Mass spectrometric characterization of a three-enzyme tandem reaction for assembly and modification of the novobiocin skeleton

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The tripartite scaffold of the natural product antibiotic novobiocin is assembled by the tandem action of novobiocin ligase (NovL) and novobiocic acid noviosyl transferase (NovM). The noviosyl ring of the tripartite scaffold is further decorated by a methyltransferase (NovP) and a carbamoyltransferase (NovN), resulting in the formation of novobiocin. To facilitate kinetic evaluation of alternate substrate usage by NovL and NovM toward the creation of variant antibiotic scaffolds, an electrospray ionization/MS assay for obtaining kinetic measurements is presented for NovL and NovM separately, in each case with natural substrate and the 3-methyl-4-hydroxybenzoic acid analog. Additionally, assays of tandem two-enzyme (NovL/NovM) and three-enzyme (NovL/NovM/NovP) incubations were developed. The development of these assays allows for the direct detection of each intermediate followed by its utilization as substrate for the next enzyme, as well as the subsequent formation of final product as a function of time. This MS tandem assay is useful for optimization of conditions for chemoenzymatic generation of novobiocin and is also suitable for evaluation of competitive usage of variant substrate analogs by multiple enzymes. The studies presented here serve as a platform for the subsequent expansion of the repertoire of coumarin-based antibiotics.

The aminocoumarin family of antibiotics, novobiocin, clorobiocin, and coumermycin A₁, is produced from various *Streptomyces* species, and each bears a noviosyl sugar component that imparts the functionality essential for its biological activity (Fig. 1) (1). This family of antibiotics exerts its antibacterial activity via the inhibition of the type II DNA topoisomerase DNA gyrase (2–5). X-ray crystallographic analysis of a 24-kDa N-terminal fragment of DNA gyrase with bound novobiocin (6–8) or clorobiocin (9) reveals that the antibiotics bind competitively at the ATP site and that the aminocoumarin bicyclic ring is the scaffold for presenting the decorated L-noviosyl sugar moiety to the target enzyme.

Widespread clinical use of novobiocin has not been achieved because of such factors as low solubility, poor pharmacokinetics, and limited activity against Gram-negative bacteria (10–12). However, reports detailing novobiocin activity against methicillin-resistant *Staphylococcus aureus* (MRSA) strains (13) have led to a renewed interest in novobiocin as an antibiotic. Therefore, it is of interest to study the novobiocin biosynthetic pathway to understand how to generate novobiocin variants. It is anticipated that structural variation in the scaffold of novobiocin will produce analogs with enhanced solubility and pharmacokinetic properties while maintaining or enhancing gyrase B inhibitory properties.

The novobiocin scaffold consists of three components: a prenylated 4-hydroxybenzoate (ring A), the 3-amino-4,7-dihydroxycoumarin nucleus (ring B), and the L-noviosyl sugar bearing a 5,5'-dimethyl moiety (ring C) (Fig. 1B). These three elements are assembled by the tandem action of two enzymes, novobiocin ligase (NovL) (14) and novobiocic acid noviosyl transferase (NovM) (1). NovL is an ATP-cleaving, amide bond-

forming ligase, joining prenylated benzoic acid and aminocoumarin to produce novobiocic acid. NovM catalyzes the transfer of L-noviose from TDP-L-noviose to the phenolic oxygen of novobiocic acid, creating the full ABC ring system. These steps are followed by two enzymatic decorations of the noviosyl ring, 4'-O-methylation catalyzed by a methyltransferase (NovP) and 3'-carbamoylation catalyzed by a carbamoyltransferase (NovN) (15–17), resulting in the formation of novobiocin (Fig. 1B). All four enzymes (NovL, NovM, NovP, and NovN) are encoded in the novobiocin biosynthetic gene cluster and can be overproduced and purified as histidine-tagged proteins from *Escherichia coli* (1, 14, 15).

Clear structural information obtained from the crystal structure of novobiocin bound to the biological target (6–8, 18–19) permits rational approaches in the search for new aminocoumarin derivatives. Recently, development of synthetic aminocoumarin compounds with gyrase-inhibiting activity has been reported (20–22). Noviosyl sugar decorations, particularly carbamoylation, are essential for the binding and inhibition of novobiocin to gyrase (6–8); however, the novobiocin scaffold can be varied without the loss of antibiotic activity (23). Therefore, the tripartite assembly of desmethyl descaramoyl novobiocin by consecutive action of NovL and NovM raises the prospect of introducing multiple variations in the novobiocin scaffold by using alternate substrates for either enzyme. The subsequent noviose decorations imparted by NovP and NovN on the scaffold variants are expected to generate novobiocin mimics as gyrase inhibitors. Consequently, rapid kinetic assays capable of characterizing individual enzymes (NovL and NovM) as well as analyzing multi-enzyme (NovL, NovM, NovP, and NovN) tandem reactions with different substrates would dramatically enhance the biosynthetic generation of coumarin-based antibiotics.

To date, all four enzymes, NovL, NovM, NovP, and NovN, have been assayed by HPLC analysis (1, 15). Recently, the development of soft ionization techniques, such as electrospray ionization (ESI), has made MS an excellent complementary technique to conventional methods for studying enzyme kinetics (24–30). A facile and broadly applicable ESI/MS assay developed by Leary and coworkers has been implemented for kinetic analyses and substrate specificity evaluation of several enzyme systems such as glutathione S-transferase (26), hexokinase (27), phosphoglucomutase (28), and NodH sulfotransferase (29, 30). Herein, a somewhat different ESI/MS technique was used to develop assays for NovL and NovM individually and in tandem. In both single- and multiple-enzyme systems, product quantification was achieved by monitoring the product ion relative to an internal standard ion by using selected ion monitoring (SIM) on

Abbreviations: ESI, electrospray ionization; LC, liquid chromatography; SAM, S-adenosyl-L-methionine.

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Fig. 1. (A) Shown above are the structures of aminocoumarin antibiotics clorobiocin and coumermycin A₁. (B) Novobiocin biosynthetic pathway is shown.

a liquid chromatography (LC)/ESI/MS instrument. This method allows different product ions generated in multiple-enzyme reaction systems to be monitored and quantified simultaneously. With this assay method, kinetic constants and catalytic mechanisms were confirmed for NovL and NovM separately. In addition, a detailed reaction profile was generated for both NovL/NovM and NovL/NovM/NovP tandem incubations, which illustrated the power of the MS technique in studying multienzyme systems. We believe this assay can be exploited in analyzing alternate substrates in tandem incubation, generating information that may greatly facilitate the discovery of aminocoumarins that target DNA gyrase.

Materials and Methods

General Materials and Methods. NovL, NovM, and NovP were overexpressed and purified as previously described (1, 14, 15). The ring A analog, 3-methyl-4-hydroxybenzoic acid (2), *S*-

adenosyl-L-methionine (SAM), and novobiocin were purchased from Aldrich or Sigma and used without further purification; 3-prenyl-4-hydroxybenzoic acid (1), 3-amino-4,7-dihydroxy-8-methyl coumarin (3), novobiocic acid (4), TDP-L-noviose, desmethyl descarbamoyl novobiocin (6), and descarbamoyl novobiocin (8) were synthesized as previously described (1, 14, 15).

All of the enzymatic reactions and LC/ESI/MS analyses were carried out at ambient temperature. Reactions were initiated by the addition of one enzyme (for NovL and NovM reactions) or a mixture of enzymes (for NovL/NovM and NovL/NovM/NovP tandem reactions) and terminated by quenching 10- μ l aliquots in 20 μ l of methanol containing an internal standard at 4°C. Quenched aliquots were incubated at -20°C for 30 min and then centrifuged at 5,000 rpm for 30 min to remove precipitated protein. The supernatant in each case was analyzed by MS.

Preparation of Novobiocic Acid Analog 5. A 5-ml reaction containing 1 mM 3-methyl-4-hydroxybenzoic acid (1) and 1 mM aminocoumarin 3 was carried out in 75 mM Tris, pH 7.5/1 mg/ml BSA/10 mM MgCl₂/5 mM ATP/10% DMSO. The reaction was initiated by the addition of NovL at a final concentration of 1 μ M and was allowed to proceed for 18 h. The product was purified by using a C18 Sep-Pak as previously described (1) and confirmed by LC/MS [$C_{18}H_{15}NO_6$ calculated, 340.09 (M+H⁺), and observed, 340.11].

MS. Data were acquired on an Agilent 1100 series LC/MSD mass spectrometer equipped with a binary pump and a standard autosampler (Agilent Technologies, Palo Alto, CA). Approximately 10 μ l of each sample solution was injected on a Polaris 5u C18-A column (150 $\times$ 3.0 mm) (Varian) by using a mixture of water and methanol at a flow rate of 0.5 ml/min. The purpose of chromatography was sample cleaning only. The column temperature was set to 40°C. The entire eluate was directed into the ESI source of the quadrupole mass spectrometer 4 min after injection by using negative ion detection. The capillary temperature and the spray voltage were 300°C and 3.0 kV, respectively. Selective ion monitoring (SIM) mode was used for quantification, and the total ion chromatogram (TIC) peak area for each SIM ion was automatically integrated by using the Agilent ChemStation data browser. The integrated peak area of the product ion and the internal standard ion were used to determine their peak area ratio (A_p/A_{is}).

Quantification Method. Calibration curves or single-point normalization factors (*R* factors) (26, 29) were used for product quantification. For each calibration curve, 11 samples (25 μ l each) containing different product concentrations were prepared. Selective ion monitoring (SIM) was performed on each sample to obtain A_p/A_{is} . Calibration curves were generated by plotting A_p/A_{is} vs. [product]/[internal standard]. *R* factors were obtained by using a sample solution containing known product concentration and were calculated by using the following equation (26, 29): $R = (A_p/A_{is})/([product]/[internal standard])$.

Descarbamoyl novobiocin (8) (5 μ M in methanol) was used as an internal standard for characterization of NovL and NovM and for analysis of the NovL/NovM reaction. Novobiocin (10 μ M in methanol) was used as an internal standard for the NovL/NovM/NovP incubation.

Characterization of NovL. Reactions were assayed in 25 μ L of NovL buffer containing 75 mM Tris, pH 7.5/1 mg/ml BSA/10 mM MgCl₂/5 mM ATP/10% DMSO. For the determination of kinetic parameters for benzoic acid moiety 1, NovL was added to 50 nM, whereas aminocoumarin 3 was kept at a constant concentration of 1 mM, and 1 was varied over a concentration range of 1–40 μ M. For the determination of kinetic parameters for benzoic acid analog 2, the concentrations used for NovL and

3 were 250 nM and 200 μ M, respectively, and acid analog **2** concentration was varied from 25 to 600 μ M. Each reaction was quenched at 2 min, and the K_m and k_{cat} values for benzoic acids **1** and **2** were determined by fitting data with GRAFIT VERSION 4.0.12 (Erithacus Software, Horley, U.K.). The mechanism of NovL was determined by using the benzoic acid and aminocoumarin components, **1** and **3**, as substrates at five different concentrations for both substrates (4, 8, 12, 20, and 30 μ M for **1** and 20, 50, 100, 200, and 400 μ M for **3**). Initial velocities were determined under each of the 25 conditions, and the catalytic mechanism of NovL was evaluated by using the GRAFIT program.

Characterization of NovM. Reactions were assayed in 25 μ L of NovM buffer (75 mM Mes, pH 6.0/10 mM $MnCl_2$ /1 mg/ml BSA/10% DMSO). NovM was added to 10 nM, and TDP-L-noviose was kept at 100 μ M. The concentration range used for novobiocic acid (**4**) and its analog **5** was 5–50 μ M and 5–80 μ M, respectively. Each reaction was quenched at 2 min, and the K_m and k_{cat} values for **4** and **5** were obtained by using the GRAFIT program. In the mechanism study for NovM, **4** and TDP-L-noviose were used as substrates at five different concentrations (5, 10, 20, 30, and 40 μ M for **4** and 2, 4, 7, 15, and 25 μ M for TDP-L-noviose). The mechanism of NovM was evaluated by using the GRAFIT program.

NovL/NovM Tandem Reaction Assay. The two-enzyme tandem reaction was carried out in 200 μ L of NovL/NovM buffer (75 mM Tris, pH 7.0/10 mM $MgCl_2$ /5 mM ATP/1 mg/ml BSA/10% DMSO). The reaction mixture contained 20 μ M acid **1**, 200 μ M aminocoumarin **3**, 200 μ M TDP-L-noviose, and 100 nM NovL and NovM. The tandem reaction was monitored from 2 to 75 min, and the amounts of novobiocic acid (**4**) and desmethyl descarbamoyl novobiocin (**6**) formed at different time points were quantified by LC/ESI/MS analysis. The concentration of substrate **1** in each sample was calculated by subtracting the total concentration of the two products **4** and **6** from its starting concentration. A reaction profile was generated by plotting the concentrations of **1**, **4**, and **6** vs. reaction time.

NovL/NovM/NovP Tandem Reaction Assay. The three-enzyme tandem reaction was carried out in 200 μ L of NovL/NovM/NovP buffer (75 mM Tris, pH 7.0/10 mM $MgCl_2$ /100 mM NaCl/5 mM ATP/1 mg/ml BSA/10% DMSO), and the reaction components were 50 μ M **1**, 500 μ M **3**, 100 μ M TDP-L-noviose, and 900 μ M SAM. The concentrations used for NovL, NovM, and NovP were 100, 100, and 500 nM, respectively. The tandem reaction was monitored from 2 to 90 min, and the amounts of **4**, **6**, and **8** at different time points were quantified. The concentration of **1** in each sample was calculated as described above. A reaction profile was generated by plotting the concentrations of **1**, **4**, **6**, and **8** vs. reaction time.

Results

Characterization of NovL. The ligase activity of NovL was characterized with both benzoic acids **1** and **2**. The monitored product ions ($[M-H]^-$) were for compounds **4** at m/z 394 and **5** at m/z 340, and these ions were quantified in relation to the internal standard ion at m/z 568.

Michaelis–Menten kinetic analysis was carried out for NovL by using substrates **1** and **2** (Fig. 2A and B). Enzyme inhibition was observed at >60 μ M acid **1** and >800 μ M acid analog **2**, so those concentration ranges were not used in fitting the Michaelis–Menten data. The average K_m , k_{cat} , and specificity constant (k_{cat}/K_m) values obtained for **1** and **2** are summarized in Table 1. The kinetic constants for **1** are in excellent agreement with those obtained by the HPLC assay, and the results for **2** are previously unreported. A 72-fold decrease in the catalytic effi-

Fig. 2. (A) Saturation plot for ring A, V_0 vs. **1**. (B) Saturation plot for **1** analog, V_0 vs. **2**. (C) Double-reciprocal plots for mechanism determination of NovL catalysis, $1/V_0$ vs. $1/[1]$ at different ring B concentrations. $\circ$, **3** = 20 μ M; $\bullet$, **3** = 50 μ M; $\square$, **3** = 100 μ M; $\blacksquare$, **3** = 200 μ M; and $\triangle$, **3** = 400 μ M.

ciency of NovL with substrate **2** was observed and is primarily the result of a 60-fold decrease in K_m relative to the natural substrate.

The catalytic mechanism of NovL was determined by fitting the data to two mechanistic models (sequential and ping-pong mechanism) of bisubstrate reactions in the GRAFIT program, and the best fit was obtained in the case of a sequential mechanism model. Fig. 2C shows the double reciprocal plot of $1/\text{initial velocity } (V_0)$ vs. $1/[\text{benzoic acid } \mathbf{1}]$ and the five lines representing different aminocoumarin **3** concentrations that all intersect at one point. In the sequential mechanism, NovL binds to the benzoic acid moiety and the aminocoumarin ring to form a ternary complex, catalyzes the ligation between substrates inside the complex, and subsequently releases the reaction products, novobiocic acid (**4**) and water.

Characterization of NovM. The glycosyltransferase activity of NovM was characterized with both the natural substrate novo-

Table 1. Kinetic constants determined for NovL and NovM enzymatic systems

Enzyme	Substrate	K_m , μM	k_{cat} , min^{-1}	k_{cat}/K_m , $\mu\text{M}^{-1}\cdot\text{min}^{-1}$
NovL	1*	8.7 ± 0.2	30.9 ± 2.1	3.6 ± 0.3
	1	$6.4 \pm 0.2^{\dagger}$	28.2 ± 0.8	4.40 ± 0.09
	2	360 ± 30	21.6 ± 0.6	0.061 ± 0.04
NovM	4	39 ± 4	219 ± 6	5.6 ± 0.7
	5	8.6 ± 0.3	290 ± 30	33 ± 2

A sequential bi-bi catalytic mechanism was determined for NovL and NovM catalysis, and mechanism results were based on two replicate experiments.

*Kinetic parameters were determined by HPLC assay.

[†]All standard deviations were calculated based on three or more sets of replicate experiments.

biocic acid (**4**) and its ring A-modified analog **5** (Fig. 1B). The monitored product ions ($[M-H]^-$) were for compounds **6** at m/z 554 and **7** at m/z 500, and they were quantified relative to the internal standard ion at m/z 568.

As reported previously, NovM inhibition was observed at $>50 \mu\text{M}$ **4** (**1**). However, no obvious substrate inhibition occurred at high concentrations of **5**. The average K_m , k_{cat} , and k_{cat}/K_m values obtained of **4** and **5** are summarized in Table 1. The kinetic parameters for **4** are consistent with those obtained by HPLC assay (**1**), and the results for **5** are previously unreported. Interestingly, the analog **5** appears to be a better substrate for NovM than the natural substrate **4**. As indicated by their specificity constant values, **5** is 6 times more active than **4**. The increased activity of **5** is attributed to its higher binding affinity to NovM (K_m) and its higher reaction turnover (k_{cat}).

A sequential bi-bi catalytic mechanism was determined for NovM catalysis. In the sequential mechanism, NovM binds to both donor and acceptor substrate (novobiocic acid and TDP-L-noviose) to form a ternary complex, during which process the noviose sugar is transferred from TDP-L-noviose to novobiocic acid. The glycosylated product, **6**, is subsequently released from the complex.

Analysis of the NovL/NovM Tandem Reaction. The two consecutive steps of the NovL/NovM two-enzyme tandem reaction for benzoic acid substrate **1** are illustrated in Fig. 1B. The intermediate **4** and final product **6** were directly monitored in one sample, and the monitored ions ($[M-H]^-$) were for compounds **4** at m/z 395 and **6** at m/z 554. The internal standard ion was at m/z 568. The reaction profile is shown in Fig. 3. The rapid depletion of benzoic acid substrate **1** under these conditions is

Fig. 3. The reaction profile for the NovL/NovM two-enzyme tandem reaction is shown; **1** (●), **4** (■), and **6** (▲) vs. reaction time.

accompanied by an increase in the novobiocic acid intermediate **4** concentration, which then undergoes conversion to the final product, desmethyl descarbamoyl novobiocin **6**. The ratio of the enzymes NovL and NovM dictates the tandem reaction profile observed and is adjusted accordingly to allow the detection of the accumulating intermediate and product.

Analysis of the NovL/NovM/NovP Tandem Reaction. In the NovL/NovM/NovP three-enzyme tandem reaction system, three species (two intermediates and a final product) were quantified in one sample. In this experiment, the substrate benzoic acid moiety **1** was first ligated to aminocoumarin **3** by the action of NovL, resulting in the formation of the first intermediate **4**, which was subsequently glycosylated by NovM to give the second intermediate **6**. In the last step, **6** was converted to the final product **8** by NovP-catalyzed methylation (Fig. 1B). Novobiocin was used as internal standard and the four $[M-H]^-$ ions monitored were compounds **4** at m/z 395, **6** at m/z 554, **8** at m/z 568, and **10** at m/z 611. Based on the kinetic properties of NovP obtained by the HPLC assay (**15**), it is known that the methylation of noviose sugar catalyzed by NovP is slow. Therefore, we adjusted the concentration of NovP to 500 nM, giving a NovL/NovM/NovP molar ratio of 1:1:5. Under these conditions, rapid conversion of the benzoic acid substrate is accompanied by accumulation of the first intermediate, novobiocic acid. The concentration of novobiocic acid reaches steady state and then rapidly decreases to form glycosylated intermediate **6**. Conversion of intermediate **6** to methylated product **8** is slow under these conditions, further demonstrating the low catalytic efficiency of NovP relative to NovL and NovM. Again, the NovL/NovM/NovP molar ratio dictates the observed reaction profile and can be adjusted to enhance the rate of product **8** formation.

Discussion

Previous structural analysis and kinetic studies of the aminocoumarin antibiotics have revealed critical interactions between the carbamoylated noviosyl ring and the ATP binding site of the gyrase B subunit (**6–8**). The strategy in the design of improved coumarin-based antibiotics is to maintain the noviose decorations necessary for binding while introducing additional functionalities to improve the inhibitory properties of the analog or modulate solubility. One approach is to generate novel antibiotics via chemoenzymatic synthesis by using four enzymes that act in tandem (NovL, NovM, NovP, and NovN) in the novobiocin biosynthetic pathway. Structural diversities of the antibiotics are introduced in the first two scaffold-assembling steps, whereas essential noviose decorations are maintained by the last two tailoring steps. Therefore, an efficient kinetic assay capable of characterizing each enzyme and analyzing the multienzyme tandem reactions will ultimately provide a foundation for chemoenzymatic approaches to novel antibiotics.

To fulfill this goal, an LC/ESI/MS assay, with an LC system for sample desalting and an ESI/MS technique for product quantification, was developed. The assay was first used to obtain the kinetic constants, determine catalytic mechanism, and to provide a foundation for initial substrate specificity studies of NovL and NovM individually. For the studies described here, the alternate substrate differs structurally from the natural substrate at the 3 position of the benzene ring in ring A, bearing a methyl group instead of a prenyl moiety. The kinetic data in Table 1 show that this structural modification is tolerated by NovL, albeit with a 72-fold reduction in the catalytic efficiency. A comparison of the K_m values suggests that acid analog **2** has a lower binding affinity to NovL, indicating important interactions between the prenyl group and the benzoic acid binding site on NovL. Surprisingly, the kinetic parameters obtained for NovM reveal that substitution at the 3 position of ring A with a methyl group results in a 6-fold enhancement in catalytic efficiency. The K_m

values suggest a higher binding affinity between NovM and the novobiocin acid analog **5**. The relative kinetic properties of NovL and NovM suggest that it is possible for individual enzymes in a multienzyme synthetic pathway to prefer different structural moieties on the substrate. Evaluation of substrate specificity for multiple enzymes in tandem will provide more relevant predictions about the prospects of chemoenzymatic synthesis of novel antibiotics.

The central aim of this study has been to develop an MS-based assay method for direct product monitoring and kinetic analysis of multienzyme tandem reactions. A distinctive feature of the LC/ESI/MS assay is that multiple species of different mass-to-charge ratios can be directly detected and quantified in one sample simultaneously, making it particularly suitable for rapid measurements of complex kinetic systems [e.g., multisubstrate (31) or multienzyme (32) systems]. As long as the consecutive action of multiple enzymes imparts mass differences to the products formed at different reaction stages, the LC/ESI/MS assay can be successfully refined for analysis of multienzyme tandem reactions.

The reaction profile of the NovL/NovM tandem incubation with benzoic acid **1** (Fig. 3) illustrates a steady-state level of the novobiocin acid intermediate (**4**) at 2.5 μM with equimolar (100 nM) concentrations of ligase and noviosyl transferase. The concentration of **4** during its steady state is lower than the concentration of substrate **1**. The relatively low accumulation of novobiocin acid suggests that the average rate constant for glycosylation (k_2) is higher than that for ligation (k_1). The observed lower catalytic activity of NovL compared with NovM is consistent with the kinetic constants measured for NovL and NovM individually. Because NovL catalysis is the rate-limiting step of the two-enzyme tandem reaction, using a higher amount of NovL in the reaction mixture may help to improve the overall formation rate of the final product **8**.

The reaction profile of the NovL/NovM/NovP (1:1:5) tandem incubation with acid **1** (Fig. 4) portrays similar reactivity for ligation and glycosylation, with the differences in the relative accumulation of novobiocin acid most likely attributed to differences in starting substrate concentrations. Accumulation of the second intermediate, desmethyl descarbamoyl novobiocin (**6**), is greater (45 μM) than that of the first intermediate **4** (11 μM), underlying a low catalytic activity of NovP, which is further verified by the slow formation of final product, descarbamoyl novobiocin (**8**), as shown in Fig. 4 *Inset*. The low catalytic activity of NovP observed in the tandem reaction system is consistent with the kinetic properties of NovP previously reported (15).

The noviosyl ring carbamoylation catalyzed by NovN is crucial for high-affinity binding between novobiocin and its bacterial target, which gives a 200-fold increase in antibiotic activity of novobiocin relative to the NovP product (**8**). Thus, it will be of

Fig. 4. Reaction profile for the NovL/NovM/NovP three-enzyme tandem reaction; **1** (●), **4** (■), **6** (▲), and **8** (◆) vs. reaction time.

particular interest to extend the current NovL/NovM/NovP system to include NovN, the last enzyme in the biosynthetic pathway. It is anticipated that kinetic information obtained from assaying the four-enzyme tandem reaction can be directly used for the optimization of chemoenzymatic synthesis of novobiocin and its analogs. In an effort to rapidly generate novel antibiotics with structural diversity, reaction specificity of alternate substrates needs to be evaluated for multiple enzymes in tandem. Introducing multiple substrates into a multienzyme system and determining their reaction specificity simultaneously may open an efficient access to the identification of novel novobiocin derivatives.

The studies described here establish a basis for multiplexing in tandem multienzyme systems. In addition to the application of this method in the rapid identification of specific pharmacophores in DNA gyrase inhibition, we expect that the LC/ESI/MS technique used here may be extended to the reconstitution of even more complex systems, because it is not essential that enzymes in tandem first be studied individually. The approach might also be used for the simultaneous analysis of multiple mutant enzymes in the processing of noncognate substrates.

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