Pantothenic acid (vitamin B₅) is the natural precursor of coenzyme A (CoA), an essential cofactor in all organisms. The pantothenic acid antimetabolite N-pentylpantothenamide inhibits the growth of *Escherichia coli* with a minimum inhibitory concentration of 2 μM. In this study, we examine the mechanism of this inhibition. Using the last five enzymes of the CoA biosynthetic pathway in *E. coli* we demonstrate that N-pentylpantothenamide does not inhibit the CoA biosynthetic enzymes but instead acts as an alternative substrate, forming the CoA analog ethyldethia-CoA. We show that N-pentylpantothenamide is converted to ethyldethia-CoA 10.5 times faster than CoA is biosynthesized from acetyl-CoA-utilizing enzymes. This mechanism of toxicity of N-pentylpantothenamide is most likely due to its biosynthetic conversion to the CoA analog ethyldethia-CoA, which may act as an inhibitor of CoA- and acetyl-CoA-utilizing enzymes.

Coenzyme A (CoA)¹ 1a is an essential cofactor in numerous metabolic pathways involving Claisen/aldol condensations and acyl substitutions, while the 4'-phosphopantetheine moiety of CoA is used as a covalently bound cofactor in fatty acid, polyketide, and non-ribosomal peptide biosynthesis. Because of this metabolic centrality, the development of new antibiotics, polyketide, and non-ribosomal peptide biosynthesis. Because of CoA is used as a covalently bound cofactor in fatty acid, acyl substitutions, while the 4'-pentylpantothenamide inhibits the growth of *Escherichia coli* with a minimum inhibitory concentration of 2 μM. In this study, we examine the mechanism of this inhibition. Using the last five enzymes of the CoA biosynthetic pathway in *E. coli* we demonstrate that N-pentylpantothenamide does not inhibit the CoA biosynthetic enzymes but instead acts as an alternative substrate, forming the CoA analog ethyldethia-CoA. We show that N-pentylpantothenamide is converted to ethyldethia-CoA 10.5 times faster than CoA is biosynthesized from acetyl-CoA-utilizing enzymes. This mechanism of toxicity of N-pentylpantothenamide is most likely due to its biosynthetic conversion to the CoA analog ethyldethia-CoA, which may act as an inhibitor of CoA- and acetyl-CoA-utilizing enzymes.

The Antibiotic Activity of N-Pentylpantothenamide Results from Its Conversion to Ethyldethia-Coenzyme A, a Coenzyme A Antimetabolite*

Erick Strauss and Tadhg P. Begley‡

From the Department of Chemistry and Chemical Biology, Baker Laboratory, Cornell University, Ithaca, New York, 14853

Pantothenic acid (vitamin B₅) is the natural precursor of coenzyme A (CoA), an essential cofactor in all organisms. The pantothenic acid antimetabolite N-pentylpantothenamide inhibits the growth of *Escherichia coli* with a minimum inhibitory concentration of 2 μM. In this study, we examine the mechanism of this inhibition. Using the last five enzymes of the CoA biosynthetic pathway in *E. coli* we demonstrate that N-pentylpantothenamide does not inhibit the CoA biosynthetic enzymes but instead acts as an alternative substrate, forming the CoA analog ethyldethia-CoA. We show that N-pentylpantothenamide is converted to ethyldethia-CoA 10.5 times faster than CoA is biosynthesized from acetyl-CoA-utilizing enzymes. This mechanism of toxicity of N-pentylpantothenamide is most likely due to its biosynthetic conversion to the CoA analog ethyldethia-CoA, which may act as an inhibitor of CoA- and acetyl-CoA-utilizing enzymes.

Coenzyme A (CoA)¹ 1a is an essential cofactor in numerous metabolic pathways involving Claisen/aldol condensations and acyl substitutions, while the 4'-phosphopantetheine moiety of CoA is used as a covalently bound cofactor in fatty acid, polyketide, and non-ribosomal peptide biosynthesis. Because of this metabolic centrality, the development of new antibiotics designed as inhibitors of CoA-utilizing enzymes (i.e. as CoA antivitamins or antimetabolites) may be feasible. However, while several CoA analogs have been characterized as inhibitors of CoA-utilizing enzymes in *vitro* (1), these analogs are not useful as antibiotics because bacteria are unable to transport CoA across the cell membrane (2, 3). Therefore the design of CoA antimetabolites must be based on pantothenic acid 2a, the most advanced cell-permeable biosynthetic intermediate (4, 5), or its precursors (see Fig. 1). Two pantothenic acid analogs have been described previously: pantoyltaurine, in which the carbonyl of pantothenic acid 2a is replaced by a sulfonate, does not inhibit the growth of *Escherichia coli* because it is not transported by pantothenate permease (6); and N-pentylpantothenamide 2b, which inhibits *E. coli* with a minimum inhibitory concentration (MIC) of ~2 μM (7).

In this study we considered two mechanisms for the antibiotic activity of N-pentylpantothenamide 2b. In the first mechanism, inhibition of CoA biosynthesis by N-pentylpantothenamide 2b or one of its metabolites would result in a decreased amount of CoA being formed in the cell and thus would retard bacterial cell growth. In the second mechanism, we considered the possibility that 2b is biosynthetically converted to the CoA analog ethyldethia-CoA, which might function as a CoA antimetabolite and inhibit CoA and acetyl-CoA-utilizing enzymes. Since the five enzymes required for the conversion of pantothenic acid to CoA (Fig. 1) have all recently been overexpressed and characterized (8–11), we were able to differentiate between these two mechanisms in this paper.

**EXPERIMENTAL PROCEDURES**

**Materials**

**General—**All chemicals and buffer components were purchased from Aldrich, Sigma or Fisher Scientific and used without further purification. 4'-Phosphopantetheine 5 was synthesized as previously described (12). 1H NMR spectra were measured on a Varian INOVA 400 MHz instrument. All HPLC analyses were performed on a HP series 1100 HPLC system with HPLC grade solvents using a Supelcosil LC-18-T 3 μm, 15 cm × 4.6 mm ID column (Supelco). ESI-MS analyses were performed at the Cornell Biotechnology Resource Center on a Bruker Esquire-LC ESI-ion trap mass spectrometer by direct infusion of the analyte mixture into the instrument at a rate of 1 μl/min.

**Synthesis of N-Pentylpantothenamide (2b)—** Sodium pantothenate (2.0 g, 8.3 mmol) was dissolved in deionized water, and the solution was washed through an Amberlite IR-120 (H⁺) column. The column was subsequently washed with two volumes with deionized water, and the combined eluates were lyophilized. The free acid obtained in this manner was dissolved in dry dimethyl formamide (10 ml), and amylamine (1.16 ml, 10 mmol) and diphenylphosphoryl azide (2.24 ml, 15 mmol) were added. The solution was cooled to 0 °C and triethylamine (1.39 ml, 10 mmol) was added. The solution was stirred at 0 °C for 2 h, followed by stirring at room temperature overnight. The volume of solvent was reduced by rotary evaporation with external heating, and the residue applied to a short silica gel column, eluting with 95:5 ethyl acetate:hexane and lyophilized to give 2b as a colorless oil. The oil was dissolved in deionized water and lyophilized to give 2b as a white powder (1.56 g, 65% yield).

**Synthesis of N-Pentylpantothenamide (2b)—** Sodium pantothenate (2.0 g, 8.3 mmol) was dissolved in deionized water, and the solution was washed through an Amberlite IR-120 (H⁺) column. The column was subsequently washed with two volumes with deionized water, and the combined eluates were lyophilized. The free acid obtained in this manner was dissolved in dry dimethyl formamide (10 ml), and amylamine (1.16 ml, 10 mmol) and diphenylphosphoryl azide (2.24 ml, 15 mmol) were added. The solution was cooled to 0 °C and triethylamine (1.39 ml, 10 mmol) was added. The solution was stirred at 0 °C for 2 h.

---

¹ This work was funded by grants from the Petroleum Research Foundation and from GlaxoSmithKline. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Chemistry and Chemical Biology, 120 Baker Laboratory, Cornell University, Ithaca, NY 14853–1401. Tel.: 607-255-7133; Fax: 607-255-4137; E-mail: tpb2@cornell.edu.

The abbreviations used are: CoA, coenzyme A; MIC, minimum inhibitory concentration; HPLC, high performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; CoA, pantothenate kinase; CoaBC, phosphopantothenoylcysteine synthetase/decarboxylase; CoaD, phosphopantetheine adenylytransferase; CoaE, dephospho-CoA kinase; DTT, dithiothreitol.
Antibiotic Activity of N-Pentylpantothenamide

**Fig. 1.** Biosynthetic conversion of pantothenic acid (2a) and its analog N-pentylpantothenamide (2b) to coenzyme A (1a) and ethyldethia-coenzyme A (1b) as catalyzed by the *E. coli* coenzyme A biosynthetic enzymes, CoaABCDE.

was filtered and added drop-wise to a solution of 2b (577 mg, 2.0 mmol) in dry pyridine (9 ml) at −40 °C (dry ice/acetonitrile). After stirring at −40 °C for 2 h, the mixture was placed in a −20 °C freezer overnight. The product was removed in vacuo and ethyl acetate (25 ml) was added. The resulting suspension was washed with 1 M H2SO4 (2 ml) and saturated Na2SO4 (1 ml), and dried (Na2SO4), and then the solvent was removed. The product was purified by flash column chromatography on silica gel with 95:5 CH2Cl2/methanol, and the fractions containing 7 were collected and concentrated in vacuo to give the product as a colorless oil (271 mg, 25% yield). 1H NMR (400 MHz, CDCl3): 8.45 (s, 1H), 7.25 (t, 1H), 7.15 (t, 1H), 3.98 (dd, 1H), 3.86 (s, 1H), 3.53 (m, 3H), 3.19 (dt, 2H), 2.41 (m, 2H), 1.46 (m, 2H), 1.27 (m, 4H), 1.04 (s, 3H), 0.86 (t, 3H), 0.78 (s, 3H).

**Synthesis of 4'-Phospho-N-pentylpantothenamide (3b)**—Pd/C (10%, 20 mg) was added to a solution of 7 (200 mg, 365 μmol) in 9:1 methanol/CHCl3/methanol, and the fractions containing 7 were collected and concentrated in vacuo to give 3b as a clear glass (110 mg, 82% yield) for 2 h. The solution was filtered, and the solvent removed in vacuo to give 3b as a colorless oil (110 mg, 82% yield). The product was dissolved in H2O, titrated to pH ~6.0 with 1 M NaOH and stored as frozen aliquots of a stock solution (60 μM) at −20 °C. 1H NMR (400 MHz, D2O): 8.35 (s, 1H), 3.63 (dd, 1H), 3.39 (dd, 1H), 2.95 (t, 2H), 2.76 (t, 2H), 1.28 (m, 2H), 1.08 (m, 4H), 0.77 (s, 3H), 0.69 (s, 3H), 0.67 (t, 3H).

**Measurement of the Minimal Inhibitory Concentration:**

**Growth Curves**

Minimal medium (0.8 mM MgSO4, 10.0 mM citric acid, 60 mM K2HPO4, 20 mM NaH2PO4, 0.5% glucose) (50 ml) was inoculated with 50 μl of an overnight culture of *E. coli* B and subsequently grown at 37 °C in the presence of varying concentrations of 2b. The A600 was periodically determined until the stationary growth phase was reached in each respective culture. The minimal concentration of 2b that retarded the onset of logarithmic growth under these conditions was regarded as the minimal inhibitory concentration (MIC).

**Enzyme Purification**

**CoaA and CoaBC**—Pure pantothenate kinase from *E. coli* (CoaA) was a gift from Suzanne Jackowski. Phosphopantothenoylcysteine synthetase/decarboxylase from *E. coli* (CoaBC) was purified as previously described.

**CoaD**—Phosphopantetheine adenylyltransferase from *E. coli* (CoaD) has previously been overexpressed from plasmid pUC/CoaD (a gift from D. Drueckhammer) and purified (8). However, as this plasmid did not encode a His-tagged protein, pUC/CoaD was treated with Ndel and BamHI, and the resulting coaD-containing fragment was cloned into Ndel/BamHI-digested pET28a expression vector (Novagen). The sequence of the resulting plasmid, designated pESC106, was verified by automated DNA sequencing (Cornell Biotechnology Resource Center) and was transformed into *E. coli* BL21(DE3) (Novagen). To obtain pure protein *E. coli* BL21(DE3) pESC106 was grown at 37 °C in 500 ml of LB broth supplemented with 15 μg/ml kanamycin sulfate to A600 ~0.6 and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. After growing overnight at 37 °C, the cells were harvested, suspended in sonication buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9, 10 mg/ml cell paste), disrupted by sonication, and centrifuged at 35,000 × g for 30 min to clarify the cell-free extract. This was applied to a 2-ml His-Bind column (Novagen). Weakly bound proteins were removed by washing with sonication buffer, followed by sonication buffer containing 60 mM imidazole. CoaD was eluted by increasing the imidazole concentration to 0.5 M. The chromatography was monitored at A280.

**CoaE**—Dephospho-CoA kinase from *E. coli* (CoaE) has previously been overexpressed as a non-fusion protein using pET/coaE (a gift from D. Drueckhammer) (11). To obtain the His-tagged protein, the coaE (previously yocE) gene was amplified by PCR using as the forward primer: 5'-CCGGGAAAATAGATATAGAGGTATAATATTTGCC-3', to introduce an Ndel site (underlined) at the start of the gene, and as the reverse primer: 5'-AAAGGACCTGATCCGATTTACGG-3' to introduce a BamHI site (underlined) at the end of the gene. The resulting PCR product was cloned into Ndel/BamHI-digested pET28a expression vector (Novagen). The sequence of the resulting plasmid, designated pESC124, was verified by automated DNA sequencing (Cornell Biotech-
Biosynthesis and Analysis of Coenzyme A Analogs

HPLC Conditions—HPLC analysis of reaction mixtures containing CoA analogs was performed using 100 mM potassium phosphate buffer, pH 6.6, containing increasing amounts of methanol as follows: 0–3 min, isocratic, 5% MeOH; 3–5 min, 5–10% MeOH; 5–10 min, 10–15% MeOH; 10–13 min, 15–30% MeOH; 13–26 min, isocratic, 30% MeOH. Chromatography was monitored at 254 nm. Retention times: CTP, CDP, CMP, 1.6–1.9 min; ATP, ADP, AMP, 2.3–3.0 min; coenzyme A (1a), 12.0 min; 3′-dephospho-coenzyme A (6a), 16.6 min; ethyldethia-coenzyme A (1b), 19.7 min; and 3′-dephospho-ethyldethia-coenzyme A (6b), 26.6 min.

HPLC Analysis of Biosynthetic Products—Each 150-μl reaction mixture contained ATP (5.0 mM), CTP (1.0 mM), t-cysteine (1.0 mM), DTT (2.0 mM), MgCl₂ (5.0 mM), CoaA (15 μM), MgCl₂ (5.0 mM), CoaA (15 μM), and CoaE (15 μM) in 50 mM Tris-HCl buffer (pH 7.6). Reactions were initiated by addition of the natural substrate 2a (0.5 mM), the analog 2b (0.5 mM), or by addition of water to the negative control samples. Reactions were incubated for 20 min at 37 °C and stopped by transferring the reaction to 95 °C for 5 min, and the precipitated protein was removed by centrifugation (13,000 rpm × 5 min). The supernatant was loaded onto a DEAE-cellulose column (1 × 25 cm) pre-equilibrated with NH₄HCO₃ (50 mM), and the column was eluted with a 600-mM gradient of NH₄HCO₃ (50–300 mM). The chromatography was monitored at A₂₅₄. The product eluted as the last fraction from the column at ~180 mM NH₄HCO₃.

Biosynthesis and Purification of Ethyldethia-CoA (1b)—A 600-μl reaction mixture contained 3b (10.5 mM), ATP (20 mM), MgCl₂ (5.0 mM), CoaD (100 μg) and CoaE (150 μg) in 50 mM Tris-HCl buffer (pH 7.6). Reactions were initiated by addition of the biosynthetic enzymes, incubated for 2 h at 37 °C and stopped by transferring the reaction to 95 °C for 5 min, and the precipitated protein was removed by centrifugation (13,000 rpm × 5 min). The supernatant was loaded onto a DEAE-cellulose column (1 × 25 cm) pre-equilibrated with NH₄HCO₃ (50 mM), and the column was eluted with a 600-mM gradient of NH₄HCO₃ (50–300 mM). The chromatography was monitored at A₂₅₄. The product eluted as the last fraction from the column at ~180 mM NH₄HCO₃.

Biosynthesis and Purification of 3′-Dephyso-ethyldethia-coenzyme A (6b)—A 600-μl reaction mixture contained 3b (10.5 mM), ATP (20 mM), MgCl₂ (5.0 mM), CoaD (100 μg) and inorganic pyrophosphatase (2 units) in 50 mM Tris-HCl buffer (pH 7.6). Reactions were initiated by addition of the biosynthetic enzymes. Four identical reaction mixtures were treated as for the purification of 1b, their supernatants combined and loaded onto a single DEAE-cellulose column (1 × 25 cm) pre-equilibrated with NH₄HCO₃ (50 mM). The column was eluted with a 500-mM gradient of NH₄HCO₃ (50–250 mM). The chromatography was monitored at A₂₅₄. The product eluted as the first fraction from the column at ~90 mM NH₄HCO₃. The product-containing fractions were combined and lyophilized, dissolved in water and lyophilized again. This was repeated until a constant weight of product was achieved. Yield: 14.8 mg (di-ammonium salt) (84%). 1H NMR (400 MHz, D₂O): δ 8.33 (s, 1H), 8.07 (s, 1H), 5.97 (d, 1H), 4.39 (s, 1H), 4.04 (s, 1H), 3.81 (s, 1H), 3.61 (m, 3H), 3.35 (m, 1H), 3.26 (m, 2H), 2.93 (m, 1H), 2.88 (t, 2H), 2.23 (t, 2H), 2.12 (m, 2H), 1.04 (m, 4H), 0.67 (s, 3H), 0.62 (t, 3H), 0.55 (s, 3H). [M + H]⁺ calculated for C₁₆H₁₄N₂O₃P₂ m/z 778.2, found 778.1; [M − ADP]⁺ = 271.2.

Antibiotic Activity of N-Pentylpantothenamide

FIG. 2. Synthetic scheme for the production of the pantothenic acid analog N-pentylpantothenamide (2b) and its 4′-phosphate (3b).

FIG. 3. Growth curves for E. coli B in minimal medium at 37 °C containing increasing amounts of the pantothenic acid analog N-pentylpantothenamide (2b). Concentrations of 2b used: □, no inhibitor; □, 1 μM; □, 2 μM; □, 3 μM; ▲, 15 μM.

FIG. 4. HPLC chromatograms showing the formation of coenzyme A (1a) and ethyldethia-coenzyme A (1b) by action of CoaABCDE on the natural biosynthetic precursor (2a) and its analog (2b). A. analysis of a reaction mixture containing 2a. B. analysis of a reaction mixture containing 2b. C. a control reaction mixture without either substrate. Identified peaks: 1) cytidine 5′-phosphates, 2) adenosine 5′-phosphates, 3) coenzyme A (1a), 4) dephyso-coenzyme A (6a), and 5) ethyldethia-coenzyme A (1b).
Antibiotic Activity of N-Pentylpantothenamide

**Enzyme Assays**

*General—All enzyme assays were based on the decrease of NADH concentration, as monitored by changes in absorbance at 340 nm. An extinction coefficient of 6220 M⁻¹ cm⁻¹ was used for NADH. Reactions were performed at 25 °C in a Hitachi U-2010 Spectrophotometer. Kinetic parameters were determined by fitting the obtained data to the Michaelis-Menten equation using Origin 6.0 (Microcal).*

**CoaA—** Pantothenate kinase activity was determined using a continuous spectrophotometric assay that coupled the production of ADP to the consumption of NADH. Each 500-µl reaction mixture contained ATP (1.5 mM), NADH (0.3 mM), phospho(enol)pyruvate (0.5 mM), MgCl₂ (10 mM), KCl (20 mM), and CoaA (5 µg) in 50 mM Tris-HCl buffer (pH 7.6). The reaction was initiated by addition of substrate (2a or 2b, at concentrations between 10 and 160 µM).

**CoaBC—** The phosphopantothenoylcysteine synthetase activity of CoaBC was assayed by the method of O’Brien (13) using the pyrophosphate detection kit available from Sigma (catalog no. P7276) that couples the production of pyrophosphate to the consumption of NADH. Each 500-µl reaction mixture contained 200 µl of pyrophosphate reagent, ATP (1.5 mM), NADH (0.3 mM), phospho(enol)pyruvate (0.5 mM), MgCl₂ (10 mM), KCl (20 mM), and CoaB-C (25 µg) in 50 mM Tris-HCl buffer (pH 7.6). The reaction was initiated by addition of substrate (3a or 3b, at concentrations between 10 and 250 µM).

**CoaD—Phosphopantetheine adenylyltransferase activity** was assayed by the method of O’Brien (13) using the pyrophosphate detection kit available from Sigma (catalog no. P7276) that couples the production of pyrophosphate to the consumption of NADH. Each 500-µl reaction mixture contained 200 µl of pyrophosphate reagent, ATP (1.5 mM), MgCl₂ (10 mM), KCl (20 mM), and CoaD (5 µg) in 50 mM Tris-HCl buffer (pH 7.6). The reaction was initiated by addition of substrate (5 or 3b, at concentrations between 10 and 250 µM).

**CoaE—** Diphospho-CoA kinase activity was determined by a modification of the published assay method (11). Each 500-µl reaction mixture contained 200 µl of pyrophosphate reagent, ATP (1.5 mM), MgCl₂ (10 mM), KCl (20 mM), pyruvate kinase (5 units), lactic dehydrogenase (5 units), and CoaE (5 µg) in 50 mM Tris-HCl buffer (pH 7.6). The reaction was initiated by addition of substrate (6a or 6b, at concentrations between 0.13 and 2.0 mM).

**Formation of 1a and 1b under Competitive Conditions—** The competitive biosynthesis of 1a and 1b was monitored as follows: the biosynthetic reaction in the 500-µl sample containing 2a (0.5 mM), 2b (0.5 mM), ATP (5.0 mM), CTP (1.0 mM), t-cysteine (1.0 mM), DTT (2.0 mM), MgCl₂ (15.0 mM), CoaA (25 µg), CoaBC (25 µg), CoaD (25 µg), and CoaE (25 µg) in 150 mM Tris-HCl buffer (pH 7.6) was pre-incubated at 37 °C, and the reaction was initiated by addition of the biosynthetic enzymes. At set time intervals 40-µl aliquots were withdrawn and immediately placed in a microcentrifuge tube preheated to 95 °C. After 5 min at 95 °C, the sample was centrifuged (13,000 rpm × 5 min) and 30 µl of the supernatant was added to 6 µl of guanosine (6 mM in 0.5 M HCl) as internal standard. The solution was mixed well, and 25 µl was injected on to the HPLC column for analysis using the same conditions as described above. The concentrations of 1a and 1b were determined using a standard curve obtained using known amounts of pure Coa co-injected with the same internal standard.

**RESULTS AND DISCUSSION**

The pantothenic acid analog N-pentylpantothenamide 2b and its phosphate 3b were obtained by the improved synthetic route shown in Fig. 2. We found that compound 2b inhibited the growth of E. coli in minimal medium (MIC = 2 µM) as previously reported (7). Analysis of the growth rate of E. coli grown in minimal medium and in the presence of varying amounts of 2b shows that the inhibitor retarded the onset of logarithmic growth, but did not abolish it entirely (Fig. 3). Furthermore, regardless of the initial amount of inhibitor present, all cultures grew to similar final cell densities. Taken together, these results show that compound 2b is bacteriostatic rather than bacteriocidal and that E. coli can overcome the inhibitory effect of N-pentylpantothenamide over time.

We considered two hypotheses for the toxicity of 2b: either it inhibits CoA biosynthesis, thus limiting the amount of CoA available to cells, or it is biosynthetically converted to ethyldethia-CoA, a potential CoA antimetabolite that could inhibit CoA utilizing enzymes.

To differentiate between these mechanisms the last five enzymes involved in CoA biosynthesis were isolated from overexpression strains and incubated with the natural precursor 2a or its analog 2b, and the reaction products were analyzed by HPLC. The chromatograms from these reaction mixtures are shown in Fig. 4. When 2a, the natural biosynthetic intermediate, was used as the substrate, the major new product formed was identified as Coa since it co-eluted with an authentic sample of 2a (Fig. 4A). When the analog 2b was used as the substrate, a new compound, eluting with a longer retention time than Coa, was formed (Fig. 4B). To determine the identity of the new compound authentic ethyldethia-CoA 1b was prepared synthetically using 3b as the starting material, which allows its purification from reaction mixtures in multimilli-gram quantities by anion exchange chromatography. The structure of the material obtained in this way was confirmed as being ethyldethia-CoA 1b by 1H NMR and ESI-MS analyses.

TABLE I

| Enzyme Substrate | kₘcat | Kₘ | kₘcat/Kₘ |
|------------------|-------|----|---------|
| CoaA 2a          | 1.07 ± 0.04 | 16.7 ± 2.0 | 64.2 ± 8.1 |
| CoaD 2b          | 1.18 ± 0.07 | 17.1 ± 3.6 | 69.2 ± 15.0 |
| CoaA 5b          | 12.3 ± 1.5 | 119 ± 30 | 102 ± 29 |
| CoaE 6a          | 14.8 ± 0.9 | 47.1 ± 8.8 | 312 ± 62 |
| CoaE 6b          | 4.01 ± 0.24 | 645 ± 115 | 6.22 ± 1.18 |
| CoaD 6a          | 12.0 ± 1.0 | 414 ± 95 | 28.8 ± 7.1 |

* CoaBC was excluded from this comparative analysis since it does not catalyze a reaction with any analog derived from the inhibitor 2b.

**FIG. 5. Competitive biosynthesis of coenzyme A (1a) and ethyldethia-coenzyme A (1b) from an equimolar mixture of pantothenic acid (2a) and N-pentylpantothenamide (2b) (500 µM each) as catalyzed by the coenzyme A biosynthetic enzymes, CoaAB-CDE, from E. coli. $\text{O}$, ethyldethia-CoA; $\text{C}$, CoA.
The compound first identified by HPLC analysis was thus shown to be ethyldethia-CoA 1b by co-elution with this authentic sample. In the same way, authentic dephospho-ethyldethia-CoA 6b was prepared by replacing the CoaE in the reaction mixtures with inorganic pyrophosphatase, and the synthetic material used to determine the identity of a compound eluting after 27 min, also by co-elution (not shown). These results demonstrate that the E. coli CoA biosynthetic enzymes (CoaABCDE) can catalyze the conversion of N-pentylpantothenamide 2b to ethyldethia-CoA 1b.

To establish the extent to which the CoA biosynthetic enzymes differentiate between the natural substrates and their respective analogs, we have determined $k_{\text{cat}}/K_m$ for both possible substrates of each enzyme (Table I). This was done using standard kinase assays for CoaA and CoaE, while an assay based on pyrophosphate release was used in the case of CoaD. These experiments demonstrate that, except in the case of CoaD where the difference is small, the analog is a better substrate than the natural precursor. In the case of CoaD, 3b is adenylylated ~3 times faster than 5, while CoaE exhibits a slightly larger preference, with the phosphorylation of 6b to give ethyldethia-CoA 1b occurring 4.6 times faster than the phosphorylation of 6a. In addition, treatment of a mixture of the natural substrate 3a (50 μM) and the analog 3b with the bifunctional enzyme phosphopantothenoylcysteine synthetase/decarboxylase (10) failed to reveal any inhibition of the synthetase activity of this enzyme with concentrations of the analog as high as 3 mM, as determined using the pyrophosphate release assay. These results demonstrate that inhibition of the CoA biosynthetic enzymes cannot be the mode of antibiotic action of N-pentylpantothenamide 2b.

To test the prediction from the kinetic parameters in Table I that the biosynthesis of ethyldethia-CoA 1b is faster than the biosynthesis of CoA, 2a and 2b (500 μM each) were incubated with purified CoaABCDE and the formation of 1a and 1b was followed by HPLC (Fig. 5). Based on initial rates, the biosynthesis of ethyldethia-CoA (1b) occurs 10.5 times faster than the biosynthesis of CoA. Since pantothenate kinase does not significantly differentiate between 2a and 2b, this rate difference is due to the preferential adenylylation of 3b over 5, the preferential phosphorylation of 6b over 6a, and the fact that the conversion of 3b to 1b requires two enzyme activities (CoaBC) less than the conversion of 3a to 1a. Thus, while the relative concentrations of the biosynthetic enzymes and the ratio of 2a to 2b in vivo is likely to be different from what was used here, this experiment suggests that the biosynthesis of ethyldethia-CoA is likely to compete effectively with the biosynthesis of CoA in the cell.

In conclusion our data show that the CoA biosynthetic enzymes are not inhibited by N-pentylpantothenamide 2b; rather this compound functions as a substrate of these enzymes, as shown by its preferential conversion to ethyldethia-CoA 1b in the competition experiment. This suggests that the toxicity of N-pentylpantothenamide results from the inhibition of CoA-utilizing enzymes by the CoA analog ethyldethia-CoA 1b. The proposed antimetabolite activity of 1b is supported by the demonstration that desulfo-CoA, which differs from 1b only in the length of the alkyl chain of the N-substituted amide, inhibits carnitine acetyl transferase, phosphotransacetylase, citrate synthase, β-hydroxy-β-methylglutaroyl-CoA synthase and α-ketoglutarate dehydrogenase (14). Our mechanistic proposal for the mode of action of 2b is analogous to the recently discovered mechanism of action of bacitracin, which is converted in vivo to methoxythiamin pyrophosphate, a thiamin pyrophosphate antivitamin (15). The facile in vivo conversion of N-pentylpantothenamide to ethyldethia-CoA overcomes the permeability problem of getting CoA analogs into cells; as a result N-pentylpantothenamide may be a useful compound for studying the physiological consequences of depriving cells of CoA and for trapping intermediates on complex enzymes such as fatty acid synthase, polyketide synthases, and non-ribosomal polypeptide synthetases, all of which utilize the CoA-derived 4′-phosphopantetheine cofactor.

Acknowledgments—We thank Dale Drueckhammer (SUNY, Stony Brook, NY) for providing plasmids pUCI/CoaD and pET/CoaE, Suzanne Jackowski (Dept. of Biochemistry, St. Jude Children’s Research Hospital, Memphis, TN) for providing CoaA, Kapila Ratnam (GlaxoSmithKline) for helpful discussions, and Jim Kerwin (Cornell Biotechnology Resource Center) for assistance with the ESI-MS analyses.

REFERENCES
1. Mishra, P. K., and Drueckhammer, D. G. (2000) Chem. Rec. 100, 3283–3309
2. F. C-Jackowski, S. (1996) in Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (Neidhardt, F. C., et al., eds) Vol. 1, pp. 687–694, American Society of Microbiology, Washington, D. C.
3. Jackowski, S., and Rock, C. O. (1984) J. Bacteriol. 158, 115–120
4. Vallari, D. S., and Rock, C. O. (1985) J. Bacteriol. 164, 136–142
5. Jackowski, S., and Alix, J. H. (1990) J. Bacteriol. 172, 3842–3848
6. Vallari, D. S., and Rock, C. O. (1985) J. Bacteriol. 162, 1156–1161
7. Clifton, G., Bryant, S. R., and Skinner, C. G. (1970) Arch. Biochem. Biophys. 137, 523–528
8. Geerlof, A., Lewendon, A., and Shaw, W. V. (1999) J. Biol. Chem. 274, 27105–27111
9. Kupke, T., Uhlele, M., Schmid, D., Jung, G., Blaeser, M., and Steinbacher, S. (2000) J. Biol. Chem. 275, 31838–31846
10. Strauss, E., Kinsland, C., Ge, Y., McLafferty, F. W., and Begley, T. P. (2001) J. Biol. Chem. 276, 13513–13516
11. Mishra, P. K., Park, P. K., and Drueckhammer, D. G. (2001) J. Bacteriol. 183, 2774–2778
12. Moffatt, J. G., and Khorana, H. G. (1961) J. Am. Chem. Soc. 83, 663–675
13. O’Brien, W. E. (1976) Anal. Biochem. 76, 425–430
14. Chase, J. P. A., Middleton, B., and Tubbs, P. K. (1966) Biochem. Biophys. Res. Commun. 23, 208–213
15. Reddick, J. J., Saha, S., Lee, J., Melnick, J. S., Perkins, J., and Begley, T. P. (2001) Bioorg. Med. Chem. Lett. 11, 2245–2248