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Antiviral agents 3. Discovery of a novel small molecule non-nucleoside inhibitor of Hepatitis B Virus (HBV)

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ABSTRACT

The discovery of a small molecule non-nucleoside inhibitor of Hepatitis B Virus is described. During our work on conocurvone derived naphthoquinone 'trimers' for the treatment of HIV, we discovered a potent inhibitor **9** of Hepatitis B Virus in an antiviral screen. During attempts to resynthesis **9** for proof of concept studies, we altered the synthesis in order to attempt to reduced side reactions and difficult to remove by-products. As a result we discovered a small molecule **19** that also was a potent inhibitor of HBV. Importantly, this small molecule inhibitor of Hepatitis B Virus is also an inhibitor of Hepatitis B Virus resistant to 3TC, a bench mark of nucleoside analogues active in the treatment of Hepatitis B Virus. The development of **19** as an agent to treat HBV infections is discussed.

Hepatitis B Virus (HBV) is one of the most prevalent viral infections of humans; it attacks the liver and can cause both acute and chronic disease. The WHO estimated 350 million chronic carriers and that about 2 billion people have been infected.[1] An estimated 600,000 to 1.2 million people die each year of HBV associated illnesses.[2,3] HBV causes a potentially life-threatening liver infection and is a major global health problem and the most serious type of Hepatitis. Twenty-five percentage of adults who were chronically infected with HBV during childhood die from liver cancer or cirrhosis.[4] HBV is more infectious than HIV and HBV is an important occupational hazard for health workers.[5] HBV is preventable with a safe and effective vaccine; its effectiveness varies and is dependant on an individual's age.[2]

Current FDA approved therapies for Hepatitis B viral infections are the biological agents Interferon-alpha (Intron A®) and Pegylated Interferon (Pegasys®); and the nucleoside analogues Lamivudine (3TC) (Zeffix®) (a cytidine analogue), Adefovir dipivoxil (Hepsera®) (an adenosine analogue), Entercavir (Baraclude®) (a guanine analogue), Telbivudine (Tyzeka®) (a thymidine analogue) and Tenofovir (Viread®) (an adenosine analogue).[3] There is no small molecule non-nucleoside inhibitor of HBV.

As part of research into naphthoquinone 'trimers' as potential HIV inhibitors[6-7] a selection of a few of naphthoquinone 'trimers', **1**–**9**,[6,7] were screened across a range of viruses. One of these compounds **9**[7] showed good inhibitory activity in an in vitro HepG2.2.15 cell assay, indicating that it had promising *anti*-Hepatitis B Virus activity. The results of the HepG2.2.15 cell assays for the naphthoquinone 'trimers' are shown in Table 1.

Table 1
Anti-Hepatitis B Virus assay. Inhibitory activity of trimeric naphthoquinones in HepG2.2.15 cells

Compound	EC_{50} (μM)	CC_{50} (μM)
1	>10	>100
2	>10	14
3	10	1367
4	3.9	1434
5	1.5	466
6	0.154	>300
7	7.9	45
8	0.096	44
9	0.009	279

Values presented were calculated using data combined from all treated cultures. EC_{50} is the concentration at which a 50% depression of HBV DNA (relative to the average of untreated cultures) was observed; CC_{50} is the drug concentration at which a 50% depression of neutral red dye uptake (relative to untreated cultures) was observed.[8]

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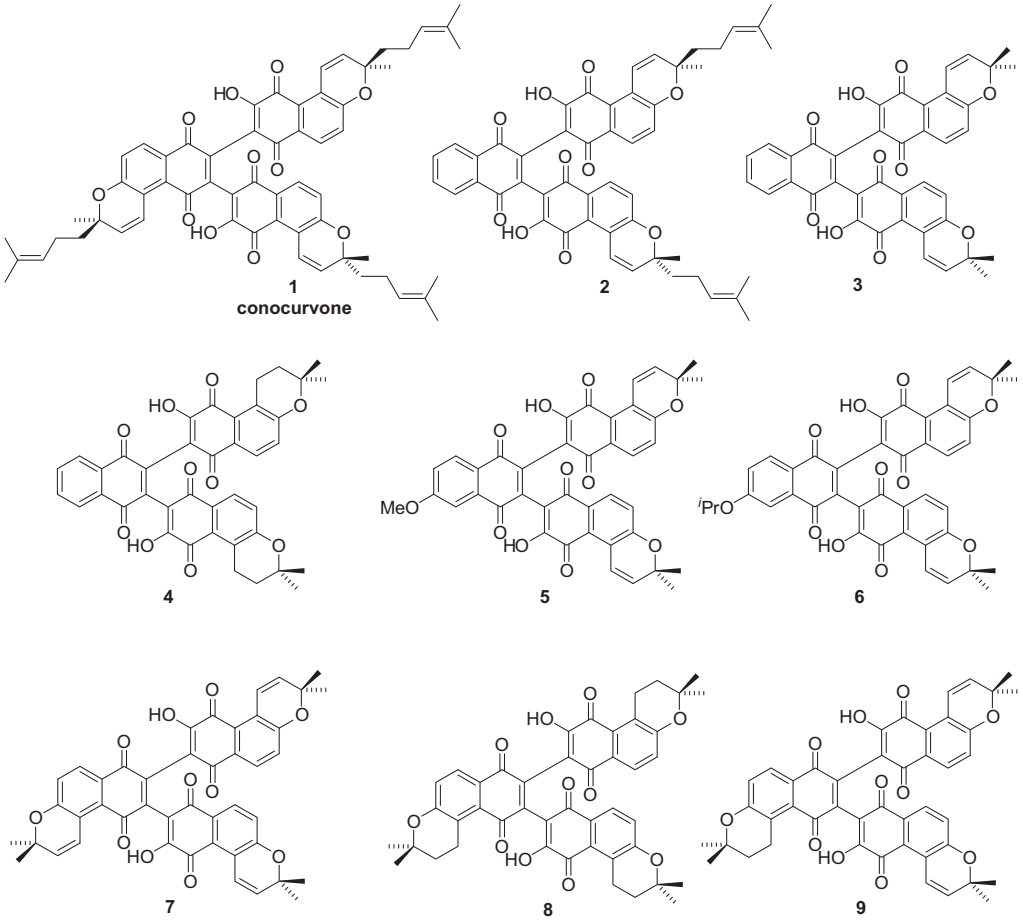
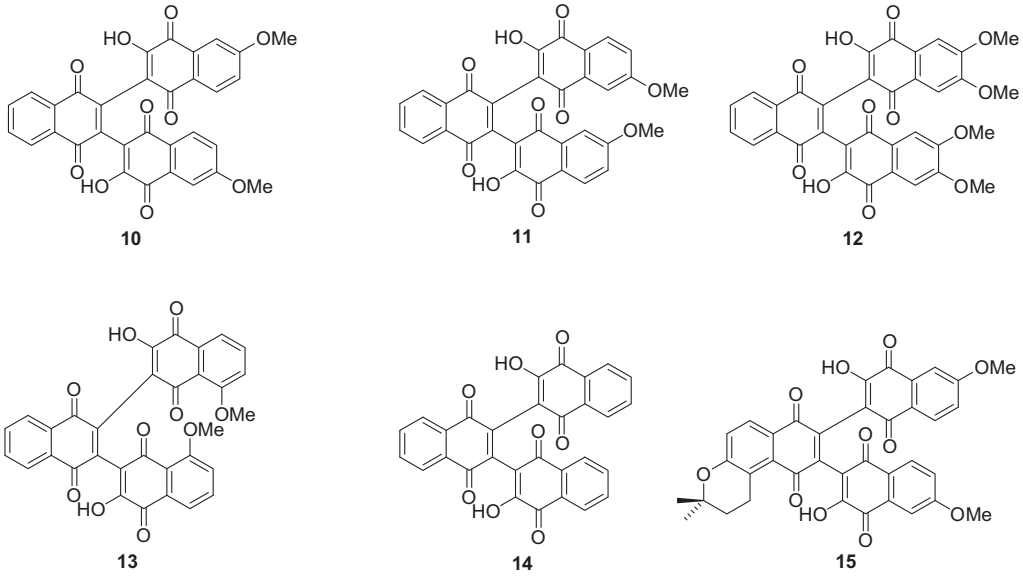


As compound **9** was the most active in the *anti*-Hepatitis B Virus assay, and the compound had a molecular weight of 750 Da, we were interested if simpler 'trimeric' naphthoquinones would also show good *anti*-Hepatitis B Virus activity. Consequently some 'trimeric' naphthoquinones were assayed (Table 2), where the pyran/dihydropyan ring(s) were replaced by methoxy groups, or deleted. The compounds screened, **10–15**,[7] were not as active as compound **9** and it was decided to synthesise larger quantities of **9** for further evaluation.



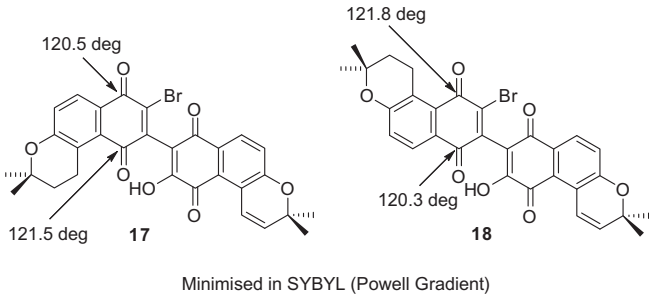


Figure 1.

The initial synthesis of compound **9** (Scheme 1) was complicated in the final coupling of the monomeric 9-hydroxy-3*H*-naphtho[2,1-*b*]pyran-7,10-dione **16** with bromodimer **17**, by self trimerisation of **16** to give **7**. This resulted in the purification of **9** having to be achieved by preparative HPLC.[7]

It was thought that distortion of the vinylogous acid bromide **17** (Fig. 1) by the *peri*-ring substituent might be a contributor in reducing the reactivity of **17**, allowing for self trimerisation of the monomeric **16** to occur resulting in the formation of **7**.

In an attempt to improve the reactivity of a vinylogous acid bromide for final coupling of **16** to give the desired **9**, the isomeric series was investigated where the coupling would use **18** (Scheme 2), as an alternative to **17** (Scheme 1).

This required the synthesis of isomeric 8-hydroxy-3*H*-naphtho[2,1-*b*]pyran-7,10-dione **19**. This was achieved by a similar sequence of reactions as for the synthesis of **16**,[6] using 2,6-dihydroxynaphthalene **20** in place of 2,7-dihydroxynaphthalene.

2,6-Dihydroxynaphthalene **20** was converted to the isomeric hydroxynaphthopyranquinone **19** using similar chemistry for the synthesis of the original monomeric hydroxynaphthopyranquinone **16**. This involved condensation of **20** with 3-methylbutenal in the presence of pyridine to give the pyranonaphthol **21**. Oxidation of **21**, using *N*,*N*-bis(salicylidene)ethylenediaminocobalt as the catalyst, gave the purple *ortho*-quinone **22**. Reduction of **22**, with sodium dithionite, followed by oxidation with an oxygen saturated solution of potassium *t*-butoxide in *t*-butanol gave the desired **19**, isomeric to the original hydroxynaphthopyranquinone **16**.

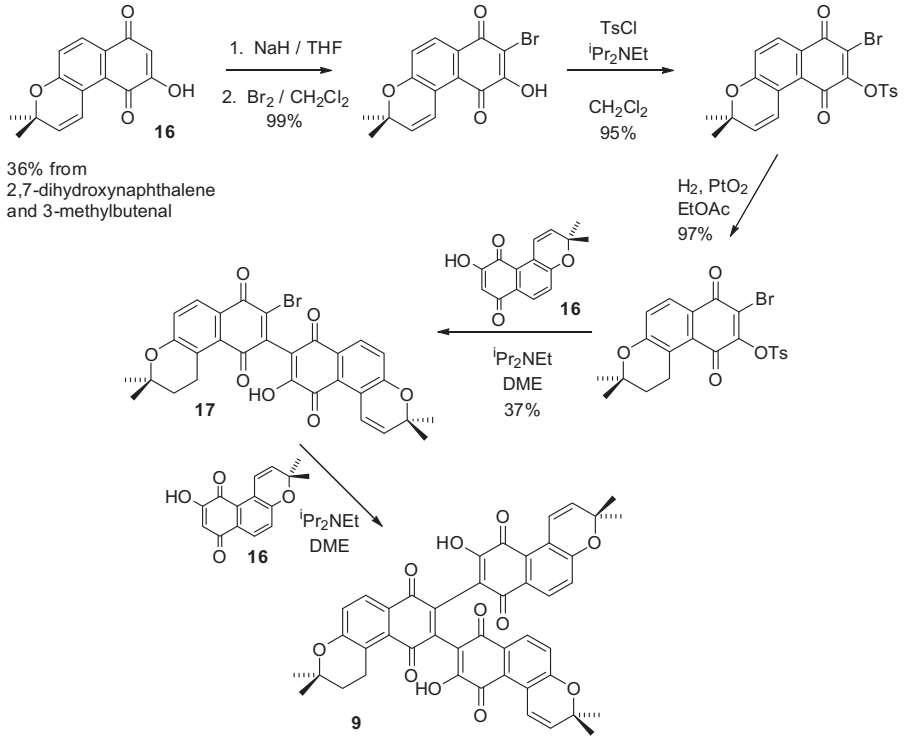
The isomeric hydroxynaphthopyranquinone **19** was hydrogenated to the give **23**, with the dihydropyran ring of the central quinone of **9**. The quinone **23** then was activated by bromination, to **24**, followed by tosylation, to the desired **25**. Reaction of **25** with **16** resulted in mostly substitution of the tosyloxy group of the hydroxynaphthopyranquinone (24%) to give the desired **18**, although there was a minor amount of bromide displacement (4%). This vinylogous acid bromide **18**, isomeric with **17**, did reacted with another molecule of **16** to give the desired **9**; although the reaction still gave significant self trimerisation of **16** to **7** and preparative HPLC would have had to be used to isolate **9**. Despite any significant improvement in the chemoselectivity of the final reaction to give **9**, the examination of the isomeric series did give an unexpected result.

The novel isomeric 8-hydroxy-3*H*-naphtho[2,1-*b*]pyran-7,10-dione **19** was a relatively potent small molecule (MW = 254.25)

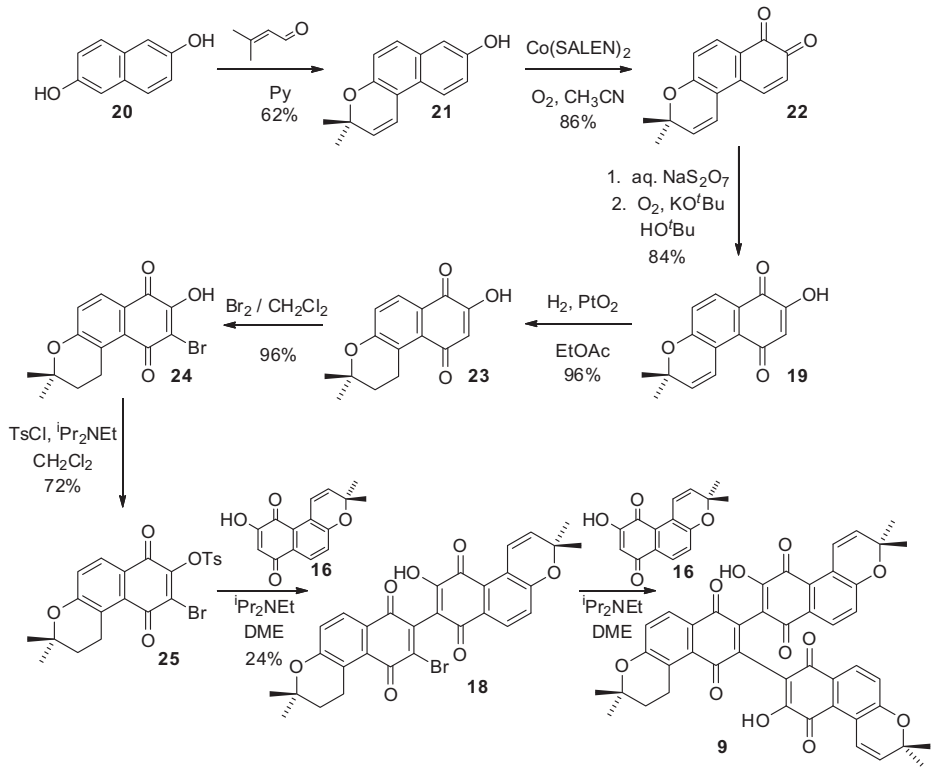
Table 2
Anti-Hepatitis B Virus assay. Inhibitory activity of trimeric naphthoquinones in HepG2.2.15 cells

Compound	EC_{50} (μM)	CC_{50} (μM)
10	0.05	58
11	56	295
12	0.40	>300
13	>10	>300
14	>10	>300
15	0.66	>300

Values presented were calculated using data combined from all treated cultures. EC_{50} is the concentration at which a 50% depression of HBV DNA (relative to the average of untreated cultures) was observed; CC_{50} is the drug concentration at which a 50% depression of neutral red dye uptake (relative to untreated cultures) was observed.[8]



Scheme 1.



Scheme 2.

non-nucleoside inhibitor of Hepatitis B Virus in the in vitro HepG2.2.15 cell assay (Table 3).[9]

Importantly, compound **19** was also found to be a potent inhibitor of wild type Hepatitis B viral replication in a transfection assay (Table 4).

Significantly was the activity of compound **19** against Hepatitis B Virus resistant to 3TC, a bench mark of nucleoside analogues active in the treatment of Hepatitis B Virus (Table 5).

As compound **19** is active against 3TC resistant Hepatitis B virus, it is perhaps not surprising that it is not a polymerase inhibitor (Supplementary data, Appendix 1) and apparently has a different mode of inhibition.

Compound **19** is not active in the usual animal models for Hepatitis B Virus (Ducks, Woodchucks) and this resulted in the development of the compound having to be carried out in primates. In a dose range finding studies, the cynomolgus monkeys used did not like the taste of the compound in Tang® formulation and plasma levels obtained were barely above the level of assay quantitation. This formulation is necessary for long term administration of the compound to primates which require dosing every few days over a long time.

Table 3
Anti-Hepatitis B Virus assay. Inhibitory activity in HepG2.2.15 cells

Compound	EC_{50} (μM)	CC_{50} (μM)
19	4.3 (n = 4)	>300 (n = 4)
3TC	0.06 (n = 4)	2128 (n = 4)
Penciclovir	3.4 (n = 3)	552 (n = 3)

Values presented were calculated using data combined from all treated cultures. EC_{50} is the concentration at which a 50% depression of HBV DNA (relative to the average of untreated cultures) was observed; CC_{50} is the drug concentration at which a 50% depression of neutral red dye uptake (relative to untreated cultures) was observed.[8]

Table 4
Wild type *anti*-Hepatitis B Virus assay. Wild type activity in Huh7 cells

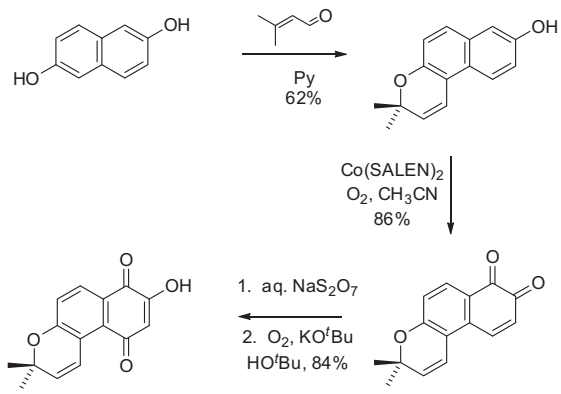
Compound	EC_{50} (μM)	CC_{50} (μM)
19	2.5–5 (n = 8)	>25 (n = 8)
3TC	0.1 (n = 10)	0.1 (n = 10)

For antiviral and cytotoxicity analyses, confluent cultures of Huh7 cells were transfected with HBV DNA and incubated for 4 h after which the supernatant media were removed and replaced with media containing serial dilutions of the assay compound. 3 days after transfection the supernatant media were again removed and replaced with media containing serial dilutions of the assay compound. 4 days after transfection cells were harvested and intracellular HBV DNA levels analysed by Southern blotting after controlled lysis. Intracellular nucleocapsids were detected by running the cell lysates on non-denaturing polyacrylamide gels and western blotting. Cytotoxicity analyses were undertaken on cells which had not been transfected but which were treated with serial dilutions of the assay compound in the same manner as described for the antiviral evaluation. Uptake of neutral red dye was used to determine toxicity on day 4.

Table 5
Anti-Hepatitis B Virus assay. Activity in HepG2 B1/HepG2 D88 cell assay. 3TC resistant cells

Compound	EC_{50} (μM)	CC_{50} (μM)
19	1.9 (n = 4)	>25 (n = 4)
3TC	>2.5 (n = 4)	>25 (n = 4)

Confluent monolayers of HepG2 B1 or HepG2 D88 cells were overlaid with serial dilutions of the assay compound and incubated for 3 days. Cell supernatants and cell lysates were then harvested and intracellular HBV DNA levels and extracellular HBV DNA levels were analysed by Southern blotting. Intracellular nucleocapsids and extracellular virions were detected by running the cell lysates and culture supernatants on non-denaturing polyacrylamide gels and western blotting. Cytotoxicity analyses were undertaken on HepG2 B1 or HepG2 D88 cells which had been treated with serial dilutions of the assay compound in the same manner as described for the antiviral evaluation. Neutral red dye uptake was used to determine toxicity on day 3.



Scheme 3.

The synthesis of **19** was scaled up in the laboratory and yields of the reactions improved, 44% overall yield (Scheme 3). Process development of the synthesis to the 100 g scale significantly improved the yield and reduced the cost of the compound.

Ongoing studies into compound **19** and its analogues include determining the mode of inhibition of the compounds of human Hepatitis B virus. Work is also being carried out to improve the acceptability of the compound, or analogues of it, by primates to allow for sufficient plasma levels of compound to be obtained.

Supplementary data

Supplementary data (synthesis and spectroscopic characterisation of previously unreported compounds **11**, **15**, **21**, **22**, **19**, **23**, **24**, **25** and **18** and Appendix 1) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.01.109.

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