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Synthesis and evaluation of novel chromogenic peptidase substrates based on 9-(4′-aminophenyl)-10-methylacridinium salts as diagnostic tools in clinical bacteriology

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Abstract—The synthesis and initial evaluation of novel chromogenic substrates with potential in the detection and differentiation of cultured bacterial colonies are described. The substrates were readily hydrolysed by specific aminopeptidase activity to release the chromogen, 9-(4′-aminophenyl)-10-methylacridinium salt, which provided a clear visual indication of the presence of the corresponding bacteria.

The ability to detect the presence of enzymes as diagnostic markers for pathogens using suitable chromogenic substrates has become a key method for the rapid identification of microorganisms in clinical[1] and food[2] samples. The incorporation of such substrates into a suitable medium can reduce the need for subculture and further biochemical tests to establish the identity of certain microorganisms. In particular, the presence of L-alanine aminopeptidase in Gram-negative bacteria can be exploited to distinguish them from Gram-positive bacteria,[3,4] which do not express this enzyme, Figure 1. In addition, chromogenic aminopeptidase substrates can be used to distinguish between certain bacterial species, for example, L-pyrrolidonyl aminopeptidase aids in the identification of enterococci, group A streptococci[5,6] and *Salmonella* spp.[7] The identification of β-alanine aminopeptidase activity in *Pseudomonas aeruginosa*[8] offers the potential for the detection of this important pathogen, for example, in specimens from patients with cystic fibrosis.

These bacterial detection systems use a chromogenic compound covalently linked to a substrate for a specific bacterial enzyme. The covalent linkage to the substrate limits the electronic resonance within the chromogen, resulting in the quenching of the colour. Action of the target bacterial enzyme(s) results in cleavage of the chromogen-substrate covalent bond, enabling resonance throughout the conjugated chromogen and the return of colour. Chromogenic substrates facilitate bacterial detection and identification when the release of the chromogenic molecule produces an intense colour that can be visualized by eye, obviating the need for expensive or specialized equipment.

Unfortunately, most substrates that have been described for visualization of peptidase activity, including *p*-nitroanilide derivatives, are unsuitable for use in agar media, due to the widespread diffusion of the chromogen through agar after hydrolysis from the substrate, and are not suitable for the visualization of bacterial colonies.

Recently, the use of acridine-based substrates was reported[9]; hydrolysis yielded chromogens that were

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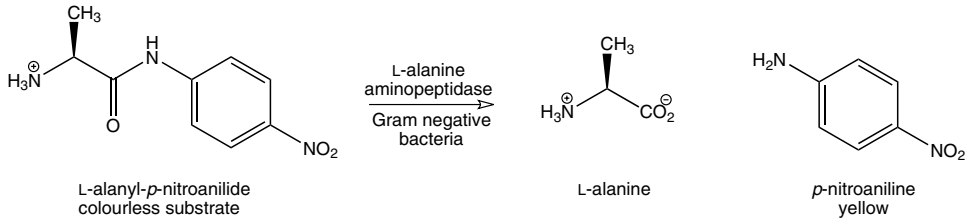
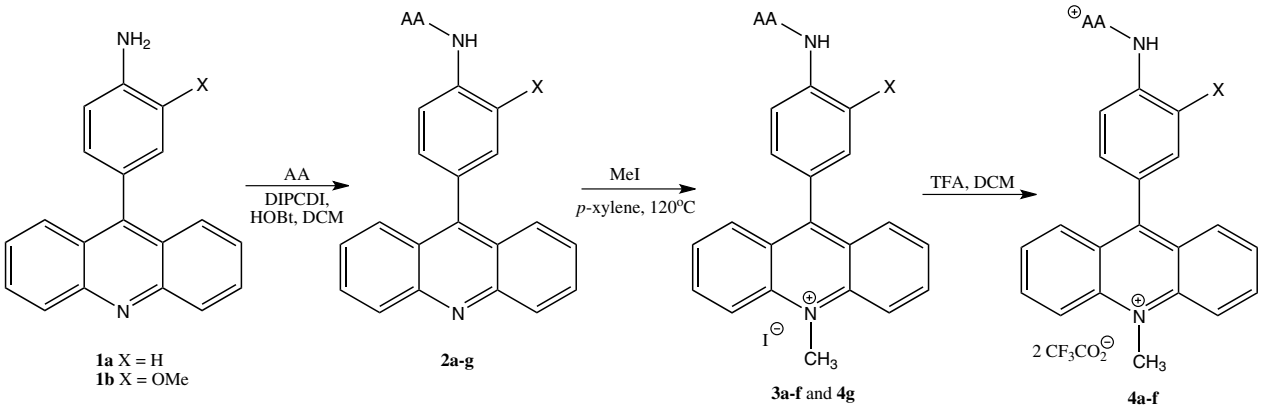


Figure 1. Differentiation of Gram-negative and Gram-positive bacteria through hydrolysis of L-alanyl-*p*-nitroanilide by L-alanine aminopeptidase.



Scheme 1. Synthesis of chromogenic substrates **4a**–**g** via precursors **2a**–**g** and **3a**–**f**.

weakly coloured when released from the substrate, but were intensely red-violet coloured when protonated by treatment of the plates with acetic acid—due to the formation of the related acridinium salt. Addition of glacial acetic acid to bacterial colonies is not ideal, not least because of the loss of viability of bacteria that may be required for further testing.

We report here the synthesis and evaluation of 9-(4′-*N*-[aminoacyl]aminophenyl)-10-methylacridinium salts **4a**–**g** as chromogenic molecules suitable for bacterial detection and identification. These compounds improve on the previously reported acridine-based substrates; they have muted colour when acylated, for example, with an amino acid, but are intensely red-orange coloured when released as the methylacridinium salts, without the requirement for the addition of acid, and they adhere to the bacterial colonies making visualization easier.

Table 1. Yields of synthesized aminoacyl-10-methylacridinium salts **4a**–**g** and related precursors **2a**–**f** and **3a**–**f**

Compound	AA	X	Yield (%)
2a	^{t}Boc-L-Ala	H	78
2b	^{t}Boc-β-Ala	H	76
2c	^{t}Boc-Gly	H	77
2d	^{t}Boc-L-Ala-L-Ala	H	79
2e	^{t}Boc-L-Ala-L-Ala-L-Ala	H	81
2f	^{t}Boc-L-Ala	OMe	81
2g	L-pyroGlu	H	80
3a	^{t}Boc-L-Ala	H	86
3b	^{t}Boc-β-Ala	H	90
3c	^{t}Boc-Gly	H	80
3d	^{t}Boc-L-Ala-L-Ala	H	89
3e	^{t}Boc-L-Ala-L-Ala-L-Ala	H	80
3f	^{t}Boc-L-Ala	OMe	92
4a	L-Ala	H	95
4b	β-Ala	H	94
4c	Gly	H	95
4d	L-Ala-L-Ala	H	94
4e	L-Ala-L-Ala-L-Ala	H	98
4f	L-Ala	OMe	98
4g	L-pyroGlu	H	91

The synthesis of 9-(4′-aminophenyl)acridine **1a** and 9-(4′-amino-3′-methoxyphenyl)acridine **1b** was achieved using a slight variation of the method of Acheson and Birtwistle,[10] as previously reported.[9]

The aminoacyl-10-methylacridinium trifluoroacetate bacterial enzyme substrates **4a**–**f** were prepared by reaction of 9-(4′-aminophenyl)acridine **1a** or **1b** with the appropriate ^{t}Boc-protected amino acid or peptide, using established peptide coupling conditions, to form the corresponding ^{t}Boc-aminoacyl-acridines **2a**–**f**. Methylation of the acridines **2a**–**f** with methyl iodide gave the ^{t}Boc protected aminoacyl-10-methylacridinium iodide salts **3a**–**f**; ^{t}Boc removal with TFA gave the expected aminoacyl-10-methylacridinium *bis*-trifluoroacetate salts **4a**–**f**, Scheme 1 and Table 1.

The L-pyroglutamyl derivative, 9-(4′-*N*-[L-pyroglutamyl]aminophenyl)-10-methylacridinium iodide **4g**, was prepared by a similar route in which ^{t}Boc protection was not required. The reaction of **1a** with L-pyroglutamate under the usual peptide coupling conditions yielded 9-(4′-*N*-[L-pyroglutamyl]aminophenyl)acridine **2g**; methylation of this intermediate, under the conditions indicated, gave the chromogenic substrate **4g**. Full details of the syntheses are provided in the Supplementary information.

The chromogenic substrates **4a**–**g** and **3e** were evaluated for their suitability as chromogenic substrates for the detection of bacterial aminopeptidase activity, for their potential to differentiate between Gram-positive and Gram-negative bacteria, and for their ability to detect *P. aeruginosa*.

An initial evaluation (results not shown) confirmed that all of the substrates tested could be hydrolysed by bacterial aminopeptidases to release the highly coloured chromogen, 9-(4′-aminophenyl)-10-methylacridinium salt, without the addition of acid. The methoxy derivative **4f** was comparable in activity to the unsubstituted analogue **4a**, but was more difficult to synthesize and was not evaluated further.

The water soluble substrates **4a**–**e**, **4g** and **3e** were incorporated into Columbia agar at 300 mg/L; 20 mL of this agar was added to each 90 mm Petri dish to give an agar depth of 4 mm. The resultant agar plates were each inoculated with nine Gram-negative and ten Gram-positive bacteria. Bacterial strains were initially cultivated on a standard nutrient agar medium. Colonies of each strain were suspended in sterile deionised water to generate a suspension with a turbidity equivalent to 0.5 McFarland units (approximately 1.5×10^8 colony forming units per ml), as confirmed with a densitometer. One microliter of this suspension was inoculated onto the agar plates containing the various substrates using an automated multipoint inoculator. All inoculated media were incubated at 37 °C for 48 h and examined visually after 24 and 48 h for the presence of growth and coloration of bacterial colonies, Table 2.

In general, the substrates were not growth inhibitory to Gram-negative bacteria and most of the substrates tested were hydrolysed by these bacteria. In contrast, the Gram-positive bacteria were either inhibited by the substrates or were unable to hydrolyse the covalent link between the amino acid and chromogen, providing a visual means to differentiate between these different classes of bacteria. The most promising substrate across the range of bacteria tested was 9-(4′-*N*-[L-Ala-L-Ala-L-Ala]aminophenyl)-10-methylacridinium *bis*-trifluoroacetate **4e**, which was readily hydrolysed by Gram-negative bacteria, while being non-inhibitory to Gram-positive bacteria—allowing the growth of all bacteria and the visualization of Gram-negative colonies through hydrolysis of the substrate with a concomitant release of the coloured chromogen as a red/orange methylacridinium salt, Figure 2.

The ^{t}Boc protected analogue, 9-(4′-*N*-[N_α-^{t}Boc-L-Ala-L-Ala-L-Ala]aminophenyl)-10-methylacridinium iodide **3e**, had a similar activity profile, presumably due to facile carbamate hydrolysis by unidentified esterase activity to yield **4e**.

The L-pyroglutamyl substrate, 9-(4′-*N*-[L-pyroglutamyl]aminophenyl)-10-methylacridinium iodide **4g**, showed promise for the differentiation of members of the enterobacteriaceae family, as it was effectively hydrolysed by *Klebsiella pneumoniae* and *Enterobacter cloacae*, but not by the strains of *Escherichia coli* and *Salmonella* spp. tested, nor by *Shigella sonnei*.

Two substrates showed potential for the detection of *P. aeruginosa*: the L-pyroglutamyl substrate, 9-(4′-*N*-

Table 2. Hydrolysis of substrates **4a–e**, **4g** and **3e** by Gram-negative and Gram-positive bacteria

Bacterial strain	Substrate[a]	4a		4b		4c		4d		4e		4g		3e	
	Reference	24 h[b]	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Gram-negative bacteria															
Escherichia coli 0157	NCTC 12079	+[c]	+	–	–	+	+	+	+	+	+	–	–	+	+
Burkholderia cepacia	NCTC 10743	+	+	–	+	+	+	+	+	+	+	–	+	+	+
Pseudomonas aeruginosa	NCTC 10662	+	+	+	+	–	+	–	+	+	+	–	+	+	+
Serratia marcescens	NCTC 10211	+	+	+	+	+	+	+	+	+	+	–	–	+	+
Salmonella poona	NCTC 4840	+	+	–	–	+	+	+	+	+	+	–	–	+	+
Salmonella typhimurium	NCTC 74	+	+	–	–	+	+	+	+	+	+	–	–	+	+
Shigella sonnei	NCTC 9774	+	+	–	–	+	+	+	+	+	+	–	–	+	+
Klebsiella pneumoniae	NCTC 10896	+	+	–	–	+	+	+	+	+	+	–	+	+	+
Enterobacter cloacae	NCTC 11936	+	+	–	–	+	+	+	+	+	+	–	+	+	+
Gram-positive bacteria															
Enterococcus faecalis	NCTC 775	NG[d]	NG	–	–	NG	NG	–	–	–	–	–	–	NG	NG
Enterococcus faecalis	NCTC 12697	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Enterococcus faecium	NCTC 7171	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Enterococcus faecium (VRE)	Wild type 141835	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Listeria monocytogenes	NCTC 11994	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Staphylococcus aureus	NCTC 6571	NG	NG	NG	NG	NG	NG	NG	NG	–	–	NG	NG	NG	NG
Staphylococcus aureus	NCTC 11939	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Staphylococcus aureus	NCTC 4163	NG	NG	NG	NG	NG	NG	–	–	–	–	–	–	NG	NG
Staphylococcus aureus	EMRSA 1729/98	NG	NG	–	–	–	–	–	–	–	–	–	–	–	–
Staphylococcus aureus	EMRSA 07924/95	NG	NG	NG	NG	NG	NG	–	–	–	–	–	–	NG	NG

[a] Each substrate was tested at a concentration of 300 mg/L.
[b] Results after 24 and 48 h of incubation at 37 °C.
[c] Positive indicates colour observed; negative indicates no colour observed.
[d] NG, no growth.

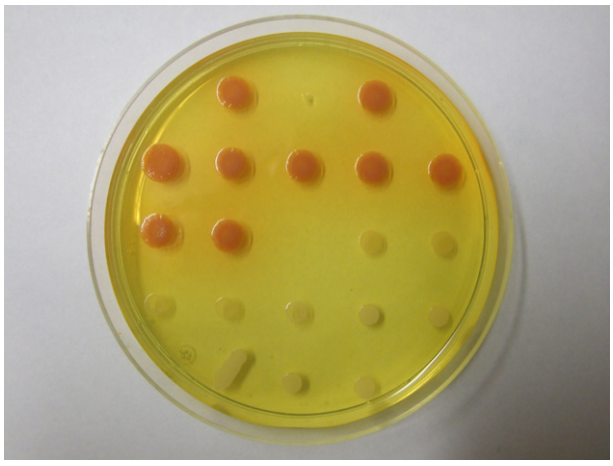


Figure 2. Evaluation of 9-(4′-*N*-[L-Ala-L-Ala-L-Ala]aminophenyl)-10-methylacridinium *bis*-trifluoroacetate **4e** with nine Gram-negative and ten Gram-positive bacterial strains.

Table 3. Percentage of bacterial strains detected by substrates **4b**, **4g** and **3e**

Bacterial strain	No. of strains	**4b**	**4g**	**3e**
Burkholderia cepacia	34	53	9	100
Burkholderia gladioli	6	0	0	83
Other *Burkholderia* spp.	9	33	56	88
Pseudomonas aeruginosa	74	100	97	100
Other *Pseudomonas* spp.	17	35	24	94
Pandoraea spp.	8	38	38	100
Ralstonia picketti	4	50	50	100
Other Ralstonia spp.	16	19	38	100
Stenotrophomonas maltophilia	2	0	0	100
Acinetobacter spp.	7	0	0	71
Brevindomonas spp.	2	0	0	100
Moraxella spp.	3	67	33	100
Sphingobacterium spiritivorum	1	100	0	100
Chryseobacterium meningosepticum	1	0	0	100
Oligella spp.	1	100	100	100

[L-pyroglutamyl]aminophenyl)-10-methylacridinium iodide **4g**, and the β-alanyl substrate, 9-(4′-*N*-[β-alanyl]-aminophenyl)-10-methylacridinium *bis*-trifluoroacetate **4b**. These substrates showed high specificity for *P. aeruginosa*, along with *Burkholderia cepacia* and *Serratia marcescens*. To evaluate the extent of their specificity, an extended range of non-fermentative bacteria were tested with these two substrates, along with 9-(4′-*N*-[N_α-^{t}Boc-L-Ala-L-Ala-L-Ala]aminophenyl)-10-methylacridinium iodide **3e** as a control substrate that is hydrolysed by most Gram-negative bacteria, Table 3.

The ability of the β-alanyl substrate, **4b**, to detect the presence of 100% of *P. aeruginosa* strains was encouraging. This substrate showed promising potential for the detection of *P. aeruginosa* in cultured samples and could be used with complementary substrates and selective agents to enhance the specificity of detection. The L-pyroglutamyl substrate, **4g** showed a similar specificity and successfully detected 97% of the *P. aeruginosa* strains tested.

In conclusion, substrates for bacterial aminopeptidases based on 9-(4′-*N*-[aminoacyl]aminophenyl)-10-methylacridinium salts were effective at differentiating between Gram-positive and Gram-negative bacteria on agar plates, due to the differential expression and activity of the appropriate aminopeptidases. 9-(4′-*N*-[L-Ala-L-Ala-L-Ala]amino-phenyl)-10-methylacridinium *bis*-trifluoroacetate **4e** and its ^{t}Boc protected derivative, 9-(4′-*N*-[N_α-^{t}Boc-L-Ala-L-Ala-L-Ala]aminophenyl)-10-methylacridinium iodide **3e**, showed greatest promise for this application. 9-(4′-*N*-[L-Pyroglutamyl]amino-phenyl)-10-methylacridinium iodide **4g** was effective in distinguishing between members of the enterobacteriaceae family, being hydrolysed by *K. pneumoniae* and *E. cloacae*, but not by the strains of *E. coli* and *Salmonella* spp. tested, nor by *S. sonnei*. Further testing is still required to evaluate this substrate's ability to distinguish *Citrobacter* species from *Salmonella* and *E. coli* species. 9-(4′-*N*-[β-Alanyl]aminophenyl)-10-methylacridinium *bis*-trifluoroacetate **4b** and 9-(4′-*N*-[L-pyroglutamyl]aminophenyl)-10-methylacridinium iodide **4g**, showed potential for the detection of *P. aeruginosa* in cultured samples; a reliable detection method for this bacterium would be valuable in the clinical management of cystic fibrosis.

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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.2007.11.018.

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