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Article

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In vitro Antiviral Activity of New Oxazoline Derivatives as Potent Poliovirus Inhibitors

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KEYWORDS. Poliomyelitis, Poliovirus, Antivirals, Oxazoline, Vaccine-derived polioviruses.

ABSTRACT: The final stages of polio eradication are proving more difficult than the early phases and the development of effective drugs and treatments is considered a priority, thus the research is ongoing. A screening of our in-house chemical library against poliovirus Sabin strains led to identification of **5** and **6** as hits active at submicromolar concentration. Derivatives of these compounds were synthesized as a preliminary structure-activity relationship study. Among them, **7** and **11** were highly active against poliovirus Sabin 1, 2 and 3. **11** was also very potent against a large panel of wild and vaccine-derived polioviruses. Time of addition

experiments suggest that **5** and **7** could be active at an early stage of viral replication, while **11** was active at same concentration at all stages of viral replication. A ligand-based approach was applied to find the common structural features shared by the new compounds and already known poliovirus inhibitors.

Introduction

Poliovirus (PV), the causative agent of poliomyelitis, is a member of the genus *Enterovirus* within the *Picornaviridae* family, a large family of small non-enveloped positive-stranded RNA viruses.[1] Although eliminated in all but two countries, it still represents a potential risk for humans until polio eradication is accomplished at global level.

Outbreaks of poliomyelitis caused by vaccine-derived polioviruses (VDPVs) that reverted to wild phenotype, unexpectedly occurred in 2000 in Hispaniola[2] followed by subsequent detections of independent emergences in other countries. Immunodeficient long-term PV excretors constitute a significant reservoir for PV post-eradication and are a major concern for the WHO. The occurrence of immunodeficiency-related VDPV (iVDPV) must be considered a potential source for outbreaks and for reemergence of polio after eradication.

At present, the only means to prevent poliomyelitis are the two type of vaccines: the Sabin live attenuated oral polio vaccines (OPVs) and the Salk inactivated polio vaccine (IPV) given to young children routinely or during supplemental immunization activities (SIAs).

Antiviral drugs would offer a promising complementary or alternative approach to the use of vaccine to control PV infections, especially for chronic persistent infections in immunodeficient individuals during the final stages of eradication and in the post-eradication era. For this reason, the WHO has supported the development of antiviral drugs against PV.

However, no antiviral agents are currently available in the market to treat infections by PV and other enteroviruses, and only the protease inhibitor AG-7404 (**1**)[3] and the uncoating inhibitor pocapavir (V-073, **2**) are in clinical trials (Figure 1).[4] Among them, the latter is the most advanced and was recently demonstrated to be active in an oral vaccine human challenge model. Additional candidates acting as uncoating inhibitors such as H1PVAT (**3**) and pirodavir (**4**) continue to be profiled and evaluated with the aim to have different antivirals

available for a combined use and to overcome the problem of possible emergence of resistant variants.[5]

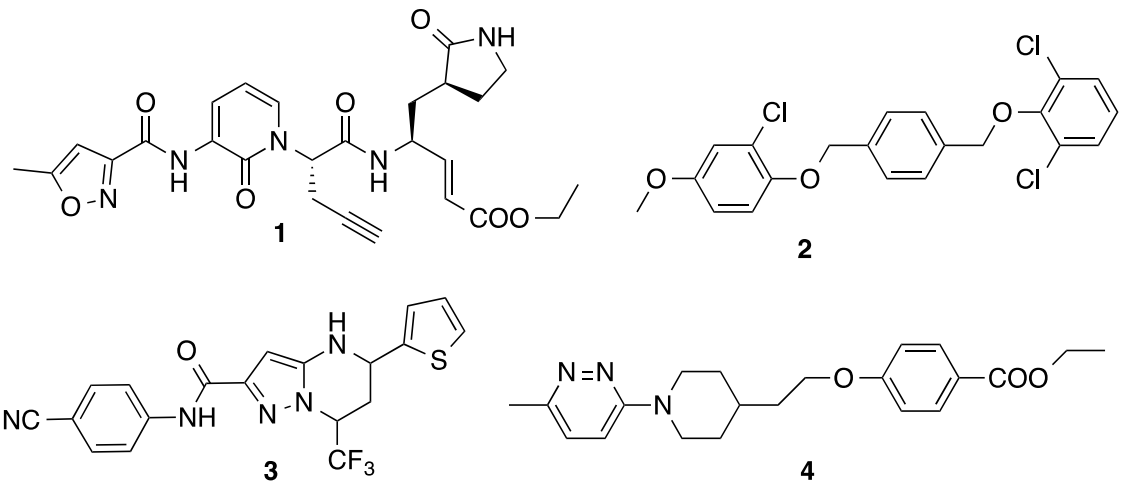


Figure 1. Structures of the anti-PV agents in clinical trials or under evaluation: the protease inhibitor **1** and the uncoating inhibitors **2**-**4**.

Recently, we reported some 3(2*H*)-isoflavenes as weak anti-PV agents that prevented viral entry with a mechanism similar to that of **2**, by interfering with receptor binding or by preventing conformational changes needed for viral capsid uncoating.[6,7] Mutations that interfered with the antiviral activity of these compounds appeared during in vitro replication of type 2 polio vaccine strains in the presence of these compounds. Some mutations acted directly by

affecting binding while others exerted their effect indirectly by altering viral capsid stability.[6,8] However, our attempts to improve the activity of these compounds or their drug-like properties were unsuccessful.

Thus, as a further attempt to discover new anti-polio agents characterized by scaffolds different from both the drugs in clinical trials and from our isoflavenes, we decided to perform a screening of our in-house library of compounds including various heterocyclic derivatives and related intermediates, previously designed as potential antitumor or antimicrobial drugs. The library includes about 1000 molecular entities but a selection has been performed picking up compounds characterized by different chemical scaffolds and excluding isoflavene derivatives. The resulting 40 compounds were tested at fixed concentration (10 μM) against Sabin 1, Sabin 2, and Sabin 3 polio strains. As **5** showed a significant activity, we decided to test also **6**, an analogue included in the original library. Both compounds were further assayed at different concentrations and showed activity at concentration ranging from about 0.2 to 91 μM (Table 1).

Results and Discussion

Compounds **5** and **6** are 5-(4-(4,5-dihydrooxazol-2-yl)phenoxy)-1-(5-methylthiophen-2-yl)pentan-1-one derivatives (Table 1) and can be structurally described as two aryl portions (one thienyl and one phenyl) separated by an oxyalkylcarbonyl linker. Thus, to some extent, these scaffolds could resemble those of compounds **2**-**4**.

To increase the potency of these hits we decided to perform some preliminary structure-activity relationship (SAR) studies. First, the aromatic portions (5-methylthiophen-2-yl and (4-(4,5-dihydrooxazol-2-yl)phenyl moieties**)** of the **5** and **6** were kept unchanged while the spacer X was modified (Figure 2). In particular, we designed and synthesized new derivatives **7-13** in which the oxygen atom of **5** and **6** was replaced by sulfur atom, sulfinyl or sulfonyl functions, or methylene, ethylene, and ethine groups. We took care to retain the distance between the phenyl ring and carbonyl group to possibly increase the lipophilicity of the new compounds. Finally, we designed two further molecules characterized by a phenyl spacer, partially mimicking the one of **2** (Figure 1

and Figure 2), resulting in compounds **14** and **15** (oxygen and sulfur derivatives, respectively) as conformationally restrained derivatives of **5** and **7**.

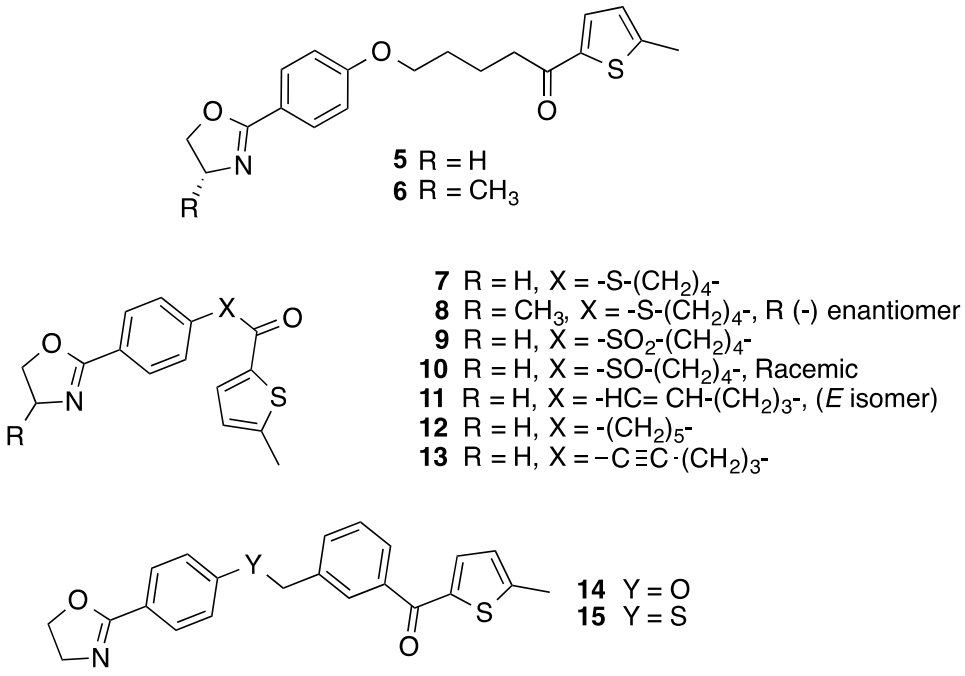
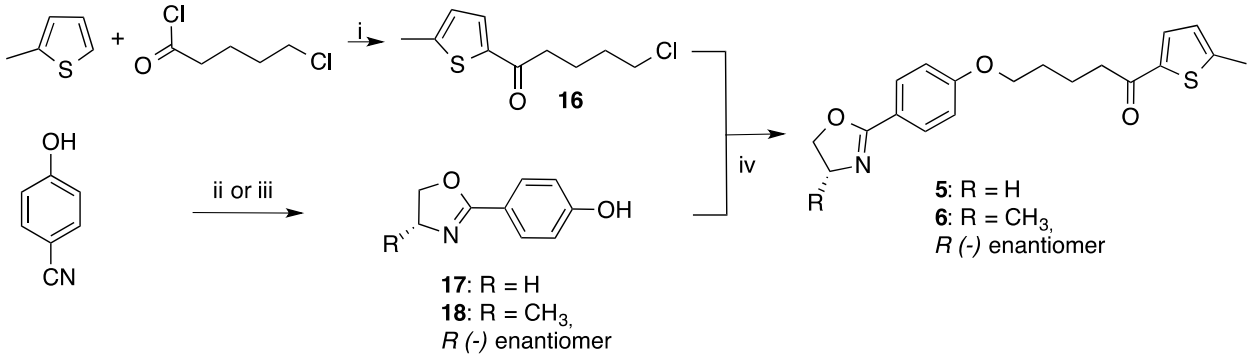


Figure 2. Structures of hit compounds **5** and **6** and the newly designed oxazolynes **7**-**15**.

Chemistry. The alkyl oxazoline derivatives **5** and **6** were synthesized as reported in Scheme 1. From 2-methylthiophene and 5-chlorovaleroyl chloride in the presence of $AlCl_3$, we synthesized the desired compound **16** that was

reacted with derivatives **17** or **18** in *N,N*-dimethylformamide (DMF) in the presence of catalytic NaI and K_2CO_3 as a base, obtaining, after chromatographic purification, **5** and **6**, respectively. Oxazolines **17** and **18** were synthesized from commercially available 4-hydroxybenzonitrile that reacted with the proper ethanolamine in anhydrous chlorobenzene in the presence of anhydrous $ZnCl_2$.

Scheme 1. Synthetic pathway for **5** and **6** derivatives[a]



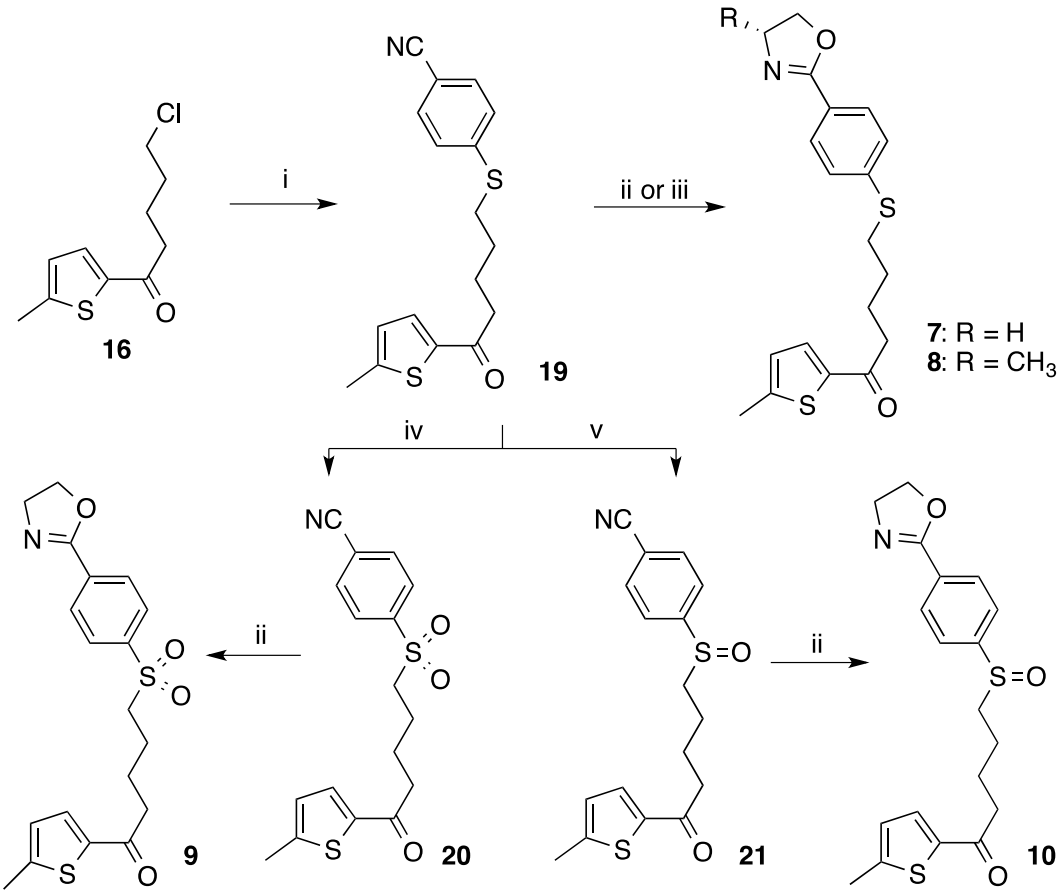
[a]Reagents and conditions: (i) $AlCl_3$, dry CH_2Cl_2, N_2, room temp, 2 h; (ii) ethanolamine, dry $ZnCl_2$, dry chlorobenzene, 160 °C, 24 h; (iii) (R)-(-)-2-amino-1-propanol, dry $ZnCl_2$, dry chlorobenzene, 160 °C, 24 h; (iv) K_2CO_3, NaI, DMF, room temp, 72 h.

The newly designed compounds **7-10** were synthesized following a multistep approach as described in Scheme 2. Compound **16** was reacted with 4-mercaptobenzonitrile in DMF in the presence of K_2CO_3 as a base to obtain the desired thioether **19.** The last was reacted in the presence of opportune ethanolamine and Si-propylsulfonic acid to obtain, after chromatographic purification, the desired oxazolines **7** and **8**. At the same time, **19** was oxidized in two different ways (with *m*-chloroperbenzoic acid, *m*CPBA, or $H_2O_2/POCl_3$) to obtain the respective sulfonyl nitrile **20** and sulfinyl nitrile **21** that were successively reacted with ethanolamine and Si-propylsulfonic acid to obtain the oxazolines **9** and **10**, respectively.

The synthesis of **11** was performed following the procedure described in Scheme 3. Compound **22** was synthesized though Heck reaction form 4-bromo-benzonitrile and 5-hexenoic acid, then reacted with oxalyl chloride in dichloromethane (DCM) in the presence of catalytic amount of DMF to obtain the acyl chloride **23**, that was rapidly reacted with 2-methylthiophene to obtain

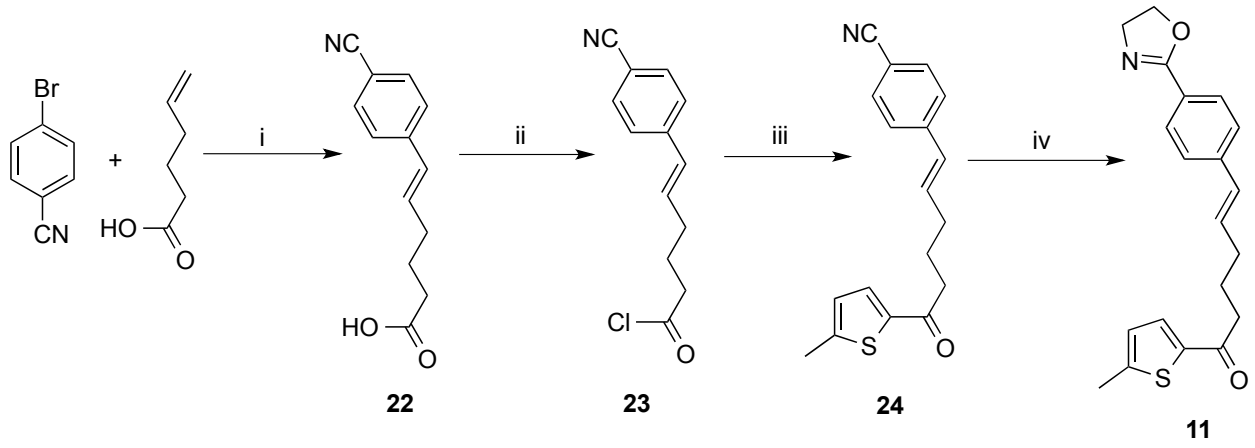
the intermediate **24**. The last compound was reacted with ethanolamine and Si-propylsulfonic acid to obtain **11**.

Scheme 2. Synthesis of the alkyl oxazolines **7-10**[a]



[a]Reagents and conditions: (i) 4-mercaptobenzonitrile, K_2CO_3, DMF, 80 °C, 6 h; (ii) ethanolamine, Si-propylsulfonic acid, 120 °C, 4 h; (iii) (R)-(-)-2-amino-1-propanol, Si-propylsulfonic acid, 120 °C, 4 h; (iv) *m*CPBA, $CHCl_3$, room temp, 30 min; (v) H_2O_2, $POCl_3$, $CHCl_3$/EtOH, room temp, 1 h.

Scheme 3. Synthesis of the alkyl oxazoline **11**[a]

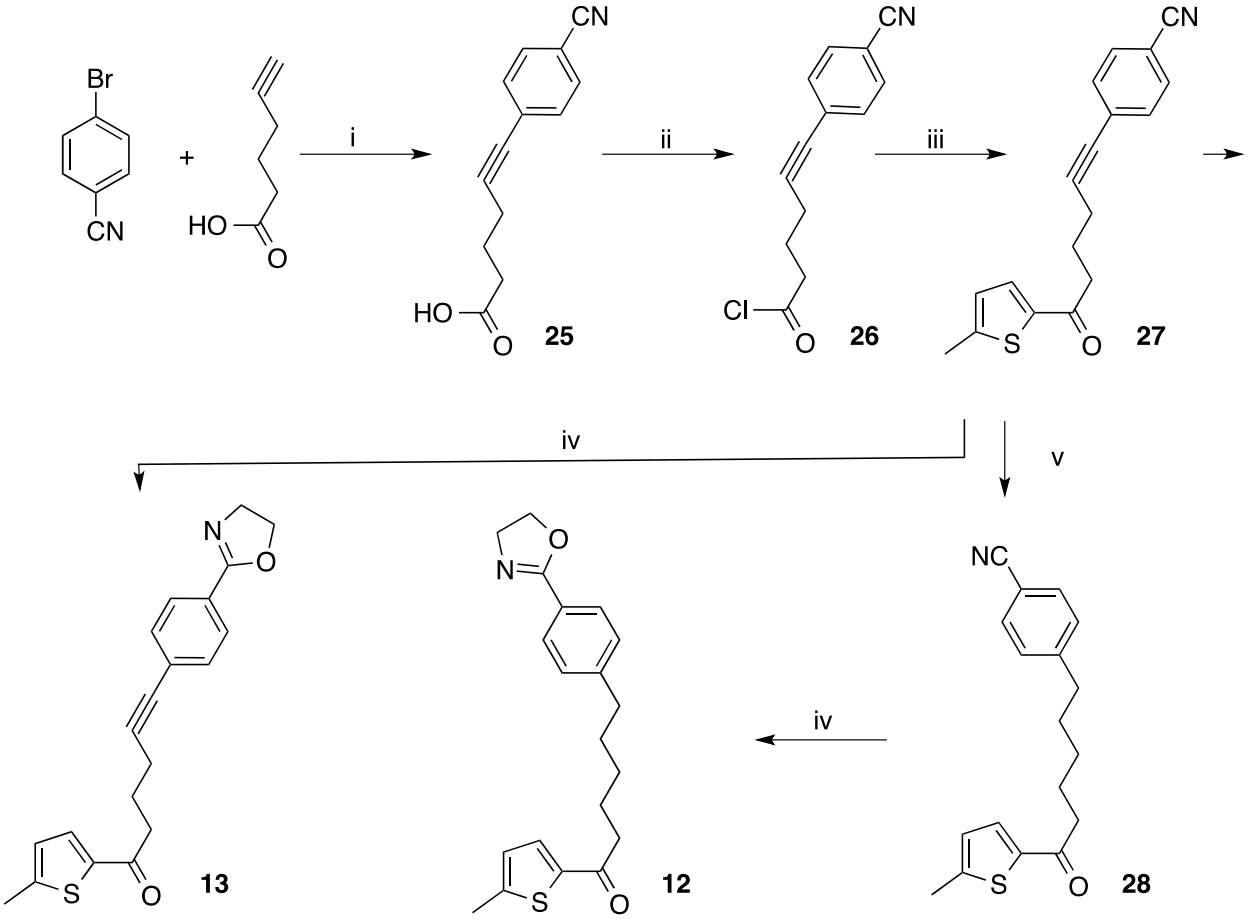


[a]Reagents and conditions: (i) TBACl, $Pd(OAc)_2$, PPh_3, KOAc, dry DMF, room temp, 7 d; (ii) oxalyl chloride, dry CH_2Cl_2, dry DMF (cat.), N_2, room temp, 2 h; (iii) 2-methylthiophene, $AlCl_3$, dry CH_2Cl_2, N_2, room temp, 2 h; ethanolamine, Si-propylsulfonic acid, 120 °C, 4 h.

The synthesis of **12** and **13** was performed following the chemical pathway reported in Scheme 4. Alkyne **25** was synthesized though Heck reaction from 4-bromo-benzonitrile and 5-hexinoic acid, then reacted with oxalyl chloride in DCM in the presence of a catalytic amount of DMF to obtain the acyl chloride **26**, that was rapidly reacted with 2-methylthiophene to obtain the intermediate **27**. The last compound was reacted with ethanolamine and Si-propylsulfonic

acid to obtain **13**. The nitrile **27** was reduced with Pd/C under a hydrogen atmosphere to obtain the saturated compound **28** that was further reacted with ethanolamine and Si-propylsulfonic acid to obtain **12** after column chromatography.

Scheme 4. Synthesis of the alkyl oxazolines **12** and **13** [a]

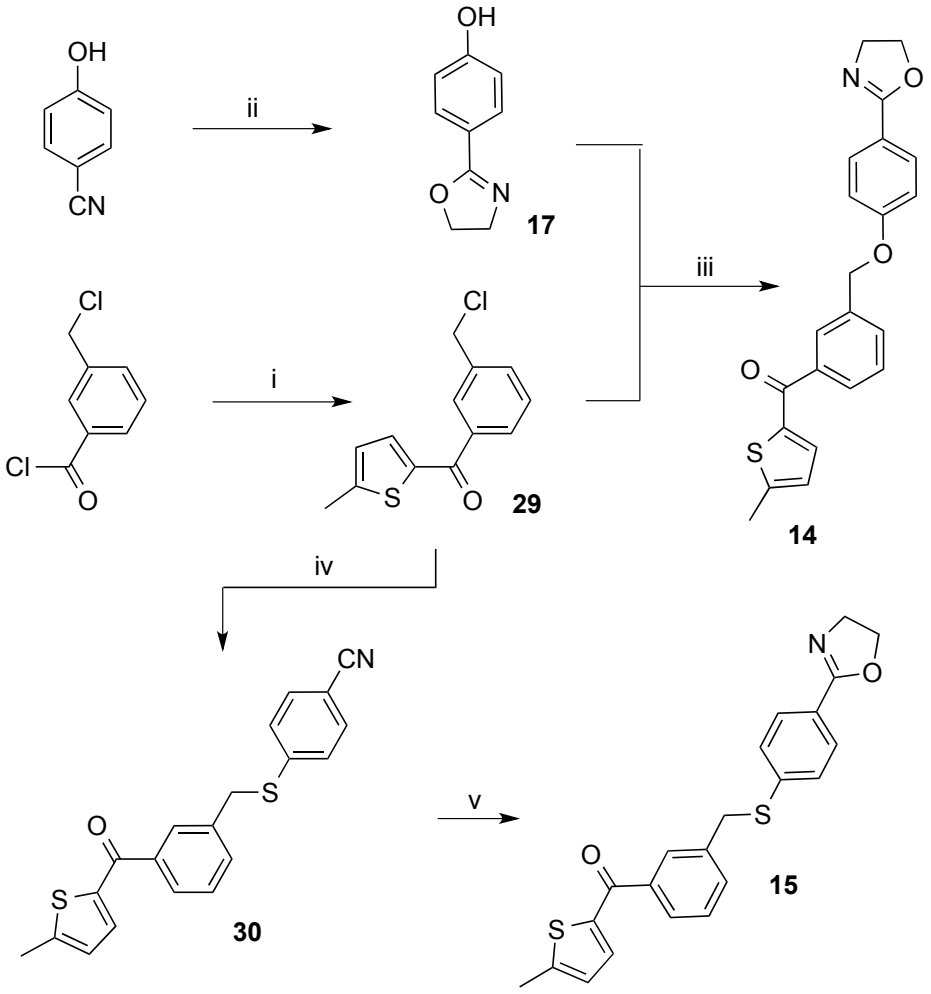


[a]Reagents and conditions: (i) TBACl, $Pd(OAc)_2$, PPh_3, KOAc, dry DMF, room temp, 7 d; (ii) oxalyl chloride, dry CH_2Cl_2, dry DMF (cat.), N_2, room temp, 2 h;

(iii) 2-methylthiophene, $AlCl_3$, dry CH_2Cl_2, N_2, room temp, 2 h; (iv) ethanolamine, Si-propylsulfonic acid, 120 °C, 4 h; (v) Pd/C cat., H_2, EtOAc, room temp, 30 psi, 2 h.

The synthesis of **14** and **15** was performed as described in Scheme 5. As the first step, 3-chloromethylbenzoyl chloride and 2-methylthiophene were reacted in DCM in the presence of $AlCl_3$ to obtain the ketone **29** that underwent nucleophilic aliphatic substitution on chloromethyl group by compound **17** in the presence of K_2CO_3 to obtain the oxazoline **14**. The intermediate oxazoline **17** was obtained by reaction of 4-cyanophenol with ethanolamine in the presence of dry $ZnCl_2$. Intermediate **29** was reacted with 4-mercaptobenzonitrile in the presence of K_2CO_3 as a base to obtain the nitrile **30** that was in turn reacted with ethanolamine and Si-propylsulfonic acid to obtain **15**.

Scheme 5. Synthesis of the alkyl oxazolines **14** and **15**[a]



[a]Reagents and conditions: (i) 2-methylthiophene, $AlCl_3$, dry CH_2Cl_2, N_2, room temp, 2 h; (ii) ethanolamine, dry $ZnCl_2$, dry chlorobenzene, 160 °C, 24 h; (iii) K_2CO_3, DMF, 80 °C, 4 h; (iv) 4-mercaptobenzonitrile, K_2CO_3, dry DMF, 80 °C, 4 h; (v) ethanolamine, Si-propylsulfonic acid, 120 °C, 4 h.

Evaluation of Biological Activities. All the newly synthesized derivatives **7-15** were assayed in vitro against the three polio Sabin strains, in parallel with **5** and **6** (Table 1). Compounds **9**, **10**, **13**, and **15** were inactive against all three Sabin strains, while derivatives **6**, **12**, and **14** were inactive against Sabin 1 but active at micromolar concentration against Sabin 2 and Sabin 3. Interestingly, compounds **5**, **7**, **8**, and **11** were active against all Sabin strains and at submicromolar concentration, with the exception of **5** against Sabin 1.

Table 1. Antiviral activities of hits **5** and **6**, newly synthesized compounds **7-15** and reference drugs **1-4** against three polio Sabin serotypes.

Compd	Sabin 1 EC_{50}[a] (μM)	Sabin 2 EC_{50} (μM)	Sabin 3 EC_{50} (μM)
5	90.800 ± 1.400	0.187 ± 0.059	0.367 ± 0.027
6	>100	0.417 ± 0.242	1.323 ± 0.205
7	0.118 ± 0.012	0.137 ± 0.010	0.330 ± 0.008
8	0.169 ± 0.018	0.302 ± 0.014	0.530 ± 0.043
9	>10	>10	>10

10	>10	>10	>10
11	0.338 ± 0.106	0.027 ± 0.002	0.053 ± 0.016
12	>10	9.273 ± 0.101	9.070 ± 0.130
13	>10	9.087 ± 0.834	9.160 ± 0.036
14	>10	>10	>10
15	>10	>10	>10
1[b]	0.173 ± 0.137	0.305 ± 0.148	0.137 ± 0.007
2[c]	0.017 ± 0.000	0.016 ± 0.012	0.020 ± 0.007
3[d]	0.010 ± 0.001[e]	0.026 ± 0.014	0.218 ± 0.162
4[e]	0.0045	0.005	0.008

[a] Effective concentration 50% (μM). [b] Reference 3. [c] Reference 4. [d] Reference 9.[e] Reference 10[f].

The most interesting hits found in the random screening, (**5**, **7** and **11**) that showed the best activity in the assays against Sabin strains, have been further assayed against a large panel of wild, iVDPV and circulating VDPV (cVDPV) (Table 2). The prototype **5** was inactive against 7 isolates, active at micromolar concentrations (2.10-67.47 μM) against 15 isolates and at submicromolar

concentrations (0.02-0.99 μM) against 22 isolates. In particular, it was very potent against 9 isolates showing nanomolar activity (Table 2).

Table 2. *In vitro* antiviral activity of **5, 7 and 11** in HeLa cells against PV Sabin, wild and VDPV polio strains.

Entry	EV Code No.	Country-Year	Serotype	Origin	**5** EC_{50}^{a} ± SD	**7** EC_{50}^{a} ± SD	**11** EC_{50}^{a} ± SD
1	10688	Taiwan-2003	PV1	iVDPV	0.04 ± 0.00	2.80 ± 1.06	0.040 ± 0.01
2	10689	Taiwan-2003	PV1	iVDPV	0.06 ± 0.00	2.03 ± 0.56	0.034 ± 0.01
3	10690	Phillipines-2004	PV1	cVDPV	48.7 ± 15.2	2.00 ± 0.36	1.26 ± 0.19
4	10691	Phillipines-2004	PV1	cVDPV	67.5 ± 19.5	0.56 ± 0.11	0.98 ± 0.14
5	10692	Phillipines-2004	PV1	cVDPV	>100 ± 0.00	>10 ± 0.00	0.78 ± 0.12
6	10693	Phillipines-2004	PV1	cVDPV	24.2 ± 7.72	>10 ± 0.00	0.98 ± 0.66
7	10694	USA-MN-2005	PV1	iVDPV	7.22 ± 1.15	6.23 ± 1.76	2.30 ± 1.09
8	10695	USA-MN-2005	PV1	iVDPV	8.41 ± 1.58	7.43 ± 0.61	0.58 ± 0.31
9	10222	USA-MD-1981	PV1	iVDPV	7.89 ± 1.79	4.90 ± 1.31	1.13 ± 0.32
10	10223	USA-IN-1982	PV1	iVDPV	0.07 ± 0.01	3.73 ± 1.31	0.02 ± 0.01
11	10224	USA-NY-1987	PV1	iVDPV	>100 ± 0.00	>10 ± 0.00	1.11 ± 0.16

12	10225	USA-NC-1990	PV1	iVDPV	35.7 ± 3.97	>10 ± 0.00	0.81 ± 0.17
13	10235	Dominican Republic-2000	PV1	cVDPV	4.61 ± 2.88	8.47 ± 0.65	0.40 ± 0.16
14	10236	Dominican Republic-2000	PV1	cVDPV	2.72 ± 1.58	0.72 ± 0.16	0.72 ± 0.12
15	10237	Haiti-2000	PV1	cVDPV	5.75 ± 5.66	6.47 ± 1.78	0.28 ± 0.05
16	10238	Dominican Republic-2000	PV1	cVDPV	2.10 ± 1.50	7.47 ± 0.67	0.19 ± 0.05
17	10239	Haiti-2001	PV1	cVDPV	0.94 ± 0.21	5.63 ± 1.50	0.22 ± 0.07
18	10240	Haiti-2001	PV1	cVDPV	3.62 ± 1.35	8.47 ± 1.25	0.27 ± 0.04
19	10241	Haiti-2001	PV1	cVDPV	3.17 ± 3.04	8.07 ± 0.35	0.37 ± 0.04
20	10242	Haiti-2001	PV1	cVDPV	37.9 ± 6.68	8.20 ± 1.40	0.02 ± 0.01
21	10219	Madagascar-2002	PV2	cVDPV	0.03 ± 0.01	1.20 ± 0.20	0.03 ± 0.01
22	10220	Madagascar-2002	PV2	cVDPV	0.04 ± 0.02	1.00 ± 0.61	0.02 ± 0.01
23	10221	USA-CA-1999	PV2	iVDPV	0.02 ± 0.02	2.37 ± 1.25	0.02 ± 0.01
24	10226	USA-PA-1991	PV2	iVDPV	>100 ± 0.00	0.13 ± 0.03	0.02 ± 0.01
25	10227	USA-PA-1991	PV2	iVDPV	0.03 ± 0.01	4.33 ± 2.00	0.04 ± 0.01
26	10228	USA-NY-1992	PV2	iVDPV	10.1 ± 1.49	9.10 ± 0.25	0.50 ± 0.17
27	10229	Egypt-1993	PV2	cVDPV	0.55 ± 0.25	2.77 ± 0.40	0.51 ± 0.18
28	10230	Egypt-1993	PV2	cVDPV	0.10 ± 0.00	5.33 ± 1.21	0.03 ± 0.00
29	10231	Egypt-1998	PV2	cVDPV	0.05 ± 0.01	5.33 ± 1.00	0.03 ± 0.00

30	10232	Egypt-1999	PV2	cVDPV	0.54 ± 0.29	6.33 ± 0.86	0.023 ± 0.01
31	10233	Egypt-1999	PV2	cVDPV	0.45 ± 0.36	8.47± 0.93	0.19 ± 0.17
32	10234	Egypt-1999	PV2	cVDPV	0.03 ± 0.01	2.47 ± 0.49	0.02 ± 0.02
33	10243	Nigeria-2002	PV2	c	0.04 ± 0.00	1.77 ± 0.67	0.03 ± 0.01
34	10804	Egypt-2007	PV3	iVDPV	>100 ± 0.00	0.44 ± 0.25	0.02 ± 0.00
35	10805	Iran-2007	PV3	iVDPV	0.27 ± 0.10	1.50 ± 0.44	0.03 ± 0.00
36	Brunhilde	USA-1939	PV1	Wild	0.99 ± 0.09	0.47 ± 0.21	0.07 ± 0.01
37	Mahoney	USA-1942	PV1	Wild	>100 ± 0.00	>10 ± 0.00	0.07 ± 0.02
38	Lansing	USA-1937	PV2	Wild	0.59 ± 0.07	2.27 ± 0.59	0.06 ± 0.01
39	MEF-1	Egypt-1942	PV2	Wild	>100 ± 0.00	1.26 ± 0.53	0.03 ± 0.00
40	P712	USA-1954	PV2	Wild	>100 ± 0.00	2.93 ± 3.01	0.24 ± 0.04
41	Saukett	USA-1962	PV3	Wild	0.35 ± 0.03	1.67 ± 0.02	0.04 ± 0.01
42	Leon	USA-1937	PV3	Wild	0.17 ± 0.12	1.11 ± 0.29	0.95 ± 0.12
43	Sabin 1	Vaccine strain	PV1	Vaccine	12.5 ± 0.78	0.70 ± 0.36	0.34 ± 0.11
44	Sabin 2	Vaccine strain	PV2	Vaccine	0.03 ± 0.00	0.16 ± 0.01	0.03 ± 0.01
45	Sabin 3	Vaccine strain	PV3	Vaccine	0.32 ± 0.04	0.33 ± 0.01	0.05 ± 0.02

[a] Effective concentration 50% (μM).

Compound **7** was inactive against 5 isolates, active at low micromolar concentrations (1.00-9.10 μM) against 32 isolates and at submicromolar concentrations (0.13-0.72 μM) against 8 isolates (Table 2).

More interestingly, **11** showed very potent activity on a wide number of strains. It was active at micromolar concentration (1.109-2.300 μM) on 3 strains and at submicromolar or nanomolar concentrations against the remaining 41 strains. In particular, it was active at nanomolar concentration against 20 strains (20-69 nM, Table 2).

Compounds **5**, **7**, and **11** were further tested in time of addition experiments against Sabin 2 to determine at what stage of the viral cycle the compounds acted (Figure 3). Interestingly, compounds **5** and **7** showed a decrease of activity after 1 h post-infection (PI). During the six hours needed for the complete PV life cycle the lost of activity is increased for **5** and stabilized for **7**. These results suggest that these compounds are active at an early stage of viral replication. Surprisingly, the derivative **11** acts at any time of viral replication, probably inhibiting or binding a target that is always present during the replication cycle.

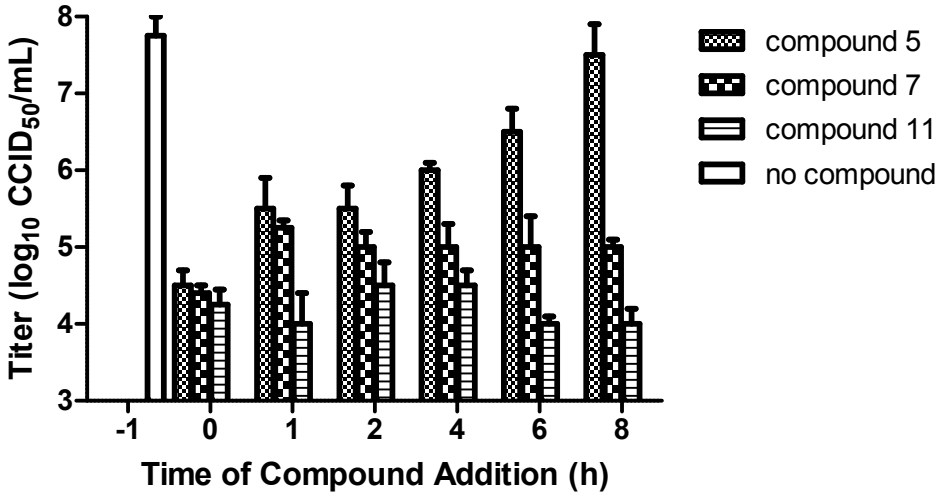


Figure 3. Effect of compounds in the time of addition assay. Time of addition experiments on **5**, **7**, and **11** performed on Sabin 2 virus. Titers indicate the presence of 1 μM **5**, **7** or **11** and 100 $CCID_{50}$ of poliovirus. Titration results were obtained using the virus titration assay.[11,12] Data represent mean values from three independent experiments.

Cytotoxicity assays on HeLa cells were also performed on the most active compounds (**5**, **7**, and **11**) to determine the selectivity of these prototypes. While **7** was slightly cytotoxic at a concentration of 20 μM, **5** and **11** were not

cytotoxic at concentrations up to 50 μM and they had selectivity indexes (SI) >1667 and >1852, respectively (Table 3).

Table 3. Cytotoxicity *in vitro* of most active compounds **5**, **7** and **11**.

Compd	Virus	EC_{50}[a] (μM)	SD	Cytotoxicity (CC_{50}[b], μM)	Date	Therapeutic ratio (CC_{50}/EC_{50})
5	Sabin 2	0.03	0.010	>50	07/09/13	>1667
7	Sabin 2	0.137	0.010	20	27/02/15	146.0
11	Sabin 2	0.027	0.002	>50	27/02/15	>1852

[a] Effective concentration 50% (μM). [b] Cytotoxic concentration 50% on Hela cells (μM).

Preliminary SAR considerations

Some preliminary SAR can be described considering the activity of **5-15** against PVs. Since the inhibitors designed in this work have been tested against a wide number of PV strains, only the activities against Sabin 2 serotype reported in Table 1 will be considered in this paragraph.

A classic bivalent bioisosteric replacement of the oxygen atom of the ether function of **5** with a methylene group led to **12** that showed a 50-fold decrease in antiviral activity (0.187 vs 9.273 μM, respectively). However, a partial rigidification of the alkyl spacer with the *trans*-styryl moiety found in **11** restored a submicromolar activity (0.027 μM) better than that of **5**. These results clearly suggested that the presence of atoms or chemical groups able to interact with the phenyl π system of these compounds caused an improvement in activity in comparison to the linear alkyl spacer.

The SAR of the new oxazolines could be explained on the basis of the different preferred conformations adopted by the phenoxy, styryl, and benzyl moieties found in **5**, **11**, and **12**, respectively. In fact, the Ph-O-CH_2- and styryl moieties are characterized by a planar arrangement consequent to a significant rehybridization and the formation of an extended π system between the phenyl ring and the oxygen lone pair of **5**, as well as between the phenyl ring and the olefinic π system in **11**. On the contrary, the sp^3 character of the corresponding alkyl carbon atom of **12** forced the overall alkyl chain to be out of the plane of

the phenyl ring and to adopt a shape significantly different from those found for **5** and **11**.

A similar trend of SAR considerations, with phenoxy and styryl compounds more active than the corresponding benzyl analogues, was previously reported for a series of phenyl oxazole inhibitors of blood platelet aggregation.[13,14]

Moreover, the additional bioisostere of **5** that contained a thiophenol moiety (**7**) also retained a submicromolar activity (0.137 μM), while its oxidation products **9** and **10** (the corresponding sulfone and sulfoxide derivatives, respectively), that differed widely in polarity and hybridization, were inactive.

Finally, the introduction of a methyl group on the oxazoline ring of **5** led to **6** that was less active if compared to the parent compound. A similar trend was found comparing the activity of the thio derivative **7** with that of its methylated counterparts **8**.

Molecular Modelling

A ligand-based approach has been applied to find the common structural features shared by the new compounds and already known PV inhibitors

(namely, **2**-**4**). As a result, a four-feature pharmacophoric model was obtained, comprised of one hydrogen bond acceptor group (HBA, red sphere, Figure 4), two aromatic rings (RA1 and RA2, orange rings), and a hydrophobic region (HY, green sphere). Derivative **5**, taken as the representative compound of the new inhibitors, was able to fulfill all the pharmacophoric features (Figure 4, a): the oxygen atom of the oxazoline ring represented the hydrogen bond acceptor, the phenyl and thiophene moieties matched the aromatic ring features, and the methyl group on the thiophene ring was accommodated within the hydrophobic region of the pharmacophore. As a comparison, **2** was also able to match the pharmacophore (Figure 4, b). In fact, the oxygen atom of its methoxy group was the hydrogen bond acceptor, the two terminal phenyl moieties fitted the aromatic ring features, and one of the two meta chlorine atoms of the *m*,*m*-disubstituted phenyl ring was located within the hydrophobic feature.

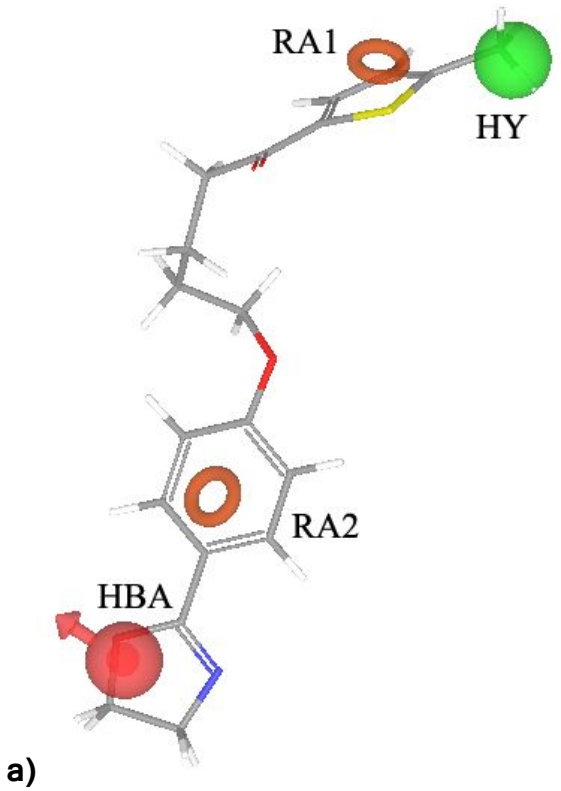
Although the three-dimensional coordinates of the VP1 capsid protein of Sabin 2 PV are not available, a rough comparison between the pharmacophoric model and the structural properties of a Sabin 1 PV homology model could be

attempted. In fact, the three-dimensional structure of Sabin 1 was recently generated by homology modeling, and its complexes with **2**-**4** have been built by docking calculations.[9] The alignment of the VP1 capsid protein of Sabin 1 (N0DLC0, 302 amino acids) with VP1 capsid protein of Sabin 2 (N0DQQ2, 301 amino acids) performed with Clustal Omega (UniProt Consortium)[15] resulted in very similar primary sequences, with 234 identical positions (77.5% identity) and 49 similar positions. Moreover, 20 (namely, Trp108, Ile110, Thr111, Tyr112, Lys113, Phe130, Met132, Phe136, Ile157, Tyr159, Pro181, Ser182, Ile183, Ile194, Val196, Val199, Tyr205, Asp236, Phe237, and Leu240) out of the 22 amino acids surrounding **4** (at a distance lower than 6 Å) in the X-ray three-dimensional structure with the VP1 capsid protein of type 1 Mahoney strain (1PO2 within the Protein Data Bank)[16] are conserved in comparison to the N0DLC0 and N0DQQ2 sequences. This suggested that the corresponding binding sites that accommodated the small molecule inhibitors could share very similar structures and interaction patterns with inhibitors.

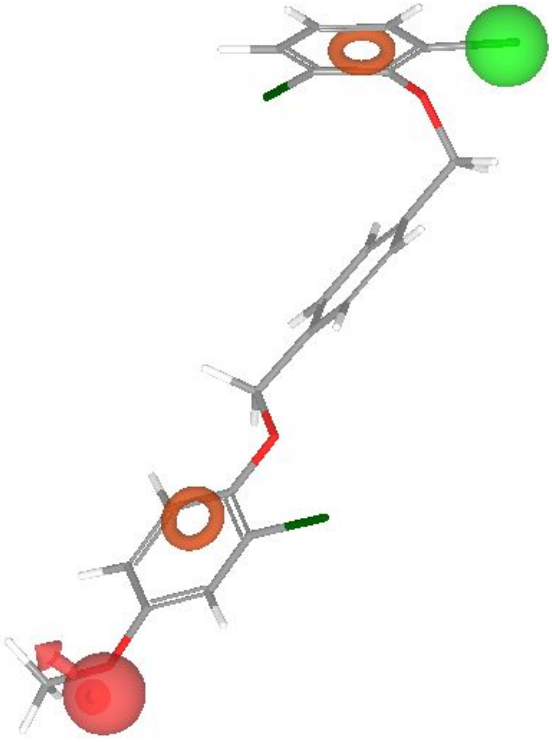
A visual inspection of the interaction pattern described for the three docked inhibitors identified several structural elements shared by the pharmacophoric model and the structure of VP1-inhibitor complexes. In particular, the hydrogen bond acceptor HBA of the pharmacophore was represented by the methoxy oxygen of **2** and by the carbonyl group of **4** that made a hydrogen bond interaction with VP1 Tyr112 (Figure 4, b and c).[9] Moreover, RA2 of the pharmacophore could be accommodated within an aromatic cage constituted by Phe130 (reported to make π interaction with the methoxyphenyl ring of **2**),[9] Phe237, Tyr205, and Tyr112, while RA1 within a hydrophobic region that included Tyr159 (described to make π interaction with the dichlorophenyl ring of **2**).[9] Finally, the same hydrophobic cavity, constituted by Leu240, Ile183, Ile194, and Ile157, also accommodated the hydrophobic feature of the pharmacophore that corresponded to the methyl group of both the new inhibitors and **4**, as well as to one of the chlorine substituents of **2**.

An additional comparison was performed between the pharmacophoric model and the binding mode of **2** in the complex with the type 2 Lansing strain

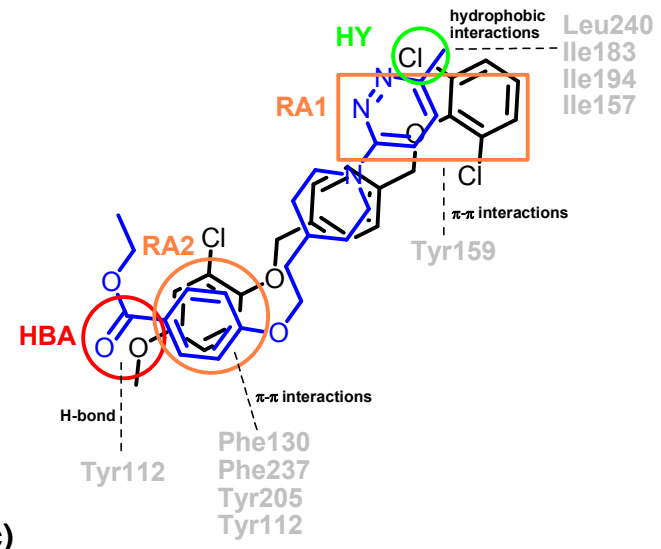
(1EAH within the Protein Data Bank).[17] The hydrophobic feature HY of the pharmacophore where one of the *m*-chlorines is accommodated (Figure 4, b) corresponded to a hydrophobic region constituted by Ile110, Leu240, Val259, Trp108, and Phe134 (Figure 4, d). Moreover, the dichlorophenyl moiety was embedded within an aromatic cage composed by Phe134, Phe136, and Tyr159, with the latter amino acid being able to give π-π (paralled displaced) interactions with the phenyl ring of the ligand. At the opposite molecular edge, an additional aromatic cage (Tyr112, Tyr205, and Phe237) accommodated the 2-chloro-4-methoxy phenyl ring. Its most profitable interactions were a T-tilted aromatic interaction with the side chain of Tyr205 and a displaced stacking with Phe237, both of them accounting for the presence of an aromatic ring feature within the pharmacophoric model. Finally, a weak electrostatic interaction that was mimic of the HBA pharmacophoric feature was found between the terminal OH group of Ser128 and the methoxy oxygen atom of **2**.


RA1
HY
RA2
HBA

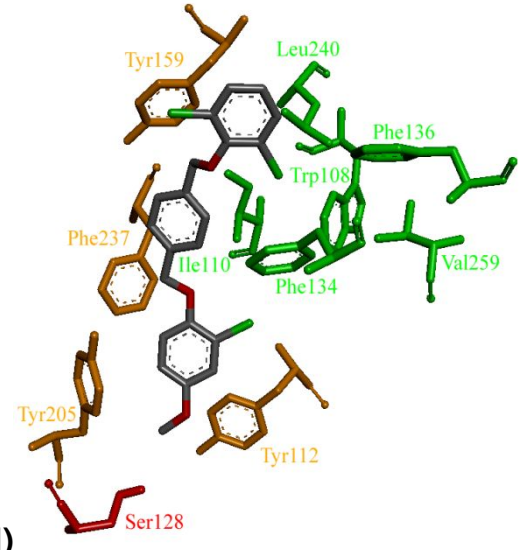
a)



b)


HY
hydrophobic interactions
Leu240
Ile183
Ile194
Ile157
RA1
Cl
N
O
Cl
π-π interactions
Tyr159
RA2
Cl
HBA
O
H-bond
Tyr112
π-π interactions
Phe130
Phe237
Tyr205
Tyr112

c)


Tyr159
Leu240
Phe136
Trp108
Phe237
Ile110
Phe134
Val259
Tyr205
Tyr112
Ser128

d)

Figure 4. Graphical representation of the superposition pattern of **5** (a) and **2** (b) into the four-feature pharmacophoric model. Features are color coded: the red sphere is the hydrogen bond group (HBA), each of the two orange circles accommodates an aromatic ring (RA1 and RA2), and the green sphere represents a hydrophobic region of space (HY); c) An overlay of the structure of **2** (black) and **4** (blue) adapted from ref. X allowed to compare the pharmacophoric features with portions of the inhibitor structures and to highlight the correspondence between the pharmacophore and amino acids (listed in grey) of the Sabin 1 PV homology model. Black dashed lines represent the major interactions between amino acids and ligand portions, as described in ref. X. d) Graphical representation of the binding mode of **2** in the complex with the type 2 Lansing strain (pdb code 1EAH).

Agreement between the pharmacophore composition and the interaction patterns found in the homology and crystallographic models provided reliability

for the pharmacophore itself and allowed us to use it for SAR analysis of the new inhibitors.

Although the six-membered chain that connected the terminal aromatic moieties of the new inhibitors did not fit any portion of the pharmacophore, its conformation played a crucial role to force the terminal edges of the molecules at the optimal distance and space orientation to fulfill the pharmacophoric features. In fact, **13** and **15**, whose spacer has been rigidified by insertion of an additional phenyl ring, showed a molecular shape significantly different from the corresponding open analogues **5** and **7**, mainly because of the quasi-planarity of the benzoyl-thiophene moiety. As a consequence, both compounds were unable to fit the pharmacophoric model well and were inactive. By contrast, **2** V-073, whose spacer was partially rigidified by a phenyl ring but maintained a certain degree of freedom due to its methylene fragments, showed activity toward PV.[9] In a similar way, insertion of the ethynyl group induced a significantly change in both length and shape of **14**, that resulted in an inactive compound.

Conclusions

Although wild PV has been controlled in most of the countries, emergence of iVDPV still represents a potential risk to polio eradication. The persistent infections of PV vaccine strains in immune deficient individuals require the design of new antiviral drugs. In this paper we have described the discovery of a new series of compounds very active against PV Sabin, wild and VDPV strains.

Two compounds were found to be very potent: namely **7** was active at submicromolar concentrations against the three Sabin vaccine strains, while **11** that was more potent than **7** against Sabin 2 and Sabin 3, was also very potent (at nanomolar concentrations) against a large panel of wild and VDPVs isolated from patients with acute flaccid paralysis or immunodeficient subjects. Both compounds **7** and **11** showed very low cytotoxicity.

Time of addition experiments performed on **5** (strictly related to **7**) suggest that it is active at an early stage of viral replication. Conversely, the same experiment performed on **11** indicated that this inhibitor can block viral

replication at different stages, thus suggesting different or additional mechanisms of action compared to that of **5**. Thus, we have identified two potent compounds, for which we hypothesize two different mechanisms of action. Further studies will be necessary to determine the specific mechanism of action, in particular for **11**.

Experiments to evaluate the toxicity of the compounds on transgenic mice expressing the human polio receptor and experiments to test whether the most active compound **11** inhibits replication in vivo in these mice are currently underway as a first step to determine whether it may be possible to use these compounds to reduce viral loads *in vivo*. Developing effective drugs/treatments for immunodeficient chronic PV excreters is a WHO priority and research is ongoing worldwide in this field.

Experimental section

Chemistry. General. Melting points were determined with a Büchi 530 capillary apparatus and are uncorrected. Compounds purity were always **>** 95%

determined by combustion analysis. Analytical results agreed to within ± 0.40% of the theoretical values. Infrared (IR) spectra were recorded on a Perkin-Elmer Spectrum-one spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker AC 400 spectrometer. The reaction solutions were purified using a solid phase extraction and filtration column (charged with silica gel pad) in a Biotage FlashVac-10 system. Merck silica gel 60 F_{254} plates were used for analytical TLC. Developed plates were visualized by UV light. Column chromatographies were performed on silica gel (Merck; 70-230 mesh) or alumina (Merck; 70-230 mesh). Concentration of solution after reactions and extractions involved the use of a rotary evaporator operating at reduced pressure of approximately 20 Torr. Analytical results agreed to within ± 0.40% of the theoretical values. Dimethylsulfoxide-d_6 99.9% (code 44,139-2) and deuterochloroform 98.8% (code 41,675-4) of isotopic purity (Aldrich) were used. Solvents were reagent grade and, when necessary, were purified and dried by standard methods. Organic solutions were dried over anhydrous sodium sulfate (Merck). Silica propylsulfonic

acid was acquired from Silicycle (particle size 10 μM, loading 0.67 mmol/g). Compounds **18**[18] and **22**[19] were synthesized as described previously.

General Procedure A (GP-A) for the Alkylation of Oxazolinyl Phenols (5, 6). The appropriate phenol derivative (1.26 mmol), NaI (0.1 mmol) and anhydrous K_2CO_3 (1.32 mmol) were added to a stirred solution of alkyl chloride **16** (1.2 mmol) in dry DMF (15 mL) at 120°C under argon atmosphere. The reaction was stirred at 120°C for 72 hours and monitored with silica gel TLC (4:1 chloroform-ethyl acetate). The reaction was quenched with water (25 mL) and extracted with ethyl acetate (3 x 30 mL). The organic phase was washed with 5% NaOH (25 mL), 5% $Na_2S_2O_3$ (25 mL), brine solution (40 mL), treated with Na_2SO_4, filtered, and evaporated under reduced pressure. The raw material was purified through silica gel column (4:1 chloroform-ethyl acetate as eluent) to obtain the final derivatives. Phenol derivative, yield (%), melting point (°C), recrystallization solvent, IR, and ^{1}H NMR are reported for each of the following compounds.

General Procedure B (GP-B) for the Synthesis of Oxazole Derivatives from the Corresponding Benzonitrile Compounds (7-12, 13, 15). Under argon atmosphere, the benzonitrile derivative (1 mmol), amino alcohol (3 mL), and silica-supported propylsulfonic acid (0.1 g) were stirred for 4 h at 120 °C. The reaction was cooled at room temperature and directly purified through silica gel column chromatography to obtain the corresponding oxazole. Benzonitrile derivative, amino alcohol, chromatography eluent, yield (%), melting point (°C), recrystallization solvent, IR, and ^{1}H NMR are reported for each of the following compounds.

General Procedure C (GP-C) for the Acylation of 2-Methylthiophene (16, 27, 29). 2-Methylthiophene (0.7 mmol) and $AlCl_3$ (0.7 mmol) were added into a well-stirred solution of the appropriate acyl chloride (0.7 mmol) in anhydrous dichloromethane (10 mL), under argon atmosphere at 0 °C. The reaction was stirred at room temperature for 2 h and then quenched with water (40 mL), extracted with dichloromethane (2 x 20 mL). The organic phase was washed with water (2 x 20 mL), brine solution (20 mL), treated with Na_2SO_4, filtered

and evaporated under reduced pressure. The raw material was purified through silica gel column chromatography to obtain the correspondent 2-acylthiophene derivatives. Acylating agent, chromatography eluent, yield (%), melting point (°C), recrystallization solvent, IR, and ^{1}H NMR are reported for each of the following compounds.

5-(4-(4,5-Dihydrooxazol-2-yl)phenoxy)-1-(5-methylthiophen-2-yl)pentan-1-one (5). Compound **5** was prepared from **17** as phenol compound by means of GP-A. Yield 53% as white solid; 129-130 °C; benzene/cyclohexane; IR ν 1631 (CO ketone) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.83-1.95 (m, 4H, CH_2), 2.50 (s, 3H, CH_3), 2.83-2.97 (m, 2H, $COCH_2$), 3.97-4.10 (m, 4H, CH_2 oxazoline and CH_2OAr), 4.30-4.40 (m, 2H, CH_2 oxazoline), 6.78 (bd, 1H, C-H thiophene), 6.87-6.91 (m, 2H, Ar), 7.53-7.56 (m, 1H, C-H thiophene). Elemental analysis calculated (%) for $C_{19}H_{21}NO_3S$: C, 66.45; H, 6.16; N, 4.08; S, 9.33. Found C, 66.437; H, 6.03; N, 4.10; S, 9.36.

***(R)*-5-(4-(4-Methyl-4,5-dihydrooxazol-2-yl)phenoxy)-1-(5-methylthiophen-2-yl)pentan-1-one (6).** Compound **6** was prepared from **18** as phenol compound

by means of GP-A. Yield 42% as pale yellow solid; 127-129 °C; benzene/cyclohexane; IR ν 1636 (CO ketone) cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.38 (d, 3H, J = 8 Hz, CH_3 oxazoline), 1.87-1.92 (m, 4H, CH_2), 2.51 (s, 3H, CH_3 thiophene), 2.91 (t, 2H, J = 4 Hz, CH_2OAr), 3.97-4.03 (m, 3H, CH_2 oxazoline and $COCH_2$), 4.38-4.42 (m, 1H, CH oxazoline), 4.56 (t, 1H, CH_2 oxazoline), 6.77 (br d, 1H, C-H thiophene), 6.88 (d, 2H, J = 8 Hz, Ar), 7.51-7.53 (m, 1H, C-H thiophene), 7.93 (d, 2H, J = 8 Hz, Ar). Elemental analysis calculated (%) for $C_{20}H_{23}NO_3S$: C, 67.20; H, 6.49; N, 3.92; S, 8.97. Found C, 67.31; H, 6.51; N, 3.99; S, 9.01.

5-((4-(4,5-Dihydrooxazol-2-yl)phenyl)thio)-1-(5-methylthiophen-2-yl)pentan-1-one (7). Compound **7** was prepared from **19** as benzonitrile compound and ethanolamine was used as amino alcohol by means of GP-B. Chloroform-ethyl acetate 4:1; yield 47% as yellow solid; 107-108 °C; methanol; IR ν 1648 (CO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.54-1.68 (m, 4H, CH_2), 2.41 (s, 3H, CH_3), 2.84 (t, 2H, J = 7 Hz, CH_2), 2.98 (t, 2H, J = 7 Hz, CH_2), 3.85 (t, 2H, J = 8 Hz, CH_2 oxazoline), 4.30 (t, 2H, J = 8 Hz, CH_2 oxazoline), 6.86 (br d, 1H, C-H

thiophene), 7.25-7.28 (m, 2H, Ar), 7.65-7.70 (m, 3H, Ar and C-H thiophene) Elemental analysis calculated (%) for $C_{19}H_{21}NO_2S_2$: C, 63.48; H, 5.89; N, 3.90; S, 17.84. Found C, 63.437; H, 5.99; N, 3.91; S, 17.80.

***(R)*-5-((4-(4-Methyl-4,5-dihydrooxazol-2-yl)phenyl)thio)-1-(5-methylthiophen-2-yl)pentan-1-one (8)**. Compound **8** was prepared from **19** as benzonitrile compound and *(R)*-(−)-2-amino-1-propanol was used as amino alcohol by means of GP-B. Chloroform-ethyl acetate 4:1; yield 30% as yellow solid; 98-99 °C; methanol; IR ν 1658 (CO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.14 (d, 3H, J = 7 Hz, CH_3 oxazoline), 1.54-1.68 (m, 4H, CH_2), 2.41 (s, 3H, CH_3), 2.84 (t, 2H, J = 7 Hz, CH_2), 2.98 (t, 2H, J = 7 Hz, CH_2), 3.82 (t, 1H, J = 8 Hz, HCH oxazoline), 4.17-4.23 (m, 1H, CH oxazoline), 4.41 (t, 2H, J = 8 Hz, HCH oxazoline), 6.86 (br d, 1H, C-H thiophene), 7.25-7.28 (m, 2H, Ar), 7.65-7.70 (m, 3H, Ar and C-H thiophene). Elemental analysis calculated (%) for $C_{20}H_{23}NO_2S_2$: C, 64.31; H, 6.21; N, 3.75; S, 17.17. Found C, 64.21; H, 6.42; N, 3.50; S, 17.20.

5-((4-(4,5-Dihydrooxazol-2-yl)phenyl)sulfonyl)-1-(5-methylthiophen-2-yl)pentan-1-one (9). Compound **9** was prepared from **20** as benzonitrile compound and ethanolamine was used as amino alcohol by means of GP-B. Ethyl acetate-methanol 99:1; yield 30% as yellow solid; 122-123 °C; methanol; IR ν 1652 (CO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.49-1.55 (m, 4H, CH_2), 2.41 (s, 3H, CH_3), 2.79 (br t, 2H, J = 7 Hz, CH_2), 3.33 (br t, 2H, J = 7 Hz, CH_2), 3.94 (t, 2H, J = 8 Hz, CH_2 oxazoline), 4.38 (t, 2H, J = 8 Hz, CH_2 oxazoline), 6.85 (br d, 1H, C-H thiophene), 7.66 (br d, 1H, C-H thiophene), 7.89 (2H, J = 8 Hz, Ar), 8.01 (2H, J = 8 Hz, Ar). Elemental analysis calculated (%) for $C_{19}H_{21}NO_4S_2$: C, 58.29; H, 5.41; N, 3.58; S, 16.38. Found C, 58.32; H, 5.39; N, 3.49; S, 16.35.

5-((4-(4,5-Dihydrooxazol-2-yl)phenyl)sulfinyl)-1-(5-methylthiophen-2-yl)pentan-1-one (10). Compound **10** was prepared from **21** as benzonitrile compound and ethanolamine was used as amino alcohol by means of GP-B. Chloroform-methanol 95:5; yield 55% as yellow solid; 128-129 °C; methanol; IR ν 1648 (CO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.30-1.60 (m, 4H, CH_2), 2.41 (s, 3H,

CH_3), 2.75-3.05 (m, 4H, CH_2), 2.98 (t, 2H, *J* = 7 Hz, CH_2), 3.85 (t, 2H, *J* = 8 Hz, CH_2 oxazoline), 4.30 (t, 2H, *J* = 8 Hz, CH_2 oxazoline), 6.86 (br d, 1H, C-H thiophene), 7.25-7.28 (m, 2H, Ar), 7.65-7.70 (m, 3H, Ar and C-H thiophene) Elemental analysis calculated (%) for $C_{19}H_{21}NO_2S_2$: C, 63.48; H, 5.89; N, 3.90; S, 17.84. Found C, 63.437; H, 5.99; N, 3.91; S, 17.80.

(E)-6-(4-(4,5-dihydrooxazol-2-yl)phenyl)-1-(5-methylthiophen-2-yl)hex-5-en-1-one (11). Compound **11** was prepared from **22** as benzonitrile compound and ethanolamine was used as amino alcohol by means of GP-B. Hexane-ethyl acetate 7:3; yield 40% as pale yellow solid; 143-144 °C; methanol; IR *ν* 1646 (CO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.66-1.74 (m, 2H, CH_2), 2.17 (br t, 2H, CH_2), 2.41 (s, 3H, CH_3), 2.86 (t, 2H, *J* = 7 Hz, CH_2), 3.86 (t, 2H, *J* = 8 Hz, CH_2 oxazoline), 4.30 (t, 2H, *J* = 8 Hz, CH_2 oxazoline), 6.35-6.45 (m, 2H, HCCH), 6.85 (br d, 1H, C-H thiophene), 7.50 (d, 2H, *J* = 8 Hz, Ar), 7.65-7.70 (m, 3H, Ar and C-H thiophene). Elemental analysis calculated (%) for $C_{20}H_{21}NO_2S$: C, 70.77; H, 6.24; N, 4.13; S, 9.44. Found C, 70.81; H, 6.20; N, 4.19; S, 9.40.

6-(4-(4,5-Dihydrooxazol-2-yl)phenyl)-1-(5-methylthiophen-2-yl)hexan-1-one (12). Compound **12** was prepared from **28** as benzonitrile compound and ethanolamine was used as amino alcohol by means of GP-B. Hexane-ethyl acetate 3:1; yield 30% as yellow solid; 83-84 °C; methanol; IR *v* 1650 (CO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.20-1.25 (m, 2H, CH_2), 1.49-1.56 (m, 4H, CH_2), 2.41 (s, 3H, CH_3), 2.53 (t, 2H, *J* = 8 Hz, CH_2), 2.77 (t, 2H, *J* = 8 Hz, CH_2), 3.85 (t, 2H, *J* = 8 Hz, CH_2 oxazoline), 4.30 (t, 2H, *J* = 8 Hz, CH_2 oxazoline), 6.85 (br d, 1H, C-H thiophene), 7.19 (d, 2H, *J* = 8 Hz, Ar), 7.66-7.70 (m, 3H, Ar and C-H thiophene). Elemental analysis calculated (%) for $C_{20}H_{23}NO_2S$: C, 70.35; H, 6.79; N, 4.10; S, 9.39. Found C, 70.40; H, 6.75; N, 4.16; S, 9.34.

6-(4-(4,5-Dihydrooxazol-2-yl)phenyl)-1-(5-methylthiophen-2-yl)hex-5-yn-1-one (13). Compound **13** was prepared from **27** as benzonitrile compound and ethanolamine was used as amino alcohol by means of GP-B. Hexane-ethyl acetate 4:1; yield 70% as pale yellow solid; 118-119 °C; methanol; IR *v* 2226 (CN), 1650 (CO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.78-1.84 (m, 2H, CH_2),

2.41-2.48 (m, 5H, CH_3 and CH_2), 2.97 (t, 2H, J = 7 Hz, CH_2), 3.87 (t, 2H, J = 8 Hz, CH_2 oxazoline), 4.32 (t, 2H, J = 8 Hz, CH_2 oxazoline), 6.87 (br d, 1H, C-H thiophene), 7.37 (d, 2H, J = 8 Hz, Ar), 7.71-7.74 (m, 3H, Ar and C-H thiophene). Elemental analysis calculated (%) for $C_{20}H_{19}NO_2S$: C, 71.19; H, 5.68; N, 4.15; S, 9.50. Found C, 71.29; H, 5.78; N, 4.20; S, 9.55.

(3-((4-(4,5-Dihydrooxazol-2-yl)phenoxy)methyl)phenyl)(5-methylthiophen-2-yl)methanone (14). To a stirred solution of **29** (0.9 mmol) in dry DMF (20 mL) at 80°C under argon atmosphere, were added anhydrous K_2CO_3 (0.9 mmol) and **28** (0.9 mmol). The reaction was stirred at 80 °C for 4 h and monitored with TLC (3:2 hexane-ethyl acetate). The reaction was quenched with 1N HCl (20 mL) and extracted with ethyl acetate (2 x 40 ml). The organic phase was washed with water (40 mL), brine solution (40 mL), treated with Na_2SO_4, filtered and evaporated under reduced pressure. The raw material was purified through column chromatography on silica gel to obtain 0.2 g of **13** as pale yellow solid (yield 55%); 116-117 °C; ethanol; IR ν 1620 (CO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 2.48 (s, 3H, CH_3), 3.83 (t, 2H, J = 8 Hz, CH_2 oxazoline), 4.28 (t,

2H, J = 8 Hz, CH_2 oxazoline), 5.21 (s, 2H, CH_2 Bz), 6.92 (br d, 1H, C-H thiophene), 7.03 (d, 2H, J = 8 Hz, Ar), 7.41 (br d, 1H, C-H thiophene), 7.51 (t, 1H, J = 8 Hz, Ar), 7.64-7.70 (m, 2H, Ar), 7.73 (d, 2H, J = 8 Hz, Ar), 7.77 (s, 1H, Ar). Elemental analysis calculated (%) for $C_{22}H_{19}NO_3S$: C, 70.01; H, 5.07; N, 3.71; S, 8.49. Found C, 70.12; H, 5.01; N, 3.79; S, 8.59.

(3-(((4-(4,5-Dihydrooxazol-2-yl)phenyl)thio)methyl)phenyl)(5-methylthiophen-2-yl)methanone (15). Compound **15** was prepared from **30** as benzonitrile compound and ethanolamine was used as amino alcohol by means of GP-B. Hexane-ethyl acetate 4:1; yield 45% as pale yellow solid; 105-106 °C; ethanol; IR ν 1607 (CO ketone) cm^{-1}. ^1H NMR (CD_3OD) δ 2.41 (s, 3H, CH_3), 3.24 (t, 2H, J = 8 Hz, CH_2 oxazoline), 3.64 (t, 2H, J = 8 Hz, CH_2 oxazoline), 4.28 (s, 2H, CH_2 bz), 6.30 (br d, 1H, C-H thiophene), 6.58 (br d, 1H, C-H thiophene), 7.05-7.16 (m, 2H, Ar), 7.36-7.40 (m, 3H, Ar), 7.44-7-50 (m, 3H, Ar). Elemental analysis calculated (%) for $C_{22}H_{19}NO_2S_2$: C, 67.15; H, 4.87; N, 3.56; S, 16.29. Found C, 67.18; H, 4.81; N, 3.53; S, 16.35.

5-chloro-1-(5-methylthiophen-2-yl)pentan-1-one (16). Compound **16** was prepared from 5-chloropentanoyl chloride as acylating agent by means of GP-C. Hexane-ethyl acetate 95:5; yield 80% as pale yellow solid; 45-47 °C; cyclohexane; IR ν 1652 (CO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.48 (m, 2H, CH_2), 1.81 (m, 2H, CH_2), 2. 35 (s, 3H, CH_3), 3.35 (t, 2H, J = 7 Hz, CH_2 CO), 3.61 (t, 2H, J = 7 Hz, CH_2Cl), 7.01 (br d, 1H, C-H thiophene), 7.82 (br d, 1H, C-H thiophene). Elemental analysis calculated (%) for $C_{10}H_{13}ClOS$: C, 55.42; H, 6.05; Cl, 16.36; S, 14.79. Found C, 55.40; H, 6.01; Cl, 16.34; S, 14.76.

4-(4,5-Dihydrooxazol-2-yl)phenol (17). To a solution of 4-hydroxybenzonitrile (8.4 mmol) in anhydrous chlorobenzene (20 mL) were added in turn ethanolamine (25 mmol) and anhydrous $ZnCl_2$ (0.84 mmol). After 24 h at 160 °C, the reaction mixture was quenched with 1N HCl (40 mL) and then extracted twice with dichloromethane (60 mL). The organic phase was washed

with water (40 mL), brine solution (40 mL), treated with Na_2SO_4, filtered and evaporated under reduced pressure to give 0.28 g of **17** as a yellow solid (20% yield). Chemical, physical, analytical and spectroscopic data of compound **17** are reported in literature.[20]

(*R*)-4-(4-methyl-4,5-dihydrooxazol-2-yl)phenol (18). Compound **18** was prepared according to literature.[18] Analytical data are herein reported.

4-((5-(5-Methylthiophen-2-yl)-5-oxopentyl)thio)benzonitrile (19). To a stirred solution of **16** (4.6 mmol) in dry DMF (20 mL) at 80°C under argon atmosphere, anhydrous K_2CO_3 (6.9 mmol) and 4-mercaptobenzonitrile (4.6 mmol) were added. The reaction was stirred at 80°C for 4 hours and monitored with TLC (4:1 hexane-ethyl acetate). The reaction was quenched with 1N HCl (20 mL) and extracted twice with ethyl acetate (40 mL). The organic phase was washed with water (40 mL) and brine solution (40 mL), treated with Na_2SO_4, filtered and evaporated under reduced pressure. The raw material was purified through column chromatography to obtain 1.2 g of **19** as yellow solid (yield 85%); 102-103°C; benzene/cyclohexane; IR ν 2225 (CN), 1652 (CO ketone) cm^{-}

^{1}. ^{1}H NMR ($CDCl_3$) δ 1.68-1.73 (m, 2H, CH_2), 1.79-1.85 (m, 2H, CH_2), 2.47 (s, 3H, CH_3), 2.81 (t, 2H, J = 7 Hz, CH_2), 2.94 (t, 2H, J = 7 Hz, CH_2), 6.72 (br d, 1H, CH thiophene), 7.19-7.24 (m, 2H, Ar), 7.43-7.46 (m, 3H, Ar and CH thiophene). Elemental analysis calculated (%) for $C_{17}H_{17}NOS_2$: C, 64.73; H, 5.43; N, 4.44; S, 20.33. Found 64.85; H, 5.52; N, 4.25; S, 20.43.

4-((5-(5-Methylthiophen-2-yl)-5-oxopentyl)sulfonyl)benzonitrile (20). *m*-Chloroperbenzoic acid (0.6 mmol) was added to a stirred solution of **19** (0.2 mmol) in chloroform (20 mL). The reaction was stirred at room temperature for 30 minutes and monitored with TLC (1:1 hexane-ethyl acetate). Tthe reaction was quenched with 10% K_2CO_3 (20 mL) and extracted twice with chloroform (20 mL). The organic phase was washed with K_2CO_3 10% (2 x 20 mL), brine solution (20 mL), treated with Na_2SO_4, filtered and evaporated under reduced pressure to obtain 0.07 g of **20** without further purification as yellow solid (yield 100%); 125-126°C; benzene/cyclohexane; IR ν 2233 (CN), 1659 (CO ketone) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.71-1.75 (m, 4H, CH_2), 2.46 (s, 3H, CH_3), 2.78 (t, 2H, J = 7 Hz, CH_2), 3.09 (t, 2H, J = 7 Hz, CH_2), 6.72 (d, 1H, J = 4 Hz, C-H

thiophene), 7.42 (d, 1H, *J* = 4 Hz, C-H thiophene), 7.80 (d, 2H, *J* = 8 Hz, Ar), 7.96 (d, 2H, *J* = 8 Hz, Ar). Elemental analysis calculated (%) for $C_{17}H_{17}NO_3S_2$: C, 58.77; H, 4.93; N, 4.03; S, 18.45. Found C, 58.70; H, 4.98; N, 4.13; S, 18.49.

4-((5-(5-Methylthiophen-2-yl)-5-oxopentyl)sulfinyl)benzonitrile (21). To a stirred solution of **19** (0.3 mmol) in chloroform (18 ml) and ethanol (18 ml), 30% H_2O_2 (0.6 mmol) and $POCl_3$ (0.6 mmol) were added. The reaction was stirred at room temperature for 1 h and monitored with TLC (1:1 hexane-ethyl acetate). The reaction was quenched with water (40 mL) and extracted twice with chloroform (20 mL). The organic phase was washed with water (2 x 20 mL), brine solution (20 mL), treated with Na_2SO_4, filtered, and evaporated under reduced pressure. The raw material was purified through column chromatography to obtain 0.1 g of **21** as yellow solid (yield 95%); 110-111 °C; benzene; IR v 2228 (CN), 1660 (CO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.30-1.60 (m, 4H, CH_2), 2.41 (s, 3H, CH_3), 2.75-3.05 (m, 4H, CH_2), 6.85 (br d, 1H, C-H thiophene), 7.65-7.70 (br d, 1H, C-H thiophene), 7.75 (d, 2H, *J* = 8 Hz,

Ar), 7.95 (d, 2H, *J* = 8 Hz, Ar). Elemental analysis calculated (%) for $C_{19}H_{21}NO_2S_2$: C, 63.48; H, 5.89; N, 3.90; S, 17.84. Found C, 63.437; H, 5.99; N, 3.91; S, 17.80.

(*E*)-6-(4-cyanophenyl)hex-5-enoic acid (22). Compound **22** was prepared according to literature.[19] Analytical data are herein reported.

***(E)*-6-(4-cyanophenyl)hex-5-enoyl chloride (23).** To a stirred solution of **22** (0.7 mmol) in anhydrous dichloromethane (10 mL) under argon atmosphere dry DMF (0.15 mL) and oxalyl chloride (3.5 mmol) were added. The reaction was stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure to obtain the desired acyl chloride **23** as yellow oil in quantitative yield and subsequently used for the reaction without further purification (IR diagnostic signals: 1816 cm^{-1} (CO, acyl chloride).

(*E*)-4-(6-(5-methylthiophen-2-yl)-6-oxohex-1-en-1-yl)benzonitrile (24). To a solution of acyl chloride **23** (0.7 mmol) in anhydrous dichloromethane (10 mL) under argon atmosphere at 0 °C, 0.7 mmol of $AlCl_3$ and 0.7 mmol of 2-methylthiophene were added. The reaction was stirred at room temperature for

2 h and monitored with TLC (9:1 hexane-ethyl acetate). The reaction was quenched with water (40 mL), extracted with dichloromethane (2 x 20 mL). The organic phase was washed with water (2 x 20 mL), brine solution (20 mL), treated with Na_2SO_4, filtered and evaporated under reduced pressure. The raw material was purified through column chromatography to obtain 0.18 g of **24** as pale brown solid (yield 90%); 62-63 °C; cyclohexane; IR ν 2226 (CN), 1646 (CO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.66-1.74 (m, 2H, CH_2), 2.17 (br t, 2H, CH_2), 2.41 (s, 3H, CH_3), 2.86 (t, 2H, J = 7 Hz, CH_2), 6.37-6.49 (m, 2H, HCCH), 6.85 (br d, 1H, C-H thiophene), 7.50 (d, 2H, J = 8 Hz, Ar), 7.65-7.70 (m, 3H, Ar and C-H thiophene). Elemental analysis calculated (%) for $C_{18}H_{17}NOS$: C, 73.19; H, 5.80; N, 4.74; S, 10.85. Found C, 73.29; H, 5.70; N, 4.70; S, 10.81.

6-(4-Cyanophenyl)hex-5-ynoic acid (25). Tetrabutylammonium chloride (hydrate, 17.8 g) was dried by azeotroping with benzene (250 mL round bottom flask equipped with a Dean-Stark apparatus). The benzene was removed in vacuo affording anhydrous tetrabutylammonium chloride (17.0 g, 61.2 mmol). To

this flask under argon were added triphenylphosphine (820 mg, 3.13 mmol), palladium acetate (703 mg, 3.13 mmol), 4-bromobenzonitrile (16.9g, 92.8 mmol), potassium acetate (36.8g, 375 mmol) and 100 mL of degassed anhydrous dimethylformamide (degassed by bubbling argon through for 10 min, dried over molecular sieves). A solution of 4-pentinoic acid (6.27 g, 62.6 mmol) and degassed anhydrous DMF (35 mL) was then added to the rapidly stirring reaction mixture at room temperature. After 5 days, the reaction mixture was poured slowly into a sodium carbonate solution (3%, 400 mL) and extracted with ethyl acetate (500 mL). The aqueous layer was treated with decolorizing carbon and filtered. Then, the aqueous layer was acidified to a pH 2 with 10% HCl which yielded **25** as a yellow-brown solid (yield 84%). This procedure led to **25** in sufficient purity to take onto the next step without complications. An analytical sample was obtained by submitting the sample to further purification by flash chromatography (3:7 ethyl acetate-methylene chloride) and recrystallization from ethyl acetate (2 times). 87-88 °C; IR ν 3230 (OH acid), 2242 (CN), 1726 (CO acid), 1650 (CO ketone) cm^{-1}. ^1H NMR (DMSO- d_6) δ

1.66-1.74 (m, 2H, CH_2), 2.30 (t, 2H, J = 6 Hz CH_2), 2.41 (s, 3H, CH_3), 7.49 (d, 2H, J = 8 Hz, Ar), 7.73 (d, 2H, J = 8 Hz, Ar), 12.06 (br s, 1H, COOH). Elemental analysis calculated (%) for $C_{13}H_{11}NO_2$: C, 73.23; H, 5.20; N, 6.57. Found C, 73.14; H, 5.10; N, 6.60.

6-(4-Cyanophenyl)hex-5-ynoyl chloride (26). To a stirred solution of **25** (0.7 mmol) in anhydrous dichloromethane (10 mL) under argon atmosphere dry DMF (0.15 mL) and oxalyl chloride (3.5 mmol) were added. The reaction was stirred at room temperature for 2 h. Then the solvent was evaporated under reduced pressure to obtain the desired acyl chloride **26** as yellow oil in quantitative yield and subsequently used for the reaction without further purification (IR diagnostic signals: 1795 cm^{-1} (CO, acyl chloride).

4-(6-(5-Methylthiophen-2-yl)-6-oxohex-1-yn-1-yl)benzonitrile (27). Compound **27** was prepared from **26** as acylating agent by means of GP-C. Hexane-ethyl acetate 9:1; yield 85% as pale brown solid; 95-96 °C; cyclohexane; IR ν 2229 (CN), 1649 (CO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.78-1.84 (m, 2H, CH_2), 2.41-2.48 (m, 5H, CH_3 and CH_2), 2.97 (t, 2H, J = 7 Hz, CH_2), 6.86 (br d, 1H,

C-H thiophene), 7.47 (d, 2H, *J* = 8 Hz, Ar), 7.65-7.70 (m, 3H, Ar and C-H thiophene). Elemental analysis calculated (%) for $C_{18}H_{15}NOS$: C, 73.69; H, 5.15; N, 4.77; S, 10.93. Found C, 73.65; H, 5.10; N, 4.73; S, 10.89.

4-(6-(5-Methylthiophen-2-yl)-6-oxohexyl)benzonitrile (28). To a solution of **27** (1.1 mmol) in ethyl acetate 10% Pd/C (0.1 mmol) was added and the resulting solution was stirred at room temperature under hydrogen atmosphere (30 psi). After 2 h the mixture was filtered on celite to remove the Pd/C and the organic phase was evaporated to obtain the desired benzonitrile **28** without further purification as pale yellow solid (yield 100%); 64-65 °C; cyclohexane; IR *v* 2230 (CN), 1649 (CO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.18-1.26 (m, 2H, CH_2), 1.47-1.57 (m, 4H, CH_2), 2.41 (s, 3H, CH_3), 2.57 (t, 2H, *J* = 8 Hz, CH_2), 2.77 (t, 2H, *J* = 8 Hz, CH_2), 6.85 (br d, 1H, C-H thiophene), 7.31 (d, 2H, *J* = 8 Hz, Ar), 7.63-7.67 (m, 3H, Ar and C-H thiophene). Elemental analysis calculated (%) for $C_{18}H_{19}NOS$: C, 72.69; H, 6.44; N, 4.71; S, 10.78. Found C, 72.60; H, 6.50; N, 4.70; S, 10.81.

(3-(Chloromethyl)phenyl)(5-methylthiophen-2-yl)methanone (29). Compound **29** was prepared from 3-(chloromethyl)benzoyl chloride as acylating agent by means of GP-C. Hexane-ethyl acetate 4:1; yield 95% as pale yellow solid; 78-79 °C; ligroin; IR ν 1629 (CO ketone) cm^{-1}. ^1H NMR (DMSO- d_6) δ 2.48 (s, 3H, CH_3), 4.80 (s, 2H, CH_2 bz), 6.94 (br d, 1H, C-H thiophene), 7.44 (br d, 1H, C-H thiophene), 7.50 (d, 1H, J = 8 Hz, Ar), 7.64 (d, 1H, J = 8 Hz, Ar), 7.68 (d, 1H, J = 8 Hz, Ar), 7.77 (s, 1H, Ar). Elemental analysis calculated (%) for $C_{13}H_{11}ClOS$: C, 62.27; H, 4.42; Cl, 14.14; S, 12.79. Found C, 62.30; H, 4.48; Cl, 14.20; S, 12.70.

4-((3-(5-Methylthiophene-2-carbonyl)benzyl)thio)benzonitrile (30). To a stirred solution of **29** (2 mmol) in dry DMF (20 mL) at 80°C under argon atmosphere, anhydrous K_2CO_3 (3 mmol) and 4-mercaptobenzonitrile (2 mmol) were added. The reaction was stirred at 80°C for 4 hours and monitored with TLC (7:3 hexane-ethyl acetate). The reaction was quenched with 1N HCl (20 mL) and extracted twice with ethyl acetate (40 mL). The organic phase was washed with water (40 mL), brine solution (40 mL), treated with Na_2SO_4, filtered and

evaporated under reduced pressure. The raw material was purified through column chromatography to obtain 0.45 g of **30** as white solid (75% yield); 132-133 °C; cyclohexane; IR ν 2223 (CN), 1623 (CO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 2.48 (s, 3H, CH_3), 4.43 (s, 2H, CH_2 Bz), 6.92 (br d, 1H, C-H thiophene), 7.32 (br d, 1H, C-H thiophene), 7.41-7.46 (m, 3H, Ar), 7.57-7.68 (m, 4H, Ar), 7.77 (s, 1H, Ar). Elemental analysis calculated (%) for $C_{20}H_{15}NOS_2$: C, 68.74; H, 4.33; N, 4.01; S, 18.35. Found C, 68.84; H, 4.35; N, 4.11; S, 18.39.

Biological methods. *Cell lines.* HeLa cells were maintained in minimal essential medium (MEM; Hyclone, GE Healthcare) supplemented with 10% fetal bovine serum (FBS; Hyclone). Cells were trypsinized and seeded into 96-well, flat-bottom, polystyrene tissue-culture plates at a density of $2x10^5$ cells/mL in 200 μL MEM-2% FBS medium. The plates were wrapped in plastic wrap and incubated for 24 hours at 37°C in a humidified 5% CO_2 incubator prior to their use in the assay.

Viruses. A panel of 45 viruses consisting of viruses of all three poliovirus serotypes which includes wild reference strains, the three Sabin vaccine strains,

representative cVDPV isolates and vaccine-derived poliovirus from iVDPV was assembled and used for susceptibility testing.

An *in vitro* cytopathic effect assay (signal-to-noise ratio $\geq$5) was used to calculate the susceptibility (EC_{50}, expressed in μM) for a given virus isolate. Compound and virus were combined with HeLa cells ($2x10^5$ cells/well) in 96-well plates, in a cross-titration format, starting at 10 μM compound. To ensure reaching endpoints from compound and virus titrations, eleven 7-point titration curves were performed in 0.5 $\log_{10}$ steps with duplicate wells for each compound-virus concentration. Three replicate tests were run for each virus, unless otherwise stated. After 72 hour incubation incubation at 37°C, cells were stained with crystal violet (0.05% crystal violet, 0.5% Tween-20, 50% ethanol, in deionized H_2O) and washed three times with deionized H_2O. Plates were air-dried and viral cytopathic effect was measured by reading absorbance at 590 nm. EC_{50} values were derived by analyzing dose-response absorbance values by four-parameter curve fitting using Prism 5.04 (GraphPad Software, Inc., La Jolla, CA). If all of the virus dilutions achieved $\geq$80% destruction of the cell

monolayer, the EC_{50} was expressed as greater than the starting compound concentration (e.g. >10 μM).

We also used a luminescence cell viability assay (ATPLite®,Perkin-Elmer, Waltham, MA) to determine cytotoxicity. Corresponding curves consisting of compound and cells only were compared to cell only wells to determine cytotoxicity. The conditions of the cytotoxicity assay were similar to the cytopathic effect assay.

Time of addition and virus titer determination assay. To synchronize the infection in the time-of-addition assay, HeLa cell monolayers were infected with poliovirus (strain Sabin 2) at 100 $CCID_{50}$ and incubated at 37°C with 5% CO_2 for 1 h. Unbound virus was removed by washing cell monolayer 3X. Drug was added to the cell monolayer at 0, 1, 2, 4, 6 or 8 hour post-infection and remained present in assay. After 12 hincubation, this newly produced virus mixture was freeze-thawed 3X and used for titer determination. The assay for virus titer measured the virus-induced CPE and was a modification (ATPLite®) of the WHO cell sensitivity assay used in the Global Polio Laboratory Network[11]

The SOLO multi-channel robotic workstation (Hudson Robotics Inc., Springfield, NJ) was used to perform 0.5 $\log_{10}$ serial dilutions from each virus infected sample (16 replicates at each dilution), starting at 1:10, and combined with HeLa cells in white 96-well plates. After five days incubation at 37°C in a humidified 5% CO2 incubator, 75 µL of cell lysis buffer and then 75 µL of reconstituted substrate solution were added to each well using the MicroFlo™ Select reagent dispenser. Viral CPE was measured by reading luminescence. The dilutions of virus that resulted in at least 80% destruction of the cell monolayer (signal-to-noise ratio $\geq$ 5) were used to calculate the virus titer in the sample (as 50% endpoint cell culture infectious dose [$CCID_{50}$], using the Kärber formula.[12] Titer values were expressed as both $CCID_{50}$ and $\log(CCID_{50})$. If none of the virus dilutions achieved the desired $\geq$ 80% destruction of the cell monolayer, the virus titer was expressed as less than the starting dilution. If all virus dilutions resulted in 100% destruction of the cell monolayer (i.e., endpoint was not reached), subsequent retesting was started at a higher virus dilution.

Modelling and structural analysis

Molecular structures were sketched with Maestro[21] and optimized with LigPrep. A systematic torsional sampling protocol was applied with OPLS-2005 force field and the implicit GB/SA distance-dependent dielectric solvent model, with water as the solvent. Conformers were then minimized using the Polak-Ribiere conjugate gradient algorithm.

Two of the new oxazolines (**11** and **12)** as well as three known inhibitors (**2**-**4**) were submitted to the common feature hypothesis generation routine of Phase[22] to build the three-dimensional pharmacophoric model.

Further details on the computational protocol have been reported previously.[23]

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website at DOI:

Virucidal activity of compounds **5**, **7** and **11**, cytotoxicity determination of compounds **5**, **7** and **11**, Determination of EC_{50} for compounds **5**, **7**, **11** against Sabin 1-3 strains.

Molecular formula strings and some data (CSV)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

PV, poliovirus; VDPVs, vaccine-derived polioviruses; iVDPVs, immunodeficiency vaccine-derived polioviruses; cVDPVs, circulating vaccine-derived polioviruses; SAR, structure-activity relationship; DMF, *N*,*N*-dimethylformamide; *m*-CPBA, *m*-chloroperbenzoic acid; DCM, dichloromethane; OPVs, oral polio vaccines; IPV, inactivated polio vaccines; SIAs, supplemental immunization activities; PI, post-infection; SI, selectivity index; HBA, hydrogen bond acceptor group; HY,

hydrophobic region; RA, aromatic ring; IR, infrared; GP, general procedure; FBS, fetal bovine serum.

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