Modeling of [ψ_{32}, ψ_{39}]-ACSL^{Phe}. The sample was dissolved in buffer containing 10 mM NaCl, 5 mM potassium phosphate, pH 6.8, and 0.05 mM EDTA. NOESY spectra of unmodified and [ψ_{32}, ψ_{39}]-modified anticodon stem-loops with mixing times 180 and 400 ms in 100% D_2O and 260 ms in 90% H_2O were compared to extract distance constraints for the double-modified molecule. The sequential assignment in the base-1' regions of both molecules is continuous and the majority of the relative NOE crosspeak intensities are similar (Table S3), indicative of very similar conformations. The NOE intensities were classified as strong, medium, weak, and very weak and converted to distance constraints. ^{31}P 1D spectra and ^{1}H-^{1}H COSY and ^{31}P-^{1}H HetCor 2D spectra were analyzed to obtain sugar pucker and phosphate backbone torsional angle (α, γ, ε, ζ) constraints. Within measurement error, these constraints were determined to be unchanged from the unmodified parent molecule, further supporting the structural similarity of the unmodified and double-modified molecules.

To more fully explore possible conformational differences between the two RNA molecules, restrained molecular dynamics calculations were performed, beginning with the average structure of the unmodified molecule and incorporating distance constraints derived from spectra of the double modified molecule. The calculations were carried out using X-PLOR 3.851. The coordinates of the average structure of the unmodified tRNA^{Phe} anticodon stem-loop (1J4Y) were used as the starting coordinates in a restrained molecular dynamics calculation. The U_{32} and U_{39} base atoms were substituted with pseudouridine base atoms to yield ψ_{32}, ψ_{39} and the G_{28}-C_{42} base pair was transposed to a C_{28}-G_{42} base pair. Using hydrogen bond constraints (G_{27}-C_{43} to ψ_{32}-A_{38}), NOE-derived distance constraints, torsional angle (α, γ, ε, ζ, and sugar pucker) constraints, and repulsive van der Waals potentials, the structure was refined with 5000 cycles of constrained minimization, 5 ps of restrained molecular dynamics at 300 K, and 10000 cycles of constrained minimization. Ten models were calculated by randomizing the initial atomic velocities. Figure 3B shows the average of five models.

Table S3. Relative NOE crosspeak intensities for ACSL ^{Phe} and [ψ_{32},ψ_{39}]-ACSL^{Phe}.

NOE Crosspeak		ACSL ^{Phe}	ψ_{32},ψ_{39}-ACSL^{Phe}
H1′ U32 (ψ32) / H6 U33		w	m
H1′ U33 / H8 G34		w	v.w.
H1′ A35 / H8 A36		w	v.w.
H2 A31/	NH1 G30	w	w
	H1′ A31	v.w.	v.w.
	H1′ U32 (ψ32)	m	-
	H3 U32 (ψ32)	s	-
	H3 U39 (ψ39)	s	s
H2 A35/	H1′ C40	m	w
	H1′ A35	w	v.w.
	H1′ A36	v.w.	n.o.
	H8 A36	v.w.	v.w.
H2 A36/	H1′ U33	w	v.w.
	H1′ G34	v.w.	-
	H1′ A35	n.o.	m
	H1′ A36	w	w
	H8 A37	v.w.	v.w.
	H2 A37	v.w.	w
	H1′ A37	m	m
H2 A37/	H1′ G34	w	v.w
	H1′ A36	w	v.w
	H1′ A38	s	s
H2 A38/	H1′ A31	n.o.	m
	H3 U32 (ψ32)	s	n.o.
	H1′ U33	s	-
	H1' A38	n.o.	w
	H1′ U39 (ψ39)	m	m
	H2 A37	v.w.	n.o.

Only three base-1’ NOE interactions have differing intensities. All NOE interactions involving the adenine H2 resonances are listed. s, <3.0 Å; m, <4.0 Å; w, <5.5 Å; v.w., <6.5 Å; n.o., not observed; -, not identifiable due to crosspeak overlap or resonance exchange.

Table S1. Chemical shifts (in p.p.m.) of the ribose proton and carbon resonances of ψ_{32},Ψ_{39}-ACSL.

Res.	H1’	C1'	H2’	C2'	H3’	C3'	H4’	C4'	H5’/5’’	C5'
G27	5.60	93.12	4.55	75.58	4.34	73.26	4.3	76.11	4.34, 4.13	66.81
C28	5.54	93.15	4.45	75.84	4.41	74.01	n.a	n.a	3.89, 3.77	64.86
G29	5.66	91.29	4.71	75.58	4.52	75.32	4.52	84.02	4.30, 4.22	68.27
G30	5.63	91.99	4.51	75.6	4.4	72.97	4.39	82.37	4.29, 4.08	68.36
A31	5.95	90.30	4.90	79.15	4.61	85.53	4.35	86.11	4.36, 3.98	67.3
ψ32	4.55	82.35	4.74	75.59	4.61	72.62	4.17	80.06	n.a	n.a
U33	5.56	92.88	4.51	74.42	4.48	73.61	4.33	78.12	4.28, 4.05	66.8
G34	5.4	91.54	4.50	76.27	4.59	75.14	4.26	84.63	4.09, 3.93	67.53
A35	5.50	91.37	4.36	77.32	4.64	76.88	3.80	84.8	3.79, 3.64	67.05
A36	5.89	93.15	4.58	75.76	4.45	72.45	4.38	82.11	4.44, 4.00	67.89
A37	5.67	92.94	4.7	76.71	4.68	75.98	4.40	85.06	4.10, 3.98	68.36
A38	5.57	93.37	4.51	74.34	4.33	73.30	4.38	77.99	4.31, 4.13	66.88
ψ39	4.42	82.12	4.33	75.56	4.52	71.98	n.a	n.a	n.a	n.a
C40	5.44	93.72	4.28	77.64	4.35	72.60	4.18	84.47	4.41, 3.93	67.42
C41	5.33	93.92	4.4	77.78	4.2	72.83	4.12	84.42	4.41, 3.95	68.84
G42	5.69	92.19	4.45	76.28	4.61	75.76	4.42	83.93	4.31, 4.13	67.05

The ^{1}H chemical shifts were measured at 25 °C and pH 6.8 and are referenced relative to DSS (0.00 ppm) and the ^{13}C chemical shifts set using the spectrometer frequency and the ^{1}H and ^{13}C gyromagnetic ratios. The uncertainties in the chemical shift values are ˜0.01 and ˜0.05 p.p.m. for ^{1}H and ^{13}C, respectively. The 5' and 5" protons are not stereospecifically assigned. n.a., Not assigned.

Table S2. Chemical shifts (ppm) of the base proton, nitrogen, phosphorus, and carbon resonances.

Residue	H6/H8	C6/C8	H5/H2	C5/C2	C2/C4	H1/H3	N1/N3	N2/N4/6	P-3’
G27									
C28	7.85	143.96	5.69	98.52					-3.40
G29	7.69	138.9				12.62			-3.72
G30	7.23	138.4				12.51			-3.69
A31	7.56	139.77	7.81	155.18			225.2 216.1	82.5	-3.81
ψ32	6.97	142.37			152.2 163.0	13.82 10.30			-3.76
U33	7.56	144.15	5.35	103.7					-3.67
G34	7.91	143.25							-3.88
A35	8.08	143.92	7.92	155.21			227.1 214.3	78.9	-3.41
A36	7.91	141.72	7.94	155.16			224.2 217.2	79.8	-3.74
A37	8.12	143.15	7.45	153.40			224.1 213.8	80.6	-3.72
A38	7.79	141.75	7.87	155.52			224.8 215.7	82.6	-3.85
ψ39	6.87	142.37			151.8 163.0	13.61 10.49			-3.91
C40	7.82	144.3	5.54	97.28					-3.96
C41	7.66	143.25	5.45	97.98					-4.00
G42	7.59	141.28							

Figure S1. ^{1}H-^{15}N spectrum of the 2D HNCCH-TOCSY experiment. The intra-base N6-H2/H8 correlations are observed for each of the five adenine residues. Participation of the adenine amino group in an intramolecular hydrogen bond leads to a downfield shift of the N6 resonance. A31 forms a Watson-Crick hydrogen base pair scheme with ψ39 and A35 and A36 form no intra-molecular hydrogen bonds. In the 32-38 bifurcated hydrogen bond, the A31 N6 would be expected to shift further down field.[10,11]