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Exploring structural motifs necessary for substrate binding in the active site of *Escherichia coli* pantothenate kinase

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ABSTRACT

The coenzyme A (CoA) biosynthetic enzymes have been used to produce various CoA analogues, including mechanistic probes of CoA-dependent enzymes such as those involved in fatty acid biosynthesis. These enzymes are also important for the activation of the pantothenamide class of antibacterial agents, and of a recently reported family of antibiotic resistance inhibitors. Herein we report a study on the selectivity of pantothenate kinase, the first and rate limiting step of CoA biosynthesis. A robust synthetic route was developed to allow rapid access to a small library of pantothenate analogs diversified at the β-alanine moiety, the carboxylate or the geminal dimethyl group. All derivatives were tested as substrates of *Escherichia coli* pantothenate kinase (*Ec*PanK). Four derivatives, all *N*-aromatic pantothenamides, proved to be equivalent to the benchmark *N*-pentylpantothenamide (N5-pan) as substrates of *Ec*PanK, while two others, also with *N*-aromatic groups, were some of the best substrates reported for this enzyme. This collection of data provides insight for the future design of PanK substrates in the production of useful CoA analogues.

1. Introduction

The biosynthesis of coenzyme A (CoA), an essential cofactor, is a process that is ubiquitously present in all living organisms. It is most commonly a five-step process from pantothenate (vitamin B_5). Pantothenate kinase (PanK, EC 2.7.1.33) is the first and rate-limiting step of this process and is characterized by the ATP-dependent transformation of pantothenate to phosphopantothenate,[1] as shown in Figure 1. Three main types of PanK have been identified, namely Types I, II and III, based on differences in structural fold, regulation, substrate affinity, and specificity.[2] Type I PanK enzymes, typically associated with bacteria, have gained significant interest due to the low sequence similarity to their human counterparts.[3] Their structural and amino acid sequence similarity is considered high (52–91% similarity), with many predicted active site residues conserved among all isoforms (Fig. S1). Numerous studies have focused on developing PanK inhibitors and substrates.[4] Of these, pantothenamides are particularly interesting due to their antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.[5–10] Although the bacteriostatic effect of pantothenamides has been recognized since the 1950s, their exact mechanism of action in bacteria has only been clarified in the last 15 years.[5–15] Interestingly, pantothenamides are substrates of *E. coli* PanK (*Ec*PanK). Their lethal effect on bacteria stems, at least in part, from their biotransformation by the CoA biosynthetic pathway into the corresponding CoA analogues. The resulting antimetabolite proceeds to

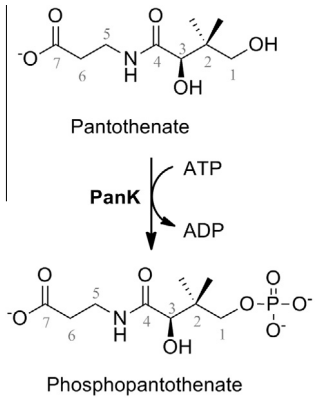

Figure 1. Reaction scheme for the PanK-catalyzed phosphorylation of pantothenate to phosphopantothenate.

Abbreviations: CoA, coenzyme A; PanK, pantothenate kinase; *Ec*PanK, PanK isoform from *Escherichia coli*; *Mt*PanK, PanK isoform from *Mycobacterium tuberculosis*; *Cb*PanK, PanK isoform from *Coxiella burnetii*; *Kp*PanK, PanK isoform from *Klebsiella pneumonia.*

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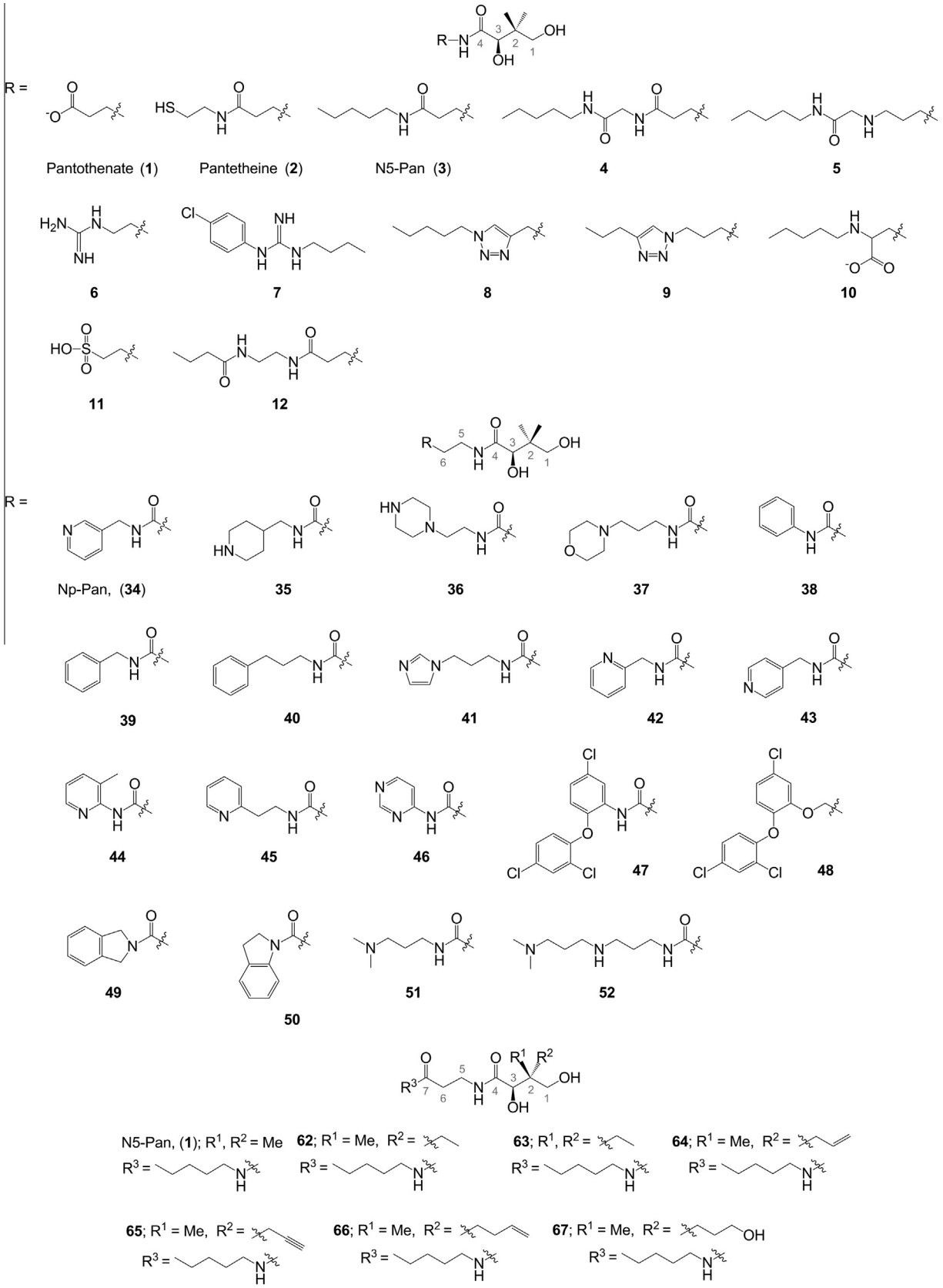


Figure 2. Pantothenate analogues described in this work.

inhibit downstream pathways, such as fatty acid biosynthesis.[8,16] Other studies have suggested that pantothenamides may, in some strains, act as inhibitors of the CoA biosynthetic pathway.[5,16]

The importance of CoA analogues not only stems from their antibacterial activity, but also from their demonstrated use as chemical biology tools and as mechanistic probes of CoA-depen-

dent enzymes. These applications include both *in vitro* and in-cell labelling of carrier proteins in polyketide and nonribosomal peptide synthesis,[17–19] as well as aminoglycoside resistance inhibitors activated by the CoA biosynthetic pathway.[20]

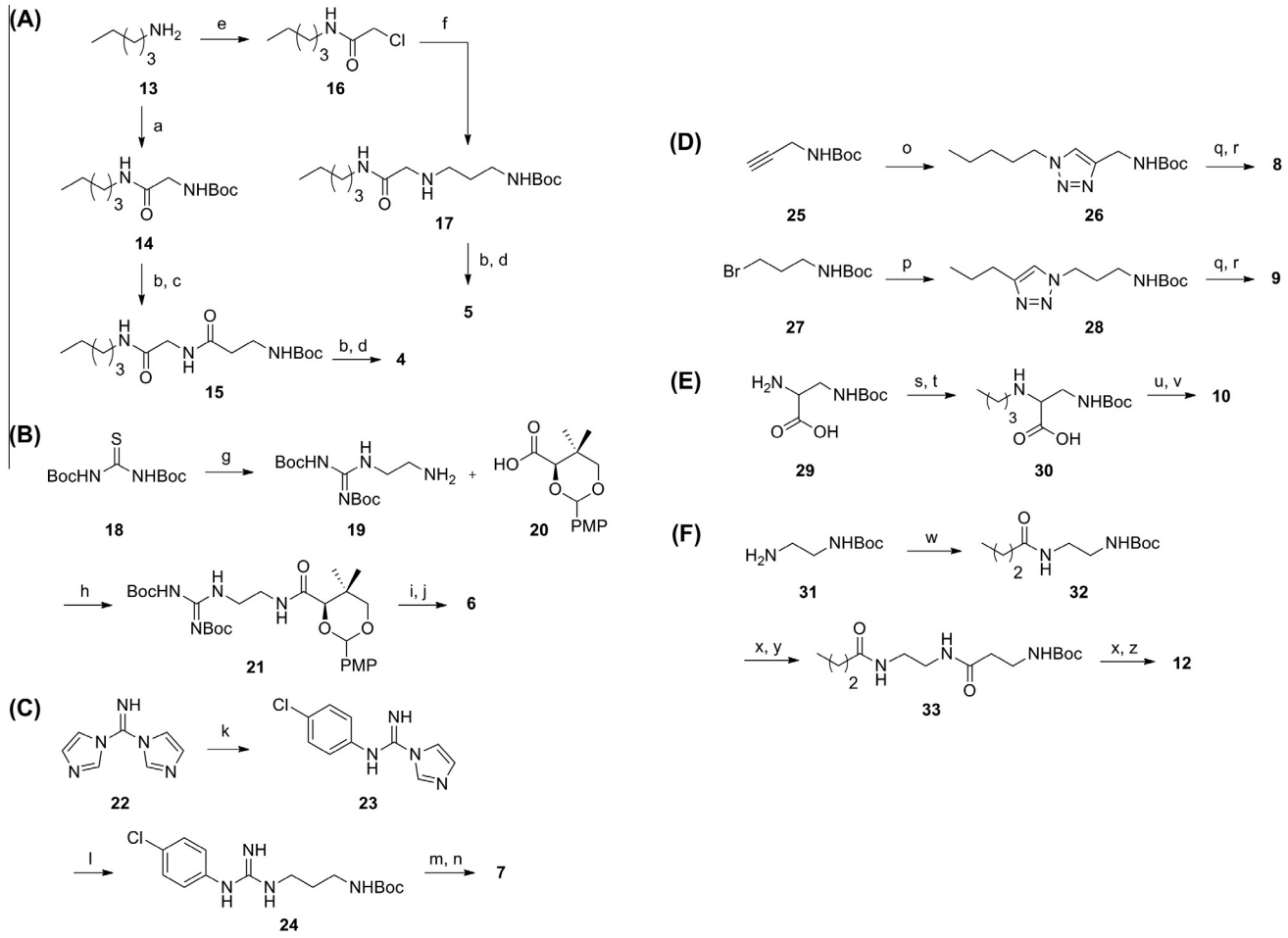
Although a wide range of pantothenamide derivatives have been reported over the years, most of these derivatives are modified at the amine moiety and less is known about the effects of modifications at other positions. Given the current and potential utility of pantothenate derivatives, a greater understanding of their effect on PanK is beneficial to future chemical biology and medicinal chemistry research. We report here on the synthesis of a library of pantothenate analogues modified at the β-alanine, the carboxylate or the geminal dimethyl group (Fig. 2), and on their structure–activity relationships as *Ec*PanK substrates.

2. Results

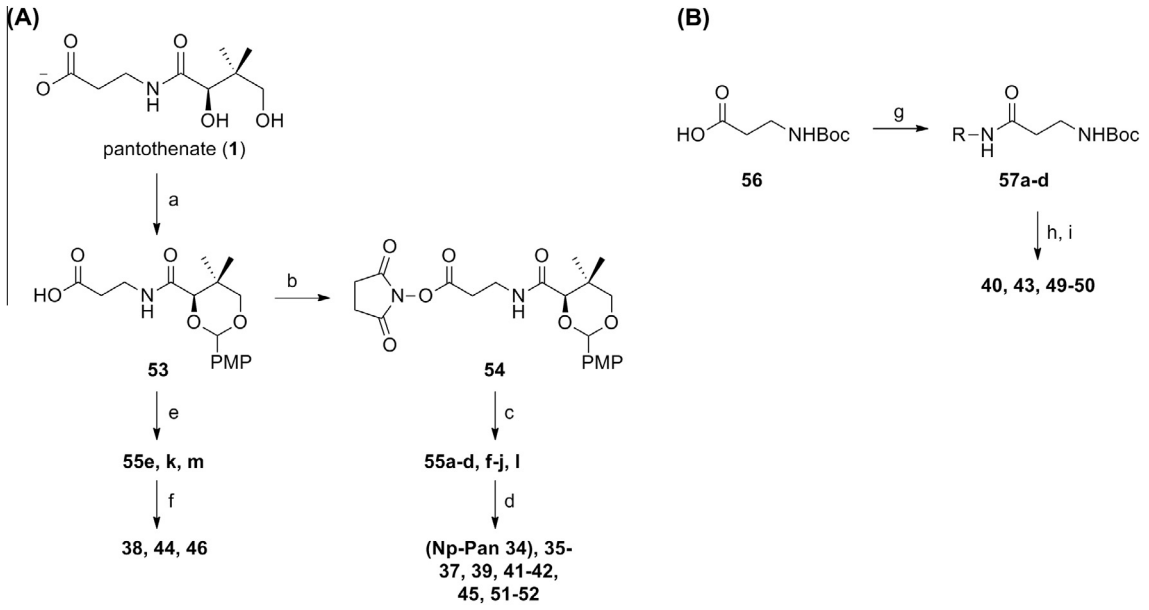
2.1. Design and synthesis

2.1.1. Compounds 4–12

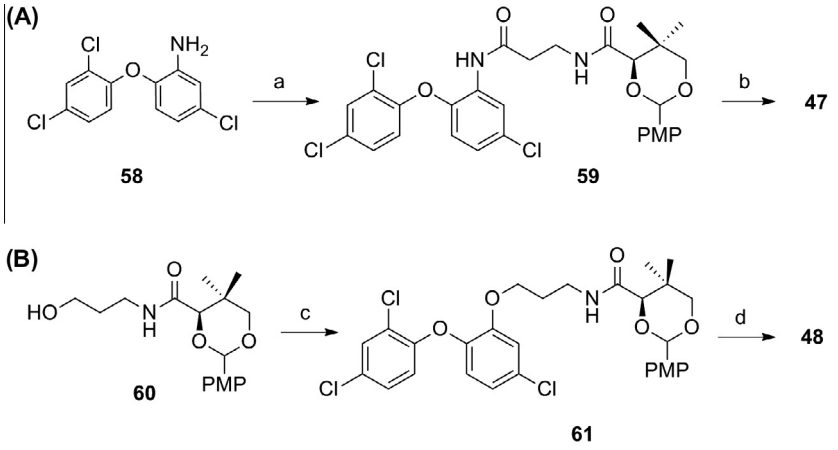
Compounds **4**–**12** are designed to probe the hydrogen bonds bridging residues Asn282 and Tyr240 to the C-7 carbonyl group of pantothenate in *Ec*PanK. Their importance was suggested by studies with CoA analogues used for carrier protein labelling studies, where C-7 carbonyl group substitutions often showed detrimental effects.[18] Synthesis of **4** and **5** (Scheme 1A) began with first obtaining the amine required for D-pantolactone ring opening. Coupling of 1-pentylamine (**13**) with Boc-glycine yielded **14**, which was coupled with Boc-β-alanine to form the Boc protected amine **15**. For the preparation of **5**, the reaction of **13** with chloroacetyl chloride first produced **16**, to which was added *tert*-butyl-3-aminopropylcarbamate to yield the Boc-protected amine **17**. With **15** and **17** in hand, subsequent Boc-deprotection followed by D-pantolactone addition afforded the desired compounds **4** and **5**. In the synthesis of guanidine-containing **6** (Scheme 1B), *N*,*N*′-di-Boc-thiourea (**18**) was first reacted with ethylenediamine to produce **19**, followed by coupling to **20** as previously reported[21] to yield **21**. Final Boc and PMP deprotection afforded **6**. For the *p*-chloroaniline *N*-linked guanidine-containing **7** (Scheme 1C), imidazole was first reacted with cyanogen bromide to form **22**.[22] Reaction of **22** with *p*-chloroaniline generated the guanidine derivative **23**, which upon addition of Boc-protected propane-1,3-diamine yielded intermediate **24**. Boc-deprotection of **24** and D-pantolactone addition finally afforded **7**. The assembly of triazole-containing derivatives **8** and **9** (Scheme 1D) involved 1,3-dipolar cycloadditions with *in situ* azide formation to form intermediates



Scheme 1. Reagents and conditions: (a) Boc-glycine, EDC, HOBt, DIPEA, THF, rt, 16 h, 94%; (b) TFA, DCM, rt, 1 h; (c) Boc-β-alanine, EDC, HOBt, DIPEA, THF, rt, 16 h, 60% over 2 steps; (d) D-pantolactone, EtOH, TEA, 80 °C, 16 h, 40% over 2 steps; (e) chloroacetyl chloride, THF, rt, 16 h, 89%; (f) *tert*-butyl 3-aminopropylcarbamate, K_2CO_3, DMF, 50 °C, 16 h, 60%; (g) ethylenediamine, DMF, rt, 30 min, 82%; (h) EDC, DIPEA, HOBt, THF, rt, 16 h, 79%; (i) TFA, DCM, rt, 3 h; (j) 80% AcOH, rt, 16 h, 66% over 2 steps; (k) *p*-chloroaniline, THF, rt, 12 h, 88%; (l) Boc-propane-1,3-diamine, THF, 65 °C, 24 h, 86%; (m) TFA, DCM, rt, 1 h; (n) D-pantolactone, EtOH, TEA, 75 °C, 24 h, 59% over 2 steps; (o) 1-bromopentane, NaN_3, $CuSO_4{\cdot}5H_2O$, sodium ascorbate, DMF, H_2O, 100 °C, 16 h, 81%; (p) 1-pentyne, NaN_3, $CuSO_4{\cdot}5H_2O$, sodium ascorbate, DMF, H_2O, 100 °C, 16 h, 26%; (q) TFA, DCM, rt, 1 h; (r) D-pantolactone, EtOH, NEt_3, 75 °C, 16 h, 14–46% over 2 steps; (s) pentanal, 1% AcOH/MeOH, rt, 5 h; (t) $NaBH_4$, MeOH, 0 °C–rt, 1 h; (u) TFA, DCM, rt, 1 h; (v) D-pantolactone, EtOH, NEt_3, 75 °C, 24 h, 52% over 4 steps; (w) butyric acid, EDC, HOBt, DIPEA, THF, rt, 16 h, 53%; (x) TFA, DCM, rt, 1 h; (y) Boc-β-alanine, EDC, DMAP, DIPEA, THF, rt, 16 h, 64% over 2 steps; (z) D-pantolactone, EtOH, NEt_3, 75 °C, 16 h, 11% over 2 steps.



Scheme 2. Reagents and conditions: (a) $PMPCH(OMe)_2$, CSA, DMF, rt, 16 h, 75%; (b) NHS, DCC, THF, rt, 6–24 h, 90%; (c) R-NH_2, DCM, rt, 16 h, 69–94%; (d) 80% AcOH, rt, 16 h, 71–94%; (e) microwave, HATU, DIPEA, DMF, 60 °C, 30 min; (f) 80% AcOH, rt, 16 h, 82–89% over 2 steps; (g) R-NH_2, EDC, HOBt, DIPEA, THF, rt, 16 h, 60–98%; (h) TFA, DCM, rt, 1 h; (i) D-pantolactone, EtOH, NEt_3, 75 °C, 16 h, 10–53% over 2 steps.



Scheme 3. Reagents and conditions: (a) Compound **53**, HATU, DMF, DIPEA, 80 °C, 16 h, 72%; (b) 80% AcOH, rt, 16 h, 90%; (c) triclosan, Ph_3P, DIAD, THF, rt, 24 h; (d) 80% AcOH, rt, 16 h, 50% over 2 steps.

26 and **28**, respectively. Deprotection and D-pantolactone addition then afforded **8** and **9**. Carboxylate-containing pantothenamide **10** (Scheme 1E) was assembled by first reductive amination of 3-*N*-Boc protected 2,3-diaminopropanoic acid **29** with pentanal to afford **30**, which was needed for nucleophilic ring opening of D-pantolactone following Boc deprotection. Synthesis of the known sulfonate-containing **11** used commercially available taurine (after NaOH treatment and precipitation) in the nucleophilic ring opening of D-pantolactone to yield **11**, as previously reported.[23] Finally, compound **12** (Scheme 1F) was prepared from two subsequent amide couplings of Boc-protected ethylenediamine (**31**) with butyric acid and with Boc-β-alanine to afford **33**. Deprotection of the Boc group followed by D-pantolactone addition yielded **12**.

2.1.2. Compounds 35–52

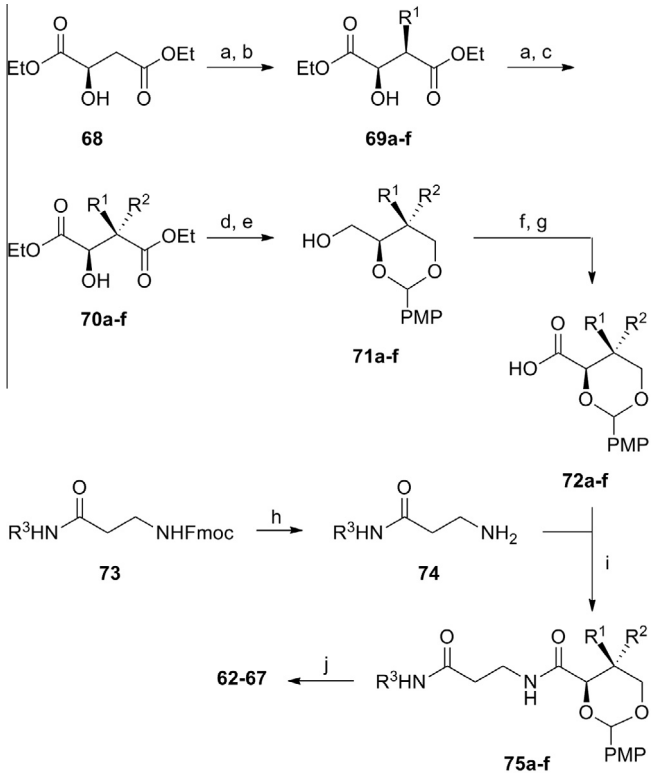
The recently reported crystal structure of *Kp*PanK bound to Np-Pan (**34**) suggests that PanK substrates may interact with the enzyme via π–π interactions with nearby Tyr180 and Phe259, and potentially with Tyr240, Tyr258, and Tyr262.[24] As such, derivatives **35**–**52** were synthesized and used in this study to probe the potential of pi–pi interactions with PanK. Np-Pan (**34**) was also re-synthesized because no kinetic data were reported.[24] For **34**, **35**–**39**, **41**–**42**, **44**–**46**, and **51**–**52** (Scheme 2A), synthesis started with the 1,3-diol protection of commercially available pantothenate as a *p*-methoxyphenyldimethylacetal to yield **53**, followed by activation to a *N*-succinamyl pantothenic ester **54**.[25] The desired commercially available amines were then reacted separately with **54**, followed by dilute acidic removal of the acetal protecting group to afford the *N*-linked derivatives **34**, **35**–**37**, **39**, **41**–**42**, **45**, and **51**–**52** in good yields. Deactivated amines such as aniline, 3-methylpyridin-2-amine, and 4-aminopyrimidine under standard reaction conditions resulted in lower yields (10–20%) of **38**, **44**, and **46**, respectively. However, under microwave irradiation at 60 °C for 30 min and using HATU as the coupling agent, higher yields (82–89%) of **38**, **44**, and **46** were achieved. In our hands, previous synthetic protocols for pantothenamide analogues using diphenylphosphorylazide to activate pantothenate were found to generate diphenyl hydrogen phosphate as a byproduct which

contaminated the final product.[7] For the synthesis of **40**, **43**, and **49**–**50** (Scheme 2B), Boc-β-alanine (**56**) was first coupled to the desired commercially available amine to produce **57a**–**d**. Subsequent deprotection followed by D-pantolactone addition then afforded the desired compounds **40**, **43**, and **49**–**50**.

Two approaches were envisaged to assemble the triclosan derivatives **47** and **48** (Scheme 3). Synthesis of the amide-linked **47** was achieved from amine **58** which was synthesized according to a literature protocol.[26] Subsequent coupling of **58** to **53** afforded **59**, followed by deprotection to yield **47**. Synthesis of the ether-linked triclosan derivative **48** on the other hand began with PMP-protected D-pantothenol **60**,[25] which underwent a Mitsunobu reaction with triclosan to afford **61**. PMP deprotection yielded the desired product **48**.

2.1.3. Compounds 62–67

Finally, a previous study with C-2 geminal dialkylpantothenamides suggests that they might be good PanK substrates.[11] Crystal structure analysis points to possible interactions of the alkyl group with a hydrophobic pocket consisting of Val97, Leu201, and Ile290. Compounds **62**–**67** were synthesized to probe this pocket and study consequent effects on substrate activity. Synthesis of C-2 geminal dialkylpantothenamide derivatives **62**–**67** was achieved by adapting a protocol previously reported by our group (Scheme 4).[11] The known diethyl ester **68**,[11] was first reacted in two successive alkylations using the method developed by Seebach and Frater[27] to generate derivatives **70a**–**f** via **69a**–**f**. Reduction to the triol followed by selective 1,3-diol protection, left the terminal alcohol of **71a**–**f** available for oxidation to the corresponding acids **72a**–**f**, via Dess–Martin Periodinane oxidation of the alcohol to the aldehyde followed by Pinnick oxidation. The acids **72a**–**f** are unstable and must be used right away. Subsequent amide coupling followed by deprotection afforded the desired pantothenamides **62**-**67**.



Scheme 4. Reagents and conditions: (a) LDA, THF, −78 °C, 15 min; (b) R_1-X, −78 °C–rt, 16 h, 67–75%; (c) R_2-X, 78 °C-rt, 16 h, 29–58%; (d) LAH, THF, 70 °C, 16 h; (e) $PMPCH(OMe)_2$, CSA, DCM, rt, 4 h, 50–86% over 2 steps; (f) DMP, DCM, rt, 2 h; (g) $NaClO_2$, $NaHPO_4$, acetone, DCM, H_2O, rt, 15 min; (h) piperidine, DMF, rt, 30 min; (i) EDC, DIPEA, HOBt, THF, rt, 16 h, 42–51% over 4 steps; (j) 80% AcOH, rt, 16 h, 64–81%.

2.2. Biological studies

Following the synthesis of **4**–**12**, **35**–**52**, and **62**–**67**, their substrate activity with *Ec*PanK was measured and the results are compiled in Table 1.

While transformation of compounds **6**–**7**, **11**, **47**–**48**, **51**–**52**, and **66**–**67** by *Ec*PanK is below our detection limit, complementary studies show that they did not inhibit the enzyme either (data not shown), suggesting that the compounds are likely not binding *Ec*PanK. All other derivatives are substrates of *Ec*PanK, yet their overall activity (k_{cat}/K_m) vary greatly from one to the other. A few compounds (**3**, **38**, **39**, **41**, **42**, **44** and **45**) show comparable activity to that of pantetheine, while **34** and **40** are some of the rare compounds to rival the preferred substrate pantothenate.

3. Discussion

Crystal structures are reported for Type I PanK isoforms from four species: Escherichia coli (*Ec*PanK),[14,28] *Mycobacterium* tuberculosis (MtPanK),[29–31] *Coxiella burnetii* (CbPanK; PDB 3TQC), and *Klebsiella pneumoniae* (*Kp*PanK).[24] The reported crystal structure

Table 1
*Ec*PanK kinetic parameters

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
*Pantothenate (**1**)*			
exp	21 ± 3	1.07 ± 0.05	50 ± 7
lit.[6]	34 ± 6	0.76 ± 0.03	21 ± 2
*Pantetheine (**2**)*			
exp	60 ± 10	1.03 ± 0.06	17 ± 3
lit.[17]	91 ± 10	0.32 ± 0.02	3.5 ± 0.4
N5-Pan (**3**)	36 ± 3	0.93 ± 0.04	26 ± 2
4	120 ± 10	1.12 ± 0.05	10 ± 1
5	1200 ± 400	0.13 ± 0.03	0.1 ± 0.1
6	NA[a]		
7	NA		
8	190 ± 20	0.96 ± 0.04	5.1 ± 0.6
9	1000 ± 200	0.49 ± 0.06	0.5 ± 0.1
10	700 ± 300	0.23 ± 0.05	0.3 ± 0.2
11	NA		
12	240 ± 30	1.09 ± 0.07	4.5 ± 0.6
34	33 ± 4	1.24 ± 0.06	38 ± 5
35	>1000	1.19 ± 0.11	0.5 ± 0.1
36	>1000	0.75 ± 0.18	0.3 ± 0.1
37	350 ± 20	1.03 ± 0.05	2.9 ± 0.3
38	43 ± 4	1.02 ± 0.05	24 ± 3
39	67 ± 6	1.15 ± 0.05	17 ± 2
40	32 ± 3	1.13 ± 0.05	35 ± 4
41	22 ± 7	0.46 ± 0.03	21 ± 6
42	50 ± 7	1.02 ± 0.05	20 ± 3
43	130 ± 30	0.94 ± 0.08	7 ± 2
44	39 ± 5	0.78 ± 0.04	20 ± 3
45	110 ± 10	1.15 ± 0.06	11 ± 1
46	450 ± 50	1.44 ± 0.09	3.2 ± 0.4
47	NA		
48	NA		
49	200 ± 100	0.45 ± 0.09	1.9 ± 0.9
50	400 ± 200	0.18 ± 0.05	0.4 ± 0.2
51	NA		
52	NA		
62	230 ± 20	1.17 ± 0.06	5.1 ± 0.5
63	1200 ± 400	0.28 ± 0.06	0.2 ± 0.1
64	590 ± 60	0.89 ± 0.06	1.5 ± 0.2
65	110 ± 10	1.04 ± 0.06	9 ± 1
66	NA		
67	NA		

[a] NA—no observable activity up to 1 μM.

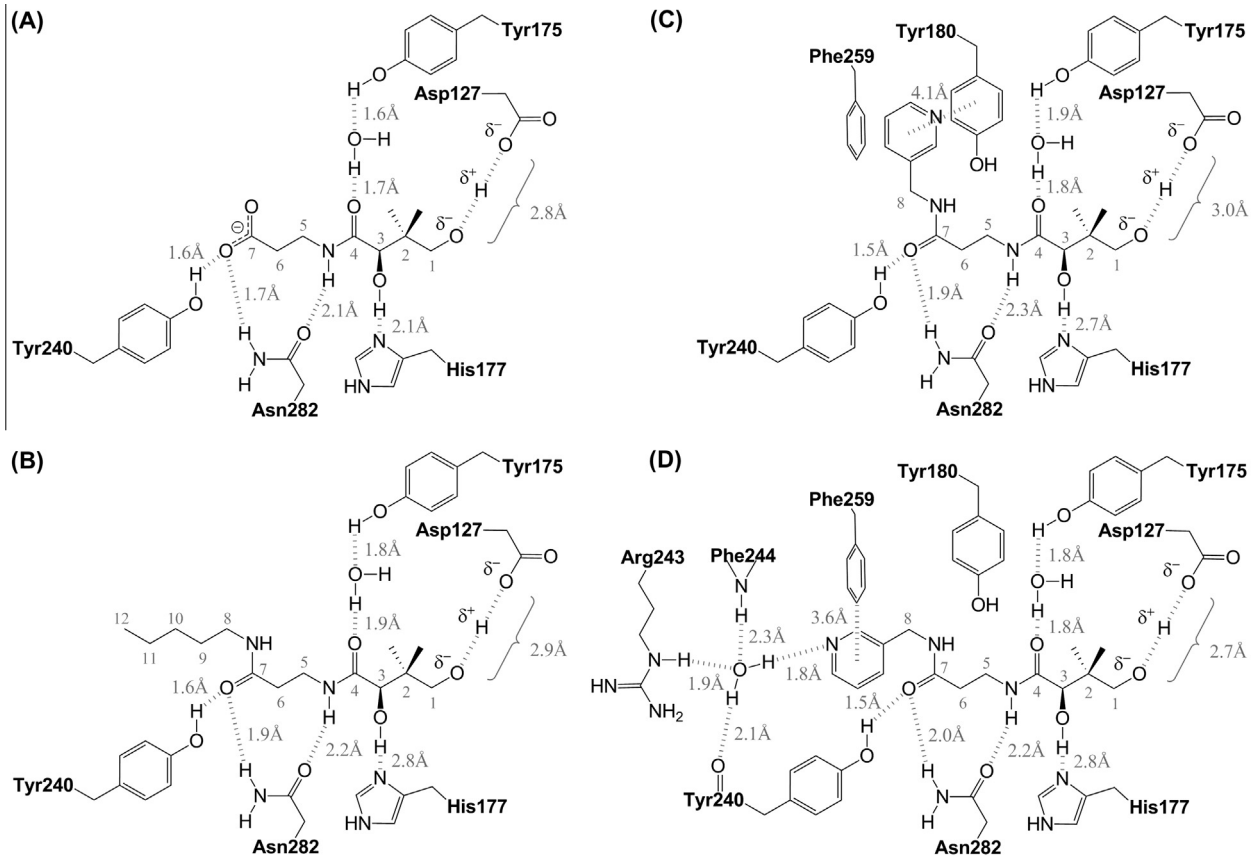


Figure 3. Highlight of the conserved active site residues involved in substrate binding; (A) *Ec*PanK (PDB 1SQ5) in complex with pantothenate (**1**); (B) *Kp*PanK (PDB 4F7W) in complex with **3**; (C) *Kp*PanK (PDB 4GI7) in complex with **34** and in a conformation where sandwich π–π stacking interactions form with Tyr180, or (D) *Kp*PanK (PDB 4GI7) in complex with **34** in a conformation where T-shaped π–π interactions form with Phe259 and water-mediated hydrogen bonding between the pyridyl nitrogen and Arg243, Phe244, and Tyr240.

of *Ec*PanK in complex with pantothenate predicts several amino acids involved in stabilizing pantothenate binding.[28] These include Asp127 and His177 forming hydrogen bonds with the C1 and C3 hydroxyl groups of pantothenate, respectively; Tyr175 hydrogen bonding with the C-4 carbonyl oxygen via H_2O; and Asn282 and Tyr240 hydrogen bonding with the terminal C-7 carboxylate of pantothenate (Fig. 3A). Recently, *Kp*PanK was also crystallized with **3** and **34**,[24] where analysis confirms similar roles of Asp127, His177, Tyr175, Asn282, and Tyr240 for substrate binding, whereas Asn282 and Tyr240 may hydrogen bond not only with the C-7 carboxylate of pantothenate but also with the C-7 amides of **3** and **34** (Fig. 3B–D). It is also interesting to note that **34** is able to adopt two different substrate conformations in the enzyme. One conformation has the pyridine moiety either positioned parallel to Tyr180 forming π–π stacking interactions (Fig. 3C), and the second conformation has the pyridine moiety positioned perpendicular to Phe259 forming T-shaped π–π interactions (Fig. 3D).

Based on the kinetic data obtained with *Ec*PanK and crystal structure analysis, we were able to identify and verify several aspects of the enzyme binding pocket that are crucial for influencing the binding affinity and activity of PanK substrates. As mentioned above, the 3D structures of PanK suggest that the hydrogen bonds between the C-7 carboxylate of pantothenate and both Asn282 and Tyr240 can be sustained by an amide group (C-7 amide of N5-Pan). This is supported by very similar K_m value (21 vs 36 μM). The importance of the C-7 carbonyl group for binding *Ec*PanK was confirmed with the 10 times difference in binding affinity between the amide-containing **4** (k_{cat}/K_m = 10 mM^{-1} s^{-1}) compared to its amide-deficient counterpart, **5** (k_{cat}/K_m = 0.1 mM^{-1} s^{-1}). This is also supported by modeling (Fig. 4A).

The next series of molecules probed whether the hydrogen bonding acceptor and donor nature of the C-7 carboxylate/amide could be mimicked with triazoles,[32,33] guanidines, or sulfonate. The results of the kinetic studies imply that guanidine groups (as in **6** and **7**) are inadequate mimics in this context. Docking **6** into *Ec*PanK suggest that the guanidine group might be better positioned near the hydroxyl group of Tyr240 (Fig. 4B). The aromatic group of **7** may however compete for Tyr240 thus preventing interaction between the guanine group of **7** and Tyr240. Triazole **8** on the other hand was a better mimic but still suffered a loss of activity (k_{cat}/K_m = 5.1 mM^{-1} s^{-1}) compared to that of **3** (k_{cat}/K_m = 26 - mM^{-1} s^{-1}). Docking studies predict that hydrogen bonding with Asn282 and Tyr240 should be preserved (Fig. 4C), and hence steric effects might explain the observed loss. This hypothesis was verified with compound **9**, which has similar steric bulk as that of **8** but with a triazole moiety not positioned to hydrogen bond with Asn282 and Tyr240 (Fig. 4C). As expected **9** showed vastly reduced activity (k_{cat}/K_m = 0.5 mM^{-1} s^{-1}) compared to **8**.

Mimicking the carboxylate group was attempted with a sulfonate, however the sulfonate-containing **11** was not a substrate of PanK. A possible explanation for this observation is that the enzyme binding pocket may not tolerate the bulky tetrahedral geometry of the sulfonate group. This is further supported by the loss of activity for **10** (k_{cat}/K_m = 0.3 mM^{-1} s^{-1}), which is a derivative of pantothenate with a side chain at C-6.

Compounds **4**, **5**, and **12** were synthesized with either C-9 or C-10 amide groups. All of these compounds (k_{cat}/K_m = 0.1–1 mM^{-1} s^{-1}) displayed reduced activity compared to **3** (k_{cat}/K_m = 26 mM^{-1} s^{-1}). This is consistent with the lack of hydrogen bond acceptors/donors in the pantothenate binding pocket besides those aligned with

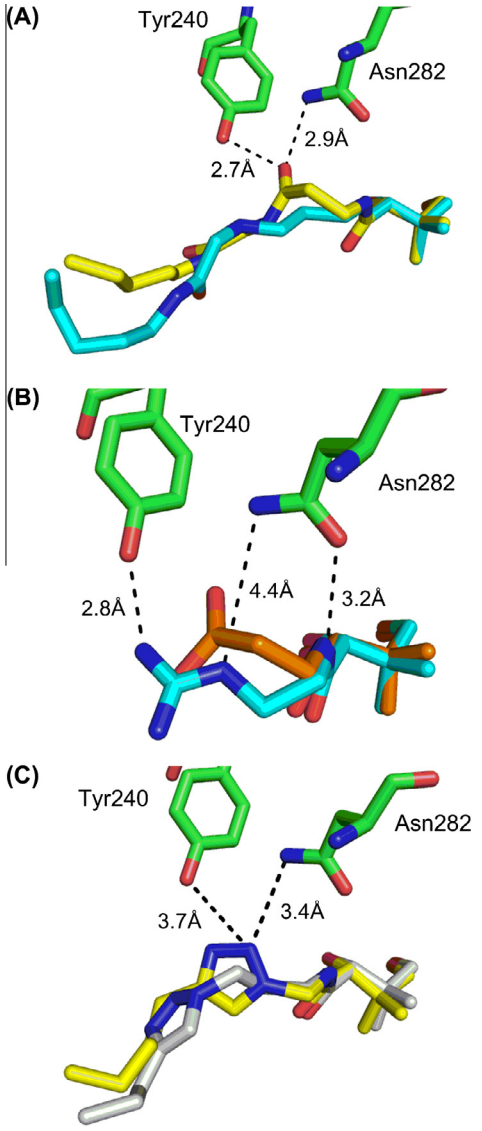


Figure 4. Highlight of the predicted hydrogen bonding interactions of Tyr240 and Asn282 from *Ec*PanK (green) with (A) **4** (yellow) and **5** (turquoise), (B) **6** (turquoise) and **1** (orange), and (C) **8** (yellow) and **9** (grey).

C-7. Indeed the majority of residues in this area are hydrophobic, aromatic residues.

A recent study shows that **34** may adopt two different substrate conformations in *Kp*PanK,[24] hinting that aromatic rings might fit better in *Ec*PanK. In fact, turnover rates were better for **34** (k_{cat}/K_m = 38 mM^{-1} s^{-1}) than for **3** (k_{cat}/K_m = 26 mM^{-1} s^{-1}). Given these results, potential π–π interactions between the enzyme and substrates were probed with additional pantothenamides containing various aromatic substituents. There was little change among binding affinities for substrates with differing chain lengths (0–3 carbons) linking the aromatic substituents to the C-7 amide, with a slight preference for 3 carbons (see **38**–**40**). A possible explanation for this result is that the high proportion of aromatic residues in the substrate binding pocket (e.g. Tyr180, Phe259, Tyr240, Tyr258, and Tyr262) allows a high degree of flexibility for π–π interactions to form. Overall, imidazole (e.g. **41**), pyridine (e.g. **34**, **42**–**45**), and phenyl groups (e.g. **38**–**40**) were all effective for this purpose. Multiring aromatics (e.g. **47**–**48**) and bulky aromatic substituents (e.g. **49**–**50**), however had a detrimental effect. This suggests that the binding pocket can only accommodate one aromatic ring.

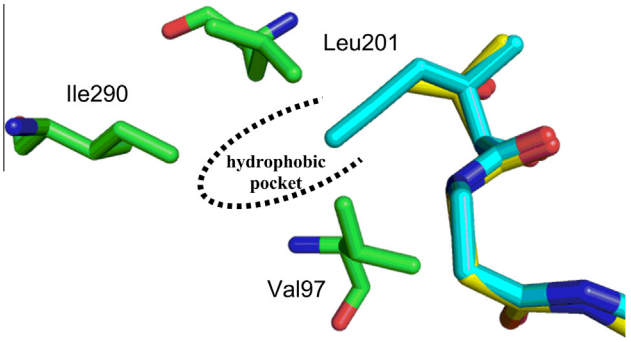


Figure 5. Highlight of the hydrophobic pocket consisting of residues Val97, Leu201, and Ile290 from *Ec*PanK (green) in complex with **3** (yellow), and a modeled conformation of **65** (turquoise) and its potential reach into the hydrophobic binding pocket of *Ec*PanK.

Another interesting observation with **34** was the position of the pyridyl nitrogen and its water-mediated hydrogen bonding with Tyr240, Phe244, and Arg243 of *Kp*PanK.[24] Our studies show that the pyridyl nitrogen brings a sizable contribution (e.g. **39**, k_{cat}/K_m = 17 mM^{-1} s^{-1}), and its position matters (e.g. **42**, k_{cat}/K_m = 20 mM^{-1} s^{-1}; **43**, k_{cat}/K_m = 7 mM^{-1} s^{-1}). The importance of the aromatic ring was further confirmed using the amine-containing, six membered ring substituted **35**, **36**, and **37** which all showed a loss of activity with *Ec*PanK (k_{cat}/K_m = 0.3–2.9 mM^{-1} s^{-1}) compared to **38**–**45**. As an extra control for the increase bulk of sp^3 over sp^2 hybridized carbons, the less bulky linear polyamine-containing **51** and **52** were also tested, and, as predicted they were not significantly transformed by *Ec*PanK.

Analysis of the various PanK crystal structures[14,24,28–31] reveals a cluster of residues (Val97, Leu201, and Ile290) forming a small hydrophobic pocket in the enzyme active site (Fig. 5). To explore whether substrates can interact with this pocket via van der Waals forces, a series of derivatives of **3** modified at the C-2 geminal dialkyl position were prepared. Emphasis was placed on modifying the C-2 alkyl substituent anti to the C-3 hydroxyl group, which is the position on the backbone of **3** that is best suited to reach into the pocket. Of these, the propynyl derivative **65** proved to be the most promising, consistent with a previous study reporting the allyl group as the best among a variety of short alkyl groups.[11] A combination of reduced bulk of the propynyl substituent and stronger van der Waals forces that alkynes experience due to the increased polarity of its π-elections as a result of its linearity, and might explain this result.

4. Conclusion

We have developed the synthetic route necessary to access pantothenate analogs diversified not only at the amine moiety but also at the β-alanine moiety, at the carboxylate, or at the geminal dimethyl group. Kinetic studies of these analogs with *Ec*PanK revealed a number of structure–activity relationships. While some of our compounds proved to be equivalent to the benchmark compound **3**, others were some of the best substrates reported for *Ec*PanK. We believe that these results will be useful to guide the future design of PanK substrates for the biosynthetic generation of CoA analogues as probes or drug candidates.

5. Experimental procedures

5.1. Docking studies

Docking was performed using FITTED via the web-based platform FORECASTER.[34]

5.2. Expression and purification of EcPanK

The expression vector pET28a/PanK was generously provided by Dr. Gerard D. Wright. Purification of *Ec*PanK followed a protocol previously described.[35]

5.3. Kinetic assay of *Ec*PanK

The enzyme assay couples the production of ADP to the consumption of NADH through the activity of pyruvate kinase and lactic dehydrogenase as previously described.[13] Reactions were performed in 96-well microtiter plates, and the decrease in NADH concentration was monitored at 340 nm every 2 s using a Molecular Devices Spectramax190 plate reader. Each reaction mixture (200 μl) contained ATP (1.5 mM), NADH (0.3 mM), phosphor(enol)pyruvate (0.5 mM), $MgCl_2$ (10 mM), KCl (20 mM), pyruvate kinase (8 units), lactic dehydrogenase (8 units) and pantothenate kinase (1.57 μM) in 50 mM Tris–HCl buffer (pH 7.6). The reaction was initiated with the addition of the desired substrate at variable concentrations (62.5–1000 μM). Kinetic parameters were determined by fitting the rate data into the Michaelis–Menten equation using GraphPad Prism 4.0 with nonlinear regressions. All data points are from duplicate experiments. For inhibition studies the same conditions were used but in the presence of pantothenate (1 mM) and at varying concentration (0, 0.625, 1.25, 2.5, 5, 10, and 20 mM) of the potential inhibitor.

5.4. Synthesis

Detailed synthetic protocols and compound characterizations are provided as Supporting information.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.04.030.

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