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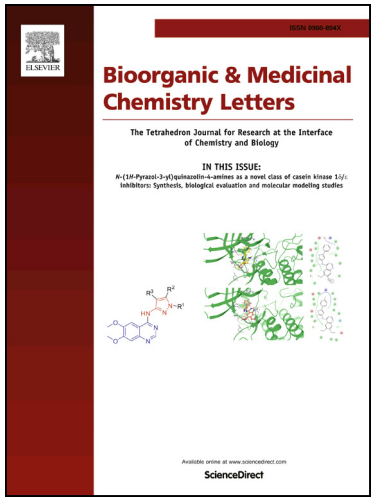

Enterovirus Inhibitory Activity of C-8-tert-Butyl Substituted 4-Aryl-6,7,8,9-tetrahydrobenzo[4,5]thieno[3,2- e][1,2,4]triazolo[4,3-a]pyrimidin-5(4H)-ones

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ABSTRACT

Members of a series of 4-aryl-6,7,8,9-tetrahydrobenzo[4,5]thieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-ones (**1**, Fig 2**)** were prepared and tested against representative enteroviruses including Human Coxsackievirus B1 (Cox B1), Human Coxsackievirus B3 (Cox B3), human Poliovirus 3 (PV3), human Rhinovirus 14 (HRV14), human Rhinovirus 21 (HRV 21) and human Rhinovirus 71 (HRV 71). The C-8-*tert*-butyl group on the tetrahydrobenzene ring in these substances was found to be crucial for their enterovirus activity. One member of this group, **1e**, showed single digit micromolar activities (1.6-8.85 μM) against a spectrum of viruses screened, and the highest selectivity index (SI) values for Cox B1 (>11.2), for Cox B3 (>11.5), and for PV3 (>51.2), respectively**.** In contrast, **1p**, was the most active analog against the selected HRVs (1.8-2.6 μM), and showed the highest selectivity indices among the group of compounds tested. The SI values for **1p** were 11.5 for HRV14, 8.4 for HRV21, and 12.1 for HRV71, respectively.

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Enteroviruses are positive-sense non-enveloped ssRNA viruses. Various viruses, such as coxsackievirus A (Cox A), coxsackievirus B (Cox B), poliovirus (PV), human rhinovirus (HRV) and enterovirus (EV), belong to this genus. Owing to frequent recombination and low replication fidelity, these viruses, like other RNA viruses, have high mutation rates.[1] Consequently various serotypes are present in each of the enteroviurses. For example, 29 serotypes of Cox A and B, and more than 160 serotypes of HRV with genetic variations are known.[2] Also PV can be divided to three serotypes (PV1, PV2 and PV3).[2] These enteroviruses cause many different types of disease such as myocarditis (Cox B), encephalitis (Cox A and B), aseptic meningitis (Cox A and B), poliomyelitis (PV), hand, foot and mouth disease (Cox A or EV71), and acute hemorrhagic conjunctivitis (EV70, Cox A24).[3] In addition, HRVs cause common colds[4] and they are responsible for the exacerbation of asthma[5] and Chronic Obstructive Pulmonary Disease (COPD).[5c,6] Even mild and generally self-limiting infections caused by HRV costs billions of dollars in terms of missed work days, medical visits and treatment.[7]

A few small molecules have been observed to inhibit the replication of these viruses.[8] For instance, Pleconaril and Vapendavir are known to bind to capsid,[8-9] which prevent their entry into the host cells. Rupintrivir inhibits 3C protease and, thus, interfers with viral polyprotein processing.[10] Enviroxime inhibits viral RNA replication by targeting 3A and related proteins.[11] However, because of insufficient efficacies or side effects observed during their clinical studies, none of these substances have been developed into drugs approved for treatment of these viral infections.[12] Therefore, a new small molecule inhibitor that shows broad-spectrum inhibition of the replication of these enteroviruses is highly desired.

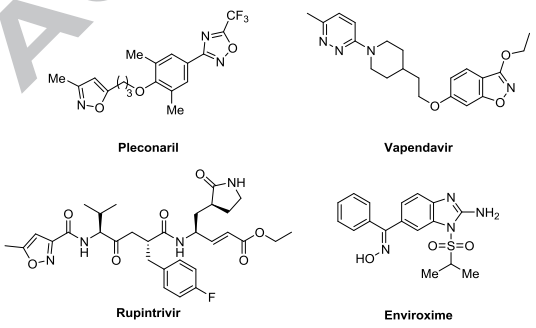


Figure 1. Known virus inhibitors

In the preliminary phase of this effort, High-Throughput Screening (HTS) of a chemical library of 100,000 compounds from ChemBank at KRICT was conducted using *in vitro* phenotypic (cytopathogenic) assays on Cox B3 and PV3 in HeLa cells. This study led to our discovery that 8-(*tert*-butyl)-4-phenyl-6,7,8,9-tetrahydrobenzo[4,5]thieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (**1)** displays a good antiviral activity profile (Figure 2). This substance **1** has 50% effective concentrations (EC_{50} values) of 8.65 μM for Cox B3 and 1.95 μM for PV3.

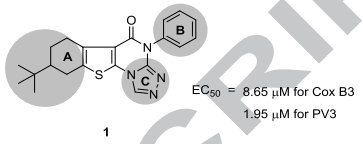
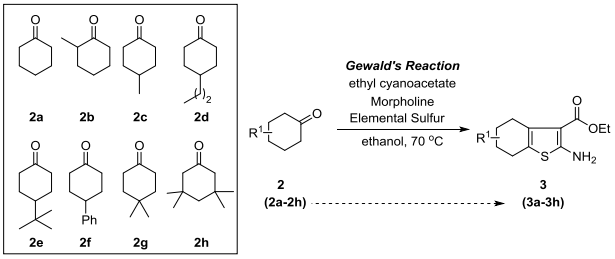


Figure 2. Initial hit compound **1** from HTS and its EC50 values against Cox B3 and PV3

In our efforts to optimize the anti-enteroviral activity of this substance, we modified cyclohexyl ring **A**, aromatic ring **B** and triazole ring **C** moieties (Figure 2). The cyclohexyl ring **A** was modified by incorporation of various alkyl groups. The thiophene amino ester intermediates **3a-h** were prepared using Gewald's reaction[13] starting with appropriate substituted cyclohexanones **2a-h** (Scheme 1). These intermediates **3** reacted with isothiocyanates to produce intermediates **4** (path I, Scheme 2). Alternatively the same intermediates **4** were obtained through path II (Scheme 2), where the thiophene amino esters **3** was converted to stable isothiocyanate derivatives **6**, which were treated with aniline to form thiourea derivatives **7**. Cyclization of **7** in mild ethanolic NaOH solution generated intermediates **4**.

Intermediates **4** were converted to hydrazine containing analogs **5** using hydrazine hydrate. The resulting hydrazines **5** were reacted with formic acid/trialkyl orthoformate to form the triazole ring containing 4-aryl-6,7,8,9-tetrahydrobenzo[4,5]thieno[3,2-*e*]triazolo[4,3-*a*]pyrimidin-5(4*H*)-ones **1a-h** possessing different R^1 substituents. Modifications of the aromatic ring **B**, conducted using the same sequence with different substituted isothiocyanates (path I) or various substituted anilines (path II), produced the desired products **1i-r** with different R^2 substitutions (Scheme 2). Substances containing R^3 substituted triazole rings (**C**, Figure 2) including Me (**1s**), Ph (**1t**), NH_2 (**1u**) and thiol (**1v**) were also prepared (Scheme 2). In addition, **1s** and **1t** were obtained by treatment of **5e** with acetic acid and benzoyl chloride. Finally, **1u** and **1v** were generated by reaction of **5e** with CNBr and CS_2.[14]

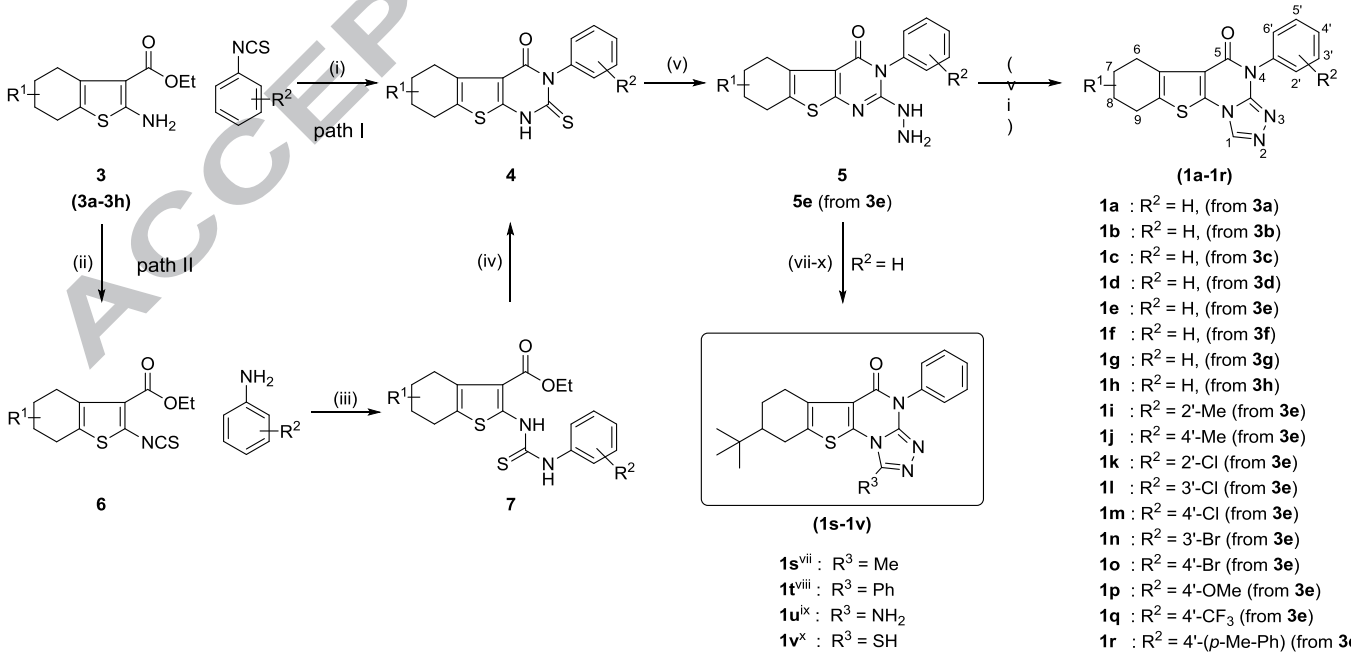


Scheme 1. Generation of intermediates **3** through using Gewald's Reaction

The inhibitory activities of **1a-v** were tested against three representative strains of enteroviruses (Cox B1, Cox B3, and PV3) (Table 1). The assays, involving measurements of the reduction of virus-induced cytotoxicity (CPE) of HeLa cells, employed the previously described method.[15] For this purpose, Pleconaril was utilized as a reference compound. In a SAR study, the effects of substituents on the cyclohexyl ring (**A**, Figure 2) on antiviral efficacy were evaluated (**1a-h,** Table 1). The data shows that only the substance bearing a *tert*-butyl group at C-8 (**1e**) displayed marginal effectiveness toward Cox B1, Cox B3 and PV3 (EC_{50} = 8.85, 8.65 and 1.95 μM, respectively). However, incorporation of other substituents such as methyl (**1c**), propyl (**1d**) or phenyl (**1f**) at this position did not have any effect on activity. Unsubstituted and differently substituted compounds (**1a**, **1b**, **1g**, and **1h**) did not display any activity. Moreover, the derivative **1e** had a very low toxicity (CC_{50} >100). Therefore, the *tert*-butyl group was incorporated at the C-8 position of the tetrahydrobenzene ring in compounds selected for the further SAR study.

The effects of the substituents on the N-4 phenyl ring (**B**, Figure 2) were determined next (Table 1 **1i-r)**. The 2'-Me (**1i**) and 4'-Me (**1j**) derivatives only showed moderate activity for inhibition of Cox B1 and Cox B3. However, when R^2 is more sterically bulky than methyl (Me), the activities decrease (**1k-r**). However, compared to those against Cox B1 and Cox B3, the inhibitory activity against PV3 is less sensitive to the size of substituents. In general, all derivatives except **1q** and **1r** possess moderate activity against PV3. Thus, when R^2 is H (**1e**) or a small group like Me (**1i** and **1j**), Cl (**1k**, **1l**, and **1m**), or OMe (**1p**), the effective activity against PV3 is moderate (EC_{50} <10 μM). However, when R^2 is Br (**1n** and **1o**), CF_3 (**1q**), or p-Me-Ph (**1r**), the PV3 activity is very low or absent (EC_{50} >10 μM). Even though the antiviral activities against all three viruses are dependent on phenyl ring at N-4substituents, the unsubstituted compound (**1e**) shows the highest activity and the highest SI values; >11.2 for Cox B1, >11.5 for Cox B3, and >51.2 for PV3.

To probe the effect of R^3 substituents on the triazole ring (**C**, Figure 2), Me (**1s**), Ph (**1t**), NH_2 (**1u**), and SH (**1v**) were introduced. However, none of these substances display enhanced activities when compared to those of the unsubstituted compound **1e** (Table 1).



(i) K_2CO_3, DMF, reflux, 8-24 h ; (ii) $CSCl_2$, TEA, THF, 0 °C, 1 h; (iii) THF, r.t., 2 h; (iv) 2% NaOH in Ethanol, reflux, 2 h; (v) Hydrazine hydrate, pyridine, reflux, 24 h; (vi) Formic acid or triethyl orthoformate, reflux, 24 h; ; (vii) AcOH, reflux, 2h; (viii) PhCOCl, reflux, 5 h; (ix) CNBr, EtOH, reflux, 5 h; (x) CS_2, 3% KOH in EtOH, reflux, 36 h.

Scheme 2. Synthetic Route to the analogs of **1**

In addition to studies with the coxsackievirus and poliovirus, we also explored the anti-rhinovirus activity of the substances against HRV14, HRV21 and HRV71 in H1 HeLa cells (Table 1) (*vide supra*). In this effort, Pleconaril was used as a reference compound. The hit compound, **1e**, has good activities against all three HRVs, but it displays a strong toxicity in H1 HeLa cells (CC_{50} 9.6 μM). Among all members of this series, the 4'-OMe derivative **1p** was found to be one of the most active against the three selected HRVs, and to display the highest selectivity. The SI values for **1p** are 11.5 for HRV14, 8.4 for HRV21, and 12.1 for HRV71.

Even though the compounds investigated in this effort are at an early stage of development, we assessed their microsomal stabilities. In vitro metabolic stabilities of **1e** were determined using human and rat liver microsomal phase I. The amount of **1e** (calculated using quantitative mass spectrometry),[16] expressed as a percentage, is given in Table 2. Buspirone was used for reference for this study. The results show that **1e** is moderately stable under these metabolic conditions.

Table 2. *In vitro* microsomal metabolic stabilities of **1e** in human and rats**.**

Compound	Human(%)[a]	Rat(%)[a]
1e	63.2	51.5
Buspirone	3.52	0.119

[a] by Mass Spectrometry

The observations made in the study described above show that **1e** and **1p** display properties that make them suitable lead compounds in further exploratory efforts aimed at developing broad spectrum anti-enteroviruse drugs. For this purpose, studies focusing on optimization of the structure of these substances to install drug-like activities and the mechanism of anti-virus action are underway in our laboratory.

Table 1. Antiviral activities of 4-aryl-6,7,8,9-tetrahydrobenzo[4,5]thieno[3,2-*e*]triazolo[4,3-*a*]pyrimidin-5(4*H*)-ones **1a-1v.**

Compound	R^1	R^2	R^3	CC_{50} (μM)[a]	EC_{50} (μM)[b]			CC_{50} (μM)[c]	EC_{50} (μM)[d]		
					Cox B1[e]	Cox B3[f]	PV3[g]		HRV14[h]	HRV21[i]	HRV71[j]
1a	H	H	H	>100	>100	>100	>100	>100	>100	>100	>100
1b	6-methyl	H	H	>100	>100	>100	>100	>100	>100	>100	>100
1c	8-methyl	H	H	78.99	>78.99	>78.99	>78.99	69	16.4	>69	>69
1d	8-propyl	H	H	41.01	>41.01	>41.01	19.97	31.7	18.1	>31.7	14
1e	8-(*tert*-butyl)	H	H	>100	8.85	8.65	1.95	9.6	1.7	2.5	1.6
1f	8-phenyl	H	H	27.48	>27.48	>27.48	>27.48	27.6	19.4	>27.6	>27.6
1g	8,8-dimethyl	H	H	54.80	>54.80	>54.80	>54.80	76.5	>76.5	>76.5	>76.5
1h	7,7,9,9-tetramethyl	H	H	9.09	>9.09	>9.09	>9.09	9.6	>9.6	>9.6	>9.6
1i	8-(*tert*-butyl)	2'-Me	H	39.11	9.83	9.22	3.44	35.4	8.8	>35.4	10.1
1j	8-(*tert*-butyl)	4'-Me	H	32.75	14.32	12.88	1.94	11.1	1.8	2.4	1.8
1k	8-(*tert*-butyl)	2'-Cl	H	26.28	19.51	16.24	1.84	20.7	2.5	14.7	3.9
1l	8-(*tert*-butyl)	3'-Cl	H	38.28	17.81	>38.28	9.63	15	2.1	>15	3.8
1m	8-(*tert*-butyl)	4'-Cl	H	36.45	>36.45	>36.45	8.35	18.8	3.7	>18.8	3.8
1n	8-(*tert*-butyl)	3'-Br	H	32.93	15.62	>32.93	13.20	26.3	3.5	20	15.9
1o	8-(*tert*-butyl)	4'-Br	H	30.47	>30.47	>30.47	12.57	11	2	>11	2.3
1p	8-(*tert*-butyl)	4'-OMe	H	39.13	>39.13	>39.13	3.63	21.9	1.9	2.6	1.8
1q	8-(*tert*-butyl)	4'-CF_3	H	35.54	>35.54	>35.54	>35.54	31.4	>31.4	>31.4	>31.4
1r	8-(*tert*-butyl)	4'-(*p*-Me-Ph)	H	>100	55.35	46.84	46.97	16.9	>16.9	>16.9	>16.9
1s	8-(*tert*-butyl)	H	Me	45.09	>45.09	>45.09	>45.09	35.9	4.3	>35.9	4.6
1t	8-(*tert*-butyl)	H	Ph	31.89	>31.89	>31.89	>31.89	8.7	>8.7	>8.7	>8.7
1u	8-(*tert*-butyl)	H	NH_2	45.85	>45.85	>45.85	>45.85	35.4	8.2	>35.4	12.8
1v	8-(*tert*-butyl)	H	SH	45.09	>45.09	>45.09	>45.09	40.1	10.1	19.4	9.1
Pleconaril	-	-	-	42.8	0.13	>42.8	0.51	53.5	0.31	0.083	0.013

[a] Concentration of chemicals required for reducing 50% of viability of normal HeLa cells. [b] Concentration of chemnicals required for improving 50% of viability of coxsackeivirus and poliovirus infected HeLa cells. [c] Concentration of chemicals required for reducing 50% of viability of H1 HeLa cells. [d] Concentration of chemicals required for improving 50% of viability of rhinovirus virus-infected H1 HeLa cells. [e] Coxsackievirus B1. [f] Coxsackievirus B3. [g] Poliovirus 3. [h] Rhinovirus 14. [i] Rhinovirus 21. [j] Rhinovirus 71

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16. See supplementary information.

Graphical Abstract

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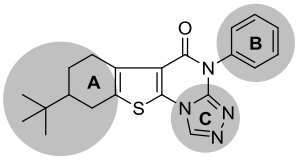

Compound **1e**

EC_{50} = 8.85 μM for CoxB1
8.65 μM for Cox B3
1.95 μM for PV3
1.7 μM for HRV14
2.5 μM for HRV21
1.6 μM for HRV71

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