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Bioorganic & Medicinal Chemistry 14 (2006) 1126–1133
Bioorganic & Medicinal Chemistry

Improving the membrane permeability of sialic acid derivatives

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Received 4 August 2005; revised 9 September 2005; accepted 13 September 2005
Available online 7 October 2005

Abstract—The potential of boronic acids to improve the bioavailability of carbohydrate derived drugs was investigated through the study of the transport of four sialic acid derivatives through a lipophilic supported liquid membrane at departure phase pH's of 7.4, 8.5 and 10.0. It was found that facilitated transport did occur in most cases, but interestingly, and in contrast to that observed with monosaccharides such as D-fructose, the lipophilic ammonium salt, Aliquat® 336, promoted higher fluxes than those of the boronic acid. The triol side chain of the sialic acid derivatives, combined with the amide at C5, appears to represent a previously unrecognised chloride binding domain which promotes extraction of these compounds into membranes containing Aliquat® 336, leading to fluxes greater than those produced by boronic acids.

1. Introduction

1.1. Membrane permeability of hydrophilic drugs

Pharmaceuticals derived from carbohydrates are used to treat a variety of diseases including HIV-AIDS and influenza,[1,2] but these drugs are often highly hydrophilic and are frequently associated with poor pharmacokinetic profiles. Classically this problem has been overcome either by the administration of excessive doses of the drug in order to force it through biological membranes via saturation or by the use of a range of 'non-oral' administration techniques. All of these approaches can lead to problems, including increased side effects and poor patient compliance.

In order to pursue oral administration for hydrophilic therapeutics, pharmaceutical additives or alternative formulations have been developed. For example, Hochman et al. achieved increased membrane permeation of hydrophilic drugs by the co-administration of palmitoylcarnitine,[3] which apparently works by altering membrane characteristics. It is believed that the palmitoylcarnitine acts by altering the membrane charge density and/or other bilayer properties such that an increased rate of passage of hydrophilic compounds through the barrier is achieved. An alternative strategy is the pro-drug approach: the drug is temporarily modified to make it more lipid soluble, with the pro-drug reverting to its active form at the site of action. A classical example of this is dipivefrin, the dipivaloyl ester of epinephrine, which is used to treat glaucoma. The active drug, epinephrine, is poorly absorbed through the cornea, but dipivefrin can be administered in the form of eye drops, taking advantage of improved membrane penetration engendered by the presence of the pivaloyl groups, and the esterase activity found in the cornea and aqueous humour.[4]

The purpose of the current study was to investigate the possibility of using boronate esters, rather than carboxylate esters, to introduce lipophilicity, and thus high membrane permeability, into hydrophilic drugs. It was thought that the ease of hydrolysis of boronate esters would mean that the success of such an approach would not require the presence of any specific enzyme activity in the vicinity of the site of action. We have previously undertaken a similar, preliminary study with nucleotides, and found that a carrier mixture of a lipophilic boronic acid and a bis(quaternary ammonium cation) greatly facilitates the passage of ribonucleoside monophosphates through liquid organic membranes.[5]

Keywords: Membrane transport; Boronic acids; Sialic acid; Anti-virals.
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doi:10.1016/j.bmc.2005.09.028

1.2. Membrane transport promoted by boronic acids

Boronic acids are a versatile class of compounds that are employed in a wide range of applications including as protective agents and catalysts in organic synthesis,[6] as bioconjugates,[7,8] in carbohydrate sensor development[9] and in the transport of sugars through artificial membranes.[10] Boronic acids themselves are not considered to be highly toxic, with their usual metabolic fate being thought to involve oxidative de-boronation in the liver, promoted by cytochrome P450 enzymes. The boron-containing by-product of this process is boric acid, which has very low mammalian toxicity. The first boronic acid-containing drug, Bortezomid® (Velcade®), was approved for use in humans by the US FDA in 2003.

Boronic acids reversibly condense with carbohydrates to form boronate esters,[6,9,10] and these esters have a number of properties that make them attractive candidates for improving the oral bioavailability of carbohydrate based drugs. First, the condensation reaction is rapid and reversible and second, the boronate ester thus formed is usually more lipophilic than the original carbohydrate and hence has a significantly different solubility profile. These properties have been especially useful in the facilitated transport of carbohydrates through liquid organic membranes,[10] two accepted mechanisms for which are shown in Figure 1. In both mechanisms, the boronic acid condenses with the carbohydrate at the interface of the departure phase and the membrane to form a boronate ester. The boronate ester then diffuses through the membrane, and when it reaches the interface with the receiving phase, the ester is hydrolysed back to the boronic acid and the original carbohydrate is released into the aqueous receiving phase. In cases where the pH of the departure phase is lower than the pK_a of the boronic acid, trigonal boronate esters are formed and a 'trigonal transport mechanism' is adopted (Fig. 1A).[11] Conversely, when the pH of the departure phase is greater than the pK_a of the boronic acid, tetrahedral boronate esters are formed and the 'tetrahedral transport mechanism' appears to dominate (Fig. 1B).[10]

In a more biologically relevant work, Smith and co-workers have investigated the boronic acid facilitated efflux of sugars, including glucose and sucrose, from liposome cell models.[12] This work parallels the study of boronic acid-promoted sugar transport through artificial liquid membranes described above, with the liquid membrane being replaced by a lipid bilayer. Similar principles appear to govern both transport processes and the boronic acids used in the latter work did not appear to adversely affect the liposomes in any way. These liposome results were thus the first to reliably suggest that boronic acids could be used to promote the passage of hydrophilic drugs through biological membranes.

1.3. Boronate esters of sialic acid derivatives

In the current study, we have applied the aforementioned boronate ester principles to the transport of compounds related to the hydrophilic anti-influenza drug Zanamivir®, which is marketed as Relenza® (**1**), and shown in Figure 2. Again we have used a hydrophobic liquid membrane as a biological membrane model. Largely due to their synthetic accessibility, precursors of Relenza® (**2**–**5**) with variation at C1 (carboxylic acid or methyl ester) and C4 (hydroxyl or azide) were chosen as test compounds for this study. In addition, a refer-

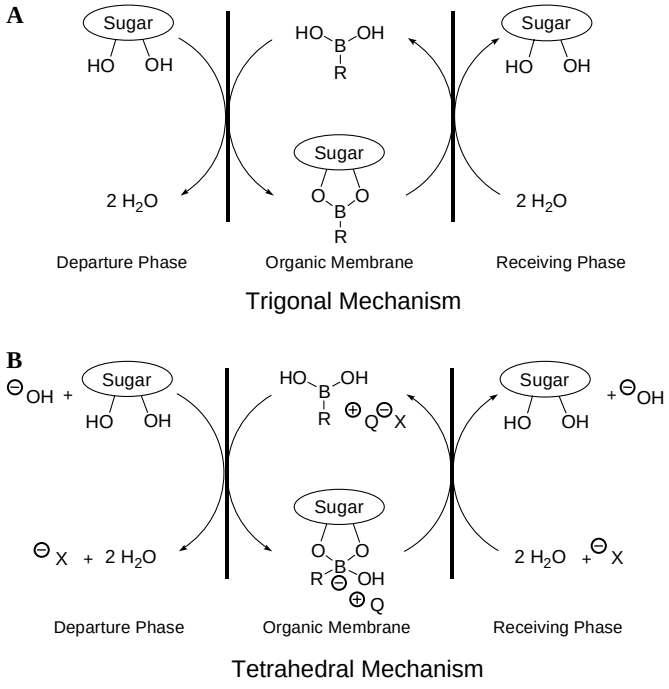
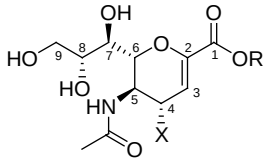


Figure 1. Accepted mechanisms for the transport of carbohydrates through liquid membranes involving trigonal (A) and tetrahedral (B) boronate ester formation.



Relenza, **1**, R = H; X= -NHC(=NH)NH_2
2, R = Me; X = OH
3, R = H; X = OH
4, R = Me; X = N_3
5, R = H; X = N_3

Figure 2. Sialic acid derivatives relevant to the current study.

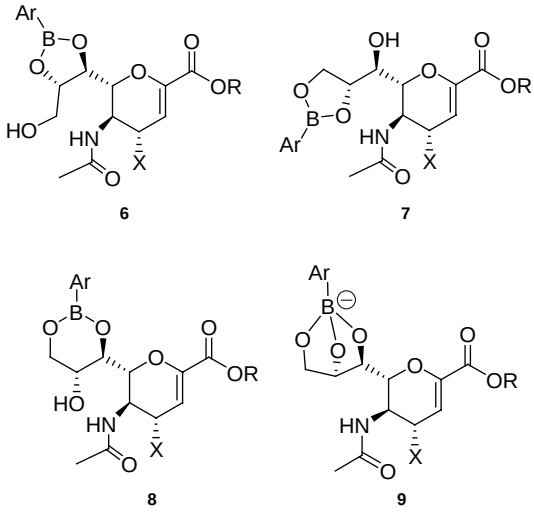


Figure 3. Likely structures of boronate esters formed between an aryl boronic acid and sialic acid derivatives (**2**–**5**).

ence compound, D-fructose, was included in the study since its boronic acid-promoted transport through lipophilic membranes has been extensively studied.[10]

An important feature of the sialic acid derivatives (**2**–**5**) is the triol side chain, the condensation of which with boronic acids to form boronate esters has been used in synthetic studies,[13] and in approaches to sialic acid sensors.[14] In principle, three different trigonal boronate esters (**6**–**8**) can be formed with this triol, as indicated in Figure 3, together with a tetrahedral boronate ester (**9**), which may act as an intermediate in the interconversion of the three possible trigonal esters.[6a] However, it should be noted that Kataoka and co-workers have proposed that the major complex formed between sialic acid and 3-(propionamido)phenylboronic acid at physiological pH is the 7,8-boronate ester shown in Figure 4.[15a] This complex is thought to predominate as a result of a B–N or B–O interaction, as shown. Interestingly, Peters and co-workers have recently concluded that a 8,9-boronate, presumably the tetrahedral equivalent to **7**, is the most abundant sialic acid boronate ester formed with phenylboronic acid at pH > 8.[15b]

In the current study, we have used the unique binding domain for boronic acids possessed by sialic acid derivatives, that is, the 7,8,9-triol region, as a handle with

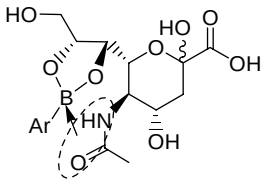


Figure 4. Major complex shown by Kataoka and co-workers[15a] to form between sialic acid and 3-(propionamido)phenylboronic acid (Ar = 3-(propionamido)phenyl).

which a lipophilic boronic acid could carry these hydrophilic carbohydrate derivatives through a lipophilic membrane. Unexpectedly, we have also discovered that the same triol is an excellent chloride receptor and allows membrane penetration by these compounds to occur in the presence of a lipophilic ammonium chloride.

2. Results and discussion

2.1. Supported liquid membrane transport experiments

The membrane support used for this study was a porous polypropylene sheet (Accurel®), which was impregnated with a mixture of *o*-nitrophenyloctyl ether (NPOE), boronic acid (NPOEBA, **10**, Fig. 5) and/or the lipophilic quaternary ammonium salt (Aliquat® 336 which is predominantly trioctylmethyl ammonium chloride), as required.[16–20] This supported liquid membrane (SLM) was clamped between aqueous departure and receiving phases, which consisted of an appropriately buffered carbohydrate solution and a phosphate buffer (pH ~ 7.4), respectively. Two departure-phase pH conditions, similar to those encountered in the human body (8.5 and 7.4, representing the jejunum and plasma, respectively), were employed, as well as a higher pH (10.0), which was more likely to promote boronic acid facilitated transport via the tetrahedral mechanism. The pH of the jejunum in particular was chosen as a focal point for this study as it is believed to be a major drug absorption site for orally administered drugs.[21]

The transport cell usually employed for this type of study requires a relatively large amount of carbohydrate, with volumes of departure and receiving phases being typically approx. 35 mL.[16–20] Due to the precious nature of the sialic acid derivatives (**2**–**5**) a new cell was designed, which allowed reduced quantities of sialic acid derivatives to be used. This included smaller volumes for both the receiving and departure phases, and the use of a peristaltic pump for continuous mixing. In addition, the volume of the receiving phase (~25.3 mL) was made twice that of the departure phase volume (~12.6 mL).

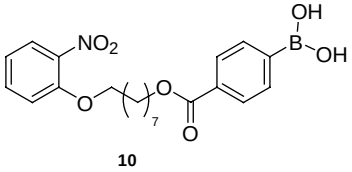


Figure 5. NPOEBA (**10**), the monoboronic acid used in this study.

It was thought that this latter modification would amplify the concentration gradient across the membrane and thus promote more rapid transport. However, this change was found to have little effect on fructose fluxes when compared with those obtained with the previously used transport cell, most likely because experiments were conducted above the saturation limit of the membrane.

Aliquots from the receiving phase were taken periodically and analysed for the carbohydrate. D-Fructose concentrations were determined via enzyme assay[16–20] and the concentrations of sialic acid derivatives were determined by HPLC. Initial fluxes were calculated from the linear plots of carbohydrate concentration versus time. The methyl esters (**2** and **4**) were omitted from experiments performed with a departure phase of 10.0 due to their rapid hydrolysis under these conditions.

2.1.1. Transport promoted by boronic acid (10) alone. The fluxes of D-fructose and the sialic acid derivatives were first determined with conditions in which the trigonal transport mechanism (Fig. 1A) was the only viable mode of transport through the membrane. The fluxes obtained under these conditions are shown in Table 1, which shows that no transport was detected for either D-fructose or the hydroxy ester (**2**). Low, but detectable, transport was observed for compounds **3**–**5**, however no clear correlation between the pH of the departure phase and flux can be seen. Significantly, the observation of transport of compounds **3**–**5** at a departure phase pH of 8.5 demonstrates the potential for the oral delivery of diol containing hydrophilic drugs utilising trigonal boronate esters. This is because this system mimics the environment of the jejunum (departure phase ~8.5) and the plasma (receiving phase ~7.4), and thus can be considered to be a simplistic model of drug absorption at this site.

2.1.2. Transport promoted by a combination of boronic acid (10) and Aliquat® 336. The factors affecting boronic acid facilitated carbohydrate transport through lipophilic membranes have been thoroughly reviewed,[10] but it is appropriate to re-state some key points here: it is commonly observed that boronic acid facilitated trigonal transport (see Fig. 1A) is much slower than the tetrahedral alternative (see Fig. 1B). This is thought in part to be due to the fact that trigonal boronate esters are less stable than their tetrahedral counterparts.[22] In addition, however, the typical boronate–ammonium ion pair involved in tetrahedral transport studies is more lipophilic than the trigonal counterpart, thus enhancing saccharide flux by improving carbohydrate extraction into the membrane. The next stage of this study thus involved testing the boronic acid facilitated transport of the sialic acid derivatives under conditions that are thought to promote tetrahedral transport.

The fluxes of D-fructose and the sialic acid derivatives (**2**–**5**) through a membrane containing **10** and Aliquat® 336 are shown in Table 2. As expected, all of the compounds tested were able to penetrate this membrane more readily than the one lacking Aliquat® 336 (Table 1). D-Fructose and the methyl esters (**2** and **4**) followed the typical trend of increasing flux with increasing pH.[10] The carboxylates (**3** and **5**), however, behaved differently, with **5** in particular displaying a significantly higher flux at pH 7.4. One complication in the transport of these latter compounds is the possible association of the carboxylate anion with the ammonium ion, which could allow boronic acid-independent transport. Aspects of this type of transport are discussed in Section 2.1.3.

The results obtained with D-fructose, shown in Table 2, provide an interesting further insight into the factors that govern carbohydrate transport promoted by boronic acids. The fact that flux does not increase significantly until the pH of the departure phase is above the pK_a of the boronic acid carrier, (pK_a ~ 8.8), rather than the pK_a of the boronate ester (pK_a D-fructose-aryl boronate ester ~4.6)[22] suggests that for efficient tetrahedral transport to occur, the boronic acid needs to be pre-ionised within the membrane. This might reflect an entropic advantage resulting from the association of the ammonium ion and the boronate ion within the membrane, prior to ester formation. This phenomenon is discussed in detail in a recent review.[10c]

2.1.3. Transport promoted by Aliquat® 336 alone. Given the high fluxes observed for the boronic acid (**10**)/Aliquat® 336 combination, it was important to discern the contribution that the ammonium salt alone was making to these fluxes. Thus, the carbohydrate permeability of a membrane that contained only Aliquat® 336 was examined and the results are shown in Table 3. As

Table 1. Carbohydrate fluxes through a supported NPOE membrane containing **10** at various departure phase pH's

	Flux (10^{-8} mol m^{-2} s^{-1})[a]		
pH:	7.4	8.5	10.0
D-Fructose	ND	ND	ND
2[b]	ND	ND	H
3	1.0 ± 0.1	1.1 ± 0.2	0.5 ± 0.1
4[b]	0.5 ± 0.1	1.0 ± 0.2	H
5	1.0 ± 0.1	0.4 ± 0.1	2.5 ± 0.3

ND = not detected.
[a] Fluxes are averages of duplicate experiments at T = 298 K.
[b] Experiments not performed for **2** and **4** at pH = 10.0 due to hydrolysis (H) of the methyl ester.

Table 2. Carbohydrate fluxes through a supported NPOE membrane containing **10** and Aliquat® 336 at varying departure phase pH's

	Flux (10^{-8} mol m^{-2} s^{-1})[a]		
pH:	7.4	8.5	10.0
D-Fructose	5.6 ± 0.5	7.0 ± 0.7	29.3 ± 2.9
2[b]	0.8 ± 0.1	1.9 ± 0.2	H
3	1.3 ± 0.1	0.6 ± 0.1	1.3 ± 0.1
4[b]	7.4 ± 1.1	48.6 ± 5.9	H
5	20.5 ± 2.8	4.2 ± 0.6	7.4 ± 1.0

ND = not detected.
[a] Fluxes are averages of duplicate experiments at T = 298 K.
[b] Experiments not performed for **2** and **4** at pH = 10.0 due to hydrolysis (H) of the methyl ester.

expected, D-fructose was not observed to pass through the membrane at any of the pH's examined, however, most remarkably, the sialic acid derivatives (**2**–**5**) all displayed higher fluxes under these conditions than those seen for the previous two membrane systems. This implies that the sialic acid derivatives can participate strongly in a transport process facilitated by Aliquat® 336 alone. This type of carbohydrate transport has been previously observed[23] and is known as mobile-site jumping transport.[23c] The process is shown schematically in Figure 6 and involves the carbohydrate initially hydrogen bonding to the anionic (chloride) component of the ammonium salt at the departure phase–membrane interface, then being drawn into the membrane through association with the highly lipophilic cation. The carbohydrate–anion complex thus formed can then either hop from cation to cation within the membrane, or the carbohydrate itself can hop from ion pair to ion pair. The overall result is the diffusion of the carbohydrate through the membrane and release into the receiving phase. Interestingly, the Aliquat® 336-promoted fluxes observed here are significantly higher than those recorded for other carbohydrates, D-fructose being one of the fastest of those previously studied.[23a] Of further interest is the fact that under most of the conditions examined, compounds **2**–**5** pass through the ammonium chloride membrane (Table 3) more readily than the ammonium boronate membrane (Table 2). This implies that mobile-site jumping transport outperforms tetrahedral boronate transport for these compounds, and that in the ammonium boronate membrane, the boronic acid may actually act to impair transport by occupying hydrogen bond donor sites preferred by the chloride. This is clearly not the case for D-fructose transport and suggests that the sialic acid derivatives (**2**–**5**) have unique properties that make them highly suited to ammonium chloride-promoted mobile-site jumping transport. This difference in behaviour may result from a greater hydrophobicity of compounds **2**–**5** and/or their stronger binding to chloride. These two possibilities were thus investigated.

Table 3. Carbohydrate fluxes through as supported NPOE membrane containing Aliquat® 336 at varying departure phase pH's

	Flux (10^{-8} mol m^{-2} s^{-1})[a]		
pH:	7.4	8.5	10.0
D-Fructose	ND	ND	ND
2[b]	3.9 ± 0.4	1.3 ± 0.2	H
3	4.5 ± 0.6	1.4 ± 0.2	2.5 ± 0.4
4[b]	28.9 ± 3.5	50.0 ± 3.6	H
5	38.9 ± 3.6	24.7 ± 3.5	7.2 ± 1.1

ND = not detected.

[a] Fluxes are averages of duplicate experiments at $T = 298$ K.

[b] Experiments not performed for **2** and **4** at pH = 10.0 due to hydrolysis (H) of the methyl ester.

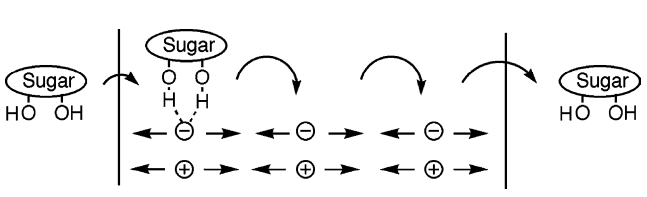


Figure 6. Schematic of mobile-site-jumping mechanism for saccharide transport mediated by lipophilic ion pairs. The saccharide 'jumps' from ion pair to ion pair and/or the saccharide–anion complex 'jumps' from cation to cation. The three-component saccharide–ion pair is also locally mobile.[23c]

2.2. *n*-Octanol/water partition coefficients (log *P*'s) and their relevance to observed fluxes

Extraction from the departure phase into the membrane and release from the membrane into the receiving phase can both be rate-limiting steps in the overall transport process. Smith and co-workers demonstrated this for boronic acid-assisted glycoside transport by showing that a bell-shaped relationship exists between flux and the extraction ability of the membrane.[24] Maximum transport was observed at intermediate levels of extraction, whereas low fluxes were observed when the membrane was either weakly or very strongly extracting. Relating that finding to the current study, one would expect that if the analyte is highly hydrophilic, extraction into the membrane will be so poor that very little transport will be observed. Conversely, transport will also be impaired if the analyte is too hydrophobic because, although extraction into the membrane will be strong, release into the receiving phase will be poor.

To obtain a measure of the relative hydrophobicities of the five test compounds, *n*-octanol/water partition coefficients (log *P* values) were determined and are displayed in Table 4. As expected, D-fructose was found to be the most hydrophilic of the five compounds tested. This supports the finding that D-fructose will only readily cross a lipophilic membrane when assisted by both a lipophilic boronic acid and a lipophilic ammonium salt. The hydroxy ester (**2**) was found to be the most hydrophobic of the five test compounds, thus suggesting that the generally poor transport observed for this compound is an indication that release into the receiving phase has become rate-limiting for this compound. Compounds **3**–**5** were found to have intermediate hydrophilicities compared to D-fructose and the hydroxy ester (**2**), which, given the above, may explain the higher fluxes observed for these compounds.

2.3. Chloride binding studies

As unexpectedly high fluxes were obtained when Aliquat® 336 alone was present in the membrane, particularly for compounds **4** and **5**, further studies were undertaken to investigate the interaction between the

Table 4. *n*-Octanol/water partition coefficients for the transported compounds

Compound	Log *P*
D-Fructose	−2.15 ± 0.05
2	0.09 ± 0.02
3	−1.36 ± 0.03
4	−0.89 ± 0.04
5	−1.42 ± 0.03

sialic acid derivatives and the chloride component of Aliquat® 336. Close examination of the structures of these carbohydrate derivatives (**2**–**5**) reveals that the presence of the amide functionality, together with the glycerol side chain, might provide a unique, and previously unrecognised anion binding domain. It was thus thought possible that the chloride component of the ammonium salt could be accommodated within the cavity formed by these four functional groups, held in place through hydrogen bonds. In order to test this hypothesis, association constants of the methyl esters, **2** and **4**, with Aliquat® 336 in DMSO-d_6 were determined, following the method of Hughes and Smith.[25] *N*-Cyclohexylacetamide (**11**), prepared from the acetylation of cyclohexylamine, was also included in this study in order to gauge the strength of association of an ordinary exocyclic acetamide with Aliquat® 336.

The Aliquat® 336 binding studies involved recording the change in the ^{1}H NMR chemical shift of the amide proton as the concentration of Aliquat® 336 was increased. Such changes were observed for all three amides studied (Fig. 7) and were assumed to result from association of the substrates with the chloride ion of the ammonium salt. Association constants between these amides were determined by an iterative curve-fitting method and are shown in Table 5.

It can be seen from Table 5 that *N*-cyclohexylacetamide (**11**) has a small, but measurable, association constant with Aliquat® 336, and by implication, chloride, in DMSO-d_6. By contrast, the two sialic acid derivatives (**2** and **4**) gave higher association constants, clearly

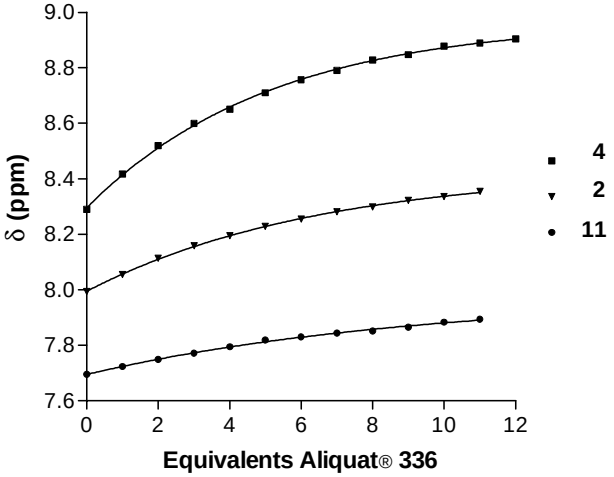
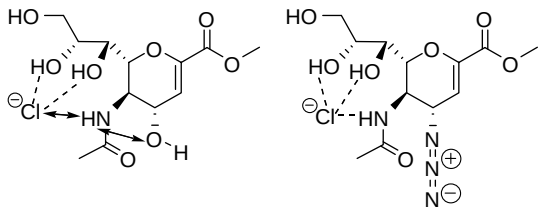


Figure 7. ^{1}H NMR N–H chemical shifts in DMSO-d_6 at 298 K for amides **2**, **4** and **11**, plotted against equivalents of added Aiquat® 336. $[\text{Amide}]_i$ = 32.8 mM.

Table 5. Binding constants (K_a) for the association of amides **3**, **5** and **11** with Aliquat® 336 in dimethylsulfoxide-d_6

Compound	K_a (M^{-1})
11	1.05 ± 0.33
2	2.57 ± 0.12
4	4.42 ± 0.24



2.Cl$^-$ **4**.Cl$^-$

Figure 8. Comparison of possible chloride binding modes for **2** and **4**.

showing that an enhancement of chloride binding can be achieved by participation of the additional functional groups present in the sialic acid derivatives (**2** and **4**). It should be noted that Kondo et al. observed a similar effect when elucidating the role of hydroxy groups in anion recognition in ligands containing sulfonamide moieties.[26] They found a more than sevenfold increase in chloride sequestering ability of ligands containing hydroxy groups compared with those lacking this functionality.

The difference in association constants between **2** and **4** may be due to the change in substitution at C4 of these compounds (Fig. 8). Methyl ester (**2**) has a hydroxy group at this position and intramolecular hydrogen bonding between the oxygen of this group and the hydrogen of the amide could compete with chloride binding, thus limiting chloride association. By contrast, compound **4** has an azide at C4, which may participate much more weakly in the analogous intramolecular hydrogen bonding suggested for the alcohol (**2**), thus allowing a higher affinity for chloride. Whatever the reason for the difference in the chloride affinities of these compounds, the observation that the azide (**4**) associates more strongly with Aliquat® 336 helps explain the increased flux observed for the C4-azides (**4** and **5**) compared to the C4-alcohols (**2** and **3**) in experiments when Aliquat® 336 alone was employed (Table 3). The fluxes of the azides are higher than those of the alcohols partly because their extraction into the membrane is better because of a stronger association with the chloride of Aliquat® 336. This conclusion is most strongly supported by the improved flux of **5** over **3**, as **5** and **3** have very similar log *P* values (Table 4) and thus should have similar solubilities in NPOE.

3. Conclusions

It was found that the boronic acid (**10**) was generally able to transport sialic acid derivatives (**2**–**5**) through a lipophilic supported liquid membrane by both a trigonal and tetrahedral mechanism. Transport experiments using **10** alone produced measurable fluxes for compounds **3**–**5**, thus demonstrating the potential for boronic acids to be used as drug delivery agents. No transport was observed for **2** under these conditions. The lipophilic nature of this compound, as shown by log *P* measurements, suggests that the rate-limiting step for its transport is release from the membrane into the receiving phase.

The combined boronic acid–ammonium membrane system, which employs both **10** and Aliquat® 336, displayed increased fluxes for all compounds relative to the membrane in which the ammonium salt was absent. This initially suggested that, as with D-fructose, the tetrahedral transport mechanism was more effective than the trigonal mechanism for the transport of sialic acid derivatives. However, a mobile-site jumping mechanism utilising Aliquat® 336 as the carrier is also possible under these conditions. The importance of this latter transport mode was investigated by studying the transport through membranes that contained Aliquat® 336 alone. This system displayed very high fluxes for the sialic acid derivatives (**2**–**5**) under conditions in which D-fructose transport was undetectable. By examination of the association constants of Aliquat® 336 to the sialic acid derivatives (**2** and **4**) in DMSO-d_6, it was concluded that the chloride component of Aliquat® 336 could hydrogen bond to the unique binding domain formed by the triol side chain and the amide moiety present in these compounds, and that the higher fluxes of the sialic acid derivatives could be partially explained by their greater affinity for chloride.

The difference in fluxes, and binding to chloride, of the hydroxy methyl ester (**2**) cf. the azide methyl ester (**4**) suggested that hydroxy substitution at C-4 in compound **2** leads to a competition between the oxygen of the hydroxyl group and chloride to accept the hydrogen bond from the amide N–H. Such competition leads to weaker chloride binding and lower transport fluxes.

Even though boronic acid facilitated transport is effective for sialic acid derivatives, the tetrahedral transport mechanism is slow compared with ion hopping facilitated by Aliquat® 336. Bearing in mind the toxicity of ammonium salts such as Aliquat® 336, further studies need to be carried out to determine if less toxic ammonium cations can be used to elicit the same effect. These results may lead to the improvement of the bioavailability of pharmaceuticals derived from sugars.

4. Experimental

4.1. General

The carrier compound NPOEBA (**10**) was prepared as previously described.[17] The sialic acid derivatives (**2**–**5**) were each prepared in six steps from sialic acid methyl ester,[27] involving initial treatment with acetyl chloride to give the 2-chloro-tetraacetate,[13] then according to procedures described by von Itzstein et al.[28] *N*-Cyclohexylacetamide (**11**) was prepared in 86% yield from cyclohexylamine by treatment with acetic anhydride and diisopropylethylamine in DMF; mp 101–103 °C (lit. 100–101 °C).[29]

HPLC chromatograms were obtained using a Waters 600 Controller, a Waters 996 Photodiode Array Detector and a Waters 717plus Autosampler. Waters Delta-Pak C18-100A (3.9 × 300 mm, 15 μm spherical) and YMC PackDiol (100 × 4.6 mm, 5.5 μm, 12 nm) columns were used for the sialic acid methyl esters (**2** and **4**) and carboxylic acids (**3** and **5**), respectively. For specific HPLC conditions, refer to Supplementary data. The results were processed with Millennium 32 software Version 3.05.01.

4.2. Transport experiments

Transport experiments were performed by dissolving the boronic acid (37.5 μmol) and/or Aliquat® 336 with 2-nitrophenyl octyl ether (250 μL) in CH_2Cl_2, concentrating under vacuum and applying the residual oil to an Accurel® type 1E (area; 8.04 cm^2, thickness; 0.1 mm, pore size; 0.1 μm obtained from Membrana GmbH, Wuppertal, Germany). The resulting membrane was then placed under vacuum for at least 12 h. The departure phase (12.5 mL) contained 0.02 M sialic acid derivative or D-fructose. This was buffered with 0.1 M sodium phosphate to obtain pH's 7.4 and 8.5, while 0.1 M sodium carbonate was used to maintain a pH of 10.0. The receiving phase (25.8 mL) was buffered at pH 7.4 with 0.1 M sodium phosphate. Both phases were continuously mixed throughout the transport experiment at a rate of 1 mL min^{-1} using a peristaltic pump. A schematic of the transport cell is shown in the Supplementary data.

Aliquots were removed at hourly intervals and analysed for the respective carbohydrate used in the given experiment. The concentration of D-fructose in the receiving phase was determined using the hexokinase-glucose-6-phosphate dehydrogenase/phosphoglucose isomerase coupled assay as described previously.[16–20] The concentrations of sialic acid derivatives in the receiving phase were determined by HPLC. Plots of [carbohydrate] versus time over a period of 6 h were used to calculate fluxes. The fluxes quoted are averages of duplicate runs. $T = 298$ K, flux uncertainty: D-fructose ±10%; sialic acid derivatives ±15%.

4.3. Water/*n*-octanol partition coefficients

n-Octanol/water partition coefficients were determined at $T = 298$ K following the method of Ahlmark et al.[30]; in each case, the selected carbohydrate was dissolved in 0.1 M sodium phosphate buffer (pH 7.4, 2 mL) to give a concentration of 50 mmol, then *n*-octanol (2 mL) was added and the resulting mixture shaken vigorously with a mechanical shaker. At hourly intervals, the mixture was centrifuged and an aliquot was taken from the aqueous phase and the carbohydrate concentration determined. This was continued until the carbohydrate concentration in the aqueous phase plateaued. The concentrations of the sialic acid derivatives were determined using UV spectroscopy and standard absorbance curves (Abs vs [sialic acid derivative]) at 240 nm. The D-fructose concentrations were determined using the aforementioned enzyme assay (Section 4.2). The difference between the initial and final carbohydrate concentrations in the aqueous phase was taken to be the concentration of carbohydrate in the *n*-octanol layer, at equilibrium.

4.4. Chloride binding experiments

Binding constants were determined according to the procedure of Hughes and Smith.[25] A solution of the host compound (1 mL, 32.8 mM) in DMSO-d_6 was placed in an NMR tube. Aliquots of a stock Aliquat® 336 solution in DMSO-d_6 (40 μL, 32.8 mM) were then added, with a ^{1}H NMR spectrum recorded after each addition. The chemical shift of the amide peak was noted and is plotted against equivalents of Aliquat® 336 added. The resultant titration curves were then fitted to a 1:1 binding model, as described by Hughes and Smith.[25]

Acknowledgments

The chloride binding constants were determined by Bradley Smith and Joseph Mahoney, University of Notre Dame. The Australian Research Council (ARC), Biota Holdings Ltd. and Monash University are acknowledged for funding. Daniel Offermann is acknowledged for originally suggesting the use of a peristaltic pump to mix the departure and receiving phases during transport experiments.

Supplementary data

Supplementary information showing conditions used in HPLC assays, a schematic of the transport cell, equations used for flux calculations and concentration versus time plots from which fluxes were determined can be found in the online version at doi:10.1016/j.bmc.2005.09.028.

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