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Original article

Efficient synthesis of 3*H*,3′*H*-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione as novel skeletons specifically for influenza virus type B inhibition

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ABSTRACT

An efficient and novel two step synthetic procedure to prepare various substituted 3*H*,3′*H*-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-diones **A**, was established from very simple and easily available starting materials. The developed method is a robust and general approach for the synthesis of these structures. The prepared compounds were tested against influenza virus type A *viz.*, A/Taiwan/1/86 (H1N1), A/Hong Kong/8/68 (H3N2) and type B *viz.*, B/Panama/45/90, B/Taiwan/2/62, B/Lee/40, B/Brisbane/60/2008. Among 31 compounds tested, some of them showed good activity (selective index values >10) against these influenza viruses preferentially for type B. The most active compound **3b** showed activity in 3.0–16.1 μM range with a selectivity index value between 30 and 166 against these type B viruses, in which it was comparable to the antiviral agent favipiravir. Also, **3b** is found to be inactive against other enveloped viruses (*viz.*, HIV and HSV) showing its specificity for influenza viruses.

1. Introduction

Influenza virus infection can be prevented by vaccination and therapeutic treatment available with viral neuraminidase (NA) or matrix protein (M2) inhibitors. Nevertheless, it has not been eradicated and has become a major cause of seasonally regional or pandemic threat to humans [1–6]. Its ability to shelter in various mammals or birds and to persistently evolve via antigenic drift or shift results in generation of uncharacterized viruses. It is also believed that they might have resistance developed to available treatments. Clinically, a series of adamantanes (M2 inhibitors) are available for the treatment of influenza type A viruses, but are ineffective against type B virus or M2-mutant viruses of H1N1 and H3N2. Also the side effects associated with them in the central nervous system are severe [7–10]. Well known NA inhibitors have broad-spectrum activity against various (sub) types of influenza viruses. Although oseltamivir phosphate (OSV-P) is safe compared with the adamantanes but increasing frequency of oseltamivir-resistant mutants and their cross-resistance to another NA inhibitor, *e.g.*, zanamivir, provoke the need for development of alternative antiviral drugs with different modes-of-action [7–10]. Thus, the discovery of novel anti-influenza agents is urgently needed. In this regard, the anti-influenza ability of various spiro substructures is worth to investigate, as they are known to be present in many biologically active [11–15] and pharmacologically important compounds [16,17]. In fact, there are well known active compounds against influenza viruses which contain spiro substructure [18].

Moreover, spiro substructures find their application in not only in medicinal chemistry but also in many other fields of chemistry [19,20]. However, despite their application in different areas of science, general and facile synthetic methods to prepare them still remain a challenge for organic chemists [21]. It requires special techniques and reactions to generate these spiro substructures mainly because of the presence of a quaternary carbon and fused rings in the structure.

Especially, 3*H*,3′*H*-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-diones **A** (Fig. 1) belongs to a class of spiro compounds which have not received a considerable attention yet. The core structure is

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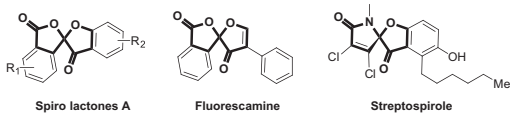


Fig. 1. Representative compounds with core spiro structure.

known to be present in synthetic compounds used for various applications such as *Fluorogens* used for the detection of amino groups (*e.g.* Fluorescamine) [22]. Lactum form of this spiro substructure is also present in Streptospirole derivatives, derived from microorganism *Streptomyces*, which are known to inhibit bacterial growth [23]. In spite of this, there are only a few methods available and all of them involve multistep synthesis [24–27].

Moreover, the previous reports available for these 3*H*,3′*H*-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-diones **A** have never attracted interest among the medicinal chemists for its application. To the best of our knowledge, there are no earlier reports of any biological activity of these 3*H*,3′*H*-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-diones **A**.

However, based on our experimental data, we found that some of these novel compounds prepared by our new method are active particularly against influenza virus type B, including B/Taiwan/2/62 (TWB), B/Lee/40 (LE), B/Panama/45/90 (PNM), and B/Brisbane/60/2008 (BB): the most potent compound having the 50% effective concentration (EC_{50}) values ranging from 3.0 to 16.1 μM against these type B viruses.

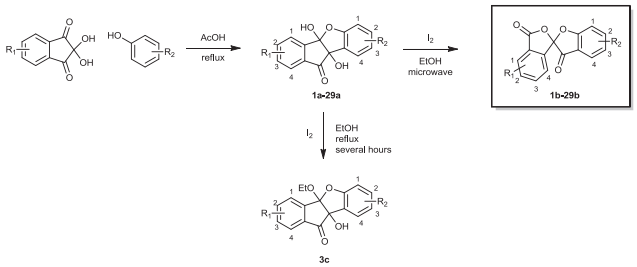
Thus, herein we report, the first microwave-assisted and molecular-iodine mediated efficient oxidative procedure to build the 3*H*,3′*H*-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione skeleton and its application into biological system as the inhibitors of influenza virus infection.

2. Results and discussion

2.1. Chemistry

A thorough literature survey reveals that, after the first synthesis of 3*H*,3′*H*-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione **1b** [24], only two reports appeared in the literature for the synthesis of similar compounds. One report shows the formation of **1b** as a side product in 14% yield during the synthesis of Wrightiadione [25]. And the other report was by Bullington and Dodd where they prepared only **30b** in a multistep route with low yield [26]. The same authors also reported a similar conversion of nitrogen analogues of these spiro compounds from 4b,9b-dihydroxy-4b,5-dihydroindeno[1,2-*b*]indol-10(9b*H*)-ones. Indeed, a few more methods to get the nitrogen analogues of these spiro lactones and respective spiro lactams are available [28,29]. But, to the best of our knowledge, there are no other reports directly targeting the synthesis of spiro lactones **A**. Also, the methods reported earlier for synthesis of **A** are not general or have drawbacks such as multistep synthesis, use of harsh reagents and conditions, low yields, and reproducibility. Thus, a general and efficient method is in need to synthesize these 3*H*,3′*H*-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-diones **A**.

We started the synthesis by the reaction of the respective ninhydrins with various phenols in acidic medium to get the 4b,9b-dihydroxy-4b*H*-indeno[1,2-*b*]benzofuran-10(9b*H*)-ones (**1a**–**31a**) in good to high yields as shown in Scheme 1 [30,31]. These compounds are known to give the corresponding alkyl ethers (*e.g.* **3c**, Scheme 1) under iodine/alcohol reflux conditions [32]. When we continued this reaction for extended time we observed the formation of the 3*H*,3′*H*-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-diones **A** as a major byproduct. In particular, we could obtain 6-isopropyl-3*H*,3′*H*-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione



Scheme 1. General synthetic scheme.

2b from 4b,9b-dihydroxy-7-isopropyl-4b*H*-indeno[1,2-*b*]benzofuran-10(9b*H*)-one **2a** with iodine (2.0 eq.)/ethanol under reflux for two days in 51% yield. The product was unambiguously characterized by ^{1}H NMR, ^{13}C NMR and its LC/MS data displayed molecular ion peak at *m*/*z* 294.1 (M^+). Finally, single crystal XRD of this compound confirmed its formation as shown in Fig. 2 (Table 1, Supporting information). Similarly, we also obtained **1b** from **1a** in 69% yield. The lack of in-depth literature precedents for the synthesis of **A**, led us to optimize the reaction conditions and further study the scope of the reaction.

Microwave reactions are well known for their high yields and shorter reaction times in comparison with classical thermal conditions. Since the reaction from **a** to **b** took several days under conventional thermal conditions and gave low chemical yields, we carried out further optimization with microwave irradiation (Table 1). The reaction with 4b,9b-dihydroxy-7,8-dimethyl-4b*H*-indeno[1,2-*b*]benzofuran-10(9b*H*)-one **3a** was used as a model system to optimize the reaction condition. Initial trials at 110 °C in ethanol gave the *O*-ethyl derivative **3c** as a major product even after 2 h of irradiation (Table 1, Entry 1). Increasing the temperature to 150 °C gave the conversion to the desired spiro-derivative **3b** in high yield with 2 h of continuous microwave irradiation (Table 1, Entry 2). Ethanol proved to be the best solvent for the reaction (Table 1, Entries 2, 3 and 4) and higher loadings of iodine shortened the reaction time (Table 1, Entries 6 and 7). We also found that a minimum of two equivalents of iodine was required to achieve high yields of **3b** as lesser equivalents showed little conversion to **3b** with the formation of **3c** (Table 1, Entry 5). Also, neither **3b** nor **3c** was obtained when the reaction was performed in absence of iodine suggesting that iodine plays an important role to facilitate the reaction (Table 1, Entry 8). Thus, 2 equivalents of iodine with 2 h of microwave irradiation were chosen as the optimum condition to avoid excessive use of iodine and to maintain appropriate reaction time.

In order to demonstrate the scope and potential for this microwave assisted synthesis of **A**, various substituents on the phenolic and ninhydrin ring were tested under the optimized reaction condition (Table 2). Thus, compounds with alkyl substituents on the phenolic ring were applied first under the optimized condition. Mono and poly-alkyl substituted derivatives were obtained in good to high yields (Table 2, **1b**, **2b**, **3b** and **4b**). The positions of these alkyl substituents were found to have no effect on the reaction and the different regioisomers of the methyl substituted derivatives

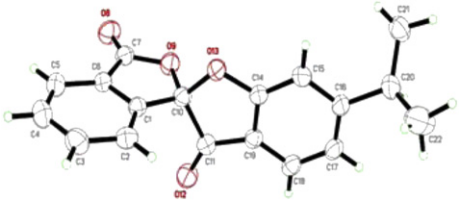
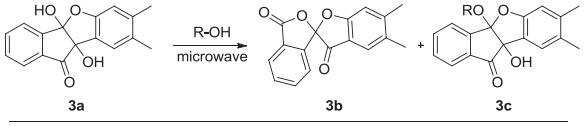


Fig. 2. ORTEP diagram of **2b**.

Table 1
Reaction condition optimization for synthesis of **3b**.

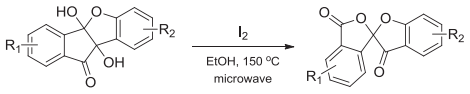


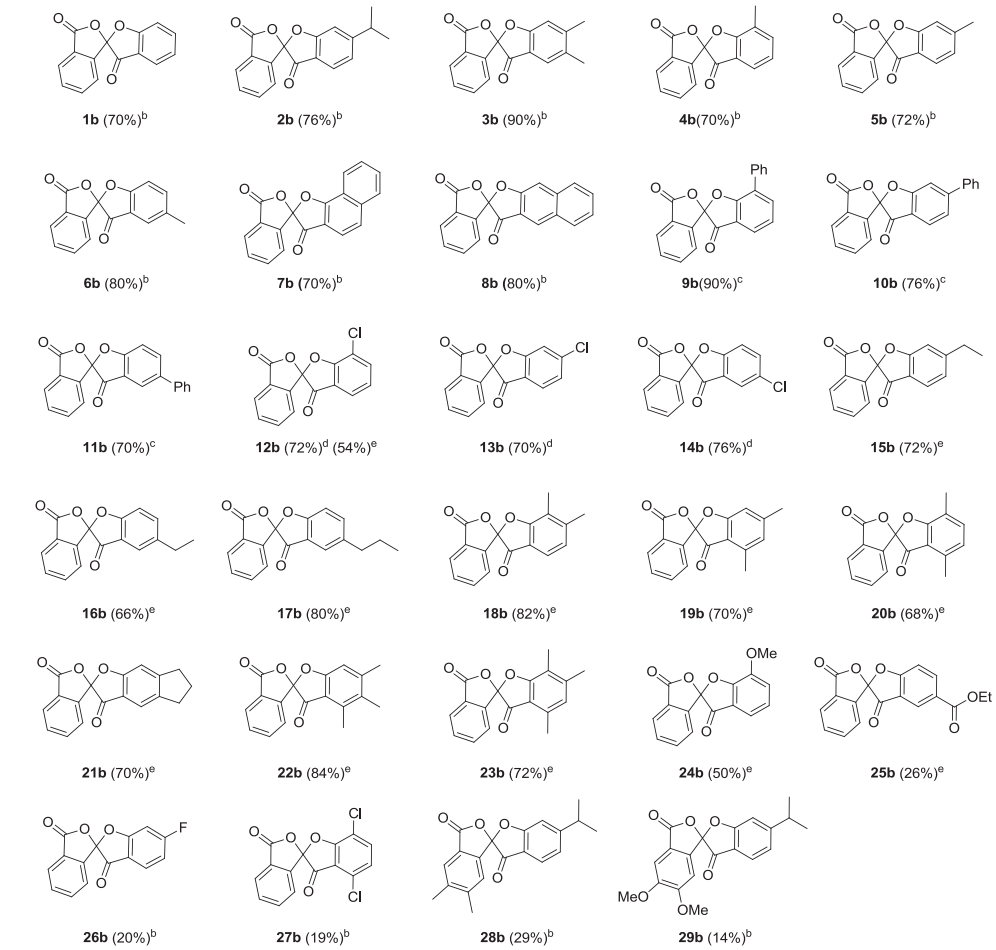
Entry	Solvent	Iodine (eq.)	Temp (°C)	Time (h)	Yield[a] (**3b**)	Yield[a] (**3c**)
1	EtOH	2.0	110	2	–	92%
2	EtOH	2.0	150	2	90%	–
3	^{i}PrOH	2.0	150	2	42%	–
4	^{t}BuOH[b]	2.0	150	2	–	–
5	EtOH	1.0	150	2	30%	28%
6	EtOH	4.0	150	1	84%	–
7	EtOH	10.0	150	0.25	80%	–
8	EtOH	0.0	150	2	–	–

[a] Isolated yield.
[b] The reaction gives a very messy TLC pattern.

were also obtained in high yields (Table 2, **4b**, **5b** and **6b**). Likewise the naphthalene ring compounds also gave good yields (Table 2, **7b** and **8b**). Interestingly, we observed that the reaction is quite sensitive other type of substituents present on the benzene rings and their positions. Particularly, in case of electron withdrawing groups on phenolic ring, we observed that the yields were decreased and the starting materials were recovered under the optimized conditions (Table 2, **26b** and **27b**). Nonetheless, with a little change in the optimized condition, we were able to obtain good yields of the products (Table 2, **9b**, **10b**, **11b**, **12b**, **13b** and **14b**). In general, electron withdrawing substituents on any ring slow down the reaction rate and higher loadings of iodine or longer reaction times were required to get high yields. Higher iodine loading was not sufficient and thus the irradiation time also has to be elongated to get high yields in some cases (Table 2, **12b**). The order of these electronic effects of substitution on phenolic ring was methyl < phenyl < chloro < fluoro ∼ ester. Transesterified product was obtained with the ester functionality on the substrate, which is in accord with the earlier published results (Table 2, **25b**) [33]. Strong electron donating substituents such as amino, hydroxy and

Table 2
Scope for the microwave assisted synthesis of the spiro derivatives of **A**.





[a] Isolated Yields. [b] with 2 eq. of Iodine for 2 h under microwave at 150 °C. [c] with 4 eq. of Iodine for 4 h under microwave at 150 °C.
[d] with 6 eq. of Iodine for 6 h under microwave at 150 °C. [e] with 4 eq. of Iodine for 2 h under microwave at 150 °C.

methoxy groups at position 2 of the phenolic ring (Scheme 1), resulted in either iodinated *O*-alkyl derivatives at lower temperature through electrophilic substitution reaction of iodine or tends to get decompose at higher temperature (see Supporting information). But, the methoxy group at position 1 on the phenolic ring gave the desired product in moderate yield (Table 2, **24b**). Substitution on the ninhydrin ring has a negative effect on the reaction (Table 2, **28b** and **29b**). As we obtained the initial results of SAR for these compounds and dimethyl group on the phenolic ring showed good activity with low toxicity (Table 3), we decided to prepare more derivatives with alkyl substitution. In these cases we found that in general using four equivalents of iodine with 2 h of irradiation gave reasonable yields with the completion of the reaction in almost every case (Table 2, **15b**–**23b**).

2.2. Mechanistic insight

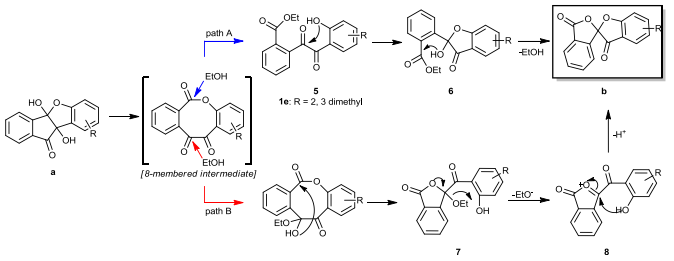
Formation of the 8-membered ring intermediate (Scheme 2) was reported earlier in the case of nitrogen analogues either through air oxidation or sodium periodate oxidation [26]. We also speculated that the same intermediate could have been formed in our case. To prove whether the 8-membered ring compound is the reactive intermediate of the reaction, we prepared two 8-membered ring compounds (Scheme 3, **1d** and **2d**) with the previously known method [26] and applied the intermediate under the microwave condition in ethanol without iodine. As a result, we were able to obtain the desired spiro products (**30b** and **31b**), suggesting that the spiro compounds could be formed through 8-membered ring intermediates.

Further, this reactive 8-membered ring intermediate may undergo two possible pathways (Scheme 2). In path A, the intermediate could be hydrolyzed to give the ester compound **5** under the reaction condition. Subsequently, the attack of phenolic hydroxyl group to the electron deficient carbonyl group may generate the 2-phenyl hydroxy furanone **6**. The hydroxy group later could cyclize with the ortho ester on the phenyl substituent and generate the lactone ring. In fact, the ester compound **1e** (Scheme 2) was isolated (in <5% yield) in our reaction conditions, when the reaction of **3a** to **3b** was quenched just after 15 min of microwave irradiation (see the Supporting information). **1e** [34] has also been converted to **3b**

Table 3
The CC_{50} and EC_{50} values of the spiro compounds against influenza A and B virus.

Compound[a]	CC_{50} (μM)	EC_{50} (μM)[b]					
		TWA[c]	HK[d]	PNM[e]	TWB[f]	LE[g]	BB[h]
1b	>500	>500	>500	88.1 ± 32.7	N.A.[i]	N.A.	N.A.
2b	314.4 ± 29.7	147.9 ± 20.7	129.3 ± 26.7	44.9 ± 3.7	N.A.	N.A.	N.A.
7b	40.4 ± 4.2	<6.2	8.9 ± 0.6	<6.2	N.A.	N.A.	N.A.
8b	>500	205.6 ± 15.0	185.7 ± 11.7	96.1 ± 57.2	N.A.	N.A.	N.A.
10b	36.3 ± 4.1	7.6 ± 1.9	>36.3	<6.2	N.A.	N.A.	N.A.
13b	>500	>500	>500	>500	N.A.	N.A.	N.A.
25b	>500	>500	>500	>500	N.A.	N.A.	N.A.
26b	>500	100.1 ± 0.5	63.8 ± 23.9	8.2 ± 2.0	N.A.	N.A.	N.A.
27b	86.0 ± 3.7	25.5 ± 19.2	17.3 ± 15.7	18.4 ± 4.0	N.A.	N.A.	N.A.
28b	>500	321.4 ± 46.2	110.0 ± 4.5	>500	N.A.	N.A.	N.A.
29b	>500	>500	>500	33.3 ± 10.1	N.A.	N.A.	N.A.
30b	305.3 ± 7.0	49.0 ± 36.0	30.0 ± 10.9	27.4 ± 0.8	N.A.	N.A.	N.A.
31b	>500	>500	>500	>500	N.A.	N.A	N.A
3b	>500	23.4 ± 0.6	19.1 ± 0.9	3.0 ± 0.1	3.6 ± 0.3	16.1 ± 0.2	4.8 ± 0.0
4b	>500	83.2 + 1.8	335.3 ± 151.6	24.7 + 2.9	53.0 + 0.0	198.7 + 27.4	103.9 + 1.8
5b	376.1 ± 41.7	27.8 ± 151.6	100.1 ± 19.6	25.7 ± 1.2	31.2 ± 5.1	59.9 ± 28.6	46.9 ± 7.3
6b	292.9 ± 81.9	32.9 ± 2.4	125.3 ± 6.0	7.6 ± 0.4	24.8 ± 0.1	32.8 ± 0.9	28.0 ± 0.0
8b	>500	205.6 ± 15.0	185.7 ± 11.7	96.1 ± 57.2	39.4 ± 5.1	159.3 ± 0.3	132.1 ± 4.6
9b	29.2 ± 0.3	12.9 ± 0.6	>29.2	4.3 ± 2.1	8.3 ± 1.1	12.2 ± 2.9	11.2 ± 3.3
11b	30.3 ± 0.4	>30.3	>30.3	6.6 ± 0.0	10.8 ± 1.7	14.1 ± 3.7	12.1 ± 1.0
12b	>500	173.8 ± 3.5	92.3 ± 0.0	21.7 ± 2.3	61.4 ± 4.4	226.6 ± 2.9	96.3 ± 6.4
14b	152.6 ± 14.3	25.2 ± 0.0	36.4 ± 7.1	7.8 ± 0.1	21.7 ± 0.2	38.8 ± 3.5	28.6 ± 1.9
15b	103.3 ± 12.6	34.6 ± 7.7	>103.3	11.2 ± 1.7	12.1 ± 0.3	34.4 ± 2.5	32.2 ± 3.7
16b	111.6 ± 2.8	28.1 ± 4.7	41.6 ± 9.8	7.5 ± 0.2	9.3 ± 0.4	31.2 ± 2.4	25.4 ± 2.4
17b	100.3 ± 0.9	30.8 ± 0.5	>100.3	9.8 ± 1.3	25.0 ± 1.5	33.7 ± 2.2	27.6 ± 0.9
18b	370.2 ± 183.6	7.1 ± 0.3	16.3 ± 3.2	9.9 ± 0.9	28.9 ± 0.5	32.0 ± 3.8	23.5 ± 3.7
19b	>500	11.4 ± 7.3	>500	>500	>500	>500	>500
20b	186.7 ± 24.8	>186.7	>186.7	>186.7	66.6 ± 9.2	>186.7	42.7 ± 4.2
21b	80.4 ± 2.7	9.8 ± 0.3	>80.4	2.1 ± 0.0	5.7 ± 9.2	10.6 ± 0.1	4.8 ± 0.1
22b	>500	>500	296.5 ± 3.6	11.0 ± 1.0	23.2 ± 4.1	254.5 ± 48.4	103.2 ± 27.8
23b	>500	6.3 ± 0.1	289.8 ± 5.3	>500	>500	>500	>500
24b	253.2 ± 1.6	126.4 ± 17.6	40.4 ± 1.0	23.6 ± 4.9	42.3 ± 2.0	58.2 ± 9.9	81.1 ± 10.0
AMT[j]	>100	0.6 ± 0.0	1.9 ± 0.4	>100	>100	>100	>100
OSV-C[k]	>100	0.05 ± 0.04	<0.01	0.06 ± 0.01	0.05 ± 0.00	0.82 ± 0.14	13.63 ± 0.53
T-705[l]	>100	6.95 ± 1.75	4.71 ± 0.98	1.94 ± 0.22	2.84 ± 0.93	1.93 ± 0.88	1.32 ± 0.23

[a] Concentration of chemicals required for reducing 50% of viability of normal MDCK cells.
[b] Concentration of chemicals required for improving 50% of viability of influenza virus-infected cells.
[c] A/Taiwan/1/86 (H1N1).
[d] A/Hong Kong/8/68 (H3N2).
[e] B/Panama/45/90.
[f] B/Taiwan/2/62.
[g] B/Lee/40.
[h] B/Brisbane/60/2008.
[i] Not applicable.
[j] Amantadine.
[k] Oseltamivir carboxylate.
[l] Favipiravir.



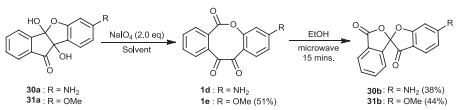
Scheme 2. Proposed mechanism.

in ethanol under microwave irradiation (see the Supporting information) without iodine. These results were supportive to corroborate our proposed mechanism. Further, in path B, which was reported by Bullington and Dodd [26], the intermediate may undergo ethanol-induced nucleophilic attack to the electron deficient carbonyl, which could result in formation of the 5-membered lactone **7** after intramolecular transesterification. The oxonium ion **8**, generated by liberating ethoxide anion, could be converted to the spiro derivative by the attack of the phenolic hydroxyl group.

2.3. Biology

Present therapeutic treatment of influenza virus is mostly based on viral NA inhibitors such as oseltamivir, zanamivir, peramivir and laninamivir, which are all sialic acid derivatives. Clinically, adamantanes such as amantadine and rimantadine as M2 protein ion channel inhibitors are also available. As a continuation of ongoing research in search of novel anti-influenza agents with entirely different modes-of-action, our in-house chemical library containing about 7000 diverse compounds was screened. The compounds **1b** and **2b** (Table 3), the initial products obtained by us under thermal conditions were identified to have some activity. Based on the structure of **1b** diverse spiro compounds (**2b**, **3b**, **7b**, **8b**, **10b**, **13b** and **25b**–**31b**) were then synthesized by the chemical route developed by us, which were subjected to test for their antiviral activity against the influenza A and B viruses. They were treated to MDCK cells infected with three different strains of influenza virus such as A/Taiwan/1/86 (H1N1) (TWA), A/Hong Kong/8/68 (H3N2) (HK) and PNM, and then reduction of the virus-induced cytotoxicity was measured by the fluorescein diacetate-based (FDA) cytopathic effect assay [35,36]. The initial activity results of these 14 compounds showed that **3b**, **26b** and **29b** have significant antiviral activity particularly against the PNM strain, with the 50% cytotoxic concentration (CC_{50}) values above 500 μM and selectivity index (the ratio of CC_{50} to EC_{50}) greater than 15. **3b** being most active and without any considerable cytotoxicity (Fig. A, Supporting information) was used as a reference for a further systematic SAR study performed with more abundant repertoire of **A**. Also, as the most predominant inhibitory effect of **3b** was against influenza virus type B (PNM) infection thus the three additional influenza B viruses (TWB, LE and BB) as well as the primarily used viral strains were tested simultaneously. Thus our further antiviral study with these spiro compounds gets focused on inhibition of the type B influenza virus infection.

Based on the antiviral test, the core compound **1a** was active against PNM but not against TWA or HK. It was not toxic to host



Scheme 3. Preparation of 8-membered intermediates and respective spiro compounds.

MDCK cells, but was not as potent as OSV-C or RBV. In the comparison analysis with **1b**, the presence of a methyl group on the phenolic ring as seen in **4b**, **5b** and **6b** showed considerably improved antiviral effect against not only PNM but also TWB, LE and BB, but cytotoxicity was exhibited at the maximum concentration of **5b** and **6b** (CC_{50} values, 376.1 and 292.9 μM, respectively). It suggests that the methyl group at position 1, 2, or 3 does not damage the function of **1b** required for inhibiting the influenza B virus infection, but substitution at position 1 is most desirable to maintain both antiviral efficacy and cell viability. The antiviral and cytotoxic profiles for the other two chloride derivatives (**12b** and **14b**) were very similar to methyl substituents. Again, chloride at position 1 of the phenolic ring has improved cell viability. Notably, upon treatment of **12b**, the cell viability even at its maximum concentration was comparable to mock cells viability. However, substitution with methoxy at the same position (**24b**) showed again cytopathicity but still was accompanied with the antiviral activity against the type B viruses. Although the compound **21b** has strong anti-flu B activity (EC_{50}, between 2.1 and 10.6 μM) its toxicity to host cells made it to have a narrow therapeutic window (data not shown). Other dimethyl substitution at positions 1 and 2, 2 and 4, or 1 and 4 (**18b**, **19b** and **20b**) was not as efficient as **3b** and trimethyl substitutions (as in **22b** and **23b**) also did not improve the antiviral property compare to **3b**. The cyclopentyl derivative (**21b**) structurally very similar to **3b** maintained the activity but induced toxicity. In accord with the initial test results (**10b**), the presence of phenyl substituents on any position of the phenolic ring (**9b** and **11b**) was a supplement to the high toxicity with CC_{50} values between 30 and 40 μM, thus resulting in reduction of their selectivity index (the ratio of CC_{50} to EC_{50}) less than 5, which is lower than that of the **1b** having about 20 for PNM. The other prepared derivatives having a higher alkyl substitution on the phenolic ring at 3 positions (**16b** and **17b**) still maintained the activity but harboured cellular toxicity (CC_{50}, about 100 μM). We also found that any substitution at position 2 on the phenolic ring with isopropyl, ethyl or fluoro group **2b**, **15b** and **26b**) still maintained the antiviral activity against the type B viruses but induced cytotoxicity of the MDCK cells.

Additionally, we could observe from Table 3 that the naphthyl rings (**7b** and **8b**) are responsible for inducing toxicity or loss of activity. The ester substitution (**25b**) showed neither antiviral activity nor toxicity. Dichloride substitution at positions 1 and 4 (**27b**) was also highly toxic. It should be mentioned here that although **26b** has CC_{50} value above 500 μM, it displayed above 20% normal cell death at the maximum concentration (data not shown). In addition, more complex substitutions at both rings 1 and 2 lost the type B-specific antiviral activity (**28b**) or showed toxicity causing cell death of about 47% at 500 μM (**29b**). Amino group at position 3 (**30b**) of the phenolic ring showed activity accompanied with toxicity whereas the methoxy group (**31b**) was neither active nor toxic.

As **3b** was the most active compounds without cytotoxicity throughout the assay, we carried out the plaque inhibition assay to confirm its efficacy against the various strains of influenza B viruses as shown (Fig. 3). Notably, it was not active against other enveloped viruses, such as herpes simplex virus (HSV) or human immunodeficiency virus (HIV) (Tables 4 and 5). Thus it was confirmed that the antiviral activity of **3b** is specific for influenza virus only.

Overall the data suggest that for spiro compounds **A** to be active and relatively safe antiviral agents for targeting the influenza B virus, it should be incorporated with the dimethyl group at positions 2 and 3 on the phenolic ring (**3b**). As an alternative and simplified approach, it can be substituted at the position 1 either with chloride (**12b**) or methyl (**4b**). In this report it can be stressed that although the activity of **3b** was less potent than the effect of OSV-C, it inhibited influenza B virus with nearly comparable

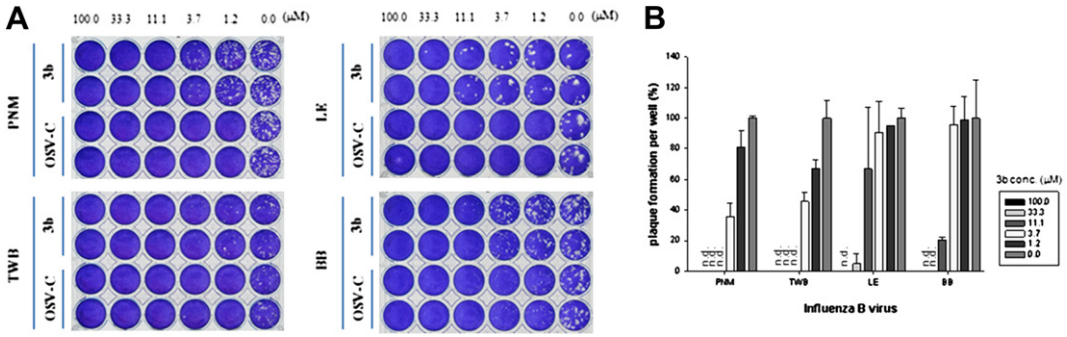


Fig. 3. A) Plaque inhibition assay of compound **3b** with MDCK cells. B) Plaque numbers were counted from crystal violet-stained plates and the percentage number relative to mock-treated, virus-infected cells (100%) was calculated. n.d., not detected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Anti-HSV activity of **3b** measured by an MTT-based CPE assay.

Compound	CC_{50} (μm)[a]	EC_{50} (μM)[b]	
		HSV-1[c]	HSV-2[d]
3b	311.7 ± 9.1	>311.7	>311.7
ACV[e]	>50.0	1.7 ± 0.3	58.6 ± 3.3

[a] Concentration of chemicals required for reducing 50% of viability of normal Vera cells.
[b] Concentration of chemicals required for improving 50% of viability of herpes simplex virus (HSV)-infected cells.
[c] HSV type 1 strain F.
[d] HSV type 2 strain MS.
[e] Acyclovir.

Table 5
Anti-HIV activity of **3b** measured by an MTT-based CPE assay.

Compound	CC_{50} (μm)[a]	EC_{50} (μM)[b]	
		HIV-1[c]	HIV-2[d]
3b	158.1 ± 5.5	>158.1	>158.1
ddC[e]	75.2 ± 7.9	0.4 ± 0.1	0.4 ± 0.1
ddI[f]	>500.0	7.8 ± 0.6	34.7 ± 4.2

[a] Concentration of chemicals required for reducing 50% of viability of normal MT4 cells.
[b] Concentration of chemicals required for improving 50% of viability of human immunodeficiency virus (HIV)-infected cells.
[c] HIV type 1 strain III-B infected in HUT78 cells.
[d] HIV-2 strain ROD.
[e] Zalcitabine.
[f] Didanosine.

antiviral activity to that of T-705 and with higher selectivity than influenza A virus. Other enveloped viruses, HSV and HIV, were not sensitive to **3b**. Thus, it is expected that the compound **3b** could be a novel specific inhibitor of influenza B viruses which are resistant to spiro adamantanes. Potentially, it may also be used as a hit compound for the discovery of novel inhibitors against influenza virus infection for which efforts are ongoing. Also, further studies to elucidate the mode-of-action of **3b** and its target molecule(s) are under progress.

3. Conclusion

In summary, we report a short and efficient synthesis of a series of 3*H*,3′*H*-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-diones **A**, by microwave-assisted and molecular iodine-mediated novel reaction pathway. This provides a new general route to prepare spiro compounds **A** in two simple steps from easily available starting materials. In addition, our synthetic methodology is advantageous as being robust and more general over the earlier reported procedures with a direct one pot conversion of **a** to the spiro derivatives **b** in good to high yields (Table 2). This novel short and efficient synthetic methodology can be usefully applied in medicinal and organic chemistry. The prepared compounds show preferential inhibition of influenza virus type B over type A in general. The SAR analysis showed that the presence of chloride or methyl substitutions at position 1 of the phenolic ring gave a potent antiviral activity without any cytotoxicity. In particular, the presence of dimethyl group at positions 2 and 3 on the ring exhibited the most enhanced activity with **3b** showing EC_{50} values between 3.0 and 16.1 μM, without any cytotoxicity at 500 μM. In addition **3b** was found to be active against only influenza viruses and showed no activity against other enveloped viruses such as HIV and HSV. Further studies to establish in-depth SAR and modes-of-action are in progress. It is expected that **3b** could be further optimized to be a candidate for development of a novel and specific therapeutics inhibiting influenza B viruses which are not sensitive to adamantanes, as well as influenza A virus to some extent.

4. Experimental section

4.1. Materials and methods in chemistry

Unless otherwise stated, all commercially available solvents and reagents were used without further purification. Melting points were recorded on Mettler Toledo MP50 apparatus and are uncorrected. IR spectra were recorded on an FT-IR Smiths IdentifyIR and the values are mentioned in cm^{-1}. The ^1H NMR and ^{13}C NMR was recorded on Varian 300 and Bruker 300 NMR spectrophotometer operating at 300 MHz, using commercial NMR solvents obtained from Aldrich with TMS as internal standard and chemical shifts are mentioned in δ ppm scale. Single crystal X-ray was performed with Bruker SMART Apex II X-ray Diffractometer. The Mass spectra were obtained over Varian 1200L quadrupole MS (EI) spectrophotometer and LCMS were recorded over Waters Acquity UPLC coupled with Micromass Quattro micro spectrophotometer. Elemental Analysis was done on Thermopack instruments. The microwave irradiation was carried on Anton Paar Synthos 3000 Microwave. The reaction progress and purity of the products was checked by Thin-Layer Chromatography (TLC) on Merck TLC Silica gel 60 F_{254} TLC plates. $KMnO_4$ and PMA stains were used as detecting agent.

4.1.1. Synthesis of 4b,9b-dihydroxy-4bH-indeno[1,2-b]benzofuran-10(9bH)-ones **1a**–**31a**

4b,9b-Dihydroxy-4b*H*-indeno[1,2-*b*]benzofuran-10(9b*H*)-ones (**1a**–**31a**) were synthesized according to previously reported approach [30,31].

*4.1.2. Preparation of 3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**1b**) under thermal condition*

4b,9b-Dihydroxy-4b*H*-benzo[*d*]indeno[1,2-*b*]furan-10(9b*H*)-one **1a** (0.50 g, 1.96 mmol) was taken in ethanol (5 mL) with iodine (0.99 g, 3.93 mmol) at room temperature. This mixture was refluxed for 72 h. Then ethanol was evaporated completely and the residue was dissolved in DCM (100 mL). This was washed with 10–15% aqueous sodium thiosulfate solution (50 mL × 2), water (50 mL) and brine (50 mL). Then it was dried over anhydrous sodium sulphate and concentrated. The crude mass obtained was then purified over silica gel column chromatography (10% ethyl acetate in hexane) to get the pure product as white solid. Yield: 0.34 g (69%); mp: 170–175 °C; IR (cm^{-1}): 1786, 1732; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.22–7.29 (m, 2H, ArH), 7.38–7.41 (m, 1H, ArH), 7.70–7.80 (m, 4H, ArH), 7.99–8.01 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 104.2, 113.6, 118.6, 122.5, 123.8, 125.8, 126.1, 127.1, 131.9, 135.1, 139.7, 142.3, 166.8, 171.2, 192.4 (C=O); HRMS (EI): mass calculated for $C_{15}H_8O_4$ (M^+), 252.0423; *m*/*z* found, 252.0435.

*4.1.3. Preparation of 6-isopropyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**2b**) under thermal condition*

4b,9b-Dihydroxy-7-isopropyl-4b*H*-benzo[*d*]indeno[1,2-*b*]furan-10(9b*H*)-one **2a** (0.50 g, 1.68 mmol) was taken in ethanol (10 mL) with Iodine (0.85 g, 3.37 mmol) at room temperature. This mixture was refluxed for 48 h. Then ethanol was evaporated completely and the residue was dissolved in DCM (100 mL). This was washed with 10–15% aqueous sodium thiosulfate solution (50 mL × 2), water (50 mL) and brine (50 mL). Then it was dried over anhydrous sodium sulphate and concentrated. The crude mass obtained was then purified over silica gel column chromatography (10% ethyl acetate in hexane) to get the pure product as white solid. Yield 250 mg (51%); mp: 132–135 °C; IR (cm^{-1}): 1775, 1730; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.32 (d, J = 6.9 Hz, 6H, CH_3), 3.05 (septet, J = 6.9 Hz, 1H, CH), 7.07 (s, 1H, ArH), 7.13 (d, J = 7.8 Hz, 1H, ArH), 7.38–7.41 (m, 1H, ArH), 7.68–7.74 (m, 3H, ArH), 7.98–8.00 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.3, 35.3, 104.6, 111.1, 116.5, 122.5, 123.0, 125.6, 126.1, 127.1, 131.8, 135.0, 142.5, 163.2, 166.9, 171.9, 191.7 (C=O); Anal. Calcd for $C_{18}H_{16}O_4$: C, 72.96; H, 5.44; O, 21.6. Found C, 73.37; H, 4.80; O, 21.58; HRMS (EI): mass calculated for $C_{18}H_{14}O_4$ (M^+), 294.0892; *m*/*z* found, 294.0893.

*4.1.4. General method for the preparation of spiro compounds under microwave condition **1b**–**29b***

4b,9b-Dihydroxy-4b*H*-indeno[1,2-*b*]benzofuran-10(9b*H*)-ones (**2a**–**ac**) (50 mg, 1.0 eq.) was taken in ethanol (1 mL) and to these specified equivalents of iodine was added. The reaction mass was then irradiated in microwave for specified time at 150 °C. Then the reaction mass was concentrated and then dissolved in DCM (100 mL). This was washed with 20% aqueous sodium thiosulfate solution (30 mL), then with water (30 mL) and brine (30 mL). This was dried with anhydrous sodium sulphate and concentrated to get the crude product which was purified over silica gel column chromatography (ethyl acetate/hexanes) to afford the spiro compounds (**1b**–**29b**).

*4.1.4.1. 3H,3′H-Spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**1b**).* Yield: 35 mg (70%). Analytical data matched as mentioned above.

*4.1.4.2. 6-Isopropyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**2b**).* Yield: 38 mg (76%). Analytical data matched as mentioned above.

*4.1.4.3. 5,6-Dimethyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**3b**).* Yield: 45 mg (90%); mp: 199–201 °C; IR (cm^{-1}): 1784, 1725; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.31 (s, 3H, CH_3), 2.41 (s, 3H, CH_3), 7.01 (s, 1H, ArH), 7.34–7.37 (m, 1H, ArH), 7.51 (s, 1H, ArH), 7.68–7.70 (m, 2H, ArH), 7.97–7.99 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 19.5, 21.8, 104.8, 114.2, 116.5, 122.6, 125.5, 126.2, 127.2, 131.9, 133.1, 135.2, 142.8, 151.6, 167.2, 170.4, 192.0 (C=O); HRMS (EI): mass calculated for $C_{17}H_{12}O_4$ (M^+), 280.0736; *m*/*z* found, 280.0737.

*4.1.4.4. 7-Methyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**4b**).* Yield: 35 mg (70%); mp: 155–157 °C; IR (cm^{-1}): 1798, 1728; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.35 (s, 3H, CH_3), 7.15 (t, J = 7.5 Hz, 1H, ArH), 7.39–7.41 (m, 1H, ArH), 7.59–7.62 (m, 2H, ArH), 7.68–7.76 (m, 2H, ArH), 7.98–8.01 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.3, 104.4, 118.3, 122.7, 123.1, 123.8, 124.0, 126.2, 127.3, 132.0, 135.2, 140.6, 142.7, 167.1, 170.1, 193.1 (C=O); HRMS (EI): mass calculated for $C_{16}H_{10}O_4$ (M^+), 266.0579; *m*/*z* found, 266.0581.

*4.1.4.5. 6-Methyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**5b**).* Yield: 36 mg (72%); mp: 185–187 °C; IR (cm^{-1}): 1787, 1724; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.41 (s, 3H, CH_3), 7.13 (d, J = 8.4 Hz, 1H, ArH), 7.37–7.39 (m, 1H, ArH), 7.56–7.59 (m, 2H, ArH), 7.66–7.74 (m, 2H, ArH), 7.97–7.99 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.0, 104.8, 113.9, 116.4, 122.7, 125.4, 125.5, 126.2, 127.2, 132.0, 135.2, 142.7, 152.6, 167.1, 171.8, 191.8 (C=O); HRMS (EI): mass calculated for $C_{16}H_{10}O_4$ (M^+), 266.0579; *m*/*z* found, 266.0580.

*4.1.4.6. 5-Methyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**6b**).* Yield: 40 mg (80%); mp: 166–168 °C; IR (cm^{-1}): 1794, 1724; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.41 (s, 3H, CH_3), 7.13 (d, J = 8.4 Hz, 1H, ArH), 7.37–7.39 (m, 1H, ArH), 7.56–7.59 (m, 2H, ArH), 7.66–7.74 (m, 2H, ArH), 7.97–7.99 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 20.8, 104.7, 113.4, 118.7, 122.7, 125.3, 126.2, 127.2, 132.0, 133.9, 135.2, 141.0, 142.6, 167.1, 169.8, 192.7 (C=O); HRMS (EI): mass calculated for $C_{16}H_{10}O_4$ (M^+), 266.0579; *m*/*z* found, 266.0580.

*4.1.4.7. 3H,3′H-Spiro[isobenzofuran-1,2′-naphtho[1,2-b]furan]-3,3′-dione (**7b**).* Yield: 21 mg (20%); mp: 187–189 °C; IR (cm^{-1}): 1794, 1713; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39–7.42 (m, 1H, ArH), 7.64–7.68 (m, 3H, ArH), 7.71–7.74 (m, 2H, ArH), 7.77–7.82 (m, 1H, ArH), 7.97 (d, J = 8.1 Hz, 1H, ArH), 8.04–8.06 (m, 1H, ArH), 8.23 (d, J = 8.7 Hz, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 105.2, 113.7, 119.3, 121.1, 122.7, 122.8, 124.4, 126.4, 127.2, 127.6, 128.8, 132.0, 132.1, 135.3, 139.6, 142.7, 167.1, 172.6, 191.9 (C=O); HRMS (EI): mass calculated for $C_{19}H_{10}O_4$ (M^+), 302.0579; *m*/*z* found, 302.0580.

*4.1.4.8. 3H,3′H-Spiro[isobenzofuran-1,2′-naphtho[2,3-b]furan]-3,3′-dione (**8b**).* Yield: 40 mg (80%); mp: 223–225 °C; IR (cm^{-1}): 1786, 1710; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36 (d, J = 9.0 Hz, 1H, ArH), 7.40–7.43 (m, 1H, ArH), 7.54–7.59 (m, 1H, ArH), 7.68–7.73 (m, 3H, ArH), 7.92 (d, J = 7.8 Hz, 1H, ArH), 7.96–8.03 (m, 1H, ArH), 8.25 (d, J = 9.0 Hz, 1H, ArH), 8.67 (d, J = 8.4 Hz, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 104.8, 111.4, 113.5, 122.7, 123.5, 126.3, 126.5, 127.3, 129.1, 129.3, 130.0, 130.9, 132.0, 135.3, 142.1, 142.6, 167.2, 174.4, 191.9 (C=O); HRMS (EI): mass calculated for $C_{19}H_{10}O_4$ (M^+), 302.0579; *m*/*z* found, 302.0580.

*4.1.4.9. 7-Phenyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**9b**).* Yield: 45 mg (90%); mp: 199–201 °C; IR (cm^{-1}): 1798, 1728; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.31–7.46 (m, 5H, ArH), 7.66–7.77 (m, 5H, ArH), 7.87–7.89 (m, 1H, ArH), 7.96–7.99 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 104.3, 119.4, 122.8, 124.4, 124.7, 126.3, 127.3, 127.7, 128.61, 128.63, 128.9, 132.0, 134.1, 135.3, 139.2, 142.5, 167.0, 168.4, 193.0 (C=O); HRMS (EI): mass calculated for $C_{21}H_{12}O_4$ (M^+), 328.0736; *m*/*z* found, 328.0735.

*4.1.4.10. 6-Phenyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**10b**).* Yield: 30 mg (60%); mp: 168–170 °C; IR (cm^{-1}): 1799, 1720; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40–7.54 (m, 6H, ArH), 7.65–

7.77 (m, 4H, ArH), 7.84 (d, J = 7.8 Hz, 1H, ArH), 7.99–8.02 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 104.9, 111.8, 117.4, 122.7, 123.4, 126.2, 126.3, 127.2, 127.6, 129.4, 129.6, 132.1, 135.3, 139.2, 142.6, 153.4, 167.1, 171.8, 192.0 (C=O); HRMS (EI): mass calculated for $C_{21}H_{12}O_4$ (M^+), 328.0736; m/z found, 328.0736.

*4.1.4.11. 5-Phenyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**11b**).* Yield: 35 mg (70%); mp: 202–204 °C; IR (cm^{-1}): 1791, 1734; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.31 (d, J = 8.4 Hz, 1H, ArH), 7.38–7.51 (m, 4H, ArH), 7.58 (d, J = 7.5 Hz, 2H, ArH), 7.69–7.76 (m, 2H, ArH), 7.97–8.02 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 104.9, 114.0, 119.2, 122.7, 123.8, 126.3, 127.1, 127.2, 128.1, 129.3, 132.1, 135.3, 137.8, 139.0, 142.5, 167.0, 170.7, 192.7 (C=O); HRMS (EI): mass calculated for $C_{21}H_{12}O_4$ (M^+), 328.0736; m/z found, 328.0735.

*4.1.4.12. 7-Chloro-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**12b**).* Yield: 36 mg (72%); mp: 200–202 °C; IR (cm^{-1}): 1789, 1735; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.20–7.25 (m, 1H, ArH), 7.41–7.44 (m, 1H, ArH), 7.69–7.79 (m, 4H, ArH), 7.99–8.02 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 104.7, 119.4, 120.5, 122.9, 124.1, 124.7, 126.4, 127.2, 132.4, 135.4, 139.5, 141.9, 166.6, 166.8, 191.9 (C=O); HRMS (EI): mass calculated for $C_{15}H_7ClO_4$ (M^+), 286.0033; m/z found, 286.0032.

*4.1.4.13. 6-Chloro-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**13b**).* Yield: 28 mg (56%); mp: 215–217 °C; IR (cm^{-1}): 1793, 1728; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.23–7.26 (m, 2H, ArH), 7.37–7.40 (m, 1H, ArH), 7.69–7.76 (m, 3H, ArH), 7.99–8.02 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 104.8, 114.4, 117.4, 122.7, 125.0, 126.4, 126.7, 127.1, 132.3, 135.4, 142.1, 146.4, 166.7, 171.4, 191.1 (C=O); HRMS (EI): mass calculated for $C_{15}H_7ClO_4$ (M^+), 286.0033; m/z found, 286.0034.

*4.1.4.14. 5-Chloro-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**14b**).* Yield: 38 mg (76%); mp: 162–165 °C; IR (cm^{-1}): 1790, 1736; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.20 (d, J = 8.4 Hz, 1H, ArH), 7.37–7.39 (m, 1H, ArH), 7.71–7.74 (m, 1H, ArH), 7.98–8.00 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 104.9, 115.2, 119.9, 122.7, 125.2, 126.4, 127.1, 129.6, 132.3, 135.4, 139.7, 142.0, 166.7, 169.6, 191.6 (C=O); HRMS (EI): mass calculated for $C_{15}H_7ClO_4$ (M^+), 286.0033; m/z found, 286.0032.

*4.1.4.15. 6-Ethyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**15b**).* Yield: 36 mg (72%); mp: 173–175 °C; IR (cm^{-1}): 1787, 1726; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.32 (t, J = 7.5 Hz, 3H, CH_3), 2.79 (q, J = 7.5 Hz, 2H, CH_3), 7.05 (s, 1H, ArH), 7.09 (d, J = 7.8 Hz, 1H, ArH), 7.38–7.41 (m, 1H, ArH), 7.63–7.74 (m, 3H, ArH), 7.97–8.00 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) 15.0, 30.1, 104.8, 112.7, 116.6, 122.7, 124.4, 125.7, 126.2, 127.3, 132.0, 135.2, 142.7, 158.8, 167.1, 172.0, 191.9 (C=O); HRMS (EI): mass calculated for $C_{17}H_{12}O_4$ (M^+), 280.0736; m/z found, 280.0736.

*4.1.4.16. 5-Ethyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**16b**).* Yield: 33 mg (66%); mp: 158–162 °C; IR (cm^{-1}): 1791, 1726; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.28 (t, J = 7.5 Hz, 3H, CH_3), 2.72 (q, J = 7.5 Hz, 2H, CH_3), 7.15 (d, J = 7.8 Hz, 1H, ArH), 7.37–7.40 (m, 1H, ArH), 7.56–7.74 (m, 4H, ArH), 7.98–8.00 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) 15.6, 28.2, 104.7, 113.5, 118.7, 122.7, 124.2, 126.3, 127.3, 132.0, 135.2, 140.2, 140.3, 142.7, 167.1, 170.0, 192.8 (C=O); HRMS (EI): mass calculated for $C_{17}H_{12}O_4$ (M^+), 280.0736; m/z found, 280.0736.

*4.1.4.17. 5-Propyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**17b**).* Yield: 40 mg (80%); mp: 156–160 °C; IR (cm^{-1}): 1784, 1735; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.97 (t, J = 7.5 Hz, 3H, CH_3), 1.61–1.74 (m, 2H, CH_2), 2.64 (q, J = 7.5 Hz, 2H, CH_2), 7.14 (d, J = 8.4 Hz, 1H, ArH), 7.38–7.40 (m, 1H, ArH), 7.53–7.60 (m, 2H, ArH), 7.66–7.74 (m, 2H, ArH), 7.97–8.00 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) 13.7, 24.6, 37.2, 104.7, 113.4, 118.7, 122.7, 124.8, 126.3, 127.3, 132.0, 135.2, 138.8, 140.6, 142.6, 167.1, 170.0, 192.8 (C=O); HRMS (EI): mass calculated for $C_{18}H_{14}O_4$ (M^+), 294.0892; m/z found, 294.0894.

*4.1.4.18. 6,7-Dimethyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**18b**).* Yield: 41 mg (82%); mp: 178–180 °C; IR (cm^{-1}): 1797, 1722; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.24 (s, 3H, CH_3), 2.43 (s, 3H, CH_3), 7.06 (d, J = 7.8 Hz, 1H, ArH), 7.37–7.40 (m, 1H, ArH), 7.51 (d, J = 7.8 Hz, 1H, ArH), 7.67–7.74 (m, 2H, ArH), 7.96–8.00 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) 10.9, 20.7, 104.9, 116.2, 122.2, 122.5, 122.7, 125.9, 126.2, 127.3, 131.9, 135.2, 142.9, 150.7, 167.2, 170.2, 192.6 (C=O); HRMS (EI): mass calculated for $C_{17}H_{12}O_4$ (M^+), 280.0736; m/z found, 280.0737.

*4.1.4.19. 5,7-Dimethyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**19b**).* Yield: 35 mg (70%); mp: 196–198 °C; IR (cm^{-1}): 1795, 1717; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.44 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 6.82 (s, 2H, ArH), 7.38–7.40 (m, 1H, ArH), 7.65–7.73 (m, 2H, ArH), 7.96–8.00 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 17.9, 22.8, 104.6, 111.0, 114.8, 122.7, 126.1, 126.6, 127.3, 131.9, 135.1, 141.1, 142.9, 151.8, 167.2, 172.0, 192.1 (C=O); HRMS (EI): mass calculated for $C_{17}H_{12}O_4$ (M^+), 280.0736; m/z found, 280.0737.

*4.1.4.20. 4,7-Dimethyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**20b**).* Yield: 34 mg (68%); mp: 136–138 °C; IR (cm^{-1}): 1791, 1722; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.29 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 6.90 (d, J = 7.5 Hz, 1H, ArH), 7.40–7.42 (m, 2H, ArH), 7.67–7.75 (m, 2H, ArH), 7.98–8.00 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.0, 17.6, 104.2, 116.5, 120.6, 122.8, 125.0, 126.2, 127.4, 131.9, 135.1, 138.5, 140.1, 142.9, 167.3, 170.1, 193.5 (C=O); HRMS (EI): mass calculated for $C_{17}H_{12}O_4$ (M^+), 280.0736; m/z found, 280.0737.

*4.1.4.21. 6,7-Dihydro-3′H-spiro[indeno[5,6-b]furan-2,1′-isobenzofuran]-3,3′(5H)-dione (**21b**).* Yield: 35 mg (70%); mp: 202–205 °C; IR (cm^{-1}): 1792, 1722; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.13–2.26 (m, 2H, CH_2), 2.94 (t, J = 7.5 Hz, 2H, CH_2), 3.02 (t, J = 7.5 Hz, 2H, CH_2), 7.06 (s, 1H, ArH), 7.36–7.39 (m, 1H, ArH), 7.57 (s, 1H, ArH), 7.68–7.73 (m, 2H, ArH), 7.96–7.99 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 26.0, 31.7, 34.2, 105.2, 109.5, 117.3, 120.4, 122.6, 126.2, 127.2, 131.9, 135.25, 140.7, 142.9, 159.6, 167.2, 171.4, 192.0 (C=O); HRMS (EI): mass calculated for $C_{18}H_{12}O_4$ (M^+), 292.0736; m/z found, 292.0735.

*4.1.4.22. 4,5,6-Trimethyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**22b**).* Yield: 84 mg (50%); mp: 164–166 °C; IR (cm^{-1}): 1785, 1720; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.19 (s, 3H, CH_3), 2.32 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 6.86 (s, 1H, ArH), 7.35–7.38 (m, 1H, ArH), 7.64–7.72 (m, 2H, ArH), 7.95–8.00 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.2, 14.6, 22.6, 104.6, 111.6, 115.0, 122.6, 126.1, 127.4, 131.6, 131.8, 135.1, 139.0, 143.1, 150.5, 167.3, 169.8, 192.7 (C=O); HRMS (EI): mass calculated for $C_{18}H_{14}O_4$ (M^+), 294.0892; m/z found, 294.0889.

*4.1.4.23. 4,5,7-Trimethyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**23b**).* Yield: 36 mg (72%); mp: 171–173 °C; IR (cm^{-1}): 1790, 1720; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.18 (s, 3H, CH_3), 2.36 (s, 3H, CH_3), 2.53 (s, 3H, CH_3), 6.81 (s, 1H, ArH), 7.38–7.40 (m, 1H, ArH), 7.65–7.73 (m, 2H, ArH), 7.97–8.00 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 10.5, 17.6, 20.6, 104.7, 114.6, 119.0, 122.8, 126.1, 127.0, 127.4, 131.8, 135.1, 137.7, 143.1, 150.0, 167.4, 170.1, 192.9 (C=O); HRMS (EI): mass calculated for $C_{18}H_{14}O_4$ (M^+), 294.0892; m/z found, 294.0889.

*4.1.4.24. 7-Methoxy-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**24b**).* Yield: 25 mg (50%); mp: 173–175 °C; IR (cm^{-1}):

1805, 1727; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.98 (m, 3H, OCH_3), 7.17–7.43 (m, 4H, ArH), 7.66–7.74 (m, 2H, ArH), 7.97–8.00 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 55.6, 104.5, 116.8, 119.9, 121.0, 122.9, 124.5, 126.3, 127.2, 132.1, 135.3, 142.3, 146.5, 161.1, 167.0, 192.7 (C=O); HRMS (EI): mass calculated for $C_{16}H_{10}O_5$ (M^+), 282.0528; *m*/*z* found, 282.0525.

*4.1.4.25. Ethyl 3,3′-dioxo-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-5-carboxylate (**25b**).* Yield: 13 mg (26%); mp: 205–208 °C; IR (cm^{-1}): 1807, 1736, 1703; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.43 (t, J = 7.2 Hz, 3H, CH_3), 4.43 (q, J = 7.2 Hz, 2H, CH_2), 7.27–7.31 (m, 1H, ArH), 7.38–7.40 (m, 1H, ArH), 7.74–7.53 (m, 2H, ArH), 8.02–8.04 (m, 1H, ArH), 8.47–8.50 (m, 2H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4, 61.8, 105.0, 113.8, 118.8, 122.7, 126.5, 126.9, 127.1, 128.1, 132.4, 135.4, 141.1, 141.9, 164.8, 166.6, 173.6, 191.8 (C=O); HRMS (EI): mass calculated for $C_{17}H_{10}O_6$ (M^+), 324.0634; *m*/*z* found, 324.0632.

*4.1.4.26. 6-Fluoro-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**26b**).* Yield: 20 mg (20%); mp: 225–227 °C; IR (cm^{-1}): 1795, 1727; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.95–7.02 (m, 2H, ArH), 7.35–7.45 (m, 1H, ArH), 7.65–7.90 (m, 3H, ArH), 7.95–8.03 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 101.6, 102.0, 105.1, 112.6, 112.9, 115.4, 122.7, 126.4, 127.1, 128.0, 128.1, 132.3, 135.4, 142.1, 166.7, 168.6, 172.1, 172.8, 172.9, 190.6 (C=O); HRMS (EI): mass calculated for $C_{15}H_7FO_4$ (M^+), 270.0328; *m*/*z* found, 270.0328.

*4.1.4.27. 4,7-Dichloro-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**27b**).* Yield: 20 mg (19%); mp: 219–221 °C; IR (cm^{-1}): 1798, 1737; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.17 (d, J = 8.4 Hz, 1H, ArH), 7.42–7.45 (m, 1H, ArH), 7.676 (d, J = 8.7 Hz, 1H, ArH), 7.71–7.76 (m, 2H, ArH), 8.00–8.02 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 104.6, 117.6, 117.7, 123.0, 125.7, 126.5, 127.1, 131.8, 132.5, 135.5, 139.3, 141.5, 166.4, 167.0, 189.1 (C=O); HRMS (EI): mass calculated for $C_{15}H_6Cl_2O_4$ (M^+), 320.9721; *m*/*z* found, 320.9718.

*4.1.4.28. 6-Isopropyl-5′,6′-dimethyl-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**28b**).* Yield: 15 mg (29%); mp: 200–202 °C; IR (cm^{-1}): 1772, 1732; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (d, J = 6.9 Hz, 6H, CH_3), 2.35 (s, 3H, CH3), 2.40 (s, 3H, CH_3), 3.06 (sept, J = 6.9 Hz, 1H, CH), 7.07 (s, 1H, ArH), 7.13 (d, J = 8.1 Hz, 2H, ArH), 7.16 (d, J = 7.8 Hz, 1H, ArH), 7.70 (d, J = 7.8 Hz, 1H, ArH), 7.75 (s, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 20.4, 20.9, 35.4, 104.8, 111.2, 116.8, 123.0, 123.3, 125.1, 125.7, 126.6, 140.7, 141.6, 145.7, 163.2, 167.5, 172.0, 192.4 (C=O); HRMS (EI): mass calculated for $C_{20}H_{18}O_4$ (M^+), 322.1205; *m*/*z* found, 322.1203.

*4.1.4.29. 6-Isopropyl-5′,6′-dimethoxy-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**29b**).* Yield: 14 mg (14%); mp: 135–137 °C; IR (cm^{-1}): 1788, 1737; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (d, J = 6.9 Hz, 6H, CH_3), 3.06 (sept, J = 6.9 Hz, 1H, CH), 3.91 (s, 3H, OCH_3), 3.997 (s, 3H, OCH_3), 6.75 (s, 1H, ArH), 7.09 (s, 1H, ArH), 7.14 (d, J = 7.8 Hz, 1H, ArH), 7.37 (s, 1H, ArH), 7.71 (d, J = 8.1 Hz, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.6, 35.4, 56.65, 56.74, 104.1, 106.6, 111.3, 116.8, 119.8, 123.1, 125.8, 136.7, 152.7, 155.7, 163.3, 167.3, 172.0, 192.3 (C=O); HRMS (EI): mass calculated for $C_{20}H_{18}O_6$ (M^+), 354.1103; *m*/*z* found, 354.1104.

*4.1.5. Preparation of 3-amino-6H-dibenzo[b,f]oxocine-6,11,12-trione (**1d**)*

The same procedure is followed as previously reported and analytical data matched [26].

*4.1.6. Preparation of 6-amino-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**30b**)*

Compound **1d** (40 mg, 0.15 mmol) was taken in ethanol (1 mL) and then irradiated with microwaves at 110 °C for 15 min. This was concentrated and then directly purified over silica gel column chromatography (50% ethyl acetate in hexanes) to get the pure product as solid. Yield: 15 mg (38%); mp: 258–260 °C; ^{1}H NMR (300 MHz, DMSO) δ 6.29 (s, 1H, ArH), 6.50 (d, J = 8.4 Hz, 1H, ArH), 7.24 (br, 2H, NH_2), 7.44 (d, J = 8.4 Hz, 1H, ArH), 7.63 (d, J = 7.5 Hz, 1H, ArH), 7.78–7.87 (m, 2H, ArH), 8.03 (d, J = 7.2 Hz, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 95.0, 105.5, 107.6, 111.6, 122.5, 125.7, 127.0, 127.4, 131.5, 134.9, 143.3, 158.7, 167.3, 173.5, 187.5 (C=O); HRMS (EI): mass calculated for $C_{15}H_9NO_4$ (M^+), 267.0532; *m*/*z* found, 267.0533.

*4.1.7. Preparation of 3-methoxy-6H-dibenzo[b,f]oxocine-6,11,12-trione (**2d**)*

Compound **31a** (410 mg, 1.44 mmol) was taken in ACN:water (1:1) (10 mL) and to this $NaOI_4$ (618 mg, 2.89 mmol) was added at r.t. This was then monitored by TLC. After 10 min of vigorous stirring the precipitated solid was filtered off and then washed with cold water and dried under vacuum to get the pure product as solid. Yield: 210 mg (51%); IR (cm^{-1}): 1754, 1686, 1656; mp: 193–195 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.96 (s, 3H, OCH_3), 6.95–6.98 (m, 2H, ArH), 7.68–7.80 (m, 2H, ArH), 7.83–7.90 (m, 2H, ArH), 8.00–8.03 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 56.4, 108.4, 114.6, 121.9, 127.7, 128.8, 131.0, 131.5, 132.5, 135.8, 139.8, 155.8, 165.1, 167.1, 191.4 (C=O), 198.6 (C=O); MS/EI: *m*/*z* (rel. intensity) 282.2 (M^+, 8.1), 254.2 (11.2), 76.1 (100).

*4.1.8. Preparation of 6-methoxy-3H,3′H-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione (**31b**)*

Compound **2d** (50 mg, 0.18 mmol) was taken in ethanol (1 mL) and then irradiated with microwaves at 110 °C for 15 min. This was concentrated and then directly purified over silica gel column chromatography (20% ethyl acetate in hexanes) to get the pure product as solid. Yield: 22 mg (44%); mp: 185–187 °C; IR (cm^{-1}): 1787, 1719; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.95 (s, 3H, OCH_3), 6.65 (s, 1H, ArH), 6.78 (d, J = 8.1 Hz, 1H, ArH), 7.39 (m, 1H, ArH), 7.66–7.69 (m, 3H, ArH), 7.97–7.99 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 56.4, 97.1, 105.4, 111.7, 113.1, 122.7, 126.2, 127.16, 127.22, 132.0, 135.2, 142.8, 167.2, 169.8, 173.9, 189 (C=O); HRMS (EI): mass calculated for $C_{16}H_{10}O_5$ (M^+), 282.0528; *m*/*z* found, 282.0527.

*4.1.9. 4b-Ethoxy-9b-hydroxy-7,8-dimethyl-4bH-indeno[1,2-b]benzofuran-10(9bH)-one (**3c**)*

4b,9b-Dihydroxy-7,8-dimethyl-4b*H*-indeno[1,2-*b*]benzofuran-10(9b*H*)-one **3a** (100 mg, 0.34 mmol) was taken in ethanol (2 mL) and to this iodine (180 mg, 0.70 mmol) was added. The reaction mass was then irradiated with microwaves for 2 h at 110 °C. Then the reaction mass was concentrated in a r.b flask and then dissolved in ethyl acetate (100 mL). This was washed with 20% $Na_2S_2O_3$ solution (30 mL) and then with water (30 mL) and brine (30 mL). This was dried with anhydrous sodium sulphate and concentrated to get the crude which was purified over silica gel column chromatography (10% ethyl acetate in hexane) to get the pure product as solid. Yield: 100 mg (92%); mp: 165–168 °C; IR (cm^{-1}): 1702; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.31 (t, J = 6.3 Hz, 3H, CH_3), 2.17 (s, 3H, CH_3), 2.18 (s, 3H, CH_3), 3.44 (s, 1H, OH), 3.94–4.14 (m, 2H, OCH_2), 6.65 (s, 1H, ArH), 7.28 (s, 1H, ArH), 7.50–7.56 (m, 1H, ArH), 7.74–7.79 (m, 2H, ArH), 7.90–7.93 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 15.9, 19.4, 20.6, 62.1, 84.0, 111.8, 112.0, 122.3, 124.1, 125.5, 125.8, 130.7, 131.2, 134.7, 136.5, 141.3, 148.2, 155.6, 199.4 (C=O); MS/EI: *m*/*z* (rel. intensity) 310.1 (M^+, 56.9), 264.1 (100).

*4.1.10. Ethyl 2-(2-(2-hydroxy-4,5-dimethylphenyl)-2-oxoacetyl) benzoate (**1e**)*

5,6-Dimethyl-3*H*,3′*H*-spiro[benzofuran-2,1′-isobenzofuran]-3,3′-dione, **3b** (500 mg, 1.60 mmol) was taken in ethanol (20 mL) and to this sodium ethoxide (0.138 g, 2.04 mmol) was added. After

10 min of stirring the reaction mass was concentrated to remove the solvent and then dissolved in ethyl acetate (100 mL) and washed with water (50 mL) and brine (20 mL). This was dried over sodium sulphate and concentrated to get the crude which was purified over silica gel column chromatography (5% ethyl acetate in hexanes) to get the pure compound as solid. Yield: 120 mg (21%); mp: 159–162 °C; IR (cm^{-1}): 1733, 1698, 1628; ^1H NMR (300 MHz, $CDCl_3$) δ 1.23–1.29 (m, 6H + 3H, CH_3), 2.93 (sept, $J = 6.9$ Hz, 1H, CH), 4.22 (q, $J = 7.2$ Hz, 3H, OCH_2), 6.88–6.91 (br, 2H, ArH), 7.61–7.71 (m, 3H, ArH), 8.05 (d, $J = 7.5$ Hz, 1H, ArH), 8.18 (d, $J = 8.4$ Hz, 1H, ArH), 11.29 (br, 1H, OH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.2, 23.3, 23.5, 34.7, 62.2, 114.7, 115.6, 118.7, 129.6, 130.41, 130.42, 131.8, 132.9, 133.4, 138.5, 160.0, 164.3, 166.7, 192.4 (C=O), 193.0 (C=O); MS (EI) m/z (rel. intensity) 340.4 (M^+, 73), 323.3 (84), 295.2 (100), 163 (67).

4.2. Antiviral assay

4.2.1. Materials and methods

Madin–Darby canine kidney (MDCK) cells (ATCC; Manassas, VA) were grown in minimum essential medium (MEM; Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) at 37 °C. Influenza virus HK were obtained from ATCC and propagated in 10-day-old chicken embryos at 37 °C. Influenza virus strain BB was obtained from the Center for Disease Control and Prevention (CDC), Atlanta, GA via Korea CDC at Cheongwon, South Korea, while TWA and PNM were obtained from the Korean CDC. The rest influenza B viruses, TWB and LE, were purchased from ATCC. All viral strains except HK were propagated via the infection of MDCK cells in serum-free medium containing 2 μg/ml of TPCK-trypsin (Sigma, St. Louis, MO) at 35 °C (LE and BB) or 33 °C (TWA, PNM, and TWB). Three days after infection, allantoic fluids harvested from the eggs and cell culture supernatants were clarified by centrifugation at 1000 rpm for 5 min.

4.2.2. Cytopathic effect reduction assay

Confluent monolayers of MDCK cells were seeded in 96-well plates and then either mock-infected or infected with approximately 50–100 plaque forming units (PFU) of influenza virus per well. After incubation for 1 h at 33 °C (mock, TWA, PNM and TWB) or 35 °C (HK, LE and BB), serial dilutions of test and standard chemicals in MEM containing 2 μg/ml TPCK-trypsin were added. On day 3 post infection (p.i.), the cell viability was measured after treatment with fluorescein diacetate (FDA; Sigma), as described previously [35,36]. In brief, the cell culture supernatants were removed and cells were incubated with 100 μL of FDA solution (300 μg/ml in PBS) at 35 °C for 20 min, and then the fluorescence intensity was measured with excitation at 485 nm and emission at 538 nm using a SpectraMax M3 plate reader (Molecular Devices, Sunnyvale, CA). The CC_{50} and EC_{50} were calculated using the SoftMax Pro Software (Molecular Devices). Compounds oseltamivir carboxylate (OSV-C; US Biological, Swampscott, MA), T-705 (favipiravir, synthesized by Dr. I.Y. Lee at KRICT) were used as standards. Antiviral assays for HSV and HIV were performed using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) according to previous reports [37,38].

4.2.3. Plaque reduction assay

MDCK cells seeded in 48-well plates confluently were infected with influenza B viruses (10–90 PFU per well) for 1 h at 33 °C (for PNM and TWB) or 35 °C (for LE and BB). And after washing with phosphate buffered saline (PBS), chemicals serially diluted in overlay medium consisting of MEM and 0.5% CMC containing 2 μg/ml of TPCK-trypsin were added to each well. On day 2 or 3 post-infection, viral plaques were stained with crystal violet. Plaque numbers were counted from crystal violet-stained plates [35] and the percentage number relative to mock-treated, virus-infected cells (100%) was calculated. The values are presented as means ± standard deviations and are representative of at least two independent experiments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.01.015.

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