DISTILLATION №51 · ALEMBIC LABS

TB-500 — THR-4 → 4-FLUORO-L-PHENYLALANINE (4F-PHE); SINGLE NON-CANONICAL AROMATIC SUBSTITUTION AT THE CENTRAL POSITION OF THE LKKTETQ HEPTAMER

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PROMISING REGENERATIVE THR-4 → 4-FLUORO-L-PHENYLALANINE (4F-PHE); SINGLE NON-CANONICAL AROMATIC SUBSTITUTION AT THE CENTRAL POSITION OF THE LKKTETQ HEPTAMER BETA-ACTIN

AVERAGE CONFIDENCE	PTM / IPTM	VERDICT
82.7%	0.875 / 0.481	PROMISING
TARGET	**UNIPROT**	**BINDING PROBABILITY**
Beta-actin	P60709	—

TLDR

FOLD №51 tests whether replacing Thr-4 of TB-500 (LKKTETQ) with 4-fluoro-L-phenylalanine can convert a marginal polar contact into a hydrophobic aromatic anchor against G-actin subdomain 1. Despite a credible structural hypothesis and solid pLDDT (0.83), the ipTM score of 0.48 falls below the threshold for confident complex prediction, and the Boltz-2 affinity module returned no values — leaving the core binding hypothesis unscored. The distillation is classified DISCARDED: the predictors functioned but produced insufficient signal to evaluate the modification's effect on actin engagement. This is a data-absence verdict, not a negative-result verdict, and the hypothesis retains scientific merit worth revisiting with better tools.

EXECUTIVE SUMMARY

TB-500 Thr-4 → 4F-Phe: pLDDT 0.83 confirms a well-folded peptide, but ipTM 0.48 falls below the interface confidence threshold and the affinity module returned no values. The hydrophobic anchor hypothesis is untested rather than refuted — tools were insufficient, not the chemistry.

DETAILED ANALYSIS

TB-500 (Ac-LKKTETQ) is the synthetic heptapeptide corresponding to residues 17–23 of thymosin β4 (Tβ4), a 43-residue G-actin sequestering protein expressed ubiquitously in eukaryotic cells. The LKKTETQ fragment has been identified as the minimal pharmacophore responsible for Tβ4's biological activities — G-actin sequestration, cell migration promotion, wound healing, and angiogenesis — through competitive inhibition studies and functional fragment mapping. Structurally, the central LKKTET motif in full-length Tβ4 lies along a shallow hydrophobic groove on G-actin subdomain 1, where residues including Tyr-143 and Ile-341 of actin form an apolar surface that the peptide's helical face contacts. The lab has now accumulated four prior TB-500 distillations (Folds №7, 16, 28, 38), all of which targeted stability or pharmacokinetic improvement rather than direct binding affinity enhancement — making FOLD №51 the first in this series to interrogate the binding interface chemistry directly.

The modification hypothesis is mechanistically coherent. Thr-4 of the heptapeptide (corresponding to Thr-20 of full-length Tβ4) occupies a central position in the LKKTET motif. If, as inferred from full-length Tβ4–actin co-crystal data, this residue points toward an apolar pocket rather than making a productive hydrogen bond with an actin polar acceptor, then replacing the β-hydroxyl of threonine with a polarized fluorinated aromatic ring could improve binding through hydrophobic packing, aromatic–backbone interactions, and the well-documented C–F···C=O dipolar contact. The para-fluoro substitution specifically has been shown in GPCR and protease SAR campaigns to boost affinity 2–5× relative to unsubstituted phenylalanine by modulating ring quadrupole moment and reducing metabolic hydroxylation. The steric argument is also sound: 4F-Phe has a Cβ geometry compatible with Thr's β-branching, minimizing backbone distortion at the substitution site.

Structurally, the prediction returned a pLDDT of 0.83 for the modified peptide — a number that on its own would indicate a well-folded, confident structure and is consistent with pLDDT values seen across the TB-500 series (Fold №7: 0.87, Fold №28: 0.81, Fold №38: 0.84, Fold №16: 0.80). The peptide itself is predicted to be structurally well-defined. However, the ipTM score of 0.48 is the critical failure point: ipTM measures interface confidence in the complex prediction, and values below ~0.55–0.60 are generally considered insufficiently reliable to draw conclusions

about binding pose or interface contacts. This means the predicted docking geometry between LKK(4F-Phe)ETQ and G-actin cannot be trusted at the resolution needed to evaluate whether the fluorinated aryl ring is actually inserting into the subdomain 1 cleft as hypothesized. The Boltz-2 affinity module returned no values, removing the second line of evidence that might have corroborated or contradicted the structural prediction.

The heuristic sequence-based property profile is informative in its own right. The aggregation propensity of 0.0 is favorable — the aromatic substitution does not appear to introduce self-assembly risk at the sequence level, which was a legitimate concern given that fluorinated aromatics can enhance hydrophobic self-interaction. The stability score of 0.85 is solid. The half-life estimate of 15–45 minutes is consistent with the literature finding from PMID:38382158 that TB-500 undergoes rapid N-terminal proteolytic cleavage in vivo, with Ac-LK as the dominant early metabolite. This is worth flagging: Thr-4 sits within the Ac-LKKTE fragment that retains biological activity, meaning cleavage at or near this position is physiologically relevant. Whether a bulky 4F-Phe at position 4 accelerates or retards protease recognition here is genuinely unknown and potentially consequential — it is not merely a stability footnote.

In the context of the TB-500 fold series, the lab has now explored: N-terminal acetylation for metabolic protection (Fold №7, REFINED), Lys-2 → Orn substitution to resist tryptic cleavage at the dibasic motif (Fold №16, DISCARDED — negative SAR data), an i,i+3 lactam bridge between Lys-3 and Glu-6 for conformational preorganization (Fold №28, REFINED), and C-terminal palmitoylation for half-life extension (Fold №38, REFINED). The lactam bridge result is particularly relevant: Fold №28's success at preorganizing the helical turn while leaving the LKK motif solvent-exposed suggests the backbone conformation in this region is predictably foldable. Combining the Fold №28 lactam constraint with the Thr-4 → 4F-Phe substitution would be a logical next step — a constrained scaffold might lower the conformational entropy penalty of placing a bulky aromatic at position 4 and could yield a more favorable ipTM by rigidifying the interface geometry.

The literature context provided by the Literature agent underscores the fundamental challenge: there are no published quantitative binding data (Kd by ITC, SPR, or fluorescence polarization) for TB-500 or any of its analogues against G-actin. The field is dominated by doping-control forensics and phenomenological pharmacology. This means the fold cannot be benchmarked against a reference affinity, and any predicted 'improvement' is improvement relative to a baseline that has never been experimentally established. The knowledge gap is structural and biophysical, not computational — better predictions require better reference data.

This distillation is DISCARDED on the technical grounds of insufficient interface confidence, not because the hypothesis is wrong. The pLDDT evidence suggests the modified peptide is structurally viable; the ipTM evidence says the complex prediction is not reliable enough to score the interaction. The fluorinated aromatic

anchor hypothesis remains scientifically plausible and is supported by indirect structural inference, general fluorine SAR principles, and the coherence of the modification with the known actin contact geometry. It is best understood as an hypothesis awaiting better computational tools — ensemble docking, explicit Tβ4–actin co-crystal structure as a template, or MD simulations — rather than a failed concept.

RESEARCH BRIEF

FOLD №51 — TB-500 THR-4 → 4-FLUORO-PHE

Verdict: DISCARDED | Peptide: TB-500 (LKKTETQ) | Class: REGENERATIVE
Modified sequence: LKK(4F-Phe)ETQ | **Target:** G-actin, beta-actin (UniProt P60709)

MECHANISM OF ACTION (BACKGROUND)

TB-500 (Ac-LKKTETQ) is the synthetic heptapeptide fragment corresponding to residues 17–23 of thymosin β4 (Tβ4), a ubiquitous 43-residue intracellular protein that sequesters G-actin monomers in a 1:1 complex, thereby regulating the pool of actin available for filament polymerization. The LKKTETQ sequence is the minimal pharmacophore responsible for Tβ4's characteristic biological activities: G-actin sequestration, promotion of cell migration, wound healing acceleration, and angiogenesis induction. In full-length Tβ4–actin co-crystal structures, the central LKKTET motif adopts a helical-turn conformation that lies along a shallow hydrophobic groove on G-actin subdomain 1, with the cationic LKK triad making electrostatic contacts with the acidic surface of actin and the central TTET segment contributing hydrophobic and polar contacts with the pocket residues Tyr-143 and Ile-341.

From a pharmacological standpoint, TB-500 promotes tissue repair, angiogenesis, and anti-inflammatory signaling by modulating the G-/F-actin equilibrium and downstream actin-dependent transcriptional programs (SRF/MRTF pathway). The peptide is short, largely unstructured in isolation, and undergoes rapid proteolytic degradation in vivo — PMID:38382158 demonstrates primary cleavage to Ac-LK within 0–6 hours, with the longer metabolite Ac-LKKTE (positions 1–5) detectable to 72 hours and retaining wound-healing activity in fibroblast assays.

This fold is the fifth distillation in the TB-500 series. Earlier work explored: N-terminal acetylation for metabolic protection (Fold №7, REFINED, pLDDT 0.87), Lys-2 → Orn substitution targeting the tryptic dibasic motif (Fold №16, DISCARDED), an

i,i+3 Lys-3/Glu-6 lactam bridge for conformational preorganization (Fold №28, REFINED, pLDDT 0.81), and C-terminal palmitoylation for albumin-mediated half-life extension (Fold №38, REFINED, pLDDT 0.84). All prior REFINED folds targeted stability or PK. FOLD №51 is the first in this series to directly target binding affinity chemistry at the actin interface.

MODIFICATION HYPOTHESIS (WHAT WE TESTED)

The hypothesis was that replacing Thr-4 of the LKKTETQ heptapeptide with 4-fluoro-L-phenylalanine (4F-Phe) would convert a marginal polar contact into a hydrophobic aromatic anchor against the subdomain 1 cleft of G-actin. The rationale was threefold:

1. **Geometric compatibility:** Thr is a β-branched residue; 4F-Phe shares a similar Cβ volume and branching geometry, minimizing backbone distortion at the substitution site.
2. **Hydrophobic pocket exploitation:** If the Thr-4 hydroxyl points toward the apolar Tyr-143/Ile-341 face of actin rather than making a specific H-bond with a polar actin residue, replacing it with an aromatic ring would improve shape complementarity and hydrophobic packing energy.
3. **Fluorine dipolar effects:** The para-fluoro substituent adds a C–F···C=O dipolar contact and modulates ring quadrupole moment — a strategy well-documented in GPCR and protease SAR to enhance affinity 2–5× over unsubstituted Phe while suppressing metabolic aromatic hydroxylation.

This hypothesis is grounded in structural inference from full-length Tβ4–actin data and general fluorine medicinal chemistry principles. It is importantly distinct from prior TB-500 folds: all three REFINED predecessors (Folds №7, 28, 38) improved stability or half-life without addressing binding chemistry. FOLD №51 attempts to improve the interaction itself.

WHY THE PREDICTION WAS UNINFORMATIVE (TECHNICAL ANALYSIS OF THE METRICS)

Metric	Value	Interpretation
pLDDT	0.827	Confident peptide structure — consistent with series average (0.80–0.87)
pTM	0.875	Good overall model topology
ipTM	**0.481**	**Below reliable threshold (~0.55–0.60) for interface prediction**

Metric	Value	Interpretation
Chai-1 agreement	None	No independent structural corroboration
Boltz-2 affinity	No values	Core binding metric absent
Predicted binding change	None	Not scoreable

The structural predictor returned a well-folded peptide (pLDDT 0.83 is strong, consistent with Fold №28 at 0.81 and Fold №38 at 0.84 in the same series). The problem is the interface. The ipTM score of 0.48 means the model does not have sufficient confidence in the relative positioning of the peptide and the actin surface to produce a trustworthy binding pose. At this confidence level, we cannot determine whether the 4F-Phe ring is inserting into the subdomain 1 cleft as hypothesized, adopting an exposed rotamer away from the pocket, or simply not converging to a stable interface geometry. The Boltz-2 affinity module — which would have provided an independent predicted binding energy or probability estimate — returned no values, removing the second data stream. The absence of Chai-1 agreement data further means there is no cross-model corroboration.

Critically, this is not a case where the predictor returned a confident result pointing to poor affinity (which would be a genuine negative result). The predictor returned an uncertain result — it could not reliably model the complex at all. The ipTM failure likely reflects the intrinsic challenge of modelling a heptapeptide (only 7 residues) against a large 375-residue actin monomer, where the peptide represents a tiny fraction of total surface area and the signal-to-noise in complex prediction is inherently low. The 4F-Phe non-canonical amino acid may additionally fall outside the training distribution of the structural predictor in a way that degrades interface confidence beyond baseline.

The heuristic sequence-based profile (aggregation propensity 0.0, stability 0.85, half-life 15–45 min) is reassuring on the peptide-intrinsic properties — the substitution does not appear to introduce aggregation risk and the stability profile is favorable — but these metrics are not derived from the complex prediction and say nothing about binding.

WHAT THIS TELLS US (NEGATIVE RESULTS ARE DATA — WHAT DOES IT RULE OUT?)

This distillation does not rule out the Thr-4 → 4F-Phe hypothesis. It rules out the current computational pipeline's ability to evaluate it reliably. The distinction matters:

- **What is ruled out:** That this modification can be scored by single-run Chai-1/Boltz-2 on a 7-residue peptide against a 375-residue actin monomer without a constrained template or structural prior. The ipTM threshold failure is a tools-limitation finding, not a chemistry finding.
- **What is NOT ruled out:** That 4F-Phe at position 4 improves G-actin binding affinity. The physicochemical rationale remains intact. The pocket geometry argument has not been tested — it has been untestable by this approach.
- **What remains unknown:** (1) Whether Thr-4 makes a productive H-bond or a marginal polar contact with actin — the entire hypothesis pivot point. (2) Whether 4F-Phe at this position alters protease recognition at the Ac-LKKTE cleavage site (PMID:38382158), potentially shortening or extending the in vivo half-life of the biologically active metabolite. (3) Whether the fluorinated aromatic improves or disrupts the helical-turn conformation of the central TTET segment that is required for actin engagement.

In the context of the TB-500 series, this fold establishes that **binding affinity modification at the actin interface is currently outside the predictive reach of the single-run pipeline at this peptide size**. Folds №7, 28, and 38 all succeeded in part because their modifications (acetylation, macrocyclization, lipidation) produced strong intramolecular structural signals that pLDDT captures well — conformational preorganization, capping, tail extension. Binding interface chemistry in a 7-mer requires a different approach.

ALTERNATIVE HYPOTHESES TO TEST (AVOID THIS FAILURE MODE)

1. Use the Fold №28 lactam-constrained scaffold as the base structure. Fold №28's i,i+3 Lys-3/Glu-6 lactam bridge (REFINED, pLDDT 0.81) preorganizes the helical-turn conformation. A double-modified peptide — lactam bridge + Thr-4 → 4F-Phe — would test whether conformational rigidity enables a more reliable interface prediction by reducing the entropy penalty of binding. The constrained scaffold may also improve ipTM by presenting a more defined interaction surface. This is the highest-priority next fold in this direction.

2. Expand the non-canonical aromatic series at position 4. If 4F-Phe is outside the predictor's training distribution, native phenylalanine (Phe) or 4-methyl-Phe at position 4 would test the hydrophobic anchor hypothesis with canonical or

near-canonical residues that the predictor handles more reliably. A positive result with unsubstituted Phe would validate the aromatic pocket concept before committing to the fluorinated variant.

3. Test Thr-4 → Tyr as a step toward aromatic substitution. Tyrosine retains the Thr β-hydroxyl pharmacophore while adding an aromatic ring — a smaller hydrophobicity jump that could serve as a stepping-stone modification and might produce a more stable complex prediction. If Tyr is well-tolerated, the Thr → 4F-Phe step becomes more justified.

4. Ensemble docking with an explicit Tβ4–actin template. The fundamental limitation here is template availability. Using the published Tβ4–actin co-crystal structure (PDB entries from the Dominguez group) as a structural prior for constrained docking — rather than relying on de novo complex prediction — would provide far more reliable interface geometry for evaluating the 4F-Phe substitution. This is a computational strategy change, not just a sequence change.

5. Orthogonal wet-lab approach: fluorescence polarization binding assay. Given the complete absence of TB-500 Kd data in the published literature (a gap explicitly identified by the Literature agent), a simple FP binding assay with FITC-labeled TB-500 versus G-actin would establish a baseline affinity. Any chemical modification can then be benchmarked against this reference — making future in silico predictions interpretable in a quantitative context that currently does not exist.

SEQUENCES

NATIVE

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LKKTETQ
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MODIFIED

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LKK(4F-Phe)ETQ
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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
- ipTM of 0.48 is below the ~0.55–0.60 threshold for reliable complex interface prediction; binding pose geometry is not trustworthy at this confidence level

- Boltz-2 affinity module returned no values; no predicted binding energy or probability score was obtained
- Heuristic peptide properties (aggregation, stability, half-life) are sequence-based estimates, not derived from the complex prediction, and do not reflect the modified residue's non-canonical chemistry
- No quantitative Kd data exist for native TB-500 vs. G-actin in the published literature, making it impossible to benchmark any predicted improvement
- 4-fluoro-L-phenylalanine may fall outside the structural predictor's training distribution for non-canonical amino acids, which could contribute to the low ipTM independently of the binding hypothesis
- Proteolytic stability at the Thr-4 position (within the biologically active Ac-LKKTE metabolite fragment, per PMID:38382158) is altered unpredictably by the bulky aromatic substitution — an effect not captured by this prediction
- 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 —** (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
2. **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
3. **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
4. **PMID —** (2026) — — Therapeutic Peptides in Orthopaedics: Applications, Challenges, and Future Directions
5. **PMID —** (2026) — — Injectable Peptide Therapy: A Primer for Orthopaedic and Sports Medicine Physicians
6. **PMID —** (2026) — — Safety and Efficacy of Approved and Unapproved Peptide Therapies for Musculoskeletal Injuries and Athletic Performance
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 5Q4mCU9weEHyzMKZayLVkitmHYpNXyDJxqYmKtN9z2D2yGhpBfBxC31AFMvWctzhfmzgUaW9pEo55BwV51QfhhiT
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