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Structure–activity relationships and enzyme inhibition of pantothenamide-type pantothenate kinase inhibitors

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Abstract—A set of novel pantothenamide-type analogues of the known *Staphylococcus aureus* pantothenate kinase (*Sa*PanK) inhibitors, *N*-pentyl, and *N*-heptylpantothenamide, was synthesized in three series. The first series of analogues (**1**–**3**) were designed as molecular probes of the PanK binding site to elucidate important structure–activity relationships (SAR). The second series of analogues (**4**–**16**) were designed using structural information obtained from the *Escherichia coli* PanK (*Ec*PanK) structure by targeting the pantothenate binding site and the adjacent phenylalanine-lined lipophilic pocket. Insight into the antimicrobial effect of *N*-pentylpantothenamide (N5-Pan) through its conversion to the antimetabolite ethyldethia-CoA and further incorporation into an inactive acyl carrier protein analogue drove the development of the third series of analogues (**17**–**25**) to enhance this effect using substrate-like substitutions. Each of the analogues was screened for enzyme inhibition activity against a panel of pantothenate kinases consisting of *Ec*PanK, *Aspergillus nidulans* (*An*PanK), *Sa*PanK, and the murine isoform (*Mm*PanK1α). Series 1 demonstrated only modest inhibitory activity, but did reveal some important SAR findings including stereospecific binding. Series 2 demonstrated a much higher inhibition rate for the entire series and significant inhibition was seen with analogues containing alkyl substituents. Series 3 demonstrated the most preferential inhibition profile, with the highest inhibitory activity against the *Sa*PanK and *Mm*PanK1α. The *Mm*PanK1α protein was inhibited by a broad spectrum of the compounds, whereas the *E. coli* enzyme showed greater selectivity. The overall activity data from these analogues suggest a complex and non-enzyme specific SAR for pantothenamide substrate/inhibitors of the different PanK enzymes.

1. Introduction

Increasing bacterial resistance to commonly used antibiotics is a great concern for the healthcare industry.[1] Infections caused by resistant bacterial strains, such as MRSA (methicillin-resistant *Staphylococcus aureus*), VRE (vancomycin-resistant enterococcus), and MDRTB (multi-drug resistant tuberculosis), have led to greatly increasing morbidity and mortality rates.[2] This has led to an urgent need to develop new, more potent antimicrobial agents with novel mechanisms of action.[3] These efforts have been greatly enhanced in recent years with the advances in structural biology, genomics, and high-throughput screening techniques, which have uncovered multitudes of potential targets.[4] Coenzyme A (CoA) biosynthesis is one pathway that has garnered much interest recently as a potential target for antimicrobial therapy.[5–7] CoA is the major acyl group carrier in biology and is an essential cofactor in intermediary metabolism.[8] CoA is assembled in a five-step pathway starting with pantothenic acid (vitamin B_5). Pantothenate kinase (PanK, the product of the *coaA* gene) catalyzes the first and regulatory step in the CoA biosynthetic pathway.[8,9] A comparison of the prokaryotic and eukaryotic PanK protein sequences reveals a very low sequence homology, leading to the hypothesis that selectivity might be possible in the development of small molecule inhibitors.[7,10]

The *N*-alkylpantothenamide class of antibacterials was first reported in 1970 as active against *Escherichia coli*,[13]

Keywords: Pantothenate kinase; Pantothenamide; Fatty acid synthase; Structure based drug design; Coenzyme A.
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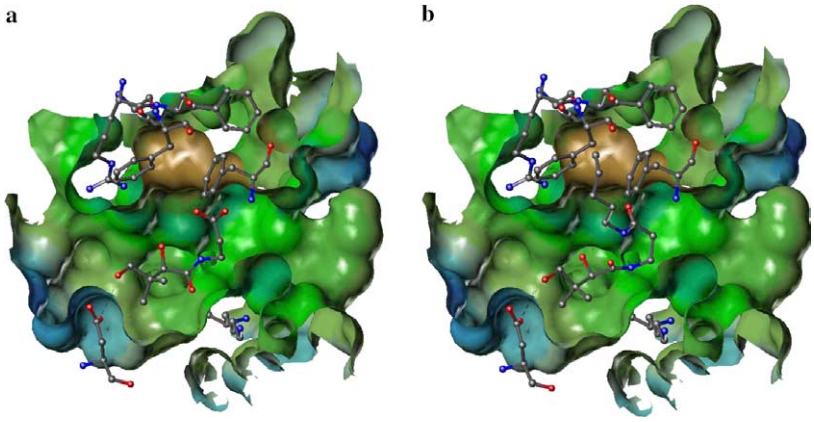


Figure 1. Cross-sectional view with MOLCAD surface of the *Ec*PanK enzyme binding pocket. Areas in brown are hydrophobic. Areas in blue are hydrophilic. And areas in green are neutral. (a) Surface of the *Ec*PanK binding site with pantothenate bound. The hydrophobic pocket (brown) extends just beyond the acid functional group of the pantothenate. (b) *N*-Pentylpantothenamide docked into the active site of *Ec*PanK. The 5-carbon alkyl chain is predicted to extend into the hydrophobic pocket. There are two phenylalanine amino acid moieties in the pocket available for possible ring stacking interaction with aromatic functional groups.

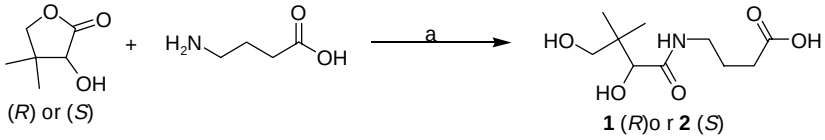
and their inhibitory activity attributed to their ability to be phosphorylated by PanK and incorporated by downstream enzymes into inactive CoA analogues.[14] Recently, these pantothenic acid analogues (**4** and **5**) were reported to be potent inhibitors of the *S. aureus* PanK;[7] however, subsequent research demonstrated that these antimetabolites are substrates for the enzyme. Furthermore, the antibacterial action of N5-Pan and N7-Pan is due to their incorporation into acyl carrier protein (ACP) and the subsequent inhibition of fatty acid biosynthesis.[6,15] Using the new binding information obtained from the latest structure for the *E. coli* PanK (Fig. 1A), N5-Pan was docked into the active site (Fig. 1B) providing a basis for the design of new inhibitors/substrates for the enzyme.[12] The aim of this work was to build on this information to develop an SAR model for PanK inhibitor/substrates, with the goal of achieving selectivity and understanding the basis for their antibacterial activity.

2. Results and discussion

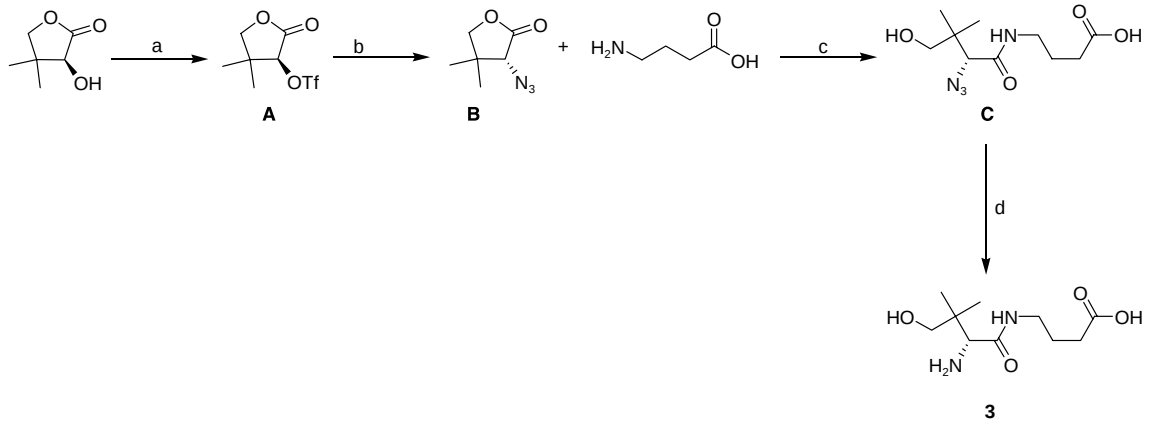
2.1. Chemistry

The synthesis of the two Ho-pantothenate (HoPan) isomers (**1** and **2**) was adapted from Kopelevich et al.[17] The syntheses of **1** and **2** were performed by the reaction of pantolactone (*R* or *S*) with 4-aminobutyric acid in the presence of diethylamine (Scheme 1). The purification of the reaction mixtures was performed by aqueous work-up to remove unreacted pantolactone and ion exchange chromatography using an Amberlite® IR-120 (H+) ion exchange column to convert the products to the free-acid form. The final products were then converted to their Ca^{2+} salts. The synthesis of compound **3** and its intermediates (**A**–**C**) was adapted from the procedure of Freskos and is detailed under the compound procedures (Scheme 2).[18]

The synthesis of the *N*-alkylpantothenamides (series 2 and 3) was adapted from the procedure of Choudhry et al.[7] Included in the series were the two known inhibitors **4** and **5**. The analogues of series 2 (**4**–**16**) were designed to probe the phenylalanine-lined hydrophobic pocket of the enzyme, which extends just beyond the acid moiety of the pantothenate binding site in *Ec*PanK. The aromatic amines (**6**–**10** and **12**) of series 2 were selected for potentially favorable ring stacking interactions between two phenylalanine residues within the hydrophobic pocket of the binding site (Table 1 and Fig. 1B). The analogues of series 3 (**17**–**25**) were designed as antimetabolite compounds to resemble the pantetheine moiety of the 4-phosphopantetheine product which is produced further down in the CoA pathway as well as the analogue ACP products demonstrated by **4** and **5**.[6,14,15] The functional groups chosen consisted of ethers, thioethers, and free hydroxyls and were intended to mimic the biosynthetic intermediates produced in the fatty acid synthase pathway. The reactions were performed under inert conditions in discrete reaction vessels. The free-acid form of pantothenate used in each of the reaction schemes was obtained by dissolving sodium pantothenate in methanol and subsequently passing through an Amberlite® IR-120 ion exchange column. The pantothenate was then dried

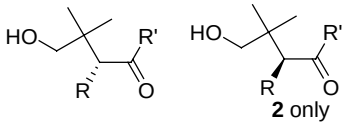


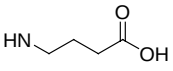
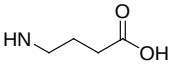
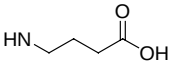
Scheme 1. Synthesis of **1** and **2**. Reagents and conditions: (a) triethylamine, methanol, 60 °C, overnight.



Scheme 2. Synthesis of **3**. Reagents and conditions: (a) trifluoro-methanesulfonic anhydride, 2,6-lutidine, DCM, −20 °C, 1 h; (b) NaN_3, DMF, 0 °C, 2 h; (c) triethylamine, methanol, 60 °C, overnight; (d) NH_4OH, pyridine, PPh_3, 6 h, ambient temperature.

Table 1. Percent of enzyme inhibition for series 1 analogues against the different PanK enzymes

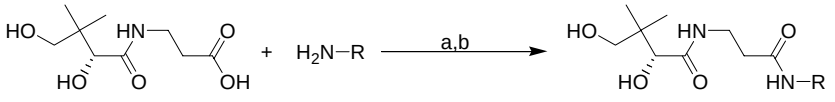


Compound	R	R′	% Inhibition at 100 μM			
			*Ec*PanK	*An*PanK	*Mm*PanK1α	*Sa*PanK
1	OH		29.4 ± 0.1	80.6 ± 0.97	2.7 ± 0.9	78.7 ± 6.0
2	OH		12.5 ± 4.3	0	0	54.6 ± 1.7
3	NH_2		11 ± 1.6	0	13.1 ± 1.6	0

under high heat and vacuum to complete dryness. The *N*-alkylpantothenamides were then prepared by reaction of the free-acid pantothenate with each of the corresponding amines in the presence of diphenylphosphoryl azide and triethylamine (Scheme 3). The amines used to form the terminal amide were hand-selected based on chemical structure and variability. For compounds **4**–**16**, each of the reaction mixtures was purified by flash chromatography. For the purification of the more polar compounds (**17**–**25**), each of the reaction mixtures was first passed over a freshly activated Amberlite® IR-400 basic ion exchange column to scavenge the diphenylphosphoric acid byproduct, as it eluted very closely with each of the desired products by TLC. Each of the compounds was then purified by flash chromatography.

3. Biology

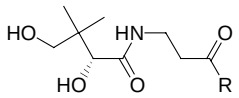
PanKs are found in all species, although their primary structures have significant differences. We selected the panel of PanKs for our analysis to cover the types of PanKs found in nature. The prototypical bacterial PanKs, typified by the *coaA* gene product (*Ec*PanK), are clearly distinct from the eukaryotic enzymes typified by the PanKs from *Aspergillus* (*An*PanK) and the mouse (*Mm*PanK1α). The enzyme from *S. aureus* (*Sa*PanK) is different from both, although it is more closely related to the metazoan enzymes than the *E. coli* enzyme. Each of the analogues was screened for inhibitory activity against the panel of PanK enzymes as determined by a decrease in enzymatic activity demonstrated by each enzyme's ability to convert pantothenate into 4′-phosphopantothenate.

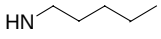
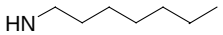
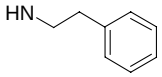
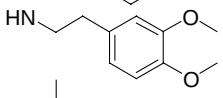
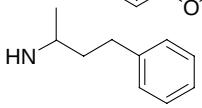
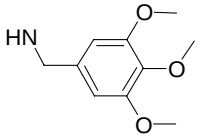
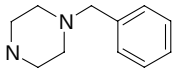
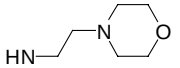
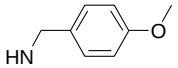
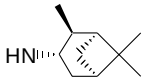
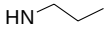
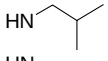
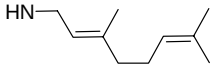
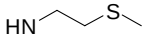
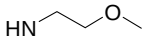
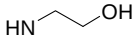
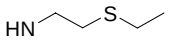
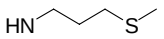
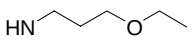
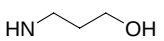
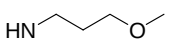
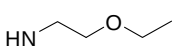


Scheme 3. Synthesis of **4**–**25**. Reagents and conditions: (a) diphenylphosphoryl azide, DMF, 0 °C; (b) triethylamine, 24 h.

In the initial series of pantothenamide-type analogues (**1**–**3**), Ho-pantothenate (HoPan), a previously reported pantothenate analogue, was synthesized in both the pure (*R*,**1**) and (*S*,**2**) isomeric forms as a starting point. The HoPan analogue contains a γ-aminobutyric acid functionality in place of the β-alanine functionality of the

Table 2. Percent of enzyme inhibition for series 2 and 3 analogues against the different PanK enzymes



Compound	R	% Inhibition at 100 μM			
		*Ec*PanK	*An*PanK	*Mm*PanK1α	*Sa*PanK
4		72.2 ± 0.7	12.6 ± 0.3	81.4 ± 1.1	98.8 ± 0.1
5		76.4 ± 0.01	10.7 ± 4.3	89.6 ± 0.6	98.2 ± 0.1
6		57.8 ± 0.6	59.5 ± 0.3	80.9 ± 0.6	89.5 ± 2.0
7		44.2 ± 0.7	35.7 ± 0.8	70.5 ± 0.3	17.7 ± 9.1
8		71.7 ± 0.2	40.7 ± 1.3	84.6 ± 2.5	86.4 ± 0.9
9		33.4 ± 7.1	28.4 ± 1.0	57.4 ± 1.9	8.7 ± 8.0
10		7.3 ± 7.6	15.9 ± 0.9	73.0 ± 0.5	9.3 ± 1.8
11		10.7 ± 6.8	13.7 ± 2.0	39.1 ± 0.2	4.2 ± 7.4
12		55.7 ± 2.5	54.7 ± 0.1	82.2 ± 0.5	53.9 ± 3.6
13		3.01 ± 2.9	71.9 ± 0.6	93.3 ± 0.3	76.6 ± 0.6
14		82.5 ± 4.4	51.4 ± 4.5	86.8 ± 0.3	99.1 ± 0.1
15		71.2 ± 0.04	46.2 ± 3.0	89.9 ± 0.6	97.1 ± 0.01
16		90.6 ± 0.4	92.2 ± 0.1	98.9 ± 0.2	97.2 ± 0.1
17		61.9 ± 5.0	49.7 ± 0.5	85.0 ± 0.01	96.5 ± 0.3
18		28.7 ± 2.6	26.8 ± 3.2	67.6 ± 0.1	90.1 ± 0.2
19		3.4 ± 10.6	25.9 ± 2.1	56.7 ± 2.4	72.2 ± 0.4
20		53.7 ± 1.2	61.0 ± 1.9	82.9 ± 0.4	97.6 ± 0.1
21		54.3 ± 3.8	72.6 ± 0.4	87.2 ± 1.9	97.7 ± 0.01
22		32.3 ± 2.6	62.1 ± 0.1	61.2 ± 0.04	96.2 ± 0.1
23		11.5 ± 1.7	36.7 ± 3.5	70.3 ± 3.7	79.9 ± 0.5
24		38.6 ± 8.5	52.9 ± 1.0	64.9 ± 1.9	94.4 ± 0.2
25		28.6 ± 5.8	41.8 ± 5.7	68.9 ± 1.2	95.1 ± 0.2

native pantothenate. Enzyme inhibition data from the two isomers revealed a distinct preference for the (*R*) conformation over the (*S*), especially with respect to the *An*PanK and *Mm*PanK1α enzymes (Table 1). Importantly, the *Ec*PanK was not inhibited by HoPan to a significant degree. The importance of the C3 hydroxyl moiety was explored through substitution of a primary amine (**3**) in place of the hydroxyl. PanK inhibition was almost completely lost following this substitution (Table 1). None of these compounds exhibited an MIC against *E. coli* (>400 μM).

A second series of pantothenamide analogues were synthesized to explore the structural diversity allowed in the alkyl chain of the known active compounds **4** and **5** (Table 2). Series 2 (**4**–**16**) was designed based on the pantothenamide scaffold of the two known inhibitors **4** and **5** using the recent structural information from *Ec*PanK.[12] The remaining analogues (**17**–**25**) are also pantothenamides, but were developed as antimetabolites to more closely mimic the structure of CoA and enhance the antibacterial effect seen with **4** and **5**.[7] The results from the PanK assays showed that many of the compounds demonstrate *Ec*PanK inhibitory activity similar to those of **4** and **5**. However, the inhibitors were not selective for the bacterial enzyme since the degree of enzyme inhibition was equal to or greater than that of *Mm*PanK1α. There were several analogues for which the *Mm*PanK1α enzyme showed the highest percent inhibition (**7**, **12**, **13**, and **16**) and a couple of instances where it demonstrated the only significant inhibition among the four enzymes (**9**–**11**). Most notably, the *Mm*PanK1α showed a greater selectivity for the aromatic amines compared to the other enzymes. The *Sa*PanK showed an almost equally high percent inhibition across the entire series. The similarity in the SAR between the *Mm*PanK1α and the *Sa*PanK reflects the closer similarity of *Sa*PanK to metazoan enzymes than to *Ec*PanK.[10,15] For compounds **13**, **19**, and **23**, *Ec*PanK was the only enzyme not inhibited to a significant degree, indicating differences in the structure of the pantothenate binding site of the *Ec*PanK compared to the other enzymes. The binding promiscuity of this group of dissimilar enzymes was disappointing with respect to the goal of identifying selective inhibitors. IC_{50} values were also obtained for the two known inhibitors (**4** and **5**) against the *Ec*PanK and *Mm*PanK1α to demonstrate an inhibition pattern for each of the series analogues consistent with single-site inhibition (Fig. 2). As indicated by the IC_{50} plots, inhibition of the enzymes by the two compounds occurs in a concentration-dependent manner.

Antimicrobial susceptibility testing of the second analogue series of compounds against the wild-type *E. coli* strain, UB1005, was conducted to explore the antibacterial action of these inhibitors. The results from this assay revealed that only **4** was active (Table 3). In a second MIC assay, each of the analogues in the second two series was screened against *E. coli* strain ANS1, which is defective in *TolC*-dependent type I secretion of small molecules and proteins (Table 3).[16] Interestingly, **5** had antibiotic activity in this *TolC*-negative screen to an

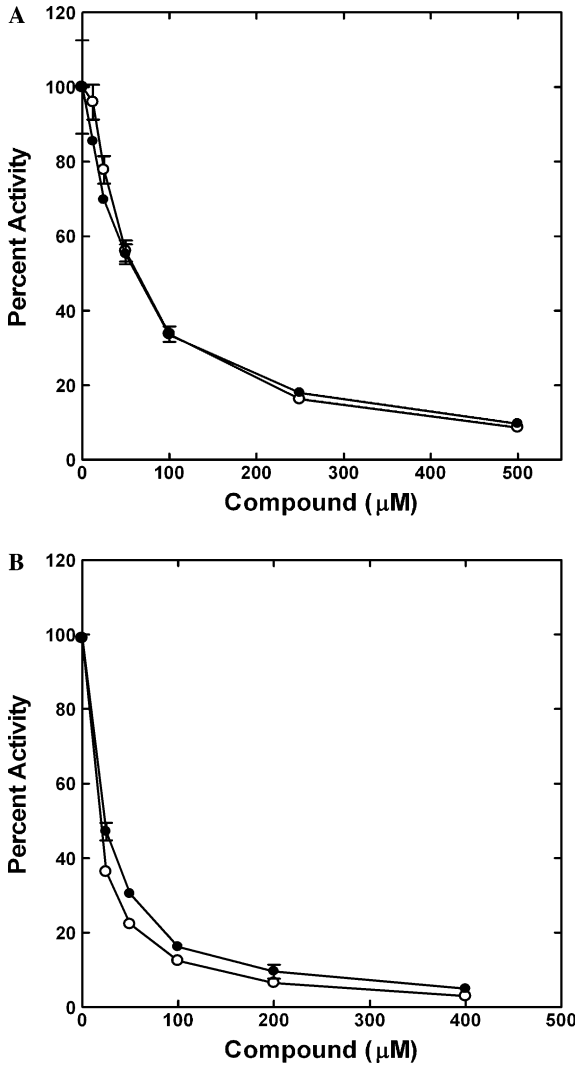


Figure 2. Inhibition of *Ec*PanK and *Mm*PanK1α by compounds **4** and **5**. Panel A, compounds **4** (○) and **5** (●) inhibited *Ec*PanK with an IC_{50} value of about 70 μM. Panel B, compounds **4** (○) and **5** (●) inhibited *Mm*PanK1α with an IC_{50} value of about 20 μM.

even lower MIC than **4**, which remained unchanged at 50 μM, indicating that **5** is a substrate for a *TolC*-dependent pump. Analogues **6**, **8**, and **14** regained detectable MIC values of 200 μM, suggesting that a *TolC* efflux system(s) was partly responsible for conferring resistance to the compounds. Only **21** from the second analogue set in the series was active with a detectable MIC of 50 μM. It was expected that **17** would show the greatest antimicrobial activity in vitro of the compounds designed as antimetabolites (**17**–**25**) as it demonstrated the highest percent inhibition against the *Ec*PanK enzyme. Furthermore, once phosphorylated by PanK, it was the most closely related structure to 4′-phosphopantetheine. However, this compound was inactive in vivo.

An MIC assay against *S. aureus* was also performed (Table 3). For this assay, six of the more active compounds from series 2 (**4**–**6** and **14**–**16**) and all of the analogues from series 3 (**17**–**25**) were screened based on their potent enzyme inhibition, including **4** and **5**, for

Table 3. MIC of pantothenamide analogues against *E. coli* and *S. aureus*

Inhibitor	MIC		
	E. coli UB1005 (μM)	*E. coli* ANS1 (μM)	*S. Aureus*
4	50	50	25 μM
5	>200	25	78 nM
6	>200	200	100 μM
7	>200	>200	ND
8	>200	200	ND
9	>200	>200	ND
10	>200	>200	ND
11	>200	>200	ND
12	>200	>200	ND
13	>200	>200	ND
14	>200	200	100 μM
15	>200	>200	200 μM
16	>200	>200	>200 μM
17	ND	>200	>200 μM
18	ND	>200	>200 μM
19	ND	>200	>200 μM
20	ND	>200	>200 μM
21	ND	50	>200 μM
22	ND	>200	>200 μM
23	ND	>200	>200 μM
24	ND	>200	>200 μM
25	ND	>200	>200 μM

which MIC values had been previously reported.[7] The data from this assay validate the previously reported potent activity of **4** and **5** against *S. aureus* (MIC 25 μM and 78 nM, respectively). None of the other analogues tested showed any significant MIC activity. Only **6**, **14**, and **15** had detectable *S. aureus* MIC values of 100, 100, and 200 μM, respectively. These results were unexpected as each of the analogues tested had significant inhibitory activity against *Sa*PanK. Many of the series 2 and 3 analogues demonstrated substantial enzyme activity relative to **4** and **5.** These results have determined that many of the compounds, which showed significant activity in the enzyme inhibition assay, are completely inactive against live cells.

One explanation for the inability of the analogues to inhibit bacterial growth was that they were not converted by the downstream enzymes to their respective CoA analogues. Alternatively, these CoA analogues may have not been substrates for AcpS, thus failing to be incorporated into ACP. We tested the first point by establishing an in vitro assay, followed by HPLC analysis that couples the phosphorylation of the analogue by *Ec*PanK to its incorporation into CoA via CoaD and CoaE, the enzymes that catalyze the last two steps in CoA biosynthesis (Fig. 3, top panel). We subsequently included AcpS and apo-ACP in the assay and we monitored the conversion of apo-ACP to the corresponding pantothenamide holo-ACPs. The reaction mixtures were analyzed by SDS–PAGE on gels containing low concentrations of urea (Fig. 3, bottom panel) that are able to resolve different ACP forms at alkaline pH.[22] Importantly, all of the designed compounds were readily metabolized by the enzymes in this system to form not only inactive analogue CoA derivatives, but also

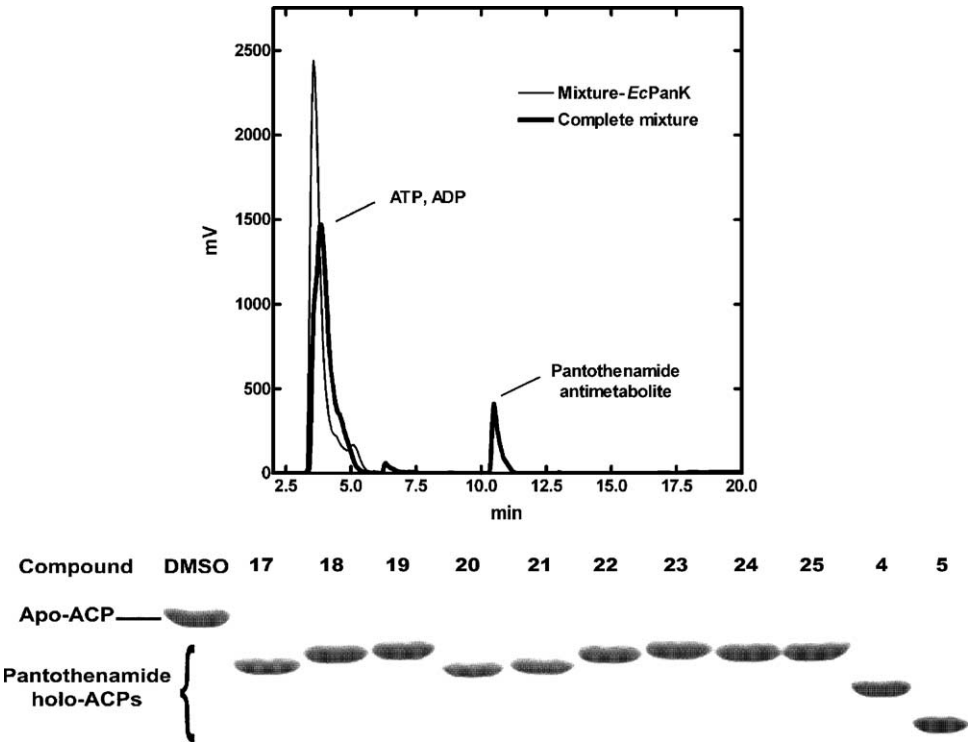


Figure 3. Conversion of compounds **4**, **5** to CoA, and **17**–**25** analogues and in vitro incorporation into ACP. Top panel: example of HPLC analysis of reaction mixtures containing one of the **17**–**25** compounds and either CoaADE (thick line) or CoaDE alone (control, thin line). Bottom panel: SDS–PAGE analysis of the holo-ACP species formed in the presence of AcpS and the CoA analogues derived from compounds **4**, **5**, and **17**–**25**. These compounds were pre-incubated with CoaADE before adding apo-ACP and AcpS, as detailed in the Section 5.

inactive ACP. These data validate our design criteria for producing compounds that would function in this pathway. Thus, we conclude that the introduction of a polar atom (O or S) into the alkyl chain must prevent the uptake of the inhibitors into the cell.

3.1. Structure–activity relationships developed for PanK enzymes

The initial structure–activity relationship for PanK was developed from the compounds in the first series of pantothenamide-type analogues and corresponds to the interactions involving *Ec*PanK (Table 1 and Fig. 4). There is some overlay between the SAR of all of the PanK enzymes tested, but each has also demonstrated unique interactions of their own. The SAR for the *Ec*PanK is the one detailed here. The primary alcohol on the C1 carbon, for which no modifications were attempted, is the site of phosphorylation. It forms an H-bond interaction with the side-chain carboxyl of Asp127 as indicated by the PanK ADP Pan ternary complex.[12] This interaction indicates that a deprotonation occurs to facilitate phosphoryl transfer. For the C3 hydroxyl, collective data from each of the PanK enzymes suggest stereoselective binding for the (*R*) isomer over the (*S*) for optimal binding. Binding was diminished for each of the enzymes with (*S*)-HoPan compared to (*R*)-HoPan, especially for *An*PanK and *Mm*PanK1α. Substitution at this position by a primary amine was also disfavored. More conclusive data are still required for the absolute SAR determination at this position for *Ec*PanK due to low-level binding of the enzyme for each analogue in series 1. However, this SAR for *Ec*PanK is supported by the known interactions determined in the PanK ADP Pan ternary complex, where the hydroxyl at this position was found to form an H-bond interaction with the imidazole ring of His177.[12] From the structure, it can also be rationalized that the reduction in binding affinity seen with the primary amine substitution is likely the result of the imidazole ring acting as an H-bond donator rather than an H-bond acceptor. A possible explanation then for the inactivity of the amine substituted (**3**) in place of the C3 hydroxyl is that the primary amine exists in the protonated state and therefore, cannot accept the proton from the H-bond donating imidazole. The C4 carbonyl and N5 amide were maintained throughout each of the series; however, they are both known to form H-bond interactions with Tyr175 and Asn282, respectively.[12] The C8 carboxyl was extended by one carbon in the case of γ-aminobutyric acid substitution (**1** and **2**). This extension of the carbon chain is not detrimental to binding, but it is reasonable to assume that a two-carbon spacer is optimal as revealed by binding interactions of the C8 carboxyl in the *Ec*PanK structure. Removal of the C8 carboxyl is disfavored as indicated by the PanK (ADP) Pan ternary complex and reduced inhibitory activity of a number of analogues designed without the C8 carboxyl functionality (data not shown). However, substitution of the carboxyl with an amide functionality, as in the pantothenamide analogues, is able to retain binding affinity. It has been determined that the C8 carboxyl forms H-bond interactions with both Asn282 and Tyr240.[12] This explains how the substitution of the carboxyl functionality with that of an amide is acceptable, as the amide is still capable of forming H-bond interactions. Although, this substitution does appear to be limited to secondary amides. The binding of a tertiary amide, compound **10**, was severely diminished except in the mammalian enzyme, perhaps indicating that an H-bond donor is required in this position. The SAR of the lipophilic pocket extending beyond the carboxylate binding site of pantothenic acid has proven to be more complex. The *Ec*PanK demonstrated more favorable binding to linear alkyl substituents that were not overly bulky or sterically hindered (**4**, **5**, **8**, and **9**). For the aromatic analogues (**6**–**9** and **12**) inhibition was slightly weaker than the linear alkyl substituents, showing no

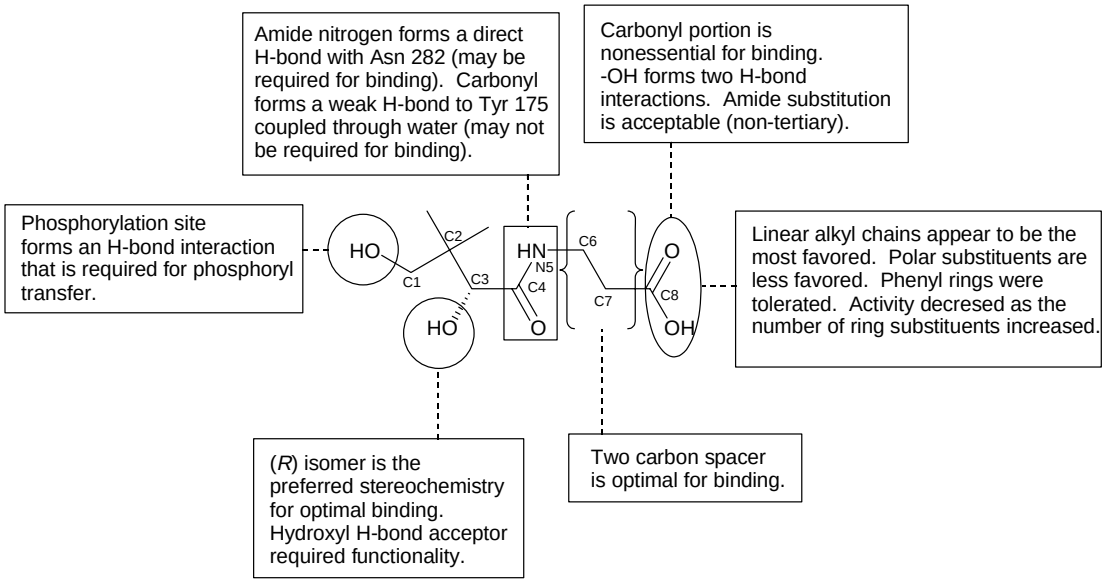


Figure 4. Developed SAR of the resolved binding interactions for inhibitors of *Ec*PanK.

enhanced binding from interactions with the phenylalanine rich pocket. Binding was greater for the larger 1-methyl-3-phenyl propyl substituent (**8**) than for the phenethyl (**6**). Methoxy-substituted phenethyl and benzyl analogue (**7**, **9**, and **12**) activities decreased with an increase in the number of methoxy substituents. Binding for this enzyme was also severely diminished for compounds containing polar functionalities (**11**, **19**, and **23**).

Some secondary SAR for the other PanK enzymes has been determined. The *An*PanK demonstrated the lowest rate of inhibition towards the series as a whole. However, it did demonstrate a higher tolerance toward steric hindrance (**13**) compared to *Ec*PanK. It also demonstrated the highest rate of inhibition toward the (*R*)-HoPan. No qualitative SAR was able to be obtained for the *An*PanK enzyme. The mammalian isoform, *Mm*PanK1α, demonstrated the highest rate of inhibition toward the series as a whole, as previously mentioned. It too did not seem to be affected by the presence of bulky or sterically hindered functionalities (**7**, **8**, **13**, and **16**). Interestingly, it was the only enzyme to demonstrate significant inhibition in the presence of a tertiary amide at N5 (**10**). The *Sa*PanK also demonstrated a high rate of inhibition. The highest percent inhibitions for this enzyme were seen with linear alkyl substituents (**4**, **5**, **14**, **20**, and **21**). Reasonable inhibition was also seen in the presence of non-substituted aromatic functionalities as well as hydroxyls (**6**, **8**, **19**, and **23**). Binding for this enzyme was most severely diminished with the addition of electron donating O-methoxy substituents placed on aromatic rings and in the case of the ethyl morpholine (**7**, **9**, and **11**). It is uncertain as to whether these effects were a result of electron density, polarity, or the increase in steric bulk.

4. Conclusion

This work reveals important SAR interactions within the pantothenate binding site of PanK. The *N*-alkylpantothenamide series of compounds has been expanded and their activities against homologues of the PanK enzyme from four species were determined. Most notable is that the absolute (*R*)-isomeric conformation of the C3 hydroxyl is required for binding to all of the enzymes and substitutions at this position are not tolerated. The hydrogen bond donor/acceptor at the C8 carboxylate is also required for binding to all non-mammalian enzymes. Probing of the lipophilic pocket beyond the pantothenate binding site has revealed distinct SAR interactions for each of the four enzymes tested. Most of the compounds in the series demonstrated significant activity against the different enzymes in an inhibition assay and were subsequently demonstrated to be substrates for the enzyme. The mammalian form of PanK proved to be highly promiscuous, demonstrating the highest degree of inhibition across the series as a whole. These initial results also suggest that selectivity will be a challenge in discovering potent inhibitors directed solely to the bacterial enzyme. MIC assays using two strains of *E. coli* as well as *S. aureus* support the theory that the active compounds with antimicrobial activity are being converted into CoA and ACP antimetabolites. In fact, most of the analogues that were inactive in the MIC assay were nonetheless substrates for the pathway enzyme in vitro. Since the one of the strains used in the test (ANS1) lacked the *TolC*-dependent efflux pumps, we conclude that these compounds most likely lack antimicrobial activity because they are unable to efficiently enter the cell. This is a somewhat surprising conclusion in light of the small difference in their structures. The inclusion of a single polar atom into the alkyl chain is sufficient to render the compound inert as an antimicrobial agent. This is difficult to reconcile with a diffusion-mediated mechanism for transport into the cell suggesting that the pantothenamides may be taken up by a protein mediated process. Current work is focused on understanding the mechanism for pantothenamide uptake since it is clear that this barrier will be a critical issue that needs to be addressed in the development of additional pantothenamide antimetabolites.

5. Materials and methods

5.1. General

All the anhydrous solvents and starting materials were purchased from Aldrich Chemical Company (Milwaukee, WI). All reagent grade solvents used for chromatography were purchased from Fisher Scientific (Sewanee, GA). Chemical reactions were monitored by thin layer chromatography (TLC) on pre-coated Merck 60 F_{254} silica gel plates and visualized by staining using a solution of ceric sulfate (1 g) and ammonium molybdate (25 g) dissolved in 500 mL of 2 M sulfuric acid. Product purification for compound **3** was performed by HPLC on a Gilson 215 HPLC (Middletown, WI) equipped with an Xterra® Prep MS C18 5 μm, 19 × 100 mm column (Milford, MA); gradient MeCN/H_2O, 0–100% (25 min) with dual wavelength detection at 215 and 254 nm. A Biotage Horizon chromatography system and silica gel cartridges were used to purify reaction mixtures **4**–**25**, Biotage Inc. (Lake Forest, VA). Purity was ascertained by analytical HPLC analysis for compounds **1**–**25** on a Shimadzu LC20 AD equipped with a Phenomenex Luna™ (Torrance, CA) 3 μm C18, 4.6 × 50 mm column; gradient MeCN/H_2O (0.01% TFA), 0–100% (10 min) with detection at 215 nm giving single peaks with retention times reported below. Melting points were obtained with a Thomas Scientific (Swedesboro, NJ) Uni-melt capillary melting point apparatus and are uncorrected. Optical rotations were obtained using a Rudolph Instruments (Fairfield, NJ) DigiPol automatic polarimeter with samples dissolved in MeOH at 10 mg/ml. All ^1H and ^{13}C NMR spectra were recorded on a Bruker ARX-300 (300 and 75 MHz for ^1H and ^{13}C NMR, respectively) or Varian INOVA-500 (500 and 125 MHz for ^1H and ^{13}C NMR, respectively) spectrometer. Chemical shifts are reported in parts per million (δ) relative to residual solvent peak or internal standard (tetramethylsilane) and coupling constants (J) are reported in hertz (Hz). Mass spectra were recorded on a Bruker Esquire (Billerica, MA) LC/MS using ESI.

5.2. General procedure for preparation of 1 and 2

In two separate 100 mL reaction vessels, 4-aminobutyric acid (340 mg, 3.3 mmol) and diethylamine (418 μL, 4 mmol) were dissolved in 20 mL methanol. The mixtures were warmed to dissolve the 4-aminobutyric acid and convert it to the diethylamine salt. Then to the first reaction vessel (*R*)-pantolactone (390 mg, 3 mmol) was added and to the second vessel (*S*)-pantolactone (390 mg, 3 mmol) was added. The reactions were then stirred overnight at 60 °C. The reaction mixtures were evaporated to dryness and dissolved in 20 mL water. Each mixture was passed over an Amberlite® IR-120 (H+) ion exchange column (2 × 10 cm) and eluted with water until neutrality was obtained. These solutions were then washed three times with dichloromethane to remove any excess pantolactone. The extracted solutions were then evaporated to dryness to yield the (*R*)-4-(2,4-dihydroxy-3,3-dimethyl-butyramido)butyric acid (**1**) and (*S*)-4-(2,4-dihydroxy-3,3-dimethyl-butyramido)butyric acid (**2**) products, respectively, as oils. The Ca^{2+} salts of each product were obtained by dissolving each in 5 mL methanol with the addition of 0.6 equiv of calcium hydroxide. The solutions were gently warmed to 40 °C and filtered to remove any undissolved solid. The filtered solutions were evaporated to dryness to yield each product as a white amorphous solid.

5.3. (*R*)-4-(2,4-Dihydroxy-3,3-dimethyl-butyrylamino)-butyric acid (1)

The synthesis was performed as detailed in the general procedure to afford 406 mg (53% yield) of **1** as a white powder. Mp 62–64 °C; $[\alpha]_D$ 33.9 (*c* 1, MeOH; *T* = 27 °C); ^{1}H NMR (500 MHz, D_2O) δ 3.84 (s, 1H), 3.37 (d, *J* = 11.2 Hz, 1H), 3.25 (d, *J* = 11.2 Hz, 1H), 3.09 (t, *J* = 6.8 Hz, 2H), 2.07 (t, *J* = 7.8 Hz, 2H), 1.63 (qin, *J* = 7.3 Hz, 2H), 0.79 (s, 3H), 0.76 (s, 3H); ^{13}C NMR (75 MHz, D_2O) δ 182.3, 174.5, 75.5, 67.9, 38.3, 38.1, 34.3, 24.9, 20.1, 18.8; MS (ESI^-) 231.9 (M−1); LC retention time 4.06 min.

5.4. (*S*)-4-(2,4-Dihydroxy-3,3-dimethyl-butyrylamino)-butyric acid (2)

The synthesis was performed as detailed in the general procedure to afford 389 mg (51% yield) of **2** as a white powder. Mp 60–62 °C; $[\alpha]_D$ −31.8 (*c* 1, MeOH; *T* = 26.2 °C); ^{1}H NMR (500 MHz, CH_3OD) δ 3.95 (s, 1H), 3.49 (d, *J* = 11.0 Hz, 1H), 3.43 (d, *J* = 11.0 Hz, 1H), 3.28 (t, *J* = 6.8 Hz, 2H), 2.26 (t, *J* = 7.1 Hz, 2H), 1.82 (qin, *J* = 6.8 Hz, 2H), 0.97 (s, 3H), 0.95 (s, 3H); ^{13}C NMR (75 MHz, D_2O) δ 181.1, 174.5, 75.5, 67.9, 38.1 (2C), 38.1, 33.5, 24.6, 20.1, 18.8; MS (ESI^-) 231.8 (M−1); LC retention time 3.93 min.

5.5. (*S*)-Trifluoro-methanesulfonic acid 4,4-dimethyl-2-oxo-tetrahydro-furan-3-yl ester (A)

In a 100 mL dry round-bottomed flask, (*S*)-pantolactone (2.1 g, 16 mmol) was dissolved in 25 mL dry DCM and 1.63 mL pyridine (20 mmol) under inert conditions. The reaction was cooled to −78 °C and triflic anhydride (5 g, 17.7 mmol) was added dropwise. The reaction mixture was stirred for 20 min at −78 °C and then for 1 h at room temperature. The reaction mixture was concentrated in vacuo and then extracted with Et_2O partitioned with 5% aqueous potassium hydrogen sulfate followed by washing with saturated sodium bicarbonate and saturated brine. The solution was dried over sodium sulfate and evaporated to complete dryness in vacuo to yield 3.85 g (91% yield) of a clear viscous oil, which crystallized upon cooling to form a white solid. Mp 29–30 °C; $[\alpha]_D$ −3.8 (*c* 1, MeOH; *T* = 25.5 °C); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.10 (s, 1H), 4.13 (dd, *J* = 9.3, 26.8 Hz, 2H), 1.34 (s, 3H), 1.25 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.9, 120.1, 85.2, 75.2, 40.0, 21.6, 19.0; MS (ESI^-) 261.1 (M−1); LC retention time 6.39 min.

5.6. (*R*)-3-Azido-4,4-dimethyl-dihydro-furan-2-one (B)

In a 100 mL dry round-bottomed flask, **A** (2.6 g, 10 mmol) was dissolved in dry DMF and cooled to 0 °C followed by the addition of NaN_3 (715 mg, 11 mmol). Under continuous stirring, the mixture was allowed to warm up to room temperature and stirred for an additional 2 h. The mixture was evaporated to dryness in vacuo to remove excess DMF and purified by flash chromatography using a linear gradient of petroleum ether/ethyl acetate 30–90% to afford 540 mg (35% yield) of **B** as a white crystalline solid. Mp 54–55 °C; $[\alpha]_D$ 103.3 (*c* 1, MeOH; *T* = 25.7 °C); ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.01 (dd, *J* = 9.0, 25.4 Hz, 3H), 1.24 (s, 3H), 1.10 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.4, 76.3, 65.9, 40.3, 22.7, 19.5; MS (ESI^+) 178.3 (M+23); LC retention time 5.68 min.

5.7. (*R*)-4-(2-Azido-4-hydroxy-3,3-dimethyl-butyryl-amino)-butyric acid (C)

In a 100 mL reaction vessel, 4-aminobutyric acid (365 mg, 3.5 mmol) and diethylamine (418 μL, 4 mmol) were dissolved in 20 mL of methanol. The mixture was warmed to dissolve the 4-aminobutyric acid and to convert it to the diethylamine salt. Intermediate **B** (0.5 g, 3.2 mmol) was then added to the solution and the reaction mixture was stirred overnight at 60 °C. The reaction mixture was evaporated to dryness and dissolved in 20 mL water. The mixture was passed over an Amberlite® IR-120 (H+) ion exchange column (2 × 10 cm) and eluted with water until neutrality was obtained. The solution was then extracted three times with dichloromethane to remove any excess pantolactone and dried in vacuo to afford 400 mg (48% yield) **C** as a clear oil. $[\alpha]_D$ 1.4 (*c* 1, MeOH; *T* = 24.8 °C); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.88–6.98 (br s, 1H), 4.04 (s, 1H), 3.60 (t, *J* = 7.1 Hz, 2H), 3.40 (q, *J* = 6.8 Hz, 2H), 2.44 (qin, *J* = 6.3 Hz, 2H), 1.99 (qin, *J* = 7.1 Hz, 2H), 1.28 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.9, 169.0, 68.5, 67.5, 38.9, 38.0, 30.9, 24.0, 20.1, 19.2; MS (ESI^-) 256.9 (M−1); LC retention time 4.35 min.

5.8. (*R*)-4-(2-Amino-4-hydroxy-3,3-dimethyl-butyryl-amino)-butyric acid (3)

In a 25 mL reaction vessel, **C** (330 mg, 1.2 mmol) was dissolved in a solution of concentrated NH_4OH (4 mL)

and pyridine (3.5 mL) followed by the addition of triphenylphosphine (0.9 g, 3.4 mmol). The reaction mixture was stirred for 6 h and evaporated to dryness in vacuo. The crude material was purified by reverse-phase HPLC with collection set at 215 nm using a linear gradient of 0.05% TFA in water to 100% acetonitrile. The appropriate fractions were pooled and dried in vacuo to afford 267 mg (62% yield) of **3** as a clear oil. $[\alpha]_D$ 21.8 (*c* 1, MeOH; $T = 24.3$ °C); ^{1}H NMR (500 MHz, CH_3OD) δ 3.75 (s, 1H), 3.53 (d, $J = 11.0$ Hz, 1H), 3.48 (d, $J = 10.7$ Hz, 1H), 3.32 (t, $J = 6.8$ Hz, 2H), 2.39 (t, $J = 7.3$ Hz, 2H), 1.85 (qin, $J = 7.1$ Hz, 2H), 1.08 (s, 3H), 1.04 (s, 3H); ^{13}C NMR (75 MHz, D_2O) δ 181.5, 167.4, 68.8, 59.5, 38.8, 36.0, 34.0, 24.5, 20.8, 18.9; MS (ESI^+) 233.1 (M+1); LC retention time 3.99 min.

5.9. General procedure for preparation of 4–25

To the free-acid form of pantothenate (6.8 mmol for **4** and **5**, 2 mmol for **6**–**16**, and 8 mmol for **17**–**25**) dissolved in 10 mL dry DMF in discrete reaction vessels under inert conditions were added diphenylphosphoryl azide (DPPA, 1.5 equiv for **4**–**16** and 1.25 equiv for **17**–**25**) and 1 of each of the 22 amines (1.5 equiv for **4**–**16** and 1.25 equiv for **17**–**25**). The reaction mixtures were then cooled to 0 °C and triethylamine (1.5 equiv for **4**–**16** and 1.25 equiv for **17**–**25**) was added to each. The reactions were stirred at 0 °C for 2 h, followed by stirring at room temperature for 12 h. The reaction volumes were reduced under vacuum at 70 °C to remove dimethylformamide. Each of the crude mixtures was purified by flash chromatography as previously detailed.

5.10. 2,4-Dihydroxy-3,3-dimethyl *N*-(2-pentylcarbamoyl-ethyl)-butyramide (4)

The synthesis was performed as detailed in the general procedure to afford 1.18 g of product (60% yield) as a viscous oil, which crystallized upon cooling. Mp 72–74 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (t, $J = 6.3$ Hz, 1H), 6.45 (t, $J = 5.5$ Hz, 1H), 4.75 (d, $J = 3.3$ Hz, 1H), 4.16 (br s, 1H), 3.98 (d, $J = 2.6$ Hz, 1H), 3.6–3.4 (m, 4H), 3.19 (q, $J = 6.2$ Hz, 2H), 2.43 (t, $J = 5.9$ Hz, 2H), 1.47 (qin, $J = 7.3$ Hz, 2H), 1.36–1.23 (m, 4H), 0.97 (s, 3H), 0.91 (s, 3H), 0.88 (t, $J = 7.0$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 170.9, 76.9, 70.3, 39.2, 38.8, 35.3, 34.8, 28.9, 28.5, 21.8, 20.8, 19.9, 13.4; MS (ESI^-) 287.2 (M−1); LC retention time 4.87 min.

5.11. 2,4-Dihydroxy-3,3-dimethyl *N*-(2-heptylcarbamoyl-ethyl)-butyramide (5)

The synthesis was performed as detailed in the general procedure to afford 1.54 g of product (71% yield) as a viscous oil, which crystallized upon cooling. Mp 74–76 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.50 (t, $J = 5.8$ Hz, 1H), 6.21 (br s, 1H), 4.47 (br s, 1H), 4.00 (d, $J = 5.1$ Hz, 1H), 3.91 (br s, 1H), 3.62–3.42 (m, 4H), 3.20 (q, $J = 6.5$ Hz, 2H), 2.43 (t, $J = 5.8$ Hz, 2H), 1.48 (t, $J = 6.9$ Hz, 2H), 1.34–1.22 (m, 4H), 0.99 (s, 3H), 0.92 (s, 3H), 0.88 (t, $J = 7.2$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 170.9, 76.7, 70.2, 39.2, 38.73, 35.3, 34.8, 31.2, 28.9, 28.4, 26.4, 22.0, 20.6, 20.0, 13.5; MS (ESI^-) 315.2 (M−1); LC retention time 5.53 min.

5.12. 2,4-Dihydroxy-3,3-dimethyl *N*-(2-phenethylcarbamoyl-ethyl)-butyramide (6)

The synthesis was performed as detailed in the general procedure to afford 408 mg of product (63% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (t, $J = 6.1$ Hz, 1H), 7.28 (t, $J = 7.6$ Hz, 2H), 7.21 (t, $J = 7.6$ Hz, 1H), 7.16 (d, $J = 7.1$ Hz, 2H), 6.58 (d, $J = 4.9$ Hz, 1H), 4.87 (br s, 1H), 4.26 (br s, 1H), 3.97 (s, 1H), 3.54–3.36 (m, 6H), 2.78 (t, $J = 7.3$ Hz, 2H), 2.37 (t, $J = 6.1$ Hz, 2H), 0.95 (s, 3H), 0.90 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.5, 171.1, 138.2, 128.1 (2), 128.1 (2), 126.0, 76.7, 70.2, 40.3, 38.8, 35.2, 35.0, 34.8, 20.6, 20.0; MS (ESI^-) 321.1 (M−1); LC retention time 4.92 min.

5.13. 2,4-Dihydroxy-3,3-dimethyl *N*-{2-[2-(3,4-dimethoxy-phenyl)-ethylcarbamoyl]-ethyl}-butyramide (7)

The synthesis was performed as detailed in the general procedure to afford 422 mg of product (55% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.46 (t, $J = 6.1$ Hz, 1H), 6.80 (d, $J = 8.6$ Hz, 1H), 6.71 (d, $J = 6.1$ Hz, 2H), 6.23 (t, $J = 5.6$ Hz, 1H), 4.45 (br d, $J = 4.6$ Hz, 1H), 3.97 (br d, $J = 4.2$ Hz, 1H), 3.90 (br s, 1H), 3.86 (d, $J = 4.4$ Hz, 6H), 3.58–3.42 (m, 6H), 2.74 (t, $J = 7.1$ Hz, 2H), 2.39 (t, $J = 6.1$ Hz, 2H), 0.99 (s, 3H), 0.91 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.3, 170.9, 148.5, 147.3, 130.7, 120.2, 111.6, 111.0, 76.9, 70.3, 55.4 (2), 40.3, 38.8, 35.2, 34.7, 34.6, 20.8, 19.9; MS (ESI^-) 381.1 (M−1); LC retention time 4.71 min.

5.14. 2,4-Dihydroxy-3,3-dimethyl *N*-[2-(1-methyl-3-phenyl-propylcarbamoyl)-ethyl]-butyramide (8)

The synthesis was performed as detailed in the general procedure to afford 471 mg of product (67% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.51 (t, $J = 4.6$ Hz, 1H), 7.27 (t, $J = 7.6$ Hz, 2H), 7.20–7.13 (m, 3H), 6.12 (t, $J = 8.3$ Hz, 1H), 4.59 (t, $J = 5.1$ Hz, 1H), 4.10–3.94 (m, 3H), 3.60–3.40 (m, 4H), 2.62 (t, $J = 8.3$ Hz, 2H), 2.42–2.30 (m, 2H), 1.81–1.68 (m, 2H), 1.14 (d, $J = 6.6$ Hz, 3H), 0.97 (s, 3H), 0.91 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.4, 170.2, 141.1, 127.9 (2), 127.7 (2), 125.4, 76.9, 70.3, 44.8, 38.8, 37.8, 35.4, 34.8, 31.9, 20.8, 20.3, 20.0; MS (ESI^-) 349.2 (M−1); LC retention time 5.30 min.

5.15. 2,4-Dihydroxy-3,3-dimethyl *N*-[2-(3,4,5-trimethoxy-benzylcarbamoyl)-ethyl]-butyramide (9)

The synthesis was performed as detailed in the general procedure to afford 480 mg of product (60% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (t, $J = 6.1$ Hz, 1H), 7.12 (t, $J = 5.4$ Hz, 1H), 6.47 (s, 2H), 4.87 (d, $J = 4.2$ Hz, 1H), 4.27 (d, $J = 5.8$ Hz, 3H), 3.94 (d, $J = 4.4$ Hz, 1H), 3.81 (s, 6H), 3.78 (s, 3H),

3.58–3.44 (m, 2H), 3.40 (q, $J = 8.6$ Hz, 2H), 2.44 (t, $J = 6.1$ Hz, 2H), 0.92 (s, 3H), 0.86 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.5, 170.9, 152.7 (2), 136.5, 133.4, 104.5 (2), 76.6, 70.0, 60.2, 55.5 (2), 43.3, 38.6, 35.1, 34.7, 20.5, 19.9; MS (ESI$^-$) 397.1 (M−1); LC retention time 4.62 min.

5.16. 2,4-Dihydroxy-3,3-dimethyl *N*-[3-(4-benzyl-piperazin-1-yl)-3-oxo-propyl]-butyramide (10)

The synthesis was performed as detailed in the general procedure to afford 402 mg of product (53% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42 (t, $J = 5.9$ Hz, 1H), 7.35–7.25 (m, 5H), 4.01 (br s, 1H), 3.99 (s, 1H), 3.66–3.52 (m, 5H), 3.52 (s, 2H), 3.47 (s, 2H), 3.43 (s, 2H), 2.54 (t, $J = 5.6$ Hz, 2H), 2.47–2.36 (m, 4H), 1.00 (s, 3H), 0.90 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.9, 169.3, 136.7, 128.6 (2), 127.8 (2), 126.8, 77.0, 70.4, 62.2, 52.3, 52.0, 44.9, 41.2, 38.9, 34.3, 32.1, 20.9, 19.8; MS (ESI$^-$) 376.3 (M−1); LC retention time 4.98 min.

5.17. 2,4-Dihydroxy-3,3-dimethyl *N*-[2-(2-morpholin-4-yl-ethylcarbamoyl)-ethyl]-butyramide (11)

The synthesis was performed as detailed in the general procedure to afford 386 mg of product (58% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.60 (t, $J = 5.9$ Hz, 1H), 6.83 (s, 1H), 3.99 (s, 1H), 3.72 (t, $J = 4.4$ Hz, 4H), 3.54 (q, $J = 5.9$ Hz, 2H), 3.48 (d, $J = 11.2$ Hz, 1H), 3.42 (d, $J = 11.0$ Hz, 1H), 3.35 (qin, $J = 6.6$ Hz, 2H), 2.56–2.41 (m, 8H), 0.96 (s, 3H), 0.92 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 171.2, 76.3, 69.8, 66.0 (2), 56.7, 52.8 (2), 38.7, 35.3, 35.2, 34.8, 20.5, 20.1; MS (ESI$^-$) 330.2 (M−1); LC retention time 4.01 min.

5.18. 2,4-Dihydroxy-3,3-dimethyl *N*-[2-(4-methoxy-benzylcarbamoyl)-ethyl]-3,3-dimethyl-butyramide (12)

The synthesis was performed as detailed in the general procedure to afford 496 mg of product (73% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.53 (t, $J = 6.1$ Hz, 1H), 7.14 (d, $J = 8.6$ Hz, 2H), 6.94 (t, $J = 5.4$ Hz, 1H), 6.82 (d, $J = 8.6$ Hz, 2H), 4.82 (d, $J = 2.9$ Hz, 1H), 4.27 (d, $J = 5.6$ Hz, 2H), 4.23, (br s, 1H), 3.93 (d, $J = 3.4$ Hz, 1H), 3.76 (s, 3H), 3.49 (qin, $J = 6.1$ Hz, 2H), 3.42 (d, $J = 11.0$ Hz, 1H), 3.37 (d, $J = 11.2$ Hz, 1H), 2.41 (t, $J = 6.1$ Hz, 2H), 0.92 (s, 3H), 0.87 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 170.9, 158.4, 129.6, 128.5 (2), 113.5 (2), 76.6, 70.0, 54.7, 42.4, 38.7, 35.1, 34.8, 20.5, 20.0; MS (ESI$^-$) 337.1 (M−1); LC retention time 4.79 min.

5.19. 2,4-Dihydroxy-3,3-dimethyl *N*-[2-(2,6,6-trimethyl-bicyclo[3.1.1]hept-3-ylcarbamoyl)-ethyl]-butyramide (13)

The synthesis was performed as detailed in the general procedure to afford 491 mg of product (69% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (t, $J = 6.1$ Hz, 1H), 6.23 (d, $J = 8.3$ Hz, 1H), 4.59 (br s, 1H), 4.24 (qin, $J = 7.9$ Hz, 1H), 4.04 (br s, 1H), 4.01 (d, $J = 4.9$ Hz, 1H), 3.56 (qin, $J = 6.1$ Hz, 2H), 3.48 (dq, $J = 11.2$, 5.4 Hz, 2H), 2.60–2.50 (m, 1H), 2.44 (t, $J = 6.1$ Hz, 2H), 2.44–2.36 (m, 1H), 1.94 (qin, $J = 2.9$ Hz, 1H), 1.86–1.74 (m, 2H), 1.50 (ddd, $J = 13.9$, 6.1, 2.2 Hz, 1H), 1.22 (s, 3H), 1.08 (d, $J = 7.3$ Hz, 3H), 1.03 (s, 3H), 1.00 (s, 3H), 0.92 (s, 3H), 0.86 (d, $J = 9.8$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.5, 170.2, 76.9, 70.3, 47.5, 47.2, 45.2, 41.0, 38.8, 37.9, 36.4, 35.5, 34.8, 34.5, 27.4, 22.8, 20.8, 20.2, 19.9; MS (ESI$^-$) 353.2 (M−1); LC retention time 5.63 min.

5.20. 2,4-Dihydroxy-3,3-dimethyl *N*-(2-propylcarbamoyl-ethyl)-butyramide (14)

The synthesis was performed as detailed in the general procedure to afford 453 mg of product (87% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.62 (t, $J = 6.4$ Hz, 1H), 6.66 (t, $J = 5.6$ Hz, 1H), 4.03 (s, 1H), 3.64–3.46 (m, 4H), 3.21 (q, $J = 6.6$, 2H), 2.48 (t, $J = 6.4$, 2H), 1.55 (sextet, $J = 7.3$ Hz, 2H), 1.01 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.5, 171.0, 76.8, 70.2, 40.9, 38.8, 35.3, 34.8, 22.1, 20.7, 19.9, 10.8; MS (ESI$^-$) 259.1 (M−1); LC retention time 4.26 min.

5.21. 2,4-Dihydroxy-3,3-dimethyl *N*-(2-isobutylcarbamoyl-ethyl)-butyramide (15)

The synthesis was performed as detailed in the general procedure to afford 423 mg of product (77% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 (t, $J = 6.1$ Hz, 1H), 7.04 (t, $J = 5.9$ Hz, 1H), 5.23 (br s, 1H), 4.61 (br s, 1H), 3.98 (s, 1H), 3.6–3.36 (m, 4H), 3.01 (t, $J = 6.4$ Hz, 2H), 2.45 (t, $J = 6.1$ Hz, 2H), 1.74 (septet, $J = 6.6$ Hz, 1H), 0.94 (s, 3H), 0.90 (s, 3H), 0.88 (d, $J = 6.6$ Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 171.1, 76.5, 70.0, 46.5, 38.7, 35.2, 34.9, 27.8, 20.9, 20.0, 19.6 (2); MS (ESI$^-$) 273.2 (M−1); LC retention time 4.52 min.

5.22. 2,4-Dihydroxy-3,3-dimethyl *N*-[2-(3,7-dimethyl-octa-2,6-dienylcarbamoyl)-ethyl]-butyramide (16)

The synthesis was performed as detailed in the general procedure to afford 326 mg of product (46% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (t, $J = 6.1$ Hz, 1H), 6.09 (t, $J = 4.6$ Hz, 1H), 5.21 (t, $J = 7.1$ Hz, 1H), 5.12 (t, $J = 6.8$ Hz, 1H), 4.05 (s, 1H), 3.87 (t, $J = 6.1$ Hz, 2H), 3.68–3.49 (m, 4H), 2.48 (t, $J = 6.1$ Hz, 2H), 2.16–2.02 (m, 4H), 1.73 (s, 3H), 1.65 (s, 3H), 1.05 (s, 3H), 0.97 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.1, 170.7, 139.7, 131.3, 123.2, 118.9, 77.0, 70.4, 38.9, 38.8, 37.1, 35.2, 34.8, 25.9, 25.1, 20.9, 19.9, 17.1, 15.7; MS (ESI$^-$) 353.2 (M−1); LC retention time 5.75 min.

5.23. 2,4-Dihydroxy-3,3-dimethyl *N*-[2-(2-methylsulfanyl-ethylcarbamoyl)-ethyl]-butyramide (17)

The synthesis was performed as detailed in the general procedure to afford 394 mg of product (17% yield) as a viscous oil, which crystallized upon cooling. Mp 61–63 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.48 (t,

J = 6.1 Hz, 1H), 6.58 (t, J = 5.6 Hz, 1H), 4.45 (br s, 1H), 3.99 (s, 1H), 3.89 (br s, 1H), 3.62–3.52 (m, 2H), 3.52–3.38 (m, 4H), 2.63 (dt, J = 1.0, 6.6 Hz, 2H), 2.46 (t, J = 6.1 Hz, 2H), 2.11 (s, 3H), 0.99 (s, 3H), 0.92 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.2, 171.0, 77.0, 70.3, 38.8, 37.3, 35.3, 34.7, 33.2, 20.9, 19.9, 14.4; MS (ESI^-) 291.0 (M−1); LC retention time 4.28 min.

5.24. 2,4-Dihydroxy-*N*-[2-(2-methoxy-ethylcarbamoyl)-ethyl]-3,3-dimethyl-butyramide (18)

The synthesis was performed as detailed in the general procedure to afford 197 mg of product (9% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.67 (t, J = 6.1 Hz, 1H), 7.28 (t, J = 5.4 Hz, 1H), 5.26 (d, J = 5.1 Hz, 1H), 4.63 (t, J = 5.6 Hz, 1H), 3.98 (d, J = 5.4 Hz, 1H), 3.57–3.36 (m, 8H), 3.34 (s, 3H), 2.46 (t, J = 6.4 Hz, 2H), 0.94 (s, 3H), 0.91 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 171.3, 76.3, 70.4, 69.8, 58.0, 38.7, 38.6, 35.1, 34.8, 20.5, 20.0; MS (ESI^-) 275.0 (M−1); LC retention time 3.98 min.

5.25. 2,4-Dihydroxy-*N*-[2-(2-hydroxy-ethylcarbamoyl)-ethyl]-3,3-dimethyl-butyramide (19)

The synthesis was performed as detailed in the general procedure to afford 710 mg of product (33% yield) as a viscous oil. ^{1}H NMR (500 MHz, CD_3OD) δ 3.91 (s, 1H), 3.61 (t, J = 5.6 Hz, 2H), 3.56–3.38 (m, 4H), 3.31 (t, J = 5.9 Hz, 2H), 2.46 (t, J = 6.6 Hz, 2H), 0.94 (s, 6H); ^{13}C NMR (75 MHz, CD_3OD) δ 174.1, 172.1, 75.4, 68.4, 59.6, 41.0, 38.4, 34.6, 34.5, 19.4, 19.0; MS (ESI^-) 261.0 (M−1); LC retention time 3.71 min.

5.26. 2,4-Dihydroxy-3,3-dimethyl *N*-[2-(2-ethylsulfanyl-ethylcarbamoyl)-ethyl]-butyramide (20)

The synthesis was performed as detailed in the general procedure to afford 709 mg of product (28% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47 (t, J = 6.1 Hz, 1H), 6.56 (t, J = 5.6 Hz, 1H), 4.41 (d, J = 5.4 Hz, 1H), 4.00 (d, J = 5.4 Hz, 1H), 3.86 (t, J = 5.9 Hz, 1H), 3.62–3.53 (m, 2H), 3.52–3.37 (m, 4H), 2.67 (dt, J = 1.7, 6.8 Hz, 2H), 2.55 (q, J = 7.3 Hz, 2H), 2.46 (t, J = 6.1 Hz, 2H), 1.26 (t, J = 7.3 Hz, 3H), 0.99 (s, 3H), 0.92 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.3, 171.0, 76.9, 70.3, 38.8, 38.0, 35.3, 34.8, 30.6, 25.0, 20.8, 19.9, 14.2; MS (ESI^-) 305.0 (M−1); LC retention time 4.53 min.

5.27. 2,4-Dihydroxy-3,3-dimethyl *N*-[2-(3-methylsulfanyl-propylcarbamoyl)-ethyl]-butyramide (21)

The synthesis was performed as detailed in the general procedure to afford 863 mg of product (34% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.62 (t, J = 6.1 Hz, 1H), 7.08 (t, J = 5.6 Hz, 1H), 5.07 (d, J = 5.1 Hz, 1H), 4.46 (t, J = 5.4 Hz, 1H), 3.99 (d, J = 4.9 Hz, 1H), 3.60–3.39 (m, 4H), 3.31 (q, J = 6.8 Hz, 2H), 2.52 (t, J = 7.1 Hz, 2H), 2.45 (t, J = 6.4 Hz, 2H), 2.09 (s, 3H), 1.80 (qin, J = 7.1 Hz, 2H), 0.96 (s, 3H), 0.92 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 171.1, 76.6, 70.1, 38.7, 38.1, 35.3, 34.8, 31.0, 28.0, 20.6, 20.0, 14.9; MS (ESI^+) 329.1 (M+23); LC retention time 4.45 min.

5.28. 2,4-Dihydroxy-3,3-dimethyl *N*-[2-(3-ethoxy-propylcarbamoyl)-ethyl]-butyramide (22)

The synthesis was performed as detailed in the general procedure to afford 1.21 g of product (48% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.50 (t, J = 6.1 Hz, 1H), 6.67 (t, J = 4.9 Hz, 1H), 4.52 (d, J = 5.4 Hz, 1H), 4.00 (d, J = 5.4 Hz, 1H), 3.96 (t, J = 5.9 Hz, 1H), 3.61–3.43 (m, 8H), 3.34 (qin, J = 5.7 Hz, 2H), 2.41 (t, J = 6.1 Hz, 2H), 1.77 (qin, J = 5.9 Hz, 2H), 1.20 (t, J = 7.1 Hz, 3H), 1.00 (s, 3H), 0.92 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.2, 170.8, 76.9, 70.3, 68.9, 65.9, 38.8, 37.7, 35.2, 34.8, 28.4, 20.9, 19.9, 14.6; MS (ESI^+) 327.2 (M+23); LC retention time 4.33 min.

5.29. 2,4-Dihydroxy-*N*-[2-(3-hydroxy-propylcarbamoyl)-ethyl]-3,3-dimethyl-butyramide (23)

The synthesis was performed as detailed in the general procedure to afford 660 mg of product (25% yield) as a viscous oil. ^{1}H NMR (500 MHz, CD_3OD) δ 3.91 (s, 1H), 3.61 (t, J = 6.4 Hz, 2H), 3.56–3.44 (m, 2H), 3.49 (d, J = 10.7 Hz, 1H), 3.41 (d, J = 11.0 Hz, 1H), 3.28 (t, J = 6.8 Hz, 2H), 2.45 (t, J = 6.8 Hz, 2H), 1.73 (qin, J = 6.8 Hz, 2H), 0.94 (s, 6H); ^{13}C NMR (75 MHz, CD_3OD) δ 174.1, 171.9, 75.4, 68.5, 58.5, 38.4, 35.5, 34.5, 34.5, 31.2, 19.4, 19.0; MS (ESI^-) 275.0 (M−1); LC retention time 3.80 min.

5.30. 2,4-Dihydroxy-*N*-[2-(3-methoxy-propylcarbamoyl)-ethyl]-3,3-dimethyl-butyramide (24)

The synthesis was performed as detailed in the general procedure to afford 1.35 g of product (57% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45 (t, J = 5.9 Hz, 1H), 6.45 (br s, 1H), 4.25 (d, J = 5.6 Hz, 1H), 3.99 (d, J = 5.4 Hz, 1H), 3.73 (t, J = 5.9 Hz, 1H), 3.60–3.53 (m, 2H), 3.51–3.44 (m, 4H), 3.40–3.28 (m, 5H), 2.42 (t, J = 6.1 Hz, 2H), 1.77 (qin, J = 6.1 Hz, 2H), 1.01 (s, 3H), 0.93 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.3, 170.9, 76.9, 70.9, 70.3, 58.2, 38.8, 37.4, 35.3, 34.8, 28.5, 20.8, 19.9; MS (ESI^-) 289.0 (M−1); LC retention time 4.11 min.

5.31. 2,4-Dihydroxy-3,3-dimethyl *N*-[2-(2-ethoxy-ethylcarbamoyl)-ethyl]-butyramide (25)

The synthesis was performed as detailed in the general procedure to afford 1.26 g of product (53% yield) as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.52 (t, J = 6.1 Hz, 1H), 6.64 (t, J = 5.1 Hz, 1H), 4.74 (d, J = 5.4 Hz, 1H), 4.10 (t, J = 5.6 Hz, 1H), 3.99 (d, J = 5.4 Hz, 1H), 3.60–3.37 (m, 10H), 2.45 (q, J = 6.1 Hz, 2H), 1.20 (t, J = 7.1 Hz, 3H), 0.98 (s, 3H), 0.92 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.4, 171.2, 76.8, 70.1, 68.2, 65.9, 38.8, 38.7, 35.2, 34.8, 20.9, 19.9, 14.5; MS (ESI^-) 289.1 (M−1); LC retention time 4.18 min.

5.32. Assay of PanK

The standard PanK assays contained D-[1-^{14}C]pantothenate (45 μM; specific activity 55 mCi/mmol), ATP (100 μM), $MgCl_2$ (10 mM), Tris–HCl (0.1 M, pH 7.5), and PanK in a total volume of 40 μL.[19,20] The compounds were dissolved in DMSO and included in the assay at 100 μM. Control assays included the equal volume of DMSO solvent. A panel of PanK enzymes was used to test the compounds: purified *E. coli* PanK (100 ng), purified *S. aureus* PanK (20 ng), and purified *A. nidulans* PanK (200 ng) and mPanK1α (40 μg cellular lysate). The mixture was incubated at 37 °C for 10 min. The reaction was stopped by adding 4 μL of 10% (v/v) acetic acid to the mix. Then 40 μL of the mixture was deposited onto a Whatmann DE81 ion exchange filter disk that was washed in three changes of 1% acetic acid in 95% ethanol (25 mL/disk; 20 min wash) to remove unreacted pantothenate. 4′-phosphopantothenate was quantitated by counting the dried disk in 3 mL of scintillation solution. A second assay was used to determine the phosphorylation of the pantothenate analogues by PanK. The reaction mix contained pantothenate or pantothenate analogue, [γ-^{32}P]ATP (0.25 mM; specific activity 1 Ci/mmol), $MgCl_2$ (10 mM), Tris–HCl (0.1 M, pH 7.5), and PanK. After incubation at 37 °C for 20 min, the reaction was stopped by adding 4 μL of 0.5 M EDTA. A 10 μL aliquot of the reaction mixture was spotted onto an activated silica gel H plate, which was developed with butanol/acetate/water (5:2:4, v/v/v). The dried plate was exposed to a storage phosphor screen, and the phosphorylated product was quantitated using ImageQuant 5.2 software (Molecular Dynamics).

5.33. Determination of the MIC

The MICs of the test compounds against *E. coli* strain ANSI and *S. aureus* strain RN4220 were determined by a broth microdilution method. ANS1 or RN4220 was grown to mid-log phase in 1% tryptone broth and then diluted 30,000-fold in the same medium. A 10 μL aliquot of the diluted cell suspension (3000–5000 colony forming units) was used to inoculate each well of a 96-well plate (U-bottom with low evaporation lid) containing 100 μL of tryptone broth with the indicated concentration of inhibitors. The plate was incubated at 37 °C for 20 h before being read with a Fusion™ Universal Microplate Analyzer (Packard, Canada) at 600 nm. The absorbance was normalized to the DMSO solvent treated control which was considered as 100%.

5.34. In vitro conversion of compounds 17–25 to CoA analogues and incorporation into ACP

E. coli ACP, holo-ACP synthase (AcpS), and *Ec*PanK were purified as previously described.[11,15,21] *E. coli* CoaD and CoaE were expressed as C-terminal and N-terminal His-tagged proteins, respectively, and purified in a single step by nickel-affinity chromatography. Stock solutions (10 or 20 mM) of compounds **4**, **5**, and **17**–**25** were prepared in DMSO and added (0.5 mM final concentration) to reaction mixtures containing Tris–HCl (50 mM, pH 7.5), ATP (5 mM), $MgCl_2$ (5 mM), DTT (2 mM), and *Ec*PanK + CoaDE (0.6 μg each) of in a total volume of 40 μL. Control reactions were set up without *Ec*PanK. Reaction mixtures were incubated at 37 °C for 30 min and then stopped by placing the tubes in boiling water for 5 min. Precipitated proteins were pelleted by centrifugation (14,000 rpm, 5 min) and the supernatants were filtered through 0.2 μm Spin-X centrifuge tube filters (Costar) before being injected (20 μL) onto the HPLC column. Products were eluted at 0.8 mL/min with K_2HPO_4 (100 mM, pH 7.0 at 4 °C) and an increasing concentration of MeOH from 5% to 60% in 16.6 min, followed by isocratic elution with 60% MeOH from t = 16.6 to 21.8 min. UV–vis products were detected at 254 nm. ATP, CoA, and dephospho-CoA standards were eluted at 3.83, 7.81, and 10.56 min, respectively. Single products were detected in complete reaction mixtures containing compounds **17** and **19**–**25** with retention times of 8.93, 5.89, 10.46, 10.08, 9.78, 6.61, 8.54, and 8.71 min, respectively. Analysis of the reaction mixture containing **18** revealed the formation of a major product with a retention time of 7.46 min and of a secondary product eluted at 10.03 min. Omission of CoaE from this reaction yielded only the product at 10.03 min and allowed its identification as the dephospho-CoA analogue derived from **2**1. N5- and N7-CoA were eluted at 12.67 and 16.86 min, respectively. No product was detected in the control reaction mixtures lacking *Ec*PanK. The formation of CoA analogues derived from compounds **4**, **5**, and **17**–**25** was monitored by HPLC at 4 °C on an AKTA Explorer 10S system (Amersham Biosciences) equipped with a micromolar RPC C2/C18 ST 4.6/100 column (Amersham Biosciences).

The in vitro incorporation of the CoA analogues into ACP was assayed by pre-incubating compounds **17**–**25** (1 mM final concentration) or DMSO (negative control) with *Ec*PanK + CoaDE (0.6 μg each) in a 40 μL reaction mixture containing Tris–HCl (50 mM, pH 7.5), ATP (5 mM), $MgCl_2$ (5 mM), and DTT (2.5 mM). Reaction mixtures were incubated for 2 h at 37 °C and then AcpS (3.5 μg) and apo-ACP (60 μg) were added to each mixture and the concentration of $MgCl_2$ was adjusted to 55 mM in a total volume of 60 μL. Reactions were incubated at 37 °C for a further 30 min and then analyzed by electrophoresis on 13% polyacrylamide gels containing 0.5 M urea.[22] Compounds **4** and **5** were included as positive controls.

References and notes

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