SUPPLEMENTARY MATERIAL

Synthesis, molecular modeling and biological evaluation of two new chicoric acid analogs

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Two conformationally constrained compounds similar to chicoric acid but lacking the catechol and carboxyl groups were prepared. In these analogs the single bond between the two caffeoyl fragments has been replaced with a chiral oxirane ring and both aromatic residues modified protecting completely or partially the catechol moiety as methyl ether. Preliminary molecular modeling studies carried out on the two analogues showed interactions near the active site of HIV integrase (IN), however, in comparison with Raltegravir, the biological evaluation confirmed that CAA-1 and CAA-2 were unable to inhibit infection at lower concentration.

Keywords: HIV-integrase, inhibitors, chicoric acid, analogs

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The following compounds are known: **2** (Soulie et al. 1992), **3** (Rottger & Waldmann 2006), **4** (Hayashi et al. 2005)

The first esterification with 3,4-dimethoxycinnamic acid was carried out trying various conditions: (NMM (*N*-methylmorpholine)/CDMT (2-chloro-4,6-dimethoxy-1,3,5-triazine) in THF (tetrahydrofuran) at rt; NMM/CDMT in DMF (dimethylformamide) at rt; DMTMM (4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride)/NMM in THF at rt; DCC (*N,N'*-dicyclohexylcarbodiimide)/DMAP (4-(dimethylamino)pyridine) in DCM at rt), the best of which was DCC/DMAP in refluxing DCM (dichloromethane).

(2S,3S)-(3,4-Dimethoxy-phenyl)-acrylic acid 2,3-epoxy-4-(tert-butyldimethylsiloxy)-butyl ester 5: To a solution of **4** (1 mmol) and 3-(3,4-dimethoxy-phenyl)-acrylic acid (1.2 mmol) in dry CH₂Cl₂ (5 mL) was added DCC (2.1 mmol) and DMAP (0.05 mmol). The resulting mixture was heated at reflux overnight. The reaction was quenched with water, washed with brine, dried with Na₂SO₄ and concentrated in vacuo. The residue was purified by flash chromatography (20% AcOEt in hexanes) to afford **5** as yellow oil (87%). *R*_f=0.42 (silica gel, EtOAc/hexanes 3:7); [*α*]_D²⁵ = -19.9 (CHCl₃, *c* = 2.5). ¹H NMR (300 MHz, CDCl₃, 25°C): δ = 0.07 (s, 6H); 0.90 (s, 9H); 3.05-3.10 (m, 1H); 3.18-3.25 (m, 1H); 3.72 (dd, *J* = 2.5, 4.6 Hz, 1H); 3.85 (m, 1H); 3.88 (s, 6H); 4.08 (dd, *J* = 7.2, 12.3 Hz, 1H); 4.51 (dd, *J* = 3.6, 12.3 Hz, 1H); 6.37 (d, *J* = 15.9 Hz, 1H); 6.85 (d, *J* = 8.3 Hz, 1H); 7.03 (d, *J* = 1.8 Hz, 1H); 7.12 (dd, *J* = 1.8, 8.3 Hz, 1H); 7.63 (d, *J* = 15.9 Hz, 1H). ¹³C NMR (75.4 MHz, CDCl₃, 25°C): δ = -5.47, 18.1, 25.7, 55.8, 55.81, 56.3, 62.4, 64.0, 109.8, 111.0, 115.0, 122.6, 127.2, 145.2, 149.2, 151.2, 166.5.

(2S,3S)-(3,4-Dimethoxy-phenyl)-acrylic acid 2,3-epoxy-4-hydroxy-butyl ester 6: To a solution of **5** (1 mmol) in THF (5 mL) was added TBAF (2 mmol) and the resulting mixture was stirred at 0°C for 50 min. The reaction mixture was quenched with water (10 mL), the organic solvent removed in vacuo and then added AcOEt (10 mL). The aqueous phase was extracted with EtOAc (2×4 mL) and the combined organic extracts washed with brine (2×5 mL), dried with Na₂SO₄, and concentrated in vacuo to afford **6** as yellow oil (96%). *R*_f=0.11 (silica gel, EtOAc/hexanes 4:6). ¹H NMR (300 MHz, CDCl₃, 25°C): δ = 2.30 (br s, 1H); 3.12-3.19 (m, 1H); 3.35-3.39 (m, 1H); 3.62 (m, 1H); 3.82-4.01 (m, 1H); 3.88 (s, 6H); 3.14 (dd, *J* = 5.4, 12.3 Hz, 1H); 4.58 (dd, *J* = 4.6, 12.3 Hz, 1H); 6.25 (d, *J* = 16 Hz, 1H); 6.82-7.11 (m, 3H); 7.62 (d, *J* = 16.0 Hz, 1H). ¹³C NMR (75.4 MHz, CDCl₃, 25°C): δ = 53.1; 56.1; 55.8; 56.3; 60.8; 61.5; 64.9; 109.8; 114.0; 115.0; 122.9; 127.2; 145.8; 147.6; 149.1; 166.8.

(2S,3S)-(3,4-Dimethoxy-phenyl)-acrylic acid 2,3-epoxy-4-[3-(3,4-dimethoxy-phenyl)-acryloyloxy]-butyl ester CAA-1: Following the same procedure described for **5**, **CAA-1** was obtained as thin yellow powder (68%). $R_f=0.42$ (silica gel, EtOAc/hexanes 3:7); ^1H NMR (300 MHz, CDCl_3 , 25°C): δ = 3.25-3.55 (m, 2H); 3.88 (s, 12H), 4.12 (dd, $J = 5.5$ 12.4 Hz, 2H), 4.53 (dd, $J = 2.8$ 12.4 Hz, 2H), 6.33 (d, $J = 15.9$ Hz, 2H), 6.83 (d, $J = 8.3$ Hz, 2H), 7.05 (d, $J = 1.9$ Hz, 2H), 7.11 (dd, $J = 1.8$, 8.3 Hz, 2H), 7.63 (d, $J = 15.9$ Hz, 2H). ^{13}C NMR (75.4 MHz, CDCl_3 , 25°C): δ = 53.3, 55.8, 55.7; 63.4, 109.52, 111.9, 114.7; 122.7, 145.5, 149.1, 151.2, 166.5.

(2S,3S)-(4-Hydroxy-3-methoxy-phenyl)-acrylic acid 2,3-epoxy-4-(tert-butyldimethylsiloxy)-butyl ester 7: To a cooled (0°C) solution of alcohol **4** (1 mmol) and ferulic acid (1 mmol) in dry THF (1 mL) were slowly added TPP (1 mmol) and DIAD (1 mmol). After stirring at room temperature for 48 h, the reaction was worked up by removal of the solvent, and the residue was purified by flash chromatography (silica gel, hexanes-EtOAc gradient from 95-5 to 1-1) to afford **7** as yellow oil (57%). $R_f=0.84$ (silica gel, EtOAc/hexanes 4:6). ^1H NMR (300 MHz, CDCl_3 , 25°C): δ = 0.04 (s, 6H); 0.82 (s, 9H); 3.06 (dt, $J = 2.3$ 4.5 Hz, 1H); 3.21 (dt, $J = 2.3$ 6.1 Hz, 1H); 3.70 (dd, $J = 4.4$ 12.1 Hz, 2H); 3.77-3.97 (m, 3H); 4.07 (dd, $J = 6.3$ 12.4 Hz, 1H); 4.52 (dd, $J = 2.9$ 12.4 Hz, 1H); 6.24 (d, $J = 15.9$ Hz, 1H); 6.52 (s, 1H); 6.83 (d, $J = 8.1$ Hz, 1H); 6.92-6.98 (m, 2H); 7.60 (d, $J = 15.9$ Hz, 1H). ^{13}C NMR (75.4 MHz, CDCl_3 , 25°C): δ = -5.5; 18.1; 25.7; 52.8; 55.8; 56.4; 62.4; 64.0; 109.4; 114.5; 114.8; 123.1; 126.7; 145.5; 146.9; 148.2; 166.8.

(2S,3S)-(4-Hydroxy-3-methoxy-phenyl)-acrylic acid 2,3-epoxy-4-hydroxy-butyl ester 8: Following the same procedure described on **5**, **8** was obtained as yellow oil (96%). $R_f=0.24$ (silica gel, EtOAc/hexanes 1:1). ^1H NMR (300 MHz, CDCl_3 , 25°C): δ = 3.12-3.18 (m, 1H), 3.22-3.35 (m, 1H), 3.61-3.78 (m, 1H); 3.85-4.05 (m, 2H), 3.88 (s, 3H), 4.12 (dd, $J = 6.3$, 12.4 Hz, 1H), 4.51 (dd, J

= 2.9, 12.4 Hz, 1H), 6.31 (d, $J = 15.9$ Hz, 1H), 6.82-7.15 (m, 4H), 7.65 (d, $J = 15.9$ Hz, 1H). ^{13}C NMR (75.4 MHz, CDCl_3 , 25°C): δ = 53.2; 56.1; 56.5; 61.3; 64.1; 109.9; 114.5; 115.2; 123.4; 126.8; 146.1; 147.2; 148.6; 167.2

(2S,3S)-(4-Hydroxy-3-methoxy-phenyl)-acrylic acid 2,3-epoxy-4-[3-(4-Hydroxy-3-methoxy-phenyl)-acryloyloxy]-butyl ester CAA-2: Following the same procedure described for **CAA-1**, **CAA-2** was obtained as thin yellow powder (26%). ^1H NMR (300 MHz, CDCl_3 , 25°C): δ = 3.31 (m, 2H); 3.78 (s, 6H), 4.15 (dd, $J = 5.6$ 11.4 Hz, 2H), 4.49 (dd, $J = 2.3$ 12.4 Hz, 2H), 6.30 (d, $J = 15.9$ Hz, 2H), 6.52 (bs, 4H); 6.82 (d, $J = 8.2$ Hz, 2H); 6.87-6.98 (m, 2H), 7.60 (d, $J = 15.9$ Hz, 2H). ^{13}C NMR (75.4 MHz, CDCl_3 , 25°C): δ = 53.2; 55.7, 56.4; 64.0, 109.8; 114.7; 115.3; 123.7; 126.8; 145.9; 147.1; 148.2; 166.5.

Molecular modeling

The docking process requires a prediction of ligand conformation and orientation (or posing) within the binding site. Energy minimization of ligands was performed with the Sybyl program, and an extensive conformational search was carried out using the Monte Carlo program (E_i -E_{min} < 5 kcal/mol, energy difference between the generated conformation and the current minimum). Representative minimum energy conformations of each compound were optimized using the *ab initio* quantum chemistry program Gaussian 03 with DFTB3LYP method and 6-311G basis set. The protein subunit A of the IN core domain in complex with 5-CITEP (PDB 1QS4) was used for all docking studies. The missing residues at positions 141-144 in this subunit were incorporated from monomer B of the IN structure PDB 1BIS after superimposition of the backbone residues 135-140 and 145-150 (Sechi et al. 2004).

Docking was performed with AutoDock version 4.02 using the new empirical free energy function and the Lamarckian protocol (Morris et al 1996, 1998). Mass-centered grid maps were generated with 60 grid points for every direction and with 0.375 Å spacing by the AutoGrid program for the whole protein target. Random starting position on the entire protein surface, random orientations, and torsions were used for the ligands. 100 independent docking runs were carried out for each ligand. The cluster analysis was computed with a cluster tolerance at less than 1.5 Å in positional root-mean-square deviation.

c) Interaction of L-chicoric acid with the HIV Integrase active side (Healy et al. 2009)

Table S1. Amino acids interactions and hydrogen bonding

Ligands	%cluster	M.B.E. ^a	E.F.E.B. ^b	E.I.C.,Ki ^c	Interaction aa, H-Bonds
CCA-1	55	-2,95	-3,17	14,78 mM	ASP64 ASP116 ASN117 PHE139ILE141 GLN148 GLY149 ILE151
CCA-2	89	-2,46	-2,70	10,45 mM	ASP64 ASP116 ASN117 ILE141GLN148 GLY149 ILE151 MG2210
L-Chicoric Acid	12	-7,35	-9,00	253,72 nM	ASP64 CYS65 THR66 HIS67ASP116 GLN148 ILE151 GLU152 ASN155 LYS156LYS159 MG2210

^aM.B.E.= Mean binding energy(kcal/mol)

^bE.F.E.B. = Estimated free energy of binding(kcal/mol)

^cE.I.C., Ki = Estimated inhibition costant, Ki

Biological evaluation

Compounds CAA-1 and CAA-2 were tested for their cytotoxic activity on monocytoid cell line U937 through an assay assessing the inhibition of mitochondrial metabolic activity. This was obtained by measuring the reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) to formazan, using a commercial colorimetric kit (Cell Titer 96 Aqueous One Solution, Promega, Madison, WI), as previously described (Balestrieri et al. 2011). In order to determine the compound concentration that induces 50% growth inhibition (IC₅₀), MOLT-3 cells were treated with the compound at the concentrations of 1000 µM, 100 µM, 10 µM and 1 µM for 3 hours.

A potential functional activity of CAA-1 and CAA-2 as antiretroviral was assessed by assaying their effect on infection of peripheral blood mononuclear cells (PBMC) with HIV. The virus was prepared by ultracentrifugation of supernatant from a chronically HIV infected cell line H-9/HIV. The amount of virus within the virus lysate was indirectly assessed by determining the amount of p24. An amount equal to 30 ng of p24 was used to infect PBMC and after 3 days, the infection was assessed on the basis of the amount of p24 released in the supernatant of infected PBMC was assessed.

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