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The Calculation and Synthesis of a Template Molecule

Stephen R. Wilson,* Wai K. Tam, Martin J. Di Grandi, and Weili Cui

Department of Chemistry
New York University
Washington Square
New York, NY 10003

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Abstract

This paper discusses an algorithm to compute the geometric requirements for a template molecule. A Program called **DESIGN** is used to calculate templates by matching vectors from a database of carbon skeleta. Applications of the approach to an epitope of myoglobin and a fragment around the active site of the enzyme thioredoxin is examined. The synthesis of the template molecule **7** is described.

INTRODUCTION

The exploitation of peptides as lead compounds and the rational design of cyclic and crosslinked analogs of bioactive peptides is an area of current interest. Its basic premise is the use of conformationally restricted molecules for the purpose of "locking" a hybrid molecule in a conformation that closely approximates the active three-dimensional structure of the native compound.[1,2] The use of computers has greatly facilitated this research and "computer-assisted drug design", the practice of modeling a molecule designed by a chemist to mimic a larger biologically active species, is now quite common.[3] This protocol, however, relies on the ability of the chemist to devise appropriate spacers, replacements, isosteres, etc., a process that is both subjective and serendipitous. We report in this account: (a) our new algorithm that actually *predicts* rigid analogs and crosslinks based on the use of a template database;[4,5] (b) the application of this program to the proteins myoglobin and thioredoxin; and (c) the synthesis of a designed template for a putative thioredoxin mimic.

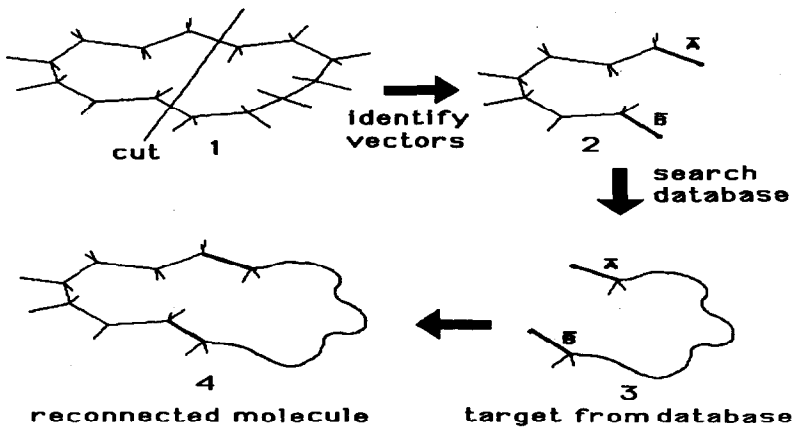


Fig. 1. General Strategy for the DESIGN Algorithm

The conceptual framework for our strategy involves consideration of the target molecule, i.e. **1**, in a minimum energy conformation (Figure 1). The ring in **1** is severed to give fragment **2** whose vectors **A** and **B** define a connection. Reconnection to a conformationally restricted template with identical vectors, i.e., **3**, should provide a new structure **4** whose conformation is now constrained to a predefined geometry. This algorithm provides the basis for designing rigid analogs for compounds of known initial geometry.

Our program called **DESIGN**[6] was devised to allow for the automatic searching of a database of templates for vectors **A** and **B** as defined in Figure 1. For peptide design, the trans amide bond is not only a convenient point for disconnection but also provides a synthetic opportunity for reconnection and well-defined vectors for identified matches. The output of our program is a list of molecules from the database that can serve as a potential template for the analyzed fragment.

Computation of Crosslinks

We now illustrate the application of **DESIGN** to the computation of crosslink stabilized antigenic sites of myoglobin. A complete immunochemical inventory of this protein has been determined and five antibody recognition sites are known.[7] Extraction of the coordinates of epitope 3 (residues 94-99) from the X-ray structure of and subsequent search of our database provided template **5** (Figure 2). Attachment of epitope 3 to **5** gave the crosslinked hybrid shown in the Figure.

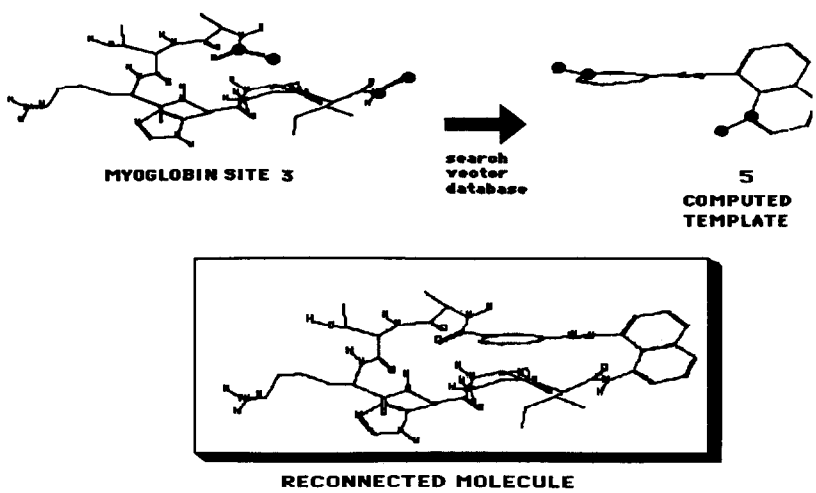


Fig. 2. Computation and Reconnection of a Template for Myoglobin Epitope 3

We next applied this methodology to thioredoxin.[8] This ubiquitous enzyme has been shown by X-ray analysis to have an active site containing two cysteine residues (^{32}Cys and ^{35}Cys) that protrude from the bulk of the folded protein. Thioredoxin has been implicated in a number of biochemical processes and references to the biological activity of model peptides based on the active site provide some evidence that its activities may be useful to treat certain disease states.[9-12] Although thioredoxin's active site has been defined to be a four residue reverse turn located on the surface of the protein, the exact source of the powerful reducing capabilities of this enzyme is not known.

The high resolution crystal structure[13] was used to define the portion of the protein surrounding the active site. Since studies on model peptides clearly demonstrated the need to go beyond the active loop, we searched twenty potential crosslinks between residues 24-42 of that includes the active site residues (Figure 3), part of an adjacent alpha-helix and part of the beta-strand that runs parallel to the helix. A search by **DESIGN**

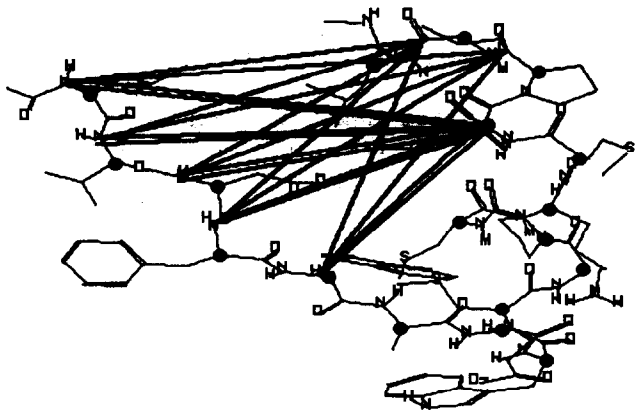


Fig. 3. Twenty Potential Crosslinks for Thioredoxin (residues 24-42)

revealed seventeen possible template molecules for nine of the original twenty crosslinks.

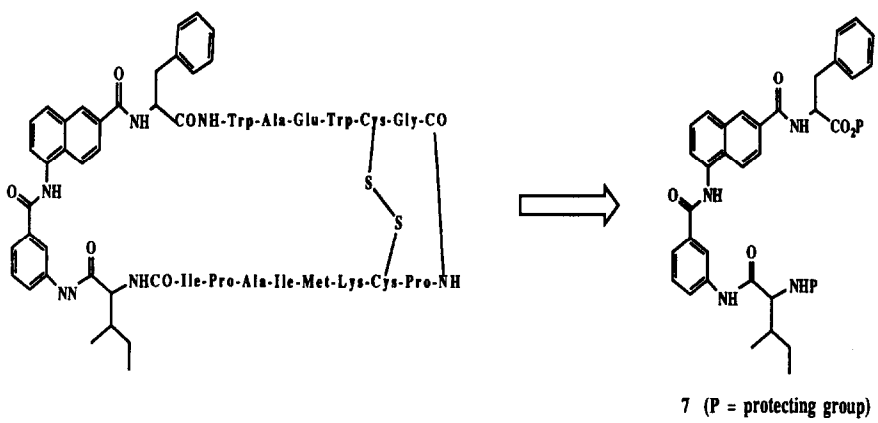
We considered several criteria to determine which of these templates predicted by **DESIGN** was the best choice. Foremost on our list of priorities was the ease with which the template could be prepared. On the basis of this and other prerequisites, we chose peptide **6**, representing residues 27-41 and **7** as our

H_2N-Phe-Trp-Ala-Glu-Trp-Cys-Gly-Pro-Cys-Lys-Met-Ile-Ala-Pro-Ile-CO_2H

6

synthetic targets. Synthesis of the organic template molecule **7** is now described.

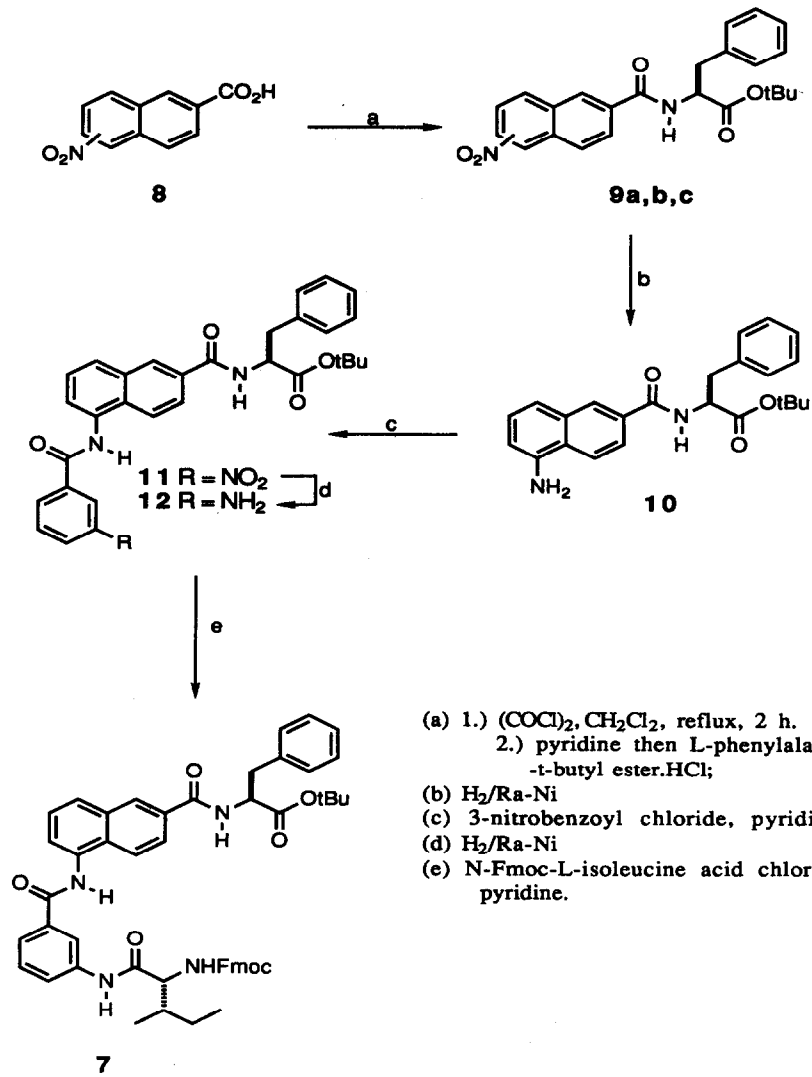
Our initial synthetic strategy involved a simple disconnection whereby the molecule would be broken into two pieces: peptide **6** and template molecule **7**. The latter would be synthesized by solid phase methods, coupled to **7** and cyclized to produce the desired thioredoxin mimetic (Figure 4).



7 (P = protecting group)

Fig. 4. Synthetic Strategy for Synthesis of Thioredoxin Template

Our synthesis starts with the commercially available 2-naphthoic acid. Nitration[14] with HNO_3 followed by two recrystallizations gave a nitro acid mixture **8**, which was converted to the corresponding acid chlorides with oxalyl chloride and treated with pyridine and the HCl salt of L-phenylalanine-t-butyl ester to give amides **9a,b,c** (scheme 1). Hydrogenation with Ra-Ni yielded the desired amine **10** after easy isomer separation by flash chromatography. With the correct isomer in hand, acylation with 3-nitrobenzoyl chloride proceeded uneventfully to give nitro amide **11**. The nitro group was again reduced (Ra-Ni) give amine **12** which was immediately acylated with N-Fmoc-L-isoleucine acid chloride, prepared by a modification of Carpino's method,[15] to yield the final product, template molecule **7**.



(a) 1.) $(COCl)_2$, CH_2Cl_2, reflux, 2 h.
2.) pyridine then L-phenylalanine -t-butyl ester.HCl;
(b) H_2/Ra-Ni
(c) 3-nitrobenzoyl chloride, pyridine
(d) H_2/Ra-Ni
(e) N-Fmoc-L-isoleucine acid chloride, pyridine.

Scheme 1

CONCLUSION

Studies using the program **DESIGN** indicate that it is quite a useful tool for suggesting target structures. We have reported the computation of tethers for the two proteins myoglobin and thioredoxin, and have also reported the synthesis of the template **7** designed to span the distance between the termini of peptide **6**. This fully protected template molecule serves as a key intermediate en route towards a putative thioredoxin mimic.

EXPERIMENTAL

General. Our prototype **DESIGN** program consists of two modules. The first, called **BUILD-LIB** , reads in an optimized Macromodel[16,17] format structure or structures and adds it to the database by loading the vector arrays with every possible amino-acid substitution pattern on the carbon skeleton. The second module, **SEARCH**, requests from the users the four distances that define vectors A and B (Figure 1) and then searches the database (Figure 5) for the best matches. A pointer to the desired molecule or molecules is thus obtained.

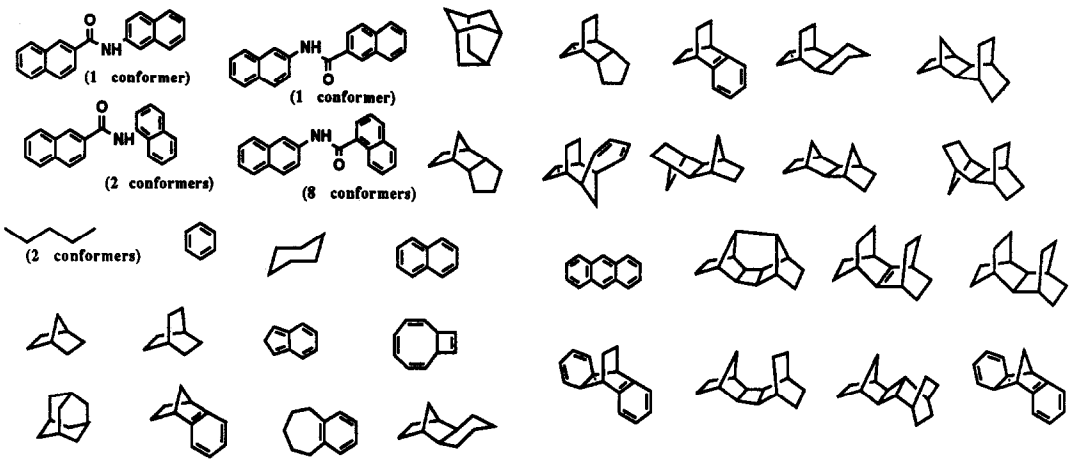


Figure 5. Partial Listing of Macromodel Structures in the Database (ref 6) Acyclics include copies of all low energy conformers.

Myoglobin site 3. Coordinates for residues 94 to 99 from the x-ray structure of myoglobin (1MYO) from the Brookhaven protein data bank were obtained by deletion in MacroModel of the balance of the structure. The ends were converted to N-Acetyl and N-CH_3 and coordinates of this fragment were saved as a MacroModel format file. Four distances from the N-Acetyl N and C and the N-CH_3 N and C were used to search our database resulting in compound **5**. Compound **5** was reconnected to site 3 using utilities available in MacroModel to give the reconnected molecule in Figure 2.

Thioredoxin. The unpublished x-ray structure at high resolution was obtained from Holmgren.[13] Residues 24 to 42 were manipulated as described for myoglobin to produce an active site fragment. Twenty possible crosslinks (Figure 3) were used to define the vectors for our search. The database was searched using these vectors to provide 17 hits. Compound **7** was selected for further investigation and was synthesized as decribed. Reconnection of the thioredoxin peptide **6** to compound **7** was carried out with MacroModel to give and excellent RMS fit to the x-ray geometry after minimization.

Synthesis of (-)-N-(5-Nitro-2-Naphthoyl)-L-Phenylalanine-t-Butyl Ester (9b). 2-Naphthoic acid (10.0 g, 58 mmol) was suspended in concentrated nitric acid (30 mL, 45 g, 0.72 mol) and heated in a 100° C oil bath for 3 h. The reaction was cooled to rt and dissolved in 10 % NaOH (850 mL). This dark brown aqueous solution was washed with EtOAc (1 x 500 mL) and then acidified with concentrated HCl to pH 2. The resulting precipitate was collected by filtration, washed with water, and recrystallized from ethanol. This yielded a pale yellow solid as a mixture of three isomeric mononitrated acids [the 4,5 and 8- Nitro-2-naphthoic acids] and one unidentified dinitrated naphthoic acid (7.4 g; 58.7 %). TLC: R_f = 0.31 (4-nitro isomer), 0.21 (5-nitro isomer), 0.10 (8-nitro isomer); (Silica gel, EtOAc:Hex/6:4). ^{1}H NMR (ppm): 7.24 - 7.33 (m, 4H), 7.65 - 7.68 (d, 1H), 7.78 - 7.95 (m, 8H), 8.14 - 8.17 (d, 2H), 8.31 (s, 2H), 8.79 (s, 1H).

The partially purified nitro acids (0.86 g, 4.0 mmol) were suspended in methylene chloride (20 mL), treated with freshly distilled oxalyl chloride (1.1 mL, 1.6 g, 13 mmol) and DMF (4 drops) and heated at reflux for 25 minutes; an aliquot quenched with dry methanol indicated by tlc analysis the presence of a higher R_f spot and no starting acid. The reaction was then cooled to rt and concentrated *in vacuo*.

The crude yellow acid chloride (approximately 4.0 mmol) was suspended in THF (20 mL) and combined with pyridine (0.81 mL, 0.79 g, 10 mmol). Ten minutes later, the HCl salt of L-phenylalanine-t-butyl ester was added (1.03 g, 4.0 mmol). Heat was then supplied to effect refluxing and an aliquot twenty minutes later indicated clean formation of the desired products. After cooling to rt, the solvent was removed *in vacuo*, and the resulting residue was suspended in water (25 mL) and extracted with EtOAc (2 x 50 mL). The combined organic layers were washed successively with 10 % HCl (2 x 25 mL), saturated $NaHCO_3$ (2 x 25 mL), brine (1 x 15 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The gummy residue was partially purified to remove the higher R_f 4-nitro adduct and used as a mixture of 5- and 8-nitro isomers in the subsequent reduction. These amines separated more cleanly by flash chromatography

TLC: R_f = 0.61 ((-)-N-(4-nitro-2-naphthoyl)-L-phenylalanine-t-butyl ester, **9a**), 0.53 ((-)-N-(5-nitro-2-naphthoyl)-L-phenylalanine-t-butyl ester, **9b**), 0.43 ((-)-N-(8-nitro-2-naphthoyl)-L-phenylalanine-t-butyl ester, **9c**), 0.29 (dinitrated naphthyl amide); (Silica gel, EtOAc: Hex/4:6).Mass Spec (low resolution, electron capture): 420 (M^+), 363 (M^+-tBu), 364 (M^+-OtBu), 87, 59.Exact Mass (Electron Capture, M^+): Calculated for $C_{24}H_{24}N_2O_5$: 420.1685; found: 420.1659.^{1}H NMR (ppm): (-)-N-(4-nitro-2-naphthoyl)-L-phenylalanine-t-butyl ester (**9a**): 1.49 (s, 9H, $-CO_2C(CH_3)_3$), 3.22 - 3.35 (ddd, 2H, -CH-CH_2-Ar), 5.00 - 5.06 (q, 1H, -CH-CH_2-Ar), 7.06 - 7.08 (d, 1H, -CO-NH-), 7.22 - 7.33 (m, 5H, phen-yl protons from Phe), 7.63 - 7.68 (t, 1H, H_7), 7.75 - 7.80 (t, 1H, H_6), 7.93 - 7.96 (d, 1H, H_5), 8.41 (s, 1H, H_3), 8.50 (s, 1H, H_1), 8.50 - 8.53 (d, 1H, H_8); (-)-N-(5-nitro-2-naphthoyl)-L-phenylalanine-t-butyl ester (**9b**): 1.47 (s, 9H, $-CO_2$-C($CH_3)_3$), 3.21 - 3.34 (ddd, 2H, -CH-CH_2-Ar), 4.99 - 5.05 (q, 1H, -CH-CH_2-Ar), 7.19 - 7.30 (m, 6H, phenyl protons from Phe, -CO-NH-), 7.49 - 7.54 (t, 1H, H_7), 7.85 - 7.88 (d, 1H, H_3), 8.01 - 8.04 (d, 1H, H_4), 8.21 - 8.23 (d, 1H, H_6), 8.23 (s, 1H, H_1), 8.41 - 8.44 (d, 1H, H_8); (-)-N-(8-nitro-2-naphthoyl)-L-phenylalanine-t-butyl ester (**9c**): 1.47 (s, 9H, $-CO_2C(CH_3)_3$), 3.22 - 3.34 (weak ddd, 2H, -CH-CH_2-Ar), 5.00 - 5.06 (q, 1H, -CH-CH_2-Ar), 7.18 -7.20 (d, 1H, -CO-NH-), 7.24 - 7.36 (m, 5H, phenyl protons from Phe), 7.46 - 7.51 (t, 1H, H_6), 7.76 - 7.84 (two overlapping d, 2H, H_4, H_7), 7.96 - 7.99 (d, 1H, H_5), 8.14 - 8.17 (d, 1H, H_3), 8.76 (s, 1H, H_1).IR (neat, cm^{-1}): 3300 (NH stretch); 1750 (C=O, ester); 1637 (C=O, amide); 1540 (NO_2), 1350 (NO_2), 1175 (C-O).

Synthesis of (-)-N-(5-Amino-2-Naphthoyl)-L-Phenylalanine -t-Butyl Ester (10) The purified nitro ester **9b** (0.600 g, 1.43 mmol) was dissolved in EtOAc:EtOH (1:1 v/v; 15 mL), a catalytic amount of Ra-Ni was added, and the heterogeneous mixture was hydrogenated under approximately 35 psi for 0.75 h. Thin-layer chromatography indicated the complete reduction of the nitro group at this point. The catalyst was removed by filtration through a plug of Florisil, and the solvent was removed *in vacuo* to yield a pale yellow syrup (0.526 g.; 94.4 %), which was used without further purification. (Note: when the nitro precursors did not separate on large scale, the amines were isolated pure by flash column

chromatography, eluting with EtOAc:Hexane/4:6.) TLC: R_f = 0.41 ((-)-N-(8-amino-2-naphthoyl)-L-phenylalanine-t-butyl ester), 0.26 ((-)-N-(5-amino-2-naphthoyl)-L-phenylalanine-t-butyl ester, **10**); (Silica gel; EtOAc: Hex/4:6). ^{1}H NMR (ppm): (-)-N-(5-amino-2-naphthoyl)-L-phenylalanine-t-butyl ester (**10**): 1.47 (s, 9H, $-CO_2C(CH_3)_3$), 3.21 - 3.34 (m, 2H, $-CH-CH_2$-Ar), 4.21 (br s, 2H, Ar-NH_2), 5.00 - 5.06 (q, 1H, $-CH-CH_2$-Ar), 6.81 - 6.83 (d, 1H, -CO-NH-Ar), 6.84 - 6.87 (dd, 1H, H_6), 7.21 - 7.38 (m, 7H, phenyl protons from Phe and H_7, H_8), 7.53 - 7.79 (dd, 1H, H_3), 7.84 - 7.87 (d, 1H, H_4), 8.19 (s, 1H, H_1). IR (neat, cm^{-1}): 3300 - 3500 (NH_2 stretch); 1730 (C=O, ester); 1650 (C=O, a-mide, NH bend); 1350 (C-N stretch); 1150 (C-O).

Synthesis of (-)-N-{[N'-(3-Nitrobenzoyl)-5-Amino]-2-Naphthoyl} -L-Phenylalanine-t-Butyl Ester (11) Oxalyl chloride (20 mL, 29 mg, 0.23 mmol) was added to a CH_2Cl_2 solution (1 mL) of 3-nitrobenzoic acid (14 mg, 0.08 mmol); the reaction was treated with DMF (1 drop) and was heated to reflux. After fifteen minutes, the reaction was cooled to rt and concentrated *in vacuo*. The crude acid chloride (approximately 0.23 mmol) was suspended in THF (1 mL) and treated sequentially with pyridine (6.4 mL, 6.3 mg, 0.08 mmol) and naphthylamine **10** (20.5 mg, 0.05 mmol). The reaction was heated to reflux, stirred at that temperature for 0.5 h and at rt overnight.

The mixture was diluted with water (10 mL) and extracted with EtOAc (2 x 25 mL); the combined organic layers were washed successively with 10 % HCl (1 x 15 mL), saturated $NaHCO_3$ (2 x 15 mL), water (1 x 15 mL), and brine (1 x 15 mL), then dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude amide was purified by prep tlc (250 mm plate), eluting with EtOAc:hexanes/4:6 to give 20.7 mg (72.7 %) purified product. TLC: R_f = 0.19 (Silica gel; EtOAc:Hex/4:6). Mass Spec (low resolution; NCI^-): 539 (M^+, base peak), 483 ($M^+-C_4H_8$), 465, 437, 148. Mass (Electron Capture, M^+): Calculated for $C_{31}H_{29}N_3O_6$: 539.2056; found: 539.2009. ^{1}H NMR (ppm): 1.45 (s, 9H, $-CO_2C(CH_3)_3$), 3.17 - 3.30 (m, 2H, $-CHCH_2$Ar), 4.93 - 4.99 (q, 1H, $-CHCH_2$Ar), 7.01 - 7.03 (d, 1H, -CO-NH-Ar), 7.17 - 7.31 (m, 7H, phenyl protons from Phe and H_7, H_8), 7.39 - 7.46 (two overlapping d, 2H, H_3, H_4), 7.52 - 7.54 (d, 1H, H_6), 7.59 - 7.65 (t, 1H, H_{13}), 7.77 (s, 1H, H_1), 8.33 - 8.37 (two overlapping d, 2H, H_{12}, H_{14}), 8.87 (s, 1H, H_{10}), 9.39 (s, 1H, -CO-NH-Ar). IR (neat, cm^{-1}): 3300 (NH stretch); 1725 (C=O, ester); 1650 (C=O, amide); 1500 (NO_2), 1350 (NO_2), 1175 (C-O).

Synthesis of (-)-N-{[N'-(3-Aminobenzoyl)-5-Amino]-2-Naphthoyl}-L-Phenylalanine-t-Butyl Ester (12) The purified nitro ester **11** (0.100 g, 0.19 mmol) was dissolved in EtOAc:EtOH (1:1 v/v; 5 mL), a catalytic amount of Ra-Ni was added, and the heterogeneous mixture was hydrogenated under approximately 35 psi for 50 minutes. The catalyst was removed by filtration through a plug of Florisil, and the solvent was removed *in vacuo*. This gave 72.4 mg (77.8 %) crude amine **12**, which was used without further purification. TLC: R_f = 0.05 (Silica gel; EtOAc:Hex/4:6). ^{1}H NMR (ppm): 1.47 (s, 9H, $-CO_2C(CH_3)_3$), 3.21 - 3.33 (m, 2H, $-CHCH_2$Ar), 3.60 - 4.05 (br s, 2H, Ar-NH_2), 4.98 - 5.04 (q, 1H, $-CHCH_2$Ar), 6.82 - 6.85 (d, 1H, H_{12}), 6.95 - 6.98 (d, 1H, -CO-NH-Ar), 7.21 - 7.32 (m, 7H, phenyl protons from Phe and H_{10}, H_{14}), 7.43 - 7.49 (t, 2H, H_7, H_{13}), 7.65 - 7.68 (d, 2H, H_3, H_4), 7.75 - 7.78 (d, 1H, H_8), 7.87 - 7.90 (d, 1H, H_6), 8.13 (s, 1H, H_1), 8.46 (s, 1H, -CO-NH-Ar). IR (neat, cm^{-1}): 3300 - 3500 (NH_2 stretch); 1750 (C=O, ester); 1650 (C=O, a-mide); 1550 (NH bend); 1350 (C-N stretch); 1150 (C-O).

Synthesis of (-)-N-{[N'-(N"-Fmoc-L-Isoleucinyl)-3-Aminobenzoyl]-5-Amino}-2-Naphthoyl-L-Phenylalanine-t-Butyl ester (7) N-Fmoc-L-isoleucine (0.131 g, 0.37 mmol) was dissolved in CH_2Cl_2 (5 mL) and treated with oxalyl chloride (0.097 mL, 0.141 g, 1.11 mmol) and DMF (1 drop). An aliquot quenched with dry methanol indicated the complete absence of the starting acid after stirring at rt for 0.5 h. The reaction was concentrated *in vacuo* and the resulting residue was used without further purification. (For larger scale syntheses, the acid chloride was purified by recrystallization from CH_2Cl_2/hexanes).

Crude N-Fmoc-L-isoleucinyl acid chloride (approximately 0.37 mmol) was taken into CH_2Cl_2 (5 mL)

and treated with pyridine (28 mL, 27 mg, 0.35 mmol); to this bright yellow homogeneous solution was added amino ester **11** (0.0734 g, 0.14 mmol) as a CH_2Cl_2 solution (2 mL). After stirring at rt for 35 minutes, tlc analysis showed the desired product along with some high R_f (minor N deprotection) and close running lower R_f impurites (possible epimerized by-products).

The reaction was diluted with water (10 mL) and saturated NaCl (5 mL) and extracted with EtOAc (2 x 25 mL); the combined organic layers were washed with saturated $NaHCO_3$ (1 x 25 mL), 5 % HCl (1 x 25 mL), brine (1 x 15 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*.

The crude product was first partially purified by elution with EtOAc:hexanes/4:6 on a 2 mm prep tlc plate to remove the high R_f impurities. The residue collected from this chromatography was then purified by radial chromatography (2 mm radial plate eluting with MeOH:DCM/1:49) to give 0.08 g purified product (65.6 %). HPLC analysis of this material revealed a single well-resolved peak indicating the purity of the final product.TLC: R_f = 0.43 (Silica gel; MeOH:DCM/1:9).Mass Spec (low resolution; NCI^-): 844 (M^+-H), 788 (M^+-tBu), 622 (M^+-Fmoc), 592, 566. The parent ion did not have the necessary intensity to run high resolution mass analysis.^{1}H NMR (ppm): 0.90 - 0.95 (t, 3H, -$CH_2\underline{CH_3}$), 1.00 - 1.02 (d, 3H, $\underline{CH_3}$CH-), 1.48 (s, 9H, -$CO_2C(\underline{CH_3})_3$), 1.58 - 1.72 (br m, 2H, -$\underline{CH_2}CH_3$), 1.88 - 2.20 (br m, 1H, -$\underline{CH}CH_2$-), 3.21 - 3.34 (m, 2H, -$CH\underline{CH_2}$Ar), 4.12 - 4.16 (t, 1H, -$\underline{CH}$-CH_2O-), 4.28 - 4.36 (m, 2H, -$CH\underline{CH_2}$O-), 4.41 - 4.47 (m, 1H, HN-$\underline{CH}$-CO), 4.99 - 5.05 (q, 1H, -$\underline{CH}CH_2$Ar), 5.95 - 6.05 (br s, 1H, -$\underline{H}$NFmoc), 7.18 - 7.85 (m, 22H, all aromatic protons (except H_1) and Ar-CO-N$\underline{H}$-), 8.14.

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