Bioorganic & Medicinal Chemistry Letters 18 (2008) 5926–5931

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Design and synthesis of a novel DNA-encoded chemical library using Diels-Alder cycloadditions ☆

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ARTICLE INFO

Article history:
Received 16 June 2008
Accepted 10 July 2008
Available online 15 July 2008

Keywords:
DNA-encoded chemical library
Diels-Alder cycloaddition
Streptavidin
Albumin
High-throughput sequencing

ABSTRACT

DNA-encoded chemical libraries are increasingly being employed for the identification of binding molecules to protein targets of pharmaceutical relevance. Here, we describe the synthesis and characterization of a DNA-encoded chemical library, consisting of 4000 compounds generated by Diels-Alder cycloaddition reactions. The compounds were encoded with unique DNA fragments which were generated through a stepwise assembly process and serve as amplifiable bar codes for the identification and relative quantification of library members.

The isolation of small organic molecules, capable of specific binding to proteins, is a central problem in chemistry, biology, and medicine. Whenever the target proteins of interest display a measurable enzymatic activity or have labeled ligands which can be used in displacement assays, large libraries of chemical compounds can be screened individually (one molecule at a time) in order to identify novel ligands. These hits are then selected for further optimization by medicinal chemistry.[1] Such high-throughput screening approaches, which are routinely used by the pharmaceutical industry and increasingly also by academic research centers, may be expensive both in terms of target protein requirements and library-associated costs (e.g., synthesis, quality controls, library management, screening assays, robotics). Thus, practical screening campaigns are limited to a few hundred thousand compounds at best. Alternative screening procedures have been proposed, such as the incubation of the target protein with a mixture of ~100 compounds, followed by the separation of molecules capable of protein interaction via gel filtration.[2] While these approaches are interesting in terms of reducing screening costs and times as well as for the identification of ligands to proteins for which biochemical screening methods are not available, they may nonetheless be limited by compound solubility, mass spectrometric detection of binding compounds, and library sizes which can realistically be screened.

The use of DNA fragments as amplifiable 'bar-codes' for the identification of chemical compounds in a library represents an attractive avenue for the synthesis and screening of large combinatorial libraries.[3–7] Several strategies can be considered for the construction of DNA-encoded chemical libraries, for example the use of self-assembling chemical libraries where each of the two complementary DNA strands carries a different chemical moiety ('dual pharmacophore chemical libraries')[8–10] or the use of individual compounds covalently attached to unique DNA fragments. In turn, these 'single pharmacophore chemical libraries' can be generated by a variety of synthetic approaches, including the transfer of chemical moieties from assisting complementary oligonucleotides,[4,11] the oligonucleotide-assisted separation of DNA derivatives followed by chemical reactions,[5] the stepwise alternated growth of chemical structures and encoding DNA fragments,[12] or simply the direct coupling of reactive chemical moieties to different amino-tagged oligonucleotides.[13,14]

In general, the construction of DNA-encoded chemical libraries is facilitated by the ability to perform chemical reactions at high yields and with good purities, using synthetic methodologies which work well in water and preserve the integrity of DNA. While until now most libraries are relying on amide bond forming reactions,[4,8,12,15] other reactions could be considered, such as reductive amination and ether bond formation.

In this Letter, we describe the construction of a DNA-encoded chemical library, consisting of 4000 compounds attached to individual double-stranded DNA fragments carrying unique DNA se-

☆ Manuscript for a special Symposium-in-Print (SIP) issue in honor of the recipient of the 2008 Young Investigator Award in Bioorganic and Medicinal Chemistry, Benjamin F. Cravatt.

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doi:10.1016/j.bmcl.2008.07.038

quences that allow the unambiguous identification of the displayed chemical moieties. The synthesis of the library was achieved using a split-and-pool procedure, which featured the following sequential steps: (i) conjugation of different dienes to distinct aminomodified synthetic oligonucleotides; (ii) pool and split; (iii) Diels-Alder reaction with suitable maleimide derivatives; (iv) encoding of the cycloaddition reaction by hybridization of partially complementary oligonucleotides followed by Klenow-mediated DNA polymerization, yielding the final compounds in a double-stranded DNA format. The purity of the intermediate steps in the library synthesis was extensively investigated using HPLC and mass spectrometry, while an assessment of library performance was provided by the decoding of selections performed against two model target proteins.

Attracted by the mild conditions at which certain Diels-Alder cycloaddition reactions can be performed in water,[16–18] we investigated the possibility to generate Diels-Alder products, which are chemically coupled to DNA. These DNA-compound conjugates were then used for the selection of binding molecules to two well-characterized target proteins: albumin and streptavidin.

Bicyclic Diels-Alder derivatives were obtained synthetically by cycloaddition from 2,4-hexadienes and maleimides as dienophiles (Fig. 1A). In order to evaluate the feasibility of library synthesis based on this experimental strategy, we synthesized a small prototype library based on *N*-Acetyl-*O*-2(*E*),4(*E*)-hexadienyl-L-hydroxyproline, which was then coupled to a synthetic 42-mer oligonucleotide, carrying a primary amino group connected with a C12-linker at the 5′ extremity (Fig. 1A). The resulting DNA-conjugate was HPLC purified and submitted in parallel to 54 different cycloaddition reactions with maleimide derivatives, which were either commercially available or generated in situ from the corresponding primary amines and maleic anhydride (Fig. 1A, supplementary methods). HPLC and ESI-MS analysis of the reaction confirmed the identity of all 54 products (supplementary Table 1), with an average yield of 64%. In a final synthetic step, the oligonucleotide conjugates were hybridized to partially complementary DNA-fragments, which carried a unique sequence for the identifi-

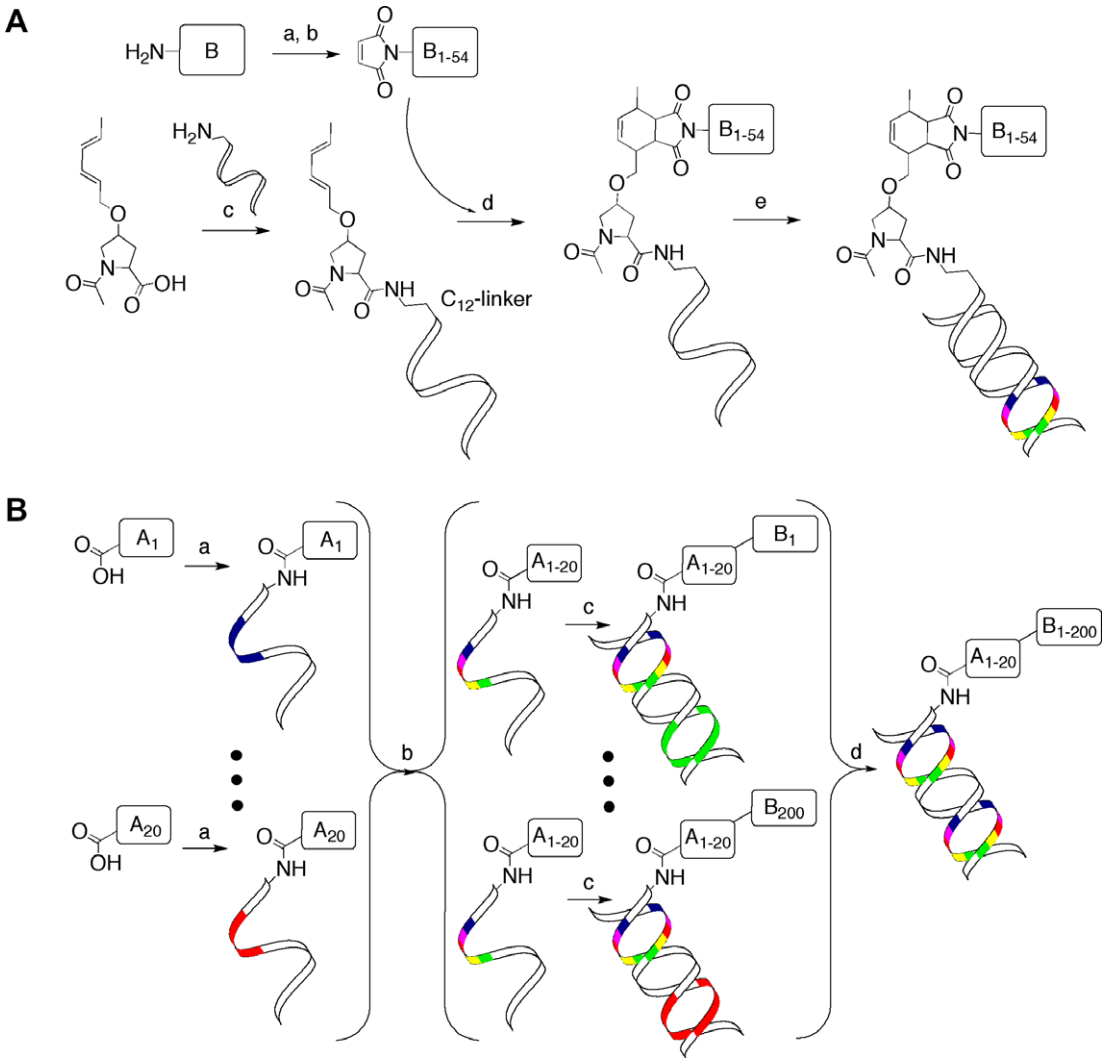

Figure 1. (A) Reaction scheme of the 54-compound prototype DNA-encoded chemical library using Diels-Alder cycloadditions between a 2(*E*),4(*E*)-hexadienyl moiety and maleimide derivatives. (a) Maleic anhydride/DMF. (b) Ac_2O or HOAc, 80 °C. (c) EDC/sulfo-NHS mediated coupling in DMSO/H_2O 75 mM TEA-Cl, pH 10. (d) Diels-Alder cycloaddition in DMF/acetate buffer, pH 4.7. (e) Klenow-Polymerase-mediated encoding of the obtained Diels-Alder structure upon hybridization with a partially complementary oligonucleotide carrying a unique sequence. (B) Schematic representation of the strategy used for library synthesis and encoding of the 4000 compound library. (a) Coupling of 20 carboxylic acids containing a 2(*E*),4(*E*)-hexadienyl moiety to 20 unique 5′-amino-modified oligonucleotides. (b) Pool and split of the 20-compound sub-library into 200 vessels. (c) 200 Diels-Alder cycloadditions with maleimides and subsequent Klenow-Polymerase encoding. (d) Pooling of the 200 reactions to yield a DNA-encoded library containing 4000 members.

cation of individual chemical moieties. Conversion into a double-stranded DNA fragment was achieved by Klenow polymerization (Fig. 1A).

To assess whether a DNA-encoded chemical library based on the bicyclic Diels-Alder scaffold could display chemical moieties capable of selective interaction with target proteins immobilized on a solid support, the 54-compound library was panned onto streptavidin–sepharose and onto albumin–sepharose. Three of the 54 building blocks used for library construction (D-desthiobiotin-, 6-ethoxy-6-oxohexanoyl-, and 4-(*p*-iodophenyl)butyl-based maleimide derivatives) had previously been characterized as suitable ligands for these target proteins.[13,14,19] In Figure 2 the results of decoding experiments are depicted, showing the relative abundance of library compounds after capture on streptavidin resin, on human serum albumin resin, or on empty resin produced from CNBr-activated sepharose, which was quenched with Tris. The DNA-encoded chemical library was submitted to an asymmetric PCR amplification using a fluorescently labeled primer. The resulting mixture was then hybridized onto an oligonucleotide microarray, carrying oligonucleotides complementary to the individual

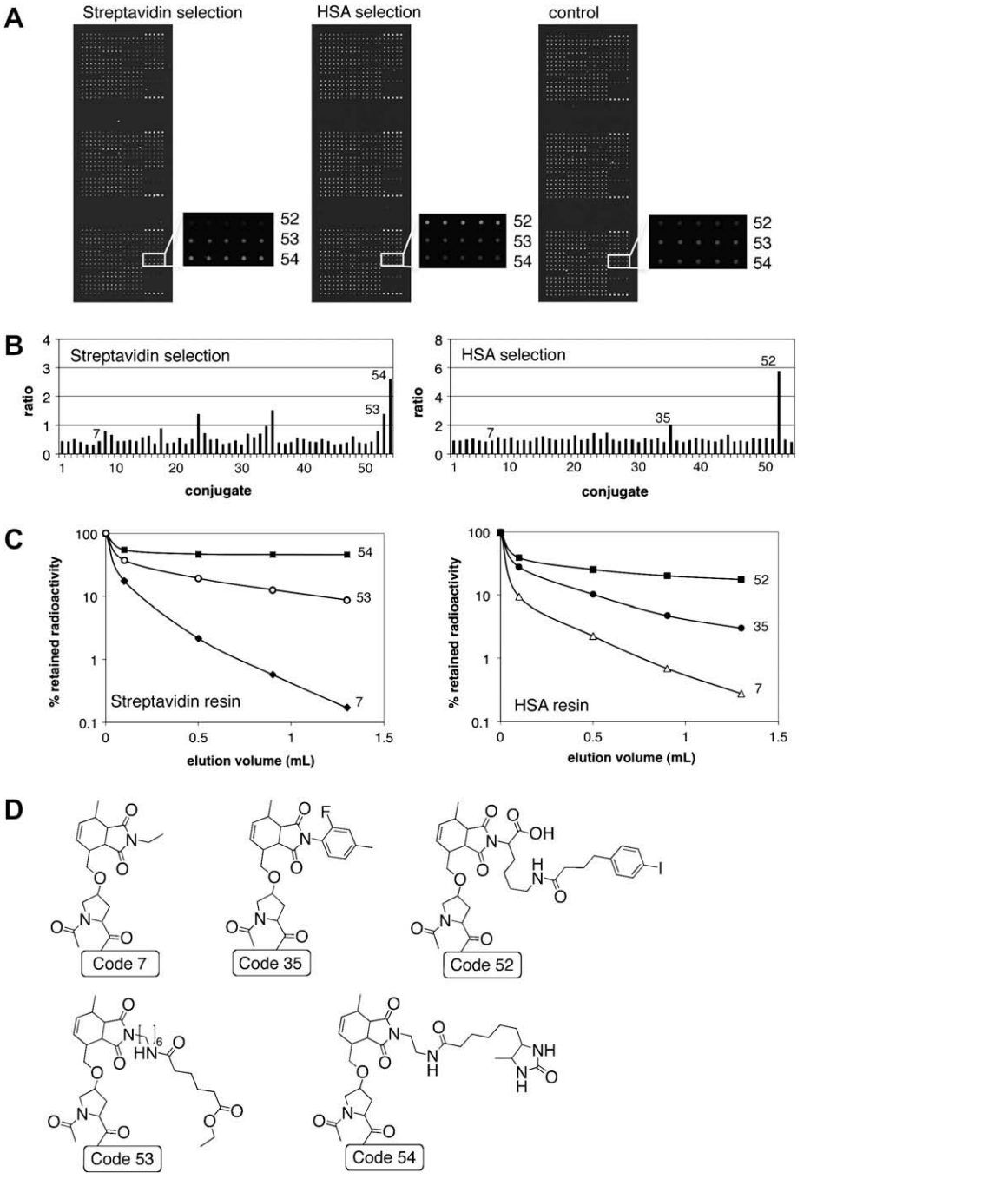

Figure 2. (A) Microarray readout after PCR amplification of selections with the 54-compound library against streptavidin, human serum albumin and of a control selection with no antigen on resin (spotted in quintuplicate). The inset highlights the signal enhancement of compounds 52–54, included as positive controls in the 54-compound library. (B) Quantitative microarray evaluation: Enrichment of oligonucleotide-compound conjugates given as a ratio of selection versus control selection. (C) Affinity chromatography of enriched (Streptavidin: conjugates 53 and 54, HSA: conjugates 35, 52) and non-enriched (conjugate 7) conjugates, each hybridized to a radiolabeled complementary oligonucleotide. The retained radioactivity was monitored during multiple washing steps. (D) Structures of control conjugates 52–54 and of a novel binder to HSA, conjugate 35.

codes of the compounds in the library.[9,10,13,14] Figure 2A shows the fluorescence images of the microarrays of three selection experiments, revealing a preferential enrichment of compounds **53** and **54** in streptavidin selections and of compounds **52** and **35** in albumin selections. Figure 2B presents a quantitative evaluation of the spot fluorescence intensities in the decoding microarrays, display-

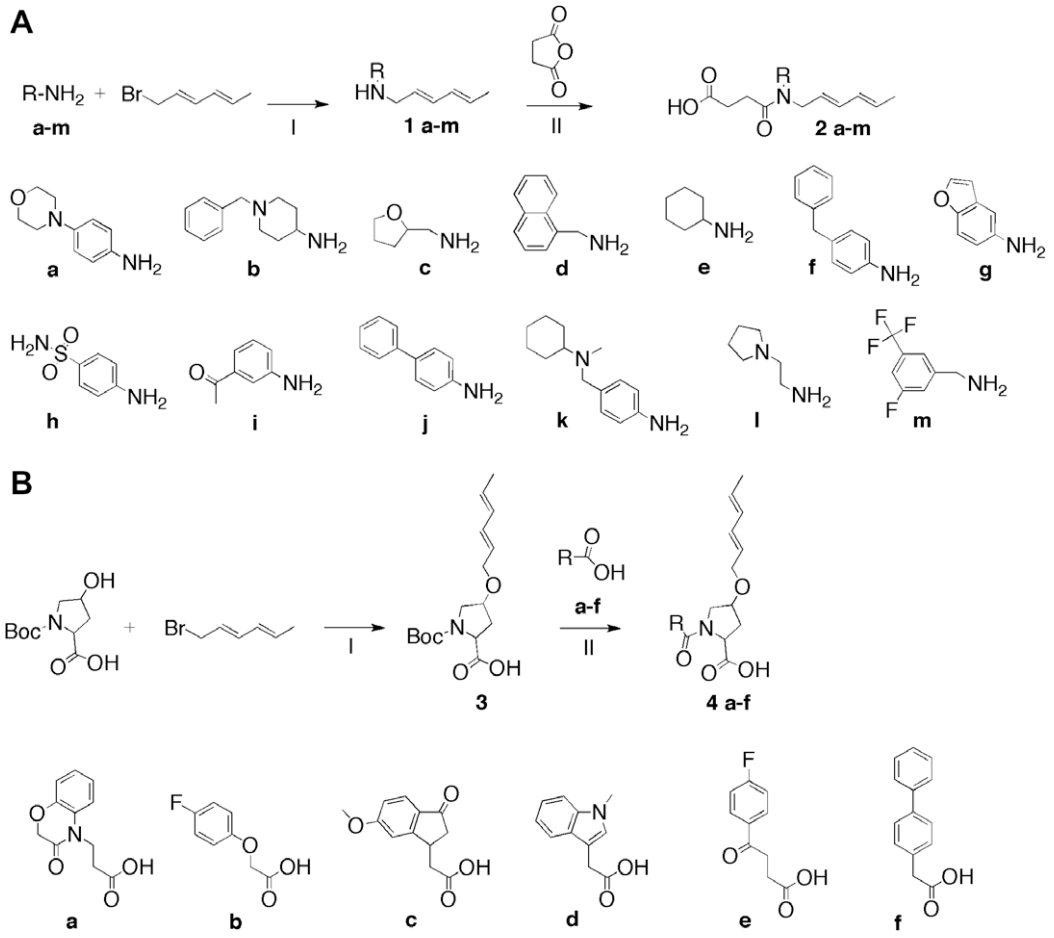

Figure 3. Synthesis scheme of 2(*E*),4(*E*)-hexadienyl derivatives. (A) Synthesis of **2a**–**m** starting from primary amines. I—Na_2CO_3, THF; II—MeCN. (B) Synthesis of **4a**–**f** starting from carboxylic acids. I—NaH, THF; II—HATU, DIPEA, DMF.

Table 1
MS characterization of 20 synthesized diene–oligonucleotide conjugates

Oligonucleotide-conjugate	2,4-Hexadienyl-derivative used for conjugation	6 base-pair code	Deconvoluted mass of diene–oligonucleotide conjugate	Calculated mass of diene–oligonucleotide conjugate	Deconvoluted mass of Diels-Alder product[a]	Calculated mass of Diels-Alder product
1	2a	CAACGC	13666.4	13661.0	13876.0	13867.3
2	2b	ACGCGC	13694.0	13689.3	13883.6	13879.6
3	2c	CACGAA	13610.6	13608.0	13792.6	13790.5
4	2d	GGCACA	13685.0	13680.1	13852.0	13846.3
5	2e	GAGGAG	13706.8	13702.1	13794.8	13788.3
6	2f	CTACCG	13663.0	13657.0	13881.6	13872.3
7	2g	CTATAT	13627.6	13621.0	13828.8	13822.2
8	2h	TTGTTT	13687.8	13682.0	13863.0	13861.2
9	2i	GCTCAT	13629.2	13624.0	13831.0	13824.4
10	2j	AAACAT	13681.6	13675.1	13865.2	13858.3
11	2k	GGGAGC	13802.8	13797.2	13913.8	13907.4
12	2l	CAGAGT	13658.6	13652.1	13808.2	13803.5
13	2m	TGCTCT	13679.2	13673.2	13887.8	13882.5
14	4a	CTGTGA	13768.6	13763.1	13930.2	13923.3
15	4b	CGGATG	13741.4	13737.1	13878.6	13872.3
16	4c	GTAGTA	13791.0	13786.1	13928.8	13922.3
17	4d	CACTTT	13671.8	13666.0	13898.4	13891.3
18	4e	TGGGCG	13784.6	13779.3	13903.2	13898.5
19	4f	GTCGCT	13735.6	13730.1	13918.8	13914.3
20	4g	TTGCGG	13576.6	13576.2	13770.4[b]	13770.4

[a] Diels-Alder cycloaddition with the maleimide product prepared from 2-phenylethylamine and maleic anhydride. Oligonculeotide code: ATC TTA.
[b] Oligonculeotide code: ATG GAG.

ing ratios of fluorescence signals in the selection experiments relative to the control selection on empty resin. In order to confirm preferential binding to the target proteins, we radiolabeled the individual DNA conjugates and measured their ability to be captured by their cognate proteins, which were immobilized on resin (Fig. 2C). The retention profiles correlated with the binding affinities of the ligands.[13,14] The relative enrichment factors are given in Figure 2B.

Encouraged by these results, we decided to synthesize a 4000 member library, based on a two-step split-and-pool strategy, as depicted in Figure 1B. Initially, twenty 2,4-hexadiene derivatives carrying a carboxylic acid moiety were synthesized (see scheme in Fig. 3 and supplementary methods) and coupled to amino-tagged oligonucleotides carrying distinctive six base codes. The resulting derivatives were HPLC purified and characterized by ESI-MS (Table 1). In all cases, yields were greater than 50% as assessed by HPLC. A representative HPLC profile and deconvoluted ESI-MS spectrum of a diene conjugate are shown in Figure 4A and B.

In order to assess the ability of the diene conjugates to efficiently undergo Diels-Alder cycloaddition with maleimide derivatives, a model reaction was performed for all 20 intermediates with 2-phenylethylamine and maleic anhydride. A representative deconvoluted ESI-MS profile for the crude single-pot reaction mixture after ethanol precipitation is displayed in Figure 4C, revealing an excellent purity. All calculated and experimental mass values for the 20 model reactions can be found in Table 1.

We then pooled the 20 diene DNA-conjugates, split them into 200 aliquots, and performed cycloaddition reactions with 200 maleimides or maleimide-generating reagents (Fig. 1B, supplementary methods). As a final step, the second code was introduced to each DNA-encoded compound by hybridization with partially complementary oligonucleotides and subsequent Klenow polymerization (Fig. 1B), in perfect analogy with the strategy described in the previous section. Figure 5A depicts in more detail the two-step encoding strategy employed for the library construction.

The 200 reactions were pooled in 20 vials and analyzed by TBE/polyacrylamide gel electrophoresis, confirming the purity and efficiency of the Klenow encoding procedure (Fig. 5B). Finally, the library was pooled in a single vial and aliquoted to a final concentration of 100 nM (total DNA content). For selection experiments, a total DNA concentration of 10 nM was used, corresponding to a 2.5 pM concentration of individual library members. The integrity of library encoding was confirmed by PCR amplification of the library with primers 5′-GCCTCCCTCGCGCCATCAGGGAGCTTGTGA ATTCTGG-3′ and 5′-GCCTTGCCAGCCCGCTCAGGTAGTCGGATCCGAC CAC-3′, complementary to the extremities of the DNA fragments in the library, followed by subcloning and sequencing. Furthermore, the suitability of the library for selection experiments was confirmed by a capture experiment on streptavidin resin, which revealed a striking preferential enrichment of library members containing a D-desthiobiotin moiety as building block B (Fig. 1B, incorporated as a positive control in the library construction, data not shown).

A Diels-Alder cycloaddition reaction represented the central step in our library synthesis, yielding bicyclic derivatives with good purities and yields. Since only a limited number of suitable maleimide derivatives were commercially available, we developed a one-pot reaction featuring the in situ formation of maleimides starting from maleic anhydride and primary amines. After dilution of the original reaction mixture with water and pH adjustment, the 2,4-hexadiene–oligonucleotide conjugates were added. This approach proved to be compatible with a variety of primary amines and 2,4-hexadiene derivatives (supplementary Table 2). We could, however, not analyze the stereochemistry of the bicyclic moiety when coupled to the DNA. Investigations in solution suggest that *endo* diastereomers may be preferentially formed.[16] When DNA-compound conjugates preferentially bind to a target protein of interest, individual compounds can be resynthesized in the absence of DNA and stereoisomers can be analyzed. It is conceivable that the bicyclic derivatives may be further optimized for medicinal chemistry applications through suitable modification strategies. As to the drug-likeliness of the Diels-Alder bicyclic scaffold, it was reassuring for us that a number of lead pharmaceutical compounds based on the Diels-Alder reaction with maleimides are currently in preclinical and clinical development.[20,21]

The DNA-encoded library presented in this article represents one of the libraries which is currently being developed in our laboratory.[8,12–14] While in principle DNA-encoded chemistry could yield libraries of huge dimensionalities, if multiple reaction steps were used within split-and-pool strategies, we have initially focused on the construction of individual libraries containing

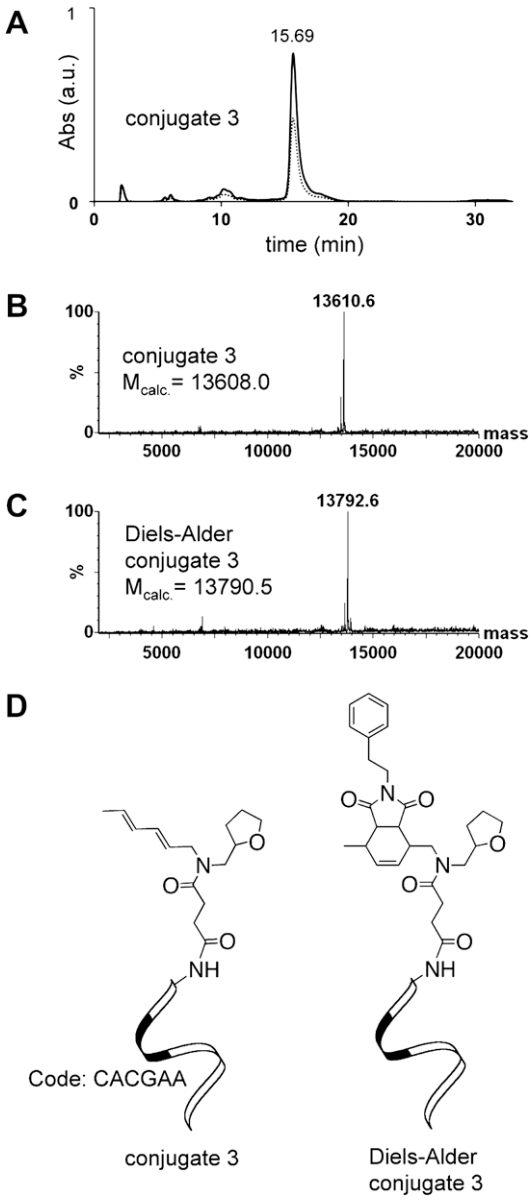

Figure 4. Example of the purification and MS-characterization of diene–oligonucleotide conjugates. (A) HPLC profile after coupling of **4c** to an 5′-aminomodifided oligonucleotide (conjugate 3) (solid line: Abs_{260} dashed line: Abs_{280}). (B) LC-ESI-MS characterization of conjugate 3 after deconvolution of the spectra. (C) LC-ESI-MS characterization of the Diels-Alder product of conjugate 3 and the maleimide prepared from 2-phenylethylamine and maleic anhydride. (D) Structure schemes.

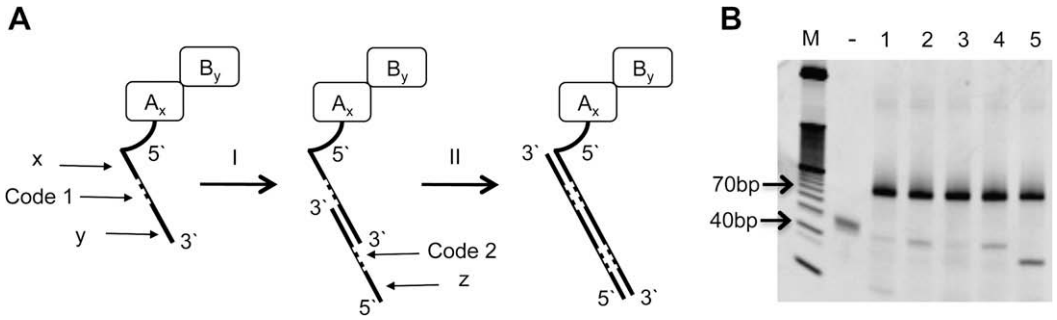

Figure 5. (A) Schematic representation of the Klenow encoding of an oligonucleotide-compound conjugate A_xB_y. *x*, forward primer site, compatible with primers for 454-high-throughput sequencing. *y*, annealing site for the partially complementary oligonucleotides carrying code 2. *z*, reverse primer site, compatible with primers for 454-high-throughput sequencing. (B) 20% polyacrylamide-TBE gel analysis of the encoding of 50 Diels-Alder reactions. M, 10 bp Marker; –, single-stranded 42-mer prior to encoding; 1–5, sub-pools after encoding of 10 reactions (68 mers). Staining was performed with Sybr green I.

1000–10,000 compounds, which featured a maximum of two synthetic steps, and concentrated on reactions, which proceed with excellent yields. Care was always taken to characterize library construction intermediates by HPLC and LC-ESI-MS, and to validate the last synthetic step with a number of model compounds. Ultimately, a direct analytical characterization of the individual components in a large library is not feasible.

In principle, the library could be expanded by the use of alternative diene or maleimide intermediates, or by further modification of a suitable functional group of moiety **A** or moiety **B** (Fig. 1B). In this case, a third code for the last reaction step can be introduced in a sequential manner.[12]

In contrast to other library screening methodologies with mixtures of compounds, for which identification procedures become more and more problematic with increasing library sizes, DNA encoding enables the use of individual library members at very low concentrations, provided that the library is captured with a biotinylated target protein at concentrations greater than K_d, or on resins coated with protein at high density (>1 mg/ml), in full analogy to peptide and antibody phage display libraries, in which rare binders can be efficiently recovered and identified.[22,23] The advent of high-throughput sequencing technologies, such as 454 sequencing,[24] opens the possibility of profiling the relative abundance of individual library members before and after a single panning step, thus providing direct information about the relative enrichment rate for all compounds in the library.[12]

In short, we have described the design, synthesis, and characterization of a DNA-encoded chemical library, containing 4000 compounds individually coupled to DNA fragments, which serve as amplifiable identification bar codes. The use of a Diels-Alder strategy with 2,4-hexadiene modified oligonucleotides and a variety of different maleimides proved to yield cycloaddition products in good yields and purities, as characterized by HPLC and LC-ESI-MS. The library is compatible with decoding strategies based on ultra-high-throughput sequencing technologies (e.g., 454 sequencing, Roche), which enables the relative quantification of individual library members before and after selection against target proteins of interest.

Acknowledgments

Financial support from the ETH Zurich, the Swiss National Science Foundation, Philochem AG, KTI (Grant No. 8868.1 PFDS-LS), and the Gebert-Rüf Foundation is gratefully acknowledged. The authors are grateful to the Functional Genomics Center Zurich for help in DNA-encoded chemical library decoding through oligonucleotide microarrays, and to Dr. Samu Melkko (Philochem AG) for reviewing the manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.07.038.

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