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Attachment of carbohydrates to methoxyaryl moieties leads to highly selective inhibitors of the cancer associated carbonic anhydrase isoforms IX and XII

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ABSTRACT

The transmembrane isoforms of carbonic anhydrase (hCA IX and XII) have been shown to be linked to carcinogenesis and their inhibition to arrest primary tumor and metastases growth. In this paper, we present a new class of C-glycosides incorporating the methoxyaryl moiety, that was designed to selectively target and inhibit the extracellular domains of the cancer-relevant CA isozymes. The glycosides have been prepared by aldol reaction of glycosyl ketones with the appropriate aromatic aldehydes. We also present the inhibition profile of our new glycomimetics, against four isozymes of carbonic anhydrase comprising hCAs I and II (cytosolic, ubiquitous isozymes) and hCAs IX and XII (tumor associated isozymes). In this study, per-O-acetylated glycoside **4**, **6** and deprotected compounds **7**, **9**, **10** and **12** were identified as potent and highly selective inhibitors of hCA IX and XII. These results confirm that attaching carbohydrate moieties to CA methoxyaryl pharmacophore improves and enhances its inhibitory activity. These CA inhibitors have developmental potential to selectively target cancer cells, leading to cell death.

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1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are the most studied members of a great family of metalloenzymes. CAs catalyze the reversible hydration of carbon dioxide and they are found in multiple organism such as vertebrates, bacteria, algae, etc.¹ Five genetically distinct CA families are known to date, the α -, β -, γ -, δ - and ζ -CAs. Mammals possess only α -CAs, while many pathogenic organisms such as bacteria and fungi encoded β -CAs. These enzymes contain a zinc ion (Zn^{2+}) in their active site, which is coordinated by three histidine residues and a water molecule/hydroxide ion (in the α -, γ - and δ -classes) or by two cysteine and one histidine residues (in the β - and ζ -CA classes), with the fourth ligand being a water molecule/hydroxide ion. Out of the 16 different CA isoforms discovered so far in the α -class, human CA isoforms hCA I and II are cytosolic enzymes that are widespread throughout the human body. Further, dimeric transmembrane glycoproteins hCA IX and XII are also human associated CA isoforms having extracellular active site and are marker for a broad

spectrum of hypoxic tumor types.^{2,3} The overexpression of these isoforms contributes to the increased acidification of extracellular hypoxic environment (pH = 6.8) in contrast to normal tissues (pH = 7.4) thus promoting tumor cell survival in an acidic condition by decreasing uptake of weakly basic anticancer drugs.⁴ Thus specifically targeting the tumor associated isoforms hCA IX and XII over the main off target isoforms hCA I and II, which have a physiological relevance, using specific inhibitors is considered to be a promising strategy in the cancer therapy.

Carbonic anhydrase inhibitors (CAIs) are classified in three main classes:

- Sulfonamides, sulfamates and sulfamides which bind in deprotonated form, as anions, to the Zn(II) ion from the enzyme active site by replacing the zinc-bound water/hydroxide ion and leading to a tetrahedral geometry of Zn(II).²
- Coumarins which exhibit a very different binding mode with no interactions between the inhibitor molecule and the active site Zn(II) ion observed, occluding the entrance to the active site cavity.⁵
- Polyamines⁶ and phenols,⁷ which bind by interacting with a water molecule/hydroxide ion coordinated to Zn(II) through hydrogen bonding.

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Methylation is one of the most common chemical modifications of the OH moiety in phenol. Only very recently, methoxyphenyl derivatives have been investigated as carbonic anhydrase inhibitors because it was considered that they do not bear any moiety normally associated with CA inhibition in their molecules.⁸ Unexpectedly, di- and tri-methoxybenzenes are rather similar or better CAIs than phenol but they showed a flat inhibition profile. Docking studies have been performed to explain the behavior of these compounds. It was found that the binding of methoxy-substituted benzene within the enzyme active site is done without interaction with the zinc ion, by means of different interactions with amino acid residues and water molecules.⁸ The compounds were located between the phenol-binding site and coumarin binding site, filling the middle of the enzyme cavity, but this hypothesis was not yet confirmed by crystallography. Thus this completely different binding mode offers the possibility of design carbonic anhydrase inhibitors with a different inhibition profile to the known inhibitors. However, up to now, only very few such derivatives have been investigated so far.⁸

One of the most successful approaches for designing CAIs targeting all isoforms known to date, was denominated 'the tail approach'. The tail approach originally consisted in attaching different tails to the scaffolds of sulfonamides to modulate the physicochemical properties of these pharmacological agents. A very good example of such 'tails' is constituted by sugars, which represent a wide range of chemotypes, leading thus to a high number of new CAIs.^{9,10} The stereochemical diversity across the carbohydrate tails provides the opportunity for interrogation of subtle differences in active site topology of CA isozymes.

Glycosidic CAIs were explored previously by our group,¹¹ Poulsen's¹² and Winum's¹³ groups and the presence of carbohydrate moieties in the molecules of such compounds was associated with effective inhibition of physiologically relevant isoforms, among which were also CA IX and XII. Recently our group has applied the 'sugar approach' to the preparation of C-glycosyl phenols, where the carbohydrate moiety is tethered to a phenol CA pharmacophore through a carbon chain.¹⁴ These compounds have been tested as inhibitors of the *Mycobacterium tuberculosis* β -CAs and have shown better inhibitory activity against mtCAs than phenol. Thus, this confirms that attaching carbohydrate moieties to phenol could improve its inhibitory activity. The antitubercular activity of the C-glycosyl phenols was investigated, allowing us to identify the first mtCA inhibitor with antimycobacterial activity.¹⁵

Thus, in the search of non-sulfonamide CAIs belonging to different classes of compounds, we report here the synthesis of a series of new C-glycosides incorporating the methoxyaryl moiety, and their inhibitory activity against the off-target hCA I and II, and tumor-associated hCA IX and XII.

2. Results and discussion

2.1. Chemistry

A set of new C-cinnamoyl glycosides (Fig. 1) was synthesized as outlined in Scheme 1. β -D-Glycosyl-propan-2-ones were prepared by Knoevenagel condensation with 2,4-pentanedione in the presence of sodium carbonate or sodium bicarbonate using water and THF as solvent.¹⁶ Crude mixtures containing the C-glycosyl ketones were acetylated and then purified to afford the peracetylated compounds in very good yields. C-Glycosides have been prepared by aldol condensation of β -C-glucosyl and β -C-galactosyl ketones with different aldehydes incorporating the methoxyaryl moiety at room temperature in the presence of pyrrolidine as catalyst.^{14,17} The crude mixtures could be easily purified by flash column chromatography and/or crystallization to afford the pure compounds 1–6 in good yields.

The ¹H NMR, ¹³C NMR, 2D COSY and HSQC were in full agreement with their structures (see Supplementary information). The *trans* double bond in the glycosides was established by the large coupling constant ($J = 16$ Hz) between the two olefinic protons. The β -configuration of the C-glycosides was established by the large coupling constant ($J = 9.7$ – 11.4 Hz) observed between H-1' and H-2'.

The O-acetate protecting groups of the carbohydrate moiety were next easily removed using triethylamine in methanol/water to afford the fully deprotected C-glycosides 7–12 in very good yields (80–98%).

The inhibitory activities of C-glycosides 1–12 against cytosolic isoforms hCA I and II as well as the membrane associated isoforms hCA IX and XII was assayed by using stopped flow assay method¹⁸ and the results are presented in Table 2.

A number of structure–activity relationships (SARs) were identified in this study and are summarized as follows:

Off-target CA isozymes.

(i) hCA I: Five of the peracetylated C-glycosides are micromolar inhibitors of the hCA I; the exception is the C-glycosyl compound 3 which is a very poor hCA I inhibitor with $K_i > 50$ μ M. A similar trend was found for the deprotected sugar analogues 7–12, where also the deprotected derivative of compound 3 (glycoside 7) is a very poor inhibitor of hCA I. It is of great interest to relate this behaviour of these compounds toward hCA I and it can be concluded that combination of a glucosyl scaffold and the 6-methoxy-2-naphthyl moiety leads to a steep decrease in the inhibitory potency of these compounds against hCA I.

(ii) The C-glycosides showed a very interesting inhibition profile against hCA II. It should be noted that all glycosyl derivatives of veratraldehyde 2, 5, 8 and 11 showed to be good inhibitors in the micromolar range. Compounds 1–3, 5, 8 and 12 are good hCA II inhibitors in the micromolar range. However being a ubiquitous, housekeeping isoform, this may not be a valuable property in another context if compounds targeting other isoforms (hCA IX and XII) should also possess activity against hCA II. Thus it is interesting to note that some of the novel compounds showed poor inhibition against hCA II while retaining potent inhibition against hCA IX and XII. C-Galactosyl derivatives 4 and 6 and their deprotected analogues 10 and 12 were very poor inhibitors of hCA II. Also C-glucosides 7 and 9 showed a highly reduced activity against this isozyme.

Cancer-associated CA isozymes.

(iii) The tumor associated target isoform hCA IX was inhibited in the submicromolar range by all the C-glycosides except compound 10, which is a micromolar inhibitor of this isozyme. The best inhibitor is compound 4 which is also a very bad hCA II inhibitor.

(iv) The second tumor-associated isoform, hCA XII, was the most inhibited isoform by compounds 2, 5, 6 and 8–12. The least effective hCA XII inhibitors were 1, 3, 4 and 7.

(v) Selectivity for inhibiting the tumor-associated isoforms (hCA IX and XII) over the widespread cytosolic forms (hCA I and II) is a key issue when designing CAIs. As can be in Table 2 several compounds showed better activity profile against hCA IX and XII over I and II which is highly desirable when only the tumor-associated isoforms would be targeted. It was observed that several C-glycoside which showed very good inhibition of isoform IX and XII were also shown to be highly selective. The most effective hCA IX inhibitor 4 showed excellent selectivity ratios over hCA I and II. Also the C-glycosides 6, 10, 12, which are nanomolar inhibitors of hCA XII, showed excellent selectivity ratios.

It is interesting to note that all the peracetylated C-glucosides 1–3 were shown to be no selective. Also C-glycosides incorporating the veratrole moiety (2, 5, 8 and 11) showed almost no selectivity and are not useful in the design of selective inhibitors. Thus, the

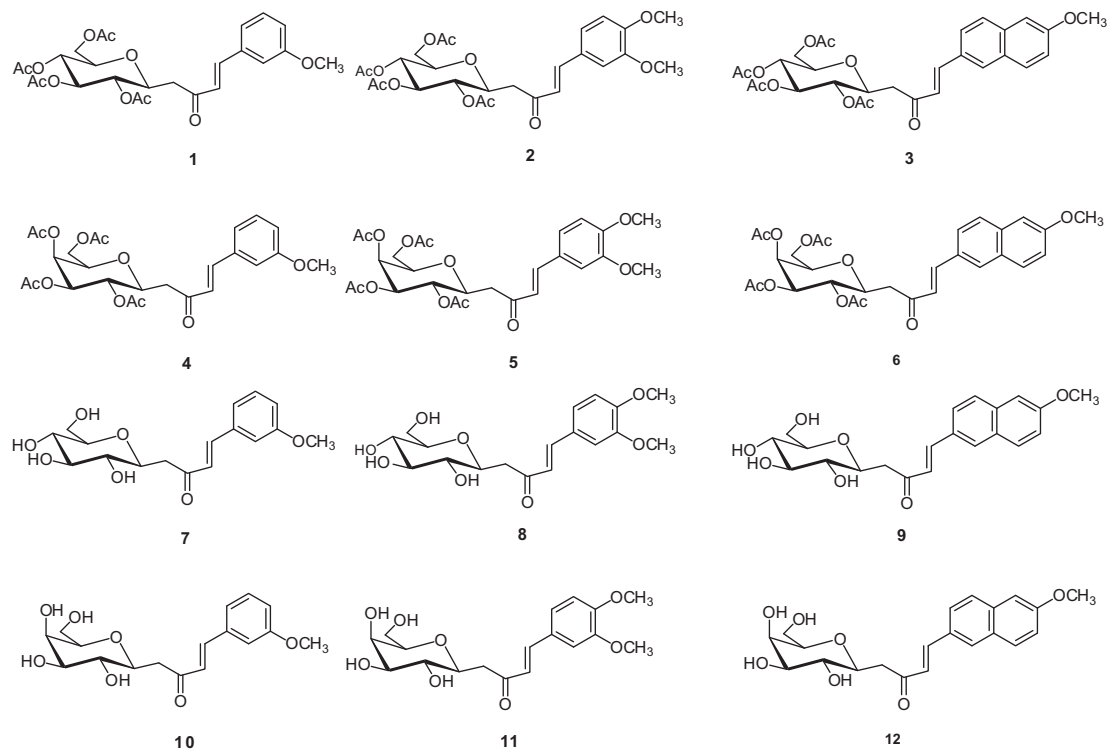
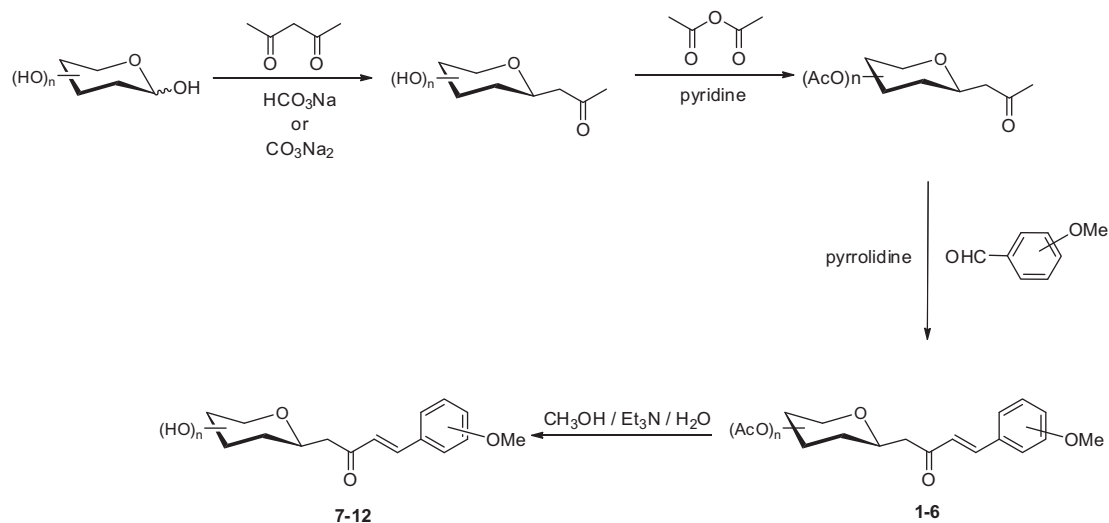


Figure 1. Peracetylated C-glycosides (1–6) and fully deprotected derivatives (7–12).



Scheme 1. Preparation of C-glycosides 1–12.

3-methoxyphenyl and 6-methoxy-2-naphthyl moieties are very useful in the design of inhibitors with high activity and selectivity.

Previously we have investigated the enzyme inhibition characteristics of a small series of C-cinnamoyl glycosides incorporating the phenol moiety.¹⁴ The inhibition profile found for those C-glycosyl compounds was flat and there was only small variations in the K_i values (micromolar range) observed across all CA isozymes investigated, leading to K_i selectivity ratios of ~ 1.0 across isozymes, that is, they were nonselective CA inhibitors. As can be seen in our present report, a small structural change, replacement of a proton by a methyl group lead to a high improvement in the activity and selectivity of the C-glycosides. It could be explained in terms of the bind mode of methoxyaryl compounds, described

above.⁸ C-Glycosides, where the carbohydrate moiety is tethered to a phenol moiety, would bind by interacting with a water molecule/hydroxide ion coordinated to Zn(II) through hydrogen bonding in the active site of carbonic anhydrase. On the other hand when a methoxyaryl was attached to the glycoside, the compounds would bind between the phenol-binding site and coumarin binding site, filling the middle of the carbonic anhydrase cavity. Thus the carbohydrate moiety should interact with amino acid residues found at the entrance of the active site cavity. As these residues are generally different between the various CA isozymes,¹⁹ this could lead to the selectivity found in this work. Up to now, we were unable to crystallize a CA isozyme in complex with one of the glycosides 1–12, thus it is not possible to confirm our hypothesis.

Table 1
Synthesis of C-glycosides

Ketone	Aldehyde	Reaction time (h)	Product	Yield (%)
C-Glucosyl	3-Methoxybenzaldehyde	78	1	76
	Veratraldehyde	23	2	66
	6-Methoxy-2-naphtaldehyde	48	3	53
C-Galactosyl	3-Methoxybenzaldehyde	70	4	72
	Veratraldehyde	24	5	65
	6-Methoxy-2-naphtaldehyde	47	6	52

Table 2
Inhibition of mammalian α -CA with the C-glycosides **1–12**^a

C-Glycoside	cLogP ^b	K_i^c (nM)				Selectivity			
		hCA I	hCA II	hCA IX	hCA XII	I/IX	II/IX	I/XII	II/XII
1	0.12	5670	421	779	794	7.3	0.5	7.1	0.53
2	-0.01	8360	541	753	126	11.1	0.7	66.3	4.3
3	1.11	>50000	413	673	589	>74.3	0.6	>85	0.7
4	0.12	8690	>50000	93	653	93.4	>538	13.3	>76
5	-0.01	4670	629	773	81	6.0	0.81	57.7	7.8
6	1.11	6650	>50000	417	80	15.9	>120	83.1	>625
7	-0.82	7440	>50000	615	682	12.1	>81	10.9	>73
8	-0.93	6510	438	840	73	12.1	0.5	89.2	6.0
9	0.19	>50000	>50000	462	236	>108	>108	>212	>212
10	-0.80	8670	>50000	3860	84	2.24	>13	103.2	>595
11	-0.93	7480	552	936	235	8.0	0.6	31.8	2.3
12	0.19	7210	>50000	485	89	14.9	>103	81.0	>562

^a All CAs are recombinant enzymes obtained in the authors' laboratory as reported earlier.⁵

^b Values calculated using ChemBioDraw Ultra 12.0.

^c Errors in the range of 5–10% of the reported value, from three different determinations.

In the development of anti-cancer compounds that target selectively the membrane bound isoform CA IX and CA XII versus the ubiquitous isoform CA II, the design of membrane non-permeant inhibitors is crucial.¹⁰ The calculated LogP (cLogP) parameter generally provides a good correlation with experimental permeability data, and molecules with cLogP values between 1 and 3 typically have good passive membrane permeability properties while those with cLogP values of <0 are more likely to have a low capacity for penetrating cell membranes.²⁰ As can be seen in Table 2, calculated LogP values for the C-glycosides show that several compounds fall within the range indicative of molecules with poor membrane permeability, the exceptions are naphthyl derivatives **3** and **6**. Values of acetylated glycosides **1–6** are consistent with the incorporated acetyl groups, decreasing the polarity of the resulting carbohydrate moiety. It is expected that the poor passive membrane permeability of the C-glycosides would enhance the preferential inhibition of CAs IX and XII over ubiquitous cytosolic hCA II.

3. Conclusion

A novel series of C-glycosides **1–12** containing the methoxyaryl scaffold have been synthesized and investigated as inhibitors against four isozymes of carbonic anhydrases comprising cytosolic, ubiquitous isozymes hCA I and II as well as the transmembrane, tumor-associated isoforms hCA IX and XII which are validated anti-tumor targets. In this study, per-O-acetylated glycoside **4**, **6** and deprotected compounds **7**, **9**, **10** and **12** were identified as potent and highly selective inhibitors of hCA IX and XII. These results confirm that attaching carbohydrate moieties to CA methoxyaryl pharmacophore improves and enhances its inhibitory activity. Also the physicochemical properties of the glycosides tested would enhance the preferential inhibition in vivo. Free glycosides could be useful for chemotherapy if they are delivered through a route of intravenous administration. For oral delivery peracetylated

C-glycosides may be used as ester prodrugs. Once in the body, the ester groups could be readily hydrolyzed by ubiquitous esterases.²¹ As it has been evidenced that isoforms IX and XII are over-expressed by tumours and are also potential targets for diagnosis and treatment of cancer, discovery of these C-glycosyl selective IX and XII inhibitors will be a promising step in the strategy for an effective cancer therapy.

4. Experimental section

4.1. General

All starting materials and reagents, were purchased from commercial suppliers. Reactions were monitored by TLC and TLC plates visualized with short wave UV fluorescence ($\lambda = 254$ nm), sulfuric acid stain (5% H₂SO₄ in methanol). Silica gel flash chromatography was performed using silica gel 60 Å (230–400 mesh). All melting points are uncorrected. ¹H and ¹³C NMR spectra were recorded on a Varian Mercury 200 (200.055 and 50.309 MHz, respectively). Chemical shifts were measured in ppm and coupling constants in Hz. High resolution mass spectra were recorded using electrospray as the ionization technique in positive ion or negative ion modes as stated. All MS analysis samples were prepared as solutions in methanol.

4.1.1. General procedure 1: synthesis of per-O-acetylated C-glycosides (**1–6**)

To a solution of per-O-acetylated β -C-glucopyranosyl or C-galactopyranosyl ketone (1.0 equiv) in dry dichloromethane was added the desired aldehyde (1.0 equiv) and pyrrolidine (0.2 equiv). The reaction was stirred at room temperature until the starting material was consumed as evidenced by TLC (see times in Table 1). The reaction mixture was concentrated and the residue diluted in ethyl acetate and washed with water (3 \times). The aqueous

extracts were combined and back extracted with ethyl acetate (1×). The organic extracts were combined, dried over NaSO₄, filtered and evaporated. The product was purified by column chromatography (eluant 3:7–1:1 hexanes–EtOAc) to give compounds **1–6**.

4.1.2. General procedure 2: deprotection of per-O-acetylated C-glycosides (**1–6** → **7–12**)

Deprotected compounds **7–12** were prepared by dissolving the corresponding per-O-acetylated precursor **1–6** in methanol/triethylamine/water (8:2:1). The reaction was kept at room temperature overnight and then concentrated. The product was purified by column chromatography (eluant 5:1 EtOAc–MeOH) to afford pure material by ¹H NMR and ¹³C NMR spectroscopy. Yields 80–98%.

4.1.2.1. (E)-1-(2',3',4',6'-Tetra-O-acetyl-β-D-glucopyranosyl)-4-(3-methoxyphenyl)but-3-en-2-one (**1**)

The title compound **1** was prepared from 1-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-propan-2-one and 3-methoxybenzaldehyde according to general procedure 1 to give a white solid. Mp = 94–95 °C. ¹H NMR (500 MHz, CDCl₃) δ 7.53 (d, 1H, J = 16.2 Hz, H-4), 7.33 (t, 1H, J = 7.9 Hz, ArH), 7.16 (d, 1H, J = 7.6 Hz, ArH), 7.08 (m, 1H, ArH), 6.98 (dd, 1H, J = 8.2, 1.9 Hz, ArH), 6.74 (d, 1H, J = 16.2 Hz, H-3), 5.25 (t, 1H, J = 9.4 Hz, H-3'), 5.09 (t, 1H, J = 9.7 Hz, H-4'), 5.01 (t, 1H, J = 9.7 Hz, H-2'), 4.28 (dd, 1H, J = 12.4, 4.9 Hz, H-6'b), 4.14 (ddd, 1H, J = 10.0, 8.5, 3.2 Hz, H-1'), 4.04 (dd, 1H, J = 12.4, 2.2 Hz, H-6'a), 3.86 (s, 3H, CH₃O), 3.74 (ddd, 1H, J = 10.1, 4.9, 2.2 Hz, H-5'), 3.04 (dd, 1H, J = 16.3, 8.5 Hz, H-1b), 2.70 (dd, 1H, J = 16.3, 3.2 Hz, H-1a), 2.05 (s, 3H, CH₃COO), 2.04 (s, 3H, CH₃COO), 2.03 (s, 6H, CH₃COO). ¹³C NMR (126 MHz, CDCl₃) δ 196.13 (C-2), 170.62 (CH₃COO), 170.22 (CH₃COO), 169.98 (CH₃COO), 169.55 (CH₃COO), 159.97 (ArC), 143.70 (C-4), 135.57 (ArC), 130.01 (ArC), 126.49 (C-3), 121.14 (ArC), 116.75 (ArC), 113.12 (ArC), 75.74 (C-5'), 74.19 (C-4'), 74.13 (C-3'), 71.70 (C-1'), 68.50 (C-2'), 62.04 (C-6'), 55.33 (CH₃O), 42.55 (C-1), 20.72 (CH₃CO), 20.66 (CH₃CO), 20.64 (CH₃CO), 20.62 (CH₃CO). HRMS *m/z*: calcd for C₂₅H₃₀O₁₁, 506.1788; found, 506.1776.

4.1.2.2. (E)-1-(2',3',4',6'-Tetra-O-acetyl-β-D-glucopyranosyl)-4-(3,4-dimethoxyphenyl)but-3-en-2-one (**2**)

The title compound **2** was prepared from 1-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-propan-2-one and veratraldehyde according to general procedure 1 to give a white solid. Mp = 135–136 °C. ¹H NMR (500 MHz, CDCl₃) δ 7.51 (d, 1H, J = 16.1 Hz, H-4), 7.16 (d, 1H, J = 8.3 Hz, ArH), 7.09 (s, 1H, ArH), 6.90 (d, 1H, J = 8.3 Hz, ArH), 6.64 (d, 1H, J = 16.1 Hz, H-3), 5.25 (t, 1H, J = 9.4 Hz, H-3'), 5.09 (t, 1H, J = 9.7 Hz, H-4'), 5.00 (t, 1H, J = 9.7 Hz, H-2'), 4.28 (dd, 1H, J = 12.4, 4.9 Hz, H-1'), 4.14 (dd, 1H, J = 18.4, 3.2 Hz, H-6'b), 4.04 (dd, 1H, J = 12.3, 1.8 Hz, H-6'a), 3.94 (s, 6H, CH₃O), 3.74 (ddd, 1H, J = 10.0, 4.8, 2.0 Hz, H-5'), 3.03 (dd, 1H, J = 16.2, 8.4 Hz, H-1b), 2.68 (dd, 1H, J = 16.2, 3.2 Hz, H-1a), 2.04 (s, 1H, CH₃COO), 2.04 (s, 1H, CH₃COO), 2.02 (s, 1H, CH₃COO), 2.02 (s, 1H, CH₃COO). ¹³C NMR (126 MHz, CDCl₃) δ 196.00 (C-2), 170.63 (CH₃COO), 170.23 (CH₃COO), 170.03 (CH₃COO), 169.57 (CH₃COO), 151.60 (ArC), 149.30 (ArC), 143.85 (C-4), 127.11 (ArC), 124.28 (C-3), 123.33 (ArC), 111.08 (ArC), 109.70 (ArC), 75.71 (C-5'), 74.21 (C-1'), 74.21 (C-3'), 71.72 (C-4'), 68.52 (C-2'), 62.04 (C-6'), 56.01 (CH₃O), 55.92 (CH₃O), 42.39 (C-1), 20.75 (CH₃CO), 20.68 (CH₃CO), 20.65 (CH₃CO), 20.63 (CH₃CO). HRMS *m/z*: calcd for C₂₆H₃₂O₁₂, 536.1894; found, 536.1898.

4.1.2.3. (E)-1-(2',3',4',6'-Tetra-O-acetyl-β-D-glucopyranosyl)-4-(6-methoxy-2-naphthyl)but-3-en-2-one (**3**)

The title compound **3** was prepared from 1-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-propan-2-one and 6-methoxy-2-naphthaldehyde according to general procedure 1 to give a yellow solid. Mp = 178–179 °C. ¹H NMR (500 MHz, CDCl₃) δ 7.93 (d, 1H, J = 8.8 Hz, ArH), 7.74 (m,

3H, ArH), 7.66 (dd, 1H, J = 8.6, 1.5 Hz, ArH), 7.20 (dd, 1H, J = 9.0, 2.5 Hz, ArH), 7.16 (d, 1H, J = 2.4 Hz, ArH), 6.83 (d, 1H, J = 16.1 Hz, H-3), 5.26 (t, 1H, J = 9.4 Hz, H-3'), 5.11 (t, 1H, J = 9.7 Hz, H-4'), 5.03 (t, 1H, J = 9.7 Hz, H-2'), 4.29 (dd, 1H, J = 12.4, 4.9 Hz, H-1'), 4.17 (dd, 1H, J = 18.3, 3.3 Hz, H-6'b), 4.05 (dd, 1H, J = 12.4, 2.1 Hz, H-6'a), 3.96 (s, 3H, CH₃O), 3.75 (ddd, 1H, J = 10.0, 4.8, 2.1 Hz, H-5'), 3.07 (dd, 1H, J = 16.2, 8.4 Hz, H-1b), 2.74 (dd, 1H, J = 16.2, 3.3 Hz, H-1a), 2.05 (s, 3H, CH₃COO), 2.05 (s, 3H, CH₃COO), 2.04 (s, 3H, CH₃COO), 2.02 (s, 3H, CH₃COO). ¹³C NMR (126 MHz, CDCl₃) δ 196.06 (C-2), 170.64 (CH₃COO), 170.24 (CH₃COO), 170.00 (CH₃COO), 169.57 (CH₃COO), 159.08 (ArC), 144.12 (C-4), 136.00 (ArC), 130.61 (ArC), 130.23 (ArC), 129.56 (ArC), 128.67 (ArC), 127.64 (ArC), 125.30 (ArC), 124.16 (C-3), 119.62 (ArC), 106.02 (ArC), 75.75 (ArC), 74.24 (C-3', C-1'), 71.76 (C-4'), 68.54 (C-2'), 62.07 (C-6'), 55.41 (CH₃O), 42.62 (C-1), 20.74 (CH₃CO), 20.68 (CH₃CO), 20.65 (CH₃CO), 20.63 (CH₃CO). HRMS *m/z*: calcd for C₂₉H₃₂O₁₁, 556.1945; found, 556.1968.

4.1.2.4. (E)-1-(2',3',4',6'-Tetra-O-acetyl-β-D-galactopyranosyl)-4-(3-methoxyphenyl)but-3-en-2-one (**4**)

The title compound **4** was prepared from 1-(2',3',4',6'-tetra-O-acetyl-β-D-galactopyranosyl)-propan-2-one and 3-methoxybenzaldehyde according to general procedure 1 to give a white solid. Mp = 111–112 °C. ¹H NMR (500 MHz, CDCl₃) δ 7.54 (d, 1H, J = 16.2 Hz, H-4), 7.33 (t, 1H, J = 7.9 Hz, ArH), 7.16 (d, 1H, J = 7.6 Hz, ArH), 7.08 (s, 1H, ArH), 6.98 (dd, 1H, J = 8.2, 2.3 Hz, ArH), 6.75 (d, 1H, J = 16.2 Hz, H-3), 5.46 (d, 1H, J = 3.2 Hz, H-3'), 5.20 (t, 1H, J = 9.9 Hz, H-4'), 5.07 (m, 1H, H-2'), 4.12 (dd, 1H, J = 11.4, 4.1 Hz, H-1'), 4.07 (m, 2H, H-6'a, H-6'b), 3.96 (m, 1H, H-5'), 3.86 (s, 3H, CH₃O), 3.09 (dd, 1H, J = 16.1, 8.4 Hz, H-1b), 2.71 (dd, 1H, J = 16.1, 3.2 Hz, H-1a), 2.17 (s, 3H, CH₃COO), 2.05 (s, 3H, CH₃COO), 2.00 (s, 3H, CH₃COO), 1.98 (s, 3H, CH₃COO). ¹³C NMR (126 MHz, CDCl₃) δ 196.45 (C-2), 170.45 (CH₃COO), 170.26 (CH₃COO), 170.24 (CH₃COO), 170.10 (CH₃COO), 159.94 (ArC), 143.66 (C-4), 135.58 (ArC), 130.01 (ArC), 126.52 (C-3), 121.15 (ArC), 116.70 (ArC), 113.15 (ArC), 74.66 (C-5'), 74.23 (C-1'), 71.99 (C-4'), 69.12 (C-3'), 67.72 (C-2'), 61.40 (C-6'), 55.34 (CH₃O), 42.75 (C-1), 20.83 (CH₃CO), 20.71 (CH₃CO), 20.62 (CH₃CO), 20.61 (CH₃CO). HRMS *m/z*: calcd for C₂₅H₃₀O₁₁, 506.1788; found, 506.1794.

4.1.2.5. (E)-1-(2',3',4',6'-Tetra-O-acetyl-β-D-galactopyranosyl)-4-(3,4-dimethoxyphenyl)but-3-en-2-one (**5**)

The title compound **5** was prepared from 1-(2',3',4',6'-tetra-O-acetyl-β-D-galactopyranosyl)-propan-2-one and veratraldehyde according to general procedure 1 to give a yellow solid. Mp = 89.5–90 °C. ¹H NMR (500 MHz, CDCl₃) δ 7.53 (d, 1H, J = 16.1 Hz, H-4), 7.16 (d, 1H, J = 8.3 Hz, ArH), 7.10 (s, 1H, J = 7.9 Hz, ArH), 6.90 (d, 1H, J = 8.3 Hz, ArH), 6.65 (d, 1H, J = 16.1 Hz, H-3), 5.46 (d, 1H, J = 3.0 Hz, H-3'), 5.21 (t, 1H, J = 9.9 Hz, H-4'), 5.09 (dd, 1H, J = 10.1, 3.4 Hz, H-2'), 4.13 (dd, 1H, J = 10.2, 4.7 Hz, H-1'), 4.11 (m, 2H, H-6'), 4.04 (dd, 1H, J = 11.2, 6.6 Hz, H-5'), 3.94 (d, 6H, J = 1.1 Hz, CH₃O), 3.08 (dd, 1H, J = 16.1, 8.4 Hz, H-1b), 2.69 (dd, 1H, J = 16.0, 3.2 Hz, H-1a), 2.18 (s, 3H, CH₃COO), 2.05 (s, 3H, CH₃COO), 2.00 (s, 3H, CH₃COO), 1.98 (s, 3H, CH₃COO). ¹³C NMR (126 MHz, CDCl₃) δ 196.27 (C-2), 170.42 (CH₃COO), 170.26 (CH₃COO), 170.23 (CH₃COO), 170.08 (CH₃COO), 151.59 (ArC), 149.29 (ArC), 143.80 (C-4), 127.14 (ArC), 124.34 (C-3), 123.33 (ArC), 111.09 (ArC), 109.71 (ArC), 74.76 (C-5'), 74.22 (C-1'), 72.00 (C-3'), 69.16 (C-4'), 67.72 (C-2'), 61.35 (C-6'), 56.01 (CH₃O), 55.92 (CH₃O), 42.59 (C-1), 20.85 (CH₃CO), 20.71 (CH₃CO), 20.62 (CH₃CO). HRMS *m/z*: calcd for C₂₆H₃₂O₁₂, 536.1894; found, 536.1878.

4.1.2.6. (E)-1-(2',3',4',6'-Tetra-O-acetyl-β-D-galactopyranosyl)-4-(6-methoxy-2-naphthyl)but-3-en-2-one (**6**)

The title compound **6** was prepared from 1-(2',3',4',6'-tetra-O-acetyl-β-D-galactopyranosyl)-propan-2-one and 6-methoxy-2-naphthaldehyde according to

general procedure 1 to give a yellow solid. Mp = 114–115 °C. ^1H NMR (500 MHz, CDCl_3) δ 7.92 (d, 1H, J = 8.2 Hz, ArH), 7.73 (m, 3H, H-4, ArH), 7.66 (dd, 1H, J = 8.6, 1.2 Hz, ArH), 7.19 (dd, 1H, J = 8.9, 2.4 Hz, ArH), 7.15 (d, 1H, J = 2.1 Hz, ArH), 6.84 (d, 1H, J = 16.1 Hz, H-3), 5.47 (d, 1H, J = 3.2 Hz, H-4'), 5.23 (t, 1H, J = 9.9 Hz, H-2'), 5.10 (dd, 1H, J = 10.1, 3.4 Hz, H-3'), 4.13 (m, 2H, H-5', H-1'), 4.06 (dd, 1H, J = 11.2, 6.4 Hz, H-6'b), 3.96 (m, 1H, H-6'a), 3.95 (s, 3H, CH_3O), 3.12 (m, 1H, H-1b), 2.75 (m, 1H, H-1a), 2.18 (s, 3H, CH_3COO), 2.05 (s, 3H, CH_3COO), 2.00 (s, 3H, CH_3COO), 1.97 (s, 3H, CH_3COO). ^{13}C NMR (126 MHz, CDCl_3) δ 196.36 (C-2), 170.42 (CH_3COO), 170.26 (CH_3COO), 170.23 (CH_3COO), 170.09 (CH_3COO), 159.06 (ArC), 144.05 (C-4), 135.98 (ArC), 130.60 (ArC), 130.22 (ArC), 129.58 (ArC), 128.66 (ArC), 127.63 (ArC), 125.34 (ArC), 124.16 (C-3), 119.61 (ArC), 106.00 (ArC), 74.71 (C-5'), 74.25 (C-1'), 72.11 (C-3'), 69.19 (C-4'), 67.75 (C-2'), 61.42 (C-6'), 55.41 (CH_3O), 42.84 (C-1), 20.84 (CH_3CO), 20.72 (CH_3CO), 20.63 (CH_3CO), 20.62 (CH_3CO). HRMS m/z : calcd for $\text{C}_{29}\text{H}_{32}\text{O}_{11}$, 556.1945; found, 556.1938.

4.1.2.7. 1-(β -D-Glucopyranosyl)-4-(3-methoxyphenyl)but-3-en-2-one (7).

The title compound **7** was prepared from compound **1** according to general procedure 2 to give a white solid. Mp = 116–117 °C. ^1H NMR (500 MHz, DMSO) δ 7.57 (d, 1H, J = 16.2 Hz, H-4), 7.35 (m, 1H, ArH), 7.29 (m, 2H, ArH), 7.00 (ddd, 1H, J = 8.2, 2.4, 1.1 Hz, ArH), 6.97 (m, 1H, H-3), 5.08 (d, 1H, J = 5.7 Hz, OH), 4.95 (t, 1H, J = 5.7 Hz, OH), 4.88 (d, 1H, J = 4.6 Hz, OH), 4.37 (t, 1H, J = 5.7 Hz, OH), 3.80 (s, 3H, CH_3O), 3.65 (dd, 1H, J = 9.2, 2.6 Hz, H-4), 3.61 (dd, 1H, J = 11.2, 4.0 Hz, H-6b), 3.41 (br s, 1H, H-6'a), 3.18 (dd, 1H, J = 8.3, 3.8 Hz, H-1'), 3.09 (m, 2H, H-2', H-5'), 2.97 (m, 2H, H-1b, H-3'), 2.82 (dd, 1H, J = 16.1, 8.8 Hz, H-1a). ^{13}C NMR (126 MHz, DMSO) δ 198.60 (C-2), 160.05 (ArC), 142.49 (C-4), 136.41 (ArC), 130.42 (ArC), 127.57 (C-3), 121.52 (ArC), 116.92 (ArC), 113.53 (ArC), 81.15 (C-5'), 78.57 (C-1'), 76.26 (C-4'), 74.04 (C-3'), 70.74 (C-2'), 61.59 (C-6'), 55.68 (CH_3O), 43.88 (C-1). HRMS m/z : calcd for $\text{C}_{17}\text{H}_{22}\text{O}_7$, 338.1366; found, 338.1378.

4.1.2.8. 1-(β -D-Glucopyranosyl)-4-(3,4-dimethoxyphenyl)but-3-en-2-one (8).

The title compound **8** was prepared from compound **2** according to general procedure 2 to give a yellow solid. Mp = 141–142 °C. ^1H NMR (500 MHz, DMSO) δ 7.54 (d, 1H, J = 16.1 Hz, H-4), 7.34 (d, 1H, J = 1.6 Hz, ArH), 7.27 (dd, 1H, J = 8.3, 1.7 Hz, ArH), 7.01 (d, 1H, J = 8.4 Hz, ArH), 6.87 (m, 1H, H-3), 5.07 (d, 1H, J = 4.9 Hz, OH), 4.95 (s, 1H, OH), 4.88 (s, 1H, OH), 4.36 (t, 1H, J = 5.7 Hz, OH), 3.82 (s, 3H, CH_3O), 3.81 (s, 3H, CH_3O), 3.62 (m, 2H, H-6'b, H-4'), 3.39 (m, 1H, H-6'a), 3.18 (t, 1H, J = 8.1 Hz, H-1'), 3.08 (m, 2H, H-2', H-5'), 2.95 (m, 2H, H-1b, H-3'), 2.79 (dd, 1H, J = 16.0, 8.8 Hz, H-1a). ^{13}C NMR (126 MHz, DMSO) δ 198.35 (C-2), 151.43 (ArC), 149.41 (ArC), 142.92 (C-4), 127.71 (ArC), 125.20 (C-3), 123.62 (ArC), 112.03 (ArC), 110.84 (ArC), 81.14 (C-5'), 78.59 (C-1'), 76.34 (C-4'), 74.06 (C-3'), 70.76 (C-2'), 61.60 (C-6'), 56.03 ($2\times\text{CH}_3\text{O}$), 43.68 (C-1). HRMS m/z : calcd for $\text{C}_{18}\text{H}_{24}\text{O}_8$, 368.1471; found, 368.1482.

4.1.2.9. 1-(β -D-Glucopyranosyl)-4-(6-methoxy-2-naphthyl)but-3-en-2-one (9).

The title compound **9** was prepared from compound **3** according to general procedure 2 to give a white solid. Mp = 130–131 °C. ^1H NMR (500 MHz, DMSO) δ 8.16 (s, 1H, ArH), 7.87 (m, 3H, H-4, ArH), 7.72 (d, 1H, J = 16.1 Hz, ArH), 7.37 (d, 1H, J = 2.3 Hz, ArH), 7.22 (dd, 1H, J = 9.0, 2.4 Hz, ArH), 7.04 (d, 1H, J = 16.2 Hz, H-3), 5.09 (d, 1H, J = 5.7 Hz, OH), 4.96 (d, 1H, J = 3.9 Hz, OH), 4.89 (d, 1H, J = 4.0 Hz, OH), 4.39 (t, 1H, J = 5.6 Hz, OH), 3.90 (s, 3H, CH_3O), 3.67 (dd, 1H, J = 9.1, 2.3 Hz, H-4'), 3.63 (m, 1H, H-6'b), 3.39 (br s, 1H, H-6'a), 3.20 (dd, 1H, J = 8.1, 5.2 Hz, H-1'), 3.10 (m, 2H, H-2', H-5'), 3.00 (dd, 2H, J = 12.7, 3.5 Hz, H-1b, H-3'), 2.83 (dd, 1H, J = 16.0, 8.8 Hz, H-1a). ^{13}C NMR (126 MHz, DMSO) δ 198.42 (C-2), 158.90 (ArC), 142.79 (C-4), 135.90 (ArC), 130.58 (ArC), 130.51 (ArC), 130.30 (ArC), 128.72 (ArC), 127.88

(ArC), 126.44 (ArC), 125.13 (C-3), 119.70 (ArC), 106.71 (ArC), 81.19 (C-5'), 78.60 (C-1'), 76.36 (C-4'), 74.08 (C-3'), 70.77 (C-2'), 61.61 (C-6'), 55.80 (CH_3O), 43.97 (C-1). HRMS m/z : calcd for $\text{C}_{21}\text{H}_{24}\text{O}_7$, 388.1522; found, 388.1531.

4.1.2.10. 1-(β -D-Galactopyranosyl)-4-(3-methoxyphenyl)but-3-en-2-one (10).

The title compound **10** was prepared from compound **4** according to general procedure 2 to give a white solid. Mp = 91–92 °C. ^1H NMR (500 MHz, DMSO) δ 7.56 (d, 1H, J = 16.2 Hz, H-4), 7.35 (t, 1H, J = 8.1 Hz, ArH), 7.29 (m, 2H, ArH), 6.99 (m, 2H, ArH, H-3), 4.89 (dd, 1H, J = 16.2, 4.6 Hz, OH), 4.70 (s, 1H, OH), 4.50 (t, 1H, J = 5.5 Hz, OH), 4.33 (dd, 1H, J = 11.9, 4.6 Hz, OH), 3.80 (s, 3H, CH_3O), 3.74 (dd, 1H, J = 21.3, 3.0 Hz, H-2'), 3.59 (td, 1H, J = 8.8, 2.5 Hz, H-1'), 3.48 (m, 1H, H-6'b), 3.38 (br s, 1H, H-6'a), 3.34 (m, 3H, H-3', H-4', H-5'), 2.96 (dd, 1H, J = 15.9, 2.5 Hz, H-1b), 2.83 (dd, 1H, J = 15.9, 8.9 Hz, H-1a).

^{13}C NMR (126 MHz, DMSO) δ 198.82 (C-2), 160.05 (ArC), 142.42 (C-4), 136.42 (ArC), 130.42 (ArC), 127.59 (C-3), 121.50 (ArC), 116.91 (ArC), 113.51 (ArC), 79.23 (C-5'), 76.89 (C-1'), 75.09 (C-4'), 70.94 (C-3'), 69.07 (C-2'), 60.87 (C-6'), 55.68 (CH_3O), 43.96 (C-1). HRMS m/z : calcd for $\text{C}_{17}\text{H}_{22}\text{O}_7$, 338.1366; found, 338.1372.

4.1.2.11. 1-(β -D-Galactopyranosyl)-4-(3,4-dimethoxyphenyl)but-3-en-2-one (11).

The title compound **11** was prepared from compound **5** according to general procedure 2 to give a yellow solid. Mp = 89–89.5 °C. ^1H NMR (500 MHz, DMSO) δ 7.53 (d, 1H, J = 16.1 Hz, H-4), 7.34 (d, 1H, J = 1.7 Hz, ArH), 7.27 (dd, 1H, J = 8.3, 1.7 Hz, ArH), 7.01 (d, 1H, J = 8.4 Hz, ArH), 6.87 (d, 1H, J = 16.2 Hz, H-3), 4.89 (d, 1H, J = 4.0 Hz, OH), 4.69 (s, 1H, OH), 4.49 (t, 1H, J = 5.6 Hz, OH), 4.33 (d, 1H, J = 4.5 Hz, OH), 3.82 (s, 3H, J = 3.4 Hz, CH_3O), 3.81 (s, 3H, CH_3O), 3.71 (d, 1H, J = 2.7 Hz, H-2'), 3.57 (td, 1H, J = 8.8, 2.5 Hz, H-1'), 3.47 (m, 1H, H-6'b), 3.36 (br s, 1H, H-6'a), 3.30 (s, 3H, J = 6.3 Hz, H-3', H-4', H-5'), 2.93 (dd, 1H, J = 15.8, 2.4 Hz, H-1b), 2.80 (dd, 1H, J = 15.9, 9.0 Hz, H-1a). ^{13}C NMR (126 MHz, DMSO) δ 198.59 (C-2), 151.42 (ArC), 149.42 (ArC), 142.86 (C-4), 127.71 (ArC), 125.24 (C-3), 123.60 (ArC), 112.04 (ArC), 110.82 (ArC), 79.21 (C-5'), 76.98 (C-1'), 75.11 (C-4'), 70.95 (C-3'), 69.06 (C-2'), 60.87 (C-6'), 56.03 ($2\times\text{CH}_3\text{O}$), 43.75 (C-1). HRMS m/z : calcd for $\text{C}_{18}\text{H}_{24}\text{O}_8$, 368.1471; found, 368.1484.

4.1.2.12. 1-(β -D-Galactopyranosyl)-4-(6-methoxy-2-naphthyl)but-3-en-2-one (12).

The title compound **12** was prepared from compound **6** according to general procedure 2 to give a yellow solid. Mp = 120–121 °C. ^1H NMR (500 MHz, DMSO) δ 8.16 (s, 1H, ArH), 7.87 (m, 3H, H-4, ArH), 7.72 (dd, 1H, J = 15.5, 7.5 Hz, ArH), 7.38 (d, 1H, J = 2.3 Hz, ArH), 7.22 (dd, 1H, J = 8.9, 2.4 Hz, ArH), 7.03 (d, 1H, J = 16.2 Hz, H-3), 4.92 (d, 1H, J = 2.8 Hz, OH), 4.71 (s, 1H, OH), 4.51 (t, 1H, J = 5.5 Hz, OH), 4.35 (d, 1H, J = 4.4 Hz, OH), 3.90 (s, 3H, J = 4.3 Hz, CH_3O), 3.72 (s, 1H, H-2'), 3.61 (dd, 1H, J = 8.8, 6.5 Hz, H-1'), 3.48 (m, 1H, H-6'b), 3.36 (br s, 1H, H-6'a), 3.33 (d, 3H, J = 6.0 Hz, H-3', H-4', H-5'), 2.99 (dd, 1H, J = 15.8, 2.3 Hz, H-1b), 2.84 (dd, 1H, J = 15.8, 9.0 Hz, H-1a). ^{13}C NMR (126 MHz, DMSO) δ 198.64 (C-2), 158.90 (ArC), 142.73 (C-4), 135.89 (ArC), 130.57 (ArC), 130.49 (ArC), 130.30 (ArC), 128.72 (ArC), 127.88 (ArC), 126.46 (ArC), 125.11 (C-3), 119.70 (ArC), 106.71 (ArC), 79.26 (C-5'), 77.00 (C-1'), 75.11 (C-4'), 70.98 (C-3'), 69.09 (C-2'), 60.88 (C-6'), 55.80 (CH_3O), 44.05 (C-1). HRMS m/z : calcd for $\text{C}_{21}\text{H}_{24}\text{O}_7$, 388.1522; found, 388.1511.

4.2. CA inhibition studies

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity as reported by Khalifah.¹⁸ Phenol red (at a concentration of 0.02 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 Na₂SO₄ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO₂ hydration reaction for a period of 10–100 s. The CO₂ 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 distilled–deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature 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, and the Cheng–Prusoff equation (Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099) as reported earlier and represent the mean from at least three different determinations.

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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.2014.07.052>.

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