ACS Combinatorial Science

Research Article

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# Screening and Identification of DNA Aptamers to Tyramine Using *in Vitro* Selection and High-Throughput Sequencing

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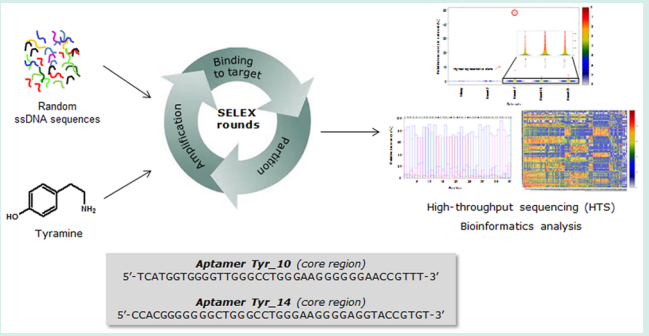
**ABSTRACT:** Aptamers are synthetic single-stranded DNA or RNA sequences that can fold into tertiary structures allowing them to interact with and bind to targets with high affinity and specificity. This paper describes the first selection and identification of DNA aptamers able to recognize the biogenic amine tyramine. To successfully isolate aptamers to this challenging small molecule target, the SELEX methodology was adapted by combining a systematic strategy to increase the selection stringency and monitor enrichment success. As the benefits of applying high-throughput sequencing (HTS) in SELEX experiments is becoming more clear, this method was employed in combination with bioinformatics analysis to evaluate the utility of the selection strategy and to uncover new potential high affinity sequences. On the basis of the presence of consensus regions (sequence families) and family similarities (clusters), 15 putative aptamers to tyramine were identified. A recently described workflow approach to perform a primary screening and characterization of the aptamer candidates by microequilibrium dialysis and by microscale thermophoresis was next leveraged. These candidate aptamers exhibited dissociation constant ($K_d$) values in the range of 0.2−152 $\mu$M with aptamer Tyr_10 as the most promising one followed by aptamer Tyr_14. These aptamers could be used as promising molecular recognition tools for the development of inexpensive, robust and innovative biosensor platforms for the detection of tyramine in food and beverages.




**KEYWORDS:** *DNA aptamers, tyramine, SELEX, high-throughput sequencing, bioinformatics analysis, molecular recognition*

## INTRODUCTION

Aptamers are artificial single-stranded oligonucleotides that can bind to specific macro- and small-molecules with high affinity. To date, aptamers have been generated against a variety of target molecules such as mycotoxins, amino acids, antibiotics, pesticides, proteins and even whole cells.[1−4] They have been used for a wide range of diagnostic, therapeutic and bioanalytical applications.[5−8] Compared to antibodies, aptamers possess several advantages mainly derived from their *in vitro* production, referred to as SELEX (systematic evolution of ligands by exponential enrichment).[9,10] Although many improvements to the original SELEX approach have been introduced over the past decade, typically, all *in vitro* selections make use of an initial chemically synthesized random oligonucleotide library consisting of a multitude of single-stranded (approximately $10^{15}$) different sequence motifs. Next, the library is subject to iterative cycles of incubation with the target, recovery of bound oligos and PCR amplification. Conventionally, at the end of selection, the enriched aptamer pool is cloned into a plasmid and tens to hundreds of individual clones are sequenced using conventional sequencing to identify individual aptamers. Finally, these aptamers are chemically synthesized to measure binding affinity toward the target of interest.[9,10]

One of the major drawbacks of conventional cloning and sequencing are that the final results may lack the most promising aptamer sequences and little to no information can be obtained about the enrichment of the sequences during the selection rounds. As a consequence, this approach may fail to identify high-affinity aptamers. In newly improved SELEX techniques, high-throughput sequencing (HTS), also known as next-generation sequencing (NGS), has been employed many times in place of classic sequencing. HTS enables comprehensive characterization of obtained aptamers, identification of their functional and rare motifs, comparison of functional motifs in each oligonucleotide population, and quantification of their abundance.[11,12] SELEX in combination with HTS, was first applied in 2002 for identification of CTF/NFI transcription factor (TF) ligands in genomic DNA. Since then and especially in the last five years, several researcher groups

**Received:** October 20, 2015
**Revised:** April 8, 2016





DOI: 10.1021/acscombsci.5b00163
*ACS Comb. Sci.* XXXX, XXX, XXX−XXX

Table 1. Main Experimental Conditions Used During the Selection Rounds

| selection round | selection mode | resin volume (μL) | input ssDNA (pmoles) | incubation time with resin (min) | eluting solution |
|---|---|---|---|---|---|
| 1 | negative[a] | 300 | 5000 | 60 | urea[b] |
| 2 | negative | 300 | 450 | 60 | urea |
| 3−7 | negative | 300 | 200 | 60 | urea |
| 8 | counter[c] (histamine) | 300 | 200 | 60 | urea |
| 9 | counter (tryptamine) | 300 | 200 | 60 | urea |
| 10 | counter (phenethylamine) | 300 | 200 | 60 | urea |
| 11 | counter (dopamine) | 300 | 200 | 60 | urea |
| 12 | counter (L-tyrosine) | 300 | 200 | 60 | urea |
| 13 | counter (L-tyrosine) | 300 | 100 | 60 | urea |
| 14 | positive | 300 | 100 | 60 | urea |
| 15 | negative | 300 | 100 | 60 | urea |
| 16 | positive | 200 | 100 | 60 | urea |
| 17−20 | positive | 100 | 100 | 45 | tyramine[d] |
| 21 | positive | 100 | 100 | 30 | tyramine |

[a]Incubation before positive selection. [b]Urea concentration: 6 M at 90 °C. [c]Competitor incubation during positive selection. [d]Tyramine concentration: 0.01 M.

improved the results obtained by different selection approaches, like microfluidic SELEX, semiautomated SELEX, CE-SELEX and capture-SELEX, with the extensive information achieved by HTS analysis.[13−16]

In this work, *in vitro* selection and HTS analysis were combined for the identification of DNA aptamers able to bind the target molecule tyramine. Tyramine is a biogenic amine (BA) mainly produced in fermented food and beverages by the decarboxylation of the amino acid tyrosine carried out by *Enterococcus* and/or *Lactobacillus* microorganisms possessing the enzyme decarboxylases.[17] Tyramine, together with histamine, are considered as the most toxic BAs and particularly relevant for food safety, although recently tyramine has been shown to be more cytotoxic than histamine.[18] Common to all BAs, an excessive oral intake of tyramine can induce adverse reactions, such as nausea, headaches, rashes, and change in blood pressure.[19] In addition, tyramine has been identified as an initiator of hypertension during treatment with monoamino oxidase inhibitor (MAOI) drugs and of dietary-induced migraine in susceptible individuals.[17] The determination of tyramine in food is of significant interest not only due to its possible toxicity, but also because it can be used as an indicator of hygienic quality and food freshness.[20] The determination of BAs are commonly achieved by separation techniques such as high-performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE).[21] Although aptamers have been selected to bind L-tyrosine[22] and L-tyrosinamide,[23] to date no aptamers are available as molecular recognition elements for tyramine. In our work, we perform twenty-one rounds of SELEX to select, for the first time, DNA aptamers targeting tyramine. The use of HTS and bioinformatics analysis allowed us to probe the diversity of the starting library as well as the enrichment evolution of the sequences throughout the selection. We also demonstrated the usefulness of this approach to categorize aptamers based on the presence of consensus regions (sequence families) and family similarities (clusters) and to identify functional sequences. Importantly, a primary screening and characterization of candidate aptamers was carried out to validate the used SELEX approach for successful isolation of novel aptamers.

## RESULTS AND DISCUSSION

### Small Molecule-Specific Aptamer Selection Strategy.

The *in vitro* selection of small molecule-binding aptamers represents an arduous challenge for researchers. While reports of failed small molecule selections are not published, we and others have frequently experienced many challenges for successfully isolating small molecule aptamers.[3,4,22−24] Evidence of the high failure rate associated with small molecule aptamers selection can be seen in the relatively low incidence of small molecule aptamers compared to protein targets and cell targets.[25]

There are two potential routes for improving the selection of high affinity aptamers. One approach is to employ a chemically modified oligonucleotide library. For example, Davies and co-workers have shown that including hydrophobic moieties in their nitrogenous bases greatly improved aptamer affinity, particularly to more challenging targets.[26] Moreover, a novel method, named click-SELEX, has been recently developed to generate nucleobase-modified aptamers with advanced recognition properties.[27] While these strategies are very powerful, the resulting cost of aptamer production is higher. The second approach is to improve the selection strategy and systematically increase stringency. Several groups have investigated and successfully employed different strategies for this purpose.[3,28] In this work, we combined numerous selection improvement strategies to successfully isolate for the first time DNA aptamers able to recognize the biogenic amine tyramine, a small molecule with a MW of 137.18 Da. Specifically, (i) a combination of negative selection rounds and counter selection rounds was employed to ensure that the sequences being enriched, as well as sequences displaying high specificity for the target of interest, were separated from background sequences; (ii) a systematic reduction of the binding incubation times was performed to challenge the library at later selection rounds, (iii) the use of two elution strategies were made to strike a balance between efficient partitioning and isolation of aptamers capable of binding the free target; (iv) finally, 21 rounds of selection were performed to ensure that we could perform all of these steps without losing all potential sequences. Details of our strategy is described below. In classical procedures of small molecule aptamers selection, target molecules have been immobilized on a solid support to allow the binding incubation with the oligonucleotide library. The successful immobilization of the


DOI: 10.1021/acscombsci.5b00163
ACS Comb. Sci. XXXX, XXX, XXX−XXX

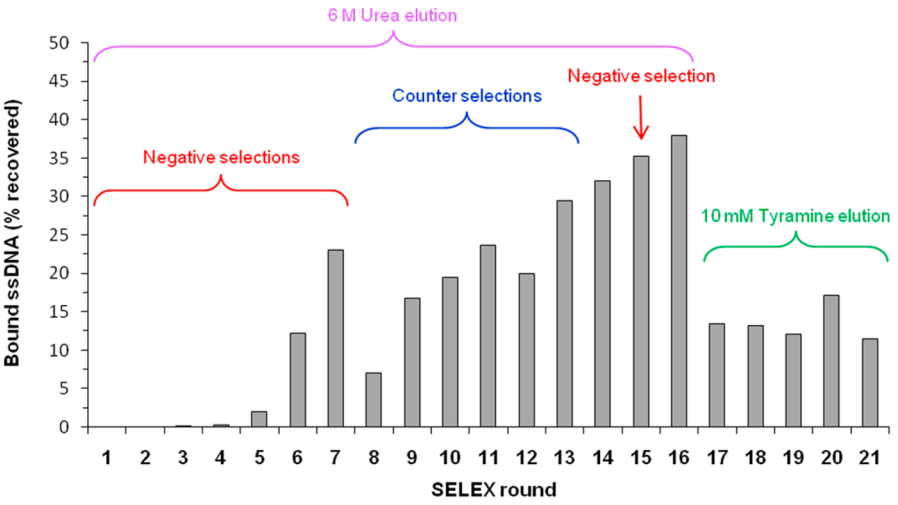



**Figure 1.** Recovery (%) of bound ssDNA to the tyramine-immobilized resin after each selection round.

target to a solid matrix surface is the key for an effective partitioning between binding and nonbinding sequences. Since tyramine contains a primary amino group, a *N*-hydroxysuccinimide (NHS)-preactivated agarose resin was used to form a chemically stable amide linkage. Using this reaction, a coupling efficiency of 20 $\mu$mol of tyramine per mg of dry material was obtained. When using a solid matrix, it is well-known that nonspecific interactions of oligonucleotides with the matrix are possible and often overtake the library. Thus, we employed stringent negative selections with unmodified resin in SELEX rounds 1 to 7 and 15. At the same time, to further increase the specificity of aptamers, the positive resin (agarose coupled with the target) of rounds between 8 and 13 was incubated with a mixture containing the single-stranded DNA (ssDNA) pool and one of the molecules chosen as competitor among the tyramine-amino acidic precursor (i.e., L-tyrosine) or other structurally related (i.e., tryptamine, phenethylamine, dopamine) or commonly occurring (i.e., histamine) biogenic amines. In this manner, sequences showing affinity for the competitors were removed by washing the column with SELEX buffer, while sequences that preferentially bound to the tyramine-immobilized resin were eluted with urea. As previous studies reported,[3,4,29,30] other conditions may be altered to increase the stringency of the selection. For example, in the selection round 1, a high volume of tyramine-immobilized resin and a high amount of ssDNA pool were incubated for 1 h to ensure a complete capture of binding sequences. As the number of rounds increased, the reagent quantities and the binding incubation time were reduced, augmenting competition between sequences (Table 1). By employing the solid-support matrix selection strategy, an efficient partitioning of the library (i.e., complete removal of nonbinding sequences and high recovery of binding sequences) was ensured. In particular, with the target irreversibly immobilized, the above-mentioned stringent washes can be easily employed. Finally, to ensure complete recovery of sequences strongly bound to the target, 6 M urea at 90 °C up to round 16 was used in the elution step. Then, the elution was performed with 0.01 M tyramine in the last five rounds to ensure that the strongly bound sequences also bind to the target free in solution rather than to the only immobilized one (Table 1).

To monitor the progress of our modified selection strategy in real-time, the fluorescence intensity of the eluted oligonucleotide fractions recovered from each round was measured and compared to that measured for the respective input amount of ssDNA incubated with the resin (Figure 1). In general, an enrichment of ssDNA was observed during the SELEX although the bound ssDNA recovered was affected by the stringent conditions applied. From these results, the major enrichment was achieved in round 16 (38%) after which the change of elution conditions negatively affected the amounts of recovered ssDNA that decreased in round 17 (13%) and remained constant until round 21 (11−17%) (Figure 1).

**Evaluating Success of Aptamer Selection through HTS and Bioinformatics Analysis.** A comprehensive study of the starting ssDNA pool properties as well as an assessment of sequence evolution during the selection process was enabled through HTS of the starting library and ssDNA pools collected during the SELEX process. Specifically, we analyzed ssDNA pools from an early enrichment round (round 2), the last round before counter selections (round 7), the last round before elution with target (round 16) and the final round of SELEX (round 21). The bioinformatics analysis of data from HTS allowed to obtain information on the number of sequences of analyzed pools, their length, the distribution of the four bases, the enrichment of sequences during the SELEX process, and finally the sequence similarity. The number of sequences yielded from each DNA pool was calculated to be greater than $4 \times 10^6$. They were sorted into sequence families on the basis of their consensus region. Although the required DNA library was 71 bases long containing a random core region of 40 random nucleotides, the HTS analysis of the starting library showed that the most abundant length of the core region was 40 nucleotides (88%) together to a low percentage (12%) of sequences with a core region slightly longer or shorter than that desired. A suitable ssDNA library should have most of the sequences occurring in a single copy and displaying the proper length and a random region containing an equal distribution of all the four bases.[12] In the starting library, 99.7% of sequences were found to be unique indicating that the initial pool was not affected by strong synthesis and/or sequencing bias. The enrichment of the DNA library throughout the selection rounds was next examined. Of particular interest, the number of unique sequences decreased to 1.9% in round 21 whereas several sequences occurred multiple times. The larger number of repeated sequences suggests that an enrichment of sequences


DOI: 10.1021/acscombsci.5b00163
ACS Comb. Sci. XXXX, XXX, XXX−XXX

**Table 2. Data Resulting from HTS Analysis**

| SELEX round | number of sequences | number of families | unique sequences | most abundant sequence 5′→3′ (ID number) | relative frequency[a] |
|---|---|---|---|---|---|
| Starting library | $1.797 \times 10^6$ | *b* | 99.7% | TGCGGTTGGGCGATCGTAGAGGTAGGCAGGGGGTTGGTTG (1) | 0.02% |
| 2 | $1.001 \times 10^7$ | 102 | 99.2% | TGCGGTTGGGCGATCGTAGAGGTAGGCAGGGGGTTGGTTG (1) | 0.05% |
| 7 | $1.109 \times 10^7$ | 80 | 2.5% | TGCGGTTGGGCGATCGTAGAGGTAGGCAGGGGGTTGGTTG (1) | 47.8% |
| 16 | $4.070 \times 10^6$ | 155 | 3.2% | TCGAGGGGGGTGGGTGGGTATGAGACAGGAGGTGGGGTAT (2) | 3.2% |
| 21 | $9.463 \times 10^6$ | 167 | 1.9% | TCGAGGGGGGTGGGTGGGTATGAGCTGTCGGAGGTGGGGCGT (3) | 4.3% |

[a]Frequency of sequences with respect to the overall number of sequences occurring in the analyzed round. [b]No consensus sequences have been identified.

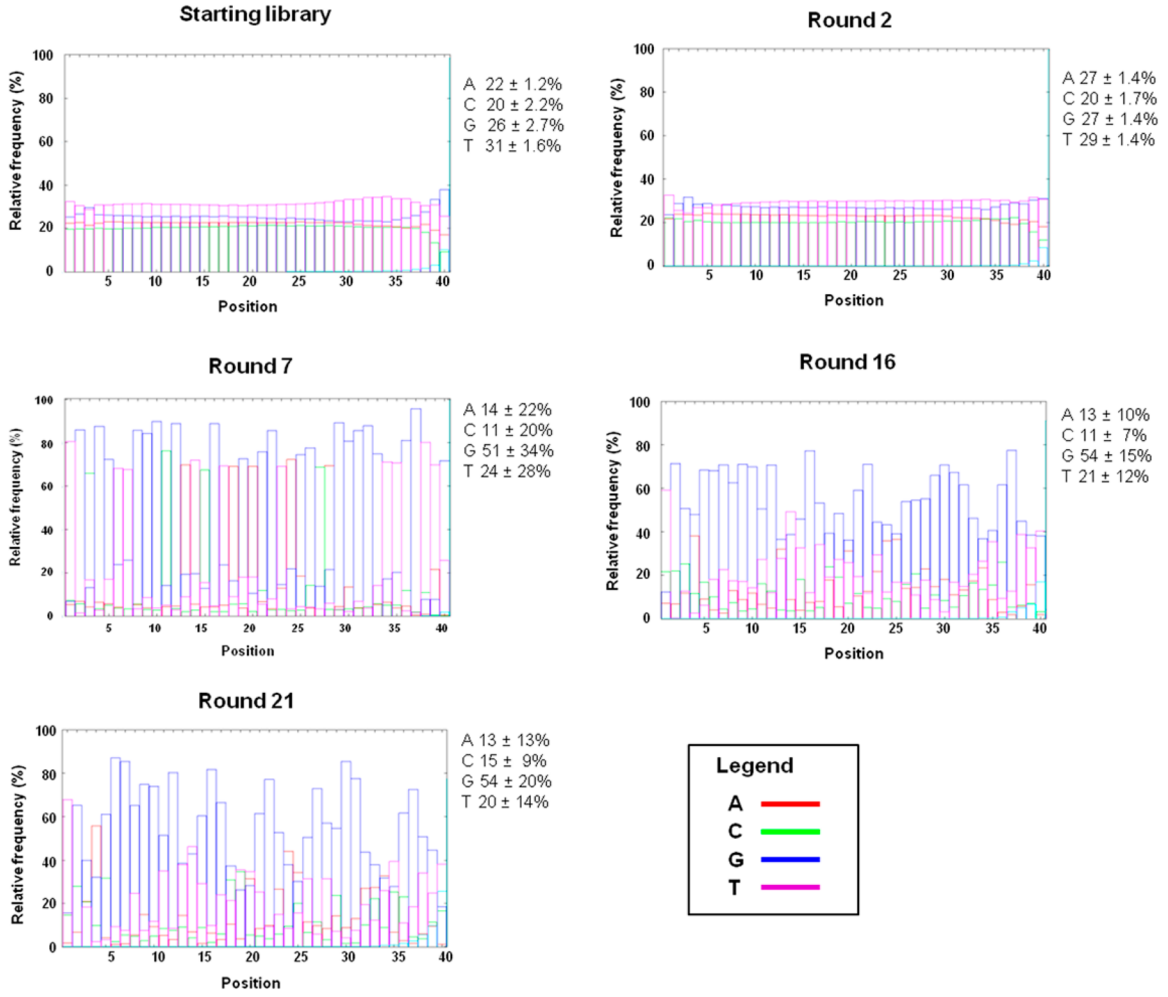



**Figure 2.** Probability of the distribution of the four bases in the random region of starting library and ssDNA pools relatives to rounds 2, 7, 16, and 21. For each round the mean and standard deviation of nucleotides (A, adenine; C, cytosine; G, guanine; T, thymine) is given on the right.

binding to tyramine occurred as the selection progressed. In particular, at the beginning of the selection, sequence 1 was the most representative one with the highest frequency recorded in round 7 (Table 2). However, as a result of the increased stringency conditions applied starting from round 8, the relative frequency of sequence 1 decreased to 1.6% in round 16 and the sequence completely disappeared in round 21. Our first hypothesis is that during the counter selections sequence 1 preferentially bound to one or more molecules used as competitors (such as histamine, tryptamine, phenethylamine, dopamine, or L-tyrosine) rather than to the target tyramine, therefore it was removed during the washing steps. In addition, the copies of the sequence #1 still occurring in the pool did not recognize tyramine used in the elution step of round 21 and were not present in the final pool of ssDNA. On the other hand, the increased incidence of others sequences was observed in the other rounds of selection. For example sequence 2 and sequence 3 showed the highest relative frequencies in rounds 16 and 21, respectively (Table 2). This suggests that the numerous rounds of selection, combined with the inclusion of counter selection strategies was critical for obtaining the desired aptamer sequences.

Another important piece of information obtained from this study was the distribution of the four bases. Figure 2 shows to



DOI: 10.1021/acscombsci.5b00163
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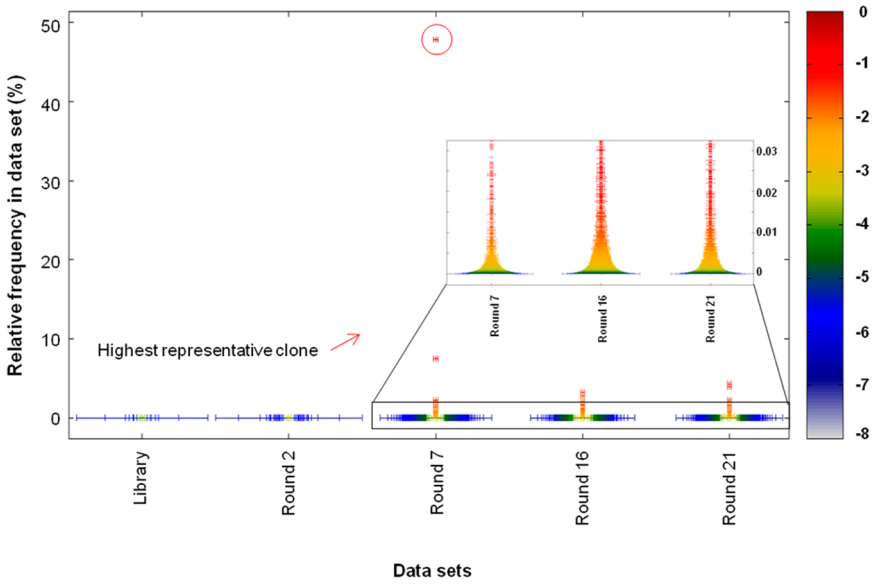


**Figure 3.** Relative frequency of individual sequences obtained by HTS of starting library and ssDNA pools relatives to rounds 2, 7, 16, and 21. The width of the data-bars correlates with the number of clones at the given frequency.

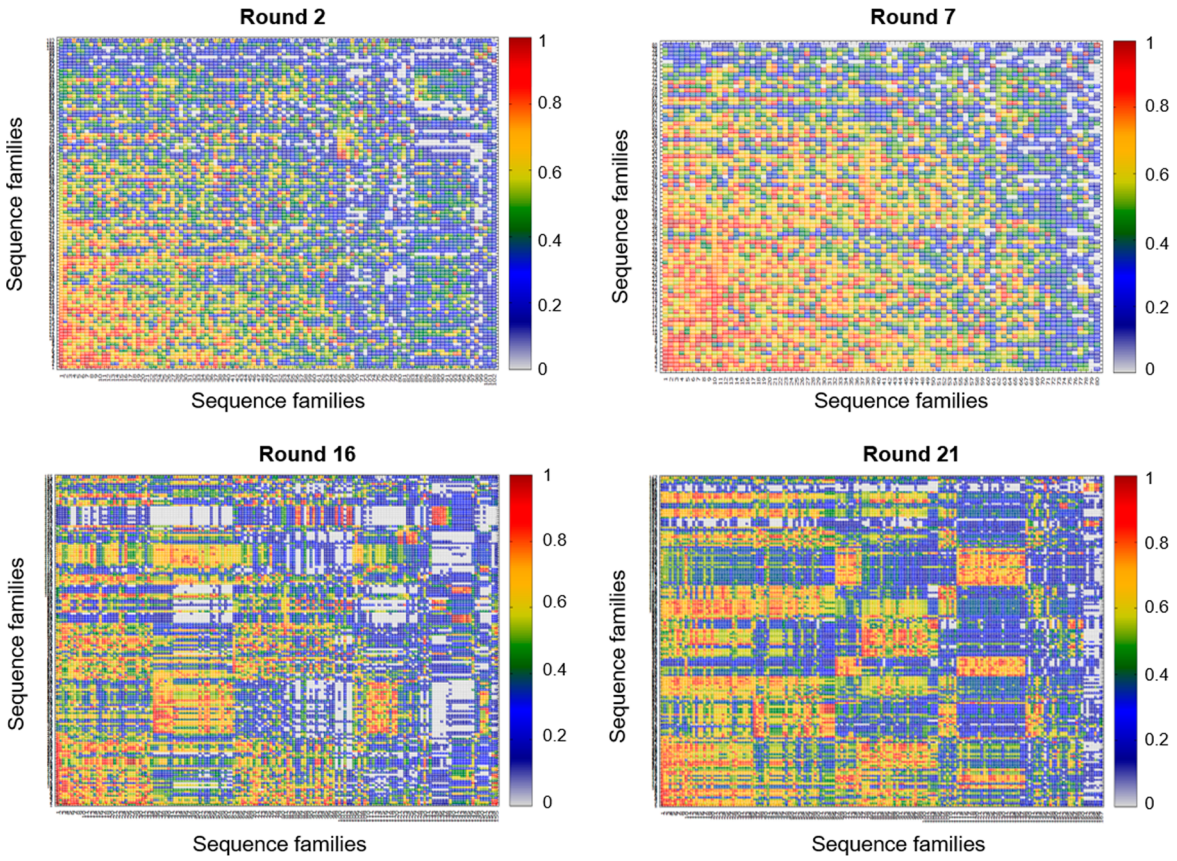


**Figure 4.** Comparative analysis of sequence families. Coloring in the graphs indicates the similarity of sequences from blue (not similar) over yellow to red (very similar).

which probability (*y*-axis) a defined position of the random sequence part (*x*-axis) is occupied by a defined nucleotide. As shown in the figure, the starting library has a quite good random distribution of the four bases (20−31%), with a slight predominance of "T" with respect to the other three bases. During the selection, the distribution considerably changed promoting the predominance of "G" that was over-represented over all positions from the round 7 onward (51%). In the final round, the ssDNA pool contained a high abundance of G (54%) . The high G content reflects a typical feature of several DNA aptamers and it could indicate the presence of G-quadruplex structures that is among the most common ssDNA structures identified by *in vitro* selection.[31] Again, by monitoring the evolution process in this way, we can confirm



DOI: 10.1021/acscombsci.5b00163
*ACS Comb. Sci.* XXXX, XXX, XXX−XXX

Table 3. Sequence Families Chosen As Aptamer Candidates for Binding Studies with Tyramine

| aptamer[a] | core region | relative frequency[b] (%) | $K_d$ ± SD ($\mu$M)[c] |
|---|---|---|---|
| Tyr_1 | TCGAGGGGGGTGGGTGGGTATGAGCTGTCGGAGGTGGGGCGTA | 10.5 | 20.5 ± 28.9 |
| Tyr_2 | TGCACGGGAGGGTTGGGTCTGGGAAGGGAGGGCACCGTGT | 5.4 | 152.4 ± 3.1 |
| Tyr_3 | TGTACGGTGGGGTTGGGCCTGGGAAGGGGGGTATCGTAT | 4.3 | 8.2 ± 8.8 |
| Tyr_4 | GGAGGGGGGTGGTGGGTGTAGGGTGTCGGGAGGAGGGGCT | 3.7 | 11.1 ± 2.7 |
| Tyr_5 | GGAGGGGGGATGGTGGTTTGATGGTGGGTGGGAAGGGGCT | 3.0 | 4.1 ± 0.5 |
| Tyr_9 | GGGGTTGCCGCGAGTAGCGGTCCGGAGTGGGGATGGGGGTGG | 1.8 | 3.7 ± 2.1 |
| Tyr_10 | TCATGGTGGGGTTGGGCCTGGGAAGGGGGGAACCGTTT | 1.7 | 0.2 ± 0.4 |
| Tyr_11 | TAGGGGGGAGTCAGGGTGGGGGTCGAATAGGTATGTGGGG | 1.6 | 1.5 ± 0.3 |
| Tyr_12 | TGCGGTTGGACGATCGTAGAGGTAGGCAGGGGGTTGGTTG | 1.3 | 11.7 ± 7.3 |
| Tyr_14 | CCACGGGGGGGCTGGGCCTGGGAAGGGGAGGTACCGTGT | 0.9 | 0.9 ± 0.6 |
| Tyr_20 | TGGAGGGGGGCGGGTGGGTTCGAGACAGGAGGTGGGGTAT | 0.6 | 1.7 ± 1.3 |
| Tyr_25 | GGCACGGGGGGGCTGGGCCTGGGAAGGGGGGGTACCGTAT | 0.5 | 15.1 ± 16.2 |
| Tyr_39 | AGGGGGGCTGCGGTTTTAAGTTGTGGGGTATTGGGTGGGG | 0.3 | 13.0 ± 2.4 |
| Tyr_30[d] | TGGCGGGTGGGTGGGTGGAATGACCGGAAGGCACAACGCAT | 0.5 | 4.8 ± 0.4 |
| Tyr_114[d] | TGGCGTCGGTGGAACTGGTGGATGGGCGGGACCGGCGGGT | 0.04 | 2.9 ± 2.7 |

[a]The number of sequence families reflects the rank position. [b]Frequency of sequences with respect to the overall number of sequences occurring in round 21. [c]Each $K_d$ value corresponds to the mean of two measurements ± standard deviation. [d]Sequences out of clusters.

that a high number of selection rounds was required to fully enrich the library for sequences with desired affinity.

Further evidence of the enrichment of G-rich sequences was obtained by studying the frequency of individual motifs in the pools. In particular, the analysis of the 15 most abundant motifs with length ranging from 2 to 8 nucleotides showed that T-rich motifs were overrepresented in the starting library whereas a greater incidence of G-rich motifs appeared as the selection progressed. In the final selection round the most abundant 8 nucleotides motif was GGGTGGGT with a frequency of 4 million copies. The diversity of the enriched populations was finally examined by the assessment of evolving sequences abundance during the SELEX process. As expected, starting library and pool of round 2 showed a high number of unique sequences (large blue bar, Figure 3). In the final rounds, a strong enrichment was observed and a greater number of high frequency sequences was achieved (Figure 3). While this HTS analysis could not be performed in real-time, importantly, our data on the population enrichment obtained by the bioinformatics analysis were in agreement with those obtained by measuring the fluorescence intensity of the eluted fractions recovered from each round in real-time. However, the combination of HTS and bioinformatic analyses offered a more comprehensive understanding of results obtained in the present study. Indeed, the most representative sequence of round 7 (relative frequency, 47.8%) (Figure 3) corresponded to the sequence 1 resulted from HTS analysis (Table 2). We suggest that both of these analysis tools are employed in future selections to ensure high-success rate of isolating aptamers to small molecule targets.

**Screening of Candidate Aptamers.** The HTS analysis resulted in more than 1 million sequence from rounds 2, 7, 16, and 21, that were sorted into hundreds of sequence families by bioinformatics analysis. Stepwise reduction of enriched sequences was obtained by clustering the sequence families into clans with defined degree of similarity (Figure 4). Sequences with high degree of similarity were already present at the beginning of the selection (round 2) and an enhancement was visualized in round 7. However, effective clusters appeared from round 16 and became more distinct in the last round of selection (Figure 4).

Thirteen sequence families, that were representative of the largest clusters of round 21, were chosen as candidate aptamers and were screened for their binding affinity toward tyramine. As a control, two additional sequence families out of clusters were also tested.

Recently McKeague and co-workers[32] proposed a workflow providing a cost-effective, efficient and rapid strategy for screening, characterizing and functionally verifying aptamers. This workflow is of particular importance with the increased application of HTS in selection experiments, given the high number of potential sequences that should be screened. In this workflow, first, a rapid binding affinity screening should be performed to provide preliminary information about the binding affinity of the large number of isolated aptamers against our target to strike a balance between reliable high throughput measurement and cost effectiveness. Although the authors recommend either fluorescence polarization, SYBR Green assay, a gold nanoparticles-based (AuNPs) assay, affinity chromatography or surface plasmon resonance, we were able to test and employ a rapid one-point micro equilibrium dialysis assay thanks to the high sensitivity of the HPLC determination of tyramine (limit of quantification 0.73 $\mu$M). For this particular target, our method was useful for screening the potential sequences and provided a quantitative measure of each sequence's affinity. Table 3 shows the sequence families that were selected for binding studies, their absolute frequencies and the $K_d$ values measured. The underlined nucleotides-sequences represent consensus regions.

Overall, the candidate aptamers showed dissociation constants ranging from 0.2 to 152.4 $\mu$M. These values are comparable to those of several small molecule-binding aptamers displaying affinities in the low to mid micromolar range.[24] Despite the broad range of $K_d$ values, there was a high degree of consensus regions among the aptamers. Interestingly, the binding affinity did not reflect the abundance as observed for the aptamers Tyr_1 and Tyr_2 that showed the highest $K_d$ values. We supposed that these sequences are the results of high affinity to partitioning matrix, PCR bias, or sequencing bias. These results were in agreement with those reported in the work of Schütze and co-workers[33] where the most abundant sequences of the final selection round did not directly coincide with the strongest binding properties. While a


DOI: 10.1021/acscombsci.5b00163
ACS Comb. Sci. XXXX, XXX, XXX−XXX

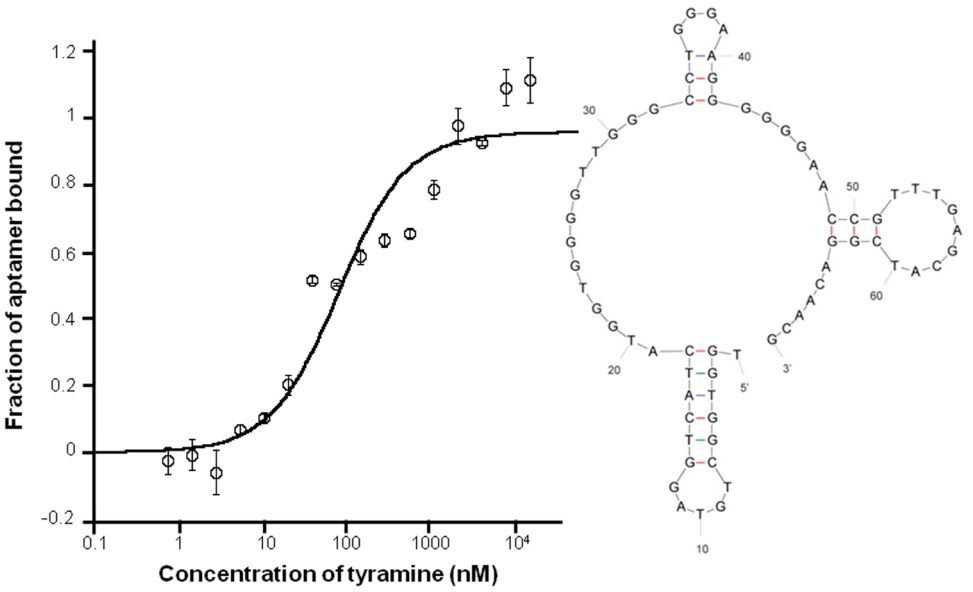


**Figure 5.** Full binding curve obtained with microscale thermophoresis for aptamer Tyr_10 and its corresponding secondary structure predicted by Mfold software.[34]

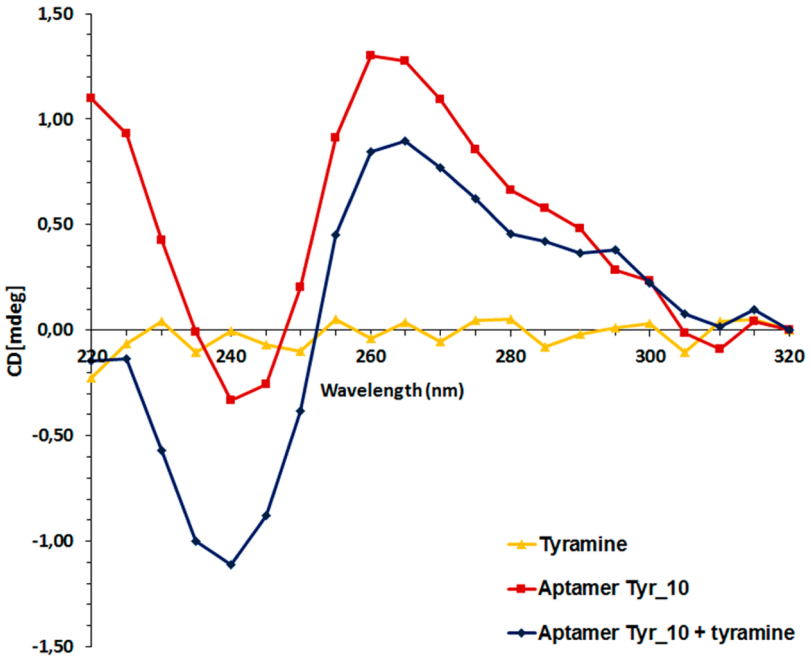


**Figure 6.** Circular dichroism spectra of tyramine (10 $\mu$M), aptamer Tyr_10 (2 $\mu$M) and aptamer Tyr_10 (2 $\mu$M) with tyramine (10 $\mu$M) after incubation at room temperature for 40 min.

large variability of results was observed in the calculated $K_d$, the information obtained from this assay was valuable and indicated the aptamer Tyr_10 as the most promising one with a $K_d$ value of 0.2 ± 0.4 $\mu$M followed by aptamer Tyr_14 with a $K_d$ value of 0.9 ± 0.6 $\mu$M. Therefore, microscale thermophoresis was used to obtain a full binding curve based on the titration of a constant concentration of aptamer (Tyr_10 or Tyr_14) with an increasing concentration of tyramine (from 1.5 nM to 50 $\mu$M). The $K_d$ values calculated by using this approach were 0.097 ± 0.037 $\mu$M for Tyr_10 and 1.25 ± 0.47 $\mu$M for Tyr_14, thus confirming the results obtained with the one point microequilibrium dialysis assay and the high affinity of the aptamer Tyr_10 toward tyramine. Figure 5 shows the full binding curve for aptamer Tyr_10 and its hypothetical secondary structure model (as predicted by Mfold software)[34] that indicates the presence of one large central loop and three small hairpins. It is important to note that the secondary structure software employed here is unable to predict the presence of a G-quadruplex motif. However, a portion of the Tyr_10 sequence matches the pattern d-($G_{3+}N_{1-7}G_{3+}N_{1-7}G_{3+}N_{1-7}G_{3+}$), where N is any base, which is generally used to predict the presence of G-quadruplexes.[35]

Since aptamer Tyr_10 showed the best binding affinity toward tyramine, for further study on conformational information, circular dichroism (CD) spectroscopy was carried out only on it. CD spectra of aptamer Tyr_10 were recorded


DOI: 10.1021/acscombsci.5b00163
ACS Comb. Sci. XXXX, XXX, XXX−XXX

before and after tyramine addition. As shown in Figure 6, the CD spectrum of pure tyramine in the SELEX buffer is near the baseline. The CD spectrum of pure aptamer Tyr_10 displays a typical G-quadruplex parallel structure, which has a characteristic positive peak centered around 260 nm, a negative peak at 240 nm and a small shoulder peak at 290 nm.[36,37] The CD spectrum of aptamer Tyr_10 incubated with tyramine displays the same positive and negative bands but with a slight decreased signal at 260 nm and increased signals at 240 and 290 nm. It could be related to conformational change of the aptamer upon binding with tyramine. In particular, the results seem to suggest that tyramine binding might shift the equilibrium toward aptamer Tyr_10 antiparallel G-quadruplexes structure.[37−39]

The preliminary specificity test for aptamer Tyr_10 was conducted against histamine and tryptamine by single point equilibrium dialysis. Histamine and tryptamine were chosen because they, together with tyramine, are the biogenic amines co-occurring at the highest concentrations in several food categories like cheese, fish sauce and fermented sausages.[17] As compared to tyramine, the aptamer Tyr_10 showed a low specificity for histamine (5 ± 1%) and tryptamine (15 ± 5%).

Following the results obtained herein, further experiments could be carried out to determine the minimal binding sequence recognizing tyramine and the robustness of aptamers Tyr_10 and Tyr_14 for their use in different application platforms.

## CONCLUSIONS

In this work, a combinatorial *in vitro* selection strategy to isolate novel small molecule- binding aptamers from large combinatorial libraries has been described. The proposed method incorporates a systematic strategy to increase the selection stringency and monitor enrichment success as well as a postselection high-throughput sequencing and bioinformatics analysis. By combining these strategies, we were able to identify DNA aptamers recognizing the biogenic amine tyramine in the low micromolar range. In addition to providing a systematic and validated selection strategy, our results represent a starting point for the identification of novel DNA aptamers to tyramine. Specifically, two high affinity aptamers, namely Tyr_10 and Tyr_14, were identified that are excellent candidates for further investigations. These aptamers will be further screened as recommended by the recently published workflow[32] to perform truncation, optimization, characterization, and functional verification aimed to their incorporation into downstream analytical applications for the detection of this target.

## EXPERIMENTAL PROCEDURES

**Reagents and Chemicals.** Phosphoramidites, modifiers, activator, deblock, capping, and oxidizing reagents were obtained from Glen Research (Sterling, VA, USA). Standard support columns and acetonitrile were purchased from BioAutomation (Plano, TX, USA). Ultra High Purity 5.0 argon was purchased from Praxair Inc. (Danbury, CT, USA). Spin-X centrifuge Tube Filters 0.22 μm cellulose acetate, tyramine, L-tyrosine, histamine dihydrochloride, dopamine hydrochloride, tryptamine and phenethylamine, formamide, *o*-phthaldialdehyde (OPA), 2-mercaptoethanol, buffering agents and salts were purchased from Sigma-Aldrich (Milan, Italy). All PCR and electrophoresis reagents were purchased from BioShop Canada Inc. (Burlington, ON, Canada). Amicon-Ultra 0.5 mL 3 kDa centrifuge units were purchased from Fisher Scientific (Ottawa, ON, Canada). Poly(ether sulfone) (PES) syringe filters 0.45 μm were purchased from Celltreat Scientific Products (Shirley, MA, USA). *N*-hydroxysuccinimide (NHS)-activated Sepharose 4 Fast Flow (supplied as a suspension in 100% isopropanol) was purchased from GE Healthcare (Mississauga, ON, Canada). Taq Core Kit and MiniElute PCR Purification Kit were from Qiagen (Milan, Italy). Fast Micro-Equilibrium Dyalizer (500 μL capacity) chambers and regenerate cellulose membranes (MWCO 5000 Da) were from Harvard (Harvard, Holliston, MA). Solvents were reagent grade or better and were from VWR International PBI (Milan, Italy). All buffers were prepared with ultrapure water produced by a Millipore Milli-Q system (EMD Millipore, Billerica, MA, USA).

**DNA Library and Primers.** The initial DNA library, standard desalting purified, was 71 bases long and contained a central region of 40 random nucleotides flanked by two primer binding sites: 5′-TGGTGGCTGTAGGTCA-N40-GAGCATCGGACAACG-3′ (Integrated DNA Technology, TemaRicerca, Bologna, Italy). The modified sense primer 5′-6-FAM-TGGTGGCTGTAGGTCA-3′ and the modified antisense primer 5′-poly dA20-HEG-CGTTGTCCGATGCTC-3′were synthesized on a MerMade 6 oligonucleotide synthesizer (BioAutomation Corporation, Plano, Texas) using standard phosphoramidite chemistry. Library and primers were purified through denaturing polyacrylamide gel electrophoresis (PAGE) (12%) according to the procedure described by Green and Sambrook[40] in order to remove the truncated DNA fragments produced in the chemical synthesis. For high-throughput sequencing (HTS) additional 5 forward primers (5′-ATCACGTGGCTGTAGGTCA-3′, 5′-TGACCACTGTAGGTCA-3′, 5′-ACAGTGGCTGTAGGTCA-3′, 5′-CAGATCGCTGTAGGTCA-3′, 5′-ACTTGAGGCTGTAGGTCA-3′) and relevant reverse primers (5′-ATCACGTTGTCCGATGCTC-3′, 5′-TGACCACCGATGCTC-3′, 5′-ACAGTGGTCCGATGCTC-3′, 5′-CAGATCGTCCGATGCTC-3′, 5′-ACTTGATGTCCGATGCTC-3′), both containing a different index for barcoding, were synthesized and HPLC purified (Bio-Fab Research srl, Rome, Italy). For microscale thermophoresis the modified Tyr_10 (5′-6-FAM-TGGTGGCTGTAGGTCATCATGGTGGGGTTGGGCCTGGGAAGGGGGGAACCGTTTGAGCATCGGACAACG-3′) and Tyr_14 (5′-6-FAM-TGGTGGCTGTAGGTCACCACGGGGGGGCTGGGCCTGGGAAGGGGAGGTACCGTGTGAGCATCGGACAACG-3′) aptamers were synthesized and HPLC purified (Bio-Fab Research srl, Rome, Italy). Library, primers and aptamers were finally dissolved in Milli-Q water to get appropriate concentrations and stored at −20 °C until use.

**Preparation of Tyramine-Modified Sepharose Resin.** Tyramine was coupled to a preactivated carboxyl-agarose NHS-sepharose according to the manufacturer's protocol. Briefly, 6.31 mg of tyramine was dissolved in 1 mL of coupling buffer (0.2 M $NaHCO_3$, 0.5 NaCl, pH 8.3) and incubate for 4 h at room temperature with 2 mL of NHS-Sepharose previously washed with 28 mL of 1 mM HCl. The supernatant was removed following a 10 min centrifugation at 10 000 rpm. The resin was washed by incubation with 4 mL of 0.1 M Tris-HCl, pH 8.5 for 1 h to block unreacted groups and then it was washed with 4 cycles of alternating washes consisting of 0.1 M Tris-HCl, pH 8.5 and 0.1 M acetate buffer, pH 4.5 containing 0.5 M NaCl. Finally, the resin was dissolved in 2 mL of SELEX buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 M NaCl, pH 7.4)


DOI: 10.1021/acscombsci.5b00163
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and stored at 4 °C until further use. Coupling efficiency was measured by determining the concentration of unbound tyramine remaining in the supernatant following the coupling reaction. In particular, it was measured the fluorescence of tyramine ($\lambda_{ex}$ = 285 nm, $\lambda_{em}$ = 317 nm) with a Fluorolog Fluorescence Spectrophotometer and a SpectrAcq controller (Horiba JobinYvon, USA). Bound tyramine was calculated as the difference between the initial and unbound concentrations. Unmodified resin necessary for negative selection was prepared by adopting the same protocol reported above by performing incubation with coupling buffer without tyramine.

**SELEX Experiments.** Prior to each selection round, NHS-activated sepharose resin (from 100 to 300 μL, depending on the selection round) was packed into a Spin-X centrifuge Tube Filter, centrifuged (at 14,000 rpm for 1 min) to remove isopropanol and then washed five times with SELEX buffer (300 μL). Before using, the random ssDNA pool was denatured at 90 °C for 5 min, cooled at 4 °C for 10 min and allowed to stand at room temperature for 15 min. In the first selection round, 5 nmol of random ssDNA pool was suspended in 200 μL of SELEX buffer, incubated with 300 μL of unmodified sepharose (negative column) in a centrifugal tube and mild shaken for 60 min. After centrifugation, the unbound ssDNA sequences (with no affinity for the negative column resin) were collected and transferred in another centrifugal tube containing 300 μL of tyramine-coupled sepharose (positive column) and incubated by mild shaking for 60 min. Following incubation, sequences with little or no affinity for tyramine were eluted from the resin by five washings using 200 μL of SELEX buffer each time. The bound sequences were eluted from the positive resin by incubating it with 200 μL of 6 M urea at 90 °C for 10 min. This process was repeated nine additional times to retrieve all traces of bound ssDNA from the resin; then all bound fractions were pooled together. The amounts of ssDNA in the loading, washing and eluting fractions were determined by fluorescence ($\lambda_{ex}$ = 490 nm, $\lambda_{em}$ = 520 nm) using a Spectrofluorometer LS55 (PerkinElmer, USA). The pooled eluted fractions were then purified (Amicon Ultra 3 kDa 0.5 mL centrifugal filters) and then amplified in 30−45 parallel PCR reactions. Each PCR reaction consisted of 100 mM Tris-HCl pH 9, 50 mM KCl, 1% Triton X-100, 2 mM $MgCl_2$, 0.2 mM dNTP each, 1 μM each primer and 5 units of Taq DNA polymerase in a volume of 100 μL. The DNA was initially melted for 10 min at 94 °C, followed by 25 cycles of 94 °C (1 min), 47 °C (1 min) and 72 °C (1 min). Final extension occurred at 72 °C for 10 min after the last cycle. PCR products were dried down, resuspended in 10 μL of $H_2O$ and heated at 90 °C for 5 min in the presence of 60% formamide. The different sized DNA strands of the PCR products were separate by a 12% denaturing PAGE.[40] The 5′-6-FAM labeled DNA strands (corresponding to the selected sequences) were identified using the MultiImage Light Cabinet (AlphaImager EC, Alpha Innotech, USA), and the relevant bands were cut out. The ssDNA was eluted from the cutted gel by using the crush and soak method with minor modification.[41] Briefly, the cutted bands were crushed with a small-bore syringe by using 3 mL of 10 mM Tris, pH 7.4 buffer/0.5 mL of gel. The sample was rapidly frozen at −80 °C for 30 min, then quickly thawed in a 50 °C hot water bath for 5 min and a soaked at 90 °C for other 5 min. The ssDNA extraction was completed after an overnight elution on a rotary shaker at 37 °C. Gel was removed via filtration through 0.45 μm PES syringe filters. The samples were rapidly frozen at −80 °C for 30 min, lyophilized and dissolved in 1−2 mL of deionized water. Finally the samples were purified using filtration with 3 kDa cutoff Amicon-Ultra centrifuge units. After concentration of the sample, the filters were rinsed four times with deionized water to remove trace amounts of urea and other remaining gel components. Purified DNA was then quantified using a Cary 300 Bio UV−visible spectrophotometer (Varian, USA) and used in the next selection round. Negative selections were performed from round 1 to 7 and in round 15. To increase specificity and selectivity, stringent conditions were used from round 8 to 13 by washing the positive column with 200 μL of SELEX buffer containing individually 5 mM of histamine, tryptamine, phenethylamine, dopamine or L-tyrosine (Table 1). By using this approach, complex and time-consuming preparation of modified resins with these molecules was avoided. Furthermore, the amounts of input ssDNA pool, positive resin and incubation time were reduced starting from round 13, 16 and 17, respectively. In the last five rounds, bound aptamers to the positive resin were incubated for 10 min with 200 μL of SELEX buffer containing 10 mM tyramine and then eluted. This step was repeated four additional times and allowed to collect sequences able to bind tyramine free in solution (Table 1).

**High-Throughput Sequencing.** The starting DNA library and DNA collected from SELEX rounds 2, 7, 16 and 21, were amplified in 14 parallel reactions using the Taq Core Kit and 5 different index-barcodes primers. Each reaction consisted of 10 μL of 10x PCR Buffer provided by the manufacter, 200 μM dNTP each, 1 μM each primer, 2.5 units of Taq DNA polymerase and 2 μL of DNA solution in a volume of 100 μL. The DNA was melted for 5 min at 94 °C, followed by 8 cycles of 94 °C (1 min), 47 to 53 °C (1 min) (depending of the annealing temperature of the used couple of primer) and 72 °C (1 min). Final extension occurred at 72 °C for 5 min after the last cycle. PCR products were cleaned using the MiniElute PCR Purification Kit according to the manufacturer's protocol and 5 pmol of each sample were examined on 2.5% agarose gel to confirm the absence of contamination. Finally, the samples corresponding to the same SELEX round were pooled for quantification with NanoDrop ND-100 spectrophotometer (Thermo Scientific, USA) and sequenced by TRON (Mainz, Germany) using an Illumina HiSeq 2000 (Illumina Inc., USA). After the sequencing was complete, the raw sequence data in FASTQ format file were processed for bioinformatics analysis by AptaIT GmBH (Munich, Germany). Fifteen sequences of the last SELEX round were selected as candidate aptamers for binding affinity studies.

**Binding Affinity ($K_d$) Measurements.** The 15 identified aptamers were screened for binding to tyramine using a one-point microequilibrium dialysis assay as described by McKeague and co-workers.[32] Specifically, 4 μM of each selected DNA aptamer was tested in the presence of 7 μM of tyramine. Each dialysis experiment was carried out in duplicate. After incubation, the tyramine content in the receiving chamber (containing unbound tyramine) was directly analyzed by HPLC-FLD after precolumn derivatization of tyramine with OPA reagent according to the method described by Soleas and co-workers,[42] with some modifications. In particular, an Agilent 1100 Series chromatographic system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, autosampler, column thermostat set at 45 °C, a fluorometric detector (model 363, $\lambda_{ex}$= 340 nm, $\lambda_{em}$= 420 nm) and the ChemStation data software (Agilent Technologies) was used for the analyses. The analytical column was a Symmetry Shield


DOI: 10.1021/acscombsci.5b00163
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C18, (4.6 × 150 mm, 5 μm; Waters) preceded by a pore-size guard filter (3 mm inner diameter, 0.45 μm; Rheodyne). The autosampler was programmed to react 50 μL of OPA with 50 μL of sample, and then injected the entire amount of the derivatized mixture onto the column. The mobile phase was a mixture of 0.05 M sodium acetate buffer:tetrahydrofuran (96 + 4, v/v) and methanol eluted at the flow rate of 1 mL $min^{-1}$. A binary gradient was applied as follow: the initial composition of the mobile phase, 53% methanol, was kept constant for 2.5 min; then the methanol content was increased up to 70% at 7 min, to 100% at 15 min and it was restored to 53% at 16 min and kept constant up to 25 min. With this mobile phase the retention time of tyramine was about 9.0 min. The limit of quantification was 0.73 μM.

The fraction of bound tyramine (f) was calculated as

$$f = (\mathrm{Tyr}_{\mathrm{TOT}} - \mathrm{Tyr}_{\mathrm{R}})/\mathrm{Tyr}_{\mathrm{TOT}}$$

where $\mathrm{Tyr}_{\mathrm{TOT}}$ is the peak area corresponding to the tyramine content in the solution loaded into the loading chamber and $\mathrm{Tyr}_{\mathrm{R}}$ is the peak area of tyramine recovered from the receiving chamber. The dissociation constant ($K_d$) was determined as

$$K_d = X/f - X$$

where $X$ is the concentration of the aptamer.

The binding affinity of the most promising aptamers (Tyr_10 and Tyr_14) was investigated by microscale thermophoresis (2bind GmbH, Regensburg, Germany) as previously described.[43] Briefly, an aliquot of 5 μL of tyramine standard was mixed with 5 μL of the fluorescent aptamers. Both tyramine standards and fluorescent aptamers were prepared in the SELEX buffer. Sixteen different tyramine concentrations were tested in the range of 1.5 nM to 50 μM in the presence of 10 nM fluorescent aptamers (duplicate measurements). The final reaction mixture was filled in capillaries and analyzed on a Monolith NT at 25 °C, with 20% LED power and 80% laser power.

**Structural Analysis and Determination of the Specificity of the Aptamers.** Secondary structure of the aptamer Tyr_10 was obtained by online program Mfold at 0.5 M NaCl, 0.005 M $MgCl_2$, and 25 °C.[34]

The specificity of the most promising aptamer (Tyr_10) was tested by using a one-point microequilibrium dialysis assay as described by McKeague and co-workers.[32] Specifically, 4 μM of the selected DNA aptamer was tested in the presence of 7 μM of histamine or tryptamine. Each dialysis experiment was carried out in duplicate. After incubation, the histamine or tryptamine content in the receiving chamber was analyzed by UPLC-ESI-MS. In particular a Waters Acquity UPLC system (Milford, MA, USA) equipped with an ESI-MS detector was used. The MS detector was a single quadrupole mass analyzer (Acquity QDa, Waters). The ESI interface was used in positive ion mode with the following settings: desolvatation temperature 600 °C, source temperature 150 °C, capillary voltage 0.8 kV, sampling frequency 5 scan $s^{-1}$. The mass spectrometer operated full scan mode ($m/z$ range 50−700). The analytical column was the Phenomenex Kinetex C18 (2.1 mm × 100 mm, 2.6 μm) with the correspondent in-line filter. The column temperature was 25 °C. The chromatographic separation was performed by a gradient elution (solvent A: water, solvent B: methanol, both containing 0.1% formic acid) as follows: the initial composition solvent B (1%) was kept constant for 10 min, then the column was washed by linearly increasing solvent B to 100% in 5 min. The mobile phase composition was then returned to the initial conditions in 1 min and the column was equilibrated for 5 min prior to the successive sample injection. The flow rate of the mobile phase was 0.5 mL $min^{-1}$ and the injection volume was 10 μL. Data acquisition and instrument control were performed by Masslynx Software (Waters, Milford, MA, USA). By using these conditions the retention time of histamine and tryptamine was 0.38 min for both. The monitored molecular ions $[M + H]^+$ were $m/z$ 112.1 for histamine and $m/z$ 161.2 for tryptamine. The limit of quantifications for histamine and tryptamine were 0.25 μM and 2 μM, respectively.

**Circular Dichroism Spectroscopy.** Aliquots of 10 μM tyramine, 2 μM aptamer Tyr_10 and 4 μM aptamer Tyr_10 mixed with 20 μM tyramine (1:1, v/v, ratio) in SELEX buffer were incubated at room temperature for 40 min. Circular dichroism spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco, Inc., Easton, MD) equipped with nitrogen purging facilities. The CD spectra of the SELEX buffer, aptamer, tyramine and the mix containing aptamer and tyramine were recorded in 0.1 cm cells (Hellma) at room temperature from 320 to 220 nm. Data gathered were the average of 5 time scans at a scanning rate of 100 nm/min. The scan of the SELEX buffer alone was subtracted from the average scans for both the aptamer and the mix with tyramine. Data were collected in units of millidegrees versus wavelength.

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### Author Contributions

The manuscript was written through contributions of all authors: A.D., M.C.D., S.V., M.M., L.C., and M.P. designed the research, S.V. and A.D. analyzed the data, and S.V, M.M., and R.S. performed the experiments. All authors have given approval to the final version of the manuscript.


### Funding

This work was supported by a dedicated grant from the Italian Ministry of Economy and Finance to the National Research Council for the project "Innovazione e Sviluppo del Mezzogiorno—Conoscenze Integrate per Sostenibilità ed Innovazione del Made in Italy Agroalimentare—Legge n. 191/2009". M.C.D. acknowledges funding from the Natural Sciences and Engineering Research Council (NSERC).


### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS

AuNPs, gold nanoparticles; BA, biogenic amine; CD, circular dichroism; CE, capillary electrophoresis; ESI-MS, electrospray ionization mass spectrometer detector; 6FAM, 6-carboxyfluorescein; HEG, hexaethylene glycol spacer phosphoramide 18; HPLC-FLD, high-performance liquid chromatography with fluorescence detection; HTS, high-throughput sequencing; $K_d$, dissociation constant; NGS, next-generation sequencing; NHS, *N*-hydroxysuccinimide; OPA, *o*-phthaldialdehyde; PAGE, denaturing polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PES, poly(ether sulfone); SD, standard deviation; SELEX, systematic evolution of ligands by exponential enrichment; ssDNA, single-stranded DNA; UPLC, ultra-performance liquid chromatography



DOI: 10.1021/acscombsci.5b00163
ACS Comb. Sci. XXXX, XXX, XXX−XXX

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DOI: 10.1021/acscombsci.5b00163
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