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Insulins with built-in glucose sensors for glucose responsive insulin release

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Abstract: Derivatization of insulin with phenylboronic acids is described, thereby equipping insulin with novel glucose sensing ability. It is furthermore demonstrated that such insulins are useful in glucose-responsive polymer-based release systems. The preferred phenylboronic acids are sulfonamide derivatives, which, contrary to naïve boronic acids, ensure glucose binding at physiological pH, and simultaneously operate as handles for insulin derivatization at LysB29. The glucose affinities of the novel insulins were evaluated by glucose titration in a competitive assay with alizarin. The affinities were in the range 15–31 mM (K_d), which match physiological glucose fluctuations. The dose-responsive glucose-mediated release of the novel insulins was demonstrated using glucamine-derived polyethylene glycol polyacrylamide (PEGA) as a model, and it was shown that Zn(II) hexamer formulation of the boronated insulins resulted in steeper glucose sensitivity relative to monomeric insulin formulation. Notably, two of the boronated insulins displayed enhanced insulin receptor affinity relative to native insulin (113%–122%) which is unusual for insulin LysB29 derivatives.

Keywords: insulin; glucose sensor; boronates; sulfonamides; glucose responsive release

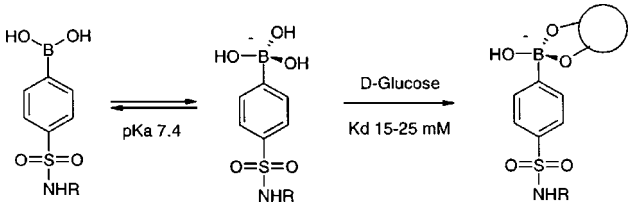
INTRODUCTION

Diabetes is associated with significant long-term risks of complications by damage to the eyes, heart, kidneys and other organs. Encouragingly, recent long-term studies have documented that tight glucose control can minimize these risks [1]. Concurrently, a number of insulin analogues have become available recently, which are helpful in the aim for tight control via daily multi-injection regimens, which can cover the need for both basal and meal-related insulin [2]. Unfortunately, even with the most stringent adherence to optimized regimens, many diabetes patients experience frequent, unexpected fluctuations in blood glucose [3].

This problem has been approached in several cases by engineering glucose-sensing polymers, which can bind or entrap insulin, and subsequently release insulin in a glucose responsive fashion. Such systems could potentially be useful as 'smart' subcutaneous insulin depots [4–8].

This paper reports a modified approach where the glucose sensor is part of the insulin molecule, i.e. not part of the polymer. Ultimately, the work is aimed at glucose responsive release from polymer-free systems, but initially this is a report of the preparative procedures for the novel insulins, and their use with a model polymer. The preferred glucose sensor is based on boronate, which is the anionic, tetragonal form of the otherwise planar boronic acid (Scheme 1). Boronates are known to bind glucose and other carbohydrates with apparent affinities in the mM range (K_d) [9–12]. This range matches the physiological range of glucose, whereas other carbohydrates physiologically occur at lower levels. The small size of the boronate building blocks allows boronated insulins to retain the overall biophysical properties of insulin. This is an advantage with respect to the predictability of insulin solubility, stability and general handling. On the contrary, if fusion proteins of insulin with protein-based glucose sensors were targeted (e.g. concanavalin A or glucose oxidase), one would likely need to establish the biophysical properties of the fusion proteins from scratch.

The carbohydrate affinity of aryl boronates have been exploited previously in construction of fluorescent glucose sensors [13], affinity columns for glycosylated proteins [14] and saccharide transport systems [15]. However, it is only the boronate form of boronic acid that binds glucose efficiently (Scheme 1) [9–12], and this is a problem for use at physiological conditions, because the pKa of simple aryl boronic acid is 8.5. Alkyl boronic acids are even less acidic and furthermore oxidatively unstable [16]. Not surprisingly, the pKa



Scheme 1

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of aryl boronic acid can be modulated by use of electron-withdrawing groups, and carboxyl and nitro groups have previously been applied for this purpose. However, neither of these groups are ideal for use in a physiological system. Carboxyl groups are not strongly electron-withdrawing, and hence the pKa values of carboxy phenylboronic acids are roughly 8 [17–19]. Nitro groups are more effective [20,21]. but these groups are not desirable from a pharmacological point of view due to general toxicity [22]. An alternative is 2-aminomethyl-phenylboronic acids, which form the desired tetragonal boronate configuration via B-N interaction above pH 5.5 [23]. However, 2-aminomethyl-phenylboronics are zwitterionic structures, which are usually scarcely soluble in water at neutral conditions, and therefore usually applied in 50% methanol [24]. Furthermore, these compounds are annoying to work with from a synthetic point of view, because they are prone to salt formation.

With these aspects in mind, sulfonamide phenylboronic acids appeared almost ideal for the given purpose. Unfortunately, sulfonamide phenylboronic acids are scarcely described, mainly because sulfonyl chlorides of phenylboronates were unknown until recently. Although there are alternative methods for the preparation of sulfonamide phenylboronic acids rather than via sulfonyl chlorides, those methods [25,26] are not compatible with the unprotected peptide functions as found in insulin. With regard to sulfonyl chlorides, they can usually be coupled with unprotected peptides to give *N*-selective sulfonylation. Luckily, sulfonyl chloride phenylboronate chemistry has recently been enabled by our description of selective lithiation of bromo phenyl N-methyl-diethanolamine boronates [27]. The pKa values of 4-sulfonamide phenylboronic acids are approximately 7.4, making them compatible with physiological conditions. The present work describes the incorporation of sulfonamide and nitro carboxy phenylboronates into insulin, and the use of such boronated insulins in a glucose responsive model release system.

MATERIALS AND METHODS

Boronic acids were detected on thin layer chromatography sheets by the use of diphenylcarbazone in methanol as a spray reagent. ^{1}H and ^{13}C NMR spectra were recorded on 300 or 400 MHz Varian instruments. Chemical shifts are reported relative to TMS at 0 ppm, and *J*-values are given in Hz. The mass spectra of insulins were recorded by electrospray mass spectrometry (ESMS).

Succinimidyl 3-pinacolborono-5-nitro-benzoate 1

3-pinacolborono-5-nitro-benzoic acid (5.26 g, 20.0 mmol, Combiblocks, San Diego, CA) in ethyl acetate (75 ml) was cooled with an ice-bath, treated with *N*,*N*′-dicyclohexylcarbodiimide (4.33 g, 21.0 mmol) and *N*-hydroxysuccinimide (2.3 g, 20.0 mmol) and left at room temperature overnight. The precipitated *N*,*N*′-dicyclohexylurea was removed by filtration, and the solvent was removed *in vacuo*. The active ester was crystallized from acetone-hexane to give **1** (6.47 g, 83%).

^{1}H-NMR ($CDCl_3$): 9.01 (m, $J_1 = 1.6$, $J_2 = 0.8$, 1H), 8.90 (m, $J_1 = 1.6$, $J_2 = 0.8$, 1H), 8.84 (m, 1H), 2.94 (s, 4H), 1.38 (s, 12H).

Lithium 4-sulfinyl N-methyl-diethanolamine phenylboronate 2

4-Bromo *N*-methyl-diethanolamine phenylboronate (26.5 g, 94 mmol, Lancaster, Lancashire, UK) in THF (800 ml) was cooled to −100 °C using an ether-nitrogen bath and treated drop-wise with butyl lithium in hexane (1.43 M, 59 ml, 70 mmol) over 10 min. After 15 min, gaseous sulfur dioxide (28 g, 0.43 mol) was added and the mixture was stirred at room temperature for 1 h. The precipitate was collected by filtration, washed with THF and dried *in vacuo*, providing 23.0 g (99%).

^{1}H NMR (DMSO-d_6): δ 7.43 (d, 2H), 7.35 (d, 2H), 3.97–3.83 (m, 4H), 3.26–3.19 (m, 2H), 2.98–2.89 (m, 2H), 2.17 (s, 3H).

tert-Butyl 4-amino-N-(4-pinacolborono-phenylsulfonyl))butyrate 3

Sulfinyl **2** (0.55 g, 2.0 mmol) was suspended in DCM (2 ml) and treated with *N*-chloro-succinimide (0.30 g, 2.2 mmol) under stirring for 1 h. *N*,*N*-diisopropylethylamine (0.23 g, 2.2 mmol) and *tert*-butyl 4-amino-butylate hydrochloride (0.43 g, 2.2 mmol) was added, and after 1 h, the organic phase was washed with 1 M hydrochloric acid solution followed by water and dried over sodium sulfate. The organic phase was filtered and pinacol (0.26 g, 2.2 mmole) and magnesium sulfate (0.26 g, 2.2 mmol) were added. The reaction mixture was stirred at room temperature for 1 h, and the organic phase was washed twice with water, dried over sodium sulfate and evaporated to provide compound **3**; 508 mg oil (60%).

^{1}H NMR ($CDCl_3$): δ 7.93 (d, $J = 8.3$, 2H), 7.83 (d, $J = 8.3$, 2H), 4.71 (t, $J = 6.0$, 1H), 2.99 (q, $J = 6.7$, 2H), 2.25 (t, $J = 7.0$, 2H), 1.75 (q, $J = 6.8$, 2H), 1.42 (s, 9H), 1.36 (s, 12H).

Succinimidyl N-(4-pinacolborono-phenylsulfonyl)-4-amino-butyrate 4

Tert-Butyl ester **3** (360 mg, 0.90 mmol) was dissolved in trifluoroacetic acid (8 ml) and cooled with an ice bath. The reaction mixture was slowly heated to room temperature and stirred at this temperature for 1 h. The reaction mixture was evaporated and the crude material was triturated with toluene. The crystalline material was filtered, dried and dissolved in dry dichloromethane, and cooled with an ice bath. *N*-Hydroxysuccinimide (104 mg, 0.90 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (185 mg, 0.90 mmole) were added and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was filtered and evaporated. The crude material was redissolved in ether and filtered to remove *N*,*N*′-dicyclohexylurea. The ether solution was evaporated to give compound **4**, 470 mg (87%).

^{1}H NMR ($CDCl_3$): δ 7.94 (d, $J = 8.7$, 2H), 7.84 (d, $J = 8.7$, 2H), 4.91 (t, 1H), 3.08 (m, 2H), 2.84 (br. s., 4H), 2.68 (t, 2H), 1.95 (m, 2H), 1.35 (s, 12H).

Sulfonyl chloride N-methyldiethanolamine phenylboronate 5

Sulfinyl **2** (8.7 mg, 32 μmol) was suspended in THF (800 μl) and treated with *N*-chloro-succinimide (4.2 mg, 32 μmol), and the mixture was stirred for 1 h to provide sulfonyl chloride **5** *in situ*.

Alternatively, sulfinyl **2** was suspended in dichloromethane (1 ml). *N*-chloro-succinimide (73 mg, 0.55 mmol) was added and the mixture was stirred at room temperature for 1 h. The organic solution was washed three times with ice cold water, dried with sodium sulphate and evaporated to isolate 80 mg (52%) of **5** as colourless crystals.

^{1}H NMR ($CDCl_3$): δ 7.94 (d, $J = 8.3$, 2H), 7.89 (d, $J = 8.3$, 2H), 4.29–4.14 (m, $J_1 = 5.6$ $J_2 = 3.0$, $J_3 = 1.9$, 4H), 3.28 (ddd, $J_1 = 4.2$ $J_2 = 1.1$, $J_3 = 2.0$, 2H), 3.06 (ddd, $J_1 = 6.8$ $J_2 = 1.5$, $J_3 = 3.4$, 2H), 2.36 (s, 3H).

LysB29-N$^{\varepsilon}$-(3-borono-5-nitro-benzoyl) DesB30 insulin 6

DesB30 insulin (400 mg, 70 μmol) was dissolved in aqueous 0.1 M sodium carbonate (4.5 ml) and treated with succinimidyl ester **1** (33 mg, 84 μmol) in acetonitrile (4.5 ml), and the mixture was slowly stirred for 30 min. Control of pH showed 10.3 initially, and 9.8 at end point. The reaction was quenched by addition of 0.2 M methylamine hydrochloride (0.8 ml). The pH was adjusted to 6 with 0.5 M HCl, and the mixture was left in a refrigerator whereby the insulin product was precipitated and isolated by centrifugation.

Insulin **6** was purified by RP-HPLC on C4 column eluted with gradient from 80% to 10%, 0.1% TFA in water/0.1% TFA, 80% acetonitrile in water, to give 143 mg (35%). Amino acid sequence analysis showed Gly/Phe 1 : 1 as the first step, which is consistent with acylation at LysB29.

ESMS: 5863.0 ($MH^+ - 2H_2O$). $C_{260}H_{376}BN_{65}O_{78}S_6$ requires 5863.5.

LysB29-N$^{\varepsilon}$-(N-(4-borono-phenylsulfonyl)-4-amino-butyroyl) DesB30 insulin 7

DesB30 insulin (1 g, 175 μmol) was dissolved in ice-cooled 50 mM sodium carbonate (50 ml) and the pH was adjusted to 10.2 with 2 N NaOH. Succinimidyl ester **4** (98.1 mg, 210 μmol) was dissolved in 50 ml acetonitrile and the insulin solution was added. The reaction mixture was slowly agitated and the pH determined as 10.4. The reaction was quenched after 30 min by addition of 0.2 M methylamine hydrochloride (19 ml) and diluted by addition of water (132 ml). The pH was adjusted to 5.5 with 2 N HCl and the resulting suspension was left at −18 °C for 2 h. The crude product was isolated by centrifugation and purified by RPLC on a C18 column eluted with a gradient from 32% to 40% (v/v) ethanol in 0.1 M phosphoric acid, 0.1 M sodium dihydrogen phosphate. The eluted fraction containing **7** was collected, desalted on a Sephadex G25 column in 0.5 M acetic acid and lyophilized to give 103.5 mg (10%). Amino acid sequence analysis confirmed LysB29-acylation (Gly/Phe 1 : 1).

ESMS: 5939.5 ($MH^+ - 2H_2O$). $C_{263}H_{384}BN_{65}O_{78}S_6$ requires 5939.7.

LysB29-N$^{\varepsilon}$-(4-borono-phenylsulfonyl) DesB30 insulin 8

DesB30 insulin (150 mg, 26 μmol) was dissolved in DMSO (3.0 ml) by slowly rotating the vial for 1 h. Triethylamine was added (36 μl, 260 μl), followed by THF solution of *in situ* generated sulfonyl chloride **5** (32 μmol), and the mixture was slowly stirred for 1 h. The reaction was quenched by addition of excess aqueous methylamine, and the insulin was isolated by aqueous dilution and adjustment of the pH to 6, followed by cooling and centrifugation.

Insulin **8** was purified by RP-HPLC similarly with insulin **6** to give 20 mg (13%). Amino acid sequence analysis confirmed LysB29-acylation (Gly/Phe 1 : 1).

ESMS: 5857.6 ($MH^+ - 2H_2O$). $C_{259}H_{377}BN_{64}O_{77}S_6$ requires 5858.6.

PEGA-succinyl-D-glucamide resin 9

Amino PEGA resin (PEG_{800}) [28] (500 mg, 0.4 mmol/g, 0.2 mmol, Novabiochem, CH or Polymer Laboratories, UK) was washed on a filter with DMF, and treated with succinic anhydride (100 mg, 1.0 mmol) in DMF (3 ml) and left for 4 h. Upon further washing with DMF, the resin was treated with O-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU, 320 mg, 10.0 mmol) and *N*,*N*-diisopropylethylamine (171 μl, 1.0 mmol). The mixture was left for 30 min and washed with DMF. D-Glucamine (181 mg, 1.0 mmol) dissolved in water-DMF (2 : 1, 3 ml) was added, and the mixture was left overnight. Resin **9** was washed with DMF and methanol and stored wet.

Glucose Responsive Insulin Release

Insulin **7** (1.0 mg, 168 nmol) was dissolved in neutral PBS buffer (0.5 ml, 10 mM phosphate, 100 mM NaCl, pH 7.4), by addition of small quantities of 0.1 M NaOH (1–2 μl), and readjustment to pH 7.4 with 0.1 M HCl (1–2 μl).

The insulin solution was added to D-glucamide resin **9** (250 mg, 0.1 mmol), and the mixture was left for 60 min. The resin was washed batch-wise with 0.5 ml portions of neutral PBS buffer, or 5, 25 or 50 mM D-glucose in neutral PBS buffer for 5 min each, and the insulin contents in the batches were evaluated by HPLC analysis (214 and 256 nm).

Glucose Responsive Insulin Release of Hexameric Insulin

Insulin **7** (1.0 mg, 168 nmol) was dissolved in neutral PBS buffer (0.5 ml, 10 mM phosphate, 100 mM NaCl, pH 7.4), by addition of small quantities of 0.1 M NaOH (1–2 μl), and readjustment to pH 7.4 with 0.1 M HCl (1–2 μl).

Zn(II) acetate was added (8.4 μl, 10 mM, 84 nmol, 3 Zn/hexamer), and the insulin hexamer was loaded on resin and eluted batch-wise with neutral buffer and glucose, as described above.

Determination of ARS : boronate Affinities

Stock alizarin solution was prepared at 50 μM in PBS buffer (10 mM phosphate, 100 mM NaCl, pH 7.4).

3-Borono-5-nitro-benzamide **10** (Combiblocks, San Diego, CA) and *N*-methyl-4-borono-benzene sulfonamide [27] **11**

were each dissolved in stock ARS solutions at 2 mM. The boronate/ARS solutions were mixed with stock ARS solution to give 14 boronate concentrations in the range 0.2 μM–4 mM with constant ARS concentration. UV/Vis spectra of the solutions were recorded and the absorption values at, e.g. 530 nm, were extracted and imported to GraphPad Prism 4.01, where curve fitting to the model of *sigmoidal dose response* gave K_d(ARS**:10**) **= 91** μM and K_d(ARS**:11**) **= 101** μM. These values were used in calculation of boronated insulin : glucose affinities by competitive ARS titration with glucose (below). The measurements were performed in duplicate.

Glucose Affinity Assay by Competitive Alizarin Titration

Boronated insulins **6**–**8** were dissolved at 100 μM in 50 μM ARS solution. Glucose was dissolved at 400 mM in the resulting ARS/insulin solutions. The glucose/ARS/insulin solutions was mixed with ARS/insulin solutions to give 14 glucose concentrations in the range 20 μM–400 mM, with constant ARS and insulin concentrations. UV/Vis spectra were recorded and the absorption values at 530 nm were extracted and imported to GraphPad Prism 4.01. Curve fitting to the model of *one site competition* using ARS K_d values as measured above gave the boronated insulin : glucose affinities as listed in Table 1. The measurements were performed in duplicate.

Insulin Receptor Affinity Assay

Insulin receptor affinity was measured with solubilized insulin receptor in a competitive scintillation proximity assay (SPA) using TyrA14-^{125}I human insulin as tracer, similarly to the described precipitation assay [29].

SPA-PVT antibody-binding beads (Amersham, UK) were applied in combination with F12 insulin receptor monoclonal antibody [30] and solubilized insulin receptor (minus exon 11) in buffer (100 mM HEPES, 100 mM NaCl, 10 mM $MgSO_4$, 0.5% human albumin, 0.025% Triton X-100, pH 7.8). The experiments were performed twice in duplicate and human insulin was used as control.

RESULTS AND DISCUSSION

Building blocks **1**, **4** and **5** (Scheme 2) were prepared as described in the experimental section. Since boronic acids are generally prone to anhydro formation, it is customary to protect and handle these in the form of esters. This point is important for characterization purposes, as NMR spectra of boronic acids in organic solvent otherwise appear complicated due to formation of mixed anhydro species. Pinacol esters (as used with **1** and **4**) are usually not recommended for protection of boronic acid, because these esters can be hard to cleave, and the released pinacol can be difficult to remove [31,32]. However, the present work shows that pinacol esters are efficiently cleaved during RP-HPLC as used for purification of the final insulin products (C4 or C18 columns). Notably, the interim robustness of pinacol esters is attractive for multi-step synthesis of building blocks, as this allows aqueous work-up procedures to be employed.

DesB30-Insulin was acylated at the LysB29 epsilon amine by reaction with succimidyl esters **1** or **4** to give insulins **6** and **7** as outlined in Scheme 3. Although insulin has three amino groups available for derivatization (A1, B1 and B29), the LysB29 epsilon amine is the most basic (pKa approximately 10), and hence the most nucleophilic at alkaline conditions (>pH 10) [33,34].

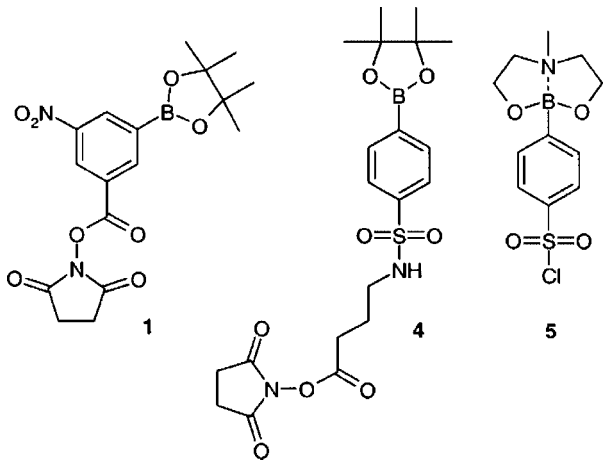
Insulin **8** was pursued under similar conditions by reaction of insulin with sulfonyl chloride **5**, but this reaction led only to formation of a complex mixture. Instead, DMSO-TEA was identified as conditions in which *N*-sulfonylation at LysB29 gave the main product.

The semi-synthetic insulins **6**, **7** and **8** were purified by RP-HPLC and characterized by electrospray mass spectrometry. Acylation at B29 was ascertained by amino acid sequence analysis and mass spectrometry following V8 endopeptidase cleavage of insulin peptide chains. The mass spectra of boronated peptides generally show dehydrated masses due to gas phase anhydro formation from the boronic acids.

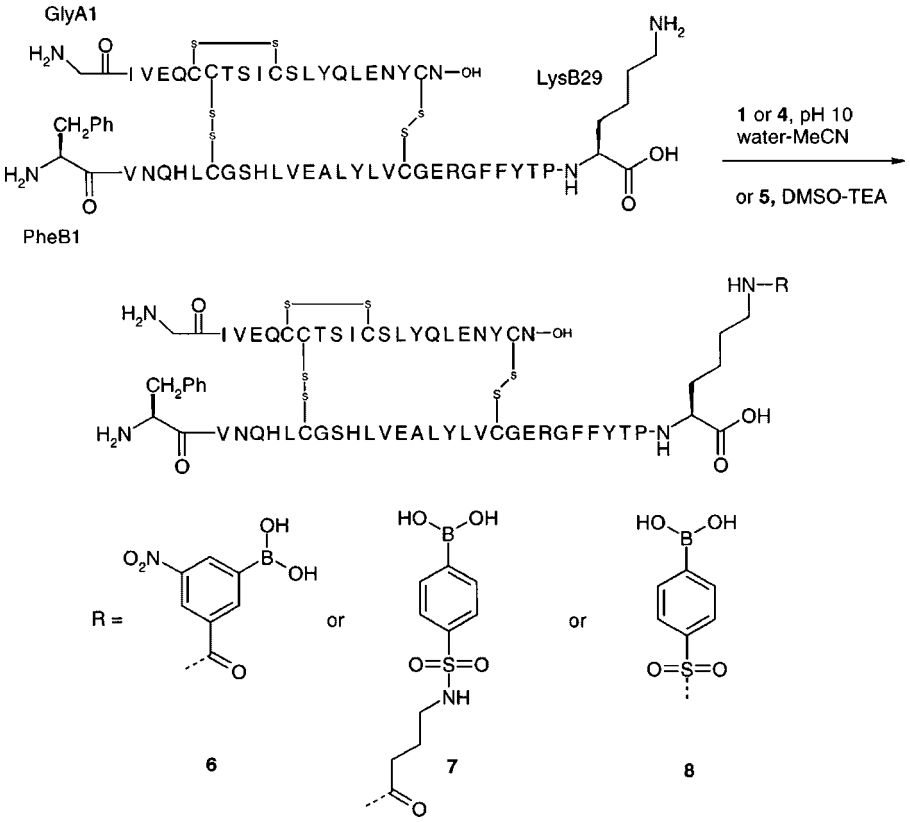
While working with the nitro boronate insulin **6**, it was noticed that this compound was partially unstable. Since nitro arenes are often photolytically reactive, it was suspected that sensitivity to light

Table 1 Glucose and Insulin Receptor Affinity of Boronated Insulins

Insulin structure	K_d D-glucose (mM)	K_d Insulin receptor (pM) (relative to insulin)
Human insulin	No binding	11 (100%)
6	15	17 (65%)
7	31	9.1 (122%)
8	26	9.7 (113%)



Scheme 2



Scheme 3

could be the problem. Indeed, by running simple stability studies with various boronate building blocks in aqueous solution (PBS buffer, pH 7.4) in daylight and in the dark, it was discovered that under daylight conditions, 3-borono-5-nitro-benzoic acid and its derivatives were transformed within 24 h to the de-boronated compounds (3-nitro-benzoic acid). On the contrary, the sulfonamide boronates were fully stable under identical conditions. Problematic stability of nitrated phenylboronic acids has a precedence in the described hydrolysis of 4-carboxy-2-nitrophenylboronic acid to 4-carboxy-2-nitrophenol at pH >9 [18].

As mentioned, the boronated insulins were expected to attain glucose affinities in the mM range (K_d). This range matches the physiological range for glucose fluctuations, which in diabetes patients fluctuates between approximately 1 and 25 mM. The matching of glucose affinity and glucose concentration is important with respect to optimal glucose sensitivity. If the glucose affinities were in the μM or nM range, the sensor would be fully saturated in the physiological range. Determinations of affinities in the mM range are, however, often problematic due to, among other things, solubility problems. Noteworthy, since D-glucose is a very small molecule, any labeling of the saccharide with a larger label would easily perturb the binding properties of glucose. With these aspects in mind, carbohydrate titration by competitive binding to Alizarin Red

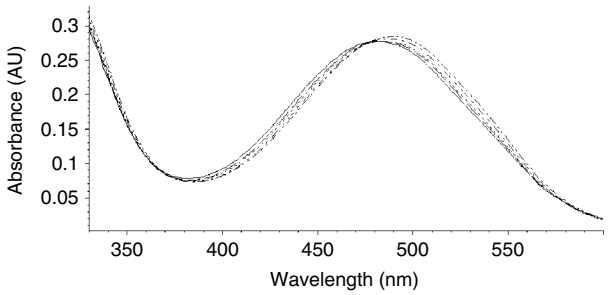


Figure 1 UV/VIS titration of boronated insulin/ARS with glucose (20 μM–400 mM) in PBS, pH 7.4.

Sodium (ARS) was preferred. Initially, ARS : boronate affinities were evaluated by titration in PBS at pH 7.4, and the absorption data at 324 or 530 nm were curve-fitting (non-linear regression) [38]. The affinities of ARS : 3-borono-5-nitro-benzamide **10** and ARS : *N*-methyl-4-borono-benzensulfonamide **11** were 91 μM and 101 μM, respectively (K_d), which matches well with literature values [35–37]. Subsequently, solutions of ARS and boronated insulins **6**, **7** and **8** were titrated with glucose (Figure 1), and the absorption data were curve-fitted by non-linear regression using the K_d values above for the ARS : boronate interaction (Figure 2). The glucose affinities were in the range 15–31 mM (Table 1), which is in accordance with literature values measured for small molecule boronates [11].

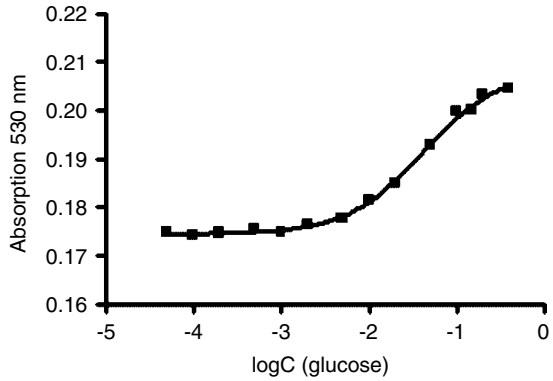


Figure 2 Curve fitting of absorption data from glucose titration.

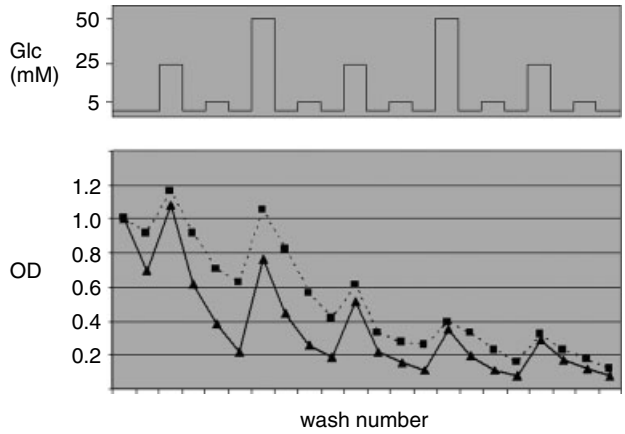


Figure 3 Insulin release from monomor (■) and Zn(II) insulin hexamer (▲) formulation on D-glucamide polymer, by PBS or D-glucose (5, 25, 50 mM) in PBS, pH 7.4.

Insulin receptor affinity of the novel insulins were measured in a competitive TyrA14-^{125}I human insulin assay [29], and were in the range 65%–122% relative to human insulin (Table 1). The insulin LysB29 position is generally tolerant with respect to derivatizations, and LysB29-derivatives usually display affinities in the range 40%–80%. The enhanced receptor affinities (>100%) of insulins **7** and **8** compared with native insulin may reflect participation of the boronate function in the receptor recognition.

A model insulin release system for the novel insulins was created by derivatizing commercial amino polyethylene glycol polyacrylamide resin (amino PEGA) [28,39] with D-glucamine via succinic acid as a linker. PEGA resin was chosen because the material swells well in aqueous solvent and allows diffusion of large molecules, such as insulin and its dimers or hexamers. Furthermore, PEGA is non-carbohydrate material. Many polymers used in biochemical applications (affinity columns, size-exclusion columns etc) are based on carbohydrate materials, but such materials may be problematic for the present purpose, due to the boronate affinity for carbohydrates. For instance, concanavalin A protein is known to bind not only to glucose, but also to sephadex [40]. Notably, boronates bind mainly to 1,2-*cis*-diols, and in e.g. Sephadex or agarose materials the majority of 1-hydroxy groups are blocked by glycoside bonds [41]. Nevertheless, since the polymer terminals are not glycosides, and since a bulk of other alcohol functions are present within such polymers, non-carbohydrate polyamide (PEGA) was preferred.

Boronated insulin **7** was loaded on the pre-swollen polymer and released by batch-wise washings with buffer or buffered glucose solutions, pH 7.4. Insulin quantification by HPLC analysis demonstrated basal release with buffer and dose-responsive release with glucose solutions of 5, 25 or 50 mM, Figure 3 (■). Since the interaction of the polymer-bound D-glucamide with boronated insulin is rather weak (Kd 0.1 mM) [42] compared with typical affinity purification columns (K_d in the μM–nM range), it is not surprising that buffer alone can partially elute the insulin. However, since some level of basal insulin release is physiologically desired, insulin 'bleeding' from the polymer is acceptable at some level. It is, however, a problem with the general principle of glucose responsive insulin release from polymers that the release profiles tend to be rather flat compared with physiological conditions. *In vivo* the insulin response to a glucose challenge is amplified relative to the challenging glucose spike [43]. This amplification will be challenging to mimic fully with a diffusion-based polymer release system. Physiological insulin release furthermore proceeds in a pulsatile fashion, but this phenomenon seems to be of less importance [44,45], and it is not complied with in traditional insulin treatment.

Shiino *et al.* seemingly improved the insulin-polymer affinity by employing doubly glyconated insulin in combination with a multi-boronated polymer (the positions of insulin acylation were not characterized) [6,42]. This setup likely improves the affinity by cooperative effects, and it probably also leads to improved glucose sensitivity. Discouragingly, A1- and/or B1-derivatives of insulin usually display unbeneficial properties such as lowered insulin receptor affinity and/or changes to peptide folding and hexamer stability [46–50].

Notably, insulins are usually formulated as hexamers via complexation with 2 or 3 zinc ions per hexamer. The zinc stabilized insulin hexamers have importance for the overall stability of insulin, which in the market must be in the order of several years. To the best of our knowledge, hexamer formulation of insulins has not previously been practised in studies of glucose responsive insulin release from polymers [51]. However, it was found that employment of the novel boronated insulins as hexamer formulations resulted in steeper insulin responses to glucose challenges relative to insulin monomer formulation, see Figure 3 (▲). The given experiments are only meant to give qualitative

information, and further work is needed to evaluate these aspects in full detail. The applied PEGA resin is anyway only meant as a model polymer, since clinical use would surely demand a biodegradable, preferably soluble, material and not a beaded resin. Such material should be identified and evaluated before further optimization makes sense. Furthermore, it is clear from Figure 3 that insulin is released from the polymer in generally smaller amounts from the hexamer formulation relative to the monomer formulation. This may reflect the larger size of insulin hexamer (36 kD), and hence more hesitant diffusion and release of insulin from the polymer network of the PEGA polymer [52,53]. The cut-off from commercially available PEGA is specified by the suppliers as approximately 35 kD (PEG_{800}; Novabiochem, CH and Polymer Laboratories, UK).

Obviously, a potential problem with the given system is the possible interference from other boronate-binding carbohydrates or diols etc. For instance fructose is known to bind to boronates stronger than does glucose. However, since the physiological glucose concentration is approximately 100 times higher than the fructose concentration (5 mM vs 50 μM) [54], this is likely not to be a serious problem. Noteworthy, distinctly glucose selective di-boronates have been described in the literature [55–57]. Unfortunately, the majority of these are not easily applicable to insulin systems, due to the lack of conjugation handles and/or non-physiological boronic acid pKa. Accordingly, investigations of insulins with built-in glucose selective di-boronates will require significant further work.

CONCLUSION

Insulins with built-in glucose sensors in the form of boronates have been prepared, and demonstrated to be useful in model glucose responsive release systems based on D-glucamine polyamide polymer. The preferred sensors are sulfonamide phenylboronic acids, which were shown to be compatible with neutral conditions, and which bind glucose in the physiologically relevant range around 20 mM (K_d). By formulating the boronated insulins as traditional zinc (II) hexamers, a steeper glucose sensitivity and release profile could be gained, and this point is important in consideration of the physiological amplification of insulin response to a given glucose challenge. With further development, boronated insulins could lead to improved control of diabetes by use in a glucose responsive release system.

REFERENCES

1. McCormack JG. New therapeutic approaches for diabetes. *Med. Chem. Res.* 2001; **10**: 480–492.
2. Lindholm A. New insulins in the treatment of diabetes mellitus. *Best Pract. Res. Clin. Gastroenterol.* 2002; **16**: 475–492.
3. Home P. The challenge of poorly controlled diabetes mellitus. *Diabetes Metab.* 2003; **29**: 101–109.
4. Brownlee M, Cerami A. Glucose-controlled insulin-delivery system: Semisynthetic insulin bound to lectin. *Nature* 1979; **206**: 1190–1192.
5. Kim SW, Pai CM, Makino K, Seminoff LA, Holmberg DL, Gleeson JM, Wilson DE, Mack EJ. Self-regulated glycosylated insulin delivery. *J. Control. Rel.* 1990; **11**: 193–201.
6. Shiino D, Murata Y, Kubo A, Kim YJ, Kataoka K, Koyama Y, Kikuchi A, Yokoyama M, Sakurai Y, Okano T. Amine-containing phenylboronic acid gel for glucose- responsive insulin release under physiological pH. *J. Control. Rel.* 1995; **37**: 269–276.
7. Hisamitsu I, Kataoka K, Okano T, Sakurai Y. Glucose-responsive gel from phenylborate polymer and poly(vinyl alcohol) — prompt response at physiological pH through the interaction of borate with amine group in the gel. *Pharm. Res.* 1997; **14**: 289–293.
8. Kost J, Langer R. Responsive polymeric delivery systems. *Adv. Drug Deliv. Rev.* 2001; **46**: 125–148.
9. James TD, Shinkai S. Artificial receptors as chemosensors for carbohydrates. *Host-Guest Chem.* 2002; **218**: 159–200.
10. Yang W, Gao X, Wang B. Boronic acid compounds as potential pharmaceutical agents. *Med. Res. Rev.* 2003; **23**: 346–368.
11. Norrild JC. An illusive chiral aminoalkylferroceneboronic acid. Structural assignment of a strong 1 : 1 sorbitol complex and new insight into boronate-polyol interactions. *J. Chem. Soc. Perkin Trans. 2* 2001; 719–726.
12. Asher SA, Alexeev VL, Goponenko AV, Sharma AC, Lednev IK, Wilcox CS, Finegold DN. Photonic crystal carbohydrate sensors: Low ionic strength sugar sensing. *J. Am. Chem. Soc.* 2003; **125**: 3322–3329.
13. James TD, Linnane P, Shinkai S. Fluorescent saccharide receptors — a sweet solution to the design, assembly and evaluation of boronic acid-derived pet sensors. *Chem. Commun.* 1996; 281–288.
14. Zanette D, Soffientini A, Sottani C, Sarubbi E. Evaluation of phenylboronate agarose for industrial-scale purification of erythropoietin from mammalian cell cultures. *J. Biotechnol.* 2003; **101**: 275–287.
15. Altamore TM, Barrett ES, Duggan PJ, Sherburn MS, Szydzik ML. Cavitand boronic acids mediate highly selective fructose transport. *Org. Lett.* 2002; **4**: 3489–3491.
16. Snyder HR, Kuck JA, Johnson JR. Organoboron Compounds II. The Reducing Action of some Organobozonic Acids. *J. Am. Chem. Soc.* 1938; **60**: 105–111.
17. Gardiner SJ, Smith BD, Duggan PJ, Karpa MJ, Griffin GJ. Selective fructose transport through supported liquid membranes containing diboronic acid or conjugated monoboronic acid-quaternary ammonium carriers. *Tetrahedron* 1999; **55**: 2857–2864.
18. Soundararajan S, Badawi M, Kohlrust CM, Hageman JH. Boronic acids for affinity chromatography: spectral methods for determinations of ionization and diol-binding constants. *Anal. Biochem.* 1989; **178**: 125–134.
19. Matsumoto A, Ikeda S, Harada A, Kataoka K. Glucose-responsive polymer bearing a novel phenylborate derivative as a glucose-sensing moiety operating at physiological pH conditions. *Biomacromolecules* 2003; **4**: 1410–1416.
20. Johnson BJB. Synthesis of a nitro benzene boronic acid substituted poly acrylamide and its use in purifying iso accepting transfer RNA. *Biochemistry* 1981; **20**: 6103–6108.
21. Mulla HR, Agard NJ, Basu A. 3-Metoxycarbonyl-5-nitrophenyl boronic acid: high affinity diol recognition at neutral pH. *Bioorg. Med. Chem. Lett.* 2004; **14**: 25–27.
22. Selassie SD, Garg R, Kapur S, Kurup A, Verma RP, Mekapati SB, Hansch C. Comparative QSAR and the radical toxicity of various-functional groups. *Chem. Rev.* 2002; **102**: 2585–2605.

23. Wulff G. Selective binding to polymers via covalent bonds. The construction of chiral cavities as specific receptor sites. *Tetrahedron* 1982; **54**: 2093–2102.
24. Arimori S, Ushiroda S, Peter LM, Jenkins ATA, James TD. A modular electrochemical sensor for saccharides. *Chem. Commun.* 2002; 2368–2369.
25. Alo BI, Kandil A, Patil PA, Sharp MJ, Siddiqui MA, Snieckus V, Josephy PD. Sequential directed ortho metalation-boronic acid cross-coupling reactions. A general regiospecific route to oxygenated dibenzo[b,d]pyran-6-ones related to ellagic acid. *J. Org. Chem.* 1991; **56**: 3763–3768.
26. Ishiyama T, Murata M, Miyaura N. Palladium(0)-catalyzed cross-coupling reaction of alkoxydiboron with haloarenes — a direct procedure for arylboronic esters. *J. Org. Chem.* 1995; **60**: 7508–7510.
27. Vedsø P, Olesen PH, Hoeg-Jensen T. Synthesis of sulfonyl chlorides of phenyl boronic acids. *Synlett* 2004; 892–894.
28. Meldal M. PEGA: A flow stable polyethylene glycol dimethyl acrylamide copolymer for solid phase synthesis. *Tetrahedron Lett.* 1992; **92**: 3077–3080.
29. Schaffer L, Brissette RE, Spetzler JC, Pillutla RC, Ostergaard S, Lennick M, Brandt J, Fletcher PW, Danielsen GM, Hsiao K, Andersen AS, Dedova O, Ribel U, Hoeg-Jensen T, Hansen PH, Blume AJ, Markussen J, Goldstein NI. Assembly of high-affinity insulin receptor agonists and antagonists from peptide building blocks. *Proc. Natl. Acad. Sci. USA* 2003; **100**: 4435–4439.
30. Brandt J, Andersen AS, Kristensen C. Dimeric fragment of the insulin receptor alpha-subunit binds insulin with full holoreceptor affinity. *J. Biol. Chem.* 2001; **276**: 12 378–12 384.
31. Matteson DS, Man HW. Hydrolysis of substituted 1,3,2-dioxaborolanes and an asymmetric-synthesis of a differentially protected syn,syn-3-methyl-2,4-hexanediol. *J. Org. Chem.* 1996; **61**: 6047–6051.
32. Malan C, Morin C, Preckher G. Two reducible protecting groups for boronic acids. *Tetrahedr Lett.* 1996; **37**: 6705–6708.
33. Kurtzhals P, Havelund S, Jonassen I, Kiehr B, Larsen UD, Ribel U, Markussen J. Albumin-binding of insulins acylated with fatty-acids — characterization of the ligand protein-interaction and correlation between binding-affinity and timing of the insulin effect *in-vivo*. *Biochem. J.* 1995; **312**: 725–731.
34. Myers SR, Yakubumadus FE, Johnson WT, Baker JE, Cusick TS, Williams VK, Tinsley FC, Kriauciunas A, Manetta J, Chen VJ. Acylation of human insulin with palmitic acid extends the time action of human insulin in diabetic dogs. *Diabetes* 1997; **46**: 637–642.
35. Springsteen G, Wang B. A detailed examination of the boronic acid-diol complexation. *Tetrahedron* 2002; **58**: 5291–5300.
36. Springsteen G, Wang B. Alizarin Red S as a general optical reporter for studying the binding of boronic acids with carbohydrates. *Chem. Commun.* 2001; 1608–1609.
37. Arimori S, Ward CJ, James TD. A D-glucose selective fluorescent assay. *Tetrahedron Lett.* 2002; **43**: 303–305.
38. GraphPad Prism 4.01. GraphPad Software, San Diego, CA, USA (www.graphpad.com).
39. Auzanneau FI, Christensen MK, Harris SL, Meldal M, Pinto BM. Synthesis and characterization of polyethylene glycol polyacrylamide copolymer (PEGA) resins containing carbohydrate ligands. Evaluation as supports for affinity chromatography. *Can. J. Chromatogr.* 1998; **76**: 1109–1118.
40. Ballerstaedt R, Ehwald R. Suitability of aqueous dispersions of dextran and concanavalin A for glucose sensing in different variants of the affinity sensor. *Biosensors Bioelectron.* 1994; **9**: 557–567.
41. Li YC, Larsson EL, Jungvid H, Galaev IY, Mattiasson B. Separation of neoglycoproteins with different degrees of glycosylation by boronate chromatography. *Chromatographia* 2001; **54**: 213–217.
42. Shiino D, Murata Y, Kataoka K, Koyama Y, Yokoyama M, Okano T, Sakurai Y. Preparation and characterization of a glucose-responsive insulin-releasing polymer device. *Biomaterial* 1994; **15**: 121–128.
43. Henquin JC. Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* 2000; **49**: 1751–1760.
44. Song SH, Kjems L, Ritzel R, McIntyre SM, Johnson ML, Veldhuis JD, Butler PC. Pulsatile insulin secretion by human pancreatic islets. *J. Clin. Endocrinol. Metab.* 2002; **87**: 213–221.
45. Tolic IV, Mosekilde E, Sturis J. Modeling the insulin-glucose feedback system: The significance of pulsatile insulin secretion. *J. Theor. Biol.* 2000; **207**: 361–375.
46. Naithani VK, Gattner HG. Preparation and properties of citraconylinsulins. *Biol. Chem. Hoppe-Seyler* 1982; **363**: 1443–1448.
47. Asada H, Douen T, Mizokoshi Y, Fujita T, Murakami M, Yamamoto A, Muranishi S. Stability of acyl derivatives of insulin in the small intestine: relative importance of insulin association characteristics in aqueous solution. *Pharm. Res.* 1994; **11**: 1115–1120.
48. Fabry M, Brandenburg D. Design and synthesis of a novel biotinylated photoreactive insulin for receptor analysis. *Biol. Chem. Hoppe-Seyler* 1992; **373**: 143–150.
49. Dodson EJ, Dodson GG, Hubbard RE, Moody PCE, Turkenburg J, Whittingham J, Xiao B, Brange J, Kaarsholm NC, Thogersen H. Insulin assembly — its modification by protein engineering and ligand-binding. *Phil. Trans. Roy. Soc. London A* 1993; **345**: 153–164.
50. Liu F, Song SC, Mix D, Baudys M, Kim SW. Glucose-induced release of glycosylpoly(ethylene glycol) insulin bound to a soluble conjugate of concanavalin A. *Bioconjugate Chem.* 1997; **8**: 664–672.
51. Kim YJ, Choi S, Koh JJ, Lee M, Ko KS, Kim SW. Controlled release of insulin from injectable biodegradable triblock copolymer. *Pharm. Res.* 2001; **18**: 548–550.
52. Meldal M, Auzanneau FI, Hindsgaul O, Palcic MM. A PEGA resin for use in the solid-phase chemical-enzymatic synthesis of glycopeptides. *Chem. Commun.* 1994; 1849–1850.
53. Auzanneau FI, Meldal M, Bock K. Synthesis, characterization and biocompatibility of PEGA resins. *J. Peptide Sci.* 1995; **1**: 31–44.
54. Shiota M, Moore MC, Galassetti P, Monohan M, Neal DW, Shulman GI, Cherrington AD. Inclusion of low amounts of fructose with an intraduodenal glucose load markedly reduces postprandial hyperglycemia and hyperinsulinemia in the conscious dog. *Diabetes* 2002; **51**: 469–478.
55. Wang W, Gao XM, Wang BH. Boronic acid-based sensors. *Curr. Org. Chem.* 2002; **6**: 1285–1317.
56. Striegler S. Selective carbohydrate recognition by synthetic receptors in aqueous solution. *Curr. Org. Chem.* 2003; **7**: 81–102.
57. Stones D, Manku S, Lu X, Hall DG. Modular solid-phase synthesis approach to optimize structural and electronic properties of oligoboronic acid receptors and sensors for the aqueous recognition of oligosaccharides. *Chem. Eur. J.* 2004; **10**: 92–100.

