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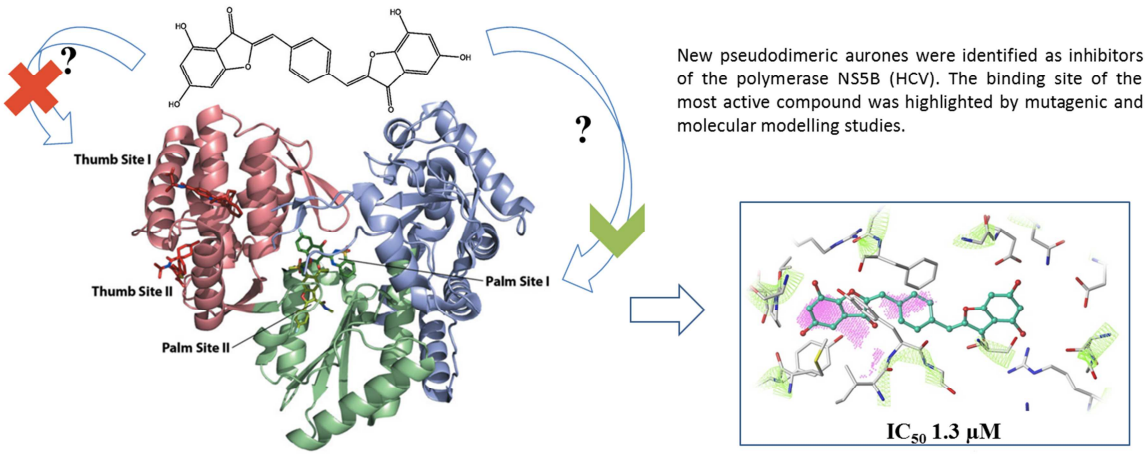


New pseudodimeric aurones were identified as inhibitors of the polymerase NS5B (HCV). The binding site of the most active compound was highlighted by mutagenic and molecular modelling studies.

New Pseudodimeric Aurones as Palm Pocket Inhibitors of Hepatitis C Virus RNA-dependent RNA Polymerase

Amel Meguellati[a,b], Abdelhakim Ahmed-Belkacem[c], Alessandra Nurisso[e], Wei Yi[a,b], Rozenn Brillet[c], Nawel Berqouch[a,b], Laura Chavoutier[a,b], Antoine Fortuné[a,b], Jean-Michel Pawlotsky[c,d], Ahcène Boumendjel[a,b] and Marine Peuchmaur[a,b,*]

[a]Univ. Grenoble Alpes, Département de Pharmacochimie Moléculaire DPM UMR 5063, 38041 Grenoble, France

[b]CNRS, DPM UMR 5063, 38041 Grenoble, France

[c]INSERM U955, Hôpital Henri Mondor, 51 Avenue du Maréchal de Lattre de Tassigny, 94010 Créteil, France

[d]National Reference Center for Viral Hepatitis B, C and Delta, Department of Virology, Hôpital Henri Mondor, Université Paris-Est, 51 Avenue du Maréchal de Lattre de Tassigny, 94010 Créteil, France

[e]School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 1211 Geneva, Switzerland

*Corresponding author Tel.: +33476635295; fax: +33476635298.

E-mail address: Marine.Peuchmaur@ujf-grenoble.fr

ABSTRACT

The NS5B RNA-dependent RNA polymerase (RdRp) is a key enzyme for Hepatitis C Virus (HCV) replication. In addition to the catalytic site, this enzyme is characterized by the presence of at least four allosteric pockets making it an interesting target for development of inhibitors as potential anti-HCV drugs. Based on a previous study showing the potential of the naturally occurring aurones as inhibitors of NS5B, we pursued our efforts to focus on pseudodimeric aurones that have never been investigated so far. Hence, 14 original compounds characterized by the presence of a spacer between the benzofuranone moieties were synthesized and investigated as HCV RdRp inhibitors by means of an *in vitro* assay. The most active inhibitor, pseudodimeric aurone **4**, induced high inhibition activity (IC_{50} = 1.3 µM). Mutagenic and molecular modelling studies reveal that the binding site for the most active derivatives probably is the palm pocket I instead of the thumb pocket I as for the monomeric derivatives.

Keywords: Aurones; Hepatitis C Virus, RNA-dependent RNA polymerase.

1. Introduction

Hepatitis C virus (HCV) infection, the major cause of liver disease worldwide, is a global health problem that affects more than 170 million people and can progress to cirrhosis, end-stage liver disease, hepatocellular carcinoma and death.[1,2]

In 2014, three drugs, leading to high HCV infection cure rates, were approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA), including the nucleotide NS5B polymerase inhibitor sofosbuvir,[3,4] the protease inhibitor simeprevir[5,6] and the NS5A polymerase inhibitor daclatasvir.[7] They can be used in combination with pegylated interferon α (IFNα) and ribavirin, or as part of sofosbuvir-based IFN-free combinations. In 2015, two additional combination, including sofosbuvir/ledipasvir and ombitasvir/paritaprevir/ritonavir plus dasabuvir were approved in the US and Europe, also yielding high cure rates. However, these new treatment regimens are extremely costly and may select multidrug resistance in patients that may lead to therapy failure. Low-income countries, where therapeutic needs are high, are unlikely to benefit from these treatments in the short- to mid-term. Therefore, the need for new direct acting antivirals (DAAs) with unique mechanisms of action is still highly desired.[8-12]

In this context, the NS5B RNA-dependent RNA polymerase (RdRp), which is absent in humans and essentially required for viral replication, is a particularly attractive target as witnessed by the increasing number of rationally designed inhibitors of this enzyme.[13-15] At the structural level, this viral polymerase is shaped as a 'right hand' with the characteristic thumb, finger and palm domains.[16-19] In addition to the catalytic site, at least four allosteric binding sites have been identified so far, including 'thumb' pockets I and II and 'palm' pockets I and II/III.[20-24] Compounds that bind to allosteric sites induce conformational changes within the enzyme, leading to loss of its catalytic activity and ultimately to a replication end of the virus.

Recently, we showed that naturally occurring aurones (2-benzylidenebenzofuran-3(2*H*)-ones, Figure 1) – small molecules belonging to the flavonoid family – have potent anti-HCV properties *in vitro*.[25,26] The investigation of aurones as potential anti-HCV drugs was motivated by the very low toxicity of naturally occurring aurones and their derivatives. In particular, a 4,6-dihydroxyaurone derivative was previously evaluated through *in vivo* oral and dermal toxicity assays and no toxicity was observed even at high concentrations.[27] The naturally occurring aureusidin (3',4,4',6-tetrahydroxyaurone, **1**, Figure 1), was first identified as an inhibitor of HCV RdRp with an IC_{50} of 5.2 µM in an enzyme assay. Further, structure-activity relationship studies allowed us to partially identify key features that are significant for the HCV RdRp inhibitory activity of this compound. Moreover, the binding site of this family of compounds was located at the thumb pocket I using site-directed mutagenesis experiments and molecular modeling.[25]

The structural analysis revealed that the targeted pocket – thumb pocket I – exhibits an unexplored hydrophobic zone and may accommodate larger structures. Hence, we assumed that pseudodimeric aurones would better fit in the thumb pocket I than the monomeric derivatives, leading to higher inhibition activity. Herein, we report the design, synthesis and activity of original pseudodimeric aurones as novel scaffolds for inhibition of NS5B polymerase. The biological evaluation, site directed mutagenesis and molecular modelling rationalization are reported.

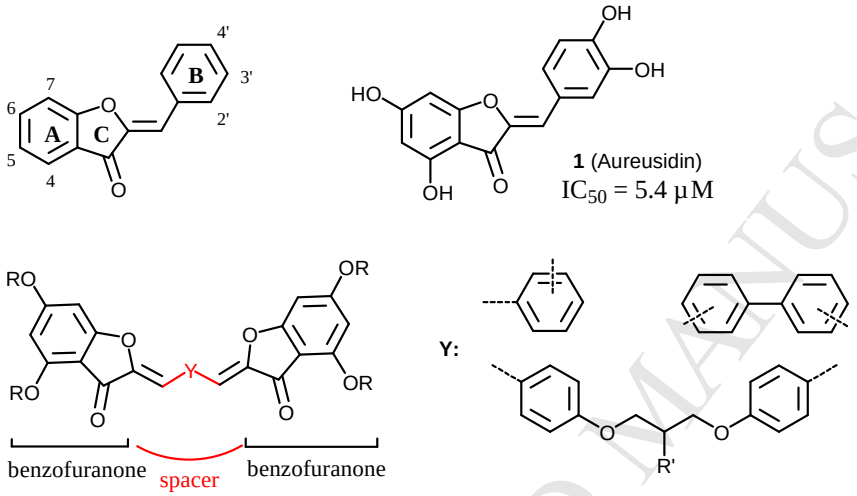


Figure 1. General structure of the aurone backbone (top left), structure and activity of the naturally occurring aureusidin **1** and structures of aurone derivatives investigated in this study (the dashed line indicate the site of linkage).

2. Results and discussion

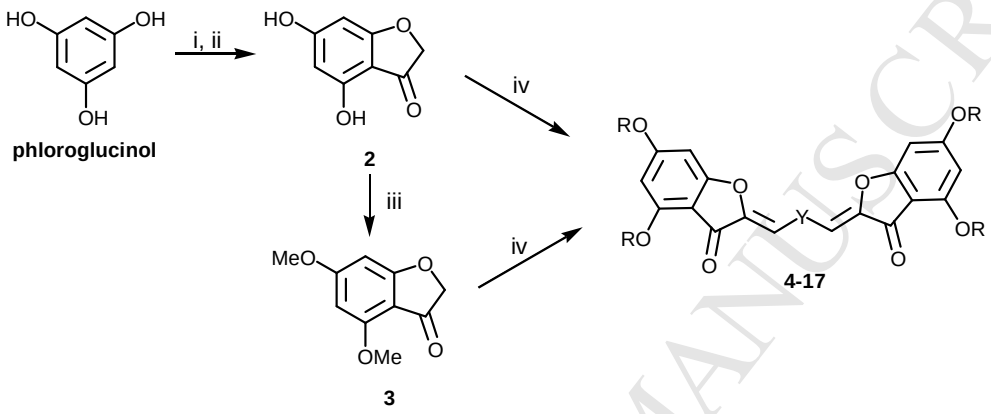
2.1. Chemistry

In this study, we focused our efforts on two novel types of structures which differ from each other by their rigidity profile. In the first, the two benzofuranone moieties are separated by a phenyl or a biphenyl group whereas in the second one, a flexible alkyl chain was inserted between the benzofuranones as shown in figure 1.

In both cases, targeted pseudodimeric aurones were obtained following our previously reported general and convenient synthetic pathway involving aldol condensation between 4,6-dihydroxy or 4,6-dimethoxybenzofuran-3(2*H*)-one (**2** or **3** respectively) and a bis-arylaldehyde under basic

conditions (Scheme 1).[28,29]. It should be highlighted that the condensation of unprotected **2** with bis-arylaldehyde provided the desired compounds in satisfactory yield and no side products formation was observed.

The 4,6-dihydroxybenzofuran-3(2*H*)-one **2** was obtained in two steps (84%) starting from phloroglucinol and was methylated in the presence of dimethyl sulfate to give 4,6-dimethoxybenzofuran-3(2*H*)-one **3** with 97% yield.

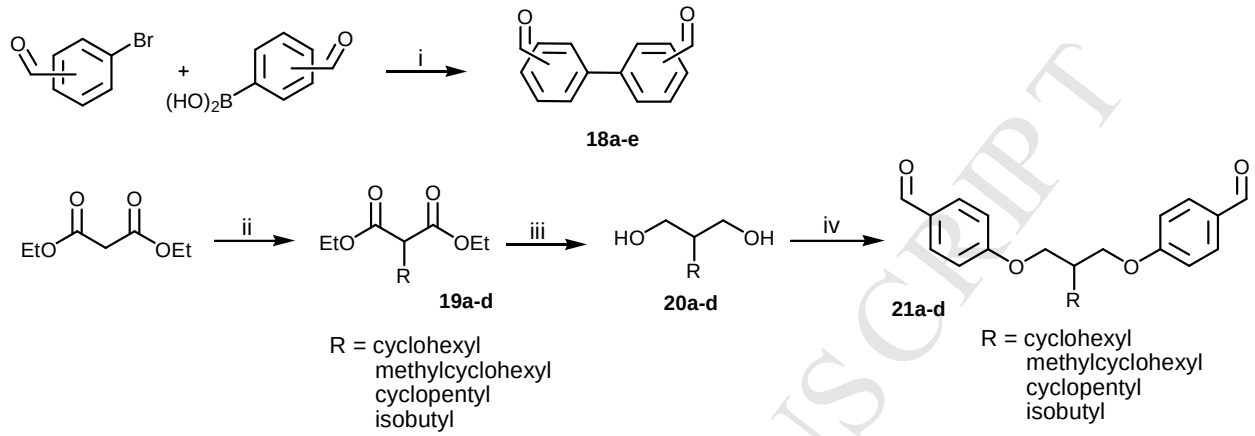


Scheme 1. General pathway for the synthesis of pseudodimeric aurones. Reagents and conditions: (i) $ClCH_2CN$, HCl, $ZnCl_2$, Et_2O, 0°C; (ii) H_2O, 100°C, 5 h, 84% (for 2 steps); (iii) Me_2SO_4, K_2CO_3, 1,2-dimethoxyethane, reflux, 5 h, 97%; (iv) bis-arylaldehyde, KOH 50% in H_2O, EtOH or MeOH, 2-15 h, 34-98%.

Among the different bisaldehydes used, the benzenedicarboxaldehydes were commercially available and the biphenyl ones **18a-e** (Scheme 2) were synthesized in one step by Suzuki-coupling reaction (61-93%) between a bromobenzaldehyde and a formylphenylboronic acid in the presence of tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) and potassium carbonate as a base (Scheme 2).

The other bisaldehydes were synthesized in one to three steps (Scheme 2). First, diethyl malonate was alkylated with a bromoalkane and sodium ethoxide in the presence of potassium iodide. The resulted diesters **19a,b** and commercial ones **19c,d** were reduced with lithium aluminium hydride in tetrahydrofuran to give the corresponding 1,3-diols **20a-d**. Finally, a Mitsunobu reaction between 4-hydroxybenzaldehyde and the synthesized 1,3-diols **20a-d** provided the desired bisaldehydes **21a-d** with 33 to 73% yield. An analogue without alkyl chain **21e** (R = H) was obtained starting from commercially available 1,3-dibromopropane and 4-

hydroxybenzaldehyde in basic media following the procedure described by W. Jiang *and al*.[30] The full series of the synthesized pseudodimeric aurones is shown in table 1.



Scheme 2. Synthesis of bisaldehydes. Reagents and conditions: (i) $Pd(PPh_3)_4$, K_2CO_3, 1,2-dimethoxyethane, reflux, 6 h, 61-93%; (ii) EtONa, RBr, KI, EtOH, reflux, 48 h; (iii) $LiAlH_4$, THF, 0°C then reflux for 48 h, 46-89%; (iv) 4-hydroxybenzaldehyde, PPh_3, DIAD, THF, rt, 12 h, 33-73%.

2.2. Assessment of HCV RdRp inhibition and structure-activity relationship.

An enzyme assay was used to assess the inhibition of the polymerase activity of a purified RdRp (NS5B protein), deleted of its 21 C-terminal amino acids in order to ensure solubility (HCV-NS5BΔ21). The J4 genotype 1b reference strand was used. This assay measures the amount of double-stranded RNA synthesized in the presence of HCV-NS5BΔ21, a homopolymeric RNA template and ATP, as previously described.[31] Initial screening was performed at 20 µM concentration of the pseudodimeric aurone followed by the measurement of IC_{50} for those that showed the highest activity at 20 µM. Prior to that, cytotoxicity of this new family was evaluated and the model compounds **4** and **6** have been shown non-toxic even at 100 µM.

Our previous SAR studies on monomeric aurones pointed out some pharmacophoric features that were crucial for the inhibitory activity of aurones on HCV RdRp: (a) a hydroxyl group at position 4 or hydroxyl groups at positions 4 and 6, (b) a 2',4'- or 3',4'-dihydroxylated B-ring or a hydrophobic and bulky substituent or alternative core at the B-ring.[25] The importance of the

hydroxylation pattern at the A-ring of benzofuranone found among monomeric aurones was also checked with these new pseudodimeric aurones: when replacing hydroxyl by methoxy (compound **6**) or by hydrogen (compound **17**), inhibitory activity was dramatically dropped.
In this study, we decided to explore the shape and the size of the targeted thumb pocket I while optimizing the polar/apolar interactions with the allosteric binding site. In order to probe the depth and the extent of the pocket, we first considered synthesizing pseudodimeric aurones with one or two aromatic phenyl groups between the benzofuranone moieties (**4**-**11**, Table 1). Because of the rigidity of these compounds, different positions for the substituents were studied. The biological evaluation of these 7 compounds revealed the importance of (a) the shape of the molecule and (b) the distance between the benzofuranone cores. The best results were obtained when the spacer between benzofuranone moieties is a simple phenyl ring with a *para*, *para* linkage (compound **4**, IC_{50} = 1.3 µM).
Then, we investigated more flexible structures in which the two benzofuranone cores were linked *via* an alkyl chain included in the spacer (**12**-**17**, Table 1). We have chosen compound **12** as a derivative to begin with because the length of the alkyl chain could allow, as stated by molecular modeling studies (cf. supporting information), new π-π interactions between the aromatic linker and the second aurone moiety with histidines 428 and 34, respectively. Given the presence of an unoccupied hydrophobic sub-pocket composed by leucine 392, alanine 395, isoleucine 424 and leucine 425 residues, analogues of compound **12** bearing an alkyl hydrophobic chain in the middle of the spacer were synthesized (compounds **13**-**16**). The biological evaluation of these new compounds showed that all substituents were globally well tolerated. Nevertheless, no significant gain in terms of inhibitory potency was observed (IC_{50} = 1.5 − 2.0 µM).

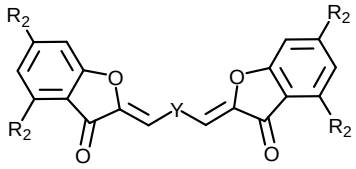
2.3. Site directed mutagenesis studies and molecular modelling

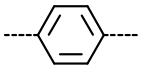
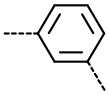
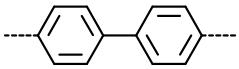
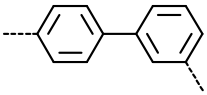
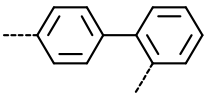
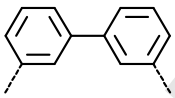
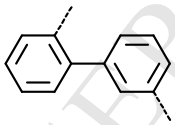
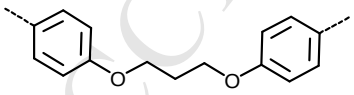
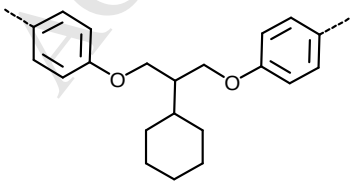
Intrigued by the fact that the addition of the hydrophobic chain did not have a positive impact on RdRp inhibitory activity, we conducted site directed mutagenesis studies in order to check the binding site localization. Taking compounds **4** and **12**-**16** as representatives, mutagenesis results indicated that thumb pocket I is not the target (Figure 2). Indeed, the inhibitory activity of compounds **4** and **12-16** on the HCV-NS5BΔ21 carrying a mutation at the thumb site I (P495L) that is known to confer resistance to reported inhibitors targeting thumb site I[24,32] was not affected by the amino acid substitution on NS5B (for compound **4**, IC_{50} = 1.3 µM for HCV-

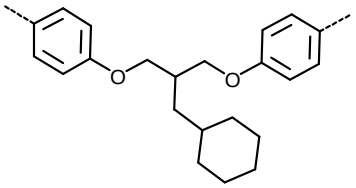
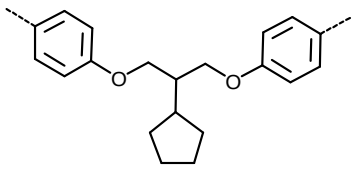
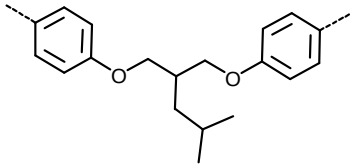
NS5BΔ21-Wt versus IC_{50} = 1.5 μM for HCV-NS5BΔ21-P495L). The latter results reveal that thumb I is probably not the site of binding. Hence, we mapped the other allosteric binding sites (thumb II, palm I and palm II/III) and selected aurone **4** for doing it. Four mutants of HCV-NS5BΔ21 were used in this study P495L, M423T, H95Q and C316Y, corresponding respectively to mutations on thumb pocket I, thumb pocket II, palm pocket I and palm pocket II/III.[24,32] The dose-response curves obtained are presented in figure 3. The amino acid substitution H95Q reduced susceptibility of compound **4** which indirectly suggests that this compound binds to (or close to) palm pocket I. Thus, molecular docking studies of compound **4** were conducted on the corresponding region of the protein, suggesting a possible favorable interaction (Figure 3A). One of the benzofuranone moieties was shown to be mainly stabilized by hydrophobic interactions in an area surrounded by apolar residues, normally occupied by palm site I inhibitors, such as Pro197, Met414, Tyr415, Ile447 and Tyr448 (Figure 3A). While the aromatic linker was further stabilized by van der Waals contacts with Phe193 and Tyr448, the second benzofuranone moiety, in contrast to the first one, occupied a more polar zone in which hydroxyl substituents may be involved in hydrogen bonds with residues such as Asn291, or with possible water molecules acting as bridges between the ligand and the protein.[33] By following the same strategy, it was also possible to determine the interaction mode of compounds **12** and **14** to this specific region. While the position of one benzofuranone core was conserved in both cases (Figures 3B-C), the presence of a longer flexible spacer determined, for **12**, the shift of the second benzofuranone moiety to a novel wide region with Ser407, Gly410, and Ile405 as possible anchoring points (Figure 3B). Concerning compound **14**, characterized by a cycloalkyl chain in the middle of the spacer, the second benzofuranone ring is now involved in polar contacts with the flexible Arg158 and Arg386 residues (Figure 3C). Indeed, the presence of a long spacer in **12** and **14** determined the possibility of more contacts with the protein *via* the two benzofuranone extremes. Nevertheless, these moieties, being exposed to wide regions in which dynamics and solvent effects may take place, could determine entropic penalties that may explain affinities comparable to **4** (Table 1).

Table 1

Structures and inhibition activity of pseudodimeric aurones. The dashed bounds indicate the sites of attachment to the methylenebenzofuranone moieties.



Compd	Y group	R_2	Yield[a] (%)	Inhibition (20 μM, %)	IC_{50} (μM)
	Dasabuvir (ABT-333)			95.7 ± 0.3	0.60 ± 0.03
4		OH	83	96.0 ± 0.2	1.3 ± 0.3
5		OH	69	97.0 ± 0.3	3.6 ± 0.8
6		OMe	55	15 ± 11	n.d.
7		OH	37	77 ± 4	n.d.
8		OH	72	99.2 ± 0.2	3.1 ± 0.5
9		OH	89	98.5 ± 0.2	6 ± 1
10		OH	98	95 ± 1	n.d.
11		OH	85	91.0 ± 0.1	n.d.
12		OH	71	96.4 ± 0.3	1.50 ± 0.07
13		OH	34	95.3 ± 0.4	2.0 ± 0.2

14		OH	78	94.9 ± 0.6	1.8 ± 0.2
15		OH	56	96.9 ± 0.1	1.90 ± 0.00
16		OH	57	97.0 ± 0.1	1.9 ± 0.1
17		H	59	0 ± 5	n.d.

n.d.: not determined

[a]Yields are given for the last step of the synthesis (condensation of bis-aldehyde and benzofuranone).

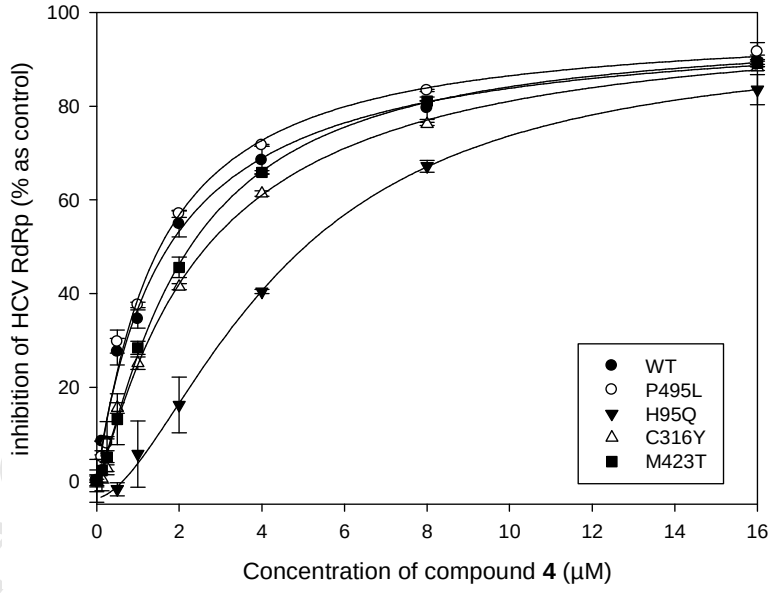


Figure 2. Inhibition curve of HCV-NS5BΔ21-WT r(wild-type), P495L, H95Q, C316Y and M423T mutants obtained with compound **4**.

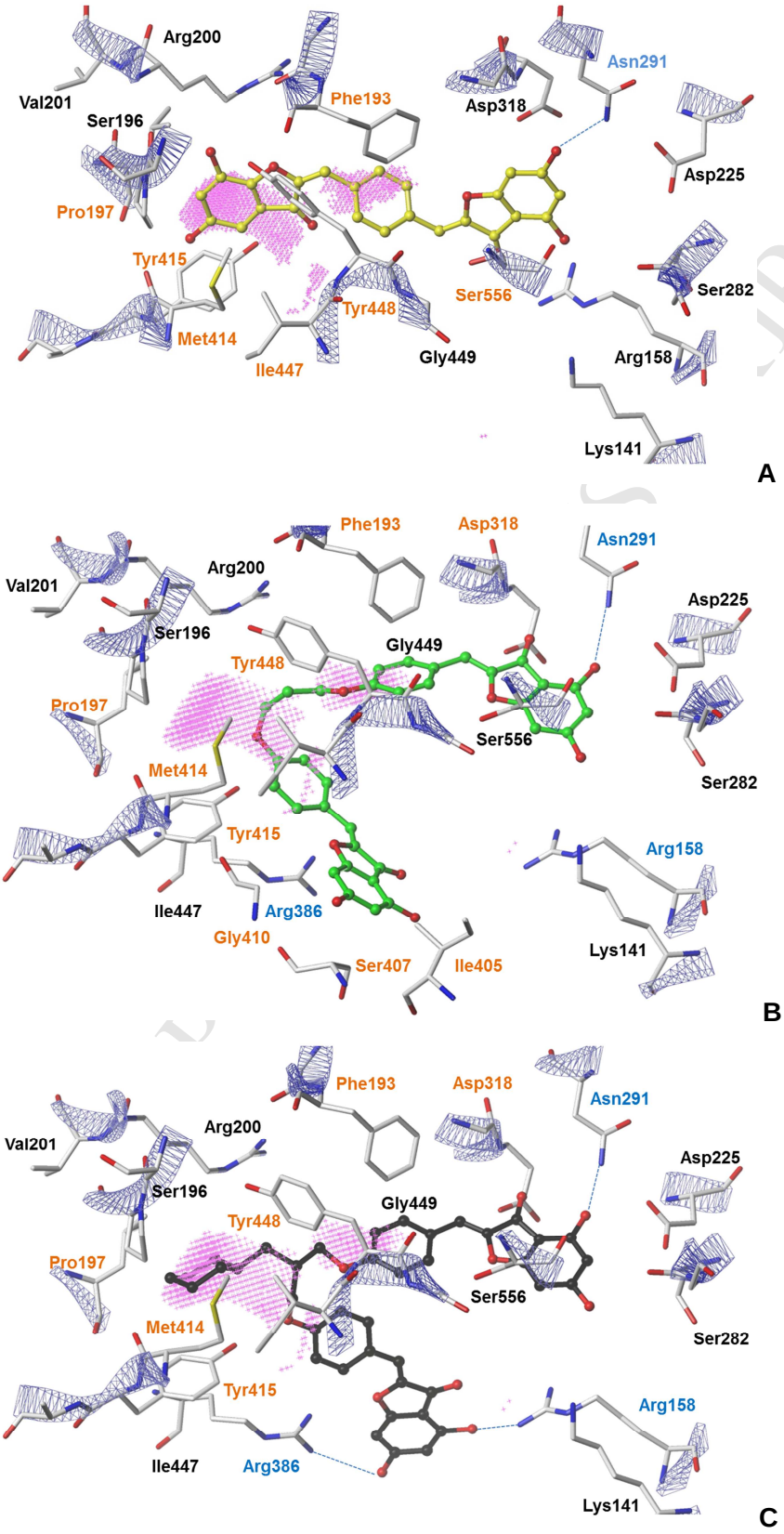

Arg200
Val201
Ser196
Phe193
Asp318
Asn291
Asp225
Pro197
Tyr415
Ser556
Ser282
Tyr448
Met414
Ile447
Gly449
Arg158
Lys141
A
Phe193
Asp318
Asn291
Arg200
Val201
Asp225
Ser196
Gly449
Tyr448
Pro197
Ser556
Met414
Ser282
Tyr415
Arg158
Ile447
Arg386
Gly410
Ser407
Ile405
Lys141
B
Phe193
Asp318
Asn291
Arg200
Val201
Asp225
Ser196
Gly449
Tyr448
Pro197
Ser556
Met414
Ser282
Tyr415
Arg158
Ile447
Arg386
Lys141
C

Figure 3. Best ranked poses of compounds **4** (A, yellow ball&sticks, ChemPLP score 62.6), **12** (B, green ball&sticks, ChemPLP score 77.7) and **14** (C, black ball&sticks, ChemPLP score 93.8) in RdRp palm pocket I represented as dark blue ribbons (PDB code 3SKE). RdRp residues involved in complex stabilization are highlighted in sticks, colored and labeled whereas hydrophobic fitting points calculated with the MLP are reported as magenta dots.

2.4. Assessment of anti-HCV activity in the genotype 2a/2a J6/JFH1 infectious model

The anti-HCV activity of compounds **4**, **12**, **13**, **14**, **16** and **17** was assessed in the J6/JFH1 (genotype 2a/2a) infectious model using ABT-333 (Dasabuvir) as reference and compound **17** as negative control (Table 2). Indeed, compound **17** that showed no inhibition of the polymerase activity (Table 1) also does not have anti-HCV properties. Compounds **4**, **12**, **13**, **14** and **16** showed here a low to moderate anti-HCV activity, the most active compound being only 4.5 less active than the Dasabuvir currently in the market. The fact that inhibitory effects of these 5 compounds are very close (1 to 2 µM, Table 1) whereas their anti-HCV activity are disparate could be explained either by a difference in the pharmacokinetic behavior of the compounds – it should be noted that this behavior was observed in many cases[34-36] – or by a genotype specificity: whereas enzymatic assays are carried out on NS5B genotype 1, anti-HCV activity involved the genotype 2a/2a J6/JFH1 infectious model. Overall, the anti-HCV activity of the tested compounds is in relative good agreement with their respective HCV RdRp inhibitory potency.

Table 2

Anti-HCV activity in the genotype 2a/2a J6/JFH1 infectious model of compounds **4**, **12**, **13**, **14**, **16**, **17** and **ABT-133** (Dasabuvir).

Compound	J6/JFH1 EC_{50} (µM)
Dasabuvir (ABT-333)	9.1 ± 0.2
4	202 ± 16
12	214 ± 21
13	42 ± 1

14	90 ± 19
16	76 ± 21
17	> 400

3. Conclusion

Based on the activity of naturally occurring aurones as inhibitors of NS5B RNA-dependent RNA polymerase (RdRp) and on the binding profile toward one of the allosteric sites, we investigated a series of pseudodimeric aurones using structural and ligand-based drug design. Herein, we reported the capacity of novel pseudodimeric aurones to inhibit HCV RdRp with IC_{50} values in the one digit micromolar order. Initially, designed to act at the thumb pocket I as the monomeric derivatives does, it was found that pseudodimeric aurones seem to target preferentially the palm pocket I as suggested by site-directed mutagenesis and molecular docking studies. The last assumption should be confirmed by co-crystallization studies. The anti-HCV activity in infectious model (genotype 2a/2a J6/JFH1) was also assessed for these new pseudodimeric aurones and results are globally correlated to their respective HCV RdRp inhibitory potency.

4. Experimental section

4.1. Chemistry

Commercially available reagents and solvents were used without further purification. Reactions were monitored by thin-layer chromatography (plates coated with silica gel 60 F254 from Merck). Silica gel 60 (70-230 mesh from Macherey-Nagel) was used for flash chromatography. Melting points were measured on a Fisher micromelting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded at room temperature in deuterated solvents on a Brüker AC-400 instrument (400 MHz). Chemical shifts (δ) are reported in parts per million (ppm) relative to TMS as internal standard or relative to the solvent [^{1}H: δ(DMSO) = 2.50 ppm, δ($CDCl_3$) = 7.24 ppm; ^{13}C: δ(DMSO) = 39.51 ppm, δ($CDCl_3$) = 77.23 ppm]. Electrospray

ionization ESI mass spectra were acquired by the Analytical Department of Grenoble University on an Esquire 300 Plus Bruker Daltonis instrument with a nanospray inlet. Accurate mass measurements (HRMS) were carried out on a TOF spectrometer, realized by the mass spectrometry analysis facilities of Orleans University (France).

4.1.1. *4,6-dihydroxybenzofuran-3(2H)-one (**2**)*

To a solution of phloroglucinol (15.0 g, 119 mmol) in diethyl ether (250 mL) at 0°C were added chloroacetonitrile (7.5 mL, 119 mmol) and dried zinc chloride (1.62 g, 11.9 mmol). The mixture was allowed to react with gaseous hydrochloric acid at 0°C for 1 h. The resulting suspension was stirred at 0°C for 1.5 h, and was allowed to react again with gaseous HCl for 30 min. The precipitate was filtered, washed with ether, dried and suspended in water (200 mL). The solution was refluxed for 5 h. After cooling, the crystals were filtered, washed with cold water and dried to afford **2** (16.6 g, 100 mmol, 84 %) as orange crystals, which were analytically pure and used without further purification. m.p. 257-259°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 4.55 (s, 2H), 5.91 (s, 2H), 10.58 (s, 1H), 10.61 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 74.8 (CH_2), 90.1 (CH), 96.1 (CH), 102.6 (C), 157.4 (C), 167.5 (C), 175.6 (C), 193.9 (C).

*4.1.2. 4,6-dimethoxybenzofuran-3(2H)-one (**3**)*

To a solution of 4,6-dihydroxybenzofuran-3(2*H*)-one **2** (2.00 g, 12.0 mmol) in 1,2-dimethoxyethane (50 mL) were added potassium carbonate (3.70 g, 26.5 mmol) and dimethyl sulfate (2.50 mL, 26.5 mmol), and the mixture was refluxed for 5 h. After cooling, solvents were removed under reduced pressure, water (40 mL) was added, and the mixture was extracted with CH_2Cl_2. The combined organic layers were washed with water then brine, dried over anhydrous $MgSO_4$, filtered off, and the filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (eluent cyclohexane/EtOAc 40:60) to afford **3** (2.25 g, 11.6 mmol, 97 %) as an orange solid. m.p. 136°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 3.86 (s, 3H), 3.90 (s, 3H), 4.59 (s, 2H), 6.01 (d, J = 1.8 Hz, 1H), 6.15 (d, J = 1.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 56.0 (CH_3), 56.1 (CH_3), 75.5 (C), 88.9 (CH), 93.0 (CH), 104.8 (C), 158.8 (C), 169.8 (C), 177.1 (C), 195.0 (C).

4.1.3. General procedure A for the synthesis of compounds (4−17)

To a solution of **2** in ethanol (7 mL/mmol) were added an aqueous solution of potassium hydroxide (50%, 5 mL/mmol) and the corresponding benzaldehyde derivative (0.45 to 0.5 equiv.). The solution was refluxed until TLC showed complete disappearance of the starting

materials (2 to 5 hours). After cooling, ethanol was removed under reduced pressure, then the residue was diluted into distilled water (50 mL/mmol) and an aqueous solution of hydrochloric acid (10%) was added to adjust the pH to 2-3. The mixture was then extracted with ethyl acetate or dichloromethane. The combined organic layers were washed with water and brine, dried over $MgSO_4$, filtered off and concentrated under reduced pressure to afford the corresponding (*Z*)-2-benzylidenebenzofuran-3(2*H*)-one derivative.

*4.1.3.1. (2Z,2'Z)-2,2'-(1,4-phenylenebis(methan-1-yl-1-ylidene))bis(4,6-dihydroxybenzofuran-3(2H)-one) (**4**)*

The crude product was prepared according to general procedure **A** starting from 4,6-dihydroxybenzofuran-3(2*H*)-one **2** (100 mg, 0.60 mmol, 1.0 equiv) and terephthalaldehyde (40 mg, 0.30 mmol, 0.5 equiv.). The pure product (108 mg, 0.25 mmol, 83%) was obtained as apowder. m.p. > 410°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 6.20 (s, 2H), 6.30 (s, 2H), 6.62 (s, 2H), 7.97 (m, 4H), 11.14 (m, 4xOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 90.7 (2xCH), 97.9 (2xC), 102.3 (2xCH), 107.2 (2xCH), 130.9 (4xCH), 133.0 (2xC), 148.3 (2xC), 158.5 (2xC), 167.6 (4xC), 178.8 (2xC); LRMS (ESI−) *m/z* (%) 429 (100) $[M-H]^-$; HRMS (ESI+) *m/z* calc. for $C_{24}H_{15}O_8$ 431.0761, found 431.0762.

*4.1.3.2. (2Z,2'Z)-2,2'-(1,3-phenylenebis(methan-1-yl-1-ylidene))bis(4,6-hydroxybenzofuran-3(2H)-one) (**5**)*

The crude product was prepared according to general procedure **A** starting from 4,6-dihydroxybenzofuran-3(2*H*)-one **2** (100 mg, 0.60 mmol, 1.0 equiv.) and isophthalaldehyde (40,2 mg, 0.60 mmol, 0.5 equiv.). The pure product (90 mg, 0.20 mmol, 69%) was obtained as an orange powder. m.p. > 410°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 6.14 (s, 2H), 6.28 (s, 2H), 6.63 (s, 2H), 7.58 (t, J = 7.6 Hz, 1H), 7.91 (d, J = 6.4 Hz, 2H), 8.35 (s, 1H), 11.02 (m, 2xOH), 11.06 (m, 2xOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 90.6 (2xCH), 97.9 (2xC), 102.3 (2xCH), 107.4 (2xCH), 129.5 (CH), 131.0 (2xCH), 132.4 (CH), 132.9 (2xC), 148.1 (2xC), 158.5 (2xC), 167.6 (2xC), 167.8 (2xC), 178.9 (2xC); LRMS (ESI−) *m/z* (%) 429 (100) $[M-H]^-$; HRMS (ESI+) *m/z* calc. for $C_{24}H_{15}O_8$ 431.0761, found 431.0763.

*4.1.3.3. (2Z,2'Z)-2,2'-(1,3-phenylenebis(methan-1-yl-1-ylidene))bis(4,6-dimethoxybenzofuran-3(2H)-one) (**6**)*

To a solution of 4,6-dimethoxybenzofuran-3(2*H*)-one **3** (100 mg, 0.51 mmol, 1.0 equiv.) in 7.5 mL of methanol were added an aqueous solution of potassium hydroxide (50%, 0.75 mL) and

isophthalaldehyde (35 mg, 0.25 mmol, 0.5 equiv.). After stirring for 2 h at rt, the reaction mixture was concentrated under vacuum. The residue was diluted in water (25 mL) and the mixture was extracted with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$, filtered off and concentrated under reduced pressure. The product (70 mg, 0.14 mmol, 55%) was obtained as a pale orange powder. m.p. > 270°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 3.91 (s, 6H), 3.93 (s, 6H), 6.38 (d, *J* = 1,6 Hz, 2H), 6.74 (d, *J* = 2.0 Hz, 2H), 6.75 (s, 2H), 7.60 (t, *J* = 8.0 Hz, 1H), 7.98 (d, *J* = 1.6 Hz, 1H), 8.00 (d, *J* = 1.6 Hz, 1H), 8.40 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 56.2 (2xCH_3), 56.5 (2xCH_3), 90.0 (2xCH), 94.4 (2xC), 103.9 (2xCH), 108.7 (2xCH), 129.6 (CH), 131.5 (2xCH), 132.8 (2xC), 133.0 (CH), 147.7 (2xC), 159.0 (2xC), 168.3 (2xC), 169.1 (2xC), 178.9 (2xC); LRMS (ESI+) *m/z* (%) 487 (100) $[M+H]^+$; HRMS (ESI+) *m/z* calc. for $C_{28}H_{23}O_8$ 487.1387, found 487.1385.

*4.1.3.4. (2Z,2'Z)-2,2'-(biphenyl-4-4'-diylbis(methan-1-yl-1-ylidene))bis(4,6-dihydroxybenzofuran-3(2H)-one) (**7**)*

The crude product was prepared according to general procedure **A** starting from 4,6-dihydroxybenzofuran-3(2*H*)-one **2** (100 mg, 0.60 mmol, 1.0 equiv.) and [1,1'-Biphenyl]-4,4'-dicarboxaldehyde **18a** (63 mg, 0.30 mmol, 0.5 equiv.). The pure product (56 mg, 0.11 mmol, 37%) was obtained as a yellow powder. m.p. > 410°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 6.16 (s, 2H), 6.29 (s, 2H), 6.67 (s, 2H), 7.87 (d, *J* = 8,0 Hz , 4H), 8.01 (d, *J* = 8,0 Hz , 4H), 11.08 (s, 2xOH), 11.10 (s, 2xOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 90.7 (2xCH), 97.9 (2xC), 102.4 (CH), 103.4 (CH), 107.6 (2xCH), 127.0 (4xCH), 128.2 (C), 128.9 (C), 131.3 (4xCH), 132.0 (2xC), 139.5 (2xC), 148.0 (2xC), 158.5 (2xC), 167.6 (2xC), 178.9 (2xC); LRMS (ESI−) *m/z* (%) 505 (100) $[M-H]^-$; HRMS (ESI+) *m/z* calc. for $C_{30}H_{19}O_8$ 507.1074, found 507.1063.

*4.1.3.5. (2Z,2'Z)-2,2'-(biphenyl-3-4'-diylbis(methan-1-yl-1-ylidene))bis(4,6-dihydroxybenzofuran-3(2H)-one) (**8**)*

The crude product was prepared according to general procedure **A** starting from 4,6-dihydroxybenzofuran-3(2*H*)-one **2** (100 mg, 0.60 mmol, 1.0 equiv.) and [1,1'-Biphenyl]-3,4'-dicarboxaldehyde **18b** (63 mg, 0.30 mmol, 0.5 equiv.). The pure product (110 mg, 0.21 mmol, 72%) was obtained as a grey powder. m.p. > 410°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 6.16 (s, 2H), 6.29 (s, 2H), 6.66 (s, 1H), 6.71 (s, 1H), 7.56-7.63 (m, 1H), 7.76 (d, *J* = 7.2 Hz, 1H), 7.84 (d, *J* = 8.2 Hz, 2H), 7.93-8.04 (m, 3H), 8.17 (s, 1H), 10.98-11.34 (m, 4xOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 90.6 (CH), 90.7 (CH), 97.8 (2xC), 102.4 (CH), 102.5 (CH), 107.6 (CH), 107.9 (CH), 127.2 (2xCH), 129.1 (2xCH), 129.7 (2xCH), 131.3 (2xCH), 131.9 (C), 133.2 (C), 139.8

(C), 139.9 (C), 148.0 (C), 148.1 (C), 158.5 (2xC), 167.5 (C), 167.6 (C), 167.7 (C), 167.8 (C), 178.9 (C), 179.0 (C); LRMS (ESI−) *m/z* (%) 505 (100) $[M-H]^-$; HRMS (ESI+) *m/z* calc. for $C_{30}H_{19}O_8$ 507.1074, found 507.1073.

*4.1.3.6. (2Z,2'Z)-2,2'-(biphenyl-2-4'-diylbis(methan-1-yl-1-ylidene))bis(4,6-dihydroxybenzofuran-3(2H)-one) (**9**)*

The crude product was prepared according to general procedure **A** starting from 4,6-dihydroxybenzofuran-3(2*H*)-one **2** (100 mg, 0.60 mmol, 1.0 equiv.) and [1,1'-Biphenyl]-2,4'-dicarboxaldehyde **18c** (63 mg, 0.30 mmol, 0.5 equiv.). The pure product (135 mg, 0.26 mmol, 89%) was obtained as a grey powder. m.p. > 410°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 6.14 (s, 1H), 6,15 (s, 1H), 6.25 (s, 1H), 6.28 (s, 1H), 6.41 (s, 1H), 6.70 (s, 1H), 7.36-7.61 (m, 5H), 8.01 (d, *J* = 6.0 Hz, 2H), 8.19-8.29 (m, 1H), 10.92-11.25 (m, 4xOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 90.5 (CH), 90.6 (CH), 97.8 (2xC), 102.3 (CH), 102.4 (CH), 105.5 (CH), 107.4 (CH), 128.1 (CH), 129.1 (CH), 129.5 (CH), 130.2 (2xCH), 130.4 (CH), 130.6 (2xCH), 131.7 (2xC), 140.4 (C), 142.1 (C), 148.1 (2xC), 158.4 (C), 158.5 (C), 167.5 (2xC), 167.7 (2xC), 178.8 (C), 178.9 (C); LRMS (ESI−) *m/z* (%) 505 (100) $[M-H]^-$; HRMS (ESI+) *m/z* calc. for $C_{30}H_{19}O_8$ 507.1074, found 507.1073.

*4.1.3.7. (2Z,2'Z)-2,2'-(biphenyl-3-3'-diylbis(methan-1-yl-1-ylidene))bis(4,6-dihydroxybenzofuran-3(2H)-one) (**10**)*

The crude product was prepared according to general procedure **A** starting from 4,6-dihydroxybenzofuran-3(2*H*)-one **2** (100 mg, 0.60 mmol, 1.0 equiv.) and [1,1'-Biphenyl]-3,3'-dicarboxaldehyde **18d** (63 mg, 0.30 mmol, 0.5 equiv.). The pure product (150 mg, 0.31 mmol, 98%) was obtained as a grey powder. m.p. > 410°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 6.15 (s, 2H), 6.26 (s, 2H), 6.73 (s, 2H), 7.53-7.82 (m, 4H), 7.90-8.07 (m, 2H), 8.12-8.27 (m, 2H), 11.00 (s, 2xOH), 11.06 (s, 2xOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 90.7 (2xCH), 97.9 (2xC), 102.4 (2xCH), 107.9 (2xCH), 127.5 (2xCH), 129.3 (2xCH), 129.4 (CH), 129.5 (CH), 129.6 (2xCH), 133.2 (2xC), 140.2 (2xC), 148.1 (2xC), 158.5 (2xC), 167.7 (2xC), 167.8 (2xC), 179.1 (2xC); LRMS (ESI−) *m/z* (%) 505 (100) $[M-H]^-$; HRMS (ESI+) *m/z* calc. for $C_{30}H_{19}O_8$ 507.1074, found 507.1070.

*4.1.3.8. (2Z,2'Z)-2,2'-(biphenyl-2-3'-diylbis(methan-1-yl-1-ylidene))bis(4,6-dihydroxybenzofuran-3(2H)-one) (**11**)*

The crude product was prepared according to general procedure **A** starting from 4,6-dihydroxybenzofuran-3(2*H*)-one **2** (100 mg, 0.60 mmol, 1.0 equiv.) and [1,1'-Biphenyl]-2,3'-dicarboxaldehyde **18e** (63 mg, 0.30 mmol, 0.5 equiv.). The pure product (130 mg, 0.25 mmol, 85%) was obtained as a grey powder. m.p. > 410°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 6.05-6.20 (m, 3H), 6.25 (s, 1H), 6.40 (s, 1H), 6.68 (s, 1H), 7.32-7.69 (m, 5H), 7.45 (d, J = 6.8 Hz, 1H), 7.50 (dd, J = 7.4, 6.8 Hz, 1H), 7.61-7.56 (m, 2H), 7.83 (s, 1H), 8.03 (d, J = 6.4 Hz, 1H), 8.26 (d, J = 7.2 Hz, 1H), 10.92-11.16 (m, 4xOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 90.6 (2xCH), 97.8 (2xC), 102.3 (2xCH), 105.5 (CH), 107.6 (CH), 128.1 (CH), 129.0 (CH), 129.1 (CH), 129.5 (CH), 129.7 (CH), 130.2 (CH), 130.3 (CH), 130.4 (CH), 131.7 (C), 132.7 (C), 140.5 (C), 142.3 (C), 148.1 (2xC), 158.4 (C), 158.5 (C), 167.6 (2xC), 167.8 (2xC), 178.8 (C), 178.9 (C); LRMS (ESI−) *m/z* (%) 505 (100) $[M-H]^-$; HRMS (ESI+) *m/z* calc. for $C_{30}H_{19}O_8$ 507.1074, found 507.1077.

*4.1.3.9. (2Z,2'Z)-2,2'-[((propane-1,3-diylbis(oxy))bis(4,1-phenylene))bis(methan-1-yl-1-ylidene)]bis(4,6-dihydroxybenzofuran-3(2H)-one) (**12**)*

The crude product was prepared according to general procedure **A** starting from 4,6-dihydroxybenzofuran-3(2*H*)-one **2** (166 mg, 1.00 mmol, 1.0 equiv.) and 4,4'-[1,3-propanediyl(oxy)]bisbenzaldehyde[37] (142 mg, 0.50 mmol, 0.5 equiv.). After purification by column chromatography on silica gel (EtOAc/MeOH/AcOH 8:1.9:0.1), the pure product (412 mg, 0.71 mmol, 71%) was obtained as a yellow powder. m.p. 271-272°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.90 (bs, 4H), 7.86 (d, J = 8.4 Hz, 4H), 7.08 (d, J = 8.4 Hz, 4H), 6.60 (s, 2H), 6.22 (s, 2H), 6.08 (s, 2H), 4.22 (s, 4H), 2.28-2.18 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 28.5 (CH_2), 64.4 (2xCH_2), 90.5 (2xCH), 97.7 (2xCH), 102.7 (2xC), 108.4 (2xCH), 115.0 (4xCH), 125.0 (2xC), 132.5 (4xCH), 146.5 (2xC), 158.3 (2xC), 159.3 (2xC), 167.2 (2xC), 167.6 (2xC), 179.0 (2xC); LRMS (ESI+) *m/z* (%) 581 (100) $[M+H]^+$, 291 (23); HRMS (ESI+) *m/z* calc. for $C_{33}H_{25}O_{10}$ 581.1442, found 581.1442.

*4.1.3.10. (2Z,2'Z)-2,2'-[((2-cyclohexylpropane-1,3-diylbis(oxy))bis(4,1-phenylene))bis(methan-1-yl-1-ylidene)]bis(4,6-dihydroxybenzofuran-3(2H)-one) (**13**)*

The crude product was prepared according to general procedure **A** starting from 4,6-dihydroxybenzofuran-3(2*H*)-one **2** (150 mg, 0.90 mmol, 1.0 equiv.) and 4,4'-[1,3-(2-cyclohexyl)propanediyl(oxy)]bisbenzaldehyde **21a** (150 mg, 0.41 mmol, 0.45 equiv.). After purification by column chromatography on silica gel (CH_2Cl_2/MeOH 9:1 to 8:2), the pure product (92 mg, 0.14 mmol, 34%) was obtained as an orange powder. m.p. > 180°C (decomposition);

RMN ^{1}H (400 MHz, CD_3OD) δ ppm 1.15-1.43 (m, 5H), 1.68-1.93 (m, 6H), 2.09-2.18 (m, 1H), 4.19 (dd, *J* = 9.7, 6.3 Hz, 2H), 4.25 (dd, *J* = 9.7, 5.1 Hz, 2H), 6.04 (d, *J* = 1.7 Hz, 2H), 6.22 (d, *J* = 1.7 Hz, 2H), 6.64 (s, 2H), 7.02 (d, *J* = 8.9 Hz, 4H), 7.83 (d, *J* = 8.9 Hz, 4H); RMN ^{13}C (100 MHz, CD_3OD) δ ppm 27.8 (CH_2), 28.0 (2xCH_2), 31.9 (2xCH_2), 38.5 (CH), 45.3 (CH), 67.8 (2xCH_2), 91.9 (2xCH), 98.8 (2xCH), 104.8 (2xC), 111.8 (2xCH), 116.2 (4xCH), 126.7 (2xC), 134.1 (4xCH), 148.5 (2xC), 160.1 (2xC), 161.8 (2xC), 169.7 (2xC), 169.8 (2xC), 183.0 (2xC); LRMS (ESI+) *m/z* (%) 663 (100) $[M+H]^+$, 359 (80), 271 (27), 193 (19); HRMS (ESI+) *m/z* calc. for $C_{39}H_{35}O_{10}$ 663.2225, found 663.2216.

*4.1.3.11. (2Z,2'Z)-2,2'-[((2-(methylcyclohexyl)propane-1,3-diylbis(oxy))bis(4,1-phenylene))bis(methan-1-yl-1-ylidene)]bis(4,6-dihydroxybenzofuran-3(2H)-one) (**14**)*

The crude product was prepared according to general procedure **A** starting from 4,6-dihydroxybenzofuran-3(2*H*)-one **2** (190 mg, 1.16 mmol, 1.0 equiv.) and 4,4'-[1,3-(2-(methylcyclohexyl))propanediyl(oxy)]bisbenzaldehyde **21b** (200 mg, 0.53 mmol, 0.45 equiv.). After purification by recrystallization in methanol, the pure product (278 mg, 0.41 mmol, 78%) was obtained as a yellow powder. m.p. > 200°C (decomposition); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.80-0.97 (m, 2H), 1.04-1.48 (m, 6H), 1.53-1.78 (m, 5H), 2.29-2.41 (m, 1H), 4.09 (d, *J* = 4.3 Hz, 4H), 6.13 (s, 2H), 6.23 (s, 2H), 6.57 (s, 2H), 7.05 (d, *J* = 8.4 Hz, 4H), 7.83 (d, *J* = 8.4 Hz, 4H), 10.95 (s, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 25.7 (2xCH_2), 26.1 (CH_2), 33.0 (2xCH_2), 34.2 (CH), 34.9 (CH_2), 35.8 (CH), 68.1 (2xCH_2), 90.5 (2xCH), 97.7 (2xCH), 102.6 (2xC), 108.3 (2xCH), 115.1 (4xCH), 125.0 (2xC), 132.5 (4xCH), 146.5 (2xC), 158.3 (2xC), 159.4 (2xC), 167.2 (2xC), 167.6 (2xC), 179.0 (2xC); LRMS (ESI+) *m/z* (%) 677 (100) $[M+H]^+$, 339 (23) $[M+2H]^{2+}$; HRMS (ESI+) *m/z* calc. for $C_{40}H_{37}O_{10}$ 677.2381, found 677.2373.

*4.1.3.12. (2Z,2'Z)-2,2'-[((2-cyclopentylpropane-1,3-diylbis(oxy))bis(4,1-phenylene))bis(methan-1-yl-1-ylidene)]bis(4,6-dihydroxybenzofuran-3(2H)-one) (**15**)*

The crude product was prepared according to general procedure **A** starting from 4,6-dihydroxybenzofuran-3(2*H*)-one **2** (210 mg, 1.25 mmol, 1.0 equiv.) and 4,4'-[1,3-(2-cyclopentyl)propanediyl(oxy)]bisbenzaldehyde **21c** (200 mg, 0.57 mmol, 0.45 equiv.). After precipitation in CH_2Cl_2, the pure product (207 mg, 0.32 mmol, 56%) was obtained as a yellow powder. m.p. > 180°C (decomposition); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.21-1.38 (m, 2H), 1.44-1.69 (m, 4H), 1.76-1.89 (m, 2H), 1.95-2.13 (m, 2H), 4.12 (dd, *J* = 9.8, 5.9 Hz, 2H), 4.19 (dd, *J* = 9.8, 3.9 Hz, 2H), 6.13 (d, *J* = 1.7 Hz, 2H), 6.23 (d, *J* = 1.7 Hz, 2H), 6.57 (s, 2H), 7.05 (d, *J* = 8.8 Hz, 4H), 7.83 (d, *J* = 8.8 Hz, 4H), 10.91 (s, 2H), 10.93 (s, 2H); ^{13}C NMR (100 MHz,

DMSO-d_6) δ ppm 24.5 (2xCH$_2$), 30.3 (2xCH$_2$), 43.0 (CH), 67.2 (2xCH$_2$), 90.5 (2xCH), 97.7 (2xCH), 102.7 (2xC), 108.4 (2xCH), 115.1 (4xCH), 125.0 (2xC), 132.5 (4xCH), 146.5 (2xC), 158.3 (2xC), 159.5 (2xC), 167.2 (2xC), 167.6 (2xC), 179.0 (2xC); LRMS (ESI+) *m/z* (%) 649 (100) [*M*+H]$^+$, 325 (15) [*M*+2H]$^{2+}$; HRMS (ESI+) *m/z* calc. for $C_{38}H_{33}O_{10}$ 649.2068, found 649.2067.

*4.1.3.13. (2Z,2'Z)-2,2'-[((2-isobutylpropane-1,3-diylbis(oxy))bis(4,1-phenylene))bis(methan-1-yl-1-ylidene)]bis(4,6-dihydroxybenzofuran-3(2H)-one) (**16**)*

The crude product was prepared according to general procedure **A** starting from 4,6-dihydroxybenzofuran-3(2*H*)-one **2** (322 mg, 1.94 mmol, 1.0 equiv.) and 4,4'-[1,3-(2-isobutyl)propanediyl(oxy)]bisbenzaldehyde **21d** (300 mg, 0.88 mmol, 0.45 equiv.). After recrystallization in methanol, the pure product (319 mg, 0.50 mmol, 57%) was obtained as an orange powder. m.p. 184-185°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.91 (d, *J* = 6.5 Hz, 6H), 1.40 (dd, *J* = 7.0, 7.0 Hz, 2H), 1.68-1.80 (m, 1H), 2.27-2.37 (m, 1H), 4.10 (d, *J* = 5.2 Hz, 4H), 6.09 (d, *J* = 1.7 Hz, 2H), 6.22 (d, *J* = 1,7 Hz, 2H), 6.58 (s, 2H), 7.06 (d, *J* = 8.8 Hz, 4H), 7.84 (d, *J* = 8.8 Hz, 4H), 10.88 (s, 2H), 10.92 (s, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 22.7 (2xCH$_3$), 24.8 (CH), 35.7 (CH), 37.2 (CH$_2$), 68.0 (2xCH$_2$), 90.5 (2xCH), 97.7 (2xCH), 102.7 (2xC), 108.4 (2xCH), 115.1 (4xCH), 125.0 (2xC), 132.5 (4xCH), 146.5 (2xC), 158.2 (2xC), 159.4 (2xC), 167.2 (2xC), 167.6 (2xC), 179.0 (2xC); LRMS (ESI+) *m/z* (%) 637 (100) [*M*+H]$^+$, 319 (18) [*M*+2H]$^{2+}$; HRMS (ESI+) *m/z* calc. for $C_{37}H_{33}O_{10}$ 637.2068, found 637.2058.

*4.1.3.14. (2Z,2'Z)-2,2'-[((2-isobutylpropane-1,3-diylbis(oxy))bis(4,1-phenylene))bis(methan-1-yl-1-ylidene)]bis(benzofuran-3(2H)-one) (**17**)*

To a solution of benzofuran-3(2*H*)-one (130 mg, 0.97 mmol, 1.0 equiv.) and 4,4'-[1,3-(2-isobutyl)propanediyl(oxy)]bisbenzaldehyde **21d** (150 mg, 0.44 mmol, 0.45 equiv.) in 15 mL of CH_2Cl_2 was added 1.6 g of Al_2O_3. The reaction mixture was stirred overnight and was then filtered off. The filtrate was concentrated under vacuum and the crude product was recrystallized in methanol to afford **17** (149 mg, 0.26 mmol, 59%) as a yellow powder. m.p. 133-134°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.93 (d, *J* = 6.5 Hz, 6H), 1.42 (dd, *J* = 7.0, 7.0 Hz, 2H), 1.68-1.82 (m, 1H), 2.30-2.40 (m, 1H), 4.14 (d, *J* = 5.3 Hz, 4H), 6.94 (s, 2H), 7.11 (d, *J* = 8.8 Hz, 4H), 7.31 (m, 2H), 7.55 (d, *J* = 8.5 Hz, 2H), 7.74-7.83 (m, 4H), 7.96 (d, *J* = 8.8 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 22.7 (2xCH$_3$), 24.8 (CH), 35.7 (CH), 37.2 (CH$_2$), 68.0 (2xCH$_2$), 112.7 (2xCH), 113.1 (2xCH), 115.3 (4xCH), 121.1 (2xC), 123.8 (2xCH), 124.1 (2xCH), 124.5 (2xC), 133.4 (4xCH), 137.3 (2xCH), 145.1 (2xC), 160.2 (2xC), 165.1 (2xC), 183.2 (2xC);

LRMS (ESI+) *m/z* (%) 573 (100) $[M+H]^+$, 287 (8) $[M+2H]^{2+}$; HRMS (ESI+) *m/z* calc. for $C_{37}H_{33}O_6$ 573.2272, found 573.2270.

4.1.4. General procedure B for the synthesis of (formylphenyl)benzaldehydes (18a−e)

To a stirred solution of the corresponding commercially available formylphenyl boronic acid (1 equiv.) and bromobenzaldehyde (1 equiv.) in 1,2-dimethoxyethane (10 mL/mmol) were added $Pd(PPh_3)_4$ (0.1 equiv.) and a solution of K_2CO_3 (6 equiv.) in water (1mL/mmol). The reaction mixture was refluxed for 6 h and then concentrated under reduced pressure. The crude product was purified by column chromatography.

*4.1.4.1. [1,1'-Biphenyl]-4,4'-dicarboxaldehyde (**18a**)*

The crude product was prepared according to procedure **B** starting from 4-bromobenzaldehyde (370 mg, 2.00 mmol) and 4-formylphenylboronic acid (300 mg, 2.00 mmol). After purification by column chromatography on silica gel (toluene/EtOAc 100:0 to 92:8), the pure product (300 mg, 1.42 mmol, 71%) was obtained as a white powder. m.p. 144−146°C, lit. 146°C;[38] ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.86 (d, J = 8.5 Hz, 4H), 8.06 (d, J = 8.5 Hz, 4H), 10.14 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 128.2 (4×CH), 130.6 (4×CH), 136.2 (2×C), 145.7 (2×C), 191.9 (2×CH).

*4.1.4.2. [1,1'-Biphenyl]-3,4'-dicarboxaldehyde (**18b**)*

The crude product was prepared according to procedure **B** starting from 4-bromobenzaldehyde (370 mg, 2.00 mmol) and 3-formylphenylboronic acid (300 mg, 2.00 mmol). After purification by column chromatography on silica gel (toluene/EtOAc 100:0 to 94:6), the pure product (365 mg, 1.74 mmol, 87%) was obtained as a white powder. m.p. 82°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.67 (dd, J = 7.7, 7.6 Hz, 1H), 7.80 (d, J = 8.4 Hz, 2H), 7.89-7.95 (m, 2H), 7.99 (d, J = 8.4 Hz, 2H), 8.15 (s, 1H), 10.08 (s, 1H), 10.12 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 127.7 (2×CH), 128.2 (CH), 129.8 (CH), 129.9 (CH), 130.4 (2×CH), 133.2 (CH), 135.7 (C), 137.1 (C), 140.6 (C), 145.5 (C), 191.8 (2×CH).

*4.1.4.3. [1,1'-Biphenyl]-2,4'-dicarboxaldehyde (**18c**)*

The crude product was prepared according to procedure **B** starting from 4-bromobenzaldehyde (370 mg, 2.00 mmol) and 2-formylphenylboronic acid (300 mg, 2.00 mmol). After purification by column chromatography on silica gel (toluene/EtOAc 100:0 to 92:8), the pure product (389 mg, 1.85 mmol, 93%) was obtained as a white powder. m.p. 99°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ

ppm 7.38 (ddd, *J* = 7.5, 1.2, 0.4 Hz, 1H), 7.47-7.52 (m, 3H), 7.61 (ddd, *J* = 7.5, 7.5, 1.4 Hz, 1H), 7.93 (d, *J* = 8.4 Hz, 2H), 7.98 (dd, *J* = 7.8, 1.2 Hz, 1H), 9.89 (s, 1H), 10.03 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) *δ* ppm 128.3 (CH), 128.6 (CH), 129.7 (2×CH), 130.6 (CH), 130.7 (2×CH), 133.6 (C), 133.8 (CH), 135.8 (C), 144.1 (C), 145.2 (C), 191.5 (CH), 191.7 (CH).

*4.1.4.4. [1,1'-Biphenyl]-3,3'-dicarboxaldehyde (**18d**)*

The crude product was prepared according to procedure **B** starting from 3-bromobenzaldehyde (247 mg, 1.33 mmol) and 3-formylphenylboronic acid (200 mg, 1.33 mmol). After purification by column chromatography on silica gel (toluene/EtOAc 100:0 to 95:5), the pure product (171 mg, 0.81 mmol, 61%) was obtained as a white powder. m.p. 94°C, lit. 90°C;[39] ^{1}H NMR (400 MHz, $CDCl_3$) *δ* ppm 6.66 (dd, *J* = 7.7, 7.6 Hz, 2H), 7.88-7.92 (m, 4H), 8.13 (d, *J* = 1.6 Hz, 2H), 10.10 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) *δ* ppm 128.1 (2×CH), 129.6 (2×CH), 129.9 (2×CH), 133.1 (2×CH), 137.2 (2×C), 140.8 (2×C), 192.2 (2×CH).

*4.1.4.5. [1,1'-Biphenyl]-2,3'-dicarboxaldehyde (**18e**)*

The crude product was prepared according to procedure **B** starting from 3-bromobenzaldehyde (247 mg, 1.46 mmol) and 2-formylphenylboronic acid (200 mg, 1.46 mmol). After purification by column chromatography on silica gel (toluene/EtOAc 100:0 to 95:5), the pure product (227 mg, 1.08 mmol, 81%) was obtained as a white powder. m.p. 57°C, lit. 54−56°C;[40] ^{1}H NMR (400 MHz, $CDCl_3$) *δ* ppm 7.40 (dd, *J* = 7.7, 0.8 Hz, 1H), 7.46-7.52 (m, 1H), 7.58-7.66 (m, 3H), 7.84-7.88 (m, 1H), 7.90-7.94 (m, 1H), 7.98 (dd, *J* = 7.7, 1.2 Hz, 1H), 9.95 (d, *J* = 0.8 Hz, 1H), 10.08 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) *δ* ppm 128.2 (CH), 128.4 (CH), 129.2 (CH), 129.4 (CH), 130.7 (CH), 130.8 (CH), 133.8 (C), 133.9 (CH), 135.8 (CH), 136.4 (C), 138.9 (C), 144.1 (C), 191.5 (CH), 191.8 (CH).

4.1.5. General procedure C for the synthesis of diethyl alkylpropanedioates (19a,b)

To a stirred suspension of NaOEt (1.5 equiv.) in ethanol (0.8 mL/mmol) were successively added diethyl malonate (1 equiv.), bromoalkane (1.5 equiv.) and KI (1.5 equiv.). The reaction mixture was refluxed for 48 h. After cooling, ethanol was removed under reduced pressure and the residue was diluted into a saturated aqueous solution of NH_4Cl (2 mL/mmol). The mixture was then extracted with ethyl acetate. The combined organic layers were washed with water and brine, dried over $MgSO_4$, filtered off and concentrated under reduced pressure to afford the corresponding crude diethyl alkylpropanedioate derivative.

*4.1.5.1. Diethyl cyclohexylpropanedioate (**19a**)*

The crude product was prepared according to procedure **C** starting from diethyl malonate (4.00 g, 24.97 mmol) and bromocyclohexane (6.08 g, 37.50 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 98:2), the pure product (1.29 g, 5.32 mmol, 21%) was obtained as a colorless liquid. RMN ^{1}H (400 MHz, $CDCl_3$) δ ppm 0.98-1.21 (m, 4H), 1.27 (t, *J* = 7.1 Hz, 6H), 1.26-1.36 (m, 1H), 1.58-1.77 (m, 5H), 2.01-2.16 (m, 1H), 3.14 (d, *J* = 9.1 Hz, 1H), 4.19 (q, *J* = 7.1 Hz, 4H); RMN ^{13}C (100 MHz, $CDCl_3$) δ ppm 14.3 (2xCH_3), 26.2 (2xCH_2), 26.3 (CH_2), 30.9 (2xCH_2), 38.1 (CH), 58.6 (CH), 61.3 (2xCH_2), 169.0 (2xC).

*4.1.5.2. Diethyl 2-(methylcyclohexyl)propanedioate (**19b**)*

The crude product was prepared according to procedure **C** starting from diethyl malonate (4.00 g, 24.97 mmol) and (bromomethyl)cyclohexane (6.64 g, 37.50 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 98:2), the pure product (1.68 g, 6.55 mmol, 26%) was obtained as a colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.82-0.93 (m, 2H), 1.06-1.20 (m, 4H), 1.23 (t, *J* = 7.1 Hz, 6H), 1.52-1.72 (m, 5H), 1.75 (dd, *J* = 7.4, 7.4 Hz, 2H), 3.40 (t, *J* = 7.7 Hz, 1H), 4.14 (q, *J* = 7.1 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 14.2 (2xCH_3), 26.2 (2xCH_2), 26.5 (CH_2), 33.0 (2xCH_2), 35.7 (CH), 36.2 (CH_2), 49.8 (CH), 61.3 (2xCH_2), 169.9 (2xC).

4.1.6. General procedure D for the synthesis of 2-alkylpropane-1,3-diols (20a−d)

To a stirred suspension of $LiAlH_4$ (3.5 equiv.) in dry THF (1.0 mL/mmol) under nitrogen atmosphere was slowly added a solution of the corresponding diethyl alkylpropanedioate (1 equiv.) in dry THF (1.0 mL/mmol) at 0°C. The reaction mixture was allowed to warm to rt and was then refluxed for 48 h before being cooled to 0°C. The reaction was quenched by careful addition of a saturated aqueous solution of NH_4Cl until neutral or acidic pH. The precipitated aluminium salts were removed by filtration through a pad of Celite® and washed with diethylether or a mixture of MeOH/CH_2Cl_2/Et_2O 1:1:1. The organic phase of the filtrate was recovered, washed with water and brine, dried over $MgSO_4$, filtered off and concentrated under reduced pressure to afford the corresponding crude product which was pure enough to be directly used in the next step.

*4.1.6.1. 2-Cyclohexylpropane-1,3-diol (**20a**)*

The crude product, prepared according to procedure **D** starting from diethyl 2-cyclohexylpropanedioate **19a** (1.20 g, 4.95 mmol), was obtained (0.36 g, 2.28 mmol, 46%) as a

white solid. m.p. 96°C, lit. 93−95°C;[41] ^{1}H NMR (400 MHz, $CDCl_3$) *δ* ppm 0.96-1.10 (m, 2H), 1.11-1.30 (m, 3H), 1.38-1.48 (m, 1H), 1.53-1.61 (m, 1H), 1.61-1.78 (m, 5H), 3.82 (dd, *J* = 10.5, 7.4 Hz, 2H), 3.88 (dd, *J* = 10.5, 3.7 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) *δ* ppm 26.7 (CH_2), 26.8 (2xCH_2), 30.8 (2xCH_2), 36.9 (CH), 47.2 (CH), 64.9 (2xCH_2).

*4.1.6.2. 2-(methylcyclohexyl)propane-1,3-diol (**20b**)*

The crude product, prepared according to procedure **D** starting from diethyl 2-(methylcyclohexyl)propanedioate **19b** (1.60 g, 6.24 mmol), was obtained (0.96 g, 5.57 mmol, 89%) as a white solid. m.p. 63−65°C; ^{1}H NMR (400 MHz, $CDCl_3$) *δ* ppm 0.80-0.94 (m, 2H), 1.08 (dd, *J* = 7.0, 7.0 Hz, 2H), 1.12-1.35 (m, 4H), 1.60-1.75 (m, 5H), 1.86-1.97 (m, 1H), 3.63 (dd, *J* = 10.6, 7.8 Hz, 2H), 3.81 (dd, *J* = 10.6, 3.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) *δ* ppm 26.5 (2xCH_2), 26.8 (CH_2), 33.8 (2xCH_2), 35.2 (CH), 35.5 (CH_2), 39.1 (CH), 67.4 (2xCH_2).

*4.1.6.3. 2-cyclopentylpropane-1,3-diol (**20c**)*

The crude product, prepared according to procedure **D** starting from commercially available diethyl 2-cyclopentylpropanedioate **19c** (2.50 g, 10.95 mmol), was obtained (1.22 g, 8.47 mmol, 77%) as a white solid. m.p. 68°C, lit. 68−69°C;[42] ^{1}H NMR (400 MHz, $CDCl_3$) *δ* ppm 1.10-1.22 (m, 2H), 1.47-1.76 (m, 6H), 1.76-1.85 (m, 2H), 3.74 (dd, *J* = 10.6, 7.5 Hz, 2H), 3.93 (dd, *J* = 10.6, 3.7 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) *δ* ppm 25.2 (2xCH_2), 31.1 (2xCH_2), 38.4 (CH), 47. 8 (CH), 66.6 (2xCH_2).

*4.1.6.4. 2-isobutylpropane-1,3-diol (**20d**)*

The crude product, prepared according to procedure **D** starting from commercially available diethyl 2-isobutylpropanedioate **19d** (4.00 g, 18.49 mmol), was obtained (1.83 g, 13.84 mmol, 75%) as a light yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* ppm 0.86 (d, *J* = 6.6 Hz, 6H), 1.02 (dd, *J* = 7.1, 7.1 Hz, 2H), 1.52-1.63 (m, 1H), 1.73-1.83 (m, 1H), 3.54 (dd, *J* = 10.7, 7.7 Hz, 2H), 3.70 (dd, *J* = 10.7, 3.8 Hz, 2H), 3.84 (bs, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) *δ* ppm 22.9 (2xCH_3), 25.5 (CH), 37.0 (CH_2), 39.7 (CH), 66.1 (2xCH_2).

4.1.7. General procedure E for the Mitsunobu reaction. Synthesis of bisaldehydes (21a−d)

To a solution of the corresponding 2-alkylpropane-1,3-diol **20a−d** (1 equiv.) in dry THF (7 mL/mmol) were added 4-hydroxybenzaldehyde (3 equiv.), PPh_3 (3 equiv.) and DIAD (3 equiv.). The reaction mixture was stirred overnight at rt and was then concentrated under reduced pressure. The crude product was purified by column chromatography.

*4.1.7.1. 4,4'-[1,3-(2-cyclohexyl)propanediyl(oxy)]bisbenzaldehyde (**21a**)*

The crude product was prepared according to procedure **E** starting from 2-cyclohexylpropane-1,3-diol **20a** (360 mg, 2.28 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 8:2), the pure product (356 mg, 0.97 mmol, 43%) was obtained as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.10-1.37 (m, 6H), 1.63-1.88 (m, 5H), 2.13-2.22 (m, 1H), 4.19 (dd, *J* = 9.4, 6.4 Hz, 2H), 4.25 (dd, *J* = 9.4, 4.8 Hz, 2H), 7.02 (d, *J* = 8.8 Hz, 4H), 7.83 (d, *J* = 8.8 Hz, 4H), 9.88 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 26.6 (CH_2), 26.7 (2xCH_2), 30.8 (2xCH_2), 36.9 (CH), 43.8 (CH), 66.7 (2xCH_2), 115.0 (4xCH), 130.2 (2xC), 132.2 (4xCH), 164.1 (2xC), 190.9 (2xCH); LRMS (ESI+) *m/z* (%) 389 (22) $[M+Na]^+$, 367 (100) $[M+H]^+$; HRMS (ESI+) *m/z* calc. for $C_{23}H_{27}O_4$ 367.1909, found 367.1915.

*4.1.7.2. 4,4'-[1,3-(2-(methylcyclohexyl))propanediyl(oxy)]bisbenzaldehyde (**21b**)*

The crude product was prepared according to procedure **E** starting from 2-(methylcyclohexyl)propane-1,3-diol **20b** (900 mg, 5.22 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 8:2), the pure product (660 mg, 1.74 mmol, 33%) was obtained as a light yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.86-0.99 (m, 2H), 1.11-1.41 (m, 4H), 1.45 (dd, *J* = 7.0, 7.0 Hz, 2H), 1.60-1.79 (m, 5H), 2.39-2.51 (m, 1H), 4.11 (d, *J* = 5.4 Hz, 4H), 7.00 (d, *J* = 8.7 Hz, 4H), 7.80 (d, *J* = 8.7 Hz, 4H), 9.85 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 26.3 (2xCH_2), 26.6 (CH_2), 33.6 (2xCH_2), 35.0 (CH), 35.6 (CH_2), 36.0 (CH), 68.4 (2xCH_2), 114.9 (4xCH), 130.1 (2xC), 132.0 (4xCH), 164.0 (2xC), 190.8 (2xCH); LRMS (ESI+) *m/z* (%) 381 (100) $[M+H]^+$; HRMS (ESI+) *m/z* calc. for $C_{24}H_{29}O_4$ 381.2066, found 381.2070.

*4.1.7.3. 4,4'-[1,3-(2-cyclopentyl)propanediyl(oxy)]bisbenzaldehyde (**21c**)*

The crude product was prepared according to procedure **E** starting from 2-cyclopentylpropane-1,3-diol **20c** (1.17 g, 8.11 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 8:2), the pure product (1.50 g, 4.25 mmol, 52%) was obtained as a light yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.15-1.29 (m, 2H), 1.45-1.68 (m, 4H), 1.78-1.89 (m, 2H), 1.96-2.11 (m, 2H), 4.13 (dd, *J* = 9.3, 5.8 Hz, 2H), 4.20 (dd, *J* = 9.3, 3.4 Hz, 2H), 6.95 (d, *J* = 8.8 Hz, 4H), 7.75 (d, *J* = 8.8 Hz, 4H), 9.79 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 24.8 (2xCH_2), 30.9 (2xCH_2), 38.8 (CH), 43.9 (CH), 67.3 (2xCH_2), 114.7 (4xCH), 129.9 (2xC), 131.8 (4xCH), 163.9 (2xC), 190.5 (2xCH); LRMS (ESI+) *m/z* (%) 375 (35) $[M+Na]^+$, 353 (100) $[M+H]^+$; HRMS (ESI+) *m/z* calc. for $C_{22}H_{25}O_4$ 353.1753, found 353.1758.

*4.1.7.4. 4,4'-[1,3-(2-isobutyl)propanediyl(oxy)]bisbenzaldehyde (**21d**)*

The crude product was prepared according to procedure **E** starting from 2-isobutylpropane-1,3-diol **20d** (1.70 g, 12.86 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 9:1 to 6:4), the pure product (3.20 g, 9.40 mmol, 73%) was obtained as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.97 (d, J = 6.6 Hz, 6H), 1.47 (dd, J = 7.2, 7.2 Hz, 2H), 1.68-1.82 (m, 1H), 2.38-2.48 (m, 1H), 4.14 (d, J = 5.6 Hz, 4H), 7.02 (d, J = 8.7 Hz, 4H), 7.83 (d, J = 8.7 Hz, 4H), 9.89 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 22.9 (2xCH_3), 25.5 (CH), 36.5 (CH), 37.6 (CH_2), 68.4 (2xCH_2), 115.0 (4xCH), 130.2 (2xC), 132.2 (4xCH), 164.1 (2xC), 190.9 (2xCH); LRMS (ESI+) *m/z* (%) 363 (25) $[M+Na]^+$, 341 (100) $[M+H]^+$; HRMS (ESI+) *m/z* calc. for $C_{21}H_{25}O_4$ 341.1753, found 341.1760.

4.2. Molecular modelling

The chemical structures of pseudodimeric aurones were prepared within SYBYL X (Tripos Inc., St. Louis, MO), assigning Tripos force field atom and bond types to each ligand. The ionization state was defined as appropriate for pH 7.4. The protein structures (PDB codes: 4DRU and 3SKE) were prepared by removing co-crystallized water molecules and ligands and by adding hydrogen atoms. Automated docking was carried out with the program GOLD, version 5.2,[43] within the MLP strategy.[44,45] Docking methodology was validated by re-docking the co-crystallized inhibitors in the corresponding pockets (RMSD < 1.5 Å, data not shown). Binding pockets were defined by considering 6 Å around the co-crystallized ligands. Preset options for the genetic search algorithm were used in calculations in which only ligand torsions were free to move. 100 solutions for each ligand-protein complex were retrieved and ranked according to the ChemPLP fitness score.

4.3. Biology

4.3.1. Cytotoxicity assays

The cytotoxicity assays were realized using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay on HEK293 cell lines. Cells were seeded in 96-well culture plates (10^4 cells/well) and allowed to attach for 24 h at 37°C in 5% CO_2. The treatment was done with a 50 µM concentration of compound in a final volume of 200 µL and allowed to grow for 72 h at 37ºC in 5% CO_2. MTT (20 µL) solution (5 mg/mL) was added and

incubated for 4 h at 37°C in 5% CO_2. The culture medium was discarded, washed with PBS followed by addition of 100 µL of DMSO and maintained under shaking for 10 min. Absorbance was measured in a microplate reader at 570 nm. Experiments were repeated three times.

4.3.2. Expression and Purification of Recombinant HCV NS5BΔ21

RdRp (NS5B protein) from the HCV J4 genotype 1b reference strain, truncated of its 21 C-terminal amino acids to ensure solubility (NS5BΔ21) and carrying a hexahistidine tag (His-Tag) at its N-terminus, was expressed in Escherichia coli C41 (DE3) and purified. Chromatography was performed on Ni-NTA columns (Qiagen, Valencia, CA). The columns were washed with a buffer containing 50 mM NaH_2PO_4 (pH 8.0), 500 mM NaCl, and 20 mM imidazole. The bound protein was eluted in 1 mL fractions with a buffer containing 50 mM NaH_2PO_4 (pH 8.0), 500 mM NaCl, and 250 mM imidazole. NS5BΔ21-enriched fractions were selected with a Bradford colorimetric assay, and NS5BΔ21 purity was determined by SDS-PAGE analysis with Coomassie staining. Purified NS5BΔ21 fractions were pooled and dialyzed against a buffer containing 5 mM Tris (pH 7.5), 0.2 M sodium acetate, 1 mM DTT, 1 mM EDTA, and 10% glycerol. NS5BΔ21 purity was >98%.

4.3.3. HCV-NS5BΔ21 Polymerase Assay.

An enzyme assay was used to measure inhibition of HCV-NS5BΔ21 polymerase activity. The cell free HCV-NS5BΔ21 polymerase assay is based on the amount of double-stranded RNA synthesized in the presence of HCV-NS5BΔ21, a homopolymeric RNA template (Poly U, GE Healthcare, Chalfont St. Giles, U.K.) and ATP, measured with an intercalating agent (SYBR Green, Applied Biosystems), as previously described.[46] Each experiment was performed in triplicate.

4.3.4. J6/JFH1 infection in cell culture

Huh7.5 cells were seeded in 48-well plates at a density of 10 000 cells/well, infected 24 h later with 100 µl of HCV infectious particles (HCVcc) and incubated overnight at 37°C. After incubation, the supernatants were removed and J6/JFH1-infected cells were washed with fresh medium. Increasing concentrations of the tested compounds were added in a medium containing 1% DMSO and the cells were incubated 72 h at 37°C. Then, the cells were washed once with Dulbecco's PBS (Invitrogen), and 75 µl Renilla lysis buffer (Promega) was added to each well. Note that 20 µl of lysate was mixed with the luciferase assay substrate, as specified by the manufacturer. Luciferase activity was measured for 10 s in a Berthold luminometer.

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Appendix A. Supplementary data

Supplementary data (^{1}H and ^{13}C NMR spectra) related to this article can be found at http://dx.doi.org/

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List of captions

Figure 1. General structure of the aurone backbone (top left), structure and activity of the naturally occurring aureusidin **1** and structures of aurone derivatives investigated in this study (the dashed line indicate the site of linkage).

Figure 2. Inhibition curve of HCV-NS5BΔ21-WT r(wild-type), P495L, H95Q, C316Y and M423T mutants obtained with compound **4**.

Figure 3. Best ranked poses of compounds **4** (A, yellow ball&sticks, ChemPLP score 62.6), **12** (B, green ball&sticks, ChemPLP score 77.7) and **14** (C, black ball&sticks, ChemPLP score 93.8) in RdRp palm pocket I represented as dark blue ribbons (PDB code 3SKE). RdRp residues involved in complex stabilization are highlighted in sticks, colored and labeled whereas hydrophobic fitting points calculated with the MLP are reported as magenta dots.

Scheme 1. General pathway for the synthesis of pseudodimeric aurones. Reagents and conditions: (i) $ClCH_2CN$, HCl, $ZnCl_2$, Et_2O, 0°C; (ii) H_2O, 100°C, 5 h, 84% (for 2 steps); (iii) Me_2SO_4, K_2CO_3, 1,2-dimethoxyethane, reflux, 5 h, 97%; (iv) bis-arylaldehyde, KOH 50% in H_2O, EtOH or MeOH, 2-15 h, 34-98%.

Scheme 2. Synthesis of bisaldehydes. Reagents and conditions: (i) $Pd(PPh_3)_4$, K_2CO_3, 1,2-dimethoxyethane, reflux, 6 h, 61-93%; (ii) EtONa, RBr, KI, EtOH, reflux, 48 h; (iii) $LiAlH_4$, THF, 0°C then reflux for 48 h, 46-89%; (iv) 4-hydroxybenzaldehyde, PPh_3, DIAD, THF, rt, 12 h, 33-73%.

Table 1

Structures and inhibition activity of pseudodimeric aurones. The dashed bounds indicate the sites of attachment to the methylenebenzofuranone moieties.

Table 2

Anti-HCV activity in the genotype 2a/2a J6/JFH1 infectious model of compounds **4**, **12**, **13**, **14**, **16**, **17** and **ABT-133** (Dasabuvir).

Highlights

- 14 pseudodimeric aurones were synthetized and characterized;
- Pseudodimeric aurones target palm pocket I of NS5B;
- Binding site was determined using site-directed mutagenesis and molecular docking studies;
- Anti-HCV activity in infectious model was assessed for these pseudodimeric aurones.