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# The broad amine scope of pantothenate synthetase enables the synthesis of pharmaceutically relevant amides†

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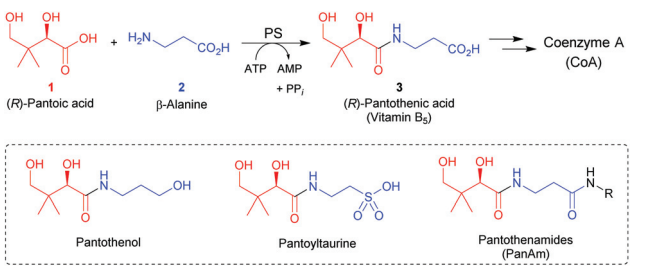
Pantothenate synthetase from *Escherichia coli* ($PS_{E.\ coli}$) catalyzes the ATP-dependent condensation of (*R*)-pantoic acid and β-alanine to yield (*R*)-pantothenic acid (vitamin $B_5$), the biosynthetic precursor to coenzyme A. Herein we show that besides the natural amine substrate β-alanine, the enzyme accepts a wide range of structurally diverse amines including 3-amino-2-fluoropropionic acid, 4-amino-2-hydroxybutyric acid, 4-amino-3-hydroxybutyric acid, and tryptamine for coupling to the native carboxylic acid substrate (*R*)-pantoic acid to give amide products with up to >99% conversion. The broad amine scope of $PS_{E.\ coli}$ enabled the efficient synthesis of pharmaceutically-relevant vitamin $B_5$ antimetabolites with excellent isolated yield (up to 89%). This biocatalytic amide synthesis strategy may prove to be useful in the quest for new antimicrobials that target coenzyme A biosynthesis and utilisation.



## 1. Introduction

Amide bond-containing natural products are some of the most important biologically active molecules.[1,2] In view of that, the development of versatile and sustainable catalytic strategies for amide bond formation is of high industrial and academic interest.[3] Biocatalysis has emerged as a sustainable catalytic methodology for amide bond formation, and several enzyme classes have been explored for their usefulness in the preparation of pharmaceutically relevant amides.[4–7] In this context, ligase enzymes that use adenosine triphosphate (ATP) to catalyze the formation of amides from carboxylic acids and amines in aqueous media may prove particularly useful.[8] A well-known example is pantothenate synthetase (PS), an enzyme that catalyzes the ATP-dependent condensation of (*R*)-pantoic acid (**1**) and β-alanine (**2**) to produce (*R*)-pantothenic acid (vitamin $B_5$, **3**), the biosynthetic precursor of the central metabolic cofactor coenzyme A (CoA) (Scheme 1).[9] The PS-catalyzed reaction proceeds *via* a pantoyl-adenylate intermediate that is attacked by β-alanine.[9]

Enzymes from the CoA biosynthetic pathway have been identified as attractive targets for new antimicrobial compounds.[10,11] One of the first strategies that was explored for the inhibition of CoA biosynthesis relied on the preparation of pantothenic acid antimetabolites,[12] such as pantothenol and pantoyltaurine (Scheme 1). These compounds maintained the core structure of the vitamin (its pantoic acid moiety) but had its β-alanine portion replaced with related amines that lacked the –COOH group that is required for synthesis of CoA. As such, they would likely be accepted as alternate substrates for CoA biosynthesis, but would block the process at a critical step. This mode of action is the same as




**Scheme 1** Biosynthesis of (*R*)-pantothenic acid (vitamin $B_5$, **3**) from (*R*)-pantoic acid (**1**) and β-alanine (**2**), serves to form the precursor to the metabolic cofactor coenzyme A (CoA). The structures of known vitamin $B_5$ antimetabolites, such as pantothenol, pantoyltaurine and the pantothenamides (PanAms), are shown in the dashed box.

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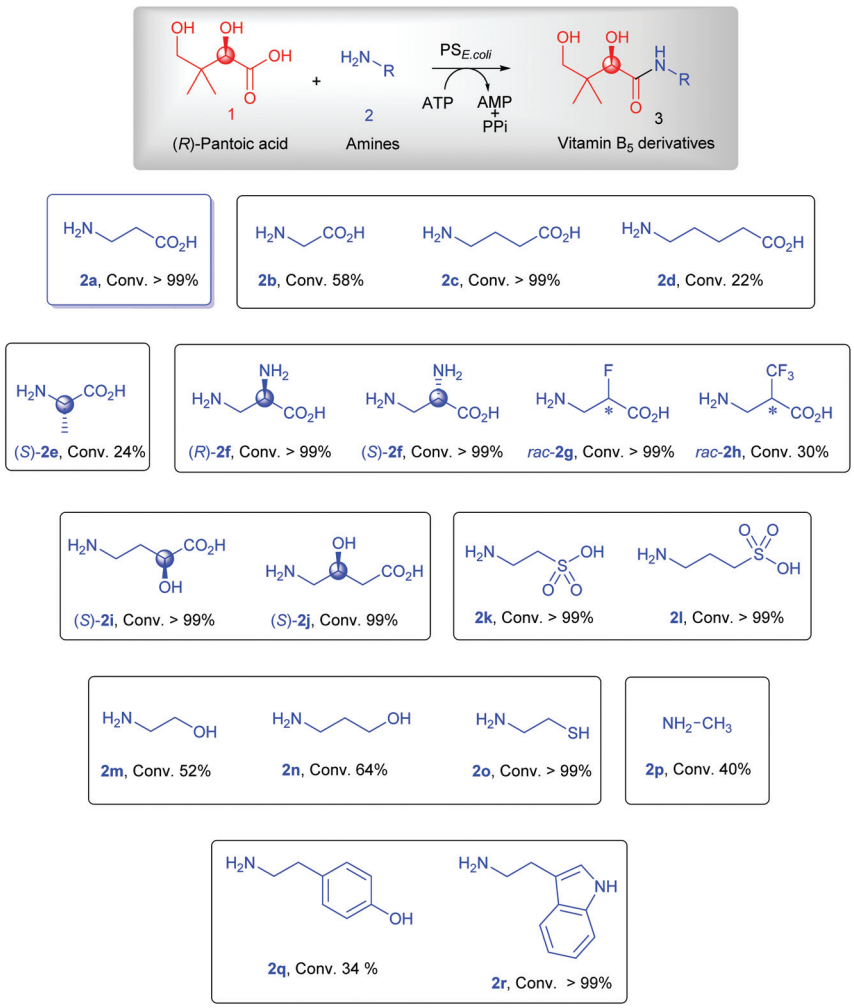
that employed by the sulphonamide antimicrobials, which mimic the *p*-aminobenzoic acid (PABA) substrate of folic acid biosynthesis.[13] Importantly, pantothenol, as well as pantoyltaurine and its amides, have shown some promise as antiplasmodial agents, with the pantoyltauramides also exhibiting selective activity against selected streptococcal infections.[10]

The pantothenamides (PanAms, Scheme 1) are a more recently developed class of pantothenic acid analogues that show potent antimicrobial activity against a variety of pathogenic microorganisms, including *Staphylococcus aureus* and the malaria parasite *Plasmodium falciparum*.[11,14] Unfortunately the PanAms, which are secondary or tertiary amides of pantothenic acid, are metabolically labile and are prone to hydrolysis by serum enzymes such as the pantetheinases.[15] Several strategies have been explored to counter or reduce this degradation to increase the clinical potential of these molecules. One that has shown some promise was the introduction of an α-methyl substituent in the β-alanine portion; the resulting α-methyl-PanAms showed increased stability (and consequently also potency) in comparison to the parent compounds.[16] Clearly, the ability to substitute the β-alanine moiety by structurally diverse amines enabling the enzyme-catalyzed production of a wide variety of structurally diverse pantothenic acids as antimetabolite antimicrobials (and precursors of such antimetabolites) is of high interest.

PS enzymes have been isolated from different organisms including bacteria, fungi, and plants.[17–20] Although several elegant studies have focused on the functional and mechanistic characterization of PS enzymes,[21–24] limited information on the substrate scope of these enzymes and their usefulness for biocatalytic synthesis of a wide variety of distinct pantothenic acid derivatives is available. Recently, we reported that pantothenate synthetase from *Escherichia coli* ($PS_{E.\ coli}$) was able to accept both the (*R*)- and (*S*)-enantiomers of α-methyl-substituted β-alanine (3-amino-2-methylpropanoic acid) as nonnative amine substrates for coupling to 1.[25] This substrate promiscuity was exploited for the enzymatic cascade synthesis of both diastereoisomers of α-methyl-substituted pantothenic acid




**Scheme 2** Amine substrate scope of $PS_{E.\ coli}$. The reaction mixture (3 mL) consisted of $PS_{E.\ coli}$ (7 μM), except for *rac*-**2h** (30 μM); (*R*)-pantoate (20 mM), except for *rac*-**2h** (10 mM); amine (10 mM), except for *rac*-**2h** (20 mM); ATP (20 mM) and $MgCl_2$ (10 mM) in Tris-HCl buffer (100 mM, pH 9). Conversion was determined by $^1$H NMR.

starting from a simple non-chiral building block. Encouraged by these findings, we aimed to systematically explore the amine substrate scope of $PS_{E.\ coli}$. Herein we show that besides the native amine substrate β-alanine, the enzyme accepts a wide range of structurally diverse amines with different functional groups for coupling to the natural carboxylic acid substrate (*R*)-pantoic acid to give various amide products with up to >99% conversion. This broad amine scope of PS thus enables the rapid synthesis of pharmaceutically relevant amides, including known vitamin $B_5$ antimetabolites.

## 2. Results and discussion

We started our investigations by testing the condensation of the native substrates **1** and **2a** using previously reported conditions.[25] With a 2-fold excess of **1** over **2a**, full conversion of **2a** into the corresponding amide product **3a** was achieved (>99%; Scheme 2 & Fig. S1†). Using the same reaction conditions, the non-native amines **2b** (glycine), **2c** (γ-aminobutyric acid, GABA), and **2d** (5-aminovaleric acid), which have different aliphatic linkers between the amino and carboxyl groups compared to **2a**, were also accepted as substrates for amidation by $PS_{E.\ coli}$. While full conversion was achieved with GABA **2c** (>99%; Fig. S3†), moderate conversion was observed with the shorter glycine **2b** (58%; Fig. S2†) and the extended 5-aminovaleric acid **2d** (22%; Fig. S4†).

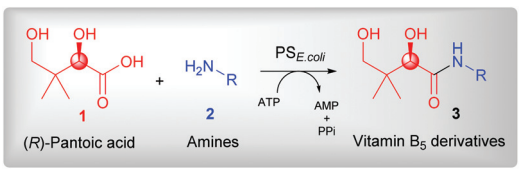
Next, the chiral amines **2e–2j** containing different substituents on the alkyl chain were tested as potential substrates for $PS_{E.\ coli}$. Whereas the enzyme was able to use (*S*)-**2e** (L-alanine) for coupling to **1** (24% conversion, Fig. S5†), the opposite enantiomer (*R*)-**2e** (D-alanine) was not accepted as a substrate (Scheme S1†), illustrating the high enantioselectivity of $PS_{E.\ coli}$ for this substrate. In contrast, both the (*R*)- and (*S*)-enantiomers of **2f** (3-amino-alanine) were accepted as substrates by the enzyme with excellent conversion (>99%; Fig. S6 & S7†). Importantly, the β-amine group of (*R*)- and (*S*)-**2f** was exclusively used for amidation, demonstrating the high regioselectivity of the enzyme. Interestingly, F- and $CF_3$-substituted β-alanines (*rac*-**2g** and *rac*-**2h**) were also accepted as substrates by $PS_{E.\ coli}$ with >99% and 30% conversion, respectively (Fig. S8 & S9†). In addition, the enzyme accepted both α- and β-hydroxyl-substituted GABAs [(*S*)-**2i** and (*S*)-**2j**] as substrates with excellent conversion (≥99%; Fig. S10 & S11†). The α-amino acids L-serine (**2s**), L-leucine (**2t**) and *rac*-3,3,3-trifluoroalanine (*rac*-**2u**) were not processed by the enzyme (Scheme S1†). Hence, it appears that α-amino acids are poor substrates for the $PS_{E.\ coli}$ enzyme, signifying that the distance between the amino and carboxyl groups is important as noted with amines **2a–2d** and **2f**.

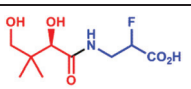
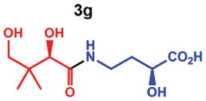
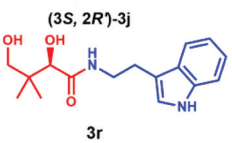
Having established that $PS_{E.\ coli}$ accepts various amino acids as non-native substrates, we investigated whether amine substrates **2k–2r** (Scheme 2), in which the carboxylic acid group has been replaced by other functional groups, can be used as unnatural substrates for coupling to **1**. Amines **2k** (taurine) and **2l** (homotaurine) are direct analogues of substrates **2a** and **2c**, respectively, and resulted in a similar enzymatic conversion (>99%; Fig. S12 & S13†). Analogues **2m** and **2n** were also accepted as non-native substrates by $PS_{E.\ coli}$ but resulted in lower conversion (52–64%; Fig. S14 & S15†). On the other hand, cysteamine (**2o**), in which the carboxyl group of **2a** was replaced by a sulfur group, gave full conversion (Fig. S16†). The simple amine **2p** (methylamine, 40% conversion, Fig. S17†) and the bulky amines **2q** (tyramine, 34% conversion, Fig. S18†) and **2r** (tryptamine, >99% conversion, Fig. S19†) were also accepted as substrates. Notably, benzylamine (**2v**) and histamine (**2w**) were not accepted as substrates by $PS_{E.\ coli}$ (Scheme S1†).

Overall, these results indicate that $PS_{E.\ coli}$ has a very broad substrate scope enabling the synthesis of various vitamin $B_5$ antimetabolites. To further demonstrate the preparative usefulness of the $PS_{E.\ coli}$ enzyme, we performed semi-preparative scale synthesis of a few selected amides (**3g**, 84 mg; **3i**, 22.1 mg; **3j**, 21.4 mg; and **3r**, 26 mg). Excellent conversions (>99%) and high product yields (up to 89%) were achieved (Table 1). Compared to previously reported chemical synthesis methods, this biocatalytic methodology offers an attractive alternative route towards difficult pantothenic acid derivatives.

In contrast to its very broad amine scope, the $PS_{E.\ coli}$ enzyme was found to be highly specific for pantoic acid, with

**Table 1** Semi-preparative-scale biocatalytic synthesis of pantothenic acid derivatives[a]




| Entry | Products | Conv.[b] (%) | Isolated yield[c] (%) | de[d] (%) |
|---|---|---|---|---|
| 1 |  **3g** | >99 | 71 | 1 : 1 |
| 2 |  **(2*S*, 2*R*′)-3i** | >99 | 88 | >99 |
| 3 |  **(3*S*, 2*R*′)-3j** | >99 | 86 | >99 |
| 4 |  **3r** | >99 | 89 | — |

[a] The reaction mixture consisted of $PS_{E.\ coli}$ (7 μM), (*R*)-pantoate [20 mM], an amine substrate [10 mM], ATP (20 mM), and $MgCl_2$ (10 mM) in 10 mL Tris-HCl buffer (100 mM, pH 9), except for *rac*-**2g** (50 mL). [b] Conversion was determined by $^1$H NMR. [c] Isolated yield after silica gel column chromatography. [d] The diastereomeric excess (de) was determined by $^1$H NMR.

various substituted 4-hydroxybutanoic acids not accepted as alternative electrophiles. Detailed insight into the structural determinants of the substrate specificity of this enzyme awaits the determination of crystal structures in complex with various non-native substrates and/or products. Such structures will also aid in the optimization of PS enzymes for synthesis of various pharmaceutically relevant amides.

# 3. Conclusions

We have demonstrated that $PS_{E.\ coli}$ has a very broad substrate scope, accepting a wide variety of amine nucleophiles in the amidation of (*R*)-pantoate. The amines the enzyme accepted included fluorinated β-alanine, α- and β-hydroxylated GABAs, and tryptamine as non-native substrates, with excellent conversions (>99%), and the corresponding amides were obtained in high isolated yields (71–89%). This attractive biocatalytic strategy for rapid synthesis of diverse pantothenic acid derivatives —including known vitamin $B_5$ antimetabolites that have proven to be difficult or tedious to synthesise—is likely to advance our ability to search for new antimicrobial compounds that target CoA biosynthesis and utilisation in a variety of pathogens.

# 4. Experimental

## 4.1 Materials and methods

The compounds β-alanine (**2a**), glycine (**2b**), γ-aminobutyric acid (**2c**), 5-aminovaleric acid (**2d**), D-alanine [(*R*)-**2e**], L-alanine [(*S*)-**2e**], 3-amino-2-fluoropropionic acid (**2g**), (*S*)-4-amino-2-hydroxybutyric acid (**2i**), (*S*)-4-amino-3-hydroxybutyric acid (**2j**), taurine (**2k**), homotaurine (**2l**), ethanolamine (**2m**), 3-amino-1-propanol (**2n**), cysteamine (**2o**), methylamine (**2p**), tyramine (**2q**), tryptamine (**2r**), L-serine (**2s**), L-leucine (**2t**), 3,3,3-trifluoro-DL-alanine (*rac*-**2u**), benzylamine (**2v**) and histamine (**2w**) were purchased from Sigma-Aldrich Chemical Co. The compounds (*R*)-2,3-diaminopropionic acid hydrochloride [(*R*)-**2f**] and (*S*)-2,3-diaminopropionic acid hydrochloride [(*S*)-**2f**] were purchased from TCI Europe N.V. The compound 3-amino-2-(trifluoromethyl)-propionic acid (*rac*-**2h**) was purchased from 1ClickChemistry Inc. (USA). Solvents were purchased from Biosolve (Valkenswaard, The Netherlands) or Sigma-Aldrich Chemical Co. Ingredients for buffers and media were obtained from Duchefa Biochemie (Haarlem, The Netherlands) or Merck (Darmstadt, Germany). Dowex 50W X8 resin (100–200 mesh) was purchased from Sigma-Aldrich Chemical Co. Ni sepharose 6 fast flow resin and a HiLoad 16/600 Superdex 200 pg column were purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden).

Pantothenate synthetase from *Escherichia coli* ($PS_{E.\ coli}$) was expressed and purified as described previously.[25] Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions on precast gels (NuPAGE™ 12% Bis–Tris protein gels). The gels were stained with Coomassie Brilliant Blue. NMR analysis was performed on a Brucker 500 MHz machine at the Drug Design Laboratory, University of Groningen. Chemical shifts ($\delta$) are reported in parts per million (ppm). Electrospray ionization orbitrap high-resolution mass spectrometry (HRMS) was performed by the Mass Spectrometry core facility, University of Groningen.

## 4.2 General procedure

**(A) Analytical scale synthesis.** The reaction mixture (3 mL) consisted of $PS_{E.\ coli}$ enzyme [7 μM; except for *rac*-**2h** (30 μM)], (*R*)-pantoate [20 mM; except for *rac*-**2h** (10 mM)], amine [10 mM; except for *rac*-**2h** (20 mM)], ATP (20 mM) and $MgCl_2$ (10 mM) in Tris-HCl buffer (100 mM, pH 9). The reaction mixture was incubated at room temperature for 24 h. The enzyme was inactivated by heating at 70 °C for 5 min and the precipitated enzyme was removed by filtration. The filtrate was evaporated under vacuum. The resulting residue was dissolved in $D_2O$ and analyzed by $^1H$ NMR spectroscopy. The conversion was estimated by comparing the NMR signals of the substrates and corresponding product (see ESI†).

**(B) Semi-preparative scale synthesis.** The initial reaction mixture (8 mL) consisted of (*R*)-pantoate [20 mM], an amine substrate [10 mM], ATP (20 mM), and $MgCl_2$ (10 mM) in Tris-HCl buffer (100 mM, pH 9). To start the reaction, freshly purified $PS_{E.\ coli}$ enzyme (7 μM) was added and the final volume of the reaction mixture was adjusted to 10 mL with the same buffer [except for *rac*-**2g** (50 mL)]. The reaction mixture was then incubated at room temperature. The progress of the enzymatic reaction was monitored by $^1H$ NMR spectroscopy. After completion of the reaction, the enzyme was inactivated by heating to 70 °C for 5 min, and the amide product was purified by silica gel column chromatography (see ESI†).

# Conflicts of interest

There are no conflicts to declare.

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