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Synthesis and Evaluation of Novel 7- and 8-Aminophenoxazinones for the Detection of β-Alanine Aminopeptidase Activity and the Reliable Identification of *Pseudomonas aeruginosa* in Clinical Samples

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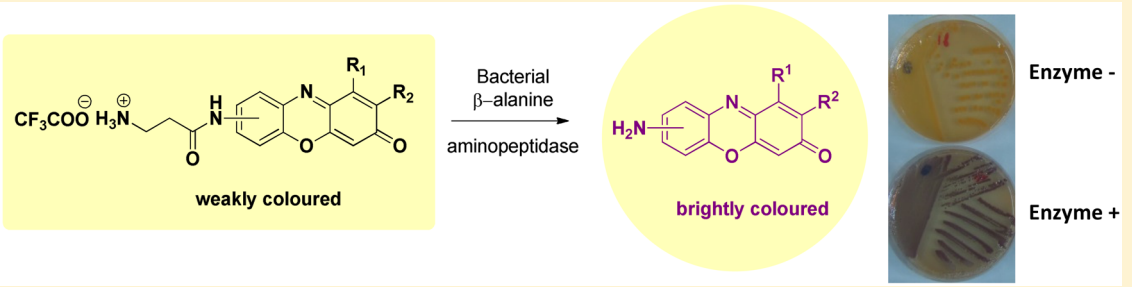
Supporting Information



ABSTRACT: A series of novel 8-aminophenoxazin-3-one and 7-aminophenoxazin-3-one chromogens and their corresponding β-alanine derivatives were synthesized and evaluated for their ability to detect β-alanyl aminopeptidase activity in bacteria known to hydrolyze β-alanine derivatized substrates. The results provided insight into the structural requirements for effective visualization of enzymatic activity and the mechanism of formation of phenoxazinon-3-ones. 8-Aminophenoxazin-3-one substrates **23c**, **23d**, and **23e** were prepared in good to high overall yield and were selective for β-alanyl aminopeptidase activity in bacteria, producing a lighter agar background coloration facilitating visualization of colored colonies, with variable localization to the colonies, but had lower sensitivities for the detection of *Pseudomonas aeruginosa* in comparison to the analogous 7-aminophenoxazin-3-one substrates. The synthetic methodology employed here allows the preparation of a range of substrates for evaluation and the establishment of structure−activity relationships. For example, the 2-pentyl substituted aminophenoxazin-3-one **22b** performed with analogous sensitivity to the corresponding 1-pentyl-7-aminophenoxazin-3-one substrate **1** used commercially, highlighting that the position of the pentyl substituent can be varied while maintaining detection sensitivity.

1. INTRODUCTION

β-Alanine derivatives of 7-aminophenoxazin-3-one are efficient markers of β-alanine aminopeptidase activity,[1] an enzyme recently identified in *Pseudomonas* sp.[2] but also suspected to be produced by *Serratia marcescens* and *Burkholderia cepacia*. Culture of these bacteria on medium containing analogues of 7-*N*-(β-alanyl)aminophenoxazin-3-one,[1] analogues of 9-(4′-*N*-[β-alanyl]aminophenyl)acridines,[3] and analogues of 9-(4′-*N*-[β-alanyl]aminophenyl)-10-methylacridinium salts[4] produce brightly colored colonies, resulting from the enzymatic hydrolysis of these chromogenic substrates.

The Gram negative rod-shaped bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen that is responsible for about 10% of hospital acquired infections in the U.S. and UK. As a result of its nutritional versatility, *P. aeruginosa* is a severe threat to immunocompromised patients, particularly neonatal and elderly patients; it is the most serious pathogenic cause of ventilator-associated pneumonia (VAP), colonizes the respiratory system of up to 80% of patients with cystic fibrosis, and is widely found in patients with AIDS and chemotherapy-induced neutropenia and organ transplant recipients. It is one of the "ESKAPE" pathogens identified as a significant clinical threat due to its ability to cause severe disease in humans.[5]

Once established, infections due to *P. aeruginosa* are difficult to treat effectively due to the intrinsic resistance afforded by the combination of thick mucus secretion, broad spectrum efflux pumps and, increasingly, acquired resistance to many antibacterial agents. Delays in the appropriately directed therapy allow this bacterium to colonize a wound or the

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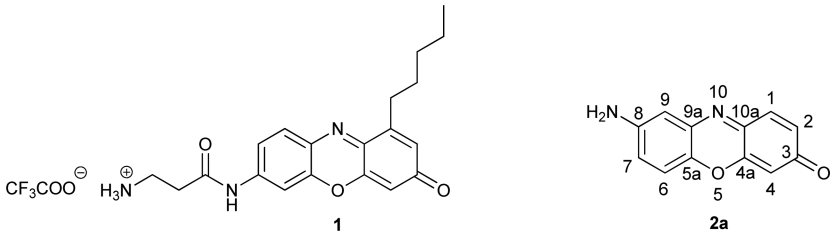
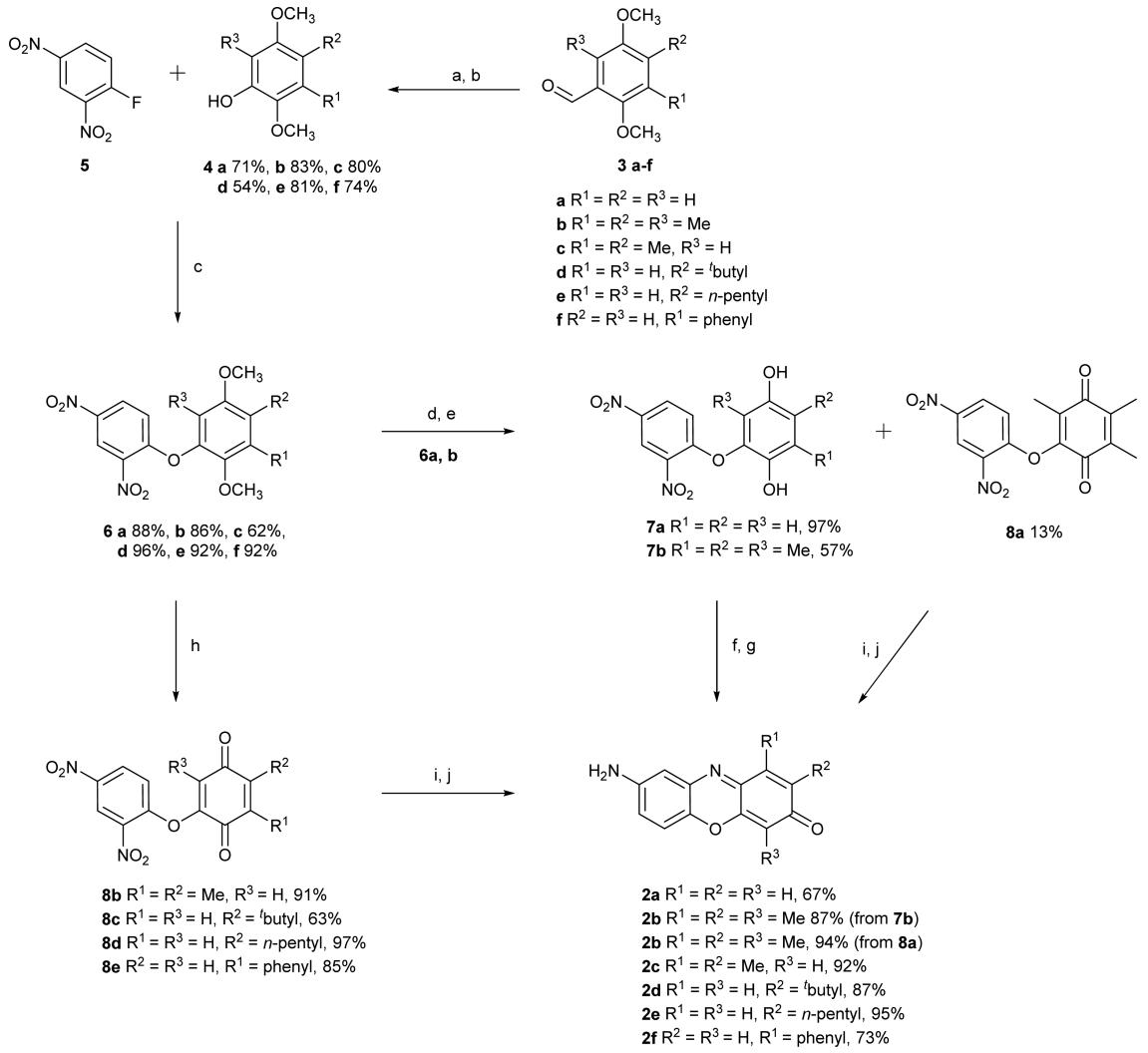


Figure 1. 7-*N*-(*β*-Alanyl)amino-1-pentylphenoxazin-3-one TFA salt **1** and 8-aminophenoxazin-3-one **2a**.

Scheme 1. Synthetic Strategy for 8-Aminophenoxazin-3-ones 2a–f[a]



[a]Reagents and conditions: (a) *m*CPBA, DCM, RT; (b) 10% aq NaOH, MeOH, RT; (c) **4a,c–f**, DMSO, Et_3N, RT, **4b**, DMSO, DBU, RT; (d) DCM, BBr_3 (1M in DCM), −78°C to RT; (e) MeOH, H_2O, 0 °C; (f) H_2, MeOH, Pd/C 5%; (g) MeOH, Et_3N, MnO_2 or Ag_2O; (h) cerium(IV) ammonium nitrate, CH_3CN, H_2O, RT; (i) H_2, Pd/C 5%, EtOAc/MeOH; (j) MeOH, Ag_2O or MnO_2.

respiratory system and to become established, resulting in poor clinical outcomes and increased patient morbidity and mortality. The production of necrosis-causing toxins, e.g., type II secreted effectors, such as ExoU and ExoT, in approximately 65% of hospital-acquired *P. aeruginosa* strains places an additional urgency on the rapid detection and appropriate treatment of this opportunistic pathogen.[6]

The substrate 7-*N*-(*β*-alanyl)amino-1-pentylphenoxazin-3-one TFA salt **1** (Figure 1) possesses excellent properties for accelerating the detection of *P. aeruginosa* in clinical samples: relatively low toxicity to *P. aeruginosa*, high sensitivity to most *P. aeruginosa* strains, and a high affinity of the released chromogen for the bacterial colonies, leading to minimal diffusion of the intense color in agar medium.[1] This substrate has been included in a chromogenic medium developed specifically for the detection of *P. aeruginosa* (chromID *P. aeruginosa*, bioMérieux)[7] and addresses the need to identify quickly and accurately *P. aeruginosa* in clinical samples. The only slight limitations associated with the use of this substrate are the background color it imparts to the agar and the nonideal synthetic route to the chromogen, which leads to mixtures of precursors that necessitate separation of mixed products at a

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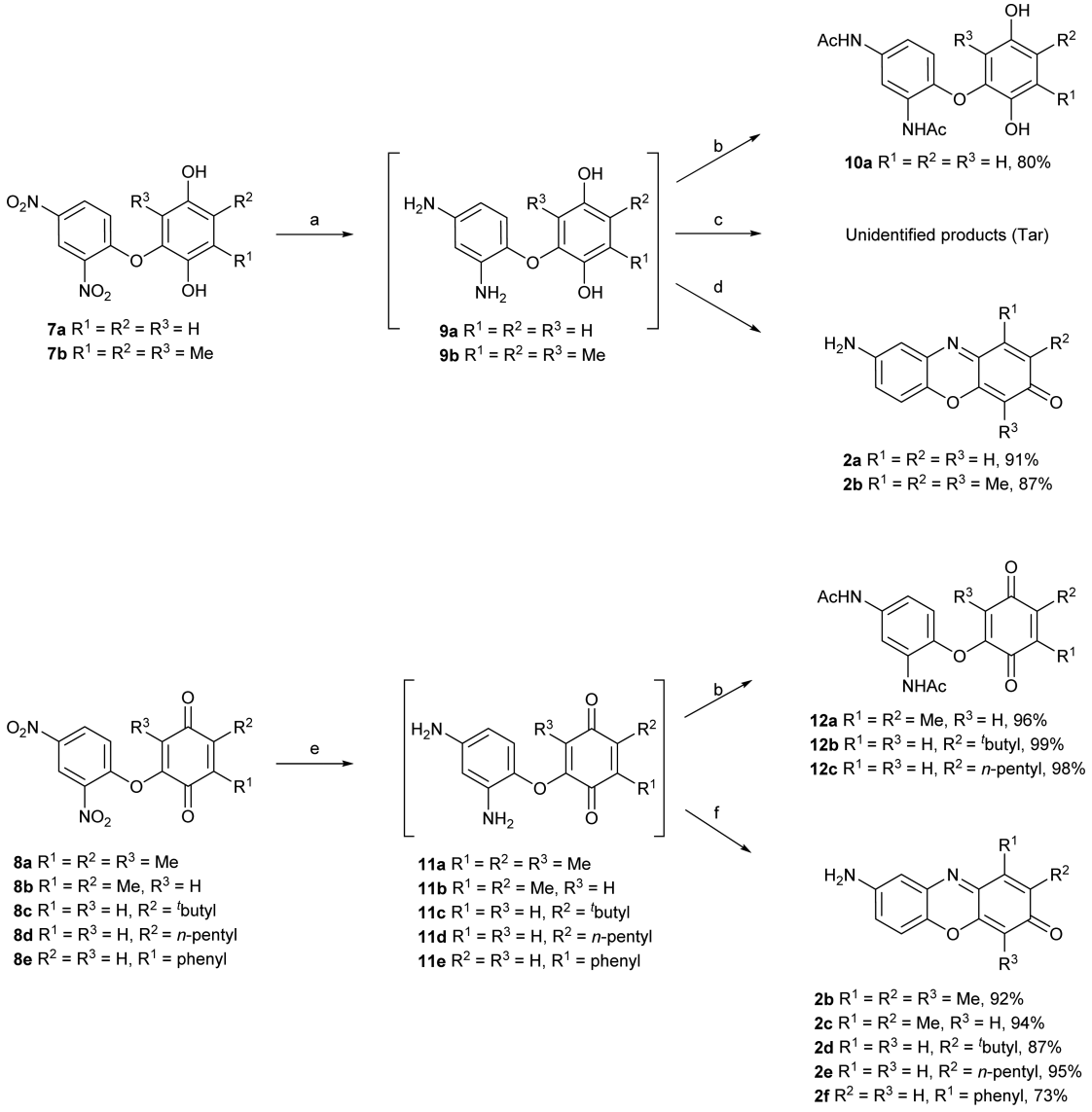
Scheme 2. Reduction and Oxidative Cyclization of Dinitrodihydroxydiphenyl Ethers 7a,b and Dinitrobenzoquinones 8a−e Proceeded through the Diamino Intermediates 9a,b and 11a−e, Respectively, to Yield 8-Aminophenoxazinones 2a−f[a]



[a]Reagents and conditions: (a) H_2, Pd/C 5%, MeOH; (b) Ac_2O, N_2, RT; (c) aerobic oxidation; (d) Et_3N, MeOH, AgO, Ag_2O, or MnO_2; (e) H_2, Pd/C 5%, EtOAc/MeOH; (f) MeOH, Ag_2O, or MnO_2.

late stage by extensive chromatography.[1] Chromogens with comparable performance that are more readily synthesized in high purity are of interest for their potential to offer new economically attractive chromogenic media for *P. aeruginosa*.

We have previously reported the synthesis of some alkyl substituted 7-aminophenoxazin-3-ones via the preparation of 2,5-dinitro-2′,5′-dimethoxydiphenylether intermediates, which, when subjected to demethylation followed by catalytic hydrogenation, spontaneously cyclized to the corresponding 7-aminophenoxazin-3-ones upon exposure to air.[1] Following the successful microbiological testing of the 7-aminophenoxazinones, we have prepared 8-aminophenoxazin-3-one **2a** (Figure 1) using analogous synthetic methodology. This substrate displayed visually interesting chromogenic properties, suggesting its substituted analogues, and their β-alanyl derivatives, would be worthy of further study. We present here the results of studies that highlight the utility of this synthetic procedure, particularly the scope for synthesis of variously substituted analogues, the good overall yields, and the ready isolation of pure and easily characterizable intermediates at each stage, culminating in the synthesis and evaluation of a series of substituted 8-*N*-(β-alanyl)aminophenoxazin-3-one derivatives of **2a** as potential chromogenic agents for the detection of *P. aeruginosa* in clinical samples, along with two novel substituted 7-*N*-(β-alanyl)-aminophenoxazin-3-one derivatives for direct comparison.

2. RESULTS AND DISCUSSION

2.1. Chemistry. The synthesis of the 8-aminophenoxazinones utilized the previous successful synthetic approach to **1**[1] and **2a**, proceeding through diphenylethers **6**, which were demethylated, then reduced and oxidatively cyclized to the desired 8-aminophenoxazinones **2** (Scheme 1).

The 2,4-dinitro-2′,5′-dimethoxydiphenylethers **6a−f** were prepared by nucleophilic aromatic substitution of 2,4-dinitro-1-fluorobenzene **5** (Sanger's reagent, DNFB)[8] by the phenols

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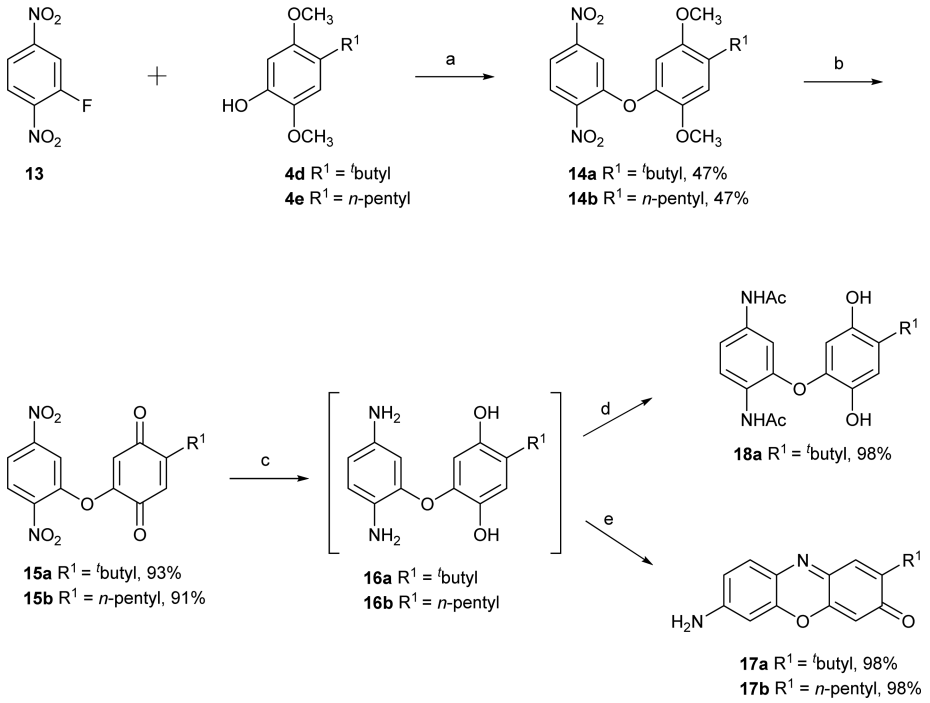
Scheme 3. Preparation of 7-Aminophenoxazinones 17a,b[a]



[a]Reagents and conditions: (a) NaH, dry DMF, RT; (b) CAN, CH_3CN, H_2O; (c) H_2, Pd/C 5%, MeOH/EtOAc; (d) Ac_2O, N_2, RT; (e) MeOH, Ag_2O, or MnO_2.

4a−f, according to the conditions previously described (Scheme 1). It was anticipated that lipophilic substituents would improve the localization of chromogenic substrates to the bacterial colonies grown on agar medium and so help to avoid the diffusion of color after substrate hydrolysis. Accordingly, substituted 2,5-dimethoxyphenols **4a−d** were prepared from the correspondingly substituted 2,5-dimethoxybenzaldehydes **3a−d**, via the Baeyer−Villiger oxidation.[9] Dimethoxyphenols **4e−f** were prepared synthetically by the introduction of alkyl or aryl substituents and an aldehyde group, followed by Baeyer−Villiger oxidation, as described in the Supporting Information.

Initially, the dealkylation of diphenylethers **6a,b,d** was attempted using boron tribromide (BBr_3).[1] The reaction was successful for diphenylethers **6a,b**; in the case of **6b**, 2,4-dinitrophenoxy-3′,4′,6′-trimethyl-2′,5′-benzoquinone **8a** was isolated in addition to the expected product **7b**. The use of BBr_3 was problematic in the case of the *tert*-butyl derivative **6d**, where none of the expected product could be isolated, possibly due to displacement of the *tert*-butyl group, resulting in multiple side reactions, but sufficient quantities of the byproducts could not be isolated for the purposes of characterization. However, oxidative dealkylation of the diphenylethers **6c−f** using cerium(IV) ammonium nitrate (CAN)[10] produced the 2,4-dinitrophenoxybenzoquinones **8b−e** in good to high yield (Scheme 1).

Following our previous synthetic route,[1] 2,4-dinitro-2′,5′-dihydroxydiphenylethers **7a−b** were hydrogenated in a Berghof apparatus with palladium on charcoal (Pd/C, 5%) as catalyst (Scheme 2). The first attempts at aerobic oxidation of the resulting 2,4-diamino-2′,5′-dihydroxydiphenylether intermediates **9a,b** did not yield the expected 8-aminophenoxazinones **2a,b** but led instead to unidentified products. The isolation of 2,4-diacetamido-2′,5′-dihydroxydiphenylether **10a**, resulting from the addition of acetic anhydride immediately after the hydrogenation of **7a**, confirmed the intermediate formation of 2,4-diamino-2′,5′-dihydroxydiphenylether **9a**. Previous work on the mechanism of oxidation of duroquinone[11] suggested that the formation of 8-aminophenoxazin-3-one would be facilitated by higher pH due to a more facile oxidation of the phenolate; however, this was not sufficient to drive the oxidative cyclization and 8-aminophenoxazinones **2a,b** were isolated in high yield only after addition of a metal oxide (AgO, Ag_2O, or MnO_2, Scheme 2).

2,4-Dinitrophenoxybenzoquinones **8a−e** were subjected to the same catalytic hydrogenation procedure (Scheme 2), resulting in rapid formation of the 8-aminophenoxazinones **2b−f**. The isolation of diacetamidophenoxybenzoquinones **12a−c**, resulting from the addition of acetic anhydride immediately after reduction of **8b−d**, established the existence of 2,4-diaminophenoxy-2′,5′-benzoquinones **11a−e** as intermediates in the formation of 8-aminophenoxazinones **2b−f** (Scheme 2).

The formation of 8-aminophenoxazinones **2** from both the dihydroxydiphenylethers **9** and phenoxybenzoquinones **11** provides evidence for the previously proposed pathway[1] (Scheme 2).

Novel 7-aminophenoxazinones **17a,b** were also prepared via the catalytic hydrogenation of 2,5-dinitrophenoxy-2′,5′-benzoquinones **15a,b** (Scheme 3). The precursor dimethoxydiphenylethers **14a,b** were obtained via coupling of 2,5-dinitro-1-fluorobenzene **13** and phenols **4d,e** using sodium hydride in DMF.[1] The yields of diphenylethers **14a,b** were moderate, presumably due to the lower electrophilic character of C-1 in **13**, compared to C-1 in Sanger's reagent **5**. CAN oxidation to the corresponding dinitrophenoxybenzoquinones **15a,b** and catalytic reduction, followed by aerial oxidation, gave the expected 7-aminophenoxazinones **17a,b** in excellent yield. As

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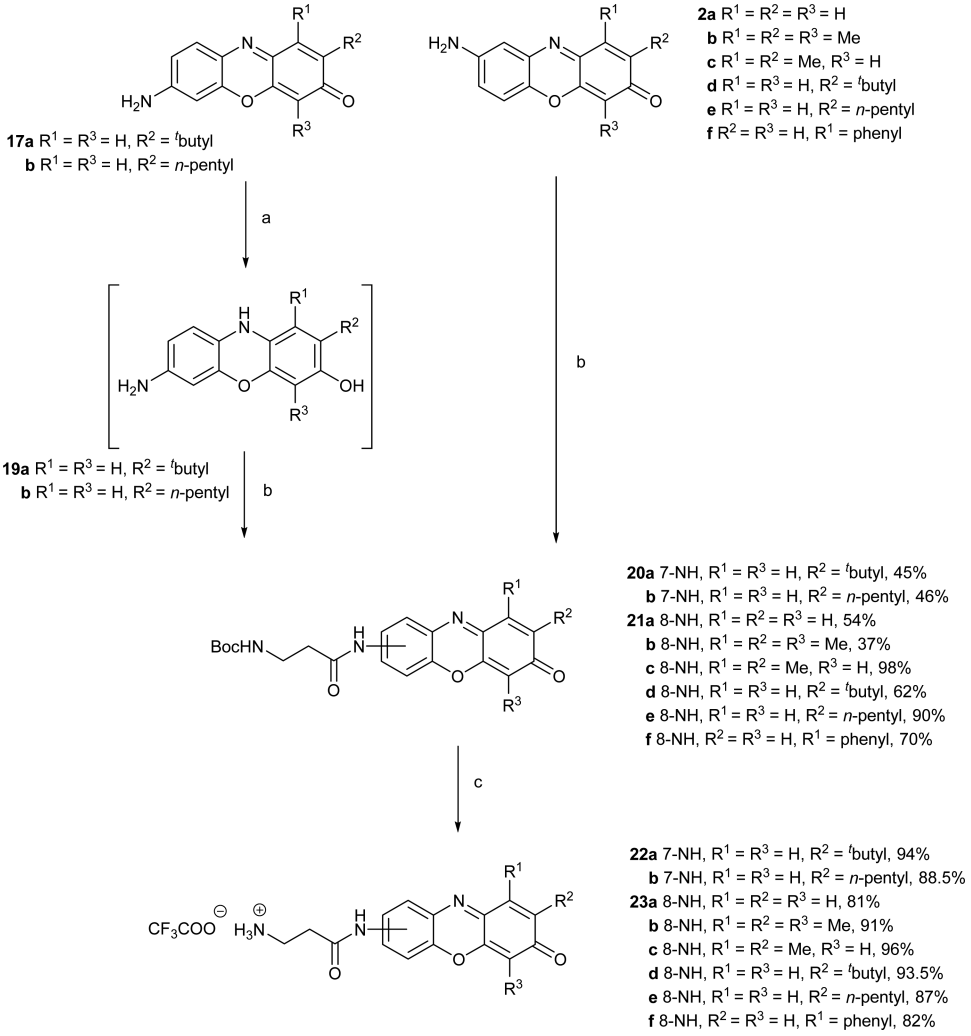
Scheme 4. Amide Bond Formation and ^{t}Boc Deprotection to Form 7-*N*-(β-Alanyl)-aminophenoxazin-3-ones 22a,b and 8-*N*-(β-Alanyl)-aminophenoxazin-3-ones 23a–f[a]



[a]Reagents and conditions: (a) H_2, Pd/C 5%, DMF; (b) ^{t}Boc-β-alanine, IBCF, dry THF, *N*-methylpiperidine, 0°C, 48 h; (c) TFA, RT, 15 min.

with the formation of **2a,b**, the addition of a metal oxide (AgO or MnO_2) during the aeration greatly accelerated the formation of **17a,b**. Addition of acetic anhydride under a protective atmosphere immediately after the hydrogenation of **15a** produced 2,5-diacetamido-2′,5′-dihydroxy-4′-^{t}butyldiphenylether **18a** in excellent yield, providing evidence for 2,5-diamino-2′,5′-dihydroxy-4′-^{t}butyldiphenylether **16a** as the direct product of hydrogenation (Scheme 3).

A facile redox couple of 2,5-diamino-2′,5′-dihydroxydiphenylether **16** and 2,5-diaminophenoxybenzoquinone (not shown in Scheme 3) accounts for the ease of formation of 7-aminophenoxazinone **17a,b**. This is not reflected by the redox couple of 2,4-diamino-2′,5′-dihydroxydiphenylether **9** and 2,4-diaminophenoxybenzoquinone **11**, which requires harsher conditions: 2,4-diamino-2′,5′-dihydroxydiphenylethers **9a,b** did not oxidize upon aerobic oxidation and 2,4-diaminophenoxybenzoquinones **11a–e** were not reduced to the corresponding dihydroxydiphenylethers under the hydrogenation conditions used (Scheme 2). It appears that the position of the amino group, whether it is *meta* or *para* to the oxygen bridging atom (with the second amino group always being *ortho* to the oxygen bridging atom), is a key determinant in the oxidation of diamino-2′,5′-dihydroxydiphenylethers and the reduction of diaminophenoxybenzoquinone species (Schemes 2 and 3). However, it remains unclear how the positioning of the amino group could affect, in this particular case, the radical process involved in oxidation and reduction.

Coupling of 7- and 8-aminophenoxazinones to β-alanine was achieved using the mixed anhydride method. While 7-aminophenoxazinones **17a,b** required reduction to 7-amino-3-hydroxyphenoxazines **19a,b** prior to amide bond formation,[1] 8-aminophenoxazinones **2a–f** reacted readily with the activated anhydride. TFA removal of the ^{t}Boc protecting group from both the 7-aminophenoxazinone **20a,b** and 8-aminophenoxazinone intermediates **21a–f** provided the desired chromogenic substrates **22a,b** and **23a–f** (Scheme 4).

2.2. Chromogenic Properties. Quantitative information, relating the chromogenic properties of analogous phenoxazinones **2d,e** and **17a,b** to the positioning of the auxochromic amino group, was obtained from their UV–visible absorbance spectra (50 μM in MeOH, Table 1). The 8-aminophenoxazin-3-ones **2d,e** yielded violet-colored methanolic solutions (λ_{max} = 528–533 nm), whereas the 7-aminophenoxazin-3-ones **17a,b** produced bright red–pink colored methanolic solutions (λ_{max} = 536–540 nm, Table 1). The auxochrome position only slightly affected the λ_{max} values for phenoxazinones **2d,e** and **17a,b** but

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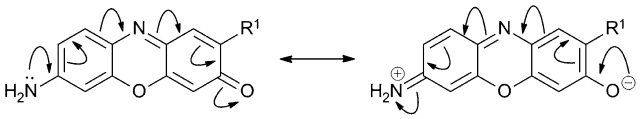
Table 1. Impact of Peptide Derivatization on λ_{max} and ε Values for Phenoxazinones 2d,e and 23a,b (50 μM in MeOH)[a]

compd	λ_{max} (nm)	$\Delta\lambda$ (nm)	A	ε ($dm^3 \cdot mol^{-1} \cdot cm^{-1}$)
2d (chromogen)	536		0.295	5900
23d (substrate)	472	64	0.348	6960
2e (chromogen)	540		0.322	6400
23e (substrate)	475	65	0.388	7760
17a (chromogen)	533		1.804	36080
22a (substrate)	458	75	0.922	18440
17b (chromogen)	528		1.554	31080
22b (substrate)	457	71	0.861	17220

[a]8-Aminophenoxazinones **a**–**c** and **f** were not included in the comparison as their corresponding 7-aminophenoxazinone analogues were not synthesized.

had a remarkable effect on the molar absorptivity (ε) values, which were, on average, almost 6-fold higher for 7-aminophenoxazinones **17a,b** compared to their corresponding 8-aminophenoxazinones **2d,e**, presumably due to the extended electron conjugation in chromogens **17a,b**, which is not possible in **2d,e** (Scheme 5).

Scheme 5. Extended Electron Conjugation in 7-Aminophenoxazin-3-one Derivatives



17a R^1 = ^{t}butyl
b R^1 = *n*-pentyl

β-Alanine derivatization of the auxochrome resulted in a hypsochromic shift in the spectra of both the 7-amino- **22a,b** (71–75 nm) and 8-aminophenoxazinones **23d,e** (64–65 nm) (Table 1). The amide derivatization most dramatically affected the ε values for 7-aminophenoxazinones **17a,b**, decreased by nearly a factor of 2, inhibiting the extended electron conjugation within the phenoxazinone ring system (Scheme 5). Conversely, the ε values of 8-aminophenoxazinones **2d,e** were slightly increased after derivatization with β-alanine (Table 1).

2.3. Microbiological Evaluation. Spot test microbiological screening, against a range of clinically relevant microorganisms, was used to compare the six β-alanyl substrates based on the 8-aminophenoxazinone core, **23a**–**f**, and the two β-alanyl substrates based on the 7-aminophenoxazinone core, **22a,b**, to the reference substrate, 7-*N*-(β-alanyl)amino-1-pentylphenoxazin-3-one **1** (Table 2). Enzymatic activity was anticipated for strains of *P. aeruginosa* and *Serratia marcescens*, which were previously shown to hydrolyze β-alanyl-derivatized chromogenic substrates; detection of *Burkholderia cepacia* was a possible, but less certain, outcome due to variable expression of β-alanyl aminopeptidase in this species.[1]

8-Aminophenoxazinone substrates **23a** (1,2,4-unsubstituted) and **23f** (1-phenyl) lacked toxicity toward the range of Gram positive and Gram negative bacteria and yeasts evaluated but failed to evidence any β-alanine aminopeptidase activity after 24 h and 48 h of incubation. Toxicity toward Gram positive bacteria and yeasts was previously observed with a range of 7-*N*-(β-alanyl)aminophenoxazinones[1] and was observed for the other substrates tested here; the absence of any toxic effect is a desirable property for a molecule used in the detection and identification of bacteria. The lack of substrate hydrolysis from **23a** and **23f** may reflect poor uptake of these substrates. Substrate **23b** (1,2,4-trimethyl) also had a poor response toward β-alanine aminopeptidase: after 24 h, a weak response could be seen for two *P. aeruginosa* strains only; after 48 h, hydrolysis could be seen for all the strains of *P. aeruginosa* and *S. marcescens* but none of the *B. cepacia* (sensitivity of detection: 77.8% after 48 h, Table 2). Although this substrate was generally nontoxic toward the microorganisms tested and the color it released was a distinguishing pink to pale purple, it diffused into the medium, making individual bacterial colony visualization difficult in this plate-based culture system. Substrate **23c** (1,2-dimethyl) showed similar results to **23b** in terms of the sensitivity of detection (77.8% after 48 h) but had an enhanced visual response after only 24 h of incubation, with less diffusion into the medium. Substrates **22a** (7-*N*,2-^{t}butyl), **23d** (8-*N*,2-^{t}butyl), **22b** (7-*N*,2-^{n}pentyl), and **23e** (8-*N*,2-^{n}pentyl) showed the best responses toward β-alanine aminopeptidase, with results similar to **1** and a positive reaction for all of the tested strains of *P. aeruginosa* and *S. marcescens* (Table 2). Except for **22b**, these substrates were also the most toxic, inhibiting the growth of most (**22a**, **23d**, and **1**) or all (**23e**) of the Gram positive strains (Table 2). Substrates **22b** and **23e** showed better localization of the released chromogen to the bacterial colonies compared to substrates **22a** and **23b**–**d**, with virtually no diffusion of the color, facilitating detection and identification of individual bacterial colonies. The prevailing trend in Table 2, when considering substrate hydrolysis and substrate toxicity, reveals that these effects correlate to the overall substrate lipophilicity according to the calculated log *P* (ChemDraw). A concomitant increase in hydrolysis in Gram negative bacteria and toxicity to Gram positive bacteria, also tend to suggest better uptake into microorganisms for lipophilic substrates, indicated by a higher cLogP, than those with lower lipophilicity.

Streaked plates (Figure 2) allowed further comparison of substrates **22b** (7-*N*,2-^{n}pentyl) and **23e** (8-*N*,2-^{n}pentyl) against the standard **1** (7-*N*,1-^{n}pentyl), after 24 h incubation, as an agent for the detection of *S. marcescens*: the presence of a lipophilic 2-^{n}pentyl chain in each of these substrates allows for good localization of the released chromogen to the bacterial colonies. Both 7-(β-alanyl)pentylaminophenoxazinones **22b** and **1** imparted a strong background color to the agar plates, which is a disadvantage as it impairs the visualization of the bacterial colonies. Although the released chromogen was not as intensely colored as that from the standard, 7-*N*-(β-alanyl)-amino-1-pentylphenoxazinone **1**, bacterial enzymatic hydrolysis of novel 8*N*-(β-alanyl)amino-2-pentylphenoxazinone substrate **23e** produced a distinctive purple color and elicited good contrast between background and bacterial colonies, offering some improvement in their visualization (Figure 2).

Finally, substrates **22b** and **23e** were tested against a wider range of 30 *P. aeruginosa* strains to evaluate their sensitivity (Table 3). Use of the medium containing substrate **22b** allowed the detection of 100% of these strains and matched the results obtained with the standard **1**, while the 8-aminophenoxazinone-based substrate **23e** detected 80% of the β-alanine aminopeptidase producing strains (Table 3).

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J. Med. Chem. XXXX, XXX, XXX–XXX

Table 2. Spot Test Screening of Substrates 23a−f, 22a,b, and 1[a] against a Range of Clinically Relevant Gram Positive and Gram Negative Bacteria and Two Strains of Yeast (cLogP, ChemDraw)

		visualization of coloration after 24 h and (48 h)[b]								
bacterial strain	reference no.	**23a**, cLogP 0.37	**23b**, cLogP 0.32	**23c**, cLogP 0.34	**23d**, cLogP 1.81	**23e**, cLogP 2.18	**23f**, cLogP 1.64	**22a**, cLogP 1.81	**22b**, cLogP 2.18	**1**, cLogP 2.18
Gram Negative Bacteria										
E. coli	ATCC 8739	− (−)[c]	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)
E. coli	ATCC 25922	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)
E. cloacae	ATCC 13047	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)
S. typhimurium	NCT C74	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)
M. morganii	CIP 103690	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)
C. freundii	CIP 103547	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)
Y. enterocolitica	NSB 29163	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)
P. rettgeri	ATCC 9250	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)
P. aeruginosa	NCTC 10662	− (−)	− (++)	+ (++)	+ (++)	− (++)	− (−)	++ (++)	− (+)	+ (++)
P. aeruginosa	NA	− (−)	± (+)	+ (+)	+ (+)	+ (+)	− (−)	++ (++)	++ (++)	++ (++)
P. aeruginosa	NA	− (−)	± (+)	+ (+)	+ (+)	+ (++)	− (−)	++ (++)	++ (++)	++ (++)
S. marcescens	NA	− (−)	− (+)	+ (++)	+ (++)	++ (++)	− (−)	++ (++)	+ (++)	+ (++)
S. marcescens	NCTC 10211	− (−)	− (+)	± (++)	− (++)	+ (++)	− (−)	++ (++)	+ (+)	++ (++)
S. marcescens	ATCC 264	− (−)	− (+)	− (+)	− (+)	− (++)	− (−)	± (+)	+ (+)	+ (++)
S. marcescens	ATCC 43861	− (−)	− (+)	− (++)	++ (++)	+ (++)	− (−)	++ (++)	+ (++)	+ (++)
B. cepacia	ATCC BA-246	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)
B. cepacia	LMG 1222	− (−)	− (−)	− (−)	− (+[e])	− (+[e])	− (−)	− (−)	− (−)	− (++[e])
B. cepacia	ATCC BAA-1911	− (−)	− (−)	− (−)	− (+[e])	− (++[e])	− (−)	− (±)	− (+)	+ (++[e])
Gram Positive Bacteria										
B. subtilis	ATCC 6633	− (−)	− (−)	+ (++)	NG (NG)	NG (NG)	− (−)	− (−)	− (−)	− (−)
MRSA	ATCC 4330	− (−)	− (−)	− (−)	NG (NG)	NG (NG)	− (−)	NG (NG)	− (−)	NG (−)
S. aureus	NCTC 6571	− (−)	− (−)	NG (NG)[d]	NG (NG)	NG (NG)	− (−)	NG (NG)	NG(NG)	NG(NG)
E. faecium	NCTC 7171	− (−)	− (−)	− (−)	− (−)	NG (NG)	− (−)	− (−)	− (−)	NG (NG)
E. faecalis	NCTC 775	− (−)	− (−)	− (−)	NG (−)	NG (NG)	− (−)	− (−)	− (−)	NG (NG)
S. pyogenes	NCTC 8306	− (−)	− (−)	NG (NG)	NG (NG)	NG (NG)	− (−)	NG (−)	− (−)	NG (NG)
L. monocytogenes	ATCC BAA-751	− (−)	− (−)	− (−)	NG (NG)	NG (NG)	− (−)	NG (−)	− (−)	NG (NG)
S. epidermidis	NCTC 11047	− (−)	− (−)	− (−)	NG (NG)	NG (NG)	− (−)	NG (NG)	NG (NG)	NG (NG)
Yeasts										
C. albicans	ATCC 90028	− (−)	− (−)	− (−)	− (−)	NG (−)	− (−)	− (−)	− (−)	NG (−)
C. glabrata	ATCC 64677	− (−)	− (−)	− (−)	− (−)	NG (−)	− (−)	− (−)	NG (NG)	− (−)

[a]Each substrate was tested at a concentration of 40 mg/L. [b]Results after 24 h and 48 h of incubation at 37 °C. [c]"−" means no coloration, " ± " means coloration with weak intensity, "+" means coloration with medium intensity, "++" means coloration with strong intensity. [d]NG, no growth. [e]This strain produces a yellow pigment.

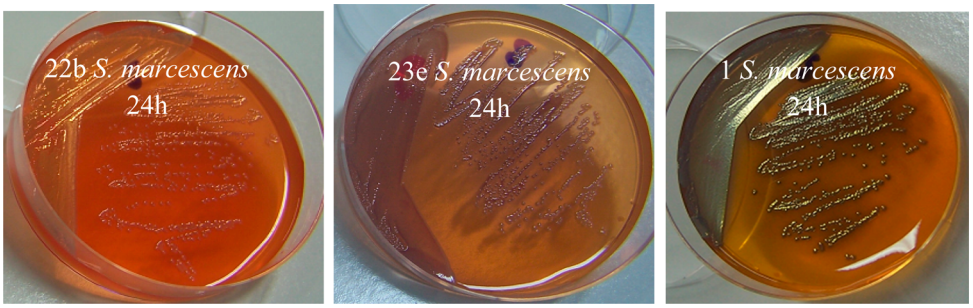

Figure 2. Comparative microbiological results for 7-*N*-(*β*-alanyl)amino-2-pentylphenoxazin-3-one **22b**, 8-*N*-(*β*-alanyl)amino-2-pentylphenoxazin-3-one **23e**, and 7-*N*-(*β*-alanyl)amino-1-pentylphenoxazin-3-one **1** (40 mg/L, 24 h incubation).

3. CONCLUSIONS

The 8-*N*-(*β*-alanyl)aminophenoxazin-3-ones **23b−e** displayed selective *β*-alanyl aminopeptidase sensitivity, producing colored bacterial colonies with *P. aeruginosa* and *S. marcescens* as expected, which was enhanced after 48 h of incubation. In particular, the 2-^{t}butyl- **23d** and 2-^{n}pentyl- **23e** 8-*N*-(*β*-alanyl)aminophenoxazin-3-ones compared well to the standard **1** after 48 h, with the advantage of lower background color, which resulted in enhanced visualization of bacterial colonies. The lack of diffusion of **23e** into the medium and its distinctive color were additional advantages, tempered by the lower sensitivity of this substrate to a wider range of *P. aeruginosa* strains. However, alternative amino acids, such as L-alanyl or L-pyroglutamyl, are used to target a range of other substrates to

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J. Med. Chem. XXXX, XXX, XXX−XXX

Table 3. Spot Test Evaluation of Substrates 22b and 23e (50 mg/L) as Substrates for *Pseudomonas aeruginosa*, Compared to the Standard 1 (% of Strain with Positive Response after 48 h of Incubation)

species	no. of strains	22b	23e	1
Pseudomonas aeruginosa	30	100%	80%	100%

specific bacteria, which have different sensitivities to that of *P. aeruginosa* to novel substrates, hence the desirable color, lack of diffusion, and low background color offered by 2-^{n}pentyl-8-*N*-aminophenoxazin-3-one, make amino acid derivatives of this molecule especially interesting for new bacterial detection applications.

The two 7-*N*-(*β*-alanyl)aminophenoxazin-3-ones **22a** and **22b** displayed similar activity to standard **1** as *β*-alanyl aminopeptidase substrates, with excellent sensitivity of **22b** to the wider range of *P. aeruginosa* strains tested, although the resulting color of the bacterial colonies was slightly less intense and, like standard **1**, there was considerable background color in the agar plates.

This study highlights the scope of this synthetic route to phenoxazinones, allowing the preparation of a range of chromogenic substrates in good overall yields such as the new 8-aminophenoxazin-3-one series reported here, facilitating structure−activity relationships and the optimization of the substrate properties. At each stage, the intermediates can be isolated and characterized, in contrast to the previous synthetic route to the 1-pentyl analogue **1**, while improvements to substrate performance may be achieved by tuning the substitution pattern of the quinonoid portion of the phenoxazinone ring. Increased hydrophobic properties achieved via alkyl substituents at the 1- and/or 2-positions enhance the color, prevent the spread of the chromogen into the medium, and also appear to increase the amount of substrate hydrolyzed by facilitating substrate uptake into the bacteria. Longer alkyl chains (C5/^{n}pentyl) improved the substrate properties; introduction of an aromatic substituent, on the other hand, completely eliminated the detection of enzymatic activity. 7-*N*-(*β*-Alanyl)amino-1-pentylphenoxazin-3-one **1** has proved an excellent substrate for the detection of *P. aeruginosa* in culture media due to the localization within bacterial colonies as a result of the incorporation of the hydrophobic pentyl chain; this work demonstrates the accessibility of a range of alkyl substituted analogues, thus enabling other applications such as in liquid media where localization within colonies is not desired. The synthetic route described here may also allow the development of a substrate which imparts lower background color to the medium.

This work has further established the utility of chromogenic substrates based on 7-aminophenoxazin-3-ones for the detection of *β*-alanyl aminopeptidase producers (e.g., *P. aeruginosa* and *Serratia marcescens*). The 8-aminophenoxazin-3-one analogues **23c−e** display distinct advantages: their coupling to *β*-alanine does not require prior reduction as they couple directly with an activated anhydride and they result in decreased background color of the agar plate (facilitating visualization of the bacterial colonies). The two novel 7-aminophenoxazin-3-ones **22a** and **22b** have comparable properties to the standard **1** and could make suitable substitutes for the detection and identification of *P. aeruginosa* in clinical samples using culture methods.

4. EXPERIMENTAL SECTION

4.1. Chemistry. The synthesis of two example chromogenic substrates **22b** and **23e** are provided below. The purity of all intermediates, except **2b,c**, was determined by elemental analysis and was within 0.4% of the calculated values. The purity of compounds **2b,c** and final substrates **22a,b** and **23a−f** was ≥95%, as determined by LC-MS. All other syntheses are detailed in the Supporting Information.

4.1.1. General. All commercially available reagents and solvents were obtained from Sigma-Aldrich, Fluka, or Riedel-de-Haan and were used without further purification. Melting points were recorded on a Reichart-Kofler hot-stage microscope apparatus and are uncorrected. Infrared spectra were recorded in the range 4000−600 cm^{-1} using a PerkinElmer Spectrum BX FT-IR spectrophotometer. NMR spectra were obtained using a Bruker Ultrashield 300 spectrometer at 300 MHz for ^1H spectra or at 75 MHz for ^{13}C spectra. Low-resolution mass spectra were recorded on a Bruker Esquire 3000plus analyzer using an electrospray source in either positive or negative ion mode. High resolution accurate mass measurements were collected by the EPSRC National Mass Spectrometry Facility at Swansea University. Elemental analyses were performed using an Exeter Analytical CE-440 elemental analyzer. Thin layer chromatography was performed on Merck silica gel $60F_{254}$. Fisons silica gel 60 (35−70 *μ*m) was used for flash chromatography; the samples were preabsorbed onto silica gel 60 (35−70 *μ*m). LC-MS was performed using an Agilent 1200 Infinity series HPLC system and an Agilent 6120 quadruple LC-MS detector (API-ES). Mobile phase A, H_2O/MeOH (90:10, with 0.1% TFA); mobile phase B, H_2O/MeOH (10:90, with 0.1% TFA), A:B (45:65), flow rate 1 mL/min, Column: ACE 5 C18 (250 mm × 4.6 mm, 5 *μ*m), column temperature was not controlled, injection volume 20 *μ*L. The data were analyzed using Agilent ChemStation.

*4.1.2. 2,5-Dimethoxy-4-pentylphenol **4e**.* 2,5-Dimethoxy-4-pentylbenzaldehyde **3e** (3.44 g, 14.58 mmol) in DCM (70 mL) was cooled to 0 °C, and *meta*-chloroperbenzoic acid (3.77 g, 24.79 mmol) was added portionwise. The reaction mixture was maintained at 0 °C for 1 h, allowed to warm to RT, and left overnight. After reflux for 6 h, it was allowed to cool to RT before being washed with a 5% aqueous solution of $NaHCO_3$ (3 × 100 mL) and dried ($MgSO_4$). The solvent was removed in vacuo. The residual oil was dissolved in methanol (20 mL) and NaOH (10%, 50 mL) added; the resulting dark solution was stirred for 2 h and then acidified (pH 1−2) with HCl (10%). The resulting mixture was extracted with DCM (3 × 80 mL), and the combined organic extracts were successively washed with $NaHCO_3$ (5%, 100 mL), water (100 mL), and brine (100 mL) and dried ($MgSO_4$). The solvent was removed in vacuo and the residue subjected to column chromatography on silica gel eluting with petroleum ether (60−80 °C):Et_2O (70:30). 2,5-Dimethoxy-4-pentylphenol **4e** was isolated as a white crystalline solid (2.66 g, 11.87 mmol, 81.4%). Recrystallization from Et_2O:petroleum ether (60−80 °C) produced white needles, mp 33−35 °C (Found: C, 69.55; H, 9.0%. $C_{13}H_{20}O_3$ requires C, 69.6; H, 9.0%.); *m*/*z* 225.0 $(MH)^+$; ν_{max}/cm^{-1} 3354 (O−H), 2953 and 2930 (C−H), 1598 and 1515 (C=C), 1192 and 1038 (C−O); δ_H (300 MHz, $CDCl_3$) 0.96 (3H, t, *J* = 6.9 Hz, 5′-H), 1.38−1.415 (4H, m, 3′-H and 4′-H), 1.58−1.63 (2H, m, 2′-H), 2.59 (2H, t, *J* = 7.5 Hz, 1′-H), 3.81 (3H, s, 5-OC*H*$_3$), 3.89 (3H, s, 2-OC*H*$_3$), 5.61 (1H, s, O*H*), 6.60 (1H, s, 6-H), 6.73 (1H, s, 3-H); δ_C (75 MHz, $CDCl_3$) 14.1 (CH_3, 5′-C), 22.6 (CH_2, 3′-C or 4′-C), 29.8 (CH_2, 1′-C), 30.1 (CH_2, 2′-C), 31.8 (CH_2, 3′-C or 4′-C), 56.1 (CH_3, 5-OCH_3), 56.8 (CH_3, 2-OCH_3), 99.5 (CH, 6-C), 113.3 (CH, 3-C), 122.3 (quat, 4-C), 140.1 (quat, 2-C), 144.1 (quat, 1-C), 152.0 (quat, 5-C).

*4.1.3. 2,4-Dinitro-2′,5′-dimethoxy-4′-pentyldiphenylether **6e**.* A solution of 2,5-dimethoxy-4-pentylphenol **4e** (2.71 g, 12.09 mmol) and Et_3N (1.69 mL, 12.09 mmol) in DMSO (50 mL) was stirred for 5 min before 2,4-dinitro-1-fluorobenzene **5** (2.25 g, 12.09 mmol) was added; the solution color turned to orange−red. After 5 h, water (200 mL) was added, resulting in the formation of a precipitate. The mixture was extracted with DCM (3 × 100 mL), and the combined DCM extracts were successively washed with NaOH (10%, 3 × 100

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J. Med. Chem. XXXX, XXX, XXX−XXX

mL), water (100 mL), and brine (150 mL) and then dried ($MgSO_4$). The solvent was removed in vacuo and the residue recrystallized from ethanol. 2,4-Dinitro-2′,5′-dimethoxy-4′-pentyldiphenylether **6e** was isolated as a yellow crystalline solid (4.36 g, 11.17 mmol, 92.4%); mp 85−87 °C (Found: C, 58.3; H, 5.7; N, 7.2%. $C_{19}H_{22}N_2O_7$ requires C, 58.5; H, 5.7; N, 7.2%.); *m/z* 390.1 $(MH)^+$, 413.1 $(MNa)^+$; ν_{max}/cm^{-1} 1608 (C═C), 1536 and 1342 (NO_2), 1212 and 1037 (C−O); δ_H (300 MHz, $CDCl_3$) 0.96 (3H, t, *J* = 6.9 Hz, 5″-H), 1.39−1.43 (4H, m, 3″-H and 4″-H), 1.62−1.67 (2H, m, 2″-H), 2.66 (2H, t, *J* = 7.5 Hz, 1″-H), 3.755 (3H, s, 2′-OC*H*$_3$), 3.82 (3H, s, 5′-OC*H*$_3$), 6.76 (1H, s, 6′-H), 6.91 (1H, s, 3′-H), 6.96 (1H, d, *J* = 9.3 Hz, 6-H), 8.31 (1H, dd, *J* = 9.3, 2.7 Hz, 5-H), 8.87 (1H, d, *J* = 3.0 Hz, 3-H); δ_C (75 MHz, $CDCl_3$) 14.1 (CH_3, 5″-C), 22.6 (CH_2, 3″-C or 4″-C), 29.6 (CH_2, 2″-C), 30.1 (CH_2, 1″-C), 31.75 (CH_2, 3″-C or 4″-C), 56.1 (CH_3, 5′-OCH_3), 56.9 (CH_3, 2′-OCH_3), 105.6 (CH, 6′-C), 115.85 (CH, 3′-C), 117.4 (CH, 6-C), 122.0 (CH, 3-C), 128.65 (CH, 5-C), 131.0 (quat, 4′-C), 138.3 (quat, 2-C), 139.3 (quat, 1′-C), 141.0 (quat, 4-C), 144.3 (quat, 2′-C), 152.2 (quat, 5′-C), 156.6 (quat, 1-C).

4.1.4. 2,5-Dinitro-2′,5′-dimethoxy-4′-pentyldiphenylether ***14b.*** To a solution of 2,5-dimethoxy-4-pentylphenol **4e** (2.07 g, 9.25 mmol) in dry DMF (30 mL), NaH (oil dispersion 60% w/w, 0.74 g, 18.50 mmol) was added in portions; when evolution of H_2 stopped, a solution of 2,5-dinitrofluorobenzene **13** (1.72 g, 9.25 mmol) in DMF (10 mL) was added. After 5 h at RT, the deep-red solution was heated at 50 °C for another 5 h. The reaction was allowed to cool to RT and quenched with brine (100 mL). The resulting mixture was extracted with DCM (3 × 80 mL) and the combined DCM layers washed with water (100 mL) and brine (2 × 100 mL) and then dried ($MgSO_4$). The solvent was removed in vacuo and the residue subjected to column chromatography, eluting with a solvent mixture of petroleum ether (60−80 °C):Et_2O (75:25). 2,5-Dinitro-2′,5′-dimethoxy-4′-pentyldiphenylether **14b** was isolated as a bright-orange solid (1.69 g, 4.33 mmol, 46.8%). Recrystallization from EtOH produced small yellow needles; mp 77−79 °C (Found: C, 58.3; H, 5.65; N, 7.2%. $C_{19}H_{22}N_2O_7$ requires C, 58.5; H, 5.7; N, 7.2%); *m/z* 391.2 $(MH)^+$, 413.2 $(MNa)^+$; ν_{max}/cm^{-1} 2853−2952 (C−H), 1629 (C═C), 1531 and 1347 (NO_2), 1209 and 1038 (C−O); δ_H (300 MHz, $CDCl_3$) 0.84 (3H, t, *J* = 6.9 Hz, 5″-H), 1.25−1.30 (4H, m, 3″-H and 4″-H), 1.48−1.55 (2H, m, 2″-H), 2.54 (2H, t, *J* = 8.1 Hz, 1″-H), 3.63 (3H, s, 2′-OC*H*$_3$), 3.70 (3H, s, 5′-OC*H*$_3$), 6.63 (1H, s, 6′-H), 6.785 (1H, s, 3′-H), 7.53 (1H, d, *J* = 2.4 Hz, 6-H), 7.83 (1H, dd, *J* = 9.0, 2.4 Hz, 4-H), 7.93 (1H, d, *J* = 8.7 Hz, 3-H); δ_C (75 MHz, $CDCl_3$) 14.0 (CH_3, 5″-C), 22.6 (CH_2, 3″-C or 4″-C), 29.6 (CH_2, 2″-C), 30.1 (CH_2, 1″-C), 31.8 (CH_2, 3″-C or 4″-C), 56.1 (CH_3, 5′-OCH_3), 56.9 (CH_3, 2′-OCH_3), 105.6 (CH, 6′-C), 112.6 (CH, 6-C), 115.9 (CH, 3′-C), 116.25 (CH, 4-C), 126.1 (CH, 3-C), 130.8 (quat, 4′-C), 139.5 (quat, 1′-C), 142.8 (quat, 5-C), 144.4 (quat, 2′-C), 150.4 (quat, 2-C), 152.2 (quat, 5′-C), 152.4 (quat, 1-C).

4.1.5. General Procedure for the Preparation of 2,4-Dinitro- and 2,5-Dinitrophenoxybenzoquinones ***8d*** *and* ***15b.*** The appropriately substituted dinitro-2′,5′-dimethoxydiphenylether (1 mol equivalent) was dissolved in CH_3CN (50 to 500 mL); cerium(IV) ammonium nitrate (3 mol equiv) was dissolved in the minimum amount of water and added slowly. The yellow solution usually darkened upon addition of the cerium(IV) ammonium nitrate solution, followed by the formation of a yellow precipitate soon after the addition was complete. The resulting mixture was stirred for a further 1 h. The CH_3CN was removed under reduced pressure and, whenever possible, the precipitate formed was collected by filtration and washed with water. It was then redissolved in DCM, the resulting solution dried ($MgSO_4$), and the solvent removed in vacuo. The resulting solid was recrystallized from ethanol. When no precipitate formed and a yellow oil/syrup was observed instead, the reaction mixture was extracted twice with DCM and the combined organic layers washed once with brine and dried ($MgSO_4$). The solvent was removed under reduced pressure and the residue either subjected to column chromatography or recrystallized from EtOH.

4.1.5.1. 2,4-Dinitrophenoxy-4′-pentyl-2′,5′-benzoquinone ***8d.*** Prepared from 2,4-dinitro-2′,5′-dimethoxy-4′-pentyldiphenylether **6e** (2.65 g, 6.79 mmol). The crude product was purified by column chromatography, eluting with petroleum ether (60−80 °C):EtOAc (70:30). 2,4-Dinitrophenoxy-4′-pentyl-2′,5′-benzoquinone **8d** was isolated as a yellow solid (2.36 g, 6.56 mmol, 96.7%). Recrystallization from EtOH produced shiny yellow plates; mp 110−114 °C (Found: C, 56.5; H, 4.4; N, 7.7%. $C_{17}H_{16}N_2O_7$ requires C, 56.7; H, 4.5; N, 7.8%.); *m/z* 383.1 $(MNa)^+$; ν_{max}/cm^{-1} 2927 (C−H), 1673 and 1649 (C═O), 1610 (C═C), 1530 and 1342 (NO_2), 1224 (C−O); δ_H (300 MHz, $CDCl_3$) 0.83 (3H, t, *J* = 6.9 Hz, 5″-H), 1.26−1.29 (4H, m, 3″-H and 4″-H), 1.43−1.48 (2H, m, 2″-H), 2.38 (2H, td, *J* = 7.8, 1.2 Hz, 1″-H), 6.03 (1H, s, 6′-H), 6.53 (1H, t, *J* = 1.5 Hz, 3′-H), 7.31 (1H, d, *J* = 9.0 Hz, 6-H), 8.43 (1H, dd, *J* = 9.0, 2.7 Hz, 5-H), 8.88 (1H, d, *J* = 2.7 Hz, 3-H); δ_C (75 MHz, $CDCl_3$) 13.9 (CH_3, 5″-C), 22.4 (CH_2, 3″-C or 4″-C), 27.6 (CH_2, 1″-C), 28.9 (CH_2, 2″-C), 31.45 (CH_2, 3″-C or 4″-C), 116.3 (CH, 6′-C), 122.5 (CH, 3-C), 123.6 (CH, 6-C), 129.4 (CH, 5-C), 130.6 (CH, 3′-C), 140.7 (quat, 2-C), 144.5 (quat, 4-C), 151.2 (quat, 4′-C), 151.8 (quat, 1-C), 155.3 (quat, 1′-C), 180.3 (quat, 2′-C), 186.6 (quat, 5′-C).

4.1.5.2. 2,5-Dinitrophenoxy-4′-pentyl-2′,5′-benzoquinone ***15b.*** Prepared from 2,5-dinitro-2′,5′-dimethoxy-4′-pentyldiphenylether **14b** (1.41 g, 3.62 mmol). The crude product was purified by column chromatography eluting with petroleum ether (60−80 °C):EtOAc (75:25). 2,5-Dinitrophenoxy-4′-pentyl-2′,5′-benzoquinone **15b** was isolated as a yellow solid (1.19 g, 3.31 mmol, 91.4%). Recrystallization from EtOH produced golden prisms; mp 91−94 °C (Found: C, 56.4; H, 4.5; N, 7.8%. $C_{17}H_{16}N_2O_7$ requires C, 56.7; H, 4.5; N, 7.8%.); *m/z* 383.2 $(MNa)^+$; ν_{max}/cm^{-1} 1676 and 1651 (C═O, quinone), 1603 (C═C), 1544 and 1343 (NO_2), 1230 (C−O); δ_H (300 MHz, DMSO-d_6) 0.79 (3H, t, *J* = 6.9 Hz, 5″-H), 1.24−1.19 (4H, m, 3″-H and 4″-H), 1.42−1.35 (2H, m, 2″-H), 2.29 (2H, t, *J* = 6.9 Hz, 1″-H), 6.18 (1H, s, 6′-H), 6.675 (1H, s, 3′-H), 8.23 (1H, dd, *J* = 9.0, 2.4 Hz, 4-H), 8.32 (1H, d, *J* = 9.0 Hz, 3-H), 8.41 (1H, d, *J* = 2.4 Hz, 6-H); δ_C (75 MHz, DMSO-d_6) 14.3 (CH_3, 5″-H), 22.3 (CH_2, 3″-C or 4″-C), 27.75 (CH_2, 2″-C), 28.6 (CH_2, 1″-C), 31.4 (CH_2, 3″-C or 4″-C), 115.1 (CH, 6′-C), 119.8 (CH, 6-C), 122.2 (CH, 4-C), 127.9 (CH, 3-C), 131.2 (CH, 3′-C), 145.25 (quat, 2-C), 146.7 (quat, 5-C), 150.2 (quat, 4′-C), 151.1 (quat, 1-C), 156.6 (quat, 1′-C), 181.0 (quat, 2′-C), 187.65 (quat, 5′-C).

4.1.6. 2,4-Diacetamido-4′-pentyl-2′,5′-benzoquinone ***12c.*** To a solution of 2,4-dinitrophenoxy-4′-pentyl-2′,5′-benzoquinone **8d** (0.46 g, 1.26 mmol) in an EtOAc:MeOH (1:1, 20 mL) was added Pd/C 5% (10% w/w of starting material). The resulting suspension was hydrogenated in a Berghof apparatus for 3 h at a H_2 pressure of 2.4 bar. Acetic anhydride (1.20 mL, 12.6 mmol) was added as soon as the hydrogenation was stopped, and the resulting mixture was stirred at rt overnight. The catalyst was removed by filtration through a pad of Celite, which was washed several times with MeOH. The filtrate was evaporated in vacuo, $NaHCO_3$ (5%) added to the residue, and the resulting mixture extracted with EtOAc (2 × 60 mL). The combined organic layers were washed with $NaHCO_3$ (5%, 70 mL) and brine (100 mL) and dried ($MgSO_4$). The solvent was removed in vacuo and the crude product purified by column chromatography on silica eluting with petroleum ether (60−80 °C):EtOAc (90:10). 2,4-Diacetamido-4′-pentyl-2′,5′-benzoquinone **12c** was isolated as a dark-orange crystalline solid (0.48 g, 1.24 mmol, 98.3%). Recrystallization from hexane:EtOAc produced red prisms, mp 90−93 °C (Found: C, 65.6; H, 6.4; N, 7.2%. $C_{21}H_{24}N_2O_5$ requires C, 65.6; H, 6.3; N, 7.3%.); *m/z* 341.0 $(M - CH_3CO)^-$, 383.0 $(M - H)^-$; ν_{max}/cm^{-1} 3357 (N−H), 3269 (N−H), 2955 (C−H), 2929 (C−H), 1726 (C═O), 1663 and 1647 (C═O, quinone), 1599 (C═C), 1206 (C−O); δ_H (300 MHz, THF-d_8) 0.93 (3H, t, *J* = 6.9 Hz, 5″-H), 1.34−1.39 (4H, m, 3″-H and 4″-H), 1.48−1.55 (2H, m, 2″-H), 2.04 (3H, s, 2-Ac or 4-Ac), 2.07 (3H, s, 2-Ac or 4-Ac), 2.40 (2H, dt, *J* = 7.5, 1.2 Hz, 1″-H), 5.67 (1H, s, 6′-H), 6.59 (1H, br s, 3′-H), 6.98 (1H, d, *J* = 8.7 Hz, 6-H), 7.91 (1H, dd, *J* = 9.0, 2.1 Hz, 5-H), 8.28 (1H, d, *J* = 2.1 Hz, 3-H), 8.63 (1H, s, 2-N*H*), 9.31 (1H, s, 4-N*H*); δ_C (75 MHz, THF-d_8) 13.3 (CH_3, 5″-C), 22.3 (CH_2, 3″-C or 4″-C), 23.0 (CH_3, 2-Ac or 4-Ac), 23.1 (CH_3, 2-Ac or 4-Ac), 27.85 (CH_2, 2″-C), 28.6 (CH_2, 1″-C), 31.5 (CH_2, 3″-C or 4″-C), 110.8 (CH, 6′-C), 112.5 (CH, 3-C), 114.9 (CH, 5-C), 120.7 (CH, 6-C), 130.45 (CH, 3′-C), 131.3 (quat, 2-C), 137.15 (quat, 1-C), 138.2 (quat, 4-C), 149.8 (quat, 4′-C), 158.2 (quat, 1′-C), 167.7 (quat,

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J. Med. Chem. XXXX, XXX, XXX−XXX

2-Ac or 4-Ac, C=O), 168.1 (quat, 2-Ac or 4-Ac, C=O), 181.0 (quat, 2′-C), 186.6 (quat, 5′-C).

4.1.7. General Procedure for the Preparation of 8-Aminophenoxazin-3-one ***2e*** *and 7-Aminophenoxazin-3-one* ***17b****.* A solution of 2,4-dinitrophenoxy-4′-pentyl-2′,5′-benzoquinone **8d** or 2,5-dinitrophenoxy-4′-pentyl-2′,5′-benzoquinone **15b** in EtOAc:MeOH (1:1) was hydrogenated in a Berghof apparatus in the presence of Pd/C 5% (10% w/w of the starting material). The H_2 pressure was set to 2.4 bar and reset to this pressure when necessary. When the H_2 pressure was steady, the reaction mixture was filtered through Celite, which was washed several times with MeOH. Either AgO or Ag_2O (3 mol equiv) was then added quickly to the filtrate, which rapidly produced a dark-purple−violet solution for 8-aminophenoxazinone derivatives and a dark-red−purple fluorescent solution for 7-aminophenoxazinone derivatives. Once the oxidative cyclization was complete (TLC monitoring), the inorganic materials were removed by filtration through Celite, which was washed successively with THF and MeOH. The combined filtrates were evaporated in vacuo and the residue subjected to column chromatography.

4.1.7.1. 8-Amino-2-pentyl-3H-phenoxazin-3-one ***2e****.* Prepared from 2,4-dinitrophenoxy-4′-pentyl-2′,5′-benzoquinone **8d** (0.38 g, 1.07 mmol); elution used a gradient mixture of petroleum ether (60−80 °C):EtOAc (50:50 to 35:65), yielding 8-amino-2-pentyl-3*H*-phenoxazin-3-one **2e** as a dark-violet solid (0.29 g, 1.01 mmol, 94.7%), mp 141−144 °C (Found: C, 71.9; H, 6.6; N, 9.65%. $C_{17}H_{18}N_2O_2$ requires C, 72.2; H, 6.4; N, 9.9%.); *m*/*z* 283.1 $(MH)^+$; ν_{max}/cm^{-1} 3400, 3337, and 3222 (N−H), 2853 and 2928 (C−H), 1639 (C=O), 1585, 1566, and 1508 (C=C); δ_H (300 MHz, DMSO-d_6) 0.87 (3H, t, *J* = 6.9 Hz, 5′-H), 1.28−1.32 (4H, m, 3′-H and 4′-H), 1.46−1.56 (2H, m, 2′-H), 2.45 (2H, t, *J* = 6.9 Hz, 1′-H), 5.41 (2H, br s, NH_2), 6.17 (1H, s, 4-H), 6.90−6.93 (2H, m, 7-H and 9-H), 7.21 (1H, d, *J* = 9.3 Hz, 6-H), 7.25 (1H, s, 1-H); δ_C (75 MHz, DMSO-d_6) 14.3 (CH_3, 5′-C), 22.4 (CH_2, 3′-C or 4′-C), 28.1 (CH_2, 2′-C), 29.5 (CH_2, 1′-C), 31.5 (CH_2, 3′-C or 4′-C), 105.0 (CH, 4-C), 111.55 (CH, 9-C), 116.55 (CH, 6-C), 120.3 (CH, 7-C), 130.6 (CH, 1-C), 134.3 (quat, 8-C), 135.3 (quat, 9a-C), 147.25 (quat, 2-C or 5a-C), 147.3 (quat, 2-C or 5a-C), 148.4 (quat, 4a-C), 149.7 (quat, 10a-C), 184.8 (quat, 3-C).

4.1.7.2. 7-Amino-2-pentyl-3H-phenoxazin-3-one ***17b****.* Prepared from 2,5-dinitrophenoxy-4′-pentyl-2′,5′-benzoquinone **15b** (0.80 g, 2.23 mmol); elution used a gradient mixture of petroleum ether (60−80 °C):EtOAc (40:60 to 0:100), yielding 7-amino-2-pentyl-3*H*-phenoxazin-3-one **17b** as a dark-violet solid (0.62 g, 2.18 mmol, 97.6%); mp 250−254 °C (Found: C, 72.0; H, 6.6; N, 9.6%. $C_{17}H_{18}N_2O_2$ requires C, 72.2; H, 6.4; N, 9.9%.); *m*/*z* 283.2 $(MH)^+$; ν_{max}/cm^{-1} 3420, 3329, and 3226 (N−H), 2923 (C−H), 1654 (C=O), 1599 and 1565 (C=C); δ_H (300 MHz, DMSO-d_6) 0.88 (3H, t, *J* = 6.9 Hz, 5′-H), 1.29−1.33 (4H, m, 3′-H and 4′-H), 1.47−1.54 (2′-H), 2.45 (2H, t, *J* = 7.5 Hz, 1′-H), 6.17 (1H, s, 4-H), 6.51 (1H, d, *J* = 2.4 Hz, 6-H), 6.71 (1H, dd, *J* = 8.7, 2.4 Hz, 8-H), 6.83 (2H, br s, NH_2), 7.24 (1H, s, 1-H), 7.48 (1H, d, *J* = 8.7 Hz, 9-H); δ_C (75 MHz, DMSO-d_6) 14.4 (CH_3, 5′-C), 22.4 (CH_2, 3′-C or 4′-C), 28.3 (CH_2, 2′-C), 29.5 (CH_2, 1′-C), 31.55 (CH_2, 3′-C or 4′-C), 97.8 (CH, 6-C), 105.1 (CH, 4-C), 113.8 (CH, 8-C), 126.4 (quat, 7-C), 130.8 (CH, 1-C), 132.1 (CH, 9-C), 140.6 (quat, 9a-C), 144.0 (quat, 4a-C), 146.7 (quat, 10a-C), 149.8 (quat, 2-C), 155.1 (quat, 5a-C), 184.3 (quat, 3-C).

4.1.8. 7-N-(N′-^{t}*Butoxycarbonyl-β-alanyl)amino-2-pentyl-3H-phenoxazin-3-one* ***20b****.* To *N*-^{t}Boc-*β*-alanine (0.96 g, 5.07 mmol) in dry THF (10 mL) at 2−3 °C (T_{int}) was added dropwise *N*-methylpiperidine (0.72 mL, 5.92 mmol), followed after 5 min by *iso*-butylchloroformate (0.66 mL, 5.07 mmol) and stirred at 3 °C for 1h. Separately, Pd/C 5% (0.05g) was added to 7-amino-2-pentyl-3*H*-phenoxazin-3-one **17b** (0.48 g, 1.69 mmol) in dry DMF (5 mL) and the mixture hydrogenated in a Berghof apparatus for 1 h at a hydrogen pressure of 2 bar. After hydrogenation, the flask was flushed with N_2 and sealed, then the suspension added via cannula to the anhydride mixture and reacted for 72 h. The reaction mixture was quenched with $NaHCO_3$ (5%) and extracted with EtOAc (3 × 70 mL). The combined organic extracts were washed successively with $NaHCO_3$ (5%, 100 mL), water (100 mL), and brine (100 mL) and dried ($MgSO_4$). The solvent was evaporated in vacuo, and the residue was subjected to column chromatography on silica, eluting with petroleum ether (60−80 °C):EtOAc (40:60), yielding 7-*N*-(*N*′-^{t}butoxycarbonyl-*β*-alanyl)amino-2-pentyl-3*H*-phenoxazin-3-one **20b** as an orange solid (0.36 g, 0.78 mmol, 46.0%). Recrystallization from EtOAc produced microcrystalline orange solid with a green metallic shine; mp 205−209 °C (Found: C, 66.35; H, 6.9; N, 9.25%. $C_{25}H_{21}N_3O_5$ requires C, 66.2; H, 6.9; N, 9.3%.); *m*/*z* 454.3 $(MH)^+$; ν_{max}/cm^{-1} 3263 and 3190 (N−H), 2951 and 2922 (C−H), 1710 (C=O), 1651 (C=O); δ_H (300 MHz, DMSO-d_6) 0.79 (3H, t, *J* = 6.9 Hz, 5′-H), 1.20−1.245 (4H, m, 3′-H and 4′-H), 1.30 (9H, s, $C(CH_3)_3$), 1.39−1.46 (2H, m, 2′-H), 2.39 (2H, t, *J* = 7.5 Hz, 1′-H), 2.47 (2H, t, *J* = 7.2 Hz, 2″-H), 3.17 (2H, q, *J* = 7.2 Hz, 3″-H), 6.20 (1H, s, 4-H), 6.80 (1H, br s, N*H*^{t}Boc), 7.22 (1H, s, 1-H), 7.40 (1H, dd, *J* = 8.7, 2.1 Hz, 8-H), 7.66 (1H, d, *J* = 8.7 Hz, 9-H), 7.82 (1H, d, *J* = 2.1 Hz, 6-H), 10.41 (1H, s, N*H*-Ar); δ_C (75 MHz, DMSO-d_6) 14.3 (CH_3, 5′-C), 22.4 (CH_2, 3′-C or 4′-C), 28.1 (CH_2, 2′-C), 28.7 (CH_2, 1′-C), 29.4 (CH_3, $C(CH_3)_3$), 31.5 (CH_2, 3′-C or 4′-C), 36.8 (CH_2, 3″-C), 37.5 (CH_2, 2″-C), 78.2 (quat, $C(CH_3)_3$), 105.3 (CH, 6-C), 106.05 (CH, 4-C), 116.8 (CH, 8-C), 129.6 (quat,7-C), 130.7 (CH, 9-C), 130.9 (CH, 1-C), 143.4 (quat, 9a-C), 144.5 (quat, 5a-C), 146.7 (quat, 10a-C and 2-C), 149.5 (quat, 4a-C), 156.0 (quat, ^{t}Boc C=O), 170.8 (quat, 1″-C), 185.1 (quat, 3-C).

4.1.9. 8-N-(N′-^{t}*Butoxycarbonyl-β-alanyl)amino-2-pentyl-3H-phenoxazin-3-one* ***21e****.* To *N*-^{t}Boc-*β*-alanine (1.01 g, 5.34 mmol) in dry THF (10 mL) at 2−3 °C (T_{int}) was added dropwise *N*-methylpiperidine (0.76 mL, 6.23 mmol). After 5 min, *iso*-butylchloroformate (0.69 mL, 5.34 mmol) was added dropwise, and the solution stirred at 3 °C for 1 h before 8-amino-2-pentyl-3*H*-phenoxazin-3-one **2e** (0.50 g, 1.78 mmol) was added. The resulting mixture was stirred under nitrogen for 48 h. The reaction mixture was quenched with $NaHCO_3$ (5%) and extracted with EtOAc (3 × 70 mL). The combined organic extracts were washed successively with $NaHCO_3$ (5%, 100 mL), water (100 mL), and brine (100 mL) and dried ($MgSO_4$). The solvent was evaporated in vacuo, and the residue was subjected to column chromatography on silica gel, eluting with a gradient mixture of petroleum ether (60−80 °C):EtOAc (35:65 to 20:80), yielding 8-*N*-(*N*′-^{t}butoxycarbonyl-*β*-alanyl)amino-2-pentyl-3*H*-phenoxazin-3-one **21e** as an orange solid (0.73 g, 1.60 mmol, 90.1%); mp 176−179 °C (Found: C, 66.2; H, 6.8; N, 9.1%. $C_{25}H_{31}N_3O_5$ requires C, 66.2; H, 6.9; N, 9.3%.); *m*/*z* 454.3 $(MH)^+$, 476.3 $(MNa)^+$; ν_{max}/cm^{-1} 3322 (N−H), 2954 and 2930 (C−H), 1684 (C=O), 1658 (C=O), 1648 (C=O), 1171 (C−O); δ_H (300 MHz, DMSO-d_6) 0.78 (3H, t, *J* = 6.6 Hz, 5′-H), 1.21−1.23 (4H, m, 3′-H and 4′-H), 1.30 (9H, s, $C(CH_3)_3$), 1.38−1.40 (2H, m, 2′-H), 2.34 (2H, t, *J* = 7.2 Hz, 1′-H), 2.42 (2H, t, *J* = 6.6 Hz, 2″-H), 3.19 (2H, q, *J* = 6.6 Hz, 3″-H), 6.095 (1H, s, 4-H), 6.77 (1H, br s, N*H*^{t}Boc), 7.15 (1H, s, 1-H), 7.28 (1H, d, *J* = 8.7 Hz, 6-H), 7.60 (1H, dd, *J* = 8.7, 2.4 Hz, 7-H), 8.00 (1H, d, *J* = 2.1 Hz, 9-H), 10.08 (1H, s, N*H*-Ar); δ_C (75 MHz, DMSO-d_6) 14.3 (CH_3, 5′-C), 22.4 (CH_2, 3′-C or 4′-C), 28.0 (CH_2, 2′-C), 28.7 (CH_3, $C(CH_3)_3$), 29.4 (CH_2, 1′-C), 31.5 (CH_2, 3′-C or 4′-C), 36.95 (CH_2, 3″-C), 37.3 (CH_2, 2″-C), 78.1 (quat, $C(CH_3)_3$), 105.8 (CH, 4-C), 116.5 (CH, 6-C), 119.0 (CH, 9-C), 124.0 (CH, 7-C), 130.7 (CH, 1-C), 133.3 (quat, 9a-C), 137.0 (quat, 8-C), 139.3 (quat, 5a-C), 147.6 (quat, 2-C), 149.2 (quat, 4a-C or 10a-C), 149.3 (quat, 4a-C or 10a-C), 156.0 (quat, ^{t}Boc C=O), 170.05 (quat, 1″-C), 185.1 (quat, 3-C).

4.1.10. General Procedure for the Removal of N-^{t}*Butoxycarbonyl Group.* The corresponding *N*-^{t}butoxycarbonyl protected compound was dissolved in an excess of neat TFA, and the resulting solution was swirled for 1 min. The TFA was evaporated in vacuo, then sequential portions of MeOH were added to the residue and evaporated. The residue was redissolved in minimal MeOH and diluted with Et_2O. After overnight standing, the microcrystalline solid that formed was collected and washed with Et_2O. The filtrate was evaporated under reduced pressure for further recovery of the product.

4.1.10.1. 7-N-(β-Alanyl)amino-2-pentyl-3H-phenoxazin-3-one TFA Salt ***22b****.* Prepared from 7-*N*-(*N*′-^{t}butoxycarbonyl-*β*-alanyl)-amino-2-pentyl-3*H*-phenoxazin-3-one **20b** (0.14 g, 0.30 mmol). 7-*N*-(*β*-Alanyl)amino-2-pentyl-3*H*-phenoxazin-3-one TFA salt **22b** was isolated as a brown−red solid with a green metallic shine (0.12 g, 0.27 mmol, 88.5%); mp 213−223 °C; HRMS (Found $(M)^+$, 354.1815.

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J. Med. Chem. XXXX, XXX, XXX−XXX

Calcd for $C_{20}H_{24}N_3O_3$: $(M)^+$, 354.1812.); m/z 354.2 (M^+) ν_{max}/cm^{-1} 3263 (N−H), 3188−3060 (N^+-H), 2928 (C−H), 1674 (C═O), 1651 (C═O); δ_H (300 MHz, DMSO-d_6) 0.88 (3H, t, J = 6.9 Hz, 5′-H), 1.30−1.33 (4H, m, 3′-H and 4′-H), 1.485−1.53 (2H, m, 2′-H), 2.45 (2H, t, J = 7.5 Hz, 1′-H), 2.82 (2H, t, J = 6.3 Hz, 2″-H), 3.15 (2H, t, J = 6.3 Hz, 3″-H), 6.25 (1H, s, 4-H), 7.26 (1H, s, 1-H), 7.50 (1H, dd, J = 8.7, 1.8 Hz, 8-H), 7.73 (1H, d, J = 8.7 Hz, 9-H), 7.89 (1H, d, J = 1.8 Hz, 6-H), 7.97 (3H, br s, NH_3^+), 10.83 (1H, s, N*H*-Ar) (Figure S10 in the Supporting Information); δ_C (75 MHz, DMSO-d_6) 14.4 (CH_3, 5′-C), 22.4 (CH_2, 3′-C or 4′-C), 28.1 (CH_2, 2′-C), 29.4 (CH_2, 1′-C), 31.5 (CH_2, 3′-H or 4′-H), 34.1 (CH_2, 2″-C), 35.2 (CH_2, 3″-C), 105.5 (CH, 6-C), 106.1 (CH, 4-C), 116.8 (CH, 8-C), 129.8 (quat, 7-C), 130.8 (CH, 9-C or 1-C), 130.9 (CH, 9-C or 1-C), 143.1 (quat, 9a-C), 144.4 (quat 5a-C), 146.7 (quat, 2-C or 10a-C), 146.8 (quat, 2-C or 10a-C), 149.4 (quat, 4a-C), 169.7 (quat, 1″-C), 185.1 (quat, 3-C).

4.1.10.2. 8-N-(β-Alanyl)amino-2-pentyl-3H-phenoxazin-3-one TFA Salt **23e**. Prepared from 8-*N*-(*N*′-^{t}butoxycarbonyl-β-alanyl)-amino-2-pentyl-3*H*-phenoxazin-3-one **21e** (0.08 g, 0.18 mmol). 8-*N*-(β-Alanyl)amino-2-pentyl-3*H*-phenoxazin-3-one TFA salt **23e** was isolated as a dark-red solid (0.07 g, 0.15 mmol, 87.0%); mp 132−139 °C; HRMS (Found $(M)^+$, 354.1813. Calcd for $C_{20}H_{24}N_3O_3$: $(M)^+$, 354.1812.); m/z 354.2 (M^+); ν_{max}/cm^{-1} 3312 (N−H), 3077 (N^+−H), 2932−2861 (C−H), 1672 (C═O), 1649 (C═O); δ_H (300 MHz, DMSO-d_6) (Figure S15 in the Supporting Information) 0.80 (3H, t, J = 6.6 Hz, 5′-H), 1.22−1.25 (4H, m, 3′-H and 4′-H), 1.425−1.47 (2H, m, 2′-H), 2.41 (2H, t, J = 8.1 Hz, 1′-H), 2.67 (2H, t, J = 6.6 Hz, 2″-H), 3.05 (2H, br s, 3″-H), 6.21 (1H, s, 4-H), 7.27 (1H, s, 1-H), 7.40 (1H, d, J = 9.0 Hz, 6-H), 7.65 (1H, dd, J = 9.0, 2.4 Hz, 7-H), 7.75 (3H, br s, NH_3^+), 8.08 (1H, d, J = 2.4 Hz, 9-H), 10.39 (1H, s, N*H*-Ar); δ_C (75 MHz, DMSO-d_6) 14.3 (CH_3, 5′-C), 22.4 (CH_2, 3′-C or 4′-C), 28.1 (CH_2, 2′-C), 29.4 (CH_2, 1′-C), 31.5 (CH_2, 3′-C or 4′-C), 33.9 (CH_2, 2″-C), 35.4 (CH_2, 3″-C), 105.9 (CH, 4-C), 116.7 (CH, 6-C), 119.2 (CH, 9-C), 124.1 (CH, 7-C), 130.9 (CH, 1-C), 133.4 (quat, 9a-C), 136.7 (quat, 8-C), 139.6 (quat, 5a-C), 147.75 (quat, 2-C), 149.4 (quat, 4a-C or 10a-C), 149.5 (quat, 4a-C or 10a-C), 169.0 (quat, 1″-C), 185.2 (quat, 3-C). LC-MS spectra are provided as Figure S7 in the Supporting Information.

4.2. Microbiology. *4.2.1. Preparation of Columbia Agar Solution.* Standard Columbia agar was prepared according to the manufacturer's recommendations: Columbia agar (41g) was dissolved by boiling in sterile deionized water (1 L). The resulting medium was sterilized by autoclaving at 121 °C for 15 min and then cooled and maintained at 50 °C in a water bath before incorporation of the substrates.

4.2.2. Preparation of Substrate-Containing Plates. A 10g/L stock solution of each substrate to be tested was prepared in DMSO or *N*-methylpyrrolidone (100 μL). The stock solutions were incorporated into Columbia agar (Oxoid, Basingstoke) to give a final substrate concentration of 50 mg/L for the spot test plates and 40 mg/L for the single bacterial species streaked plates. The final media were poured into separate sterile Petri dishes and left to set into a gel.

4.2.3. Bacterial Suspension Preparation. The bacterial strains were obtained from the American Type Culture Collection (ATCC, Cockeysville, USA), the National Collection of Type Cultures (NCTC, Colindale, UK), or isolated from clinical material obtained from patients in the Freeman Hospital (Newcastle-upon-Tyne). In this case, the microorganisms were identified according to standard procedures. Colonies of each strain were suspended in sterile deionized 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.

4.2.4. Spot Test Plates. The agar plates (**4.2.2**) were each inoculated with 10 Gram negative bacteria, eight Gram positive bacteria, and two yeasts (from the National Collection of Type Cultures, London, UK). All inoculated plates were incubated at 37 °C for 24 h and then examined visually for the presence of growth and coloration of bacterial colonies.

4.2.5. Individual Bacterial Species Streaked Plates. For each strain tested, a bacterial suspension was prepared in saline 0.85% at a standard inoculum of 0.5 McFarland (1.5×10^8 colony forming units per mL), using a densitometer. Using a sterile loop, 10 μL of the bacterial suspension was inoculated onto a test plate containing a particular substrate. The plates were then incubated aerobically at 37 °C and visual inspections made at 24 h and 48 h of incubation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b01591.

Synthetic methods and spectral characterization of synthetic intermediates and products; selected LC-MS and NMR spectra of key intermediates and products (PDF)

Molecular formula strings (CSV)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ESKAPE *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.; VAP ventilator-associated pneumonia; DNFB 2,4-dinitro-1-fluorobenzene (Sanger's reagent)

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J. Med. Chem. XXXX, XXX, XXX−XXX

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J. Med. Chem. XXXX, XXX, XXX−XXX