

Peer Review File

Manuscript Title: β -NAD as a Building Block in Natural Product Biosynthesis

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

This manuscript describes in detail a novel route to secondary metabolites starting with the PLP mediated 2+3 fusion of common metabolites NAD and SAM. This is an unexpected role for NAD, and the relevant enzyme that catalyses this reaction appears relatively widespread among bacteria. The authors present a thorough analysis using both in vivo and in vitro approaches using isolated enzymes, isotopic labelling and a range of putative precursors to unambiguously establish the overall biosynthetic route towards compound 1 as outline in Fig 1.

While the pathway has many steps of interest, the most novel aspect here is the initial reaction between NAD and SAM, as mediated by the enzyme SbzP. The authors present a mechanism as outlined in Fig 2 based upon mechanistic considerations/reported properties of distantly related PLP enzymes. In absence of detailed SbzP structure or kinetic studies, this particular Fig 2 needs to be seen as a hypothetic proposal only (at this stage). While some spectroscopic data has been collect on SbzP (FigS18), this reviewer is unconvinced by the quality of the spectra shown (significant background drift, lack of any real feature at 520nm) to directly support the proposed mechanism.

Alternative interpretations/mechanistic proposals regarding the timing and manner of the C-C bond formation steps could be made (for example a more concerted addition, or lacking the unusual geminal amine/imine adducts). As such the statements with respect to the mechanism ie : "SbzP, however, exhibits a novel reaction mode and activates the amino acid substrate in an inverted manner, catalyzing a nucleophilic- Cg-, and electrophilic-driven Ca alkylation (Fig.2b)" need to make clear this is subject to experimental investigation/validation or such data needs to be proposed here (ie kinetic spectroscopy, MS data on enzyme/PLP adducts formed, structure of enzyme in complex with relevant ligands). One would suspect this might well form the subject of a follow on paper, but it is important not to overstretch the interpretation at this stage.

Furthermore, for the sake of clarity, this reviewer would prefer Fig 2 to explicitly define removal/addition of protons.

Additional comments:

- 1) Did any of the compounds tested in SFig5 act as (competitive) inhibitors?
- 2) SFig27 reveals the SbzP protein is relatively impure (as compared to other samples reported here), this might hint at instability and thus difficulties to study the enzyme in vitro?
- 3) the authors might wish to include in their discussion transient covalent adducts made with b-NADH in certain enzyme <https://www.nature.com/articles/nchembio.1385>, while bNAD is also used sacrificially in (poly)-ADP ribosylation.

Referee #2 (Remarks to the Author):

Bara, Awakawa, and coworkers describe a novel bacterial biosynthetic pathway which uses both SAM and NAD+ cofactors as building blocks to construct the 6-azatetrahydroindane scaffold found in several sulfonamides. The authors establish the unusual biosynthetic origins of this scaffold through a series of isotopic labeling and NMR studies. The biosynthesis of the 6-

azatetrahydroindane scaffold is probed by creating a series of sbz gene deletions as well as biochemical studies on the encoded enzymes. These experiments do an excellent job building upon the previous work characterizing this biosynthetic gene cluster (Ref. 14) and clarifies the timings of several downstream modifications in this biosynthetic pathway. The highlight of this paper is SbzP, a novel PLP-dependent enzyme which is broadly distributed amongst various strains of bacteria and which catalyzes the novel tandem C α and C γ alkylation between SAM and NAD⁺, generating the 6-azatetrahydroindane moiety.

Overall, the manuscript is well written, and conveys information very clearly and efficiently. I believe that the findings described in the manuscript would be of significant interest to those in the fields of chemistry, natural products and enzymology. The major issue holding the manuscript back from publication in Nature is the lack of evidence to describe how SbzP catalyzes its reaction. Without these experiments and crystal structures of SbzP to explain this, the manuscript does not rise to the level of similar previous work in this journal. Without this additional work, the current manuscript is well suited for publication in Nature Communications or Nature Chemical Biology.

Major Comment:

Figure 2a – Given the emphasis placed on SbzP in the manuscript I find that there is minimal data to support the SbzP-catalyzed reaction. The proposed mechanism for SbzP is thinly supported by a UV-Vis experiment, which has a very small shift at ~510 nm, diagnostic of a quinonoid or more extended conjugated intermediate.

As drawn in Figure 2a, I think the initial steps of the reaction are fine, and the nucleophilic addition to C4 of NAD⁺ could be thought of resembling hydride transfers normally associated with NAD⁺. The first thing I find unusual is the formation of a geminal diamine in intermediate I in order to push the electron back up. Though there are some instances of such geminal diamines being relatively stable (Cook and Holden, 2007; Di Salvo et al. 2013; Sekula et al. 2018), however these kinds of intermediates are often fairly transient, and generally occur at the end of the reaction. Therefore, it seems unlikely that such a species would remain stable over multiple steps of the reaction. One alternative might be to have the NAD attack at C α directly, as depicted in the next intermediate.

Would the deprotonation of C5 of intermediate II be spontaneous as depicted? This seems unlikely to me as it does not seem to be very acidic, and loss of a proton does not generate an aromatic species. This might be an enzyme catalyzed step instead.

In the interest of discussion I have brainstormed one alternative mechanism that could occur from the β,γ -unsaturated quinonoid intermediate. In this mechanism, the NAD⁺ substrate could be oriented with C5 of NAD⁺ positioned closely to the C α of the quinonoid intermediate and C4 would be positioned near to C γ of the β,γ -unsaturated quinonoid intermediate. Then the β,γ -unsaturated quinonoid intermediate could then react via a canonical nucleophilic C α alkylation between C α and C5 of NAD⁺. This step would be coupled with a canonical electrophilic C γ alkylation between C4 of NAD⁺ and C γ and a reprotonation at C β . After this step, C5 could be deprotonated by the nearby PLP nitrogen (or another enzymatic base). This more typical mechanism would also eliminate the need to form a geminal diamine until after the cyclization is complete. The one issue here is that NAD⁺ C5 is not as positive at C4 but it may still be possible to favor such a reaction within the active site of the enzyme.

To aid in the study of the SbzP mechanism, its crystal structure could be obtained, which might provide insights into how SAM and NAD are oriented with respect to each other and PLP. The SbzP-catalyzed reaction could also be carried out using deuterated substrates or solvents (similar to Ref. 29), and the results of such experiments might provide additional mechanistic insights. It would also be interesting to test NADH to see if it inhibits the reaction or SbzP has some mechanism to discriminate between NADH and NAD⁺ or even convert the former to the latter. The authors could also consider probing the mechanism with studies on variant enzymes,

and specifically interrogating some of the proposed steps in the mechanism using a variety of common spectroscopic and other methods.

Minor comments:

Quinoid is used throughout manuscript instead of quinonoid.

Figure 3a – The top trace is labeled 'WT', and it should be more clearly defined what 'WT' represents. Additionally, isolated compound 4 should be used as a standard for this figure.

Figure 3b – The font of the enzyme annotations is small and difficult to read. Some arrow tips also appear to be extending into the end of adjacent genes. There is a lot of information in this panel. A simplified color scheme for the sbz gene cluster might convey the similarities and/or differences between the other gene clusters. For example, sbzB/C/D/K could all be colored the same to indicate production of β -methyl phenylalanine in a cryptic gene cluster.

Extended Figure 2b – masses don't match Supplementary Figure 10

Supplementary Figure 10 – The turnover here by SbzQ appears to be very low ~2000 counts. The assay conditions could be modified (run longer than 1 hr for example) to improve the results here.

Supplementary Figures 19/20 – It is a bit repetitive to have the PQ homologs in Fig 19 and then separate them in Figure 20. Perhaps the P homologs and the PQ homologs could be split into two figures. Additionally, given the similarities between SbzP and cysthionine- γ -synthase/lyases it might also be informative to create an alignment between the two types of enzymes, and this might highlight other conserved residues. For example, the conserved Asp (-DECH-) which coordinates pyridinium nitrogen in these type I PLP-dependent enzymes is not highlighted in Figure 19. Additionally, are there conserved putative iron/ α -KG binding residues in the 'Q' portion of the PQ homologs?

Page S35 – HR-MS data should be reported with ppm error.

Spelling/grammar/formatting errors in:

Supplementary Figures 5, 24

Supplementary Note 2

Referee #3 (Remarks to the Author):

Barra et al. have investigated the biosynthesis of altemicidin on a genetic and enzymatic level, with structural characterisation of all pathway intermediates. One of the steps along the pathway is absolutely spectacular, which is the formation of an unusual dinucleotide from the building blocks NAD⁺ and SAM. These compounds are usually used as reagents, NAD⁺ for oxidations and SAM as methyl donor, but not as building blocks themselves (with some exceptions, e. g. SAM serves as precursor for spermidin / spermin).

The unusual dinucleotide is generated by a PLP-dependent enzyme (SbzP) that is also mechanistically most astonishing, because it catalyses two C-C bond formations in a formal [2+3]-cycloaddition. A detailed mechanistic proposal is discussed in the manuscript and supported by site-directed mutagenesis. Something like the reaction of this enzyme has never been seen in Nature before.

Besides this remarkable step the authors also functionally characterised all other enzymes along the biosynthetic pathway, and showed that the PLP-dependent enzyme SbzP makes up a new enzyme family that is likely involved in the biosynthesis of several other unusual natural products. This was further underpinned in the present work by the functional characterisation of SbzP homologs from several other biosynthetic gene clusters by heterologous expression that all catalyse the same reaction.

Overall, this is a brilliant study that leaves no open questions. Still, a bit work on the text could

increase the readability. In the current form, the manuscript text appears quite technical, e. g. page 3, first paragraph of the Gene deletions section:

„LC-MS analysis of the culture extracts of gene deletion strains showed accumulation of specific metabolites (...). Purification and structural characterisation revealed the presence of 12 ($m/z = 361.0823$) in the delta-szbE strain (...).“

More simple: „The delta szbE strain accumulated compound 12.“, details such as detected MS signals should not be given in the main text. This is just an example, the whole manuscript should be cleaned up and could be significantly shortened. The authors should focus on their interesting story, rather than giving to many experimental details.

The authors also make excessive usage of „we“ and similar, which is arguably not a good style of scientific writing. Neutral / passive forms should be preferred. Also phrases like „Interestingly, ...“ (page 5), „Intriguingly, ...“ (page 6) should be avoided.

The Conclusions should also be rewritten, because they are mainly a summary of the main findings of the present work. Here the authors should focus more on the impact of their findings for future work.

Other minor comments:

1. Page 3, left, top: What means „The quaternisation of the ring nitrogen, resulting in a permanently positively charged pyridinium moiety...“? There does not seem to be an intermediate that fits to this statement?
2. Page 3, right, middle: rephrase „strict substrate specificity“. Only 7 and 4 were tested, 7 was accepted, 4 was not. But it is not known if compounds more similar to 7 would be accepted.
3. Page 5, left, middle: rephrase „highly conjugated“. Double bonds are either conjugated or not, they cannot be more or less conjugated (or only if torsion angles are to be discussed, but this is not the case here). The authors mean that the conjugated system spans a large part of the intermediate.
4. Page 5, right, bottom: The compound name should read „7,8-diaminopelargonic acid“.

Author Rebuttals to Initial Comments:

Reviewer #1

This manuscript describes in detail a novel route to secondary metabolites starting with the PLP mediated 2+3 fusion of common metabolites NAD and SAM. This is an unexpected role for NAD, and the relevant enzyme that catalyses this reaction appears relatively widespread among bacteria. The authors present a thorough analysis using both in vivo and in vitro approaches using isolated enzymes, isotopic labelling and a range of putative precursors to unambiguously establish the overall biosynthetic route towards compound 1 as outline in Fig 1.

While the pathway has many steps of interest, the most novel aspect here is the initial reaction between NAD and SAM, as mediated by the enzyme SbzP. The authors present a mechanism

as outlined in Fig 2 based upon mechanistic considerations/reported properties of distantly related PLP enzymes. In absence of detailed SbzP structure or kinetic studies, this particular Fig 2 needs to be seen as a hypothetical proposal only (at this stage).

We truly appreciate the thoughtful comment and addressed the concerns by conducting a series of additional mechanistic experiments, including kinetic studies to demonstrate a Ping-Pong Bi-Bi mechanism (Supplementary Note 4).

While some spectroscopic data has been collect on SbzP (FigS18), this reviewer is unconvinced by the quality of the spectra shown (significant background drift, lack of any real feature at 520nm) to directly support the proposed mechanism.

As pointed out by the reviewer, recombinant SbzP exhibits poor stability in vitro, which presumably caused the background drift and low quality of the previously obtained UV spectra. To overcome this limitation, we utilized SbzP homolog PsePQ, which we found to exhibits increased stability. Additionally, we employed a stopped-flow apparatus (SX. 18MV-R; Applied Photophysics), allowing for the measurement of time-resolved spectral changes. The improved spectra which now clearly show the formation of 520 nm absorption over time has been newly added in Figure 2b.

Alternative interpretations/mechanistic proposals regarding the timing and manner of the C-C bond formation steps could be made (for example a more concerted addition, or lacking the unusual geminal amine/imine adducts).

We have added a corresponding discussion on the suggested alternative reaction mechanism for C-C bond formation events (Supporting Figure 29) and sentences as follows.

"The proposed sequential C4 and C5 alkylation of β -NAD is in line with the typical reactivity of N-substituted pyridinium salts, which are activated for nucleophilic addition to C4, generating reactive non-conjugated enamines (resembling intermediate I), which readily react with electrophiles to yield 3,4-dihydropyridinium species, (such as intermediate II)²¹. Furthermore, a recently reported total synthesis of **1**, utilized the same overall strategy of dearomative, stepwise [3+2]-cycloaddition to pyridinium salts to construct the 6-azatetrahydroindane scaffold²¹. However, an alternative mechanism, in which C α of the β,γ -unsaturated quinonoid directly reacts with C5 of β -NAD, followed by C γ -C4 bond formation (either step-wise or concerted) cannot be ruled out at this stage (Supplementary Figure 30)." (Page5)

As such the statements with respect to the mechanism ie : "SbzP, however, exhibits a novel reaction mode and activates the amino acid substrate in an inverted manner, catalyzing a nucleophilic- C γ -, and electrophilic-driven C α alkylation (Fig.2b)" need to make clear this is subject to experimental investigation/validation or such data needs to be proposed here (ie kinetic spectroscopy, MS data on enzyme/PLP adducts formed, structure of enzyme in complex with relevant ligands). One would suspect this might well form the subject of a follow on paper, but it is important not to overstretch the interpretation at this stage.

According to the reviewer's suggestion, and as also stated above, we have provided further experimental data to support the mechanistic proposal, as well as included a discussion on mechanistic alternatives (Supporting Figure 30).

Furthermore, for the sake of clarity, this reviewer would prefer Fig 2 to explicitly define removal/addition of protons.

We have modified the Figure 2a, as suggested.

1) Did any of the compounds tested in SFig5 act as (competitive) inhibitors?

Thank you for the interesting suggestion. All of the compounds in SFig5 were tested, but did not show any inhibitory activities (Supplementary Fig. 20). Additionally, 5'-(methylthio)adenosine (AdoSMe) was evaluated and found to inhibit the reaction. The kinetic values of the reaction with and without AdoSMe were collected and revealed that AdoSMe does not act as a competitive inhibitor, as can be seen from the obtained K_m and V_{max} values. The inhibitor study further underpinned the proposed Ping-Pong Bi-Bi mechanism (see Supplementary Note 4 for further detail).

2) SFig27 reveals the SbzP protein is relatively impure (as compared to other samples reported here), this might hint at instability and thus difficulties to study the enzyme in vitro?

Thank you for the suggestion. We indeed struggled with severe stability problems during the course of the investigation of SbzP, although several attempts were undertaken to improve the stability by buffer optimization. The best condition was found to be KPB buffer (pH 8.0, 300 mM NaCl and 5% glycerol), which allowed us to evaluate the function of SbzP in vitro. Due to the

stability problems, we used SbzP homolog PsePQ for several experiments conducted in the course of the revision experiments, which exhibits improved stability.

3) *the authors might wish to include in their discussion transient covalent adducts made with β -NADH in certain enzyme <https://www.nature.com/articles/nchembio.1385>, while β NAD is also used sacrificially in (poly)-ADP ribosylation.*

Thank you very much for the thoughtful suggestion. We have added these points to the discussion in the conclusion part.

"This study reports the first secondary metabolite biosynthetic pathway, in which the central electron carrier β -NAD serves as a primary building block for the generation of a novel class of natural products. The gatekeeping enzyme SbzP represents the first example of a biosynthetic enzyme which accepts the β -NAD as a substrate. β -NAD is also known to be sacrificially used as an ADP-ribose donor for protein modifications (PARPs)⁴⁹, and transient, covalent adducts between nicotinamide dinucleotides and substrates in oxidoreductase enzymes have been observed⁵⁰..." (Page 7)

Reviewer #2

Bara, Awakawa, and coworkers describe a novel bacterial biosynthetic pathway which uses both SAM and NAD⁺ cofactors as building blocks to construct the 6-azatetrahydroindane scaffold found in several sulfonamides. The authors establish the unusual biosynthetic origins of this scaffold through a series of isotopic labeling and NMR studies. The biosynthesis of the 6-azatetrahydroindane scaffold is probed by creating a series of sbz gene deletions as well as biochemical studies on the encoded enzymes. These experiments do an excellent job building upon the previous work characterizing this biosynthetic gene cluster (Ref. 14) and clarifies the timings of several downstream modifications in this biosynthetic pathway. The highlight of this paper is SbzP, a novel PLP-dependent enzyme which is broadly distributed amongst various strains of bacteria and which catalyzes the novel tandem C α and C γ alkylation between SAM and NAD⁺, generating the 6-azatetrahydroindane moiety.

Overall, the manuscript is well written, and conveys information very clearly and efficiently. I believe that the findings described in the manuscript would be of significant interest to those in the fields of chemistry, natural products and enzymology.

Thank you very much for the reviewer's positive comments.

The major issue holding the manuscript back from publication in Nature is the lack of evidence to describe how SbzP catalyzes its reaction. Without these experiments and crystal structures of SbzP to explain this, the manuscript does not rise to the level of similar previous work in this journal. Without this additional work, the current manuscript is well suited for publication in Nature Communications or Nature Chemical Biology.

We truly appreciate the critical thoughts and mechanistic suggestion from the reviewer. We conducted a series of additional experiments to support the proposed SbzP mechanism, including: a) stopped-flow analysis of the SbzP reaction to trace transient β,γ -unsaturated quinonoid species; b) steady-state kinetic studies that revealed a Ping-Pong Bi-Bi mechanism; c) isotopic labelling experiments; and d) homology model-based mutational studies. The results are in line with the proposal, which we believe represents the intrinsic reactivity of N-substituted pyridinium salts (such as β -NAD) and the experimentally detected β,γ -unsaturated quinonoid (Fig. 2b). However, we have also added a discussion on mechanistic alternatives, as suggested by the reviewer (see also the response to reviewer 1). The instability of the SbzP-like proteins, has so far hampered us from crystal structure-based investigations, which we believe is caused by a family-specific, N-terminal domain region, predicted as strongly disordered. We prepared a series of SbzP truncated variants, which showed that the N-terminal domain is essential for enzyme function, as truncation of only 10 amino acids lead to complete abolished enzyme activity. We have included a discussion on this family-specific structural feature (Main Text, page 8, Supplementary Note 9).

Figure 2a – Given the emphasis placed on SbzP in the manuscript I find that there is minimal data to support the SbzP-catalyzed reaction. The proposed mechanism for SbzP is thinly supported by a UV-Vis experiment, which has a very small shift at ~510 nm, diagnostic of a quinonoid or more extended conjugated intermediate.

We were able to improve the spectroscopic data by utilizing a stopped-flow apparatus in combination with using PsePQ, a more stable homolog of SbzP (please also see the response to Reviewer 1).

The first thing I find unusual is the formation of a geminal diamine in intermediate I in order to push the electron back up. Though there are some instances of such geminal diamines being relatively stable (Cook and Holden, 2007; Di Salvo et al. 2013; Sekula et al. 2018), however these kinds of intermediates are often fairly transient, and generally occur at the end of the reaction. Therefore, it seems unlikely that such a species would remain stable over multiple steps of the reaction. One alternative might be to have the NAD attack at C α directly, as depicted in the next intermediate.

Thank you very much for the criticism. We have added a corresponding discussion to the manuscript (page 7, Supplementary Figure 30), as elaborated above.

Would the deprotonation of C5 of intermediate II be spontaneous as depicted? This seems unlikely to me as it does not seem to be very acidic, and loss of a proton does not generate an aromatic species. This might be an enzyme catalyzed step instead.

Many thanks for the thoughtful comment. We have adjusted the mechanistic proposal, according to the suggestion (Figure 2a).

In the interest of discussion I have brainstormed one alternative mechanism that could occur from the β,γ -unsaturated quinonoid intermediate. In this mechanism, the NAD $^{+}$ substrate could be oriented with C5 of NAD $^{+}$ positioned closely to the C α of the quinonoid intermediate and C4 would be positioned near to C γ of the β,γ -unsaturated quinonoid intermediate. Then the β,γ -unsaturated quinonoid intermediate could then react via a canonical nucleophilic C α alkylation between C α and C5 of NAD $^{+}$. This step would be coupled with a canonical electrophilic C γ alkylation between C4 of NAD $^{+}$ and C γ and a reprotonation at C β . After this step, C5 could be deprotonated by the nearby PLP nitrogen (or another enzymatic base). This more typical mechanism would also eliminate the need to form a geminal diamine until after the cyclization is complete. The one issue here is that NAD $^{+}$ C5 is not as positive at C4 but it may still be possible to favor such a reaction within the active site of the enzyme.

As discussed above, we thank the reviewer for the discussion and have added alternative reaction mechanism for the C-C bond formation events (Supporting Figure 29) with corresponding discussion sentences in Page 5 (please also see the response to Reviewer 1).

To aid in the study of the SbzP mechanism, its crystal structure could be obtained, which might provide insights into how SAM and NAD are oriented with respect to each other and PLP.

As discussed above and in the letter to editor, the SbzP-like protein family exhibits poor stability in vitro, especially when concentrated above 20 μ M, significantly hampering us from obtaining suitable protein crystals for X-ray analysis. Crystallization attempts constituted: utilization of four crystal screening kits (PEG ion, Crystal Screen I & II, Wizard I & II) at three temperatures (4, 10, 20°C), and testing of three enzyme concentrations (8, 10, 12 mg/ml). We speculate that the difficulties arise from the above mentioned N-terminal domain, spanning about 100 amino acids, which is predicted as strongly disordered (X-talPred-RF). In attempts to overcome these problems, we prepared a series of SbzP truncations. However, even the longest possible truncation (10 amino acids, compare Supplementary Note 9), led to completely abolished enzyme function. Furthermore, by considering the experimentally evaluated ping-pong mechanism, we believe that a complex structure with both substrates (β -NAD and SAM), or analogs thereof will be highly difficult to obtain, to clarify their orientation.

The SbzP-catalyzed reaction could also be carried out using deuterated substrates or solvents (similar to Ref. 29), and the results of such experiments might provide additional mechanistic insights. It would also be interesting to test NADH to see if it inhibits the reaction or SbzP has some mechanism to discriminate between NADH and NAD $^{+}$ or even convert the former to the latter. The authors could also consider probing the mechanism with studies on variant enzymes, and specifically interrogating some of the proposed steps in the mechanism using a variety of common spectroscopic and other methods.

We thank the reviewer for the suggestion and enzymatically prepared (^{2} H $_6$)-SAM from commercial (2,3,3,4,4, methyl- ^{2} H $_5$)-L-methionine. Unlabelled SAM and the prepared isotopologue were utilized as substrates for the PsePQ reaction in H $_2$ O and ^{2} H $_2$ O (Supplementary Figure 23).

To verify the regiospecificity of the reprotonation (proposed to occur at C β of SAM (C2 in enzyme product 7)), the obtained isotopologs of 7 were further subjected to the PsePQ-mediated hydroxylation by subsequent addition of α -ketoglutarate (equivalent to SbzQ reaction). The results were found to be in line with the proposed mechanism. To verify the stereospecificity of the reprotonation step, we also obtained the enzyme product prepared in ^{2} H $_2$ O in preparative

scale, leading to isolation of (S)-(2-²H)-7, supported by NMR analysis (Supplementary Figure 25 and 26). The results therefore suggest reprotonation of proposed intermediate I from the Si-face. Based on this insight, we evaluated a homology model of SbzP to identify a possible acidic residue responsible for the reprotonation step. As a result, the conserved acidic residue D139 was identified and probed by point mutation. (Figure 2a and Supplementary Note 7).

We probed NADH as a possible substrate of SbzP, but did not find any detectable consumption. On the other hand, NADH was found to act as an inhibitor for the SbzP-mediated reaction. The inhibitory activity was analyzed by kinetic investigations, revealing non-competitive inhibition.

Minor comments:

Quinoid is used throughout manuscript instead of quinonoid.

Thank you very much for the careful review. We have corrected the mistake.

Figure 3a – The top trace is labeled 'WT', and it should be more clearly defined what 'WT' represents. Additionally, isolated compound 4 should be used as a standard for this figure.

We have added a more precise description to the Figure and added the chromatogram of compound 4 as reference.

Figure 3b – The font of the enzyme annotations is small and difficult to read. Some arrow tips also appear to be extending into the end of adjacent genes. There is a lot of information in this panel. A simplified color scheme for the sbz gene cluster might convey the similarities and/or differences between the other gene clusters. For example, sbzB/C/D/K could all be colored the same to indicate production of β-methyl phenylalanine in a cryptic gene cluster.

As the reviewer suggested, the fonts of gene product annotations have been enlarged and the genes grouped, according to their function and adapted the color-code.

Extended Figure 2b – masses don't match Supplementary Figure 10

Thank you very much for the notice. We have fixed Supplementary Figure 10.

Supplementary Figure 10 – The turnover here by SbzQ appears to be very low~2000 counts. The assay conditions could be modified (run longer than 1 hr for example) to improve the results here.

The substrate was completely consumed under the employed conditions. However, the signal intensity of substrate and product (probably caused by condition of the ESI-source) were underrepresented. We repeated the assay under the same conditions to improve the data and the Figure has been updated.

Supplementary Figures 19/20 – It is a bit repetitive to have the PQ homologs in Fig 19 and then separate them in Figure 20. Perhaps the P homologs and the PQ homologs could be split into two figures.

According to the suggestion, we have separated the P homologs and the PQ homologs (Supplementary Figures 29 and 31).

Additionally, given the similarities between SbzP and cystathionine-γ-synthase/lyases it might also be informative to create an alignment between the two types of enzymes, and this might highlight other conserved residues. For example, the conserved Asp (-DECH-) which coordinates pyridinium nitrogen in these type I PLP-dependent enzymes is not highlighted in Figure 19. Additionally, are there conserved putative iron/α-KG binding residues in the 'Q' portion of the PQ homologs?

We thank the reviewer for the interesting insight. Members of the cystathionine-γ-synthase/lyase subfamily, which also activate C_γ of the amino acid substrate (but proceed via a vinyl glycine ketimine in the catalytic cycle, which is attacked by a nucleophile) is functionally and phylogenetically distinct from the discovered SbzP-like family and thus appears on a separate branch in the phylogenetic analysis (Fig. 2c). To further underline this difference, we have added a CGL-protein sequence to the protein sequence alignment and the results further illustrate the difference between the subfamilies, as the SbzP-like proteins seem to be more closely related to the AT-subfamily (such as ACCS and ATT-I). We have highlighted the conserved residue for PLP and carboxylate group binding, which can now be also seen in the homology model (Supplementary Figure 27 and 29). We have also highlighted the conserved putative iron/α-KG binding residues in Supplementary Figure 31.

Page S35 – HR-MS data should be reported with ppm error.

We have made revision.

Spelling/grammar/formatting errors in:
Supplementary Figures 5, 24
Supplementary Note 2
Thank you very much. We have revised them.

Reviewer #3

Barra et al. have investigated the biosynthesis of alternicidin on a genetic and enzymatic level, with structural characterisation of all pathway intermediates. One of the steps along the pathway is absolutely spectacular, which is the formation of an unusual dinucleotide from the building blocks NAD⁺ and SAM. These compounds are usually used as reagents, NAD⁺ for oxidations and SAM as methyl donor, but not as building blocks themselves (with some exceptions, e. g. SAM serves as precursor for spermidin / spermin). The unusual dinucleotide is generated by a PLP-dependent enzyme (SbzP) that is also mechanistically most astonishing, because it catalyses two C-C bond formations in a formal [2+3]-cycloaddition. A detailed mechanistic proposal is discussed in the manuscript and supported by site-directed mutagenesis. Something like the reaction of this enzyme has never been seen in Nature before. Besides this remarkable step the authors also functionally characterised all other enzymes along the biosynthetic pathway, and showed that the PLP-dependent enzyme SbzP makes up a new enzyme family that is likely involved in the biosynthesis of several other unusual natural products. This was further underpinned in the present work by the functional characterisation of SbzP homologs from several other biosynthetic gene clusters by heterologous expression that all catalyse the same reaction. Overall, this is a brilliant study that leaves no open questions.

Thank you very much for the reviewer's positive comments and support.

Still, a bit work on the text could increase the readability. In the current form, the manuscript text appears quite technical, e. g. page 3, first paragraph of the Gene deletions section: „LC-MS analysis of the culture extracts of gene deletion strains showed accumulation of specific metabolites (...). Purification and structural characterisation revealed the presence of 12 (m/z = 361.0823) in the delta-szbE strain (...).“ More simple: „The delta szbE strain accumulated compound 12.“, details such as detected MS signals should not be given in the main text. This is just an example, the whole manuscript should be cleaned up and could be significantly shortened. The authors should focus on their interesting story, rather than giving to many experimental details. According to the reviewer's comment, we have modified the text (Page 3).

The authors also make excessive usage of „we“ and similar, which is arguably not a good style of scientific writing. Neutral / passive forms should be preferred. Also phrases like „Interestingly, ...“ (page 5), „Intriguingly, ...“ (page 6) should be avoided. We have revised the text as the reviewer suggested.

The Conclusions should also be rewritten, because they are mainly a summary of the main findings of the present work. Here the authors should focus more on the impact of their findings for future work. We have revised the conclusion to emphasize on the impact of the work (Page 7).

1. Page 3, left, top: What means „The quaternisation of the ring nitrogen, resulting in a permanently positively charged pyridinium moiety...“? There does not seem to be an intermediate that fits to this statement? We have made changes to clarify the meaning.

2. Page 3, right, middle: rephrase „strict substrate specificity“. Only 7 and 4 were tested, 7 was accepted, 4 was not. But it is not known if compounds more similar to 7 would be accepted. We have rephrased it as follows.

“Compound 7 and nucleoside analog 4 were not accepted as substrates, suggesting that preceding C5 hydroxylation by SbzQ is essential for substrate recognition.” (Page 3)

3. Page 5, left, middle: rephrase „highly conjugated“. Double bonds are either conjugated or not, they cannot be more or less conjugated (or only if torsion angles are to be discussed, but this is

not the case here). The authors mean that the conjugated system spans a large part of the intermediate.

We have rephrased it as follows.

"a β,γ -unsaturated quinonoid, which possesses an extended conjugated π -system with nucleophilic character at C γ " (Page 5)

4. Page 5, right, bottom: The compound name should read „7,8-diaminopelargonic acid“.

Thank you very much. We have revised it.

Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

The reviewed manuscript is much improved, and provides significantly more supporting data for the mechanistic proposal(s) made in Fig 2.

As the mechanism of this enzyme is one of the key features, I would suggest the presentation of this in Fig 2 (but also Supp Fig 22/30) is done with great care. Although some steps are shown better compared to the first version, it would be good to very clearly stipulate in each step where both electrons and protons are going to. For example the various protonation states of K334 are often unclear/not well defined. In Supp Fig 22, panel e shows an acid-base couple, not isomers.

Referee #2 (Remarks to the Author):

The authors have accomplished a lot in this paper, and the amount of work reported represents multiple manuscripts. Additionally, the manuscript is improved upon the original submission. However, the paper remains hard to read, and the audience is likely to be restricted to specialists. These issues can be addressed if the authors work with the editor to ensure all statements are logical and connected with one another, and the discovery is clearly stated.

Additional comments:

- The new sentence: "Based on the obtained insights and given that PLP enzymes typically activate α -amino acids, the possibility of an SbzP-mediated [3+2]-annulation reaction between an aspartic acid-derived amino acid and nicotinamide mononucleotide (b-NMN) appeared most likely" may need further explanation for non-specialist readers, as their feeding experiment did not involve NMN. Similarly, once they find the in vitro substrates this result should be linked back to the feeding?
- For the sentence, "Although it appeared less likely" The authors need to clarify why it appeared less likely.
- The sentence that begins "Whereas the delta-sbzE strain ..." can be reworded with help from the editor to function as a more normal introductory sentence.
- "suggesting a penultimate F420-dependent reduction step ..." the structure of 11 compared to 12 (to me) suggests a reduction, but it doesn't necessarily suggest SbzF must be F420-dependent. I believe they are implying (but not stating) that the annotation implies the F420-dependence.
- The authors stated that they used a homologous enzyme for assays, however, when they switched to this enzyme is not mentioned in the main text, and I do not see an accession number for PsePQ.
- led to 85% enzyme activity loss – what do the authors conclude from this result? It appears Asp should not be essential for this reaction? Please state conclusion.
- Supplementary Figure 1 legend lists SbzJ twice. SbzK is not shown in the figure, so should be removed from the legend.
- Supplementary Note 1 doesn't indicate percent incorporation or compare to the other precursors they might have tested.
- Extended Data Fig 1 – part c – what the "control" is needs to be stated

- The program/software used to generate the homology model of SbzP is not stated anywhere in the manuscript.
- PctIV should be PctV
- In discussion of K334A Fig S29 (sequence alignment) is referenced, instead of experimental data for this variant (like Fig S28)?
- Figure 3 Caption – grouped
- Page 7 - ...accepts the b-NAD as a substrate...
- Page 7 - a group of novel b-NAD tailoring enzyme family...
- Figures in Supplementary Note 1 still all have quinoid in them.

Author Rebuttals to First Revision:

Reviewer #1

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better compared to the first version, it would be good to very clearly stipulate in each step where both electrons and protons are going to. For example the various protonation states of K334 are often unclear/not well defined. In Supp Fig 22, panel e shows an acid-base couple, not isomers.

We have modified Fig.2, Supplementary Figs. 22, and 30 accordingly. We also corrected the label for chemical structures shown in Supplementary Fig.22e to "internal aldimine acid-base pair".

Reviewer #2

The authors have accomplished a lot in this paper, and the amount of work reported represents multiple manuscripts. Additionally, the manuscript is improved upon the original submission. However, the paper remains hard to read, and the audience is likely to be restricted to specialists. These issues can be addressed if the authors work with the editor to ensure all statements are logical and connected with one another, and the discovery is clearly stated.

As outlined above and below, we have addressed the advice and made changes in the manuscript.

Additional comments:

** The new sentence: "Based on the obtained insights and given that PLP enzymes typically activate α -amino acids, the possibility of an SbzP-mediated [3+2]-annulation reaction between an aspartic acid-derived amino acid and nicotinamide mononucleotide (β -NMN) appeared most likely" may need further explanation for non-specialist readers, as their feeding experiment did not involve NMN. Similarly, once they find the *in vitro* substrates this result should be linked back to the feeding?*

We have modified the explanation for the retrobiosynthetic analysis, and added information about the metabolic origin of β -NMN to make the connection to our feeding experiment result clearer, as follows: "Based on these insights and the molecular structure of **4**, a plausible biosynthetic scenario appeared to be an SbzP-mediated [3+2]-annulation reaction between an aspartic acid-derived amino acid (fragment C) and nicotinamide mononucleotide (β -NMN), itself metabolically derived from aspartic acid and glyceraldehyde 3-phosphate (fragment A and B)¹. The electrophilic *N*-ribosyl pyridinium moiety of β -NMN presumably provides sufficient reactivity towards nucleophilic addition at C4¹⁴. The corresponding alkyl nucleophile on the other hand, could be generated by a PLP-mediated β,γ -elimination on an α -amino acid with $C\gamma$ -leaving group^{15,16}, generating a β,γ -unsaturated quinonoid (Extended Data Fig.1b). To test this hypothesis, recombinant SbzP was incubated with β -NMN and nine candidate α -amino acid substrates, exhibiting a $C\gamma$ -leaving group (Supplementary Fig.5)." (page 2-3)

** For the sentence, "Although it appeared less likely" The authors need to clarify why it appeared less likely.*

We have modified the sentence as follows: "Although *in vivo* accumulation of **4** suggested a mononucleotide metabolic origin, we next hypothesized, that dinucleotide analogs of β -NMN, namely β -nicotinamide adenine dinucleotide (β -NAD, **5**) or nicotinamide adenine dinucleotide phosphate (β -NADP), exhibiting the same electrophilic pyridinium moiety, could be utilized by SbzP." (page 3)

** The sentence that begins "Whereas the delta-sbzE strain ..." can be reworded with help from the editor to function as a more normal introductory sentence.*

We have reworded it as follows: "To gain insight into subsequent enzymatic steps in the *sbz* pathway, gene deletion strains were constructed in order to identify specific pathway intermediates. Deletion of *sbzE* led to accumulation of compound **12**, indicative for a terminal SAM-dependent methylation to generate **1** (Fig.1c, Extended Data Fig.2a)." (page 3)

** "suggesting a penultimate F420-dependent reduction step ..." the structure of **11** compared to **12** (to me) suggests a reduction, but it doesn't necessarily suggest SbzF must be F420-dependent. I believe they are implying (but not stating) that the annotation implies the F420-dependence.*

We have reworded it as below: "Detection of 4,5-dihydropyridine analog **11** in the Δ sbzF strain suggested a penultimate imine reduction step catalyzed by SbzF." (page 3)

• *The authors stated that they used a homologous enzyme for assays, however, when they switched to this enzyme is not mentioned in the main text, and I do not see an accession number for PsePQ.*

We have added a sentence: "Due to higher protein stability, SbzP homolog PsePQ (WP_185022109) was utilized for kinetic, photospectroscopic and labelling experiments." (page 5)

• *led to 85% enzyme activity loss – what do the authors conclude from this result? It appears Asp should not be essential for this reaction? Please state conclusion.*

As the reviewer pointed out, D139 is not essential, but we believe that it should play a role in the catalysis based on our homology modeling, the shown significant decrease of the activity of D139A mutation, as well as the conserved nature of this residue in all SbzP homologs. Thus, we describe it as "a possible catalytic acid residue". We would like to leave this description.

• *Supplementary Figure 1 legend lists SbzJ twice. SbzK is not shown in the figure, so should be removed from the legend.*

Done.

• *Supplementary Note 1 doesn't indicate percent incorporation or compare to the other precursors they might have tested.*

We have added the relative incorporation rates in Supplementary Figs 2 and 3.

• *Extended Data Fig 1 – part c – what the "control" is needs to be stated*

We have added a sentence: "Control" indicates incubation of substrates without enzyme."

• *The program/software used to generate the homology model of SbzP is not stated anywhere in the manuscript.*

We used i-Tasser platform to construct the homology model. We have added this information to the methods section (page 8) and added corresponding references.

• *PctIV should be PctV*

We have deleted this description during the course of manuscript editing process.

• *In discussion of K334A Fig S29 (sequence alignment) is referenced, instead of experimental data for this variant (like Fig S28)?.*

A corresponding chromatogram has been added to Supplementary Fig.28.

• *Figure 3 Caption – grouped*

We have reworded it by "are grouped".

• *Page 7 - ...accepts the β -NAD as a substrate...*

• *Page 7 - a group of novel β -NAD tailoring enzyme family...*

These sections have been deleted, during the course of the manuscript editing process.

• *Figures in Supplementary Note 1 still all have quinoid in them.*

Supplementary Note 1 does not contain any description for quinoid, but we believe reviewer 2 might refer to Figures in Supplementary Note 8, where we have corrected the mistake.