Glucose-based spiro-oxathiazoles as in vivo anti-hyperglycemic agents through glycogen phosphorylase inhibition

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The design of glycogen phosphorylase (GP) inhibitors targeting the catalytic site of the enzyme is a promising strategy for a better control of hyperglycaemia in the context of type 2 diabetes. Glucopyranosylidene-spiro-heterocycles have been demonstrated as potent GP inhibitors, and more specifically spiro-oxathiazoles. A new synthetic route has now been elaborated through 1,3-dipolar cycloaddition of an aryl nitrile oxide to a glucono-thionolactone affording in one step the spiro-oxathiazole moiety. The thionolactone was obtained from the thermal rearrangement of a thiosulfinate precursor according to Fairbanks’ protocols, although with a revisited outcome and also rationalised with DFT calculations. The 2-naphthyl substituted glucose-based spiro-oxathiazole Sh, identified as one of the most potent GP inhibitors (K = 160 nM against RMGPb) could be produced on the gram-scale from this strategy. Further evaluation in vitro using rat and human hepatocytes demonstrated that compound Sh is a druggable anti-hyperglycaemic compound performing slightly better than DAB used as a positive control. Investigation in Zucker fa/fo rat model in acute and subchronic assays further confirmed the potency of compound Sh since it lowered blood glucose levels by ~36% at 30 mg/kg and ~43% at 60 mg/kg. The present study is one of the few in vivo investigations for glucose-based GP inhibitors and provides data in animal models for such drug candidates.

Introduction

Diabetes mellitus is one of the most severe global health problems of the 21st century. According to the International Diabetes Federation (IDF) 425 million people, ~9% of the adult population suffer from this disease, many of them may even remain undiagnosed. The number of diabetic patients is predicted to reach close to 700 million by 2045 with the most significant increase in low and medium income territories of Asia, Africa and South-America. In 2017 USD ~727 billion was spent for diabetes treatments representing more than 12% of the global health expenditures. Most of the diabetic patients (>90%) belong to the so-called type 2 diabetes mellitus (T2DM) when the organism does not produce insulin in sufficient quantities and/or the peripheral cells are more or less resistant to the insulin action to promote uptake of blood glucose. Due to the constantly high levels of glycaemia, short and especially long term complications are developed being frequent causes of death, thereby diabetes is one of the main contributors to global mortality. At present no causal therapy is known, treatment regimes aim at maintaining blood sugar levels around the normoglycemic value of ~6.1 mM by applying various medications.3, 4 Hepatic glucose output is elevated in T2DM patients. This glucose production consists of glycogenolytic and gluconeogenic components and the latter is known to be recycled through the glycogen pool.3, 4 Therefore, liver glycogen phosphorylase, the rate limiting enzyme of glycogen degradation has become a validated target to find potential new therapeutical possibilities against T2DM.4, 5

A broad variety of synthetic compounds and natural products have been tested as inhibitors of glycogen phosphorylase (GP)6-12 mainly with the most easily available rabbit muscle GPb (RMGPb).13 Many of these compounds’ complexes with the GP enzyme were also studied by X-ray crystallography. From the so discovered seven GP binding sites the most investigated one is the catalytic (or active) site which can accommodate D-glucose (the physiologib inhibitor of the enzyme) and a large array of glucose derivatives.14, 15 The most

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efficient glucose derived inhibitors belong to three main compound categories: glucopyranosylidene-spiroheterocycles, \(^{14,17}\) N-acyl-\(^{-}\)N\(^{-}\)-\(^{-}\)O-glucopyranosyl ureas, \(^{14,15}\) and \(\beta\)-d-glucopyranosyl heterocycles. \(^{18-26}\) In each category several inhibitors have submicromolar and even low nanomolar inhibitor constants. Some of these glucose analog GP inhibitors (GPIs) were subjected to various physiological investigations to show significant blood sugar diminishing effects in streptozotocin-induced diabetic rats, \(^{27}\) restoration of whole body insulin sensitivity, \(^{28}\) triggering of mitochondrial oxidation and mTORC2 (mammalian target of rapamycin complex 2) signaling, \(^{29}\) and improvement of pancreatic \(\beta\)-cell function. \(^{30}\)

Among the anomeric spiroworoles the most efficient inhibitors are the glucopyranosylidene-spiro-isoxazolines \(^{31,32}\) (K \(i\) = 0.63 \(\mu\)M against RMGPb for a 2-naphthyl derivative) and the glucopyranosylidene-spiro-oxathiazoles \(^{33}\) (K \(i\) = 0.16 \(\mu\)M against RMGPb for a 2-naphthyl derivative) while their xylopyranosylidene counterparts remained inactive. \(^{34}\) The glucose derived isoxazolines were shown to have significant inhibitory effects in rat and human hepatocytes, and also diminished hepatic glucose production in Zucker \(fa/\)fa rats by single dose oral administration. \(^{35}\) In this paper a new synthesis of the glucopyranosylidene-spiro-oxathiazoles, their \(in\) \(vitro\) enzymatic evaluation, as well as cellular and \(in\) \(vivo\) evaluations of the best inhibitor as an antihyperglycaemic agent are described.

**Synthesis**

Our previously explored strategy \(^{36}\) was developed for the synthesis of glycosylidene-spiro-oxathiazoles. The key step was the oxidative spirocyclization of O-peracetylated glycosyl hydroximothioates \(^{37}\) (e.g. 2) to glycosylidene-spirooxathiazoles 3 and 4 (Scheme 1), a transformation achieved readily upon treatment with NBS in refluxing halogenated solvents (CCl\(_4\), CHCl\(_3\)). When the hydroximothioate moiety displayed an aryl substituent, our earlier study showed that the cyclization proceeded well whatever the configuration of the pyranose ring (\(\alpha\)-gluco, \(\beta\)-galacto) or that of the anumeric centre (\(\alpha/\beta\)). Moreover, the cyclization was stereoselective, yielding preferentially \(1S\)-spiro-oxathiazoles in the D-galacto series. Later on, this methodology was applied for preparing D-glucos \(^{33,34}\) or \(\alpha\)-xylo \(^{34}\) analogues. In this last series, the cyclization was shown to occur with the opposite stereoselectivity, thus favouring formation of the \(1R\)-configured spiro-oxathiazoles. The compounds studied in more details displayed the aromatic pharmacophores a–l (Scheme 1). \(^{35,38}\) For the first step of the sequence, earlier studies \(^{37}\) showed that, under basic conditions, 2,3,4,6-tetra-O-acetyl-1-thio-\(\beta\)-d-glucopyranose 1 reacted readily with hydroximoyl chlorides to afford the corresponding glucosyl hydroximothioates 2. For improving access to glucosinolates, \(^{39}\) a procedure involving the \(in\) \(situ\) formation of an oximoyl chloride from the oxime using inexpensive bleach, which is then reacted directly under basic conditions with thioglycopyranose has been reported. However, comparison of these procedures showed that the conventional method to hydroximothioates resulted in higher yields. \(^{34}\) Therefore, the two-step conventional method has been used to prepare new analogs in the present work (\(Ar = p\)-CO\(_2\)-H\(_2\)N\(_2\)H\(_2\) 2-quinolinyl, 9-phenanthryl) with either hydrogen bond donors or acceptors or expanded aromatic moieties for better interactions of the aglycons in the \(\beta\)-pocket of the enzyme’s catalytic site.

![Scheme 1. Synthesis of glucose-based spirow-oxathiazoles \(^{33,38}\) by oxidative spirocyclization.](image-url)

Glucosyl hydroximothioates precursors 2j-I were obtained in variable yields (Table 1), their spirocyclization proceeded as generally observed, yielding preferentially the \(1S\)-configured spirobicycles 3j-I. The anomeric configurations for compounds 3j-I were deduced from NMR spectroscopy which revealed significantly different chemical shifts for the H3 and H5 pyranosylidene protons, and the C1 spiro carbon. \(^{33,34,36,38}\) As noted earlier for \(1S\)-configured analogs, these signals were respectively close to 5.65, 4.45, and 122.5 ppm. The protected \(1S\)-spirobicycles were decacylated in high yield under Zeppelin conditions.

Enzyme kinetic studies (IC\(_{50}\) and/or K\(_i\) measurements) were then performed to evaluate whether the prepared spirobicycles were GP inhibitors. Then, the most potent candidates were further evaluated through \(in\) \(vitro\) and \(in\) \(vivo\) biological assays to demonstrate their pharmacological interest. Therefore, substantial quantities of material were needed, typically gram- to multi-gram-scale. This called for the design and development of an unprecedented route to \(\alpha\)-glucose based spiro-oxathiazoles, with the hope that a stereoselectivity higher than that of the NBS-mediated cyclization could be achieved.
A new route to spiro-oxathiazoles by 1,3-dipolar cycloaddition of nitrile oxides to gluconothionolactone

The design of a new access to spiro-oxathiazoles was inspired by our reported synthesis of glycosylidene-spiro-isoxazolines based on 1,3-dipolar cycloaddition of nitrile oxides to exomethylene glycals (pyranoid exo-glycals).\textsuperscript{31, 32, 35} Concerted reactions are advantageous as regard to atom economy, experimental conditions and yield, so they are of particular interest in carbohydrate chemistry. We and others could demonstrate that such cycloadditions occurred with a high stereocontrol, the dipole attacking the exomethylene carbohydrate from the α-face of the α-glucopyranosylidene ring,\textsuperscript{36} while for steric and electronic reasons, regioselectivity was equally high, favouring attack of the anomic carbon by the oxygen atom of nitrile oxides in all cases studied. The more complex cycloaddition of nitrones (E and/or Z configurations, endo and/or exo transition states) to pyranoid exo-glycals led to mixtures of cis/trans and α/β products, but the α-face approach usually predominated, or was even exclusive in some cases.\textsuperscript{41, 42}

Based on this 1,3-dipolar cycloaddition analogy, the known α-glucono-δ-thionolactone \textsuperscript{12}\textsuperscript{43, 45} appeared as the required precursor towards spiro-oxathiazoles. Moreover, benzylated α-glucono-δ-thionolactone \textsuperscript{12} appeared to be a readily accessible substrate, based on the one-pot two-step synthetic route by the group of Fairbanks.\textsuperscript{46, 47} 1-Thiosugars reacting with tert-butyl-sulfinyl chloride in the presence of triethylamine were converted into glycosyl S-tert-butyl thiosulfimates (within 10 min at room temperature), which upon thermolysis (10-20 min in refluxing toluene) yielded various glycono-thionolactones, and in particular α-glucono-δ-thionolactone \textsuperscript{12} in a notable 85% yield.\textsuperscript{47} Glycono-δ-thionolactones have been shown to react with dienes, diazoalkanes, and carbenoids,\textsuperscript{48} and the thermolysis of the obtained dihydro-1,2,3-, and -1,3,4-thiadiazoles has been investigated.\textsuperscript{49}

We developed this new route for synthesizing compound \textsuperscript{5h} on the multi-gram scale considering its remarkable inhibitory properties. The direct synthesis of dithiocarbonate \textsuperscript{8} from the commercially available hemiacetal \textsuperscript{6} using tosyl chloride and potassium O-ethyl dithiocarbonate under phase-transfer conditions\textsuperscript{50} gave us no results. However, the two-step protocol in one-pot reported by Vasella\textsuperscript{55} (treatment of hemiacetal \textsuperscript{6} with CCl\textsubscript{4} and HMPT, then with potassium O-ethyl dithiocarbonate at -40°C, for which basic conditions dictate the choice of base-stable protective groups) afforded the desired dithiocarbonate \textsuperscript{8} via the intermediate chloride \textsuperscript{7} (Scheme 2). Subsequent methanolation delivered the 2,3,4,6-tetra-o-benzyl-1-thio-α-glucopyranose \textsuperscript{9} in an 85% overall yield. Thiol \textsuperscript{9} was reacted with tert-butyl-sulfinyl chloride\textsuperscript{47} to afford the β-configured thiosulfinate \textsuperscript{10} in 90% yield as a ~1:1 S(S),S(R) diastereoisomeric mixture. While mass spectrometry displayed a main peak as expected (m/z = 661 [M+H]\textsuperscript{+}), the formed diastereoisomers \textsuperscript{10} were visible as two close spots on TLC plates. They were purified by column chromatography but could not be fully separated in pure form. The \textsuperscript{1}H NMR spectra of this mixture displayed two singlets for the diastereotopic tert-butyl groups resonating at 1.40 and 1.58 ppm, respectively.

Table 1: Stereoselective cyclization of β-0-glucosyl thiohydrotimates 2 to glucose-based spiro-oxathiazoles 3-4 and their deacetylation to S.\textsuperscript{31, 34}

<table>
<thead>
<tr>
<th>Ar</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>isolated yields [%] \textsuperscript{a}</th>
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<tbody>
<tr>
<td>a</td>
<td>90 \textsuperscript{f}</td>
<td>46 \textsuperscript{f}</td>
<td>11</td>
<td>90 \textsuperscript{f}</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>60 \textsuperscript{f}</td>
<td>33 \textsuperscript{f}</td>
<td>16</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>48</td>
<td>15</td>
<td>7</td>
<td>84</td>
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</tr>
<tr>
<td>d</td>
<td>65</td>
<td>30</td>
<td>16</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>e</td>
<td>71 \textsuperscript{f}</td>
<td>69 \textsuperscript{f}</td>
<td>17</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>f</td>
<td>85</td>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>g</td>
<td>40</td>
<td>61 \textsuperscript{f}</td>
<td>18</td>
<td>97</td>
<td></td>
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<tr>
<td>h</td>
<td>78</td>
<td>36 \textsuperscript{f}</td>
<td>16</td>
<td>94</td>
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<tr>
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<td>78</td>
<td>0</td>
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<td>k</td>
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<td>l</td>
<td>86</td>
<td>49</td>
<td>-</td>
<td>&gt;95%</td>
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\textsuperscript{a} Yields were calculated based on the isolated yields and on the amount of product present in the 1S5-mixtures (ratio measured by \textsuperscript{1}H NMR unresolved by column chromatography.)
Following Fairbanks’ procedure, thermal elimination was carried out in refluxing toluene with either the crude thiosulfinate 10 (two-step one-pot process) or the purified diastereoisomeric mixture, but disappointingly, the yields recorded for the thionolactone 12 were around 45%, significantly lower than the 85% yield reported.47 As a byproduct, the glucono-δ-lactone 11 was identified and actually isolated in an equal amount when the reaction time was increased thus allowing for the complete conversion of both thiosulfinate diastereoisomers 10. Formation of an undesired analogous δ-lactone was observed by Fairbanks (>20% yield) during the thermal elimination at 120°C of a rhamno-configured thiosulfinate.46 Presence of di-tert-butylthiosulfinate as a byproduct (vide infra, text and Fig. 3, Fig. 4) has been noted as another limitation of the method.47 These disappointing and intriguing observations led to repeated trials for enhancing the yield of thiolactone 12, but without success. TLC monitoring of the reaction revealed that the more mobile thiosulfinate diastereoisomer 10 was converted faster than the other one, and while its spot decreased visually on TLC plates, that of the thionolactone 12 increased. Seemingly, after complete conversion of the more reactive thiosulfinate, there was no increase in the amount of α-glucono-δ-thionolactone 12 from the qualitative analysis of TLC, and disappointingly, the isolated yields never exceeded 45%. Analysis of MS data collected for the fractions recovered from column chromatography confirmed the formation of α-glucono lactone 11 [m/z (M+Na)⁺ = 561], and showed the presence of an unidentified product (m/z = 607 M+H⁺) (vide infra, text and Fig. 1). After inquiring about these discrepancies, Prof. A.J. Fairbanks kindly provided us with the investigator’s original report. It appeared that the 85% yield reported47 for the thionolactone 12 was overestimated as, from the report, it corresponded to a crude product « contaminated with tert-butyl species, possibly di-tert-butyl thiosulfinate. Yields projected to approximately 54-56% ». Presence of byproducts, allegedly mixed disulfides or disproportionation products, was also supposed, but not proven. A note clearly stated that the yield estimated by ¹H NMR for the thionolactone 12 were 54 and 56% (close to the 43-53% yields observed in the α-galacto series),47 so that these data corroborated ours, suggesting the occurrence of at least one unwanted reaction detrimental to the yield of the desired glycono-thionolactone.

D-Glycono-thionolactones are subject to epimerization at C-2 or 2,3-elimination under basic conditions. Therefore, D-glucono-δ-thionolactone 12 was often used without further purification for the 1,3-dipolar cycloaddition. With the crude thionolactone 12, this step proceeded smoothly in the presence of 2-naphthyl hydroximoyl chloride to afford the spiro-oxathiazole 13 in high yield (~95%) and stereoselectivity (9:1 S/R mixture). The diastereoismeric mixture could be resolved by silica gel column chromatography to afford the desired spiro-oxathiazole 13 with a S-configuration at the spiro-anomeric center (85% isolated yield) and the minor R-epimer (<10% yield).

Not surprisingly, the final debenzylating step proved to be problematic, and standard hydrogenolysis in the presence of palladium did not proceed at all, while Et₃SiH/H₂ resulted in partial deprotection only. Treatments with bromine or NBS and water under various free radical conditions (NBS, CCl₄, hv, with or without CaCO₃; NaBrO₂/Na₂S₂O₅) resulted in complex mixtures, due to partial debenzylations,53, 54 and/or formation of oxidized products as evidenced by mass spectrometry: in addition to the expected fragments corresponding to the partial or total debenzylation, fragments corresponding to M+14 indicated the oxidation of benzylic to benzoates, while others (M+16 and M+32) suggested oxidation to sulfoxides and sulfones. Fortunately, debenzylation could be achieved by treatment with BCl₃ at –60°C55 with careful TLC monitoring to quench the reaction immediately after disappearance of the starting material to avoid further unwanted reactions. Purification by column chromatography delivered the desired final O-unprotected spiro-oxathiazole 5h (815 mg, 65% yield).

In conclusion, the designed 1,3-dipolar cycloaddition approach to tetra-O-benzyl α-glucono-δ-thionolactone 12 was most efficient for synthesizing the corresponding α-glucose-based 15S-3-(2-naphthyl)-spiro-1,4,2-oxathiazole 13. Both regio-, and stereo-selectivities were excellent. In contrast to literature reports, the limiting step of this approach was the synthesis of tetra-O-benzyl α-glucono-δ-thionolactone 12 by thermolysis of glucopyranosyl tert-butyl sulfinate precursors 10. The discrepancies between our results and those of Fairbanks as reported and detailed in an internal document prompted the investigation of the thermolysis mechanism by DFT calculations to better understand the possible path to the desired thionolactone.

Proposed mechanisms for the thermal elimination of thiosulfinates 10 to thionolactone 12

Various possible modes of reaction were envisaged (Scheme 3) since tert-butyl thiosulfinates may undergo thermal elimination by hydrogen atom abstraction through five-membered cyclic transition states (TS). Glycono-thionolactones being reportedly obtained sometimes in excellent yields,46, 47 elimination involving the anomeric proton (path a) was privileged (anomeric effect), thus delivering the desired thionolactone 12 and tert-butyl sulfenic acid. However, elimination might also involve one of the nine equivalent hydrogen atoms of the tert-butyl moiety (path b), which would afford isobutene and the 3-D-glucopyranosyl-thiosulfoxyl acid 14 as other name: 3-D-glucopyranosyl-1-oxa-trisulfane). Considering early and recent reports on the thermolysis of di-tert-thiosulfinate also termed di-tert-butylisulfane-S-oxide, tBuS(O)S₅Bu,57 these speculations were reasonable, but the total weight of lactones 11 and 12 (ca 90%, 1:1 ratio) measured after full conversion of both thiosulfinates indicated that path b was only a marginal process.
The puzzling formation of α-glucolonactone 11 might be due to hydrolysis by water present in trace amount or to adventitious reactions, as discussed recently, but since 11 was detected on TLC plates even at the initial stage of the reaction, and as use of a purified mixture of diastereomeric thiosulfinate 10 led to similar lactone distribution, this hypothesis was uncertain. As the glycophosphoryl tert-buty thiosulfinate 10 must achieve a favorable 5-membered cyclic TS for the thermal elimination to proceed, and because of probable steric clashes between the tert-buty group and the 2-O-benzyl group for the S(S)-diastereoisomer, most probably the S(R)- and S(S)-tert-buty thiosulfinate underwent elimination at different rates, the (R)-diastereoisomer reacting faster to deliver thionolactone 12 through path a. Still, another hypothesis was needed to explain the presence of glyconolactone 11. The thermal rearrangement of α-configured glycophosphoryl xanthates to α-glycosyl dithiocarbonates has been reported to proceed without inversion of configuration at moderate temperatures (50-65°C). This reported example of the Freudenberg-Schönberg rearrangement was supposed to occur by an intramolecular, 1,3-shift through a 4-membered cyclic TS. An analogous thermal rearrangement could occur with the less reactive S(S) thiosulfinate (path c), and the rearranged product with a O-glycosidic bond [OS(S) moiety] would be susceptible to afford by thermal elimination the lactone 11 and tert-buty thiosulfonic acid (BuSSH). Interestingly, the relative enthalpies for thiosulfonic acid isomers [HS(O)SH, and HS(S)OH] estimated by theoretical studies at 298 K in the gas phase were found to be almost the same, suggesting that isomers 10 and 10’ might have similar energies. While the rearrangement of xanthates to dithiocarbonates is stability driven, the proposed pathway to glyconolactone 11 might benefit from the activation of the anomer center (rarrangement favoured) and, in lactone 11, the stabilization of C=O bond (162 kcal.mol$^{-1}$) by ca. 50 kcal.mol$^{-1}$ compared to the C=S thiocarbonyl bond (115 kcal.mol$^{-1}$) in thionolactone 12. Eventually, calculations were carried out to identify the reaction pathway leading to the glucolonactone byproduct (hydrolysis versus thermal rearrangement followed by elimination).

**DFT Calculations**

As a representative model, the tetra-O-methyl analog of 10 was chosen (See supporting information for further details) to have reasonable computational time. The numbering of molecules applied is the same for both O-benzyl- (synthetic work) and O-methyl-protected (computational approach) analogues, but using italicized types in the last case. Our calculations addressed first the three reaction pathways mentioned above (Scheme 3), namely the two possible direct internal eliminations (E1, path a, path b), then path c, in which an internal S,S-rearrangement by 1,3-shift was supposed to occur prior to the Ei elimination. The stereochemistry at the sulfur atom did not influence the calculations and the results are presented here for a single diastereoisomer at the sulfur atom.

Starting from 10, the two divergent internal eliminations (E1) discussed above were envisaged (Fig. 1). Deprotonation of the anomeric position (path a) was found to be easy (TS-a, $\Delta$G$^\circ$ = 21.2 kcal.mol$^{-1}$), leading to the D-glucono-thionolactone 12 and formation of tert-buty sulfenic acid. In the case of path b, which involves one of the nine hydrogen atoms of the tert-buty groups, and leads to product 14 and isobutene, a higher activation barrier of $\Delta$G$^\circ$ = 24.9 kcal.mol$^{-1}$ (TS-b) was calculated. This is in agreement with the synthetic work, as the thermal $\Delta$-elimination delivered the thionolactone 12 in moderate yield while the benzyl-protected product 14 was not observed.

As a possibility for the puzzling formation of 11 from 10, path c was envisioned (Fig. 2). For this two-step pathway, a 1,3 shift of the tert-buty thiosulfinate group led first to 10’ (TS-c, $\Delta$G$^\circ$ = 36.3 kcal.mol$^{-1}$), which subsequently could react through an Ei elimination (TS-d, $\Delta$G$^\circ$ = 10.9 kcal.mol$^{-1}$) leading to 11 and tert-buty thiosulfinic acid. Due to the high activation energy of TS-c, this pathway can thus be excluded.
At this point, since the formation of the experimentally observed $\delta$-gluconolactone 11 cannot be explained through path c, we wondered whether lactone 11 could be formed due to hydrolysis of $\delta$-glucono-thionolactone 12. In this event, since the reaction was conducted under anhydrous conditions, water had to be formed as the reaction proceeded. Interestingly, Block et al. reported the dismutation of tert-butyl sulfenic acid, giving rise to tert-butyl-2-methyl-propane-2-sulfinitoate (or thiosulfinate) and water (0.5 eq. each, Fig. 3). The computed thermodynamics for the dismutation ($\Delta G = -4.4$ kcal mol$^{-1}$) were in agreement with the experimental observations of Block. Subsequently, water, assisted by the coordination of the thiosulfinate, can trigger the hydrolysis of compound 12 through TS-e ($\Delta G^e = 30.6$ kcal mol$^{-1}$), providing gluconolactone 11 and hydrogen sulfide (0.5 eq. each). This proposed mechanism is in agreement with the experimental formation of lactones 11 and 12 in a 1:1 ratio.

To further confirm that lactone 11 came from the hydrolysis of thionolactone 12 (Fig. 4), we computed the attack of di-tert-butyl thiosulfinate on substrate 12 (Fig. 4). TS-f ($\Delta G^f = 41.5$ kcal mol$^{-1}$) could lead indeed to the formation of 11 and tert-butyl-2-methylpropane-2-sulfinitoate (or dithiosulfinate) without the assistance of a water molecule. Since the $\Delta G^o$ between TS-e and TS-f was 10.9 kcal mol$^{-1}$ in favor of the former, water hydrolysis pathway was confirmed to be the most energetically reasonable route.

In summary, these calculations showed a good agreement with the synthetic results, as they indicated that the thermal elimination via 5-membered transition states preferentially involved the anomeric proton rather than one of the nine tert-butyl hydrogen atoms, thus yielding the target gluconothionolactone. Simultaneous formation of gluconolactone through a 1,3-shift of the thiosulfinate moiety, followed by Ei can be ruled out. Based on the reported dismutation of tert-butyl sulfenic acid which yielded di-tert-butyl thiosulfinate and water (0.5 eq. each), the calculations predicted that 12 can be hydrolysed into 11 (0.5 eq.). Thus, in order to optimize the yield of the target gluconothionolactone 12, water, formed while the thermal elimination proceeds, has to be trapped by efficient means, for example by adding activated molecular sieves to the reaction medium.  

**Enzyme kinetics**

Previous structure-activity relationship studies revealed that phenyl oxathiazole 5a was a moderate inhibitor of GP ($K_i = 26 \mu M$). Other para-substituted phenyl analogues (R = phenyl, NO$_2$, CN) exhibited lower inhibitory properties with IC$_{50}$ values in the high micromolar range for 5b,c,g. The para-fluoro and para-methoxy analogues 5d and 5e had similar and slightly better inhibitory potential than 5a ($K_i = 28$ and 8 $\mu M$, respectively). As observed for several other classes of glucose-based GP inhibitors, the 2-naphthyl oxathiazole derivative 5h was the best inhibitor among the series of compounds studied, and one of the best among glucose-based GP inhibitors. Substitution by a para-carboxy group on the phenyl (5j) resulted in a good inhibitory potency ($K_i = 238 \mu M$), while the 2-quinolinyl analogue 5k was more potent ($K_i = 26 \mu M$), although much less potent than the 2-naphthyl analogue 5h. In spite of the size of the 9-phenanthryl residue, compound 5l displayed moderate inhibitory properties.
Table 2: Kinetic data measured for spiro-oxathiazoles $5a$-$e, g, h, i, j$ for the inhibition of RMGPb

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
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</tr>
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<tr>
<td>$5b$</td>
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<tr>
<td>$5e$</td>
<td><img src="image" alt="Structure" /></td>
<td>$K_i = 8 \pm 0.9^{33}$</td>
</tr>
<tr>
<td>$5g$</td>
<td><img src="image" alt="Structure" /></td>
<td>$IC_{50} = 25\mu$M</td>
</tr>
<tr>
<td>$5h$</td>
<td><img src="image" alt="Structure" /></td>
<td>$K_i = 0.16 \pm 0.04^{33, 34}$</td>
</tr>
<tr>
<td>$5j$</td>
<td><img src="image" alt="Structure" /></td>
<td>$K_i = 238.5$</td>
</tr>
<tr>
<td>$5k$</td>
<td><img src="image" alt="Structure" /></td>
<td>$K_i = 26.02$</td>
</tr>
<tr>
<td>$5l$</td>
<td><img src="image" alt="Structure" /></td>
<td>$IC_{50} = 32.4 \pm 1.1$</td>
</tr>
</tbody>
</table>

**In vitro pharmacological evaluations**

Guided by the kinetic results and for an in depth evaluation of his inhibitory property, the glucose-based GP inhibitor $5h$ with the best $K_i$ value was assayed in vitro with rat and human hepatocytes in primary culture (see Table S1). The potent in vitro GP inhibitor 1,4-dideoxy-1,4-imino-b-arabinotiol (DAB, $K_i = 400$ nM)\(^{33}\) was selected as the reference compound for its established in vivo activity in a GP-dependent glycaemia study.\(^{33, 69, 70}\) An evaluation of glucose release after glucagon stimulation in primary rat hepatocytes was performed. Further evaluation in human hepatocytes for the candidate $5h$ was accomplished by measuring both glucose release and intracellular glycogen for an assessment of species specificity.

IC\(_{50}\) Values calculated for glucose release (product of GP-mediated glycogen depolymerisation) or intracellular glycogen content (substrate of GP-mediated glycogen depolymerisation) are similar (2-5 µM, Table 3) in human hepatocytes. In the studied cellular model, this result demonstrates that compound $5h$ affected glycogenolysis via GP inhibition.

Table 3: In vitro IC\(_{50}\) for compound $5h$ based on glucose release and intracellular glycogen content after glucagon stimulation in rat and human primary hepatocytes in primary cultures

<table>
<thead>
<tr>
<th>Compound</th>
<th>Glucose release $IC_{50}$ (µM)(^a)</th>
<th>Intracellular glycogen $IC_{50}$ (µM)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rats</td>
<td>Humans</td>
</tr>
<tr>
<td>DAB</td>
<td>$2.08 \pm 1.51$</td>
<td>$4.88 \pm 1.20$</td>
</tr>
<tr>
<td>$5h$</td>
<td>$6.7$</td>
<td>$3.37 \pm 1.00$</td>
</tr>
</tbody>
</table>

\(^a\) IC\(_{50}\) values are average of three to five measurements (see supporting information).

\(^b\) IC\(_{50}\) values are average triplicate measurements for three human hepatocyte cultures (see supporting information).

The concentration-response curves obtained for glucose release (Fig. 5) and observed intracellular glycogen content (Fig. 6) provided a basis for a comparison against DAB. Compound $5h$ performed slightly better than DAB in terms of concentrations to obtain 50% inhibition on glucose release in rat and human hepatocytes. Such concentrations are compatible with potential pharmacological applications.

Figure 5: Glucose release from rat and human hepatocytes after glucagon stimulation in vitro measured in the presence of compound $5h$ versus DAB as the reference compound. Top: Rat hepatocytes, bottom: human hepatocytes
**In vivo pharmacological evaluations**

The glucagon challenge test in the Zucker fa/fa rat model was performed to measure the glucose-lowering effect *in vivo* with compound 5h. This animal model, displaying hyperphagia and insulin resistance with hyperinsulinemia, was chosen due to its high hepatic glycogen content. We used glucagon (200 µg/kg, in a single subcutaneous “SC” administration) as hyperglycemic agent. A dose-dependent effect was clearly observed when compound 5h was introduced orally in a single administration (Figure 7).

Liver glucose output kinetics (Fig. 7A) and their areas under the curves (AUC) for 45 min (Fig. 7B) pointed to a dose-dependent decrease in liver glucose production ranging from 7.5 to 60 mg/kg (Fig. 7B). The rate of endogenous glucose production being elevated in type 2 diabetes renders this effect on liver output relevant for therapeutic applications. Compound 5h was then selected for a subchronic oral administration with a dose corresponding to the first significantly effective in the acute *in vivo* glucagon challenge test (30 mg/kg). In a glucagon challenge test performed after 4 days of treatment, a nearly 33% reduction of hepatic glucose production (p<0.05) was observed (Fig. 8A-B). It is worth pointing out that the activity of glycogen phosphorylase was preserved, in spite of the subchronic treatment, since hepatic glucose production was not further reduced.

As a summary, the *in vitro* and *in vivo* pharmacological effects of glucose-based spiro-oxathiazole 5h were analyzed and they validated its potent GP inhibition both *in vitro*, in cell assays and in a diabetic rat model (*in vivo*). In rat and human hepatocytes, compound 5h reduced glucagon-stimulated glucose output through glycogenolysis inhibition (Table 3, Fig.
Identification of this candidate, multigram-scale preparations for in vivo validation called for a robust and faster synthetic route to the target glucose-based spiro-oxathiazole.

Conflicts of interest
There are no conflicts to declare

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Notes and references
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