

SUPPORTING INFORMATION

Protein-nucleic acid conjugation with sterol linkers using hedgehog autoprocessing

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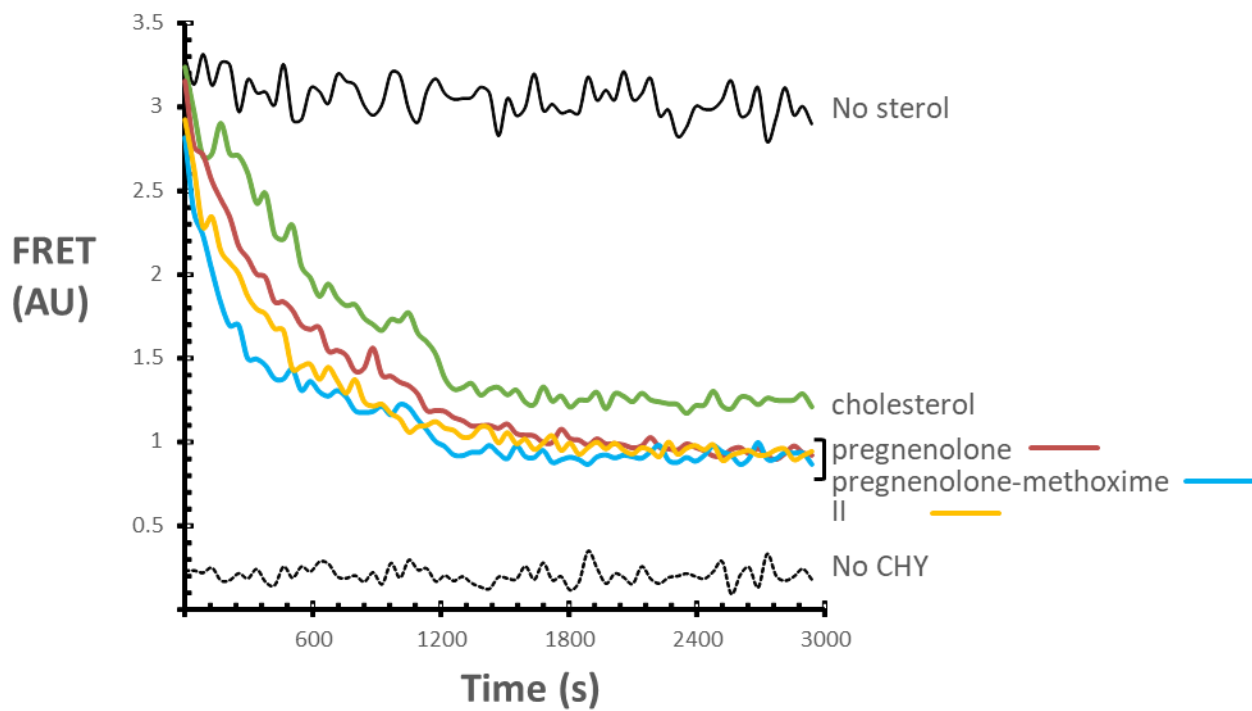
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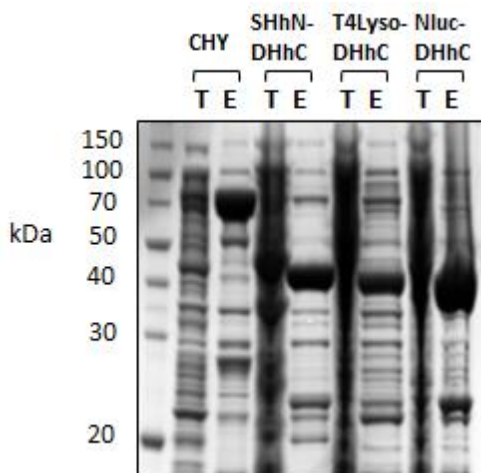


Supporting Figure 1. Substrate activity of pregnenolone and its oxime adducts are comparable to cholesterol. Sterols were assayed using the FRET active precursor, C-H-Y. Sterol concentration, 100 μ M.

Chemicals: All chemical reagents were obtained from commercial supplier and used directly unless otherwise noted: Acetonitrile, Pregnenolone, Phosphate buffer, Terrific Broth, TCEP, Tris Base, EDTA (Fisher Scientific Inc.); Arabinose, Cholesterol, Chloramphenicol, HEPES, n-butanol, Luria Bertani Broth (Sigma); Chloroform-d, D₂O (Cambridge Isotope Laboratories); TEAA (Calbiochem); Cyclopentanyl acetone, pyrazine (Alfa Aesar); DNase and restriction enzymes (NEB Biolabs); Fos-Choline-12 (Anatrace); Ampicillin, IPTG, Methoxyamine (MP Biomedicals); KCl, MgCl₂ (VWR); Lysozyme (Invitrogen); MeOH (Macron Fine Chemicals); Ni-NTA column (GE Health); Bis-aminooxy-PEG₃ (BroadPharm).

Protein Constructs and Expression: FRET-active C-H-Y was expressed from plasmid, pBAD33 using arabinose induction in *E. coli* MC1061 and purified by Ni-NTA chromatography, as described (1). The three other HhC precursor constructs (SHhN-DHhC, T4-lysozyme-HhC, and Nluc-HhC) were expressed from the pET-22b using IPTG induction in *E. coli* BL21 DE3, and then purified by Ni-NTA chromatography (2).

Inductions for all constructs were carried out at 16 °C for 18-20 hours. Cells were collected by centrifugation at 10000 RPM, resuspended in lysis buffer (0.5 % Triton X-100, 0.05 M K₂HPO₄, 0.4 M NaCl, 0.1 M KCl, 10 % glycerol, 0.01 M imidazole, pH=7.3), and then subjected to several freeze (-80 °C) / thaw cycles and sonication. Insoluble material was removed by centrifugation at 10,000 RPM and to the cleared lysate we added, DNase-I and an equal volume of cold 2x NiNTA bind buffer (1 M NaCl, 0.04 M Na₂HPO₄, 0.06 M imidazole, 20 % glycerol, pH=7.5). Ni-NTA spin columns were used to purify protein according to the manufacturer's protocol. Purified protein was stored at -80 °C in elution buffer (0.02 M Na₂HPO₄, 0.5 M NaCl, 0.5 M imidazole, 10% glycerol, pH=7.3) with added TCEP (5×10^{-3} M). All proteins were expressed in the soluble fraction and purified under native conditions, as shown below: (T) total soluble; (E) elution.



FRET assay: HhC activity assays were carried out as described (1) with minor modifications. Briefly, activity of C-H-Y was monitored with a BioTek H1 plate reader at 30 °C. On a 96 well plate, each well contained C-H-Y (2×10^{-7} M) in BisTris buffer (0.05 M, pH 7.1), ethylenediaminetetraacetic acid

(EDTA, 5×10^{-4} M), NaCl (0.1 M), Tris(2-carboxyethyl) phosphine hydrochloride (TCEP, 5×10^{-3} M), Fos-Choline-12 (1.5×10^{-3} M). The samples were incubated for 10 min at 30 °C. Then cholesterol from an ethanol stock was added to initiate cholesterylation reactions. The final concentration of ethanol was 4% (V/V). Cholesterolysis of C-H-Y was monitored continuously by the decrease in the FRET ratio with excitation at 400 nm. ($\text{FRET} = \frac{\text{emission } 540\text{nm}}{\text{emission } 460\text{nm}}$)

Equation for calculating K_M :

$$V_0 = \frac{V_{max} \times [S]}{K_m + [S]}$$

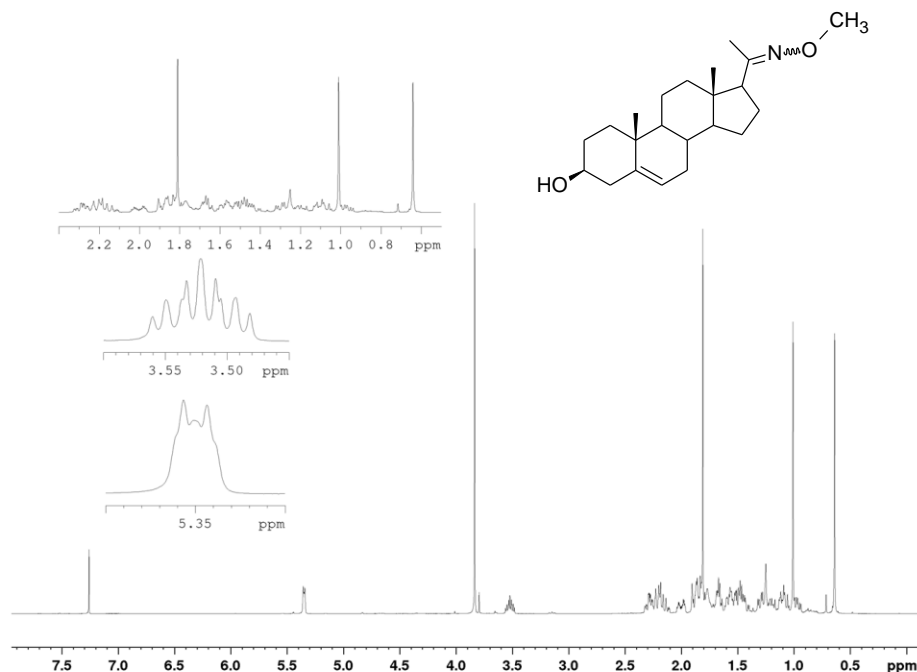
Equation for calculating k_{max} :

$$\text{FRET} = \text{FRET}_0 e^{-kt} + C$$

Preparation of pregnenolone methoxime: To 10 ml of 90% methanol and 10% 1 M TEAA, 31.8 mg (0.1 mmol) of pregnenolone and 33.4 mg (0.4 mmol) of methoxyamine were added. The mixture was stirred at room temperature for 24 h. The binary solvent was evaporated in vacuo. The remaining mixture was washed with ethyl acetate (5 ml) for three times. The organic phase was washed with phosphate buffer (pH=7.0), dried over Na_2SO_4 , and evaporated in vacuo to afford pregnenolone methoxime (33 mg, 95%) as a white powder.

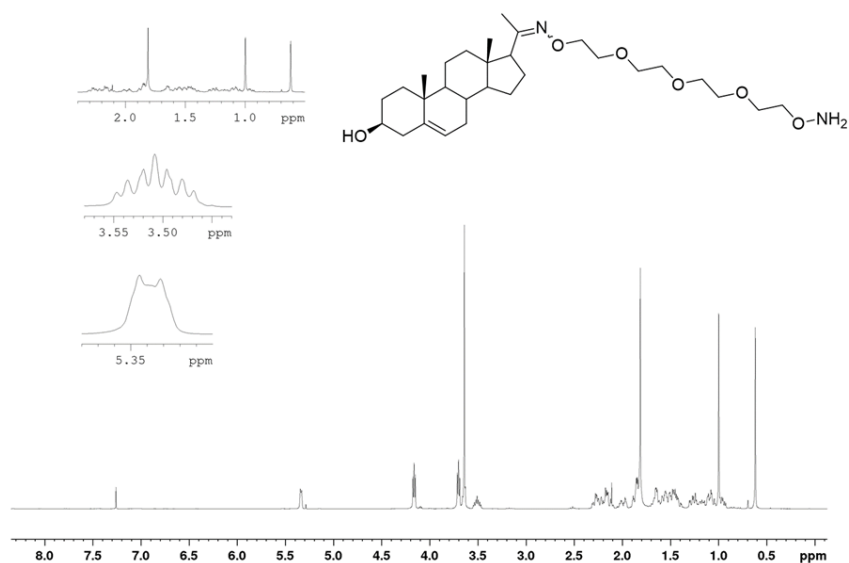
^1H NMR spectra were acquired with Bruker Avance III HD 400 (400MHz) spectrometer at 25 °C CDCl_3 (cambridge isotope laboratories) was used as NMR solvent. ^1H chemical shifts are reported as δ in units of parts per million (ppm) relative to chloroform-d (7.26, s)

^1H NMR (400MHz, CDCl_3) δ 5.35 (dd, 1H), 3.83 (s, 3H), 3.52 (m, 1H), 2.19 (dd, 1H), 1.81 (s, 3H), 1.67 (m, 2H), 1.01 (s, 3H), 0.64 (s, 3H). ^{13}C NMR (100MHz, CDCl_3) δ 157.70, 140.80, 121.50, 71.75, 61.22, 56.60, 43.78, 36.56, 24.33, 19.45, 15.60, 13.17.



Preparation of II: To 1 ml of 90% methanol and 10% 1 M TEAA, 31.8 mg (0.1 mmol) of pregnenolone and 44.9 mg (0.2 mmol) of Bis-aminooxy-PEG₃ were added. The mixture was stirred at room temperature for 16h. After that the mixture was washed with ethyl acetate (5 ml) for three times. Then the organic phase was combined and washed with H₂O (5 ml) for three times, dried over Na₂SO₄, and evaporated under N₂ flow to afford pregnenolone oximes II (47 mg, 90%) as a white solid.

¹H NMR (400MHz, CDCl₃) δ 5.34 (dd, 1H), 4.16 (t, 2H), 3.70 (t, 2H), 3.51 (m, 1H), 2.17 (dd, 1H), 1.81 (s, 3H), 1.66 (m, 2H), 1.00 (s, 3H), 0.62 (s, 3H). ¹³C NMR (100MHz, CDCl₃) δ 158.00, 140.82, 121.50, 74.77, 72.55, 71.73, 69.89, 56.73, 43.66, 36.56, 24.27, 19.42, 15.93, 13.21.



Kinetics and equilibrium of ketoxime formation using a model reaction: We used the condensation of methoxyamine (MP Biochemicals) and cyclopentanyl acetone in phosphate buffered D₂O to determine rates of oxime formation and hydrolysis **at pH 7, 22 °C**. Reaction progress was monitored by ¹H NMR using pyrazine (Alfa Aesar) as an internal integration standard. To determine the overall equilibrium constant, methoxyamine (2×10^{-3} M) and cyclopentanyl acetone (2×10^{-3} M) were combined in phosphate buffer (0.2 M). Approach to equilibrium was monitored over a period of 24 hours by following the disappearance of methoxyamine and cyclopentanyl acetone and the accumulation of oxime. In a separate set of experiments, kinetics of ketoxime formation were determined under pseudo first order conditions, with cyclopentanyl acetone at 2×10^{-3} M, and methoxyamine in excess, either 0.02 M, 0.03 M, 0.04 M, 0.05 M. Reaction progress curves, following the disappearance of cyclopentanyl acetone, were fit to first order exponential equation. The second order rate constant of ketoxime formation was obtained from the slope from plotting k_{obs} vs methoxyamine concentration. The rate of ketoxime hydrolysis at pH 7 was calculated from: $k_{\text{hyd}} = k_{\text{oxime}} / K_{\text{eq}}$.

$$\frac{[\text{pyrazine}] \times V \times 4}{[\text{compound}] \times V \times 3} = \frac{\text{Integral of pyrazine's peak}}{\text{Integral of compound's peak}}$$

$$[\text{compound}] = \frac{4 \times \text{Integral of compound's peak} \times [\text{pyrazine}]}{3 \times \text{Integral of pyrazine's peak}}$$

$$[a] = \frac{4 \times 0.4430 \times 0.001}{3 \times 1} = 5.9 \times 10^{-4} M$$

$$[b] = \frac{4 \times 0.4533 \times 0.001}{3 \times 1} = 6.0 \times 10^{-4} M$$

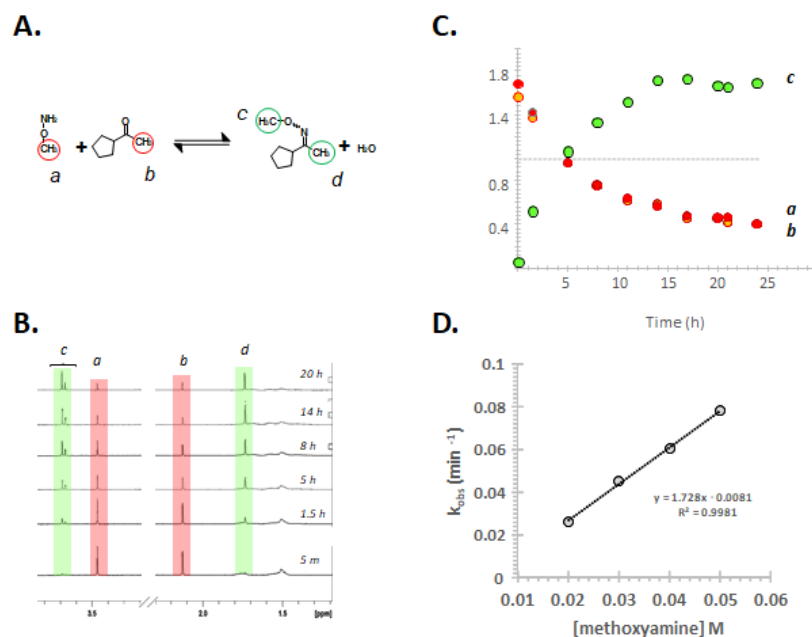
$$[c] = \frac{4 \times 1.6784 \times 0.001}{3 \times 1} = 2.2 \times 10^{-3} M$$

$$K_{eq} = \frac{[c]}{[a][b]} = \frac{2.2 \times 10^{-3}}{5.9 \times 10^{-4} \times 6.0 \times 10^{-4}} = 6.2 \times 10^3 M^{-1}$$

Kinetics of ketoxime formation were determined under pseudo first order conditions, with cyclopentanyl acetone at $2 \times 10^{-3} M$, and methoxyamine in excess, either 0.02 M, 0.03 M, 0.04 M, 0.05 M. The second order rate constant of ketoxime formation was obtained from the slope from plotting k_{obs} vs methoxyamine concentration.

$$k_{hyd} = \frac{k_{oxime}}{K_{eq}} = \frac{1.7}{6.2 \times 10^3} = 2.7 \times 10^{-4} min^{-1}$$

$$t_{1/2} = \frac{\ln 2}{k_{hyd}} = \frac{0.693}{2.7 \times 10^{-4}} = 2566 min = 42.8 h$$



Supporting Figure 2. (A) Condensation of methoxyamine and cyclopentanyl acetone (B) Reaction progress was monitored at selected intervals by ^1H NMR by following the loss of starting materials (red) and appearance of product (green). (C) Approach to equilibrium. Integrals of specific methyl group peaks, as in (A), plotted as a function of time. (D) Observed (pseudo) first order rates of oxime formation plotted as function of increasing methoxyamine concentration.

General Procedure for Steramer preparation: In 200 μL of 90 % 0.05 M TEAA and 10% methanol, pregnenolone mono-oxime PEG₃ derivative (II) was added to a final concentration of 0.05 M, followed by addition of benzaldehyde modified oligonucleotide (5×10^{-5} M). After 16 h at room temperature on a rotisserie mixer, the reaction was quenched by adding 1 mL of n-butanol followed by a brief vortex (15-30 seconds). The resulting solution was incubated at -80 °C for 1-3 hours. Next, the precipitated steramer was collected by centrifugation at 14000 rpm for 30 mins. The supernatant was decanted, and the pellet was washed with another 1 mL of N-butanol and then collected centrifugation. This procedure was repeated 3x. To remove last traces of N-butanol the pellet dried under Nitrogen stream, then brought up in TE buffer pH7.4 (Tris 0.01 M, EDTA 1×10^{-3} M; Fisher Scientific)

Steramer separation by RP-HPLC: To remove unreacted oligonucleotide, steramers were purified over a Phenomenex Clarity 3 μm Oligo-RP LC column (50x4.6 mm) using a gradient elution from 90%/10% buffer A (5 %ACN in 0.05 M TEAA)/Buffer B (MeOH) to 40% A /60% B over 20 minutes. The flow rate was 1 mL/min and eluate was monitored at 260 nm. Sample injection volume was 20 μL .

General Reaction Conditions for ProNAC biocatalysis by HhC: Precursor protein (2×10^{-6} M, final), Fos-choline 12 (1.5×10^{-3} M, final), Tris(2-carboxyethyl) phosphine hydrochloride (TCEP, 5×10^{-3} M, final), BiseTris buffer (0.05 M, final, pH 7.1), ethylenediaminetetraacetic acid (EDTA, 5×10^{-4} M, final), NaCl (0.1 M, final) were combined. To that mixture, steramer (2×10^{-5} M, final) was added to initiate the reaction. The reaction mixture was incubated for 3 h at room temperature. Then the reaction mixture was analyzed with SDS-PAGE electrophoresis. The extent of reaction was calculated based on gel bands intensity.

$$\% \text{Precursor Consumed} = 1 - \frac{[\text{Remaining "SHhN-DHhC"}]}{[\text{SHhN:cholesterol}] + [\text{DHhC}] + [\text{Remaining "SHhN-DHhC"}]}$$

Nanoluciferase ProNAC preparation, SEC, and Enzymatic Assay: In 30 μL , we combined Nluc-HhC (1.8×10^{-5} M, final) with steramer (1.5×10^{-4} M, final) with Fos-choline 12 (1.5×10^{-3} M, final). After overnight incubation at 4 °C, reactions were passed over HisPur Ni-NTA spin column (Thermo Scientific), and the flow through was injected (20 μL) over TSKgel G3000SW_{xl} column (7.8 mm x 30 cm) with running buffer of 1xPBS buffer, pH 7 at a flow rate of 1ml/min and 4.1-4.3 MPa. Eluate was monitored at 280 nm. Apparent molecular weight determinations were made by comparison to calibration standards (RNase 13 kDa; Carbonic anhydrase 30 kDa; Albumin 66 kDa; Alcohol dehydrogenase 150 kDa; β -amylase 200 kDa) Fractions containing luciferase were assayed for enzymatic activity using the Marker Gene Gaussia Luciferase cellular assay kit (Promega).

Protein and Oligonucleotide Sequences. FRET active C-H-Y (1), and chimeric SHhN-DHhC (2) have been described.

T4Lysozyme-HhC-His6

MNIFEMLRIDERLRLKIYKDTEGYTIGIGHLLTKSPSLNAAKSELDKAIGRNCNGVITKDEAEK
 LFNQDVDAAVRGILRNAKLKPVYDSLDAVRRCALINMVFQMGETGVAGFTNSLRMLQQKRW
 DEAAVNLAKEIWNQTPNRAKRVITTFRTGTWDAYKNLGPSPSGSGHG**CFTPESTALLES****GV**
KPLGELS**IGDRVLSMTANGQAVYSEVILEMDRNLEQM****QNFVQLHTDGGAVLTVTPAHLVS**
WQPESQKLTFVFADRIEKNQVLVRDVETGELRPQRVVKVGSVRSKGVVAPLTREGTIVN
SVAASCYAVINSQSLAHWGLAPMRLSTLEAWLPAKEQLHSSPKVVSSAQQQNGIHWYANA
LYKVKDYVLPQSWRHDGSGHHHHHH

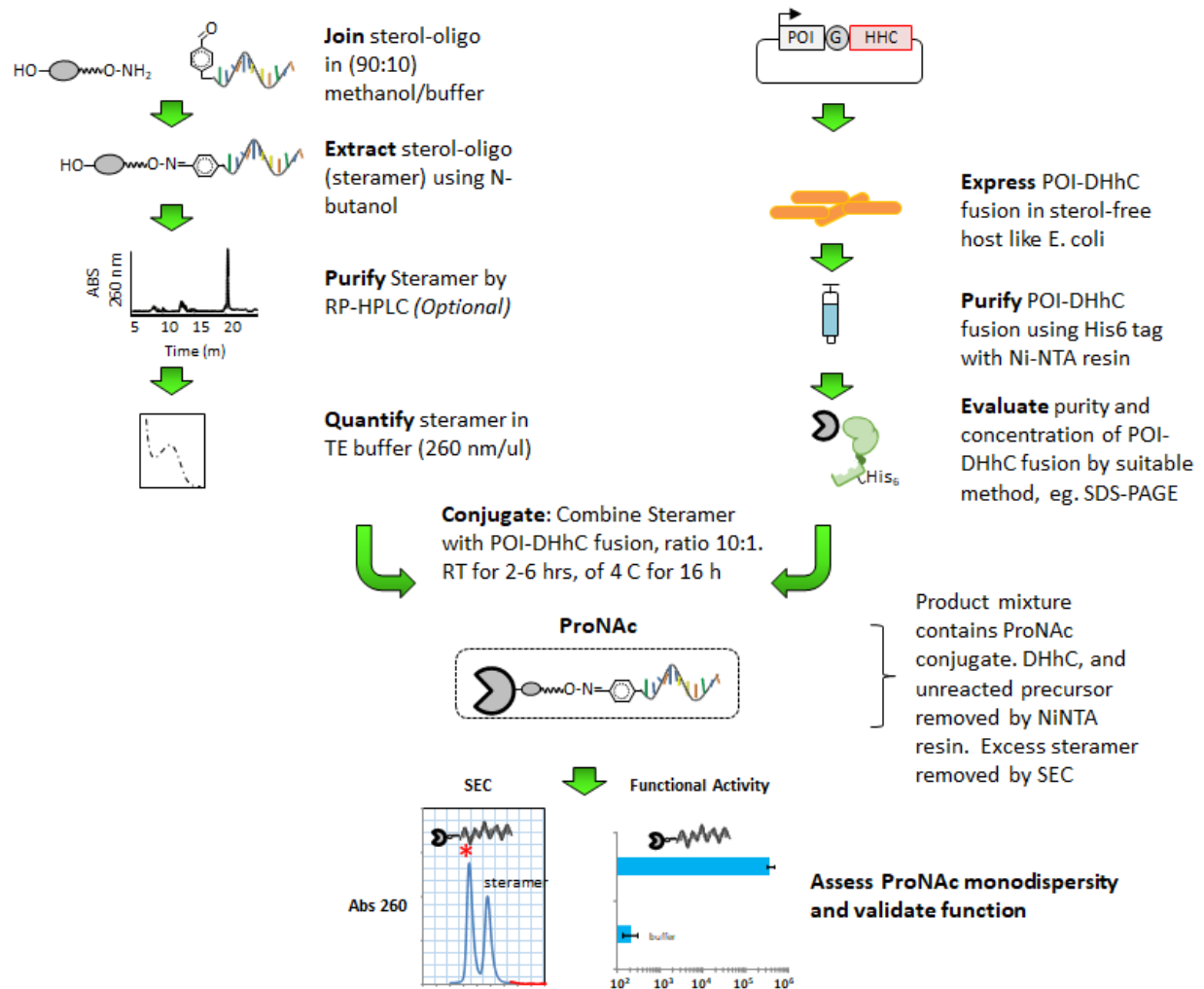
N_{Luc}-HhC-HiS6

MAGVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVII
 PYEGLSGDQMGMQIEKIFKVVPVDDHHFKVILHYGTLVIDGVTNPMIDYFGRPYEGIAVFDGK
 KITVTGTLWNGNKIIDERLINPDGSLLFRVTINGVTGWRLCERILATGHG**CFTPESTALLES****GV**
RKPLGELS**IGDRVLSMTANGQAVYSEVILEMDRNLEQM****QNFVQLHTDGGAVLTVTPAHLVS**
VWQPESQKLTFVFADRIEKNQVLVRDVETGELRPQRVVKVGSVRSKGVVAPLTREGTIV
NSVAASCYAVINSQSLAHWGLAPMRLSTLEAWLPAKEQLHSSPKVVSSAQQQNGIHWYAN
ALYKVKDYVLPQSWRHDGSGHHHHHH

Oligonucleotide Sequences

Name	Sequence	5' modification	Vendor	MW
S-dT ₁₀	TTTTTTTTTTT	C6Ald	TriLink	3291.3
S-dT ₂₀	TTTTTTTTTTTTTTTTTTTTT	C6Ald	TriLink	6333.3
S-dT ₃₀	TTTTTTTTTTTTTTTTTTTTTTTTTTTTT	C6Ald	TriLink	9375.3
S-dHP ₃₉	AATGATGATAACACCTTCTACACCTCCATAAT CATCATT	SAhMC2	IDT	12041. 9

Supporting Figure 3. Preparation of ProNAc Conjugates Using HhC/Steramer System



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2. Owen, T. S., Xie, X. J., Laraway, B., Ngoje, G., Wang, C., and Callahan, B. P. (2015) Active site targeting of hedgehog precursor protein with phenylarsine oxide, *Chembiochem* 16, 55-58.