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Pyrazolo[1,5-*a*]pyrimidin-7-ones as promising antimicrobial scaffolds: In vitro and in vivo evaluation.

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ABSTRACT

This study presents a family of pyrazolo[1,5-*a*]pyrimidin-7-ones as potential antimicrobial agents. Initial screening against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* identified compound **3** as active against *S. aureus* (MIC_{50} 1.8 μM). A structure activity relationship study of 35 analogues yielded the more potent analogues (MIC_{50} 1.2 μM, MIC_{80} 6.4 μM). Hit compounds were further evaluated against Methicillin-Resistant *S. aureus* (MRSA) and *Pseudomonas aeruginosa*, with pyrimidinone **12** showing the strongest activity against MRSA (MIC_{50} 1.88 μM, MIC_{80} 2.93 μM). In vivo toxicity assessment using *Galleria mellonella* larvae indicated that the leading active compounds were non-toxic. Importantly, in an in vivo *G. Melonella* MRSA infection model, compound **12** improved survival by 25 % compared to untreated controls (95 % vs. 75 %, $p < 0.05$). These strong in vivo results highlight pyrazolo[1,5-*a*]pyrimidin-7-ones as promising scaffolds for the development of new antibacterial agents.

1. Introduction

4H-Pyrazolo[1,5-*a*]pyrimidin-7-one is a fused nitrogen-containing heterocycle that serves as both a core scaffold in various medicinal compounds and a versatile synthetic intermediate. Derivatives of this structure have demonstrated a broad range of pharmacological activities, including anticancer, antiviral, anti-obesity, anti-inflammatory, and anti-schistosomal properties [1–8].

Despite these diverse applications, to the best of our knowledge, no antimicrobial studies on pyrazolo[1,5-*a*]pyrimidin-7-ones have been reported. In contrast, related structures such as pyrazolo[1,5-*a*]pyrimidines and pyrazolo[3,4-*d*]pyrimidines have shown notable antimicrobial activity. These include antifungal effects against *Fusarium oxysporum*, *Aspergillus niger*, *Candida albicans*, and *Aspergillus fumigatus* [9–12], as well as antibacterial activity against both Gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*) [5,6] and Gram-negative (*Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*) strains [10–12].

Several synthetic routes to pyrazolo[1,5-*a*]pyrimidin-7-ones have been described. Marjani et al. reported the condensation of 1,3-diketones with substituted 5-aminopyrazoles in the presence of H_2SO_4 at room temperature (4–8 h) [13]. Gavrin et al. prepared four 4H-pyrazolo[1,5-*a*]pyrimidin-7-ones via reaction of aminopyrazoles with the sodium salt of ethyl formyl acetate at room temperature over 16 h [2]. We previously reported a one-pot, microwave-assisted synthesis involving a ketonitrile, hydrazine, and methanol heated under microwave conditions (100 W, 150 °C) for 5 min, followed by addition of acetic acid and a ketoester, and further microwave heating for 2 h (Scheme 1) [1].

Herein, we report the development of the pyrazolo[1,5-*a*]pyrimidin-7-one scaffold as a novel antimicrobial chemotype, supported by promising in vitro and in vivo activity, including therapeutic efficacy demonstrated in a *G. mellonella* MRSA infection model.

2. Results and discussion

We have a strong interest in microbial systems and the discovery of novel antimicrobial agents [14–20]. Part of our work involves an antimicrobial screening programme, using structural diverse molecules, in an effort to discover new scaffolds for further optimisation. As part of

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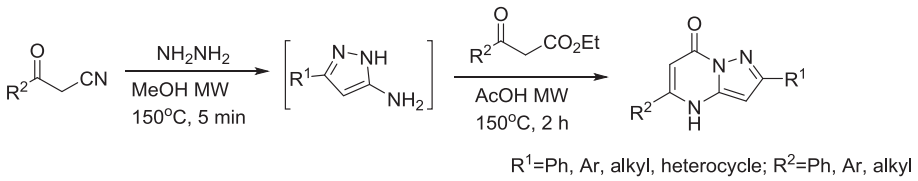


Scheme 1. Synthesis of 4-*H*-pyrazolo[1,5-*a*]pyrimidin-7-ones via a one pot microwave assisted method as reported by Kelada et al. [1]

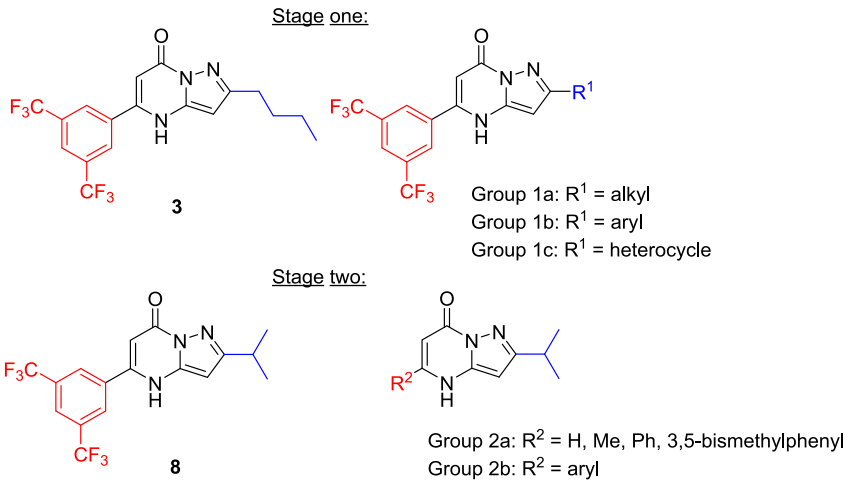


Fig. 1. Compound groups of pyrazolo[1,5-*a*]pyrimidin-7-ones used in the two-stage antimicrobial study. In Stage 1, variation at R^1 led to the identification of hit compound **8**. Stage 2 used compound **8** as the scaffold, with structural variation introduced at R [2].

this programme, the pyrazolopyrimidinone core was identified as a possible new scaffold for antimicrobial agents with compound **3** (Fig. 1) generating an MIC_{50} of 1.8 μM against *S. aureus*. Compounds **3** outperformed three reference antibiotics: ampicillin trihydrate (MIC_{50} = 21.8 μM), tetracycline (MIC_{50} = 11.0 μM), and streptomycin sulfate (MIC_{50} = 5.2 μM (see Supporting Information).

In order to probe the structure activity relationship, a total of 35 pyrazolo[1,5-*a*]pyrimidin-7-one analogues were screened in two stages. In Stage 1, R^1 substituents were varied (alkyl, aryl, heterocyclic; groups 1a, 1b, and 1c; Fig. 2), leading to the identification of compound 8 (R^1 = i-Pr) as a new hit with enhanced activity (MIC_{50} = 1.2 μM). In Stage 2, the R^1 group (i-Pr) was retained, and modifications at the R^2 position were explored (groups 2a and 2b; Fig. 1). Antimicrobial testing was performed using the broth microdilution method following CLSI guidelines [21]. Full experimental details are provided in the Supporting Information.

2.1. Assessment of anti-microbial activity against S. aureus

To investigate the effect of alkyl variation at R,[1] a series of pyrazolo [1,5-*a*]pyrimidin-7-ones were synthesized, with the 3,5-bis(trifluoromethyl)phenyl group fixed at R,[2] group 1a (Table 1). Linear (methyl, ethyl, *n*-propyl) and branched (isopropyl, *tert*-butyl, isobutyl) alkyl chains were evaluated (Table 1). No clear trend was observed in relation to chain length or steric bulk. However, the isopropyl analogue (compound **8**) demonstrated the highest activity, with an MIC_{50} of 1.2 μM and

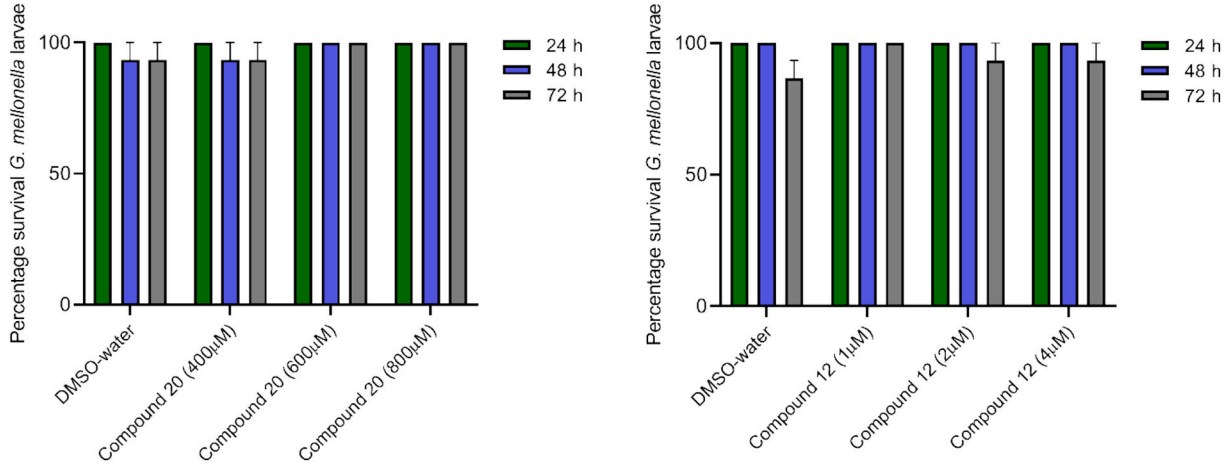
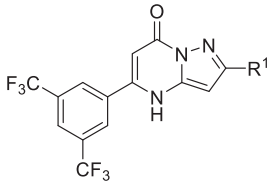
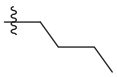


Fig. 2. Percentage survival of *G. mellonella* larvae: (**A**) Treatment with either a 5:95 (*v*/v) DMSO:distilled water mix or compound **20**; (**B**) Treatment with either a 5:95 (v/v) DMSO:distilled water mix or compound **12**. Survival was assessed for 24, 48 and 72 h at 37 °C. A 5:95 (v/v) DMSO:distilled water mix was used as a control. Data are presented as the mean ± se of three independent experiments and were analysed using a two-tailed unpaired Student's *t*-test.

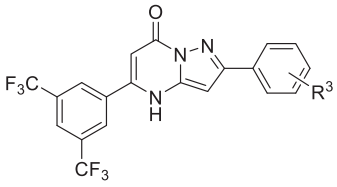
Table 1
Antibacterial (*S. aureus*) activity of group 1a and 1c pyrazolo[1,5-*a*]pyrimidin-7-ones as MIC_{50}/MIC_{80} values.



Number	R^1	*S. aureus*	
		MIC_{50} (μM)	MIC_{80} (μM)
3		1.8	a
5	Me	85	a
6	Et	1.8	10
7		35	a
8		1.2	6.4
9		47	a
10		2.2	a
16		12	a
17		520	a

a Compound was not sufficiently active to generate a MIC_{80} value.

Table 2
Antibacterial (*S. aureus*) activity of group 1b pyrazolo[1,5-*a*]pyrimidin-7-ones as MIC_{50} values.

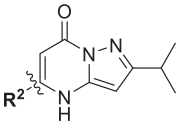


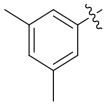
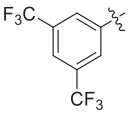
R^3 = *p*-CF_3 (**12**), *p*-F (**34**), *p*-Cl (**35**), *p*-Br (**36**), *p*-NO_2 (**37**), *p*-OMe (**38**), *m*-Cl (**13**), *m*-NO_2 (**15**), *m*-OMe (**39**), *o*-Cl (**11**), *o*-OMe (**14**)

Number	R^3	*S. aureus*
		MIC_{50} (μM)
11	*o*-Cl	2.8
12	*p*-CF_3	3.6
13	*m*-Cl	291
14	*o*-OMe	299
15	*m*-NO_2	300

Only group 1b compounds that generated an MIC_{50} are included in the table. (see SI for results from all other group 1b compounds).

Table 3
Antibacterial *(S. aureus and E. coli)* activity of group 2a pyrazolo[1,5-*a*]pyrimidin-7-ones as MIC_{50}/MIC_{80} values.



Number	R^2	*S. aureus*		*E. coli*	
		MIC_{50} (μM)	MIC_{80} (μM)	MIC_{50} (μM)	MIC_{80} (μM)
18	H	662	a	626	a
19	Me	698	a	373	733
20	Ph	90	a	373	733
21		b	a	594	a
8		1.2	6.4	584	a

a Compound was not sufficiently active to generate a MIC_{80} value. b Compound was not sufficiently active to generate a MIC_{50} value.

an MIC_{80} of 6.4 μM against *S. aureus*, surpassing all other group 1a analogues and outperforming the reference antibiotics—ampicillin trihydrate (MIC_{80} = 1.09 mM), tetracycline (MIC_{80} = 12.6 μM), and streptomycin sulfate (MIC_{80} = 210.9 μM). Compound **6** (ethyl) also produced a low MIC_{50} value of 1.8 μM, with an MIC_{80} of 10 μM.

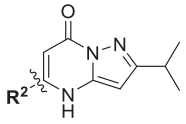
Next, aryl groups with various substituents (electron-withdrawing, electron-donating, and halogens) at different positions were introduced at R,[1] while maintaining R^2 as 3,5-bis(trifluoromethyl)phenyl, group 1b (Table 2). Five compounds exhibited activity, all with higher MIC_{50} values than compound **8**. The most active aryl derivative, compound **11** (R^1 = ortho-chlorophenyl), had an MIC_{50} of 2.8 μM, but none of the group 1b compounds produced an MIC_{80}.

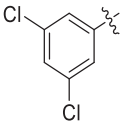
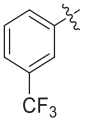
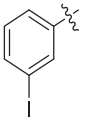
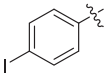
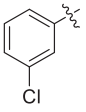
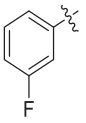
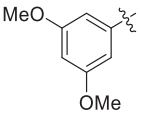
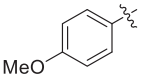
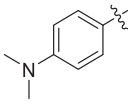
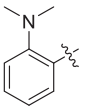
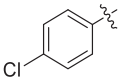
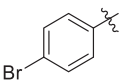
Two group 1c analogues, with heterocycles at R^1 (2-furan, **16**; 2-thiophene, **17**) were assessed, (Table 1 and Table S5 in Supporting Information). Compound **16** showed modest activity (MIC_{50} = 12 μM), while compound **17** was considerably less active (MIC_{50} = 520 μM). Neither generated an MIC_{80}. Overall, these data confirmed isopropyl as the optimal R^1 group, with compound **8** emerging as the lead candidate for further SAR exploration at R^2 [2].

To probe the contribution of the 3,5-bis(trifluoromethyl)phenyl group at R,[2] compound **8** was modified to yield analogues with hydrogen (**18**), methyl (**19**), or phenyl (**20**) at R^2, keeping R^1 as isopropyl, group 2a (Table 3). All modifications significantly reduced antibacterial activity. Compound **20** (R^2 = Ph) was the most active among them (MIC_{50} = 90 μM).

Notably, substitution with two methyl groups at the 3- and 5-positions of the aryl ring (compound **23**, Table 3) did not yield an active compound, as neither MIC_{80} nor MIC_{50} values could be determined. The inactivity of the bis-CH_3 analogue, compared to the potent bis-CF_3 derivative, likely arises from a combination of steric, electronic, and physicochemical differences, which is consistent with established trends in medicinal chemistry [22]. The trifluoromethyl (CF_3) group is both more lipophilic and sterically demanding than a methyl group, with Hansch π values of +0.88 and + 0.56, respectively. Increased lipophilicity can enhance membrane permeability and, consequently, biological activity. Additionally, CF_3 is strongly electron-withdrawing, whereas CH_3 is mildly electron-donating and electronic effects that can significantly influence target binding [23–25]. These collective factors likely contribute to the activity observed with bis-CF_3

Table 4
Antibacterial (*S. aureus* and *E. coli*) activity of group 2b pyrazolo[1,5-*a*]pyrimidin-7-ones as MIC_{50}/MIC_{80} values.



Number	R^2	*S. aureus*		*E. coli*	
		MIC_{50} (μM)	MIC_{80} (μM)	MIC_{50} (μM)	MIC_{80} (μM)
22		2.2	a	641	a
23		2.3	a	b	a
24		4.2	a	b	a
25		8.4	a	b	a
26		10	a	631	a
27		19	360	193	410
28		250	370	310	420
29		375	a	643	a
30		415	a	719	a
31		483	a	146	a
32		b	a	702	a
33		b	a	717	a

a Compound was not sufficiently active to generate a MIC_{80} value. b Compound was not sufficiently active to generate a MIC_{50} value.

substitution.

Further aryl substitutions at R^2 included electron-withdrawing (CF_3, NO_2), electron-donating (OMe, NMe_2), and halogen substituents, exploring different positional isomers, group 2b (Table 4). Compound **22** (3,5-dichlorophenyl) showed the highest activity within this group (MIC_{50} = 2.2 μM), but remained less potent than compound **8**. Electron-donating substituents (compounds **28**–**31**) tended to reduce activity. Compound **23**, a mono-CF_3 analogue of **8**, had a higher MIC_{50} (2.3 μM), reinforcing the importance of dual CF_3 substitution for optimal activity.

The combined results from groups 1a–1c and 2a–2b indicate that the presence of an isopropyl group at R^1 and a 3,5-bis(trifluoromethyl) phenyl group at R^2 yields the most potent antibacterial pyrazolo[1,5-*a*]pyrimidin-7-one scaffold (compound **8**) against *S. aureus*.

2.2. Assessment of anti-microbial activity against *E. coli* and *C. albicans*

The most active compound against *E. coli* was **27** (R^1 = i-Pr, R^2 = m-FC_6H_4) from group 2b, with an MIC_{50} of 193 μM and MIC_{80} of 410 μM. While several pyrazolo[1,5-*a*]pyrimidin-7(4H)-ones were more effective than ampicillin trihydrate (MIC_{50} = 2.55 mM), they were generally less potent than tetracycline (MIC_{50} = 56.3 μM) and streptomycin sulfate (MIC_{50} = 6.2 μM). Most compounds from groups 2a and 2b showed limited or no activity, and all were considerably less effective against *E. coli* compared to *S. aureus* (Tables 3 and 4). For instance, compound **8** (MIC_{80} = 6.4 μM against *S. aureus*) was 64 times more potent than compound **27** against *E. coli*. This difference likely reflects the increased barrier function of the Gram-negative outer membrane, which restricts the uptake of hydrophobic compounds. A similar trend was observed with the reference antibiotics.

The antifungal activity against *C. albicans* was modest. No clear structure–activity relationship (SAR) emerged. Compound **3** (group 1a) showed the best activity in its group (MIC_{50} = 190 μM, MIC_{80} = 350 μM), while the most active overall was compound **11** (group 1b, MIC_{50} = 149 μM, MIC_{80} = 182 μM). These values remain significantly higher than fluconazole (MIC_{50} = 3.27 μM) reported by Clancy et al. [26]

2.3. Assessment of anti-microbial activity against MRSA and *P. aeruginosa*

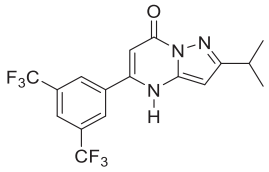
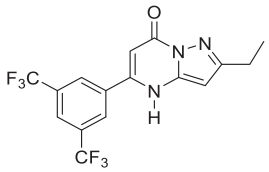
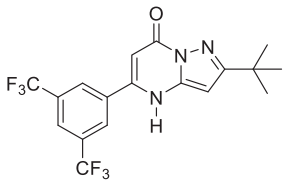
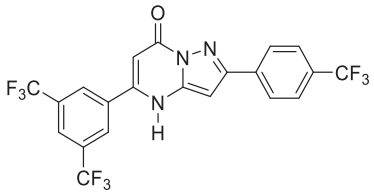
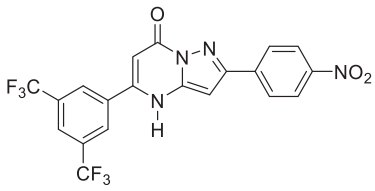
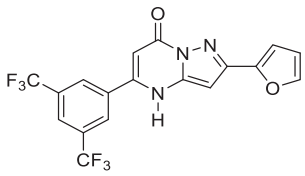
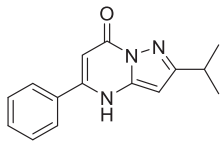
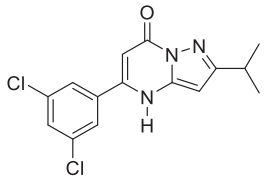
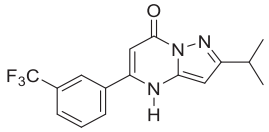
Given the superior activity of compound **8** against *S. aureus*, a subset of derivatives from groups 1a–c and 2a–b was selected for evaluation against MRSA and *P. aeruginosa*. MRSA and *P. aeruginosa* are clinically important multidrug-resistant pathogens (see Table 5 and Supporting Information) [27,28].

From group 1a (alkyl R^1), compounds **6** (Et), **8** (i-Pr), and **10** (*t*-Bu) showed strong activity against MRSA, with low micromolar MIC_{50} values (3.01 μM, 8.01 μM, 3.68 μM respectively). All three outperformed ampicillin (MIC_{50} = 2.33 mM) and tetracycline (MIC_{50} = 292 μM), both of which failed to produce an MIC_{80}. In group 1b, compound **12** (R^1 = *p*-$CF_3C_6H_4$) emerged as the most potent overall, with MIC_{50} = 1.88 μM and MIC_{80} = 2.93 μM comparable to vancomycin (MIC_{90} = 1.35 μM) [29,30]. In contrast, compound **37** (R^1 = *p*-$NO_2C_6H_4$), inactive against *S. aureus*, also showed no anti-MRSA activity.

To probe the role of the 3,5-bis-CF_3 aryl group at R^2, compounds **23** (one CF_3 replaced with H), **20** (both CF_3 replaced with H), and **22** (CF_3 replaced with Cl) were evaluated. Compound **23** showed the highest anti-MRSA activity among these, suggesting partial retention of potency with one CF_3, but compound **12**, which carries three CF_3 groups (R^1 = *p*-$CF_3C_6H_4$, R^2 = 3,5-bis-$CF_3C_6H_3$), was the most active. These findings point to a key role for CF_3 driven hydrophobicity in enhancing MRSA activity, likely by improving membrane permeability.

All compounds tested against *P. aeruginosa* were markedly less active than against MRSA. The most active, compound **20** (group 2a), showed an MIC_{80} of 692 μM, compared to 2.93 μM for compound **12** against MRSA. As with *E. coli*, the poor activity is likely due to the Gram-negative outer membrane. Streptomycin sulfate, used as a control, had

Table 5
Antibacterial activity of pyrazolo[1,5-*a*]pyrimidin-7-ones against MRSA as MIC_{50}/MIC_{80} values.

Number	Group	Structure	MIC_{50} (μM)	MIC_{XX} (μM)
8	1a		8.01	MIC_{71} 105
6	1a		3.01	MIC_{66} 155
10	1a		3.68	MIC_{75} 17.6
12	1b		1.88	MIC_{80} 2.93
37	1b		a	A
16	1c		10.42	MIC_{74} 23.42
20	2a		224.35	MIC_{64} 375
22	2b		32.51	MIC_{60} 46.9
23	2b		4.83	MIC_{77} 8.78

[a]Bacterial growth was not inhibited by this compound.

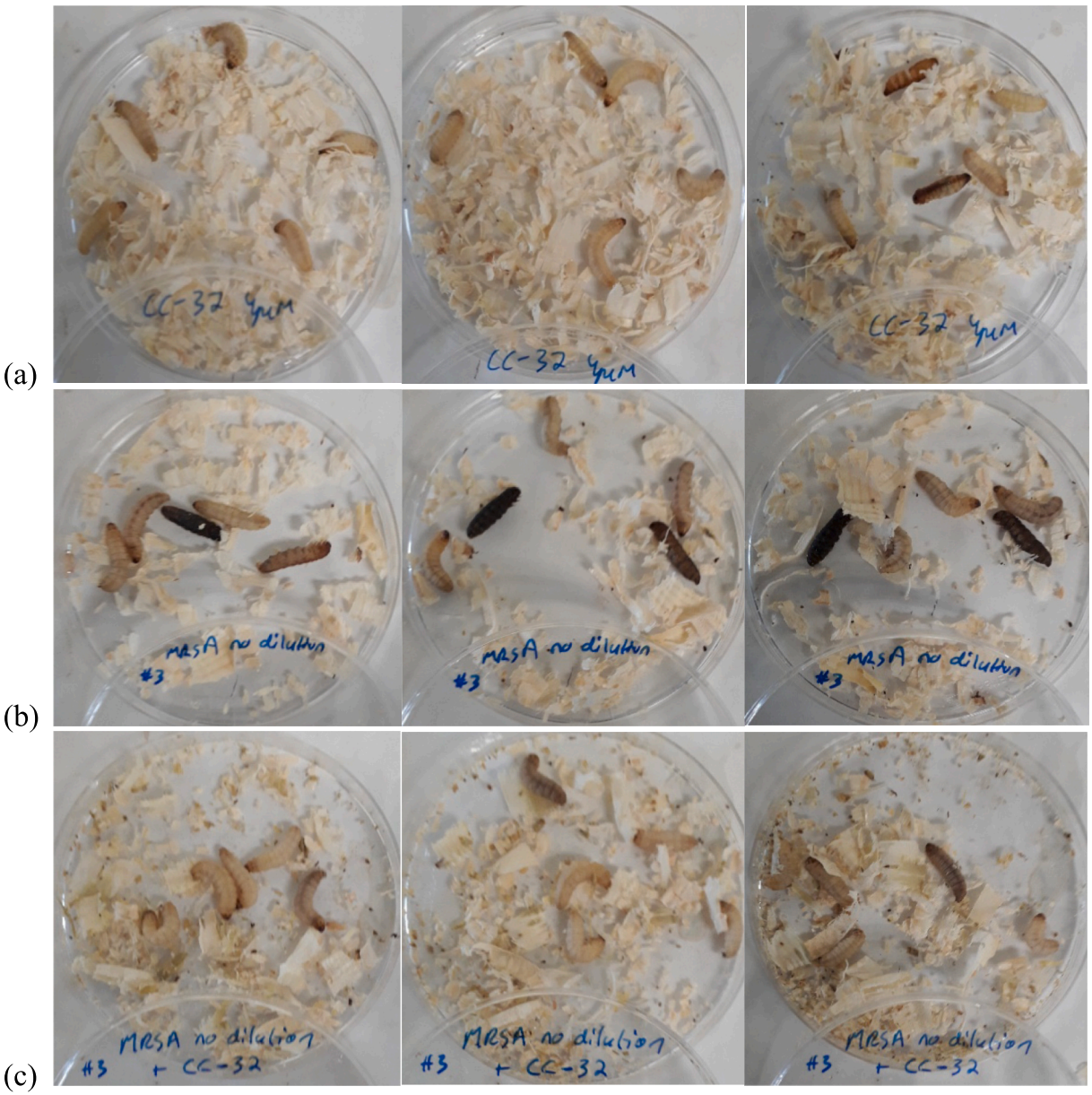


Fig. 3. *G. mellonella* larvae injected with 4 μM compound **12** as observed after a 24-, 48- and 72-h incubation period, reading from left to right. **(b)** *G. mellonella* larvae injected with a MRSA culture without dilution, as observed after a 24-, 48- and 72-h incubation period, reading from left to right. **(c)** *G. mellonella* larvae injected with a MRSA culture without dilution, left for 30 min then injected with 4 μM compound **12**, as observed after a 24-, 48- and 72-h incubation period, reading from left to right.

MIC_{50} and MIC_{80} values of 6.4 μM and 40.4 μM, respectively, substantially more potent than any pyrazolo[1,5-*a*]pyrimidin-7-one tested.

2.4. *In vivo toxicity and therapeutic evaluation*

To assess potential toxicity, an in vivo study was conducted using *Galleria mellonella* larvae, a widely accepted non-mammalian infection model that offers low cost, ethical acceptability, and immune system parallels with mammals [31–36]. *G. mellonella* larvae have been successfully used to study pathogen virulence and the in vivo tolerance and efficacy of antimicrobial agents [37–40].

We selected two lead compounds for evaluation: compound **20** (most active against *P. aeruginosa*) and compound **12** (most active against MRSA). Each compound was injected (10 μL) into five healthy sixth-instar *G. mellonella* larvae at the highest non-toxic dose, followed by incubation at 37 °C for 72 h. Larvae were monitored daily for survival and melanisation (see Fig. 2 and Supplementary Information).

No significant differences in survival were observed between treated and control groups, indicating that both compounds were well tolerated in vivo.

Next, we evaluated the therapeutic efficacy of **20** and **12** in *G. mellonella* infection models. To establish a working infectious dose, *P. aeruginosa* and MRSA cultures were titrated to identify concentrations that resulted in ~50 % mortality. As expected, *G. mellonella* exhibited higher sensitivity to *P. aeruginosa*, consistent with prior reports of its low LD_{50} (<10 CFUs) [40,41]. Based on screening data, the infectious dose for *P. aeruginosa* was set at a 1:1,000,000 dilution of the stationary-phase culture in PBS, while undiluted MRSA broth was used for the MRSA model (see Supplementary Information).

Following infection, larvae were allowed to rest for 30 min before receiving treatment. For the *P. aeruginosa* model, larvae were injected with 10 μL of 800 μM compound **20** (in 5 % DMSO, 95 % water). For

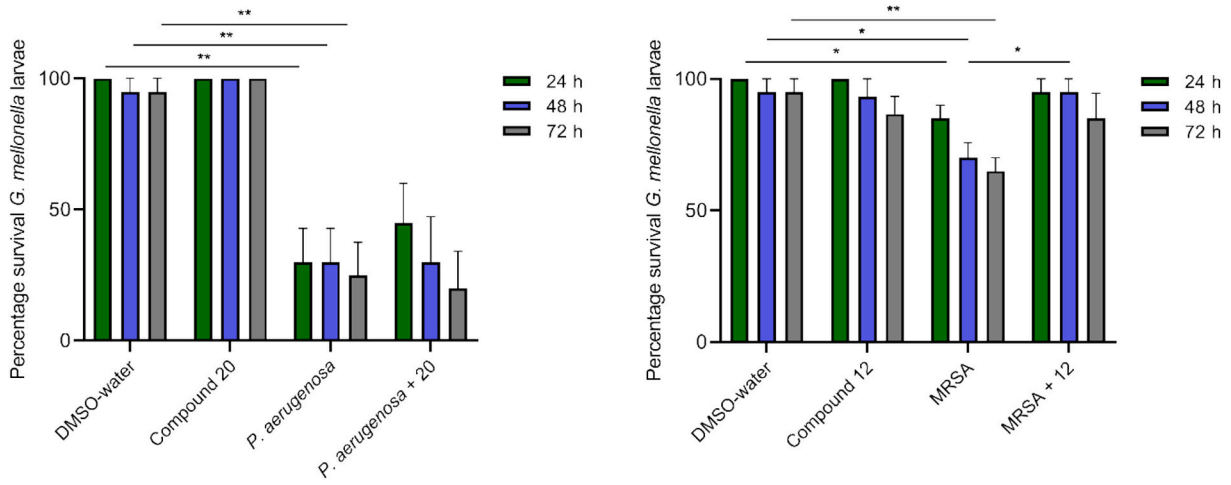


Fig. 4. Percentage survival of *G. mellonella* larvae: (**A**) Post infection with *P. aeruginosa* and subsequent treatment with compound **20** (800 μM) after 30 min; (**B**) post infection with MRSA and subsequent treatment with compound **12** (4 μM) after 30 min. Larvae were treated and survival was assessed for 24, 48 and 72 h at 37 °C. A 5:95 (*v*/v) DMSO:distilled water mix was used as a control. Data are presented as the mean ± se of four independent experiments and were analysed using a two-tailed unpaired Student's *t*-test, $^*P < 0.05$.

MRSA, the treatment consisted of 10 μL of 4 μM compound **12**. All groups were incubated at 37 °C and monitored for 72 h (see Fig. 3).

As shown in Fig. 3, *P. aeruginosa* infection significantly reduced larval survival, and treatment with compound **20** did not improve survival relative to untreated infected controls. This suggests that while **20** shows in vitro antibacterial activity and is non-toxic in vivo, it lacks therapeutic efficacy under these conditions. Such discrepancies between in vitro and in vivo results are common [42] and may arise from factors such as host immune interactions or protein binding that are absent in vitro. In *G. mellonella*, innate immunity consists of cellular and humoral components [43], which may inactivate compound **20**.

In contrast, compound **12** demonstrated promising in vivo efficacy against MRSA. Infection with MRSA significantly reduced larval survival, but treatment with **12** increased survival by 25 % at 48 h compared to the untreated group (control = 75 %, treated = 95 %, $p < 0.05$; Fig. 3, Fig. 4). These results highlight **12** as a promising candidate for further preclinical evaluation.

3. Conclusion

We report the discovery of a series of pyrazolo[1,5-*a*]pyrimidin-7-ones with promising antimicrobial properties. Initial screening identified compound **3** as a hit against *Staphylococcus aureus* ($MIC_{50} = 1.8$ μM). A structure activity relationship study of 35 analogues revealed enhanced antibacterial activity over antifungal activity and led to the identification of compound **8** (R^1 = i-Pr, R^2 = 3,5-bis(CF_3)Ph) as a new lead, with superior potency ($MIC_{50} = 1.2$ μM) compared to commercial antibiotics.

Select compounds were further evaluated against MRSA and *P. aeruginosa*. Compound **12** (R^1 = *p*-$CF_3C_6H_4$, R^2 = 3,5-bis(CF_3)Ph) showed the strongest activity against MRSA ($MIC_{50} = 1.88$ μM, $MIC_{80} = 2.93$ μM), comparable to vancomycin ($MIC_{90} = 1.35$ μM). In vivo toxicity studies using *G. mellonella* larvae confirmed all compounds tested to be non-toxic. Moreover, compound **12** significantly improved survival in *G. mellonella* infected with MRSA, indicating in vivo efficacy.

These findings, and the in vivo data in particular, underscore the potential of pyrazolo[1,5-*a*]pyrimidin-7-ones as a novel antimicrobial scaffold.

CRediT authorship contribution statement

Amanda A. Doyle: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Mark Kelada:** Investigation. **Clara Charleton:** Investigation. **Robert Devine:** Investigation. **John M. D. Walsh:** Investigation. **Ronan Bergin:** Data curation. **Kevin Kavanagh:** Writing – review & editing, Supervision, Formal analysis. **John C. Stephens:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rechem.2025.102403.

Data availability

Data will be made available on request.

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