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# Biosynthesis of the thiamin pyrimidine: the reconstitution of a remarkable rearrangement reaction

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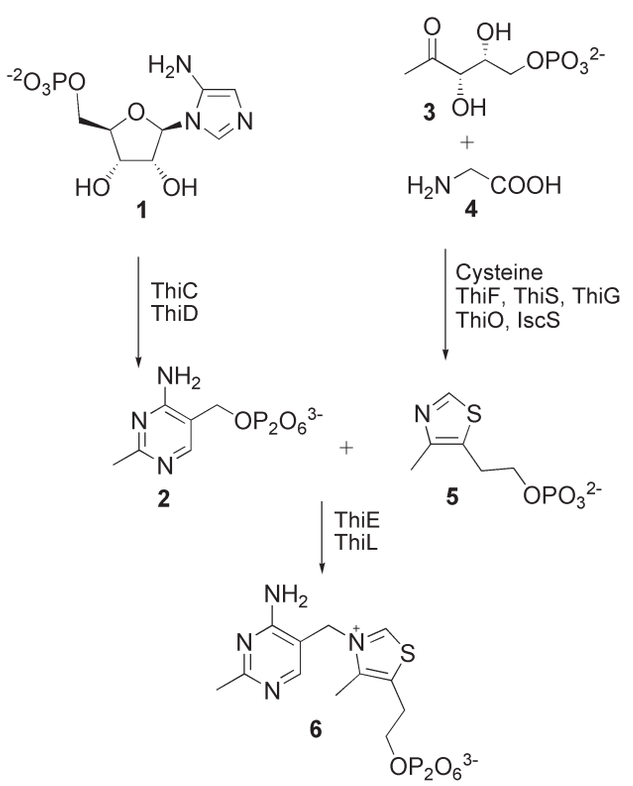
The conversion of 5-aminoimidazole ribonucleotide (AIR) into 4-amino-2-methyl-5-hydroxymethylpyrimidine (HMP) is a fascinating reaction on the thiamin biosynthetic pathway in bacteria and is probably the most complex unresolved rearrangement in primary metabolism. We have successfully reconstituted this reaction in a cell-free system. The *E. coli thiC* gene product and an additional unidentified *E. coli* protein are required for the reaction. In addition, SAM and nicotinamide cofactors are required for full activity. Labeling studies to determine the origin of most of the atoms in the pyrimidine are described. Based on these studies, a new mechanism for HMP formation is proposed.

## Introduction

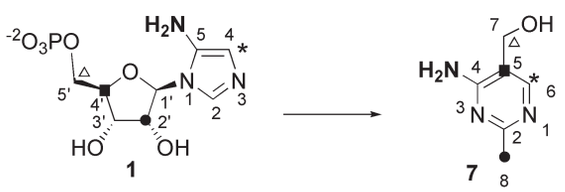
Thiamin pyrophosphate (TPP, **6**)[1] is an essential cofactor in all living systems and plays a central role in metabolism. The biosynthesis of the vitamin in *B. subtilis* is outlined in Scheme 1.[2–6] 4-(β-Hydroxyethyl)-5-methylthiazole phosphate (Thz–P, **5**), formed in a complex condensation from 1-deoxy-D-xylulose-5-phosphate (**3**), glycine (**4**), and cysteine,[7] is coupled to 4-amino-2-methyl-5-hydroxymethylpyrimidine pyrophosphate (HMP–PP, **2**) to give thiamin phosphate (TMP, **8**). A phosphorylation catalyzed by ThiL provides the active cofactor TPP (**6**).




**Scheme 1**

The precursor to 4-amino-2-methyl-5-hydroxymethylpyrimidine (HMP, **7**) has been identified as 5-aminoimidazole ribonucleotide (AIR, **1**), an intermediate of purine nucleotide biosynthesis.[8] While AIR is phosphorylated, it is unclear whether the direct product of the pyrimidine-forming reaction is HMP or HMP phosphate (HMP–P). However, since ThiD can catalyze the phosphorylation of HMP to HMP–P and of HMP–P to HMP–PP,[9] we will assume here that HMP is the first product of the pyrimidine biosynthesis.

Isotopic labeling studies, using whole cells and labeled precursors to AIR, indicate that all of the carbon and nitrogen atoms of HMP (**7**) originate from AIR (**1**).[10–14] These labeling studies (Scheme 2) suggest that C5 of the pyrimidine originates from C4′ of AIR, that the C2 methyl substituent originates from C2′ of AIR, and that the nitrogens of the aminoimidazole are retained in the pyrimidine. However, because these labeling studies suggest that HMP is formed by complex chemistry that is without precedent in the biosynthetic or organic chemistry literature, this proposal must be viewed as tentative until the labeling studies can be verified in a defined cell-free system using labeled AIR instead of metabolic precursors.



**Scheme 2**

The *thiC* gene complements all HMP requiring mutants in *E. coli*, *S. typhimurium*, and *B. subtilis*.[15,16] Here we describe the overexpression of *thiC* and the first reconstitution of the rearrangement of AIR to HMP in a cell-free system. An initial biochemical characterization of the reaction and a comprehensive set of labeling experiments are presented.

## Results

### Overexpression of *thiC*

The *E. coli thiC* gene was cloned into pET17b for overexpression in the *purC*⁻ *E. coli* strain CAG18470 (DE3). SDS-PAGE analysis demonstrated that ThiC can be highly expressed as a soluble 68 kDa protein (Fig. 1). Purification of ThiC resulted in inactive protein. Activity could not be restored by adding cell-free extract to the purified protein. Therefore, cell-free extract derived from the *thiC* overexpression strain was used as the source of ThiC for biochemical assays.

### Reconstitution of the AIR (1) to HMP (7) conversion

A sensitive assay to detect the formation of HMP was developed (Scheme 3), using HMP–P kinase (ThiD)[8] and thiamin phosphate synthase (ThiE)[17] to convert HMP to TMP (**8**). TMP (**8**) was then oxidized to thiochrome phosphate (Thc–P, **9**) which is highly fluorescent and easy to detect.[7,18] Thc–P was isolated and quantified using HPLC with fluorescence detection.

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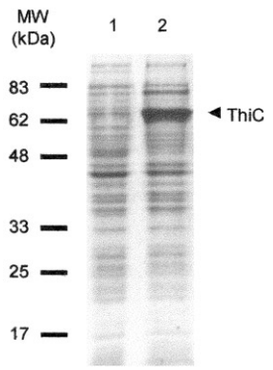
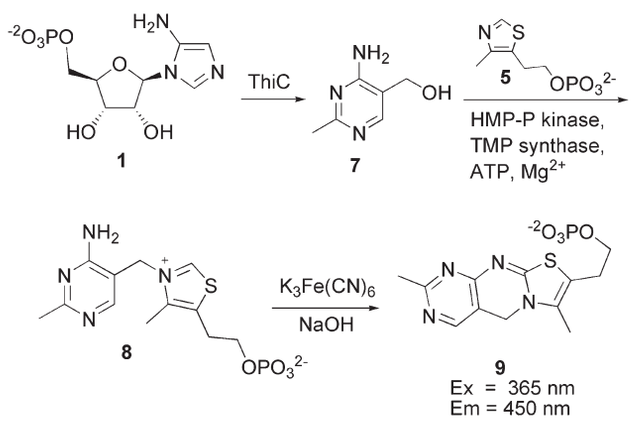



**Fig. 1** SDS-PAGE analysis of the overexpression of ThiC. Lane 1 = soluble extract from CAG18470 (DE3), Lane 2 = soluble extract from the *thiC* overexpression strain.




**Scheme 3**

In our reconstitution reaction, the ThiC protein preparation was incubated with AIR, SAM, NADH, NADPH, and an *E. coli* protein preparation lacking ThiC.[19] The reaction was analyzed for HMP formation as shown in Scheme 3. Under these conditions, HMP was formed in a time dependent fashion (Fig. 2a). After 3 h, 4 nmol of HMP were formed (20% conversion of 20 nmol of AIR). When AIR or ThiC is removed from the reaction mixture, no HMP synthesis can be detected. Furthermore, full activity is dependent on NADH, NADPH and SAM (Fig. 2b). NAD could also fulfill the nicotinamide cofactor requirement. HMP formation is also dependent on an additional *E. coli* protein in a concentration dependent manner (Fig. 2c). When the reaction was carried out under oxygen depleted conditions, no change in activity was observed (Fig. 2b).

### The methyl group of SAM is not incorporated into HMP

SAM generally functions as a methyl donor, so we considered the possibility that a carbon of HMP could originate from SAM. When SAM was replaced with [methyl-$^{14}$C]SAM (5.5 μCi) in the reaction mixture and the thiochrome was purified by HPLC and analyzed by scintillation counting, radioactivity associated with the thiochrome peak was equivalent to background levels (60 cpm).

### 5′-Deoxyadenosine is not present in HMP forming reactions

An alternative role for SAM is that it serves as a source of the 5′-deoxyadenosyl radical. Several examples of reactions mediated by a SAM-derived adenosyl radical have been identified, and these enzymes generate 5′-deoxyadenosine during the course of catalysis.[20] Thus, we examined the HMP forming reaction for the presence of 5′-deoxyadenosine. In a reconstitution mixture containing [2,8-$^{3}$H]SAM (87 μCi), 1.3 nmol of HMP were formed after 3 h. An identical reaction mixture was subjected to HPLC analysis to detect the formation of [2,8-$^{3}$H]5′-deoxyadenosine.

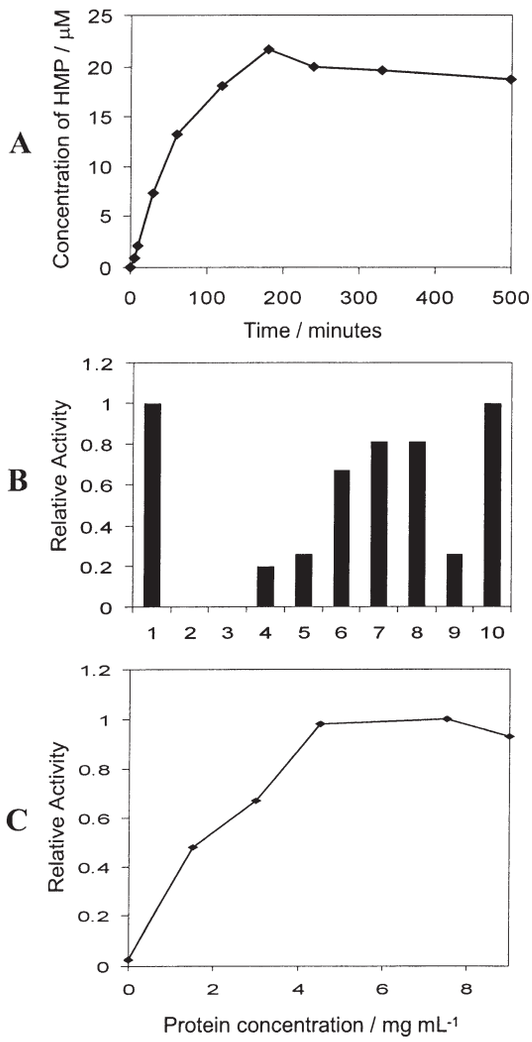



**Fig. 2** *In vitro* reconstitution of HMP biosynthesis from AIR. Panel A: Time-dependent production of HMP. Panel B: Reaction requirements. 1 = AIR, SAM, NADH, NADPH, ThiC, and an *E. coli* protein extract lacking ThiC,[19] 2 = Reaction 1 without ThiC, 3 = Reaction 1 without AIR, 4 = Reaction 1 without SAM, 5 = Reaction 1 without NADH and NADPH, 6 = Reaction 5 + NADPH, 7 = Reaction 5 + NADH, 8 = Reaction 5 + NAD, 9 = Reaction 1 without *E. coli* protein extract, 10 = Reaction 1 without oxygen. Panel C: Reaction 9 + varying amounts of *E. coli* protein extract.[19]

We could not detect any radioactivity associated with the peak corresponding to 5′-deoxyadenosine. Based on a conservative limit of detection of 1500 cpm for [2,8-$^{3}$H]5′-deoxyadenosine (and an observed 15% counting efficiency for [2,8-$^{3}$H]SAM), less than 0.01 nmol of 5′-deoxyadenosine were formed. This result suggests that SAM is not being cleaved to the adenosyl radical. Thus, the role of SAM in the HMP biosynthetic reaction is unclear.

### Synthesis of $^{13}$C-,$^{15}$N-, and $^{2}$H-labeled AIR

Labeled AIR was synthesized as shown in Scheme 4. Following closely the method of Ramsden,[21] 1,2,3,5-tetraacetyl ribofuranose (**13**) was coupled with the 4-nitroimidazole silver salt (**11**) to form the glycoside bond. Deprotection and reduction of the nitro group afforded 5-aminoimidazole ribonucleoside (AIRs), which was directly phosphorylated using Leonard's method.[22] [2′-$^{13}$C]AIR, [5′-$^{13}$C]AIR, [1′-$^{2}$H]AIR, [2′-$^{2}$H]AIR, [3′-$^{2}$H]AIR, [4′-$^{2}$H]AIR, and [5′,5′-$^{2}$H$_2$]AIR (Fig. 3) were obtained by starting with appropriately labeled D-ribose. For the synthesis of [amino-$^{15}$N]AIR (Fig. 3), imidazole was nitrated using H$^{15}$NO$_3$, and the silver salt of the resulting [nitro-$^{15}$N] 4-nitroimidazole was coupled to the protected ribose.

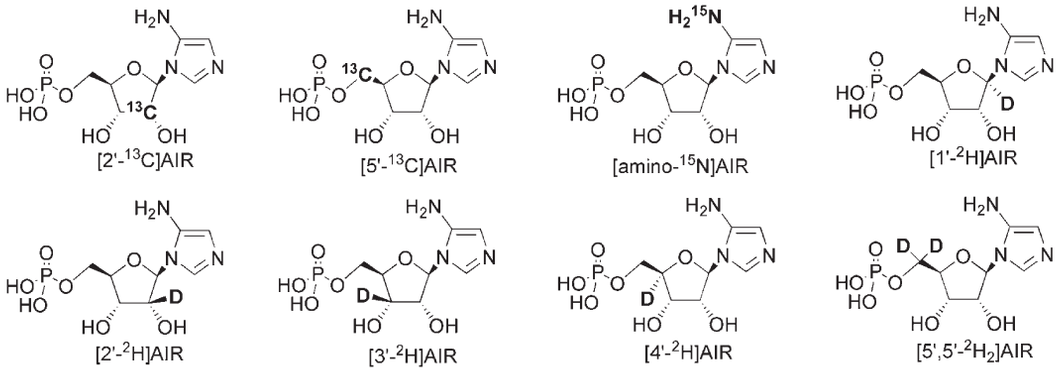
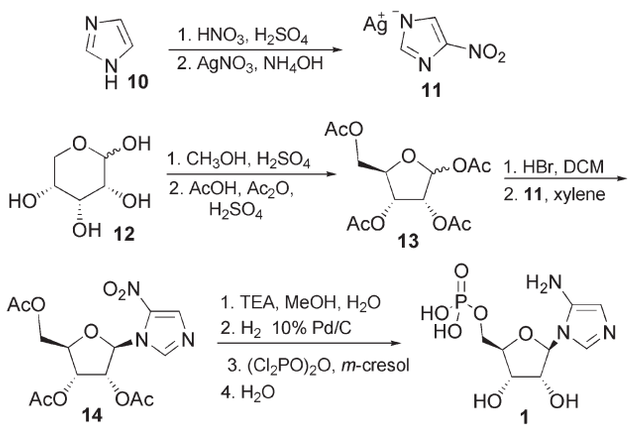



**Fig. 3** Structures of labeled 5-aminoimidazole ribonucleotides used to identify the atom transfer from AIR to HMP.




**Scheme 4**

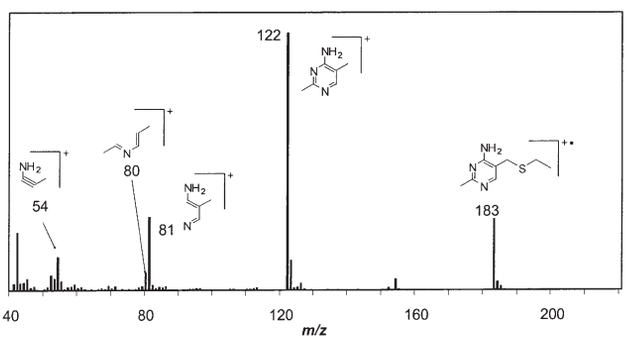
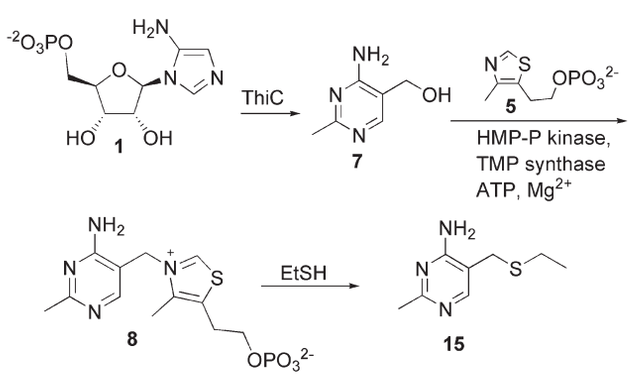



**Fig. 4** Mass spectrum of unlabeled 2-methyl-4-amino-5-[(ethylthio)-methyl]pyrimidine (ETMP, **15**).

## Label incorporation from AIR into HMP

To confirm and extend the labeling pattern of Scheme 2, labeled AIR (**1**) was treated under the conditions described for the *in vitro* formation of HMP (**7**), and the resulting HMP (**7**) was converted *in situ* to TMP (**8**). The thiamin phosphate in the reaction mixture was cleaved with ethanethiol to provide 2-methyl-4-amino-5-[(ethylthio)methyl]pyrimidine (ETMP, **15**)(Scheme 5), which was extracted into methylene chloride and analyzed by GC-MS.[23] NMR analysis was not possible because our reconstitution system does not yet give sufficient quantities of the pyrimidine. Fig. 4 illustrates a typical mass spectrum obtained for ETMP (**15**) synthesized from AIR (**1**), and the identities of the major fragments (*m*/*z* = 54, 80, 81, 122) are indicated.[23]

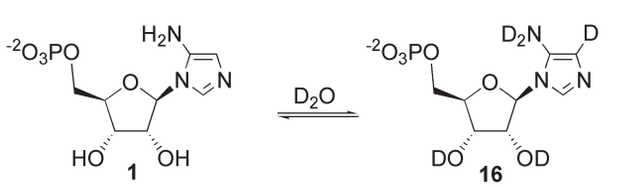



**Scheme 5**

In the *m*/*z* = 122 fragment, resulting from loss of EtS, all of the atoms originating from HMP are retained. The *m*/*z* = 81 fragment contains N1, C4, C5, C6, C7 and the amino group of the pyrimidine. Atoms N1, C2, C5, C6, C7, and C8 of the pyrimidine are retained in the *m*/*z* = 80 fragment while the *m*/*z* = 54 fragment contains only atoms C4, C5, C7, and the amino group. Isotopic label incorporation into the assigned fragments thus provides information regarding the position of labeled atoms on HMP.

The results of the AIR labeling studies are presented in Fig. 5 and 6. When [2′-$^{13}$C]AIR is used in the *in vitro* reconstitution system, $^{13}$C-ETMP is formed (Fig. 5a). Only the fragment corresponding to *m*/*z* = 80 is labeled, thus the C2′ carbon of AIR must be incorporated into either C2 or C8 of the pyrimidine. This ambiguity is clarified below. Using [5′-$^{13}$C]AIR, $^{13}$C-ETMP is also formed (Fig. 5b). All of the major ions in the spectrum are shifted by *m*/*z* = 1. Since C5 and C7 are the only carbons retained in each of the identified fragment ions, the label must be on one of these atoms. This ambiguity is also clarified below. When [amino-$^{15}$N]AIR is incubated under HMP forming conditions and derivatized, $^{15}$N-ETMP is formed (Fig. 5c). Analysis of its mass spectrum shows that all of the fragments except for *m*/*z* = 80 are labeled. Thus, the 4-amino nitrogen of the pyrimidine comes from the 5-amino nitrogen of AIR.

When the pyrimidine-forming reaction was run in deuterated buffer, GC-MS analysis of the resulting ETMP demonstrated the incorporation of two deuterium atoms into the pyrimidine moiety (Fig. 6a). The fragmentation pattern is consistent with the localization of one deuterium on C6 and one on C8. The deuterium at C6 is expected to arise from a deuterium at C4 of AIR (which rapidly exchanges with solvent, Scheme 6). That only one deuterium from solvent is incorporated into the methyl substituent is particularly interesting as it is contrary to previous mechanistic predictions for the reaction[2,4,24] and suggests the possibility that one of the hydrogen atoms of the methyl group originates from C2′ of AIR. In support of this, when [2′-$^{2}$H]AIR was used in the reconstitution reaction, $^{2}$H-ETMP was formed (Fig. 6b). The position of the label can be reliably assigned to C8 of the pyrimidine, as only the *m*/*z* = 80 fragment is labeled. This result also demonstrates that the C2′ carbon of AIR is transferred to C8 rather than C2 of the pyrimidine.




**Scheme 6**

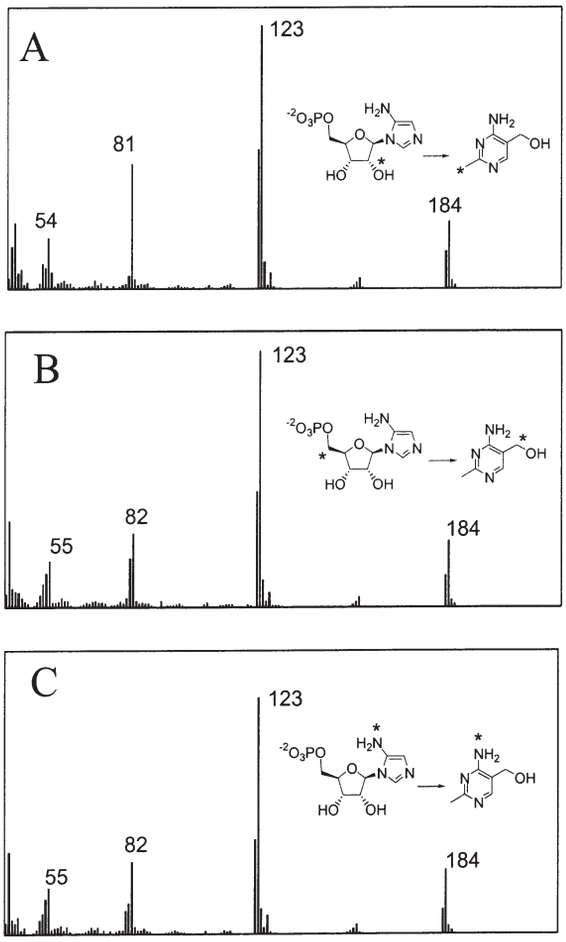



**Fig. 5** Mass spectral analysis of 2-methyl-4-amino-5-[(ethylthio)methyl ]pyrimidine (ETMP, **15**). Panel A: ETMP derived from [2′-$^{13}$C]AIR. Panel B: ETMP derived from [5′-$^{13}$C]AIR. Panel C: ETMP derived from [amino-$^{15}$N]AIR. The relatively high signal corresponding to non-labeled ETMP results from backgroud TMP and TPP in assay mixtures.

In order to determine whether C5′ of AIR provides C5 or C7 of the pyrimidine, [5′,5′-$^{2}$H]AIR was used as the enzymatic substrate. Surprisingly, only one deuterium was observed in the product pyrimidine (Fig. 6c). Since the $m/z = 54$ fragment has shifted to $m/z = 55$, the deuterium is clearly on C7 of the pyrimidine, and this supports the assignment that C7 of the pyrimidine arises from C5′ of AIR.

Since the origin of one hydrogen atom at C7 and one at C8 of HMP were still undetermined, we hypothesized that some of the other hydrogen atoms of AIR might be transferred to HMP. When [3′-$^{2}$H]AIR was used as the enzymatic substrate, the deuterium was observed in the resulting ETMP (Fig. 6d). Only the $m/z = 80$ fragment was labeled, indicating that the C3′ hydrogen of AIR is transferred to C8 of HMP. Unlabeled ETMP ($M^+ = 183$) is formed from reaction mixtures containing [1′-$^{2}$H]AIR or [4′-$^{2}$H]AIR (spectra not shown).

In all of our labeling experiments, a relatively high background signal from non-labeled ETMP is observed. This ETMP results from background thiamin in the reactions that is converted to non-labeled ETMP during the assay procedure. Specifically, TMP bound to thiamin phosphate synthase (ThiE) and protein-bound TPP from cell-free extracts contaminate the reactions. In each labeling experiment, the quantity of non-labeled ETMP corresponds exactly to the amount of background thiamin in the reaction mixture and does not represent dilution of the isotopic label during HMP biosynthesis.

### None of the HMP hydrogens originate from NADH

When the nicotinamide cofactors in the reaction mixture were replaced with [4*R*-$^{2}$H]NADH or [4*S*-$^{2}$H]NADH, the mass of

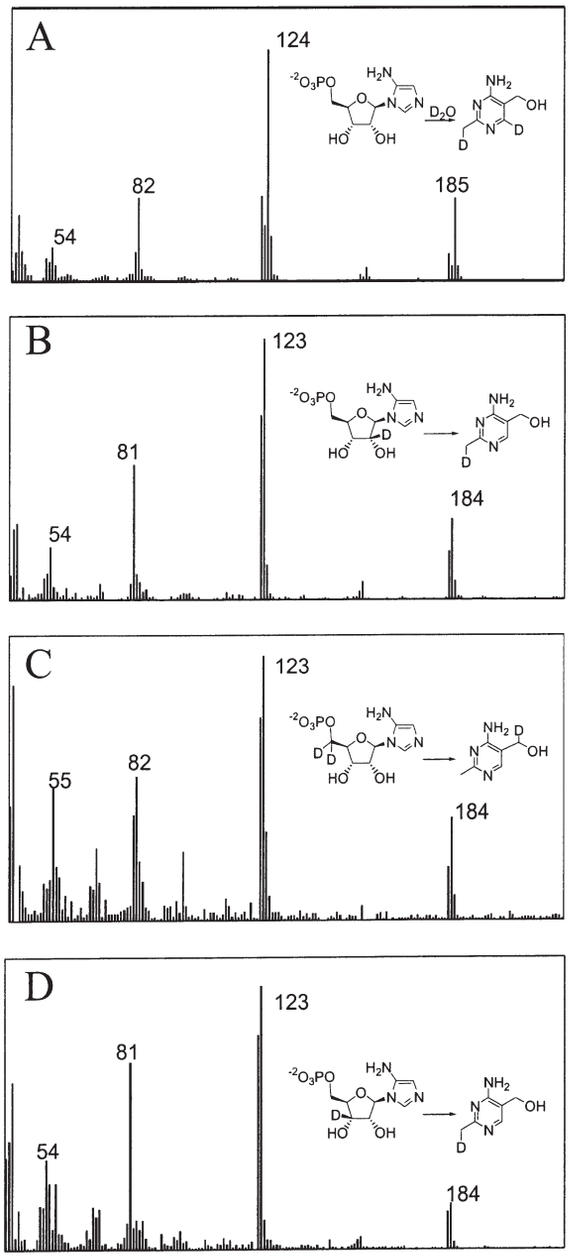



**Fig. 6** Mass spectral analysis of 2-methyl-4-amino-5-[(ethylthio)methyl]-pyrimidine (ETMP, **15**). Panel A: ETMP derived from unlabeled AIR in $D_2O$. Panel B: ETMP derived from [2′-$^{2}$H]AIR. Panel C: ETMP derived from [5′,5′-$^{2}$H$_2$]AIR, Panel D: ETMP derived from [3′-$^{2}$H]AIR. The relatively high signal corresponding to non-labeled ETMP results from background TMP and TPP in assay mixtures.

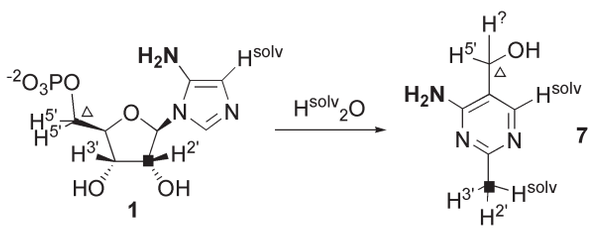
the resulting ETMP corresponded to the unlabeled compound ($M^+ = 183$)(spectra not shown). This demonstrates that under our assay conditions none of the HMP protons are derived from NAD(P)H. This result does not rigorously exclude the possibility of a hydride transfer from NADH to HMP because the cell-free extract may contain dehydrogenases that catalyze the exchange of the deuterium from the labeled cofactor. In an attempt to prevent alcohol dehydrogenase-mediated deuterium-to-hydrogen exchange of [4-$^{2}$H]NADH, $D_5$-ethanol (1% v/v) was added to the reaction mixture. However, deuterium incorporation into HMP was still not observed.

## Discussion

The biosynthesis of the pyrimidine moiety of thiamin from AIR is a complex process. In this paper, we describe the first successful reconstitution of this biosynthesis in a cell-free system. The reconstitution requires the ThiC protein and is enhanced by the nicotinamide cofactors and SAM (Fig. 2B). In addition, HMP

formation is enhanced by adding cell-free extract from *E. coli* grown in the presence of thiamin to repress transcription of the *thiC* gene (Fig. 2C). This suggests that an additional protein may be required for HMP biosynthesis.

The labeling pattern for the conversion of AIR (**1**) to HMP (**7**) is remarkable and there is no biochemical or chemical precedent for a transformation of this kind (Scheme 2). Since most of the previous labeling studies were carried out using labeled glucose in whole cells, the possibility that the unusual labeling pattern was due to an unanticipated reaction in the conversion of glucose to HMP had to be rigorously excluded. The availability of a cell-free pyrimidine biosynthesis system enabled us to do this and to expand on these labeling experiments. Our studies using $^{13}$C-, $^{15}$N-, and $^{2}$H-AIR and deuterated buffer are summarized in Scheme 7 and are in complete agreement with the labeling studies in the literature demonstrating that C8 of the pyrimidine originates from C2′ of AIR,[12] C7 of the pyrimidine originates from C5′ of AIR,[10] and the amino nitrogen of AIR is retained as the amino nitrogen in HMP.[13] In addition, our studies demonstrate that one of the C5′ protons of AIR is removed and replaced with a proton of still unknown origin. We also demonstrate that the pyrimidine methyl protons are derived from the C2′ hydrogen of AIR, the C3′ hydrogen of AIR, and the reaction buffer. These new results are not consistent with any of the previous mechanistic proposals for HMP formation.[2,4,24]



**Scheme 7**

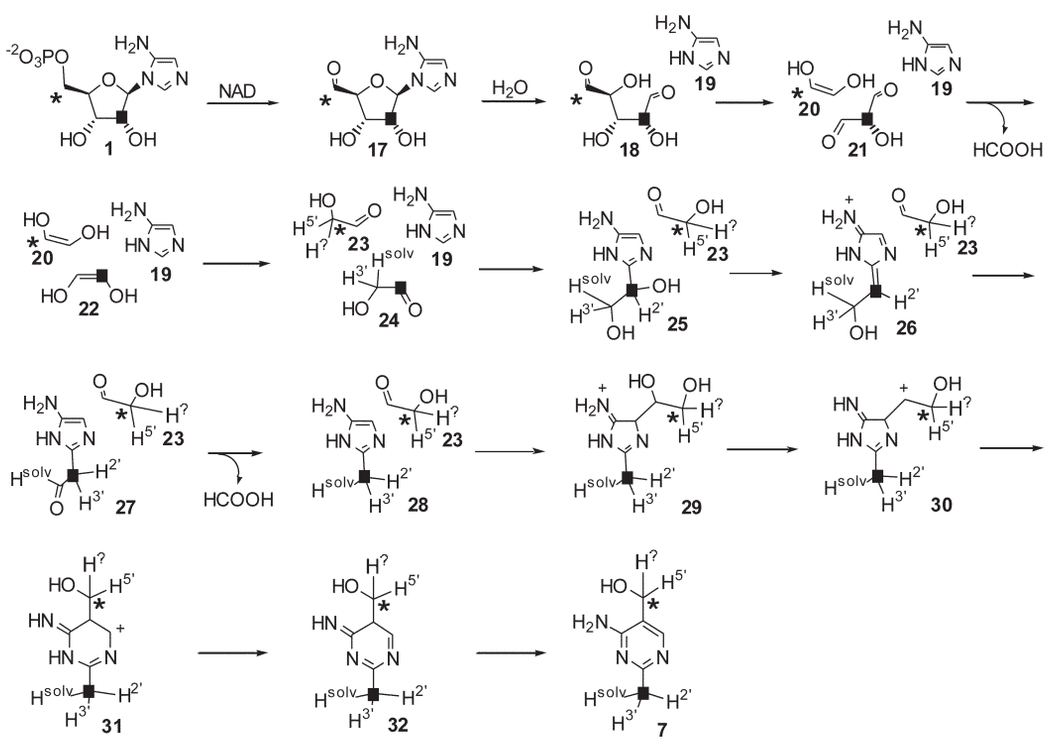
The labeling studies demonstrate that the conversion of AIR (**1**) to HMP (**7**) involves extensive fragmentation of the ribose. In a formal sense, the C2′ carbon of the ribose is reduced and used to methylate the C2 carbon of the aminoimidazole. The C4′ carbon of the ribose is inserted into the C4–C5 bond of the aminoimidazole, thus expanding the five membered imidazole ring to a six membered pyrimidine ring.

A number of mechanisms can be proposed that are consistent with our labeling studies. However, in the absence of experimental data to differentiate between them, we will only consider one of these here (Scheme 8). In this mechanism, dephosphorylation of AIR (**1**) followed by oxidation of the resulting alcohol gives aldehyde **17**. This oxidation is consistent with the loss of one C5′ hydrogen from AIR during pyrimidine formation. N–glycosyl bond cleavage[25] followed by a retroaldol reaction[26] gives **19**, **20**, and **21**. Loss of C1′ as formate followed by tautomerization of enediols **20** and **22** generates the C5′- and C2′-containing carbon fragments required for the assembly of HMP. Electrophilic substitution at C2 of the aminoimidazole by **24** gives **25**. This reaction connects the C2′ carbon of the ribose and the C2 carbon of the imidazole as required by the labeling studies.[27] Loss of water to generate the stabilized carbocation **26** followed by a hydride shift would give **27**. This hydride shift is consistent with the observed transfer of the H3′ proton of AIR to the methyl group of HMP (**7**). Loss of formate from **27**, with the observed incorporation of one proton from solvent, completes the biogenesis of the methyl group of HMP. The next set of steps in our proposal address the mechanism of the ring expansion reaction. Addition of aldehyde **23** to C4 of the aminoimidazole would give **29**.[27] Loss of water to form cation **30** followed by a 1,2-shift would give **31**. Aromatization of **31** would complete the formation of HMP (**7**). This mechanistic proposal predicts that one of the methylene protons on HMP should come from the buffer and we do not yet understand why we do not observe this. In addition, our mechanistic proposal does not explain the SAM requirement of the reaction. The proposal does predict that aldehyde **17**, and aminoimidazoles **25**, **27** and **29** are intermediates and we are currently synthesizing these compounds.

## Experimental

### Materials

Chemicals were purchased from Sigma-Aldrich except as noted. Deuterium oxide, [2′-$^{13}$C]D-ribose, [5′-$^{13}$C]D-ribose, and [$^{15}$N] nitric acid were purchased from Cambridge Isotope Laboratories, and [1′-$^{2}$H]D-ribose, [2′-$^{2}$H]D-ribose and [3′-$^{2}$H]D-ribose, [4′-$^{2}$H]D-ribose and [5′,5′-$^{2}$H$_2$]D-ribose were from Omicron Biochemicals. *S*-adenosylmethionine hydrogen sulfate *p*-toluenesulfonate salt



**Scheme 8**

was purchased from USB. [Methyl-$^{14}$C]*S*-adenosylmethionine was obtained from ICN pharmaceuticals. [2,8-$^{3}$H] ATP was purchased from Amersham Biosciences. AdoMet synthetase was prepared from the overproducing strain DM22(pK8) as described previously.[28] HMP (**7**), Thz–P (**5**), thiamin phosphate synthase, and HMP–P kinase were prepared as described previously.[9,17,29] Unlabeled AIR (**1**) was synthesized from 5-aminoimidazole-4-carboxamide ribonucleotide (Sigma) as described previously.[30] Yeast alcohol dehydrogenase, glutamate dehydrogenase (bovine liver Type III), alkaline phosphatase, and lysozyme were from Sigma. The *purC*$^{-}$ *E. coli* strain CAG18470 (*purC*80::Tn10 *rph-1*), obtained from CGSC, was converted to CAG18470 (DE3) using Novagen's λ(DE3) lysogenization kit. Oligonucleotide synthesis and DNA sequencing were carried out at the Cornell BioResource Center.

### Cloning

The *thiC* gene was amplified by PCR from the plasmid pVJS716[31] using the following primers: 5′-CAGTCTGCGGCTTGAGT-3′ and 5′-CCTTAACTTTTTGGAATGACATATGTCTGCAA-CAAAACTG-3′. The 5′-primer inserts an *Nde*I restriction site (underlined) directly upstream of the *thiC* gene. The first 500 bp of *thiC* from the PCR product were cloned into pET17b (Novagen) using the *Nde*I/*Eco*RI sites. The remainder of the *thiC* gene (derived from pVJS722[31]) was inserted into the expression vector. The bulk of the PCR-derived DNA was exchanged with the corresponding *thiC* from pVJS722 using the *SmaI*/*EcoRI* restriction sites. The remaining 40 bps of PCR derived DNA were sequenced and shown to contain no base mutations. A representative plasmid, named pC816, was transformed into *E. coli* CAG18470 (DE3).

### Overexpression of *thiC* and preparation of *E. coli* protein extract

*E. coli* CAG18470 (DE3) carrying pC816 was grown in 0.5 L of minimal media (E salts, 0.5% glucose) supplemented with adenosine (118 mg L$^{-1}$), guanosine (8.5 mg L$^{-1}$), uracil (11 mg L$^{-1}$), tetracycline (15 μg mL$^{-1}$) and ampicillin (200 μg mL$^{-1}$) at 37 °C until the $OD_{600\ nm}$ = 0.5, at which time the temperature was decreased to 20 °C and adenosine (400 mg) was added to de-repress the thiamin biosynthetic genes.[32] Twenty minutes later, IPTG (1 mM) was added to induce expression of the recombinant *thiC*. Two hours after induction, cells were harvested and stored at −80 °C. Immediately prior to assaying for HMP formation, the cell pellet was resuspended in 3 mL of assay buffer (100 mM Tris–HCl, 5 mM $MgCl_2$ pH 8.0) and lysed by treatment with lysozyme (5 mg, 15 min on ice) followed by sonication. After centrifugation, the cleared lysate was buffer exchanged on a PD-10 column (Amersham Biosciences) using assay buffer, and the resulting protein preparation (10–15 mg mL$^{-1}$ total protein) was used as the source of ThiC in biochemical assays. An *E. coli* protein extract (lacking ThiC) was prepared similarly from CAG18470 (DE3) except growth was carried out in LB media (Gibco BRL) supplemented with tetracycline (15 μg mL$^{-1}$) at 37 °C without addition of IPTG. The cells were harvested 5 h after inoculation ($OD_{600\ nm}$ ~ 1.5) and stored at −80 °C.

### *In vitro* reconstitution of HMP biosynthesis

Enzymatic reactions were carried out in a 200 μL total volume containing (optimized concentrations) AIR (0.1 mM), SAM (1 mM), NADH (2 mM), NADPH (1 mM), Thz–P (0.5 mM), HMP–P kinase (0.1 mg mL$^{-1}$), thiamin phosphate synthase (0.1 mg mL$^{-1}$), 100 μL of the ThiC cell extract (10–15 mg mL$^{-1}$) and 72 μL of the *E. coli* protein preparation (10–15 mg mL$^{-1}$) described above. The reactions were initiated by addition of the protein extracts followed by thorough mixing. Following incubation at 37 °C for 3 h (except as noted), the reactions were quenched by the addition of 200 μL of 10% trichloroacetic acid. Protein was removed by centrifugation followed by filtration through a Microcon 10 membrane (Millipore). 100 μL of the filtrate was neutralized with 50 μL of 4 M potassium acetate then subjected to thiochrome derivitization by addition of 50 μL of alkaline $K_3Fe(CN)_6$ (30 mg mL$^{-1}$ in 7 M NaOH) and incubation at room temperature for 1 min. The sample was then neutralized with 57 μL of 6 M HCl. 100 μL of the resulting sample was analyzed by HPLC using a Supelcosil LC-18-T 15 cm × 4.6 mm, 3 μm column equilibrated with 5 mM $K_2HPO_4$ pH 6.6, 0.4 mM tetrabutylammonium hydrogen sulfate, 5% methanol. Thiochrome phosphate (**9**) eluted after 21.2 min by increasing the methanol as follows: 0–3 min (5–10%), 3–20 min (10–40%), 20–24 min (40%). Chromatography was carried out at a flow rate of 1 mL min$^{-1}$ and monitored by in-line fluorescence detection (excitation = 365 nm, emission = 450 nm). A standard curve for quantitating HMP concentration was developed using reaction mixtures containing chemically synthesized HMP.

### *In vitro* HMP formation under oxygen depleted conditions

Buffers and reaction components were de-aerated by repeated vacuum/argon fill cycles or by bubbling argon through solutions. Cells were grown and harvested under anaerobic conditions, then under argon, cells were resuspended in de-aerated buffer, lysed and the lysate cleared by centrifugation. The clarified lysate was moved into a glove box and buffer exchanged on a PD-10 column into de-aerated assay buffer. The proteins were then added to the other reaction components and the mixtures incubated at 37 °C for 3 h in the anaerobic chamber, then quenched and analyzed as described above.

### Assay for $^{14}$C incorporation from [methyl-$^{14}$C]SAM into HMP

Enzymatic conversion of AIR to HMP was carried out as described above, except the SAM was replaced with 1 mM [methyl-$^{14}$C]SAM (58.5 mCi mmol$^{-1}$). After derivatization and neutralization, the thiochrome phosphate was purified from radioactive impurities as follows: The reaction mixture was loaded onto a Supelcosil LC-18-T 25 cm × 10 mm, 5 μm HPLC column equilibrated with 10 mM $K_2HPO_4$ pH 6.6, 0.4 mM tetrabutylammonium hydrogen sulfate. The fluorescent thiochrome phosphate, which was eluted using a gradient of equilibration buffer and methanol, was collected and concentrated to 3 mL *in vacuo*. The pH was adjusted to 10 with 4.5 M NaOH (45 μL), then $MgCl_2$ and alkaline phosphatase (2 mg, 140,000 units) were added and the mixture incubated overnight at room temperature. 1 mL of brine was added and the thiochrome extracted into isobutanol (3 × 2 mL). The combined extracts were evaporated to dryness under a stream of argon and the residue was taken up in water (400 μL) and injected onto a Supelcosil LC-18-T 25 cm × 10 mm, 5 μm HPLC column equilibrated with 10 mM $K_2HPO_4$ pH 6.6. The thiochrome was collected during elution using a gradient of the equilibration buffer and methanol. After evaporation of solvent *in vacuo*, the thiochrome was analyzed by HPLC as described above (retention time = 18 min) using in-line fluorescence detection followed by in-line scintillation counting.

### Synthesis of [2,8-$^{3}$H] *S*-adenosylmethionine

[2,8-$^{3}$H]SAM was prepared from [2,8-$^{3}$H]ATP and methionine with AdoMet synthetase by modifying a previously described procedure.[28,33] A 0.9 mL reaction mixture containing [2,8-$^{3}$H]ATP (1.11 mM, 1 Ci mmol$^{-1}$), methionine (2.22 mM), inorganic pyrophosphatase (0.25 units), 100 mM Tris–HCl pH 8.0, 50 mM KCl, 26 mM $MgCl_2$, 1 mM EDTA, 8% β-mercaptoethanol, and AdoMet synthetase extract (140 μL, 1.5 mg total protein) was incubated at room temperature for 5 h. The reaction was quenched by addition of 90 μL of 1 N HCl. After centrifugation to remove precipitated protein, the supernatant was loaded onto a Dowex 50WX8-100 column (0.7 mL bed volume) that had been charged with 6 N HCl and equilibrated with water. The column was washed with 10 mL of 1 N HCl, then SAM was eluted with 10 mL of 6 N HCl. The solvent was removed by rotary evaporation (40 °C) and the residue was dissolved in water and lyophilized. The resulting [2,8-$^{3}$H]-SAM (0.63 μmol, 1 Ci mmol$^{-1}$) co-eluted with authentic SAM (retention time = 5.5 min) and was 90% radiochemically pure

by HPLC analysis with in-line scintillation counting (Supelcosil LC-18-T 15 cm × 4.6 mm, 3 μm, 100 mM $K_2HPO_4$ pH 6.6, isocratic).

### Assay for [2,8-$^3$H]5′-deoxyadenosine

Enzymatic conversion of AIR to HMP was carried out as described above, except that SAM was replaced with [2,8-$^3$H]SAM (0.5 mM, 1 Ci mmol$^{-1}$). After a 3 h incubation, 5′-deoxyadenosine (20 nmol) was added and the mixture was filtered through a Microcon 10 membrane (Millipore). The filtrate was loaded onto an HPLC column (Supelcosil LC-18-T 15 cm × 4.6 mm, 3 μm) equilibrated with 50 mM $K_2HPO_4$ pH 6.6, 10% methanol. The column was washed with equilibration buffer for 15 min, then the methanol was increased linearly to 20% over 15 min. Under these conditions, SAM eluted after 2–5 min and 5′-deoxyadenosine eluted after 19–20 min. Chromatography was carried out at a flow rate of 1 mL min$^{-1}$ and monitored by UV absorption (254 nm) and in-line scintillation counting.

### Synthesis of labeled NADH

**[4*R*-$^2$H]NADH.** [4*R*-$^2$H]NADH was prepared from NAD (200 mg) and $D_6$-ethanol (1 mL) using yeast alcohol dehydrogenase (1750 units) in 20 mL of 0.5 M Tris (pH 10) at room temperature, as described previously.[34] When the reaction reached completion (5 h), enzyme was removed using a 10,000 MWCO membrane and [4*R*-$^2$H]NADH was purified on a MonoQ 5/5 column (Amersham) as described previously.[35] Reaction progress was monitored by HPLC (Supelcosil LC-18-T 15 cm × 4.6 mm, 3 μm column). The $^1$H NMR spectrum of [4*R*-$^2$H]NADH was consistent with that previously reported.[35]

**[4-$^2$H]NAD.** [4*R*-$^2$H]NADH (100 mg) was dissolved in 20 mL of 50 mM Tris pH 7.5. α-Ketoglutarate (58 mg), ammonium acetate (462 mg), and L-glutamate dehydrogenase (100 units) were added and incubation was carried out at room temperature. After 12 h the reaction was complete (by HPLC), and enzyme was removed using a 10,000 MWCO membrane. The crude mixture containing [4-$^2$H]NAD was used for subsequent reactions.

**[4*S*-$^2$H]NADH.** To the [4-$^2$H]NAD (100 mg) preparation described above was added Tris (100 mg), ethanol (0.5 mL), and yeast alcohol dehydrogenase (875 units) followed by a 3 h incubation at room temperature. [4*S*-$^2$H]NADH was purified on a MonoQ 5/5 column (Amersham) as previously described. The $^1$H NMR spectrum of [4*S*-$^2$H]NADH was consistent with that previously reported.[35]

### Synthesis of labeled AIR

**1,2,3,5-Tetra-*O*-acetyl ribofuranose (13).** 1,2,3,5-Tetraacetyl ribofuranose was prepared from D-ribose in 48% yield using a standard protocol and purified by flash column chromatography (1 : 3 cyclohexane : ether).[36,37]

[2-$^{13}$C] Tetraacetyl ribofuranose: $^1$H NMR ($CDCl_3$) $\delta$ 6.41 (m, 0.4, H1α), 6.15 (s, 0.6, H1β), 5.2–5.4 (m, 2, H2, H3), 4.0–4.5 (m, s, H4, H5, H5′) 2.10 (s, 3, $CH_3CO$), 2.07 (s, 3, $CH_3CO$), 2.06 (s, 3, $CH_3CO$), 2.04 (s, 3, $CH_3CO$). $^{13}$C NMR ($CDCl_3$) $\delta$ 74.4 ($^{13}$C enriched, C2β), 70.4 ($^{13}$C enriched, C2α).

[5-$^{13}$C] Tetraacetyl ribofuranose: $^1$H NMR ($CDCl_3$) $\delta$ 6.40 (d, 0.4, H1α), 6.13 (s, 0.6, H1β), 5.2–5.4 (m, 2, H2, H3), 4.2–4.6 (m, 3, H4, H5, H5′) 2.10 (s, 3, $CH_3CO$), 2.07 (s, 3, $CH_3CO$), 2.06 (s, 3, $CH_3CO$), 2.04 (s, 3, $CH_3CO$).

[1-$^2$H] Tetraacetyl ribofuranose: $^1$H NMR ($CDCl_3$) $\delta$ 5.2–5.4 (m, 2, H2, H3), 4.0–4.5 (m, H4, H5, H5′), 2.12 (s, 3, $CH_3CO$), 2.10 (s, 3, $CH_3CO$), 2.08 (s, 3, $CH_3CO$), 2.06 (s, 3, $CH_3CO$).

[2-$^2$H] Tetraacetyl ribofuranose: $^1$H NMR ($CDCl_3$) $\delta$ 6.41 (d, 0.3, H1α), 6.15 (s, 0.7, H1β), 5.3 (d, 0.7, H3β), 5.2 (d, 0.3, H3α), 4.1–4.5 (m, 3, H4, H5, H5′) 2.10 (s, 3, $CH_3CO$), 2.07 (s, 3, $CH_3CO$), 2.06 (s, 3, $CH_3CO$), 2.04 (s, 3, $CH_3CO$).

[3-$^2$H] Tetraacetyl ribofuranose: $^1$H NMR ($CDCl_3$) $\delta$ 6.41 (d, 0.3, H1α), 6.15 (s, 0.7, H1β), 5.2–5.3 (m, 1, H2), 4.1–4.5 (m, 3, H4, H5, H5′), 2.10 (s, 3, $CH_3CO$), 2.08 (s, 3, $CH_3CO$), 2.06 (s, 3, $CH_3CO$), 2.04 (s, 3, $CH_3CO$).

[4-$^2$H] Tetraacetyl ribofuranose: $^1$H NMR ($CDCl_3$) $\delta$ 6.41 (d, 0.3, H1α), 6.15 (s, 0.7, H1β), 5.2–5.5 (m, 2, H2, H3), 4.1–4.5 (m, 2, H5, H5′), 2.10 (s, 3, $CH_3CO$), 2.08 (s, 3, $CH_3CO$), 2.06 (s, 3, $CH_3CO$), 2.04 (s, 3, $CH_3CO$).

[5,5-$^2H_2$] Tetraacetyl ribofuranose: $^1$H NMR ($CDCl_3$) $\delta$ 6.40 (d, 0.3, H1α), 6.13 (s, 0.7, H1β), 5.2–5.4 (m, 2, H2, H3), 4.42 (d, 0.3, H4α), 4.36 (d, 0.7, H4β) 2.10 (s, 3, $CH_3CO$), 2.07 (s, 3, $CH_3CO$), 2.06 (s, 3, $CH_3CO$), 2.04 (s, 3, $CH_3CO$).

**[Nitro-$^{15}$N] 4-nitroimidazole.** 40% Aqueous [$^{15}$N] nitric acid (5 mL, 43.5 mmol) was added to imidazole (821 mg, 12.1 mmol) dropwise over 10 min at room temperature. The solution was cooled to 0 °C and concentrated $H_2SO_4$ (1.43 mL) was added in 300 μL portions over 10 min. The reaction mixture was heated at reflux for 19 h, then cooled and poured over ice (22 g). The precipitate was filtered, washed with cold water, and allowed to dry to a pale yellow solid (300 mg, 22%). $^1$H NMR ($D_6$-DMSO) $\delta$ 13.2 (br s, 1, NH), 8.3 (s, 1, H5), 7.8 (s, 1, H2).

**4-Nitroimidazole silver salt (11).** 4-Nitroimidazole silver salt was prepared as described previously.[21] To 4-nitroimidazole (300 mg, 2.6 mmol) in conc. $NH_4OH$ (2.6 mL) was added a silver nitrate solution (440 mg, 2.6 mmol in 2.6 mL of water) and the mixture was stirred for 15 min at room temperature. The yellow precipitate was filtered, washed (EtOAc/ether), then dried under vacuum at 56 °C to give 463 mg of yellow solid (81%).

**5-Nitro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazole[21] (14).** Tetraacetyl ribofuranose (178 mg, 0.56 mmol) was dissolved in 3 mL of dry $CH_2Cl_2$ and anhydrous HBr was bubbled through the mixture for 20 min at room temperature. The solvent was removed *in vacuo* and the resulting syrup was dissolved in 3 mL of dry xylene. 0.5 g of 4 Å molecular sieves were added to remove dissolved HBr. After 10 min, the solution was added dropwise to a mixture of 4-nitroimidazole silver salt (197 mg, 0.9 mmol) in xylene (7 mL) at reflux. The reaction mixture was refluxed for 1 h, cooled, and filtered. The solvent was evaporated *in vacuo* (45 °C bath) and the residue dissolved in 5 mL $CHCl_3$. The solution was washed ($NaHCO_3$, $H_2O$), dried ($Na_2SO_4$), and concentrated, and the product was purified by flash column chromatography (1 : 1 ethyl acetate : hexanes) to give 45 mg of a colorless oil (22%).

[2′-$^{13}$C] 5-Nitro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazole: $^1$H NMR ($CDCl_3$) $\delta$ 8.15 (s, 1, H2), 8.05 (s, 1, H4), 6.45 (t, 1, H1′), 5.70 (d of d, 0.5, H2′), 5.3 (m, 1.5, H2′, H3′), 4.5 (m, 1, H4′), 4.4 (d, 2, H5′, H5″), 2.15 (s, 6, 2 × $CH_3CO$), 2.05 (s, 3, $CH_3CO$). $^{13}$C NMR ($CDCl_3$) $\delta$ 75.1 ($^{13}$C enriched, C2′).

[5′-$^{13}$C] 5-Nitro-1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)imidazole: $^1$H NMR ($CDCl_3$) $\delta$ 8.15 (s, 1, H2), 8.05 (s, 1, H4), 6.46 (d, 1, H1′), 5.50 (m, 1, H2′), 5.32 (m, 1, H3′), 4.62 (d, 1, H5′), 4.47 (d of d, 1, H4′), 4.15 (d, 1, H5′), 2.16 (s, 6, 2 × $CH_3CO$), 2.07 (s, 3, $CH_3CO$). $^{13}$C NMR ($CDCl_3$) $\delta$ 61.8 ($^{13}$C enriched, C5′)

[Nitro-$^{15}$N] 5-nitro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazole: $^1$H NMR ($CDCl_3$) $\delta$ 8.15 (s, 1, H2), 8.05 (s, 1H), 6.43 (d, 1, H1′), 5.5 (d of d, 1, H2′), 5.3 (m, 1, H3′), 4.5 (m, 1, H4′), 4.4 (d, 2, H5′, H5″) 2.16 (s, 6, 2 × $CH_3CO$), 2.06 (s, 3, $CH_3CO$).

[1′-$^2$H] 5-Nitro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazole: $^1$H NMR ($CDCl_3$) $\delta$ 8.15 (s, 1, H2), 8.05 (s, 1, H4), 5.5 (d, 1, H2′), 5.3 (m, 1, H3′), 4.5 (m, 1, H4′), 4.4 (m, 2, H5′, H5″), 2.16 (s, 6, 2 × $CH_3CO$), 2.05 (s, 3, $CH_3CO$).

[2′-$^2$H] 5-Nitro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazole: $^1$H NMR ($CDCl_3$) $\delta$ 8.15 (s, 1, H2), 8.05 (s, 1, H4), 6.43 (s, 1, H1′), 5.3 (d, H3′), 4.5 (m, 1, H4′), 4.4 (m, 2, H5′, H5″), 2.16 (s, 6, 2 × $CH_3CO$), 2.05 (s, 3, $CH_3CO$).

[3′-$^2$H] 5-Nitro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazole: $^1$H NMR ($CDCl_3$) $\delta$ 8.15 (s, 1, H2), 8.05 (s, 1, H4), 6.43 (d, 1, H1′), 5.5 (d, 1, H2′), 4.5 (m, 1, H4′), 4.4 (m, 2, H5′, H5″), 2.15 (s, 6, 2 × $CH_3CO$), 2.05 (s, 3, $CH_3CO$).

[4′-$^2$H] 5-Nitro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazole: $^1$H NMR ($CDCl_3$) $\delta$ 8.15 (s, 1, H2), 8.05 (s, 1, H4), 6.45 (d, 1, H1′), 5.5 (d of d, 1, H2′), 5.3 (d, 1, H3′), 4.4 (s, 2, H5′, H5″), 2.15 (s, 6, 2 × $CH_3CO$), 2.05 (s, 3, $CH_3CO$).

[5′,5′-$^2H_2$] 5-Nitro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazole: $^1$H NMR ($CDCl_3$) $\delta$ 8.15 (s, 1, H2), 8.05 (s, 1, H4), 6.43 (s, 1, H1′), 5.45 (m, 1, H2′), 5.3 (m, 1, H3′), 4.42 (d, 1, H4′), 2.16 (s, 6, 2 × $CH_3CO$), 2.06 (s, 3, $CH_3CO$).

**5-Nitro-1-(β-D-ribofuranosyl)imidazole[21].** 5-Nitro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl) imidazole (15 mg, 0.04 mmol) was dissolved in methanol (0.8 mL), then water (0.1 mL) and triethylamine (0.1 mL) were added. The mix was stirred for 12 h at room temperature, then solvent was removed *in vacuo*. The residue was dissolved in 2 mL of water and lyophilized to provide 10 mg (99%) of a pale yellow oil.[21]

[2′-$^{13}$C] 5-Nitro-1-(β-D-ribofuranosyl)imidazole: $^1$H NMR ($CD_3OD$) $\delta$ 8.6 (s, 1, H2), 8.0 (s, 1, H4), 6.3 (m, 1, H1′), 4.4 (m, 0.5, H2′), 4.25 (m, 1, H3′), 4.1 (m, 1, H4′), 4.02 (m, 0.5, H2′), 4.0 (m, 1H, H5′), 3.8 (m, 1H, H5″). $^{13}$C NMR ($CD_3OD$) $\delta$ 77.5 ($^{13}$C enriched, C2′).

[5′-$^{13}$C] 5-Nitro-1-(β-D-ribofuranosyl)imidazole: $^1$H NMR ($CD_3OD$) $\delta$ 8.6 (s, 1, H2), 8.0 (s, 1, H4), 6.3 (s, 1, H1′), 4.4 (m, 1, H2′), 4.25 (m, 1, H3′), 4.1 (m, 1, H4′), 3.7 (d of d, 1, H5′), 3.5 (d of d, 1, H5″). $^{13}$C NMR ($CD_3OD$) $\delta$ 60.8 ($^{13}$C enriched, C5′).

[Nitro-$^{15}$N] 5-nitro-1-(β-D-ribofuranosyl)imidazole: $^1$H NMR ($CD_3OD$) $\delta$ 8.6 (s, 1, H2), 8.0 (s, 1, H4), 6.3 (s, 1, H1′), 4.2 (m, 2, H2′, H3′), 4.1 (m, 1, H4′), 4.0 (m, 1, H5′), 3.8 (m, 1, H5″).

[1′-$^2$H] 5-Nitro-1-(β-D-ribofuranoyl)imidazole: $^1$H NMR ($CD_3OD$) $\delta$ 8.6 (s, 1, H2), 8.0 (s, 1, H4), 4.2 (m, 2, H2′, H3′), 4.1 (m, 1, H4′), 4.0 (m, 1, H5′), 3.8 (m, 1, H5″)

[2′-$^2$H] 5-Nitro-1-(β-D-ribofuranoyl)imidazole: $^1$H NMR ($CD_3OD$) $\delta$ 8.6 (s, 1, H2), 8.0 (s, 1, H4), 6.3 (s, 1, H1′), 4.2 (m, 1, H3′), 4.1 (m, 1, H4′), 4.0 (m, 1, H5′), 3.8 (m, 1, H5″).

[3′-$^2$H] 5-Nitro-1-(β-D-ribofuranoyl)imidazole: $^1$H NMR ($CD_3OD$) $\delta$ 8.6 (s, 1, H2), 8.0 (s, 1, H4), 6.3 (d, 1, H1′), 4.2 (d, 1, H2′), 4.1 (m, 1, H4′), 4.0 (m, 1, H5′), 3.8 (m, 1, H5″)

[4′-$^2$H] 5-Nitro-1-(β-D-ribofuranoyl)imidazole: $^1$H NMR ($CD_3OD$) $\delta$ 8.6 (s, 1, H2), 8.0 (s, 1, H4), 6.3 (s, 1, H1′), 4.2 (m, 2, H2′, H3′), 4.0 (d, 1, H5′), 3.8 (d, 1, H5″).

[5′,5′-$^2H_2$] 5-Nitro-1-(β-D-ribofuranosyl)imidazole: $^1$H NMR ($CD_3OD$) $\delta$ 8.6 (s, 1, H2), 8.0 (s, 1, H4), 6.3 (s, 1, H1′), 4.2 (m, 2, H2′, H3′), 4.1 (m, H4′).

**5-Amino-1-(β-D-ribofuranosyl)imidazole[21].** 5-Nitro-1-(β-D-ribofuranosyl)imidazole (9.8 mg, 0.04 mmol) was dissolved in anhydrous methanol (5 mL). 12 mg of 10% Pd/C was added and the mixture was hydrogenated at atmospheric pressure and room temperature for 3 h. The catalyst was filtered and solvent removed *in vacuo* to give a colorless oil quantitatively.[21]

[2′-$^{13}$C] 5-Amino-1-(β-D-ribofuranosyl)imidazole: $^1$H NMR ($D_2O$) $\delta$ 7.6 (s, 1, H2), 6.4 (s, 1, H4), 5.6 (d of d, 1, H1′), 4.8 (m, 0.5, H2′) 4.3 (m, 1.5, H2′, H3′), 4.1 (m, 1, H4′), 3.8 (m, 2, H5′, H5″). $^{13}$C NMR ($D_2O$) $\delta$ 70.6 ($^{13}$C enriched, C2′).

[5′-$^{13}$C] 5-Amino-1-(β-D-ribofuranosyl)imidazole: $^1$H NMR ($D_2O$) $\delta$ 7.6 (s, 1, H2), 6.4 (s, 1, H4), 5.6 (d, 1, H1′), 4.5 (t, 1, H2′), 4.3 (m, 1, H3′), 4.2 (m, 1, H4′), 4.0 (m, 1, H5′), 3.7 (m, 1, H5″). $^{13}$C NMR ($D_2O$) $\delta$ 58.3 ($^{13}$C enriched, C5′).

[Amino-$^{15}$N] 5-amino-1-(β-D-ribofuranosyl)imidazole: $^1$H NMR ($D_2O$) $\delta$ 7.6 (s, 1, H2), 6.4 (s, 1, H4), 5.6 (d, 1, H1′), 4.5 (t, 1, H2′), 4.3 (m, 1, H3′), 4.1 (m, 1, H4′), 3.8 (m, 2, H5′, H5″).

[1′-$^2$H] 5-Amino-1-(β-D-ribofuranosyl)imidazole: $^1$H NMR ($D_2O$) $\delta$ 7.6 (s, 1, H2), 6.4 (s, 1, H4), 4.5 (d, 1, H2′), 4.3 (m, 1, H3′), 4.1 (m, 1, H4′), 3.8 (m, 2, H5′, H5″).

[2′-$^2$H] 5-Amino-1-(β-D-ribofuranosyl)imidazole: $^1$H NMR ($D_2O$) $\delta$ 7.6 (s, 1, H2), 6.4 (s, 1, H4), 5.6 (s, 1, H1′), 4.3 (d, 1, H3′), 4.1 (m, 1, H4′), 3.8 (m, 2, H5′, H5″).

[3′-$^2$H] 5-Amino-1-(β-D-ribofuranosyl)imidazole: $^1$H NMR ($D_2O$) $\delta$ 7.6 (s, 1, H2), 6.4 (s, 1, H4), 5.6 (d, 1, H1′), 4.5 (d, 1, H2′), 4.1 (m, 1, H4′), 3.8 (m, 2, H5′, H5″).

[4′-$^2$H] 5-Amino-1-(β-D-ribofuranosyl)imidazole: $^1$H NMR ($D_2O$) $\delta$ 7.6 (s, 1, H2), 6.4 (s, 1, H4), 5.6 (d, 1, H1′), 4.5 (t, 1, H2′), 4.3 (d, 1, H3′), 3.8 (m, 2, H5′, H5″).

[5′,5′-$^2H_2$] 5-Amino-1-(β-D-ribofuranosyl)imidazole: $^1$H NMR ($D_2O$) $\delta$ 7.6 (s, 1, H2), 6.4 (s, 1, H4), 5.6 (d, 1, H1′), 4.5 (t, 1, H2′), 4.3 (m, 1, H3′), 4.1 (d, 1, H4′).

**5-Amino-1-(β-D-ribofuranosyl)imidazole 5′-phosphate (AIR, 1).** AIR was prepared from AIRs by the method of Leonard.[22] A solution of 5-amino-1-(β-D-ribofuranosyl)imidazole (5 mg, 0.024 mmol) in *m*-cresol (0.8 mL) was cooled to 0 °C, pyrophosphoryl chloride (20 mg, 0.08 mmol) was added and the mixture was stirred at 0 °C. After 4.5 h, the mixture was poured into 10 mL of ice water and extracted with ether (4 × 10 mL). The aqueous layer was adjusted to pH 7.5 with 2 M NaOH and applied to a DEAE-cellulose column (1.0 × 15 cm) pre-equilibrated with 25 mM ammonium bicarbonate pH 7.5. The column was washed with water (20 mL) and then eluted with a linear gradient of $NH_4HCO_3$ (25 mM to 600 mM over 50 mL). AIR-containing fractions (UV 254 nm) were pooled and lyophilized to give 8 mg of crude AIR contaminated with salts. HPLC analysis (Supelcosil LC-18-T 15 cm × 4.6 mm, 3 μm column, 10 mM $K_2HPO_4$ pH 6.6 isocratic, retention time = 2.0 min) indicated the mixture contained 0.004 mmol of AIR (17%) and no UV absorbing impurities. In addition, $^1$H-NMR revealed no impurities.

[2′-$^{13}$C] 5-Amino-1-(β-D-ribofuranosyl)imidazole 5′-phosphate: $^1$H NMR ($D_2O$) $\delta$ 8.4 (s, 1, H2), 6.6 (s, 1, H4), 5.7 (m, 1, H1′), 4.7 (m, 0.5, H2′), 4.2 (m, 2.5, H2′, H3′, H4′), 4.0 (m, 2, H5′, H5″). $^{13}$C NMR ($D_2O$) $\delta$ 74.1 ($^{13}$C enriched, C2′).

[5′-$^{13}$C] 5-Amino-1-(β-D-ribofuranosyl)imidazole 5′-phosphate: $^1$H NMR ($D_2O$) $\delta$ 8.4 (s, 1, H2), 6.6 (s, 1, H4), 5.7 (d, 1, H1′), 4.4 (t, 1, H2′), 4.2 (m, 2, H3′, H4′), 4.0 (m, 1, H5′), 3.8 (m, 1, H5″). $^{13}$C NMR ($D_2O$) $\delta$(d, $^{13}$C enriched, C5′).

[Amino-$^{15}$N] 5-amino-1-(β-D-ribofuranosyl)imidazole 5′-phosphate: $^1$H NMR ($D_2O$) $\delta$ 8.4 (s, 1, H2), 6.6 (s, 1, H4), 5.7 (d, 1, H1′), 4.4 (t, 1, H2′), 4.25 (t, 1, H3′), 4.2 (m, 1, H4′), 3.9 (m, 2, H5′, H5″).

[1′-$^2$H] 5-Amino-1-(β-D-ribofuranosyl)imidazole 5′-phosphate: $^1$H NMR ($D_2O$) $\delta$ 8.4 (s, 1, H2), 6.6 (s, 1, H4), 4.4 (d, 1, H2′), 4.25 (t, 1, H3′), 4.2 (m, 1, H4′), 3.9 (m, 2, H5′, H5″).

[2′-$^2$H] 5-Amino-1-(β-D-ribofuranosyl)imidazole 5′-phosphate: $^1$H NMR ($D_2O$) $\delta$ 8.4 (s, 1, H2), 6.6 (s, 1, H4), 5.7 (s, 1, H1′), 4.2 (m, 2, H3′, H4′), 4.0 (m, 2, H5′, H5″).

[3′-$^2$H] 5-Amino-1-(β-D-ribofuranosyl)imidazole 5′-phosphate: $^1$H NMR ($D_2O$) $\delta$ 8.4 (s, 1, H2), 6.6 (s, 1, H4), 5.7 (d, 1, H1′), 4.4 (d, 1, H2′), 4.2 (m, 1, H4′), 3.9 (m, 2, H5′, H5″).

[4′-$^2$H] 5-Amino-1-(β-D-ribofuranosyl)imidazole 5′-phosphate: $^1$H NMR ($D_2O$) $\delta$ 8.4 (s, 1, H2), 6.6 (s, 1, H4) 5.7 (d, 1, H1′), 4.4 (t, 1, H2′), 4.25 (d, 1, H3′), 3.9 (m, 2, H5′, H5″).

[5′,5′-$^2H_2$] 5-Amino-1-(β-D-ribofuranosyl)imidazole 5′-phosphate: $^1$H NMR ($D_2O$) $\delta$ 8.4 (s, 1, H2), 6.6 (s, 1, H4), 5.7 (d, 1, H1′), 4.4 (t, 1, H2′), 4.2 (m, 2, H3′, H4′).

### Incorporation of labels from AIR and NADH into the pyrimidine

Reactions were carried out in a 2.5 mL total volume containing AIR (0.1 mM), SAM (1 mM), NADH (2 mM), NADPH (1 mM), Thz–P (0.5 mM), HMP–P kinase (0.1 mg $mL^{-1}$), thiamin phosphate synthase (0.1 mg $mL^{-1}$), 1.25 mL of the ThiC cell extract (10–15 mg $mL^{-1}$) and 0.9 mL of the *E. coli* protein preparation (10–15 mg $mL^{-1}$) described above. The reactions were initiated by addition of the protein extracts followed by thorough mixing. Incubation at 37 °C was carried out for 3 h. 200 μL of each reaction mixture was quenched and analyzed for TMP as described above. The remaining 2.3 mL was quenched by addition of 50 μL of 6 N HCl and the thiamin converted to 2-methyl-4-amino-5-[(ethylthio)-methyl]pyrimidine (ETMP, **15**) as follows:[23] the pH was adjusted to 5 with 2 M NaOH (~100 μL) and the precipitated proteins removed by centrifugation. The supernatant was transferred to a screw-top vial, 1 mL of ethanol and 2 mL of ethanethiol were added, and the

vial was sealed and heated to 100 °C. After 2.5 h, the reaction mix was cooled and EtOH and EtSH were removed under a stream of argon. The remaining solution was acidified with 0.2 mL of 6 N HCl and extracted with $CH_2Cl_2$. The aqueous layer was neutralized (0.2 mL of 6 N NaOH), saturated with $NaHCO_3$, then extracted with $CH_2Cl_2$ (3 × 5 mL). The organic extracts were combined, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was dissolved in 10 μL of ethyl acetate and 1 μL of this solution was used for GC-MS analysis as described previously.[23]

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## References

1 Abbreviations: AIR, 5-aminoimidazole ribonucleotide; HMP, 4-amino-2-methyl-5-hydroxymethylpyrimidine; SAM, *S*-adenosylmethionine; TPP, thiamin pyrophosphate; Thz–P, 5-methyl-4-(β-hydroxyethyl)thiazole phosphate; HMP–PP, 4-amino-2-methyl-5-hydroxymethylpyrimidine pyrophosphate; TMP, thiamin phosphate; HMP–P, 4-amino-2-methyl-5-hydroxymethylpyrimidine phosphate; Thc–P, thiochrome phosphate; NADH, nicotinamide adenine dinucleotide, reduced form; NADPH, nicotinamide adenine dinucleotide 3′-phosphate, reduced form; NAD, nicotinamide adenine dinucleotide; AIRs, 5-aminoimidazole ribonucleoside; ETMP, 2-methyl-4-amino-5-[(ethylthio)methyl]-pyrimidine.
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