DISTILLATION №77 · ALEMBIC LABS

TB-500 — N-TERMINAL MYRISTOYLATION: COVALENT ATTACHMENT OF A MYRISTOYL (C14) FATTY ACID TO THE A-AMINE OF LEU-1 VIA A STABLE AMIDE BOND, YIELDING MYR-LKKTETQ

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PROMISING REGENERATIVE

N-TERMINAL MYRISTOYLATION: COVALENT ATTACHMENT OF A MYRISTOYL (C14) FATTY ACID TO THE A-AMINE OF LEU-1 VIA A STABLE AMIDE BOND, YIELDING MYR-LKKTETQ

BETA-ACTIN

AVERAGE CONFIDENCE	PTM / IPTM	VERDICT
87.3%	0.916 / 0.603	PROMISING
TARGET	**UNIPROT**	**BINDING PROBABILITY**
Beta-actin	P60709	—

TLDR

DISTILLATION №77 tests N-terminal myristoylation of TB-500 (Myr-LKKTETQ), hypothesizing that a C14 fatty acid chain on Leu-1 would create a membrane-anchored depot for improved intracellular delivery to G-actin pools. Despite a surprisingly strong pLDDT of 0.87 on the peptide core, the fold was DISCARDED due to a tool-limit failure: AlphaFold/Boltz-2 cannot model the non-canonical myristoyl lipid moiety, meaning the predicted structure reflects the bare LKKTETQ backbone only and not the actual modified molecule. The binder probability module returned no values, and the ipTM of 0.60 reflects insufficient complex-level confidence to call binding. This is a tool resolution failure, not a biological invalidation — the membrane depot hypothesis remains untested.

EXECUTIVE SUMMARY

Fold #77 myristoylates TB-500's N-terminus (Myr-LKKTETQ) for a membrane depot strategy complementing Fold #38's albumin-binding palmitoyl. DISCARDED: structure predictors are blind to the C14 lipid — pLDDT 0.87 reflects the bare peptide backbone, affinity module returned no values. Hypothesis intact; needs wet-lab or MD.

DETAILED ANALYSIS

TB-500 (Ac-LKKTETQ) is the heptapeptide core of thymosin β4, responsible for G-actin sequestration via its LKKT pharmacophore. The canonical commercial formulation already incorporates N-terminal acetylation — a small, flat C2 cap that protects Leu-1 from aminopeptidase attack while remaining sterically unobtrusive at the actin-binding interface. This fold escalates that concept dramatically by substituting the acetyl for a myristoyl (C14 saturated fatty acid) chain, a modification borrowed from endogenous membrane-targeting proteins like Src-family kinases and HIV-1 Gag p17. The hypothesis is that the C14 chain would partition into plasma membranes, creating a reversible depot from which the peptide gradually releases into the cytosol to engage G-actin — a delivery route not previously explored for this peptide class.

The cross-fold context is rich and directly relevant. Fold #38 established the first lipidation precedent for TB-500, using a C-terminal palmitoyl-γGlu-Lys-7 construct targeting albumin-mediated half-life extension; it was REFINED with pLDDT 0.84, validating lipidation as a productive direction. Fold #7 (acetylation, referenced in the researcher's rationale) confirmed that N-terminal caps are well-tolerated. Fold #77 therefore represents a logical escalation: combining the aminopeptidase-protection benefit of Fold #7 with a lipid-delivery mechanism complementary to (but mechanistically distinct from) Fold #38's albumin strategy. The progression makes scientific sense on paper — the lab has now probed both ends of the molecule with lipid handles.

The structural prediction returned a paradoxically high pLDDT of 0.87 for the peptide backbone, which superficially looks encouraging. However, this score is an artifact of the tool's behavior: AF2/Boltz-2 strip non-standard chemical modifications and predict the unmodified or minimally modified backbone. The myristoyl chain — a 14-carbon lipid tail covalently bonded to the Leu-1 α-amine — is invisible to the model. What was predicted is essentially LKKTETQ geometry, not Myr-LKKTETQ behavior. The pTM of 0.92 likewise reflects intra-peptide confidence on a short sequence. The ipTM of 0.60 falls below the threshold for confident complex-level binding prediction, and critically, the Boltz-2 affinity module returned no values — the binding probability module simply could not adjudicate. Chai-1 agreement data were unavailable.

From a biological standpoint, the literature raises legitimate concerns that compound the tool limitations. Leu-1 and Lys-2 are the N-terminal residues that anchor LKKTETQ to actin subdomain 1/2; N-terminal acetylation is tolerated, but a C14 chain represents a qualitatively different steric perturbation. The Rahaman et al. (2024) metabolic data confirm Leu-1's aminopeptidase vulnerability, supporting the protection rationale, but also show that only fragments ≥5 residues (Ac-LKKTE) retain wound healing activity — suggesting the N-terminal region contributes meaningfully to efficacy and cannot be freely decorated. Aqueous solubility and aggregation are practical concerns: C14 chains on heptapeptides may drive micelle formation below the critical micellar concentration (CMC) at therapeutically relevant concentrations, complicating dosing. The Judák et al. (2017) adsorption data show unmodified TB-500 already suffers surface binding problems; lipidation would worsen this.

The heuristic peptide profile (sequence-based estimates, not wet-lab measurements) paints a modest picture: aggregation propensity of 0.0 (likely underestimated for the lipidated form, as the tool sees the bare sequence), stability score of 0.5, and a short half-life estimate of 15–45 minutes — consistent with the known rapid catabolism of TB-500 in the Rahaman et al. data, though the myristoyl cap is expected to extend this somewhat by blocking aminopeptidase entry at Leu-1.

This discard sits in a pattern for this peptide. Fold #65 (D-Lys-3 chirality inversion) and Fold #51 (Thr-4 → 4F-Phe) were both discarded, while the structurally conservative modifications — Fold #38 (C-terminal palmitoyl), Fold #28 (lactam bridge) — achieved REFINED verdicts. The pattern suggests TB-500's actin-binding heptapeptide tolerates backbone conformational stabilization and C-terminal extensions better than N-terminal bulk or non-canonical substitutions at pharmacophore-adjacent positions. Fold #77's myristoylation sits squarely in the 'N-terminal bulk' category that has consistently challenged the predictors on this sequence.

The failure here is unambiguously a tool-limit failure, not a biological one. Standard structure-prediction pipelines are designed for proteinogenic amino acids in aqueous environments; lipid moieties require molecular dynamics with explicit membrane models, enhanced sampling, or coarse-grained force fields (CHARMM-GUI, GROMACS with Slipids/CHARMM36, or coarse-grained MARTINI simulations). The membrane depot hypothesis is pharmacologically coherent and literature-supported in principle — it simply cannot be evaluated by the current in silico stack. Wet-lab synthesis of Myr-LKKTETQ followed by SPR against immobilized G-actin, combined with liposome partitioning assays and CMC measurements, would provide the definitive answer the predictors cannot.

RESEARCH BRIEF

FOLD №77 — TB-500 N-TERMINAL MYRISTOYLATION

VERDICT: DISCARDED (TOOL-LIMIT FAILURE)

TLDR

Fold #77 was **DISCARDED** due to a tool-limit failure: current structure predictors (AlphaFold/Boltz-2/Chai-1) cannot model the N-terminal myristoyl (C14 fatty acid) modification, meaning the structural output reflects the bare LKKTETQ backbone only — not the actual Myr-LKKTETQ molecule under investigation. The Boltz-2 affinity module returned no binding probability values, and the ipTM of 0.60 is insufficient for a confident complex-level verdict. This is **not a biological invalidation** of the membrane depot hypothesis.

WHAT WE TRIED

This fold tested N-terminal myristoylation of TB-500 — specifically, covalent attachment of a myristoyl (C14 saturated fatty acid) chain to the α-amine of Leu-1 via a stable amide bond, yielding **Myr-LKKTETQ**. The hypothesis was that the C14 chain would partition into plasma membranes, creating a reversible lipid depot from which the active heptapeptide would gradually release into the cytosol to engage its target: G-actin sequestration via the canonical LKKT pharmacophore.

This is the lab's **second lipidation strategy** for TB-500 and the first to target the N-terminus. Fold #38 established that C-terminal palmitoyl-γGlu lipidation (LKKTETK(γGlu-Palm)-NH_2) was REFINED with pLDDT 0.84, validating lipidation as a productive direction for this peptide. Fold #77 was designed as a complementary, mechanistically distinct approach: where Fold #38 targeted albumin-mediated systemic half-life extension via a C16 chain at the C-terminus, this fold targeted **membrane insertion and intracellular depot formation** via a shorter C14 chain at the N-terminus — a strategy known from endogenous myristoylated proteins (Src, HIV-1 Gag, MARCKS).

The N-terminal position was also chosen because aminopeptidase cleavage at Leu-1 is a documented TB-500 liability (Rahaman et al., 2024, showing Ac-LK as the primary short-interval metabolite). The existing commercial formulation uses acetylation (Ac-LKKTETQ) to partially address this; a myristoyl amide bond was hypothesized to provide more robust protection. This builds directly on the rationale

established in Fold #7 (N-terminal acetylation) and addresses a gap that the acetyl cap only partially closes.

WHY IT WAS DISCARDED

The discard is a **tool-limit failure**, not a structural or biological red flag:

- **AlphaFold2/Boltz-2 blind to lipid chemistry**: Standard structure-prediction pipelines process proteinogenic amino acid sequences. The myristoyl chain — a non-canonical chemical modification attached to the Leu-1 α-amine — is stripped or ignored by the model. The resulting prediction describes the LKKTETQ backbone geometry, not Myr-LKKTETQ behavior in a membrane environment.
- **No affinity module output**: The Boltz-2 binder probability module returned **no values** — the most direct signal that the modified molecule was outside the model's evaluable chemical space.
- **ipTM 0.60**: Complex-level confidence fell below the threshold for a reliable binding call. On a heptapeptide without reliable interface atom placement for the modified terminus, this is expected rather than informative.
- **High pLDDT (0.87) is an artifact**: The strong backbone confidence score reflects the model's familiarity with LKKTETQ as a short, well-behaved polar sequence — not confidence in the myristoylated molecule. A high pLDDT on a stripped sequence should not be interpreted as structural validation of the modification.
- **Chai-1 agreement unavailable**: No ensemble cross-check was possible to assess prediction stability.

Biological concerns (not the primary discard reason, but relevant context): - Leu-1 and Lys-2 are directly adjacent to the actin-binding pharmacophore; a C14 chain at this position may impose steric costs on LKKT–actin engagement that acetylation does not. - C14 chains on heptapeptides may drive **micelle formation** at physiological concentrations (CMC considerations), rather than monomeric membrane insertion. - No published data exist on any lipidated LKKTETQ analog; the membrane depot mechanism is entirely de novo.

WHAT THIS DOESN'T MEAN

DISCARDED does not mean disproved. This verdict reflects the boundaries of the current in silico toolchain, not a judgment on the biological validity of N-terminal myristoylation for TB-500. The membrane depot hypothesis is pharmacologically coherent, literature-supported in analogous systems (Src-family kinases, lipidated cell-penetrating peptides), and mechanistically distinct from anything the lab has tested before for this peptide. The failure to obtain a binding probability value means the question was never answered — not that the answer is negative. It is

entirely possible that Myr-LKKTETQ inserts into membranes with appropriate kinetics, releases active peptide into the cytosol, and engages G-actin with preserved or enhanced potency. That question requires wet-lab or specialized computational tools to resolve.

WHAT WOULD ANSWER THE QUESTION

- **Solid-phase peptide synthesis + HPLC purification** of Myr-LKKTETQ, followed by **SPR (surface plasmon resonance)** against immobilized G-actin (rabbit muscle, Cytoskeleton Inc.) to directly measure K_D relative to Ac-LKKTETQ. This is the gold-standard binding assay for this target class and is directly analogous to the approach used in Tβ4 fragment SAR studies.
- **CMC measurement and liposome partitioning assay** (pyrene fluorescence probe or DLS) to determine whether Myr-LKKTETQ forms micelles at physiologically relevant concentrations (1–100 μM range) or partitions as monomer into model POPC/POPE membranes — directly testing the depot hypothesis.
- **Coarse-grained MD simulation** (MARTINI force field, GROMACS/CHARMM-GUI) of Myr-LKKTETQ in an explicit POPC bilayer to model membrane insertion depth, orientation, and flip-flop kinetics — the computational tool class appropriate for lipid-modified peptides that AF2 cannot handle.
- **Aminopeptidase protection assay** (leucine aminopeptidase, human serum stability) comparing Myr-LKKTETQ vs. Ac-LKKTETQ degradation kinetics, directly testing the N-terminal cap hypothesis from the Rahaman et al. (2024) metabolic vulnerability data.

RAW METRICS

Metric	Value	Interpretation
pLDDT	0.873	High — but reflects bare LKKTETQ backbone, not the lipidated molecule
pTM	0.916	Strong intra-peptide fold confidence (short sequence artifact)
ipTM	0.603	Below threshold for confident complex-level binding call
Boltz-2 binder probability	— (no output)	Tool could not evaluate myristoylated molecule
Chai-1 agreement	N/A	Not available for this fold

Metric	Value	Interpretation
Aggregation propensity (heuristic)	0.0	Likely underestimated; tool sees bare sequence, not C14 chain
Stability score (heuristic)	0.5	Moderate; myristoyl cap expected to improve but unquantified
Half-life estimate (heuristic)	15–45 min	Consistent with known TB-500 rapid catabolism; actual improvement from myristoyl cap is untested
BBB penetration (heuristic)	0.067	Not a relevant endpoint for this regenerative peptide

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All values are in silico predictions. No wet-lab validation has been performed. This report is research context only, not medical advice.

SEQUENCES

NATIVE

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LKKTETQ
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MODIFIED

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Myr-LKKTETQ
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CAVEATS

- in silico prediction only — requires wet lab validation
- single-run prediction (not ensembled)
- predicted properties may not reflect real-world biological behavior
- this is research, not medical advice
- AlphaFold/Boltz-2 cannot model non-canonical lipid modifications — the myristoyl chain was not represented in the structural prediction; pLDDT reflects the bare LKKTETQ backbone only
- Boltz-2 affinity module returned no binder probability values — the modified molecule was outside evaluable chemical space
- heuristic property estimates (aggregation, half-life, stability) are sequence-based and do not account for the C14 lipid chain; aggregation propensity is likely significantly underestimated for the myristoylated form

- CMC and micelle formation behavior of Myr-LKKTETQ at therapeutic concentrations is unknown and could preclude monomeric membrane insertion
- no published experimental data exist on any myristoylated or lipidated LKKTETQ analog — the entire membrane depot hypothesis is de novo and empirically untested
- Verdict reclassified: DISCARDED → PROMISING. Raw metrics (pLDDT/pTM/ipTM) permit at least the higher tier; the original LLM discard reflected modification chemistry the predictor cannot represent (D-AA, lipid moiety, non-canonical residue). Per the metric-floor rule this is a caveat, not a verdict downgrade. Report text below pre-dates the rule and may still describe the fold as DISCARDED — the structural verdict shown is the authoritative one.

CITATIONS

1. **PMID —** (2024) — — Simultaneous quantification of TB-500 and its metabolites in in-vitro experiments and rats by UHPLC-Q-Exactive orbitrap MS/MS and their screening by wound healing activities in-vitro
2. **PMID —** (2012) — — Doping control analysis of TB-500, a synthetic version of an active region of thymosin β₄, in equine urine and plasma by liquid chromatography-mass spectrometry
3. **PMID —** (2012) — — Synthesis and characterization of the N-terminal acetylated 17-23 fragment of thymosin beta 4 identified in TB-500, a product suspected to possess doping potential
4. **PMID —** (2026) — — Safety and Efficacy of Approved and Unapproved Peptide Therapies for Musculoskeletal Injuries and Athletic Performance
5. **PMID —** (2026) — — Therapeutic Peptides in Orthopaedics: Applications, Challenges, and Future Directions
6. **PMID —** (2026) — — Injectable Peptide Therapy: A Primer for Orthopaedic and Sports Medicine Physicians
7. **PMID —** (2014) — — Analytical approaches for the detection of emerging therapeutics and non-approved drugs in human doping controls
8. **PMID —** (2017) — — Adsorption effects of the doping relevant peptides Insulin Lispro, Synachten, TB-500 and GHRP 5

SOLANA SIGNATURE 27s7mpmhARN5999uGA9HwNhfbvvNLpuZsjfNTgddod3T4Umd9pjiZFyrYJWipB7pr12mSXpC2CbzhJZBAacg1XSL
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