Enzyme and metabolic engineering offer the potential to develop biocatalysts for converting natural resources into a wide range of chemicals. To broaden the scope of potential products beyond natural metabolites, methods of engineering enzymes to accept alternative substrates and/or perform novel chemistries must be developed. DNA synthesis can create large libraries of enzyme-coding sequences, but most biochemistries lack a simple assay to screen for promising enzyme variants. Our solution to this challenge is structure-guided mutagenesis in which optimization algorithms select the best sequences from libraries based on specified criteria (i.e. binding selectivity). Our computational procedure was demonstrated by tuning substrate binding of the highly-active ‘TesA thioesterase in Escherichia coli in favor of medium-chain (C6-C12) lengths. Specifically, the Iterative Protein Redesign & Optimization procedure (IPRO) was used to design ‘TesA variants with enhanced C12- or C8-specificity while maintaining high activity. After four rounds of structure-guided mutagenesis, we identified three thioesterases with enhanced production of dodecanoic acid (C12) and twenty-seven thioesterases with enhanced production of octanoic acid (C8), the fatty acid products of thioesterase-mediated catalysis. The top variants reached up to 49% C12 and 50% C8 while exceeding native levels of total free fatty acids. A similar sized library created through random mutagenesis failed to identify medium-chain specific, highly-active variants. The chain length-preference of ‘TesA and the best mutant were confirmed in vitro using acyl-CoA substrates. Molecular dynamics simulations, confirmed by resolved crystal structures, of ‘TesA variants suggest that hydrophobic forces govern ‘TesA substrate specificity. In this work, we not only successfully modified ‘TesA substrate preference but in doing so, we identified the third most C12-specific and tenth most C8-specific thioesterase characterized to date. These results are significant because medium-chain fatty acids are limited in natural abundance relative to long-chain fatty acids. This limited supply leads to high costs of C6-C10 oleochemicals such as fatty alcohols, amines, and esters. We expect that the new thioesterase variants will be useful to metabolic engineering projects aimed at sustainable production of medium-chain oleochemicals. Furthermore, we anticipate that the lessons learned from both successful and failed computational designs can guide algorithmic advancements aiding with future enzyme engineering endeavors.