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Inhibition of porcine liver carboxylesterase by a new flavone glucoside isolated from *Deverra scoparia*

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Abstract

An endemic North African Saharan plant from of the Apiaceae family, *Deverra scoparia*, used locally for medicinal preparations, showed a strong inhibitory effect on porcine liver carboxylesterase. The active compound from the aerial part of the plant was purified by semi-preparative HPLC and photodiode array detection, and structurally determined by ^{1}H, ^{13}C NMR and mass spectroscopy methods. This compound was identified as flavone-3,4′,7-trihydroxy-3′-methoxy-7-glucoside and it was found to be a powerful competitive inhibitor of porcine liver carboxylesterase with a inhibition constant value of 16 μM. Based on the structural features of the inhibitor and the enzyme active site region, it seems that the flavonoside binds to the surface of the enzyme. The low K_i value suggests some physiological significance of such inhibitory activity, especially concerning the bio-transformation of xenobiotics.

Keywords: Carboxylesterase; Flavonoids; *Deverra scoparia*; HPLC

1. Introduction

Carboxylesterases (EC 3.1.1.1) are serine esterases present at high levels in a large array of animal tissues, including stomach, small intestine, colon, blood plasma, liver, heart, brain, lung, pancreas, spleen, testis and prostate [1]. They play a key biological role as they are able to hydrolyse numerous endogen and xenobiotic ester-containing substances. This group of esterases is in fact not clearly delimited, as compared to other esterases, such as lipases, acetylcholinesterase and cholesterol esterase, due to its large substrate specificity.

Carboxylesterase-mediated hydrolysis is used in the design of many drugs and pharmacophores, as for example, the chemotherapeutic agents CPT-11 [2,3], lovastatin, which is used for cholesterol managing [4]. Carboxylesterases (CE) also hydrolyse cocaine and heroin [5], as well as aspirin [6] and contribute to the detoxication of the pesticides pyrethroid [7] and carbamate [8], thus sparing acetylcholinesterase, which is targeted by these pesticides [9]. Pyrethroids are weakly toxic for mammals due their efficient CE activity [10]. Also, pest resistant strains of insects were found to have elevated levels of carboxylesterases [11,12]. Thus, controlling the activity of these enzymes would be highly useful for management of the biolog-

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ical impact of ester compounds consumed by humans through different ways. The most potent inhibitors of carboxylesterase found to date are synthetic compounds, such as trifluoromethyl ketone or nitrophyl derivatives [13–17].

Phenolics are in many cases the active principles of medicinal plants. Several beneficial properties have been attributed to these substances, including inhibition of several enzymes. We have recently reported inhibitory effects of flavonoids extracts from plants used in Arab folk medicine on porcine liver and rat intestinal carboxylesterase [18]. Here, a new flavone glucoside was purified from an endemic arabic-saharan plant of the Apiceae family, *Deverra scoparia* used in popular medicine to facilitate the digestion. This compound was found to inhibit porcine liver CE enzyme in a competitive manner at micromolar concentration level. The binding mode of the inhibitor is discussed based on the structural data.

2. Materials and methods

2.1. Materials

Purified porcine liver CE (19 unit/mg of proteins on ethylbutyrate) and all reagents were purchased from Sigma Aldrich. Enzyme solutions were prepared in 0.1 M Tris–HCl buffer (pH 7.4) and stored in the freezer until use. Plants were collected from a local herbalist in flowering times between March and May in Saharan Atlas of Algeria (at 40 km north of Laghouat). Samples were identified at the Agronomic National Institute of Alger, and the voucher specimens were deposited at the laboratory of Fundamental Sciences, University of Laghouat.

2.2. Extraction and isolation

Air-dried aerial parts of plants were finely powdered. 100 g of powder were homogenised in 1 L of cold aqueous methanol (80%). Three successive extractions with methanol (80%) were carried out at room temperature for 24 h. After removing methanol under vacuum at 40 °C, ammonium sulfate (20%) and metaphosphoric acid (2%) were added to the aqueous phase. Pigments and lipids were removed by three successive extractions with petroleum ether (2:1, v/v). Phenolic compounds were then extracted three times with dichloromethane then with ethyl acetate (1:1, v/v). The organic phases were combined and were dried with anhydrous sodium sulfate, and then evaporated to dryness under vacuum at 40 °C. The residue was dissolved in methanol (2 mL) and kept at −20 °C. The filtered extract (0.45 μm Millipore filters) was analysed by HPLC.

2.3. Analyses of phenolics

Chromatographic analysis of phenolics was performed with a Waters Alliance™ System consisting of a Waters 2690 XE separation module and a Waters 996 photodiode array detector controlled by the Waters Millenium™ Chromatography manager software. UV spectra were recorded in the range 220–600 nm to identify the different classes of phenolics. Their amounts in plant extracts were calculated from the characteristic absorbance maxima. Hydroxybenzoic compounds were quantified at 280 nm as gallic acid, hydrocinnamic compounds at 320 nm as caffeic acid and flavonoids at 360 nm as quercetin.

2.4. Analytical HPLC

Reverse phase HPLC was carried out at 30 °C on a purosphere RP-18 column (250 mm × 4 mm; 5 μm; Merck) using a gradient (1 mL min^{-1}) based on solvent A (0.1% TFA in water) and solvent B (MeOH/ACN: 50/50, v/v): 0–20 min, 20–40% B; 20–25 min, 40–60% B; 25–35 min, 60% B; 35–40 min, 60–70% B.

2.5. Semi-preparative HPLC

Semi-preparative HPLC was performed at 30 °C on a purosphere RP-18 column (250 mm × 10 mm; 10 μm; Merck) using a gradient (3 mL min^{-1}): 0–25 min, 10–40% B; 25–35 min, 40–60% B; 35–40 min, 60% B. Fractions were collected on a microplate sampler and freeze dried.

2.6. General NMR and ESI-MS procedures

NMR spectra were recorded at 300 and 75 MHz for ^{1}H and ^{13}C, respectively. Samples were dissolved in MeOD and chemical shifts are expressed in δ (ppm) referring to TMS.

The EI-MS in negative and positive mode was taken on a triple stage quadruple API III Plus (Sciex, Canada) spectrometer. A solution of the compounds was directly injected into the sprayer of the ES source. The nebuliser gas flow was set to 0.6 μL min^{-1} and the desolvation gas flow to 5 μL min^{-1}. The ESI voltage was 5 kV above the acceleration potential for the spectrometer (ca. 5000 V). For operation in the MS/MS mode, collision gas was Argon with a pressure of 1×10^{-3} mbar in the collision

cell. The MS/MS spectra were recorded for energy of collision of 10, 20 or 30 eV.

2.7. Enzyme inhibition assay

The enzymatic activity of CE from porcine liver was determined using *p*-nitrophenyl acetate as substrate. The liberated *p*-nitrophenol is monitored at 414 nm using a microplate reader (iEMS-Labsystem S.A., France). The enzyme assay was performed using 96-well plates (250 μL/well) at 37 °C in 20 mM Tris, pH 7.4 using porcine liver carboxylesterase (1 μg/mL) and three concentrations of substrate (0.2, 0.5 and 1.0 mM). Data were recorded at 15 s intervals for up to 5 min. Inhibition kinetics were performed using varying concentration of phenolic extracts or purified flavonoid in the assay mixture. Reciprocal initial velocity plots versus inhibitor concentration were used to determine the mode of inhibition. The inhibition constants (K_i) were calculated according to the Dixon model [19] from curve fits using GraphPad Prism software.

3. Results and discussion

Twenty plants known for their therapeutic properties in traditional Arab medicine were tested on the enzymatic activity of porcine liver CE. This enzyme was chosen because it plays a key role in toxication–detoxication processes. It showed normal Michaelis kinetics on *p*-nitrophenyl acetate and, for each extract, the IC_{50} values were calculated from the plot of the enzyme activity as a function of phenolics concentration arbitrary expressed as μM of quercetin, gallic acid or caffeic acid, according to the phenolic class of the extract (data not shown).

Among all plant phenolic extracts, only that of *D. scoparia* was found to inhibit significantly, in a concentration-dependent manner, the CE activity. So far, no phytochemical studies were done on this plant which is very locally distributed in the septentrional ecoregion of the Algerian Atlas. We have then managed the isolation, purification and identification of the active compound(s) in *D. scoparia* as regards carboxylesterase activity.

The major components of the phenolic extract of *D. scoparia* were separated by analytical HPLC as described in Section 2. The corresponding chromatograms exhibited only peaks with flavonol derivative. According to this analytical chromatogram five fractions (as shown in Fig. 1) were collected by semi-preparative HPLC and tested on porcine carboxylesterase activity. Our results show that only fraction III which account for 80.7% of the total phenolics, significantly inhibits the enzyme activity. From these results, the inhibitory fraction was used for detailed spectroscopic analysis and structure elucidation. The molecular structure of the isolated fraction was unambiguously determined by mass, ^{1}H and ^{13}C NMR spectroscopy. Thus, mass spectroscopy analysis demonstrated the presence of a molecular ion at *m/z* 478.1111 Da corresponding to a component possessing a $C_{22}H_{22}O_{12}$ molecular formula. A fragment ion at *m/z* 162 Da corresponding to a neutral molecule is also observed and can be attributed to a glycosyl unit. This assumption is supported by the interpretation of the ^{1}H and ^{13}C NMR spectra indicating the presence of an *O*-linked glycosyl unit with a characteristic ^{1}H proton doublet at 4.40 ppm exhibiting a coupling constant of 6.0 Hz. Moreover, a signal at 58.69 ppm assigned to the CH_2 group can be noticed as well as a chemical shift δ 162.25 ppm characteristic to the C-9 carbon bearing the glycosyl unit.

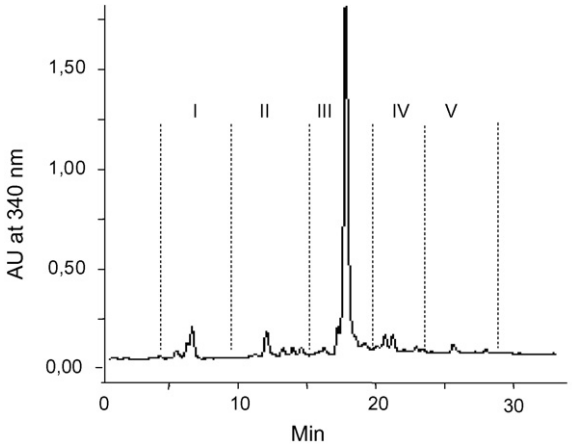

Fig. 1. Analytical HPLC chromatogram of phenolics extract of *Deverra scoparia*. Fractions I to V were collected by semi-preparative HPLC.

The remaining fragment of the molecule possesses a $C_{16}H_{10}O_7$ molecular formula presenting an ion at *m/z* 314.04 Da. ^{13}C NMR spectrum analysis led us to the assignment at 175.51 ppm of a carbonyl group characteristic to such a flavonoid structure. A signal at 131.10 ppm pertaining to C-3 (C-OH moiety) led to the confirmation of this proposal. Examination of ^{1}H and ^{13}C NMR spectra demonstrates the presence of two phenol moieties (OH) at 8.63 and 9.72 ppm and 144.96 and 154.48 ppm, respectively. The presence of a methoxy group is also well determined by the presence of a signal at δ 53.77 ppm.

The molecular structure of compound shown in Fig. 2, which is in accordance with the mass and NMR data, is therefore 3,5-dihydroxy-2-(3-hydroxy-4-meth-

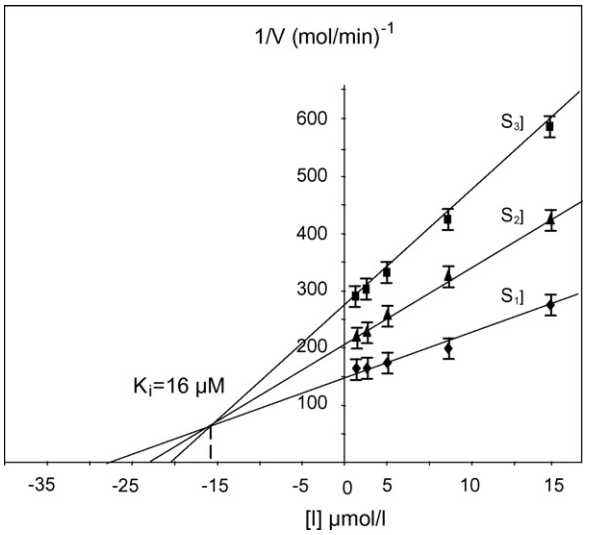

Fig. 2. Dixon plots of inhibition of porcine carboxylesterase by the flavonol derivative isolated from *Deverra scoparia* expressed in molar equivalent (μM) of quercetin. Substrate concentrations: 0.25 mM (S_1), 0.50 mM (S_2) and 1.00 mM (S_3). The graph represents the means of three experiments.

oxy-phenyl)-7-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydropyran-2-yloxy)-chromen-4-one 1: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 11.5$ (s, 1H), 9.72 (s, 1H), 8.63 (s, 1H), 7.12 (s, 1H), 6.80 (s, 1H), 6.36–6.33 (m, 1H), 5.78–5.75 (m, 1H), 5.28 (s, 1H), 5.06 (s, 1H), 4.42–4.40 (d, $J = 6$ Hz, 1H), 4.22–1.94 (m, 12 H). ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 175.51$, 162.25, 159.35, 154.48, 147.48, 144.96, 131.10, 120.08, 119.16, 113.25, 111.54, 106.26, 102.15, 98.95, 96.78, 91.73, 78.37, 75.48, 74.51, 72.40, 67.88, 58.70, and 53.77.

This compound described as flavone-3,4′,7-trihydroxy-3′-methoxy-7-glucoside forms part of a known family of flavonoid but was never isolated until now.

This flavonoid was found to inhibit CE in competitive manner with an inhibition constant of 16 μM (Fig. 2). This corresponds to about 8 mg/L, a concentration at least two magnitude lower than the ingested total phenolics. It is possible that the medicinal properties of this plant are linked to this competitive activity on carboxylesterase. Additional studies are needed for responding to this question.

Carboxylesterase belong to the group of serine dependent enzymes, some having lipolytic and others proteolytic activities, sharing a catalytic cycle in which the nucleophilic attack on the carbonyl carbon by OH group of serine residue with a concomitant transfer of the proton to histidine residue leads to the formation of a tetrahedral intermediate.

Porcine and human CE have similar substrate hydrolysis preferences for some prodrugs and share high-sequence homology (about 80% sequence identity and 88% similarity). Three crystal structures of human and rabbit liver CE have been reported to date [20–22]. The active site of the human CE containing the catalytic triad, is a 10–15 Å deep hydrophobic pocket at the interface of three domains. Similarly, computer modelling of human CE and rabbit liver CE has shown that the catalytic amino acids are buried at the bottom of a long deep gorge [1,23]. The active site is lined with aromatic residues, and thus hydrophobic molecules will be preferentially accommodated.

Elsewhere, an active-site model of pig liver esterase showed that the binding regions controlling specificity are composed of two hydrophobic and two hydrophilic pockets [24]. The larger hydrophobic pocket, which interacts with aliphatic or aromatic hydrocarbons has a volume of about 30 Å^3, while the smaller hydrophobic pocket has a volume of about 6 Å^3. The hydrophilic pockets are located at the front and back of the active site. These data suggest the enzyme may accommodate more or less large ester-containing compounds. The active site gorge diameter for all models is in the range of 3–4 Å [23].

The structure of the flavonoside isolated in this study from *D. scoparia* (Fig. 3) shows that electrostatic interactions are not involved to its binding to the enzyme. One may expect that exposed oxygen atoms of the inhibitor form hydrogen bonds with hydrophilic residues present within the active site gorge or at the binding surface of the enzyme. They consequently prevent the substrate to access to the catalytic amino acids. The hydropho-

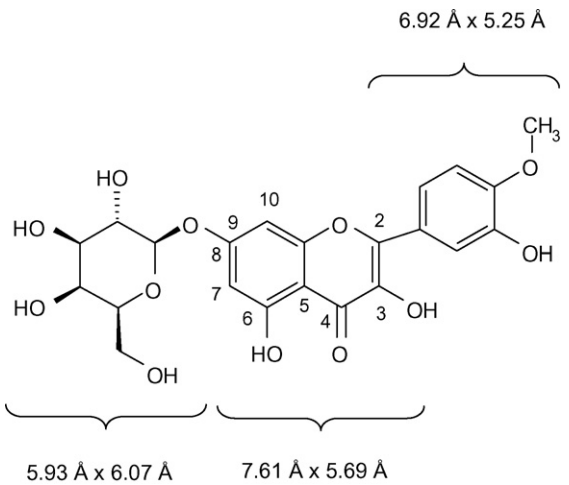

Fig. 3. Molecular structure and dimensions from a planar model of isolated fraction from phenolic extract of *Deverra scoparia*.

bicity is also a determinant of enzyme specificity for carboxylesterase inhibitors, and for many esterases the most potent inhibitor have large log P values [25]. As the flavonoside is weakly lipophilic with a log P value of 1.77, one may suppose that hydrophobic interactions, engaging the pyranose and phenol rings, play only a minor role in interaction with the enzyme. In addition, the width of the flavonoside shown in Fig. 3 is more than 5 Å and is larger than the substrate-binding regions of CE. It should therefore cover the catalytic pocket, thus sterically hindering the binding of the substrate to the active site. Additional studies are necessary for testing these suppositions. This will throw additional light on how does work this enzyme.

4. Conclusion

In this study, we have isolated the flavonoside flavone-3,4′,7-trihydroxy-3′-methoxy-7-glucoside from a medicinal plant, *D. scoparia*. This compound was found to be a potent competitive inhibitor of porcine carboxylesterase ($K_i = 16$ μM). The structural data of the enzyme and the flavonoside clearly show that the latter cannot profoundly enter the active site or the binding pockets, but rather stays at the surface of the enzyme closing the access of the substrate to the catalytic site. Hydrogen and other low energy interactions may contribute to the binding of the inhibitor to the enzyme, rather than hydrophobic interactions. Beside these mechanistic considerations one should question about the link between the carboxylase-inhibitory activity of the flavonoside and the medicinal properties of the plant from which it was isolated.

References

[1] T. Satoh, M. Hosokawa, Annu. Rev. Pharmacol. Toxicol. 38 (1998) 257.
[2] P.D. Senter, H. Marquardt, B.A. Thomas, B.D. Hammock, I.S. Frank, H.P. Svensson, Cancer Res. 56 (1996) 1471.
[3] A. Kojima, N.R. Hackett, A. Ohwada, R.G. Crystal, J. Clin. Invest. 101 (1998) 1789.
[4] B.K. Tang, W. Kalow, J. Clin. Pharmacol. 47 (1995) 449.
[5] E.V. Pindel, N.Y. Kedishvili, T.L. Abraham, M.R. Brzezinski, J. Zhang, R.A. Dean, W.F. Borson, J. Biol. Chem. 272 (1997) 14769.
[6] F.M. Williams, E.M. Mutch, E. Nicholson, H. Wynne, P. Wright, D. Lambert, M.D. Rawlins, J. Pharm. Pharmacol. 41 (1989) 407.
[7] J.E. Casida, D.W. Gammon, A.H. Glickman, L.J. Lawrence, Annu. Rev. Pharmacol. Toxicol. 23 (1983) 413.
[8] R.C. Gupta, W.D. Dettbarn, Chem. Biol. Interact. 87 (1993) 295.
[9] D.M. Maxwell, Toxicol. Appl. Pharmacol. 114 (1992) 306.
[10] A.H. Glickman, J.E. Casida, Neurobehav. Toxicol. Teratol. 4 (1982) 793.
[11] F.J. Byrne, K.J. Gorman, M. Cahill, I. Denholm, A.L. Devonshire, Pest Manage. Sci. 56 (2000) 867.
[12] C.M. Conyers, A.D. MacNicoll, N.R. Price, Insect Biochem. Mol. Biol. 28 (1998) 435.
[13] A. Székàcs, B. Bordàs, B.D. Hammock, Rational Approaches to structure, Activity and Ecotoxicology of Agrochemicals, CRC Press, Boca Raton, FL, 1992, pp. 219–249.
[14] T.L. Huang, T. shiotsuki, T. Uematsu, B. Borhan, Q.X. Li, et al., Pharm. Res. 13 (1996) 1495.
[15] M.B.A. Ashour, B.D. Hammock, Biochem. Pharmacol. 36 (1987) 1869.
[16] C.E. Wheelock, T.F. Sverson, B.D. Hammock, Chem. Res. Toxicol. 14 (2001) 1563.
[17] K.J.P. Yoon, J.L. Hyatt, C.L. Morton, R.E. Lee, P.M. Potter, M.K. Danks, Mol. Cancer Ther. 3 (2004) 903.
[18] P. Stocker, M. Yousfi, O. Djerridane, J. Perrier, R. Amziani, S. El Boustani, A. Moulin, Biochimie 86 (2004) 919.
[19] M. Dixon, Biochem. J. 55 (1953) 170.
[20] C.L. Bencharit, J.L. Morton, P. Hyatt, M.K. Kuhn, P.M. Danks, M.R. Potter, M.R. Redinbo, Chem. Biol. 10 (2003) 341.
[21] S. Bencharit, C.L. Morton, Y. Xue, P.M. Potter, M.R. Redinbo, Nat. Struct. Biol. 10 (2003) 349.
[22] S. Bencharit, C.L. Morton, E.L. Howard-Williams, M.K. Danks, P.M. Potter, M.R. Redinbo, Nat. Struct. Biol. 9 (2002) 337.
[23] R.M. Wadkins, C.L. Morton, J.K. Weeks, L. Oliver, M. Wierdl, M.K. Danks, P.M. Potter, Mol. Pharmacol. 60 (2001) 355.
[24] E.J. Toone, M.J. Werth, J. Jones, J. Am. Chem. Soc. 112 (1990) 4946.
[25] C.E. Wheelock, M.E. Colvin, I. Uemura, M.M. Olmstead, J.R. Sanborn, Y. Nakagawa, D. Jones, D. Bruce, Hammock, J. Med. Chem. 45 (2002) 5576.