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Short communication

Synthesis of new benzimidazole–coumarin conjugates as anti-hepatitis C virus agents

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Abstract

Nineteen new conjugated compounds were successfully synthesized by a one-flask method from benzimidazole and coumarin derivatives. A methylenethio linker was used to connect these two kinds of derivatives. In addition, substituted benzimidazol-2-thiones were also coupled with β-D-glucose peracetate; the resultant glucosides were further converted to the corresponding 2-(methylthio)coumarin derivatives. Their activity against the hepatitis C virus was tested; two of the most potent compounds 2-[(6′-bromocoumarin-3′-yl)methylenethio]-5-fluorobenzimidazole (**4i**) and its derivative 1-[(2″,3″,4″,6″-tetra-*O*-acetyl)glucopyranos-1″-yl]-2-[(6′-bromocoumarin-3′-yl)methylenethio]benzimidazole (**7c**) showed EC_{50} values of 3.4 μM and 4.1 μM, respectively. At a concentration of 5.0 μM, compound **7c** inhibited HCV RNA replication by 90% and had no effect on cell proliferation. Given these data, a structure–activity relationship was established.

Keywords: Benzimidazole; Coumarin; Conjugate; *N*-Glucosides; Anti-HCV

About 3% of the current world population (~170 million people) are chronically infected by the chronic hepatitis C virus (HCV), which causes a progressive liver disease (Lauer and Walker, 2001). The infection can lead to lethal complications, such as cirrhosis and hepatocellular carcinoma (Penin et al., 2004). Liver failure from chronic hepatitis C is the leading cause for liver transplantation in the West (Armstrong et al., 2000; Condron et al., 2005). Current therapies result in only 50–60% of the patients in a sustained virological response (Bretner, 2005a). There is thus an unmet need for potent antiviral compounds (Pawlotsky and Gish, 2006; Neyts, 2006).

Some benzimidazole (Andrzejewska et al., 2002; Gümüs et al., 2003) and coumarin (Ito et al., 2003; Nam et al., 2002) derivatives show diverse biological activities with significant clinical potential, including the treatment of leukaemia (Demirayak et al., 2002; Garuti et al., 2000) and cancer (Antonini et al., 1988; Lukevics et al., 2001). These compounds may also possess potent antiviral activity (Curini et al., 2003; Devivar et al., 1994). Examples include a series of non-nucleoside benzimidazoles reported by Beaulieu et al. (Beaulieu et al., 2004a,b; Beaulieu and Llinàs-Brunet, 2002; McKercher et al., 2004). Recently 2-arylbenzimidazole-5-carboxylic acids were shown to inhibit the HCV NS5B RNA polymerase (Hirashima et al., 2006; Ishida et al., 2006). To the best of our knowledge, hybridized molecules containing benzimidazole and coumarin have never been reported as anti-HCV agents (cf. Bretner et al., 2005b). We here report that benzimidazole–coumarin conjugates with a methylenethio linker and the corresponding *N*-glucosides exhibit antiviral activity against HCV.

1. Synthesis of new benzimidazoles 4a–k and the corresponding glucopyranosides 7a–e

We treated various substituted phenylenediamines **1a–f** with carbon disulfide (Klimesová et al., 2002) and ethanolic KOH in H_2O to give intermediates **2a–f** (see Scheme 1). Subsequently, aqueous NH_4OH (35%) and 3-chloromethylcoumarins **3a–c** were added in situ to afford the desired benzimidazole–SCH_2–coumarin derivatives **4a–k**, respectively, which were purified by HPLC. Their overall yields were 53–86% and purity was >99.8% as checked by GC and HPLC.

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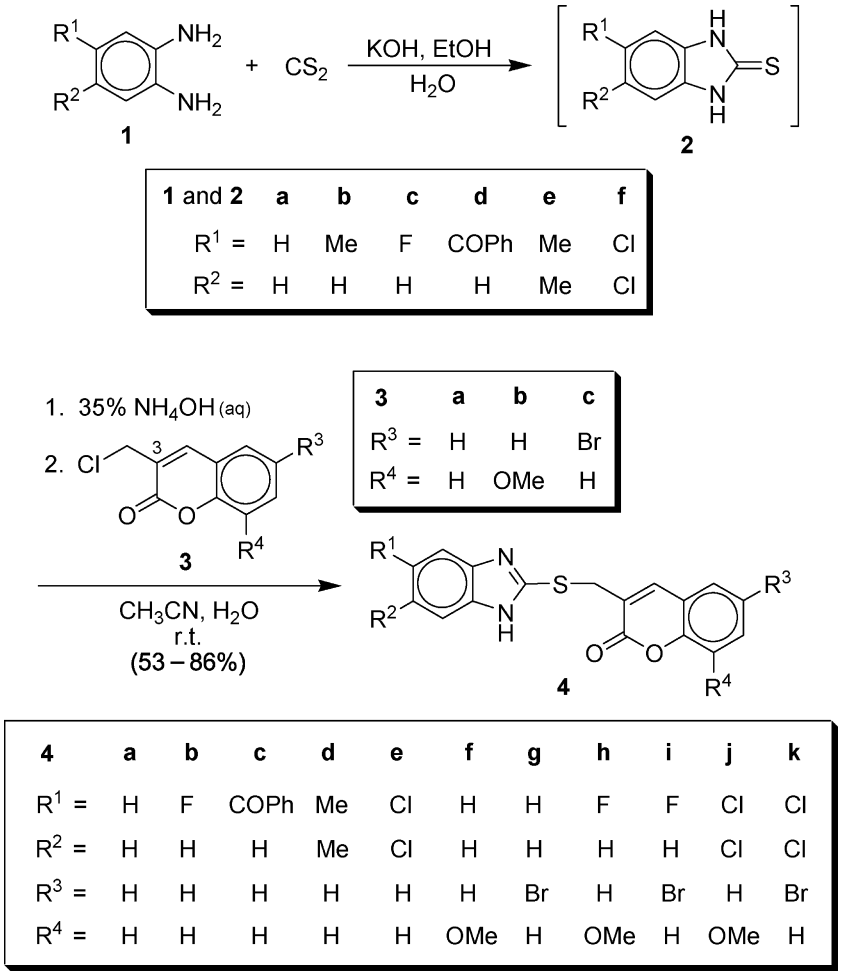


1 and 2	a	b	c	d	e	f
R^1 =	H	Me	F	COPh	Me	Cl
R^2 =	H	H	H	H	Me	Cl

3	a	b	c
R^3 =	H	H	Br
R^4 =	H	OMe	H

4	a	b	c	d	e	f	g	h	i	j	k
R^1 =	H	F	COPh	Me	Cl	H	H	F	F	Cl	Cl
R^2 =	H	H	H	Me	Cl	H	H	H	H	Cl	Cl
R^3 =	H	H	H	H	H	H	Br	H	Br	H	Br
R^4 =	H	H	H	H	H	OMe	H	OMe	H	OMe	H

Scheme 1. A "one-flask" method for the synthesis of benzimidazole–SCH_2–coumarin conjugates.

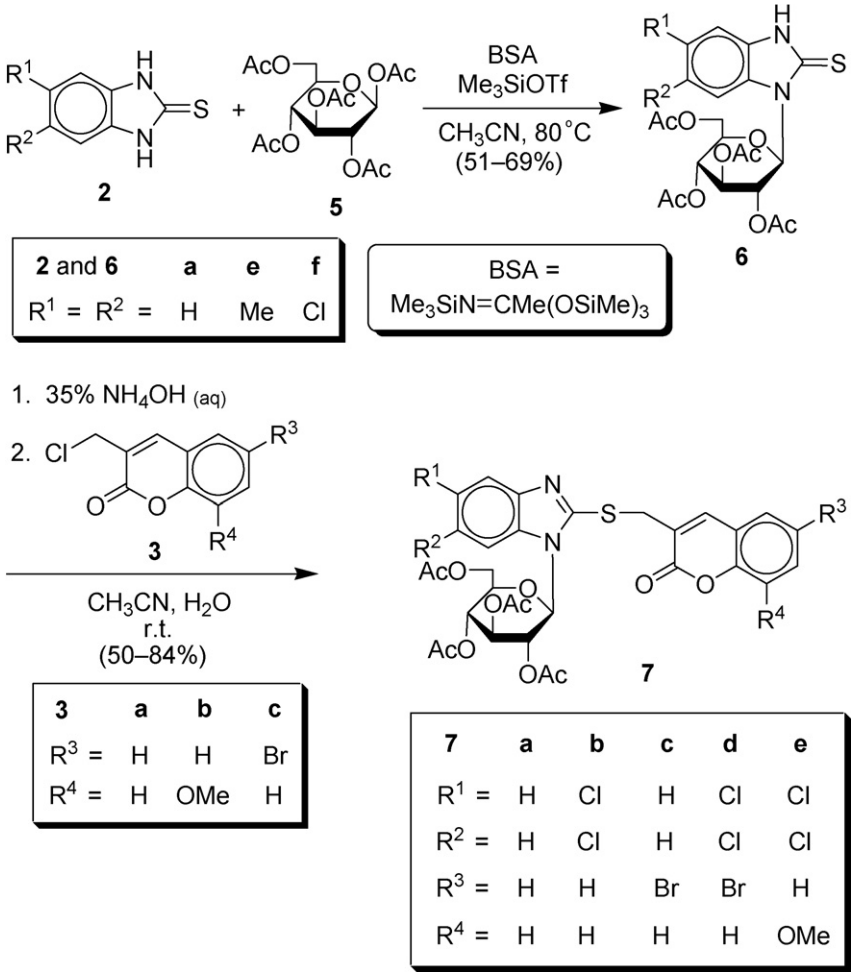
Structures of the conjugated products were identified by spectroscopic methods. For example, the ^{1}H NMR spectrum of conjugate **4i** showed a singlet at 4.33 ppm for the SCH_2 protons and a singlet at 7.95 ppm for the CH=C–COO proton in the coumarin moiety. Its ^{13}C NMR spectrum exhibited resonance at 30.66 ppm for the SCH_2 carbon and 149.94 ppm for the –N=C(–N)(–S) carbon. For the elemental analysis of **4i**, the calculated percentages are C 50.50, H 2.49, Br 19.54, F 4.70, N 6.93, O 7.92, S 7.91; which were found C 50.39, H 2.53, Br 19.48, F 4.66, N 6.97, O 7.89, S 7.86.

Our design by using the sulfur atom in the –SCH_2– linker to attach the benzimidazole ring directly was to facilitate tautomerization of the imidazole nucleus. Consequently, the regioisomeric problem for $R^1 \neq R^2$ in compound **4** can be circumvented during the preparation of **4b**, **4c**, **4h**, and **4i**.

Xu et al. (Duan et al., 2004) isolated *O*-galloyl-β-D-glucoses from traditional Chinese medicine *Galla Chinese*. Three glucose esters in this family are identified as inhibitors of hepatitis C virus NS3 protease. Consequently we considered to synthesize glucosides **7** by coupling the heterocycles **4** with a glucose ester. Unfortunately, direct coupling of conjugated heterocycles **4a**–**k** with *O*-peracetylpyranose **5** met with failure. Therefore, we decided to silylate 2-thiones **2** with *N*,*O*-bistrimethylsilylacetamide (BSA) (Bhan et al., 1997; Khodair et al., 2003); the intermediates were then coupled with *O*-peracetylpyranose **5** in the presence of Me_3SiOTf at 80 °C (Scheme 2).

In the reactions **2** + **5** → **6**, a mixture of regioisomers might be produced when unsymmetrical 2-thiones **2b**–**d** were used as the starting materials. To circumvent the potential problems of separation and structural identification, we adopted the symmetrical benzimidazole-2-thiones **2a**, **2e**, and **2f** to react with pyranose **5** individually. Accordingly, compounds **6a**, **6e**, and **6f** were isolated as the exclusive products. These glucosidic 2-thiones were alkylated with various 3-(chloromethyl)coumarins **3a**–**c** in the presence of aqueous NH_4OH (35%) (Khodair et al., 2003) at room temperature to give the desired *N*-glucopyranosyl conjugates **7a**–**e** with purity >99.7%, which were purified and checked by HPLC.

The structures of **7a**–**e** were satisfactorily characterized by spectroscopic methods. For example, the high-resolution mass of glucoside **7d** was found 783.9896, which is consistent with the theoretical value 783.9899 for $C_{31}H_{27}BrCl_2N_2O_{11}S$. Its ^{13}C NMR spectrum showed 31 peaks for every carbon atoms therein in full. In the ^{1}H NMR spectrum, the glycosidic proton resonated at the 5.55 ppm as a doublet with $J = 8.4$ Hz, which indicates



Scheme 2. A synthetic pathway to generate *N*-glucosides of benzimidazole–SCH_2–coumarin conjugates.

the β configuration of glycoside (Zhang et al., 2005). The two $ClCH_2$–hydrogens in the starting material **3c** at 4.53 ppm as a singlet was shifted to 4.40 ppm and 4.84 ppm as two doublets with $J = 11$ Hz for the two diastereotopic SCH_2 hydrogens in the product **7d**. Appearance of these peaks with an AB pattern indicates the success of the coupling reaction **6f** + **3c** → **7d**. Furthermore, the elemental analysis results of **7d** are listed below: the calculated percentages are C 47.45, H 3.47, Br 10.07, Cl 8.92, N 3.57, O 22.44, S 4.08; they were found C 47.51, H 3.44, Br 9.99, Cl 8.88, N 3.61, and S 4.11.

2. Antiviral activity tests

We performed antiviral assays with the 19 synthesized conjugated compounds against 15 viruses in four different cell lines. They included HIV-1_{IIIB} and HIV-2_{ROD} in MT-4 cells; herpes simplex virus-1 (KOS), thymidine kinase-deficient (TK^-), herpes simplex virus-2 (G), vaccinia virus, and vesicular stomatitis virus in E_6SM cells; respiratory syncytial virus in HeLa cells; as well as parainfluenza-3 virus, reovirus-1, Sindbis virus, Coxsackie B4 virus, and Punta Toro virus in Vero cell cultures. None of the compounds proved active against any of the aforementioned viruses.

3. Antiviral evaluation in the hcv genotype 1b subgenomic replicon

The potential antiviral activity of the compounds was initially evaluated in the Huh 5-2 replicon system, which was kindly provided by Prof. R. Bartenschlager, University of Heidelberg, Germany (Lohmann et al., 1999). The antiviral assays and cytostatic determination assays have been described in detail before (Paeshuyse et al., 2006). Among the 19 newly synthesized benzimidazole–coumarin conjugates, some were found to inhibit HCV subgenomic replicon replication in the Huh 5–2 cell line. The 50% inhibitory concentrations for viral replication (EC_{50}) and host cell growth (CC_{50}), as well as the selectivity indices ($SI = CC_{50}/EC_{50}$), are presented in Table 1. Conjugate **4i** and glucoside **7c** inhibited HCV replication with EC_{50} values of 3.4 μM and 4.1 μM, respectively. To exclude that the observed antiviral effect was caused by inhibition of the luciferase activity in the replicon assay, or to particular characteristics of this Huh 5-2 replicon construct, we further explored the antiviral activity of compounds **4i** and **7c** by means of a quantitative RT–PCR assay in three other HCV replicon models (Coelmont et al., 2006; Paeshuyse et al., 2006). The activity of the compounds was confirmed in these assays (see Table 2).

Table 1
Inhibitory effects of conjugated compounds **4**, **6**, and **7** on HCV subgenomic replicon replication in Huh 5-2 cells

Compound[a]	R^1	R^2	R^3	R^4	CC_{50} (μM)[b,c]	EC_{50} (μM)[c,d]	SI[e]
4a	H	H	H	H	90	27	3.4
4b	F	H	H	H	45	11	3.9
4c	COPh	H	H	H	37	6.7	5.6
4d	Me	Me	H	H	67	15	4.4
4e	Cl	Cl	H	H	74	15	4.8
4f	H	H	H	OMe	27	10	2.8
4g	H	H	Br	H	42	4.0	10
4h	F	H	H	OMe	42	16	2.6
4i	F	H	Br	H	27	3.4	8.0
4j	Cl	Cl	H	OMe	11	3.6	3.1
4k	Cl	Cl	Br	H	44	2.3	19
6a	H	H	H	H	104	104	1.0
6e	Me	Me	H	H	98	100	0.98
6f	Cl	Cl	H	H	60	26	2.3
7a	H	H	H	H	76	11	6.7
7b	Cl	Cl	H	H	20	3.1	6.3
7c	H	H	Br	H	43	4.1	10
7d	Cl	Cl	Br	H	>64.8	4.1	>16
7e	Cl	Cl	H	OMe	33	4.3	7.6

[a] Interferon α-2b was used as a (positive) reference compound at 10,000 units/well and reduced the signal in the viral RNA (luciferase) assay to background levels without any cytotoxic activity.
[b] Cytostatic concentration: the concentration required to reduced cell proliferation by 50%.
[c] The values obtained as the average of triplicate determinations.
[d] Effective concentration: the concentration required to inhibit HCV RNA replication by 50%.
[e] Selectivity index (ratio of CC_{50} to EC_{50}).

A dose–response effect of **7c** on HCV RNA replication in Huh 5-2 cells and of their effect on host cell proliferation is depicted in Fig. 1. For example, at a concentration of 5.0 μM, compound **7c** inhibited HCV RNA replication by 90%, whereas at this concentration it had almost no effect on cell proliferation. At a concentration of 16 μM, compound **7c** inhibited HCV replicon replication by 99% whereas cell growth was reduced only by ∼20%. These data clearly indicate that the antiviral activity is specific because potent inhibition was observed at concentrations that have no, or little, effect on the cell proliferation.

4. Structure–activity relationship

Different substituents including F, Cl, Br, Me, OMe, and COPh were placed at various positions of the benzimidazole–SCH_2–coumarin conjugates (see **4a**–**k**). By analyzing the EC_{50} and CC_{50} values shown in Table 1, we can deduce the following structure–activity relationships: (a) introduction of an F-, Cl-, or Me-substituent on the benzimidazole ring (i.e., **4b**, **4e** and **4d** *versus* **4a**) showed little effect on their HCV inhibitory activity; (b) introduction of a Br group on the coumarin ring (e.g., **4g** *versus* **4a**) enhanced HCV inhibition by 6.7-fold and also the selectivity by 2.9-fold; (c) introduction of an OMe group on the coumarin ring (e.g., **4j** *versus* **4e**) further improved the antiviral activity; (d) enhancement of the selectivity resulting from substituents in the coumarin nucleus (cf. **4f**, **4a**, and **4g**) followed the order OMe < H < Br; (e) attachment of the $–CH_2$–coumarin moiety onto the benzimidazole-2-thione nucleus connected with a pyranose (e.g., **6a** *versus* **7a**) enhanced

Table 2
Inhibitory effects of conjugated compounds **4i** and **7c** on HCV subgenomic replicon replication in Huh 6, Huh 9-13, and Huh mono cells

Cell	Compound	CC_{50} (μM)[a]	EC_{50} (μM)[b]	SI[c]
Huh 6	**4i**	23 ± 6.0	3.0 ± 1.6	7.7
Huh 6	**7c**	25 ± 7.2	6.0 ± 0.8	4.2
Huh 9-13	**4i**	26 ± 3.6	4.6 ± 1.0	5.7
Huh 9-13	**7c**	73 ± 2.9	0.8 ± 0.5	91
Huh mono	**4i**	26 ± 6.0	9.0 ± 8.0	2.9
Huh mono	**7c**	31 ± 14	12 ± 7.0	2.6

[a] Cytostatic concentration: the concentration required to reduced cell proliferation by 50%. Data are mean values from two independent experiments.
[b] Effective concentration: the concentration required to inhibit HCV RNA replication by 50%. Data are mean values from two experiments.
[c] Selectivity index (ratio of CC_{50} to EC_{50}).

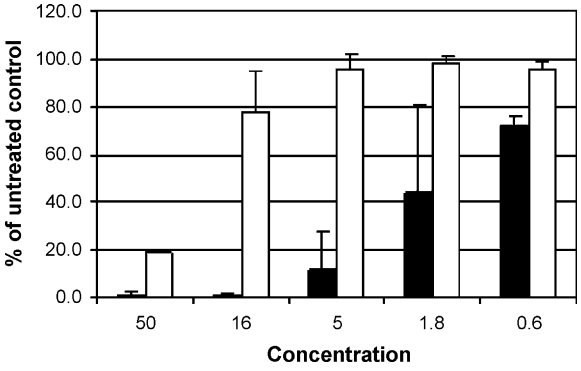


Fig. 1. Dose–response effect of compound **7c** on HCV subgenomic replication and cell proliferation in Huh 5-2 cells. Black bars represent the effect on HCV replicon content, open bars the effect on host cell proliferation. Data are mean values ± S.D. for two independent experiments.

the HCV inhibitory activity by >9.4-fold and also the selectivity by 6.7-fold; (f) incorporation of a β-D-glucose peracetate moiety into the benzimidazole–coumarin conjugate (e.g., **4e** *versus* **7b**) resulted in a 4.8-fold increase in anti-HCV activity.

In conclusion, a series of substituted benzimidazole–coumarin conjugated compounds were synthesized and evaluated for their activities against various viruses. In this compound library, several new conjugates resulted in an inhibitory effect on HCV replication: the conjugate **4i** displayed an EC_{50} of 3.4 μM. The *N*-glucoside **7c** inhibited HCV RNA replication by 90% and 99% at concentrations of 5.0 μM and 16 μM, respectively. These conjugates may be considered as potential lead anti-HCV compounds for further selectivity optimization.

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