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Bioorganic & Medicinal Chemistry Letters 14 (2004) 3257–3261
Bioorganic & Medicinal Chemistry Letters

The monoethyl ester of meconic acid is an active site inhibitor of HCV NS5B RNA-dependent RNA polymerase

Paola Pace,* Emanuela Nizi, Barbara Pacini, Silvia Pesci, Victor Matassa,† Raffaele De Francesco, Sergio Altamura and Vincenzo Summa

Department of Medicinal Chemistry and Biochemistry, IRBM-MRL Rome, Via Pontina, Km 30.600, 00040 Pomezia (Rome), Italy

Received 21 January 2004; revised 24 March 2004; accepted 29 March 2004

Abstract—Screening of the in-house sample collection for compounds with HCV NS5B RNA dependent RNA polymerase inhibition led to the identification of a new lead. Afterwards, we discovered that the screening lead, rather than containing the expected structure **1**, was comprised of roughly a 1:1 mixture of meconic acid **2** and its monoethyl ester **3**, with all inhibitory potency residing with **3**. We propose that this compound shares critical common features for activity with α,γ-diketoacids inhibitors previously discovered by our group. SAR around this molecule will be presented to provide an improved basis for structure-based ligand design.

1. Introduction

The lack of a highly effective and safe treatment option for the hepatitis C virus (HCV) highlights the necessity of developing more efficient means of combating and, ultimately, curing this viral disease. A primary focus is currently on finding inhibitors of the NS5B polymerase, an enzyme absolutely required for replication of the virus and exhibiting important differences with cellular polymerases.[1,2]

Recent discoveries from our laboratories of the biological activity of α,γ-diketoacids[3] as low nanomolar inhibitors of NS5b HCV polymerase further stimulated our interest in this area. A new screening of our in-house sample collection identified another low micromolar lead as a specific and reversible inhibitor of NS5B HCV polymerase, competitive with the diketoacid. The compound was inactive at 200 μM against HIV-RT and poliovirus RNA-dependent RNA polymerase. Surprisingly, a second batch of the screening hit was inactive in the HCV polymerase assay and provided spectral data inconsistent with that obtained from the original sample. ^{1}H, ^{13}C NMR and mass analyses showed that, while the second batch had the expected structure **1**, the original screening compound was in fact a roughly 1:1 mixture of meconic acid (poppy acid or 3-hydroxy-4-oxo-4*H*-pyran-2,6-dicarboxylic acid)[4] **2** and its monoethyl ester **3**. Compound **3** was therefore prepared and shown to provide an NMR spectrum, which when combined with the spectrum of commercial meconic acid, accounted for the observed NMR spectrum of the first batch of screening compound. The meconic ester derivative was therefore identified as the active component of this mixture, having an $IC_{50} = 2.25\ \mu M$ against HCV NS5B polymerase (Fig. 1).

Our preliminary structure–activity studies are summarized in Table 1 and demonstrate the absolute requirement of the 2-carboxylic acid functionality. Decarboxylation or esterification abolish the activity

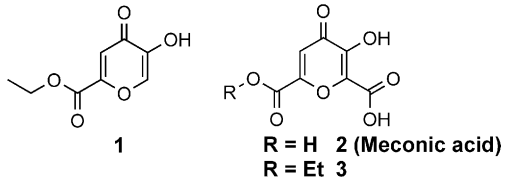

Figure 1. Screening lead for HCV NS5b Polymerase inhibition (see text).

Keywords: Meconic acid; HCV polymerase inhibitors.
* Corresponding author. Tel.: +39-0691093313; fax: +39-0691093654; e-mail: paola_pace@merck.com
† Present address: Graffinity Pharmaceutical AG Im, Neuenheiner Feld 518, 69120 Heidelberg, Germany.

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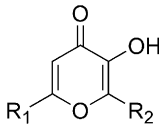
doi:10.1016/j.bmcl.2004.03.087

Table 1. Inhibitory potency of meconic acids derivatives



Compd	R_1	R_2	IC_{50} (μM)[a]
1	CO_2Et	H	>100
2	CO_2H	CO_2H	>100
3	CO_2Et	CO_2H	2.25
4	CO_2Et	CO_2Et	>100
5	CO_2H	CO_2Me	>100
6	H	CO_2H	51
7	CONHEt	CO_2H	24
8	$CONEt_2$	CO_2H	>100
9	CH_2OEt	CO_2H	16.2
10	CH_2OBn	CO_2H	>100
11	CO_2tBu	CO_2H	36

[a] See Ref. 5 for polymerase assay protocol. Data are the mean of two to four independent experiments.

(compounds **1** and **4**). Altering the position of the carboxylate with respect to the ester gave inactive **5**. Our previous experience with α,γ-diketoacids suggests a common paradigm for HCV NS5B inhibition based on the shared presence of a carbonyl, an enolic OH and a carboxylate functionality. This similarity allows a very plausible overlay of the two structures with the three oxygen atoms in a similar spatial arrangement (Fig. 2).

Studies by ourselves,[6] and other groups,[7] showed that in the NS5B polymerase active site there are two strictly conserved aspartic and a glutamic acid residues that chelate Mg^{2+} ions required for nucleotide incorporation. Recently, we reported that diketoacids are active site inhibitors and they are capable of interacting directly with the metal ions.[8] Therefore, some of the binding energy of the meconic acid derivative too could be derived from metal ion interaction in the active site. This hypothesis is supported by a mutually exclusive binding of the compounds to the enzyme, demonstrated by competition experiments using tritiated diketoacid (DKA) as probe (Fig. 3). We measured the ability of our lead to compete for 50% of the bound [^{3}H]-DKA (DC_{50}). For **3** we obtained a $DC_{50} = 1.2$ and 0.3 μM for the DKA.

As for the diketoacids and consistent with the direct interaction with the active-site metal ions, the inhibitory

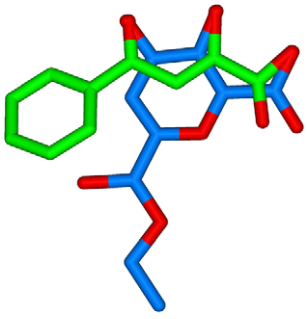


Figure 2. Overlay of a diketoacid (green) and meconic acid monoethyl ester 2 (blue).

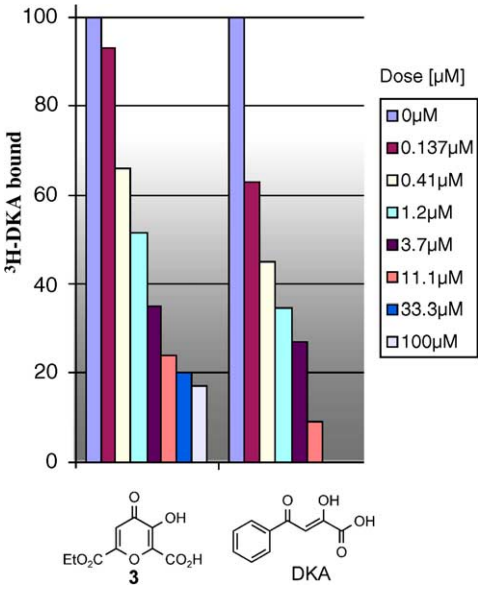


Figure 3. Displacement of ^{3}H-DKA from NS5b HCV polymerase.

potency is dependent on the type of metal used in the assay (Mg^{2+} or Mn^{2+}, see Table 2).

Although Mg^{2+} is the actual cofactor in vivo, the highest inhibitory potency measured in the presence of Mn^{2+} allowed us to readily calculate an IC_{50} for compounds, like meconic acid **2**, whose inhibitory potency was virtually undetectable under standard assays conditions.

Further SAR studies were undertaken to evaluate the effects of the 6-ethylcarboxylate portion. Meconic acid (Table 1, compound **2**) itself, having the 6-carboxylic acid, is inactive in the standard assay conditions (Mg^{2+}). Moreover (Table 1, compounds **6**–**10**), decarboxylation, replacement with an ethyl- or diethyl-amide, with an ethylether or a benzylether was accompanied by substantial or complete loss of inhibitory potency, despite the apparent conservation of the metal binding pattern. The more bulky *tert*butylester **11** proved 10-fold less active than the ethyl ester **3**.

It seems reasonable that all these modifications had a secondary effect in the ionization and tautomerization behaviour of these analogues, which are likely to show

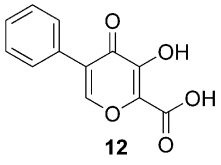
Table 2. Inhibition of NS5b HCV pol by meconic acid derivatives

Compound	Metal ion	IC_{50} (μM)[a]
2	Mg^{2+}	>100
2	Mn^{2+}	18
3	Mg^{2+}	2.25
3	Mn^{2+}	0.165
4	Mg^{2+}	>100
4	Mn^{2+}	4.0
9	Mg^{2+}	16.2
9	Mn^{2+}	0.8
13	Mg^{2+}	23
13	Mn^{2+}	1.9

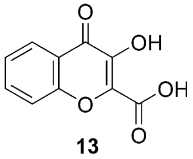
[a] See Ref. 6 for polymerase assay protocol. Data are the mean of two to four independent experiments.

different pK_a values for the enolic OH and the carboxylic acid. This hypothesis is consistent with results from Choux and Benoit,[9] who investigated the transmission of the substituent effect on a series of substituted 3-hydroxy-4-pyrones by measuring the pK_a values and establishing correlation with other properties.

Structural suggestion derived from our previous SAR studies on diketoacids inhibitors[3] (see also Fig. 2), which had revealed the presence of an aromatic moiety to be essential to improve binding affinity, prompted us to place a phenyl ring in the 5-position (**12**) or fused in a chromene system (**13**). However, instead of an increase in activity, the chromene compound **13** ($IC_{50} = 23\,\mu M$) lost 10-fold in potency and the 5-phenyl derivative **12** showed a somewhat larger decrease in activity.



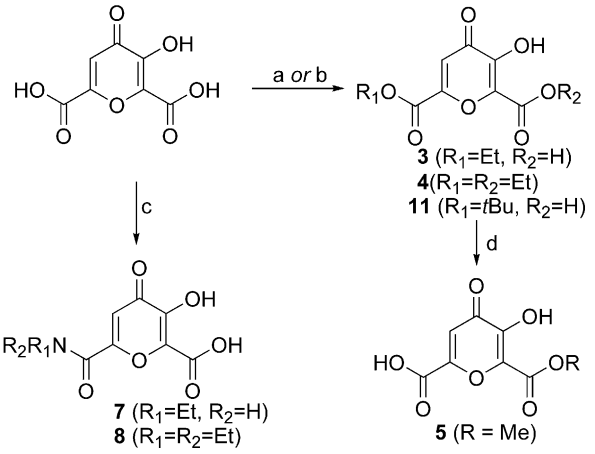
12



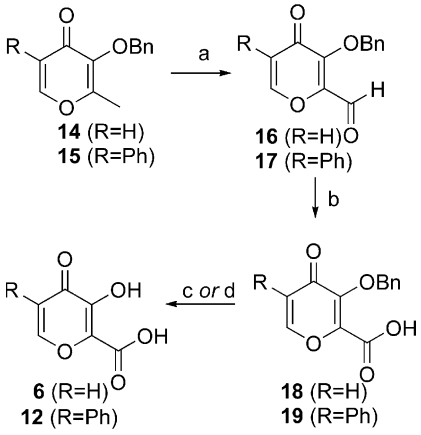
13

Unfortunately, none of the compounds active in the HCV NS5B polymerase assays displayed either significant inhibition of HCV RNA replication in a cell-based assay ($EC_{50} > 50\,\mu M$) or toxicity at the same concentration.

An issue when working with meconic acid is its stability.[10] Verkade[11] has described the partial decarboxylation of meconic acid with hydrochloric acid and has proven that the carboxyl lost during the reaction is from the position adjacent to the hydroxyl group. The solution to this will be the subject of further publications.



Scheme 1. Synthesis of meconic derivatives. Reagents and conditions: (a) CH_3COCl (10 equiv), MeOH or EtOH, 0 °C to room temp, 24 h or 4 days; (b) *i*-PrN=C(O-*t*-Bu)NH-*i*-Pr (2 equiv), CH_2Cl_2, room temp, 24 h; (c) $EtNH_2$ or Et_2NH, HOBT (1 equiv), EDCI (1 equiv), iPr_2EtN (2 equiv), DMF, 0 °C to room temp, 12 h; (d) 1 N NaOH (10 equiv), MeOH, 45 min.



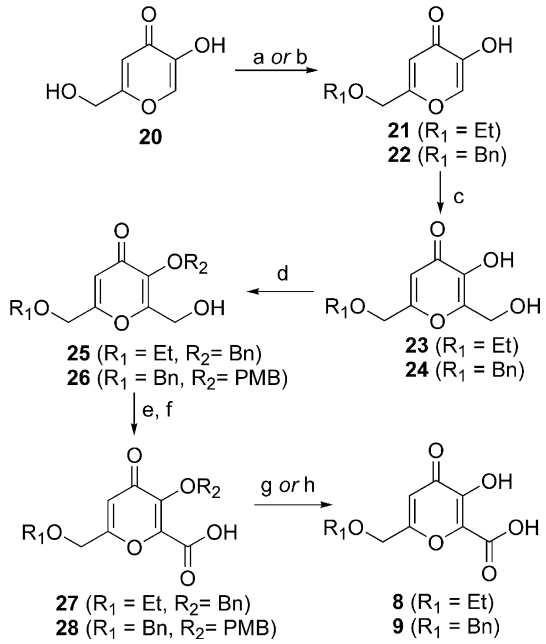
Scheme 2. Synthesis of compounds **6** and **12**. Reagents and conditions (see text): (a) SeO_2 (3 equiv), PhBr, 160 °C, 12 h; (b) $NaClO_2$ (1.5 equiv), H_2O_2 30%, NaH_2PO_4 (0.2 equiv), MeCN/H_2O (1/1 v/v), room temp, 1 h; (c) 18 → 6: $TMSCH_2N_2$, MeOH, room temp; 3 N HCl, reflux, 6 h; 1 N NaOH, MeOH, room temp; (d) 19 → 12: TMSI (1.3 equiv), MeOH, 1 h.

2. Synthesis

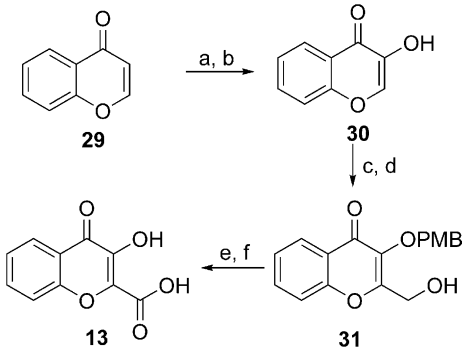
In order to identify the chemical structure of the screening sample and further study its biological properties, we prepared compound **3** and the related **4**, **5**, **7**, **8** and **11** as in Scheme 1. Reactivity of the two carboxylate groups of meconic acid is dependent on both electronic as well as steric factors. In particular, esterification with the appropriate alcohol or *O*-*tert*-butyl-*N*,*N*′-diisopropylisourea, as well as the amidification, occurs primarily at the 6-carboxylic acid.[12] Diester **4** can be achieved by prolonged reactions times, while upon saponification, it reverts to the free 6-carboxylic acid **5**. Similarly, compounds **7** and **8** were obtained by exposing meconic acid to the appropriate amine in the presence of coupling reagents.

Compound **6** was prepared from O-benzylmaltol **14** as in Scheme 2. Following literature precedents and after trying several conditions, we used SeO_2 for the oxidation of the 2-methyl to the aldehyde **16**, and $NaClO_2$ for further oxidation to the carboxylic acid **18**.[13] For stability reasons, we converted the carboxylate into its methyl ester before removal of the O-benzyl group. A similar protocol was followed also for the synthesis of **12**. The starting material **15** was prepared from maltol by a three step literature procedure, involving its 5-bromination,[14] O-benzylation and Suzuki coupling with phenylboronic acid.[15] Then, the two-step oxidation followed by TMSI cleavage of the benzylether afforded **12**.[16]

Compounds **9** and **10** were prepared form Kojic acid **20**, as depicted in Scheme 3.[17] Synthesis according to literature procedures allowed preparation of compounds **23** and **24**. Subsequent two step oxidation of the hydroxymethyl group required protection of the enolic function as a benzyl or *p*-methoxybenzyl ether.



Scheme 3. Synthesis of compounds **8** and **9**. Reagents and conditions: (a) BnBr (1.1 equiv), Cs_2CO_3 (1 equiv) DMF, 60 °C, 3 h; EtI (1.5 equiv), NaH (1.1 equiv), DMF, room temp, 1 h; 4 N HCl, reflux, 0.5 h; (b) PMBCl (1.1 equiv), Cs_2CO_3 (1 equiv), DMF; BnBr (1.1 equiv), NaH (1.1 equiv), DMF; CH_2Cl_2/TFA (9/1 v/v); (c) HCHO 35% sol (1.5 equiv), 1 N NaOH, room temp, 6 h; (d) BnBr or PMBCl (1 equiv), Cs_2CO_3 (1 equiv), DMF, 60 °C, 3 h; (e) MnO_2 (3 equiv), CH_2Cl_2, room temp, 24 h; (f) Ag_2O (10 equiv), NaCN (5 equiv), MeOH, room temp, 1 h; (g) 4 N HCl, reflux, 0.5 h; (h) CH_2Cl_2/TFA (9/1 v/v), room temp, 1 h.



Scheme 4. Synthesis of compound **13**. Reagents and conditions: (a) mCPBA (1 equiv), benzene, reflux, 3 h; (b) 1 N HCl, reflux, 1 h; (c) HCHO 35% sol (1. 5 equiv), 1 N NaOH, room temp, 6 h; (d) PMBCl (1.1 equiv), Cs_2CO_3 (1 equiv), DMF; (e) MnO_2 (30 equiv), CH_2Cl_2, room temp, 2 h; (f) Ag_2O (2 equiv), NaOH 10%, MeOH, room temp, 1 h.

For the synthesis of compound **13**, commercially available 4*H*-chromen-4-one **29** was selected as starting material (Scheme 4). Epoxidation and subsequent acid opening of the epoxide allowed for introduction of the hydroxyl group of **30**.[18] Synthesis of **11** from **30** utilized the same synthetic procedure previously reported.

3. Conclusion

The monoethyl ester of meconic acid was identified as new inhibitor of NS5B HCV polymerase. Although more potent and stable analogues will aid future studies, interpretation of our data in light of molecular modeling analysis and our previous experience with diketoacids inhibitors, led to important findings concerning the molecular determinants of HCV NS5B inhibitory activity.

Further efforts in modifying these lead structures are in progress and the results will be reported in due course.

Acknowledgements

The authors thank Mauro Cerretani, Nadia Gennari and Sergio Serafini for determination of the IC_{50} values for NS5b, Fabio Bonelli for mass analysis of the screening leads and of all the compounds, Uwe Koch for the modeling and Michael Rowley for valuable discussions. This work was supported in part by a grant from the MIUR.

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5. The NS5B polymerase was expressed as a C55 truncation in *E. coli* and purified to homogeneity. Reactions were carried out in 50 μL buffer containing 20 mM Tris/HCl (pH 7.5), 0.05% Triton X-100, 2% glycerol, 50mM NaCl, 1 mM DTT, 5 mM $MgCl_2$ and 5 nM purified NS5B_Δ C55 enzyme. Poly(rA)/oligo(rU) template/primer was present at a final concentration of 20 μg/mL and 3H-UTP (Amersham) at 10 μM concentration. The NS5B_Δ C55 protein was preincubated with template RNA template for 20 min at 23 °C in reaction buffer. The reaction was started by the addition of UTP and incubated at 23 °C for 30 min. The activity was measured as the radioactivity present in the acid-insoluble material. Compounds were dissolved in 100% DMSO and serial dilutions made in DMSO. IC_{50} values were calculated using three parameters logistic equation and inhibition data were fitted by Kaleidagraph software.
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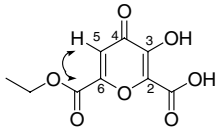
correlation between the (5)C*H* and the (6)*C*OOEt but not with the (2)*C*OOH.



Similar experiments corroborated also structural assignement of the other ester and amide derivatives.

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