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C_2-Symmetric azobenzene-amino acid conjugates and their inhibition of Subtilisin Kexin Isozyme-1

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ABSTRACT

C_2-Symmetric azobenzene-amino acid/peptide hybrids containing stable *E*-azo moiety have been synthesized. Upon irradiation with long wavelength UV, these compounds isomerized to the *Z*-form, whose thermal reisomerization to the *E* isomer slowed down considerably. These compounds exhibited in vitro moderate to strong inhibition of mammalian cellular protease Subtilisin Kexin Isozyme-1, also called Site 1 Protease, which plays vital roles in cholesterol synthesis, lipid metabolism, bone formation, and viral infections.

Symmetry has a crucial role in many chemical and biological processes.[1] In asymmetric synthesis, C_2-symmetric catalysts play a crucial role.[2,3] There are many proteins and enzymes which are biologically active in homo dimeric form possessing C_2-symmetry. Presence of C_2-symmetry is a common feature of restriction enzymes[4] (e.g., *Hin*dIII) which enables them to recognize palindrome sequence of *ds* DNA. HIV-protease is another example of C_2-symmetric aspartyl protease enzyme which functionally active in homo dimeric form.[5] Other proteolytic enzymes have been shown to recognize substrates with C_2-symmetry or pseudo C_2-symmetry thus pointing out complementarity with the active site of the enzyme. One such family is the Proprotein Convertases (PCs), currently known as Proprotein Convertase Subtilisin/Kexin-type (PCSKs).[6,7] So far there are nine members in this family, of which seven, namely PC1/PC3 (PCSK1), PC2 (PCSK2), Furin (PCSK3), PC4 (PCSK4), PACE4 (PCSK5), PC5/PC6 (PCSK6), and PC7/PC8/LPC (PCSK7) are Kexin-type and cleave a variety of inactive precursor proteins at the consensus sequence (K/R)-X*n*-(K/R)↓, where X is any amino acid except Cys and $n = 0, 2, 4, 6$. The eighth member is a pyrolysin type and is called Subtilisin Kexin-Isozyme-1 (SKI-1)[8]/Site 1 Protease (S1P)[9]/(PCSK8) while the ninth or the last member is known as Neural Apoptosis-Regulated Convertase (NARC-1)/PCSK9 is a Proteinase K subtype.[10] While SKI-1 has been shown to cleave inactive proproteins at the motif R-X-(hydrophobic)-Z↓, Z being mostly L. In rare cases, P1 residue Z can be His (CREB-H), Arg (Luman), Lys (SKI-1, Machupo and Tacaribe viruses GPC) (REF). PCSK9 has been shown only to cleave its own prodomain at V-F-A-Q↓S-I-P site. While most PCSK enzymes exist in monomeric form, a few of them have been shown to exist also in dimeric form with enhanced enzymatic and/or functional activity in most cases.[11–13] Owing to various functional and physiological roles, PCs including SKI-1 enzyme are considered as important targets for intervention of a number of diseases including viral infections and cancer.[14,15] Since there is a pseudo C_2-symmetry around the active site (motif: Arg/Lys/His-X-Leu/Ile-Leu)[12,16] of SKI-1, substrates with C_2-symmetry are attractive targets for studying their interactions with these enzymes. Based on this idea and the preference of hydrophobic residue at P1/P2 by SKI-1, we have designed synthesized azobenzene-amino acid (or peptide) hybrids with C_2-symmetry (Fig. 1) and studied their protease inhibitory activities in vitro against SKI-1. In this paper we describe our results.

The syntheses of the target compounds **1a–d** are depicted in Scheme 1. The key steps involved the linking of the protected bromoacylated amino acid benzyl esters **3a–c** to the azobenzene via bis O-alkylation. The resulting products **2a–c** were hydrolyzed by LiOH to the corresponding free acids. The other compound **1d** containing a bis dipeptide unit was similarly prepared (Scheme 2)

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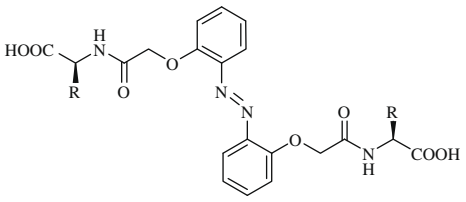
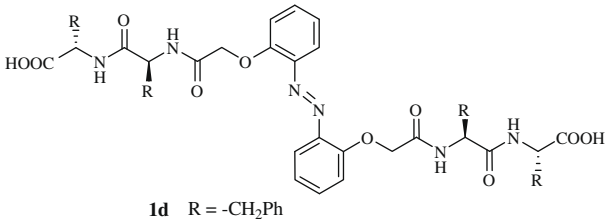


1a-c For **a**, R = -^{i}Pr, for **b**, R = CH_2Ph, for **c**, R = H

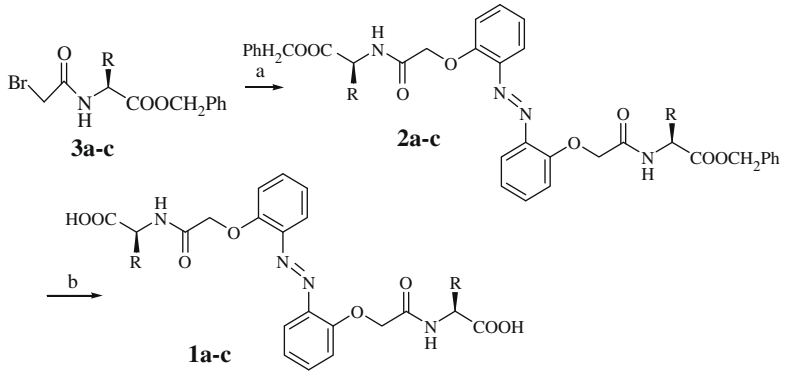


1d R = -CH_2Ph

Figure 1. Target azobenzene-amino acid/peptide hybrids.

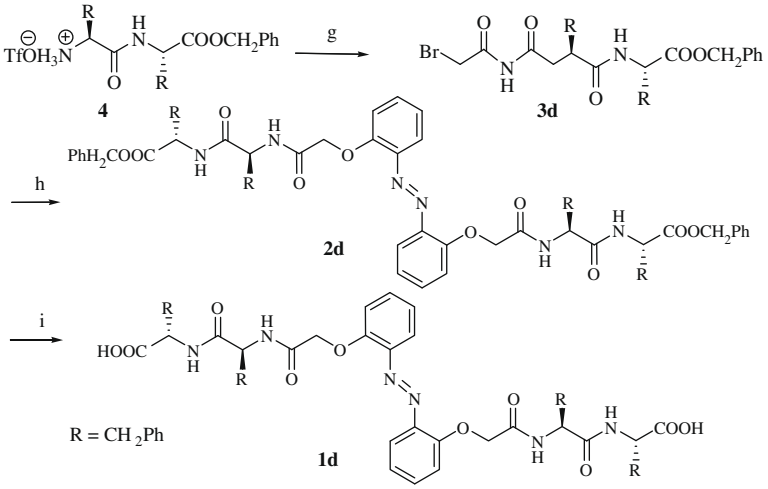
by O-alkylation with the bromoacylated dipeptide **3d**. The compounds **2a–d** when isolated by column chromatography generally remain in the *trans* (*E*) form which was deduced from strong UV absorption between wave length 354–361 nm. Moreover, the appearance of the aromatic hydrogens at relatively downfield (well above δ 7.2) as compared to the known *cis* (*Z*) azo compounds[17] supported the *E*-configuration of N=N moiety. One of the compounds **1a** gave a single crystal which allowed us to confirm the structure by X-ray crystallography. It was elaborated in a bis enediyne derivative as reported earlier.[17]

The dibenzyl esters **2a–d** were then examined for their thermal reisomerization kinetics. The photoisomerization studies were performed in dichloromethane with UV-irradiation at 365 nm for 3–4 h. The thermal reisomerization for *Z* to *E* conversion of all the series at 30 °C showed first order kinetic as shown in Table 1 which reveals that the half lives for various azobenzene esters follow the order: **2c > 2b > 2a > 2d**. That the peptide bonds on the two arms of the azobenzene have rendered some stability to the *Z*-isomer is clear from the larger $t_{1/2}$ values as compared to the azobenzene derivatives without such unit. The stability is, however, maximum for glycine substituted compound **2c**, followed by the phenyl alanine and valine-based compounds **2b** and **2a**, respectively, clearly demonstrating the higher stability for compounds with minimum steric strains. The incorporation of phenyl or isopropyl substituent, led to an increase in steric strain thus lowering the half life of the *Z*-isomer. The greater stability of the phenyl



a) 2,2'-dihydroxyazobenzene, anh. Cs_2CO_3, dry CH_3CN, 40-50° C, 12 h, 50%; **b**) LiOH, THF/H_2O, 18 h, 60%; **for a, R = -^{i}Pr; for b, R = -CH_2Ph; for c, R = H**

Scheme 1. Synthesis of azobenzene-amino acid hybrids.



g) $BrCH_2COCl$, Et_3N, DCM, 12-15 m; **h**) 2, 2'-dihydroxy azo benzene, Cs_2CO_3, CH_3CN (dry), 12 h, 60%; **i**) LiOH, THF/ H_2O, 16-18 h, 50%

Scheme 2. Synthesis of azobenzene-amino acid derivatives.

Table 1
Rate constants and half lives of *Z*-azo isomers

Compound	2a	2b	2c	2d
K (h^{-1})	0.022	0.010	0.009	0.024
$t_{1/2}$ (h)	31.5	69.3	77.0	28.9

alanine derivative over the valine counterpart may be due to the π-stacking interaction between the two phenyl rings in the *Z*-form. The incorporation of more peptide bonds, however, caused a lowering of half life. Whether or not this is a general phenomenon is still to be addressed because of lack of number examples.

With the compounds in hands, inhibition studies in vitro against human (h) recombinant soluble SKI-1 enzyme[12,16] were carried out. For this study, the fluorogenic substrate Q-GPC$_{251–263}$ (Abz-$_{251}$Asp-Ile-Tyr-Ile-Ser-Arg-Arg-Leu-Leu-↓-Gly-Thr-Phe Thr$_{263}$-Tyx-Ala-CONH$_2$ where Abz = 2-amino benzoic acid, Tyx = 3-nitrotyrosine) was used. For each study the activity of the enzyme used was nearly identical, releasing 10 pmol of Abz containing N-terminal peptide fragment per microliter of enzyme sample in one hour following incubation at 37 °C.[18] The progress of enzyme inhibition was monitored by stop time assay.

The inhibition assay was performed only with the thermally stable *E*-form because of instability of *Z*-form.[19] End point assay was conducted by measuring relative fluorescence units (RFU) (λ_{ex} = 320 nm and λ_{em} = 420 nm) at various times starting from 0 to 6 h after incubation of SKI-1 (10 μL) with Q-GPC$_{251–263}$

Table 2
Measured inhibition constant values

Compound	Inhibition constant K_i (μm)
2a	125 ± 15
2b	11.8 ± 1.5
1a	75 ± 8
1b	225 ± 18
1c	265 ± 11
1d	140 ± 12

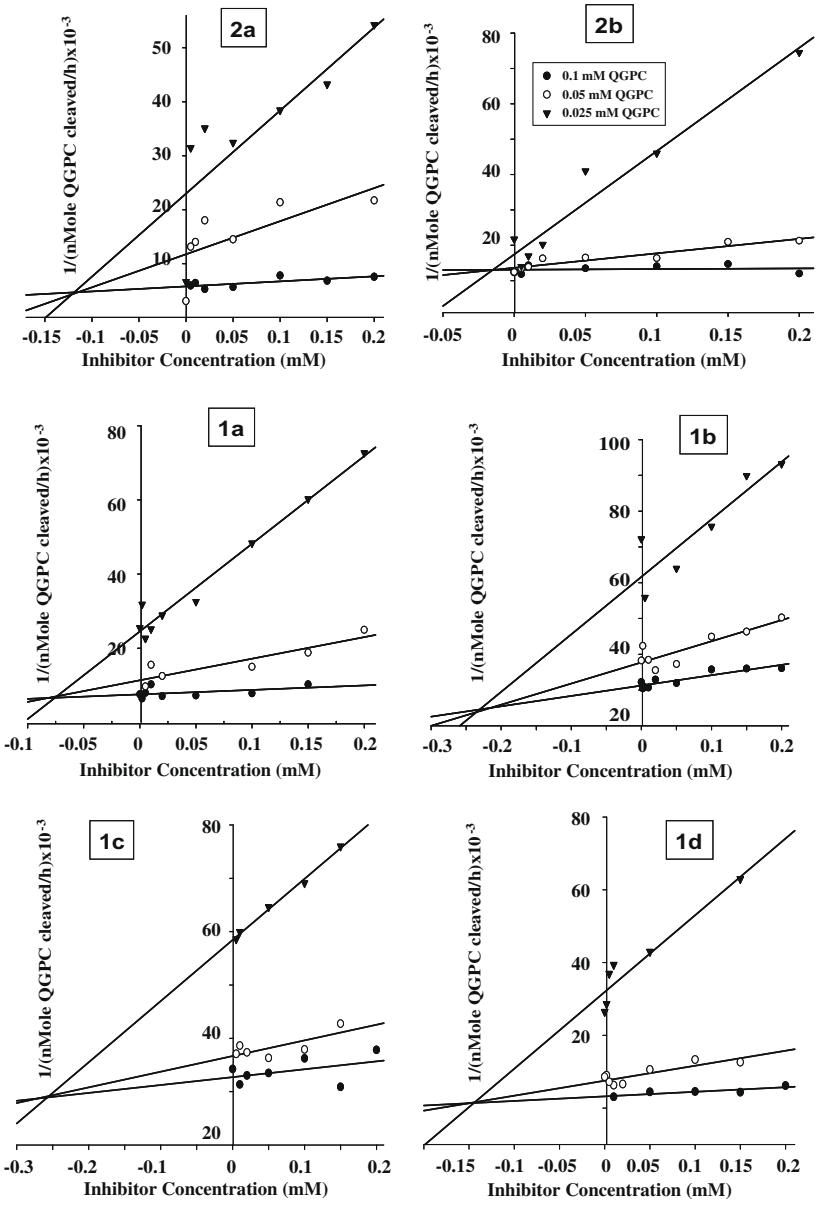


Figure 2. Inhibition kinetics of various azo compounds against SKI-1 enzyme using Dixon plots.

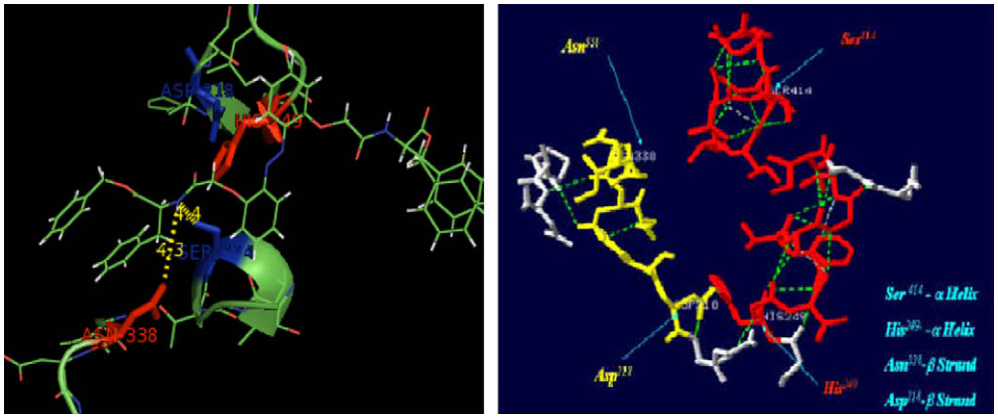


Figure 3. Models showing SKI-1 catalytic domain (right) and the docking of compound **2b** with SKI-1 catalytic region (left).

(100 μM) in buffer [25 mM Tris + 25 mM Mes + 2 mM $CaCl_2$, pH 7.4] in a total volume of 100 μL in 96-well plate. For determination of K_i values, each inhibitor concentration was varied over a range wide enough to yield residual activities of 20–80% of control value following incubation with SKI-1 enzyme. Typically h-SKI-1 sample (10 μL) and substrate (10 μL) were incubated in buffer (total volume 100 μL) with each inhibitor (2 μL) of varying concentrations in 96-well micro-titer plate at 37 °C. The rate of substrate hydrolysis was obtained from the changes in fluorescence readings and the values transformed into amounts of μmol/h of peptide cleaved by using standard curve and the measured quenching corrections as we did previously.[16] Non-linear regression analysis of plots of the hydrolysis rate versus the inhibitor concentration was used. In all cases the inhibition is mostly competitive in nature as determined by Dixon plots (Fig. 2). The inhibition constant K_i (Table 2) was measured using three different concentrations (100, 50, and 25 μM) of Q-$GPC_{251–263}$.

From this table we find that all the compounds have inhibition with IC_{50} or K_i in the micromolar range with compound **1b** being the most potent inhibitor. Unlike the highly reactive peptidyl-cmk (chloromethyl ketone) derivatives,[20] the azo-compound derivatives did not exhibit any significant inhibition of Furin at 100 μM or lower concentrations. We have also performed the docking study so as to compare the in vitro and in silico results. For that, we used Autodock version 3.0.5 program. It was observed from the Swiss PDB Viewer that no direct H-bonding interactions occurred with SKI-1 active site residues, but other stabilizing interactions occurred in the active site region especially with the catalytic $_{218}$Asp, $_{249}$His, $_{414}$Ser, and $_{338}$Asn residues (Fig. 3). H-bonding and other polar interactions occurred with nearby residues (not shown in the figure). As docking occurred in the active site region of SKI-1 (in case of compound **2b** as an example), it suggested to a competitive inhibition. In reality also, this compound showed competitive inhibition. Thus our docking experiment supported in vitro study. Figure 3 represents theoretical active site structure of the SKI-1 enzyme (right) and the docking result with compound **2b** (left).

Thus we have successfully synthesized novel C_2-symmetric azobenzene-amino acid conjugates which showed modest to good inhibitory activities against SKI-1 enzyme in a competitive manner. Efforts are currently underway to evaluate their ex vivo activities using specific cell lines. Previously it was demonstrated that similar C_2-symmetric bis-azo compounds such as 2,2′-azobis(2-amidinopropane hydrochloride can cause peroxidation of rat liver microsomes[21] but no report of their protease inhibitory activity towards SKI-1 was ever reported. Therefore this may provide an alternate strategy for design of more selective and potent inhibitors of SKI-1 that may find useful therapeutic and biochemical applications.

Acknowledgments

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Spectral data of selected compounds (IR data taken in KBr pellet and expressed in cm^{-1}, ^1H NMR and ^{13}C NMR were recorded at 400 and 100 MHz, respectively, in $CDCl_3$ unless stated otherwise):

1a: υ_{max} 3376, 2966, 1728, 1646, 1542, 1285, 1056; δ_H (d_6-DMSO) 12.87 (2H, br s), 7.83 (2H, d, *J* = 8.8 Hz), 7.57 (2H, d, *J* = 7.6 Hz), 7.42 (2H, t, *J* = 7.6 Hz), 7.00 (2H, d, *J* = 8.4 Hz), 6.95 (2H, d, *J* = 7.6 Hz), 4.76 (4H, s), 4.23 (2H, m), 1.99 (2H, m), 0.76 (6H, d, *J* = 6.8 Hz), 0.71 (6H, d, *J* = 6.8 Hz); δ_C (d_6-DMSO) 173.0, 168.0, 155.6, 142.1, 133.3, 121.9, 117.0, 115.4, 67.9, 56.8, 30.6, 19.4, 17.9; mass (ES^+)

m/*z* 529.31 (MH^+); HRMS Anal. Calcd for $C_{26}H_{32}N_4O_8$ 529.2298. Found 529.2300.

1b: υ_{max} 3401, 1723, 1661, 1529, 1486, 1439, 1233, 1120, 759; δ_H (d_6-DMSO) 13.0 (2H, br s), 8.26 (2H, m), 7.49 (2H, d, *J* = 8.0 Hz), 7.41 (2H, t, *J* = 8.0 Hz), 7.18 (10H, m), 7.01 (4H, m), 4.72 (4H, s), 4.58 (2H, m), 3.09 (2H, dd, *J* = 13.2, 4.8 Hz) , 2.90 (2H, dd, *J* = 13.2, 4.8 Hz); δ_C (d_4-MeOH) 172.5, 169.1, 155.3, 142.5, 136.0, 132.5, 129.1, 127.9, 126.4, 122.3, 117.4, 115.3, 68.5, 52.9, 37.0; mass (ES^+) *m*/*z* 625.23 (MH^+); HRMS Anal. Calcd for $C_{34}H_{32}N_4O_8$ 645.2278. Found 625.2282..

2c: υ_{max} 2963, 1745, 1660, 1376, 759; δ_H (200 MHz) 7.80–7.61 (4H, m, aromatic-H), 7.44–7.21 (10H, m, aromatic-H), 7.11–7.02 (6H, m, aromatic-H, –CONH), 5.12 (4H, m, $-CO_2CH_2Ph$), 4.76 (4H, s, $-OCH_2CO$), 4.11 (4H, d, *J* = 5.2 Hz, $-NHCH_2COOCH_2Ph$); δ_C (50 MHz) 169.2, 168.8, 155.2, 135.0, 132.9, 128.6, 128.5, 128.4, 128.3, 128.3, 128.2, 123.0, 67.1, 40.8, 29.6; mass (ES^+) *m*/*z* 625.25 (MH^+); HRMS Anal. Calcd for $C_{34}H_{32}N_4O_8$ 625.2278. Found 625.2283.

1c: υ_{max} 2966, 1728, 1662, 1217, 1056, 735; δ_H (d_6-DMSO) 8.39–8.33 (2H, m), 7.64–7.62 (2H, m), 7.50 (2H, t, *J* = 8.0 Hz), 7.30–7.23 (2H, m), 7.12–7.08 (2H, m), 4.79 (4H, s), 3.85 (4H, s); δ_C (d_6-DMSO) 172.9, 168.7, 155.5, 138.1, 131.7, 123.7, 121.4, 119.5, 67.8, 43.0; mass (ES^+) *m*/*z* 445.13 (MH^+); HRMS Anal. Calcd for $C_{20}H_{20}N_4O_8$ 445.1359. Found 445.1362.

2d: υ_{max} 2965, 1748, 1662, 1578, 759; δ_H 7.43 (2H, m), 7.36–7.35 (6H, m, aromatic-H), 7.30–7.24 (12H, aromatic-H), 7.18–7.15 (4H, aromatic-H), 7.10–6.89 (16H, m, aromatic-H, –CONH), 6.25 (2H, d, *J* = 8.0 Hz, –CONH), 5.10 (4H, s, $-CO_2CH_2Ph$), 4.78 (2H, m, α-H), 4.66–4.62 (6H, m, α-H, $-OCH_2CO$), 3.04–2.92 (8H, m, 2× $-CH_2Ph$); δ_C ($CDCl_3$): 170.7, 169.8, 168.3, 155.2, 136.3, 135.4, 135.0, 133.0, 129.2, 129.2, 129.1, 128.8, 128.7, 128.6, 128.6, 128.5, 127.1, 126.9, 123.1, 118.0, 115.4, 67.3, 54.1, 53.4, 37.8, 37.7, 29.7; mass (ES^+) *m*/*z* 1099.5 (MH^+); HRMS Anal. Calcd for $C_{66}H_{62}N_6O_{10}$ 1099.4605. Found 1099.4608.

1d: υ_{max} 2976, 1728, 1667, 1217, 1056, 735; δ_H (d_6-DMSO): 7.52 (2H, br s), 7.33 (2H, s), 7.18–7.14 (16H, m), 6.99–6.97 (4H, m), 6.86 (4H, m), 4.59 (6H, m, α-H), 4.33 (2H, br s), 3.01–2.83 (8H, m); δ_C (d_6-DMSO) 170.2, 170.0, 167.2, 155.3, 138.5, 138.0, 137.7, 132.7, 129.5, 129.4, 129.2, 128.3, 128.1, 127.9, 126.3, 126.0, 121.5, 67.6, 55.0, 53.4, 37.9, 37.4; mass (ES^+) *m*/*z* 919.4 (MH^+); HRMS Anal. Calcd for $C_{52}H_{50}N_6O_{10}$ 919.3666. Found 919.3670.