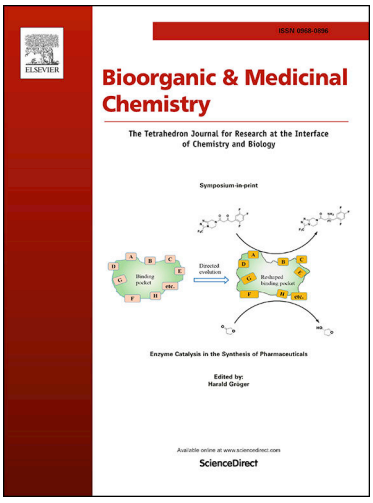

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Novel substituted *N*-benzyl(oxotriazinoindole) inhibitors of aldose reductase exploiting ALR2 unoccupied interactive pocket

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Abstract

Recently we have developed novel oxotriazinoindole inhibitors (**OTIs)** of aldose reductase (ALR2), characterized by high efficacy and selectivity. Herein we describe novel **OTI** derivatives design of which is based on implementation of additional intermolecular interactions within an unoccupied pocket of the ALR2 enzyme. Four novel derivatives, **OTI-(7-10),** of the previously developed *N*-benzyl(oxotriazinoindole) inhibitor **OTI-6** were synthetized and screened. All of them revealed 2 to 6 times higher ALR2 inhibitory efficacy when compared to their non-substituted lead compound **OTI-6**. Moreover, the most efficient ALR2 inhibitor **OTI-7** (IC_{50} = 76 nM) possesses remarkably high inhibition selectivity ($S_F \geq$ 1300) in relation to structurally related aldehyde reductase (ALR1). Derivatives **OTI-(8-10)** bearing the substituents -$CONH_2$, -COOH and -CH_2OH, possess 2-3 times lower inhibitory efficacy compared to **OTI-7**, but better than the reference inhibitor **OTI-6**. Desolvation penalty is suggested as a possible factor responsible for the drop in ALR2 inhibitory efficacy observed for derivatives **OTI-(8-10)** in comparison to **OTI-7**.

Keywords: aldose reductase (ALR2); interactive pocket; *N*-benzyl(oxotriazinoindole) ALR2 inhibitors; desolvation penalty

1. Introduction

Diabetes mellitus (DM), a wide-spread human disorder, has been recognized as a main cause of health problems such as blindness, neuronal diseases, hearth and kidney failures.[1] One of the most important therapeutic targets, implicated in late diabetic complications, is the polyol pathway. This pathway involves two steps, where *D*-glucose is converted to *D*-fructose *via D*-sorbitol. (Fig. 1) In the first step, aldose reductase (ALR2) catalyses reduction of *D*-glucose to *D*-sorbitol while NADPH is oxidized to $NADP^+$.[2] Deficiency of NADPH hampers regeneration of glutathione (GSH) from its dimer (GSSG), causing high excess of intracellular reactive oxygen species (ROS) in various tissues including the heart, neurons, eyes and kidneys.[3] In addition, high concentration of highly polar *D*-sorbitol results in osmotic imbalances and cell swelling.[4] *D*-Fructose, formed in the second step of the polyol pathway, causes excessive glycation of proteins and lipids and enhances formation of advanced glycation and lipoxidation end products (AGEs and ALEs), also involved in inflammation and oxidative stress. According to all these facts, inhibition of ALR2 activity can indirectly suppress above mentioned pathological imbalances and slow down late diabetic complications.

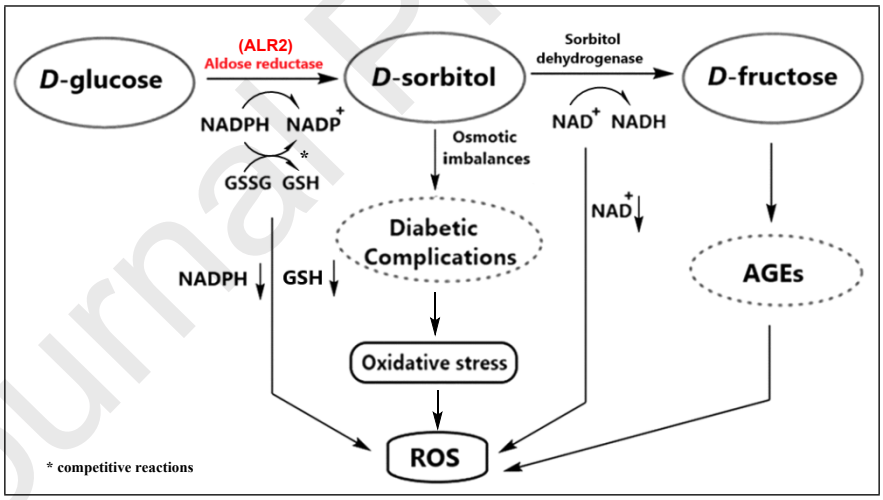
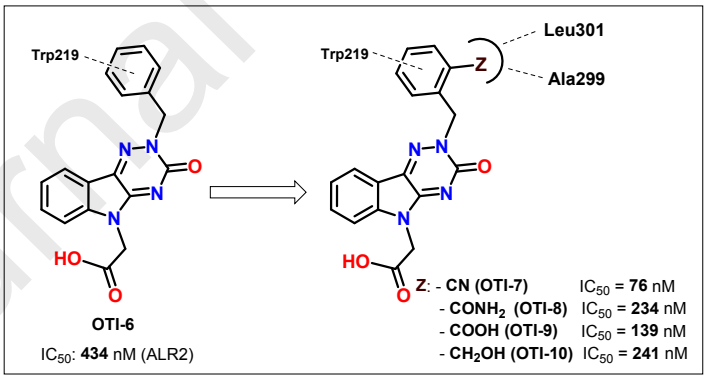


Figure 1. An illustration of the polyol pathway and its implication in the diabetic complications.

Reduction of the carbonyl functional group of lipid peroxidation products, namely 4-hydroxynonenal (HNE), and its glutathione adduct (GS-HNE) by ALR2 may generate intermediates contributing to activation of pro-inflammatory signaling. It points to aldose reductase inhibitors (ARIs) as promising agents in therapy of chronic inflammatory disorders and even several types of tumors related to chronic inflammation.[5-9] Moreover, interventions

with ARIs may have potential in attenuation of pathological consequences of several genetic metabolic disorders including galactosemia and sorbitol dehydrogenase deficiency.[10]

Over the last 40 years, numerous ARIs have been developed, but only **epalrestat** as ARI is therapeutically available in Japan, China and India.[11] Most of structurally different ARIs have been refused in clinical trials mainly for their low *in vivo* efficacies, pharmacokinetic drawbacks and/or side effects. The largest class of ARIs are carboxylic acids where carboxylate anion moiety occupies an anion binding pocket of the active site of ALR2. Štefek et al. developed the carboxymethylated thioxotriazinoindole **cemtirestat (CMTI)**, 2-(3-thioxo-2*H*-[1,2,4]triazino[5,6-*b*]indol-5(3*H*)-yl)acetic acid, as a novel lead-like candidate with excellent ALR2 inhibitory efficacy, selectivity and antioxidant activity.[12] Very recently, the inhibition efficacy and selectivity of **CMTI** was improved by isosteric replacement of sulphur with oxygen, which led to the discovery of the oxotriazinoindole inhibitor **OTI**, 2-(3-oxo-2*H*-[1,2,4]triazino[5,6-*b*]indol-5(3*H*)-yl)acetic acid, with markedly increased inhibition selectivity.[13] In this study, we aimed to exploit a previously unoccupied ALR2 pocket consisting of Trp219, Ala299 and Leu301 (PDB: 4QX4). In order to utilize this pocket to ensure additional interactions novel *N*-benzyl(oxotriazinoindole) derivatives were proposed. Besides the leading unsubstituted *N*-benzyl derivative **OTI-6** previously described by us,[13] herein we proposed, synthesized and screened its substituted derivatives **OTI-(7-10)**, containing different functional groups in an *ortho* position on a benzyl aromatic ring. (Scheme 1)



Scheme 1. Design of novel benzyl derivatives **OTI-(7-10)** from **OTI-6**.

2. Results and discussion

In this study, we aimed to explore an unoccupied ALR2 pocket in close proximity to the triazine ring of **CMTI** inhibitor (PDB: 4QX4). This pocket contains important amino acid residues e.g. Trp219, Ala299 and Leu301. In order to utilize this pocket for additional ligand-enzyme

interactions, novel *N*-benzyl(oxotriazinoindole) derivatives **OTI-(7-10)** have been designed and developed.

Predictions and interaction diagrams of OTI-(7-10)

Proposed derivatives **OTI-(7-10)** have been docked into the ALR2 protein conformer taken from the complex PDB: 4QX4. As expected, proposed derivatives possess additional H-bonds in the ALR2 unoccupied lipophilic pocket with Ala299 or Leu301 from backbone and two π-π interactions of OTI´s benzyl moiety with Trp219. Furthermore, the other important interactions e.g. of an acetate group in an anion binding pocket are crucial for a high ALR2 activity of all **OTI** derivatives (ionic interactions: with His110 and Lys77 and H-bonds with His110, Tyr48 and Trp111). Predicted interactions of **OTI-(7-10)** inhibitors are depicted in the diagrams (Fig. 2) as well as in the *Supporting information of this paper*.

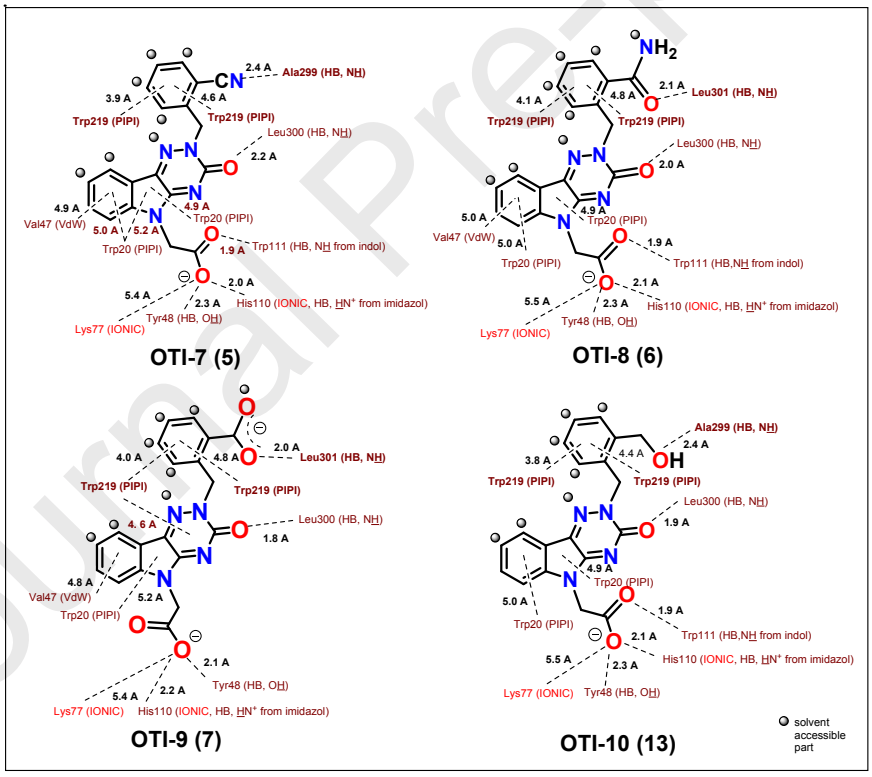
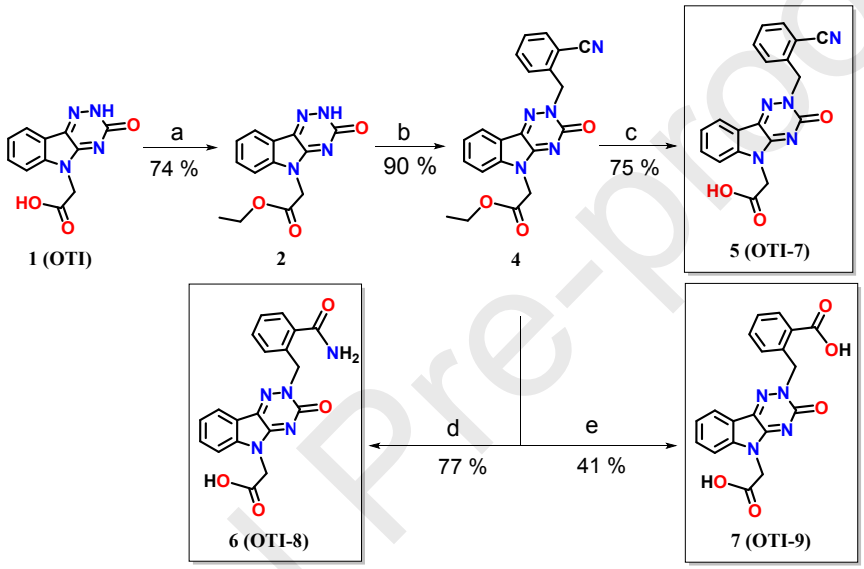


Figure 2. Predicted **OTI-(7-10)** interactions in a binding site of ALR2 enzyme (PDB: 4QX4).

Synthesis of OTI-(7-10)

Compound **1** (**OTI**)[13] represented a joint starting material for synthesis of all proposed inhibitors **OTI-(7-10)**. In the first step, **OTI** underwent acidic esterification by EtOH (abs) in

the presence of cat. amount of H_2SO_4 (conc). Obtained ester **2** was alkylated to **4** by CaH_2 in DMF, followed by addition of 2-(bromomethyl)benzonitrile **3** at 80 °C. Obtained *N*-(2-cyanobenzyl) derivative **4** was used as an intermediate for the synthesis of all proposed compounds **OTI-(7-9)**. Hydrolysis of an ester group of **4** by aq solution of NaOH in a mixture of MeOH / H_2O (1 / 1) at reflux within 30 min provided the *N*-(2-cyanobenzyl)acetate **OTI-7**. Partial hydrolysis of *N*-(2-cyanobenzyl)ester **4** with TFA / H_2SO_4 (conc) (4 / 1) at 70 °C within 6 h afforded *N*-(2-carbamoylbenzy)acetate **OTI-8**. Biscarboxylic acid **OTI-9** was prepared by complete hydrolysis of **4** in a mixture of H_2O / H_2SO_4 (conc) (1 / 1) at 100 °C within 8 days. (Scheme 2)

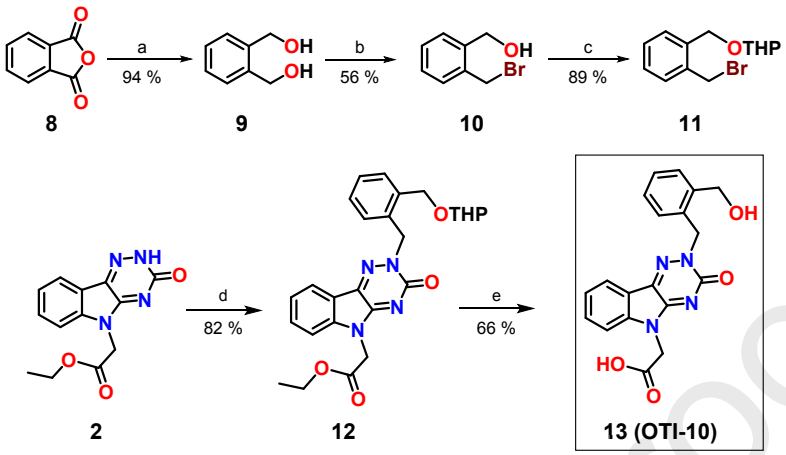


Scheme 2. Preparation of **OTI-(7-9)** from **OTI**.

Reagents and conditions: (a) EtOH (abs), cat H_2SO_4 (conc), reflux, 6 h; (b) (1) CaH_2 (1.19 mol eq), DMF (abs), 80 °C, 30 min, (2) 2-(bromomethyl)benzonitrile (**3**) (1.19 mol eq), from rt to 80 °C, 1 h; (c) NaOH (2.00 mol eq), MeOH / H_2O (1 / 1) reflux, 30 min; (d) TFA / H_2SO_4 (conc) (4 / 1), 70 °C, 6 h; (e) H_2O / H_2SO_4 (conc) (1 / 1), 100 °C, 8 d.

Synthesis of hydroxymethyl derivative **OTI-10** required THP protected (bromomethyl)phenylmethanol **11**, which was prepared in three steps starting from phthalic anhydride (**8**). Compound **8** was reduced to diol **9** by $LiAlH_4$ in THF (abs) at 0 °C to rt for 16 h in 94 % yield. Afterwards, treatment of diol **9** by conc HBr (48 %) in toluene at 70 °C within 20 min provided product **10** and traces of bis-brominated side product. After FLC separation, required compound **10** was obtained in 56 % yield. Finally, *O*-protected compound **11** was obtained in 89 % yield by reaction of 3,4-dihydropyrane (DHP) in a presence of cat amount of pyridinium *p*-toulenesulfonate (PPTS) in refluxing DCM within 30 min. Afterwards, previously

prepared ester **2** was alkylated with **11** by CaH_2 in DMF at 80 °C within 30 min to perform an intermediate **12**. Compound **12** was hydrolysed to desired *N*-(2-hydroxymethylbenzyl) derivative **OTI-10** by aq HCl in dioxane at 90 °C within 8 h in 66 % yield. (Scheme 3)



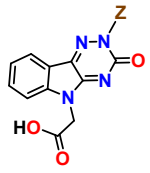
Scheme 3. Preparation of hydroxymethyl derivative **OTI-10**.

Reagents and conditions: (a) $LiAlH_4$ (2.00 mol eq), THF (abs), from 0 °C to rt, 16 h; (b) HBr (48 %) (1.12 mol eq), toluene, 70 °C, 20 min; (c) 3,4-dihydropyran (DHP) (1.16 mol eq), PPTS (pyridinium *p*-toulenesulfonate) (0.10 mol eq), DCM (abs), reflux, 30 min; (d) (1) CaH_2 (1.19 mol eq), DMF (abs), 80 °C, 30 min, (2) **11** (1.47 mol eq), from rt to 80 °C, 1 h; (e) HCl (conc) / H_2O (1 / 10), dioxane, 90 °C, 8 h.

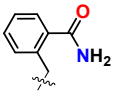
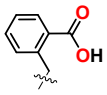
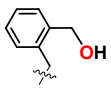
Determination of enzyme inhibitory activities

Inhibitors **OTI-(7-10)** were tested for their ability to inhibit the reduction of *D,L*-glyceraldehyde using ALR2 isolated from the rat eye lenses. Unsubstituted benzyl analogue **OTI-6** was used as a reference inhibitor. To assess selectivity we used a structurally related detoxification enzyme (an antitarget), aldehyde reductase (ALR1) isolated from the rat kidneys.[13]

Table 1. Results of *in vitro* enzyme assays of the novel ALR2 inhibitors **OTI-(7-10)** in comparison with the unsubstituted parent compound **OTI-6** and previously developed inhibitors **Cemtirestat** and **Epalrestat**.



OTI-(6-10)

Z:							
compound	OTI-6	OTI-7 (5)	OTI-8 (6)	OTI-9 (7)	OTI-10 (13)	Cemtirestat[a]	Epalrestat[b]
ALR2 IC_{50} [μM]	0.434 ± 0.014	**0.076 ± 0.006**	0.236 ± 0.005	0.139 ± 0.005	0.244 ± 0.003	0.116 ± 0.008	0.227 ± 0.019
ALR1 I (%, 100 μM)	38 ± 5	51 ± 1	50 ± 2	62 ± 1	33 ± 5	35.0 ± 1.6	n.d.
ALR1 IC_{50} [μM]	> 100	> 100	> 100	59 ± 6	> 100	n.d.	n.d.
S_F (selectivity factor)	> 230	**> 1 333**	> 424	424	> 410	302	n.d.

[a]Hlaváč et al. (2020),[13] [b]Majekova et al. (2017) (measured in 1 % DMSO),[26] n.d.-not determined. Results are means ± SD from minimum of three independent repetitions. *I* is the % of enzyme inhibition observed at 100 μM inhibitor concentration. Selectivity factor: $S_F = IC_{50}$ (ALR1) / IC_{50} (ALR2).

According to the results we found out that all substituted *N*-(benzyl) derivatives **OTI-(7-10)** exhibited from 2 to 6-fold better inhibitory efficacy than unsubstituted analogue **OTI-6**. In addition, they also revealed low inhibition of ALR1 antitarget, which resulted from a good to an excellent enzyme selectivity. (Table 1) Obtained results confirmed proposed additional interactions of substituted *N*-(benzyl) derivatives **OTI-(7-10)** within the interactive pocket of ALR2. The best ALR2 inhibition (IC_{50} = 76 nM) and selectivity relative to ALR1 (S_F > 1333) was obtained for **OTI-7** derivative containing a *N*-(2-cyanobenzyl) group. Predicted binding position of **OTI-7** in an active site of ALR2 shows a H-bond of a cyano group with Ala299 from backbone (2.4 Å). Moreover, the aromatic ring of a benzyl moiety forms two π-π interactions with Trp219 (3.9 and 4.6 Å). (Fig. 3)

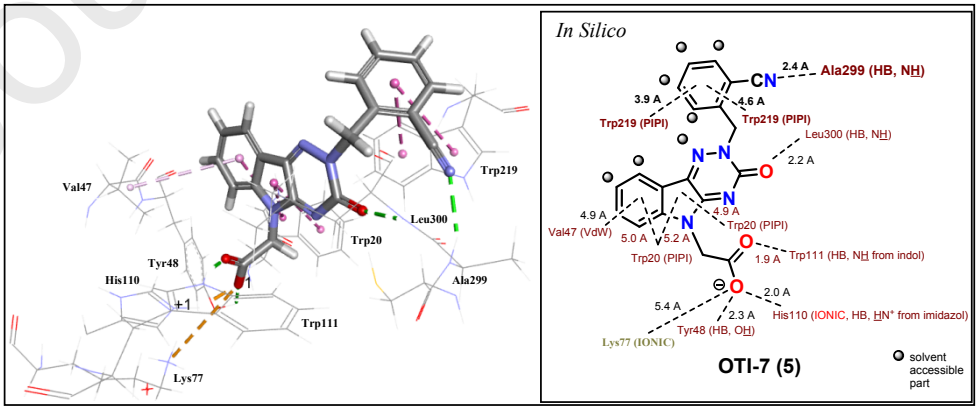


Figure 3. Predicted pose and interactions of **OTI-7 (5)** in a binding site of an ALR2 (PDB: 4QX4).

In spite of predicted similar positions and interactions of the derivatives **OTI-10** and **OTI-7**, **OTI-10** exhibited about 3-times lower inhibitory activity characterized with IC_{50} = 241 nM. The additional reason of lower activity of **OTI-10** could be desolvation and rotation penalty caused by a -CH_2OH group. Desolvation penalty effect has been a subject of our recent publication.[14] Derivatives **OTI-(8,9)** (-$CONH_2$, -COOH, resp.) revealed 2 to 3-fold lower inhibitory activity in comparison to **OTI-7** (-CN). Besides desolvation penalty, the lower activity of a carbamoyl derivative **OTI-8** could be also caused by conformational penalty of -$CONH_2$ group. Symmetry of delocalized carboxylate group in **OTI-9** (-COO^-) exhibited better inhibition activity than **OTI-8** (-$CONH_2$) and **OTI-10** (-CH_2OH). In addition, outer part of a studied pocket and a benzyl group are well water accessible (Fig. 4) and hence a benzyl group could provide two conformers. A first conformer allows formation of the predicted H-bond with an orientation of a polar group inside the pocket and a second conformer prefers orientation toward the solvent. Consequently, very polar groups would not be oriented inside the pocket, where the predicted H-bonds could be formed, but owing to solvation they would remain oriented toward a water environment out of the pocket. Therefore significant H-bond with Ala299 or Leu301 would not be formed as was predicted and thus the least solvated derivative **OTI-7** revealed the highest inhibitory activity.

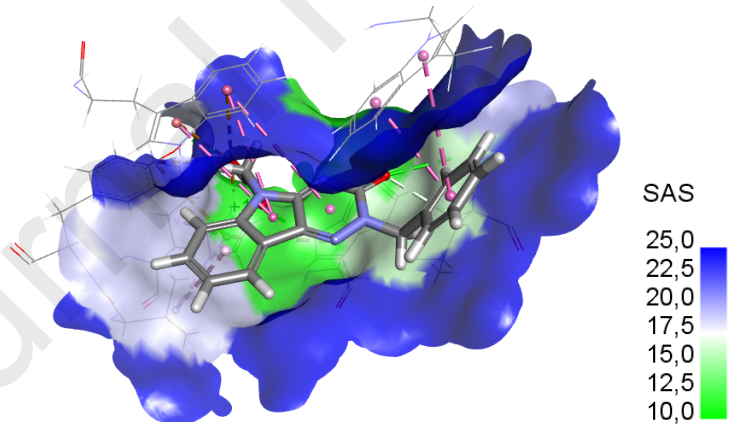


Figure 4. Solvents Accessible Surface of the unsubstituted benzyl derivative **OTI-6**. Blue colour indicates water accessible part.

In order to determine drug-like parameters, molecular obesity indices for compounds **OTI-(6-10)** were calculated (Table 2). The parameters take into account size, inhibitory efficacy and lipophilicity.

Table 2. Physicochemical properties and molecular obesity indices calculated for compounds **OTI-(6-10)**.

Compound	**MW** <500[h]	**pKa**[b]	**pIC$_{50}$**	**LogP**[a] <5[h]	**LogD**[a]	**LE**[c] >0.3[h]	**BEI**[d] >14.7[h]	**LLE**[e] >3.8[i]	**LELP**[f] <7.5[h]	**TPSA**[g] 60-140[h]
OTI6	334.33	4.14	6.36	2.04	-0.98	0.36	19.05	4.32	5.72	90.01
OTI7	359.34	4.14	7.12	1.61	-1.24	0.37	19.83	5.51	4.37	113.8
OTI8	377.35	4.14	6.63	1.05	-1.25	0.33	17.58	5.57	3.18	133.1
OTI9	378.34	4.14/4.15*	6.86	1.39	-2.50	0.34	18.14	5.47	4.05	127.31
OTI10	364.35	4.14	6.61	1.31	-1.53	0.34	18.17	5.30	3.82	110.24

[a]Calculated with MedChem Designer™ [b]Calculated with Pallas 3.112, log *D* represents the logarithm of the distribution ratio in octanol-buffer [pH 7] [c]Ligand efficiency, LE = -1.4log(IC_{50})/*N*, *N*: number of heavy atoms [d]Binding efficiency index, BEI = pIC_{50}/MW [e]Lipophilic ligand efficiency, LLE = pIC_{50} - logP [f]Ligand efficiency-dependent lipophilicity, LELP = log *P*/LE. [g]Topological polar surface area, TPSA calculated with MedChem Designer™ [h]Optimal drug values.[15-17] [i]Mean value for successful lead.[17] *BnCOOH.

Calculated values of the ligand efficacy (LE) and the binding efficiency index (BEI) for all inhibitors **OTI-(6-10)** were found in the range of optimal values (>0.3 and >14.7, resp). In addition, all novel compounds exhibited favourable lipophilic ligand efficiency parameter (LLE) values above the limit 3.8, important for a successful lead. All compounds **OTI-(6-10)** showed ligand-efficiency-dependent lipophilicity (LELP) in recommended range (<7.5), which implies good drug-likeness of all developed inhibitors. Moreover, all compounds have appropriate topological polar surface area (TPSA) (60–140 Å^2) predicting good oral absorbtion.

Based on the results from screening and the SAR conclusions, we have proposed other appropriate *N*-benzyl(oxotriazinoindole) analogues shown in Fig. 5. These derivatives should preserve similar interactions as the parent compounds within a studied ALR2 pocket and they should have lower desolvation and/or conformational penalties. Acetyl derivative **OTI-8a** (an analogue to amide derivative **OTI-8**) possesses lower polarity and solvation. Methyl ester **OTI-9a** is a less polar analogue to carboxylic acid **OTI-9**. Besides its lower solvation, it could also possess better penetration through the cell membrane in contrast to **OTI-9**. In addition, nitro derivative **OTI-9b** should be less solvated because of its lower polarity and with no conformational penalty considering its symmetric delocalized structure (Fig. 5) although -NO_2 group is less favorable in drug development due its potential toxicity. More detailed predicted interaction diagrams of newly proposed analogues **OTI-(8a-9a,b)** are described in the *Supporting information of this paper.*

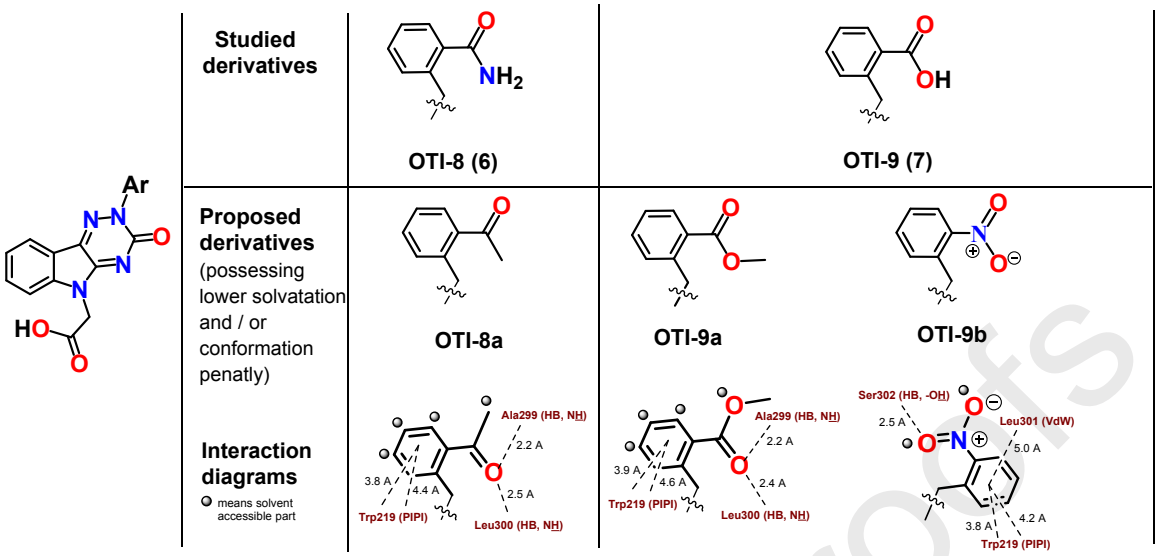


Figure 5. The structures of studied inhibitors **OTI-(8,9)** and their proposed promising analogues **OTI-(8a-9a,b)**.

Apart from **OTI-(8a-9a,b)** analogues, we proceeded with a proposal of other *N*-substituted analogues possessing lipophilic naphthalene derivatives **OTI-(11-13)** with favourable predictions comprising four π-π interactions with Trp219 and Van der Waals interactions with Ala299 or Leu301. (Fig. 6)

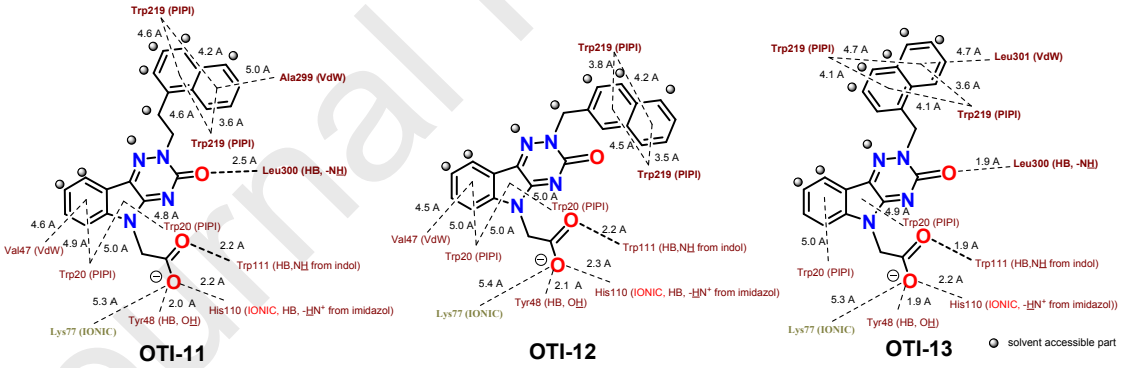


Figure 6. Proposed naphthalene derivatives **OTI-(11-13)**, their predicted poses and interactions.

Other polycyclic derivatives with favourable predictions and lower solvation include **OTI-14** and **OTI-15** derivatives. They contain 3,4-dihydronaphtalen-2(1*H*)-one and 2,3-dihydro-1*H*-inden-1-one moiety, resp. Besides their π-π interactions with Trp219, they also revealed H-bond with Ala299 and **OTI-15** also with Leu300. (Fig. 7)

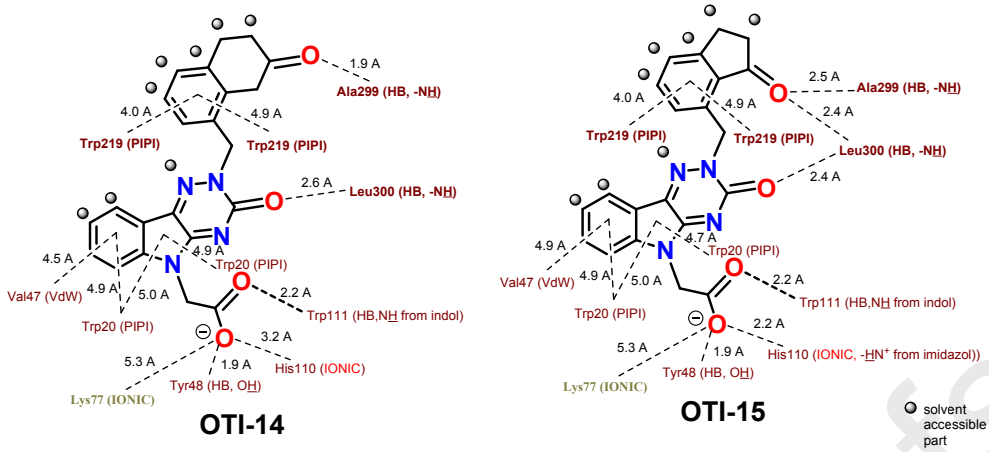


Figure 7. Predicted ALR2 poses and interactions of proposed polycyclic derivatives **OTI-14** and **OTI-15**.

Conclusions

We have developed four novel *N*-benzyl(oxotriazinoindole) inhibitors **OTI-(7-10)**, possessing polar functional groups (-CN, -$CONH_2$, -COOH, -CH_2OH, resp.) in *ortho* position on a benzyl group. They exhibited 2 to 6-fold better inhibitory activity than their *N*-(benzyl) lead compound **OTI-6**. Our findings confirmed expected additional interactions of novel **OTI** derivatives in a studied binding pocket of ALR2. Moreover, the novel derivatives also exhibited ALR1 inhibitory activity in high micromolar concentrations, which resulted in good to excellent ALR1 / ALR2 selectivity for developed compounds **OTI-(7-10)**. The most efficient ALR2 inhibitor **OTI-7** (IC_{50} = 76 nM) contains a planar and less solvated -CN functional group on a benzyl ring. Other derivatives **OTI-(8-10)** (-$CONH_2$, -COOH and -CH_2OH, resp.) revealed 2 to 3-fold lower ALR2 inhibitory activity than **OTI-7**. The reason of lower activity of derivatives **OTI-(8-10)** was assigned to a desolvation and possibly also to a conformational penalty. Considering these effects, we have proposed novel analogues **OTI-(8a-9a,b)** which preserve the favorable interactions of their parent compounds within ALR2 while possessing less polar substituents. Therefore, improved inhibition efficacies and drug-like properties of the novel derivatives are expected. Several polycyclic *N*-substituted derivatives **OTI-(11-15)** have also been selected due to their favorable proposed interactions in ALR2 pocket.

Experimental

Computer predictions

The structure of hu-ALR2 protein in a complex with NADP+ (PDB: 4QX4)[12] was used for our predictions. The optimized conformers of the proposed ligands were docked into the enzyme-cofactor complex by UCSF software Dock Blaster methodology.[18] The predicted poses of ligands in an active site of ALR2 enzyme were analysed and used for composition of **OTI-(7-10)** intermolecular interactions diagrams. (Fig. 2-3 and 5-6) The protein conformer of a human recombinant enzyme AKR1B1 from the complex (PDB: 4QX4), possessing two 3E2 (cemtirestat) ligands and NAP ($NADP^+$) cofactor, was used for modelling, grid generation and docking. The structures of ligands were proposed by molecular modelling in the Discovery Studio Visualizer software (release 2019) from Biovia.[19] The optimal 3D structures of proposed ligands together with their protomers, tautomers and isomers were generated by a Ligand Preparation tool of the Maestro software (release 2016) from Schrodinger at software standard predifined conditions and pH = 7.0 / ± 1.[20] The target protein was prepared for docking in a Protein Preparation Wizard tool of the Maestro software by standard predefined conditions at pH 7.0, while the present water molecules were removed. The target hydrogens were minimized, invalid bond order corrected, missing side chains added and detected amino acid alternative positions selected. Afterwards, a Receptor Glide Grid Generation tool of the Maestro software was used at standard predefined conditions exploiting binding position of 3E2 402 ligand, while the second 3E2 403 ligand was removed before grid generation. The optimal conformers of the ligands were docked into the enzyme-cofactor complex by a Glide Docking tool of the Maestro software at standard predefined conditions in an extra precision (XP) mode with flexible ligand sampling, rewarding intramolecular hydrogen bonds and enhancing planarity of conjugated pi groups. Only the best pose for each ligand was recorded. The most promising ligands were selected based on their score from docking experiments and the results of their intermolecular interaction analysis with target amino acid residues.

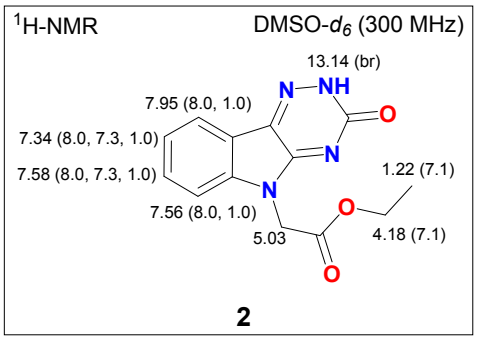
Chemistry

Melting points were measured by Barnstead Electrothermal IA9200 and are uncorrected. ^{1}H- and ^{13}C-NMR spectra were recorded on Varian Gemini (300 / 600 MHz and 75 / 150 MHz, resp.), chemical shifts are given in parts per million (ppm), tetramethylsilane was used as an internal standard. DMSO-d_6 and $CDCl_3$ were used as a solvent, unless otherwise specified. IR spectra were acquired on FT-IR-ATR REACT IR 1000 (ASI Applied Systems) with diamond probe and MTS detector. Mass spectra were performed on LC-MS (Agilent Technologies 1200 Series equipped with Mass spectrometer Agilent Technologies 6100 Quadrupole LC-MS). The

course of the reactions was followed by TLC analysis (Merck Silica gel 60 F254). UV lamp (254 nm) and iodine vapours were used for visualization of TLC spots. Starting chemicals were purchased from Sigma-Aldrich, Fluorochem, AlfaAesar or Acros vendors. All tested compounds **OTI-(6-10)** possess purity over 95 %. Their purity was determined by combustion analysis, HPLC and melting point. Combustion analysis was measured on vario MICRO cube (elemental analysis found values for carbon, hydrogen, and nitrogen within 0.4 % of the calculated values for their molecular formulas). Detailed descriptions of the intermediates and the final products **OTI-(7-10)**, their predicted poses in ALR2 and spectra figures are deposited in the *Supporting information* of this paper.

Ethyl 2-(3-oxo-2,3-dihydro-5*H*-[1,2,4]triazino[5,6-*b*]indol-5-yl)acetate (2)

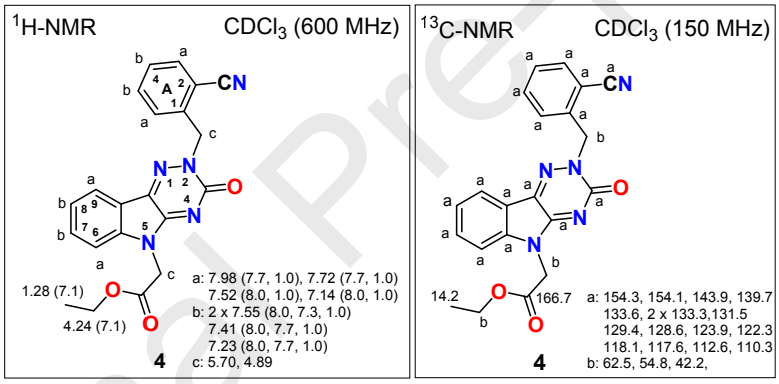
To a solution of 100 mg (0.41 mmol, 1.00 mol eq) of **1** (**OTI**) dissolved in 30 ml of EtOH (abs), 1.00 ml of H_2SO_4 (conc) was added and the reaction stirred at reflux under Ar. After 6 h TLC analysis confirmed a presence of a new product without any starting compound. The mixture was cooled down and excess of EtOH evaporated by RVE. Crude product was dissolved in 30 ml of DCM, washed with water (5 x 30 ml) and saturated aq solution of $NaHCO_3$ (3 x 30 ml). Separated organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure yielding 85.0 mg (0.31 mmol, 76 %) of ester **2** as an orange solid product. Novelty: ethyl 2-(3-oxo-2,3-dihydro-5*H*-[1,2,4]triazino[5,6-*b*]indol-5-yl)acetate (**2**) is described in the literature by its M.P., ^{1}H-NMR, ^{13}C-NMR, IR, MS and elemental analysis.[13] M.p.: 247.0 - 253.0 °C [DCM], lit.: 248.0 - 252.0 °C [MeOH / DCM].[13]



^{1}H NMR (300 MHz, DMSO-d_6): δ 13.14 (br s, 1H, -NH-), 7.95 (dd, 1H, *J*(8,9) = 8.0 Hz, *J*(7,9) = 1.0 Hz, H-C(9)), 7.58 (ddd, 1H, *J*(6,7) = 8.0 Hz, *J*(7,8) = 7.3 Hz, *J*(7,9) = 1.0 Hz, H-C(7)), 7.56 (dd, 1H, *J*(6,7) = 8.0 Hz, *J*(6,8) = 1.0 Hz, H-C(6)), 7.34 (ddd, 1H, *J*(8,9) = 8.0 Hz, *J*(7,8) = 7.3 Hz, *J*(6,8) = 1.0 Hz, H-C(8)), 5.03 (s, 2H, NCH_2COOEt), 4.18 (q, 2H, *J*(CH2,CH3) = 7.1 Hz, $-OCH_2CH_3$), 1.22 (t, 3H, J(CH_2,CH_3) = 7.1 Hz, $-OCH_2CH_3$).

Ethyl 2-[2-(2-cyanobenzyl)-3-oxo-2,3-dihydro-5*H*-[1,2,4]triazino[5,6-*b*]indol-5-yl]acetate (4)

To a solution of 100.0 mg (0.37 mmol, 1.00 mol eq) **2** in 3 ml DMF (abs), 18.5 mg (0.44 mmol, 1.19 mol eq) of CaH_2 was added and the reaction stirred at 80 °C for 30 min. Then, the mixture was cooled to rt and 86.5 mg (0.44 mmol, 1.19 mol eq) of benzonitrile **3** was added portionwise and the mixture stirred at 80 °C within 1 h. After consumption of **2** (confirmed by a TLC analysis) the reaction was cooled down in an ice bath, 10 ml of water added and the reaction stirred for 10 min. Resulting mixture was extracted with DCM (3 x 15 ml), a combined organic phase washed with brine (3 x 20 ml), dried over Na_2SO_4, filtered and concentrated under reduced pressure. A crude product was purified by FLC (SiO_2, Hex / EA = 1 / 5) to give 128 mg (0.33 mmol, 90 %) of **4** as an orange solid material. Novelty: ethyl 2-[2-(2-cyanobenzyl)-3-oxo-2,3-dihydro-5*H*-[1,2,4]triazino[5,6-*b*]indol-5-yl]acetate (**4**) has not been described in the literature. M.p.: 175.0 - 180.0 °C [Hex / EA].

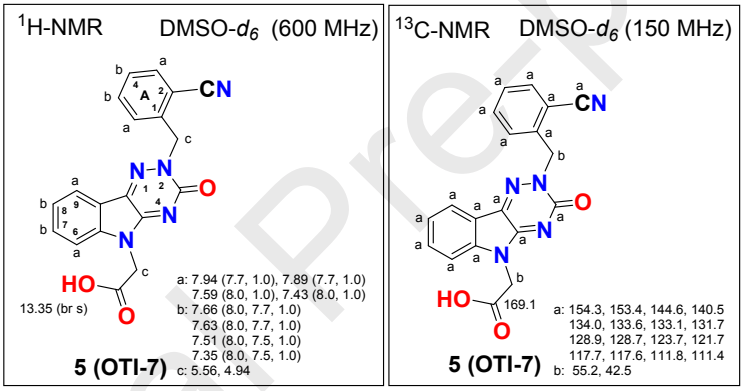


^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.98, 7.72, 7.52 and 7.14 (4 x dd, 4 x 1H, J(6,7 or 8,9 or A_3,A_4 or A_5,A_6) = 7.7 or 8.0 Hz, J(6,8 or 7,9 or A_3,A_5 or A_4,A_6) = 1.0 Hz, H-C(6, 9, A_3 and A_6)), 2 x 7.55, 7.41 and 7.23 (4 x ddd, 4 x 1H, J(7,8 or A_4,A_5) = 8.0 Hz, J(6,7 or 8,9 or A_3,A_4 or A_5,A_6) = 7.7 or 7.3 Hz, J(6,8 or 7,9 or A_3,A_5 or A_4,A_6) = 1.0 Hz, H-C(7, 8, A_4 and A_5)), 5.70 and 4.89 (2 x s, 2H, -C$\underline{H}_2$COOEt and -C$\underline{H}_2$Ar), 4.24 (q, 2H, J(CH_2,CH_3) = 7.1 Hz, -OC$\underline{H}_2$$CH_3$), 1.28 (t, 3H, J(CH_2,CH_3) = 7.1 Hz, -$OCH_2C\underline{H}_3$). ^{13}C-NMR (150 MHz, $CDCl_3$): 166.7 (-$\underline{C}$OOEt), 154.3, 154.1, 143.9, 139.7, 133.6, 2 x 133.3, 131.5, 129.4, 128.6, 123.9, 122.3, 118.1, 117.6, 112.6, 110.3, 62.5, 54.8, 42.2 and 14.2 (-$OCH_2\underline{C}H_3$). FT-IR (solid, cm^{-1}): 2983 (w), 2931 (w), 2228 (m, -CN), 1738 (s), 1662 (s), 1633 (s), 1600 (s), 1568 (s), 1498 (m), 1478 (s), 1408 (s), 1373 (m), 1314 (w), 1274 (m), 1200 (s), 1131 (w), 1104 (s), 1052 (s), 1015 (m), 955 (m), 936 (m), 874 (w), 786 (m), 755 (s), 730 (m), 697 (m), 670 (m), 557 (m), 486 (w), 465 (w), 430 (s). MS

(ESI m/z): 387.0 (95 %) [M-e$^-$]. Anal. calcd for $C_{21}H_{17}N_5O_3$ (387.39): C, 65.11; H, 4.42; N, 18.08; Found: C, 64.36; H, 4.49; N, 18.45.

2-(2-(2-Cyanobenzyl)-3-oxo-2,3-dihydro-5*H*-[1,2,4]triazino[5,6-*b*]indol-5-yl)acetic acid 5 (OTI-7)

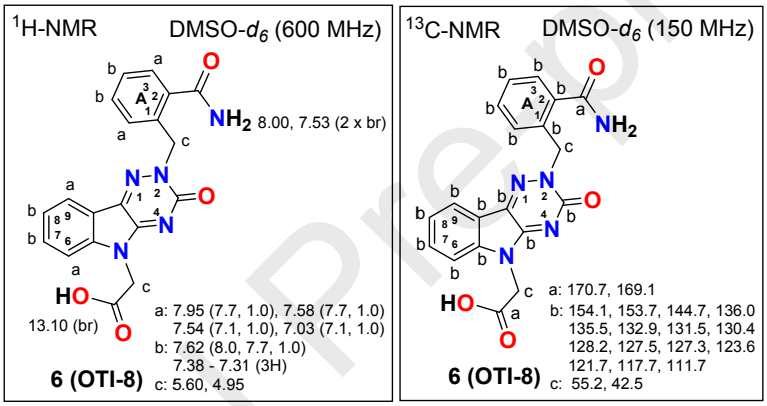
To a solution of 100 mg (0.26 mmol, 1.00 mol eq) ester **4** in 20 ml of MeOH, 20.6 mg (0.52 mmol, 2.00 mol eq) of NaOH in 3 ml water was added and the mixture refluxed for 30 min. After consumption of starting material **4** (confirmed by TLC analysis) the reaction mixture was cooled down in an ice bath and acidified with 1 M HCl to pH = 3. After 10 min of stirring, formed precipitate was filtered off, washed with ice water and dried under reduce pressure to yield 70.0 mg (0.19 mmol, 75 %) of acid **5 (OTI-7)** as a brown solid product. Novelty: 2-[2-(2-cyanobenzyl)-3-oxo-2,3-dihydro-5*H*-[1,2,4]triazino[5,6-*b*]indol-5-yl]acetic acid **5** (**OTI-7**) has not been described in the literature. M.p.: 280.9 - 284.5 °C [MeOH / H_3O^+].



^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.35 (br s, 1H, -COOH), 7.94, 7.89, 7.59 and 7.43 (4 x dd, 4 x 1H, J(6,7 or 8,9 or A_3,A_4 or A_5,A_6) = 8.0 or 7.7 Hz, J(6,8 or 7,9 or A_3,A_5 or A_4,A_6) = 1.0 Hz, H-C(6, 9, A_3, A_6)), 7.66, 7.63, 7.51 and 7.35 (4 x ddd, 4 x 1H, J(7,8 or A_4,A_5) = 8.0 Hz, J(6,7 or 8,9 or A_3,A_4 or A_5,A_6) = 7.7 or 7.5 Hz, J(6,8 or 7,9 or A_3,A_5 or A_4,A_6) = 1.0 Hz, H-C(7, 8, A_4, A_5)), 5.56 and 4.94 (2 x s, 2 x 2H, -C$\underline{H}_2$COOH and -C$\underline{H}_2$Ar). ^{13}C-NMR (150 MHz, DMSO-d_6): 169.1 (-COOH), 154.3, 153.4, 144.6, 140.5, 134.0, 133.6, 133.1, 131.7, 128.9, 128.7, 123.7, 121.7, 117.7, 117.6, 111.8, 111.4, 55.2, 42.5. FT-IR (solid, cm^{-1}): 3300 - 2800 (br, COOH) 2226 (w), 1762 (m), 1626 (m), 1604 (s), 1572 (s), 1502 (m), 1468 (m), 1420 (m), 1381 (m), 1341 (m), 1316 (w), 1278 (m), 1229 (m), 1207 (m), 1162 (w), 1134 (w), 1115 (w), 1084 (w), 1058 (w), 1039 (w), 994 (w), 934 (w), 885 (w), 788 (s), 767 (s), 668 (m), 611 (m), 584 (m), 556 (m), 499 (w), 457 (w), 433 (s). MS (ESI m/z): 358.1 (80 %) [M-H$^+$], 314.0 (100 %) [M-CO_2-H$^+$]. Anal. calcd for $C_{19}H_{13}N_5O_3$ (359.35): C, 63.51; H, 3.65; N, 19.49 Found: C, 63.30; H, 3.60; N, 19.50.

2-[2-(2-Carbamoylbenzyl)-3-oxo-2,3-dihydro-5*H*-[1,2,4]triazino[5,6-*b*]indol-5-yl]acetic acid 6 (OTI-8)

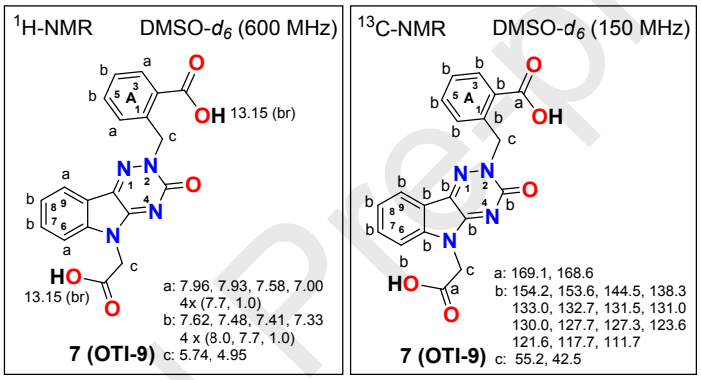
A solution of 20.0 mg (0.05 mmol, 1.00 mol eq) ester **4** in 1 ml of CF_3COOH (TFA) and 0.25 ml of H_2SO_4 (conc) was stirred at 70 °C for 6 h. After complete consumption of the starting compound **4** (confirmed by a TLC analysis), the reaction was cooled down and 20 ml of H_2O and 20 ml of brine added, and the mixture extracted with EA (5 x 15 ml). Combined organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. A crude product **6** (**OTI-8)** was purified by trituration with a mixture of Hex / EA to give 15.0 mg (0.04 mmol, 77 %) of acid **6 (OTI-8)** as a solid compound. Novelty: 2-[2-(2-carbamoylbenzyl)-3-oxo-2,3-dihydro-5*H*-[1,2,4]triazino[5,6-*b*]indol-5-yl]acetic acid **6** (**OTI-8**) has not been described in the literature. M.p.: 221.3 - 225.1 °C [EA].



^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.10 (br s, 1H, -COOH), 8.00 and 7.53 (2 x br s, 2 x 1H, -ArCON$\underline{H}_2$), 7.95, 7.58, 7.54 and 7.03 (4 x dd, 4 x 1H, J(6,7 or 8,9 and A_3,A_4 or A_5,A_6) = 7.7 or 7.1 Hz, J(6,8 or 7,9 or A_3,A_5 or A_4,A_6) = 1.0 Hz, H-C(6, 9, A_3 and A_6)), 7.62 and 7.38 - 7.31 (1 x ddd and m, 1H and 3H, J(7,8 or A_4,A_5) = 8.0 Hz, J(6,7 or 8,9 or A_3,A_4 or A_5,A_6) = 7.7 Hz, J(6,8 or 7,9 or A_3,A_5 or A_4,A_6) = 1.0 Hz, H-C(7, 8, A_4 and A_5)), 5.60 and 4.95 (2 x s, 2 x 2H, -C$\underline{H}_2$COOH and -C$\underline{H}_2$Ar). ^{13}C-NMR (150 MHz, DMSO-d_6): 170.7, 169.1, 154.1, 153.7, 144.7, 136.0, 135.5, 132.9, 131.5, 130.4, 128.2, 127.5, 127.3, 123.6, 121.7, 117.7, 111.7, 55.2 and 42.5. FT-IR (solid, cm^{-1}): 3357 (m), 3191 (m), 2959 (m), 2928 (m), 2858 (w), 2342 (w), 1736 (s), 1671 (s), 1606 (s), 1578 (s), 1498 (m), 1465 (m), 1413 (m), 1375 (w), 1282 (m), 1199 (s), 1122 (s), 941 (m), 748 (s), 678 (m), 631 (m), 504 (w), 438 (m). MS (ESI m/z): 378.0 (100 %) [M+H$^+$], 400.1 (20 %) [M+Na$^+$], 777.2 (80 %) [(2 x M) + Na$^+$]. Anal. calcd for $C_{19}H_{15}N_5O_4$ (377.36): C, 60.48; H, 4.01; N, 18.56; Found: C, 60.85; H, 4.39; N, 18.75.

2-{[5-(Carboxymethyl)-3-oxo-3,5-dihydro-2*H*-[1,2,4]triazino[5,6-*b*]indol-2-yl]methyl}benzoic acid 7 (OTI-9)

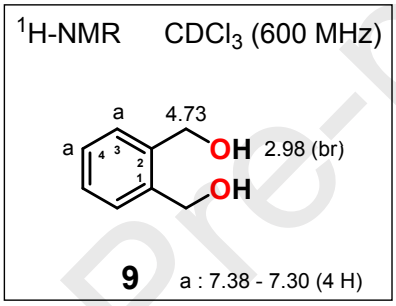
A solution of 30.0 mg (0.08 mmol, 1.00 mol eq) of ester **4** in 2 ml of H_2SO_4 (conc) and 2 ml of H_2O was stirred at 100 °C within 8 d. After complete consumption of starting compound **4** and its amidic intermediate (confirmed by a TLC analysis), the reaction was cooled down, 20 ml of H_2O added and the resulting mixture extracted with EA (5 x 15 ml). Combined organic layer was dried over Na_2SO_4, filtered and concentrated to a half of its volume. Then, the solution was placed into a refrigerator, where the product **7** (**OTI-9)** precipitated. After filtration, crude product was triturated with a mixture of Hex / EA to give 12.0 mg (0.03 mmol, 41 %) of bisacid **7** (**OTI-9)** as a yellow solid compound. Novelty: 2-{[5-(carboxymethyl)-3-oxo-3,5-dihydro-2*H*-[1,2,4]triazino[5,6-*b*]indol-2 yl]methyl}benzoic acid **7** (**OTI-9)** has not been described in the literature. M.p.: 260 - 277 °C (dec) [EA].



^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.15 (2 x br s, 2 x 1H, 2 x -COOH), 7.96, 7.93, 7.58 and 7.00 (4 x dd, 4 x 1H, J(6,7 or 8,9 or A_3,A_4 or A_5,A_6) = 7.7 Hz, J(6,8 or 7,9 or A_3,A_5 or A_4,A_6) = 1.0 Hz, H-C(6, 9, A_3 and A_6)), 7.62, 7.48, 7.41 and 7.33 (4 x ddd, 4 x 1H, J(7,8 or A_4,A_5) = 8.0 Hz, J(6,7 or 8,9 or A_3,A_4 or A_5,A_6) = 7.7 Hz, J(6,8 or 7,9 or A_3,A_5 or A_4,A_6) = 1.0 Hz, H-C(7, 8, A_4 and A_5)), 5.74 and 4.95 (2 x s, 2 x 2H, -C$\underline{H_2}$COOH and -C$\underline{H_2}$Ar). ^{13}C-NMR (150 MHz, DMSO-d_6): 169.1, 168.6, 154.2, 153.6, 144.5, 138.3, 133.0, 132.7, 131.5, 131.0, 130.0, 127.7, 127.3, 123.6, 121.6, 117.7, 111.7, 55.2 and 42.5. FT-IR (solid, cm^{-1}): 3000 - 2200 (br, -COOH) 2822 (m), 2606 (m), 2088 (w), 1725 (m), 1688 (m), 1635 (m), 1602 (s), 1569 (s), 1499 (m), 1467 (m), 1406 (m), 1313 (m), 1225 (s), 1203 (s), 1134 (w), 1107 (m), 1056 (w), 968 (m), 940 (s), 827 (w), 788 (s), 733 (s), 663 (s), 614 (w), 582 (w), 554 (w), 483 (w), 436 (s). MS (ESI m/z): 377.0 (25 %) [M-H$^+$]$^-$, 365.1 (100 %) [M-CO_2-H$^+$+CH_3OH]$^-$, 333.1 (100 %) [M-CO_2-H$^+$], 755 (30 %) [(2 x M)-H$^+$]. Anal. calcd for $C_{19}H_{14}N_4O_5$ (378.34): C, 60.32; H, 3.73; N, 14.81; Found: C, 60.55; H, 4.05; N, 14.97.

1,2-Phenylenedimethanol (9)

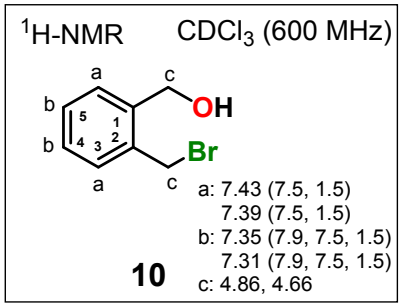
A solution of phthalic anhydride **8** (440 mg, 2.97 mmol, 1.00 mol eq) in 15 ml of THF (abs) was added dropwise at 0 °C to the stirred suspension of $LiAlH_4$ (226 mg, 5.96 mmol, 2.00 mol eq) in 20 ml of THF (abs) within 30 min. Then, the mixture was stirred overnight at room temperature. Afterwards, the reaction was cooled to 0 °C and 10 ml of isopropanol added slowly, followed by methanol (10 ml) and water (10 ml). Obtained wax-like precipitate was filtered through Celite and obtained filtrate washed with EA and DCM. Organic solution was evaporated and aqueous mixture extracted with EA (3x 20 ml) and DCM (3x 10 ml). Combined organic layer was dried over Na_2SO_4, filtered and concentrated over reduced pressure totally yielding 385 mg (2.79 mmol, 94 %) of diol **9** as a colourless oil, which solidified by storing in a refrigerator. Novelty: 1,2-phenylenedimethanol (**9**) is described in the literature by its M.P., ^{1}H-NMR, ^{13}C-NMR, IR and HRMS.[21] M.p.: 52.0 - 57.7 °C [EA / DCM], lit: 61 - 63 °C [EA][21].



^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.38 - 7.30 (m, 4H, 2 x H-C(3 and 4)), 4.73 (s, 4H, 2 x -C$\underline{H_2}$OH), 2.98 (br s, 2H, -CH$_2$O$\underline{H}$).

2-(Bromomethyl)phenyl]methanol (10)

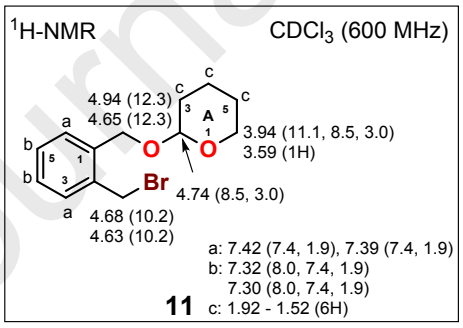
A solution of 105 mg (0.76 mmol, 1.00 mol eq) 1,2-phenylenedimethanol (**9**) in 20 ml of toluene was heated to 70 °C. At this temperature, 80 µl (0.85 mmol, 1.12 mol eq) of 48 % solution of HBr was added dropwise. After 20 min, TLC analysis confirmed a presence of product **10** and a small amount of probably bis brominated side product. Reaction mixture was cooled down, neutralized with saturated aq solution of Na_2CO_3 and extracted with diethyl ether (3 x 15 ml). Combined organic layer was evaporated and resulted mixture purified by FLC (Hex / EA = 5 / 1) to afford 80.0 mg (0.40 mmol, 56 %) of desired product **10** as a white solid compound. Novelty: 2-(bromomethyl)phenyl]methanol (**10**) is described in the literature by its M.P., ^{1}H-NMR, IR[22] and MS spectra.[23] M.p.: 61.0 - 63.8 °C [Et_2O], lit: 55 °C [hexane].[22]

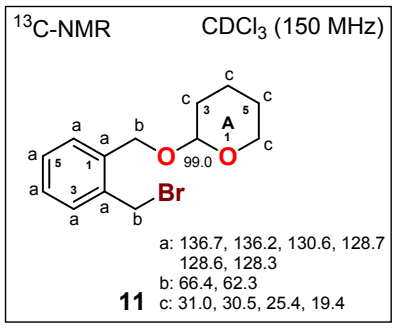


^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.43 and 7.39 (2 x dd, 2 x 1H, J(3,4 or 5,6) = 7.5 Hz, J(3,5 or 4,6) = 1.5 Hz, H-C(3 and 6)), 7.35 and 7.31 (2 x ddd, 2 x 1H, J(4,5) = 7.9 Hz, J(3,4 or 5,6) = 7.5 Hz, J(3,5 or 4,6) = 1.5 Hz, H-C(4 and 5)), 4.86 and 4.66 (2 x s, 2 x 2H, -C$\underline{H_2}$OH and C$\underline{H_2}$Br).

2-{[2-(Bromomethyl)benzyl]oxy}tetrahydro-2*H*-pyran (11)

A solution of 245 mg (1.22 mmol, 1.00 mol eq) of arylmethanol **10** in 20 ml of DCM (abs), 130 µl (1.42 mmol, 1.16 mol eq) of 3,4-dihydro-2*H*-pyran (DHP) and 30.6 mg (0.12 mmol, 0.10 mol eq) of PPTS (pyridinium *p*-toulenesulfonate), was stirred under Ar for 30 min at reflux. Subsequent TLC analysis confirmed presence of a new product and traces of two side products. Then, 20 ml of $NaHCO_3$ (sat aq sol) was added and resulting mixture extracted with DCM (3 x 15 ml). Combined organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure yielding 310 mg (1.09 mmol, 89 %) of tetrahydropyran **11** as a colourless oily product. Novelty: 2-{[2-(bromomethyl)benzyl]oxy}tetrahydro-2*H*-pyran (**11**) is described in the literature by its ^{1}H-NMR and crystal property description.[24]



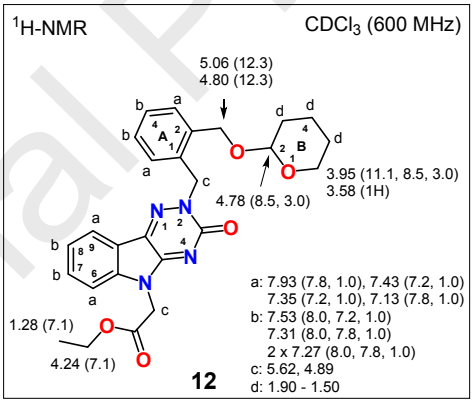


^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.42 and 7.39 (2 x dd, 2 x 1H, J(3,4 or 5,6) = 7.4 Hz, J(3,5 or 4,6) = 1.9 Hz, H-C(3 and 6)), 7.32 and 7.30 (2 x ddd, 2 x 1H, J(4,5) = 8.0 Hz, J(3,4 or 5,6) = 7.4 Hz, J(3,5 or 4,6) = 1.9 Hz, H-C(4 and 5)), 4.94 (d, 1H, J_{gem} =12.3 Hz, -C$\underline{H_2}$OTHP), 4.74 (dd, 1H, $J(A_2,A_3)$ = 8.5 Hz, $J(A_2,A_3)$ = 3.0 Hz, H-C_A(2)), 4.68 (d, 1H, J_{gem} = 10.2 Hz, -CH_2Br), 4.65 (d, 1H, J_{gem} = 12.3 Hz, -C$\underline{H_2}$OTHP), 4.63 (d, 1H, J_{gem} = 10.2 Hz, -CH_2Br), 3.94 (ddd, 1H, J_{gem} = 11.1 Hz, $J(A_5,A_6)$ = 8.5 Hz, $J(A_5,A_6)$ = 3.0 Hz, H-C_A(6)), 3.59 (m, 1H, H-C_A(6)), 1.92 - 1.52 (m, 6H, H-C_A(3-5)). ^{13}C-NMR (150 MHz, $CDCl_3$): 136.7, 136.2, 130.6, 128.7, 128.6,

128.3, 99.0 (-OCHO-), 66.4, 62.3, 31.0, 30.5, 25.4 and 19.4. FT-IR (solid, cm-1): 2939 (m), 2867 (w), 1723 (w), 1454 (m), 1385 (m),1260 (w), 1200 (m), 1118 (s), 1076 (m), 1022 (s), 971 (m), 904 (m), 869 (m), 814 (w), 760 (m), 606 (m), 533 (w)

Ethyl 2-{3-oxo-2-{2-{[(tetrahydro-2*H*-pyran-2-yl)oxy]methyl}benzyl}-2,3-dihydro-5*H*-[1,2,4]triazino[5,6-*b*]indol-5-yl}acetate (12)

To a solution of 200.0 mg (0.74 mmol, 1.00 mol eq) **2** in 5 ml DMF (abs), 37.0 mg (0.88 mmol, 1.19 mol eq) of CaH_2 was added and the mixture stirred at 80 °C under Ar within 30 min. Then, the reaction was cooled to rt, 310 mg (1.09 mmol, 1.47 mol eq) of tetrahydropyran **11** was added dropwise and the solution was stirred at 80 °C within 1 h under Ar. After consumption of **2** (confirmed by a TLC analysis) the reaction was cooled down, 10 ml of water added, and the mixture stirred for 10 min in an ice bath. Resulting mixture was extracted with DCM (3 x 15 ml), washed with brine (3 x 20 ml), dried over Na_2SO_4, filtered and concentrated under reduced pressure. A crude product was purified by FLC (SiO_2, Hex / EA = 1 / 5) to give 286 mg (0.60 mmol, 82 %) of **12** as red oil. Novelty: ethyl 2-{3-oxo-2-{2-{[(tetrahydro-2*H*-pyran-2-yl)oxy]methyl}benzyl}-2,3-dihydro-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl}acetate (**12**) has not been described in the literature.

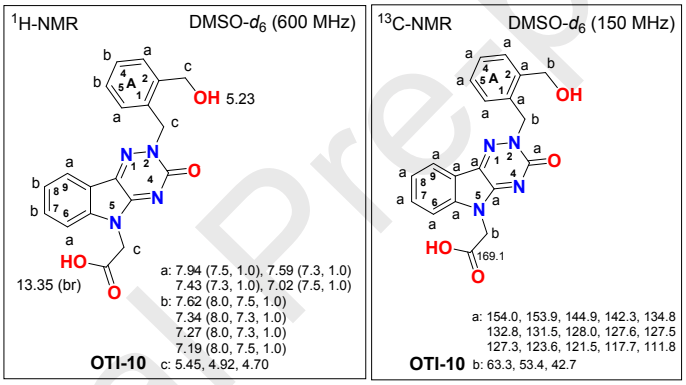


^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.93, 7.43, 7.35 and 7.13 (4 x dd, 4 x 1H, J(6,7 or 8,9 or A_3,A_4 or A_5,A_6) = 7.8 and 7.2 Hz, J(6,8 or 7,9 or A_3,A_5 or A_4,A_6) = 1.0 Hz, H-C(6, 9, A_3 and A_6)), 7.53, 7.31, 2 x 7.27 (4 x ddd, 4 x 1H, J(7,8 or A_4,A_5) = 8.0 Hz, J(6,7 or 8,9 or A_3,A_4 or A_5,A_6) = 7.8 and 7.2 Hz, J(6,8 or 7,9 or A_3,A_5 or A_4,A_6) = 1.0 Hz, H-C(7, 8, A_4 and A_5)), 5.06 (d, 1H, J_{gem} = 12.3 Hz, -OC$\underline{H}_2$Ph), 5.62 and 4.89 (surprisingly 2 x s, 2 x 2H, -C$\underline{H}_2$COOEt and -C$\underline{H}_2$Ph), 4.80 (d, 1H, J_{gem} = 12.3 Hz, -OC$\underline{H}_2$Ph), 4.78 (dd, 1H, J(B_2,B_3) = 8.5 Hz and J(B_2,B_3) = 3.0 Hz, H-C_B(2)), 4.24 (q, 2H, J(CH_2,CH_3) = 7.1 Hz, -OC$\underline{H}_2$CH$_3$), 3.95 (ddd, 1H, J_{gem} = 11.1 Hz, J(B_5,B_6) = 8.5 Hz, J(B_5B_6) = 3.0 Hz, H-C_B(6)), 3.58 (m, 1H, H-C_B(6)), 1.90 - 1.50 (m, 6H, 3 x -CH_2- from C(B_3-B_5)), 1.28 (t, 3H, J(CH_2,CH_3) = 7.1 Hz, -OCH$_2$C$\underline{H}_3$). Due to moisture

instability of the **12**, its other spectra and analysis were not recorded and compound **12** was used directly in the next step.

2-{2-[2-(Hydroxymethyl)benzyl]-3-oxo-2,3-dihydro-5*H*-[1,2,4]triazino[5,6-*b*]indol-5-yl}acetic acid 13 (OTI-10)

To a solution of 50.0 mg (0.11 mmol, 1.00 mol eq) ester **12** in 10 ml of dioxane, 1 ml of H_2O and 0.1 ml of HCl (37 % w/w) were added and resulting solution stirred at 90 °C for 8 h. After that, TLC analysis confirmed presence of a new product without side products. Reaction mixture was cooled down, 30 ml of water added and extracted with DCM (3 x 15 ml). Combined organic layer was dried over Na_2SO_4, filtered and concentrated under reduce pressure yielding 25.0 mg (0.07 mmol, 66 %) of acid **13** (**OTI-10**) as a yellow solid compound. Novelty: 2-{2-[2-(hydroxymethyl)benzyl]-3-oxo-2,3-dihydro-5*H*-[1,2,4]triazino[5,6-b]indol-5-yl}acetic acid **13** (**OTI-10**) has not been described in the literature. M.p.: 220.0 - 238.0 °C (dec) [DCM].



^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.35 (br s, 1H, -COOH), 7.94, 7.59, 7.43 and 7.02 (4 x dd, 4 x 1H, J(6,7 or 8,9 or A_3,A_4 or A_5,A_6) = 7.5 or 7.3 Hz, J(6,8 or 7,9 or A_3,A_5 or A_4,A_6) = 1.0 Hz, H-C(6, 9, A_3 and A_6)), 7.62, 7.34, 7.27 and 7.19 (4 x ddd, 4 x 1H, J(7,8 or A_4,A_5) = 8.0 Hz, J(6,7 or 8,9 or A_3,A_4 or A_5,A_6) = 7.5 or 7.3 Hz, J(6,8 or 7,9 or A_3,A_5 or A_4,A_6) = 1.0 Hz, H-C(7, 8, A_4 and A_5)), 5.45, 4.92 and 4.70 (3 x s, 3 x 2H, -C$\underline{H_2}$COOEt, -C$\underline{H_2}$Ph and -C$\underline{H_2}$OH), 5.23 (s, 1H, -OH). ^{13}C-NMR (150 MHz, DMSO-d_6): 169.1 (-COOH), 154.0, 153.9, 144.9, 142.3, 134.8, 132.8, 131.5, 128.0, 127.6, 127.5, 127.3, 123.6, 121.5, 117.7, 111.8, 63.3, 53.4 and 42.7. FT-IR (solid, cm^{-1}): 3207 (w), 2922 (w), 2712 (w), 2602 (w), 2522 (w), 2108 (w), 2087 (w), 1728 (s), 1606 (s), 1577 (s), 1502 (m), 1467 (m), 1414 (s), 1374 (m), 1228 (s), 1203 (s), 1116 (w), 1087 (w), 1045 (m), 997 (w), 954 (w), 937 (w), 893 (w), 786 (m), 751 (s), 670 (m), 610 (m), 585 (m), 478 (w), 434 (s). MS (ESI m/z): 363.0 (95 %) [M-H$^+$], 319.1 (95 %) [M-CO_2-H$^+$]. Anal. calcd for $C_{19}H_{16}N_4O_4$ (364.36): C, 62.63; H, 4.43; N, 15.38 Found: C, 62.75; H, 4.49; N, 15.55.

Animals: Male Wistar rats 8 - 9 weeks old, weighing 200 - 230 g, were used as organ donors. The animals came from the Breeding Facility of the Institute of Experimental Pharmacology Dobrá Voda (Slovak Republic). The study was approved by the Ethics Committee of the Institute and performed in accordance with the Principles of Laboratory Animal Care (NIH publication 83 - 25, revised 1985) and the Slovak law regulating animal experiments (Decree 289, Part 139, July 9th 2003).

Enzyme assays: Preparation of ALR2 from the rat lens and ALR1 from the rat kidneys was previously reported.[25] Enzyme activities were assayed spectrophotometrically by determining NADPH consumption at 340 nm and were expressed as decrease of the optical density (O.D.) / s/mg protein.[25] The experimental procedures are in detail described in *Supporting information* to this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://xxx. These data include spectra of compounds described in this article together with predicted poses and interaction analyses for all developed inhibitors. Included are also experimental procedures comprising isolation of rat kidney ALR1, rat eye lens ALR2 and enzyme activity assays.

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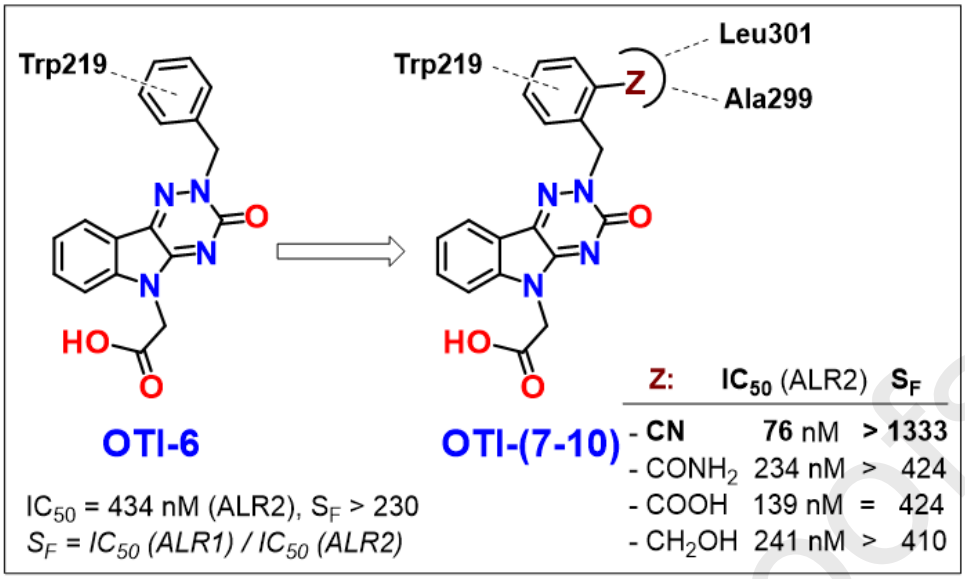

Highlights:

- Four novel aldose reductase inhibitors exploiting an unoccupied enzyme pocket were developed
- All novel inhibitors revealed higher inhibitory efficacy and selectivity compared to their lead
- Additional inhibitor-enzyme interactions were suggested to be responsible for the improved biological activity
- Based on this study, several novel promising derivatives were proposed

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: