S-Adenosyl-l-Methionine Salvage Impacts Psilocybin Formation in “Magic” Mushrooms

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Psychotrophic Psilocybe mushrooms biosynthesize their principal natural product psilocybin in five steps, among them a photosynthesis and two methyltransfer reactions, which consume one equivalent of S’-adenosine triphosphate (ATP) and two equivalents of S-adenosyl-l-methionine (SAM). This short but co-substrate-intensive pathway requires nucleoside cofactor salvage to maintain high psilocybin production rates. We characterized the adenosine kinase (AdoK) and S-adenosyl-l-homocysteine (SAH) hydrolase (SahH) of Psilocybe cubensis. Both enzymes are directly or indirectly involved in regenerating SAM. qRT-PCR expression analysis revealed an induced expression of the genes in the fungal primordia and carpophores. A one-pot in vitro reaction with the N-methyltransferase PsIM of the psilocybin pathway demonstrates a concerted action with SahH to facilitate biosynthesis by removal of accumulating SAH.

Introduction

The principal natural product of psychotropic “magic” mushrooms is psilocybin (Scheme 1), first isolated by Albert Hofmann and colleagues.[1] It has produg character as it represents the stable precursor of the dephospho analogue psilocin (Scheme 1), the actual neuroactive compound. Psilocybin is under consideration in advanced clinical trials as a pharmaceutical to treat therapy-resistant depression.[2] In pioneering work, a biosynthetic cascade was suggested.[3] Recently, the corresponding genetic locus and the enzymes were identified.[4] Psilocybin biosynthesis requires four enzymes (Scheme 1): l-tryptophan decarboxylation and hydroxylation at C-4 are catalyzed by the decarboxylase PsID and the monooxygenase PsIH, respectively, to form 4-hydroxytryptamine. Subsequently, the S’-adenosine triphosphate (ATP)-dependent S-methylthioribokinase-like enzyme PsIK phosphorylates 4-hydroxytryptamine.[5] The biosynthesis is completed by the methyltransferase PsIM which installs the tertiary amine and requires two equivalents of S-adenosyl-l-methionine (SAM). Psilocybin titers can reach up to 2% of the fungal dry biomass.[6]

Consequently, SAM must be efficiently regenerated to keep up the high psilocybin production rates. Hence, we expected 1) enzymes of the SAM salvage cycle and 2) adenosine kinase (AdoK) that supports this cycle to be critical for psilocybin biosynthesis (Scheme 1). Adenosine kinases catalyze the phosphorylation of adenosine (Ado) to adenosyl-S’-monophosphat (AMP) using ATP as phosphate donor[6] and they play an intrinsic role in purine salvage metabolism, primarily to regenerate Ado to AMP, eventually to provide new ATP in a three-step reaction (Scheme 1).[7] Most importantly, they are indirectly involved in the SAM regeneration system as well.[8]

The methyl donor SAM is regenerated by the concerted action of three enzymes: S-adenosyl-l-homocysteine hydrolase (SahH) cleaves SAH to release Ado (the AdoK substrate) and l-homocysteine (HCy), which is subsequently methylated to l-methionine (l-Met) by a 5’-methyltetrahydrofolate-dependent l-methionine synthase (MetS). Lastly, l-Met is adenosylated by SAM synthetase (SamS) to complete the regeneration cycle. Typically, SahH enzymes are homotetramers that noncovalently bind nicotinamide adenine dinucleotide (NAD+)* via an N-terminal domain.[9] The enzymatic cleavage of SAH requires NAD”-dependent oxidation/re-reduction steps.[10] In vitro, the equilibrium of the SahH reaction favors the synthetic direction, yet the hydrolytic direction is supported by continuous enzymatic removal of Ado under physiological conditions.[11] Ado, 2’-deoxy-Ado and adenedine (Ade) derivatives bind tightly to SahH, and accumulation of these compounds inhibits its hydrolytic activity.[12] Hence, removal of Ado by AdoK is likely to maintain SahH’s activity. Collectively, both AdoK and SahH activity is required to provide sufficient methyl donor to dimethyl norbaceocystin to psilocybin (Scheme 1).

Herein we report the characterization of AdoK and SahH from the psilocybin producer Psilocybe cubensis. Kinetic parameters and substrate specificities were investigated. In addition,
the impact on psilocybin production due to the metabolic interplay of both enzymes is highlighted. Information on basidiomycete primary metabolism is scanty, and neither enzyme has genetically or biochemically been characterized for any basidiomycete, that is, a division of 30,000 species. Given the poorly studied interface of primary and secondary metabolism in basidiomycetes, this study has pilot character beyond psilocybin biosynthesis.

Results and Discussion
Gene expression analysis
The genes encoding adenosine kinase (adoK) and the SAM salvage pathway (sahH, metS, samS) were identified in the *P. cubensis* genome(s) using the genes of experimentally proven enzymes from *Saccharomyces cerevisiae* or *Homo sapiens* as query (Table 1). First, an expression analysis was carried out from fungal mycelium derived from submerse (shake flasks) and

| Gene | Length [bp]/(number of introns) | CDS [bp](a) | Gene product(b) | Gene locus(c) |
|------|--------------------------------|-------------|-----------------|--------------|
| adoK | 1426/5                         | 1041        | adenosine kinase | NODE_5658: 141,215–142,640 |
| sahH | 1919/10                        | 1290        | S-adenosyl-l-homocysteine hydrolase | NODE_599: 19,244–21,162 |
| metS | 2709/7                         | 2295        | l-methionine synthetase | NODE_7121: 231,799–234,507 |
| samS | 1449/5                         | 1182        | S-adenosyl-l-methionine synthetase | NODE_6392: 312,966–314,414 |

(a) Sequences were deposited in the NCBI GenBank under accession numbers MN380460 (adoK) and MN380461 (sahH). (b) CDS: coding sequence excluding introns, which were verified for adoK and sahH, and bioinformatically predicted for metS and samS. (c) Experimentally verified for AdoK and SahH, predicted for MetS and SamS.

Table 1. *P. cubensis* genes involved in the SAM salvage pathway.
emergence of submersed mycelium, primordia and carpophores from *P. cubensis*. The genes *psiD*, *psiH*, *psiK*, *psiM* as well as genes for tryptophan synthase (*trpB*)[13] adenosine kinase (*adoK*) and the SAM salvage cycle (*sahH, samS, metS*) were tested.

A differential stage specific gene, *mtdA*, was included to verify the switch from asexual to sexual lifestyle. Its homolog *mtd1*, encoding a putative peptide transporter in the mushroom *Schizophyllum commune*, was previously characterized as carpophore-specific marker gene.[14] As expected, *mtdA* was induced in primordia and in fruiting bodies of *P. cubensis*, but not in undifferentiated mycelium (Figure 1A). The *trpB* gene is sixfold upregulated in emergent mycelium and during mating which is consistent with an increased l-tryptophan demand during psilocybin biosynthesis. In contrast, the psilocybin biosynthetic gene cluster (*psiD, psiH, psiK, psiM*) was only poorly expressed in any tested mycelial stage, but its transcription was induced during fruiting body formation which is consistent with previous observations.[15] The gene for the gateway enzyme PsID is most upregulated (395-fold in primordia relative to submersed mycelium). However, except for *psiK*, expression of the *psi* genes is threefold downregulated in mature carpophores relative to primordia suggesting that the main biosynthetic performance occurs during the primordial stage. Moreover, all genes involved in the SAM salvage pathway including *adoK* and *sahH* were upregulated five- to 16-fold in primordia and mature fruiting bodies. These results pointed to a correlation of the SAM cycle activity and psilocybin production and matched the high psilocybin content found in primordia and fruiting bodies (0.4 g g⁻¹ dry biomass), but not in mycelia (Figure 1B). Similar developmental stage-specific production rates were observed for α- and β-amanitin in *Amanita exitialis*. In this species, the toxin concentrations in the mycelium were only about 10% of those in fruiting bodies.[16] Co-expression of *adoK* and *sahH* with the *psi* gene cluster under psilocybin-producing conditions suggested that both enzymes might be beneficial for psilocybin production. Hence, we recombinantly produced and biochemically characterized *P. cubensis* AdoK and SahH (Figure S1 in the Supporting Information).

Biochemical characterization of AdoK

Heterologous gene expression yielded a 36.9 kDa monomeric protein, according to size exclusion chromatography (Figure S2) which is a typical feature of eukaryotic AdoKs.[17] Enzymatic activity was measured with a coupled fluorometric assay. Optimal turnover was detected at 42 °C and between pH 7.5–8.5 (Figure S3). These values are similar to wheat AdoK and to that of the parasite *Toxoplasma gondii*, which were optimally active at 37 °C.[18] The observed slightly alkaline pH optimum correlates with previously described activities of AdoK from plants, but contrasts mammalian enzymes which prefer acidic reaction conditions.[19] Mg²⁺ is essential for AdoK’s activity with a broad optimal concentration ranging from 0.5 to 5 mM (Figure S4). With the exception of Zn²⁺, various divalent cations can replace Mg²⁺ at < 5 mM to a different extent (Mn²⁺ > Mg²⁺ > Co²⁺ > Ca²⁺ > Zn²⁺; Figure 2A). A supportive effect of Mn²⁺ has been described for AdoKs isolated from human plasmodia, *Lupinus luteus* seeds or the protist *Leishmania donovani*.[19b, 20] However, Mn²⁺ inhibits *P. cubensis* AdoK activity at concentrations > 1 mM.

In hamster AdoK, Mg²⁺ interacts with asparagine (N239) and glutamate (E242) within a highly conserved NXE motif,[21] which is present in all as yet characterized AdoK enzymes including *P. cubensis* AdoK. We generated a mutant gene for an AdoK variant in which this particular glutamate residue (E222) was replaced by alanine (AdoK222A). This amino acid exchange resulted in an inactive enzyme, regardless of the cation concentration (Figure S4).

Monovalent cations had minor effects on enzyme activity. A cooperative effect of K⁺ in the binding ATP binding pocket has been described for mammalian AdoKs.[20c, 22] In the case of *P. cubensis* AdoK, K⁺—but not Na⁺ or Li⁺—slightly stimulated activity (up to 140%) at 10 mM, but were not essential for activity (Figure S5). In the crystal structure of murine AdoK, K⁺—
is retained by amino acid residues D310, N312, I346, G351, and R349, which form an anion hole.

An according mutant of Psilocybe AdoK lacking the potential K\(^{+}\) binding aspartate (AdoK\(^{D294A}\)) was generated. It did not show turnover, independent of the K\(^{+}\) or Mg\(^{2+}\) concentration (Figures S4 and S6), suggesting a similar involvement of K\(^{+}\) in AdoK activity.

Sequence alignment with experimentally characterized representatives from various species revealed that P. cubensis AdoK clusters with the sole characterized fungal AdoK from baker’s yeast S. cerevisiae and, more distantly, with those of the protists L. donovani and T. gondii (Figure S7). While most, if not all, mammalian and plant AdoKs are inhibited by Ado (at five- to 20-fold \(K_M\)) by a negative feedback mechanism,\[19b, d, 23\] the above-mentioned evolutionary branch comprises AdoKs, the activity of which is not or only slightly impaired by excess Ado.\[19b, 24\]

We next investigated the kinetic properties of AdoK. It followed a Michaelis–Menten type response, with a \(K_M = 33.6 \text{ mM}\) for Ado, the \(k_{cat} = 1.4 \times 10^3 \text{ s}^{-1}\) (Figure 2B, Table 2). The \(K_M\) value for AdoK’s second substrate ATP was 11.6 \(\mu\text{M}\) (Figure 2C), which is lower than values obtained for human, plant, and other fungal representatives (200–2000 \(\mu\text{M}\)),\[19b, 24b\] although, enzymes with \(K_M\) values of \(< 0.05 \text{ \mu M}\) (human erythrocytes, wheat germ) have been reported.\[18a, 19b\]

We then interrogated P. cubensis AdoK’s substrate specificity. The enzyme was active with Ado but with other naturally occurring nucleosides, such as inosine, uridine, guanosine, and thymidine (Figure S8), which reflects the substrate profile of other plant, fungal, and mammalian AdoKs.\[20a, 24b, 25\] None of the tested nucleosides severely repressed enzymatic activity, even when present in 100-fold excess over Ado (10 \(\mu\text{M}\) vs. 100 \(\mu\text{M}\), Figure S8). In contrast to various bacterial, plant, or other eukaryotic AdoKs,\[19b, d, 26\] the Psilocybe enzyme is not inhibited by Ado at 500 \(\mu\text{M}\) (Figure 2B). The enzyme also phosphorylated the cytokinin precursor \(N^6-(\Delta^2\text{-isopentenyl})\text{adenosine}\), a zeatin derivative, as demonstrated both in our fluorimetric assay and by activity testing by UHPLC–MS (Figures 2D and S9). Phosphorylation of plant hormones by AdoKs has been demonstrated for plant AdoKs from Physcomitrella and Arabidopsis.\[19b\] The psilocybin congeners 4-hydroxytryptamine and psilocin did not serve as substrates (Figure 2D).

**Figure 2.** Characterization of P. cubensis AdoK. A) Impact of divalent metal cations on AdoK activity. Mn\(^{2+}\), Mg\(^{2+}\), Co\(^{2+}\), Ca\(^{2+}\), and Zn\(^{2+}\) were tested between 0.1 and 5 \(\mu\text{M}\). Values are given in ratios relative to activity at 5 \(\mu\text{M}\) MgCl\(_2\). B) Michaelis–Menten kinetics for Ado. C) Michaelis–Menten kinetics for ATP. D) Substrate specificity of AdoK at 0.1–1000 \(\mu\text{M}\). Ado, adenosine; \(N^6\)-ip-Ado: \(N^6-(\Delta^2\text{-isopentenyl})\text{adenosine}\).

**Table 2.** Kinetic parameters of P. cubensis AdoK and SahH.

| Enzyme | Substrate | \(K_M\) [\(\mu\text{M}\)] | \(k_{cat}\) [\(\text{s}^{-1}\)] | \(k_{cat}/K_M\) [\(\text{M}^{-1}\text{s}^{-1}\)] |
|--------|-----------|------------------|-----------------|-----------------|
| AdoK   | Ado       | 33.6             | \(1.4 \times 10^3\) | 4.2 \(\times 10^3\) |
|        | ATP       | 11.6             | \(1.4 \times 10^3\) | 1.2 \(\times 10^3\) |
| SahH   | SAH       | 57.2             | 0.2             | 4.1 \(\times 10^3\) |
|        | Ado       | 56.7             | 1.8             | 3.1 \(\times 10^4\) |
|        | Hcy       | 19.8             | 1.8             | 9.1 \(\times 10^4\) |

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Biochemical characterization of SahH

Heterologous production of \textit{P. cubensis} SahH yielded a 46.8 kDa protein. Size-exclusion chromatography indicated a homotetramer (Figure S2) which was previously found as well for other SAH proteins.\(^{10}\) SAH hydrolysis was assayed by detecting the free thiol group of the product \(\text{L-homocysteine},\) using Ellman’s reagent in a colorimetric assay.\(^{9b}\) SahH catalyzed the hydrolysis of SAH in HCy and Ado (Figure 3A). Similar to our findings for AdoK, SahH is thermostable up to 50 \(^\circ\)C, with an optimum turnover at 34 \(^\circ\)C (Figure S10). Thermal stability is a common feature of SahH enzymes\(^{27}\) and is most likely caused by the cofactor-binding C-terminal helix-18.\(^{10}\) This helix is critical for NAD\(^+\) association via K426 and Y430 in the structures of human and \textit{Trypanosoma} SahH\(^{10}\) and is present in all characterized eukaryotic SahHs including \textit{P. cubensis} SahH. Activity of >90\% was detected in a range between pH 5.5 and 7.5, yet rapidly dropped above pH 8.0 (Figure S10). Optimum turnover was at pH 7.0–7.5. We further determined catalytic parameters of SahH in hydrolytic and synthetic direction (Figure 3A–C). All substrates are accepted with similar affinity, according to their \(K_M\) values (Table 2). \(K_M\) values for the hydrolytic direction are slightly above the values obtained from other characterized enzymes (1–21 \(\mu\)M).\(^{28}\) The \(k_{cat}\) values are in a similar range as previously published enzymes \((k_{cat} = 0.2 \text{ s}^{-1} \text{ (for SAH, hydrolytic direction), } k_{cat} = 1.8 \text{ s}^{-1} \text{ (Ado, HCy, synthetic direction))}.\(^{29}\) Most importantly, catalytic efficiency \((k_{cat}/K_M)\) for Ado or HCy is 8- or 22-fold higher than for SAH, respectively, suggesting that 1) SAH synthesis might be the kinetically favored reaction, at least in vitro, and, conversely, 2) SAH hydrolysis requires the activity of Ado removing enzymes, such as AdoK.

The crystal structures of rat and \textit{Plasmodium} SahH identified histidine (H54) as essential for SAH binding at the ribose moiety and to abstract a proton from ribose at C-4' prior to elimination of HCy.\(^{9a, 30}\) We mutated the codon for H51, that is, the equivalent residue in \textit{P. cubensis} SahH, into an alanine (SahH\(_{H51A}\)). The in vitro activity assay showed a severely impaired catalytic capacity (<5\% of native SahH activity, Figure S11). The co-substrate NAD\(^+\) binds noncovalently to the enzyme and is essential for its activity (Figure 3D). Mechanistically, NAD\(^+\) first oxidizes the 3'-OH group of SAH to facilitate efficient hydrolysis of SAH and the release of HCy. After addition of a water molecule to the remaining 3'-keto-4',5'-dihydroadenosine, the final reduction of the 3'-keto group requires NADH + H\(^+\) to give Ado.\(^{31}\) Previous works suggested the C-terminal lysine K425 (from the adjacent subunit) to properly position the nucleoside moiety of NAD\(^+\) via 2'-OH and 3'-OH.\(^{9a, 11}\) We verified the preserved NAD\(^+\) binding site\(^{9a}\) in \textit{Psilocybe} AdoK. A variant carrying an alanine at the C-terminal NAD\(^+\) binding site, rather than the canonical NAD\(^+\)-binding lysine (K424 in \textit{P. cubensis} SahH), was created, heterologously
produced, and tested for activity in vitro. Independent of the NAD$^+$ concentration, SahH$_{K424A}$ is virtually catalytically inactive (Figure S12).

SAH hydrolysis is strongly inhibited by elevated Ado concentrations and—to a lower extent—by tenfold excess of Ade over the SAH substrate (Figure S13). Feedback-inhibition by adenosine derivatives has been described for several SahH enzymes. However, the enzyme’s activity is not product-inhibited by AMP (Figure S13). As Ado is released during SAH hydrolysis, we reasoned that turnover by SahH may be increased when its product Ado is removed by AdoK-mediated phosphorylation of Ado to AMP. A one-pot enzymatic assay with SahH and AdoK showed a 2.5-fold increased HCy release by SahH in an ATP-concentration dependent manner (Figure S14). AdoK itself is not inhibited by high Ado concentrations (Figure 2A), which allows for efficient conversion from SAH via Ado to AMP.

Adenosine metabolite processing increases in vitro psilocybin production

A biocatalytic in vitro route for psilocybin production from 4-hydroxy-L-tryptophan was proposed and includes a three-enzyme, one-pot reaction, using PsiD, PsiK, and PsiM. The iterative methylation of norbaeocystin to psilocybin (Figure 4A) by PsiM is thought to be a rate-limiting critical step since N-methyltransferases are comparably slow enzymes with $k_{cat}$ values ranging between 0.12 h$^{-1}$ to 0.58 s$^{-1}$. Previous studies demonstrated increased psilocybin yields, when Escherichia coli SAH nucleosidase and adenine deaminase were added to the PsiM reaction to irreversibly remove SAH from the chemical equilibrium. Hence, we hypothesized SAH could inhibit PsiM and ran assays in the presence of SAH, ranging between 1–16 mM. SAH competitively inhibited PsiM’s activity in a concentration-dependent manner (Figures 4A and B).

We further calculated the PsiM-mediated production rates of the monomethylated product baecystin in presence of SahH and AdoK. When solely SahH was added to the PsiM reaction, a threefold increase of enzymatic norbaeocystin methylation was observed (Figure 4C). However, when both AdoK and SahH were present, a fivefold increase of product formation was detected indicating that the chemical equilibrium of SAH hydrolysis is on the side of SAH. Yet, the hydrolytic reaction is preferred in the presence of the SAH-degrading activity of AdoK. The robust supporting effect of AdoK/SahH was likewise evident in the presence of inhibitory amounts of SAH (Figure 4C). Interestingly, a simple two-enzyme reaction with AdoK and PsiM elevated the norbaeocystin levels 2.5-fold. We correlated this to a spontaneous hydrolysis of released SAH, which may be enhanced by downstream AdoK activity. In sum, this report provides evidence that 1) the genes of the SAM salvage cycle are co-expressed with the psilocybin biosynthetic gene cluster and 2) SahH and AdoK can support the final step of psilocybin biosynthesis in vitro.

Conclusion

Psilocybin has entered clinical trials as low side effect medication against depression. Its simple four-step enzymatic biosynthetic route requires continuous recycling of ATP and SAM to reach high metabolite titers. AdoK and SahH are critical players in the psilocybin pathway and their genes are expressed in Psilocybe fruiting bodies. SahH is required to efficiently remove SAH, a competitive inhibitor of the PsiM-mediated methylation reaction. The strong product inhibition of SahH by Ado is in turn bypassed by the activity of AdoK. This close enzymatic interplay of PsiM, SahH, and AdoK allows for the efficient methyl transfer to norbaeocystin which completes psilocybin biosynthesis. This report provides insight into the primary/secondary metabolism interface in P. cubensis but also suggests novel strategies to improve yields for biocatalytic psilocybin production.

Experimental Section

Microbiological methods: P. cubensis FSU12407 was maintained on MEP (15 g L$^{-1}$ malt extract, 3 g L$^{-1}$ peptone, 18 g L$^{-1}$ agar) plates. To collect biomass from emerse cultures, P. cubensis was cul-
tivated for 7 days on MEP agar plates at 25 °C. For submerser cultures, the fungus was incubated in MEP liquid medium for 4 days at 25 °C and 140 rpm. Carphophore formation was induced as described. Melia were collected, shock-frozen in liquid nitrogen and lyophilized prior to RNA isolation or metabolite quantification.

RNA isolation, cDNA synthesis and expression analysis: Fungal mycelium was lysed with 0.2 mM glass beads using a FastPrep cell disruptor. RNA was isolated with the SV Total RNA Isolation System (Promega) using the manufacturer’s protocol. RNA (1 mg) was reverse transcribed to cDNA by using the RevertAid RT kit (Thermo) using an anchored oligo-(dT) 18 primer. Expression analysis was carried out with an AnalytikJena qTower 1 using the qPCR Mix Eva-Green (Bio&SSELL) and primers with a minimum primer efficiency of 91 % (Table S1, Figure S15). Amplification protocol: initial denaturation at 95 °C, 15 min followed by 40 cycles of amplification (95 °C, 15 s; 60 °C, 20 s; 72 °C, 20 s). A melting curve was obtained by heating from 60 to 95 °C. Genes encoding α-actin (actA), glyceraldehyde-3-phosphate dehydrogenase (gpdA) and enolase (enoA) served as internal housekeeping reference genes. Gene expression levels were determined by a described method.[5]

Metabolite quantitation: Lyophilized fungal mass (0.2–1 g) was ground to a fine powder and extracted twice with 50 mL methanol (MeOH). The pooled fractions were evaporated to dryness and the residue was dissolved in 10 mL water. After defatting in 10 mL cyclohexane, 9 mL of the aqueous phase were lyophilized. The residue was dissolved in 4 mL water/acetonitrile (ACN, 99:1), filtered and subjected to UHPLC–MS. Analyses were carried out on an Agilent Infinity II 1290 instrument, equipped with a diode array and a mass detector (6130 quadrupole) and a Phenomenex Luna Omega polar C 18 column (100 × 2.1 mm, 1.6 μm). ELuent A was 0.1% formic acid in water, eluent B was ACN. The following gradient was applied: 0 min, 1% B; within 3 min to 100 % B; within another min to 100 % B; held at 100 % B for 2 min. The flow rate was 0.5 mL min⁻¹ and the chromatogram was extracted at λ = 280 nm. Psilocybin, baeocystin and norbaeocystin were quantified against a calibration curve with respective authentic reference standards.

Heterologous protein production: The construction of plasmids encoding AdoK, SahH and their respective mutants, and oligonucleotide primers, are described in the Supporting Information, and Tables S1–S2. Production of the N-terminally His₆-tagged proteins was carried out in E. coli BL21 in 400 mL LB medium, supplement- ed with 50 μg mL⁻¹ kanamycin, at 37 °C and 180 rpm. After reaching an OD₆₀₀ of 0.6–0.8, the temperature was shifted to 16 °C, and gene expression was induced by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). After 16 h, cells were harvested by centrifugation and the pellet was resuspended in binding buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0). After cell lysis by a sonifier, the protein crude extract was centrifuged, and the cell-free supernatant was transferred to an equilibrated gravity affinity column with 1 mL Protino Ni⁺⁺-NTA resin. After incubation on ice for 30 min, the resin was washed with binding buffer containing concentrations of imidazole (20 and 40 mM) and the proteins were eluted with binding buffer with 500 mM imidazole. The enzymes were re-buffered in reaction buffer (50 mM TRIS-HCl, 20 mM NaH₂PO₄, Triton X-100 0.01%, pH 7.8) by size exclusion chromatography (PD10, GE Healthcare). After supplementation with 10% glycerol, enzymes were stored at −80 °C without loss of activity for up to 2 months. Protein production of PsIM was carried out as described.[36] For size-exclusion chromatography (SEC), the native and denatured (95 °C, 10 min) enzymes were re-buffered in phosphate buffer (10 mM sodium dihydrogen phosphate, 140 mM NaCl, pH 7.5). SEC was performed by FPLC on an Akta Pure 25 instrument with a Superdex 200 increase 10/300 GL column (GE Healthcare).

Enzymatic assays: AdoK activity was determined in kinase buffer (reaction buffer + 10 mM MgCl₂) as described.[29] To determine kinetic parameters, alternative substrates and activity of mutant proteins, a continuous assay was performed: 70 μL master mix (2 U mL⁻¹ horseradish peroxidase, 1 U mL⁻¹ pyruvate oxidase, 20 U mL⁻¹ pyruvate kinase, 300 μM phosphoenolpyruvate, 100 μM thiamine pyrophosphate, 10 μM flavin adenine dinucleotide, 35 μM AmplifluRed in kinase buffer) was mixed with 10 μL adenosine or alternative phosphate acceptors (0–1000 μM final concentration) and 10 μL ATP (0–100 μM final). The reaction was started by adding 10 μL (34 nM final) AdoD or AdoK mutants. Fluorescence detection was carried out in black 96-well plates at λₘₜₜ = 545 nm and λₘₜₜ = 600 nm at 30 °C for 60 min in a ClarioStar plate reader (BMG Labtech). To determine temperature and pH optima, and metal cation dependence, a discontinuous assay was performed: ATP (200 μM), Ado (200 μM) and AdoK (34 nM) were mixed in kinase buffer, adjusted to pH 4–9 (in 50 mM acetate or TRIS buffer), or amended with divalent cations (0.01–5 mM CaCl₂, MgSO₄, MnCl₂, or ZnCl₂) or monovalent cations (0.1–100 mM NaCl, KCl, LiCl). The reaction was incubated for 1, 5, or 30 min at 42 °C (for temperature optimum varied between 4–60 °C). The reactions were then placed on ice and the enzyme was removed by filtering through Sartorius VIVAspin 100 spin columns (10 kDa cutoff). The ADP concentration in a 20 μL aliquot of the enzyme-free flow-through was determined by addition of 80 μL master mix as described above.

SahH activity was determined in hydroxase buffer (reaction buffer + 100 μM NAD⁺). SAH hydrolysis was recorded by detecting free thiols of group HCY by using Ellman’s reagent (5,5-dithiobis-(2-nitrobenzoic acid), DTNB) as described.[30] A continuous assay was used to determine activity, kinetic parameters, pH optimum, NAD⁺-dependence, and the activity of mutant proteins. The reactions contained 64 μM SahH, 200 μM DTNB and 0–100 μM SAH in hydroxase buffer and were recorded at λₘₜₜ = 412 nm in transparent 384-well plates at 34 °C for 30 min. The temperature optimum was determined discontinuously: the reaction was incubated for 30 min at 4–60 °C, briefly chilled on ice and filtered through a VIVAspin 100 column (10 kDa cutoff). The HCY concentration in a 100 μL aliquot of the enzyme-free flow-through was determined at λₘₜₜ = 412 nm as described above.

PsIM reactions were performed in TRIS-HCl buffer (50 mM, pH 8.0) by quantitative detection of the products baeocystin and psilocybin by UHPLC–MS against calibrated authentic standards. The assay for SAH inhibition comprised 3 mM SAM, 1 mM norbaeocystin, 1 μM PsIM, and SAH concentrations varied between 1 and 16 mM. The coupled enzymatic assay with PsIM, SahH and AdoK was carried out in the absence of inhibiting SAH concentrations (0–2 mM) to determine if the PsIM reaction increases. The assay was composed of 3 mM SAM, 2 mM SAH, 1 mM norbaeocystin, 6 mM ATP, 6 mM MgCl₂, and 100 μM NAD⁺. In additional reactions, SahH (64 μM) and AdoK (34 nM) were added. Reactions were started by addition of 1 μM PsIM. After incubation for 2 and 24 h at 25 °C, reaction mixtures were frozen and lyophilized. The residue was dissolved in 50 μL MeOH and subjected to UHPLC/MS as described.[36]

Chemical synthesis and analysis of N⁺(α-isopentenyl)adenosine: The alternative AdoK substrate, N⁺(α-isopentenyl)adenosine was synthesized according to a published protocol in modified form.[31] Details are given in the Supporting Information. The identi-
ty of the compound was confirmed by NMR spectroscopy data (Table S3) that is compatible with previously published values.179

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Conflict of Interest

The authors declare no conflict of interest.

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