

Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- | | |
|-------------------------------------|--|
| n/a | Confirmed |
| ☐ | ☒ The exact sample size (<i>n</i>) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☐ | ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☒ | ☐ The statistical test(s) used AND whether they are one- or two-sided
<i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i> |
| ☒ | ☐ A description of all covariates tested |
| ☒ | ☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☐ | ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☒ | ☐ For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i>) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted
<i>Give P values as exact values whenever suitable.</i> |
| ☒ | ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☒ | ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☒ | ☐ Estimates of effect sizes (e.g. Cohen's <i>d</i> , Pearson's <i>r</i>), indicating how they were calculated |

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	All data were collected using specified instruments and their associated commercially available software. The nuclear magnetic resonance (NMR) spectra were obtained using a Bruker AVANCE III HD 500 MHz. ESI mass spectra were acquired on an Agilent 6560 Accurate Mass Q-TOF LC/MS. MALDI mass spectra were obtained using FlexControl v2.0 (Bruker). High-resolution mass spectrometry (HRMS) data were collected using an Agilent 6530 Accurate Mass Q-TOF LC/MS system. No in-house algorithms or software were used for data collection.
Data analysis	All data were analyzed using the specified instruments and their associated commercially available software. NMR data were processed using TopSpin v4.3.0 (Bruker). ESI mass spectra data were processed using Compass DataAnalysis Viewer v6.1 (Bruker). No in-house algorithms or software were used for data analysis.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Human research participants

Policy information about [studies involving human research participants and Sex and Gender in Research](#).

Reporting on sex and gender

n/a

Population characteristics

n/a

Recruitment

n/a

Ethics oversight

n/a

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences

☐ Behavioural & social sciences

☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

The quantification of flexizyme-mediated tRNA acylation yield (%) presented in the supplementary materials was derived from the mean of multiple (n = 1-3) independent reactions. Similarly, all cyclic backbone synthesis data were obtained from multiple (n = 1-3) independent in vitro protein expression reactions. The protein expression was conducted using a commercially available in vitro protein expression kit (PURExpress, NEB), which was validated by the vendor. The produced peptides were identified via MALDI analysis using multiple (n > 3) samples. Cyclic backbones produced from multiple experiments were collected and analyzed simultaneously. This is standard practice for characterizing acylation yield and peptides produced in a cell-free protein synthesis system. The mass spectrometric analysis confirmed the site-specific synthesis of cyclic backbones in peptides, indicating that the sample size did not impact quantification.

Data exclusions

No data were excluded from the analysis.

Replication

All attempts at flexizyme-mediated tRNA charging and in vitro cyclic backbone synthesis during replication were successful.

Randomization

All cyclic backbones containing non-canonical chemical substrates were analyzed by mass spectrometry. Therefore, no randomization was applied in this study.

Blinding

Blinding was not relevant because all substrates were successfully incorporated into peptides in vitro and fully characterized.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

MagStrep type3 XT Beads 5% suspension (iba GmbH) was used for the purification of reporter peptides.

Validation

We used commercially available Strep-Tactin protein-coated ferrimagnetic beads for the purification of produced peptides containing a StrepII tag (WSHPQFEK) and a non-canonical chemical substrate. This product was validated by the vendor. No secondary antibodies were used.