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Synthesis and biological evaluation of 2-oxonicotinonitriles and 2-oxonicotinonitrile based nucleoside analogues

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A B S T R A C T

Drug resistance and emergence of new pathogens highlight the need for developing new therapeutic agents. We focused on 2-oxonicotinonitrile (2-ONN) as derivative of the natural product 2-pyridinone.[1] Herein, we describe the synthesis of 2-ONNs bearing two aryl groups, which we coupled with organo-halides, including three glycosyl bromides, to prepare the nucleoside analogues. Coupling occurred mostly at the 2-ONN ring nitrogen to give the aimed targets, and in a few cases, it happened at the 2-oxo position giving *O*-alkylation products. Free 2-ONNs and their acetylated nucleosides were tested against a number of viruses. The nucleoside analogue **2a_{Ac}** showed good anti SARS-CoV and anti influenza A (H_5N_1) activities. Additionally, **7b** had good activity against Gram positive bacterium, *Bacillis subtilis*.

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1. Introduction

Drug resistance in microbial and viral infections has become a marked problem causing the failure of many treatments. The constant search for new drugs to be used alone or in combination with existing market drugs has become a necessity. In continuation of our efforts in finding such drug candidates, we have focused on the investigation of 2-oxonicotinonitrile (2-ONN) derivatives containing two aryl groups at the four- and six- positions of the ring. The 2-ONN ring contains the biologically important, naturally occurring 2-pyridinone motif [1,2]. This motif has been found in compounds that are human immune deficiency virus 1 (HIV-1) nonnucleoside reverse transcriptase inhibitors [3—5], anti-herpes simplex virus 1 (HSV-1) agents [6], hepatitis C virus (HCV) NS5B polymerase inhibitors [7], and anti-hepatitis B virus (HBV) agents [8]. In addition to their antiviral activity, 2-pyridinone derivatives are known to possess activity against wild type [9,10] and resistant [11] bacterial strains. The derivatives have also been shown to be antifungal [12] and antiprotozoal agents [13,14].

Herein, we discuss the synthesis of three 2-ONN diaryl derivatives (**1a**—**c**, Fig. 1) for antiviral and antimicrobial activity testing. For enhancing their potency as antiviral agents, our 2-ONN compounds were coupled with different halo-sugars and halo-alkyl derivatives, to make the corresponding nucleoside and acyclic nucleoside analogues. Nucleoside analogues are well known to exhibit potent antiviral activity [15].

2. Results and discussion

2.1. Chemistry

2-ONN derivatives **1a**—**c** (Fig. 1) were synthesized by one-pot condensation [16,17] of ethyl cyanoacetate with the corresponding aromatic ketone and aromatic aldehyde, in presence of ammonium acetate in refluxing ethanol (see Scheme 1). The 2-ONN ring was attached to 2-pyridyl (Py) and *p*-chlorophenyl (PhCl) groups in **1a**, to 2-thienyl (Th) and 4-isopropylphenyl (PhPr) groups in **1b**, and to two Th groups in **1c**. Compounds **1a** [18] and **1c** [19] were reported to be prepared through their respective chalcones in two steps from the aldehyde and ketone. However, the one-pot method that we adapted afforded a shorter route for their synthesis. Compounds **1a**—**c** were obtained as the amido tautomer as suggested by the presence of bands in the IR spectrum characteristic for NH and C=O of the amide functional group. This was also

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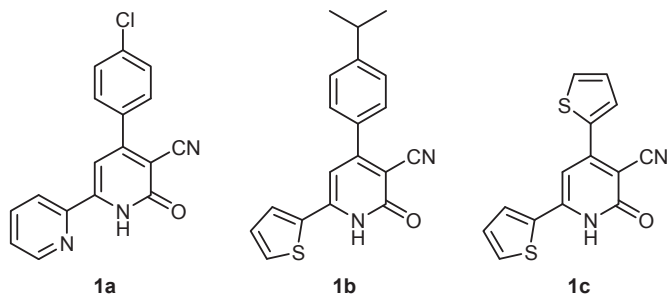[1] 2-ONN: 2-oxonicotinonitrile.

Fig. 1. Structures of three synthesized 2-ONN derivatives.

emphasized by the presence of an ^{1}H NMR peak at a chemical shift higher than 12 ppm, corresponding to the amide NH. Obtaining the amido tautomer rather than the imido was explained by a recent study showing that the amido tautomer was more stable and hence predominant [20].

Derivatives **1a**−**c** were coupled with different organohalides in presence of K_2CO_3 in N,N-dimethylformamide (DMF) in order to give the aimed nucleoside analogues (Table 1). A range of these coupling agents were O-acetylated (Table 1, entries 1−6), in which case the coupling reaction was followed by deacetylation with triethylamine (TEA) in H_2O/MeOH. Products before and after deacetylation were separated by crystallization from ethanol for biological evaluation. Structures of these separated coupling products were confirmed by IR, NMR, and elemental analyses. Table 1 shows the structure of the final products after deacetylation and lists the yields for the coupling reaction itself.

Entries 1−3 of Table 1 show the synthesis of seven 2-ONN based nucleoside analogues. Compounds **1a**−**c** were coupled with both peracetylated α-D-glucopyranosyl and α-D-galactopyranosyl bromides leading to the formation of the corresponding N-glycosides **2a**−**c** (entry 1) and **3a**−**c** (entry 2), respectively, after deacetylation. Similarly, **1a** was coupled with peracetylated α-D-lactosyl bromide (entry 3) to give the respective N-lactoside (**4**) after deacetylation. In all these seven cases, the acetylated N-β-glycosides were obtained in yields ranging from 51% to 87%, indicating that the coupling reaction occurred mainly at the nitrogen of the 2-ONN ring. Formation of the N-glycoside was confirmed by an IR band in the range of 1640−1660 cm^{-1} corresponding to an amide C=O functional group for each compound. Those resulting nucleosides had β-configuration as indicated by the ^{1}H NMR coupling constant of the anomeric proton ($J > 7.5$ Hz) due to diaxial coupling with H-2′.

Preparation of acyclic nucleoside analogues were attempted by coupling our 2-ONNs with five different substituted alkyl halides. Coupling with 3-hydroxypropyl chloride led to the N- alkylation, as desired, to give **8a**−**c** in good yields (entry 7). The 2-ONN ring of **1a**−**c** reacted with 2-bromomethoxyethyl acetate at the nitrogen atom as well to give the N- alkylation products **7a** and **7b** after deprotection and the unprotected **7c$_{Ac}$** (entry 6). However, when **1a**−**c** were coupled with 4-bromobutyl acetate, results varied. **1b** and **1c** underwent N- alkylation to give **6b** and **6c** as the final and major products, respectively (entry 5). On the other hand, **1a** underwent coupling at the 2-oxo position with the same reagent and

reaction conditions in a good yield to give, after deprotection, the O-alkylation product **5** (entry 4). This was judged by the absence of the IR band corresponding to the amide C=O functional group. The coupling at the 2-oxo position of **1a** was also seen with allyl bromide and propargyl bromide to give the O-alkylation products **9** (entry 8) and **10** (entry 9), respectively.

Based on the above results, only **1a** among the three 2-ONN compounds under study was glycosylated at the 2-oxo position with a few simple organohalides. In all other cases coupling occurred mainly at the ring N atom. In fact, these results were in contrast to the ones we observed in our previous work [21,22], where we have examined the coupling of the 2-ONN derivative **1d** (Fig. 2) with 10 different glycosyl and substituted alkyl halides under the same reaction conditions. In all cases, alkylation occurred at the 2-oxo position of the 2-ONN ring of **1d** and the corresponding O-glycosylation and O-alkylation products were separated in yields ranging from 48% to 87%. Compounds **1a**−**c** differ from **1d** by changing one of the aryl groups attached to the 2-ONN ring, while retaining the other one. Other published work showed that a very close 2-ONN structure (**1e**, Fig. 2) is also glycosylated at the 2-oxo position under the same conditions [23]. Furthermore, the similar 2-ONN compound **1f** (Fig. 2) was also reported to couple with ethyl bromoacetate at the 2-oxo position under similar reaction condition to the one used in our work [24].

Interestingly, reported results [25] showed that a compound different from **1d**, only in that the positions of the PhCl and Th groups relative to the 2-ONN ring are interchanged, undergoes N-glucosylation under similar reaction conditions to ours. Keeping these contradicting results in mind, one can envision that regioselectivity of the reaction depends on the size of both the coupling agent and the substituents on the 2-ONN ring. Additionally, the type and relative positions of the aryl substituents on the ring induce an electronic effect that contributes to the relative electron density at the ring N and 2-oxo positions, which in turn controls the regioselectivity. It is to be noted that careful chromatographic separation of products of such reactions show that both N- and O-glycosylation/alkylation can occur, with one of them as the major product [26].

2.2. Biology

2.2.1. Evaluation of antimicrobial activities

Our new 2-ONN derivatives were screened in vitro for antibacterial activity against Bacillis subtilis and Escherichia coli, representing Gram positive and Gram negative bacteria, respectively, and antifungal activity against the fungi Aspergillus niger and Aspergillus virdi-nutans. Zone of inhibition screening tests based on well diffusion method were used to examine both the antibacterial and antifungal activities. Table 2 shows the diameter of inhibition zone in mm for the 2-ONN compounds against the selected bacteria and fungi. The results were compared to Ampicillin as a reference antibacterial drug and Dermatin as a reference antifungal drug to evaluate the potency of the tested compounds. This antimicrobial study was conducted for both the final deprotected form and O-acetylated form of the 2-ONN derivatives. As mentioned above, O-acetylated forms were originally obtained as synthetic intermediates. The latter were more lipophilic and could have different biological properties than the deacetylated counterparts. Derivatives in Table 2 that were variants of 2-ONN compounds from Table 1 by acetyl protection were denoted by the addition of Ac to the number of each compound. Since the tested samples were prepared as N,N-dimethylformamide (DMF) solutions, wells containing only the microorganism and DMF were used as a negative control. DMF resulted in no inhibition zone.
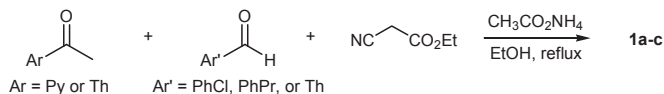
Py: 2-pyridyl, Th: 2-thienyl, PhCl: P-chlorophenyl, and PhPr: 4-isopropylphenyl

Scheme 1. Preparation of 2-ONN derivatives **1a**−**c**.

Among the tested compounds, **7b** showed the highest antibacterial activity (23 mm) against *B. subtilis*, which was even higher than the standard drug Ampicillin (20 mm). However, it had no activity against the Gram negative bacteria *E. coli*. In fact, all the tested compounds except **3b** showed no activity against *E. coli*. **3b** had small antibacterial activity of 5 mm compared to that of Ampicillin (18 mm). Compounds **2b** and **7c_Ac** showed moderate antibacterial activity against *B. subtilis*. Compounds **2b_Ac**, **2c_Ac**, **3a_Ac**, **3b_Ac**, **2c**, **3a**, **3b**, **3c**, **5_Ac**, **5**, **8a** and **9** showed lower antibacterial activity against Gram positive bacteria compared to Ampicillin. These data proved that there was no obvious trend suggesting that increasing lipophilicity by addition of acetyl groups in the 2-ONN structure had an effect on the antibacterial activity. Antifungal zone of inhibition studies showed that all the tested compounds had moderate antifungal activity against *Asperigillus niger* and *Asperigillus virdi-nutans* compared to the standard drug Dermatin.

2.2.2. In vitro antiviral screening

A panel of different viral species was used to evaluate the *in vitro* antiviral activity of some of the 2-ONN compounds in this report. The panel consisted of HCV, HBV, HSV-1, HSV-2, severe acute respiratory syndrome coronavirus (SARS-CoV), West Nile virus (WNV), influenza A virus (serotypes H_3N_2, H_5N_1 and H_1N_1) and influenza B virus. The acetylated 2-ONN glucosides **2a_Ac**–**c_Ac**, and the acetylated galactoside of **1c** (**3c_Ac**), which we designed as nucleoside

analogues, in addition to uncoupled 2-ONN derivatives **1a**–**c** were selected for this study. The compounds were evaluated for their antiviral activity in comparison to known drugs for each virus as controls. Antiviral activity was given as 50% effective concentration (EC_{50}), which is the concentration of the compound in μM required to inhibit 50% of viral infection. Cytotoxic concentration (CC_{50}) was determined as the concentration of the compound in μM at which 50% of cells become dead. Selectivity index (SI) was calculated as the ratio of CC_{50} to IC_{50} values, and was used along with the EC_{50} values to evaluate the bioactivity of 2-ONN derivatives.

Anti HCV and HBV activities were assessed using RNA replicon and DNA virion control assays, respectively. Anti HBV activity was compared to that of Lamivudine. Anti HSV-1 and HSV-2 as two additional representatives of DNA virus families were compared to acyclovir as the control drug. Similarly, anti HCV activity of the compounds was compared to that of human interferon-alpha (HIA) and 2′C-methylcytidine (2′C-MC). As summarized in Table 3 results showed that among the tested compounds, **1c** showed the best antiviral activity against HSV-1 and HSV-2 ($EC_{50} > 12$ μM), while **2b_Ac**, **2c_Ac** and **3c_Ac** were moderately active against HSV-1 and HSV-2 with an EC_{50} of 67.2, >60, and >60 μM, respectively. However, the SI values are much lower than reference drugs. All tested compounds were inactive against HCV and HBV as the EC_{50} values are higher than 100 μM. Anti SARS-CoV and WNV activities were investigated in comparison with $M_{128}8_{533}$ and infergen, respectively

Table 1
Coupling of 2-ONN derivatives **1a**–**c** with different organohalides.

Entry	Deriv.[a]	Coupling agent	Product[a,b]	% Yield[c]
1	**1a**–**c**		**2a-c**	**2a**: 66 **2b**: 58 **2c**: 62
2	**1a**–**c**		**3a-c**	**3a**: 87 **3b**: 70 **3c**: 51
3	**1a**		**4**	66
4	**1a**		**5**	80
5	**1b,c**		**6b,c**	**6b**: 57 **6c**: 53

Table 1 (*continued*)

Entry	Deriv.[a]	Coupling agent	Product[a,b]	% Yield[c]
6	**1a–c**	AcO⌒O⌒Br	**7a,b, 7cAc**	**7a**: 76 **7b**: 62 **7cAc**: 45
7	**1a–c**	HO⌒⌒Cl	**8a-c**	**8a**: 74 **8b**: 88 **8c**: 82
8	**1a**	⌿Br	**9**	82
9	**1a**	Br	**10**	73

[a] a: Ar = Py; Ar′ = PhCl, b: Ar = Th; Ar′ = PhPr, c: Ar = Ar′ = Th.
[b] Glc: glucosyl, Gal: galactosyl, Lac: lactosyl.
[c] Coupling yield before deprotection.
[d] Coupling product was not deacetylated for **7cAc**.

as control drugs. Results were obtained using both visual and neutral red tests. Among the tested compounds, only **2aAc** was slightly active by visual, but not neutral red test for SARS-CoV.

Anti influenza activity was screened using ribavirin as the control drug. Cytopathic effect and cytotoxicity were used as control assays and the resulting data were reported in Table 4. The screened compounds against influenza B virus showed that all tested compounds were not active. On the other hand, **2aAc** was slightly active against H_5N_1 influenza A virus. The 2-ONN derivatives were moderately active against H_1N_1 serotype.

3. Conclusion

A series of 2-oxonicotinonitrile based analogues of nucleosides and acyclic nucleosides were prepared by coupling 2-ONNs with

Fig. 2. Structures of 2-ONNs in the literature.

bromosugars and other organohalides. Some attempts of the coupling resulted in reaction at the 2-oxo position instead of the 2-ONN ring nitrogen. Seemingly, a combination of steric and electronic factors had an effect on the regioselectivity of the coupling reaction. Changing the coupling reaction conditions, such as the type of the base used, could lead to different selectivity [24]. Among the synthesized 2-ONN derivatives and their nucleosides, **7b**

Table 2
Antibacterial and antifungal inhibitory activities of 2-ONN derivatives.

2-ONN derivative (250 μg/disc)	Diameter of inhibition zone (mm)			
	Bacillus subtilis	*Escherichia coli*	*Asperigillus niger*	*Asperigillus virdi-nutans*
2bAc	1	0	7	7
2cAc	2	0	8	6
2b	10	0	7	8
2c	2	0	8	6
3aAc	1	0	7	5
3bAc	1	0	9	6
3a	2	0	7	7
3b	3	5	8	6
3c	4	0	8	8
5Ac	3	0	9	7
5	2	0	7	5
7cAc	10	0	8	7
7b	23	0	7	6
8a	3	0	9	9
9	1	0	7	7
DMF	0	0	0	0
Ampicillin	20	18	–	–
Dermatin	–	–	22	31

Table 3
Antiviral activity of compounds **1a–c**, **2a–c$_{Ac}$**, and **3c$_{Ac}$**.

2-ONN derivative	HCV EC$_{50}$[a]	HBV EC$_{50}$[a]	HSV-1		HSV-2		SARS-CoV		WNV EC$_{50}$[a]
			EC$_{50}$[a]	SI	EC$_{50}$[a]	SI	EC$_{50}$[a]	SI	
1a	>100	>100	>300	1	>300	1	>0.32	ND	>2.2
1b	>100	>100	>300	1	>300	1	>26	ND	>2.2
1c	>100	>100	>12	<4.7	>12	<4.8	>100	ND	>4.6
2a$_{Ac}$	>100	>100	>300	1	>300	1	8.3	4.3	>5.1
2b$_{Ac}$	>100	>100	67.2	>4.4	60.3	>4.9	>100	ND	>4.7
2c$_{Ac}$	>100	>100	>60	<4	>60	<4	>32	ND	>3
3c$_{Ac}$	>100	>100	>60	<2.4	>60	<2.4	10	1.9	>3.6
HIA	2	–	–	–	–	–	–	–	–
2′C-MC	1.6	–	–	–	–	–	–	–	–
Lamivudine	–	0.035	–	–	–	–	–	–	–
Acyclovir	–	–	3.3	>30.3	9.6	>10.4	–	–	–
M$_{128}$533	–	–	–	–	–	–	0.59	>170	–
Infergen	–	–	–	–	–	–	–	–	0.00004

[a] EC$_{50}$ is measured in μM.

showed good activity against the Gram positive bacterium *B. sub-tilis*, and **2a$_{Ac}$** was active against SARS-CoV and influenza A H$_5$N$_1$ virus. The 2-ONN compounds showed moderate antifungal activity.

4. Experimental results and protocols

4.1. Materials, instruments and general considerations

Starting materials and reagents were purchased from Aldrich and had a minimum purity of 97%. DMF was dried by distillation from CaCl$_2$. 4-Bromobutyl acetate [27] and 2-bromom ethoxyethyl acetate [28] were prepared according to literature from tetrahydrofuran and 1,3-dioxane, respectively. Other solvents and reagents were purchased from commercial sources and used as received. Reaction progress was monitored by TLC analysis using aluminium-backed plates pre-coated with Merck silica gel 60 F$_{254}$. Spots separated on the TLC plates were visualized by UV light and/or charring with a solution of 10% H$_2$SO$_4$ in EtOH. Column chromatography was carried out on Whatman™ 60–120 mesh silica. Melting points were measured using an Electrothermal IA 9100 apparatus. IR spectra (KBr disc) were recorded on either a Pye Unicam Sp-3-300 or a Shimadzu FTIR 8101 PC infrared spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded on JEOL-JNM-LA 300 MHz spectrometer. Chemical shifts (δ) were reported in ppm and were referenced to tetramethylsilane as a standard (0.00 ppm). Chemical shifts for ^{13}C NMR were referenced relative to DMSO (39.50 ppm). Elemental microanalysis was done on a Perkin Elmer 240 analyzer. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values.

Table 4
Anti influaza activity of **1a–c**, **2a–c$_{Ac}$**, and **3c$_{Ac}$**.

2-ONN derivative	Type-B		Type-A					
			H$_3$N$_2$		H$_5$N$_1$		H$_1$N$_1$	
	EC$_{50}$[a]	SI	EC$_{50}$[a]	SI	EC$_{50}$[a]	SI	EC$_{50}$[a]	SI
1a	>36	ND	>2.6	0	>100	>0	26	>3.8
1b	46	>2.2	22	>4.5	32	>3.1	ND	–
1c	>100	ND	16	>4.2	>100	>0	32	>3.1
2a$_{Ac}$	>3.2	ND	24	>4.2	3.2	1.3	ND	–
2b$_{Ac}$	40	>2.2	33	>3	32	1	ND	–
2c$_{Ac}$	32	1	40	>2.5	>100	>0	ND	–
3c$_{Ac}$	>12	ND	>32	ND	>19	ND	>28	ND
Ribavirin	2.4	>130	2	>160	4.2	>76	10	>32

[a] EC$_{50}$ is measured in μM.

4.2. Chemistry

4.2.1. General procedure for the preparation of 2-ONN **1a–c**

A mixture of the aromatic ketone (0.01 mol), the aromatic aldehyde (0.01 mol), ethyl cyanoacetate (1.13 g, 0.01 mol), and ammonium acetate (6.16 g, 0.08 mol) in ethanol (30 mL) was refluxed for 12 h. After cooling, the precipitate was filtered off, washed with ethanol, and dried. Recrystallization from DMF/Ethanol (1:10 v:v) afforded the 2-ONN derivatives **1a-c**.

4.2.1.1. 4-(4-Chlorophenyl)-2-oxo-6-(2-pyridyl)nicotinonitrile (**1a**).

This compound was prepared from 2-acetylpyridine (1.21 g) and 4-chlorobenzaldehyde (2.4 g) according to the general procedure described above, and was obtained as a green powder (1.55 g, 50.4% yield), mp 318–320 °C (lit [18]. mp 209–214 °C). IR (KBr, cm^{-1}): 3294 (NH), 2217 (C≡N), 1655 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.32 (s, 1H, Ar–H, 2-ONN ring), 7.59 (t, 1H, J = 5.1 Hz, Ar–H, Py), 7.68 (d, 2H, J = 8.7 Hz, Ar–H), 7.78 (d, 2H, J = 8.7 Hz, Ar–H), 8.04 (t, 1H, J = 7.5 Hz, Ar–H, Py), 8.30 (d, 1H, J = 8.1 Hz, Ar–H, Py), 8.77 (d, 1H, J = 4.8 Hz, Ar–H, Py), 12.38 (br, 1H, NH). Anal. Calcd for C$_{17}$H$_{10}$ClN$_3$O (307.73): C, 66.35; H, 3.28; N, 13.65. Found: C, 66.34; H, 3.28; N, 13.66.

4.2.1.2. 4-(4-Isopropylphenyl)-2-oxo-6-(2-thienyl)nicotinonitrile (**1b**).

This compound was prepared from 2-acetylthiophene (1.26 g) and 4-isopropylbenzaldehyde (1.48 g) according to the general procedure described above, and was obtained as a yellow powder (1.2 g, 38% yield), mp 310–311 °C. IR (KBr, cm^{-1}): 3278 (NH), 2210 (C≡N), 1636 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.24 (d, 6H, J = 6.9 Hz, CH$_3$(i-Pr)), 2.98 (m, 1H, CH (i-Pr)), 7.23 (s, 1H, 2-ONN ring), 7.29 (t, 1H, J = 3.9 Hz, Th), 7.43 (d, 2H, J = 8.1 Hz, Ar–H), 7.64 (d, 2H, J = 8.1 Hz, Ar–H), 7.87 (d, 1H, J = 5.1 Hz, Th), 8.06 (d, 1H, J = 4.8 Hz, Th), 12.74 (br, 1H, NH). Anal. Calcd for C$_{19}$H$_{16}$N$_2$OS (320.41): C, 71.22; H, 5.03; N, 8.74. Found: C, 71.23; H, 5.04; N, 8.73.

4.2.1.3. 2-Oxo-4,6-di(2-thienyl)nicotinonitrile (**1c**).

This compound was prepared from 2-acetylthiophene (1.26 g) and thiophene-2-carbaldehyde (1.12 g) according to the general procedure described above, and was obtained as a yellow powder (1.2 g, 43% yield), mp > 300 °C (lit [19]. mp 318 °C dec.) IR (KBr, cm^{-1}): 3273 (NH), 2208 (C≡N), 1636 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.22 (s, 1H, 2-ONN ring), 7.26 (t, 1H, J = 3.9 Hz, Th), 7.32 (t, 1H, J = 3.9 Hz, Th), 7.88 (d, 1H, J = 5.1 Hz, Th), 7.98 (m, 2H, Th), 8.07 (d, 1H, J = 4.8 Hz, Th), 12.74 (br, 1H, NH). Anal. Calcd for C$_{14}$H$_8$N$_2$OS$_2$ (284.36): C, 59.13; H, 2.84; N, 9.85. Found: C, 59.12; H, 2.85; N, 9.86.

4.2.2. General procedure for glycosylation and alkylation

The 2-ONN derivatives **1a**–**c** (0.010 mol) were stirred with potassium carbonate (1.38 g, 0.010 mol) in dry DMF (15 mL) for 1 h, followed by the addition of the appropriate peracetylated glycosyl bromide (0.011 mol) or alkyl halide (0.010 mol) in portions. Except for allyl and propargyl derivatives, the reaction mixture was stirred at room temperature overnight, filtered to remove insoluble materials, and then the solvent was evaporated under reduced pressure. For allyl and propargyl derivatives, the reaction mixture was refluxed for 5 h after stirring overnight; and after cooling it was filtered, and the filtrate was poured into ice-water to give the crude product as a precipitate, which in turn was filtered off and dried. The crude product obtained in all cases was purified by recrystallization from 95% ethanol.

4.2.3. General procedure for deacetylation

TEA (1 mL) was added to a solution of the acetylated compound (0.01 mol) in MeOH (20 mL) and 3 drops of water. The mixture was stirred overnight at room temperature, and then the solvent was evaporated under reduced pressure. The residue was repeatedly co-evaporated with MeOH until TEA was removed, and then purified by crystallization from 95% ethanol.

4.2.4. Compounds prepared by glycosylation/alkylation followed by deacetylation

4.2.4.1. 4-(4-Chlorophenyl)-1-(β-D-glucopyranosyl)-2-oxo-6-(2-pyridyl)nicotinonitrile (2a).
Compound **1a** (3 g) was reacted with peracetylated glucosyl bromide (3.2 g) according to the general procedure described for glycosylation, to give a yellow powder (4.2 g, 66% yield), mp 220–222 °C. IR (KBr, cm^{-1}): 2225 (C≡N), 1747 (C=O, acetoxy), 1662 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.78, 1.94, 1.99 and 2.03 (4s, 12H, 4CH$_3$CO), 4.08 (dd, 1H, $J_{5',6'}$ = 4.67, $J_{6',6''}$ = 12.22 Hz, H-6'), 4.22 (dd, 1H, $J_{5',6''}$ = 4.9, $J_{6',6''}$ = 12.2 Hz, H-6''), 4.39 (m, 1H, H-5'), 5.07 (t, 1H, $J_{3',4'}$ = 9.4 Hz, H-4'), 5.23 (t, 1H, $J_{1',2'}$ = 8.2, $J_{2',3'}$ = 9.6 Hz, H-2'), 5.63 (t, 1H, $J_{2',3'}$ = 9.6, $J_{3',4'}$ = 9.3 Hz, H-3'), 6.72 (d, 1H, $J_{1',2'}$ = 8.1 Hz, H-1'), 7.32 (s, 1H, Py), 7.59 (t, 1H, J = 5.1 Hz, Py), 7.68 (d, 2H, J = 8.7 Hz, Ar–H), 7.78 (d, 2H, J = 8.7 Hz, Ar–H), 8.05 (t, 1H, J = 7.5 Hz, Py), 8.25 (d, 1H, J = 8.1 Hz, Py) 8.64 (d, 1H, J = 4.8 Hz, Py). Anal. Calcd for C$_{31}$H$_{28}$ClN$_3$O$_{10}$ (638.02): C, 58.36; H, 4.42; N, 6.59. Found: C, 58.34; H, 4.43; N, 6.58. This powder (6 g) was deprotected according to the general procedure described for deacetylation, to give **2a** as a grey powder (3.4 g, 88% yield), mp 300–302 °C. IR (KBr, cm^{-1}): 3394 (broad, 4OH), 2223 (C≡N), 1660 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$/D$_2$O): δ 3.24–3.63 (m, 6H, H-6', H-6'', H-5', H-4', H-3', and H-2'), 6.13 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 7.23 (s, 1H, 2-ONN ring), 7.43 (t, 1H, J = 5.1 Hz, Py), 7.60 (d, 2H, J = 8.7 Hz, Ar–H), 7.65 (d, 2H, J = 8.7 Hz, Ar–H), 7.95 (t, 1H, J = 7.5 Hz, Py), 8.23 (d, 1H, J = 8.1 Hz, Py), 8.68 (d, 1H, J = 4.8 Hz, Py). Anal. Calcd for C$_{23}$H$_{20}$ClN$_3$O$_6$ (469.87): C, 58.79; H, 4.29; N. 8.94. Found: C, 58.78; H, 4.29; N, 8.93.

4.2.4.2. 1-(β-D-Glucopyranosyl)-4-(4-isopropylphenyl)-2-oxo-6-(2-thienyl)nicotinonitrile (2b).
Compound **1b** (3.2 g) was reacted with peracetylated glucosyl bromide (4.1 g) according to the general procedure described for glycosylation to give a yellow powder (3.78 g, 58% yield), mp 225–227 °C. IR (KBr, cm^{-1}): 2215 (C≡N), 1744 (C=O, acetoxy), 1643 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.24 (d, 6H, J = 6.9 Hz, CH$_3$ (i-Pr)), 1.78, 1.85, 1.98 and 2.03 (4s, 12H, 4CH$_3$CO), 2.98 (m, 1H, CH (i-Pr)), 4.09 (dd, 1H, $J_{5',6'}$ = 4.7, $J_{6',6''}$ = 12.2 Hz, H-6'), 4.33 (dd, 1H, $J_{5',6''}$ = 4.9, $J_{6',6''}$ = 12.2 Hz, H-6''), 4.33 (m, 1H, H-5'), 5.03 (t, 1H, $J_{3',4'}$ = 9.4, H-4'), 5.20 (t, 1H, $J_{1',2'}$ = 8.2, $J_{2',3'}$ = 9.6 Hz, H-2'), 5.62 (t, 1H, $J_{2',3'}$ = 9.6, $J_{3',4'}$ = 9.0 Hz, H-3'), 6.42 (d, 1H, J = 8.7 Hz, H-1'), 7.23 (t, 1H, J = 3.9 Hz, Th), 7.42 (d, 2H, J = 8.1 Hz, Ar–H) 7.56 (s, 1H, 2-ONN ring), 7.62 (d, 2H, J = 8.1 Hz, Ar–H), 7.84 (d, 1H, J = 5.1 Hz, Th),

8.03 (d, 1H, J = 4.8 Hz, Th). ^{13}C NMR (75 MHz, DMSO-d6): δ 20.12 (CH$_3$(i-Pr), 20.25, 20.26, 20.28 and 20.30 (4CH$_3$CO), 34.2 (CH(i-Pr)), 61.5 (C-6'), 68.0 (C-4'), 68.8 (C-3'), 71.4 (C-2'), 72.2 (C-5'), 93.8 (C-1'), 115.2 (C≡N), 118.2, 128.5, 129.8, 130.2, 130.8, 131.2, 132.2, 132.8, 134.5, 135.2, 142.2, 151.2 (Ar–C), 161.5, 168.0, 169.4, 170.2 and 170.6 (5 C=O). Anal. Calcd for C$_{33}$H$_{34}$N$_2$O$_{10}$S (650.70): C, 60.91; H, 5.27; N, 4.31. Found: C, 60.92; H, 5.27; N, 4.30. This powder (6.6 g) was deprotected according to the general procedure described for deacetylation to give **2b** as a yellow powder (4.58 g, 96% yield), mp 298–300 °C. IR (KBr, cm^{-1}): 3391 (broad, 4OH), 2215 (C≡N), 1644 (C=O, amide). ^{1}H NMR (300 MHz, CDCl$_3$/D$_2$O): δ 1.26 (d, 6H, J = 6.9 Hz, CH$_3$ (i-Pr)), 2.88 (m, 1H, CH (i-Pr)), 3.14 (m, 3H, H-3', H-6' and H-6''), 3.89 (m, 3H, H-2', H-4' and H-5'), 6.18 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 7.27 (t, 1H, J = 3.9 Hz, Th), 7.38 (d, 2H, J = 8.1 Hz, Ar–H), 7.51 (d, 2H, J = 8.1 Hz, Ar–H), 7.62 (s, 1H, 2-ONN ring), 7.64 (d, 1H, J = 5.1 Hz, Th), 8.21 (d, 1H, J = 4.8 Hz, Th). Anal. Calcd for C$_{25}$H$_{26}$N$_2$O$_6$S (482.55): C, 62.23; H, 5.43; N, 5.81. Found: C, 62.24; H, 5.44; N, 5.80.

4.2.4.3. 1-(β-D-Glucopyranosyl)-2-oxo-4,6-di(2-thienyl)nicotinonitrile (2c).
Compound **1c** (2.8 g) was reacted with peracetylated glucosyl bromide (4.1 g) according to the general procedure described for glycosylation to give a green powder (3.78 g, 62% yield), mp 225–226 °C. IR (KBr, cm^{-1}): 2213 (C≡N), 1748 (C=O, acetoxy), 1639 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.84, 1.98, 1.99 and 2.02 (4s, 12H, 4CH$_3$CO), 4.05 (dd, 1H, $J_{5',6'}$ = 4.6, $J_{6',6''}$ = 12.2 Hz, H-6'), 4.17 (dd, 1H, $J_{5',6''}$ = 4.9, $J_{6',6''}$ = 12.2 Hz, H-6''), 4.35 (m, 1H, H-5'), 5.03 (t, 1H, $J_{3',4'}$ = 9.4 Hz, H-4'), 5.23 (t, 1H, $J_{1',2'}$ = 8.4 Hz, H-2'), 5.65 (t, 1H, $J_{2',3'}$ = 9.0, $J_{3',4'}$ = 9.6 Hz, H-3'), 6.43 (d, 1H, $J_{1',2'}$ = 8.7 Hz, H-1'), 7.25 (t, 1H, J = 3.9 Hz, Th), 7.31 (t. 1H, J = 3.9 Hz, Th), 7.52 (s, 1H, 2-ONN ring), 7.87 (d, 1H, J = 5.2 Hz, Th), 7.98 (m, 2H, Th), 8.05 (d, 1H, J = 4.8 Hz, Th). Anal. Calcd for C$_{28}$H$_{26}$N$_2$O$_{10}$S$_2$ (614.64): C, 54.71; H, 4.26; N, 4.56. Found: C, 54.72; H, 4.25; N, 4.55. This powder (6.1 g) was deprotected according to the general procedure described for deacetylation to give **2c** as a green powder (4.41 g, 91.4% yield), mp 300–302 °C. IR (KBr, cm^{-1}): 3360 (broad, 4OH), 2216 (C≡N), 1640 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$/D$_2$O): δ 3.36–3.40 (m, 6H, H-6', H-6'', H-5', H-4', H-3' and H-2'), 5.98 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 7.23 (t, 1H, J = 3.9 Hz, Th), 7.29 (d, 1H, J = 5.2 Hz, Th), 7.65 (s, 1H, 2-ONN ring), 7.82 (d, 1H, J = 5.2 Hz, Th), 7.94 (m, 2H, Th), 7.98 (d, 1H, J = 4.8 Hz, Th). Anal. Calcd for C$_{20}$H$_{18}$N$_2$O$_6$S$_2$ (446.50): C, 53.80; H, 4.06; N, 6.27. Found: C, 53.81; H, 4.08; N, 6.26.

4.2.4.4. 4-(4-Chlorophenyl)-1-(β-D-galactopyranosyl)-2-oxo-6-(2-pyridyl)nicotinonitrile (3a).
Compound **1a** (3.07 g) was reacted with peracetylated galactosyl bromide (4.1 g) according to the general procedure described for glycosylation a pale yellow powder (5.59 g, 87% yield), mp 184–186 °C. IR (KBr, cm^{-1}): 2229 (C≡N), 1753 (C=O, acetoxy), 1650 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.63, 1.84, 1.87 and 2.05 (4s, 12H, 4CH$_3$CO), 3.87 (dd, 1H, $J_{5',6'}$ = 6.0, $J_{6',6''}$ = 11.1 Hz, H-6'), 4.01 (dd, 1H, $J_{5',6'}$ = 6.3, $J_{6',6''}$ = 11.1 Hz, H-6''), 4.56 (m, 1H, H-5'), 5.23 (t, 1H, $J_{3',2'}$ = 10.2, $J_{3',4'}$ = 2.8 Hz, H-3'), 5.38 (t, 1H, $J_{2',1'}$ = 8.4, $J_{2',3'}$ = 10.3 Hz, H-2'), 5.43 (t, 1H, $J_{4',3'}$ = 2.8, $J_{4',5'}$ = 2.7 Hz, H-4'), 6.51 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 7.33 (s,1H, 2-ONN ring), 7.43 (t, 1H, J = 5.1 Hz, Py), 7.53 (d, 2H, J = 8.7 Hz, Ar–H), 7.64 (d, 2H, J = 8.7 Hz, Ar–H), 7.90 (t, 1H, J = 7.5 Hz, Py), 8.12 (d, 1H, J = 8.1 Hz, Py), 8.45 (d, 1H, J = 4.8 Hz, Py). ^{13}C NMR (75 MHz, DMSO-d$_6$): δ 20.78, 20.84, 20.89 and 20.91 (4CH$_3$CO), 62.1 (C-6'), 68.06 (C-4'), 68.15 (C-2'), 70.66 (C-3'), 71.72 (C-5'), 94.64 (C-1'), 114.4, 115.8 (C≡N), 122.6, 126.3, 129.4, 130.6, 130.9, 134.8, 135.8, 138.1, 150.2, 153.1, 156.2, 156.4 (Ar–C), 162.3, 169.4, 170.0, 170.3 and 170.5 (5 C=O). Anal. Calcd for C$_{31}$H$_{28}$ClN$_3$O$_{10}$ (638.02): C, 58.36; H, 4.42; N, 6.59. Found: C, 58.35; H, 4.43; N, 6.58. This powder (6.3 g) was deprotected according to the general procedure described for

deacetylation to give **3a** as a yellow powder (4 g, 91% yield), mp 230–232 °C. IR (KBr, cm^{-1}): 3416 (broad, 4OH), 2220 (C≡N), 1659 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$/D$_2$O): δ 3.55 (m, 3H, H-3′, H-6′ and H-6″), 3.83 (m, 3H, H-2′, H-4′ and H-5′), 6.09 (d, 1H, $J_{1',2'}$ = 7.8 Hz, H-1′), 7.23 (s, 1H, 2-ONN ring), 7.53 (t, 1H, J = 5.1 Hz, Py), 7.62 (d, 2H, J = 8.7 Hz, Ar−H), 7.71 (d, 2H, J = 8.7 Hz, Ar−H), 7.99 (t, 1H, J = 7.5 Hz, Py), 8.41 (d, 1H, J = 8.1 Hz, Py), 8.70 (d, 1H, J = 4.8 Hz, Py). Anal. Calcd for C$_{23}$H$_{20}$ClN$_3$O$_6$ (469.87): C, 58.79; H, 4.29; N, 8.94. Found: C, 58.79; H, 4.28; N, 8.93.

4.2.4.5. 1-(β-D-Galactopyranosyl)-4-(4-isopropylphenyl)-2-oxo-6-(2-thienyl)nicotinonitrile (3b). Compound **1b** (3.2 g) was reacted with peracetylated galactosyl bromide (4.1 g) according to the general procedure described for glycosylation to give a yellow powder (4.58 g, 70% yield), mp 160–161 °C. IR (KBr, cm^{-1}): 2218 (C≡N), 1754 (C=O, acetoxy), 1644 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.23 (d, 6H, J = 6.9 Hz, CH$_3$ (*i*-Pr)), 1.63, 1.84, 1.88 and 2.04 (4s, 12H, 4CH$_3$CO), 2.88 (m, 1H, CH (*i*-Pr)), 3.87 (dd, 1H, $J_{5',6'}$ = 6.0, $J_{6',6''}$ = 11.1 Hz, H-6′), 4.01 (dd, 1H, $J_{5',6'}$ = 6.3, $J_{6',6''}$ = 11.1 Hz, H-6″), 4.43 (m, 1H, H-5′), 5.19 (t, 1H, $J_{3',2'}$ = 10.2, $J_{3',4'}$ = 2.8 Hz, H-3′), 5.26 (t, 1H, $J_{2',1'}$ = 8.4, $J_{2',3'}$ = 10.3 Hz, H-2′), 5.44 (t, 1H, $J_{4',3'}$ = 2.80, $J_{4',5'}$ = 2.78 Hz, H-4′), 6.26 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1′), 7.30 (t, 1H, J = 3.9 Hz, Th), 7.48 (d, 2H, J = 8.1 Hz, Ar−H) 7.50 (s, 1H, 2-ONN ring), 7.54 (d, 2H, J = 8.1 Hz, Ar−H), 7.76 (d, 1H, J = 5.1 Hz, Th), 8.01 (d, 1H, J = 4.8 Hz, Th). ^{13}C NMR (75 MHz, DMSO-d$_6$): δ 20.12 (CH$_3$ (*i*-Pr)), 20.84, 20.85, 20.87 and 20.94 (4CH$_3$CO), 33.82 (CH (*i*-Pr)), 61.9 (C-6′), 68.03 (C-4′), 68.24 (C-2′), 70.60 (C-3′), 71.81 (C-5′), 94.7 (C-1′), 114.2, 115.6 (C≡N), 127.3, 128.7, 129.1, 129.6, 132.1, 133.4, 142.5, 151.3, 153.1, 154.2, 157.1 (Ar−C), 162.2, 169.4, 169.9, 170.3 and 170.5 (5 C=O). Anal. Calcd for C$_{33}$H$_{34}$N$_2$O$_{10}$S (650.70): C, 60.91; H, 5.27; N, 4.31. Found: C, 60.90; H, 5.28; N, 4.32. This powder (6.5 g) was deprotected according to the general procedure described for deacetylation to give **3b** as a yellow powder (1.56 g, 85.5% yield), mp 220–222 °C. IR (KBr, cm^{-1}): 3418 (broad, 4OH), 2216 (C≡N), 1642 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$/D$_2$O): δ 1.21 (d, 6H, J = 6.9 Hz, CH$_3$ (*i*-Pr)), 2.93 (m, 1H, CH (*i*-Pr)), 3.34–3.48 (m, 3H, H-3′, H-6′ and H-6″), 3.52–3.61 (m, 3H, H-2′, H-4′ and H-5′), 5.92 (d, 1H, $J_{1',2'}$ = 8.1 Hz, H-1′), 7.19 (t, 1H, J = 3.9 Hz, Th), 7.41 (d, 2H, J = 8.1 Hz, Ar−H), 7.62 (d, 2H, J = 8.1 Hz, Ar−H), 7.66 (s, 1H, 2-ONN ring), 7.73 (d, 1H, J = 5.1 Hz, Th), 7.99 (d, 1H, J = 4.8 Hz, Th). Anal. Calcd for C$_{25}$H$_{26}$N$_2$O$_6$S (482.55): C, 62.23; H, 5.43; N, 5.81. Found: C, 62.24; H, 5.43; N, 5.82.

4.2.4.6. 1-(β-D-Galactopyranosyl)-2-oxo-4,6-di(2-thienyl)-nicotinonitrile (3c). Compound **1c** (2.8 g) was reacted with peracetylated galactosyl bromide (4.1 g) according to the general procedure described for glycosylation to give a colourless solid (3.05 g, 51% yield), mp 210–212 °C. IR (KBr, cm^{-1}): 2221 (C≡N), 1745 (C=O, acetoxy), 1634 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.69, 1.82., 1.88 and 2.04 (4s, 12H, 4CH$_3$CO), 3.91 (dd, 1H, $J_{5',6'}$ = 6.0, $J_{6',6''}$ = 10.1 Hz, H-6′), 3.99 (dd, 1H, $J_{5',6'}$ = 6.3, $J_{6',6''}$ = 10.1 Hz, H-6″), 4.44 (m, 1H, H-5′), 5.21 (t, 1H, $J_{3',2'}$ = 10.23, $J_{3',4'}$ = 2.8 Hz, H-3′), 5.28 (t, 1H, $J_{2',1'}$ = 8.4, $J_{2',3'}$ = 10.3 Hz, H-2′), 5.43 (t, 1H, $J_{4',3'}$ = 2.8, $J_{4',5'}$ = 2.78 Hz, H-4′), 6.23 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1′), 7.20 (s, 1H, 2-ONN ring), 7.24 (t, 1H, J = 3.9 Hz, Th), 7.29 (t, 1H, J = 5.2 Hz, Th), 7.82 (d, 1H, J = 5.2 Hz, Th), 7.86 (m, 2H, Th), 8.05 (d, 1H, J = 4.8 Hz, Th). ^{13}C NMR (75 MHz, DMSO-d$_6$): δ 20.84, 20.85, 20.88 and 20.93 (4CH$_3$CO), 61.9 (C-6′), 68.06 (C-4′), 68.23 (C-2′), 70.59 (C-3′), 71.82 (C-5′), 94.79 (C-1′), 112.5, 115.6 (C≡N), 129.1, 129.6, 129.7, 131.1, 131.7, 132.3, 132.5, 136.6, 142.3, 148.7 and 153.2 (Ar−C), 162.7, 169.4, 169.9, 170.3 and 170.5 (5 C=O). Anal. Calcd for C$_{28}$H$_{26}$N$_2$O$_{10}$S$_2$ (614.64): C, 54.71; H, 4.26; N, 4.56. Found: C, 54.72; H, 4.26; N, 4.57. This solid (6.14 g) was deprotected according to the general procedure described for deacetylation to give **3c** as a yellow powder (3.96 g, 89% yield), mp 245–246 °C. IR (KBr, cm^{-1}): 3420 (broad,

4OH), 2218 (C≡N), 1645 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$/D$_2$O): δ 3.47–3.78 (m, 6H, H-6′, H-6″, H-5′, H-4′, H-3′ and H-2′), 5.95 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1′), 7.23 (t, 1H, J = 3.9 Hz, Th), 7.31 (d, 1H, J = 5.2 Hz, Th), 7.56 (s, 1H, 2-ONN ring), 7.81 (d, 1H, J = 5.1 Hz, Th), 7.93 (m, 2H, Th), 8.07 (d, 1H, J = 4.8 Hz, Th). Anal. Calcd for C$_{20}$H$_{18}$N$_2$O$_6$S$_2$ (446.50): C, 53.80; H, 4.06; N, 6.27. Found: C, 53.82; H, 4.04; N, 6.26.

4.2.4.7. 4-(4-Chlorophenyl)-1-[O$^{4'}$-(β-D-galactopyranosyl)-β-D-glu-copyranosyl]-2-oxo-6-(2-pyridyl)nicotinonitrile (4). Compound **1a** (3.07 g) was reacted with peracetylated O^4-(β-D-galactopyranosyl)-β-D-glucopyranosyl bromide (7.2 g) according to the general procedure described for glycosylation to give a yellow powder (5.2 g, 66% yield), mp 220–221 °C. IR (KBr, cm^{-1}): 2223 (C≡N), 1747 (C=O, acetoxy), 1662 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.77, 1.86, 1.87, 1.89, 1.90, 1.98 and 2.05 (7s, 21H, 7CH$_3$CO), 3.87 - 4.01 (m, 3H, H-2′b, H-6′a and H-6′b), 4.14 (dd, 1H, $J_{6''a,6'a}$ = 11.4, $J_{6''a,5'a}$ = 5.6 Hz, H-6″a), 4.36 (m, 1H, H-5′b), 4.68 (dd, 1H, $J_{6''b,5'b}$ = 6.3 Hz, H-6″b), 4.87 (m, 1H, H-5′a), 4.95 (dd, 1H, $J_{1'b,2'b}$ = 7.6 Hz, H-1′b), 5.04 (dd, 1H, $J_{4'b,3'b}$ = 3.1, $J_{4'b,5'b}$ = 3.8 Hz, H-4′b), 5.21 (dd, 1H, $J_{2'a,1'a}$ = 8.7, $J_{2'a,3'a}$ = 8.4 Hz, H-2′a), 5.27 (dd, 1H, $J_{4'a,3'a}$ = 9.1, $J_{4'a,5'a}$ = 9.9 Hz, H-4′a), 5.30 (d, 1H, $J_{3'b,4'b}$ = 3.1 Hz, H-3′b), 5.38 (dd, 1H, $J_{3'a,2'a}$ = 8.4, $J_{3'a,4'a}$ = 9.1 Hz, H-3′a), 6.54 (d, 1H, $J_{1'a,2'a}$ = 8.7 Hz, H-1′a), 7.23 (s, 1H, 2-ONN ring), 7.42 (t, 1H, J = 5.1 Hz, Py), 7.60 (d, 2H, J = 8.7 Hz, Ar−H), 7.66 (d, 2H, J = 8.7 Hz, Ar−H), 7.93 (t, 1H, J = 7.5 Hz, Py), 8.49 (d, 1H, J = 8.1 Hz, Py), 8.61 (d, 1H, J = 4.8 Hz, Py). Anal. Calcd for C$_{43}$H$_{44}$ClN$_3$O$_{18}$ (926.27): C, 55.76; H, 4.79; N, 4.54. Found: C, 55.77; H, 4.78; N, 4.55. This powder (9.2 g) was deprotected according to the general procedure described for deacetylation to give **4** as a yellow powder (4.8 g, 75% yield), mp 275–277 °C. IR (KBr, cm^{-1}): 3410 (broad, 7OH), 2220 (C≡N), 1655 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$/D$_2$O): δ 3.40–3.76 (4m, 12H, H-2′b, H-3′b, H-4′b, H-5′b, H-6′b, H-6″b, H-2′a, H-3′a, H-4′a, H-5′a, H-6′a and H-6″a), 5.81 (d, 1H, $J_{1'b,2'b}$ = 7.8 Hz, H-1′b), 5.92 (d, 1H, $J_{1'a,2'a}$ = 8.9 Hz, H-1′a), 7.24 (s, 1H, 2-ONN ring), 7.43 (t, 1H, J = 5.1 Hz, Py), 7.62 (d, 2H, J = 8.7 Hz, Ar−H), 7.68 (d, 2H, J = 8.7 Hz, Ar−H), 7.92 (t, 1H, J = 7.5 Hz, Py), 8.48 (d, 1H, J = 8.1 Hz, Py), 8.60 (d, 1H, J = 4.8 Hz, Py). Anal. Calcd for C$_{29}$H$_{30}$ClN$_3$O$_{11}$ (632.01): C, 55.11; H, 4.78; N, 6.65. Found: C, 55.12; H, 4.77; N, 6.65.

4.2.4.8. 4-(4-Chlorolphenyl)-2-(4-hydroxybutyloxy)-6-(2-pyridyl)nicotinonitrile (5). Compound **1a** (3.07 g) was reacted with 4-bromobutyl acetate (1.95 g) according to the general procedure described for alkylation to give a green powder (3.4 g, 80% yield), mp 130–132 °C. IR (KBr, cm^{-1}): 2220 (C≡N), 1739 (C=O, acetoxy). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.83 (m, 2H, CH$_2$), 1.99 (s, 3H, CH$_3$CO), 2.94 (m, 2H, CH$_2$),, 4.08 (t, 2H, J = 5.7 Hz, CH$_2$), 4.64 (t, 2H, J = 6.3 Hz, CH$_2$), 7.43 (t, 1H, J = 5.1 Hz, Py), 7.65 (d, 2H, J = 8.7 Hz, Ar−H), 7.78 (d, 2H, J = 8.7 Hz, Ar−H), 8.10 (s, 1H, 2-ONN ring), 8.12 (t, 1H, J = 7.5 Hz, Py), 8.43 (d, 1H, J = 8.1 Hz, Py), 8.60 (d, 1H, J = 4.8 Hz, Py). ^{13}C NMR (75 MHz, DMSO-d$_6$): δ 20.65 (CH$_3$CO), 24.75 (CH$_2$), 24.87 (CH$_2$), 63.42 (CH$_2$), 66.85 (CH$_2$), 93.85, 113.4, 114.8 (C≡N), 121.6, 125.4, 128.9, 130.1, 134.4, 135.0, 137.5, 149.5, 152.9, 154.9, 156.0, 163.9 and 170.2 (Ar−C, C=N and C=O). Anal. Calcd for C$_{23}$H$_{20}$ClN$_3$O$_3$ (421.88): C, 65.48; H, 4.78; N, 9.96. Found: C, 65.47; H, 4.79; N, 9.98. This powder (4.21 g) was deprotected according to the general procedure described for deacetylation to give **5** as a yellow powder (2.6 g, 86% yield), mp 145–146 °C. IR (KBr, cm^{-1}): 3418 (broad, OH), 2221 (C≡N). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.63 (m, 2H, CH$_2$), 1.87 (m, 2H, CH$_2$), 3.28 (t, 1H, J = 4.5 Hz, OH), 3.48 (t, 2H, J = 5.7 Hz, CH$_2$), 4.62 (t, 2H, J = 6.3 Hz, CH$_2$), 7.53 (t, 1H, J = 5.1 Hz, Py), 7.68 (d, 2H, J = 8.7 Hz, Ar−H), 7.77 (d, 2H, J = 8.7 Hz, Ar−H), 8.0 (t, 1H, J = 7.5 Hz, Py), 8.12 (s, 1H, 2-ONN ring), 8.43 (d, 1H, J = 8.1 Hz, Py), 8.74 (d, 1H, J = 4.8 Hz, Py). Anal. Calcd for

$C_{21}H_{18}ClN_3O_2$ (379.84): C, 66.40; H, 4.78; N, 11.06. Found: C, 66.41; H, 4.78; N, 11.08.

4.2.4.9. 1-(4-Hydroxybutyl)-4-(4-isopropylphenyl)-2-oxo-6-(2-thienyl)nicotinonitrile (6b).

Compound **1b** (3.2 g) was reacted with 4-bromobutyl acetate (1.95 g) according to the general procedure for alkylation to give a yellow powder (2.55 g, 57% yield), mp 240–242 °C. IR (KBr, cm^{-1}): 2221 (C≡N), 1731 (C=O, acetoxy), 1643 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.25 (d, 6H, J = 6.9 Hz, CH$_3$ (*i*-Pr)), 1.84 (m, 2H, CH$_2$), 1.99 (s, 3H, CH$_3$CO), 2.94 (m, 2H, CH$_2$), 3.10 (m, 1H, CH (*i*-Pr)), 4.09 (t, 2H, J = 5.7 Hz, CH$_2$), 4.53 (t, 2H, J = 6.3 Hz, CH$_2$), 7.21 (t, 1H, J = 3.9 Hz, Th), 7.43 (d, 2H, J = 8.1 Hz, Ar–H), 7.62 (d, 2H, J = 8.1 Hz, Ar–H), 7.76 (s, 1H, 2-ONN ring), 7.81 (d, 1H, J = 5.1 Hz Hz, Th), 8.09 (d, 1H, J = 4.8 Hz, Th). Anal. Calcd for $C_{25}H_{26}N_2O_3S$ (434.55): C, 69.10; H, 6.03; N, 6.45. Found: C, 69.11; H, 6.02; N, 6.45. This powder (4.34 g) was deprotected according to the general procedure described for deacetylation to give **6b** as a yellow powder (3.5 g, 92% yield), mp 260–262 °C. IR (KBr, cm^{-1}): 3450 (OH), 2218 (C≡N), 1643 (C=O, amide). Anal. Calcd for $C_{23}H_{24}N_2O_2S$ (392.51): C, 70.38; H, 6.16; N, 7.14. Found: C, 70.37; H, 6.17; N, 7.13.

4.2.4.10. 1-(4-Hydroxybutyl)-2-oxo-4,6-di(2-thienyl)nicotinonitrile (6c).

Compound **1c** (2.8 g) was reacted with 4-bromobutyl acetate (1.95 g) according to the general procedure described for alkylation to give a yellow powder (2.1 g, 53% yield), mp 210–212 °C. IR (KBr, cm^{-1}): 2212 (C≡N), 1731 (C=O, acetoxy), 1644 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.76 (m, 2H, CH$_2$), 1.84 (m, 2H, CH$_2$), 1.99 (s, 3H, CH$_3$CO), 4.06 (t, 2H, J = 5.7 Hz, CH$_2$), 4.53 (t, 2H, J = 6.3 Hz, CH$_2$), 7.23 (t, 1H, J = 3.9 Hz, Th), 7.31 (d, 1H, J = 5.2 Hz, Th), 7.82 (d, 1H, J = 5.2 Hz, Th), 7.85 (s, 1H, 2-ONN ring), 7.97 (m, 2H, Th), 8.14 (d, 1H, J = 4.8 Hz, Th). Anal. Calcd for $C_{20}H_{18}N_2O_3S_2$ (398.50): C, 60.28; H, 4.55; N, 7.03. Found: C, 60.27; H, 4.56; N, 7.02. This powder (4 g) was reacted according to the general procedure described for deacetylation to give **6c** as a yellow powder (2 g, 86% yield), mp 260–262 °C. IR (KBr, cm^{-1}): 3450 (OH), 2212 (C≡N), 1638 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.68 (m, 2H, CH$_2$), 1.85 (m, 2H, CH$_2$), 3.45 (t, 1H, J = 4.48 Hz, OH), 3.55 (t, 2H, J = 5.7 Hz, CH$_2$), 4.50 (t, 2H, J = 6.3 Hz, CH$_2$), 7.16 (t, 1H, J = 3.9 Hz, Th), 7.26 (d, 1H, J = 5.2 Hz, Th), 7.68 (d, 1H, J = 5.2 Hz, Th), 7.85 (m, 2H, Th), 7.90 (s, 1H, 2-ONN ring), 7.96 (d, 1H, J = 4.8 Hz, Th). Anal. Calcd for $C_{18}H_{16}N_2O_2S_2$ (356.46): C, 60.65; H, 4.52; N, 7.86. Found: C, 60.65; H, 4.53; N, 7.85.

4.2.4.11. 4-(4-Chlorophenyl)-1-[(2-hydroxyethoxy)methyl]-2-oxo-6-(2-pyridyl)nicotinonitrile (7a).

Compound **1a** (3.07 g) was reacted with 2-bromomethoxyethyl acetate (1.97 g) according to the general procedure described for alkylation to give a yellow powder (3.2 g, 76% yield), mp 220–222 °C. IR (KBr, cm^{-1}): 2221 (C≡N), 1734 (C=O, acetoxy), 1661 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 2.02 (s, 3H, CH$_3$CO), 3.38 (t, 2H, J = 5.4 Hz, CH$_2$), 4.43 (t, 2H, J = 5.4 Hz, CH$_2$), 4.72 (s, 2H, NCH$_2$O), 7.52 (t, 1H, J = 5.1 Hz, Py), 7.63 (d, 2H, J = 8.7 Hz, Ar–H), 7.74 (d, 2H, J = 8.7 Hz, Ar–H), 8.02 (t, 1H, J = 7.5 Hz, Py), 8.12 (s, 1H, 2-ONN ring), 8.48 (d, 1H, J = 8.1 Hz, Py), 8.72 (d, 1H, J = 4.8 Hz, Py). Anal. Calcd for $C_{22}H_{18}ClN_3O_4$ (423.85): C, 62.34; H, 4.28; N, 9.91. Found: C, 62.33; H, 4.29; N, 9.92. This powder (4.2 g) was deprotected according to the general procedure described for deacetylation to give **7a** a yellow powder (1.2 g, 86% yield), mp 260–262 °C. IR (KBr, cm^{-1}): 3426 (OH), 2219 (C≡N), 1659 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 3.40 (t, 2H, J = 5.3 Hz, CH$_2$), 3.83 (t, 2H, J = 5.0 Hz, CH$_2$), 4.63 (t, 1H, J = 5.3 Hz, OH), 5.98 (s, 2H, NCH$_2$O), 7.55 (t, 1H, J = 5.1 Hz, Py), 7.61 (d, 2H, J = 8.7 Hz, Ar–H), 7.69 (d, 2H, J = 8.7 Hz, Ar–H), 7.98 (t, 1H, J = 7.5 Hz, Py), 8.05 (s, 1H, 2-ONN ring), 8.40 (d, 1H, J = 8.1 Hz, Py),

8.72 (d, 1H, J = 4.8 Hz, Py). Anal. Calcd for $C_{20}H_{16}ClN_3O_3$ (381.81): C, 62.91; H, 4.22; N, 11.01. Found: C, 62.92; H, 4.23; N, 11.00.

4.2.4.12. 1-[(2-Hydroxyethoxy)methyl]-4-(4-isopropylphenyl)-2-oxo-6-(2-thienyl)nicotinonitrile (7b).

Compound **1b** (3.2 g) was reacted with 2-bromomethoxyethyl acetate (1.97 g) according to the general procedure described for alkylation to give a yellow powder (3.3 g, 62% yield), mp 180–182 °C. IR (KBr, cm^{-1}): 2218 (C≡N), 1735 (C=O, acetoxy), 1643 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.24 (d, 6H, J = 6.9 Hz, CH$_3$ (*i*-Pr)), 2.02 (s, 3H, CH$_3$CO), 2.97 (m, 1H, CH (*i*-Pr)), 3.34 (t, 2H, J = 5.4 Hz, OCH$_2$ (c)), 4.45 (t, 2H, J = 5.4 Hz, OCH$_2$ (d)), 4.72 (s, 2H, NCH$_2$O (a)), 7.22 (t, 1H, J = 3.9 Hz, Th), 7.43 (d, 2H, J = 8.1 Hz, Ar–H), 7.62 (d, 2H, J = 8.1 Hz, Ar–H), 7.79 (s, 1H, 2-ONN ring), 7.82 (d, 1H, J = 5.1, Hz, Th), 8.11 (d, 1H, J = 4.8 Hz, Th). ^{13}C NMR (75 MHz, DMSO-d$_6$): δ 20.6 (CH$_3$CO), 23.6 (CH$_3$ (*i*-Pr)), 33.3 (CH (*i*-Pr)), 61.9 (CH$_2$O (d)), 65.1 (OCH$_2$ (c)), 91.2 (NCH$_2$O), 112.2, 115.2 (C≡N), 126.7, 128.2, 128.6, 129.0, 131.2, 133.0, 133.4, 142.4, 150.6, 152.5, 156.2 (Ar–C) and 163.6, 170.3 (2C = O). Anal. Calcd for $C_{24}H_{24}N_2O_4S$ (436.92): C, 66.03; H, 5.54; N, 6.42. Found: C, 66.02; H, 5.53; N, 6.44. This powder (4.3 g) was deprotected according to the general procedure described for deacetylation to give **7b** as a yellow powder (2.78 g, 85% yield), mp 200–201 °C. IR (KBr, cm^{-1}): 3428 (OH), 2221 (C≡N), 1662 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.23 (d, 6H, J = 6.9 Hz, CH$_3$ (*i*-Pr)), 2.99 (m, 1H, CH (*i*-Pr)), 3.57 (t, 2H, J = 5.3 Hz, CH$_2$), 3.80 (t, 2H, J = 5.0 Hz, CH$_2$), 4.52 (t, 1H, J = 5.3 Hz, OH), 5.40 (s, 2H, NCH$_2$O), 7.23 (t, 1H, J = 3.9 Hz, Th), 7.45 (d, 2H, J = 8.1 Hz, Ar–H), 7.63 (d, 2H, J = 8.1 Hz, Ar–H), 7.65 (s, 1H, 2-ONN ring), 7.80 (d, 1H, J = 5.1 Hz, Th), 7.85 (d, 1H, J = 4.8 Hz, Th). Anal. Calcd for $C_{22}H_{22}N_2O_3S$ (394.49): C, 66.98; H, 5.62; N, 7.10. Found: C, 66.99; H, 5.62; N, 7.11.

4.2.5. Compounds prepared by alkylation without deacetylation

4.2.5.1. 1-[(2-Acetoxyethoxy)methyl]-2-oxo-4,6-di(2-thienyl)nicotinonitrile (7c$_{Ac}$).

This compound was prepared from **1c** (2.8 g) and 2-bromomethoxyethyl acetate (1.97 g) according to the general procedure described above for alkylation and was obtained as a yellow powder (1.8 g, 45% yield), mp 160–161 °C. IR (KBr, cm^{-1}): 2214 (C≡N), 1732 (C=O, acetoxy), 1643 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 2.06 (s, 3H, CH$_3$CO), 3.38 (t, 2H, J = 5.4 Hz, CH$_2$), 4.44 (t, 2H, J = 5.4 Hz, CH$_2$), 4.72 (s, 2H, NCH$_2$O (a)), 7.23 (t, 1H, J = 3.9 Hz, Th), 7.33 (d, 1H, J = 5.2 Hz, Th), 7.79 (m, 2H, Th), 7.82 (s, 1H, 2-ONN ring), 7.83 (d, 1H, J = 5.2 Hz, Th), 8.15 (d, 1H, J = 4.8 Hz, Th). Anal. Calcd for $C_{19}H_{16}N_2O_4S_2$ (400.47): C, 56.98; H, 4.03; N, 7.00. Found: C, 56.99; H, 4.04; N, 7.02.

4.2.5.2. 4-(4-Chlorolphenyl)-1-(3-hydroxypropyl)-2-oxo-6-(2-pyridyl)nicotinonitrile (8a).

This compound was prepared from **1a** (3.07 g) and 3-hydroxypropyl chloride (0.94 g) according to the general procedure described above, and was obtained as a yellow powder (2.7 g, 74% yield), mp 174–176 °C. IR (KBr, cm^{-1}): 3328 (OH), 2221 (C≡N), 1652 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.95 (m, 2H, CH$_2$), 3.31 (t, 2H, J = 5.3 Hz Hz, CH$_2$), 3.62 (t, 2H, J = 5.0 Hz, CH$_2$), 4.69 (t, 1H, J = 5.3 Hz, OH), 7.33 (t, 1H, J = 5.1 Hz, Py), 7.53 (d, 2H, J = 8.7 Hz, Ar–H), 7.69 (d, 2H, J = 8.7 Hz, Ar–H), 8.01 (t, 1H, J = 7.5 Hz, Py), 8.12 (s, 1H, 2-ONN ring), 8.44 (d, 1H, J = 8.1 Hz, Py), 8.75 (d, 1H, J = 4.8 Hz, Py). Anal. Calcd for $C_{20}H_{16}ClN_3O_2$ (365.81): C, 65.67; H, 4.41; N, 11.49. Found: C, 65.68; H, 4.40; N, 11.48.

4.2.5.3. 1-(3-Hydroxypropyl)-4-(4-isopropylphenyl)-2-oxo-6-(2-thienyl)nicotinonitrile (8b).

This compound was prepared from **1b** (3.2 g) and 3-hydroxypropyl chloride (0.94 g) according to the general procedure described for alkylation and was obtained as a yellow powder (3.3 g, 88% yield), mp 180–182 °C. IR (KBr, cm^{-1}): 3384 (OH), 2219 (C≡N), 1660 (C=O, amide). Anal. Calcd for

$C_{22}H_{22}N_2O_2S$ (378.49): C, 69.81; H, 5.86; N, 7.40. Found: C, 69.82; H, 5.85; N, 7.42.

4.2.5.4. 3-Cyano-1-(3-hydroxypropyl)-4,6-di(2-thienyl)-2-pyridone (8c). This compound was prepared from **1c** (2.8 g) and 3-hydroxypropyl chloride (0.94 g) according to the general procedure described for alkylation and was obtained as a yellow powder (2.8 g, 82% yield), mp 180–181 °C. IR (KBr, cm^{-1}): 3460 (OH), 2217 (C≡N), 1643 (C=O, amide). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.93 (m, 2H, CH$_2$), 3.33 (t, 2H, J = 5.3 Hz, CH$_2$), 3.63 (t, 2H, J = 5.0 Hz, CH$_2$), 4.56 (t, 1H, J = 5.3 Hz, OH), 7.23 (t, 1H, J = 3.9 Hz, Th), 7.32 (d, 1H, J = 5.2 Hz, Th), 7.63 (d, 1H, J = 5.2 Hz, Th), 7.83 (s, 1H, 2-ONN ring), 7.97 (m, 2H, Th), 8.14 (d, 1H, J = 4.8 Hz, Th). Anal. Calcd for $C_{17}H_{14}N_2O_2S_2$ (342.44): C, 59.63; H, 4.12; N, 8.18. Found: C, 59.62; H, 4.13; N, 8.17.

4.2.5.5. 2-(Allyloxy)-4-(4-chlorolphenyl)-6-(2-pyridyl)nicotinonitrile (9). This compound was prepared from **1a** (2.36 g) and allyl bromide (0.93 g) according to the general procedure described for alkylation and was obtained as a yellow powder (2.19 g, 82% yield), mp 176–177 °C. IR (KBr, cm^{-1}): 2222 (C≡N). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 5.15 (d, 2H, J = 5.4 Hz, CH$_2$), 5.32 (d, 1H, J = 10.2 Hz), 5.56 (d, 1H, J = 17.1 Hz), 6.17 (m, 1H), 7.33 (t, 1H, J = 5.1 Hz, Py), 7.53 (d, 2H, J = 8.7 Hz, Ar–H), 7.75 (d, 2H, J = 8.7 Hz, Ar–H), 8.01 (t, 1H, J = 7.5 Hz, Py), 8.13 (s, 1H, 2-ONN ring), 8.43 (d, 1H, J = 8.1 Hz, Py), 8.74 (d, 1H, J = 4.8 Hz, Py). Anal. Calcd for $C_{20}H_{14}ClN_3O$ (347.80): C, 69.07; H, 4.06; N, 12.08. Found: C, 69.08; H, 4.06; N, 12.09.

4.2.5.6. 4-(4-Chlorophenyl)-2-(prop-2-ynyloxy)-6-(2-pyridyl)nicotinonitrile (10). This compound was prepared from **1a** (3.05 g) and propargyl bromide (1.18 g) according to the general procedure described for alkylation and was obtained as a yellow powder (2.5 g, 73% yield), mp 180–182 °C. IR (KBr, cm^{-1}): 2224 (C≡N). ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 3.68 (s, 1H, ≡CH), 5.30 (d, 2H, J = 7.2 Hz, CH$_2$), 7.32 (t, 1H, J = 5.1 Hz, Py), 7.54 (d, 2H, J = 8.7 Hz, Ar–H), 7.76 (d, 2H, J = 8.7 Hz, Ar–H), 8.02 (t, 1H, J = 7.5 Hz, Py), 8.13 (s, 1H, 2-ONN ring), 8.44 (d, 1H, J = 8.1 Hz, Py), 8.74 (d, 1H, J = 4.8 Hz, Py). Anal. Calcd for $C_{20}H_{12}ClN_3O$ (345.78): C, 69.47; H, 3.50; N, 12.15. Found: C, 69.46; H, 3.52; N, 12.16.

4.3. Biology

4.3.1. Antimicrobial screening

The 2-ONN compounds were screened against the specified microorganisms using the well diffusion method [29]. In this method, bacteria were grown on the nutrient agar and fungi were grown on the Czapek's-Dox agar medium slants. The spores were inoculated in sterilized media, then shaken to make their dispersion homogeneous, and then poured in plates and left to solidify. Solutions of the tested compounds in DMF were prepared in a concentration of 250 μg/mL. Wells of 1 cm diameter were punched into the agar medium and filled with 1 mL of the compound solution. The plates were left for 24 h in a refrigerator to allow diffusion. Next, the plates were incubated at 35 °C for 48 h, in case of antibacterial screening, and at 28 °C for 5–7 days, in case of antifungal screening. The diameter of inhibition zones was measured in mm at the end of an incubation period. Ampicillin and Dermatin were used as references for bacteria and fungi, respectively.

4.3.2. In vitro antiviral screening

In vitro activity screening against viruses was carried out by Health Specialist, Virology Branch, Division of Microbiology and Infection Diseases (DMID) at the National Institute of Allergy and Infection Diseases (NIAID) and the National Institute of Health (NIH), USA. The compounds were evaluated for their antiviral activity against HCV and HBV using RNA Hybridization (Replicon) and DNA Hybridization (virion) control assays. Cytopathic effect and neutral red control assays were used with the other viruses.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.12.055.

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