The Structure and Biosynthesis of New Tetrahydropyrimidine Derivatives in Actinomycin D Producer *Streptomyces parvulus*

USE OF $^{13}$C- AND $^{15}$N-Labeled L-Glutamate AND $^{13}$C AND $^{15}$N NMR SPECTROSCOPY*

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Two novel compounds, 2-methyl, 4-carboxy, 5-hydroxy-3,4,5,6-tetrahydropyrimidine (THP(A)) and 2-methyl, 4-carboxy-3,4,5,6-tetrahydropyrimidine (THP(B)) have been identified in the pool of *Streptomyces parvulus* by in vivo and in vitro studies. $^{13}$C and $^{15}$N were introduced into the compounds by feeding *S. parvulus* with $^{15}$N- and $^{13}$C-labeled l-glutamate. High resolution $^{13}$C and $^{15}$N NMR have been applied to elucidate their structure and biosynthesis in *S. parvulus*. The splitting patterns and coupling constants of adjacent nitrogen-carbon molecular fragments enable us to unravel their molecular structure. Two different glutamate pools are responsible for their biosynthesis, THP(A) carbon skeleton derives from the extracellular l-$^{13}$C-glutamate, whereas THP(B) stems from d-fructose via the intracellular glutamate. During cell growth, THP(A) is synthesized and becomes the major constituent of the intracellular pool. It is consumed after THP(B) is accumulated intracellularly. The onset of THP(A) and THP(B) synthesis seems correlated to the time of actinomycin D synthesis. Their high cellular concentrations during actinomycin D synthesis suggest that they may function as nitrogen storage. Other possible functions of THP molecules within the cell are discussed.

In our recent paper (1), $^{13}$C and $^{15}$N NMR techniques have been used to measure metabolic regulation operating in *Streptomyces parvulus* during actinomycin D synthesis. We followed the fate of nutrient incorporation intracellularly and the relationship of primary metabolites and actinomycin D synthesis. Two previously unknown metabolites have been found to accumulate intracellularly throughout cell growth and actinomycin D production. Much research has been done on the biosynthesis of actinomycin D in *Streptomyces*, and most enzymes associated with the antibiotic synthesis have been identified (2-7). But little is known about the intracellular pool metabolites, their function in the cell life cycle, and the general regulation of antibiotic synthesis. Timing of antibiotic synthesis and rate of its production may also be affected by the availability of intracellular pool precursors (8).

In the present study, the molecular structure of the new metabolites, tetrahydropyrimidine derivative, was under taken. In addition, attempts have been made to reveal the pathways leading to the new compounds in order to understand the biogenesis of pyrimidine-like compounds and their association with actinomycin D synthesis in *S. parvulus.* $^{13}$C and $^{15}$N were incorporated into the new compounds by culturing *S. parvulus* with $^{13}$C- and $^{15}$N-labeled L-glutamate. High resolution $^{13}$C and $^{15}$N NMR spectroscopy directed toward elucidating their structure and biosynthesis have been performed by in vivo and in vitro studies. $^{13}$C and $^{15}$N chemical shifts, $^{1}H-^{13}$C, $^{13}$C-$^{13}$C, $^{1}H-^{16}$N, and $^{13}$C-$^{15}$N splitting patterns and coupling constants enabled us to reveal the molecular structures of the two new metabolites: 2-methyl, 4-carboxy, 6-hydroxy-3,4,5,6-tetrahydropyrimidine (THP(A)) and 2-methyl, 4-carboxy-3,4,5,6-tetrahydropyrimidine (THP(B)).

EXPERIMENTAL PROCEDURES

Materials—$^{13}$C- and $^{15}$N-labeled L-glutamate were prepared in our laboratory. L-$^{13}$C Glutamic acid (60 atom % enriched) was produced microbiologically from [2-$^{13}$C]acetate (95 atom % enriched). The isotopic enrichments of its carbons were determined by GC-MS analysis as previously described (9) and found to be C-2, C-3, and C-5, 70% enriched. C-1 was 36% enriched, and C-5 was less than 1% enriched. The synthesis of L-$^{15}$N glutamate (95 atom % enriched) was produced microbiologically from $^{15}$NHCl (95% enriched) as described elsewhere (10).

$^{15}$NHCl (95%) was purchased from Cambridge Isotope Laboratories. Trifluoroacetic anhydride, used for derivatization of amino acids for GC-MS studies, was purchased from Fluki AD. All other materials were of reagent grade.

Strains and Culture—*S. parvulus* (ATCC 12434) (kept on soil culture at 4 °C) was grown on NZ amine medium for 2 days at 30 °C in a gyratory shaking incubator. After centrifugation and washing twice with 0.2% KCl, a suspension of the mycelium (3%) served as a source for the new metabolites. Medium 1 consisted of 40 g D-fructose, 1.0 g of KH₂PO₄, 25 mg ZnSO₄, 25 mg of CaCl₂·2H₂O; 25 mg of MgSO₄·7H₂O; 25 mg of FeSO₄·7H₂O and 2.1 g of L-glutamic acid/100 ml of deionized water at pH 7.1 (growth medium 1).

Medium 2 is as medium 1 in which L-glutamate was replaced by L-$^{13}$C glutamate. Medium 3 is as medium 1 in which L-glutamate was replaced by L-$^{15}$N glutamate.

Cell and Cell Extract Preparations for NMR Measurement—Cell extracts of nonlabeled *S. parvulus* cells were prepared from 1 liter of *S. parvulus* growth culture. Cells were harvested by centrifugation (at 5,000 × g for 5 min) at 4 °C. Cells were washed twice with 0.2% KCl solution to remove traces of culture medium. Intracellular extracts were obtained either by suspending washed cell pellets in 10 ml of water and heating them for 15 min at 100 °C as previously described (12) or by the perchloric acid procedure. The supernatant was sep-

1 The abbreviations used are: THP(A), 2-methyl, 4-carboxy, 5-hydroxy-3,4,5,6-tetrahydropyrimidine (metabolite A); THP(B), 2-methyl, 4-carboxy-3,4,5,6-tetrahydropyrimidine (metabolite B); GC-MS, gas chromatography-mass spectroscopy; NOE, nuclear Overhauser effect; TMS, trimethylsilane.

2 A. Lapidot, manuscript in preparation.
rated by centrifugation at 15,000 x g for 15 min and concentrated under reduced pressure to 1 ml. No significant changes of cell extracts components have been noted by the NMR or other analytical methods using the two procedures for cell extraction.

For 13C NMR studies of isotopically labeled cells, S. parvulus cells were incubated at 30 °C for 72 h (mid-log phase) in growth medium containing L-[13C]glutamate (medium 2). Three-ml cell culture samples were harvested by centrifugation and washed twice with 0.2% KCl before transferring to the NMR tube. For 15N NMR studies, 200 ml of cell culture were harvested after 48 h of incubation with L-[15N]glutamate (medium 3). Cell extracts were prepared as described above and transferred to the 10-mm NMR tube. The NMR measurements were carried out at 7 °C.

Separation of THP(A) and -(B) from S. parvulus Cell Extracts—Cell extract samples were mixed with 4 volumes of 1 M acetic acid before separation by Dowex 50 W chromatography. The columns were washed with 10 ml of deionized water to remove carbohydrates and polyols. The amino acids were eluted with 5 ml of 3 M NH4OH, this fraction contained also the two new compounds THP(A) and -(B). Ammonia was evaporated to dryness, and the residue was brought to pH 5 and separated on Dowex 1 anion-exchange chromatography. Acidic amino acids such as glutamic acid remained attached to the anion exchange. THP(A) and -(B) were eluted with 10 ml of water, as was shown by 13C NMR spectroscopy. Glutamic acid was eluted by 10 ml of 1 N HCl.

Basic Hydrolysis of THP(A) and -(B)—The water fraction eluted from Dowex 1 anion-exchange column, which contains THP(A) and -(B), was concentrated to 1 ml; 1 ml of 20% KOH was added, and the mixture was heated in sealed tubes at 110 °C for 3 h. After cooling the mixture was analyzed by NMR, GC-MS, and amino acid analyzer.

13C NMR Spectroscopy—13C NMR spectra at 67.89 MHz were obtained with a Bruker WH-270 MHz spectrometer operating in the FT mode (at 7 °C). Proton broad band-decoupled 13C NMR spectra were recorded with a Bruker AM-400 spectrometer operating at 100.62 MHz were operated with power-gated proton decoupling to reduce effects from dielectric heating and to maintain the sample temperature at about 10 °C. Proton-decoupled 13C NMR spectra and gated 1H decoupling with full nuclear Overhauser effect (NOE) were obtained, with the following spectrometer conditions: 60° pulses, 23.8 KHz spectral width, 2-s repetition time, and 16K Fourier data transform. Sample tube of 10 mm outer diameter were used with both instruments.

15N spectra were obtained at 27.37 MHz with a Bruker WH 270 spectrometer. Proton-decoupled 15N NMR spectra and gated 1H decoupling with full NOE were obtained with pulse width 60 μs, 6 KHz spectral width, 16K data points, and a recycle time of 6.3 s. These NMR conditions did not result in saturation in differential NOEs of most of the resonances.

GC-MS Measurements—GC-MS analyses were performed on a Finnigan 4500 quadrupole GC-MS interfaced to an INCOS data system. The mass spectrometer was operated in the chemical ionization mode with isobutane as reactant gas. Samples were introduced through the GC-MS inlet system. Measurements of isotopic abundance were made using computer-controlled selected ion monitoring. Purified cell extracts were hydrolyzed for 3 h in 10% KOH solution at 110 °C in sealed tubes. Samples were evaporated to dryness under a stream of nitrogen gas. Last traces of water were removed by azotropic distillations of methylene chloride. The dry samples were derivatized to N-trifluoroacetyl-n-butyl esters as described previously (13). The mixture of trifluoroacetate-n-butyl esters was injected into a glass column packed with Texporse Hac or into a 30 m fused silica capillary SE 54 column (J & W Scientific Inc.). The separation conditions of the GC were: pressure, 12 p.s.i.; split, 15 ml/min (ratio 1:25); injection temperature, 220 °C; temperature program, 100 °C isothermal for 1 min, then 100-200 °C at 5 °C/min. MS conditions were: transfer line temperature, 250 °C; ion source temperature, 150 °C; manifold temperature, 100 °C; ionizing energy, 70 eV; multiple ion voltage supply, 1.5 kV; and emission current, 0.5 mA. Calculations of isotopically abundance were made as described previously (14) and are presented as atom % excess.

Amino Acid Analysis—Intracellular pool of S. parvulus grown on different media (1, 2, and 3) were measured for their amino acid contents. Cell extract hydrolysates were also analyzed by amino acid analyzer (Dionex D-500).

**Fig. 1.** Proton-decoupled natural abundance 13C NMR spectra (67.39 MHz) of cell extract, taken from S. parvulus cells grown in medium GF (medium 1), early-log phase (a) and after separation from glutamate and carbohydrates (b). Each spectrum consists of 2000 accumulations 2 s repetition time. The 13C NMR peaks include the following: Glu, glutamate; Ala, alanine; PC, phosphatidylcholine derivative; N-(CH3); F, fructose; M, mannitol; A and B, new metabolites.
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FIG. 2. Proton broad band-decoupled (a) and gated-decoupled, with full NOE, natural abundance $^{13}$C NMR spectrum (100.6 MHz) of partially purified A and B metabolites (b) (2000 and 7000 accumulations, respectively). The extended view of the region 15–23 ppm corresponds to -CH$_2$ groups of metabolites A and B.

### TABLE I

$^{13}$C and $^{15}$N NMR chemical shift assignments and spin-spin coupling constants of tetrahydropyrimidine derivatives

The suggested structures which are compatible with the $^{13}$C and $^{15}$N NMR studies are: metabolite A, 2-methyl, 4-carboxy, 5-hydroxyl-3,4,5,6-tetrahydropyrimidine; and metabolite B, 2-methyl, 4-carboxyl-3,4,5,6-tetrahydropyrimidine.

| Pyrimidine derivative (A) | C-2 | C-2' | C-3 | C-4 | C-4' | C-5 | C-6 |
|--------------------------|-----|------|-----|-----|------|-----|-----|
| Chemical shifts ($\delta$ ppm)* | 262.3 | 161.5 | 19.0 | 267.8 | 60.6 | 175.3 | HOCH | HNCH$_2$ |
| $^3$J$^{13}$C,$^1$H (Hz) | N.S. | 47.0 | 47.0 | 96.3 | 53.0 | N.S. | 6.0 |
| $^3$J$^{15}$N,$^1$H (Hz) | N.S. | 15.5 | | | | |
| C-N groups | HNC=N | HCNH= | HCNH= | |
| $^3$J$^1$N,$^1$H (Hz) | 258.9 | 161.5 | 19.1 | 263.5 | 53.6 | 177.0 | CH$_2$ | HNCH$_2$ |
| C-2 | C-2' | C-3 | C-4 | C-4' | C-5 | C-6 |
| Pyrimidine derivative (B) | HNC=N | -CH$_2$ | HNCH= | COOH | HOCH | HNCH$_2$ |
| Chemical shifts ($\delta$ ppm) | 15.5 | | | | | | |
| $^3$J$^{13}$C,$^1$H (Hz) | | | | | | | |
| C-N groups | HNC=N | | | | | | |
| $^3$J$^{15}$N,$^1$H (Hz) | | | | | | | |
| C-2 | C-2' | C-3 | C-4 | C-4' | C-5 | C-6 |

a $^{13}$C chemical shifts are in parts/million down-field from tetramethylsilane. $^{15}$N chemical shifts are in parts/million up-field from HNO$_3$.

b Not separated.

RESULTS

Natural Abundance $^{13}$C NMR Spectrum of S. parvulus Cell Extract—The intracellular pool of S. parvulus cell culture, grown in chemically defined medium (GF) (medium 1), without adding any isotopically labeled precursor, was analyzed by $^{13}$C NMR spectroscopy. The natural abundance proton-decoupled spectrum of cell extract consists of numerous sharp resonances that have been assigned on the basis of chemical shifts previously reported (1, 12, 15). The spectrum reflects
the accumulation of several primary metabolites and possible actinomycin D precursors (Fig. 1a). The region between 10 to 60 ppm shows resonances associated with Krebs cycle intermediate: glutamate (Glu), alanine (Ala), and a derivative of choline –N+(CH₃)₃ (PC) group (not resolved), which resonates at 53.5 ppm. Large pools of new metabolites A and B, which have not been previously reported, are also observed. The polyol resonances region (60–100 ppm) arise from mannitol (M) and fructose (F). The resonances in the region between 160 and 190 ppm are originating from carboxylic acids and
other nonprotonated carbon groups.

Amino acid analysis confirmed our results that glutamic acid constitutes >80% and alanine ~15% of the total amino acids pool level after 48 h growth of S. parvulus in GF medium.

$^{13}$C NMR of the Unknown Metabolites A and B—The natural abundance proton-decoupled spectra of partially purified metabolites A and B is shown in Fig. 1b. Resonances of alanine (Ala C-3 and Ala C-2) and choline derivative group -N'-(CH$_3$)$_2$ (PC) are also observed. However, the intense resonances of carbohydrate F and M carbons at the region 63-80 ppm, and of glutamate observed in Fig. 1a, are not seen in the $^{13}$C NMR spectrum obtained after removing carbohydrates (by cation-exchange chromatography) and glutamate (by anion-exchange chromatography) (Fig. 1b). In both spectra the intracellular level of metabolite B is 3-fold higher than that of metabolite A. The different chemical shifts which reflect the electronic distribution surrounding the observed nucleus are used for structural characterization of the measured molecule.

The use of gated-decoupling technique ([H] on between data acquisitions) can regain some sensitivity due to the NOE and in the same time give information on $^{13}$C-$^1$H coupling constants as observed in $^{13}$C NMR experiments done without $^1$H decoupling. From the signal multiplicities observed in the gated-decoupled spectrum, it is possible to discriminate between methyl (quartet), methylene (triplet), methine (doublet), and quaternary carbon (singlet) resonances. Fig. 2 is the $^{13}$C NMR of the power-gated (Fig. 2a) and gated-decoupled spectra (Fig. 2b) (100.6 MHz), of S. parvulus cell extract, partly purified metabolites A and B. Six different carbon species are summarized in Table I for metabolite A: methyl group at 19 ppm, CH$_2$ group at 43.5 ppm, and two resonances at 60.6 and 60.3 ppm, which might correspond to -CH-NH-and -CH-0H species. The resonance at 161.5 ppm arises from a nonprotonated carbon, and the resonance at 175.3 ppm corresponds to -COOH group. Metabolite B carbons resonate at: -CH$_3$ group at 19.1 ppm, -CH$_2$ group at 23 ppm, another -CH$_3$ group at 38.0 ppm, -CH group at 53.5 ppm, nonprotonated carbon at 161.5 ppm, and -COOH group at 177.0 ppm.

Proton-decoupled $^{13}$C NMR Spectra of S. parvulus Cells Grown on $^{13}$C-Labeled Glutamate—The $^{13}$C-$^1$C splitting patterns of enriched products can be used to assign the carbon resonances by the multiplet pattern produced. In the present study highly enriched L-$^{12}$C[glutamate was used as the labeled precursor (medium 2). The $^{13}$C spectrum obtained from intact S. parvulus cells grown in the presence of $^{13}$C-labeled glutamate and unlabeled d-fructose is shown in Fig. 3. The most intense peaks are those of metabolite A. The splitting pattern, of metabolite A carbon resonances appear as multiplets, similar to its origin, L-$^{13}$C[glutamate (see L-$^{13}$C[glutamate splitting pattern, Fig. 3b). The weak resonances arising from trehalose do not show $^{13}$C-$^1$C coupling, indicating that they are not originating from adjacent $^{13}$C-$^{13}$C fraction of the $^{13}$C-labeled glutamate. The $^{13}$C-$^{13}$C splitting pattern provides information on the carbon fragment condensation (9, 16). Each carbon is represented by several peaks which are the super position of spectra from all the $^{13}$C isotopomers present. As a result of spin-spin coupling, $^{13}$C nuclei that are directly bonded to each other appear as doublets; $^{13}$C nuclei that are bonded only to $^{12}$C nuclei are singlet peaks. The same $^{13}$C-$^{13}$C coupling constants appear twice, thus allowing the identification of two adjacent carbons. Fig. 3a, upper trace, is an expanded region of the resonances at 19.0, 43.5, and 161.5 ppm corresponding to metabolite A carbons. The triplet resonances centered at 19 ppm correspond to $^{13}$C-methyl group with a typical $^{13}$C-$^{12}$C coupling constant of $J_{C-C} = 47$ Hz. The triplet resonances centered at 161.5 ppm is of identical $^{13}$C-$^{13}$C coupling constant ($J_{C-C} = 47$ Hz). Thus, the methyl group at 19.0 ppm is coupled to the nonprotonated carbon at 161.5 ppm. The $^{13}$C-$^{13}$C coupling constant of the triplet resonances centered at 43.5 ppm is of 36 Hz. The $^{13}$C chemical shifts and $^{13}$C-$^{13}$C coupling constant of this fragment might be consistent with N-amino acetyl group (-CH$_2$NHCOCH$_3$). However, the stability of the cell extract sample to strong acidic solutions (6 N HCl, 110 °C for 15 h), argued against the possibility of amide bonds in these molecules.

$^{13}$N NMR Studies from S. parvulus Grown on $^{15}$N-Labeled Glutamate—$^{15}$N NMR was applied to identify the nitrogen species of the new metabolite A and B. S. parvulus cells were grown in media containing L-$^{15}$N[glutamate (95% enriched) as the sole nitrogen source. The cells were harvested at the mid-log phase, and cell extracts were prepared for $^{15}$N NMR studies. The proton broad band-decoupled 27 MHz $^{15}$N NMR spectrum of cell extract of S. parvulus is shown in Fig. 4a. The intense resonance at high field 335 ppm corresponds to glutamic acid (17). The intense resonances at lower field 258.9 and 263.5 ppm were assigned to metabolite B, and the weak
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FIG. 5. Proton-decoupled natural abundance $^{13}$C NMR spectrum (67.39 MHz) of S. parvulus partially purified metabolites A and B (5000 accumulations). Cells were grown in the presence of L-$[^{13}$N]glutamate (95% enriched) and harvested at mid-log phase (lower trace). The expanded region of the resonances at 38.0, 43.5, 53.6, 60.3, 60.6, and 161.5 ppm of metabolites A and B is shown in the upper trace. The resonances at 38, 43.5, 53.6, and 60.6 ppm are doublets due to $^{15}$N-$^{12}$C coupling $J_{C-N}=6-7$ Hz, corresponding to C-NH species. The triplet resonance centered at 161.5 ppm corresponds to amidine group HNC=NH $J_{C-N}=13.5$ Hz.

Resonances at 267.8 ppm and 262.3 ppm arise from metabolite A. The different intensities are in accordance with their intracellular concentrations. The proton-coupled spectrum, as shown in Fig. 4b, presents four doublets centered at 258.9, 262.3, 263.5, and 267.8 ppm, indicating two NH species per molecule. After removal of glutamate by cation exchange chromatography, the $^{15}$N resonances observed are only those of metabolite A and metabolite B.

The $^{15}$N labeling of S. parvulus cell pool metabolites facilitated the determination of $^{15}$N-$^{13}$C coupling constants. These constants are most useful for signal assignments of carbon-nitrogen molecular fragments and molecular structure elucidation. $^{13}$C-$^{15}$N splittings are directly observed in natural abundance $^{13}$C proton-decoupled NMR experiments of $^{15}$N-enriched samples. $^{13}$C NMR spectrum (at 67.89 MHz) of $^{15}$N-enriched metabolite A and metabolite B is shown in Fig. 5. The expanded region of the resonances 43.5 and 60.6 ppm correspond to metabolite A, and the resonances at 38.0 and 53.6 ppm correspond to metabolite B. All the four resonances appear as doublets due to $^{13}$C-$^{15}$N spin-spin coupling. Their coupling constants, $J_{C-N}$, are of 6-7 Hz and arise from a C-NH species. Most $^{13}$C NMR spectra were obtained under conditions where signals from quaternary carbons are partially saturated and thus attenuated, such as C-2 of metabolites A and B (161.5 ppm), C-4 of metabolite A (175.3 ppm), and C-4 of metabolite B (177.0 ppm). The triplet resonances centered at 161.5 ppm correspond to amidine residue (N=CN) with coupling constant, $J_{C-N}$, of 13.5 Hz (Table I).

From the above results, derived from $^{13}$C and $^{15}$N NMR spectroscopy, proton-coupled and decoupled techniques, $^{13}$C and $^{15}$N chemical shifts, $^{13}$C-$^{1}H$, $^{15}$N-$^{1}H$, $^{13}$C-$^{13}$C, and $^{15}$C-$^{15}$N coupling constants, the molecular structure of the two stable tetrahydropyrimidine derivatives could be elucidated. The high resolution $^{13}$C proton-coupled natural abundance spectrum is consistent with six different carbon groups: methyl (CH$_3$), methylene (CH$_2$), two methane (CH), and two quaternary carbon species for metabolite A and six different carbon species, methyl, two methylene, methane, and two quaternary carbons, for metabolite B.

$^{13}$C NMR of $^{13}$C-enriched metabolite A revealed that the methyl carbon resonate at 19 ppm is coupled to the quaternary carbon at 161.5 ppm. $^{15}$N chemical shifts and proton-coupled $^{15}$N-$^{1}H$ spectrum confirm the existence of two NH groups in one of the new metabolites. The availability to identify adjacent carbons to nitrogenous group, from their spin-spin coupling patterns and coupling constants, revealed that C-6 (at 43.5 ppm) and C-4 (at 60.6 ppm) of metabolite A are adjacent to different nitrogenous groups. In metabolite B, C-6 (at 38.0 ppm) and C-4 (at 53.6 ppm) are adjacent to two different nitrogenous groups. The quaternary carbons at 161.5 ppm, in both metabolites A and B are coupled to the two nitrogens in an amidine bond (N=CNH). Summary of $^{13}$C and $^{15}$N chemical shifts, $^{13}$C-$^{1}H$, $^{15}$N-$^{1}H$, $^{13}$C-$^{13}$C, and $^{15}$C-$^{15}$N coupling constants of the two tetrahydropyrimidine derivatives are summarized in Table I. The suggested molecular structure for metabolites A is 2-methyl, 4-carboxy, 5-hydroxy-3,4,5,6-tetrahydropyrimidine.
The suggested structure for metabolite B is 2-methyl-4-carboxy-3,4,5,6-tetrahydropyrimidine.

Confirmation of the Molecular Structure of the Tetrahydropyrimidine Derivatives by Basic Hydrolysis, NMR, and GC-MS Studies—The suggested molecular structures of the two new metabolites are consistent with the stability of cyclic amidines to acidic condition. Hydrolysis at a significant rate appears only at high pH, as shown in Scheme 1. Indeed, when partially purified THP(A) and -(B) were treated with 10% KOH (at 100 °C for 3 h), they hydrolyzed to amino acids. Their times of elution, obtained by amino acid analyzer, were similar to the expected elution time of basic amino acids, such as lysine and ornithine. The molecular structures of the two hydrolysates were confirmed by 

\[ \text{\textsuperscript{13}C NMR spectrum shown in (Fig. 6b) is significantly different from the THP(A) and -(B) spectrum (Fig. 6a). New resonances at 24.3 and 182.0 ppm arise from acetate carbons as a result of ring opening and hydrolysis of the tetrahydropyrimidine derivatives (Scheme 1).}

The carbon 13 resonances of the hydrolysates A' and B' are in agreement with the structures suggested for A' and B': 2,4-diamino-3-hydroxybutyric acid (A') and 2,4-diaminobutyric acid (B') (Table III). These compounds are consistent with their origins, tetrahydropyrimidine derivatives. C-5 of both pyrimidine molecules THP(A) and -(B) are shifted downfield upon hydrolysis, C-3 of 2,4-diamino-3-hydroxybutyric acid resonates at 67.4 ppm (instead of 60.3 ppm in THP(A)). Similar \textsuperscript{13}C chemical shifts are noted as a result of hydrolysis of THP(B), C-3, corresponding to C-5 of the heterocyclic molecule, is shifted downfield to 29 ppm in the 2,4-diaminobutyric acid (B').

The \textsuperscript{15}N NMR spectrum of the amino acids A' and B' is shown in Fig. 7. The \textsuperscript{15}N chemical shifts at 334.9 and 347.8 ppm and those at 335.5 and 342.4 ppm correspond to free amino groups NH₂, two for each of the hydrolysate products A' and B', respectively.

Chemical ionization GC-MS was carried out to determine the molecular weights of the basic hydrolysate samples. They were derivatized to N-trifluoroacetyl-n-butyl esters before injection to the GC-MS. Chemical ionization GC-MS revealed molecular ions (M + 1) m/z 383 and (M + 1) m/z 366 of the N-trifluoroacetyl-n-butyl ester derivatives 2,4-diamino-3-hydroxybutyric acid and 2,4-diaminobutyric acid, respectively.

Analysis of the Labeling Pattern of THP(A) from L-\textsuperscript{13}C Glutamate—Analysis of \textsuperscript{13}C multiplets of a complex pattern

\begin{table}[h]
\centering
\caption{\textsuperscript{13}C and \textsuperscript{15}N NMR chemical shifts assignments of tetrahydropyrimidine derivatives hydrolysates A' and B'}
\begin{tabular}{cccccc}
\hline
 & C-1 & C-2 & C-3 & C-4 & N₂ \hline
A' & 172.1 & 58.2 & 334.9 & 67.4 & 43.0 & 347.8 \\
B' & 174.3 & 53.3 & 335.5 & 29.0 & 37.5 & 342.4 \hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{THP(A) \textsuperscript{13}C enrichments obtained from 72-h incubation with L-\textsuperscript{13}C glutamate (medium no. 2)}
\begin{tabular}{cccc}
\hline
Carbons & \textsuperscript{13}C enrichment* & Doublet/singlet \hline
C-2 & 16.5 & 3.9 \\
C-2' & 17.0 & 4.1 \\
C-4 and C-5 & 22.7 & N.S. \\
C-6 & 13.3 & 2.4 \hline
\end{tabular}
\end{table}

\* \textsuperscript{13}C enrichments determined from relative peak areas of the \textsuperscript{13}C resonances in comparison to glutamate C-3 and C-4 in Fig. 3a. Glutamate C-3 and C-4 \textsuperscript{13}C enrichments were analyzed by GC-MS as previously described (9). 
‡ The attenuated signal intensity was corrected by normalizing to natural abundance compound. 
§ Not separated.
its doublet to singlet ratio is only 2.4. Whereas the doublet to
tion via malate-fumarate in trichloroacetic acid cycle. Indeed
C-3 and C-4 carbons. The significant lower 13C enrichment of
from glutamate C-5 (zero labeled) and C-2 after randomiza-
enrichments. The decreased 13C enrichment of C-6 may arise
comparison to their origins, pyrimidine derivatives metabo-

C-4 and C-5 (each one is 23% W-enriched). These carbons
be used to determine the relative 13C enrichment in various
nonrandom labeled distribution. NMR peak intensities can
enables one to follow if the precursor fragment is incorporated
intact. The pattern at a specific site give information from
which one can determine the relative amount of labeled and
unlabeled carbons at the neighboring position and to identify
nonrandom labeled distribution. NMR peak intensities can
be used to determine the relative 13C enrichment in various
carbons. The 13C enrichment of THP(A) carbons were deter-
mined by comparison of their peak areas with those of intra-
cellular metabolite, THP(A). This metabolite is the major 13C-
enriched compound observed by 13C NMR of L-[13C]glutamate
fed S. paruulus cells or cell extract. Its 13C NMR splitting
pattern is similar to the exogenous L-[13C]glutamate (Fig. 3).
THP(B) could not be observed by 13C NMR of L-[13C]gluta-
mate fed S. paruulus cells, or cell extracts, but have been observed together with THP(A) by natural abundance 13C
NMR of cell extract derived from D-fructose and L-glutamate
fed S. paruulus (Fig. 1) (see "Experimental Procedures").
Therefore, we conclude that two different pools serve as
carbon precursor for the two THP compounds. THP(A) is
derived from the exogenous glutamate pool, whereas THP(B)

FIG. 7. The time course of actinomycin D production de-
picted as the rate of increments of actinomycin D concentra-
tion (C). The uptake of L-glutamate from the chemically defined GF
medium (●) was determined by amino acid analyzer. Intracellular
concentrations of glutamate (C), metabolites A (●) and B (○), as
time-dependent, derived from 13C NMR signal intensity (spectra not
shown).

FIG. 8. The time course of actinomycin D production de-
picted as the rate of increments of actinomycin D concentra-
tion (C). The uptake of L-glutamate from the chemically defined GF
medium (●) was determined by amino acid analyzer. Intracellular
concentrations of glutamate (C), metabolites A (●) and B (○), as
time-dependent, derived from 13C NMR signal intensity (spectra not
shown).
is originating from the new intracellular glutamate pool, derived from D-fructose catabolism.

Significant difference of intracellular mobilities of the new THP molecules are noted. THP(B) NMR resonances are attenuated and broadened beyond detection in the intact cells (1). Only following cell membrane and/or cell wall rupture, during intracellular cell extraction, this metabolite is released and can be detected by $^{13}$C and $^{15}$N NMR spectroscopy.

Times of synthesis and consuming of THP(A) and -(B) during cell life cycle were followed by natural abundance $^{13}$C NMR of cell extracts. The extracellular L-glutamate is consumed by the cell during the first 30 h of cell growth, during this period we have found that THP(A) is synthesized and becomes the major constituent of the intracellular pool. THP(B) NMR resonances are originating from the new intracellular glutamate pool. They are slowly catabolized during actinomycin D synthesis (Fig. 8). They are slowly catabolized during actinomycin D synthesis (Fig. 8). They are slowly catabolized during actinomycin D synthesis (Fig. 8). They are slowly catabolized during actinomycin D synthesis (Fig. 8). They are slowly catabolized during actinomycin D synthesis (Fig. 8).

The onset of THP(B) synthesis seems correlated to that of trehalose, a carbohydrate storage material, found in this bacteria and in other microorganisms (1, 12, 19–21). THP(A) and -(B) could be also involved in metabolic regulation of actinomycin D synthesis. It is known that cyclic AMP (cAMP) is involved in catabolic repression in several organisms. Foster and Katz (4) concluded that neither cAMP nor cyclic GMP had any effect on relieving glutamate repression in S. parvulus. It is likely that some other nucleotides take the place of cAMP. THP molecules, found in the present study, could be involved in relieving glutamate repression and regulating actinomycin D synthesis in S. parvulus. Other possible function of THP molecules within the cell may be a defensive role for the organism. Further studies are planned to ascertain the role of these new metabolites.

We anticipate that knowledge of the structures and biosynthesis of new pyrimidine derivatives will contribute to better understanding of the control mechanisms of antibiotic biosynthesis.

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