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Novel synthesis of dihydropyrimidines for α-glucosidase inhibition to treat type 2 diabetes: *In vitro* biological evaluation and *in silico* docking

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ABSTRACT

A convenient and efficient new method has been established for the synthesis of dihydropyrimidines by inexpensive and non-toxic N-acetyl glycine (NAG) catalysed reaction of aromatic aldehydes with ethyl acetoacetate and urea/thiourea. This method is applicable for various substituted aldehydes as well as urea and thiourea. It has also been used to synthesize bicyclic oxygen-bridged pyrimidine derivatives (**4d**, **4j**). The biological assay revealed that the majority of compounds synthesized displayed modest inhibitory activity against α-glucosidase at low micro-molar concentrations. Molecular docking studies were also performed on the most active compound, **4f** (with IC_{50} value 112.21 ± 0.97 μM), to show the enzyme – inhibitor interactions.

1. Introduction

α-Glucosidase (EC3.2.1.20) is an important enzyme found in the brush border surface of cells in the small intestine. During the digestion of food this enzyme hydrolyzes carbohydrates and produces α-D-glucose, which is absorbed in the blood stream, increases postprandial blood glucose levels and causes diabetes. Thus, for the control and prevention of diabetes, α-glucosidase inhibitors are of particular interest as they can help to reduce the carbohydrate digestion and subsequent monosaccharide absorption [1,2]. Blood glucose levels is very critical for diabetes mellitus management and levels should be maintained within an acceptable range (70–100 mg/dl) [3,4]. In addition to diabetes prevention, balanced levels of monosaccharides within the blood stream may also benefit to avoid hyperlipidemia, hyperlipoproteinemia and obesity [5]. α-Glucosidase also enables monosaccharide removal from viral glycoproteins, thus, its inhibitors could alter cell-to-cell signaling, virus recognition by the cell and could be used in the treatment of viral diseases, cancers and immune-regulations [6–10]. Acarbose, deoxynojirimycin, miglitol and voglibose are among the key known candidates which are used extensively for inhibiting the α-glucosidases (Fig. 1). Due to side effects and absorptivity problems associated with these inhibitors [11,12], new potent α-glucosidase inhibitors are highly desired.

Dihydropyrimidines (DHPMs) are considered pharmacologically very important molecules due to their number of biological activities, such as antiviral, antihypertensive, antibacterial, and antagonists [13–17]. Pyrimidines also occupy a unique position in the medicinal chemistry, as being part of nucleic acids [14]. Recently, pyrimidine derivatives have been reported as potent inhibitors of the enzymes responsible for diabetes [18], and particularly, pyrimidine fused heterocycles are identified as specific α-glucosidase inhibitors (Fig. 2) [19].

The Biginelli reaction is a multi-component one pot reaction which affords an efficient synthesis of DHPMs. Various synthetic methods have been reported for the preparation of DHPMs [20–22], however, to achieve better results in most of the reports metal catalysis has been extensively explored [23–27]. Metal catalysts are expensive, environmentally harmful and some of the

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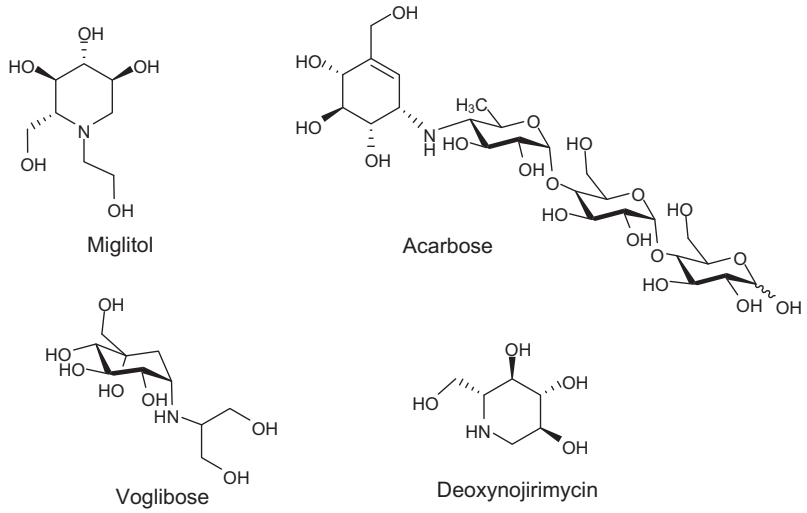


Fig. 1. Structures of acarbose, deoxynojirimycin, miglitol and voglibose.

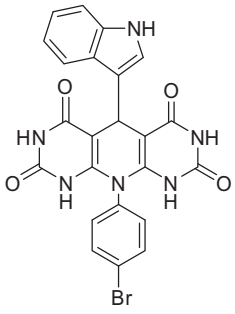


Fig. 2. The molecular structure of a pyrimidine based α-glucosidase inhibitor.

reported conditions require prolonged reaction time, and complete removal of metal traces from products is usually problematic.

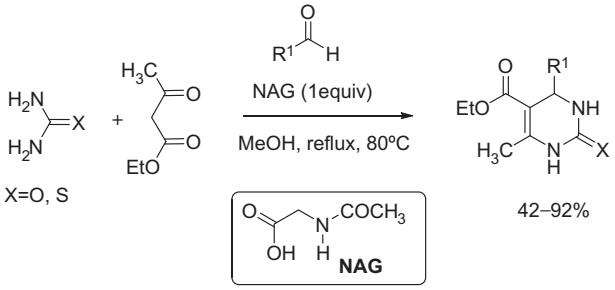
Metal free, simple and environmentally benign reaction conditions have attracted much attention [28–30]. Discrete organocatalysts can effectively accelerate chemical transformations with easy handling due to their moisture and oxygen stability. In addition, their syntheses are easily available with inexpensive costs and as well non-toxic nature. For this reason they are good candidates for synthesis of pharmaceutical products when compared to transition metal catalysts. We planned to search for a potential new organocatalyst which should give an efficient synthesis of dihydropyrimidines with the advantage of providing an easy separation workup. This led us to amino acids and especially *N*-protected amino acids; such as N-acetyl glycine (NAG). In this paper, we disclose the novel and a facile synthesis of dihydroprymidines under mild reaction conditions using NAG. *In vitro* α-glycosidase inhibitory activity for all the synthesized compounds and docking of the most active compound into the active site of the targeted protein to demonstrate the binding pattern are also described.

2. Results and discussion

2.1. Chemistry

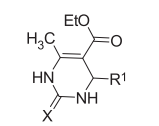
We began our studies from the optimization of mole equivalents requirement of NAG. Different amounts (0.2–2 equivalents) of NAG were tested and one equivalent of NAG [31] gave high yield (92%) of the required pyrimidine **4a** from benzaldehyde **1**, urea **2** and ethyl acetoacetate **3**. Whereas, 0.2 and 0.5 equivalents of NAG afforded relatively poor yields and excess amount of catalyst (2 equiv) resulted almost equal yield (93%). Hence, one equivalent of the NAG was found to be the optimum amount to obtain the good yield. To demonstrate the scope of our new method, a number of pyrimidines bearing different substituents were accomplished in significant to excellent yields (40–92%) from urea or thiourea, selected aldehydes and ethyl acetoacetate, in the presence of NAG as a catalyst (Scheme 1). It was also found that urea could easily lead to cyclized product relatively in shorter period of time (Table 1, entries 1–5) while thiourea was less effective, producing dihydropyrimidine derivatives in moderate yields (Table 1, entries 6–10). The best results were obtained using 1 equiv. NAG, ethanol as solvent at 80 °C for 3 h. Notably, a variety of functional groups (OH, OCH_3, Cl) were tolerated under newly optimized reaction conditions.

According to previous reports, the use of salicylaldehyde in Biginelli reaction gave 4-(2-hydroxyphenyl) pyrimidines [32–38] but, in recent studies accomplished by various research groups have shown that oxygen-bridged pyrimidine derivatives are formed [39–43]. In order to explore the behavior of salicylaldehyde under our newly optimized reaction conditions and to compare with traditional reaction conditions (HCl in MeOH) [32–38]. We made two attempts (Scheme 2), and we observed the formation of 1,4-conjugate products **4d** and **4j** under our new optimized conditions instead of dihydropyrimidines **4m** which was obtained in traditional conditions. It is concluded that NAG can promote 1,4-conjugate addition by activating the α,β-unsaturated ester, while HCl could not support conjugate addition. The structures for compounds **4d**, **4j** and **4m** and relative stereochemistry in case of **4d** and **4j** were confirmed from the single crystal XRD (see Figs. 3–5).

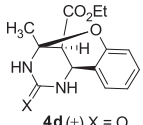


Scheme 1. Synthetic protocol of dihydropyrimidines derivatives **4a–4l**.

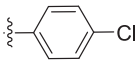
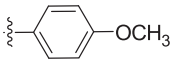
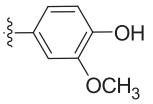
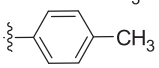
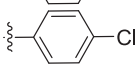
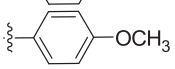
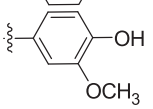
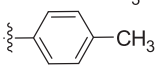
Table 1
Reactions time, %yields, α-glucosidase inhibition activity and IC_{50} values (mean ± sem, $n = 3$) of dihydropyrimidines derivatives (ND, not determined).

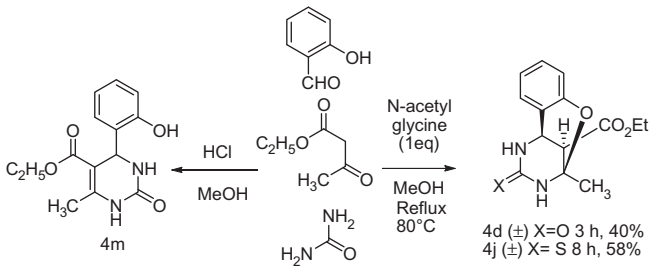


4a-4c, 4e, 4f-4i, 4k-4l



4d (±) X = O
4j (±) X = S

Entry	Products	R^1	X	Time (h)	Yield (%)	%Inhibition (0.5 mM)	IC_{50} (μM)
1	**4a**		O	2.3	92	89.54 ± 1.42	143.25 ± 1.39
2	**4b**		O	3	45	83.72 ± 1.76	212.51 ± 1.57
3	**4c**		O	3	55	ND	ND
4	**4e**		O	7	74	82.36 ± 1.41	305.32 ± 1.37
5	**4f**		O	5	77	88.42 ± 0.98	112.21 ± 0.97
6	**4g**		S	5	40	36.92 ± 0.76	–
7	**4h**		S	7	59	25.36 ± 0.52	–
8	**4i**		S	7	63	12.08 ± 0.14	–
9	**4k**		S	8	74	23.70 ± 0.31	–
10	**4l**		S	8	65	83.56 ± 0.75	125.12 ± 0.42
11	**4d**	–	O	–	40	91.26 ± 1.32	122.43 ± 1.27
12	**4j**	–	S	–	58	13.50 ± 0.21	–
13	Acarbose					92.23 ± 0.14	38.25 ± 0.12



Scheme 2. Study of the effect of catalysts in the Biginelli reaction while using salicylaldehyde.

Another advantage of this new methodology, along with the simple reaction conditions, is the easy separation of the catalyst (NAG) from the reaction mixture by washing the mixture with an aqueous solution of $NaHCO_3$. Although this procedure was initially suspected to cause the formation of the 1, 4-conjugate addition products (**4d** and **4j**), where salicylaldehyde was used (Scheme 2), it was eventually ruled out as both **4d** and **4j** were obtained when $NaHCO_3$ was not used in the work up.

2.2. XRD

The structures of the compounds **4d**, **4j** and **4m** and relative stereochemistry of compounds **4d** and **4j** were confirmed by single

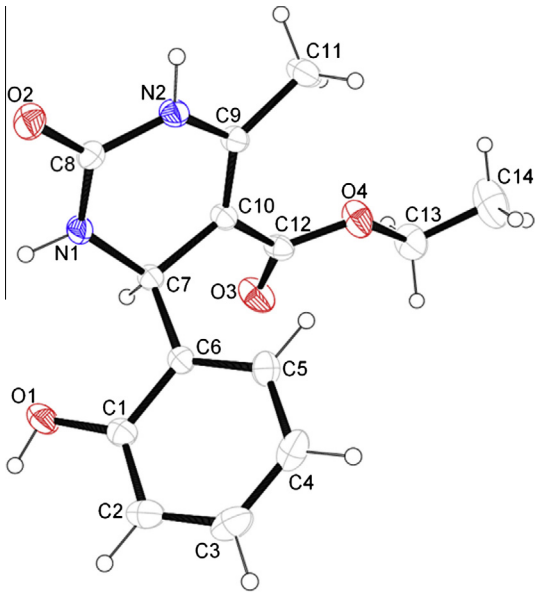


Fig. 3. An ORTEP III diagram of **4m** (using HCl as catalyst) showing the atom numbering scheme. The thermal ellipsoids are drawn at 30% probability.

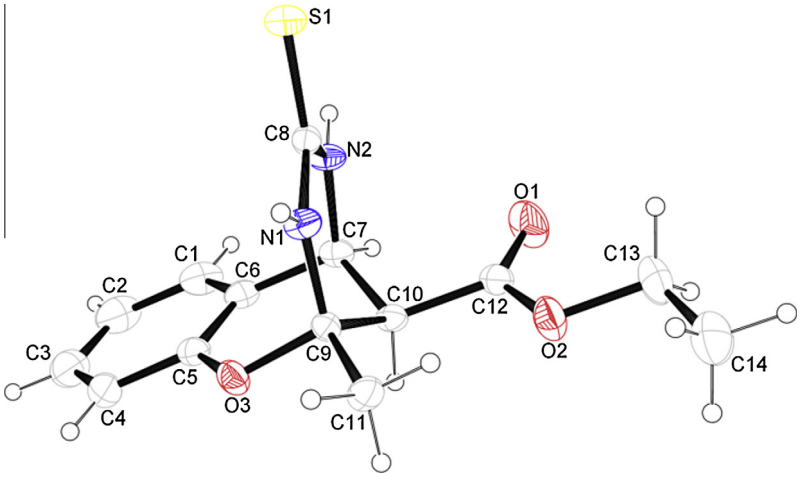


Fig. 4. An ORTEP III diagram of **4j** showing the atom numbering scheme. The thermal ellipsoids are drawn at 30% probability.

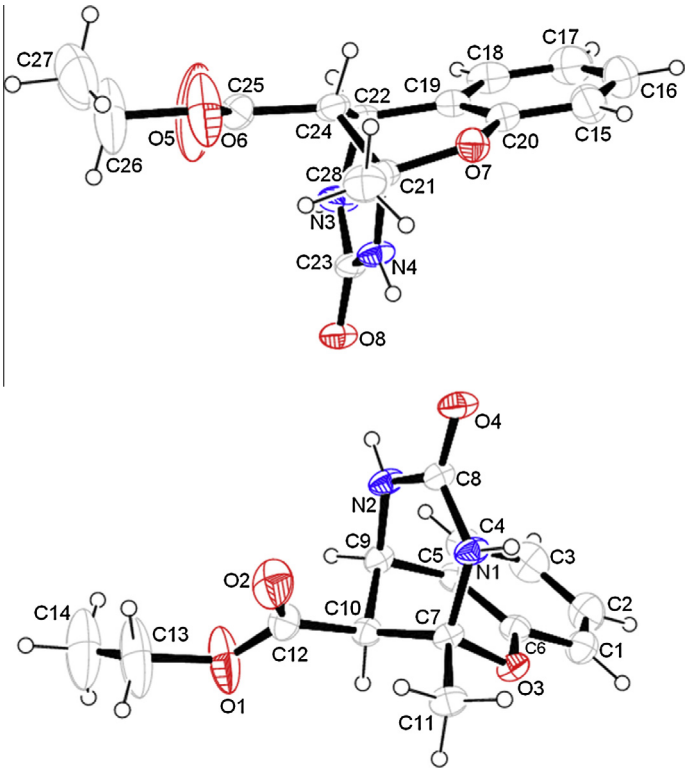


Fig. 5. An ORTEP III diagram of **4d** (using NAG as catalyst) showing the atom numbering scheme. The thermal ellipsoids are drawn at 30% probability.

crystal X-rays diffraction studies (Figs. 3–5 and Supplementary information).

2.3. Biological activities

In order to exploit the α-glucosidase inhibition activity of dihydropyrimidines, a 'one-pot' synthetic approach was developed to produce a library of dihydropyrimidine derivatives **4a–4j** (Schemes 1 and 2). Compound **4f** bearing hydrophobic 4-methyl phenyl group was found to be the most active α-glucosidase inhibitor with IC_{50} value of 112.21 ± 0.97 μM (entry 5, Table 1). The presence of hydroxyl and methoxy substituents on the phenyl ring (**4e, 4k**) resulted in a substantial loss in inhibitory activity. Introduction of an electron-donating group with less electronegative atom in the 4-position of the phenyl ring for example CH_3 (**4f, 4l**) is responsible to display more inhibitory activity (IC_{50} = 112.21 ± 0.97 μM and 125.12 ± 0.42 μM) compared with the hydroxyl and methoxy substituted phenyl derivatives (**4c, 4e, 4i, 4k**). Moreover, the presence of carbonyl (C=O) moiety in the dihydropyrimidine ring system (**4a, 4b, 4d, 4e, 4f**) retained inhibitory activity, whereas thiocarbonyl (C=S) moiety resulted in large loss in inhibitory activity with the exception of 4-methyl phenyl derivative **4l**. Overall, both the hydrophobic group (CH_3—) in the *para*-position of R^1 and presence of carbonyl (C=O) moiety on dihydropyrimidine are favorable for better enzyme inhibitory activity. In general, analogs derived from urea have shown better inhibition activities than

compounds obtained from thiourea, except compound **4l**, where we believe again 4-methyl in the phenyl ring is playing a great role for such high activity.

The overall order of synthesized compounds based on IC_{50} value of α-glucosidase was as follows:

4f > **4d** > **4l** > **4a** > **4b** > **4e**

2.4. Molecular modeling studies

The novel compounds **4a–b** and **4d–l** were tested towards the activity against α-glucosidase (EC3.2.1.20, maltase, MAL12) from baker's yeast. Results of that assay revealed that derivatives **4a–b**, **4d–f** and **4l** were weak inhibitors with the most active compound **4f** which was 3-fold less potent than the acarbose (IC_{50} 112.21 ± 0.97 μM vs. 38.25 ± 0.12 μM). Therefore, we decided to perform molecular modeling studies to identify possible binding mode and to find the reasons of observed low activity.

There are some published crystal structures of α-glucosidase from different sources (human, *Bacteroides*, mouse) but *Saccharomyces cerevisiae* one has not been crystallized so far [44]. This made us build homology model of the enzyme in the beginning. Isomaltase (EC 3.2.1.10, oligo-1,6-glucosidase, MALX3) from baker's yeast was found to be the most suitable template because it shared 71% identity and 84% similarity with our target. Sequence alignment of both proteins is presented in Fig. 6. Based on that alignment and isomaltase crystal structure (PDB code: 3A4A, [44]) homology models of α-glucosidase were built. All models were characterized by high quality due to the high sequence homology with the template. From the whole series of obtained structures, a model with the lowest modeler objective function was selected for further analyses. Superimposition of this model and the template is shown in Fig. 7. Both structures fit to each other very well. The active site contains important, from the viewpoint of hydrolysis, amino acids: two histidines, two aspartates and one glutamate. In case of α-glucosidase these are: His111, Asp214, Glu276, His348 and Asp349.

Before the analysis of the binding mode of the most active compound **4f**, the docking method was simply validated, based on reference inhibitor. Acarbose was docked into α-glucosidase and its binding mode was compared to the one, presented in the complexes 1LWJ and 3W37 [44]. The 1LWJ structure is indeed 4-α-glucanotransferase from *Thermotoga maritima*, in a complex with derivative of acarbose, and 3W37 – sugar beet α-glucosidase with acarbose. They share relatively low homology with baker's yeast α-glucosidase (23% and 16% identity, respectively) but the main interactions of the ligand are quite similar. The most important for the binding with the enzyme, part of acarbose was substituted cyclohexenylamine moiety which due to the presence of hydroxyl groups and amine group provided hydrogen bonds with His111, Asp214, Glu276, His348, Asp349 and Arg439. The detailed view of reference inhibitor bound with the target is shown in Fig. 8.

Finally, compound **4f** was docked into α-glucosidase. Both enantiomers of this compound were taken into account because crystallographic data revealed that for these derivatives (e.g. **4m**) racemic mixture was obtained during synthesis.

The large differences in the manner of interaction of both enantiomers with the enzyme were observed (Fig. 9). The R isomer occupied the area where cyclohexenylamine fragment of acarbose was previously located and formed the essential interactions with

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MAL12_YEAST   1    MTISD-HPET EPKWWKEATI YQIYPASFKD SNNDGWGDLK GITSKLQYIK DLGVDAIWVC PFYDSPQQDM   69
MALX3_YEAST   1    MTISSAHPET EPKWWKEATF YQIYPASFKD SNDDGWGDMK GIASKLEYIK ELGADAIWIS PFYDSPQDDM   70

MAL12_YEAST   70   GYDISNYEKV WPTYGTNEDC FELIDKTHKL GMKFITDLVI NHCSTEHEWF KESRSSKTNP KRDWFFWRPP   139
MALX3_YEAST   71   GYDIANYEKV WPTYGTNEDC FALIEKTHKL GMKFITDLVI NHCSSEHEWF KESRSSKTNP KRDWFFWRPP   140

MAL12_YEAST   140  KGYDAEGKPI PPNNWKSFFG GSAWTFDETT NEFYLRLFAS RQVDLNWENE DCRRAIFESA VGFWLDHGVD   209
MALX3_YEAST   141  KGYDAEGKPI PPNNWKSYFG GSAWTFDEKT QEFYLRLFCS TQPDLNWENE DCRKAIYESA VGYWLDHGVD   210

MAL12_YEAST   210  GFRIDTAGLY SKRPGLPDSP IFDKTSKLQH PNWGSHNGPR IHEYHQELHR FMKNRVKDGR EIMTVGEVAH   279
MALX3_YEAST   211  GFRIDVGSLY SKVVGLPDAP VVDKNSTWQS SDPYTLNGPR IHEFHQEMNQ FIRNRVKDGR EIMTVGEMQH   280

MAL12_YEAST   280  GSDNA--LYT SAARYEVSEV FSFTHVEVGT SPFFRYNIVP FTLKQWKEAI ASNFLFINGT DSWATTYIEN   347
MALX3_YEAST   281  ASDETKRLYT SASRHELSEL FNFSHTDVGT SPLFRYNLVP FELKDWKIAL AELFRYINGT DCWSTIYLEN   350

MAL12_YEAST   348  HDQARSITRF ADDSPKYRKI SGKLLTLLEC SLTGTLYVYQ GQEIGQINFK EWPIEKYEDV DVKNNYEIIK   417
MALX3_YEAST   351  HDQPRSITRF GDDSPKNRVI SGKLLSVLLS ALTGTLYVYQ GQELGQINFK NWPVEKYEDV EIRNNYNAIK   420

MAL12_YEAST   418  KSFGKNSKEM KDFFKGIALL SRDHSRTPMP WTKDKPNAGF TGPDVKPWFL LNESFEQGIN VEQESRDDDS   487
MALX3_YEAST   421  EEHGENSEEM KKFLEAIALI SRDHARTPMQ WSREEPNAGF SGPSAKPWFY LNDSFREGIN VEDEIKDPNS   490

MAL12_YEAST   488  VLNFWKRALQ ARKKYKELMI YGYDFQFIDL DSDQIFSFTK EYEDKTLFAA LNFSGEEIEF SLPREGASLS   557
MALX3_YEAST   491  VLNFWKEALK FRKAHKDITV YGYDFEFIDL DNKKLFSFTK KYNNKTLFAA LNFSSDATDF KIPNDDSSFK   560

MAL12_YEAST   558  FILGNY--DD TDVSSRVLKP WEGRIYLVK  584
MALX3_YEAST   561  LEFGNYPKKE VDASSRTLKP WEGRIYISE  589
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Fig. 6. Sequence alignment of baker's yeast α-glucosidase (MAL12_YEAST) and oligo-1,6-glucosidase (MALX3_YEAST, isomaltase). Both enzymes share 71% identity and 84% similarity. The identical residues are colored in green. The most important amino acids for catalytic activity are marked by red rectangles.

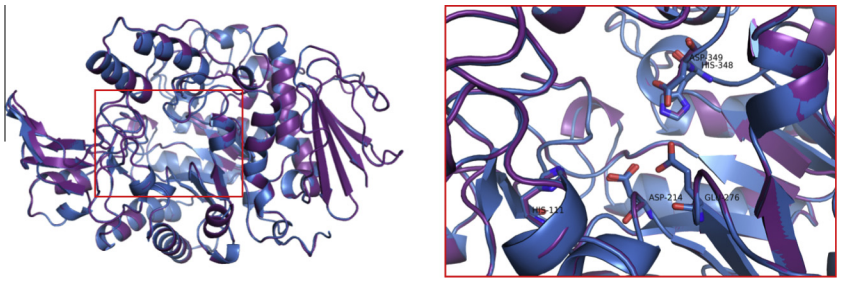

Fig. 7. Superimposed structures of α-glucosidase (target, selected in blue) and oligo-1,6-glucosidase (template, selected in violet). Red rectangle indicates the active site (left). View of the active side with the most important amino acids (right). The residue numbers concern α-glucosidase.

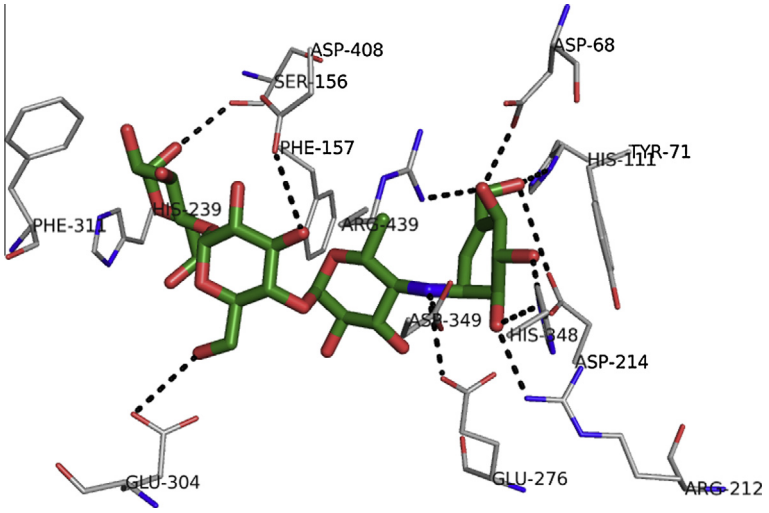

Fig. 8. Binding mode of acarbose into the active gorge of *Saccharomyces cerevisiae* α-glucosidase. Black dotted lines represent hydrogen bonds. All hydrogen atoms are hidden.

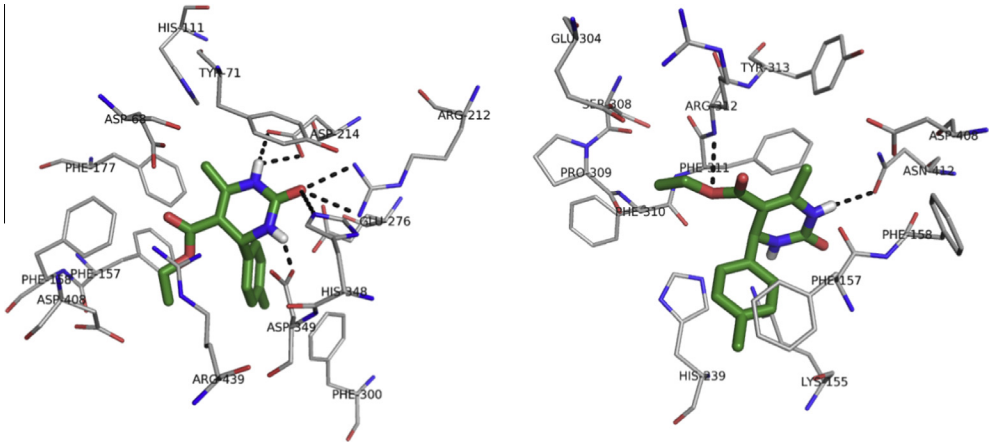

Fig. 9. Binding mode of R (on the left) and S (on the right) isomers of compound **4f** into the active gorge of *Saccharomyces cerevisiae* α-glucosidase. Black dotted lines represent hydrogen bonds. Only polar hydrogens for ligand were shown.

catalytic amino acids. The crucial features in binding mode were observed for urea fragment in the 2-pyrimidone ring. The NH group in position 1 created hydrogen bonds with Asp214, the carbonyl group – H-bonds with two residues: Arg212 and His348, and the NH in position 3 – with Asp349. This fragment of molecule played key role and any replacement or change of it led to decrease in activity e.g. thione group instead of carbonyl could not interact via H-bonds with His348 and Arg212. The other parts of (R)-**4f** were less important but they also interacted with the enzyme: *p*-tolyl (4-methylphenyl) created hydrophobic interactions with Phe300, methyl group in position 6 with Phe177 and ethyl chain from ester substituent with Phe158.

It was also observed that there were some possibilities to improve the activity, for example: increase of the length of substituent in position 6 and introduction of hydrogen bond acceptor at the end of this chain could provide interaction with His111; introduction of linker (one or two CH_2 groups) between phenyl and pyrimidone rings could result in π–π stacking between phenyl and Phe300; chain in ester group could be expanded to reach Asp68 and Arg439.

The S isomer was located on the opposite site, in the area, occupied previously by the last sugar ring of acarbose and did not interact with any catalytic His, Asp or Glu, responsible for oligosaccharide hydrolysis. It formed only H-bond between NH in position 1 and —$CONH_2$ of Asn412, H-bond between oxygen atom from ester group and backbone of Arg312, and π–π stacking His239 – phenyl ring in position 4 – Phe157.

Those differences between both stereoisomers enabled us to formulate hypothesis that (R)-**4f** was responsible for the activity (eutomer) meanwhile (S)-**4f** was an inactive ingredient of the racemic mixture (distomer).

Comparison of scoring function (GoldScore) values for R and S isomers did not reveal any significant differences but both ones were much lower assessed than acarbose.

3. Conclusion

An efficient new method has been developed for the synthesis of dihydropyrimidines and oxy bridged pyrimidines by using an inexpensive N-acetyl glycine. The influence of N-acetyl glycine on the formation and diastereoselectivity of oxy-bridged pyrimidines from salicylaldehyde has been explored and the relative stereochemistry of the oxy-bridged pyrimidines has been confirmed by single crystal XRD. This new method accommodates a range of substrates and avoids the use of any expensive metal catalysts. The synthesized compounds have shown promising α-glucosidase activity and SAR demonstrated that the activity is very much dependent on the nature and position of substituents in the R[1]

and X. Compound **4f**, bearing the 4-methyl phenyl substituent presented the highest inhibitory activity with IC_{50} value 112.21 ± 0.97. The bicyclic oxygen-bridged pyrimidine derivatives (**4d, 4j**) have been identified as a new class of α-glucosidase inhibitors. Molecular modeling not only demonstrated the binding pattern of the more active compound **4f**, but it also revealed the importance of the substituents in the most active compound. This research opens up new avenues for the development of more potent α-glucosidase inhibitors that can be used as treatment for diabetes mellitus type II and for the discovery of new antiviral drugs.

4. Experimental

4.1. General methods

Reagents were purchased from common commercial suppliers and were used without further purification. Solvents were purified and dried by standard procedures, when necessary. TLC was performed on silica coated aluminum plates ($6F_{254}$, 0.2 mm). ^1H NMR spectra were recorded on Bruker NMR 300 MHz. IR spectra were recorded on a Jasco A-302 IR spectrophotometer.

4.2. General procedure for the synthesis of dihydropyrimidines

Ethyl acetoacetate (1.2 equiv.), desired aldehyde (1 equiv.) and NAG (1 equiv.) were added to a stirred solution of urea/thiourea (1.5 equiv.) in ethanol at RT. Then the reaction mixture was heated under reflux at 80 °C for time period shown in Table 1. At the end of reaction, formation of precipitates took place in the reaction flask. The precipitates were filtered and washed with diethyl ether and then with aqueous $NaHCO_3$ solution to remove catalyst. The solid product was then dried and recrystallized from hot ethanol to obtain the required product.

4.2.1. Ethyl 6-methyl-2-oxo-4-phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate (**4a**)

According to general procedure, **4a** was obtained from benzaldehyde, urea, and ethyl acetoacetate in 2.5 h. and 92% yield as white crystalline solid: m.p. 200–202 °C (lit. [45] 202–204 °C); R_F 0.37 (n-hexane: EtOAc, 1:1); δ_H (300 MHz, DMSO-d6):9.16 (s, 1H, NH), 7.71 (s, 1H, NH), 7.26 (m, 5H, Ar—H), 5.12 (d, 1H, CH), 3.96 (q, 2H, CH_2), 2.23 (s, 3H, CH_3), 1.14 (t, 3H, CH_3); ν_{max}/cm^{-1} (KBr):3245 (N—H), 3118 (Ar—C—H), 2982 (C—H), 1714 (C=O), 1647 (NH_2—C=O), 1466 (Ar—C=C), 1297 (CH_3), 1223 (CO—O—CH_2); Anal. Calcd. for $C_{14}H_{16}N_2O_3$: C, 64.60; H, 6.20; N, 10.76; Found: C, 64.57; H, 6.22; N, 10.71.

4.2.2. Ethyl 4-(4-chlorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (**4b**)

According to general procedure, **4b** was obtained from 4-chlorobenzaldehyde, urea, and ethyl acetoacetate in 3 h. and 45% yield as white crystalline solid: m.p. 214–216 °C (lit. [46] 213–215 °C); R_F 0.39 (n-hexane: EtOAc, 1:1); δ_H (300 MHz, DMSO-d6): 9.22 (s, 1H, NH), 7.74 (s, 1H, NH), 7.37 (t, 2H, Ar—H), 7.23 (d, 2H, Ar—H), 5.12 (s, 1H, CH), 3.37 (q, 2H, CH_2), 2.23 (s, 3H, CH_3), 1.082 (t, 3H, CH_3); ν_{max}/cm^{-1} (KBr): 3241 (N—H), 3119 (Ar—C—H), 2978 (C—H), 1713 (C=O), 1648 (NH_2—C=O), 1461 (Ar—C=C), 1291 (CH_3), 1224 (CO—O—CH_2), 784 (Ar—Cl); Anal. Calcd. for $C_{14}H_{15}ClN_2O_3$: C, 57.05; H, 5.13; N, 9.50; Found: C, 57.08; H, 5.09; N, 9.49.

4.2.3. Ethyl 4-(4-methoxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (**4c**)

According to general procedure, **4c** was obtained from 4-methoxybenzaldehyde, urea, and ethyl acetoacetate in 3 h. and 55% yield as white solid: m.p. 197–200 °C (lit. [47] 198–200 °C) R_F 0.43 (n-hexane: EtOAc, 1:1); δ_H (300 MHz, DMSO-d6):9.13 (s, 1H, NH), 7.65 (s, 1H, NH), 7.10 (t, 2H, Ar—H), 6.85 (m, 2H, Ar—H), 5.07 (s, 1H, CH), 3.96 (q, 2H, CH_2), 3.70 (s, 3H, CH_3) 2.22 (s, 3H, CH_3),1.09 (t, 3H, CH_3); ν_{max}/cm^{-1} (KBr):3230 (N—H), 3111 (Ar—C—H), 2944 (C—H), 1711 (C=O), 1653 (NH_2—C=O), 1460 (Ar—C=C), 1282 (CH_3), 1229 (CO—O—CH_2); Anal. Calcd. for $C_{15}H_{18}N_2O_4$: C, 62.06; H, 6.25; N, 9.65; Found: C, 62.02; H, 6.24; N, 9.69.

4.2.4. (±)2,6-Methano-4-oxo-3,4,5,6-tetrahydro-2H-[1,3,5]benzoxadiazocines (**4d**)

According to general procedure, **4d** was obtained from 2-hydroxybenzaldehyde, urea, and ethyl acetoacetate in 3hr. and 40% yield as off white solid: m.p. 199–200 °C (lit. [48] 200–202 °C); R_F 0.40 (n-hexane: EtOAc, 1:1); δ_H (300 MHz, DMSO-d6): 9.17 (s, 1H, NH), 7.71 (s, 1H, NH), 7.32 (q, 2H, Ar—H), 7.22 (q, 2H, Ar—H), 5.12 (d, 1H, CH), 3.96 (q, 2H, CH_2), 2.23 (s, 3H, CH_3),1.08 (t, 3H, CH_3); ν_{max}/cm^{-1} (KBr): 3490 (O—H), 3234 (N—H), 3117 (Ar—C—H), 2937 (C—H), 1714 (C=O), 1646 (NH_2—C=O), 1471 (Ar—C=C), 1294 (CH_3), 1223 (CO—O—CH_2), 1092 (C—N); Anal. Calcd. for $C_{14}H_{16}N_2O_4$: C, 60.86; H, 5.84; N, 10.14; Found: C, 60.81; H, 5.89; N, 10.09.

4.2.5. Ethyl 4-(4-hydroxy-3-methoxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (**4e**)

According to general procedure, **4e** was obtained from 3-methoxy-4-hydroxybenzaldehyde, urea, and ethyl acetoacetate in 7 h. and 74% yield as white solid: m.p. 231–233 °C (lit. [49] 230–232 °C); R_F 0.26 (n-hexane: EtOAc, 1:1); δ_H (300 MHz, DMSO-d6): 9.09 (s, 1H, NH), 7.60 (s, 1H, NH), 6.604 (m, 4H, Ar—H), 5.04 (s, 1H, CH), 3.98 (q, 2H, CH_2), 3.70 (s, 3H, CH_3), 2.22 (s, 3H, CH_3), 1.10 (t, 3H, CH_3); ν_{max}/cm^{-1} (KBr): 3523 (O—H), 3228 (N—H), 3108 (Ar—C—H), 2955 (C—H), 1702 (C=O), 1647 (NH_2—C=O), 1490 (Ar—C=C), 1278 (CH_3), 1224 (CO—O—CH_2), 1094 (C—N); Anal. Calcd. for $C_{15}H_{18}N_2O_5$: C, 58.82; H, 5.92; N, 9.15; Found: C, 58.85; H, 5.88; N, 9.10.

4.2.6. Ethyl 6-methyl-4-(4-methylphenyl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (**4f**)

According to general procedure, **4f** was obtained from 4-methylbenzaldehyde, urea, and ethyl acetoacetate in 5 h. reflux and 77% yield as white solid: m.p. 212–215 °C (lit. [50] 215–216 °C); R_F 0.45 (n-hexane: EtOAc, 1:1); δ_H (300 MHz, DMSO-d6): 9.13 (s, 1H, NH), 7.66 (s, 1H, NH), 7.10 (s, 4H, Ar—H), 5.08 (d, 1H, CH), 3.96 (q, 2H, CH_2), 2.23 (d, 6H, CH_3), 1.08 (t, 3H, CH_3); ν_{max}/cm^{-1} (KBr): 3238 (N—H), 3111 (Ar—C—H), 2933 (C—H), 1707 (C=O), 1653 (NH_2—C=O), 1474 (Ar—C=C), 1286 (CH_3), 1230 (CO—O—CH_2); Anal. Calcd. for $C_{15}H_{18}N_2O_3$: C, 65.68; H, 6.61; N, 10.21; Found: C, 65.64; H, 6.60; N, 10.26.

4.2.7. Ethyl 6-methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (**4g**)

According to general procedure, **4g** was obtained from benzaldehyde, thiourea, and ethyl acetoacetate in 5 h. and 40% yield as white solid: m.p. 206–209 °C (lit. [51] 208–210 °C); R_F 0.48 (n-hexane: EtOAc, 1:1); δ_H (300 MHz, DMSO-d6): 10.31 (s, 1H, NH), 9.63 (s, 1H, NH), 7.27 (m, 5H, Ar—H), 5.15 (d, 1H, CH), 4.03 (m, 2H, CH_2), 2.27 (s, 3H, CH_3),1.09 (t, 3H, CH_3); ν_{max}/cm^{-1} (KBr):3324 (N—H), 3174 (Ar—C—H),2982 (C—H), 1671 (C=O), 1474 (Ar—C=C), 1281 (CH_3), 1189 (CO—O—CH_2), 1114 (C—N), 764 (C=S); Anal. Calcd. for $C_{14}H_{16}N_2O_2S$: C, 60.85; H, 5.84; N, 10.14; Found C, 60.81; H, 5.88; N, 10.12.

*4.2.8. Ethyl 4-(4-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5 carboxylate (**4h**)*

According to general procedure, **4h** was obtained from 4-chlorobenzaldehyde, thiourea, and ethyl acetoacetate in 7 h. and 59% yield as yellow crystalline solid: m.p. 190–192 °C (lit. [51] 192–194 °C); R_F 0.54 (n-hexane: EtOAc, 1:1); δ_H (300 MHz, DMSO-d6): 10.37 (s, 1H, NH), 9.65 (s, 1H, NH), 7.40 (d, 2H, Ar—H), 7.21 (d, 2H, Ar—H), 5.15 (s, 1H, CH), 3.99 (q, 2H, CH_2), 3.32 (s, 3H, CH_3) 2.28 (s, 3H, CH_3), 1.08 (t, 3H, CH_3); ν_{max}/cm^{-1} (KBr): 3324 (N—H), 3181 (Ar—C—H), 3014 (C—H), 1673 (C=O), 1466 (Ar—C=C), 1281 (CH_3), 1189 (CO—O—CH_2), 1113 (C—N), 829 (Ar—Cl), 752 (C=S); Anal. Calcd. for $C_{14}H_{15}ClN_2O_2S$: C, 54.10; H, 4.86; Cl, 11.41; N, 9.01; Found: C, 54.12; H, 4.82; N, 8.99.

*4.2.9. Ethyl 4-(4-methoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (**4i**)*

According to general procedure, **4i** was obtained from 4-methoxybenzaldehyde, thiourea, and ethyl acetoacetate in 7 h. and 63% yield as white solid: m.p. 150–154 °C (lit. [51] 152–152 °C); R_F 0.65 (n-hexane: EtOAc, 1:1); δ_H (300 MHz, DMSO-d6): 10.27 (s, 1H, NH), 9.58 (s, 1H, NH), 7.16 (m, 2H, Ar—H), 6.88 (d, 2H, Ar—H), 5.09 (s, 1H, CH), 3.98 (q, 2H, CH_2), 3.71 (s, 3H, CH_3), 2.27 (s, 3H, CH_3), 1.06 (t, 3H, CH_3); ν_{max}/cm^{-1} (KBr): 3310 (N—H), 3186 (Ar—C—H), 2986 (C—H), 1668 (C=O), 1461 (Ar—C=C), 1272 (CH_3), 1186 (CO—O—CH_2), 1115 (C—N), 771 (C=S); Anal. Calcd. for $C_{15}H_{18}N_2O_3S$: C, 58.80; H, 5.92; N, 9.14; Found: C, 58.78; H, 5.90; N, 9.13.

*4.2.10. (±)2,6-Methano-4-thioxo-3,4,5,6-tetrahydro-2H-[1,3,5]benzoxadiazocines (**4j**)*

According to general procedure, **4j** was obtained from 2-hydroxybenzaldehyde, thiourea, and ethyl acetoacetate in 7 h. and 58% yield as white crystals: m.p. 205–207 °C (lit. [48] 203–205 °C); R_F 0.68 (n-hexane: EtOAc, 1:1); δ_H (300 MHz, DMSO-d6): 9.13 (s, 1H, NH), 7.19 (m, 2H, Ar—H), 6.92 (t, 1H, Ar—H), 6.83 (d, 1H, Ar—H), 4.56 (q, 1H, CH), 4.16 (m, 2H, CH_2), 1.77 (s, 3H, CH_3),1.22 (t, 3H, CH_3); ν_{max}/cm^{-1} (KBr): 3523 (O—H)3362 (N—H), 3172 (Ar—C—H), 2995 (C—H), 1727 (C=O), 1483 (Ar—C=C), 1326 (CH_3), 1233 (CO—O—CH_2), 1092 (C—N), 761 (C=S); Anal. Calcd. for $C_{14}H_{16}N_2O_3S$: C, 57.52; H, 5.52; N, 9.58; Found: C, 57.50; H, 5.48; N, 9.57.

*4.2.11. Ethyl 4-(4-hydroxy-3-methoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (**4k**)*

According to general procedure, **4k** was obtained from 4-hydroxy-3-methoxybenzaldehyde, thiourea, and ethyl acetoacetate in 8 h. and 74% yield as white crystalline solid: m.p. 197–199 °C; R_F 0.56 (n-hexane: EtOAc, 1:1); δ_H (300 MHz, DMSO-d6): 10.24 (s, 1H, NH), 9.54 (d, 1H, OH), 9.00 (s, 1H, NH), 6.63 (m, 3H, Ar—H), 5.06 (s, 1H, CH), 4.01 (q, 2H, CH_2), 3.71 (s, 3H, CH_3) 2.26 (s, 3H, CH_3), 1.10 (t, 3H, CH_3); ν_{max}/cm^{-1} (KBr): 3610 (O—H), 3412 (N—H), 3194 (Ar—C—H), 3003 (C—H), 1689 (C=O), 1474 (Ar—C=C), 1271 (CH_3), 1195 (CO—O—CH_2), 1124 (C—N), 753 (C=S); Anal. Calcd. for $C_{15}H_{18}N_2O_4S$: C, 55.88; H, 5.63; N, 8.69; Found: C, 55.84; H, 5.61; N, 8.67.

*4.2.12. Ethyl 6-methyl-4-(4-methylphenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (**4l**)*

According to general procedure, **4l** was obtained from 4-methylbenzaldehyde, thiourea, and ethyl acetoacetate in 8 h. and 65% yield as yellow crystalline solid: m.p. 176–178 °C (lit. [52] 177–179 °C); R_F 0.87 (n-hexane: EtOAc, 1:1); δ_H (300 MHz, DMSO-d6): 10.28 (s, 1H, NH), 9.59 (s, 1H, NH), 7.10 (q, 4H, Ar—H), 5.11 (d, 1H, CH), 3.99 (q, 2H, CH_2), 2.26 (d, 6H, CH_3),1.09 (t, 3H, CH_3); ν_{max}/cm^{-1} (KBr): 3324 (N—H), 3174 (Ar—C—H), 2982 (C—H), 1671 (C=O), 1474 (Ar—C=C), 1281 (CH_3), 1189 (CO—O—CH_2), 1114 (C—N), 764 (C=S); Anal. Calcd. for $C_{15}H_{18}N_2O_2S$: C, 62.04; H, 6.25; N, 9.65; Found: C, 62.09; H, 6.19; N, 9.72.

4.3. Glucosidase assay procedure

The α-glucosidase inhibition assay was performed with slight modifications according to the method of Pierre et al. [53]. Total volume of the mixture was 100 μL, contained 70 μL 50 mM Na_2HPO_4 buffer, pH 6.8, 10 μL (0.5 mM) test compound, followed by the addition of 10 μL enzyme (0.0234 units, Sigma Inc.). The contents were mixed, pre-incubated for 10 min at 37 °C and pre-read at 400 nm. The reaction was initiated by the addition of 10 μL of 0.5 mM substrate (p-nitrophenyl glucopyranoside, Sigma Inc.). After 30 min of incubation at 37 °C, absorbance of the yellow color produced due to the formation of p-nitrophenol was measured at 400 nm using Synergy HT (BioTek, USA) using 96-well micro-plate reader. Acarbose was used as positive control. The percent inhibition was calculated by the following equation

$$\text{Inhibition } (\%) = (\text{abs of control} - \text{abs of test/abs of control}) \times 100$$

IC_{50} values (concentration at which there is 50% enzyme inhibition) were determined after suitable dilutions of the active compounds and data computed using EZ-Fit Enzyme Kinetics Software (Perrella Scientific Inc. Amherst, USA).

4.4. Molecular modeling studies

4.4.1. Homology modeling

Sequence of baker's yeast α-glucosidase was obtained from Uniprot (No. P53341) [54] then Blast 2.2.25+ program [55] was utilized to search for homologous proteins. Isomaltase from baker's yeast was found to be the most identical and similar to our target. Full sequence of template and its 3D structure were downloaded from Uniprot (No. P53051) [54] and Protein Data Bank (3A4A), respectively. Sequence alignment of target and template was performed with ClustalW2, using BLOSUM62 matrix, gap opening penalty 10 and gap extension penalty 0.2 values [56]. Automodel from Modeller 9.10 was applied to obtain models of the enzyme [57]. It included HETATM residues (glucose from template) in the structures of target and provided very fast refinement, based on conjugate gradients optimization and molecular dynamics simulations at different temperatures. From the five generated models, the one with the lowest modeler objective function upon visual inspection was selected for docking.

4.4.2. Ligand docking

The three-dimensional structure of compound **4f** was prepared on the basis of crystal structure of derivative **4m** in Sybyl X-1.1 [58]. All atoms and bond types, protonation states were checked and subsequently Gasteiger–Marsili charges were added. Acarbose molecule was built on the basis of data from PDB crystal structures (3W37, 3K8M complexes) and prepared in the same way as our ligand. The homology model of α-glucosidase, developed in the above described way, was used for dockings with Gold 5.1 software [59]. All histidine residues were set as Nε (HSE) tautomers and ligand (glucose which was taken into account during model building) was removed. The active site was defined as all amino acid residues within 15 Å from that glucose molecule. Dockings were performed using genetic algorithm with default settings. GoldScore function and analysis of binding mode were applied to find final ligand poses. Results were viewed in PyMOL 0.99rc2 [60].

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Appendix A. Supplementary material

CCDC 923604-923606 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif. Supplementary material associated with paper can be found, in the online version. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2014.05.003.

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