One-pot preparation of coenzyme A analogues *via* an improved chemo-enzymatic synthesis of pre-CoA thioester synthons†

Marianne van Wyk and Erick Strauss*

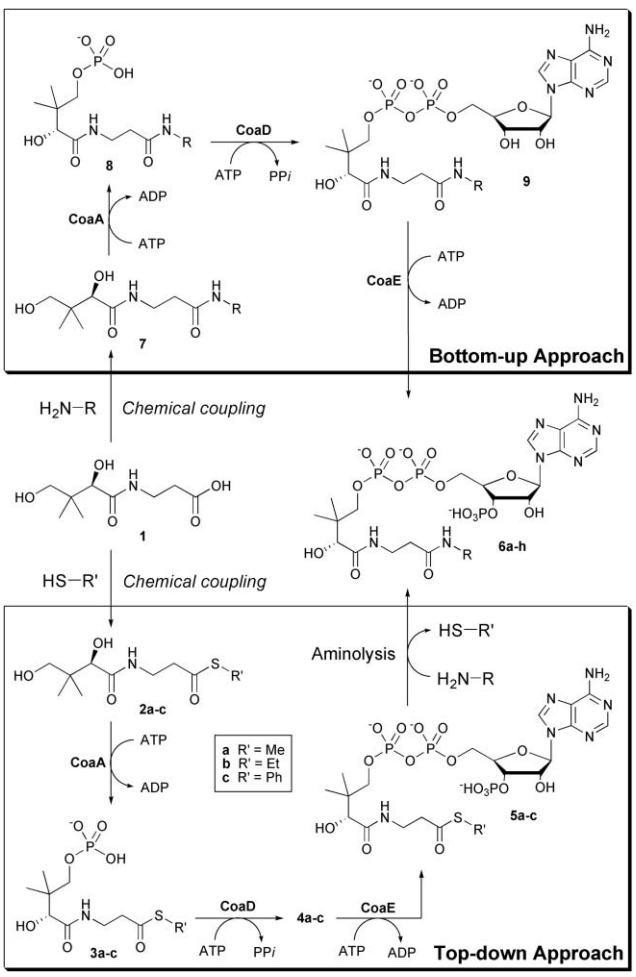
Received (in Cambridge, UK) 18th September 2006, Accepted 24th October 2006
First published as an Advance Article on the web 14th November 2006
DOI: 10.1039/b613527g

Coenzyme A analogues are synthesized in a one-pot preparation by biotransformation of pantothenate thioesters through the simultaneous use of three CoA biosynthetic enzymes, followed by aminolysis.

Analogues of the acyl group-carrying cofactor coenzyme A (CoA) have been widely used as mechanistic probes or as inhibitors of enzymatic activity.[1] Recently their utility has expanded even further through the demonstration that analogues of CoA, which normally serve as the source of the 4′-phosphopantetheine prosthetic group of acyl carrier proteins (ACPs), are also accepted as alternate substrates by promiscuous phosphopantetheinyl transferase (PPTase) enzymes.[2] This allows the preparation of so-called *crypto*-ACPs in which the normal pantetheine prosthetic group is replaced by a variety of pantothenate-based analogues; these may subsequently serve as alternate substrates for mechanistic studies, as fusion protein tags, or as reporter labels.[3]

The biggest obstacle to the widespread use of CoA analogues remains the tedious synthetic preparation of the analogue itself.[1] A major advancement in this regard was made when it was shown that the adenylylation and final phosphorylation steps of the synthesis can be performed by the last two enzymes of the CoA biosynthetic pathway (CoaD and CoaE); this allowed the preparation of CoA analogues **6** using almost any phosphopantetheine analogue **8** as substrate (Scheme 1).[1,4] Recent developments have also seen the introduction of the first kinase in the pathway (pantothenate kinase, CoaA) to the method to perform the problematic initial phosphorylation reaction.[5] However, since CoaA acts as the rate-limiting enzyme in CoA biosynthesis through its feedback inhibition by CoA itself,[1] chemo-enzymatic preparations that make use of this enzyme require the sequential application of the three biosynthetic enzymes to limit the negative effect this inhibition may have on yield.

The greatest drawback of the methods outlined above lies in their bottom-up approach: each CoA analogue must be prepared by the chemo-enzymatic transformation of the corresponding pantetheine analogue, which is usually prepared synthetically.[6] In contrast, a preferred method would allow the preparation of CoA analogues in a top-down manner from a single common precursor, ideally in a single step (Scheme 1). Such an approach was first developed by Drueckhammer and co-workers, who used a single CoA thioester analogue (**5**, R′ = Pr) to prepare CoA and acetyl-CoA analogues through aminolysis reactions with various amines.[7] Although this methodology offered significant advantages, it also had some shortcomings: first, the chemo-enzymatic preparation of the CoA thioester analogue was a low yielding process, mostly due to the chemical synthesis of the phosphopantothenate thioester precursor (**3**, R′ = Pr); and second, the propyl thioester of the analogue was relatively stable towards aminolysis, requiring a large excess of (often costly) amine to drive the reaction to completion.



Scheme 1 Chemo-enzymatic preparation of CoA analogues *via* biotransformation of pantothenamides **7** (bottom-up approach) or *via* aminolysis of pre-CoA thioester synthons **5** (top-down approach). CoaA: pantothenate kinase; CoaD: phosphopantetheine adenylyltransferase; CoaE: dephospho-CoA kinase.

Department of Chemistry, Stellenbosch University, Matieland, 7602, South Africa E-mail: estrauss@sun.ac.za; Fax: +27-21-808-3360
† Electronic supplementary information (ESI) available: Detailed experimental methods, and analytical data (NMR spectra and LC-MS traces). See DOI: 10.1039/b613527g

We set out to address these shortcomings by establishing an improved method for the top-down synthesis of CoA analogues using the method of Drueckhammer as our starting point. To address the low overall yield of the preparation we decided to investigate the feasibility of the enzymatic phosphorylation of the thioester precursors of the CoA thioester analogues **5** (which we will refer to as pre-CoA synthons). To increase the reactivity of the original propyl thioester analogue toward aminolysis we prepared the equivalent but sterically less hindered methyl and ethyl thioesters, as well as the activated phenyl thioester. However, since the aminolysis reactions are performed in aqueous solutions, we were keenly aware that these changes might also increase the rate of hydrolysis of the thioester, a competitive side reaction that takes place under these conditions.[8] Our aim was thus to find a balance between low reactivity and a high rate of hydrolysis.

The *S*-methyl (**2a**), *S*-ethyl (**2b**) and *S*-phenyl (**2c**) thiopantothenate precursors were subsequently all successfully synthesized in a simple one-step thioesterification procedure using unprotected pantothenic acid as starting material.‡

Our initial investigation of the enzymatic phosphorylation of the pantothenate thioesters **2a–c** was focused on making use of the CoaA enzyme from *Escherichia coli* (*Ec*CoaA). However, when this enzyme was assayed for its ability to catalyze the ATP-dependant phosphorylation of its native substrate pantothenate **1** compared to the thioesters **2a–c**, no evidence of kinase activity with the unnatural substrates was found (results not shown). This was surprising, since *Ec*CoaA is known to accept a variety of pantothenamides as alternate substrates.[9,10] However, when the same analysis was performed using the recently characterized CoaA protein from *Staphylococcus aureus* (*Sa*CoaA) the enzyme exhibited activity towards all the substrates with specificity constants within an order of magnitude of that of the natural substrate (Table 1). This result was particularly encouraging, not only because it confirmed *Sa*CoaA's lack of substrate specificity, but also because this particular pantothenate kinase enzyme has been shown to be refractory towards feedback inhibition by CoA.[11] This specific trait suggested that *Sa*CoaA could be used simultaneously with the CoaD and CoaE enzymes in the biotransformation reactions, without any concern about the impact it may have on yield. We set out to demonstrate that this was indeed the case, using the chemo-enzymatic preparation of the pre-CoA synthons **5a–c** as a model.

Pantothenate thioesters **2a–c** were subsequently treated with *Sa*CoaA in the presence of ATP and the reaction mixtures were analyzed by LC-MS for the formation of the respective 4′-phosphopantothenate thioesters **3a–c** as products of the phosphorylation reaction. This was followed by LC-MS analyses of the reaction mixtures of each of the thioesters **2a–c** in the presence of *Sa*CoaA, *Ec*CoaD (*i.e.* CoaD from *E. coli*) and ATP

Table 1 The kinetic parameters of *Sa*CoaA with pantothenic acid **1** in comparison to the pantothenate thioesters **2a–c** as substrates

Substrate	k_{cat}/s^{-1}	$K_M/\mu M$	$k_{cat}K_M^{-1}/mM^{-1}\ s^{-1}$
Pantothenic acid **1**	1.41	76.2 ± 18.5	18.5 ± 6.30
S-Methyl thiopantothenate **2a**	2.55	826.5 ± 108.4	3.09 ± 1.49
S-Ethyl thiopantothenate **2b**	2.39	1205.5 ± 347.2	1.99 ± 1.13
S-Phenyl thiopantothenate **2c**	0.98	936.8 ± 250	1.05 ± 0.53

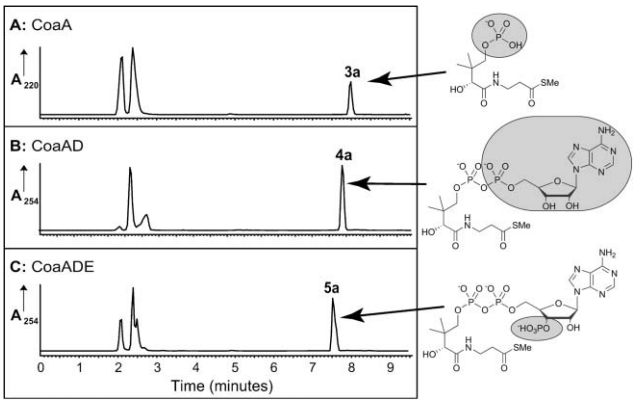
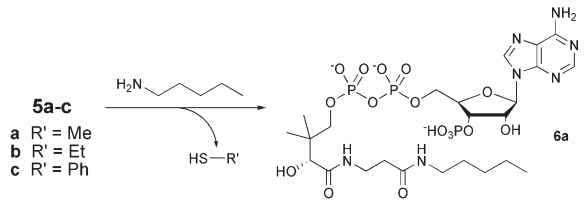


Fig. 1 Three-step biosynthesis of pre-CoA synthon **5a** from *S*-methyl thiopantothenate **2a** as monitored by HPLC. **A**: Reaction mixture of **2a** and *Sa*CoaA in the presence of ATP forms **3a**. **B**: Reaction mixture of **2a**, *Sa*CoaA, *Ec*CoaD and ATP forms **4a**. **C**: Reaction mixture of **2a**, *Sa*CoaA, *Ec*CoaD, *Ec*CoaE and ATP forms the pre-CoA synthon **5a**. The peaks between 2 and 3 min represent excess ATP and the ADP formed during the course of the reaction.

to confirm the formation of the 3′-dephospho-CoA analogues **4a–c**, and finally by analysis of reaction mixtures in which the thioester substrate, the three biosynthetic enzymes (*Sa*CoaA, *Ec*CoaD and *Ec*CoaE) and ATP are all incubated together to form the expected thioester synthons **5a–c**. Fig. 1 shows HPLC traces of these analyses for the methyl thioester as representative of the obtained results: panel A shows the formation of **3a** as a single product, panel B the formation of **4a**, and panel C the formation of the methyl pre-CoA synthon **5a**. Similar results were obtained for the ethyl and phenyl pre-CoA synthons, although longer incubation times were required in the case of the latter.§ LC-MS analyses were also used to determine if the thioesters are stable under these conditions; however, no hydrolysis products could be detected in any of the reaction mixtures.

Previous attempts at preparing CoA analogues by aminolysis of a relatively stable propyl pre-CoA synthon required a large excess of amine (up to 400 mol equivalents) and prolonged incubation times (up to 48 hours). These reactions were also performed at pH ~10, which increased the rate of the undesired hydrolytic cleavage of the pre-CoA synthon.[7c] In contrast, we decided to optimize the aminolysis conditions of the pre-CoA synthons by using no more than 10 equivalents of amine, an incubation time of less than 6 h and a reaction pH of 9. To determine which of the three pre-CoA synthons **5a–c** would completely be converted under these conditions, aminolysis reactions were initially performed using the simple primary amine, pentylamine (Scheme 2). This amine was chosen since we had previously prepared the expected CoA



Scheme 2 Aminolysis of pre-CoA synthons **5a–c** with pentylamine to form ethyldethia-CoA **6a**.

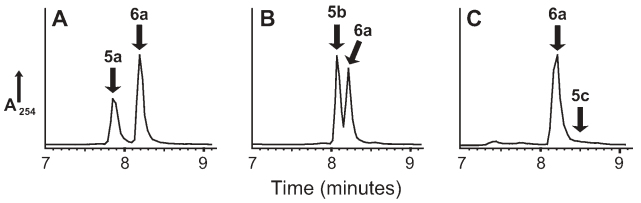
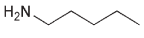
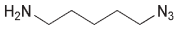
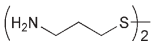
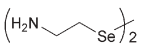
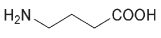
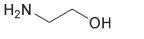


Fig. 2 Aminolysis of pre-CoA synthons **5a–c** with 10 equivalents pentylamine to form ethyldethia-CoA **6a**. Reaction mixtures (in 50 mM HEPES buffer, pH 9.0) were incubated for 6 h at 50 °C and analyzed by LC-MS. **A**: Aminolysis of **5a** shows partial conversion to **6a**. **B**: Aminolysis of **5b** shows partial conversion to **6a**. **C**: Aminolysis of **5c** shows its complete disappearance, and formation of **6a**.

Table 2 Aminolysis of the pre-CoA synthon **5c** with a variety of amines to form the corresponding CoA analogues **6a–h**

Amine	CoA analogue	Conversion[a] (%)
	Negative control	0 (100% hydrolysis)
	**6a**	78
	**6b**	85
	**6c**	58
	**6d**	94
	**6e**	>50[b]
	**6f**	>50[b]
Biotin–cadaverine	**6g**	>95
Dansylcadaverine	**6h**	>25[b,c]

[a] Conversion values relate the relative amount of aminolysis product (compared to hydrolysis) for the reactions performed at pH 9.0. [b] Overlap of the chromatographic signals representing the hydrolysis product and ATP/ADP prevented the accurate determination of the % conversion to these analogues. Instead, conversion was conservatively estimated by comparison to the other samples. See ESI† for details. [c] Low conversion to **6h** is presumed to be a result of the low aqueous solubility of dansylcadaverine, not because of poor aminolysis activity.[13]

analogue product (*i.e.* ethyldethia-CoA, **6a**) using the traditional bottom-up approach;[10] it was thus available for use as standard in the subsequent analyses.

The three pre-CoA synthons **5a–c** were subsequently prepared separately by incubating the appropriate pantothenate thioester precursor with *Sa*CoaA, *Ec*CoaD, *Ec*CoaE and ATP. Upon completion of the biotransformation the enzymes were removed, and 10 mol equivalents of pentylamine were added to the reaction mixture. The aminolysis reaction was allowed to proceed at 50 °C for 6 h before the mixtures were analyzed for the disappearance of the pre-CoA synthon and the formation of the expected product **6a**. The results, as summarized in Fig. 2, show that while all three pre-CoA synthons underwent some degree of aminolysis, only the activated phenyl thioester **5c** disappeared completely while forming the expected aminolysis product. We thus decided to use the phenyl pre-CoA synthon **5c** for all subsequent reactions.

We finally set out to demonstrate the general utility of the method by extending it to amines with diverse functional groups and of increasing complexity. While initial results showed the complete disappearance of **5c**, accompanied by formation of the expected aminolysis products, a control reaction in which the poor aminolysis substrate *tert*-butyl amine was used indicated that under these conditions the pre-CoA synthon may also undergo extensive hydrolysis. Consequently the effective conversion of **5c** in the presence of each amine was determined by integration of the peak areas of the hydrolysis and the aminolysis products, each identified by LC-MS analysis. The results show that while conversion is dependent on the functional groups in the amine's side chain, size and complexity are not necessarily impediments to the aminolysis reaction, as demonstrated by the successful preparation of the biotinylated analogue **6g** (Table 2). Other analogues that were similarly prepared are the azide-containing **6b** (ideally suited for "click" chemistry-based reporter labeling[12,13]), homocysteamine-CoA **6c**,[2b] seleno-CoA **6d**,[14] the transition-state analogue **6e**,[7c] oxy-CoA **6f**[4a] and the fluorescent-labeled **6h**.

In summary, we have presented a general method for the one-pot preparation of a diverse array of CoA analogues from a single activated pre-CoA thioester synthon. Owing to its simplicity and ease of use, this method should greatly aid the expansion of the increasing number of applications of CoA analogues.

Notes and references

‡ Thioesterification reactions were performed using pantothenic acid, the corresponding thiol (or its sodium salt) and diphenylphosphoryl azide or diethylphosphoryl cyanide as coupling agents. See ESI† for details.
§ The formation of the three pre-CoA synthons were subsequently confirmed by preparation of the thioesters on a preparative scale, followed by their purification on C_{18} solid phase extraction cartridges and ^{1}H NMR and HRMS analysis of the pure compounds. See ESI† for details.

1 P. K. Mishra and D. G. Drueckhammer, *Chem. Rev.*, 2000, **100**, 3283.
2 (*a*) J. J. La Clair, T. L. Foley, T. R. Schegg, C. M. Regan and M. D. Burkart, *Chem. Biol.*, 2004, **11**, 195; (*b*) A. M. Gehring, R. H. Lambalot, K. W. Vogel, D. G. Drueckhammer and C. T. Walsh, *Chem. Biol.*, 1997, **4**, 17.
3 (*a*) J. Yin, F. Liu, X. Li and C. T. Walsh, *J. Am. Chem. Soc.*, 2004, **126**, 7754; (*b*) B. N. Cook and C. R. Bertozzi, *Bioorg. Med. Chem.*, 2002, **10**, 829; (*c*) K. M. Clarke, A. C. Mercer, J. J. La Clair and M. D. Burkart, *J. Am. Chem. Soc.*, 2005, **127**, 11234; (*d*) A. S. Worthington and M. D. Burkart, *Org. Biomol. Chem.*, 2006, **4**, 44; (*e*) N. George, H. Pick, H. Vogel, N. Johnson and K. Johnson, *J. Am. Chem. Soc.*, 2004, **126**, 8896.
4 (*a*) M. Dai, Y. Feng and P. J. Tonge, *J. Am. Chem. Soc.*, 2001, **123**, 506; (*b*) E. Strauss and T. P. Begley, *ChemBioChem*, 2005, **6**, 284.
5 I. Nazi, K. P. Koteva and G. D. Wright, *Anal. Biochem.*, 2004, **324**, 100.
6 A. L. Mandel, J. J. La Clair and M. D. Burkart, *Org. Lett.*, 2004, **6**, 4801.
7 (*a*) K. W. Vogel and D. G. Drueckhammer, *J. Am. Chem. Soc.*, 1998, **120**, 3275; (*b*) D. P. Martin, R. T. Bibart and D. G. Drueckhammer, *J. Am. Chem. Soc.*, 1994, **116**, 4660; (*c*) R. T. Bibart, K. W. Vogel and D. G. Drueckhammer, *J. Org. Chem.*, 1999, **64**, 2903.
8 (*a*) W. Yang and D. G. Drueckhammer, *Org. Lett.*, 2000, **2**, 4133; (*b*) W. Yang and D. G. Drueckhammer, *J. Am. Chem. Soc.*, 2001, **123**, 11004.
9 K. G. Virga, Y.-M. Zhang, R. Leonardi, R. A. Ivey, K. Hevener, H.-W. Park, S. Jackowski, C. O. Rock and R. E. Lee, *Bioorg. Med. Chem.*, 2006, **14**, 1007.
10 E. Strauss and T. P. Begley, *J. Biol. Chem.*, 2002, **277**, 48205.
11 R. Leonardi, S. Chohnan, Y.-M. Zhang, K. G. Virga, R. E. Lee, C. O. Rock and S. Jackowski, *J. Biol. Chem.*, 2005, **280**, 3314.
12 R. Manetsch, A. Krasinski, Z. Radic, J. Raushel, P. Taylor, K. B. Sharpless and H. C. Kolb, *J. Am. Chem. Soc.*, 2004, **126**, 12809.
13 J. L. Meier, A. C. Mercer, H. Rivera, Jr. and M. D. Burkart, *J. Am. Chem. Soc.*, 2006, **128**, 12174.
14 J. Seravalli, W. Gu, A. Tam, E. Strauss, T. P. Begley, S. P. Cramer and S. W. Ragsdale, *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 3689.