Supplementary Information

Non-canonical amino acids as a useful synthetic biological tool for lipase-catalysed reactions under hostile environments

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Contents

1. TTL cloning and amino acid composition

The gene of the lipase from the anaerobic thermophilic bacterium *Thermoanaerobacter thermohydrosulfuricus* (TTL) was cloned into the vector pQE80L (Qiagen, Hilden, Germany) as previously described (pQE80L-TTL-H6).[1] The translated sequence of TTL, with highlighted canonical amino acid (cAA) residues (coloured) that are replaced by noncanonical ones together with a C-terminal His-Tag (brown colour), is the following:

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1     MQKAVEITYN GKTLRGMMHL PDDVKGKVPM VIMFHGFTGN KVESHFIFVK MSRALEKVGI
61    GSVRFDFYGS GESDGDFSEM TFSSELEDAR QILKFVKEQP TTDPERIGLL GLSMGGAIAG
121   IVAREYKDEI KALVLWAPAF NMPELIMNES VKQYGAIMEQ LGFVDIGGHK LSKDFVEDIS
181   KLNIFELSKG YDKKVLIVHG TNDEAVEYKV SDRILKEVYG DNATRVTIEN ADHTFKSLEW
241   EKKAIEESVE FFKKELLKGG SHHHHHH
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2. TTL 3D structural model.

To gain insight into the distribution of the cAA residues within TTL, we performed a 3D structure homology model as described in detail elsewhere (Fig S1).[1]

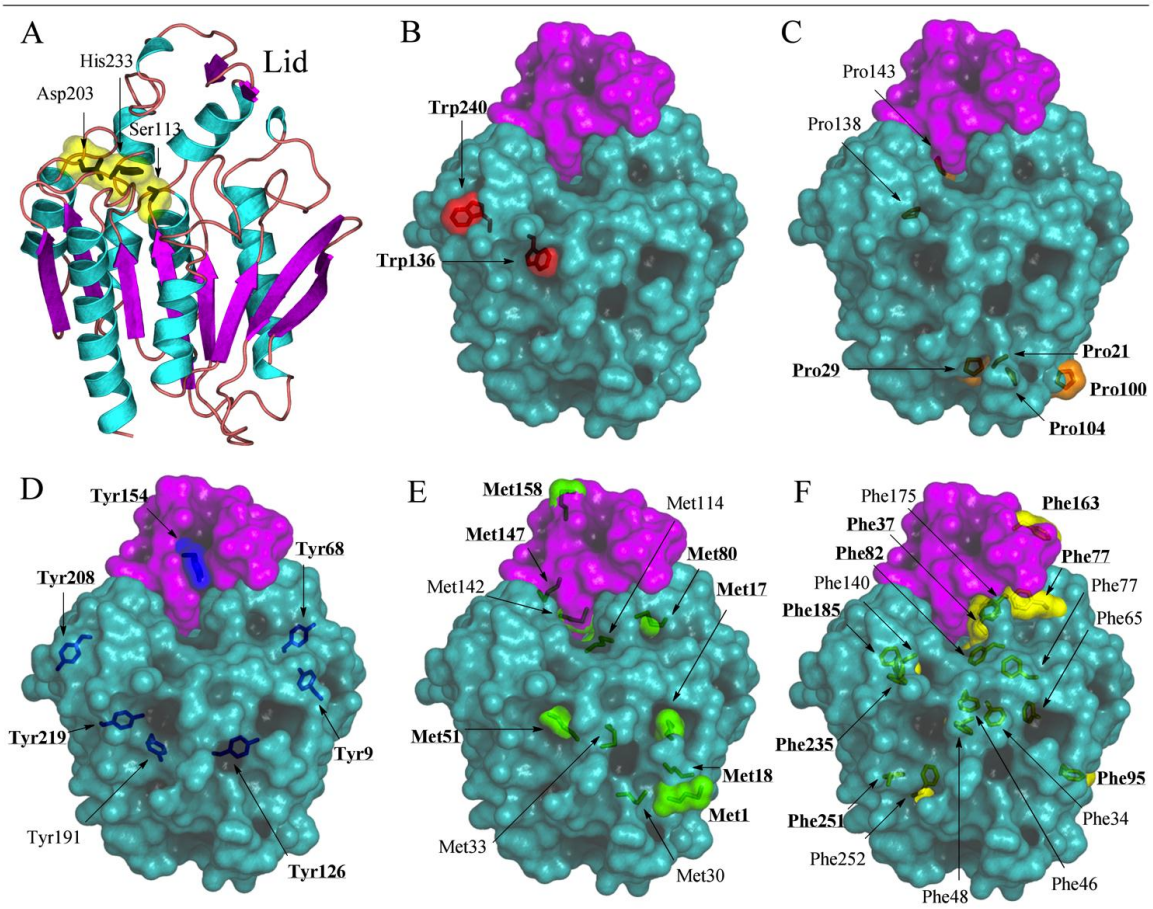

Fig. S1. TTL 3D structure homology model: The model reveals a canonical α/β-hydrolase fold[2], which is characterised by eight central and parallel β-sheet strands surrounded by α-helices, and the presence of the catalytic triad that consists of a nucleophile that can be serine, aspartate or cysteine (in TTL Ser113), a histidine (in TTL His233) and a catalytic acid that can be aspartate or glutamate (in TTL Asp203; A). In addition, there is a flexible domain (as rendered by the model construction) between the residues Gln153 and Leu171, indicating the presence of a lid or loop structure that covers the substrate binding cavity and the active site (violet domain B-F), a common feature present in many lipases.[3] TTL contains 2 tryptophan (B), 6 proline (C), 7 tyrosine (D), 11 methionine (E), and 16 phenylalanine (F) residues buried or surface exposed (non-bold/-underlined or **bold/underlined**, resp.). In the case of B-F, the lid is shown in violet, and the remaining surface-mode region in cyan. The images were rendered with PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.

3. Functional properties of parent TTL and its congeners

Upon noncanonical amino acid (ncAA) incorporation of the tryptophan (**1**) analogues 4-aminotryptophan (**1a**), 4-fluorotryptophan (**1b**), and 7-azatryptophan (**1c**) into TTL, protein expression, purification and analytical characterization (see section 7: Protein protocols), the hydrolytic activity was measured as reported previously (see section 9: Spectrophotometric assays)[1] at different temperatures, times and pH values (see section 10: TTL[**1a**/**1b**/**1c**] congeners data). As listed in Table S1, the TTL congeners derived from **1** were obtained in good yields and homogeneity; for comparison, our previously described enzymes are included. The TTL congeners with **1** analogues displayed highest (optimal) activity at different temperature, pH as well as diverse activation values. As other lipases[4], TTL is prone to thermal activation, *i.e.* the increase of activity upon heat, most likely due to the structural rearrangement on a flexible lid that covers the active site. Thermal activation may be a form of interfacial activation[4, 5]; that is, the increase of lipase activity upon contact with lipid aggregates, a phenomenon often observed in lipases.[6]

Table S1. General properties of TTL congeners.

TTL	Yield (mg/L)	TIE[a]	T_{opt} (°C)[b]	pH_{opt}[c]	Activity (mU/μg)[d]		TAF[e]	Reference
					Basal	Max.		
1	20	-	70	8	2.4	32	14	Hoesl et al.[1]
1a	42	High	65	8	2.4	25	11	This work
1b	37	High	55	9	2.8	31	11	This work
1c	18	High	65	8	4.0	10	3	This work
2a	23	High	50	9	3.0	8.3	3	Hoesl et al.[1]
2b	29	Low	50	9	2.6	6.3	2	Hoesl et al.[1]
2c	20	Low	50	9	3.5	6.6	1	Hoesl et al.[1]
2d	20	Low	55	9	7.2	10.4	2	Hoesl et al.[1]
3a	6	High	70	8	4.6	30.3	7	Hoesl et al.[1]
3b	13	High	50	8	2.3	3.7	2	Hoesl et al.[1]
4a	20	High	70	7	26.5	30.8	1	Hoesl et al.[1]
4b	20	High	65	7	0.51	8.9	17	Hoesl et al.[1]
5a	50	Low	70	8	6.3	39.7	6	Hoesl et al.[1]
5b	67	Low	55	7	6.5	13.2	2	Hoesl et al.[1]

[a] Total Incorporation Efficiency (TIE) of ncAAs in place of their analogues based to the intensity of the protein masses by each single species (see section 8: Protein protocols): A high TIE means fully or >50% substituted residues, whereas low TIE indicates <50%. [b] Optimal temperature displaying the highest lipase activity using *p*-nitrophenyl palmitate as substrate as described in section 9 (Spectrophotometric assays). [c] Optimal pH where the lipase exhibits the highest activity as determined with tricaprylin as substrate (described in section 9: Spectrophotometric assays). [d] The maximal (highest) lipase activity was observed at 80°C/60 min for TTL[**1**], 75°C/40 min for TTL[**1a**]; 75°C/60 min for TTL[**1b**] and 75°C/60 min for TTL[**1c**] (see section 10: TTL[**1a**/**1b**/**1c**] congeners data). [e] Thermal Activation Factor: Ratio between maximum (Max.) and non-activated (basal) lipase activity (see section 10: TTL[**1a**/**1b**/**1c**] congeners data). [b,c,d,e] Values are an average of three independent experiments.

A way to estimate whether a lipase has been thermally activated is by relating the basal and the maximal activity upon heat, which can be obtained by incubating TTL at various temperatures over long time periods (Fig. S7). While an activation of 14-fold was required in case of TTL[**1**], 11-fold was necessary for TTL[**1a**] as well as TTL[**1b**] but only 3-fold in the case of TTL[**1c**] (Table S1). However, the maximal activity of TTL[**1c**] was highly impaired (from 32 to 10 mU/μg), and to a lesser extent TTL[**1a**] (from 32 to 25 mU/μg) in comparison to the parent TTL[**1**] and TTL[**1b**], whose maximal activities are practically the same (~32 mU/μg). Thus the incorporation of **1a** and **1c** into TTL affects hydrolysis, whereas **1b** slightly activates it (3-fold) without affecting catalysis. Moreover, the introduction of two fluor atoms into TTL[**1b**] shifted the optimal temperature by 15°C (70 to 55) and pH by 1 unit (8 to 9) when compared to the parent TTL. Similar effects without compromising activity have been only reported for TTL[**3a**], TTL[**4a**] and TTL[**5a**] by 7-, 1- and 6-fold, resp. (Table S1).

4. Effects of various metal cations on parent TTL and its congeners (Table S2)

TTL	Relative Lipase Activity[a,b,c]															
	-	Ag+	Ni2+	Cu2+	Co2+	Rb2+	Zn2+	Sr2+	Mn2+	Mg2+	Fe2+	Fe3+	Ca2+	K+	Na+	Cr3+	Al3+
1	1.0	0.7	0.0	0.3	0.1	0.9	0.0	0.6	0.2	0.7	0.0	0.1	0.6	0.9	1.2	0.1	0.3
1a	1.0	0.8	0.0	0.5	0.3	1.1	0.0	0.6	0.4	0.6	0.0	0.2	0.4	1.0	0.9	0.0	0.0
1b	1.0	0.6	0.0	0.2	0.2	0.8	0.0	0.3	0.4	0.4	0.0	0.3	0.0	1.0	0.9	0.3	0.8
1c	1.0	0.6	0.1	0.0	0.4	0.7	0.0	0.1	0.3	0.3	0.0	0.3	0.3	1.2	1.2	0.2	0.2
2a	1.0	0.7	0.0	0.2	0.3	0.7	0.0	0.5	0.4	0.5	0.0	0.3	0.4	1.0	1.0	0.1	0.4
2b	1.0	0.8	0.1	0.3	0.6	0.8	0.1	0.3	0.7	0.4	0.1	0.6	0.5	1.0	1.0	0.1	0.6
2c	1.0	0.7	0.1	0.1	0.4	0.8	0.0	0.2	0.3	0.3	0.1	0.3	0.3	0.9	0.9	0.1	0.1
2d	1.0	0.6	0.1	0.2	0.4	0.8	0.0	0.1	0.3	0.4	0.1	0.3	0.3	0.9	1.0	0.2	0.2
3a	1.0	0.6	0.0	0.1	0.1	0.7	0.1	0.1	0.3	0.2	0.0	0.2	0.2	0.9	0.8	0.2	0.1
3b	1.0	0.9	0.0	0.5	0.2	0.9	0.1	0.7	0.3	0.7	0.1	0.2	0.6	1.0	1.0	0.2	0.1
4a	1.0	0.4	0.0	0.1	0.2	0.2	0.1	0.0	0.2	0.0	0.1	0.2	0.1	0.9	1.2	0.1	0.0
4b	1.0	0.7	0.0	0.3	0.4	0.7	0.0	0.2	0.5	0.5	0.0	0.3	0.1	1.3	1.1	0.0	0.0
5a	1.0	0.6	0.0	0.3	0.5	0.6	0.0	0.0	0.4	0.3	0.2	0.4	0.3	1.3	1.2	0.2	0.5
5b	1.0	0.8	0.0	0.0	0.4	0.7	0.0	0.1	0.2	0.4	0.1	0.2	0.3	0.9	0.9	0.1	0.1

[a] The TTL congeners were incubated for 1 hour at 25°C with 10 mM of the metal cations. [b] To correct for *p*-nitrophenol auto-hydrolysis due to chemical agent exposure and buffer background, samples without lipase and without metal cation were respectively included. Lipase activity was measured in triplicate for all cases at the optimal temperature and pH 8.0 as described in section 9: Spectrophotometric assays. The values were normalized to an absolute value of 1.0 for each TTL congener without metal cation to assess its effect in each particular case. [c] Negative effects are displayed in white background (less than 1.0), whereas neutral and positive effects are shown on light (1.0) and dark (more than 1.0) grey backgrounds, resp. Without metal cation treatment, parent TTL and congeners display a typical basal activity as indicated in Table S1 above, and in our previous study.[1]

5. Effects of surfactants, protein denaturing, reducing and alkylating agents as well as inhibitors on parent TTL and its congeners (Table S3)

	Relative Lipase Activity[a,b,c]														
TTL	No chemical agent	SDS	Triton X-100	Tween 20	Tween 80	EDTA	PVA	CHAPS	Mercaptoethanol	Dithiothreitol	2-Iodoacetate	GdnCl	Urea	PMSF	Pefabloc
1	1.0	0.1	0.9	0.3	0.1	2.6	3.4	1.7	0.8	0.8	0.8	0.0	0.9	0.8	0.01
1a	1.0	0.3	0.1	0.1	0.0	2.3	2.4	1.9	1.1	1.1	1.2	0.7	1.2	1.0	0.1
1b	1.0	0.0	0.1	0.1	0.0	1.7	2.6	2.3	0.9	1.0	1.1	0.3	1.1	1.0	0.3
1c	1.0	0.0	0.3	0.4	0.0	0.0	3.3	4.1	1.3	1.2	1.6	0.0	1.9	1.2	0.0
2a	1.0	0.1	0.0	0.3	0.0	1.0	1.8	1.9	0.8	1.0	1.2	0.3	1.5	1.4	0.4
2b	1.0	0.1	0.2	0.7	0.4	0.5	1.6	7.1	0.9	1.2	1.4	0.0	1.3	1.2	0.0
2c	1.0	0.2	0.0	0.0	0.0	0.3	1.9	9.9	0.8	1.0	1.2	0.0	1.3	1.0	0.0
2d	1.0	0.0	0.6	1.3	0.1	1.1	4.6	16.3	1.4	1.0	1.2	0.0	1.4	0.8	0.1
3a	1.0	0.0	0.1	0.0	0.0	0.5	0.5	5.2	0.7	1.1	1.0	0.4	1.5	1.0	0.0
3b	1.0	0.0	0.4	0.0	0.1	1.1	1.6	11.7	0.2	1.0	1.0	0.4	1.5	1.2	0.4
4a	1.0	0.0	0.1	0.2	0.0	0.2	1.3	1.0	1.3	1.1	1.0	0.5	1.0	0.8	0.0
4b	1.0	0.0	0.0	0.1	0.0	0.5	2.4	1.9	0.9	1.1	1.1	0.2	1.0	1.0	0.0
5a	1.0	0.1	0.0	0.3	0.0	1.0	1.8	1.9	0.9	1.0	1.1	0.7	1.0	1.0	0.0
5b	1.0	0.1	0.2	0.0	0.0	1.5	2.8	3.7	1.1	1.0	1.0	0.0	1.3	0.9	0.0

[a] TTL congeners were incubated for 1 hour at 25°C with 10% SDS, Triton X-100, Tween 20, Tween 80, EDTA (Ethylenediaminetetraacetic acid), PVA/polyvinyl alcohol, and CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate); as well as various concentrations of protein denaturing agents and inhibitors: 10 mM (Mercaptoethanol, Dithiothreitol, and 2-Iodoacetate), 0.5 M GdnCl, 2 M Urea, 0.1 mM PMSF (phenylmethylsulfonyl fluoride) and Pefabloc (AEBSF, [4-(2-Aminoethyl) benzenesulfonyl fluoride). [b] To correct for *p*-nitrophenol auto-hydrolysis due to chemical agent exposure and buffer background, samples without lipase and without chemical agent were respectively included. Lipase activity was measured in triplicate for all cases at the optimal temperature and pH 8.0 as described in section 9: Spectrophotometric assays. The values were normalized to an absolute value of 1.0 for each TTL congener without chemical agent to assess its effect in each particular case. [c] Negative effects are displayed in white background (less than 1.0), whereas neutral and positive effects are shown on light (1.0) and dark (more than 1.0) grey backgrounds, resp. Without chemical agent treatment, parent TTL and congeners display a typical basal activity as indicated in Table S1 above, and in our previous study.[1]

6. Chemicals and strains

All chemicals were bought from Merck KGaA (Darmstadt, Germany) or Sigma (Steinheim, Germany) unless otherwise indicated. The L-isomers were used of all amino acids. The tryptophan (**1**) analogues 4-aminotryptophan (**1a**), 4-fluorotryptophan (**1b**), and 7-aza-tryptophan (**1c**) were prepared by enzymatic condensation of the corresponding indole molecules and L-serine with tryptophan synthase as described elsewhere.[7] 4-amino- and 7-azaindole were obtained from Biosynth AG (Staad, Switzerland), and 4-fluoroindole from Molekula Deutschland Ltd. (Vaterstetten, Germany). The proline (**2**) analogues *cis*-4-fluoroproline (**2a**) and *trans*-4-fluoroproline (**2b**) were obtained from BachemAG (Bubendorf, Switzerland) and *cis*-4-hydroxyproline (**2c**) as well as *trans*-4-hydroxyproline (**2d**) from Sigma. The tyrosine (**3**) analogues *ortho*-fluorotyrosine (**3a**) and *meta*-fluorotyrosine (**3b**) were respectively obtained from SynQuest Labs, Inc. (Alachua, FL) and Sigma. The methionine (**4**) analogues norleucine (**4a**) and azidohomoalanine (**4b**) were respectively obtained from Sigma and by chemical synthesis as previously described.[8] Finally, the phenylalanine (**5**) analogues *meta*-fluorophenylalanine (**5a**) and *para*-fluorophenylalanine (**5b**) were purchased from Sigma.

The following *E. coli* strains were used: i) **1** (Trp) auxotrophic strain ATCC 49980 with the WP2 genotype[9] (*trp, uvrA, malB*) supplemented with **1a**, **1b,** and **1c**. ii) **2** (Pro) auxotrophic strain CAG18515 (*LAM- rph-1 proA3096::Tn10kan*) (CGSC# 7331) supplemented with **2a**, **2b**, **2c**, and **2d.** iii) **3** (Tyr) auxotrophic strain AT2471 (*LAM- e14- tyrA4 relA1 spoT1 thi-1*) (CGSC# 4510) supplemented with **3a** and **3b**. iv) **4** (Met) auxotrophic strain CAG18491 (*LAM- rph-1 metEo-3079::Tn10*) (CGSC *E. coli* Genetic Resources at Yale, CGSC# 7464) supplemented with **4**, **4a,** and **4b**. v) **5** (Phe) auxotrophic strain DG30 (*proA2 aspC13 hisG4 ilvE12 argE3 thi-1 tyrB507 hsdS14 hppT29 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 supE44 recB21 recC22 sbcB15* λ^-) (*E. coli* Strain National BioResource Project, Japan, strain ME8600) supplemented with **5a** and **5b**.

7. Protein protocols

7.1 Noncanonical amino acid incorporation

The pQE80L-TTL-H6 expression vector was introduced into the auxotrophic *E. coli* strains by electroporation according to standard methods.[10] Clones containing the plasmid were selected and propagated in LB media containing 100 mg L^{-1} ampicillin. For expressing the TTL congeners, the cells were grown in New Minimal Medium (NMM)[11] containing a limited amount of **1**, **2**, **3**, **4**, or **5**, whose depletion was indicated by growth arrest at mid-log phase (OD_{600} 0.6–0.8). Then all ncAAs were added to a final concentration of 0.5 mM in the media of the respective strains. In the particular case of **1a**, **1b**, and **1c** the reaction mixtures were added to the cells without further purification with a final concentration of the corresponding indoles of 0.8 mM (see above). In the case of **2c** and **2d**, 500 mM NaCl was added to the cells 30 min prior to induction. In the case of parent TTL, the Met auxotrophic strain was used with **4** in the same conditions as the TTL congeners. After 10-15 min of adding the ncAAs, TTL expression was induced by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG; Applichem, Darmstadt, Germany) and performed for 4-6 h at 30 °C with vigorous shaking.

7.2 Protein purification

After expression, the cells were harvested by centrifugation (3,200 xg/4 °C/10 min) and the cell pellets were resuspended in sodium phosphate buffer (50 mM NaH_2PO_4/300 mM NaCl/pH 8.0) with 10 mM imidazole and 0.1 % Triton X-100. After adding 1 mg/ml DNase (Roche, Mannheim, Germany), RNase (Sigma), and lysozyme (Sigma), cells were ruptured by sonication and the homogenate cleared from cell debris by centrifugation (30,000 xg/30 min/4 °C). The lysate was loaded onto a 1 ml HiTrap Chelating HP column (GE Healthcare, Munich, Germany), followed by 10 column volumes of washing with sodium phosphate buffer containing 20 mM imidazole, followed by 5 column volumes of high salt wash buffer (50 mM NaH_2PO_4/2 M NaCl/20 mM imidazole/pH 8.0), and also 5 column volumes of sodium phosphate buffer with 20 mM imidazole. The proteins were eluted with an imidazole gradient of 20-500 mM) in sodium phosphate buffer. The elution fractions were analyzed by SDS-PAGE[12], and the enriched in the desired variants were pooled, dialysed against 50 mM Tris-HCl/pH 8.0, and concentrated by ultrafiltration (Vivaspin 20 MWCO 10,000; Sartorius AG, Goettingen, Germany). The concentrated samples were assayed for protein content by Bradford[13] using Bovine Serum Albumin for calibration. Fig. S2 shows the purity (close to 90%) of TTL[**1**/**1a**/**1b**/**1c**] congeners. A similar purity of the remaining TTL congeners is reported elsewhere.[1]

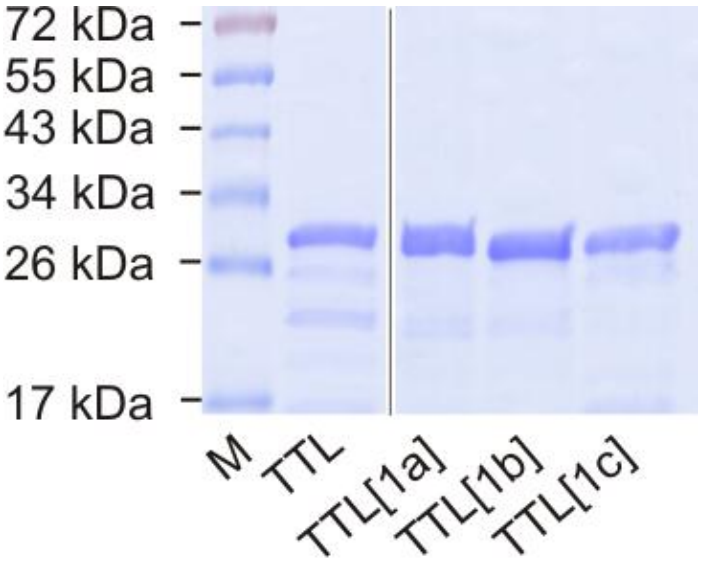

Fig S2. Purity of Trp-derived TTL congeners: A 12% SDS-PAGE was carried out to assess congener purity.

7.3 Protein analytical characterization

To assess the incorporation efficiency of the ncAAs into TTL congeners, the samples were submitted to Electrospray-Ionisation Mass Spectrometry (ESI-MS). While ESI-MS allows examining the incorporation of ncAAs at the primary TTL structure, Circular Dichroism Spectrometry (CDS) permits to explore the degree of alteration of the secondary TTL structure upon ncAA incorporation.

For ESI-MS, 20 µL aliquots of the purified variants were separated on a Waters RP C4 column (300 Å pore size; 3.5 µm particle size; 100 x 2.1 mm; Waters GmbH, Eschborn, Germany) by eluting with a gradient from 20 to 90 % B [0.05 % (v/v) TFA in acetonitrile] in A [0.05 % (v/v) TFA in water] within 20 min and with a flow rate of 250 µl min^{-1}. The molecular masses of the fractions were analyzed on a MicrOTOF ESI-MS (Bruker Daltonics, Bremen, Germany). The proportion of parent protein in the TLL congener samples was calculated as follows: The peak intensities of the fully and partially un-substituted protein species were taken from the corresponding mass spectra, summed up, and the peak intensity of the parent protein was expressed as the percentage of the sum of the peak intensities. Fig. S3 displays the masses of the Trp congeners. The masses of the remaining TTL congeners can be found in our previous publication.[1]

In the case of CDS, 6.5 µM of TTL parent protein and congeners in 10 mM Tris-HCl/pH 8.0 were measured thrice on a dichrograph JASCO J-715 (JASCO, Gross-Umstadt, Germany). The spectra are plotted as the mean residue molar ellipticity ([V]R) from 200 up to 250 nm at the optimal temperature of the proteins. Fig. S4 shows the secondary structures of the Trp-derived TTL congeners, while the CD spectra of the remaining TTL congeners can be found in our previous publication.[1] In all cases, the secondary structures of the TTL congeners seem to be unchanged or slightly affected upon ncAA incorporation.

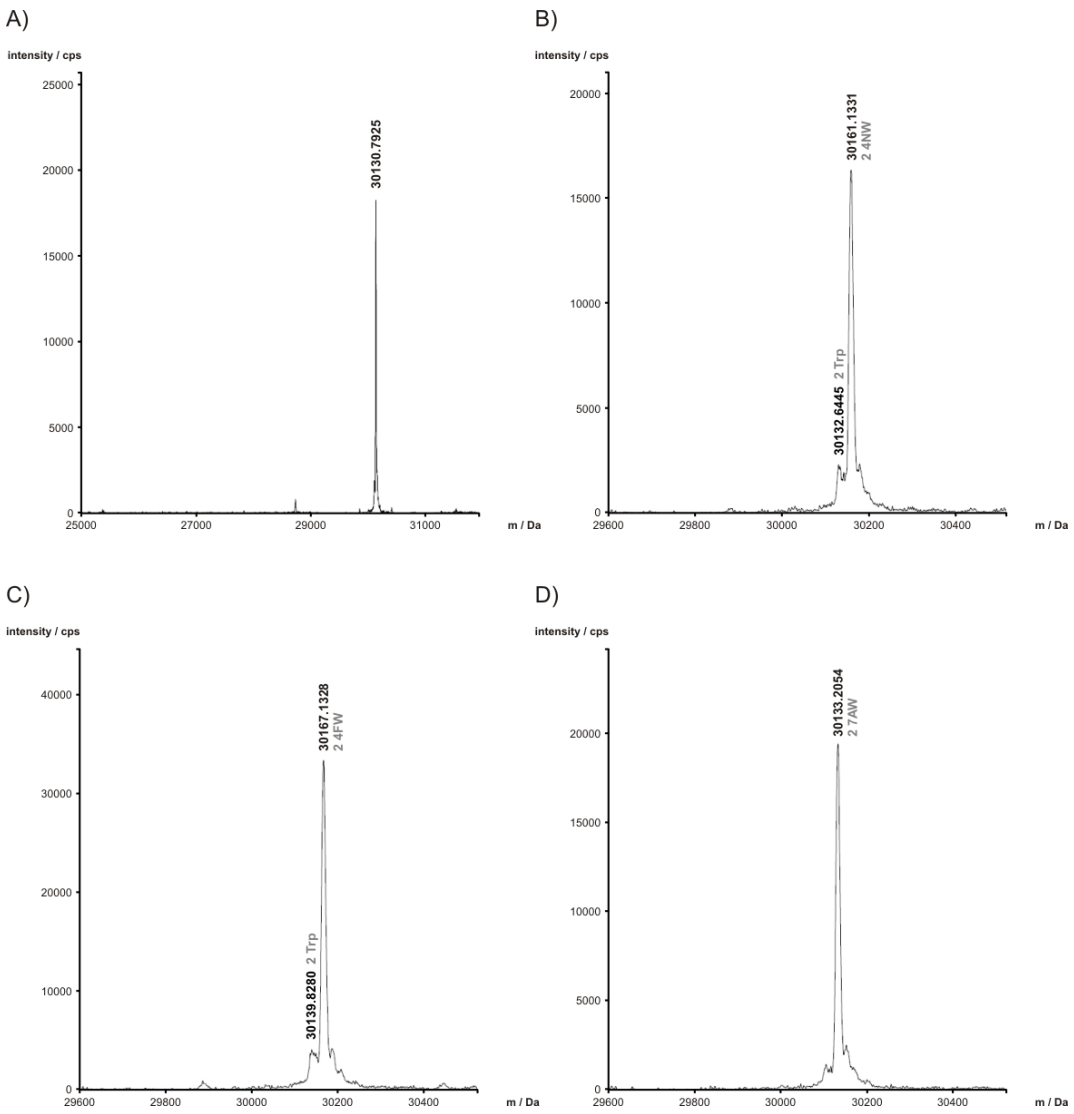

Fig S3. Experimentally calculated masses of TTL congeners: The theoretical mass of parent TTL is 30130.24 Da; those of TTL[**1a**], TTL[**1b**], and TTL [**1c**] are 30161.13, 30167.13, and 30133.20 Da, respectively.

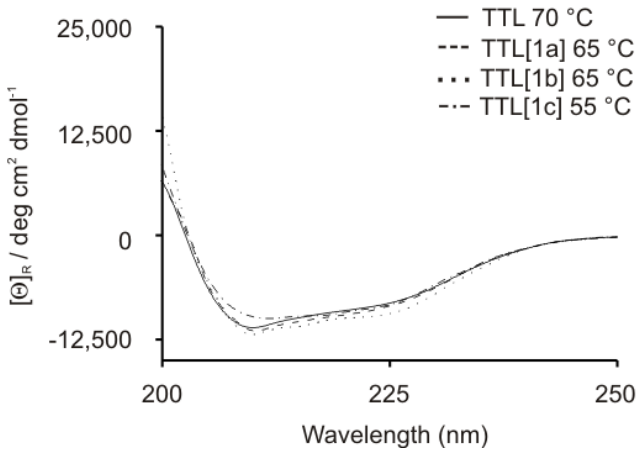

Fig S4. Circular dichroism spectra of Trp-derived TTL congeners at their T_{opt}.

8. Spectrophotometric assays

We used two spectrophotometric assays. On one hand, the standard lipase assay based on *p*-nitrophenyl palmitate (*p*NPP) hydrolysis was used as a reliable and fast method to determine lipase activity.[14] On the other hand, to determine optimal pH, the tricaprylin-based assay, which is based on the formation of copper soaps for detection of free fatty acids[15], was used.

In the case of the standard *p*NPP lipase assay, the cleavage of 0.9 mM *p*NPP (Sigma) was measured at the desired temperature in a buffer of 50 mM Tris-HCl/pH 8.0 with 1 mg mL^{-1} Arabic gum (Acros Organics, Geel, Belgium) that was pre-homogenized at 22,000 rpm using an Ultra-Turrax (IKA Werke GmbH & Co. KG, Staufen, Germany) for 4 min at room temperature. The reaction was started by mixing 900 µl buffered *p*NPP suspension with 100 µl of the TTL congeners. *p*NPP auto-hydrolysis was assessed by including a blank with buffer instead of enzyme. The reactions were incubated at the desired temperature for 10 (thermal treatment) or 15 (chemical treatment) min with vigorous shaking and stopped by adding 100 µl of 1 M Na_2CO_3 and chilling on ice for 1 min. After spinning at 20,000 xg for 10 min at room temperature, the absorption of the supernatant was measured at 410 nm in a spectrophotometer (Ultrospec 6300 pro, Amersham Biosciences). All values were determined in triplicate and corrected for autohydrolysis. One unit (1 U) of lipase activity is defined as the amount of enzyme required to liberate 1 µmol of *p*NPP (extinction coefficient $\varepsilon_{pH\ 8.0} = 12.75 \times 10^6\ M^{-1}\ cm^{-1}$) per minute under the aforementioned conditions.

In the case of the tricaprylin assay, 10 mM tricaprylin (1,2,3-trioctanoylglycerol, Sigma) and 5 g L Arabic gum were used in 40 mM universal pH buffer[16] pre-emulsified with a homogenizer at maximum speed for 1 min at room temperature. An aqueous Copper(II)-acetate-l-hydrate solution (58 mg mL^{-1}) was used as the copper reagent (pH 6.1 adjusted with pyridine). The dye reagent contained 1 mg ml^{-1} diethyldithiocarbamate (Sigma) dissolved in 99.8 % (v/v) ethanol. Then 200 µl of substrate solution were mixed with 100 µl of congener and mixed vigorously at the optimal temperature of the lipase for 18 h. The reaction was stopped by addition of 125 µl 3 M HCl followed by extraction with 1.5 ml isooctane by vigorously mixing for 10 min. Phases were separated by spinning in a table-top centrifuge at maximum speed for 10 min at room temperature. Thereafter, 1.25 ml of the organic phase were removed and mixed with 250 µl of copper reagent. The emulsion was mixed at 1400 rpm for 5 min and centrifuged again at maximum speed for 5 min. 1 ml of the organic phase was withdrawn and mixed with 200 µl dye reagent. After 5 min, the absorption at 430 nm was measure. Auto-hydrolysis was assessed by including a blank without enzyme. All reactions were done in triplicate.

9. Functional properties of congeners TTL[1a/1b/1c]

To assess the physicochemical differences among the Trp-derived TTL congeners, the temperature as well as pH profiles and stability upon temperature exposure over time were measured. The temperature profiles (Fig. S5) were performed between 40 and 90 °C and pH 8.0 for 10 min using the standard lipase assay based on *p*NPP as indicated above, whereas the pH profiles (Fig. S6) were determined using the tricaprylin assay for 18 h between pH 4 and 12. From Fig. S5 and S6 it becomes easy to identify the optimal temperature (T_{opt}) and pH (pH_{opt}) where the TTLs display the highest lipase activity. The stability upon temperature exposure over time was measured using the standard *p*NPP assay at pH 8.0 and 100 µl of TTL incubated at 75, 80, 85, 90, or 95 °C for different time intervals between 10 and 180 min (Fig. S7). As for the remaining TTL congeners, the original data can be consulted in the respective publication.[1]

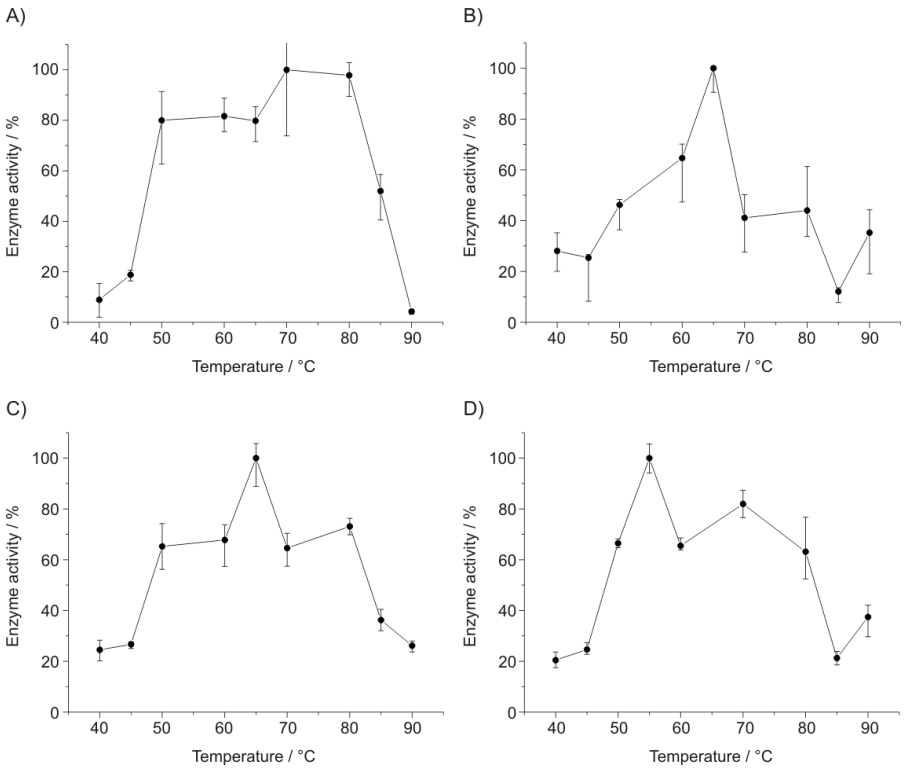

Fig S5. Effect of temperature on lipase activity: A) TTL[**1**], B) TTL[**1a**], C) TTL[**1b**], D) TTL[**1c**].

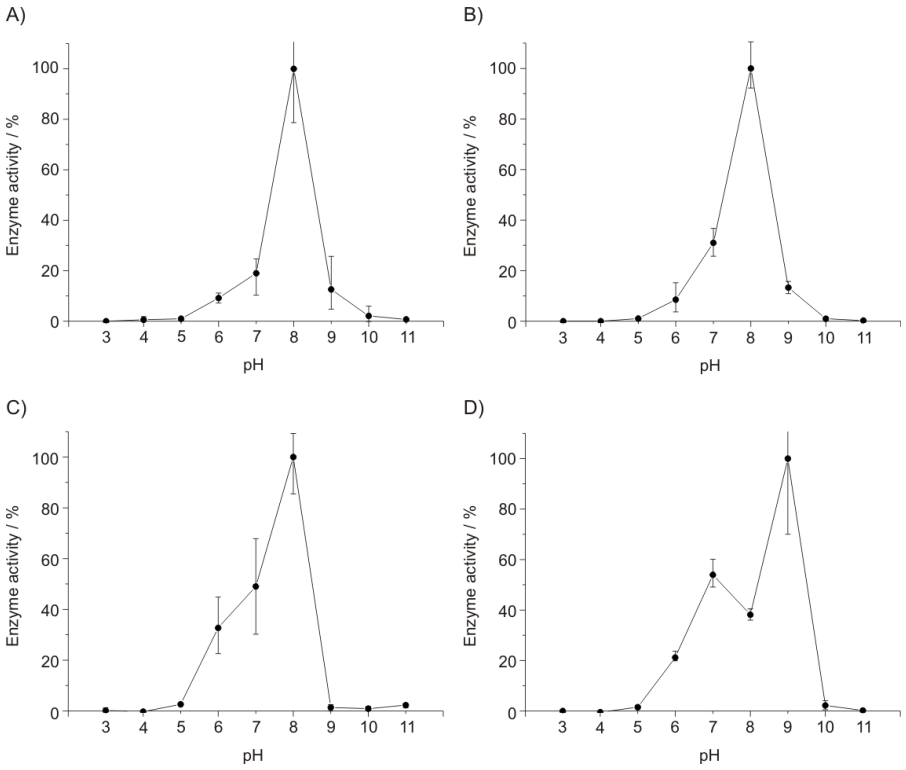

Fig S6. Effect of pH on lipase activity: A) TTL[**1**], B) TTL[**1a**], C) TTL[**1b**], D) TTL[**1c**].

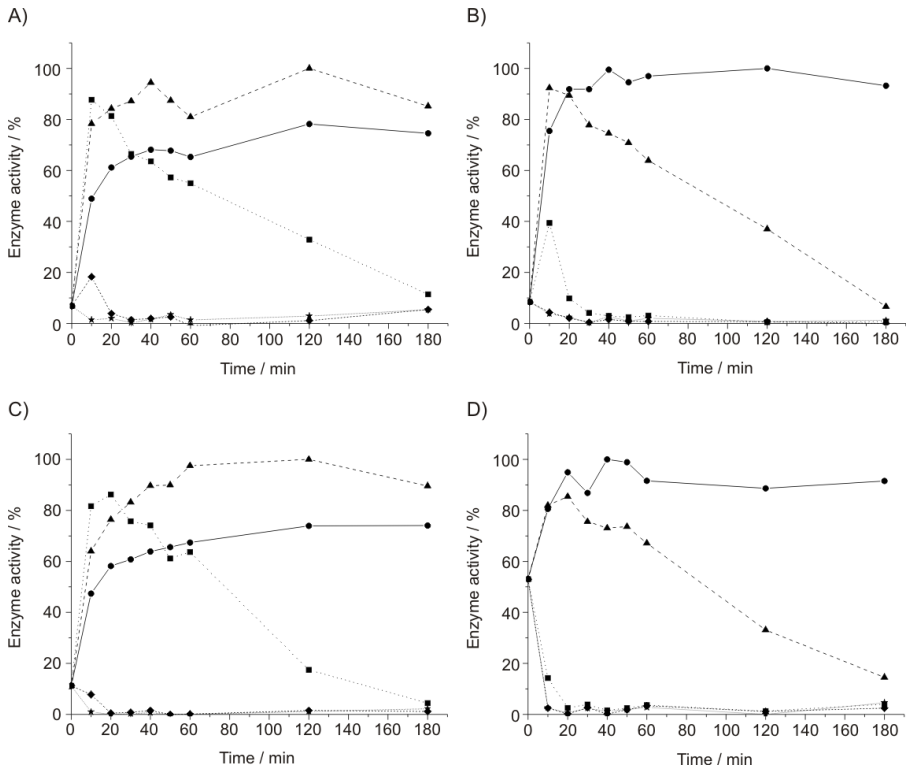

Fig S7. Dependence of enzyme activity upon temperature exposure: The congeners A) TTL[**1**], B) TTL[**1a**], C) TTL[**1b**], and D) TTL[**1c**] were incubated at 75°C (–●–), 80°C (--▲--), 85°C (· ·■· ·), 90°C (- -♦- -), and 95°C (···□···). Thereafter, lipase activity was measured at different time points.

10.References

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