Expression and assay of an N-methyltransferase involved in the biosynthesis of a vancomycin group antibiotic

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An N-methyltransferase responsible for methylating the N-terminal leucine of a vancomycin group antibiotic has been expressed, and its activity assayed against a series of putative vancomycin precursors.

Vancomycin (1) and teicoplanin are the drugs of choice against methicillin-resistant Staphylococcus aureus (MRSA). The emergence of vancomycin-resistant enterococci (VRE) has highlighted the need for the development of new antibiotics. We have sequenced the DNA containing a gene cluster assumed to encode enzymes for the biosynthesis of chloroeremomycin. This assumption is strongly supported by the expression of a glycosyl transferase within the cluster, which was able to add glucose to an aglycone form of chloroeremomycin. Recently, gene disruption in another glycopeptide producing strain has indicated the oxidative genes involved in cross-linking the aromatic groups along the peptide backbone, and led to the isolation of a linear heptapeptide precursor of a vancomycin group antibiotic.

We now report the successful expression and assay of an N-methyltransferase from this cluster (MtfA, previously referred to as ORF 16) responsible for N-methylating the N-terminal leucine of chloroeremomycin. MtfA has significant sequence homology to the N-methyltransferase EryCVI which is involved in the synthesis of the desosamine moiety of erythromycin D. The MtfA gene was amplified from cosmid pCZA361 using primers which introduced suitable restriction sites for cloning into the expression vector pET28a(+) (Novagen). The protein was then expressed in E. coli BL21 (DE3) under the control of the T7lac promoter as a His6-tagged fusion protein which allowed one-step purification using Novagen His-Bind Quick 900 Cartridges. The relative molecular mass of the purified protein (which gave rise to a single band following SDS-PAGE) was found to be 32.52 kDa (using ESI-MS) which was in excellent agreement with that calculated (32.519 kDa) from the protein sequence.

We assayed MtfA against four different substrates. Each substrate represents a putative precursor corresponding to a different stage of the pathway. The first substrate was N-demethylvancomycin (3) which is identical in structure to N-demethylchloroeremomycin with the exception that the latter has an additional residue 6 amino-sugar (4-epi-vancosamine) and the residue 4 sugar is epi-vancosamine rather than vancomamine. The second substrate was the aglycone of 3 (4). These substrates allow assays for putative late stage methylation in the biosynthetic pathway. The third substrate was the linear heptapeptide d-Leu-d-Tyr-l-Asn-d-Phpg-d-Phpg-l-Tyr-l-Dhp-OH (5). This has the same linear peptide sequence as vancomycin but has no cross-linking between the aromatic side chains, no chlorine atoms or benzylic hydroxy groups and lacks the residue 4 sugars. This substrate allows an assay for a methylation step near to the middle of the biosynthetic pathway. The final substrates were the R- and S-enantiomers of leucine. These substrates allow assays for methylation at the start of the biosynthetic pathway. Although the N-terminal leucine of chloroeremomycin is the R-isomer, the possibility of post-methylation epimerisation cannot be ruled out.

Assays were performed in 50 mM Tris–HCl buffer, pH 7.5 at 25 °C. The concentrations of substrate (100 μM), (S)-adenosyl-l-methionine (200 μM) and enzyme (1 mg) were identical in each assay, as was the incubation time (24 h). Reaction mixtures were then subjected to analytical reverse phase HPLC and fractions analysed by FT-ICR mass spectrometry. The extent of methylation was determined by comparing the ratios of the intensities of the peaks corresponding to the singly charged parent ions of the substrates and their methylated products (Table 1). Appropriate controls were carried out.

Fig. 1 FT-ICR spectra showing the MH+ peaks due to substrate and methylated product(s) following MtfA assays for (a) 3, (b) 5 and (c) 4.

Table 1 Extent of methylation of various substrates by MtfA determined by FT-ICR spectrometry and HPLC analysis (parentheses). Each assay was performed under identical conditions of 100 μM substrate, 200 μM (S)-adenosylmethionine and 1 mg of enzyme for 24 h.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate dimethylation (%)</th>
<th>Substrate monomethylation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>95 (&gt; 95)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>(R)-Leucine</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(S)-Leucine</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

HPLC retention times of products and substrates did not overlap significantly, peak integration was also used to determine the extent of methylation (Table 1). Leucine and N-methylleucine have very weak UV absorption properties and mass spectrometry was used for their detection.13

Approximately 95% of 3 was monomethylated by MtfA to vancomycin [Table 1 and Fig. 1(a), cf. peaks at m/z 1434 and 1448]. In Fig. 1(a) the fragmentation peak at m/z 1305 corresponds to the loss of the residue 4 vancosamine from the methylated product, indicating that N-methylation occurs at the N-terminal leucine and not the amino group of vancosamine. Species 4 also proved a good substrate for MtfA with over 95% methylation. Interestingly, 30% underwent a second methylation to form a dimethylated product [Table 1 and Fig. 1(c), cf. peaks at m/z 1129, 1143 and 1157]. In contrast, only 30% of 5 was monomethylated [Fig. 1(b), cf. peaks at m/z 1049 and 1035] and no methylation of (R)- or (S)-leucine was detected. Each substrate was additionally assayed in the absence of MtfA and no methylation was observed.

We also carried out competition experiments in which an equimolar concentration of two substrates (100 μM each) was incubated with enzyme (2 mg) and (S)-adenosyl-L-methionine (200 μM) [Table 2].14 In the competition experiment between substrates 3 and 5, approximately 95% of 3 was monomethylated whereas no methylation of 5 was detected, confirming that 3 is a much better substrate than 5. In the competition experiment between substrates 3 and 4, 90% of 4 was monomethylated whereas only 30–35% of 3 was monomethylated. No dimethylated products were detected. The lack of a second methylation of 4 in this competition experiment implies that the N-methylated aglycone is a poorer substrate for the enzyme than is 3. Most importantly, this result also implies that 4 is a better substrate than 3 and hence the best substrate of those tested.

These results indicate that the order of specificity of the substrates tested for MtfA is 4 > 3 > 5 > (R)- and (S)-leucine. This order suggests that N-methylation is a late step in the biosynthetic pathway of chloroeremomycin, taking place after the oxidative crosslinking of the heptapeptide backbone but before the addition of the ring 4 disaccharide. However, the importance of the chlorine atoms on substrate specificity is yet to be determined.

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Notes and references

9. This peptide was synthesised using a combination of solution phase and solid phase methodology. Phgp and Dhp refer to 4-hydroxyphenylglycine and 3,5-dihydroxyphenylglycine, respectively.
10. 14 Equimolar mixtures of (R)-N-methylleucine and (R) leucine, vancomycin and N-demethylvancomycin and vancomycin aglycone and N-demethylvancomycin aglycone were subjected to FT-ICR mass spectrometry. For a given solution, the intensities of the singly and doubly charged parent ion peaks of each component were the same to within 10%.
11. Fragmentation peaks corresponding to N-demethylvancomycin, N-demethylvancomycin aglycone and (R)-leucine were not observed in the FT-ICR spectra of vancomycin, vancomycin aglycone and (R)-N-methylleucine, respectively.
12. Solutions of leucine and N-methylleucine were subjected to reverse phase HPLC and fractions were collected at intervals of 30 s. Each fraction was then subjected to FT-ICR mass spectrometry to test for the presence of the corresponding compound. By progressively narrowing the time interval between collections the retention time of the compounds was determined to within five seconds. For each reaction assay, samples corresponding to these retention times were collected and subjected to FT-ICR mass spectrometry.
13. All values refer to monomethylation only, since no dimethylation was observed in either experiment.