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A Pair of Residues That Interactively Affect Diterpene Synthase Product Outcome

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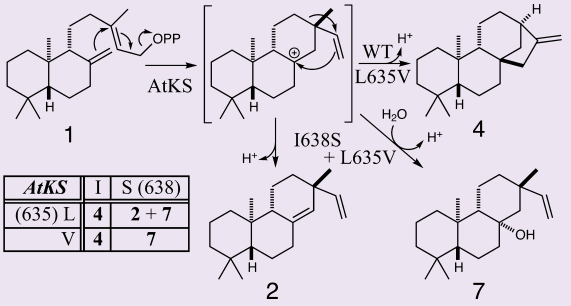
Supporting Information

ABSTRACT: The labdane-related diterpenoids (LRDs) are an important superfamily of natural products whose structural diversity critically depends on the hydrocarbon skeletal structures generated, in large part, by class I diterpene synthases. In the plant kingdom, where the LRDs are predominantly found, the relevant class I diterpene synthases are clearly derived from the *ent*-kaurene synthase (KS) required in all land plants for phytohormone biosynthesis and, hence, are often termed KS-like (KSL). Previous work, initiated by the distinct function of two alleles of a KSL from rice, OsKSL5, identified a single residue switch with a profound effect on not only OsKSL5 product outcome but also that of land plant KSs more broadly, specifically, replacement of a key isoleucine with threonine, which interrupts formation of the tetracyclic *ent*-isokaurene at the tricyclic stage, leading to production of *ent*-pimaradiene instead. Here, further studies of these alleles led to discovery of another, nearby residue that tunes product outcome. Substitution for this newly identified residue is additionally shown to exert an epistatic effect in KSs, altering product distribution only if combined with replacement of the key isoleucine. On the other hand, this pair of residues was found to exert additive effects on the product outcome mediated by distantly related KSLs from the eudicot castor bean. Accordingly, it was possible to use a rational combination of substitutions for this pair of residues to engineer significantly increased (dominant) selectivity for novel 8α-hydroxy-*ent*-pimar-15-ene product outcome in the KS from the dicot *Arabidopsis thaliana*, demonstrating the utility of these results.



AtKS	I	S (638)
(635) L	4	2 + 7
V	4	7

The labdane-related diterpenoids (LRDs) form a large superfamily of natural products, with ∼7000 known compounds, which are characterized by a decalin core structure.[1] While this bicyclic core is generated from the acyclic general diterpenoid precursor (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP) by class II diterpene cyclases, substantial additional structural diversity is generated by the subsequently acting class I diterpene synthases. In the plant kingdom, where the LRDs are predominantly found, these enzymes seem to have most directly evolved *via* gene duplication and neo-functionalization of the *ent*-kaurene synthases (KSs) required in all vascular plants for gibberellin hormone biosynthesis.[2] Accordingly, these enzymes are often termed KS-like (KSL) and form a distinct subfamily within the plant terpene synthase family.[3]

Like class I terpene synthases more generally, the KS(L)s carry out catalysis in a highly conserved α-helical bundle domain that contains two signature motifs. These DDxxD and NDxx(S/T)xxxE sequences are involved in ligation of a trinuclear Mg^{2+} cluster to promote substrate binding and the subsequent initiating ionization of the allylic diphosphate ester bond.[4,5] This is typically followed by formation of a series of carbocation intermediates, leading to the creation of distinct diterpene backbones, largely guided by the initial configuration of the substrate imposed by the steric confines of the active site.[6] Moreover, terpene synthases additionally seem to utilize electrostatic interactions to further guide these carbocation cascades, including not only the pyrophosphate anion coproduct but also electron-rich amino acid side-chains.[7] Finally, the reaction is terminated by deprotonation, most often using the pyrophosphate anion coproduct,[8] which generally yields an olefin, although prior addition of water can occur, yielding a hydroxylated product. Notably, such hydroxylation also is a key step in terpenoid biosynthesis, as this increases both solubility and the ability to specifically bind molecular targets, which is required for biological activity.[2,9]

Particularly relevant here are various single residue "switches" between aliphatic and hydroxylated side-chains at a key position, which profoundly affects product outcome in KS(L)s. This "switch" position was initially identified by discovery of two functionally distinct alleles of the rice (*Oryza sativa*) OsKSL5, which react with *ent*-copalyl diphosphate (**1**, *ent*-CPP), with that from subspecies *japonica* (OsKSL5j) producing *ent*-pimara-8(14),15-diene (**2**) and that from subspecies *indica* (OsKSL5i) producing *ent*-(iso)kaur-15-ene (**3**) instead.[10,11] Subsequently, of the three differences in the active site between these two orthologs, it was found that mutation of an isoleucine in **3** producing OsKSL5i to the threonine occupying that position in **2** producing OsKSL5j was sufficient to completely

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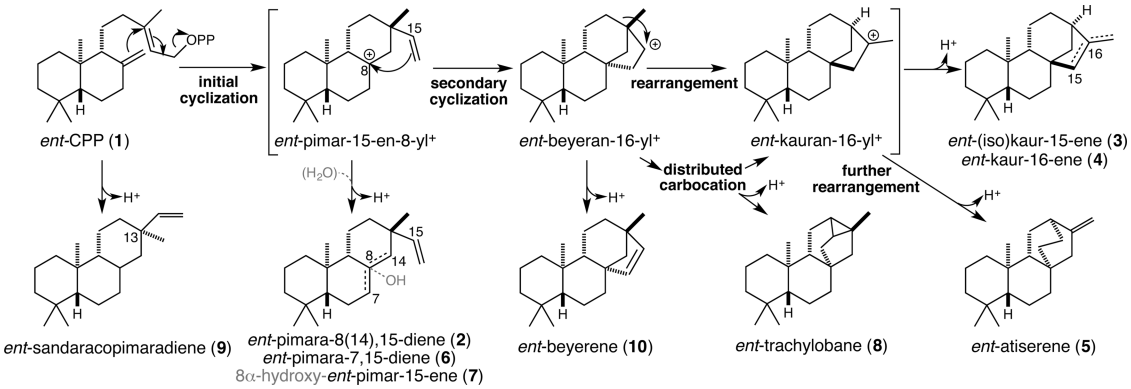
Scheme 1. Cyclization Mechanism for KS(L)s Investigated Here[a]



[a]All KSs produce **4**. OsKSL5j produces **5**, while OsKSL5i produces **2**. RcKSL2 produces largely **8**. RcKSL3 produces **10**. RcKSL4 produces **9**. Initial cyclization of *ent*-CPP (**1**) leads to the *ent*-pimar-15-en-8-yl$^+$, as C13 epimers, which may be followed by either direct deprotonation, addition of water, or secondary cyclization, most often followed by further rearrangement, prior to terminating deprotonation. Compound numbering corresponds to that defined in the text.

switch product outcome, with the resulting OsKSL5i:I664T mutant yielding only **2**. Furthermore, this Ile was noted as being conserved across all the known plant KSs, and substitution of Thr was found to similarly alter product outcome with the KS from not only rice (OsKS) but also the eudicot *Arabidopsis thaliana* (AtKS), leading to predominant production of **2** rather than *ent*-kaur-16-ene (**4**).[12] Later work demonstrated similar effects with both the KS from a gymnosperm[13] as well as those from earlier diverging bryophytes.[14] This effect on product outcome represents abbreviation of the sequential bicyclization and rearrangement required for formation of the tetracyclic *ent*-kaurane backbone found in **3** and **4** after initial cyclization to a tricyclic *ent*-pimar-15-en-8-yl$^+$ intermediate, which is then directly deprotonated at carbon-14 to yield **2** (Scheme 1). Notably, the importance of Thr at this position to enable pimaradiene production was demonstrated in another rice KSL, OsKSL4, as substitution of the Thr at this position by Ile led to predominant production of a tetracycle resulting from further cyclization and rearrangement of the initially formed *syn*-pimar-15-8-yl$^+$ intermediate that is otherwise directly deprotonated by the wild-type enzyme.[15] However, most KSLs do not contain such a hydroxyl containing residue at this position, demonstrating that alternative mechanisms exist for altering product outcome. In addition, even in the original work with OsKSL5,[12] it was noted that substitution of Ile for the Thr found at this position in OsKSL5j did not lead to complete specificity for production of **3**, as the resulting OsKSL5j:T664I also produces small amounts of **4**, along with more substantial amounts of *ent*-atiserene (**5**), resulting from alternative rearrangement of the *ent*-beyeran-12-yl$^+$ intermediate formed by the second cyclization step (Scheme 1).

Here is reported further investigation of OsKSL5 that led to identification of a secondary residue switch that further tunes product outcome. Notably, this residue also is conserved in plant KSs, where it seems to exert an epistatic effect, only altering product outcome in the context of changes to the originally identified key Ile. Interestingly, the residues at both positions were found to exert additive effects on product outcome in distantly related eudicot KSLs. Building on the realization that these can act in an additive fashion, rational engineering of AtKS was carried out to enable predominant production of a novel hydroxylated diterpene.

RESULTS AND DISCUSSION

Further Investigation of OsKSL5. It was previously found that of the three differences in active site residues between the orthologous OsKSL5j and OsKSL5i, switching the Ile at position 664 in OsKSL5i to the Thr found in OsKSL5j was sufficient to "short circuit" the production of **3**, as OsKSL5i:I664T specifically produces **2** instead. However, the reciprocal residue switch led to production of a mixture of tetracycles **3**–**5** (*i.e.*, by OsKSL5j:T664I), rather than the almost exclusive production of **3** observed with OsKSL5i.[12] Hypothesizing that changes to the other active site residues that differ between these two orthologs might be sufficient to increase catalytic specificity for the production of **3**, the corresponding double residue switch constructs, OsKSL5j:T664I + L661V or V718I, were made. The product outcome of these two double mutants was investigated by incorporation into a previously described modular metabolic engineering system—*i.e.*, these were expressed in *E. coli* also engineered to produce their substrate **1**.[16] Relative to the original OsKSL5j:T664I single residue switch mutant, while the addition of V718I did not alter product outcome, the addition of L661V led to much more specific production of **3** (Figure 1a). Indeed, the product outcome mediated by this OsKSL5j:L661V/T664I double residue switch mutant is essentially identical to that mediated by OsKSL5i. Moreover, the reciprocal single residue switch, OsKSL5i:V661L, led to reduced catalytic specificity and production of a mixture of **3**–**5**, closely resembling that mediated by the OsKSL5j:T664I mutant, although it does not affect the specific production of **2** by the originally reported OsKSL5i:I664T residue switch (Figure 1b). Thus, these two residues together seem to fully account for the specific production of **2** versus **3** catalyzed by these two functionally distinct alleles of OsKSL5.

However, as previously reported,[12] switching this residue alone did not affect the production of **2** by OsKSL5j, nor did the reciprocal double residue switch further affect product outcome (*i.e.*, both OsKSL5j:L661V and OsKSL5i:V661L/I664T still specifically produce **2**). Hence, the presence of the hydroxyl containing Thr at the original/primary position (i.e., residue 664) masks the effect of the newly identified/secondary position (i.e., residue 661) in OsKSL5, and this pair of residues then seems to exhibit an epistatic interaction (Figure 1c). In particular, the presence of leucine instead of valine at the

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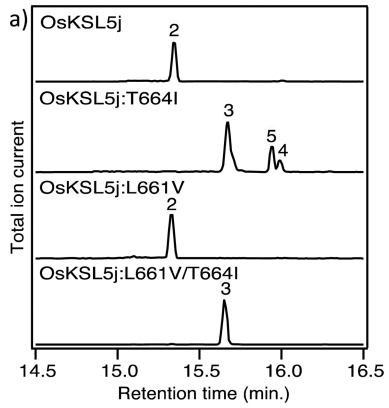
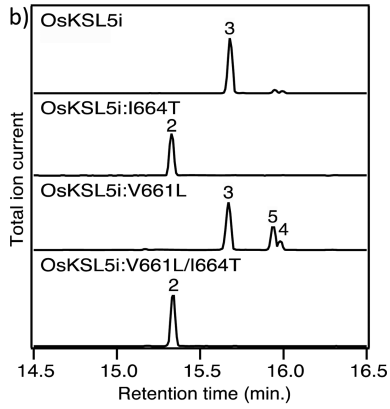
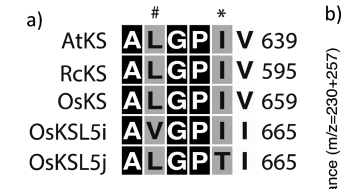


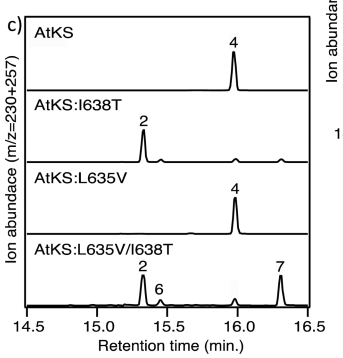


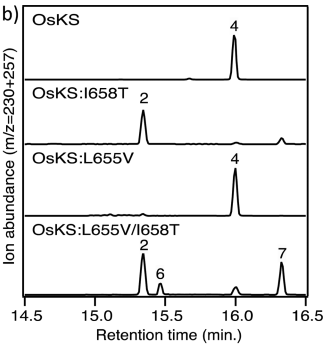
c)

OsKSL5	(664) I	T
(661) L	**3** – **5**	**2**
V	**3**	**2**

Figure 1. Epistatic effect of the original/primary and newly identified/secondary pair of residues on OsKSL5 product outcome. (a) Chromatograms from GC–MS analysis of wild-type OsKSL5j and the indicated mutants. (b) Chromatograms from GC–MS analysis of wild-type OsKSL5i and the indicated mutants. (c) Punnett square representation of the epistatic effect of the primary (664) and secondary (661) residues on product outcome in OsKSL5. Numbers correspond to the chemical structures defined in the text. Enzymatic products were identified by comparison of both retention time and mass spectra to authentic standards (Figure S1).







d)

KSs	I	T (prim.)
(sec.) L	**4**	**2**
V	**4**	**2**, **6**, **7**

Figure 2. Epistatic effect of the original/primary and newly identified/secondary pair of residues on KS product outcome. (a) Partial sequence alignment of representative KSs and KSLs with an asterisk (*) above the primary (original) position and a pound sign (#) above the secondary (new) position. Residues are numbered as in the full-length proteins from the NCBI database accession numbers given in the Materials section. (b) Chromatograms from GC–MS analysis of wild-type OsKS and the indicated mutants. (c) Chromatograms from GC–MS analysis of wild-type AtKS and the indicated mutants. (d) Punnett square representation of the epistatic effect of the primary (prim.) and secondary (sec.) residues on product outcome in KSs. Numbers correspond to the chemical structures defined in the text. Enzymatic products were identified by comparison of both retention time and mass spectra to authentic standards (Figure S1).

secondary position (661) decreases the specific production of **3** in the presence of Ile at the primary position, although it does not affect progression of the reaction through the second cyclization step (*i.e.*, all the products are tetracycles; see Scheme 1). On the other hand, even in the closely related (89% amino acid sequence identity) **3** producing OsKSL6, substitution of Leu for the Val found at this secondary position only slightly decreases catalytic fidelity. The resulting OsKSL6:V661L mutant still predominantly produces **3**, with only slight increases in the relative amounts of **4** and **5** (Figure S2), leaving in question the broader impact of this secondary position on product outcome.

Investigation of the Secondary Position in KSs. Notably, the newly identified/secondary position seems to be completely conserved as a Leu in land plant KSs (Figure 2a), which are specific for the production of **4**. This contrasts to the results reported above with OsKSL5 and -6, where Leu instead of Val at this position negatively impacts catalytic specificity. Substitution of Val for this Leu has only a minor effect on product outcome in either the rice OsKS or AtKS—*i.e.*, both OsKS:L655 V and AtKS:L635V still produce almost entirely **4** (Figure 2b,c). Nevertheless, when combined with substitution of Thr for Ile at the primary position, such substitution more dramatically impacts catalytic fidelity, leading to less specific production of **2**, with small increases in the relative amounts of **4**, as well as the double-bond isomer of **2**, *ent*-pimara-7,15-diene (**6**), and a more significant increase in the amount of 8α-hydroxy-*ent*-pimar-15-ene (**7**; resulting from addition of water prior to terminating deprotonation). Thus, the effect of Leu versus Val at the secondary position is largely masked in these two KSs, with substantial changes in product outcome only evident upon substitution for the Ile at the primary position. Accordingly, the residues at these two positions in the KSs seem to exhibit an essentially epistatic relationship as well, albeit with opposite effect as observed with OsKSL5 (c.f., Figures 1c and 2d).

Application in Distant Eudicot KSLs. While monocots seem to have particularly expanded upon LRD biosynthesis, with multiple KSLs present in these species,[2] there are a number of eudicots that similarly contain small families of KSLs. This includes castor bean (*Ricinus communis*), which contains three KSLs only distantly related to those found in monocots and that further exhibit an intriguing range of catalytic functionality.[17] In particular, all three RcKSLs react with **1**. However, RcKSL2 produces a mixture of pentacyclic *ent*-trachylobane (**8**) with smaller amounts of tetracyclic **4**, while RcKSL3 produces essentially only the tricyclic *ent*-sandaracopimaradiene (**9**), and RcKSL4 produces predominantly the tetracyclic *ent*-beyerene (**10**). As expected, the *R. communis* KS, RcKS, contains an Ile at the primary switch

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position (Figure 3a), and substitution of Thr has a similar effect to that in other KSs, largely short-circuiting the complex dual

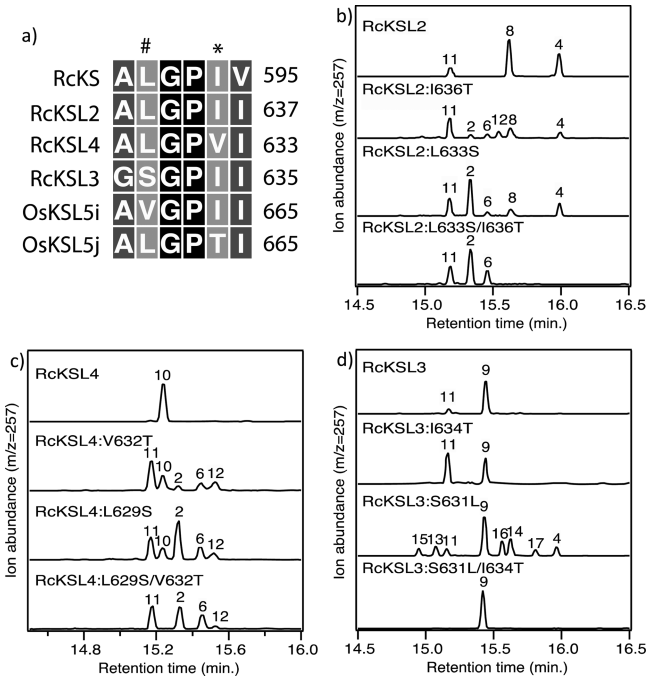

Figure 3. Additive effect of the original/primary and newly identified/secondary pair of residues on RcKSL product outcome. (a) Partial sequence alignment of RcKS(L)s and OsKSLs with an asterisk (*) above the primary (original) position and a pound sign (#) above the secondary (new) position. Residues are numbered as in the full-length proteins from the NCBI database accession numbers given in the Materials section. (b) Chromatograms from GC−MS analysis of wild-type RcKSL2 and the indicated mutants. (c) Chromatograms from GC−MS analysis of wild-type RcKSL4 and the indicated mutants. (d) Chromatograms from GC−MS analysis of wild-type RcKSL3 and the indicated mutants. Numbers correspond to the chemical structures defined in the text. Enzymatic products were identified by comparison of both retention time and mass spectra to authentic standards (Figure S1).

cyclization and rearrangement reaction leading to **4** (Figure S3). The resulting RcKS:I594T mutant produces small amounts of **4**, along with a mixture of pimaradienes **2** and **6**, as well as **7**, plus the labdatriene double-bond isomers *ent*-sclarene (**11**) and (*Z*)-biformene (**12**), which result from immediate deprotonation of the allylic carbocation formed upon initiating diphosphate ionization (*i.e.*, without any cyclization). The RcKSLs contain an Ile (RcKSL2 and -3) or Val (RcKSL4) at the primary switch position (Figure 3a). Consistent with the retained production of **4** following substitution of Val for the corresponding Ile in AtKS,[14] substitution of Ile for the Val at this position in RcKSL4 did not affect product outcome—*i.e.*, the resulting RcKSL4:V632I still predominantly produces **10** (Figure S4). Substitution of Thr at this primary switch position seems to most significantly reduce catalytic activity, as indicated by the reduced overall yield of diterpenes from expression of the corresponding mutants in the metabolic engineering system, not only with RcKS, but also RcKSL2−4. Nevertheless, such substitution also still abbreviated the catalyzed cyclization reactions, although it has a less dramatic effect. The resulting RcKSL2:I636T mutant still produces substantial amounts of **8** and **4**, although the major products are the labdatrienes **11** and **12** (Figure 3b). Similarly, the resulting RcKSL4:V632T still produces substantial amounts of **10** (Figure 3c). Even in the tricycle **9** producing RcKSL3, the resulting I634T mutant still produces substantial amounts of **9**, along with the labdatriene **11** (Figure 3d).

Intriguingly, while the other two RcKSLs contain Leu (as found in KSs), RcKSL3 contains serine at the secondary switch position instead (Figure 3a). Hypothesizing that this residue might exert an effect on product outcome, this Ser was substituted with Leu. Interestingly, the resulting RcKSL3:S631L mutant produces a small amount of the tetracyclic **4**, along with a number of other diterpenes (**9**, **11**, **13**−**17**), although **9** is still the major product. However, also substituting Thr for the Ile at the primary position restores almost exclusive production of **9** by the RcKSL3:S631L/I634T double mutant (Figure 3d). On the other hand, substituting Ser for the Leu at this secondary position in RcKSL2 was more effective at preventing the normally catalyzed second cyclization step, with the resulting RcKSL2:L633S mutant producing less of **8** and **4** than RcKSL2:I636T. Notably, combining these changes led to almost complete abbreviation of the catalyzed reaction after initial cyclization, with the RcKSL2:L633S/I636T double mutant largely producing pimaradienes **2** and **6**, along with the labdatriene **11** (Figure 3b). Similarly, such substitution in RcKSL4 also was more effective at abbreviating further cyclization, as the resulting RcKSL4:L629S mutant produced less **10** than RcKSL4:V632T. Again, combining these changes led to almost complete abbreviation of catalysis, with the RcKSL4:L629S/V632T double mutant producing mostly the tricyclic **2** and **6**, along with substantial amounts of the bicyclic **11** (Figure 3c). Given that substitution of alanine for this Leu in both RcKSL2 and -4 has a lesser effect than substitution of Ser, the observed change in product outcome seems to depend more on the presence of the hydroxyl group than the change in steric volume (Figure S5). Accordingly, these two positions seem to have an additive effect on product outcome, with the presence of residues with hydroxyl containing side-chains promoting abbreviation of the normally catalyzed cyclization reaction.

Rational Combination in AtKS for Specific Generation of 8α-Hydroxy-*ent*-pimar-15-ene. It has previously been reported that substitution of smaller residues (Ala or Ser) for the Ile in the primary position in AtKS leads to increased production of the hydroxylated pimarene **7**, although **2** remains the major product.[14] Given the additive effects observed with the RcKSLs above, and the significant increase in relative production of **7** by addition of the L635V mutation to AtKS:I638T (*i.e.*, by AtKS:L635V/I638T; see Figure 2c), it was hypothesized that a further optimized combination of changes at these two positions might lead to even more specific production of **7**. Indeed, combining the L635V change at the secondary position with either Ala or Ser substitution for the Ile at the primary position led to increased production of **7**, with the AtKS:L635V/I638S double mutant exhibiting the highest specificity, such that **7** is the dominant product (Figure 4).

Conclusions. The results presented here demonstrate that the residues at the original/primary and newly identified/secondary switch positions interactively affect KS(L) product outcome. However, the exact nature of the interaction differs, as does their effects. These differences seem to reflect the phylogenetic relationships between the various KS(L)s investigated here. Conservation of Leu at the secondary position in KSs is consistent with both the ability of this

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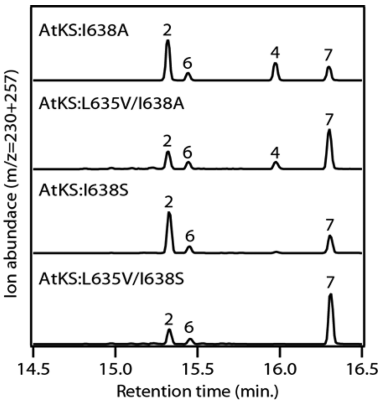

Figure 4. Application of the additive effect between the original/primary and newly identified/secondary pair of residues on AtKS product outcome to engineer predominant production of a hydroxylated diterpene. Chromatograms from GC−MS analysis of the wild-type AtKS and the indicated mutants. Numbers correspond to the chemical structures defined in the text. Enzymatic products were identified by comparison of both retention time and mass spectra to authentic standards (Figure S1).

residue to increase the catalytic rate and specificity for production of **4**, as well as functional conservation of KSs as representative of the ancestral activity in the KS(L) subfamily. The more pronounced effect on catalytic specificity observed with Val substitution for the Leu at the secondary position in the presence of Thr substitution for the Ile at the primary position provides some evidence for how these residues interactively affect product outcome. Specifically, substitution of the smaller Val for Leu at the secondary position presumably opens up the active site, which seems to decrease the confinement of the key *ent*-pimar-15-en-8-yl$^+$ intermediate within the active site. As a result, this carbocation is no longer as precisely positioned relative to the residue at the primary position—*e.g.*, for stabilization by the hydroxyl group of Thr, enabling alternative product outcomes.

By contrast, OsKSL5 belongs to an early diverging KSL lineage within monocots[18] and is relatively distantly related to any KSs (Figure 5). This is consistent with the distinct effects on product outcome observed upon interchanging Leu and Val at the secondary position (Figure 2d). Moreover, it seems likely that these enzymes arose *via* parallel re-evolution of the ability to produce the *ent*-kauranyl$^+$ intermediate relative to KSs (albeit with specific terminating deprotonation to yield **3** rather than

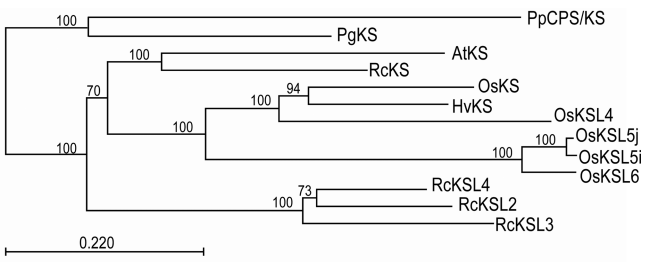

Figure 5. Phylogenetic tree for the KS(L)s biochemically characterized here (constructed from protein sequence alignment with the Neighbor Joining algorithm, 100 replicates, using CLC Sequence Viewer 6.9.1). The nonvascular plant derived PpCPS/KS and gymnosperm derived PgKS serve as the outgroup rooting the tree. For utilized sequences, see the NCBI protein database accession numbers referred to in the Materials section.

4). In particular, there seems to be distinct positioning of not only the *ent*-pimarenyl$^+$ intermediate in the active site of these OsKSLs relative to the KSs, as evidenced by the lack of effect of such interchange on production of **2**, but also of the *ent*-kauranyl$^+$ intermediate, as evidenced by the observed effect on production of **3** instead (Figures 1 and 2).

Similarly, RcKSL2−4 belong to an early diverging KSL lineage within eudicots[17] and also are relatively distantly related to any KSs (Figure 5). This is consistent with the clearly additive effect of altering the residues at the primary and secondary position in these KSLs. The presence of a hydroxylated side-chain containing residue in either position seems to at least partially block progression of the reaction past initial cyclization. In the case of RcKSL3, whose product **9** is epimeric (at carbon-13) to the other pimarane products reported here (i.e., **2**, **6**, **7**), the presence of either Ser at the secondary position or Thr at the primary position is sufficient for specific production of **9**, while a Leu/Ile pairing (respectively) enables some further cyclization and rearrangement (at least to a small extent; Figure 3d). On the other hand, in RcKSL2 and -4 the effect of the analogous substitution with Ser/Thr (respectively) is only partial, with complete blockage of further cyclization only observed if both are present (Figure 3b and c). This suggests that the *ent*-pimar-15-en-8-yl carbocation is positioned roughly equidistant from these residues, enabling this additive effect.

Notably, realization that the effect of the residues at these two positions can exert additive effects led to rational engineering. Specifically, for production of the hydroxylated pimarene **7** by AtKS. Previous results had demonstrated that substitution of Ala or Ser for the Ile at the primary switch position led to substantial production of **7**.[14] Given the increase in relative amounts of **7** produced by the addition of Val substitution for the Leu at the secondary switch position to the original Thr for Ile primary switch (see Figure 2c), it seemed intuitively obvious that combining these changes might lead to specific production of **7**. Indeed, the AtKS:L635V/I638S double mutant produces **7** as its major/predominant product (Figure 4). Thus, the newly identified/secondary switch position and interactive effect between this and the previously identified/primary position has already been shown to have practical implications for engineering product outcome. It will be of interest to further investigate the utility of these residues for altering catalytic specificity in other KSLs and plant terpene synthases more broadly.

METHODS

Materials. Unless otherwise noted, chemicals were purchased from Fisher Scientific and molecular biology reagents from Invitrogen. Sequence alignments and phylogenetic analysis were performed with CLC sequence viewer 6.9.1 using the default parameters. The NCBI protein database accession numbers of proteins included in this study: PpCPS/KS, *Physcomitrella patens* KS (BAF61135); PgKS, *Picea glauca* KS (ADB55711); AtKS, *Arabidopsis thaliana ent*-KS (AAC39443); RcKS, *Ricinus communis ent*-KS (XM_002533648); OsKS, *Oryza sativa ent*-KS (BAE72099); HvKS, *Hordeum vulgare ent*-KS (Q673F9); OsKSL4, *Oryza sativa* kaurene synthase-like enzyme 4 (Q66QH3); OsKSL5i, *Oryza sativa* kaurene synthase-like enzyme 5i (A4KAG7); OsKSL5j, *Oryza sativa* kaurene synthase-like enzyme 5j (Q6Z5J6); OsKSL6, *Oryza sativa* kaurene synthase-like enzyme 6 (ABH10733); RcKSL2, *Ricinus communis* kaurene synthase-like enzyme 2 (XM_002525795); RcKSL3, *Ricinus communis* kaurene synthase-like enzyme 3 (XM_002525790); RcKSL4, *Ricinus communis* kaurene synthase-like enzyme 4 (XM_002525796).

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Mutant Construction. The constructs utilized here encode pseudomature enzymes suitable for recombinant expression in *Escherchia coli* (*i.e.*, without the N-terminal transit peptide sequence that leads to the necessary plastid import in planta), although residue numbering is based on the full-length proteins. Previous work has shown that RcKSL2 contains an Ala in the "middle" position of the NDxx(S/T)xxxE motif, and that the A676T mutant was observed previously to produce the same product profiles with the wild type but with significantly higher efficiency (about 60-fold increase) in our metabolic engineering system,[17] so in this study, all the relevant mutants were generated in an RcKSL2:A676T background. Site-directed mutants were constructed by whole-plasmid PCR amplification of the relevant pENTR/SD/d-TOPO constructs using the relevant primers (Table S1) and AccuPrime Pfx DNA Polymerase. All mutants were verified by complete gene sequencing and then transferred *via* directional recombination to the T7-based, N-terminal GST-fusion expression vector pDEST15.

Enzymatic Analyses. To determine catalyzed product outcome, each pDEST15 based construct was cotransformed with a previously described pGGeC vector containing both a GGPP synthase and *ent*-CPP synthase,[16] along with a previously reported pIRS plasmid that increases metabolic flux toward terpenoids,[19] into the OverExpress C41 strain of *E. coli* (Lucigen). The resulting recombinant strains were cultured in 50 mL of TB medium (pH = 7.0), with appropriate antibiotics, in 250 mL Erlenmeyer flasks. These cultures were first grown by shaking at 37 °C to mid log phase (OD_{600} ~ 0.7); then the temperature dropped to 16 °C for 0.5 h prior to induction with 1 mM isopropylthiogalactoside (IPTG) and supplementation with 40 mM pyruvate and 1 mM $MgCl_2$. The induced cultures were further shaken at 16 °C for an additional 72 h before extraction with an equal volume of hexanes, with the organic phase then separated, and concentrated under N_2 when necessary.

Product Analyses. Gas chromatography with mass spectral detection (GC-MS) was carried out on a Varian 3900 GC with a Saturn 2100T ion trap mass spectrometer in electron ionization (70 eV) mode, using an Agilent HP-5MS column (Agilent, 19091S-433) with 1.2 mL/min helium flow rate. Samples (1 uL) were injected in splitless mode using an 8400 autosampler, with the injection port set at 250 °C. The following temperature program was used: the oven temperature started at 50 °C, which was maintained for 3 min, and then increased at a rate of 15 °C/min to 300 °C, where it was held for another 3 min. The mass spectrum was recorded by mass-to-charge ratio (m/z) values in a range from 90 to 650, starting from 13 min after sample injection until the end of the run. Enzymatic products were identified by comparison of retention time and mass spectra to those of authentic standards.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b01075.

Supplemental figures and table (PDF)

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Notes

The authors declare no competing financial interest.

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