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Transformation of Isopropylamine to L-Alaninol by *Pseudomonas* sp. Strain KIE171 Involves *N*-Glutamylated Intermediates

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***Pseudomonas* sp. strain KIE171 was able to grow with isopropylamine or L-alaninol [*S*-(+)-2-amino-1-propanol] as the sole carbon source, but not with D-alaninol. To investigate the hypothesis that L-alaninol is an intermediate in the degradation of isopropylamine, two mini-Tn*5* mutants unable to utilize both isopropylamine and L-alaninol were isolated. Whereas mutant KIE171-BI transformed isopropylamine to L-alaninol, mutant KIE171-BII failed to do so. The two genes containing a transposon insertion were cloned, and the DNA regions flanking the insertions were sequenced. Two clusters, one comprising eight *ipu* (isopropylamine utilization) genes (*ipuABCDEFGH*) and the other encompassing two genes (*ipuI* and *orf259*), were identified. Comparisons of sequences of the deduced Ipu proteins and those in the database suggested that isopropylamine is transported into the cytoplasm by a putative permease, IpuG. The next step, the formation of γ-glutamyl-isopropylamide from isopropylamine, ATP, and L-glutamate, was shown to be catalyzed by IpuC, a γ-glutamylamide synthetase. γ-Glutamyl-isopropylamide is then subjected to stereospecific monooxygenation by the hypothetical four-component system IpuABDE, thereby yielding γ-glutamyl-L-alaninol [γ(L-glutamyl)-L-hydroxy-isopropylamide]. Enzymatic hydrolysis by a hydrolase, IpuF, was shown to finally liberate L-alaninol and to regenerate L-glutamate. No gene(s) encoding an enzyme for the next step in the degradation of isopropylamine was found in the *ipu* clusters. Presumably, L-alaninol is oxidized by an alcohol dehydrogenase to yield L-2-aminopropionaldehyde or it is deaminated by an ammonia lyase to propionaldehyde. Genetic evidence indicated that the aldehyde formed is then further oxidized by the hypothetical aldehyde dehydrogenases IpuI and IpuH to either L-alanine or propionic acid, compounds which can be processed by reactions of the intermediary metabolism.**

Isopropylamine is used as a solvent and as a raw material in manufacturing various chemicals. The compound occurs as a constituent of the herbicides atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine] and propachlor (2-chloro-*N*-isopropylacetanilide), from which it is liberated during microbial degradation (16, 21, 27). Little information is available on the microbial metabolism of isopropylamine. Studies with an isopropylamine-utilizing *Mycobacterium* strain suggested that degradation is initiated by an inducible amine dehydrogenase that yields ammonium and acetone as the first intermediate. However, the further metabolism of acetone has not been investigated in this organism (5). Recently, aerobic gram-negative bacteria were isolated that utilize isopropylamine but not acetone, thus leading to the conclusion that acetone is not a reaction intermediate in isopropylamine degradation (W. Dilling, W. R. Knauber, and B. Schink, Abstr. Frühjahrstagung VAAM, abstr. PU046, 1997).

The preliminary observations mentioned above make it appear likely that, at least in some organisms, isopropylamine degradation is initiated by monooxygenation to yield alaninol (2-amino-1-propanol), which may be further oxidized to alanine or deaminated to propionaldehyde. Alaninol is a chiral compound, and a degradative pathway involving L-alaninol would be of interest for the biotransformative production of this versatile synthon from the cheap prochiral compound isopropylamine. This prompted us to isolate an organism, *Pseudomonas* sp. strain KIE171, which grows with isopropylamine and L-alaninol but not with D-alaninol. In the present work we provide genetic and biochemical evidence that isopropylamine degradation in this strain proceeds via γ-glutamyl-isopropylamide and γ-glutamyl-L-alaninol to L-alaninol.

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MATERIALS AND METHODS

Materials. Reagents for molecular biology were obtained from Fermentas (Vilnius, Lithuania) and New England Biolabs. γ-(L-Glutamyl)-isopropylamide and γ-(L-glutamyl)-L-1-hydroxy-isopropylamide were obtained from Bachem (Bubendorf, Switzerland). All other chemicals were reagent grade or better and were obtained from Aldrich, Fluka, or Sigma.

Growth media and bacterial strains. *Pseudomonas* sp. strain KIE171 and its derivatives were cultivated aerobically at 30°C in the mineral salts medium described by Kulla et al. (15) with a 10 or 20 mM carbon source. Cells were grown in 500-ml Erlenmeyer flasks containing 100 ml of liquid medium and shaken at 140 rpm. *Escherichia coli* was grown aerobically with shaking (140 rpm) in Luria-Bertani medium (22). Growth was monitored as turbidity at 650 nm. Solid medium contained 15 g of agar/liter. Ampicillin was added at 200 μg/ml, and kanamycin was added at 50 μg/ml.

Pseudomonas sp. strain KIE171 has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as DSM 12360. The isopropylamine- and L-alaninol-nonutilizing mutants KIE171-B, KIE171-BI, and KIE171-BII carry the Deutsche Sammlung von Mikroorganismen und Zellkulturen numbers DSM 11521, DSM 11629, and DSM 13380, respectively. *E. coli* strains DH5α (GIBCO/BRL Life Technologies) and XL1-Blue (Stratagene) were used for cloning, and *E. coli* BL21(DE3) (Novagen) was used as the host for the overex-

pression of proteins. *E. coli* strain S17-1 λ*pir* (17) was the donor in biparental matings.

Enrichment and isolation of an isopropylamine utilizer. Ten milliliters of sewage sludge from the Lonza AG wastewater treatment plant was mixed with 90 ml of mineral salts medium containing 20 mM isopropylamine, and the mixture was incubated at 30°C under nonsterile conditions without shaking. After growth had occurred, 1-ml aliquots of the enrichment were transferred into 99 ml of the same medium and incubated under the above-mentioned conditions. After four subcultures in sterile medium with shaking, the final enrichment was serially diluted and spread onto plates containing mineral salts medium with 20 mM isopropylamine. The majority of the colonies were of one type. Purification of a typical colony yielded a pure culture of the isopropylamine utilizer *Pseudomonas* sp. strain KIE171.

Mutagenesis. Chemical mutagenesis of strain KIE171 was performed with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) according to the protocol of Foster (10). A suspension of mutagenized cells containing approximately 50% survivors was plated on minimal medium containing 20 mM L-glutamate. A total of 2,000 colonies were replica plated on minimal plates containing 20 mM isopropylamine or 20 mM L-alaninol as the sole carbon source. One mutant, strain KIE171-B, unable to grow with these compounds, was chosen for further study.

Transposon mutagenesis was carried out by using the mini-Tn*5* system (7). The transposon was introduced into strain KIE171 by plate conjugation on Luria-Bertani medium at 30°C for 8 h with a donor/recipient ratio of 1:1 . *E. coli* S17-1 λpir (pUT-miniTn*5Km*) was used as the donor strain. Exconjugants were selected on mineral salts agar containing 10 mM L-lactate and 10 mM L-alanine plus 50 μg of kanamycin per ml. A total of 3,000 kanamycin-resistant strain KIE171 exconjugants were then replicated onto mineral salts medium containing 50 μg of kanamycin per ml and a 20 mM concentration of either isopropylamine, L-alaninol, L-alanine, or L-lactate as the carbon source. Mutants impaired in growth with one or several of these carbon sources were chosen for further study.

DNA manipulations. Isolation of plasmids and of genomic DNA, restriction enzyme digestion, agarose gel electrophoresis, Southern analysis, and transformation of *E. coli* were carried out using standard methods (2).

RNA isolation and primer extension analysis. Isolation of RNA and primer extension were performed as described before (3). Oligonucleotide ipupe (5′-C CAGATCATATTCTTTGGCGTTGCCTCAT-3′) was used to prime the reverse transcription reaction. The plasmid used for generation of a sequencing ladder was pME4771, which was constructed by insertion of a 0.7-kb *Apa*I/*Pst*I fragment containing the *ipu* promoter region in pBluescript II KS.

Construction of an *ipuH*::*xylE* fusion strain. Plasmid pME4268 contains a 2.7-kb *Sma*I/*Sal*I fragment harboring the *ipuH* gene (S. I. de Azevedo Wäsch, unpublished data). For the construction of an *ipuH*::*xylE* fusion in strain KIE171, the 2.4-kb *Sma*I fragment from pX1918GT (24) was inserted in the blunted *Bam*HI site of pME4268 to give pME4762. The *Sma*I/*Hin*dIII fragment from pME4762 was then cloned in the vector pEX18Tc (24), resulting in pME4763. Plasmid pME4763 was introduced in strain KIE171 by conjugation, and integrants were selected on Luria-Bertani plates containing tetracycline (15 μg/ml). To select for second crossover events, integrants were subsequently plated on Luria-Bertani medium containing 5% sucrose. Sucrose-resistant colonies were obtained, and one such colony was designated KIE171-BV. PCR was used to verify whether correct replacement had occurred.

Construction of *ipuC* and *ipuF* expression plasmids. For the production of IpuC as an N-terminal histidine-tagged fusion protein, the *ipuC* gene was amplified by PCR from genomic DNA of strain KIE171 with the oligonucleotide primers IPUC-NT (5′-AACAGGTGATACATATGAGCGAAG-3′) and IPUC-CT (5′-TTTGAAGCTTAGGATCTGGGCG-3′), with the changes to introduce *Nde*I and *Hin*dIII restriction sites, respectively, underlined. The 1.4-kb PCR product was digested with *Nde*I and *Hin*dIII, and the resulting fragment encompassing *ipuC* was ligated into *Nde*I-*Hin*dIII-digested pET-28a(+) (Novagen), resulting in plasmid pME4275, in which *ipuC* is under the control of the T7 polymerase promoter. The *ipuC* sequence of plasmid pME4275 was sequenced to confirm that no changes had been introduced during PCR amplification. For the production of the wild-type IpuC, the *Nde*I-*Hin*dIII *ipuC* insert of pME4275 was placed under the control of the T7 promoter of vector pET24a(+) (Novagen), generating plasmid pME4277.

For the production of IpuF as an N-terminal histidine-tagged fusion protein, the *ipuF* gene was amplified by PCR from plasmid pME4259, which carries a 22-kb *Xho*I fragment obtained by transposon rescue from strain KIE171-BII in pBluescript II KS(+) (Stratagene). The oligonucleotide primers used were IPUF-NT (5′-GGGCCAGCTAGCATGGAAAAGCTTAG-3′) and IPUF-CT (5′-TCTACTGAGCTCTACTACTGCTAC-3′), with the changes to introduce *Nhe*I and *Sac*I restriction sites, respectively, underlined. The 909-bp PCR product was digested with *Nhe*I and *Sac*I, and the resulting 891-bp fragment containing *ipuF* was cloned into the vector pET28a(+), resulting in plasmid pME4751. To confirm that no changes had been introduced during PCR amplification, the *ipuF* gene of plasmid pMR4751 was sequenced. Plasmid pME4756, which expresses wild-type IpuF, was obtained by cloning the *Nhe*I-*Sac*I insert of pME4751 into the expression vector pET24b(+).

Enzyme assays. γ-Glutamylamide synthetase (IpuC) activity was assayed by measuring the substrate-dependent formation of inorganic phosphate from ATP as described for the assay of glutamine synthetase activity (25). The reaction mixture (0.4 ml) contained 10 mM ATP, 10 mM substrate,10 mM L-glutamate, 50 mM $MgCl_2$, 50 mM imidazole-HCl (pH 7.0), 3.5 mM NaCl, and 25 μg of enzyme preparation. The reaction was started by the addition of enzyme, and it was run at 25°C. To stop the reaction, 0.9 ml of ferrous sulfate reagent (0.8% $FeSO_4 \cdot 7H_2O$ in 15 mM H_2SO_4) and 0.075 ml of ammonium molybdate reagent [6.6% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 7.5 M H_2SO_4] were added to 0.1 ml of incubation mixture. The sample was mixed vigorously and color was allowed to develop at room temperature for 2 min, after which absorbance was measured at 660 nm. One unit was defined as the amount of enzyme forming 1 μmol of inorganic phosphate per min under standard assay conditions.

γ-Glutamylamide hydrolase (IpuF) activity was assayed by measuring the L-glutamate formed from γ-glutamylamide substrates. The reaction mixture (4 ml) contained 17% (vol/vol) glycerol, 100 mM $MgCl_2$, 200 mM Tris-HCl (pH 8.0), and 10 to 100 mM substrate. The reaction was started by the addition of enzyme (100 to 200 μg), and it was run at 30°C. At 30-s intervals, samples of 0.4 ml were taken, boiled for 2 min, and centrifuged. The concentration of L-glutamate in the supernatant was determined enzymatically as described (30) or it was determined by high-pressure liquid chromatography (HPLC) analysis as described below. One unit was defined as the amount of enzyme forming 1 μmol of glutamate per min under standard assay conditions.

Catechol-2,3-dioxygenase activity was measured as described before (14).

Production and purification of histidine-tagged IpuC and IpuF proteins. For the production of His_6-IpuC in *E. coli*, strain BL21(DE3) harboring plasmid pME4275 was grown at 30°C in Luria-Bertani medium. When the culture had reached an A_{650} of 0.6, expression was induced by addition of isopropyl-β-D-1-thiogalactopyranoside to a final concentration of 100 μM, and the culture was incubated for another 3 h to a final A_{650} of 1.5. The same procedure was used for production of wild-type IpuC in *E. coli* strain BL21(DE3) harboring the expression plasmid pME4277. To obtain His_6-IpuF, the induction of strain BL21(DE3) carrying the expression plasmid pME4751 was performed at 18°C for 5 h to a final A_{650} of 1. The same procedure was used for the production of wild-type IpuF from the expression plasmid pME4756.

Induced *E. coli* BL21(DE3) cells (0.5 g) expressing His_6-IpuC or His_6-IpuF were suspended in 4 ml of the appropriate lysis buffer containing DNase I (10 μg/ml). Cell extract was obtained by two passages through a French pressure cell at 5.5 MPa and subsequent centrifugation at 40,000 × *g* for 30 min. His_6-IpuC was purified by metal chelate-affinity chromatography on a 2.5-ml HisBind Resin column (Novagen) according to the manufacturer's instructions, and the pure protein was stored at −20°C in 200 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, 20%(vol/vol) glycerol (pH 8.0).

Crude extract containing His_6-IpuF was supplemented with glycerol to a final concentration of 15% (vol/vol). His_6-IpuF was purified on a 1-ml Ni-nitrilotriacetic acid agarose column (Qiagen) as described by the manufacturer and stored at −20°C in 250 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, 15%/vol/vol) glycerol (pH 8.0).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Mini-PROTEAN II system (Bio-Rad) with 12% polyacrylamide gels under denaturing conditions (22). The broad range molecular weight markers used were from Bio-Rad or from New England Biolabs. Protein concentrations were measured using the method of Bradford (4) with Bio-Rad dye concentrate. Bovine serum albumin was used as a standard.

HPLC. HPLC was carried out with an alliance HPLC system (Waters), using MILLENNIUM software. Phenyl-isothiocyanate (PITC)-derivatized amines, including isopropylamine, L-alaninol, γ-glutamylamides, L-glutamate, ATP, and ADP, were separated on a Nucleosil-C_{18} reversed-phase column (250 by 4.6 mm; particle size, 7 μm) by applying a step gradient from 5 mM potassium phosphate buffer, pH 6.5, at the start to 80% (vol/vol) methanol–20%(vol/vol) 5 mM potassium phosphate buffer, pH 6.5, at 27 min. The flow rate was 1 ml/min. Compounds eluting from the column were detected by measuring the A_{254} and identified by cochromatography. For derivatization with PITC, 15 μl of sample was mixed with 15 μl of ethanol and subsequently with 140 μl of derivatization mixture (70% [vol/vol] ethanol, 20%[vol/vol] triethylamine, 10% [vol/vol] PITC). After 10 min at room temperature, the sample was lyophilized, resuspended in

500 μl of 5 mM potassium phosphate, pH 6.5, and passed through a filter with a pore size of 0.2 μm.

DNA sequencing and analysis. DNA was sequenced on both strands by using PCR methods with fluorescent dideoxynucleotide terminators and an ABI Prism automatic sequencer (Perkin-Elmer). DNA sequences and derived amino acid sequences were analyzed using the Genetics Computer Group Wisconsin package, version 10. Similarity searches were performed using the gapped BLAST program (1) against public protein and gene databases.

Nucleotide sequence accession numbers. The sequence of the *ipuI orf259* gene cluster of *Pseudomonas* sp. strain KIE171 has been deposited in the GenBank database under accession number AJ311161. The sequence of the *ipuABC-DEFGH* gene cluster carries accession number AJ311159 and that of the strain KIE171 16S rRNA gene has the accession number AJ11160.

RESULTS

Properties of *Pseudomonas* sp. strain KIE171. *Pseudomonas* sp. strain KIE171 was isolated from sludge of the Lonza AG wastewater treatment plant by enrichment on minimal medium containing 20 mM isopropylamine as the sole carbon source. Isopropylamine, L-alaninol, L-alanine, D-alanine, L-lactate, propionic acid, aminoethanol, propane-1,2-diol, and L-glutamate supported growth, whereas D-alaninol, D,L-alaninol, and acetone did not. Based on its 16S ribosomal DNA (rDNA) sequence and its profile of fatty acids, strain KIE171 was classified within the γ-subdivision of the proteobacteria into rRNA similarity group 1 of *Pseudomonas* (18). The 16S rDNA sequence of strain KIE171 was 99% identical to that of *Pseudomonas citronellolis*, whereas its physiological characteristics matched most closely those of *Pseudomonas aeruginosa* (95.8% 16S rDNA identity). A clear attribution of strain KIE171 to a particular species of RNA group 1 thus was not obtained.

Accumulation of L-alaninol by an isopropylamine-nonutilizing mutant. To establish that L-alaninol is an intermediate in the degradation of isopropylamine, a mutant defective in the utilization of these compounds was generated by mutagenesis with MNNG and isolated as described in Materials and Methods. This mutant, strain KIE171-B, exhibited no growth after 5 days of incubation in minimal medium containing either 20 mM isopropylamine or 20 mM L-alaninol. Whole cells of strain KIE171-B suspended in minimal medium with 20 mM isopropylamine formed 8 mM L-alaninol within 40 h. Based on HPLC analysis, the supernatant of the medium after 40 h of incubation did not contain D-alaninol. Incubation of the cell suspension for more than 40 h resulted in the disappearance of L-alaninol (not shown). This effect is believed to be due to the activity of a putative aldehyde dehydrogenase encoded by *ipuH* (see below).

Identification of genes involved in isopropylamine utilization. For the identification of the genes encoding the enzymes of the isopropylamine degradation pathway, we isolated miniTn*5* transposon insertion mutants of *Pseudomonas* sp. strain KIE171 that are unable to grow with isopropylamine. Two Ipu$^-$ (isopropylamine utilization-negative) mutants were obtained that displayed no growth with isopropylamine or L-alaninol as the sole carbon source but displayed normal growth with all other carbon sources tested. Of these mutants, strain KIE171-BI transformed isopropylamine to L-alaninol, whereas strain KIE171-BII failed to do so. The genes whose insertional inactivation caused loss of the ability to grow with isopropylamine were isolated by selection of the kanamycin resistance gene present on the minitransposon (7). The DNA fragments carrying a transposon insertion were sequenced and found to represent two apparently unlinked loci. These were termed cluster A (mutant KIE171-BI) and cluster B (mutant KIE171-BII) and appear to constitute two transcriptional units comprising two and eight genes, respectively. A schematic representation of the 10 open reading frames (ORFs) identified in the DNA sequences of clusters A and B is shown in Fig. 1.

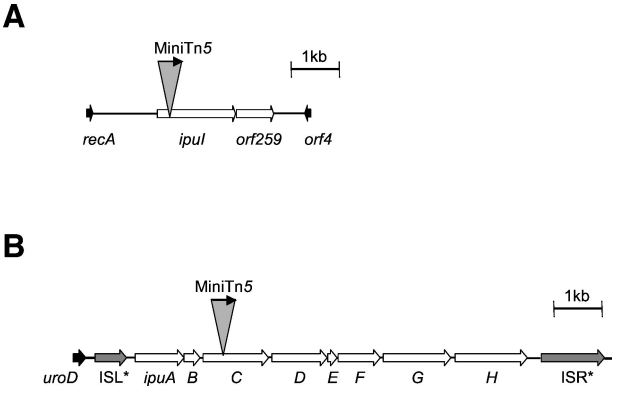

FIG. 1. Schematic presentation of gene clusters A and B of *Pseudomonas* sp. strain KIE171. The position and orientation of the transposon insertions in the genomes of mutants KIE171-BI (A) and KIE171-BII (B) are shown. Transposon insertions occurred in *ipuI*, the gene encoding a putative aldehyde dehydrogenase, and in *ipuC*, which encodes γ-glutamyl-isopropylamide synthetase. *, ISL and ISR show sequence similarity to the insertion elements IS *2* and IS *3* of *E. coli*, respectively.

With the exception of that encoded by *orf259*, the polypeptides encoded in clusters A and B displayed significant sequence identity to proteins of known function (Table 1). Based on the function of their homologs, these proteins can be arranged in a hypothetical pathway for the degradation of isopropylamine that is presented in Fig. 2. In this pathway isopropylamine is thought to be transported into the cytoplasm by IpuG, which shows weak sequence similarity to amino acid permeases of the amino acid-polyamine-organocation (APC) superfamily (13). The degradation of isopropylamine is then initiated by IpuC. This protein exhibits about 30% sequence identity to glutamine synthetases. Preliminary characterization revealed that IpuC catalyzes the ATP-dependent conversion of isopropylamine and L-glutamate to γ-glutamyl-isopropylamide and that ammonium is not a substrate (see below). IpuC thus is a γ-glutamyl-isopropylamide synthetase.

The next step in the hypothetical pathway is catalyzed by a four-component monooxygenase system that is proposed to stereospecifically hydroxylate γ-glutamyl-isopropylamide to γ-glutamyl-L-alaninol. It is thought to be composed of IpuD, which in its sequence is 30% identical to P-450$_{CAM}$ of *Pseudomonas putida* (23), and of IpuA, IpuB, and IpuE. IpuA exhibits 28% sequence identity to various thioredoxin reductases. Sequence alignments revealed that the two active-site cysteines typical for thioredoxin reductases (29) are absent in IpuA. Since the two flavin adenine dinucleotide binding domains and the NAD(P) binding site are present, it seems to be a NAD(P)H-dependent ferredoxin reductase, but not a thioredoxin reductase. IpuB and IpuE show strong sequence identity (48 and 40%, respectively) to ferredoxins. A [4Fe-4S] and a [3Fe-4S]

TABLE 1. Genes in DNA regions associated with isopropylamine utilization

Gene or ORF	Length (amino acids)	Gene		Inferred function	Sequence comparison of of representative hit[a]	% Identity[b]
		Start (bp)	End (bp)			
Cluster A						
ipuI	546	1471	3108	Aldehyde dehydrogenase	AldA (*Emericella nidulans*) (P08157)	30.8 (481)
orf259	259	3108	3884	Unknown	ORF 222 (*Pseudomonas aeruginosa*) (A82958)	27.4 (212)
Cluster B						
ipuA	327	1362	2342	NAD(P)H-dependent reductase	TrxB (*Streptomyces clavuligerus*) (Q05741)	28.1 (327)
ipuB	113	2342	2680	Ferredoxin	FdxA (*Streptomyces griseus*) (Q10839)	48.0 (100)
ipuC	460	2743	4122	γ-Glutamyl-isopropylamide synthetase	GlnA (*Thermotoga maritima*) (P36205)	33.5 (421)
ipuD	387	4194	5354	γ-Glutamyl-isopropylamide monooxygenase	CamA (*Pseudomonas putida*) (P00183)	30.9 (340)
ipuE	65	5371	5565	Ferredoxin	FdxA (*Pyrococcus furiosus*) (P29603)	40.3 (64)
ipuF	296	5589	6476	γ-Glutamyl-L-alaninol hydrolase	GuaA (*Escherichia coli*) (P04079)	24.3 (173)
ipuG	477	6533	7963	Isopropylamine permease	PheP (*Escherichia coli*) (P24207)	21.5 (455)
ipuH	508	8051	9574	Aldehyde dehydrogenase	ALDH2 (*Homo sapiens*) (P05091)	43.9 (481)

[a] Accession numbers from PIR, SwissProt, and Trembl databases are shown in parentheses.
[b] The numbers of amino acids considered for comparison are given in parentheses.

cluster are present in IpuB, whereas IpuE contains a single [3Fe-4S] cluster. This suggests that IpuB and IpuE function as electron transport components and, together with IpuA and IpuD, constitute a four-component cytochrome P-450-based monooxygenase system responsible for the stereospecific hydroxylation of γ-glutamyl-isopropylamide.

In a further step of the proposed pathway, γ-glutamyl-L-alaninol is hydrolyzed to L-alaninol and L-glutamate. This reaction was shown to be catalyzed by purified IpuF (see below). IpuF is related to the N-terminal part of guanosine 5′-phosphate synthetase. This enzyme catalyzes the final step in guanine ribonucleotide biosynthesis, and its N-terminal domain is responsible for the hydrolysis of glutamine, thus providing ammonium for the ATP-dependent formation of guanosine 5′-phosphate from xanthosine 5′-phosphate by the C-terminal synthetase domain (31).

None of the ORFs encoded in clusters A and B is similar to an enzyme reacting with an amino-alcohol such as L-alaninol. The reactions proposed to be involved in the degradation of this compound (Fig. 2) thus are entirely speculative. L-Alaninol is possibly oxidized by an alcohol dehydrogenase to L-2-aminopropionaldehyde, or it is possibly deaminated by an ammonia lyase to propionaldehyde. Evidence for the formation of an aldehyde as an intermediate in the degradation of L-alaninol is provided by the growth properties of mutant KIE171-BI. Its inability to grow with isopropylamine and L-alaninol is due to insertional inactivation of *ipuI* in cluster A, a gene encoding a protein with 30% sequence identity to NAD-dependent aldehyde dehydrogenases. It appears unlikely that the phenotype of strain KIE171-BI is caused by a polar effect on the expression of *orf259*, which encodes a polypeptide similar to a hypothetical protein of unknown function from *P. aeruginosa* (Table 1). Another protein with sequence similarity to NAD-dependent aldehyde dehydrogenases is encoded by *ipuH*, the most-downstream gene in cluster B. Degradation of L-alaninol thus appears to involve two aldehyde dehydrogenases. This view is supported by our observation that, in order to obtain a strain stably accumulating L-alaninol from isopropylamine, it is necessary to inactivate both *ipuH* and *ipuI* (de Azevedo Wäsch et al., unpublished).

Evidence for the formation of γ-glutamyl-isopropylamide by IpuC. In the pathway suggested by analysis of the *ipu* genes (Fig. 2), IpuC is postulated to catalyze the ATP-dependent formation of γ-glutamyl-isopropylamide from isopropylamine and L-glutamate. To verify this reaction, IpuC was expressed in *E. coli* as an N-terminally histidine-tagged fusion protein (His_6-IpuC) and purified in one step by metal chelate-affinity chromatography (Fig. 3). A crude *E. coli* extract containing His_6-IpuC had a specific activity of 0.67 U/mg of protein, while a crude extract from *E. coli* expressing wild-type IpuC exhibited an activity of 0.75 U/mg of protein (Fig. 4). When purified His_6-IpuC was incubated for 5 h in 1 ml of standard incubation mixture, 6.5 μmol of isopropylamine, 7.1 μmol of L-glutamate, and 4.7 μmol of ATP were consumed, and the products detected were 5.6 μmol of γ-glutamyl-isopropylamide, 4.9 μmol of ADP, and 5.6 μmol of inorganic phosphate. Except for inorganic phosphate, which was determined by a colorimetric assay, substrates and products were measured by HPLC analysis and identified by cochromatography with pure compounds (see Materials and Methods). 4-Aminobutyrate, glutarate, D-glutamate, L-aspartate, and L-2-aminoadipate were not accepted as substrates by the enzyme. However, IpuC showed a broad substrate range with respect to primary amines. Amino-alkanes; amino-alcohols, including L-alaninol and D-alaninol; and amino-esters were substrates (Table 2). Ammonium and primary amines with a positive or a negative charge on the side chain were not.

IpuF is a γ-glutamyl-L-alaninol hydrolase. IpuF catalyzes the third step in the proposed pathway, the hydrolysis of γ-glutamyl-L-alaninol to L-alaninol and L-glutamate (Fig. 2.) An N-terminally histidine-tagged fusion protein of IpuF (His_6-IpuF) was expressed in *E. coli* and purified by metal chelate-affinity chromatography (not shown). Purified His_6-IpuF catalyzed the hydrolysis of γ-glutamyl-L-alaninol, γ-glutamyl-isopropylamide, γ-glutamyl-ethylamide, L-glutamine, and γ-glutamyl-*p*-nitroanilide but did not react with reduced glutathione or with γ-glutamyl-L-alanine. Since γ-glutamyl-isopropylamide was the best substrate, enzyme activity was routinely determined with this compound. Crude extract containing wild-type IpuF exhibited about the same specific activity for

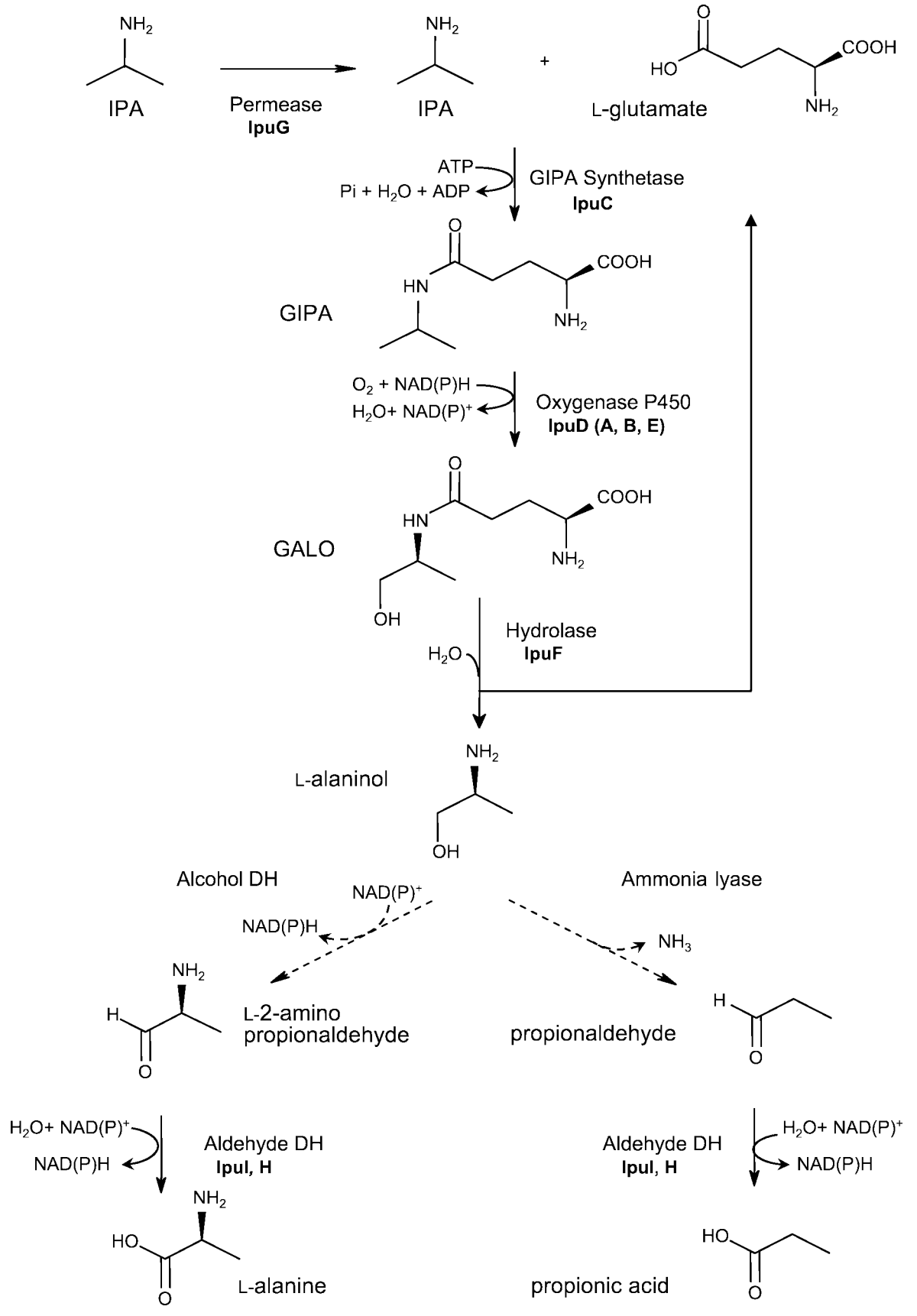

FIG. 2. Postulated pathway for the degradation of isopropylamine by *Pseudomonas* sp. strain KIE171. Arrows with broken lines indicate hypothetical reactions, for which no experimental evidence is available. Abbreviations: GIPA, γ-glutamyl-isopropylamide; GALO, γ-glutamyl-L-alaninol.

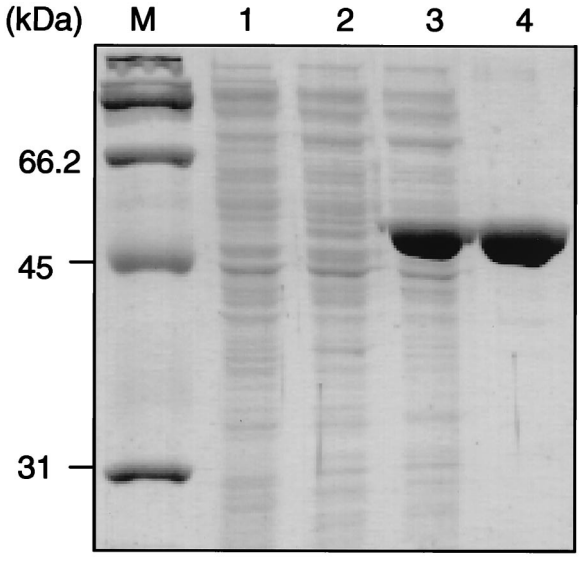

FIG. 3. Purification of histidine-tagged γ-glutamylamide synthetase (His_6-IpuC) from *E. coli* BL21(DE3)(pME4275). Protein samples (15 μg) were analyzed by SDS-PAGE on 12% acrylamide gels and stained with Coomassie brilliant blue. Lane M, molecular mass markers; lane 1, crude extract of uninduced *E. coli* BL21(DE3); lane 2, crude extract of uninduced *E. coli* BL21(DE3)(pME4275); lane 3, crude extract of induced *E. coli* BL21(DE3)(pME4275); purified His_6-IpuC after metal chelate-affinity chromatography.

γ-glutamyl-isopropylamide as crude extract containing His_6-IpuF, that is, 1.44 and 1.35 U/mg of protein, respectively. Purified His_6-IpuF was stable for several weeks upon storage at −20°C in buffer containing 20% (vol/vol) glycerol. However, during incubation at 30°C, the activity of the enzyme dropped with a half-life of 19 min. The enzyme showed a Michaelis-Menten-type saturation curve in response to increasing concentrations of γ-glutamyl-isopropylamide, with an estimated K_m of 65 mM and a V_{max} of 13.4 U/mg of protein. For γ-glutamyl-isopropylamide the K_m was 65 mM, with a V_{max} of 1.5 U/mg of protein. IpuF thus catalyzed the hydrolysis of γ-glutamyl-L-alaninol postulated in the pathway (Fig. 2), although with a high K_m and at a rate 1 order of magnitude below that observed for γ-glutamyl-isopropylamide.

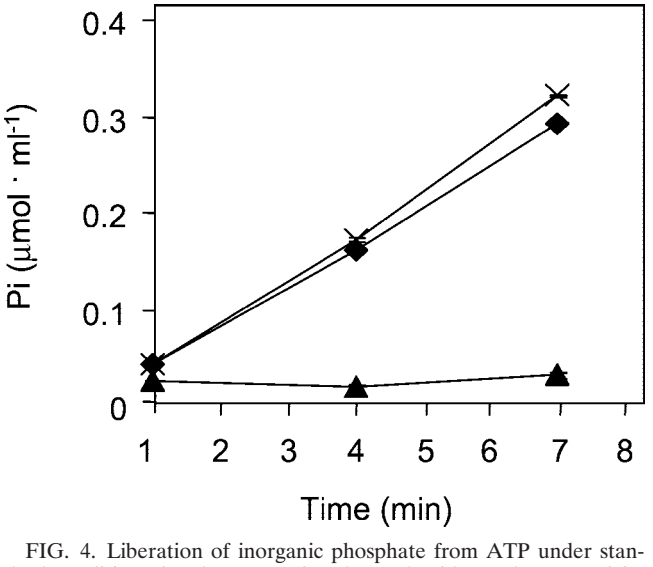

FIG. 4. Liberation of inorganic phosphate from ATP under standard conditions for the assay of γ-glutamylamide synthetase activity with isopropylamine as substrate. Each vial contained in a total volume of 400 μl of complete assay mixture 25 μg of protein from one of the following crude extracts: *E. coli* BL21(DE3)(pME4277) (×), *E. coli* BL21(DE3)(pME4275) (◆), or *E. coli* BL21(DE3) (▲).

TABLE 2. Substrate range of purified histidine-tagged γ-glutamyl-amide synthetase (His_6-IpuC)

Substrate	Relative activity (%)[a]
Methylamine	90
Ethylamine	145
Propylamine	72
Butylamine	62
Isopropylamine	100
Isobutylamine	100
s-Butylamine	70
t-Butylamine	74
Ethanolamine	146
1-Amino-2-propanol	186
3-Amino-2-propanol	140
R-2-Amino-1-propanol	142
S-2-Amino-1-propanol	140
S-2-Amino-1-butanol	133
R-2-Amino-1-butanol	132
Glycine methyl ester	50
4-Aminobutyric acid methyl ester	42
2-Amino-1,3-propanediol	79

[a] A relative specific activity of 100% corresponds to 1.16 U/mg of protein.

Regulation of expression of the *ipuABCDEFGH* genes. In order to investigate the regulation of expression of the *ipuABC DEFGH* genes, we constructed strain KIE171-BV, which harbors a chromosomally encoded transcriptional *ipuH*::*xylE* fusion, and measured the levels of catechol-2,3-dioxygenase activity. KIE171-BV exhibited an ~22-fold-higher catechol-2,3-dioxygenase specific activity when grown with glutamate plus isopropylamine than when grown with glutamate alone (not shown).

Primer extension analysis confirmed that expression of *ipu* is regulated at the level of transcription. The transcriptional start of the *ipu* gene cluster was located 28 bp upstream of the translational start of the *ipuA* gene (Fig. 5).

DISCUSSION

Analysis of the genes responsible for the utilization of isopropylamine as a carbon source by *Pseudomonas* sp. strain KIE171 leads us to propose a pathway that converts the substrate in three enzyme-catalyzed steps to L-alaninol. This compound is then further degraded by as yet unknown reactions, of which at least one appears to be catalyzed by two aldehyde dehydrogenases, whose genes have been identified (Fig. 2). The pathway from isopropylamine to L-alaninol involves two *N*-glutamylated intermediates, and its occurrence was supported by the following lines of physiological and biochemical evidence. Resting cells of a mutant of strain KIE171, unable to grow with L-alaninol, quantitatively accumulated L-alaninol from isopropylamine. The purified enzymes γ-glutamyl-isopropylamide synthetase (IpuC) and γ-glutamyl-L-alaninol hydro-

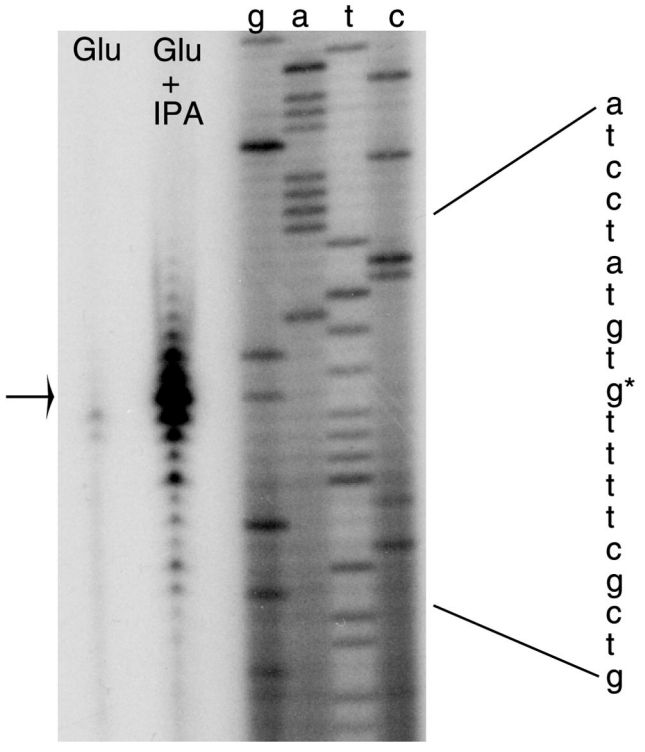

FIG. 5. Identification of the transcriptional start of the *ipuABCDEFGH* operon by primer extension analysis. RNA was isolated from glutamate (Glu)-grown cells or from cells grown with glutamate and isopropylamine (Glu + IPA) as carbon sources and reverse transcribed. The sequencing ladder obtained with plasmid pME4771 as a template is shown. The major primer extension product is indicated by an arrow, and the position of the transcriptional start is marked by an asterisk.

lase (IpuF) catalyzed in vitro reactions one and three of the proposed pathway. When the transformation of isopropylamine by resting cells was performed in the presence of $^{18}O_2$, label was detected in the L-alaninol accumulated in the medium, thereby supporting the notion that the second step of the pathway is catalyzed by a monooxygenase (de Azevedo Wäsch, unpublished data). Finally, in biotransformation experiments using dense cell suspensions, we have observed by HPLC analysis, in addition to L-alaninol, the accumulation of γ-glutamyl-isopropylamide and γ-glutamyl-L-alaninol from isopropylamine (de Azevedo Wäsch et al., unpublished data).

The active-site cavities of prokaryotic and eukaryotic glutamine synthetases are lined by 15 invariant amino acid residues that participate in the ATP-dependent conversion of ammonium and glutamate to glutamine (9). Sequence alignments of IpuC with prokaryotic glutamine synthetases show that 13 of these residues as well as the conserved tyrosine in the adenylylation site of prokaryotic glutamine synthetases are present in γ-glutamyl-isopropylamide synthetase. In the enzyme of *Salmonella enterica* serovar Typhimurium, whose structure has been solved (9), residue Ser-53 interacts with Glu-327, which stabilizes the tetrahedral glutamine adduct in the transition state and accepts a proton from the adduct to form glutamine (9). These residues, which are central to the enzymatic mechanism of glutamine synthetases, are changed in γ-glutamyl-isopropylamide synthetase to glutamate (Glu-74 in IpuC) and to tryptophan (Trp-324 in IpuC), respectively. In contrast to glutamine synthetases, IpuC accepted a range of amines as substrates. For example, ethylamine was glutamylated by the enzyme to γ-glutamyl-ethylamide (theanine), the active ingredient of green tea (12), and this reaction may provide the basis for developing a production process for a compound of commercial interest (6).

The pathway from isopropylamine to L-alaninol consumes 1 mol of ATP per mol of substrate degraded. A more energy-efficient version of the pathway would involve transfer of the glutamyl residue of γ-glutamyl-L-alaninol onto isopropylamine, rather than its hydrolytic removal. Based on amino acid sequence analysis it is unlikely that IpuF is able to catalyze such a glutamyl-transfer reaction. However, it cannot be excluded that a host enzyme encoded outside of the *ipu* gene clusters acts as glutamyl-transferase and thereby participates in isopropylamine degradation. The IpuF enzyme is about half the size of guanosine 5′-phosphate synthetase of *E. coli*, and its sequence aligns with the N-terminal part, the amidotransferase domain (31), of the latter. The catalytically important residues in amidotransferases, Cys-86 and His-181 (*E. coli* numbering), are present in IpuF as Cys-104 and His-200, thus suggesting that the mechanism of hydrolysis of γ-glutamyl-L-alaninol is similar to that for the hydrolysis of glutamine of amidotransferase enzymes (20).

The *ipuABCDEFGH* gene cluster was found to be flanked by two putative IS elements (Fig. 1), features reminiscent of a catabolic transposon for isopropylamine degradation. ISL, the left-hand element, exhibited sequence similarity to IS *2* (8), but inverted repeats and the start codon of a putative transposase gene could not be identified. The sequence of ISR was similar to that of IS *3* (8). It consisted of a 1,334-bp DNA sequence with similarity to genes encoding transposition proteins. This sequence was interrupted by several frameshifts, and it contained flanking 30-bp imperfect inverted repeats. These features and the absence of target site duplications resulting from transposon insertion make it appear unlikely that the apparent *ipu* transposon is functional. However, the possibility that it becomes transposable by site-specific recombination with an element containing a functional transposase gene cannot be excluded (28). Support for the view that the *ipu* genes have been introduced into *Pseudomonas* sp. strain KIE171 by lateral transfer is provided by their GC content of 51%. This value differs significantly from the overall GC content of members of the genus *Pseudomonas*, which varies between 58 and 68% (19).

Sequencing of the genes involved in aniline degradation by *P. putida* (11) and *Acinetobacter* sp. strain YAA (26) has revealed ORFs that encode, in addition to a putative aniline oxygenase, a protein with homology to glutamine synthetases (TdnQ and AtdA1, respectively) and one with homology to the amidotransferase domain of GMP synthetases (TdnT and AtdA2, respectively). These proteins were proposed to transfer the amino group of the substrate to an unknown acceptor or to release ammonia (11). However, by analogy to the isopropylamine degradation pathway described in the present study, it seems more likely that they are involved in the *N*-glutamylation of aniline and in the subsequent hydrolytic removal of glutamate, respectively. The principle of protecting an amino

group by a glutamyl residue prior to hydroxylation of a neighboring carbon atom thus appears to be followed in at least one other degradative pathway for a primary amine.

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