2H, m, PCH_2-CH_2CH_2O), 1.90–1.70 (4H, m, PCH_2C$H_2$$CH_2$O), 1.20 (6H, t, $J = 7$ Hz, C$H_3$$CH_2$OP); δ_C (75.42 MHz, D_2O), 168.78, 154.24, 144.43, 104.83, 92.36, 85.65, 76.48, 73.19 (d, $J = 18$ Hz, PO*C*H_2-CH_3), 72.43, 72.01, 65.92 (d, $J = 6$ Hz, PCH_2CH_2*C*H_2), 24.36, 22.30 (m, P*C*H_2*C*H_2), 18.21 (d, $J = 6$ Hz, $POCH_2$*C*H_3).

5′-*O*-3-{3-[(Ethoxy)sodiooxyphosphoryl]propyl}uridine 19

5′-*O*-{3-[(Diethoxy)phosphoryl]propyl}uridine (**18**) (135 mg crude, <0.32 mmol) was treated with 1 M NaOH (1 mL) and stirred at 50 °C overnight. Analytical RP-HPLC analysis (Phenomenex ODS3, 3 mm × 250 mm, 0.1 M NH_4OAc–H_2O→0.1 M NH_4OAc 1:1 H_2O–MeCN, 30 min gradient, 1.0

mL min^{-1}, observing 260 nm) showed only a new product (R_t 24.0 min) and so the reaction was neutralised with 2 M HCl, filtered to remove a yellow solid and lyophilised to yield a white solid. The product was taken up in the minimum amount of water and applied to a Sephadex G-10 gel filtration column and fractions showing UV absorbance at 260 nm were combined and lyophilised. The crude product was purified by semi-prep RP-HPLC and lyophilisation to constant mass yielded the title compound as a white solid, 11 mg, 14% yield, R_t 21.0 min: LR-ESMS (+ve ion) MH^+ 395.2; δ_H (300 MHz, D_2O) 7.78 (1H, d, $J = 8$ Hz, uracil 6-H), 5.81 (1H, d, $J = 4$ Hz, 1′-H), 5.79 (1H, d, $J = 8$ Hz, uracil 5-H), 4.28–4.10 (3H, m), 3.78 (2H, quintet, $J = 7$ Hz, CH_3CH_2OP), 3.72 (1H, dd, $J = 12$, 2 Hz) and 3.61 (1H, dd, $J = 12$, 4 Hz) (5′-CH_2 in AB system), 3.60–3.45 (2H, m), 1.80–1.40 (4H, m), 1.10 (3H, t, $J = 7$ Hz, CH_3CH_2OP); δ_C (75.42 MHz, d_6-DMSO) 168.82, 154.29, 144.42, 104.98, 92.11, 85.74, 76.43, 74.41 (d, $J = 17$ Hz, $POCH_2CH_3$), 72.53, 72.05, 63.11 (d, $J = 6$ Hz, $PCH_2CH_2CH_2$), 26.53, 25.71, 24.54 (m, PCH_2CH_2), 18.62 (d, $J = 6$ Hz, $POCH_2CH_3$).

Characterisation of inhibitors in the radiochemical assay

Aqueous solutions of the inhibitors were prepared at known concentrations, as determined by UV absorbance at 262 nm ($\varepsilon_{uridine} = 10^4$ M^{-1} cm^{-1}). *Escherichia coli* translocase 1 was prepared from overexpression construct JM109/pBROC525, as previously described,[3] and was solubilised in 0.5% Triton X-100. Radiochemical assays were performed, as previously described,[3] in the presence of 10 μL solubilised *E. coli* translocase I (4 mg mL^{-1} protein), 12.5 μM ^{14}C-UDP-MurNAc-pentapeptide (64 nCi $nmol^{-1}$) and 10 μM dodecaprenyl phosphate. All assays were performed in triplicate. % Inhibition was calculated as {(cpm in the absence of inhibitor − cpm in presence of inhibitor)/cpm in the absence of inhibitor} × 100. IC_{50} values were determined as the concentration of inhibitor required to reduce the cpm of the *n*-butanol layer by 50% as compared to an inhibitor-free control assay.

Time course inhibitor assays with phosphonate 19 and amine 9

At $t = 0$ min 160 μL solubilised enzyme (0.5% Triton X-100), 80 μL of an aqueous solution of inhibitor (final concentrations: 1.5 mM **19**; 85 μM **9**) and 400 μL buffer (200 mM Tris·HCl pH 7.5, 50 mM $MgCl_2$) were mixed and held on ice. At time points from 0–120 min 40 μL aliquots were removed and treated with 10 μL of 62.5 μM (4 nCi μL^{-1}, 64 nCi $nmol^{-1}$) ^{14}C-UDP-MurNAc-pentapeptide + 50 μm dodecaprenyl phosphate. This allowed radiochemical assays to be performed as described above. A control assay of solubilised enzyme incubated in the absence of inhibitor was also performed at each time point and activities were expressed as a percentage of this total count.

Recovery of activity after removal of inhibitor by dialysis

Five incubations were set up and held on ice, containing 250 μL solubilised enzyme, 625 μL buffer (200 mM Tris·HCl pH 7.5, 50 mM $MgCl_2$) and: (i) 250 μL H_2O; (ii) 125 μL of 0.1 mM UDP-MurNAc-pentapeptide (final concentration 11 μM) + 125 μL H_2O; (iii) 150 μL of 12.4 mM phosphonate **19** (final concentration 1.32 mM) + 100 μL H_2O; (iv) 125 μL of 16.9 mM amine **9** (final concentration 1.88 mM) + 125 μL H_2O; (v) 125 μL of 16.9 mM amine **9** (final concentration 1.88 mM) + 125 μL of 0.1 mM UDP-MurNAc-pentapeptide (final concentration 11 μM). After 2 h 200 μL aliquots were removed and kept on ice. The remainder of each incubation was dialysed against 500 mL buffer (200 mM Tris·HCl pH 7.5, 50 mM $MgCl_2$) overnight at 4 °C. 3 × 40 μL aliquots were removed from both the dialysed and undialysed incubations and treated with 10 μL of 62.5 μM (4 nCi μL^{-1}, 64 nCi $nmol^{-1}$) ^{14}C-UDP-MurNAc-pentapeptide + 50 μM dodecaprenyl phosphate. This allowed radiochemical assays to be performed as described above. All assays were performed in triplicate. Activities were expressed as a percentage of the total count observed in incubation (i).

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