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

USE OF 13C- AND 15N-LABELED L-GLUTAMATE AND 13C AND 15N NMR SPECTROSCOPY*

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**EXPERIMENTAL PROCEDURES**

Materials—13C- and 15N-labeled L-glutamate were prepared in our laboratory. L-13C[Glutamic acid (50 atom % enriched)] was produced microbiologically from [2-13C]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-4, 70% enriched. C-1 was 36% enriched, and C-5 was less than 1% enriched. The synthesis of L-15N[Glutamate (95 atom % enriched)] was produced microbiologically from 15NH4Cl (95% enriched) as described elsewhere (10).

15NH4Cl (95%) was purchased from Cambridge Isotope Laboratories. Trifluoroacetic anhydride, used for derivatization of amino acids for GC-MS studies, was purchased from Fluka AD. All other materials were of reagent grade.

Strain 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 inoculum for the chemically defined growth medium (GF) (11) which contained glucose (1%), potassium succinate (0.2%), 0.01 M MgSO4·7H2O, 0.02 M K2HPO4, MgSO4·7H2O (6 g/l), CaCl2·2H2O (100 mg/l), and 2.1 g of L-glutamic acid/1000 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-15N-glutamate. Medium 3 is as medium 1 in which L-glutamate was replaced by L-13C-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 described previously (12) or by the perchloric acid procedure. The supernatant was sepa-

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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, tetramethylsilane.

‡L. 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 $^{13}$C NMR studies of isotopically labeled cells, S. paruulus cells were incubated at 30 °C for 72 h (mid-log phase) in growth medium containing L-$^{[13]}$C]glutamate (medium 2). Three-ml cell culture samples were harvested by centrifugation and washed twice with 0.2% acetic acid. The cell pellets were re-suspended in 1 ml of 20% KOH solution before separation by Dowex 1 anion-exchange chromatography. The columns were packed with Tetrasorb Hac or into a glass column packed with Tabsorb Hac or into a glass column packed with Tabsorb Hac or into a glass column packed with Tabsorb Hac or into a glass column packed with Tabsorb Hac or into a glass column packed with Tabsorb Hac.

Separation of THP(A) and -(B) from S. paruulus 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 polysaccharides. The amino acids were eluted with 5 ml of 3 M NH$_4$OH, 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. Acids were derivatized to N-trifluoroacetoyl-n-butyl esters as described previously (13). The mixture of trifluoroacetate-n-butyl esters was injected into a glass column packed with Tabsorb Hac or into a 30 m fused silica capillary SE 54 column (J & W Scientific Inc.). The separation conditions of the GC were: pressure, 15 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, 200 °C; manifold temperature, 150 °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. paruulus grown on different media (1, 2, and 3) were measured for their amino acids contents. Cell extract hydrolysates were also analyzed by amino acid analyzer (Dionex D-500).

High resolution $^{13}$C NMR spectra obtained with a Bruker WH 270 MHz spectrometer operating at 100.82 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 $^{13}$C NMR spectra and gated H 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 was used with both instruments.

$^{14}$N spectra were obtained at 27.37 MHz with a Bruker WH 270 spectrometer. Proton-decoupled $^{15}$N NMR spectra and gated H decoupling with full NOE were obtained with pulse width 60 μs, 7 KHz spectral width, 60K 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 reagent 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 azetropic distillation 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 Tabsorb Hac or into a 30 m fused silica capillary SE 54 column (J & W Scientific Inc.). The separation conditions of the GC were: pressure, 15 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, 200 °C; manifold temperature, 150 °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. paruulus grown on different media (1, 2, and 3) were measured for their amino acids contents. Cell extract hydrolysates were also analyzed by amino acid analyzer (Dionex D-500).

Fig. 1. Proton-decoupled natural abundance $^{13}$C NMR spectra (67.39 MHz) of cell extract, taken from S. paruulus 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 $^{13}$C NMR peaks include the following: Glu, glutamate; Ala, alanine; PC, choline derivative, -N'-(CH$_3$)$_2$; F, D-fructose; M, mannitol; A and B, new metabolites.
Biosynthesis of Tetrahydropyrimidine Derivatives by $^{13}$C and $^{15}$N NMR

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.

<table>
<thead>
<tr>
<th>Pyrimidine derivative (A)</th>
<th>N-1</th>
<th>C-2</th>
<th>C-2'</th>
<th>N-3</th>
<th>C-4</th>
<th>C-4'</th>
<th>C-5</th>
<th>C-6</th>
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<tbody>
<tr>
<td>Chemical shifts (δ ppm)$^a$</td>
<td>262.3</td>
<td>161.5</td>
<td>19.0</td>
<td>267.8</td>
<td>60.6</td>
<td>175.3</td>
<td>60.3</td>
<td>43.5</td>
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<tr>
<td>$J_{13C,1H}$ (Hz)</td>
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<td>47.0</td>
<td>N.S.$^a$</td>
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<td>N.S.</td>
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<td>C-N groups</td>
<td>13.5</td>
<td>HNC=N</td>
<td>HCNH=</td>
<td>HCNH=</td>
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<tr>
<td>$J_{15N,1H}$ (Hz)</td>
<td>96.3</td>
<td>HNC=N</td>
<td>6</td>
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<table>
<thead>
<tr>
<th>Pyrimidine derivative (B)</th>
<th>N-1</th>
<th>C-2</th>
<th>C-2'</th>
<th>N-3</th>
<th>C-4</th>
<th>C-4'</th>
<th>C-5</th>
<th>C-6</th>
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<tr>
<td>Chemical shifts (δ ppm)$^a$</td>
<td>258.9</td>
<td>161.5</td>
<td>19.1</td>
<td>263.5</td>
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<td>HNC=N</td>
<td>HCNH=</td>
<td>HCNH=</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C-N groups</td>
<td>100.7</td>
<td>132.7</td>
<td>96.3</td>
<td>135.5</td>
<td>145.5</td>
<td>135.5</td>
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</tr>
</tbody>
</table>

$^a$ $^{13}$C chemical shifts are in parts/million down-field from tetramethylsilane. $^{15}$N chemical shifts are in parts/million up-field from HN0$_3$.

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...
Biosynthesis of Tetrahydropyrimidine Derivatives by $^{13}$C and $^{15}$N NMR

**Fig. 3.**

**a.** Proton-decoupled $^{13}$C NMR spectrum (67.39 MHz) of *S. parulis* cells grown in the presence of L-$[^{13}$C$]$glutamate, taken at the late-log phase. Spectral condition is as in Fig. 1; it consists of 20,000 accumulation. The washed cells spectrum include the $^{13}$C-labeled peaks: A, metabolite A; Glu, glutamate; PC, choline derivative; Tre, trehalose. The extended view of the resonances at 19, 43.5 and 161.5 ppm are those of metabolite A. The triplet resonances are due to $^{13}$C-$^{13}$C couplings.

**b.** Proton-decoupled $^{13}$C NMR spectrum (67.39 MHz) of cell culture medium containing $^{13}$C-enriched L-glutamate and nonenriched D-fructose. 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$^+(CH_3)_3$ (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. 13C NMR of the Unknown Metabolites A and B—The natural abundance proton-decoupled spectrum 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'(CH3)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 13C 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 ([1H] on between data acquisitions) can regain some sensitivity due to the NOE and in the same time give information on 13C-1H coupling constants as observed in 13C NMR experiments done without 1H 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 13C NMR of the power-gated (Fig. 2a) and gated-decoupled spectra (Fig. 2b) (at 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, CH2 group at 43.5 ppm, and two resonances at 60.6 and 60.3 ppm, which might correspond to C-H-NH- and C-H-OH species. The resonance at 161.5 ppm arises from a nonprotonated carbon, and the resonance at 175.3 ppm corresponds to C=O group. Metabolite B carbons resonate at: -CH3 group at 19.1 ppm, -CH2 group at 23 ppm, another -CH2 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 13C NMR Spectra of *S. parvulus* Cells Grown on L-[13C]-Labeled Glutamate—The 13C-13C 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-[13C]glutamate was used as the labeled precursor (medium 2). The 13C spectrum obtained from intact *S. parvulus* cells grown in the presence of 13C-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-[13C]glutamate (see L-[13C]glutamate splitting pattern, Fig. 3b). The weak resonances arising from trehalose do not show 13C-13C coupling, indicating that they are not originating from adjacent 13C-13C fraction of the 13C-labeled glutamate. The 13C-13C 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 13C isotopomers present. As a result of spin-spin coupling, 13C nuclei that are directly bonded to each other appear as doublets; 13C nucleus that are bonded only to 12C nuclei are single peaks. The same 13C-13C 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 13C-methyl group with a typical 13C-13C coupling constant of J_{C,C} = 47 Hz. The triplet resonances centered at 161.5 ppm is of identical 13C-13C 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 13C-13C coupling constant of the triplet resonances centered at 43.5 ppm is of 36 Hz. The 13C chemical shifts and 13C-13C coupling constant of this fragment might be consistent with N-amino acetyl group (-CH2NHCOCH3). 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.

Proton-decoupled 15N NMR (27.37 MHz) (a) and gated 1H decoupling with full NOE (a') (2600 and 5000 accumulations, respectively) of 15N-enriched *S. parvulus* cell extracts spectra. *S. parvulus* cells were cultured in the presence of L-[15N]glutamate and harvested at the mid-log phase. Spectrum b (2000 accumulations) is of cell extracts after removing carbohydrates and glutamate. The labeled 15N peaks include glutamate (Glu) and two 15N resonances of each of the new metabolites A and B.

![Image](image-url)

**Fig. 4.** Proton-decoupled 15N NMR (27.37 MHz) (a) and gated 1H decoupling with full NOE (a') (2600 and 5000 accumulations, respectively) of 15N-enriched *S. parvulus* cell extracts spectra. *S. parvulus* cells were cultured in the presence of L-[15N]glutamate and harvested at the mid-log phase. Spectrum b (2000 accumulations) is of cell extracts after removing carbohydrates and glutamate. The labeled 15N peaks include glutamate (Glu) and two 15N resonances of each of the new metabolites A and B.
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 258.5, 262.3, 263.5, and 267.8 ppm, indicating two NH species per molecule. After removal of glutamate by cation exchange chromatography, the 15N resonances observed are only those of metabolite A and metabolite B.

The 15N labeling of S. parvulus cell pool metabolites facilitated the determination of 13C-15N coupling constants. These constants are most useful for signal assignments of carbon-nitrogen molecular fragments and molecular structure elucidation. 13C-15N splittings are directly observed in natural abundance 13C proton-decoupled NMR experiments of 15N-enriched samples. 13C NMR spectrum (at 67.89 MHz) of 15N-enriched metabolite A and metabolite B is shown in Fig. 5. The expanded region of the resonances at 38.0, 43.5, 53.6, and 60.6 ppm correspond to amidine group HNC=NH with coupling constant, $J_{C15N} = 13.5$ Hz. The triplet resonance centered at 161.5 ppm corresponds to amidine group HNC=NH with coupling constant, $J_{C2H} = 6-7$ Hz, corresponding to C-NH species. The triplet resonance centered at 161.5 ppm corresponds to amidine group HNC=NH with coupling constant, $J_{C2H} = 13.5$ Hz.

From the above results, derived from 13C and 15N NMR spectroscopies, proton-coupled and decoupled techniques, 13C and 15N chemical shifts, $J_{H13C}$, $J_{H15N}$, $J_{13C13C}$, and $J_{13C15N}$ coupling constants, the molecular structure of the two stable metabolites A and B could be elucidated. 13C NMR spectroscopy clearly reveals six carbons in each metabolite. The high resolution 13C proton-coupled natural abundance spectrum is consistent with six different carbon groups: methyl (CH3), methylene (CH2), 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.

13C NMR of 13C-enriched metabolite A revealed that the methyl carbon resonate at 19 ppm is coupled to the quaternary carbon at 161.5 ppm. 15N chemical shifts and proton-coupled 15N-$^1$H spectrum confirm the existence of two NH groups in each 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 53.6 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 13C and 15N chemical shifts, $J_{H13C}$, $J_{H15N}$, $J_{13C13C}$, and $J_{13C15N}$ 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 conditions. Hydrolysis at a significant rate appears only at high pH, as shown in Scheme 1.

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 13C, 15N NMR, and molecular weights were determined from mass spectrometry analysis.

The 13C 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 I). 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 13C 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').

**TABLE I**

<table>
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<th>Carbons</th>
<th>13C enrichment*</th>
<th>Doublet/singlet</th>
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<tr>
<td>C-2</td>
<td>15.5</td>
<td>3.6</td>
</tr>
<tr>
<td>C-3</td>
<td>17.0</td>
<td>4.1</td>
</tr>
<tr>
<td>C-4 and C-5</td>
<td>22.7</td>
<td>N.S.*</td>
</tr>
<tr>
<td>C-6</td>
<td>13.7</td>
<td>2.4</td>
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</tbody>
</table>

* 13C enrichments were determined from relative peak areas of the 13C resonances in comparison to glutamate C-2 and C-4 in Fig. 3a. The attenuated signal intensity was corrected by normalizing to natural abundance compound.

**TABLE II**

**13C and 15N NMR chemical shifts assignments of tetrahydropyrimidine derivatives hydrolysates A' and B'**

<table>
<thead>
<tr>
<th>Carbons</th>
<th>13C</th>
<th>15N</th>
</tr>
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<tbody>
<tr>
<td>C-2</td>
<td>172.1</td>
<td>58.2</td>
</tr>
<tr>
<td>C-2'</td>
<td>174.3</td>
<td>53.3</td>
</tr>
</tbody>
</table>

Analysis of the Labeling Pattern of THP(A) from L-[13C] Glutamate—Analysis of 13C multiplts of a complex pattern...
The glutamate used in this study consists of three adjacent triplets of singlets, and its doublet to singlet ratio of the pair C-2 and C-2' is 4.0 (Table III), indicating that both carbons are derived from intact glutamate C-3, C-4 and is further diluted by unlabeled pool of pyruvate (or acetyl-CoA) originating from the unlabeled D-fructose catabolism.

**DISCUSSION**

In the present investigation we have shown that *S. paruulus* cells have the capacity to maintain high levels of previously unknown pyrimidine derivative.

The goal of this study was to define the molecular structure of the new metabolites discovered in the process of actinomycin D synthesis. The molecular structure of the THP(A) and THP(B) have been deduced from $^{13}$C and $^{15}$N NMR chemical shifts, $^{13}$C-$^1$H, $^{15}$N-$^1$H, and $^{13}$C-$^{13}$C spin-spin splitting patterns and coupling constants. But only the results derived from $^{13}$C-$^{13}$C spin-spin splitting pattern and coupling constants allowed us to eliminate other possible molecular structure than those suggested for THP(A) and THP(B): 2-methyl, 4-carboxy, 5-hydroxy-3,4,5,6-tetrahydropyrimidine and 2-methyl, 4-carboxy, 3,4,5,6-tetrahydropyrimidine, respectively. The molecular weights of THP(A) and THP(B) hydrolysates, measured by chemical ionization GC-MS technique, substantiate the suggested structures for THP(A) and THP(B). The stereochemistry of THP(A) and THP(B) are currently being studied.

The labeling experiments here described confirm our recent observations that intracellular glutamate pool did not originate from culture medium glutamate (1). Instead, the exogenous L-$[^{13}$C$]glutamate retains its carbons in the new intracellular metabolite, THP(A). This metabolite is the major $^{13}$C-enriched compound observed by $^{13}$C NMR of L-$[^{13}$C$]glutamate fed *S. paruulus* cells or cell extract. Its $^{13}$C NMR splitting pattern is similar to the exogenous L-$[^{13}$C$]glutamate (Fig. 3). THP(B) could not be observed by $^{13}$C NMR of L-$[^{13}$C$]glutamate fed *S. paruulus* cells, or cell extracts, but have been observed together with THP(A) by natural abundance $^{13}$C 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) is derived from labeled metabolites provide means for quantifying the proportion of each labeled species in the mixture of isotopomers (9, 16, 18). Adjacent carbons that have predominant multiplet pattern resonances are originated from the same precursor fragment. The $^{13}$C-$^{13}$C splitting pattern 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 the nonrandom labeled distribution. NMR peak intensities can be used to determine the relative $^{13}$C enrichment in various carbons. The $^{13}$C enrichment of THP(A) carbons were determined by comparison of their peak areas with those of intracellular glutamate C-3 (and/or C-4). The $^{13}$C enrichments of glutamate carbons have been analyzed by chemical and electron ionization GC-MS measurements as previously described (9). THP(A) $^{13}$C enrichments are summarized in Table III. The glutamate used in this study consists of three adjacent triplets of singlets, and its doublet to singlet ratio of the pair C-2 and C-2' is 4.0 (Table III), indicating that both carbons are derived from intact glutamate C-3 and C-4 carbons. The significant lower $^{13}$C enrichment of this fragment (17%) suggests that THP(A) C-2, C-2' fragment, stems from pyruvate or acetyl-CoA carbons derived from glutamate C-3, C-4 and is further diluted by unlabeled pool of pyruvate (or acetyl-CoA) originating from the unlabeled D-fructose catabolism.

**Fig. 7.** The time course of actinomycin D production depicted as the rate of increases of actinomycin D concentration ([3]). The uptake of L-glutamate from the chemically defined GF medium (●) was determined by amino acid analyzer. Intracellular concentrations of glutamate (O), metabolites A (●) and B (△), as time-dependent, derived from $^{13}$C NMR signal intensity (spectra not shown).

**Biosynthesis of Tetrahydropyrimidine Derivatives by $^{13}$C and $^{15}$N NMR**

16021
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 $^{13}\text{C}$ and $^{15}\text{N}$ NMR spectroscopy.

Times of synthesis and consuming of THP(A) and -(B) during cell life cycle were followed by natural abundance $^{13}\text{C}$ 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(A) is consumed after THP(B) is accumulated intracellularly. The onset of THP(B) synthesis seems correlated to the time of onset of actinomycin D synthesis, after the release of THP(B), this metabolite is released into the medium-like actinomycin D. Their nitrogen pool is at least twice that of the endogenous glutamate pool during actinomycin D synthesis (Fig. 8). They are slowly catabolized during actinomycin D synthesis. Their role might be similar to that of trehalose, a carbohydrate storage material, found in 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.

REFERENCES