MOTS-C — ALL-HYDROCARBON I,I+4 STAPLE BETWEEN POSITIONS 5 AND 9: MET-5 → (S)-2-(4'-PENTENYL)ALANINE (S5) AND TYR-9 → (S)-2-(4'-PENTENYL)ALANINE (S5), FOLLOWED BY RUTHENIUM-CATALYZED RING-CLOSING METATHESIS TO FORM A COVALENT HYDROCARBON BRIDGE ACROSS ONE HELICAL TURN

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DISCARDED | LONGEVITY

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5'-AMP-ACTIVATED PROTEIN KINASE CATALYTIC SUBUNIT ALPHA-2

AVERAGE CONFIDENCE	PTM / IPTM	VERDICT
60.4%	0.566 / 0.227	DISCARDED
TARGET	**UNIPROT**	**BINDING PROBABILITY**
5'-AMP-activated protein kinase catalytic subunit alpha-2	P54646	—

TLDR

DISTILLATION №30 tests an all-hydrocarbon i,i+4 stapled variant of MOTS-c designed to pre-organize residues 5–9 into a defined helical turn for improved AMPK alpha-2 engagement. Despite sound chemical rationale, the structural prediction returned a global pLDDT of 0.60 — statistically indistinguishable from the native baseline of ~0.62 across prior folds — and a very low ipTM of 0.23, indicating Boltz-2 could not confidently model the peptide docked against the AMPK alpha-2 interface. The staple did not deliver the predicted local helical stabilization, and the fold is discarded as biologically uninformative in silico. The negative result narrows the design space: hydrocarbon constraint at positions 5/9 does not rescue the structural ambiguity that has persisted across all five MOTS-c folds in this lab.

EXECUTIVE SUMMARY

MOTS-c i,i+4 hydrocarbon staple (Met-5/Tyr-9 → S5): pLDDT 0.60, ipTM 0.23 — no improvement over the native baseline and no confident AMPK interface docking. Four consecutive MOTS-c folds now cluster near the same structural ceiling, pointing toward a fundamental prediction limit rather than a solvable design problem.

DETAILED ANALYSIS

MOTS-c is a 16-amino acid mitochondrial-derived peptide (MDP) encoded within the 12S rRNA of the mitochondrial genome, first characterized by Lee et al. (2015) as a regulator of insulin sensitivity and metabolic homeostasis through inhibition of the folate cycle and de novo purine biosynthesis. The resulting AICAR accumulation activates AMPK — a validated master energy sensor — making MOTS-c a compelling longevity target. Despite strong biological interest, MOTS-c presents a persistent structural challenge in this lab: it is short (16 residues), intrinsically disordered, and has returned pLDDT values clustering tightly around 0.62–0.63 across every modification tested (Folds #5, #19, #25), suggesting the sequence itself resists confident fold prediction regardless of point substitutions or lipidation.

Fold #30 pursued a qualitatively different strategy: all-hydrocarbon i,i+4 stapling between positions 5 and 9, replacing Met-5 and Tyr-9 with (S)-2-(4'-pentenyl)alanine (S5) non-natural residues followed by ruthenium-catalyzed ring-closing metathesis. The design logic was rigorous. Hydrocarbon stapling at i,i+4 spacing is the canonical approach to enforce a single helical turn while leaving side chains at non-stapled positions free for receptor engagement. Positioning the staple on the solvent-exposed face — away from the proposed cationic C-terminal patch (R12/K13/R16) and aromatic residues Trp-3 and Phe-10 — was intended to preserve the AMPK-engaging surface characterized computationally in Fold #19. Replacing Met-5 also

addressed the oxidation liability flagged in the Nle substitution rationale of Fold #5, making this a two-for-one design.

The structural prediction, however, did not support the hypothesis. Global pLDDT returned at 0.60 — marginally lower than the native baseline and within noise for a single-run prediction — and critically, the ipTM of 0.23 indicates Boltz-2 could not produce a confident model of the stapled peptide engaging the AMPK alpha-2 interface. This inter-chain confidence score is the most diagnostic metric for a docking-dependent hypothesis: values below ~0.40 typically indicate the interface geometry is not predicted with meaningful confidence. The Boltz-2 affinity module returned no values, and no Chai-1 agreement run was available, leaving the prediction unsupported by any orthogonal structural scorer.

The literature context from the LITERATURE agent deepens the interpretive challenge. The dominant mechanistic model for MOTS-c's AMPK activation is indirect — through AICAR accumulation — rather than direct AMPK binding. No experimental structure of MOTS-c in any conformation has been published; no SAR data exists at residue resolution; and the specific hypothesis that the C-terminal cationic patch directly contacts the AMPK alpha-2 regulatory interface originates from Fold #19's computational modeling, not from published biochemistry. The literature agent also identifies a meaningful concern the researcher acknowledged but did not fully resolve: Tyr-9 carries a hydroxyl and aromatic side chain that may contribute to binding in the native peptide, and its wholesale replacement with a pentenyl-alanine stapling residue eliminates both features simultaneously.

Across the five MOTS-c folds in this lab (#5, #19, #22 Humanin for comparison, #25, #30), no modification has broken through the 0.63 pLDDT ceiling. The pattern is now statistically meaningful rather than incidental: point substitutions (K13R, Nle-1), lipidation (myristoylation), and now covalent constraint (hydrocarbon staple) all return structurally similar, low-confidence predictions. This is consistent with the literature agent's conclusion that MOTS-c may function through multiple conformational modes across cytoplasm, nucleus, and membrane compartments — a biologically flexible peptide for which conformational constraint may be philosophically misaligned with the actual mechanism.

Heuristic sequence-based estimates from the structural run show an aggregation propensity of 0.054 (low — favorable), a stability score of 0.461 (moderate), and a half-life estimate in the moderate range (~30 min–2 hrs). These are not elevated relative to the native peptide and do not compensate for the structural prediction failure. BBB penetration probability of 0.13 is low, consistent with MOTS-c's primary role in peripheral metabolic tissues.

The honest assessment is that this fold delivers a meaningful negative result: covalent helical pre-organization via hydrocarbon stapling at i,i+4 (Glu-5/Lys-9 face) does not rescue MOTS-c's structural ambiguity in silico. Whether this reflects a genuine absence of stable helical propensity in the native sequence, a limitation of Boltz-2 in handling non-natural stapling residues (S5), or both, cannot be resolved

from a single prediction run. The negative result should redirect future efforts away from conformation-locking strategies and toward functional surface engineering, indirect pathway modulation, or alternative delivery approaches building on Fold #25's lipidation precedent.

RESEARCH BRIEF

DISTILLATION №30 — DISCARDED

MOTS-C I,I+4 STAPLED HELIX (GLU-5/LYS-9)

Verdict: DISCARDED — structural prediction uninformative **pLDDT:** 0.60 | **ipTM:** 0.23 | **pTM:** 0.57

MECHANISM OF ACTION (BACKGROUND)

MOTS-c is a 16-amino acid mitochondrial-derived peptide (MDP) encoded within the 12S rRNA of the mitochondrial genome. Its primary established mechanism (Lee et al., 2015, PMID:25738459) is inhibition of the folate cycle and de novo purine biosynthesis, leading to accumulation of AICAR (5-aminoimidazole-4-carboxamide ribonucleotide). AICAR is a well-validated indirect activator of AMPK — the master cellular energy sensor — which drives glucose uptake, fatty acid oxidation, and metabolic homeostasis. The AICAR–AMPK axis is the consensus signaling route in the literature (PMID:36677050, PMID:36761202), placing MOTS-c's AMPK engagement primarily in the indirect category.

Beyond metabolic regulation, MOTS-c has demonstrated activity in gestational diabetes, bone metabolism, pulmonary fibrosis, ovarian cancer (where it competes for protein-protein interaction surfaces with LARS1/USP7), and cardiovascular conditions. Nuclear translocation under stress conditions has been documented, implicating multiple conformational modes and binding partners across cellular compartments. No experimental structure of MOTS-c — free or bound — has been published, and no residue-level SAR data exists.

MODIFICATION HYPOTHESIS (WHAT WE TESTED)

Fold #30 introduced an all-hydrocarbon i,i+4 staple between positions 5 and 9 of MOTS-c, replacing Met-5 and Tyr-9 with (S)-2-(4'-pentenyl)alanine (S5) non-natural residues followed by ruthenium-catalyzed ring-closing metathesis:

```
Native:  M  R  W  Q  E  M  G  Y  I  F  Y  P  R  K  L  R
         1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16
Stapled: M  R  W  Q  E [S5] G  Y  I [S5] F  Y  P  R  K  L  R
```

The design rationale was threefold: 1. **Conformational pre-organization**: Enforce a single helical turn spanning residues 5–9 to reduce the entropic cost of AMPK engagement, exploiting the well-established principle that intrinsically disordered peptides bind targets with lower affinity due to the conformational entropy penalty. 2. **Oxidation resistance at Met-5**: Replacing oxidation-prone Met-5 with a non-oxidizable S5 stapling residue addressed the same stability liability flagged in the Nle substitution of **Fold #5** — a two-for-one modification. 3. **Preservation of the binding surface**: The staple was positioned on the solvent-exposed face (Met-5/Tyr-9), leaving the cationic C-terminal patch (R12/K13/R16, the AMPK-engaging surface explored computationally in **Fold #19**) and aromatic residues Trp-3/Phe-10 unperturbed.

This was the first application of hydrocarbon stapling to MOTS-c or any MDP in the literature, and the first stapled peptide in this lab's longevity series.

WHY THE PREDICTION WAS UNINFORMATIVE

Global structural confidence did not improve. pLDDT returned at 0.60 — marginally lower than the native MOTS-c baseline of ~0.62 observed across Folds #5, #19, and #25. For a single-run prediction, this difference is within noise. The predicted improvement to >0.65–0.70 in the stapled region did not materialize.

Inter-chain docking confidence collapsed. The ipTM of 0.23 is the most diagnostic metric here. For a peptide–protein complex prediction, ipTM values below ~0.40 indicate that the model cannot confidently place the peptide at the target interface. Boltz-2 essentially could not predict a meaningful bound geometry for the stapled MOTS-c against the AMPK alpha-2 catalytic subunit. The affinity module returned no values, consistent with this failure mode.

The predicted helical turn was not observed. Despite the staple spanning residues 5–9, the structural output did not show a clearly defined α-helical segment with elevated local pLDDT in that region. The C-terminal cationic patch (R12/K13/R16) and aromatic residues appeared structurally unperturbed — consistent with design intent — but absence of disruption at the retained surface is not evidence of functional improvement.

Possible technical contributors to the failure: - Boltz-2 and similar structure predictors are trained primarily on natural amino acids. The S5 non-natural stapling residue (pentenyl side chain with a covalent hydrocarbon bridge) is chemically exotic and may not be modeled accurately, potentially explaining why the expected local helical stabilization was not predicted. - The staple is represented as a sequence-level modification, but the covalent constraint geometry requires accurate

modeling of the metathesis-derived bridge — a feature that current AlphaFold-family tools handle imperfectly at best. - Single-run prediction without ensemble or multiple seeds cannot distinguish whether the low confidence reflects genuine disorder or prediction uncertainty from the non-natural residue representation.

WHAT THIS TELLS US (NEGATIVE RESULTS ARE DATA)

The structural ambiguity of MOTS-c is not resolved by i,i+4 hydrocarbon stapling at positions 5/9 — at least not in a way current predictors can detect. Across five MOTS-c folds in this lab:

Fold	Modification	pLDDT	Verdict
#5	Met-1 → Nle	0.62	PROMISING
#19	Lys-13 → Arg	0.63	PROMISING
#25	N-terminal myristoylation	0.63	PROMISING
#30	i,i+4 hydrocarbon staple (5/9)	0.60	DISCARDED

The 0.62–0.63 pLDDT cluster is now a reproducible feature of MOTS-c predictions regardless of modification class (point substitution, lipidation, covalent constraint). This consistent ceiling suggests either: (a) the sequence intrinsically lacks helical propensity that current tools can amplify; (b) the AMPK alpha-2 complex geometry is not well-captured for this peptide class; or (c) both.

The negative result also raises a literature-grounded concern: **if MOTS-c's primary AMPK mechanism is indirect** (via AICAR accumulation through folate/purine pathway inhibition), then conformational optimization for direct AMPK binding may be chasing the wrong target entirely. Locking MOTS-c into a single helical conformation could actively impair its multi-compartment function (cytoplasmic metabolism, nuclear translocation, membrane interaction), consistent with the literature agent's assessment that conformational flexibility may be required for MOTS-c's broad activity profile.

The Tyr-9 → S5 substitution introduces a specific concern that deserves explicit note: Tyr-9 is an aromatic residue with a hydroxyl group that could contribute to hydrogen bonding at a binding interface. Replacing it with a hydrophobic pentenyl chain eliminates both features, a modification that could reduce biological activity independent of the helical constraint effect.

ALTERNATIVE HYPOTHESES TO TEST

Given the consistent structural prediction ceiling for MOTS-c and the indirect-mechanism concerns from the literature:

1. **Shorter, truncated analogs**: Rather than constraining the full 16-mer, identify a minimal active fragment (e.g., the C-terminal cationic tail, residues 12–16, as a standalone peptide) and test whether a shorter sequence achieves better structural confidence. The conformational entropy problem scales with sequence length.
2. **Extend the lipidation strategy from Fold #25**: Myristoylation returned PROMISING at pLDDT 0.63 with a delivery-focused rationale. Testing a palmitoyl (C16) or stearoyl (C18) variant, or adding a PEG spacer between the fatty acid and Met-1, may improve membrane engagement metrics without disrupting the functional sequence. This parallels the palmitoylation work in Fold #27 on FOXO4-DRI.
3. **Lactam stapling as an alternative constraint chemistry**: The TB-500 lactam cyclization fold in the recent lab history demonstrated that ring-forming strategies can produce interpretable outputs. A Glu-5/Lys-9 or Asp-5/Lys-9 lactam bridge — avoiding the non-natural amino acid representation problem that may have confounded this fold — could be attempted as a chemically distinct conformational constraint on the same helix-spanning positions.
4. **C-terminal modification building on Fold #19**: K13R delivered a modest but consistent PROMISING signal. A double substitution (K13R + R12 methylation or R16 modification) targeting the cationic patch more aggressively, without touching the central region, avoids the conformational disruption risk entirely.
5. **Abandon direct AMPK targeting; explore AICAR pathway mimicry**: If MOTS-c acts primarily via AICAR, designing an analog optimized for folate cycle enzyme inhibition (direct binding to ATIC or MTHFD enzymes) rather than AMPK engagement may be mechanistically better aligned. This would require target switching and a new structural hypothesis.

SEQUENCES

NATIVE

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MRWQEMGYIFYPRKLR
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MODIFIED

```
MRWQE[S5]GYI[S5]FYPRKLR
```

CAVEATS

- in silico prediction only — requires wet lab validation
- single-run prediction (not ensembled) — confidence metrics should be interpreted with caution
- predicted properties may not reflect real-world biological behavior
- this is research, not medical advice
- Boltz-2 and related structure predictors are trained primarily on natural amino acids; the (S)-2-(4'-pentenyl)alanine (S5) stapling residue and covalent hydrocarbon bridge geometry may be modeled inaccurately, potentially explaining the absence of predicted helical stabilization
- the covalent RCM-derived staple constraint cannot be faithfully represented as a simple sequence substitution — the geometric rigidity of the bridge is likely undermodeled
- no Chai-1 agreement run was available for orthogonal structural validation; ipTM of 0.23 is based on Boltz-2 alone
- heuristic property estimates (aggregation propensity, stability score, half-life, BBB penetration) are sequence-based approximations only and do not account for the non-natural stapling residues or the covalent bridge
- the dominant MOTS-c mechanism in the literature is indirect AMPK activation via AICAR — whether direct AMPK alpha-2 binding occurs at all remains unresolved experimentally
- no SAR data for MOTS-c exists in the published literature; the functional consequences of Met-5 and Tyr-9 substitution are entirely unknown

CITATIONS

1. **PMID —** (2015) — — The mitochondrial-derived peptide MOTS-c promotes metabolic homeostasis and reduces obesity and insulin resistance
2. **PMID —** (2023) — — MOTS-c Functionally Prevents Metabolic Disorders
3. **PMID —** (2023) — — MOTS-c: A promising mitochondrial-derived peptide for therapeutic exploitation
4. **PMID —** (2019) — — MOTS-c: A Mitochondrial-Encoded Regulator of the Nucleus
5. **PMID —** (2024) — — Mitochondrial-Derived Peptide MOTS-c Suppresses Ovarian Cancer Progression by Attenuating USP7-Mediated LARS1 Deubiquitination

6. **PMID —** (2022) — — The mitochondrial-derived peptide MOTS-c relieves hyperglycemia and insulin resistance in gestational diabetes mellitus
7. **PMID —** (2023) — — Role of MOTS-c in the regulation of bone metabolism
8. **PMID —** (2023) — — MOTS-c: A potential anti-pulmonary fibrosis factor derived by mitochondria
9. **PMID —** (2025) — — Redefining Mitochondrial Therapy for ME/CFS: The Case for MOTS-c
10. **PMID —** (2026) — — Humanin and MOTS-c Attenuate Atrial Fibrillation by Suppressing Fibrosis and Mitochondrial Dysfunction

SOLANA SIGNATURE 5m5hK3hhVyMEQFNNuQb4cNkCffRmz8xTvHLV86DnbbcxKTuHwhNynhta3imtUnP3snJS2MNc8R8kbdYGwQHtb4Qj
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