Protein Chemistry

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Modular Assembly of Macrocyclic Organo–Peptide Hybrids Using Synthetic and Genetically Encoded Precursors**

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Macrocyclic peptides and peptide-containing molecules are attractive molecular scaffolds for the development of bioactive compounds to modulate biomolecular interactions. These structures combine a high degree of functional complexity with restricted conformational flexibility, which make them well-suited to achieve selective and tight binding to extended biomolecular interfaces, such as those mediating protein–protein and protein–nucleic acid complex formation.[1] Compared to linear peptides, conformationally constrained peptide-based ligands often exhibit higher proteolytic stability,[2] enhanced cell permeability,[3] and higher affinity towards the target biomolecule,[4] which render them valuable as probes and potential pharmacological agents. Indeed, many cyclic and lariat peptides isolated from natural sources[5] exhibit potent biological activities and have provided a source of viable drugs.[1d]

Both biosynthetic[6] and synthetic[7] methods have been implemented to afford peptides in cyclic or conformationally constrained configurations. Genetic encoding offers the unrivalled advantage that vast molecular libraries (10^8–10^{10}) can be rapidly created by combinatorial mutagenesis and readily explored using genetic selection or ultrahigh-throughput screening methods.[6,8] However, the pool of building blocks available for construction of biological peptide libraries remains restricted, limiting the degree of ligand diversity achievable through these approaches. In contrast, synthetic methods can draw upon a much broader spectrum of precursor structures, including non-natural amino acids,[9] peptoids,[10] and amino acid unrelated scaffolds,[11] which can be exploited to confer improved or novel conformational and target-binding properties to peptide-based ligands.

Integrating the advantages of biological and synthetic approaches would open unprecedented opportunities for ligand diversification and molecular discovery. Towards this goal, we have developed a method that allows the embedding of non-proteogenic synthetic moieties into genetically encoded peptidic frameworks. This strategy enables the modular assembly of macrocyclic organo–peptide hybrids (MOrPHs), the size and composition of which can be readily diversified by varying the nature of the synthetic and biosynthetic precursors (Scheme 1).

Our approach takes advantage of the reactivity of intein proteins[12] and the opportunity to introduce bioorthogonal functionalities into proteins by amber stop codon suppression.[13] We envisioned that incorporating an alkyne-bearing non-natural amino acid within the N-terminal portion of an intein-fused polypeptide would yield a recombinant protein carrying two functional groups with orthogonal reactivity, namely the alkyne moiety and the thioester bond transiently formed at the junction with the intein by reversible N→S acyl transfer. A tandem chemoselective reaction could thus be exploited to mediate coupling of this biosynthetic precursor (BP) to an azide/hydrazide-containing synthetic precursor (SP) and promote the formation of an organo–peptide macrocycle (Scheme 1).

To test our design, we first constructed a plasmid (pBP_MG6) encoding for a 6mer target sequence (TS6: TGSYGT) preceded by Met, Gly, and the amber stop codon TAG and fused at the C terminus to the N-terminal cysteine of intein GyrA from *Mycobacterium xenopi*.[14] This construct was expressed in *E. coli* in the presence of *O*-propargyltyrosine (OpgY) and a previously described mutant $tRNA_{CUA}$ ($Mj$$tRNA_{CUA}$)/tyrosyl-tRNA synthetase (MjTyrRS) pair[15] encoded by a second vector (pEVOL[16]). The latter allow the site-selective incorporation of OpgY at the N-terminal end of the target sequence in the biosynthetic precursor by stop codon suppression. The resulting protein, called MG6, was purified by nickel-affinity chromatography and its identity confirmed by MALDI-TOF (Figure 1a). To test the macrocyclization reaction, the bifunctional synthetic precursor **1** was synthesized and coupled to MG6 by Cu^I-catalyzed azide–alkyne 1,3-dipolar cycloaddition[17] (CuAAC; 20 min) followed by removal of the copper catalyst and excess **1** by fast buffer exchange (2 min). Formation of the MG6-**1** adduct occurred quantitatively and was followed by complete splicing of the GyrA intein after 16 h as indicated by MALDI-TOF analysis (Figure 1a). This process was accompanied by the accumulation of a product with molecular mass (m/z 1016.3) corresponding to the desired organo–peptide macrocycle **7**, as revealed by LC-MS (Figure 1b). Along with the major macrocyclic product, the formation of a small amount (ca. 20%) of the acyclic peptide H_2N-G(OpgY-**1**)TGSYGT-COOH (**8**; m/z 1034.3) was also observed, indicating that hydrolysis of the MG6-**1** adduct competes to a minor extent with the macrocyclization process. The cyclic backbone of the predominant product (**7**) was further evidenced by MS/MS analysis (Figure 1c), which showed few fragments as a result of multiple ring-opening pathways leading to acylium ions of

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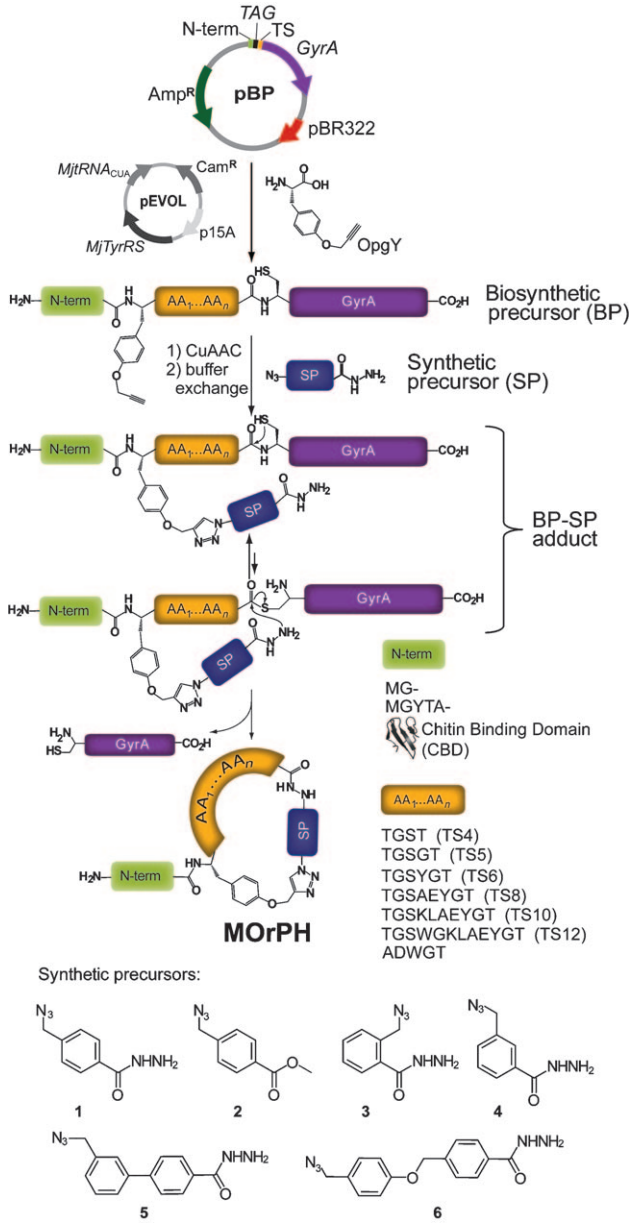

Scheme 1. Strategy for the modular synthesis of macrocyclic organo–peptide hybrids (MOrPHs). The vector pBP encodes for a linear polypeptide (biosynthetic precursor) comprising an N-terminal tail (green), O-propargyl tyrosine (OpgY), a target sequence TS (yellow), and GyrA intein (purple). Upon coupling of this protein to a synthetic precursor (blue) by Cu^{I}-catalyzed alkyne–azide cycloaddition (CuAAC), the thioester bond at the intein junction is intercepted by the nucleophilic hydrazide to yield an organo–peptide macrocycle. The various N-terminal tails, target sequences, and synthetic precursors described in this study are indicated.

the same m/z as observed in cyclic peptides.[18] In comparison, the minor acyclic product (**8**) exhibited a fragmentation pattern typical for a linear peptide (Figure 1d).

Control experiments were carried out to confirm the mechanism and specificity of the reaction. Omitting the copper catalyst from the reaction with **1** resulted in no macrocycle formation and much reduced splicing of the intein fusion protein (ca. 15%, 16 h), the latter deriving from background hydrolysis of the protein as indicated by observation of the linear peptide H_2N-G(OpgY)TGSYGT-COOH by LC-MS (Supporting Information, Figure S1). The reaction was then carried out using an analogue of **1** (compound **2**), which carries a methyl ester in place of the hydrazide. After coupling **2** to MG6, only background splicing of the MG6-**2** adduct (ca. 20%, 16 h) and accumulation of the hydrolysis product H_2N-G(OpgY-**2**)TGSYGT-COOH were observed (Supporting Information, Figure S2), which confirmed the direct involvement of the hydrazide in **1** in macrocyclization. Finally, the regioselectivity of the CuAAC was investigated by coupling **1** (and the other SPs described later) to N-Boc-protected *O*-propargyltyrosine methyl ester under identical conditions used for MOrPH synthesis (50 mM KPi, pH 7.5). This reaction afforded the disubstituted 1,4-triazole product as single regioisomer, as determined by ^{1}H NMR and NOE experiments (Supporting Information), confirming the excellent regioselectivity of this reaction.[17] Altogether, these studies demonstrate that the assembly of the hybrid macrocycle occurred with the expected regiochemistry and according to the envisioned route; that is, by intramolecular attack of the nucleophilic hydrazide on the thioester linkage after formation of the BP-SP adduct.

Encouraged by these results, we investigated the viability of this strategy to assemble diverse MOrPHs by varying the genetically encoded portion of the macrocycle. To this end, biosynthetic precursors comprising shorter (TS4, TS5) and longer (TS8, TS10, TS12) target sequences (Scheme 1) were prepared (named MG4, MG5, MG8, MG10, and MG12, respectively). As for TS6, these target sequences were randomly chosen with the exception of the I-1 position (preceding the intein), where a threonine was introduced as this substitution was reported to induce minimal self-splicing of GyrA fusion proteins during expression in *E. coli*.[19] After coupling with **1**, the desired macrocycle formed as the almost exclusive product (95–100%) from all the reactions except that involving MG4, which yielded an approximately 1:1 ratio of macrocycle and acyclic product (Figure 2a) as estimated from the corresponding LC-MS extracted-ion chromatograms. Tandem mass spectrometry further confirmed the cyclic structure of the produced MOrPHs (Supporting Information, Figure S3). Importantly, these results indicated that macrocyclization is strongly favored over the competing thioester hydrolysis (leading to the acyclic product) across target sequences from five up to twelve amino acids, allowing for the efficient assembly of MOrPHs of variable ring size (Supporting Information, Figure S3). In these reactions, splicing of the BP-SP adduct was found to be higher (90–100%) in the context of MG4, MG5, and MG6 compared to MG8, MG10, and MG12 (50–75%) after overnight incubation at room temperature (Figure 2b). This trend can be rationalized considering that in the BPs with shorter target sequences, the protein-bound SP is positioned closer to the intein, favoring the nucleophilic attack of the hydrazide on the thioester linkage. As observed with MG6, CuAAC coupling proceeded quantitatively with the various biosynthetic precursors, as judged from MALDI-TOF spectra acquired immediately after the coupling reaction. Based on

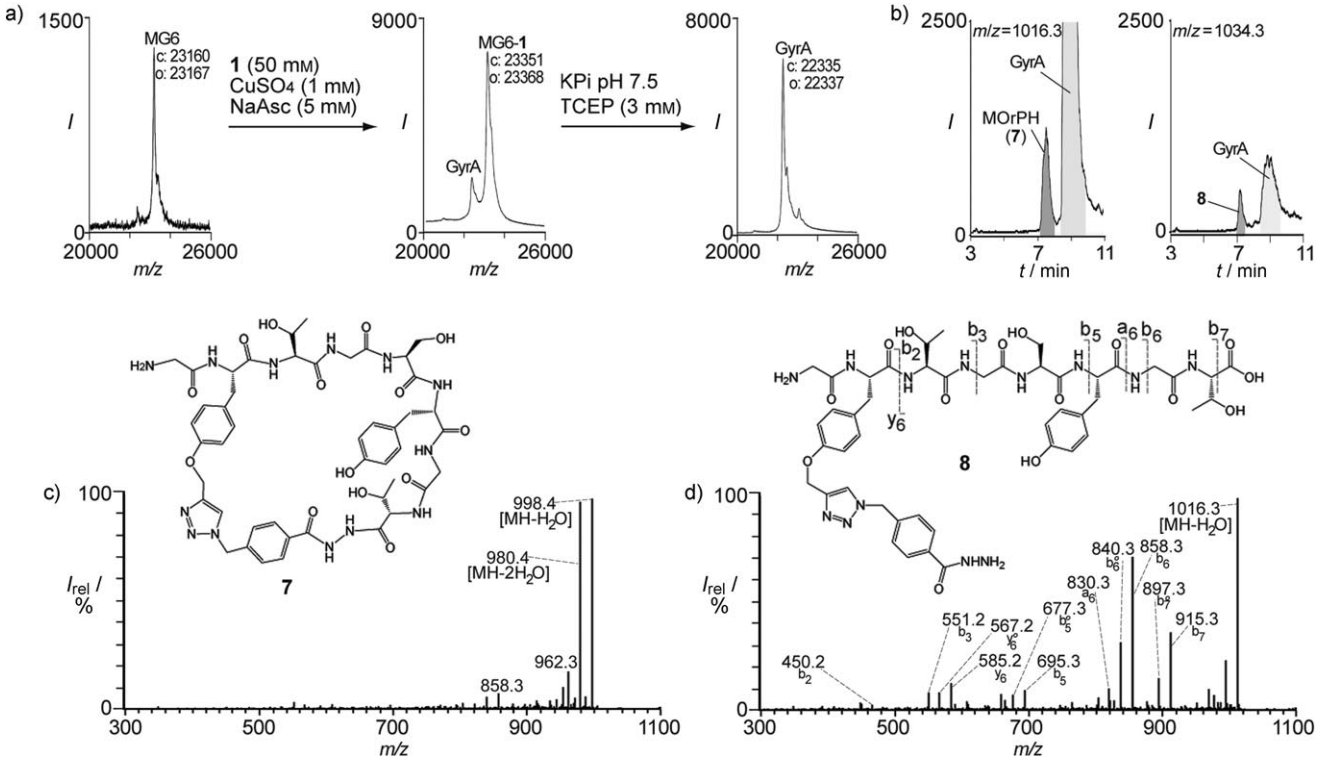

Figure 1. a) MALDI-TOF spectrum of biosynthetic precursor MG6 after purification (left), after coupling to **1** by CuAAC (middle), and after overnight incubation at room temperature (right). The observed (o) and calculated (c) *m/z* values ($[M+H]^+$ species) are indicated. NaAsc = sodium ascorbate, TCEP = tris(2-carboxyethyl)phosphine. b) Extracted-ion chromatograms for *m/z* corresponding to the macrocycle (left) and the acyclic product (right) as obtained from LC-MS analysis of the reaction mixture after 16 h. Light gray peak: unrelated multicharged ions from spliced GyrA intein. c) MS/MS spectrum of the MOrPH product **7** (precursor ion: *m/z* 1016.3). d) MS/MS spectrum of the acyclic product **8** (precursor ion: *m/z* 1034.3) with assignment of the fragment ions.

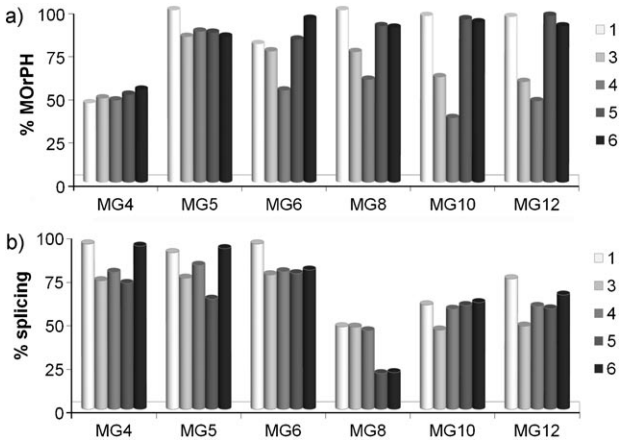

Figure 2. Reactions between SP **1**, **3**, **4**, **5**, and **6** and the MG biosynthetic precursors. a) Fraction of MOrPH formed ([MOrPH]/([MOrPH] + [acyclic product]) and b) percentage of splicing of the corresponding BP-SP adducts ([GyrA]/([GyrA] + [unspliced BP-SP])) as determined by LC-MS (16 h).

this, the percentage of MOrPH produced, and the extent of protein splicing (Figure 2), the overall yield for MOrPH formation in the reaction of **1** with the MG constructs was estimated to range from about 50% (MG4, MG8, MG10, MG12) to more than 80% (MG5, MG6).

Next, we investigated the possibility of diversifying MOrPH architecture by varying the structure of the synthetic precursor. To this end, compounds **3**–**6** (Scheme 1) were synthesized, which incorporate phenyl, biphenyl, and diphenyl scaffolds. Bi- and diaryl structures are, among others, recurring motifs ("privileged structures") in small molecules with biological activity,[20] including those found to inhibit protein–protein interactions.[21] These SPs were also designed to evaluate the effect of the distance between the azide and the hydrazide on the efficiency of macrocyclization. Such distance increases in the order **3** (5.5 Å), **4** (6 Å), **1** (7 Å), **5** (9–11 Å), **6** (12–15 Å), as calculated based on energy-minimized conformations of these molecules (MM2 force-field). Reactions of **3**–**6** with the BPs containing target sequences of varying length were performed as described for **1** and analyzed by LC-MS. Notably, the desired macrocyclic product was obtained for all 30 combinations tested (Supporting Information, Figure S4), demonstrating the functionality of the method across widely different SP structures and its versatility to afford diverse MOrPHs by varying the synthetic and peptidic portion of these structures.

The ratio of macrocycle versus acyclic product produced in these reactions was analyzed to assess the relative efficiency of macrocyclization in the context of the various SP/BP pairs (Figure 2a). Interestingly, MOrPH assembly was found to occur with highest efficiency (> 85%) with the 5mer

target sequence and all the SPs and with the 6mer, 8mer, 10mer, and 12mer target sequences and **1**, **5**, and **6**. Synthetic precursors with closely spaced azide/hydrazide (less than 6 Å as in **3** and **4**) were suboptimal for cyclization with target sequences longer than six amino acid residues, while the shortest target sequence tested (4mer) yielded an approximately 1:1 mixture of macrocycle and acylic product regardless of the SP structure. With respect to the extent of BP-SP adduct splicing, the trend observed in the reactions with **1** was reproduced in the reactions with the other four SPs as well (Figure 2b), supporting our conclusions regarding the higher reactivity of BPs with shorter target sequences.

To investigate the kinetics of MOrPH formation, splicing of MG5-**1** and MG10-**1** adducts, which produce MOrPH almost exclusively, was monitored over time. These studies revealed that adduct splicing (and thus MOrPH formation) occurs in large part within the first two hours from the coupling reaction (Figure 3). In contrast, a high concentration

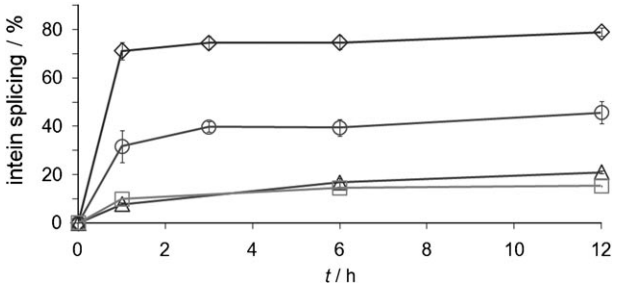

Figure 3. Time course measurement of GyrA splicing for MG5-**1** adduct (◇), MG10-**1** adduct (○), MG5 in the presence of **1** at 50 mM (△), and MG5 alone (□) as determined by LC-MS analysis. Error bars are calculated from experiments carried out in duplicate.

of unbound **1** (50 mM) caused a negligible amount of protein splicing over background hydrolysis, even over extended periods of time (12 h), which is consistent with the slow kinetics observed for intein-mediated ligations using nucleophiles other than thiols.[12b] Overall, these results denoted the large rate acceleration in the hydrazide-induced intein splicing reaction for the intramolecular versus intermolecular mechanism. An important consequence of this rate difference is that hydrazide-dependent splicing occurs exclusively after tethering of the synthetic precursor to the protein, providing an excellent control over the undesired intermolecular reaction.

To determine whether milligram amounts of pure MOrPH product could be obtained using the described method, a scaled-up reaction was carried out using **1** and about 50 mg of purified MG6 protein. After the reaction, the low-molecular-weight products could be isolated from the reaction mixture by filtration followed by solid-phase extraction, yielding about 2 mg of a mixture of **7** and **8** in about 80:20 ratio and 90% purity. MOrPH **7** was then successfully isolated in more than 95% purity by further purification using C_{18} reverse-phase HPLC (Supporting Information, Figure S5).

Various bioactive peptides and depsipeptides found in nature display a lariat backbone, where an N- or C-terminal tail is connected to a cyclized portion of the peptide sequence.[7e,h,22] To explore the scope of the method to prepare MOrPHs in lariat configuration, the five synthetic precursors (**1**, **3**–**6**) were reacted with the biosynthetic precursor Lar5, which consists of a pentamer N-terminal tail (MGYTA) and a pentamer target sequence (ADWGT). Macrocyclization was found to proceed efficiently in all cases, as indicated by the extent of splicing of the BP-SP adducts (50–60%) and the observation of the desired lariat macrocycles as the sole product by MALDI-TOF (Figure 4).

These results suggested that modification of the N-terminal portion in the biosynthetic precursor was compatible with MOrPH formation. To further investigate this aspect, the six target sequences TS4 to TS12 were fused to a larger N-terminal tail consisting of the 71 amino acid chitin binding domain (CBD) of chitinase A1 from *Bacillus circulans*. The resulting CBD fusion biosynthetic precursors were tested in reactions with **1** and **3**–**6**. As illustrated by the MALDI-TOF spectra (Figure 5; Supporting Information, Figures S6–S9), the desired CBD-tethered MOrPHs were obtained as the sole

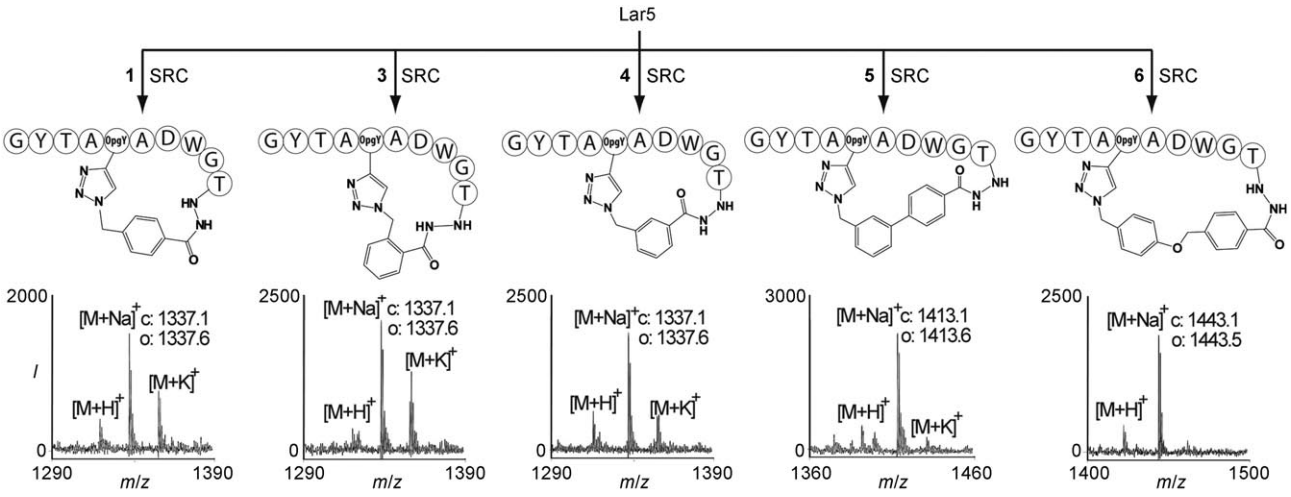

Figure 4. MALDI-TOF spectra of the lariat MOrPHs. Calculated (c) and observed (o) *m/z* values for the sodium adduct are indicated along with the peaks corresponding to the proton and potassium adducts. SRC = standard reaction conditions (see the Supporting Information).

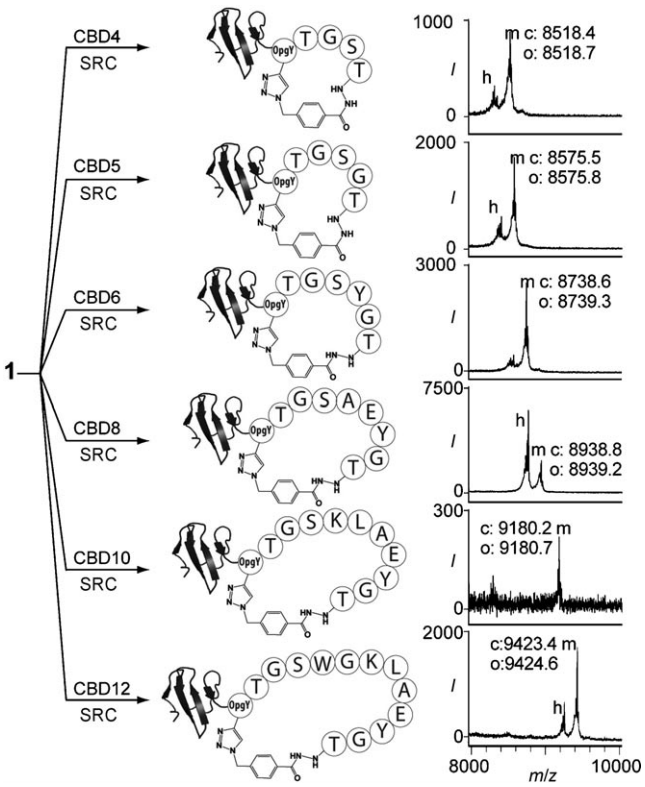

Figure 5. MALDI-TOF spectra of the CBD-tethered MOrPHs (m) obtained with **1**. h = small MW fragment resulting from hydrolysis of unmodified biosynthetic precursor.

or the predominant product from the majority of these reactions. Coupling of the SPs to the CBD constructs by CuAAC proceeded efficiently in most cases (70–100%) and could not be achieved in only one case (**6** + CBD4). As observed with the MG constructs, **1**, **5**, and **6** were highly efficient precursors for MOrPH assembly, as judged by the occurrence of no or little acyclic byproduct. Altogether, these studies showed that the present method can be readily extended to afford structurally diverse MOrPHs linked to the C terminus of a protein.

In summary, we have established a new method for constructing conformationally constrained organo–peptide hybrids by combining a genetically encoded polypeptide and a synthetic precursor. Using this strategy, MOrPHs of molecular weight from 700 to 1800 Da and featuring different ring size, structure, and composition could be rapidly (< 2 h) and efficiently prepared (50–80% yields). The efficiency of MOrPH synthesis across largely different synthetic precursors (for example, **1**, **5**, and **6**) and 5 to 12 amino acid peptidic moieties suggests that MOrPH libraries could be accessed through combinatorial variation of these elements, which will be the object of future investigations. The method is amenable to scale-up to isolate the desired MOrPH in high purity for further testing and it offers the versatility to enable the preparation of organo–peptide hybrids in cyclic or lariat configuration as isolated entities, or tethered to a protein of interest. The latter feature has clear implications with respect to enabling the immobilization of these macrocycles on a solid support or a cellular/viral structure for screening purposes. In particular, we envision that coupling MOrPH synthesis with a display method will provide a powerful tool to discover valuable MOrPH compounds with tailored protein-binding properties.

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