

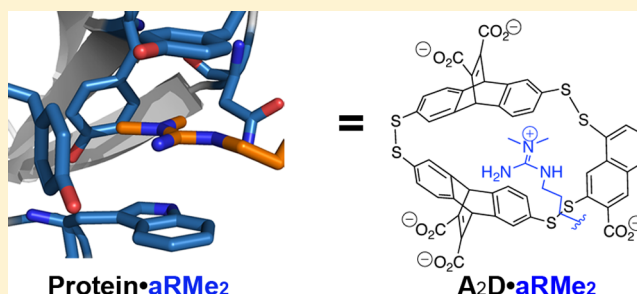
A Synthetic Receptor for Asymmetric Dimethyl Arginine

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Supporting Information

ABSTRACT: Dynamic combinatorial chemistry was utilized to identify a novel small molecule receptor, **A₂D**, for asymmetric dimethyl arginine (aRMe₂), which is a post-translational modification (PTM) in proteins. It is known to play a role in a number of diseases, including spinal muscular atrophy, leukemia, lymphoma, and breast cancer. The receptor exhibits 2.5–7.5-fold selectivity over the isomeric symmetric dimethyl arginine, depending on the surrounding sequence, with binding affinities in the low micromolar range. The affinity and selectivity of **A₂D** for the different methylated states of Arg parallels that of proteins that bind to these PTMs. Characterization of the receptor–PTM complex indicates that cation– π interactions provide the main driving force for binding, loosely mimicking the binding mode found in the recognition of dimethyl arginine by native protein receptors.



INTRODUCTION

Methylation of arginine (Arg) is a common post-translational modification (PTM) found in both cytoplasmic and nuclear proteins that plays a critical role in many cellular functions, including transcriptional regulation, signal transduction, protein translocation, mRNA splicing, and DNA repair.¹ Protein Arg methyltransferases (PRMTs) methylate Arg (Figure 1),

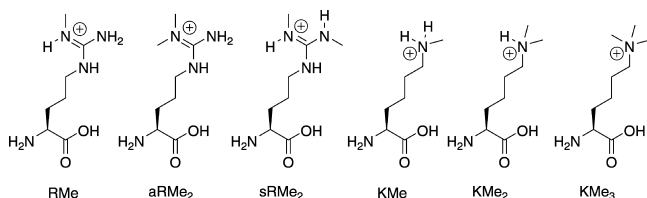


Figure 1. Methylation states of Arg and Lys that are found in proteins.

resulting in either monomethyl Arg (RMe), asymmetric dimethylarginine (aRMe₂), or symmetric dimethylarginine (sRMe₂) depending on the enzyme's specific function.¹ Dysregulation of Arg methylation has been linked to a number of different diseases, including spinal muscular atrophy, leukemia, lymphoma, and breast cancer.¹ Arg methylation has been shown in some cases to inhibit protein–protein interactions, while in other cases it induces protein–protein interactions via specific recognition of the methyl Arg by a ‘reader’ protein, in turn controlling various downstream biological functions. To date, crystal or NMR structures have been obtained of four tudor domains bound to sRMe₂ or aRMe₂.² In each case, RMe₂ is bound in an aromatic cage of the tudor domain via a combination of CH– π interactions with the CH₃(δ^+) groups, cation– π and π – π stacking of the guanidinium group with two aromatic rings, NH– π inter-

actions, and hydrogen bonds (Figure 2),² with binding affinities ranging from 5 μ M to >200 μ M.^{2,3}

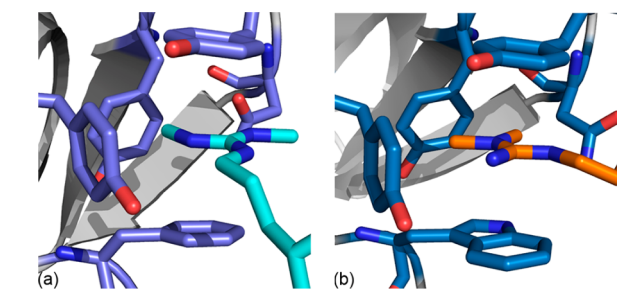


Figure 2. (a) Crystal structure of the SND1 extended Tudor domain bound to sRMe₂ (cyan) of PIWIL1 (pdb: 3OMG). (b) NMR structure of the SMN Tudor domain bound to aRMe₂ (orange) (pdb: 4A4G).

With the goals of gaining a better understanding of methylarginine recognition and developing new methods for sensing these PTMs, we set out to develop small molecule synthetic receptors for methylated Arg. Previously we reported using dynamic combinatorial chemistry (DCC)⁴ to develop a synthetic receptor, **A₂B** (both *rac*- and *meso*-), for another methylated amino acid, trimethyl Lys (KMe₃) (Figure 3). **A₂B** displays affinity and selectivity that is comparable to a native KMe₃ protein receptor.⁵ Herein we demonstrate that small structural changes to one of the building blocks that make up **A₂B** result in a new molecule, **A₂D** (Figure 3), that binds aRMe₂ with low micromolar affinity and selectively recognizes aRMe₂ over sRMe₂ and RMe.

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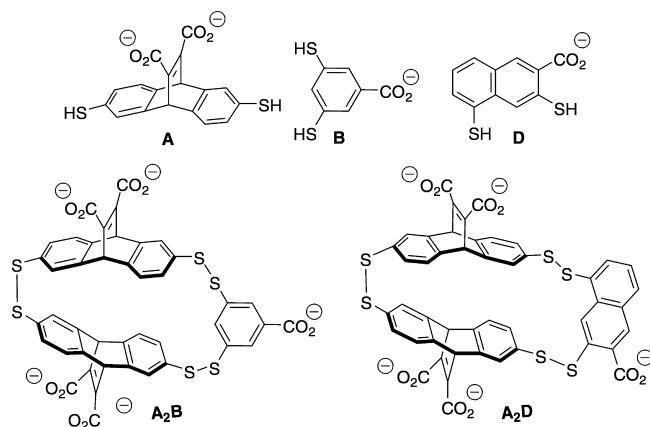


Figure 3. (a) Structure of monomers investigated in DCLs with methylated Lys and Arg guests. Monomer C (not shown) was reported previously.⁵ (b) Structure of **A₂B** (only the *rac*-stereoisomer is shown) that binds **KMe₃** and **A₂D** that binds **aRMe₂** and **KMe₃**.

RESULTS

System Design. DCC was used to screen for novel receptors for the different methylation states of Lys and Arg. DCC is an attractive alternative to the rational design of synthetic receptors in that it allows molecular recognition to guide the synthesis of complex host systems from simple building blocks.⁴ These building blocks react reversibly to produce an equilibrium mixture of potential receptors. In the presence of a molecular target, favorable host–guest binding interactions drive the synthesis and amplification of favorable receptors at the expense of other species. DCC also allows for parallel screening of the same library of building blocks against all Lys and Arg methylation states, providing a rapid approach to screen for selective recognition of the different PTMs. Disulfide exchange was chosen as the reversible reaction to link the monomeric building blocks because it occurs in aqueous solution at close to neutral pH and is stable toward most biological functional groups.⁶ In addition, exchange can be quenched under acidic conditions, allowing for analysis of the library under static conditions.

The previously reported receptor, **A₂B**, forms an aromatic binding pocket with preference for **KMe₃** over the lower methylation states of Lys and all of the methylation states of Arg.⁵ Binding occurs via cation– π interactions between the tetraalkyl ammonium group of **KMe₃** and the aromatic pocket. The preference for **KMe₃** arises from a number of factors including a better fit in the binding pocket and lower desolvation cost than the lower methylation states of Lys as well as favorable $\text{CH}(\delta^+)-\pi$ interactions.⁷ We sought to explore structure–function relationships in **A₂B** by replacing monomer **B** with a larger naphthalene-based dithiol, monomer **D**, in a dynamic combinatorial library (DCL) with monomer **A** and evaluating the impact on recognition of methylated Lys and Arg. We hypothesized that incorporation of monomer **D** would provide larger macrocycles which may interact with PTMs that are sterically occluded by **A₂B**, such as **aRMe₂** and **sRMe₂**. Additionally, the naphthyl ring of **D** was expected to provide better stacking interactions with the planar Arg side chain than the phenyl ring in **B**. Lastly, similar to **A₂B**, an aromatic pocket was expected to minimize binding to lower methylation states of Lys and Arg due to unfavorable desolvation costs.⁸

Dynamic Combinatorial Chemistry. Eight separate DCLs biased toward the formation of **A₂D** were prepared in

which building blocks **A** and **D** were mixed in a 2:1 ratio, respectively, and each was combined with a dipeptide guest, **Ac-KMe_x-G-NH₂** ($x = 0-3$) or **Ac-RMe_y-G-NH₂** ($y = 0-2$), or no guest in the case of the untemplated library. This simple sequence was chosen to minimize interactions with neighboring amino acids, with the hope of amplifying species that bind primarily to the modified side chain. The composition of each library was monitored by LC-MS, and after reaching equilibrium, the receptors that were amplified in the presence of each guest as compared to the untemplated library were identified. Unmethylated Lys and Arg peptides were used to evaluate receptor selectivity for methylated residues over unmodified residues.

Upon templation with **aRMe₂**, **A₂D** is strongly amplified, while the amplification by **sRMe₂** is quite weak in comparison (Figure 4). There are two sets of possible **A₂D** diastereomers, however the amplification of only a single **A₂D** diastereomer in the presence of methylated PTMs was observed. It should be noted that only one **A₂D** isomer exists in high enough concentration to be detected in the untemplated library, indicating significant differences in thermodynamic stabilities between the isomers of **A₂D**. The amplification of **A₂D** in the

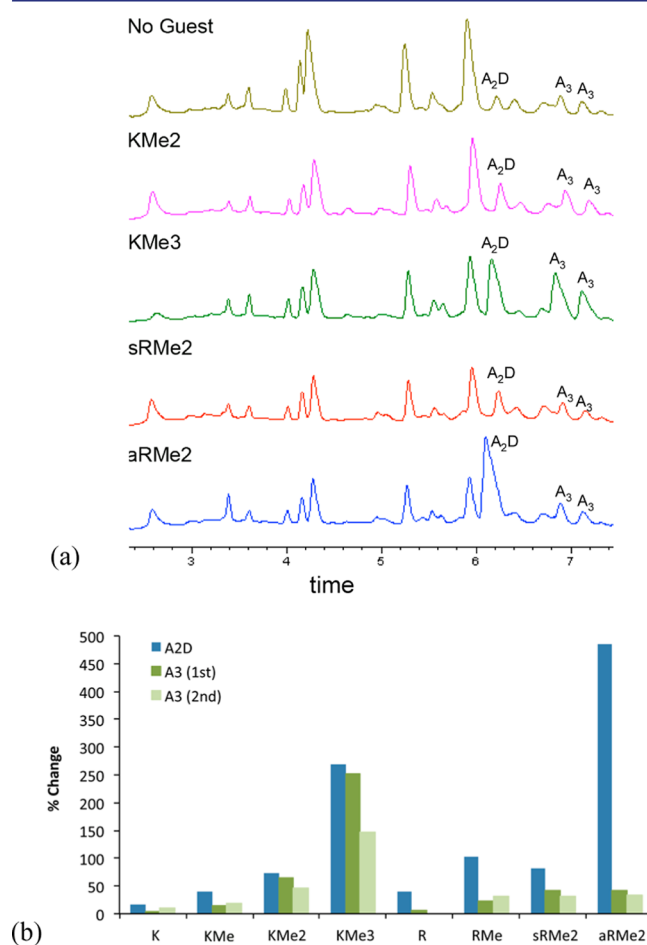


Figure 4. (a) The analytical HPLC traces at 254 nm of DCLs consisting of monomers **A** (5 mM), **D** (2.5 mM), and 7.5 mM guest. The y-axis is absorbance in arbitrary units and all traces are on the same scale. (b) Percent amplification of **A₂D** biased libraries templated with Lys and Arg dipeptide guests relative to the untemplated library. The extent of amplification of **A₂D** (blue) and both **A₃** isomers (green) is shown.

biased library is also observed with KMe_3 , but to a lesser extent than with aRMe_2 (Figure 4). Furthermore, templation with KMe_3 also results in a similar degree of amplification of two A_3 isomers.⁹

Structural Characterization. A_2D was synthesized on a preparative scale via DCC and purified by RP-HPLC as described in the Experimental Procedures section. NMR studies, including 1D ^1H spectra, NOESY, TOSCY, and COSY experiments, were undertaken to characterize the structure of the A_2D receptor in both the absence and presence of guest.¹⁰ In characterizing the receptor alone, most notable were the chemical shifts of protons 2 and 3 of the naphthyl ring (Figure 5a), which were shifted to ~ 3.1 and 2.9 ppm,

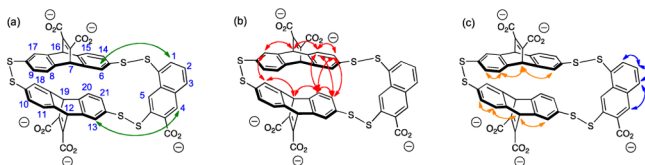


Figure 5. A_2D NOESY cross peaks: (a) NOEs between A and D subunits; (b) NOEs between A subunits; and (c) NOEs within subunits.

respectively (see Supporting Information). This is almost 5 ppm further upfield from where these proton signals appear in the spectra of monomer D. Based on observed NOEs (Figure 5a) and the significant upfield shift of these naphthalene protons, it is proposed that this portion of the naphthalene ring is directed toward the inside of the receptor cavity, sandwiched between both A subunits and packed into the aromatic pocket. Furthermore, the observed A_2D isomer is predicted to be the RR,SS/SS,RR pair of enantiomers due to the presence of NOE cross peaks between four monomer A aromatic doublets (protons 14, 15, 20, and 21 in Figure 5), suggesting that they are all in close proximity on one edge of the macrocycle (Figure 5b). While the A subunits appear to be in close proximity on one edge of the molecule, no NOEs were observed between protons on the other edge (protons 6–13), suggesting that they are further apart from each other, creating a binding cleft.

NMR was also used to investigate the recognition of aRMe_2 and sRMe_2 by A_2D by evaluating changes in the ^1H spectra of the dipeptides, $\text{Ac-aRMe}_2\text{-G-NH}_2$ and $\text{Ac-sRMe}_2\text{-G-NH}_2$, upon binding (Table 1). Experiments were performed with 600 μM peptide and a 3–4-fold excess of A_2D so that the peptide is in the fully bound state. The peaks for A_2D broaden significantly

Table 1. Change in Chemical Shifts of $\text{Ac-aRMe}_2\text{-G-NH}_2$ and $\text{Ac-sRMe}_2\text{-G-NH}_2$ in the Presence of an Excess of A_2D Relative to the Unbound State^a

position	$\Delta\delta$ aRMe_2	$\Delta\delta$ sRMe_2
Ac	0.03	−0.06
H α	0.03	−0.16
H β 1	−0.13	−0.22
H β 2	−0.16	−0.21
H γ	−0.22	−0.22
H δ	−0.39	−0.39
NCH ₃	−0.96	−0.44
Gly	0.03	−0.06

^aConditions: 25 °C, 10 mM $\text{Na}_2\text{DPO}_4/\text{NaD}_2\text{PO}_4$, pD 8.4 (uncorrected).

upon mixing, indicating that the rate of exchange is on the NMR time scale. Interestingly, the side chain of both aRMe_2 and sRMe_2 is also broadened, suggesting that binding can occur in more than one conformation (see Supporting Information for spectra). In the case of aRMe_2 , more significant upfield shifting is observed for protons closer to the guanidinium group, with the methyl protons shifted to the greatest extent, indicating that they are packed against the face of an aromatic ring. In contrast, upfield shifting is observed to some degree at every position in the sRMe_2 peptide, including the Gly protons. While a greater degree of upfield shifting is observed at the α and β positions of sRMe_2 than aRMe_2 , less upfield shifting is observed at the methyl groups. These data suggests that aRMe_2 binding stems primarily from interaction with the guanidinium methyl groups, whereas sRMe_2 interacts with the receptor at multiple positions along the side chain but with less interaction of the methyl groups. The broadening of the sRMe_2 proton signals bound to A_2D suggests that it interconverts between inserting one methyl group or the other into the receptor binding pocket, unlike aRMe_2 , for which the methyl groups give a sharp peak.

Binding Studies. Binding of A_2D to histone 3 peptides containing Arg methylation at position 8¹¹ or KMe_3 at position 9¹² was determined to evaluate the selectivity for the different PTMs within the same sequence (Chart 1).¹³ To determine the

Chart 1. Peptide Sequences Used for ITC Binding Measurements Containing R, RMe, aRMe_2 , sRMe_2 , or KMe_3 as well as a YGG Sequence at the N- or C-Terminus for Concentration Determination

H3 R8Me _X (X = 0–2):	Ac-YGG-QTA(RMe _X)KSTG-NH ₂
H3 K9Me ₃ :	Ac-YGG-QTAR(KMe ₃)STG-NH ₂
H3 R2Me ₂ :	A(RMe ₂)TKQTA-GGY-NH ₂
RUNX1 R210Me ₂ :	Ac-YGG-TAM(RMe ₂)VSP-NH ₂

influence of the peptide sequence on recognition of aRMe_2 , two additional biologically relevant Arg methylation sites were investigated, including histone 3 R2¹⁴ and RUNX1 R210 (also known as AML1)¹⁵ (Chart 1). Isothermal titration calorimetry (ITC) was performed to measure binding affinities (Table 2). The peptides were synthesized containing YGG at the N- or C-terminus for concentration determination by UV. We have

Table 2. Dissociation Constants (K_d) and $\Delta G_{\text{binding}}$ for A_2D Binding to the Peptides Shown in Chart 1 As Measured by ITC^a

entry	peptide	charge	K_d (μM)	ΔG_1 (kcal/mol)
1	H3 aR8Me ₂	+2	5.1 ± 0.6	-7.2 ± 0.1
2	H3 sR8Me ₂	+2	38.4 ± 4.8	-6.0 ± 0.1
3	H3 R8Me ^{b,c}	+2	26.0 ± 3.0	-6.3 ± 0.1
4	H3 K9Me ₃ ^c	+2	3.9 ± 0.5	-7.4 ± 0.1
5	H3R8K9 ^{b,c}	+2	≥ 60	≤ -5.7
6	H3 aR2Me ₂ ^c	+3	0.93 ± 0.01	-8.2 ± 0.1
7	H3 sR2Me ₂	+3	2.3 ± 1.7	-7.7 ± 0.1
8	RUNX1 aR210Me ₂	+1	6.4 ± 0.9	-7.1 ± 0.1
9	RUNX1 sR210Me ₂	+1	18.1 ± 0.8	-6.5 ± 0.1

^aAverage of three runs, except where noted (see Supporting Information). Data fit to a two-site binding model. Error is from the standard deviation. Conditions: 25 °C, 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 8.4. ^bData fit to a one-site binding model. ^cAverage of two runs.

shown that Tyr in the context of an unmodified peptide does not bind to **A₂D**, although we cannot exclude the possibility that the Tyr tag in the peptides used for ITC contributes to binding.¹⁶ With the exception of H3 R8K9 and H3 R8Me, the ITC data were best fit with a model for two independent sites, giving two binding affinities representing a tight binding event and a weaker binding event that we expect is due to nonspecific interactions (see Supporting Information).¹⁷

The original receptor, **A₂B**, exhibits a binding affinity for H3 K9Me₃ of approximately 25 μ M,⁵ as measured by fluorescence anisotropy, with binding affinities >600 μ M for all of the methylation states of H3 R8 (see Supporting Information). In contrast, **A₂D** exhibits a binding affinity of 5 μ M for H3 aR8Me₂, with greater than 7-fold selectivity over H3 sR8Me₂ and more than 10-fold selectivity over the unmodified H3 peptide, H3 R8K9. The selectivity over H3 R8Me is slightly less, at about 5-fold, suggesting that the smaller RMe side chain can be accommodated better than the bulky sRMe₂. These results are qualitatively consistent with the amplifications observed in the DCLs (Figure 4b) and indicate that **A₂D** exhibits similar selectivity to many methyl Arg binding proteins (Table 3).

Table 3. Binding Affinities of Different Reader Proteins to Methyl Arg Containing Peptides

reader protein	methylation mark	K_d (μ M)	ref
eTud11	Aub sR11Me ₂	71	2a
eTud11	Aub sR13Me ₂	48	2a
eTud11	Aub sR15Me ₂	5.4	2a
SMN Tudor	sRMe ₂ ^a	476	2b
SMN Tudor	aRMe ₂ ^a	1025	2b
SPF30 Tudor	sRMe ₂ ^a	652	2b
SPF30 Tudor	aRMe ₂ ^a	1006	2b
SND1 eTudor	PIWIL1 sR4Me ₂	10	2c
SND1 eTudor	PIWIL1 aR4Me ₂	42	2c
SND1 eTudor	PIWIL1 R4Me	19	2c
SND1 eTudor	PIWIL1 R4	97	2c
TDRD1 TD2	sR45Me ₂	55	3a
TDRD1 TD2	sRMe ₂ ^a	172	3a
TDRD1 TD3	sR74Me ₂	35	3a
TDRD1 TD3	sRMe ₂ ^a	353	3a
TDRD3	PIWIL sRMe ₂	>300	3b
TDRD3	PIWIL aRMe ₂	>150	3b

^aReported K_d is for the amino acid alone.

Surprisingly, the binding affinity of **A₂D** for H3 K9Me₃ was found to be equivalent to that of H3 aR8Me₂, showing no distinction between the two modifications. It is worth noting that the similar affinity of **A₂D** for aRMe₂ and KMe₃ is not apparent from the **A₂D** amplification data alone. This can be explained by the fact that KMe₃ amplifies both **A₂D** and **A₃**, which compete for monomer **A** in the DCL. Thus, the extent of **A₂D** amplification by KMe₃ is lower than in the presence of aRMe₂, which does not significantly amplify either **A₃** receptor. This highlights the possible breakdown of the correlation between amplification and binding efficiency as a result of complex competing equilibria that minimize the overall free energy of the system.⁹

The binding affinities of **A₂D** to aRMe₂ and sRMe₂ were compared across all three peptides in Chart 1 to gain insight into the influence of the surrounding sequence on binding. While **A₂D** binds aRMe₂ more strongly than sRMe₂ in every

case, the degree of selectivity appears to be somewhat influenced by the surrounding sequence, ranging from about 2.5- to 7.5-fold. This is likely due to variation in additional contacts made between the receptor and the peptide. The data in Table 2 suggest a modest influence of the sequence on binding based on net charge of the peptide, but the trend is not strong (compare entries 1, 6, and 8 and 2, 7, and 9). The peptides with a +3 charge bind more tightly than the others, but the peptides with a +1 and +2 charge are not significantly different. Thus, it may be that the location of the charge also influences binding affinity, through its ability to make a direct contact with the carboxylates on the exterior of the receptor. For example, if the H3 aR8Me₂ peptide adopts an extended conformation, K9 would be directed away from aR8Me₂, which may reduce its influence on binding, in contrast to K4 in the H3 aR2Me₂ peptide, which would be directed toward the receptor.

DISCUSSION AND CONCLUSIONS

Combined, these data provide insight into the binding affinity and selectivity of **A₂D** for methylated Arg. The selectivity for the different methylation states of Arg can be explained by differences in size, surface area, and desolvation costs. Binding of unmethylated Arg in aqueous solution has been reported with several synthetic receptors.^{8,18} However, cation- π interactions alone are typically not strong enough to result in binding due to the high desolvation cost of binding Arg in an aromatic pocket; generally, electrostatic interactions are also needed to achieve binding in aqueous solution.⁸ Thus, as the carboxylates in monomer **A** are held outside of the binding pocket and the carboxylate in monomer **D** is in the same plane as the naphthalene, which is not optimal for binding Arg,^{8,19} weak binding of unmethylated Arg by **A₂D** was expected.

Methylation of Arg reduces its desolvation cost by both reducing the number of hydrogen-bond donors on the guanidinium group and preventing the formation of bifurcated hydrogen bonds with water.²⁰ Additionally, increased methylation of Arg has been shown in other systems to make stacking interactions with aromatic rings more favorable.²¹ Thus, binding of methylated arginines in an aromatic pocket of the appropriate size is expected to be able to overcome the desolvation cost.⁸ Binding of aRMe₂ by **A₂D** is about 0.5 – 1.2 kcal/mol more favorable than binding of sRMe₂, depending on the peptide sequence. The magnitude of a cation- π interaction is equivalent for the two isomers of RMe₂, and so that cannot account for the selectivity of **A₂D** for aRMe₂.²¹ It may be that sRMe₂ is too large to effectively fit into the binding pocket, such that sRMe₂ can only insert one methyliminium group into the pocket. This is consistent with the NMR data, which exhibits half as much shifting of the methyl groups in sRMe₂ as observed for aRMe₂ as well as broadening of the methyl groups in sRMe₂. This would result in one less methyl- π interaction than aRMe₂ and potentially place an N-H in the pocket instead, resulting in a higher desolvation penalty than for aRMe₂. The binding of RMe to **A₂D** is also weaker than the binding of aRMe₂. RMe can make fewer van der Waals contacts than aRMe₂, and it also has a higher desolvation cost, which can account for the 0.9 kcal/mol difference in binding affinities.

Interesting conclusions can also be drawn from a comparison of **A₂D** and **A₂B**. While **A₂B** exhibits a 2 kcal/mol difference in binding affinity for K9Me₃ relative to aR8Me₂, replacement of the phenyl ring in **A₂B** with the naphthyl ring in **A₂D** abolishes this selectivity. This gain in binding energy for aRMe₂ by **A₂D**

can be attributed to both the formation of a larger binding pocket, which can accommodate aRMe_2 and additional π – π stacking interactions between aRMe_2 and the naphthyl ring.

The carboxylic acids in A_2B and A_2D do not appear to play a direct role in binding to the methylated side chain. It is well established that the carboxylates on monomer **A** are held rigidly outside of the cavity and do not contribute directly to binding of ammonium or guanidinium cations.⁸ Additionally, the carboxylates on the aromatic rings of **B** and **D** are not oriented properly to interact directly with the cation in the binding pocket, as has been shown in other synthetic receptors for Lys and Arg.^{8,19} Thus, it appears that a combination of cation– π and van der Waals interactions is the primary driving force for binding both methylated side chains.²² However, charged residues in the surrounding peptide appear to contribute additional electrostatic interactions with the carboxylates in a sequence-dependent manner. Modification of the carboxylates to create a neutral water-soluble receptor would likely abrogate any sequence selectivity and is currently under investigation.

The structures of protein binding pockets that recognize RMe_2 are all quite similar, consisting of a cube in which four faces consist of aromatic residues, the fifth face is a hydrogen-bonding group (typically Asn), and the sixth face is open to allow for entry of the RMe_2 side chain into the pocket (Figure 2).² A_2D loosely mimics this binding format, as it provides an aromatic pocket with five aromatic rings, although it lacks the hydrogen-bonding group directed into the pocket. Nonetheless, A_2D is still able to bind aRMe_2 more tightly than several of the RMe_2 binding proteins (see Table 3). This suggests that the aromatic cages in the native RMe_2 binding proteins are not necessarily optimal for binding of RMe_2 but rather provide the necessary degree of binding to result in the desired phenotype. Additionally, a comparison of A_2D to the binding pockets of RMe_2 binding proteins suggests that the introduction of a hydrogen bonding group in the appropriate location may further increase binding affinity and selectivity. This is an ongoing effort in our group.

In conclusion, we have identified a novel small molecule receptor, A_2D , for the recognition of aRMe_2 . With the exception of antibodies,²³ to the best of our knowledge this is the only known synthetic receptor that recognizes aRMe_2 with selectivity over sRMe_2 . A_2D exhibits comparable affinities and selectivities for the different methylation states of Arg as native methyl Arg binding domains. The low micromolar affinity of aRMe_2 by A_2D is impressive, as the primary driving force for binding is the interaction with the modified side chain, unlike many of the native proteins which recognize the surrounding residues and protein backbone as well. The preference for aRMe_2 over the other methylation states can be attributed to differences in the van der Waals contacts, cation– π interactions, and desolvation penalties. The results presented here demonstrate the ability to develop and fine tune a receptor by making subtle changes to the building blocks used in DCC and suggest opportunities to further optimize function. Receptors of this type are promising for applications involving differential sensing of PTMs, such as pattern recognition assays or affinity chromatography.^{24,25}

EXPERIMENTAL PROCEDURES

Synthesis of Monomer D. Synthesis of monomer **D** was achieved following a modified procedure for the synthesis of an isomeric compound, as described in the Supporting Information.²⁶

Dynamic Combinatorial Chemistry. The relevant building blocks were individually dissolved in water, adding sufficient 1.0 M aqueous NaOH to fully deprotonate the thiols and carboxylic acids on the building blocks, using sonication when necessary. The pH of each solution was then adjusted to 8.5 using 1.0 M aqueous HCl and 1.0 M aqueous NaOH. Aliquots of each monomer solution were combined in a 2 mL LC-MS vial to reach a final concentration of 5 mM **A** and 2.5 mM **D**, respectively. When necessary, an aliquot of the appropriate peptide guest dissolved in water was added to the reaction to reach a final concentration of 7.5 mM peptide. Any remaining volume was made up with water. The vials were capped and analyzed by HPLC-MS at various time points.

Analytical LC-MS. LC-MS was carried out on an Agilent Rapid Resolution LC-MSD system equipped with an online degasser, binary pump, autosampler, heated column compartment, and diode array detector. All separations were performed using 5 mM NH_4OAc H_2O –acetonitrile gradients at pH 5 and a Halo C18 column (4.6×100 mm, $2.7 \mu\text{m}$). The MS was performed using a single quad mass spectrometer. Mass spectra (ESI-) were acquired in ultrascan mode by using a drying temperature of 350°C , a nebulizer pressure of 45 psi, a drying gas flow of 10 L/min, and a capillary voltage of 3000 V. The reactions were monitored weekly (3 μL injections) until equilibrium was reached after about 3 weeks.

Synthesis of A_2D . Biased libraries were prepared on a 0.05 mmol scale (**A**: 35.6 mg, 0.1 mmol, 6.67 mM; **D**: 11.8 mg, 0.05 mmol, 3.33 mM) templated with methylisoquinoline iodide (41.0 mg, 0.15 mmol, 10 mM). Methylisoquinoline iodide was used as the template as it is less expensive than the aRMe_2 peptide and also templates A_2D formation. Upon equilibration the libraries were neutralized, and the receptors were isolated by semipreparative HPLC. Approximately 0.3 mL injections were chromatographed using buffered mobile phases **A** (10 mM NH_4OAc in water) and **B** (10 mM NH_4OAc in 9:1 ACN to water) using a gradient (0–35% **B** from 0 to 5 min, then 35–70% **B** from 5 to 20 min) with a flow rate of 4.0 mL/min. A sharper A_2D peak was achieved with a column heater set to 40°C , but this was not necessary for separation from the library. The A_2D peak at 13.5 min was collected and analyzed for purity by analytical LC-MS, giving a 45% yield (see Supporting Information). Purified A_2D was lyophilized to powder and stored under nitrogen.

NMR Characterization of A_2D . All 1D and 2D NMR samples were prepared and analyzed on a Varian Inova 600 MHz instrument similar to those described previously, unless noted otherwise. For characterization, a 600 μM A_2D sample was prepared in 50 mM borate buffer at pD 9.25 containing 0.05 mM DSS. The 1D spectrum of A_2D was collected at 5°C using 32 K data points and 900 scans with a 3 s presaturation. 2D NMR experiments used for structural analysis included COSY, TOCSY, and NOESY experiments. ^1H NMR (600 MHz, D_2O , borate buffer, pD 9.25, uncorrected): δ = 8.20 (s, 1H), 8.18 (s, 1H), 8.07 (s, 1H), 8.04 (d, 1H), 7.99 (s, 1H), 7.97 (d, 1H), 7.81 (d, 1H), 7.73 (s, 1H), 7.61 (d, 1H), 7.05 (d, 1H), 6.82 (s, 1H), 6.46 (d, 1H), 5.95 (s, 1H), 5.75 (d, 1H), 5.72 (s, 1H), 5.58 (s, 1H), 5.34 (d, 1H), 5.30 (s, 1H), 5.22 (s, 1H), 3.10 (dd, 1H), 2.93 (d, 1H).

NMR Analysis of Binding Interactions. NMR experiments were performed on 600 MHz Bruker NMR at 25°C , in 10 mM $\text{Na}_2\text{DPO}_4/\text{NaD}_2\text{PO}_4$ (pD 8.0 = pH 8.4) buffer with DSS (2.0 mM) as an internal standard. Peptide stock solutions were made (20–24 mM), and concentration determined with respect to DSS by NMR. Peptides were diluted to a concentration of 600 μM . An NMR of each pure peptide was obtained for comparison, and the 600 μM peptide stock solution was then used to dissolve A_2D (3.2–3.5 equiv) to give a final concentration of 2–2.2 mM A_2D in 600 μM peptide. 1D spectra were collected for each sample with 128–512 scans. All Arg protons were assigned using TOCSY analysis with and without A_2D present.

Isothermal Titration Calorimetry. ITC titrations were performed on a Microcal AutoITC200. Titrations were carried out at 298 K in buffered H_2O (10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 8.4). The concentration of A_2D was determined by measuring the UV–vis absorbance at 267 nm, using a NanoDrop2000 with a xenon flash lamp, 2048 element linear silicon CCD array detector, and 1 mm path length. A 1–3 mM solution of peptide was titrated into an 80–200

μM solution of A_2D , using 2.0 μL increments every 3 min. Heats of dilution were subtracted prior to fitting. Binding curves were produced using the supplied Origin software and fit using one- or two-site binding models.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis, NMR characterization, and binding data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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