Why are some alcohols easy to glucosylate with β-glucosidases while others are not? A computational approach

B. Mattheus de Roode,[a] Han Zuilhof,*†[a] Maurice C. R. Franssen,*†[a] Albert van der Padt[b] and Aede de Groot[a]

[a] *Laboratory of Organic Chemistry, Wageningen University, Dreijenplein 8, 6703 HB Wageningen, The Netherlands*
[b] *Food and Bioprocess Engineering Group, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands*

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A method is presented for predicting the reactivity of alcoholic aglycons in the β-glucosidase mediated glucosylation reaction. The successful enzymatic glucosylation of an aglycon appears to be mainly dependent on the nucleophilicity of the aglycon. Vinylic and phenolic aglycons are not nucleophilic enough to be glucosylated enzymatically, although their chemical glucosylation is facile. By using PM3 and AM1 semi-empirical methods, the magnitude of this nucleophilicity can be calculated and was found to correlate with the charge on the reacting atom of the aglycon. Based on this trend, the aglycons can be classified as reacting or non-reacting. The orbital related parameters seem to have a limited influence on the reaction behaviour. In addition to these calculations, the energy of the transition state of two enzymatic reactions has been calculated using a simplified model of the enzyme active site for both an experimentally reacting and an experimentally non-reacting aglycon (cyclohexanol and phenol, respectively). The activation energy for the cyclohexanol complex was computed to be 1.3 kcal mol^{-1}, while the calculated activation energy for the phenol complex is 15.8 kcal mol^{-1}. This difference can indeed explain the fact that cyclohexanol is easily glucosylated while phenol is not.

Finally, it is pointed out that facile and fast calculation methods can be used to make a confident prediction about the reaction behaviour of aglycons without performing the actual laboratory experiments.

Introduction

β-Glucosidases

β-Glucosidases are widely applied in food technology, biotechnology and organic chemistry. These enzymes have been used for the hydrolysis of glucosides and for the glucosylation of various alcohols. In the hydrolysis reaction, β-glucosidases are commonly used to screen glycosidically bound volatiles in fresh plant material.[1] Through hydrolysis of the corresponding glucosides, a variety of aglycons like monoterpenols[2,3] or phenols[4,5] are easily released. By tuning the reaction conditions, for example, by working at high substrate concentrations, β-glucosidase can also glucosylate alcohols.[6–9] However, this enzymatic pathway suffers from low yields in comparison to chemical glucosylation.

If we compare the aglycons that are used for the enzymatic glucosylation reaction with the volatiles that are released in the enzymatic hydrolysis reaction, it is apparent that the hydrolysis reaction is not in all cases reversible. Such reported glucosylation reactions mainly deal with linear alcohols[10–12] and sugars,[13,14] while phenols have never been enzymatically glucosylated to our knowledge. In line with this, only the aliphatic hydroxy group of hydroxyalkyl phenols is glucosylated,[15] whereas phenolic glucosides are readily hydrolysed. To understand this one-sided behaviour of β-glucosidase, the mechanism for the reaction, proposed in 1953,[16] has to be examined (see schematic view in Fig. 1).

In the first step, the capacity of the aglycon to act as a leaving group is enhanced by protonation, after which the anomeric centre of the glycon is attacked by a nucleophilic group from the enzyme. Formation of the covalent *α*-glucose–enzyme intermediate[17] (proved for an *Alcaligenes faecalis* β-glucosidase) proceeds *via* a transition state with some oxocarbonium ion character[18,19] (determined from kinetic isotope effect and *ab initio* studies). The nucleophilic attack of an aglycon or water upon the anomeric centre proceeds *via* a transition state with a more pronounced oxocarbonium ion character[20–22] (derived from kinetic isotope effect and inhibition studies). The newly formed β-glucoside leaves the active site, returning the enzyme to its original state. Studies on almond β-glucosidase[23,24] show evidence of a similar mechanism. In a recent study of the reaction mechanism the position of the acidic amino acid in the active site (AH in Fig. 1) is discussed. It seems that the aglycon is not protonated "from above" (as in Fig. 1), but "from the side" (the non-dissociated amino acid is positioned at the same side as the basic amino acid B).[25]

In the proposed reaction mechanism, which has been substantiated by several studies,[19,26] the nature of both glycon and aglycon is important. The glycon is important for the recognition of the glucoside by the enzyme, whereas the aglycon mainly determines the reaction rate in both hydrolysis and glucosylation reactions. In studies with an *A. faecalis* β-glucosidase, the first step in the reaction mechanism was found to be rate-determining for the hydrolysis of glucosides having relatively poor leaving groups ($pK_a > 8$).[20] If the leaving group is a relatively strong acid ($pK_a < 7$), the second step becomes rate-determining, as was shown with almond β-glucosidase.[23]

It is assumed that the second step in the mechanism proceeds *via* an oxocarbonium ion-like transition state, in which the

† Correspondence on computational chemistry: Tel: +31-317-482367, Fax: +31-317-484914, E-mail: Han.Zuilhof@phys.oc.wau.nl; correspondence on enzyme catalysis: Tel: +31-317-482976, Fax: +31-317-484914, E-mail: Maurice.Franssen@bio.oc.wau.nl

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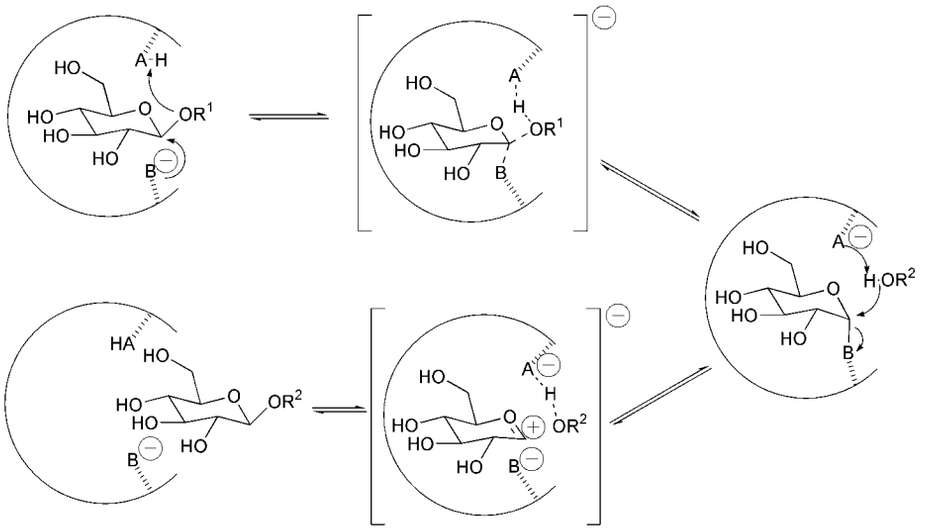

Fig. 1 Schematic view of the reaction mechanism of β-glucosidase. R^1OH = water or aglycon, R^2OH = aglycon or water; AH = acidic amino acid side chain; B^- = basic amino acid side chain.

charge development on the oxygen atom can be, to some degree, substrate dependent. This may lead to the conclusion that the differences in the aglycon reactivity should not be as extreme (reacting or non-reacting) as they appear to be. The nucleophilicity of the aglycon clearly is important in this step of the mechanism. Therefore, a method to determine the nature of the nucleophilicity of the aglycon would be helpful to explain why some aglycons cannot be glucosylated. With the aid of computational electronic structure methods it is possible to calculate a set of parameters which are related to the nucleophilicity of the aglycon.

Computational quantum chemistry in enzyme catalysis

Computational quantum chemistry plays an increasingly important role in enzyme catalysis. *Ab initio* and semi-empirical calculation methods are mainly used to elucidate the transition state of the enzyme reaction.[27] Studies on the mechanisms of β-lactamase,[28] glucosaminidase,[29] glutathione reductase,[30] serine hydrolase[31] and glycosidases[32] are typical recent examples of these types of calculations. Another type of study involves structure–activity relationships based on computationally derived reactivity parameters. In many cases, the reactivity can be correlated with frontier orbital parameters,[33] *i.e.* the energy and relevant orbital coefficients of the HOMO (Highest Occupied Molecular Orbital) and LUMO (Lowest Unoccupied Molecular Orbital), and the charges on the atoms directly involved in the reaction. HOMO or LUMO energies of the reactants display a good correlation with the enzymatic reaction rates of peroxidase,[34] glutathione *S*-transferase,[35] catechol-1,2-dioxygenase[36] and cytochrome P450.[37]

Although the mechanism of β-glucosidase has been thoroughly investigated in the literature, to the best of our knowledge, the glucosylation reaction has never been studied from a theoretical point of view. The aim of this paper is to give a theoretical background for the observed differences in reactivity of β-glucosidase towards aglycons in glucosylation reactions. To this end, the orbital coefficients and LUMO and HOMO energies will be calculated, completed with electrostatic potential charge and orbital-based atomic charges for 16 nucleophiles which have been subjected to the glucosylation reaction. Furthermore, ESP-based (ElectroStatic Potential) and orbital-based atomic charges will be calculated for a variety of successfully glucosylated aglycons from the literature. The importance of the different parameters with respect to the glucosylation reaction will be evaluated. In addition, the reaction path for a successfully glucosylated aglycon and a non-reacting aglycon will be simulated. From these data the transition state for the glucosylation of both systems will be calculated. Finally, we will show that the calculation of these parameters is a useful tool to rapidly predict the behaviour of aglycons in the β-glucosidase-mediated glucosylation reaction.

Experimental

Calculations

The structures of all aglycons were pre-optimised using the MM2 force field as implemented in the ChemOffice Ultra program (Cambridge Scientific Computing). These structures were subsequently fully optimised with semi-empirical methods (both AM1 and PM3), to obtain the HOMO/LUMO energies, orbital coefficients, orbital-related net atomic charges, and the electrostatic potential charges. In all cases solvent models were used to mimic the effects of water, with a relative permittivity $\varepsilon = 78.5$. Within MOPAC97 (part of ChemOffice Ultra) this was performed using the COSMO routine.[38] The AM1 results differed only marginally from those calculated with the PM3 hamiltonian, and the same trend was observed. Therefore, the results from the AM1 calculations will not be discussed.

For two cases transition state optimisations were performed (PM3 computation in GAUSSIAN94[39]). First, a reaction path for the stepwise approach of the reacting oxygen atom of the aglycon anion and the reacting carbon atom of the glucose–glutamic acid complex was calculated (from 3.0 to 1.4 Å, stepsize r(C–O) = 0.1 Å). Secondly, transition state optimisations were carried out with the QST2 option implemented in GAUSSIAN94. The transition state was in both cases characterised by performing vibrational frequency analysis (one imaginary frequency, corresponding to movements along the reaction path, was found). The transition states of the complexes with neutral aglycons were estimated by following the reaction path for the stepwise approach of the reacting oxygen atom of the neutral aglycon and the reacting carbon atom of the glucose–glutamic acid adduct (from 1.70 to 1.65 Å, stepsize r(C–O) = 0.01 Å). The energies between the optimised structures where the $C–O_{leaving\ group}$ bond was broken ($C–O_{leaving\ group}$ distance equals 1.55 Å for both structures) were used as a representative estimate of the transition state energy.

Chemicals

Glucose, cyclohexanol and nerol were obtained from Sigma. Ethyl acetate, petroleum ether, acetonitrile (HPLC-grade) and methanol (HPLC-grade) were obtained from Lab-Scan. *tert*-

Amyl alcohol, *p*-methoxyphenol and geraniol were obtained from Acros. Citronellol, vanillin, eugenol, cinnamyl alcohol, phenethyl alcohol, benzyl alcohol, furaneol and 4-hydroxybenzaldehyde were obtained from Aldrich. Hexan-1-ol and octan-1-ol were obtained from Merck. Phenol was obtained from Brocades. Deionized water was used in all experiments. Citric acid and sodium phosphate from Sigma were used to prepare the buffer solution.

Citrate–phosphate buffer pH 6.0 was prepared by dissolving 7.74 g citric acid and 45.23 g sodium phosphate in 1 l water.

Enzyme

Almond β-glucosidase (E.C.3.2.1.21) was obtained from Sigma (5.6 U mg^{-1}).

Equipment

Incubations were carried out in thermostatted New Brunswick Scientific G24 or Innova 4080 incubator shakers. The HPLC system was composed of a Gyncotek pump and auto sampler with a Gyncotek UV-detector and a Sedex Evaporative Light Scattering Detector. Samples were eluted with acetonitrile–water (90 : 10 v/v%) on a Spherisorb S5-amino column (150 × 4.6 mm). Activity measurements were carried out using a Perkin-Elmer Lambda 18 UV/Vis spectrophotometer. NMR measurements were carried out on a Bruker AC 200. Preparative scale separations were carried out with Bond Elut LRC NH_2 columns from Varian.

Enzymatic glucosylation

The enzymatic reaction was carried out in a well-capped 20 ml vial containing 0.5 ml of citrate–phosphate buffer pH 6.0 (0.16 M) with an almond β-glucosidase activity of 0.4 to 30 U ml^{-1} buffer solution, 500 g glucose per kg of buffer solution and an organic phase (4.5 ml) consisting of pure aglycon or of various concentrations of the aglycon in *tert*-amyl alcohol. The two-phase system was shaken at approximately 280 rpm at 50 °C. At regular time intervals samples for HPLC analysis were taken from the organic layer of the reaction mixture.

Preparative glucosylation of geraniol, nerol and citronellol

The enzymatic reactions were carried out as described above, using 4.5 ml of pure aglycon and 20 U enzyme ml^{-1} buffer solution. After reaching equilibrium, the reactions were stopped. The two-phase system was evaporated under reduced pressure at 40 °C. The liquid residue was filtered over a funnel with a plug of cotton in order to remove insolubles. The clear mixture was separated over a preparative amino column. The column was activated with 3 ml $CHCl_3$ after which 3 ml of the reaction mixture were applied. Then, the column was rinsed with three portions of 1 ml $CHCl_3$. Finally, 3 ml of methanol were used to elute the glucoside from the column. This methanol phase was evaporated to yield the pure glucoside.

Geranyl β-D-glucopyranoside. FAB-MS: 339 (M + Na), 163, 145, 137, 113, 95, 81, 69; ^{1}H NMR (200 MHz, D_2O) δ 1.39 (s, 3H, $H_{9'}$), 1.46 (s, 6H, $H_{8'}$, $H_{10'}$), 1.90 (br s, 4H, $H_{4'}$, $H_{5'}$), 3.02 (t, J = 8.3 Hz, 1H, H_2), 3.13 (br s, 1H, H_5), 3.43 (m, 2H, H_3, H_4), 3.65 (br s, 2H, H_6), 4.10 (d, J = 7.4 Hz, 2H, $H_{1'}$), 4.22 (d, J = 7.9 Hz, 1H, H_1), 4.96 (m, 1H, $H_{6'}$), 5.15 (m, 1H, $H_{2'}$).

Neryl β-D-glucopyranoside. FAB-MS: 339 (M + Na), 315 (M − H), 163, 137, 95, 81, 69; ^{1}H NMR (200 MHz, D_2O) δ 1.39 (s, 3H, $H_{9'}$), 1.55 (s, 6H, $H_{8'}$, $H_{10'}$), 1.95 (br s, 4H, $H_{4'}$, $H_{5'}$), 3.00 (t, J = 8.3 Hz, 1H, H_2), 3.18 (br s, 1H, H_5), 3.50 (m, 2H, H_3, H_4), 3.65 (br s, 2H, H_6), 4.10 (d, J = 7.7 Hz, 2H, $H_{1'}$), 4.21 (d, J = 7.9 Hz, 1H, H_1), 5.00 (m, 1H, $H_{6'}$), 5.18 (m, 1H, $H_{2'}$).

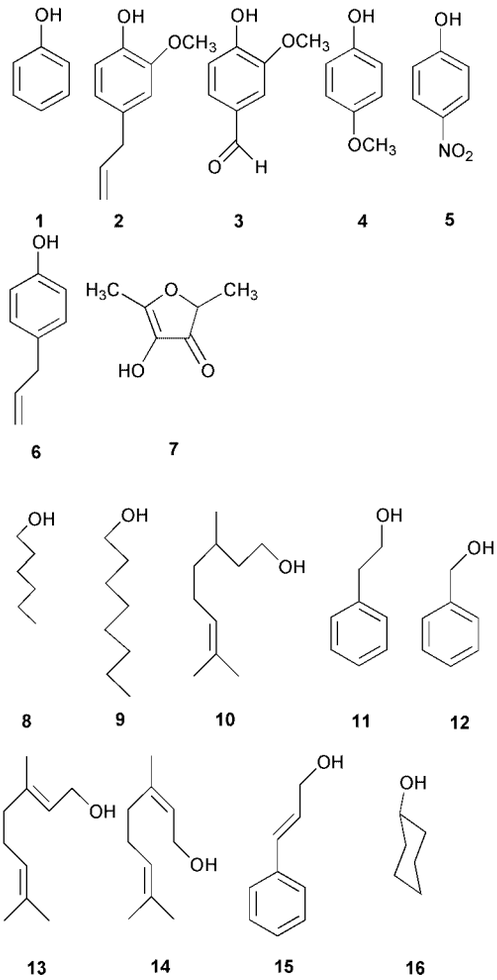

Fig. 2 16 Aglycons which have been subjected to the glucosylation reaction with β-glucosidase. **1** Phenol, **2** eugenol, **3** vanillin, **4** *p*-methoxyphenol, **5** *p*-nitrophenol, **6** chavicol, **7** furaneol, **8** hexan-1-ol, **9** octan-1-ol, **10** citronellol, **11** phenethyl alcohol, **12** benzyl alcohol, **13** geraniol, **14** nerol, **15** cinnamyl alcohol, **16** cyclohexanol.

Citronellyl β-D-glucopyranoside. ^{1}H NMR (200 MHz, D_2O) δ 0.89 (s, 3H, $H_{9'}$), 1.42 (s, 6H, $H_{8'}$, $H_{10'}$), 1.47 (m, 1H, $H_{2'}$), 1.65 (br s, 4H, $H_{4'}$, $H_{5'}$), 3.01 (t, J = 8.2 Hz, 1H, H_2), 3.18 (br s, 1H, H_5), 3.50 (m, 2H, H_3, H_4), 3.65 (br s, 2H, H_6), 4.17 (d, J = 7.9 Hz, 2H, H_1), 4.36 (d, J = 7.1 Hz, 1H, H_1), 4.95 (m, 1H, $H_{6'}$).

Activity measurements

All non-reacting aglycons were monitored for their inhibitory effect on the enzyme. For this purpose, enzyme reactions were carried out as described above. At regular time intervals, samples were taken from the water phase for activity measurements. The activity of β-glucosidase was measured by monitoring the hydrolysis of *p*-nitrophenol glucoside (pNP glucoside) with UV–vis spectrometry. A known amount of β-glucosidase was added to 2.5 ml of a 2 mM solution of pNP glucoside in a citrate–phosphate buffer pH 6.0. The increase of *p*-nitrophenol with time was measured at 405 nm at 40 °C.

Results

Glucosylation

First, enzymatic glucosylation experiments with phenol, eugenol and vanillin were performed (see Fig. 2, structures **1**, **2** and **3**), but no glucosides could be obtained from these reactions.

The successful glucosylation of primary (Fig. 2, structures **8** to **12**), allylic (Fig. 2, structures **13** to **15**) and secondary (Fig. 2,

structure **16**) alcohols induced us to search for the reason why the first group of aglycons could not be glucosylated. The nucleophilic character of the non-reacting aglycons was modified by substituting the phenol ring with electron-donating (Fig. 2, structure **6**) or electron-withdrawing (Fig. 2, structures **4** and **5**) groups. These modifications did not lead to the desired glucosides either. In addition, an enolic furan derivative (Fig. 2, structure **7**) was tried, but this was also not glucosylated.

These results suggest that the nucleophilicity of the aglycon is an important reactivity parameter. Therefore, to investigate the nature of the nucleophilicity of all tested aglycons, we have calculated the charges and relevant orbital-related parameters of the atoms that are directly involved in this reaction for the ground state of the molecules in Fig. 2.

Semi-empirical calculations

As there is no unambiguous quantum mechanical operator to calculate the charge on an atom, several methods to approximate this charge are available. Since the charge on the oxygen atom of the aglycon is likely to be important for the reactivity, and to minimise the possibility of any method-dependent result, two very different electron-density division schemes were used to obtain two types of charges: net atomic charges (based on an orbital-division technique) and ESP charges (derived from the electrostatic potential around the molecule). The calculated charges on the oxygen atom of the aglycons and the relevant parameters for the active frontier orbitals are shown in Table 1.

The charge on the reactive atoms was also calculated for successfully glucosylated aglycons from the literature (see Table 2).

Table 1 Calculated values of net atomic and ESP charge (Q), orbital coefficient of the reacting atom in the HOMO (c) and the molecular orbital energy (E_{HOMO}) for different aglycons. **Bold** = successfully glucosylated by β-glucosidase, plain = unable to glucosylate with β-glucosidase

Aglycon	Q_{nuc}		c_{nuc}^2 (−)	E_{HOMO}/ eV
	Net atomic	ESP		
Octanol	−0.408	−0.417	0.364	−11.08
Hexanol	−0.407	−0.420	0.359	−11.08
Citronellol	−0.405	−0.417	0.003	− 9.52
Geraniol	−0.404	−0.405	0.006	− 9.56
Cinnamyl alcohol	−0.403	−0.426	0.010	− 9.25
Phenethyl alcohol	−0.403	−0.415	0.002	− 9.77
Cyclohexanol	−0.400	−0.430	0.260	−11.08
Nerol	−0.399	−0.398	0.002	− 9.62
Benzyl alcohol	−0.397	−0.362	0.020	− 9.94
p-Methoxyphenol	−0.300	−0.314	0.107	− 9.08
Phenol	−0.299	−0.302	0.141	− 9.47
Chavicol	−0.299	−0.321	0.126	− 9.33
Eugenol	−0.284	−0.269	0.125	− 9.35
Vanillin	−0.279	−0.281	0.104	− 9.31
p-Nitrophenol	−0.278	−0.291	0.155	− 9.89
Furaneol	−0.266	−0.261	0.114	− 9.60

Determination of the enzyme model

The applicability of initial state calculations to explain the reactivity of different aglycons is, of course, limited to reactions in which the initial state resembles the transition state to a sufficient degree for the purpose at hand. However, for reactions with an early transition state, this technique allows a very fast determination of reactivity trends.[44–46] The optimisation of the geometry of the transition state itself provides more direct information, and can give a more detailed insight into the observed differences in the aglycon reactivity. Therefore, a model of the enzyme–glucose intermediate has to be made. For this, it is important to determine to what extent the active site of the enzyme can be simplified, without losing specific reaction characteristics. Various studies concerning the active site of β-glucosidases have been performed to elucidate the role of the non-covalent interactions between the enzyme and the substrate glycon. The importance of hydrogen bonding at the different positions of the glycon has been studied for *A. faecalis* β-glucosidase and compared with earlier studies on β-glucosidases from *E. coli*, *A. wertii*, *A. oryzae* and mammalian lactase.[47] Interactions with all oxygen atoms of the glycon play a role in the recognition of the substrate, the stabilisation of the ground state and the transition state of the substrate–enzyme intermediate. However, the most important interaction for stabilising the transition state for all β-glucosidases seems to be the interaction at the 2-position of the glycon.[47]

Mutagenesis experiments with *A. faecalis* β-glucosidase[48] and the crystal structure of a white clover β-glucosidase[49] have identified glutamate as the attacking nucleophile in the active site. Based on this information, approximate models of the structure of the glucose–enzyme intermediate can be made, and two of these (model systems **I** and **II**) are presented in Fig. 3.

Using these enzyme models, the reaction path of an aglycon approaching the glucose–enzyme intermediate can be simulated. Since a simplified model of the glucose–enzyme intermediate is used, the obtained energy levels of the different stationary points of the glucosylation reaction will be approximations, yet useful for a comparison between the different aglycons and to indicate which parts of the model system are important for the nucleophilic substitution reaction. For this

Table 2 Calculated net atomic and ESP charges on the reactive oxygen atom of aglycons successfully glucosylated with β-glucosidase according to the literature

β-Glucosidase source	Aglycon	Q_{nuc}	
		Net atomic	ESP
Almond[40]	2-Acetamidopropanol	−0.399	−0.402
	Methyl 2-acetamido-3-hydroxypropanoate	−0.393	−0.348
	trans-1-Acetyl-4-hydroxy-L-proline	−0.391	−0.406
	Methyl 2-azido-3-hydroxypropanoate	−0.387	−0.360
	Methyl 2-acetamido-3-methyl-3-hydroxypropanoate	−0.383	−0.401
Almond[41]	Pent-3-en-2-ol	−0.403	−0.409
	But-3-en-2-ol	−0.402	−0.420
	Pent-1-en-3-ol	−0.400	−0.420
	Hex-1-en-3-ol	−0.395	−0.421
Sulfolobus solfatoricus[42]	3-(Hydroxymethyl)-4-hydroxypentanol	−0.403	−0.402
Almond[12]	6-(Trifluoroacetamido)hexanol	−0.407	−0.413
Almond[43]	2-(Trimethylsilyl)ethanol	−0.419	−0.420
	Hexane-1,6-diol	−0.407	−0.420
	Pent-4-en-1-ol	−0.402	−0.411

Table 3 Calculated values of the orbital and charge parameters of the anomeric carbon atom for two models of the covalent β-glucosidase intermediate

Model	Q_{elec}	c_{elec}^2 (−)	E_{LUMO}/eV
Glucose	0.197	0.174	1.920
I	0.200	0.008	0.331
II	0.186	0.004	0.361

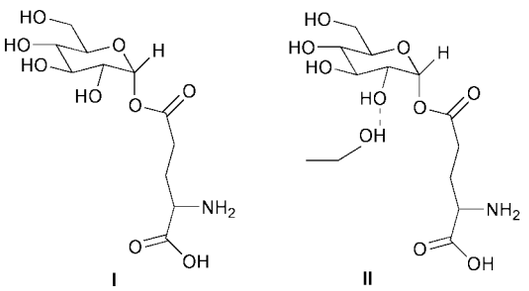

Fig. 3 Two models of the covalent glucose–enzyme intermediate as used in the calculations. **I** γ-(α-glucosyl) glutamate; **II**, **I** with a hydrogen bond on the 2-position of glucose. For reasons of simplicity, ethanol was taken as the hydrogen bond donor/acceptor.

purpose the relevant charges and orbital parameters on the reacting carbon atom of these two systems and glucose are given in Table 3.

The differences between the orbital parameters of the two model systems **I** and **II** are minimal. However, compared to glucose there is a large difference. Apparently, the covalent bond with glutamic acid affects the electron distribution on the anomeric centre, while the hydrogen bond barely influences this distribution. The charges on the anomeric centre of model **I** and glucose are almost identical. The hydrogen bond does have a small effect on the charge of the anomeric carbon atom, as was expected. This difference will have an influence on the results of the calculations of the transition state, but since this effect is similar for all approaching aglycons this will not significantly affect our final conclusions. These models represent the electrophile as present in glucosidase better than glucose. However, since the difference in charge and orbital parameters on the anomeric centre of the two models is small, further calculations have been done with intermediate model **I** to obtain a compromise between completeness of representation and computational efficiency.

Transition state optimisation

Although the reaction mechanism of the glucosylation by β-glucosidase has been investigated thoroughly with a variety of experimental techniques, the degree of concertedness of bond breaking and bond making in the transition state is still unclear.[25] Evidence based on kinetic isotope effect studies exists for a transition state with an S_N2-like character in the first step of the mechanism.[18,21] For the second step in the mechanism larger kinetic isotope effects were observed, supporting a more oxocarbonium-like intermediate.[20] Therefore, it is difficult to predict the character of the transition state *a priori*. The transition state of the glutamic acid–glucose–aglycon complex was optimised with a non-reacting (phenolate) and a reacting aglycon (cyclohexanolate). These anions were chosen based on their similar steric effects (van der Waals areas of 62.5 and 63.5 Å^2, respectively), but significantly different reactivity.

First, the reaction path of both aglycons reacting with model system **I** was determined, *via* a stepwise reduction of the bond length between the oxygen atom of the aglycon and the anomeric carbon atom with full optimisation of all the geometrical features (see Fig. 4).

Table 4 Deformation and activation energy and resonance energy (in kcal mol^{-1}) for the phenolate and the cyclohexanolate complexes. The deformation energy was split into an aglycon contribution and the enzyme model contribution

Complex	ΔE_{def}	$\Delta E^{\ddagger}$	B (=$\Delta E_{def} - \Delta E^{\ddagger}$)
Phenolate	42.6	15.8	26.8
Aglycon/enzyme model	1.1/41.5		
Cyclohexanolate	32.8	1.3	31.5
Aglycon/enzyme model	0.8/32.0		

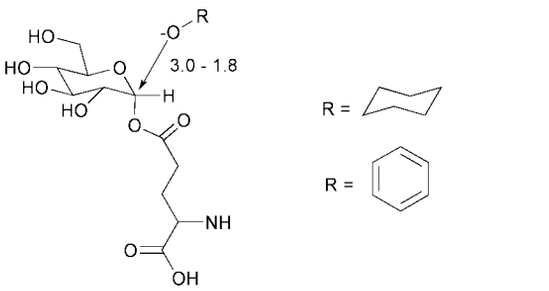

Fig. 4 Approach of a deprotonated aglycon towards the anomeric centre of the enzyme–glucose model (r(C–O) in ångström).

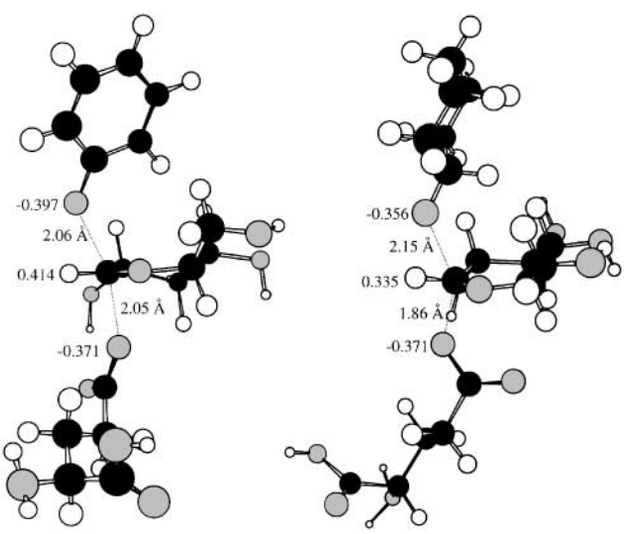

Fig. 5 Presentation of the optimised transition states of a nucleophilic attack on the β-glucosidase model. The bond lengths between the reactive atoms and the net atomic charges on the relevant atoms are given in the figure. Left: phenolate as the nucleophile; right: cyclohexanolate as the nucleophile.

The structure with maximum enthalpy on this approximate reaction path was used as a starting point for the full geometry optimisation of the corresponding transition states. Optimised transition states are presented in Fig. 5, together with selected geometrical features.

In order to determine if the difference between the phenolate and the cyclohexanolate transition state originates in the difference in bond distortion and non-bonded repulsion in the two transition states (*i.e.*, the total deformation energy: ΔE_{def}) or in the difference in the quantum mechanical resonance energy (B), the heat of formation of the reaction components in the ground states and in the transition states was calculated.[50] The deformation energy was characterised as the difference between ground state and transition state energies of the components. The quantum mechanical resonance energy (also called the degree of avoided crossing) was defined as the difference between deformation and activation energy[51] (see Fig. 6 and Table 4).

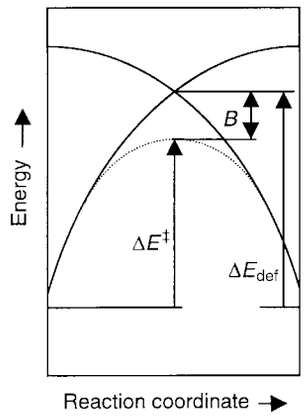

Fig. 6 A state correlation diagram for an identity exchange reaction. The relevant features (activation energy: $\Delta E^{\ddagger}$, deformation energy: ΔE_{def} and quantum mechanical resonance energy: B) are reproduced in the figure.

Discussion

Unsuccessful glucosylation

The unsuccessful glycosylation of phenols by a β-glucosidase that does glucosylate aliphatic alcohols can be due to two factors: (a) Phenolic substrates can deactivate the enzyme. Vanillin and eugenol are known deactivators of numerous enzymes.[52,53] To monitor this effect, the enzyme activity was measured during the reactions with all phenolic aglycons. The enzyme showed a residual activity of at least 40% after 200 h of incubation, ruling out inactivation as a possible explanation for failure of the reaction. (b) Phenolic substrates are significantly worse nucleophiles than aliphatic alcohols. Although the conjugation of the hydroxy group with a phenyl ring will diminish its nucleophilicity, several cases are known in which phenols can be glucosylated efficiently. For example, the chemical glycosylation of tyrosine has been successful in an acid-catalysed condensation reaction between peracetylated glucose and a suitably protected tyrosine residue[54] and a variety of phenols have been glucosylated based on the same glucose donor, using boron trifluoride etherate as catalyst.[55] Since this nucleophilicity factor did not yield a clear answer, it was investigated in more detail.

Calculation of parameters related to the nucleophilicity of the aglycons

The nucleophilicity of the tested aglycons can be described with a limited number of parameters that can easily be calculated with semi-empirical quantum chemical calculations. From the parameters in Table 1, a correlation between the charge on the reacting atom of the aglycon and the reactivity thereof can be inferred. Apparently, vinylic and phenolic aglycons are not nucleophilic enough to be glucosylated by almond β-glucosidase. This is clearly caused by the reaction path taken by the enzyme, since chemical glycosylation of all substrates is facile. The charge on the directly involved oxygen atom in reactive aglycons is in all cases calculated to be more negative than that in non-reacting aglycons, and this conclusion is independent of any of the three methods of charge calculation. In fact, the computed charge differences between reactive and non-reactive aglycons are quite substantial: the net atomic charges on the oxygen atoms of the reacting aglycon with the lowest negative charge (benzyl alcohol) and the non-reacting aglycon with the highest negative charge (*p*-methoxyphenol) differ by almost 0.1. Similarly, for the ESP charge, a threshold negative charge is found for reacting aglycons. Apparently, if the negative charge on the reacting atom of the aglycon is above a threshold value (*vide infra*) glucosylation becomes energetically unfavourable.‡

The orbital coefficient and the energy of the HOMO do not show any correlation with success of glucosylation. On the basis of frontier MO theory it is to be expected that a high orbital coefficient and a E_{HOMO} which is close to the E_{LUMO} of the electrophile are favourable for the reaction.[33] However, aglycons with a high orbital coefficient and a relatively high E_{HOMO} have been found among the group of non-reacting aglycons (*e.g.*, vanillin, phenol and thiophenol). Conversely, aglycons with a low orbital coefficient and a low E_{HOMO} have been found among the reacting aglycons (*e.g.*, geraniol and benzyl alcohol). The fact that these two orbital-related parameters do not correlate with the reactivity of the aglycons is in agreement with the principles of hard and soft acids and bases.[56] Since both the negatively charged nucleophilic aglycons and the positively charged electrophilic enzyme–glucose intermediate are hard in terms of the HSAB theory, the reaction is expected to be charge controlled.[33] Therefore, the parameter with the major influence on the reaction is the charge on the reacting atom of the aglycon. The other parameters have only minor influence on the reactivity.

This correlation between successful glycosylation and the negative charge on the directly involved oxygen atom is also perfect for all available literature data as tabulated in Table 2. The difference between the lowest net atomic charge values on the reactive oxygen atom (−0.383 for methyl 2-acetamido-3-methyl-3-hydroxypropanoate) and the first non-reacting aglycon is over 0.08. Based on the results from Tables 1 and 2, the (PM3-calculated) threshold values for successful glucosylation are −0.383 for the net atomic charge and −0.348 for the electrostatic potential charge.

Transition state optimisation

In both transition states, presented in Fig. 5, glucose is in a skew boat conformation, which is in accordance with the proposed conformation in the reaction mechanism[20,22] and *ab initio* calculations on ring distortion.[32] The phenolate transition state differs from the cyclohexanolate transition state both in structure and in energy with respect to the separate reactants. These differences are expressed in the distances between the reactive atoms and in the charges on the reactive atoms. The charge on the anomeric carbon atom in the transition state is 0.335 with cyclohexanolate, while it is computed to be 0.414 in the case of phenolate. This difference in charge is related to the $C–O_{leaving\ group}$ distance in the transition state: 1.86 and 2.05 Å, respectively. The charge on the $O_{nucleophile}$ decreases with a decreasing $C–O_{nucleophile}$ distance. This is in accordance with calculations for charges on the oxygen atom of the aglycon in the hydrolysis reaction of a glucoside. The charge on the oxygen atom (in the hydrolysis reaction the $O_{leaving\ group}$) decreases from −0.5 to −0.79 with a decreasing $C–O_{leaving\ group}$ distance.[21]

The difference in the $C–O_{leaving\ group}$ distance between the two transition states seems to be in contradiction with the proposed oxocarbonium-like character of this step in the mechanism, since the $C–O_{leaving\ group}$ distance in an S_N1 reaction should be independent of the strength of the nucleophile. However, the results show that a more nucleophilic aglycon induces a decreased $C–O_{leaving\ group}$ distance, which seems to support more S_N2 character of the transition state in this step of the reaction mechanism.

‡ This outcome is independent of the computational method used. The electron density on the oxygen atom of benzyl alcohol and *p*-methoxyphenyl was also calculated as NPA- and Merz-Kollman (ESP) charges by B3LYP/6-31G(d) and B3LYP/6-31+G(d) computations in combination with the PCM solvation model as implemented in GAUSSIAN98. Numerically the values differed from those obtained with the semi-empirical calculations discussed in the text, but the same trend was observed, although in a far more time-consuming manner.

The computed activation energies of both complexes show a striking difference. The activation energy for the phenolate complex is 15.8 kcal mol^{-1}, while the activation energy for the cyclohexanolate complex is only 1.3 kcal mol^{-1}, which readily explains the different reactivity of the aglycons. The analogous calculation of the activation energies of the complexes with neutral aglycons resulted in higher activation energies but still showed a difference of 7.6 kcal mol^{-1} between those for the reacting and the non-reacting aglycon. Therefore, the direct computation of the transition state yields a result that is completely in line with the initial state computations presented in Tables 1 and 2.

The origin of the difference in activation energy

As can be seen in Table 4, the energetic effect of the geometrical perturbation of the nucleophiles in the transition state is minimal. The difference between the ground state energy and the energy of the nucleophile in the transition state is only 0.8 and 1.1 kcal mol^{-1} for cyclohexanolate and phenolate, respectively. In contrast, the perturbation of the reactant state of the glucose–glutamic acid intermediate is much larger in the phenolate transition state than in the cyclohexanolate transition state (41.5 and 32.0 kcal mol^{-1} respectively). In addition to this, the (energy-lowering) quantum mechanical resonance energy in the cyclohexanolate transition state is larger than for the phenolate transition state (31.5 and 26.8 kcal mol^{-1} respectively). Hence, the difference in activation energy of both complexes is a result of both a larger resonance energy in the cyclohexanol transition state and a larger deformation energy in the phenol transition state. The perturbation of the enzyme model is the main contributor to the deformation energy.

Conclusions

The reactivity or lack thereof of almond β-glucosidase towards 16 aglycons was explained using quantum chemical methods. A clear relation between the computed charge on the oxygen atom of the nucleophile and the success of glucosylation of the aglycon was found. If the negative charge on the reacting atom of the aglycon is above a threshold value, glucosylation becomes unfavourable. This difference in reactivity was also expressed in the difference in the computed activation energy of >15 kcal mol^{-1} between a reacting and a non-reacting aglycon approaching an enzyme model.

Use of this method predicted that glucosylation of nerol, geraniol and citronellol by β-glucosidase would be successful, in contrast with literature reports.[57] Subsequent experimental testing indeed showed this prediction to be borne out (see Experimental section), which shows that calculation of the charge on the reacting atom—by either semi-empirical or density functional methods—is a reliable method of predicting the aglycon reactivity.

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