Supporting Information

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SI Materials and Methods

General Coupling Protocol for Isotopic Peptides. Six isotopic peptides (Table S1) were synthesized for kinetic isotope effect (KIE) analysis. The reference (light isotope, remotely radiolabeled) peptides were acetylated with either [^{14}C]- or [^{3}H]-Ac_2O. The carbonyl carbon, amide nitrogen, α-hydrogen, and carbonyl oxygen corresponding to the scissile peptide bond were isotopically substituted by incorporation of Fmoc-[1-^{14}C]-Phe-OH, Fmoc-[^{15}N]-Pro-OH, Fmoc-[2-^{3}H]-Phe-OH, and Fmoc-[^{18}O]-Phe-OH, respectively. The ^{15}N and ^{18}O peptides were remotely radiolabeled via acetylation with [^{3}H]-Ac_2O.

Peptides were synthesized on cross-linked ethoxylate acrylate resin (CLEAR)-amide resin (100–200 mesh 0.43 mmol/g). Coupling was achieved by reacting each amino acid with 1H-Benzotriazolium 1-[bis(dimethylamino)methylene]-5chloro-,hexafluorophosphate (1-),3-oxide (HCTU); 6-Chloro-1-Hydroxybenzotriazole (6-Cl-HOBt); and N,N-diisopropylethylamine (DIPEA) in N,N-Dimethylformamide (DMF) for 0.5–2 h at room temperature. Fluorenylmethoxycarbonyl (Fmoc) deprotection was accomplished at each step by reacting the growing peptide chain with 30% piperidine in DMF for 20 min. Between each coupling and deprotection step, the resin was washed three times sequentially with DMF, isopropanol, and methylene chloride. After the final amino acid coupling and Fmoc deprotection, each peptide was acetylated by reacting the peptidyl-resin with Ac_2O and DIPEA for 0.5–3 h.

Amino acids bearing stable isotopes were coupled with three equivalents (eq) amino acid, 2.9 eq HCTU, 2.9 eq 6-Cl-HOBt, and 5.8 eq DIPEA. Radiolabeled amino acids were coupled with 1.1 eq amino acid (80–125 μCi), 1.1 eq HCTU, 1.1 eq 6-Cl-HOBt, and 2.2 eq DIPEA. The ^{14}C-remotely labeled peptide was acetylated with 1.2 eq [^{14}C]-Ac_2O(50–250 μCi) and 2.4 eq DIPEA. ^{3}H-remotely labeled peptides were acetylated with 1.5 eq [^{3}H]-Ac_2O (1–5 mCi) and 3 eq DIPEA. Peptides not requiring a remote label were acetylated with 10 eq Ac_2O and 20 eq DIPEA.

Amino acid side chain deprotection and cleavage of the peptide from the resin were achieved by reacting the peptidyl-resin with 95:2.5:2.5 trifluoroacetic acid (TFA), H_2O, and triisopropylsilane (TIS). The peptides were filtered from the resin, precipitated with ice-cold diethyl ether, and then centrifuged. The precipitates were air dried and purified by semipreparative reverse-phase HPLC using a linear gradient (3–40% acetonitrile in water with 0.1% TFA over 40 min). The peak corresponding to the desired peptide was collected, frozen, and lyophilized. The purified nonradioactive peptides were confirmed by electrospray ionization mass spectrometry (MS-ESI) to be 831 Da, and radioactive peptides were confirmed by coelution with the nonradioactive peptide using analytical reverse-phase HPLC over the same linear gradient.

Detailed Synthesis of Fmoc-[^{18}O]-Phe-OH. Phenylalanine (40 mg), dried with P_2O_5 overnight, was dissolved in 1.0 ml $H_2{}^{18}O$ (97%). Dry HCl gas—generated by reacting 1 mg NaCl with H_2SO_4 (dropwise)—was slowly bubbled into the solution, and the vial was sealed with a Teflon-line cap. The exchange solution was placed in an oil bath at 60–65 ° C for 24 to 72 h. A measure of 10 μL aliquots were removed at various time intervals to follow the extent of oxygen exchange by MS-ESI. Final incorporation of ^{18}O into Phe-OH was measured to be 93.4% and 6.5% into 170 Da ([$^{18}O_2$]-Phe-OH) and 168.01 Da ([^{18}O, ^{16}O]-Phe-OH) isotopes, respectively, corresponding to a total ^{18}O incorporation of 96% Once completed, the obtained [$^{18}O_2$]-Phe-OH was stored with P_2O_5 in a vacuum to keep dry.

Fmoc protection of [$^{18}O_2$]-Phe-OH was carried out by dissolving the amino acid in 1.0 ml $H_2{}^{18}O$ (97%), followed by addition of $NaHCO_3$ to bring the reaction pH to 9.0. Fmoc-Osu (121 mg, 1.5 eq) was dissolved in 1.5 mL cold acetone and added slowly to the amino acid solution while stirring on ice. Reaction progress was followed by thin-layer chromatography (TLC) (8:3:0.4 $CH_2Cl_3/CH_2OH/H_2O$) analysis using ninhydrin reactivity for detection, and after 2 h no [$^{18}O_2$]-Phe-OH was detected. Complete conversion to Fmoc-[^{18}O]-Phe-OH was confirmed by mass spectrometry, corresponding to a molecular weight of 833 Da. Subsequently, the reaction mixture was evaporated, washed twice with benzene, and vacuum dried.

Synthesis of Fmoc-[2-^{3}H]-Phe-OH. [2-^{3}H]-Phe-OH was synthesized by reductive amination of phenylpyruvate through a series of enzymatic ^{3}H transfer reactions (described in the main text and illustrated in Fig. S1). The final yield of [2-^{3}H]-Phe-OH was determined to be approximately 80 μCi—based on scintillation counting of a small aliquot—corresponding to an estimated chemical yield of 1.0 μmole. The purified [2-^{3}H]-Phe-OH was vacuum dried and dissolved in 1.0 mL water for subsequent Fmoc protection.

For Fmoc protection, 2 mg of H-Phe-OH were added to the 1.0 mL [2-^{3}H]-Phe-OH solution. Then 5.25 mg of $NaHCO_3$ was added to bring the reaction pH to 9.0. Fmoc-Osu (3.7 mg, 1.1 eq) in acetone at 5 °C was then added to the amino acid mixture and stirred on ice for 1 h, and the solution was allowed to warm to room temperature overnight. The reaction was followed by TLC analysis and ninhydrin reactivity. Once the reaction was complete, the solution was evaporated, rinsed twice with benzene, and vacuum dried.

GAMT-NEDT Reaction Buffer for KIE Measurements. 50 mM glycine, 50 mM sodium acetate, 50 mM Tris-HCl, pH 6.0, 0.2 M NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100 (v/v).

Scintillation Counting for KIE Measurements. Scintillation counting was performed using 12 × 20 mL vial racks. A measure of 10 mL scintillation fluid was added to each sample, and samples were counted sequentially for 10 min, each over ten cycles of counting. Each counting cycle included (*i*) a water control (blank), (*ii*) a ^{14}C-remote-labeled substrate peptide (15,000 cpm), (*iii*) unreacted heavy/light mixture (R_0), (*iv*) approximately 70% reacted heavy/light mixture (R_f), (*v*)-(*n*)…replicates of (*iii*) and (*iv*) for *n* reactions. To correct for overlap of signals between ^{3}H and ^{14}C channels, the ratio of signal of the ^{14}C-remote-labeled substrate in channel 1 to channel 2 was determined [Eq. **S1**]:

$$r = \text{channel 1}/\text{channel 2}. \qquad \textbf{[S1]}$$

Corrected values for ^{3}H and ^{14}C cpm were then obtained from Eqs. **S2** and **S3**:

$$\text{cpm } {}^{3}\text{H} = \text{channel 1} - \text{channel 2} \times r \qquad \textbf{[S2]}$$

$$\text{cpm } {}^{14}\text{C} = \text{channel 2} \times (1 + r). \qquad \textbf{[S3]}$$

Tables of Calculated Isotope Effects from the Fixed Parameter Methods.

Tables S1–S7 give examples (from the many exploratory structures calculated) of the redundancies in the predicted isotope effects (IEs) for the mechanism of HIV protease. The IEs were calculated from theoretical structures using our own *n*-layered integrated molecular orbital and molecular mechanics (ONIOM) (M06-2X/6-31+G**:am1) and (B3LYP/6-31G*:am1) methods in Gaussian 09. The model systems are similar to those shown in the manuscript. The bond making/breaking distance for each step of the reaction was varied, and the grid of IEs is shown below. IEs with the heading KIE are derived from a structure with one imaginary frequency, and those shown under equilibrium isotopic effect (EIE) are derived from structures with no negative frequencies. Intermediates and transition structures for the protonation of the amide and scissile bond breaking are electronically similar; therefore, a grid of predicted IEs using the fixed parameter method gives several matches to the experimental values. To better explore the system, the enzyme pocket was included in the system and the transition structure was calculated without imposing constraints. Unconstrained structures were used for the current study, and IEs are shown in Table 1 of the main text.

Geometries for Structures from Fig. 3

Structure **8**—attack of water on the carbonyl

ONIOM (M06-2X/6-31+G**:am1)
m06BBts1uncon
E(RAM1) = −1.10909212843
E(RM062X) = −1391.31252779
E(RAM1) = −0.657278136005
Zero-point correction = 0.847231 (Hartree/Particle)
Thermal correction to energy = 0.904747
Thermal correction to enthalpy = 0.905691
Thermal correction to Gibbs free energy = 0.749790
Sum of electronic and zero-point energies = −1390.917111
Sum of electronic and thermal energies = −1390.859595
Sum of electronic and thermal enthalpies = −1390.858650
Sum of electronic and thermal free energies = −1391.014552
E (Thermal) CV S
KCal/Mol Cal/Mol-Kelvin Cal/Mol-Kelvin
Total 567.737 208.677 328.122
C,0,3.2021921446,0.497159343,3.4795558309
C,0,3.7546027942,1.7362993951,2.7928538076
O,0,4.0170691655,2.7752047814,3.433181906
C,0,1.7055026577,0.4796697228,3.4590629443
C,0,1.0611322799,0.1603806038,2.096774233
O,0,1.7703070488,−0.0881724735,1.0911172549
O,0,−0.2057574016,0.1836041289,2.1020075747
N,0,4.0136474368,1.6353869772,1.4337114938
C,0,4.3041347785,2.7735249585,0.6268044692
C,0,3.1754685588,3.3621417447,−0.232039206
O,0,3.3719379575,4.4867756622,−0.7562510903
N,0,1.9963624833,2.6907066825,−0.4348531816
C,0,0.9933251134,3.1968757004,−1.3226128748
C,0,0.057766165,4.2646630643,−0.728692657
O,0,−1.1820655726,4.14986163,−0.8704891443
N,0,0.6100338353,5.3479412909,−0.0967000781
C,0,−0.1710704506,6.3972928681,0.473093489
C,0,3.1909957407,−0.5911099835,−3.4399256062
C,0,4.0548276887,−1.2392101958,−2.3712947914
O,0,5.1413477156,−1.7814016516,−2.6656333577
C,0,1.8910337877,−1.310191749,−3.6204948238
C,0,0.8429121497,−0.9729939082,−2.5539054686
O,0,0.9421878646,0.122467774,−1.9415191938
O,0,−0.0787680214,−1.8204107212,−2.3955701419
N,0,3.6083097463,−1.171457407,−1.0662750341
C,0,4.2469845384,−1.8881024247,−0.0098022237
C,0,3.5162555264,−3.1577835631,0.4635167219
O,0,4.1477507921,−4.2240357669,0.6130607104
N,0,2.1721663628,−3.0604957276,0.7516499271
C,0,1.4323587429,−4.1587044919,1.2860751996
C,0,0.1940426925,−4.6162070022,0.5018140178
O,0,−0.6378476558,−5.3373387142,1.1090742982
N,0,0.0376968789,−4.2981581036,−0.818114968
C,0,−1.1231752386,−4.6763299201,−1.5662304233
C,0,−4.4090024751,3.9310777616,−0.8114248278
C,0,−4.4232634459,2.479484808,−1.2332711424
O,0,−5.4849013739,1.886277272,−1.4286282553
N,0,−3.2141688541,1.8816657317,−1.3319622709
C,0,−3.0516314816,0.4855710342,−1.6685288885
C,0,−2.4356650671,−0.32374254,−0.5162835132
C,0,−2.2957618055,0.3389500166,−2.985411597
O,0,−2.0264271839,−1.5499377626,−0.8231903428
O,0,−0.9383126602,0.6303772011,−0.2889459766
N,0,−3.063897619,−0.2849655208,0.692205596
C,0,−2.9766916144,−1.43818069,1.5830768027
C,0,−3.7140035597,−2.6439950831,1.0070069531
O,0,−4.7795938551,−2.5393124648,0.4119835847
N,0,−3.169867048,−3.8448836629,1.3115416362
O,0,−6.3544145166,−0.1167623954,0.5482838725
H,0,1.2975464045,1.441431421,3.802467527
H,0,1.3355162173,−0.2706117298,4.1732186604
H,0,3.6011020713,−0.4332730065,2.9939154109
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H,0,1.4778366283,3.6627081075,−2.2299774446
H,0,0.3376357137,2.3349644768,−1.6510172647
H,0,1.8466579282,1.7643416954,−0.0810664854
H,0,1.6017212037,5.4435503244,−0.0689883269
H,0,1.4477136162,−1.0190321245,−4.584835511
H,0,2.0267465969,−2.397750907,−3.6566598819
H,0,3.0011080369,0.482473093,−3.1674768145
H,0,5.2786813998,−2.2109331952,−0.3359930828
H,0,2.7324872208,−0.725759238,−0.8686689602
H,0,1.0586694741,−3.8982629277,2.3201451491
H,0,2.1024806993,−5.0657089112,1.3694434523
H,0,1.730503633,−2.163168253,0.7350673977
H,0,0.6096326977,−3.5963496536,−1.2381130894
H,0,−1.9280426634,−1.6808966939,1.7963129561
H,0,−2.2000157272,−3.92347085,1.5869985264
H,0,−4.0600034568,0.0686576559,−1.7718594325
H,0,−1.2512364097,−1.586818991,−1.5076200186
H,0,−0.169020822,0.3823318518,−0.9177046892
H,0,−6.1899163532,0.4684214686,−0.2058000097
H,0,−5.8130343178,−0.9093448056,0.3941448288
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H,0,−2.2167356811,−0.7060572444,−3.2887467994
H,0,−5.1504598919,4.4725450147,−1.40032966
H,0,−4.7127956446,3.9735165604,0.2389421051
H,0,−3.4251342727,4.398510904,−0.9161974077
H,0,−3.6011100607,−4.6626138019,0.905845086
C,0,−3.4082395118,0.9547111246,1.4194574845
H,0,−2.6849312344,1.7386683057,1.1817159474
H,0,−4.4242857641,1.2709967675,1.1589577813
C,0,−3.3169423709,0.5369269963,2.8896128524
H,0,−3.9896977118,1.1248894361,3.519992449
H,0,−2.2860356885,0.6538525492,3.2332167168
C,0,−3.6895118231,−0.9440521918,2.8516186563
H,0,−4.7720743847,−1.059266189,2.7217288453
H,0,−3.3681633561,−1.5026367412,3.7343080461
H,0,−1.867585715,−5.1991637327,−0.9089033489

H,0,−0.8203916953,−5.3708288355,−2.3958096871
H,0,−1.5993194949,−3.7607986858,−2.0148425
H,0,−1.2626503096,6.1374792783,0.4344770098
H,0,0.1294889064,6.5580032027,1.5438212047
H,0,−0.0072687378,7.3566034996,−0.0898290436
H,0,4.3323078576,−1.2147602811,0.8929562887
H,0,5.1221574386,2.4975653262,−0.1021089806
H,0,3.7785370715,−0.611925587,−4.3963067848
H,0,3.5682348972,0.5197611152,4.5409469626
H,0,−2.3661370682,2.4077833124,−1.1353190722
H,0,−0.6317967088,0.4147301779,0.6713816273
Structure **9**—*gem*-diol intermediate
ONIOM (M06-2X/6-31+G**:am1)
m06BBpxfer115diolint.2
E(RAM1) = −1.14900902578
E(RM062X) = −1391.33450508
E(RAM1) = −0.707329611739
Zero-point correction = 0.848601 (Hartree/Particle)
Thermal correction to energy = 0.906855
Thermal correction to enthalpy = 0.907799
Thermal correction to Gibbs free energy = 0.749748
Sum of electronic and zero-point energies = −1390.927584
Sum of electronic and thermal energies = −1390.869330
Sum of electronic and thermal enthalpies = −1390.868385
Sum of electronic and thermal free energies = −1391.026436
E (Thermal) CV S
KCal/Mol Cal/Mol-Kelvin Cal/Mol-Kelvin
Total 569.060 209.926 332.646
C,0,2.0187893222,3.1660920278,2.6525221658
C,0,1.6852313853,4.1699811871,1.5596292892
O,0,1.7437827533,5.3977737558,1.785609326
C,0,0.8102225893,2.4225250394,3.1257685202
C,0,0.4643925737,1.2213269133,2.2459522563
O,0,1.2432822964,0.7484914514,1.4314915807
N,0,1.3382966544,3.6730878236,0.3224909735
C,0,1.0031732933,4.5274026144,−0.7728889067
C,0,−0.4799024953,4.4745544903,−1.1823770181
O,0,−1.1694384826,5.519089056,−1.2032810156
N,0,−0.9962643989,3.2637362219,−1.5726091361
C,0,−2.3600933067,3.0963568975,−1.961037246
C,0,−3.3507651694,2.9594048988,−0.7887230052
O,0,−4.0538129545,1.9297776314,−0.686278317
N,0,−3.4527522544,4.0115888391,0.0845706882
C,0,3.9509584012,1.8992503289,−2.2303376075
C,0,4.7430495136,1.3236452659,−1.0686810016
O,0,5.9856966372,1.2119748188,−1.1225698492
C,0,3.1216342647,0.8470247263,−2.9037635105
C,0,1.9692751684,0.3592407412,−2.0073535104
O,0,1.2685425667,1.2412934644,−1.4362068916
O,0,1.8115716118,−0.8812326808,−1.9020246795
N,0,4.033523867,0.9709801308,0.066945139
C,0,4.6269775937,0.2173303947,1.1227021755
C,0,4.2842194063,−1.2809637729,1.1759303309
O,0,5.1738170114,−2.1004486855,1.5054030644
N,0,2.9933355646,−1.6877888612,0.9503497504
C,0,2.5855064509,−3.0440391172,1.1421552379
C,0,2.802016147,−3.9878183979,−0.0539576433
O,0,1.8483269031,−4.6784915426,−0.4757239551
N,0,4.0712083166,−4.0853547799,−0.5671950083
C,0,4.3750040075,−4.8381452838,−1.7404923364
C,0,−5.5363380526,−0.4956830156,−2.0308455269
C,0,−4.3716849175,−1.455136243,−1.9632127912
O,0,−4.5405829668,−2.668620972,−2.1226607577
C,0,−2.0240125385,−1.7787027663,−1.364637062
C,0,−1.1418072064,−1.0983558304,−0.277220635
C,0,−1.221277096,−2.0734367578,−2.6298986446
O,0,0.0977296159,−1.7071346555,−0.1813917074
O,0,−1.0610966859,0.2598372956,−0.6021575008
N,0,−1.7024242189,−1.1717957543,1.0981896655
C,0,−1.4109094651,−2.3672008523,1.9224830253
C,0,−1.8871846791,−3.6803040835,1.2825291417
O,0,−3.0227660711,−4.1140399451,1.4753454638
C,0,−2.1960639122,−2.067525605,3.2099989142
N,0,−0.9798343413,−4.3310066904,0.5284234693
O,0,−5.6357284866,−3.2686494662,0.4932796476
H,0,−0.0675862894,3.0774496575,3.1987553813
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H,0,1.3384032937,2.6834270388,0.1618571697
H,0,−2.6888284405,3.9737202295,−2.589381845
H,0,−2.4453331219,2.146943475,−2.5645079547
H,0,−0.4409556916,2.4364778827,−1.48499966
H,0,−2.8611584812,4.8042302107,−0.0419558786
H,0,2.6726082272,1.2842992087,−3.8070405941
H,0,3.7343443952,−0.0095385517,−3.2082973263
H,0,3.2859685727,2.7267814666,−1.8606404044
H,0,5.7520688367,0.2888391153,1.054447756
H,0,3.0337059533,1.0326694666,0.0482859797
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H,0,3.1381012042,−3.4862534505,2.021161652
H,0,2.3201315584,−1.0675807694,0.5431900189
H,0,3.4788372918,−5.4320066496,−2.0641152385
H,0,4.7845209108,−3.4950348094,−0.1987378764
H,0,−6.1364295523,−0.6619535099,−1.1300409059
H,0,−1.536672119,−1.5484478418,3.9094473951
H,0,−0.3333752825,−2.4105724584,2.0873045582
H,0,−0.1381569956,−3.857739993,0.2089649389
H,0,−2.4337147325,−2.7170974578,−0.9751380509
H,0,−0.7528334429,−1.1590154231,−3.0048973567
H,0,−0.4330074116,−2.8058567388,−2.4386213781
H,0,0.7201328459,−1.3939929901,−0.9194674947
H,0,−0.1179376889,0.5514418909,−0.7387130145
H,0,−5.4090186535,−3.2854520316,−0.4494902954
H,0,−4.8549210584,−3.6369589469,0.9331106667
C,0,−3.1443180195,−0.8972637679,1.2799521451
H,0,−3.3589105553,0.1299772555,0.9604204875
H,0,−3.7650235755,−1.5890071653,0.6998103848
C,0,−3.3626788818,−1.1441932772,2.7794819063
H,0,−4.3362817894,−1.6124309022,2.9423873464
H,0,−3.3276175555,−0.2044157354,3.3363123762
O,0,−0.7362419058,0.7589176082,2.4968623946
H,0,−1.0303521598,−0.0332320393,1.850262179
H,0,−6.1489472134,−0.738221027,−2.9003641912
H,0,1.6119278443,4.2286347009,−1.6758033258
H,0,4.2881189022,0.6482055592,2.1102988269
H,0,5.2247588238,−5.5422836868,−1.5282499878
H,0,−2.5488637104,−2.9907748319,3.6727258285
N,0,−3.1652493119,−0.9264596281,−1.6736373798
H,0,−3.0660136874,0.0687418439,−1.495213585
H,0,−5.2269462437,0.5528975904,−2.0599703614
H,0,2.4638902462,3.7476222042,3.5041556456
H,0,4.6882942702,2.3363460692,−2.9542632181
C,0,−4.2859589024,3.981201607,1.2428299014
H,0,−4.8445918043,3.0086050213,1.296475055
H,0,−3.6648102758,4.0957652572,2.1724503147
H,0,−5.0260569432,4.8261201706,1.2032511633
H,0,−1.9036362406,−2.470700361,−3.3866254047
H,0,−1.2886805011,−5.1580596636,0.0375486059
H,0,4.6796850443,−4.1556270184,−2.5801537491
Structure **10**–proton transfer from Asp25 to proline
ONIOM (M06-2X/6-31+G**:am1)
onpxferm06BBgp
E(RAM1) = −1.13096461452
E(RM062X) = −1391.32878389
E(RAM1) = −0.684577621041

Zero-point correction = 0.845716 (Hartree/Particle)
Thermal correction to energy = 0.903571
Thermal correction to enthalpy = 0.904516
Thermal correction to Gibbs free energy = 0.747134
Sum of electronic and zero-point energies = −1390.929455
Sum of electronic and thermal energies = −1390.871599
Sum of electronic and thermal enthalpies = −1390.870655
Sum of electronic and thermal free energies = −1391.028037
E (Thermal) CV S
KCal/Mol Cal/Mol-Kelvin Cal/Mol-Kelvin
Total 567.000 208.847 331.238
C,0,2.0901226619,3.0988660876,2.6387860746
C,0,1.7835566831,4.1126460253,1.5484669804
O,0,1.8807932396,5.3391769979,1.770424724
C,0,0.8657425882,2.3727752294,3.0981865132
C,0,0.4847526532,1.1852803326,2.2026012743
O,0,1.2766605588,0.7244057288,1.3764314564
N,0,1.4198418722,3.6262192471,0.3109863313
C,0,1.1023235801,4.4902672177,−0.7808007992
C,0,−0.3839910491,4.4753854384,−1.1818555721
O,0,−1.0611311548,5.5276759057,−1.1628255834
N,0,−0.9197741327,3.2855100446,−1.6126516871
C,0,−2.2906715304,3.1510587624,−1.9878364813
C,0,−3.2659275149,2.9761488141,−0.8075968537
O,0,−3.980970057,1.9509527278,−0.7400878818
N,0,−3.342390491,3.990357994,0.1109431891
C,0,3.9967809079,1.7885260745,−2.254762021
C,0,4.7711332339,1.2037914364,−1.0853786191
O,0,6.0113985691,1.0630515572,−1.1360260156
C,0,3.149653857,0.7487353414,−2.9252245661
C,0,1.9763317844,0.3019729849,−2.0358312132
O,0,1.2954019411,1.2061777195,−1.4794657995
O,0,1.7827233382,−0.9347004401,−1.9227490323
N,0,4.0497471383,0.8734806485,0.0475057917
C,0,4.6241196861,0.1256999818,1.1177606585
C,0,4.2655696098,−1.3690964394,1.1698374029
O,0,5.1536017779,−2.2067410773,1.4544387994
N,0,2.96158359,−1.7544649981,0.9834012241
C,0,2.5442247251,−3.1086732697,1.1649903802
C,0,2.7039021636,−4.0285840941,−0.0589336085
O,0,1.7245713378,−4.6945057646,−0.4629197488
N,0,3.9531003515,−4.1356893853,−0.6163020632
C,0,4.2019141841,−4.857648535,−1.8218129364
C,0,−5.5253340301,−0.3796954438,−2.0892774771
C,0,−4.4115400066,−1.3904892847,−1.9514436743
O,0,−4.6400138033,−2.6024140181,−2.0215591009
C,0,−2.0709551945,−1.7829460887,−1.3609793617
C,0,−1.1429211164,−1.0793433372,−0.330207514
C,0,−1.288530024,−2.175291926,−2.6128541667
O,0,0.0731916011,−1.7188939613,−0.2137629957
O,0,−1.0378089113,0.2534454096,−0.7067474644
N,0,−1.6990368312,−1.0826701436,1.0709070843
C,0,−1.4294948273,−2.2626628481,1.9399162688
C,0,−1.9341908963,−3.5822859506,1.3363360897
O,0,−3.0745993474,−3.9880771668,1.5556355226
C,0,−2.2062070953,−1.9050780505,3.2174051851
N,0,−1.0444568308,−4.2721298493,0.5994011041
O,0,−5.6784068145,−3.0558754448,0.6458044709
H,0,0.0023872109,3.0455706735,3.1826480594
H,0,1.0334735129,1.9652547598,4.1063434104
H,0,2.8558738407,2.3662563219,2.2618395689
H,0,1.3559121462,5.5577299609,−0.5140499158
H,0,1.3784229682,2.6357340765,0.1570165818
H,0,−2.6165744059,4.0586923326,−2.5731915613
H,0,−2.3970588493,2.230023645,−2.6302280696
H,0,−0.3803632948,2.4466486252,−1.5399976313
H,0,−2.7357366954,4.77614504,0.0142203715
H,0,2.7212007106,1.1852528879,−3.8387805296
H,0,3.7442975565,−0.1262826567,−3.212450833
H,0,3.3473699049,2.6323474539,−1.894122385
H,0,5.7504282949,0.1857973909,1.0629438769
H,0,3.0519220486,0.9694029416,0.0337746354
H,0,1.4456230798,−3.1031929231,1.4198780209
H,0,3.1255249621,−3.5759961122,2.0121859339
H,0,2.2792679209,−1.1109570923,0.6299898848
H,0,3.2923847006,−5.4456556625,−2.1173927547
H,0,4.6857700678,−3.5587501435,−0.2640215302
H,0,−6.1275495151,−0.4385995575,−1.1767677699
H,0,−1.5356898277,−1.3739897396,3.8953152192
H,0,−0.3516842031,−2.3115249236,2.0976812378
H,0,−0.1974901061,−3.8241426198,0.2564677785
H,0,−2.5137863362,−2.6819580063,−0.9183426851
H,0,−0.7807665182,−1.3018678509,−3.0318286708
H,0,−0.5324732835,−2.9311331021,−2.3857464151
H,0,0.7204500898,−1.4123337812,−0.9453936177
H,0,−0.0857103551,0.5508929541,−0.7871451846
H,0,−5.4905910503,−3.1123597659,−0.3039217738
H,0,−4.9071762577,−3.4635015016,1.0670237341
C,0,−3.1415047964,−0.7720312949,1.2555586313
H,0,−3.331450665,0.24833457,0.9026163986
H,0,−3.7631934098,−1.4749373352,0.694041582
C,0,−3.3559774477,−0.9722088108,2.7614320261
H,0,−4.3384983081,−1.416678522,2.9367994228
H,0,−3.2971326755,−0.0179667343,3.2894330135
O,0,−0.702600691,0.7367847778,2.4342734443
H,0,−1.1028601427,−0.1482945609,1.6805049958
H,0,−6.1560417317,−0.6561139141,−2.9352754476
H,0,1.6995069414,4.1782311682,−1.6869583042
H,0,4.2748734282,0.5665040125,2.0971711094
H,0,5.0619145541,−5.5641214236,−1.6667970836
H,0,−2.5760625326,−2.8079005521,3.7058576227
N,0,−3.1787304851,−0.9016760784,−1.7044897204
H,0,−3.0302407353,0.0976918125,−1.597316729
H,0,−5.1600689809,0.6448737426,−2.2016641177
H,0,2.542154634,3.6673882526,3.4951191802
H,0,4.7465776476,2.2049799274,−2.978011307
C,0,−4.1438639527,3.9111856205,1.2896140128
H,0,−4.7114444672,2.9425586105,1.3112488507
H,0,−3.4965370121,3.9738711976,2.2062180516
H,0,−4.875306005,4.7641724555,1.310420914
H,0,−1.9908378082,−2.5785071354,−3.3476135925
H,0,−1.3688311578,−5.1137842168,0.1445910372
H,0,4.4652115072,−4.1538113847,−2.6579002658

Structure **11**—protonated amide intermediate
ONIOM (M06-2X/6-31+G**:am1)
m06BBprotamideint
E(RAM1) = −1.12461532094
E(RM062X) = −1391.32412580
E(RAM1) = −0.672861955514
Zero-point correction = 0.848969 (Hartree/Particle)
Thermal correction to energy = 0.906976
Thermal correction to enthalpy = 0.907920
Thermal correction to Gibbs free energy = 0.750712
Sum of electronic and zero−point energies = −1390.926910
Sum of electronic and thermal energies = −1390.868903
Sum of electronic and thermal enthalpies = −1390.867959
Sum of electronic and thermal free energies = −1391.025167
E (Thermal) CV S
KCal/Mol Cal/Mol-Kelvin Cal/Mol-Kelvin
Total 569.136 209.360 330.873
C,0,2.207306525,3.0776066102,2.6437227331
C,0,1.9245303285,4.0846002959,1.542246621
O,0,2.0756059244,5.3106604739,1.7378095719
C,0,0.9658071871,2.3939384376,3.1217366911
C,0,0.5272257914,1.2050708769,2.2439488996
O,0,1.307420437,0.733399232,1.3966581201

N,0,1.5238288252,3.5932463231,0.3169815438
C,0,1.2264337343,4.4537912185,−0.7822014321
C,0,−0.2628223531,4.4824049414,−1.1713237825
O,0,−0.9189833908,5.5466811231,−1.1256067572
N,0,−0.8269965399,3.3132224966,−1.6261277555
C,0,−2.2051956275,3.2155572242,−1.9850752244
C,0,−3.1674355468,3.0257104695,−0.796462424
O,0,−3.904417369,2.0138009988,−0.7512749721
N,0,−3.2092638116,4.0112723884,0.1537450798
C,0,4.0217721622,1.670281839,−2.2430690842
C,0,4.7713134662,1.0650418034,−1.0674385376
O,0,6.0063235067,0.8800859947,−1.1154939755
C,0,3.1551494832,0.6528104157,−2.9220124634
C,0,1.9609419122,0.234406941,−2.0486329271
O,0,1.3112024463,1.1496949474,−1.4747904556
O,0,1.7153990678,−0.996962418,−1.9689056055
N,0,4.0346085315,0.7645391935,0.0615339246
C,0,4.581788187,0.0227685703,1.1499136944
C,0,4.1957487208,−1.4650801343,1.2057893553
O,0,5.0718217453,−2.3249292885,1.4597208897
N,0,2.8801038352,−1.8218538325,1.0436645659
C,0,2.4429925502,−3.1707127682,1.2148878633
C,0,2.5659711742,−4.0752476133,−0.0252589002
O,0,1.5677806806,−4.7191025606,−0.4209616493
N,0,3.8020007335,−4.1958848122,−0.6067014293
C,0,4.0160672369,−4.9044836174,−1.826973796
C,0,−5.5485607922,−0.2383104337,−2.1069120818
C,0,−4.473686168,−1.2875153819,−1.9487773083
O,0,−4.7485273245,−2.4912685027,−1.979938216
C,0,−2.1416398465,−1.7529841804,−1.3781472627
C,0,−1.1727668934,−1.0562309918,−0.384573825
C,0,−1.3859008248,−2.2082572826,−2.6248823109
O,0,0.0176197675,−1.7294194127,−0.253308837
O,0,−1.0369034157,0.2610907301,−0.7751123011
N,0,−1.7299833586,−1.019156436,1.0364780624
C,0,−1.4897327951,−2.1933078982,1.9335971854
C,0,−2.0418552627,−3.501241417,1.3479198351
O,0,−3.1953594859,−3.8604219101,1.5767081466
C,0,−2.2451772576,−1.7857127427,3.2085310399
N,0,−1.1770870537,−4.2327293454,0.6246121155
O,0,−5.7714501389,−2.8257983586,0.7049147363
H,0,0.1257770515,3.0952472871,3.2111366059
H,0,1.1332944088,1.9917535168,4.1322227749
H,0,2.9435591163,2.314989926,2.2677407035
H,0,1.5159154988,5.5157137169,−0.5296307283
H,0,1.427694214,2.6024668057,0.1868372281
H,0,−2.5207392755,4.1457757646,−2.5398212056
H,0,−2.3386353386,2.3157830855,−2.6518012017
H,0,−0.30933782,2.4607825748,−1.5581771283
H,0,−2.5820997788,4.7832883003,0.0756992322
H,0,2.7475754557,1.098638324,−3.8407250314
H,0,3.7299213726,−0.237117476,−3.2037107639
H,0,3.391217616,2.5304500773,−1.8876237722
H,0,5.7094513415,0.0624625031,1.1111601255
H,0,3.0415796863,0.9078183455,0.0527182253
H,0,1.3502678587,−3.1546717286,1.4911063304
H,0,3.0324870479,−3.6608616635,2.0436943799
H,0,2.1966270106,−1.1491184067,0.7502904926
H,0,3.092869876,−5.4762135061,−2.1117406171
H,0,4.5508533452,−3.6354164886,−0.2610177388
H,0,−6.158993628,−0.263240571,−1.1985659634
H,0,−1.5510202379,−1.2692172519,3.8723627043
H,0,−0.412233183,−2.2634654314,2.0785721592
H,0,−0.309447595,−3.8256150941,0.2815133376
H,0,−2.6111623302,−2.6232238649,−0.9065803072
H,0,−0.8516417935,−1.3664013869,−3.0740350629
H,0,−0.6546897956,−2.9826265061,−2.3796601899
H,0,0.6835391697,−1.4383730225,−0.9865903466
H,0,−0.0737183665,0.5500947175,−0.8274044759
H,0,−5.607716723,−2.9194201633,−0.2463674582
H,0,−5.0209233603,−3.2736825529,1.1222243704
C,0,−3.1637647203,−0.6532659874,1.2358483776
H,0,−3.3207702305,0.3634901986,0.8591507535
H,0,−3.8005688914,−1.3549326916,0.6925414738
C,0,−3.3600339317,−0.8132397178,2.7472589799
H,0,−4.3590477501,−1.2085927309,2.9455273451
H,0,−3.2437957279,0.1484163439,3.250382191
O,0,−0.6471919792,0.7801396932,2.4966405519
H,0,−1.1594309154,−0.2116971939,1.5520503349
H,0,−6.1830812874,−0.5022559199,−2.9540892311
H,0,1.8067479183,4.1136639554,−1.6892223506
H,0,4.2230384483,0.4797479782,2.1183517335
H,0,4.869388389,−5.6242780797,−1.6988937554
H,0,−2.6483927688,−2.6681439314,3.7076196593
N,0,−3.2197388355,−0.8397696507,−1.7302864378
H,0,−3.0322596331,0.1558157807,−1.6515103154
H,0,−5.1446413028,0.7703348839,−2.2298384225
H,0,2.6871345674,3.6427032031,3.4868168483
H,0,4.788107213,2.0673629547,−2.9598839403
C,0,−3.9817575147,3.9039069772,1.349964951
H,0,−4.5698106577,2.9473835093,1.3500545314
H,0,−3.3098477182,3.917576308,2.2508172073
H,0,−4.6933180655,4.7707970907,1.4208021756
H,0,−2.1089962884,−2.6083732,−3.3406886177
H,0,−1.526058803,−5.0753183475,0.1902695642
H,0,4.2716509381,−4.1925334628,−2.6585476465
Structure **12**—scissile bond cleavage
ONIOM (M06-2X/6-31+G**:am1)
m06BBCNuncon
E(RAM1) = −1.14073599711
E(RM062X) = −1391.32068879
E(RAM1) = −0.696826030057
Zero−point correction = 0.849199 (Hartree/Particle)
Thermal correction to energy = 0.907243
Thermal correction to enthalpy = 0.908187
Thermal correction to Gibbs free energy = 0.750929
Sum of electronic and zero-point energies = −1390.915400
Sum of electronic and thermal energies = −1390.857356
Sum of electronic and thermal enthalpies = −1390.856411
Sum of electronic and thermal free energies = −1391.013670
E (Thermal) CV S
KCal/Mol Cal/Mol-Kelvin Cal/Mol-Kelvin
Total 569.304 208.825 330.978
C,0,1.0894572547,3.8949561378,2.0737293404
C,0,0.0611495571,4.6998227962,1.2970947095
O,0,−0.4116144514,5.7600840109,1.7589276164
C,0,0.5372367803,2.6033096692,2.5999207289
C,0,0.3148957671,1.5426286166,1.4909218708
O,0,1.2782345252,0.7869564174,1.2046802351
N,0,−0.2567509665,4.2581204668,0.0222808684
C,0,−1.2918846086,4.8744839152,−0.7453272869
C,0,−2.5273321066,4.0348533985,−1.0986844163
O,0,−3.6124178666,4.626881594,−1.3146128195
N,0,−2.4069078239,2.6798124812,−1.2934466044
C,0,−3.4948198243,1.8898051152,−1.7784967648
C,0,−4.4988164115,1.4173647701,−0.7127581065
O,0,−4.7367265681,0.1945764562,−0.5848775232
N,0,−5.1401841356,2.3742330133,0.0302008169
C,0,3.2821272209,3.3173176881,−1.421590075
C,0,4.3697226977,2.7397767518,−0.5284065338
O,0,5.5637947744,3.0776382653,−0.6757673744
C,0,2.7853559992,2.3082460514,−2.4128612042
C,0,1.7500778745,1.3505475774,−1.8229829814
O,0,0.5802535072,1.7027106743,−1.6724920371
O,0,2.2107368813,0.1691051713,−1.5457109819
N,0,3.9688379024,1.8665352003,0.4567396571

C,0,4.8972402843,1.1823703365,1.2971949777
C,0,4.9468634911,−0.3451224364,1.1384037328
O,0,6.0554569615,−0.9285734255,1.1813111104
N,0,3.774246611,−1.0524337405,1.0404012054
C,0,3.7603642116,−2.4819610525,1.0555646453
C,0,3.9766822102,−3.160117567,−0.3096051274
O,0,3.1399488778,−3.9823526894,−0.7397035711
N,0,5.1357375874,−2.8648016573,−0.9840884319
C,0,5.433055719,−3.3814130145,−2.2795873787
C,0,−5.252653366,−2.9484459356,−0.7502959183
C,0,−3.8489227036,−3.3404973616,−1.1405823619
O,0,−3.4903198878,−4.5222584103,−1.1275236333
C,0,−1.5821675041,−2.6195222374,−1.6259156479
C,0,−0.7304155304,−1.4423860723,−1.173875705
C,0,−1.2373775807,−2.9501647122,−3.0815895629
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Structure **13**—proton transfer from Asp25 to proline with $r_{(N\text{-}H)}$ bond length at 1.20 Å

ONIOM (M06-2X/6-31+G**:am1)
E(RAM1) = −1.12705151579
E(RM062X) = −1391.32678196
E(RAM1) = −0.678445029838
Zero-point correction = 0.846194 (Hartree/Particle)
Thermal correction to energy = 0.903883
Thermal correction to enthalpy = 0.904827
Thermal correction to Gibbs free energy = 0.748185
Sum of electronic and zero-point energies = −1390.929194
Sum of electronic and thermal energies = −1390.871506
Sum of electronic and thermal enthalpies = −1390.870561
Sum of electronic and thermal free energies = −1391.027204
E (Thermal) CV S
KCal/Mol Cal/Mol-Kelvin Cal/Mol-Kelvin
Total 567.195 208.493 329.682
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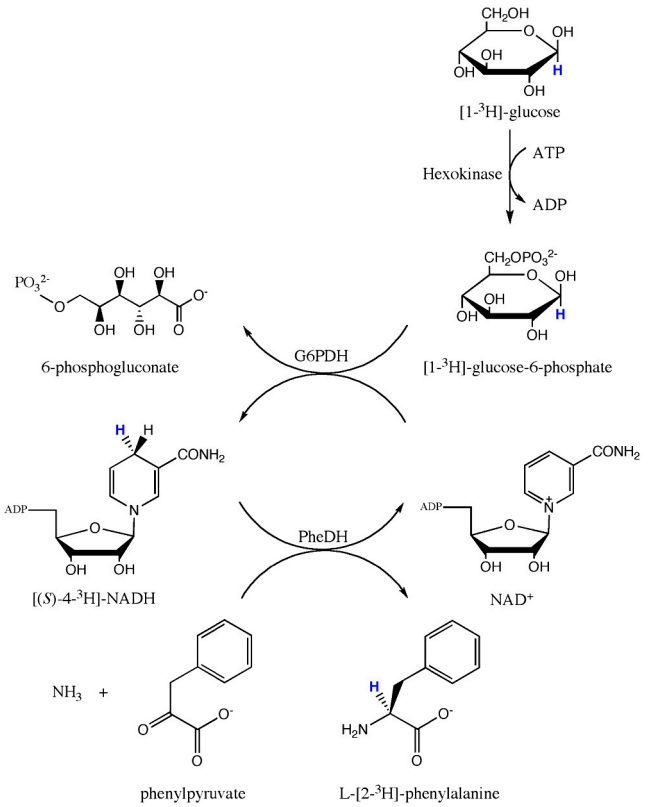

Fig. S1. Synthesis of L-[2-^{3}H]-phenylalanine. ^{3}H is transferred from [1-^{3}H]-glucose to [2-^{3}H]-Phe-OH via a sequence of ^{3}H-transfer reactions. First, ATP-dependent hexokinase catalyzes conversion of[1-^{3}H]-glucose to [1-^{3}H]-glucose-6-phosphate. ^{3}H is then transferred from [1-^{3}H]-glucose-6-phosphate to NAD^+ by glucose-6-phosphate dehydrogenase (G6PDH), reducing it to [(*S*)-4-^{3}H]-NADH and forming 6-phosphogluconate. Finally, phenylalanine dehydrogenase (PheDH) is used to produce 2-^{3}H-Phe-OH from [(*S*)-4-^{3}H]-NADH and NH_3 by reductive amination of phenylpyruvate.

Table S1. Transition state for attack of water (M06-2X/6-31+G full model system)**

	EIE	KIE	KIE	KIE	KIE	EIE	EIE	EIE
r(C-O) distance (Å)	1.7	1.8	1.9	2	2.1	2.2	2.3	2.4
[$^3H_3$]Ac-SQNF*[^{15}N]PVV-NH_2	1.001	1.000	0.998	0.997	0.997	0.998	0.998	0.998
Ac-SQN[1-^{14}C]F*PVV-NH_2	1.078	1.072	1.061	1.047	1.029	1.017	1.011	1.007
[$^3H_3$]Ac-SQN[^{18}O]F*PVV-NH_2	1.039	1.045	0.990	0.990	0.990	0.999	1.000	1.000
[$^3H_3$]Ac-SQN[^{18}O]F*PVV-NH_2	0.992	0.991	1.051	1.056	1.026	1.026	1.026	1.028
Ac-SQN[α-^{3}H]F*PVV-NH_2	0.932	0.938	0.945	0.954	0.958	0.960	0.963	0.967

KIE, kinetic isotope effect; EIE, equilibrium isotopic effect.

Table S2. *gem*-diol intermediate (M06-2X/6-31+G full model system)**

	EIE
[$^3H_3$]Ac-SQNF*[^{15}N]PVV-NH_2	1.002
Ac-SQN[1-^{14}C]F*PVV-NH_2	1.017
[$^3H_3$]Ac-SQN[^{18}O]F*PVV-NH_2	0.995
[$^3H_3$]Ac-SQN[^{18}O]F*PVV-NH_2	1.002
Ac-SQN[α-^{3}H]F*PVV-NH_2	0.922

EIE, equilibrium isotopic effect.

Table S3. Transition state for proton transfer (M06-2X/6-31+G full model system)**

	EIE	KIE	KIE	EIE	KIE
r(N-H) distance (Å)	1.1	1.2	1.3	1.4	unconstrained
[$^3H_3$]Ac-SQNF*[^{15}N]PVV-NH_2	0.992	0.995	0.998	1.000	0.996
Ac-SQN[1-^{14}C]F*PVV-NH_2	1.019	1.025	1.021	1.018	1.020
[$^3H_3$]Ac-SQN[^{18}O]F*PVV-NH_2	0.994	0.995	0.995	0.995	0.994
[$^3H_3$]Ac-SQN[^{18}O]F*PVV-NH_2	0.998	1.000	1.000	1.001	1.000
Ac-SQN[α-^{3}H]F*PVV-NH_2	0.910	0.917	0.920	0.922	0.919

EIE, equilibrium isotopic effect; KIE, kinetic isotope effect.

Table S4. Protonated amide intermediate (M06-2X/6-31+G full model system)**

	EIE
[$^3H_3$]Ac-SQNF*[^{15}N]PVV-NH_2	0.992
Ac-SQN[1-^{14}C]F*PVV-NH_2	1.019
[$^3H_3$]Ac-SQN[^{18}O]F*PVV-NH_2	0.994
[$^3H_3$]Ac-SQN[^{18}O]F*PVV-NH_2	0.998
Ac-SQN[α-^{3}H]F*PVV-NH_2	0.908

EIE, equilibrium isotope effects.

Table S5. Transition state for scissile bond breaking (M06-2X/6-31+G full model system)**

	EIE	KIE	KIE	KIE
r(C-N) distance (Å)	1.9	2.1	2.3	2.5
[$^3H_3$]Ac-SQNF*[^{15}N]PVV-NH_2	1.012	1.016	1.017	1.018
Ac-SQN[1-^{14}C]F*PVV-NH_2	1.072	1.056	1.017	0.997
[$^3H_3$]Ac-SQN[^{18}O]F*PVV-NH_2	0.994	0.993	0.998	0.999
[$^3H_3$]Ac-SQN[^{18}O]F*PVV-NH_2	0.994	0.990	0.990	0.989
Ac-SQN[α-^{3}H]F*PVV-NH_2	0.922	0.867	0.891	0.914

EIE, equilibrium isotope effect; KIE, kinetic isotope effect.

Table S6. Transition state for proton transfer (B3LYP/6-31+G* full model system)

	EIE	KIE	KIE
r(N-H) distance (Å)	1.1	1.2	1.3
[$^3H_3$]Ac-SQNF*[^{15}N]PVV-NH_2	0.996	0.999	1.000
Ac-SQN[1-^{14}C]F*PVV-NH_2	1.030	1.034	1.027
[$^3H_3$]Ac-SQN[^{18}O]F*PVV-NH_2	0.996	0.997	0.997
[$^3H_3$]Ac-SQN[^{18}O]F*PVV-NH_2	0.999	1.001	1.002
Ac-SQN[α-^{3}H]F*PVV-NH_2	0.910	0.912	0.914

EIE, equilibrium isotope effect; KIE, kinetic isotope effect.

Table S7. Transition state for scissile bond breaking (B3LYP/6-31+G* full model system)

	EIE		KIE			
r(C-N) distance (Å)	1.7	1.8	1.9	2	2.1	2.2
[$^{3}H_{3}$]Ac-SQNF*[^{15}N]PVV-NH_2	0.996	1.003	1.003	1.011	1.013	1.015
Ac-SQN[1-^{14}C]F*PVV-NH_2	1.031	1.044	1.044	1.069	1.066	1.059
[$^{3}H_{3}$]Ac-SQN[^{18}O]F*PVV-NH_2	0.996	0.996	0.996	0.998	0.996	0.995
[$^{3}H_{3}$]Ac-SQN[^{18}O]F*PVV-NH_2	0.999	0.997	0.997	0.999	0.997	0.995
Ac-SQN[α-^{3}H]F*PVV-NH_2	0.911	0.936	0.936	0.928	0.933	0.936

EIE, equilibrium isotope effect; KIE, kinetic isotope effect.