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# Development of an LC–MS based enzyme activity assay for MurC: application to evaluation of inhibitors and kinetic analysis

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**Abstract**

An enzyme activity assay, based on mass spectrometric (MS) detection of specific reaction product following HPLC separation, has been developed to evaluate pharmaceutical hits identified from primary high throughput screening (HTS) against target enzyme *Escherichia coli* UDP-*N*-acetyl-muramyl-L-alanine ligase (MurC), an essential enzyme in the bacterial peptidoglycan biosynthetic pathway, and to study the kinetics of the enzyme. A comparative analysis of this new liquid chromatographic–MS (LC–MS) based assay with a conventional spectrophotometric Malachite Green (MG) assay, which detects phosphate produced in the reaction, was performed. The results demonstrated that the LC–MS assay, which determines specific ligase activity of MurC, offers several advantages including a lower background (0.2% versus 26%), higher sensitivity (≥10 fold), lower limit of quantitation (LOQ) (0.02 μM versus 1 μM) and wider linear dynamic range (≥4 fold) than the MG assay. Good precision for the LC–MS assay was demonstrated by the low intraday and interday coefficient of variation (CV) values (3 and 6%, respectively). The LC–MS assay, free of the artifacts often seen in the Malachite Green assay, offers a valuable secondary assay for hit evaluation in which the false positives from the primary high throughput screening can be eliminated. In addition, the applicability of this assay to the study of enzyme kinetics has also been demonstrated.


*Keywords:* Mass spectrometric detection; Enzyme activity assay; Hit evaluation; Compound screening; Kinetic analysis

*Abbreviations:* MurC, UDP-*N*-acetylmuramyl-L-alanine ligase; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; UNAM, uridine diphosphate-*N*-acetylmuramic acid; UNAM-Ala, uridine diphosphate-*N*-acetylmuramyl-L-alanine; UNAM-Ala-Glu, uridine diphosphate-*N*-acetylmuramyl-L-alanine-D-glutamate; RP-HPLC, reversed-phase high-performance liquid chromatography; DMSO, dimethyl sulfoxide; ESI-MS, electrospray ionization mass spectrometry; PK, pyruvate kinase; LDH, lactate dehydrogenase; MG, Malachite Green; HTS, high throughput screening; MRM, multiple reaction monitoring; SIM, selected ion monitoring

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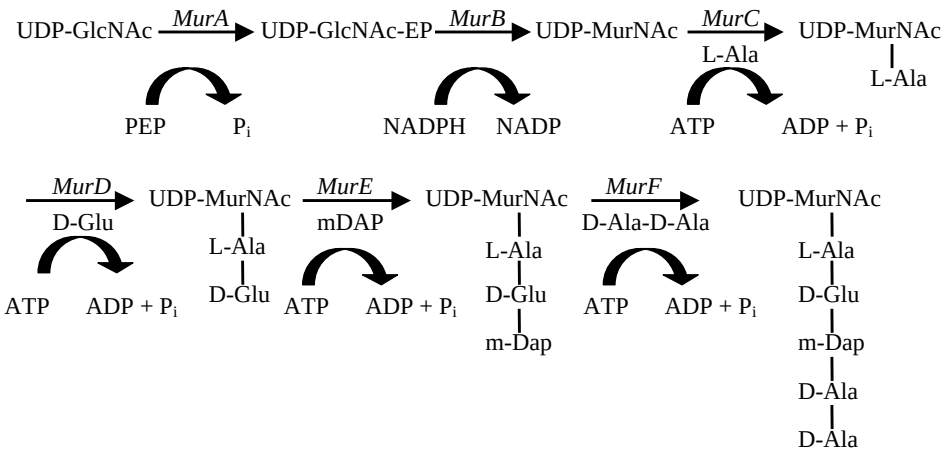
## 1. Introduction

Uridine diphosphate *N*-acetylmuramate: L-alanine ligase (UNAM:Ala ligase or MurC) catalyzes the third chemical step in cytoplasmic stage of bacterial cell wall biosynthesis and is an attractive target for antibacterial drug discovery [1] (Scheme 1). The enzyme is a nonribosomal peptide ligase, which utilizes ATP to create an amide bond between L-alanine and uridine diphosphate-*N*-acetylmuramic acid (UNAM) to form UNAM-Ala, ADP, and inorganic phosphate ($P_i$). Measurements of ADP or inorganic phosphate are commonly used to determine the activity of this enzyme. Detection of $P_i$ includes methods such as the Malachite Green (MG) assay [2] and methylthioguanosine (MESG) assay [3], while a commonly used method for ADP detection is pyruvate kinase/lactate dehydrogenase (PK/LDH) coupled assay [4]. The MG assay is based on the conversion of $P_i$ to a phosphomolybdate complex, which can be quantified spectrophotometrically at 650 nm. The assay is discontinuous and amenable to automation, making it attractive for primary high throughput screening (HTS). The MESG assay is based on a coupled reaction in which $P_i$ released from the primary reaction reacts with MESG, catalyzed by a purine nucleoside phosphorylase, to form a purine base and a ribose-1-phosphate. In the PK/LDH coupled assay, ADP produced by a target enzyme reaction is converted to ATP by pyruvate kinase in the presence of phosphoenol pyruvate (PEP). The pyruvate generated in the PK reaction is then reduced to lactate by NADH in the presence of LDH. In both cases, the target reaction can be monitored by absorbance (MG or MESG assay) or fluorescence (PK/LDH assay). While these assays are amenable to and used for HTS, orthogonal and secondary assays are critical for identifying false positives and confirming hits found in the primary HTS. False positives can arise with spectrophotometric and spectroflurometric assays as these assays often suffer from interfering signals produced by reagents required for the reaction and compounds being screened. For example, signal enhancements or attenuations in MG assays have been observed due to interaction between the compounds and phosphomolybdate complex and/or Malachite Green. In coupled assays, such as PK/LDH or MESG, assay interference may arise if the compounds being screened are substrate-mimics or potent inhibitors of the coupling enzymes used in the assay. In addition, non-productive ATP hydrolysis activities of some ligases involved in peptidoglycan biosynthesis have been observed [5]. Neither $P_i$ nor ADP detection can differentiate between ligase activity and the ATPase activity of an enzyme. Due to these limitations of such conventional assays, there is a need for development of orthogonal, secondary assays to facilitate the hit evaluation process.

The use of mass spectrometry to study enzyme kinetics and mode of inhibition has been recognized as this method detects reaction products directly and




Scheme 1. Cytoplasmic stage of peptidoglycan biosynthesis (MurA: UDP-GlcNAc-enolpyruvyltransferase; MurB: Flavin dependant UDP-*N*-acetylglucosamine enolpyruvate reductase; MurC, MurD, MurE, and MurF: ATP-dependant amino acid ligases).

quantitatively, eliminating the need for substrate modification or secondary enzymatic reactions irrelevant to the target enzyme reaction [6–12]. The application of mass spectrometry as a drug discovery tool in screening potential pharmaceutical compounds has also been reported [13–16]. In this paper, we report the development of a liquid chromatographic mass spectrometric (LC–MS) based MurC enzyme assay for secondary screening, for measuring the potency ($IC_{50}$) of inhibitors, and for studying enzyme kinetics.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Malachite Green hydrochloride and $MgCl_2$ were purchased from J.T. Baker. Extra pure glacial acetic acid was from GmbH & Co. Ammonium molybdate-Malachite Green reagent was prepared as described [17] except that glass containers were replaced with plastic containers. All other reagents and organic solvents were purchased from Sigma-Aldrich.

*Escherichia coli* MurC was over expressed and purified as reported earlier [18]. Stock concentrations of the enzyme were determined by amino acid analysis. UNAM, UNAM-Ala, and UDP-*N*-acetylmuramyl-L-alanine-D-glutamate (UNAM-Ala-Glu) were enzymatically synthesized and purified as described earlier [19] except that an additional ion-exchange chromatographic step was used in purification. The synthesis of the phosphinate inhibitor 1, a transition state analogue, has been published [20].

### 2.2. Instrumentation

Reversed-phase HPLC was performed on an HP 1100 system equipped with a temperature-controlled well-plate autosampler (Agilent). Mass spectrometry was performed using an LCQ$^{deca}$ ion-trap mass spectrometer (ThermoFinnigan). A Valco two-position valve integrated in the LCQ$^{deca}$ was used to divert the early eluting salts to waste in order to prevent contamination of the LC–MS interface. Column eluates were transferred via a PEEK tubing (0.005 in.) through the Valco divert valve and approximately one-third of the total flow was directed, using a T-splitter, into the atmospheric pressure ionization source of the mass spectrometer. A contact-closure was used to trigger mass spectra acquisition upon HPLC injection. The mass spectrometer was calibrated using the vendor's calibration solution and tuned using UNAM-Ala, injected by a built-in infusion pump on the LCQ$^{deca}$ and a sheath flow of HPLC solvents. The mass accuracy was typically 50–100 ppm in the mass range of *m*/*z* 50–2000. The HPLC autosampler was kept at 4 °C for the entire analysis period.

### 2.3. Malachite Green assay

MG assay was performed using 96-well, half area microtiter plates (non-treated, non-sterile, polystyrene flat bottom plate from Costar, Inc.). For inhibition studies, *E. coli* MurC was preincubated with inhibitors (dissolved in 1% DMSO) at room temperature for 10 min. Reactions were initiated by the addition of MurC substrates. The final concentrations of components in a typical assay were 50 mM Tris–HCl, pH 8.0, 2.5 mM DTT, 10 mM $MgCl_2$, 20 mM ammonium formate, and 24 nM *E. coli* MurC. Each substrate was used at a concentration appropriately five times its apparent $K_m$: 300 μM ATP, 275 μM L-alanine, and 100 μM UNAM. After a 15-min incubation, the reactions were stopped by addition of ammonium molybdate-Malachite Green reagent in 1 M HCl at 1.5 volume of the assay solution. Absorbance was read at 650 nm, 5 min after the quench.

### 2.4. LC–MS assay

The LC–MS based MurC assay, an end point assay that measures UNAM-Ala production, was performed in a U-shaped 96-well microtiter plate (Agilent) with a reaction volume of 100 μl. The reaction conditions were identical to those described above for the MG assay except that no MG reagent was added. Enzyme reactions were quenched by addition of 6 μl of 50% acetic acid, followed by the addition of 10 μl of 67.5 μM UNAM-Ala-Glu, used as an internal standard.

A 20 μl volume of the quenched reaction mixture (pH ≈ 3) was injected onto a reversed-phase column (YMC Pro C18, 3 μm, 120 Å, 4 mm × 50 mm, from Waters) and eluted using a $NH_4Ac/H_2O$/MeOH gradient (solvent A: 10 mM $NH_4Ac$ in water, pH 5.6; solvent B: $NH_4Ac$ (1 M)-MeOH (1:99, v/v, pH 5.6).

The HPLC conditions were as follows: 8% solvent B for 0.5 min followed by a gradient to 95% solvent B in 1.0 min. Solvent B was kept at 95% for 0.5 min followed by a gradient to 8% B in 0.1 min. The column was then equilibrated at 8% B for 4.9 min before the next injection. The flow rate was kept constant at 250 μl/min. Mass spectrometric detection was carried out in the negative-ion mode using selected ion monitoring (SIM). Full MS scan experiments were initially performed to check possible in-source fragmentation of ions being detected and to select MS mode. Typical mass spectrometric conditions were as follows: heated capillary temperature, 280 °C; spray voltage, 4.5 V; desolvation gas ($N_2$), 60 l/h; auxiliary gas ($N_2$), 10 l/h. Selected ion current (SIC) chromatograms of UNAM-Ala and internal standard UNAM-Ala-Glu were plotted and integrated using LCQuan incorporated in Xcalibur software (ThermoFinnigan). The area ratios between chromatographic peaks of the ions derived from UNAM-Ala and UNAM-Ala-Glu ($\mathbf{A}_{\mathrm{UNAM-Ala}}/\mathbf{A}_{\mathrm{UNAM-Ala-Glu}}$) were used to evaluate enzyme activity.

The linearity of UNAM-Ala concentration versus mass spectrometric signal ($\mathbf{A}_{\mathrm{UNAM\text{-}Ala}}/\mathbf{A}_{\mathrm{UNAM\text{-}Ala\text{-}Glu}}$) was determined with purified UNAM-Ala. Each sample contained 50 mM Tris–HCl, pH 8.0, 2.5 mM DTT, 10 mM $MgCl_2$, 20 mM ammonium formate, 300 μM ATP, 275 μM L-alanine, 100 μM UNAM, 24 nM *E. coli* MurC (prequenched with acetic acid, final concentration of acetic acid was 3%), 6 μM UNAM-Ala-Glu, and 0–100 μM UNAM-Ala.

The reproducibility of the LC–MS assay was evaluated by a 100% activity control (in the absence of an inhibitor) and a 50% activity control (in the presence of a control inhibitor, AMP-PCP, at its $IC_{50}$ concentration). The LC–MS signals of quenched reaction mixtures were measured 12 times on the same day as well as on four different days.

### 2.5. Apparent $K_M$($K_{M,app}$) and maximum velocity ($V_{max}$) for *E. coli MurC* substrates

Determination of $K_{\mathrm{M,app}}$ and $V_{\max}$ for each substrate (ranged from 15 to 300 μM for Ala and ATP, respectively, and from 5 to 100 μM for UNAM) was performed using the LC–MS assay under saturating conditions of the other two substrates as follows: [ATP] = 740 μM, [UNAM] = 228 μM, [L−Ala] = 660 μM. Data were collected in duplicates and averaged. Other reaction components were 50 mM Tris–HCl, pH 8.0, 2.5 mM DTT, 10 mM $MgCl_2$, 20 mM ammonium formate. Initial velocity ($V_0$) was calculated based on ESI-MS data and a normalization factor [9,10]. $V_{\max}$ and $K_{\mathrm{M,app}}$ values for each substrate were obtained from Lineweaver–Burk plots.

### 2.6. Dose–response curve of known inhibitors

The inhibition properties of β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP) and phosphinate inhibitor 1 [20] on the activity of *E. coli* MurC were assessed at 12 inhibitor concentrations spread at two-fold intervals. For the MG assay, the $A_{650}$ values at each compound concentration were background corrected. Background values were obtained in parallel with the assays in the same plate except that MurC was omitted. The inhibitory effect was calculated based on Eq. (1), and the $IC_{50}$ value (inhibitor concentration required for 50% inhibition) was determined by semi-log plot of inhibitor concentrations versus remaining enzyme activity as determined by $A_{650}$ (for the MG assay) or area ratio of reaction product (UNAM-Ala) to internal standard (UNAM-Ala-Glu) for the LC–MS assay. Grafit 4.0 software was used to perform non-linear regression to the Hill equation, which yielded $IC_{50}$ values. The final concentrations of reaction components were 50 mM Tris–HCl, pH 8.0, 2.5 mM DTT, 10 mM $MgCl_2$, 20 mM ammonium formate, 300 μM ATP, 275 μM L-alanine, 100 μM UNAM, 24 nM *E. coli* MurC.

### 2.7. Compound screening

A plate containing 96 compounds from a compound library was screened at 10 μM concentration using both the LC–MS assay and the MG assay. The assay conditions were as described above in Sections 2.3 and 2.4. The percent inhibition was calculated using Eq. (1):

$$\%\,\mathrm{Inhibition} = \frac{\text{corrected signal for 100\% activity} - \text{corrected signal for compound assay}}{\text{corrected signal for 100\% activity}} \times 100 \quad (1)$$

In the above equation, the "corrected signal for 100% activity" is the difference of signals between 100% activity control (activity in the absence of an inhibitory compound) and 0% activity control (MurC activity pre-quenched). For the LC–MS assay, the "corrected signal for compound assay" is the difference of the signal produced in the presence of the compound from that observed in the 0% activity control run. For the MG assay, this term refers to the difference in the signal produced in the presence of the compound from that observed in the compound background control (obtained in parallel with the assay except that the enzyme was omitted).

### 2.8. ADP and $P_i$ inhibitions of E. coli MurC

The effect of ADP or $P_i$ on the LC–MS detection of UNAM-Ala was evaluated with solutions containing constant concentrations of UNAM-Ala (12 μM) and UNAM-Ala-Glu (6 μM), varied concentrations of ADP (0–20 mM) or $P_i$ (0–200 mM) and a pre-quenched (with acetic acid, 3% final) MurC reaction mixture. The effect of ADP on the MG assay was evaluated with solutions containing constant concentrations of $P_i$ (10 μM), varied concentrations of ADP, and a MurC reaction mixture (MG reagent was added before addition of reaction substrates). The $IC_{50}$ values of ADP and $P_i$ for *E. coli* MurC were measured by varying ADP and $P_i$ concentrations in the range of 0–40 and 0–200 mM, respectively, using procedures as described for known inhibitors.

## 3. Results and discussion

### 3.1. Assay development and validation

This LC–MS assay detects the formation of UNAM-Ala catalyzed by MurC reaction. UNAM-Ala is fairly hydrophilic and poorly retained on a conventional reversed-phase column. It co-elutes with salts and other low molecular weight components required for the enzyme reaction. These small molecules significantly suppress the UNAM-Ala ion signal during mass spectrometric detection, resulting in decreased sensitivity. To efficiently separate UNAM-Ala from other reaction components, several commercially available columns, including XTerra MS C18 (Waters), AQUA C18 (Phenomenex), Nova C18 (Waters), Targa C18 (Higgins Analytical Inc.), Extented C18 (Zorbax), YMC ODS-AQ (Waters), and YMC ProC18 (Waters), were investigated. Among them, YMC Pro C18, a high-coverage C18 bonding with a unique end-capping, appeared to be the most appropriate column for efficient desalting with a relatively short run time. An ammonium acetate/methanol based mobile phase at pH 6.5 was selected to achieve good HPLC performance as well as good volatility and appropriate pH for negative ion detection. Reproducible performance was achieved with a 7-min HPLC run in a gradient mode. For optimal performance, the column was cleaned with 95% solvent B for about 20 min after every two plates of sample injections.

Typical ion chromatograms of two ions detected by SIM are illustrated in Fig. 1. Two reaction progress experiments performed under the same reaction conditions as described in Section 2.4 showed that the reaction was linear up to 20-min reaction time ($Y = 0.078X - 0.083$; $R^2 = 0.9983$) and up to 50 nM MurC concentration ($Y = 0.091X - 0.26$; $R^2 = 0.9973$). MurC activity was measured in the presence of 24 nM enzyme, by quantifying UNAM-Ala generated in a 15-min reaction to ensure that the initial velocity data were collected. Although the muropeptide can be detected as both positive and negative ions, negative ion mode was selected due to a cleaner spectrum observed in a full MS scan experiment (data not shown). Under the conditions applied, the level of source fragmentation of the detecting ions can be ignored, as determined by the full MS scan experiment. To eliminate possible errors due to variation of instrument performance such as varied injection volumes delivered by the HPLC auto-sampler, sample evaporation, degradation and adsorptive losses, a chemical analog of UNAM-Ala, UNAM-Ala-Glu, was used as an internal standard. UNAM-Ala-Glu is a product of MurD reaction, the reaction immediately following the MurC reaction in the peptidoglycan biosynthesis pathway (Scheme 1). To ensure efficient ionization, only one-third of the LC flow was injected into the ionization source.

*E. coli* MurC loses activity at pH < 5.5 and pH > 10.5 (data not shown). Several volatile acids, including TFA, formic acid, and glacial acetic acid were evaluated for their effectiveness in quenching the MurC reaction. For a 100 μl reaction, 6 μl of 50% (v/v) of

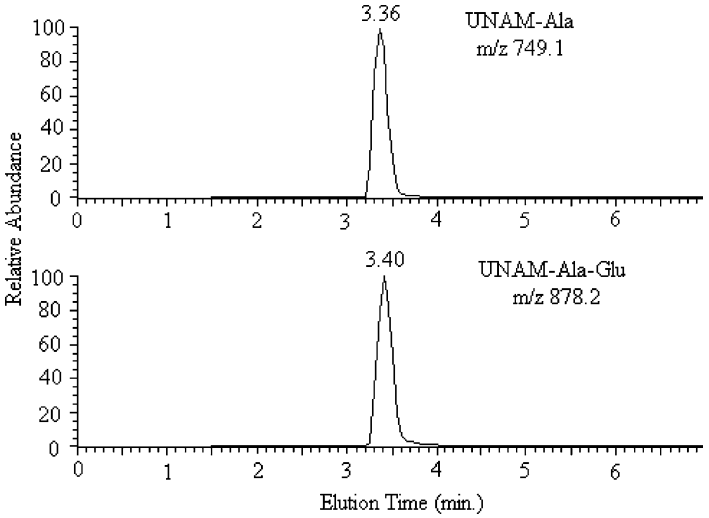



Fig. 1. Ion chromatogram of MurC reaction monitored in the SIM mode. Analyte: UNAM-Ala (*m*/*z* 749.1); internal standard: UNAM-Ala-Glu (*m*/*z* 878.2). LC–MS conditions were as described in Section 2.4.

each acid was sufficient to quench the reaction. Acetic acid was chosen for its compatibility with the subsequent LC–MS analysis.

Assay reproducibility was evaluated with 100 and 50% activity controls (Section 2.4). The relatively low intraday and interday coefficients of variation (CV) (3 and 6%, respectively) indicated satisfactory method precision. UNAM-Ala was stable in the quenched reaction mixture for up to 4 days at 4 °C without significant loss of signal (about 10% signal reduction). The stability of the reaction product allows for batch processing of a large number of samples.

To evaluate the background level of LC–MS assay, the activities of two MurC controls, 0% activity (i.e. 100% inhibition) and 100% activity (0% inhibition), were measured in parallel with the MG assay. The background signals generated by the LC–MS assay and by the MG assay were about 0.2 and 26% of their respective 100% activity control signals. The HPLC separation and ion selection process in mass spectrometer filtered out the chemical noise, resulting in the superior selectivity.

The linearity of the LC–MS based assay with respect to UNAM-Ala concentration was evaluated using serial concentrations of UNAM-Ala in a pre-quenched MurC reaction mixture. As shown in Fig. 2, the MS signal was linear over a wide range of UNAM-Ala concentrations (0.015–100 μM; $Y = 0.224X - 0.0108$; $R^2 = 0.999$). In comparison, the linear range for $P_i$ concentration in the MG assay was significantly narrower (1–25 μM of $P_i$).

### 3.2. Evaluation of potential enzyme inhibitors

The signal relative to the sum of noise and background level is a critical measure of the reliability of an assay. Assay reliability is usually assessed by assay window, which is calculated based on the Eq. (2), where "Avg. 100% activity" and "Avg. 0% activity" are the average signals obtained for 100% and 0% activity controls, respectively. "Stdev 100%" and "Stdev 0%" are the standard deviations calculated for the 100 and 0% activity controls. A method with an assay window of ≥10 is generally considered reliable for compound screening.

$$\text{Assay window} = \frac{\text{Avg. 100\% activity} - \text{Avg. 0\% activity}}{\sqrt{(\text{Stdev 100\%})^2 + (\text{Stdev 0\%})^2}} \quad (2)$$

The assay window of the LC–MS assay for the MurC reaction was evaluated at a fixed substrate concentration ($5\times K_M$) and varied enzyme concentrations and compared with those obtained for the MG assay. Data were collected in duplicates and averaged. As shown in Fig. 3, better assay windows were obtained

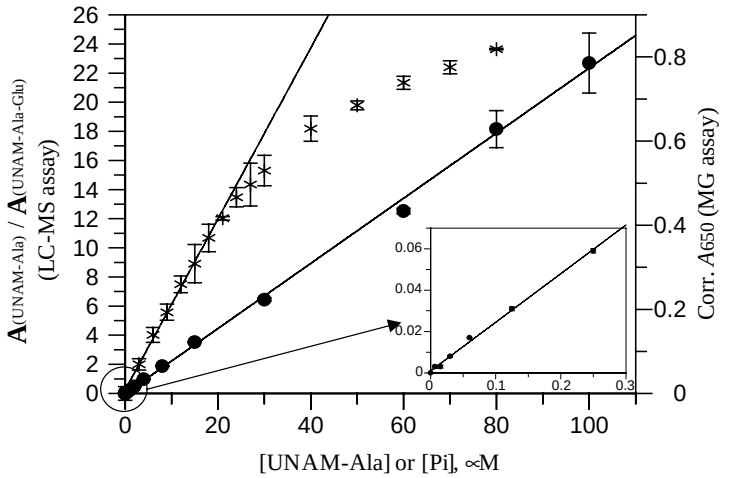



Fig. 2. The linearity of UNAM-Ala detection by LC–MS assay (●) and $P_i$ detection by Malachite Green assay (✳). Each UNAM-Ala concentration was analyzed in triplicates using LC–MS conditions described in Section 2.4. Corr. $A_{650}$ is the background corrected absorbance at 650 nm in the MG assay. Linear regression was performed using an average of three measurements. $R^2$ value is 0.999 for both the MG and the LC–MS assays. The inset is the expanded region from 0.015 to 0.25 μM UNAM-Ala.

for the LC–MS assay than for the MG assay over a wide enzyme concentration range. This is due to the low background of the LC–MS assay, in which the background (signal measured at 0% enzyme activity) essentially arises only from instrument noise, provided that the mass of the detecting ion is specific and desalting by HPLC is efficient. In spectrophotometric assays, however, both chemical and instrumental noises contribute to the background, resulting in a reduced dynamic range and decreased sensitivity for product detection. A even more selective LC–MS/MS experiment performed on a triple quadrupole mass spectrometer (API 4000, Applied Biosystems) operated in the multiple reaction monitoring (MRM) mode, detecting transition of UNAM-Ala to UDP (*m/z* 749.1 → 403.1) and UNAM-Ala-Glu to UDP (*m/z* 878.1 → 403.1), showed an even wider linear dynamic range and better sensitivity (LLOQ = 0.02 pmol on column), compared to the LC–MS assay.

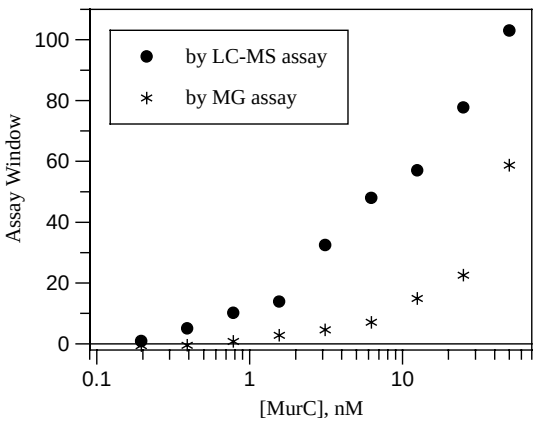



Fig. 3. Assay windows measured at different enzyme concentrations. Each sample contained 50 mM Tris–HCl, pH 8.0, 2.5 mM DTT, 10 mM $MgCl_2$, 20 mM ammonium formate, 300 μM ATP, 275 μM L-alanine, and 100 μM UNAM. The concentration of *E. coli* MurC in each sample is as indicated by the *x*-axis.

Before implementing the LC–MS assay for compound screening, the assay was evaluated using two known inhibitors, AMP-PCP and the phosphinate inhibitor 1, which were known to pose no interference to $P_i$ detection in the MG assay (data not shown). Dose–response curves from which the $IC_{50}$ values were calculated are illustrated in Fig. 4. Under identical assay conditions, the $IC_{50}$ values of AMP-PCP measured by the LC–MS assay (41 ± 6.5 μM) and by the MG assay (44 ± 4.0 μM) were in good agreement. This was also true for the phosphinate inhibitor 1, which gave an $IC_{50}$ of 37 ± 1.1 nM by the LC–MS assay and 42 ± 2.7 nM by the MG assay. These results suggest that the LC–MS assay can be reliably used

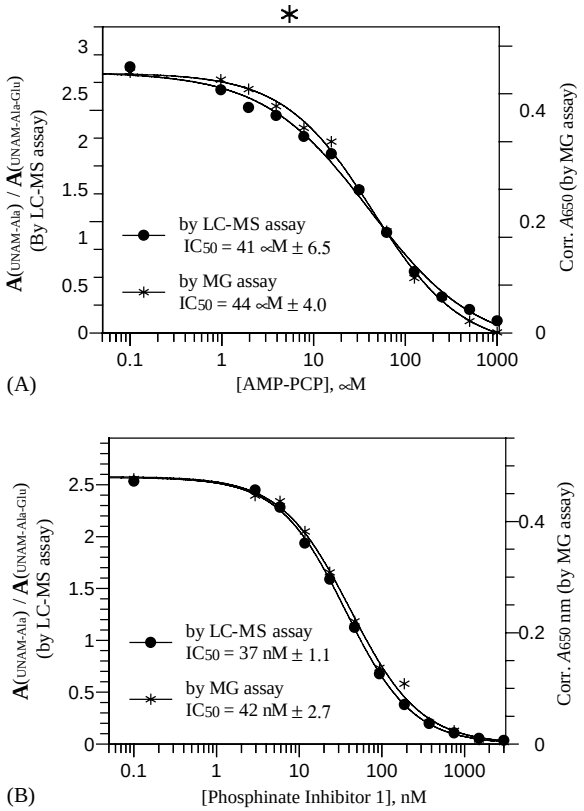



Fig. 4. Dose–response curves for AMP-PCP (A) and phosphinate inhibitor 1 (B) measured by LC–MS assay (●) and by MG assay (✳). Final concentrations of other reaction ingredients were as described in Section 2.6.

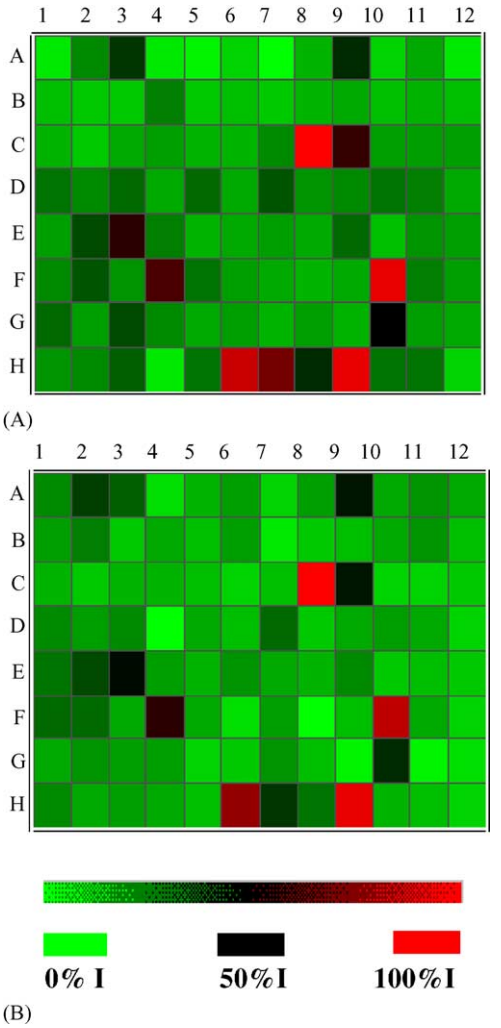



Fig. 5. Compound screening by the LC–MS assay (A) and by MG assay (B). The percent inhibition by each compound is demonstrated in heat maps.

as an orthogonal, secondary assay for confirming HTS hits.

The effectiveness of the LC–MS assay in rapid compound screening was demonstrated by screening a representative plate of compounds at a single compound concentration (10 μM) using the MurC reaction (Fig. 5A). The screening was also performed in parallel with the MG assay (Fig. 5B). In general, good agreement was observed between the two methods. Any discrepancy seen in the two methods was likely due to interference of $P_i$ signal (signal attenuation or enhancement) in the MG assay by the compounds. For example, the compound located in H7 position in the microtiter plate (Fig. 5) showed 34% inhibition by the MG assay and 73% inhibition by the LC–MS assay. A separate experiment showed that the compound caused signal enhancement in the MG assay (data not shown). Although background correction is routinely performed in the MG assay, the background cannot be fully corrected when there is optical interference from a compound being screened. It should be noted that, in the MG assay, the interaction of a compound with the phosphomolybdate complex cannot be quantitatively corrected since in the background solution, no phosphate is produced and, therefore, no phosphomolybdate complex is formed. This interference is usually qualitatively evaluated by addition of an arbitrary, fixed amount of $P_i$ into a prequenched

reaction mixture containing the screening compound. Any attenuation or enhancement of the $P_i$ signal in the presence of a compound indicates interference by the compound. However, this information cannot be quantitatively implemented into compound screening results since the accurate amount of $P_i$ generated in a reaction in the presence of a screening compound is unknown.

The LC–MS assay has a lower throughput than the MG assay. Higher throughput could be achieved by shortening the run time with further optimized HPLC separation conditions to ensure efficient desalting so that high sensitivity and accuracy are maintained. When a reaction requires the addition of large amounts of substrates while substrate conversion needs to be controlled at a low level, some separation between the reaction product and substrates may be necessary to ensure sensitivity. For the *E. coli* MurC assay described here, about 200 compounds can be screened per day with high sensitivity and accuracy. The throughput is expected to increase if a triple quadrupole mass spectrometer is used. The MRM capability of a triple quadrupole instrument allows efficient quantitation of trace amount of reaction product in complex reaction mixtures.

### 3.3. Study of enzyme kinetics

The utility of the LC–MS assay in measuring kinetic parameters was evaluated by measuring $K_{M,app}$ and $V_{max}$ values for each *E. coli* MurC substrate. Initial velocity ($V_0$) was calculated based on ESI-MS data and a normalization factor, which was obtained using a published method [9,10]. An average normalization factor of 2.63 was obtained from nine measurements using a series of pre-quenched reaction mixtures containing fixed (5.8 μM) internal standard concentration but different concentrations of UNAM-Ala (0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 15.0, 30.0 μM) (Table 1). Fig. 6 shows the saturation plot ($V_0$ versus [substrate]) and corresponding double-reciprocal plot ($1/V_0$ versus 1/[substrate]) for each substrate. The $K_{M,app}$ and $V_{max}$ values for each substrate obtained from the plots are summarized in Table 2. The $K_{M,app}$ value for ATP is in good agreement with reported value ($61.4 \pm 4.6$ μM) measured using the MG assay under the same conditions [18]. The discrepancy between the LC–MS assay and the MG assay in determining $K_{M,app}$ values for UNAM and L-Ala (Table 2) was due to ATP interference at high ATP concentrations in the MG assay (measurements of $K_{M,app}$ values for UNAM and L-Ala require saturating ATP concentration). The $K_{M,app}$ values for L-Ala and UNAM obtained by the LC–MS assay agreed well with $K_M$ values obtained from a global fit analysis ($20.7 \pm 5.3$ μM and $35.5 \pm 5.2$ μM, respectively) where full substrate saturation of the enzyme was assumed [18]. The LC–MS assay allowed a wide range of substrate concentrations to be investigated without signal interference.

The applicability of the LC–MS assay to studying product inhibition of *E. coli* MurC by ADP and $P_i$ was investigated. In a separate study, an attempt using the MG assay to study ADP inhibition of the enzyme was unsuccessful due to severe ADP interference. To investigate the interference, a fixed amount of $P_i$ (10 μM) and variable amounts of ADP (0–10 mM, spread at two-fold intervals) were spiked to pre-quenched MurC reaction mixtures and the added $P_i$ was detected by the

Table 1
Normalization factor (NF)

| [UNAM-Ala] (μM) | 0.125 | 0.25 | 0.5 | 1.0 | 2.2 | 4.0 | 8.0 | 15.0 | 30.0 | Average NF | CV% |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NF | 2.74 | 2.60 | 2.58 | 2.73 | 2.86 | 2.72 | 2.55 | 2.49 | 2.42 | 2.63 | 5.3 |

Table 2
$K_{M,app}$ and $V_{max}$ for *E. coli* MurC substrates

| | ATP | L-Ala | UNAM |
|---|---|---|---|
| $K_{M,app}$(μM) | 63.8 ± 6.1 (61.4 ± 4.6)[a] | 22.9 ± 2.0 (56 ± 0.58)[a] | 34.3 ± 6.2 (19.2 ± 12.6)[a] |
| $V_{max}$ (μM min$^{-1}$) | 0.26 ± 0.01 | 0.49 ± 0.02 | 0.36 ± 0.02 |

[a] $K_{M,app}$ values obtained earlier by MG assay [18] are indicated in parentheses for comparison.

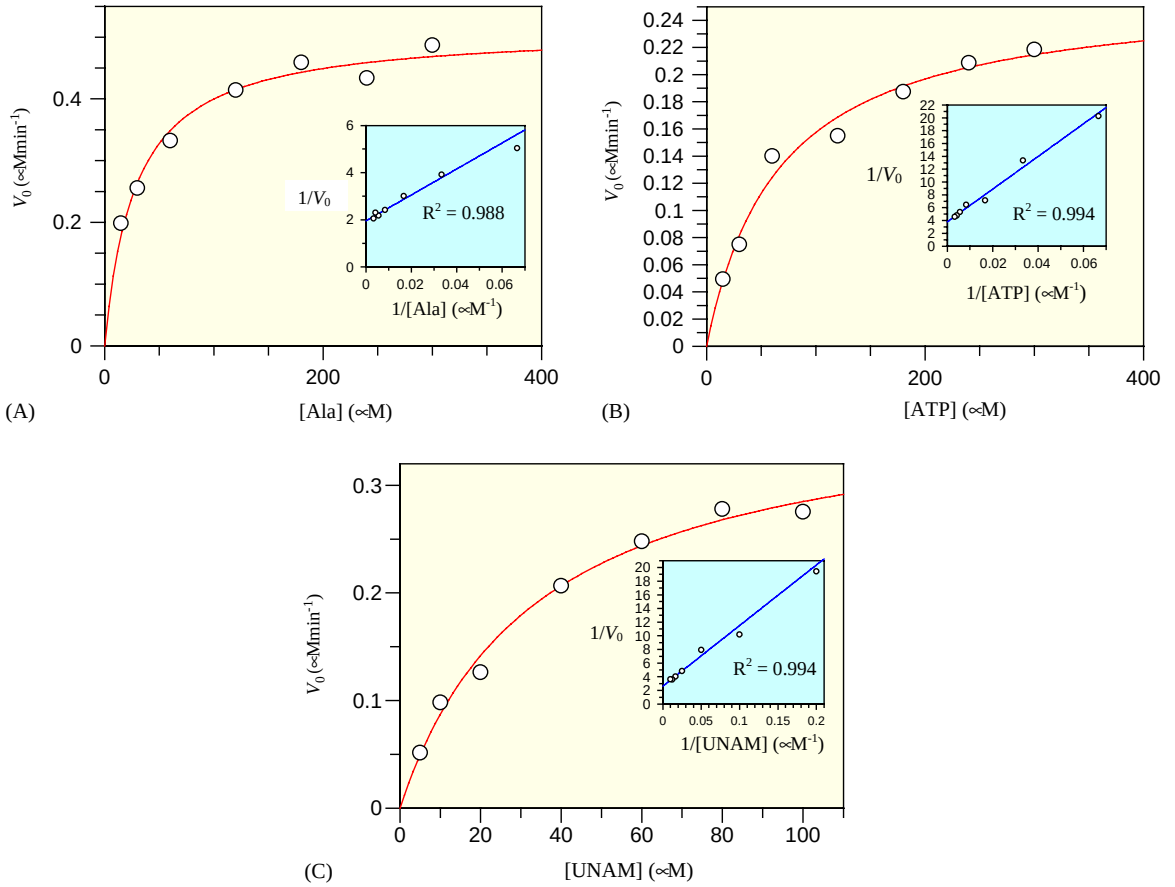



Fig. 6. Saturation plots of $V_0$ vs. [Ala] (A), $V_0$ vs. [ATP] (B), and $V_0$ vs. [UNAM] (C). Each inset is the corresponding double-reciprocal plot.

MG assay. Signal enhancement in the MG assay was observed at ADP concentrations ≥0.15 mM and the enhancement became more severe as ADP concentration increased (Fig. 7). At ADP concentrations above 5 mM significant precipitation was formed in the reaction mixture, preventing absorbance measurements. In the LC–MS assay, a fixed amount of UNAM-Ala (12 μM) and variable amounts of ADP (0–10 mM) were spiked into pre-quenched MurC reaction mixtures and the added UNAM-Ala was detected by the LC–MS assay. No ADP interference was observed within experimental error (Fig. 7). Although some ion suppression at high concentrations of added ADP was observed due to incomplete separation of the abundant ADP by the reversed phase column used (data not shown), the levels of ion suppression to UNAM-Ala and to the internal standard UNAM-Ala-Glu were

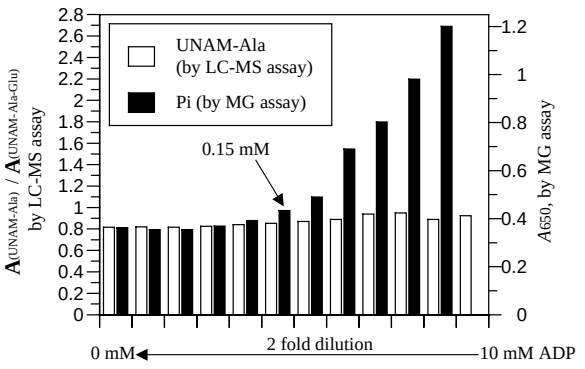



Fig. 7. The effect of ADP on $P_i$ detection (by the MG assay) and on UNAM-Ala detection (by LC–MS assay). The signal enhancement became obvious at an ADP concentration of ∼0.15 mM (black arrow) in the MG assay.

comparable due to similar physical chemical properties of the two ions. Therefore, the area ratio used to evaluate enzyme activity was essentially unaffected in the range of ADP concentrations investigated. The inhibition of *E. coli* MurC by ADP was investigated in the range of 0–40 mM ADP. The resulting $IC_{50}$ was 3.6 mM under the conditions used.

MG assay cannot be used to study $P_i$ inhibition due to its limited dynamic range for $P_i$ detection. The LC–MS assay detects UNAM-Ala produced in the MurC reaction and, therefore, can be used to study the inhibition of MurC by $P_i$. A fixed amount of UNAM-Ala (12 μM) and variable amounts of $P_i$ (0–200 mM) were spiked into pre-quenched *E. coli* MurC reaction mixtures and the added UNAM-Ala was detected by the LC–MS assay. No signal interference was observed within experimental error (data not shown). The measured $IC_{50}$ for $P_i$ was 38 mM under the conditions used. The $IC_{50}$ values measured for ADP and $P_i$ for *E. coli* MurC are high compared to the substrate $K_M$ values, suggesting that neither ADP nor $P_i$ release is rate limiting for the enzyme turnover. Detailed product inhibition studies for this enzyme is beyond the scope of this work and will be investigated separately.

The activity of non-productive ATP hydrolysis by some ligases involved in peptidoglycan biosynthesis has been observed [5]. $P_i$ detection by MG or MESG assay, or ADP detection by PK/LDH coupled assay measures total $P_i$ or ADP production including that generated by the non-productive ATP hydrolysis, therefore, these assays do not specifically measure ligase activity of such enzymes. In contrast, good assay specificity can be achieved by the LC–MS assay as it determines only ligase activity of the enzyme by specifically detecting the ligated product. To investigate possible ATPase activity of *E. coli* MurC, reaction progress was monitored by both LC–MS and MG assays. The reaction rates measured under assay conditions of the two methods were essentially the same within experimental error (12.9 and 11.7 nM/s, respectively), suggesting that *E. coli* MurC has no detectable ATPase activity under these conditions.

## 4. Conclusion

The LC–MS based assay developed here allows direct, rapid, and quantitative measurement of the activity of *E. coli* MurC. This assay has greater specificity, sensitivity, and accuracy than the conventional spectrophotometric MG assay. Although this MS based method is not readily amenable to a high throughput screening of a large compound library, it is valuable as an orthogonal, secondary assay for following up on hits from a primary HTS campaign. The assay is also valuable in the lead optimization stage of drug discovery.

This assay specifically measures the ligase activity of the *E. coli* MurC. Application of this assay in studying enzyme kinetics has been demonstrated by its ability to monitor reaction progress and measure $V_{max}$ and $K_{M,app}$ of *E. coli* MurC substrates accurately. Measurements of $IC_{50}$ values of ADP and $P_i$ suggest that this assay could be used for product inhibition studies.

This assay may be adopted for activity assessment of other enzymes involved in the early stages of peptidoglycan biosynthesis (Scheme 1), since the products of these reactions share physical chemical and structural similarities. A substrate in an upstream reaction or a product in a downstream reaction can be used as the internal standard. MS detection based assays do not require the reaction product to contain an intrinsic or extrinsic chromophore or fluorophore or a radioactive probe nor does it require introduction of a coupled enzymatic reaction step. Therefore, problems associated with the use of additional steps or probes, which are not relevant to the enzyme reaction of interest, can be avoided.

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