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Macrocyclic Inhibitors of the Bacterial Cell Wall Biosynthesis Enzyme Mur D

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Abstract—Computer-based molecular design has been used to produce a series of new macrocyclic systems targeted against the bacterial cell wall biosynthetic enzyme MurD. Following their preparation, which involved a novel metathesis-based cyclisation as the key step, these systems were found to show good inhibition when assayed against the MurD enzyme.

The widespread emergence of bacterial resistance to existing antibiotics has generated an urgent need for the discovery and development of novel antibacterial agents active against previously unexploited targets. The later stages of bacterial peptidoglycan synthesis have been extensively exploited as drug targets,[1] most notably through the development of β-lactam- and glycopeptide antibiotics. However, early biosynthetic steps have received relatively little attention as potential drug targets even though this part of the biosynthetic pathway offers essential enzymes that have no mammalian counterparts.[2,3]

Peptidoglycan contains chains of alternating *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) residues, cross-linked through pentapeptide side chains attached to MurNAc. The pentapeptide chain sequence is L-Ala (occasionally L-Gly)-D-Glu-X-D-Ala-D-Ala where X is usually a diamino acid and often *meso*-diaminopimelic acid (*m*-Dpm) or L-Lys. The biosynthesis of peptidoglycan is a complex three stage process. The first stage involves the assembly of the uridine diphosphate–MurNAc–pentapeptide **1** by enzymes located in the cytoplasm, or at the inner surface of the cytoplasmic membrane.[4] The D-glutamate ligase or MurD enzyme catalyses the addition of D-glutamate to UDP-MurNAc-L-Ala **2** to give UDP-MurNAc-L-Ala-D-Glu **3** which is then converted to **1** by the successive actions of MurE and MurF (Scheme 1).

Previous work by Tanner[5] and Gegnas[6] has shown that several phosphinates developed as transition state isosteres for the amide bond formed by the ligase have potentially useful inhibitory properties. We were intrigued by the possibility of applying a molecular design approach using the available X-ray crystallographic data for MurD[7] to produce a novel class of MurD inhibitor and report here the successful preparation and biological evaluation of such systems.

Our approach was inspired by an initial inspection of the active site within the X-ray crystal structure of MurD containing the bound substrate **2**[7] which suggested that it may be possible to omit much of the sugar portion of **2** to yield **4** (Scheme 2) whilst still retaining binding due to the presence of the ligand/protein contacts within the remaining part of the molecule.

The active site was further analysed using the de novo molecular design programme SPROUT.[8] This revealed the presence of a hydrophobic region within the binding cavity (Fig. 1) which was not being utilized in binding of the natural substrate. Further analysis using SPROUT indicated that the binding of the simplified substrate analogues **4** could be increased by placing alkyl groups within this hydrophobic region. An attractive entropically favorable extension would be to incorporate these

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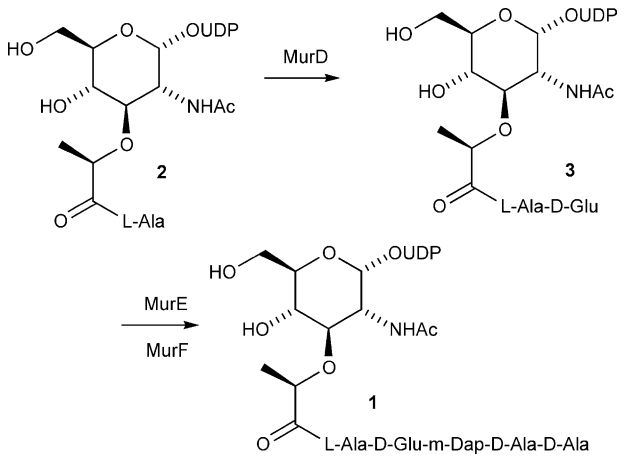
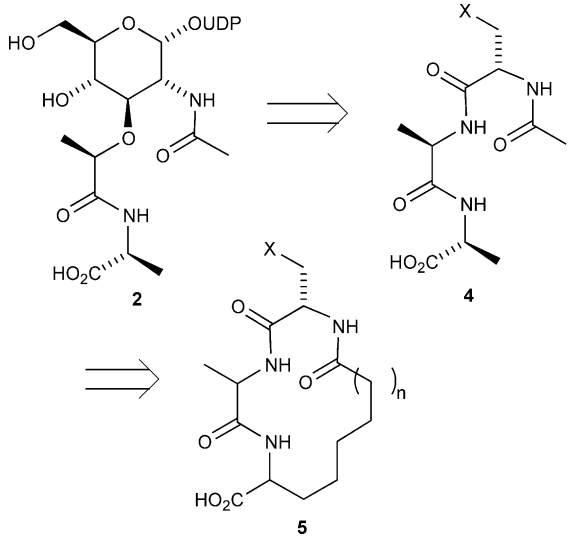


Scheme 1. Biosynthesis of peptidogylcan.



Scheme 2. Simplification of MurD substrate reveals macrocyclic structures **5**.

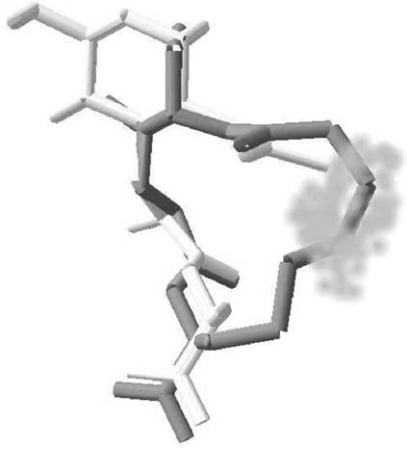
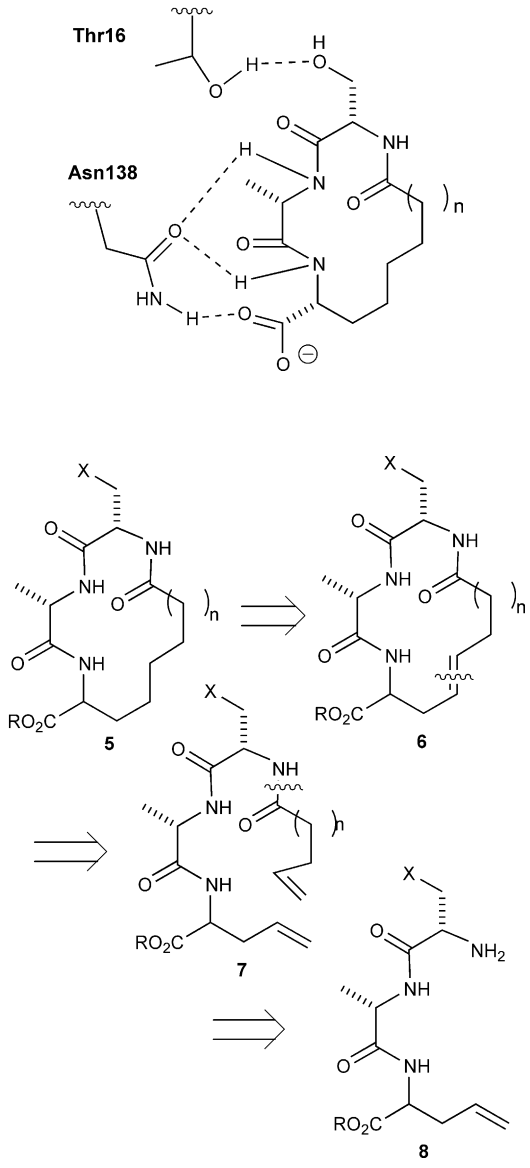


Figure 1. Overlay of natural substrate **2** (light) and macrocyclic inhibitor **5** ($n=1$) showing the position of the hydrophobic region (shaded).

as part of a chain connecting the original *N*-acetyl- and L-alanyl groups, respectively, as in **5** (Scheme 2).

Further modelling studies predicted that medium sized ring systems based on structure **5** (R=H) would adopt conformations in the active site that would favour contacts between the ligand and residues Thr16 and Asn138 (Fig. 2).

This modelling also indicated that the stereochemical configuration at the carbon bearing the methyl group was not important for binding and it was decided to synthesise macrocycles possessing (*S*) absolute stereochemistry at this centre (as in Fig. 2). It was reasoned that macrocycles **5** should be accessible via hydrogenation of the double bond in olefins **6**. These should be accessible from dienes **7** via use of an olefin metathesis type ring closure. The required dienes are derived from *N*-acylation of tripeptides **8** which are based on the commercially available allyl glycine (Scheme 3).

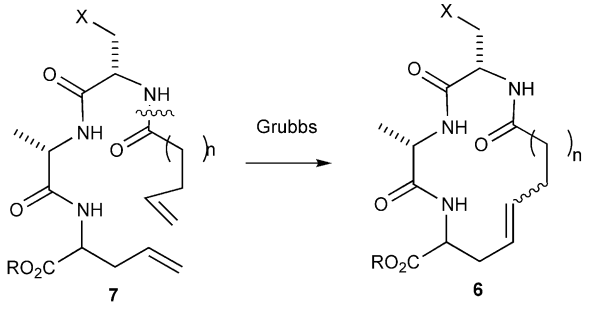


Scheme 3. Retrosynthetic analysis of macrocycles **5**.

According to the modelling studies, it appeared that binding of macrocycles **5** should be optimum for X=OH, due to a hydrogen bonding contact of this hydroxyl with Thr16 (Fig. 2). Additionally, this binding model indicated that an '*R*' absolute stereochemistry at the exocyclic carboxyl residue in **5** would also favour binding. It was decided to produce a range of heterocycles **5** which could test these predictions.

The required dienes **7** were obtained in good yields via coupling of either hex-5-en- or pent-6-enioc acids with either alanine- (leading to X = H) or serine (giving X = OH) derived esters respectively followed by further coupling of an L-alanine residue to yield the corresponding dipeptides. Coupling of these to either D- or L-allyl glycine esters (leading to '*R*' or '*S*' absolute stereochemistry respectively at the exocyclic carboxyl in macrocycles **5**) gave dienes **7**. Carboxylic acids were protected as either methyl or *t*-butyl esters. Methyl esters were cleaved using 1N lithium hydroxide and t-butyl esters were deprotected with a 50% trifluoroacetic acid in dichloromethane solution. Cleavages with lithium hydroxide proceeded in yields of 60–70% and with trifluoroacetic acid in greater than 90%. All couplings were performed in solution using HATU and standard peptide coupling protocols. Yields for the individual couplings were consistently over 90%.

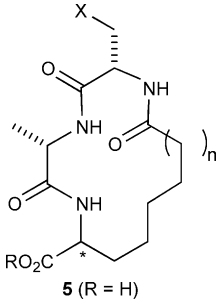
For the synthesis of macrocycles **5**, ring closing metathesis of dienes **7** was performed using Grubbs catalyst[9] and following an initial column-based chromatographic purification, the cyclised systems **6** (R = Me) were obtained in pure form following HPLC. Hydrogenation of the resulting alkene from the metathesis reaction was facile and generally could be accomplished either directly over the Grubbs catalyst or subsequently by using activated palladium on charcoal (Scheme 4). Final ester hydrolysis gave the desired macrocycles **5** (R = H).



n	Chirality at (*)	X	Yields of **5** (R = Me) (%)
2	S	H	21
2	R	H	55
3	S	H	58
3	R	H	58
2	S	O^{t}Bu	22
2	R	O^{t}Bu	25

Scheme 4. Metathesis-based cyclisations.

Table 1. Binding of macrocycles **5** (R = H) to MurD



5 (R = H)

Entry	*n*	Chirality at (*)	X	IC_{50} (μM)
1	2	*S*	H	5.1 (±1.4)
2	2	*R*	H	1.5 (±0.1)
3	3	*S*	H	3.2 (±0.1)
4	3	*R*	H	1.5 (±0.1)
5	2	*S*	OH	2.3 (±1.2)
6	2	*R*	OH	0.7 (±0.3)
7	2	*S*	O^{t}Bu	9.0 (±0.1)
8	2	*R*	O^{t}Bu	1.9 (±0.2)

Biological evaluation[10] of macrocycles **5** (R = H) was performed against isolated *Escherichia coli* MurD enzyme and is summarised in Table 1.

Analysis of the data in Table 1 reveals some interesting trends. In all cases, systems having '*R*' absolute stereochemistry at the exocyclic carboxyl (and thus derived from D-allyl glycine) display strongest affinity for MurD (entries 2, 4, 6 and 8) compared to the corresponding S-derivatives in keeping with the modeling predictions. Additionally, in the '*S*' series (X = H), affinity increases upon increasing ring size which may reflect the increased hydrophobic contribution to binding resulting from the location of alkyl chain fragments within the hydrophobic region of the cavity. The system possessing an hydroxy group (X = OH, derived from a serine amino acid) and '*R*' absolute stereochemistry at the exocyclic carboxyl exhibits the greatest affinity for MurD (entry 6). This lends further support to the proposed mode of binding of these macrocycles which predicts a hydrogen-bonding contact of this hydroxyl group to Thr16 within the enzyme. The observation that the *tert*-butyl ethers in both the '*R*' and '*S*' series not only show affinities lower than the corresponding methyl-containing compounds (entries 1 and 2) but also lower than the corresponding alcohols (entries 5 and 6) may suggest that the *tert*-butyl groups are too bulky for them to be favourably located in the part of the cavity containing Thr16.

In conclusion, molecular modeling techniques have been used in conjunction with X-ray crystallographic data to design a novel class of MurD inhibitor based on a cyclised peptidic framework. A straightforward synthetic approach from readily available precursors and based on an olefin metathesis strategy has been developed. In vitro binding studies of a number of these systems with isolated MurD enzyme reveals them to posses useful affinity for the enzyme and supports the model of binding of these systems within the active site of the enzyme.

Acknowledgements

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9. See Zuercher, W. J.; Hashimoto, M.; Grubbs, R. H. *J. Am. Chem. Soc.* **1996**, *118*, 6634. In a typical procedure, to a solution of the acyclic diene (1 mmol) in anhydrous dichloromethane was added a solution of Grubbs catalyst, [bis(tricyclohexylphosphine)benzylidine ruthenium (IV) dichloride] (40 mol%) in dichloromethane (10 mL). The mixture was heated to reflux for 48 h, cooled and concentrated in vacuo, followed by purification using column chromatography.
10. The gene encoding MurD from *E. coli* JM109 was amplified by PCR using the primers MurDF 5′-TATTAACCATGGCTGATTATCAGGGT-3′ and MurDR 5′-TATATACTCGAGTCAA CCTAACTCCTTCG-3′ which introduced a 5′ *Nco*I site and a 3′ *Xho*I site. The resulting product was cloned into the *Sma*I site of pBS SK(+) and sequenced. The fragment encoding MurD was excised with *Nco*I and *Xho*I, cloned into the *Nco*I- *Xho*I site of pET28b(+) and transformed into *E. coli* BL21. For overexpression, the resulting strain was grown and lysed as described in the pET manual (Novagen). The lysate was loaded on a HiLoad Phenyl Sepharose column (Amersham) equilibrated with buffer A (100 mM Tris, pH 8.2, 2 mM DTT, 1.5 M $(NH_4)_2SO_4$), and bound protein was eluted during a linear gradient with buffer B [as A, without $(NH_4)_2SO_4$]. Eluate containing MurD activity was desalted using a HiPrep desalting column (Amersham) in buffer B and loaded on a pre-equilibrated Q-Sepharose column using the same buffer. Bound protein was eluted using a linear gradient with buffer C (as B, with 1 M NaCl). Fractions containing MurD activity were concentrated and applied on a HiLoad Superdex 200pg column equilibrated in Buffer B. MurD protein was again concentrated and stored at −20 °C in buffer B with 50% glycerol.
UDP-MurNac-L-Ala was purified as described by: Auger, G.; Martin, L.; Bertrand, J.; Ferrari, P.; Fanchon, E.; Vaganay, S.; Petillot, Y.; van Heijenoort, J.; Blanot, D.; Dideberg, O. *Protein Expr. Purif.* **1998**, *13*, 23.
The D-glutamate-adding enzyme activity of MurD was monitored by the detection of orthophosphate generated during the reaction based on the colourimetic malachite green method described by Walsh, A. W.; Falk, P. J.; Thanassi, J.; Discotto, L.; Pucci, M. J.; Ho, H.-T. *J. Bact.* **1999**, *181*, 5395. All assays were carried out at 37 °C. To measure MurD inhibition, 2-fold dilution series of the inhibitor compound (10 μL) was incubated with 0.32 μM MurD (15 μL) for 10 min. 25 μL of enzyme substrate (400 μm ATP, 100 μm D-glutamic acid, 80μM UDP-MurNac-L-Ala in assay buffer [50 mM Hepes, pH 8, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$)] was added and incubated for 20 min. 50-μL malachite green-molybdate (0.045% w/v malachite green hydrochloride and 4.2% w/v ammonium molybdate in 4 N HCL mixed in a 3:1 ratio for 30 min and then passed through a Millipore 0.22 μm syringe driven filter unit) was added and incubated for 10 min. Absorbance at 660 nm was then measured with a Molecular Devices SpectraMax 250 plate reader. An accompanying series of orthophosphate standards was run for each plate. It was established that under these conditions, the production of orthophosphate versus time was linear.