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Design, synthesis, and structure–activity relationships of pyrazolo[3,4-*d*]pyrimidines: a novel class of potent enterovirus inhibitors

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Abstract—A series of pyrazolo[3,4-*d*]pyrimidines were synthesized and their antiviral activity was evaluated in a plaque reduction assay. It is very interesting that this class of compounds provide remarkable evidence that they are very specific for human enteroviruses, in particular, coxsackieviruses. Some derivatives proved to be highly effective in inhibiting enterovirus replication at nanomolar concentrations. SAR studies revealed that the phenyl group at the N-1 position and the hydrophobic diarylmethyl group at the piperazine largely influenced the in vitro antienteroviral activity of this new class of potent antiviral agents. It was found that the pyrazolo[3,4-*d*]pyrimidines with a thiophene substituent, such as compounds **20**–**24**, in general exhibited high activity against coxsackievirus B3 ($IC_{50} = 0.063$–$0.089\,\mu M$) and moderate activity against enterovirus 71 ($IC_{50} = 0.32$–$0.65\,\mu M$) with no apparent cytotoxic effect toward RD (rhabdomyosarcoma) cell lines ($CC_{50} > 25\,\mu M$).

1. Introduction

Enteroviruses comprise more than 60 distinct serotypes within the family of *picornaviridae*. The enteroviruses are frequently classified by their sub-genus names: polioviruses, coxsackieviruses A and B, echoviruses, and the newer numbered enteroviruses.[1] Human enteroviruses are the most common cause for viral infections in humans along with rhinoviruses. Symptoms for enteroviral infections range from nonspecific upper respiratory infection and mild fever to central nervous system infections particularly viral meningitis, encephalitis, and severe myocarditis.[1] Children are considered to be relatively immunodeficient, therefore enteroviral infections of neonates can be lethal and life threatening, with high risk for morbidity, and mortality.

Enteroviral infection is a worldwide distribution. It is estimated that worldwide they cause over 5 billion enteroviral infections annually, and many outbreaks have been reported from Asian countries.[2] In 1998, the epidemic outbreak of enterovirus 71 infection in Taiwan resulted in almost 80 fatalities and 405 severe cases.[3] Unfortunately, there is no effective antiviral drug for treatment of enteroviral disease. This highlights the urgency and significance for developing antienteroviral agents.

In 1966, a novel antienteroviral agent, pleconaril **A** (Fig. 1), was made available for treatment of life threatening enteroviral infections, such as meningitis, encephalitis, myocarditis.[4] It is under development by Viropharma, Inc. for the treatment of severe enteroviral infection in phase 2 clinical trials. But it is worth to mention that pleconaril exhibited no activity against enterovirus 71. In our previous study, we reported the synthesis, antiviral and cytotoxic properties of imidazolidinones **B** (Fig. 1).[5] This is a novel class of potent and selective human enterovirus 71 inhibitors. Further SAR studies of this class of compounds are currently under active investigation. Recently, an antiviral screening program was initiated to search for new enterovirus inhibitors via plaque reduction assay[6] in our laboratory. Interestingly, a novel pyrazolo[3,4-*d*]pyrimidine, compound **1**, was identified as a potential antienteroviral agent, the

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Keywords: Enterovirus inhibitor.

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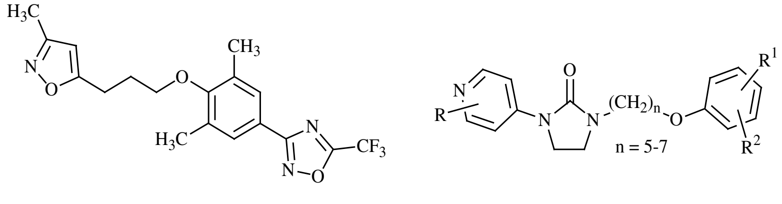
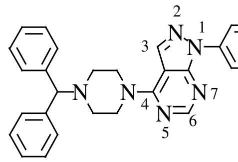


Pleconaril **A** Imidazolidinones **B**



Compound **1**

Figure 1. Structures of pleconaril **A**, imidazolidinones **B**, and compound **1**.

structure of which is illustrated in Figure 1. This compound was found to possess antiviral activity against three genotypes (A, B, and C) of enterovirus 71, coxsackieviruses A9, A10, A16, A24, B1–B6, echovirus 9, and enterovirus 68. Therefore many structurally related pyrazolo[3,4-*d*]pyrimidines were synthesized and evaluated against enterovirus 71 (EV71) and coxsackievirus B3 (CVB3). Details of this investigation will be described herein.

2. Results and discussion

A variety of synthetic procedures for pyrazolopyrimidine derivatives have been reported in the literature.[7] To the best of our knowledge, there is no report concerning the synthesis of these interesting piperazine-containing pyrazolo[3,4-*d*]pyrimidines. We report here an efficient synthesis of the novel antiviral pyrazolo[3,4-*d*]pyrimidines **1**–**24** via the key nucleophilic substitution[8] of compound **26**[9] or **36**[10] with various diarylmethylpiperazines **30**[11] and **34**[11] (Schemes 1–3). The parental compound **1** was subjected to evaluation against a variety of viruses. The results of this biological evaluation are illustrated in Table 1. Preliminary structure–activity relationship against EV71 and CVB3 are also reported (Tables 2–4).

3. Chemistry

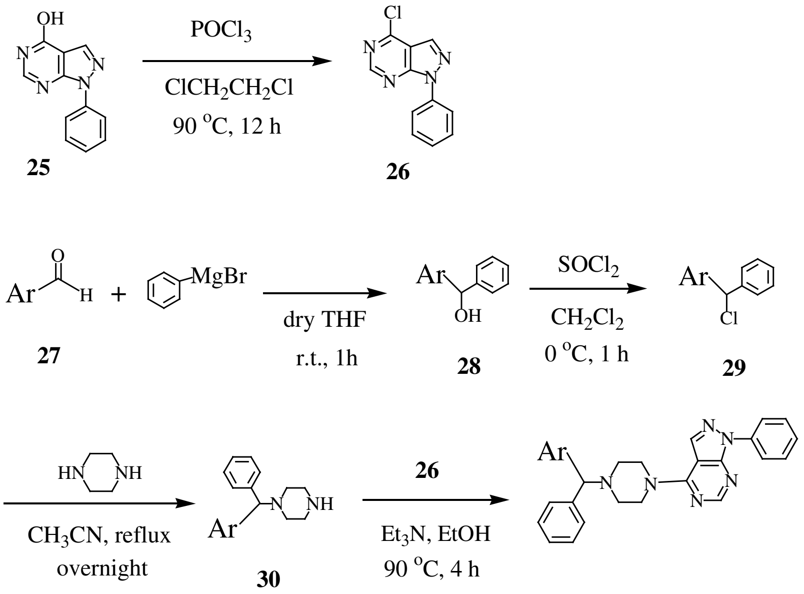
Pyrazolo[3,4-*d*]pyrimidines **1**–**8** and **14**–**20** were prepared by the method summarized in Scheme 1. The key intermediate **26**, 4-chloro-1-phenyl-1*H*-pyrazolo[3,4-*d*]-pyrimidine, was obtained in 90% yield by treating phosphoryl chloride with compound **25** in dichloroethane at refluxing temperature. Reaction between compound **27** and phenylmagnesium bromide gave compound **28**. Compound **28** was subsequently treated with thionyl chloride to give the corresponding chloro compound **29** which was directly treated with piperazine in acetonitrile to give compound **30**. The treatment of compound **30** with compound **26** and triethylamine in refluxing ethanol gave the target compounds **1**–**8** and **14**–**20**.

In similar fashion, the thiophene-containing compounds **21**–**24** were synthesized in a short convergent synthetic route as shown in Scheme 2. Reaction of 2-bromo-3-methylthiophene **31** with turning magnesium in dry THF followed by nucleophilic addition with the aldehyde gave compound **32**. Compound **32** was subsequently treated with thionyl chloride to give the corresponding chloro compound **33**, which was directly treated with piperazine in acetonitrile to give compound **34**. The treatment of compound **34** with compound **26** and triethylamine in refluxing ethanol gave the target compounds **21**–**24**.

Other pyrazolo[3,4-*d*]pyrimidines **9**–**13** were prepared by the reported method summarized in Scheme 3.[10] Compound **36**, 4-chloro-1*H*-pyrazolo[3,4-*d*]pyrimidine, is commercially available or conveniently prepared by chlorination of allopurinol **35** with phosphoryl chloride in *N*,*N*-dimethylaniline at refluxing temperature. Nucleophilic substitution of compound **36** with 1-benzhydrylpiperazine gave compound **9**. Compound **9** was subsequently treated with alkyl iodide and cesium carbonate to give target compounds **10**–**13**. The new pyrazolo[3,4-*d*]pyrimidines **1**–**24** were characterized by ^{1}H NMR, ESMS, and FT-IR.[12,13]

4. Bioactivity

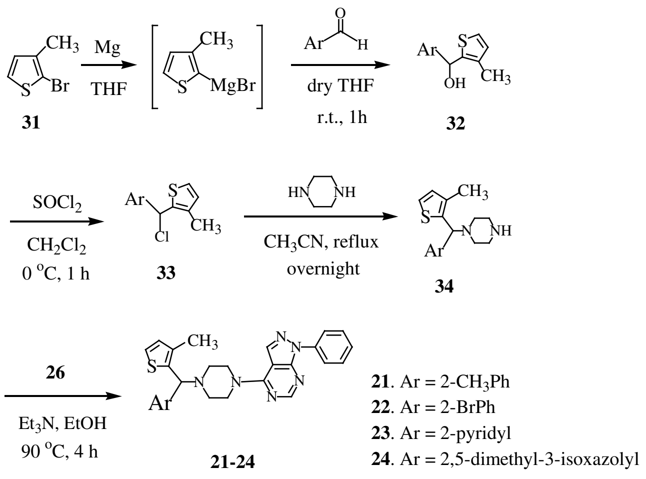
Pyrazolo[3,4-*d*]pyrimidines described herein were tested in a plaque reduction assay[6] under a standard procedure. The parental compound **1** was subjected to evaluation against a variety of viruses, including coxsackieviruses (10 serotypes), echoviruses (2 serotypes), human enteroviruses 68 and 71, influenza A and B viruses, herpes simplex virus 1 (HSV-1), human rhinovirus 2 (HRV 2), and 14 (HRV 14). As shown in Table 1, this compound exhibited significant activities



1. Ar = Ph
2. Ar = 4-ClPh
3. Ar = 4-BrPh
4. Ar = 4-CF_3Ph
5. Ar = 4-PhPh
6. Ar = 4-CNPh
7. Ar = 3-CNPh
8. Ar = 2-CH_3Ph

14. Ar = 3-pyridyl
15. Ar = 4-pyridyl
16. Ar = 2-pyridyl
17. Ar = 2-thiazole
18. Ar = 3-furanyl
19. Ar = 2,5-dimethyl-3-isoxazolyl
20. Ar = 5-methyl-2-thienyl

Scheme 1. General synthetic route to pyrazolo[3,4-*d*]pyrimidines **1**–**8** and **14**–**20**.



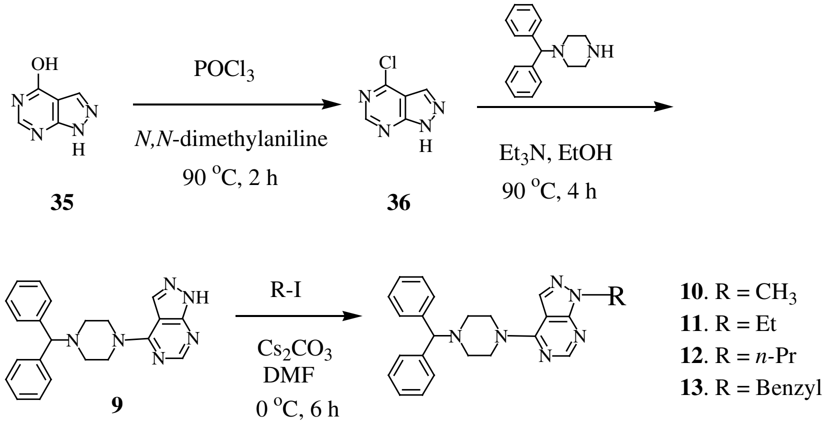
21. Ar = 2-CH_3Ph
22. Ar = 2-BrPh
23. Ar = 2-pyridyl
24. Ar = 2,5-dimethyl-3-isoxazolyl

Scheme 2. General synthetic route to pyrazolo[3,4-*d*]pyrimidines **21**–**24**.

against human enterovirus 68 ($IC_{50} = 0.18\,\mu M$), human enterovirus 71 ($IC_{50} = 0.35$–$0.52\,\mu M$), echovirus 9 ($IC_{50} = 0.30\,\mu M$), and coxsackieviruses A and B ($IC_{50} = 0.05$–$0.94\,\mu M$). However, it showed no activity against influenza A and B viruses, HSV-1, HRV 2, HRV 14, and echovirus 29 up to the concentration tested ($25\,\mu M$). We observed that the parental compound **1** is highly active against most of the serotypes of enteroviruses tested in the submicromolar range and also possess a very low cytotoxic effect on the uninfected rhabdomyosarcoma (RD) host cells ($IC_{50} > 25\,\mu M$). It was therefore of interest to explore the SAR of pyrazolo[3,4-*d*]pyrimidines.

In preliminary SAR studies, a series of pyrazolo[3,4-*d*]pyrimidines **1**–**13** were synthesized according to Schemes 1 and 3 then submitted for anti-EV71 and anti-CVB3 testing as well as cytotoxicity evaluation in RD cell lines. These significant biological results were summarized in Table 2. As shown in Table 2, compared to the parental compound **1**, when a substituent (Br, Cl, CF_3, CN, Me, Ph) was introduced on one of the phenyl rings of the diphenylmethyl group, compounds **2**–**8** showed a slight decrease in activity against EV71, retained activity against CVB3, and showed no cytotoxicity against RD cell lines. This effect might be due to their distinct differences of hydrophobic properties. On the other hand, replacement of the phenyl group at the N-1 position with either a hydrogen or alkyl substituent (Me, Et, *n*-Pr, benzyl), such as compounds **9**–**13**, resulted in a complete loss of antiviral activity against both EV71 and CVB3. However, this unexpected biological result is not fully understood and is worthy of further study.

According to Scheme 1, a variety of heteroaryl-containing pyrazolo[3,4-*d*]pyrimidines **14**–**20** were synthesized. The results are shown in Table 3 and are



Scheme 3. General synthetic route to pyrazolo[3,4-*d*]pyrimidines **9**–**13**.

Table 1. Antiviral activity of compound **1** and imidazolidinone **B** ($R = R^1 = H$, $R^2 = 4\text{-Br}$, $n = 6$) against various viruses

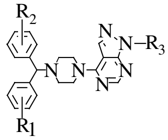
	IC_{50} (μM)[a]	
	Imidazolidinone **B** ($R = R^1 = H$, $R^2 = 4\text{-Br}$, $n = 6$)	Compound **1**
Enterovirus 68	2.13 ± 0.19	0.18 ± 0.08
Enterovirus 71 (1743) genotype B	0.60 ± 0.02	0.47 ± 0.17
Enterovirus 71 (2231) genotype C	1.15 ± 0.05	0.52 ± 0.17
Enterovirus 71 (BrCr) genotype A	1.26 ± 0.05	0.35 ± 0.02
Coxsackievirus A9	0.77 ± 0.05	0.26 ± 0.02
Coxsackievirus A10	5.88 ± 0.34	0.39 ± 0.01
Coxsackievirus A16	>25	0.23 ± 0.06
Coxsackievirus A24	0.55 ± 0.06	0.05 ± 0.01
Coxsackievirus B1	5.10 ± 0.05	0.69 ± 0.02
Coxsackievirus B2	>25	0.08 ± 0.01
Coxsackievirus B3	>25	0.15 ± 0.01
Coxsackievirus B4	>25	0.16 ± 0.01
Coxsackievirus B5	>25	0.44 ± 0.04
Coxsackievirus B6	>25	0.94 ± 0.34
Echovirus 9	2.82 ± 0.01	0.30 ± 0.04
Echovirus 29	>25	>25
Influenza A virus (WSN)	>25	>25
Influenza B virus (HK)	>25	>25
Herpes simplex virus 1	>25	>25
Human rhinovirus 2	>25	>25
Human rhinovirus 14	11.60 ± 0.12	>25

[a] Mean of triplicate well values. All experiments were performed at least twice. Plaque reduction assay was employed.

compared to the parental compound **1**. Replacement of one of the phenyl rings of the diphenylmethyl group with either a 3-pyridine ring (**14**), 4-pyridine ring (**15**), or 3-furan ring (**18**) slightly lowered activity against both EV71 and CVB3; but replacing the phenyl ring with a 2-pyridine ring (**16**) retained activity. However, replacement of the phenyl ring of diphenylmethyl group with either a 2-thiazole ring (**17**), 2,5-dimethyl-3-isoxazole ring (**19**), or 5-methyl-2-thiophene ring (**20**) resulted in a significant improvement in activity against CVB3, but retained activity against EV71. These encouraging results prompt us to further investigate this new class of pyrazolo[3,4-*d*]pyrimidines.

In this study, we observed that the substituted thiophene-containing compound **20**, in terms of potency and selectivity index, appear to be the most promising candidate for further development as an antienteroviral agent. On the basis of the skeleton of compound **20**, several 5-methyl-2-thiophene derivatives **21**–**24** were thus synthesized (Scheme 2). The results are shown in Table 4 and are compared to the compound **20**. Replacement of one of the phenyl rings of the diarylmethyl group with either a 2-methylphenyl ring (**21**), 2-bromophenyl ring (**22**), 2-pyridine ring (**23**), or 2,5-dimethyl-3-isoxazole ring (**24**) provide a slight improvement in activity against both EV71 and CVB3. It is very interesting to note that this series of compounds exhibited very high activity against CVB3 ($IC_{50} = 0.063$–0.089 μM) and moderate activity against EV71 ($IC_{50} = 0.32$–0.65 μM) with no cytotoxicity up to the concentration of 25 μM. These significant results demonstrated that the substituted thiophene moiety of the pyrazolo[3,4-*d*]pyrimidines seem to play a very

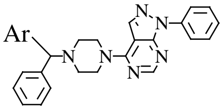
Table 2. Antienteroviral activity and cytotoxicity for compounds **1**–**13**

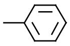
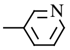
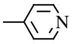
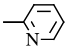
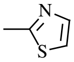
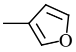
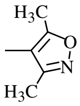
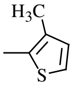


Compound	R$_1$	R$_2$	R$_3$	IC$_{50}$ (μM)[a]	IC$_{50}$ (μM)[a]	CC$_{50}$ (μM)[b]
				EV71	CVB3	RD
1	H	H	Ph	0.41 ± 0.08	0.15 ± 0.01	>25
2	H	4-Cl	Ph	1.97 ± 0.012	0.26 ± 0.07	>25
3	H	4-Br	Ph	0.91 ± 0.28	0.49 ± 0.04	>25
4	H	4-CF$_3$	Ph	1.65 ± 0.26	0.30 ± 0.05	>25
5	H	4-Ph	Ph	1.90 ± 0.96	0.31 ± 0.01	>25
6	H	4-CN	Ph	0.94 ± 0.42	0.18 ± 0.02	>25
7	H	3-CN	Ph	0.78 ± 0.08	0.11 ± 0.05	>25
8	H	2-CH$_3$	Ph	1.98 ± 1.56	0.13 ± 0.01	>25
9	H	H	H	>25	>25	>25
10	H	H	CH$_3$	>25	>25	>25
11	H	H	Et	>25	>25	>25
12	H	H	*n*-Pr	>25	>25	>25
13	H	H	Benzyl	>25	>25	>25

[a] Mean of triplicate well values. All experiments were performed at least twice. Plaque reduction assay was employed.
[b] Mean of triplicate well values. All experiments were performed at least twice.

Table 3. Antienteroviral activity and cytotoxicity for compounds **14**–**20**



Compound	Ar	IC$_{50}$ (μM)[a]	IC$_{50}$ (μM)[a]	CC$_{50}$ (μM)[b]
		EV71	CVB3	RD
1		0.41 ± 0.08	0.15 ± 0.01	>25
14		4.60 ± 0.23	0.53 ± 0.02	>25
15		0.78 ± 0.02	0.30 ± 0.003	>25
16		0.37 ± 0.02	0.17 ± 0.002	>25
17		0.34 ± 0.06	0.11 ± 0.05	>25
18		1.25 ± 0.06	0.27 ± 0.11	>25
19		0.58 ± 0.27	0.095 ± 0.008	>25
20		0.46 ± 0.14	0.089 ± 0.033	>25

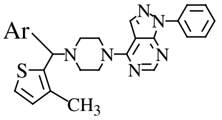
[a] Mean of triplicate well values. All experiments were performed at least twice. Plaque reduction assay was employed.
[b] Mean of triplicate well values. All experiments were performed at least twice.

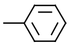
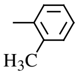
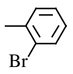
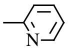
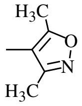
important role in enhancing the anti-CVB3 activity. This effect might be due to their drastically conformational change and steric requirement between the two adjacent aromatic rings of diarylmethyl group.

5. Conclusion

In summary, a novel pyrazolo[3,4-*d*]pyrimidine, compound **1**, was found to possess significant antiviral

Table 4. Antienteroviral activity and cytotoxicity for compounds **21**–**24**



Compound	Ar	IC_{50} (μM)[a]	IC_{50} (μM)[a]	CC_{50} (μM)[b]
		EV71	CVB3	RD
20		0.46 ± 0.14	0.089 ± 0.033	>25
21		0.41 ± 0.17	0.084 ± 0.041	>25
22		0.32 ± 0.26	0.072 ± 0.005	>25
23		0.36 ± 0.09	0.063 ± 0.013	>25
24		0.65 ± 0.35	0.081 ± 0.002	>25

[a] Mean of triplicate well values. All experiments were performed at least twice. Plaque reduction assay was employed.

[b] Mean of triplicate well values. All experiments were performed at least twice.

activity against human enteroviruses in our screening program. Preliminary studies revealed that this compound is very specific for human enteroviruses, in particular, coxsackieviruses. We have developed an efficient synthesis of these new pyrazolo[3,4-*d*]pyrimidines in an attempt to evaluate their antienteroviral activity in a plaque reduction assay. Compound **1**–**24** were synthesized and evaluated against EV71 and CVB3. According to our SAR investigation, the phenyl group at the N-1 position and the hydrophobic diarylmethyl group at the piperazine largely influenced the in vitro antienteroviral activity of this new class of potent antiviral agents. On the basis of these biological results, a series of thiophene-containing compounds **20**–**24** were found to exhibit high antiviral activity against CVB3 ($\text{IC}_{50} = 0.063$–$0.089\,\mu\text{M}$) and moderate antiviral activity against EV71 ($\text{IC}_{50} = 0.32$–$0.65\,\mu\text{M}$) with very low cytotoxic effect toward RD cell lines. Further SAR studies and mechanistic studies on this new class of antiviral compounds are currently under active investigation and will be reported in due course.

Acknowledgements

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12. All the new compounds gave satisfactory spectral data consistent with their proposed structures. Selected spectral data for compounds **1**, **16**, and **23**. Compound **1**: mp 215–216 °C; IR ($CHCl_3$) ν_{max} 2810, 1573, 1547, 1501 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.41 (s, 1H), 8.07 (dd, 2H, $J = 6.3$ Hz, $J = 0.9$ Hz), 8.04 (s, 1H), 7.52–7.44 (m, 6H), 7.33–7.21 (m, 7H), 4.29 (s, 1H), 4.04 (br s, 4H), 2.57 (br s, 4H); ESMS 447.3 [M+1]. Anal. Calcd for $C_{28}H_{26}N_6$: C, 75.31; H, 5.87; N, 18.82. Found: C, 75.33; H, 5.80; N, 18.85. Compound **16**: mp 185–186 °C; IR ($CHCl_3$) ν_{max} 2958, 2817, 1571, 1547, 1501 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.54 (d, 1H, $J = 4.5$ Hz), 8.41 (s, 1H), 8.08 (dd, 2H, $J = 8.1$ Hz, $J = 1.2$ Hz), 8.04 (s, 1H), 7.66–7.46 (m, 6H), 7.34–7.23 (m, 4H), 7.15 (t, 1H, $J = 0.9$ Hz), 4.49 (s, 1H), 4.04 (t, 4H, $J = 5.1$ Hz), 2.68–2.56 (m, 4H); ESMS 448.2 [M+1], 470.2 [M+23]. Anal. Calcd for $C_{27}H_{25}N_7$: C, 72.46; H, 5.63; N, 21.91. Found: C, 72.41; H, 5.56; N, 21.85. Compound **23**: mp 330 °C (decomposed); IR ($CHCl_3$) ν_{max} 2913, 2811, 1571, 1547, 1501 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.55 (d, 1H, $J = 0.6$ Hz), 8.40 (d, 1H, $J = 1.8$ Hz), 8.08 (d, 2H, $J = 8.1$ Hz), 8.01 (s, 1H), 7.64–7.55 (m, 2H), 7.49 (t, 2H, $J = 7.2$ Hz), 7.30–7.12 (m, 3H), 6.71 (dd, 1H, $J = 4.2$ Hz, $J = 1.2$ Hz), 4.83 (s, 1H), 4.00 (br s, 4H), 2.73–2.53 (m, 4H), 2.27 (s, 3H); ESMS 468.4 [M+1], 490.4 [M+23]. Anal. Calcd for $C_{26}H_{25}N_7S$: C, 66.78; H, 5.39; N, 20.97. Found: C, 66.86; H, 5.48; N, 20.95.
13. Selected spectral data for compounds **9** and **11**. Compound **9**: mp 216–217 °C; IR (KBr) ν_{max} 3425, 3200, 3111, 2995, 2820, 1597, 1563, 1521, 1490 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) δ 8.20 (s, 1H), 7.96 (s, 1H), 7.44–7.42 (m, 5H), 7.30–7.18 (m, 5H), 4.28 (s, 1H), 3.99 (br s, 4H), 2.57 (br s, 4H); ESMS 371.2 [M+1], 393.2 [M+23]. Anal. Calcd for $C_{22}H_{22}N_6$: C, 71.33; H, 5.99; N, 22.69. Found: C, 71.30; H, 5.91; N, 22.66. Compound **11**: mp 169–170 °C; IR (KBr) ν_{max} 2974, 2852, 2816, 2766, 1547, 1499, 1478, 1450 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.34 (s, 1H), 7.87 (s, 1H), 7.45 (dd, 4H, $J = 7.2$ Hz, $J = 1.2$ Hz), 7.32–7.19 (m, 6H), 4.43 (q, 2H, $J = 7.2$ Hz), 4.29 (s, 1H), 4.00 (q, 4H, $J = 4.8$ Hz), 2.57 (t, 4H, $J = 4.8$ Hz), 1.49 (t, 3H, $J = 7.2$ Hz); ESMS 399.2 [M+1]. Anal. Calcd for $C_{24}H_{26}N_6$: C, 72.33; H, 6.58; N, 21.09. Found: C, 72.25; H, 6.53; N, 21.07.