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Exploring a *Streptomyces* wax synthase using acyl-SNACs as donor substrates†

Federica Casolari,^a Saad Alrashdi,^{ab} Reuben Carr^c and Hai Deng ^a

The demand of fragrance and food industries for short/branched wax esters is increasing due to their rich scent and low toxicity. Wax synthase and acyl-CoA:diacylglycerol O-acyltransferase (WS/DGAT) are a family of bacterial enzymes capable of catalysing the production of wax esters. Here, we report that a WS/DGAT from *Streptomyces coelicolor* is able to mediate the reactions between alcohol acceptors and synthetic acyl-donor mimics, acyl-SNACs. The enzyme displayed considerable substrate tolerance towards acyl-donors with structural diversity. Structural modelling-guided site directed mutagenesis resulted in a variant, L25F, the catalytic efficiency of which was improved toward aromatic, short-linear, and branched acyl-donors compared to the wild type.

Introduction

Wax esters (WEs) are considered key commercial products due to their numerous applications as biofuels, surface coatings, pharmaceuticals, and lubricants.^{1,2} While wax esters are primarily used in the solvent and biofuel industries, new applications are starting to emerge. The global flavours and fragrances market is expected to grow and was estimated at 29.15 billion US dollars in 2022. This spiking product market is being driven by the global demand and consumption of processed food and cosmetics.³ Interestingly, recent studies have demonstrated that branched wax esters can augment the skin-feel of lotions and other wax ester-containing cosmetics.⁴ Moreover, short chain esters, such as hexyl or ethyl acetate, can be utilised as both food additives and fragrances, while they can also function as drop-in fuels or lubricants.^{5–7} Long-chain wax esters are not only exploited as cosmetics or biodiesels, but also as

commercial vegetable oils and fats.^{8,9} Additionally, both butyl and hexyl benzoate were reported as key esters responsible for the sweet aroma of different fruit species.^{10,11} The majority of these valuable wax esters are currently prepared from fossil derived feedstock which requires energy intensive processing and is considered by many as non-sustainable for the future. Therefore, biotransformation of WEs is an attractive route to a more sustainable supply of these chemicals under mild conditions.

Generally, two enzyme families have been explored for the production of WEs. The first enzyme family is alcohol acyl-transferases (AATs or ATFs, EC 2.3.1.84) reported mainly in plants and yeast. They are able to metabolise short-chain alcohols and acyl coenzyme A (acyl-CoA) to yield volatile esters for the fragrance profile of many fruits or flowers, as well as the flavour palate found in beer or wine production.^{12,13}

The second enzyme family is wax ester synthases. They are bifunctional enzymes behaving as wax synthase (EC 2.3.1.75) and acyl-CoA:diacylglycerol O-acyltransferase (EC 2.3.1.20) (WS/DGAT) having the ability to combine acyl-accepting compounds (*i.e.* fatty alcohol or diacylglycerol) and acyl-donors (*i.e.* acyl-CoA or acyl-ACP) while producing the corresponding wax esters or triacylglycerols (TAGs) (Scheme 1).^{14,15} It has been well-documented that WSs possess a conserved motif (HHXXXDG) in the binding pocket containing two catalytic histidine residues which are responsible for ester bond formation.¹⁶

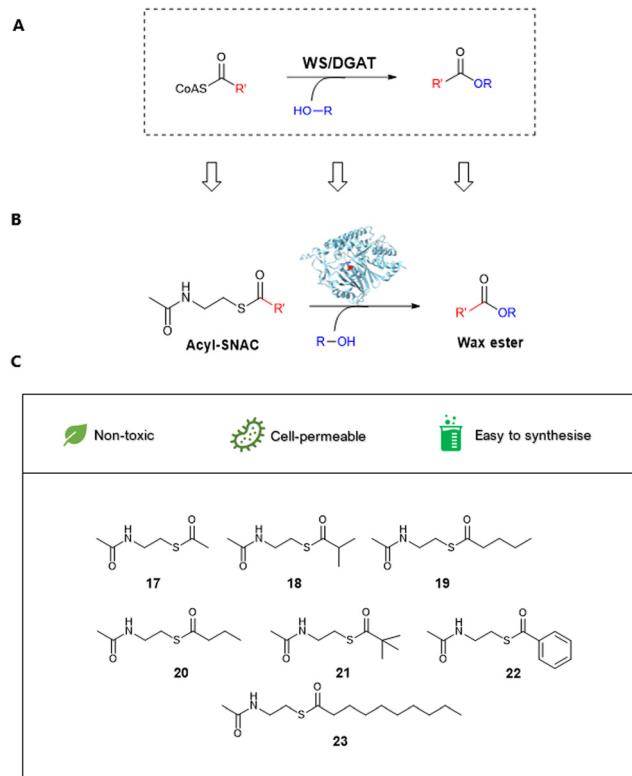
Bacteria are among the most abundant sources of the WS/DGAT family, as wax esters are pivotal in their biosynthesis of storage lipids.¹⁷ As such, bacterial WS/DGATs have been extensively studied to explore their biocatalytic potential. For example, wax synthases from *Marinobacter* have shown substrate promiscuity and exhibit good selectivity towards short and medium chain acyl-CoA, using hexanol and decanol as acyl acceptors to generate medium to long acyl-chain wax esters, such as 6,10,14-trimethylpentadecan-2-one, an intrinsic taste compound present in celery, oregano and sweet basil.^{18–20} Structure-guided site mutagenesis leading a variant of the *Marinobacter* genus (*Ma*-WS-A144F) displayed significantly increased selectivity towards C6 acyl-CoA (caproyl-CoA) compared to palmitoyl-CoA (C7 acyl-CoA) since such mutation in the binding

^a Department of Chemistry, University of Aberdeen, Aberdeen AB24 3UE, UK.
E-mail: h.deng@abdn.ac.uk

^b College of Science and Arts in Gurayat, Jouf University, King Khaled Road, Saudi Arabia

^c Ingenna Ltd Scotland, UK

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Scheme 1 (A) WS/DGAT mechanism reported in the literature. (B) Proposed mechanism of WS-SCo (*Streptomyces coelicolor* A32) with CoA synthetic mimics, acyl-SNACs. (C) Diverse selection of acyl-SNACs synthesised and used in this study.

pocket was likely to decrease the binding affinity of longer chains.²¹ Analogous studies have also described how the selection of the mutation site was driven by its proximity to the conserved HHXXXXDG motif of wax ester synthases, and how altering its surrounding could modify the enzyme substrate affinity.^{22,23}

Streptomyces species are able to synthesize various types of WEs and TAGs. For example, WE and TAG contents occupy nearly 9% (wt/wt) of the cell dry weight (CDW) in *S. lividans*.²⁴ As a result, WSs from *Streptomyces* have been well-studied to demonstrate their abilities to generate various DGATs.²⁵ Notably, *Streptomyces* WS/DGAT naturally metabolise long aliphatic-chain substrates, catalysing the esterification reaction from C12 to C18 acyl-donors, such as myristoyl-CoA.²⁶

In this study, we report characterization of a WS from *Streptomyces coelicolor* (WS-SCo) as a potential biocatalyst for WE production. We explore its catalytic capacity using different acyl-SNACs (17–23, Scheme 1) as acyl acceptors and C6 and C4 alcohols as acyl donors. To our knowledge, this is the first report to use synthetic mimics of CoAs for wax ester synthesis. The wild-type enzyme displays considerable substrate preference toward branched short chain and aromatic acyl-SNACs when hexanol is used, while showing substrate specificity toward straight acyl-SNACs after the addition of butanol, suggesting that different acyl donors affect substrate selectivity toward acyl acceptors, a similar phenomenon observed in other WS systems.^{27,28} Further structural modelling-guided site

directed mutagenesis (SDM) allow generation of an enzyme variant, L25F, which displays a similar trend that the variant shows substrate selectivity toward aromatic acyl SNACs when hexanol is used, while toward straight chain acyl-SNACs when butanol is added.

Results and discussion

Considering that the catalytic capacities of WSs from *Streptomyces* as potential biocatalysts have been largely overlooked, we chose a putative WS (WS-SCo) (GenBank: QFI41200.1) from *S. coelicolor* A3(2), the model *Streptomyces* strain, to explore its substrate tolerance. In this regard, we first sought to overexpress the gene encoding a putative WS/DGAT (WS-SCo) in *E. coli* BL21 (pGro7). The enzyme was expressed as an N-terminal His₆-recombinant protein that was purified to near homogeneity by Ni-NTA chromatography and gave an estimated molecular weight of 47 kDa, as observed in SDS-PAGE analysis (Fig. S1, ESI†). To assay the activity of the recombinant protein, the commercially available acetyl-CoA 24 was used for the initial examination. Incubation of the purified WS-SCo (10 μM) with acetyl CoA 24 (1 mM) and hexanol 25 (2.5 mM) as a common alcohol acceptor resulted in the production of hexyl acetate esters as observed in HR-LCMS analysis (Fig. S4, ESI†). In the control assay with the boiled enzyme, the formation of the ester was not observed. This indicated that WS-SCo is indeed a WS/DAG that can accept short-chain acyl-CoA species.

To obtain the kinetic data of WS-SCo, we monitored the CoA release using the DTNB (5,5'-dithiobis(2-nitrobenzoic acid), Ellman's reagent) derivatization assay.²⁹ The kinetic constants were the values of $K_m = 73.294 \pm 0.021 \mu\text{M}$ and $k_{\text{cat}} = 0.171 \pm 0.022 \text{ min}^{-1}$, suggesting that WS-SCo is a competent WS/DAG that has the capacity of transforming short-chain acyl-CoA with hexanol into the corresponding ester. Many WS/DAGs from other organisms have been examined for their substrate tolerances using various straight chain saturated or unsaturated acyl-CoAs as substrates.^{30,31} However, few have been tested with branch chain or aromatic acyl-CoAs due to their commercial unavailability.

Structurally simplified CoA analogues such as *N*-acetyl-cysteamine thioesters (SNACs) have been developed to study thiolate-templated enzymes such as biosynthetic pathways to fatty acids, polyketides and non-ribosomal peptides.³² Therefore, we decided to examine whether WS-SCo could accept acyl-SNACs, the acyl-CoA synthetic mimic, as its substrates. To this end, we synthesized seven acyl-SNACs (acetyl-17, isobutryl-18, valeryl-19, butyryl-20, *tert*-butyl-21, phenyl-22 and decanoyl-23) with structural diversity using our previously described method.³³ Briefly, the one-pot synthesis started with the commercially available stable cysteamine reacting with acetic anhydride under alkali conditions to provide *N,N*-diacetyl-cysteamine. *In situ* reduction by TCEP and *S*-acylation using chosen anhydrides gave the corresponding acyl-SNACs (Fig. S5, ESI†). The products were confirmed by HR-LCMS, ¹H-NMR spectroscopy and ¹³C-NMR spectroscopy analyses (Fig. S6–S29, ESI†). To validate this, kinetic data were obtained as previously described. WS-SCo displayed a

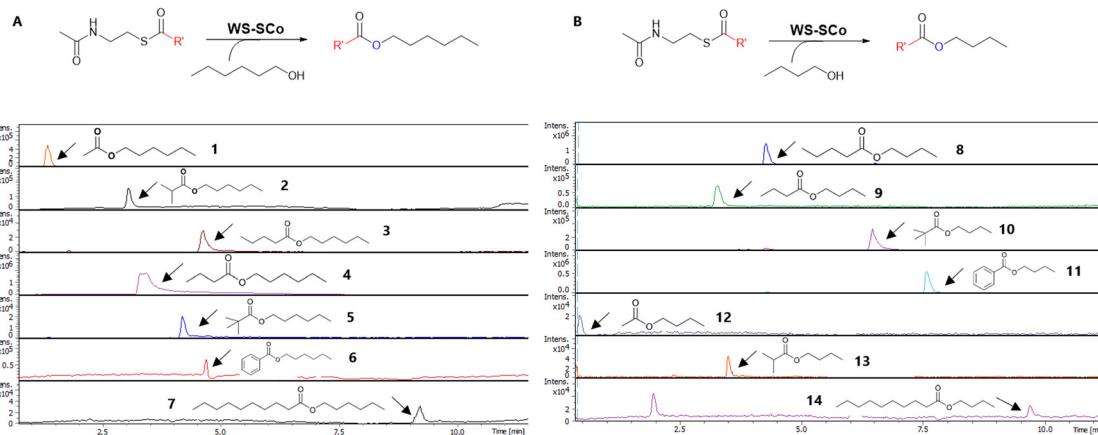


Fig. 1 Extracted ion chromatograms of ESI-Q-ToF-LCMS analysis displaying WS-SCo-produced short esters (**1–14**) with acyl-SNACs (**17–23**) as acyl-donors and hexanol **25** (A) and butanol **26** (B) as acyl-acceptors.

much larger affinity toward acetyl-SNAC compared to its CoA analogue, as indicated by a low K_m value of $28.261 \pm 0.104 \mu\text{M}$ and a k_{cat}/K_m constant ($4.897 \pm 1.093 \text{ mM min}^{-1}$) which was twice that of acetyl-CoA ($2.392 \pm 0.394 \text{ mM min}^{-1}$). This suggested that acyl-SNACs have the potential to replace to acyl-CoAs as enzymatic substrates for wax ester bio-catalysis.

With acyl-SNACs available, we then set out to probe the overall substrate tolerance of WS-SCo. Incubation of the purified enzyme ($10 \mu\text{M}$) with acyl-SNACs (1 mM) and alcohol (2.5 mM) (hexanol **25** and butanol **26**) resulted in the production of the corresponding esters, **1–14**, as shown by HR-LCMS analysis (Fig. 1). This is the first report to demonstrate that bacterial WS-SCo is able to process acyl-CoA synthetic mimics, acyl-SNACs, thus allowing us to evaluate various acyl-donors with structural diversity. Interestingly, WS-SCo appears to display similar reactivity towards aromatic and branched chain acyl-SNACs compared to straight-chain SNACs when **25** or **26**

was used as acyl-acceptors. However, when decanol **27** was used, no corresponding esters were observed, indicating that WS-SCo is unable to process C10 or longer-alcohols.

Encouraged by this observation, detailed kinetic studies of WS-SCo were performed. We measured the reactions by monitoring SNAC release using the DTNB derivatisation assay. Incubation of WS-SCo ($10 \mu\text{M}$) with hexanol or butanol (2.5 mM) and various concentrations ($25 \mu\text{M}$ to 1 mM) of acyl-SNACs provided the corresponding hexyl or butyl esters (Table 1).

When hexanol is present, the enzyme exhibits decreasing selectivity from longer (decanoyle-SNAC $K_m = 15.206 \pm 0.001 \mu\text{M}$) to shorter linear chains while appearing to favour small-branched substrates (isobutyryl-SNAC $K_m = 5.229 \pm 0.003 \mu\text{M}$) in comparison to aromatics. Similar conclusions are supported by the catalytic efficiency (k_{cat}/K_m) data, which exhibit highest values for long ($40.548 \pm 0.343 \text{ mM min}^{-1}$) and branched ($23.333 \pm 5.072 \text{ mM min}^{-1}$) acyl chains. This reveals a degree

Table 1 Heat map of kinetic constants for WS-SCo with hexanol and butanol as acyl-acceptors and acyl-SNACs as acyl-donors. Michaelis–Menten kinetics were measured using non-linear regression, varying the acyl-SNAC concentration between $25 \mu\text{M}$ and 1 mM and keeping alcohol concentrations at saturating levels (2.5 mM)

Acyl-SNACs	$K_m (\mu\text{M} \pm \text{STD Dev})$		$K_{\text{cat}} (\text{min}^{-1} \pm \text{STD Dev})$		$K_{\text{cat}}/K_m (\text{mM} \cdot \text{min}^{-1} \pm \text{STD Dev})$	
	Hexanol	Butanol	Hexanol	Butanol	Hexanol	Butanol
Acetyl-SNAC	Yellow	Red	Red	Light Blue	Light Brown	Brown
Isobutyryl-SNAC	Green	Yellow	Red	Red	Light Brown	Brown
Valeryl-SNAC	Yellow	Yellow	Light Blue	Red	Light Purple	Brown
Butyryl-SNAC	Yellow	Light Green	Dark Blue	Red	Dark Purple	Light Brown
tert-butyl-SNAC	Yellow	Light Green	Dark Blue	Light Red	Dark Purple	Light Brown
Phenyl-SNAC	Yellow	Light Green	Light Red	Light Red	Light Purple	Light Brown
Decanoyle-SNAC	Green	Yellow	Dark Blue	Light Red	Dark Purple	Brown

Low K_m ,
High selectivity
for substrate
High K_m ,
Low selectivity
for substrate
Low k_{cat} ,
low substrate
turnover
High k_{cat} ,
high substrate
turnover
Low k_{cat}/K_m ,
low catalytic
efficiency
High k_{cat}/K_m ,
high catalytic
efficiency



of variance in substrate acceptance which can be explained by the promiscuous nature of bacterial WS.³⁴

When butanol was added in the reactions, the preference of selectivity and efficiency of WS-SCo is less apparent because it lacks the ability to selectively convert long, short linear (acetyl-SNAC) and short branched (isobutyl-SNAC) acyl-donors into butyl esters. The enzyme still displays good enzymatic efficiency towards branched (*tert*-butyl-SNAC $k_{cat}/K_m = 6.019 \pm 2.654 \text{ mM min}^{-1}$), aromatic (phenyl-SNAC $k_{cat}/K_m = 6.284 \pm 0.872 \text{ mM min}^{-1}$) and linear chains (butyryl-SNAC $k_{cat}/K_m = 5.041 \pm 2.648 \text{ mM min}^{-1}$), indicating its ability to efficiently produce *tert*-butyl, phenyl and butyryl C4-esters.

Clearly, there is a discrepancy of kinetic profiles between hexanol and butanol as acyl-acceptors, which has been previously reported.²⁷ This may be due to a key conformational change in the active site when an acyl-donor is bound in its corresponding binding pocket, as observed in previous studies.^{35,36}

To the best of our knowledge, this is the first instance of a bacterial WS/DGAT demonstrating its catalytic capacity toward branched/aromatic acyl-SNACs to process the corresponding esters. To further investigate whether the key residues in the acyl-CoA pocket of WS-SCo can affect the length of acyl-SNACs, we generated a site-specific variant and compared its activity with that of the wild type of WS-SCo.

Previous crystallography and mutagenesis studies of bacterial WS/DGATs allowed the identification of key residues that can influence substrate selectivity.^{21,30} For example, it was found that mutations of Ala144 to phenylalanine, Gly25 to valine and Ala148 to isoleucine improved the selectivity of a WS/DGAT from a *Marinobacter* sp. towards shorter acyl-CoAs. This is because these residues are positioned in the acyl-CoA pocket and increasing the amino acid side chain would decrease the binding affinity of longer-chain acyl-CoAs.

With this in mind, we performed structural modelling analysis of WS-SCo using AlphaFold (DeepMind), searching for the potential location of the acyl-donor active site.^{37,38} WS-SCo displays high structural similarity to *Ma*-WS/DGAT (Fig. S30, ESI†). Interestingly, structural comparison with WT-*Ma*-WS/DGAT indicated that, although enzymes contain an Ala residue in positions 143 and 144, respectively, WS-SCo possesses Phe138 in the acyl-CoA pocket while *Ma*-WS/DGAT has Thr132. In the latter, these two residues (Ala144 and Thr132) act as gatekeepers to interact with the hydrocarbon chains of acyl-CoAs. Changing Ala144 to a bulky side chain such as phenylalanine resulted in good selectivity toward short chain acyl-CoA. Additionally, WS-SCo possesses Ala27, which is equivalent to Gly25 in *Ma*-WS/DGAT. The variations of Phe138 and Ala27 in WS-SCo may explain the reason for the good selectivity of WS-SCo toward branched short chain/aromatic acyl-SNACs. However, WS-SCo still displays good catalytic efficiency toward longer-chain acyl-SNACs (*i.e.* decanoyl-SNAC), suggesting that, although possibly not fully extended, the acyl chain can still be folded into the pocket for the esterification. Another interesting observation is that WS-SCo possesses Leu25 (Fig. 2), instead of Val23 in *Ma*-WS/DGAT, which is intrinsically close to His142, responsible for the WS/DGAT mechanism. It

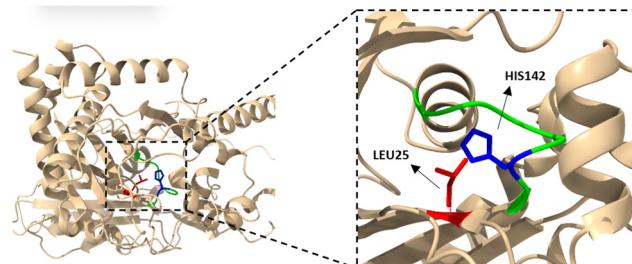


Fig. 2 Screen caps of modelling of WS-SCo and its acyl-donor binding pocket. Mutation site (leucine25, red) and conserved motif HHALADG (green) containing catalytic histidine142 (blue) are highlighted here.

was hypothesized that changing this residue to one with a “bulkier” and hydrophobic side chain (such as phenylalanine) may affect the binding of acyl-chains. For this reason, a WS-SCo-L25F variant was generated and expressed in the same system.

To assess the impact of L25F mutation on enzymatic activity, we conducted a comparison analysis of both wild type and L25F variant using available acyl-SNACs and alcohols. Again, WS-SCo activity seems to be varying according to the alcohol used. When hexanol is present, Leu25Phe mutation improves the enzyme activity to bind with aromatic (285.5%, phenyl-SNAC) and long chains (207.6%, decanoyl-SNAC), while it decreases for all other substrates. This behaviour could be attributed to stacked aromatic interactions between the rings of phenyl-SNAC and Phe25 and with the long chain of decanoyl-SNAC being not fully extended. Significant low activity (24.4%) for “bulkier” branched acyl chains, such as *tert*-butyl-SNAC, suggests that the third methyl group could be blocked by the aromatic ring of Phe25, inhibiting its binding.

Conversely, WS-SCo-L25F betters its activity towards linear chains, from longer (C10) to shorter (C1) linear substrates (acetyl-SNAC > butyryl-SNAC > decanoyl-SNAC), when butanol is used. Under the same conditions, a significant decrease in

Table 2 Activity comparison assay between WS-SCo-WT and WS-SCo-L25F. Activity was expressed as the difference in $\text{Abs}_{412\text{nm}}$ during 30 min of reaction, standardised with the extinction coefficient of TNB²⁻ ($14150 \text{ M}^{-1} \text{ cm}^{-1}$), of the wild type (100%) and the mutant for each substrate tested. Results are shown as percentage (%) of at least duplicates samples

Acyl-SNACs	WS-SCo-L25F (%)	
	Hexanol	Butanol
Acetyl-SNAC	41.9 \pm 2.0	211.8 \pm 2.3
Isobutyl-SNAC	88.9 \pm 0.5	86.0 \pm 0.3
Valeryl-SNAC	95.9 \pm 2.0	25.6 \pm 11.5
Butyryl-SNAC	80.7 \pm 0.3	175.0 \pm 0.3
<i>tert</i> -butyl-SNAC	24.4 \pm 0.6	70.9 \pm 1.7
Phenyl-SNAC	285.5 \pm 2.9	63.6 \pm 0.4
Decanoyl-SNAC	207.6 \pm 3.2	141.2 \pm 0.3

Low activity High activity



activity for branched acyl-SNACs, such as *tert*-butyl (70.9%) and aromatic (63.6%) also becomes apparent (Table 2).

Although previously reported mutations in the acyl-CoA binding pocket achieved higher selectivity towards smaller acyl-donors, an active preference for longer alcohol chains could still be noticed, which could support our reported substrate variation.²¹ Additionally, a recent study described a change in the structural configuration occurring in the pocket during the binding of the acyl-donor, preventing the correct reactivity with the alcohol.²⁸ Therefore, WS-SCo-L25F could potentially lose the selective binding to the alcohol if the binding site is too narrow. In conclusion, our findings indicate that although the Leu25Phe mutation does not improve activity towards branched acyl chains, it does significantly increase WS-SCo reactivity with linear and aromatic SNACs. Future crystallography studies may help us improve our understanding of WS-SCo towards branched and aromatic acyl-SNACs.

Conclusions

In summary, we have demonstrated that WS-SCo from *Streptomyces coelicolor* A3² is a considerably promiscuous wax ester synthase capable of catalysing the production of numerous small wax esters. To our knowledge, WS-SCo is the first enzyme of its family to be examined towards CoA analogues, synthetic acyl-SNACs, where its substrate affinity varies according to the alcohol tested (butanol or hexanol). Moreover, the enzymatic activity assay revealed that the L25F mutated enzyme favoured aromatic and linear acyl-donors. When butanol was used as the acyl-acceptor, WS-SCo-L25F also displayed improved activity towards aromatic/long-chain acyl acceptors. Overall, WS-SCo is capable of yielding a multitude of small wax esters, such as hexyl and butyl acetate (1 and 12), isobutyrate (2 and 13), *tert*-butyrate (5 and 10) and benzoate (6 and 11), which are esters commonly exploited by the food and fragrance industries.

Author contributions

FC carried out all experiments, designed the study and drafted the manuscript. SA participated in the design of the study and synthesised all acyl-SNACs. HD conceived the study, participated in its design and helped in drafting the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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