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Enzymatic Characterization and Inhibitor Discovery of a New Cystathionine γ-Synthase from *Helicobacter pylori*

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Cystathionine γ-synthase (CGS) catalyses the first step of the transsulfuration pathway that converts L-cysteine to L-homocysteine in bacteria, whereas this pathway is absent in human. In this report, we identified a new *metB* gene from *Helicobacter pylori* strain SS1, and the recombinant *H. pylori* Cystathionine γ-synthase (HpCGS) was successfully cloned, expressed and purified in *Escherichia coli* system. Enzymatic study of HpCGS indicated that the K_m and k_{cat}/K_m values against the substrate *O*-succinyl-L-homoserine (L-OSHS) were 3.02 mM and 98.7 $M^{-1}s^{-1}$, respectively. Moreover, four natural products (α-lapachone, 9-hydroxy-α-lapachone, Paulownin and Yangambin, Fig. 1) were discovered to demonstrate inhibitory activities against HpCGS with IC_{50} values of 11 ± 3, 9 ± 1, 19 ± 2 and 27 ± 6 μM, respectively. All these four inhibitors prevent the binding of L-OSHS to HpCGS in a non-competitive fashion. *In vitro* antibacterial assays further indicated that these four discovered compounds could highly inhibit the growth of *H. pylori* and exhibited strong inhibitory specificity against *H. pylori* related to *E. coli*.

Key words: cystathionine γ-synthase, *Helicobacter pylori*, inhibitor, inhibitor type, minimum inhibition concentration, transsulfuration.

Abbreviations: CGS, cystathionine γ-synthase; HpCGS, *Helicobacter pylori* cystathionine γ-synthase; EcCGS, *E. coli* cystathionine γ-synthase; L-OSHS, *O*-Succinyl-L-homoserine; HO-HxoDH, D-2-Hydroxyisocaproate Dehydrogenase; MIC, minimum inhibition concentration; APPA, DL-E-2-amino-5-phosphono-3-pentenoic acid; PPCA, 3-(phosphonomethyl)pyridine-2-carboxylic acid; CTCPO, 5-carboxymethylthio-3-(3′-chlorophenyl)-1,2,4-oxadiazol; Ni-NTA, nickel-nitrilotriacetic acid; PCR, polymerase chain reaction.

It is known that different organisms display distinct spectra of transsulfuration enzymes. Most plants and microbes employ only the forward pathway from cysteine to homocysteine and methionine, and in mammals only the reverse transsulfuration is carried out, while fungi take up transsulfuration in both directions (*1*). Cystathionine γ-synthase (CGS; EC2.5.1.48), encoded by the *metB* gene, is a pyridoxal 5′-phosphate (PLP)-dependent enzyme responsible for the γ-replacement reaction of an activated form of L-homoserine with L-cysteine, leading to L-cystathionine. For microorganisms, such a reaction is the first step involved in the transsulfuration pathway that converts L-cysteine into L-homocysteine. Since CGS is absent in non-ruminant animals that require a dietary source of L-homocysteine or L-methionine (*2*, *3*), CGS has been regarded as an attractive target for antibiotics discovery (*4*). Based on the research in many species, *O*-succinyl-L-homoserine (L-OSHS) and *O*-acetyl-L-homoserine are discovered to be the substrates of CGS in some bacteria and fungi (*5*, *6*), while *O*-phospho-L-homoserine is the substrate in plants (*7*). Apart from the above physiologically relevant reaction, CGS also catalyses a γ-elimination reaction in the absence of L-cysteine (Scheme 1). It has been found that the γ-elimination of succinate from L-OSHS is common to both the γ-replacement and γ-elimination reactions. The steady-state mechanisms of CGS γ-replacement and γ-elimination reactions in *Escherichia coli* have been thoroughly investigated (*2*). Since it is difficult to use the γ-replacement reaction to detect the inhibition of CGS by a continuous assay (*2*), and L-cysteine is the second substrate binding to the derivative yielded by the first-substrate reaction with the PLP cofactor in γ-replacement reaction (*8*, *9*), we adopted the γ-elimination reaction as an alternative way for investigating CGS kinetics and inhibition.

Recently, bacterial diseases and antibiotic-resistant infections are globally increasing. *Helicobacter pylori* is a gram-negative, microaerophilic, motile, spiral-shaped bacterium, which is associated with a number of human diseases, including gastritis, peptic ulceration and gastric cancer (*10*). *Helicobacter pylori* has been recognized as a pathogenic bacterium that chronically infects about 50% of the world's human population (*11*). It is confirmed that the rapid infection of *H. pylori* is a severe threat to

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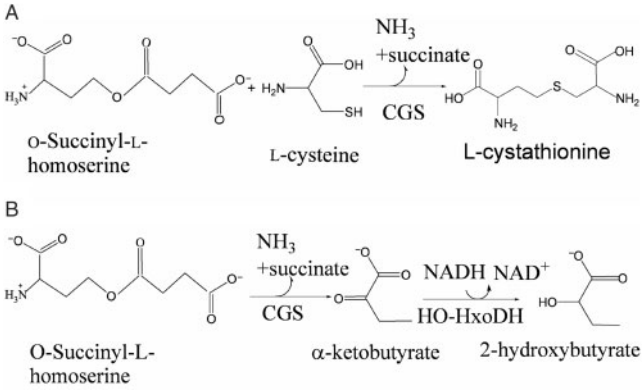

Scheme 1. **γ-Replacement and γ-elimination reactions of CGS.** (A) γ-Replacement reaction of CGS. (B) γ-Elimination reaction of CGS and the continuous coupled spectrophotometric assay involving HO-HxoDH.

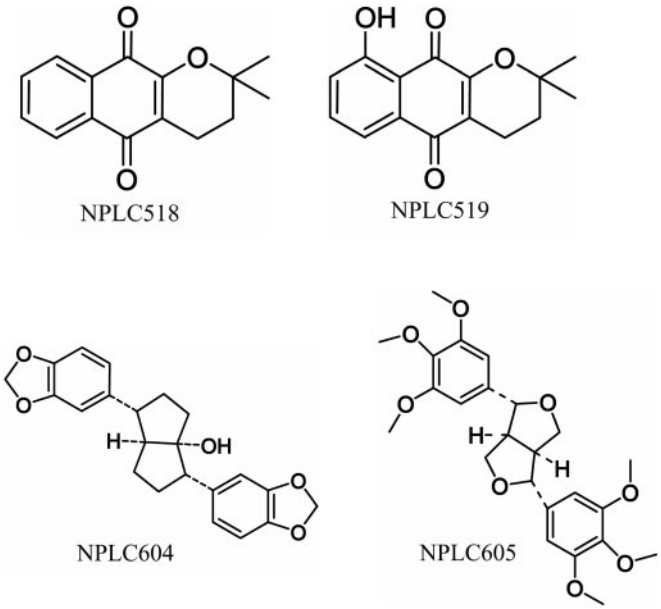

Fig. 1. **Structures of the four discovered HpCGS inhibitors: α-lapachone (NPLC518), 9-hydroxy-α-lapachone (NPLC519) Paulownin (NPLC604) and Yangambin (NPLC605).**

human health. Usually, the treatment of *H. pylori* infections involves the administration of various combinations of several drugs. However, the overuse and misuse of antibacterial agents have resulted in the generation of antibiotic resistant strains. For example, in UK, the metronidazole resistance was found by 31.7% of *H. pylori* isolates (*12*). Accordingly, the alarming rise of antibiotics resistance among the key bacterial pathogens is strongly stimulating an urgency to discover novel antibacterial agents acting on new drug targets. Fortunately, the mapping of the complete genome of *H. pylori* has been accomplished (*13*, *14*), which has provided investigators with powerful tools to identify novel antibacterial targets (*15*, *16*). At the same time, comparison of bacterial target genes with human genes will be also necessary because a good antimicrobial drug target should have the least homologs with mammalian cells to avoid side-effects.

Here we identified a new *metB* gene from *H. pylori* strain SS1 (Genbank™ accession number AY904357). The recombinant *H. pylori* CGS (HpCGS) was cloned, expressed and purified in *E. coli* system, and its enzymatic properties were characterized. Moreover, based on the constructed high-throughput screening platform against the natural product library in our lab, we have successfully screened out four natural products, α-lapachone (NPLC518), 9-hydroxy-α-lapachone (NPLC519), Paulownin (NPLC604) and Yangambin (NPLC605) (Fig. 1), which demonstrated not only strong specific inhibition activities toward HpCGS enzyme, but also the growth of *H. pylori* bacterium *in vitro*. However, it is noticed that Salama *et al*. (*17*) ever reported that *metB* gene seems not to be essential for *H. pylori* growth. Although it is unclear whether or not the inhibition against *H. pylori* from these discovered compounds is through the HpCGS enzyme inhibition, our current work is expected to provide useful information for better understanding the CGS features of *Helicobacter pylori*, and the discovered inhibitors might be used as potential lead compounds in the discovery of anti-*H. pylori* agents.

MATERIALS AND METHODS

Materials—Helicobacter pylori strain SS1 and *E. coli* strain JM109 were maintained in our institute. *Lactobacillus delbrueckii subsp. Bulgaricus* was provided from the Institute of microbiology, CAS. *Escherichia coli* host strain BL21(DE3) and BL21(DE3)pLysS were purchased from Stratagene. The natural products (NPLC518, 519, 604 and 605) were from our lab-established chemical library containing 15,000 compounds. All chemicals used were of reagent grade or ultrapure quality.

Cloning of H. Pylori and E. coli metB—Based on the genome sequences of *H. pylori* strains 26,695 and J99 (Genbank™ Accession Numbers NC_000915 and NC_000921, NCBI), two polymerase chain reaction (PCR) primers: 5′-GGGGCTTTGACTTTGGTGTG-3′ and 5′-CAGCCAGGTTTTAACCATTTTCC-3′ were designed to amplify the corresponding region including *metB* gene on the chromosome of *H. pylori* strain SS1. The genomic DNA of *H. pylori* strain SS1 was prepared as a template by using Genomic DNA Extraction Kit (Sangon). PCR conditions used were: 1 × (94°C 5 min), 30 × (94°C 1 min, 50°C 1 min, 72°C 2 min 22 s), 1 × (72°C 10 min). The amplified DNA segment was purified and subjected to nucleotide sequencing. According to the sequencing result, a pair of PCR primers (sense: 5′-CAGCT CATATGCACATGCAAACCAAA-3′ and antisense: 5′-TATCTCGAGGCTTATTTTAGCGAACG-3′) with *Nde I* and *Xho I* sites underlined, respectively, were synthesized to amplify *metB* gene from *H. pylori* strain SS1. The amplification was performed as follows: 1 × (94°C 5 min), 30 × (94°C 1 min, 55°C 1 min, 72°C 2 min), 1 × (72°C 10 min). The PCR products and vectors [pET-28b(+), Novagen] were digested with the restriction enzymes *Nde I* and *Xho I* (New England Biolabs), ligated

with T4 DNA ligase (New England Biolabs), transformed into *E. coli* strain BL21(DE3). The recombinant clone pET28b-*metB* was sequenced and found to be identical to the sequencing result of PCR products. The nucleotide sequence of *metB* gene from *H. pylori* strain SS1 was submitted to GenBank database under the accession number AY904357.

The PCR primers of *E. coli metB* were (sense: 5′-CAGCTCATATGACGCGTAAACAGGC-3′ and antisense: 5′-TAACTCGAGCCCCTTGTTTGCAGC-3′, with *Nde I* and *Xho I* sites underlined, respectively) synthesized and the cloning process was the same as that of *H. pylori metB*.

Expression and Purification of HpCGS and EcCGS—The *H. pylori* or *E. coli metB* cloning products were cultured in LB media supplemented with 50 μg/ml of kanamycin at 37°C. When the OD_{600} reached around 0.6, the cultures were induced with 0.4 mM IPTG and further incubated for 4 h. Cells were harvested at 5,000 rpm for 30 min, and the resultant pellet was stored at −80°C until further use. The pellet was resuspended in buffer A (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 10 mM imidazole, 10 μM PLP), sonicated on ice, centrifuged at 15,000 rpm for 20 min to yield a clear supernatant, which was loaded onto a column with Ni-NTA resin (Qiagen) pre-equilibrated in Buffer A. The column was washed with Buffer B (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 60 mM imidazole) for several times and eluted with Buffer C (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 120 mM imidazole), the eluted fractions of HpCGS were pooled with 1/10 volume Buffer D (1 M Tris–HCl, pH 9.0, 500 mM NaCl), whereas the eluted fractions of EcCGS were pooled in 1.5 ml Eppendorf tube directly. The purified protein was dialysed against Buffer E (20 mM Tris–HCl, pH 9.0, 500 mM NaCl, 10 μM PLP) to remove imidazole.

Expression and Purification of D-2-Hydroxyisocaproate Dehydrogenase (HO-HxoDH)—The *hdhD* gene encoding HO-HxoDH was cloned from the genomic DNA of *Lactobacillus delbrueckii subsp. Bulgaricus* as the template, which was prepared by using Genomic DNA Extraction Kit (Sangon) with the primers designed to introduce restriction sites: 5′-ACCAGGATCCATG ACTAAAATTGCCA-3′ and 5′-TATACTCGAGCAGGTTA ACGATGCTT-3′, with *BamH I* and *Xho I* sites underlined, respectively. The *hdhD* gene was introduced into the pGEX-4T-1 vector (Amersham) and transformed into *E. coli* strain BL21(DE3)pLysS. The recombinant clone pGEX4T-1-*hdhD* was sequenced and found to be identical to the sequencing result of PCR products. The cloning products were cultured in LB media supplemented with 100 μg/ml ampcillin at 37°C. When the OD_{600} reached about 0.6, the cultures were induced with 0.4 mM IPTG and further incubated for 4 h. Cells were harvested at 5,000 rpm for 30 min, and the resultant pellet was stored at −80°C.

The pellet was resuspended in PBS lysis buffer and sonicated before centrifugation at 15,000*g* for 20 min at 4°C. The lysate was applied to a GSH resin (Amersham) equilibrated with PBS buffer. The resin was washed with PBS followed by buffer containing 40 mM GSH. Protein fractions were concentrated by ultrafiltration.

Mass Spectrometry—During the mass spectrometry assay, the LC/MS system was used, which was a combination of HP1100 (Agilent) LC system with LCQ-DECA mass spectrometer (Thermo Finnigan). The protein sample was injected into the column by an autosampler and separated at a flow rate of 0.2 ml/min. The peptide fraction was detected by PDA (TSP UV6000) and directly introduced on-line into ESI source. The operating condition was optimized with standard solution, and the working parameters of ion source were as follows: capillary temperature, 200°C; spray voltage, 5 kV; capillary voltage, 15 V; and sheath gas flow rate, 20 arb. The scan mass range was from *m/z* 200 to *m/z* 2,000.

Enzymatic Assays—HpCGS or EcCGS activity was evaluated by using the published continuous coupled spectrophotometric assay involving HO-HxoDH (*2*, *18*). In this method, L-OSHS was catalysed by CGS into α-ketobutyrate, followed by the production of 2-hydroxybutyrate with the oxidation of NADH to NAD^+ by HO-HxoDH (Scheme 1). All assays at 25°C were conducted in a 96-well plate system on a Benchmark Plus™ microplate spectrophotometer (BIO-RAD) by measuring the decrease in absorbance at 340 nm ($\varepsilon_M = 6180\ M^{-1}cm^{-1}$). The assay solution contained 20 mM Tris–HCl (pH 9.0), 20 μM PLP, 0.2–4.0 mM L-OSHS, 0.7 mM NADH, 21 μM HO-HxoDH and CGS at desired concentrations. The reaction was initiated by the addition of the diluted HpCGS enzyme. By fitting the data to the Michaelis–Menten equation, the K_m toward L-OSHS was obtained. During the assay of pH effects on HpCGS, the enzymatic activity was measured from pH 5.0 to pH 11, with three different buffers, 20 mM MES (pH 5.0–6.0), 20 mM Tris–HCl (pH 7.0–9.0) and 20 mM Glycine (pH 10–11). While for evaluation of the temperature effects, the enzymatic activity assays were carried out from 25 to 80°C.

Inhibitor Screening—During the inhibitor screening, we used the lab-constructed chemical library containing 15,000 compounds to explore the possible novel small molecular inhibitors against HpCGS enzyme. In the enzyme activity assay, the initial velocities of the enzyme activity were determined with the presence of various concentrations of inhibitors dissolved in dimethyl sulfoxide (Me_2SO). The final Me_2SO concentration in assays with compound concentrations $<10^{-5}$ M was 0.1% (v/v), while with the compound concentrations between 3×10^{-4} and 10^{-5} M was 3% (v/v). HpCGS (0.4 μM) was incubated with varied concentrations of inhibitors for 30 min prior to starting the assays. IC_{50} values of HpCGS were obtained by fitting the data to a sigmoid dose-response equation using Origin software. Inhibitor type and inhibition constants K_i and αK_i were determined by the double-reciprocal (Lineweaver–Burk) plot and plots of the V_{max}s and slopes of the lines from the double-reciprocal plot as a function of inhibitor concentrations. In double-reciprocal plot, the enzyme activity was measured with three or four different concentrations of each inhibitor as a function of the substrate concentration (0.2, 0.3, 0.6, 1.0 and 3.0 mM). The V_{max}s and slopes of the double-reciprocal lines of every inhibitor were thus plotted as

a function of the inhibitor concentrations to gain αK_is and K_is.

During the assay of the possible inhibition of the discovered HpCGS inhibitors against EcCGS, all the experimental conditions and methods were carried out similarly to those for HpCGS inhibitor evaluation.

Antibiotic Susceptibility Tests—Paper Discus Method was used to evaluate the bacterial growth inhibition activity for the compounds. Dimethyl sulfoxide paper was used as negative control and ampicillin paper was used as positive control. The MIC values were determined by the standard agar dilution method using Columbia agar supplemented with 10% sheep blood containing 2-fold serial dilutions of agents. The plates were inoculated with a bacterial suspension (10^8 cfu/ml) in Brain Heart Infusion broth with a multipoint inoculator (Sakuma Seisakusho). Compound-free Columbia agar media were used as controls. Inoculated plates were incubated at 37°C under microaerobic conditions and examined after 3 days. The MIC was generally defined as the lowest concentration of antimicrobial agent that completely inhibits visible bacterial growth.

Surface Plasmon Resonance (SPR) Technology-Based Analysis—The binding affinities of the discovered inhibitors against HpCGS were investigated by Surface Plasmon Resonance (SPR) Technology-Based Biacore 3000 (Biacore Inc.). Protein immobilization, binding affinity assay, and data analysis were carried out according to the pre-existing templates supplied with the instrument's software. The HpCGS enzyme to be covalently coupled to the surface matrix of CM5 sensor chip (Biacore) was diluted in 10 mM sodium acetate buffer (pH 5.0) to a final concentration of 22 μg/ml. Equilibration of the baseline was completed by a continuous flow of HBS-EP running buffer [10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.005% (v/v) surfactant P20, 3% Me_2SO, pH 7.4] through the chip for 1–2 h. Compounds were automatically injected into the flow cells with concentration increased gradually. All the Biacore data were collected at 25°C with HBS-EP as running buffer at a constant flow of 20 μL/min. The association rate (k_{on}), dissociation rate (k_{off}) and equilibrium dissociation (K_D, $K_D = k_{off}/k_{on}$) constants were obtained from sensorgram by fitting to 1:1 Langmuir binding model from the BIAevaluation 3.1 software.

RESULTS

Hp-metB Gene Sequence Analysis—The *metB* gene from *H. pylori* strain SS1 (Hp-*metB*) was successfully cloned based on the available genome sequences of *H. pylori* strains 26,695 and J99, and the recombinant expression plasmid pET28b-HpmetB was generated by inserting the amplified *Hp-metB* fragment into the vector pET28b. The results showed that the *Hp-metB* gene was 1143-bp fragment including the stop codon.

Figure 2 shows the results of the amino acid sequence alignment of HpCGS with CGS enzymes from *E. coli* and *N. tabacum*. It was found that HpCGS shared about 40 and 38% identity to *E. coli* and *N. tabacum* CGS, respectively. HpCGS contains the essential catalytic residues as conserved in the well-characterized *E. coli* and *N. tabacum* CGSs (*1*, *19*). For an apolar residue is needed (*1*), the *H. pylori* Leu323 (*E. coli* Leu327, *N. tabacum* Phe389) is also conserved. As indicated in the CGS monomer crystal structures from *E. coli* and *N. tabacum*, both the structures showed three distinct domains including an N-terminal domain (*E. coli*, residues 1–51; *N. tabacum*, residues 48–109), a large PLP-binding domain (*E. coli*, residues 52–247; *N. tabacum*, residues 110–310) and a C-terminal domain (*E. coli*, residues 248–385; *N. tabacum*, residues 311–445) (*1*, *19*). It seems that HpCGS might also contain these three domains as suggested by the alignment result (Fig. 2). However, as shown in Fig. 2, the Phe35 and Phe38 of EcCGS that are bound to a hydrophobic pocket on the C-terminal domain influence the active site and the cofactor binding (*1*), different from the case for the residues Gln33 and Ile36 of HpCGS, which might make the two enzymes differ in catalytic characters.

Preparation of HpCGS and HO-HxoDH—After directly one-step purification of nickel-affinity chromatography (His-band resin, Novagen), the recombinant HpCGS enzyme, coupled with an N-terminus and C-terminus six-histidine tags, was purified as confirmed by SDS–PAGE (see supporting information Fig. S1). The LC/MS spectrometry gives a 44,250 kDa molecular mass of recombinant HpCGS (see supporting information Fig. S2), in good agreement with the 44,248 kDa predicted from its amino acid sequence. In addition, for EcCGS enzymatic characterization purpose as stated above, we also prepared HO-HxoDH protein, which was obtained by directly one-step purification of glutathione sepharose-4B affinity chromatography (Pharmacia) (see supporting information Fig. S3).

Enzyme Kinetic Analysis—The catalytic properties of HpCGS and the effects of pH and temperature on HpCGS were completely investigated. As shown in Fig. 3A and B, the enzymatic activity of HpCGS increases from pH 5.0 to pH 7.5 and decreases from pH 7.5 to pH 11, while the enzymatic activity gradually increases between 25 and 45°C and decreases from 45 to 80°C.

The kinetic parameters K_m and V_{max} of HpCGS and EcCGS were calculated by varying the substrate concentration and fitting initial velocity data to the Michaelis–Menten equation by non-linear fitting analysis using Origin software (Fig. 3C). For HpCGS, by the continuous coupled spectrophotometric assay, against the substrate L-OSHS, a K_m of 3.02 mM was determined with a specific activity at 403 nmol/min/mg, and k_{cat} was calculated as 0.297 s^{-1} with k_{cat}/K_m at 98.7 $M^{-1}s^{-1}$. For EcCGS, a K_m of 1.29 mM was determined (Fig. 3D), in good agreement with the reported value (1.3 mM) (*2*).

Inhibitor Discovery and Binding Affinity Assay—By screening against our constructed natural compound library, four natural products, α-lapachone (NPLC518), 9-hydroxy-α-lapachone (NPLC519), Paulownin (NPLC604) and Yangambin (NPLC605) (Fig. 1), were identified to show strong inhibitory activities against HpCGS with IC_{50}s at 11 ± 3, 9 ± 1, 19 ± 2 and 27 ± 6 μM, respectively (Table 1). Figure 4 depicts the dose-dependent inhibitions of HpCGS by these four

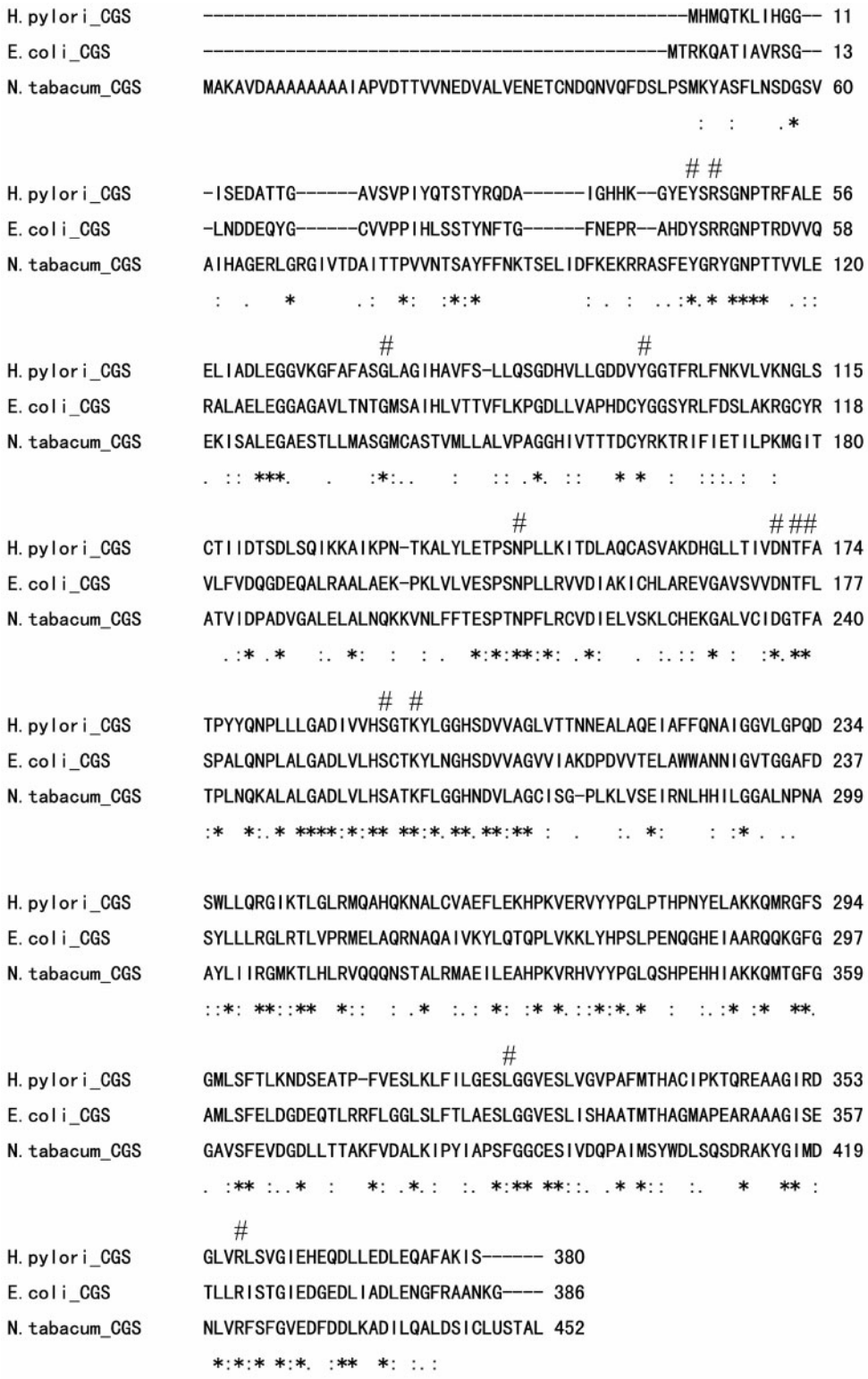

Fig. 2. **Multiple amino acid sequence alignment of *H. pylori* CGS (*H. pylori*_CGS) with *Escherichia coli* CGS (*E. coli*_CGS) and *Nicotiana tabacum* CGS (*N. tabacum*_CGS).** The conserved active site residues are indicated by '#'. Strictly conserved residues are marked with asterisk; conserved substitutions are represented by ':'; the '.' symbol means the semi-conserved substitutions are observed. Alignment was performed by using Clustal W program at http://www.ebi.ac.uk/clustalw/index.html website.

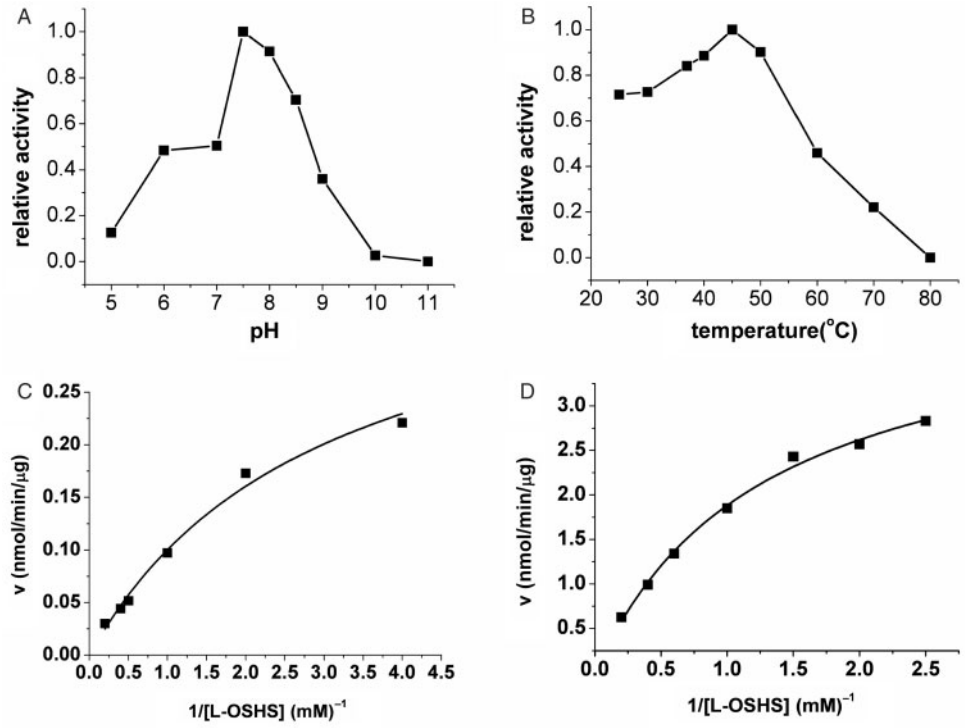

Fig. 3. **Effects of pH (A) and temperature (B) on HpCGS activity and kinetic analysis of HpCGS (C) and EcCGS (D) enzymes.** The initial velocities of the product formation were determined with the increasing concentrations of the substrate L-OSHS. The data were analysed by non-linear curve fitting (Michaelis–Menten equation). The obtained K_m value of HpCGS and EcCGS are 3.02 and 1.29 mM, respectively.

Table 1. **Inhibition data of the four determined HpCGS inhibitors.**

Compound	Inhibition mode	IC_{50} (μM)	K_i (μM)	αK_i (μM)	MIC (μg/ml)
α-Lapachone	Noncompetitive	11 ± 3	19 ± 3	26 ± 4	2.6
9-Hydroxy-α-lapachone	Noncompetitive	9 ± 1	16 ± 2	58 ± 17	2.5
Paulownin	Noncompetitive	19 ± 2	60 ± 4	83 ± 55	15
Yangambin	Noncompetitive	27 ± 6	31 ± 0.3	91 ± 8	10

compounds. Moreover, in a separate control experiment, by directly using 2 mM α-ketobutyrate as the substrate, these four inhibitors showed no any inhibition activities against HO-HxoDH at concentrations even up to 50 μM, further confirming that these four compounds are HpCGS inhibitors. The very low inhibition activity for these four compounds against EcCGS demonstrated their inhibition specificity against HpCGS relative to EcCGS (see supporting information Table S1).

The binding affinities of the determined inhibitors to HpCGS were further investigated by using SPR technology-based Biacore 3000 instrument. As indicated in Fig. 5 and Table 2, these four inhibitors showed moderately binding affinities against HpCGS with equilibrium dissociation constants (K_Ds) at 10.9, 3.4, 17.8 and 27 μM for NPLC518, 519, 604 and 605, respectively.

Anti-Bacterial Assay—These four discovered inhibitors were also applied to the antibacterical activity assay against *H. pylori* and *E. coli*. The results showed that all the inhibitors, NPLC518, 519, 604 and 605, displayed significant activities in the inhibition of *H. pylori* strain SS1 growth with MICs of 2.6, 2.5, 15 and 10 μg/ml (Table 1), respectively, but exhibited no any antibacterical activities in *E. coli* strain JM109 growth. These results thus further confirmed the highly inhibitory specificity against HpCGS for the four discovered inhibitors.

Inhibitor Type Characterization—To further inspect the inhibition modes of the discovered inhibitors against HpCGS, we used Lineweaver–Burk plot for determining the inhibitor type. During the assay, each inhibitor was preincubated with HpCGS enzyme for 30 min at room temperature. In Lineweaver–Burk plot method, we chose five different concentrations of the substrate L-OSHS (0.2, 0.3, 0.6, 1.0 and 3.0 mM) to measure the enzyme activity. The inhibitor concentrations were: for NPLC518 at 0, 5 and 10 μM; for NPLC519 at 0, 7, 8 and 9 μM, for NPLC604 at 0, 20, 30 and 40 μM and for NPLC605 at 0, 30 and 50 μM (Fig. 6). It is discovered that all the four inhibitors prevented the binding of L-OSHS

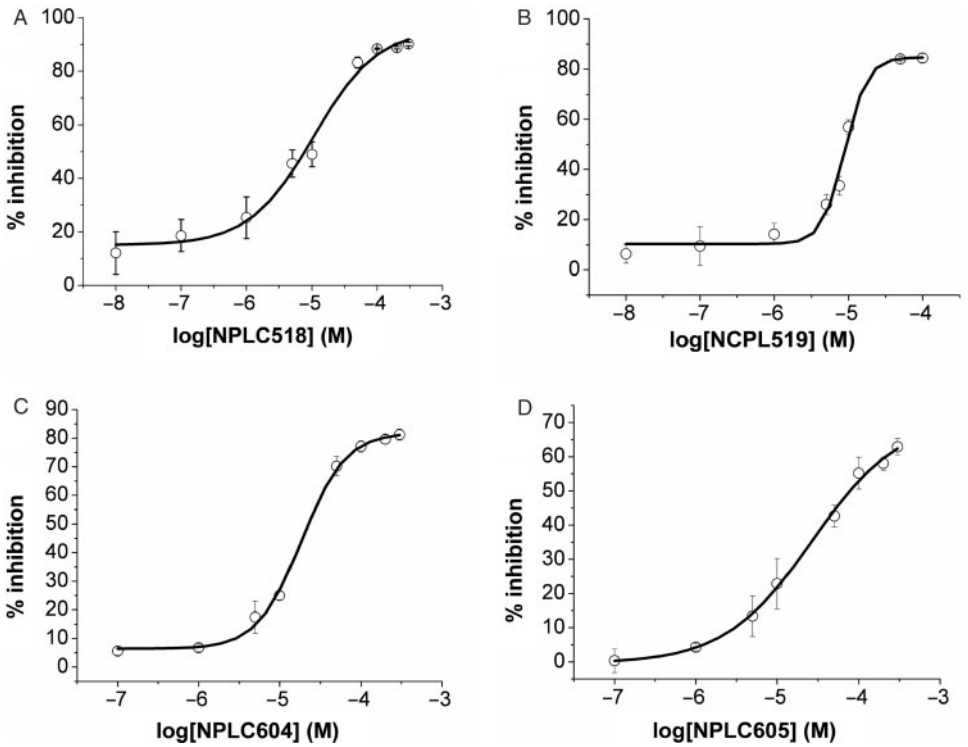

Fig. 4. **Dose-response curves of enzyme inhibition.** (A) NPLC518 ($IC_{50} = 11 \pm 3\,\mu M$); (B) NPLC519 ($IC_{50} = 9 \pm 1\,\mu M$); (C) NPLC604 ($IC_{50} = 19 \pm 2\,\mu M$); (D) NPLC605 ($IC_{50} = 27 \pm 6\,\mu M$).

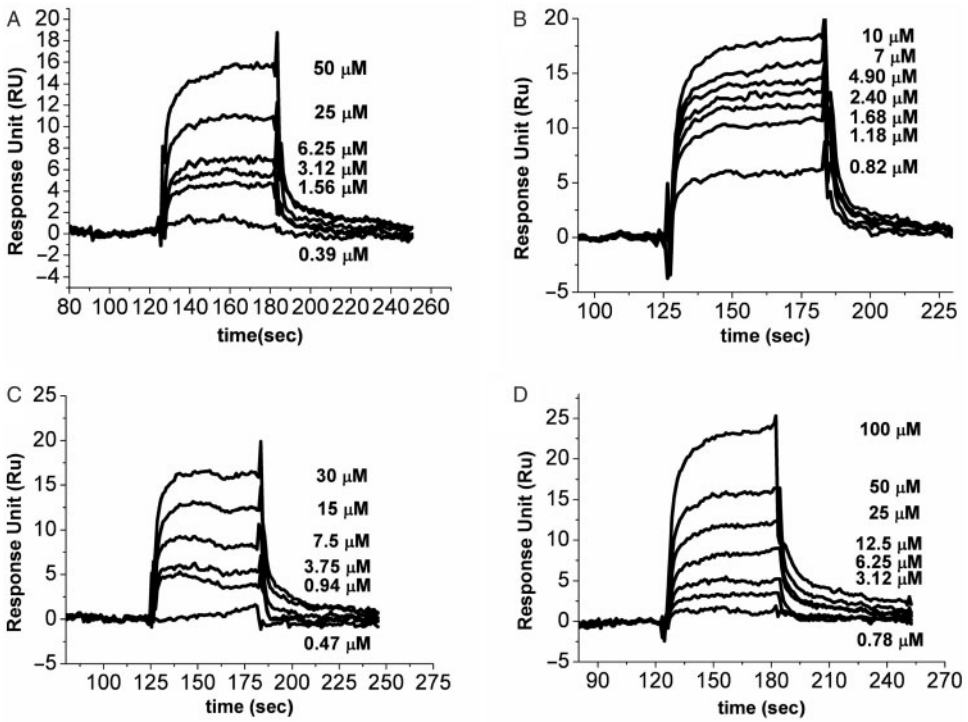

Fig. 5. **Representative sensorgrams of the four discovered inhibitors binding to HpCGS as investigated by SPR technology-based Biacore 3000.** (A) NPLC518, with concentrations at 50, 25, 6.25, 3.12, 1.56 and 0.39 μM; (B) NPLC519, with concentrations at 10, 7, 4.9, 2.40, 1.68, 1.18 and 0.82 μM; (C) NPLC604, with concentrations at 30, 15, 7.5, 3.75, 0.94 and 0.47 μM; (D) NPLC605, with concentrations at 100, 50, 25, 12.5, 6.25, 3.12 and 0.78 μM. Association was monitored for 180 s, and dissociation was monitored for more than 150 s. The 1:1 Langmuir binding model was used for kinetic parameter determination.

to HpCGS in a noncompetitive fashion. In addition, by using the secondary plot in which the V_{max}s and slopes of the double-reciprocal lines were plotted as a function of the inhibitor concentrations, the αK_i and K_i values for the four inhibitors NPLC518, 519, 604 and 605 could be thus evaluated (Table 1).

DISCUSSION

Recently, the rapid infection of *H. pylori* has become a severe threat to human health, and the discovery of new effective drugs has attracted more and more attention. The genome study of *H. pylori* has confirmed the presence of the *metB*-encoded CGS, whereas this gene lacks in mammals.

In the current work, we applied the HpCGS γ-elimination reaction with a continuous coupled spectrophotometric assay involving HO-HxoDH (Scheme 1) to study the HpCGS kinetics and inhibition. The rate of NADH reduction to NAD^+ could be recorded in spectral absorbance. The enzymatic results showed that the optimum temperature for this reaction is 45°C, and the optimum pH is 7.5, similar to that of CGS from spinach leaves (*Spinacia oleracea L.*) (*20*), and lower than that of *Salmonella* CGS (pH 8.2) (*21*). HpCGS was active with K_m at 3.02 mM and k_{cat} 0.297 s^{-1} toward the substrate L-OSHS. Such a K_m is around 2-fold greater and the k_{cat} value is 6-fold smaller than the corresponding reported values of *E. coli* CGS, and k_{cat}/K_m is thus reduced by approximately 1 order of magnitude (*2*). It is suggested that such a difference in the specific activity may be attributed to the different analysis systems or the growth rate of the individual organisms. Faster growth rate of *E. coli* would require more efficient homocysteine synthesis, while the much slower growth rate of *H. pylori* would reduce the need for rapid homocysteine synthesis, allowing for the HpCGS involving much lower specific activity (*22*).

In our work, four natural inhibitors of HpCGS, α-lapachone, 9-hydroxy-α-lapachone, Paulownin, and

Table 2. **Summary of the kinetic parameters of the four determined inhibitors binding to HpCGS evaluated by SPR technology based Biacore 3000[a].**

Compound	k_{on} (1/Ms)	k_{off} (1/s)	K_D (μM)
α-Lapachone	2620	0.028	10.9
9-Hydroxy-α-lapachone	20 300	0.0689	3.4
Paulownin	2280	0.0405	17.8
Yangambin	889	0.024	27.0

[a]The association rate (k_{on}), dissociation rate (k_{off}), and equilibrium dissociation (K_D, $K_D = k_{off}/k_{on}$) constants were determined from the sensorgrams (Fig. 5) by fitting to the 1:1 Langmuir binding model from the BIAevaluation 3.1 software.

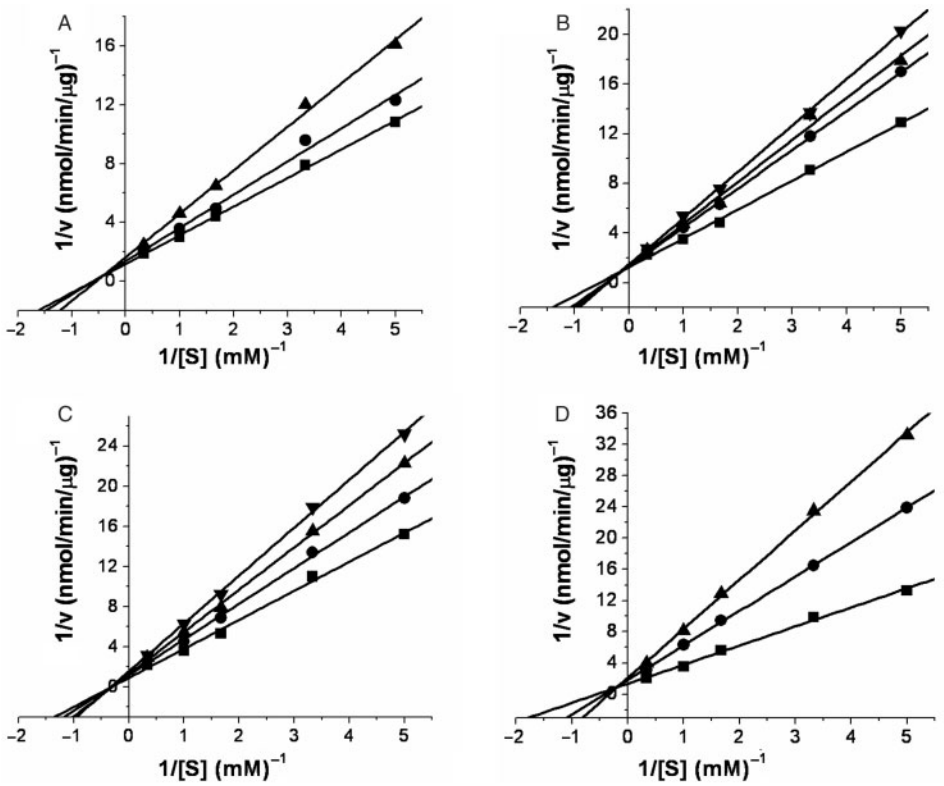

Fig. 6. **Inhibition type determination of the four determined inhibitors.** (A) NPLC518, with concentrations at 0 (filled square), 5(filled circle) and 10 (filled traingle) μM; (B) NPLC519, with concentrations at 0 (filled square), 7 (filled circle), 8 (filled traingle) and 9 (filled inverted triangle) μM; (C) NPLC604, with concentrations at 0 (filled square), 20 (filled circle), 30 (filled triangle) and 40 (filled inverted triangle) μM. (D) NPLC605, with concentrations at 0 (filled square), 30 (filled circle) and 50 (filled triangle) μM. For all assays, L-OSHS was used as the substrate and the lines are discovered to be the best fit to the non-competitive inhibition.

Yangambin, were discovered. They all prevented the binding of L-OSHS to HpCGS in a non-competitive fashion. Moreover, it is discovered that these four compounds exhibited very low inhibition activities against EcCGS, implying their inhibition specificity against HpCGS relative to EcCGS.

Recently, there are reports regarding CGS inhibitor exploration. For example, three *Nicotiana tabacum* CGS inhibitors, DL-E-2-amino-5-phosphono-3-pentenoic acid (APPA), 3-(phosphonomethyl)pyridine-2-carboxylic acid (PPCA) and 5-carboxymethylthio-3-(3′-chlorophenyl)-1,2,4-oxadiazol (CTCPO), have been described (*8*). APPA, mimicking the substrate *O*-phospho-L-homoserine, is a competitive inhibitor with its affinity ($K_i = 27\,\mu M$) (*23*) lower than those of the compounds α-lapachone ($K_i = 19\,\mu M$) and 9-hydroxy-α-lapachone ($K_i = 16\,\mu M$) discovered in our work, and PPCA has much lower affinity ($K_i = 200\,\mu M$) compared with the four discovered inhibitors in our work. CTCPO was identified to be a new competitive inhibitor of *Nicotiana tabacum* CGS with high affinity ($K_i = 2\,\mu M$). However, this compound is an inhibitor against plant CGS and has not been identified to exert phytotoxic effect. An irreversible inhibitor Propargylglycine, produced from *Streptomycetes* (*24*), is a potent inhibitor of CGS with some phytotoxic effect (*25*). Two synthesized CGS inhibitors, 4-(phosphonomethyl)-pyridine-2-carboxylic acid and Z-3-(2-phosphonoethen-1-yl)pyridine-1-acetic acid (*26*), are reversible competitive inhibitors with low affinities ($K_i = 45$ and $40\,\mu M$, respectively) (*27*). Although several kinds of CGS inhibitors have been found, there are few reports involving their strong antibacterial activities. Therefore, it is still significant to search for potent CGS inhibitors with high antibacterial activity.

More importantly, further research has indicated that all these four inhibitors demonstrated strong antibacterial activities against *H. pylori* in vitro whereas no antibacterial activities against *E. coli* in vitro. To our knowledge, these four determined inhibitors might be the firstly discovered natural active compounds acting at CGS enzyme. However, it is noticed that *Hp-metB* gene was suggested to be not essential for *H. pylori* growth as reported by Salama *et al*. (*17*) using a genome-saturating mutant library of *Helicobacter pylori* and MATT (microarray tracking of transposon mutants) technique. Although it is uncertain whether or not there are other targeting enzymes or proteins for these discovered four natural products besides HpCGS enzyme, their strongly specific anti-*H. plori* activities might supply useful information for providing potential inhibitor structure information, and the four discovered inhibitors could obviously be used as potential lead compounds for further research. Moreover, our current work is also expected to help better understand the CGS features of *H. pylori* strain.

In conclusion, we have cloned, expressed and purified a novel CGS enzyme from *H. pylori*. The biochemical characterization of HpCGS was also investigated. By high-throughput screening methodology, we have discovered and characterized four natural inhibitors of HpCGS enzyme, which demonstrated strong inhibitory activities against *H. pylori*. Representing two new chemical scaffolds, these four determined inhibitors could be available for further chemical modification in the development of CGS inhibitors with better specificity and antibacterial activity.

Supplementary data are available at JB online.

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