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Thiazolides as Novel Antiviral Agents. 1. Inhibition of Hepatitis B Virus Replication

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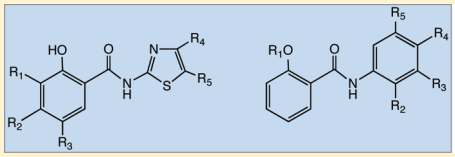
S *Supporting Information*

ABSTRACT: We report the syntheses and activities of a wide range of thiazolides [viz., 2-hydroxyaroyl-*N*-(thiazol-2-yl)-amides] against hepatitis B virus replication, with QSAR analysis of our results. The prototypical thiazolide, nitazoxanide [2-hydroxybenzoyl-*N*-(5-nitrothiazol-2-yl)amide, NTZ] **1** is a broad spectrum antiinfective agent effective against anaerobic bacteria, viruses, and parasites. By contrast, 2-hydroxybenzoyl-*N*-(5-chlorothiazol-2-yl)amide **3** is a novel, potent, and selective inhibitor of hepatitis B replication ($EC_{50} = 0.33\ \mu m$) but is inactive against anaerobes. Several 4′- and 5′-substituted thiazolides show good activity against HBV; by contrast, some related salicyloylanilides show a narrower spectrum of activity. The ADME properties of **3** are similar to **1**; viz., the *O*-acetate is an effective prodrug, and the *O*-aryl glucuronide is a major metabolite. The QSAR study shows a good correlation of observed EC_{90} for intracellular virions with thiazolide structural parameters. Finally we discuss the mechanism of action of thiazolides in relation to the present results.



EC_{50} (VIR) 0.15 -5.0 μM

INTRODUCTION

Hepatitis B Virus. It is currently estimated that two billion people worldwide have been affected by hepatitis B virus (HBV) and that of these around 360 million are chronically affected.[1] The so-called Dane particle, observed in the blood of patients with hepatitis, was identified as the viral agent in 1970.[2] The virion is characterized by the surface antigen HBsAg, originally known as the "Australia antigen" and related to HBV by the pioneering research of Blumberg.[3] While vaccination is often effective as a preventative measure,[4] treatment of patients with chronic HBV requires chemotherapy. Since HBV, like HIV, replicates via reverse transcriptase through an RNA template and since HBV is common in patients who develop AIDS, structurally related nucleoside analogues may have valuable activity against both these viruses: the HBV polymerase and the HIV-1 reverse transcriptase have significant homology.[5]

Indeed, the earliest chemotherapeutic agents for HBV were viral DNA polymerase inhibitors such as lamivudine, adefovir, and entecavir. All these agents were effective, with submicromolar anti-HBV IC_{50}, but as usual with such agents resistant strains quickly appeared.[6−8] HBV displays a particularly fast mutation rate (10^{10-11} point mutations per day in individuals with active replication),[9] and additionally cross-resistance has been noted between chemical agents from different structural groups.

Among other approaches, agents that target the encapsidation step (viz., assembly of viral RNA, polymerase, and core into the nucleocapsid prior to replication) have been developed, notably heteroaryldihydropyrimidines[10] and phenopropenamides.[11] Since the HBsAg is a heavily glycosylated protein, inhibitors of the N-glycosylation pathway have also been studied[12] and found effective both in vitro and in an experimental in vivo infection model.[13] Finally, other nucleic acid-based approaches have been studied, notably antisense nuceotides[14] and gene therapy employing short interfering RNA (siRNA), which has shown promising results in model infection.[15] In summarizing new approaches to HBV therapy, including immune modulation, Loomba and Liang[16] commented that "an improved understanding of virus−host interactions" was a key need in the development of new therapies. Indeed there is a clear need for effective new small molecule drugs as anti-HBV agents, ideally ones that would act through a mechanism not leading to rapid generation of resistant viral mutants. We now report that a series of thiazolide analogues

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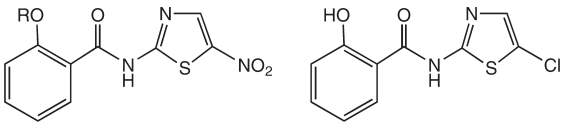
[2-hydroxyaroyl-*N*-(thiazol-2-yl)amides] appear to fulfill these requirements.

Thiazolides. The aminothiazole derivative nitazoxanide **1** (Figure 1) was first developed as an antiparasistic agent, particularly indicated against *Cryptosporidium parvum*[17] and has been marketed in the U.S. since 2002. Indeed **1** is a broad-spectrum antiinfective agent, also active against anaerobic bacteria[18] and as an antiprotozoal[19] and anthelmintic agent. It was in the late 1990s that the antiviral activity of nitazoxanide was first noted[20] during its use in the treatment of AIDS patients who had developed cryptosporidiosis. In most of its indications, nitazoxanide **1** behaves as a prodrug for the free phenol, tizoxanide **2**, which is the effective circulating drug in vivo.

There is strong evidence that **1** owes its activity against anaerobic bacteria and parasites to inhibition of the enzyme pyruvate/ferredoxin reductase (PFOR),[21] whereby production of acetyl CoA within the anaerobe is prevented. Nevertheless, the nitro group is not directly involved (e.g., by reduction), nor is it essential for broader antiinfective activity. For instance, halothiazolides are also effective against the parasites *S. neurona* and *C. parvum*.[17b,22] We used nitazoxanide as the starting point for a program of synthesis and evaluation of a wide range of thiazolide analogues, particularly as selective antiviral agents. We have already presented data showing that nitazoxanide and a few close analogues are effective inhibitors of HBV and in some cases of hepatitis C virus (HCV) replication in cell cultures.[23] Further testing has revealed that **1** is active against a range of both DNA and RNA viruses, and it has been evaluated in clinical trials.[24]



1 R = Ac, **2** R = H **3**

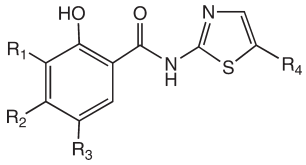
Figure 1. Basic thiazolide structures **1**−**3**.

In this paper we present a full account of the synthesis and structure−activity relationships of a wide range of thiazolides, plus some related salicyloyl anilides, against HBV. We also present a quantitative structure−activity relationship (QSAR) study, showing that the activities of thiazolides against HBV demonstrate excellent correlation for intracellular virions. Our results have led to the identification of 2-hydroxybenzoyl-*N*-(5-chlorothiazol-2-yl)amide **3** as a potent, selective anti-HBV agent. The *O*-glucuronide of **3**, the primary in vivo metabolite of this analogue, has been independently synthesized and evaluated.

CHEMISTRY

Coupling Methods. The synthesis of nitazoxanide **1** and analogues (Tables 1 and 2) without further aryl substitution is generally achieved straightforwardly from commercially available acetylsalicyloyl chloride (Scheme 1, X = H) or a substituted salicyloyl chloride. In the case of nitazoxanide **1** itself,[25] anhydrous coupling with 2-amino-5-nitrothiazole (Et_3N−THF) is necessary in view of the amine's low nucleophilicity. More nucleophilic 2-aminothiazoles, e.g., 5-Br and 5-Cl (both commercially available), can be successfully coupled in a two-phase system (CH_2Cl_2/aqueous $NaHCO_3$). Analogues **3**−**13** (Table 1) were made using one or

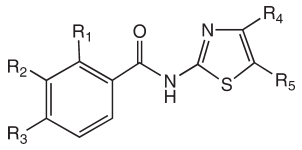
Table 1. Activities of 5′-Nitro and 5′-Halothiazolides against HBV Replication



compd	R_1	R_2	R_3	R_4	CC_{50},[a] μM	EC_{50}[b](VIR), μM	EC_{50}[c](RI), μM	EC_{90}[d] (VIR), μM	EC_{90}[e] (RI), μM	SI[f](VIR)	SI[g](RI)
2	H	H	H	NO_2	>100	0.15	0.46	0.58	1.20	>172	>83
3	H	H	H	Cl	38	0.33	1.00	0.89	2.90	43	13
4	Me	H	H	NO_2	85	0.64	2.10	3.5	12.0	24	7.1
5	H	Me	H	NO_2	>100	1.30	4.20	6.90	15.0	>14	>6.7
6	H	H	H	Br	>100	1.20	2.90	4.00	8.7	>25.0	>12.0
7	Me	H	H	Br	>100	3.50	7.60	9.0	22.0	>11	>4.5
8	Cl	H	H	Br	30.0	0.32	0.59	2.00	3.90	15	7.7
9	H	F	H	Br	12.0	2.10		5.60		2.1	
10	H	H	Cl	Br	45.0	0.21	3.50	1.10	11.0	41	4.1
11	Me	H	H	Cl	100	>10.0	>10.0	>10.0	>10.0		
12	H	H	Me	Cl	100	0.33	0.90	0.83	2.0	>120	>51
13	H	Me	H	Cl	100	1.00	2.70	3.30	6.3	>30	>16
14	H	H	H	F	51	3.1		12.0		4.3	

[a] Drug concentration at which a 2-fold lower level of neutral red dye uptake is observed relative to average level in untreated cultures.[34] [b] Drug concentration required to reduce extracellular (virion) HBV DNA by 50% relative to untreated cells. All EC_{50} and EC_{90} values were determined in triplicate, with standard deviations of ±20% of the value quoted. [c] Drug concentration required to reduce intracellular HBV DNA by 50%. [d] Drug concentration required to reduce extracellular (virion) HBV DNA by 90%. [e] Drug concentration required to reduce intracellular HBV DNA by 90%. [f] Selectivity index: CC_{50}/EC_{90} for extracellular HBV. [g] Selectivity index: CC_{50}/EC_{90} for intracellular HBV.

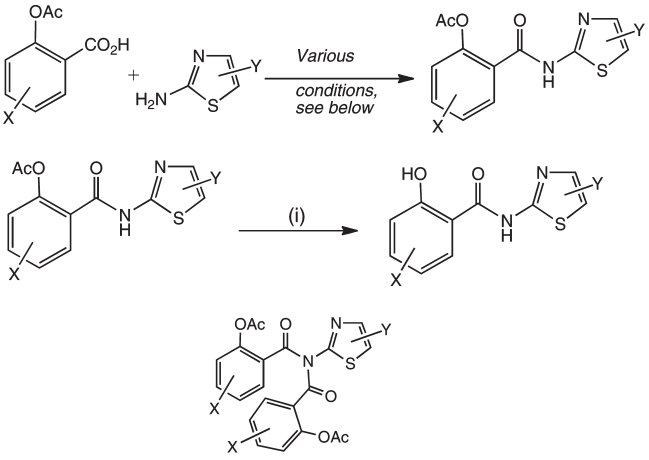
Table 2. Activities of Other Thiazolides Including Prodrugs against HBV Replication



compd	R_1	R_2	R_3	R_4	R_5	CC_{50},[a] μM	EC_{50}[b] (VIR), μM	EC_{50}[c] (RI), μM	EC_{90}[d] (VIR), μM	EC_{90}[e] (RI), μM	SI[f] (VIR)	SI[g] (RI)
1	AcO	H	H	H	NO_2	>100	0.12	0.59	0.83	2.10	>121	>48
16	OH	H	H	H	H	16.0	>10.0		>10.0			
17	OH	H	H	H	*i*-Pr	>100	>10.0	>10.0	>10.0	>10.0		
18	OH	H	H	H	C_6H_4Cl	>100	>10.0	>10.0	>10.0	>10.0		
19	OH	H	H	Ph	H	22.0	5.60		21.0		1.0	
20	OH	Me	H	Ph	H	>100	0.22	7.80	0.73	30.0	>137	3.3
21	OH	Cl	H	Ph	H	>100	0.15		0.60		>166	
22	H	H	OH	H	NO_2	>100	>10.0		>10.0			
23	OH	H	H	H	NHAc	>100	>10.0	>10.0	>10.0	>10.0		
24	OH	H	H	SO_2Me	H	>100	>10.0		>10.0			
25	OH	H	H	H	SO_2Me	>100	>10.0	>10.0	>10.0			
26	OH	H	H	H	CN	>100	3.2	5.5	9.4	14.0	>11	>7.1
27	OH	H	H	H	CO_2Me	15.0	>10.0		>10.0			
28	OH	H	H	H	CF_3	>100	3.8	9.1	11.0	27.0	>9.1	>3.7
29	EtO_2CO	H	H	H	NO_2	9.9	0.21	0.54	0.69	1.80	13	5.5

[a] Drug concentration at which a 2-fold lower level of neutral red dye uptake is observed relative to average level in untreated cultures.[34] [b] Drug concentration required to reduce extracellular (virion) HBV DNA by 50% relative to untreated cells. All EC_{50} and EC_{90} values were determined in triplicate, with standard deviations of ±20% of the value quoted. [c] Drug concentration required to reduce intracellular HBV DNA by 50%. [d] Drug concentration required to reduce extracellular (virion) HBV DNA by 90%. [e] Drug concentration required to reduce intracellular HBV DNA by 90%. [f] Selectivity index: CC_{50}/EC_{90} for extracellular HBV. [g] Selectivity index: CC_{50}/EC_{90} for intracellular HBV.

Scheme 1. General Synthetic Procedures for Thiazolides[a]



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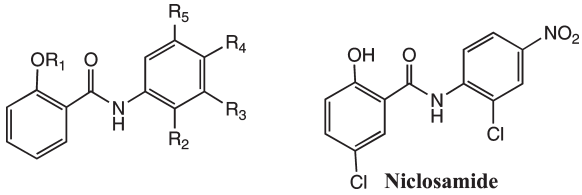
[a] (i) Coupling step: Carboxylic acid reacted with $SOCl_2$/pyridine or $(COCl)_2$/DMF, DCM. Then acid Cl was *either* added to amine in aqueous $NaHCO_3$−organic solvent with stirring *or* added to amine in dry THF with Et_3N. Alternatively the carboxylic acid and amine were reacted with BrPyBOP or BOP-Cl and NMM in CH_2Cl_2. Deprotection: aqueous HCl, 60 °C or aqueous NH_3.

other of these procedures. The fluoro analogue **14** required the prior synthesis of 2-amino-5-fluorothiazole (see below), followed by acylation as above. When the two-phase acylation method is used, bis-acylated products of type **15** may also be isolated. Their amounts decrease with time, in keeping with their base instability. Indeed, the imides have proved to be more base-labile than the phenolic acetates. The imides are inactive under the assay conditions described.

Similarly compounds **16**−**22** and **26**−**28** (Table 2) were obtainable by coupling of the appropriate 2-aminothiazole with the appropriate acid chloride. 2-Amino-4-phenylthiazole is also commercially available. Analogue **23** was prepared directly from nitazoxanide, and special chemistry was used for the sulfone analogues **24** and **25** (see below for these analogues). Finally, the salicyloylanilides **30**−**37** (Table 3) were made by acylation of the appropriate aniline with acetylsalicyloyl chloride. Compounds **35** and **37** were commercially available.

For aryl-substituted analogues where the acid chloride is not commercially available, e.g., 3-methylsalicylic acid, standard acid chloride forming conditions may be used from the *O*-acetyl acids [$(COCl)_2$/catalyst DMF or $SOCl_2$/pyridine]. In general, carbodiimide-based methods lead to very sluggish reactions even when HOBt and/or DMAP is added. However, both BrPyBOP[26] and BOP-Cl[27] gave reasonable yields with 2-amino-4-phenylthiazole, using NMM as base, though the reactions were slow (3−4 days at 20 °C). Both 2-acetoxy-3-methylbenzoic acid (see analogues **19** and **20**, Table 2) and 4-acetoxybenzoic acid could be coupled in this way: in the case of 2-acetoxy-3-chlorobenzoic acid, a reasonable yield was obtained using 3 equiv of BOP-Cl for an extended period. These conditions are less satisfactory for 2-amino-5-bromo- or 2-amino-5-chlorothiazole, however, with generally

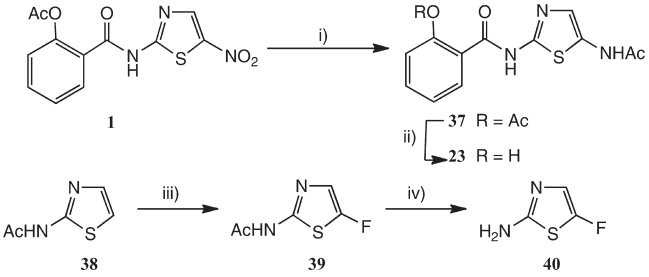
Table 3. Activities of Salicyloyl Anilides against HBV Replication



compd	R_1	R_2	R_3	R_4	R_5	CC_{50},[a] μM	EC_{50}[b] (VIR), μM	EC_{50}[c] (RI), μM	EC_{90}[d] (VIR), μM	EC_{90}[e] (RI), μM	SI[f] (VIR)	SI[g] (RI)
30	Ac	H	H	Cl	H	>100	3.50		9.7		11	
31	H	H	H	Cl	H	>100	>10		>10			
32	Ac	H	H	Br	H	>100	0.76	2.10	3.00	6.80	>33	>15
33	Ac	H	H	I	H	>100	0.20	0.73	1.20	3.30	>83	>30
34	H	H	H	NO_2	H	>100	>10		>10			
35	H	Cl	H	H	CF_3	>100	3.70		13.0			
36	Ac	H	CF_3	H	CF_3	>100	3.80		14.0		>7.1	
37	H	Me	H	NO_2	Cl	89.0	0.28	0.66	0.73	1.7	122	52
niclosamide						>100	>10.0		>10.0			

[a] Drug concentration at which a 2-fold lower level of neutral red dye uptake is observed relative to average level in untreated cultures.[34] [b] Drug concentration required to reduce extracellular (virion) HBV DNA by 50% relative to untreated cells. All EC_{50} and EC_{90} values were determined in triplicate, with standard deviations of ±20% of the value quoted. [c] Drug concentration required to reduce intracellular HBV DNA by 50%. [d] Drug concentration required to reduce extracellular (virion) HBV DNA by 90%. [e] Drug concentration required to reduce intracellular HBV DNA by 90%. [f] Selectivity index: CC_{50}/EC_{90} for extracellular HBV. [g] Selectivity index: CC_{50}/EC_{90} for intracellular HBV.

Scheme 2. Syntheses of 5′-Acetamido and 5′-Fluorothiazolides[a]



[a] (i) H_2−Raney Ni, Ac_2O; (ii) aqueous NH_3; (iii) SelectFluor, MeCN, heat; (iv) aqueous HCl/MeOH.

slow reactions resulting. In general the rapid acid chloride method is preferred here. Carpino's reagent HATU[28] gives a good yield when coupling 2-amino-5-chlorothiazole with 2-acetoxy-3-methylbenzoic acid but gives a very sluggish coupling between 2-amino-4-phenylthiazole and the same acid. In the Experimental Section we have only described the acid chloride methods in detail.

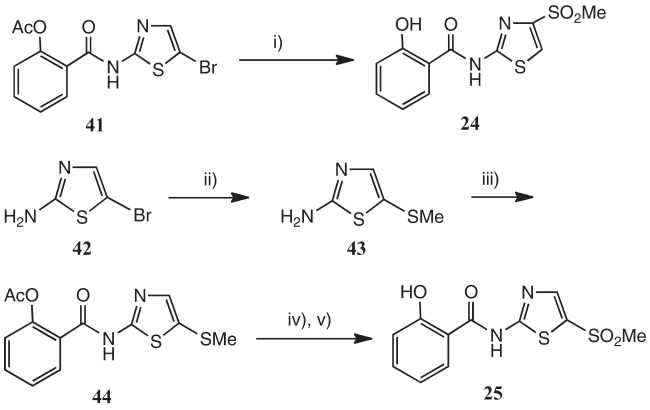
Other Thiazoles. Earlier results suggested that the nitro group was essential for activity against anaerobes, via inhibition of PFOR (see above), but not for antiviral activity. It was therefore of interest to study the reduction of nitazoxanide **1** (Scheme 2). This was achieved using Raney Ni/H_2, as reported for 2-acetamido-5-nitrothiazole.[29] The free 5-amino compound could not be isolated, but when the reaction was performed in Ac_2O, the diacetate **37** resulted in high yield; mild base-catalyzed deprotection afforded the free phenol **23**. This compound was antivirally inactive.

To prepare the 5′-F analogue (Scheme 2), 2-acetamidothiazole **38** was reacted with excess of SelectFluor[30] in MeCN. Optimum reaction (∼40% conversion to **39**) was achieved after about 3 h, with longer reaction leading to decomposition. Hydrolysis of the acetyl group afforded free amine **40**. After we had used this procedure, a report appeared confirming our result.[31] These authors, however, found that on a larger scale, treatment of Boc-2-aminothiazole with 2 equiv of ^{t}BuLi followed by addition of *N*-fluorobenzenesulfonimide and then acidolysis with HCl was a more reliable route to **40**. Coupling of **40** with acetylsalicyloyl chloride followed by standard deprotection gave the 5′-F analogue **14**.

Syntheses of the 4′- and 5′-sulfone analogues led to some interesting chemistry (Scheme 3). Heating the 5′-Br intermediate **41** with sodium sulfinate under CuI catalysis in DMF afforded a sulfone product in 37% yield. However, this product proved to be the 4′-sulfone **24** (concomitant deacetylation occurs) and not the 5′-isomer.[32] Several variations of this procedure, however, failed to reproduce the result; complex mixtures of products resulted. Subsequent rigorous comparison of **24** and the 5′-sulfone **25**, prepared unambiguously as described below, using HPLC and ^{1}H/^{13}C NMR studies confirmed that they were indeed isomeric products. Their physical properties are almost identical, but there are small characteristic differences in their ^{13}C NMR shifts and HPLC retention times.

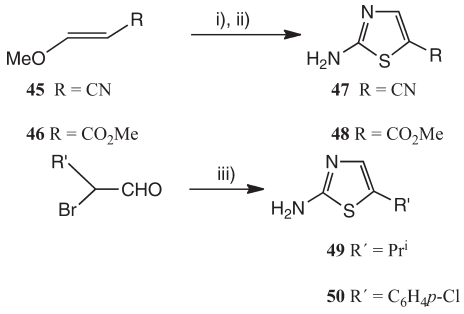
A reasonable mechanism for the sulfinate displacement reaction involves addition at C(4′), followed by a hydride shift and loss of Br^-. In fact, the 5′-sulfone may be unambiguously prepared by displacement of Br^- from 2-amino-5-bromothiazole **42** using MeSNa (Scheme 3), giving 2-amino-5-methylthiothiazole **43**. Reaction of **43** with acetylsalicyloyl chloride followed by peracid oxidation of the resulting 5′-SMe thiazolide **44** and then deprotection afforded **25**. Rather surprisingly, both these analogues were inactive against HBV (the 5′-methylthiothiazolide

Scheme 3. Syntheses of 4′-and 5′-Methanesulfonylthiazolides[a]



[a] (i) $MeSO_2Na$, CuI, DMF, heat; (ii) MeSNa, EtOH; (iii) acetylsalicyloyl chloride; (iv) $3\text{-}ClC_6H_4CO_3H$ (2 equiv); (v) aqueous NH_3.

Scheme 4. Syntheses of 5′-Aryl-, Alkyl-, Cyano-, and Methoxycarbonylthiazolides[a]



[a] (i) *N*-Bromosuccinimide, MeOH; (ii, iii) $(H_2N)_2C{=}S$.

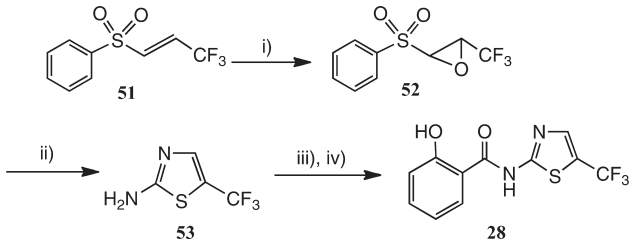
intermediate was also inactive) though as we have reported[23] the 4′-sulfone **24** is a potent inhibitor of HCV.

To prepare the 5′-cyano- and 5-methoxycarbonyl analogues **26** and **27**, methyl 3-cyano- **45** and methyl 3-methoxyacrylate **46** (Scheme 4) were treated with NBS and the crude bromo products condensed directly with thiourea. After purification, the 2-amino-5-substituted thiazole products **47** and **48** were condensed with acetylsalicyloyl chloride to give **26** and **27** after deacetylation. Recently both **47** and **48** have become commercially available. A similar Hantzsch synthesis via the appropriate α-bromoaldehyde (Scheme 4) was used for the synthesis of the isopropyl and 4-chlorophenyl analogues **17** and **18** via the appropriate thiazoles **49** and **50**.

Further Prodrugs. Although in general the *O*-acetyl derivatives serve as efficient prodrugs for the corresponding free phenols, we studied a range of other prodrugs made by acylation of tizoxanide **2**. The *O*-ethyl carbonate **29** (Table 2) is given as an example. A variety of other sterically bulkier prodrugs (not shown) were less effective in cell culture; nevertheless, their potential utility in animal PK and in vivo studies is being pursued.

Deacetylation. In general, free phenols are used in the biological assays for hepatitis B, although the *O*-acetates are equiactive. The acetates of the precursors may be cleaved using acid or base

Scheme 5. Synthesis of 5′-Trifluoromethylthiazolide 28[a]



[a] (i) MCPBA, K_2CO_3, CH_3CN, room temp to 40 °C; (ii) $NH_2C(=S)NH_2$ $(H_2N)_2CS$ (2 equiv), DMF, 75 °C; (iii) acetylsalicyloyl chloride, Et_3N, CH_2Cl_2; (iv) HCl, H_2O, THF, 50 °C.

catalysis: we have generally employed mild heating in aqueous HCl, as given in Scheme 1, but aqueous ammonia is also effective, as for the sulfone analogue **25**. Traditional Zemplen deprotection using catalytic methoxide is rather inefficient, probably because of the highly acidic NH proton.

Finally, the 5-trifluoromethyl analogue **28** was prepared (Scheme 5) from the αβ-unsaturated sulfone **51** which was itself obtained by a known procedure.[33] Base-catalyzed epoxidation of **51**, using MCPBA−K_2CO_3 in MeCN rather than ^{n}BuLi−TBHP in THF as described, followed by treatment of the epoxide **52** with thiourea afforded the desired 2-amino-5-trifluoromethylthiazole **53** in good yield.[34] Without further purification, **53** was coupled with acetylsalicyloyl chloride. The intermediate was deacetylated as described above (acid conditions) to afford the desired analogue **28**.

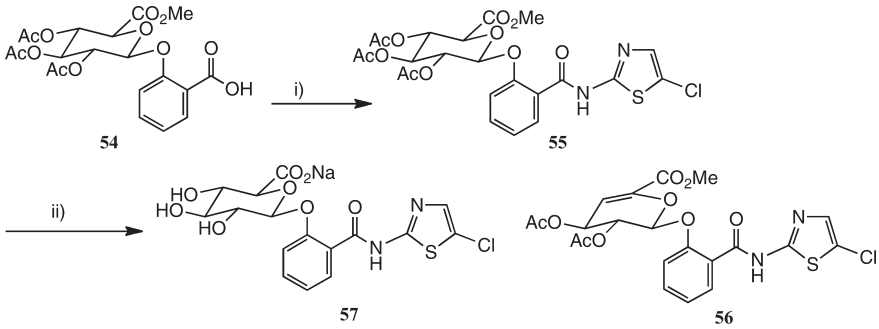
Glucuronide Metabolite. Once it was established that the *O*-aryl glucuronide was a general in vivo metabolite for this class, as already known for tizoxanide itself, it was important to prepare a reference sample of the glucuronide of the lead candidate **3**. The protected glucuronide of salicylic acid **54** prepared by us before[35] was condensed with 2-amino-5-chlorothiazole using EDCI and DMAP as catalyst (Scheme 6), giving a reasonable yield of the protected glucuronide **55**. It is important not to use too strong a base: when Et_3N was employed instead of DMAP, significant amounts of the unsaturated glucuronide **56** were obtained. This elimination is well-known in the literature,[36] but bases stronger than Et_3N have generally been used. Finally, hydrolysis of **55** led to the free glucuronide **57** which was isolated as its sodium salt.

RESULTS

Antiviral Activity. In general terms, the best activity against HBV was observed for thiazolides with electron-withdrawing groups at C(5′), especially 5′-nitro and 5′-halo, and these data, obtained in Hep G2 (2.2.15 cells),[37] are summarized in Table 1. Here the therapeutic (selectivity) index, SI, is shown for each analogue as a ratio of the cytotoxicity (CC_{50}) to efficacy (EC_{90}, the drug concentration at which a 10-fold depression of intracellular HBV DNA was observed relative to the average levels in untreated cultures). Both intracellular HBV replication and extracellular virus production were measured, using the virion (VIR) and replication intermediate (RI) assays, respectively.

Considering first tizoxanide **2** and other nitro analogues, introduction of a methyl group at position 3, 4, or 5 (not shown) or halogenation at these positions led to 5- to 10-fold loss of activity and selectivity, as typified in compounds **4** and **5**. Ortho-substitution as in **4** did not have such a drastic effect as in the

Scheme 6. Synthesis of the *O*-Glucuronide of 3[a]



[a] (i) 2-Amino-5-chlorothiazole hydrochloride, NMM, EDCI, DMAP, HOBt, CH_2Cl_2; (ii) NaOH, aqueous MeOH, then pH 6.

antiparasitic screens, where, as we have noted,[38] activity against a *Neospora* sp. was virtually abolished by a 3-Me group. In the 5′-bromo series, a reasonable level of activity, though at a lower level than the nitro compounds, was observed in compounds **6**−**10**. Here again a loss of activity for the 3-Me analogue **7** was noted: the 4- and 5-Me compounds (not shown) were superior but offered no advantage over **6**. The 3- and 5-chloro analogues **8** and **10** were rather more potent than **6**, but there were concerns over their reduced SI. By contrast, fluorinated analogues typified by **9** were significantly less active and demonstrated low selectivity.

The 5′-chloro analogues **3** and **11**−**13** showed significantly better activity than the bromo compounds and retained high SI values (as noted above, the direct 5′-Br analogues of **12** and **13** are not shown but were less active). Here the loss of activity in the 3-methyl analogue was more severe. Compound **11** was essentially inactive, but both the unsubstituted compound **3** and the 5-methyl analogue **12** exhibited submicromolar EC_{90} values. The 4-methyl compound **13** was less active than **3** or **12**. Nominally the activity of **12** was slightly superior to **3**, but it later proved to have an unfavorable metabolic/toxicity profile, probably owing to CYP oxidation to a reactive *p*-quinonemethide; this is discussed in more detail in the next section. The *p*-cresol structural element is indeed now regarded as a "structural alert".[39] The 5′-fluoro analogue **14** was significantly less active than **2**, **3**, or **6**, a trend also noted in the activities of halo analogues against *C. parvum*.[17b]

We screened a large number of other thiazolides, and the most interesting of these in structure−activity terms are summarized in Table 2. As stated above, nitazoxanide **1** is a prodrug of tizoxanide **2** and their activities are virtually identical in this assay. This may not be true under the conditions of all antiviral assays, depending on the local availability of esterases in each case. From a range of other possible prodrugs, the ethyl carbonate **29** was the most effective, though its SI appears less than that of **1**. The unsubstituted thiazole **16**[40] showed complete loss of HBV activity, and nonpolar, electronically neutral alkyl and aryl groups at C(5′) were also inactive, compounds **17** and **18**.

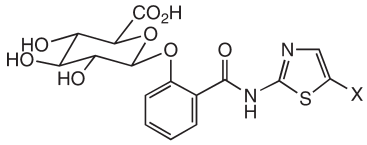
The 4′-phenyl analogues **19**, **20**, and **21** were of interest. With no further substitution in ring A, compound **19**, a modest level of activity was seen but with a low selectivity index. Introduction of a methyl or chloro group at R_2, however, led to a significant improvement in both potency and selectivity, as in compounds **20** and **21**. It would appear nevertheless that these compounds are significantly less effective against intracellular HBV RNA replication intermediates. Thus, the ratio of efficacy EC_{50}(VIR)/EC_{50}(RI) at about 1:35 is much less than that seen with the 5′-chloro analogues, about 1:3 (compare compound **3**, Table 1, with compound **20**, Table 2). Replacement of the 2-hydroxy group by 4-hydroxy as in **22**[41] led to complete loss of HBV activity.

Other heteroatom and electron-withdrawing C(5′) substituents were also screened, compounds **23**−**28**. The cyano and trifluoromethyl analogues **26** and **28** retained slight activity, but the 5-methoxycarbonyl analogue **27** was inactive, as were the acetamido analogue **23** and sulfonyl analogues **24** and **25**.

Finally, we prepared and screened a set of salicyloyl anilides and the activities of the most interesting, compounds **30**−**37**, are summarized in Table 3. In general the activity profile was more restricted than the thiazolides, and among monosubstituted analogues only the 8-bromo **32** and 8-iodo **33** analogues showed useful activity. Here the 8-nitro compound **34** was inactive. Solubility problems are probably responsible for the lack of activity of compound **31** (cf. acetate analogue **30**, which has low potency) and in these analogues the EC_{50} values of the *O*-acetates are probably more reliable. Among other analogues, the mono- and bis-trifluoromethyl derivatives **35** and **36** showed moderate activity and the trisubstituted **37** showed good activity. From a practical point of view, however, these molecules are close in structure to that of niclosamide (Table 3), which is inactive against HBV and has poor aqueous solubility and oral absorption. Additionally the scope for finding new compounds is much less; e.g., the free phenol form of **31** is known.[42]

In summary, the 5′-chloro analogue **3** was selected for further evaluation in view of its good activity against both extra- and intracellular HBV replication, EC_{90}/EC_{50} of about 3:1, and its good selectivity index (viz., low cell toxicity).

Drug Absorption, Distribution, Pharmacokinetics, and Metabolism. Nitazoxanide **1** and the thiazolides generally are rather insoluble compounds, and the free phenolic forms are highly protein bound (see below). Nevertheless, their log *P* (e.g., clogP = 1.24 for **1**) and other physicochemical parameters, notably H-bond donors (two) and acceptors (seven), are favorable for oral absorption according to well-known guidelines.[43,44] Indeed, nitazoxanide is well absorbed from the gastrointestinal tract:[45,46] when administered after food, the oral bioavailability rises to about 50%. This behavior contrasts with the poor absorption of the related series of *N*-salicyloylanilides, typified by the anthelmintic agent niclosamide (Table 3). In clinical trials vs hepatitis C, nitazoxanide is generally administered as two 500 mg tablets per day; recent clinical trial experience has helped to define the optimum dosing schedule.[47] Nitazoxanide, tizoxanide, and the newer thiazolides are strongly bound to plasma proteins,



57 X = Cl

58 X = NO_2

Figure 2. Thiazolide *O*-glucuronides.

typically >95%, but as we have reported,[23] the consequent raising of EC_{90} does not prevent an effective therapeutic concentration being achieved.

In ADME/Tox studies, compound **12** was not an inhibitor of 14 different CYP isozymes and showed complete stability in human blood and plasma after a 1 h incubation period. However, it was found to be >99% protein bound and was extensively metabolized by human liver microsomes and S9 fraction, with only 16% and 6% of the parent compound remaining after a 60 min incubation period. In rat and dog PK studies, **12** is not orally absorbed. For this reason, compound **3** is the preferred new generation thiazolide, as it shows no adverse effects against a panel of CYPs, nor does it show unfavorable drug−drug interactions. Full details of the pharmacokinetic study will be published elsewhere.

Deacetylation of **1** by blood plasma esterases is rapid ($t_{1/2}$ = 6 min), and subsequently tizoxanide **2** is extensively metabolized in the liver as its *O*-arylglucuronide **58**[35] (Figure 2), which is excreted in both urine and bile. This behavior is also general for the new thiazolides. We have reported that **58** retains some moderate activity against anaerobic bacteria, but it is essentially devoid of antiviral activity.[35] As noted above, the glucuronide **57** of compound **3** has also been independently synthesized as a standard. Pharmacokinetic studies for the new generation thiazolides, including prodrugs, are underway, and the results will be reported elsewhere.

Quantitative Structure−Activity Relationships. *Correlation of EC_{90} RI (Inhibition of Replication within Cells).* By use of the data in Tables 1−3, quantitative structure−activity relationships for the drug concentration required to reduce intracellular HBV DNA by 90%, pEC_{90} RI, were constructed with the GA-MLR method using autoscaled and filtered subset of the descriptor set generated by the Pipeline Pilot,[48] DRAGON,[49] and CDK[50] descriptor generation software as described in the Experimental Section.

Recent guidelines recommend that a training set for QSAR model development must contain at least 10 data points/compounds,[51] and other sources strongly recommend that an external test set should contain a minimum of 5 data points/compound.[52] As the EC_{90} RI data set contained 11 compounds, there were not a sufficient number data points to split into a training and test set and still have an adequate number for either; therefore, internal leave-one-out, leave-many-out and bootstrap internal validations were performed on the constructed models.

The quality of each model was evaluated by calculation of the coefficient of determination (r^2), the adjusted coefficient of determination (r^2_{adj}), mean absolute error (MAE), standard deviation of regression, *F*, average fold error, leave-one-out r^2 (r^2_{LOO}), 10-fold leave-many-out r^2 (r^2_{LMO}) averaged over 1000 runs, and bootstrap r^2 (r^2_{BS}) averaged over 5000 runs. Table 5 shows the contributing terms and performance statistics for pEC_{90} RI QSAR models generated with the three different fitness functions.

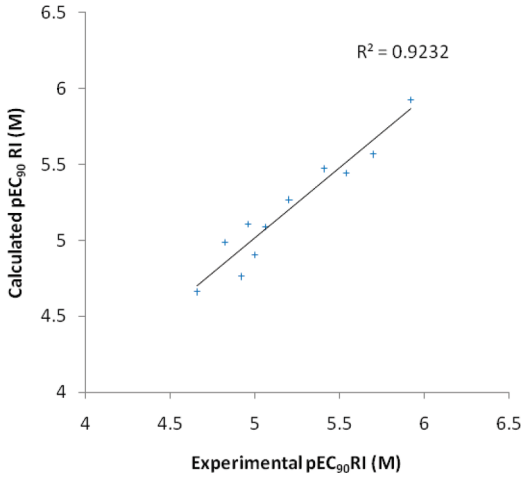


Figure 3. Predicted pEC_{90} RI against experimentally determined pEC_{90} RI for model 01A.

The QSAR models using r^2_{adj} and r^2_{adj} in leave-one-out validation as the fitness function yielded the same model as did the best model using average r^2 for bootstrap validation as the fitness function. This convergence of the descriptors that were selected by different fitness functions empirically indicates that this model is likely to be the best available with respect to this data set and set of descriptors. The descriptors within this model are IDDE, the mean information content on the distance degree equality,[53] BEHp7, the highest eigenvalue 7 of the Burden matrix weighted by atomic polarizabilities BCUT descriptors,[54] and ATSc3, the Moreau−Broto autocorrelation descriptors using partial charges.[55] Figure 3 shows a graph for predicted pEC_{90} RI against experimentally determined pEC_{90} RI for model 01A: it can be seen that there are no outliers as the residuals are of the same magnitude for all predicted data points.

The performance measures (Table 4) indicate a robust and predictive model with most measures falling within the recommended guidelines.[56] In particular r^2 is greater than 0.7, mean absolute error is less than 0.1, r^2_{LOO} is greater than 0.5, and the r^2_{BS} internal validation performance parameter is >0.5 which indicates a robust model.

Aside from giving an empirical model of pEC_{90} RI activity, these descriptors are not readily physically interpretable; therefore, a correlation was sought with other descriptors that are more physically interpretable. Descriptors with >0.75 correlation with the above three descriptors were identified (Table 2, Supporting Information), showing the identity of the descriptors as well as their correlation coefficient. For the BEHp7 descriptor, which is negatively correlated with the activity, there are a number of physically interpretable descriptors that are highly positively correlated to it. These are number of atoms, number of bonds, number of non-hydrogen atoms, number of non-hydrogen bonds, number of rotatable bonds, and rotatable bond fraction. This indicates that a small, rigid molecule is more likely to have activity.

Correlation of EC_{90} VIR (Inhibition of Virions Released from the Cell). Quantitative structure−activity relationships for the drug concentration required to reduce extracellular HBV DNA by 90%, pEC_{90} VIR, were constructed with the GA-MLR method using autoscaled and filtered subset of the descriptor set

Table 4. QSAR Models and Their Performance Statistics for the pEC_{90} RI (M) End-Point, As Determined by GA-MLR Using Different Fitness Functions for Subjective Descriptor Selection

	Subjective Descriptor Selection Fitness Function		
	model 01A r^2_{adj}	model 01B r^2_{adj} in LOO validation	model 01C r^2_{adj} in bootstrap validation
model activity EC_{90} RI, M	21.31−1.85 (IDDE)	21.31−1.85 (IDDE)	21.31−1.85 (IDDE)
	−3.30 (BEHp7)	−3.30 (BEHp7)	−3.30 (BEHp7)
	−23.63(ATSc3)	−23.63 (ATSc3)	−23.63 (ATSc3)
r^2	0.923	0.923	0.923
r^2_{adj}	0.904	0.904	0.904
MAE	0.088	0.088	0.088
SD of regression, M	0.131	0.131	0.131
F-value	28.046	28.046	28.046
av fold error	1.013	1.013	1.013
r^2_{LOO}	0.767	0.767	0.767
r^2_{LMO}	0.763	0.763	0.763
r^2_{BS}	0.528	0.528	0.528

Table 5. QSAR Models and Their Performance Statistics for the pEC_{90} VIR (M) End-Point, As Determined by GA-MLR Using Different Fitness Functions for Subjective Descriptor Selection

	Subjective Descriptor Selection Fitness Function		
	model 02A r^2_{adj}	model 02B r^2_{adj} in LOO validation	model 02C r^2_{adj} in bootstrap validation
model activity, EC_{90} RI, M	15.27−1.53 (BEHe8)	4.37 + 1.85 (X5sol)	74.81−63.96 (MATS6m)
	−70.97 (BEHp7)	−15.27 (VC-5)	+31.83 (ATSc2)
	+0.50 (khs.sCl)		
r^2	0.721	0.415	0.551
r^2_{adj}	0.666	0.361	0.511
MAE	0.185	0.288	0.231
SD of regression, M	0.273	0.376	0.329
F-value	7.762	3.540	6.153
av fold error	1.035	1.053	1.043
r^2_{LOO}	0.443	0.125	0.281
r^2_{LMO}	0.436	0.109	0.270
r^2_{BS}	−1.200	−1516.856	0.041

generated by the Pipeline Pilot, DRAGON, and CDK descriptor generation software as defined in the Experimental Section.

In analogy to the EC_{90} RI data set, the EC_{90} VIR data set contained 13 compounds; therefore, there were insufficient data points to split into training and test sets and still have an adequate number for either. Hence internal leave-one-out, leave-many-out and bootstrap internal validations were performed on the constructed models. Table 5 shows the contributing terms and performance statistics for pEC_{90} VIR QSAR models generated with the three different subjective fitness functions. All QSAR models constructed for the pEC_{90} VIR end point fall short of thresholds recommended for the mean absolute error and internal validation r^2 statistics. The performance of these models indicates that a robust model could not be constructed for the pEC_{90} VIR end point.

In summary, our SAR data were substantiated by a QSAR study based on the activities against intracellular virions which showed an excellent correlation. In the future we will aim to employ the QSAR to inform the molecular design of novel derivatives.

DISCUSSION AND MECHANISM OF ACTION

In summary, we have synthesized a range of thiazolides [2-hydroxyaroyl-*N*-(thiazol-2-yl)amides] together with some related salicyloyl anilides and screened them for inhibition of HBV replication. A number of the thiazolides exhibited submicromolar EC_{50} against both extracellular HBV virions and intracellular replication intermediates; the salicyloyl anilides were generally less potent. In general thiazolides with an electron-withdrawing substituent at C(5′), especially nitro and chloro, were most potent in this assay; substitution in the phenyl ring had less effect, with a 3-methyl group generally causing loss of potency. The introduction of a 3-chloro substituent improved potency at the cost of selectivity. Other electron-withdrawing 5′-substituents were significantly less effective, as were 5′-alkyl or aryl groups. Our results were substantiated by a QSAR study based on the activities against intracellular virions that showed an excellent correlation.

Analogues with a 4′-phenyl group were of interest. Although the activity of the unsubstituted aryl analogue was modest, here the introduction of a 3-methyl or 3-chloro substituent dramatically improved activity, the likely drawback being a significant increase in log *P* for this series. Finally a number of salicyloyl anilides, analogues of niclosamide, were screened. In general these compounds were less active, though it is interesting that here again analogues with electron-withdrawing substituents

were the most effective, particularly the *p*-iodo compound. The known poor oral absorption of this class of drug, in addition to the reduced scope for novelty, makes them less attractive for development than the thiazolides.

From the analogues described, 2-hydroxybenzoyl-*N*-(5-chlorothiazol-2-yl)amide **3** was selected for further development. In activity terms, this was very similar to the 5-methyl analogue **12**, but the latter proved to have an unfavorable metabolic profile. After scale-up, the pharmacokinetic behavior of the two chloro analogues, as their acetate prodrugs, will be determined in both rats and dogs and compared to that of nitazoxanide **1**. The results of these studies will be reported in due course.

The mechanism of action of the thiazolides is still under active investigation, but over the past few years some important findings have appeared. The inhibition of PFOR as a mechanism of action against anaerobes alluded to above[21] is postulated to be due to mimicry of TPP anion by the anion of nitazoxanide, without involving direct redox reactions of the nitro group. This does not explain, however, why other thiazolides with an acidic NH [in particular, those with strong electron-withdrawing groups at C(5)] apparently lack significant anaerobic activity. A very recent publication[57] reported the activity of a number of analogues of **1**, including some other heterocycles, against anaerobic bacteria. Here again activity was essentially confined to nitro analogues, including a dinitrothiophene amide. Also, it has been shown by affinity chromatography that **1** inhibits the action of a nitroreductase in *Giardia lamblia*.[58]

Turning to antiviral activity, studies in rotavirus have shown that nitazoxanide is cytoprotective;[24a] that is, it acts at a postentry level, as shown specifically in MDCK cells infected with influenza A virus.[59] Thus, when infected cells were treated with nitazoxanide between 0 and 6 h postinfection, viral replication was effectively inhibited and a single administration remained effective for up to 48 h. Pretreatment of cells with **1** prior to infection, however, was not cytoprotective. In the same study, it was shown that **1** effects post-translational modification of hemeagglutinin (HA), specifically by inhibition of the maturation of HA glycoprotein at a stage before resistance to endoglycosidase H digestion. However, it must be noted that in the same studies **1** was not found to be an inhibitor of cellular glycosidation pathways.[59]

In the case of HBV, it is known that **1** induces reductions in key HBV proteins, especially the surface antigen HBsAg, also HBeAg and HBcAg,[23] and in view of the observation with HA, it is probably significant that all these proteins are heavily glycosylated. However, there is no effect of **1** on levels of HBV RNA transcription, consistent with a post-translational mechanism. The difference in mechanism is valuable in that **1** has been shown to be synergistic with lamivudine and adefovir against HBV, and indeed it maintains equivalent activity against HBV strains resistant to those agents.[23]

Recent studies including clinical trial results have shown that **1** is active against different genotypes of the hepatitis C virus (HCV) and is also synergistic with interferon (with and without ribavirin), telaprevir (an HCV protease inhibitor), and 2′C-methylcytidine (an HCV RNA polymerase inhibitor).[47,60] In the most recent trials, phase II clinical studies carried out in 511 experienced and naive patients with chronic hepatitis C genotypes 1 (240 subjects) and 4 (271 subjects) combined with pegylated interferon-α 2a (Pegasys) with or without ribavirin showed that NTZ **1** added a 40−50% increase in the SVR rate of the standard of care combining pegylated interferon-α 2a and ribavirin.[61]

Here too, equivalent activity is maintained by **1** against representative drug-resistant strains of HCV.[60] HCV replicon-containing cell lines resistant to **1** have been isolated, but the resistance phenotype was not found to be transferred to naive cells by HCV replicons isolated from these cell lines, indicating that a cellular mechanism is most likely responsible for this property.[62] In other studies in HCV containing cell lines, **1** was observed to enhance the phosphorylation of eukaryotic initiation factor-2 α (eIF2α).[63] In the same report, **1** was found capable of enhancing the activity of PKR in enzymatic assays, a protein kinase activated by double-stranded RNA that is part of innate cell defense mechanisms and activates eIF2α via phosphorylation.[63] The weight of evidence therefore supports a mechanism involving stimulation of the host cell immune system. Most recently, it was discovered that in peripheral blood mononuclear cells from human volunteers thiazolides showed potent immunomodulating effect of both the innate and adaptive immune systems.[64]

In summary, it may be said that the mechanism of antiinfective action of nitazoxanide and other thiazolides is complex and most likely involves more than one pathway. The complete elucidation of the mechanism is a work in progress. In the case of antiviral activity, an increasing body of evidence is consistent with a post-translational, host-cell-mediated effect that may either stimulate innate cell defense processes or inhibit maturation of key viral proteins. What is beyond doubt is that the thiazolides are highly effective antiviral agents both in vitro and in vivo, as we have demonstrated here for HBV, and in further publications we shall report on their structure−activity properties against other important viruses.

EXPERIMENTAL SECTION

Chemical Procedures. Organic extracts were washed finally with saturated aqueous NaCl and dried over anhydrous Na_2SO_4 prior to rotary evaporation at <30 °C. Analytical thin-layer chromatography was performed using Merck Kieselgel 60 F 254 silica plates. Preparative column chromatography was performed on Merck 938S silica gel. Unless otherwise stated, ^{1}H and ^{13}C NMR spectra were recorded on $CDCl_3$ solutions using either Bruker 250 or 400 MHz (100 MHz for ^{13}C) instruments with tetramethylsilane as internal standard. The NMR data at 500 MHz (^{1}H) were obtained using a Varian instrument and the 125 MHz (^{13}C) using a Bruker AV2 500 MHz instrument operating at 125 MHz. Both low- and high-resolution mass spectra were obtained by direct injection of sample solutions into a Micromass LCT mass spectrometer operated in the electrospray mode, +ve or −ve ion as indicated. CI mass spectra (NH_3) were obtained on a Fisons Instruments Trio 1000. All compounds tested were analyzed by HPLC using an Agilent 1100 system, eluting with a variable percentage of MeCN in water containing 0.1% CF_3CO_2H, and were of at least 97% peak area purity.

HPLC data was collected on Agilent 1100HPLC systems with the following columns and conditions: A: Agilent Zorbax C8 75 × 4.6 mm 5 micron column (Part #993967-906) maintained at 30 °C. Solvent A Water (0.1% TFA); Solvent B Acetonitrile (0.07% TFA), Gradient 5 min 95% A to 95% B; 2 min hold; then recycle; UV Detection @ 210 and 250 nm. B: Agilent Zorbax Eclipse XDB-C18 50 × 4.6 mm 1.8 micron column (Part #927975-902) maintained at 30 °C. Solvent A Water (0.1% TFA); Solvent B Acetonitrile (0.07% TFA), Gradient 5 min 95% A to 95% B; 1 min hold; 1 min recycle; 30 sec hold. UV Detection @ 210 and 254 nm with no reference. C: Agilent Zorbax C8 150 × 4.6 mm 5 micron column maintained at 30 °C. Solvent A Water (0.1% TFA); Solvent B Acetonitrile (0.07% TFA), Gradient 10 min 95%

A to 95% B; 2 min hold; then recycle. UV Detection @ 210 and 254 nm with no reference.

Antiviral assays were performed as described previously.[37,65] In summary, confluent cultures of the chronically HBV-producing cell line, 2.2.15, were maintained on 96-well flat-bottomed tissue culture plates (confluence in this culture system is required for active, high levels of HBV replication equivalent to that observed in chronically infected individuals[37,65]). Cultures were treated with nine consecutive daily doses of the test compounds. HBV DNA levels were assessed by quantitative blot hybridization 24 h after the last treatment. Cytotoxicity was assessed by uptake of neutral red dye 24 h following the last treatment.

General Procedures for Acid Chloride Couplings and *O*-Acetate Deprotections. Acetylsalicyloyl chloride was used directly; substituted versions were made from the *O*-acetates as indicated below.

(1) Two-Phase Method. To a stirred solution of a protected salicylic acid (1 mmol) in dry Et_2O (10 mL) at 0 °C was added pyridine (1.2 mmol) followed by dropwise addition of thionyl chloride (1.2 mmol). Stirring was continued at 0 °C for 4 h. Then the white precipitate formed was filtered off, and the filtrate was concentrated under vacuum to give the acid chloride as an oil which was used without further purification. The appropriate 2-aminothiazole (1 mmol) was added to a vigorously stirred two-phase mixture of $NaHCO_3$ (3 mmol for a free base, 4 mmol for an HCl or HBr salt) in H_2O (3 mL/mmol) and EtOAc (3 mL/mmol). A solution of the above acid chloride in EtOAc (2 mL/mmol) was then added with vigorous stirring. The reaction mixture was stirred at 20 °C for 12 h. The layers were separated, and the aqueous layer was extracted once with EtOAc. The combined organic extracts were washed with 0.5 N HCl (2×), followed by brine. The organic layer was dried and concentrated under vacuum to give a pale yellow solid which was chromatographed on silica, eluting with EtOAc−hexane mixtures. In favorable cases trituration of crude product with Et_2O followed by drying the resulting solid gave the *O*-acetyl intermediate directly. This material was heated in concentrated aqueous HCl (3 mL/mmol) at 50 °C for 24 h. The reaction mixture was cooled to ambient temperature, then filtered and the solid washed with H_2O (distilled) until the washings were at neutral pH. The solid was dried under vacuum to give the product. On a small scale it was more efficient to extract the final product into EtOAc, followed by washing with H_2O (3×) and brine, then drying and evaporation. Chromatography of the final product, if necessary, was again carried out using EtOAc−hexane mixtures.

*2-Hydroxy-3-methylbenzoyl-N-(5-bromothiazol-2-yl)amide (**7**).* Yield: 75 mg, 66%. Mp 187−188 °C. Anal. ($C_{11}H_9BrN_2O_2S$) C, H, N. ^{1}H NMR [400 MHz, $(CD_3)_2SO$] 2.21 (3H, s, CH_3Ar), 6.86 (1H, d, *J* = 7.7 Hz, ArH), 7.39 (1 H, d, *J* = 7.2 Hz, ArH), 7.73 (1 H, s, 4′-H), and 7.95 (1 H, d, *J* = 7.8 Hz, ArH). ^{13}C NMR [100 MHz$(CD_3)_2SO$] 15.6, 101.9, 114.3, 118.8, 126.2, 126.6, 135.7, 136.3, 158.4, 159.9, and 168.8. MS (CI) *m/z* 313 and 315 (M^+ for ^{79}Br and ^{81}Br, respectively). HRMS, found, *m/z* 312.9641, $C_{11}H_{10}BrN_2O_2S$ (MH^+ for ^{79}Br) requires *m/z*, 312.9646.

(2) Anhydrous Conditions. Either the free amine was used directly or the HBr or HCl salt of the appropriate 2-aminothiazole was partitioned between dilute aqueous NaOH and EtOAc. Then the organic layer was separated, dried, and evaporated to dryness. A solution of the thiazole (1 mmol) in THF (3 mL) was added to a stirred solution of salicyloyl chloride (1 equiv) in THF (3 mL/mmol) at 0 °C before the addition of Et_3N (1 equiv). The solution was stirred at room temperature until reaction was complete. Then the reaction mixture was poured into water and extracted with ethyl acetate (2×). The organic layer was washed with 1 M HCl, water, dried, and evaporated. The product was purified by column chromatography to give the intermediate *O*-acetate, which was hydrolyzed as above to deliver the product.

*2-Hydroxybenzoyl-N-(5-chlorothiazol-2-yl)amide (**3**).* Mp 227−228 °C (dec). ^{1}H NMR [500 MHz, $(CD_3)_2SO$] 7.00 (1 H, t, ArH), 7.04 (1 H, d, ArH), 7.48 (1 H, t, 4-H), 7.60 (1 H, s, 4′-H), and 7.96 (1 H, d, 6-H). ^{13}C NMR [125 MHz, $(CD_3)_2SO$] 116.5, 117.1, 118.5, 119.7, 130.3, 134.6, 135.4, 155.8, 157.3, and 164.7. *m/z* (ES +ve ion mode) 277 (MNa^+, 100%). Found: *m/z*, 276.9806; $C_{10}H_7{}^{35}ClN_2O_2SNa$ requires *m/z*, 276.9809.

*3-Chloro-2-hydroxybenzoyl-N-(5-bromothiazol-2-yl)amide (**8**).* Yield: 0.116 g, 48%. Mp 200 °C. Found: *m/z*, 332.90930. $C_{10}H_7BrClN_2O_2S$ (MH^+) requires *m/z*, 332.91003. ^{1}H NMR [400 MHz, $(CD_3)_2SO$] 7.02 (1 H, t, *J* = 7.9 Hz, ArH), 7.69 (1 H, dd, *J* = 7.9 and 1.4 Hz, ArH), 7.84 (1 H, s, 4′-H) and 8.02 (1 H, dd, *J* = 7.9 and 1.4 Hz, ArH). *m/z* (CI, NH_3) 333 (MH^+, 35%).

*2-Hydroxy-5-methylbenzoyl-N-(5-chlorothiazol-2-yl)amide (**12**).* Mp 212−213 °C. Anal. ($C_{11}H_9ClN_2O_2S$) C, H, N. ^{1}H NMR [$(CD_3)_2SO$] 2.27 (3 H, s, CH_3Ar), 6.94 (1 H, d, *J* = 8.3 Hz, ArH), 7.29 (1 H, d, *J* = 8.1 Hz, ArH), 7.59 (1 H, s, 4′-H), and 7.78 (1 H, s, ArH). ^{13}C NMR [$(CD_3)_2SO$] 19.9, 99.1, 115.7, 117.0, 118.5, 128.5, 130.1, 135.3, 135.7, 155.0, and 164.1. MS (CI) *m/z* 269, 271 (MH^+ for ^{35}Cl, ^{37}Cl, respectively). HRMS, found, *m/z* 269.0149, $C_{11}H_{10}ClN_2O_2S$ (MH^+ for ^{35}Cl) requires *m/z*, 269.0151.

*2-Hydroxy-3-methylbenzoyl-N-(4-phenylthiazol-2-yl)amide (**20**).* Mp 180 °C. ^{1}H NMR (400 MHz, $CDCl_3$) 7.94 (1H, d, *J* = 8.1, ArH), 7.83 (2H, d, *J* = 8.5, 2 × ArH), 7.52−7.41 (4H, m, ArH), 7.2 (1H, s, ArH), 6.69 (1H, t, *J* = 7.7, ArH), 2.31 (3H, s, CH_3). ^{13}C NMR [125 MHz, ($CDCl_3$] 15.8, 108.4, 112.0, 118.7, 123.2, 126.0, 128.2, 128.8, 134.1, 136.4, 150.2, 157.2, 160.4, and 167.7. *m/z* (CI) 311 (35%, $[M + H]^+$); (ES +ve ion mode) 333 (MNa^+, 100%). Found: *m/z*, 333.0659; $C_{17}H_{14}N_2O_2SNa$ requires *m/z*, 333.0668.

See Supporting Information for the characterization of all other analogues made using either of the above methods. The thiazole precursors of compounds **17** and **18** are known.[66,67]

Acetate Deprotection, Basic Conditions. Mild base hydrolysis using aqueous ammonia is also used for deacetylation, as in the following example.

*2-Hydroxybenzoyl-N-(5-acetamidothiazol-2-yl)amide (**23**).* To a solution of nitazoxanide **1** (615 mg, 2.0 mmol) in acetic anhydride (~70 cm^3), Raney Ni was added with vigorous stirring. The reaction mixture was evacuated and twice refilled with hydrogen, then allowed to stir at room temperature under an atmosphere of hydrogen until the theoretical volume (~135 cm^3, 6.0 mmol) was consumed after 30 min. The reaction mixture was stirred for a few minutes and flushed with N_2 to allow the escape of hydrogen present in the flask. The solution was filtered through a sinter followed by the evaporation of solvent in vacuo. The crude product was redissolved in EtOAc (50 cm^3) and washed with excess saturated aqueous $NaHCO_3$. The organic fraction was again evaporated, and the crude product was dissolved in acetone (10 cm^3) and stirred for 2 h with 1 N HCl (5 cm^3) to hydrolyze overacetylated material. The reaction was neutralized with satd. aq. $NaHCO_3$ and extracted with EtOAc (4 × 20 cm^3). The combined organic fractions were evaporated, and the *O*-acetate of **23** was purified through flash column chromatography as a white amorphous solid (548 mg, 86%). Mp 176.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$) 2.08 (3H, s, CH_3), 2.25 (3H, s, CH_3), 7.16 (1H, s, CH), 7.26 (1H, dd, *J* = 0.6, 8.1 Hz, ArH), 7.39 (1H, td, *J* = 1.0, 7.6 Hz, ArH), 7.60 (1H, td, *J* = 1.6, 8.0 Hz, ArH), 7.75 (1H, dd, *J* = 1.6, 7.6 Hz, ArH), 11.0 (1H, s, NH). ^{13}C NMR (100 MHz, $CDCl_3$) 21.1, 22.6, 123.7, 126.1, 127.3, 130.0, 131.0, 132.7, 148.8, 166.8, 169.1, 169.4, 184.5, 201.8. *m/z* EI $[M + Na]^+$ 342 [$(M + Na)^+$, 100%]. Found: *m/z*, 342.0524; $C_{14}H_{13}N_3O_4NaS$ requires 342.0538. To a solution of this *O*-acetate (319 mg, 1.0 mmol) in acetone (5 cm^3), 20 cm^3 of aqueous NH_3 was added. Then the mixture was stirred overnight at room temperature, followed by evaporation. The crude mixture was dissolved in EtOAc (20 mL) and was washed with 1 M HCl. The desired product **23** was purified through flash column chromatography as a white solid (0.25 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) 2.10 (3H, s, CH_3), 6.98 (1H, t, *J* 7.0 Hz, ArH), 7.03 (1H, d, *J* 8.0 Hz, ArH), 7.18 (1H, s, ArH), 7.45 (1H, td, *J* = 1.75, 7.0 Hz, ArH), 8.01 (1H, dd,

J = 1.75, 8.0 Hz, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) 22.7, 117.3, 117.5, 119.8, 130.5, 130.7, 134.5, 158.2, 167.0. m/z CI $[M + H]^+$ 278 [100%]. Found: m/z, 278.05983; $C_{12}H_{12}N_3O_3S$ requires 278.05994.

*Ethyl 2-[(5-Nitro-1,3-thiazol-2-yl)carbamoyl]phenylcarbonate (**29**).* A solution of ethyl chloroformate (0.114 g, 1.11 mmol) in dry THF (3 mL) was added to a bright yellow solution of tizoxanide **2** (0.250 g, 0.942 mmol) and Et_3N (0.118 g, 1.16 mmol) in dry THF (6 mL), yielding a white precipitate. The mixture was stirred at 20 °C for 6 h, then concentrated in vacuo, and the residue was partitioned between water and dichloromethane. The organic layer was washed with water (2×), then with brine, dried, and concentrated to give crude **14** (0.343 g, >100%) as a yellow solid. The crude product was purified by chromatography using a gradient of 0−50% ethyl acetate in hexane. Appropriate fractions were combined and evaporated to give **29** (0.254 g, 80%) as a tan solid. Mp 159−161 °C. HPLC area purity 100% (conditions C). ^{1}H NMR [400 MHz, $(CD_3)_2SO$] δ 1.25 (t, J = 7 Hz, 3H), 4.22 (q, J = 7 Hz, 2H), 7.43 (dd, J = 1, 8 Hz, 1H), 7.48 (td, J = 1, 8 Hz, 1H), 7.71 (td, J = 2, 8 Hz, 1H), 7.88 (dd, J = 2, 8 Hz, 1H), 8.71 (s, 1H), and 13.69 (br s, 1H). MS (ES +ve mode) m/z 360.0 $(M + Na)^+$, 338.0 $(M + H)^+$and (ES −ve mode) m/z 336.1 $(M - H)^-$.

*Methyl 1-[2-N-(5-Chlorothiazol-2-yl)carboxamido]phenyl-2,3,4-tri-O-acetyl-β-D-glucopyranuronate (**55**).* Methyl 1-(2-carboxyphenyl)-2,3,4-tri-*O*-acetyl-β-D-glucopyranuronate **54**[35] (0.45 g, 1 mmol) was dissolved with 2-amino-5-chlorothiazole, hydrochloride (0.17 g, 1 mmol), DMAP (0.12 g, 1 mmol), and anhydrous 1-hydroxybenzotriazole (0.14 g, 1 mmol) in anhydrous CH_2Cl_2 (5 mL). The clear solution was stirred and cooled to 0 °C. Then *N*-methylmorpholine (0.11 mL, 1 mmol) and EDCI (0.19 g, 1 mmol) were added. The pale yellow solution was stored at 0 °C for 72 h, then diluted with EtOAc (30 mL) and washed with 5% aqueous citric acid (25 mL), backwashing with EtOAc. Then the combined organic phases were washed with saturated aqueous $NaHCO_3$, water, and brine, dried, and evaporated to give the crude product which was purified by chromatography, eluting first with a gradient of 30−100% EtOAc in hexane, then with 10% MeOH−$CHCl_3$. Appropriate fractions were combined and evaporated to give reasonably pure product as a pale yellow solid (0.31 g, 54%) which was recrystallized from CH_2Cl_2−EtOH−hexane to give **55** as off-white crystals (0.169 g, homogeneous by TLC). Concentration gave a second crop (0.032 g), also of good purity. Mp 245−246.5 °C (dec). ^{1}H NMR [500 MHz, $(CD_3)_2SO$] 1.91, 1.95, 2.03 (9 H, 3s, 3 × CH_3CO), 3.66 (3 H, s, CH_3O), 4.77 (1 H, d, J = 10.0 Hz, 5′-H), 5.00−5.10 (2 H, m), and 5.48 (1 H, m, 2′-H + 3′-H + 4′-H), 5.66 (1 H, d, J = 7.9 Hz, 1′-H), 7.18−7.25 (2 H, m, ArH), 7.50−7.60 (2 H, m, ArH), 7.58 (1 H, s, thiazole 4-H), and 12.6 (1 H, br s, NH). ^{13}C NMR [125 MHz, $(CD_3)_2SO$] 20.2, 20.3 (×2), 52.6, 68.9, 69.9, 70.9 (×2), 97.7, 115.4, 118.2, 122.9, 124.8, 128.9, 132.3, 135.7, 153.4, 155.7, 164.8, 167.0, 168.6, and 169.3(×2). m/z (ES +ve mode) 593 (MNa^+, 100%). Found: m/z, 593.0603. $C_{23}H_{23}ClN_2O_{11}S$ Na requires m/z, 593.0603.

Any traces of elimination byproduct **56** were distinguished by ^{1}H NMR [500 MHz, $CDCl_3$] 2.02, 2.13 (6 H, 2s, 2xCH_3CO), 3.76 (3 H, s, CH_3O), 5.40 (1 H, m), 5.44 (1 H, m), 6.10 (1 H, brs), 6.42 (1 H, m), 7.26 (1 H, s, thiazole 4-H), 7.32, 7.46, 7.62, and 8.13 (4 H, 4 m, ArH). See also ref 33.

*1-[2-N-(5-Chlorothiazol-2-yl)carboxamido]phenyl-β-D-glucopyranosiduronic Acid (**57**).* The ester **55** (0.159 g, 0.28 mmol) suspended in MeOH (1.5 mL) was stirred at 0 °C with 2.5 M NaOH (0.56 mL, added dropwise). After 2 h, allowing the mixture to regain ambient temperature, the reaction appeared complete by TLC. Glacial AcOH was added dropwise to achieve a pH of 5.6. Then EtOH (4 mL) was added and the mixture cooled to 0 °C. The resulting light beige solid was filtered, washed with ether, and dried to give substantially pure product (0.113 g, 90%). Analytical material was obtained by chromatography on Lichroprep (Merck), eluting with a gradient of 0−60% MeCN in H_2O. Appropriate fractions were combined and evaporated to give an off-white solid which was triturated with a few drops of moist EtOH and excess ether to afford the product, which was filtered, washed with ether, and dried to give highly pure **57** (0.066 g) as Na salt. ^{1}H NMR [400 MHz, $(CD_3)_2SO$ + D_2O] 3.25−3.40 (3 H, m, 2′-H + 3′-H + 4′-H), 3.65 (1 H, m, 5′-H), 5.15 (1 H, m, 1′-H), 7.22 (1 H, m, ArH), 7.41 (1 H, m, ArH), 7.59 (1 H, s, thiazole 4′-H), 7.61 (1 H, m, Ar H), and 7.84 (1 H, m, ArH). ^{13}C NMR[125 MHz, $(CD_3)_2SO$ + D_2O] 71.7, 73.3, 74.4, 76.1, 101.4, 116.6, 118.5, 121.6, 122.7, 130.8, 134.0, 135.9, 155.3, 155.6, 163.7, and 171.1. m/z (ES +ve mode) 452 (100%, M^+ for Na salt). HPLC analytical purity 98.4% at 280 nm (C_{18} reverse-phase column, MeCN−H_2O). m/z (ES +ve mode) 475 (100%). Found: m/z, 474.9945. $C_{16}H_{14}ClN_2O_8SNa_2$ requires m/z, 474.9949.

Quantitative Structure−Activity Relationship Methods. In order to assist in analyzing and interpreting the structure−activity relationships (SAR) associated with the 5′-nitro and 5′-halothiazolide compounds, quantitative structure−activity relationship (QSAR) models were developed for some of the biological end points/activities against HBV replication.

QSAR models were developed and validated for data concerning the drug concentration required to reduce intracellular HBV DNA by 90% (EC_{90} RI) and the drug concentration required to reduce extracellular HBV DNA by 90% (EC_{90} VIR). These data were chosen for their amenability toward modeling in terms of range of values and distribution of data (vide infra). The EC_{90} RI data set contains 11 compounds, and the EC90 VIR data set contains 13 compounds (see Tables 4 and 5). The range for the EC_{90} RI is 1.20−22.00 μM, while the EC_{90} VIR data spans from 0.58 to 12.0 μM. Both data sets are spread relatively evenly. The biological end points for these assays are reported in μM. For the development of QSAR models, however, the activities were converted to pEC_{90} values in M ($pEC_{90} = -\log_{10}(EC_{90}/10^6)$. These end-points offer the greatest range of activity of the biological end points for data sets containing low-micromolar or submicromolar activities. Given the above characteristics, these data sets were subject to QSAR analysis.

In total, 685 zero-, one-, and two-dimensional molecular descriptors/properties were calculated for the set of compounds using Pipeline Pilot Student Edition,[48] DRAGON Web version 3.0,[49] and CDK.[50]

The combined descriptor set of 685 variables was autoscaled and filtered using two objective selection methods. First, descriptors that had the same value for 80% of the data set were removed, as these contained minimal information. This left 554 and 557 descriptors for the EC_{90} RI and EC_{90} VIR data sets, respectively. Second, the CHORCHOP procedure[68] was used to eliminate one of a pair of descriptors that exhibited very high intercorrelation (r > 0.99). The procedure removed the descriptor whose distribution deviated the most from normal (as defined by maximum kurtosis 100). This left 37 descriptors for the EC_{90} RI data set and 46 descriptors for the EC_{90} VIR data set.

The multiple linear regression machine learning method coupled with genetic algorithm subjective descriptor selection (GA-MLR) as implemented in the PHAKISO program was used to relate the activities (Y) of a set compounds to their molecular descriptors (X) using a linear equation.[69,70]

The genetic algorithm was set to have population size 50, replacement rate 0.6, crossover rate 1.0, and maximum number of generations 100. The maximum number of descriptors allowed was set to 3 in order to follow the recommended 5:1 ratio of number of descriptors to molecule to minimize the occurrence of chance correlations. The subjective fitness function for descriptor selection in this case was chosen to be either the highest adjusted r^2 for the training set, leave-one-out validation, or bootstrap validation (averaged of 1000 repeats each validation). For the bootstrap method a number of descriptor selection runs were performed, and the best model of the multiple runs was selected. QSAR models were developed using all three adjusted R^2 criteria as a subjective fitness function, and the model that gave the best performance for training set and internal validation tests was selected for further analysis if minimum performance thresholds were met.

■ ASSOCIATED CONTENT

Supporting Information. Spectroscopic data for compounds **4**−**6**, **9**−**11**, **13**, **14**, **17**−**19**, **21**, **24**−**28**, **31**−**33**, and **35**−**37** and results from C, H, and N analyses for selected examples. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

BOP-Cl, bis(2-oxo-3-oxazolidinyl)phosphonic chloride; EDCI, *N*-(3-Dimethylamino)propyl-*N*′-Ethyl carbodiimide, hydrochloride; GA-MLR, genetic algorithm and multiple linear regression; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HBsAg, hepatitis B surface antigen; NMM, *N*-methylmorpholine; NTZ, nitazoxanide [2-hydroxybenzoyl-*N*-(5-nitrothiazol-2-yl)amide]; PFOR, pyruvate ferredoxin reductase; PyBroP, bromotripyrrolidinophosphonium hexafluorophosphate; QSAR, quantitative structure−activity relationship; SelectFluor, 1-chloromethyl-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane bis(tetrafluoroborate)

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