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Synthesis and evaluation of novel chromogenic aminopeptidase substrates for microorganism detection and identification

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Abstract—The amides **8a**–**e** and **10a**–**c** were prepared as chromogenic aminopeptidase substrates. A range of microorganisms were grown in the presence of these compounds and coloured colonies were produced in several cases after addition of acetic acid—thus giving potential methods for the detection of aminopeptidase activity and for microorganism identification.
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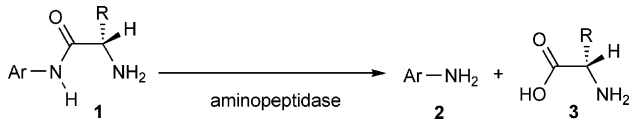
Aminopeptidase activity in microorganisms can often be detected by observing the change in colour when the amide bond in a weakly coloured amide substrate **1** is hydrolysed by the aminopeptidase giving a strongly coloured amine product **2** (Scheme 1). The strong colour of the amine is a consequence of delocalisation of the amine-nitrogen lone-pair throughout the aromatic ring system. The taxonomic utility of aminopeptidases for identification and/or discrimination of bacteria has long been recognised. L-Pyrrolidonyl peptidase has proven to be a useful test in schemes for the rapid identification of *Salmonella* spp.,[1] enterococci and Group A streptococci,[2,3] whereas L-γ-glutamic acid aminopeptidase can be used to differentiate *Shigella* spp.[4] Detection of proline aminopeptidase has been used for the identification of *Neisseria meningitidis*[5] as well as a diagnostic aid in the confirmation of bacterial vaginosis.[6] There has been longstanding interest in the detection of L-alanine aminopeptidase for the differentiation of Gram-positive and Gram-negative bacteria. This is due to the fact that Gram-negative bacteria readily demonstrate the presence of this enzyme whereas Gram-positive bacteria do not. Alanine aminopeptidase has been shown to be a periplasmic enzyme in *Pseudomonas aeruginosa*.[7] Differentiation is usually achieved using L-alanyl-*p*-nitroanilide as a chromogenic substrate[8] although fluorogenic peptidase substrates have also been applied to this task in commercially available assays.[9] The last 15 yr has seen an expansion in the use of chromogenic agar media in diagnostic microbiology for the rapid detection of pathogenic bacteria in both clinical samples[10] and food.[11] Such media exploit chromogenic substrates that can be hydrolysed to yield chromogens that remain restricted to bacterial colonies and do not diffuse in agar. This allows isolation and detection of target pathogens within polymicrobial cultures. Chromogenic media have relied on esters of various non-diffusible chromogens such as glycosides of indoxyl and its halogenated derivatives.[12] To date, no chromogenic media have exploited the diagnostic potential of peptidase substrates, largely due to the lack of appropriate substrates that yield a coloured product that does not diffuse in agar.



Scheme 1. Aminopeptidases.

In this paper, the preparation of a selection of novel L-alanine and β-alanine acridine-based substrates is reported and their potential application for detecting and identifying a range of clinically important microorganisms is described. The substrates we have synthesised and evaluated are the L-alanine **8a**–**e** and β-alanine **10a**–**c** derivatives of the 9-(4′-aminophenyl)acridines **6a**–**e** (Scheme 2). Upon hydrolysis by aminopeptidases, these

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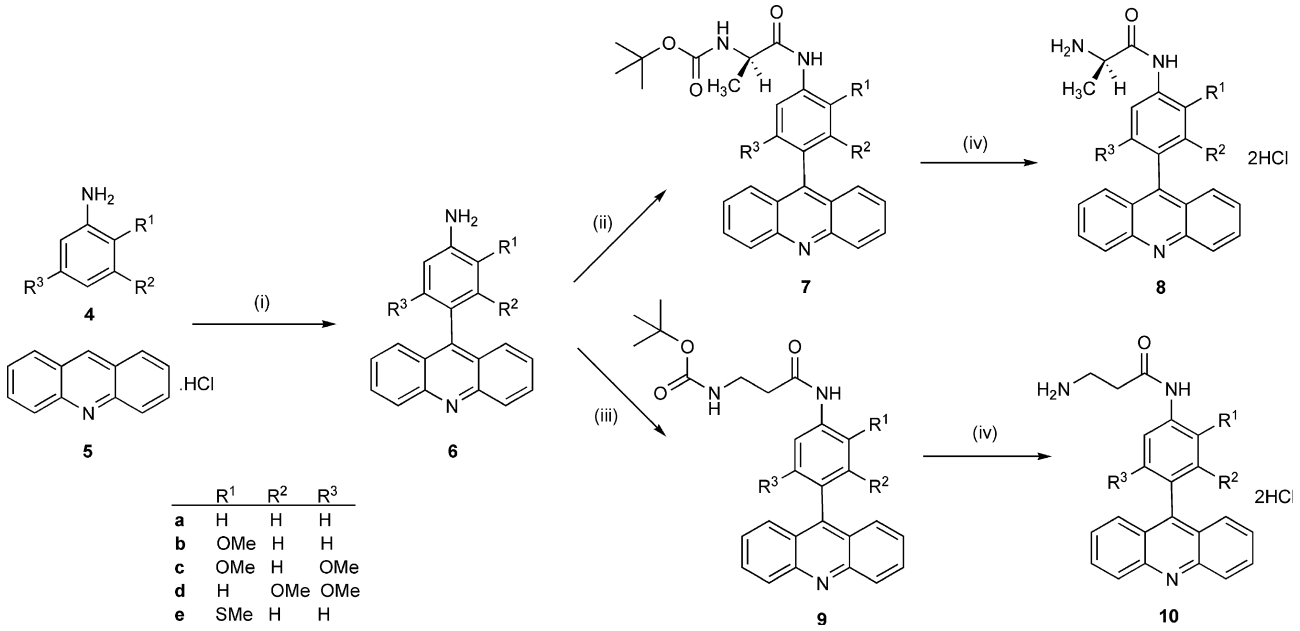
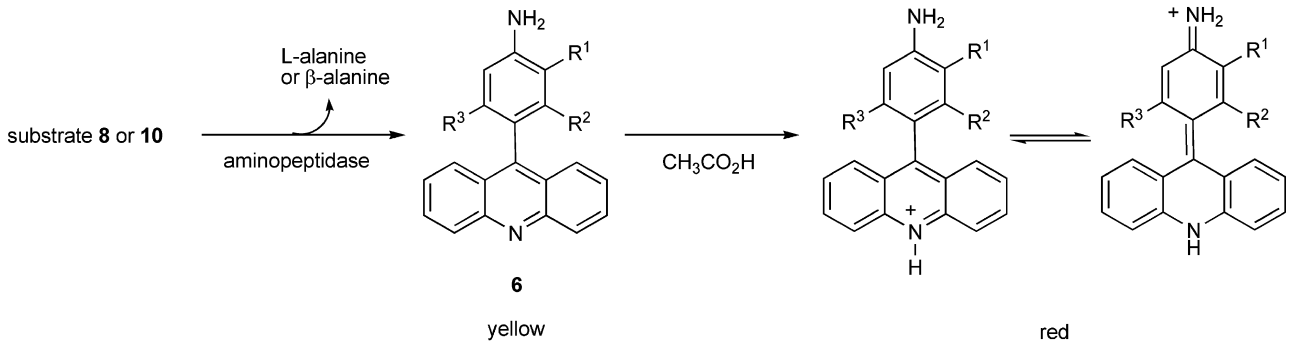


Scheme 2. Synthesis of the substrates **8** and **10**. Reagents and conditions: (i) S^8, heat; (ii) BOC-L-alanine, NMM, IBCF, −10 to −15 °C, then add **6**; (iii) BOC-β-alanine, NMM, IBCF, −10 to −15 °C, then add **6**; (iv) EtOAc, HCl. BOC, *tert*-butyloxycarbonyl; NMM, *N*-methylmorpholine; IBCF, *iso*-butylchloroformate.



Scheme 3. Hydrolysis of the substrates **8** and **10**.

substrates afford the 9-(4′-aminophenyl)acridines **6a**–**e** which are pale yellow at physiological pH but give red colours when protonated by acetic acid (Scheme 3).[13]

The 9-(4′-aminophenyl)acridine derivatives **6** that provide the aromatic amine fragment of the substrates were prepared by heating the appropriately substituted anilines **4** with acridine hydrochloride **5** in the presence of elemental sulfur. This method of preparing 9-(4′-aminophenyl)acridine derivatives **6**, which has been used previously by other workers, afforded the known compounds **6a**[14,15] and **6b**[16] and the novel products **6c**–**e** in 42–65% yield. Reaction of the amines **6a**–**e** with BOC-L-alanine afforded the protected amides **7a**–**e**, respectively (76–83%), from which the substrates **8a**–**e** (65–76%) were obtained as their dihydrochloride salts by deprotection with hydrogen chloride. The dihydrochloride salts of the β-alanyl substrates **10a**–**c** (67–71%) were similarly prepared from the corresponding protected amides **9a**–**c** which in turn were synthesised from amines **6a**–**c** and BOC-β-alanine (75–80%). Full details of the synthetic work are given in the Supplementary information.

The aminopeptidase substrates **8a**–**e** and **10a**–**c** were evaluated against the range of clinically important microorganisms shown in Table 1. In general, the L-alanyl substrates **8a**–**d** did not inhibit growth of the Gram-negative organisms and cream or yellow colonies were produced in the presence of the substrate. Addition of acetic acid resulted in the production of various shades of red-coloured colonies (see Scheme 3) due to protonation of the amino-acridines **6a**–**d** which are produced by hydrolysis of the substrates. The L-alanyl substrate **8e** inhibited the growth of the Gram-negative organisms *Escherichia coli* and *Klebsiella pneumoniae*. Figure 1 shows hydrolysis of substrate **8c** by colonies of *E. coli* (NCTC 10418). Yellow colonies become red-violet within seconds on addition of glacial acetic acid.

In order to demonstrate that the development of colour was due to specific enzyme activity, substrates **8a**–**d** were incubated with purified L-alanine aminopeptidase. Substrates **8a**–**d** all generated an immediate pink-orange colouration on addition of acetic acid only in the tubes containing enzyme. No change of colour was observed

Table 1. Colour formation as a result of aminopeptidase activity in a range of microorganisms

Substrate	Organism	Organism growth: substrate absent[a]	Organism growth: substrate present[a]	Colony colour in presence of substrate	Colony colour after acidification
8a	*Escherichia coli*[b]	++	++	Cream	Pink-orange
	Klebsiella pneumoniae[b]	++	++	Cream	Pink-orange
	Pseudomonas aeruginosa[b]	++	++	Cream	Diffuse orange
	Burkholderia cepacia[b]	Not evaluated			
	Staphylococcus aureus[c]	++	++	Pale yellow	Yellow
	Enterococcus faecalis[c]	++	++	Yellow	Yellow
8b–d	*Escherichia coli*	++	++	Yellow	Red-violet
	Klebsiella pneumoniae	++	++	Yellow	Red-brown
	Pseudomonas aeruginosa	++	++	Yellow	Orange-brown
	Burkholderia cepacia	++	++	Yellow	Orange-brown
	Staphylococcus aureus	++	No growth	—	—
	Enterococcus faecalis	++	No growth[d]	—	—
8e	*Escherichia coli*	++	Trace	None	Red-violet
	Klebsiella pneumoniae	++	+	None	Red-brown
	Pseudomonas aeruginosa	++	++	None	Pale orange
	Burkholderia cepacia	++	++	None	Orange-brown
	Staphylococcus aureus	++	No growth	—	—
	Enterococcus faecalis	++	No growth	—	—
10a	*Escherichia coli*	++	No growth	—	—
	Klebsiella pneumoniae	++	++	None	None
	Pseudomonas aeruginosa	++	++	None	Orange-brown
	Burkholderia cepacia	++	++	None	None
	Staphylococcus aureus	++	No growth	—	—
	Enterococcus faecalis	++	No growth	—	—
10b	*Escherichia coli*	++	No growth	—	—
	Klebsiella pneumoniae	++	+	None	None
	Pseudomonas aeruginosa	++	++	Pale yellow	Red-brown
	Burkholderia cepacia	++	++	Yellow	Orange
	Staphylococcus aureus	++	No growth	—	—
	Enterococcus faecalis	++	No growth	—	—
10c	*Escherichia coli*	++	No growth	—	—
	Klebsiella pneumoniae	++	+	Pale yellow	Orange
	Pseudomonas aeruginosa	++	++	Yellow	Red-brown
	Burkholderia cepacia	++	++	Yellow	Red-brown
	Staphylococcus aureus	++	No growth	—	—
	Enterococcus faecalis	++	No growth	—	—

[a] ++, Good growth ; +, moderate growth; +/–, poor growth.
[b] Gram-negative organism.
[c] Gram-positive organism.
[d] Moderate growth with substrate **8d**.

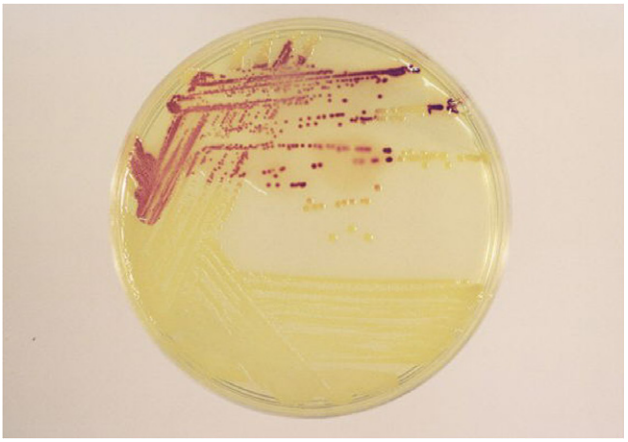


Figure 1. Hydrolysis of substrate **8c** by colonies of *E. coli* (NCTC 10418) after addition of glacial acetic acid.

in any tubes without the enzyme demonstrating that colour generation was associated with hydrolysis by a specific enzyme. Figure 2 shows the colour resulting from hydrolysis of substrate **8c** by purified L-alanine aminopeptidase following acidification.

The β-alanyl substrates **10a–c** inhibited the growth of the Gram-negative organism *E. coli* and with *K. pneumoniae* inhibition was also noted with substrates **10b** and **10c**. No significant colour change in the colonies produced by these organisms was observed on exposure to acetic acid suggesting that hydrolysis of the substrates had not occurred. In contrast, *P. aeruginosa* and *Burkholderia cepacia* produced coloured colonies with substrates **10b** and **10c** in the presence of acetic acid. Substrates **10a–c** did not produce any colour change in the presence of purified L-alanine aminopeptidase.

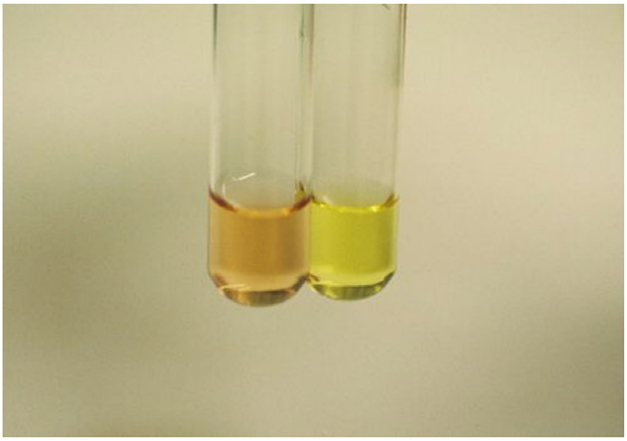


Figure 2. Colour resulting from hydrolysis of substrate **8c** by L-alanine aminopeptidase following acidification (left, substrate with enzyme; right, substrate without enzyme).

In contrast to the Gram-negative organisms described above, and with the exception of substrate **8a**, the other substrates described in this paper inhibited the growth of the Gram-positive organisms *Staphylococcus aureus* and *Enterococcus faecalis*. In cases where growth had occurred with substrate **8a**, no significant change in colony colour was observed on addition of acetic acid. This suggests that the substrates had not been hydrolysed by aminopeptidases.

For the bacterial strains that generated coloured colonies on addition of acetic acid, it was of particular value to note that the colour remained tightly localised on the colony and was not seen in the surrounding agar. This may be due to either the relatively large molecular size and lipophilic nature of the chromogen or localisation of the chromogen on or within the microorganism. This indicates that such substrates have the potential to discriminate aminopeptidase-producing pathogens within heavily mixed polymicrobial cultures.

In conclusion, a series of novel aminopeptidase sensitive substrates have been prepared, characterised and evaluated. Many of the substrates proved useful for the detection of Gram-negative microorganisms. Particularly interesting was the inhibitory effect of most of the substrates on the growth of Gram-positive organisms and this could have practical applications in suppressing the growth of Gram-positive organisms in the presence of Gram-negative ones and also as a method of distinguishing between these two types of organisms.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.11.088.

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