

## RESEARCH ARTICLE

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2019, 10, 1438

## Synthetic studies on the reverse antibiotic natural products, the nybomycins†‡

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Antimicrobial resistance (AMR) is a serious issue that could have severe consequences if steps are not taken. The nybomycin natural products have the potential to extend the clinical efficacy of the marketed fluoroquinolone class of antibiotics through a 'reverse antibiotic' approach. However, only very limited structure–activity relationships are known for these fascinating compounds, in part due to challenges with their synthesis. Here we report a new scalable and robust synthetic route to the nybomycin natural products to aid in the development of this series. Through this synthesis, we report the antibiotic activity of novel analogues of this family confirming the selectivity for fluoroquinolone resistant bacteria and potential future opportunities for further optimisation.

Received 7th April 2019,  
Accepted 22nd May 2019

DOI: 10.1039/c9md00207c

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Antibiotic resistance is a worldwide issue. Globally, AMR causes around 700 000 deaths per year at a low estimate.<sup>1</sup> This figure is likely to be an underestimate as incidences are hard to attribute and sometimes not reported. However, with the rise of AMR and the lack of new clinical options, this figure is projected to rise to around 10 million deaths in 2050.<sup>1</sup> That equates to one death every 3 seconds.

The quinolone class of antibiotics, which include ciprofloxacin and levofloxacin, are used to treat a variety of bacterial infections including from both Gram-positive and Gram-negative organisms.<sup>2</sup> They are one of the most prescribed classes of antibiotics.<sup>3</sup> The targets for these antibiotics are the homologous type II topoisomerase enzymes, DNA gyrase and topoisomerase IV.<sup>4,5</sup> Both of these enzymes relieve the strain (or amount of supercoiling) generated in DNA as a consequence of its unwinding during DNA replication.<sup>6–8</sup> Fluoroquinolones function by stabilising the cleaved complex of DNA gyrase (or topoisomerase IV) and DNA, leading to fragmentation of the DNA and death of the bacterial cells.<sup>8–10</sup> The main cause of resistance to this class of antibiotics occurs when there is a mutation in one of the key residues in the quinolone-resistance determining region (QRDR) of the

enzyme.<sup>9,11</sup> These mutations disrupt a water-mediated magnesium bridge linking the quinolone to the protein *via* the non-catalytic Mg<sup>2+</sup> ion, resulting in a significant loss of affinity of the fluoroquinolone to the enzyme.<sup>6,9,12,13</sup>

Resistance to fluoroquinolones was noted rapidly upon initial clinical usage and the resistance rate continues to increase in most bacteria species.<sup>14</sup> Given the success of these antibiotics, new approaches are needed to re-establish their clinical effectiveness. One emerging approach is the use of a 'reverse antibiotic',<sup>15,16</sup> a concept experimentally verified *in vitro* with the fluoroquinolone ciprofloxacin and the natural product deoxynybomycin.<sup>16</sup> In this approach (Fig. 1), deoxynybomycin was identified to be active against fluoroquinolone-resistant bacteria through binding to QRDR mutant type II topoisomerase enzymes, S84L gyrA and E84K parC. Importantly however, resistance to deoxynybomycin occurred by a back-mutation to the wild-type type II topoisomerase enzymes, thus re-sensitising the bacteria to fluoroquinolones. In theory, the combined usage of fluoroquinolones and nybomycin natural products could trap bacteria in an 'evolutionary loop' where they will always be sensitive to one of the antibiotics. This approach breaks the cycle of needing to constantly develop new antibiotics for resistant bacteria and may be a powerful way to tackle AMR, especially once further developed. Furthermore, development of the nybomycin series may provide alternative antibiotics against this validated bacterial target class, given some of the side effects observed for the fluoroquinolones.<sup>17,18</sup>

Deoxynybomycin belongs to the nybomycin series of natural products. Both nybomycin and deoxynybomycin are reported to be highly active against the fluoroquinolone resistant bacteria strains (FQR) but inactive against the

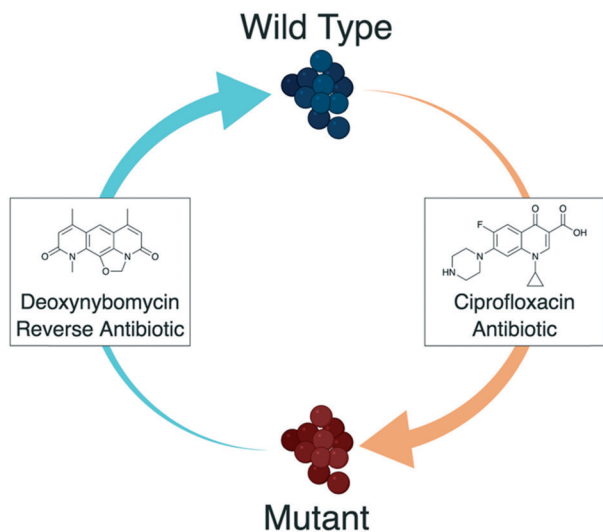
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† Raw data files are openly available DOI: 10.14469/hpc/5498

‡ Electronic supplementary information (ESI) available: Experimental procedures, characterisation data, NMR spectra, further X-ray data. CCDC 1908213. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c9md00207c

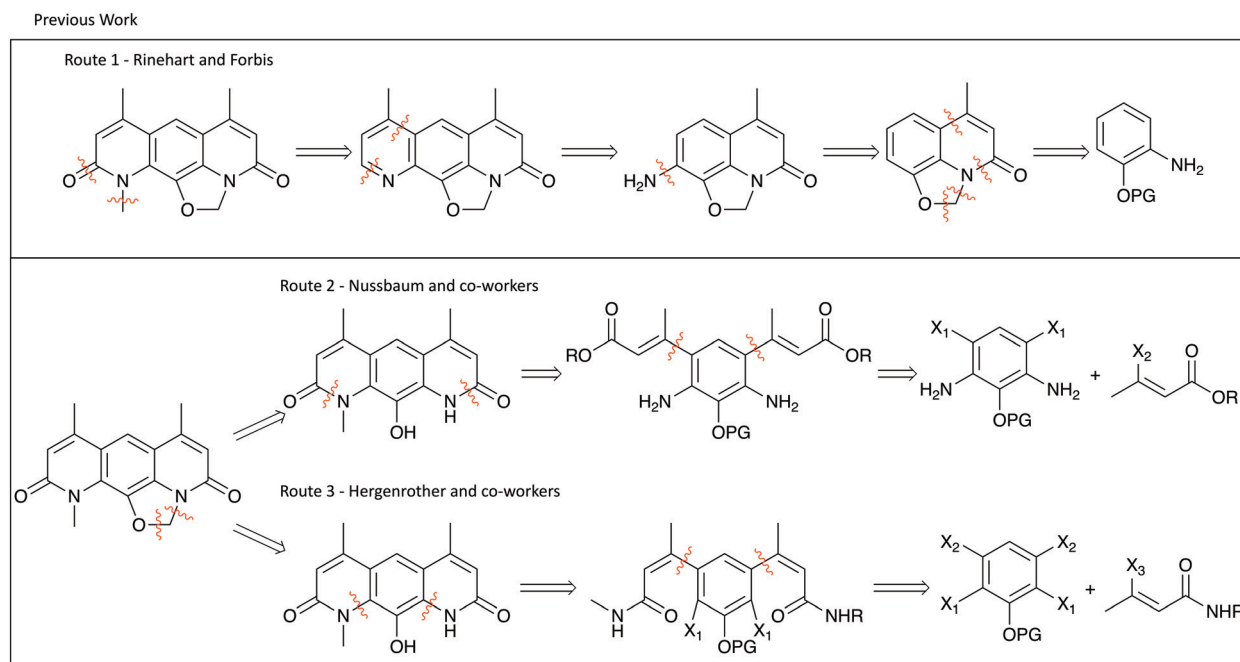


**Fig. 1** Diagrammatic representation of the 'reverse antibiotic' concept. Ciprofloxacin (a fluoroquinolone antibiotic) is an effective treatment of wild type *S. aureus* shown in blue. Target mutations lead to a mutant strain, shown in red, that is resistant to ciprofloxacin. Deoxynybomycin (a reverse antibiotic) can then be used to treat the mutant *S. aureus*. Importantly, the mutation required to gain resistance to deoxynybomycin reverts back to the wild-type target, which resensitises the *S. aureus* to fluoroquinolone antibiotics. This could be an important approach in breaking the constant need to discover new antibiotics with novel modes of action.<sup>15,16</sup> Image created with BioRender.com.

fluoroquinolone sensitive strains (FQS) and are, thus far, the only example of a 'reverse antibiotic'.<sup>15,16</sup> Whilst the nybomycins show a lack of activity against Gram-negative bacteria,<sup>19</sup> it has been shown that structural modification of

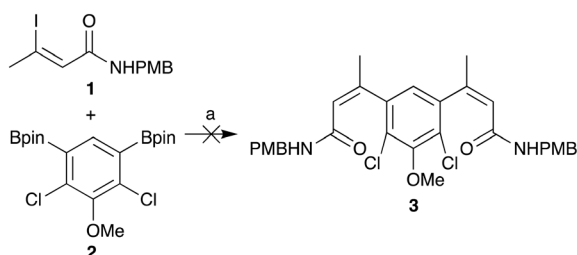
the scaffold can lead to broad spectrum activity.<sup>19</sup> Despite the key importance this places upon this natural product series, very limited medicinal chemistry exploration of the nybomycin scaffold has been reported.<sup>16,19</sup> Such medicinal chemistry studies are clearly needed: both nybomycin and deoxynybomycin have poor aqueous solubility and selectivity issues over the human topoisomerase isoforms.<sup>20</sup> Optimisation will be needed to alleviate these liabilities, in order for this class of antibiotics to progress into clinical exploration. Here we report our synthetic studies on the nybomycins, aiming to develop a reliable and reproducible synthesis that is amenable to analogue synthesis. Through this route, we report some initial structure activity relationships (SAR) of the nybomycin series', identifying novel structural determinants for their antibiotic activity.

Although nybomycin and deoxynybomycin are natural products that were first reported in 1955,<sup>21</sup> their isolation is far from straight-forward<sup>22</sup> and the fermentation process is inefficient (1000 L is reported to give 350–200 mg of nybomycin).<sup>23</sup> The previously reported total syntheses are also relatively low yielding (13%,<sup>24</sup> 11%,<sup>16</sup> 0.84%<sup>25,26</sup> overall yield). An overview of the prior synthetic approaches is shown in Fig. 2. Route 1, developed by Rinehart and Forbis,<sup>25,26</sup> disregards the symmetry of the molecule and constructs the central framework sequentially. Given this, in addition to some low yielding steps, an overall yield of 0.84% is obtained for the synthesis of deoxynybomycin, which is clearly not ideal for analogue synthesis. Routes 2 and 3, developed by Nussbaum and co-workers<sup>24</sup> and Hergenrother and co-workers<sup>16,27,28</sup> respectively, better exploit the symmetrical nature of the molecule, reducing the overall number of linear steps.



**Fig. 2** Previously reported routes to deoxynybomycin.

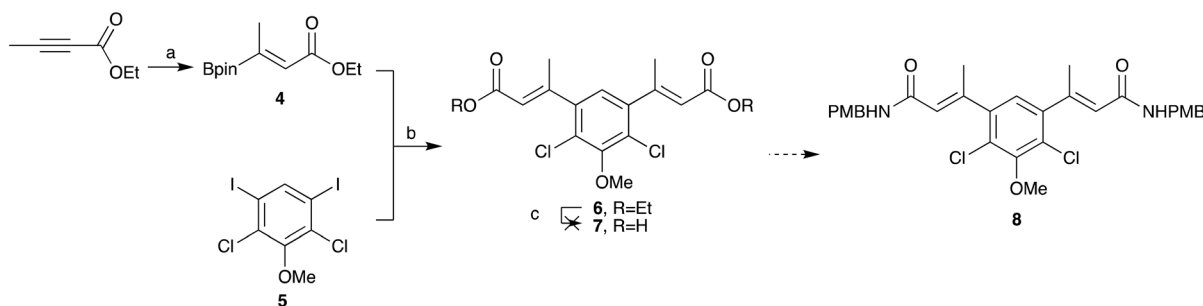
Route 3 was identified as the preferred route for further medicinal chemistry as it had the best overall yield and offered the most reliable synthesis of deoxybomycin. The *cis*-iodo-alkene **1** and borylated core **2** intermediates, shown in Scheme 1, were synthesised in accordance with the literature protocols.<sup>27–29</sup> However, the reported Suzuki-Miyaura cross-coupling failed to yield any of the desired compound **3** in our hands. A screen for alternative reaction conditions was therefore carried out; altering the catalyst, ligand, base and solvent. Regrettably, all conditions surveyed failed to yield any of the desired cross-coupled product. Due to the relatively unactivated nature of aryl boronic ester **2** and the base instability of the iodo-alkene **1**, we proposed that swapping the coupling partners would improve the robustness of this capricious cross-coupling reaction.



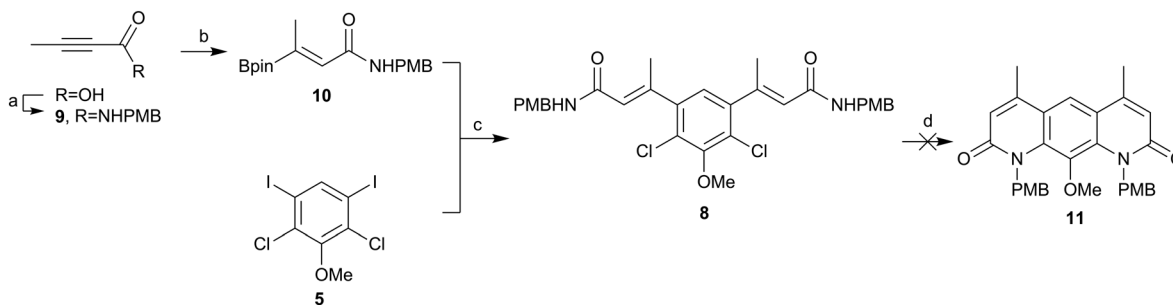
**Scheme 1** Unsuccessful Suzuki-Miyaura cross-coupling of **1** and **2** to form **3** using the previously reported conditions.<sup>27–29</sup> (a) Pd(dppf)Cl<sub>2</sub>·CH<sub>2</sub>Cl<sub>2</sub>, K<sub>2</sub>CO<sub>3</sub>, DME, H<sub>2</sub>O, 85 °C.

Borylated-alkene **4** was synthesised as shown in Scheme 2. This was achieved in a high yield in accordance with the work of Aggarwal and co-workers from ethyl but-2-ynoate using bis(pinacolato)diboron, sodium *tert*-butoxide and copper(II) chloride.<sup>30</sup> Determination of the stereoisomer formed in this reaction was challenging, but mechanistically it was more likely to be the *Z*-isomer following *syn*-addition: incorrect stereochemistry for the latter cyclisation. This material was progressed in the hope that *in situ* isomerisation would occur, either heat or transition metal mediated, during palladium catalysed amination. Pleasingly, cross-coupling of the borylated alkene **4** and the iodo core **5** progressed in an excellent yield (97%). This validated the approach to exchange to coupling partners in order to further optimise this chemistry. However, prior to cyclisation, the ethyl ester **6** needed to be converted to the amide **8**, *via* the carboxylic acid **7**. Unfortunately, base-mediated hydrolysis of the di-ester **6** proved problematic and the desired product could not be obtained due to degradation.

In order to avoid such degradation, the chemistry was repeated using *N*-(4-methoxybenzyl)but-2-ynamide (**9**). The formation of the alkyne amide **9** was achieved from but-2-ynoic acid using propylphosphonic anhydride (T3P) in good yields (Scheme 3). Alkyne amide **9** then underwent hydroboration as before, to yield the (presumably) *syn*-hydroborated alkene amide **10**.<sup>30</sup> Product **10** was then successfully coupled with the iodo core **5** using the previous conditions, again in an excellent yield (86%). The next step was to cyclise the



**Scheme 2** Attempted formation of amide **8**. (a) B<sub>2</sub>pin<sub>2</sub>, NaO<sup>t</sup>Bu, Cu(II)Cl<sub>2</sub>, xantphos, MeOH, THF, rt, 77%, (b) Pd(dppf)Cl<sub>2</sub>·CH<sub>2</sub>Cl<sub>2</sub>, K<sub>2</sub>CO<sub>3</sub>, DME, H<sub>2</sub>O, 85 °C, 97%. (c) NaOH, H<sub>2</sub>O, THF.

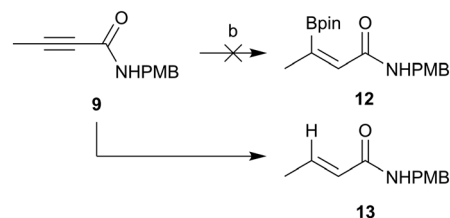


**Scheme 3** Attempted synthesis of **11**. (a) NH<sub>2</sub>PMB, T3P, NEt<sub>3</sub>, THF, rt, 84%, (b) B<sub>2</sub>pin<sub>2</sub>, NaO<sup>t</sup>Bu, Cu(II)Cl<sub>2</sub>, xantphos, MeOH, THF, rt, 97%, (c) Pd(dppf)Cl<sub>2</sub>·CH<sub>2</sub>Cl<sub>2</sub>, K<sub>2</sub>CO<sub>3</sub>, DME, H<sub>2</sub>O, 85 °C, 86%, (d) Pd G2 Xphos, Xphos, K<sub>2</sub>CO<sub>3</sub>, *i*-PrOH, 90 °C.

compound to form the core of deoxynybomycin **11**. Using the Buchwald-Hartwig conditions reported by Hergenrother and co-workers,<sup>28,29</sup> no evidence of cyclised material was observed and only the starting amide remained. A screen of alternative reaction conditions was carried out varying the catalyst, base and solvent, as well as alternative pathways such as nucleophilic aromatic substitution (S<sub>N</sub>Ar), but none of these conditions successfully delivered the product. We attribute the failure of these reactions to the incorrect stereochemistry of the alkene and lack of *in situ* isomerisation. We therefore instead explored alternative methods to access the correct stereochemistry to facilitate cyclisation.

Hydroboration of an alkyne typically occurs to provide the *anti*-Markovnikov adduct with *syn*-selectivity *via* a four-membered transition state. Although such regiochemistry is required for our coupling intermediate, the stereochemistry is incorrect. Given our failed attempt above, *E*-stereochemistry of the hydroboration product would be required in order for the resultant coupled product to be in the correct stereochemistry to cyclise. Sawamura and co-workers have previously reported an *anti*-hydroboration of alkynyl esters to give hydroborated alkenes with the desired *regio*- and stereochemistry.<sup>31</sup> Given the previous challenges with ester hydrolysis once attached to the core, synthesis of the amide coupling partner **12** was attempted (Scheme 4). Unfortunately the *anti*-hydroboration chemistry developed by Sawamura and co-workers did not work for substrate **9**. Instead of hydroboration, reduction was the preferred pathway and the major isolated product was (*E*)-*N*-(4-methoxybenzyl)but-2-enamide (**13**).

Therefore, we returned to an ester coupling partner, shown in Scheme 5. Pleasingly, ethyl but-2-ynoate was hydroborated in a reasonable yield (60%) and ~9:1 *anti*:*syn*. Evidence for the alternative stereochemistry of this reaction came from the <sup>1</sup>H NMR data. There was a downfield shift of 0.43 ppm in the alkene proton and 0.22 ppm in the alkene methyl group when compared to the previously synthesised addition product, assumed to be *syn*. Coupling of this material **14** to iodo core **5** progressed in excellent yield (98%). However, base-mediated hydrolysis once again proved to be problematic. Conditions using different hydroxide sources, reaction times, temperatures, solvents and solvent ratios were screened, shown in the ESI.† The best conditions – using NaOH (3M)

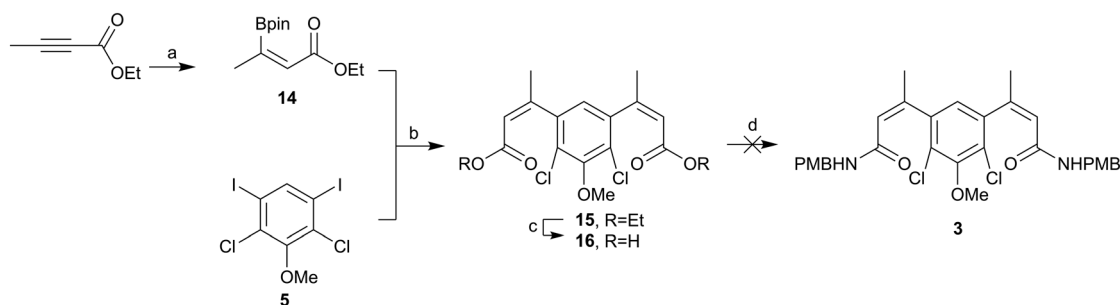


**Scheme 4** Attempted synthesis of amide coupling partner **12**. (a) HBpin, P(Bu)<sub>3</sub>, 60 °C, MeCN, 47%.

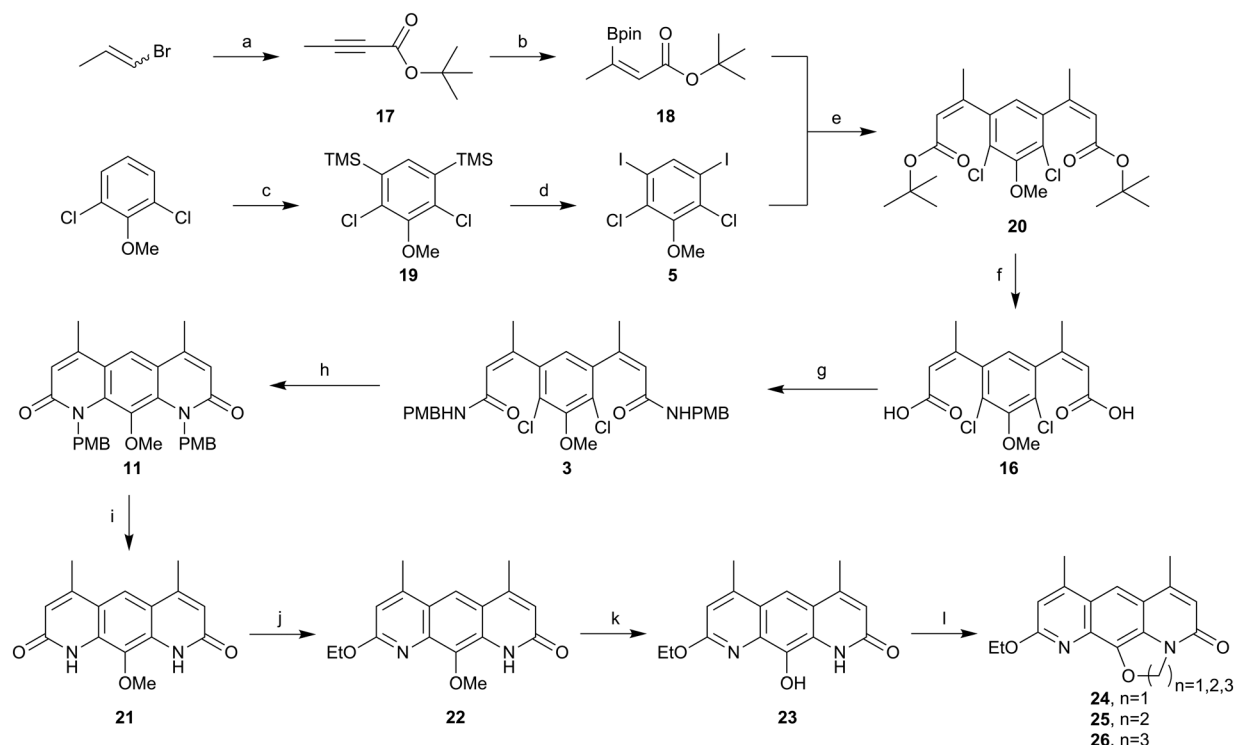
in MeOH – yielded small amounts of the di-acid **16**, but purifying this compound proved problematic and telescoping the crude material into the amide formation using T3P as the coupling agent or *via* the formation of the acyl chloride did not yield any of the desired di-amide **3**. Direct amination of ester **15** using trimethylaluminum also failed.

Given the apparent base lability of the intermediate **15**, we changed to the use of a *tert*-butyl ester, which would allow formation of carboxylic acid **16** through acid-mediated *tert*-butyl cleavage (Scheme 6). Alkyne **17** was synthesised as reported previously in the literature,<sup>32</sup> *via* an elimination and deprotonation of a mixture of *cis*/*trans* 1-bromopropene with *n*BuLi, then trapping the lithiated alkyne anion formed with di-*tert*-butyl dicarbonate. Pleasingly, the hydroboration developed by Sawamura and co-workers<sup>31</sup> worked well on alkyne **17** to give alkene **18** in an acceptable yield (52%). This material was purified by distillation and crystals were grown from a mixture of pentane and diethyl ether to allow for determination of the geometry across the double bond by a single-crystal X-ray diffraction. As evident from the X-ray structure of alkene **18** (Fig. 3), the desired *anti*-addition product was indeed obtained. This information compared to the <sup>1</sup>H NMR data let us unequivocally assign the stereochemistry of alkene **4** as *Z*.

The key coupling step proceeded cleanly and efficiently to yield the di-ester **20** in a good yield (75%) and proved to be robust and suitable to gram scale synthesis (Scheme 6). Treatment of **20** with trifluoroacetic acid afforded the desired di-acid **16** in an excellent yield (95%), which was cleanly converted to the di *para*-methoxybenzyl (PMB) amide **3** by first formation of the di-acyl chloride and then treatment with excess PMB amine. Amide **3** was cyclised using the



**Scheme 5** Attempted synthesis of amide **3**. (a) HBpin, P(Me)<sub>3</sub>, THF, rt, 60%, (b) Pd(dppf)Cl<sub>2</sub>·CH<sub>2</sub>Cl<sub>2</sub>, K<sub>2</sub>CO<sub>3</sub>, DME, H<sub>2</sub>O, 85 °C, 98%, (c) NaOH, H<sub>2</sub>O, MeOH, rt, (d) (i) (COCl)<sub>2</sub>, rt, (ii) NH<sub>2</sub>PMB, NEt<sub>3</sub>, DCM, rt.

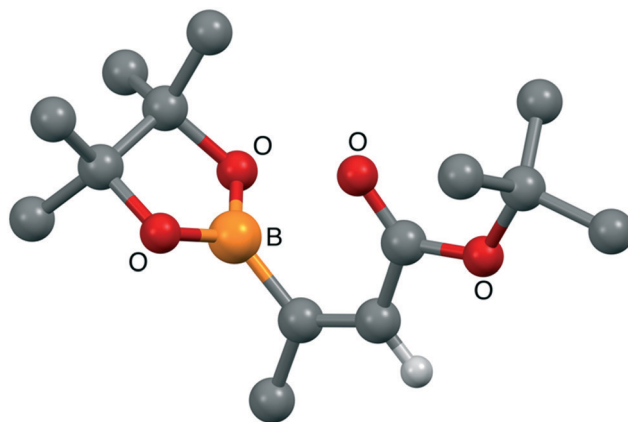


**Scheme 6** Synthesis of ethoxy-pyridine analogues of **24**, **25** and **26**. (a) *n*-BuLi, Boc<sub>2</sub>O, THF, −78 °C, 98%, (b) HBpin, P(Me)<sub>3</sub>, THF, rt, 52%, (c) LDA, TMSCl, THF, −78 °C, quant, (d) ICl, DCM, rt, 86%, (e) Pd(dppf)Cl<sub>2</sub>·CH<sub>2</sub>Cl<sub>2</sub>, K<sub>2</sub>CO<sub>3</sub>, DME, H<sub>2</sub>O, 85 °C, 75%, (f) TFA, DCM, rt, 95%, (g) (i) (COCl)<sub>2</sub>, rt, (ii) NH<sub>2</sub>-PMB, NEt<sub>3</sub>, DCM, rt, 96%, (h) Pd G2 Xphos, Xphos, K<sub>2</sub>CO<sub>3</sub>, i-PrOH, 90 °C, 99%, (i) TFA, rt, 98%, (j) EtI, K<sub>2</sub>CO<sub>3</sub>, DMSO, 70 °C, 59%, (k) BBr<sub>3</sub>, DCM, rt, 41%, (l) CH<sub>2</sub>Br<sub>2</sub>, K<sub>2</sub>CO<sub>3</sub>, DMSO, 85 °C, 14% for **24**, BrCH<sub>2</sub>CH<sub>2</sub>Br, K<sub>2</sub>CO<sub>3</sub>, DMSO, 85 °C, 13% for **25**, BrCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Br, K<sub>2</sub>CO<sub>3</sub>, DMF, 110 °C, 18% for **26**.

Buchwald–Hartwig amination conditions used by Hergenrother and co-workers<sup>27,28</sup> to yield **11**. Deprotection of the PMB protecting groups was achieved using trifluoroacetic acid, resulting in **21**. The solubility of compound **21** was very poor in a range of organic solvents, but ethylation using iodoethane in DMSO at 85 °C with potassium carbonate as the base gave a more soluble product. After analysis of the product by <sup>13</sup>C NMR and comparison with similar compounds in the literature,<sup>16</sup> this reaction was found to have afforded the *O*-alkylated ethoxy-pyridine **22** as the major reaction product. The chemoselectivity of this alkylation was contradictory to the literature, which suggested *N*-alkylation to be preferred.<sup>33–35</sup> However, we took this as an opportunity to explore the antibacterial effect of these novel nybomycin-like analogues and probe novel structure–activity relationships. Chemoselective demethylation of anisole **22** was achieved using BBr<sub>3</sub> and the product **23** could be isolated *via* precipitation with water and filtration. The resulting off-white solid **23** was cyclised using 1,1-dibromomethane for **24**, 1,2-dibromoethane for **25** and 1,3-dibromopropane for **26**, to access three novel nybomycin-like analogues for anti-bacterial testing.

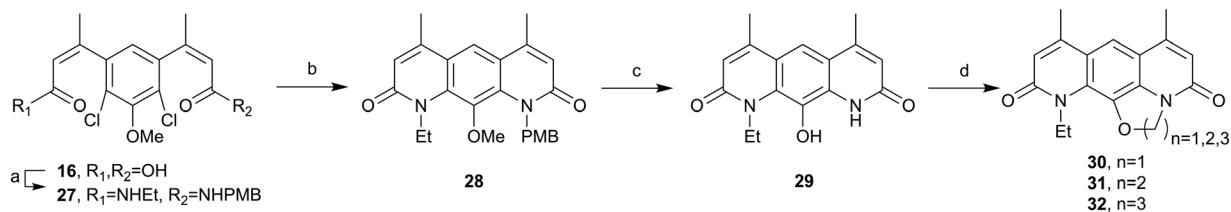
Attention then returned to the synthesis of **30**. To avoid *O*-ethylation the *N*-ethyl *N*-PMB amide **27** was prepared, prior to cyclisation, as shown in Scheme 7. Di-acid **16** underwent an unsymmetrical amide coupling with a 1:1 mixture of ethylamine and PMB amine to give the statistical mixture of the amide products. These products could be separated by

column chromatography affording the desired amine **27** in a 42% yield and the undesired di-PMB amide **3** which could be recycled for the synthesis of the ethoxy-pyridines above. The desired amide **27** was cyclised under Buchwald–Hartwig amination conditions using Xphos Pd G2.<sup>36</sup> This gave good conversion (85%) to the cyclic product **28** which was then globally deprotected using HBr at reflux. Treatment of the reaction mixture with water gave an off-white precipitate which was isolated *via* filtration to give **29**. This material could then



**Fig. 3** Structural conformation of **18**. X-ray structure of **18**, confirming *anti*-hydroboration had occurred and the coupling partner had the desired conformation (*E*).





**Scheme 7** Synthesis of deoxynybomycin analogues **30**, **31** and **32**. (a) (i)  $(\text{COCl})_2$ , rt, (ii)  $\text{NH}_2\text{Et}$ ,  $\text{NH}_2\text{PMB}$ ,  $\text{NEt}_3$ , DCM, rt, 42%, (b) Pd G2 Xphos,  $\text{K}_2\text{CO}_3$ , i-PrOH, 85 °C, 85%, (c) HBr, 110 °C, 91%, (d)  $\text{CH}_2\text{Br}_2$ ,  $\text{K}_2\text{CO}_3$ , DMSO, 85 °C, 33% for **30**,  $\text{BrCH}_2\text{CH}_2\text{Br}$ ,  $\text{K}_2\text{CO}_3$ , DMSO, 110 °C, 28% for **31**,  $\text{BrCH}_2\text{CH}_2\text{CH}_2\text{Br}$ ,  $\text{K}_2\text{CO}_3$ , DMF, 110 °C, 44% for **32**.

be cyclised using 1,1-dibromomethane for **30**, 1,2-dibromoethane for **31** and 1,3-dibromopropane for **32**, to two novel nybomycin analogues for anti-bacterial testing and comparison with the previously described compound **30**.<sup>16</sup>

The antibiotic activity of **30** and novel analogues (**24**, **25**, **26**, **31** and **32**) were determined using the Kirby–Bauer disc diffusion assay<sup>37</sup> using the protocol described by Sridhar and co-workers,<sup>38</sup> with the fluoroquinolone sensitive (FQS) *S. aureus* strain SH1000 and the fluoroquinolone resistant (FQR) *S. aureus* strain USA300 JE2 (Table 1). *S. aureus* USA300 JE2 has a mutated DNA gyrase (S84L) and topo-

isomerase IV (S80Y) which causes resistance to ciprofloxacin as well as additional mutations that confer resistance to other antibiotic classes.<sup>39</sup>

As previously reported,<sup>15,16</sup> compound **30** showed the desired mutant selectivity, resulting in effective killing of USA300 JE2. Ring expanded analogues **31** and **32** gave comparable activity, with the 6 membered ring (**31**) tentatively the most active. This initial result will have to be confirmed using an additional susceptibility testing method.

Interestingly, compounds **24**, **25**, and **26**, where the oxygen of the pyridone was alkylated rather than the nitrogen, showed greatly reduced activity compared to **30**, **31**, and **32**. **24** was inactive at a concentration of 32 mmol in both the FQR strain and FQS strain (data not shown). However, compound **25** with the 6-membered ring, showed some activity against the FQR strain at 10 mmol. This novel SAR is intriguing and suggests that further investigation may be warranted. Such work is ongoing and will be reported in due course.

## Conclusions

In summary we have developed a robust and scalable route to the nybomycin scaffold which can facilitate the synthesis diverse array of analogues. The key differentiating step of our approach was to switch the coupling partners of the capricious Suzuki–Miyaura cross-coupling, resulting in scalable coupling yields up to 98%. Our route only involves the use of column chromatography three times which further enables scalability. We believe this route should allow for much needed medicinal chemistry exploration of the nybomycin ‘reverse antibiotics’. Indeed, we have initially surveyed five novel analogues which indicate key structure determinants for antibiotic activity and opportunities for further optimisation.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

MJF would like to thank the EPSRC for an Established Career Fellowship (EP/R00188X/1). The Aragon laboratory is supported by the UK Medical Research Council. OBC was supported by a Cross Faculty Collaborative studentship from

**Table 1** Zone of inhibitions (in cm) of known compounds ciprofloxacin and **30** and novel analogues **31**, **32**, **24**, **25** and **26**. SH1000 was used as the FQS strain and USA300 JE2 was used as the FQR strain. Compounds were tested at 1 and 10 mmol using the Kirby–Bauer disc diffusion assay with 7 replicates

Compound	Structure	Zone of inhibition (cm)			
		FQS		FQR	
		1 mmol	10 mmol	1 mmol	10 mmol
Ciprofloxacin		1.8	3.1	0	1.2
<b>30</b>		0	1.8	1.4	2.9
<b>31</b>		0	2.2	1.8	3.2
<b>32</b>		0	2.3	0	2.7
<b>24</b>		0	0	0	0
<b>25</b>		0	0	0	1.0
<b>26</b>		0	0	0	0

the London Institute of Medical Research (LMS), which receives its core funding from the UK Medical Research Council. The authors would like to give thanks Dr Andrew Edwards for providing the SH1000 and USA300 JE2 bacterial strains, Peter Haycock for NMR support, Adam Jarmuz for microbiology support and Dr. Lisa Haigh for mass spectrometry.

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