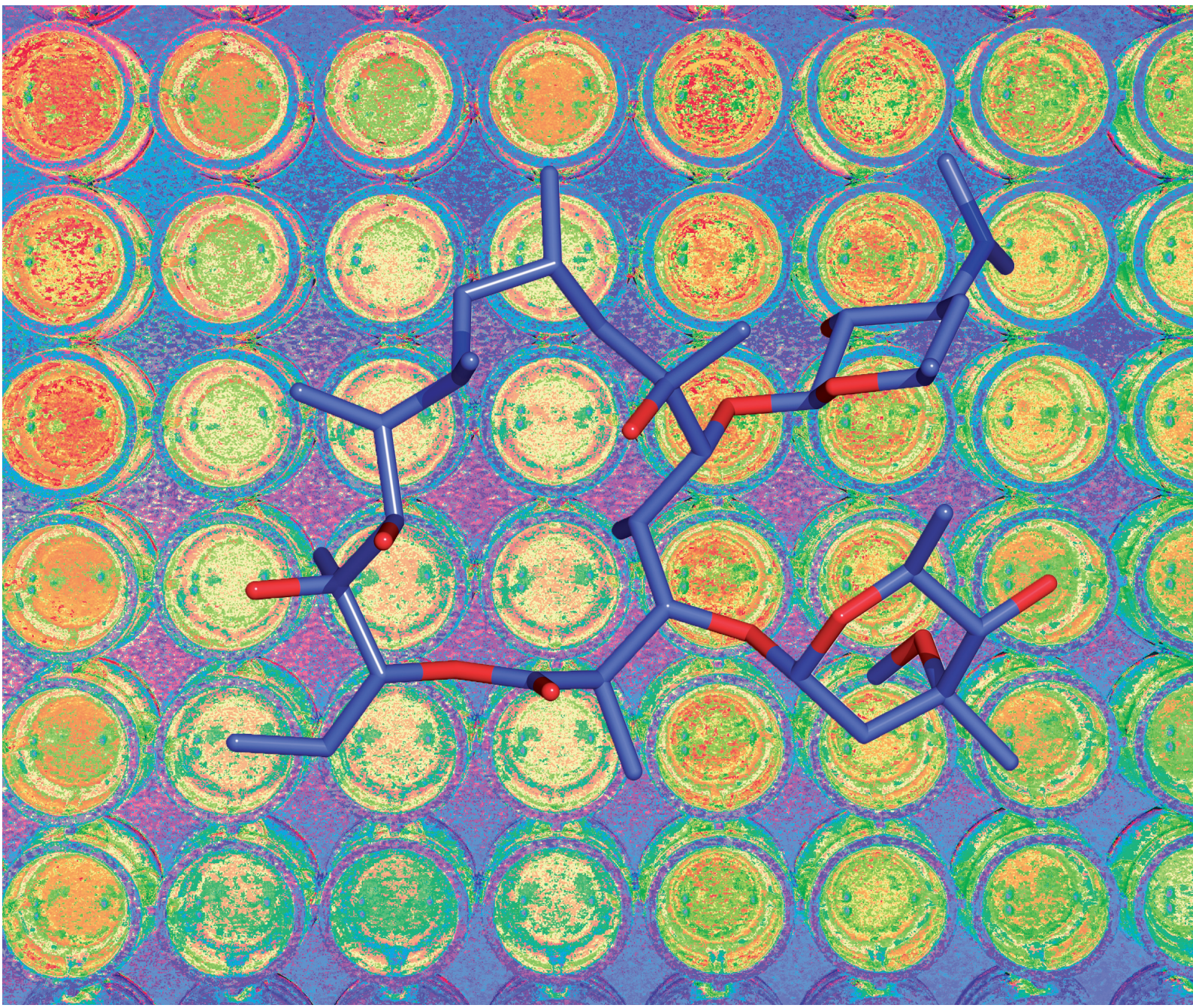
Organic & Biomolecular Chemistry

www.rsc.org/obc

Volume 6 | Number 22 | 21 November 2008 | Pages 4069–4272



ISSN 1477-0520

FULL PAPER

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Chemical Science

In this issue...

1477-0520(2008)6:22;1-I

RSC Publishing

Using chemical probes to investigate the sub-inhibitory effects of azithromycin†

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Received 30th July 2008, Accepted 19th August 2008
First published as an Advance Article on the web 19th September 2008
DOI: 10.1039/b813157k

The antibacterial drug azithromycin has clinically beneficial effects at sub-inhibitory concentrations for the treatment of conditions characterized by chronic *Pseudomonas aeruginosa* infection, such as cystic fibrosis. These effects are, in part, the result of inhibition of bacterial biofilm formation. Herein, the efficient synthesis of azithromycin in 4 steps from erythromycin and validation of the drug's ability to inhibit biofilm formation at sub-MIC (minimum inhibitory concentration) values are reported. Furthermore, the synthesis of immobilized and biotin-tagged azithromycin analogues is described. These chemical probes were used in pull-down assays in an effort to identify azithromycin's binding partners *in vivo.* Results from these assays revealed, as expected, mainly ribosomal-related protein binding partners, suggesting that this is the primary target of the drug. This was further confirmed by studies using a *P. aeruginosa* strain containing plasmid-encoded *ermC*, which expresses a protein that modifies 23S rRNA and so blocks macrolide entry to the ribosome. In this strain, no biofilm inhibition was observed. This work supports the hypothesis that the sub-inhibitory effects of azithromycin are mediated through the ribosome. Moreover, the synthesis of these chemical probes, and proof of their utility, is of value in global target identification in *P. aeruginosa* and other species.

Introduction

Antibiotics have had an essential role in the global increase in quality of life and life expectancy. The macrolide[1] class of antibiotics was discovered in the 1950s, and, as a result of their clinical efficacy and safety, now represents 20% of all prescribed antibiotics.[2] Macrolides show good *in vitro* activity against Gram positive bacteria and have been shown to inhibit protein synthesis by interacting with bacterial ribosomes.[3] Azithromycin (**1**) (Fig. 1),[4] approved for clinical use in 1992 and marketed as Zithromax® by Pfizer, is one of the most commonly prescribed macrolides and is used to treat a range of bacterial infections including pneumonia and acute bronchitis.[5] However, azithromycin does not have a bacteriostatic or bactericidal effect *in vivo* against Gram negative bacteria, such as *Pseudomonas aeruginosa* (minimum inhibitory concentration [MIC] value >1000 μg ml^{-1}).[6] Nevertheless, over the last decade, azithromycin at sub-inhibitory concentrations (*i.e.* sub-MIC) has been shown to have clinically beneficial effects in cystic fibrosis (CF) sufferers.[7] In CF,[8] and other related pulmonary tract infections,[5] chronic *P. aeruginosa* infection is the major cause of morbidity and mortality.[9] Although azithromycin is known to inhibit protein synthesis by blocking the protein exit tunnel in bacterial ribosomes,[10] its effects at lower concentrations, where it is neither bacteriostatic or bactericidal, are less well understood. Herein, we report the synthesis of azithromycin (**1**), the

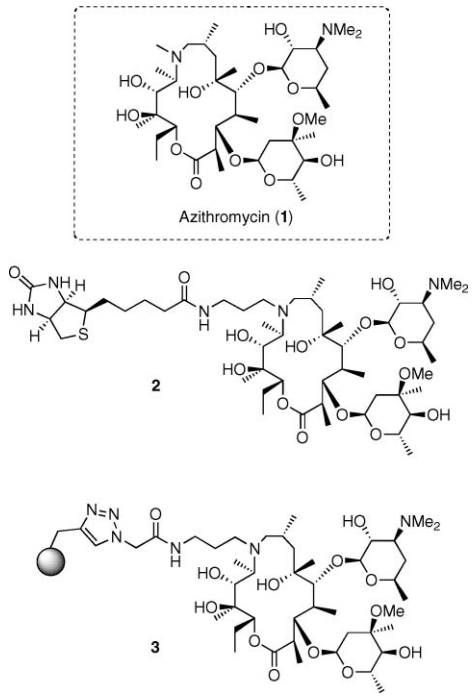

Fig. 1 The structures of azithromycin (**1**), the biotin-tagged analogue **2**, and the immobilized equivalent **3**.

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† Electronic supplementary information (ESI) available: Full experimental procedures, characterization and NMR spectra for the compounds obtained, and experimental details for the biological assays performed (*i.e.* MIC assay, biofilm assays and pull-down assays). Biofilm inhibition assays for the intermediates towards azithromycin synthesis are also reported. See DOI: 10.1039/b813157k

biotin-tagged analogue **2**, and the immobilized equivalent **3** (Fig. 1) in an effort to address this issue.

A number of groups have investigated the effects of azithromycin on *P. aeruginosa* at sub-MIC values.[11,12] The effects of azithromycin are known to be multiple and are proposed to include: reduced bacterial motility; interruption of quorum sensing; reduction in virulence factor production; increased susceptibility to stationary phase killing; and, of key significance to this study, impaired ability to form mature biofilms.[13] The formation of bacterial mature biofilms has consequences in terms of both their ability to cause infection and also to resist antibiotic treatment.[14] The transition from a dispersed 'planktonic' state to a mature biofilm occurs with a defined development profile. This complex process requires coordination such that different cells throughout the colony express different sets of genes in a spatial and time dependent fashion (Fig. 2).[15] The formation of mature biofilms confers on *P. aeruginosa* protection from chemical intervention in CF sufferers and has been cited as a major cause for treatment failure.[16] Bacterial drug-resistance has led to new technologies to discover antibacterial treatments.[17]

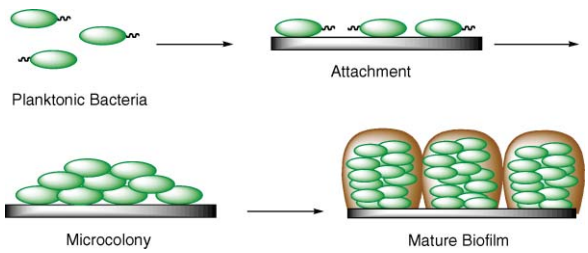


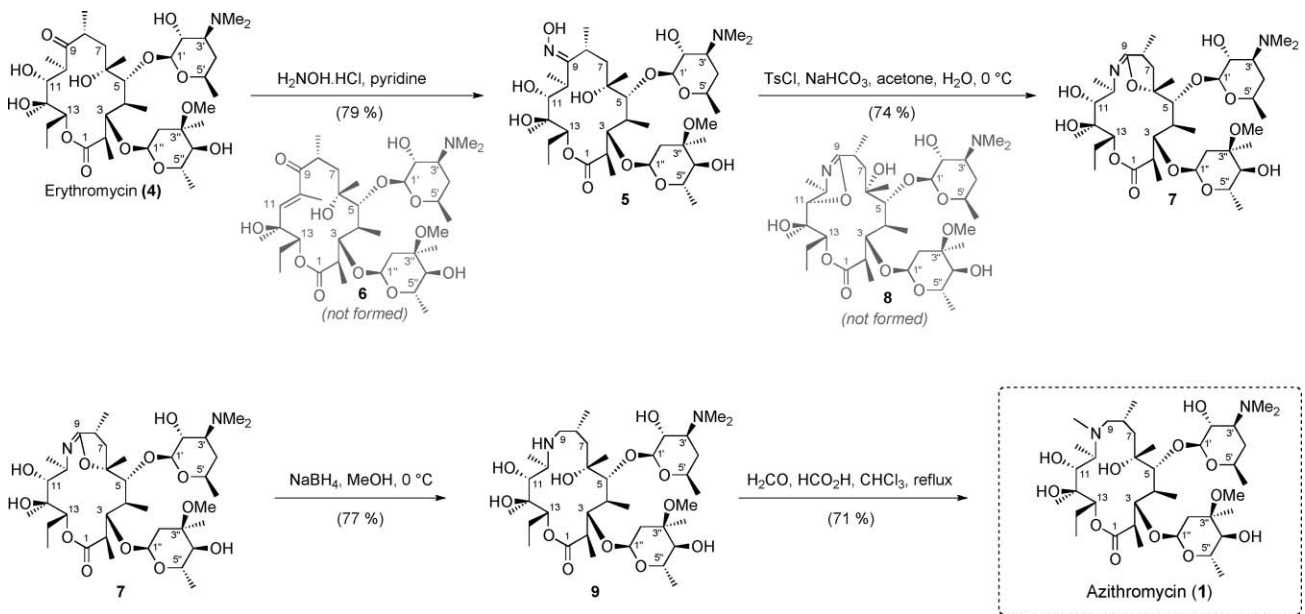
Fig. 2 The formation of mature biofilms in *P. aeruginosa* is a major cause of treatment failure in CF sufferers. It is a complicated process and occurs with a defined development profile requiring coordinated gene expression.[15]

Results and discussion

Azithromycin causes therapeutic effects at sub-inhibitory concentrations that are clinically relevant. The unresolved question is whether these sub-inhibitory effects are due to azithromycin targeting the ribosome or (as yet) unidentified protein(s).[18] We sought to test the hypothesis that azithromycin targets a non-ribosome related protein using the tagged analogues **2** and **3** in pull–down assays.

By adapting known methodologies[19] we were able to synthesize azithromycin from erythromycin (**4**) in an overall yield of 32% over 4 steps (Scheme 1). Initially, erythromycin (**4**) was converted to the (*E*)-oxime **5** in 79% yield by a reaction with hydroxylamine in pyridine.[20] Compared to alternative methods investigated,[21] this reaction furnished exclusively the desired *E*-isomer and did not lead to formation of the α,β-unsaturated by-product **6**. Using TsCl and $NaHCO_3$ then facilitated the formation of the cyclic 6,9-iminoester **7** *via* an intercepted Beckman rearrangement of **5**.[22] Again, under these conditions, selective formation of **7** was observed in the absence of the alternative 9,11-iminoester product **8**.[23] The subsequent reduction of **7** to the secondary amine **9** could be achieved by H-Cube® hydrogenation or more productively with $NaBH_4$. The synthesis of azithromycin was then completed under Eschweiler–Clarke methylation conditions.[24] The reactions all proceeded in good yield (>70%) and represent an optimized synthesis of azithromycin.[25]

In order to confirm that the biofilm inhibition assays were conducted at sub-MIC values, cultures of *P. aeruginosa* PAO1 (wild-type) were grown in the presence of azithromycin (3.9 μg ml^{-1} to 1000 μg ml^{-1}). Under these conditions, using alanine-glycerol-salts (AGS) media,[26] no growth inhibition was observed (*i.e.* MIC_{50} >1000 μg ml^{-1}). Full experimental details can be found in the ESI.† Biofilm inhibition assays with azithromycin were carried out in the same growth medium (Fig. 3a). These assays involved incubating



Scheme 1 The four step synthesis of azithromycin (**1**) from erythromycin (**4**). The compounds shown in grey (**6** and **8**) were not formed under the conditions reported but did form under alternative conditions. The numbers shown correspond to the NMR assignments in the experimental section (see ESI†).

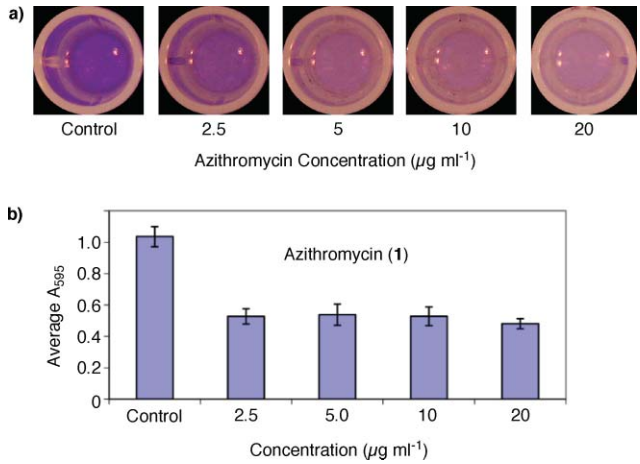
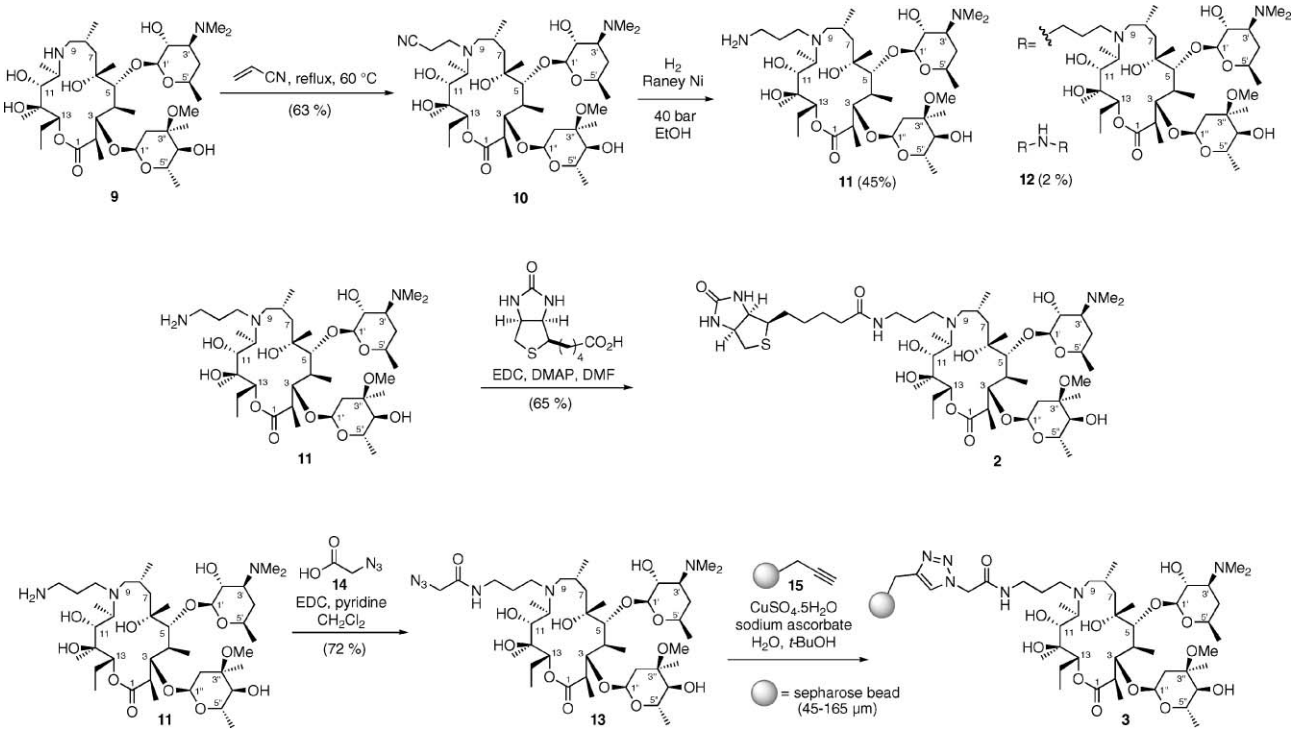


Fig. 3 (a) Biofilm assays at various concentrations of azithromycin. After staining with crystal violet and sequential washing, any biofilm present retains the purple stain. The more purple the appearance of the well, the greater the biofilm formation. (b) Graph showing the absorption at 595 nm (A_{595}) against azithromycin concentration. The lower the absorption, the less biofilm is present. Both these figures show that azithromycin, at the concentrations investigated, inhibited biofilm formation.

the *P. aeruginosa* PAO1 cultures with azithromycin (2.5 μg ml^{-1} to 20 μg ml^{-1}) for 5 days at 37 °C in microtitre plates. The biofilms that resulted could then be visualized by crystal violet staining (purple) and the (relative) amounts quantified by analyzing the absorption from each sample at 595 nm (A_{595}). Azithromycin was shown to have a marked effect on the biofilms of *P. aeruginosa* and inhibition was observed at all of the concentrations investigated (Fig. 3b, biofilm inhibition results in the wells appearing less purple).

In an effort to identify the molecular targets of azithromycin, the analogues **2** and **3** were synthesized for use in pull-down (affinity-chromatography) assays. The use of affinity methods is a common approach to target identification.[27] The synthetic route to both **2** and **3** initially involved the conjugate addition of the azithromycin precursor **9** with acrylonitrile to give **10** followed by Raney nickel reduction (using an H-Cube®) to give the tethered amine compound **11** (Scheme 2).[28] Although a small amount of the dimer **12** (2%) was formed, these reactions proceeded in acceptable yields and provided a suitable site for attaching either biotin or a solid support. The biotinylated compound **2** was formed directly *via* an EDC–DMAP coupling of **11** with D-biotin (Scheme 2).[29] To attach **11** to the solid support, two further steps were required. The amine **11** was initially converted to the α-azoamide **13** in an EDC–DMAP coupling with the acid **14**.[30] The solid support **15** was derivatized with an alkyne functionality. The azide compound **13** could therefore be immobilized using 'click chemistry'.[31] The highly efficient copper-catalyzed Huisgen 1,3-dipolar cycloaddition[32] thus furnished the immobilized azithromycin compound **3** (Scheme 2).[33]

In an effort to test the hypothesis that azithromycin acts to inhibit biofilm formation in *P. aeruginosa* by acting on a protein target other than the ribosome (maybe one involved in quorum sensing),[15] pull-down investigations were performed using suitably designed negative controls to account for non-specific binding interactions. The biotin-tagged compound **2**, immobilized *via* binding to solid supported streptavidin,[29] gave inconclusive results due to the tag binding to various biotin-binding proteins in the cell. However, the immobilized azithromycin analogue **3** gave more promising results and the resulting 1D PAGE gel showed clear enrichment of specific bands (Fig. 4) compared with samples using control sepharose beads such as **15**. A small number of



Scheme 2 The synthesis of the biotin-tagged and immobilized azithromycin compounds **2** and **3**.

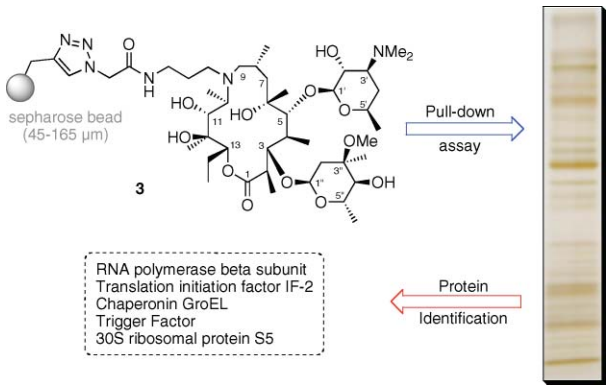


Fig. 4 The pull-down assay using the cell-extract of *P. aeruginosa* and the immobilized azithromycin analogue **3**. Most of the identified proteins are associated with translation or the folding of nascent proteins.

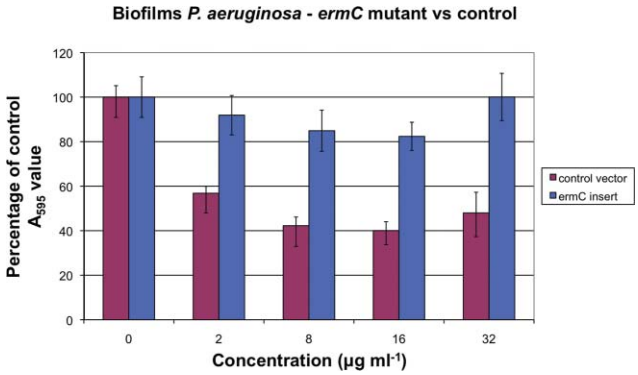


Fig. 5 A graphical representation of the biofilm assays performed at various concentrations of azithromycin using *P. aeruginosa* containing either a control vector or the *ermC* plasmid. The A_{595} values for the mutant are shown as a percentage of the value obtained in the absence of azithromycin. In the p*ermC*-containing strain the inhibition of biofilm formation was greatly reduced compared to the control vector strain.

strongly-stained bands, presumably corresponding to highly-abundant captured proteins, were excised from the gels and digested with trypsin prior to MS/MS analysis to determine the identity of the protein(s). Most of the proteins identified this way were directly associated with ribosome function, translation or the folding of nascent proteins. However, we also found that the immobilized azithromycin captured RNA polymerase β subunit, although this might not be unexpected in a tightly coupled prokaryotic transcription–translation system such as that found in many rapidly growing species like *P. aeruginosa.*

Since there was no evidence that analogue **3** was binding any proteins other than those associated with translation, this suggested that indeed *only* the ribosome was critical to azithromycin's ability to inhibit biofilm formation at sub-MIC values. To further test this hypothesis we performed biofilm inhibition assays in a strain of *P. aeruginosa* that contained the plasmid-encoded *ermC.* Plasmid-encoded *ermC* (p*ermC*) was constructed by sub-cloning the *ermC* gene from p*ermC*T into pUCPKS to enable stable maintenance in *P. aeruginosa.*[34] The *ermC* gene encodes a methylase which acts to modify the ribosome at a known macrolide binding site (specifically a single adenine residue (A2058 in *Escherichia coli*) in the 23S rRNA).[34] This modification makes the bacteria less susceptible to the action of macrolides and is one of the modes of action by which bacteria become resistant to this type of antibiotic (the other is *via* increased drug-efflux). Performing the biofilm assays under the same conditions as before (Fig. 2) but using the p*ermC*-containing *P. aeruginosa* strain gave notable results: little or no biofilm inhibition was observed (Fig. 5). This result clearly indicated that an azithromycin–ribosome interaction is required for biofilm formation to be affected at sub-MIC values. Similar results have been observed previously using the *P. aeruginosa* PAO1 strain expressing the 23S methylase ErmBP.[11]

Conclusions

An efficient synthesis of azithromycin was developed, and this material was used to validate the drug's ability to inhibit biofilm formation at sub-MIC values for *P. aeruginosa.* Biotin-tagged and immobilized azithromycin derivatives were synthesized and exploited in pull-down assays in an effort to identify binding partners and hence illuminate its sub-inhibitory mode of action. Results from these assays uncovered mainly ribosomal proteins and polypeptides involved in translation or folding of the nascent polypeptide. The hypothesis that azithromycin mediates sub-MIC effects through binding to the ribosome was further substantiated by studies using a p*ermC*-containing *P. aeruginosa* strain. The *ermC* gene encodes a methylase, which methylates the ribosome at a known macrolide binding site. In these strains little or no biofilm inhibition was observed. Although additional interactions with as yet undisclosed proteins may be involved, these effects appear subordinate to any ribosome mediated activities. Although this ribosome dependence has been observed by other groups,[11] the realization of the synthesis of immobilized azithromycin **3**, and its ability to pull-down proteins with little background noise, provides an important chemical probe to further investigate the molecular targets of azithromycin in *P. aeruginosa* and other species.

Acknowledgements

This work was supported by funding from the EPSRC, BBSRC, MRC, Syngenta and Augustus and Harry Newman Foundation. We thank Tammo Winkler (Syngenta, Basel) for help with NMR interpretation, Alexander Mankin (University of Illinois) for supplying plasmid-encoded *ermC*, and Barbara Iglewski (U. Rochester, USA) for supplying the *P. aeruginosa* strain PAO1. We also acknowledge the EPSRC National Mass Spectrometry Service Centre, Swansea, for providing mass spectrometric data.

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