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Design and synthesis of *N*-(3,5-difluoro-4-hydroxyphenyl) benzenesulfonamides as aldose reductase inhibitors

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Abstract—*N*-(3,5-Difluoro-4-hydroxyphenyl)benzenesulfonamide (**4**) and its derivatives **5**–**7** were prepared as putative bioisosteres of the previously reported aldose reductase inhibitors, which are the *N*-benzenesulfonylglycine derivatives **I**–**IV**. The in vitro aldose reductase inhibitory activity of the prepared compounds is higher than that of the respective glycine derivatives. Furthermore, the parent compound **4** reveals high antioxidant potential. Additionally, the intestine permeability of **4** is determined, and there is initial evidence that there is an operating influx mechanism. Overall, the data indicate that the presented chemotype could serve as a core structure for the design of putative pharmacotherapeutic agents, aiming to the long-term complications of diabetes mellitus.

1. Introduction

Diabetes mellitus is a complex metabolic disorder that affects between 6 and 20% of the population in Western industrialized societies, with an estimated worldwide prevalence of 150 million people in 2000. This number is expected to increase to 220 million people in 2010.[1,2] Furthermore, taking into account its present rate of increase, within few decades, it will be one of the world's most common diseases and one of the biggest public health problems, with an estimated minimum of half a billion cases.[3] The diabetic individual is prone to late onset complications that are largely responsible for the morbidity and mortality observed in the patients.[4] It has been demonstrated that the more severe and sustained the degree of hyperglycemia, the more likely it is that the chronic complications of diabetes will develop.[5,6] Pharmaceutical intervention of hyperglycemia induced diabetic complications is actively pursued since it is very difficult to maintain normoglycemia by any means in patients with diabetes mellitus.[7,8]

Aldose reductase enzyme (AR, ALR2, E.C. 1.1.1.21, alditol/$NADP^+$ oxidoreductase) of the polyol metabolic pathway was first found to be implicated in the etiology of the secondary complications of diabetes.[8] Aldose reductase inhibitors (ARIs) have therefore been noted as possible pharmacotherapeutic agents. Although several ARIs have progressed to the clinical trials' level, only one is currently on the market and only in Japan (Epalrestat, ONO Pharmaceutical, Osaka, Japan).[9,10] However, the inhibition of the polyol pathway is considered to be a promising approach to control diabetic complications. Thus, attention is currently targeted to discover ARIs of distinct chemical structures, being derivatives of neither hydantoin nor carboxylic acid that are known to cause either toxicity or possess narrow spectrum of tissue activity.[11,12]

In the present work, the synthesis and the AR inhibitory activity of *N*-(3,5-difluoro-4-hydroxyphenyl)benzenesulfonamide (**4**) and its derivatives **5**–**7** are presented. The design of the compounds **4**–**7** was based on the previously reported ARIs **I**–**IV**, which are *N*-benzenesulfonylglycines,[13] taking into account the proposal that the 2,6-difluorophenol ring could be a lipophilic isostere for molecules containing a carboxylic acid group.[11,14] The structures of the compounds **4**–**7** and **I**–**IV** are shown in Figure 1.

Keywords: Antidiabetic aldose reductase inhibitors; Non-classical bioisosterism; Antioxidant activity; Permeability.
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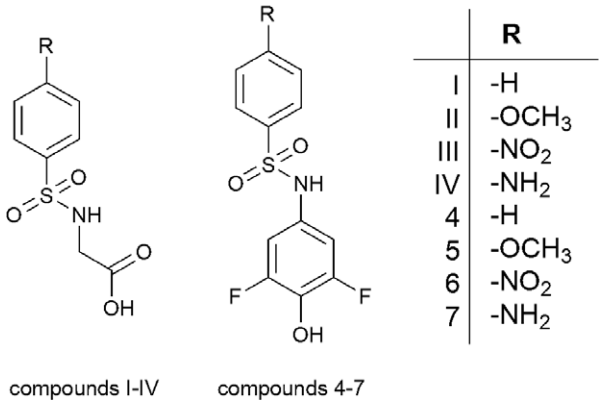


	R
I	-H
II	-OCH_3
III	-NO_2
IV	-NH_2
4	-H
5	-OCH_3
6	-NO_2
7	-NH_2

Figure 1. Structures of *N*-benzenesulfonylglycines **I–IV** and *N*-(3,5-difluoro-4-hydroxyphenyl)benzenesulfonamides **4–7**.

Furthermore, the antioxidant potential of compound **4** was evaluated both in a homogeneous 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay system,[15–18] as well as in a heterogeneous unilamellar L-α-phosphatidylcholine dioleoyl (DOPC) liposomes' assay system.[18,19] Oxidative stress is well established as an important biochemical factor implicated in the long-term complications of diabetes mellitus.[12,20]

Finally, the permeability of compound **4** was determined in vitro using rat jejunum mounted in side-by-side diffusion cells. We considered that these results could be an initial indication of the permeability potential of the presented chemotype.[10]

2. Results and discussion

2.1. Chemistry

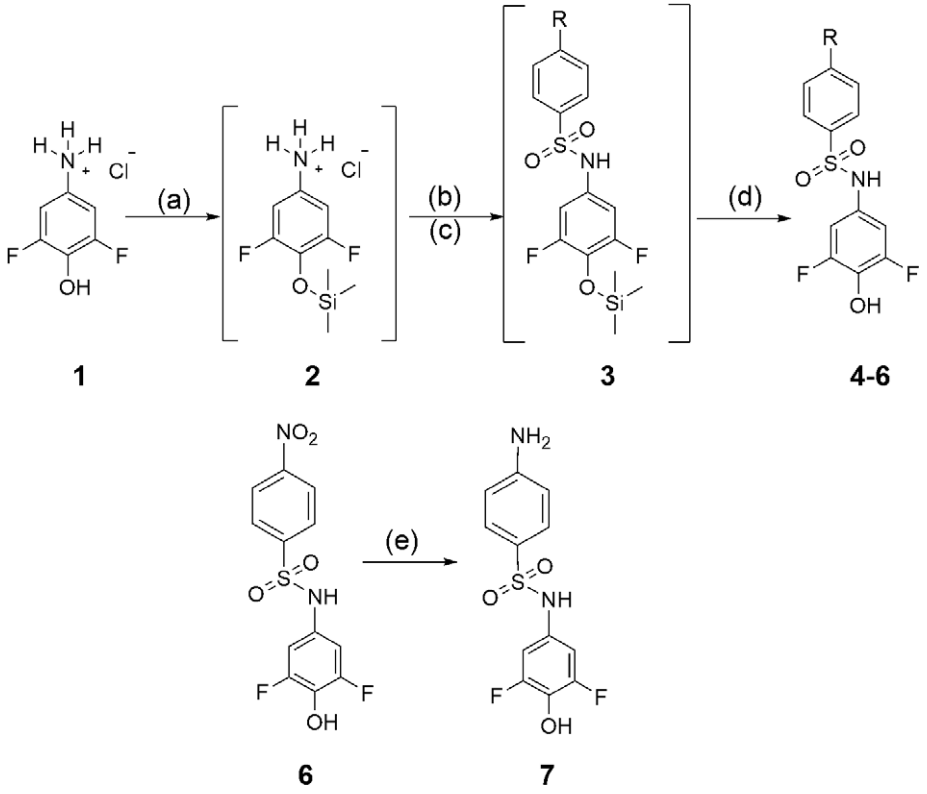
4-Amino-2,6-difluorophenol has been reported to be unstable and could be isolated only as its hydrochloride salt **1**.[11] We propose that the degradation of the non-protonated aniline form of **1** is probably due to an auto oxidative process via an iminoquinone intermediate. Thus, in order to overcome the above mentioned instability, a one-pot reaction (Scheme 1) was implemented for the formation of the *N*-(3,5-difluoro-4-hydroxyphenyl)benzenesulfonamides (**4–6**). This involved the following consecutive steps: (i) protection of the phenolic hydroxyl-group as its corresponding trimethylsilyl ether, (ii) formation of the sulfonamide bond by the reaction with the appropriate aryl-sulfonyl chloride, in the presence of a catalytic amount of *N*,*N*-dimethylpyridin-4-amine (DMAP), (iii) addition of an equimolar amount of triethylamine in respect to the staring fluorophenol derivative **1**, and (iv) a hydrolytic process in order to obtain the target compounds **4–6**. For the above synthetic route, we suggest an operating cyclic mechanism based on the high affinity of oxygen to silicon as well as the hypernucleophilic properties of DMAP.[21–24] The addition of triethylamine at the latter stage of the reaction was found to increase the overall yield. It is worth noting that the addition of triethylamine in higher amounts or at the start of the reaction resulted in the formation of 4-amino-2,6-difluorophenyl arylsulfonates as side products, with a significant lower yield of the target compounds **4–6**.

For the formation of compound **7**, we investigated the heterogeneous catalytic transfer hydrogenation in the presence of cyclohexene[11] in refluxing either ethanol (57% yield) or isopropanol (77% yield).

2.2. In vitro results

2.2.1. Aldose reductase inhibitory activity. The target compounds **4–7** were tested for their ability to inhibit rat lenses AR. It has been shown that there is an approximately 85% sequence similarity between rat lenses and human AR, while the proposed active sites of both enzymes are identical.[25] The performed assay was based on the spectrophotometric monitoring of NADPH oxidation, which is proven to be a reliable method.[26] Results are shown in Table 1 and for comparison, results previously reported for compounds **I–IV** in a similar assay are also included.[13] It was found that the putative bioisosteres of compounds **I–IV**, the difluorophenol derivatives **4–7**, had improved potency. Although the activities of **4–7** are not at a submicromolar range, they could be considered as putative hit inhibitors towards the AR enzyme.[27] Inspection of the low-energy conformations (SPARTAN SGI, version 5.1.3)[28] reveals that the distances' range of the geometric (unweighted) centers of the aromatic areas of the aryl-sulfonamide substituents of **4–7** and **I–IV** (centroids) from the phenolic or carboxylic acidic hydrogens are quite similar (6.91–6.95 and 7.09–7.11 Å, respectively). Furthermore, the analogous distances of the centroids of the heavy atoms (i.e., excluding the hydrogens) of these molecules from the phenolic or carboxylic acidic hydrogens are also quite similar (4.66–4.99 and 4.55–5.15 Å, respectively). As a working hypothesis, we propose that the better inhibitory activities of **4–7** in comparison to **I–IV** could be due to their higher molecular surface area [calculated electron density surface area range (SPARTAN SGI, version 5.1.3)[28] for compounds **4–7** is 254.73–283.67 $Å^2$ while for compounds **I–IV** is 211.06–239.34 $Å^2$]. A number of works have previously indicated the importance of the molecular surface area of the compounds for the inhibition of the AR enzyme.[29–33]

2.2.2. DPPH scavenging activity. The radical scavenging potential of the title compound **4** was assessed in vitro by using the model reaction with the stable free radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The scavenging activity data are expressed as IC_{50} values, EC_{50} ratios, as well as an initial rate of interaction. Results are summarized in Table 2. In this homogeneous system of the ethanol solution of DPPH, antioxidant activity stems from an intrinsic chemical reactivity towards radicals. DPPH, as a weak hydrogen radical atom abstractor, could be considered as a good kinetic model for peroxyl $ROO^{\cdot}$ radicals.[34] According to the derived data, the tested compound **4** possesses high antioxidant activity, similar to that of trolox,[16,35,36] which is a known reducing agent for DPPH.[37] It must be noted that there is a considerable difference in the kinetics of the reaction



Scheme 1. Synthesis of *N*-(3,5-difluoro-4-hydroxyphenyl)benzenesulfonamides **4**–**7**. Reagents: (a) $(CH_3)_3SiCl$/THF; (b) $ArSO_2Cl$, DMAP/THF; (c) Et_3N/THF; (d) H_2O; (e) cyclohexene, Pd/C/$CH_3CH(OH)CH_3$.

Table 1. Aldose reductase inhibitory activity data

Compound	IC_{50} (±SD[a]) μM	IC_{50}[b] (μM)
4	32.9 (±0.4)	134
5	15.5 (±0.1)	31
6	44.4 (±1.9)	79
7	14.1 (±0.5)	16
Sorbinil	0.249 (±0.006)[c]	

[a] $n = 3$.
[b] Reported IC_{50} values (μM) for the respective glycine derivatives by De Ruiter et al.[13]
[c] Quoted representative IC_{50} values: 0.07–0.9 μM by Zaher et al.[44]

with the DPPH between these two molecules. In the case of compound **4**, the reaction rate is significantly slower than with trolox, and the steady state is achieved after 270 min. In the case of trolox, the steady state is achieved after 30 min and it is constant after 270 min. Furthermore, the approximate stoichiometry of the reactions (σ, number of reduced DPPH molecules per one molecule of antioxidant) were defined as $1/(2 \times EC_{50})$,[38] that in both tested compounds was found to be greater than one and apparently not equal to their available hydroxyl groups. A putative mechanism of a more complex reaction, justifying such a stoichiometry, could involve further reactions of the generated phenoxyl radicals of compound **4** or trolox, such as coupling, fragmentation and intermolecular addition. However, it should be pointed out that in the case of trolox this proposed more complex reaction with DPPH

Table 2. Antioxidant and permeability data

Compound	Homogeneous system of DPPH			Heterogeneous system of DOPC liposomes	P_{app}[a] ($\times 10^{-6}$ cm/s)		
	IC_{50} (±SD[b]) $\times 10^{-6}$ M	EC_{50}	Initial rate[c] (±SD[b])	IC_{50} (±SD[b]) $\times 10^{-6}$ M	M–S (±SD[b])	S–M (±SD[b])	R[d]
4	49 (±0.01)	0.25	0.498 (±0.005)	95.9 (±8.5)	6.34 (±0.17)	2.91 (±0.45)	0.46[e]
Trolox	37 (±0.09)	0.19[f]	1.809 (±0.004)	93.5[g]			
Epalrestat					4.34[h]	10.90[h]	2.5[h]

[a] Apparent permeability coefficient in mucosal to serosal (M–S) and serosal to mucosal (S–M) direction.
[b] $n = 3$.
[c] Absorbance decrease at 517 nm at 30 s.
[d] R factor is the ratio between S–M/M–S.
[e] $p = 0.025$.
[f] Reported $EC_{50} = 0.19$ by Ancerewicz et al.[16]
[g] Reported by Rackova et al.[19]
[h] Reported by Sturm et al.[10]

takes place in a shorter period, and probably in parallel with the initial step of the interaction, while for compound **4** it is significantly more time-dependent.

2.2.3. Inhibition of lipid peroxidation. In membranes, the relative antioxidant reactivity is probably different from a homogeneous system since it is determined by additional factors, such as location of the antioxidant and radicals and ruled predominantly by the partition ratios between water and lipophilic compartments. As an indicative heterogeneous assay, the antioxidant inhibitory efficiency of compound **4** was evaluated in the system of unilamellar DOPC liposomes oxidatively stressed by peroxyl radical generated in the aqueous phase by thermal decomposition of the hydrophilic azo initiator 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH). Results are shown in Table 2. For comparison, results previously reported for trolox in an identical assay are also included.[19] It is apparent from the inhibition data, which are expressed as an IC_{50} value, that the tested compound **4** is a potent antioxidant, comparable to that of the known antioxidant trolox.[19]

2.2.4. Permeability. Apparent permeability coefficients (P_{app}) in mucosal-to-serosal (M–S) and serosal-to-mucosal (S–M) direction were determined for compound **4**. Results are tabulated in Table 2. For comparison, results previously reported for epalrestat in an identical assay are also included.[10] The tested compound exhibited asymmetrical transport properties and its P_{app} value in the M–S direction is higher than in the S–M direction. We propose that there is an operating influx process. However, at this point, we cannot suggest a molecular mechanism of this active transport. According to the Center for Drug Evaluation and Research (CDER) proposed permeability standards (atenolol as low permeable drug and metoprolol as high permeable drug)[39,40] and the M–S P_{app} data previously obtained for various drugs,[10] the tested compound **4** was classified into biopharmaceutical classification system (BCS) to estimate its in vivo effective permeability in humans.[41] It can be categorized in intermediate permeability class with the degree of absorption between 40% and 85% and its bioavailability would not be limited by the permeability.[40]

3. Conclusion

N-(3,5-Difluoro-4-hydroxy-phenyl)benzenesulfonamide (**4**) as well as its derivatives **5**–**7** exhibits a comparatively improved aldose reductase inhibitory activity. Additionally, the parent compound **4** exhibits a high antioxidant potential. It should be noted at this point that the antioxidant activity is considered as an additional indication for the ability of a compound to inhibit the formation of advanced glycation end products (AGEs),[42] that are implicated in the development of the long-term complications of diabetes.[11] Finally, compound **4** can be characterized as an intermediate membrane permeable chemical entity. We propose that the presented experimental data could comprise the basis for the design of novel chemotypes, as pharmacotherapeutic agents for the treatment of the long-term diabetic complications.

4. Experimental

4.1. General Notes

Unless otherwise stated, all commercial reagents were available from Aldrich or Fluka. Melting points were determined in open glass capillaries using a Mel-Temp II apparatus. UV spectra were recorded either on a Perkin-Elmer 554 or on a Hitachi U-2001 spectrophotometer. IR spectra were obtained on a Shimadzu spectrophotometer, and ^{1}H NMR spectra on a Bruker AW-80 spectrometer with internal TMS standard. Elemental analyses were performed in Galbraith Laboratories, Inc., Knoxville, TN, USA. Diffusion analyses were performed on EasyMount side-by-side chambers; equipped with a multi channel voltage–current clamp, model VCC MC6, Physiologic Instruments, San Diego, USA. HPLC analyses were performed with a Hewlett-Packard system, Series 1100, Waldbrom, Germany. Statistical analyses were performed using SPSS v.12.0.

4.2. Chemistry

4.2.1. General procedure for the preparation of *N*-(3,5-difluoro-4-hydroxyphenyl)benzenesulfonamides (4–6). To a suspension of 4-amino-2,6-difluorophenol hydrochloride (**1**)[11] (275 mg, 1.5 mmol) in THF (78 mL), chlorotrimethylsilane (0.25 mL, 1.9 mmol) was added and the mixture was vigorously stirred under a nitrogen atmosphere for 48 h. Then, aryl-sulfonyl chlorides (i.e., benzenesulfonyl chloride, 4-methoxybenzenesulfonyl chloride and 4-nitrobenzenesulfonyl chloride) (0.26 mL, 393 mg and 421 mg, respectively, 1.9 mmol) and *N*,*N*-dimethylpyridin-4-amine (DMAP, 48 mg, 0.4 mmol) were added and the mixture was vigorously stirred under a nitrogen atmosphere for 24 h. Triethylamine (0.21 mL, 1.5 mmol) in THF (11.5 mL) was then added dropwise and the stirring was continued under a nitrogen atmosphere for 24 h. Finally, the reaction mixture was poured into a stirred H_2O/ice mixture (~270 mL), and after 2 h of stirring it was extracted with CH_2Cl_2 (3× 50 mL). The organic layer was collected and the aqueous layer, after saturation with NaCl, was further extracted with CH_2Cl_2 (2× 20 mL). The combined organic phases were washed with saturated aqueous NaCl solution, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure and temperature <40 °C. The residues were flash chromatographed with petroleum ether/EtOAc 2.5:1 (compounds **4** and **6**) or 2:1 (compound **5**). Analytical samples were prepared by recrystallization from CH_2Cl_2/acetonitrile/petroleum ether.

4.2.1.1. *N*-(3,5-Difluoro-4-hydroxyphenyl)benzenesulfonamide (4). 225 mg, yield 52%, mp 178.5–180 °C, IR (Nujol): 3367–3269 cm^{-1} (–OH and –NH), 1170 cm^{-1} ($–SO_2NH–$), ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 6.7 (d, 2H, difluorophenyl-2,6*H*, J = 8.4 Hz), 7.4–7.9 (m, 6H, phenyl-*H* and phenyl-O*H*), 9.6–9.8 (br s, 1H, $–SO_2NH–$), Anal. Calcd for $C_{12}H_9F_2NO_3S$: C, 50.52; H, 3.18; N, 4.91. Found: C, 50.34; H, 3.19; N, 4.84.

4.2.1.2. *N*-(3,5-Difluoro-4-hydroxyphenyl)-4-methoxybenzenesulfonamide (5). 278 mg, yield 58%, mp 165–

166 °C, IR (Nujol): 3474–3047 cm^{-1} (–OH), 1260 cm^{-1} (C–O), 1162 cm^{-1} (–SO_2NH–), ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 3.8–4.0 (s, 3H, –OC*H*$_3$), 6.6–7.5 (m, 5H, methoxyphenyl-3,5*H*, difluorophenyl-2,6*H* and phenyl-*OH*), 7.7 (d, 2H, methoxyphenyl-2,6*H*, J = 5.6 Hz), 9.3–9.6 (br s, 1H, –SO_2N*H*–), Anal. Calcd for $C_{13}H_{11}F_2NO_4S$: C, 49.52; H, 3.52; N, 4.44. Found: C, 49.18; H, 3.50; N, 4.37.

4.2.1.3. *N*-(3,5-Difluoro-4-hydroxyphenyl)-4-nitrobenzenesulfonamide (6). 300 mg, yield 60%, mp 182–183 °C, IR (Nujol): 3517–3132 cm^{-1} (–OH), 1163 cm^{-1} (–SO_2-NH–), ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 6.7 (d, 2H, difluorophenyl-2,6*H*, J = 8.1 Hz), 7.3–8.4 (m, 5H, phenyl-O*H* and phenyl-*H*), 9.8–10.1 (br s, 1H, –SO_2N*H*–), Anal. Calcd for $C_{12}H_8F_2N_2O_5S$: C, 43.64; H, 2.44; N, 8.48. Found: C, 43.69; H, 2.44; N 8.56.

4.2.2. 4-Amino-*N*-(3,5-difluoro-4-hydroxyphenyl)benzenesulfonamide (7). To a solution of *N*-(3,5-difluoro-4-hydroxyphenyl)-4-nitrobenzenesulfonamide (**6**) (500 mg, 1.51 mmol) in isopropanol (12.5 mL), cyclohexene (1.15 mL, 11.36 mmol) and Pd/C (375 mg) were added. The mixture was refluxed under a nitrogen atmosphere for 4 h, cooled to room temperature, filtered through Celite, and evaporated under reduced pressure. The residue was recrystallized from CH_2Cl_2/acetonitrile/petroleum ether to provide compound **7** (348 mg, yield 77%): mp 158–159 °C, IR (Nujol): 3410–3196 cm^{-1} (–OH and –NH_2), 1151 cm^{-1} (–SO_2NH–), ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 3.4–4.4 (br s, 2H, phenyl-N*H*$_2$), 6.4–6.8 (m, 5H, phenyl-O*H*, aminophenyl-3,5*H*, difluorophenyl-2,6*H*), 7.6 (d, 2H, aminophenyl-2,6*H*, J = 8.0 Hz), 9.2–9.4 (br s, 1H, –SO_2N*H*–), Anal. Calcd for $C_{12}H_{10}F_2N_2O_3S(0.4CH_2Cl_2)$: C, 44.56; H, 3.26; N, 8.38. Found: C, 44.39; H, 3.22; N, 8.89.

4.3. In vitro assays

4.3.1. In vitro aldose reductase enzyme assay. The target compounds **4**–**7** as well as the reference compound sorbinil ($C_{11}H_9FN_2O_3$, Phizer, Inc., Central Research Division, Groton, CT, USA) were dissolved in 10% aqueous solution of DMSO. Lenses were quickly removed from Fischer-344 rats of both sexes following euthanasia. The experiments conform to the law for the protection of experimental animals (Republic of Greece) and are registered at the Veterinary Administration of the Republic of Greece. The enzyme preparation and assay were performed as previously described.[11,33] Compounds **4**–**7** were tested at five concentrations, the log (dose)–response curves were then constructed from the inhibitory data, and the IC_{50} values were calculated by least-square analysis of the linear portion of the log (dose) versus response curves ($0.952 < r^2 < 0.994$). The experiments were performed in triplicate.

4.3.2. DPPH assay. To investigate the antiradical activity of the title compound **4** in a homogeneous system, a method based on the scavenging of the stable free radical DPPH was used.[15–18] Compound **4** or, as a reference compound, 6-hydroxy-2,5,7,8-chroman-2-carboxylic acid (trolox) was dissolved in 1 mL of absolute ethanol (0.4–0.025 mM) and added to 1 mL solution of DPPH in absolute ethanol (0.4 mM) to give final concentrations of 0.2–0.0125 mM and 0.2 mM for the tested compounds and DPPH, respectively. The continual absorbance decrease of the ethanol solution of the stable free radical at 517 nm, in the presence of the tested compounds, was measured. The IC_{50} values were determined by least-square analysis of the linear portion of the log C versus % scavenging of DPPH curves ($0.904 < r^2 < 0.977$) of nine concentrations of the tested compounds after 270 min (steady state). The effective concentration value (EC_{50}, that is, the IC_{50} value divided by the concentration of DPPH) was also calculated.[16] Finally, the initial rate of the reaction was estimated from the approximately linear absorbance decrease during the initial 30 s, of equimolar concentrations (0.2 mM) of the tested compounds and DPPH.[18] The experiments were performed in triplicate.

4.3.3. Liposome preparation, incubation and LOOH determination. The experimental protocol is based on a previously described methodology.[18,19] A suspension of unilamellar L-α-phosphatidylcholine dioleoyl (C18:1, [*cis*]-9; DOPC; 99% grade) liposomes (1 mM) in phosphate buffer (20 mL, 20 mM, pH 7.4) was prepared. The liposomes (final concentration 0.8 mM DOPC) were incubated in the presence of different concentrations of compound **4** (10–300 μM) with the water-soluble initiator AAPH (final concentration 10 mM) at 50 °C for 80 min. Aliquots (1 mL) of the incubation mixtures were extracted with 2 mL portions of an ice-cold mixture of $CHCl_3$/MeOH (2:1, v/v) containing 2,6-di-*tert*-butyl-*p*-cresol (BHT) (0.05%). The lipid hydroperoxide content was determined by the thiocyanate method by sequentially adding the $CHCl_3$/MeOH (2:1, v/v) mixture (1.4 mL) and the thiocyanate reagent (0.1 mL).[43] The thiocyanate reagent was prepared by mixing equivalent volumes of a methanolic solution of KSCN (3%) and a ferrous ammonium sulfate solution (45 mM in 0.2 mM HCl). After the mixture had been left at ambient temperature for at least 5 min, the absorbance at 500 nm was recorded. The lipid peroxide value was determined using a calibration curve prepared with standard cumene hydroperoxide. The value of IC_{50} was obtained by least-square analysis of the linear part of the semi logarithmic plot of I (%, percentage of inhibition) versus antioxidant concentration ($0.965 < r^2 < 0.971$). The experiment was performed in triplicate.

4.3.4. In vitro transport study across rat jejunum. The study was performed in a similar manner as described previously.[10] The experiments conform to the law for the protection of experimental animals (Republic of Slovenia) and are registered at the Veterinary Administration of the Republic of Slovenia. Rat jejunum was obtained from starved male Wistar rats (250–320 g) after euthanasia and laparotomy. The tissue was opened along the mesenteric border, stretched onto special inserts with exposed tissue area of 1 cm^2 and then placed between two compartments of side-by-side diffusion chambers. Each compartment was filled with 2.5 mL of incubation saline containing 10 mM glucose and 10 mM mannitol on serosal and mucosal side of the tis-

sue, respectively. After preincubation for 25 min, the incubation salines on mucosal side, if studying mucosal-to-serosal (M–S) transport, or serosal side, if studying serosal-to-mucosal (S–M) transport, were replaced by 2.5 mL donor solution containing 0.479 mM of the title compound **4**, 10 μM fluorescein as a paracellular transport marker and 10 mM glucose (S–M) or 10 mM mannitol (M–S) in Ringer buffer. Samples of 250 μL were withdrawn from acceptor side every 25 min up to 175 min and replaced by fresh Ringer buffer containing all necessary ingredients at appropriate concentrations. The diffusion chambers were equipped with two pairs of Ag/AgCl electrodes, connected to the chambers via 3 M KCl/3.5% agar bridges for measuring transepithelial potential difference (PD) and short circuit current (I_{sc}) with multi channel voltage–current clamp in order to check/control the tissue viability and integrity by monitoring PD, I_{sc} and transepithelial electrical resistance (TEER) every 25 min. The viability was additionally checked by recording the rise of I_{sc} and PD after the addition of concentrated glucose solution to the mucosal compartment at the end of the experiment. Tissue integrity was assessed also by determining fluorescein permeability coefficient. The samples were analyzed by HPLC. A Chromolith C8 100×4.6 mm column was used. The mobile phase was composed of 0.05 M KH_2PO_4 (adjusted to pH 3.5 by orthophosphoric acid) and acetonitrile with 80:20 portions, respectively (flow = 2.2 mL/min). For detection, UV diode array detector was used at 220 nm. Fluorescence detector Tecan GENious was used for fluorescein detection, and the apparent permeability coefficient (P_{app}) was calculated.

Acknowledgments

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