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Bioorganic & Medicinal Chemistry 12 (2004) 2973–2983
Bioorganic & Medicinal Chemistry

Saccharides as efficacious solubilisers for highly lipophilic compounds in aqueous media

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Received 29 July 2003; accepted 16 March 2004
Available online 22 April 2004

Abstract—The bioavailability of lipophilic substrates is critical for biotransformations with isolated enzymes as well as with whole cells. With the example of a series of lipophilic ketones the suitability of saccharides as potent solubilisers for highly lipophilic substrates was demonstrated. Best results were obtained for D-glucose, which increased substrate solubility up to 50 times. In whole-cell biocatalysis the sugar acts both as solubiliser and as carbon source for which reason this procedure does not impair cell physiology and is unique in being environmentally benign. The capability of saccharides to solubilise lipophilic compounds in aqueous media sources from their ability to form hydrophilic and lipophilic domains at hydrophobic interfaces, thus forming cyclodextrin-like structures around the lipophilic substrate.

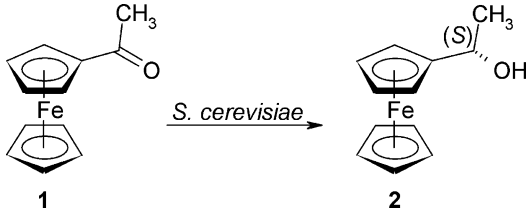
1. Introduction

The importance of stereoisomerically pure alcohols in chiral drug synthesis has led to the development of a series of sophisticated asymmetric synthetic methodologies, and of these stereoselective microbial biotransformations of ketones have emerged as one of the most powerful tools.[1–5] However, most compounds of interest are lipophilic, and as biocatalytic protocols mostly require reductive biotransformations be done in aqueous media, bioavailability of a ketone substrate is of major importance for the outcome of a bioprocess.

The problem of solubilising lipophilic substrates in aqueous media is as old as the history of biotransformations for which reason a multitude of solubilisation techniques has been introduced, such as addition of solubilisers,[6–8] emulsifiers,[9] co-solvents,[10] biphasic reaction media[11,12] or pseudo-crystalline fermentation.[13] Nevertheless, each technique will to some extent affect cell integrity, mostly due to interaction with the cell membrane of a micro-organism.[14–16] These detrimental effects become especially prominent with highly lipophilic substrates, where substrate solubility in water is extremely poor and large amounts of a solubilising agent need to be added to the cell culture.

One example perfectly outlining these intricacies is acetyl ferrocene (**1**), a precursor of chiral ferrocenyl diphosphin ligands,[5,17] which exhibit extraordinary properties in a series of applications.[18–20] Their synthesis is typically realised via (*S*)-1-ferrocenyl ethanol (**2**), the enantiopure access to which emerged as the critical and yield-limiting step.[21] In search for a synthetically superior alternative to currently applied lipase catalysis, the whole-cell biotransformation of commercially available acetyl ferrocene (**1**) with *Saccharomyces cerevisiae* was investigated (Scheme 1).

Yet, the whole-cell approach inherently suffers from the extreme low bioavailability of acetyl ferrocene (**1**), as in water solubility of the solid does not exceed 8 mg/L at 30 °C. When attempts were undertaken to solubilise the substrate by adding Tween 80® (3%), as reported for the trifluoroacetyl ferrocene analogue,[22] solubility increased to 258 mg/L at 30 °C. On the other hand this solubiliser severely affects cell wall integrity as a result of which



Scheme 1. Microbial reduction of acetyl ferrocene (**1**) by *S. cerevisiae*.

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doi:10.1016/j.bmc.2004.03.041

there is uncontrolled influx of cytotoxic **1** into the cell. The consequence was complete loss of cell viability and a meagre enantioselectivity (<20% ee), for which reason a sophisticated, cell-physiologically benign solubilisation technology for the highly lipophilic substrate needed to be developed that does not impair cell viability.

2. Results and discussion

A solution of 20% (v/v) ethanol in water, which previously also had been described as good solubiliser for trifluoroacetyl ferrocene[23] proved cytotoxic already in the absence of **1**. Moreover ethanol concentrations above 5% (v/v) impaired cell viability significantly as a consequence of which conversion dropped below 5%.

With dimethyl sulfoxide (DMSO) no significant improvement was achieved. The reaction mixture was inhomogeneous with concentrations of DMSO $\leqslant$30% (v/v) (Table 1). However, while solubilisation of **1** increased with augmenting DMSO fractions, substrate toxicity became progressively more effective until there was no substrate conversion detectable with 30% (v/v) DMSO, where the ternary mixture of water, DMSO and **1** became homogeneous. In substrate-free control experiments 30% (v/v) DMSO did not affect cell viability. Alternatively, the addition of differently concentrated solutions of **1** in DMSO was tested. In all cases substrate conversion was less than 10%.

Also biphasic biotransformations with the organic solvents ethyl acetate, toluene and isopropyl palmitate were unsuccessful. Heterogeneous biotransformations with solid substrate failed due to its low bioavailability.

When instead of co-solvents cyclodextrins (CD) were tested for solubilisation of **1**, with 110 mg/L γ-CD brought about the so far best substrate solubility—without cell impairment (Table 1).

Hence, there was a considerable solubilising effect by γ-CD, and there were no toxic effects on the yeast cells. However, CD-concentrations above 1% (w/v) were not applicable due to precipitate formation upon addition of the substrate. This effect was observed also for other substrates. It is owed to the fact that CD-complex solubility does not exceed that of pure CD alone, for which reasons attempts had been undertaken to improve complex solubilities by polar modification of the CD-torus.[24,25] Therefore and with regard to the non-optimal solubilising power of γ-CD we searched for a superior alternative.

Table 1. Solubility of acetyl ferrocene (**1**) in the presence of solubilisers

Entry	Solubiliser	Solubility in water at 30 °C (mg/L)
1	None	8
2	Tween 80® 3% (v/v)	258
3	Ethanol 5% (v/v)	28
4	Ethanol 10% (v/v)[a]	76
5	Ethanol 20% (v/v)[a]	95
6	DMSO 10% (v/v)[b]	8
7	DMSO 30% (v/v)[a]	588
8	α-Cyclodextrin 1% (w/v)[c]	8
9	β-Cyclodextrin 1% (w/v)[c]	8
10	γ-Cyclodextrin 1% (w/v)[c]	110

[a] Heterogeneous.
[b] Cytotoxic.
[c] Precipitate formation with $c(\mathrm{CD}) > 1\%$ (w/v).

Cyclodextrins are toroidally shaped cyclic saccharides characterised by strong intramolecular proton bridges, what renders the outside of the torus highly polar and the inside a lipophilic cavity. But owed to the strong polar interactions in the CD molecule, the cyclic sugar possesses a rather rigid structure and a strong solvent cage exists around the molecule and its inclusion compounds.[26] For this reason, uptake as well as liberation of the highly lipophilic species **1** from CD inclusion compounds is kinetically disfavoured. Hence, a structure with superior solubilisation properties to a high degree combines the desired bipolar structure of the cyclic octasaccharide with good substrate liberation properties. In other words, the inclusion compound is required to be less rigid.

It is a common motive in molecular recognition that saccharides form hydrophilic and lipophilic domains as a result of hydrophobic interactions.[27–29] The idea was to take advantage of this circumstance by adding solid acetyl ferrocene (**1**) to aqueous solutions of disaccharides. We assumed that hydrophobic interactions of a sugar with the substrate induce the formation of hydrophilic and hydrophobic domains, what should finally result in the formation of a water-soluble complex. Initially, disaccharides were tested for whether CD inclusion compound-like structures are formed with **1**, which are flexible enough to exhibit satisfactory substrate solubilisation and liberalisation properties in aqueous media. In fact there was a substantial increase in substrate solubility at 30 °C in water accompanied by good substrate liberation properties, and without any cytotoxic effects on the cells. Table 2 depicts the solubilising action of three disaccharides for **1**.

The observed effect is initiated by hydrophobic interactions of a disaccharide molecule with the substrate at the hydrophobic interface between acetyl ferrocene (**1**) and the aqueous phase, triggering the disaccharide to build

Table 2. Solubility of acetyl ferrocene (**1**) in disaccharide solutions

Entry	Solubiliser	Solubility in water at 30 °C (mg/L)
1	None	8
2	D-Maltose 5% (w/v)	76
3	D-Maltose 10% (w/v)	103
4	D-Maltose 15% (w/v)	78
5	D-Sucrose 5% (w/v)	123
6	D-Sucrose 10% (w/v)	172
7	D-Sucrose 15% (w/v)	108
8	D-Lactose 5% (w/v)	63
9	D-Lactose 10% (w/v)	73
10	D-Lactose 15% (w/v)	58

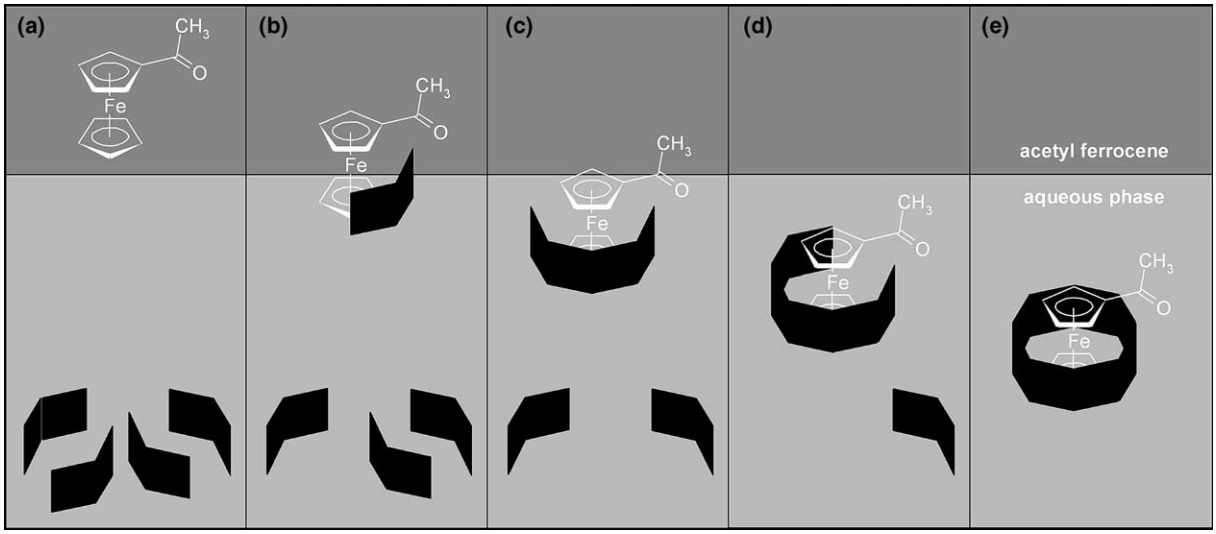


Figure 1. Complex formation between acetyl ferrocene (**1**) and D-maltose induced by hydrophobic interactions.

intramolecular hydrogen bonds, which divide the sugar into a hydrophilic and a hydrophobic domain (Fig. 1b). Consecutively, three more disaccharides complete the growing complex (Fig. 1c–e).

Induced by supramolecular interactions the system disaccharide/acetyl ferrocene is self-organising, with **1** acting as a template around which four disaccharide molecules arrange. The toroidal structure is stabilised by substrate-induced hydrophobic interactions and hydrogen bonds. Interconnection of the four disaccharides by hydrogen bonding as well as the formation of intramolecular proton bridges render the outer sphere of the complex hydrophilic and the inner sphere hydrophobic. Figure 2 compares the structures of the γ-CD inclusion compound **3** with complex **4** formed from D-maltose and **1**. The only marked difference between these two species is four covalent bonds missing in **4** rendering this structure sufficiently flexible to display good substrate uptake and liberation properties. For these reasons D-maltose appeared as an ideal candidate for solubilising highly lipophilic substrates in aqueous media.

The solubilising effect of the disaccharide is also effective with other lipophilic substrates. As can be derived from Table 2, 10% (w/v) solutions of a sugar exhibit the best solubilisation properties. As was nicely demonstrated with the example of phenolphthalein (Fig. 4), for the formation of structures of type **4** a critical saccharide concentration is required [∼10% (w/v)], as non-complex forming sugar molecules stabilise this structure. Below this value, simply too little sugar is present to realise full solubilisation power. Above, complexing abilities are less pronounced due to mutual interactions by the sugar molecules. For these reasons it is advantageous to continuously replace the sugar consumed by the whole-cell biocatalyst.

In complementary experiments with monosaccharides D-glucose emerged as the even most powerful solubiliser. Obviously, solubilisation power is rather determined by flexibility and mobility of the sugar, which is to form complexes with a lipophilic substrate than the γ-CD-like structural preorganisation of a disaccharide.

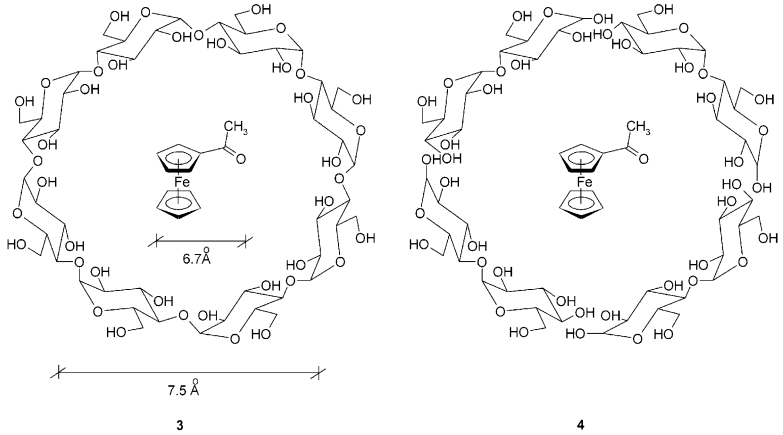
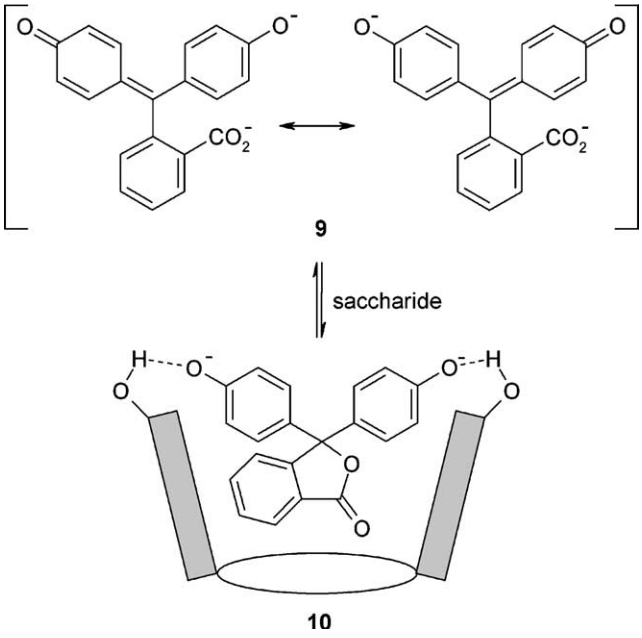


Figure 2. γ-CD inclusion complex (**3**) of acetyl ferrocene (**1**) and D-maltose complex (**4**) with **1**.

Aside from steric requirements and host flexibility, the hydrophobic effect represents the most important factor governing host–guest interactions in the saccharide series. Concomitantly, the release of water from the solvent cage of the sugar and from the hydrophobic hydration sphere of the lipophilic substrate to be incorporated into the bulk aqueous phase must be considered as the main entropic factor favouring complex formation.[30–32] However, this favourable contribution to entropy is usually over-compensated by the loss of degrees of freedom from combining 5 to 9 independent molecules into one complex. Hence, the complexation appears as an enthalpically driven process. The sum of all these factors is reflected in the increased bioavailabilities of lipophilic substrates in 10% (w/v) D-glucose. The greatest solubilising effect was obtained for **1**, where solubility increased by a factor of 48 (Table 3).

From whole-cell experiments there was no indication for decreased conversion rates caused by a sugar, what is an expression of the good substrate liberation properties of complexes with open-chained saccharides, due to which substrate mobility is generally not reduced upon complexation. Moreover it was evident that for 10% (w/v) saccharide solutions no detrimental effects were observed.



Scheme 2. Formation of the lactonoid *leuko*-form **10** upon complexation.

2.1. Phenolphthalein complexes

Further evidence for saccharides forming a lipophilic environment around hydrophobic compounds was provided by solubilisation studies with phenolphthalein (**9**) according to the method by Vikmon.[33] The phenomenon of complexation with sugars involves changes in photophysical and photochemical properties of the guest molecules caused by the environmental differences between the interior of the saccharide complex and the aqueous medium.[34,35] As a consequence the spectra of chromogenic guest molecules are usually perturbed in the presence of saccharides (vide infra). The triphenyl methane indicator dye phenolphthalein changes from colourless to purple in alkaline media. However, upon complexation by cyclodextrins the colour instantly disappears, even at pH 10, what is caused by formation of the lactonoid *leuko*-form **10**, which is less polar and therefore more stable in a lipophilic environment (Scheme 2).[36]

When this method was applied to photometrically compare complexation abilities of several sugars, for all saccharide solutions a decrease of colour intensity was observed, which was maximal with β-CD. Of the open-chained saccharides D-glucose exhibited the strongest effect, what is in line with the findings for the example compounds **1** and ethyl 2-oxocyclopentanoate (**5**) (Fig. 3).

The absorbance decrease shows that complexation with open-chained sugars, whereupon **9** undergoes the chemical transformation to lactone **10**, does not merely provide a lipophilic environment so as to derive the carboxylate group of **9** into the lactonoid ring. Instead, the intrinsic binding energy is primarily responsible for the distortion of a trigonal sp^2-conjugated **9** towards the formation of a tetrahedral sp^3 central carbon, even though the two phenolic moieties remain ionised at pH 10. These results essentially reflect the situation reported for **9** with CD.[37] They further outline the enthalpy driven character of complex formation with saccharides (vide supra) the energy gain of which is reinvested into structural reorganisation of the guest molecule towards a more lipophilic species, what results in increased stabilising hydrophobic host–guest interactions. The findings also serve well to explain the spectroscopic behaviour of the hydrophobic substrates

Table 3. Solubility of lipophilic substrates in 10% (w/v) D-glucose solutions compared with that of γ-CD

Entry	Substrate	Solubility in water at 30 °C (mg/L)	Solubility in 10% (w/v) D-glucose at 30 °C (mg/L)	Solubility with 1% γ-CD at 30 °C (mg/L)[a]
1	Acetyl ferrocene (**1**)	8	383	110
2	Ethyl 2-oxocyclopentanoate (**5**)	2700	27,500	7000
3	Ethyl 2-oxocyclohexanoate (**6**)	1300	2700	600[b]
4	2,2,2-Trifluoroacetophenone (**7**)	12,200	14,700	18,000
5	Ethyl 2-chloroacetoacetate (**8**)	8900	11,200	8900

[a] γ-CD precipitates in concentrations >1% (w/v).
[b] Precipitate formation.

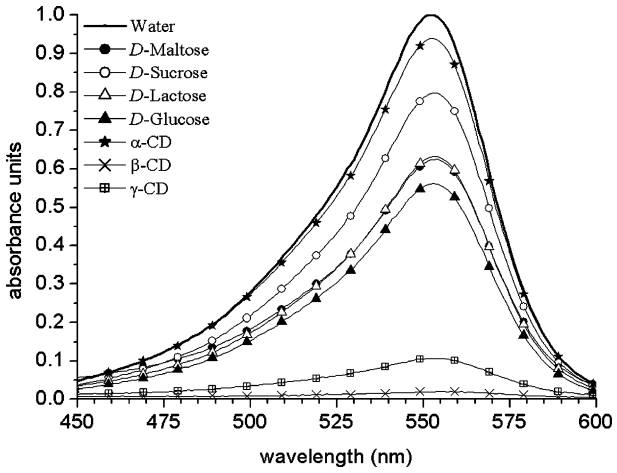


Figure 3. Decrease in colour intensity upon complexation of 0.03 mM phenolphthalein (**9**) by saccharides at pH 10.

upon complexation by mono- and disaccharides, since ^{1}H NMR, fluorescence and UV-absorption experiments revealed considerable similarities to the respective CD inclusion complexes.

Phenolphthalein complexes further were ideal models to study complex stabilities between the dye and β-CD on one and D-glucose on the other hand. In solution, inclusion complexes are entities which, obeying the law of mass action, form an equilibrium with their constituents. Primarily, their existence is consequence of full inclusion of the guest molecule into the host compound; that is, the spatial arrangement is decisive for complex formation.[38]

We investigated the equilibrium of phenolphthalein **9** with its *leuko*-form **10** in β-CD and D-glucose solution with the purpose of proving that host (saccharide) and guest (dye) form an equilibrium, which obeys the law of mass action. According to the method of Broser and Lautsch[38] the complex stability constants were photometrically determined by variation of saccharide (complex forming agent) concentration. The S-shaped curve obtained from plotting the measured extinction values versus $\log c_{\text{Saccharide}}$ experimentally confirms the existence of a true inclusion compound (Fig. 4).

From the curves the K_F values were calculated as 7.1×10^{-5} mol/L for β-CD, and as 6.0×10^{-1} mol$^{3/2}$/L$^{3/2}$ for D-glucose. Hence, one β-CD molecule forms a complex with one molecule phenolphthalein. On the other hand, from the odd-numbered exponent the multifaceted nature of the D-glucose complex is evident. As will be shown by ^{1}H NMR experiments (vide infra), only a fraction of the D-glucose molecules is directly involved in complex formation. Obviously there is a secondary stabilising effect exerted by other D-glucose molecules, what finds further expression in the experimentally observed maximum solubility of lipophilic compounds in 10% (w/v) saccharide solution.

2.2. ^{1}H NMR spectroscopy

Proton NMR studies were done with ethyl 2-oxocyclopentanoate (**5**) instead of **1**, since the cyclic β-keto ester is sufficiently soluble in water to allow the detection of solubilisation effects.

Upon complexation by D-glucose and D-maltose, respectively, ^{1}H NMR signals of pure **5** in D_2O show significant downfield shifts ranging between 4 and 14 Hz, what is in good agreement with the CD-inclusion complexes of **5** (4–15 Hz). Typically ^{1}H NMR resonance shifts of guest molecule signals range between 2 and 16 Hz.[39] In addition, upon complexation a prominent change occurs at the anomeric carbon of D-maltose. In aqueous solution the sugar is made up of 5.9% α-anomer and 94.1% β-anomer. In the presence of **5**, as a result of substrate complexation 28.2% of D-maltose consist of the α-anomer, what is exactly the configuration of glycosidic bonds in cyclodextrins. This effect was not observed with D-glucose, what is reasonable, as monosaccharide complexes are much more flexible than the

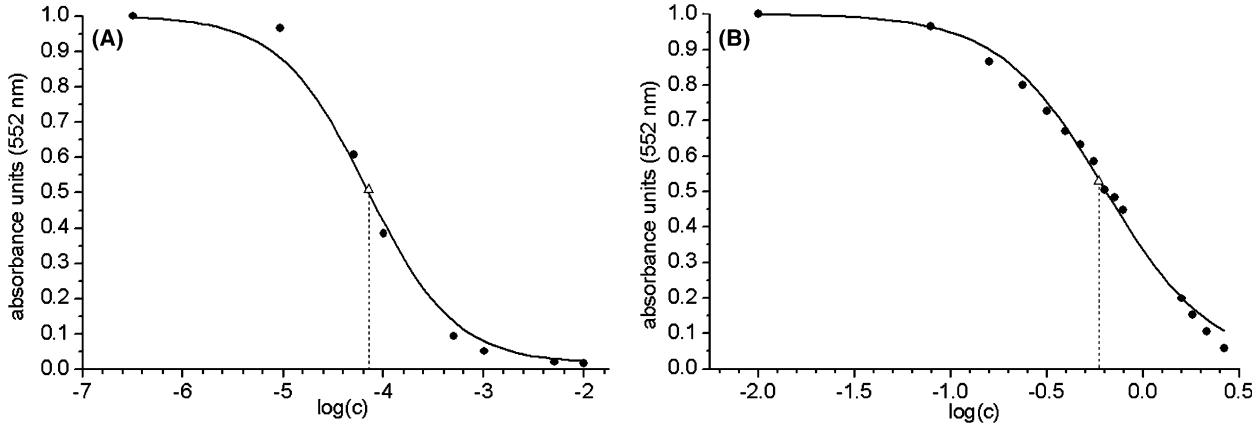


Figure 4. Complex formation constant is strongly dependent on the concentration of the complex forming saccharide (A: β-CD, B: D-glucose). The S-shape of the curves provide clear evidence that phenolphthalein forms true complexes with both β-CD and D-glucose. The white triangle (△) denotes $\log K_F$. Note that at $\log(c) = 0.43$ maximum solubility of D-glucose in water is reached.

disaccharide analogues and therefore much more subject to dynamic exchange of complex forming D-glucose molecules.

The observed resonance shifts of the signals reflect the extent of structural reorganisation of a sugar upon complex formation with the lipophilic β-keto ester. Figure 5 compares the effects of different sugars on ^{1}H NMR resonance of **5**. Generally, the resonance shifts of the mono- and disaccharide complexes are in the same order of magnitude as reported for CD inclusion complexes of lipophilic compounds.[40]

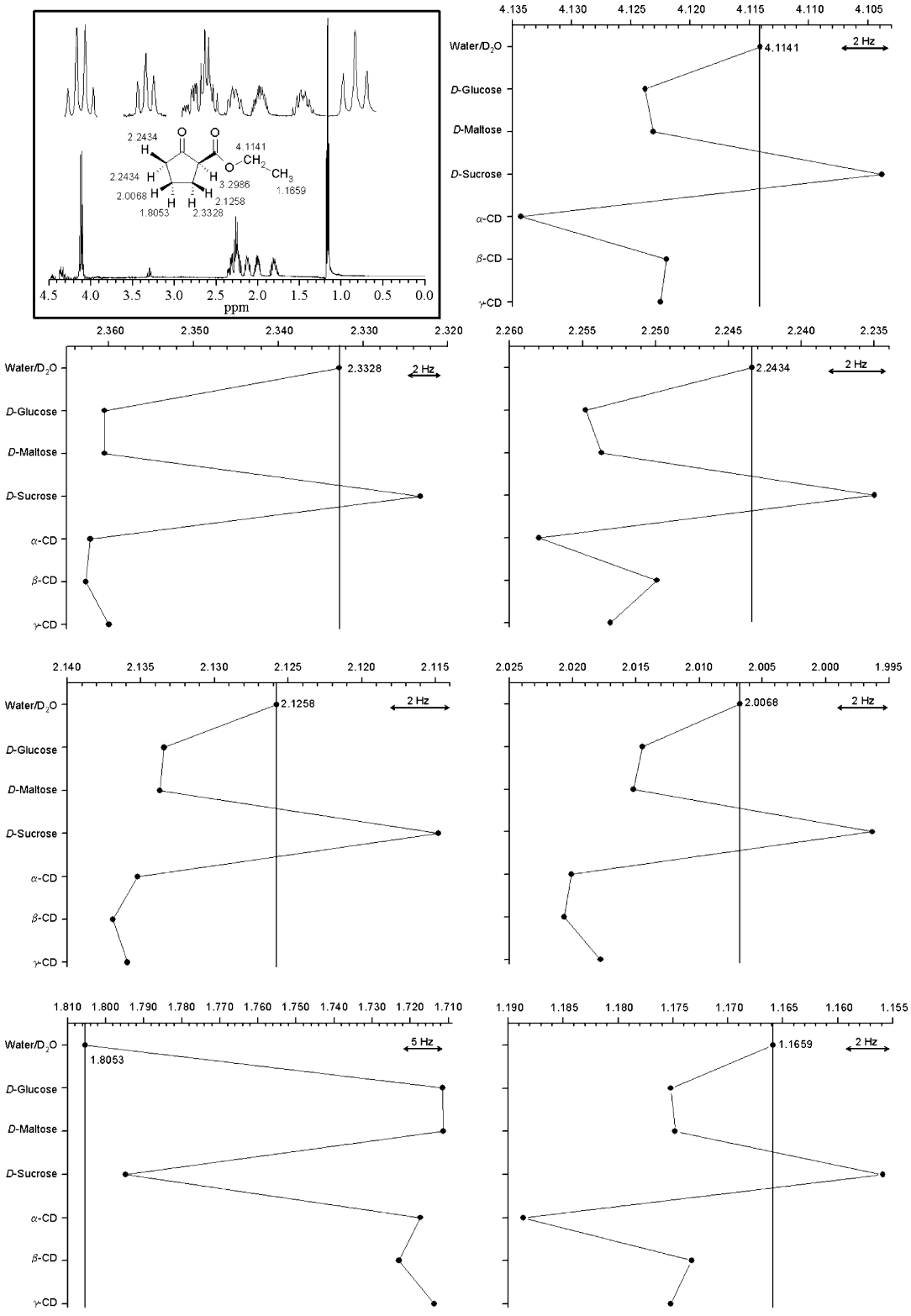


Figure 5. ^{1}H NMR resonance shifts of **5** observed upon complexation by different sugars.

Expectedly, compared to CD the signal shifts are less pronounced with open-chained saccharides, since these systems are less rigid and the substrate possesses more degrees of freedom. Phenomenologically this is expressed in the superior substrate solubilisation and liberation properties observed for the more flexible saccharide complexes.

With D-sucrose ^{1}H NMR signals are shifted up-field, what is in sharp contrast to all other saccharides tested. This remarkable phenomenon is ascribed to the structure of D-sucrose complexes, which due to the D-fructose moiety cannot adopt the optimal γ-CD-like structure and therefore exhibit differing ^{1}H NMR spectroscopic behaviour.

2.3. Fluorescence–emission spectroscopy

The incorporation of a guest molecule into a host compound is accompanied by fluorescence enhancement caused by a decrease in rotational freedom and elimination of water molecules surrounding the fluorescent guest molecule.[41] On this basis, fluorescence enhancement is a function of substrate accommodation in the cavity.

After excitation at 270 nm fluorescence enhancement of acetyl ferrocene inclusion complexes at $\lambda_{max} = 330$ nm was most prominent with the α- and β-CD inclusion complexes, where for steric reasons **1** is buried only partially in the cavity.[42] Due to the resulting strong interaction emission increased by factor 20 and 19, respectively. As open-chained sugar complexes are flexible systems and γ-CD possesses the largest cavity, rotational freedom of the guest molecules was not affected substantially, and substrate fluorescence increased merely by a factor of 1.25.

Even more pronounced effects were observed with the host D-maltose. On the basis that the strong emitting disaccharide ($\lambda_{max} = 342$ nm) shows significant fluorescence, which is largely quenched upon complex formation with a lipophilic substrate, D-maltose provides strong fluorescence-spectroscopic evidence for substrate complexation. Fluorescence quenching is due to structural reorganisation of the sugar molecules in order to adopt a torus-like geometry, which possesses different photochemical properties. Here, fluorescence decreased by 32% when **1** was added and 81%, respectively, when **5** was added (Fig. 6). Fluorescence quenching was generally observed, when complexes with lipophilic substrates were formed.

2.4. UV-absorption spectroscopy

On the basis that in CDs electron density is increased inside the cavity, for the O-atoms of the glycosidic bonds are directed inwards, inclusion compounds were tested for whether they experience a red-shift in the absorption maximum ($\Delta\lambda$) and an augmentation of the molar absorption coefficient ($\Delta\varepsilon(\lambda)$).[36,43] Although complex formation is not usually accompanied by major changes in the UV–VIS spectra of the guest,[44] this effect was evident in all cases (Table 4).

The very good quality of complexation properties displayed by open-chained saccharides can be conceived from comparison with the changes reported for CD complexes of comparable substrates with which the observed values are in good agreement.[45–47]

A striking observation was made for D-maltose complexes, which also show red-shifts, but which exhibited absorbance decreases instead of enhancements. This unexpected finding appears reasonable taking into account UV-spectroscopic properties of the sugar. Pure D-maltose displays strong absorption in water ($\lambda_{max} = 197$ nm, $\varepsilon_{max}(197) = 10.14 \times 10^5$ L/mol cm). However, when upon complex formation with a lipophilic substrate the disaccharide molecules are structurally reorganising in order to adopt a torus-like geometry, UV-absorption of the sugar species drops to a

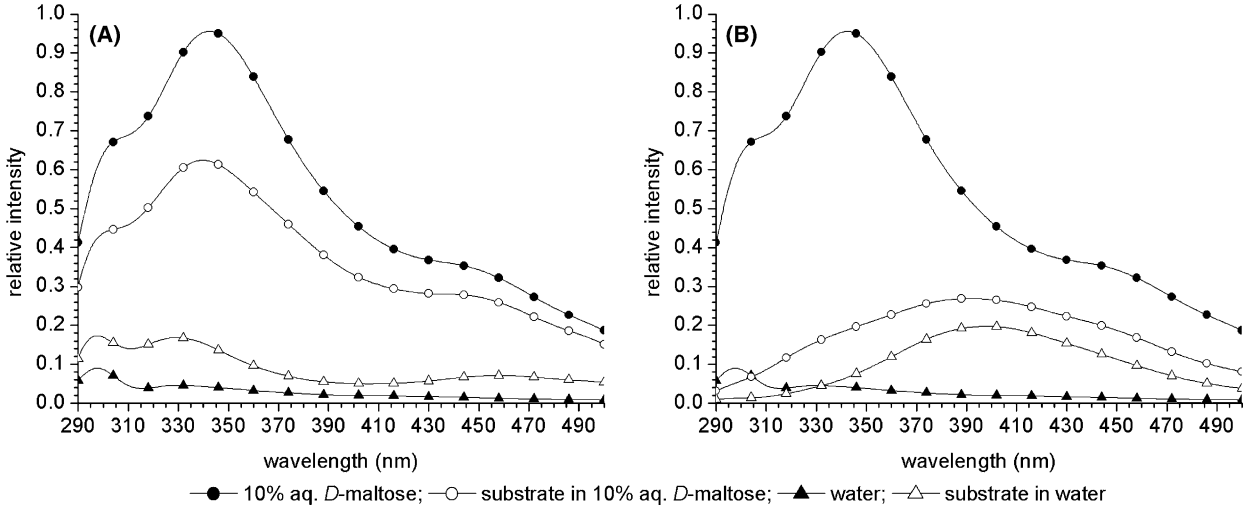


Figure 6. Fluorescence quenching of the host D-maltose upon complex formation with 10 μg/mL **1** after excitation at 270 nm (A) and 10 mg/mL **5** after excitation at 301 nm (B).

Table 4. Solubilities in water at 30 °C, red-shifts and absorbance enhancements upon aggregation

Entry	Substrate	Complexant	λ_0[a] (nm)	λ_1[b] (nm)	$\varepsilon(\lambda_0)$[c] (L/mol cm)	$\varepsilon(\lambda_1)$[d] (L/mol cm)
1	Acetyl ferrocene (**1**)	α-CD	271	295	8.44×10^5	1.14×10^6
2		β-CD	271	292	8.44×10^5	1.32×10^6
3		γ-CD	271	293	8.44×10^5	1.21×10^6
4		D-Glucose	271	292	8.44×10^5	9.12×10^5
5		D-Maltose	271	295	8.44×10^5	n.d.[e]
6		D-Sucrose	271	294	8.44×10^5	1.07×10^6
7		D-Lactose	271	293	8.44×10^5	1.37×10^6
8	Ethyl 2-oxocyclopentanoate (**5**)	α-CD	285	285	4.34×10^3	4.34×10^3
9		β-CD	285	285	4.34×10^3	4.34×10^3
10		γ-CD	285	285	4.34×10^3	4.34×10^3
11		D-Glucose	285	285	4.34×10^3	2.90×10^3
12		D-Maltose	285	285	4.34×10^3	n.d.[e]
13		D-Sucrose	285	285	4.34×10^3	4.70×10^3
14		D-Lactose	285	285	4.34×10^3	4.34×10^3
15	Ethyl 2-oxocyclohexanoate (**6**)	α-CD	258	258	1.40×10^4	2.54×10^4
16		β-CD	258	260	1.40×10^4	4.70×10^4
17		γ-CD	258	260	1.40×10^4	2.29×10^4
18		D-Glucose	258	258	1.40×10^4	1.78×10^4
19		D-Maltose	258	260	1.40×10^4	n.d.[e]
20		D-Sucrose	258	259	1.40×10^4	1.65×10^4
21		D-Lactose	258	258	1.40×10^4	1.65×10^4
22	Trifluoroacetophenone (**7**)	α-CD	205	206	3.21×10^5	4.25×10^5
23		β-CD	205	207	3.21×10^5	6.04×10^5
24		γ-CD	205	206	3.21×10^5	4.84×10^5
25		D-Glucose	205	207	3.21×10^5	4.95×10^5
26		D-Maltose	205	209	3.21×10^5	n.d.[e]
27		D-Sucrose	205	207	3.21×10^5	6.26×10^5
28		D-Lactose	205	206	3.21×10^5	4.90×10^5
29	Ethyl 2-chloroacetoacetate (**8**)	α-CD	263	266	1.06×10^4	3.07×10^4
30		β-CD	263	265	1.06×10^4	1.76×10^4
31		γ-CD	263	264	1.06×10^4	1.28×10^4
32		D-Glucose	263	264	1.06×10^4	1.23×10^4
33		D-Maltose	263	264	1.06×10^4	n.d.[e]
34		D-Sucrose	263	263	1.06×10^4	1.14×10^4
35		D-Lactose	263	263	1.06×10^4	1.14×10^4

[a] λ_{max} in water.
[b] λ_{max} in saccharide complex.
[c] Molar absorption coefficient $\varepsilon(\lambda)$ in water.
[d] Molar absorption coefficient $\varepsilon(\lambda)$ in saccharide complex.
[e] Not determined (see text).

minimum value. As UV-absorption spectra were recorded against a solution of the respective sugar in the reference beam, phenomenologically there is less absorption measured for the sample than for the pure substrate. Therefore, UV-absorbance decrease of a sugar is another strong evidence for its complex forming activity.

An exception was found for the strong absorber acetyl ferrocene (**1**). With increasing wavelength D-maltose molar extinction coefficient $\varepsilon(\lambda)$ asymptotically strives towards a minimum. At 295 nm molar extinction of acetyl ferrocene (**1**) exceeds that of D-maltose ($\varepsilon(295) = 7.56\times10^3$ L/mol cm) by two orders of magnitude (Table 4).

Hence, for whole-cell biotransformations D-glucose concurrently serves both as cheap carbon source and as efficacious solubiliser for highly lipophilic substrates, what is environmentally benign and economically attractive. Of further advantage is that the use of sugars instead of conventional solubilisers like Tween® or Triton® does not impair living cells.

Finally, experiments with yeast alcohol dehydrogenase (EC 1.1.1.1), pig liver esterase (EC 3.1.1.1) and *Candida antarctica* lipase A (EC 3.1.1.3) demonstrated that disaccharides are finely suited as solubilisers for biotransformations of highly lipophilic substrates with isolated enzymes, too. After long-term incubation ($\geqslant$15 h) no decrease of enzyme activity was detected. Moreover, with up to 9% rate enhancement substrate bioconversion proceeded even somewhat faster, what again reflects the good substrate bioavailability mediated by open-chained saccharide complexes. And, in contrast to conventional amphiphilic solubilisers or organic solvents there is no risk of denaturing the biocatalyst.

3. Conclusion

We have demonstrated that saccharides are powerful solubilising agents for lipophilic substrates. Induced by hydrophobic interactions with the substrate they form cyclodextrin-like structures, which exhibit good sub-

strate solubilisation and liberation properties. As saccharides exhibit no detrimental effects on both whole-cells and isolated enzymes, they are a cheap and environmentally benign alternative to common solubilisers such as Tween® or Triton®, which markedly impair cell viability. The benefit is obvious: higher substrate concentrations can be realised without impairment of the microbial biocatalyst.

Current studies aim to replace the use of pure substrates by the addition of their saccharide complexes to cell suspensions, with the purpose to increase effective substrate concentrations, as substrate bioavailability recently was shown to markedly contribute to the outcome of stereoselective biotransformations.[49]

4. Experimental

Substrates were purchased from Fluka and used as supplied. Organic solvents were purchased from Acros. Cyclodextrins were provided by Wacker GmbH, Burghausen, Germany, and enzymes were supplied by JFC-Jülich Fine Chemicals GmbH, Jülich, Germany. Yeast was the product of Societé de levure FALA S.A., Strasbourg, France.

Absorption and fluorescence experiments were conducted on an Amersham Pharmacia Biotech Ultrospec 2100pro UV–VIS spectrophotometer and on a Hitachi F-4500 spectrofluorimeter, respectively. ^{1}H NMR spectra were recorded in D_2O with a Bruker DRX-500 spectrometer at 500 MHz. Chemical shifts are quoted in ppm from internal TSP. HPLC analyses were carried out isocratically with $H_2O/CH_3CN = 20{:}80$ on a Knauer HPLC system (LiChrospher 100 RP-18/5 μm) equipped with a Wellchrom diode array detector K-2800.

4.1. Solubility studies

According to the Higuchi and Connors method,[48] a 5 mM solution of substrate in *tert*-butyl methyl ether (0.5 mL) was added to 1 mL of a solution of saccharide (5%, 10% or 15% w/v) or the CD (1% w/v), respectively, in water at 30 °C, and the solutions were allowed to react in a rotary shaker (300 rpm) at 30 °C in an Eppendorf tube. After reaching equilibrium (30 min), volatile components were removed in the N_2-stream, and the sample was submitted to centrifugation in order to ensure that the sample was free of suspended, emulsified or colloidal dissolved material. The concentration of substrate in the aqueous phase was determined spectrophotometrically at 310 nm with reference to a suitably constructed standard curve.

4.2. ^{1}H NMR experiments

To a solution of the saccharide (10% w/v) or the CD (1% w/v), respectively, in D_2O (0.7 mL) substrate was added according to maximum substrate solubility as determined above. For purposes of calculating resonance shifts induced by complexation and to provide reference spectra, the spectra of pure substrate and pure sugar were obtained using the same conditions. To determine torus-shaped complex formation the characteristic signals of anomeric protons were used where possible.

4.3. Fluorescence–emission experiments

The samples prepared as described above were measured in a 0.5 cm cuvette. Spectra were recorded after excitation at 270 nm for **1**, 301 nm for **5** and 245 nm for **7**, respectively, after optimal excitation frequency had been determined by 3D fluorescence spectroscopy. Due to its low solubility in water **6** displayed too weak fluorescence, **8** is fluorescence inactive in water. To provide reference spectra, the spectra of pure substrates and pure sugars were obtained using the same conditions. Where complexation effects of D-maltose were determined, due to strong self-fluorescence the emission spectrum of pure D-maltose was subtracted from the sample in order to unquestionably differentiate between host fluorescence and complexation effects.

4.4. UV-absorption experiments

The samples prepared as described above were measured in a 1 cm cuvette. To provide reference spectra, the spectra of pure substrates and pure sugars were obtained using the same conditions. Where complexation effects were determined, due to strong absorbance by sugars the UV-absorption spectrum of pure sugar was subtracted from the sample in order to unquestionably differentiate between sugar absorbance and complexation effects.

4.5. Determination of complex formation constants K_F

Complexing agent concentration dependent stability constants K_F were determined according to the method by Broser and Lautsch[38] with phenolphthalein as substrate, and with β-CD and D-glucose as complexing agents.

4.6. Whole-cell biotransformations

Bio-reductions were performed in a 15 L Infors HT ISF200 fermenter according to Ref. 49.

4.7. Biotransformations with isolated enzymes

In order to demonstrate the suitability of saccharides as solubilisers for biotransformations with isolated enzymes, yeast alcohol dehydrogenase (EC 1.1.1.1), pig liver esterase (EC 3.1.1.1), and *C. antarctica* lipase A (EC 3.1.1.3) were added to a solution of 10% (w/v) saccharide (5 g) in 0.5 M potassium phosphate buffer

(50 mL) at 30 °C and pH 6.6. D-Maltose, D-sucrose, D-lactose and D-glucose were tested for these purposes. After incubation at 30 °C for 15 h, enzyme activities were determined. Yeast alcohol dehydrogenase activity was determined in accordance with Ref. 50. Pig liver esterase, and *C. antarctica* lipase A activities were assayed according to Ref. 51 with **5** as substrate at $\lambda_{max} = 287$ nm.

Acknowledgements

We gratefully acknowledge Prof. Dr. K.-H. van Pée for continuous discussions, encouragement and support, as well as financial support by the Graduate Research Centre 'Heterocycles' and the German National Merit Foundation. Cyclodextrins were the generous gift of Wacker Chemie GmbH, Burghausen, Germany, and enzymes were kindly provided by JFC—Jülich Fine Chemicals GmbH, Jülich, Germany. Yeast was kindly provided by FALA Hefe GmbH, Kesselsdorf, Germany. We thank Dr. M. Gruner and A. Rudolph for recording NMR spectra.

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