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Investigations of Coronatine Biosynthesis. Overexpression and Assay of CmaT, a Thioesterase Involved in Coronamic Acid Biosynthesis

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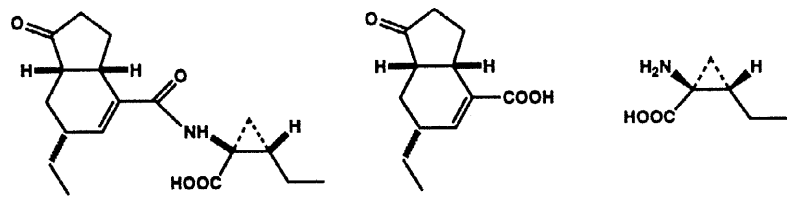
Abstract:

The protein (CmaT) encoded by the *cmaT* gene of the coronamic acid biosynthetic gene cluster has been overexpressed in *Escherichia coli* in soluble and active form fused to the carboxyl terminus of MalE, the maltose-binding protein. CmaT was also overexpressed in *E. coli* as an N-terminal His-tagged protein. The N-terminal His-tagged form of CmaT was produced in insoluble form, but it could be refolded to obtain CmaT in soluble and highly active form. Both the MalE-CmaT fusion protein and the refolded His-tagged CmaT protein exhibited esterase activity.

Keywords: Enzymes and enzyme reactions, Hydrolysis, Thiolesters

INTRODUCTION

Coronatine (**1**) (Figure 1) is a chlorosis-inducing phytotoxin of novel structure produced by several pathovars of *Pseudomonas syringae* including pv. *atropurpurea*, pv. *glycinea*, and pv. tomato.[1-4] Coronatine also exhibits other biological activities including distortion of leaf growth, inhibition of root elongation, and induction of hypertrophy when applied to the cut surface of potato tubers.[5,6] Structural and functional homologies have recently been found between coronatine, methyl jasmonate, and 12-oxophytodienoic acid suggesting that coronatine may function as a molecular mimic of the octadecanoid signaling molecules of higher plants.[7,8]



1 2 3

Figure 1. The structures of coronatine (**1**), coronafacic acid (**2**), and coronamic acid (**3**).

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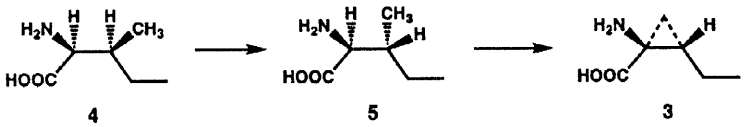
The coronatine molecule is composed of two moieties with distinct biosynthetic origins, coronafacic acid (**2**), which is a polyketide, [9,10] and coronamic acid (**3**) (Figure 1). Coronamic acid is derived from L-isoleucine (**4**) via L-alloisoleucine (**5**) (eq. 1).[9] The genes for coronatine production in *P. syringae* pv. *glycinea* PG4180 are encoded within a 30-kb region that is present on a 90-kb plasmid designated p4180A.[11] Detailed genetic investigations have shown that the genes required for the biosynthesis of coronamic acid are contained within a 6.9-kb region of the coronatine gene cluster. Sequencing of this region revealed the presence of four open reading frames: *cmaA*, *cmaB*, *cmaT*, and *cmaU*.[12,13] A region within the protein (CmaA) coded by *cmaA* encompassing 500 amino acid residues exhibits significant homology to the modules



(1)

of peptide synthetases and contains the conserved spatial arrangement of six core sequences that are found in peptide synthetase modules. The core 6 sequence contains a serine residue which is usually the attachment site of a 4'-phosphopantetheine moiety that is involved in thiolester formation. The deduced amino acid sequence for *cmaB* exhibits the highest similarity to the C-terminal region of SyrB, a protein involved in the biosynthesis of the lipodepsipeptide phytotoxin syringomycin.[14] The amino acid sequence for the protein (CmaT) encoded by *cmaT* shares a strong sequence similarity with thioesterases of both prokaryotic and eukaryotic origin, and it contains two highly conserved core sequences that are present in known thioesterases. No significant similarities were found between the nucleotide and deduced amino acid sequence of *cmaU* and current entries in the data bases. The sequence similarities exhibited by CmaA suggest that L-isoleucine or L-alloisoleucine is activated by adenylation and linked to CmaA via a thiolester with a 4'-phosphopantetheine moiety where it is then cyclized to the thiolester of coronamic acid, perhaps with the assistance of CmaB and/or CmaU. The postulated role for CmaT is to release enzyme-bound coronamic acid by hydrolysis of the thiolester moiety linking it to CmaA.

Identification of the genes required for coronamic acid biosynthesis should allow the biosynthesis of this unusual amino acid to be investigated at the enzymatic level. The initial stages of this investigation, which involve the overexpression and enzymatic assay of CmaT, are described here.

RESULTS AND DISCUSSION

The initial efforts to overproduce CmaT were carried out by PCR amplification of the structural gene from plasmid pSAY12, which contains all of the genes required for coronamic acid biosynthesis.[15] The purified PCR product was then ligated into the expression vector pMal-c2 and transformed into *E. coli* DH5α. *E. coli* clones bearing the desired construct (pMCmaT) were identified and used for expression studies. For overproduction of MalE-CmaT, *E. coli* DH5α cells containing pMCmaT were grown in "terrific broth"[16] at 37 °C to an A_{600} of about 0.6 followed by addition of IPTG to a concentration of 1 mM to induce CmaT production. The cells were harvested 3 h after induction, and the total soluble protein isolated by sonication of the cells in Tris buffer and removal of cell debris by centrifugation. Purification of MalE-CmaT was

accomplished by binding the protein to an amylose column followed by elution with maltose. SDS-PAGE analysis showed a discrepancy in the predicted (92.5 kDa) and the experimental molecular mass (67-70 kDa) of the expressed protein. DNA sequence analysis revealed an error in the published DNA sequence at position 5195, where an additional TA base pair was observed. This additional TA base-pair changes the reading frame downstream from this point and leads to the generation of a stop codon (TGA) adjacent to this point (Figure 2). The introduction of the stop codon changes the predicted molecular mass of CmaT from 49.6 kDa to 27.0 kDa. The predicted molecular mass of the fusion protein generated as a result of the presence of this new stop codon (69.9 kDa) is consistent with the experimentally observed mass.

Published Sequence:	**GGC AGT GAG ACG**
Amino Acid	**G S E R**
Corrected Sequence:	**GGC AGT TGA GAC G**
Amino Acid	**G S Stop**

Figure 2. Position of the stop codon in the corrected DNA sequence for *cmaT*.

The MalE-CmaT fusion protein was assayed for esterase activity using p-nitrophenolate esters of acetic acid, propionic acid, and butyric acid by continuous monitoring at A_{400} for the p-nitrophenolate anion.[17] The MalE-CmaT fusion protein catalyzed the hydrolysis of all three substrates at rates comparable to those exhibited for the hydrolysis of some p-nitrophenolate esters by the overproduced ACP-TE domain of DEBS3 (Table I).[17] The ability of the MalE-CmaT fusion protein to catalyze the hydrolysis of the N-acetylcysteamine (NAC) thiolesters of propionic acid, butyric acid, and cyclopropane carboxylic acid and of coenzyme A thiolesters of propionic, butyric, and isobutyric acid was also examined by a discontinuous assay using DTNB. Although the N-acetylcysteamine thiolesters and the CoA thiolesters should be better mimics of the 4'-phosphopantetheine moiety by which an amino acid is presumably attached to CmaA, the rates of hydrolysis of all of the thiol esters were significantly slower than those observed with the three p-nitrophenolate esters (Table I). Similar behavior has been observed for NAC esters with the overproduced ACP-TE domain of DEBS3.[17] The slower rates observed for all of the thiolester substrates relative to the p-nitrophenolate esters is presumably a reflection of the fact that the p-nitrophenolate anion is a better leaving group than the thiolate anion. It appears difficult to draw any clear conclusions about the binding preferences of CmaT from the data in Table I. The NAC esters were synthesized by standard methods.[18,19]

In the current hypothesis for coronamic acid biosynthesis, it is postulated that the role of CmaT is to release coronamic acid bound to CmaA as a thiolester. The size of the MalE protein (42.9 kDa) relative to that of CmaT (27.0 kDa) raised concerns that future studies of CmaA might be jeopardized because the MalE-CmaT fusion protein might fail to interact properly with CmaA. Since attempts to remove the MalE fusion partner of CmaT by treatment of the fusion protein with the protease Factor Xa were unsuccessful, the overproduction of an N-terminal His-tagged version of CmaT was investigated. A DNA segment corresponding to the corrected open reading frame for *cmaT* was amplified from plasmid pSAY12 by PCR and the purified PCR product was ligated into the expression vector pProEx-HTa and transformed into *E. coli* DH5α. *E. coli* clones bearing the desired construct (pProCmaT) were recovered and used for expression

Table I. Rate of Hydrolysis of Esters and Thiolesters by MalE-CmaT and His-tagged CmaT

Substrate	Rate (nmol/min-mg)
p-Nitrophenyl acetate	0.457
p-Nitrophenyl propionate	0.638 (5.21)[a]
p-Nitrophenyl butyrate	0.356 (2.48)[a]
N-Acetyl-S-propionylcysteamine	0.087
N-Acetyl-S-butyrylcysteamine	0.111
N-Acetyl-S-cyclopropylcarbonylcysteamine	0.013
Propionyl coenzyme A	0.042
n-Butyryl coenzyme A	0.059
i-Butyryl coenzyme A	0.055

[a]Values for His-tagged CmaT

studies. The expression of the N-His-tagged CmaT fusion protein was carried out by growth of the recombinant *E. coli* cells in LB broth at 37 °C until an A_{550} of 0.4-0.5 was reached followed by induction by addition of IPTG to a final concentration of 0.5 mM. The cultures were then grown for an additional 3-4 h before harvesting. The harvested cells were sonicated in Tris buffer and the soluble and insoluble protein fractions analyzed by SDS-PAGE. PAGE analysis indicated that all of the His-tagged CmaT fusion protein had been expressed in insoluble form. In subsequent preparations, the sonicated cell suspension was fractionated by centrifugation at 32, 000 x g for 15 minutes at 4 °C. The supernatant was discarded and the insoluble pellet, which consisted largely of inclusion bodies formed from overexpressed His-tagged CmaT fusion protein was washed and the washed inclusion bodies were resuspended in a solubilization buffer and allowed to dissolve slowly overnight at 4 °C. Insoluble material was removed by centrifugation at 32, 000 x g at 4 °C and the supernatant was applied to a Talon column that had been equilibrated with solubilization buffer. The column was washed with solubilization buffer containing 10 mM imidazole. Bound protein was then eluted with solubilization buffer containing 100 mM imidazole. The eluent was transferred to dialysis tubing (MW cut-off 10, 000) and dialyzed against refolding buffer containing 6 M urea. The denaturant urea was removed by sequential dialysis against refolding buffer containing decreasing concentrations of urea, in 2 M steps. To completely remove the urea, the sample was further dialysed against refolding buffer containing no urea. The refolded N-terminal His-tagged form of CmaT was assayed for enzymatic activity and found to exhibit significantly higher activity with p-nitrophenolate esters (Table I). This result suggests that the MalE component of the MalE-CmaT fusion protein may interfere with the proper folding of CmaT.

In an attempt to gain additional insight into the binding requirements of CmaT, cyclopropane carboxylic acid, 1-amino-1-cyclopropane carboxylic acid, and (±)-coronamic acid were evaluated as inhibitors of the hydrolysis of p-nitrophenyl propionate catalyzed by MalE-CmaT and His-tagged CmaT. All three

compounds were found to be very weak inhibitors of the hydrolysis reaction (see Experimental). This may reflect the relatively poor binding of a carboxylate anion relative to a neutral ester or thiolester.

In conclusion, these studies have confirmed the hypothesis that CmaT is a thiolesterase, and they have provided a method for the overproduction of a soluble and active form of the enzyme which should be suitable for further investigations of coronamic acid biosynthesis at the enzymatic level.

EXPERIMENTAL

Standard chemical reagents were purchased from Sigma/Aldrich Chemical Co. Microbiological media were purchased from Difco, Inc. (±)-Coronamic acid was prepared by the method of Baldwin.[20] The vector pProEX-HTa was obtained from Gibco-BRL, while the vector pMal-c2, Vent polymerase, and amylose resin were purchased from New England Biolabs. TALON metal affinity resin was purchased from Clontech. The detergent Nonidet P40 was obtained from U. S. Biochemical Corp. PCR primers were obtained from Integrated DNA Technologies, Inc. PCR experiments were conducted with a Biometra PCR Personal Cycler. The QiaQuick DNA purification kit was obtained from Qiagen, Inc. Sonication was carried out with a Branson Model 250 Sonifier. NMR spectra were obtained with a Bruker AC250 spectrometer using deuterochloroform as solvent and with TMS as an internal standard. Mass spectra were measured in the EI mode at 70eV with a Finnigan MAT 95 spectrometer. Ultraviolet-visible spectra were obtained with a Hewlett-Packard HP 8452A Diode Array spectrophotometer. SDS-PAGE was performed according to the method of Laemmli[21] and protein bands were visualized by staining with Coomassie blue. Protein concentrations were determined by the method of Bradford[22] with bovine serum albumin as standard. Transformations were carried out with commercially available competent *Escherichia coli* cells following procedures recommended by the manufacturers. *E. coli* strains were grown in Luria-Bertani (LB) medium or "terrific broth " at 37 °C. Selection was made with 150 μg of ampicillin per mL in LB agar or liquid medium. Column chromatography utilized Merck silica gel, type 60A, 230-400 mesh. Automated DNA sequencing was performed with an Applied Biosystems DNA sequencer at the Molecular Genetics Core Facility, University of Texas at Houston Medical School by using universial and synthetic oligonucleotide primers.

Construction of Expression Plasmids.

Cloning of *cmaT* into pMal-c2

The coding sequence originally reported for *cmaT* was amplified by PCR using a plasmid carrying the biosynthetic pathway for coronamic acid (pSAY12) as template, gene-specific synthetic oligonucleotides primers designed with *Eco* RI restriction sites (described below), and Vent DNA polymerase. The PCR product was purified with a QiaQuick Kit using the procedure described by the manufacturer. The purified PCR product was digested overnight with *Eco* RI at 37 °C. The*Eco* RI fragment containing *cmaT* was gel purified using the QiaQuick kit, ligated into pMAL-c2 cleaved with *Eco* RI and dephosphorylated with calf intestinal alkaline phosphatase, and transformed into *E.coli* strain DH 5α using standard techniques. Recombinant clones were identified by agarose gel electrophoresis and clones with the correct orientation of the insert with respect to the regulatory signals in the pMal-2c vector were identified by restriction analysis with *Pst* I. Clones with the correct size and orientation (pMCmaT) were used for expression following procedures described by New England Biolabs, and the results were analyzed by SDS/PAGE.

Primers Used For PCR:

PCR Primer, Primer 1	GACGAATTCATGGCCGATCCTTTTGTG
PCR Primer, Primer 2	ACTGAATTCCACTAGGTAGATTTCGAC

The PCR reaction employed 100 pmoles of each PCR primer, 200 μM dNTP, 4 mM $MgSO_4$, 1x Vent reaction buffer, and distilled H_2O to 100 μL. The PCR conditions used thirty cycles of 1 min at 97 °C, 1 min at 52 °C, and 1 min at 72 °C followed by 5 minutes at 72 °C. All the components were added and mixed in a 0.7 mL thin walled PCR tube and a single colony of *E.coli* strain DH5α containing the plasmid pSAY12 was picked and resuspended in the PCR mix. The PCR reaction was initiated by the addition of 3U of Vent DNA polymerase.

Cloning of *cmaT* into pProEx-HTa

A similar procedure to that used with pMAL-c2 was employed for cloning of *cmaT* into pProEX-HTa. In this instance, the PCR primers were designed based upon the corrected DNA sequence for *cmaT* (see below), with a *Nco* I site in the 5' Primer and a *Spe* I site in the 3' primer. The PCR product was purified with a QiaQuick Kit using the procedure described by the manufacturer and then digested with SpeI followed by purification using a QiaQuick kit and digestion with *Nco* I. The vector pProEX-HTa was also sequentially digested with *Spe* I and *Nco* I. The doubly-digested vector was then subjected to gel purification. The doubly-digested PCR fragment was ligated into the doubly-digested vector and transformed into *E. coli* strain DH5α using standard techniques. Clones containing the correct insert were identified by agarose electrophoresis and analyzed for expression by SDS/PAGE.

Primers Used For PCR:

PCR Primer, CmaT-*Nco* I	GACCCATGGATGGCCGATCCTTTTGTG
PCR Primer, CmaT-*Xho*I	GACTCTCGAGCTAGGTAGATTTCGGAC ACG

The PCR reaction employed 100 pmoles of each PCR primer, 200 μM dNTP, 4 mM $MgSO_4$, 1x Vent reaction buffer, and distilled H_2O to 100 μL. The PCR conditions used thirty cycles of 1 min at 97 °C, 1 min at 56 °C and 1 min at 72 °C followed by 5 minutes at 72 °C. All the components were added and mixed in a 0.7 mL thin walled PCR tube and a single colony of *E.coli* strain DH5α containing the plasmid pSAY12 was picked and resuspended in the PCR mix. The PCR reaction was initiated by the addition of 3U of Vent polymerase.

Overproduction and Purification of MalE-CmaT Fusion Protein

Overproduction of MalE-CmaT Fusion Protein

Freezer stock of *E. coli* DH5α containing the expression plasmid pMCmaT was used to inoculate a 50 mL culture of "terrific broth"[16] containing 100 μg/mL of ampicillin. This culture was grown at 37 °C for 18 h. Ten mL aliquots of this culture were used to inoculate four 100 mL cultures of terrific broth containing 0.2% (w/v) glucose and 100 μg/mL of ampicillin. Cultures were grown at 37 °C to an A_{600} of about 0.6. IPTG was added to a 1 mM concentration and growth was allowed to continue for 3 h. Cells were collected by centrifugation at 12,000 x g for 10 min at 4 °C. The supernatant was decanted and the cell paste was stored at -20 °C overnight.

Purification of MalE-CmaT Fusion Protein

Cell paste (4 g) was resuspended in column buffer (20 mM Tris HCl, pH 7.4, containing 200 mM NaCl and 1 mM Na_2EDTA) to give a final volume of 100 mL The cells were disrupted by sonication (65% power for 4 min on ice followed by cooling for 2 min, then an additional 4 min of sonication). Cell debris was removed by centrifugation at 24, 000 x g for 35 min at 4 °C. The supernatant was carefully decanted and applied to an amylose resin column (3.7 x 2.5 cm, 18 mL bed volume) equilibrated in column buffer. The column was washed with 12 column bed volumes of buffer to remove non-binding material. The column was eluted with column buffer containing 10 mM maltose to elute bound CmaT. Determination of the protein concentration by the Bradford procedure gave a concentration of 3.5 mg/mL. SDS-PAGE analysis showed a discrepancy in the predicted (92.5 kDa) and the experimental molecular mass (67-70 kDa) of the expressed protein. DNA sequence analysis revealed an error in the published DNA sequence at position 5195 where an additional TA base pair was observed. This additional TA base-pair changes the reading frame downstream of this point and leads to the generation of a "new" stop codon (TGA) adjacent to this point (Figure 2). The predicted molecular mass of the truncated fusion protein generated as a result of the presence of this new stop corresponded with the experimentally observed mass. Attempts to remove the MalE fusion partner of CmaT by treatment of the fusion protein with the protease Factor Xa according to the New England Biolabs protocol were unsuccessful.

Overproduction, Purification, and Refolding of the N-Terminal His-Tagged Fusion Protein.

Overproduction of N-Terminal His-Tagged Fusion Protein

A single colony of *E.coli* strain DH5α containing the plasmid pProCmaT was used to inoculate 10 mL of LB Broth supplemented with 150 μg/mL of ampicillin and the culture was grown overnight with shaking at 37 °C. Two milliliter aliquots of the overnight culture were used to inoculate five 200 mL aliquots of LB broth supplemented with 150 μg/mL of ampicillin. The cultures were grown until an OD A_{550} of 0.4-0.5 was reached and then induced by addition of IPTG to a final concentration of 0.5 mM. The cultures were grown for a further 3-4 h and then harvested by centrifugation at 8,000 x g for 10 minutes at 4 °C. The cell pellet was resuspended in 10 mL per gram wet weight of sonication buffer (50 mM Tris HCl, 100 mM NaCl, pH 8.0) and placed on ice for a short period. After the cell suspension was chilled, it was subjected to 5-10 cycles of sonication for 2 minute intervals and chilling for 5 minutes, while lysis was monitored by microscopy. The sonicated cell suspension was fractionated by centrifugation at 32,000 x g for 15 minutes at 4 °C. The supernatant was discarded and the insoluble pellet, which consisted largely of inclusion bodies formed from overproduced CmaT, was washed once with sonication buffer and 3 times with sonication buffer containing 2 M Urea and 0.5% Nonidet P40; with each washing, the inclusion bodies were harvested by centrifugation at 32,000 x g for 10 minutes at 4 °C. The washed inclusion bodies were resuspended in 20 ml of solubilization buffer (6 M guanidine HCl, 50 mM Tris HCl, 100 mM NaCl and 0.5% Nonidet P40) and allowed to dissolve slowly overnight at 4 °C. Insoluble material was removed by centrifugation at 32,000 x g for 15 minutes at 4 °C and the supernatant was applied to a 10 mL Talon column that had been equilibrated with solubilization buffer. The column was washed with 5 volumes of solubilization buffer containing 10 mM imidazole. Bound protein was then eluted with 3 column volumes of solubilization buffer containing 100 mM imidazole. The eluent was transferred to dialysis tubing (MW cut-off 10,000) and dialyzed against refolding buffer (50 mM

Tris HCl, 100 mM NaCl) containing 6 M urea. The denaturant was removed by sequential dialysis for 12-16 h against refolding buffer containing decreasing concentrations of urea, in 2 M steps. To completely remove the urea, the sample was further dialysed against 4 liters of refolding buffer containing no urea.

Enzymatic Assay of p-Nitrophenyl Esters

p-Nitrophenyl acetate (PNPA), p-nitrophenyl propionate (PNPP) and p-nitrophenyl butyrate (PNPB) were evaluated as substrates. Sufficient substrate was dissolved in 3.0 mL of methanol to produce a 33.3 mM solution. A 0.1 mL aliquot of this solution was immediately added to 4.9 mL of distilled water to give a 0.67 mM substrate stock solution. Esterase assays consisted of 800 μL of buffer (50 mM Tris HCl, pH 8.0), 100 μL of substrate stock solution, and 100 μL of enzyme (0.35 mg). The assay mixture was incubated at 30 °C and the change in A_{400} was continuously monitored for 30 min. Hydrolysis rates were calculated using a molar extinction coefficient of 18,300 $cm^{-1}M^{-1}$for the p-nitrophenolate anion.[23] The hydrolysis rates were corrected for spontaneous hydrolysis. Background hydrolysis rates were determined by the same method, but with omission of enzyme. Cyclopropane carboxylic acid, 1-amino-1-cyclopropane carboxylic acid and (±)-coronamic acid were evaluated as inhibitors of the CmaT-catalyzed hydrolysis of PNPP. In these experiments, a portion of the buffer in this assay was replaced by an equal volume of inhibitor stock solution (10 mM in buffer) and the change in A_{400} was determined as above. All three compounds were found to be weak inhibitors. At a concentration of 5 mM, cyclopropane carboxylic acid, 1-amino-1-cyclopropane carboxylic acid, and (±)-coronamic acid caused 38% inhibition, 37% inhibition, and 12% inhibition, respectively, of PNPP hydrolysis catalyzed by MalE-CmaT, while at the same concentration, 1-amino-1-cyclopropane carboxylic acid caused 42% inhibition of the hydrolysis of PNPP catalyzed by His-tagged CmaT.

Enzymatic Assay of Thiolesters

Point assays were used for thiolesterase activity measurements since 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNB) was found to inhibit PNPP hydrolysis by CmaT. Stock solutions of thiolester substrates (10 mM in distilled water) were used. Thiolesterase assays consisted of 800 μL of buffer (50 mM Tris HCl, pH 7.4), 100 μL of thiolester substrate, and 100 μL of enzyme (0.35 mg). The assay mixture was incubated at 30 °C for 15 h. The reaction was terminated and the protein removed by means of a Millipore Ultrafree-MC filter unit (10, 000 MW cutoff) at 4 °C. Aliquots (150 μL) of the filtrate were placed in a cuvette along with 100 μL of 1.0 mM DTNB and 750 μL of buffer. After mixing, the change in A_{412} was monitored for 30 min at room temperature to ensure complete reaction between free thiols and DTNB. Hydrolysis rates were calculated using a molar extinction coefficient of 13,600 $cm^{-1}M^{-1}$ for the 3-carboxy-4-nitro-1-thiophenolate anion.[23] The hydrolysis rates were corrected for spontaneous hydrolysis. Background hydrolysis rates were determined by the same method, but with enzyme omitted.

Synthesis of N-Acetylcysteamine Thiolesters

The N-acetylcysteamine thiolesters of propionic acid and cyclopropane carboxylic acid were prepared by the procedure of Lee *et al.* [19] The yield of N-acetyl-S-propionylcysteamine was 68% after chromatography on silica gel with 4:1 ethyl acetate-hexane. N-acetyl-S-propionylcysteamine was a colorless oil: ^1H NMR δ 1.18 (t, 3H, CH_3), 1.98 (s, 3H, $COCH_3$), 2.61 (q, 2H, CH_2CO), 3.01 (t, 2H, SCH_2), 3.43 (q, 2H, NCH_2), 6.06 ppm (bs, 1H, NH); ^{13}C NMR δ 9.84, 23.36, 28.57, 37. 63, 39.86, 170.5, 201.0 ppm; HRMS found: 175.0663, theory: 175.0667. The preparation of (1-^{13}C) N-acetyl-S-propionylcysteamine has been previously

reported.[19] The yield of N-acetyl-S-cyclopropylcarbonylcysteamine was 76% after chromatography on silica gel with 4:1 ethyl acetate-hexane. N-acetyl-S-cyclopropylcarbonylcysteamine was a colorless oil: ^{1}H NMR δ 1.00 (m, 2H, C-3, C-4 H trans to thiolester), 1.16 (m, 2H, C-3, C-4 H cis to thiolester), 1.97 (s, 3H, $COCH_3$), 2.05 (m, 1H, C-2 H), 3.05 (t, 2H, SCH_2), 3.45 (q, 2H, NCH_2), 6.06 (bs, 1H, NH); ^{13}C NMR δ 11.3, 22.9, 23.4, 28. 7, 40.0, 170.5, 200.1ppm; HRMS found: 187.0666, theory: 187.0667.

The N-acetylcysteamine ester of butyric acid was synthesized by a modification of the method of Dhaon *et al.*[18] Butyric acid (174 mg, 1.97 mmol), N-acetylcysteamine (274 mg, 2.30 mmol), and 4-dimethylaminopyridine (24 mg, 0. 196 mmol) were dissolved in dry CH_2Cl_2 (7 mL) and the solution was cooled to 0 °C under an argon atmosphere. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (422 mg, 2.20 mmol) was added with stirring and the reaction mixture was then stirred on ice for 45 min. The ice bath was removed and the mixture was stirred for an additional hr. The colorless solution was diluted with ethyl acetate (25 mL), and the organic phase was extracted once with water (10 mL) and once with sat. aq. $NaHCO_3$ (10 mL). The washed organic phase was dried over $MgSO_4$ and concentrated in vacuo to give a colorless oil. Purification by silica gel chromatography using 80-85% ethyl acetate in hexane yielded 310 mg (83%) of the pure thiolester as a colorless oil: ^{1}H NMR δ 0.96 (t, 3H, C-4, CH_3), 1.69 (sextet, 2H, C-3 CH_2), 1.98 (s, 3H, COCH3), 2.59 (t, 2H, CH_2CO), 3.03 (t, 2H, SCH_2), 3.42 (q, 2H, NCH_2), 6.34 (bs, 1H, NH); ^{13}C NMR δ 13.3, 19.0, 23.0, 28.2, 39.5, 45.8, 170.3, 199.8 ppm; HRMS found: 189.0826, theory: 189.0824.

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REFERENCES

(1) Ichihara, A.; Shiraishi, K.; Sato, H.; Sakamura, S.; Nishiyama, K.; Sakai, R.; Furusaki, A.; Matsumoto, T. *J. Am. Chem. Soc.* **1977**, *99*, 636-637.

(2) Mitchell, R. E. *Physiol. Plant Pathol.* **1982**, *20*, 83-89.

(3) Mitchell, R. E.; Hale, C.; Shanks, J. *Physiol. Plant Pathol.* **1983**, *23*, 315-322.

(4) Mitchell, R. *Phytochem.* **1978**, *17*, 2028-2029.

(5) Gnanamanickam, S. S.; Starratt, A. N.; Ward, E. W. B. *Can. J. Bot.* **1982**, *60*, 645-650.

(6) Sakai, R. *Ann. Phytopathol. Soc. Jpn.* **1980**, *46*, 499-503.

(7) Feys, B. J. F.; Benedetti, C. E.; Penfold, C. N.; Turner, J. G. *The Plant Cell* **1994**, *6*, 751-759.

(8) Weiler, E. W.; Kutchan, T. M.; Gorba, T.; Brodschelm, W.; Niesel, U.; Bublitz, F. *FEBS Lett.* **1994**, *345*, 9-13.

(9) Parry, R. J.; Mhaskar, S. V.; Lin, M.-T.; Walker, A. E.; Mafoti, R. *Can. J. Chem.* **1994**, *72*, 86-99.

(10) Parry, R. J.; Jiralerspong, S.; Mhaskar, S.; Alemany, L.; Willcott, R. *J. Am. Chem. Soc.* **1996**, *118*, 703-704.

(11) Bender, C.; Liyanage, H.; Palmer, D.; Ullrich, M.; Young, S.; Mitchell, R. *Gene* **1993**, *133*, 31-38.

(12) Ullrich, M.; Bender, C. L. *J. Bacteriol.* **1994**, *176*, 7574-7586.

(13) Parry, R. J.; Patel, J. unpublished observations.
(14) Zhang, J. H.; Quigley, N. B.; Gross, D. C. *J. Bacteriol.* **1995**, *177*, 4009-4020.
(15) Young, S. A.; Park, S. K.; Rodgers, C.; Mitchell, R.; Bender, C. L. *J. Bacteriol.* **1992**, *174*, 1837-1843.
(16) Sambrook, S.; Fritsch, E. F.; Maniatis, T. Molecular Cloning, Second Ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989.
(17) Weissman, K. J.; Smith, C. J.; Hanefield, U.; Aggarwal, R.; Bycroft, M.; Staunton, J.; Leadlay, P. F. *Angew. Chem. Int. Ed.* **1998**, *37*, 1437-1440.
(18) Dhaon, M. K.; Olsen, R. K.; Ramasamy, K. *J. Org. Chem.* **1982**, *47*, 1962-1965.
(19) Lee, M. S.; Qin, G.; Nakanishi, K.; Zagorski, M. G. *J. Am. Chem. Soc.* **1989**, *111*, 6234-6241.
(20) Baldwin, J. E.; Adlington, R.; Rawlings, B. *Tetrahedron Lett.* **1985**, *26*, 481-484.
(21) Laemmli, U. K. *Nature* **1970**, *227*, 680-685.
(22) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248-254.
(23) Dawson, R. M. C.; Elliott, D. C.; Elliott, W. H.; Jones, K. M. Data for Biochemical Research, Third Ed.; Oxford University Press: Oxford, 1986.