Thioester Mediated Biocatalytic Amide Bond Synthesis with *In Situ* Thiol Recycling

Christian Schnepel[1], Laura Rodriguez-Perez[1], Yuqi Yu[1], Antonio Angelastro[1], Rachel S. Heath[1], Max Lubberink[1], Francesco Falcioni[2], Keith Mulholland[3], Martin A. Hayes[4], Nicholas J. Turner[1], and Sabine L. Flitsch[1]*

[1] Department of Chemistry, The University of Manchester, Manchester Institute of Biotechnology, 131 Princess Street, Manchester M1 7DN (UK).

[2] Early Chemical Development, Pharmaceutical Sciences, R&D, AstraZeneca, Cambridge (UK).

[3] Chemical Development, Pharmaceutical Technology & Development, Operations, AstraZeneca, Macclesfield SK10 2NA (UK).

[4] Compound Synthesis and Management, Discovery Sciences, R&D, AstraZeneca, Gothenburg (SE).

* Corresponding author: Prof Sabine L. Flitsch, sabine.flitsch@manchester.ac.uk

Abstract

The conversion of carboxylic acids to thioesters is a key step in the biosynthesis of natural products, resulting in activation of the acyl groups for subsequent reactions, *e.g.* acylation of nucleophiles including carbon-carbon bond formation. For example, thioesters of Coenzyme A (CoA-SH; *e.g*. acetyl-S-CoA) are intermediates in many metabolic pathways, and are increasingly recognised as important cofactors for epigenetic post-translational modifications, such as *N-, O*- and *S*-acylations of proteins. However, the limited availability of a broad range of structurally diverse thioesters has limited their wider exploitation in biochemistry, cell biology and biotechnology. Furthermore, the high cost of CoA-SH impairs its use in stoichiometric quantities. To address these challenges we show that the adenylation (A-) domain of the carboxylic acid reductase (CAR) from *Segniliparus rugosus* (CAR*sr*-A) can function as a broad spectrum acyl-S-CoA synthetase, to generate acyl-S-CoA intermediates from a wide range of carboxylic acids. In addition, CAR*sr*-A was able to generate thioesters from structurally simpler thiols such as pantetheine. The resulting thioesters were then used as substrates for acyltransferases to synthesise a wide range of amides, including the more difficult to prepare, but pharmaceutically relevant aryl amides. Importantly, CoA-SH is recycled during the reaction and can be used in sub-stoichiometric quantities. This approach has also been applied to acylate a histone peptide H4-20 with a range of carboxylic acids, including non-natural chemical labels, by employing a lysine acetyltransferase (HATp300). Overall, this combination of a broad spectrum biocatalyst for thioester synthesis, together with *in-situ* CoA-SH recycling, provides a generic platform for thioester-dependent cell-free synthesis, with potential applications beyond amide bond formation.

Introduction

The cell-free application of cofactor-dependent enzymes in biotechnology has been made possible by the development of generic recycling systems,[1] such as *in situ* regeneration of ATP from inexpensive polyphosphate[2], NAD(P)H regeneration,[3] or the recently introduced recycling of *S*-adenosylmethionine.[4] However, such generic recycling systems are not available for thioester cofactors, which are key substrates of many enzymes in metabolic and biosynthetic pathways, including crotonases,[5,6] thiolases,[7] oxidases,[8] carboxylases,[9,10] and acyltransferases[11,12] In addition, specific cognate activating enzymes are required for conversion of each class of carboxylic acid substrate. For example, acetyl-S-CoA synthetase catalyses the condensation of acetate and CoA-SH (**1**) to give acetyl-S-CoA (**8**).[11,13] This high specificity for the carboxylic acid is particularly challenging and limits the broader application of acyl-S-CoA biocatalysis.[14,15] Attempts to broaden the substrate scope have been explored using protein engineering,[16,17] or database mining,[11] with limited success so far. The acetylation of CoA-SH (**1**) using vinyl acetate has only been used for acetyl-S-CoA,[18] and chemical strategies were also reported. However, these approaches cannot be used to generate the thioester *in situ*, nor are they compatible with recycling systems.[19] *N*-acetylcysteamine (SNAC, **2**) thioesters have been explored as inexpensive alternatives to CoA-SH, but SNAC thioesters are often poor surrogates for subsequent enzymes.[20] The demand for a more generic thioester generation/recycling system is particularly acute in biocatalytic amide bond formation, given that many acyltransferases are available but their use is prohibited by the supply of thioesters.[11,21–23]

Here we report a robust, generic, *in situ* thioester generation/recycling system that (i) has a broad substrate range towards carboxylic acids; (ii) can generate simpler thioesters such as acyl-pantetheinates and (iii) can be coupled with acyltransferases for amide bond synthesis, thereby resulting in the use of CoA-SH in sub-stoichiometric amounts. We demonstrate the versatility of this platform via the one-pot synthesis of amides involving acyl-S-CoA intermediates, formed directly from a diverse range of carboxylic acids.[11,21–23] In addition, we show an application in histone peptide acylation, a key post-translational modification widely observed in epigenetic transcriptional regulation, which is catalysed by lysine acetyltransferases (KATs)[24], to introduce both labelled acetic acid and larger carboxylic acids containing clickable functional groups (Figure 1).[25]

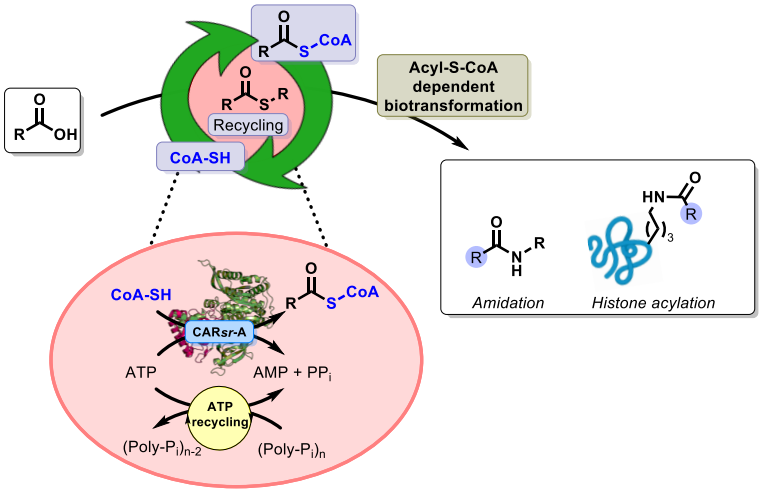

Figure 1. Carboxylic acid reductase A-domain (CAR*sr*-A) was established as a promiscuous thioester synthetase that functions as a generic thioester regeneration system. By combination with biotransformations acting on acyl-S-CoA substrates, release of CoA-SH (**1**) enables *in-situ*-regeneration of acyl-CoA by CAR*sr*-A while a (poly-P_i) kinase regenerates ATP.

Identification of acid-thiol ligase activity of CAR*sr*-A

We have previously shown that carboxylic acid reductases can be used for alternative *N*-acylation reactions *via* formation of the acyl-AMP intermediate.[22,26] CAR A-domains can function as standalone enzymes for amide and ester bond formations in water with broad substrate specificities for carboxylic acids and both amines or alcohols as nucleophiles.[23,27,28] The PCP domain of full-length CARs carries a phosphopantetheinyl moiety, which forms a thioester intermediate by intercepting the acyl-adenylate, in order to transfer the acyl group from the A- to the reduction domain. Previous analysis of the crystal structure of CARs suggests similarity of their A-domains to acyl-S-CoA synthetases (**1**; Supplementary Figure 1).[29] To explore the similarities between CAR A-domain and acyl-S-CoA synthetases more broadly, a sequence similarity network was generated by using a variety of acyl-CoA synthetases collected from BRENDA database,[30] as well as sequences of CAR A-domains (Supplementary Figure 2). It turned out that A-domain sequences resemble 4-coumarate-S-CoA synthetase, and *in-silico*-docking of **1** into CAR*sr*-A revealed a binding site for **1** proximal to the ATP- and acid-binding site. Moreover, docking suggested that **1** is able to interact with the protein by forming several polar interactions, thus providing a pocket to accommodate the thiol in the A-domain near the acyl-adenylate (Figure 2a, left).

Inspired by these initial findings, the adenylation domain of CAR*sr* (CAR*sr*-A) covering sequence part [1-658] of the full-length enzyme was designed, subcloned and employed as a biocatalyst. The catalytic activity of CAR*sr*-A was first tested using the previously described direct amide bond formation. Using 5 mM benzoic acid (**4**) and 150 mM methylamine along with purified CAR*sr*-A resulted in 90% conversion into the product amide (Supplementary Figure 3).[23] After confirming the activity of the isolated A-domain of CAR*sr*, thiols were tested as external nucleophiles for thioester formation, a reaction that had not been examined previously. Using an excess of SNAC (**2**) (150 mM) as a thiol and benzoic acid (**4**) (5 mM), the corresponding product, benzoyl-SNAC (**7**), was detected by LC-MS with nearly quantitative conversion of acid (>95%) (Supplementary Figure 4). Reducing the SNAC concentration to less than 50 mM dramatically decreased the conversion. Based on these initial results, a range of biologically relevant thiols was further investigated at different concentrations (Figure 2b/c). We were pleased to see that pantetheine (**3**) and CoA-SH (**1**) were found to be considerably better substrates with good conversions being observed even in the presence of a small excess of thiols **1** and **3** (5-6 mM) over acid **4**. These findings were further corroborated by molecular docking revealing a binding mode for **3** similar to the pose suggested for **1**, whereas **7** is located distant from the ATP site (Supplementary Figure 5). Thus, the formation of **5** and **6** appears to be much more favourable compared to both amide and ester syntheses described previously, both requiring a significant excess of nucleophile.[23,28] These observations suggest that the binding pocket for the pantetheinyl group in the active site in CAR*sr*-A is easily accessible to external thiols.

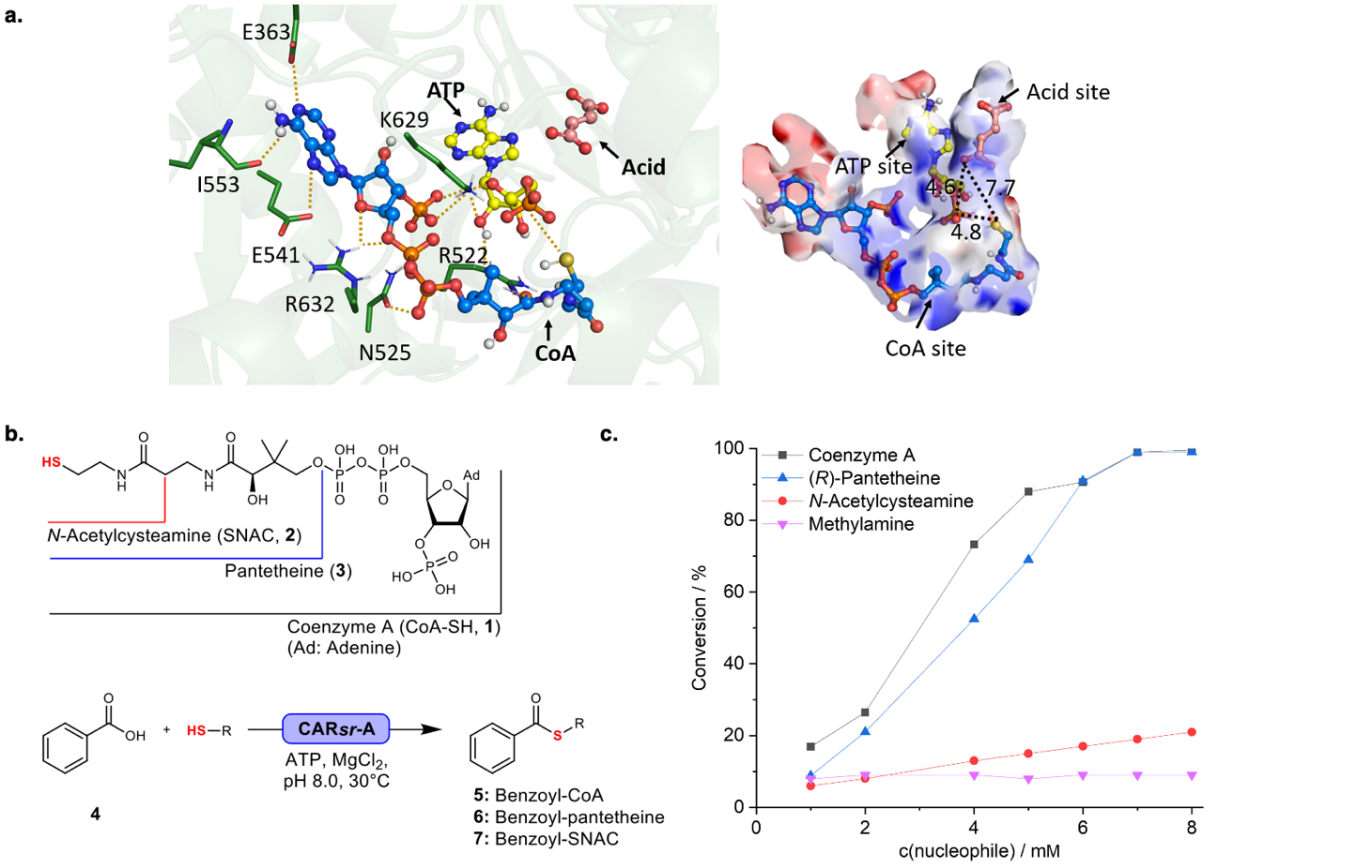

Figure 2. (a) *In-silico* docking reveals a CoA-SH binding site proximal to the ATP and acid sites in CAR*sr*-A. CoA-SH (**1**), ATP and carboxylic acid are shown as ball and stick representations; key residues interacting with **1** are shown in green. Negatively charged surface area is coloured in red and positively charged area is coloured in blue. (b) CAR*sr*-A catalysed formation of thioesters. (c) Coupling of benzoic acid (**4**, 5 mM) to different nucleophiles supplied in varying concentrations indicates that **1** and pantetheine (**3**) can be coupled in equimolar ratios of thiol to benzoic acid in high conversions. General reaction conditions: 5 mM benzoic acid, 20 mM ATP, 50 mM $MgCl_2$, 25 µM CAR*sr*-A, 100 mM HEPES (pH 8.0). Conversions were determined by substrate depletion assays using UPLC-MS after incubation for 20 h at 30 °C.

Profile of CAR*sr*-A towards acyl-S-CoA thioester formation

It was previously shown that full-length CAR*sr* has a broad carboxylic substrate scope.[31] This feature was also observed for CAR*sr*-A, which catalysed the formation of a large variety of acyl-S-CoA thioesters (Figure 3a). Short- to medium-chain aliphatic carboxylic acids (**8**-**12**) were efficiently transformed into the corresponding acyl-S-CoA esters. Further functionalisation and sterically more demanding carboxylic acids were well tolerated. Moreover, **11** and **18** offer handles for further modification using established bioorthogonal labelling methods.[32] Similarly, high conversions were achieved for aryl carboxylic acids. Several functionalised benzoate derivatives, *e.g.*, **20**, **21**, and **22**, were readily accepted by the enzyme. The dicarboxylic acids **13** and **25** were selectively transformed into the corresponding mono-thioesters, while the second carboxyl group remained unmodified, which is difficult to achieve using conventional chemical synthesis. Interestingly, polar amino substituents attached to the benzene moiety gave no notable conversion. Representative heterocyclic and bulkier bicyclic derivatives were also good substrates of CAR*sr*-A, *e.g.*, giving access to heterocyclic thioesters **27**-**29**, providing interesting moieties for natural product and pharmaceutical synthesis and have been less represented in approaches towards enzymatic acyl-S-CoA formation.

In order to make CAR*sr*-A catalysed acyl-S-CoA formation more economically viable, the reaction was adapted towards using cell-free extracts (CFEs). The previously described polyphosphate kinase CHU[33] was implemented for *in-situ*-regeneration of ATP starting from the cost-effective precursors AMP and polyphosphate. As in previous biotransformations using purified adenylation domain, benzoylation of **1** succeeded with comparable efficiency (Supplementary Figure 6). With this optimised system in hand, [^{13}C]-labelled benzoyl-CoA (**5**) was generated, simply by using the labelled carboxylic acid [^{13}C]-**4** as a precursor. Formation of [^{13}C]-**5** was discontinuously monitored by ^{13}C-NMR revealing a new signal at δ 195 ppm that corresponds to the chemical shift of the thioester's carbonyl-C atom (Figure 3b). Moreover, single ion monitoring (SIM) by UPLC-MS to specifically detect substrate- and product-related ions further confirmed the formation of isotopically labelled thioester [^{13}C]-**5**.

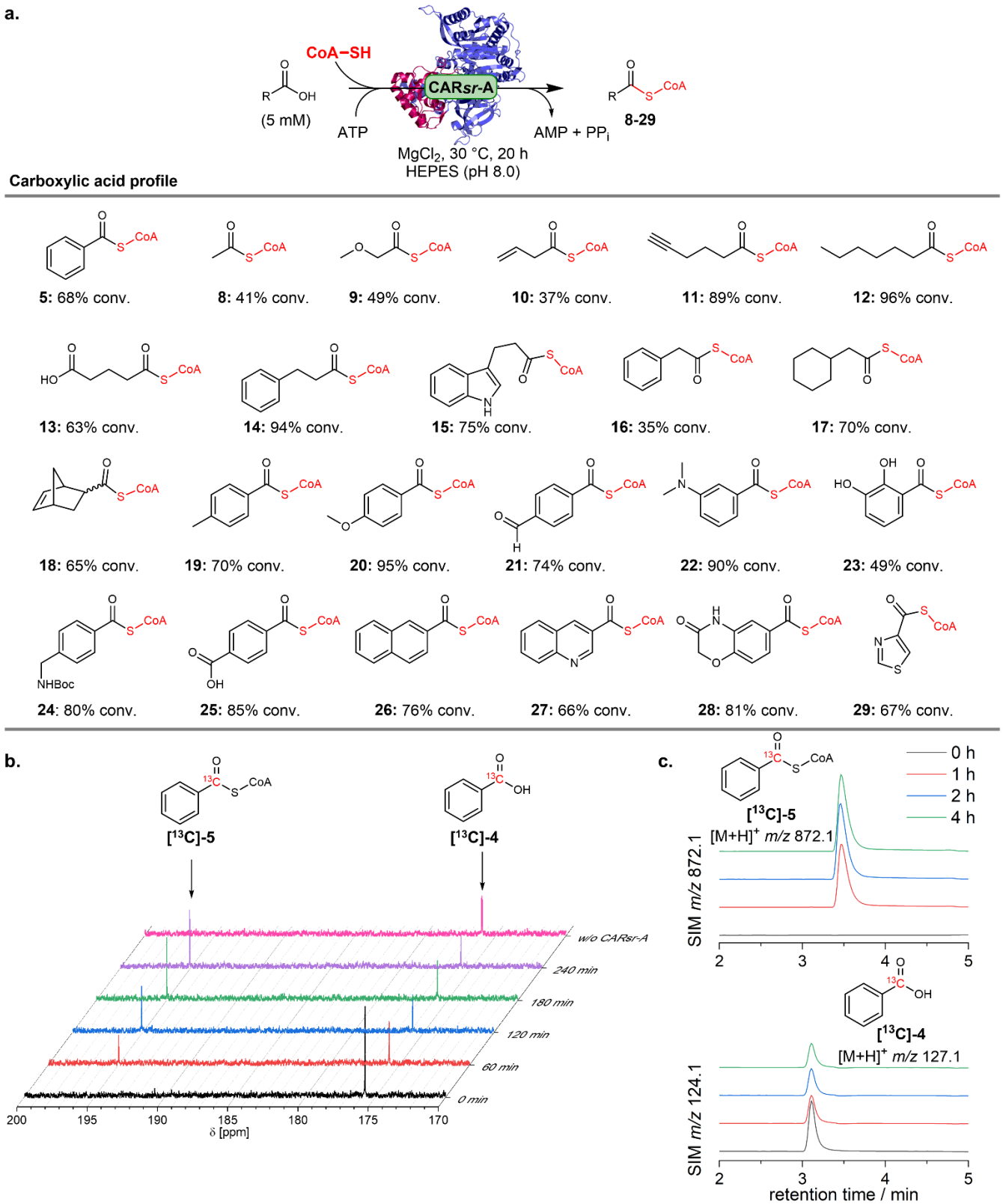

Figure 3. Profiling of acyl-CoA thioesters (**8**-**29**) formed by CAR*sr*-A. Representative carboxylic acid substrates were tested and analysed by UPLC-MS. Quantification was based on substrate depletion. Reaction conditions: 25 µM CAR*sr*-A (purified), 5 mM carboxylic acid, 5 mM CoA-SH, 20 mM ATP, 50 mM $MgCl_2$, 100 mM HEPES (pH 8.0). Conversions were quantified in substrate depletion assays upon incubation for 20 h at 30 °C. (b) ^{13}C-NMR time progress of formation of [^{13}C]-labelled benzoyl-S-CoA (**5**) using CAR*sr*-A (cell-free extract) along with concomitant ATP regeneration (100 MHz, reference: methanol-d_4). (c) Time course of CAR*sr*-A catalysed formation of [^{13}C]-benzoyl-CoA monitored by UPLC-MS using SIM for detecting $[M+H]^+$ ions of substrate, [^{13}C]-**4** (*m/z* 124.1), and thioester, [^{13}C]-**5** (*m/z* 872.1). Reaction conditions: 5 mM [^{13}C]-benzoic acid (**4**), 5 mM CoA-SH (**1**), 10 mM AMP, 20 mg mL^{-1} sodium hexametaphosphate, 5 mg mL^{-1} CAR*sr*-A (CFE), 2 mg mL^{-1} CHU, 100 mM $MgCl_2$, 100 mM HEPES (pH 8.0), 30 °C.

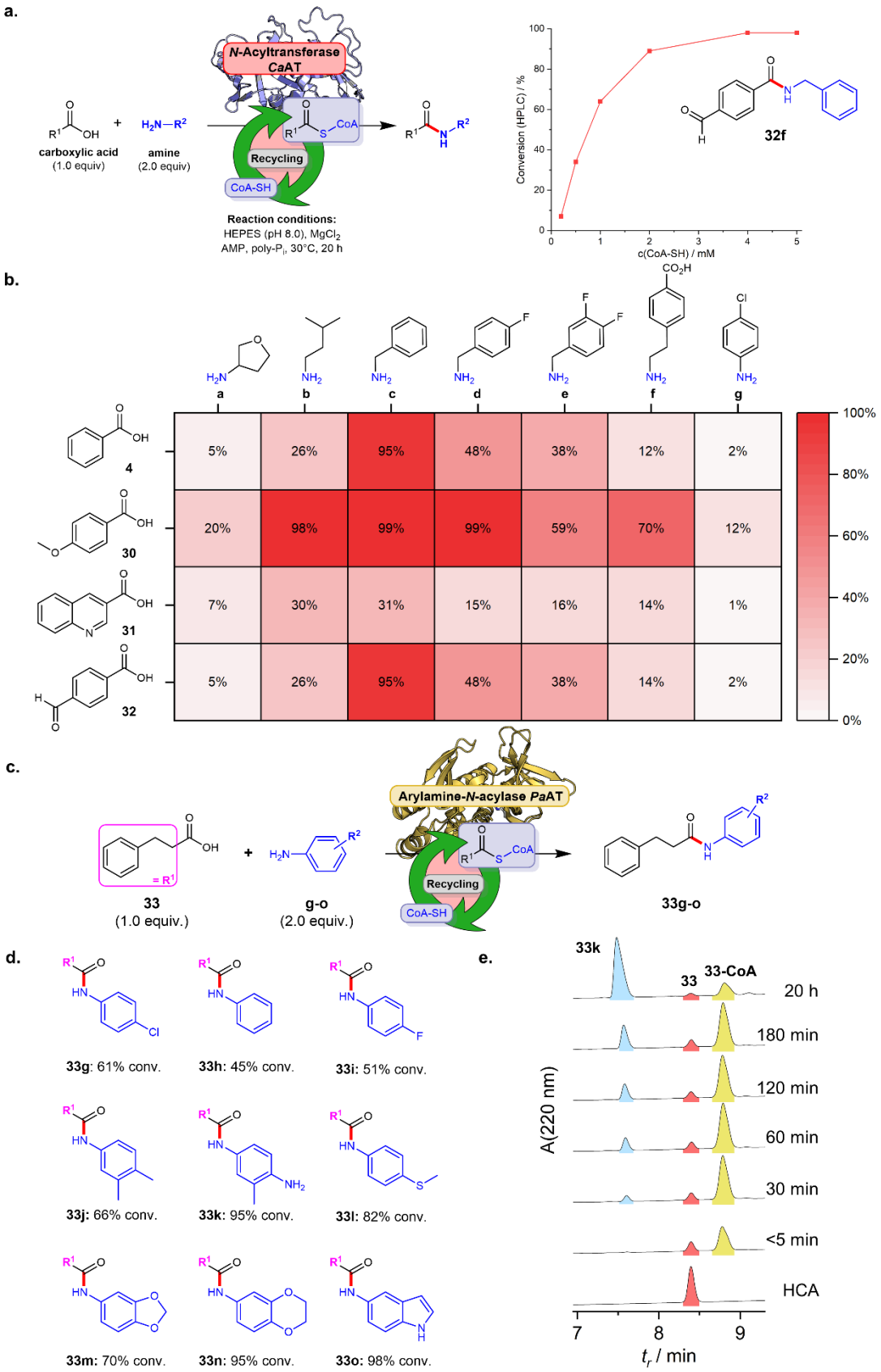

Figure 4. (a) Biocatalytic formation of amides by combining *N*-acyltransferases with CAR*sr*-A for the regeneration of acyl-CoA derivatives. (a) *Ca*AT/CAR*sr*-A/CHU-catalysed transformation of 4-formylbenzoic acid (**32**) (5 mM) and benzylamine (**f**, 10 mM) into amide **32f** independent of CoA-SH concentration. (b) Contour plot of substrate profile of *Ca*AT (conversions determined by UPLC-UV/MS) combined with CAR*sr*-A's regeneration of acyl-CoA. (c) *N*-acylation of arylamines catalysed by *Pa*AT along with *in-situ*-supply of acyl-CoA. (d) Acylation of different arylamines using 3-phenylpropionic (**33**) under optimised conditions. (e) Time progress of the formation of amide **33k** analysed by HPLC-UV.

Reaction conditions (a-e): 5 mM Carboxylic acid, 10 mM amine **a**-**o**, 2 mM CoA-SH, 10 mM AMP, 20 mg mL^{-1} sodium hexametaphosphate, Enzymes (CFE): 5 mg mL^{-1}, CAR*sr*-A, 5 mg mL^{-1}, *Ca*AT (a / b) or *Pa*AT (c / d), 2 mg mL^{-1} CHU, 100 mM $MgCl_2$, 100 mM HEPES (pH 8.0). Reactions were analysed by UPLC-UV/MS after incubation at 30°C for 20h.

Formation of small-molecule amides applying *in-situ*-CoA-SH recycling

N-Acyltransferases (ATs) catalyse amide bond formations by transferring the activated acyl group of acyl-S-CoA onto amines. In previous work, pairs of CoA ligases and ATs had to be identified in order to catalyse a specific amidation, which can become a laborious procedure.[11] The broad substrate scope of CAR*sr*-A allowed its use as an all-round acyl-CoA synthetase together with amide bond-forming ATs. To demonstrate its impact, we particularly focused on more challenging amine substrates that had been less successful previously. Initially, plant-derived tyramine-*N*-hydroxycinnamoyl acyltransferase, *Ca*AT, was chosen as a representative example for combination in one-pot reactions with CAR*sr*-A.[34] Using CFEs of *Ca*AT, along with CAR*sr*-A and CHU, the amidation of 5 mM 4-formylbenzoic acid (**32**) with benzylamine (**c**) (10 mM, 2.0 equiv) (Figure 4A) in the presence of CoA-SH (**1**) gave the desired amide **32c** in quantitative conversion, demonstrating full compatibility of all components of the *in situ* recycling system. Given the cost of CoA-SH (**1**), the scope for using sub-stoichiometric amounts of **1** was examined. By applying this reaction setup formation of amide **32c** showed little decrease of conversion down to 2 mM of CoA-SH (**1**) while keeping acid and amine concentration constant. Furthermore, amide **32c** was synthesised on a small preparative scale (5 mM acid, 30 mL reaction volume) to confirm the presence of the desired amide by NMR upon isolation and purification by semi-preparative HPLC (Supplementary Data). Next, the scope of the regeneration system with a range of enzymatic acylations was explored using different substrates and ATs. With CAR*sr*-A / *Ca*AT a broad range of aryl carboxylic acids led to moderate to high conversions with amine **c** (Supplementary Figure 7). Specifically, the *para*-substituted benzoic acids such as **30** and **32** turned out to be the more preferred substrates. With regard to the amine scope, *Ca*AT exhibited a preference for bulkier aliphatic amines and benzylamines that are usually difficult to couple in aqueous solution such as electron-poor amines **e** and **f** (Figure 4b). NMR analysis of a scaled-up reaction of **30** with bifunctional substrate **f** showed that the latter compound exclusively functions as an amine in the cascade. As CAR*sr*-A does not catalyse formation of its thioester, **38f** only gave one constitutional isomer without the need for protecting groups.

Very few enzymatic approaches are currently known to catalyse acylation of anilines due to poor nucleophilicity and low solubility of arylamines compared to aliphatic amines. The amide ligase McbA was reported to accept aniline (**h**) and 2,4-dimethylaniline (**j**) albeit in low substrate concentrations and with a limited acid scope.[21,22] Alternatively, arylamine-*N*-acyltransferases adopt an important role in detoxification processes by catalysing acetylation of anilines using acetyl-S-CoA. However, difficulties accessing other acyl thioesters have restricted the synthetic use of these enzymes,[35] an issue that we sought to address using CAR*sr*-A with the previously described acyltransferase *Pa*AT (Figure 4c).[11] A range of anilines, including electron-poor as well as more sterically demanding amines, was studied. Promising conversions from 45 to 98% were achieved without further optimisation of catalyst loading and amine equivalents (Figure 4d). Even the more electron-deficient 4-fluoroaniline (**i**) was acylated in an acceptable conversion, whereas electron-rich and bulkier amines turned out to be even more preferred. Preparative-scale synthesis of **33k** and **33m** enabled to confirm the identity of the isolated amides by NMR. For instance, diamine **k** was exclusively acylated in the sterically better accessible C4 position. The time course analysis of the cascade demonstrated that the thioester intermediate **14** formed quickly after reaction initiation, leading to a maximum concentration within the initial 60 min (Figure 4e). Amide **33k** could be clearly detected after 30 min and continuously accumulated thereafter. Apart from **33** a range of other carboxylic acids was accepted by *Pa*AT in the cascade (Supplementary Figure 8). Yet we observed that *Pa*AT was a more reluctant catalyst towards benzoic acid (**7**) and related carboxylic acids. In light of the broad substrate scope of CAR*sr*-A for these compounds, it can be anticipated that engineering of *Pa*AT will help to resolve this bottleneck and thus broaden the scope of the modular system. In analytical-scale reactions, the substrate loading was further increased to 40 mM carboxylic acid along with 80 mM amine: As exemplified for the formation of amide **33m**, a conversion of 61% resulted, which further reduced the CoA-SH loading to 5 mol% (Supplementary Figure 9). These data point towards the application of this modular cascade that implements recycling of non-natural acyl-S-CoA, *e.g.*, to provide amide building blocks for pharmaceutical synthesis.

Non-native acylation of histone H4 peptide

Having demonstrated the applicability of acyl-S-CoA regeneration by CAR*sr*-A in small molecule synthesis, we envisaged establishing its use with lysine acetyltransferases (KATs), which are of great interest for modulating gene expression through acetylation and acylation of histone tails using acyl-S-CoA.[36,37] Apart from histone, manifold other cellular proteins have been shown to be subject to post-translational *N*-,[38–40] *O*-,[41,42] and *S*-acetylation.[43] In particular, misregulation of KATs leads to a variety of diseases such as cancer[44] or neurological disorders.[45] Identification of new protein substrates has been facilitated by the incorporation of bioorthogonal handles and labelling through the use of chemical biology tools.[32,46–49] However, this is often hampered by difficulties in accessing acyl-S-CoA derivatives for non-natural bioorthogonal substrates.[25,50] Therefore, we envisioned exploiting CAR*sr*-A to generate CoA-SH thioesters *in situ* along with modification of potential peptide targets by KAT p300. Therefore, a 20-mer peptide derived from the N-terminal segment of histone H4 (H4-20, **34**) was chosen as a model substrate for acetylation using KAT p300 in a one-pot reaction (Figure 5a). By making use of the acyl-S-CoA regeneration system employing CAR*sr*-A along with CoA-SH and ATP, isotopically labelled [$^2H_3$]-acetyl-S-CoA was generated *in situ* from [$^2H_3$]-acetic acid (**38**). MALDI-TOF analysis showed that **34** was modified *via* [$^2H_3$]-acetyl-S-CoA acetylating up to five lysine residues, hence yielding the labelled peptide **38a**-**e** in agreement with previous studies using acetyl-S-CoA directly (Figure 5b; Supplementary Information).[51,52]

Furthermore, CAR*sr*-A and p300 could be harnessed to acylate peptide **34** with bioorthogonal chemical reporters directly from commercially available acids. Consequently, CAR*sr*-A was employed to generate these substrates *in situ*, allowing one-pot

functionalisation of **34** using p300 (Figure 5b). Starting from 4-pentynoic acid (**39**), the peptide was acylated at up to four lysine residues, resulting in **39a-d**. Up to five acylations were observed when 3-azidopropionic acid (**40**) was employed, correlating with detection of **40a-e** by MALDI-TOF in agreement with p300 activities observed in previous studies.[46] In conclusion, these results show that CAR*sr*-A can become a valuable tool to study epigenetic acylation processes providing easy access to thioesters for heavy atom labelling and bioorthogonal *in-situ* labelling techniques directly using commercially available carboxylic acids.[53]

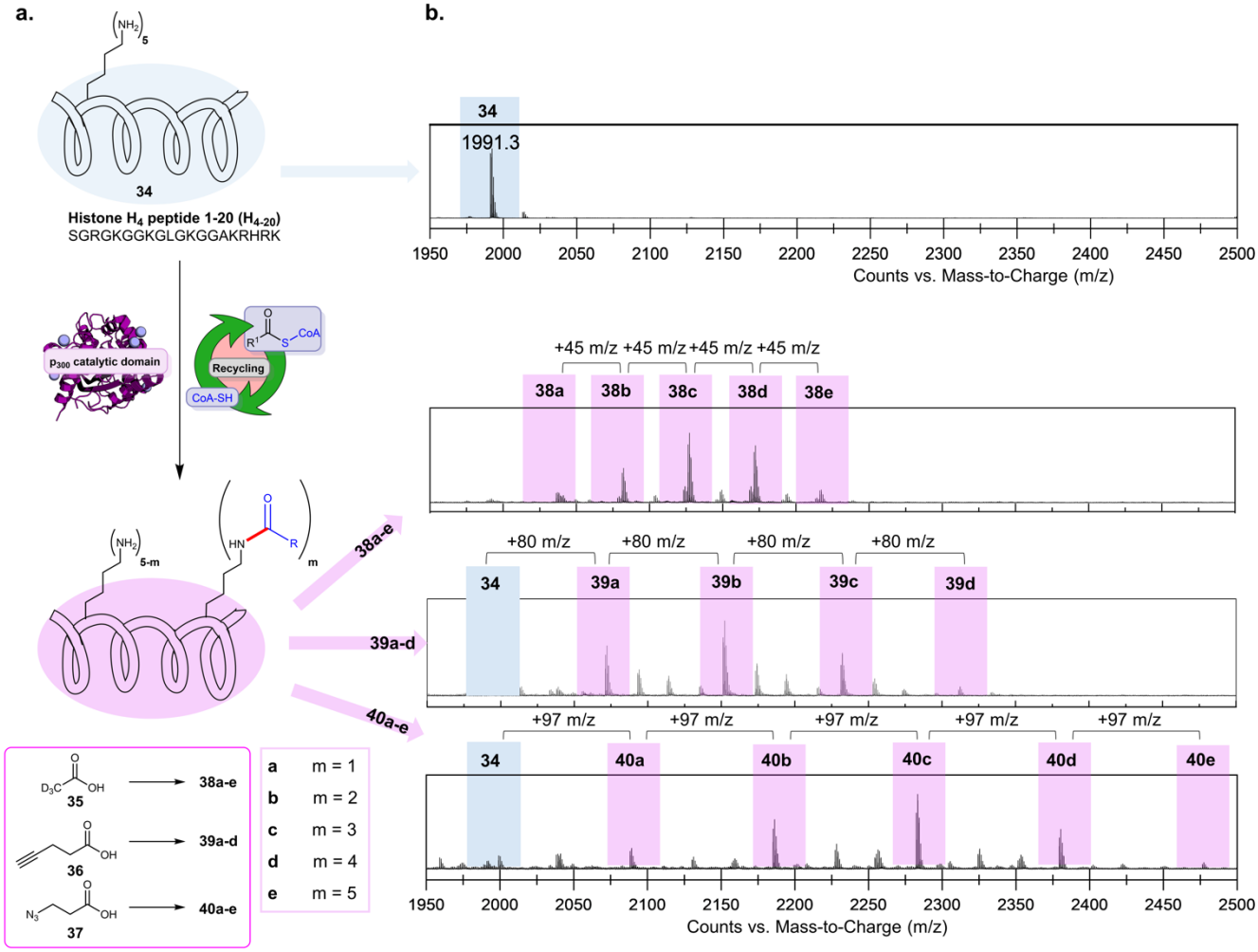

Figure 5. (a) Enzymatic acylation of histone H4 peptide (1-20) **34** with different carboxylic acids (**35-37**) using the catalytic domain of KAT p300 along with acyl-CoA generation by CAR*sr*-A. (b) MALDI-TOF MS spectra (positive ionisation) of different biotransformations using p300 and CAR*sr*-A. The increase in *m/z*-ratios between signals corresponding to the expected increase due to acylation is indicated on top of the spectra. Complete peak assignment can be found in the Supplementary Information. Reaction conditions: 0.1 mM peptide **34**, 5 mM acid **35-37**, 0.5 mM CoA-SH, 1 mM $MgCl_2$, 5 mM ATP, 35 μM CAR*sr*-A, 11 μM p300, 50 mM HEPES (pH 8.0). Reactions were analysed by MALDI-TOF MS upon incubation at 37 °C overnight.

Discussion and Conclusion

In summary, we have shown that the CAR*sr* A-domain can be used as a robust, generic thioester synthetase, particularly for acyl-S-CoA and acyl-pantetheine derivatives. The enzyme offers a broad substrate scope towards carboxylic acids and can be applied for *in-situ*-regeneration of acyl-S-CoA in one-pot enzymatic acylation reactions using diverse acyltransferases and starting from easily accessible carboxylic acids. The expensive cofactor CoA-SH can be used in sub-stoichiometric amounts (down to 5 mol%), and combination with an ATP-regeneration system makes scale-up of amidations feasible. The system was demonstrated to be applicable both for small molecule amide targets and side-chain acylated histone peptides using the epigenetic 'writer' KAT p300.[25] In addition to natural acylation, unnatural acyl chains, including isotopic labels and bioorthogonal handles, could be introduced into the peptides, which will provide tools for analysis of epigenetic processes. This *in-situ*-recycling system makes thioester intermediates accessible as biochemical tools and intermediates in biocatalysis and should stimulate the exploitation of the plethora of acyl-S-CoA dependent reactions in biology beyond acylation reaction described here.

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Author contributions

S.L.F., N.J.T, M.A.H., K.M., and F.F. managed and supervised the project. S.L.F, N.J.T., M.A.H., and C.S. devised the concept. C.S. performed cloning, biocatalyst production, enzyme assays, and thioester scope studies. C.S. undertook and optimised cascade reactions and screenings. L.R.P. and A.A. carried out KAT reactions and MALDI-TOF analysis. Y.Y. performed bioinformatics. C.S. and A.A. synthesised and purified biotransformation products. C.S. and M.L. designed A-domain and did initial activity tests. C.S. and R.S.H. carried out enzyme purifications. C.S., S.L.F., L.R.P., N.J.T., and Y.Y. wrote the manuscript and generated the figures.

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