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# DESIGN AND SYNTHESIS OF REAGENTS FOR PHAGE DISPLAY SCREENING OF DEHALOGENASES

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**Abstract:**

Bifunctional molecules containing both a biotin and a substrate unit have been designed and synthesized for phage display screening of mutant libraries of haloalkane dehalogenase enzymes. The molecules were assembled using a convergent modular synthetic strategy. One molecule was synthesized to evaluate the concept of covalent capture and a second for screening of phage libraries for enantioselectivity. 

To optimize the properties of proteins and peptides is a goal with important ramifications in many areas of research, ranging from chemistry to medicine. An attractive approach to achieve this is the use of random mutagenesis. In this context phage display has proven very useful as a method for screening large libraries of mutant peptides and proteins especially because of convenient amplification and evaluation.[1] Although phage display studies thus far have been mostly concerned with the binding properties of peptides and antibodies, the method holds great promise for engineering of improved biocatalysts such as enzymes and catalytic antibodies. Several approaches towards this goal are under development. They are based on different answers to the main question: 'How to screen for catalysis.' It is important to physically retain the phage particles with improved mutants on a solid support. One possibility is to use non-covalent binding to relevant inhibitors.[2] Alternatively, covalent capture can be achieved by using suicide inhibitors,[3] the generation of reactive leaving groups,[4] or by taking advantage of different pH dependencies of individual steps in a covalent catalysis mechanism.[5]

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We are involved in a program to optimize a haloalkane dehalogenase with optimal activity for short-chain haloalkanes (designated DhlA[6]), and a dehalogenase with activity for long-chain haloalkanes (DhaA[7]). Both of these enzymes are capable of converting haloalkanes to alcohols and hold potential for biocatalalytic applications. This potential lies in the kinetic resolution of racemic substrates, which represents a pathway to optically pure haloalkanes and alcohols, both of which are valuable synthetic building blocks. Enantioselectivity of the wild type enzymes with chiral substrates[8] is insufficient for practical purposes with E-values ranging from 1 to 5.[9] We envision improvement of the enantioselectivity of these enzymes by screening phage libraries of mutant enzymes in competition experiments between a biotin-linked enantiomer (for streptavidin capture) and its non-biotin antipode. Several rounds of such screening should lead to pools of mutants with improved enantioselectivity.

The mechanism of DhlA is well-studied and involves an Asp-His-Asp catalytic triad and two discrete reaction steps. The mechanism of DhaA is expected to be very similar. First nucleophilic attack of an aspartate residue occurs with expulsion of the halide and formation of a substrate-enzyme ester intermediate. In the second step this ester is hydrolyzed by means of histidine mediated general base catalysis.[10] By screening with a phage library in which all variants lack this crucial histidine residue, mutants which can carry out the alkylation half-reaction are covalently captured. We thus select for improved binding and the first reaction step, which are likely selectivity determining.[11] To obtain improved functional enzymes the histidine of the catalytic triad has to be reintroduced at the end.

This phage display system requires molecules which contain chiral substrates tethered to a solid phase or which can be captured by a solid phase after reaction. We chose the biotin-streptavidin interaction, which is commonly used in phage display panning experiments. The generic structure of the molecules needed for such a system is shown in Figure 1. The molecules contain three parts, namely a biotin unit, a spacer and a substrate unit.

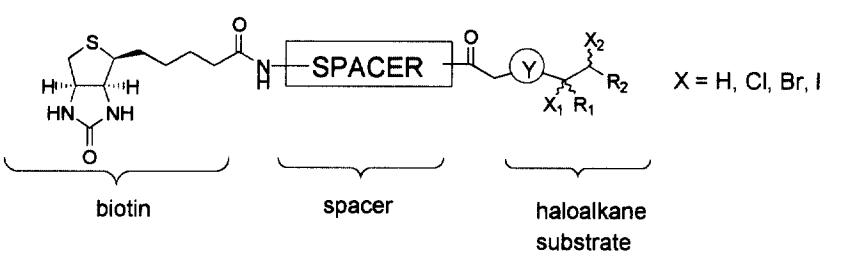
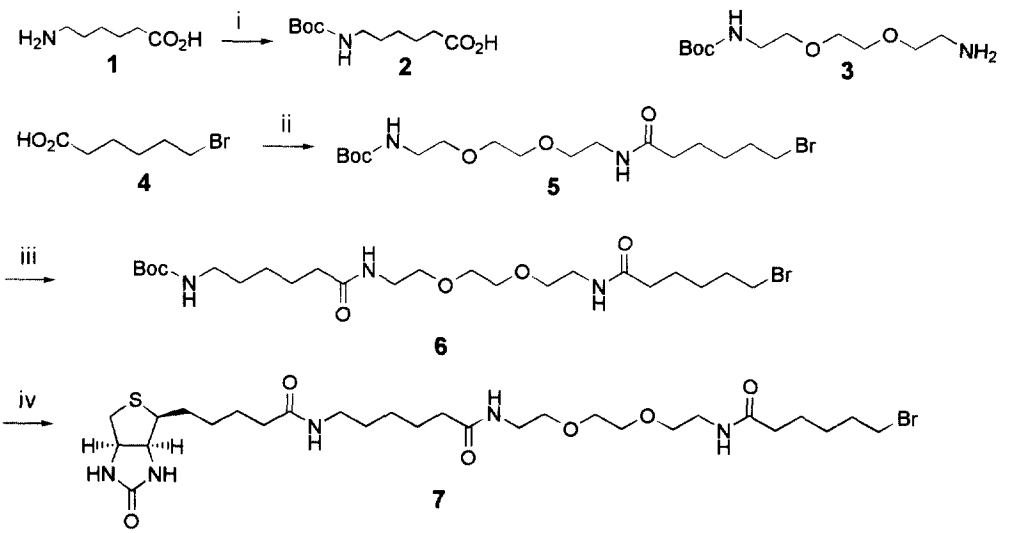



**Figure 1.** Generic target structure of molecules for phage display screening of dehalogenases.

The first biotin/substrate molecule chosen for synthesis was 7. This molecule was made to prove the concept of the covalent capture strategy described above. For this reason it contains pentyl bromide, which is known to be a substrate for the wild-type DhlA and DhaA. The length of the spacers was chosen to avoid steric interference between the DhlA, when covalently attached to the substrate end of the molecule, and

streptavidin, when it is bound to the biotin end. The required length was estimated by examination of their respective crystal structures.[9,12] On the biotin end it was estimated that about 4 to 5 atoms (in a linear spacer) beyond the biotin carbonyl would be required. This number is consistent with other studies involving the streptavidin-biotin interaction.[13] The active site of DhlA is buried deeply inside the enzyme. The distance from the bound halide to the protein surface was estimated to be 17Å, a distance which can be covered by a linear C15 spacer or its equivalent.




**Scheme 1** i) $(Boc)_2O$, $NEt_3$, DMF, 80 °C, 2 h, 86%; ii) a. $SOCl_2$, Δ, 2 h; b. **3**, $NEt_3$, $CH_2Cl_2$, 1 h, 94%; iii) a. TFA/$CH_2Cl_2$, 1 h; b. **2**, TBTU, DMF, 14 h, 86%; iv) a. TFA/$CH_2Cl_2$, 1 h; b. biotin N-hydroxysuccinimidyl ester, $NEt_3$, DMF, 44%.

The chosen molecule 7 was prepared as shown in Scheme 1. The amino group of 6-aminocaproic acid **1** was protected with a Boc group yielding **2**. The mono protected 2,2'-(ethylenedioxy)diethylamine **3**[4b] was coupled to the acid chloride of 6-bromohexanoic acid, which after deprotection and coupling to **2** using the coupling reagent TBTU[14] gave **6**. Removal of the Boc protecting group and coupling the amine to the succinimidyl ester of biotin lead to the desired target 7.[15]

To see whether or not the design strategy was valid, the molecule was added to the enzymes DhlA and DhaA. It was found that these enzymes are both capable of converting 7 at good rates of only about one order of magnitude slower than the excellent substrate 1-bromobutane, indicating that biotin and spacer do not prevent the enzyme catalysis. After these encouraging observations we investigated whether a mutant lacking the catalytic triad histidine forms a stable covalent linkage to 7. Formation of a stable ester intermediate of 1,2-dibromoethane by the His-289-Gln DhlA mutant was demonstrated by fluorescence quenching and mass spectrometry.[16] Quenching of the fluorescence of two active site tryptophans of DhlA is indicative of the presence of compounds in the active site. Such a quenching was observed over time when 7 was added to a

solution of the His mutant. The quenching was similar to that caused by 1-bromohexane and persisted even after the small molecules were dialyzed away, indicating the formation of a stable ester intermediate crucial for the screening strategy (Figure 2). Panning experiments with phage-expressed enzymes to validate the concept further are currently being optimized and will be reported elsewhere.

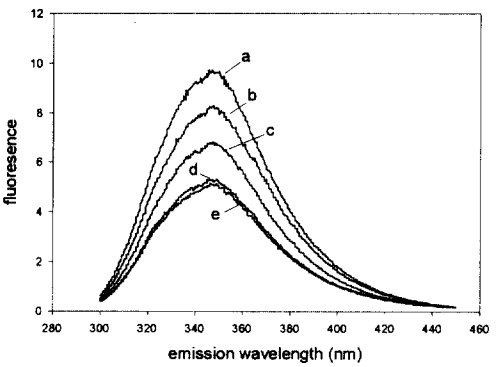
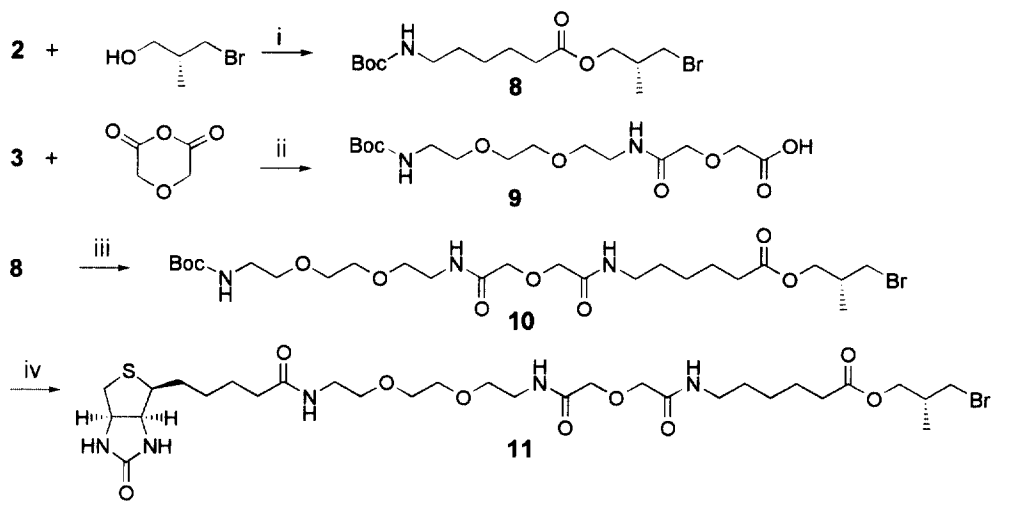



**Figure 2.** Fluorescence quenching of His-289-Gln DhlA mutant (1.4 μM) by 7 (200 μM). a) before addition of 7; b) 2 min. after addition; c) 15 min.; d) 131 min.; e) quenching experiment with 1-bromohexane (fully equilibrated) for reference.

We also completed the synthesis of a chiral substrate attached to biotin and spacer, compound **11**, which is intended for phage screening of mutant libraries for enantioselectivity. To this end we used the commercially available (R)-(+)-3-bromo-2-methyl-1-propanol. The racemate of this compound is non-selectively converted by DhaA and therefore represents a good test case for the introduction of enantioselectivity. Compound **11** was synthesized as shown in Scheme 2. It contains the chiral unit, and the distance between halide and biotin is kept close to that of 7. The chiral alcohol (R)-(+)-3-bromo-2-methyl-1-propanol was coupled by DCC to acid **2** giving the ester **8**. Amine **3** was used to open diglycolic anhydride leading to acid **9**. After deprotection of **8** it was coupled to **9** using TBTU. The resulting **10** was deprotected and coupled to activated biotin to give the target compound **11**.[17]

In summary, we have prepared two molecules for phage display screening. The first compound (7) contains an achiral haloalkane unit and has been used for evaluation of the concept of using covalent capture to screen for catalysis. It was shown that indeed covalent capture occurs with 7 and the His-289-Gln mutant of DhlA. The second molecule **11** contains a chiral substrate portion for phage display screening for enantioselectivity with the dehalogenases DhlA and DhaA



**Scheme 2** i) DCC, DMAP, THF, 14h; ii) py, dioxane, 90 °C, 4h, 91%; iii) a. TFA/$CH_2Cl_2$, 1h; b. **9**, TBTU, DMF, 25% (from **2**); iv) a. TFA/$CH_2Cl_2$, 1 h; b. biotin N-hydroxysuccinimidyl ester, $NEt_3$, DMF, 80%..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8. Chiral haloalkanes used are e.g. 1,2-dibromopropane, 1,2-dichloropropane, 1,3-dichlorobutane, 1,3-dibromobutane, methyl-3-bromo-2-methyl propionate, 3-bromo-2-methyl-1-propanol.
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14. TBTU: O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate.
15. Compound **7**: $^1$H-NMR (300 MHz, DMSO-$d_6$): δ 7.86 (t, 1H, $J$ = 5.8 Hz), 7.82 (t, 1H, $J$ = 5.5 Hz), 7.73 (t, 1H, $J$ = 5.5 Hz), 6.42 (s, 1H), 6.36 (s, 1H), 4.29 (dd, 1H, $J$ = 7.5, 5.1 Hz), 4.11 (ddd, 1H, $J$ = 7.7, 4.3, 1.5 Hz), 3.52 (t, 2H, $J$ = 6.6 Hz), 3.48 (s, 4H), 3.37 (t, 2H, $J$ = 5.5 Hz), 3.37-3.29 (m, 2H), 3.17 (app. q, 4H, $J$ = 5.9 Hz), 3.12-3.04 (m, 1H), 2.98 (app. q, 2H, $J$ = 6.3 Hz), 2.81 (dd, 1H, $J$ = 12.5, 5.1 Hz), 2.56 (d, 1H, $J$ = 12.5 Hz), 2.09-1.99 (m, 6H), 1.82-1.72 (m, 2H), 1.67-1.12 (m, 16H); Electrospray MS (MeOH, $NH_4OAc$): *m/z* 664 (100%, $M$ + H), 681 (25%, $M + NH_4^+$).
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17. Compound **11**: $^1$H-NMR (300 MHz, DMSO-$d_6$): δ 8.05 (t, 1H, $J$ = 5.9 Hz), 8.02 (t, 1H, $J$ = 5.9 Hz), 7.83, (t, 1H, $J$ = 5.7 Hz), 6.41 (s, 1H), 6.35 (s, 1H), 4.29 (dd, 1H, $J$ = 7.7, 5.1 Hz), 4.12 (ddd, 1H, $J$ = 7.7, 4.4, 1.8 Hz), 3.95 (d, 2H, $J$ = 6.6 Hz), 3.92, (s, 2H), 3.90 (s, 2H), 3.54-3.04 (m, 17 H), 2.80 (dd, 1H, $J$ = 12.5, 5.1 Hz), 2.56 (d, 1H, $J$ = 12.5 Hz), 2.31 (t, 2H, $J$ = 7.3 Hz), 2.18-2.06 (m, 1H), 2.05 (t, 2H, $J$ = 7.3 Hz), 1.65-1.18 (m, 12H), 0.96 (d, 3H, $J$ = 7.0 Hz); Electrospray MS (MeOH, $NH_4OAc$): *m/z* 738 (100%, $M$ + H), 755 (45%, $M + NH_4^+$).