

Protein Ligands with Nanomolar Affinity from β -peptide Libraries

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I. Synthesis and Characterization of β -Peptide Libraries Suitable for On-Bead Screening

We selected Tentagel HMB Macrobeads resin (~150 μ m, ~0.4 mmol/g, Rapp Polymere) containing a 4-hydroxymethylbenzoic acid (HMBA) chemical handle for the synthesis of one-bead-one- β -peptide libraries. These beads are suitable for use on a COPAS automated bead sorting instrument (Union Biometrica), and the HMBA handle enables independent side chain deprotection and cleavage steps (under strongly acidic and basic conditions, respectively). Several modifications were made to previously reported β -peptide synthesis protocols¹⁻³ to boost the yield and purity one-bead-one-compound β^3 -peptide (OBO β) libraries. Specifically, β^3 -peptide libraries

were synthesized manually on a 35 μ mole scale using standard Fmoc chemistry and Tentagel HMBA resin loaded with β^3 -homoglutamic acid as described.³ One cycle of peptide elongation consisted of the following steps. First, the loaded resin was washed with N-methyl-2-pyrrolidone (NMP) (3 x 30 sec) and the terminal Fmoc protecting group was removed with 20% piperidine/DMF (1 x 2 min, 2 x 8 min). Notably, after five residues had been attached to the resin this deprotection step was supplemented with a second deprotection step using diazabicyclo[5.4.0]undec-7-ene (DBU) as follows: 1 x 2 min 20% piperidine/DMF, 2 x 8 min 20% piperidine/DMF, 2 x 8 min piperidine/DBU/NMP (2:2:96), 2 x 8 min 20% piperidine/DMF. After deprotection, the resin was washed with NMP (6 x 30 s) and, if necessary, the resin was split into separate pools for coupling different β^3 -amino acids. Coupling of the next β^3 -amino acid was performed for 90 minutes using 2 equiv of the appropriate β^3 -amino acid, 2 equiv PYBOP[®], 2 equiv HOBt, and 5.3 equiv diisopropylethylamine (DIEA). This coupling was then repeated with fresh reagents for another 90 minutes. The resin was then washed once with NMP (1 x 30 s) and re-pooled if the resin had been split prior to coupling. Unreacted amino groups were acetylated upon treatment with 6% v/v acetic anhydride and 6% v/v NMM in NMP (8 min), and the capped resin was washed with NMP (2 x 30 s). The elongation cycle was repeated until the β -peptide sequence was complete. Once the final Fmoc protecting group had been removed, the resin was washed with NMP (8 x 30 s) and methylene chloride (8 x 30 s), dried 20 min under N₂, and then treated for 90 min with a side chain deprotection cocktail composed of 2% v/v water and 2% v/v tri-isopropylsilane in trifluoroacetic acid (TFA). Resin was washed with methylene chloride (8 x 30 s) and methanol (8 x 30 s), then dried under vacuum and stored dry at 4 °C. Negligible degradation (as detected by analytical HPLC of β -peptide cleaved from 100-200 beads of a non-library synthesis and by MALDI-TOF MS of β -peptide cleaved from single library beads) of the Tentagel HMB resin or attached β -peptide was observed after 4 months of storage.

Protocols for the synthesis of β -peptide libraries were compared by cleaving the β -peptide from a pool of 100-200 beads and analyzing the products by analytical reverse-phase HPLC (Figure SI-1). HPLC was performed using a Vydac C8 analytical column using a flow rate of 1 mL/min, with a gradient of 20% to 100% acetonitrile in water (with 0.1% TFA) over 30 minutes.

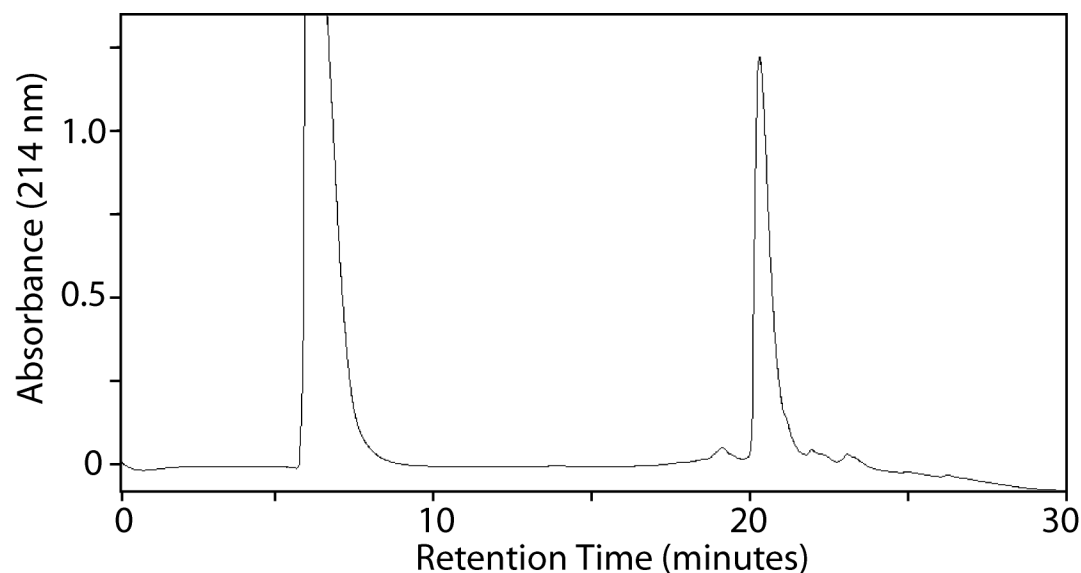


Figure SI-1. Representative HPLC chromatogram illustrating the products obtained upon synthesizing β 53-1 on Tentagel beads using optimized library synthesis protocols, then cleaving a pool of 100-200 beads as described above. HPLC was performed using a Vydac C8 analytical column using a flow rate of 1 mL/min, with a gradient of 20% to 100% acetonitrile in water (with 0.1% TFA) over 30 minutes. The peak at 7 min is the injection peak and the peak at 21 min is β 53-1.

II. Optimization of On-Bead Assay and Screening

Various blocking and washing conditions were evaluated to optimize the on-bead assay, including permutations of various buffers, salts, ionic detergents, non-ionic detergents, and

blocking agents. Conditions that proved optimal were 10 mM Tris-Cl buffer (pH 8.0), with 0.1% Tween-20, 0.15 M NaCl, and 2.5 mg/mL gelatin (subsequently referred to as “BW buffer”). Significantly, independently increasing the concentrations of each BW buffer component led to a decrease in overall signal of β 53-1 beads compared to β NEG beads (Figure SI-2).

Figure SI-2. Effects of blocking and washing buffer components on signal intensity in on-bead assays. A mixture of β 53-1 and β NEG beads were incubated with 2.0 μ M ^{Bio}hDM2 and 5 nM Qdots-SA605 as described, using a blocking and washing buffer containing 10 mM Tris-Cl (pH 8.0) and the indicated concentrations of gelatin, NaCl and Tween-20.

Screening assays were performed on a small scale using 96-well PVC plates (Falcon 353911, Becton Dickinson) as follows. Beads were wetted with methanol, and ~25 beads were removed to a well containing 100 μ L BW buffer. Beads were incubated in wells for 5 minutes at room temperature, shaking on a tabletop orbital shaker. BW buffer was then removed from beads using flat-tip gel-loading pipette tips (BioDOT Universal Fit, Dot Scientific), which were found not to permit passage of swelled beads. Fresh BW buffer was added, and beads were blocked in BW buffer for 30 minutes. After removing BW buffer, target protein (2.0 μ M biotinylated hDM2 (^{Bio}hDM2) or other control, pre-incubated in BW buffer for 10 minutes) was added in 50 μ L total volume and incubated for 60 minutes. Beads were then washed (2 x 2 min) with 100 μ L fresh BW buffer. Streptavidin-coated quantum dots (5 nM Qdots-SA605, Quantum Dot Corp; these were

also pre-incubated for 10 minutes in BW buffer) were then added in 25 μ L total volume and incubated for 30 minutes. Beads were then washed (2 x 2 min) with 100 μ L fresh BW buffer. Finally, beads were visualized using an Olympus CKX41 fluorescence microscope using the standard blue/green filter set (excitation at 460-490 nm, emission barrier at 515 nm). Beads were sorted by eye, and “hit” beads were determined to be those with substantially greater orange fluorescence than the bulk of the screened beads. Hit beads were picked using standard 10 μ L pipette tips and washed extensively with NMP and methanol prior to cleavage. Pictures were obtained using manual camera settings and a 1/5 second exposure, which generated images identical to those observed in the objective lens.

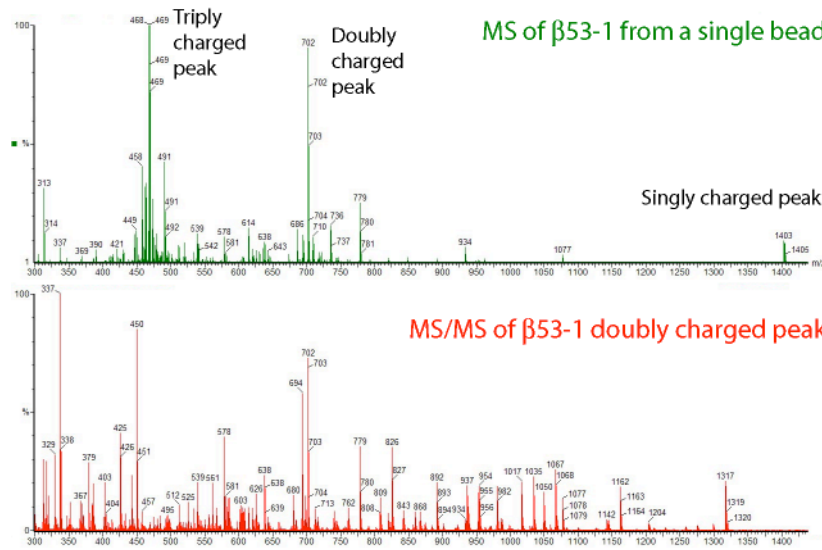
In addition to optimizing buffer and wash conditions, this test protocol was used to perform several controls to ensure the screen detected specific interactions between hDM2 and immobilized peptide. A mixture of β 53-1 and β NEG beads were treated as described above, with 2.0 μ M of ^{Bio}hDM2, unbiotinylated hDM2₁₋₁₈₈, biotinylated BSA (^{Bio}BSA), or no protein added at the target incubation step. Notably, no signal was observed for beads treated with no protein, ^{Bio}BSA, or unbiotinylated hDM2₁₋₁₈₈ (**Figure SI-3**).

Figure SI-3. Controls using various proteins as recognition agents. A mixture of β 53-1 and β NEG beads were screened as described, using (a-c) 2.0 μ M of the indicated protein or (d) no protein at the target incubation step. All steps were performed in BW buffer.

III. Cleavage, Desalting, and Sequencing of Individual Beads

After screening, β -peptides were cleaved from the resin using 1.0 M sodium methoxide in methanol followed by addition of water and aqueous acidification using 20% formic acid. Cleaved β -peptides were desalted using C18-packed 10 μ L micropipette tips (ZipTips, Millipore) according to the manufacturer instructions. MALDI mass spectra were obtained using 5% of the cleaved sample diluted in α -cyano-4-hydroxycinnamic acid (CHCA) matrix on a Voyager MALDI-TOF mass spectrometer (Applied Biosystems). For MS/MS analysis, 20 to 60% of the cleaved β -peptide was diluted with 50% $\text{CH}_3\text{CN}/50\% \text{H}_2\text{O}/0.1\%$ formic acid and directly infused into a Q-ToF Micro (Waters, Milford, MA) mass spectrometer with an off-axis electrospray ion source at 1 $\mu\text{L}/\text{min}$ flow rate. The parent ion of the peptide, usually the doubly charged peak, was identified manually. Subsequently, the parent ion was transmitted to the collision cell (pressurized with argon and collision energy optimized at ~ 30 V) to produce the MS/MS spectrum (see Figure SI-4). Raw data processing was performed using the MaxEnt 3 algorithm (MassLynx) to produce centroided and “deisotoped” mass spectra. Each amino acid sequence was ascertained by manual interpretation of a processed ion spectrum and also by automated *de novo* sequencing using the commercially available software PEAKS (Bioinformatics Solutions).

Figure SI-4. Representative mass spectral data obtained from β -peptides cleaved from single beads. (Top) ESI-MS spectrum of β 53-1 cleaved from a single Tentagel bead. Note that the resulting peptide was isolated as a methyl ester. (Bottom) MS/MS spectrum of the doubly charged



peak observed in the ESI-MS spectrum.

IV. Screening of the 1000-member Library

The 1000-member library was screened in a manner analogous to that described for

the control screen above using an augmented BW buffer, termed BW+ buffer. BW+ buffer consisted of 10 mM Tris-HCl (pH 8.0) supplemented with 0.1% Tween-20, 0.5 M NaCl, and 5 mg/mL gelatin. Methanol-wetted beads were scooped into an empty 10 mL polypropylene column, washed once (2 min) with 4 mL BW+ buffer, and incubated for 30 minutes with 4 mL fresh BW+ buffer. Incubations were performed at room temperature with end-over-end rotisserie mixing (LabQUAKE). The BW+ buffer was drained and the beads were incubated with 500 nM BiohDM2 in BW+ buffer for 30 minutes. Beads were washed (2 x 2 min), then incubated in 4 mL BW+ buffer with 5 nM Qdots-SA605. Beads were washed again (2 x 2 min), pipetted onto 96-well PVC plate lids (Falcon 353913, Becton Dickinson) and visualized as before. A second screen of the 1000-member library was performed in an identical manner except it used twice as many beads, a lower concentration of BiohDM2 (200 nM) and more stringent washes (5 x 5 min washes) at each washing step.

V. Synthesis and Characterization of β 53-8-10

β 53-8-10 were synthesized, purified and characterized as described.^{1,3} Expected and observed mass data are tabulated below. CD spectra are illustrated in **Figure SI-5**. The overexpression and purification of hDM2₁₋₁₈₈ used in FP competition assays, and the synthesis and purification of β 53-1 have been described previously.¹

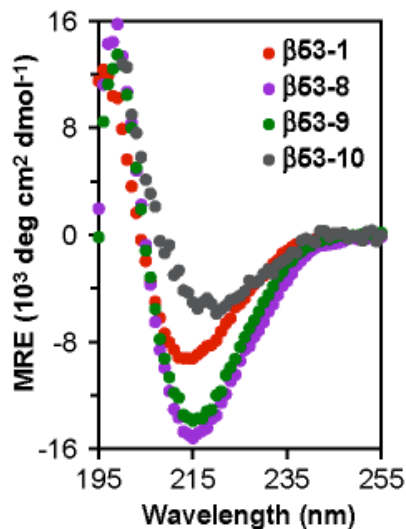
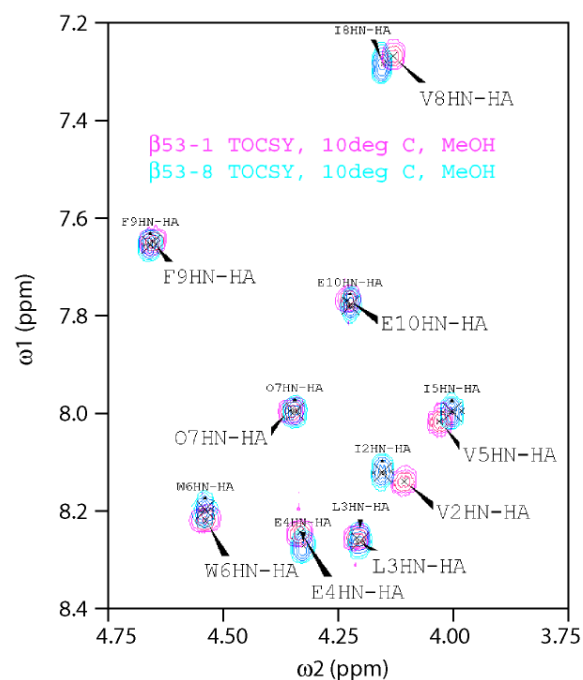


Figure SI-5. Circular dichroism (CD) spectroscopy of β 53-1 and β 53-8-10. CD spectroscopy was performed and processed as described.² CD spectra were obtained in 1 mM sodium phosphate/citrate/borate buffer, pH 7.0, at 25 °C. CD spectra were obtained at 80 μ M peptide concentration for β 53-1, β 53-8, and β 53-9, and 18 μ M peptide concentration for β 53-10 due to the poor aqueous solubility of this molecule.

	Expected M+H	Observed M+H
β 53-8	1431.8	1430.0
β 53-9	1417.8	1416.9
β 53-10	1538.9	1537.3

VI. Methanol Solution Structure of β 53-8



Sample preparation and NMR data collection for $\beta 53-8$ in methanol at 10 °C were identical to procedures described for $\beta 53-1$,⁴ except that a 2D NOESY experiment (pulse sequence from the Varian Biopack user library) was used to derive distance constraints in place of a 2D ROESY experiment. Assignment was straightforward as most resonances were nearly identical to corresponding resonances in $\beta 53-1$ (see Figure SI-6; resonances are listed in Table SI-1). A NOE

buildup curve was established by performing identical NOESY experiments with mixing times of 100, 200, 400 and 600 ms; data from the 200 ms NOESY was used to derive 116 distance constraints (listed in Table SI-2). The final low-energy ensemble of structures was calculated using DYANA⁵ as described.⁴ No constraints were violated among the 20 lowest-energy structures. The average RMS deviation from mean for backbone atoms was $0.21 \pm 0.06 \text{ \AA}$, and the average RMS deviation from mean for all heavy atoms was $0.98 \pm 0.27 \text{ \AA}$, indicating an ensemble of highly analogous structures was obtained.

Figure SI-6. Overlay of portions of the 2D TOCSY spectra of $\beta 53-1$ (magenta peaks, large type labels) and $\beta 53-8$ (cyan peaks, small type labels) in methanol at 10 °C. Figure was generated by overlaying data visualized using the SPARKY program.⁶

Table SI-1. Chemical shift assignments relative to DSS for β 53-8 in CD₃OH at 10 °C:

Residue	Atom	Resonance (ppm)	Residue	Atom	Resonance (ppm)	Residue	Atom	Resonance (ppm)
1	CA	51.852	4	HB2	1.77	7	HB3	1.501
1	CB	31.766	4	HB3	1.588	7	HD2	2.838
1	CD	41.577	4	HEQ	2.364	7	HD3	2.797
1	CG	26.018	4	HG3	2.246	7	HEQ	2.37
1	CME	39.781	4	HN	8.345	7	HG3	1.57
1	HA	3.547	5	CA	53.511	7	HN	8.05
1	HAX	2.836	5	CB	43.038	8	CA	53.258
1	HB2	1.784	5	CD1	13.592	8	CB	41.928
1	HB3	1.734	5	CG1	28.365	8	CD1	13.586
1	HD3	2.942	5	CG2	16.81	8	CG1	28.282
1	HEQ	2.562	5	CME	40.706	8	CG2	16.376
1	HG3	1.71	5	HA	4.007	8	CME	39.56
2	CA	53.258	5	HAX	2.511	8	HA	4.156
2	CB	41.963	5	HB	1.297	8	HAX	2.127
2	CD1	13.014	5	HEQ	2.234	8	HB	1.336
2	CG1	28.24	5	HG12	1.426	8	HEQ	2.332
2	CG2	16.954	5	HG13	0.905	8	HG12	1.405
2	CME	40.071	5	HN	8.052	8	HG13	0.947
2	HA	4.16	5	QD1	0.774	8	HN	7.29
2	HAX	2.562	5	QG2	0.783	8	QD1	0.797
2	HB	1.418	6	CA	50.791	8	QG2	0.797
2	HEQ	2.353	6	CB	34.078	9	CA	49.574
2	HG12	1.417	6	CD1	125.175	9	CB	43.829
2	HG13	1.045	6	CE3	120.868	9	CD1	131.844
2	HN	8.185	6	CH2	123.474	9	CE1	130.564
2	QD1	0.82	6	CME	42.649	9	CME	42.045
2	QG2	0.823	6	CZ2	113.501	9	CZ	128.698
3	CA	46.921	6	CZ3	120.842	9	HA	4.667
3	CB	48.08	6	HA	4.548	9	HAX	2.232
3	CD1	24.277	6	HAX	2.823	9	HB3	2.675
3	CD2	24.411	6	HB2	2.991	9	HD1	7.079
3	CG	27.26	6	HB3	2.849	9	HE1	7.005
3	CME	42.887	6	HD1	6.944	9	HEQ	2.464
3	HA	4.208	6	HE1	10.373	9	HN	7.686
3	HAX	2.622	6	HE3	7.369	9	HZ	6.979
3	HB2	1.346	6	HEQ	2.423	10	CA	47.777
3	HB3	1.118	6	HH2	6.959	10	CB	32.583
3	HEQ	2.321	6	HN	8.266	10	CG	32.587
3	HG	1.457	6	HZ2	7.21	10	CME	41.263
3	HN	8.336	6	HZ3	6.831	10	HA	4.228
3	QD1	0.818	7	CA	48.189	10	HAX	2.452
3	QD2	0.772	7	CB	35.203	10	HB2	1.779
4	CA	47.957	7	CD	42.154	10	HB3	1.528
4	CB	33.472	7	CG	26.971	10	HEQ	2.517
4	CG	32.979	7	CME	42.77	10	HG2	2.199
4	CME	42.648	7	HA	4.349	10	HG3	2.158
4	HA	4.332	7	HAX	2.414	10	HN	7.819
4	HAX	2.788	7	HB2	1.389			

Table SI-2. List of upper-distance limits used for structure determination:

Residue 1	Atom 1	Residue 2	Atom 2	Constraint (Å)	Residue 1	Atom 1	Residue 2	Atom 2	Constraint (Å)
7	BRN+	9	BPHE	3.7	4	BGU-	7	BRN+	3.3
7	BRN+	10	BGU-	3.45	7	BRN+	7	BRN+	2.9
5	BILE	8	BILE	3.39	3	BLEU	3	BLEU	2.93
1	BRN+	2	BILE	4.17	10	BGU-	10	BGU-	3.05
2	BILE	5	BILE	3.39	2	BILE	2	BILE	3.02
2	BILE	4	BGU-	4.04	5	BILE	8	BILE	3.8
6	BTRP	8	BILE	3.52	7	BRN+	10	BGU-	3.39
3	BLEU	5	BILE	4.14	2	BILE	2	BILE	2.96
4	BGU-	6	BTRP	3.39	2	BILE	5	BILE	2.77
3	BLEU	6	BTRP	3.24	2	BILE	2	BILE	3.83
6	BTRP	9	BPHE	3.49	5	BILE	5	BILE	3.55
8	BILE	9	BPHE	4.26	3	BLEU	3	BLEU	3.73
8	BILE	10	BGU-	5.07	3	BLEU	3	BLEU	3.89
7	BRN+	8	BILE	3.83	7	BRN+	7	BRN+	3.64
8	BILE	8	BILE	3.83	8	BILE	8	BILE	3.92
8	BILE	8	BILE	2.9	9	BPHE	9	BPHE	4.6
7	BRN+	8	BILE	2.77	6	BTRP	6	BTRP	5.19
9	BPHE	9	BPHE	3.08	6	BTRP	6	BTRP	4.69
9	BPHE	10	BGU-	4.35	9	BPHE	9	BPHE	5.5
10	BGU-	10	BGU-	2.9	6	BTRP	9	BPHE	5.5
9	BPHE	9	BPHE	2.83	6	BTRP	9	BPHE	5.5
8	BILE	9	BPHE	2.74	6	BTRP	9	BPHE	5.5
9	BPHE	10	BGU-	2.65	6	BTRP	6	BTRP	5.5
10	BGU-	10	BGU-	3.52	9	BPHE	9	BPHE	5.5
6	BTRP	7	BRN+	2.71	9	BPHE	9	BPHE	5.5
4	BGU-	5	BILE	2.4	3	BLEU	6	BTRP	5.5
1	BRN+	2	BILE	2.9	3	BLEU	6	BTRP	5.5
7	BRN+	7	BRN+	3.33	6	BTRP	9	BPHE	5.5
2	BILE	2	BILE	3.18	6	BTRP	6	BTRP	5.1
7	BRN+	7	BRN+	3.7	8	BILE	8	BILE	3.58
5	BILE	5	BILE	3.45	6	BTRP	6	BTRP	5.47
2	BILE	2	BILE	3.89	10	BGU-	10	BGU-	4.11
3	BLEU	3	BLEU	4.07	4	BGU-	5	BILE	3.49
3	BLEU	3	BLEU	3.49	7	BRN+	7	BRN+	3.42
5	BILE	6	BTRP	3.02	5	BILE	5	BILE	5.5
2	BILE	3	BLEU	2.8	4	BGU-	5	BILE	5
3	BLEU	4	BGU-	2.77	2	BILE	3	BLEU	3.67
4	BGU-	4	BGU-	3.67	9	BPHE	9	BPHE	2.96
6	BTRP	6	BTRP	3.33	6	BTRP	6	BTRP	2.87
6	BTRP	6	BTRP	2.87	4	BGU-	5	BILE	4.94
6	BTRP	9	BPHE	3.24	5	BILE	8	BILE	5.5
9	BPHE	9	BPHE	2.96	5	BILE	5	BILE	4.2
3	BLEU	6	BTRP	2.74	5	BILE	5	BILE	3.21
3	BLEU	6	BTRP	4.73	2	BILE	5	BILE	5.5
3	BLEU	6	BTRP	5.5	7	BRN+	10	BGU-	5.5
1	BRN+	4	BGU-	3.64	10	BGU-	10	BGU-	4.11
4	BGU-	4	BGU-	2.99	5	BILE	5	BILE	5.5

Residue 1	Atom 1	Residue 2	Atom 2	Constraint (Å)
3 BLEU	HB3	3 BLEU	HEQ	3.76
4 BGU-	HB3	4 BGU-	HEQ	4.17
7 BRN+	HB3	7 BRN+	HEQ	3.95
4 BGU-	HB2	4 BGU-	HEQ	4.17
2 BILE	HA	2 BILE	QD1	4.85
8 BILE	HA	8 BILE	QD1	4.67
5 BILE	QD1	8 BILE	HA	5.57
5 BILE	HA	5 BILE	QD1	4.3
2 BILE	QD1	5 BILE	HA	4.98
2 BILE	QD1	2 BILE	HAX	5.19
5 BILE	QD1	5 BILE	HAX	5.54
2 BILE	QD1	2 BILE	HEQ	4.95
8 BILE	QD1	8 BILE	HEQ	4.85
5 BILE	QD1	5 BILE	HEQ	4.3
8 BILE	QD1	8 BILE	HAX	5.72
1 BRN+	QB	1 BRN+	HAX	3.82
4 BGU-	QB	4 BGU-	HEQ	3.79
4 BGU-	QB	4 BGU-	HAX	3.64
6 BTRP	HN	6 BTRP	QB	3.74
7 BRN+	HN	7 BRN+	QG	6.29
7 BRN+	QG	7 BRN+	HAX	6.01
8 BILE	QG1	8 BILE	HEQ	6.38

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