Bioorganic & Medicinal Chemistry 21 (2013) 2163–2176

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and biological evaluation of helioxanthin analogues

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ARTICLE INFO

Article history:
Received 27 August 2012
Revised 20 November 2012
Accepted 21 November 2012
Available online 5 December 2012

Keywords:
Helioxanthin derivatives
Hepatitis B virus
Anti-HBV agents
Structure–activity relation

ABSTRACT

Helioxanthin and analogues have been demonstrated to suppress gene expression of human hepatitis B virus. In the continuous attempt to optimize antiviral activity, various structural motifs were grafted on the helioxanthin scaffold. Many such analogues were synthesized and evaluated for their anti-hepatitis B virus activity. Structure–activity relationships of these helioxanthin derivatives are also discussed. Among these new compounds, **15** exhibits the highest activity against HBV (EC_{50} = 0.06 μM). This compound can suppress viral surface antigen and DNA expression. Furthermore, viral RNA is also diminished while the core promoter is deactivated upon treatment by **15**. A plausible working mechanism is postulated. Our results establish helioxanthin lignans as potent anti-HBV agents with unique mode of action. Since their antiviral mechanism is distinct from current nucleoside/nucleotide drugs, helioxanthin lignans constitute a potentially new class of anti-HBV agents for combination therapy.

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1. Introduction

The hepatitis B virus (HBV) is a major world health problem. According to the world health report released by WHO in 1997, at least 300 million of are chronically infected with HBV despite the availability of an effective vaccine.[1] The majority of cases occur in developing country in which nearly 10% of the population are infected. Long term HBV infection can result in acute and fulminant liver failure. Many chronic patients latter develop liver cirrhosis and eventually hepatocellular carcinomas. It has been estimated that HBV-related complications cause 600,000–1.2 million deaths every year. No therapeutic strategy that can completely eradicate HBV from the host is hitherto available. Yet, to diminish virus replication is still crucial for the patients, since it would not only prevent further infection but also attenuated inflammation response to viral expression.

Knowledge of the viral life cycle and pathogenesis can be an important guide in the development of effective therapies. The infection of HBV begins with the internalization viral DNA which is a circular 3.2 kb genome composed of a partially double-stranded DNA.[2] Its genome encodes for four main genes by a series of overlapping reading frames. The core gene encodes for core antigen (HBcAg) and the precore gene encodes for the e antigen (HBeAg). The three envelope genes PreS1, PreS2 and S encode for the large, middle, and small envelope proteins, respectively. Finally, the polymerase gene encodes for the multifunctional polymerase, and the X gene encodes for the X protein. After translocated into the hepatocyte nucleus, the genome is converted into covalently closed circle DNA [cccDNA] which serves as the template for the transcription of all viral mRNA. During the subsequent replication, the mRNA not only serves as the template for reverse transcription but also encodes the viral core protein and the HBV polymerase. In the cytoplasm, the mRNA is translated to make viral proteins. After encapsulation and maturation, the viral particles are released from the infected cells.

Interferon (INF) treatment is the first approved therapy for HBV related liver disease.[3] This class of cytokine can bind to receptors on the hepatocyte membrane and trigger a cascade of intracellular immune response to combat HBV proliferation.[4] However, the incidence of adverse effect in INF treatment, ranging from fatigue to decrease in platelets, makes it unsuitable for some patients. In addition, INFs need to be administrated by weekly injection which diminishes its practicality in area with limited medical resources. Recently, several nucleoside and nucleotide analogues were launched to treat chronic HBV infection. This class of compound suppresses HBV reproduction by inhibiting the viral polymerase.[5] Lamivudine[6] [(−)-L-2′,3′-dideoxy-3′-thiacytidine] is the first nucleotide analogue licensed for chronic hepatitis B infection. Since then, adefovir[7,8] [PMEA, 9-(2-phosphonylmethoxyethyl)adenine], entecavir[9] (BMS-200475, carbocyclic 2′-deoxyguanosine), telbivudine[10] (synthetic L-thymidine nucleoside analogue), and levudine[11] [L-FMAU, 1-[(2*S*,3*R*,4*S*,5*S*)-3-fluoro-4-hydroxy-5-(hydroxymethyl) oxolan-2-yl]-5-methyl-pyri-midine-2,4-dione] were also approved for clinical use. Unfortunately, drug resistant mutant often emerge

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http://dx.doi.org/10.1016/j.bmc.2012.11.037

when the target of the drug is polymerase. Indeed, resistant and cross-resistant strand against lamivudine (3TC) and adefovir appeared after only one to 2 years of treatment.[12,13] To overcome the drug-resistance problem, it is essential to develop non-nucleotide drugs that can block viral replication at a different stage during its life cycle. One successful example of this strategy is the heteroarylpyrimidine Bay 41-4109 reported by Deres and Schroder.[14] The authors demonstrated that this drug impedes HBV replication by inhibiting the assembly of nucleocapsids. Such approach not only opens unique opportunities in anti-HBV research, but may also provide new insights to the development of antiviral agent in general (Fig. 1).

Natural products constitute an inexhaustible source of inspiration and challenge for many disciplines in science. For medicinal chemistry, natural products provide instrumental lead structures that can be developed into useful pharmacological targets against pathogenic agents. Arylnaphthalene lignans are a subclass of botanical lignans. Their basic structural feature is a naphthalene core with an aryl group at 1 position. A diverse range of medicinal potentials, including antitumor[15–17] and antiviral activities,[18] were discovered for this class of compounds. Helioxanthin is an arylnaphthalene lactone lignan isolated from *Taiwania cryptomerioides* Hayata[19] and *Heliopsis scabra* Dunal.[20] It was found helioxanthin and its derivatives can inhibit HBV production in various strand of HBV infected cellular models with sub-micromolar concentration.[21,22] We have also reported helioxanthin can effectively inhibit HBV gene expression and replication in 3TC-resistance strand of human hepatoacyte.[23] Many helioxanthin congeners were synthesized and two derivative, lactam **5-4-2** and phthalazin dione **8-1**, are even more potent than the parent compound against HBV.[24] Furthermore, we have verified that such compounds most likely target the viral mRNA production machinery instead of the polymerase. With this antiviral mechanism, relapse associated with drug resistance is much less likely. In our previous study, we have synthesized a wide range of helioxanthin analogues and tested their anti-HBV activities. A preliminary structure–activity relation (SAR) model was also proposed.[23] In our ongoing venture to discover new anti-HBV agents and investigate their mechanism, a new series of nearly 40 helioxanthin derivatives were synthesized and their anti-HBV activities assessed. Here, we report the synthetic strategy and the results of anti-HBV testing. A new compound with potency exceeding that of the parent helioxanthin was revealed. We also put forward a refined SAR model to guide future investigation.

2. Results

2.1. Synthesis of helioxanthin analogues

In our effort to explore various anti-HBV agents, we constantly go back helioxanthin analogues for inspiration. Our strategy is to identify essential structural motifs that enable a helioxanthin analogue to block HBV production. These key structural elements will then be combined on the arylnaphthalene scaffold to produce more potent helioxanthin derivatives. In our earlier studies, we have discovered a few compounds with comparable antiviral activity to the parent compound.[23] The present study is an extension of our previous effort with similar approach.

There are several different synthetic strategies to arylnaphthalene lignans in the literature, including the dimerization of dehydro-cinnamate,[25,26] Pd-catalyzed benzannulation,[27] and Diels–Alder approach.[28] Although each methodology has its merits, we found the Diels–Alder approach developed by Charlton has the widest substrate scope and therefore enables us to generate a library of derivatives in a few routine steps. The synthesis (Scheme 1) starts with piperonal. The aldehyde was first protected as acetal before *ortho*-metallation was carried out with *n*-butyl lithium. The aryl lithium thus generated can react with various aldehydes to furnish the hydroxyacetal **2**. When heated in acetic acid solution, **2** was converted into bezo[*c*]furan in situ to react with maleic anhydride in the key Diels–Alder cycloaddition. The adduct underwent spontaneous aromatization under the reaction condition to give **3** which was then reduced to give the lactone helioxanthin **4** (separated from the regioisomer retro-**4**). When we substituted fumaronitrile for maleic anhydride as the dienophile, dinitrile **5** is produced instead (Scheme 2).

By employing various aldehydes after the lithiation step, we synthesized helioxanthin analogues of which the [1,3]dioxole rings on the aryl moiety were opened. Lactones (**12**, **17**, **22**, **26** and **29**) and their regioisomers (**13**, **18**, and **23**) were prepared as described previously.[23] Phenolic hydroxyl groups were integrated as benzyl protected ether and deprotected after the anhydrides were reduced to lactones. Compounds **14** and **19** thus produced are acetylated to give **15** and **20**, respectively. **16**, **21**, and **25** were

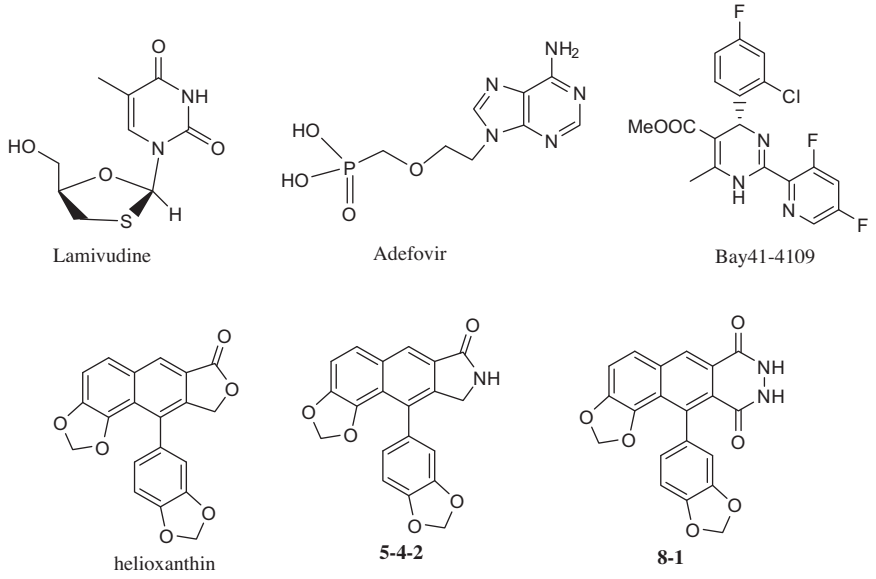
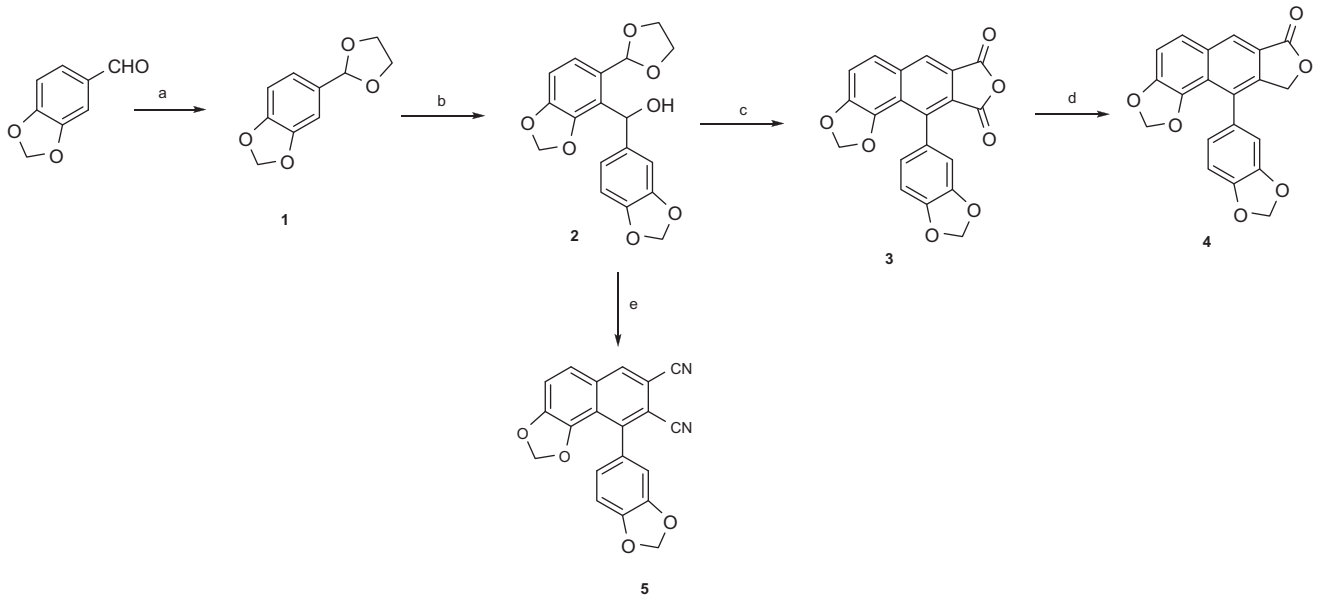


Figure 1. Examples of anti-HBV compounds.



Scheme 1. Reagents and conditions: (a) ethylene glycol, *p*-toluenesulfonic acid, benzene, reflux; (b) *n*-BuLi, piperonal, THF, −78 °C to room temperature; (c) maleic anhydride, AcOH, Ac_2O, CH_2Cl_2, 140 °C, 24 h; (d) $NaBH_4$, THF, room temperature, 2 h; (e) fumaronitrile, AcOH, Ac_2O, CH_2Cl_2, 140 °C, 24 h.

produced alone side their regioisomers **14**, **19**, and **24** (previously unpublished). In hope to improve antiviral activity through fluorination, compound **27** was synthesized by using 2,2-difluorobenzo[*d*][1,3]dioxole-5-carbaldehyde as the electrophilic aldehyde after the lithiation step. In an attempt to identify the target protein of helioxanthin, we synthesized **30** and **31** with functionalized long alkyl chain attached to the phenyl group. We perceive such compounds can be used as probes that may reveal the action site for this class of agents through various in vivo bioconjugative methodologies. We also envision that similar compounds with an anchoring group can be used as stationary phase in affinity based separation techniques.

In our earlier investigation, lactone **4** was reduced with excess $LiAlH_4$ to provided diol **32**. In the same study, we found that **32** and its acetate derivatives **32**(**OAc**) and **32**(**OAc**)**2** are quite active against HBV. By combining the modification at phenyl moiety with this diol motif, we wish to generate another series of active analogues. To fulfill this purpose, lactone **14**, **19**, **24**, **26** were first reduced to **35**, **39**, **45**, and **50**, respectively. The free hydroxyl groups in these compounds were then esterified with acetic anhydride to produce **36**, **37**, **40**, **41**, **46**, **47**, and **51**–**53**. Compound with higher hydrophobicity (**33**, **34**, **38**, **42**–**44**, **49**, and **54**) can be synthesized by using hexanoic anhydride in the esterification step. In these reactions, mixtures of products with different degrees of esterification are inevitably produced. Fortunately, enough samples can be purified by flash chromatography for subsequent testing.

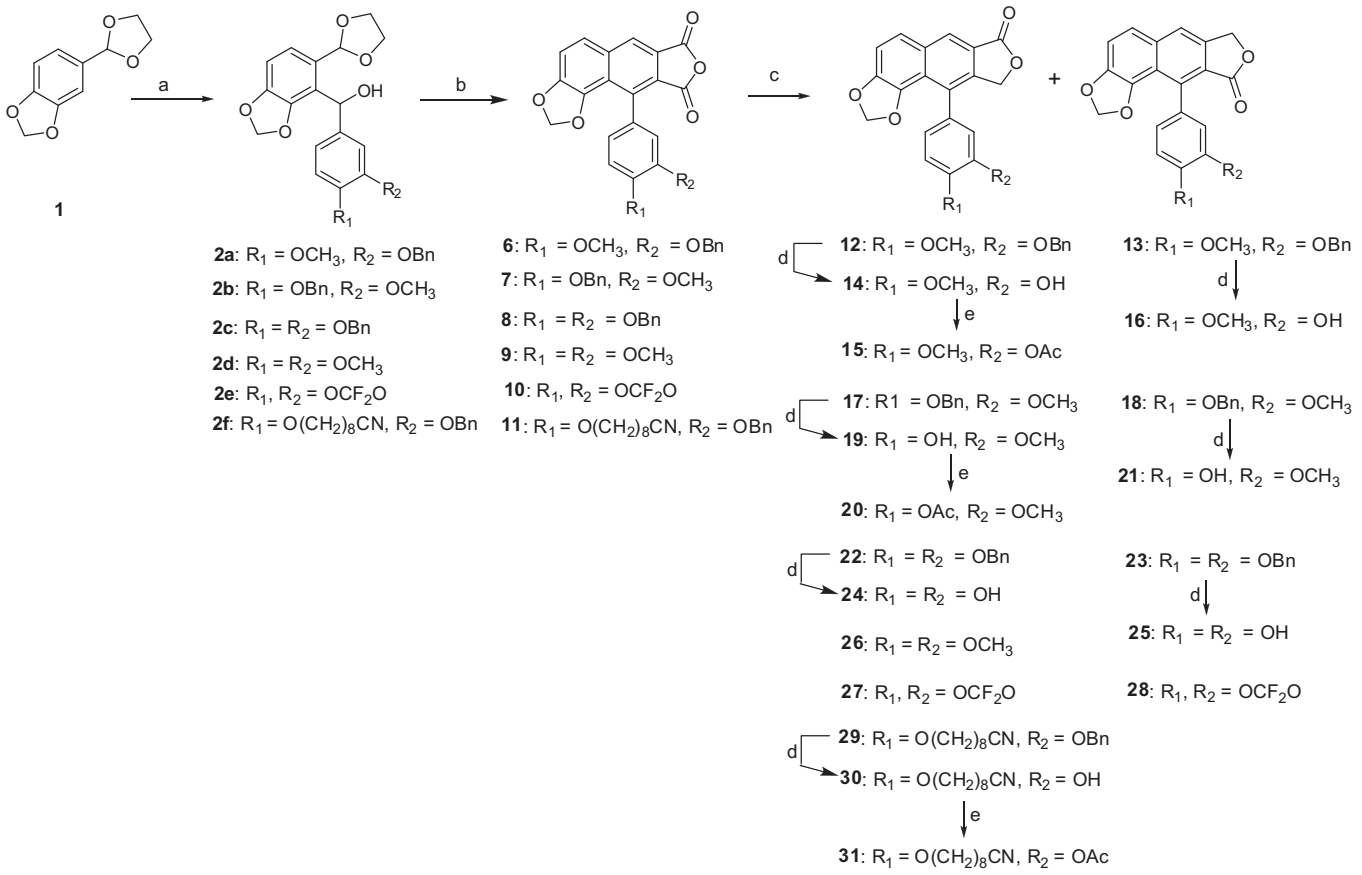
Lactam **5-4-2** reported by Cheng's group is among the most potent anti-HBV helioxanthin analogues in the literature. A few N-alkylated derivatives of **5-4-2** were also reported.[18] this line, we devised a convenient and versatile synthetic scheme to this class of compounds (Scheme 4). Starting from **32**, dicarbaldehyde **55** was produced via Swern oxidation. After **55** was treated with 3-aminopropionitrile and TMSCl, lactam **56** was obtained in good regioselectivity via an intramolecular Cannizarro reaction.[29] Through similar mechanism, **55** also undergoes rapid cyclization reaction with glycine in boiling acetic acid[30] to give the N-acetic acid lactam **57** and its regioisomer in 5:1 ratio. Although this mixture of free acid isomers cannot be easily separated, desired isomers can be isolated in pure form after the mixture underwent alkylation with alkyl iodides under basic condition to give **58–60**. Ester **58** was reduced with DIBAL-H to furnish aldehyde **61**, which was further reduced with $NaBH_4$ to provided alcohol **62** (Scheme 4).

2.2. Evaluation of anti-HBV activities

The synthetic analogues were evaluated against human hepatitis B virus in human hepatoma cell line, HepA2. Since our prior studies have revealed that none of the benzylated or anhydride compounds exhibit any antiviral activity, these compounds were also skipped in this study. Among 38 new compounds tested, 16 of them were found active. These results are listed in Table 1 together with some published data (**4**, **14**, **19**, **32**, **32**(**OAc**), and **32**(**OAc**)**2**) for comparison. Among the active compounds, **15** exhibits the highest EC_{50} (0.06 μM), which is about three times more active than the parent helioxanthin **4**. Good activities were also found for compound **27** (EC_{50} = 0.38 μM), **39** (EC_{50} = 0.67 μM), **40** (EC_{50} = 0.49 μM), **41** (EC_{50} = 0.65 μM) and **58** (EC_{50} = 0.86 μM). The activities of these compounds are only slightly inferior to that of helioxanthin **4**. Compound **16** (EC_{50} = 1.74 μM), **33** (EC_{50} = 1.90 μM), **34** (EC_{50} = 1.90 μM), and **42** (EC_{50} = 1.97 μM) are moderately active, about ten times less active than helioxanthin. Finally, compound **21** (EC_{50} = 3.94 μM), **25** (EC_{50} = 3.40 μM), **36** (EC_{50} = 3.70 μM), **37** (EC_{50} = 3.03 μM), **43** (EC_{50} = 3.33 μM), **45** (EC_{50} = 2.73 μM), and **48** (EC_{50} = 2.77 μM) exhibit detectable activities, yet are considerably less active (>20 times) than helioxanthin. The rest of the compounds are found to be inactive against HBV activity within the concentration limits. Most compounds in the current study have very low cytotoxicity. Except for **14** and **59**, no detrimental effect on cell viability was observed for any other compounds. (For CC_{50} values, see Supplementary data)

2.3. Compound 15 suppressed HBsAg production in HepA2 cells

The suppression of HBsAg production in HepA2 cells is the major essay to screen for anti-HBV activity.[31] As mentioned before, compound **15** possesses the lowest EC_{50} against HBsAg production in this study, even exceeding that of helioxanthin. Further tests



Scheme 2. Reagents and conditions: (a) *n*-BuLi, 3-benzyloxy-4-methoxybenzaldehyde (for **7**), 4-benzyloxy-3-methoxybenzaldehyde (for **8**), 3,4-dibenzyloxybenzaldehyde (for **9**), 2,2-difluorobenzo[*d*][1,3]dioxole-5-carbaldehyde (for **10**), 2,2-difluorobenzo[*d*][1,3]dioxole-5-carbaldehyde (for **11**), and 9-(2-(benzyloxy)-4-formylphenoxy)nonanenitrile (for **12**), THF, −78 °C to room temperature; (b) maleic anhydride, AcOH, Ac_2O, CH_2Cl_2, 140 °C, 24 h; (c) $NaBH_4$, THF, room temperature, 2 h; (d) BBr_3, CH_2Cl_2, −78 °C, 2 h; (e) Ac_2O, pyridine, room temperature, 12 h.

Table 1
The suppression of HBV surface antigen (HBsAg) by helioxanthin derivatives in HepA2 cells

	EC_{50} (μM)		EC_{50} (μM)
4	0.16 ± 0.03	**40**	0.49 ± 0.066
5	>6.0	**41**	0.65 ± 0.03
14	0.14 ± 0.02	**42**	1.97 ± 0.21
15	0.06 ± 0.01	**43**	3.33 ± 0.70
16	1.74 ± 0.09	**44**	>12.5
19	1.89 ± 0.21	**45**	2.73 ± 0.76
20	>6.0	**46**	>12.5
21	3.94 ± 0.81	**47**	>12.5
25	3.4 ± 0.53	**48**	2.77 ± 0.35
26	>6.0	**49**	>12.5
27	0.38 ± 0.02	**50**	>12.5
30	>12.5	**51**	>12.5
31	>12.5	**52**	>12.5
32	1.41 ± 0.31	**53**	>12.5
32(OAc)	0.42 ± 0.21	**54**	>12.5
32$(OAc)_2$	1.22 ± 0.87	**56**	>6.0
33	1.9 ± 0.40	**57**	>6.0
34	1.9 ± 0.66	**58**	0.86 ± 0.0057
35	>12.5	**59**	>12.5
36	3.7 ± 0.70	**60**	>6.0
37	3.03 ± 0.20	**62**	>6.0
38	>12.5		
39	0.67 ± 0.04		

Cultured HepA2 cells were seeded on 96-well culture plates and treated with various concentrations of compounds in DMEM for 2 days. Cultured cells were collected and the number of cells was examined using MTT assay.
EC_{50}: concentration of suppression of HBsAg at 50% of untreated cells.

were therefore conducted on this compound. HepA2 cells were seeded in a 24-well culture plate to which a range of concentrations of compound **15** were added. After an incubation period of 48 h, HBsAg in the media were determined by ELISA assay. The result clearly revealed compound **15** potently suppressed the endogenously expressed HBsAg production in a dose-dependent manner (Fig. 2). In all concentrations tested, the efficacy of **15** is superior to helioxanthin, which was used as a positive control throughout this study. It is worth noting that the suppressive activity of **15** on HBsAg production in HepA2 cells is highly specific and not due to general cytotoxic effect. The treated cells were viable and continued to proliferate throughout the incubation period. Structurally, compound **20** is almost identical with **15**, yet inactive. This compound, when used as the negative control, does not show any suppression activity in HepA2 cells. We can therefore conclude the suppression of HBsAg by the lactone **15** must be highly structure-specific.

2.4. Compound 15 suppressed viral replication of HBV

We next examined the effect of **15** on HBV DNA in 1.3ES2 cells (stably transfected with a 1.3-fold wide-type HBV ayw strain genome in HepG2 cells[32]). In this investigation, 1.3ES2 cells were treated with various concentrations of **15**, helioxanthin **4**, and **20** for 72 h. After the treatment, the amount of viral-associated HBV DNA released into the medium was determined using quantitative real-time PCR (Fig. 3). Like the result on the suppression of HBsAg, compound **15** reduced HBV DNA in a dose-dependent manner in

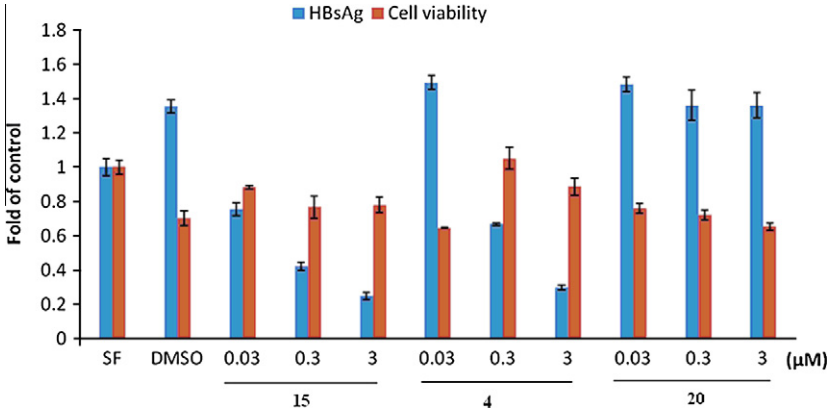


Figure 2. Compound **15** suppressed the HBsAg production in HepA2 cells. HepA2 cells were seeded on 24-well plates at a density of 1×10^5 cells/cm^2 in DMEM with 10% fetal calf serum and allowed to attach overnight. The cells were then washed twice with phosphate-buffered saline (pH 7.0) and treated with various concentrations of compound **15** or **4** and **20** in serum-free DMEM for 48 h. The amounts of HBsAg in the culture medium were determined by enzyme immunoassay. Viable cells in each well were determined by the MTT assay. Data are expressed as mean ± S.D. (n = 3).

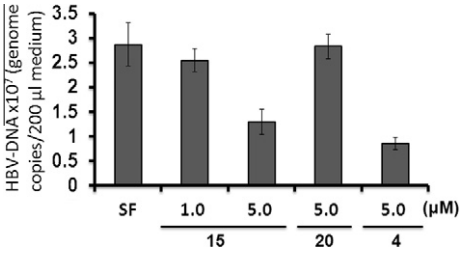


Figure 3. Effect of of compound **15** on the HBV DNA level and its analogs in the media on 1.3ES2 cells. Quantitative real time PCR was used to detect wild type HBV titer in the media of 1.3ES2 cells. Cultured cells were seeded on 100 mm culture dishes and treated with various concentration of compound **15** (0, 1.0, and 5.0 μM) or **20** (5.0 μM) and helioxanthin **4** (5.0 μM) in serum-free (SF) DMEM for 72 h and media collected for real-time PCR analysis using primer pair HBV DNA as template. Data are expressed as mean ± S.D. (n = 3).

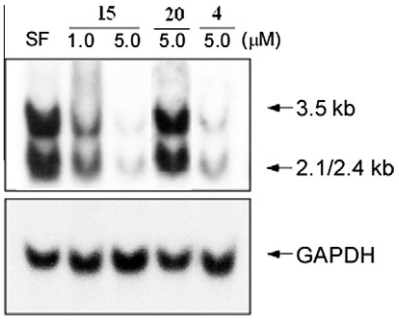


Figure 4. Effects of compound **15** on steady-state mRNA level of HBV RNAs in 1.3ES2 cells. 1.3ES2 cells were seeded on 100 mm culture dishes and treated with various concentration of compound **15** (0, 1.0, and 5.0 μM), or **20** (5.0 μM) and **4** (5.0 μM) in serum-free DMEM medium for 48 h. Total RNA was extracted from serum free (SF) and compounds-treated cells and analyzed by Northern hybridization with HBV DNA probe as described in Section 2.5. The mRNA of the glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene was used as an internal marker.

1.3ES2 cells. In contrast, **20** exhibited no such effect on HBV DNA replication under any concentration.

2.5. Compound 15 suppressed all HBV transcripts in 1.3ES2 cells

To determine whether the suppression of viral replication by **15** is mediated through interfering with the expression of viral transcripts, total cellular RNA was extracted and examined by Northern blot analysis. 1.3ES2 cells were grown in DMEM with 10% fetal calf serum till confluence. The medium was then changed to serum free DMEM with various concentrations of **15**, heliothanxin **4**, and **20**. After 48 h. we found compound **15** significantly decreased the 3.5-kb (HBeAg mRNA and pregenomic RNA) and 2.4/2.1-kb (large HBsAg mRNA and middle/major HBsAg mRNA) HBV RNA levels in a dose-dependent manner in 1.3ES2 cells (Fig. 4). On the other hand, **20** had no such effect on HBV RNA levels.

2.6. Compound 15 significantly suppresses viral core promoter activity in HepA2 cells

Having demonstrated **15** can effectively reduce the level of viral transcripts, we suspect that **15** might operate through regulating the viral promoter. Since HBV core promoter region contains many binding sites for hepatocyte nuclear transcriptional factor, we proceed to construct plasmids containing the core promoters (CP) followed by the luciferase reporter gene to examine the effect of **15** on HBV promoter activity. As shown in Figure 5, we found that

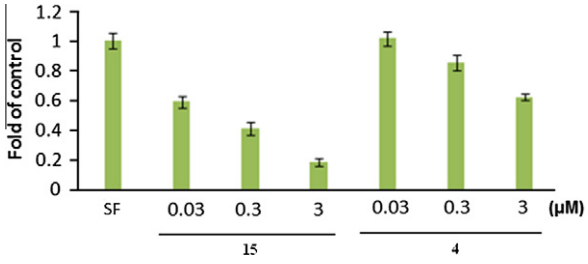


Figure 5. Compound **15** suppressed the HBV core promoter activity in HepA2 cells. HepA2 cells were transfected with CP (C) of HBV promoter region with luciferase reported gene. After transfection, HepA2 cells were treated with serum free (SF), compounds **15** or **4** (0, 0.03, 0.3 and 3.0 μM) in serum-free DMEM for 24 h, respectively. The transfection efficiency was corrected by cotransfecting β-gal expression vector and assayed β-gal activity simultaneously. Data were expressed as mean ± S.D. (n = 3).

compound **15** suppresses CP activities in HepA2 cells in a dose-dependent manner. Although this effect is also observed in helioxanthin **4**, **15** exerts much stronger inhibition with 40% suppression at 0.03 μM. Conversely, with much higher concentration at 3.0 μM, **4** can only attain 30% suppression.

3. Discussion

3.1. Refine SAR model

Our previous study convincingly established that helioxanthin analogues with slight modifications at the lactone or [1,3]dioxole moieties can retain anti-HBV activities. On the contrary, functionalization around the naphthalene rim always results in inactive compounds. Under this guideline, all compounds tested in the present report are modified at the lactone area, the phenyl area, or both. By carefully correlating compound structures with their anti-HBV activities, a refined SAR model was developed to account for the observations. To simplify the discourse, compounds without acetate or hexanoate derivatization will be discussed first. Those esterified derivatives will be considered separately.

Compounds only modified at the [1,3]dioxole ring (**14**, **19**, **24**, **26**, **27**, and **30**) will be discussed first. When the [1,3]dioxole ring is replaced by a hydroxyl group and a methyl ether, the resulting compounds **14** and **19** are both active. Yet, despite their structural similarity, the former possesses potency near that of helioxanthin **4** while the latter is only one tenth as active. Two more [1,3]dioxole-ring-opened analogues, dihydroxy **24** and dimethoxy **26**, are reported in this study. Although these two compounds show very different sizes and polarities at the phenyl moiety, both are weakly active. The closest structural analogue to **4**, the [1,3]dioxole-fluorinated **27**, has about one quarter activity as **4**. Unfortunately, the intended probe compound **30** is inactive within the concentration tested. These results reveal the delicate nature of the interaction site with phenyl [1,3]dioxole. This site must be quite size selective to distinguish the tight binding **14** and **4** from their smaller and larger congener **24** and **26**. Such selectivity definitely attributes to the lack of activity in **30** and renders the phenyl site unsuitable for tagging labels. Furthermore, the discrepancy between **14** and **19** clearly demonstrated this bind site is not only size selective but also sensitive to the surface potential of the substrates. Both the size selectivity and surface potential matching probably attributed to the slightly diminished activity of **27** compared to **4**.

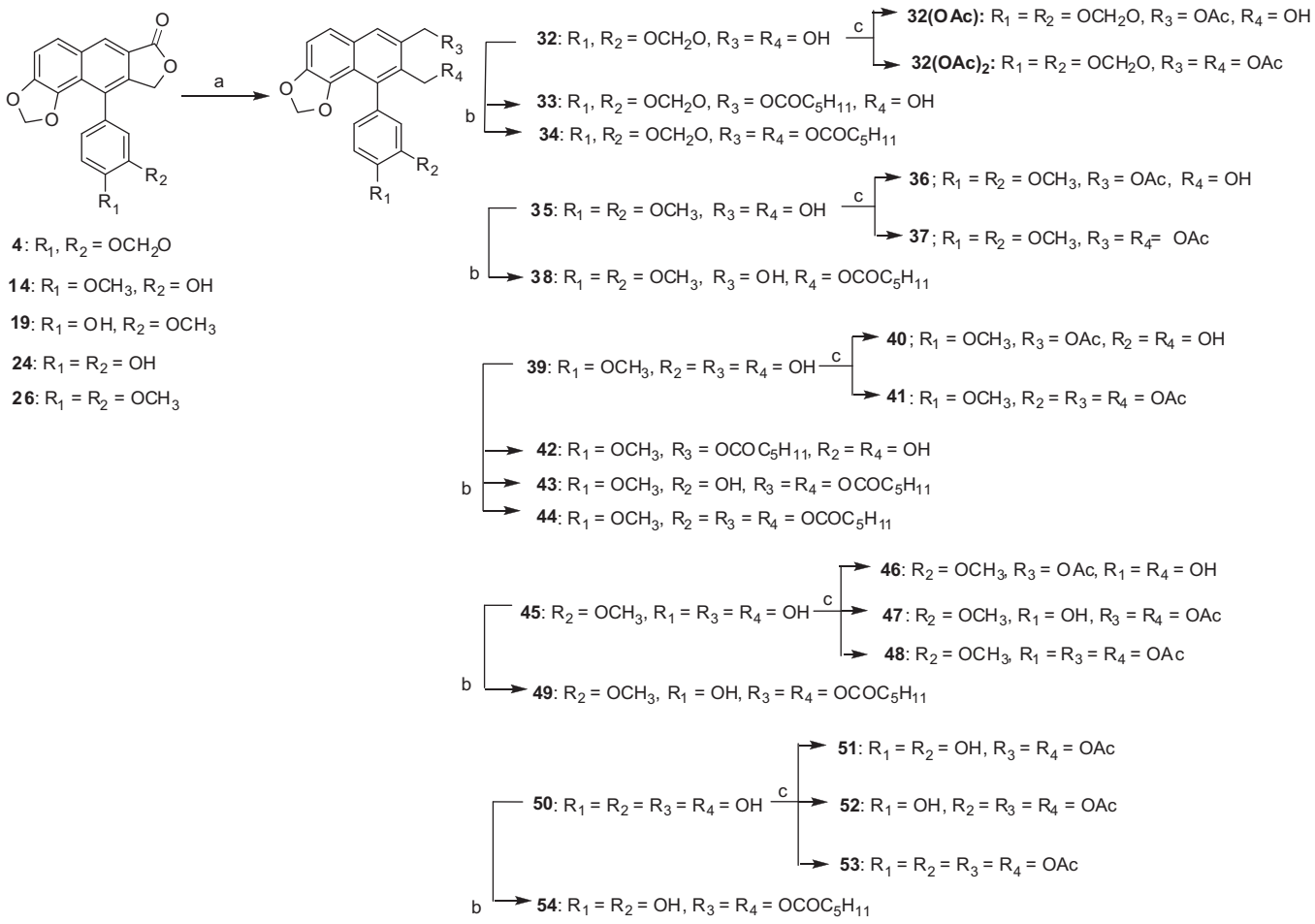
We next examined the derivatives only with their lactone portion modified (retro-**4**, **5**, **32**, **56–62**). The earlier study has established that retro-**4** is moderately active. Lactone **4** can be reduced to acyclic diol **32** yet remain moderately active. More remarkably, lactam **5-4-2** is more active than **4** despite a non-hydrogen bonding lactone oxygen atom is replaced by a strong hydrogen bond donor N–H. The structural diversity of these active derivatives seems to suggest the anti-HBV activities might survive even with more drastic alteration at the lactone site. Unfortunately, the present results confirm the exact opposite. The activities of these lactone modified compounds are extremely sensitive to the size and polarity of the groups incorporated. For example, the acyclic dinitrile **5**, unlike acylic **32**, is completely inactive. The most unexpected failure is from the N-alkylated lactam series (**56–62**). The parent **5-4-2** is so highly potent that we anticipated at least moderate activities from its derivatives. However, the derivatives are inactive whether the *N*-alkyl group carries a neutral nitrile (**56**), an ionizable carboxylate (**57**), or a polar hydroxyl (**62**). The only exception is the *N*-methyl acetate **58** which is somewhat less active than **4**. The activities disappear when the length of the alkyl chain increases beyond five carbons. Since this delicate size effect was also observed by Cheng's group, it can therefore be postulated the binding site of the lactone moiety is a rigid pocket with a hydrophobic bottom. Hydrophilic end groups in compound **56**, **57**, and **62** are therefore excluded from the site. Derivative with nonpolar substitution up to four atoms as in **58** can still fit in and therefore exhibits activity. On the other hand, the pocket cannot accommodate longer alkyl chains and therefore renders **59** and **60** inactive.

The third class of compounds are modify both at the [1,3]dioxole and the lactone ring. Based on the structures at the original lactone region, these hybrid compounds can be categorized into two sub-types, the retro isomer family (**16**, **21**, and **26**) and the diol family (**35**, **39**, **45**, and **50**). Compound **16** and **39** are the most active compounds in the retro family and diol family, respectively. The phenyl groups in both compounds carry the same substitution pattern, a *para* methoxy and a *meta* hydroxyl. Interestingly, the same substitution pattern is also found on **14**, the most active dioxole modified helioxanthin. Compounds with other substitution patterns are much less active. Based on such resemblance in SAR trends, we postulate the binding modes of these derivatives are quite similar to the parent helioxanthin.

We next examine the SAR of acetate and hexanoate derivatives, including **15**, **20** and most compounds in Scheme 3. At first sight, these results seem to contradict our previous SAR model. We already stated the binding sites of both [1,3]dioxole and lactone must be quite sensitive to the size of the substrates. Therefore, esterification at either the diol or the phenolic sites should render most of these compounds inactive because these binding sites cannot accommodate bulky ester groups. However, in most instances, the acetylated derivatives exhibit similar or improved activities when compared with the parent alcohols. Compound **15** is even more active then helioxanthin itself. The striking effect of acetylation is also manifested when **35** is compared to acetylated derivative **36** and **37** as the inactive parent diol was 'activated' by acetylation. More surprisingly, many hexanoate esters still exhibit weak yet detectable activities. These observations strongly suggest substitution size is relatively unimportant in these ester derivatives. Bearing in mind the unambiguous size effect witnessed in the unesterified compounds, we hereby propose a model to harmonize the discrepancy between in these two series of compounds. We believe, instead of working directly on the target, the esters act as prodrugs that undergo hydrolysis in vivo to regenerate the active diol or phenol compounds. In such a model, the ester compounds are masked forms of their parent alcohols. The lower activity found for the hexanoates might reflect their resistance to hydrolysis. However, this model cannot answer why many esters possess higher activities than the parent alcohols. To account for this peculiarity, we hypothesize that esterification might improve the bio-viability of this class of compounds. Since the free diols are more hydrophilic and less soluble than the ester derivatives, it is likely that esterification makes the compounds more permeable to membranes and less prone to aggregate; both important factors in optimizing drug candidates. It is also possible that the acetylation protect the phenolic hydroxyl against oxidation and therefore increase its life time in vivo.

3.2. Anti-HBV mechanism of compound 15

In order to establish the antiviral mechanism of these helioxanthin analogues, we conduct further tests with the most active compound, **15**, in this study. In these investigations, it is demonstrated that **15** does not only decrease the HBsAg and HBV DNA level, but the viral RNA expression is also clearly suppressed. Similar phenomenon is also observed when the infected cells are treated with helioxanthin or **8-1**. Such observation strongly indicates that the viral life cycle is intercepted by this class of agents before the reverse transcription from RNA to DNA takes place. According to this conception, helioxanthin derivatives must operate through a totally different mechanism from the nucleotide and nucleotide mimic drugs, which inhibit the viral polymerase and therefore only reduce the viral DNA. On the other hand, helioxanthin analogues are also distinct from those potent heteroaryldihydropyrimidine

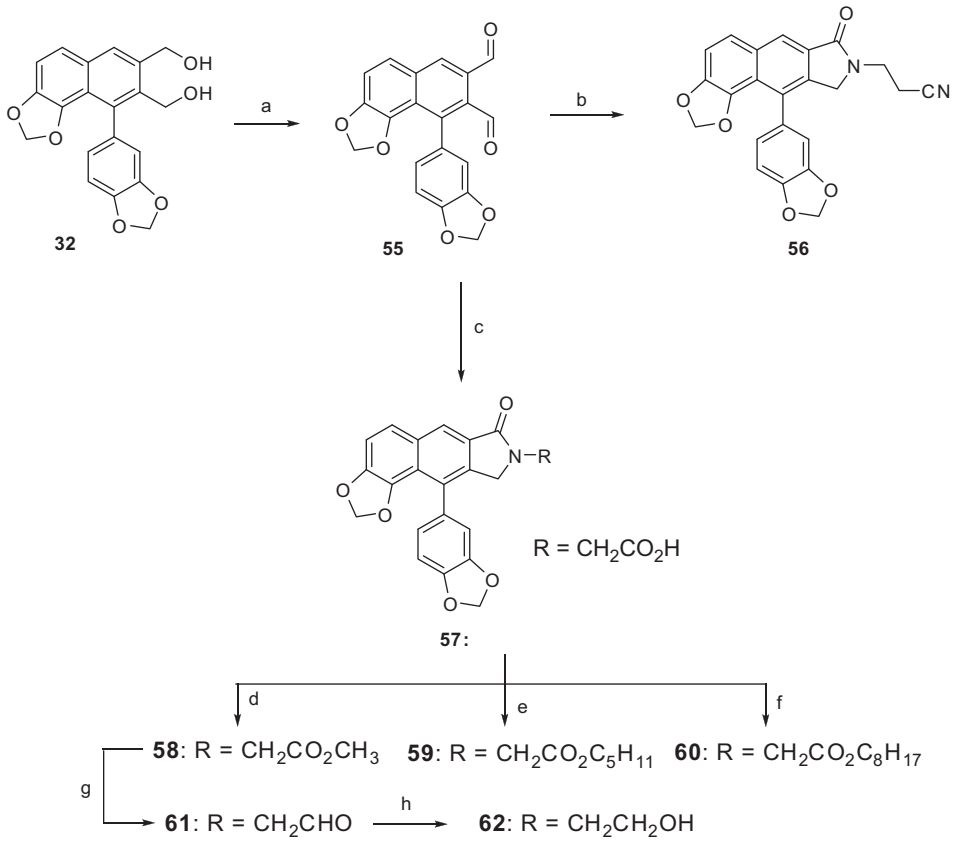


Scheme 3. Reagents and conditions: (a) LAH, THF, 0 °C, 1 h; (b) hexanoic anhydride, pyridine, room temperature, 12 h; (c) Ac_2O, pyridine, room temperature, 12 h

compounds which interfere with the viral capsid assembly and therefore should also have little effect on RNA level.

Such comprehensive suppression of viral transcripts and proteins and at every levels of life cycle is the signature of helioxanthin and its analogues. Since the 3.5-kb pregenomic RNA does not only encode the core and polymerase proteins but also serves as a template for reverse transcription, we can reasonably infer that compound **15** suppresses viral proliferation by inhibiting the production of this key RNA transcript at a very upstream event. Since the viral core promoter plays a critical role in viral gene expression[33–35] we next examined the viral core promoter (CP) activity using luciferase as the reporter assay (Fig. 5). Indeed, we found **15** effectively inhibit CP activity with potency comparable to the most active congener, **5-4-2**. Based on these results, we conclude the anti-HBV mechanism of **15** is most likely though interference with the transcriptional machinery, much like the parent helioxanthin **4**, **t**he phthalazine dione derivative **8-1** and lactam **5-4-2**. In earlier studies, hepatocyte nuclear factor HNF-4-1 and HNF-4-2 are implicated as the target of **8-1**. We too believe **15** may down regulate similar transcriptional factor. This series of studies firmly establishes the family of helioxanthin derivatives as effective anti-HBV drugs or lead compounds. It is also noteworthy that **4**, **14**, **8-1**, and **5-4-2** have all been found active against lamivudine-resistant strand that contains L515M and M539V double mutations in the polymerase. As our results indicate, **15** is probably a precursor of **14** and it targets viral CP rather than polymerase. We are confident that many drug resistant strands that emerge after the administration of nucleotide analogue will still respond to treatment by **15**. This highlights the potential of these natural product analogues in long-term anti-HBV therapy or combination therapy.

In conclusion, we have synthesized a new series of helioxanthin analogues. Skeletal variations include modifications at [1,3]dioxole ring, lactone ring, and both. Further derivatizations are carried out with esterification of various alcohols. Compound **15** was revealed to be even more active than the parent helioxanthin. By correlating the molecular structures with anti-HBV activities, a refined model of SAR was proposed. By assuming that the esterified compounds can serve as the precursors of the free alcohol, the apparent contradictory SAR results from these two classes of derivatives was resolved. We believe these SAR insights can provide vital clues in designing more active lead compounds with good bio-viability. For example, a hypothetical compound that contains the lactam motif in **5-4-2** and the opened [1,3]dioxole in **15** can exceed both predecessors in anti-HBV potency. As expected, **15** does not only reduce HBV DNA but also decreases RNA like its parent compounds. Since the viral core promoter is deactivated by **15**, we infer this class of structures can interrupt the transcriptional machinery in hepatocyte and thus nullify HBV reproduction. This mechanism is distinct from the existing nucleotide analogue therapeutics like lamivudine and adefovir, which target the viral polymerase. Such uniqueness mode of action makes **15** a worthy candidate for further investigation in combination therapy. For example, a cocktail of **15**, lamivudine, and Bay 41-4109 might eradicate HBV infection



Scheme 4. Reagents and conditions: (a) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −78 °C, 12 h; (b) 3-aminopropionitrile, CH_3CN/DMF, TMSCl, room temperature, 12 h; (c) glycine, AcOH, reflux, 5–10 min; (d) CH_3I, K_2CO_3, DMF, room temperature, 2 h; (e) $C_5H_{11}I$, K_2CO_3, DMF, room temperature, 2 h; (f) $C_8H_{17}I$, K_2CO_3, DMF, room temperature, 2 h; room temperature, 2 h; (g) DIBAL-H, toluene, −40 °C, 2 h; (h) $NaBH_4$, THF, room temperature, 1 h.

by intercepting viral life cycle at many stages. Alone this direction, we are currently testing these compounds as novel anti-HBV agents in HBV transgenic mice.

4. Synthetic procedures

4.1. General protocols for synthesis

^1H and ^{13}C NMR spectra were obtained on Bruker AC 300, AMX 400, AV 400, or DRX 500 MHz spectrometer. Chemical shifts of ^1H were calibrated with the residual proton peak(s) in deuterated solvent as the internal references ($CHCl_3$ at δ 7.24 ppm, CH_3COCH_3 at δ 2.49 ppm, and CH_3OH at δ 4.78 ppm). Chemical shifts of ^{13}C spectrum were calibrated accordingly (the central peak of $CHCl_3$ at δ 77.00 ppm, carbonyl of CH_3COCH_3 at 206.00 ppm, and CH_3OH at δ 49.15 ppm). Mass spectra were recorded on a JMS-700 double focusing mass spectrometer (JEOL, Tokyo, Japan). Commercially available solvents and reagents were used without further purification except for THF and CH_2Cl_2 which are dried over Na/benzophenone and CaH_2, respectively and distilled before use. All reactions were carried out under a dry N_2 atmosphere and thoroughly mixed with magnetic stirs. Acetal **1**, hydroxyacetal **2**, anhydride **3**, helioxanthin **4**, **12–14**, **17–19**, **22–24**, **32**, **32**(**OAc**) and **32**(**OAc**)$_2$ were prepared as published before.

4.1.1. 9-(Benzo[*d*][1,3]dioxol-5-yl)naphtho[1,2-*d*][1,3]dioxole-7,8-dicarbonitrile (5)

Mixture of hydroxyacetal **2** (3 g, 8.71 mmol), fumaronitrile (680 mg, 8.71 mmol), acetic anhydride (12.7 ml), and acetic acid (4.7 ml) was heated at 140 °C for 24 h. After this period, the reaction mixture was cooled to room temperature, diluted with CH_2Cl_2 (50 ml), and washed with 5% $NaHCO_3$ solution (3 × 50 ml). The organic fraction was dried over $MgSO_4$ and concentrated to furnish yellow solid compound **6** (1.85 g, 62%). ^1H NMR (400 MHz, $CDCl_3$) δ 8.21 (s, 1H), 7.58 (d, 1H, J = 8.5 Hz), 7.40 (d, 1H, J = 8.5 Hz), 6.89 (d, 1H, J = 7.8 Hz), 6.83–6.80 (m, 2H), 6.05 (AB, 2H, J = 18.6 Hz), 5.97 (AB, 2H, J = 12.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.73, 148.50, 147.37, 144.20, 143.04, 136.00, 129.64, 129.07, 124.27, 123.17, 119.07, 116.30, 115.39, 114.52, 111.17, 109.79, 108.06, 107.52, 102.48, 101.48; IR (CH_2Cl_2, cast) 2227, 1622, 1488, 1444, 1318, 1298, 1235, 1094, 1039 cm^{-1}; FAB-HRMS Calcd for $C_{20}H_{10}O_4N_2$ m/z (M^+) 342.0641, found 342.0644.

4.1.2. 10-(3-Benzyloxy-4-methoxy-phenyl)-furo[3′,4′:6,7]naphtho[1,2-*d*][1,3]dioxol-7,9-dione (6), 10-(4-Benzyloxy-3-methoxy-phenyl)-furo[3′,4′:6,7]naphtho[1,2-*d*][1,3]dioxol-7,9-dione (7), 10-(3,4-Bis-benzyloxy-phenyl)-furo[3′,4′:6,7]naphtho[1,2-*d*][1,3]dioxole-7,9-dione (8), 10-(3,4-Dimethoxyphenyl)-furo[3′,4′:6,7]naphtho[1,2-*d*][1,3]dioxole-7,9-dione (9), 10-(2,2-difluorobenzo[*d*][1,3]dioxol-5-yl)furo[3′,4′:6,7]naphtho[1,2-d][1,3]dioxole-7,9-dione (10) and 9-(2-(Benzyloxy)-4-(7,9-dioxo-7,9-dihydrofuro[3′,4′:6,7]naphtho[1,2-*d*][1,3]dioxol-10-yl)phenoxy)nonanenitrile (11)

Anhydrides **6–11** were synthesized as described for **3**. The acetal **1** was dissolved in dry THF under nitrogen and cooled to −78 °C. n-BuLi (2.5 M in hexanes) was added drop wise over 5 min. The mixture was stirred for another 15 min before the addition of appropriate aldehydes (3-benzyloxy-4-methoxybenzaldehyde for

6, 4-benzyloxy-3-methoxybenzaldehyde for **7**, 3,4-dibenzyloxy benzaldehyde for **8**, 3,4-dimethoxybenzaldehyde for **9**, 2,2-difluorobenzo[*d*][1,3]dioxole-5-carbaldehyde for **10**, and 9-(2-(benzyloxy)-4-formylphenoxy) nonanenitrile for **11**). After stirred for 30 min, the solution was gradually warmed to room temperature and was stirred for another 2.5 h. The reaction was quenched with water and the resulting mixture was extracted with ether. The ether extract was dried over $MgSO_4$ and concentrated to give corresponding hydroxyacetals. The crude hydroxyacetals were mixed with maleic anhydride, acetic anhydride, and acetic acid in CH_2Cl_2 and the mixture was heated to 140 °C for 24 h. After workup, the anhydrides were filtered as yellow solids and used in the subsequent reaction without further purification or characterization.

4.1.3. 2-Methoxy-5-(7-oxo-7,9-dihydrofuro[3′,4′:6,7] naphtho[1,2-*d*][1,3]dioxol-10-yl) phenyl acetate (15)

To a solution of **14** (20 mg, 0.056 mmol) in pyridine (3 ml) was added acetic anhydride (6 mg, 0.056 mmol) and the resulting mixture was stirred at room temperature overnight. After the completion of the reaction, 10% HCl (5 ml) was added and the mixture was extracted with ether (10 ml). The ether extract was washed with saturated $NaHCO_3$ (5 ml), dried over $MgSO_4$, and concentrated. After column chromatography (hexane/CH_2Cl_2 = 3:1), **15** (16 mg, 73%) was isolated. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.39 (s, 1H), 7.67 (d, 1H, J = 8.7 Hz), 7.28 (d, 1H, J = 8.7 Hz), 7.18 (dd, 1H, J = 2.1, 8.3 Hz), 7.03–7.00 (m, 2H), 5.92 (s, 2H), 5.21 (AB, 2H, J = 15.2 Hz), 3.89 (s, 3H) 2.30 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.10, 168.85, 150.91, 146.96, 141.75, 139.72, 139.19, 130.69, 129.09, 128.16, 127.50, 127.01, 125.39. 124.29, 121.31, 121.12, 111.80, 111.65, 101.69, 69.53, 55.89, 20.70; EI-HRMS Calcd for $C_{22}H_{16}O_7$ m/z (M^+) 392.0896, found 392.0892.

4.1.4. 10-(3-Hydroxy-4-methoxyphenyl)furo[3′,4′:6,7] naphtho[1,2-*d*][1,3]dioxol-9(7*H*)-one (16)

To a solution of **13** (40 mg, 0.09 mmol) in anhydrous CH_2Cl_2 (3.0 ml) at −78 °C was slowly added a solution of BBr_3 (1 M in CH_2Cl_2, 0.09 ml) and the mixture was stirred for 2 h at the same temperature. After this time, MeOH (4 ml) was added to the reaction mixture and the resulting solution was warmed back to room temperature and concentrated in vacuo. The residue was dissolved in ether (30 ml) and washed with saturated $NaHCO_3$ (20 ml), water (20 ml), and brine (20 ml). The organic extract was dried over $MgSO_4$ and concentrated. After column chromatography (hexane/CH_2Cl_2 = 1:2), **16** (24 mg, 75%) was isolated. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (s, 1H), 7.50 (d, 1H, J = 8.6 Hz), 7.32 (d, 1H, J = 8.6 Hz), 6.90–6.88 (m, 2H), 6.83 (dd, 1H, J = 2.0, 8.2 Hz), 5.86 (AB, 2H, J = 6.0 Hz), 5.60 (s, 1H), 5.34 (d, 2H, J = 1.0 Hz), 3.94 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.22, 146.37, 144.99, 144.41, 143.59, 138.10, 137.54, 132.73, 128.57, 122.20, 121.41, 120.85, 120.69, 120.10, 116.09, 113.56, 109.23, 101.45, 67.77, 55.74; FAB-HRMS Calcd for $C_{20}H_{15}O_6$ m/z ([M+H]$^+$) 351.0869, found 351.0875.

4.1.5. 2-Methoxy-4-(7-oxo-7,9-dihydrofuro[3′,4′:6,7] naphtho[1,2-*d*][1,3]dioxol-10-yl)phenyl acetate (20)

To a solution of **19** (20 mg, 0.056 mmol) in pyridine (3 ml) was added acetic anhydride (6 mg, 0.056 mmol) and the reaction mixture was allowed to proceed as described for **15**. After column chromatography (hexane/CH_2Cl_2 = 1:2), pure **20** (28 mg, 72%) was isolated. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.43 (s, 1H), 7.70 (d, 1H, J = 8.7 Hz), 7.30 (d, 1H, J = 8.7 Hz), 7.10 (d, 1H, J = 8.4 Hz), 6.92–6.90 (m, 2H), 5.90 (AB, 2H, J = 8.5 Hz), 5.21 (AB, 2H, J = 15.2 Hz), 3.80 (s, 3H) 2.34 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.00, 168.87, 150.67, 147.01, 141.71, 139.64, 139.61, 135.47, 130.70, 128.72, 127.71, 125.50, 122.43, 121.20, 121.13, 113.57, 111.89, 101.62, 69.53, 56.05, 20.73; IR (CH_2Cl_2, cast) 1761, 1630, 1507, 1460, 1273, 1249, 1198, 1127, 1073 cm^{-1}; FAB-HRMS Calcd for $C_{22}H_{16}O_7$ m/z (M^+) 392.0896, found 392.0898.

4.1.6. 10-(4-Hydroxy-3-methoxyphenyl)furo[3′,4′:6,7] naphtho[1,2-*d*][1,3]dioxol-9(7*H*)-one (21)

Compound **21** was synthesized as described for **16** in 72% isolated yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (s, 1H), 7.51 (d, 1H, J = 8.6 Hz), 7.33 (d, 1H, J = 8.6 Hz), 6.94 (d, 1H, J = 8.0 Hz), 6.86 (d, 1H, J = 1.6 Hz), 6.83 (dd, 1H, J = 1.6, 8.0 Hz), 5.85 (s, 2H), 5.76 (s, 1H), 5.34 (s, 2H), 3.84 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.27, 145.46, 145.40, 144.97, 143.53, 138.31, 137.58, 132.77, 127.14, 122.85, 122.25, 120.77, 120.65, 120.14, 113.55, 113.30, 112.68, 101.38, 67.75, 55.97; FAB-HRMS Calcd for $C_{20}H_{15}O_6$ m/z ([M+H]$^+$) 351.0869, found 351.0870.

4.1.7. 10-(3,4-Dihydroxyphenyl)furo[3′,4′:6,7]naphtho[1,2-*d*][1,3]dioxol-9(7*H*)-one (25)

Compound **25** was synthesized as described for **16** in 69% isolated yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (s, 1H), 7.48 (d, 1H, J = 8.6 Hz), 7.31 (d, 1H, J = 8.6 Hz), 6.81–6.79 (m, 2H), 6.68 (dd, 1H, J = 1.9, 8.1 Hz), 5.84 (AB, 2H, J = 5.8 Hz), 5.35 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.55, 145.11, 144.09, 143.72, 142.93, 138.72, 137.34, 132.76, 127.67, 122.13, 121.98, 120.64, 120.41, 120.15, 116.74, 114.40, 113.78, 101.55, 68.30; ESI-HRMS Calcd for $C_{19}H_{12}O_6Na$ m/z ([M+Na]$^+$) 359.0532, found 359.0542.

4.1.8. 10-(2,2-Difluorobenzo[*d*][1,3]dioxol-5-yl)furo[3′,4′:6,7] naphtho[1,2-*d*][1,3]dioxol-7(9*H*)-one (27) and 10-(2,2-Difluorobenzo[*d*][1,3]dioxol-5-yl)furo[3′,4′:6,7]naphtho[1,2-*d*][1,3]dioxol-9(7*H*)-one (28)

Anhydride **10** (500 mg, 1.1 mmol) was reduced with $NaBH_4$ (130 mg, 3.43 mmol) in dry THF (20 ml) as described before. After column chromatography (hexane/CH_2Cl_2 = 2:3), pure **27** (340 mg, 70%) and **28** (100 mg, 20%) were isolated. Compound **27**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (s, 1H), 7.70 (d, 1H, J = 8.6 Hz), 7.31 (d, 1H, J = 8.6 Hz), 7.12 (d, 1H, J = 8.6 Hz), 7.05–7.03 (m, 2H), 5.92 (AB, 2H, J = 15.2 Hz), 5.16 (AB, 2H, J = 15.2 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.73, 147.15, 143.53, 141.54, 139.79, 132.73, 131.72, 130.64, 128.07, 127.71, 125.63, 124.30, 121.18, 121.10, 112.04, 110.57, 109.05, 101.70, 69.18; IR (CH_2Cl_2, cast) 1762, 1632, 1498, 1459, 1443, 1239, 1151, 1072, 1031 cm^{-1}; FAB-HRMS Calcd for $C_{20}H_{10}O_6F_2$ m/z (M^+) 384.0445, found 384.0442. Compound **28**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (s, 1H), 7.54 (d, 1H, J = 8.6 Hz), 7.35 (d, 1H, J = 8.6 Hz), 7.09–7.01 (m, 3H), 5.87 (AB, 2H, J = 11.2 Hz), 5.37 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.13, 145.22, 143.56, 143.25, 142.96, 137.43, 136.20, 132.68, 131.75, 131.29, 124.95, 122.49, 121.48, 121.16, 119.78, 113.89, 110.99, 108.22, 101.61, 68.00; IR (CH_2Cl_2, cast) 1763, 1618, 1499, 1457, 1376, 1314, 1239, 1155, 1023, cm^{-1}; FAB-HRMS Calcd for $C_{20}H_{10}O_6F_2$ m/z (M^+) 384.0445, found 384.0442.

4.1.9. 9-(2-(Benzyloxy)-4-(7-oxo-7,9-dihydrofuro[3′,4′:6,7] naphtho[1,2-*d*][1,3]dioxol-10-yl)phenoxy)nonanenitrile (29)

Compound **11** (500 mg, 1.1 mmol) was reduced with $NaBH_4$ (130 mg, 3.43 mmol) in dry THF (20 ml) as described before. After workup, mixture of lactone isomers was used in the subsequent reaction without further purification or characterization.

4.1.10. 9-(2-Hydroxy-4-(7-oxo-7,9-dihydrofuro[3′,4′:6,7] naphtho[1,2-*d*][1,3]dioxol-10-yl)phenoxy)nonanenitrile (30)

To a solution of **29** (50 mg, 0.096 mmol) in anhydrous CH_2Cl_2 (5 ml) at −78 °C was added slowly a solution of BBr_3 (1 M in CH_2Cl_2, 0.22 ml) and the debenzylation was performed and worked up as described before. After column chromatography (CH_2Cl_2/methanol = 19:1), **30** (24 mg, 77%) was isolated. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.39 (s, 1H), 7.67 (d, 1H, J = 8.6 Hz), 7.28 (d,

1H, J = 8.6 Hz), 6.90 (d, 1H, J = 2.0 Hz), 6.88 (d, 1H, J = 8.2 Hz), 6.78 (dd, 1H, J = 2.0, 8.2 Hz), 5.92 (AB, 2H, J = 5.5 Hz), 5.71 (s, 1H), 5.18 (AB, 2H, J = 15.1 Hz), 4.12–4.07 (m, 2H), 2.34 (t, 2H, J = 7.0 Hz), 1.87–1.84 (m, 2H), 1.68–1.65 (m 2H), 1.50–1.46 (m, 4H), 1.41–1.37 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.23, 146.86, 145.77, 145.17, 141.80, 139.71, 130.78, 129.85, 129.21, 127.25, 125.40, 121.47, 121.08, 120.70, 119.75, 115.45, 111.73, 110.81, 101.53, 69.62, 68.83, 29.25, 29.08, 28.68, 28.54, 25.95, 25.28, 17.14; FAB-HRMS Calcd for $C_{28}H_{27}O_6N$ m/z (M^+) 473.1838, found 473.1835.

4.1.11. 2-((8-Cyanooctyl)oxy)-5-(7-oxo-7,9-dihydrofuro [3′,4′:6,7]naphtho[1,2-d][1,3]dioxol-10-yl)phenyl acetate (31)

To a solution of **30** (20 mg, 0.056 mmol) in pyridine (3 ml) was added acetic anhydride (6 mg, 0.056 mmol) and the reaction mixture was allowed to proceed as described for **15**. After column chromatography (hexane/CH_2Cl_2 = 1:2), **31** (28 mg, 72%) was isolated. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (s, 1H), 7.68 (d, 1H, J = 8.6 Hz), 7.29 (d, 1H, J = 8.6 Hz), 7.15 (dd, 1H, J = 2.1, 8.4 Hz), 7.02 (d, 1H, J = 2.1 Hz), 6.99 (d, 1H, J = 8.4 Hz), 5.92 (s, 2H), 5.21 (AB, 2H, J = 15.2 Hz), 4.04–4.02 (m, 2H), 2.33 (t, 2H, J = 7.1 Hz), 2.29 (s, 3H), 1.80–1.79 (m, 2H), 1.67–1.64 (m 2H), 1.49–1.44 (m, 4H), 1.37–1.36 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.12, 168.77 150.41, 146.97, 141.77, 139.74, 139.45, 130.73, 128.98, 128.26, 127.49, 126.99, 125.41, 124.13, 121.35, 121.14, 119.76, 112.53, 111.81, 101.68, 69.55, 68.57, 29.16, 29.04, 28.75, 28.58, 25.85, 25.33, 20.62, 17.14; IR (CH_2Cl_2, cast) 2858, 2245, 1767, 1631, 1511, 1461, 1370, 1271, 1072, 1015 cm^{-1}; FAB-HRMS Calcd for $C_{30}H_{29}O_7N$ m/z (M^+) 515.1944, found 515.1948.

General procedure for the preparation of ***32,***[1] ***35***, ***39***, ***45*** *and* ***50***: As described for **32**, a solution of lithium aluminum hydride in THF was cooled to 0 °C in an ice bath and a THF solution of lactone (**4**, **15**, **20**, **23** and **26**) was added. The mixture was stirred for 1 h at the same temperature. Water and a few drops of concentrated sulfuric acid were then slowly added at 0 °C. The mixture was then extracted with CH_2Cl_2. The organic fraction was dried over $MgSO_4$ and concentrated under reduced pressure to give desired product.

4.1.12. (9-(3,4-Dimethoxyphenyl)naphtho[1,2-*d*][1,3]dioxole-7,8-diyl)dimethanol (35)

Yield 91%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71(s, 1H), 7.37 (d, 1H, J = 8.5 Hz), 7.14 (d, 1H, J = 8.5 Hz), 6.86 (d, 1H, J = 7.9 Hz), 6.82–6.80 (m, 2H), 5.72 (AB, 2H, J = 7.2 Hz), 4.82 (AB, 2H, J = 12.2 Hz), 4.53 (AB, 2H, J = 11.8 Hz), 3.91 (s, 3H), 3.78 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.11, 147.88, 144.79, 141.91, 135.70, 135.06, 132.01, 129.57, 129.28, 122.12, 122.04, 119.61, 113.46, 110.99, 110.07, 100.86, 65.13, 59.86, 55.86, 55.76; IR (CH_2Cl_2, cast) 3406, 1725, 1508, 1450, 1262, 1070, 1024, 797, 734 cm^{-1}; FAB-HRMS Calcd for $C_{21}H_{20}O_6$ m/z (M^+) 368.1260, found 368.1256.

4.1.13. (9-(3-Hydroxy-4-methoxyphenyl)naphtho[1,2-*d*][1,3] dioxole-7,8-diyl)dimethanol (39)

Yield 92%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (s, 1H), 7.38 (d, 1H, J = 8.5 Hz), 7.15 (d, 1H, J = 8.5 Hz), 6.86 (d, 1H, J = 8.2 Hz), 6.82 (d, 1H, J = 2.0 Hz), 6.75 (dd, 1H, J = 2.0, 8.2 Hz), 5.75 (s, 2H), 5.70 (br s, 1H), 4.88 (AB, 2H, J = 12.2 Hz), 4.57 (AB, 2H, J = 12.0 Hz), 3.94 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 145.90, 144.83, 144.67, 141.99, 135.65, 135.12, 132.71, 129.63, 129.41, 122.10, 121.54, 119.61, 116.28, 111.06, 109.67, 100.92, 65.32, 60.00, 55.92; IR (CH_2Cl_2, cast) 3385, 1506, 1450, 1323, 1249, 1227, 1138, 1070, 1025 cm^{-1}; FAB-HRMS Calcd for $C_{20}H_{18}O_6$ m/z (M^+) 354.1103, found 354.1105.

4.1.14. (9-(4-Hydroxy-3-methoxyphenyl)naphtho[1,2-*d*][1,3] dioxole-7,8-diyl)dimethanol (45)

Yield 91%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77 (s, 1H), 7.41 (d, 1H, J = 8.6 Hz), 7.17 (d, 1H, J = 8.6 Hz), 6.93 (d, 1H, J = 8.0 Hz), 6.80 (d, 1H, J = 1.6 Hz), 6.77 (dd, 1H, J = 1.6, 8.0 Hz), 5.75 (s, 2H), 5.64 (br s, 1H), 4.90 (s, 2H), 4.60 (s, 2H), 3.84 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 148.17, 146.89, 146.08, 143.35, 137.41, 137.19, 136.55, 132.78, 131.50, 128.91, 124.10, 123.37, 121.03, 115.44, 115.31, 111.87, 102.11, 64.26, 59.91, 56.58; IR (CH_2Cl_2, cast) 3442, 1643, 1630, 1509, 1449, 1417, 1376, 1106 cm^{-1}; FAB-HRMS Calcd for $C_{20}H_{18}O_6$ m/z ([M+H]$^+$) 354.1103, found 354.1106.

4.1.15. 4-(7,8-bis(Hydroxymethyl)naphtho[1,2-*d*][1,3]dioxol-9-yl)benzene-1,2-diol (50)

Yield 89%, ^{1}H NMR (400 MHz, CD_3OD) δ 7.84 (s, 1H), 7.44 (d, 1H, J = 8.6 Hz), 7.16 (d, 1H, J = 8.6 Hz), 6.78 (d, 1H, J = 8.0 Hz), 6.71 (d, 1H, J = 2.0 Hz), 6.60 (dd, 1H, J = 2.0, 8.0 Hz), 5.73 (AB, 2H, J = 9.6 Hz), 5.48 (s, 1H), 4.91 (s, 2H), 4.55 (AB, 2H, J = 11.4 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 145.88, 145.53, 145.24, 143.23, 137.18, 137.09, 136.33, 132.77, 131.31, 128.72, 123.13, 122.78, 120.89, 118.58, 115.30, 111.65, 101.93, 64.18, 59.78; IR (CH_2Cl_2, cast) 3390, 1504, 1449, 1322, 1272, 1218, 1070, 1047 cm^{-1}; FAB-HRMS Calcd for $C_{19}H_{16}O_6$ m/z ([M]$^+$) 340.0947, found 340.0942.

General procedure for the preparation of ***33–54:*** To a solution of above appropriate alcohol in pyridine was added acetic anhydride or hexanoic anhydride and the resulting mixture was stirred at room temperature for 12 h. For the synthesis of monoesters, 1 equiv of anhydride is used while 2–3 equiv are required for multiple esterification. After the completion of the reaction, 10% HCl was added and the mixture was extracted with ether. The ether extract was washed with saturated $NaHCO_3$, dried over $MgSO_4$, and concentrated. After column chromatography, the desired products were isolated.

4.1.16. (9-(Benzo[*d*][1,3]dioxol-5-yl)-8-(hydroxymethyl) naphtho[1,2-*d*][1,3]dioxol-7-yl)methyl hexanoate (33)

Yield 80%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.80 (s, 1H), 7.40 (d, 1H, J = 8.5 Hz), 7.16 (d, 1H, J = 8.5 Hz), 6.82 (d, 1H, J = 7.8 Hz), 6.77 (d, 1H, J = 1.2 Hz), 6.73 (dd, 1H, J = 1.2, 7.8 Hz), 6.03 (s, 1H), 5.99 (s, 1H), 5.77 (AB, 2H, J = 7.0 Hz), 5.41, (s, 2H), 4.54 (AB, 2H, J = 11.9 Hz), 2.35 (t, 2H, J = 7.6 Hz), 1.66–1.60 (m, 2H), 1.29–1.26 (m, 4H), 0.85 (t, 3H, J = 6.8 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.52, 146.89, 146.83, 144.98, 141.88, 135.59, 135.47, 132.95, 130.39, 130.11, 129.48, 123.07, 122.28, 119.81, 111.16, 110.49, 107.42, 100.98, 64.90, 59.21, 34.37, 31.24, 24.57, 22.26, 13.86; IR (CH_2Cl_2, cast) 3455, 1731, 1635, 1602, 1488, 1444, 1373, 1323, 1272, 1234, 1171, 1069, 1043, 933, 799 cm^{-1}; FAB-HRMS calcd for $C_{26}H_{26}O_7$ m/z (M)$^+$ 450.1679, found 450.1685.

4.1.17. (9-(Benzo[*d*][1,3]dioxol-5-yl)naphtho[1,2-*d*][1,3]dioxole-7,8-diyl)bis(methylene)dihexanoate (34)

Yield 80%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.81 (s, 1H), 7.42 (d, 1H, J = 8.5 Hz), 7.18 (d, 1H, J = 8.5 Hz), 6.78 (d, 1H, J = 7.9 Hz), 6.71 (d, 1H, J = 1.4 Hz), 6.67 (dd, 1H, J = 1.4, 7.9 Hz), 6.02 (S, 1H), 5.98 (S, 1H), 5.78 (AB, 2H, J = 6.9 Hz), 5.27, (s, 2H), 4.97 (s, 2H), 2.34 (t, 2H, J = 7.5 Hz), 2.24 (t, 2H, J = 7.5 Hz), 1.64–1.62 (m, 2H), 1.58–1.55 (m, 2H), 1.29–1.23 (m, 8H), 0.86 (t, 6H, J = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.53, 173.29, 146.92, 146.83, 145.10, 141.96, 137.28, 132.43, 130.67, 130.51, 129.87, 129.76, 123.08, 122.32, 119.72, 111.47, 110.44, 107.34, 101.05, 100.98, 64.49, 61.09, 34.29, 34.25, 31.28, 24.65, 24.59, 22.29, 13.87; FAB-HRMS calcd for $C_{32}H_{36}O_8$ m/z (M)$^+$ 548.2410, found 548.2413.

4.1.18. (9-(3,4-Dimethoxyphenyl)-8-(hydroxymethyl) naphtho[1,2-*d*][1,3]dioxol-7-yl)methyl acetate (36)

Yield 72%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.82 (s, 1H), 7.42 (d, 1H, J = 8.5 Hz), 7.17 (d, 1H, J = 8.5 Hz), 6.89 (d, 1H, J = 8.1 Hz), 6.85 (dd, 1H, J = 1.6, 8.1 Hz), 6.82 (d, 1H, J = 1.6 Hz), 5.74 (AB, 2H, J = 11.0 Hz), 5.42 (s, 2H), 4.53 (AB, 2H, J = 11.7 Hz), 3.94 (s, 3H), 3.82 (s, 3H), 2.11 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.74, 148.26, 148.00, 145.03, 141.97, 135.96, 135.38, 131.84, 130.24, 130.13, 129.52, 122.32, 122.02, 119.88, 113.41, 111.19, 110.12, 101.98, 65.17, 59.31, 55.94, 55.82, 21.17; IR (CH_2Cl_2, cast) 3497, 1736, 1508, 1451, 1372, 1324, 1230, 1247, 1072 cm^{-1}; FAB-HRMS Calcd for $C_{23}H_{22}O_7$ m/z (M^+) 410.1366, found 410.1362.

4.1.19. (9-(3,4-Dimethoxyphenyl)naphtho[1,2-*d*][1,3]dioxole-7,8-diyl)bis(methylene) diacetate (37)

Yield 75%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.82 (s, 1H), 7.43 (d, 1H, J = 8.5 Hz), 7.19 (d, 1H, J = 8.5 Hz), 6.86 (d, 1H, J = 7.8 Hz), 6.77–6.79 (m, 2H), 5.74 (AB, 2H, J = 11.5 Hz), 5.28 (s, 2H), 4.99 (s, 2H), 3.93 (s, 3H), 3.80 (s, 3H), 2.10 (s, 3H), 2.00 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.72, 170.36, 148.36, 147.91, 145.17, 142.05, 137.63, 131.30, 130.41, 130.24, 130.05, 129.76, 122.34, 122.04, 119.78, 113.41, 111.52, 110.05, 101.05, 64.81, 61.44, 55.88, 55.79, 21.05, 20.91; IR (CH_2Cl_2, cast) 1738, 1509, 1452, 1373, 1324, 1232, 1138, 1075 cm^{-1}; FAB-HRMS Calcd for $C_{25}H_{24}O_8$ m/z (M^+) 452.1471, found 452.1464.

4.1.20. (9-(3,4-Dimethoxyphenyl)-8-(hydroxymethyl) naphtho[1,2-*d*][1,3]dioxol-7-yl)methyl hexanoate (38)

Yield 75%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.82 (s, 1H), 7.42 (d, 1H, J = 8.5 Hz), 7.17 (d, 1H, J = 8.5 Hz), 6.90 (d, 1H, J = 8.0 Hz), 6.85 (dd, 1H, J = 1.6, 8.0 Hz), 6.82 (d, 1H, J = 1.6 Hz), 5.74 (AB, 2H, J = 11.1 Hz), 5.42 (s, 2H), 4.53 (AB, 2H, J = 11.1 Hz), 3.94 (s, 3H), 3.82 (s, 3H), 2.35 (t, 2H, J = 7.5 Hz), 1.65–1.61 (m, 2H), 1.29–1.25 (m, 4H), 0.85 (t, 3H, J = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.55, 148.25, 148.00, 145.00, 142.50, 135.93, 135.44, 131.86, 130.41, 130.06, 129.54, 122.32, 122.05, 119.90, 113.44, 111.17, 110.12, 100.97, 64.98, 59.32, 55.94, 55.83, 34.40, 31.27, 24.60, 22.29, 13.88; IR (CH_2Cl_2, cast) 3413, 1711, 1634, 1507, 1451, 1378, 1322, 1262, 1137 cm^{-1}; FAB-HRMS Calcd for $C_{27}H_{30}O_7$ m/z (M^+) 466.1992, found 466.2001.

4.1.21. (9-(3-Hydroxy-4-methoxyphenyl)-8-(hydroxymethyl) naphtho[1,2-*d*][1,3]dioxol-7-yl)methyl acetate (40)

Yield 72%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (s, 1H), 7.39 (d, 1H, J = 8.5 Hz), 7.16–7.13 (m, 2H), 6.99–6.97 (m, 2H), 5.76 (AB, 2H, J = 5.9 Hz), 4.87 (s, 2H), 4.58 (AB, 2H, J = 12.0 Hz), 3.89 (s, 3H), 2.28 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.14, 150.31, 144.89, 141.92, 138.69, 135.80, 135.25, 134.70, 131.92, 129.60, 129.51, 128.00, 124.80, 122.09, 119.48, 111.24, 111.10, 101.04, 65.26, 59.88, 55.89, 20.75; IR (CH_2Cl_2, cast) 3403, 1762, 1505, 1451, 1371, 1322, 1269, 1211, 1070 cm^{-1}; FAB-HRMS Calcd for $C_{22}H_{20}O_7$ m/z (M^+) 396.1209, found 396.1203.

4.1.22. (9-(3-Acetoxy-4-methoxyphenyl)naphtho[1,2-*d*][1,3] dioxole-7,8-diyl)bis(methylene) diacetate (41)

Yield 25%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (s, 1H), 7.41 (d, 1H, J = 8.6 Hz), 7.18 (d, 1H, J = 8.6 Hz), 7.09 (dd, 1H, J = 2.1, 8.3 Hz), 6.94–6.96 (m, 2H), 5.77 (AB, 2H, J = 5.8 Hz), 5.27 (s, 2H), 5.01 (AB, 2H, J = 12.0), 3.88 (s, 3H), 2.27 (s, 3H), 2.09 (s, 3H), 1.99 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.73, 170.44, 168.77, 150.51, 145.24, 142.01, 138.84, 136.61, 131.16, 130.50, 130.26, 130.17, 129.69, 127.72, 124.91, 122.25, 119.66, 111.55, 111.08, 101.20, 64.84, 61.23, 55.85, 21.04, 20.86, 20.70; FAB-HRMS Calcd for $C_{26}H_{24}O_9$ m/z (M^+) 480.1420, found 480.1418.

4.1.23. (9-(3-Hydroxy-4-methoxyphenyl)-8-(hydroxymethyl) naphtho[1,2-*d*][1,3]dioxol-7-yl)methyl hexanoate (42)

Yield 71%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (s, 1H), 7.40 (d, 1H, J = 8.5 Hz), 7.17–7.13 (m, 2H), 6.96–6.99 (m, 2H), 5.77 (AB, 2H, J = 6.0 Hz), 4.88 (s, 2H), 4.59 (AB, 2H, J = 12.0 Hz), 3.88(s, 3H), 2.55 (t, 2H, J = 7.5 Hz), 1.75–1.71 (m, 2H), 1.38–1.33 (m, 4H), 0.89 (t, 3H, J = 7.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.05, 150.36, 144.89, 141.95, 138.79, 135.79, 135.27, 134.78, 131.89, 129.61, 129.47, 127.84, 124.84, 122.07, 119.49, 111.24, 111.09, 101.05, 65.29, 59.90, 55.86, 34.06, 31.19, 24.70, 22.31, 13.92; IR (CH_2Cl_2, cast) 3393, 1760, 1646, 1505, 1452, 1375, 1322, 1268, 1070 cm^{-1}; FAB-HRMS calcd for $C_{26}H_{28}O_7$ m/z (M)$^+$ 452.1835, found 452.1842.

4.1.24. (9-(3-Hydroxy-4-methoxyphenyl)naphtho[1,2-*d*][1,3] dioxole-7,8-diyl)bis(methylene) dihexanoate (43)

Yield 60%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (s, 1H), 7.40 (d, 1H, J = 8.5 Hz), 7.17–7.14 (m, 2H), 6.99–6.97 (m, 2H), 5.76 (AB, 2H, J = 5.5 Hz), 5.41 (s, 2H), 4.53 (AB, 2H, J = 11.9 Hz), 3.88 (s, 3H), 2.54 (t, 2H, J = 7.5 Hz), 2.35 (t, 2H, J = 7.5 Hz), 1.75–1.70 (m, 2H), 1.65–1.59 (m, 2H), 1.40–1.33 (m, 4H), 1.31–1.23 (m, 4H), 0.89 (t, 3H, J = 7.0 Hz), 0.85 (t, 3H, J = 7.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.53, 171.97, 150.41, 145.02, 141.94, 138.80, 135.55, 134.92, 131.72, 130.44, 130.17, 129.46, 127.83, 124.86, 122.20, 119.69, 111.20, 111.15, 101.09, 64.93, 59.18, 55.85, 34.39, 34.05, 31.26, 31.20, 24.70, 24.59, 22.31, 22.28, 13.92, 13.87; IR (CH_2Cl_2, cast) 3361, 1736, 1633, 1457, 1376, 1234, 1168, 1073 cm^{-1}; FAB-HRMS calcd for $C_{32}H_{38}O_8$ m/z (M)$^+$ 550.2567, found 550.2575.

4.1.25. (9-(3-(Hexanoyloxy)-4-methoxyphenyl)naphtho[1,2-*d*][1,3]dioxole-7,8-diyl)bis (methylene)dihexanoate (44)

Yield 22%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (s, 1H), 7.40 (d, 1H, J = 8.5 Hz), 7.17 (d, 1H, J = 8.5 Hz), 7.07 (dd, 1H, J = 2.1, 8.3 Hz), 6.91–6.93 (m, 2H), 5.76 (AB, 2H, J = 6.0 Hz), 5.27 (d, 2H, J = 2.8 Hz), 5.00 (AB, 2H, J = 12.0 Hz), 3.86 (s, 3H), 2.53 (t, 2H, J = 7.4 Hz), 2.33 (t, 2H, J = 7.4 Hz), 2.23 (t, 2H, J = 7.4 Hz), 1.74–1.71 (m, 2H), 1.65–1.54 (m, 4H), 1.40–1.33 (m, 4H), 1.31–1.23 (m, 8H), 0.91–0.84 (m, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.53, 173.18, 171.65, 150.55, 145.17, 142.03, 138.94, 136.60, 131.20, 130.61, 130.47, 129.97, 129.71, 127.64, 124.94, 122.19, 119.65, 111.46, 111.02, 101.20, 64.35, 61.07, 55.81, 34.30, 34.21, 34.02, 31.32, 31.21, 24.73, 24.60, 22.33, 22.30, 13.93, 13.89; IR (CH_2Cl_2, cast) 1736, 1505, 1452, 1374, 1321, 1271, 1162, 1075, 1048 cm^{-1}; FAB-HRMS calcd for $C_{38}H_{48}O_9$ m/z (M)$^+$ 648.3298, found 648.3293.

4.1.26. (9-(4-Hydroxy-3-methoxyphenyl)-8-(hydroxymethyl) naphtho[1,2-*d*][1,3]dioxol-7-yl)methyl acetate (46)

Yield 72%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (s, 1H), 7.42 (d, 1H, J = 8.5 Hz), 7.17 (d, 1H, J = 8.5 Hz), 7.04 (d, 1H, J = 8.0 Hz), 6.90 (d, 1H, J = 1.8 Hz), 6.87 (dd, 1H, J = 1.8, 8.0 Hz), 5.75 (s, 2H), 4.90 (s, 2H), 4.61 (s, 2H), 3.84 (s, 3H), 2.34 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.00, 150.19, 139.10, 135.44, 135.25, 135.15, 133.92, 130.12, 129.69, 129.62, 122.19, 121.74, 119.35, 118.10, 114.47, 113.49, 111.19, 100.97, 65.27, 59.97, 56.01, 20.75; IR (CH_2Cl_2, cast) 3405, 1762, 1505, 1451, 1370, 1322, 1268, 1210, 1071 cm^{-1}; FAB-HRMS Calcd for $C_{22}H_{20}O_7$ m/z ([M]$^+$) 396.1209, found 396.1217.

4.1.27. (9-(4-Hydroxy-3-methoxyphenyl)naphtho[1,2-*d*][1,3] dioxole-7,8-diyl)bis(methylene) diacetate (47)

Yield 48%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.83 (s, 1H), 7.42 (d, 1H, J = 8.5 Hz), 7.17 (d, 1H, J = 8.5 Hz), 7.04 (d, 1H, J = 8.0 Hz), 6.92 (d, 1H, J = 1.5 Hz), 6.87 (dd, 1H, J = 1.5, 8.0 Hz), 5.74 (s, 2H), 5.42 (s, 2H), 4.54 (s, 2H), 3.77 (s, 3H), 2.34 (s, 3H), 2.11 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.73, 168.98, 150.15, 145.05, 141.86,

139.04, 137.98, 135.34, 135.04, 130.32, 129.44, 122.32, 122.19, 121.70, 119.48, 114.46, 111.26, 101.00, 65.06, 59.21, 56.00, 21.16, 20.75; IR (CH_2Cl_2, cast) 3477, 1738, 1640, 1501, 1453, 1369, 1216, 1116, 1044, 797 cm^{-1}; FAB-HRMS Calcd for $C_{24}H_{22}O_8$ m/z ($[M]^+$) 438.1315, found 438.1306.

4.1.28. (9-(4-Acetoxy-3-methoxyphenyl)naphtho[1,2-*d*][1,3] dioxole-7,8-diyl)bis(methylene) diacetate (48)

Yield 30%, ^1H NMR (500 MHz, $CDCl_3$) δ 7.83 (s, 1H), 7.42 (d, 1H, J = 8.5 Hz), 7.19 (d, 1H, J = 8.5 Hz), 7.03 (d, 1H, J = 8.0 Hz), 6.87 (d, 1H, J = 1.7 Hz), 6.83 (dd, 1H, J = 1.7, 8.0 Hz), 5.75 (s, 2H), 5.27 (s, 2H), 5.02 (s, 2H), 3.76 (s, 3H), 2.33 (s, 3H), 2.09 (s, 3H), 1.99 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.69, 170.22, 168.82, 150.13, 145.22, 141.93, 139.23, 137.41, 136.98, 130.31, 130.24, 130.2, 129.66, 122.32, 122.18, 121.68, 119.40, 114.41, 111.61, 101.10, 64.76, 61.18, 55.95, 21.02, 20.85, 20.73; IR (CH_2Cl_2, cast) 1738, 1598, 1503, 1452, 1370, 1232, 1120, 1074 1026 cm^{-1}; FAB-HRMS Calcd for $C_{26}H_{24}O_9$ m/z ($[M]^+$) 480.1420, found 480.1416.

4.1.29. (9-(4-Hydroxy-3-methoxyphenyl)naphtho[1,2-*d*][1,3] dioxole-7,8-diyl)bis(methylene) dihexanoate (49)

Yield 63%, ^1H NMR (400 MHz, $CDCl_3$) δ 7.83 (s, 1H), 7.42 (d, 1H, J = 8.6 Hz), 7.17 (d, 1H, J = 8.6 Hz), 7.03 (d, 1H, J = 8.0 Hz), 6.91 (d, 1H, J = 1.8 Hz), 6.88 (dd, 1H, J = 1.8, 8.0 Hz), 5.74 (s, 2H), 5.42 (s, 2H), 4.54 (s, 2H), 3.76 (s, 3H), 2.60 (t, 2H, J = 7.5 Hz), 2.35 (t, 2H, J = 7.5 Hz), 1.79 (m, 2H), 1.64 (m, 2H), 1.40 (m, 4H), 1.30–1.27 (m, 4H), 0.93 (t, 3H, J = 7.0 Hz), 0.86 (t, 3H, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.55, 171.93, 150.20, 141.89, 140.19, 139.14, 137.89, 135.37, 135.12, 130.46, 130.25, 129.46, 122.29, 122.19, 121.74, 119.48, 114.44, 111.23, 101.02, 64.88, 59.23, 55.97, 34.39, 34.04, 31.26, 31.23, 24.75, 24.59, 22.36, 22.29, 13.97, 13.88; IR (CH_2Cl_2, cast) 3453, 1733, 1641, 1503, 1454, 1273, 1166, 749 cm^{-1}; ESI-HRMS Calcd for $C_{32}H_{38}O_8Na$ m/z ($[M+Na]^+$) 573.2464, found 573.2455.

4.1.30. (9-(3,4-Dihydroxyphenyl)naphtho[1,2-*d*][1,3]dioxole-7,8-diyl)bis(methylene) diacetate (51)

Yield 43%, ^1H NMR (400 MHz, $CDCl_3$) δ 7.79 (s, 1H), 7.40 (d, 1H, J = 8.5 Hz), 7.20 (m, 2H), 7.16 (d, 1H, J = 8.5 Hz), 7.14 (br s, 1H), 5.76 (AB, 2H, J = 5.7 Hz), 4.88 (AB, 2H, J = 12.2 Hz), 4.59 (s, 2H), 2.32 (s, 3H), 2.25 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.36, 168.25, 144.95, 141.80, 141.28, 141.03, 137.94, 135.60, 135.31, 134.05, 129.81, 129.55, 127.98, 125.31, 122.21, 122.13, 119.04, 111.23, 101.08, 65.17, 59.81, 20.72; IR (CH_2Cl_2, cast) 3423, 1766, 1637, 1503, 1451, 1369, 1267, 1209, 1174, 1105 cm^{-1}; FAB-HRMS Calcd for $C_{23}H_{20}O_8$ m/z ($[M]^+$) 424.1158, found 424.1147.

4.1.31. (9-(3-Acetoxy-4-hydroxyphenyl)naphtho[1,2-*d*][1,3] dioxole-7,8-diyl)bis(methylene) diacetate (52)

Yield 22%, ^1H NMR (500 MHz, $CDCl_3$) δ 7.83 (s, 1H), 7.41 (d, 1H, J = 8.5 Hz), 7.21 (m, 2H), 7.17–7.16 (m, 2H), 5.77 (AB, 2H, J = 6.1 Hz), 5.42 (s, 2H), 4.54 (s, 2H), 2.32 (s, 3H), 2.25 (s, 3H), 2.11 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.73, 168.31, 168.23, 145.11, 141.79, 141.33, 141.03, 137.77, 135.31, 134.23, 130.54, 130.34, 129.38, 127.96, 125.34, 122.27, 122.19, 119.26, 111.30, 101.13, 65.01, 59.05, 21.15, 20.72; FAB-HRMS Calcd for $C_{25}H_{22}O_9$ m/z ($[M]^+$) 466.1264, found 466.1269.

4.1.32. 4-(7,8-Bis(acetoxymethyl)naphtho[1,2-*d*][1,3]dioxol-9-yl)-1,2-phenylene diacetate (53)

Yield 12%, ^1H NMR (500 MHz, $CDCl_3$) δ 7.83 (s, 1H), 7.42 (d, 1H, J = 8.5 Hz), 7.21–7.17 (m, 2H), 7.15 (dd, 1H, J = 1.8, 8.2 Hz), 7.12 (d, 1H, J = 1.8 Hz), 5.77 (s, 2H), 5.27 (s, 2H), 5.02 (AB, 2H, J = 12.1 Hz), 2.30 (s, 3H), 2.24 (s, 3H), 2.09 (s, 3H), 1.99 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.71, 170.35, 168.10, 167.94, 145.32, 141.90, 141.53, 141.21, 137.19, 135.94, 130.58, 130.39, 130.15, 129.61, 127.70, 125.32, 122.27, 122.21, 119.30, 111.67, 101.25, 64.78, 60.99, 21.03, 20.81, 20.73, 20.67; FAB-HRMS Calcd for $C_{27}H_{24}O_{10}$ m/z ($[M]^+$) 508.1369, found 508.1378.

4.1.33. (9-(3,4-Dihydroxyphenyl)naphtho[1,2-*d*][1,3]dioxole-7,8-diyl)bis(methylene) dihexanoate (54)

Yield 61%, ^1H NMR (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.40 (d, 1H, J = 8.6 Hz), 7.19 (m, 2H), 7.16 (d, 1H, J = 8.6 Hz), 7.13–7.12 (m, 1H), 5.77 (AB, 2H, J = 6.0 Hz), 4.88 (AB, 2H, J = 12.2 Hz), 4.58 (s, 2H), 2.56 (t, 2H, J = 7.5 Hz), 2.49 (t, 2H, J = 7.5 Hz), 1.78–1.67 (m, 4H), 1.42–1.32 (m, 8H), 0.92 (t, 3H, J = 7.1 Hz), 0.88 (t, 3H, J = 7.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.33, 171.20, 144.93, 141.33, 141.10, 137.81, 135.62, 135.36, 134.12, 130.93, 129.77, 129.56, 127.84, 125.34, 122.26, 122.10, 119.02, 111.21, 101.12, 65.16, 59.80, 34.08, 31.28, 31.23, 24.64, 24.55, 22.34, 22.29, 13.90, 13.86; IR (CH_2Cl_2, cast) 3420, 1766, 1632, 1503, 1456, 1316, 1270, 1152, 1111, 1023 cm^{-1}; ESI-HRMS Calcd for $C_{31}H_{36}O_8Na$ m/z ($[M+Na]^+$) 559.2308, found 559.2313.

4.1.34. 9-(Benzo[*d*][1,3]dioxol-5-yl)naphtho[1,2-*d*][1,3]dioxole-7,8-dicarbaldehyde (55)

To a dichloromethane (2.0 ml) solution of oxalyl chloride (0.3 ml, 5 equiv) was added dropwise DMSO (0.5 ml, 10 equiv in 1.0 ml CH_2Cl_2) at −78 °C, and the resulting mixture was stirred for 15 min. A dichloromethane solution of **32** (250 mg in 5.0 ml) was added dropwise at −78 °C. After the mixture was stirred for 12 h, triethylamine (1 ml, 20 equiv) was added dropwise, and the reaction mixture was then slowly warmed to room temperature and stirred for 1 h. The mixture was quenched with water (50 ml) and extracted with dichloromethane (50 ml × 2). The combined organic layer was washed with brine, dried ($MgSO_4$), filtered, and concentrated in vacuo. 230 mg (93%) of dicarbaldehyde was obtained as almost pure. This was used directly without further purification.

4.1.35. 3-(10-(Benzo[*d*][1,3]dioxol-5-yl)-7-oxo-7*H*-[1,3] dioxolo[4′,5′:3,4]benzo[1,2-f]isoindol-8(9*H*)-yl)propanenitrile (56)

Compound **55** (50 mg, 0.14 mmol) and 3-aminopropionitrile (10 mg, 0.14 mmol) were placed in a round-bottomed flask. Mixture of CH_3CN/DMF (2:1/3 ml) was used as reaction medium; TMSCl (13 mg, 0.12 mmol) was added and the mixture was stirred for 12 h at room temperature. The mixture was extracted with ethyl acetate. The combined organic layer was evaporated in vacuum. The isolation of the product 20 mg (32%) was accomplished by flash column chromatography (EA/Hexane = 1/1). ^1H NMR (500 MHz, $CDCl_3$) δ 8.28 (s, 1H), 7.63 (d, 1H, J = 8.7 Hz), 7.25 (d, 1H, J = 8.7 Hz), 6.86 (d, 1H, J = 7.8 Hz), 6.79 (d, J = 1.4 Hz), 6.78 (dd, 1H, J = 1.4, 7.8 Hz), 6.03 (AB, 2H, J = 15.2 Hz), 5.90 (AB, 2H, J = 9.6 Hz), 4.40 (AB, 2H, J = 16.5 Hz), 3.85 (t, 2H, J = 6.5 Hz), 2.74 (t, 2H, J = 6.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.70, 147.29, 147.23, 146.01, 141.69, 135.73, 131.15, 130.46, 129.74, 127.29, 124.74, 124.56, 122.40, 120.75, 117.96, 111.29, 109.77, 107.93, 101.34, 101.18, 50.54, 39.28, 17.21; IR (CH_2Cl_2, cast) 2896, 1691, 1630, 1492, 1441, 1272, 1235, 1062, 1035 cm^{-1}; FAB-HRMS Calcd for $C_{23}H_{16}O_5N_2$ m/z (M^+) 400.1059, found 400.1052.

4.1.36. 2-(10-(Benzo[*d*][1,3]dioxol-5-yl)-7-oxo-7*H*-[1,3] dioxolo[4′,5′:3,4]benzo[1,2-*f*]isoindol-8(9*H*)-yl)acetic acid (57)

To a solution of **55** (48 mg, 0.14 mmol) in acetic acid (3 ml) was added glycine (10 mg, 0.14 mmol) and the mixture was boiled for 5–10 min. After this period, acetic acid was removed under reduced pressure. Inseparable mixture of **57** and the retro-isomer **57a** was obtained (34 mg, 61%, **57**:**57a** = 5:1). The reported spectrum was extracted from the mixture spectrum based on peak intensity. Compound **57**: ^1H NMR (400 MHz, CD_3OD) δ 8.25 (s,

1H), 7.72 (d, 1H, J = 8.7 Hz), 7.29 (d, 1H, J = 8.7 Hz), 6.89–6.83 (m, 3H), 6.00 (AB, 2H, J = 9.8 Hz), 5.89 (AB, 2H, J = 11.5 Hz), 4.43 (AB, 2H, J = 16.9 Hz), 4.17 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.59, 148.82, 148.76, 147.44, 143.04, 138.25, 132.95, 132.12, 131.04, 129.51, 125.91, 125.06, 124.01, 122.06, 112.18, 111.15, 108.87, 102.66, 102.58, 71.49, 51.90; IR (CH_2Cl_2, cast) 3419, 1736, 1631, 1487, 1439, 1373, 1320, 1273, 1232, 1039 cm^{-1}; **57+57a:** ESI-HRMS calcd for $C_{22}H_{14}NO_7$ m/z ([M−H]$^-$) 404.0770, found 404.0776.

4.1.37. Methyl 2-(10-(benzo[*d*][1,3]dioxol-5-yl)-7-oxo-7*H*-[1,3]dioxolo[4′,5′:3,4]benzo[1,2-*f*]isoindol-8(9*H*)-yl)acetate (58)

To a DMF solution of **57** (25 mg, 0.06 mmol 2.0 ml) was added K_2CO_3 (17 mg, 0.12 mmol) and CH_3I (18 mg, 0.13 mmol). The mixture was stirred 2 h. After completion of the reaction, the reaction mixture was washed with water (10 ml × 2) and extracted with dichloromethane (20 ml). After purified by column chromatography (DCM/Hexane = 2/1), 22 mg (81%) of **58** and 4 mg (15%) of **58a** were obtained. Compound **58:** ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.31 (s, 1H), 7.63 (d, 1H, J = 8.6 Hz), 7.23 (d, 1H, J = 8.6 Hz), 6.85 (d, 1H, J = 7.8 Hz), 6.81 (d, 1H, J = 1.5 Hz), 6.79 (dd, 1H, J = 1.5, 7.8 Hz), 6.02 (AB, 2H, J = 17.4 Hz), 5.89 (AB, 2H, J = 8.2 Hz), 4.36 (s, 2H), 4.35 (AB, 2H, J = 16.1 Hz), 3.72 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.37, 168.64, 147.19, 147.11, 145.89, 141.63, 136.20, 131.39, 130.48, 129.48, 127.28, 124.73, 122.52, 120.74, 111.13, 109.86, 107.83, 101.29, 101.13, 52.32, 50.18, 43.83; IR (CH_2Cl_2 cast) 1744, 1692, 1635, 1486, 1459, 1434, 1273, 1234, 1061, 1064, 927, 803 cm^{-1}; ESI-HRMS Calcd for $C_{23}H_{17}NO_7$Na m/z ([M+Na])$^+$ 442.0903, found 442.0902.

4.1.38. Pentyl 2-(10-(benzo[*d*][1,3]dioxol-5-yl)-7-oxo-7*H*-[1,3]dioxolo[4′,5′:3,4]benzo[1,2-*f*]isoindol-8(9*H*)-yl)acetate (59) and octyl 2-(10-(benzo[*d*][1,3]dioxol-5-yl)-7-oxo-7*H*-[1,3]dioxolo[4′,5′:3,4]benzo[1,2-f]isoindol-8(9*H*)-yl)acetate (60)

To a solution of **57** in DMF was added K_2CO_3 and $C_5H_{11}I$ (for the synthesis of **59**) and $C_8H_{17}I$ (for **60**) and the reactions were treated as described for **58**. After column chromatography **59** (68%) and **59a** (13%); **60** (66%) and **60a** (14%) were isolated, respectively. Compound **59:** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.31 (s, 1H), 7.63 (d, 1H, J = 8.6 Hz), 7.24 (d, 1H, J = 8.6 Hz), 6.85 (d, 1H, J = 7.8 Hz), 6.80 (d, 1H, J = 1.4 Hz), 6.78 (dd, 1H, J = 1.4, 7.8 Hz), 6.02 (AB, 2H, J = 14.0 Hz), 5.89 (AB, 2H, J = 6.6 Hz), 4.35 (s, 2H), 4.35 (AB, 2H, J = 16.2 Hz), 4.11 (t, 2H, J = 6.6 Hz), 1.60–1.57 (m, 2H), 1.36–1.30 (m, 2H), 0.89 (t, 3H, J = 7.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.98, 168.63, 147.18, 147.09, 145.86, 141.63, 136.25, 131.42, 130.47, 129.44, 127.36, 124.71, 122.51, 120.73, 111.11, 109.85, 107.82, 101.28, 101.12, 65.32, 50.17, 43.92, 30.51, 19.05, 13.63; IR (CH_2Cl_2, cast) 1740, 1696, 1633, 1490, 1460, 1373, 1273, 1234, 1038 cm^{-1}; ESI-HRMS Calcd for $C_{26}H_{23}NO_7$Na m/z ([M+Na])$^+$ 484.1372, found 484.1368. **60:** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.31 (s, 1H), 7.64 (d, 1H, J = 8.6 Hz), 7.24 (d, 1H, J = 8.6 Hz), 6.85 (d, 1H, J = 7.8 Hz), 6.81 (d, J = 1.3 Hz), 6.79 (dd, 1H, J = 1.6, 7.8 Hz), 6.02 (AB, 2H, J = 14.4 Hz), 5.89 (AB, 2H, J = 6.6 Hz), 4.35 (s, 2H), 4.35 (AB, 2H, J = 16.2 Hz), 4.10 (t, 2H, J = 6.7 Hz), 1.61–1.58 (m, 2H), 1.25–1.22 (m, 10H), 0.84 (t, 3H, J = 6.9 Hz); ^{13}C NMR (400 MHz, $CDCl_3$) δ 168.97, 168.64, 147.19, 147.10, 145.87, 141.64, 136.26, 131.44, 130.49, 129.45, 127.37, 124.72, 122.52, 120.74, 111.11, 109.86, 107.83, 101.28, 101.12, 65.63, 50.18, 43.95, 31.72, 29.11, 28.49, 25.83, 22.59, 14.04; IR (CH_2Cl_2, cast) 1681, 1635, 1443, 1413, 1378, 122, 1039, 764 cm^{-1}; ESI-HRMS Calcd for $C_{30}H_{31}NO_7$Na m/z ([M+Na])$^+$ 540.1998, found 540.1985.

4.1.39. 2-(10-(Benzo[*d*][1,3]dioxol-5-yl)-7-oxo-7H-[1,3]dioxolo [4′,5′:3,4]benzo[1,2-*f*]isoindol-8(9*H*)-yl)acetaldehyde (61)

To a solution of **58** (40 mg, 0.095 mmol) in dry toluene (3 ml) was added dropwise DIBALH (1 M in toluene, 0.16 ml) at −40 °C. The mixture was stirred 2 h at same temperature. After completion of the reaction, the reaction mixture was washed with water (20 ml), extracted with dichloromethane (20 ml) and dried over $MgSO_4$. After evaporation of solvents, 30 mg (81%) of **61** obtained and was used further without purification and characterization.

4.1.40. 10-(Benzo[*d*][1,3]dioxol-5-yl)-8-(2-hydroxyethyl)-8,9-dihydro-7*H*-[1,3]dioxolo[4′,5′:3,4]benzo[1,2-f]isoindol-7-one (62)

Compound **61** (30 mg, 0.077 mmol) and $NaBH_4$ (10 mg, 0.26 mmol) were stirred in dry THF (3 ml) for 1 h at room temperature. After this period, 10% HCl was added cautiously to the reaction mixture and the reaction was stirred for another 30 min. The mixed solution was extracted with ether. The organic fraction was dried over $MgSO_4$ and concentrated. After column chromatography, 23 mg (76%) of **62** isolated. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (s, 1H), 7.62 (d, 1H, J = 8.6 Hz), 7.23 (d, 1H, J = 8.6 Hz), 6.86 (d, 1H, J = 7.7 Hz), 6.79–6.77 (m, 2H), 6.03 (AB, 2H, J = 11.7 Hz), 5.89 (AB, 2H, J = 6.7 Hz), 4.35 (AB, 2H, J = 16.8 Hz), 3.88 (t, 2H, J = 4.9 Hz), 3.72 (t, 2H, J = 4.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.63, 147.22, 147.13, 145.82, 141.64, 136.23, 131.40, 130.49, 129.38, 128.02, 124.65, 124.26, 122.48, 120.59, 111.11, 109.82, 107.86, 101.28, 101.14, 61.67, 51.33, 46.52; IR (CH_2Cl_2, cast) 3353, 1677, 1619, 1492, 1438, 1374, 1294, 1230, 1036 cm^{-1}; FAB-HRMS Calcd for $C_{22}H_{18}O_6N$ m/z ([M+H]$^+$) 392.1134, found 392.1133.

5. Materials and methods for cell culture

5.1. Reagents

Hepatitis B surface antigen (HBsAg) enzyme immunoassay (EIA) kits were purchased from Bio-Rad (Hercules, CA, USA). Fetal calf serum was obtained from Hyclone (Logan, UT, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco/BRL (Gaithersbung, MD, USA). MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), was purchased from Sigma Chemical Co. (St. Louis. MO, USA).

5.2. Cell culture

Human hepatoblastoma HepA2 cell line is derived from HepG2 cells by transfecting with two tandem repeats of the HBV genome and continually secretes HBsAg and HBeAg into the culture medium.[36] The 1.3ES2 cell line is a clonal derivative of HepG2 cells in which the 1.3 copies of the entire HBV genome was stably integrated in the host genome.[26] These cell lines were used to assess the antiviral activity of helioxanthin and its analogues. Stock cultures of human hepatoma cells HepA2 and 1.3ES2 were maintained in DMEM supplemented with 10% fetal calf serum and antibiotics (100 IU/ml each of penicillin and streptomycin) in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. The cultures were passaged by trypsinization every 4 days. For bioassays, cells were plated either in 24-well plates at a density of 1×10^5 cells/well or 96-well plates at a density at of 3×10^4 cells/well in 100-mm culture dishes at a density of 1.5×10^6 cells/dish in DMEM medium containing 10% fetal calf serum.

5.3. Quantification of HBsAg

Cells were seeded either in 96-well plates at a density of 3×10^4 cells/well or in 24-well plates at a density of 1×10^5 cells/well in DMEM medium containing 10% fetal calf serum. After 24 h of incubation, the cells were washed twice with phosphate-buffered saline (PBS), pH 7.0, and treated with various concentrations of drugs in serum-free DMEM for 48 h. The amount of HBsAg

production in the culture medium was determined by enzyme immunoassay (EIA) kits (Bio-Rad, CA, USA). The viability of cells was determined by a MTT cell proliferation assay.[37]

5.4. Quantitative detection of HBV DNA by real-time light cycler PCR

We have used dilutions of known amounts of HBV-DNA from plasmid as a control. The standard curve showed a good linear range when 10^3–10^7 copies of plasmid DNA were used as templates (data not shown). Cells were seeded in 100 mm culture dishes at a density of 5×10^6 cells/well before treated with various concentrations of drug in serum-free DMEM for 72 h. For quantification of HBV DNA, viral DNA was extracted from culture media using High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The PCR primers used were purchased from Tib-Molbiol (Berlin, Germany). The oligonucleotide sequences of primers were: HBV Forward: 5′-CAGGTCTGTGCCAAG-3′ (the accession number of GenBank: V01460, nt 1168–1182) HBV Reverse: 5′-TGCGGGATAGGACAA-3′ (nt 1359–1345). The PCR cycling program consisted of an initial denaturing step at 95 °C for 10 min, followed by 45 amplification cycles at 95 °C for 12 s, 54 °C for 20 s.

5.5. RNA isolation and Northern blot analysis

Total RNA was extracted from the cells using the phenol and guanidium isothiocyanate method.[38] The RNA (20 mg) was denatured by 2.2 M formaldehyde, separated on a denaturing formaldehyde 1.2% agarose gel, and transferred to a nylon membrane (Hybond-XL, Amersham). The membrane was hybridized with a ^{32}P-radiolabelled full-length HBV probe. The relative amount of total RNA applied was normalized to that of glyceraldehyde-3-phosphate dehydrogenase.

5.6. Transient transfections and luciferase assay

HepA2 cells were transfected with pCP-Luc plasmid using lipofectamine 2000 transfection reagent (Invitrogen). The transfected cells were changed to a serum-free DMEM with drug for 48 h. To prepare total cell lysate from transfected cells for luciferase activity measurements, the medium was aspirated from the cell culture and the cells were gently rinsed with PBS. Cells were scrapped from the plates and collected through centrifugation. The supernatant was collected for protein and luciferase activity measurements immediately following lysate preparation. Protein concentrations of the resultant cell lysates were measured by the Bradford method. Lysates prepared from transfected cells were analyzed for luciferase activity using a luminometer and the Promega Luciferase Assay System as described by the manufacturer (Promega). Luciferase activities were normalized to the amount of protein in each lysate. For all transient transfections with promoter–luciferase reporter construct, the level of luciferase activity was determined without drug treatment to be set to one. The transfection efficiency was normalized using the activity of β-galactosidase as an internal control. The values are means plus and minus the standard error of the mean of at least three independent experiments.

5.7. Plasmid

The HBV sequence used in this study is of *ayw* subtype.[39] The accession number of GeneBank: V01460, Eco RI site as nucleotide 1. The pCP-Luc, plasmid was generous gift from Dr. Chungming Chang (National Health Research Institutes, Taiwan).

Acknowledgments

The authors thank Dr. Chungming Chang for providing the pCP-Luc plasmid. This work was supported by Grants from Academia Sinica, National Science Council (NSC 98-2320-B-010-024-MY3), and Ministry of Education, Republic of China (Aim for the Top University Plan).

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.11.037.

References and notes

1. World Health Organization. Hepatitis B. World Health Organization Fact Sheet 204 (Revised August 2008). (http://www.who.int/mediacentre/factsheets/fs204/en/index.html).
2. Robinson, W. S. *Annu. Rev. Microbiol.* **1977**, *31*, 357.
3. Lok; Mc Mahon *Hepatology* **2007**, *45*, 2.
4. Pang, K. R.; Wu, J. J.; Huang, D. B.; Tyring, S. K.; Baron, S. *Methods Mol. Med.* **2005**, *116*, 1.
5. Zoulim, F. *J. Clin. Virol.* **2001**, *21*, 243.
6. Dienstag, J. L.; Perril, N. *Engl. J. Med.* **1995**, *333*, 1657.
7. Walsh, K. M.; Woodlo, R. P.; Schiff, E. R.; Bartholomew, M.; Vicary, C.; Rubin, Mall, T.; Lamy, P.; Wight, D. G.; Bloor, S.; Alexander, G. J. *Gut* **2001**, *49*, 436.
8. Franco, J.; Saeian, K. J. *Vasc. Interv. Radiol.* **2002**, *13*, S191.
9. Langley, D. R.; Walsh, A. W.; Baldick, C. J.; Eggers, B. J.; Rose, R. E.; Levine, S. M.; Kapur, A. J.; Colonno, R. J.; Tenney, D. J. *J. Virol.* **2007**, *81*, 3992.
10. Ruiz-Sancho, A.; Sheldon, J.; Soriano, V. *Expert. Opin. Biol. Ther.* **2007**, *7*, 751.
11. Lee, H. S.; Chung, Y. H.; Lee, K. S.; Byun, K. S.; Paik, S. W.; Han, J. Y.; Yoo, K.; Yoo, H. W.; Lee, J. H.; Yoo, B. C. *Hepatology* **2006**, *43*, 982.
12. Yeh, C. T.; Chien, R. N.; Chu, C. M.; Liaw, Y. F. *Hepatology* **2000**, *31*, 1318.
13. Locarnini, S.; Mason, W. S. *J. Hepatol.* **2006**, *44*, 422.
14. Deres, K.; Schroder, C. H.; Paessens, A.; Goldmann, S.; Hacker, H. J.; Weber, O.; Kramer, T.; Niewohner, U.; Pleiss, U.; Stoltefuss, J.; Graef, E.; Koletzki, D.; Masantschek, R. N.; Reimann, A.; Jaeger, R.; Gross, R.; Beckermann, B.; Schlemmer, K. H.; Haebich, D.; Helga, R. W. *Science* **2003**, *299*, 893.
15. Sanghyun, L.; Hyun, S. B.; Yong, P. K.; Bak-Kwang, K.; Seon, H. C.; Kazuo, O.; Hyun, S. *Phytother. Res.* **2005**, *19*, 103.
16. Kuo, Y. H.; Wu, M. D.; Hung, C. C.; Huang, R. L.; Yang Kuo, L. M.; Shen, Y. C.; Ong, C. W. *Bioorg. Med. Chem.* **2005**, *13*, 1555.
17. Cheng, Y. C.; Ying, C. X.; Leung, C. H.; Li, Y. *J. Clin. Virol.* **2005**, *34*, S147.
18. Yeo, H.; Li, Y.; Fu, L.; Zhu, J. L.; Gullen, E. A.; Dutschman, G. E.; Lee, Y.; Chung, R.; Huang, E. S.; Austin, D. J.; Cheng, Y. C. *J. Med. Chem.* **2005**, *2*, 534.
19. He, K.; Zeng, L.; Shi, G.; Zhao, G. X.; Kozlowski, J. F.; McLaughlin, J. L. *J. Nat. Prod.* **1997**, *60*, 38.
20. Burden, R. S.; Crombie, L.; Whiting, D. A. *Tetrahedron Lett.* **1968**, 1035.
21. Tseng, Y. P.; Kuo, Y. H.; Hu, C. P.; Jeng, K. S.; Janmanchi, D.; Lin, C. H.; Chou, C. K.; Yeh, S. F. *Antiviral Res.* **2008**, *77*, 206.
22. Li, Y.; Fu, L.; Yeo, H.; Zhu, J. L.; Chou, C. K.; Kou, Y. H.; Yeh, S. F.; Gullen, E.; Austin, D.; Cheng, Y. C. *Antiviral Chem. Chemother.* **2005**, *16*, 193.
23. Janmanchi, D.; Tseng, Y. P.; Wang, K. C.; Huang, R. L.; Lin, C. H.; Yeh, S. F. *Bioorg. Med. Chem.* **2010**, *18*, 1213–1226. Ying, C. X.
24. Li, Y.; Leung, C. H.; Robek, M. D.; Cheng, Y. C. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 8526.
25. Holmes, T. L.; Stevenso, R. *J. Chem. Soc. C-Org.* **1971**, 2091.
26. Stevenson, R.; Weber, J. V. *J. Nat. Prod.* **1989**, *52*, 367.
27. Mizufune, H.; Nakamura, M.; Mitsudera, H. *Tetrahedron Lett.* **2001**, *42*, 437.
28. Charlton, J. L.; Oleschuk, C. J.; Chee, G. L. *J. Org. Chem.* **1996**, *61*, 3452.
29. Wan, J.; Wu, B.; Pan, Y. *Tetrahedron* **2007**, *63*, 9338.
30. Grigg, R.; Gunaratne, H. Q. N.; Sridharan, V. *J. Chem. Soc., Chem. Commun.* **1985**, 1183.
31. Yeh, S. F.; Hong, C.Y.; Huang, Y. L.; Liu, T. Y.; Choo, K. B.; Chou, C. K. *Antiviral Res.* **1993**, *20*, 185–193.
32. Chou, Y. C.; Jeng, K. S.; Chen, M. L.; Liu, H. H.; Liu, T. L.; Chen, Y. L.; Liu, Y. C.; Hu, C. P.; Chang, C. *J. Virol.* **1813**, *2005*, 79.
33. Gilbert, S.; Galarneau, L.; Lamontagne, A.; Roy, S.; Ɓelanger, L. *J. Virol.* **2000**, *74*, 5032.
34. Li, J.; Ou, J. H. *J. Virol.* **2001**, *75*, 8400.
35. Yu, X.; Mertz, J. E. *J. Virol.* **2003**, *77*, 2489.
36. Chang, C. M.; Jeng, K. S.; Hu, C. P.; Lo, S. J.; Su, T. S.; Ting, L. P.; Chou, C. K.; Han, S. H.; Pfaff, E.; Salfeld, J. *EMBO J.* **1987**, *6*, 675.
37. Denizot, F.; Lang, R. *J. Immunol. Methods* **1986**, *89*(2), 271.
38. Gruffat, D.; Piot, C.; Durand, D.; Bauchart, D. *Anal. Biochem.* **1996**, *242*, 77.
39. Galibert, F.; Mandart, E.; Fitoussi, F.; Tiollais, P.; Charnay, P. *Nature* **1979**, *281*, 646.