Probing the ligand preferences of the three types of bacterial pantothenate kinase

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Abstract

Pantothenate kinase (PanK) catalyzes the transformation of pantothenate to 4′-phosphopantothenate, the first committed step in coenzyme A biosynthesis. While numerous pantothenate antimetabolites and PanK inhibitors have been reported for bacterial type I and type II PanKs, only a few weak inhibitors are known for bacterial type III PanK enzymes. Here, a series of pantothenate analogues were synthesized using convenient synthetic methodology. The compounds were exploited as small organic probes to compare the ligand preferences of the three different types of bacterial PanK. Overall, several new inhibitors and substrates were identified for each type of PanK.

1.Introduction

Antibacterial resistance is growing to become a severe health threat in all parts of the world.[1] To address this global crisis, there is an urgent need to validate new antibacterial targets and develop novel antibacterial agents.[2] Coenzyme A (CoA) is an essential cofactor for all living organisms. It participates in over 100 different reactions in central metabolic processes such as the tricarboxylic acid cycle, lipid biosynthesis and catabolism, amino acid biosynthesis, and as carrier of the acetyl group in a range of acetylation reactions.[3,4] Pantothenate (Figure 1), also known as vitamin B_5, is the precursor for CoA biosynthesis. Its ATP-dependent phosphorylation to produce 4′-phosphopantothenate is catalyzed by pantothenate kinase (PanK), and is the first and committed step of the universal CoA biosynthetic pathway.[5] This is followed by four additional steps to finally produce CoA.[3–5] The CoA biosynthetic steps are conserved across all kingdoms, but the structure and mechanism of the enzymes involved vary significantly. The utilization of pantothenate has therefore been suggested as a novel target for the development of antimicrobials with low cytotoxicity.[6–7]

Since the discovery of pantothenate by Williams *et al.* in 1933,[8] many pantothenate antimetabolites have been reported to inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*.[7] Of these, the pantothenamides are arguably the most studied.[6–7] It has been demonstrated that their mode of action largely depends on the PanK type found in the bacterium. For bacteria harboring the promiscuous type I PanK, such as *E. coli*, pantothenamides are believed to impede growth after being transformed by the CoA biosynthetic enzymes into the corresponding CoA antimetabolites,[9–13] which subsequently may interfere with a series of essential CoA-related pathways. In *S. aureus*, the only bacterium known to express a type II PanK, the pantothenamides have been shown to act through different modes of action.[9,14–18] Apart from being transformed into inhibitory CoA antimetabolites, it has been demonstrated that the pantothenamides' inhibitory effect also results from the pantothenamides being trapped on the *S. aureus* PanK after their transformation into the corresponding 4′-phosphopantothenamides.[9,16–18] Finally, pantothenamides have no effect on bacteria that have only a type III PanK such as *Pseudomonas* spp., *Clostridium* spp., *Neisseria* spp., or *Helicobacter* spp., because the amide substituent is not accommodated in the tighter pantothenate binding sites of most type III PanKs. In fact, the binding sites of type III PanKs are very specific, and to date these enzymes have been shown to accept only pantothenate as a substrate. It is worth noting that although most bacteria carry only one type of PanK, some bacteria harbor genes for two different types of PanK; for example, some mycobacteria and bacilli have genes coding for both a type I and a type III PanK.[19]

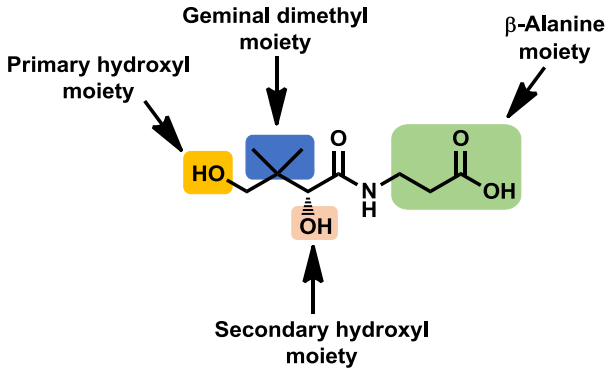

Figure 1. Pantothenic acid, showing the four sites where modifications were introduced to produce the molecules presented here.

Apart from pantothenate antimetabolites, several inhibitors have also been reported for type I and II PanKs.[9,18,20–22] In contrast, only one inhibitor of a type III PanK has been reported: an ATP mimic with a K_i of 164 ± 3 μM (a 3-fold improvement relative to the K_m of ATP which is 510 μM) for the inhibition of *Bacillus anthracis* PanK,[23] however, no bacterial growth inhibition was reported for this compound.

In light of this background, it is clear that the design of novel pantothenate antimetabolites and pantothenate-related PanK inhibitors can benefit from a better understanding of the ligand preferences of all three types of bacterial PanK. We report here new pantothenate analogues and their use as probes to study the specificity of the pantothenate binding pockets of the type I, II and III PanKs. The study of ATP binding pockets was not pursued, mainly because type III PanKs show poor affinity for ATP (with K_m values in the millimolar range), and because of the concern of generating non-specific inhibitors that may also inhibit many other ATP-dependent enzymes.

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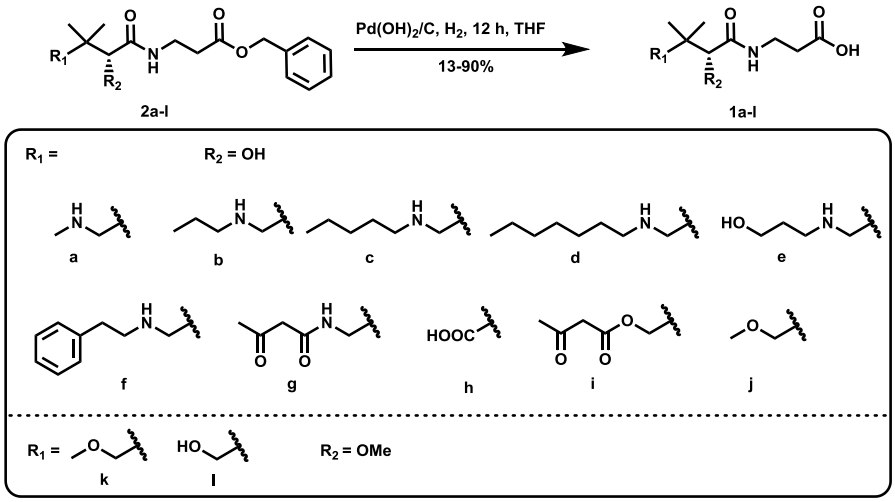
The pantothenate analogues presented here can be categorized into four classes, depending on where the modification is located (Figure 1): 1) the primary hydroxyl moiety; 2) the geminal-dimethyl group; 3) the secondary hydroxyl group; or 4) the β-alanine moiety. These modifications allowed not only the identification of novel inhibitors and substrates, but also revealed important differences in the binding preferences of the three types of PanK. This information should provide guidance in the design of ligands that selectively target a specific PanK type.

2. Results and discussion

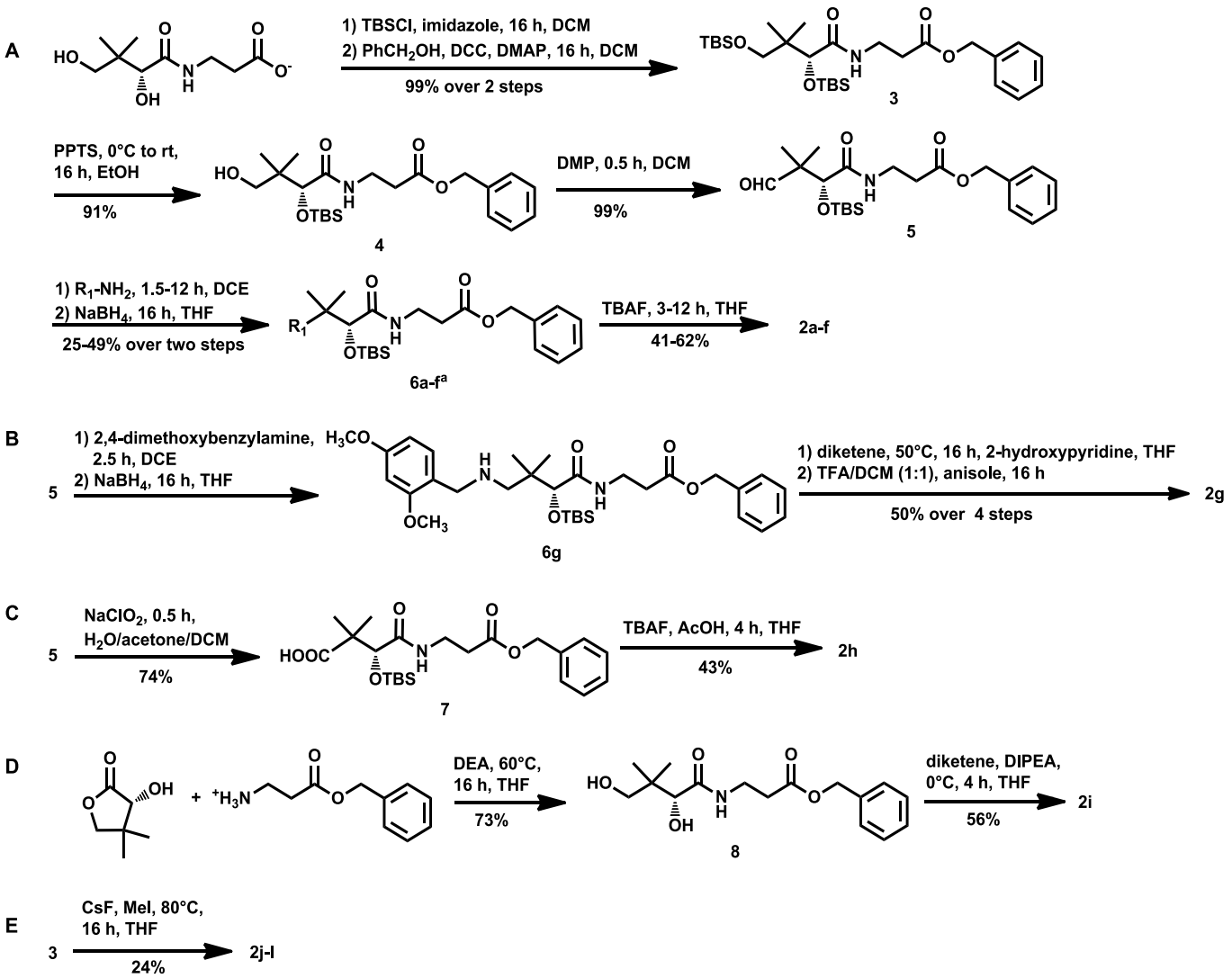
2.1 Synthesis

Pantothenate analogues **1a**–**j** that were modified at the primary hydroxyl group, and **1k**–**l** that were modified at the secondary hydroxyl group, were prepared from intermediates **2a**–**l** via benzyl deprotection in the presence of $Pd(OH)_2/C$ (Scheme 1). For the synthesis of the intermediates, D-pantothenate was used as a starting material in all cases, except in the case of **2i** where D-pantolactone was used (Scheme 2).

Intermediates **2a**–**f** were prepared as follows: the two hydroxyl groups of pantothenate were each protected with a TBS group (Scheme 2A), before protection of the carboxylate as a benzyl ester to generate compound **3**. Next, 1.1 equivalent of PPTS was used to selectively deprotect the TBS group on the primary hydroxyl group and yield compound **4**. Subsequent oxidation with DMP produced aldehyde **5**. To synthesize the amines **2a**–**f**, aldehyde **5** was condensed with the desired amine before $NaBH_4$ reduction to yield compounds **6a**–**f**. Compounds **2a**–**f** were isolated after TBAF-mediated TBS-deprotection. Similarly, **2g** was also generated via reductive amination of aldehyde **5**, but this was followed with ring opening of diketene in the presence of 2-hydroxylpyridine, and full deprotection using TFA (Scheme 2B). To synthesize the carboxylate **2h** (Scheme 2C), aldehyde **5** was oxidized to carboxylic acid **7** by Pinnick oxidation, followed by TBS deprotection using TBAF. Synthesis of **2i** (Scheme 2D) started by combining D-pantolactone and the 4-toluenesulfonate salt of β-alanine benzyl ester in the presence of diethylamine at 60°C, to produce compound **8**. This compound was next used as a nucleophile to open the ring of diketene and generate **2i**. Compounds **2j**–**l** were prepared from compound **3** (Scheme 2E). Both TBS groups were first removed using CsF, generating the negatively charged alkoxides, to which methyl iodide was added *in situ* to form **2j**–**l** in one pot.



Scheme 1. Synthesis of compounds **1a**–**l**.



Scheme 2. Synthesis of compounds **2a**–**l**. [a]R_1 is the same as in Scheme 1, except for compound **6e**, in which R_1 is . TBSCl: *tert*-butyldimethylsilyl chloride; DCC: dicyclohexylcarbodiimide; DMAP: 4-(dimethylamino)pyridine; PPTS: pyridium *p*-toluenesulfonate; DMP: Dess-Martin periodinane; TBAF: tetrabutylammonium fluoride; TFA: trifluoroacetic acid; DEA: diethylamine; DIPEA: *N*,*N*-diisopropylethylamine.

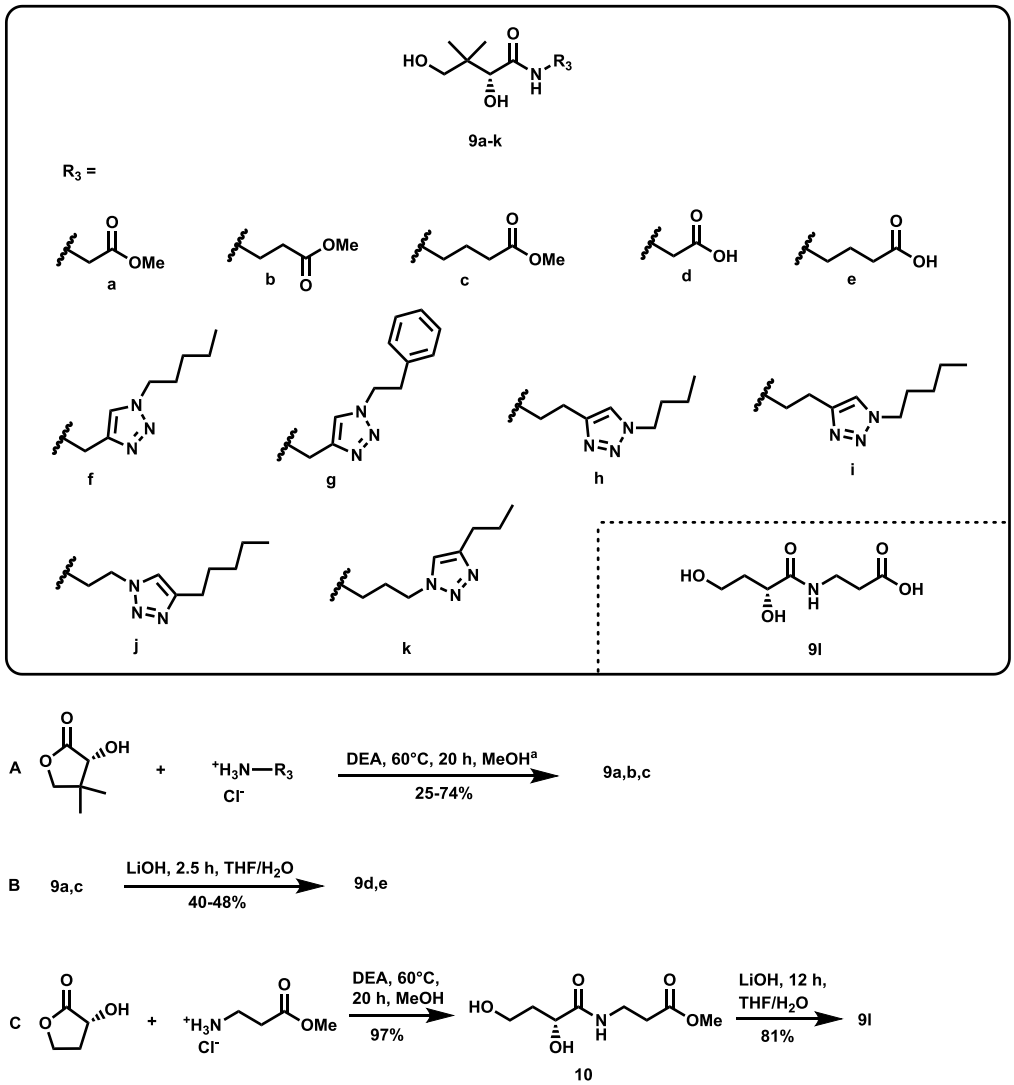
Compounds modified at the β-alanine moiety (**9a**–**k**) or at the geminal-dimethyl group (**9l**) were synthesized as shown in Scheme 3. Methyl esters **9a**–**c** and **10** were prepared directly from the relevant lactones via ring-opening with the desired amine. Hydrolysis of **9a**,**c** and **10** separately with lithium hydroxide quickly generated compounds **9d**,**e** and **9l** respectively. Compounds **9f**–**k** were designed to mimic known pantothenamides, with the triazole ring acting as a bioisostere of the amide group that is susceptible to hydrolysis by enzymes present in the serum of the host.[24] Specifically, **9f** and **9g** were designed as analogues of *N*-pentyl and *N*-phenethyl α-pantothenamide respectively, **9h** as an analogue of *N*-butyl pantothenamide (N4-Pan), **9i** and **9j** as two regioisomeric analogues of *N*-pentyl pantothenamide (N5-Pan), and **9k** as an analogue of *N*-propyl homopantothenamide.[9] They were synthesized as reported elsewhere.[25–26]

1.1. Structure-activity relationship (SAR) studies for differentiated inhibition of PanK types

For all enzyme activity and inhibition studies, *E. coli* pantothenate kinase (*Ec*PanK$_I$, *i.e.* a type I PanK), and *P. aeruginosa* pantothenate kinase (*Pa*PanK$_{III}$, *i.e.* a type III PanK) were selected as representatives to study these PanK types. *S. aureus* pantothenate kinase (*Sa*PanK$_{II}$) is the only known bacterial type II PanK, and was used here to represent this type of PanK.

We first tested all compounds that cannot act as PanK substrates because they either lack the primary hydroxyl that is phosphorylated by PanK (as is the case for compounds **1a**–**d**,**f**–**h)**, or this hydroxyl is blocked (as seen for **1i**–**k**). These compounds were screened at a fixed concentration of 200 μM to determine their ability to inhibit the activity PanK enzymes acting on 25 μM (for *Ec*PanK$_I$ and *Sa*PanK$_{II}$) or 30 μM (for *Pa*PanK$_{III}$) pantothenate. As shown in Figure 2, compound **1g** exhibits weak inhibition of all three PanK types. We propose that the acetoacetamide moiety of **1g** may mimic the polar diphosphate group of ATP in the ternary complex or the transition state. The acetoacetate group has previously been shown to mimic diphosphate moieties.[27] For *Ec*PanK$_I$, compounds **1c**,**f**,**j** also show some inhibition, and **1a** to a lesser extent, suggesting that this enzyme can tolerate either N or O at the primary alcohol position. In contrast, *Pa*PanK$_{III}$ seemed to accommodate an amino group at this carbon better than ethers (e.g. **1a** vs **1j**). Interestingly, the diacid **1h** shows inhibition only for *Sa*PanK$_{II}$, even though the crystal structure of *Sa*PanK$_{II}$ bound to 4′-phospho-N5-Pan (PDB: 4M7Y) does not show any residues in the vicinity of the introduced carboxylate that would likely form stabilizing interactions with this group.[17]

When compounds **1e**,**l** and **9a**–**l** were studied as potential inhibitors of the PanK enzymes, none of them showed any inhibition at ≥100 μM. These compounds were also characterized for their ability to act as substrates of the respective PanKs (*vide infra*).



Scheme 3. Synthesis of compounds **9a**–**l**. [a]Alternatively: 115°C for 2 hours in a microwave reactor.

2.3 SAR studies for PanK types showing differentiated activity

All the compounds containing a primary alcohol at (**1l**, **9a**–**l**) or near C-4′ (**1e**) were next tested for their ability to act as substrates of the three PanKs. In those cases where activity was observed, the relevant kinetic parameters were determined and the results are summarized in Table 1.

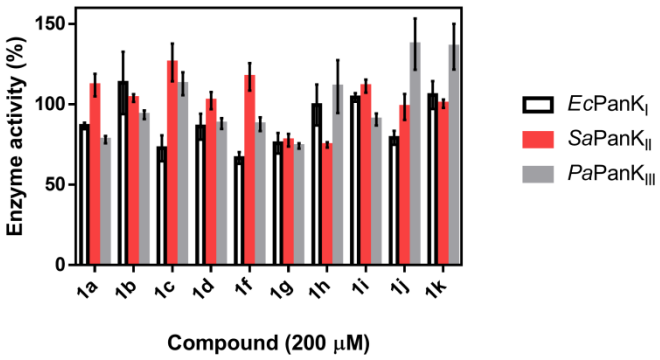


Figure 2. Activity of the respective PanK enzymes acting on 25 μM (for *Ec*PanK$_I$ and *Sa*PanK$_{II}$) or 30 μM (for *Pa*PanK$_{III}$) pantothenate in the presence of 200 μM of the indicated compounds **1a**–**d**,**f**–**k**. The activity is reported relative to that of a control reaction without inhibitor, which was set at 100%.

Table 1. Kinetic parameters of the PanK enzymes acting on compounds **1e**,**l**, **9a**–**l** as substrates.[a]

	Ec PanK$_I$			*Sa* PanK$_{II}$			*Pa* PanK$_{III}$		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}.s^{-1}$)
Pantothenate	17 ± 1	1.29 ± 0.02	76 ± 5	19 ± 1	2.16 ± 0.05	115 ± 10	20 ± 5	0.39 ± 0.01	20 ± 5
N5-Pan	36 ± 3[26]	0.93 ± 0.04[26]	26 ± 2[26]	<1.5[9]	0.44 ± 0.03[9]	>290[9]			
1e									
1l	16 ± 3	0.49 ± 0.02	31 ± 6						
9a	156 ± 26	0.91 ± 0.04	5.8 ± 1.0	36 ± 2	4.0 ± 0.1	115 ± 12			
9b	292 ± 65	0.80 ± 0.06	2.7 ± 0.6	ns	ns	8.3 ± 0.2[b]			
9c				ns	ns	7.1 ± 0.2[b]			
9d	62 ± 12	1.59 ± 0.07	26 ± 5						
9e				60 ± 2	3.4 ± 0.1	57 ± 3	683 ± 72	0.98 ± 0.04	1.4 ± 0.2
9f	190 ± 20[26]	0.96 ± 0.04[26]	5.1 ± 0.6[26]	64 ± 5	3.4 ± 0.1	54 ± 6			
9g	25 ± 3	1.09 ± 0.02	44 ± 5	126 ± 29	4.7 ± 0.5	37 ± 12			
9h	50 ± 8	1.08 ± 0.04	22 ± 4	7.1 ± 0.4	0.82 ± 0.02	115 ± 9			
9i	33 ± 5	0.72 ± 0.01	22 ± 3	6.8 ± 0.7	0.73 ± 0.04	107 ± 16			
9j	21 ± 5	1.11 ± 0.06	53 ± 13	8.5 ± 1.2	0.82 ± 0.05	97 ± 19			
9k	1000 ± 200[26]	0.49 ± 0.06[26]	0.49 ± 0.11[26]	369 ± 381	7.6 ± 4.3	21 ± 33			
9l							64 ± 14	0.32 ± 0.02	5.0 ± 1.1

[a]Blank cells mean that the particular PanK did not show activity toward the indicated compound under the tested conditions;

[b]value of $k_{cat}/K_{0.5}$ estimated from the linear slope ($v = k_{cat}/K_{0.5}\cdot[E]\cdot[S]$ when $[S] << K_{0.5}$);

ns: not saturated at 200 μM.

Compound **1e** contains a primary hydroxyl group which is shifted four atoms away from that of the natural substrate. It was not found to act as a substrate for any of the PanK tested. On the other hand, when the secondary hydroxyl group of pantothenate is blocked with a methyl substituent, as in the case of **1l**, the molecule was only accepted as an *Ec*PanK$_I$ substrate, with a K_m value similar to that of pantothenate. This suggests that methylation does not significantly disrupt the interaction between this hydroxyl group and His-177 of *Ec*PanK$_I$ (Figure 3A), and that there is space for modifications near this secondary hydroxyl group.[28] The crystal structure of *Sa*PanK$_{II}$ bound to 4′-phospho-N5-Pan (Figure 3B) does not indicate any interactions between the secondary hydroxyl group and the enzyme, suggesting that the lack of activity is

likely due to steric hindrance. In *Pa*PanK$_{III}$, the secondary hydroxyl group of pantothenate forms two hydrogen bonds with the carboxylate of Asp-101.[29] In this case, methylation likely disrupted at least one of these interactions, and/or cannot be accommodated in the tight active site, leading to no activity being observed for **1l**. Overall, these differences in how the PanK enzymes interact with the secondary hydroxyl group of the pantothenoyl moiety suggests that modifications at this position may allow for the development of pantothenate antimetabolites or inhibitors that are selective for *Ec*PanK$_I$.

When modifications were introduced at the β-alanine end of the molecule (e.g. **9a**–**k**), both *Ec*PanK$_I$ and *Sa*PanK$_{II}$ accepted most of these molecules as substrates, while *Pa*PanK$_{III}$ did not. This was expected based on results with pantothenamides which are often tolerated by *Ec*PanK$_I$ and *Sa*PanK$_{II}$, but not by *Pa*PanK$_{III}$.[9] Only the carboxylate **9e** was found to act as a substrate for *Pa*PanK$_{III}$, which might be explained by the flexibility of the three-carbon linker present in compound **9e** (compared to the two-carbon linker in the β-alanine moiety of pantothenate), which may allow **9e** to be accommodated in the binding pocket without radically disturbing the interactions with the carboxylate group. The importance of these interactions is highlighted by the finding that simply converting the acid to a methyl ester (as in **9b**) was sufficient to decrease activity with *Pa*PanK$_{III}$ below detection, indicating that the interactions between the terminal carboxylate group of pantothenate and Thr-180′, Tyr-92, Arg-102 and Gly-99 residues (Figure 3C) contribute a large proportion of the affinity. For *Ec*PanK$_I$, both one or two carbon-linkers between the amide and the carboxylate are well tolerated, as demonstrated with compounds **9a**,**b**,**d** and **9f**–**j**. These are all good *Ec*PanK$_I$ substrates, a finding that is in agreement with previous studies of pantothenamide analogues.[9] In contrast, the compounds containing a three-carbon-linker (**9c**,**e**,**k**) are poorly or not accepted as substrates by *Ec*PanK$_I$, in agreement with earlier studies of **9e**.[28,30–31] This suggests that regardless of the identity of the terminal group (whether a methyl ester in **9a**–**c**, a carboxylate in **9d**–**e**, or a substituted triazole in **9f**–**k**), the linker length is determinant for binding affinity to *Ec*PanK$_I$. The tolerance of this enzyme for methyl esters such as **9a**,**b** can be rationalized in light of the crystal structure of the pantothenate–*Ec*PanK$_I$ complex (PDB: 1SQ5), which shows that only one oxygen in the carboxylate group of pantothenate is involved in hydrogen bonds with the side chains of residues Tyr-240 and Asn-282 (Figure 3A).[28] In contrast, the crystal structure of the pantothenate–*Pa*PanK$_{III}$ complex (PDB: 2F9W) suggests that both oxygen atoms of the carboxylate group of pantothenate participate in hydrogen bonds with the protein (Figure 3C).[29] In the case of *Sa*PanK$_{II}$, the enzyme was found to tolerate substrates with a one- (**9a**,**f**,**g**), two- (**9b**,**h**–**j**), or three-carbon-linker (**9c**,**e**,**k**), in agreement with previous reports for **9e**.[30–32] The only exception is the free acid **9d**, with a one-carbon-linker; however, its analogous methyl ester (**9a**) is accepted, indicating that for the shorter compounds ionization interferes with binding.

Several important features of the *Sa*PanK$_{II}$ pantothenate binding site were recently discovered in a SAR study of pantothenamides.[17] This study defined a binding pocket in which the *N*-substituent of these compounds are accommodated, and in which several hydrophobic, π-stacking and hydrogen bonding interactions are possible. Apart from these interactions, a recent study on modifications to the pantothenoyl amide indicated that interactions with this amide are also crucial for determining activity with *Sa*PanK$_{II}$.[18] Interestingly, we show here that the same SARs apply when a triazole group is used as an amide bioisostere (e.g. compounds **9f**–**k**). The results show that triazoles attached to the pantoyl amide with two carbon-linkers (**9h**–**j**) show high affinity for the enzyme (low $K_{0.5}$ values), but poor turnover (low k_{cat} values). This is similar to what is observed with the corresponding pantothenamides that were found to be phosphorylated and then trapped in *Sa*PanK$_{II}$'s active site as the phosphorylated products.[17] In contrast, the triazoles attached to the pantothenoyl amide via a one or three carbon-linker (**9f**,**g**,**k**) show much better turnover, although at the cost of significantly reduced affinity. This suggests that due to their comparatively poor interactions with the enzyme, these compounds are phosphorylated but then released. Docking results of compounds **9f**, **9h** and **9k** (which only differ in the relative location of the triazole) with *Sa*PanK$_{II}$ (PDB 4M7Y) are in agreement with our enzymatic data. They suggest that all three compounds can be accommodated in the binding pocket, with compound **9h** showing the lowest ΔG and a better alignment for hydrogen bonds between the enzyme's active site residues and the triazole N-3 and CH, consistent with the triazole acting as a functional mimic of the amide bond (Figure S1).

A) *Ec*PanK$_I$ B) *Sa*PanK$_{II}$ C) *Pa*PanK$_{III}$

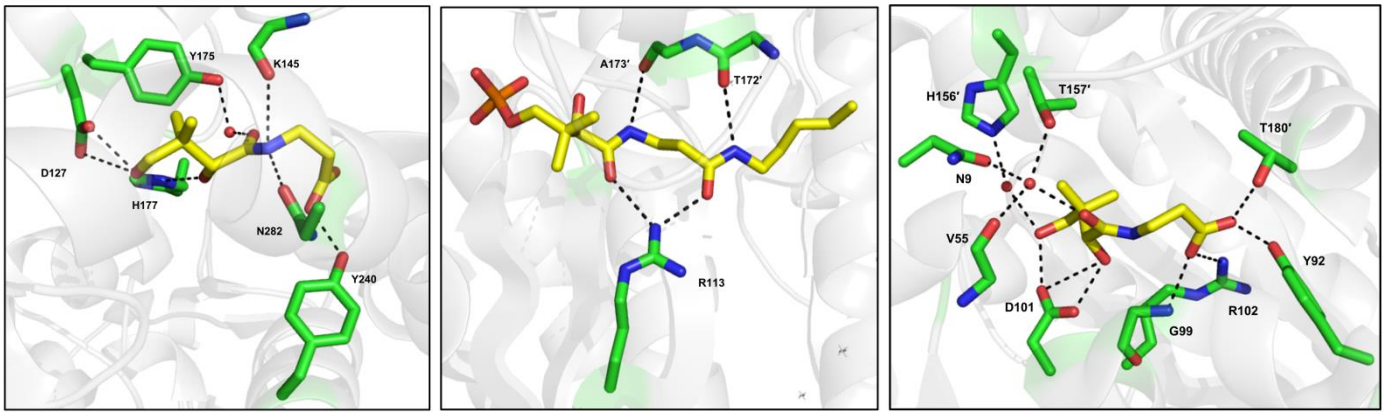


Figure 3. Crystal structures of PanKs emphasizing ligand interactions observed in some crystallized complexes. A) *Ec*PanK$_{I}$ in complex with pantothenate (PDB: 1SQ5);[28] B) *Sa*PanK$_{II}$ in complex with phosphorylated N5-Pan (PDB: 4M7Y);[17] C) *Pa*PanK$_{III}$ in complex with pantothenate (PDB: 2F9W).[29]

It is worth noting that although the *N*-substituent binding pockets of *Ec*PanK$_{I}$ and *Sa*PanK$_{II}$ are both able to accommodate a range of variations—an advantage for the design of pantothenate antimetabolites—our results demonstrate that it is possible to target either enzyme specifically. An example of such targeted specificity is seen for compound **9c** that is only a substrate for *Sa*PanK$_{II}$, while **9d** is only acted on by *Ec*PanK$_{I}$.

Finally, only *Pa*PanK$_{III}$ tolerated the removal of both geminal methyl groups of pantothenate. Compound **9l** was accepted as a substrate by *Pa*PanK$_{III}$, although with some loss in affinity. This is consistent with the binding pocket of *Pa*PanK$_{III}$ being tight and more dependent on hydrogen bonding and ionic interactions than hydrophobic ones, while the opposite trend is observed for *Ec*PanK$_{I}$ and *Sa*PanK$_{II}$. This result also implies that it may be possible to design *Pa*PanK$_{III}$-specific pantothenate antimetabolites and/or inhibitors.

1.2. Antimicrobial activity

The antibacterial activity of compounds **1a**–**l** and **9a**–**l** against *E. coli* (Gram-negative with a type I PanK), *S. aureus* (Gram-positive with a type II PanK) and *P. aeruginosa* (Gram-negative with a type III PanK) was next measured in rich media. *Enterococcus faecium* was also included in the antibacterial study, as an example of a Gram-positive bacterium expressing a type I PanK. No potent growth inhibitors were discovered, suggesting that any inhibition of the PanKs or other CoA-dependent targets were likely offset by the pantothenate present in the rich media. Nonetheless, the results obtained with *S. aureus* are interesting (Table S1). The most active compounds were found to be triazoles **9h**–**j** that all have a two carbon-linker between the triazole and the pantoyl amide.[25] These compounds were designed to mimic N5-Pan and N4-Pan, two known pantothenamides with antibacterial activity against *S. aureus* (Figure 4).[9,25,33] In comparison, the triazoles with a one carbon-linker (**9f**,**g)** or with a three carbon-linker (**9k**) show negligible growth inhibition (Table S1). This finding is in agreement with the analysis that the compounds showing antistaphylococcal activity bind to enzyme in a manner that leads to the trapping of the phosphorylated products on the enzyme (*i.e.* compounds show high affinity and slow turnover)—as is the case for the antistaphylococcal N5-Pan.[17–18] Compounds **9a**,**e** show better antistaphylococcal activity than **9b**–**d**,**l** (Table S1). Since compounds **9a**,**e** are better *Sa*PanK$_{II}$ substrates than **9b**–**d**,**l** (Table 1), their superior antistaphylococcal activity might be related to their better activity as *Sa*PanK$_{II}$ substrates, which would likely translate into a more efficient formation of the corresponding CoA antimetabolites. These compounds will cause growth inhibition by interfering with downstream CoA-related pathways.

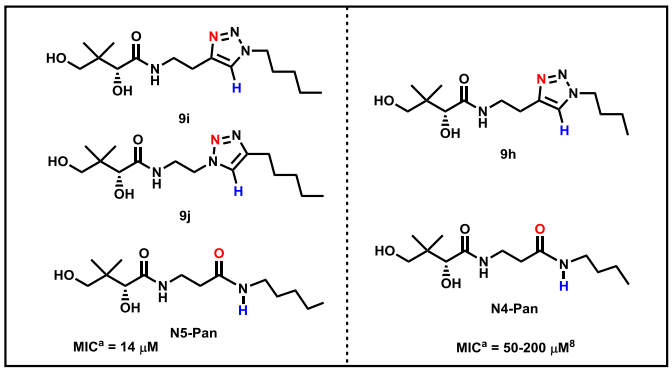


Figure 4. Two-carbon linker triazoles possibly mimicking the pantothenamides N5-Pan and N4-Pan. Red highlights the hydrogen bond acceptors, and blue highlights the hydrogen bond donors. [a]MICs shown are for *S. aureus* in rich medium.

3. Conclusion

Overall, a series of pantothenate analogues modified at different positions were synthesized with convenient synthetic methodology. Various substituents were introduced at the primary hydroxyl group; which led to a molecule, **1g**, able to inhibit all three PanK types. Specific inhibitors were also discovered for two of the PanK types: **1c**,**f**,**j** for *Ec*PanK$_{I}$ and **1h** for *Sa*PanK$_{II}$. Our results suggest that when modifications are introduced at the primary hydroxyl group of pantothenate, *Sa*PanK$_{II}$ might prefer negatively charged moieties. In contrast, *Ec*PanK$_{I}$ has no preference between nitrogen or oxygen at this position, while *Pa*PanK$_{III}$ prefers a nitrogen.

Modifications at the secondary hydroxyl group of pantothenate allow selectivity for *Ec*PanK$_I$. Interestingly, when the geminal dimethyl groups of pantothenate were replaced with hydrogen atoms, the interaction of the resulting molecule (**9l**) with both *Ec*PanK$_I$ and *Sa*PanK$_{II}$ were greatly affected, while the effect was small for *Pa*PanK$_{III}$, yielding a substrate that is selective for this enzyme.

Modifications at the β-alanine moiety of pantothenate was a more versatile strategy to differentiate activity and produced both molecules that are specific for one type of PanK, as well as compounds accepted as substrates by more than one PanK type. Consistent with the crystal structures,[28–29] the kinetic profiles of these compounds imply that the pocket of *Pa*PanK$_{III}$ around the terminal carboxylate of pantothenate is fairly tight and well-defined, while that of *Ec*PanK$_I$ and *Sa*PanK$_{II}$ is more promiscuous, with the latter likely larger.

In recent years, research has revealed the important role of the gut microbiome in health and disease. It is increasingly recognized that broad-spectrum antibacterial agents lead to severe imbalances in the normal gut microbiome, which is linked to various diseases,[34] and even to secondary infections by *Clostridium difficile* (which harbors a type III PanK).[35] The advantages of narrow-spectrum antibacterials are evident.[36] The utilization of pantothenate has been suggested as a possible target for the development of new antimicrobials.[6–7] As a key enzyme in the CoA biosynthetic pathway, PanK has attracted increasing interest in recent years. Our use of pantothenate analogues as probes to study the ligand preferences of PanK suggests possible paths to design inhibitors or antimetabolites selective for an organism with a particular type of PanK.

Competing interests statement

The authors declare no competing interests.

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Supplementary Material

The following file is available free of charge. Supporting Information.docx contains experimental protocol, compound characterization data and ^{1}H NMR and ^{13}C NMR spectra.

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