Ancestral diterpene cyclases show increased thermostability and substrate acceptance

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Bacterial diterpene cyclases are receiving increasing attention in biocatalysis and synthetic biology for the sustainable generation of complex multicyclic building blocks. Herein, we explore the potential of ancestral sequence reconstruction (ASR) to generate remodeled cyclases with enhanced stability, activity, and promiscuity. Putative ancestors of spiroviolene synthase, a bacterial class I diterpene cyclase, display an increased yield of soluble protein of up to fourfold upon expression in the model organism Escherichia coli. Two of the resurrected enzymes, with an estimated age of approximately 1.7 million years, display an upward shift in thermostability of 7–13 °C. Ancestral spiroviolene synthases catalyze cyclization of the natural C20-substrate geranylgeranyl diphosphate (GGPP) and also accept C15 far- nesyl diphosphate (FPP), which is not converted by the extant enzyme. In contrast, the consensus sequence generated from the corresponding multiple sequence alignment was found to be inactive toward both substrates. Mutation of a nonconserved position within the aspartate-rich motif of the reconstructed ancestral cyclases was associated with modest effects on activity and relative substrate specificity (i.e., \( k_{\text{cat}}/K_{\text{M}} \) for GGPP over \( k_{\text{cat}}/K_{\text{M}} \) for FPP). Kinetic analyses performed at different temperatures reveal a loss of substrate saturation, when going from the ancestor with highest thermostability to the modern enzyme. The kinetics data also illustrate how an increase in temperature optimum of biocatalysis is reflected in altered entropy and enthalpy of activation. Our findings further highlight the potential and limitations of applying ASR to biosynthetic machineries in secondary metabolism.

Introduction

The increased awareness of the impact of human activities on environment and climate has intensified research in biocatalysis [1–3]. Today, enzyme engineering through directed evolution is a cornerstone in generating biocatalysts with extended substrate scope and novel reactivities [4], which are prerequisites to enable accelerated biosynthesis of sustainable building blocks. In particular, generation of renewable,
multicyclic scaffolds with potential applications [5–7] as biofuels, fine chemicals, medicines, and novel polymers by synthetic biology efforts is currently receiving significant attention [8]. Herein, we explore the potential of applying ancestral sequence reconstruction (ASR) [9–11] as an enzyme engineering tool to obtain biological polycyclization catalysts with enhanced stability and promiscuity. The importance of these two key features for efficient biocatalysis and extended reaction scope has been discussed in the literature [1,2].

Among other renewable building blocks, terpenes are currently in the spotlight as a large pool of sustainable hydrocarbons [5]. Terpenes constitute the most diversified family of natural products, comprising a myriad of complex polycyclic compounds with important functions in all forms of life [6,12]. Biosynthesis of terpenes is founded on the assembly of elongated polyisoprenes from the universal C5 building blocks dimethyl allyl diphosphate and isopentenyl diphosphate. The cyclization of relatively simple, linear polyisoprenoids, catalyzed by terpene cyclase enzymes, is a key for the generation of terpene diversity [13]. Terpene cyclases are classified according to their isoprenoid substrate carbon content: mono- (C10), sesqui- (C15), di- (C20), sester- (C25), tri- (C30), sesquar- (C35), and tetra- (C40) terpene cyclases have been described [6,14]. In addition, a mechanistic distinction for substrate activation can be made [13]: class I enzymes initiate the cyclization sequence by metal ion-dependent cleavage of a labile allylic-diphosphate bond, whereas the class II mechanism is dependent on protonation of an isoprene (or oxirane) group of the prefolded substrate; a process which can be facilitated by hydrogen tunneling [15]. Biocatalysts harboring separate class I and class II active sites for sequential cyclization within the same protein also exist [13]. The carbocation generated by either mechanism propagates in the substrate through C–C bond formations, ring expansions and/or methyl and hydride shifts, throughout the partially concerted cyclization sequence chaperoned within the enzyme active site [16]. Termination of cyclization occurs through deprotonation by a suitable base or by water addition [17].

Bacterial diterpene cyclases have recently emerged as potent biocatalysts both by contributing to enhanced understanding of fundamental secondary metabolism [18] and in the context of synthetic biology programs [8]. At present, the availability of bacterial diterpene cyclase sequences is significantly limited [19], and our fundamental understanding of associated cyclization mechanisms remains incomplete [18]. We reasoned that ASR of class I enzymes would constitute an interesting opportunity to expand terpene cyclase sequence space, and to investigate the potential, as well as limitations, of ASR when applied to an essentially unexplored family of important biosynthetic enzymes. We speculated that this enzyme engineering strategy could provide access to biocatalysts with enhanced promiscuity and stability, since both traits are generally observed in reconstructed ancient enzymes [20,21]. This phenomenon is often explained by the hypothesis that extinct ancestral enzymes were general catalysts, which evolved toward a more specialized function over time [22,23].

In order to test these hypotheses, we reconstructed ancestral class I diterpene cyclases and characterized the putative ancestral enzymes in comparison to their modern counterpart with respect to promiscuity, activity, and thermostability. The consensus sequence, generated from the same multiple sequence alignment used for phylogenetic analysis, displayed intermediate stability, yet was found to be inactive. The loss of substrate saturation that was observed when going from ancestor to modern enzyme is in accordance with an increase in turnover number at the expense of $K_M$ during the course of evolution. Our results indicate the importance of the ancestral protein background in directing substrate acceptance and underline the high potential of applying ASR as a sequence-based enzyme engineering tool on biocatalysts within secondary metabolism.

**Results**

**Reconstruction of ancestral class I diterpene cyclases**

To investigate the feasibility of using ASR to generate cyclase enzymes with extended catalytic versatility, we turned our attention toward spiroviolene synthase (SvS, EC 4.2.3.158); a recently discovered class I diterpene cyclase from *Streptomyces violens* [18]. SvS catalyzes the cyclization of prefolded C20 geranylgeranyl diphosphate (GGPP) into tetracyclic spiroviolene (Fig. 1A).

The amino acid sequence of SvS from *S. violens* was used as a query to generate a multiple sequence alignment, and corresponding phylogenetic tree, of 29 homologous enzymes (Fig. 2). We used maximum likelihood statistics in MEGA7 [24,25] to reconstruct the most likely ancestral sequences at three nodes, A1–A3, which are all located within the subtree of *Streptomyces* species (Fig. 3A). The probabilities of predicted residues are given in Fig. 3B. The putative ancestral sequences differed from the *S. violens* sequence by 6%
The associated minimum ages of the ancestral enzymes were estimated to be between 0.04 million years (SvS-A3) and 1.7 million years (SvS-A1), using available calibration points [26] and the relative divergence time estimation function in MEGA7 [24]. Despite their high similarity (only 12 residues differ out of 361), both SvS-A1 and SvS-A2 were included in our analyses, in order to investigate the robustness of ASR in this particular case. We hypothesized that comparing these two ancestors could yield insights into the method, given the limited pool of available closely related sequences, and the fact that only one sequence diverged from SvS-A1 before SvS-A2. As we were interested in comparing ancestral enzymes to the consensus sequence, all *Streptomyces* sequences that descend from SvS-A1 were aligned using MUSCLE [27] and the corresponding consensus sequence was created using the Unipro UGENE software [28]. The consensus sequence differs from modern SvS by 28% (Fig. 3C).

During the reconstruction process, we noticed a change in the conserved aspartate-rich DDxx(x)D motif typical for class I diterpene cyclases (Fig. 3D) [13]. This highly conserved motif is, together with the so-called NSE/DTE motif, involved in the binding of three divalent metal ions, which in turn position the substrate in the active site and facilitate catalysis by interaction with the negatively charged diphosphate group [29]. Mutations within, and in proximity of, this key motif have previously been shown to impact product specificity [17]. To
investigate the significance of this position for substrate specificity and catalysis, three ancestral sequences with single point mutations were designed, in addition to the originally predicted sequences. In the mutated sequences, all residues in the motif were kept conserved with respect to SvS from *S. violens*, resulting in mutants SvS-A1_A89H, SvS-A2_A89H, and SvS-A3_N89H. Enzyme sequences (Fig. S1) were designed with an N-terminal 6xHis-tag, cloned into a pET22b- (+) vector and expressed in *Escherichia coli* BL21(DE3) cells.

**Ancestral enzymes display enhanced thermostability and increased yield of soluble protein**

To assess the expression of the ancestral proteins in *E. coli*, a comparative experiment was performed in which all four enzymes were purified and the final yield of soluble protein in milligram per liter of cell culture was calculated (Fig. 4).

In addition to a 2- to 4-fold increase in protein yield for the two oldest ancestors, we observed that
these enzymes could be stored for up to 3 weeks at 4 °C before they precipitated, as opposed to less than 1 week for the modern enzyme and SvS-A3. Moreover, SvS-A1 and SvS-A2 could be frozen and thawed without precipitation, as opposed to modern SvS. The consensus enzyme was found to generate a lower yield of soluble protein of less than 1 mg/L. These findings suggested that SvS-A1 and SvS-A2 are generally more stable proteins [30]. To confirm this, we established melting curves for all enzymes by monitoring intrinsic tryptophan fluorescence at 330 and 350 nm using nanoDSF (Table 1). Indeed, we found that the two oldest ancestral enzymes had increased melting temperatures compared to SvS from S. violens, with $T_m$ being up to 13 degrees higher. This was confirmed by complementary DSF measurements using a fluorescent dye (Table 1, values given in brackets). The consensus sequence displayed an increase in $T_m$ of ca. 6 degrees compared to the modern enzyme, similar to that of SvS-A1.

**Ancestral enzymes are active toward GGPP and accept truncated substrate FPP**

Analysis of relative activities for formation of spiroviolene showed that the three resurrected terpene cyclases were active (Table 2). To investigate promiscuity of ancestral cyclases, enzymes were incubated with SvS’ native substrate GGPP, as well as two additional truncated substrates (Fig. 1B): C15-substrate farnesyl diphosphate (FPP) and C10-substrate geranyl diphosphate (GPP). As reported previously [18] SvS from S. violens did not convert FPP or GPP. Interestingly, analysis by GC/MS demonstrated that all three ancestral enzymes were able to form products from both GGPP and FPP (Figs S2–S6), albeit FPP was associated with a 5– to 300-fold lower initial reaction rate (Table 2, right column). No product could be detected from reactions of ancestral enzymes with GPP. Based on comparison of mass spectra and retention times of product peaks to those of previously published

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**Table 1.**

| Enzyme          | Count |
|-----------------|-------|
| SvS S. violens | 100   |
| SvS-A3          | 94    |
| SvS-A2          | 77    |
| SvS-A1          | 76    |
| SvS-consensus   | 72    |

**Fig. 3.** Reconstruction of ancestral spiroviolene synthases. (A) A rooted subtree showing the homologous enzymes from *Streptomyces* species only. Ancestral nodes A1, A2, and A3 are marked with blue dots. (B) Histogram of probabilities of correctly inferred ancestral residues for SvS-A1, SvS-A2, and SvS-A3. (C) Percentage identity matrix showing the shared sequence identity between modern SvS, ancestral enzymes, and SvS consensus. (D) A section (residues 78–100, S. violens numbering) of aligned SvS from S. violens, ancestors A1–3, and the *Streptomyces* consensus sequence, including the aspartate-rich motif highlighted in gray (full sequences in Fig. S1). The nonconserved position 89 is highlighted in cyan.
### Table 1. Melting temperatures of modern and reconstructed enzymes.

|           | T<sub>m</sub> (°C) |
|-----------|------------------|
| SvS        | 57.2 ± 0.7 (57.0 ± 0.3) |
| SvS-A3     | 55.5 ± 0.4 (±)        |
| SvS-A2     | 70.5 ± 0.3 (70.3 ± 0.3) |
| SvS-A1     | 64.1 ± 0.1 (65.0 ± 0.2) |
| SvS consensus | 63.1 ± 0.6 (±)    |

The complementary assay did not yield a melting curve of good quality for SvS-A3, possibly because the dye interacts with the folded protein and thermal denaturation can thus not be detected.

In order to investigate relative second-order rate constants, we carried out a series of one-pot reactions in which GGPP and FPP were mixed at different concentration ratios, keeping a fixed total substrate concentration of 150 μM. Although all ancestral enzymes prefer GGPP as their main substrate, relative

### Table 2. Initial reaction rates for conversion of GGPP and FPP by modern enzyme and ancestors show the impact of mutation in aspartate-rich motif on substrate specificity.

|           | V<sub>0</sub><sub>GGPP</sub> (s<sup>-1</sup>)<sup>a</sup> × 10<sup>-3</sup> | V<sub>0</sub><sub>FPP</sub> (s<sup>-1</sup>)<sup>b</sup> × 10<sup>-4</sup> | Apparent<sup>c</sup> K<sub>cat</sub>/K<sub>M</sub>GGPP/K<sub>cat</sub>/K<sub>M</sub>FPP |
|-----------|-----------------|-----------------|-----------------|
| SvS S. violens | 1 ± 0.07 | _d<sup>a</sup> | _d<sup>a</sup> |
| SvS-A3 | 2 ± 0.15 | 4 ± 0.3 | 6 |
| SvS-A3_N89H | 4 ± 1.15 | 3 ± 0.3 | 16 |
| SvS-A2 | 0.6 ± 0.03 | 0.7 ± 0.04 | 8 |
| SvS-A2_A89H | 0.6 ± 0.03 | 0.5 ± 0.04 | 12 |
| SvS-A1 | 0.3 ± 0.02 | 0.01 ± 0.005 | 231 |
| SvS-A1_A89H | 0.3 ± 0.02 | 0.01 ± 0.001 | 308 |

<sup>a</sup> Initial reaction rate for formation of spiroviolene (Fig. S2).

<sup>b</sup> Initial reaction rate for formation of reference product from FPP (Fig. S5).

<sup>c</sup> Relative specificity was determined according to Eqn 1 and is based on analysis of the main products (Figs S2 and S5). Small deviations occur due to rounding.

<sup>d</sup>ΔSvS from S. violens does not react with FPP.

ancestors (Fig. S2) displayed an m/z of 272 upon GC/MS analysis (Fig. S4), as could be expected for isomers. For conversion of FPP by the ancestors (Fig. S5), we compared the mass spectrum and retention time of the main product with those of reference compounds trans-trans-farnesol, trans-nerolidol, and isomers of trans-farnesene, which represent mechanistically possible linear products [31]. This comparison (Fig. S6) showed that likely a cyclic product was formed, which could also be in accordance with the reaction mechanism of SvS (Fig. 1A). No product could be detected upon incubation of the Streptomyces consensus sequence with either GGPP, FPP, or GPP.

In order to investigate relative second-order rate constants, we carried out a series of one-pot reactions in which GGPP and FPP were mixed at different concentration ratios, keeping a fixed total substrate concentration of 150 μM. Although all ancestral enzymes prefer GGPP as their main substrate, relative k<sub>cat</sub>/K<sub>M</sub> ratios for GGPP over FPP of down to six were observed (Table 2, right column). Moreover, changing a single residue in the aspartate-rich motif from the predicted ancestral residue to the histidine occurring in SvS from S. violens (position 89) did not obstruct acceptance of FPP as a substrate and only resulted in small effects on relative activity and promiscuity (Table 2).
Kinetics assays of modern SvS and SvS-A2 reveal shift in temperature optimum

Due to the increased melting temperature of SvS-A2, which could be of interest from a biocatalytic perspective, an extended kinetic investigation of this enzyme scaffold was undertaken and compared to the modern enzyme, using GGPP as a substrate (Fig. 5, Table 3). The apparent \( k_{\text{cat}}/K_M \) for generation of spiroviolene displayed by ancestor SvS-A2 was up to twofold higher compared to that of modern SvS (Table 3). We found that the observed shift in melting optimum is accompanied by a shift in temperature optimum for catalysis of 10 degrees, whereas SvS from \( S. violens \) displayed maximum activity at 20 °C, the temperature optimum for SvS-A2 remains at 30 °C. As product release has previously been shown to be rate limiting for some class I terpene cyclases [32,33], it is possible that our kinetic data reflect this step. The low \( k_{\text{cat}} \) value displayed by the ancestral enzyme (Table 3) is comparable to that of other terpene cyclases [17].

In addition, we observed a loss of substrate saturation for GGPP when going from SvS-A2 to the modern enzyme (Fig. 5). This is aligned with previous hypotheses regarding an increase in turnover number at the expense of \( K_M \) during evolution [34].

Kinetic and (quasi)thermodynamic analyses were performed based on determined apparent \( k_{\text{cat}}/K_M \) values at different temperatures below the respective optima, according to transition state theory [35] (Fig. 6). As Gibbs free energy of activation is \( \Delta G^* = \Delta H^* - T^*\Delta S^* \), SvS-A2 would benefit from a higher entropy of activation compared to that of the extant enzyme, to sustain biocatalysis at its higher temperature optimum. Aligned with this notion, and under the assumption of equal relative abundance of active enzyme, the associated change in entropy of activation for ancestral SvS compared to that of modern enzyme (\( T^*\Delta S_{\text{SvS,A2-S.violens}}^* \)) was around 2.5 kcal mol\(^{-1}\) (at 20 °C). The corresponding change in enthalpy of activation (\( \Delta H_{\text{SvS,A2-S.violens}}^* \)) was around 2 kcal mol\(^{-1}\). Overall, our data demonstrates small changes in enthalpy and entropy of activation, which is consistent with the relatively young age of SvS-A2.

Table 3. Kinetic constants of modern SvS and SvS-A2. Values are shown for formation of spiroviolene from GGPP, based on data shown in Fig. 5. The errors shown are standard deviations with \( n = 3 \).

| \( \tau \) (°C) | \( k_{\text{cat}}/K_M \) (s\(^{-1}\)M\(^{-1}\)) | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( K_M \) (μM) | \( k_{\text{cat}}/K_M \) (s\(^{-1}\)M\(^{-1}\)) |
|---|---|---|---|---|
| 11.7 | 88 ± 4 | 0.0015 ± 2.9\( \times \)10\(^{-4}\) | 15 ± 1.1 | 97 ± 22 |
| 13.7 | 97 ± 7 | – | – | – |
| 17.9 | 106 ± 3 | – | – | – |
| 20.4 | 127 ± 4 | 0.0025 ± 2.0\( \times \)10\(^{-4}\) | 18 ± 1.7 | 139 ± 20 |
| 25.0 | 112 ± 2 | 0.0046 ± 1.1\( \times \)10\(^{-4}\) | 24 ± 0.9 | 192 ± 10 |
| 30.0 | 109 ± 10 | 0.0065 ± 0.6\( \times \)10\(^{-4}\) | 27 ± 0.6 | 239 ± 7 |

\(^{a}\) Deviations may occur due to rounding.

Discussion

The aim of this study was to investigate the potential of using ASR as a tool to obtain biocatalytic scaffolds within secondary metabolism that display enhanced promiscuity and stability. Despite their relatively young estimated age, putative ancestral spiroviolene synthases show increased stability, which is reflected in melting temperatures of up to 13 degrees higher...
The general increase in stability of ancestral enzymes has previously been attributed to a bias toward the consensus sequence during the ancestral reconstruction process [38]. According to this hypothesis, the consensus sequence is stable per se, as it represents the most common, and thus tolerable, residue at each position within the protein. When comparing sequence identities (Fig. 3C), the two most stable ancestors SvS-A1 and SvS-A2 are indeed closest to the consensus sequence. However, the $T_m$ of SvS-A2 is in fact 7 degrees higher than that of the consensus sequence, indicating that sequence bias is not the only underlying factor for the increased stability. Moreover, we could not detect any activity of the enzyme corresponding to the consensus sequence toward any of the substrates investigated. The lack of evolutionary context awareness of the consensus approach, which accounts for every position individually and thus might fail to incorporate coevolution of certain sites, could possibly interfere with enzymatic activity. Similar to the consensus approach, the maximum likelihood method used for the ancestral reconstruction has a preference to select the most frequent residue in one particular position, as those will be present in a higher degree over the course of evolution. As a result, there might be a bias to automatically select the most stabilizing residue for each position within a certain clade of the tree [39]. High protein stability has recently been connected to higher abundance of soluble protein in the cell [30]. This is in accordance with our findings, namely that the two oldest ancestral terpene cyclases are readily expressed in E. coli with up to four times higher protein yield compared to their modern counterpart (Fig. 4). This phenomenon has previously been reported for ancestral serum paraoxonase enzymes, which also displayed large increases in melting temperature [38]. Overall, these results show the potential of ASR for engineering proteins with increased stability, which may also facilitate purification and crystallization [40].

The promiscuity displayed by ancestral terpene cyclases is in line with other studies, which suggest that compared to the modern enzyme, as well as an increased yield of soluble protein upon expression in E. coli. Ancestral enzymes also showed promiscuous activity: they accept an additional truncated substrate that is not converted by the modern enzyme.

It has been suggested that evolution selects for biochemical function of enzymes rather than their stability, yet a certain level of stability is needed to allow accumulation of destabilizing mutations that may benefit catalytic function [36]. High protein stability has therefore been identified as an evolvability determinant, which could partially explain why this trait is typically observed in ancestral proteins, including relatively young ancestors such as those investigated in the present work. Another possible explanation for this observed thermostability is the distinction between an active evolutionary pressure to maintain activity at lower temperatures versus a passive drift of decreasing thermostability over time [37]. In contrast to the traditionally proposed activity/stability trade-off, this scenario substantiates the possibility to find or design highly active enzymes with increased stability compared to modern enzymes. Aligned with this notion, SvS-A2 displays increased thermostability and higher activity compared to the extant terpene cyclase (Tables 1 and 3). A recent study on ancestral adenylate kinases describes the importance of modulating transition-state heat capacity to sustain high catalytic turnover on a cooling Earth [37]. Our data shows that SvS-A2 and modern SvS display different temperature dependence of catalysis (Fig. 6), resulting in an increased entropy of activation for the ancestral enzyme that is active at higher temperature. An attempt to fit our experimental data to the modified equation of TS theory [37], that take the change in heat capacity into consideration (i.e., $\Delta C_p$), resulted in nonoptimal fits.

The general increase in stability of ancestral enzymes has previously been attributed to a bias toward the consensus sequence during the ancestral reconstruction process [38]. According to this hypothesis, the consensus sequence is stable per se, as it represents the most common, and thus tolerable, residue at each position within the protein. When comparing sequence identities (Fig. 3C), the two most stable ancestors SvS-A1 and SvS-A2 are indeed closest to the consensus sequence. However, the $T_m$ of SvS-A2 is in fact 7 degrees higher than that of the consensus sequence, indicating that sequence bias is not the only underlying factor for the increased stability. Moreover, we could not detect any activity of the enzyme corresponding to the consensus sequence toward any of the substrates investigated. The lack of evolutionary context awareness of the consensus approach, which accounts for every position individually and thus might fail to incorporate coevolution of certain sites, could possibly interfere with enzymatic activity. Similar to the consensus approach, the maximum likelihood method used for the ancestral reconstruction has a preference to select the most frequent residue in one particular position, as those will be present in a higher degree over the course of evolution. As a result, there might be a bias to automatically select the most stabilizing residue for each position within a certain clade of the tree [39]. High protein stability has recently been connected to higher abundance of soluble protein in the cell [30]. This is in accordance with our findings, namely that the two oldest ancestral terpene cyclases are readily expressed in E. coli with up to four times higher protein yield compared to their modern counterpart (Fig. 4). This phenomenon has previously been reported for ancestral serum paraoxonase enzymes, which also displayed large increases in melting temperature [38]. Overall, these results show the potential of ASR for engineering proteins with increased stability, which may also facilitate purification and crystallization [40].

The promiscuity displayed by ancestral terpene cyclases is in line with other studies, which suggest that
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modern, specific enzymes originate from evolvable, more general, ancestors [20,22]. Interestingly, SvS-A3 is promiscuous toward FPP, even though all its descendants in the tree have been experimentally characterized in a previous study and are reported to not accept this substrate [18]. The observed drop in substrate promiscuity of SvS-A1, reflected in a higher relative substrate specificity ratio of GGPP over FPP (Table 2) compared to both SvS-A2 and SvS-A3, caused us to critically evaluate the method of ASR in this particular case. As can be seen from the phylogenetic tree in Fig. 2, only one sequence diverges from SvS-A1 before SvS-A2. When closely examining this sequence from Streptomyces aidingensis, we noted that, among all Streptomyces sequences in the subtree, it has the lowest sequence identity compared to the Streptomyces consensus (59% for S. aidingensis vs. 70–93% for all other modern sequences). Moreover, it is the only Streptomyces sequence included in the multiple sequence alignment in which the DDxx(x)D motif is not entirely conserved (DDACCG for S. aidingensis, characteristics of the NSE/DTE motif are present). Upon closer inspection, it was found that the S. aidingensis genome carries another sequence with lower sequence identity toward other members of the tree, but with a conserved DDxx(x)D motif. Thus, it is possible that the sequence included in the tree may be a paralog to the actual enzyme, which may reduce the bootstrap value for the node of SvS-A2. Therefore, we hypothesize that the overall topology of the clade descending from SvS-A2 is in fact robust, but the results we obtained from experiments with SvS-A1 may be less relevant than those of SvS-A2 and SvS-A3. Overall, bootstrap values in the phylogenetic tree are high – only three nodes have values below 70 – which implies a robust starting point for ancestral reconstruction (Fig. 2). We also found that the predicted probabilities of ancestral residues in our reconstruction (Fig. 3B) were comparable to those of other studies [37].

With respect to substrate promiscuity, we investigated the influence of a single residue in the – otherwise conserved – aspartate-rich DDxx(x)D motif. Position 89 (numbering of Svs from S. violens) is occupied by a histidine in the enzyme from S. violens and one additional sequence in the tree (S. ochraceisceroticus). Mutation of this position in ancestors to histidine did not abolish activity toward FPP and was further associated with small effects on relative substrate specificities (Table 2, approximately up to twofold change in relative $k_{cat}/K_M$ values). The most common residue in this position is alanine (48% or 14 out of 29 sequences), followed by valine (17%). Several other amino acids are tolerated, such as arginine (10%), glutamate (7%), leucine (7%), and asparagine (3%). Previous studies have shown that the aspartate-rich motif in class I terpene cyclases is highly important for establishing the cluster of divalent cations – in this case Mg$^{2+}$ – responsible for interaction with the diphosphate group of the substrate [41]. It has been shown that mutating amino acids within the DDxx(x)D motif affects product specificity of terpene cyclases [42,43]. Ohnuma et al. [44] performed random mutagenesis on an FPP synthase, an enzyme from the prenyltransferase family, from Bacillus steaothermophilus. Screening of different variants yielded several mutants that were able to synthesize GGPP in addition to FPP – in contrast to the native enzyme. They identified one key mutation in close proximity to the DDxx(x)D motif, which is also conserved in that enzyme class and fulfills an analogous function, namely coordination of the diphosphate moiety via metal ion interaction. Since replacement of a tyrosine by a histidine in this position allowed for GGPP synthesis, Ohnuma et al. hypothesized that the tyrosine side chain may be blocking further elongation of FPP. Therefore, it is possible that the small changes observed in substrate promiscuity through a single point mutation in the aspartate-rich motif of ancestral diterpene cyclases are caused by steric effects. However, as mutation of the respective ancestral residues in this position to histidine did not completely abolish acceptance of the substrate FPP, the small effects on activity and specificity observed herein highlight the importance of the ancestral background for substrate promiscuity.

In summary, we have successfully implemented ASR on an emerging and relatively uncharacterized family of biosynthetic enzymes. To the best of our knowledge, ASR has previously not been performed on diterpene cyclases. It can be concluded that ASR can yield enzyme scaffolds that display significantly enhanced protein stability; on par with the 12 °C increase in thermostability of a sesquiterpene cyclase obtained through directed evolution [45]. Especially this feature of ancestral enzymes can be attractive for various reasons, such as increased yield of soluble protein or facilitated purification [40]. We observed enhanced substrate promiscuity of ancestors and detailed kinetic analyses revealed an increased $k_{cat}/K_M$ for the ancestor with highest thermostability, as well as a loss of substrate saturation for the modern enzyme. We believe that our findings underline the potential of ASR as an approach to exploring sequence space of enzymes within secondary metabolism. Finally, by exploring both ASR and the consensus approach, we address the previously identified lack...
of comparison between the two methods [46], which further corroborates the potential of ASR in enzyme engineering.

Experimental procedures

Ancestral sequence reconstruction

A protein BLAST search was performed via NCBI in the nonredundant database using the sequence of SvS from *S. violens* [18] as a query (GenBank accession no. WP_051831532.1). The 50 closest homologues were selected, and duplicates and mutant proteins were removed, before aligning the sequences in *MEGA7* [24] using the MUSCLE algorithm [27]. Based on the multiple sequence alignment, a number of sequences were removed to reduce possible gaps in ancestral sequences, resulting in a selection of 27 homologues. Two homologous sequences from *Pseudomonas* species were included as outgroup, bringing the total number of sequences to 29. A bootstrapped phylogenetic tree (250 replicates) was constructed in *MEGA7* [25] using maximum likelihood statistics and the LG+F model [47], including a gamma distribution for rate variation across sites (number of discrete gamma categories: 5). The resulting tree was rooted using the *Pseudomonas* outgroup as a reference (Fig. 2). Three ancestral nodes were identified between the species in the tree. The most likely sequences were inferred for these three nodes using Maximum Likelihood statistics in *MEGA7*. The three ancestral sequences were aligned to the query sequence (Fig. S1) and three gap positions were identified which were filled with the residue from *S. violens* in the corresponding position (A68, L359, and G360 in all ancestral sequences, and G346 in SvS-A1). In addition to the original reconstructed ancestral sequences, three mutated sequences were designed. In these sequences all residues in the DDxx(x)D motif were kept conserved with respect to SvS from these sequences all residues in the DDxx(x)D motif were.

Site-directed mutagenesis

To substitute the histidine in position 89 with the respective ancestral residues in order to obtain the originally predicted ancestral sequences, site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara CA, USA), according to the manufacturers protocol. The following primers were designed and ordered from Integrated DNA Technologies (Coralville, IA, USA): 5'-GTT TAT TGG GTT TTT GCA TTT GAT GAC CGT TGT GAT GCC CGT TGT GAT AAT AAT GG-3' (SvS-A1/A2 forward), 5'-GTT GCT CAG CGG ACC ATT ATC ACA ACG GGC ATC ATC AAA TGC-3' (SvS-A1/A2 reverse), 5'-GTC GTG TTA TTA TTC GGT GGG TGT TGC ATT GTA AAA CGG TTG TGA TAA ACG ACG AAA GTG-3' (SvS-A3 forward), and 5'-CGG GTG TTA CTA AAC GGA CCA TTA TCA CAA CGG TTA TCA TCA AAT GC-3' (SvS-A3 reverse).

Protein expression and purification

*Escherichia coli* BL21 (DE3) cells were transformed with pET22b(+) vectors containing sequences of SvS from *S. violens*, SvS-A1/2/3 (and mutants), and SvS consensus. A single colony from each construct was inoculated in 25 mL of 2YT medium (16 g L\(^{-1}\) tryptone, 10 g L\(^{-1}\) yeast extract, 5 g L\(^{-1}\) NaCl) containing 100 μg mL\(^{-1}\) of ampicillin and incubated at 180 rpm in 37 °C over night. The overnight cultures were diluted to an OD\(_{600}\) of 0.07 in 600 mL of 2YT medium in 2-L flasks containing 100 μg mL\(^{-1}\) of ampicillin, and were incubated at 160 rpm in 37 °C. The cultures were induced with 0.4 mm of isopropyl β-D-thiogalactopyranoside (IPTG) at OD\(_{600}\) ≈ 0.5 and the expression was performed for 22 h at 160 rpm in 18 °C. Cells were harvested by centrifugation for 15 min (4 °C, 2264 g). The supernatant was discarded. For the characterization of activity toward GPP, FPP, and GGPP, as well as relative specificity, affinity purification was performed as follows: cell pellets were resuspended to 0.2 g mL\(^{-1}\) in binding buffer (20 mM Na\(_2\)HPO\(_4\)/NaH\(_2\)PO\(_4\), 500 mM NaCl, 20 mM imidazole, 1 mM MgCl\(_2\), pH 7.4). Cells were lysed by ultrasonication on ice for 6 × 50 s (1 s on, 1 s off, amplitude 50%), and the soluble fractions were obtained after centrifugation for 30 min (4 °C, 14 610 g). Protein purification was performed by incubation of the soluble enzyme fraction with 2 mL of Ni\(^{2+}\)-NTA beads (Qiagen, Hilden, Germany) through end-over-end mixing at 4 °C, and subsequent affinity chromatography using 5-mL polypropylene gravity columns (Qiagen). The columns were washed with 10 column volumes (CV) binding buffer and protein elution was performed in 10 CV using elution buffer (20 mM Na\(_2\)HPO\(_4\)/NaH\(_2\)PO\(_4\), 500 mM NaCl, 500 mM imidazole, 1 mM MgCl\(_2\), pH 7.4). Elution fractions with enzyme were concentrated by centrifugation for 15 min.
(4 °C, 2264 g) in Amicon Ultra centrifugal filter units (MWCO 10 kDa; Sigma-Aldrich, St. Louis, MO, USA), and re-suspended in storage buffer (20 mM Na2HPO4/NaH2PO4, 1 mM MgCl2, pH 7.4). Due to low purity and yield of modern SvS from the affinity purification method, anion exchange was used for purification for kinetics assays as well as for all characterization of SvS consensus. Cell pellets (kinetics assays as well as for all characterization of SvS consensus. Cell pellets (~3.5 g) were re-suspended in 30 ml of buffer A (20 mM Tris-HCl, pH 8.5) and lysed by ultrasonication on ice for 2 °C 30 min (4 °C, 100%). The mixtures were centrifuged at 30 215 g, 1 °C, for 30 min (4 °C, 48 298 g). The supernatant (soluble fraction) was filtered in a Sartobran 300 0.45 + 0.2 μm (Sartorius AG, Göttingen, Germany) before being loaded onto a 10-ml Hi-Trap QHP column (GE Healthcare, Chicago, IL, USA) using an ÄKTA Explorer (GE Healthcare). Protein was eluted with buffer B (20 mM Tris-HCl, 500 mM NaCl, pH 8.5), after which buffer was changed to storage buffer. All enzyme concentrations were determined by Bradford assays, and a western blot was performed to confirm presence of His-tagged proteins of the correct size. In case of SvS consensus, its correct elution fraction was confirmed by MALDI-TOF (MALDI TOF/TOF 5800 series; Sciex AB, Framingham, MA, USA).

**Yield of soluble protein**

In order to compare the yield of soluble protein of SvS from *S. violens* and the ancestral enzymes, cells were grown in 20 mL of 2YT medium in 100-mL shake flasks (100 μg·mL−1 ampicillin) at 37 °C, 180 rpm. Cells were induced with 0.4 mM IPTG at OD600 = 0.5, and expression was performed in 18 °C at 160 rpm for 22 h. Cells were harvested by centrifugation for 15 min (4 °C, 2264 g). Pellets were re-suspended in 1 mL binding buffer and lysed by sonication (3 × 50 s, 1 s on 1 s off, 30% amplitude) on ice. The lysed mixtures were centrifuged for 30 min (4 °C, 20 215 g). The supernatant was mixed with 250 μL of NIT-A beads through 3 s of vortexing and left for end-over-end shaking on ice for 1 h. Mixtures were centrifuged for 2 min (4 °C, 1947 g) and the supernatant was removed. Beads were washed with 700 μL of binding buffer (2 × 3 s of vortexing followed by centrifugation for 2 min at 4 °C, 1947 g), and the protein was eluted by addition of 1000 μL of elution buffer (three times). The elution fractions were pooled, the protein concentration was determined by Bradford assay using a BSA standard for reference, and the pools were analyzed on SDS. For each pool, 15 μL of protein was loaded, corresponding to 10–15 μg of protein. Purity was estimated using automatic lane detection in image processing software from Bio-Rad (Hercules, CA, USA) and the amount of soluble protein in milligram per liter of culture was then calculated using the respective purity and protein concentration (Fig. 4B).

**Relative specificity toward GGPP/FPP**

Reactions to determine relative substrate specificity were carried out in a total reaction volume of 200 μL storage buffer, with a final enzyme concentration of 10 μM and a constant total substrate concentration of 150 μM. Substrates were purchased from Sigma-Aldrich at the highest possible purity. Three different ratios of geranylgeranyl diphosphate (GGPP) to FPP were tested: 1 : 1, 1 : 4, and 1 : 8. Reactions were left for 3 h shaking in a Thermomixer (Eppendorf, Hamburg, Germany) at 1200 rpm in 30 °C, after which products were extracted with 2 × 300 μL of hexane spiked with 20 μM of decane as an internal standard. The solvent phase was analyzed by GC/FID on a GCMS-QP2010 Ultra (Shimadzu Corporation, Kyoto, Japan) equipped with a Rxi-5ms capillary column (30 m long, 0.25 μm thick, 0.25 mm inner diameter; Restek Corporation, Bellefonte, PA, USA). GC/FID was used with the following temperature program: the oven temperature was set at 50 °C, then raised to 250 °C at a speed of 5 °C·min−1, and finally increased to 350 °C with a rate of 15 °C·min−1. Relative specificity toward GGPP over FPP was determined by using the following equation:

\[
\frac{V_{GGPP}}{V_{FPP}} = \frac{[GGPP]}{[FPP]} 
\]

Here, \(V\) refers to the initial reaction rate for respective substrate.

**Kinetics and thermodynamics**

Kinetics assays for SvS from *S. violens* and SvS-A2 were carried out in reactions with a total volume of 200 μL in 2-mL Eppendorf tubes, incubated in thermomixers (Eppendorf), while shaking at 1200 rpm for 30, 60, and 90 min. Reactions contained 185 μL of reaction buffer (50 mM Tris-HCl, 1 mM MgCl2, pH 7.4) and 4 μL of 25 μM enzyme stock solution in storage buffer (which yielded a final enzyme concentration 500 nM). GGPP (≥ 95 % in 7 : 3 MeOH/NH4OH; Sigma–Aldrich) concentrations were varied between 5 and 120 μM. The methanol content was held constant by at 3.9% (V/V) in all reactions by addition of methanol mixed with storage buffer in a 7 : 3 ratio. The reactions were started by addition of enzyme to preheated tubes at the desired temperature (as verified by an external thermometer). Reactions were quenched by addition of 2 × 300 μL hexane spiked with 10 μM decane as an internal standard for product extraction. The solvent phase was analyzed by GC/FID. For thermodynamic analyses, kinetics assays were performed at different temperatures.

**Thermostability measurements**

Initial thermostability measurements were performed on a Prometheus NT.48 nanoDSF instrument (NanoTemper
technologies, münchen, germany). Protein unfolding was monitored by following the ratio of intrinsic protein fluorescence at 350 to 330 nm over time, increasing the temperature from 20 °C to 80 °C with 1 degree per minute. Additional dsf measurements were performed on a CFX96 Real-Time Thermal Cycler (Bio-Rad). Protein unfolding was monitored by measuring fluorescence of SYPRO Orange stain (Sigma-Aldrich) that was added to the protein prior to heating. The temperature was increased with 1 degree per minute from 20 °C to 90 °C.

Product analysis on GC/MS
All qualitative MS analyses were performed on a GCMS-QP2010 Ultra (Shimadzu) equipped with a Rxi-5 ms capillary column (30 m long, 0.25 μm thick, 0.25 mm inner diameter; Agilent Technologies). The following temperature program was used: the oven temperature was set at 50 °C, then raised to 250 °C at a speed of 8 °C/min⁻¹, and finally increased to 330 °C with a rate of 15 °C/min⁻¹. The injection volume was 3 μL and the split ratio was 3. Helium was used as a carrier gas. All chemicals, solvents and reference compounds were purchased at the highest possible purity from Sigma-Aldrich.

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Conflict of interest
Erik Nordling is employed by Swedish Orphan Biovitrum AB. Natalie Hendrikse is funded by the Swedish Foundation for Strategic Research (SSF) and jointly employed by the KTH Royal Institute of Technology and Swedish Orphan Biovitrum AB. The authors declare no conflict of interest.

Author contributions
POS designed most of the study and supervised the work. EN designed part of the study and supervised the work. GC performed part of the experiments. NHS designed part of the study, performed most of the experiments and wrote the major part of the manuscript.

References
1 Bornscheuer UT, Huisman GW, Kazlauskas RJ, Lutz S, Moore JC & Robins K (2012) Engineering the third wave of biocatalysis. Nature 485, 185–194.
2 Reetz MT (2013) Biocatalysis in organic chemistry and biotechnology: past, present, and future. J Am Chem Soc 135, 12480–12496.
3 Nestl BM, Hammer SC, Nebel BA & Hauer B (2014) New generation of biocatalysts for organic synthesis. Angew Chem Int Ed Engl 53, 3070–3095.
4 Arnold FH (2017) Directed evolution: bringing new chemistry to life. Angew Chem Int Ed Engl 56, 2–8.
5 Zhu Y, Romain C & Williams CK (2016) Sustainable polymers from renewable resources. Nature 540, 354–362.
6 Oldfield E & Lin FY (2012) Terpene biosynthesis: modularity rules. Angew Chem Int Ed Engl 51, 1124–1137.
7 Bohlmann J & Keeling CI (2008) Terpenoid biomaterials. Plant J 54, 656–669.
8 Andersen-Ranberg J, Kongstad KT, Nielsen MT, Jensen NB, Pateraki I, Bach SS, Hamberger B, Zerbe P, Staerk D, Bohlmann J et al. (2016) Expanding the landscape of diterpene structural diversity through stereochemically controlled combinatorial biosynthesis. Angew Chem Int Ed Engl 55, 2142–2146.
9 Merkl R & Sterner R (2016) Ancestral protein reconstruction: techniques and applications. Biol Chem 397, 1–21.
10 Gumulya Y & Gillam EMJ (2017) Exploring the past and the future of protein evolution with ancestral sequence reconstruction: the ‘retro’ approach to protein engineering. Biochem J 474, 1–19.
11 Thornton JW (2004) Resurrecting ancient genes: experimental analysis of extinct molecules. Nat Rev Genet 5, 366–375.
12 Rabe P, Rinkel J, Nubbemeyer B, Köllner TG, Chen F & Dickschat JS (2016) Terpene cyclases from social amoebae. Angew Chem Int Ed Engl 55, 15420–15423.
13 Christianson DW (2006) Structural biology and chemistry of the terpenoid cyclases. Chem Rev 106, 3412–3442.
14 Sato T, Hoshino H, Yoshida S, Nakajima M & Hoshino T (2011) Biofunctional triterpene/sesquiterpene cyclase: tetraprenyl-β-curcumene cyclase
is also squalene cyclase in Bacillus megaterium. J Am Chem Soc 133, 17540–17543.
15 Eriksson A, Kürten C & Syrén PO (2017) Protonation-initiated cyclization by a class II terpene cyclase assisted by tunneling. ChemBioChem 18, 2301–2305.
16 Chen M, Harris GG, Pemberton TA & Christianson DW (2016) Multi-domain terpenoid cyclase architecture and prospects for proximity in bifunctional catalysis. Curr Opin Struct Biol 41, 27–37.
17 Christianson DW (2017) Structural and chemical biology of terpenoid cyclases. Chem Rev 117, 11570–11648.
18 Rabe P, Rinkel J, Dolja E, Schmitz T, Nubbemeyer B, Luu TH & Dickschat JS (2017) Mechanistic investigations of two bacterial diterpene cyclases: spiroviolene synthase and tsukubadiene synthase. Angew Chem Int Ed Engl 56, 2776–2779.
19 Yang Y-L, Zhang S, Ma K, Yuxing X, Tao Q, Renata H, Wang ZJ & Arnold FH (2015) Expanding activity and stability of ancestral diterpene cyclases in bacteria and fungi. Angew Chem Int Ed Engl 56, 4749–4752.
20 Devamani T, Rauwerdink AM, Lunzer M, Jones BJ, Mooney JL, Tan MAO, Zhang ZJ, Xu JH, Dean AM & Kazaurskas RJ (2016) Catalytic promiscuity of a new family of diterpene cyclases in bacteria and fungi. Angew Chem Int Ed Engl 56, 4794–4797.
21 Risso VA, Gavira JA, Mejia-Carmona DA, Gaucher EA & Sanchez-Ruiz JM (2013) Hyperstability and substrate promiscuity in laboratory resurrections of precambrian β-lactamases. J Am Chem Soc 135, 2899–2902.
22 Baier F, Copp JN & Tokuriki N (2016) Evolution of enzyme superfamilies: comprehensive exploration of sequence-function relationships. Biochemistry 55, 6375–6388.
23 Renata H, Wang ZJ & Arnold FH (2015) Expanding the enzyme universe: accessing non-natural reactions by mechanism-guided directed evolution. Angew Chem Int Ed Engl 54, 3351–3367.
24 Kumar S, Stecher G & Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 33, 1870–1874.
25 Hall BG (2013) Building phylogenetic trees from molecular data with MEGA. Mol Biol Evol 30, 1229–1235.
26 Marin J, Battistuzzi FU, Brown AC & Hedges SB (2016) The timetree of prokaryotes: new insights into their evolution and speciation. Mol Biol Evol 34, 437–446.
27 Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32, 1792–1797.
28 Okonechnikov K, Golosova O, Fursov M, Varlamov A, Vaskin Y, Efremov I, German Grehov OG, Kandrov D, Rasputin K, Syabro M et al. (2012) Unipro UGENE; a unified bioinformatics toolkit. Bioinformatics 28, 1166–1167.
29 Baer P, Rabe P, Fischer K, Citron CA, Klapschinski TA, Groll M & Dickschat JS (2014) Induced-fit mechanism in class I terpene cyclases. Angew Chem Int Ed Engl 53, 7652–7656.
30 Leuenberger P, Ganscha S, Kahraman A, Cappelletti V, Boersema PJ, von Mering C, Claassen M & Picotti P (2017) Cell-wide analysis of protein thermal unfolding reveals determinants of thermostability. Science 355, 794–805.
31 Baer P, Rabe P, Citron CA, De Oliveira Mann CC, Kaufmann N, Groll M & Dickschat JS (2014) Hedycaryol synthase in complex with nerolidol reveals terpene cyclase mechanism. ChemBioChem 15, 213–216.
32 Cane DE, Chiu HT, Liang PH & Anderson KS (1997) Pre-steady-state kinetic analysis of the trichodiene synthase reaction pathway. Biochemistry 36, 8332–8339.
33 Mathis JR, Back K, Starks C, Noel J, Poulter CD & Chappell J (1997) Pre-steady-state study of recombinant sesquiterpene cyclases. Biochemistry 36, 8340–8348.
34 Bar-Even A, Noor E, Savir Y, Liebermeister W, Davidi D, Tawfik DS & Milo R (2011) The moderately efficient enzyme: evolutionary and physicochemical trends shaping enzyme parameters. Biochemistry 50, 4402–4410.
35 Eyring H & Stearn AE (1939) The application of the theory of absolute reaction rates to proteins. Chem Rev 24, 253–270.
36 Bloom JD, Labthavikul ST, Otey CR & Arnold FH (2006) Protein stability promotes evolvability. Proc Natl Acad Sci U S A 103, 5869–5874.
37 Nguyen V, Wilson C, Hoemberger M, Stillar JB, Agafonov RV, Kutter S, English J, Theobald DL & Kern D (2017) Evolutionary drivers of thermoadaptation in enzyme catalysis. Science 355, 289–294.
38 Trudeau DL, Kaltenbach M & Tawfik DS (2016) On the potential origins of the high stability of reconstructed ancestral proteins. Mol Biol Evol 33, 2633–2641.
39 Williams PD, Pollock DD, Blackburne BP & Goldstein RA (2006) Assessing the accuracy of ancestral protein reconstruction methods. PLoS Comput Biol 2, 0598–0605.
40 Romero-Romero ML, Risso VA, Martinez-Rodriguez S, Ibarra-Molero B & Sanchez-Ruiz JM (2016) Engineering ancestral protein hyperstability. Biochem J 473, 3611–3620.
41 Rinkel J, Lauterbach L & Dickschat JS (2017) Spata-13,17-diene synthase – an enzyme with sesqui-, di-, and sesiterpene synthase activity from Streptomyces xinghaiensis. Angew Chem Int Ed Engl 56, 16385–16389.
42 Cane DE & Xue Q (1996) Trichodiene synthase – enzymatic formation of multiple sesquiterpenes by alteration of the cyclase active site. *J Am Chem Soc* **118**, 1563–1564.
43 Cane DE, Xue Q, Van Epp JE & Tsantrizos YS (1996) Enzymatic formation of isochamigrene, a novel sesquiterpene, by alteration of the aspartate-rich region of trichodiene synthase. *J Am Chem Soc* **118**, 8499–8500.
44 Ohnuma SI, Nakazawa T, Hemmi H, Hallberg AM, Koyama T, Ogura K & Nishino T (1996) Conversion from farnesyl diphosphate synthase to geranylgeranyl diphosphate synthase by random chemical mutagenesis. *J Biol Chem* **271**, 10087–10095.
45 Lauchli R, Rabe KS, Kalbarczyk KZ, Tata A, Heel T, Kitto RZ & Arnold FH (2013) High-throughput screening for terpene-synthase-cyclization activity and directed evolution of a terpene synthase. *Angew Chem Int Ed Engl* **52**, 5571–5574.
46 Risso VA & Sanchez-Ruiz JM (2017) Resurrected ancestral proteins as scaffolds for protein engineering. In *Directed Enzyme Evolution: Advances and Applications* (Alcalde M, ed.), 1st edn, pp. 229–255. Springer International Publishing AG, Basel.
47 Le SQ & Gascuel O (2008) An improved general amino acid replacement matrix. *Mol Biol Evol* **25**, 1307–1320.

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Sequences of the investigated enzymes.

**Fig. S2.** Gas chromatogram showing product peaks from reactions with GGPP.

**Fig. S3.** MS analysis of main product from reactions with GGPP.

**Fig. S4.** MS analysis of other products from reactions with GGPP.

**Fig. S5.** Gas chromatogram showing product peaks from reactions with FPP.

**Fig. S6.** MS analysis of main product from reactions with FPP and reference compounds suggests that a cyclic product was formed by ancestors.