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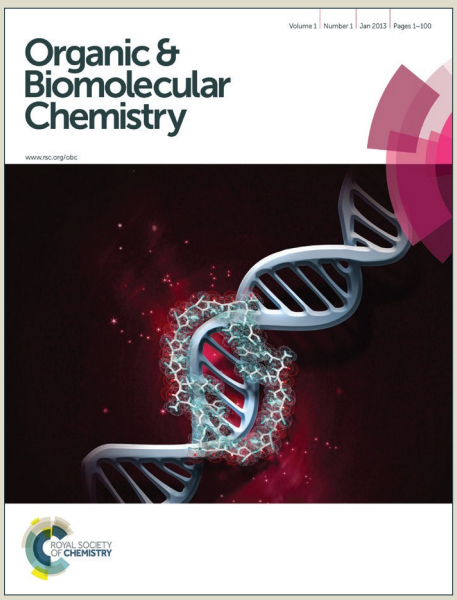

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ARTICLE

Selective photoregulation of the activity of glycogen synthase and glycogen phosphorylase, two key enzymes in glycogen metabolism

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Glycogen is a polymer of α-1,4- and α-1,6-linked glucose units that provides a readily available source of energy in living organisms. Glycogen synthase (GS) and glycogen phosphorylase (GP) are the two enzymes that control, respectively, the synthesis and degradation of this polysaccharide and constitute adequate pharmacological targets to modulate cellular glycogen levels, by means of the inhibition of their catalytic activity. Here we report on the synthesis and biological evaluation of a selective inhibitor that consists of an azobenzene moiety glycosidically linked to the anomeric carbon of a glucose molecule. In the ground state, the more stable (*E*)-isomer of the azobenzene glucoside had a slight inhibitory effect on rat muscle GP (RMGP, IC_{50} = 4.9 mM) and *Escherichia coli* GS (*Ec*GS, IC_{50} = 1.6 mM). After irradiation and subsequent conversion to the (*Z*)-form, the inhibitory potency of the azobenzene glucoside did not significantly change for RMGP (IC_{50} = 2.4 mM), while its effect on *Ec*GS increased by 50-fold (IC_{50} = 32 μM). Sucrose synthase 4 from potato, a glycosyltransferase that does not operate on glycogen, was only slightly inhibited by the (*E*)-isomer (IC_{50} = 0.73 mM). These findings could be rationalized on the basis of kinetic and computer-aided docking analysis, which indicated that both isomers of the azobenzene glucoside mimic the *Ec*GS acceptor substrate and exert their inhibitory effect by binding to the glycogen subsite in the active center of the enzyme. The ability to selectively photoregulate the catalytic activity of key enzymes of glycogen metabolism may represent a new approach for the treatment of glycogen metabolism disorders.

Introduction

Photoswitchable compounds are becoming increasingly popular for a number of biological applications based on the reversible photocontrol of structure of biomolecules, *e. g.* DNA[1-2] and proteins,[3-4] and of various biological functions such as biocatalysis,[5-6] ion transport,[7-8] cell adhesion[9] and protein folding.[10-11] In these examples, the biological activity of the modified molecules is altered by a light-triggered change of their molecular structure.[12] Most investigations involve chemical modification of nucleotides, peptides, proteins and lipids employing azobenzenes as photoswitchable chromophores.[5,7,10-14] Random-chemical substitution in undefined sites of biomolecules was the strategy of the past, whereas the novel concepts embark on the strategy of site specific incorporation and defined conformational transitions of structural elements.[15-18]

We are interested in the regulation of glucose and glycogen metabolism. Glycogen is a multibranched polymer of glucose that serves as carbohydrate and energy reservoir in bacteria, fungi and animals. The polysaccharide is synthesized by the action of glycogen synthase (GS), which catalyzes the successive addition of glucose units, arising from adenosine 5'-diphosphoglucose (ADPG) or uridine 5'-diphosphoglucose (UDPG), to the non-reducing end of a growing glycogen molecule. Glycogen phosphorylase (GP) is a key enzyme in glycogen degradation. Using inorganic phosphate as co-substrate, GP catalyzes the release of glucose-1-phosphate from the non-reducing end of the polysaccharide. Some metabolic disorders, such as diabetes, or rare diseases, like Lafora disease, are characterized by an abnormal accumulation of glycogen. Therefore, compounds that could selectively modulate the activity of GS or GP would be useful as potential drugs for the treatment of diseases related to glycogen metabolism.

Both GS and GP belong to a very large family of enzymes, namely glycosyltransferases (GTs), which catalyze the transfer of glycosyl residues from activated donors to diverse acceptors

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and play crucial roles in a great variety of biological processes, ranging from carbon and energy metabolism and cellular and molecular recognition to signal transduction and cell wall formation. Many of these enzymes use sugar diphosphonucleotides as donors and they mainly differ in the nature of the acceptor molecule. The design and synthesis of competitive inhibitors of GTs is fraught with difficulty, since such inhibitors are required to mimic a reaction transition state that effectively involves three distinct species: the glycosyl acceptor, the nucleoside diphosphate, and the glycosyl donor.[19-20] Therefore, alternative strategies to inhibition of GTs that act upon and produce polymeric molecules have been employed. For example, partial inhibition of the biosynthesis of cellulose[19] or the galactan component of the Mycobacterium tuberculosis cell wall[21] has been attained by the use of glycosyl donor analogues that act as chain terminators. Nevertheless, it seems reasonable that a putative competitive inhibitor aimed to act selectively on GS or GP should possess a structure resembling that of glycogen instead of mimicking the sugar diphosphonucleotide donor molecule.

With this objective in mind, our goal was to design, synthesize and analyze a molecule that might potentially resemble a short branch of glycogen and that additionally included a molecular switch, whose structure could be altered by an external stimulus such as light. Azobenzene and spiropyran are the most commonly used photoswitches in the design of molecules with photomodulated biological activity.[22-28] We have synthesized the glucoside **1** (Fig. 1) around the azobenzene photoswitch. Irradiation of the more stable (*E*)-form with UV light causes the isomerization across the N=N double bond to the bent (*Z*)-isomer, which by thermal equilibration or by exposure to visible light relaxes back to the (*E*)-isomer. If the *Z*→*E* back transition is slow enough and one conformation of the azobenzene glucoside is found to decrease the enzyme activity significantly more than the other, this allows for the spatial and temporal resolution of the biological activity of the inhibitor.

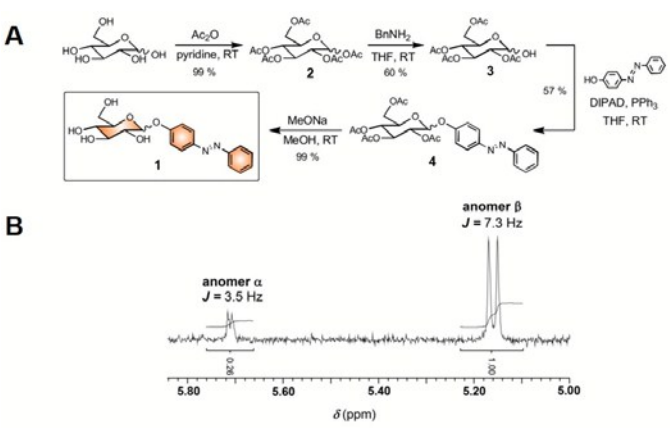


Fig. 1 A) Synthesis of the azobenzene glucoside **1**. B) Partial ^{1}H NMR spectrum of **1** in D_2O, showing the resonances of the anomeric protons of the α and the β anomer and their relative integrals.

We have studied the photochromic properties of azobenzene glucoside **1** and its potency and selectivity as inhibitor of the enzymatic activity of three purified GT preparations that were readily available to us: *Escherichia coli* GS (*Ec*GS) and rabbit muscle GP *a* (RMGP*a*) were studied as representatives of the enzymes responsible for the synthesis and degradation of glycogen, respectively. Sucrose synthase 4 (SuSy 4) from potato, which catalyzes the conversion of UDPG and fructose into sucrose and UDP, was included in the study as an example of a GT that does not act on glycogen.

Results and discussion

Synthesis of the azobenzene glucoside 1

Synthesis of compound **1** (Fig. 1A) started with the quantitative peracetylation of D-glucose with acetic anhydride in pyridine.[29] Next, the anomeric acetyl group of 1,2,3,4,6-penta-*O*-acetyl-D-glucopyranoside (**2**) was selectively cleaved using benzylamine in THF at room temperature to furnish **3**,[30] which was employed for the glycosylation of commercially available 4-hydroxyazobenzene, by means of the Mitsunobu reaction.[31] The resulting 4-(phenylazo)phenyl-2,3,4,6-tetra-O-acetyl-D-glucopyranoside (**4**) was deacetylated with MeONa/MeOH to give 4-(phenylazo)phenyl-D-glucopyranoside (**1**) in quantitative yield.[32-33] The azoglucoside **1** was obtained after four steps with an overall yield of 34 % as a mixture of the α and β anomers. Integration of resonance of the anomeric proton in the ^{1}H NMR spectrum of compound **1** showed that the α:β ratio was approximately 1:4 (Fig. 1B).

Photochromic properties of the azobenzene glucoside 1

Fig. 2A shows the changes observed in the absorption spectrum of the azobenzene glucoside **4** after irradiation with UV light in HEPES buffer, pH 7 at 30 °C. The spectrum of the more stable (*E*)-isomer exhibits the typical bimodal absorption profile of azobenzenes, which consists of a strong symmetry-allowed π–π* transition with a maximum at 339 nm and a weaker symmetry-forbidden n–π* transition centred at *ca*. 430 nm. UV light induced *E*→*Z* photoisomerization of the azobenzene glucoside causes a large decrease of the intensity of the 339 nm band. Assuming that the (*Z*)-isomer does not contribute to the absorption at this wavelength and that the spectrum obtained before UV irradiation corresponds to 100 % of (*E*)-isomer, the apparent fractional amount of (*Z*)-form present in the system, Yapp, can be determined by Equation 1:[34]

$$Y_{app} = 1 - \frac{A_{ph}}{A_0} \times 100 \qquad (1)$$

where A_0 and A_t stand for the initial absorbance (100 % (*E*)-isomer) and the absorbance at after a given irradiation time, respectively, both measured at 339 nm. In our system, the photostationary state, in which no further change of the electronic spectrum was observed, was reached after

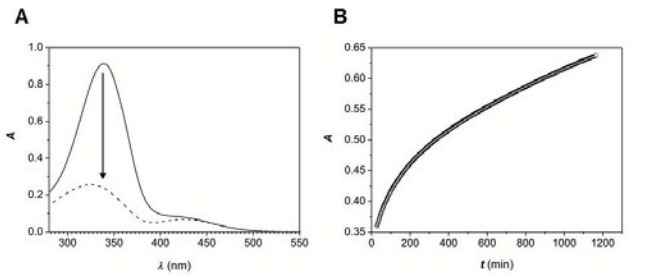


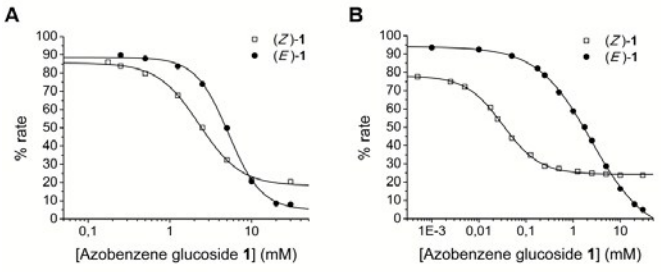
Fig. 2 A) UV/Vis absorption spectrum of 1 (30 μM in HEPES buffer pH 7, 30 ºC) before (solid line) and after (dashed line) irradiation with UV-light (320 nm < λ_{irrad} < 390 nm) for 10 minutes. B) Absorbance (339 nm) *versus* time trace for the thermally-activated *Z* →*E* back reaction of a solution of 1 (30 μM in HEPES buffer pH 7, 30 ºC), after the photo-stationary state had been reached. The trace was fitted to a biexponential function (R^2 = 0.99995), from which the values of the respective amplitudes (A_1 = 0.10 ± 0.01; A_2 = 0.42 ± 0.01) and first order decay constants (k_1 = 1.57 ± 0.02 × 10^{-4} s^{-1}; k_2 = 9.57 ± 0.09 × 10^{-6} s^{-1}) were estimated

approximately 10 minutes of irradiation. The apparent extent of *E*→*Z* photoisomerization for the azobenzene glucoside **1** was 75 %, a value lower than those previously reported for the isolated α- and β-azobenzene glucosides, which were estimated to be higher than 95 %.[35] It must be noted, however, that we have analyzed the extent of photoconversion in aqueous solution whereas the reported values were determined in DMSO.

When illumination ceased and the sample was kept in the dark, the thermally-activated *Z*→*E* reaction lead back to the thermodynamically stable (*E*)-isomer. This process was followed by monitoring the absorbance at 339 nm of a sample of **1** irradiated with UV light until the photostationary state was reached and then kept in the dark at 30 °C (Fig. 2B). The kinetic data obtained could be fitted to a biexponential function with a ratio of the amplitudes for the two distinct phases of 1:4, which closely matches the α:β ratio obtained from the ^{1}H NMR spectrum of compound **1**. Thus, the data for the smaller amplitude phase can be ascribed to the thermal *Z*→*E* back isomerization of the α anomer, while the larger amplitude phase derives from the more abundant β anomer of the azobenzene glucoside **1**. Both anomers showed slow *Z*→*E* back isomerization reactions, with first order rate constants of 1.57 × 10^{-4} s^{-1} and 9.57 × 10^{-6} s^{-1}, which correspond to half-life times of 1.22 h and 20.1 h, for the α and β anomer, respectively. Again, these half-life values are somewhat lower than those estimated for the separate α- and β-azobenzene glucosides in DMSO.[35] Both the large extent of *E*→*Z* conversion attained at the photostationary state and the long half-life times of the generated (*Z*)-isomers, allowed us to test the biological activity of these isomers without the need of a continuous light source illuminating the sample.

Biological activity of the azobenzene glucoside 1

The inhibitory potential of the azobenzene glucoside **1** on the enzymes responsible of the degradation and synthesis of glycogen was tested using purified preparations of commercially available RMGP*a* and recombinantly produced *Ec*GS, respectively. Recombinant SuSy 4 from potato was also included in the study as a representative of a non-glycogen metabolizing GT. RMGP*a* activity was assayed in the direction of phosphorolysis and *EcGS* and SuSy 4 in the direction of synthesis of glycogen and sucrose, respectively. All the assays were performed using saturating concentration of the respective substrates in HEPES buffer, pH 7.0 (10 mM Pi and 4 mg mL^{-1} glycogen for RMGP*a*; 2 mM ADPG and 4 mg mL^{-1} glycogen for *Ec*GS; 5 mM UDPG and 30 mM fructose for SuSy 4). A stock solution of (*E*)-**1** was serially diluted and each one of the dilutions was irradiated for 20 minutes to reach the photostationary state. The effect of either photoisomer of the azobenzene glucoside **1** as GT inhibitor was measured in a concentration-dependent manner. Thereby, sigmoidal curves were obtained (Fig. 3A and 3B), from which IC_{50} values for each isomer were deduced (Fig. 3C). It must be noted that the actual IC_{50} values of the (*Z*)-isomer must be somewhat lower than those shown in Fig. 3C, since, for the sake of simplicity, calculations were done assuming 100 % photoconversion of the (*E*)-isomer into the (*Z*)-form upon irradiation. This analysis revealed that the ground state (*E*)-azobenzene glucoside is a moderate SuSy 4 inhibitor (IC_{50} = 0.73 mM), while the (*Z*)-form showed to be a weak inhibitor, such that its IC_{50} could not be determined (not shown). RMGP*a* was moderately inhibited by both photoisomers, with IC_{50} values of 4.9 mM and 2.4 mM for the (*E*) and (*Z*)-forms, respectively. In contrast, this initial evaluation showed that while (*E*)-form of **1** is a moderately strong *Ec*GS inhibitor (IC_{50} = 1.6 mM), the inhibitory potency of the bent (*Z*)-isomer increased more than 50-fold (IC_{50} = 31.5 μM). Thus, the azobenzene glucoside **1** is an excellent photoswitchable selective inhibitor for the regulation of *Ec*GS activity (Fig. 3C).



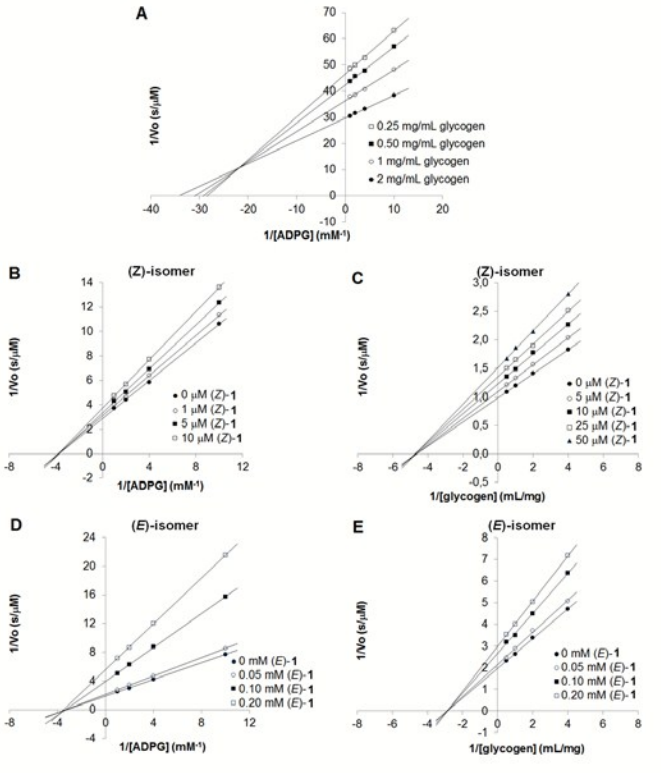
C

Target enzyme	Compound	IC_{50} value
SuSy 4	(*Z*)-**1**	0.73 ± 0.01 mM
	(*E*)-**1**	-*
RMGP*a*	(*Z*)-**1**	2.4 ± 0.1 mM
	(*E*)-**1**	4.9 ± 0.2 mM
*Ec*GS	(*Z*)-**1**	31.5 ± 0.1μM
	(*E*)-**1**	1.6 ± 0.1 mM

Fig. 3 Relative RMGP*a* (A) and *Ec*GS activities (B) at varying concentrations of the (*Z*) (□) and (*E*) (●) photoisomers of azobenzene glucoside **1**. IC_{50} values of (*E*)-**1** and (*Z*)-**1** for SuSy 4, RMGP*a* and *Ec*GS inhibition (C). * IC_{50} of (*Z*)-**1** for SuSy 4 could not be determined, since this form does not inhibit the enzyme to half of its initial value at the highest concentration tested. Values are the mean of three independent experiments.

We next examined the kinetic mechanism of the inhibition exerted by compound **1** on *Ec*GS. The double reciprocal plots of *Ec*GS activity under pseudo single substrate conditions converges to a negative value, which is characteristic of a sequential binding mechanism[36] of the two substrates, ADPG and glycogen, excluding a ping-pong mechanism (Fig. 4A). Additionally, double reciprocal analyses in the presence of varying concentrations of the inhibitor molecule (Fig. 4B-4E) showed that the (*Z*)-form of **1** is a non-competitive inhibitor with respect to both ADPG and glycogen, with observed inhibitory constants of 38 ± 5 nM and 67 ± 4 nM, respectively.

The (*E*)-isomer was also found to be a non-competitive inhibitor with respect to ADPG (K_i = 122 ± 21 μM) and glycogen (K_i = 352 ± 32 μM) (Fig. 4F). Together these results dictate that *Ec*GS follows an ordered bi-bi kinetic mechanism, where ADPG binds first to the free enzyme, followed by glycogen that binds to the ADPG-enzyme complex.[36-37] Moreover, this kinetic analysis indicated that both isomers of the azobenzene glucoside **1** mimic the acceptor substrate and occupy the glycogen subsite in the catalytic center of *Ec*GS.



F

Target enzyme	Compound	Substrate	Type of Inhibition	K_i value	Kinetic mechanism
*Ec*GS	(*Z*)-**1**	ADPG	Non-competitive	38 ± 5 nM	Ordered *bi-bi*
		glycogen	Non-competitive	67 ± 4 nM	
	(*E*)-**1**	ADPG	Non-competitive	122 ± 21 μM	
		glycogen	Non-competitive	352 ± 32 μM	

Fig. 4 Double reciprocal plot of *Ec*GS activity under single substrate conditions (A). Double reciprocal analyses of *Ec*GS activity in the presence of varying concentrations of (*Z*)-isomer (B and C) or (*E*)-isomer (D and E) of azobenzene glucoside 1. Inhibition constants of (*E*)-**1** and (*Z*)-**1** on *Ec*GS activity (F). Values are the mean of three independents experiments.

Docking of azobenzene glucoside 1 into the glycogen-binding site of *E. coli* glycogen synthase

Compound **1** is a 1:4 mixture of the α- and β-anomers. This raises the question as to which of the two anomers is the actual *Ec*GS inhibitor. To try to answer this question and to visualize complexation of the different forms of the azobenzene glucoside within the active site of GS, computer-aided docking of these molecules to the available X-ray structure of *Ec*GS was performed. The crystal structure of *Ec*GS mutant E377A in complex with ADPG and maltopentaose as an acceptor analogue of glycogen (PDB 3CX4)[38] was employed for docking the four geometric isomers of the azobenzene glucoside, α-(*E*), β-(*E*), α-(*Z*) and β-(*Z*), to the site normally occupied by glycogen in the active site (Fig. 5). The structures of the four isomers were previously optimized at the B3LYP/3-21G level of theory.[39] For these calculations, the (*E*)-isomer was characterized by an inversion angle α (the N=N-C angle) of 120° and a rotation angle ω (the dihedral angle C-N=N-C describing a rotation around the central N=N bond) of 180°. For the (*Z*)-form the inversion angle α was fixed to 120° and the rotation angle ω to 10°, since this dihedral angle at the N=N double bond for azobenzene is experimentally found to range from 8 to 12°, using X-ray methods and magnetic resonance spectroscopy techniques.[40-41]

In the best docked conformation, only the β-(*Z*)-isomer of compound **1** presents a good overlap of the glucosyl moiety and the two aromatic rings with the first three glucose residues of the crystallographic ligand maltopentaose (Fig. 5C). This analysis further shows that the C2 hydroxyl group of the glucose residue is within hydrogen bonding distance (2.9 Å) with the side chain of Asp137 and the C6 hydroxyl group with the backbone amide of Gly17 (2.8 Å). The aromatic ring next to the glucose moiety is also well oriented to stablish π-stacking interactions with the Tyr95 side chain (3.1 Å).

Finally, the second N of the N=N double bond can form a 2.9 Å hydrogen bond with the side chain of His96. In the remaining three geometric isomers, the aromatic ring distal to the glucose unit resides in a position distant from that occupied by the third glucose moiety of bound maltopentaose and they present several unfavorable interactions with various amino acid residues of the glycogen-binding site. Thus, docking analysis provides a reasonable explanation for the distinct inhibitory potency exhibited by the isomers of compound **1** on *Ec*GS activity and allows us to propose that the more abundant β-anomer with a (*Z*)-configuration of the azobenzene moiety is the actual inhibitor since it is the molecule that most resembles a bound glycogen molecule.

Conclusions

We have designed and synthesized the azobenzene glucoside **1**, which in its β-(*Z*)-form structurally resembles three terminal glucose residues of a glycogen branch. The molecule incorporates an azobenzene photoswitch whose conformation can be significantly altered by irradiation with UV light. The large attainable extent of photoconversion of compound **1**

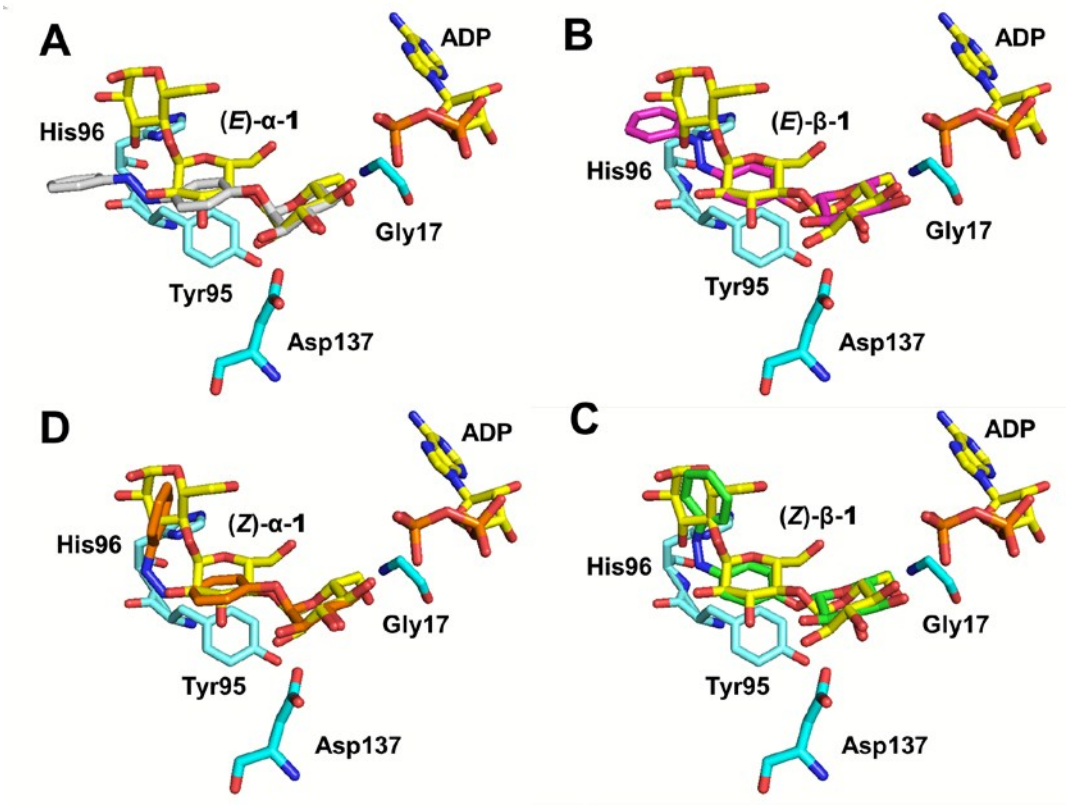


Fig. 5 Computer modelled docking of the azobenzene glucoside **1** to the glycogen binding site of *Ec*GS. Figure shows the α-(*E*) (A, in grey), β-(*E*) (B, in pink), α-(*Z*) (C, in orange) and β-(*Z*) (D, in green) forms of compound **1**. Selected *Ec*GS amino acids are shown in blue and three glucose residues from bound maltopentaose and ADP are shown in yellow.

from the ground state (*E*)-isomer to the bent (*Z*)-form, and the long life of the later allowed us to analyze the inhibitory properties of each photoisomer separately.

While both isomers were modest inhibitors of RMGP*a*, and only the (*E*)-form moderately inhibited SuSy 4, inhibitory potency in front of *Ec*GS increased by more than 50 fold upon irradiation, and the (*Z*)-isomer showed an IC_{50} in the μM range and kinetic inhibitory constants in the nM range. To the best of our knowledge, the azobenzene glucoside **1** is, as the kinetic and the docking analysis indicate, the first GS inhibitor that mimics the acceptor substrate of the enzyme, namely glycogen. Although compound **1** was synthesized as a 1:4 mixture of the α- and β-anomers, docking analysis suggests that the more abundant β-anomer is the one responsible for the observed inhibition. These observations, and the fact that compound **1** conformation can be photomodulated, open up the possibility of further development of drugs that act selectively on glycogen metabolic enzymes and whose biological effects can be spatially and temporally resolved.

Experimental section

Kinetic parameters of the photoinduced *E→Z* and thermal *Z→E* isomerization of the azobenzene glucoside

Solutions of the azobenzene glucoside **1** *c.a.* 3×10^{-5} M in 1 cm optical path quarz cells were irradiated with a Philips high-pressure mercury lamp (500 W nominal power) and using a 0.5 M solution of $Co(NO_3)_2$ as optical filter. Irradiation was pursued until no changes were observed in the electronic spectrum of the sample. Afterwards solution was thermostated in the dark at the desired temperature and the thermal *Z→E* isomerization was monitored by the change in the electronic spectrum of the sample. Observed rate constants and phase amplitudes were derived by fitting a biexponential function to the absorbance at 339 nm *versus* time trace, using standard software packages.

Cloning, expression and purification SuSy 1 from *Solanum Tuberosum L.*

The full length cDNA encoding for potato SuSy 4 cloned into the pET28a vector (Novagen) was kindly provided by Prof. Javier Pozueta-Romero, from Instituto de Agrobiotecnología y Recursos Naturales, Universidad Pública de Navarra/CSIC, Spain. This construct attaches a 6xHis tag sequence to the N-terminus of SuSy 4. *E. coli* BL21(DE3) cells harboring the pET28a/SuSy 4 plasmid were grown at 37 ^{o}C up to OD_{600} ~0.8 in LB medium supplemented with kanamycin (30 μg mL^{-1}). At that point, 0.8 mM IPTG was added and the induction was carried out at 22 ^{o}C for 16-18 h. The cells were collected by centrifugation and resuspended in 50mM Tris-HCl buffer (pH 7.4) plus 1 mM PMSF. Cell disruption was carried out by sonication of the ice-cooled suspension, after which the

insoluble material was separated by centrifugation at 13000 *g* for 45 min. The supernatant was loaded onto a HisTrap column (GE Healthcare), previously equilibrated with 50 mM Tris-HCl, pH 7.4. Recombinant SuSy 4 was eluted with a linear gradient of 0-250 mM imidazole in the same buffer and loaded on a Superdex 200 gel filtration column (GE Healthcare). The eluted sample was analyzed by SDS-PAGE, and the fractions that contained homogenous enzyme, as determined by SDS-PAGE, were pooled, concentrated with Centriprep YM-30 (Millipore) and stored at -80 ^{o}C in 50 mM Tris-HCl (pH 7.4) containing 10% (v/v) of glycerol.

Expression and purification of *Ec*GS

BL21(DE3) cells harboring the pET-23a/*Ec*GS plasmid[42] were grown at 37 ^{o}C in LB medium containing ampicillin (50 μg mL^{-1}) up to an optical density at 600 nm of ~0.8. At that point, 0.8 mM IPTG was added and the culture was grown at 23 ^{o}C for an additional 14-16 h. Cells were collected by centrifugation and resuspended in 50mM Tris-HCl buffer pH 7.4 plus 1 mM PMSF. Cell disruption was carried out by sonication of the ice-cooled suspension. The insoluble material was separated by centrifugation at 13,000 *g* for 45 min, and the supernatant was loaded into a nickel affinity column (HisTrap HP, GE Healthcare). The column was washed with 50 mM Tris-HCl pH 7.4 and His-tagged *Ec*GS was eluted with a linear gradient of 0-1 M imidazole and loaded on a Superdex 200 gel filtration column (GE Healthcare). The fractions that contained homogenous enzyme, as determined by SDS-PAGE, were pooled, concentrated with Centriprep YM-30 (Millipore) and stored at -80 ^{o}C in 50 mM Tris-HCl pH 7.4, containing 10% (v/v) of glycerol.

Assay of SuSy 4 and *Ec*GS activity

Sucrose and glycogen synthase activities were measured at 30 ^{o}C using the method reported by Morell and Copeland[43] in which the UDP or ADP, respectively, produced by the enzymes in the synthesis direction is coupled to NADH oxidation via pyruvate kinase and lactate dehydrogenase. For SuSy activity measurements the reaction mixture (100 μL) contained 200 mM HEPES buffer (pH 7.0), 30 mM fructose, 5 mM UDPG, 0.7 mM phosphoenolpyruvic acid, 0.6 mM NADH, 50 mM KCl, 13 mM $MgCl_2$, pyruvate kinase (7.5 U), lactate dehydrogenase (15 U) and an appropriate amount of enzyme. The same reaction mixture was used for *Ec*GS, except that 30 mM fructose and 5 mM UDPG were replaced with 4 mg mL^{-1} glycogen and 2 mM ADPG, respectively. One unit of enzyme activity is defined as the amount of the enzyme producing 1 μmol of UDP or ADP in 1 min at 30 ^{o}C.

Assay of rabbit muscle glycogen phosphorylase activity

Glycogen phosphorylase activity was measured in the direction of glycogen degradation (phosphorolysis) by determination of NADPH in an assay coupled to phosphoglucomutase and glucose-6-phosphate dehydrogenase as described by Schinzel and Palm[44] with a slightly modification. The enzyme activity was assayed at 30 °C in a 200 mM HEPES buffer pH 7.0 containing $NADP^+$ (2.2 mM), $MgCl_2$ (1 mM), glucose 1,6-bisphosphate (5 μM), glucose-6-phosphate dehydrogenase (1 unit), phosphoglucomutase (1 unit), 10 mM NaH_2PO_4 and 4 mg mL^{-1} glycogen, and an appropriate amount of enzyme. The reaction was started by the addition of the enzyme and the increase in absorbance at a wavelength of 340 nm, due to the formation of NADPH, was followed continuously using a Bio-Rad Benchmark Plus Microplate Spectrophotometer for 60 min at 30 ^{o}C. One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of glucose-1-phosphate in 1 min at 30 ^{o}C.

Enzyme kinetic analysis

Plots of initial rates (μM s^{-1}) *versus* substrate concentration were fitted to Michaelis-Menten equation by nonlinear least square regression using OriginPro 8.0. Alternatively, data was linearized and fitted to a straight by representing the inverses of initial rates *versus* inverses of substrate concentration.

Docking studies

The structures of (*E*)- and (*Z*)-isomers of the azobenzene glucoside **1** were optimized at the B3LYP/3-21G level of theory using Gaussian 03W version 6.0. The coordinates and refinement restraint files for both (*E*)-**1** and (*Z*)-**1** were prepared using the PRODRG of CCP4 program suite 6.3.0. Computer-aided docking of the optimized structures was performed by using Coot 0.7.0. Docking studies were based on the X-ray structure of the *E. coli* glycogen synthase (PDB 3CX4).[38]

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