Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Supporting Information for:

Inner residues of macrothiolactone in autoinducer peptides I/IV circumvents spontaneous *S*-to-*O* acyl transfer to the upstream serine residue

Masanobu Nagano, Satoshi Ishida, Hiroaki Suga*
Graduate School of Science, The University of Tokyo, Japan 113-0033

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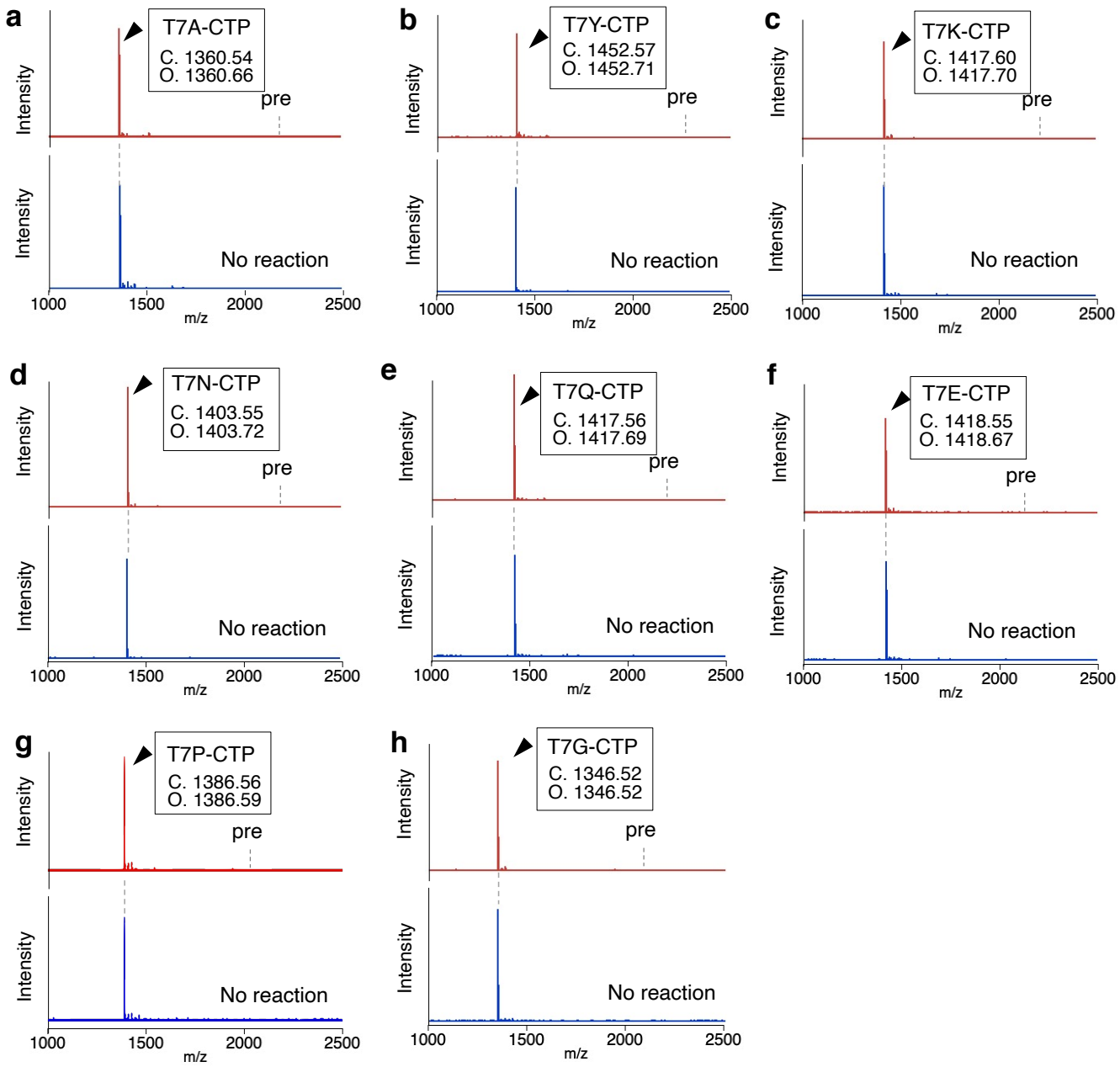

Figure S1. MALDI-TOF-MS of *in vitro*-translated AIP-Ia analogs mutated at the position 7 and its IAA labeling. Mutational scanning the STCD motif in AIP-I. Thr7 in pre-AIP-Ia was mutated to eight distinct amino acids (X = A, Y, K, N, Q, E P and G). Red spectra: samples after 30 min of transcription/translation. Blue spectra: samples after IAA alkylation of free thiol group of the SXCD motif. Pre: linear precursor peptide.

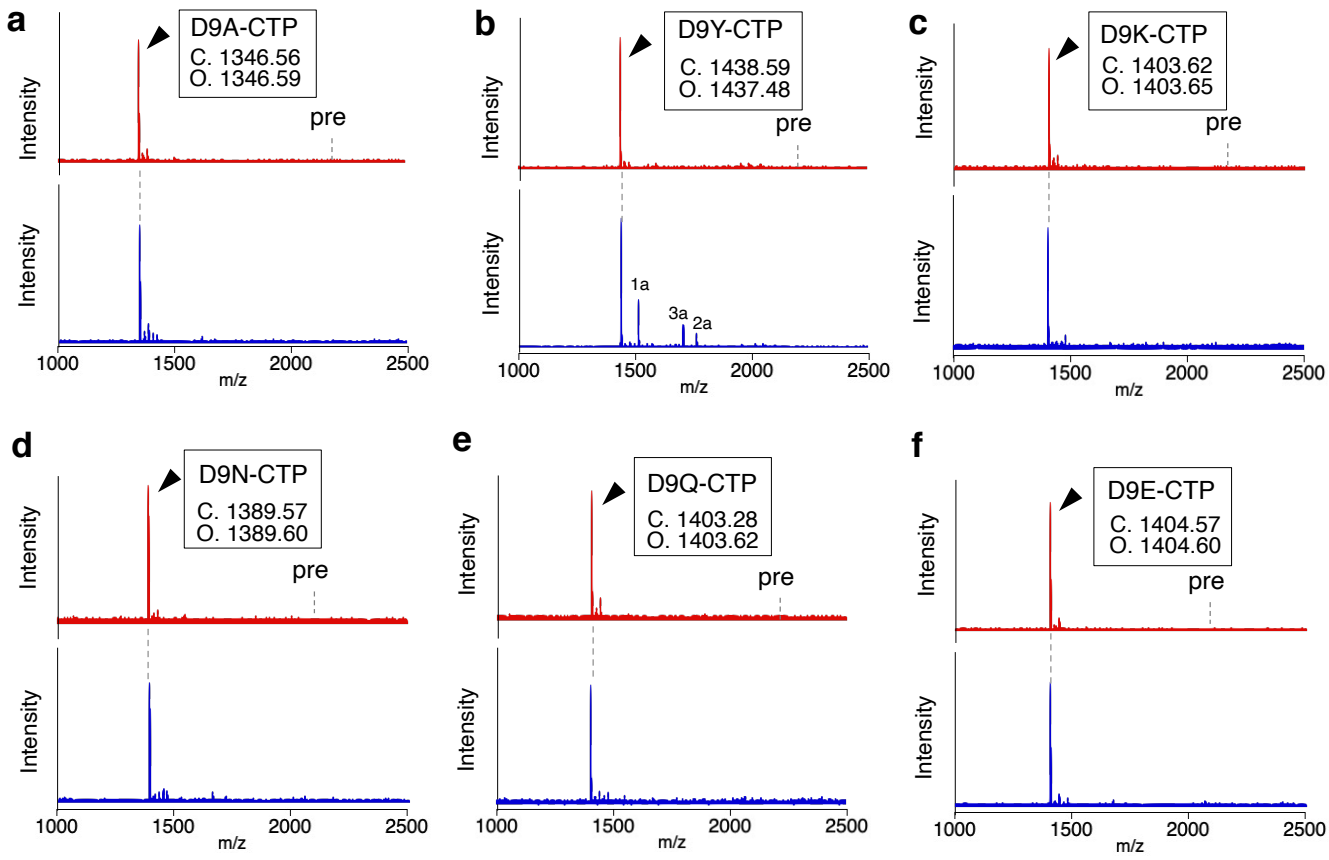

Figure S2. MALDI-TOF-MS of *in vitro*-translated AIP-Ia analogs mutated at the position 9 and its IAA labeling. Mutational scanning the STCD motif in AIP-I. Asp9 in pre-AIP-Ia was mutated to six distinct amino acids (X = A, Y, K, N, Q, E). Red spectra: samples after 30 min of transcription/translation. Blue spectra: samples after IAA alkylation of free thiol group of the STCX motif. Side products - 1a; alkylated hydrolyzed peptide, 2a; tri-alkylated DTT adduct, 3a; bi-alkylated Cys adduct, whch were attributed to the lower stability of the thioester in the *in vitro* translation mixture (*Ref-17*). Pre: linear precursor peptide.

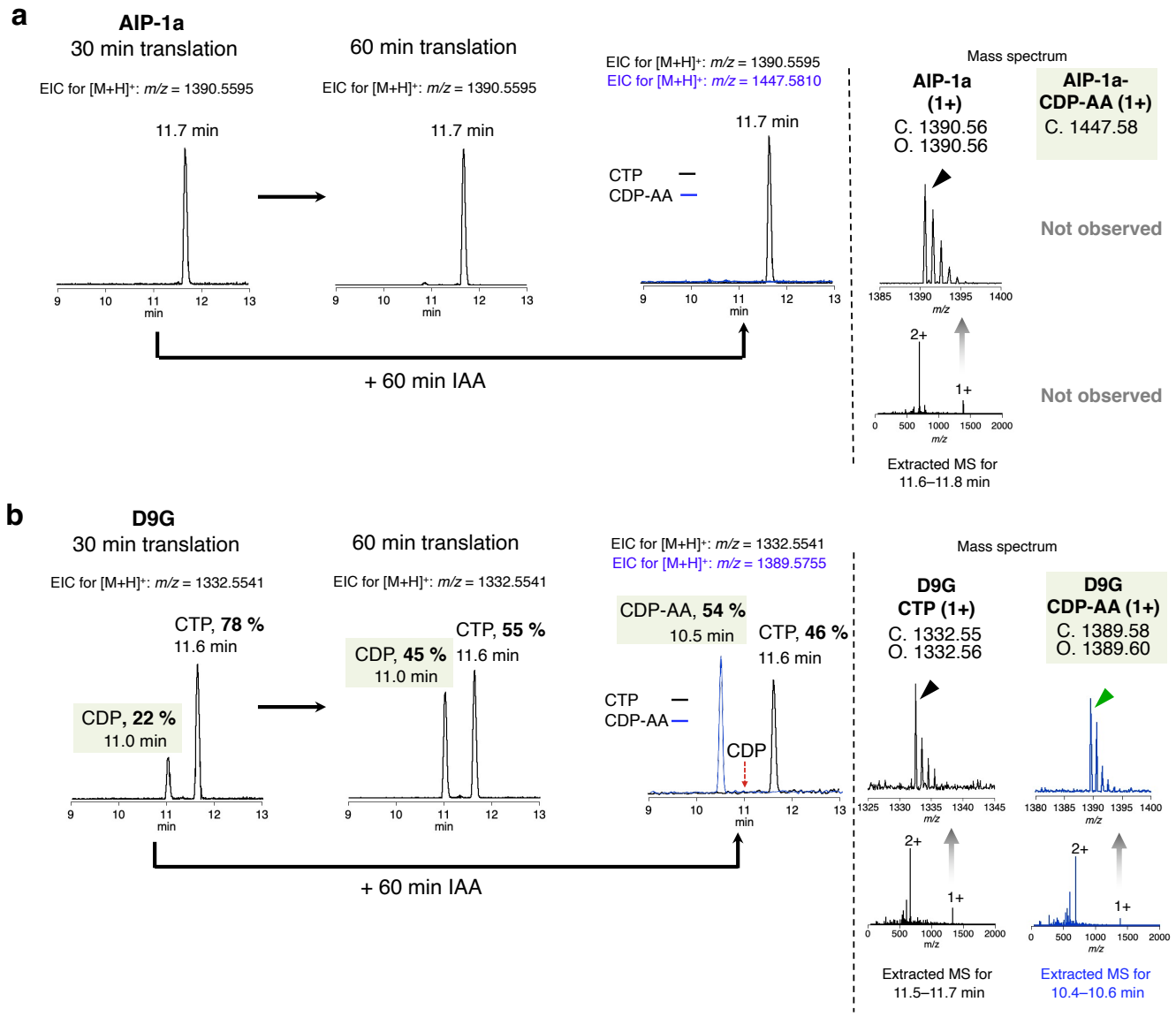

Figure S3. LC-MS of *in vitro* expressed AIP-1a and its mutants, D9G. Precursor peptide was *in vitro*-expressed by FIT system via incubation for 30 or 60 min, then analyzed by LC-MS. After the 30 min reaction point was also applied to IAA alkylation for 60 min and analyzed by LC-MS. Displayed are EIC (extracted ion chromatogram) LC-MS chromatograms and composite mass spectra of desired AIP-1a or the mutant in the corresponding chromatograms. EIC chromatogram corresponding to $[M+H]^+$ of CDP-AA is drawn in blue. Each EIC chromatogram was exhibited by searching calculated m/z in +/- 0.05 *m/z* range. Note, no precursor peptides were detected by LC-MS.

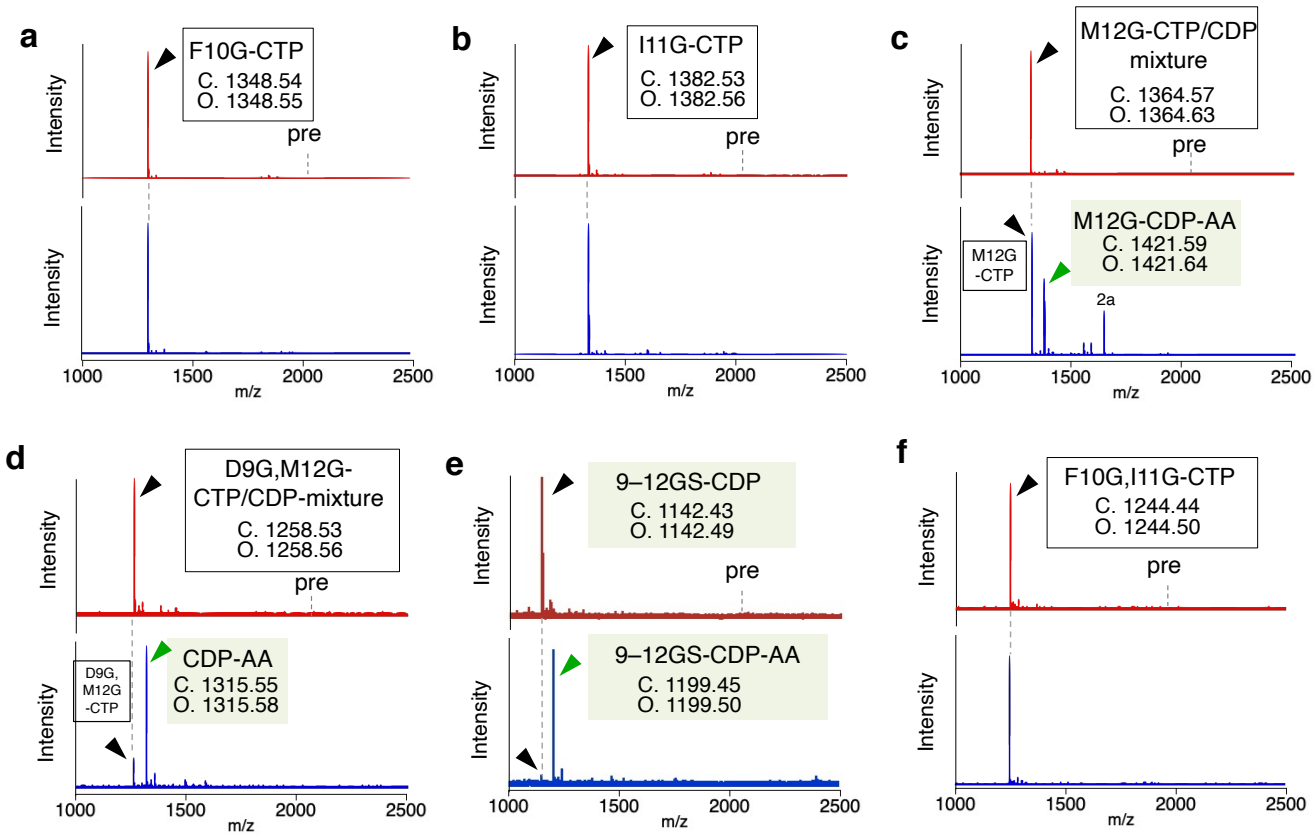

Figure S4. MALDI-TOF-MS of *in vitro*-translated AIP-Ia analogs mutated in a cyclic part and its IAA labeling. Mutational scanning of the C-terminal residue flanking the backbone thioester by MALDI-TOF-MS and IAA labeling of *in vitro* expressed cyclic depsipeptides. Red spectra: samples after 30 min of transcription/translation. Blue spectra: samples after IAA alkylation of free thiol group of the Cys in the STCX motif. 2a; tri-alkylated DTT adduct, which was attributed to the lower stability of the thioester in the *in vitro* translation mixture (*Ref-17*).Pre: linear precursor peptide.

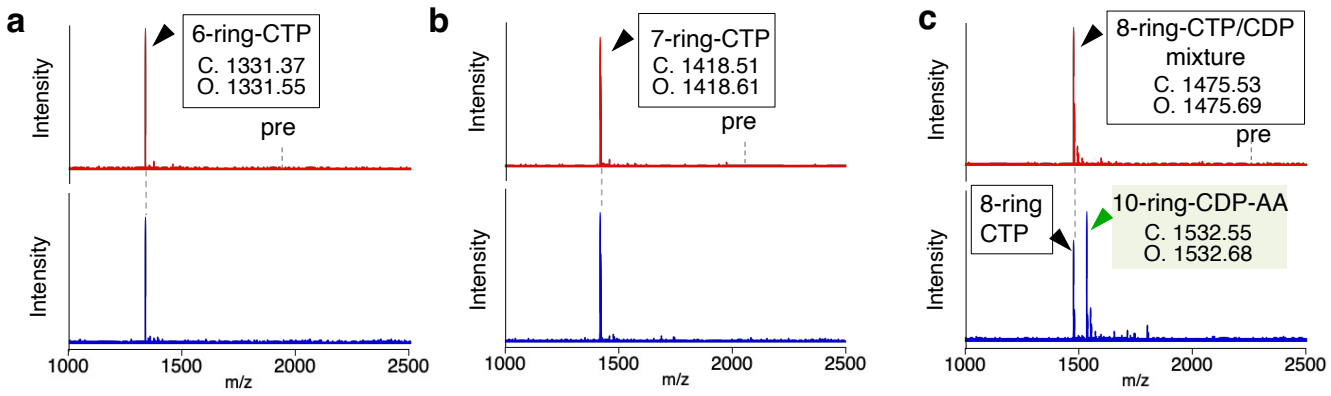

Figure S5. MALDI-TOF MS and IAA labeling analysis of *in vitro* expressed AIP-Ia with varying ring sizes. MALDI-TOF MS analysis of *in vitro* expressed peptides. Red spectra: samples after 30 min of transcription/translation. Blue spectra: samples after IAA alkylation of free thiol group of the SPCG motif.

Table S1. Peptide list and the summary of the MALDI-TOF MS analyses of CDP formation. The product names in brackets correspond to minor peaks. No pre was detected after 30 min translation.

a

Entry	Precursor peptide (pre)	Sequence (7 9 13)	Product after IAA labeling
1	preAIP-1a	fMRGGYSTCDFIM^{S}F^{4Cl}YRA	CTP
2	T7A	-----SAC----^{S}F^{4Cl}---	CTP
3	T7Y (preAIP-4a)	-----SYC----^{S}F^{4Cl}---	CTP
4	T7K	-----SKC----^{S}F^{4Cl}---	CTP
5	T7N	-----SNC----^{S}F^{4Cl}---	CTP
6	T7Q	-----SQC----^{S}F^{4Cl}---	CTP
7	T7E	-----SEC----^{S}F^{4Cl}---	CTP
8	T7G	-----SGC----^{S}F^{4Cl}---	CTP
9	T7P	-----SPC----^{S}F^{4Cl}---	CTP
10	D9A	-----S-CA---^{S}F^{4Cl}---	CTP
11	D9Y	-----S-CY---^{S}F^{4Cl}---	CTP
12	D9K	-----S-CK---^{S}F^{4Cl}---	CTP
13	D9N	-----S-CN---^{S}F^{4Cl}---	CTP
14	D9Q	-----S-CQ---^{S}F^{4Cl}---	CTP
15	D9E	-----S-CE---^{S}F^{4Cl}---	CTP
16	D9G	-----S-CG---^{S}F^{4Cl}---	**CDP-AA** (CTP)

b

Entry	Precursor peptide (pre)	Sequence (9 12 13)	Product(s) after IAA labeling
1	preAIP-1a	fMRGGYSTCDFIM^{S}F^{4Cl}YRA	CTP
2	D9G	-----S-CG---^{S}F^{4Cl}---	CDP-AA (CTP)
3	F10G	-----S-C-G--^{S}F^{4Cl}---	CTP
4	I11G	-----S-C--G-^{S}F^{4Cl}---	CTP
5	M12G	-----S-C---G^{S}F^{4Cl}---	CTP (**CDP-AA**)
6	D9G,M12G	-----S-CG--G^{S}F^{4Cl}---	**CDP-AA** (CTP)
7	9–12GS	-----S-CGGSG^{S}F^{4Cl}---	**CDP-AA**

c

Entry	Precursor peptide (pre)	Sequence (9 12)	Product(s) after IAA labeling
1	preAIP-1a	fMRGGYSTCDFIM^{S}F^{4Cl}YRA	CTP
2	5-ring	-----S-CDGGM^{S}F^{4Cl}YRA	CTP
3	6-ring	-----S-CDGSGM^{S}F^{4Cl}YRA	CTP
4	7-ring	-----S-CDGSGSM^{S}F^{4Cl}YRA	CTP
5	8-ring	-----S-CDGSGSGM^{S}F^{4Cl}YRA	**CDP-AA** (CTP)

Table S2. Known AIP-thiolactones from various Gram-positive species. In the sequence, a thiol sidechain of the Cys is thiolactonized at the C-terminal, respectively. Discovered important positions in the thiolactone avoiding *S*-to-*O* acyl transfer are highlighted in green. S, Streptococcus; *B, Bacillus; C, Crostridium; L, Listeria.*

Entry	AIPs bearing a thiolactone structure	Matured sequence	SX_1CX_2 motif
1	*S. aureus* AIP-I	YSTCDFIM	+
2	*S. aureus* AIP-II	GVNACSSLF	–
3	*S. aureus* AIP-III	INCDFLL	–
4	*S. aureus* AIP-IV	YSTCYFIM	+
5	*S. saprophyticus* AIP	INPCFGYT	–
6	*S. arlettae* AIP	VNPCGGWF	–
7	*S. auricularis* AIP-I	AKTCTVLY	–
8	*S. auricularis* AIP-II	TKTCTVLY	–
9	*S. capitis* AIP-I	ANPCQLYY	–
10	*S. capitis* AIP-II	ANPCALYY	–
11	*S. carnosus* AIP	YNPCVGYF	–
12	*S. cohnii cohnii* AIP	GKVCSAYF	–
13	*S. cohnii urealyticus* AIP	VKPCTGFA	–
14	*S. gallinarum* AIP	ARPCGGFF	–
15	*S. lugdunensis* AIP-I	DICDAYF	–
16	*S. lugdunensis* AIP-II	DMCDGYF	–
17	*S. epidermidis* AIP-I	DSVCASYF	+
18	*S. epidermidis* AIP-II	NASKYNPCSDYL	–
19	*S. epidermidis* AIP-III	NAAKYNPCASYL	–
20	*S. argenteus* AIP (*S. aureus* AIP-I)	YSTCDFIM	+
21	*S. schweitzeri* AIP (*S. aureus* AIP-IV)	YSTCYFIM	+
22	*S. caprae* AIP-I	YSTCSYYF	+
23	*S. caprae* AIP-II	YRTCNTYF	–
24	*S. lugdunensis* AIP-II	DMCNGYF	–
25	*S. schleiferi* AIP	KYPFCIGYF	–
26	*S. simulans* AIP-I	KYNPCLGFL	–
27	*S. simulans* AIP-II	YYPCFGYF	–
28	*S. saphrophyticus* AIP	INPCFGYT	–
29	*S. hiycus* AIP	KINPCTVFF	–
30	*S. chromogenes* AIP	SINPCTGFF	–
31	*S. warneri* AIP	YSPCTNFF	+
32	*S. vitulinus* AIP	VIRGCTAFL	–
33	*S. hominis* AIP	TYSTCYGYF	+
34	*S. haemolyticus* AIP	SFTPCTTYF	+
35	*B. cereus* AIP	EKLCIGFG	–
36	*B. sphaericus* AIP	HNFCVLYS	–
37	*C. beijerincki* AIP	KCCFSGGL	–
38	*C. botulinum* AIP-I	SSACYWCV	+
39	*C. botulinum* AIP-II	DSACVVGI	+
40	*C. difficile* AIP-I	NSTCPWII	+
41	*L. welshimeri* AIP	SKACFMFV	–

Table S3. Primer list corresponding to the AIP peptides. O1-F48 was used as a forward primer in all steps of extension, PCR1 and PCR2. F: Forward primer, R: Reverse primer.

Encoded	Step	Oligo name	Sequence (5' -> 3')
AIP-Ia	Extension	O1−F48	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATG
	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O2−R46	GTACCACATAATAAAGTCACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O3−R42	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAAGTC
T7A	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O4−R44	TACCACATAATAAAGTCACAAGCTGAGTACCCGCCACGCATATG
	PCR2	O3−R42	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAAGTC
T7Y	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O5−R44	TACCACATAATAAAGTCACAGTATGAGTACCCGCCACGCATATG
	PCR2	O3−R42	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAAGTC
T7K	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O6−R44	TACCACATAATAAAGTCACATTTTGAGTACCCGCCACGCATATG
	PCR2	O3−R42	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAAGTC
T7N	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O7−R44	TACCACATAATAAAGTCACAGTTTGAGTACCCGCCACGCATATG
	PCR2	O3−R42	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAAGTC
T7Q	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O8−R44	TACCACATAATAAAGTCACACTGTGAGTACCCGCCACGCATATG
	PCR2	O3−R42	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAAGTC
T7E	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O9−R44	TACCACATAATAAAGTCACATTCTGAGTACCCGCCACGCATATG
	PCR2	O3−R42	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAAGTC
T7G	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O10−R44	TACCACATAATAAAGTCACATCCTGAGTACCCGCCACGCATATG
	PCR2	O3−R42	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAAGTC
T7P	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O11−R44	TACCACATAATAAAGTCACATGGTGAGTACCCGCCACGCATATG
	PCR2	O3−R42	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAAGTC
D9A	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O12−R51	GCACGGTACCACATAATAAAGTAACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O13−R39	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAA
D9Y (AIP-IVa)	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O14−R51	GCACGGTACCACATAATAAAGTAACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O12−R39	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAA
D9K	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O15−R51	GCACGGTACCACATAATAAAAGCACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O12−R39	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAA
D9N	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O16−R51	GCACGGTACCACATAATAAAGTTACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O12−R39	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAA
D9Q	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O17−R51	GCACGGTACCACATAATAAACTGACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O12−R39	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAA
D9E	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O18−R51	GCACGGTACCACATAATAAATTCACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O12−R39	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAA
D9G	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O19-R51	GCACGGTACCACATAATAAAACCACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O12−R39	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATAAA

Table S4. Primer list corresponding to the AIP analogs. O1-F48 was used as a forward primer in all steps of extension, PCR1 and PCR2. F: Forward primer, R: Reverse primer.

Encoded	Step	Oligo name	Sequence (5' -> 3')
F10G	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O20-R51	GCACGGTACCACATAATACCGTCACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O21−R39	TTTCCGCCCCCCGTCTTATGCACGGTACCACATAATACC
I11G	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O22−R51	GCACGGTACCACATACCAAAGTCACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O23−R39	TTTCCGCCCCCCGTCTTATGCACGGTACCACATACCAAA
M12G	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O24−R51	GCACGGTACCAACCAATAAAGTCACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O25−R39	TTTCCGCCCCCCGTCTTATGCACGGTACCAACCAATAAA
F10G-I11G	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O28−R51	GCACGGTACCACATTCCACCGTCACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O29−R39	TTTCCGCCCCCCGTCTTATGCACGGTACCACATTCCACC
D9G-M12G	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O30−R51	GCACGGTACCATCCAATAAAACCACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O31−R39	TTTCCGCCCCCCGTCTTATGCACGGTACCAACCAATAAA
9-12GS	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O32−R51	ACGGTACCAACCTGATCCACCACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O33−R39	TTTCCGCCCCCCGTCTTATGCACGGTACCAACCTGATCC
6-ring	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O36−R51	CGGTACCACATTCCTGAACCGTCACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O37−R42	TTTCCGCCCCCCGTCTTATGCACGGTACCACATTCCTGAACC
7-ring	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O38−R51	TACCACATTGATCCTGAACCGTCACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O39−R45	TTTCCGCCCCCCGTCTTATGCACGGTACCACATTGATCCTGAACC
8-ring	Extension	O1−R40	GTACCCGCCACGCATATGTATATCTCCTTCTTAAAGTTAA
	PCR1	O40−R52	CAACCCATTGATCCTGAACCGTCACAAGTTGAGTACCCGCCACGCATATGT
	PCR2	O41−R48	TTTCCGCCCCCCGTCTTATGCACGGTACCAACCCATTGATCCTGAACC