Supplementary Material

The hyperthermophilic partners *Nanoarchaeum* and *Ignicoccus* stabilize their tRNA T-loops via different but structurally equivalent modifications

Simon Rose,[1] Sylvie Auxilien,[2] Jesper F. Havelund,[1] Finn Kirpekar,[1]

Harald Huber,[3] Henri Grosjean,[2] & Stephen Douthwaite[1]

[1] Department of Biochemistry & Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark.

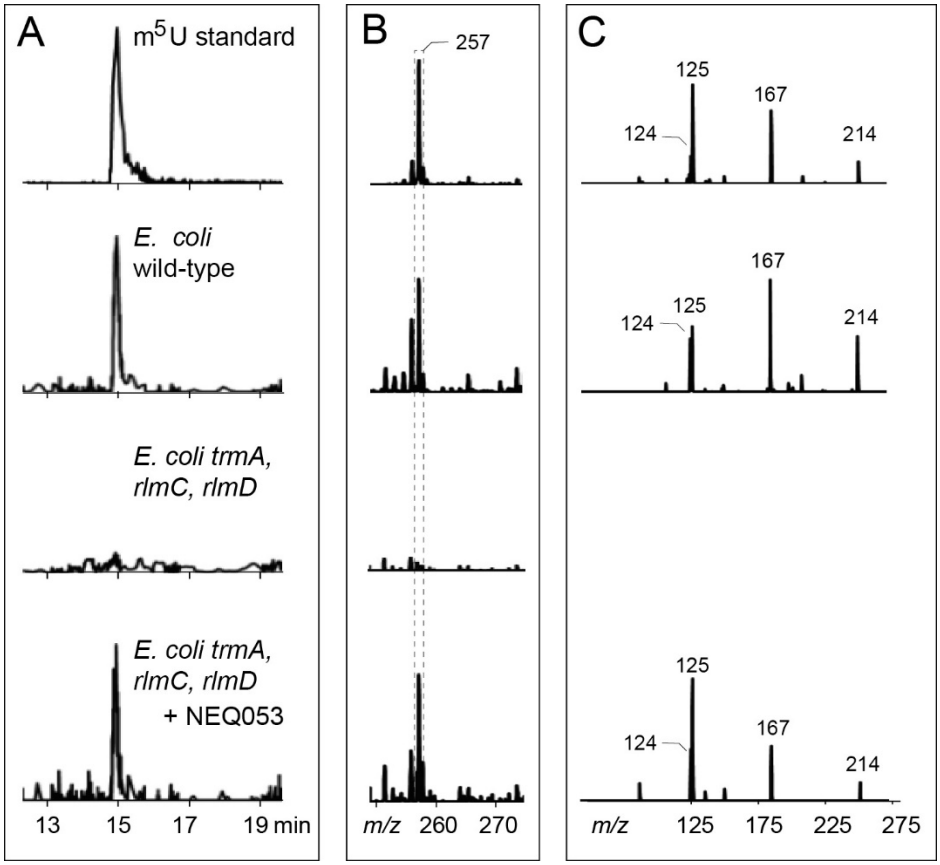
[2] Université Paris-Saclay, CEA, CNRS, Institute for Integrative Biology of the Cell (I2BC), 91198, Gif-sur-Yvette, France.

[3] Lehrstuhl für Mikrobiologie und Archaeenzentrum, Universität Regensburg, Universitätsstraße 31, D-93053 Regensburg, Germany.

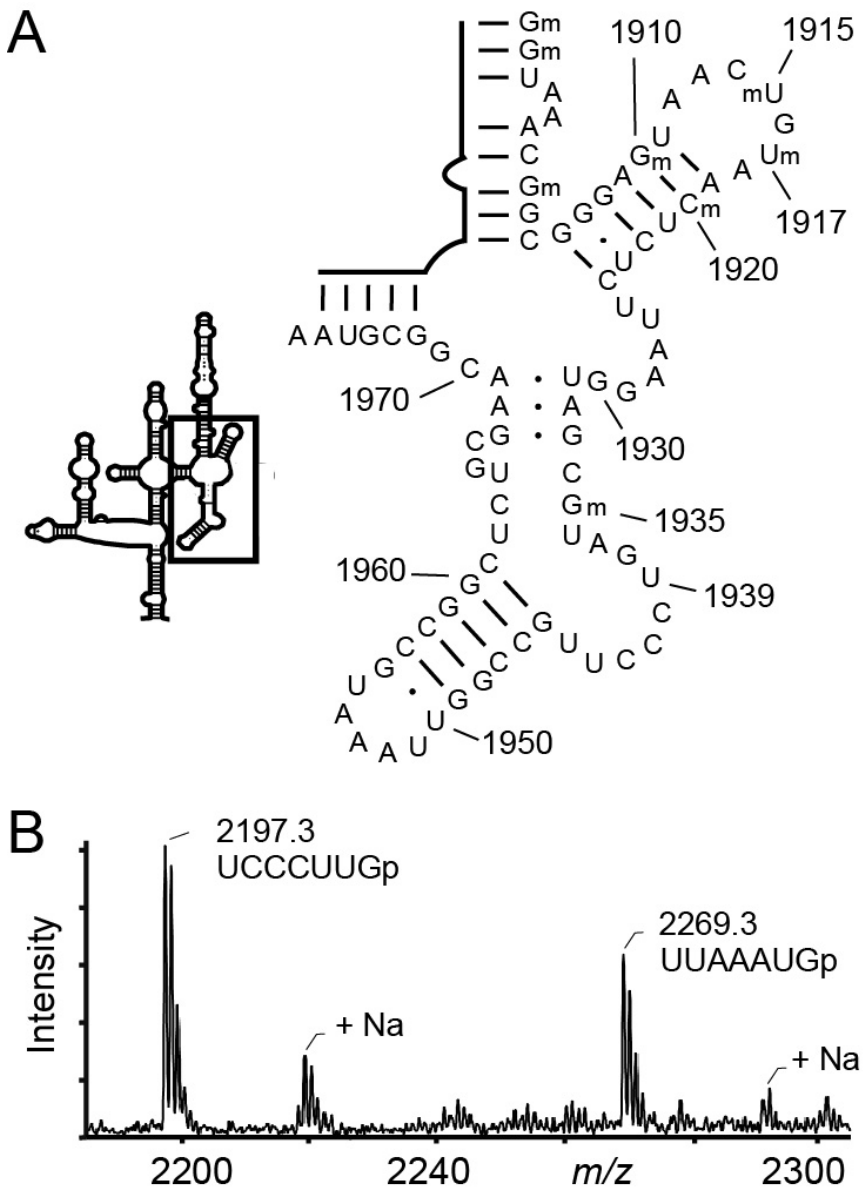
Supplementary Table S1. Matching the experimentally determined mass data for compound #6 with putative atomic compositions.

Formula	Score	Difference (ppm)
$C_{10}N_2O_5SH_{14}$	98.70	2.01
$C_{11}N_6OSH_{10}$	96.65	-2.50
$C_{11}N_2S_3H_{18}$	86.06	-0.64
$C_8N_5O_4SH_{12}$	85.45	7.29
$C_{13}N_3O_2SH_{12}$	83.25	-7.74

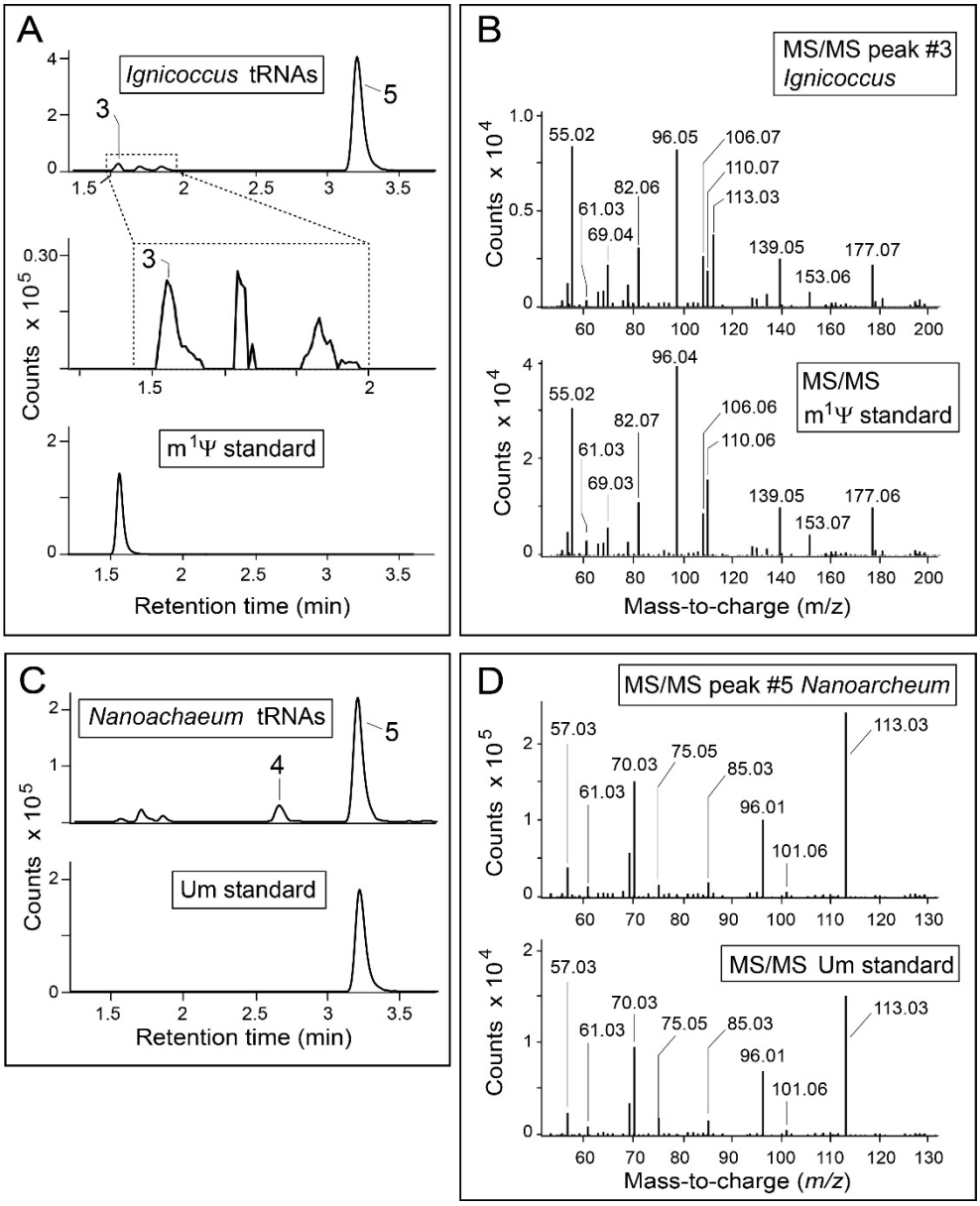
The software *Masshunter* (Agilent) was used to calculate the most plausible formula for compound #6 based on its neutral mass and isotope distribution. Sulphur has a measurable proportion of the ^{34}S isotope generating a characteristic M+2 signal (Figure S4), and is thus present in each of the five top scoring putative formulas for the compound. The formula for $m^1s^4\Psi$ ($C_{10}N_2O_5SH_{14}$) has the highest score, and is the only atomic composition compatible with a nucleoside. The differences (in ppm) are shown between the measured mass of compound #6 and the theoretical masses of the putative compounds. The same composition analyses also fit for compound #7 (m^5s^2U) and compound #8 (s^2Um).



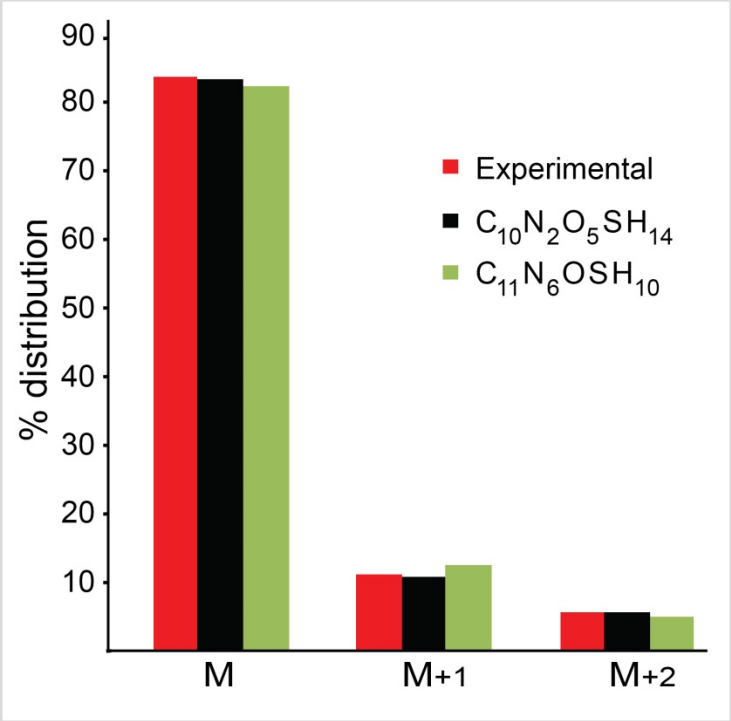
Supplementary Figure S1: Expression of NEQ053 *in vivo* in *E. coli.* (A) Nucleoside separation by LC on a Phenomenex Luna C18 column of showing the fractions eluting around 15 min. Elution profiles show (from the top down): a chemically synthesized m^5U standard; total nucleosides from digestion of wild-type *E. coli* tRNAs; nucleosides from digestion of total tRNAs from the *E. coli trmA, rlmC, rlmD* null-strain; and nucleosides from digestion of total tRNAs from the *E. coli trmA, rlmC, rlmD* null-strain complemented with NEQ053. **(B)** ESI-MS spectra of the corresponding LC fractions at 15 min run in negative ion mode on with ion-trap selection of the *m/z* 257 peak. For the null-mutant, no signal above background was registered. **(C)** Collision-induced fragmentation of m^5U in negative ion mode. Breaking the uracil base results in loss of NHCO comprising *N3* and the *C2* carbonyl group, seen as the *m/z* 214.07 fragment. Opening of the sugar and loss of $C_3H_6O_3$ (with the base remaining intact and retaining the ribose *C1´* and *C2´-O*) results in the *m/z* 167.05 fragment, and loss of the entire ribose is seen in the *m/z* 125.05 fragment. The adjacent peak at *m/z* 124.04 has lost NHCO from the base and $C_3H_6O_3$ from ribose, while retaining the *C5* methyl group. The *m/z* 124 and 167 fragments were isolated and their compositions confirmed by further fragmentation into smaller structures including those obtained using an alternative MS approach (Figure 3).



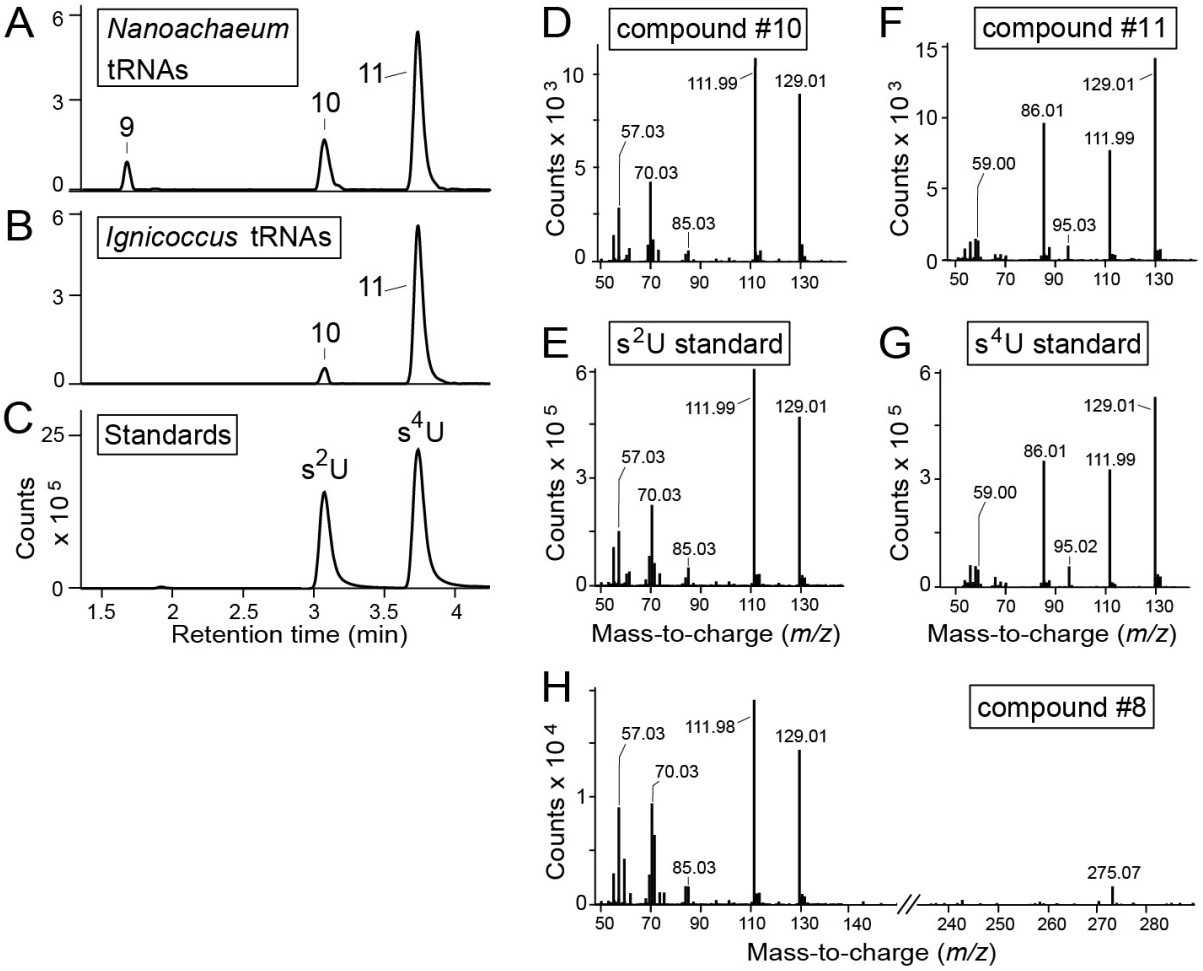
Supplementary Figure S2: Nucleotide modifications in *N. equitans* 23S rRNA. (A) Structure of the U1939 region in *N. equitans* 23S rRNA, where eight nucleotides in this region were shown by MS and primer extension to be 2′-*O*-methylated *in vivo* (at the sites indicated). **(B)** Partial MALDI spectrum of authentic 23S rRNA isolated from *N. equitans* cells and digested with RNase T1. The UCCCUUGp fragment at *m/z* 2197 (nucleotides 1934-1940) shows that there is no methylation *in vivo* at U1939 in *N. equitans*. The UUAAAUGp fragment stems from the adjacent sequence 1950-1955. Adducts of these fragments with sodium (+Na) are 22.0 Da greater in mass. Comparable analyses of the *N. equitans* 23S rRNA regions around nucleotide U747 (not shown) also revealed an absence of any m^5U modification (and a lack of 2′-*O*-methylation).



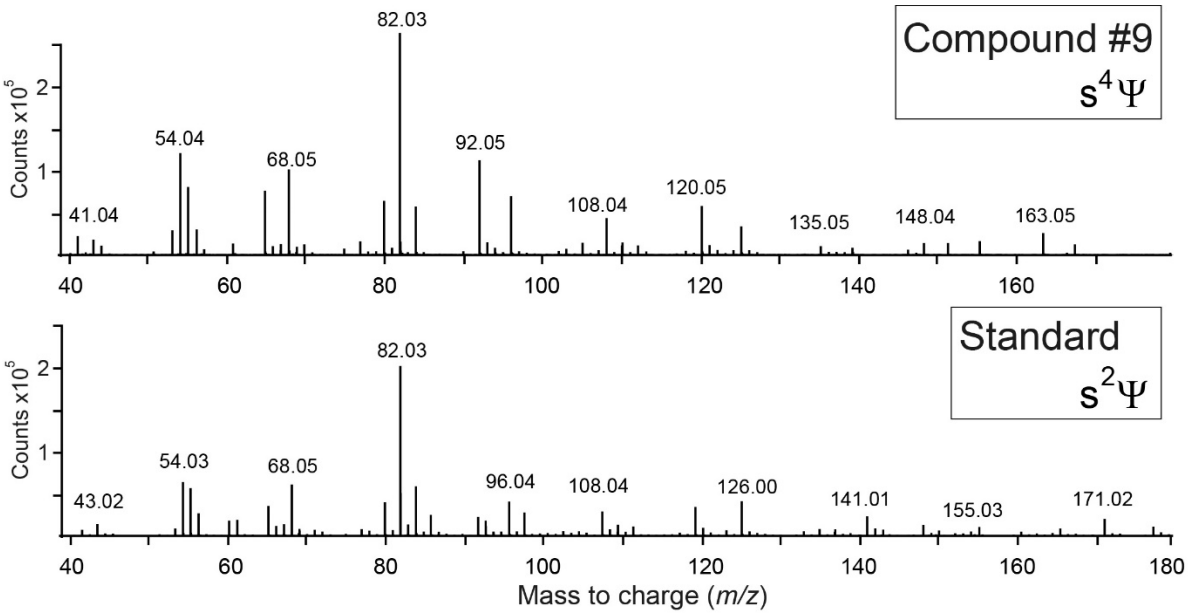
Supplementary Figure S3: Tandem MS fragmentation analyses of compounds #3 and #5. **(A)** Expansion of the region around 1.5 minute in the *I. hospitalis* nucleoside LC profile (Figure 3), and **(B)** fragmentation analysis of compound #3 in positive ion mode compared to a $m^1\Psi$ standard. **(C)** *N. equitans* nucleosides (Figure 3) and **(D)** fragmentation of compound #5 in positive ion mode compared to a Um standard.



Supplementary Figure S4: Application of accurate mass determination and isotopic patterns to elucidate a compound's formula. The calculated isotopic patterns of the two highest scoring formulas from Table S1 are compared to the experimentally determined spectral pattern for compound #6. The theoretical pattern for the formula $C_{10}N_2O_5SH_{14}$ comes closest to that obtained experimentally, and fits with the composition of a ribonucleoside. M is the monoisotopic signal; M+1 and M+2 result from the natural isotope distributions of the elements listed.



Supplementary Figure S5: Nucleosides registered at *m/z* 261.05 in positive ion mode correspond to thiolated uridine. Chromatogram profiles from **(A)** *N. equitans* and **(B)** *I. hospitalis* tRNA nucleosides compared to **(C)** a mixture of s^2U and s^4U standards. **(D)** MS/MS fragmentation in positive ion mode of compound #10 from the *N. equitans* eluents and **(E)** the s^2U standard show release of a thiolated uracil base (at *m/z* 129.01), loss of NH_3 (at *m/z* 111.99) and a series of smaller fragments indicative of s^2U. The MS/MS fragmentation in positive ion mode of **(F)** *N. equitans* compound #11 and **(G)** the s^4U standard also show release of the thiolated uracil base loss of NH_3 (at *m/z* 111.99) followed by a distinctly different set of smaller fragments indicative of s^4U. **(H)** Analyses of the *I. hospitalis* compound #8 at *m/z* 275.07 with the loss of a methylated ribose to leave the *m/z* 129.01 thiolated nucleobase, which showed a fragmentation pattern identical to s^2U. This confirmed that compound #8 is s^2Um. The analysis of compound #9 is shown in Supplementary Figure S6.



Supplementary Figure S6: Tandem MS spectra of nucleoside compound #9 in positive ion mode, comparing its fragmentation pattern with the $s^2\Psi$ standard. The two compounds have stable a glycosidic bond, consistent with derivatives of pseudouridine; they have the same mass, identical with thiolated (pseudo)uridine. However, despite being closely related, their fragmentation patterns show that they are not identical, and fragmentation of compound #9 in negative ion mode (as in Figure 5) indicates that is most likely $s^4\Psi$.