Supporting Information for

"Structure-Activity Analysis of Human Ghrelin *O*-Acyltransferase Reveals Chemical Determinants of Ghrelin Selectivity and Acyl Group Recognition"

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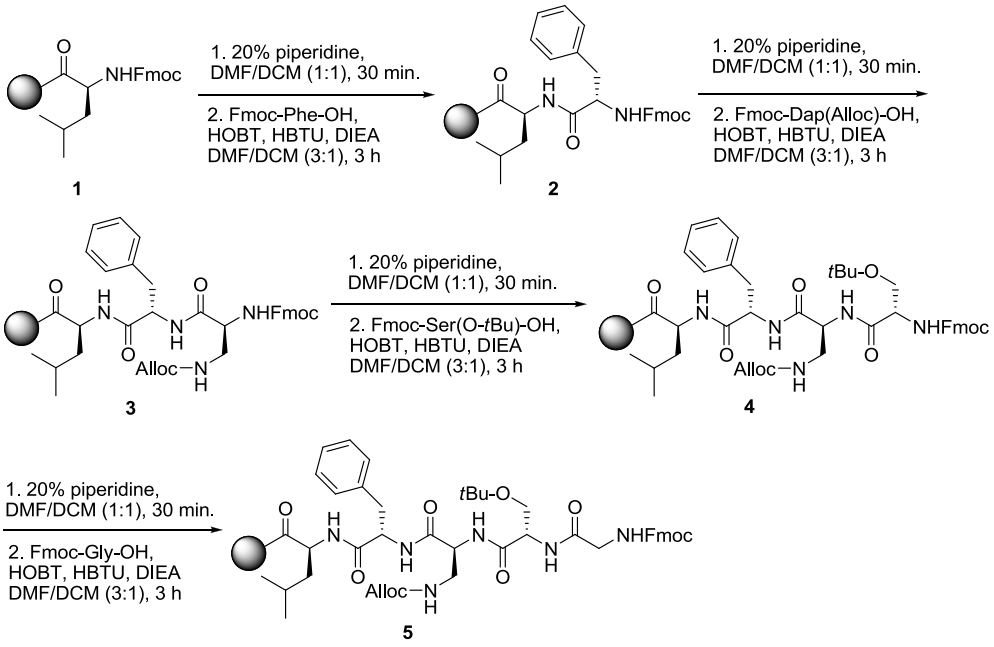
Figure S4. Sequence-activity assessment of potential hGOAT substrates in the human genome.

Synthetic procedure for acylated peptide inhibitors

General procedure:

All Fmoc protected amino acids, coupling reagents and resin were purchased from Chem-Impex Int'l Inc or Nova Biochem. All solvents were purchased from Acros or Sigma Aldrich unless specifically noted. Dichloromethane (DCM) was distilled before use. MALDI spectra were recorded on an AB4800 MALDI-TOF mass spectrometer using an α-cyano-4-hydroxycinnamic acid (4-CHCA) matrix. HPLC purification was performed on Agilent Technologies 1200 series HPLC (flow rate 5 mL/min; UV monitoring at 218 nm and 254 nm) using a semipreparative Agilent Zorbax 300SB-C18 column (9.4 x 250mm, 5µM particle size) and Agilent 1260 series Quat Pump with a variable wavelength UV-Vis detector. Solvents for HPLC purification are 0.1% trifluoroacetic acid in ultrapure H_2O (A) and acetonitrile (CH_3CN) (B).

Solid Phase Peptide Synthesis:



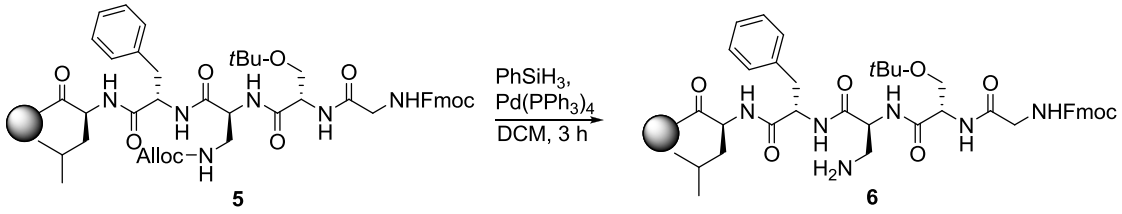
Fmoc-L-Leu-Rink amide AM resin **1** (0.286 g, 0.35 meq/g, 0.1 mmol) was swelled in DMF (5 mL) for 5 minutes in a peptide vessel. The resin was then swelled in DCM (5 mL) for 5 minutes. Liquid was drained and the resin was ready for the following procedure.

General procedure for Fmoc-deprotection:[1]

A solution of piperidine (1 mL) in anhydrous DMF (2 mL) and DCM (2 mL) was added to the peptide vessel containing the swelled resin (0.1 mmol). The mixture was allowed to rock for 30 minutes at room temperature. The solution was drained, and the resin was rinsed with DMF (3 mL x 2). A solution of 1:1 DCM/DMF (3 mL x 2) was added to the resin and the peptide vessel was allowed to rock for 3 minutes. The solution was drained, and DMF (3 mL) was added. The vessel was rocked for 3 minutes, and the solvent was drained.

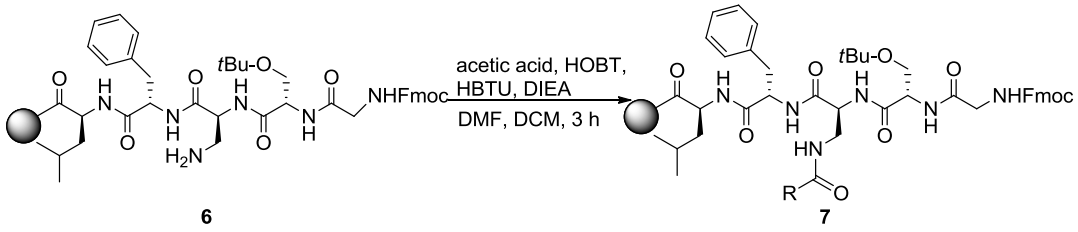
General procedure for peptide synthesis:[1]

Fmoc-amino acid (0.5 mmol), HOBT (0.068 g, 0.5 mmol), and HBTU (0.190 g, 0.5 mmol) were dissolved in anhydrous DMF (3 mL) and DCM (1 mL). DIEA (0.087 mL, 0.5 mmol) was added and the resulting solution was added to the peptide vessel containing the resin (0.1 mmol). The vessel was allowed to rock for 3 hours at room temperature, the solution was drained, and the resin was rinsed with DMF (3 mL x 2). A solution of 1:1 DCM/DMF (3 mL x 2) was added to the resin and the peptide vessel was allowed to rock for 3 minutes. The solution was drained, and DMF (3 mL) was added. The vessel was rocked for 3 minutes, and the solvent was drained.



Orthogonal alloc group deprotection:[2]

The resin **5** (0.05 mmol) was suspended in anhydrous DCM (3 mL) in a peptide vessel. $Pd(PPh_3)_4$ (0.046 g, 0.04 mmol) and phenylsilane (0.12 mL, 1.0 mmol) were added. The vessel was allowed to rock for 2 hours, and the solution was drained under vacuum. The resin was rinsed with DCM (3 mL x 2), followed by washing the resin with DCM (3 mL x 2) by rocking for 3 minutes. Generation of the side chain primary amine to generate resin-bound peptide **6** was confirmed by orange resin coloration upon testing with TNBS.[3]



*General procedure for acylation of peptide **6**:*

Alkyl carboxylic acid (0.25 mmol), HOBT (0.034 g, 0.25 mmol), and HBTU (0.095 g, 0.25 mmol) were dissolved in anhydrous DMF (3 mL) and DCM (1 mL). DIEA (0.044 mL, 0.25 mmol) was added and the resulting solution was added to the peptide vessel containing the resin **6** (0.05 mmol). The vessel was allowed to rock for 3 hours, and the solution was drained. The resin was rinsed with DMF (3 mL x 2). A solution of 1:1 DCM/DMF (3 mL x 2) was added to the resin and the

peptide vessel was allowed to rock for 3 minutes. The solution was drained, and DMF (3 mL) was added. The vessel was rocked for 3 minutes, and the solvent was drained. Acylation of the side chain primary amine was confirmed by a negative TNBS test, with the resin exhibiting a pale gray color.[3]

Procedure for peptide cleavage from the resin:

The resin-bound peptide **7** (0.05 mmol) was deprotected and cleaved from the resin support per previously reported protocols.[1] The Fmoc group was removed according to the general procedure for Fmoc-deprotection described above. Following Fmoc removal, a cleavage cocktail of TFA/H_2O/TIPS/anisole (4.5 mL/0.25 mL/0.125 mL/0.125 mL) was added to the vessel, and the vessel was allowed to rock for 3 hours. The solution was collected, and the resin was rinsed with TFA (1 mL x 2) with the rinse added to the collected solution.

Ice cold ether (10 mL) was added to a separatory funnel. The combined cleavage solution was poured into the funnel, followed by 5% acetic acid in water (5 mL). The resulting organic layer was extracted with 5% acetic acid in water (5 mL x 3). The aqueous layer and washes were combined and the remaining TFA and acetic acid were removed under reduced pressure. The acylated peptide product **8** was obtained by lyophilization and purified by reverse phase HPLC using a gradient of 20% B for 10 minutes followed by 35% B for 30 min. Acylated peptide masses were confirmed by MALDI as indicated below.

Acetylated peptide: Acetylated peptide is prepared from acylation of peptide **6** with acetic acid. HRMS (MALDI) calculated for $C_{25}H_{39}N_7O_7$ 572.2809 $(M+Na)^+$; Found, 572.3545.

Butyrylated peptide: Butyrylated peptide is prepared from acylation of peptide **6** with butyric acid HRMS (MALDI) calculated for $C_{27}H_{43}N_7O_7$ 600.3122 $(M+Na)^+$; Found, 600.3056.

Hexanoylated peptide: Hexanoylated peptide is prepared from acylation of peptide **6** with hexanoic acid. HRMS (MALDI) calculated for $C_{29}H_{47}N_7O_7$ 628.3435 $(M+Na)^+$; Found, 628.3759.

Heptanoylated peptide: Heptanoylyated peptide is prepared from acylation of peptide **6** with heptanoic acid. HRMS (MALDI) calculated for $C_{30}H_{49}N_7O_7$ 642.3591 $(M+Na)^+$; Found, 642.3752.

Octanoylated peptide: Octanoylated peptide is prepared from acylation of peptide **6** with octanoic acid. HRMS (MALDI) calculated for $C_{31}H_{51}N_7O_7$ 656.3748 $(M+Na)^+$; Found, 656.4725.

Nonanoylated peptide: Nonanoylated peptide is prepared from acylation of peptide **6** with nonanoic acid. In the nonanoic acid acylation step, HCTU was used in place of HBTU as a coupling reagent. After the peptide was cleaved from the resin, the solution was collected, and the resin was washed by TFA (1 mL x 2) with the rinse added to the collected solution. Solvent was removed under reduced pressure at room temperature overnight, and the obtained crude peptide was purified by reverse phase HPLC. HRMS (MALDI) calculated for $C_{32}H_{53}N_7O_7$ 670.401

$(M+Na)^+$; Found, 670.472.

Decanoylated peptide: Decanoylated peptide is prepared from acylation of peptide **6** with decanoic acid. HRMS (MALDI) calculated for $C_{33}H_{55}N_7O_7$ 684.4061 $(M+Na)^+$; Found, 684.4306.

Table S1. Calculated and observed m/z values of hGOAT peptide substrates as determined by MALDI-TOF mass spectrometry.

Peptide	Observed Species	Calculated mass (Da)	Experimental Mass (Da)
$GSSFLC_{AcDan}$	$[M+Na]^+$	858.98	859.84
Sarcosine-$SSFLC_{AcDan}$	$[M+K]^+$	889.11	888.89
N,*N*-dimethyl-$GSSFLC_{AcDan}$	$[M+K]^+$	903.14	903.04
Betaine-$SSFLC_{AcDan}$	$[M]^+$	880.08	879.22
Methoxyacetic-$SSFLC_{AcDan}$	$[M+K]^+$	890.10	889.47
$GDSFLC_{AcDan}$	$[M+K]^+$	903.09	902.26
$GPSFLC_{AcDan}$	$[M+K]^+$	885.12	885.22
$GHSFLC_{AcDan}$	$[M+K]^+$	925.15	924.05
$GKSFLC_{AcDan}$	$[M+H]^+$	879.10	877.02
$GSTFLC_{AcDan}$	$[M+K]^+$	889.11	889.07
$GSSALC_{AcDan}$	$[M+K]^+$	798.99	799.13
$GSSTLC_{AcDan}$	$[M+Na]^+$	812.91	812.30
$GSSELC_{AcDan}$	$[M+Na]^+$	840.92	840.58
$GSSVLC_{AcDan}$	$[M+Na]^+$	810.94	810.46
$GSSQLC_{AcDan}$	$[M+K]^+$	856.04	856.16
$GSSHLC_{AcDan}$	$[M+H]^+$	827.96	826.44
$GSSKLC_{AcDan}$	$[M+H]^+$	819.00	817.56
$GSSYLC_{AcDan}$	$[M+Na]^+$	874.98	874.77
$GSSWLC_{AcDan}$	$[M+K]^+$	914.12	914.14
$GSS[Cha]LC_{AcDan}$	$[M+Na]^+$	865.03	864.92
$GSS[Nit\text{-}F]LC_{AcDan}$	$[M+Na]^+$	903.98	904.07
$GSS[Nit\text{-}Y]LC_{AcDan}$	$[M+Na]^+$	919.98	919.96

Table S2. Quantitation of peptide substrate octanoylation by hGOAT following one hour incubation.

Peptide	Integrated Substrate Fluorescence (AU)	Integrated Product Fluorescence (AU)	Increase in retention time upon peptide octanoylation (min)
$GSSFLC_{AcDan}$	25 ± 4	3.3 ± 0.3	6.5
Sarcosine-$SSFLC_{AcDan}$	15 ± 5	2.5 ± 0.3	6.5
N,*N*-dimethyl-$GSSFLC_{AcDan}$	23 ± 9	*Not detected*	-
Betaine-$SSFLC_{AcDan}$	32 ± 3	*Not detected*	-
Methoxyacetic-$SSFLC_{AcDan}$	200 ± 100	*Not detected*	-
$GDSFLC_{AcDan}$	50 ± 10	*Not detected*	-
$GPSFLC_{AcDan}$	27 ± 2	*Not detected*	-
$GVSFLC_{AcDan}$	41 ± 7	0.44 ± 0.09	7
$GHSFLC_{AcDan}$	15 ± 1	0.13 ± 0.03	5
$GKSFLC_{AcDan}$	16 ± 2	0.32 ± 0.05	5
$GFSFLC_{AcDan}$	25 ± 4	0.20 ± 0.04	6.5
$GSTFLC_{AcDan}$	26 ± 8	0.41 ± 0.08	6.5
$GSSALC_{AcDan}$	15 ± 1	1.13 ± 0.09	6.5
$GSSTLC_{AcDan}$	8 ± 2	0.9 ± 0.2	6.5
$GSSELC_{AcDan}$	9.9 ± 0.6	*Not detected*	-
$GSSVLC_{AcDan}$	16 ± 2	0.2 ± 0.1	7
$GSSQLC_{AcDan}$	7.8 ± 0.6	0.48 ±0.02	6.5
$GSSHLC_{AcDan}$	11.7 ± 0.6	0.4 ± 0.1	5
$GSSKLC_{AcDan}$	8 ± 3	*Not detected*	-
$GSSYLC_{AcDan}$	10 ± 3	0.6 ± 0.1	6
$GSSWLC_{AcDan}$	26.4 ± 0.2	1.77 ± 0.07	6
GSS[Cha]LC_{AcDan}	21 ± 5	0.7 ± 0.2	7
GSS[Nit-F]LC_{AcDan}	2.5 ± 0.7	0.32 ± 0.02	6.5
GSS[Nit-Y]LC_{AcDan}	3 ± 1	0.25 ± 0.05	6

Table S3. K_m values for hGOAT octanoylation of peptide substrates

Peptide	K_m (μM)
$GSSFLC_{AcDan}$	0.5 ± 0.1
$GVSFLC_{AcDan}$	< 1
$GHSFLC_{AcDan}$	> 5
$GKSFLC_{AcDan}$	> 5
$GFSFLC_{AcDan}$	1 ± 0.5
$GSSALC_{AcDan}$	> 5
$GSSTLC_{AcDan}$	> 5
$GSSVLC_{AcDan}$	< 1
$GSSQLC_{AcDan}$	> 5
$GSSHLC_{AcDan}$	> 5
$GSSYLC_{AcDan}$	> 5
$GSSWLC_{AcDan}$	> 5
$GSS[Cha]LC_{AcDan}$	0.9 ± 0.3
$GSS[Nit\text{-}F]LC_{AcDan}$	> 5
$GSS[Nit\text{-}Y]LC_{AcDan}$	> 5

Table S4. IC_{50} values for inhibition of hGOAT-catalyzed octanoylation by acylated peptide inhibitors.

Acyl Chain Length	IC_{50} (nM)[a]
0	> 10,000[b]
2	5800 ± 2300
4	3200 ± 750
6	1050 ± 150
7	65 ± 29
8	22 ± 6
9	441 ± 53
10	1470 ± 260

[a] IC_{50} values were determined as described in the Methods section. IC_{50} values are the average of a minimum of three independent determinations, with standard deviation reported as error.

[b] No inhibition was observed with the GSAFL (0 acyl length) inhibitor up to a concentration of 10 μM.

Supporting Figure Legends

Figure S1. Effect of coincubation of *N*,*N*-dimethylglycine and betaine substrates on hGOAT-catalyzed octanoylation of the $GSSFLC_{AcDan}$ substrate. Coincubation of either the *N*,*N*-dimethylglycine or betaine substrates (1.5 μM) with the parent $GSSFLC_{AcDan}$ substrate did not reduce $GSSFLC_{AcDan}$ octanoylation. Assays were performed as described for inhibitor trials in the Methods section, with the dimethylglycine or betaine substrate peptide used in place of the acyl inhibitors.

Figure S2. Representative time courses for peptide substrate octanoylation by hGOAT. (a) Plot of octanoylated product formation versus time for the $GSSFLC_{AcDan}$ substrate. (b) Plot of octanoylated product formation versus time for the $GKSFLC_{AcDan}$ substrate. (c) Plot of octanoylated product formation versus time for the $GSS[Cha]LC_{AcDan}$ substrate, [Cha] = cyclohexylalanine. Assays were performed as described in the Methods section with 1.5 μM peptide substrate and quenched at various time points by addition of 20% acetic acid in isopropanol. Reactions were analyzed by HPLC as described in the Methods section. All reaction times courses are fit linearly.

Figure S3. Representative K_m curves for peptide substrate octanoylation by hGOAT. For all plots, product fluorescence is normalized to product fluorescence measured for reactions with 5 μM peptide substrate. (a) Dependence of peptide octanoylation on $GSSFLC_{AcDan}$ substrate concentration. (b) Dependence of peptide octanoylation on $GKSFLC_{AcDan}$ substrate concentration.

(c) Dependence of peptide octanoylation on GSS[Cha]LC_{AcDan} substrate concentration, [Cha] = cyclohexylalanine. (d) Dependence of peptide octanoylation of $GFSFLC_{AcDan}$ concentration. Assays were performed and analyzed as described in the Methods section.

Figure S4. Sequence-activity assessment of potential hGOAT substrates in the human genome. Starting with the human genome open reading frames annotated in the Swiss-Prot database (20,265 as of April 2014), bioinformatics analysis yields ghrelin as the only likely hGOAT substrate within the human genome.

Figure S1.

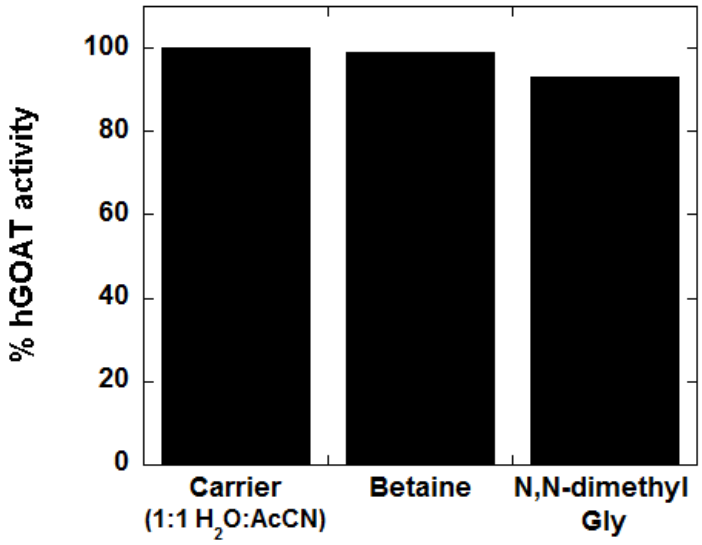

% hGOAT activity
100
80
60
40
20
0
Carrier
(1:1 H2O:AcCN)
Betaine
N,N-dimethyl
Gly

Figure S2.

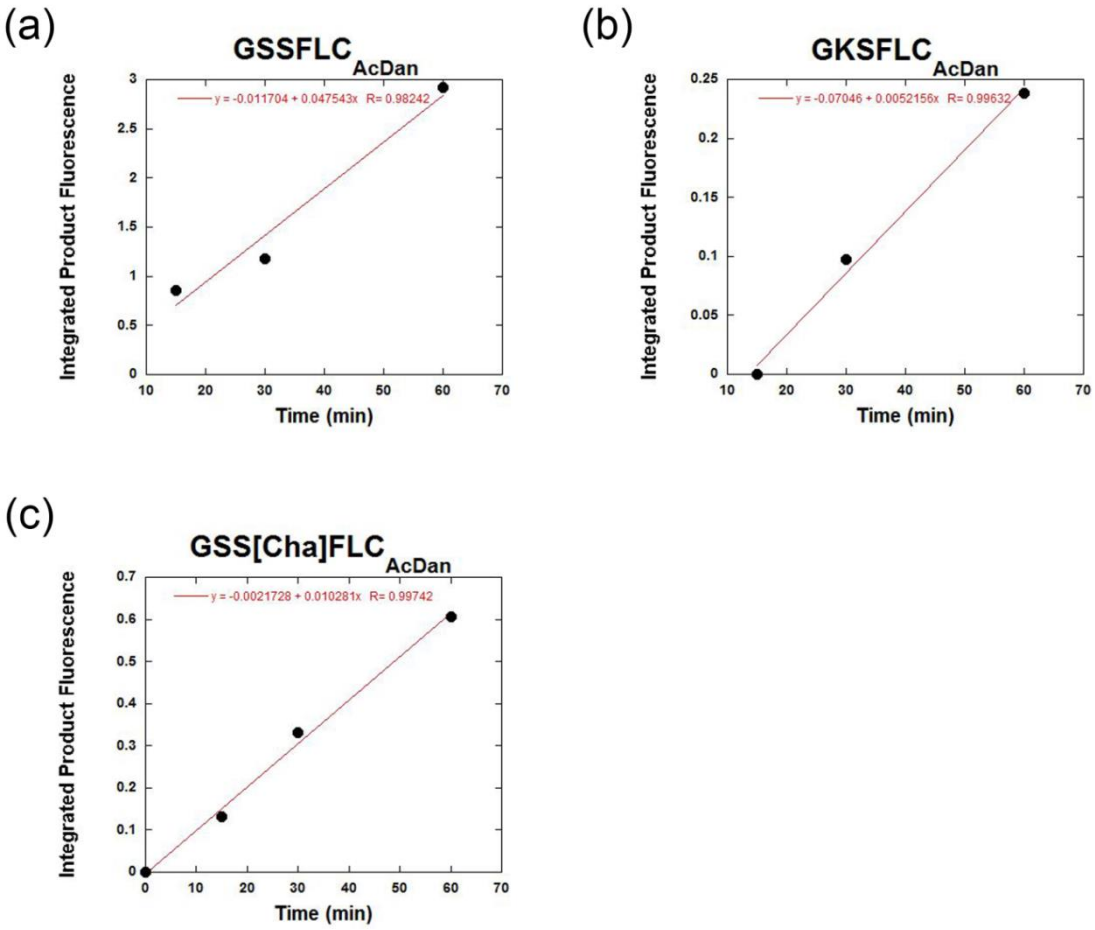

(a)
GSSFLC AcDan
y = -0.011704 + 0.047543x R= 0.98242
Integrated Product Fluorescence
Time (min)
(b)
GKSFLC AcDan
y = -0.07046 + 0.0052156x R= 0.99632
Integrated Product Fluorescence
Time (min)
(c)
GSS[Cha]FLC AcDan
y = -0.0021728 + 0.010281x R= 0.99742
Integrated Product Fluorescence
Time (min)

Figure S3.

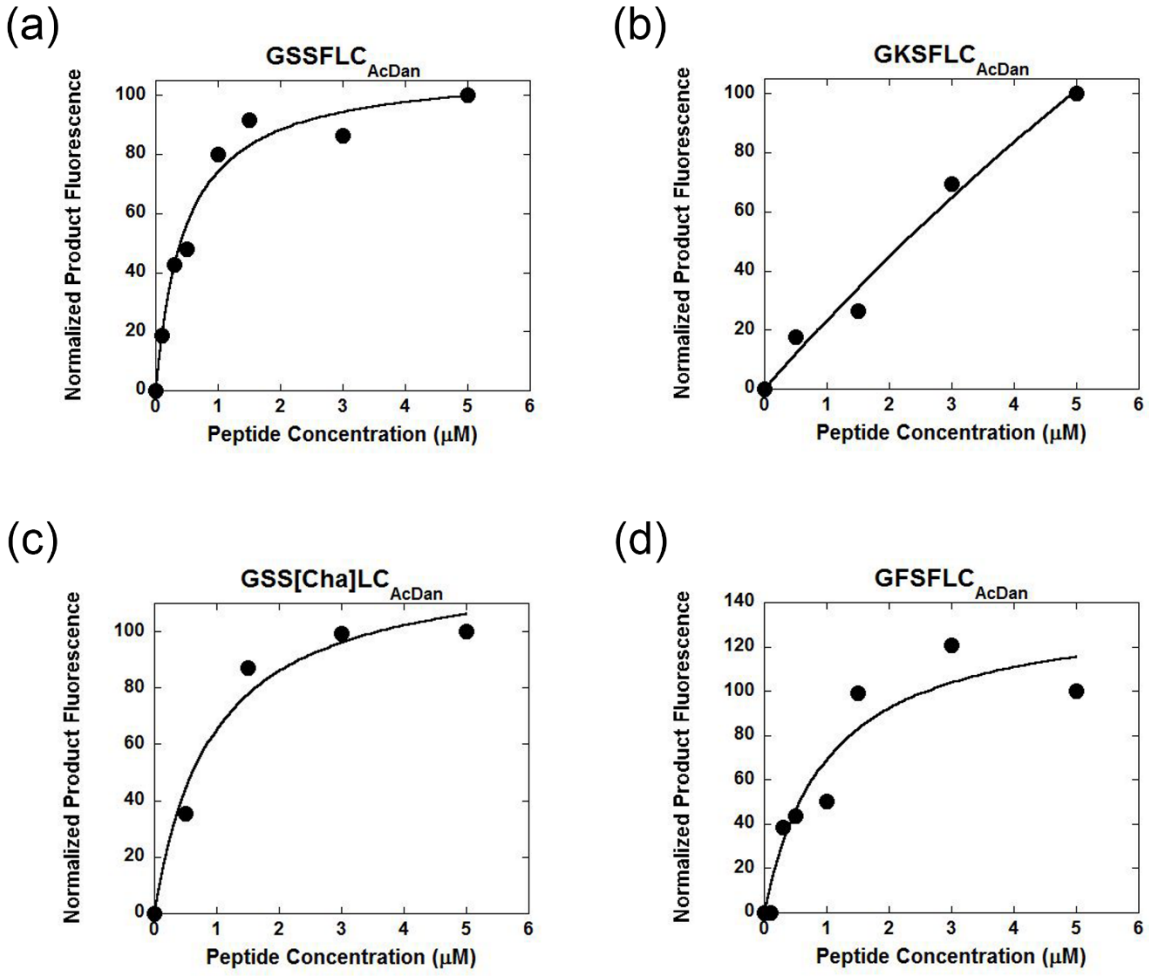
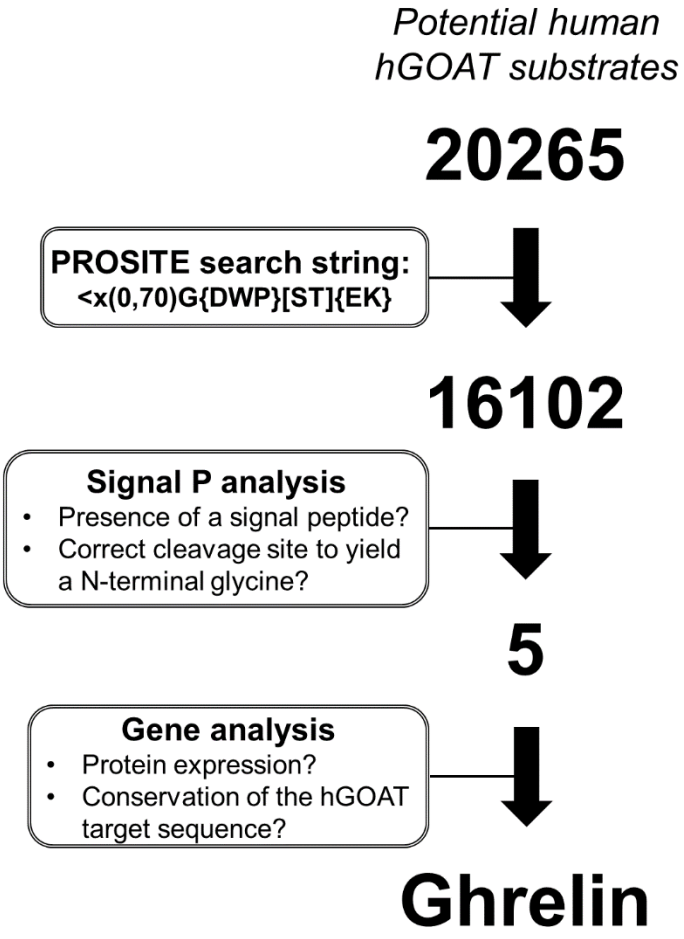

(a)
GSSFLC AcDan
Normalized Product Fluorescence
Peptide Concentration (μM)
(b)
GKSFLC AcDan
Normalized Product Fluorescence
Peptide Concentration (μM)
(c)
GSS[Cha]LC AcDan
Normalized Product Fluorescence
Peptide Concentration (μM)
(d)
GFSFLC AcDan
Normalized Product Fluorescence
Peptide Concentration (μM)

Figure S4.


Potential human
hGOAT substrates
20265
PROSITE search string:
<x(0,70)G{DWP}[ST]{EK}
16102
Signal P analysis
• Presence of a signal peptide?
• Correct cleavage site to yield a N-terminal glycine?
5
Gene analysis
• Protein expression?
• Conservation of the hGOAT target sequence?
Ghrelin

References

1. Krzysiak, A. J., Scott, S. A., Hicks, K. A., Fierke, C. A., and Gibbs, R. A. (2007) Evaluation of protein farnesyltransferase substrate specificity using synthetic peptide libraries, *Bioorg. Med. Chem. Lett. 17*, 5548-5551.
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3. Hancock, W. S., and Battersby, J. E. (1976) A new micro-test for the detection of incomplete coupling reactions in solid-phase peptide synthesis using 2,4,6-trinitrobenzenesulphonic acid, *Anal. Biochem. 71*, 260-264.