Sofosbuvir Thio-analogues: Synthesis and Antiviral Evaluation of the First Novel Pyridine- and Pyrimidine-Based Thioglycoside Phosphoramidates

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ABSTRACT: The synthesis and antiviral screening of the first reported series of pyridine- and pyrimidine-based thioglycoside phosphoramidates are herein reported. They were prepared through two synthetic steps: The first step is via coupling of mercapto-derivatized heterocyclic bases with the appropriate $\alpha$-bromo per-acetylated sugars. The second one is the hydrolysis of the acetate esters under basic conditions that were consequently conjugated with the phosphoramidating reagent to afford the desired thioglycoside prodrugs. Eight compounds were evaluated for their antiviral activities against different viral cell lines, namely, adenovirus 7, HAV (hepatitis A) HM175, Coxackievirus B4, and HSV-1 (herpes simplex virus type 1), in addition to the antiviral bioassay against ED-43/SG-Feo (VYG) replicon of HCV (hepatitis C virus) genotype 4a. Both compounds $5b$ and $11$ showed notable antiviral activity against Coxackie virus B4, reflected from the $CC_{50}$ values of 17 and 20 $\mu$g/100 $\mu$L and $IC_{50}$ values of 4.5 and 6.0 $\mu$g/100 $\mu$L, respectively. Same two compounds elicited remarkable activities toward herpes simplex virus type 1, represented by $CC_{50}$ values of 17 and 16 $\mu$g/100 $\mu$L and $IC_{50}$ values of 6.3 and 6.6 $\mu$g/100 $\mu$L, respectively. Combination of $11$ with acyclovir elicited a notable synergistic activity in comparison with acyclovir alone, as inferred from herpes simplex polymerase enzyme inhibitory assay values of 2.64 and 4.78 $\mu$g/100 mL, respectively. Only compound $11$ elicited a remarkable activity against HCV. Potential promising activities of compound $11$ have been shown with respect to $CC_{50}$, $IC_{50}$, and enzyme assay inhibitory activities.

1. INTRODUCTION

A considerable number of various illnesses are accounted for by DNA and RNA viruses in humans and animal hosts. Herpes simplex virus type 1 (HSV-1), which is a type of DNA virus belonging to the herpes virus family, is incriminated in the infection of mucocutaneous epithelial cells and in the establishment of some ganglionic sensory latencies. Acyclovir (Figure 1) has been referred to as the gold standard medication for the treatment of HSV infections.

In addition, human adenoviruses, which are another class of DNA-containing viruses, are responsible for various ocular, respiratory, and gastrointestinal tract infections. Although there is no current FDA-approved medication protocol for the treatment of adenovirus infections, the broad-spectrum antiviral drug cidofovir could be used in some responsive patients.

On the other hand, Coxackievirus B4 (CV B4), hepatitis A virus (HAV), and hepatitis C virus (HCV) are a few examples of enveloped single-stranded RNA-containing viruses. Coxackieviruses are commonly accused of causing viral myocarditis, accompanied with the development of pancreatitis,encephalitis, meningitis, pleurodynia, and hepatitis. While there are curative direct-acting antivirals (DAAs) for HCV, there is no specific medication for both CV B4 and HAV. Among the DAAs, curative treatments of HCV are the protease inhibitor simprevir, NSSA inhibitor daclatasvir, and polymerase NSSB inhibitor sofosbuvir.

About half of the number of the clinically used antiviral drugs is nucleos(t)ides; hence, the improvement of the overall tolerability and pharmacokinetic profile is of a major priority in the conceptual design and development of newly synthesized antiviral agents. One of the most successful strategies to surmount the poor bioavailability of nucleosides and to deliver the therapeutically active S-monophosphate is to mask the polar S-hydroxyl functionality with arylxy triester phosphoramidate. This methodology is known as “Protide” technology in which the bioavailable phosphoramidate prodrug, after absorption and distribution, releases its nucleoside monophosphate after two sequential hydrolytic events by an esterase-type enzyme such as cathepsin A and a phosphoramidase-type...
Thioglycosides, where the anomeric carbon of the sugar motif is connected to the proper heterocyclic or any other aglycon by a sulfur atom through a thioether bond, have proven to be of biological interest. As a part of our current project to explore synthetic methods for the preparation of S-glycosylated derivatives of heterocyclic nitrogen bases, we have recently reported on the synthesis and anticancer and antiviral activities of a number of acyclic and heterocyclic thioglycosides that have an interesting cytotoxic activity such as cyanoethylene thioglycosides, pyridine thioglycosides, pyrimidine thioglycosides, imidazole thioglycosides, pyrazole thioglycosides, triazole thioglycosides, oxadiazole thioglycosides, thiophene thioglycosides, quinoline thioglycosides, thienopyrazole thioglycosides, and pyrazolopyrimidine thioglycosides. We have reported that the thioglycosides of dihydropyridine shows a strong P-glycoprotein (Pgp) antagonist and has activity against human colon carcinoma cells. In light of these findings and our previous reports, the purpose of this work was to design and synthesize the novel and first reported pyridine- and pyrimidine-based thioglycoside phosphoramidates as sofosbuvir thio-analogues.

Owing to the endless mutations and the emergence of numerous multidrug-resistant viral strains, the arena of developing novel broad-spectrum, less toxic, and bioavailable antiviral medicines is warmly welcoming new frontiers. Driven by the aforementioned facts and aiming to explore novel
antiviral drug candidates of promising potency, selectivity, bioavailability, and safety profile, we have incorporated the phosphoramidate functionality to pyridine- and pyrimidine-based thioglycosides, exploiting the molecular hybridization and protide strategic approaches in the first reported thioglycoside phosphoramidates. It was suggested that such a combination is believed to give some insights into the efficacy of the regioselective phosphoramidate coupling was successfully achieved via the alkoxide generation of all of the unprotected hydroxyl groups of the sugar part of the nucleosides using the proper molar ratio of a strong base. In the current work, it was anticipated that the Grignard reagent isopropyl magnesium chloride would be sufficiently basic to abstract the protons from the free hydroxyl groups of the deacetylated nucleosides to furnish the desired alkoxide anions. The preferential regioselectivity was essentially based on the differential nucleophilicity of the formed alkoxide. The most nucleophilic alkoxide that is capable of performing the required nucleophilic attack on the electrophilic center of the phosphoramidate phosphorus would be the primary alkoxide due to both electronic and steric effects that are believed to play roles in orientation of the phosphoramidate tail of the targeted nucleotides through the $S_N\text{2}$ reaction, as proposed in Scheme 3.

2. RESULTS AND DISCUSSION

2.1. Chemistry. The per-acetylated pyridine and pyrimidine-based thioglycosides 3a, 3b, 10, 14a, 14b, 18a, and 18b have been synthesized through nucleophilic substitution coupling of tautomeric thiol-containing heterocyclic bases 1, 13, or 17 with the corresponding $\alpha$-bromo-sugar 2a, 2b, or 9. The glycosidic linkage that connects the heterocyclic base to the anomeric carbon of the sugar was through the sulfur atom of the thioamide moiety of the base rather than through the nitrogen atom, as depicted in Scheme 1. This thioglycosidic connection was elucidated and confirmed by X-ray crystallography of compounds 5a and 14b, as shown in Figures 2 and 3.

![Figure 2. X-ray crystal structure of compound 5a.](image1)

![Figure 3. X-ray crystal structure of compound 14b.](image2)

The unprotected thioglycosides 5a, 5b, 11, 15a, 15b, 19a, and 19b were uneventfully obtained by ammonolysis of the acetate esters under basic conditions of methanolic ammonia. The following regioselective phosphoramidate coupling was successfully performed using the proper molar ratio of a strong base. In the current work, it was anticipated that the Grignard reagent isopropyl magnesium chloride would be sufficiently basic to abstract the protons from the free hydroxyl groups of the deacetylated nucleosides to furnish the desired alkoxide anions. The preferential regioselectivity was essentially based on the differential nucleophilicity of the formed alkoxide. The most nucleophilic alkoxide that is capable of performing the required nucleophilic attack on the electrophilic center of the phosphoramidate phosphorus would be the primary alkoxide due to both electronic and steric effects that are believed to play roles in orientation of the phosphoramidate tail of the targeted nucleotides through the $S_N\text{2}$ reaction, as proposed in Scheme 3.

2.2. Antiviral Screening. A preliminary antiviral screening was initially performed for eight different nucleos(t)ides of the newly synthesized analogs that comprise both pyridine and pyrimidine thioglycosides at either the free unprotected or phosphoramidated levels. These tested compounds are 5b, 8a, 8b, 11, and 12 of the pyridine-based analogs, among which compounds 5b and 11 represent the unprotected nucleosides, while compounds 8a, 8b, and 12 are from the protide type. On the other hand, compounds 19a, 20a, and 20b are the tested ones from the pyrimidine-based congeners, of which only compound 19a represents the free unprotected analog, while both 20a and 20b are the phosphoramidate nucleotide analogs. These compounds were tested on both DNA- and RNA-containing viruses. Both adenovirus and HSV-1 are representatives of the DNA-containing viruses, while Coxackie virus B4, hepatitis A virus, and HCV are representatives of the RNA-containing viruses.

2.2.1. Cytotoxicity Assay. Cytotoxicity assay was carried out via cell morphology evaluation using an inverted light microscope and cell viability test applying the trypan blue dye exclusion method where the nontoxic doses of the tested compounds were determined based on the assay on FRHK-4, Hep2, BGM, Vero, and Huh 7.5 cell lines prior the application on the targeted viruses. The values of the nontoxic doses of the tested compounds ranged from 70 to 100 μg/mL, as shown in Table 1 and Figure 4. It could be inferred from the tabulated results of the nontoxic dose determination of the tested compounds that both compounds 5b and 11 showed the highest safety profile that could participate in a relatively wider therapeutic index compared to the rest of compounds. The higher the nontoxic dose value, the higher the safety profile; therefore, compound 11 exhibited the safest profile among all the tested compounds with 100 μg/mL nontoxic dose against all the tested cell lines, except against BGM cell line, which showed a 90 μg/mL value. The second safest compound is 5b that showed a 90 μg/mL nontoxic dose against all the tested cell lines, except against Hep2 cell line, which showed a 100 μg/mL value. On the other side, both compounds 19a and 20b showed the least safe dosing profile among the test compounds with 70–80 μg/mL nontoxic dose values against all the tested cell lines. The application of the previously determined nontoxic dilutions on different doses of the BGM cell lines measured the initial and final viral titers of Coxackie virus B4 and, hence, the mean percentage reduction of viral titers. As shown in Table 2, it could be revealed that both compounds 5b and 11 exhibited the most promising antiviral activities among...
the tested compounds with 70% mean percentage reduction of the viral titer compared to 10–23.5% of the rest of the evaluated candidates. These two compounds underwent further investigations by determining their CC50 and IC50 values, showing very close activity profiles with 17 and 20 μg/100 μL values for CC50 and 4.5 and 6 μg/100 μL values for IC50 of compounds 5b and 11, respectively (Table 3).

2.2.3. Determination of HAV HM175 and Adenovirus 7 Titers Using Plaque Assay. The values of mean percentage reduction of all the tested compounds against both hepatitis A virus HM 175 strain and adenovirus 7 did not reflect notable activity. These values were ranging between 10 and 30 mean percentage reductions against hepatitis A virus HM 175 strain and between 10 and 20 mean percentage reductions against adenovirus 7, as shown in Table 2.

2.2.4. Determination of Herpes Simplex Virus Type 1 Titers Using Plaque Assay. Furthermore, both compounds 5b and 11 showed the most prominent activity among all the tested compounds against herpes simplex virus type 1 with mean percentage reduction values of 90 and 83.3%, respectively, as depicted in Table 2. For these two most active compounds, CC50, IC50, and SI values were assessed in comparison with acyclovir. The CC50 values of compounds 5b and 11 were 17 and 16 μg/100 μL, respectively, versus 0.28 μg/100 μL for acyclovir. On the other hand, the IC50 values of 5b and 11 were 6.3 and 6.6 μg/100 μL, respectively, versus 0.07 μg/100 μL for acyclovir, as shown in Table 4.

2.2.4.1. Herpes Simplex Polymerase (HSP) Enzyme Assay for Compounds 5b, 11, and Acyclovir. Despite showing lower potency of both compounds 5b and 11 than acyclovir in the enzyme assay against herpes simplex polymerase, the combination of 11 with acyclovir elicited a notable synergistic activity in comparison with acyclovir alone (Table 5). This finding of potential synergism could recommend a highly effective combination therapy.

2.2.5. Antiviral Bioassay of Tested Materials against ED-43/SG-Feo (VYG) Replicon of Hepatitis C Virus Genotype 4a. Compound 11 exhibited a moderate antiviral activity against
HCVcc genotype 4 with just 50% mean percentage reduction for which both CC50 and IC50 values were assessed as 19 and 10 μg/100 μL, respectively, as tabulated in Table 6.

Furthermore, both compounds 5b and 11 were evaluated for their DDA (direct-acting antiviral activity) by measuring enzyme-inhibitory activities against two potential targets of HCV, namely, NS5B (nonstructural protein 5B) and HCV protease, in comparison to sofosbuvir. These enzyme assay results were in accordance to the previously mentioned data with respect to the superiority of compound 11 over 5b with respect to its potency and potential efficacy against HCV-RNA genome (Tables 7 and 8).
The remarkable synergistic potentiation upon combining compound 11 and sofosbuvir recommends a promising
combination therapy that may lead to a pan-genotypic combotherapy.

3. CONCLUSIONS

We have achieved the first report of pyrindine and pyrimidine thioglucoside phosphoramidates as sofosbuvir thio-analogues. The structures of the synthesized compounds were confirmed by the spectral data, and the thioglucosidic linkage was elucidated by X-ray crystallography. The compounds were evaluated for their antiviral activities against different viral cell lines, namely, adeno virus 7, HAV (hepatitis A) HM175, Coxsackievirus B4, and HSV-1 (herpes simplex virus type 1) in addition to the antiviral bio assay against ED-43/S-G Feo (VYG) replicon of HCV (hepatitis C virus) genotype 4a. Compounds 5b and 11 showed notable antiviral activity against Coxsackievirus B4 and herpes simplex. Only compound 11 showed a moderate activity against HCV especially with the synergistic combination with sofosbuvir that may lead to a powerful combination therapy. All the tested thioglycoside prodtides showed low to moderate activities against the tested viruses; however, retaining some antiviral potential especially for 5b and 11, which are the prodrugs of 5b and 11, respectively, recommends their prolonged activities and the expected better pharmacokinetic profile. This may recommend further investigational pharmacokinetic studies in the combinations of sofosbuvir with the corresponding phosphoramidate prodrugs 5b and 11.

4. EXPERIMENTAL PART

All melting points were measured on a Gallenkamp melting point apparatus. The 1H NMR and 13C NMR spectra were measured on a Jeol-500 MHz spectrometer in DMSO-d6 or CDCl3 using Si(CH3)4 as an internal standard at the Faculty of Pharmacy, Ain Shams University, Cairo, Egypt. Elemental analyses were carried out at the Microanalytical Unit, Faculty of Science, Cairo University. Progress of the reactions was monitored by TLC using aluminum sheets coated with silica gel F254 (Merck). Viewing under a short-wavelength UV lamp effected detection. X-ray data were measured at the Institut fur Anorganische und Analytische Chemie, Technische Universität Braunschweig, Braunschweig, Germany. Pyridine-2-(1H)-thiones 1, 13, and 17 and their corresponding thioglucosides were prepared following our literature procedures.

4.1. General Procedure for the Synthesis of Acetylated Thioglucosides 3a, 3b, 10, 14a, 14b, 18a, and 18b. To a solution of pyridinethione 1 (0.01 mol) in dry DMF (20 mL), NaH (15 mmol) was added portion-wise through 15 min and the solution was stirred at room temperature for another 30 min. Then, a solution of 2,3,4,6-tetra-O-acetyl-a-D-gluco (or galacto)pyronosyl bromide was dropped within 30 min and the reaction mixture was stirred at room temperature until completion (TLC, 3−6 h). After completion, the reaction mixture was poured on ice water to remove the potassium bromide formed. The product was filtered off, dried, and crystalized from ethanol.

4.2. General Procedure for the Synthesis of Unprotected Pyridine Thioglucosides 5a, 5b, and 11 and Pyrimidine Thioglucosides 15a, 15b, 19a, and 19b. Dry gaseous ammonia was passed through a solution of protected glycosides 3a, 3b, 10, 14a, 14b, 18a, or 18b in dry methanol (20 mL) at 0 °C for 10 min. Then, the mixture was stirred at 0 °C for 2 to 6 h. The mixture was evaporated under reduced pressure at 60 °C to give a solid residue, which was crystallized from ethanol.

4.3. General Procedure for the Synthesis of Pyrimidine/Pyridine Phosphoramidate Nucleotides. A flame-dried flask kept under a nitrogen atmosphere was loaded with the unprotected pyridine thioglucosides 5a, 5b, and 11 or pyrimidine thioglucosides 15a, 15b, 19a, and 19b (1 molar ratio) at 25 °C THF, the formed suspension was allowed to stir and cool down to −5 °C, and then iPrMgCl (1.88 M in THF) was added in a dropwise manner through a dropping funnel without exceeding 0 °C for a period of 1 h. At the end of the addition, bring the temperature to 15−18 °C in about 30 min and keep the reaction mixture under these conditions for 30 min. Cool down to 0−5 °C and, in the meantime, prepare solution of 7 in THF by adding solution 7 to the main reaction mixture through a dropping funnel without exceeding 5 °C in a period of 1−2 h. At the end of dropping, wash the dropping funnel with THF and keep the reaction mixture under stirring at 0−5 °C for about 18−22 h, as monitored in TLC. When the reaction is over, add to the reaction mixture at 0−5 °C a solution of ammonium chloride, leave the reaction temperature to increase to about 10 °C, and then add a few drops of HCl. Bring the mixture to 25 °C, separate the phases, separate the upper THF organic layer, distill it out under vacuum, then add it to the residue DCM, wash it six times with 2% sodium carbonate, and then wash it with 0.5 N HCl and with brine. Filter the organic layer on Celite/charcoal.

Table 5. HSP Enzyme Assay % Inhibition and IC50 Values for Compounds 5b and 11 and Their Combination with Acyclovir

| compound no. | % inhibition | HSP IC50 (μg/mL) |
|--------------|--------------|-----------------|
| 5b           | 77           | 9.99            |
| 11           | 83           | 7.92            |
| acyclovir    | 84           | 4.78            |
| 5b + acyclovir| 84.6         | 4.34            |
| 11 + acyclovir| 88           | 2.64            |

Table 6. CC50 and IC50 Values of Compound 11 against HCVcc Genotype 4

| compound no. | mean % reduction | non-toxic dose (μg/100 μL) | CC50 (μg/mL) | IC50 (μg/mL) | SI |
|--------------|------------------|----------------------------|--------------|--------------|----|
| 11           | 50%              | 10                         | 19           | 10           | 1.9|

Table 7. HCV NS5B Enzyme Assay % Inhibition and IC50 Values for Compounds 5b and 11 and Their Combination with Sofosbuvir

| compound no. | % inhibition | HCV NS5B IC50 (μg/mL) |
|--------------|--------------|------------------------|
| 5b           | 52           | 17.83 ± 1.09           |
| 11           | 83           | 4.48 ± 0.9             |
| sofosbuvir (sov.) | 87           | 2.06 ± 0.34           |
| 5b + sov.    | 68           | 7.65 ± 0.72            |
| 11 + sov.    | 90           | 1.81 ± 0.29            |

Table 8. HCV Protease Enzyme Assay % Inhibition and IC50 Values for Compounds 5b and 11 and Their Combination with Sofosbuvir

| compound no. | % inhibition | HCV protease IC50 (μg/mL) |
|--------------|--------------|---------------------------|
| 5b           | 41           | 19.54 ± 1.3               |
| 11           | 85           | 3.18 ± 0.27               |
| 5b + sov.    | 61           | 8.23 ± 0.61               |
| 11 + sov.    | 91           | 1.04 ± 0.08               |
pad and wash the filter with DCM, distill it out under vacuum, and crystallize the residue from the appropriate solvent.

4.3.1. 3-Cyano-4,6-diethyl-2-((β-β-glucopyranosylthio)-pyrimidine (5a). White powder; EtOH; yield, 75%; mp 226–228 °C; IR (KBr, cm⁻¹): ν 3462 (OH), 2968 (CH), 2206 (CN), 1601 (C=Н); 1H NMR (400 MHz, DMSO-д₆): δ 2.48 (s, 3H, CH₃), 2.53 (s, 3H, CH₃), 3.14–3.18 (m, 2H, H-6, H-6'), 3.23–3.26 (m, 2H, H-4, H-4'), 3.99–3.41 (m, 1H, H-3, 3H'), 3.59–3.61 (m, 1H, H-2), 4.49 (t, 1H, Jᵣ₋ₓ₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋之內容


4.3.2. 6-Dimethyl-2-((β-β-glucopyranosylthio)-pyrimidine (5b). White powder; EtOH; yield, 82%; mp 199–201 °C; IR (KBr, cm⁻¹): ν 3452 (OH), 2939 (CH), 2211 (CN), 1597 (C=Н); 1H NMR (500 MHz, DMSO-д₆): δ 2.41 (s, 3H, CH₃), 2.49 (s, 3H, CH₃), 3.42–3.49 (m, 2H, 2H-6), 3.53–3.59 (m, 1H, H-3), 3.75 (t, 1H, Jᵣ₋ₓ₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~
Hz, J1 − J4 = 3.1 Hz, H-3′), 3.74–3.75 (m, 1H, CH), 4.49–4.52
(m, 2H, CH2), 4.94 (s, D2O exch., 3H, 2′-OH, 3′-OH, and
4′-OH), 5.12 (m, 1H, CH), 5.32 (s, 1H, OH), 5.36 (s, 1H, OH),
5.54 (m, 2H, H-3′), 7.24–7.35 (m, 5H, C6H5), 8.16 (s, 1H, pyridine H-5), 10.26 (s, 1H, NH).

Anal. calcd. for C24H34N3O9PS (571.58): C, 52.43; H, 5.75; N,
7.06; S, 5.53%. Found: C, 52.35; H, 6.16; N, 7.35; S, 5.61%.

3.14. Isopropyl-2-(((3R,3S,5R,6R)-6-ethyl-
((hydroxymethyl)-2H-pyran-2-ylthio)-4-methyl-6-phenylpyr-
imidine-5-carboxylate)-3,4,5-trihydroxytetrahydro-2H-
pyran-2-yl)methoxy)(phenoxy)phosphoryl)amino)-propanoate
(20b). White powder; yield, 98.5%; mp 134 °C; 1H NMR
(500 MHz, DMSO-d6): δ 1.24 (d, 3H, CH3), 1.32 (d, 6H,
2CH3), 2.44 (q, 2H, CH2), 3.34 (s, 1H, OH), 3.45 (s, H, OH),
3.52 (s, H, OH), 3.91 (m, 2H, 2H-6′), 4.23–4.25 (m, 1H, H-1′),
5.25–5.54 (m, 2H, H-3′, H-1′), 5.64 (d, 1H, H-2′), 7.35–7.75 (m,
10H, Ar-H), 10.28 (s, 1H, NH). Anal. calcd. for C34H37N5O11PS
(705.71): C, 54.46; H, 5.71; N, 5.95; S, 4.54%. Found: C, 54.36;
H, 5.65; N, 5.84; S, 4.42%.

4.4. Antiviral Screening Methods. 4.4.1. Cytotoxicity
Test. It was done according to Simões et al.,40 where samples (50
mg) were dissolved in 1 mL of DMSO. Decontamination of
samples was done by adding 24 μL of 100X of the antibiotic–
antimycotic mixture to 1 mL of each sample. Afterward, bifid
dilutions were carried out to 100 μL of original dissolved
samples and 100 μL of each dilutions was inculated in Hep-2,
Vero, BGM, FRHK4, and Huh 7.5 cell lines (obtained from the
Holding Company for Biological Products & Vaccines
VACSDRA, Egypt) previously cultured in 96-well multwell plates
(Greiner Bio-One, Germany) to estimate the nontoxic dose of
the tested samples. Cytotoxicity assay was done through cell
morphology evaluation using an inverted light microscope and
cell viability test applying the trypsin blue dye exclusion method.

4.4.2. Cell Morphology Evaluation by Inverted Light
Microscopy. Hep-2, Vero, BGM, FRHK4, and Huh 7.5 cell
cultures (2 × 105 cells/mL) were prepared separately in 96-well
tissue culture plates (Greiner Bio-One, Germany). After 24 h
incubation at 37 °C in a humidified 5% (v/v) CO2 atmosphere,
cell monolayers were confluent, and the medium was removed
from each well and replenished with 100 μL of bifold dilutions
of different samples tested prepared in DMEM (GIBCO BRL).

For cell controls, 100 μL of DMEM without samples was added. All
cultures were incubated at 37 °C in a humidified 5% (v/v) CO2
atmosphere for 72 h. Cell morphology was observed daily for
microscopically detectable morphological alterations, such as
loss of confluence, cell rounding and shrinking, and cytoplasm
granulation and vacuolization. Morphological changes were
scored (Simões et al.).

4.4.3. Cell Viability Assay. It was done through the trypsin
blue dye exclusion method (Walum et al.).11 Hep-2, Vero,
BGM, FRHK4, and Huh 7.5 cell cultures (2 × 105 cells/mL)
were grown in 12-well tissue culture plates (Greiner Bio-One,
Germany). After 24 h incubation, the same assay described
above for tested samples cytotoxicity was followed by applying
100 μL of tested samples dilutions (bifold dilutions) per well.

After 72 h, the medium was removed, cells were trypsinized, and
an equal volume of 0.4% (w/v) trypsin blue dye aqueous solution
was added to the cell suspension. Viable cells were counted under
the phase contrast microscope.

4.4.4. Determination of Adenovirus 7, HAV HM175,
Coxsackievirus B4, and Herpes Simplex Virus Type 1 Titters
Using Plaque Assay. Nontoxic dilutions were mixed (100 μL)
with 100 μL of different doses of adenovirus 7, HAV HM175,
Coxsackievirus B4, and herpes simplex virus type 1 (1 × 105,
1 × 106, and 1 × 107). The mixture was further incubated for half an
hour at 37 °C. The inoculation of (100 μL) 10-fold dilutions of
treated and untreated adenovirus 7, HAV HM175, Coxsacki-

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ievirus B4, and herpes simplex virus type 1 was carried out separately into Hep-2, FRHK4, BGM, and Vero cell lines, respectively, in 12-multwell plates. After 1 h of incubation for adsorption at 37 °C in a 5% CO₂-water vapor atmosphere without constant shaking, the plates were shaken intermittently to keep the cells from drying. After the appropriate incubation period, the cells were stained with 0.4% crystal violet after formalin fixation, and the number of plaques was counted. The viral titers were then calculated and expressed as plaque-forming units per milliliter (pfu/mL) (Schmidtke et al.).

4.4.5. Antiviral Bioassay of Tested Materials against ED-43/SG-Feo (VYG) Replicon of HCV Genotype 4a. ED-43/SG-Feo (VYG) replicon of HCV genotype 4a was treated with the nontoxic dose of the tested materials. HCV RNA was quantified in algal extract-treated Huh 7.5-infected cells using qRT-PCR (Taqman probe kit, Qiagen) and according to the manufacturer’s instructions to show a dose-dependent decrease in subgenomic RNA copies according to Saeed et al.45

### ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01364.

All spectral analysis such as IR, ¹H NMR, and ¹³C NMR spectra for the newly synthesized compounds (PDF)

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Notes

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