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Natural product hybrid and its superacid synthesized analogues: Dodoneine and its derivatives show selective inhibition of carbonic anhydrase isoforms I, III, XIII and XIV

Hélène Carreyre [a], Jean-Marie Coustard [a], Grégoire Carré [b], Clarisse Vandebrouck [b], Jocelyn Bescond [b], Maurice Ouédraogo [c], Jérôme Marrot [d], Daniella Vullo [e], Claudiu T. Supuran [f,*], Sébastien Thibaudeau [a,*]

[a] Superacid Group in Organic Synthesis Team—Université de Poitiers, CNRS UMR 7285 IC2MP, 4, Avenue Michel Brunet, F-86022 Poitiers Cedex, France
[b] Institut de Physiologie et Biologie Cellulaires, Université de Poitiers, FRE 3511, 1 rue Georges Bonnet, F-86022 Poitiers Cedex, France
[c] Université de Ouagadougou, 03 BP 7021, Ouagadougou 01, Burkina Faso
[d] Institut Lavoisier de Versailles, UMR CNRS 8180, Université de Versailles St-Quentin, 45 avenue des Etats Unis, 78035 Versailles Cedex, France
[e] Università degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Rm 188, Via della Lastruccia 3, I-50019 Sesto Fiorentino (Firenze), Italy
[f] Università degli Studi di Firenze, Neurofarba Department, Section of Pharmaceutical Sciences, Via Ugo Schiff 6, I-50019 Sesto Fiorentino (Firenze), Italy

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ABSTRACT

The natural product dodoneine (a dihydropyranone phenolic compound), extracted from African mistletoe *Agelanthus dodoneifolius*, has been investigated as inhibitor of several human carbonic anhydrase (hCA, EC 4.2.1.1) isoforms. By using superacid chemistry, analogues of the lactone phenolic hybrid lead compound have been synthesized and tested as CA inhibitors. Small chemical modifications of the basic scaffold revealed strong changes in the selectivity profile against different CA isoforms. These new compounds selectively inhibited isoforms CA I (K_Is in the range of 0.13–0.76 μM), III (K_Is in the range of 5.13–10.80 μM), XIII (K_Is in the range of 0.34–0.96 μM) and XIV (K_Is in the range of 2.44–7.24 μM), and can be considered as new leads, probably acting as non-zinc-binders, similar to other phenols/lactones investigated earlier.

1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are widespread enzymes in all organisms. These metalloenzymes catalyse CO_2 hydratation to bicarbonate and protons.[1] CO_2, bicarbonate and protons are essential molecules/ions in many important physiological/pathologic processes. The catalytic mechanism of CAs is understood in detail. In all enzyme classes, a metal hydroxide species (L_3–Zn^{2+}–OH^-) of the enzyme is the catalytically active species, acting as a strong nucleophile (at neutral pH) on the CO_2 molecule bound in a hydrophobic pocket nearby.[2] So far, 16 different α-CA isoforms were isolated and characterized in mammals, where they play important physiological roles.[3] Many mammalian CAs are well established therapeutic targets, with the potential to be inhibited to treat a wide range of disorders, including glaucoma, epilepsy, obesity, hyperglycemia and more recently cancer.[4] In the quest of CA inhibitors (CAIs), beside the classical primary sulfonamides,[5] new families were recently discovered to act as good inhibitors.[6] In the design of new drugs, the selective inhibition of CAs became an essential parameter, especially considering the high number of isozymes in mammals and their widespread distribution in the organism.[4] Recently, a novel strategy to attain good selectivity was developed: designing of mechanism-based inhibitors that may interact in other part of the enzyme than the classical zinc binding zone. Such a strategy led to the discovery of new chemotypes that selectively inhibit some targeted hCA isoforms. For instance coumarins, thiocoumarines[7] and more recently sulfocoumarins,[8] located at the entrance of the enzyme active site, were found to act as selective inhibitors of several isoforms, such as hCA IX isozyme, a validated anti-cancer target, and some of these compounds are currently under clinical trials. Recently, these and other groups discovered two new classes of inhibitors which do not bind to the metal ion within the CA active site. Phenol derivatives[9] and lactones[10] were found to be low micromolar CAIs. Phenol was found anchored to the Zn(II)-coordinated water molecule, binding through two hydrogen bonds to this molecule and the OH of Thr199,[11] and can be considered as a non-zinc binding inhibitor. In the same time, our group recently isolated and characterized a new dihydropyranone phenolic compound named dodoneine, **1**, from an African mistletoe *Agelanthus dodoneifolius* (Fig. 1).[12]

* Corresponding authors. Tel.: +39 055 4573005; fax: +39 055 4573385 (C.T.S.); tel.: +33 549454588; fax: +33 549453501 (S.T.).
E-mail addresses: claudiu.supuran@unifi.it (C.T. Supuran), sebastien.thibaudeau@univ-poitiers.fr (S. Thibaudeau).

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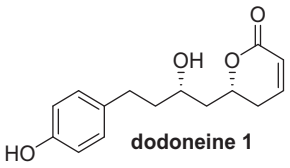


Figure 1.

Considering the fact that this compound incorporates both the phenol and lactone chemotypes known to act as CAIs, dodoneine may be considered as an interesting natural product hybrid which might possess CA inhibitory action.[13] A question arises as to whether combining these two moieties in the natural product hybrid molecule dodoneine, could be of interest to access to novel selective CAIs. Here we investigated this possibility, and report the evaluation of dodoneine and its superacid synthesized analogues as CAIs. Among the tested compounds, new potent hCA I, hCA III, mCA XIII and hCA XIV (h = human, m = murine isoforms) selective inhibitors have been identified.

2. Results and discussion

2.1. Chemistry

Initially, dodoneine **1** was tested against all the catalytically active mammalian CA isoforms, hCA I–XIV (Table 1).

Dodoneine showed inhibition in the range of 5.5–10.4 μM against isoforms I, III, IV, XIII and XIV and did not inhibit the other isoforms. To the best of our knowledge, in terms of selectivity, the inhibition profile of this compound was really original and let us envisages that dodoneine **1** could be considered as a new lead compound in the design of selective CAIs. Beside its micromolar level of inhibition toward isoforms known for their low to very low catalytic activity, this compound inhibits isoforms differing strongly for subcellular localization (hCA I, III and XIII are cytosolic, hCA IV is localized in plasma membrane and hCA XIV is a membrane bounded isozyme). This inhibitory profile let us envisage a new mode of action, results which deserve to test other analogues. However, since the discovery of dodoneine, even if various groups elegantly performed its synthesis,[14] the difficulty to access easily and efficiently to a variety of analogues, probably discouraged ambitious projects in this direction.

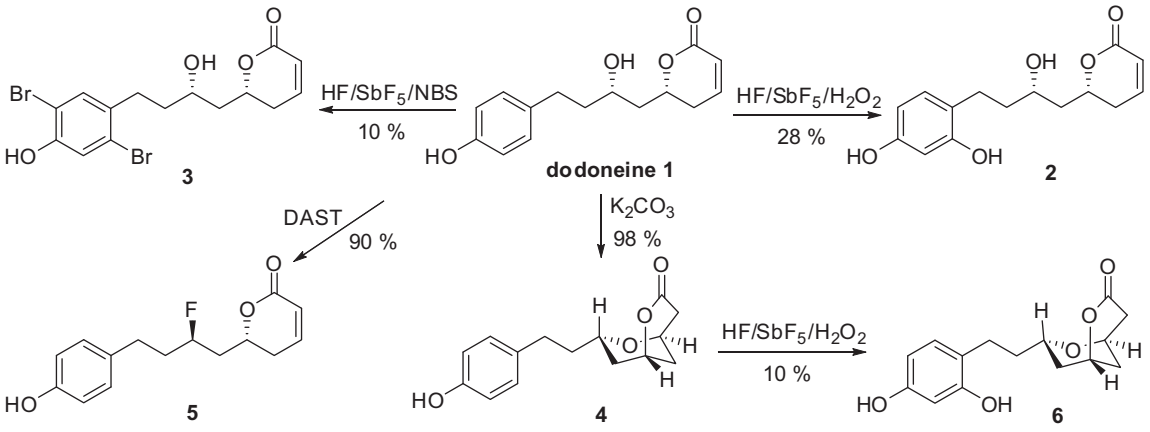
The possibility to selectively access to novel compounds starting from usually non reactive substrates, via superacid-catalyzed reactions[15] (e.g., CH activation,[16] fluorination,[17] hydroxylation,[18] Friedel–Crafts and other reactions)[19] has been largely documented. On account of the exceedingly nature of such catalysts, under HF/SbF_5 superacid conditions, natural products react in their (poly)protonated forms (one could consider the substrate being protected by protonation). As a consequence, novel modifications, than are observed with conventional media, can be performed directly to the elaborated products, without suffering from long synthetic routes, non compatible with rare natural products modifications. This strategy has been previously applied to indole, Cinchona and Vinca alkaloids.[20] The commercialization of the difluorinated anticancer agent vinflunine (Javlor®)[21] synthesized in superacid HF/SbF_5 additionally demonstrates that this technology is already operational at an industrial scale. This chemistry was applied to the synthesis of new dodoneine analogues **2** and **3** (Scheme 1).

Starting from dodoneine **1** after superelectrophilic activation[22] in the presence of H_2O_2,[23] the hydroxylated analogue **2** was synthesized. Interestingly, this compound was also found in very small quantity in the plant methanolic extract,[24] confirming that superacid electrophilic hydroxylation can be considered as a new direct tool to synthesize hydroxylated metabolites of natural products. Bromination of dodoneine **1** by using HF/SbF_5/NBS procedure[25] led to the formation of dibrominated analogue **3**.[26]

Table 1
hCA inhibition data of dodoneine and analogues by a stopped flow CO_2 hydrase assay[33]

Compound	K_i[a] (μm)											
	hCA I	hCA II	hCA III	hCA IV	hCA Va	hCA Vb	hCA VI	hCA VII	hCA IX	hCA XII	mCA XIII	hCA XIV
1	5.48	—[b]	10.35	9.61	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	9.27	9.34
2	0.38	—[b]	—[b]	4.12	21.6	13.7	—[b]	—[b]	32.6	24.5	8.13	7.24
3	0.76	21.8	—[b]	13.7	8.55	6.32	—[b]	—[b]	15.7	10.8	7.89	12.5
4	—[b]	—[b]	10.80	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	0.91	—[b]
5	0.13	36.9	—[b]	5.36	7.13	1.36	—[b]	24.9	3.57	1.48	0.96	2.44
6	—[b]	—[b]	5.13	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	0.34	—[b]

[a] Errors in the range of ±5% of the reported data from three different assays.
[b] Not active >100.



Scheme 1. Superacid chemistry on dodonein **1** leading to derivatives **2**–**6**.

2.2. CA inhibition

Dodonein 1, and its analogues **2**–**6** were evaluated as hCA inhibitors and tested against the hCA I–hCA XIV isoforms (Table 1). Interestingly the modification of the aromatic ring substitution strongly modifies the inhibition profile of the compound. Hydroxylated analogue **2** inhibits hCA I at nanomolar level and hCA IV, V, IX, XII, XIII and XIV at low micromolar level (Table 1). However compound **2** was found to be inactive toward hCA II, III, VI and VII. The substitution of the aromatic ring with halogen atoms dramatically changes inhibition properties. Against isozymes hCA III, VI and VII, compound **3** was inactive, and the other isoforms were inhibited at micromolar level by this dibrominated analogue. These results confirmed that these compounds could interact differently within the active sites of the enzyme. Back to the dodoneine extract study, we identified a bicyclic analogue of dodoneine (compound **4**) present in low quantity in the methanolic extract. To test it, the compound was synthesized from dodoneine. Under basic conditions, **1** underwent an easy intramolecular cyclization by conjugate addition of the hydroxyl group to the double bond, to afford the thermodynamically stable bicyclic lactone **4**, metabolite of dodoneine.[12,27] Compound **4** was found to be the first highly selective hCA III (K_i = 10.8 μM) and mCA XIII inhibitor (K_i = 910 nM) reported to date.

Among the 15 human isoforms, hCA III is by far, the least understood and investigated.[28] However, the recently found physiologic/pathologic functions of hCA III make this enzyme a target of choice in the quest of new drugs.[29] To evaluate whether dodoneine could be considered as a prodrug of **4**, the cyclisation process in physiological conditions was evaluated. After 12 h half of dodoneine was converted to its bicyclic analogue **4**.[24] However, the different inhibition profile of both compounds does not confirm this hypothesis. To pursue this investigation we envisaged the synthesis of a dodoneine analogue, which could not metabolizes to the bicyclic analogue. Carbon–fluorine bond being really strong and fluorine atom being classically used to stabilize compound against metabolic modification,[30] fluorinated analogue **5** was synthesized by fluorodehydroxylation reaction in the presence of DAST, and evaluated as carbonic anhydrase inhibitor (Table 1).[31] As mentioned above, again, the different inhibitory profile of the fluorinated analogue **5** inhibiting at low micromolar level all hCA isoforms, despite hCA III and VI, seems to refute the hypothesis that dodoneine **1** could be considered as a prodrug of the bicyclic highly selective inhibitor **4**. However, the modification of the alcohol function of the aliphatic chain strongly modifies the the inhibitor properties of these compounds, a result which emphasizes the importance of the aliphatic core structure on the inhibition profile. Considering the previously reported vasodilatator effect of dodoneine[12] and its CA inhibitory profile, further investigations could be pursue to evaluate possible correlations.[32] To confirm that the phenolic bicyclic lactone is the essential pharmacophore to attain the CA III and CA XIII inhibition selectivity, an hydroxylated analogue of **4** was synthesized and evaluated. Compound **6** also inhibited selectively hCA III (K_i = 5.1 μM) and mCA XIII (K_i = 340 nM) confirming the high potential of this new chemotype.

3. Conclusion

In conclusion, several natural product hybrids have been synthesized by using direct superacid catalyzed reactions on natural product dodoneine extracted from *Agelanthus dodoneifolius*, and evaluated as new CA inhibitors. The interesting inhibitory profile diversity of these new compounds make them important leads in the quest of new non-zinc-binding selective inhibitors, probably interfering differently in the enzymes active sites. Two new highly selective hCA III and XIII inhibitors were discovered. These new hybrid molecules become targets of choice for further therapeutic evaluations.

4. Experimental part

4.1. Chemistry

4.1.1. General procedure

The authors draw the reader's attention to the dangerous features of superacidic chemistry. Handling of hydrogen fluoride and antimony pentafluoride must be done by experienced chemists with all the necessary safety arrangements in place.

Reactions performed in superacid were carried out in a sealed Teflon® flask with a magnetic stirrer. No further precautions have to be taken to prevent mixture from moisture (test reaction worked out in anhydrous conditions leads to the same results as expected).

Yields refer to isolated pure products.

^{1}H, ^{13}C and ^{19}F NMR were recorded on a 400 MHz Bruker Advance DPX spectrometer using $CDCl_3$, or CD_3OD as solvent. COSY ^{1}H–^{1}H and ^{1}H–^{13}C experiments were used to confirm the NMR peaks assignments.

All separations were done on preparative TLC plate.

High Resolution Mass Spectrometry (HRMS) spectra were performed at the Centre Régional de Mesures Physiques de l'Ouest of the University of Rennes 1, France or at the Institut de Chimie Organique et Analytique of the University of Orleans, France.

4.1.2. Preparation of HF/SbF_5 mixture

After condensation of the desired quantity of hydrogen fluoride in a Teflon® reactor at −30 °C, antimony pentafluoride was slowly added to the reactor at the same temperature and the reactor was then sealed and maintained at reaction temperature. In these conditions, the SbF_5 molar percentage was determined accordingly: for example, 1 mL of SbF_5 (13.8×10^{-3} mol) was added to 3 mL of anhydrous liquid HF (0.15 mol) to give a HF/SbF_5 solution with a SbF_5 molar percentage of 8.4 mol %.

4.1.3. Synthesis of compounds 2 and 6

Optimized procedure in superacidic media: to a mixture of HF/SbF_5 (4 mL, SbF_5 mol % = 8.4) maintained at −20 °C, was added substrate and H_2O_2 by dropwise addition of 0.6 mmol. The mixture was magnetically stirred at the same temperature during 30 min. The reaction mixture was then hydrolyzed in ice and neutralized with Na_2CO_3 until pH 6.5, extracted with dichloromethane (×2) and then with ethyl acetate (×2). The combined organic phases were dried ($MgSO_4$) and concentrated in vacuo. Products were then purified by preparative TLC plate.

4.1.3.1. Compound 2: (*R*)-6-[(*S*)-2-hydroxy-4-(3,4-dihydroxyphenyl)butyl]-5,6-dihydropyran-2-one. This compound was obtained from dodoneine **1** (100 mg, 0.38 mmol) following the optimized procedure. The reaction crude was purified with CH_2Cl_2/EtOH: 95/5 as eluent. Compound **2** (29 mg) was eluted (R = 28%). ^{1}H NMR (CD_3OD, 400 MHz, ppm) δ: 1.55–1.70 (m, 2H, H-9), 1.70–1.90 (m, 2H, H-7), 2.20–2.30 (ddd, 1H, J = 18.6 Hz, J = 11.6 Hz, J = 2.6 Hz, H-5), 2.35 (m, 1H, H-5), 2.45–2.60 (m, 2H, H-10), 3.60 (m, 1H, H-8), 4.5 (m, 1H, H-6), 5.83 (m, 1H, H-3), 6.11 (dd, 1H, J = 8.1 Hz, J = 2.5 Hz, H-6′), 6.16 (d, 1H, J = 2.4 Hz, 1H, H-3′), 6.77 (d, 1H, J = 8.1 Hz, H-5′), 6.93 (m, 1H, H-4). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ: 26.5 (CH_2, C-10), 30.1 (CH_2, C-5), 39.2 (CH_2, C-9), 43.0 (CH_2, C-7), 68.3 (CH, C-8), 77.8 (CH, C-6), 103.5 (CH, C-3′), 107.5 (CH, C-6′), 120.6 (C, C-1′), 121.3 (=CH, C-3), 131.5 (CH, C-5′), 148.4 (=CH, C-4), 157.0 (C, C-4′), 157.5 (C,

C-2′), 167.1 (CO, C-2). HRMS (ESI, MeOH): Calcd for $C_{15}H_{18}O_5$ $[M+Na]^+$: 301.1052, found 301.1053.

4.1.3.2. Compound 6: (1*S*,5*R*,7*S*)-7-(2,4-dihydroxyphenethyl)-2,6-dioxabicyclo[3.3.1]nonan-3-one. This compound was obtained from bicyclic lactone **4** (100 mg, 0.38 mmol) following the optimized procedure. The reaction crude was purified with CH_2Cl_2/EtOH: 95/5 as eluent. Compound **6** (10 mg) was eluted (R = 10%). ^{1}H NMR (CD_3OD, 400 MHz, ppm) δ: 1.51 (m, 1H, H-8), 1.61 (m, 2H, H-10), 1.83 (m, 1H, H-8), 1.88 (m, 2H, H-9), 2.40–2.55 (m, 2H, H-11), 2.65 (d, 1H, J = 19.3 Hz, H-4), 2.77 (dd, 1H, J = 19.3 Hz, J = 5.3 Hz, H-4), 3.56 (m, 1H, H-7), 4.22 (m, 1H, H-5), 4.85 (m, 1H, H-1), 6.11 (dd, 1H, J = 8.1 Hz, J = 2.5 Hz, H-6′), 6.15 (d, 1H, J = 2.4 Hz, 1H, H-2′), 6.73 (d, 1H, J = 8.1 Hz, H-5′). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ: 26.2 (CH_2, C-11), 30.4 (CH_2, C-9), 37.0 (CH_2, C-4), 37.3 (CH_2, C-10), 38.1 (CH_2, C-8), 66.1 (CH, C-7), 67.3 (CH, C-5), 75.3 (CH, C-1), 103.5 (CH, C-3′), 107.3 (CH, C-6′), 120.3 (C, C-1′), 131.6 (CH, C-5′), 157.0 (C, C-4′), 157.5 (C, C-2′), 173.1 (CO, C-3). HRMS (ESI, MeOH): Calcd for $C_{15}H_{18}O_5$ $[M+Na]^+$: 301.1052, found 301.1053.

4.1.4. Synthesis of compound 3

4.1.4.1. Compound 3: (*R*)-6-[(*S*)-2-hydroxy-4-(2,5-dibromo-4-hydroxyphenyl)butyl]-5,6-dihydropyran-2-one. To a mixture of HF/SbF_5 (4 mL, SbF_5 mol % = 3.8) maintained at −20 °C, was added *N*-bromosuccinimide (1.2 equiv) and dodoneine **1** (50 mg, 0.19 mmol). The mixture was magnetically stirred at the same temperature during 30 min. The reaction mixture was then hydrolyzed in ice and neutralized with Na_2CO_3 until pH 6–7, extracted with dichloromethane (×2) and then with ethyl acetate (×2). The combined organic phases were dried ($MgSO_4$) and concentrated in vacuo. The reaction crude was purified with preparative TLC plate and CH_2Cl_2/EtOH: 95/5 as eluent. Compound **3** (8 mg) was eluted (R = 10%). ^{1}H NMR ($CDCl_3$, 400 MHz, ppm) δ: 1.75 (m, 2H, H-9), 1.85 (m, 2H, H-7), 2.01 (m, 1H, H-7), 2.40 (m, 2H, H-10), 2.75 (m, 1H, H-5), 2.85 (m, 1H, H-5), 3.90 (m, 1H, H-8), 4.68 (m, 1H, H-6), 5.65 (br s, 1H, OH), 6.03 (m, 1H, H-3), 6.90 (m, 1H, H-4), 7.21 (s, 1H, H-3′), 7.35 (s, 1H, H-6′). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 29.6 (CH_2, C-10), 31.0 (CH_2, C-5), 37.7 (CH_2, C-9), 42.0 (CH_2, C-7), 68.6 (CH, C-8), 77.2 (CH, C-6), 109.2 (C, C-5′), 120.1 (CH, C-3′), 121.3 (=CH, C-4), 123.7 (C, C-2′), 132.7 (CH, C-6′), 134.6 (C, C-1′), 145.1 (=CH, C-3), 151.2 (C, C-4′), 163.8 (CO, C-2). HRMS (ESI, MeOH): Calcd for $C_{15}H_{17}Br_2O_4$ $[M+NH_3]^+$: 435.97507, found 435.97536.

4.1.5. Synthesis of compound 5

4.1.5.1. Compound 5: (*R*)-6-[(*R*)-2-fluoro-4-(4-hydroxyphenyl) butyl)-5,6-dihydropyran-2-one. To a mixture of CH_2Cl_2 (2 mL) and dodoneine **1** (50 mg, 0.19 mmol) maintained at −40 °C under nitrogen, was added DAST (0.15 mL, 1.14 mmol). The mixture allowed to reach room temperature after addition, was magnetically stirred during 5 h. The reaction crude was directly purified with preparative TLC plate and CH_2Cl_2/EtOH: 95/5 as eluent. Compound **5** (45 mg) was eluted (R = 90%). ^{1}H NMR ($CDCl_3$, 400 MHz, ppm) δ: 1.75 (m, 2H, H-9), 1.85 (m, 2H, H-7), 2.37 (m, 2H, H-10), 2.70 (m, 2H, H-5), 4.65 (m, 1H, H-6), 4.75 (br s, 1H, OH), 4.85 (dm, 1H, J = 48.0 Hz, H-8), 6.03 (ddd, 1H, J = 9.7 Hz, J = 2.5 Hz, J = 1.2 Hz, H-3), 6.76 (d, 2H, J = 6.0 Hz, H-3′ and H-5′), 6.89 (ddd, 1H, J = 9.7 Hz, J = 5.7 Hz, J = 2.7 Hz, H-4), 7.05 (d, 2H, J = 6.0 Hz, H-2′ and H-6′). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 29.8 (d, J = 6 Hz, CH_2, C-5), 30.3 (d, J = 17 Hz, CH_2, C-10), 37.7 (d, J = 21 Hz, CH_2, C-9), 41.1 (d, J = 20 Hz, CH_2, C-7), 74.4 (CH, C-6), 89.3 (d, J = 167 Hz, CFH, C-8), 115.4 (CH, C-2′ and C-6′), 121.5 (=CH, C-4), 129.5 (CH, C-3′ and C-5′), 133.23 (C, C-1′), 144.9 (=CH, C-3), 153.9 (C, C-4′), 164.0 (CO, C-3). ^{19}F{^{1}H} NMR (396 MHz, $CDCl_3$, ppm) δ: −184.5. HRMS (ESI, MeOH): Calcd for $C_{15}H_{17}O_3F$ $[M+Na]^+$: 287.10594, found 287.1058.

4.1.6. Conversion of dodoneine to compound 4

4.1.6.1. Compound 4: (1*S*,5*R*,7*S*)-7-(4-hydroxyphenethyl)-2,6-dioxabicyclo[3.3.1]nonan-3-one. To a mixture of dodoneine **1** (10 mg, 0.04 mmol) in methanol (1.5 mL) was added Na_2CO_3 (5.3 mg, 0.05 mmol). The mixture was magnetically stirred at room temperature during 2 h. After evaporation of methanol, the crude was solubilized in dichloromethane and filtered. The organic phase was dried ($MgSO_4$) and concentrated in vacuo. Compound **4** (9.5 mg) was obtained without further purification (R = 95%). ^{1}H NMR ($CDCl_3$, 400 MHz, ppm) δ: 1.65 (m, 1H, H-8), 1.73 (m, 2H, H-10), 1.95 (m, 1H, H-8), 2.0 (m, 2H, H-9), 2.65 (m, 2H, H-11), 2.72 (d, 1H, J = 18.9 Hz, H-4), 2.9 (dd, 1H, J = 19.2 Hz, J = 5.3 Hz, H-4), 3.66 (m, 1H, H-7), 4.34 (m, 1H, H-5), 4.9 (m, 1H, H-1), 6.71 (d, J = 8.5 Hz, 2H, H-3′ and H-5′), 7.00 (d, J = 8.4 Hz, 2H, H-2′ and H-6′). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 30.4 (CH_2, C-9), 31.4 (CH_2, C-11), 37.1 (CH_2, C-4), 38.0 (CH_2, C-8), 39.0 (CH_2, C-10), 65.7 (CH, C-7), 67.3 (CH, C-5), 75.2 (CH, C-1), 116.2 (CH, C-3′ and C-5′), 130.4 (CH, C-2′ and C-6′), 133.8 (C, C-1′), 156.5 (C, C-4′), 173 (C=O, C-3). HRMS (ESI, MeOH): Calcd for $C_{15}H_{18}O_4$ $[M+Na]^+$: 285.11028, found 285.1093.

4.2. CA inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity.[33] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled–deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min–6 h at room temperature (15 min) or 4 °C (6 h) prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[34] and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier.[35,36]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.04.041.

References and notes

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