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Fluorogenic/chromogenic enzyme substrates constructed around the 2-amino-7-nitrofluorene core structure for the detection of aminopeptidase activities in clinically important microorganisms

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ABSTRACT

Chromogenic/fluorogenic enzyme substrates have been widely employed for the detection and identification of pathogenic microorganisms. In this paper we describe the synthesis and evaluation in solid (agar) media of a series of novel enzyme substrates for the detection of aminopeptidase activities in clinically important microorganisms. Amino acid derivatives of 2-amino-7-nitrofluorene and its 9,9-dimethyl analogue were prepared and these substrates produced yellow coloured/fluorescent colonies when aminopeptidase activity was present. The L-alanylaminopeptidase substrates 2-(L-alanyl)-amino-7-nitrofluorene (**18a**) and 2-(L-alanyl)-amino-7-nitro-9,9-dimethylfluorene (**18b**) could differentiate between Gram-negative and Gram-positive bacteria and the β-alanylaminopeptidase substrates 2-(β-alanyl)-amino-7-nitrofluorene (**19a**) and 2-(β-alanyl)-amino-7-nitro-9,9-dimethylfluorene (**19b**) were effective in detecting *Pseudomonas aeruginosa*, a microorganism commonly encountered in people with cystic fibrosis. A homologue of substrate **19b**, 2-(γ-aminobutyryl)-amino-7-nitro-9,9-dimethylfluorene (**20b**), was also prepared and evaluated for the detection of *Burkholderia cepacia* (also associated with people with cystic fibrosis).

1. Introduction

Chromogenic/fluorogenic enzyme substrates have been widely utilised in diagnostic microbiology and an important health-care application of these substrates encompasses the detection and identification of clinically relevant, pathogenic microorganisms [1–5]. The substrates are designed to target specific types of enzymatic activity associated with the microorganism of interest and one area of endeavour in which substrates of general structure **1** (Scheme 1) have been utilised is for the detection of microbial aminopeptidase activity. These substrates are generally designed to be weakly coloured/fluorescent, but after hydrolysis by an appropriate aminopeptidase, strongly coloured/fluorescent amines **2** are liberated and hence aminopeptidase activity can be visualised. Representative examples of structures **1** include derivatives of 4-nitroaniline **3** [6,7], 9-(4-aminophenyl)acridinium salts **4** [8], 4-(4-aminostyryl)lepidinium salts **5** [9], 7-aminophenoxazin-3-ones **6** [10,11], 7-amino-4-methylcoumarins **7** [7], 2-(2-aminophenyl)benzoxazoles (X = O) and benzothiazoles (X = S) **8** [12] and 2-amino-*N*-benzylacridones **9** [13] (Fig. 1). Interestingly, this last substrate exhibits a strong blue fluorescence, but after hydrolysis to the corresponding amine a notable change in the fluorescence emission wavelength (to yellow) is observed.

For applications in solid culture media (agar), both the substrate and its hydrolysis product should be non-inhibitory to the microbial

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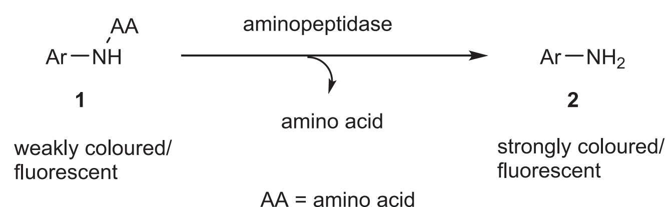


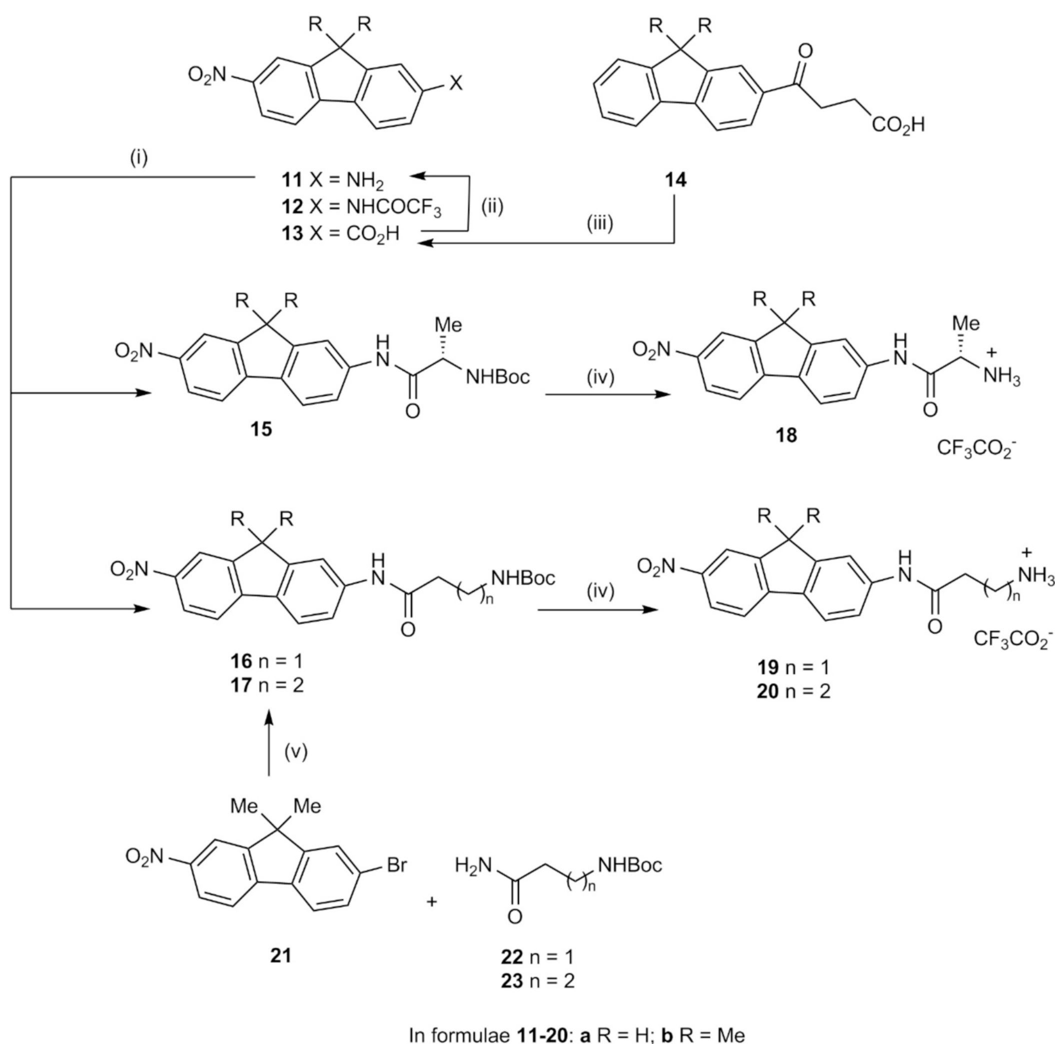
Scheme 1. Design of chromogenic/fluorogenic aminopeptidase substrates

pathogens being targeted. The substrate should be hydrolysed to generate a chromophore/fluorophore that does not diffuse into the culture medium but remains highly localised within the microbial colonies. This enables clear differentiation of microorganisms producing the target enzyme from those that do not, which is an important consideration when the detection of specific microorganisms within polymicrobial cultures is attempted [2]. Clinical samples may contain several other microbial species in addition to the microorganism of interest and hence it is often necessary to employ a culture medium that is both selective (*e.g.* by the use of antimicrobials to inhibit the growth of unwanted microorganisms) and differential (by detecting specific enzymes).

Of direct relevance to the work described in this paper is the differentiation of Gram-negative bacteria from Gram-positive bacteria by utilising a suitable L-alanylaminopeptidase substrate because L-alanylaminopeptidase activity is ubiquitous in Gram-negative bacteria but is generally undetected in most Gram-positive bacteria [6,7]. Also of interest to us is the detection of β-alanylaminopeptidase activity which is associated with the common respiratory pathogen *Pseudomonas aeruginosa*, a pernicious microorganism that frequently colonises the lungs of people with cystic fibrosis [10,11].

In this paper we describe the synthesis and evaluation of the

Fig. 1. Representative examples of known chromogenic and fluorogenic aminopeptidase substrates and the structures of novel substrates prepared in this work (AA = amino acid).



Scheme 2. Synthesis of aminopeptidase substrates. Reagents and conditions: (i) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC.HCl), 1-hydroxybenzotriazole, Boc-protected amino acid, CH_2Cl_2, 2 days, rt. (11–75%); (ii) (a) $SOCl_2$ then aq. NaN_3, (b) toluene, 100 °C, 1 h, (c) H_2O (49% over 3 steps); (iii) HNO_3, H_2SO_4 (48%) (iv) TFA, CH_2Cl_2, rt., 4–6 h (84–100%), (v) Buchwald-Hartwig reaction, see ref. 17 (38–61%).

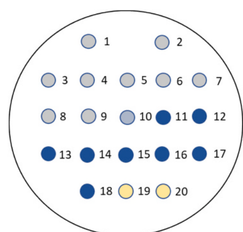


Fig. 2. Arrangement of microorganisms on a Columbia agar plate with the microorganisms numbered in the sequence shown in the evaluation Tables. Spots 1–10 represent Gram-negative bacteria, spots 11–18 depict Gram-positive bacteria and spots 19 and 20 define the yeast species.

aminopeptidase substrates **10** associated with the 2-amino-7-nitrofluorene core structure. Substrates **10** were conceived as 'extended' versions of the 4-nitroaniline substrates **3** in which the biphenyl moiety, and hence the conjugated π-system, is planar by virtue of the fluorene's methylene bridge. Substrates **3** are unsuitable for use in solid (agar) media because the 4-nitroaniline chromophore diffuses away from the microorganism colonies and we anticipated that diffusion in agar media could be minimised by using these 'larger', extended substrates **10**. Additionally, the fluorene's methylene bridge is amenable to substitution and hence diffusion could be further restricted by appropriate choice of substituents (*e.g.* alkyl groups) at this position.

The synthetic routes adopted for the preparation of substrates **18-19a,b** and **20b** are depicted in Scheme 2. The mono-reduction of 2,7-dinitrofluorene using palladium on carbon and formic acid [14] was initially attempted as a route to 2-amino-7-nitrofluorene **11a**, but in our hands, a low yield of product was obtained after chromatography. Compound **11a** was therefore prepared using an alternative literature procedure [15] from 2-aminofluorene *via* nitration of its *N*-trifluoroacetamide derivative **12a** followed by removal of the trifluoroacetamide group. The 9,9-dimethyl analogue **11b** was similarly prepared from 2-amino-9,9-dimethylfluorene. During our work, a milligram scale preparation of compound **11b** (62% yield) contaminated by an isomer (5-amino-2-nitro-9,9-dimethylfluorene, 19% yield) from 2-nitro-9,9-dimethylfluorene was reported using a photocatalytic amination procedure [16]. The condensation of amines **11a,b** with an appropriate Boc-protected amino acid gave the series of amides **15a,b**, **16a,b** and **17b** which were then deprotected using trifluoroacetic acid

Table 1
Evaluation of the L-alanylaminopeptidase substrates **18a** and **18b** in Columbia agar. Inoculum = 100,000 colony-forming units (cfu)/spot.

	Substrate	18a									18b								
	Substrate concentration	100 mgL^{-1}			50 mgL^{-1}			25 mgL^{-1}			100 mgL^{-1}			50 mgL^{-1}			25 mgL^{-1}		
	Growth[a] and Colour/ fluorescence intensity[b]	Growth	Colour (yellow)	Fluor. (yellow)	Growth	Colour (yellow)	Fluor. (yellow)	Growth	Colour (yellow)	Fluor. (yellow)	Growth	Colour (yellow)	Fluor. (yellow)	Growth	Colour (yellow)	Fluor. (yellow)	Growth	Colour (yellow)	Fluor. (yellow)
Microorganism/Reference[c]																			
Gram-negative microorganisms																			
1	*Escherichia coli* NCTC 10418	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
2	*Raoultella planticola* NCTC 9528	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
3	*Providencia rettgeri* NCTC 7475	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	*Enterobacter cloacae* NCTC 11936	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	*Serratia marcescens* NCTC 10211	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	*Salmonella typhimurium* NCTC 74	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	+	+
7	*Pseudomonas aeruginosa* NCTC 10662	+/–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	*Yersinia enterocolitica* NCTC 11176	+	+	+	+	+	+/–	+	+	+/–	+	+	+	+	+	+	+	+	+
9	*Burkholderia cepacia* NCTC 10743	+	+	+	+	+	+/–	+	+	+/–	+	+	+	+	+	+	+	+	+
10	*Acinetobacter baumannii* NCTC 12156	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+
Gram-positive microorganisms																			
11	*Streptococcus pyogenes* NCTC 8306	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+/–	–	+/–
12	*Staphylococcus aureus* (MRSA) NCTC 11939	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+/–	–	+/–
13	*Staphylococcus aureus* (MSSA) NCTC 6571	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+/–	Grey	+
14	*Staphylococcus epidermidis* NCTC 11047	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
15	*Listeria monocytogenes* NCTC 11994	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	+/–	–	+/–
16	*Enterococcus faecium* NCTC 7171	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	+/–	–	+/–
17	*Enterococcus faecalis* NCTC 775	+	Tr.	+	+	–	–	+	–	–	–	–	–	–	–	–	+/–	–	+/–
18	*Bacillus atrophaeus* ATCC 9372	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	Orange	–
Yeasts																			
19	*Candida albicans* ATCC 90028	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Tr.	–	–
20	*Candida glabrata* NCPF 3943	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] + good growth, +/– weak growth, Tr. trace of growth, – no growth.
[b] + strong colour/fluorescence, +/– weak colour/fluorescence, Tr. trace of colour/fluorescence, – no colour/fluorescence.
[c] NCTC: National Collection of Type Cultures; ATCC: American Type Culture Collection; NCPF: National Collection of Pathogenic Fungi.

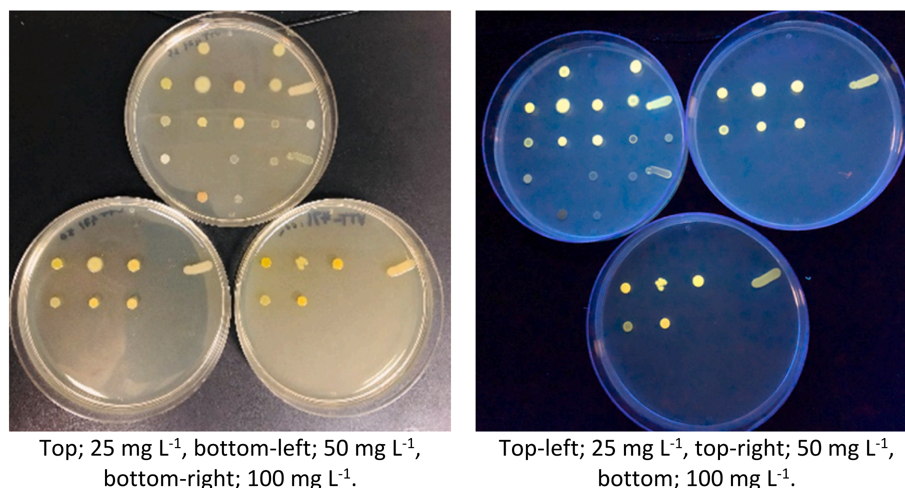


Fig. 3. Columbia agar plates showing 20 microorganisms in the presence of substrate **18b** at various concentrations. Left-hand image viewed under ambient light and right-hand image viewed under UV illumination (λ = 360 nm). Inoculum = 100,000 cfu/spot.

(TFA) giving the required substrates **18a,b**, **19a,b** and **20b** respectively. As part of some of our other ongoing studies in fluorene chemistry, we had prepared the fluorene derivative **14b** through Friedel-Crafts acylation of 9,9-dimethylfluorene with succinic anhydride. Nitration of compound **14b** introduced a nitro-group into the fluorene core as expected, but under the reaction conditions an oxidative cleavage of the succinyl moiety occurred and the carboxylic acid derivative **13b** was produced. The identity of this product was confirmed through its conversion into the amine **11b** *via* a Curtius rearrangement. This synthetic sequence, which has not been optimised, provides a serendipitous alternative for accessing the starting material **11b**. An alternative, shorter synthesis of the intermediate **16b** was also developed and published by us which exploited a Buchwald-Hartwig reaction (38% yield) between 2-bromo-7-nitro-9,9-dimethylfluorene **21** and Boc-protected 3-aminopropanamide **22** using catalytic quantities of $Pd_2(dba)_3$ and 'Xantphos' in the presence of Cs_2CO_3 [17]. A similar procedure using the homologue **23** of amide **22** enabled an alternative preparation of the Boc-derivative **17b** (61% yield).

Each substrate was evaluated simultaneously on a single plate in Columbia agar medium (Thermo Fisher Scientific, Loughborough, UK; pH 7.3; 37 °C in air for 18 h) against a selection of 20 clinically important microorganisms which included 10 Gram-negative bacteria, 8 Gram-positive bacteria and 2 yeasts. The growth of the microorganisms was compared with a control plate in which no substrate was present. The arrangement of the microorganisms on each plate is depicted in Fig. 2 and the numbering (1−20) corresponds with the sequence shown in the appropriate evaluation Tables.

The L-alanyl substrates **18a** and **18b** (Table 1, Fig. 3) were evaluated at three concentrations (100 mg L^{-1}, 50 mg L^{-1}, and 25 mg L^{-1}) against the panel of 20 microorganisms. At a substrate concentration of 100 mg L^{-1} both moderately intense, yellow-coloured colonies and intense, yellow fluorescent colonies were obtained with most Gram-negative bacteria. The growth of *Escherichia coli* and *Raoultella planticola* was inhibited by these substrates and *Acinetobacter baumannii* was also inhibited by substrate **18b**. The growth of most Gram-positive bacteria was inhibited by both substrates at a concentration of 100 mg L^{-1}. Substrates are expected to become less inhibitory at lower concentrations, and this was exemplified by substrate **18b** at a concentration of 25 mg L^{-1} which allowed the growth of all Gram-negative bacteria and the associated production of yellow-coloured/fluorescent colonies. Most Gram-positive bacteria also grew at this substrate concentration, albeit weakly, apart from *Staphylococcus epidermidis* which was inhibited. The formation of coloured colonies was not observed with the Gram-positive bacteria at this lower substrate concentration. The growth of the two yeast species was inhibited by both substrates at all concentrations, with the exception of *Candida albicans* which showed slight growth at 25 mg L^{-1} in the presence of substrate **18b**, producing a colourless and non-fluorescent colony.

Previous studies have revealed that within the selection of 10 Gram-negative bacteria used in this study, β-alanylaminopeptidase substrates will be hydrolysed by the Gram-negative bacteria *Serratia marcescens*, *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex [18]. In accordance with expectations these three microorganisms produced intense, yellow fluorescent colonies in the presence of the substrates **19a** and **19b** at substrate concentrations of 100 mg L^{-1} (Table 2, Fig. 4). These substrates were also evaluated at lower concentrations (50 mg L^{-1} and 25 mg L^{-1}), and intense, yellow fluorescent colonies were still observed at 25 mg L^{-1} except for *Serratia marcescens* and substrate **19a**. Additionally, substrate **19b** produced yellow-coloured colonies with these three microorganisms at all substrate concentrations. In contrast, only a trace of colour was apparent from *Pseudomonas aeruginosa* and *Burkholderia cepacia* and substrate **19a** at the higher concentration (100 mg L^{-1}) and no coloured colonies were generated by *Serratia marcescens* at the lower substrate concentrations. The growth of most Gram-positive bacteria and yeasts was generally inhibited by these substrates. In view of the potential application of β-alanylaminopeptidase substrates for the detection of *Pseudomonas aeruginosa* in people with cystic fibrosis, a novel chromogenic medium using substrate **19b** was developed and reported by our group for culture of *Pseudomonas aeruginosa* from respiratory samples of people with cystic fibrosis [17]. A total of 198 respiratory samples were cultured onto this medium and two other commercially available media. The sensitivity and the positive predictive value of this novel medium was greater than that of currently available commercial media, it was shown to be highly effective for the isolation and specific detection of *Pseudomonas aeruginosa* from respiratory samples, and the colour produced was found to be stable and localised only to the microbial colonies after at least 72 h of incubation.

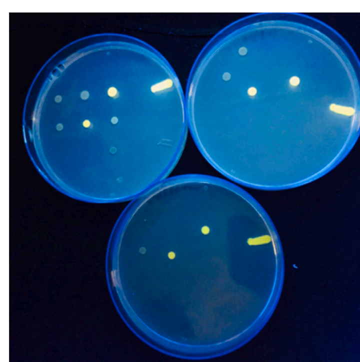
In view of our general interest in the application of aminopeptidase substrates in microbial diagnostics, the homologue of the β-alanyl substrate, compound **20b**, was also prepared (Table 3). The same Gram-negative bacteria (*Providencia rettgeri*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, and *Burkholderia cepacia*) which grew in the presence of the β-alanyl substrate **19b** also grew in the presence of substrate **20b** at the same substrate concentration (100 mg L^{-1}). However, within this group of bacteria, only *Burkholderia cepacia* produced coloured/fluorescent colonies with substrate **20b**. In view of the association of the *Burkholderia cepacia* complex with people with cystic fibrosis, it was therefore of interest to examine the efficacy of substrate

Table 2
Evaluation of the β-alanylaminopeptidase substrates **19a** and **19b** in Columbia agar. Inoculum = 100,000 cfu/spot.

	Substrate	19a									19b								
	Substrate concentration	100 mgL^{-1}			50 mgL^{-1}			25 mgL^{-1}			100 mgL^{-1}			50 mgL^{-1}			25 mgL^{-1}		
	Growth[a] and Colour/ fluorescence intensity[b]	Growth	Colour (yellow)	Fluor. (yellow)	Growth	Colour (yellow)	Fluor. (yellow)	Growth	Colour (yellow)	Fluor. (yellow)	Growth	Colour (yellow)	Fluor. (yellow)	Growth	Colour (yellow)	Fluor. (yellow)	Growth	Colour (yellow)	Fluor. (yellow)
Microorganism/Reference[c]																			
Gram-negative microorganisms																			
1	*Escherichia coli* NCTC 10418	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	*Raoultella planticola* NCTC 9528	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	*Providencia rettgeri* NCTC 7475	+	–	–	+	–	–	+	–	–	–	–	–	+	–	+/–	+	–	+/–
4	*Enterobacter cloacae* NCTC 11936	+	–	–	+	–	–	+	–	–	–	–	–	–	–	–	+	–	+/–
5	*Serratia marcescens* NCTC 10211	+	+	+	+	–	–	+	–	–	+	+	+	+	+	+	+	+	+
6	*Salmonella typhimurium* NCTC 74	+	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–
7	*Pseudomonas aeruginosa* NCTC 10662	+/–	Tr.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	*Yersinia enterocolitica* NCTC 11176	+	–	–	+	–	–	+	–	–	+	–	–	+	–	–	+	–	+/–
9	*Burkholderia cepacia* NCTC 10743	+	Tr.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	*Acinetobacter baumannii* NCTC 12156	+	–	–	+	–	–	+	–	–	–	–	–	–	–	–	+	–	+/–
Gram-positive microorganisms																			
11	*Streptococcus pyogenes* NCTC 8306	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	*Staphylococcus aureus* (MRSA) NCTC 11939	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	*Staphylococcus aureus* (MSSA) NCTC 6571	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
14	*Staphylococcus epidermidis* NCTC 11047	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
15	*Listeria monocytogenes* NCTC 11994	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	+/–	Grey	–
16	*Enterococcus faecium* NCTC 7171	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
17	*Enterococcus faecalis* NCTC 775	+	Tr.	+	+	–	–	+	–	–	–	–	–	–	–	–	+/–	Grey	–
18	*Bacillus atrophaeus* ATCC 9372	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Yeasts																			
19	*Candida albicans* ATCC 90028	Tr.	–	–	+	–	–	+	–	–	–	–	–	–	–	–	Tr.	–	–
20	*Candida glabrata* NCPF 3943	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–

[a] + good growth, +/− weak growth, Tr. trace of growth, − no growth.
[b] + strong colour/fluorescence, +/− weak colour/fluorescence, Tr. trace of colour/fluorescence, − no colour/fluorescence.
[c] NCTC: National Collection of Type Cultures; ATCC: American Type Culture Collection; NCPF: National Collection of Pathogenic Fungi.

Top-left; 25 mg L^{-1}, top right; 50 mg L^{-1}, bottom; 100 mg L^{-1}.



Top-left; 25 mg L^{-1}, top right; 50 mg L^{-1}, bottom; 100 mg L^{-1}.

Fig. 4. Columbia agar plates showing 20 microorganisms in the presence of substrate **19b** at various concentrations. Left-hand image viewed under ambient light and right-hand image viewed under UV illumination (λ = 360 nm). Inoculum = 100,000 cfu/spot. *Pseudomonas aeruginosa*, *Serratia marcescens* and *Burkholderia cepacia* produce clearly visible yellow colonies, which fluoresce under UV. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Evaluation of substrate **20b** in Columbia agar. Inoculum = 100,000 cfu/spot.

	Substrate	20b		
	Substrate concentration	100 mg L^{-1}		
	Growth[a] and Colour/ fluorescence intensity[b]	Growth	Colour (yellow)	Fluor. (yellow)
Microorganism/Reference[c]				
Gram-negative microorganisms				
1	*Escherichia coli* NCTC 10418	–	–	–
2	*Raoultella planticola* NCTC 9528	–	–	–
3	*Providencia rettgeri* NCTC 7475	+	–	–
4	*Enterobacter cloacae* NCTC 11936	–	–	–
5	*Serratia marcescens* NCTC 10211	+	–	–
6	*Salmonella typhimurium* NCTC 74	–	–	–
7	*Pseudomonas aeruginosa* NCTC 10662	+	–	–
8	*Yersinia enterocolitica* NCTC 11176	+	–	–
9	*Burkholderia cepacia* NCTC 10743	+	+	+
10	*Acinetobacter baumannii* NCTC 12156	–	–	–

[a] + good growth, – no growth.
[b] + strong colour/fluorescence, – no colour/fluorescence.
[c] NCTC: National Collection of Type Cultures.

20b with a wider selection of species that are part of the *Burkholderia cepacia* complex (Table 4). However, substrate **20b** was not deemed suitable for differentiation of the *Burkholderia cepacia* complex from other species as approximately half of the microorganisms tested did not demonstrate substrate hydrolysis within 48 h. However, substrate **20b** may be useful as a complementary substrate to differentiate between the most important pathogenic species within the *Burkholderia cepacia* complex such as *B. cenocepacia, B. multivorans* and *B. vietnamiensis*. In people with cystic fibrosis, *B. multivorans* and *B. cenocepacia* are the dominant species whereas several other species (*e.g.* including *B. dolosa*, *B. ambifaria*, *B. anthina* and *B. pyrrocinia*) are rarely encountered [19].

In conclusion, the 2-amino-7-nitrofluorene based substrates resulted in the production of both brightly coloured and strongly fluorescent microbial colonies when appropriate aminopeptidase activity was present, a distinct advantage of these substrates. The non-diffusible yellow product is uncommon among diagnostic reagents and some of these substrates would prove useful when combined with others that produce complementary colours. The L-alanylaminopeptidase substrates **18a,b** were effective at differentiating Gram-negative bacteria from Gram-positive bacteria and yeasts at 25 mg L^{-1}, though **18a** was found to be inhibitory to *E. coli* and *R. planticola.* The β-alanylaminopeptidase substrates **19a,b** were effective for the detection of *P. aeruginosa* and *B. cepacia* at 25 mg L^{-1}, and substrate **19b** also enabled detection of *S. marcescens*. The efficacy of substrate **19b** for the detection of

Table 4
Evaluation of substrate **20b** against a panel of 26 isolates belonging to the *Burkholderia cepacia* complex in Oxoid Columbia agar at 37 °C. Substrate concentration = 50 mg L^{-1}, inoculum = 10^5 cfu/spot.

	Reference[a]	Species	24 h		48 h	
			Growth[b]	Colour[c] (yellow)	Growth[b]	Colour[c] (yellow)
1	LMG 1222	*Burkholderia cepacia*	+	+ [d]	+	+ [d]
2	LMG 10929	*Burkholderia vietnamiensis*	+	–	+	–
3	LMG 13010	*Burkholderia multivorans*	+	–	+	–
4	LMG 14191	*Burkholderia pyrrocinia*	+	+	+	+
5	LMG 14294	*Burkholderia stabilis*	+	+	+	+
6	LMG 16232	*Burkholderia vietnamiensis*	+	–	+	–
7	LMG 16660	*Burkholderia multivorans*	+	–	+	–
8	LMG 16656	*Burkholderia cenocepacia*	+	+/–	+	+
9	LMG 17588	*Burkholderia multivorans*	+	–	+	–
10	LMG 18821	*Burkholderia cepacia*	+	+ [e]	+	+ [f]
11	LMG 18822	*Burkholderia multivorans*	+	–	+	–
12	LMG 18824	*Burkholderia multivorans*	+	–	+	–
13	LMG 18828	*Burkholderia cenocepacia*	+	+	+	+
14	LMG 18829	*Burkholderia cenocepacia*	+	+	+	+
15	LMG 18830	*Burkholderia cenocepacia*	+	+/–	+	+ [f]

(continued on next page)

Table 4 (*continued*)

	Reference[a]	Species	24 h		48 h	
			Growth[b]	Colour[c] (yellow)	Growth[b]	Colour[c] (yellow)
16	LMG 18832	*Burkholderia cenocepacia*	+	+	+	+
17	LMG 18835	*Burkholderia vietnamiensis*	+	–	+	–
18	LMG 18863	*Burkholderia cenocepacia*	+	+/−	+	+
19	LMG 18870	*Burkholderia stabilis*	+	+	+	+
20	LMG 18943	*Burkholderia dolosa*	+	–	+	–
21	LMG 19182	*Burkholderia ambifaria*	+	–	+	+
22	LMG 19467	*Burkholderia ambifaria*	+	–	+	+
23	LMG 20980	*Burkholderia anthina*	+	–	+	+
24	LMG 20983	*Burkholderia anthina*	+	+/−	+	+
25	LMG 21820	*Burkholderia dolosa*	+	–	+	–
26	LMG 21824	*Burkholderia pyrrocinia*	+	–	+	Tr.

[a] Belgium National Collection - Laboratory of Microbiology, Ghent.
[b] + good growth.
[c] + strong colour, +/− weak colour, Tr. trace of colour, − no colour.
[d] Yellow pigment on substrate-free control.
[e] Pink colour.
[f] Brown colour that diffused into the agar.

P. aeruginosa has enabled the development of a new medium for detection of this microorganism in people with cystic fibrosis [17]. In evaluation of the homologue of substrate **19b**, **20b**, only *Burkholderia spp.* produced coloured/fluorescent colonies and may be useful as a complementary substrate to differentiate pathogenic species within *B. cepacia* complex.

CRediT authorship contribution statement

Valérie Chalansonnet: Methodology, Investigation. **Charlotte E. Marsh:** Investigation, Formal analysis. **Sylvain Orenga:** Project administration, Methodology, Investigation, Conceptualization. **John D. Perry:** Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Stephen P. Stanforth:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Hannah E. Sykes:** Investigation, Formal analysis. **Thang V. Truong:** Writing – original draft, Investigation, Formal analysis. **Graeme Turnbull:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis. **Alexander Twist:** Investigation, Formal analysis. **Andrey Zaytsev:** Methodology, Investigation, Formal analysis.

Declaration of competing interest

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Authors contribution

Conceptualisation; JDP, SO, SPS, synthetic work and data analysis; CEM, HES, SPS, TVT, AT, GT, AZ, microbiological work and data analysis, VC, SO, JDP; project management JDP, SO, SPS, GT, writing manuscript JDP, TVT, SPS, GT.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2026.110093.

Data availability

All data is provided in the manuscript and supplementary information.

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