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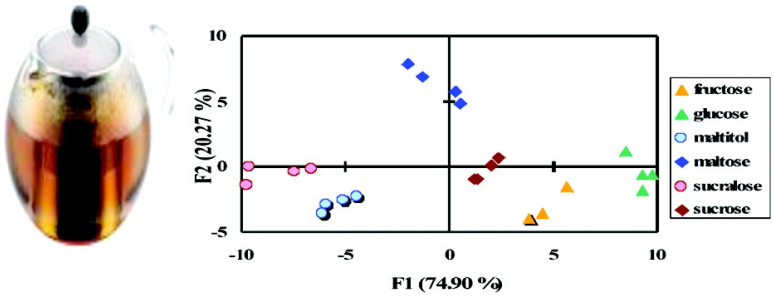
J. Am. Chem. Soc., **2007**, 129 (44), 13575-13583• DOI: 10.1021/ja073939u • Publication Date (Web): 10 October 2007



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Boronic Acid Based Peptidic Receptors for Pattern-Based Saccharide Sensing in Neutral Aqueous Media, an Application in Real-Life Samples

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Abstract: The advent of the alternative sweeteners market has signaled a demand for chemosensors which target multiple saccharides and saccharide derivatives, in aqueous media at physiological pH. This demand has largely been unmet as existing molecular receptors for saccharides have generally not shown sufficient degrees of affinity and selectivity in aqueous media. A chemosensor array for saccharides and saccharide derivatives, fully operational in aqueous media at physiological pH, has been developed and is reported herein. Boronic acid based peptidic receptors, derived from a combinatorial library, served as the cross-reactive sensor elements in this array. The binding of saccharides to these receptors was assessed colorimetrically using an indicator uptake protocol in the taste-chip platform. The differential indicator uptake rates of these receptors in the presence of saccharides were exploited in order to identify patterns within the data set using linear discriminant analysis. This chemosensor array is capable of classifying disaccharides and monosaccharides as well as discriminating compounds within each saccharide group. Disaccharides have also been distinguished from closely related reduced-calorie counterparts. This linear discriminant analysis set was then employed as a training set for identifying a specific saccharide in a real-world beverage sample. The methodology developed here augers well for use in other real-world samples involving saccharides as well as for sensing other desired analytes.

Introduction

There is considerable interest in developing sensors for saccharides due to their broad utility in wide-ranging applications. Saccharide sensors are needed anywhere from the food and cosmetic industries to medicinal and academic arenas. For applicability in biological and medicinal arenas, it is crucial that these sensors operate in aqueous media at physiological pH. Difficulties associated with developing saccharide sensors result from the fact that saccharides contain one kind of recognition unit (the hydroxyl functional group) and that they lack a spectroscopic handle, such as a chromophore or fluorophore, whose modulation can be harnessed in a sensing scheme. Although myriad biosensors[1] have been reported, many lack the durability necessary for widespread usage in real-world settings. By contrast, chemosensors[2] are being increasingly used in real-world settings, offering the advantage of not only greater durability than their biological counterparts but also flexibility in their design: signaling units affording various sorts of readouts (circular dichroism, colorimetric, fluorescent, and electrochemical) are readily incorporated, and their design can be synthetically optimized and modified.

Two main approaches have been employed in the design of synthetic receptors, starting points for the chemosensing of saccharides. The first approach involves the use of supramolecular hosts tailored with complementary hydrogen-bonding binding sites with saccharides primarily in nonaqueous media.[3] This approach has mainly been used in academic arenas in the development of biomimetic systems for studying protein−carbohydrate hydrogen bonding interactions. It has found limited utility in real-world settings, hampered by the difficulty in synthesizing these receptors and effecting molecular recognition in aqueous media.

The boronic acid moiety is featured in the second approach, either appended to elaborate scaffolds or used in simple constructs.[4] Reversible covalent cyclic ester formation of these moieties with diols of saccharides is the basis of this recognition motif. In contrast to the first approach, recognition of saccharides can be readily effected in aqueous media in the basic pH range,

[†] The Department of Chemistry and Biochemistry.
[‡] The McCombs School of Business.

(1) Jelinek, R.; Kolusheva, S. *Chem. Rev.* **2004**, *104*, 5987−6016.
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(4) (a) James, T. D. In *Boronic Acids Preparation, Applications in Organic Synthesis and Medicine: Boronic Acid-based Receptors and Sensors for Saccharides*; Hall, D., Ed.; Wiley-VCH: Weinheim, 2005; pp 441−479. (b) James, T. D.; Phillips, M. D.; Shinkai, S. *Boronic Acids in Saccharide Recognition*; The Royal Society of Chemistry: Cambridge, 2006.

and with additional design elements, at physiological pH. These systems, however, routinely lack a high degree of selectivity for a particular saccharide, with the exception of fructose, due to the promiscuity in reactivity of the boronic acid moiety, and are often difficult to synthesize and purify.

The current challenges are to continue to develop sensors for saccharides which meet the criteria necessary for application in real-world settings. They must be relatively easy to synthesize, operate at constant physiological pH in aqueous media, and target multiple saccharides and saccharide derivatives with high selectivity. The latter, arising from the advent of the alternative sweeteners market, is exemplified by the commonplace usage of saccharide derivatives such as sucralose (Splenda) and various other substitutes in commercial products. Applications of these sensors in an industrial setting include their use in the quality control of food and dietary products and monitoring the fermentation of beverages and extracts.

Pattern-based discrimination of analytes derived from array sensing schemes holds great promise in meeting the challenges faced by new saccharide sensors. This research area, coined as "differential sensing", has emerged as a powerful tool for detecting chemically diverse analytes, discriminating subtly different compounds within each class of analytes, and detecting mixtures thereof.[5] Discrimination is achieved through the use of an array of cross-reactive sensor elements which interact with analytes in a differential fashion, to produce a composite signal or fingerprint for the analyte or mixture of interest. The design principles of array sensing are based on the mammalian olfaction and gustation systems, in which receptors on the tongue or in the nose produce a fingerprint for each odorant/tastant.[6] These receptors are biased toward a particular class (display a reasonable degree of affinity) but do not display high specificity for any particular analyte within that class. These receptors on their own exert little discriminatory power but work in tandem to create the fingerprint or pattern. Thus, moderately selective boronic acid based receptors such as those described previously are well-suited for this approach, but the process of identifying a series of suitable receptors with varying degrees of selectivity for targeted saccharides is complex and time-consuming. Irrespective of this, some studies of pattern-based discrimination of saccharides has been recently achieved. Severin and co-workers have used an indicator displacement assay consisting of a rhodium complex and gallocyanine at different pH values for the discrimination of amino sugars and aminoglycosides.[7] Chang et. al have reported the discrimination of 23 saccharides (mainly monosaccharides and disaccharides) using boric and boronic acids with pH indicators.[8a] Last, Singaram also recently used boronic acid bipyridinium salts to differentiate sugars.[8b] But, in these three cases real-world samples of unknowns were not analyzed.

In this paper, we report the development of a novel chemosensor array for saccharides and saccharide derivatives which operates in aqueous media at physiological pH. Boronic acid based pentapeptidic receptors, derived from a combinatorial library, served as the cross-reactive sensing elements in the array. A randomly chosen set of receptors from this library were incorporated in the taste-chip platform[9] and used for targeting saccharides (sucrose, maltose, fructose, and glucose) and saccharide derivatives (sucralose and maltose). The sensory and discriminatory properties of this chemosensor array were assessed using the linear discriminant data set of responses. In this detailed study, the very first set of randomly chosen receptors, without any subsequent sequence characterization, were found sufficient to discriminate monosaccharides from disaccharides, and discrimination of the isomers within each saccharide group was achieved. The discrimination of disaccharides and their closely related reduced calorie counterparts was also achieved. Importantly, we demonstrated that the use of linear discriminant analysis, with these saccharides and saccharide derivatives as a training set, led to the identification of sucralose in a real-world beverage sample. This is different from simply distinguishing complex mixtures such as coffee and tea; rather the identification of a specific analyte in the mixture is the focus. Due to the success of the approach, we questioned if a second set of randomly chosen receptors could perform a similar analysis. Indeed we found in a quick and less detailed study that a totally new set of receptors could discriminate some of the same saccharides, as well as completely new structures. The lesson is that the approach is very general and applicable to a wide range of carbohydrates.

Results and Discussion

Design, Synthesis, and Characterization of Pentapeptide Library 1. The pentapeptide library **1** (theoretical diversity = 19^5) containing all randomized positions was constructed on tentagel resin using standard Fmoc/TBTU protocols and the "split and pool" method (Figure 3).[10] Tentagel was the resin of choice due to its chemical robustness and excellent swelling properties in a variety of solvents. All natural amino acids (except lysine and cysteine) and diamino butanoic acid (DAB) were used as building blocks for this library. DAB, a lysine derivative bearing a side chain with two less carbon atoms, was expected to reduce the conformational flexibility of the pentapeptide, hypothesized to aid in the recognition of analytes. It is for that reason that this synthetic amino acid was chosen as the entry point to the phenylboronic acid moiety via the reductive amination with 2-formylphenyl boronic acid. DAB was present in three times the concentration of all other amino acids at the start of the synthesis and in subsequent coupling steps. Consequently, although not changing the diversity of the library, the probability of DAB being designated as the randomized amino acid in the library increased from 23% to 54%. This was done to ensure that the phenylboronic acid moiety was adequately represented in the library. The phenylboronic acid moiety (Figure 1, right panel) has a benzylic amine in the ortho position which undergoes hydrolysis to form a

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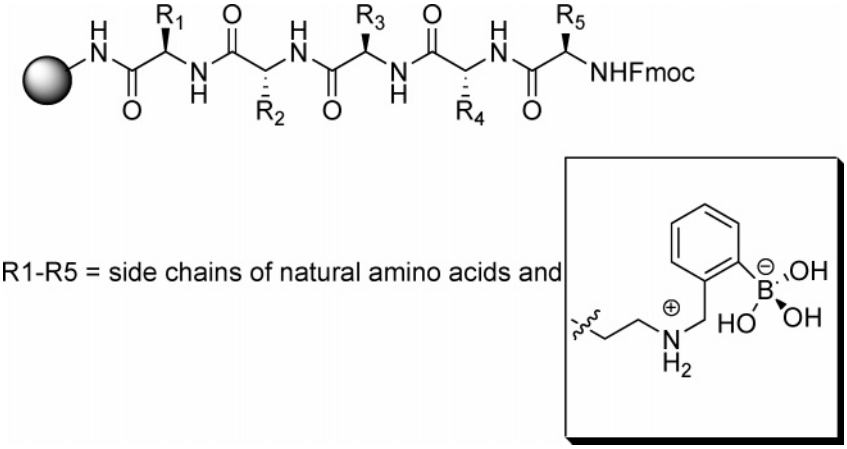

Figure 1. General structure of receptors from combinatorial pentapeptide library **1**.

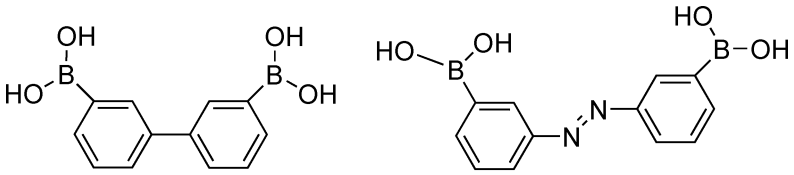

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Figure 2. Receptors **2** and **3**.

zwitterionic amino boronate species, facilitating cyclic ester formation with saccharides in aqueous solution at physiological pH.[11]

By introducing the phenylboronic acid moiety in a combinatorial fashion, the number of these moieties and their position on the peptide chain was varied. These variations were expected to result in the main differential response of these receptors in an array setting. Intuitively, this should be the case, but receptors in which two boronic acids moieties are arranged in different orientations provide elegant examples of this concept. Binding constants of monosaccharides with receptor **1** (Figure 2) are significantly larger than the binding constants of disaccharides.[12] This receptor forms 1:1 complexes with monosaccharides via cyclic ester formation with two diol groups on opposite ends of the saccharide, termed the head-to-tail orientation. The distance between the boronic acid moieties of receptor **2** is too small for head-to-tail association with disaccharides, and this is manifested in lower binding constants. Receptor **3**, on the other hand, in which the distance between boronic acid moieties is larger than that in receptor **2**, participates in head-to-tail association with disaccharides and gives larger binding constants with disaccharides compared to monosaccharides.[13] Additional differential responses could result from the participation of saccharides in cyclic ester formation with boronic acid moieties and in noncovalent interactions with other groups on neighboring peptides of the same resin bead. For example, saccharides are expected to participate in hydrogen bonding interactions with polar amino acid side chains or with the amides on the protein backbone. X-ray crystallographic data and solution-phase studies of protein−carbohydrate complexes demonstrate that carbohydrate recognition by proteins is mediated by an intermolecular hydrogen-bonding network consisting of polar amino acid side chains[14] and/or the amides of the peptide backbone[15] with the hydroxyl groups and oxygen atoms of saccharides. Hydrophobic contacts between amino acids possessing aromatic side chains with nonpolar groups of saccharides also govern protein−carbohydrate recognition.[16] The combined effects of these variations in **1** was intended to result in the overall differential response of these receptors.

To optimize the reductive amination step in the synthesis of library **1** and to characterize the receptors from this library, model pentapeptide **4** (Scheme 1) was prepared. It consists of a central DAB that is flanked by a mixture of polar and hydrophobic residues and was synthesized on tentagel resin containing an *o*-nitro benzyl linker. This linker could be cleaved photolytically and permit solution-phase characterization of the peptide. Pentapeptide **5** (Scheme 1) was synthesized by a modification of the method described by Meldal and coworkers.[17] The optimal conditions which were obtained by us for the reductive amination of model pentapeptide **4** with 2-formylphenyl boronic acid are shown in Scheme 1. 2-Formylphenyl boronic acid (7 equiv) was added to **4**, and the reaction proceeded at 50 °C for 1 h. This condensation step was repeated

(11) Zhu, L.; Shabbir, S. H.; Gray, M.; Lynch, V. M.; Sorey, S.; Anslyn, E. V. *J. Am. Chem. Soc.* **2006**, *128*, 1222−1232.
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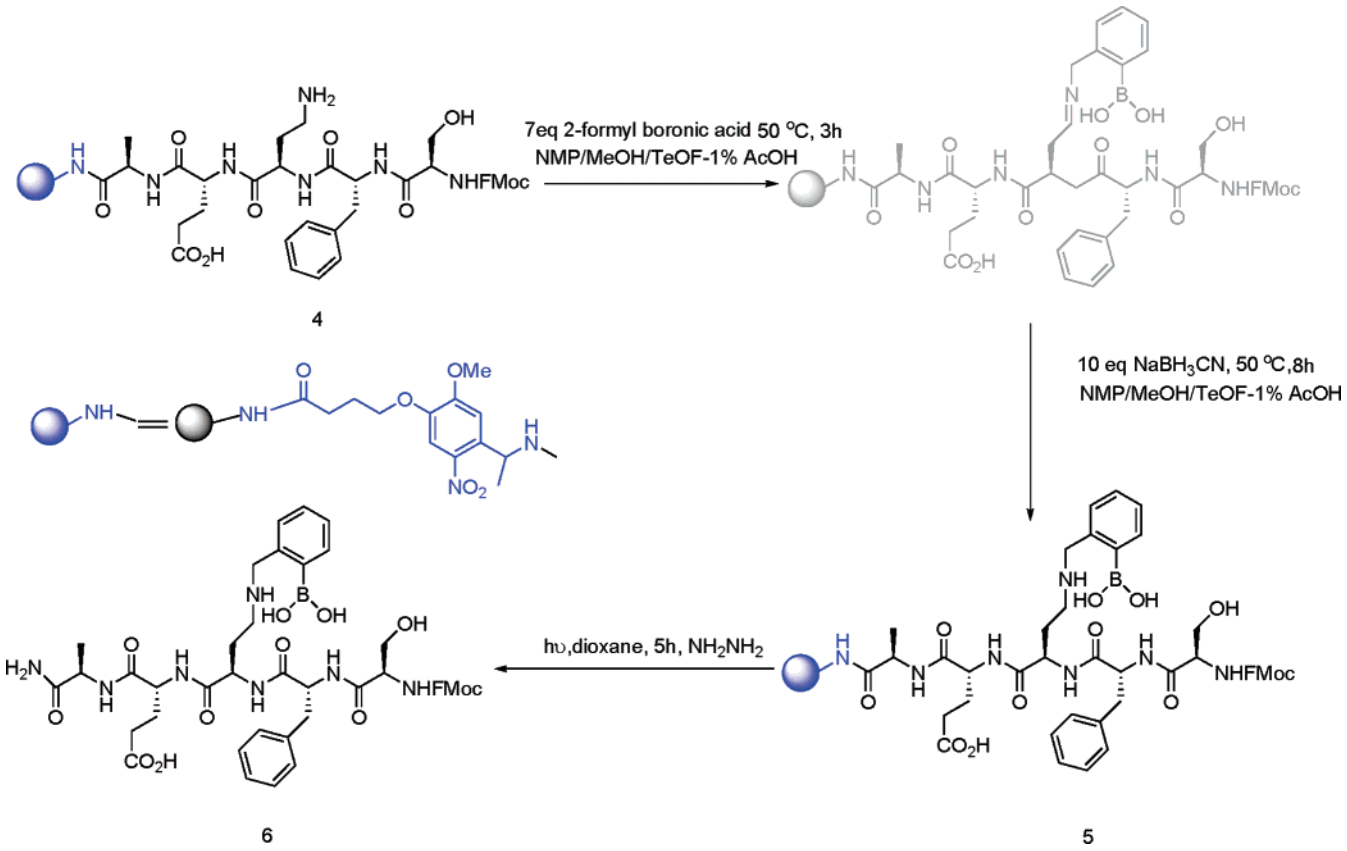
Scheme 1. Synthesis of Modified Model Pentapeptide **5**



twice in order to drive imine formation to completion. A product which was negative to the ninhydrin test, presumed to be resin-bound peptide **5**, was obtained following the addition of $NaBH_3CN$ (10.5 equiv) at 42 °C overnight. A solvent system consisting of 1:1:1 (NMP/MeOH/TEOF) in the presence of 1% AcOH was used for the reaction. This solvent system proved to be effective for the reductive amination as it contains an excellent solvent for swelling the resin beads (220 μM for tentagel) (NMP), a solvent for removing the water formed as a byproduct in the reaction (TEOF), and a solvent which is common in classical reductive amination schemes (MeOH). Finally, photolytic cleavage of the C-terminus peptide amide from the resin in dioxane in the presence of hydrazine yielded a product of 922.4 *m*/*z* (LC-ESI/MS), corresponding to **6** in 74% yield (HPLC). The MALDI-TOF MS/MS fragmentation pattern of **6** was also consistent with the amino acid sequence of this peptide.[18]

To obtain characterization of the resin-bound reductive amination product, Edman degradation was carried out prior to photolytic cleavage of resin-bound peptide **5**. Key features of the Edman degradation (cycle 3) of this resin-bound peptide were the disappearance of the starting phenylthiohydantoin (PTH)-derivatized DAB and the emergence of hydrophobically shifted peaks of low intensity (Supporting Information). Concrete assignments of the structures of these PTH-derivatized products cannot be made, but it can be speculated that they are remnants of a more intense peak due to breakdown of the PTH-derivatized reductive amination product which is not stable under Edman degradation conditions.[19]

Following the synthesis of library **1** using the optimized reductive amination conditions, Edman degradation was used to characterize this library. Sequencing results and the solution-phase studies of the model system, taken together, provided a means to do this. The low-intensity peaks which were observed in Edman degradation cycle 3 of the model pentapeptide were regarded as spectroscopic signatures of the desired secondary amine, and therefore, their presence in the cycles of Edman degradation indicated that DAB had been successfully modified.

Creation of the Saccharide Training Set. The completion of the synthesis facilitated the evaluation of the sensing and discriminatory properties of an array of resin-bound receptors (RBRs) from library **8** toward various saccharides and saccharide derivatives. The University of Texas taste-chip platform,[9] coupled with an indicator uptake signaling protocol,[20] was used to generate responses from this array toward this group of analytes. These responses were then analyzed using linear discriminant analysis (LDA).[21] With the analysis of real-world

(18) For references on the characterization of peptides by mass spectrometry (ESI and MALDI), see for example: (a) Truhlar, S. M. E.; Croy, C. H.; Torpey, J. W.; Koeppe, J. R.; Komives, E. A. *J. Am. Soc. Mass. Spectrom.* **2006**, *17*, 1490−1497. (b) Zhao, Y.; Lin, Y. H. *Proteomics* **2005**, *5*, 853−855. (c) Wang, X.; Zhu, Y.-L. *Shengwu Jishu Tongxun* **2006**, *17*, 465−467. (d) Yamaguchi, M.; Nokihara, K. *Shimadzu Hyoron* **1996**, *53*, 43−53. (e) Tomoya; K.; Masayuki; K.; Ohashi, M. *Yuki Gosei Kagaku Kyokaishi* **1996**, *54*, 375−383. (f) Gillece-Castro, B. L.; Stults, J. T. *Methods Enzymol.* 1996, 271 427−448.

(19) The instability of ornithine, a lysine derivative bearing a side chain of one less carbon atom, to Edman degradation conditions should be noted. See for example: Walk, T. B.; Sussmuth, R.; Kempter, C.; Gnau, V.; Jack, R. W.; Jung, G. *Biopolymers* **1999**, *49*, 329−40.

(20) (b) Wright, A. T.; Anslyn, E. V.; McDevitt, J. T. *J. Am. Chem. Soc.* **2005**, *127*, 17405−17411. (c) Wright, A. T.; Griffin, M. J.; Zhong, Z.; McClesky, S. C.; Anslyn, E. V.; McDevitt, J. T. *Angew. Chem., Int. Ed.* **2005**, *44*, 6375−6378.

(21) Rencher, A. C. In *Methods of Multivariate Analysis*; Bloomfield, P., Cressie, N. A. C., Fisher, N. I., Johnstone, I. M., Kadane, J. B., Ryan, L. M., Scott, D. W., Silverman, B. W., Smith, A. F. M., Teugels, J. T., Eds.; Wiley-Interscience: New York, 2002; pp 156−247, 270−298.

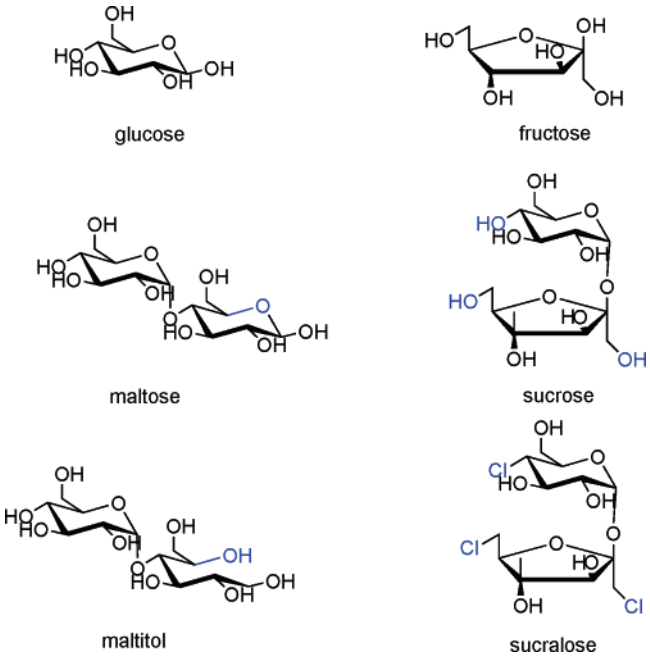


Figure 3. Saccharides and saccharide derivatives used in training set.

samples as the goal in sight, the aim was to create a training set which provided a good representation of saccharides and saccharide derivatives in commercial products, yet a challenging test of the discriminatory properties of this array of receptors. To this end, we chose monosaccharides (fructose and glucose) and disaccharides (sucrose and maltose) which have traditionally been used as sweeteners in commercial products including beverages. We also selected saccharide derivatives (maltitol and sucralose) which are used ubiquitously as low-calorie alternatives in the billion-dollar weight loss industry (Figure 3).

The objective of this study was to determine if this array could classify monosaccharides and disaccharides and whether the discrimination of isomers within each of these saccharide groups was possible. A further objective was to determine whether traditional sweeteners could be distinguished from their closely related low calorie alternatives. Namely, maltose and maltitol whose structural difference is equated to the difference between a CHO and a CH_2OH group as well as sucrose and sucralose where three hydroxyl groups have been replaced by Cl atoms. This training set comprised of colorimetric responses from these saccharides and saccharide derivatives was ultimately used for identifying saccharides in real-world beverage samples.

Generation of Colorimetric Responses for Saccharides Using Receptors from Library 1 in Conjunction with an Indicator Uptake Protocol within the Taste-Chip Platform. Although presented in detail elsewhere,[9] a brief description of the taste-chip platform is given here. At the core of the taste-chip platform is a silicon wafer chip anisotropically etched to consist of an array of inverted cavities. These truncated cavities house individually addressable beads that are spatially isolated in array configurations of 3×4 and 5×7. The array is put in a microfluidics flow cell consisting of a pair of polymethyl methacrylate (PMMA) discs fitted with peek tubing. The PMMA discs permit the continuous flow of solutions from an external fluid delivery system to each cavity in the array. This microfluidics flow cell is placed on top of a stereoscope equipped with a Charge-Coupled Device (CCD) camera. Colorimetric data in the form of Red Green Blue (RGB) pixels are obtained for each cavity in the array by illuminating the bottom of the flow cell with white light from the CCD camera.

An indicator-uptake assay (Figure 4) was used to assess the binding of saccharides to RBRs within the taste-chip platform. Randomly selected RBRs (30) from library **1** and *N*-acetylated blank resin beads (5) were placed in the 5×7 microchip array and sealed in the microfluidics flow cell. Each resin bead was then given an index number from 1 to 35. Henceforth, RBR followed by a number refers to a unique RBR which was placed in that indexed slot in the array, spatially isolated from all other RBRs. A solution of saccharide (80 μM in 500 μM HEPES Buffer at pH =7.4 in water) was delivered to the flow cell at a rate of 0.25 mL/min. This was followed by a brief buffer wash and delivery of an indicator (8 μM in 500 μM HEPES buffer at pH = 7.4 in water) at a flow rate of 0.5 mL/min. Presumably, during analyte delivery, saccharide binds to a certain number of sites on the RBRs. The buffer wash removes any weakly bound saccharides, and the indicator will then bind to the remaining unoccupied sites on these RBRs. The rate of indicator uptake of each receptor is generally regarded to be an indirect measure of the affinity of the targeted saccharide for that RBR. Snapshots of the array were taken at 5 s intervals during the indicator delivery stage using the CCD camera. Extensive acid and base washes (300 mM HCl for 80 min at 2 mL/min and 150 mM NaOH for 10 min at 2 mL/min) were then done to remove the analyte and indicator bound to the RBRs. In so doing, the system was regenerated for further analysis. Acid/base washes of this sort have been effective in our previous studies, showing that the bead−receptor conjugates are stable to such conditions.[9,20] Four experimental replicates were obtained for each saccharide used in the training set. Absorbance data were extracted from the CCD images by defining an area of interest (AOI) for each resin bead in the array at the time intervals at which the snapshots were taken. RGB intensities ($I_{R,A}$, $I_{G,A}$, $I_{B,A}$ for RBRs and $I_{R,N}$, $I_{G,N}$, $I_{B,A}$ for *N*-acetylated blank resin beads) are obtained for each AOI and converted to absorbance values (A_R, A_G and A_B) using the following equations:

$$A_R \approx -\log\{I_{R,A}/I_{R,N}\} \quad (1)$$

$$A_G \approx -\log\{I_{G,A}/I_{G,N}\} \quad (2)$$

$$A_B \approx -\log\{I_{B,A}/I_{B,N}\} \quad (3)$$

Bromopyrogallol red (BPR) (**7**) was chosen as the indicator as it contains a *cis* 1,2 diol and hence is capable of forming cyclic esters with boronic acid moieties of the receptors. It displayed non-complex kinetics with acid and base in the absence of analytes and was easily removed via acid and base washes.

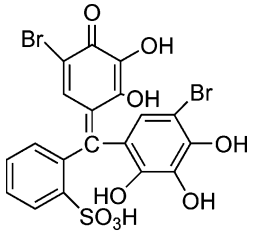


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Figure 4. General scheme of the indicator uptake assay: saccharide (represented as white hexagons) is introduced to the RBRs in each of the pyramidal pits in the first step, followed by indicator (represented as pink hexagons) in the second step. Saccharide and indicator are then removed from the RBRs using acid and base washes.

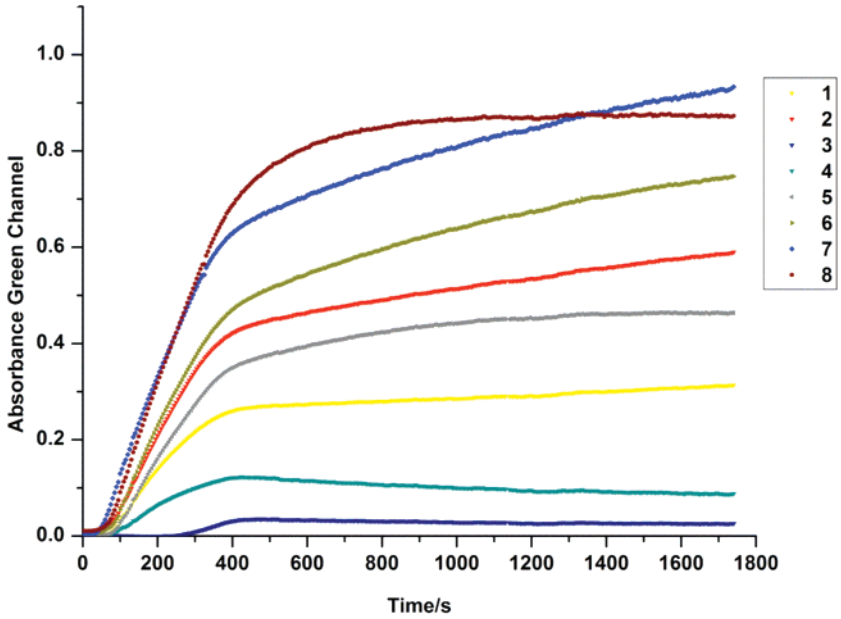


Figure 5. Differential indicator uptake displayed by eight receptors with BPR (8 μM) in the presence of glucose (80 μM).

Kinetic Analysis and General Trends. Images obtained of the microarray for the indicator uptake of RBRs in this work were similar for all saccharides/saccharide derivatives, and hence it became mandatory to use chemometric analysis to determine any differences between the targeted saccharides/saccharide derivatives. To select an appropriate chemometric pre-processing algorithm to characterize the response of the sensor array, several mathematical expressions were explored. Curve fitting algorithms (polynomial: 3−5 degrees), an equilibrium measurement (differences obtained between the initial and final absorbance values), and kinetic measurements (rate constant expressions and composites of linear slopes) were all considered. Varying degrees of pattern-based discrimination of the targeted analytes were obtained using these methods (data not shown). We chose to exploit differences in the rate of indicator uptake for the receptors in the presence of all analytes for this purpose. RBRs (15 total) that showed indicator uptake in the presence of at least one of the analytes were selected for kinetic analysis. Pseudo first-order rate constants were approximated for the uptake of bromopyrogallol red by the RBRs by the nonlinear least-squares fitting[22] of the absorbance vs time profiles to the following equation:

$$y = c - a \bullet \exp(-bt) \qquad (4)$$

where y is the absorbance at time t, c is the final absorbance, a is the difference between the final and initial absorbance values, and b is the pseudo-first-order rate constant (k_ψ). An optimization procedure was employed in fitting the absorbance vs time profiles in which the constants obtained were such that the squared difference between the data points of the fit and $c - a \bullet \exp(-bt)$ was minimized.

Under the conditions of the assay described previously, pseudo first order rate constants obtained for the uptake of BPR with the RBRs were determined to vary over the range of an order of magnitude: 0.0012 s^{-1} (RBR **12** in the presence of fructose) to 0.017 s^{-1} (RBR **8** in the presence of glucose). RBRs **3** and **12** displayed the lowest rates of indicator uptake, whereas RBR **5** displayed the highest rates of indicator uptake in the presence of the entire set of analytes. No correlation between the rates of indicator uptake and analytes was ascertained.

A truncated data set obtained for eight RBRs, representative of the entire set of RBRs, was used. Differential indicator uptake kinetics, i.e., different rates of indicator uptake, was observed for all RBRs in the presence of all targeted analytes. A representative absorbance vs time profile of an experimental replicate for the uptake of BPR (8 μM) by RBRs in the presence of glucose (80 μM) is shown in Figure 5. This kinetic behavior was very similar between experimental replicates of the same saccharide group but varied from one saccharide group to the next. This variation in indicator uptake kinetics among saccharide groups is best demonstrated by a histogram (Figure 6, *xz* plane) of average rate constants for the uptake of BPR in the presence of the saccharide groups against RBRs. A unique pattern of average rate constants for the saccharide set was created by each RBR, indicating that each receptor in the array elicited a unique response to the group of saccharides/saccharide derivatives. Of further note in Figure 6 is the *yz* plane of the histogram, depicting average rate constants for the uptake of

(22) Implemented in MATLAB 7.2. This program is available from *www.Mathworks.com*.

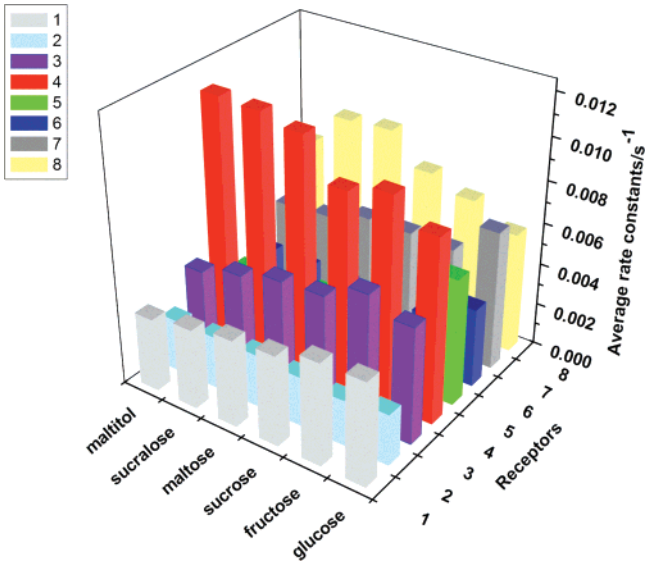


Figure 6. Average rate constants (dimension 1) determined for all receptors for the indicator uptake of BPR in the presence of all analytes versus saccharides/saccharide derivatives (dimension 2) and receptors (dimension 3).

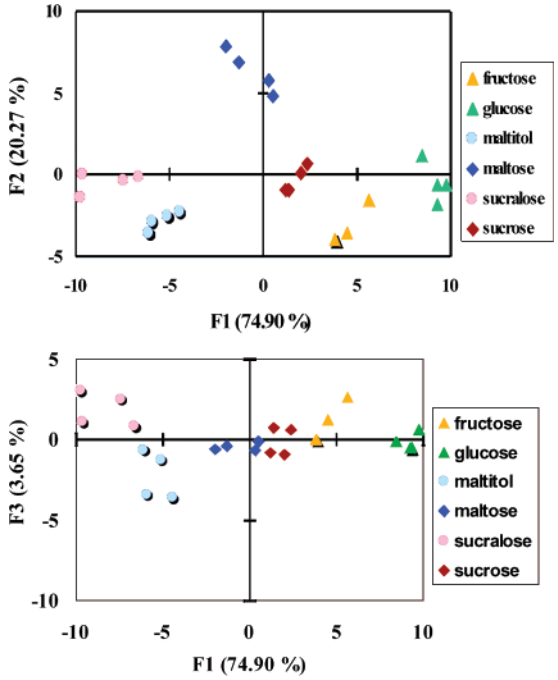


Figure 7. Discriminant scores plots of F2 vs F1 and F3 vs F1.

BPR in the presence of the saccharide groups against the saccharide/saccharide derivatives. Unique patterns of average rate constants were created for all saccharide/saccharide derivatives, and these patterns facilitated evaluation of the data set by LDA.

LDA of Kinetic Data. A training set composed of rate constants for the indicator uptake of BPR with all analytes exhibited by the 15 RBRs was generated. Chemometric analysis was carried out to reduce the dimensionality of the training set in order to identify patterns associated with the analytes. LDA, a multivariate analytical technique aimed at minimizing the separation within the replicates of a group while maximizing the separation between groups, was employed. Functions that are linear combinations of discriminating variables are created and used to describe the existing data set. The number of functions which are created is one less than the number of groups which are being separated. They are of the unstandardized form:

$$Z = A1X1 + A2X2 + A3X3 + ... + AkXk + C \qquad (5)$$

where Z = discriminant score (used to predict group membership), Ak = discriminant weight (measure of the discriminating ability of variable Xk), Xk = discriminating variable, and C = discriminant constant.

When performed in a stepwise manner, variables used to compute the linear functions are selected based on the Wilks' lambda method.[21] Wilks' lambda (Λ) is defined as the ratio of the within-group sum of the squares to the total sum of the squares

$$\Lambda = \frac{\sum (Z_{ij} - \bar{Z}_i)^2}{\sum (Z_j - \bar{Z})^2} \qquad (6)$$

where $\bar{Z}_j$ = mean discriminant score for group j, $\bar{Z}$ = mean of the discriminant score for all the groups, and Z_i = individual discriminant score. Therefore, this procedure chooses the variables that maximize the separation between the groups while minimizing the separation between the observations associated with each group. Wilks' lambda can be approximated to the partial F-statistic of each variable. It is defined as follows:

$$F = 1\left\{\frac{-(\Lambda_{k-1})/(\Lambda k)}{(\Lambda_{k-1})/(\Lambda k)}\right\} \bullet \left\{\frac{(N-g-1)}{(g-1)}\right\} \qquad (7)$$

where Λ = Wilks' Lambda, N = total sample size, g = number of groups, and k = number of discriminant variables in discriminant functions.

Forward stepwise discriminant analysis models of data are governed by specific F value or partial F statistic (F-value in the presence of all other variables) values necessary for variable entry and exit into the model. In the first step, the statistically most significant variable as determined by the univariate ANOVA F-test[23] is entered in the model. In essence, this is the variable in the data set which is best able to discriminate the groups in the absence of all other variables. In subsequent steps, the partial F-statistic (F-value in the presence of all other variables) of each variable is calculated, and those with the largest partial F-statistic enter the model. These are the variables which provide the maximum amount of additional separation in the presence of all other variables. The process is repeated until the partial F-statistic of the variable which is selected is below the threshold F value necessary for entry into the model.

Forward stepwise discriminant analysis was performed using XLSTAT[24] on this training set matrix consisting of analytes (rows) and RBRs (columns) and yielded the following functions (Table 1):

Table 1 reveals that five functions, linear composites of six RBRs, were formed. The maximum amount of the variance (99%) is described by the first three functions; RBRs **7**, **1**, and **14** were determined to be the most discriminating variables in

(23) Johnson, K. J.; Synovec, R. E. *Chemom. Intel. Lab. Syst.* **2002**, *60*, 225. Ciosek, P.; Brzózka, Z.; Wróblewski, W. *Sens. Actuators B* **2004**, *103*, 76.
(24) This program is available from *www.xlstat.com*.

Table 1. Standardized Coefficients of the Discriminant Functions (*Ak* Values)

discriminating variable *resin-bound receptors*	1	2	3	4	5
1	0.877	1.707	0.604	1.607	0.365
3	1.138	3.089	0.347	0.158	0.564
6	0.074	2.013	0.990	1.931	0.604
7	2.321	0.360	0.556	1.910	0.213
10	1.202	1.248	0.423	0.428	0.428
14	1.353	0.387	1.348	1.182	0.074
% variance	79.4	18.5	1.2	0.9	0.2

these functions. Score plots of Function 2 (F2) vs Function 1 (F1) and Function 3 (F3) vs Function 1 (F1) show that excellent classification of all saccharide groups is achieved: large spatial separation between saccharide groups and clustering of experimental replicates within each saccharide group (Figure 7). The extraordinary discriminatory capabilities of this array which was reduced to a mere six RBRs are summarized as follows. Disaccharides and monosaccharides are separated, and discrimination of the isomers within each saccharide group was achieved. Additionally, sucrose and maltose are differentiated from their closely related low-calorie alternatives (sucralose and maltitol). Of further interest is the fact that saccharides/saccharide derivatives are distributed along F1 in accordance with their saccharide group: monosaccharides appear at high F1 discriminant scores, disaccharides appear at intermediate F1 values, and the saccharide derivatives appear at low F1 discriminant scores. F1 is able to discriminate maltose and maltitol whereas F2 and F3 are able to discriminate sucrose and sucralose. The mere fact that axes dependencies are observed bears testimony to the remarkable discriminatory properties of this array. In other words, replacing three hydroxyl groups with three Cl atoms (sucralose and sucrose) and the difference between a CH_2OH and CHO group (maltose and maltitol) have resulted in a large spatial separation between these saccharide groups.

Validation of this training set was carried out using a resubstitution method (reported as a confusion matrix) and a leave-one-out method (reported as a jack-knife matrix).[21] In the former, factor scores associated with each replicate (observation) are submitted to the classification functions and assigned to a group. The number of correct classifications and misclassifications are counted. Using this method, 100% correct classification was achieved.

The leave-one-out method is an iterative method which reports values of a simulation in which all but one of the observations is treated as the training set and the group membership of the omitted observation is predicted based on this training set. This is done by calculating the mean discriminant scores associated with the saccharide groups, termed the function centroids. The closeness of the unknown discriminate scores to those of the function centroids was used to determine the identity of the unknown sample based on this training set. This procedure is repeated until all the observations are classified in this manner. Using the leave-one-out method, 91% correct classification was achieved: two fructose replicates were reassigned to sucrose and glucose groups. The high rates of classification which were obtained using these two methods attested to the soundness of this training set.

Illustration of the Methodology of Identifying Saccharides in Real-World Beverage Samples Using Tea Sweetened with Splenda. The analysis of real-world samples is the ultimate goal of this study. In that vein, a tea sample sweetened with Splenda (contains sucralose) was prepared, and the ability of this training set to predict/identify the saccharide present in this tea sample was tested. This was done by brewing Celestial Seasonings peppermint tea in about 200 mL of boiling distilled water and then adding approximately 60 mg of the contents of a Splenda packet. The tea sample was allowed to cool to room temperature and then diluted to a concentration of approximately 80 μM (in total saccharide content) with 500 mM HEPES buffer at pH = 7.4. Rate constants of the indicator uptake with BPR were obtained in a similar fashion to those obtained for the saccharides and saccharide derivatives. Three experimental replicates of this sample were obtained.

The discriminant analysis of this training set was repeated, but in this instance, rate constants obtained for the tea sample were included as unknown saccharide group membership values. The identification process was carried out in a similar fashion to that used for the leave-one-out method. Discriminant scores of the tea sample in all three experimental replicates were closest to the sucralose group. Based on these findings, the training set validated that the tea was sweetened with Splenda. Given that it was compulsory that this training set designate group membership of the tea sample replicates, a legitimate concern arose as to whether training set predictions were based on the mere chance of sucralose group membership assignment. The chance of sucralose membership assignment of each tea sample replicate is 17%, a percentage which represented the probability of assignment to any of the six saccharide groups present in the training set, in the absence of discriminant score considerations. This relatively low chance percentage for each of the three tea sample replicates dispelled any notion of training set predictions based on chance.

To our knowledge this is the first array sensor that predicts the identity of a sweetner in a real-life beverage. Humans can distinguish artificial sweetners from their natural analogues in taste tests, and commonly it is a matter of acquired taste to like, accept, and, with some people, prefer an artificial sweetener. A trained array, obviously, has no perceptions, biases, or feelings. Currently, the answer from our DA analysis possesses neither quality aspects nor the ability to make a judgment call predicting or mimicking the likely response of a human test panel to a particular sweetener. Such an extension of our work is clearly a future direction that will be attempted by us and others, which will require very sensitive and subtle differentiation of the complex mixture content. For now, we simply note that the concentration of all saccharides used in this study are orders of magnitude lower that the values obtained for the human taste detection threshold for these saccharides.[25]

Extension to Other Saccharides and Saccharide Derivatives. Encouraged by the successful discrimination of the closely related compounds in the training set, assessing the generality of this saccharide chemosensing approach became the next mission. In essence, we sought to answer three questions: First, could a second diverse set of saccharide/saccharide derivatives be targeted? Second, could other sets of randomly selected RBRs

(25) *Compilation of odor and taste threshold values and data*; Stahl, W. H., Ed.; McCormick and Co., Inc.: Baltimore, MD, 1973.

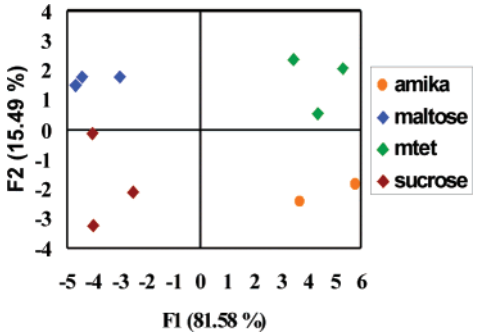
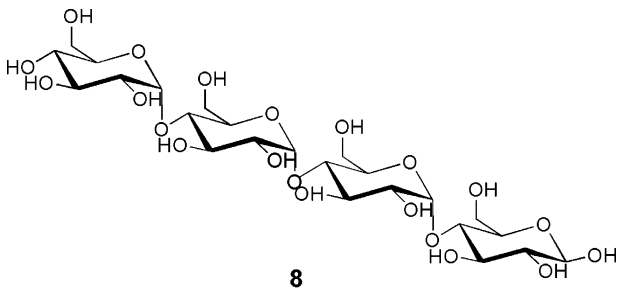


Figure 8. Discriminant score plot of F2 vs F1. Fewer points were collected intentionally to demonstrate a quick analysis and the generality of the approach.

exert discriminatory power similar to the previous set of RBR, or was the discriminatory power limited to a lucky first set of RBRs (peptides with those specific sequences)? Third, is the technique general and robust; i.e., could we rapidly uncover saccharide discrimination without extensive training and array analysis? To answer these questions we undertook another study, but now we intentionally were not as rigorous in the method development. We used another set of randomly selected RBRs from library **1**. The analyte set now included the new targets maltotetraose **8** (a tetrasaccharide, heavily utililized in pharmaceuticals and fine chemical industries due to its low sweetness and superior moisture-retention ability), amikacin **9** (an aminoglycoside, known better as its trademark name Amiglyde-V used to treat severe hospital-acquired infections), and two of the earlier targets, maltose and sucrose. A quickly obtained score



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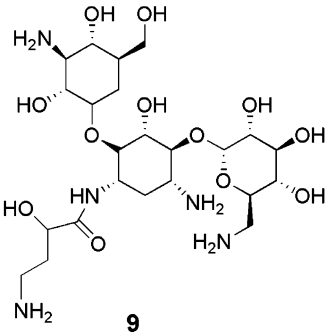

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plot (few replicates) using the same indicator-uptake protocol for this data set (Figure 8) again features classification of all saccharides. Thus, the methodology developed here not only has been successfully implemented in the sensing and a first set of randomly chosen receptors but also was found to perform similarly with a second totally different set of randomly chosen receptors. The implication is that the first set of receptors was not just a fortuitous result but that repeated use of a random set of differential library **1** leads to a general approach for saccharide differentiation. This further means that one does not need to sequence and characterize the structures of the receptors as a means of reproducing the array but instead that one can generally rely on each randomly chosen receptor set to give a new discriminate score plot having an acceptable degree of discrimination.

Summary

A chemosensor was constructed by incorporating the pentapeptidic receptors possessing boronic acids in the taste-chip platform. An indicator uptake protocol with BPR was used to assess the binding of saccharides to these receptors. The patterns obtained by the differential indicator uptake of these receptors in the presence of saccharides were exploited in LDA, which was done to evaluate the discriminatory properties of this chemosensor array. The discriminatory properties are summarized as follows. Monosaccharides and disaccharides were discriminated, and the discrimination within each saccharide group was achieved. Differentiation of disaccharides (sucrose and maltose) and closely related saccharide derivatives (sucralose and maltitol) is also achieved. This LDA data set was then employed as a training set for identifying sucralose in a real-world beverage sample. This was one of the first examples where a specific target in a complex beverage was identified via supramolecular pattern-based sensing, rather than the simpler classification of different beverages such as tea and coffee. Next, to show the generality of the technique, we undertook a second very quick test of the concept with a new set of receptors and analytes and found similar discriminatory possibilities. Hence, the technique is unique to neither one particular set of receptors nor a particular set of analytes. Future designs based on this chemosensor array could involve the use of receptors from directed combinatorial libraries to create more competent chemosensor arrays, extending this methodology to the analysis of other real-world saccharide samples, and targeting other desired analytes.

Acknowledgment. We thank Dr. Klaus Linse for Edman degradation characterization of peptides and Mr. Aravind Gowrisankar for his assistance with MATLAB programming. We would also like to acknowledge Dr. Aaron Wright and Mr. Michael Griffin for their assistance with the acquisition and analysis of data obtained from the taste-chip platform. This work was supported by the Welch Foundation, the National Institute of Health (EB0059), and the Department of the Navy, Office of Naval Research (N00014-05-1857). Any opinions, findings, and conclusions or recommendations expressed in this material are the views of the authors and do not necessarily reflect the views of the Office of Naval Research.

Supporting Information Available: Experimental procedures and characterization of modified peptides (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA073939U